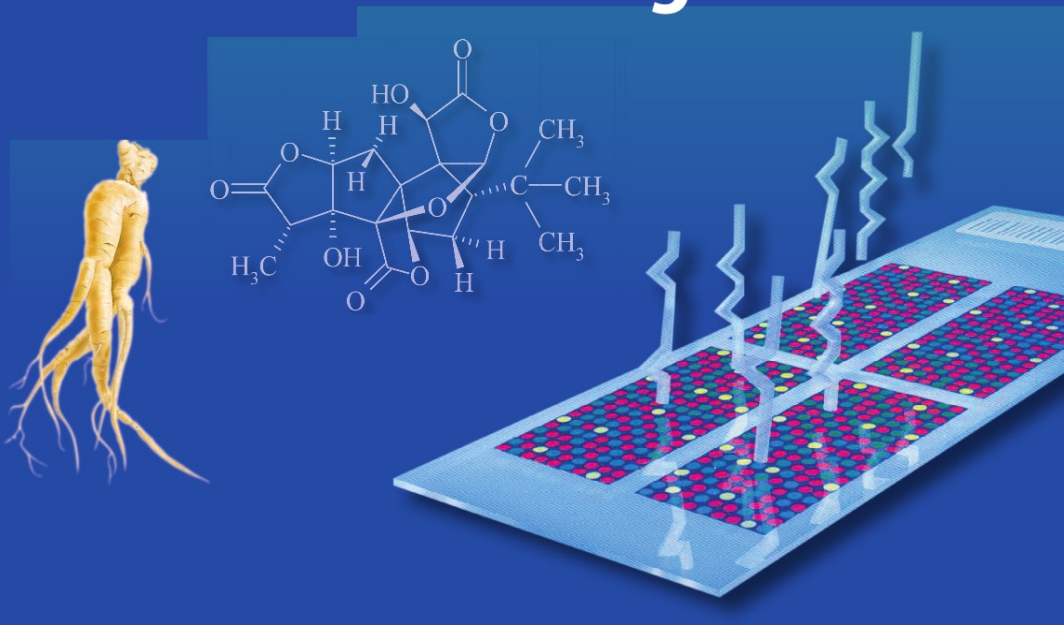
Hildebert Wagner · Gudrun Ulrich-Merzenich
Editors

Evidence and Rational Based Research on Chinese Drugs



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Preface

After the successful introduction of acupuncture to western medicine, great advances in analytical chemistry, molecular biology, genomic technology and system biology have again brought herbal drugs into the focus of basic research on Traditional Chinese Medicine (TCM).

A growing number of Western physicians become trained in Traditional Chinese Medicine and advocate Chinese medicinal preparations. However, as daily use increases, concerns about the effectiveness and safety of TCM drugs are raised by regulatory authorities and parts of the medical community which are still alien to TCM. Also China itself shows a growing interest in developing its traditional herbal medicine into contemporary drugs of global relevance. This is supported through an encouraging number of government-funded research projects in TCM all over China as well as on international level.

The authors of this book are confident that a development of TCM drugs towards a contemporary and global medicine can be achieved successfully through the application of modern research methodology. This book provides an up-to-date compilation of ongoing efforts in the further development of TCM. It covers a broad range of vital issues, starting with fundamental aspects like the present status in analytic drug monographies of TCM drugs for a quality proof and the DNA-based authenticity proof of TCM plants as prerequisites for a novel drug development. The following chapters are dedicated to individual plants, to their chemical constituents, and to their potential application. The disease indications range from cancer, stroke, mental, or gynecological disorders and describe the chemical and pharmacological aspects and the significant efforts which have already been undertaken in the development of compounds like artemisinin or its derivates to a potential anticancer drug or Gingko preparations as treatment prevention and for the prevention and treatment of Dementia and Alzheimer's disease.

In the closing chapter, the editors have summarized the results obtained over the last 10 years in all areas of basic and applied research on Chinese drugs. The progress and the improvements are impressive, but the remaining shortcomings

cannot be overlooked. Therefore the editors have attempted to provide some guidelines and recommendations for the challenges which have to be overcome in the next years.

The editors regret that several Chinese experts who were asked to contribute to this book could not accept our invitation. Nevertheless, there is no question that the tremendous task of modernizing Traditional Chinese Medicine requires joint cooperation between experts from China and the West supported by appropriate resources on all levels.

We thank all authors who are eminent scientists with a high dedication to TCM. May their contributions foster a further contemporary development of TCM drugs.

Munich, Germany H. Wagner
Bonn, Germany G. Ulrich-Merzenich

Acknowledgments

- The editors wish to express their sincere thanks to all colleagues who contributed with their special expertise in Chinese Medicine to the successful outcome of this book. They express their deep graditude to Prof. Xiao Pei-gen, Acadamy of Medical Sciences, Beijing for advices concerning the Quality Proof and Medicinal Application of Chinese Drugs.
- We are deeply indebted to our numerous coworkers for technical assistance. In particular, we would like to thank Mrs. Magdalena Heimhilger and Stefanie Koch for their computer work and assistance in literature research.
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Chapter 1
Development of New Analytical Monographs of Herbal Drugs from TCM for Quality Proof and Development of New Phytopharmaceuticals

H. Wagner

1.1 Introduction

China possesses the world's largest resource of medicinal plants, which are documented in several Chinese medicinal books and Pharmacopoeias. Around 5,000 herbal, animal and mineral drugs have been used in China alone (Xiao 1983). In Vol. 1 of the 2010 Chinese Pharmacopoeia, 527 of the total number of monographs inclusive those of animals and minerals are devoted to herbal drugs. In the Appendix of the same Pharmacopoeia, formulas (fixed herbal drug combinations, so-called Chinese fixed patent Medicines) are also listed. In western countries (Europe, the USA and other countries), from 75 to 150 herbal drugs of Chinese origin are most frequently used and are obtained either through markets or prescriptions by doctors trained in Chinese medicine.

In China, TCM drugs have the status of drugs or dietary supplements (foods). In the USA, they are classified exclusively as dietary supplements or functional foods. In Germany, they are defined as conventional drugs. Therefore, the health authority insists on the performance of special quality and safety tests according to the criteria of a good manufacturing practice (CMP). Independent of specific national regulations, however, there is also an international consensus that all TCM drugs must meet certain stipulated high-quality standards. Additionally, it must be guaranteed that all TCM drugs prescribed by physicians are safe for patients.

In the last 5 years, many reviews and original papers have appeared in Chinese, German and European Journals which tackled this theme and issued various proposals of how these aims could be achieved. The unanimous conclusion is that the present methods stipulated in the monographs of the Chinese Pharmacopoeia are not sufficient to meet the high standards of the official European regulatory

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Drug Authority. Therefore, further analytical efforts are necessary to satisfy the demands of high quality and safety standards for Chinese drugs (Wagner and Bauer 2007; Vlietinck et al. 2009; Yuan et al. 2011; Bauer and Franz 2010; Wagner et al. 2011). Because of the belated adaptation of the analytical methods to the present state of scientific art and technique, our Institute at the University of Munich was charged by the Bavarian Drug Regulatory authority with the task of performing quality proofs on important Chinese drugs for the first Chinese Hospital in Germany in Bad Kötzting, with the aim of developing new analytical monographs. Following 15 years of comprehensive laboratory work, two volumes have recently been published by Springer Publishing Company (http://www.springer.com/biomed/pharmaceutical+science/book/978-3-7091-0762-1?changeHeader).

These two volumes describe the methods prescribed for the TLC and HPLC chemical fingerprints of the 80 Chinese Herbal drugs used most frequently in the Bad Kötzing TCM hospital (Wagner et al. 2011). A third volume containing a further 40 or 45 herbal drug monographs will be completed by the beginning of 2013.

Meanwhile, the Department of Health of the Hong Kong Special Administrative Region of the People's Republic of China published its special Hong Kong Chinese Materia Medica (HKCMM) standards, in which recommendations for safety and quality control of 125 herbal materials used commercially in Hong Kong are presented (http://www.dh.gov.hl/english/main/main_cm/main_cm_hkcmms.html).

1.2 Modernisation of Quality Proof of Chinese Herbal Drugs

1.2.1 Identity and Safety Proof

Among the various prerequisites for a perfect quality proof of herbal drugs, the *identity* (authenticity) and *safety proofs* take first precedence. Identity means botanical authenticity. A safety proof aims mainly to exclude any kind of adulterations or falsifications of herbal drugs which might, for example, contain toxic constituents, possess minor general quality or have a quite different pharmacological activity in comparison with that of the official herbs.

The detection of non-permitted amounts of "Foreign Matters" e.g. "Pesticides residues", "Heavy metals", "Aflatoxin B1", "Ochratoxin", "Radioactive Contamination", or microbiological impurity are internationally stipulated and therefore not the topic of this chapter. The corresponding necessary tests require special laboratories in which they are performed prior to all other quality investigations.

For the European Herbal Drug Regulatory Authority, the following limits for heavy metals are obligatory: lead 5 mg/kg, cadmium 0.9 mg/kg, mercury 0.2 mg/kg, arsenic 2.0 mg/kg and copper 20.0 mg/kg. These standards are not mandatory for China, except for extracts used for injections, which must meet the international standards.

The new monographs also include the morphological (macroscopic) and microscopic botanical identity proofs of the herbal drugs, but it must be taken into

consideration that without the necessary pharmacognostic experience and the use of authentic standard drugs, the authenticity of a drug cannot be guaranteed with certainty by botanical methods alone.

1.2.2 TLC and HPLC Fingerprinting

Based on experience gained by the Munich research group from the first TLC fingerprinting of herbal drugs (Wagner and Bladt 2001), we have decided to use the chromatographic TLC and HPLC-fingerprint analytical techniques, which meet both the requirements of a science-based authenticity proof of the Chinese drugs and the high standards of the European Drug Regulatory Authority. This method enables researchers, for the first time, to detect the complex entities of all main low molecular constituents of a plant drug, with the advantage that the single constituents can be made visible in coloured TLC photographs and in a quantifiable HPLC peak profiling. At the same time, for safety reasons, these new techniques can also be used to exclude possible falsifications and adulterations of herbal drugs. The chromatographic TLC, HPLC and GC fingerprint analytical techniques described in the monographs are unique in that they have never been used in any Pharmacopoeia (except the American Pharmacopoeia in which some the Chinese Herbal drugs have yet been monographed) although they are the most comprehensive, non-sophisticated chromatographic methods for a science-based identity and stability proof of Chinese herbal drugs. Using online recordable UV spectra with the diode array technique, it is also possible to gain information about the chemical structure of single constituents. Each monograph of the new manual also contains a description of the macroscopic features, an updated list of all the main bioactive constituents of a drug identified to date and the pharmacological and biological activities of the single herbal drugs and their therapeutic applications. A comprehensive reference list informs the reader about new analytical topics and trends. The contents of 80 herbal drug monographs contained in Vol. I (1–466 p.) and Vol. II (467–1024 p.) are listed in Table 1.1 (Wagner et al. 2011).

The applicability of the monograph has been approved comprehensively for the quality control of imported Chinese herbal drugs for the Chinese Hospital in Bad Kötzting.

At the same time, the chromatographic fingerprint method used for the quality proof of Chinese Drugs has also been advocated by Chinese experts in this field as the best presently available, non-sophisticated and feasible method (Liang et al. 2010). Accordingly, this fingerprint technology was also recently accepted as the favoured method in the frame work of the international ISO standardisation[1] of the "Quality and Safety of TCM".

[1] Resolution 18 of the second plenary meeting of ISO/TC 249 held in the Hague, the Netherlands, on May 2–4th 2011 (Establishment of the working group "Quality and Safety of TCM products", convened under German auspices) http://www.iso.org and http://www.din.de.

Table 1.1 Alphabetic list of monographs of Vol. I and Vol. II (Wagner et al. 2011, Springer Wien, New York)

Lat. name	Chin. name	Page	Volume
Acanthopanax senticosus	Ciwujia	415	I
Aconitum kusnezoffii	Zhicaowu	977	II
Aconitum lateralis	Fuzi	977	II
Acorus calamus	Zangchangpu	777	II
Acorus tatarinowii	Sichangpu	777	II
Alisma orientalis	Zexie	467	II
Amomum compactum	Doukou	335	I
Amomum kravanh		335	I
Amomum longiligulare		335	I
Amomum villosum		335	I
Amomum xanthioides		335	I
Andrographis paniculata	Chuanxinlian	273	I
Anemarrhena asphodeloides	Zhimu	403	I
Angelica biserrata	Duhuo	99	I
Angelica dahurica	Baizhi	171	I
Angelica formosana		171	I
Angelica pubescens	Duhuo	99	I
Angelica sinensis	Danggui	161	I
Artemisia capillaris	Yinchen	967	II
Artemisia scoparia		967	II
Asarum heterotropoides	Xixin	45	I
Asarum mandshuricum		45	I
Asarum seoulense		45	I
Asarum sieboldii		45	I
Astragalus chrysopterus	Huangqi	83	I
Astragalus floridus		83	I
Astragalus membranaceus		83	I
Astragalus mongholicus		83	I
Astragalus tongolensis		83	I
Atractylodes chinensis	Cangzhu	691	II
Atractylodes lanceae		691	II
Atractylodes macrocephala	Baizhu	113	I
Belamcanda chinensis	Shegan	127	I
Bupleurum chinense	Chaihu	1	I
Bupleurum scorzonerifolium		1	I
Camellia sinensis	Cha-yeh	951	II
Carthamus tinctorius	Honghua	475	II
Cassia obtusifolia	Juemingzi	935	II
Cassia tora		935	II
Chaenomeles speciosa	Mugua	767	II
Cimicifuga dahurica	Shengma	559	II
Cimicifuga heracleifolia		559	II
Cimicifuga racemosa		559	II
Cimicufuga foetida		559	II
Cinnamomum cassia	Rougui	991	II

(continued)

Table 1.1 (continued)

Lat. name	Chin. name	Page	Volume
Cinnamomum ceylanicum		991	II
Citrus reticulata	Chenpi	647	II
Citrus reticulata viride	Qingpi	647	II
Clematis chinensis	Weilingxian	355	I
Clematis hexapetala		355	I
Clematis manschurica		355	I
Cnidium monnieri	Shechuangzi	499	II
Codonopsis modesta	Dangshen	233	I
Codonopsis pilosula		233	I
Codonopsis tangshen		233	I
Coptis chinensis	Huanglian	301	I
Coptis deltoides		301	I
Coptis teeta		301	I
Corydalis yanhusuo	Yanhusuo	665	II
Curcuma kwangsiensis	Ezhu/Yujin	601	II
Curcuma longa	Jianghuang/Yujin	601	II
Curcuma phaeocaulis	Ezhu/Yujin	601	II
Curcuma wenyujin		601	II
Curcuma xanthorrhiza	JAPAN	601	II
Dioscorea futschauensis	Mianbixie	615	II
Dioscorea hypoglauca	Fenbixie	615	II
Dioscorea nipponica	Chuanshanlong	615	II
Dioscorea opposita	Shanyao	615	II
Dioscorea septemloba	Mianbixie	615	II
Dipsacus asperoides	Xuduan	677	II
Drynaria fortunei	Gusuibu	211	I
Eclipta prostrata	Mohanlian	263	I
Epimedium acuminatum	Yinyanghuo	485	II
Epimedium brevicornum		485	II
Epimedium koreanum		485	II
Epimedium pubescens		485	II
Epimedium sagittatum		485	II
Epimedium wushanense		485	II
Eucommia ulmoides	Duzhong	831	II
Evodia bodineiri	Wuzhuyu	391	I
Evodia officinalis		391	I
Evodia rutaecarpa		391	I
Forsythia suspensa	Lianqiao	381	I
Frittilaria cirrhosa	Chuanbeimu	13	I
Frittilaria delavayi	Chuanbeimu	13	I
Frittilaria hupehensis	Hubeibeimu	13	I
Frittilaria pallidiflora	Yibeimu	13	I
Frittilaria przewalskii	Chuanbeimu	13	I
Frittilaria thunbergii	Zhebeimu	13	I
Frittilaria unibraceteata	Chuanbeimu	13	I
Frittilaria ussuriensis	Pingbeimu	13	I

(continued)

Table 1.1 (continued)

Lat. name	Chin. name	Page	Volume
Frittilaria verticillata	Zhebeimu	13	I
Frittilaria walujewii	Yibeimu	13	I
Ganoderma lucidum	Lingzhi	633	II
Ganoderma sinensis		633	II
Gardenia jasminoides	Zhizi	245	I
Gastrodia elata	Tianma	255	I
Houttuynia cordata	Yuxingxao	59	I
Isatis indigotica	Banlangen	791	II
Leonurus japonicus	Yimucao	707	II
Ligusticum chuanxiong	Chuanxiong	181	I
Lonicera confusa	Shanyinhua	587	II
Lonicera hypoglauca		587	II
Lonicera japonica	Jinyinhua/Rendongteng	587	II
Lonicera marcanthoides	Shanyinhua	587	II
Lycium barbarum	Digupi/Gouqizi	509/521	II
Lycium chinense		509/521	II
Lycopus lucidus	Zelan	141	I
Magnolia biloba	Houpo	203	I
Magnolia biondii	Xinyi	719	II
Magnolia denudata		719	II
Magnolia liliiflora		719	II
Magnolia officinalis	Houpo	203	I
Magnolia sprengeri	Xinyi	719	II
Morus alba	Sangbaipi/Sangye	535/549	II
Notopterygium forbesii	Qianghuo	151	I
Notopterygium incisum		151	I
Ophiopogon japonicus	Maidong	819	II
Paeonia lactiflora	Baishoa	281	I
Paeonia veitchii	Baishoa/Chishao	281	I
Panax ginseng	Renshen	875	II
Panax notoginseng	Sanqi	843	II
Panax quinquefolium	Xiyangshen	875	II
Phellodendron amurense	Guanhuangbo	573	II
Phellodendron chinensis	Huangbo	573	II
Pinellia ternata	Banxia	71	I
Piper longum	Bibo	729	II
Polygonum multiflorum	Heshouwu	439	I
Poria cocos	Fuling	923	II
Pueraria lobata	Gegen	221	I
Rehmannia glutinosa	Dihuang	23	I
Rehmannia hueichingensis		23	I
Rehmannia lutea		23	I
Rehmannia purpurea		23	I
Rheum officinale	Dahuang	857	II
Rheum palmatum		857	II
Rheum tanguticum		857	II

(continued)

Table 1.1 (continued)

Lat. name	Chin. name	Page	Volume
Salvia miltiorrhiza	Danshen	903	II
Schisandra chinensis	Wuweizi	45	I
Scrophularia ningpoensis	Xuanshen	427	I
Scutellaria baicalensis	Huangqin	755	II
Siegesbeckia glabrescens	Xixiancao	893	II
Siegesbeckia orientalis		893	II
Siegesbeckia pubescens		893	II
Sinomenium acutum	Qingfengteng	369	I
Sinomenium cinereum		369	I
Sophora flavescens	Kushen	743	II
Sophora japonica	Huaihua/Huaimi	291	I
Stephania tetrandra	(Han)Fangji	311	I
Tribulus terrestris	Jili (Baijili)	805	II
Uncaria hirsuta	Gouteng	343	I
Uncaria macrophylla		343	I
Uncaria rhynchophylla		343	I
Uncaria sessilifructus		343	I
Uncaria sinensis		343	I
Uncaria tomentosa		343	I
Zanthoxylum armatum	Huajiao	191	I
Zanthoxylum bungeanum		191	I
Zanthoxylum piperitum		191	I
Zanthoxylum schinifolium		191	I
Ziziphus jujuba	Suanzaoren	325	I

1.3 Facts and Perspectives

1.3.1 Botanical Diversity

Since the introduction of the binominal nomenclature of plants by C. von Linne` (1753), all plants of the world are designated with a Latin genus- and species name e.g. *Chamomilla* (genus) and *recutita* or inodora (species) which are used for description and references in herbaria. Modern analytical and molecular methods have revealed that we must also differentiate between so-called subspecies and chemical races which, based on genetic or morphological and microscopic characteristics, can lead to a different qualitative or quantitative chemical composition of constituents. This great botanical diversity is unquestionably present in Chinese plants as well and of crucial importance for the identity (authenticity) proof of all herbal drugs.

Therefore, we must conclude that assessing the botanical identity proof of Chinese herbal drugs as we do for all western drugs is a basic requirement and takes first priority before a plant can be officially integrated into an official Pharmacopoeia.

Hence, those herbal drugs which have not yet been subjected to identification cannot be admitted for evidence and rationally based therapies. In many monographs of the 2010 Chinese Pharmacopoeia, more than two species (sometimes up to nine species) are listed and often labelled as synonyms or subvarieties.

For example, the monograph for *Fritillariae bulbus* lists 11 species, along with five species in monographs of *Epimedii herba*, while one monograph for *Uncariae ramulus* c. *uncis* includes five species, without any evidence that their chemical compositions are qualitatively equivalent such that they can be substituted for one another. In the new manual (Wagner et al. 2011), the TLC and HPLC fingerprints provided show clearly that often several species from one genus exhibit quite deviating patterns of chemical constituents. Correspondingly, it must be suggested that not all species, subspecies or sub-varieties possess equivalent pharmacological activities and therapeutic efficacies. In this context, a series of very comprehensive HPLC fingerprint analytical studies were submitted for five *Epimedium* species of different origin (Li et al. 2008; Guo et al. 2008; Huang et al. 2003; Wang et al. 2006; Xie et al. 2010). For their demonstrations, researchers used the HPLC fingerprinting analysis or the so-called chemometric score projection plot method. For example, they could divide 46 samples of *Epimedium* spp. into four main types and nine subtypes, and a deviating composition of the characteristic isoprenylated flavonoids was recently described by Xu et al. (2010) for 12 authentic samples and 26 commercial samples of Fructus Aurantii immaturi (FAI) from different Chinese sources. The corresponding HPLC fingerprints and score projections plot of the principle components analysis for the 38 samples of this herbal drug based on their chromatographic fingerprints are described in full details by Liang et al. (2010). They confirm the aforementioned inconsistencies among the chemical compositions of constituents in the various species within one monograph.

In this context, it may be of interest to show the results of our TLC and HPLC fingerprint analyses of the three official *Curcuma* spp. listed in three individual monographs in the 2010 Chinese Pharmacopoeia, namely, Rhizoma Curcumae longae (Jianghuang), Rhizoma Curcumae (Ezhu) and Radix Curcumae (Yujin). The first rhizome should originate from the species *Curcuma longa* (syn = *C. domestica*). The second rhizome can stem from the three species *C. phaeocaulis*, *C. kwangsiensis* or *C. wenyujin*. For the third, Radix Curcumae, all four of these named species are described. A further species, the Javanese turmeric *Rhizoma xanthorrhizae* Roxb., is the official turmeric rhizome of some European Pharmacopoeias (Wagner et al. 2011) (see Fig. 1.1).

As shown in Fig. 1.1, all samples of Rhizoma Curcumae longae (1–6) show, in UV 365 nm, the prominent yellow-green fluorescent zones of curcumin (T) at R*f* 0.67, directly below demethoxycurcumin at R*f* 0.58 and bisdemothoxycurcumin at R*f* 0.39. In contrast, in all extract samples of Radix Cucumae wenyujin (7–9) only a weak blue fluorescent zone (dihydrocurcumin?) at R*f* 0.6 can be detected. The botanical origins of the root samples 10 and 11 were not determined, but it can be suggested that they also originate from *Curcuma wenyujin*. Obviously, the roots of this species are devoid of any of the characteristic curcuminoids for Curcuma roots. In the extract sample of Rhizoma Curcumae Kwangsiensis (12), curcuminoids are also absent. The Rhizoma

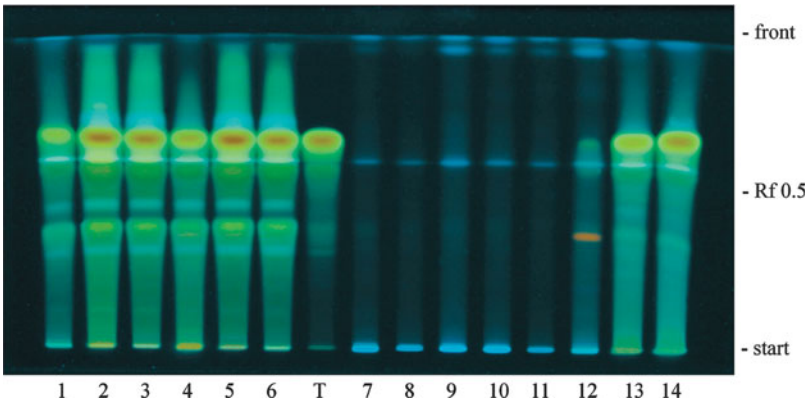


Fig. 1.1 TLC fingerprint analysis of MeOH extracts from roots/rhizomes of various *Curcuma* species in UV 366 nm (Wagner et al. 2011, Vol. 2, p. 609, Springer Wien, New York). 1–6 Rhizoma Curcumae longae extract samples, 7–9 Radix Curcumae wenyujin extract samples, 10/11 *Curcuma* samples not determined, 12 Rhizoma Curcumae Kwangsiensis and 13/14 Rhizoma Curcumae xanthorrhizae

Curcumae xanthorrhizae extract samples (13, 14) contain a high amount of Curcumin (T, R*f* 0.67) and a small amount of demethoxycurcumin. Bisdemethoxycurcumin (R*f* 0.39) characteristic for all Rhizoma Curcumae longae samples (1–6), could not be detected (Wagner et al. 2011).

In this context, it is of interest that the terpenoid composition of the essential oil of Curcuma wenyujin (7–9) also differs distinctly from that of the Rhizoma Curcumae longae (1–6), (Wagner et al. 2011). The therapeutic effects and indications of the various Curcuma rhizoma roots described for TCM are not congruent with the distinct deviations of their chemical compositions.

1.3.2 Uncertain Botanical Nomenclature/Falsifications or Adulterations

The non-uniform nomenclature for the same plant in various regions of China and elsewhere in East Asia can further complicate the correct taxonomic determination. For example, according to Hempen and Fischer 2009, "Bianxu" used in the mainland of China is supposed to be the herb from *Polygonum aviculare* (family = Polygonaceae). In Taiwan, one can obtain the herb labelled under the same Chinese name as *Euphorbia thymifolia* (family Euphorbiaceae) and it is further reported that this herb was imported from a herbal drug company in Hong Kong labelled as *Belamcanda chinensis* herb (family Iridaceae) (Hsu 1987; Bauer and Franz 2010). Another example is the Chinese drug *Zicao*, which can be derived from the root of *Arnebia euchroma* (Ruan Zicao), *Lithospermum erythrorrhizon* (Zicao) or *Onosma paniculatum* (Dian Zicao). Which one is used in practice is unknown or uncertain (Hu et al. 2006).

This uncertainty can cause impermissible substitutions, falsifications or adulterations, as occurred 15 years ago when the root of *Stephania tetrandra* (Hanfangji) was mistaken for the root of *Aristolochia fangji* (Guanfangji) and administered to women as tea medication that produced severe nephrotoxic side effects (Reginster et al. 1997; Nortier and Vanherweghem 2002). The *Aristolochia* herbal drug contains the carcinogenic aristolochic acid. After detection of this falsification, the drug was banned from the Chinese Pharmacopoeia in 2002. Meanwhile, special TLC and HPLC fingerprint methods were developed which allow the detection of even micrograms of these acids in an herbal drug or drug mixtures (see Monographs of Radix Stephaniae Vol. I, p. 311; Radix Clematidis Vol. I, p. 355; Caulis Sinomenii Vol. I, p. 369, in Wagner et al. 2011).

The damage to the reputation of TCM drugs by this intoxication event immediately initiated a search for further plant species within the *Aristolochia* family which are supposed to contain also the cancerogenic aristolochic acid. Wu et al. (2007) have summarised all the results in an comprehensive article, concluding that "three categories of nomenclature could be identified (1) one-to-one (one plant part from one species): the herb guan mutong refers to the root of *Aristolochia manshuriensis*; (2) multiple-to-one (multiple plant parts from the same species serve as different *Aristolochia debilis*) and (3) one-to-multiple (one herb refers to multiple species): the herb fangji refers to the root of either *Aristolochia fangchi*, *Stephania tetrandra* or *Cocculus trilobus*. In this case, the first belongs to a different family (Aristolochiaceae) as the latter two (Menispermaceae), and only the first contains aristolochic acid (AA), as demonstrated by independent analytical data provided in this article".

The confusion is not herewith totally excluded because in TCM herbal medicine, mutong (*Akebia quinata*) is also allowed to be substituted with either guan mutong (*Aristolochia manshuriensis*) or eluan mutong (*Clematis armandii*) and mufangji (*Cocculus trilobus*) by guang fangchi (*Aristolochia fangchi*) or hanzhong fangji (*Aristolochia heterophylla*). Therefore the authors "advocate the importance" of using "pharmaceutical names which define the species name, the plant part and sometimes the special process performed on the herb, including cultivation conditions".

Therefore, TLC and HPLC fingerprint analysis according to the method described in the new monographs (Wagner et al. 2011) is imperative for safety and the benefit of patients.

In the article cited above, further examples are given for the three categories. Of special interest is category 3, in which multiple species are assigned to one Chinese herbal name in Pinyin and in Chinese characters (Table 1.2).

Through development of the new monographs, another example of special biodiversity was detected in the Apiaceae family (Zschocke et al. 1998). Comparative TLC and HPLC/MS investigations of the roots of *Angelica sinensis* and multiple related species [*Angelica dahurica*, *Angelica pubescens*, *Ligusticum Chuanxiong*, *Levisticum officinale* and the fruits of *Cnidium monniera*] all belonging to the Apiaceae family, including *Angelica acutiloba* which in Japan is used as a substitute for *A. sinensis*, showed great similarity in their main constituents. All these herbal drugs, with the

Table 1.2 Multiple-to-one: multiple plant parts from the same species serve as different herbs (Wu et al. 2007)

Chinese herbal name in Pinyin	Herbal name in Chinese character	Plant part	Species name (Family name)	Species name in Pinyin and in Chinese character
Gualou ('heat-clearing, phlegm-resolving')	栝楼	Fruit	*Trichosanthes kirilowii* Maxim. (Cucurbitaceae)	Gualou 栝楼
Tianhuafen ('heat-clearing, thirst-quenching')	天花粉	Root		
Gualouzi ('lung-clearing, Chi-regulating')	栝楼子	Seed		
Gualoupi ('cough-suppressing')	栝楼皮	Pericarpium		

exception of *Cnidium* spp., contained, in addition to essential oils, alkylated furano- or benzocoumarins, coniferylferulate and polyacetylenes. The latter compounds are suggested to be produced primarily from symbiosis with the plant's living phytofungi (see below). Because the microscopic and macroscopic features of these herbs are also very similar, a distinct discrimination from the other species mentioned above may be impossible even for experienced botanists or pharmacognosists. A clear solution to this great handicap in botanical authentication can likely only be expected from a thorough barcode DNA-analytical screening (see Chap. 2 in this book).

In the course of our work on the development of the new manual, we faced a very similar problem when we investigated the chemical composition of the Chinese rhizome of *Acorus tatarinowii*, formerly known as *Acorus gramineus*. In the essential oil of this species, we detected an uncommonly high amount (up to 80 %) of the cancerogenic β-Asarone (Wagner et al. 2011), Vol. II, p. 777. The other species, *Acorus calamus* var. *americanus*, distributed in North America, contains in 2–6 % essential oil 0–0.5 % β-Asarone, whereas the *Acorus calamus* usually marked in Europe contained around 3 %–13 % β-Asarone in 2–6 % oil.

Interestingly, there seems to be a conspicuous correlation of the β-Asarone content with (Table 1.3) the chromosome count. *A. calamus* is classified as diploid variety ($2n = 2x = 24$) distributed mainly in North America and triploid variety ($2n = 3x = 36$) in some European countries. The tetraploid varieties ($2n = 4x = 48$) were found preferred in East Asia, India and in Japan and there is even a hexaploid variety ($2n = 6x = 72$) in the Kashmir area. So the species are defined primarily on the basis of genomic differences. In the family Araceae as a whole, the basic chromosome number ranges from 8 to 22. The different species appear to

Table 1.3 Content of essential oil and β-Asarone of various *Acorus* species (Wagner et al. 2011, Vol. 2, p. 779, Springer Wien, New York)

Acorus calamus L.	Origin	*n*	Essential oil (%)	β-Asarone content[a] (%)
var. *americanus* WULFF	USA	Diploid	2–6	0–0.5
var. *calamus* L.	Europe	Triploid	2–6	3–13
var. *angustata* ENGLER	East Asia	Tetraploid	Up to 7	Up to 80

[a]Wagner and Bauer (1999)

follow a geographical pattern of distribution with respect to ploidy level (Ogra et al. 2009; Zhang 2005). If the tetraploid chromosomal feature can be generally confirmed for all *Calamus* species labelled as *Calamus tatarinowii*, it will be imperative to remove this species from the Chinese Pharmacopoeia and, correspondingly, to ban it from the Chinese market and from any export to other countries.

1.4 Processing of TCM Herbal Drugs

Apart from the simple cutting and cleaning of the raw drugs described in the Chinese Pharmacopoeia, many other types of pre-treatment or processing unknown to western Pharmacopoeias are used. In the 2005 Chinese Pharmacopoeia (People's Republic of China, English Edition, Vol. I, Appendix II A-25), the processing is to be defined "to fulfil the requirements of drugs", whatever that may mean for each single drug. In one recent publication (Zhao et al. 2010), the expressed purpose of processing is "to alter the appearance, the physical characteristic and chemical constituents of a herbal drug". In none of the monographs except those for crude drugs containing toxic constituents is the necessity of the various processing techniques rationalised or clearly substantiated. According to the Chinese Pharmacopoeia, processing can be achieved primarily through the following methods: roasting and boiling, scalding, calcining, carbonising, steaming, boiling, processing with wine, vinegar or salt water and different kinds of stir baking. In Monograph Vol. II, No. 79, p. 977 and Vol. I, No. 9, p. 71 we have chromatographically investigated two processed TCM drugs and compared the TLC and HPLC fingerprints with those of the unprocessed raw drugs.

The first example is the herbal drug Radix Aconiti kusnezoffii praeparata, the root of the Aconit species *Aconitum kusnezoffii* which, in TCM, is usually used only in this processed form. The plant aconit is also known in western countries but is not used because it contains very toxic *Aconitum* diterpen alkaloids. In western countries, in homoeopathy, the *Aconitum* extracts are used in very diluted water–alcoholic Aconit tinctures (e.g. D4-10) for the treatment of rheumatic arthralgia and bad colds. The processed roots of *A. kusnezoffii* obtained from our Chinese supplier were pre-treated with boiling water. As listed in Table 1.4, the LD 50 values (mg/kg) of Aconitine and Mesacontine were the most toxic among the roots, recorded at 1.00–1.80 and 1.90 mg/kg, respectively, whereas the other concomitant alkaloids possessed a much lower toxicity.

Table 1.4 Content (μg/g) of Aconitine, Mesaconitine, and Hypaconitine in raw and processed Radix Aconiti kusnezoffii[a]

	Aconitum kusnezoffii, raw		*Aconitum kusnezoffii*, processed	
	Min	Max	Min	Max
Aconitine	19.78	1,580.75	8.81	34.54
Mesaconitine	19.23	1,216.57	10.24	144.18
Hypaconitine	122.71	640.41	7.87	142.16

[a]Qiao et al. (2009)
Wagner et al. 2011, Vol. 2, p. 981, Springer Wien, New York

As shown through comparison of the HPLC graphs of the non-processed and processed roots (Fig. 1.2a, b), the alkaloids concentration of Aconitine (Peak 7) and Mesaconitine (Peak 6) were strongly degraded through the processing procedure used. This was the aim of this processing procedure.

This example shows that the HPLC fingerprint analytical technique is the most reliable and ideal candidate to replace the methods of the Chinese Pharmacopoeia with a modern technique that has the possibility to quantify the corresponding alkaloids according to the safe limits stipulated by toxologists and first proposed by Singhuber et al. (2009).

The second herbal drug example follows a very similar purpose. Rhizoma Pinelliae, the dried tuber of *Pinellia ternata* (Wagner et al. 2011, Vol. I, p. 71), is used internally in TCM as an anti-asthmatic, antiemetic expectorant and cytostatic drug. The raw drug is used for external treatment only, but must be pre-treated ("processed") for internal use. The LD50 value of unprocessed Rhiz. Pinelliae ternatae injected i.p. into mice is approx. 13 g/kg. The raw drug contains the protein "pinellin" which possesses abortive properties (Kano et al. 1987). It is destroyed by pre-treatment with alum solution, ginger, lime water or long decoction. The phenolics, e.g. homogentisic acid, its glucoside (Hasegawa 1958) and 3,4-dihydroxybenzaldehyde (Suzuki 1969), possess skin- and mucous membrane-irritating properties and can be destroyed by cooking and drying (Hegnauer 1986). The HPLC chromatographic analysis of unprocessed and processed Pinelliae rhizoma extracts shows clearly that the main components are, to a great extent, still detectable in the raw rhizome, whereas they were degraded to a great extent in the processed rhizome (Fig. 1.3) (Wagner et al. 2011).

1.5 Endo(Phyto) Fungi in Chinese Medicinal Plants

1.5.1 Occurrence/Examples

During the development of the new monographs, we discovered a conspicuous occurrence of lipophilic acetylene compounds of the falcarin(di)ol type among the most prominently used Chinese root drugs (Fig. 1.4). They could be detected during our HPLC fingerprint analysis using Diode ARRAYdetector 4500 A, which permits the online registration of the UV spectrum of each single HPLC peak. The acetylene

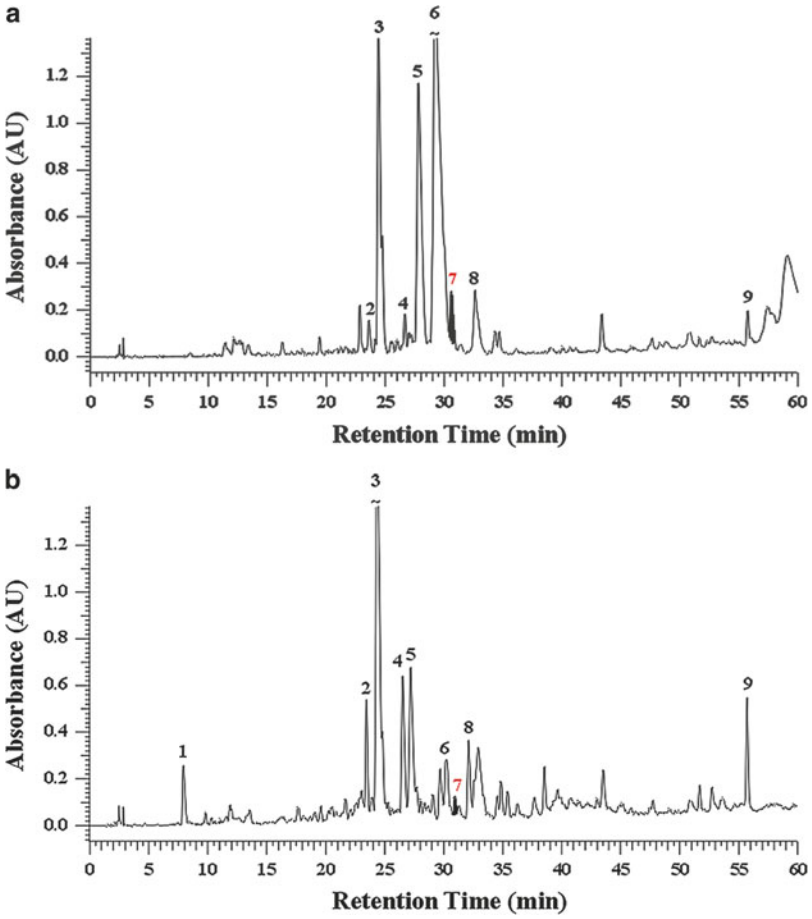


Fig. 1.2 HPLC fingerprint analysis of the ether extract of raw (**a**) and processed (**b**) Radix Aconiti kusnezoffii (6 = Mesaconitine, 7 = Aconitine) (Wagner et al. 2011, Vol. 2, p. 987, Springer Wien, New York)

compounds, when present in even very low concentrations, showed a characteristic triple band accumulation in the Rt range of 25–30 min.

The first acetylene compounds of the falcarin(di)ol-type were described for Ginseng root/rhizome drugs from the plants *Panax notoginseng*, *Panax Ginseng* and *Panax quinquefolium*.

In Table 1.5, nine further occurrences of falcarin(di)ols acetylene compounds are listed. With the one exception of Fructus Foeniculi, all others were detected exclusively in the roots or rhizomes of these plants.

Initially, we considered these constituents to be biosynthesized from the plants. In the meantime, however, several publications appeared in which the original source of these compounds was identified as endo(phyto) fungi which are living symbiotically in different parts of the Chinese plants (Strobel and Daisy 2003; Li et al. 2007).

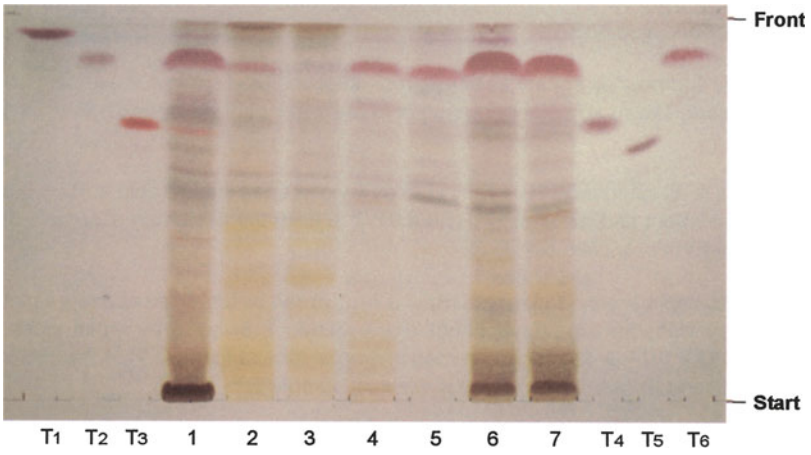


Fig. 1.3 TLC fingerprint analysis of MeOH extracts of raw and processed Rhizoma Pinelliae ternatae (Wagner et al. 2011, Vol. 1, p. 76, Springer Wien, New York). 1 authentic Pinelliae rhizome sample (Korea), 2 commercial Pinelliae rhizome sample (China), 3 commercial Pinelliae rhizome pre-treated with alum, 4 commercial Pinelliae rhizome pre-treated with ginger, 5 commercial Pinelliae rhizome pre-treated with licorice and lime, 6 Arisaematis rhizoma sample (China), 7 *Arisaematis amurensis* rhizome (Korea)

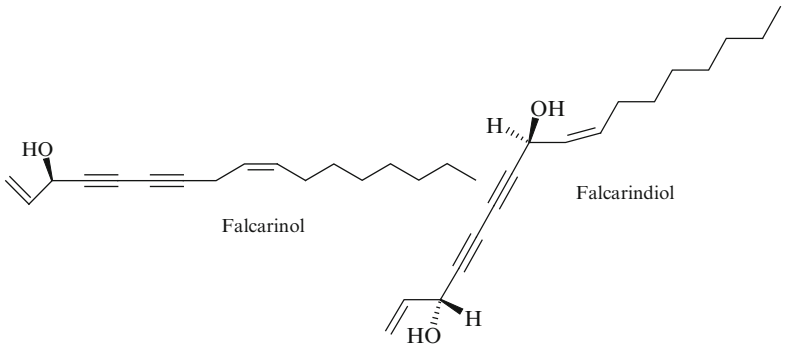


Fig. 1.4 Acetylene compounds

The most famous example of the production (biosynthesis) of a long-known terpene alkaloid by an endo(phyto) fungus is the *Taxus brevifolia* tree, the bark of which contains the symbiotic living fungus *Taxomyces andreanae*. This fungus is able to biosynthesize the same terpene alkaloid, paclitaxel, as the Taxus tree (Stierle et al. 1993). Which organism, the fungus or the plant, first produced paclitaxel and was the gene supplier for the other organism is not known. What do we know about the taxonomic identification of the endo(phyto) fungi living in Chinese plants?

Among the plants listed in Table 1.5, the falcarindiol-producing fungus associated with *Panax Ginseng* was identified as a *Paecilomyces* spp. (Xu et al. 2009). The ether extract (oil) of the cultured fungus produced 38 compounds,

Table 1.5 TCM—drugs containing falcarinol and falcarindiol

Species	Drug	Chinese name	Wagner et al. (2011)	Literature
Angelica dahurica	Radix Angelicae Dahuricae	Baizhi	Vol. I, p. 171	Wang et al. (2010), Choi et al. (2005), Lechner et al. (2004)
Angelica pubescens	Radix Angelicae Pubescentis	Duhuo	Vol. I, p. 99	Liu et al. (1998)
Angelica sinensis	Radix Angelicae Sinensis	Danggui	Vol. I, p. 161	Deng et al. (2008, 2006), Chen et al. (2007), Wang et al. (2005)
Daucus carota	Fructus Carotae	Nanheshi	(ChAB 2005)	Roman et al. (2011), Rai et al. (2011), Rawson et al. (2011), Kjellenberg et al. (2010), Søltoft et al. (2010), Schmiech et al. (2009, 2008), Purup et al. (2009), Metzger et al. (2008), Christensen et al. (2007), Baranska et al. (2005), Zidorn et al. (2005), Czepa et al. (2004, 2003)
Foeniculum vulgare	Fructus Foeniculi	Xiaohuixiang	(ChAB 2005)	Zidorn et al. (2005)
Glehnia littoralis	Radix Glehniae	Beishashen	(ChAB 2005)	Satoh et al. (1996)
Ligusticum chuanxiong	Rhizoma Chuanxiong	Chuanxiong	Vol. I, p. 181	Chang et al. (2007)
Notopterygium forbesii/incisum	Rhizoma et Radix Notopterygii	Qianghuo	Vol. I, p. 151	Kou et al. (2010), Ohnuma et al. (2009), Ma et al. (2008), Zschocke et al. (1997)
Panax ginseng	Radix et Rhizoma Ginseng	Renshen	Vol. II, p. 875	Washida and Kitanaka (2003), Liu et al. (2007), Xu et al. (2009)
Panax notoginseng	Radix et Rhizoma Notoginseng	Sanqi	Vol. II, p. 843	Rao et al. (1997)
Panax quinquefolium	Radix Panacis Quinquefolii	Xiyangshen	Vol. II, p. 875	Wang et al. (2000)
Saposhnikovia divaricata	Radix Saposhnikoviae	Fangfeng	(ChAB 2005)	Wang et al. (2000)

including falcarinol as one active antitumoral and antifungal compound. A comparative analysis of the ether extract of the host plant *Panax Ginseng* showed the presence of 51 compounds that also included, apart from falcarindiol, falcarinol, called panaxynol. Falcarinol was found to exhibit a hepatoprotective effect (Hisashi et al. 1998) and showed selective in vitro cytotoxicity against L-1210, MK-1, B-16 and L-929 cancer cell lines as compared to normal cell cultures (Santos et al. 2005). In this study, Xu et al. (2009) observed that falcarinol was present in the ether extract of ginseng in a concentration of 2.87 %, whereas in the ether extract of *Paecilomyces* spp., only 1.38 % falcarinol could be determined. This could provide some evidence that the *Paecilomyces* spp. produces similar or the same metabolites as the host Ginseng. It must also, however, be considered that the other main compounds detected in the phytofungus besides falcarinol were an Indane-1,3-dione, -derivative (40.01 %), Androst-2-en-4-one, 17-(tetra-hydropyran-3-yl) oxy-quinoline (7.76 %), -Xanthatin (4.41 %) and Isotanshinone II (3.03 %), which could not be found in the Ginseng ether extract. In contrast, the Ginseng ether extract consisted mainly of a Decalin-derivative (27.26 %), Retinol (12.68 %), ± *trans* Neridol (3.96 %), falcarinol (2.87 %), β-Panasinsene (1 %) and other compounds.

As described in the next paragraph, multiple quite structurally different antitumoral and antifungal compounds have been isolated from phytofungi of other TCM-herbal drugs over the past decade or so.

1.5.1.1 Curcuma wenyujin

The endophytic fungus strain L 18 isolated from the leaves was identified as *Chaetomium globosum* Kunze, based on morphological characteristics and sequence data for the international transcribed spacer (ITS-5,8 S-ITS2) of the nuclear ribosomal DNA F (Wang et al. 2012). Ongoing research led to the isolation of chaetoglobosin, together with the three known compounds, Ergosterol, Ergosterol 5α, 8-Peroside and 2-Methyl-3-hydroxy-indole. Chaetoglobosin A was identified as 18-Dimethyl-(7s13e, 16s, 17e, 19r, 21c)-ol-3-yl)-1; (13)cytochalasa-13, 17, 21-triene-6,7 epoxy-19-hydroxy-10-(1h-indolin) with the molecular weight of 528.64 and the sum formula $C_{12}H_{36}N_2O_5$ (Fig. 1.5).

Chaetoglobosin possesses strong fungistatic activities on plant pathogenic fungi and inhibits the growth of mouse forestomach carcinoma cell (MFC) and mouse hepatocellular carcinoma cell (H22).

1.5.1.2 Rehmannia glutinosa

In investigations of the endophytes of the wild plant *Rehmannia glutinosa*, the fungal secondary metabolites *Massarigenin D*, *Spiromassaritone* and *Paecilospirone* (Fig. 1.5) were isolated from a strain identified as *Massarina tunicate* (Oh et al. 2003; Hirota et al. 1991). These rare spiro-5,6-lactones showed remarkable antifungal

activities, comparable with those of griseofulvin and ketoconazole, and also a remarkable cytotoxic activity as investigated by the MTT assay (Sun et al. 2011). Spiromassaritone displayed the strongest cytotoxicity, with an IC50 value of 5.6 μg/ml against HepG-2 and of 6.8 μg/ml against breast carcinoma cell MCF-7).

Seven TCM plants which live in symbiosis with endophytic fungi also deserve mention: *Curcuma wenyujiin*, *Salvia miltiorrhiza* and *Hypercia serrata*, *Cordyceps sinensis*, *Artemisia annua* and *Camptotheca acuminata*.

1.5.1.3 Salvia miltiorrhiza

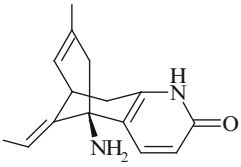
The isolation of the endophytic fungus *Trichoderma atroviride* from the root of *S. miltiorrhiza* led to the detection that this fungus produces the same tanshinone I and tanshinone II A (Fig. 1.5) as characteristic compounds of this plant (Ming et al. 2011). Since the concentration of these diterpene quinones measured in the mycological medium were, surprisingly, very high, the possibility of an industrial production of these very valuable compounds for the prevention and therapy of cardiovascular and ischemic stroke seems to be very promising.

1.5.1.4 Huperzia serrata

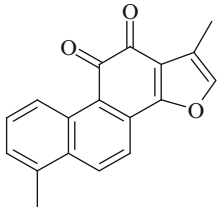
Huperzin A (HupA, Fig. 1.5) a Lycopodium alkaloid with cholinesterase inhibitory (AChEI) activity was isolated from the whole plant of *Huperzia serrata* (Quian Ceng Ta) Thunb. ex Murray Trev., in the 1980s (Zhu et al. 2010a, b). It possesses better anti-AChE activity, higher oral bioavailability and fewer toxic effects than the AChE inhibitors tacrine, donepezil, rivastigmine and galantamine that are used currently for the treatment of Alzheimer's disease (AD).

Unfortunately, the amount of huperzin available per isolation was very low (c. 0.007 %) and all efforts to increase the alkaloid content by special cultivation methods using other *Lycopodium* species failed. The total synthesis of HupA has also been reported, but no industrially feasibly method has yet been developed. An attempt to produce the alkaloid in higher amounts using in vitro propagated cell issues was also not successful. Meanwhile, however, a promising solution of this problem seems to be forthcoming. In 2010, Zhu et al. (2010a) were able to identify five isolates of the genus *Shiraia* from among 69 isolated phytofungi strains of a natural population of *H. serrata*, using DNA sequence data, which produced Hup. A. One of them, the strain S2f14, produced about 327.8 μg/2^{-1} Hup A.

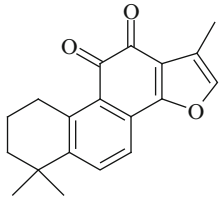
In our efforts to develop a new monograph for the herb *Lycopodium japonicum*, we failed to detect HupA in five herb samples obtained from China and from some German firms. The reason for this failure could be the very low concentration of HupA produced by the plant (0.007 %, see above) or the possibility that HupA is produced from only selected living phytofungi, among which the Shiraia strain SZf14 is the main alkaloid-producing fungus.



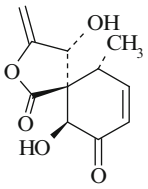
Huperzin A
Zhu et al. (2010)



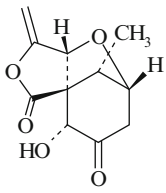
Tanshinone I



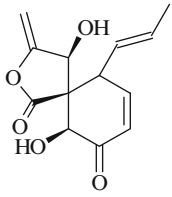
Tanshinone IIA
Ming et al. (2011)



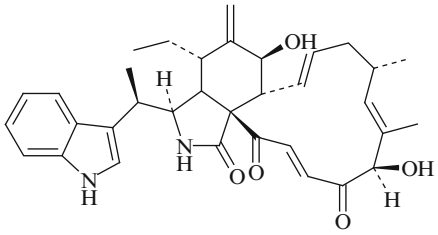
Massarigenin D



Spiromassaritone
Sun et al. (2011)



Paecilospirone



Chaetoglobosin A
Wicklow et al. (2011)

Fig. 1.5 Metabolites produced by symbiotic living endo(phyto) fungi in the Chinese plants *Curcuma wenyujin*, *Rehmanniae glutinosa*, *Salvia miltiorrhiza* and *Huperzia serrata*

1.5.1.5 *Cordyceps sinensis* (*Dongchong xiacao*)

Although derived from a fungus family, which parasitised on the larva of some insects (family Hepialidae) and the dead worm caterpillars, we include the fungus here because it is one of the most valued traditional "Chinese herbs" described in a monograph of the Chinese Pharmacopoeias. Mycologists have isolated more than 10 fungal species from *C. sinensis*. The most important species were identified as *Paecilomyces hepiali* and *Hirsutella sinensis* (Zhu et al. 2010b; Chen et al. 2006; Wei et al. 2006).

The fungus *Cordyceps sinensis* was recently re-named *Ophiocordyceps sinensis* (Berkely) (Sung et al. 2007).

According to the monograph in the 2010 Chinese Pharmacopoeia, the fungus also known as "winter arm" or "summer grass", grows in a highly specific environment in the high altitude areas of the Qinghai-Tibetan Plateau. The drug is collected in early summer after the stromata have come up out of the ground but before the spores have ejected, partly dried in the sun, removed from the attached fibrous matter and other impurities and dried in the sun at low temperature (Chinese Pharmacopoeia 2005/2010).

In the Chinese Pharmacopoeia, the action of the fungus is described as "tonifying the lung and the kidney, arresting bleeding and dissolving phlegm". For medicinal use in TCM, the following indications are listed: chronic cough, asthma, hemoptysis in phthisis, impotence and seminal emission with aching of loins and knees. The physiological and pharmacological effects are characterised as immune-stimulating, lipid-decreasing, bronchial-dilatative antibiotic and applicable as adjuvant for the treatment of tuberculosis and pneumonia (Hempen and Fischer 2009). To date, only the endogenous purin nucleotide Adenosine and some polysaccharides could be isolated. From a scientific point of view, none of the enumerated pharmacological effects or applications to humans or patients have been substantiated by evidence.

1.5.1.6 Artemisia annua

An endophytic fungus *Colletotrichum* sp. with strong antimicrobial activity against the bacteria *B. subtilis*, *S. aureus*, *S. lutea* and *Pseudomonas* sp. was isolated from *Artemisia annua*. The new metabolites, namely 6-isoprenylindolo-3-carboxylic acids and several ergostadiene derivatives, exhibited antimicrobial activities with MIC values ranging from 25 to 75 μg/ml (Lu et al. 2000).

1.5.1.7 Camptotheca acuminate

Camptothecine and 10-hydroxycamptothecine, two precursors for the synthesis of the clinically useful Topotecan and Irinotecan, were isolated from the wood of this tree. Meanwhile, 9-methoxycamptothecine and 10-hydroxycamptothecine could be obtained from strains of the endophytic fungus *Fusarium solani* (Kusari et al. 2009).

1.5.2 Perspectives

Each year, further occurrences of symbiotic living phytofungi in Chinese plants are reported. Over the last 10 years, studies have shown that phytofungi possess an

extraordinary biodiversity, estimated at around one million naturally occurring endophytic fungi (Petrini 1991). Huang et al. (2008) reported that, in Hong Kong, 1,160 endophytic fungal strains were isolated alone from 29 TCM plants alone. They could be classified into 31 fungal groups including a total of 73 distinct morphospecies. *Alternaria*, *Colletotrichum*, *Phomopsis* and *Xylariales* were the predominant fungal taxa in 29 host plants. In this context, the question arises of what implications this abundant occurrence of phytofungi and the compounds they produce may have for the quality proof of TCM drugs and for pharmacological and therapeutic utilisation in general. If they are biosynthesized only in traces or very small amounts, they cannot be used as marker compounds for quality or identity proofs. If such compounds emerge as absolutely new compounds which have never before been detected in wild collected plants or in those originated from cultivations, we cannot exclude the possibility that they also contribute to the overall pharmacological and therapeutic activity of one herbal drug preparation. This aspect deserves special attention. As described in the preceding chapter, it is noteworthy that many compounds produced from endophytic fungi possess remarkable antitumoral, antibiotic, antimycotic or immunosuppressive activities (Strobel and Daisy 2003; Mitchell et al. 2008). Today, the problem of producing significant amounts of these compounds from isolated strains can be regarded as solved. Many microbial fermentation processes and geneting engineering methods are available to produce valuable drugs on an industrial scale. In two comprehensive review articles written by Gunatilaka (2006) and Radić and Štrukelj (2012), the advantage of the biotransformation process in general and the role of endofungi in particular are explained for the production of anticancer, antimicrobial and antioxidant compounds.

Since China possesses one of the greatest pools of medicinal plants which correspondingly may contain an immense plethora of phyto(endo) fungi, we can envision a significant contribution towards combating world wide the currently increasing threat of cancer, multi-infectious syndromes and all kinds of degenerative diseases.

References

Baranska M, Schulz H (2005) Spatial tissue distribution of polyacetylenes in carrot root. Analyst 130:855–859

Baranska M, Schulz H, Baranski R, Nothnagel T, Christensen LP (2005) In situ simultaneous analysis of polyacetylenes, carotenoids and polysaccharides in carrot roots. J Agric Food Chem 53:6565–6571

Bauer R, Franz G (2010) Modern European monographs for quality control of Chinese herbs. Planta Med 76:2004–2011

Chang XL, Ma YB, Zhang XM, Jiang ZY, Chen JJ (2007) Studies on chemical constituents of rhizomes of *Ligusticum chuanxiong*. Zhongguo Zhong Yao Za Zhi 32:1533–1536

Chen J, Zhang W, Lu T, Li J, Zheng Y, Kong L (2006) Morphological and genetic characterization of a cultivated *Cordyceps sinensis* fungus and its polysaccharide component possessing antioxidant property in H-22 tumor bearing mice. Life Sci 78:2742–2748

Chinese Pharmacopoeia (2005/2010) Pharmacopoeia of the People's Republic of China, English edn. People's Medical Publishing House, Beijing, China

Choi SY, Ahn EM, Song MC, Kim DW, Kang JH, Kwon OS, Kang TC, Baek NI (2005) In vitro GABA-transaminase inhibitory compounds from the root of *Angelica dahurica*. Phytother Res 19:839–845

Christensen LP, Kreutzmann S (2007) Determination of polyacetylenes in carrot roots (*Daucus carota* L.) by high-performance liquid chromatography coupled with diode array detection. J Sep Sci 30:483–490

Czepa A, Hofmann T (2004) Quantitative studies and sensory analyses on the influence of cultivar, spatial tissue distribution, and industrial processing on the bitter off-taste of carrots (*Daucus carota* l.) and carrot products. J Agric Food Chem 52:4508–4514

Czepa A, Hofmann T (2003) Structural and sensory characterization of compounds contributing to the bitter off-taste of carrots (*Daucus carota* L.) and carrot puree. J Agric Food Chem 51:3865–3873

Deng S, Chen SN, Yao P, Nikolic D, van Breemen RB, Bolton JL, Fong HH, Farnsworth NR, Pauli GF (2006) Serotonergic activity-guided phytochemical investigation of the roots of *Angelica sinensis*. J Nat Prod 69:536–541

Deng S, Wang Y, Inui T, Chen SN, Farnsworth NR, Cho S, Franzblau SG, Pauli GF (2008) Anti-TB polyynes from the roots of *Angelica sinensis*. Phytother Res 22:878–882

Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69:509–526

Guo BL, Pei LK, Xiao PG (2008) Further research on taxonomic significance of flavonoids in *Epimedium* (Berberidaceae). J Syst Evol 46:874–885

Hasegawa C (1958) Jap J Biochem Soc 30(706)

Hegnauer R (1986) Chemotoxonomie der Pflanzen Bd. VII. Birkhäuser Verlag, Basel, pp 581–591

Hempen CH, Fischer T (2009) A materia medica for Chinese medicine, 2nd edn. Churchill Livingstone, Elsevier

Hirota A, Nakagawa M, Hirota H (1991) Structure of paecilospirone, a new antibiotic from *Paecilomyces*. Agric Biol Chem 55:1187–1188

Hisashi M, Toshiyuki M, Tadashi K, Kiyofumi N, Iwao T, Norihisa N, Masayuki Y (1998) Hepatoprotective and nitric oxide production inhibitory activities of coumarin and polyacetylene constituents from the roots of *Angelica furcijuga*. Bioorg Med Chem Lett 8:2191–2196

Hsu HY (1987) A study of Chinese homonymic medicinal herbs. Orient Heal Arts Int Bull 12:387–408

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M (2008) Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 33:61–75

Hu Y, Jiang Z, Leung KS, Zhao Z (2006) Simultaneous determination of naphthoquinone derivatives in Boraginaceous herbs by high-performance liquid chromatography. Anal Chim Acta 577:26–31

Huang CY, Zhao LH, Mei LH (2003) Studies on the HPLC fingerprints of *Epimedium brevicornum* crude drug. Chin J Nat Med 1:146–148

Kano Y, Arimoto Y, Cho CD, Tamura K, Yasuda M (1987) CA 109(2):11813n. Shoyakugaku Zasshi 41:282–288

Kjellenberg L, Johansson E, Gustavsson KE, Olsson ME (2010) Effects of harvesting date and storage on the amounts of polyacetylenes in carrots, *Daucus carota*. J Agric Food Chem 58:11703–11708

Kou G, Zhang Y, Yang X, Rong R (2010) O-methylnotopterol, a new natural product from the roots and rhizomes of *Notopterygium incisum*. Zhongguo Zhong Yao Za Zhi 35:1134–1136

Kusari S, Zählke S, Spiteller M (2009) An endophytic fungus from Camtotheca that produces camptothecin and analogues. J Nat Prod 72:2–7

Lechner D, Stavri M, Oluwatuyi M, Pereda-Miranda R, Gibbons S (2004) The anti-staphylococcal activity of *Angelica dahurica* (Bai Zhi). Phytochemistry 65:331–335

Li WC, Zhou J, Guo SY, Guo LD (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. Fungal Divers 25:69–80

Li PK, Guo BI, Huang WH (2008) Studies on fingerprinting and identification of main species of *Herba Epimedii*. Chin J Chin Mater Med 38:1662–1668

Liang Y-Z, Xie P-S, Chan K (2010) Perspective of chemical fingerprinting of Chinese herbs. Planta Med 76:1997–2003

Liu JH, Lee C-S, Leung K-M, Yan Z-K, Shen B-H, Zhao Z-Z, Jiang Z-H (2007) Quantification of two polyacetylenes in radix ginseng and roots of Related *Panax* Species using a gas chromatography-mass spectrometric method. J Agric Food Chem 55:8830–8835

Liu JH, Zschocke S, Reininger E, Bauer R (1998) Inhibitory effects of *Angelica pubescens* f. *biserrata* on 5-lipoxygenase and cyclooxygenase. Planta Med 64:525–529

Lu H, Zou WX, Meng JC, Hu J, Tan RX (2000) New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. Plant Sci 151:67–73

Ma Z, Xu W, Liu-Chen LY, Lee DY (2008) Novel coumarin glycoside and phenethyl vanillate from *Notopterygium forbesii* and their binding affinities for opioid and dopamine receptors. Bioorg Med Chem 16:3218–3223

Metzger BT, Barnes DM, Reed JD (2008) Purple carrot (*Daucus carota* L.) polyacetylenes decrease lipopolysaccharide-induced expression of inflammatory proteins in macrophage and endothelial cells. J Agric Food Chem 56:3554–3560

Mitchell AM, Strobel GA, Hess WM, Vargas PN, Ezra D (2008) Miscodor crispans, a novel endophyte from Ananas anassoides in the Bolivian Amazon. Fungal Divers 31:37–43

Ming Q, Han T, Li W, Zhang Q, Zhang H, Zheng C, Huang F, Rahman K, Qin L (2011) Tanshinone IIA and tanshinone I production by *Trichoderma atroviride* D16, an endophytic fungus in *Salvia miltiorrhiza*. Phytomedicine 19:330–333

Nortier JL, Vanherweghem JL (2002) Renal interstitial fibrosis and urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). Toxicology 181–182:577–580

Oh H, Swenson DC, Gloer JB, Shearer CA (2003) New bioactive rosigenin analogues and aromatic polyketide metabolites from the freshwater aquatic fungus *Massarina tunicate*. J Nat Prod 66:73–79

Ohnuma T, Komatsu T, Nakayama S, Nishiyama T, Ogura K, Hiratsuka A (2009) Induction of antioxidant and phase 2 drug-metabolizing enzymes by falcarindiol isolated from *Notopterygium incisum* extract, which activates the Nrf2/ARE pathway, leads to cytoprotection against oxidative and electrophilic stress. Arch Biochem Biophys 488:34–41

Ogra RK, Mohanpuria P, Sharma UK, Sharma M, Sinha AK, Ahuja PS (2009) Indian calamus (*Acorus calamus* L.): not a tetraploid. Curr Sci 97:1644–1647

Petrini O (1991) Fungal endophytes of three leaves. In: Andrews JH, Hirane SS (eds) Microbiol ecology of leaves. Springer Publishing Company, New York, pp 179–197

Purup S, Larsen E, Christensen LP (2009) Differential effects of falcarinol and related aliphatic C(17)-polyacetylenes on intestinal cell proliferation. J Agric Food Chem 57:8290–8296

Qiao YL, Zhang YH, Zhang W, Zhang JL (2009) A rapid resolution liquid chromatographic method for fingerprint analysis of raw and processed caowu (Aconitum kusnezoffii). J AOAC Int 92:653–662

Radić N, Štrukelj B (2012) Endophytic fungi—The treasure chest of antibacterial substances. Phytomedicine 19:1270–1284

Rai DK, Brunton NP, Koidis A, Rawson A, McLoughlin P, Griffiths WJ (2011) Characterisation of polyacetylenes isolated from carrot (*Daucus carota*) extracts by negative ion tandem mass spectrometry. Rapid Commun Mass Spectrom 25:2231–2239

Rao G, Wang X, Jin W (1997) Polyacetylene compounds from *Panax notoginseng*. Zhong Yao Cai 20:298–299

Rawson A, Tiwari BK, Tuohy MG, O'Donnell CP, Brunton N (2011) Effect of ultrasound and blanching pretreatments on polyacetylene and carotenoid content of hot air and freeze dried carrot discs. Ultrason Sonochem 18:1172–1179

Reginster F, Jadoul M, van Ypersele de Strihou C (1997) Chinese herbs nephropathy presentation, natural history and fate after transplantation. Nephrol Dial Transplant 12:81–86

Roman M, Dobrowolski JC, Baranska M, Baranski R (2011) Spectroscopic studies on bioactive polyacetylenes and other plant components in wild carrot root. J Nat Prod 74:1757–1763
Santos PAG, Figueiredo AC, Oliveira MM, Barroso JG, Pedro LG, Deans SG, Scheffer JJC (2005) Growth and essential oil composition of hairy root cultures of *Levisticum officinale* W.D.J. Koch (lovage). Plant Sci 168:1089–1096
Satoh A, Narita Y, Endo N, Nishimura H (1996) Potent allelochemical falcalindiol from *Glehnia littoralis* F. Schm. Biosci Biotechnol Biochem 60:152–153
Schmiech L, Alayrac C, Witulski B, Hofmann T (2009) Structure determination of bisacetylenic oxylipins in carrots (*Daucus carota* L.) and enantioselective synthesis of falcarindiol. J Agric Food Chem 57:11030–11040
Schmiech L, Uemura D, Hofmann T (2008) Reinvestigation of the bitter compounds in carrots (*Daucus carota* L.) by using a molecular sensory science approach. J Agric Food Chem 56:10252–10260
Singhuber J, Zhu M, Prinz S, Kopp B (2009) *Aconitum* in Traditional Chinese Medicine – a valuable drug or an unpredictable risk? J Ethnopharmacol 126:18–30
Søltoft M, Eriksen MR, Träger AW, Nielsen J, Laursen KH, Husted S, Halekoh U, Knuthsen P (2010) Comparison of polyacetylene content in organically and conventionally grown carrots using a fast ultrasonic liquid extraction method. J Agric Food Chem 58:7673–7679
Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260:214–216
Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502
Sung GH, Hywel-Jones NL, Sung JM, Luangsa-Ard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. Stud Mycol 57:5–59
Sun Z-L, Zhang M, Zhang J-F, Feng J (2011) Antifungal and cytotoxic activities of the secondary metabolites from endophytic fungus *Massrison* sp. Phytomedicine 18:859–862
Suzuki M (1969) Studies on the irritating substance of *Pinellia ternata* Breitenbach (Araceae). Arzneimittelforschung 19:1307–1309
Vlietinck A, Pieters L, Apers S (2009) Legal requirements for the quality of herbal substances and herbal preparations for the manufacturing of herbal medicinal products in the European Union. Planta Med 75:683–688
Wagner H, Bauer R (2007) Quality proof of herbal drugs from Traditional Chinese Medicine (TCM drugs). Urban & Fischer, Elsevier, Munich, pp 917–949, Appendix in: Hempen C.H., Fischer T. (2009) A Materia Medica for Chinese Medicine, 2nd edn
Wagner H, Bladt S (2001) Plant drug analysis, 2nd edn. Springer, Wien
Wagner H, Bauer R, Melchart D, Xiao PG, Staudinger A (2011) Chromatographic fingerprint analysis of herbal medicines: thin-layer and high performance liquid chromatography of Chinese drugs, Vol. 1 and 2, 2nd edn. Springer, Wien, p 1024
Wang CN, Shiao YJ, Kuo YH, Chen CC, Lin YL (2000) Inducible nitric oxide synthase inhibitors from *Saposhnikovia divaricata* and *Panax quinquefolium*. Planta Med 66:644–647
Wang J, Chen Z, Wu Y, Fan G (2010) Fast determination of active components in *Angelica dahurica* extract using capillary electrochromatography with methacrylate ester-based monolithic columns. Se Pu 28:240–246
Wang LX, Wang CZ, Geng XD (2006) Studies on the RPLC fingerprints and quality evaluation of *Epimedium* L. crude drugs. Acta Chim Sin 64:551–555
Wang Y, Xu L, Ren W, Zhao D, Zhu Y, Wu X (2012) Bioactive metabolites from *Chaetomium globosum* L18, an endophytic fungus in the medicinal plant *Curcuma wenyujin*. Phytomedicine 19:364–368
Wang Y, Zeng QG, Zhang ZB, Yan RM, Wang LY, Zhu D (2011) Isolation and characterization of endophytic huperzine A-producing fungi from *Huperzia serrata*. J Ind Microbiol Biotechnol 38:1267–1278

Wang YL, Liang YZ, Chen BM, He YK, Li BY, Hu QN (2005) LC-DAD-APCI-MS-based screening and analysis of the absorption and metabolite components in plasma from a rabbit administered an oral solution of danggui. Anal Bioanal Chem 383:247–254

Wei XL, Yin XC, Guo YL, Shen NY, Wei JC (2006) Analysis of molecular systematics on *Cordyceps sinensis* and its related toxa. Mycosystema 25:192–220

Washida D, Kitanaka S (2003) Determination of polyacetylenes and ginsenosides in *Panax* species using high performance liquid chromatography. Chem Pharm Bull 51:1314–1317

Wicklow DT, Rogers KD, Dowd PF, Gloer JB (2011) Bioactive metabolites from *Stenocarpella maydis*, a stalk and ear rot pathogen of maize. Fungal Biol 115:133–142

Wu KM, Farrelly JG, Upton R, Chen J (2007) Complexities of the herbal nomenclature system in traditional Chinese medicine (TCM): lessons learned from the misuse of *Aristolochia*-related species and the importance of the pharmaceutical name during botanical drug product development. Phytomedicine 14:273–279

Xiao PG (1983) Recent developments on medicinal plants in China. J Ethnopharmacol 7:95–109

Xie PS, Yan YZ, Guo BL, Lam CWK, Chui SH, Yu QX (2010) Chemical pattern-aided classification to simplify the intricacy of morphological taxonomy of *Epimedium* species using chromatographic fingerprinting. J Pharm Biomed Anal 52:452–460

Xu LL, Han T, Wu JZ, Zhang QY, Zhang H, Huang BK, Rahman K, Qin LP (2010) Comparative research of chemical constituents, antifungal and antitumor properties of ether extracts of *Panax ginseng* and its endophytic fungus. Phytomedicine 16:609–616

Xu XN, Jiang JH, Liang YZ, Yi LZ, Cheng JL (2010) Chemical fingerprint analysis for quality control of *Fructus Aurantii Immaturus* based on HPLC-DAD combined with chemometric methods. Anal Methods 2:2002–2010

Yuan HL, Zhang TT, Xiao XH (2011) Quality control approaches for Chinese herbal medicines. Chin Herb Med 3:17–22

Zhang W (2005) Pharmacognostic study of *Acorus* – from a phylogenetic perspective. Ph.D. Thesis, Hongkong Baptist University

Zhao Z, Liang Z, Chan K, Lu G, Lee LM, Chen H, Li L (2010) A unique issue in the standardization of Chinese materia medica: processing. Planta Med 76:1975–1986

Zhu D, Wang J, Zeng Q, Zhang Z, Yan R (2010a) A novel endophytic Huperzine A-producing fungus, *Shiraia* sp. Slf14, isolated from *Huperzia serrata*. J Appl Microbiol 109:1469–1478

Zhu J-S, Gao L, Li X-H, Yao Y-S, Zhoo J-Q, Zhou Y-J, Lu J-H (2010b) Maturational altervation of oppositely oriented rDNA and differential proliferation of GC- and AT-biased genotypes of *Ophiocordyceps sinensis* and *Paecilomycea hepiali* in natural *Cordyceps sinensis*. Am J Biomed Sci 2:217–338. doi:10. 5099/aj100300217

Zidorn C, Jöhrer K, Ganzera M, Schubert B, Sigmund EM, Mader J, Greil R, Ellmerer EP, Stuppner H (2005) Polyacetylenes from the Apiaceae vegetables carrot, celery, fennel, parsley, and parsnip and their cytotoxic activities. J Agric Food Chem 53:2518–2523

Zschocke S, Lehner M, Bauer R (1997) 5-Lipoxygenase and cyclooxygenase inhibitory active constituents from Qianghuo (*Notopterygium incisum*). Planta Med 63:203–206

Zschocke S, Liu JH, Stuppner H, Bauer R (1998) Comparative study of roots of *Angelica sinensis* and related umbelliferous drugs by thin layer chromatography, high-performance liquid chromatography and liquid chromatography – mass spectrometry. Phytochem Anal 9:283–290

Chapter 2
DNA-Based Authentication of TCM-Plants: Current Progress and Future Perspectives

G. Heubl

2.1 Introduction

Traditional Chinese Medicine (TCM), with its long history, is deeply rooted in the Chinese culture and represents one of the oldest forms of medical therapy in the world. TCM has been used for thousands of years in China for health maintenance, disease prevention, and used to a lesser extent for the application of a variety of clinical therapies. Today, the global use of TCM is rapidly increasing and over 130 countries in the world are using Chinese Herbal Medicine (Hsiao 2007). The majority of drugs harvested and processed in China are of plant origin. A highly developed industry delivers medical plant preparations to the local Chinese customers and exports plant-based drugs to Asia and developing markets in Europe, Canada, and the USA.

Increasing interest by multinational pharmaceutical companies in herbal-based medicine is contributing to the significant economic growth of the global TCM market. It is estimated that 70–80 % of people worldwide rely chiefly on traditional herbal remedies as their primary form of health care (Farnsworth and Soejarto 1991; Pei 2001). Herbal medicine is becoming more main-stream and fashionale in richer countries. Therefore the TCM market has been growing at 10–20 % annually in Europe and North America over recent years (Hamilton 2003; Ten Kate and Laird 1999). In addition, there are many related botanical products sold as health foods, food supplements, herbal teas, and for various other purposes related to health and personal care. In terms of the number of species, the use of plants as medicine represents by far the most important application area of natural resources. Plants provide the predominant ingredients of medicine in most medical traditional

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systems of healing and have been the source of inspiration for several new drug search endeavors of major pharmaceutical companies (Schippmann et al. 2002).

2.2 Biodiversity and Medicinal Plant Resources in China

China is one of the world's megabiodiverse countries with 32,308 species of seed plants belonging to 363 families and 3,427 genera (Ding 2002). The country spans a huge geographical area, is characterized by enormous variations in geographical, climatological, and topographical features and covers five climatic zones (cold-temperate, temperate, warm-temperate, subtropical, and tropical). The geology and geography is very complex, comprising the highest mountain ranges on Earth (the Himalayas), vaste plateaus such as Qinghai-Xizang (Tibetan) or Pamir, and arid basins such as Tarim, which contains the largest desert in China (Taklamakan). Main rivers of Asia such as Mekong, Brahmaputra, Yangtze, and Yellow River originate in Qinghai-Xizang Plateau (Lopez-Pujol and Zhao 2004). All features contribute to the enormous diversity of biomes (from rainforests to deserts) found in China, as well as to the enormous species diversity including many medicinal plants (Lopez-Pujol et al. 2006).

There is no reliable data for the total number of medicinal plants on earth and available information for countries and regions vary greatly. Estimates for the number of plant species used medicinally include 35,000–70,000 worldwide (Farnsworth and Soejarto 1991; Schippmann et al. 2002), 7,295–11,146 in China (Pei-Gen 2007; Hamilton 2004; Huang et al. 2002a, b). According to statistics there are 12,807 kinds of remedies in Chinese medicine. Of these, 11,146 (80 %) are plant based, 1,581 are animal based, and 80 are mineral based (Gao et al. 2002). Regarding the diversity of Chinese medicinal plants, only 500–600 species are commonly used in Traditional Chinese Medicine and prescribed by Chinese medical practitioners, 1,430 in Mongolian Medicine, and 1,106–3,600 in Tibetan Medicine (Pei 2001, 2002b). In the 2005 edition of the Chinese Pharmacopoeia 1,146 monographs and 538 herbal drugs have been included. The improved version of the People's Republic of China Pharmacopoeia 2010 contains 1,174 monographs (in total 4,567 monographs) and enumerates more than 4,600 varieties of species. In Europe and USA 75–150 herbal drugs are mostly used by TCM practitioners (Table 2.1).210648_Wagner

The Chinese medicinal industry represents a significant portion of the pharmaceutical industry in China. There are 1,200 Chinese medicinal, industrial enterprises that manufacture approximately 8,000 Chinese herbal medicine products. The total annual sales of functional food, TCM preparations, medicinal plant extracts, and other processed materials exceed US $ 40 billion (Liu et al. 2011). Today, it is a big challenge to protect the medicinal plant resources from overexploitation and habitat destruction (Srivastava et al. 1996). For example, 70–80 % of natural materials were collected from the wilderness to meet the annual demand of approximate one million tons (Balunas and Kinghorn 2005). The annual sales of these natural resources have

Table 2.1 Diversity and inventory of the Chinese flora with data on medicinal plants

Resource	Total number of species	Medicinal plant species number
• Algae	5,000[a]/8,997[d]/12,500[e]	115[a]
• Mosses	2,200[a,c,d,e,f]	43[a]
• Ferns	2,300[e]/2,600[a,b,d,f]	456[a]
• Seed plants	25,000[a]/30,000[b,c,d]	10,188[a]
Gymnosperms	192[e]/200[a]/250[b]/270[d]	
Angiosperms	25,000[f]/30,000[c,d]/31,500[g]	
Total plants	34,692–47,570	10,802
• Lichens	2,000[c]	52[a]
• Fungi	8,000[f,d]	292[a]

[a]Gao et al. (2002)
[b]Lopez-Pujol and Zhao (2004)
[c]Xue (1997)
[d]SEPA (1998)
[e]Li (2003)
[f]NEPA (1994)
[g]Flora of China Project Missouri Botanical Garden (http://www.mobot.org/press/Assets/FP/flora_china.asp). Estimated numbers of other medicinal resources in China: Medicinal animals 1,581; medicinal mine 80 (Huang et al. 2002a, b)

increased to more than 100 times in relation to the levels of 1980. Many of these natural resources are derived from species that are threatened or have become rare or endangered by large-scale exploitation (Nalawade et al. 2003; Cole et al. 2007). With the rapid increase of consumer demand for crude drugs and natural health products, many medicinal plant species are threatened with extinction due to overexploitation and habitat destruction. To ensure the sustainable use of medicinal plant resources, a conservation framework consisting of conservation strategies, cultivation practices, and various technologies has been developed. Conservation strategies include establishing in situ and ex situ conservation centers, setting up government policies and regulations, establishing methods for resource surveying and trade monitoring as well as establishing and enforcing Good Agricultural Practices (Liu et al. 2011).

About 140 new drugs have originated directly or indirectly from Chinese medicinal plants by means of modern scientific methods, confirming that these plants are an important resource (Liu and Yaniv 2005; Lee et al. 2005). The first compound derived from Chinese herbal remedies to enter the Western pharmacopoeia was Ephedrine, an amphetamine-like stimulant isolated from the Chinese medicinal herb "Ma-Huang" (*Ephedra sinica*). The next significant pharmaceutical breakthrough derived from Chinese medicine was the isolation of Artemisinin from "Qing-Hao" (*Artemisia annua*). Researchers found that Artemisinin was beneficial for fever and killed even chloroquine-resistant strains of *Plasmodium*, the parasite that causes malaria. Recent work suggests that Artemisinin may also have anticancer properties, a hypothesis which was also established for *Coix lacryma-jobi* var. *ma-yuen* the Job's Tear. Modern pharmacological studies have demonstrated that the Coix "seed" (a bony utricle of the single female flower enclosing the fruit) and its preparation-KLT (Kanglaite Injection) possess extensive pharmacological

activities (Li 2001; Lu et al. 2008). Active components have been isolated and defined in many other Chinese herbs, for example, anthraquinone glycosides in rhubarb (*Rheum officinale*), gingerols in ginger (*Zingiber officinale*), ginkgolides in Ginkgo (*Ginkgo biloba*) or berberine, an antibacterial component from Chinese goldthread (*Coptis chinensis*) (Wang et al. 2007a).

2.3 Adulterants, Substitutes, and Confused Species

Although many Chinese medicinal plants have been used successfully in China for a long period of time they have never been subject to the stringent quality standards and regulations for pharmaceutical products in Europe or USA. To make traditional and innovative plant products acceptable for modern medicine, it is necessary to have reliable botanical, phytochemical and molecular identification tools for the identification of medicinal plant species (Liang et al. 2004).

A severe problem of the global TCM market is that many erroneous substitutes and adulterants of Chinese medicinal plants are traded due to their lower costs or due to the misidentification of species with similar morphological features.

In recent times the use and demand of herbal preparations has been growing in Western countries where medicinal plants have gained popularity and attention among physicians and patients. Among the alternative medicines TCM is becoming one of the most widely used therapies throughout the world (Normile 2003). One of the reasons for the increasing interest in herbal medicines is the belief that, being natural and traditionally used, they are hence safe and do not possess the potential for negative side effects. This coupled with lower costs compared to conventional medications is the major attraction of these treatments. Nevertheless, their natural origin is not a guarantee of safety. In literature, many reports point out the risks associated with the use of herbal products (Chan 1997; Ernst 2004; Ng et al. 2009). The quality of a herbal drug can be negatively affected by the use of inherent toxic herbs, by fraudulent action due to substitution or adulteration, by contamination, by misidentification, by confusion of species, and by inappropriate labeling. There are several incidents of Chinese herbs which document that adulterants or substitutes caused serious intoxications and even deaths (But 1994; Graham-Brown 1992; Ng et al. 2009; Chan 2003; Mazzanti et al. 2008; Zhao et al. 2007a; Chan and Critchley 1996; Gertner et al. 1995; But et al. 1996; Chen et al. 2002; McIntyre 1998; Yang and Chen 1998).

A case of encephalopathy and neuropathy was reported following ingestion of a decoction supposedly prepared from "Long Dan Cao" (*Gentiana rigescens* radix). Investigation showed that the toxicity was in fact due to adulteration of the herb with the roots of *Podophyllum emodi* or *P. hexandrum* which contain the neurotoxin podophyllotoxin in high concentration (But 1994; Ng et al. 2009). Several cases of renal damage attributed to "Fang-Ji" (*Stephania tetrandra*) in a weight-loss preparation were actually caused by "Guang Fang Ji" (*Aristolochia fangchi*) that contains the highly toxic aristolochic acid, a known nephrotoxin which causes renal failure

and urothelial carcinoma. The confusion in the latter case has obviously arisen from the similar Chinese names (Fugh-Berman 2000). Several other poisoning cases are documented and resulted from erroneous substitution of herbs.

One further example of species confusion, which had serious consequences, involves the common name "Bai Mao Teng." This name has been used for *Solanum lyratum* and *Aristolochia mollissima*. Apparently, these herbs belong to two different families but look similar. While *S. lyratum* is not harmful, *A. mollissima* contains the toxic aristolochic acid that can cause kidney failure and cancer of the urinary tract (Zhao et al. 2006).

Another incident refers to commercial "Mu Tong," a Chinese diuretic drug, which is associated with five species: *Clematis armandii* and *Clematis montana* ("Chuan Mu Tong"), *Akebia quinata* ("Wu Ye Mutang"), *A. trifoliata* ("San Ye Mu Tong"), *Aristolochia manshuriensis* ("Guan Mu Tong"), and *A. moupinensis*/*A. kaempferi* ("Huai Mu Tong"). This has led to a serious confusion with the consequence that substitution with *Aristolochia manshuriensis* caused renal failure (Zhu 2002; Cheung et al. 2006; Debelle et al. 2008).

Very often the high price of some herbs is an incentive for criminals to make quick profit by manufacturing counterfeit products. The more precious and rare the crude drug is, the more likely the counterfeit products will find their way into the market. A classic example is *Cordyceps sinensis* (recently renamed as *Ophiocordyceps sinensis*), a fungus parasitizing the larva of some species of insects and the dead caterpillar (Dong and Yao 2010). This scarce drug is found only in fairly inaccessible alpine regions on the Tibetan Plateau and is one of the best-known traditional Chinese medicinal products, with great benefits to human health and with a huge economic value. There are many substitutes of so-called *Cordyceps* that are traded worldwide, such as *Cordyceps militaris* (the most commonly used substitute), *C. martialis*, *C. hawkesii*, *C. liangshanensis*, *C. barnesii*, *C. cicadicola*, *C. gracilis*, *C. ramose*, *C. ophioglossoides*, and *C. gunnii*. In addition, there are counterfeit plant products of the fungus and mimics such as *Stachys geobombycis*, *Stachys sieboldii*, and *Lycopus lucidus* that have been found on the market. Consequently there is a serious problem for authentication and quality control of *Cordyceps* (Li et al. 2006).

A review published by Zhao et al. (2006) enumerates further examples and summarizes the facts and reasons for confusion in the current Hong Kong medicinal herb market.

2.4 Authentication of Herbal Material on the DNA Level

The authentication of Chinese medicinal plants, depending on the correct identification of species, is an essential prerequisite to ensure safety, herbal drug quality, and therapeutic efficacy (Zhao et al. 2006). Identification of herbal materials, which commonly consist of dried or processed parts, is generally difficult because many useful diagnostic characters are lost during the drying process. This is particularly

true when one herb has more than one common name, or where one common name is used for more than one herb (Zhao and Li 2004). In practice, the identification of medicinal plants relies mainly on morphological, anatomical, and phytochemical characters. Many pharmacopoeias refer to macroscopic and microscopic evaluation (morphology, histology) and chemical profiling (TLC-, HPLC-, GC-fingerprinting) for quality control and standardization of raw and processed herbs (Chan 2003; Siow et al. 2005; Wagner et al. 2011). However, chemical variability within the plant material often hinders the confirmation of its botanical identity as the chemical composition is affected by growth and storage conditions as well as by the harvesting process. Otherwise microscopic examination of drugs requires botanical expertise for the unequivocal authentication, as related species often possess similar features.

With the improvements in molecular biotechnology and plant genetics in the past decades, genetic tools are considered to provide more reliability for authentication of herbal materials at the DNA level (Kumar et al. 2009). Thus in the meanwhile, various DNA-based molecular marker techniques are applied in many fields and their application is remarkably increasing for species characterization in medicinal plants (Shaw et al. 2002, 2009; Joshi et al. 2004; Zhang et al. 2007; Sucher and Carles 2008). This is especially useful in case of those species that are frequently substituted or adulterated with other species or in case of varieties that are morphologically and/or phytochemically almost indistinguishable.

Benefiting in the first place from PCR techniques, DNA markers have become a powerful tool for identification and authentication of plant, animal, fungal, and bacterial species (Yip et al. 2007; Kaplan et al. 2004; Pereira et al. 2008; Hao et al. 2010). Contrary to chemical fingerprinting which is strongly influenced by the age of the sample, physiological conditions, environmental factors, cultivation area, harvesting period, drying, and storage conditions, DNA is an extremely stable macromolecule that is not affected by external factors and therefore can be recovered from fresh, dried, and even processed biological material. Additionally the marker molecules are not tissue specific and thus can be detected at all stages of organism development. Moreover, only a small amount of a sample is sufficient for analysis.

This review provides an overview of DNA-based technologies and most commonly used assays with an emphasis on those that are based on DNA hybridization, restriction enzymes, random PCR amplifications, species-specific PCR primers, and DNA sequencing. A critical evaluation of all methods is presented focusing on their discriminatory power, sensitivity, reproducibility, user-friendliness, and costs (see Table 2.3).

The previously described incidents highlight the importance of a correct botanical classification and the need of an adequate knowledge of morphological characteristics of herbal drugs in order to perform a proper identification. As more and more people worldwide use Chinese herbs, authentication becomes an increasing problem because adulterated and substituted Chinese medicinal materials are widely common in the market (Mills and Bone 2005).

Therefore authentication of Chinese medicinal materials is the key for safety, appropriate use and maximum therapeutic potency, minimization of trading fraud,

and last but not least for the increase of consumer confidence in Chinese medicine. Identification at species level is required for quality assurance, which includes both identifying the crude plant product and evaluating its pharmaceutical quality (Wagner et al. 2011). Thus, authentication is a fundamental step for the successful, reliable clinical application and for accurate experimental studies of TCM plants. This will ensure the safe and effective use of Chinese medicinal herbs throughout the world.

2.4.1 Types of DNA Markers Used in Plant Genome Analysis

There are various types of DNA-based molecular techniques that are used to evaluate DNA polymorphism in order to authenticate plant taxa (Sucher and Carles 2008; Shaw et al. 2009; Yip et al. 2007; Kaplan et al. 2004; Pereira et al. 2008; Heubl 2010). These are hybridization-based methods, polymerase chain reaction (PCR)-based methods, and sequencing-based methods. In recent times the use of multilocus sequence analysis (MLSA), which is commonly used in phylogenetic studies, has proven its discriminatory power. Additionally DNA microarrays that contain thousands of probes are a promising new development for sensitive and high-throughput taxon identification (Trau et al. 2002; Schena et al. 1998).

2.4.1.1 RAPD (Randomly Amplified Polymorphic DNA)

The RAPD technology utilizes short synthetic oligonucleotides (10 bp long) of random sequences as primers to generate a high number of anonymous DNA fragments via PCR reaction. The large number of amplification products is generally separated on agarose gels and stained with ethidium bromide or SyBRgreen.

Using an appropriate annealing temperature in the PCR cycle, oligonucleotide primers bind to several priming sites on the complementary sequences in the template genomic DNA. If these priming sites are within an amplifiable distance discrete DNA fragments are generated. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Polymorphism of amplified fragments is caused by (1) base substitutions or deletions in the priming sites, (2) insertions that render priming sites too distant to support amplification, or (3) insertions or deletions that change the size of the amplified fragment (Weising et al. 2005).

Because of the simplicity (no prior sequence information is necessary), low costs, efficiency in developing a large number of DNA markers in a short time, and requirement for less sophisticated equipment, RAPDs have found a wide range of applications. Although the RAPD method is easy to perform, the issue of reproducibility has been an important concern. In fact, the RAPD reaction is far more sensitive than conventional PCR because of the length of a single and arbitrary primer, which is used to amplify anonymous regions of a given genome. Special

care is needed for keeping out contaminant DNA (from infections and parasites) in the material to avoid misleading patterns. RAPDs are inherited as dominant-recessive characters which mean that homozygotes and heterozygotes cannot be distinguished.

RAPD markers have found a wide range of applications in the authentication of medicinal plants. The technique has been applied in many plant groups like *Glycyrrhiza* (Yamasaki et al. 1994), *Atractylodes* (Kohjyouma et al. 1997; Chen et al. 2001), *Astragalus* (Cheng et al. 2000), *Amomum* (Wang et al. 2000), *Scutellaria* (Hosokawa et al. 2000), *Panax* (Cheung et al. 1994; Shim et al. 2003; Cui et al. 2003; Lim et al. 2007), *Aconitum* (Cole and Kuchenreuther 2001), *Ginkgo* (Fan et al. 2004), *Anectochilus* (Cheng et al. 1998), *Lycium* (Zhang et al. 2001a), *Angelica* (Watanabe et al. 1998), *Bupleurum* (Liang et al. 2002), *Dendrobium* (Zhang et al. 2001b), *Magnolia* (Guo et al. 2001), *Asarum* (Huang et al. 1998), *Apocynum* (Lu et al. 2010a), *Trollius* (Li et al. 2010a), *Phyllanthus* (Dnyaneshwar et al. 2006), *Indigofera* (Zhang et al. 1997), *Coptis* (Cheng et al. 1997), *Codonopsis* (Zhang et al. 1999), *Taraxacum* (Cao et al. 1996a), *Elephantopus* (Cao et al. 1996b), and *Rehmannia* (Cheng et al. 2002).

2.4.1.2 AP-PCR (Arbitrary Polymerase Chain Reaction)

AP-PCR (or Arbitrarily Chosen Primers ACP-PCR) is a special variation of RAPD which is using single primers approximately 10–50 bp in length (Welsh and McClelland 1990). In AP-PCR the amplification follows three steps. In the first two cycles annealing is under nonstringent conditions. Higher primer concentrations are used in the first cycle. Often primers of variable length are used and products are mostly analyzed on polyacrylamide gels. AP-PCR has been applied to various groups for identification of species and analysis of genetic variation (Munthali et al. 1992; Kersten et al. 2007). Similar to RAPDs, reproducibility can be a problem for fingerprints generated by a single primer, because small changes in annealing conditions can affect banding pattern.

2.4.1.3 DAF (DNA Amplification Fingerprinting)

DNA Amplification Fingerprinting (DAF) is a variant of the RAPD technique and was developed by Caetano-Anollés et al. (1991a). For PCR amplification, very short oligonucleotide primers of arbitrary sequence (mostly five nucleotides) are used to amplify short fragments of genomic DNA resulting in a very complex banding pattern (Caetano-Anollés et al. 1991a, b). DAF uses low stringency amplification conditions so that primers can anneal arbitrarily at multiple sites on each template DNA strand and initiate DNA synthesis. The method needs careful optimization of parameters and only two temperature cycles are required. DAF products are routinely separated by polyacrylamide gels and detected by silver staining (Chawla 2002). Another approach called ASAP (arbitrary signatures from

amplification profiles) was developed to enhance the level of informativeness of DAF reactions by using primers which contain both a 5′ mini-hairpin sequence and a short "core" arbitrary 3′ sequence. These arbitrary mini-hairpin primers increase detection of polymorphic DNA and direct the controlled amplification of small template molecules, thereby generating "sequence signatures" from PCR-amplified fragments (Caetano-Anollés and Gresshoff 1996).

2.4.1.4 ISSR (Inter Simple Sequence Repeat)

In higher plants, Inter Simple Sequence Repeat or ISSR markers are frequently applied because they are known to be abundant, very reproducible, highly polymorphic and easy to use (Zietkiewicz et al. 1994; Bornet et al. 2002). ISSR, also known as anchored simple sequence repeat (ASSR), has been used in genetic fingerprinting, gene tagging, phylogenetic analysis, species and cultivar identification, and assessment of hybridization (Kurane et al. 2009).

The ISSR technique is nearly identical to RAPD except that ISSR primers are designed from microsatellite regions and are longer (approximately 14 bp or more) than RAPD primers. Microsatellites are very short stretches of DNA that are "hypervariable," expressed as different variants within populations and among different species. ISSR uses the presence of Simple Sequence Repeats (SSRs) which are characterized by mono-, di-, or trinucleotide repeats (e.g., AA... or AG... CAG...) that have 4–10 repeating units side-by-side. These SSRs are ubiquitous, abundant, and highly polymorphic. The primers used can be 5′ or 3′ anchored by 1–3 selective nucleotides to prevent internal priming and to amplify only a subset of the targeted inter-repeat regions. ISSR markers access variations in the numerous microsatellite regions dispersed throughout the genome. The PCR products are mostly separated on agarose gels and stained with ethidium bromide. Alternatively amplified DNA fragments can also be screened and detected using capillary electrophoresis which significantly increases the amount of information compared to the traditional agarose gel electrophoresis. Since ISSRs are dominant markers, the amplified fragments are scored as diallelic. Presence (0/1) of loci can be used for genetic similarity or cluster analysis. Changes in the amplified products can arise through structural changes in the region (insertions or deletions) or the loss of primer binding sites.

ISSR markers overcome the weakness of low reproducibility of RAPDs, the high costs of AFLPs, the complexity of SSRs and thus this technique is less time consuming, more cost-efficient, use of radioactivity is not required, no prior sequence information is necessary, and it shows high polymorphism.

ISSRs have been used for screening genetic diversity and authentication of *Dendrobium* (Shen et al. 2006), *Cistanche* (Shi et al. 2009), *Fritillaria* (Li et al. 2009a), *Salvia* (Song et al. 2010), *Rehmannia* (Wang et al. 2005), *Vitex* (Hu et al. 2007), *Cannabis* (Kojoma et al. 2002), *Rhodiola* (Xia et al. 2007), *Cymbidium* (Wang et al. 2009), *Ammopiptanthus* (Ge et al. 2005), *Swertia* (Joshi and Dhawan 2007), *Glycyrrhiza* (Yao et al. 2008), and *Houttuynia* (Wu et al. 2005).

2.4.1.5 AFLP (Amplified Fragment Length Polymorphism)

Amplified fragment length polymorphism (AFLP) originally developed by Zabeau and Vos (1993) is a powerful tool for DNA fingerprinting of organismal genomes and it combines the use of RFLP and PCR techniques. This multilocus approach needs no prior sequence information, it is highly reproducible, with the ability to screen a large number of loci (ca. 50–100 fragments per reaction) for polymorphisms. It is a very useful technique for DNA fingerprinting, especially when very little information on the genome of the plant under study is available (Mueller and Wolfenbarger 1999; Blears et al. 1998).

The procedure of this technique (Vos et al. 1995) is a multistep process (1) Digestion of total genomic DNA with two restriction enzymes. One restriction enzyme is a frequent cutter (four-base recognition site, e.g., MseI), the second restriction enzyme is a rare cutter (six-base recognition site, e.g., EcoRI). (2) Adapters specific to the restriction sites are ligated to the fragment ends which serve as binding sites for selective primers in PCR amplification. (3) A first PCR (preselective amplification) is performed. The PCR primers consist of a core sequence (part of the adapter), a restriction enzyme specific sequence, and 1–5 selective nucleotides (the higher the number of selective nucleotides, the lower the number of bands obtained per profile). (4) Preselective amplification products undergo another PCR run, and again a subset of those fragments is selected. Usually, for the second selective amplification, two extra nucleotides are added to the primers. (5) Amplified fragments, labeled with fluorescent or radioactive tags, are separated on acrylamide gels or with automated genetic analyzers and dominant markers are scored as the presence/absence of loci (Weising et al. 2005).

The advantages of AFLPs lie in their high genomic abundance, considerable reproducibility, in their generation of many informative bands per reaction, in their wide range of applications, and in the fact that no sequence data are required for primer construction. Compared with the widely used RFLP, AFLP is faster, less labor intensive, and provides more information.

Disadvantages include the need for purified, high molecular weight DNA, the dominance of alleles, and the possible nonhomology of comigrating fragments belonging to different loci. Because of the highly informative fingerprinting profiles, which are usually obtained as results, AFLPs can be applied in studies involving genetic identity, parentage, identification of clones and cultivars, and in phylogenetic studies of closely related species.

AFLP analyses have been used in *Panax* (Choi et al. 2008; Ha et al. 2002), *Actaea* (Zerega et al. 2002), *Plectranthus* (Passinho-Soares et al. 2006), *Caladium* (Loh et al. 1999), *Cannabis* (Datwyler and Weiblen 2006), and *Rehmannia* (Qi et al. 2008).

2.4.1.6 RAMPO (Randomly Amplified Microsatellite Polymorphism)

This method, also termed RAHM (random amplified hybridization microsatellite) or RAMS (randomly amplified microsatellites), combines arbitrarily primed PCR (RAPD) with microsatellite hybridization to produce polymorphic genetic fingerprints (Weising et al. 2005; Weising and Kahl 1998). No prior sequence information is needed. Genomic DNA is first amplified with a single arbitrary 10-mer primer (as in RAPDs) or microsatellite-complementary 15- to 16-mer PCR primer. After electrophoretic separation and staining of the PCR products, the gel is either dried or blotted onto a nylon membrane and subsequently hybridized to a radiolabeled mono-, di-, tri-, or tetranucleotide repeat probe such as [GT]8, [GA]8, or [CAA]5. Subsequent autoradiography reveals reproducible, probe-dependent fingerprints that are completely different from the ethidium bromide staining patterns and that are polymorphic at an intraspecific level. An advantage of the RAMPO technique is the low complexity of banding patterns, which is considerably facilitating the detection of species-specific bands. RAMPO bands appear to be less sensitive to misinterpretation than RAPD bands, because not only the size but also the hybridization signal intensity of two bands (i.e., the presence and copy number of a certain microsatellite) are criteria for homology. The method is mainly used for identification and discrimination of genotypes within and among populations, cultivates, and germplasm e.g., in *Ficus* (Chatti et al. 2007) and *Phoenix dactylifera* (Soumaya et al. 2008). RAMPOs share their fate with other marker technologies that are partly or totally based on blot hybridization. These methods are barely used anymore, because more convenient marker systems are available for most purposes.

2.4.1.7 RFLP (Restriction Fragment Length Polymorphism)

RFLP analysis was one of the first techniques to be widely used for detecting variations at the DNA level. The principle of this method is based on the comparison of banding patterns from DNA sequences digested with specific restriction enzymes (e.g., HaeIII, EcoRI, BamHI). Restriction enzymes are endonucleases produced by bacteria with the function to cut specific DNA sequence motifs of invading DNA molecules. Each enzyme has a specific, typically palindromic recognition sequence. Consequently it recognizes and cuts DNA in a predictable way, resulting in a reproducible set of DNA fragments of different lengths. If two organisms (strains, individuals, or species) differ in the distance between sites of cleavage of a particular restriction endonuclease, then also the length of the fragments produced differs. Consequently RFLP is a result of (a) point mutation creating or destroying a restriction site and (b) insertion/deletions altering the size of a given restriction fragment. These differences in fragment lengths can be detected by gel electrophoresis, hybridization, and visualization. To detect specific fragments, the DNA restriction fragment profile of the agarose gel is transferred to

a nitrocellulose or nylon membrane. Afterwards a single-stranded DNA probe is conveniently labeled, using any standard method (e.g., a radioisotope or digoxygenin) and hybridized with the target DNA, which is stuck to the membrane. Polymorphisms are detected by the presence or absence of bands. RFLP is a robust methodology; the markers are relatively polymorphic, codominantly inherited, and highly reproducible. The method also provides opportunity to simultaneously screen numerous samples. The technique is time consuming, costly, labor intensive and requires a large quantity of good quality or undegraded DNA (Weising et al. 2005).

RFLP combined with DNA hybridization has mainly been used for phylogenetic studies in the past e.g., in *Lupinus* (Yamazaki et al. 1993), *Hedysarum* (Trifi-Farah and Marrakchi 2001), *Triticum* (Mori et al. 1997), *Musa* (Gawel et al. 1992), and for detection of *Dendrobium* (Li et al. 2005) and *Fritillaria* (Tsoi et al. 2003).

2.4.1.8 Microsatellites or SSR (Simple Sequence Repeats)

Microsatellites also known as simple sequence repeats (SSRs), short tandem repeats (STRs), or simple sequence length polymorphisms (SSLPs) are the smallest class of simple repetitive DNA sequences (Litt and Luty 1989; Gupta et al. 1996). Based on tandem repeats of short (2–6 bp) DNA sequences, these markers are highly polymorphic due to variation in the number of repeat units and dispersed throughout most eukaryotic genomes. The large number of alleles and the high levels of variability among closely related organisms made PCR-amplified microsatellites the marker system of choice for a wide variety of applications like population genetic studies, genome mapping, and marker-assisted breeding (Valdes et al. 1993; Akkaya et al. 1995; Schuler et al. 1996). It is meanwhile proven that the predominant mutation mechanism in microsatellite tracts is slipped-strand mispairing (Levinson and Gutman 1987). The repeat length at specific SSR loci is easily assayed by PCR using primers specific to conserved regions flanking the repeat. PCR fragments are usually separated on polyacrylamide gels or capillary sequencers in combination with fluorescent detection systems. The reason for the wide usage of nuclear microsatellites is their high abundance, enormous extent of allelic diversity, and suitability for automatization. Meanwhile many SSR primers deduced from flanking sequences of known microsatellites are deposited in DNA databases.

In addition to nuclear microsatellites, chloroplast microsatellites are also particularly effective markers for analysis of the genetic diversity (Clark et al. 2000) and phylogeography of plant populations (Chen et al. 2011), studying mating systems, gene flow via both pollen and seeds, detection of hybridization, and introgression (Agarwal 2008). One limitation of the approach is the need of sequence data for primer construction. Primer sequences flanking chloroplast microsatellites are usually inferred from fully or partially sequenced chloroplast genomes. In general, these primer pairs produce polymorphic PCR fragments from the species of origin and their close relatives, but transportability to more distant taxa is limited. Attempts to design universal primers to amplify chloroplast microsatellites have

resulted in a set of consensus chloroplast microsatellite primers (ccmp1–ccmp10) that aims at amplifying cpSSR regions in the chloroplast genome of dicotyledonous angiosperms (Weising and Gardner 1999). Most of the primer pairs derived from A or T mononucleotide repeats ($n = 10$) identified in the tobacco chloroplast genome, were functional as genetic markers in the Actinidiaceae, Brassicaceae, and Solanaceae (Chung and Staub 2003). Universal primers for the amplification of chloroplast microsatellites in grasses (Poaceae) have also been developed (Provan et al. 2004).

A major limitation of SSRs are the time and high development costs required to isolate and characterize each locus when preexisting DNA sequence is not available. Typically, this process requires the construction and screening of a genomic library of size-selected DNA fragments with SSR-specific probes, followed by DNA sequencing of isolated positive clones, PCR primer synthesis, and testing. With the availability of large numbers of ESTs and other DNA sequence data, development of EST-based SSR markers is less time consuming and expensive. The methodology and applications of nuclear microsatellite markers in plants and other organisms has been subjected to numerous reviews, including Goldstein and Schlötterer (1999), Zane et al. (2002), Li et al. (2002), Varshney et al. (2005), and Squirrell et al. (2003).

Microsatellites have been applied in *Panax* (Kim et al. 2007a; Jo et al. 2009), *Acanthopanax* (Kim and Chung 2007), *Dendrobium* (Fan et al. 2009), *Cymbopogon* (Kumar et al. 2007), *Bupleurum* (Chun et al. 2009), and *Schisandra* (Boqian et al. 2009).

2.4.1.9 SAMPL (Selective Amplification of Microsatellite Polymorphic Loci)

The SAMPL technique was introduced by Morgante and Vogel (1994). It combines the high and controllable multiplexing rate of the AFLP technique with the high levels of microsatellite polymorphism by using AFLP-type primers together with compound microsatellite primers (Weising et al. 2005).

SAMPLs differ from AFLPs by using primers with compound microsatellite motifs in combination with oligonucleotides complementary to the end-ligated adapters for the selective amplification step (Paglia et al. 1998). In brief, genomic DNA is digested with restriction enzymes (commonly *EcoRI* and *MseI*), and the resulting fragments are ligated to adapters. Afterwards a preamplification reaction for all ligated DNA fragments is carried out with primers annealing to the adapters. These preamplified products are then used as templates for a selective SAMPL-polymerase chain reaction (PCR) reaction that uses the adapter-primer (*EcoRI* oligo-1) in combination with an end-labeled microsatellite-based 15-mer oligonucleotide (Karp and Edwards 1997) to amplify a group of fragments from those fragments that were restricted, ligated, and preamplified. A disadvantage of multilocus SSR profiling is the capture of only some of the polymorphism associated with microsatellites due to the prevalence of dominant markers and difficulty in identifying allelic fragments in complex DNA fingerprints. This

multiplexing genome profiling technique has not been used adequately in plant genomics, although a few reports have already documented its potential for detecting polymorphisms (Molina and Kahl 2002). This method was used for analysis of genetic diversity in *Cicer* (Winter et al. 1999), *Lactuca* (Witsenboer et al. 1997), and *Tribulus* (Sarwat et al. 2008).

2.4.1.10 DAMD (Directed Amplification of Minisatellite-Region DNA)

DAMD is a DNA fingerprinting method based on amplification of the regions rich in minisatellites at relatively high stringencies by using previously found VNTR core sequences as primers (Heath et al. 1993; Somers and Demmon 2002). Minisatellites also known as variable number of tandem repeats (VNTR) or hypervariable repeats (HVR) are similar to microsatellites (SSR) except that the tandem repeat DNA sequences are longer and generally consist of 10–60-bp motifs. Extreme variations in the tandem repeat copy number of minisatellite loci are responsible for the polymorphism observed. By using the VNTR core sequences as primers, the directed amplification of minisatellite-region DNA (DAMD) with PCR is capable of producing RAPD-like results for the identification of species (Silva et al. 2001). They are also used to generate highly variable probes for DNA fingerprinting. This method is more reproducible than RAPD due to the longer primers used.

Recently, DAMD-PCR has been applied successfully for genotyping of wheat cultivars and rice species (Zhou et al. 1997). The method has been used for authentication of *Panax* (Ha et al. 2002), *Capsicum* (Ince et al. 2009), *Salvia* (Karaca et al. 2008), and *Morus* (Bhattacharya et al. 2005).

2.4.1.11 SNP (Single Nucleotide Polymorphism)

Single nucleotide polymorphisms (SNPs) are widely observed between individuals, ecotypes, and species, serving as efficient molecular markers particularly in genetic analysis and breeding programs, also including ecological and evolutionary studies.

SNPs are single-base pair positions in the genomes of two (or more) individuals, at which different sequence alternatives (alleles) exist. Polymorphisms result from point mutations (either transition or transversion events) causing single base-pair differences between DNA sequences. According to most recent estimates, one SNP occurs every 100–300 bp (or every 1,000 bp) in any genome (Kwok 2001). SNPs are codominant, single-locus, biallelic markers and they are the most abundant molecular markers known so far. The major SNP genotyping techniques fall into at least six groups (1) direct sequencing, (2) restriction enzyme digestion (cleaved amplified polymorphic sequences = CAPS), (3) allele-specific PCR, (4) allele-specific primer extension, (5) allele-specific oligonucleotide hybridization, and (6) allele-specific oligonucleotide ligation (Weising et al. 2005).

Several molecular markers such as restriction fragment length polymorphism (RFLP), cleaved amplified polymorphic sequence (CAPS), amplification refractory mutation system (ARMS), and single-strand confirmation polymorphism (SSCP) are based on SNPs.

Many biotechnology companies are marketing DNA microarrays that can test a sample DNA for thousands of SNP sequences.

SNPs have been applied in authentication of *Perilla* varieties (Luo et al. 2006a), *Dendrobium officinale* (Ding et al. 2008), *Panax* cultivars (Wang et al. 2010a), *Boehmeria* varieties (Li et al. 2010b).

2.4.1.12 ARMS (Amplification Refractory Mutation System)

ARMS, also known as allele-specific polymerase chain reaction (AS-PCR) is a simple, timesaving, and effective method for detecting any mutations involving single base changes (SNPs) or small deletions. It has become a standard technique that allows the discrimination of alleles (Newton et al. 1989). The basis of ARMS is that oligonucleotides with a mismatched 3′-residue will not function as primers in the PCR. ARMS allows amplification of test DNA only when the target allele is contained within the sample and it does not amplify the nontarget allele. Following an ARMS reaction, the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. A main advantage of ARMS is that the amplification step and the authentication step are combined, in a way that the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. The method provides a quick screening assay that does not require any form of labeling as the amplified products are visualized simply by agarose gel electrophoresis and ethidium bromide staining. Multiplex ARMS or MARMS are a similar approach but there are several primer combinations to be optimized simultaneously, which increases the complexity of the procedure. The ARMS technique has been applied in authentication of *Alisma* (Li et al. 2007), *Panax* (Zhu et al. 2004; Diao et al. 2009), *Rheum* (Yang et al. 2004), *Dendrobium* (Ding et al. 2008; Qian et al. 2008), and *Curcuma* (Sasaki et al. 2002).

2.4.1.13 CAPS or PCR-RFLP (Cleaved Amplified Polymorphic Sequence)

CAPS, originally named PCR-RFLP, is a combination of PCR of target DNA and subsequent digestion with a restriction enzyme (Maeda et al. 1990; Lum et al. 2005). CAPS markers are generated in two steps. In the first step of a standard CAPS experiment, a defined sequence is amplified using specific 20–25 bp primers. In the second step, the PCR-product is digested with a restriction enzyme usually with a four-base recognition specificity. The digested fragments are separated on agarose gels and stained with ethidium bromide. To identify suitable combinations of amplicons and restriction enzymes, a wide range of PCR primer pairs and restriction enzymes need to be screened during the initial phase of a CAPS

project, using a small set of templates. Combinations that reveal informative polymorphisms are then applied to the full set of organisms under investigation. However, the ability of CAPS to detect DNA polymorphism is not as high as SSRs or AFLPs because nucleotide changes affecting restriction sites are essential for the detection of DNA polymorphism by CAPS. Furthermore, the development of CAPS markers is only possible where mutations disrupt or create a restriction enzyme recognition site. Advantages of CAPS include the involvement of PCR requiring only low quantities of template DNA, the codominance of alleles, and the high reproducibility. The results can be easily scored and interpreted. Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of Southern blot hybridization and radioactive detection procedures. However, in comparison with RFLP analysis, CAPS polymorphisms are more difficult to find because of the limited size of the amplified fragments. Furthermore, sequence data are needed to design PCR primers.

PCR-RFLP has been used for authentication of *Alisma* (Li et al. 2007), *Angelica* (Watanabe et al. 1998), *Sinopodophyllum* and *Dysosma* (Gong et al. 2006), *Ephedra* (Guo et al. 2006), *Fritillaria* (Wang et al. 2005, 2007b), *Artemisia* (Lee et al. 2009), *Panax* (Diao et al. 2009; Do et al. 2001; Lu et al. 2010b; Um et al. 2001), *Actinidia* (Zhao et al. 2007b), *Atractylodes* (Mizukami et al. 2000), *Glehnia* (Mizukami et al. 1993a), *Astragalus* (Lu et al. 2009), *Dendrobium* (Zhang et al. 2005a), *Duboisia* (Mizukami et al. 1993b), and *Codonopsis* (Fu et al. 1999).

2.4.1.14 SCAR (Sequence Characterized Amplified Region)

In 1993, Paran and Michelmore introduced a new type of RAPD-derived molecular marker, which circumvented several of the drawbacks inherent to RAPDs. A SCAR marker can be used to rapidly amplify a diagnostic nucleic acid from herbal materials using a pair of specific oligonucleotide primers designed from polymorphic RAPD (Semagn et al. 2006; McDermott et al. 1994) or ISSR (Albani et al. 2004) fragments.

Polymorphic fragments from RAPDs or ISSR are selected among amplified fingerprints. After cloning and sequencing for the selected polymorphic regions, pairs of internal primers are designed to amplify a unique and specific sequence designed as a SCAR marker. SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into codominant markers (Paran et al. 1991). Prior sequence information (i.e., sequencing the polymorphic fragments) is required for designing the primers flanking the polymorphic region. As PCR inhibitory effects of ingredients can lead to false negative results, amplification of a control fragment using the same DNA template should be performed to ensure that the quality of sample DNA is suitable for PCR. The concept of generating locus-specific SCARs from anonymous PCR fragments is not restricted to RAPDs but was applied to other multilocus marker techniques such as ISSR or AFLP (Shan et al. 1999; Xu et al. 2001).

The SCAR technique has been used for authentication of *Panax* (Wang et al. 2001; Choi et al. 2008) and for discrimination of species of *Artemisia* (Lee et al. 2006a), *Phyllanthus* (Dnyaneshwar et al. 2006; Theerakulpisut et al. 2008), *Pueraria* (Devaiah and Venkatasubramanian 2008a), *Sinocalycanthus* (Ye et al. 2006), *Embelia* (Devaiah and Venkatasubramanian 2008b), and *Lycium* (Sze et al. 2008).

2.4.1.15 SSCP (Single-Strand Confirmation Polymorphism)

SSCP is a powerful mutation detection system. The principle of this technique is that under neutral condition, the single-stranded DNA (ssDNA) folds into a tertiary structure. Differences in DNA sequences (often a single base pair) alter the single stranded DNA in the tertiary conformation (by differential folding), which in turn affect the mobility of the ssDNA in a gel. Based on their mobility differences, SNPs can be detected (Orita et al. 1989).

F-SSCP is an adapted version of SSCP analysis involving amplification of the target sequence using fluorescent primers (Makino et al. 1992).

The method is not frequently applied for authentication e.g., in *Boesenbergia* (Techaprasan et al. 2008).

2.4.1.16 MSAP (Methylation-Sensitive Amplified Polymorphism)

This tool is a modification of the AFLP technique and was developed for monitoring the state of genomic DNA methylation. Genomic DNA is double-digested with one of the methylation-sensitive enzymes *Hpa*II or *Msp*I and then with the methylation-insensitive *Eco*RI. The resulting fragments are ligated with the corresponding double-stranded adapters, a first preselective amplification is carried out followed by a selective amplification step.

MSAP was first developed to determine DNA methylation events in dimorphic fungi (Reyna-Lopez et al. 1997) and later adapted for the detection of cytosine methylation in the rice genome (Xiong et al. 1999), pepper (Portis et al. 2004), apple (Xu et al. 2000), and Siberian ginseng (Chakrabarty et al. 2003).

2.4.1.17 LAMP (Loop-Mediated Isothermal Amplification)

Loop-mediated isothermal amplification (LAMP), which amplifies target nucleic acids with high specificity, efficiency, and rapidity under isothermal conditions was developed by Notomi et al. (2000). This method relies on auto-cycling strand displacement DNA synthesis performed by a DNA polymerase with high strand displacement activity. A specially designed set of two inner and two outer primers is used. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template

for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycles, one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA and a new stem–loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity (Nagamine et al. 2001, 2002).

This technique was applied to detect *Panax ginseng* (Sasaki et al. 2008), the botanical source of Ginseng (Ginseng Radix), and to distinguish this species from *Panax japonicus*. It was also used for the detection of *Lophophora willamsii* (Sasaki et al. 2009) and *Curcuma longa* (Sasaki and Nagumo 2007).

2.4.1.18 SDA (Subtracted Diversity Array)

PCR-based plant identification techniques are often limited by their low throughput, whereas hybridization-based microarray technology represents a rapid and high-throughput tool for genotype identification. Using an innovative technique, a "Subtracted Diversity Array" (SDA) was constructed from a pooled genomic DNA library of 49 angiosperm species, from which pooled non-angiosperm genomic DNA was subtracted (Jayasinghe et al. 2007). The subtraction was carried out using the Clontech PCR-Select cDNA Subtraction Kit. This new SDA method was shown to be superior to conventional molecular identification methods in terms of accuracy, sensitivity, and efficiency, as well as capacity for high-throughput and broad application. The SDA technique was validated for potential genotyping use. The results indicated a successful subtraction of non-angiosperm DNA. This study demonstrates the potential of establishing a highly informative, reliable, and high-throughput microarray-based technique for the novel application of sequence-independent genotyping of major angiosperm clades.

Niu et al. (2011) showed that SDAs technique is suitable to differentiate two ginseng species, *Panax ginseng* and *Panax quinquefolius*, that are frequently mixed for adulteration. Further, SDA was sensitive enough to detect a deliberate adulteration of 10 % *P. quinquefolius* in *P. ginseng*. Thirty-nine species-specific features including 30 *P. ginseng* specific and nine *P. quinquefolius* specific were obtained. This resulted in a feature polymorphism rate of 10 % from the 376 features used for fingerprinting the two ginseng species. The functional characterization of 14 *Panax* species-specific features by sequencing revealed one putative ATP synthase, six putative uncharacterized proteins, and two retroelements to be different in these two species.

2.4.1.19 MLPA (Multiplexed Ligase-Dependent Probe Amplification)

An assay well suited to medicinal plant species identification is the Multiplexed Ligase-dependent Probe Amplification (MLPA) assay (Barthelson 2009; Shen and Wu 2009). MLPA is a semi-quantitative PCR-based technique initially developed by Schouten et al. (2002). It uses the sensitivity of the polymerase chain reaction, but increases the specificity by including a key ligation step for those MLPA probes that hybridize to a DNA sequence. Several key features distinguish this technique from other PCR-based techniques. First, the amplification is ligation dependent as amplification of non-ligated oligoes does not take place. Since ligation occurs at high temperatures, specificity is further ensured. Second, the amplification is highly multiplexed, allowing the detection of up to 50 targets in one single tube assay. This further allowed mutation testing at the exonic and sub-exonic level for a single gene or multiple genes in one assay, thus becoming a cost-effective medium-throughput test. Third, although this is not a real-time PCR because the primers are in excess of templates and the amplification is in linear range, the amount of amplicons generated at the end is in proportion to the templates (ligation products). Last and more importantly, a common PCR primer is used for the amplification of all target sequences, which is a key feature to ensure the relative quantification of each target with respect to a control sample. Due to its low costs, excellent sensitivity, reliability, and ease of development and implementation, the MLPA technique has become a very popular research and diagnostic tool.

2.4.1.20 Real-Time PCR

Real-time quantitative PCR allows the sensitive, specific, and reproducible quantitation of nucleic acids. Since its introduction, real-time quantitative PCR has revolutionized the field of molecular diagnostics and has become the main technical platform for nucleic acid detection in research and development, as well as in routine diagnostics (Klein 2002; Arya et al. 2005). The intention of real-time PCR is the detection of a specific DNA sequence in a sample by measuring the accumulation of amplified products during the PCR using fluorescent technology. Consequently it allows the researcher to better determine the amount of starting DNA in the sample before the amplification process. Present day real-time methods generally involve fluorescence labeling to show the amount of DNA present at each cycle of PCR. In a few cases real-time PCR has recently been used in identification of Chinese medicinal plants (Matsuyama and Nishi 2011; Slanc et al. 2006).

DNA sequence analysis of rDNA internal transcribed spacer (ITS) and fluorescence melting curve analysis using real-time PCR were applied for authentication of the traditional Chinese medicinal plant *Cimicifuga foetida* from four substitutes: *C. heracleifolia*, *C. dahurica*, *C. acerina*, and *C. simplex*. According to the melting temperature—which is a function of the GC/AT ratio, length, and nucleotide sequences of the amplified product—*C. foetida* was differentiated from all other

species (Ying et al. 2009). Based on real-time PCR technology there are further studies for authentication in the genera *Euphorbia* (Xue et al. 2008a), *Gentiana* (Xue et al. 2008b), and *Drynaria* (Xue and Xue 2008) species from adulterants.

The ongoing development of quantitative DNA-based methods using real-time PCR could enable a quantitative analysis of species composition in mixed plant materials and products in the future (Table 2.2).

2.5 DNA Sequencing Analysis

DNA sequencing is the process of determining the precise order of the nucleotides or bases (A,T,G,C) in a particular DNA molecule. The most common approach used for DNA sequencing is the dideoxy or Sanger method which was developed in the mid 1970s. It mimics the basic process used to copy DNA in a cell during chromosomal replication, except that the procedure is done in a tube or microtiter plate using a minimal set of components. Normally the length of the "sequence read" can vary from about 50 to more than 1,000 bases. If the region to be sequenced exceeds the length of a typical sequencing read internal primers have to be used to generate overlapping in order to reconstruct the complete sequence of a longer DNA region. Most large-scale DNA sequencing facilities use fluorescent dyes (rather than a radioactivity isotope) to label and detect the four bases, and capillary electrophoresis to separate DNA molecules on the basis of size. Because the end of each terminated molecule contains a dye-labeled base, the sequence of the strand complementary to the template can be determined. Modern Capillary Sequencer can run plates of 96 samples in a couple of hours and can produce read lengths of more than 700 bases. Using software provided by the manufacturers the signal of the dyes is determined for each position so that the proper base can be identified. The order of the bases is displayed in a chromatogram or trace file.

In the last decade, instrumentation for DNA sequencing has improved dramatically in terms of increasing read length and accuracy, high throughput, and decreasing costs. Meanwhile there are several next-generation DNA sequencing platforms, such as Roche's (454) pyrosequencing system, Illumina's Solexa Genome Sequencer or Applied Biosystem's SOLiD Genome Sequencer.

Currently DNA sequencing is applied in various fields as in analysis of phylogenetic relationship, population genetics, systematics, and evolution (Baldwin et al. 1995). DNA polymorphisms are revealed by determining the nucleotide sequence in a defined region of the genome and aligning the sequence with homologous regions of related organisms (Alvarez and Wendel 2003).

By choosing appropriate regions of the nuclear, plastidal, or mitochondrial genome this approach provides a highly reproducible analysis at various taxonomic ranks to differentiate TCM plants from its substitutes or adulterants.

In order to ensure a correct species identification based on DNA sequence data it is necessary to have herbarium specimen for verification or a reliable database that guarantees that the reference specimen was correctly identified by a taxonomic

Table 2.2 Comparison of DNA profiling techniques for herbal authentication and quality control

	Importance for authentication	Reproducibility	Quantity of DNA required	Level of polymorphism	Locus specificity	Technical demand	Sequence information required	Automation	Running costs	Development costs
RAPD	+	Low	Low	Medium	No	Low	No	Yes	Low	Low/medium
ISSR	++	Medium/high	Low	Medium	No	Low/medium	No	Yes	Low/ medium	Low
AFLP	++	High/medium	Medium	Medium	No	Medium	No	Yes	Medium	Low
RFLP	+	High	High	Medium	Yes	High	Yes	No	High	Medium/high
ARMS	++	High	High	Low	Yes	Low	Yes	Yes	Low	Medium
SSR	+	High/medium	Low	High/medium	No	Low/medium	Yes/no	Yes	Medium	High
CAPS	++	High	Low	Low/medium	Yes	Low/high	Yes	Yes	Low	Medium
SCAR	++	High	Low	Low/medium	Yes	Low/medium	Yes	Yes	Low	Medium
RAMPO	+	Medium	Low	Medium	Yes	High	No	Yes	Medium	Medium
CAPS	++	High	Low	Low/medium	Yes	Low	No	Difficult	Medium	Medium
SSCP	+	Medium	Low	Low	Yes	Medium/high	Yes	No	Low/ medium	High
LAMP	+	High	Low	Low	Yes	Medium	Yes	Yes	Low	Medium
SNP	+	High	Low	High	Yes	Medium	Yes	Yes	Low	High
Sequencing DNA-barcoding	+++	High	Low	Low/medium	Yes	Medium	Yes	Yes	Medium	Medium
Microarray	+++	High	Low	High	Yes	High	Yes	Yes	High	High

The table is based on the most frequent molecular tools and does not represent all DNA-based molecular techniques. Strength and weakness of DNA methods are also summarized by Weising et al. (2005), Yip et al. (2007), Pereira et al. (2008), Hao et al. (2010), Mondini et al. (2009), and Agarwal (2008)

expert. Additionally the sequence should be obtained in independent studies including related taxa. A common way to assign a particular sequence to a taxon is to perform a BLAST search (Basic Local Alignment Search Tool) in the databases of NCBI (GenBank), BOLD, MMDBD. However, care must be taken when assigning the questioned sequence to the species with the highest similarity, because several gaps and false sequences are known to be present in these databases (Heubl 2010).

There are many studies concerning the application of DNA sequence-based markers to differentiate medicinal taxa used in TCM from its substitutes or adulterants.

Sequencing analyses based on nuclear ITS have been applied to *Panax* (Ngan et al. 1999; Kim et al. 2007b), *Asarum* (Kelly 1998; Liu et al. 2005; Yamaji et al. 2007), *Astragalus* (Dong et al. 2003; Yip and Kwan 2006), *Dendrobium* (Xu et al. 2006; Ding et al. 2002; Lau et al. 2001; Zhang et al. 2003), *Fritillaria* (Wang et al. 2005), *Leonurus* (Yang et al. 2006), *Perilla* (Luo et al. 2006b), *Phyllanthus* (Lee et al. 2006b), *Rehmannia* (Albach et al. 2007), *Salvia* (Wang et al. 2005), *Swertia* (Xue et al. 2006a), *Plantago* (Sahin et al. 2007), *Bupleurum* (Yang et al. 2007), and *Euphorbia* (Xue et al. 2006b).

Another frequently used marker is the nuclear 5S rDNA intergenic spacer used for authentication of *Adenophora* (Zhao et al. 2003a), *Aconitum* (Carles et al. 2005), *Angelica* (Zhao et al. 2003b), *Astragalus* (Dong et al. 2003; Ma et al. 2000), *Curcuma* (Xia et al. 2005), *Epimedium* (Sun et al. 2004), *Fritillaria* (Cai et al. 1999), *Crocus* (Ma et al. 2001), *Ligularia* (Zhang et al. 2005b), *Pueraria* (Sun et al. 2007), *Saussurea* (Chen et al. 2008).

From nuclear DNA also 18S rDNA has been tested in *Dioscorea* (Liu et al. 2001a), *Pinellia* (Liu et al. 2001b), and *Panax* (Zhu et al. 2003).

From chloroplast DNA a couple of markers including genes, intergenic spacers, or introns are applied. The atpB-rbcL region was used for differentiation of *Phyllanthus* (Lee et al. 2006b), trnC-trnD in *Panax* (Lee and Wen 2004), trnL-F in *Pueraria* (Albach et al. 2007), *Rheum* (Yang et al. 2001) and *Ephedra* (Long et al. 2004), rpl16 in *Swertia* (Xue et al. 2006b), rpl16-rpl14 spacer in *Scutellaria* (Hosokawa et al. 2005), atpF-atpA in *Angelica* (Hosokawa et al. 2006), trnD-trnT in *Dyosma* (Gong et al. 2006), trnK in *Actinidia* (Zhao et al. 2007c), *Atractylodes* (Mizukami et al. 2000) and *Curcuma* (Sasaki et al. 2002), matK in *Agastache* (Luo et al. 2002), *Panax* (Komatsu et al. 2001), rbcL in *Dryopteris* (Zhao et al. 2007c), *Cnidium* (Kondo et al. 1996), or *Pinellia* (lin et al. 2006).

Recently sequencing analysis has been applied to proof the possibility of DNA-based authentication of plant extracts. Internal transcribed spacer (ITS) was successfully amplified from different extracts types from *Echinacea* species and *Matricaria chamomilla* (Novak et al. 2007).

2.6 DNA Barcoding

DNA barcoding, a term first created by Hebert et al. (2003a) is a novel molecular and bioinformatical tool designed to provide rapid, accurate, automatable, and cost-effective identification of species. Contrary to other molecular methods, it can be used on a large scale and with high reliability. For DNA barcoding, the unique nucleotide sequence patterns of small DNA fragments (400–800 bp) are used as specific reference collections to identify specimens and to discover cryptic taxa (Vijayan and Tsou 2010).

DNA barcoding uses a short genetic marker from a standard locus (alternatively from nuclear, mitochondrial, or plastidal DNA) of an organism. An ideal and successful DNA barcode marker should be suitable for a wide range of taxa (breadth of taxonomic application), routinely retrievable with a universal primer pair, be short enough to be accessible to bidirectional sequencing, and provide a unique sequence for maximal discrimination among species which means high variation between species but conserved within the species, so that the intra-specific variation will be insignificant (CBOL 2009). Additionally these markers should be flanked by evolutionary conserved regions so that universal primers can be used and they should be free of insertions or deletions to be easily alignable (Shneyer 2009) (Fig. 2.1).

2.6.1 Barcoding and Herbal Monographs

DNA authentication of medicinal species used in TCM is of major importance because there are many cases in the Chinese Pharmacopoeia where several species are listed under one common name or listed as synonyms, subspecies, or varieties in herbal monographs.

A prime example is the genus *Dendrobium* (Orchidaceae) which is represented in the Flora of China by 78 species (14 endemic). Overlapping distribution ranges coupled with high morphological variation makes proper species identification difficult. Based on processing methods, Herba Dendrobii is classified into fresh *Dendrobium*, "Fengdou Shihu," and "Huangcao Shihu," the latter being the predominant form of Herba Dendrobii on Chinese herbal medicine market.

As source material for Herba Dendrobii the Pharmacopoeia of the People's Republic of China (2010 edition) lists four species, *D. catenatum* Lindl. (syn. *D. officinale* K. Kimura and Migo), *D. nobile* Lindl., *D. chrysotoxum* Lindl., and *D. fimbriatum* Hook. as the authorized plant sources. However in the Chinese Materia Medica Dictionary (2006 edition), 16 further species are enumerated as medicinal plants: *D. aduncum* Wall. ex Lindl., *D. bellatulum* Rolfe, *D. chrysanthum* Wall. *ex* Lindl., *D. chryseum* Rolfe, *D. crepidatum* Lindl. *ex* Paxt, *D. cucullatum* R. Br., *D. densiflorum* Lindl. ex Wall., *D. devonianum* Paxt, *D. hancokii* Rolfe, *D. henryi* Schltr., *D. hercoglossum* Rchb. fil., *D. linawianum* Rchb. fil., *D. loddigesii* Rolfe, *D. lohohense* Tang et Wang, *D. longicornum* Lindl., and *D. moniliforme* (L.) Sw. Further *Dendrobium* species that are not mentioned in either of these references are

Table 2.3 Plant DNA barcoding markers tested for their suitability and recommendations for barcoding in land plants and Chinese medicinal plants (Ycf5 is also known as ccsA)

DNA segment tested for suitability	Proposed/recommended DNA markers	References
nrITS, atpB-rbcL, psbM–trnD, trnC-ycf6, trnH-psbA, trnL-F, trnK-rps16, trnV-atpE rpl36-rps8, ycf6-psbM	nrITS + trnH-psbA	Kress et al. (2005)
ITS1, accD, ndhJ, matK, trnH-psbA, rbcL, rpoB, rpoC1, ycf5	rbcL + trnH-psbA	Kress and Erickson (2007)
atpF-atpH, atpH-atpI, rps15-ycf1, ndhG-ndhI, psbK-psbI, petA-psbJ, trnH-psbA	atpF-atpH + psbK-psbI	Lee et al. (2007)
rpoC1, rpoB, matK, trnH-psbA, nrITS, trnL-F	rpoC1 + rpoB + matK or rpoC1 + matK + trnH-psbA	Chase et al. (2007)
nrITS, accD, ndhJ, matK, trnH-psbA, rpoB, rpoC1, ycf5	nrITS	Sass et al. (2007)
accD, matK, trnH-psbA, rbcL, rpoB, rpoC1, UPA	matK + trnH-psbA	Newmaster et al. (2008)
TrnL (UAA) intron	trnL (UAA) intron	Taberlet et al. (2007)
matK, trnH-psbA, psbK-psbI, atpF-atpH	matK or matK + trnH–psbA + psbK-psbI	Lahaye et al. (2008a)
accD, matK, trnH-psbA, rbcL, rpoB, rpoC1, ycf5, ndhJ	matK or matK + trnH–psbA	Lahaye et al. (2008b)
Cox1, 23S rDNA, rpoB, rpoC1, rbcL, matK, trnH-psbA, atpF-atpH, psbK-psbI	matK and psbK-psbI + trnH-psbA + atpF-atpH	Fazekas et al. (2008)
trnH-psbA, rbcL, rpoC, CO1, rpoB, matK	trnH-psbA + rbcL or rpoB or rpoC	Erickson et al. (2008)
atpF-atpH, rpoB, rpoC1, rbcL, matK, psbK-psbI, trnH-psbA	rbcL + matK	Hollingsworth et al. (2009)
accD, matK, ndhA, ndhJ, ndhK, rpl22, rpoB, rpoC1, rpoC2, ycf2, ycf5, ycf9	rpoC1 + rpoB + matK	Ford et al. (2009)
atpF-atpH, matK, rbcL, rpoB, rpoC1, psbK-psbI, trnH-psbA	rbcL + matK	CBOL-PWG (2009)
psbA-trnH, matK, rbcL, rpoC1, ycf5, ITS2, and ITS	ITS2 + psbA-trnH	Chen et al. (2010)

Proposed markers are based on universality, success of PCR amplification, interspecific sequence variability, and species discriminatory power/identification capability. Several proposed combinations of loci have been summarized by Pennisi (2007), Shneyer (2009), and Vijayan and Tsou (2010). The combination *rbcL* + *trnL-F* (intron and IGS) has been proposed as a two-locus barcode for ferns (De Groot et al. 2011). The best performing single loci for mosses were the *rbcL* and *rpoC1* coding regions (Liu et al. 2010b)

additionally processed. A survey of the medicinal *Dendrobium* species revealed that in total 27 other *Dendrobium* species are also used clinically as substitutes for "Huangcao Shihu" at various places in China, a situation which may cause inconsistent therapeutic effects or even endanger safety of consumers (Li et al. 1986, 1991; Ma et al. 1995). The stems of *Dendrobium* species, have very similar morphological and anatomical characteristics, and the traditional authentication

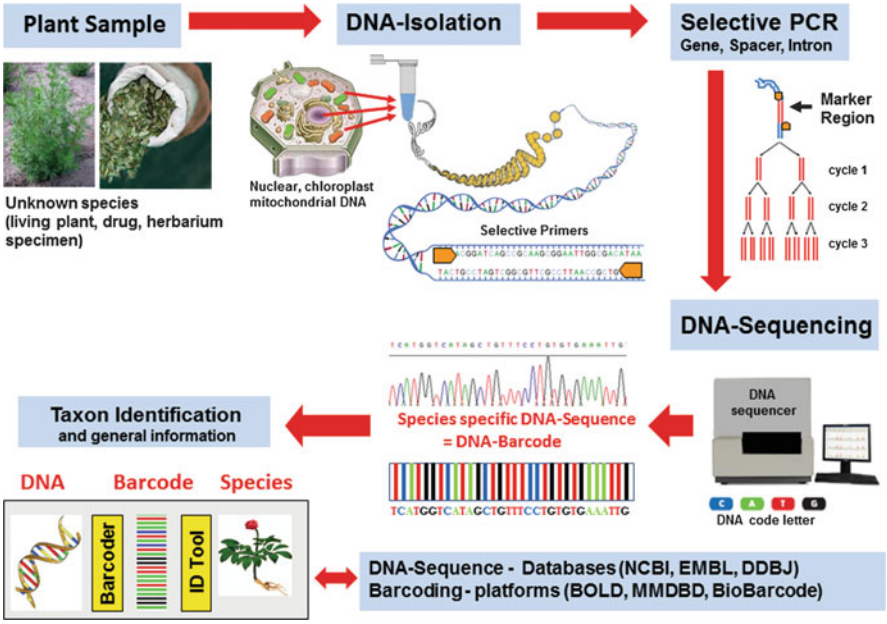

Fig. 2.1 Major steps in the DNA barcoding process. These procedures include tissue sampling, DNA extraction, polymerase chain reaction and marker amplification, PCR product check, and sequencing. The end product of this procedure will be a unique, species-specific "barcode" sequence of the marker (gene, spacer, intron) that can be used for species identification and submitted to a DNA database (e.g., GenBank-NCBI, Bold). The original specimen is kept as a voucher. All collateral information (identification, collection data, etc.) are stored along with the DNA barcode sequence

of different "Huangcao Shihu" samples is therefore far from reliable. In addition, the chemical constituents of many *Dendrobium* species are still unknown, as proper chemical analysis methods have not been developed. However, the determination of the botanical origins of different "Huangcao Shihu" samples and their quality control through morphological and chemical studies is fraught with difficultly. Consequently DNA barcoding may offer an alternative method for the identification of the used *Dendrobium* species for herbal medicines (Xu et al. 2006; Takamiya et al. 2011).

A further remarkable example where multiple species are used in TCM is the genus *Fritillaria* (Liliaceae) which includes 24 species (15 endemic) in China. Bulbs of various *Fritillaria* species (Bulbus Fritillariae) are among the most popular herbal medicines in China and have been used as antitussive and expectorant herbs. According to the Chinese Phamacopoeia, herbal "Beimu" is derived from the bulbs of nine *Fritillaria* species. These include *Fritillaria thunbergii* Miq. ("Zhebeimu"), *F. cirrhosa* D. Don. (Chuanbeimu), *F. unibracteata* Hiao et Hsia, *F. przewalskii* Maxim ex Batal, *F. delavayi* Franch, *F. ussuriensis* Maxim. (Pingbeimu), *F. walujewii*, *F. pallidiflora* Schrenk ("Yibeimu"), and *F. hupehensis* Hsiao et K.C ("Hubeibeimu").

Furthermore other *Fritillaria* species (e.g., *Fritillaria anhuiensis* S.C. Chen et S.F. Yin, *Fritillaria puqiensis* G.D. Yu et G.Y. Chen) are often used in different regions in China as substitutes. These distinctions can be very confusing for consumers. As there are no specific microscopic characteristics and the pattern and concentration of chemical components are often unstable, for quality control and standardization of *Fritillaria* derived herbal medicine, exact species identification using DNA barcoding is the only reliable method (Wang et al. 2007b).

Another example where the Chinese Phamacopoeia allows some interchangeable use of herbals is the genus *Epimedium* (Berberidaceae) which is one of the most popular herbal drug ("Yinyanhuo") comprising 41 species (40 endemic) in China. Herba Epimedii is prepared from aerial parts of five *Epimedium* species listed in the Chinese Pharmacopoeia: *E. brevicornum* Maxim., *E. koreanum* Nakai, *E. sagittatum* (Sieb. et Zucc.) Maxim., *E. pubescens* Maxim., and *E. wushanense* T. S. Ying. In common with many traditional medicinal plants, a major problem with *Epimedium* is the absence of a rigorous method to authenticate species. Taxonomists have variably reported numbers ranging from 20 to 50 species (Sun et al. 2005). Traditional herbalists do not differentiate among *Epimedium* species, but rather use a mixture of species together as *Herba Epimedii*. These species differ significantly in concentrations of major and minor constituents (Wu et al. 2003). The extensive variation in morphological traits make the species difficult to classify taxonomically and there is some confusion due to lack of scientific research.

Considering the examples mentioned above there are numerous monographs in the Chinese Pharmacopoeia where multiple species (further examples *Dioscorea* and *Uncaria* each with five species listed) are a legitimate source for the preparation of herbal medicines. As these species have a different composition of bioactive compounds and are quantitatively and qualitatively not equivalent, the therapeutic efficacy of a TCM medication can vary to a high extent. For species-rich genera lacking morphological characters or taxa of controversial botany, DNA barcoding as a new tool is a prerequisite to clearly distinguish the authentic herb from the substitute/adulterant. If the barcode DNA-analysis of all frequently used

Chinese drugs becomes available in the near future, the application of this method and further DNA fingerprinting techniques should be included in monographs to optimize the quality and safety proof of the drugs (Fig. 2.2).

2.6.2 Standard Markers for Barcoding

In certain animal groups the mitochondrial cytochrome *c* oxidase subunit 1 (CO1, *cox1*) gene sequence (ca. 650 bp in length) is currently used as a universal DNA barcode (Hebert et al. 2003b; Waugh 2007). It has been introduced as standard marker as it can be easily sequenced and provides greater than 97 % species-level specificity in different animal phyla, e.g., birds (Hebert et al. 2004), mammals (Luo et al. 2011), fishes (Ward et al. 2009), amphibians (Vences et al. 2005), and various groups of arthropods (Virgilio et al. 2010). Based on this mitochondrial

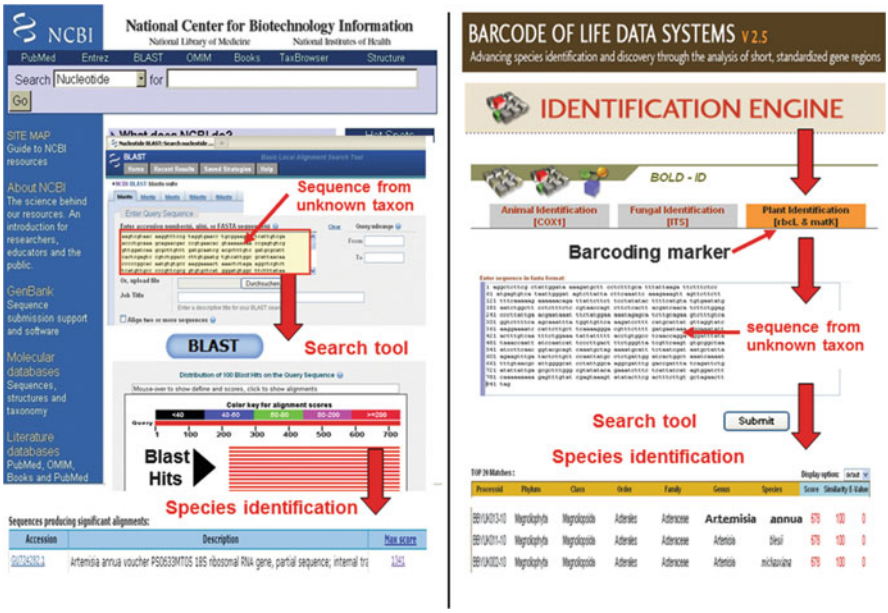

Fig. 2.2 DNA sequence BLAST search against the databases of GenBank (NCBI) and BOLD using the barcoding markers *ITS* and *rbcL* + *matK* sequences of *Artemisia annua* as a query. NCBI (http://www.ncbi.nlm.nih.gov) offers the BLAST search tool to perform fast searching with rigorous statistical methods for judging the significance of matches. The Barcode of Life Data Systems (BOLD; http://www.boldsystems.org/views/login.php) and its Identification System (IDS) for *rbcL* and/or *matK* is an identification tool for plant species. The ID engine uses all sequences uploaded to BOLD to locate the closest match. It returns a species-level identification when possible

gene, DNA barcoding is progressing rapidly in many groups of animals, which can be reviewed online via the Canadian Barcode of Life (http://www.bolnet.ca) and the Consortium for the Barcode of Life (CBOL; http://www.barcoding.si.edu). Other alternative mitochondrial regions such as cytochrome b, 12S, 18S (SSU), 28S (LSU) rRNA, and the nuclear ITS2 region have been proposed (but not established) for animal identification (Wong et al. 2001; Chen et al. 2010; Ferri et al. 2009; Yao et al. 2010).

However, the cytochrome c oxidase marker, which is widely used in animal barcoding, seems promising only for algae (Robba et al. 2006; Lane et al. 2007) but it is not suitable for higher plants because of a much slower substitution rate in cox1 which is two- to threefold lower than in chloroplast genes and 10- to 20-fold lower than in nuclear genes (Wolfe et al. 1987; Drouin et al. 2008). Furthermore the mitochondrial genome in plants is characterized by the occurrence of large structural rearrangements and often nonfunctional copies of mitochondrial genes (pseudogenes) which cause erroneous results. Consequently in plants appropriate barcoding regions have been analyzed, tested, and selected for discriminating

among the 260,000–422,000 species of seed plants (Govaerts 2001; Thorne 2002), but until now no agreement exists which marker is the most promising, though most researchers agree that more than one region is necessary. Consequently a multilocus approach based on the plastid genome with a focus on coding and noncoding regions (introns or spacers) is currently the most effective strategy for species identification in plants (Chase et al. 2005, 2007; Kress et al. 2005; Newmaster et al. 2006, 2008; Cowan et al. 2006; Ford et al. 2009; Kress and Erickson 2007; Lahaye et al. 2008a, b; Fazekas et al. 2008).

In order to promote the use of DNA barcoding for all eukaryotic organisms, the Consortium for the Barcode of Life (CBOL, http://barcoding.si.edu) was established in 2004 at the National Museum of Natural History in Washington, which currently includes more than 120 organizations from 45 nations.

In 2005 the Plant Working Group (PWG CBOL) reported that five very promising regions were chosen for further barcoding studies. These regions were *matK* (encodes a maturase and is located within the *trnK* gene), *rpoB* (RNA polymerase subunit), *rpoC1* (RNA polymerase subunit), *accD* (subunit of acetyl-CoA carboxylase), and *ccsA* (previously known as *ycf5*; the gene encoding a protein involved in cytochrome *c* biosynthesis).

In a subsequent work in 2007, additional candidate plastid DNA regions were tested in different groups (gymnosperms, angiosperms, ferns, equisetes, and mosses).

Based on assessments of recoverability, sequence quality, and levels of species discrimination, a core two-locus combination of *rbcL* and *matK* as the plant barcode was recommended. This combination was shown to successfully discriminate among 907 samples from 550 species at the species level with a probability of 72 % (CBOL Plant Working Group 2009). The group admits that the two-locus barcode is far away from perfection due to the limited identification rate, and thus further research for other appropriate candidates is necessary. Additional combinations of noncoding and coding plastid regions have been tested for barcoding purposes (Fazekas et al. 2008; Pennisi 2007; Ford et al. 2009).

Other combinations involving three plastid regions have also been proposed by various working groups that include the Royal Botanical Gardens, Kew, UK http://www.rbgkew.org.uk/barcoding (Chase et al. 2007).

In view of the available markers as a core barcode to identify land plants a combination of two loci from plastidal DNA, *rbcL* + *matK* was adopted as "the plant barcode" by the Executive Committee of the Consortium for the Barcoding of Life (CBOL Plant Working Group 2009) after much deliberation (e.g., Kress et al. 2005; Hollingsworth et al. 2009). They agreed that *rbcL* and *matK* are approved and the most suitable barcode regions for land plants. Based on these markers, the BOLD Identification System (BOLD-IDS) provides a species identification tool that accepts DNA sequences with a minimum sequence length of 500 bp from these two barcode region and returns a taxonomic assignment at the species level (a list of the nearest matches) when possible. The group admits that the two-locus barcode is far away from perfection due to the low identification rate and that the search is not over.

Additionally the plastidal *psbA-trnH* intergenic spacer and internal transcribed spacer region (ITS1-5.8S rRNA-ITS2) of nuclear ribosomal DNA have been tested as supplementary barcodes. Kress et al. (2005) suggested that these two noncoding regions might have potential as universal plant barcodes. Generally the ITS, which is part of the ribosomal operon, is organized in large blocks (tandem arrays of nearly 50–100 copies) in the chromosome of the nuclear organizer region. It is one of the most commonly sequenced DNA regions (ca 700 bp) used in plant phylogenetic studies at the generic and species level. The advantage of these spacers lies in their high variability. They are adjacent to the conserved 5.8S rRNA region and flanked by conserved 18S and 26S rRNA genes, which facilitate the development of primers. Meanwhile more than 50,000 plant ITS sequences have been deposited in GenBank (Hajibabaei et al. 2007). As nuclear ITS is arranged in multiple copies, paralogs may occur due to hybridization and subsequent incomplete concerted evolution. As a consequence in some groups the presence of several functional copies was detected (Alvarez and Wendel 2003). Not only the sequence is an effective barcode but also the secondary structure of the ITS2 region could provide useful information for species identification and has potential as a molecular morphological characteristic (Yao et al. 2010).

The *trnH-psbA* spacer, though short in length (ca. 450 base pairs), is one of the most variable plastid regions in angiosperms and can be easily amplified across a broad range of land plants (Shaw et al. 2007). In general intergenic spacers and introns tend to be more variable than genes and are therefore considered as better identifiers.

Recently, Chen et al. (2010) compared seven candidate DNA barcodes from Chinese medicinal plant species. This study including a total of 4,800 species from 753 distinct genera revealed that the Internal Transcribed Spacer 2 (ITS2) of nuclear rDNA and the *psbA-trnH* locus are promising universal barcodes for plant identification. A test of the discrimination ability of ITS2 showed that the rate of successful identification at species level was 92.7 % for ITS2 and 72.8 % for *psbA-trnH*.

Shaw et al. (2007) compared chloroplast genomes of Solanaceae, Fabaceae, and Poaceae and showed that no less than nine intergenic spacers, which were practically not used in molecular phylogenetic studies (*rpl32-trnL*, *trnQ-rps16*, *trnV-ndhC*, *ndhF-rpl32*, *psbD-trnT*, *psbJ-petA*, *rps16-trnK*, *atpI-atpH*, *petL-psbE*), were far more variable than the most variable ones of those spacers used earlier. Probably these regions have potential as DNA barcoding markers, if not universal, then suitable for studies on higher taxonomic level.

Moreover, further DNA barcode markers have been used to identify medicinal plants from different plant families and genera (Chen et al. 2010; Song et al. 2009; Luo et al. 2010; Pang et al. 2010; Gao et al. 2010a; Sun et al. 2010; He et al. 2010) including also medicinal Pteridophytes (Ma et al. 2010) (Table 2.3).

In 2008 several DNA barcoding projects have been initiated in China when the country became a central node of the International Barcode of Life project (*iBOL*). The Barcoding Chinese Plants Project is closely associated with the Germplasm Bank of Wild Species (GBOWS, Kunming Institute) which was founded to

safeguard and barcode about 6,000 species of vascular plants of the flora of China. Based on a nationwide seed collecting program a large data set, involving 6,286 individuals representing 1,757 species in 141 genera of 75 families of seed plants, was assembled by the China Plant BOL Group. Comprehensive analysis of the dataset was made to assess the universality, sequence quality, and discriminatory power of the chosen barcoding markers (Li et al. 2011a).

Meanwhile an integrated DNA barcode database (MMDBD) is under construction (Lou et al. 2010). This platform contains ca. 1,300 species of Chinese medicinal plants listed in the Pharmacopoeia of the People's Republic of China and it provides information on storage, retrieval, comparison, and analysis of DNA sequences, for distinguishing medicinal materials from their common substitutes and adulterants (see http://www.cuhk.edu.hk/icm/mmdbd.htm).

In combination with phylogenetic analyses, in recent years barcoding activities have been intensified in many genera including Chinese medicinal plants. Barcoding projects based on nuclear and/or plastidal markers has been applied in different plant families e.g., Rosaceae (Pang et al. 2011), Caprifoliaceae (Liu et al. 2010a), Asteraceae (Gao et al. 2010b), Loranthaceae (Li et al. 2009b), Euphorbiaceae (Pang et al. 2010), Fabaceae (Gao et al. 2010b), Polygonaceae (Song et al. 2009), Rutaceae (Luo et al. 2010), Myristicaceae (Newmaster et al. 2008), Lemnaceae (Wang et al. 2010b), Lamiaceae (Han et al. 2009), as well as in the genera *Panax* (Zuo et al. 2011), *Dendrobium* (Asahina et al. 2010), *Lonicera* (Sun et al. 2010), *Paris* (Zhu et al. 2010), *Aconitum* (He et al. 2010), Taxillus (Li et al. 2010c), *Amomum* (Zhen-Yan and Ling 2010), *Astragalus* (Guo et al. 2010), *Paeonia* (Zhang et al. 2009), in Cycadales (Sass et al. 2007), and also in medicinal Pteridophytes (Ma et al. 2010).

Molecular authentication of herbal medicinal materials has increased enormously in the last decade and its advantages are undisputed. This new tool has been included for the first time in the *Pharmacopoeia of the People's Republic of China* and its online Supplementary Note 2 as a standard method for the identification of some traditional Chinese medicines (Li et al. 2011b).

Once fully developed, DNA barcoding has the potential to completely revolutionize our knowledge of plant diversity. In the future scientists will be able to quickly identify known species and retrieve information about them. Compiling a public library of sequences linked to vouchered specimens and their scientific binomial names will make barcoding a practical and efficient tool for the identification of species, including all Chinese medicinal plants (Fig. 2.3).

2.7 DNA Microarrays (DNA Chip Technology)

The DNA chip technology developed by Fodor et al. (1991) enables the production of a "biochip" designed to identify fluorescent-labeled DNA or RNA fragments through their hybridization to oligonucleotide probes. DNA microarrays are a high-throughput technology for simultaneous analysis of multiple genes in many taxa or

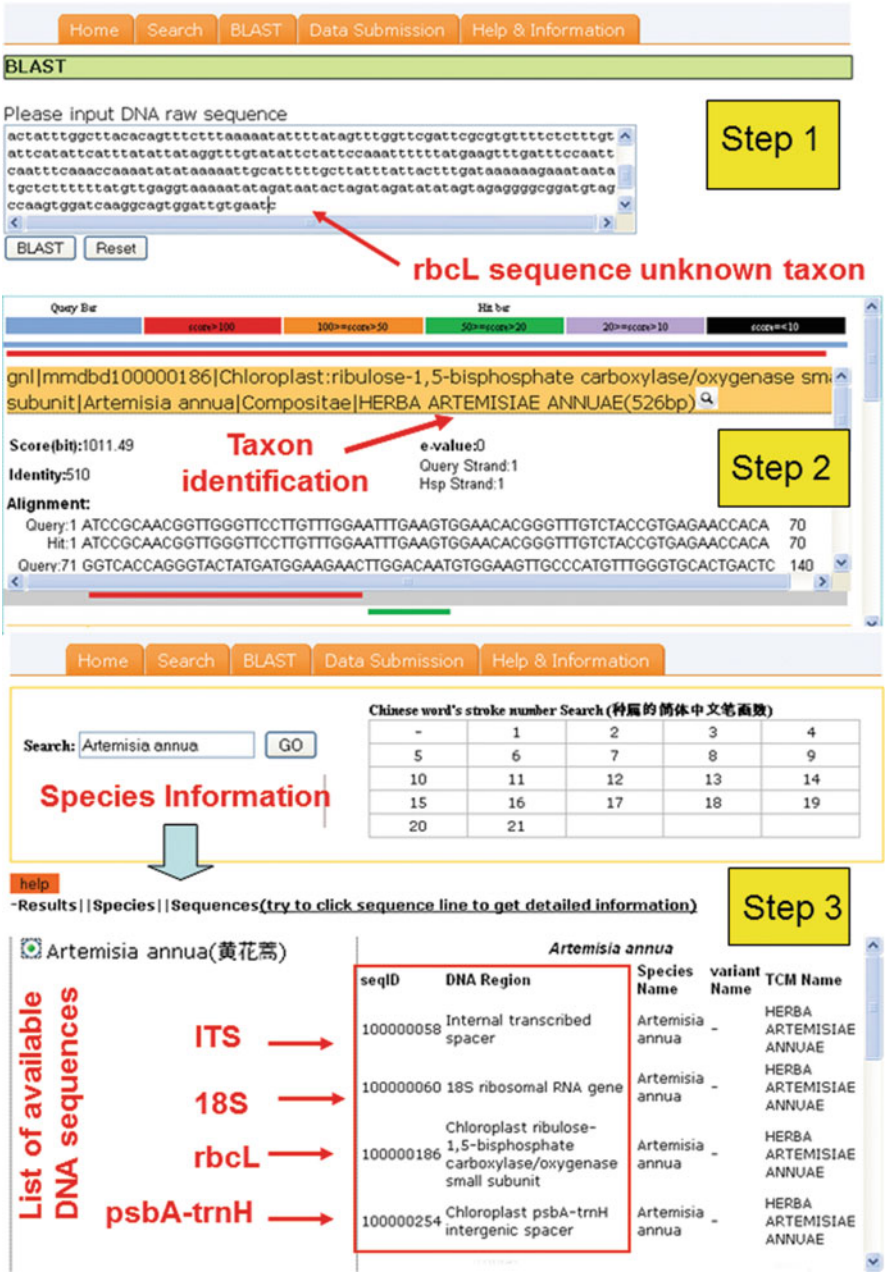

Fig. 2.3 (continued)

Fig. 2.3 Steps in the identification process using the platform MMDBD (Medicinal Materials DNA Barcode Database—http://137.189.42.34/mherbsdb/index.php). Search and result page with sequence similarity BLAST search using the *rbcL* sequence of *Artemisia annua* for query (steps 1–3). Information page for medicinal materials including herb name, species name, family name, medical part, pharmacopoeia information, status in CITES, adulterant, DNA sequence (with voucher), and key reference (step 4)

samples (Fodor et al. 1993; Gershon 2002). To apply this technique for identification and authentication of herbal material, it is necessary to identify a distinct DNA sequence that is unique to each species (Preeti et al. 2006a, b). The DNA sequence information is then used to synthesize a corresponding probe on a chip. These probes are capable of detecting complementary target DNA sequences if present in the test sample being analyzed. These immobilized DNA fragments are arranged in

a regular pattern on a microarray by fixation on glass slides, silicon, or nylon membranes (Gebauer 2004).

DNA extracted from the target sample and labeled with a specific fluorescent molecule is then hybridized to the microarray DNA. A positive hybridization result is detected by the intensity of the fluorescence, which reflects the stability of the hybridization between the oligonucleotide probe and the target sequence, and it is visualized with fluorescence scanning or imaging equipment. Each hybridization surface may contain a very large number of unique oligonucleotide probes (up to 400,000), permitting several thousand individual nucleotide positions to be characterized at the same time. The DNA microarray field is a combination of several technologies, including automated DNA sequencing, DNA amplification by PCR, oligonucleotide synthesis, nucleic acid labeling chemistries, and bioinformatics.

Recently this technique has been applied for the identification of various species of *Fritillaria* (Tsoi et al. 2003), *Dendrobium* (Li et al. 2005; Zhang et al. 2003), and *Bupleurum* (Lin et al. 2008). Previously, the nucleotide sequences of the nuclear 18S rRNA gene of 13 *Panax* taxa were determined. On the basis of the nucleotide differences, a DNA microarray (PNX array) was developed for the identification of various *Panax* drugs (Zhu et al. 2008). A silicon-based DNA microarray was designed and fabricated for the identification of toxic traditional Chinese medicinal plants. Species-specific oligonucleotide probes were derived from the 5S ribosomal RNA gene of *Aconitum carmichaeli*, *A. kusnezoffii*, *Alocasia macrorrhiza*, *Croton tiglium*, *Datura inoxia*, *D. metel*, *D. tatula*, *Dysosma pleiantha*, *Dy. versipellis*, *Euphorbia kansui*, *Hyoscyamus niger*, *Pinellia cordata*, *P. pedatisecta*, *P. ternata*, *Rhododendron molle*, *Strychnos nux-vomica*, *Typhonium divaricatum*, and *T. giganteum* (Carles et al. 2005).

The analyses demonstrated that DNA microarray-based technology can provide a rapid, high throughput tool for correct botanical identification, for authentication of crude plant materials, standardization, and for quality control being used for hundreds of samples simultaneously (Debouck and Goodfellow 1999).

This application of DNA microarrays will not only benefit the herbal drug industry but can also facilitate the identification of herbal products by regulatory authorities (Fig. 2.4).

2.8 Limitations of Genetic Markers in Herbal Drug Technology

Molecular authentication methods in comparison to macroscopic, microscopic, and phytochemical analyses have several advantages, which make them suitable for the identification of plants used in TCM. The DNA-based techniques are not affected by environmental factors, independent from the physical form of the plant material, and requite only a low amount of material. Although DNA analysis is currently considered to be cutting-edge technology, it has certain limitations:

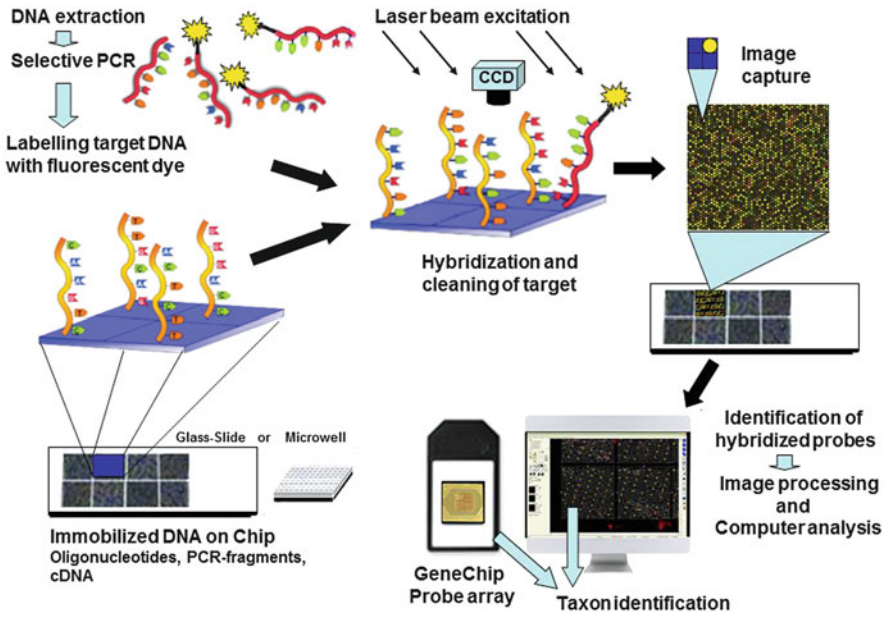

Fig. 2.4 Workflow overview with major steps of an automated and high throughput DNA microarray platform for species identification

- The applicability of a DNA-based method depends generally on the quality and quantity of the DNA. When plant drugs are processed under extreme conditions DNA degradation (fragmentation) is observed, making PCR amplification impossible. Drug processes involving mechanical stress, high temperature, pH variations, enzymatic activities, and fermentations affect the primary structure of DNA and cause often hydrolysis, oxidation, and deamination of the DNA. Plant-derived polyphenolics are also capable of oxidative DNA damage particularly in the presence of transition metal ions (Bauer et al. 2003; Malik et al. 2003; Novak et al. 2007).
- The isolation of high quality genomic DNA being essential for molecular applications becomes difficult for many medicinally used plants due to the presence of secondary compounds which serve mainly for chemical defense against herbivores. High concentrations of these secondary compounds (polysaccharides, tannins, essential oils, polyphenolics, alkaloids, etc.) often influence DNA extraction, PCR reaction, or restriction digestion. In tissues of medicinal plants, secondary compounds generally get accumulated and the problem becomes increasingly severe as the material gets older. Polysaccharide contaminations are particularly problematic as they can inhibit the activity of many commonly used enzymes, such as polymerases, ligases, and restriction endonucleases. Polyphenol contaminations of DNA make it resistant to restriction enzymes and interact irreversibly with proteins and nucleic acids. Choosing

the most suitable DNA extraction procedure may help to eliminate the PCR inhibitors (Friar 2005; Pirttilä et al. 2001; Fleischmann and Heubl 2010).

- Sometimes plant materials are contaminated with symbiotic or pathogenic microbes. Especially endophytic fungi often occur as symbionts living within the tissues of their angiosperm hosts. DNA isolation techniques for obtaining genomic plant DNA do not discriminate between plant and fungal DNA and PCR primers with broad applicability also amplify DNA contaminants. Fingerprinting using RAPD analyses are particularly vulnerable to these fungal contaminants because of the short length of their primers. Although the amount of endofungal DNA is presumed small as compared to the host genomic DNA, researchers need to consider its potential presence. Problems with endophytic fungi can be eliminated with plant-specific primer design for nuclear markers (e.g., ITS) (Saar et al. 2001).
- The orthology of characters is one of the fundamental and implicit assumptions in the use of DNA sequence data to reconstruct phylogeny or to establish barcodes for species. However, some studies revealed the presence of some degree of intra-individual variations among the copies of ITS1 and ITS2 sequences of the nuclear ribosomal cistron (Feliner and Rosselló 2007). Various reasons such as recent hybridization, lineage sorting, recombination among copies, high mutation rate, and pseudogene formation (nonfunctional paralogous) are considered to be the reasons for such variations (Song et al. 2008). Additionally nonfunctional copies of plastidal or mitochondrial pseudogenes, which have been sometimes transferred to the nucleus, have been detected in various eukaryotic organisms, too. For DNA barcoding as a practical molecular method to identify species only orthologous DNA sequences can be used. Consequently cloning of PCR products is sometimes inevitable (Bailey et al. 2003; Buckler et al. 1997).

2.9 Perspectives for Authentication Using DNA Barcoding

The principle of DNA barcoding is that a standardized, fast-evolving short segment of the genome from chloroplast, mitochondrial, or nuclear DNA can be used to quickly and easily identify any organism. For the process of identification and authentication, different aspects of classical taxonomy, laboratory practice, and data management must be considered.

The first step in authentication of medicinal plants is sampling of the correct taxon and determination using identification keys as provided in the Flora of China. The designated species should match the description of the "type" specimen in all relevant diagnostic features. Confirmation of the determined taxon by a plant taxonomist/systematist or expert in the field of pharmacognosy is favorable. The researcher should also give detailed information for all specimens that are used for barcoding, such as latin taxon name, collector, collection date and number, locality, geospatial coordinates (GPS data), as well as information on infraspecific variants

(variety, chemotype, hybrid) in case of cultivated plant material. These data combined with high resolution images should be deposited in public databases (e.g., BOLD, MMDBD). Special emphasis should be placed on unambiguous association of DNA barcodes with individual specimens and corresponding data records. Thus the specimens that are used for DNA barcoding need to be preserved individually, each with a unique identifying number, or sample ID. Consequently deposition of voucher specimens in local herbaria or natural history collections is important for comparison and verification (see sampling instructions BOLD/CCDB).

For barcoding studies it is also essential to reconstruct molecular phylogenies (using software e.g., PAUP, MrBayes, etc.) including a maximum of species of a taxonomic group. There are many genera of medicinal plants in China which exhibit an extraordinary diversity as *Astragalus* (401 species), *Aconitum* (211 species), *Artemisia* (186 species), *Berberis* (215 species), *Clematis* (147 species), *Polygonum* (113 species), or *Salix* (275 species) and phylogenetic information is limited or missing. Consequently in these genera misidentification is very common. In this respect the knowledge of closely related taxa and sister species relationships, the primary candidates that show low interspecific divergence and often share haplotypes, is of main interest. It is conspicuous that DNA barcoding studies generally neglect or underestimate intraspecific genetic variability and variation in the barcode region is not considered (Moritz and Cicero 2004). Thus phylogenetic reconstructions using comprehensively sampled groups are fundamental to explore intraspecific variation and interspecific divergence. For this purpose adequate sampling of several individuals across the geographic range of a species is required. There is also need to examine groups in more detail with frequent hybridization, recent radiations, introgressions, or incomplete lineage sorting (Li et al. 2011b; China Plant BOL Group).

Concerning laboratory practice and improvements, a cost-effective long-term storage for plant tissue samples (e.g., fresh tissue storage in liquid nitrogen or silica gel desiccation) has to be tested and used for DNA preservation. Furthermore DNA extraction from (old) herbarium specimens, increase of sequencing success, particularly from samples containing long mononucleotide repeats, is required. With application of new polymerases with higher fidelity and processivity or using mixtures that include repair enzymes amplification of degraded DNA can be overcome (Mitchell et al. 2005; Hajibabaei et al. 2005). Additionally development and optimization of DNA barcoding primers for a certain taxonomic group or highly degraded DNAs will greatly improve PCR success and multiplex PCR reactions that can routinely amplify barcode markers will significantly reduce laboratory costs. To add more reliability in the identification of species and to complement the barcoding regions more suitable, nuclear barcode loci (low-copy nuclear regions as ADH, waxy, leafy) must be developed. Future barcoding activities should be focused on the discriminating power of standard markers (*rbc*L, *mat*K, ITS, *trn*H-*psb*A) in species-rich angiosperm families as Asteraceae, Fabaceae, Rosaceae, Ranunculaceae, Lamiaceae, and Apiaceae that contain many important Chinese medicinal plants. Next-generation sequencing technologies will

provide exciting new opportunities for barcoding of multiple samples or even entire plastid genomes that can be used as the 'next-generation plant barcode' (Chen et al. 2010; Sucher et al. 2012; Hollingsworth et al. 2011).

In the field of data management, sophisticated tools are required to automatically query segments of individual sequences, to check for different affinities or incongruence within markers, to decode the peaks in over-laid traces of sequencing chromatograms, to check for editing errors or pseudogenes, and to detect microinversions. Furthermore there is demand for improved bioinformatic search routines because sequence identification methods that use local pairwise alignments (e.g., BLAST) are unable to accurately differentiate between highly similar sequences and are not designed to cope with hierarchic phylogenetic relationships or within taxon variability (Little 2011).

Comprehensive databases containing voucher specimens, macro and microscopic data, chemical profiling, and DNA barcoding information would clearly be beneficial for the authentication of medicinal plants and for providing consumers with a safe product. The molecular detection technologies therefore undoubtedly contribute to the research and development of herbal drugs.

2.10 Guidelines for Authentication of Chinese Medicinal Plants

- For ensuring the safety, efficacy, and quality of traditional Chinese herbal medicines classical botanical methodologies have to be applied including collection and conservation of the botanical material (herbarization of plants and silica gel desiccation of leaves for DNA extraction). It is important to supply representative portions of the plant for correct identification, particularly flowering parts, fruits, and seeds. Large specimens should be dissected and mounted to show the main distinguishing characteristics.
- Additionally proper *documentation* is required: who collected the plant, date of collection, collection site (preferably by GPS coordinates), a unique collection number or code, and habitat information (surrounding vegetation, soil, exposition, and landscape). Supplementary photographs of the growing plant in its habitat should be provided. It is advisable to collect several sets of the same specimen so that, following correct identification, one can retain a specimen for later reference. The plant should be determined by an expert (taxonomist) and given a legitimate *Latin binominal name* (including genus, species, subspecies/variety, and author) according to the guidelines and rules of the International *Code of Botanical Nomenclature* (ICBN) (McNeill et al. 2006).
- Online *taxonomic sources* such as the Flora of China Checklist, The Plant List (TPL), The International Plant Names Index (IPNI), World Checklist of selected plant families or Medicinal Plant Names Index (MPNI) offer access to accepted names. If available, the local and/or English common names and relevant information, such as the cultivar name, ecotype, chemotype should also be provided.

- From the collected and properly dried plant material (one or several representative individuals) a *voucher specimen* should be prepared by mounting the plant(s) on a herbarium sheet in a format suitable for conserving the morphological key characters (e.g., flowers, fruits) of the specimen. This voucher which serves as a permanent record and reference of an individual plant species in time and space must be labeled with scientific name and detailed collection data (as mentioned above) and deposited in a registered public herbarium, museum, or repository of a certified research institute. It is appropriate to prepare two or more duplicate voucher specimens ("back ups") that can be sent to taxonomic experts (plant taxonomists) anywhere in the world for confirmation of the species identification. Detailed information on procedures to properly collect, press, and prepare voucher specimens are available (Hildreth et al. 2007; Smillie and Khan 2010; Eisenmann et al. 2012).
- Seeds or other propagation materials should be specified, and all necessary information relating to the identity, quality, and performance of their products, as well as their breeding history should be provided. For the identification of plants grown from seed material it is essential to prepare a voucher specimen. Due to the need for herbal drugs of consistent quality and reliable supply, methods for commercial field cultivation and post-harvest processing should be developed to guarantee high standards with regard to the required bioactive constituents. To achieve genetically improved or new cultivars breeding programs should be developed.
- For *chemical standardization* it is useful to collect bulk material from the population of the reference sample to reduce potential collecting errors. These plants should be harvested during the appropriate season or time period to ensure the best possible quality of source materials. Collection practices should ensure the long-term survival of wild populations and their associated habitats. Medicinal plants/herbal drugs from species that are listed as endangered (CITES, Convention on International Trade in Endangered Species of Wild Fauna and Flora) must not be collected unless the relevant competent authority has given its authorization.
- Using DNA information for species identification, DNA sequences have to be generated to detect differences in nucleotide positions that are a prerequisite for sequence similarity search in databases. This function allows the user to conduct homology searches between the sequence of interest and the data in the public sequence databases (GenBank; MMDBD; BOLD). To make identification reliable, it is necessary to have complete information of all species of a plant genus. This can only be achieved by conducting phylogenetic reconstructions (using a combination of universal DNA barcoding markers of the nuclear and chloroplast genome) including a comprehensive sampling of species from genera of frequently used TCM plants. Only this molecular phylogenetic approach provides evidence for the identification of genetically closely related species or cryptic taxa. All analyses should include voucher numbers of samples, primer sequences, and PCR conditions for generating barcode sequences. All sequences

should be deposited in *DNA databases (GenBank; MMDBD; BOLD)* for future data retrieval, similarity search, and species identification.

- The focus of DNA barcoding analyses should be on these medicinal plant species which are worldwide and the most frequently used official Chinese plant drugs. Special attention should be paid to those medicinal plants that are frequently substituted or adulterated with other species that are morphologically and/or phytochemically indistinguishable. Based on available sequence information (barcodes), other diagnostic molecular identification tools (PCR-RFLP, ARMS, SCAR, etc.) with high discrimination power should be developed for easy, cheap, and reliable identification of species, varieties, and cultivars.
- To examine correlations between *DNA barcoding* and *chemical profiling*, chromatographic techniques (e.g., TLC, HPLC, GC as well as IR, MS, and NMR spectrometry) should be applied in an integrated strategy using the same particular plant source.
- In view of the amount of DNA barcodes deposited meanwhile in databases, it is highly recommended that molecular authentication should be implemented as a standard method for identification of Chinese herbal medicinal materials in future editions of the Pharmacopoeia of the People's Republic of China.
- Beyond that *interdisciplinary workshops/conferences* are necessary to exchange ideas, to highlight perspectives of future research, to stimulate new work in the field of TCM, and to enhance the dialogue between scientists from plant taxonomy, molecular biology, agriculture, phytochemistry, pharmacognosy, and medicine. In addition a *global information network on TCM research* is required which integrates available databases. A modernization of Traditional Chinese Medicine has become necessary and urgent to increase the worldwide acceptance.

2.11 Future Developments

New innovative automated assays and specific tools for DNA analysis are emerging and will contribute to the next generation of technologies. These are minisequencing (Pastinen et al. 1996; Cai et al. 2000), nanoscale DNA sequencing (Pastinen et al. 1996), Microsphere-based suspension arrays (Lowe 2000), and next-Generation Sequencing (Mardis 2008; Lerner and Fleischer 2010). Further extremely promising developments are the nanopore technology for identification of DNA bases with very high confidence and the arrayed primer extension reaction (APEX) which is an enzymatic genotyping method to analyze hundreds to thousands of variations in the genome simultaneously in a single multiplexed reaction (Fortina et al. 2005; Pirrung et al. 2000). Another upcoming method for large-scale multiplex analysis of nucleic acid sequences is the oligonucleotide ligation assay (OLA), which can be applied for the detection of known single nucleotide polymorphisms (SNPs) and allelic discrimination in highly polymorphic genes (Kurg et al. 2000).These novel approaches to DNA sequencing promise complete genomic analysis and have a

high multiplexing capacity and great potentials for genotyping and future taxon identification (Grossmann et al. 1994).

New high-throughput sequencing (HTS) technologies enable application of new molecular approaches. The recent introduction of massive parallel sequencing technology producing millions of DNA sequence reads (in total 0.5–60 giga base pairs) in a single run has revolutionized genomic research in biology and medicine. These so-called next-generation sequencing platforms, such as Roche/454, Illumina/Solexa, Helicos, and ABI/Solid system can sequence DNA faster and at much lower costs in comparison to the conventional 96-capillary system of Sanger sequencing.

Next-generation sequencing techniques (NGS) can be used to address new and long-standing questions previously inhibited by technological and financial limitations (Kircher and Kelsko 2010). Although no "third-generation" platform has been made commercially available yet, several companies have prototype technologies in active development.

References

Agarwal M (2008) Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Rep 27:617–631

Akkaya MS, Shoemaker RC, Specht JE, Bhagwat AA, Cregan PB (1995) Integration of simple sequence repeat and markers into a soybean linkage map. Crop Sci 35:1439–1445

Albach DC, Li HQ, Zhao N, Jensen SR (2007) Molecular systematics and phytochemistry of Rehmannia (Scrophulariaceae). Biochem Syst Ecol 35:293–300

Albani MC, Battey NH, Wilkinson MJ (2004) The development of ISSR derived SCAR markers around the seasonal flowering locus (SFL) in Fragaria vesca. Theor Appl Genet 109:571–579

Álvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol 29:417–434

Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR (2005) Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 5(2):209–219

Asahina H, Shinozaki J, Masuda K et al (2010) Identification of medicinal Dendrobium species by phylogenetic analyses using matK and rbcL sequences. J Nat Med 64(2):133–138

Bailey CD, Carr TG, Harris SA, Hughes CE (2003) Characterization of angiosperm nrDNA polymorphism, paralogy, and pseudogenes. Mol Phylogenet Evol 29:435–455

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Missouri Bot Gard 82:247–277

Balunas MJ, Kinghorn AD (2005) Drug discovery from medicinal plants [J]. Life Sci 78 (5):431–441

Barthelson RA (2009) Protocols for in vitro cultures and secondary metabolite analysis of aromatic and medicinal plants. Identification of medicinal plants and plant sequences: multiplexed MLPA assay. Methods Mol Biol 547(3):277–288

Bauer T, Weller P, Hammes WP, Hertel C (2003) The effect of processing parameters on DNA degradation in food. Eur Food Res Technol 217:338–343

Bhattacharya E, Dandin SB et al (2005) Single primer amplification reaction methods reveal exotic and indigenous mulberry varieties are similarly diverse. J Biosci 30:669–677

Blears M, De Grandis S, Lee H, Trevors J (1998) Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. J Ind Microbiol Biotechnol 21:99–114

Boqian Y, Jing W, Guopei C, Ting W (2009) Isolation and characterization of polymorphic microsatellite loci in a traditional Chinese medicinal plant, Schisandra sphenanthera. Cons Genetics 10:615–617

Borne B, Goraguer F, Joly G, Branchard M (2002) Genetic diversity in European and Argentinian cultivated potatoes (*Solanum tuberosum* subsp. *tuberosum*) detected by inter-simple sequence repeats (ISSRS). Genome 45:481–484

Bornet B, Branchard M (2004) Use of ISSR fingerprints to detect microsatellites and genetic diversity in several related Brassica taxa and Arabidopsis thaliana. Hereditas 140:245–248

Buckler ES, Ipplito SA, Holtsford TP (1997) The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. Genetics 145:111–125

But PPH (1994) Herbal poisoning caused by adulterants or erroneous substitutes. J Trop Med Hyg 97:371–374

But PP, Tomlinson B, Cheung KO, Yong SP, Szeto ML, Lee CK (1996) Adulterants of herbal products can cause poisoning. Br Med J 313:117

Caetano-Anollés G, Gresshoff PM (1996) Generation of sequence signatures from DNA amplification fingerprints with mini-hairpin and microsatellite primers. Biotechniques 20:1044–1056

Caetano-Anollés G, Bassam BJ, Gresshoff PM (1991a) DNA amplification fingerprinting: a strategy for genome analysis. Plant Mol Biol Rep 9:294–307

Caetano-Anollés G, Bassam BJ, Gresshoff PM (1991b) DNA amplification using very short arbitrary oligonucleotide primers. Biotechnology 9:553–557

Cai ZH, Li P, Dong TT, Tsim KW (1999) Molecular diversity of 5S-rRNA spacer domain in Fritillaria species revealed by PCR analysis. Planta Med 65:360–364

Cai H, White PS, Torney D et al (2000) Flow cytometry-based minisequencing: a new platform for high-throughput single-nucleotide polymorphism scoring. Genomics 66:135–143

Cao H, But PPH, Shaw PC (1996a) Authentication of the Chinese drug "ku-di-dan" (herba elephantopi) and its substitutes using random-primed polymerase chain reaction (PCR). Acta Pharm Sin 31:543–553

Cao H, But PPH, Shaw PC (1996b) Identification of herba taraxaci and its adulterants in Hong Kong market by DNA fingerprinting with random primed PCR. China J Chin Mater Med 22:197–200

Carles M, Cheung MK, Moganti S, Dong TT, Tsim KW, Ip NY et al (2005) A DNA microarray for the authentication of toxic traditional Chinese medicinal plants. Planta Med 71:580–584

CBOL Plant Working Group (2009) A DNA barcode for land plants. Proc Natl Acad Sci 106 (31):12794–12797

Chakrabarty D, Yu KW, Paek KY (2003) Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (Eleuterococcus senticosus). Plant Sci 165:61–68

Chan TYK (1997) Monitoring the safety of herbal medicine. Drug Saf 17:209–215

Chan K (2003) Some aspects of toxic contaminants in herbal medicines. Chemosphere 52:1361–1371

Chan TY, Critchley JA (1996) Usage and adverse effects of Chinese herbal medicines. Hum Exp Toxicol 15:5–12

Chan TYK, Chan JCN, Tomlinson B, Critchley JA (1993) Chinese herbal medicines revisited: a Hong Kong perspective. Lancet 342:18–25

Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RO et al (2005) Land plants and DNA barcodes: short-term and long-term goals. Philos Trans R Soc Lond B 360:1889–1895

Chase MW, Cowan RS, Hollingsworth PM, van den Berg C, Madrinan S et al (2007) A proposal for a standardised protocol to barcode all land plants. Taxon 56:295–299

Chatti K, Saddoud O, Salhi Hannachi A, Mars M, Marrakchi M, Trifi M (2007) Analysis of genetic diversity and relationships in a Tunisian Fig (Ficus carica) germplasm collection by random amplified microsatellite polymorphisms. J Integr Plant Biol 49:386–391

Chawla HS (2002) Introduction to plant biotechnology, 2nd rev. edn. Science Publishers, Enfield, NH

Chen KT, Su YC, Lin JG, Hsin LH, Su YP, Su CH, Li SY, Cheng JH, Mao SJ (2001) Identification of Atractylodes plants in Chinese herbs and formulations by random amplified polymorphic DNA. Acta Pharm Sin 22:493–497
Chen J, Chen L, An Z, Shi S, Zhan Y (2002) Non-technical causes of fakes existing in Chinese medicinal material markets. Zhongyaocai 25:516–519
Chen F, Chan HYE et al (2008) Authentication of Saussurea lappa and endangered medicinal material, by ITS DNA and 5S rRNA sequencing. Planta Med 74:889–892
Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X, Luo K, Li Y, Li X, Jia X, Lin Y, Leon C (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLOS ONE 5(1):e86137
Cheng KT, Chang HC, Su CH, Hsu FL (1997) Identification of dried rhizomes of Coptis species using random amplified polymorphic DNA. Bot Bull Acad Sin 38:241–244
Chen JH, Zhou Y, Yang YP (2011) Isolation and characterization of 20 new microsatellite loci in *Coriaria nepalensis* (Coriariaceae). Am J Bot 98(6):e141–e143
Cheng KT, Fu LC et al (1998) Identification of Anoectochilus formosanus and Anoectochilus koshunensis species with RAPD markers. Planta Med 64:46–49
Cheng KT, Su B, Chen CT, Lin CC (2000) RAPD analysis of Astragalus medicines marketed in Taiwan. Am J Chin Med 28:273–278
Cheng JL, Huang LQ, Shao AJ, Lin SF (2002) RAPD analysis on different varieties of Rehmannia glutinosa. Zhongguo Zhongyao Zazhi 27:505–508
Cheung KS, Kwan HS, But PPH, Shaw PC (1994) Pharmacognostical identification of American and Oriental ginseng roots by genomic fingerprinting using arbitrarily primed polymerase chain reaction (AC-PCR). J Ethnopharmacol 42:67–69
Cheung TP, Xue C, Leung K et al (2006) Aristolochic acids detected in some raw Chinese medicinal herbs and manufactured herbal products – a consequence of inappropriate nomenclature and imprecise labelling? Clin Toxicol 44:371
Chiou SJ, Yen JH et al (2007) Authentication of medicinal herbs using PCR-Amplified ITS2 with specific primers. Planta Med 73:1421–1426
Choi YE, Ahn CH, Kim BB, Yoon ES (2008) Development of species specific AFLP-derived SCAR marker for authentication of Panax japonicus C. A. MEYER. Biol Pharm Bull 31:135–138
Chun S, Jian-He W, Shi-Lin C, Huai-Qiong C, Cheng-Min Y (2009) Development of genomic SSR and potential EST-SSR markers in Bupleurum chinense DC. Afr J Biotechnol 8:6233–6240
Chung SM, Staub JE (2003) The development and evaluation of consensus chloroplast primer pairs that possess highly variable sequence regions in a diverse array of plant taxa. Theor Appl Genet 107:757–776
Clark CM, Wentworth TM, Malley DMO (2000) Genetic discontinuity revealed by chloroplast microsatellites in eastern North American Abies (Pinaceae). Am J Bot 87:774–778
Cole CT, Kuchenreuther MA (2001) Molecular markers reveal little genetic differentiation among Aconitum noveboracense and A. columbianum (Ranunculaceae) populations. Am J Bot 88:337–347
Cole IB, Saxena PK, Murch SJ (2007) Medicinal biotechnology in the genus Scutellaria. In Vitro Cell Dev Biol Plant 43(4):318–327
Cowan RS, Chase MW, Kress WJ, Savolainen V (2006) 300,000 species to identify: problems, progress, and prospects in DNA barcoding of land plants. Taxon 55:611–616
Cui XM, Lo CK, Yip KL, Dong TT, Tsim KW (2003) Authentication of Panax notoginseng by 5S-rRNA spacer domain and random amplified polymorphic DNA (RAPD) analysis. Planta Med 69:584–586
Dasmahapatra KK, Mallet J (2006) DNA barcodes: recent successes and future prospects. Heredity 97:254–255
Datwyler SL, Weiblen GD (2006) Genetic variation in hemp and marijuana (Cannabis sativa L.) according to amplified fragment length polymorphisms. J Forensic Sci 51:371–375

De Groot GA, During HJ, Maas JW, Schneider H, Vogel JC et al (2011) Use of rbcL and trnL-F as a two-locus DNA barcode for identification of NW European ferns: an ecological perspective. PLoS One 6(1):e16371

Debelle FD, Vanherweghem JL, Nortier JL (2008) Aristolochic acid nephropathy: a worldwide problem. Kidney Int 74:158–169

Debouck C, Goodfellow PN (1999) DNA microarrays in drug discovery and development. Nat Genet 21:48–50

Desmarais E, Lanneluc I, Lagnel J (1998) Direct amplification of length polymorphisms (DALP), or how to get and characterize new genetic markers in many species. Nucleic Acids Res 26:1458–1465

Devaiah KM, Venkatasubramanian P (2008a) Development of SCAR marker for authentication of Pueraria tuberosa (Roxb. ex. Willd.) DC. Curr Sci 94:1306–1308

Devaiah KM, Venkatasubramanian P (2008b) Genetic characterization and authentication of Embelia ribes using RAPD-PCR and SCAR marker. Planta Med 74:194–196

Diao Y, Lin XM et al (2009) Authentication of Panax ginseng from its adulterants by PCR-RFLP and ARMS. Planta Med 75:557–560

Dictionary of Chinese Materia Medica (2006), 2nd edn. Shanghai Scientific & Technical, Shanghai

Ding T (2002) The botany database: seed plants of China (SPCH), and its applications. Geoscience and Remote Sensing Symposium, 2002. IGARSS '02. 2002 I.E. International

Ding X, Xu L, Wang Z, Zhou K, Xu H, Wang Y (2002) Authentication of stems of Dendrobium officinale by rDNA ITS region sequences. Planta Med 68:191–192

Ding G, Zhang DZ et al (2008) SNP, ARMS and SSH authentication of medicinal Dendrobium officinale Kimura et Migo and application for identification of Fengdou drugs. Biol Pharm Bull 31:553–557

Dnyaneshwar W, Preeti C, Kalpana J, Bhushan P (2006) Development and application of RAPD-SCAR marker for identification of Phyllanthus emblica L. Biol Pharm Bull 29:2313–2316

Do KR, Hwang WJ, Lyu YS, An NH, Kim HM (2001) Molecular authentication of Panax ginseng species by RAPD analysis and PCR-RFLP. Biol Pharm Bull 24:872–875

Dong CH, Yao YJ (2010) On the reliability of fungal materials used in studies on Ophiocordyceps sinensis. J Ind Microbiol Biotechnol. doi:10.1007

Dong TT, Ma XQ, Clarke C, Song ZH, Ji ZN, Lo CK et al (2003) Phylogeny of Astragalus in China: molecular evidence from the DNA sequences of 5S rRNA spacer, ITS, and 18S rRNA. J Agric Food Chem 51:6709–6714

Drouin G, Daoud H, Xia J (2008) Relative rates of synonymous substitutions in the mitochondrial, chloroplast and nuclear genomes of seed plants. Mol Phylogenet Evol 49(3):827–831

Ernst E (2004) Risks of herbal medicinal products. Pharmacoepidem. Drug Saf. 13(11):767–71

Eisenmann SW, Struwe L, Tucker AO (2012) Voucher specimens are essential for documenting source material used in medicinal plant investigations. J Med Active Plants 1(1):30–43

Erickson DL, Spouge J, Resch A, Weigt LA, Kress WJ (2008) DNA barcoding in land plants: developing standards to quantify and maximize success. Taxon 57:1304–1316

Fan XX, Shen L, Zhang X, Chen XY, Fu CX (2004) Assessing genetic diversity of Ginkgo biloba L. (Ginkgoaceae) populations from China by RAPD markers. Biochem Genet 42:269–278

Fan WJ, Luo YM et al (2009) Development of microsatellite markers in Dendrobium fimbriatum Hook, an endangered Chinese endemic herb. Mol Ecol Res 9:373–375

Farnsworth NR, Soejarto DD (1991) Global importance of medicinal plants. In: Akerele O, Heywood V, Synge H (eds) The conservation of medicinal plants. Cambridge University Press, Cambridge, pp 25–51

Fazekas AJ et al (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS One 3(7):e2802

Feliner GN, Rosselló JA (2007) Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species level evolutionary studies in plants. Mol Phylogenet Evol 44:911–919

Ferri G, Alù M, Corradini B, Licata M, Beduschi G (2009) Species identification through DNA "barcodes". Genet Test Mol Biomarkers 13:421–426
Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251:767–773
Fleischmann A, Heubl G (2009) Overcoming DNA extraction problems from carnivorous plants. Anal Jardín Bot Madrid 66(2):209–215
Fodor SP, Rava RP, Huang XC, Pease AC, Holmes CP, Adams CL (1993) Multiplexed biochemical assays with biological chips. Nature 364:555–556
Ford C, Ayres KL, Toomey N, Haider N et al (2009) Selection of candidate coding DNA barcoding regions for use on land plants. Bot J Linn Soc 159(1):1–11
Fortina P, Kricka LJ, Surrey S, Grodzinski P (2005) Nanobiotechnology: the promise and reality of new approaches to molecular recognition. Trends Biotechnol 23:168–173
Friar EA (2005) Isolation of DNA from plants with large amounts of secondary metabolites. Methods Enzymol 395:3–14
Fu RZ, Wang J, Zhang YB, Wang ZT, But PP, Li N, Shaw PC (1999) Differentiation of medicinal Codonopsis species from adulterants by polymerase chain reaction-restriction fragment length polymorphism. Planta Med 65:648–650
Fugh-Berman A (2000) Herb–drug interactions. Lancet 355:134–138
Gao WY, Jia W, Duan HQ, Huang L, Xiao X, Xiao P, Paek KY (2002) Good agriculture practice (GAP) and sustainable resource utilization of Chinese materia medica. Plant Biotechnol 4(3):103–107
Gao T, Yao H, Song JY, Liu C, Zhu YJ, Ma XY, Pang XH, Xu HX, Chen SL (2010a) Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2. J Ethnopharmacol 130:116–121
Gao T, Yao H, Song J, Zhu Y, Liu C, Chen S (2010b) Evaluating the feasibility of using candidate DNA barcodes in discriminating species of the large Asteraceae family. BMC Evol Biol 10:324
Gawel NJ, Jarret RL, Whittemore AP (1992) Restriction fragment length polymorphism (RFLP)-based phylogenetic analysis of Musa. Theor Appl Genet 84:286–290
Ge XJ, Yu Y, Yuan YM, Huang HW, Cheng YAN (2005) Genetic diversity and geographic differentiation in endangered Ammopiptanthus (Leguminosae) populations in desert regions of Northwest China as revealed by ISSR analysis. Ann Bot 95:843–851
Gebauer M (2004) Microarray applications: emerging technologies and perspectives. Drug Discov Today 9:915–917
Gershon D (2002) Microarray technology: an array of opportunities. Nature 416:885–891
Gertner E, Marshall PS, Filandrinos D, Potek AS, Smith TM (1995) Complications resulting from the use of Chinese herbal medications containing undeclared prescription drugs. Arthritis Rheum 38:614–617
Goldstein DB, Schlötterer C (1999) Microsatellites: evolution and applications. Oxford University Press, Oxford, UK
Gong W, Fu CX, Luo YP, Qiu YX (2006) Molecular identification of Sinopodophyllum hexandrum and Dysosma species using cpDNA sequences and PCR-RFLP markers. Planta Med 72:650–652
Govaerts R (2001) How many species of seed plants are there? Taxon 50:1085–1090
Graham-Brown R (1992) Toxicity of Chinese herbal remedies. Lancet 340:673–674
Grossmann PD, Bloch W, Brinson E et al (1994) High-density multiplex detection of nucleic acid sequences: oligonucleotide ligation assay and sequence-coded separation. Nucleic Acids Res 22:4527–4534
Guo BL, Wu M, Si JP, Li JS, Xiao PG (2001) Research on DNA molecular marker of Magnolia officinalis Rehd. et Wils. RAPD study on certified species. Yao Xue Xue Bao 36:386–389
Guo Y, Tsuruga A, Yamaguchi S, Oba K, Iwai K, Sekita S et al (2006) Sequence analysis of chloroplast chlB gene of medicinal Ephedra species and its application to authentication of Ephedra herb. Biol Pharm Bull 29:1207–1211

Guo HY, Wang WW, Yang N et al (2010) DNA barcoding provides distinction between Radix Astragali and its adulterants. Sci China Life Sci 53(8):992–999

Gupta PK, Balyan HS, Sharma PC, Ramesh B (1996) Microsatellites in plants: a new class of molecular markers. Curr Sci 70:45–54

Gupta AR, Patra RC, Das DK, Gupta PK, Swarup D, Saini M (2001a) Sequence characterization and polymerase chain reaction-restriction fragment length polymorphism of the mitochondrial DNA 12S rRNA gene provides a method for species identification of Indian deer. Mitochondrial DNA 19:394–400

Gupta PK, Roy JK, Prasad M (2001b) Single nucleotide polymorphisms: a new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants. Curr Sci 80(4):524–535

Ha WY, Yau FC, But PP, Wang J, Shaw PC (2001) Direct amplification of length polymorphism analysis differentiates Panax ginseng from P. quinquefolius. Planta Med 67:587–589

Ha WY, Shaw PC, Liu J, Yau FC, Wang J (2002) Authentication of Panax ginseng and Panax quinquefolius using amplified fragment length polymorphism (AFLP) and directed amplification of minisatellite region DNA (DAMD). J Agric Food Chem 50:1871–1875

Hajibabaei M, deWaard JR, Ivanova NV, Ratnasingham S, Dooh RT, Kirk SL, Mackie PM, Hebert PDN (2005) Critical factors for assembling a high volume of DNA barcodes. Philos Trans R Soc B 360(1462):1959–1967

Hajibabaei M, Singer GA, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet 23(4):167–172

Hamilton AC (2003) Medicinal plants and conservation: issues and approaches. Paper presented to International Plants Conservation Unit World Wildlife Foundation, UK

Hamilton AC (2004) Medicinal plants, conservation and livelihoods. Biodivers Conserv 13:1477–1517

Han J, Shi L, Song J et al (2009) Testing potential DNA barcoding regions in the Labiatae medicinal plants. Planta Med 75:407

Hao DC, Chen SL, Xia PG, Peng Y (2010) Authentication of medicinal plants by DNA-based markers and genomics. Chin Herb Med 2(4):250–261

Hayashi K (1992) PCR-SSCP—rapid and easy detection of DNA sequence changes. Hum Cell 5:180–184

Hayden MJ, Nguyen TM, Waterman A, Chalmers KJ (2008) Multiplex-ready PCR: a new method for multiplexed SSR and SNP genotyping. BMC Genomics 9:80

He J, Wong KL, Shaw PC, Wang H, Li DZ (2010) Identification of the medicinal plants in Aconitum L. by DNA barcoding technique. Plant Med 76:1622–1628

Heath DD, Iwama GK, Devlin RH (1993) PCR primed with VNTR core sequences yields species specific patterns and hypervariable probes. Nucleic Acids Res 21:5782–5785

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003a) Biological identification through DNA barcodes. Proc R Soc Lond B 270:313–321

Hebert PDN, Ratnasingham S, DeWaard JR (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B 270(suppl):96–99

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS Biol 2(10):e312

Heubl G (2010) New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. Planta Med 76(17):1963–1975

Hildreth J, Hrabeta-Robinson E, Applequist W, Betz J, Miller J (2007) Standard operating procedure for the collection and preparation of voucher plant specimens for use in the nutraceutical industry. Anal Bioanal Chem 389:13–17

Hollingsworth ML, Clark A, Forrest LL, Richardson JE, Pennington RT, Long D, Cowan R, Chase MW, Gaudeul M, Hollingsworth PM (2009) Selecting barcoding loci for plants: evaluation of seven candidate loci with species level sampling in three divergent groups of land plants. Mol Ecol Resour 9:439–457

Hollingsworth PM, Graham SW, Little DP (2011) Choosing and using a plant DNA barcode. PLoS One 6(5):e19254

Hosokawa K, Minami M, Kawahara K, Nakamura I, Shibata T (2000) Discrimination among three species of medicinal Scutellaria plants using RAPD markers. Planta Med 66:270–272

Hosokawa K, Minami M, Nakamura I, Hishida A, Shibata T (2005) The sequences of the plastid gene rpl16 and the rpl16-rpl14 spacer region allow discrimination among six species of Scutellaria. J Ethnopharmacol 99:105–108

Hosokawa K, Hishida A, Nakamura I, Shibata T (2006) The sequences of the spacer region between the atpF and atpA genes in the plastid genome allows discrimination among three varieties of medicinal Angelica. Planta Med 72:570–571

Hsiao JIH (2007) Patent protection for Chinese herbal medicine product invention in Taiwan. J World Intell Prop 10(1):1–21

Hu Y, Zhang Q, Xin H, Qin LP, Lu BR, Rahman K et al (2007) Association between chemical and genetic variation of Vitex rotundifolia populations from different locations in China: its implication for quality control of medicinal plants. Biomed Chromatogr 21:967–975

Huang L, Wang M, Zhou C, Li N, He X, Yang B (1998) Problems and solutions in the use of RAPD to the identification of the Chinese drugs "xi-xin" (Herba asari) and its substitutes. Yao Xue Xue Bao 33:778–784

Huang LQ, Cui GH, Chen ML, Feng CQ, Yang B, Wang YY, Dai RW (2002a) Study on complex system of Chinese material medica GAP fulfilling-the situation, problems, and of Chinese material medica germplasm. China J Chin Mater Med 27:481–483

Huang LQ, Cui GH, Dai RW (2002b) Study on complex system of Chinese Material Medica GAP fulfilling. China J Chin Mater Med 27:1–3

ICBN, International Code of Botanical Nomenclature (2006) 2006 Electronic version of the original English text. Prepared and edited by McNeill J, Barrie FR, Burdet HM, Demoulin V, Hawksworth DL, Marhold K, Nicolson DH, Prado J, Silva PC, Skog JE, Wiersema JH, and Turland NJ. Adopted by the Seventeenth International Botanical Congress, Vienna, Austria, July 2005. Last updated 09.03.2007

Ince AG, Karaca AGM et al (2009) Development and utilization of diagnostic DAMD-PCR markers for Capsicum accessions. Genet Res Crop Evol 56:211–221

Jayasinghe R, Kong S, Coram TE, Kaganovitch J, Xue CC, Li CG, Pang EC (2007) Construction and validation of a prototype microarray for efficient and high-throughput genotyping of angiosperms. Plant Biotechnol J 5(2):282–289

Jo BH, Suh DS et al (2009) Characterization of polymorphic microsatellite loci in cultivated and wild Panax ginseng. Genes Genomics 31:119–127

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, et al (1993) Theor Appl Genet 85:985–993

Joshi P, Dhawan V (2007) Assessment of genetic fidelity of micropropagated Swertia chirayita plantlets by ISSR marker assay. Biol Plantarum 51(1):22–26

Joshi K, Chavan OP, Warude D (2004) Molecular markers in herbal drug technology. Curr Sci 87 (2):159–165

Kaplan J, Chavan P, Warude D, Patwardhan B (2004) Molecular markers in herbal drug technology. Curr Sci 87:159–165

Karaca M, Ince AG et al (2008) PCR-RFLP and DAMD-PCR genotyping for Salvia species. J Sci Food Agric 88:2508–2516

Karp A, Edwards KJ (1997) DNA markers, a global overview. In: Caetano-Anollés G, Gresshoff PM (eds) DNA markers, protocols, applications, and overviews. Wiley-Liss, New York

Kelly LM (1998) Phylogenetic relationships in Asarum (Aristolochiaceae) based on morphology and ITS sequences. Am J Bot 85:1454–1467

Kersten T, Daniel C, König GM, Knöß W (2007) The potential of PCR-related methods to identify medicinal plants in herbal medicinal products. Planta Med 73:256

Kim J, Chung KW (2007) Isolation of new microsatellite-containing sequences in Acanthopanax senticosus. J Plant Biol 50:557–561

Kim J, Jo BH, Lee KL, Yoon ES, Ryu GH, Chung KW (2007a) Identification of new microsatellite markers in Panax ginseng. Mol Cells 24:60–68

Kim OT, Bang K, Dong S, Lee JW, Kim YC, Shin YS, Hyun DY, Lee SS, Cha SW, Seong NS (2007b) Molecular authentication of ginseng cultivars by comparison of internal transcribed spacer and 5.8S rDNA sequences. Plant Biotechnol Rep 1:163–167

Kircher M, Kelsko J (2010) High-throughput DNA sequencing-concepts and limitations. Bioessays 32(6):524–536

Klein D (2002) Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8(6):257–260

Kohjyouma M, Nakajima S, Namera A, Shimizu R, Mizukami H, Kohda H (1997) Random amplified polymorphic DNA analysis and variation of essential oil components of Atractylodes plants. Biol Pharm Bull 20:502–506

Kojoma M, Lida O, Makino Y, Sekita S, Satake M (2002) DNA fingerprinting of Cannabis sativa using inter-simple sequence repeat (ISSR) amplification. Planta Med 68:60–63

Komatsu KS, Zhu S et al (2001) Phylogenetic analysis based on 18S rRNA gene and matK gene sequences of Panax vietnamensis and five related species. Planta Med 67:461–465

Kondo K, Terabayashi S et al (1996) Phylogenetic relationship of medicinally important Cnidium officinale and Japanese Apiaceae based on rbcL sequences. J Plant Res 109:21–27

Korea Herbal Pharmacopoeia (2002) Ministry of Health Family Welfare of South Korea, Seoul, Korea

Kress WJ, Erickson DL (2007) A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS One 2(6):e508

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci USA 102:8369–8374

Kumar J, Verma V et al (2007) Development of simple sequence repeat markers in Cymbopogon species. Planta Med 73:262–266

Kumar P, Gupta VK, Misra AKL, Modi DR, Pandey BK (2009) Potential of molecular markers in plant biotechnology. Plant Omics J 2(4):141–162

Kurane J, Shinde V, Harsulkar A (2009) Application of ISSR marker in pharmacognosy: current update. Phcog Rev 3(6):216–228

Kurg A, Tõnisson N, Georgiou I, Shumaker J, Tollett J, Metspalu A (2000) Arrayed primer extension: solid-phase four-color DNA resequencing and mutation detection technology. Genet Test 4:1–7

Kwok PY (2001) Methods for genotyping single nucleotide polymorphisms. Annu Rev Genom Human Genet 2:235–258

Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G, Maurin O, Duthoit S, Barraclough TG, Savolainen V (2008a) DNA barcoding the floras of biodiversity hotspots. Proc Natl Acad Sci USA 105:2923–2928

Lahaye R, Savolainen V, Duthoit S, Maurin O, van der Bank M (2008) A test of psbK–psbI and atpF–atpH as potential plant DNA barcodes using the flora of the Kruger National Park as a model system (South Africa). Nature Proc hdl:10101/npre.1896.1

Lane CE, Lindstrom SC, Saunders GW (2007) A molecular assessment of Northeast Pacific Alaria species (Laminariales, Phaeophyceae) with reference to the utility of DNA barcoding. Mol Phylogenet Evol 44(2):634–648

Lau DT, Shaw PC, Wang J, But PP (2001) Authentication of medicinal Dendrobium species by the internal transcribed spacer of ribosomal DNA. Planta Med 67:456–460

Lee C, Wen J (2004) Phylogeny of Panax using chloroplast trnC-trnD intergenic region and the utility of trnC-trnD in interspecific studies of plants. Mol Phylogenet Evol 31:894–903

Lee HH, Itokawa H, Kozuka M (2005) Asian herbal products: the basis for development of high-quality dietary supplements and new medicines. In: Shi J, Ho CT, Shahidi F (eds) Asian functional foods. CRC, Taylor & Francis Group, Boca Raton, pp 21–72

Lee MY, Doh EJ, Park CH, Kim YH, Kim ES, Ko BS et al (2006a) Development of SCAR marker for discrimination of Artemisia princeps and A. argyi from other Artemisia herbs. Biol Pharm Bull 29:629–633

Lee SK, Li PT, Lau DT, Yung PP, Kong RY, Fong WF (2006b) Phylogeny of medicinal Phyllanthus species in China based on nuclear ITS and chloroplast atpB-rbcL sequences and multiplex PCR detection assay analysis. Planta Med 72:721–726

Lee HL, Yi DK, Kim JS, Kim KJ (2007) Development of plant DNA barcoding markers from the variable noncoding regions of chloroplast genome. Abstract presented at the second international barcode of life conference. Academia Sinica, Taipei, Taiwan. 18–20 September 2007

Lee JH, Lee JW, Sung JS, Bang KH, Moon SG (2009) Molecular authentication of 21 Korean Artemisia species (Compositae) by polymerase chain reaction-restriction fragment length polymorphism based on trnL-F region of chloroplast DNA. Biol Pharm Bull 32(11):1912–1916

Lerner HRL, Fleischer RC (2010) Prospects for the use of next-generation sequencing methods in ornithology. BioOne 27(1):4–15

Levinson G, Gutman GA (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol 4:203–221

Li DP (2001) Progress on mechanism of KLT injection antitumous effect. Traditi Chin Drug Res Clin Pharmacol 2:122–124

Li ZY (2003) Advances in plant resource conservation in China: sustainable development of plant resources in China. Acta Bot Sin 45:124–130

Li Y, Ding WL (2010) Genetic diversity assessment of Trollius accessions in China by RAPD markers. Biochem Genet 48:34–43

Li MF, Xu GJ, Xu LS, Jin RL (1986) Survey and identification of commercial example of Shihu (*Dendrobium*) (I). Chin Trad Herbal Drugs 17:33–37

Li MF, Xu GJ, Xu LS, Jin RL (1991) Survey and identification of commercial example of Shihu (*Dendrobium*) (II). Chin Trad Herbal Drugs 22:173–178

Li YC, Korol AB, Fahima T, Beiles A, Nevo E (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Mol Ecol 11(12):2453–2465

Li T, Wang J, Lu Z (2005) Accurate identification of closely related Dendrobium species with multiple species-specific gDNA probes. J Biochem Biophys Methods 62:111–123

Li SP, Yang FQ, Tsim WK (2006) Quality control of Cordyceps sinensis, a valued traditional Chinese medicine. J Pharm Biomed Anal 41:1571–1584

Li X, Ding X, Chu B, Ding G, Gu S, Qian L et al (2007) Molecular authentication of Alisma orientale by PCR-RFLP and ARMS. Planta Med 73:67–70

Li K, Wu W, Zheng Y, Dai Y, Xiang L, Liao K (2009a) Genetic diversity of Fritillaria from Sichuan province based on ISSR. Zhongguo Zhongyao Zazhi 34:2149–2154

Li YH, Ruan JL, Chen SL, Lu D, Zhu KX, Zhao MH, Pei HH (2009b) Study on medicinal plants of Loranthaceae resources in China. World Sci Technol Mod Tradit Chin Med 5:665–669

Li CI, Chiou SJ, Tong TS, Lee CY, Lee LT, Cheng CM (2010a) Development and validation of molecular markers for characterization of Boehmeria nivea var. nivea and Boehmeria nivea var. tenacissima. Chin Med 5:40

Li YH, Ruan JL, Chen SL et al (2010b) Authentication of Taxillus chinensis using DNA barcoding technique. J Med Plant Res 4(24):2706–2709

Li CI, Shu-Jiau Chiou SJ, Tong TS, Lee CY, Lee LT, Cheng CM (2010c) Development and validation of molecular markers for characterization of Boehmeria nivea var. nivea and Boehmeria nivea var. tenacissima. Chin Med 5:40

Li DZ, Liu JQ, Chen ZD, Wang H, Ge XJ, Zhou SL, Gao LM, Fu CX, Chen SL (2011a) Plant DNA barcoding in China. J Systemat Evol 49(3):165–168

Li DZ, Gao LM, Li HT, Wang H, Liu JQ, Chen ZD, Zhou SL, Chen SL, Yang YB, Fu CX, Zeng CX, Yan HF, Zhu YJ, Sun YS, Chen SY, Zhao L, Wang K, Yang T, Daun GW (2011b) Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc Natl Acad Sci USA 108:19641–19646

Liang ZT, Qin MJ, Wang ZT, Huang Y, Wang NH (2002) Identification of Bupleurum L. plants by RAPD technology. Zhongcaoyào 33:1117–1119
Liang YZ, Xieb PS, Chan K (2004) Quality control of herbal medicines. J Chromatogr B 812:53–70
Lim W, Mudge KW et al (2007) Utilization of RAPD markers to assess genetic diversity of wild populations of North American ginseng (Panax quinquefolium). Planta Med 73:71–76
Lin J, Zhou X, Gao S, Wu W, Liu X, Sun X et al (2006) Authentication of Pinellia ternata and its adulterants based on PCR with specific primers. Planta Med 72:844–847
Lin WY, Chen LR, Lin TY (2008) Rapid authentication of Bupleurum species using an array of immobilized sequence-specific oligonucleotide probes. Planta Med 74:464–469
Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet 44:397–401
Little DP (2011) DNA barcode sequence identification incorporating taxonomic hierarchy and within taxon variability. PLoS One 6(8):e20552
Liu CX, Yaniv Z (2005) Research and development of new drugs originating from Chinese plants. In: Yaniv Z, Bachrach U (eds) Handbook of medicinal plants. Food Products– Haworth, pp 61–96
Liu Y, Cao H, Wang X (2001a) Application of gene technology in quality control of Chinese drugs (I) – identification of Pinellia ternata species from Yuncheng, Shandong using DNA sequencing. Chin J Pharm Anal 21:423–426
Liu Y, He B, Cao H (2001b) Application of gene technology in quality control of Chinese drugs (II) – identification of Chinese yam (Dioscorea polystachia rhizome) using DNA sequencing. Chin J Tradit Herb Drugs 32:113–117
Liu CS, Bai GB, Yan YN (2005) Studies on the botanical sources and DNA molecular identification of Herba Asari based on ITS sequence. Zhongguo Zhongyao Zazhi 30:329–332
Liu Y, Yan HF, Cao T, Ge XJ (2010a) Evaluation of 10 plant barcodes in Bryophyta (Mosses). J Systemat Evol 48(1):36–46
Liu Z, Chen K, Luo K, Pan H, Chen S (2010b) DNA barcoding in medicinal plants Caprifoliaceae. Zhongguo Zhongyao Zazhi 35(19):2527–2532
Liu C, Yu H, Chen L (2011) Framework for sustainable use of medicinal plants in China. Plant Divers Resour 33(1):65–68
Loh JP, Kiew R, Kee A, Gan LH, Gan YY (1999) Amplified fragment length polymorphism (AFLP) provides molecular markers for the identification of Caladium bicolor cultivars. Ann Bot (Lond) 84:155–161
Long C, Kakiuchi N, Takahashi A, Komatsu K, Cai S, Mikage M (2004) Phylogenetic analysis of the DNA sequence of the non-coding region of nuclear ribosomal DNA and chloroplast of Ephedra plants in China. Planta Med 70:1080–1084
Lopez-Pujol J, Zhao AM (2004) China: a rich flora needed of urgent conservation. Orsis 19:49–89
Lopez-Pujol J, Zhang FM, Song GE (2006) Plant biodiversity in China: richly varied, endangered, and in need of conservation. Biodivers Conserv 15:3983–4026
Lou SK, Wong KL, Li M et al (2010) An integrated web medicinal materials DNA database: MMDBD (Medicinal Materials DNA Barcode Database). BMC Genomics 11:402
Lowe CR (2000) Nanobiotechnology: the fabrication and applications of chemical and biological nanostructures. Curr Opin Struct Biol 10:428–434
Lu Y, Li CS, Dong Q (2008) Chinese herb related molecules of cancer-cell-apoptosis: a minireview of progress between Kanglaite injection and related genes. J Exp Clin Cancer Res 27:31
Lu KT, Lee HC, Liu FS et al (2009) Discriminating Astragali Radix from Hedysarum Radix in Chinese medicine preparations using nested PCR and DNA sequencing methods. J Food Drug Anal 17:380–385
Lu C, Zhang W, Peng X, Gu G, Chen M, Tang Z (2010a) Development of randomly amplified polymorphic DNA-sequence characterized amplified region marker for identification of Apocynum venetum LINN. from A. pictum SCHRENK. Biol Pharm Bull 33:522–526

Lu KT, Lee HC, Liu FS et al (2010b) Identification of Ginseng Radix in Chinese medicine preparations by nested PCR-DNA sequencing method and nested PCR-restriction fragment length polymorphism. J Food Drug Anal 18:58–63

Lum MR, Potter E, Dang T, Heber D, Hardy M, Hirsch AM (2005) Identification of botanicals and potential contaminants through RFLP and sequencing. Planta Med 71:841–846

Luo JP, Cao H, Liu YP (2002) DNA sequencing and molecular identification of Patchouli and its substitute wrinkled gianthyssop. Yao Xue Xue Bao 37:739–742

Luo YM, Zhang WM, Ding XY, Shen J, Bao SL, Chu BH et al (2006a) SNP marker and allele-specific diagnostic PCR for authenticating herbs of Perilla. Acta Pharm Sin 41:840–845

Luo YM, Zhang WM, Ding XY, Shen J, Bao SL, Chu BH, Mao SG (2006b) SNP marker and allele-specific diagnostic PCR for authenticating herbs of Perilla. Yao Xue Xue Bao 41 (9):840–845

Luo K, Chen SL, Chen KL, Song JY, Yao H, Ma XY, Zhu YJ, Pang XH, Yu H, Li XW, Liu Z (2010) Assessment of candidate plant DNA barcodes using the Rutaceae family. Sci China Life Sci 53:701–708

Luo A, Zhang A, Ho SY, Xu W, Zhang Y, Shi W, Cameron SL, Zhu C (2011) Potential efficacy of mitochondrial genes for animal DNA barcoding: a case study using eutherian mammals. BMC Genomics 12:84

Ma GX, Xu GJ, Xu LS, Li MF (1995) Survey and identification of commercial example of Shihu (*Dendrobium*) (III). Chin Trad Herbal Drugs 26:370–372

Ma XQ, Duan JA, Zhu DY, Dong TT, Tsim KW (2000) Species identification of Radix Astragali (Huangqi) by DNA sequence of its 5S-rRNA spacer domain. Phytochemistry 54:363–368

Ma XQ, Zhu DY, Li SP, Dong TT, Tsim KW (2001) Authentic identification of stigma Croci (stigma of Crocus sativus) from its adulterants by molecular genetic analysis. Planta Med 67:183–186

Ma YS, Yu H, Li YY, Yan H, Cheng X (2008) a study of genetic structure of Stephania yunnanensis (Menispermaceae) by DALP. Biochem Genet 46:227–240

Ma XY, Xie CX, Liu C et al (2010) Species identification of medicinal pteridophytes by a DNA barcode marker, the chloroplast psbA-trnH intergenic region. Biol Pharm Bull 33 (11):1919–1924

Maeda M, Uryu N, Murayama N, Ishii H, Ota M, Tsuji K, Inoko H (1990) A simple and rapid method for HLA-DP genotying by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Hum Immunol 27:111–121

Makino R, Yazyu H, Kishimoto Y, Sekiya T, Hayashi K (1992) F-SSCP (fluorescence-based polymerase chain reaction-singlestrand conformation polymorphism (PCR-SSCP)) analysis. PCR Methods Appl 2:10–13

Malik A, Azam S, Hadi N, Hadi SM (2003) DNA degradation by water extract of green tea in the presence of copper ions: implications for anticancer properties. Phytother Res 17(4):358–363

Mardis ER (2008) The impact of next-generation sequencing technology on genetics. Trends Genet 24(3):133–141

Matsuyama S, Nishi K (2011) Genus identification of toxic plant by real-time PCR. Int J Legal Med 125(2):211–217

Mazzanti G, Battinelli L, Daniele C, Costantini S, Ciaralli L, Evandri MG (2008) Purity control of some Chinese crude herbal drugs marketed in Italy. Food Chem Toxicol 46:3043–3047

McDermott JM, Brandle U, Dutly F, Haemmerli UA, Keller S, Muller KE, Wolf MS (1994) Genetic variation in powdery mildew of barley: development of RAPD, SCAR and VNTR markers. Phytopathology 4:1316–1321

McIntyre M (1998) Chinese herbs: risk, side effects, and poisoning: the case for objective reporting and analysis reveals serious misrepresentation. J Altern Complement Med 4 (1):15–16

Mills S, Bone K (2005) The essential guide to herbal safety. Churchill Livingstone, London

Mitchell D, Willerslev E, Hansen A (2005) Damage and repair of ancient DNA. Mutat Res 571:265–276

Mizukami H, Ohbayashi K, Kitamura Y, Ikenaga T (1993a) Restriction fragment length polymorphisms (RFLPs) of medicinal plants and crude drugs. I. RFLP probes allow clear identification of Duboisia interspecific hybrid genotypes in both fresh and dried tissues. Biol Pharm Bull 16:388–390

Mizukami H, Ohbayashi K, Umetsu K, Hiraoka N (1993b) Restriction fragment length polymorphism of medicinal plants and crude drugs. II. Analysis of Glehnia littoralis of different geographical origin. Biol Pharm Bull 16:611–612

Mizukami H, Okabe Y, Kohda H, Hiraoka N (2000) Identification of the crude drug Atractylodes rhizome (Byaku-jutsu) and Atractylodes lancea rhizome (So-jutsu) using chloroplast trnK sequence as a molecular marker. Biol Pharm Bull 23:589–594

Molina C, Kahl G (2002) Genomics of two banana pathogens, genetic diversity, diagnostics, and phylogeny of Mycosphaerella fijiensis and M. musicola. In: Jain SM (ed) Banana improvement, cellular and molecular biology, and induced mutations. FAO/IAEA, Vienna

Mondini L, Noorani A, Pagnotta MA (2009) Assessing plant genetic diversity by molecular tools. Diversity 1:19–35

Morgante M, Vogel J (1994) Compound microsatellite primers for the detection of genetic polymorphisms. US Patent Application 08/326456

Mori N, Moriguchi T, Nakamura C (1997) RFLP analysis of nuclear DNA for study of phylogeny and domestication of tetraploid wheat. Genes Genet Syst 72:153–161

Moritz C, Cicero C (2004) DNA barcoding: promise and pitfalls. PLoS Biol 2(10):e354

Mueller UG, Wolfenbarger L (1999) AFLP genotyping and fingerprinting. Trends Ecol Evol 14:389–394

Munthali M, Ford-Lloyd BV, Newbury HJ (1992) The random amplification of polymorphic DNA for fingerprinting plants. PCR Methods Appl 1:274–276

Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T (2001) Loop-mediated isothermal amplification reaction using a nondenatured template. Clin Chem 47:1742–1743

Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223–229

Nakai R, Shoyama Y, Shiraishi S (1996) Genetic characterization of *Epimedium* species using random amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (RFLP) diagnosis. Biol Pharm Bull 19(1):67–70

Nalawade SM, Sagare AP, Lee CY et al (2003) Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Bot Bull Acad Sin 44:79–98

NEPA National Environmental Protection Agency (1994) China biodiversity conservation action plan. China Environmental Science Press, Beijing

Newmaster SG, Fazekas AJ, Ragupathy S (2006) DNA barcoding in the land plants: evaluation of rbcL in a multigene tiered approach. Can J Bot 84:335–341

Newmaster SG, Fazekas AJ, Steeves RAD, Janovec J (2008) Testing candidate plant barcode regions with species of recent origin in the Myristicaceae. Mol Ecol Notes 8:480–490

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N et al (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 17:2503–2516

Ng KY, Cheng CI, Xu HX (2009) Safety issues of Chinese medicine: a review of intoxication cases in Hong Kong. Chin Herb Med 1(1):29–39

Ngan F, Shaw P, But P, Wang J (1999) Molecular authentication of Panax species. Phytochemistry 50:787–791

Niu L, Mantri N, Li CG, Xue C, Wohlmuth H, Pang EC (2011) Detection of Panax quinquefolius in Panax ginseng using subtracted diversity array. J Sci Food Agric 18. doi:10.1002/jsfa.4319

Normile D (2003) The new face of traditional Chinese medicine. Science 299:188–190

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12):E63

Novak J, Grausgruber-Gröger S, Luka B (2007) DNA-based authentication of plant extracts. Food Res Int 40(3):388–392

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. Proc Natl Acad Sci USA 86:2766–2770

Paglia GE, Olivieri AM, Morgante M (1998) Towards second-generation STS (sequence-tagged sites) linkage maps in conifers, a genetic map of Norway spruce (Picea abies K.). Mol Gen Genet 258:466–478

Pang XH, Song JY, Zhu YJ, Xie CX, Chen SL (2010) Using DNA barcoding to identify species within Euphorbiaceae. Planta Med 76:1784–1786

Pang X, Songa J, Zhua Y, Xub H, Huanga L, Chena S (2011) Applying plant DNA barcodes for Rosaceae species identification. Cladistics 27:165–170

Paran I, Michelmore RW (1993) Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993

Paran I, Kesseli R, Michelmore R (1991) Identification of restriction fragment-length-polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near isogenic lines. Genome 34:1021–1027

Passinho-Soares H, Felix D, Kaplan MA, Margis-Pinheiro M, Margis R (2006) Authentication of medicinal plant botanical identity by amplified fragmented length polymorphism dominant DNA marker: inferences from the Plectranthus genus. Planta Med 72:929–931

Pastinen T, Partanen J, Syvanen AC (1996) Multiplex, fluorescent, solid-phase minisequencing for efficient screening of DNA sequence variation. Clin Chem 42:1391–1397

Pastinen T, Raitio M, Lindroos K et al (2000) A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays. Genome Res 10:1031–1042

Pei S (2001) Ethnobotanical approaches of traditional medicine studies: some experiences from Asia. Pharmaceutical Botany 39:74–79

Pei S (2002a) A brief review of ethnobotany and its curriculum development in China. In: Shinwari ZK, Hamilton A, Khan AA (eds)In: Proceedings of a Workshop on Curriculum Development in Applied Ethnobotany, Nathiagali, 2–4 May 2002. WWF-Pakistan, Lahore, Pakistan

Pei S (2002b) Ethnobotany and modernisation of Traditional Chinese Medicine. Paper at a Workshop onWise Practices and Experiential Learning in the Conservation and Management of Himalayan Medicinal Plants, Kathmandu, Nepal, 15–20 December 2002, supported by the Ministry of Forest and Soil Conservation, Nepal, the WWF-Nepal Program, MMAPPA and PPI

Pei-Gen X (2007) Ethnopharmacological investigation of Chinese medicinal plants. In: Chadwick DJ, Marsh J (eds) Etrnobotany and search for new drugs. Ciba Foundation Symposium 185 – Ethnobotany and the Search for New Drugs

Pennisi E (2007) Wanted: a barcode for plants. Science 318:190–191

Pereira F, Carneiro J, Amorim A (2008) Identification of species with DNA-based technology: current progress and challenges. Recent Pat DNA Gene Seq 2:187–200

Pharmacopoeia of the People's Republic of China (2005) State pharmacopoeia committee. Chemical Industry Publisher, Beijing, China

Pharmacopoeia of the People's Republic of China (2010) 2010 edn. China Medical Science and Technology Press, Beijing

Pirrung MC, Connors RV, Odenbaugh AL, Montague-Smith MP, Walcott NG, Tollett JJ (2000) The arrayed primer extension method for DNA microchip analysis. Molecular computation of satisfaction problems. J Am Chem Soc 122:1873–1882

Pirttilä AM, Hirsikorpi M, Kämäräinen T, Jaakola L, Hohtola A (2001) DNA isolation methods for medicinal and aromatic plants. Plant Mol Biol Rep 19:273a–f

Portis E, Acquadro A, Comino C, Lanteri S (2004) Analysis of DNA methylation during germination of pepper (Capsicum annuum L.) seeds using methylation-sensitive amplification polymorphism (MSAP). Plant Sci 166:169–178

Preeti C, Kalpana J, Bhushan P (2006) DNA microarrays in herbal drug research. Advance Access Publication

Preeti C, Kalpana J, Bhushan P (2006b) DNA microarrays in herbal drug research. Evid Based Complement Altern Med 3(4):447–457

Provan J, Biss PM, McMee D, Mathews S (2004) Universal primers for the amplification of chloroplast microsatellites in grasses (Poaceae). Mol Ecol Notes: Primer Note

Qi JJ, Li XE et al (2008) Genetic relationships among Rehmannia glutinosa cultivars and varieties. Planta Med 74:1846–1852

Qian L, Ding G et al (2008) Molecular authentication of Dendrobium loddigesii Rolfe by amplification refractory mutation system (ARMS). Planta Med 74:470–473

Qin J, Leung FC, Fung Y, Zhu D, Lin B (2005) Rapid authentication of ginseng species using microchip electrophoresis with laser-induced fluorescence detection. Anal Bioanal Chem 381:812–819

Reyna-Lopez GE, Simpson J, Ruiz-Herrera J (1997) Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphism. Mol Gen Genet 253:703–710

Robba L, Russell SJ, Barker GL, Brodie J (2006) Assessing the use of the mitochondrial cox1 marker for use in DNA barcoding of Red algae (Rhodophyta). Am J Bot 93(8):1101–1108

Saar DE, Polans NO, Sörensen PD, Duvall MR (2001) Angiosperm DNA contamination by endophytic fungi: detection and methods of avoidance. Plant Mol Biol Rep 19:249–260

Sahin FP, Yamashita H et al (2007) DNA authentication of Plantago herb based on nucleotide sequences of 18S-28S rRNA internal transcribed Spacer region. Biol Pharm Bull 30:1265–1270

Sarwat M, Das S et al (2008) Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in Tribulus terrestris, a medicinal herb. Plant Cell Rep 27:519–528

Sasaki Y, Nagumo S (2007) Rapid identification of Curcuma longa and C. aromatica by LAMP. Biol Pharm Bull 30:2229–2230

Sasaki Y, Fushimi H et al (2002) Sequence analysis of Chinese and Japanese Curcuma drugs on the 18S rRNA gene and trnK gene and the application of amplification-refractory mutation system analysis for their authentication. Biol Pharm Bull 25:1593–1599

Sasaki Y, Komatsu K, Nagumo S (2008) Rapid detection of Panax ginseng by loop-mediated isothermal amplification and its application to authentication of Ginseng. Biol Pharm Bull 31 (9):1806–1808

Sasaki Y, Fujimoto T, Aragane M, Yasuda I, Nagumo S (2009) Rapid and sensitive detection of Lophophora williamsii by loop-mediated isothermal amplification. Biol Pharm Bull 32 (5):887–891

Sass C, Little DP, Stevenson DW, Specht CD (2007) DNA barcoding in the Cycadales: testing the potential of proposed barcoding markers for species identification of Cycads. PLoS One 2:e1154

Schadt EE, Turner S, Kasarskis A (2010) A window into third-generation sequencing. Hum Mol Genet 19:R227–R240

Schena M, Heller RA et al (1998) Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol 16(7):301–306

Schippmann U, Leaman DJ, Cunningham AB (2002) Impact of cultivation and gathering of medicinal plants on biodiversity: global trends and issues. Inter-Department Working Group on Biology Diversity for Food and Agriculture, Food and Agricultural Organisation of the United Nations, Rome, Italy

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30(12):e57

Schuler GD, Boguski MS, Stewart EA et al (1996) A gene map of the human genome. Science 274:540–546

Semagn K, Bjørnstad A, Ndjiondjop MN (2006) An overview of molecular marker methods for plants. Afr J Biotechnol 5:2540–2568

SEPA State Environmental Protection Administration (1998) China's biodiversity: a country study. China Environmental Science Press, Beijing

Shan X, Blake TK, Talbert LE (1999) Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat. Theor Appl Genet 98:1072–1078

Shaw PC, Ngan FN, But PPH, Wang J (2002) Molecular markers in Chinese medicinal materials. In: Shaw PC, But PPH (eds) Authentication of Chinese medicinal material by DNA technology. World Scientific Publishing, Singapore

Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am J Bot 94(3):275–288

Shaw PC, Wong KKL, Chan AWK, Wong WC, But PPH (2009) Patent applications for using DNA technologies to authenticate medicinal herbal material. J Chin Med 4:1–11

Shen Y, Wu BL (2009) Designing a simple multiplex ligation-dependent probe amplification (MLPA) assay for rapid detection of copy number variants in the genome. J Genet Genomics 36(4):257–265

Shen J, Ding XY, Ding G, Liu DY, Tang F, He J (2006) Studies on population difference of Dendrobium officinale II establishment and optimization of the method of ISSR fingerprinting marker. Zhongguo Zhongyao Zazhi 31:291–294

Shi HM, Wang J, Wang MY, Tu PF, Li XB (2009) Identification of Cistanche species by chemical and inter-simple sequence repeat fingerprinting. Biol Pharm Bull 32:142–146

Shim YH, Choi JH, Park CD et al (2003) Molecular differentiation of Panax species by RAPD analysis. Arch Pharm Res 26:601–605

Shneyer VS (2009) DNA Barcoding is a new approach in comparative genomics of plants. Russ J Genet 45(11):1267–1278

Silva LM, Montes de Oca H, Diniz CR, Fortes-Dias CL (2001) Fingerprinting of cell lines by directed amplification of minisatellite-region DNA (DAMD). Braz J Med Biol Res 34:1405–1410

Siow YL, Gong Y, Au-Yeung KK, Woo CW, Choy PC (2005) Emerging issues in traditional Chinese medicine. Can J Physiol Pharmacol 83:321–334

Slanc P, Ravnikar M, Strukelj B (2006) Identification of individual herbal drugs in tea mixtures using restriction analysis of ITS DNA and real-time PCR. Pharmazie 61(11):912–915

Smillie TJ, Khan IA (2010) A comprehensive approach to identifying and authenticating botanical products. Clin Pharmacol Ther 87:175–186

Society of Japanese Pharmacopoeia (2001) Japanese pharmacopoeia (English version), 14th edn. Junkudo Book Shop, Tokyo

Somers DJ, Demmon G (2002) Identification of repetitive, genome-specific probes in crucifer oilseed species. Genome 45:485–492

Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc Natl Acad Sci USA 105(36):13486–13491

Song JY, Yao H, Li Y, Li XW, Lin YL, Liu C, Han JP, Xie CX, Chen SL (2009) Authentication of the family Polygonaceae in Chinese pharmacopoeia by DNA barcoding technique. J Ethnopharmacol 124:434–439

Song Z, Li X, Wang H, Wang J (2010) Genetic diversity and population structure of Salvia miltiorrhiza Bge in China revealed by ISSR and SRAP. Genetica 138:241–249

Soumaya R, Dakhlaoui-Dkhil S, Salem AOM, Zehdi-Azouzi S, Rhouma A, Marrakchi M, Trifi M (2008) Genetic diversity and phylogenic relationships in date-palms (Phoenix dactylifera L.) as assessed by random amplified microsatellite polymorphism markers (RAMPOs). Scientia Hort 117:53–57

Squirrell J, Hollingsworth PM, Woodhead M, Russell J, Lowe AJ, Gibby M, Powell W (2003) How much effort is required to isolate nuclear microsatellites from plants? Mol Ecol 12:1339–1348

Srivastava J, Lambert J, Vietmeyer N (1996) Medicinal plants: an expanding role in development [M]. World Bank Publications, Washington, DC

Sucher JN, Carles MC (2008) Genome-based approaches to the authentication of medicinal plants. Planta Med 74:603–623

Sucher NJ, Hennel JR, Carles MC (2012) Plant DNA fingerprinting and barcoding. Methods Mol Biol 862:13–22

Sun Y, Fung KP, Leung PC, Shi D, Shaw PC (2004) Characterization of medicinal Epimedium species by 5S rRNA gene spacer sequencing. Planta Med 70:287–288

Sun Y, Fung KP, Leung PC, Shaw PC (2005) A phylogenetic analysis of *Epimedium* (Berberidaceae) based on nuclear ribosomal DNA sequences. Mol Phylogenet Evol 35(1):287–291

Sun Y, Shaw PC, Fung KP (2007) Molecular authentication of Radix Puerariae Lobatae and Radix Puerariae Thomsonii by ITS and 5S rRNA spacer sequencing. Biol Pharm Bull 30:173–175

Sun ZY, Gao T, Yao H, Shi LC, Zhu YJ, Chen SL (2010) Identification of Lonicera japonica and its related species using the DNA barcoding method. Plant Med. doi:10.1055/s-0030-1250324

Sze SCW, Song JX, Wong RNS, Feng YB, Ng TB, Tong Y, Zhang KYB (2008) Application of SCAR (sequence characterized amplified region) analysis to authenticate Lycium barbarum (wolfberry) and its adulterants. Biotech Applied Biochem 51:15–21

Taberlet P et al (2007) Power and limitations of the chloroplast *trn*L(UAA) intron for plant DNA barcoding. Nucleic Acids Res 35:e14

Takamiya T, Wongsawad P, Tajima N, Shioda N, Lu JF, Wen CL, Wu JB, Handa T, Iijima H, Kitanaka S, Yukawa T (2011) Identification of Dendrobium species used for herbal medicines based on ribosomal DNA internal transcribed spacer sequence. Biol Pharm Bull 34(5):779–82

Techaprasan J, Klinbunga S, Jenjittiku T (2008) Genetic relationships and species authentication of *Boesenbergia* (Zingiberaceae) in Thailand based on AFLP and SSCP analyses. Biochem Syst Ecol 36(5–6):408–441

Ten Kate K, Laird S (1999) The commercial use of biodiversity: access to genetic resources and benefit-sharing. Commission of the European Communities and Earthscan, London, p 398

Theerakulpisut P, Kanawapee N, Maensiri D, Bunnag S, Chantaranothai P (2008) Development of species-specific SCAR markers for identification of three medicinal species of Phyllanthus. J Syst Evol 46:614–621

Thorne RF (2002) How many species of seed plants are there? Taxon 51:511–522

Trau D, Lee TM, Lao AI, Lenigk R, Hsing IM, Ip NY et al (2002) Genotyping on a complementary metal oxide semiconductor silicon polymerase chain reaction chip with integrated DNA microarray. Anal Chem 74:3168–3173

Trifi-Farah N, Marrakchi M (2001) Hedysarum phylogeny mediated by RFLP analysis of nuclear ribosomal DNA. Gen Res Crop Evol 48:339–345

Tsoi PY, Wu HS, Wong MS, Chen SL, Fong WF, Xiao PG et al (2003) Genotyping and species identification of Fritillaria by DNA chip technology. Acta Pharm Sin 4:185–190

Um JY, Chung HS, Kim MS, Na HJ, Kwon HJ, Kim JJ, Lee KM, Lee SJ, Lim JP, Do KR, Hwang WJ, Lyu YS, An NH, Kim HM (2001) Molecular authentication of Panax ginseng species by RAPD analysis and PCR-RFLP. Biol Pharm Bull 24:872–875

Valdes AM, Slatkin M, Freimer NB (1993) Allele frequencies at microsatellite loci – the stepwise mutation model revisited. Genetics 133:737–749

Varshney RV, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23:28–55

Vences M, Thomas M, Bonett RM, Vieites DR (2005) Deciphering amphibian diversity through DNA barcoding: chances and challenges. Philos Trans R Soc London Ser B 360:1859–1868

Vijayan K, Tsou CH (2010) DNA barcoding in plants: taxonomy in a new perspective. Curr Sci 99 (11):1513–1541

Virgilio M, Backeljau T, Nevado Band De Meyer M (2010) Comparative performances of DNA barcoding across insect orders. BMC Bioinformatics 11:206

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wagner H, Bauer R, Melchart D, Xiao PG, Staudinger A (2011) Chromatographic fingerprint analysis of herbal medicines: thin-layer and high performance liquid chromatography of Chinese drugs, Vol I and Vol II, 2nd edn. Springer, Wien

Wang H, Wang Q (2005) Analysis of rDNA ITS sequences of Radix et Rhizoma Salviae miltiorrhizae and plants of Salvia L. Chin Tradit Herb Drugs 36:1381–1385

Wang P, Huang F, Zhou L, Cao L, Liang S, Xu H, Liu J (2000) Analysis of Amomun villosum species and some adulterants of Zingiberaceae by RAPD. Zhong Yao Cai 23:71–74

Wang J, Ha WY, Ngan FN, But PP, Shaw PC (2001) Application of sequence characterized amplified region (SCAR) analysis to authenticate Panax species and their adulterants. Planta Med 67:781–783

Wang CZ, Li P, Ding JY, Jin GQ, Yuan CS (2005) Identification of Fritillaria pallidiflora using diagnostic PCR and PCR-RFLP based on nuclear ribosomal DNA internal transcribed spacer sequences. Planta Med 71:384–386

Wang CZ, Li P, Ding JY et al (2007a) Simultaneous identification of Bulbus Fritillariae cirrhosae using PCR-RFLP analysis. Phytomedicine 14:628–632

Wang MW, Hao XJ, Chen KX (2007b) Biological screening of natural products and drug innovation in China. Philos Trans R Soc Lond B Biol Sci 362(1482):1093–1105

Wang Y, Li XE, Li XD, Qi JJ, Sun P, Zhou LL (2008) Analysis of genetic diversity of wild Rehmannia glutinosa by using RAPD and ISSR markers. Zhongguo Zhongyao Zazhi 33(22):2591–2595

Wang HZ, Wu ZX, Lu JJ et al (2009) Molecular diversity and relationships among Cymbidium goeringii cultivars based on inter-simple sequence repeat (ISSR) markers. Genetica 136 (3):391–399

Wang HT, Sun H, Kwon WS, Jin H, Yang DC (2010a) A PCR-based SNP marker for specific authentication of Korean ginseng (Panax ginseng) cultivar "Chunpoong". Mol Biol Rep 37:1053–1057

Wang W, Wu Y, Yan Y, Ermakova M, Kerstetter R et al (2010b) DNA barcoding of the Lemnaceae, a family of aquatic monocots. BMC Plant Biol 10(1):205

Ward RD, Hanner R, Hebert PDN (2009) The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol 74:329–356

Watanabe A, Araki S, Kobari S, Sudo H, Tsuchida T, Uno T et al (1998) In vitro propagation, restriction fragment length polymorphism, and random amplified polymorphic DNA analyses of Angelica plants. Plant Cell Rep 18:187–192

Waugh J (2007) DNA barcoding in animal species: progress, potential and pitfalls. Bioessays 29 (2):188–197

Weising K, Gardner RC (1999) A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome 42:9–11

Weising K, Kahl G (1998) Hybridization-based microsatellite fingerprinting of plants. In: Caetano-Anolles G, Gresshoff PM (eds) DNA markers: protocols, applications, and overviews. Wiley, New York, pp 238–243

Weising K, Bybom H, Wolff K, Kahl G (2005) DNA fingerprinting in plants. Principles, methods, and application, 2nd edn. Taylor and Francis, Boca Raton

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Winter E, Pfaff T, Udupa SM, Hiittel B, Sharma PC, Sahi S, Arreguin-Espinoza R, Weigand F, Muehlbauer FJ, Kahl G (1999) Characterization and mapping of sequence-tagged microsatellite sites in the chickpea (Cicer arietinum L.) genome. Mol Gen Genet 262:90–101

Witsenboer H, Vogel J, Michelmore RW (1997) Identification, genetic localization, and allelic diversity of selectively amplified polymorphic loci in lettuce and wild relatives (Lactuca spp.). Genome 40:923–936

Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84(24):9054–9058

Wong KL, Wang J, But PPH, Shaw PC (2001) Application of ctyochrome b DNA sequences for the authentication of endangered snake species. Forensic Sci Int 139:49–55

Wu H, Lien EJ, Lien LL (2003) Chemical and pharmacological investigations of *Epimedium* species: a survey. Prog Drug Res 60:1–57

Wu W, Zheng YL, Chen L, Wei YM, Yang RW, Yan ZH (2005) Evaluation of genetic relationships in the genus Houttuynia Thunb. In China based on RAPD and ISSR markers. Biochem Syst Ecol 33:1141–1157

Xia Q, Zhao KJ, Huang ZG, Zhang P, Dong TT, Li SP et al (2005) Molecular genetic and chemical assessment of Rhizoma Curcumae in China. J Agric Food Chem 53:6019–6026

Xia T, Chen S, Chen S, Zhang D, Zhang D, Gao Q et al (2007) ISSR analysis of genetic diversity of the Qinghai-Tibet plateau endemic Rhodiola chrysanthemifolia (Crassulaceae). Biochem Syst Ecol 35:209–214

Xiao PG (1994) Ethnopharmacological investigation of Chinese medicinal plants. Ciba Found Symp 185:169–173

Xiao PG, Yong P (1998) Ethnopharmacology and research on medicinal plants in China. In: Prendergast HDV, Etkin NL, Harris DR, Houghton PJ (eds) Plants for food and medicine. Proceedings from a Joint Conference of the Society for Economic Botany and the International Society for Ethnopharmacology, London, Royal Botanic Gardens, Kew, UK, pp 31–39

Xiong LZ, Xu CG, Saghai-Maroof MA, Zhang QF (1999) Pattern of cytosine methylation in an elite rice hybrid and its potential lines, detected by a methylationsensitive amplification polymorphism technique. Mol Gen Genet 261:439–446

Xu M, Li X, Korban SS (2000) AFLP-Based detection of DNA methylation. Plant Mol Biol Rep 18:361–368

Xu M, Huaracha E, Korban SS (2001) Development of sequence-characterized amplified regions (SCARs) from amplified fragment length polymorphism (AFLP) markers tightly linked to the Vf gene in apple. Genome 44:63–70

Xu H, Wang Z, Ding X, Zhou K, Xu L (2006) Differentiation of Dendrobium species used as "Huangcao Shihu" by rDNA ITS sequence analysis. Planta Med 72:89–92

Xue D (1997) China's national report on implementation of the Convention on Biological Diversity. China Environmental Science Press, Beijing

Xue CY, Xue HG (2008) Application of real-time scorpion PCR for authentication and quantification of the traditional chinese medicinal plant Drynaria fortunei. Planta Med 74:e765–e770

Xue CY, Li DZ, Lu JM, Yang JB, Liu JQ (2006a) Molecular authentication of the traditional Tibetan medicinal plant Swertia mussotii. Planta Med 72:1223–1226

Xue HG, Zhou SD, He XJ, Yu Y (2006b) Molecular authentication of the traditional Chinese medicinal plant Euphorbia pekinensis. Planta Med 73:91–93

Xue CY, Li DZ, Wang QZ (2008a) Identification and quantification of the traditional Chinese medicinal plant Gentiana macrophylla using Taqman real-time PCR. Planta Med 74 (15):1842–1845

Xue HG, Wang H, Li DZ, Xue CY, Wang QZ (2008b) Differentiation of the traditional Chinese medicinal plants Euphorbia humifusa and E. maculata from adulterants by TaqMan real-time polymerase chain reaction. Planta Med 74:302–304

Yamaji H, Fukuda T, Yokoyama J, Pak JH, Zhou C, Yang C et al (2007) Reticulate evolution and phylogeography in Asarum sect. asiasarum (Aristolochiaceae) documented in internal transcribed spacer sequences (ITS) of nuclear ribosomal DNA. Mol Phylogenet Evol 44:863–884

Yamasaki M, Sato A, Shimomura K, Saito K, Murakoshi I (1994) Genetic relationships among Glycyrrhiza plants determined by RADP and RFLP analyses. Biol Pharm Bull 17:1529–1531

Yamazaki M, Sato A, Saito K, Murakoshi I (1993) Molecular phylogeny based on RFLP and its relation with alkaloid patterns in Lupinus plants. Biol Pharm Bull 16:1182–1184

Yang HY, Chen CF (1998) Pharmacology and toxicology of herbal medicine: subacute toxicity of commonly used Chinese drugs. J Toxicol Sci 23(Suppl 2):229–233

Yang ZY, Ling Z (2010) Screening potential DNA barcode regions in Amomum (Zingiberaceae). Acta Bot Yunnan 32(5):393–400

Yang M, Zhang D, Liu J, Zheng J (2001) A molecular marker that is specific to medicinal rhubarb based on chloroplast trnL/trnF sequences. Planta Med 67:784–786
Yang DY, Hirotoshi F, Shao-Qing C, Katsuko K (2004) Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system (ARMS) analyses of medicinally used rheum species and their application for identification of Rhei rhizoma. Biol Pharm Bull 27:661–669
Yang ZY, Chao Z, Huo KK, Wu BY, Pan SL (2006) Nuclear ribosomal DNA internal transcribed spacer 1 sequences of 4 Leonurus species. Nan Fang Yi Ke Da Xue Xue Bao 26:1593–1595
Yang ZY, Chao Z et al (2007) ITS sequence analysis used for molecular identification of the Bupleurum species from northwestern China. Phytomedicine 14:416–422
Yao H, Zhao Z, Chen DF, Chen JK, Zhou TS (2008) ISSR primer screening and preliminary evaluation of genetic diversity in wild populations of Glycyrrhiza uralensis. Biol Plantarum 052(1):117–120
Yao H, Song J, Liu C, Luo K, Han J et al (2010) Use of ITS2 region as the universal DNA barcode for plants and animals. PLoS One 5(10):e13102
Ye Q, Qiu YX, Quo YQ, Chen JX, Yang SZ, Zhao MS, Fu CX (2006) Species-specific SCAR markers for authentication of Sinocalycanthus chinensis. J Zhejiang Univ Sci B 7:868–872
Ying XC, Zhu D, Wang Q, Zhong WQ (2009) Application of Light Cycler polymerase chain reaction and melting curve analysis to the authentication of the traditional Chinese medicinal plant Cimicifuga foetida. Planta Med 75(8):873–874
Yip PY, Kwan HS (2006) Molecular identification of Astragalus membranaceus at the species and locality levels. J Ethnopharmacol 106:222–229
Yip PY, Chau CF, Mak CY, Kwan HS (2007) DNA methods for identification of Chinese medicinal materials. J Chin Med 2:1–19
Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application number 92402629.7. Publication number 0534858A1
Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. Mol Ecol 11:1–16
Zerega NJC, Mori S, Lindqvist C, Zheng Q, Motley TJ (2002) Using amplified fragment length polymorphisms (AFLP) to identify black cohosh (Actaea racemosa). Econ Bot 56:154–164
Zhang R, Zhang B, Ye H (1997) Identification of the Chinese herbs of Indigoferae L. by RAPD analysis. Hongguo Zhong Yao Za Zhi 22:72–73
Zhang YB, Ngan FN, Wang ZT, Wang J, But PPH, Shaw PC (1999) Differentiation of Codonopsis pilosula using random amplified polymorphic DNA. Planta Med 65:57–60
Zhang KYB, Leung HW et al (2001a) Differentiation of Lycium barbarum from its related Lycium species using random amplified polymorphic DNA. Planta Med 67:379–381
Zhang M, Huang HR, Liao SM, Gao JY (2001b) Cluster analysis of Dendrobium by RAPD and design of specific primer for Dendrobium candidum. Zhongguo Zhong Yao Za Zhi 26:442–447
Zhang YB, Wang J, Wang ZT, But PP, Shaw PC (2003) DNA microarray for identification of the herb of Dendrobium species from Chinese medicinal formulations. Planta Med 69:1172–1174
Zhang M, Zhang DZ, Xu XH, Zhang T, Wang ZT (2005a) 5S rRNA gene spacer sequences from Ligularia medicinal plants and the identification of HPAs-containing species. Chin J Nat Med 3:38–40
Zhang T, Xu LS, Wang ZT, Zhou KY, Zhang N, Shi YF (2005b) Molecular identification of medicinal plants: Dendrobium chrysanthum, Dendrobium fimbriatum and their morphologically allied species by PCR-RFLP analyses. Yao Xue Xue Bao 40:728–733
Zhang YB, Shaw PC, Sze CW, Wang ZT, Tong Y (2007) Molecular authentication of Chinese herbal materials. J Food Drug Anal 15:1–9
Zhang JM, Wang JX, Xia T, Zhou SL (2009) DNA barcoding: species delimitation in tree peonies. Sci China C Life Sci 52(6):568–578
Zhao ZZ, Li TYS (2004) Hong Kong commonly confused Chinese medicines. Chinese Medicine Merchants Association, Hong Kong

Zhao KJ, Dong TT, Cui XM, Tu PF, Tsim KW (2003a) Genetic distinction of radix adenophorae from its adulterants by the DNA sequence of 5S-rRNA spacer domains. Am J Chin Med 31:919–926

Zhao KJ, Dong TT, Tu PF, Song ZH, Lo CK, Tsim KW (2003b) Molecular genetic and chemical assessment of radix Angelica (Danggui) in China. J Agric Food Chem 51:2576–2583

Zhao Z, Hu Y, Liang Z, Yuen J, Jiang Z, Leung KSY (2006) Authentication is fundamental for standardization of Chinese medicines. Planta Med 72:865–874

Zhao YP, Qiu YX et al (2007a) Authentication of Actinidia macrosperma using PCR-RFLP based on trnK sequences. Bot Studies 48:239–242

Zhao ZL, Leng CH et al (2007b) Identification of Dryopteris crassirhizoma and the adulterant species based on cpDNA rbcL and translated amino acid sequences. Planta Med 73:1230–1233

Zhao ZZ, Yuen JPS, Wu J, Yu T, Huang W, Zhao ZZ, Li TYS (2007c) Easily confused Chinese medicines in Hong Kong. Ann Acad Med 35(11):764–769

Zhen-Yan Y, Ling Z (2010) Screening potential DNA barcode regions in Amomum (Zingiberaceae). Acta Bot Yunnan 32(5):393–400

Zhou Z, Bebeli PJ et al (1997) Direct amplification of minisatellite-region DNA with VNTR core sequences in the genus Oryza. Theor Appl Genet 95:942–949

Zhu YP (2002) Toxicity of the Chinese herb mu tong (Aristolochia manshuriensis). What history tells us. Adverse Drug React Toxicol Rev 21(4):171–177

Zhu S, Fushimi H, Cai S, Komatsu K (2003) Phylogenetic relationship in the genus Panax: inferred from chloroplast trnK gene and nuclear 18S rRNA gene sequences. Planta Med 69:647–653

Zhu S, Fushimi H, Cai S, Komatsu K (2004) Species identification from Ginseng drugs by multiplex amplification refractory mutation system (MARMS). Planta Med 70:189–192

Zhu S, Hirotoshi Fushimi H, Komatsu K (2008) Development of a DNA microarray for authentication of ginseng drugs based on 18S rRNA gene sequence. J Agric Food Chem 56 (11):3953–3959

Zhu YJ, Sl C, Hui Y et al (2010) DNA barcoding the medicinal plants of the genus Paris. Yaoxue Xuebao 45(3):376–382

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20(2):176–183

Zuo YJ, Chen ZJ, Kondo K et al (2011) DNA barcoding of Panax species. Planta Med 77 (2):182–187

Chapter 3
Newest Results on the Chemistry and Pharmacology of TCM Drugs Containing Triterpene and Steroid Saponins

Marie-Aleth Lacaille-Dubois

3.1 Introduction

Nature has provided traditional therapies in all cultures since the beginning of civilization, but only recently has human technology caught up with nature. The long-term objectives of these therapies include the discovery of new pharmacologically active agents using traditional herbal drugs as a guide. In this context, traditional Chinese medicine (TCM) could serve as a source of inspiration for drug development (Verpoorte et al. 2009). Research combining phytochemical and phytopharmacological techniques provides an excellent opportunity to identify novel natural compounds of biological interest.

The saponins are a group of steroidal and triterpenoid glycosides possessing a broad spectrum of biological and pharmacological activities (immunomodulating, immunoadjuvant, cytotoxic, antitumor, antihepatotoxic, anti-inflammatory, hypoglycemic, antimicrobial, and others), as has been highlighted in several review articles (Lacaille-Dubois 2005; Podolak et al. 2010; Dinda et al. 2010). They are among the main bioactive components in TCM. Some saponin drugs are reported in the Chinese Pharmacopoeia (2005, 2010) and several Chinese drug monographs and analyses have been produced in order to provide a scientific, pharmaceutical characterization of single Chinese herbal drugs and their compounds (Wagner and Bauer 1996–2010). Furthermore, the analytical investigation of 80 Chinese herbal drugs, among them more than ten saponin-containing TCM drugs which are most frequently in use, has been described in a detailed overview (Wagner et al. 2011). The authors demonstrated that chromatographic fingerprint analysis by TLC and HPLC provides a rational approach to the quality assessment of Chinese drugs.

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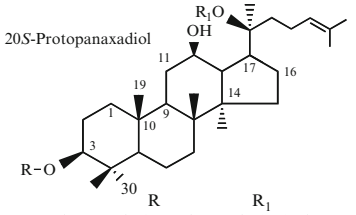
This review focuses on the latest chemical, pharmacological, and clinical perspectives on the most important triterpenoidal and steroidal saponin drugs in TCM, some of which were outlined in Wagner et al. (2011) from an analytical and biological point of view: *P. ginseng* N° 72, Vol II, p875, *P. quinquefolium* N° 72, Vol II, p843, *P. notoginseng* N° 70, Vol II, p843, *Astragalus* ssp. N°8, Vol I, p83, *Bupleurum* ssp. N°1, Vol I, p1, *Lonicera macranthoides* N° 51, Vol II, p587, *Clematis* ssp. N° 33, Vol I, p355, *Anemarrhena asphodeloides* N° 37, Vol I, p403, *Dioscorea* ssp. N° 53, Vol II, p615, *Tribulus terrestris* N°67, Vol II, p805, *Ophiopogon japonicus* N°68, Vol II, p819, and *Rhizoma Cimicifugae* "*Shengma*" N° 49, Vol II, p559.

We will first introduce the newest techniques for obtaining and analyzing the saponins from complex mixtures of drugs used in TCM. The structures and bioactivity of the new and unusual saponins will be presented together with the pharmacological results in a second part of this review. It seems that the most relevant examples considered TCM as sources of new bioactive saponins in the field of immunology and cancerology. Therefore, the second part will highlight this aspect with some modern pharmacological experiments related to immunology, cancer, and, to a lesser extent, other bioactivities (cardiovascular and central nervous system, anti-inflammatory, antidiabetic, just to mention a few). A third part discusses a few clinical studies.

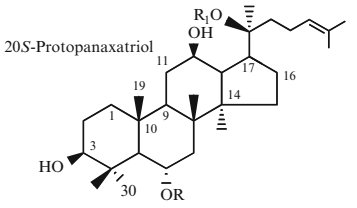
3.1.1 Chemistry of Saponins from TCM Drugs

3.1.1.1 Extraction, Isolation

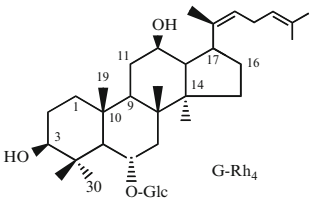
Several classes of compounds have been isolated from TCM drugs, among them the triterpene or steroid saponins suggested to contribute in part to the pharmacological activities. Among some promising advances in extraction methods, ultrasound-assisted extraction of saponins has been found to be more efficient and three times faster than conventional soxhlet extraction (Angelova et al. 2008). This method is usually carried out at lower temperatures than other forms of extraction, which helps avoid the degradation of thermally unstable constituents in plant material. Microwave-assisted extraction is also more efficient than the conventional method. In a recent study, Qi et al. (2010b) developed a fast and efficient method for isolation of four known ginsenosides (Rf, Rd, Re, and Rb1) from *Panax ginseng* (Asian ginseng) by high-performance countercurrent chromatography (HPCCC) coupled with evaporative light scattering detection (ELSD) (Fig. 3.1). The purity was assessed by HPLC coupled with a diode array detector (DAD) and ELSD (HPLC–DAD–ELSD) and the structures were characterized by electrospray ionization mass spectrometry (ESI-MS) and compared with standard. The study of the heat-processed leaves of *P. ginseng* led to the isolation and structural elucidation of three new dammarane-type glycosides with the unusual peroxyl group at C-23, called ginsenosides SL1–SL3 (Fig. 3.1), as well as 11 known compounds (Tung et al. 2010). This study completed previous work on the leaves describing the isolation and structural elucidation of two new dammarane-type saponins, Ki and



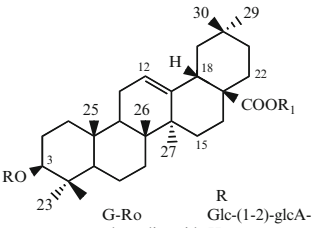
	R	R_1
G-Rb1	Glc-(1-2)-glc-	Glc-(1-6)-glc-
G-Rb2	Glc-(1-2)-glc-	Ara-(1-6)-glc-
G-Rc	Glc-(1-2)-glc-	Araf-(1-6)-glc-
G-Rh2	Glc-	H
G-Rg3	Glc-(1-2)-glc-	H
G-Rs3	6Acglc-(1-2)-glc-	H
G-F2	Glc-	Glc-
G-Rd	Glc-(1-2)-glc-	Glc-
G-Rb3	Glc-(1-2)-glc-	Xyl-(1-6)-glc-



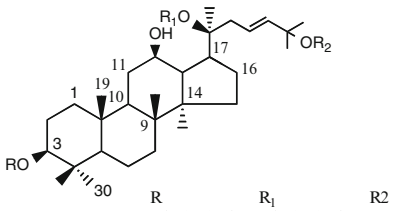
	R	R_1
G-Re	Rha-(1-2)-glc-	Glc-
G-Rf	Glc-(1-2)-glc-	H
G-Rh1	Glc-	H
G-Rg1	Glc-	Glc-
G-Rg2	Rha-(1-2)-glc-	H
NG-R1	Xyl-(1-2)-glc-	Glc-



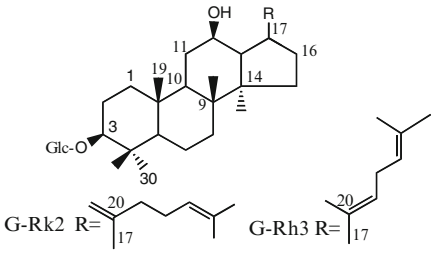
$G\text{-}Rh_4$



	R	R_1
G-Ro	Glc-(1-2)-glcA-	Glc-
oleanolic acid	H	H



	R	R_1	R2
NG-K	Glc-(1-2)-glc-	Glc-(1-6)-glc-	H
Quinquefoloside -Lc	Glc-(1-2)-glc-	Xyl-(1-6)-glc-	CH_3



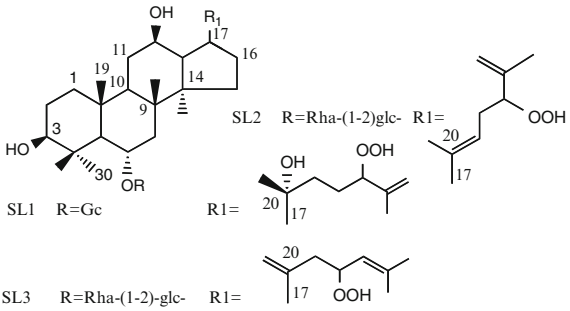


Fig. 3.1 Representative examples of *Panax* saponins

Km, together with 15 known ones (Tung et al. 2009). Compared to the long history of use and widespread research on Asian ginseng, the study of American ginseng (*Panax quinquefolius*, also reported as *P. quinquefolium*) is relatively limited. In a recent review, the different structures of the ginsenosides in American ginseng are described, including naturally occurring compounds and those resulting from steaming or biotransformation (Qi et al. 2011). A total of 98 ginsenosides, mainly protopanaxadiol and protopanaxatriol glycosides, have been identified. Differences in sugar types, quantities, and attachment positions provide diversity in ginsenoside structures. Highlighted are the chemical and pharmacological diversity and potential structural–activity relationship of ginsenosides.

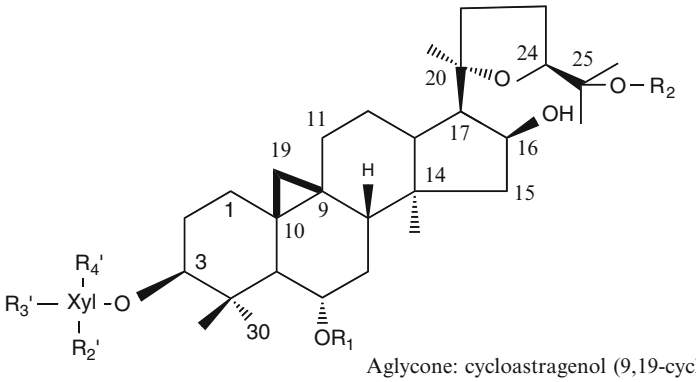
High-speed countercurrent chromatography coupled with ELSD (HSCCC-ELSD) is a powerful technique used, e.g., for separating and purifying four triterpene saponins astragalosides I, II, IV, and acetylastragaloside I (Fig. 3.2) from *Astragalus membranaceus* roots, using stepwise elution with a pair of solvent systems composed of *n*-hexane–ethyl acetate–ethanol–water (1:0.6:0.6:1 and 1:1:1:1). The compounds were identified by comparison of NMR, and MS data with literature data of standards compounds (Peng et al. 2008).

Centrifugal partition chromatography (CPC) coupled with ELSD detection (CPC-ELSD) was applied to separate saikosaponins a and c preparatively from *Bupleurum falcatum* roots (Yoon and Kim 2009) (Fig. 3.3). The two-phase solvent system composed of ethyl acetate/*n*-butanol/methanol/water (15:1:3:15) was used to yield saikosaponins a and c from a saponin-rich extract with 96.6 and 97.3 % of purity, respectively. Structural identification of these compounds was accomplished by comparison of spectroscopic data from ESI-MS, ^{1}H NMR, and ^{13}C NMR studies with those reported previously.

An efficient and rapid method of isolation of saponins from *Gypsophila paniculata* was achieved for the first time by using high-speed countercurrent chromatography (*n*-hexane–*n*-butanol–methanol–0.02 % TFA, 1:9:1:9) and compared with preparative-HPLC in order to integrate their advantages to improve separation efficiency (Yao et al. 2008). Thus, five known triterpene saponins were isolated and characterized.

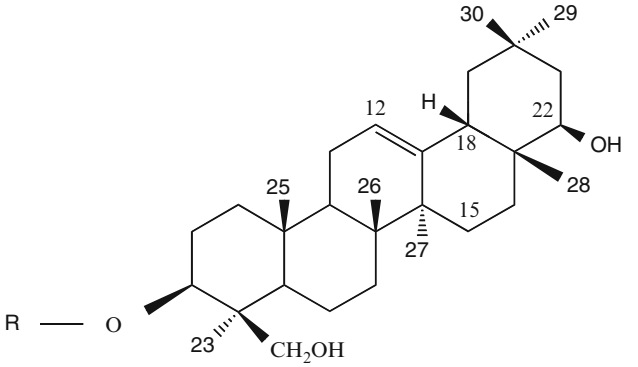
Dioscin derivatives from the *Dioscorea villosa* roots were isolated and purified by CPC-ELSD. A solvent system composed of chloroform–methanol–isopropanol–water (7:6:1:4) was used to obtain prosapogenin A of dioscin, dioscin, deltonin, and diosgenin 3-*O*-[α-L-rhamnopyranosyl(1→2)]-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)]-β-D-glucopyranoside from a saponin-rich extract from the roots of *D. villosa* (Yoon and Kim 2008) (Fig. 3.4).

There are many literature reports on the isolation of saponins from *Paris polyphylla*, and, as these components are very valuable, it is very important to use an effective extraction method. A new method of ultrahigh pressure extraction (UPE) was used to extract steroid saponins at room temperature (Zhang et al. 2007b). The optimum extraction is achieved with ethanol 90 %, a pressure of 400 MPa, 2 min, and a liquid to solid ratio of 40:1. The UPE methodology showed higher efficiency at room temperature than the usual room temperature extraction at normal pressure, ultrasound-assisted extraction (frequency: 50 Hz; power: 250 W), microwave-assisted extraction (frequency: 2,450 Hz; radiation source: 300 W), and soxhlet extraction.



Aglycone: cycloastragenol (9,19-cyclo lanostane-type)

	R1	R2	R2'	R3'	R4'
Acetyl-astragaloside I	Glc	H	Ac	Ac	Ac
Astragaloside I	Glc	H	Ac	Ac	H
Isoastragaloside I	Glc	H	Ac	H	Ac
Astragaloside II	Glc	H	Ac	H	H
Isoastragaloside II	Glc	H	H	Ac	H
Astragaloside III	H	H	Glc	H	H
Astragaloside IV	Glc	H	H	H	H
Isoastragaloside IV	H	Glc	H	H	H
Astragaloside V	H	Glc	Glc	H	H
Astragaloside VI	Glc	H	Glc	H	H
Astragaloside VII	Glc	Glc	H	H	H

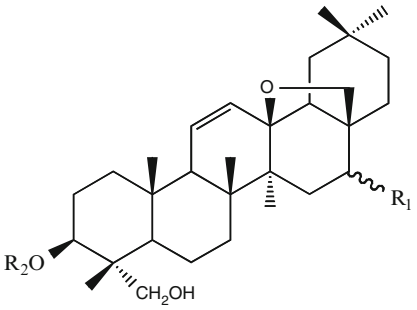


Aglycone: Soyasapogenol B (Δ^{12} oleanene-type)

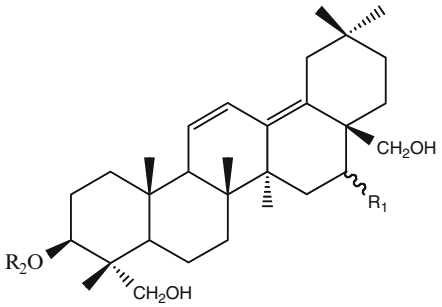
Astragaloside VIII	R = Rha-(l-2)-xyl-(l-2)-GlcA-
Soyasaponin I	R = Rha-(l-2)-Glc-(l-2)-GlcA-

Fig. 3.2 Representative examples of *Astragalus* saponins

Ophiopogon japonicus Ker-Gawler (Liliaceae) is used in TCM as an expectorant, antitussive, and tonic agent, and its tubers are often used for the treatment of cardiovascular and cerebrovascular diseases in combination with *Panax ginseng*.



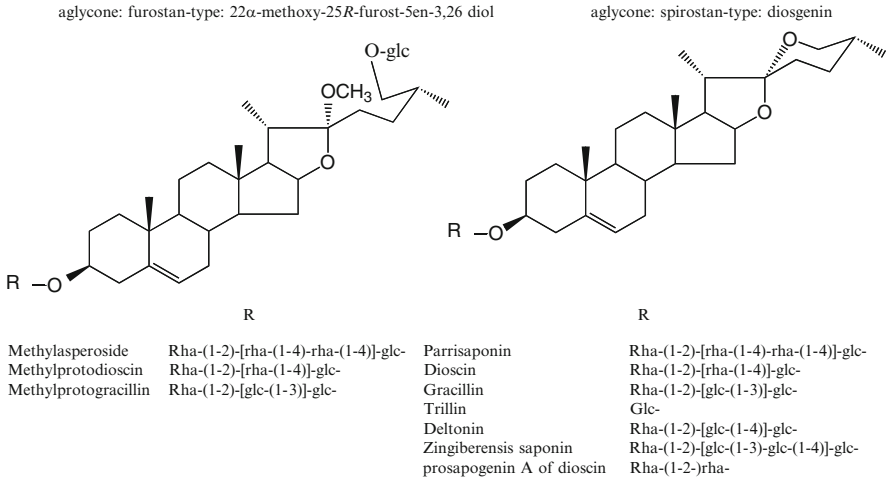
	R1	R2
Saikosaponin a	β-OH	Glc-(1-3)-fuc-
Saikosaponin c	β-OH	Rha-1-4)-[glc-(1-6)]-glc-
Saikosaponin d	α-OH	Glc-(1-3)-fuc-



	R1	R2
Saikosaponin b1	β-OH	Glc-(1-3)-fuc-
Saikosaponin b2	α-OH	Glc-(1-3)-fuc-

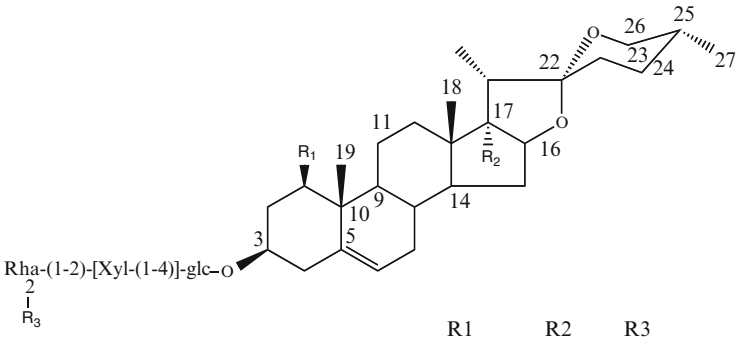
Fig. 3.3 Representative examples of *Bupleurum* saponins

Steroidal saponins are the main active constituents and three new steroidal saponins were isolated recently from the fibrous roots and characterized as derivatives of ruscogenin, diosgenin, and pennogenin (Duan et al. 2010) (Fig. 3.5). However, because of their high polarity, being non-chromophores, and low content in plants, steroidal saponins are difficult to isolate from *O. japonicus* using conventional phytochemical methods. Therefore a method of HPLC/ESI-MS(n) was used successfully, yielding a total of eight steroidal saponins and important structural information on aglycone types, sugar types, and saccharide sequences (Wang et al. 2011). An original method of extraction has been achieved with 75–85 % ethanol in an ultrasonic extractor (ultrasonic wave with a frequency of 20–150 kHz at 20–80° for 20–200 min), filtering, concentration, and partition against *n*-butanol. This method has the advantages of short extraction period, low temperature extraction, reduced solvent consumption, and high extraction efficiency (Cai et al. 2010).



R		R	
Methylasperoside	Rha-(1-2)-[rha-(1-4)-rha-(1-4)]-glc-	Parrisaponin	Rha-(1-2)-[rha-(1-4)-rha-(1-4)]-glc-
Methylprotodioscin	Rha-(1-2)-[rha-(1-4)]-glc-	Dioscin	Rha-(1-2)-[rha-(1-4)]-glc-
Methylprotogracillin	Rha-(1-2)-[glc-(1-3)]-glc-	Gracillin	Rha-(1-2)-[glc-(1-3)]-glc-
		Trillin	Glc-
		Deltonin	Rha-(1-2)-[glc-(1-4)]-glc-
		Zingiberensis saponin	Rha-(1-2)-[glc-(1-3)-glc-(1-4)]-glc-
		prosapogenin A of dioscin	Rha-(1-2-)rha-

Fig. 3.4 Representative examples of *Dioscorea* saponins



	R1	R2	R3
Ruscogenin triglycoside	OH	H	OH
Diosgenin triglycoside	H	H	OAc
Pennogenin triglycoside	H	OH	OAc

Fig. 3.5 Representative examples of *Ophiopogon* saponins

3.1.1.2 Identification

The structural elucidation of the saponins is based on the 1D- and 2D-NMR experiments (COSY, TOCSY, NOESY, HSQC, HMBC) and mass spectroscopy (MALDI-TOF-MS, ESI-TOF-MS, HR-FAB-MS). In recent years, a number of important analytical methods have been reported to provide excellent separation and good structural characterization abilities, suitable to the analysis of complex TCM extracts. An overview of TLC and HPLC fingerprint analytical techniques, allowing the identification of saponins in more than 10 TCM drugs, was recently reported (Wagner et al. 2011).

"Ginseng" (Asian, American, Japanese, Himalayan, Siberian ginseng) refers to a wide spectrum of distinct species with different medicinal qualities. Many factors, including growing and storage conditions, can influence the quality of ginseng products. Recent reviews summarized the most recent development of ginseng analysis, in particular novel approaches in sample pretreatment and the use of HPLC-MS (Angelova et al. 2008; Jia and Zhao 2009). Often, total content in ginsenosides and Rg1/Rb1 ratio which differ among species are used for the standardization in ginseng. For quality control purposes, numerous ginsenosides such as Rb1, Rb2, Rc, Rd, Re, and Rf (Fig. 3.1) have been chosen as reference standards. A HPLC–ELSD method was developed for simultaneous determination of 11 major triterpene saponins in *Panax notoginseng* (namely, notoginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3, Rd, and Rg3) (Fig. 3.1). The method provided good reproduction and sensitivity (Wan et al. 2006). In recent years, ultra-performance liquid chromatography has provided new possibilities for fast separation, enhanced resolution and sensitivity, high-speed detection, and reduction in the consumption of mobile phase. Thus the UPLC-ESI-MS methodology was used successfully to tentatively identify 21 major saponins and to quantify 15 of these in roots of *P. notoginseng* (Dan et al. 2009). This method gives limits of detection and quantification within the range of 0.015–0.382 and 0.052–1.124 μg/ml, respectively, for 15 studied saponins.

In traditional Chinese medicine, *Gynostemma pentaphyllum* (Thunb.) Makino is an herbal drug that has been extensively researched in China. Dammarane saponins isolated from this drug, namely, gypenosides or gynosaponins, were believed to be responsible for various phamacological properties (Razmovski-Naumovski et al. 2005). Over 100 saponins were isolated and characterized from this drug by scientists in Japan and China since 1976. Some of them are the same protopanaxadiol-type ginsenosides Rb1 (gypenoside III), Rb3 (Gypenoside IV), and Rd (Gypenoside VIII) found in *Panax ginseng* by direct comparison with authentic standard samples (Razmovski-Naumovski et al. 2005; Tsai et al. 2010). This constitutes the first example of ginsenosides found outside of the Araliaceae family. Authors have characterized the main gypenosides in the ethanol extract by HPLC with ELSD or MS detection as gypenosides LXIII, IV (ginsenoside Rb3), and VIII (ginsenoside Rd) (Fig. 3.6) which might be responsible for inhibition of the proliferation of the glioma cells (Schild et al. 2010, Fig. 3.6). An appropriate extraction, purification, and HPLC-MS method was developed to determine saponins and flavonoids in *G. pentaphyllum*. A total of 34 saponins were separated within 34 min using a Gemini C18 column and a gradient mobile phase of acetonitrile and 0.1 % formic acid in water, in which 18 saponins were identified by LC/MS with ESI mode and Q-TOF (LC/MS/MS) (Kao et al. 2008).

Many analytical methods have been used for the quality assessment of *Astragalus membranaceus* through LC-MS of HPLC–ELSD of saponins, which is suitable for the routine analysis of TCM compounds (Yu et al. 2007). A rapid, efficient, and accurate LC-MS/MS method was used for the separation and simultaneous quantification of astragalosides I–IV (Fig. 3.2) in samples of Radix *Astragali* (Zu et al. 2009). Chromatographic separation was achieved on an Agilent Eclipse XDB

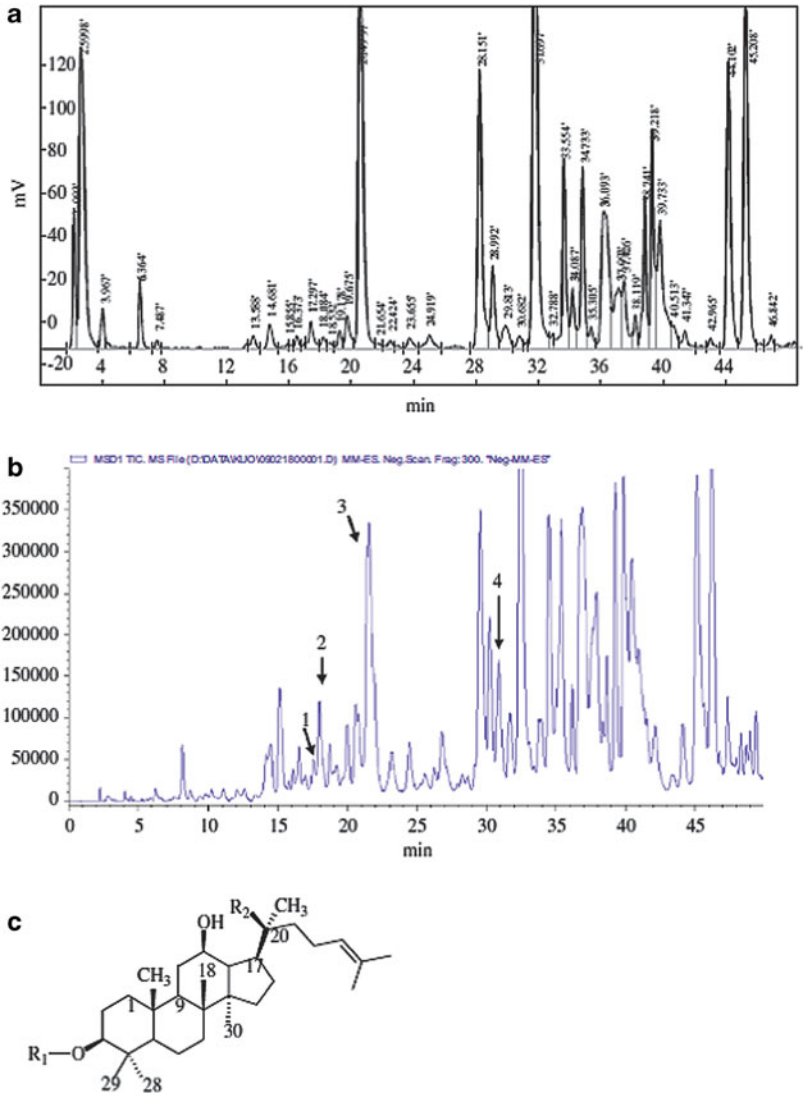


Fig. 3.6 Characterization of the ethanolic *Gynostemma pentaphyllum* extract. (**a**) HPLC chromatogram of saponins from *G. pentaphyllum* extract with ELSD detection. *Peak 1*: Gypenoside LXIII; *peak 2*: Gypenoside LXIII; *peak 3*: Gypenoside IV (Ginsenoside Rb3); *peak 4*: Gypenoside VIII (Ginsenoside Rd). (**b**) HPLC chromatogram of saponins from *G. pentaphyllum* extract with MS detection. *Peak 1*: Gypenoside LXIII; *peak 2*: Gypenoside LXIII; *peak 3*: Gypenoside IV (Ginsenoside Rb3); *peak 4*: Gypenoside VIII (Ginsenoside Rd). (**c**) A common structure of gypenosides (dammarane-type triterpene glycosides) partly identical with Ginseng saponins (with permission of the authors Schild et al. 2010)

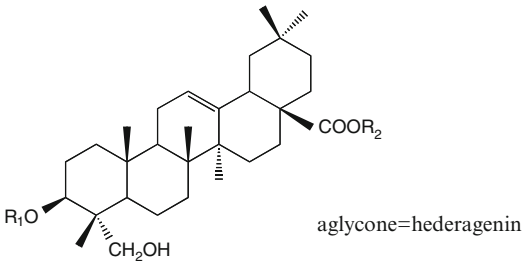
(ODS)-C18 column with a mobile phase consisting of acetonitrile and 0.05 % formic acid aqueous solution by the use of an efficient 17-min program, which can be applied for quality control of Radix *Astragali* and related medicinal products. A triple quadrupole mass spectrometer was operated in positive ionization mode with multiple reaction monitoring for the detection of four astragalosides.

Oleanane-type triterpene saponins were characterized from *Glycyrrhiza uralensis* using a rapid-resolution liquid chromatography with time-of-flight mass spectrometry (RRLC-TOF-MS) method. The major diagnostic ions and fragmentation pathway have been characterized for the first time, allowing the unambiguous identification of 13 saponins from *G. uralensis* (Zheng et al. 2010b). Similarly, an RRLC method coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) has been developed for analysis of oleanane triterpene saponins from *Achyranthes bidentata* (Li et al. 2010f). A retro Diels–Alder rearrangement from the oleanane aglycone skeleton in the MS/MS process yielded characteristic fragment ions in positive-ion mode, which were helpful in predicting the aglycone structure. Losses of monosaccharide sequences and the sugar-chain fragment ions provided important information on sugar types and attachment sequences. A total of 22 oleanane-type saponins were rapidly identified by this method and opened possibilities for similar studies on other herbal medicines.

A recent work mentioned a sensitive HPLC-ESI-MS method for the determination of asperosaponin VI, a triterpenoid saponin extracted from *Dipsacus asper* (Dipsacaceae) in rat plasma (Li et al. 2010e). Chromatographic separation was achieved on a C(18) column with a mobile phase of 10 mM ammonium acetate buffer containing 0.05 % formic acid–methanol (32:68). The validated method was successfully applied in a pharmacokinetic study of rats administered asperosaponin VI orally (Fig. 3.7).

To ensure the clinical efficacy of the Chinese herbal drug *Flos Lonicerae* (*Lonicera japonica*, Caprifoliaceae), a new HPLC–ELSD method has been developed that allowed the simultaneous quantification of seven major saponins in the drug. The separation was achieved on a C18 analytical column eluted by a mobile phase consisting of acetonitrile–acetic acid (95:0.5) and 0.5 % acetic acid using a gradient elution. The drift tube of temperature of ELSD was set at 106°C with a nitrogen flow rate of 2.6 l/min (Chai et al. 2005). A detailed review reported the application of a HPLC-MS method in the phytochemical analysis of TCMs (Yang et al. 2009b). Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are the two commonly used ion sources, whereas triple quadrupole, ion trap, Fourier transform ion cyclotron resonance (FTICR), and time-of-flight (TOF) mass spectrometers were used as online analyzers. A fast liquid chromatography method with diode array detection (DAD) and time-of-flight mass spectrometry (TOF-MS) has been newly developed for the analysis of constituents in *Flos Lonicerae,* a TCM (Qi et al. 2009).

An ultrahigh-performance liquid chromatography method coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UHPLC/ESI-Q-TOF-MS/MS) was developed to separate and identify triterpenoid



	R_1	R_2
Akebia saponin D = asperosaponin VI	Glc-(1-4)-glc-(1-3)-rha-(1-2)-ara-	Glc-(1-6)-glc-

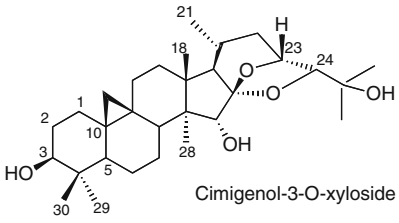
Fig. 3.7 Representative examples of *Dipsacus* saponins

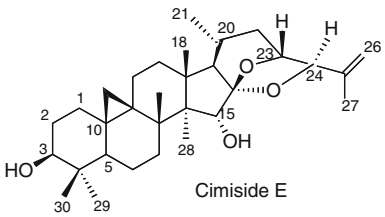
saponins in crude extract of the stem bark of *A. julibrissin* (Han et al. 2011). On the basis of the analysis of the spectra, fragmentation rules were proposed, which led to the identification of 28 compounds, including eight new saponins. This method offers advantages in resolution, speed, reproducibility, and sensitivity of analysis and provided a rapid and accurate procedure of the identification of triterpenoid saponins in crude extract of *Albizia julibrissin*. The combination of UHPLC and Q-TOF technique provides excellent separations and good structural elucidation, which make it suitable for analyzing complex TCM extracts.

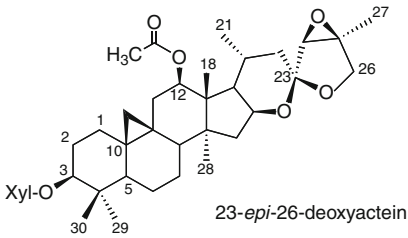
The genus *Actea* (including *Cimicifuga*) has been a source of around 200 cycloartane triterpenes. While they are major bioactive constituents of complementary and alternative medicines, their structural similarity is a major dereplication problem. New tools including qNMR enabled rapid dereplication of more than 170 known triterpenes and facilitated elucidation of new compounds (Qiu et al. 2012). An analysis by LC/turbo ion spray (TIS)-mass spectrometry (MS) method was developed to examine the finger profile of seven *Cimicifuga* herbs and six *Cimicifuga racemosa* commercial products (Wang et al. 2005). This method provides a tool for the rapid identification of *Cimicifuga* plants and quality control for the manufacture of *C. racemosa*. Triterpene glycosides were selected as chemical markers because they appear as major phytochemical class in *Cimicifuga* species (Wang et al. 2005). Among them, actein, 23-epi-26-deoxyactein, and cimigenol-3-*O*-xyloside (Fig. 3.8) were found to be the main constituents of *Cimicifuga* rhizoma used in TCM (Wagner et al. 2011).

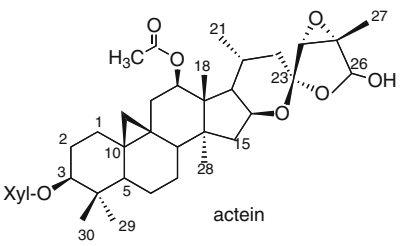
A simple and accurate method of HPLC–ELSD was developed for simultaneous determination of five triterpene saponins in *Clematis* ssp. for the first time. Using this method, 10 samples from *Clematis* ssp. were analyzed on a Zorbax SB-C (18) column eluted by a gradient elution of acetonitrile and water with 0.1 % formic acid (Sun et al. 2008a). A method of HPLC coupled with ESI multistage mass spectrometry [HPLC-ESI-MS(n)] was successfully applied to characterize triterpene saponins from nine *Clematis* ssp. crude extracts (Sun et al. 2007a).

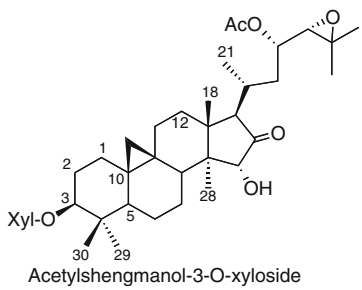
A UPLC-ESI-Q-TOF-MS/MS method was applied to the separation and characterization of steroidal saponins, the major bioactive constituents, from crude extracts of *Dioscorea zingiberensis*. According to the summarized fragmentation











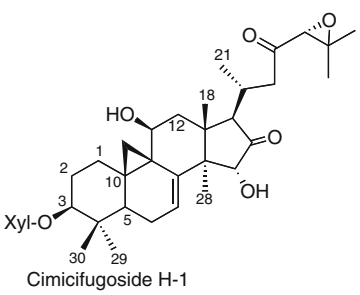


Fig. 3.8 Representative examples of *Cimicifuga* ssp. saponins

patterns, the identification of a total of 31 saponins with five aglycone skeletons from *D. zingiberensis* could be achieved, even when reference standards were unavailable, based on their retention times, mass spectrometric fragmentation patterns, and MS and MS/MS data (Zhu et al. 2010). An effective, sensitive, and rapid analytical method as an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed to determine the diosgenin content in the rhizome of *Dioscorea opposita*. This method constitutes a valuable tool for quality control of diosgenin-containing medicinal plants (Li et al. 2010d). A sensitive and simple

HPLC–ELSD method was developed for the quality control of 31 commercial samples belonging to seven species of *Dioscorea* L. from different areas (*D. spongiosa*, *D. hypoglauca*, and *D. nipponica*), commonly used in TCM (Pharmacopeia Committee of P. R. China 2010), *D. tokoro*, *D. tenuipes*, *D. gracillima*, and *D. zingiberensis* (Shen et al. 2011). Thus, for the first time four bioactive components were determined simultaneously (dioscin, gracillin, protoneodioscin, and protoneogracillin) (Fig. 3.4) and their contents were quite different among the seven species of *Dioscorea* L. A HPLC-ESI-MS/MS method was successfully employed for the simultaneous identification and quantification of steroidal saponins in the rhizomes of *Paris polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* (Zhang et al. 2010). The characteristic fragmentation pattern of diosgenin- and pennogenin-type steroidal saponins was investigated using ESI-MS (n) in negative-ion mode and provided useful information for the identification of steroid saponins in both of these species.

Another review focussed on the identification of the phytochemical constituents in the crude extracts of TCM drugs using HPLC/MS, which facilitate the convenient control of traditional medicines and their pharmacological preparations (Yang et al. 2009b). A number of examples of phytoconstituents were given, among them saponins from *Panax* ssp., *Astragalus* ssp., and *Dioscorea* ssp.

3.1.2 Pharmacology of Saponins from TCM Drugs

3.1.2.1 Triterpene Saponins

TCM Sources of New Saponins with Immunoadjuvant Activities

Since the saponins are well known to act as adjuvant when given together with an antigen in a vaccine, considerable efforts have been made to identify active components responsible for this effect. The mechanism of action of most adjuvants still remains only poorly understood, since immunization often activates a complex cascade of responses. Adjuvant functions include an induction of a mixed Th1/Th2 response with its typical cytokines (interferon (IFN)-γ, Il 2 and Il 4, or Il 10) and antibodies of the IgG1 and IgG2a class in mice or IgG2 in dogs (Lacaille-Dubois 2005). The aldehyde group at C-4 of the aglycone constitutes an important structural feature involved in direct T-lymphocyte stimulation, inducing a Th 1 protective response. The strongest adjuvant active saponins were isolated from *Quillaja saponaria* (Rosaceae), a Chilean tree, of which QS 21 in particular has been evaluated in a large number of parenterally administered vaccines in phase I and phase II human clinical trials, including cancer immunotherapies (melanoma, breast, and prostate), HIV recombinant envelope, and malarial antigens. After the saponins from *Quillaja saponaria* and their anologs have been extensively studied as immunoadjuvants, few saponins from other sources have been reported to possess this property.

Recently, increased research has been carried out on plant-derived saponins in the search for new adjuvants from TCM herbs, mainly *Panax ginseng* and *P. notoginseng, Astragalus* ssp., *Momordica cochinchinensis*, *Glycyrrhiza uralensis*, *Achyranthes bidentata*, and *Platycodon* ssp. (Song and Hu 2009).

Panax ginseng

The roots of *Panax ginseng* C.A. Meyer (Araliaceae) have been used traditionally for more than 2,000 years in Asian countries. The name of the genus *Panax* is derived from the Greek *pan* (all), *akos* (cure), meaning "cure all". The roots are recognized as a general tonic in the Pharmacopoeia of the People's Republic of China (2005). Its beneficial effects have been analyzed in extensive preclinical and epidemiological studies. It exhibited various pharmacological effects including anti-inflammatory, antioxidant, antistress, immunostimulant, antitumor, as well as stimulant effects on the central nervous system. The ginseng saponins, called ginsenosides, are dammarane-type saponins (Fig. 3.1) and are traditionally considered the most important and representative bioactive constituents in the drug (Lacaille-Dubois 2005). The use of biochemical and molecular–biological techniques allowed the determination of pharmacological actions and possible mechanism of the main ginsenosides, as reviewed by Xiang et al. (2008). More than 40 ginsenosides have been characterized in *Panax ginseng* which can be classified into protopanaxadiol and protopanaxatriol glycosides. In a 2007b study by Sun et al., the ginsenosides showed various immune responses when co-administered with ovalbumin (OVA) in mice. The compounds were investigated for their adjuvant effects on the immune response to OVA in mice by measuring specific IgG isotypes, lymphocyte proliferation, and production of IFN-γ and Il 5. Of the eight ginsenosides, Rg1, Re, Rg2, Rg3, and Rb1 (Fig. 3.1) showed higher adjuvant effects than the others in terms of inducing specific antibody responses. This indicates that they could be considered the major compounds contributing to the adjuvant properties of total ginseng saponins. Adjuvant properties might be attributed to the different molecular conformation determined by the sugar chains linked to the dammarane skeleton. Namely, the lack of sugar at C-20 or the presence of only one sugar might characterize a relative hydrophobicity of the saponin which affects the adjuvant activity. Among them, ginsenoside Rg1 (Fig. 3.1) has been found to act synergistically with aluminum hydroxide in inducing antibody responses as well as Il5 and IFN-γ against OVA in mice (Sun et al. 2008b).

In order to study the adjuvant active compounds from the total saponins from *P. notoginseng*, ginsenoside Rh_4 was isolated and characterized as glucopyranosyl-6-protopanaxadiol-20,24 diene (Fig. 3.1). This compound in the splenocyte-proliferation assay was shown to enhance the mitogen- and OVA-induced splenocyte proliferation in OVA-immunized mice, especially at a dose of 25 μg, in comparison with the OVA control group. The OVA-specific serum IgG, IgG1, and IgG2b antibody levels were also significantly enhanced by Rh_4 at a dose of 25 μg in comparison to the OVA control group. These results indicate that

ginsenoside Rh4 significantly increased the activation potential of both T and B cells in mice immunized with OVA (Yang et al. 2007). Under the experimental conditions, Rh4 showed a slight hemolytic effect and this suggests that Rh_4 could be safely used as an adjuvant, with low side effects. In a screening based on immunological bioassay reported by Qin et al. (2006), six immunologically active adjuvant saponins, notoginsenosides K, R1, and U and ginsenosides Rd, Re, and Rh_4, were isolated. As ginsenoside Rh_4, notoginsenoside K (NG-K) (Fig. 3.1) was shown to possess a slight hemolytic effect and to enhance significantly a specific antibody and cellular response against OVA in mice, suggesting its safe use as an adjuvant (Qin et al. 2006).

Astragalus Species

The root of several *Astragalus* species, including *A. membranaceus* (Fisch) Bge var. *mongolicus* (Bge) and *A. membranaceus* (Fish) Bge, has a long history of TCM use for the treatment of nephritis, diabetes, and cancer. It is now used as an immunomodulating agent in some herbal preparations for the treatment of common cold, diarrhea, fatigue, anorexia, and cardiac diseases. Modern pharmacological studies have shown various activities such as immunostimulant, tonic (adaptogenic), hepatoprotective, diuretic, antidiabetic, analgesic, expectorant, and sedative properties. Extensive chemical investigations revealed that polysaccharides, flavonoids, and saponins of *Astragalus membranaceus* were considered pharmacologically active compounds. A 2011 review provides comprehensive information on the history, traditional uses, phytochemistry, pharmacological research and toxicology, and clinical application of *Astragalus radix* to explore their therapeutic potential and future research opportunities (Liu et al. 2011). The phytochemical studies reported that about 40 saponins were isolated from the roots in recent years. Many saponins belonged to cycloartane tetracyclic triterpenoids, including the structure without and with furan ring, while a few saponins belonged to oleanane triterpenoids (Fig. 3.2).

The adjuvant potential of *A. membranaceus* saponins (AMS) on the cellular and humoral immune responses of mice against OVA was investigated (Yang et al. 2005). AMS enhanced the Con-A, LPS-, and OVA-induced splenocyte proliferation in the OVA-immunized mice at a dose of 100 μg. OVA-specific IgG, IgG1, and IgG2 antibody titers in serum were also enhanced by AMS compared with OVA control group. These results, together with the low hemolytic properties, suggest that AMS could be used safely as adjuvant. These results corroborated those showing prominent Il-2 inducing activity of triterpene saponins from *Astragalus* species (Yesilada et al. 2005). IL-2 is a cytokine produced by activated T cells, which has shown powerful immunostimulatory properties. Accordingly, the IL-2 inducing activity of the triterpene saponins might be the mechanism involved in order to explain the immunomodulatory effects of *Astragalus* species.

Bupleurum ssp.

In the Chinese Pharmacopoeia, the official drugs are represented by *Bupleurum chinense* D.C. and *B. scorzonerifolium* Willd. They are often used in combination with other drugs as antihepatotoxic, antipyretic, analgesic, sedative, and antidepressant agents, in case of menstrual complaints, sudden loss of hearing, and malaria.

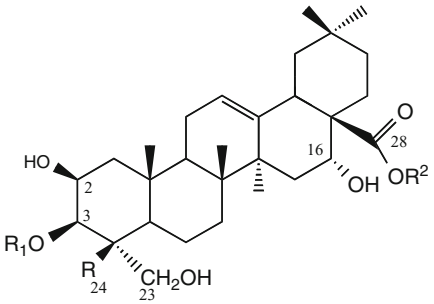
The crude saponin mixture was shown to significantly enhance the ConA-, LPS-, and OVA-induced splenocyte proliferation in the OVA-immunized mice at a dose of 100 mg. The OVA-specific IgG, IgG1, and IgG2b antibody levels in serum were also significantly enhanced by the saponin of *B. chinensis* compared with OVA control group. These results suggested that this extract could be safely used as adjuvant with low or non-hemolytic effect (Sun 2006a). The main constituents are triterpene saponins of the oleanane-type saikosaponins a, c, d, b1, and b4 (Fig. 3.3). Saikosaponin d (Ssd) exhibits a variety of pharmacological and immunomodulatory activities, including anti-inflammatory, antibacterial, antiviral, and anticancer effects. A recent study described the effects of Ssd on activated mouse T lymphocytes through the NF-kappaB, NF-AT, and AP-1 signaling pathways, cytokine secretion, and IL-2 receptor expression (Wong et al. 2009). The results demonstrated that Ssd not only suppressed OKT3/CD28-costimulated human T cell proliferation, but also inhibited phorbol 12-myristate 13-acetate (PMA)-, PMA/Ionomycin-, and Con A-induced mouse T cell activation in vitro.

Platycodon grandiflorum

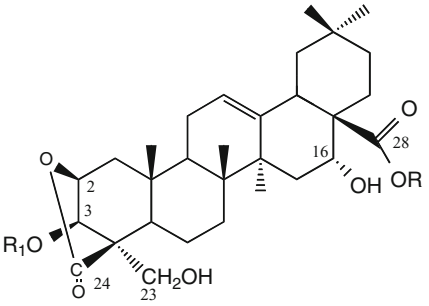
The roots of *P. grandiflorum* A. D.C. (Campanulaceae) provide a well-known pharmaceutical preparation used as a remedy for respiratory disorders. The extract possesses a wide range of pharmacological functions such as immunomodulatory, antitumor, anti-inflammatory, hepatoprotective, antihyperlipidemic, anti-adiposity, hypoglycemic, analgesic, and neuroprotective activities (Xie et al. 2008). The oleanane-type saponins proved to be the main bioactive principles responsible for the above-mentioned activities. These compounds are derivatives of four types of aglycones, platycodigenin, platycogenic acid A, platycogenic acid A-lactone, and polygalacic acid. From a chemical point of view, the most interesting saponins from *P. grandiflorum* are platycosides M1–M3 possessing an unusual structure containing a gamma-lactone in A-ring (Fu et al. 2006) (Fig. 3.9).

The studies of immunological-adjuvant properties of TCM led to the conclusion that the total saponin (PGS) from the roots of *P. grandiflorum* exhibits more distinctive adjuvant potentials on a specific antibody and cellular response against ovalbumin (OVA) in mice.

The total saponin fraction was separated on silica gel column, affording four fractions A, B, C, and D. It was shown that PGS and its fractions elicit both Th1 and Th2 responses as associated with an enhancement of IgG2b and IgG1 levels. More recently, a study of Xie et al. (2010) led to the conclusion that the pure saponin, platycodin D, improves the immunogenicity of the Newcastle disease virus-based recombinant avian influenza (rL-H5) vaccine by enhancing both humoral and



	R	R_1	R_2
Platycodigenin	CH_2OH	H	H
Platycogenic acid A	COOH	H	H
Polygalacic A	CH_3	H	H
Platycodin D	CH_2OH	Glc	Api-(1-3)-Xyl-(1-4)-Rha-(1-2)-Ara-



	R	R1
Platyconic acid A lactone	H	H
Platycoside M1	Glc	H
Platycoside M2	Glc	Rha-(1-2)-ara-
Platycoside M3	Glc	Xyl-(1-4)-rha-(1-2)-ara-

Fig. 3.9 Representative examples of *Platycodon* saponins

cellular immune response in mice and that this compound is a promising adjuvant for influenza vaccines.

Additionally, other TCM drugs such as *Achyranthes bidentata* (Sun 2006b), *Glycyrrhiza uralensis* (Sun and Pan 2006), and *Gynostemma pentaphyllum* (Sun and Zheng 2006) were also found to be interesting sources of saponins having adjuvant activity in the immune response to ovalbumin in mice (Xie et al. 2008).

TCM Sources of Saponins with Cancer-Related Activities

Panax ginseng

A pharmacological investigation has shown that ginsenoside 20 (*S*)-protopanaxadiol, one of the metabolites of ginseng saponins, is able to inhibit the invasiveness of

human fibrosarcoma HT 1080 cells significantly in vitro and this action may be due to downregulation of the expression of matrix metalloproteinase-2. Increased expression of this enzyme in tumor cells mainly contributes to the proliferation, invasion, and metastasis of cancer cells. This suggested that the metabolite ginsenosid 20(S)-protopanaxadiol may be applied as a potential therapeutic agent in the prevention and treatment of cancer (Li et al. 2006).

Ginsenosides Rh3 and Rk2 (Fig. 3.1), obtained from the heat-processed leaves of *P. ginseng*, showed potent cytotoxic effects with IC_{50} of 0.8 and 0.9 μM against human leukemia cells HL-60 by using the 3-(dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay. Furthermore, ginsenosides SL3, 20*S*-Rg2, F4, and 20*S*-Rh2 displayed strong activities with IC_{50} ranging from 7.5 to 9.0 μM, which were close to the positive control mitoxanthrone (IC_{50} 7.9 μM) (Tung et al. 2010).

Ginsenoside Rg3 combined with low-dose gemcitabine, an antiangiogenic drug, may significantly inhibit angiogenesis and growth of lung cancer and improve survival and quality of life in Lewis lung carcinoma-bearing mice (Liu et al. 2009). Another interesting study showed that a combination of Rg3 (50 μM) with cisplatin (10 μM) and doxorubicin (2 μM) was also more effective in the inhibition of prostate cancer cell growth and NF-kappaB activity than those treated with Rg3 or chemotherapeutics alone (Kim et al. 2010). Recent studies supplied valuable data to demonstrate the antitumor effect and the tentatively identified mechanism of action of Rg3 in B16 melanoma cells, in HCT 116, SW480, and HT-29 colorectal cancer cells, in AGS cells, the most common human gastric adenocarcinoma cell line, and in MDA-MB-231, a breast cancer cell line (Chen et al. 2008a, 2011; Lee et al. 2009; Kim et al. 2011).

Recently, ginsenoside Rh2 treatment significantly inhibited viability of both MCF-7 and MDA-MB-231 human breast cells in a concentration-dependent manner, which correlated with mitochondria-mediated apoptosis. Rh2-induced apoptosis was accompanied by the downregulation of antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. It also caused induction of the proapoptotic members Bak, Bax, and Bim leading to mitochondrial translocation of Bax and activation of caspases. Moreover, Rh2-induced apoptosis was partially, yet significantly, protected by transient transfection of MCF-7 cells with Bax- and Bak-targeted siRNAs. Oral gavage of 5 mg Rh2/kg of mouse (three times a week) caused apoptosis of MDA-MB-231 xenografts significantly (Choi et al. 2011).

In PC-3, DU145, and C4-2 prostate cancer cells, combinations of Rh2 or a PPD with docetaxel were predominantly additive or synergistic. Combinations of Rh2 + docetaxel and a PPD + docetaxel caused established PC-3 tumors to regress from their initial size by 15 % and 27 %, respectively (Musende et al. 2010). (20*S*)-20-*O*-β-D-glucopyranosyl-protopanaxadiol, one of the metabolites detected in blood after oral administration of ginsenoside Rb1, Rb2, or Rc, is the main form of protopanaxadiol saponins absorbed from the intestine. This metabolite is responsible for various biological activities such as anti-inflammatory, antiallergic, hepatoprotective, and antitumor properties. The structure of this compound was solved by X-ray diffraction and the compound was tested in vivo after ip administration, towards the inhibition of Lewis lung carcinoma growth (Zhou et al. 2009a).

It showed a dose-dependent inhibitory effect on tumor growth of 25.7, 19, and 13.2 % after 3 weeks with administration of 10, 5, and 0.5 mg/kg, respectively, as compared to the negative control whereas the positive control cyclophosphamide (CTX) inhibited the tumor growth by 37.5 % at 10 mg/kg. The antitumor efficacy of the metabolite (0.5, 5, and 10 mg/kg) + CTX (10 mg/kg) in combination therapy resulted in significant greater tumor-growth inhibition than CTX alone in a dose-dependent manner (Zhou et al. 2009a).

Panax notoginseng

The antiproliferative effects of major saponins in *P. notoginseng*, notoginsenoside R1, ginsenosides Rb1, Rb3, and Rg1 (Fig. 3.1) were evaluated on SW480 human colorectal cancer cells, and the observed effects supported the pharmacological activity of the extracts of roots, rhizomes, flowers, and berries (Wang et al. 2009a). It was shown that after steaming the *P. notoginseng* roots, ginsenoside Rg3 content increased significantly, an effect that was partially responsible for the increase of the antiproliferative activity in SW480 human colon cancer cells (Sun et al. 2010). A comparative study of the ginsenosides was achieved amongst white (air-dried) and red (steamed) roots of notoginseng (*Panax notoginseng*), Asian ginseng (*Panax ginseng*), and American ginseng (*Panax quinquefolius*) through quantification of 19 major ginsenosides by HPLC/UV at 202 nm and comparison of their anticancer-related activities (antiproliferative effect and apoptosis-inducing activity). It was shown that the white notoginseng possessed higher ginsenoside content two- to fivefold higher than white American and Asian ginseng. During the steaming process, less polar compounds were obtained in red ginseng. Among the three red ginsengs, red notoginseng exhibited the best anticancer activity on colorectal cancer cell lines HCT 116 and the red American ginseng had a slightly higher antiproliferative effect than the red notoginseng on SW480 cell lines (Sun et al. 2011).

Panax quinquefolium

American ginseng (*Panax quinquefolius*) has a long history of use in TCM. These roots are often used in combination with other herbal ingredients with the goal of improving the effectiveness of the TCM preparations on the central nervous, cardiovascular endocrine, and immune systems. It has been used to treat stress and fatigue characterized by insomnia, poor apettite, and nervousness, and also in the regulation of various metabolic disturbances including blood sugar and lipid levels.

A new compound, named quinquefoloside-Lc (Fig. 3.1), was isolated from the leaves of *Panax quinquefolium*, together with nine known compounds, and its structure was elucidated as a tetraglycoside of 3β,12β,20*S*-trihydroxy-25-methoxydammar-23-ene on the basis of MS, 1D-, and 2D-NMR experiments as well as by chemical degradation. The cytotoxicity of these compounds against human breast cancer MCF-7 cell line was tested using the MTT method (Qiu et al. 2009). The results revealed that ginsenosides bearing three or four glycosyl units showed weaker activity than ginsenosides possessing one glucosyl unit.

Novel ginsenosides are continually being isolated and identified with improved techniques. A recent review briefly reported anticancer effects and their mechanism of action, the possible structural–function relationship in cancer chemoprevention. The considerations may produce insights into chemical and pharmacological approaches for enhancing the chemopreventive effects of ginsenosides and for developing novel anticancer agents (Qi et al. 2010a). Ginsenoside Rg3 (Fig. 3.1) isolated from *P. ginseng* and *P. quinquefolius* was shown to inhibit cell proliferation and viability in colon cancer cells in vitro. This effect may be mediated at least in part by blocking nuclear translocation of the β-catenin protein in colon cancer cells (He et al. 2011). These investigations may lead to the development of novel therapies in which Rg3 can be used as an effective adjuvant for the treatment of colorectal cancers.

Astragalus ssp.

Astragalus membranaceus has been used to ameliorate the side effects of antineoplastic drugs. Recently, it was reported that total *Astragalus* saponins (AST) induced growth inhibition and promoted apoptosis in the human hepatocellular HepG2 cells via an extracellular signal-regulated protein kinase (ERK)-independent NF-kB signaling pathway. It was established that AST downregulated expression of the human hepatocellular carcinoma (HCC) tumor marker a-fetoprotein and suppressed HepG2 cell growth by inducing apoptosis (Auyeung et al. 2009).

Furthermore, AST was shown to promote apoptosis in HT-29 human colon cancer cells through caspase activation, DNA fragmentation, and nuclear chromatin condensation. This antitumorigenic effect was also observed in vivo in HT-29 nude mice xenograft, and was comparable with that produced by conventional chemotherapeutic drug 5-fluorouracil (Tin et al. 2007). A study to elucidate the mechanism of action led to the suggestion that AST induces the extrinsic apoptotic cascade and causes cell cycle arrest in HT-29 cells by modulation of both mammalian target of rapamycin (m-TOR) and extracellular signal-regulated kinases (ERK) signaling pathway, in which inhibition of NF-kB is important in the latter mechanism (Auyeung et al. 2010). For the first time three different natural compounds, isolated from hairy roots of *Astragalus membranaceus*, cultivated in airlift bioreactor (astragalosides I, II, and III, 1.64 %, 1.12 %, 1.08 %, Fig. 3.2), were tested for their cytotoxic potential and apoptosis induction in a panel of human tumor cell lines and can be used as means of reliable supply of cycloartane saponins to allow extension of the research to human clinical studies (Ionkova et al. 2010).

Gynostemma pentaphyllum

Gynostemma pentaphyllum (Thunb.) Makino, a traditional Chinese herb of the Cucurbitaceae family, has shown a variety of interesting activities, including antitumor, cholesterol-lowering, immunopotentiating, antiulcer, antioxidant, antihypertensive, antithrombotic properties, just to mention a few (Chen et al. 2009a; Schild et al. 2010). In the following, we will summarize recent results obtained with the aim

of clarifying the mechanism of action on tumor cells. *G. pentaphyllum* contains many biologically active compounds such as dammarane-type glycosides (Shi et al. 2010) called gypenosides, which are related structurally to ginseng saponins (Razmovski-Naumovski et al. 2005). Gypenosides (Gyp), a mixture of saponins, were shown to induce apoptosis in human colon cancer colo 205 cells (Chen et al. 2006), lung cancer cell line A549 (Lu et al. 2008a), human tongue cancer SCC-4 cells (Chen et al. 2009a), mouse leukemia WEHI-3 cells (Hsu et al. 2011), and human leukemia HL-60 cells (Lin et al. 2011). It was shown that gypenosides induced endoplasmic reticulum (ER) stress and production of reactive oxygen species and Ca^{2+}, changed the ratio of Bax/Bcl-2 (increased the levels of Bax but decreased the levels of Bcl-2), resulting in a decrease in the levels of the mitochondrial membrane potential in a time- and dose-dependent manner, cytochrome *c* release, and activation of caspases-3 and -9, before leading to apoptosis (Lin et al. 2011; Fig. 3.10). Oral consumption of Gyp increased the survival rate of mice injected with WEHI-3 cells used as a mouse model of leukemia (Hsu et al. 2011). An interesting discovery was that gypenosides administered orally reduced HL-60 tumors in a xenograft animal model. Tumors in mice receiving Gyp at 5 and 20 mg/kg showed a reduction of tumor size and weight by 34 and 57 %, respectively, as compared with a control group (Lin et al. 2011). Furthermore, Gyp inhibits invasion and migration of human tongue SCC-4 cells by downregulating proteins associated with these processes, resulting in reduced metastasis (Lu et al. 2008b).

Recently a study has shown that an ethanolic extract from *G. pentaphyllum* increased the superoxide dismutase activity and the cellular H_2O_2 concentration in C6 glioma. It inhibited cell proliferation by inducing activation of caspase 3, but no effect was observed on the proliferation of astrocytes. From these results, the authors suggested that the ethanol extract of *G. pentaphyllum* may selectively change the concentration of H_2O_2 to toxic doses only in tumor cells due to increased SOD activity (Schild et al. 2010). However, these results do not allow conclusions concerning the effect of single saponins on proliferation of C6 glioma tumor cells. However, the most representative components of the ethanolic extract are outlined in Fig. 3.6. In another study, the authors showed a preparative column chromatography method on a Cosmosil $75C_{18}$-OPN with a mobile phase constituted of 100 % EtOH used to isolate saponins from *G. pentaphyllum*, and evaluated their antiproliferative effect on hepatoma cells Hep3B (Tsai et al. 2010). A total of 30 saponins were separated within 40 min, of which 17 were identified, including gypenoside IV (ginsenoside Rb3) and gypenoside VIII (ginsenoside Rd).

Dipsacus asper

It was reported that methanol extracts of the roots of *Dipsacus asper* Wall (Dipsacaceae) exhibited apoptosis-inducing activities in a dose-dependent manner in U 937 (human monocyte-like histiocytic) cells (Jeong et al. 2008). The analysis of the active BuOH fraction led to the isolation and characterization of *Akebia* saponin D (ASD) (Fig. 3.7). This molecule induced apoptosis in a dose-dependent manner in U 937 cells, and is highly cytotoxic against human and murine leukemia cells. The subG1 cell population and expression of p53 and Bax gene were

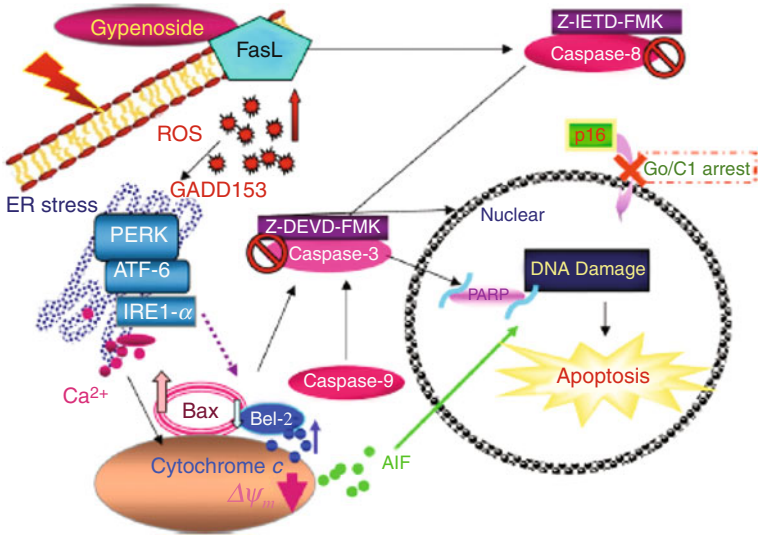
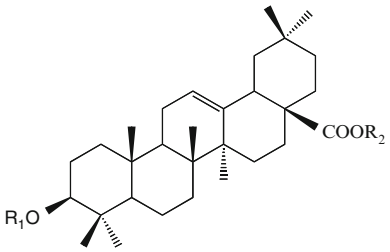


Fig. 3.10 The proposed signaling pathways of Gyp-induced Go/G1 arrest and apoptosis in HL-60 cells (with permission of the authors Lin et al. 2011)

significantly increased and ASD enhanced NO production from RAW264.7 macrophage cells. All these results indicate that ASD may exert apoptosis-inducing activity through activation of NO production and apoptosis-related p53 and Bax gene expression providing a scientific basis on the usefulness of *D. asper* as a chemopreventive agent (Jeong et al. 2008).

Lonicera macranthoides

The dried flower buds of *L. macranthoides* (Caprifoliaceae) are commonly used in TCM for the treatment of fever, headache, bacterial infections, colds, dysentery, enteritis, pain, swelling, and syphilopathy (Wang et al. 2009c). This plant was newly added to the Pharmacopoeia (2005 edition) together with *L. hypoglauca* Miq and *L. confuse* D.C. Among the saponins isolated from this drug (Chen et al. 2008c; Jia et al. 2007), macranthoside B, a 3-*O*-tetraglycoside of hederagenin (Fig. 3.11), was shown to inhibit the proliferation of various kinds of tumor cells with IC_{50} values in the range of 10–20 μM and suppress the growth of HepG2 human hepatocarcinoma in xenograft tumors in athymic BALB/cA nude mice, which was similar to the cyclophosphamide as a positive control (Wang et al. 2009c). Additional investigations have shown that macranthoside B induced apoptosis in HepG2 cells as determined by Annexin V and PI double-staining assays and amplification of the caspase cascade resulting in the cleavage of poly-ADP-ribose polymerase (PARP). In order to perform pharmacokinetic studies in biological samples, a LC/ESI/MS method has been developed and validated for identification and quantification of



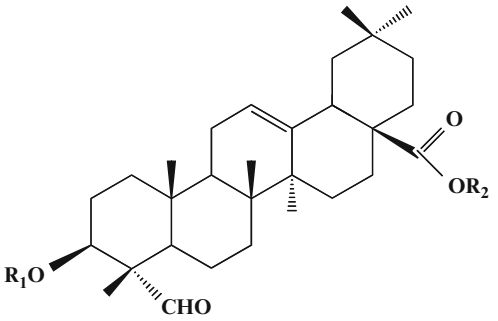
	R_1	R_2
Macranthoidin B	Glc-(1-4)-glc-(1-3)-rha-(1-2)-ara-	Glc-(1-6)-glc-
Macranthoidin A	Glc-(1-3)-rha-(1-2)-ara-	Glc-(1-6)-glc-
Dipsacoside B	Rha-(1-2)-ara-	Glc-(1-6)-glc-
Macranthoside B	Glc-(1-4)-glc-(1-3)-rha-(1-2)-ara-	H

Fig. 3.11 Representative examples of *Lonicera* saponins

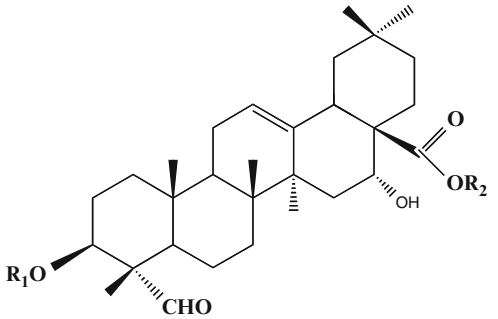
four major bioactive saponins (macranthoside B, macranthoidin A, macranthoidin B, and dipsacoside B) in rat plasma after oral administration of an extract of saponins from *L. macranthoides* (Chen et al. 2009b).

Gypsophila ssp.

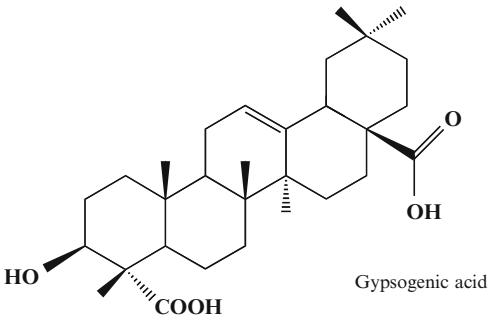
The *Gypsophila* genus belonging to the Caryophyllaceae family includes about 150 species around the world, distributed widely across Asia and Europe. Many of the 18 species growing in China, especially in Xinjiang province, have applications either in TCM or as folklore herbs to treat fever, consumptive disease, and infantile malnutrition syndrome (Nie et al. 2010). Among the Caryophyllaceae family, the genus *Gypsophila* has the highest accumulation of gypsogenin. It appears threefold more often than gypsogenic acid or quillaic acid (Boettger and Melzig 2011). Recently, it was shown that the cytoxicity of cellular membrane-impermeable type I ribosome-inactivating protein (type I RIP), such as saporin, was drastically enhanced by a factor of 3,85,000 upon combination with a complex mixture of triterpene saponins from *Gypsophila paniculata* L. (*Saponinum album*, SAP; Merck N° 7695) in cell culture experiments (Weng et al. 2009). SAP consists mainly of the saponin gypsoside A (30 %) (Fig. 3.12) and further saponins with an identical aglycone (40 %). As pure saponins are necessary for use in medical applications, a convenient method combining mild alkaline hydrolysis, dialysis, and prep-HPLC was developed, resulting in the isolation of three saponins directly from the roots of *Gypsophila paniculata* (Weng et al. 2010). The cytotoxicity was evaluated on ECV-304 cells which were incubated with or without saporin (6 nM). All saponins showed significant toxicity-enhancing properties on saporin without causing toxicity by themselves at 20 μg/ml, as indicated by the comparison to the cytotoxicity of saporin alone. SAP was shown to enhance the cytotoxicity of a targeted chimeric toxin (composed of saporin, the toxic moiety linked by a cleavable adapter to



	R_1	R_2
Gypsogenin	H	H
Gypsoside A	Gal-(1-4)-glc-(1-2)-[ara-(1-3)]-glcA-	Xyl-(1-3)-xyl-(1-2)-[xyl-(1-3)-fuc-(1-4)]-rha-
Gypsophila saponin 2	Gal-(1-2)-[Xyl-(1-3)]-glcA-	Glc-(1-3)-[xyl-(1-4)]-rha-(1-2)-fuc-
Gypsophila saponin 3	Gal-(1-2)-[Xyl-(1-3)]-glcA-	Glc-(1-3)-[xyl-(1-3)-xyl-(1-4)]-rha-(1-2)-fuc-



	R1	R2
Quillaic acid	H	H
Gypsophila saponin 1	Gal-(1-2)-[Xyl-(1-3)]-glcA-	Glc-(1-3)-[xyl-(1-4)]-rha-(1-2)-fuc-
Gypoldoside A	Gal-(1-2)-[Xyl-(1-3)]-glcA-	Ara-(1-2)-ara-(1-3)-xyl-(1-4)-rha-(1-2)-fuc-



Gypsogenic acid

Fig. 3.12 Representative examples of *Gypsophila* saponins

human epidermal growth factor (EGF), a targeting moiety), more than 13,600-fold compared to controls cells, decreasing the IC_{50} values from 2.4 nM to 0.18 pM in HER-14 cells [Swiss mouse embryo NIH-3 T3 cells transfected with human EGF receptor (EGFR)] (Bachran et al. 2006).

Another similar study showed that the cytotoxicity of a chimeric toxin on murine TSA mammary adenocarcinoma cells transfected with human EGFR was enhanced 20,000-fold by low saponin concentration in a synergistic manner (Bachran et al. 2009). This effect was confirmed in vivo by subcutaneous application of SAP and a chimeric toxin in BALBc mice bearing a solid EGFR transfected TSA cell tumor, resulting in 94 % tumor volume reduction with a 50-fold chimeric toxin concentration compared with treatment without saponin. The side effects were moderate and usually reversible. Targeting the EGF receptor is a very promising approach, since it is overexpressed in about 90 % of cervical cancers. Therefore the toxic effect of the plant toxin saporin and EGR as targeting moiety was evaluated on human cervical carcinoma cell lines (Hela, CaSki, SiHa, PHCC1, and PHCC2) in combination with SAP which exhibited synergistic activities in previous studies (Bachran et al. 2010). The cells, except SiHa, revealed high sensitivity to the toxin with 50 % cell survival in the range of 5–24.5 nM. After combination with SAP, the cytotoxicity was remarkably increased from 9,000- to 2,500,000-fold and the cytotoxicity was clearly target receptor specific. The important role of saponins from *G. paniculata* as tool for improved targeted tumor therapies is described in a review of Fuchs et al. (2009).

A new saponin, gypoldoside A isolated from *Gypsophila oldhamania*, was reported to be highly active in comparison with cisplatin as positive control, against three different human cancer cell lines HT-29, SGC7901, and PLC/PRF/5, with IC_{50} values of 1.31 ± 0.71, 1.22 ± 0.87, and 0.55 ± 0.21 μM, respectively (Bai et al. 2007).

Platycodon grandiflorum

It was shown that Platycodin D represents a novel chemical class of microtubule-disrupting agents (Kim et al. 2008). Namely, the authors demonstrated the effectiveness of this triterpene saponin as an inhibitor of human leukemia cell proliferation (U937, THP-1, K562 cells) and identified a novel mechanism of action. Treatment of synchronized leukemia cells with varying concentrations of platycodin D (Fig. 3.9) resulted in significant mitotic arrest and endoreduplication via downregulation of Cdc2/Cdk2 protein and upregulation of wee1 expression, and elevated the Cdk2 protein via downregulation of p21 after 48 h. Furthermore platycodin D was shown to possess an anticancer activity by disrupting the dynamics of microtubule assembly, thus perturbing the formation and function of the mitotic spindle apparatus and arresting cells in mitosis. Platycodin D at 200, 400, and 800 μM significantly increased microtubule polymerization, similar to paclitaxel (3 μM) causing cell arrest in the G2/M phase, blocking cell cycle progression (Kim et al. 2008). Finally, platycodin D was shown to induce apoptosis in U937 cells through activation of caspase 3. Taken together, these results suggest the platycodin D might provide a new approach to cancer therapy.

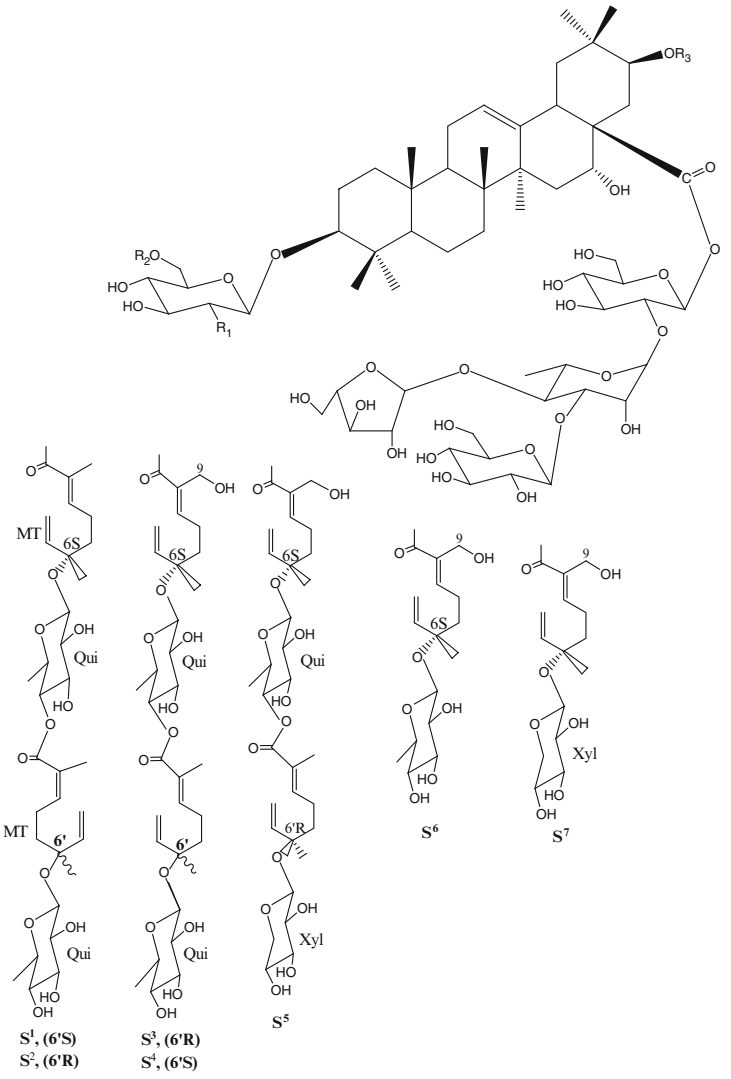
Albizia julibrissin

Albizia julibrissin Durazz (Leguminosae) is a plant widely distributed all over the world. As a traditional Chinese medicine, it is recorded in Chinese Pharmacopoeia as a sedative agent and an anti-inflammatory drug to treat swelling, pain in the lungs, skin ulcer, and wounds. It is widely used for treating insomnia together with other Traditional Chinese Medicine. The isolation and structure elucidation of some complex triterpenoid saponins from this plant have been well documented between 1996 and 2006 and these compounds were shown to possess inhibitory activities against some cancer cells in vitro (Zou et al. 2010; Lacaille-Dubois et al. 2011). Recently, some new minor triterpenoid saponins julibrosides J_{16}, J_{17}, J_{21} (Zou et al. 2010), J_{32}, J_{35}, and J_{36} and a prosapogenin as a new natural product (Zheng et al. 2010a) were isolated and characterized (Fig. 3.13). J_{16}, J_{17}, and J_{21} were tested for inhibitory activity against human cancer cell lines (H-60, PC-3, MIE-8, BGC823, MDA-MB-435, BEL 742, and Hela) and showed good inhibitory activity on BEL 742 (Zou et al. 2010). A bioassay-guided fractionation of a 70 % ethanolic extract from the stem bark yielded julibroside J8 which was evaluated on the growth of human microvascular endothelial cells (HMEC-1), four human tumor cell lines, and a normal cell line (MRC-5) by the MTT assay. Julibroside J8 at 0.5–4 μg/ml dose-dependently inhibits the growth, migration, and tube formation in HMEC-1 cells (Hua et al. 2009). It also inhibited the formation of microvessels on chicken chorioallantoic membrane (CAM) at a concentration of 10–50 μg/egg. Furthermore, in transplanted colon carcinoma cells in a nude mice neovascularization model, julibroside J8 reduced vessel density within tumor at a concentration of 0.5–3 mg/kg. This effect was higher than the positive control ginsenoside Rg3. These results suggested that julibroside 8 could be considered a potent antiangiogenic and cytotoxic drug (Hua et al. 2009).

Cimicifuga ssp

In Traditional Chinese Medicine, Rhizoma Cimicifugae (Shengma) refers to the rhizomes of *Cimicifuga* species *C. heracleifolia* Kom, *C. dahurica* Turcz, and *C. foetida L.* (Ranunculaceae), which are listed in the Pharmacopoeia of the People's Republic of China. They are used as antipyretic, analgesic, and anti-inflammatory drug for the treatment of febrile diseases (e.g., influenza infection) and inflammations of the upper respiratory tract (Wagner et al. 2011). *C. racemosa*, commonly known as "black cohosh", is a herb used among Native Americans to treat a variety of ailments, including diarrhea, sore throat, and rheumatism. It is best known in Europe and Western countries for its health benefit in treating menopausal disorders, and reducing the risk of osteoporosis. Although many pharmacological effects and clinical uses, including relief of hot flash, anti-osteoporosis, anti-HIV, anti-inflammatory, antidiabetes, antimalaria, and vasoactive properties, have been discovered in *Cimicifuga* ssp. as reviewed by Li and Yu (2006), many studies remain to be explored for the biological actions of each individual components of this herb. Most of recent studies concern cancer-related activities.

The growth inhibitory activity of compounds from *C. racemosa* and related Asian species has been investigated on human breast cancer cell line MDA-MB-453.



R_1	R_2	R_3	compounds	References
OH	Xyl-^{2}Fuc-	S^4	Julibroside J_8	Hua et al. 2009
OH	Xyl-^{2}Fuc-	S^2	Julibroside J_{16}	Zou et al., 2010
OH	Xyl-^{2}Ara-	S_3	Julibroside J_{17}	Zou et al., 2010
OH	Xyl-^{2}Fuc-	S_3	Julibroside J_{21}	Zou et al., 2010
O-Glc	Xyl-^{2}Fuc-	S_6	Julibroside J_{32}	Zheng et al., 2010
OH	Xyl-^{2}Ara-	S5	Julibroside J_{35}	Zheng et al., 2010
O-Glc	Xyl-^{2}Fuc-	S_3 (CH_2-9)	Julibroside J_{36}	Zheng et al., 2010

Fig. 3.13 Representative examples of *Albizia* saponins

The activity appears to be related to their triterpene glycoside composition (Einbond et al. 2008). The most active compound was 25-acetyl-7,8 didehydrocimigenol 3-*O*-β-D-xylopyranoside having a 25 acetyl function. (IC_{50} 5 μM). Furthermore, actein was found to induce apoptosis. Taken together these results indicate that actein and related compounds may be useful in the prevention and treatment of human breast cancer. Cimiside E isolated rom *C. heracleifolia* was shown to possess apoptotic action on gastric cancer cells with IC_{50} of 14.58 μM (Guo et al. 2009).

A recent study has shown that cimigenol, shengmanol, and dahuricol derivatives isolated from *C. dahurica* were tested against human HL-60, SMMC-7721, A549, SK-BR-3, PANC-1, and HepG2 cell lines and three of them exhibited a broad spectrum and moderate cytotoxic activity with IC_{50} ranging from 6.20 to 22.74 μM (Nian et al. 2011; Tian et al. 2007). In addition, the total glycosides from the aerial part of *C. dahurica* inhibited the growth of the implanted mouse H22 tumor in a dose-dependent manner (Tian et al. 2007). After this study and previous results, some structure–activity relationships of compounds with a cimigenol-skeleton can be proposed.

Most of the fifteen 9,19-cycloartane triterpene glycosides isolated from the roots of *C. foetida* showed more selective and higher cytotoxicity against the human HepG2 cell line than against MCF7, HT-29, and MKN28 cell lines (Nian et al. 2010). Three of them, 25-*O*-acetylcimigenol-3-*O*-[4′-*O*-(E)-2-butenoyl]- β-D-xylopyranoside, 25-*O*-acetylcimigenol-3-*O*-[3′-*O*-acetyl]β-D-xylopyranoside, and 3′ -*O*-acetyl-23-epi-26-deoxyactein, exhibited significant cytotoxicity against HepG2 cells, with IC_{50} values of 1.29, 0.71, and 1.41 μM, respectively.

TCM Sources of Triterpene Saponins with Various Bioactivities

Panax ginseng, P. notoginseng, and P. quinquefolius

Ginsenoside Rg3 (Fig. 3.1), one of the most effective ginseng saponins, has anti-inflammatory and anticancer effects. Rg3 was shown to inhibit tumor necrosis factor-α (TNF- α)-induced protein and mRNA expression of two cell adhesion molecules, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in EVC 304 human endothelial cells. In addition, expression of two pro-inflammatory cytokines, TNF- α and interleukin-1β (IL-1 β), was suppressed by Rg3. These results indicate that Rg3 may have anti-inflammatory and anti-atherosclerotic activities in the vasculature, which is mediated partly by downregulation of the expression of cell adhesion molecules and pro-inflammatory cytokines in endothelial cells (Hien et al. 2010). Ginsenoside Rg3 is also effective in attenuating brain infarction after cerebral ischemia, but the detailed mechanism is not known (Tian et al. 2009). A study showed that 20 (*S*)-ginsenoside Rg3 (2–16 μM) inhibited Ca^{2+}- and H_2O_2-induced swelling of mitochondria isolated from rat brains and the addition of Ca^{2+} generated reactive oxygen species (ROS) in isolated mitochondria. These results suggest that ginsenoside Rg3 inhibits the opening of mitochondrial permeability transition pores (MPTP) by free radical scavenging action in rat brain. This effect may contribute to the neuroprotective

activity of ginsenoside Rg3 (Tian et al. 2009). Other studies showed that ginsenoside Rd exerts neuroprotective effect in an animal model of focal cerebral ischemia (Ye et al. 2011). Rd (50 mg/kg) significantly reduced the infarct volume by 52 % in rats which were subjected to transient middle cerebral artery occlusion (MCAO). To evaluate the underlying mechanism of action of Rd against stroke, brain tissues were assayed for mitochondrial enzyme activities, production of reactive oxygen species (ROS) energy metabolites, and apoptosis. The results demonstrated that the neuroprotective effects of Rd in transient focal ischemia may involve an integrated process of the mitochondrial protection, energy restoration, and inhibition of apoptosis.

Ginsenoside Rg1 is the major compound of *Panax notoginseng*, a Chinese drug widely used in TCM to improve learning and memory. It was shown to have protective effects against β-amyloid peptide (Abeta)-induced neurotoxicity in PC 12 cells. Abeta is a molecule which plays a central role in the pathophysiology of Alzheimer's disease (AD). Cell death, LDH release, NO release, ROS production, lipid peroxidation, and intracellular calcium elevation are associated events induced by Abeta. The authors concluded that Rg1 may be a promising agent for AD, and the mechanism is related to β-secretase inhibition and protection against Abeta-induced cytotoxicity (Wang and Du 2009). Similar conclusions were observed with ginsenoside Rg2 which was able to inhibit the formation of Abeta 1–40 (Li et al. 2007).

Ginsenoside Rb1, one of the active components in the ginseng root, has been demonstrated to have neuroprotection for cerebral ischemia (Gao et al. 2010), and recently a study showed that the promotion of the neurogenesis and regulation of the expressions of brain-derived neurotrophic factor (BDNF) and caspase-3 by ginsenoside Rb1 may be involved in the neuroprotection against cerebral ischemia (Gao et al. 2010). The neuroprotective differences between American and Asian ginseng have not been reported. Since American ginseng has a lower ratio of Rg1/Rb1, it seems to calm the central nervous system, whereas Asian ginseng appears to stimulate the CNS (Qi et al. 2011).

Furthermore, antidiabetic effects have been demonstrated for some ginsenosides (Rb1, Rb2, Rh2, and the aglycone 20*S* PPT) in animal models. A recent study showed that ginsenoside Rd, a purified saponin from *P. notoginseng*, may prevent the development of atherosclerosis through mechanisms resulting in the reduction of ox-LDL uptake and cholesterol accumulation in macrophages (Li et al. 2011a).

A comparison between the effects of protopanaxadiol and protopanaxatriol glycosides isolated from *P. quinquefolius* showed that the protopanaxadiol glycosides inhibited the pancreatic lipase activity in a dose-dependent manner at the concentration of 0.25–1 mg/ml, whereas the protopanaxatriol derivatives were inactive (Liu et al. 2010).

The antiobesity effect of ginsenoside Rg3 was shown to involve the AMP-activated protein kinase (AMPK) signaling pathway and PPAR-gamma inhibition (Hwang et al. 2009). It is well known that AMPK plays a role in maintaining health in the context of diseases such as diabetes, obesity, and cancer. It was demonstrated that the antiobesity effects of the crude saponins from stems and leaves of *Panax quinquefolium* in high-fat diet-treated mice may be due to the inhibition of intestinal absorption of dietary fat by ginsenosides Rc, Rb1, and Rb2 (Liu et al. 2008b).

Furthermore, some studies made in comparison with diazepam have shown the anxiolytic effect of *P. quinquefolium* in mice, suggesting that it might be a potential candidate for use as anxiolytic drug (Wei et al. 2007).

Acanthopanax senticosus, also known as *Eleutherococcus senticosus* or Siberian ginseng, is one of the traditional Chinese medicines in the Pharmacopoeia of the People's Republic of China. It is used as an adaptogen like *Panax ginseng*. A recent review has shown the advances in botany, chemistry, pharmacology, study of adverse reactions, and clinical trials of this plant in the last decade (Huang et al. 2011). The chemical studies reported around 40 triterpene saponins, some of them showing pancreatic lipase inhibitory activity in vitro. Intensive pharmacological experiments in vitro and in vivo demonstrated that *A. senticosus* possessed antistress, antiulcer, anticancer, anti-inflammatory, and hepatoprotective activities.

Astragalus ssp.

A. membranaceus extract (AME) is used widely for the treatment of cardiovascular diseases in China. The cardioprotective effect and the molecular mechanism related to angiogenesis were studied in vitro and in vivo (Zhang et al. 2011). At 75 μg/ml AME significantly increased the proliferation, migration, and tube formation on human umbilical vein endothelial cells (HUVECs). Another study was performed on rats with ligation of left anterior descending artery to study the cardiac protective effect of AME (50 and 100 mg/kg for 3, 7, and 14 days). AME was shown to inhibit cardiac fibrosis, to reduce infarct size, and to increase capillary and arteriole densities. The activation of AKT/GSK3b and AKT/mTOR pathways and the increased expression of VEGF may contribute to the promoted neovascularization by AME (Zhang et al. 2011). Five saponins, astragalosides VII, V, IV, III, and soyasaponin (Fig. 3.2), were characterized among the constituents of AME by HPLC/MS. Astragaloside IV is often used as standard in quality assessment because it showed many pharmacological activities such as improvment of endothelial function, cardiomyocyte damage, memory function, hepatic fibrosis, experimental Parkinson disease, just to mention a few. Astragaloside IV was also reported for its antitumor and antiviral properties (Liu et al. 2011). Astragaloside IV is commonly used in degenerative bone disease such as osteoporosis, and Bian et al. (2011) investigated the osteogenetic effect of this molecule under the conditions of centrifugating pressure in preosteoblasts, OCT-1 cells. The results showed that astragaloside IV plus centrifugation of 500 rpm for 3 days and plus 200 rpm for 5 days significantly induced osteogenesis-related protein and gene expression (Bian et al. 2011).

Gynostemma pentaphyllum

The antihyperglycemic effect of an extract of *Gynostemma pentaphyllum* Makino (GPE), containing standardized concentrations of gypenosides, was evaluated in

C57BL/KSJ-db/db mice. These results suggest that the supplementation of high-dose GPE (0.01 %) in the diet lowers the blood glucose level by altering the hepatic glucose metabolic enzyme activities (Yeo et al. 2008). In addition, *G. pentaphyllum* has been shown to reduce both hyperglycemia and hyperlipidemia in diabetic Zucker fatty rats (Megalli et al. 2006). Phanoside (21,23-epoxy-3β,20-,21-trihydroxydammaran-24-ene-3-*O*-([α-D-rhamnopyranosyl (1–2)]-[β-glucopyranosyl(1–3)]- β-D-lyxopyranoside)) and four stereoisomers differing in configurations at positions 21 and 23 isolated and characterized from *G. pentaphyllum* were found to stimulate insulin release from isolated rat pancreatic islets. At 500 μM phanoside stimulates insulin release in vitro tenfold at 3.3 mM glucose and potentiates the release almost fourfold at 16.7 mM glucose. At these glucose levels, 2 μM glibenclamide stimulates insulin release only twofold (Norberg et al. 2004). Also, when given orally to rats, phanoside (40 and 80 mg/ml) improved glucose tolerance and enhanced plasma insulin levels at hyperglycemia. This effect was shown in islets not only of normal Wistar rats, but also of diabetic Goto-Kazkizaki (GT) rats, and this effect seems to be exerted distal to K-ATP channels and L-type Ca2+ channels, which is on the exocytotic machinery of the pancreatic B-cells. Similar to sulfonylureas, the effect of phanoside is not glucose-dependent (Khanh Hoa et al. 2007). A study has suggested that the supplementation with gypenosides improved insulin resistance in high-fat diet mice at least in part by increasing glucose utilization, which seems to be mediated via elevation of glucokinase activity and hepatic glycogen concentration (Zhang et al. 2009). In particular, a synergic effect was observed between a procyanidin fraction and gypenosides on improvement of insulin resistance and decrease of serum triglyceride level in high-fat diet fed mice. Such a combination might be of interest for patients showing clinically manifested insulin resistance (Zhang et al. 2009). Four gypenosides were identified from an ethanol extract of *G. pentaphyllum*, which was shown to present neuroprotective effects in a 6-hydroxydopamine-lesioned rat model of Parkinson's disease without any sign of toxicity during the 28 days of treatment. These results suggest that this extract might be helpful in the prevention of Parkinson's disease (Choi et al. 2010). Furthermore, a study has shown that gypenosides TN-2 and XXLIV reduced memory and learning deficits in scopolamine-induced memory-deficient mice (Hong et al. 2011; Joh et al. 2010).

Furthermore, the cardiovasular effects of the aqueous extract (2.5, 5.0, 10.0 mg/kg) and two isolated gypenosides III (0.7 mg/kg) and VIII (0.3 mg/kg) of *G. pentaphyllum* leaves were investigated in the anesthetized guinea pigs in comparison with verapamil (1 mg/kg), a well-known Ca-antagonistic drug. It was shown that the intravenous administration of the extract and the pure gypenosides produced a protective effect against vasopressin-induced coronary spasm, arrhythmias, and pressor response together with a protective and suppressive effect on ouabain-induced arrhythmias in the anesthetized guinea pigs. These results showed that *G. pentaphyllum* extract possesses significant cardiovascular properties similar to those exhibited by verapamil and suggested that gypenoside III and gypenoside VIII are two of the compounds responsible for these effects, which might be mediated through their ability to inhibit calcium overload (Circosta et al. 2005).

Clematis ssp.

Clematis chinensis Osbeck (Ranunculaceae), widely distributed in the south of China, is used with *C. hexapetala* and *C. mandshurica* as a traditional Chinese herbal drug called Weilingxian in the Chinese Pharmacopoeia for its antitumor, anti-inflammatory, and analgesic activity. These plants are rich in saponins and seven new triterpene saponins, claematochinensosides A–G having hederagenin and oleanolic acid as aglycone, were isolated and characterized from the roots and rhizomes of *C. chinensis*. Six of them—claematochinensosides A and C–G—showed inhibitory activities against COX-1 and COX-2 enzymes (Fu et al. 2010). Furthermore, a saponin fraction from *C. chinensis* (SFC) was shown to be efficient against an osteoarthritis model in rats (Wu et al. 2010). Osteoarthritis was induced by intraarticular injection of monosodium iodoacetate into knee joints of rats. It was observed that SFC (50, 100, 200 mg/kg) dose-dependently reduced cartilage injury and proteoglycan degradation whereas diclofenac (4 mg/kg) only slightly alleviated them. SFC also prevented monosodium iodoacetate-induced rabbit chondrocyte impairment. These results showed that SFC improved joint destruction and cartilage erosion in monosodium iodoacetate-induced osteoarthritis in rats and the mechanism of action is through preventing extracellular matrix degradation and chondrocyte injury. An acetone extract of *C. chinensis* containing the most total saponins showed significant and dose-dependent inhibitory effects on PGE2, MMP-3, -13, and COX-2 productions by lipopolysaccharide-stimulated primary human chondrocytes (Hsieh et al. 2010). Furthermore, a new analysis method was established to evaluate the antiarthritic effects in vivo. Namely, the acetone extract showed inhibitory effect on 2-^{18}F-fluoro-2-deoxy-D-glucose (^{18}F-FDG) uptake when assessed by positron emission tomography (PET) uptake in the joints and serum PGE2 of rabbits.

Dipsacus asper

Asperosaponin VI (Fig. 3.7), also named *Akebia* saponin D, a triterpene saponin isolated from *Dipsacus asper* Wall, has shown cardioprotective effects. However, the effect on cardiomyocytes apoptosis is poorly understood. The aim of the study of Li et al. (2010a) was to investigate the cardioprotective role of Asperosaponin VI and the underlying mechanisms in hypoxia-induced cardiomyocyte apoptosis. They showed that this saponin inhibited apoptosis in hypoxia-induced cardiomyocytes by increasing the Bcl-2/Bax ratio and decreasing caspase-3 expression, as well as enhancing of p-Akt and p-CREB (Li et al. 2010a). In consequence, asperosaponin was shown to protect cardiac myocytes from hypoxia-induced apoptosis via activation of the PI3K/Akt and CREB pathways. Another study reported the possible protective role of asperosaponin VI on acute myocardial infarction in rats (Li et al. 2010b). The mechanism might be attributed to scavenging lipid peroxidation products and reactive oxygen species, increasing antioxidant defense enzymes

and preventing mitochondrial damage. A study reported the neuroprotective capacity of *Akebia* saponin D from *Dipsacus asper* to antagonize the amyloid-β peptide ($A\beta_{25-35}$)-induced cytotoxicity in PC 12 cells (Zhou et al. 2009b). This research suggests that *Dipsacus asper* may represent a potential treatment strategy for Alzheimer's disease.

Platycodon grandiflorum

A study showed that platycodin D (Fig. 3.9) was found to inhibit intracellular triglyceride accumulation in 3 T3-L1 cells with an IC_{50} value of 7.1 μM (Lee et al. 2010). The mechanism for the antiobesity activity of Platycodin D was tentatively elucidated at the molecular and cellular levels. The treatment of the cells with this saponin induced a significant downregulation of the genes involved in lipid metabolism such as fatty-acid binding protein 4 and lipoproteine lipase. The treatment resulted also in a reduction of peroxysome proliferator-activated receptor γ expression and its binding to target DNA sequence and an upregulation of KLF2 (Kruppel-like factor, an anti-adipogenic factor).

Gypsophila ssp.

Seven new triterpene saponins were isolated from the roots of *Gypsophila paniculata* (Yao et al. 2010). They include 2,28-*O*-bidesmosides with or without a 4-methoxycinnamoyl group and 3-*O*-monoglycosides. All the saponins have been tested for their α-glucosidase inhibitory activity, in order to find compounds which are able to retard the absorption of dietary carbohydrates and thus suppress postprandial hyperglycemia. Compound 1 (Fig. 3.12) (IC_{50} 100 ± 3.3 μM) and its aglycone were more potent than ascarbose, the positive control (IC_{50} 398.1 ± 9.6 μM). The other compounds were found to be almost inactive in this test ($IC_{50} > 2{,}000$ μM).

Gypsophila oldhamania has been used as a substitute for the TCM Yin-Chai-Hu (roots of *Stellaria dichotoma* var. *lanceolata* Bge) for the treatment of fever, consumptive disease, infantile malnutrition syndrome, and diabetes. In order to find new natural lipase inhibitors, the chemical investigation of this plant led to the isolation of three new triterpenoid saponins: gypsosaponins A–C having gypsogenin, quillaic acid, and gypsogenic acid as aglycones (Fig. 3.12). They showed inhibitory activity against pancreatic lipase of 58.2, 99.2, and 50.3 % at concentration of 1 mg/ml, respectively (Zheng et al. 2007). Furthermore seven new triterpene saponins have been isolated and elucidated from the roots of *G. oldhamania* together with five known saponins (Luo et al. 2008). These saponins classified into three series: 3-*O*-monoglycosides, 28-*O*-monoglycosides, and 3,28-*O*-bidesmosides were evaluated for their α-glucosidase inhibitory activity. The results showed that the 28-*O*-monoglycosides were more active than the two other classes, compound 4 (gypsogenin 28-*O*-glycoside having a tetrasaccharide chain

constituted of three glucose and one galactose unit) being the most active with IC_{50} of 15.2 ± 1.8 μM in comparison with acarbose (388.0 ± 9.6 μM).

Recently, new information regarding the mode of action of *Cimicifuga* rhizome for anti-inflammatory action in endothelial cells has been reported for two isolated compounds cimiside E and 23-*O*-acetylshengmanol-3-xyloside (Moon et al. 2011) (Fig. 3.8). These compounds were shown to selectively inhibit expression of vascular cell adhesion molecule-1 (VCAM-1) in human endothelial cells activated with inflammatory cytokine, TNF-α. Therefore these compounds may be useful for the treatment of pathologic inflammation disorders such as atherosclerosis (Moon et al. 2011).

Two new cyclolanostane diglycosides isolated from *C. foetida* effectively inhibited the proliferation of murine splenocytes induced by Con A, with IC_{50} values ranging from 12.7 to 33.3 nM (Pan et al. 2009).

A study explored the toxicological effect of cimicidol-3-*O*-xyloside, a main triterpenoid of *Cimicifuga* rhizoma after oral administration (50 mg/kg/day), over a 7-day period in female SD rats using metabolomic analyses of ^{1}H NMR spectra of urine, serum, and liver tissue extracts. Histopathological studies of liver and analyses of blood biochemical parameters such as alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, and creatinine revealed that cimicidol-3-*O*-xyloside had no negative impacts on liver and kidney (He et al. 2012).

After a study investigating the effects of *C. heracleifolia* on bone loss in ovariectomized mice, the authors concluded that this drug can prevent bone loss in mice induced by ovariectomy (Ahn et al. 2012).

3.1.2.2 Steroid Saponins

Anemarrhena asphodeloides Bge

A. asphodeloides Bge (Liliaceae) is a well-known traditional Chinese medicinal herb and is officially listed in the Chinese Pharmacopoeia. It has been widely traditionally used in China for its antidepressant, antidiabetic, anti-inflammatory, antiplatelet aggregation, and antipyretic effects. The constituents of the rhizomes include xanthones and steroid saponins. Among them, timosaponin A-III (Fig. 3.14) was shown to inhibit the proliferation of human colorectal cancer HT-15 cells with cell cycle arrest in the GO/G1 and G2/M phase and induction of apoptosis (Kang et al. 2011). The latter was evidenced by DNA fragmentation, activation of caspases, induction of cleaved poly(ADP ribose) polymerase, and suppression of Bcl-xL and Bcl-2 expression. In an in vivo murine xenograft model, this compound at a dose of 2 or 5 mg/kg, 3 times/week, ip administration for 4 weeks significantly suppressed tumor growth in athymic nude mice bearing HCT-15 cells, without toxicity (Kang et al. 2011).

Only the constituents successfully assimilated into blood and kept at a considerable concentration level in targets organs have the possibility to be responsible for the curative effects. Ma et al. (2008) studied the constituents absorbed into rat urine

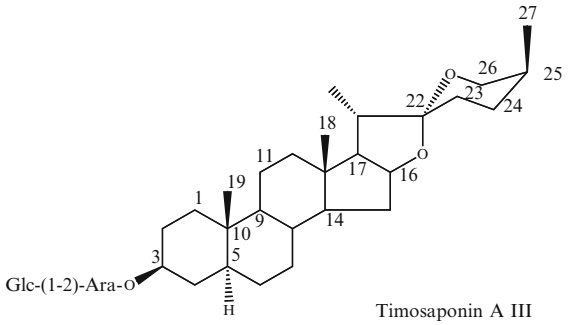


Fig. 3.14 A representative example of *Anemarrhena* saponins

and their metabolites after oral administration of the rhizome decoction by using a solid-phase extraction and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS/MS) methodology. A total of 11 compounds, including six steroid saponins, were identified in rat urine sample, which indicated that this kind of compound in this drug was assimilated easily in vivo.

Paris polyphylla

P. polyphylla var. *yunnanensis* Hand-Mazz (PPY), distributed in the southwest of China, is a traditional Chinese medicinal herb well documented in the Chinese Pharmacopoeia since 1985. This plant is rich in steroid saponins, some of them were identified as polyphyllin D, dioscin, formosanin C, methylprotogracillin, and trillin (Figs. 3.4 and 3.15) by liquid chromatography tandem multistage mass spectrometry (Man et al. 2009a). They were characterized as the main antitumor active constituents of the drug, used for treating cancer for thousands of years, but a few studies have yet investigated their effect on pulmonary metastasis. Therefore, Man et al. (2009b) studied the underlying mechanism of antitumor effects of the saponin fraction on T739 bearing LA 795 mice using histopathology, immunohistochemistry, and reverse transcription polymerase chain reaction. They showed that this saponin extract has a powerful antiproliferative effect by inducing apoptosis and inhibition of the pulmonary metastasis by reducing expression of MMP-2 and MMP-9 and upregulating level of TIMP-2. Wound healing and migration assays were used to detect the anti-invasive effect of formosanin C, a saponin isolated from *Paris polyphylla*, on LA795 cells (Man et al. 2011). Through the gelatin zymography assay and immunofluorescence analysis, formonasin C was shown to suppress the enzyme activity of MMP-2 and MMP-9, and protein expression of MMP-1, -2, -3, -9, and -14 excreted from LA795 cells. This activity, superior to that of cisplatin, might have a potential therapeutic application in the treatment of lung tumors.

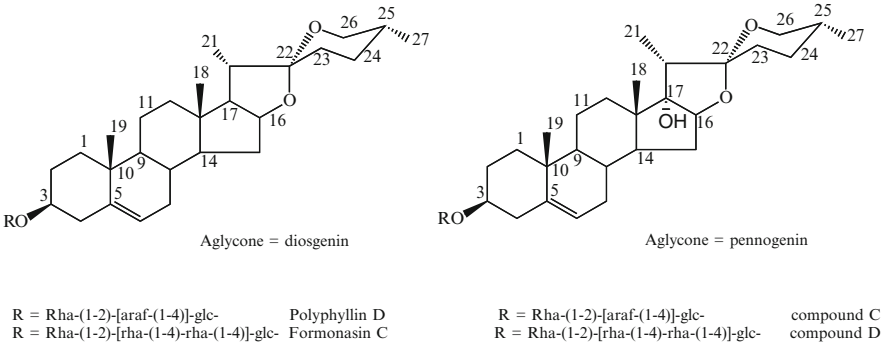


Fig. 3.15 Representative examples of *Paris* saponins

The mechanism of action of Polyphillin D, another saponin from *P. polyphylla* on human non-small cell lung cancer (NSCLC) cell line NCI-H460, was studied by bioinformatics, proteomic, and transcriptomic analyses (Siu et al. 2008). The latter revealed that polyphillin D induced the cytotoxic effect through a mechanism initiated by endoplasmic reticulum stress followed by mitochondrial apoptotic pathway with activation of caspase-9 and caspase-3.

Dioscorea ssp.

The tubers of yam (*Dioscorea* genus, Dioscoreaceae family) which contains many nutrients and physiologically active components such as steroid saponins are consumed as food and used widely in TCM (Sautour et al. 2007; Tang et al. 2007) as anticancer (*D. colletii* var. *hypoglauca*), cardio-, cerebrovascular-, gastropathy-protective, and curative agents (*D. panthaica*), and as anti-rheumatism agents (*D. nipponica, D. futschauensis*). Sautour et al. (2007) have summarized some of the important reports on the chemistry and the biological activities of *Dioscorea* steroid saponins during 2000–2006. These discoveries became possible as a result of the scientific development of isolation, structure elucidation, and in vitro assays. Over 50 steroid saponins of furostane-, spirostane-, and pregnane-type skeleton have been discovered and characterized from 13 *Dioscorea* species (Fig. 3.4). The main biological and pharmacological properties of *Dioscorea* saponins concerned cytotoxic and antifungal activity, which were highlighted.

Dioscorea nipponica has been used for a long time to prevent and treat coronary heart disease in TCM and some steroidal saponins were believed to be responsible for the activity (Qinga et al. 2010). Furthermore, these authors showed that human serum albumin (HSA) functionalized magnetic nanoparticles (MNPs) were used to isolate and identify saponin ligands that bind to HSA from *D. nipponica* extract. Electrospray ionization mass spectrometry (ESI-MS) was used for compound identification and semi-quantification. Three saponins, i.e., dioscin, gracillin, and pseudo-protodioscin, were isolated effectively from the

extract showing affinity to HSA-MNPs. Among the three saponins, dioscin bound to HSA much stronger than gracillin and pseudo-protodioscin did, resulting in first structure/activity relationships.

Total steroid saponins extracted from the rhizomes of *Dioscorea zingiberensis* were shown to inhibit thrombosis by both improving the anticoagulation activity and inhibiting platelet aggregation activity, suggesting that the total saponins of *D. zingiberensis* have the potential to reduce the risk of cardiovascular diseases by antithrombotic action (Li et al. 2010c). A chemical fingerprint method was first established and validated to quantify and standardize the extract's constituents, including parvifloside, protodeltonin protodioscin, protogracillin, zingiberensis saponin, deltonin, dioscin, and trillin (Fig. 3.4).

Tribulus terrestris

The fruits of *T. terrestris* (Zygophyllaceae) have been used in TCM for treatment of eye problems, edema, abdominal distention, and sexual dysfunction. Among the new saponins, which have been recently isolated and characterized from *T. terrestris* (Xu et al. 2009b, 2010; Wang et al. 2009b; Su et al. 2009a), the five most original compounds are the aglycones (23*S*,25*S*)-5α-spirostane-24-one-3β,23-diol, (24*S*,25*S*)-5α-spirostane-3β,24-diol, (25*R*)-5α-furostan-2α,3β,22α,26-tetraol, (25*R*)-5α-furostan-20(22)-en-2α,3β,26-triol, and (25*S*)-5α-furostan-12-one-22-methoxy-3β,26-diol (Su et al. 2009b) (Fig. 3.16). When the five saponins were evaluated against HL-60 leukemia cell lines, they showed a moderate cytotoxicity with IC_{50} values in the range of 41 ± 3.4 μM and 49.45 ± 7.2 μM. A mixture of saponins of *Tribulus terrestris* L. (TTLS) was evaluated on apoptosis in cortical neurons induced by hypoxia–reoxygenation in rats. The apoptosis rate was analyzed quantitatively by flow cytometry with Annexin V-FITC and propidium iodide staining. TTLS can decrease the apoptosis induced by hypoxia and reoxygenation. The mechanism might be related to stabilization of mitochondrial membrane potential, inhibition of caspase activity, and reduction of Bax protein expression (Liu et al. 2008a).

3.1.3 Clinical Studies

If the chemical and pharmacological studies of the saponin-containing TCM drugs have been largely developed in recent years, clinical studies are relatively scarcely reported in several randomized, double-blinded, placebo-controlled pilot trials. It could be due to the toxic effect of some saponins after intravenous administration. Most clinical studies concern the ginseng saponins, and to a lesser extent those of *Gynostemma pentaphyllum* and *Astragalus membranaceus*.

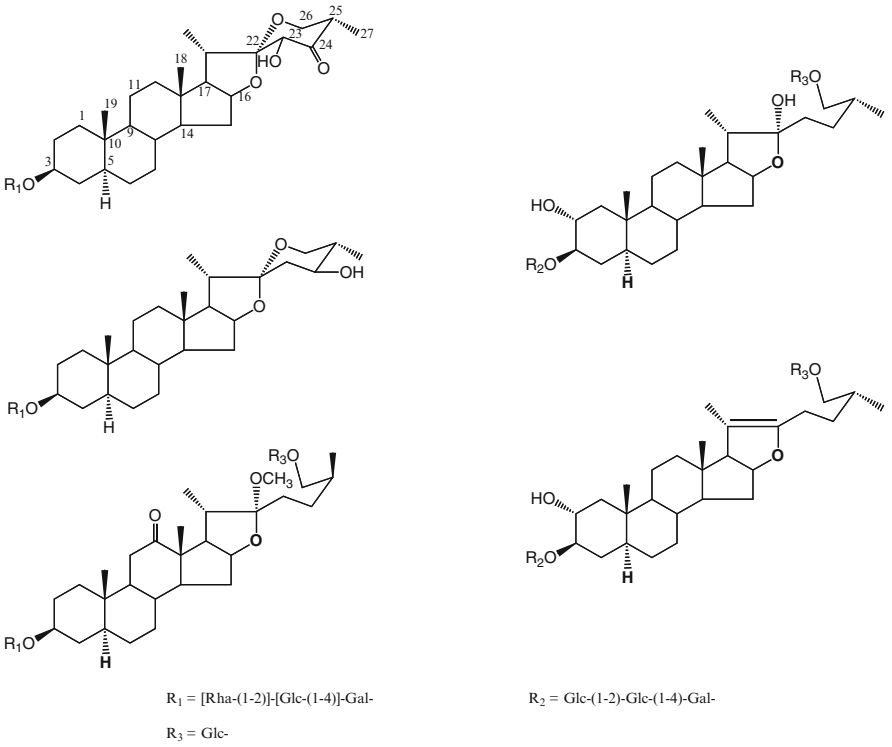


Fig. 3.16 Representative examples of *Tribulus* saponins

Despite the scientific studies demonstrating in vitro and in vivo positive effects of ginseng in a wide range of pathological conditions, the quality of most clinical trials on ginseng is quite poor and reliable clinical data in humans are still missing (Xiang et al. 2008). However, some studies have been completed and will be presented below. A recent review reported several randomized clinical studies on ginseng with no conclusive evidence that ginseng itself can cure cancer. Its roles in cancer treatment should be viewed as a supplementary therapy to enhance host immune response to cancer and patient quality of life (Jia and Qian 2011).

The therapeutic effect and possible mechanism of action of total notoginseng saponins (PNS) were evaluated for treatment of rheumatoid arthritis in 84 patients. They were divided into two groups of patients, one receiving all the routine therapy, diclofenac, leflunomide, and prednisone and the other receiving PNS additionally. Clinical efficacy and numerous parameters were observed (Zhang et al. 2007a). It was concluded that PNS significantly improved the conditions of patients, enhancing the therapeutic effect in treating rheumatoid arthritis, through regulating the disordered immunity and improving the anti-inflammatory and analgesic effect ($p < 0.05$ and $p < 0.01$).

Recently, a study following a single or multiple intravenous dose of ginsenoside Rd in healthy Chinese volunteers showed that this compound was well tolerated with no pattern of dose-related adverse events (Zeng et al. 2010). It had a favorable pharmacokinetic and safety profile that enables the drug to be explored in future clinical studies that target patients with acute ischemic stroke.

A preparation called "Shenmai", a mixture of Radix *Ginseng rubra* and Radix *Ophiopogon* (Pharmacopoeia of the People's Republic of China 2005), has been used for raising tumor patients' immunity and for treating cardiac emergency for a long time. Ginsenoside Rg1, the major constituent of *G. rubra*, was considered responsible for the efficacy of this injection. A pharmacokinetic study of Rg1 was acheived in ten healthy volunteers, receiving an intravenous single dose of "Shenmai". The rapid distribution and elimination of Rg1 assessed by a quantitative determination using LC-ESI-MS/MS was consistent with the rapid effectiveness of this herbel preparation. Furthermore, this work gives valuable informations for the clinical application of "Shenmai" injection, proving its safety and efficacy (Yang et al. 2009a).

The antidiabetic effect of *Gynostemma pentaphyllum* tea was investigated in type-2 diabetic patients (Huyen et al. 2010) in a trial with tea or placebo over 12 weeks. After 12 weeks' treatment, fasting plasma glucose levels, glycosylated hemoglobin [HbA(1C)], and insulin resistance decreased significantly in the *Gynostemma* group as compared to the control group. Furthermore, no hypoglycemia and no adverse effects in kidney and liver parameters or gastrointestinal functions were reported. This study shows a rapid improvement of glycemia and insulin sensitivity, providing a basis for a novel, effective, and safe approach using *Gynostemma pentaphyllum* to treat type-2 diabetic patients.

Recently, many basic studies have provided evidence supporting the preventative and therapeutic effects of *Astragalus membranaceus* in diabetic nephropathy. Astragaloside IV can ameliorate high glucose-induced podocyte adhesion by upregulating $\alpha_3\beta_1$ integrin and inhibiting the activation and overexpression of integrin-linked kinase (Chen et al. 2008b). A review aimed to systematically report the randomized and semi-randomized control trials to ascertain the role of *Astragalus* in the treatment of diabetic nephropathy (Li et al. 2011b). Twenty-five studies included a total of 1,804 patients (945 in treatment group and 859 in control group). *Astragalus* could reduce urea nitrogen, serum creatinine, urine protein, and increase serum albumin in CKD patients, and could significantly improve creatin clearance in CKD patients. Even acknowledging that most of these trials were not of very high quality, the potential value of this traditional herb should not be dismissed. The clinical studies on *Cimicifuga* ssp. concern mainly *C. racemosa* which has been reviewed in this issue by Wuttke and Seidlova-Wuttke (2012).

3.2 Conclusion

The goal of this review is to provide a useful reference for chemists and biologists researching saponin-containing TCM drugs and will open the door to discovery of drug agents. In the past 5 years, some promising advances have been achieved in the

analytical and biological methods, resulting in the characterization of a great diversity of saponins often considered the main bioactive constituents of some TCM drugs. Of the 80 drugs which are highlighted in TCM (Wagner et al. 2011), approximately 15 saponin drugs have been analyzed. Thus, chromatographic fingerprint analysis of TCM drugs by GC, HPLC, and HPTLC represented a comprehensive approach in the quality assessment of TCM drugs. On the basis of these literature data, we decided to report in this review on the newest results in the chemistry, pharmacology, and clinical studies of saponin-containing TCM drugs. It appears that the literature on triterpene saponins is more abundant than that on steroid saponins, and ginseng occupies a prominent position in the list of TCM drugs which are widely studied. However, other TCM drugs belonging to the genera *Astragalus, Gynostemma, Bupleurum, Clematis, Dipsacus, Platycodon, Gypsophila, Anemarrhena, Lonicera, Paris, Dioscorea, Tribulus, and Ophiopogon* are also well documented in recent years. From a chemical point of view, promising advances have been achieved in the methodology of extraction and isolation (ultrasound- or microwave-assisted extraction, ultrahigh pressure extraction, prep-HPLC, high-speed countercurrent chromatography, centrifugal partition chromatography). The newest methods of analysis using HPLC and UPLC coupled with sophisticated detection methods (DAD, ELSD, ESI-MS, TOF-MS) have allowed researchers to characterize saponins in complex mixtures, and spectroscopic methods such as 1D- and 2D-MNR techniques have allowed us to determine structures of unusual pure saponins. From a pharmacological point of view, the most relevant results have been obtained with the cancer-related activity of saponins, namely, cytotoxicity against a large panel of tumor cells by inducing apoptosis and the remarkable synergistic cytotoxic effect of saponins with chemotherapeutic agents such as the gypsogenin derivatives of *Gypsophila paniculata* (Fuchs et al. 2009). Furthermore, neuroprotective properties of some drugs are of interest, such as that of *Akebia* saponin IV from *Dipsacus asper* which may represent a potential strategy for Alzheimer's disease. From a clinical point of view, there are few recent studies on saponin-containing TCM drugs, due to high toxic effects of some of them after intravenous administration, but some extracts administrated per os such as the *Gynostemma pentaphyllum* tea showed an interesting antidiabetic effect in type-2 diabetic patients.

From all these observations, it can be concluded that saponin-containing TCM drugs are of great interest for further successful innovative chemical and pharmacological developments.

References

Ahn BS, Yang M, Jang H, Lee HJ, Kim JC, Moon C, Jung U, Jo SK, Jang JS, Kim SH (2012) Evaluation of the antiosteoporotic potential of *Cimicifuga heracleifolia* in female mice. Phytother Res 26:663–668

Angelova N, Kong HW, Van der Heijden R, Yang SY, Choi YH, Kim HK, Wang M, Hankemeier T, Van der Greef J, Xu G, Verpoorte R (2008) Recent methodology in the phytochemical analysis of ginseng. Phytochem Anal 19:2–16

Auyeung KKW, Law PC, Ko JKS (2009) *Astragalus* saponins induce apoptosis via an ERK-independent NF-kB signaling pathway in the human hepatocellular HepG2 cell line. Int J Mol Med 23:189–196

Auyeung KKW, Mok NL, Wong CM, Cho CH, Ko JKS (2010) *Astragalus* saponins modulate mTOR and ERK signaling to promote apoptosis through the extrinsic pathway in HT-29 colon cancer cells. Int J Mol Med 26:341–349

Bachran C, Sutherland M, Heisler I, Hebestreit P, Melzig MF, Fuchs H (2006) The saponin-mediated enhanced uptake of targeted saporin-based drugs is strongly dependent on the saponin structure. Exp Biol Med (Maywood) 231:412–420

Bachran C, Dürkop H, Sutherland M, Bachran D, Müller C, Weng A, Melzig MF, Fuchs H (2009) Inhibition of tumor growth by targeted toxins in mice is dramatically improved by Saponinum album in a synergistic way. J Immunother 32:713–725

Bachran D, Schneider S, Bachran C, Urban R, Weng A, Melzig MF, Hoffmann C, Kaufmann AM, Fuchs H (2010) Epidermal growth factor receptor expression affects the efficacy of the combined application of saponin and a targeted toxin on human cervical carcinoma cells. Int J Cancer 127:1453–1461

Bai H, Zhong YY, Wang YS, Liu L, Zhou L, Wang J, Mu YL, Zuo CX (2007) A major triterpenoid saponin from *Gypsophila oldhamania*. Chem Biodivers 4:955–960

Bian Q, Huang JH, Liang QQ, Shu B, Hou W, Xu H, Zhao YJ, Lu S, Shi Q, Wang YJ (2011) The osteogenetic effect of astragaloside IV with centrifugating pressure on the OCT-1 cells. Pharmazie 66:63–68

Boettger S, Melzig MF (2011) Triterpene saponins of the Caryophyllaceae and Illecebraceae family. Phytochem Lett 4:59–68

Cai H, Li J, Zhou S, Ge F, Zhou J, Huang Y (2010) Method for preparing total saponins from *Ophiopogon japonicus* based on ultrasonic -aided extraction. Faming Zhuanli Shenqing CN 101780213 A 20100721.

Chai XY, Li SL, Li P (2005) Quality evaluation of *Flos Lonicerae* through a simultaneous detemination of seven saponins by HPLC with ELSD. J Chromatogr A 1070:43–48

Chen JC, Lu KW, Lee JH, Yeh CC, Chung JG (2006) Gypenosides induced apoptosis in human colon cancer cells through the mitochondria-dependent pathways and activation of caspase-3. Anticancer Res 26:4313–4326

Chen J, Peng H, Ou-Yang X, He X (2008a) Research on the antitumor effect of ginsenoside Rg3 in B16 melanoma cells. Melanoma Res 18:322–329

Chen J, Gui D, Chen Y, Mou L, Liu Y, Huang J (2008b) Astragaloside IV improves high glucose-induced podocyte adhesion dysfunction via alpha $\alpha_3\beta_1$ integrin upregulation and integrin-linked kinase inhibition. Biochemical Pharmacol 76:796–804

Chen Y, Feng X, Jia X, Wang M, Liang J, Dong Y (2008c) Triterpene glycosides from Lonicera isolation and structural elucidation of seven glycosides from flower buds of *Lonicera macranthoides*. Chem Nat Compd 44:39–43

Chen JC, Lu KW, Tsai ML, Hsu SC, Kuo CL, Yang JS, Hsia TC, Yu CS, Chou ST, Kao MC, Chung JG, Gibson Wood W (2009a) Gypenosides induced G0/G1 arrest via CHk2 and apoptosis through endoplasmic reticulum stress and mitochondria-dependent pathways in human tongue cancer SCC-4 cells. Oral Oncol 45:273–283

Chen CY, Qi LW, Xi L, Li P, Wen XD (2009b) Liquid chromatography-mass spectrometry analysis of macranthoside B, macranthoidin A, dipsacoside B and macranthoside B in rat plasma for the pharmacokinetic investigation. J Chromatogr B 877:159–165

Chen XP, Qian LL, Jiang H, Chen JH (2011) Ginsenoside Rg3 inhibits CXCR(4) expression and related migrations in a breast cancer cell line. Int J Clin Oncol 16:519–523

Chinese Pharmacopoeia Commission (2005) Pharmacopeia of the People's Republic of China (English Edition), vol 1. Chemical Industry Press, Beijing

Choi HS, Park MS, Kim SH, Hwang BY, Lee CK, Lee MK (2010) Neuroprotective effects of herbal ethanol extracts from *Gynostemma pentaphyllum* in the 6-hydroxydopamine-lesioned rat model of Parkinson' disease. Molecules 15:2814–2824

Choi S, Oh JY, Kim SJ (2011) Ginsenoside Rh2 induces Bcl-2 family proteins-mediated apoptosis *in vitro* and in xenografts *in vivo* models. J Cell Biochem 112:330–340

Circosta C, De Pasquale R, Occhiuto F (2005) Cardiovascular effects of the aqueous extract of *Gynostemma pentaphyllum* Makino. Phytomedicine 12:638–643

Dan M, Xie G, Gao X, Long X, Su M, Zhao A, Zhao T, Zhou M, Qiu Y, Jia W (2009) A rapid ultra-performance liquid chromatography-electrospray ionisation mass spectrometric method for the analysis of saponins in the adventious roots of *Panax notoginseng*. Phytochem Anal 20:68–76

Dinda B, Debmath S, Chandra Mohanta B, Harigaya Y (2010) Naturally occurring triterpene saponins. Chem Biodivers 7:2327–2567

Duan CL, Li YJ, Jiang Y, Liu JX, Tu PF (2010) Spirostanol saponins from the fibrous roots of *Ophiopogon japonicus* (Thunb.) Ker-Grawl. Helv Chim Acta 93:227–232

Einbond LS, Wen-Cai Y, He K, Wu H, Cruz E, Roller M, Kronenberg F (2008) Growth inhibitory activity of extracts and compounds from *Cimicifuga* species on human breast cancer cells. Phytomedicine 15:504–511

Fu WW, Shimizu N, Takeda T, Dou DQ, Chen B, Pei YH, Chen YJ (2006) New A-ring lactone triterpenoid saponins from the roots of *Platycodon grandiflorum*. Chem Pharm Bull 54:1285–1287

Fu Q, Zan K, Zhao M, Zhou S, Shi S, Jiang Y, Tu P (2010) Triterpene saponins from *Clematis chinensis* and their potential antiinflammatory activity. J Nat Prod 73:1234–1239

Fuchs H, Bachran D, Panjideh H, Scellmann N, Weng A, Melzig MF, Sutherland M, Bachran C (2009) Saponins as tool for improved targeted tumor therapies. Curr Drug Targets 2:140–151

Gao XQ, Yang CW, Chen GJ, Wang GY, Chen B, Tan SK, Liu J, Yuan QL (2010) Ginsenoside Rb1 regulates the expressions of brain-derived neurotrohic factor and caspase −3 and induces neurogenesis in rats with experimental cerebral ischemia. J Ethnopharmacol 132:393–399

Guo LY, Joo EJ, Son KH, Jeon SJ, Jang S, Shin EM, Zhou HY, Kim Y (2009) Cimiside E arrests cell cycle and induces apotosis in gastric cancer cells. Arch Pharm Res 32:1385–1392

Han L, Pan G, Wang Y, Song X, Gao X (2011) Rapid profiling and identification of triterpenoid saponins in crude extracts from *Albizia julibrissin* Durazz by ultra high-performance liquid chromatography coupled with electrospray ionization quadrupole time of flight tandem mass spectrometry. J Pharm Biomed Anal 55:996–1009

He BC, Gao JL, Luo X, Luo J, Shen J, Wang L, Zhou Q, Wang YT, Luu HH, Haydon RC, Wang CZ, Du W, Yuan CS, He TC, Zhang BQ (2011) Ginsenoside Rg3 inhibits colorectal tumor growth through the dow-regulation of Wnt/b-catenin signaling. Int J Oncol 38:437–445

He CC, Dai YQ, Hui RR, Hua J, Chen HJ, Luo QY, Li JX (2012) NMR-based metabolomic approach on the toxicological effects of a Cimicifuga triterpenoid. J Appl Toxicol 32:88–97

Hien TT, Kim ND, Kim HS, Kang KW (2010) Ginsenoside Rg3 inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells. Pharmazie 65:699–701

Hong SW, Yang JH, Jof EH, Kim HJ, Kim DH (2011) Gypenoside TN-2 ameliorates scopolamine-induced learning deficit in mice. J Ethnopharmacol 134:1010–1013

Hsieh MS, Wang KT, Tseng SH, Lee SJ, Chen CH, Wang CC (2010) Using 18 F-FDG microPET Imaging to measure the inhibitory effects of *Clematis chinensis* Osbeck on the proinflammatory and degradative mediators associated with inflammatory arthritis. J Ethnopharmacol 38:343–358

Hsu HY, Yang JS, Lu KW, Yu CS, Chou ST, Lin JJ, Chen YY, Lin ML, Chueh FS, Chen SS, Chung JG (2011) An experimental study on the antileukemia effects of gypenosides in vitro and in vivo. Integr Cancer Ther 10:101–112

Hua H, Feng L, Zhang XP, Zhang LF, Jin J (2009) Anti-angiogenic activity of julibroside J8, a natural product isolated from *Albizia julibrissin*. Phytomedicine 16:703–711

Huang LZ, Zhao HF, Huang BK, Zheng C, Peng W, Qin LP (2011) *Acanthopanax senticosus*: review of botany, chemistry and pharmacology. Pharmazie 66:83–97

Huyen VT, Phan DV, Thang P, Hoa NK, Ostenson CG (2010) Antidiabetic effect of *Gynostemma pentaphyllum* tea in randomly assigned type-2 diabetic patients. Horm Metab Res 42:353–357

Hwang JT, Lee MS, Kim HJ, Sung MJ, Kim HY, Kim MS, Kwon DY (2009) Antiobesity effect of ginsenoside Rg3 involves the AMPK and PPAR-gamma signal pathways. Phytother Res 23:262–266

Ionkova I, Momekov G, Proksch P (2010) Effects of cycloartane saponins from hairy roots of *Astragalus membranaceus* Bge. on human tumor cell targets. Fitoterapia 81:447–451

Jeong SI, Zhou B, Bae JB, Kim NS, Kim SG, Kwon J, Kim DK, Shin TY, Jeon H, Lim JP, Kim H, Kim HK, Oh CH (2008) Apoptosis-inducing effect of Akebia saponin D from the roots of Dipsacus asper Wall in U937 cells. Arch Pharm Res 31:1399–1404

Jia L, Qian K (2011) An evidenced-base perspective of *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (American ginseng) as a preventing or supplementary therapy for cancer patients. In: Cho WCS (ed) Evidenced-based anticancer materia medica (Chap. 4). Springer, Heidelberg, pp 85–96

Jia L, Zhao Y (2009) Current evaluation of the millennium phytomedicine- Ginseng (I):etymology, pharmacognosy, phytochemistry, market and regulations. Curr Med Chem 16:2475–2484

Jia XD, Feng X, Dong YF, Zhao XZ, Wang M, Zhao YY, Sun H (2007) Saponins from flower buds of *Lonicera macranthoides*. Chin Traditional Herbal Drugs 38:1452–1455

Joh EH, Yang JW, Kim DH (2010) Gypenoside LXXIV ameliorates scopolamine-induced learning deficit in mice. Planta Med 76:793–795

Kang YJ, Chung HJ, Nam JW, Park HJ, Seo EK, Kim YS, Lee D, Lee SK (2011) Cytotoxic and antineoplastic activity of timosaponin A III for human cancer cells. J Nat Prod 74:701–706

Kao TH, Huang SC, Inbaraj BS, Chen BH (2008) Determination of flavonoids and saponins in *Gynostemma pentaphyllum* (Thunb) Makino by liquid chromatography-mass spectrometry. Anal Chim Acta 626:200–211

Khanh Hoa N, Norberg A, Sillard R, Van Phan D, DuyThuan N, Ngoc Dzung DT, Jörnvall H, Östenson CG (2007) The possible mechanisms by which phanoside stimulates insulin secretion from rat islets. J Endocrinol 192:389–394

Kim MO, Moon DO, Choi YH, Lee JD, Kim ND, Kim GY (2008) Platycodin D induces mitotic arrest in vitro, leading to endoreduplication, inhibition of proliferation and apoptosis in leukemia cells. Int J Cancer 122:3674–3681

Kim SM, Lee SY, Cho JS, Choi SS, Yun YP, Yoo HS, Yoon do Y, Oh KW, Han SB, Hong JT (2010) Combination of ginsenoside Rg3 with docetaxel enhances the susceptibility of prostate cancer cells via inhibition of NF-kappa B. Eur J Pharmacol 631:1–9

Kim BJ, Nah SJ, Jeon JH, So I, Kim SJ (2011) Transient receptor potential melastatin 7 channels are involved in ginsenoside Rg3-induced apoptosis in gastric cancer cells. Basic Clin Pharmacol Toxicol 109:233–239

Lacaille-Dubois MA (2005) Bioactive saponins with cancer related and immunomodulatory activity: recent developments. In: Atta-Ur-Rahman (ed) Studies in natural products chemistry series, vol 32. Elsevier, Amsterdam, pp 209–246

Lacaille-Dubois MA, Pegnyemb DE, Noté OP, Mitaine-offer AC (2011) A review of acacic acid-type saponins from Leguminoseae-Mimosoideae, as potent cytotoxic and apoptosis inducing agents. Phytochem Rev 10:565–584

Lee SY, Kim GT, Roh SH, Song JS, Kim HJ, Hong SS, Kwon SW, Park JH (2009) Proteomic analysis of the anticancer effect of 20*S*-ginsenoside Rg3 in human colon cancer cell lines. Biosci Biotechnol Biochem 73:811–816

Lee H, Kang R, Kim YS, Chung SI, Yoon Y (2010) Platycodin D inhibits adipogenesis of 3 T3-L1 cells by modulating Kruppel-like factor 2 and peroxisome proliferator-activated receptor g. Phytother Res 24:S161–S167

Li JX, Yu ZY (2006) *Cimicifugae* rhizoma: from origins, bioactive constituents to clinical outcomes. Curr Med Chem 13:2927–2951

Li G, Wang Z, Son Y, Liu K, Wang Z (2006) Ginsenoside 20 (S)-protopanaxadiol inhibits the proliferation and invasion of human fibrosarcoma HT 1080 cells. Basic Clin Pharmacol Toxicol 98:588–592

Li N, Liu B, Dluzen DE, Jin Y (2007) Protective effects of ginsenoside Rg2 against glutamate-induced neurotoxicity in PC 12 cells. J Ethnopharmacol 111:458–463

Li C, Tian J, Li G, Jiang W, Xing Y, Hou J, Zhu H, Xu H, Zhang G, Liu Z, Ye Z (2010a) Asperosaponin VI protects cardiac myocytes from hypoxia-induced apoptosis via activation of the PI3K/Akt and CREB pathway. Eur J Pharmacol 649:100–107

Li C, Liu Z, Tian J, Li G, Jiang W, Zhang G, Chen F, Lin P, Ye Z (2010b) Protective role of asperosaponin VI, a triterpene saponin isolated from *Dipsacus asper* Wall on acute myocardial infarction in rats. Eur J Pharmacol 627:235–241

Li H, Huang W, Wen Y, Gong G, Zhao Q, Yu G (2010c) Antithrombotic activity and chemical characterisation of steroidal saponins from *Dioscorea zingiberensis* C.H. Wright. Fitoterapia 81:1147–1156

Li J, Yang D, Yu K, He J, Zhang Y (2010d) Determination of diosgenin content in medicinal plants with enzyme-linked immunosorbent assay. Planta Med 76:1915–1920

Li K, Ding L, Yang ZL, Liu EH, Qi LW, Li P, Hu YZ (2010e) Determination of asperosaponin VI in rat plasma by HPLC-ESI-MS and its application to preliminary pharmacokinetic studies. Biomed Chromatogr 24:550–555

Li YJ, Wei HL, Qi LW, Chen J, Ren MT, Li P (2010f) Characterization and identification of saponins in *Achyranthes bidentata* by rapid-resolution liquid chromatography with electrospray ionization quadrupole time of flight tandem mass spectrometry. Rapid Commun Mass Spectrom 24:2975–2985

Li J, Xie ZZ, Tang YB, Zhou JG, Guan YY (2011a) Ginsenoside Rd, a purified component from *Panax notoginseng* saponins, prevents atherosclerosis in apoE knockout mice. Eur J Pharmacol 652:104–110

Li M, Wang W, Xue J, Gu Y, Lin S (2011b) Meta-analysis of the clinical value of *Astragalus membranaceus* in diabetic nephropathy. J Ethnopharmacol 133:412–419

Lin JJ, Hsu HY, Yang JS, Lu KW, Wu RSC, Wu KC, Lai TY, Chen CY, Ma CY, Wood WG, Chung JG (2011) Molecular evidence of antileukemia activity of gypenosides on human myeloid leukemia HL-60 cells in vitro and in vivo using a HL-60 cells murine xenograft model. Phytomedicine 18:1075–1085

Liu XM, Huang QF, Zhang YL, Lou JL, Liu HS, Zheng H (2008a) Effects of *Tribulus terrestris* L. saponins on apoptosis of cortical neurons induced by hypoxia-reoxygenation in rats. Zhong Xi Yi Jie He Xue Bao 6:45–50

Liu W, Zheng Y, Han L, Wang H, Saito M, Ling M, Kimura Y, Feng Y (2008b) Saponins (ginsenosides) from stems and leaves of *Panax quinquefolium* prevented high-fat diet-induced obesity in mice. Phytomedicine 15:1140–1145

Liu TG, Huang Y, Cui DD, Huang XB, Mao SH, Ji LL, Song HB, Yi C (2009) Inhibitory effect of ginsenoside Rg3 combined with gemcitabine on angiogenesis and growth of lung cancer in mice. BMC Cancer 9:250

Liu R, Zhang JZ, Liu WC, Kimura Y, Zheng YN (2010) Antiobesity effects of protopanaxadiol types of ginsenosides isolated from the leaves of American ginseng (*Panax quinquefolius* L.) in mice fed with a high-fat diet. Fitoterapia 81:1079–1087

Liu J, Zhao ZZ, Chen HB (2011) Review of *Astragali* radix. Chin Herbal Medicines 3:90–105

Lu HF, Chen YS, Yang JS, Chen JC, Lu KW, Chiu TH, Liu KC, Yeh CC, Chen GW, Lin HJ, Chung JG (2008a) Gypenosides induced GO/G1 arrest via inhibition of cyclin E and induction of apoptosis via activation of capspases −3 and −9 in human lung cancer A-549 cells. In Vivo 22:215–221

Lu KW, Tasi ML, Chen JC, Hsu SC, Hsia TC, Lin MW, Huang AC, Chang YH, Ip SW, Lu HF, Chung JG (2008b) Gypenosides inhibited invasion and migration of human tongue cancer SCC4 cells through down-regulation of NFkappaB and matrix metalloproteinase-9. Anticancer Res 28:1093–1099

Luo JG, Ma L, Kong LY (2008) New triterpenoid saponins with strong inhibitory activity from the roots of *Gypsophila oldhamania*. Bioorg Med Chem Lett 16:2912–2920

Ma C, Wang L, Tang Y, Fan M, Xiao H, Huang C (2008) Identification of major xanthones and steroidal saponins in rat urine by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry technology following oral administration of rhizoma *Anemarrhena* decoction. Biomed Chromatogr 22:1066–1083

Man SL, Gao WY, Zhang YJ, Jin X, Yan LL, Ma CY, Huang X et al (2009a) Characterisation of steroidal saponins in saponin extract from *Paris polyphylla* by liquid chromatography tandem multi-stage mass spectrometry. Anal Bioanal Chem 395:495–505

Man SL, Gao WY, Zhang YJ, Yan LL, Ma CY, Liu CX, Huang LQ (2009b) Antitumor and antimetastatic activities of Rhizoma *Paridis* saponins. Steroids 74:1051–1056

Man S, Gao W, Zhang Y, Liu Z, Yan L, Huang L, Liu C (2011) Formosanin C-inhibited pulmonary metastasis through repression of matrix metalloproteinases on mouse lung adenocarcinoma. Cancer Biol Ther 11:592–598

Megalli S, Davies NM, Roufogalis BD (2006) Anti-hyperlipidemic and hypoglycemic effects of *Gynostemma pentaphyllum* in the Zucker fatty rat. J Pharm Pharmaceutic Sci 9:281–291

Moon L, Ha YM, Jang HJ, Kim HS, Jun MS, Kim YM, Lee YS, Lee DH, Son KH, Kim HJ, Seo HG, Lee JH, Kim YS, Chang KC (2011) Isoimeratorin, cimiside E and 23-*O*-acetylshengmanol-3-xyloside from Cimicifugae rhizome inhibit TNF-α-induced VCAM-1 expression in human endothelial cells: involvement of PPAR-γ upregulation and PI3K, ERK1/2, and PKC signal pathways. J Ethnopharmacol 133:336–344

Musende AG, Eberding A, Jia W, Ramsay E, Bally MB, Guns ET (2010) Rh2 or its aglycone a PPD in combination with docetaxel for treatment of prostate cancer. Prostate 70:1437–1447

Nian Y, Zhang YL, Chen JC, Lu L, Qiu MH, Qing C (2010) Cytotoxic chemical constituents from the roots of *Cimicifuga foetida*. J Nat Prod 73:93–98

Nian J, Zhang XM, Li Y, Wang YY, Chen JC, Lu L, Zhou L, Qiu MH (2011) Cycloartane triterpenoids from the aerial parts of *Cimicifuga foetida*. Phytochemistry 72:1473–1481

Nie W, Luo JG, Kong LY (2010) Triterpenoids from roots of *Gypsophila pacifica*. Chinese Herbal Medicines 2:145–147

Norberg A, Khanh Hoa N, Liepinsh E, Van Phan D, Duy Thuan N, Jörnvall H, Sillard R, Östenson CG (2004) A novel insulin-releasing substance, phanoside, from the plant *Gynostemma pentaphyllum*. J Biol Chem 279:41361–41367

Pan RL, Chen DH, Si JY, Zhao XH, Li Z, Cao L (2009) Immunosuppressive effects of new cyclolanostane triterpene diglycosides from the aerial part of *Cimicifuga foetida*. Arch Pharm Res 32:185–190

Peng J, Dong F, Qi Y, Han X, Xu Y, Xu L, Xu Q, Liu K (2008) Preparative separation of four triterpene saponins from radix Astragali by high-speed counter-current chromatography coupled with evaporative light scattering detection. Phytochem Anal 19:212–217

Pharmacopeia Committee of P. R. China (2010) Pharmacopoeia of People's Republic of China. China Medical Science and Technology Press, Beijing

Podolak I, Galanty A, Sobolewska D (2010) Saponins as cytotoxic agents: a review. Phytochem Rev 9:425–474

Qi LW, Chen CY, Li P (2009) Structural characterisation and identification of iridoid glycosides saponins, phenolic acids and flavonoids in flos *Lonicerae japonicae* by a fast liquid chromatography method with diode-array detection and time of flight mass spectrometry. Rapid Commun Mass Spectrom 23:3227–3242

Qi LW, Wang CZ, Yuan CS (2010a) American ginseng: potential structure-function relationship in cancer chemoprevention. Biochem Pharmacol 80:947–954

Qi X, Ignatova S, Luo G, Liang Q, Jun FW, Wang Y, Sutherland I (2010b) Preparative isolation and purification of ginsenosides Rf, Re, Rd, and Rb1 from the roots of *Panax ginseng* with a salt/containing solvent system and flow step-gradient by high performance counter-current chromatography coupled with an evaporative light scattering detector. J Chromatogr A 26:1995–2001

Qi LW, Wang CZ, Yuan CS (2011) Ginsenosides from American ginseng: chemical and pharmacological diversity. Phytochemistry 72:689–699

Qin F, Ye YP, Sun HX (2006) Haemolytic activity and adjuvant effect of notoginseng K from the roots of *Panax notoginseng*. Chem Biodivers 3:1144–1152

Qinga LS, Xue Y, Zheng Y, Xiong J, Liao X, Ding LS, Li BG, Liu YM (2010) Ligand fishing from *Dioscorea nipponica* extract using human serum albumin functionalized magnetic nanoparticles. J Chromatogr A 1217:4663–4668

Qiu YK, Dou DQ, Cai LP, Jiang HP, Kang TG, Yang BY, Kuang HX, Li MZ (2009) Dammarane-type saponins from *Panax quinquefolium* and their inhibition activity on human breast cancer MCF-7 cells. Fitoterapia 80:219–222

Qiu F, Imai A, McAlpine JB, Lankin DC, Burton I, Karakach T, Farnsworth NR, Chen SN, Pauli GF (2012) Dereplication, residual complexity, and rational naming: the case of the *Actea* triterpenes. J Nat Prod 75:432–443

Razmovski-Naumovski V, Huang HWT, Tran VH, Qian Li G, Duke CC, Roufogalis BD (2005) Chemistry and pharmacology of *Gynostemma pentaphyllum*. Phytochem Rev 4:197–219

Sautour M, Mitaine-Offer AC, Lacaille-Dubois MA (2007) *Dioscorea* genus: a review of bioactive steroid saponins. J Nat Med 61:91–101

Schild L, Chen BH, Makarov P, Kattengell K, Heinitz K, Keilhoff G (2010) Selective induction of apoptosis in glioma tumour cells by a *Gynostemma pentaphyllum* extract L. Phytomedicine 18:2–10

Shen Z, Zhang WT, Zhao WL, Huang W (2011) Simultaneous determination of four major steroidal saponins in seven species of *Dioscor*ea L. by HPLC-ELSD. Chin Herbal Medicines 3:70–74

Shi L, Cao JQ, Li W, Zhao YQ (2010) Three triterpene saponins from *Gynostemma pentaphyllum*. Helv Chim Acta 93:1785–1794

Siu FM, Ma DL, Cheung YW, Lok CN, Yan K, Yang Z, Yang M, Xu S, Ko BC, He QY, Che CM (2008) Proteomic and transcriptomic study on the action of a cytotoxic saponin (Polyphyllin D): induction of endoplasmic reticulum stress and mitochondria-mediated apoptotic pathways. Proteomics 8:3105–3117

Song X, Hu S (2009) Adjuvant activities of saponins from traditional Chinese medicinal herbs. Vaccine 27:4883–4890

Su L, Feng SG, Qiao L, Zhou YZ, Yang RP, Pei YH (2009a) Two new steroidal saponins from *Tribulus terrestris*. J Asian Nat Prod Res 11:38–43

Su L, Chen G, Feng SG, Wang W, Li ZF, Chen H, Liu YX, Pei YH (2009b) Steroidal saponins from *Tribulus terrestris*. Steroids 74:399–403

Sun HX (2006a) Haemolytic activities and adjuvant effect of *Bupleurum chinense* saponins on the immune response to ovalbumine in mice. Vaccine 24:1324–1331

Sun HX (2006b) Adjuvant effect of *Achyranthes bidentata* saponins on specific antibody and cellular response to ovalbumine in mice. Vaccine 24:3432–3439

Sun HX, Pan HJ (2006) Immunological adjuvant effect of *Glycyrrhiza uralensis* saponins on the immune responses to ovalbumine in mice. Vaccine 24:1914–1920

Sun XH, Zheng QF (2006) Haemolytic activities and adjuvant effect of *Gynostemma pentaphyllum* saponins on the immune responses to ovalbumin in mice. Phytother Res 19:895–900

Sun F, He Q, Shi P, Xiao P, Cheng Y (2007a) Characterization and identification of triterpenoid saponins in crude extracts from *Clematis* spp. by high performance liquid chromatography/electrospray ionization with multi-stage tandem mass spectrometry. Rapid Commun Mass Spectrom 21:3743–3750

Sun J, Hu S, Song X (2007b) Adjuvant effects of protopanaxadiol and protopanaxatriol saponins from ginseng roots on the immune responses to ovalbumine in mice. Vaccine 25:1114–1120

Sun F, He Q, Xiao P, Muhammad I, Cheng Y (2008a) Simultaneous quantification of five triterpenoid saponins in *Clematis* L. ssp. by High-performance liquid chromatography with evaporative light scattering detection. Phytochem Anal 19:40–45

Sun J, Song X, Hu S (2008b) Ginsenoside Rg1 and aluminium hydroxide synergistically promote immune response to ovalbumine in BALB/c mice. Clin Vaccine Immunol 15:303–307

Sun S, Wang CH, Tong R, Li XL, Fishbein A, Wang Q, He TC, Du W, Yuan CS (2010) Effects of steaming the root of *Panax notoginseng* on chemical composition and anticancer activities. Food Chem 118:307–314

Sun S, Qi LW, Du GJ, Menhendale SR, Wang CZ, Yuan CS (2011) Red notoginseng: higher ginsenoside content and stronger anticancer potential than Asian and American ginseng. Food Chem 125:1299–1305

Tang SR, Yang RT, Pan FS, Zhao AM, Pang ZJ (2007) Steroidal saponins and steroidal sapogenins in Chinese *Dioscorea* L. Zhiwu Ziyuan Yu Huanjing Xuebao 16:64–72

Tian Z, Si J, Chang Q, Zhou L, Chen S, Xiao P, Wu E (2007) Antitumor activity and mechanisms of action of total glycosides from aerial part of *Cimicifuga dahurica* targeted against hepatoma. BMC Cancer 7:237

Tian J, Zhang S, Li G, Liu Z, Xu B (2009) 20 (S)-ginsenoside Rg3, a neuroprotective agent, inhibits mitochondrial permeability transition pores in rat brain. Phytother Res 23:486–491

Tin MMY, Cho CH, Chan K, James AE, Ko JKS (2007) *Astragalus* saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft. Carcinogenesis 28:1347–1355

Tsai YC, Lin CL, Chen BH (2010) Preparative chromatography of flavonoids and saponins in *Gynostemma pentaphyllum* and their antiproliferation effect on hepatoma cell. Phytomedicine 18:2–10

Tung NH, Song GY, Park YJ, Kim YH (2009) Two new dammarane-type saponins from the leaves of *Panax ginseng*. Chem Pharm Bull 12:1412–1414

Tung NH, Song GY, Minh CV, Kiem PV, Jin LG, Boo HJ, Kang HK, Kim YH (2010) Steamed ginseng-leaf component enhance cytotoxic effects on human leukemia HL-60 cells. Chem Pharm Bull 58:1111–1115

Verpoorte R, Crommelin D, Danhof M, Gilissen LJWJ, Schuitmaker H, van der Greef J, Witkamp RF (2009) Commentary: "A systems view on the future of medicine: Inspiration from Chinese Medicine ?". J Ethnopharmacol 121:479–481

Wagner H, Bauer R, (eds) Chinese drug monographs and analysis. Kötzting, Verlag für Ganzheitliche Medizin. Dr. Erich Wühr GmbH (1996–2010)

Wagner H, Bauer R, Melchart D, Xiao PG, Staudinger A (eds) (2011) Chromatographic fingerprint analysis of herbal medicines–Thin-layer and high performance liquid chromatography of Chinese drugs, vol 1 & 2, 2nd edn. Springer, Vienna, Austria

Wan JB, Li P, Li S, Wang Y, Dong TTX, Tsim KWK (2006) Simultaneous determination of 11 saponins in *Panax notoginseng* using HPLC-ELSD and pressurized liquid extraction. J Sep Sci 29:2190–2196

Wang YH, Du GH (2009) Ginsenoside Rg1 inhibits beta-secretase activity in vitro and protects against Abeta-induced cytotoxicity in PC 12 cells. J Asian Nat Prod Res 11:604–612

Wang HK, Sakurai N, Shih CY, Lee KH (2005) LC/TIS/MS fingerprint profiling of *Cimicifuga* species and analysis of 23-epi-26-deoxyactein in *Cimicifuga racemosa* commercial products. J Agric Food Chem 53:1379–1386

Wang CZ, Xie JT, Fishbein A, Aung HH, He H, Mehendale SR, He TC, Du W, Yuan CS (2009a) Antiproliferative effects of different plant parts of *Panax notoginseng* on SW480 human colorectal cancer cells. Phytother Res 23:6–13

Wang J, Zu X, Jiang Y (2009b) Five furostanol saponins from fruits of *Tribulus terrestris* and their cytotoxic activities. Nat Prod Res 23:1436–1444

Wang J, Zhao XZ, Qi Q, Tao L, Zhao Q, Mu R, Gu HY, Wang M, Feng X, Guo QL (2009c) Macranthoside B, a hederagenin saponin extracted from *Lonicera macranthoides* and its anti-tumor activities in vitro and in vivo. Food Chem Toxicol 47:1716–1721

Wang Y, Xu J, Qu H (2011) Structure characterization and identification of steroidal saponins from *Ophiopogon japonicus* ker-gawler (Liliaceae) by high-performance liquid chromatography with ion trap mass spectrometry. Phytochem Anal 22:166–171

Wei XY, Yang JY, Wang JH, Wu CF (2007) Anxiolytic effect of saponins from *Panax quinquefolium* in mice. J Ethnopharmacol 111:613–618

Weng A, Bachran D, Görik C, Vachran C, Fuchs H, Melzig MF (2009) A simple method for isolation of *Gypsophila* saponins for the combined application of targeted toxins and saponins in tumor therapy. Planta Med 75:1421–1422

Weng A, Jenett-Siems K, Schmieder P, Bachran D, Bachran C, Görick C, Thakur M, Fuchs H, Melzig MF (2010) A convenient method for saponin isolation in tumor therapy. J Chromatogr B 878:713–718

Wong VK, Zhou H, Cheung SS, Li T, Liu L (2009) Mechanistic study of saikosaponin-d (Ssd) on suppression of murine T lymphocyte activation. J Cell Biochem 107:303–315

Wu W, Xu X, Dai Y, Xia L (2010) Therapeutic effect of the saponin fraction from *Clematis chinensis* Osbeck roots on osteoarthritis induced by monosodium iodoacetate through protecting articular cartilage. Phytother Res 24:538–546

Wuttke W, Seidlova-Wuttke D (2012) Complementary and traditional chinese medicine (TCM) methods in the treatment of gynecological diseases. In: Wagner H, Ulrich-Merzenich G (eds) "Evidence and rational based research on Chinese drugs" (in press)

Xiang YZ, Shang HC, Gao XM, Zhang BL (2008) A comparison of the ancient use of ginseng in traditional Chinese medicine with modern pharmacological experiments and clinical trials. Phytother Res 22:851–858

Xie Y, Pan H, Sun H, Li D (2008) A promising balanced Th1 and Th2 directing immunological adjuvant, saponins from the roots of *Platycodon grandiflorum*. Vaccine 26:3937–3945

Xie Y, Sun HX, Li D (2010) Platycodin D improves the immunogenicity of newcastle disease virus-based recombinant avian influenza vaccine in mice. Chem Biodivers 7:677–689

Xu L-L, Han T, Wu J-Z, Zhang Q-Y, Zhang H, Huang B-K, Rahman K, Quin L-P (2009a) Comparative research of chemical constituents, antifungal and antitumor properties of ether extracts of *Panax ginseng* and its endophytic fungus. Phytomedicine 16:609–616

Xu T, Xu Y, Liu Y, Xie S, Si Y, Xu D (2009b) Two new furostanol saponins from *Tribulus terrestris*. Fitoterapia 80:354–357

Xu YJ, Xu TH, Zhou HO, Li B, Xie SX, Si YS, Liu Y, Liu TH, Xu DM (2010) Two new furostanol saponins from *Tribulus terrestris*. J Asian Nat Prod Res 12:349–354

Yang ZG, Sun HX, Fang WH (2005) Hemolytic activities and adjuvant effect of *A. membranaceus* saponins on the immune response to ovalbumin in mice. Vaccine 23:5196–5203

Yang Z-G, Ye Y-P, Sun H-X (2007) Immunological adjuvant effect of ginsenoside Rh_4 from the roots of *Panax notoginseng* on specific antibody and cellular response to ovalbumin in mice. Chem Biodivers 4:232–240

Yang L, Xu SJ, Wu ZF, Liu YM, Zeng X (2009a) Determination of ginsenoside Rg1 in human plasma and its application to pharmacokinetic studies following intravenous administration of « Shenmai » injection. Phytother Res 23:65–71

Yang M, Sun J, Lu Z, Chen G, Guan S, Liu X, Jiang B, Ye M, Guo DA (2009b) Phytochemical analysis of traditional Chinese medicine using liquid chromatography coupled with mass spectrometry. J Chromatogr A 1216:2045–2062

Yao S, Luo J, Huang X, Kong L (2008) Application of preparative high-speed counter-current chromatography/preparative high-performance liquid chromatography mode in rapid separation of saponins. J Chromatogr B Analyt Technol Biomed Life Sci 864:69–77

Yao S, Ma L, Luo JG, Wang JS, Kong LY (2010) New triterpene saponins from the roots of *Gypsophila paniculata*. Helv Chim Acta 93:361–374

Ye R, Zhang X, Kong X, Han J, Yang Q, Zhang Y, Chen Y, Li P, Shi M, Xiong L, Zhao G (2011) Ginsenoside Rd attenuates mitochondrial dysfunction and sequential apoptosis after transient focal ischemia. Neuroscience 178:169–180

Yeo J, Kang YJ, Jeon SM, Jung UJ, Lee MK, Song H, Choi MS (2008) Potential hypoglycemic effect of an ethanol extract of *Gynostemma pentaphyllum* in C57BL/KsJ-db/db mice. J Med Food 11:709–716

Yesilada E, Bedir E, Calis I, Takaishi Y, Ohmoto Y (2005) Effect of triterpene saponins from *Astragalus* species on in vitro cytokine release. J Ethnopharmacol 96:71–77

Yoon KD, Kim J (2008) Preparative separation of dioscin derivatives from *Dioscorea villosa* by centrifugal partition chromatography coupled with evaporative light scattering detection. J Sep Sci 31:2486–2491

Yoon KD, Kim J (2009) Application of centrifugal partition chromatography coupled with evaporative light scattering detection for the isolation of saikosaponins-a and -c from *Bupleurum falcatum* roots. J Sep Sci 32:74–78

Yu QT, Qi LW, Li P, Yi L, Zhao J, Bi Z (2007) Determination of seventeen main flavonoids and saponins in the medicinal plant Huang-qi (Radix *Astragali*) by HPLC-DAD-ELSD. J Sep Sci 30:1292–1299

Zeng X, Deng Y, Feng Y, Liu Y, Yang L, Huang Y, Sun J, Liang W, Guan Y (2010) Pharmacokinetics and safety of ginsenoside Rd following a single or multiple intravenous dose in healthy Chinese volunteers. J Clin Pharmacol 50:285–292

Zhang JH, Wang JP, Wang HJ (2007a) Clinical study on effect of total *Panax notoginseng* saponins on imune related inner environment imbalance in rheumatoid arthritis patients. Zhongguo Zhong Xi Yi Jie He Za Zhi 27:589–592

Zhang SQ, Zhang JS, Wang CZ (2007b) Extraction of steroid saponins from *Paris polyphylla* Sm var. Yunnanensis using novel ultrahigh pressure extraction technology. Pharmaceut Chem J 41:424–429

Zhang HJ, Ji BP, Chen G, Zhou F, Luo YC, Yu HQ, Gao FY, Zhang ZP, Li HY (2009) A combination of grape seed-derived procyanidins and gypenosides alleviates insulin resistance in mice and HepG2 cells. J Food Sci 74:H1–H7

Zhang T, Liu H, Liu XT, Xu DR, Chen XQ, Wang Q (2010) Qualitative and quantitative analysis of steroidal saponins in crude extracts from *Paris polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* by high performance liquid chromatography coupled with mass spectrometry. J Pharm Biomed Anal 51:114–124

Zhang L, Yang Y, Wang Y, Gao X (2011) *Astragalus membranaceus* extract promotes neovascularisation by VEGF pathway in rat model of ischemic injury. Pharmazie 66:144–150

Zheng Q, Li W, Han L, Koike K (2007) Pancreatic lipase-inhibiting triterpene saponins from *Gypsophila oldhamania*. Chem Pharm Bull 55:646–650

Zheng L, Zheng J, Zhang QY, Wang B, Zhao YY, Wu LJ (2010a) Three new oleanane triterpenoids saponins acetylated with monoterpenoid acid from *Albizia julibrissin*. Fitoterapia 81:859–863

Zheng YF, Qi LW, Zhou JL, Li P (2010b) Structural characterisation and identification of oleanane-type triterpene saponins in *Glycyrrhiza uralensis* Fisher by rapid-resolution liquid chromatography coupled with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 24:3261–3270

Zhou W, Feng M, Li X, Yan Q, Zhou C, Li J, Zhou P (2009a) X-Ray structure investigation of (20*S*)-20-*O*-β-D-glucopyranosyl-protopanaxadiol and antitumor effect in Lewis Lung carcinoma in vivo. Chem Biodivers 6:380–388

Zhou YQ, Yang ZL, Xu L, Li P, Hu YZ (2009b) Akebia saponin D, a saponin component from *Dipsacus asper* Wall, protects PC 12 cells against amyloid-β induced cytotoxicity. Cell Biol Int 33:1102–1110

Zhu J, Guo X, Fu S, Zhang X, Liang X (2010) Characterization of steroidal saponins in crude extracts from *Dioscorea zingiberensis* C. H. Wright by ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry. J Pharm Biomed Anal 53:462–474

Zou K, Zhang QY, Wang B, Cui JR, Zhao YY, Zhang RY (2010) Cytotoxic triterpenoid saponins acylated with monoterpenoid acid from *Albizia julibrissin*. Helv Chim Acta 93:2100–2106

Zu YG, Yan MM, Fu YJ, Liu W, Zhang L, Gu CB, Effert T (2009) Determination and quantification of astragalosides in radix Astragali and its medicinal products using LC-MS. J Sep Sci 32:517–525

Chapter 4
Efficacy of *Andrographis paniculata* in Upper Respiratory Tract Infectious Diseases and the Mechanism of Action

Alexander Panossian and Georg Wikman

Abbreviations and Definitions

APE	*Andrographis paniculata* extract
ESE	*Eleutherococcus senticosus* root extract
KJ	Kan Jang fixed combination of *Andrographis paniculata and Eleutherococcus senticosus* extracts
Polyvalence	the range of biological activities that an extract may exhibit which contribute to the overall effect observed clinically or in vivo

4.1 Introduction

A recent interest of Western medicine has been focused on the use of herbs as treatment or adjuvant therapy in various diseases. Particularly, the knowledge that antibiotics are often over-prescribed for common infections, thus causing increased bacterial resistance, has instilled interest in herbal therapy for the uncomplicated

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upper respiratory tract (URT) infections (Roxas and Jurenka 2006). The leaves and aerial parts of *Andrographis paniculata* (Burm. f.) Nees[1] (Acanthaceae) have been used for prophylactic and symptomatic treatment of respiratory infections, such as common cold, influenza with fever, soar throat, acute and chronic cough, sinusitis, bronchitis, and pharyngotonsillitis (Herba Andrographidis 2002; Herba Andrographis (Chuanxinlian) 2010; Carr and Nahata 2006; Kligler et al. 2006; Poolsup et al. 2004; Coon and Ernst 2004). Commonly found in tropical and subtropical Asia (mainly in China, Thailand, and India), *A. paniculata* is currently one of the most used medicinal plants in Southeast Asia. It has been commonly used in TCM and Ayurvedic systems as an antipyretic treatment effective against a variety of infections diseases, including urinary infection with difficult painful urination, tonsillitis, dysentery, oedema, bacillary dysentery, bronchitis, carbuncles, colitis, coughs, dyspepsia, malarial and intermittent fever, hepatitis, mouth ulcers, sores, tuberculosis, colic, otitis media, vaginitis, pelvic inflammatory disease, chickenpox, and eczema. Plant is effective for carbuncles, sores, venomous snake bites, ulcers in the mouth or on the tongue, liver disorders, burns, and traumatic infection (Herba Andrographis (Chuanxinlian) 2010; Herba Andrographidis 2002; Akbar 2011; Kunwar et al. 2010). Efficacy for prophylaxis and symptomatic treatment of upper respiratory infections, such as the common cold, uncomplicated sinusitis, bronchitis, and pharyngotonsillitis; urinary tract infections; and acute diarrhoea has also been supported by clinical trials (Herba Andrographidis 2002).

The functional claims of *A. paniculata* dietary supplements [Consolidated list of Article 13 health claims of the European Food Safety Authority (EFSA) http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_article13.htm] are related mainly to "respiratory health", "immune function", and "body defences against external agents". They are usually formulated as following—"helps support the body's natural "immunity", "contributes to the resistance of the organism, supports the natural defence mechanism, especially at the level of the upper respiratory tract", "helps to soften respiratory troubles like cough, sore throats in natural way", etc.

In China, (Herba Andrographis (Chuanxinlian) 2010), and "Common Andrographis tablets", containing 1,000 mg of Andrographis herba (Chuanxinlian Pian (Tabellae Andrographitis) 2010), are included in the Pharmacopoeia of the People's Republic of China and indicated in influenza with fever, soar throat, acute and chronic cough, and other infectious diseases.

In this article we review evidence from pharmacological studies, related to pharmacological activity and clinical efficacy of Herba Andrographidis and to the mechanism of action of the main active principles related to inflammation.

[1] Synonyms: *Justicia latebrosa* Russ., *J. paniculata* Burm. f., *J. stricta* Lam. ex Steud. Common names: chuā̄n-xī̄n-lián (China), kalmegh, bhunimbah, kirattiktah, mahatita (India), teetakaa (Nepal), quasab-uz-zarirah (Pakistan), fah-talai-jone (Thailand), sambiloto (Indonesia), sunbiroat (Japan), king of bitters, green chiretta, creat, creyat root (England), Andrographidis Kraut (Germany).

Reviewing the available bibliographic documentation on clinical efficacy of andrographis (Kligler et al. 2006; Poolsup et al. 2004; Coon and Ernst 2004), it is practically impossible not to pay attention to the significant number of clinical trials on the combination of *A. paniculata* with *Eleutherococcus senticosus* (Kan Jang™), developed in Europe by Swedish herbal institute and extensively used in Scandinavia for an early intervention treatment of URT infections since 1970s (Cáceres et al. 1997, 1999; Melchior 1996; Melchior et al. 2000; Spasov et al. 2004; Gabrielian et al. 2002; Shakhova et al. 2003). Initially, Kan Jang fixed combination has been marketed in Scandinavia as a dietary supplement and, from 1979, as a medicinal product "naturmedel". Currently, Kan Jang has "well-established" status in Denmark and has shown efficacy for the treatment of sinusitis (Gabrielian et al. 2002), familial Mediterranean fever (Amaryan et al. 2003), and influenza (Kulichenko et al. 2003) as well. Eleutherococcus is a typical adaptogen, which is known to increase the state of nonspecific resistance against a wide variety of environmental assaults and emotional conditions (Radix Eleutherococci 2002; ESCOP Monographs 2003; EMEA/HMPC/244569/2006 2007). In TCM it is used to reinforce *qi*, invigorate the function of the spleen and kidney, and anchor the mind (Radix et, Rhizoma seu Caulis Acanthopanacis Senticosi (Ciwaujia) 2005). Moreover, large body of evidence indicates possible benefits of Eleutherococcus in treatment of URT infections (Tables 4.1 and 4.2). In this review we have made an attempt to assess the real benefits of the multitarget therapy concept on the combination of Andrographis with Eleutherococcus used for the treatment of URT infections (Sect. 4.3) by comparison of the results of clinical trials of Kan Jang and the monodrug Andrographis.

4.2 Active Principles

Several groups of bioactive compounds have been isolated and identified from the herb—diterpene lactones (over 20 andrographolides), their glycosides, flavonoids (over 20 compounds), sesquiterpenelactones Paniculides A,B,C, dimeric bis-andrographolides, and polyphenols (Fig. 4.1) (Wagner et al. 2011; Buckingham 1993; Chao and Lin 2010).

The main active compounds of Andrographis are diterpene lactones:

- Andrographolide (AND, I) (Deng et al. 1982; Madav et al. 1996; Handa and Sharma 1990; Shukla et al. 1992; Puri et al. 1993; Visen et al. 1993; Kapil et al. 1993; Amroyan et al. 1999; Chiou et al. 1998, 2000; Shen et al. 2000; Panossian et al. 2002; Peng et al. 2002; Shen et al. 2002; Rajagopal et al. 2003; Kumar et al. 2004; Hsu et al. 2004; Tsai et al. 2004; Xia et al. 2004; Wiart et al. 2005; Cheung et al. 2005; Hidalgo et al. 2005; Iruretagoyena et al. 2005; Kim et al. 2005; Zhou et al. 2006; Thisoda et al. 2006; Qin et al. 2006; Xu et al. 2007; Sheeja et al. 2007; Abu-Ghefreh et al. 2009; Suebsasana et al. 2009; Sulaiman et al. 2010; Parichatikanond et al. 2010; Chao et al. 2010; Chandrasekaran et al. 2011)

Table 4.1 Clinical studies of *E. senticosus* extract, ESE

Number of patients	Sex	Age (years)	Route	Dose (ml)	Frequency	Duration course	Short summary of results	References
100	Male	N.S.	p.o.	1.5	Three times daily	N.S.	Miners with chronic bronchitis received ES extract plus other therapy. No side effects were reported.	Farnsworth et al. (1985)
NDA	Male	N.S.	p.o.	N.S.	N.S.	N.S.	Patients with pneumoconiosis and chronic bronchitis were given the ES extract plus other forms of therapy. No side effects were reported.	Farnsworth et al. (1985)
22	Male	N.S.	p.o.	1.5	Three times daily	N.S.	The ES extract was given to miners with pneumoconiosis and was reported to increase lung capacity. No side effects were mentioned.	Farnsworth et al. (1985)
42	Male	N.S.	p.o.	N.S.	N.S.	N.S.	Miners ill with pneumoconiosis were treated with ES extract and followed with electrocardiograms. The extract decreased changes in heart rhythm activity and sinus bradycardia or arrhythmia, and inhibited introduction of stimulation on the right leg of the Bundle of His. No side effects were reported.	Farnsworth et al. (1985)
28	Male	N.S.	p.o.	2.5	Three times daily	5 days 1	The ES extract was given to patients with stage 1 pneumoconiosis who were institutionalised. Respiration was improved with no side effects being reported.	Farnsworth et al. (1985)
64	Both	9–15	p.o.	1 drop for each year of age	Once daily	6 weeks 1	Administration to children with abating forms of pulmonary tuberculosis. Duration of illness was 2–4 years. Initial 6 weeks of dosing was interrupted for 2 weeks and then	Farnsworth et al. (1985)

							continued for 3½ months total. Improvement in physical exercises was used as a criterion of effectiveness in restoration of good health. No side effects were reported in the treated patients.	
1,376	Both	NDA	p.o.	2 ml of ES or placebo	Once daily	2 months 1	Prophylaxis against influenza virus infections and other acute respiratory diseases investigated in a double-blind, placebo-controlled study. Morbidity rates were considered lower in the ES group than in the placebo group but not statistically significant. Significantly lower frequency of complications (pneumonia, bronchitis, maxillary sinusitis, otitis) ($p < 0.05$)	Shadrin et al. (1986)
NDA	Both	2–7	p.o.	2 drops for each year of age	Once daily	2 months 1	Prophylaxis against viral infections in children under school age with immune-cellular deficiencies (T and B cells and/or increased number of lymphocytes) in a double-blind, placebo-controlled study. Morbidity rates of acute viral infections and influenza decreased by 9.8 % and pneumonia by 40 % in the ES group compared to placebo.	Kozlov (1986)
247 (130—ES, 117—contr.)	Both	1–7	p.o.	1 drop per each year of age	Once daily	5 days 1	Administration to creche–kindergarten infants during incidences of influenza, angina, and acute respiratory and adenoviral infections. ES resulted in 3.6-fold decrease in indices compared to establishment nearby.	Barkan et al. (1980)

(continued)

Table 4.1 (continued)

Number of patients	Sex	Age (years)	Route	Dose (ml)	Frequency	Duration course	Short summary of results	References
517 (265—ES, 252—contr.)	Both	1–7	p.o.	1 drop per each year of age	Once daily	1 month	Incidences of respiratory viral infections were investigated in a larger group of children in a controlled study. The incidences decreased threefold.	Barkan et al. (1980)
195	Both	Children	p.o.	0.3–0.5 ml/kg body weight	Once daily	1 week	ES used as an adjuvant with penicillin in treatment of meningococcal infections. Decreased frequency of acoustic organ complications and other complications reduced by a factor of 3 and hospitalisation reduced by 5–6 days.	Kovalenko and Vereshchagin (1994)
?x100	Male	28–55	p.o.	1.4	Once daily for 8 years	For 4–10 month 8 years	The frequency of illness (common cold) of miners in Vorkuta and Inti in North Siberia was compared. Miners of Vorkuta were prophylactically treated with ESE, while miners in Inti were control group. ES prevents frequency of common cold for 25.3 %	Kalashnikov (1986)
154 (54—ESE, 59—CT, 41—CT + dibasol)	Both	4–14	p.o.	5–15 drops	Three times daily	NDA	Blood lymphocytes/neutrophils ratio normalisation and DNA/RNA of lymphocytes in the course of recovery of children with acute pneumonia was observed. ESE potentiates the recovery of these blood parameters.	Kim (1992)
815 (440—ESE, 375—control)	NDA	NDA	NDA	2 drops	NDA	10–25 days	Morbidity rate decreased in ESE group 1.5–1.6 times, the duration of the disease was 0.9 days shorter, post-influenza complications were less frequent.	Gagarinova et al. (1995)

CT conventional therapy

- 14-Deoxy-11,12-didehydro-andrographolide (DDDAND, II) (Deng et al. 1982; Kumar et al. 2004; Wiart et al. 2005; Thisoda et al. 2006)
- Neoandrographolide (NAND, III) (Deng et al. 1982; Kapil et al. 1993; Batkhuu et al. 2002; Kamdem et al. 2002; Wiart et al. 2005; Cheung et al. 2005; Liu et al. 2007a, b; Parichatikanond et al. 2010) (Figs. 4.2–4.4)

which are structurally similar to cortisol and formally might be considered as seco-corticosteroid-like compounds.

Several minor diterpene lactones, such as 14-deoxyandrographolide, 14-deoxy-14,15-didehydro-andrographolide, andrograpanin, iso-andrograpanin, 14-acetyl-andrographolide, 19-acetyl-anhydro-andrographolide, as well as flavanones and flavones contributes to the overall effects of Herba Andrographidis extract on the immune system (Chao et al. 2010).

4.3 Pharmacological Activity and Mechanism of Action

Andrographis paniculata extracts and its active constituents have actions targeting multiple mediators involved in:

- Life cycle of viruses and bacteria
- Acute inflammatory host response to infectious agent

Polyvalent action of Andrographis (Table 4.3) includes:

- Anti-inflammatory activity associated with modulation of immune response of innate immune system by inhibition of NF-κB (Xia et al. 2004; Hidalgo et al. 2005; Bao et al. 2009; Chao et al. 2009a, b, 2010; Parichatikanond et al. 2010) (Fig. 4.5), platelet-activating factor (PAF) antagonism (Amroyan et al. 1999; Burgos et al. 2005a), inhibition of NO production in macrophages (Chiou et al. 1998, 2000; Batkhuu et al. 2002; Liu et al. 2007a, b, 2008; Chao et al. 2009a, b, 2010; Chandrasekaran et al. 2010, 2011), inhibition of histamine and proinflammatory interleukins (Panossian et al. 2002; Puri et al. 1993; Kumar et al. 2004; Iruretagoyena et al. 2005; Chandrasekaran et al. 2010), prevention of ROS production, and neutrophil adhesion (Shen et al. 2000, 2002; Kamdem et al. 2002; Sheeja et al. 2006; Yu et al. 2003)
- Antiviral activity (associated with the direct inhibition of replication of viruses, and indirect antiviral effect via activation of the immune system including cytokine formation) (Glatthaar-Saalmüller et al. 2001; Protasova and Zykov 1984)
- Antibacterial activity (associated with the direct bacteriostatic and bactericidal effects and indirect antimicrobial effect via activation of the innate and adaptive immune response)

The pharmacology of *A. paniculata* extracts and their active principles, primarily andrographolides I–III, has been studied in different animal models using

Table 4.2 Pharmacological activity of Eleutherococcus associated with URT infections

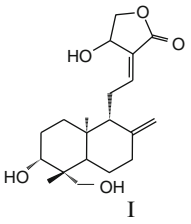
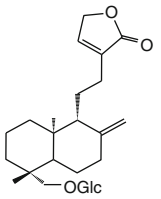
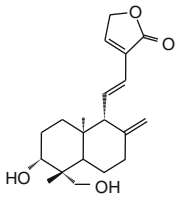
Pharmacological activity of radix Eleutherococci	Effect in vivo and in vitro experiments
Immunosupporting	Improve nonspecific and humoral immune response and modulate interleukines formation (Bohn et al. 1987; Chubarev et al. 1989; Drozd et al. 2002; Gladchun 1983; Kimura and Sumiyoshi 2004; Kormosh et al. 2006; Kupin and Polevaia 1986; Rogala et al. 2003; Schmolz et al. 2001; Shen et al. 1991; Steinmann et al. 2001; Wagner et al. 1984; Yu et al. 2003)
	Stimulate host innate immunity via upregulation of Hsp70 (Asea 2005; Asea and Pedersen 2010; Panossian and Wikman 2010; Panossian et al. 2010; Radons and Multhoff 2005; Tsan and Gao 2004a, b, c)
	Modulate release of arachidonic acid and biosynthesis of eicosanoids
	In isolated human PMNL (Panossian et al. 1982)
Anti-inflammatory/antiallergy	Antihistamine-like effect (Jeong et al. 2001; Yi et al. 2002)
	Inhibition of Akt and JNK pathways in macrophage (Jung et al. 2007; Radix Eleutherococci 2002)
	Inhibition of NO formation (Panossian et al. 2007)
	Antioxidant effect (Yu et al. 2003)
Antiviral	Direct inhibition of replication of rhinovirus, respiratory syncytial virus, and influenza A virus in cell culture (Glatthaar-Saalmüller et al. 2001)
Anti-fatigue/stress-protective	Modulates cortisol, stress proteins (Hsp70, JNK) formation/ Anti-fatigue (Asea and Pedersen 2010; Panossian and Wikman 2009, 2010; Panossian et al. 2009, 2010)

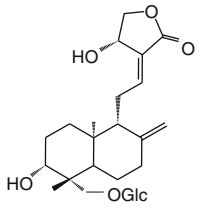
different modes of administration (oral, intravenous, subcutaneous) and different species including mouse, rat, pigs, rabbit, dog, and monkey, isolated tissues from different species, and bacteria in vitro assays (Deng et al. 1982; Deng 1985; Madav et al. 1995; Vedavathy and Rao 1991; Zhu and Nedichin 1998; Tang and Eisenbrand 1992; Sheeja et al. 2006; Suebsasana et al. 2009; Sulaiman et al. 2010).

These studies have demonstrated an effect on:

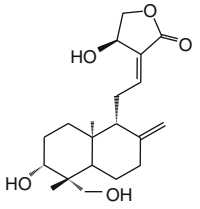
- *The immune system*—anti-inflammatory (including antipyretic, analgesic, and antiallergic) effect, IFNγ-mediated antibacterial and antiviral effects, and NF-κB, TNF-α, IL-1, IL-2, IL-6, IL-12, COX-2, PAF, LTB_4 and NO mediated effects on respiratory system
- *The neuroendocrine system*—analgesic effect
- *The infectious agent*—antiviral and antimicrobial effects

Andrographis paniculata is known as an immunostimulatory botanical (Denzler et al. 2010). At the same time, it is used for the relief of symptoms of inflammation (Poolsup et al. 2004). This "dual" effect of Andrographis is well documented in many pharmacological studies, however it is difficult to give rationale for this "dualism". It can be due to:

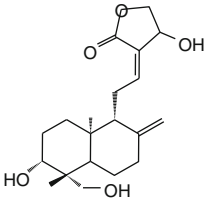
Chemical structure	 I	 II	 III
Chemical name	3, 14, 19-Trihydroxy-8(17), 12-labdadien-16, 15-olide;	19-O-β-D-gluco-pyrano-8(17)-13-labdadien-16,15-olide	3, 19-Dihydroxy-8(17), 11E,13-labdatrien-16, 15-olide; 4-deoxy-11,12-didehydro-andrographolide
Synonym	Andrographolide	Neoandrographolide	
Molecular formula	$C_{20}H_{30}O_5$	$C_{26}H_{40}O_8$	$C_{20}H_{28}O_4$
Relative molecular mass	350.454	480.597	332.439
CAS Registry Number:	5508-58-7	27215-14-1	42895-58-9



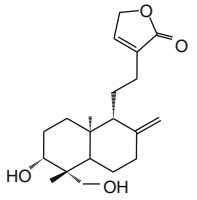
3 ,14,19-Trihydroxy-8(17),12-labdadien-16,15-olide,19-O-.beta.-D-Glucopyranoside
Synonym: Andrographiside



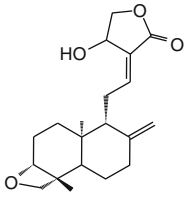
3,14,19-Trihydroxy-8(17),12-labdadien-16,15-olide, Variant: (ent-3.beta.,12E,14S)-form
Synonym: 14-Epiandrographolide



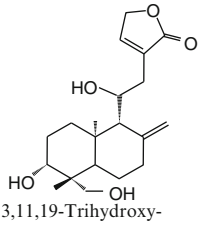
3,14,19-Trihydroxy-8(17),12-labdadien-16,15-olide Variant: (ent-3.beta.,12Z,14.xi.)-form
Synonym: Isoandrographolide



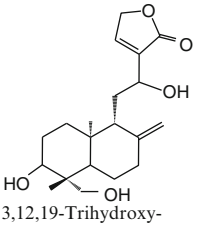
19-Hydroxy-8(17),13-labdadien-16,15-olide, Variant: ent-form
Synonym: Andrograpanin



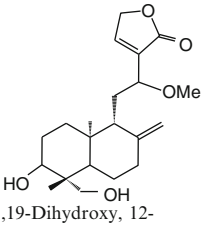
3,19-Epoxy-14-hydroxy-8(17),12-labdadien-16,15-olide, Variant: (ent-3.beta.,14R)-form



3,11,19-Trihydroxy-8(17),13-labdadien-16,15-olide Variant: (ent-3.beta.,11S)-form
Synonym: 14-Deoxy-11-hydroxyandrographolide



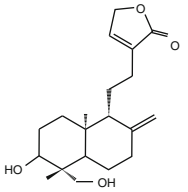
3,12,19-Trihydroxy-8(17),13-labdadien-16,15-olide, Variant: (ent-3.beta. 12.xi.)-form
Synonym: 14-Deoxy-12-hydroxyandrographolide



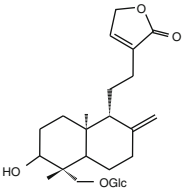
3,19-Dihydroxy, 12-methoxy-8(17),13-labdadien-16,15-olide, Variant: (ent-3.beta.,12.xi.)-form
Synonym: 14-Deoxy-12-methoxyandrographolide

Fig. 4.1 (continued)

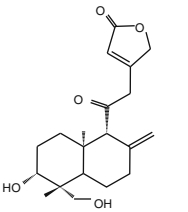
- The dose-dependent reversal effect on the components of immune system
- The various phases or/and the strength of an adaptive immune response to infection depending on what is more important for host homeosthasis - to kill virus/bacteria by activating the immune response, or to suppress the overreaction of the immune system in order to relieve the inflammation



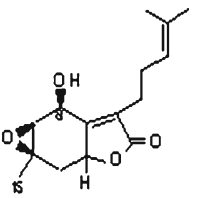
3,19-Dihydroxy-8(17),13-labdadien-16,15-olide, Variant: (ent-3.beta.)-form
Synonym: 14-Deoxyandrographolide



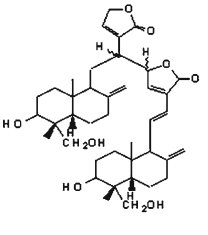
3 ,19-Dihydroxy-8(17),13-labdadien-16,15-olide-19-O-.beta.-D-Glucopyranoside
Synonyms: Andropanoside. Ninandrographolide. 14-Deoxyandrographoside



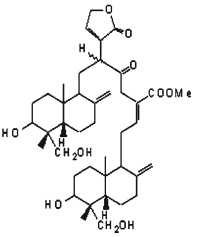
3,19-Dihydroxy-11-oxo-8(17),13-labdadien-15,16-olide
Synonym: 14-Deoxy-11-oxoandrographolide



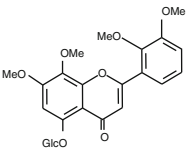
Paniculide A
Paniculide B: 15-Hydroxy-Paniculide A
Paniculide C: 15-Hydroxy, 8-keto-Paniculide A



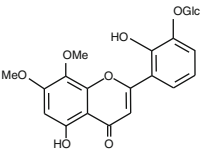
Bisandrographolides A, B and C (diastereoisomers)



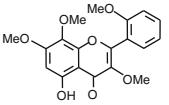
Bisandrographolide D



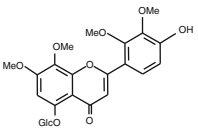
2',3',7,8-Tetramethoxy, 5-hydroxyflavone, 5-O-.beta.-D-glucopyranoside
Synonym: Andrographidin D



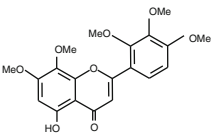
7,8-Di-methoxy-2',3',5-trihydroxyflavone -3'-O-.beta.-D-glucopyranoside
Synonym: Andrographidin B



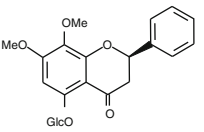
5-Hydroxy-2',3,7,8-tetramethoxyflavone



2',3',7,8-Tetra-methoxy-,4',5-di-hydroxyflavone-5-O-.beta.-D-glucopyranoside
Synonym: Andrographidin F



5-Hydroxy-2',3',4',7,8-pentamethoxyflavone.
Synonym: Serpyllin



5-Hydroxy-7,8-Dimethoxy-(2R)-flavanone, 5-O-.beta.-D-glucopyranoside
R-form

Fig. 4.1 (continued)

These questions are exemplified below: the changes in the levels of mRNA transcripts by andrographolides (I or III) were measured using human cDNA microarrays. Among the altered gene expressions, genes involved in immune and inflammation processes were selectively *downregulated*, such as cytokines and cytokine receptors (TNFSF14, TNF, TNFRSF6, and IL1A), chemokines (CCL8 and CXCL11), JAK/STAT signalling (JAK3 and STAT5A), TLRs family (TLR4 and TLR8), and NF-κB (NFKB1). Andrographolide and neoandrographolide exhibited an anti-inflammatory effect by inhibition of COX-1 in ionophore A23187-induced *isolated human platelets* and inflammatory cytokines in the concentration of about 30.1 μM (10 μg/ml). The underlying mechanisms of

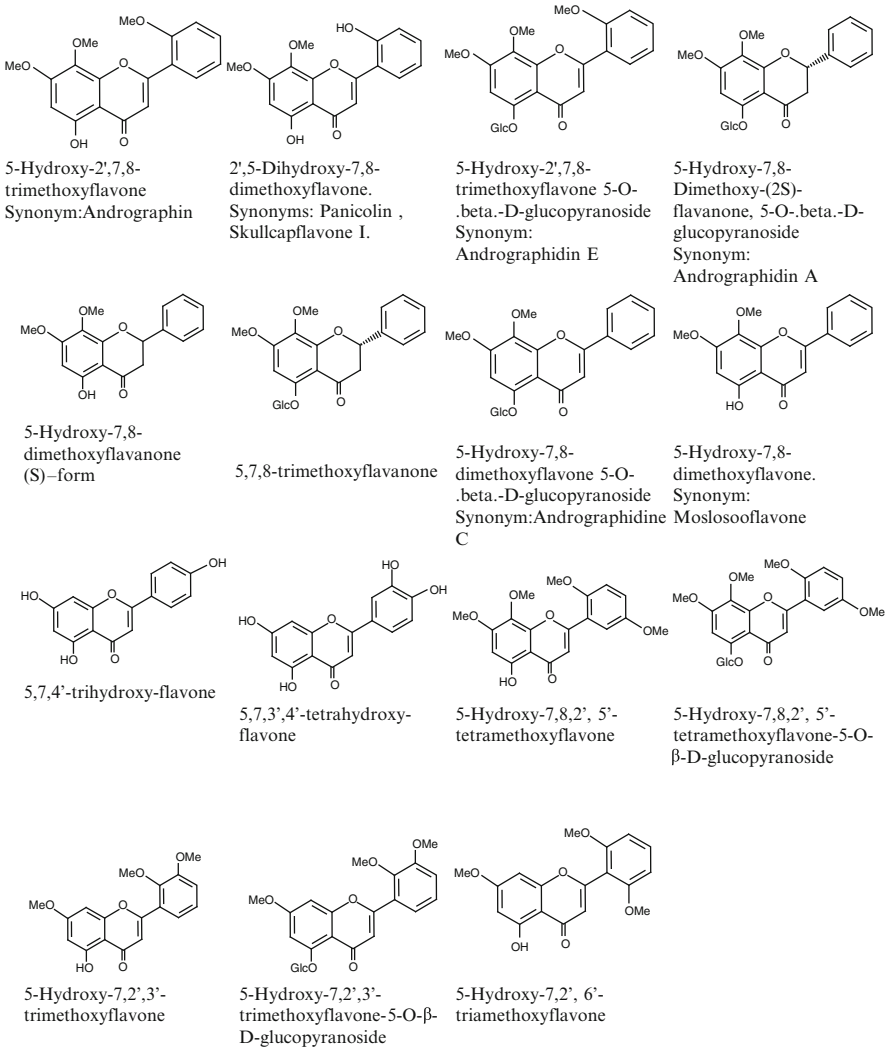


Fig. 4.1 Chemical structures and names of the compounds isolated and identified in Andrographis paniculata

andrographolide may be related to down-expression of genes involved in inflammatory cascade (Parichatikanond et al. 2010). It should be mentioned that this concentration of andrographolide is almost 15-fold higher than the concentration of andrographolide in blood of human subjects after oral administration of Kan Jang tablets (approx. 1.9 μM) (Panossian et al. 2000).

However, in a similar study the microarray analysis of the isolated cellular RNA from peripheral blood mononuclear cells (*a mixed population of T-lymphocytes, B-lymphocytes*, *natural killer cells*, *monocytes*, *macrophages*, *and dendritic cells*)

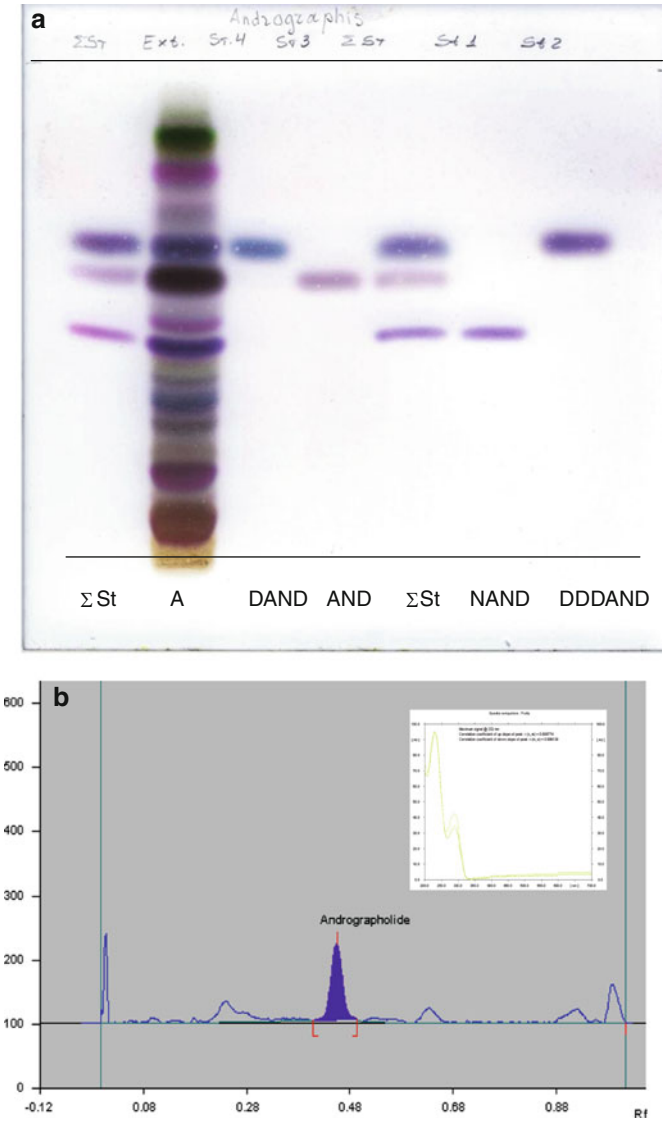


Fig. 4.2 The upper panel (**a**) presents TLC fingerprint of Herba Andrographidis and reference standards: AND—andrographolide, standard (30 µl, $c = 1$ mg/ml); DAND—deoxyandrographolide, standard (30 µl, $c = 1$ mg/ml); DDDAND—14-deoxy-11,12-didehydroandrographolide (30 µl, $c = 1$ mg/ml); NAND—neoandrographolide (30 µl. $c = 0.6$ mg/ml); ΣSt—Reference standards (each of 20 µl); A—Extract of *A. paniculata*, siccum (40 µl, $c = 150$ mg/ml); *Stationary phase:* Silica gel 60 F_{254} precoated TLC plates; *Mobile phase:* ethyl acetate:methanol:water—77:15:8, by volume; *Detection:* in daylight after spraying the plate with vanillin–sulphuric acid reagent. The lower panel (**b**) presents the HPTLC chromatogram of *A. paniculata* herb methanolic extract (detection at 232 nm) and UV spectra of andrographolide (modified from Misra et al. 2009)

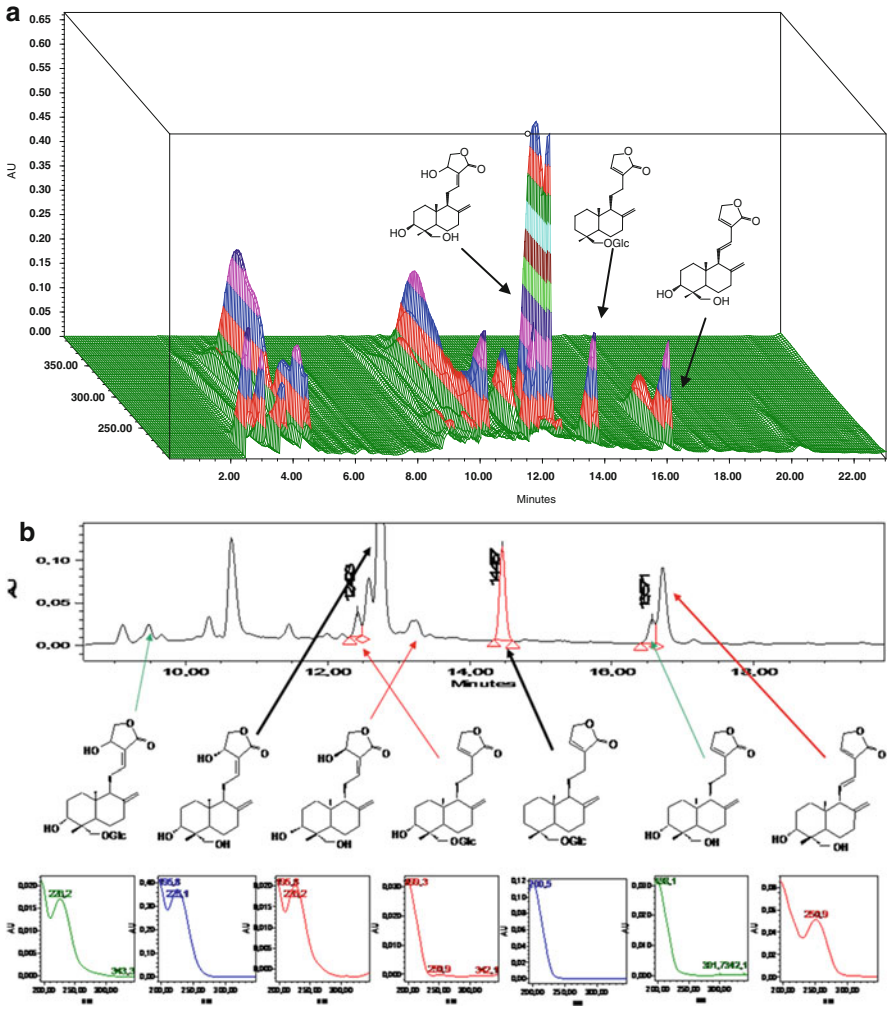


Fig. 4.3 The upper panel (**a**) presents 3D HPLC fingerprint of Kan Jang Andrographis, detection from 200 to 400 nm. Chromatography conditions: stationary phase—octadecyl silicagel Agilent Hypersil ODS (250 × 4.6 mm) 5μm particle size, precolumn LiChrospher RP-18 (4 × 4) 5 μm; mobile phase—gradient (table below) of acetonitrile (solvent B) in 0.2 % phosphoric acid (solvent A); flow rate—1 ml/min; gradient of solvent B in solvent A. The lower panel (**b**) presents the fragment of 2D plot of the same sample detected at 205 nm

showed that Andrographis extract (in the concentrations corresponding to the therapeutic dose in humans) *induced* many *immunostimulatory* genes involved in the genetic expression of cytokines leading to innate and adaptive immune and inflammatory responses, including the induction of prostaglandin-endoperoxide synthase 2 (COX-2) (Denzler et al. 2010).

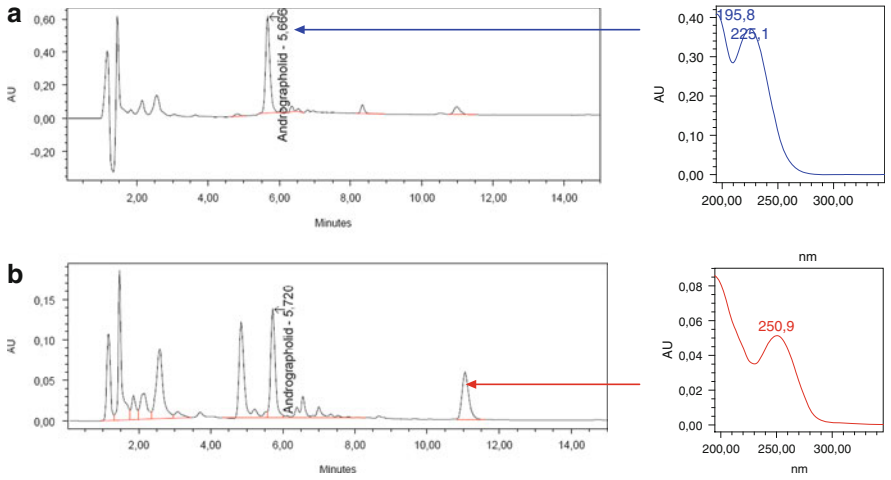


Fig. 4.4 The upper panel (**a**) presents the HPLC chromatogram of *Herba Andrographidis* extract (detection at 225 nm) and online UV spectra of andrographolide. The lower panel (**b**) presents the HPLC chromatogram of the same (detection at 254 nm) and UV spectra of 14-deoxy-11,12-didehydroandrographolide. These two compounds are normally used for quantitative analysis and standardisation of herbal substance, herbal preparations, and herbal medicinal products, as it is described in current Chinese Pharmacopoeia, for instance (Herba Andrographis (Chuanxinlian), 2010). The content of these two andrographolides must be not less than 0.8 % in herbal substance and about 10 % in the dry native ethanolic extract. The recommended daily dose of these two andrographolides is 48–72 mg (assuming the content of 0.8 % in crude drug) that corresponds to 6–9 g of crude drug or 480–720 mg of dry native ethanolic extract (Herba Andrographis (Chuanxinlian), 2005). Normally the content of andrographolide is about 4 % in dried whole plant, 0.8–1.2 % in stem, and 0.5–6 % in leaf extracts (Chao et al. 2010). Misra et al. (2009) found only 1.178 % andrographolide in whole plant powder; however, their method of analysis was not validated for accuracy and the recovery of andrographolide has not been estimated

A proper generation of inflammatory cytokines helps the innate immune response, but an overproduction results in endotoxemia in the acute phase and causes tissue injury, septic shock, and even death. It has been shown that suppression of proinflammatory cytokines may not necessarily benefit survival of "infected" mice. This might be the reason why a low dose of Andrographis increases survival, while the high dose increases mortality (Chao et al. 2009a, b).

Stimulation of the immune system by Andrographis helps the organism to cope with virus/bacteria. Overreaction to viral/bacterial challenge might be harmful and it is necessary to decrease the inflammatory response on the other hand (Fig. 4.5).

The most important *extracellular* mediators of inflammation are histamine, interleukins, prostaglandins, leukotrienes, and platelet-activating factor (PAF) (Naclerio et al. 1988; Proud et al. 1994; Zhu et al. 1997; Pinckard et al. 1988). They cause dilatation and leakage of blood vessels, mucous gland secretion, stimulation of nociceptors (pain sensory receptors), and an activation of sneeze and cough reflexes. The most important *intracellular* mediators of inflammation, involved in the stimulus response coupling, are PAF, nitric oxide (NO), and nuclear

Table 4.3 Pharmacological activity of Andrographis paniculata associated with URT infections

Pharmacological activity	Effect (Reference)
Immunosupporting	Stimulation of immunostimulatory/inflammatory gene expression in blood mononuclear cells (Denzler et al. 2010)
	Modulation of antigen specific and nonspecific immune function by induction of lymphocytes, natural killer cells, macrophage function (Abu-Ghefreh et al. 2009; Iruretagoyena et al. 2005; Ji et al. 2005; Kumar et al. 2004; Naik and Hule 2009; Pinckard et al. 1988; Puri et al. 1993; Sheeja and Kuttan 2007a, b; Xu et al. 2007)
	Modulation of cytokine formation (Abu-Ghefreh et al. 2009; Burgos et al. 2005b; Chandrasekaran et al. 2010, 2011; Iruretagoyena et al. 2005; Ji et al. 2005; Panossian et al. 2002; Parichatikanond et al. 2010), including activation of gamma interferon formation (Panossian et al. 2002)
	Selective downregulation of expressions of the genes involved in immune and inflammation processes, such as cytokines and cytokine receptors (TNFSF14, TNF, TNFRSF6, and IL1A), chemokines (CCL8 and CXCL11), JAK/STAT signalling (JAK3 and STAT5A), TLR family (TLR4 and TLR8), and NF-κB (NFKB1) (Parichatikanond et al. 2010)
Anti-inflammatory/ antiallergy	Inhibition of endotoxin- or 2,4 dinitrophenol-induced fever, carrageenin-, kaolin-, egg white-, and nystatin-induced paw oedema, croton oil-induced acute exudation, xylene- or acetic acid-induced cutaneous or peritoneal capillary permeability (Deng et al. 1982; Deng 1985; Madav et al. 1995, 1996; Sheeja and Kuttan 2007b; Suebsasana et al. 2009; Sulaiman et al. 2010)
	Restores phenylephrine-induced vasoconstriction (Chuanxinlian Pian (Tabellae Andrographitis) 2010)
	Inhibition of NF-kB (Bao et al. 2009; Chao et al. 2009a, 2010; Jung et al. 2007; Wiart et al. 2005; Yamamoto and Gaynor 2001)
	Inhibition of PAF (Amroyan et al. 1999; Burgos et al. 2005a; Liu et al. 2007b; Thisoda et al. 2006)
	Inhibition of iNOS (Batkhuu et al. 2002; Chandrasekaran et al. 2010; Chao et al. 2009b; Chiou et al. 2000; Chuanxinlian Pian (Tabellae Andrographitis) 2010; Liu et al. 2007a, b
	Inhibition of oxidative stress (Sheeja et al. 2006; Shen et al. 2000) inhibiting inflammatory (PGE_2 and TXB_2) and allergic (LTB_4) mediators (Chandrasekaran et al. 2010; Chao et al. 2009b; Liu et al. 2007a)
	Inhibition of COX-1 and COX-2 in human blood (Parichatikanond et al. 2010)
	Downregulation of MAPK pathways (Liu et al. 2008)
	Analgesic and antipyretic effects (Suebsasana et al. 2009; Sulaiman et al. 2010)
Antiviral/antibacterial	Effective against influenza A Virus in vivo and in vitro (Chen et al. 2009; Ko et al. 2006)
	Inhibition of pro-protein convertases (Basak et al. 1999)
	Inhibition of bacterial growth of both Gram-negative and Gram-positive bacteria (Mishra et al. 2009; Singha et al. 2003; Zaidan et al. 2005)

factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulates genes responsible for both the innate and adaptive immunity.

Almost all of these mediators, involved in the inflammatory response to viral/bacterial challenge, are molecular targets of several constituents of Andrographis (Table 4.3).

NF-κB activation has been implicated in many human diseases, including rhinovirus-induced infectious diseases and common cold (Zhu et al. 1997; Pande and Ramos 2005). Numerous inhibitors of NF-κB pathways (Fig. 4.5) as well as specific inhibitors which could directly prevent NF-κB-DNA binding have been found during last decades (Pande and Ramos 2005; Yamamoto and Gaynor 2001).

The inhibition of NF-κB transcriptional activity by andrographolide has been demonstrated in many in vivo experiments on mice and in isolated cell cultures—human cell lines of transformed embryonic kidney 293 cells, promyeloid cells, mouse fibroblast cells, endothelial cells, neutrophils, peritoneal macrophages, lymphocytes, and thoracic lymph node cells (Xia et al. 2004; Hidalgo et al. 2005; Bao et al. 2009; Chao et al. 2009a, b, 2010).

It has been shown that andrographolide attenuated the TNF-induced binding of p50 subunit of NF-κB to ^{32}P-labelled NF-κB oligonucleotide in a dose-dependent manner ($IC_{50} = 15$ μM). It was demonstrated that andrographolide inhibits NF-κB activation by blocking the binding of NF-κB oligonucleotide to nuclear proteins, through interaction with a cysteine residue of NF-κB protein (Fig. 4.5). Andrographolide–p50 protein complex was isolated and analysed. The mass spectrum of p50 showed a peak of $m/z = 40{,}561$, which corresponded to the calculated molecular mass of p50, while the mass spectrum of the covalent adduct had peak of $m/z = 40{,}881$ (Xia et al. 2004). As cysteine 62 residue is located in the loop L1 (the DNA-binding pocket) of p50, it was suggested that covalent conjugation of Andrographolide to this residue will abrogate its binding activity for NF-κB oligonucleotide (Xia et al. 2004).

The results of this study are consistent with other publications (Hidalgo et al. 2005; Bao et al. 2009; Chao et al. 2009a, b, 2010) with one exception. *Xia et al. reported that andrographolide fails to inhibit IkB degradation, p50 and p65 nuclear translocation, and cell growth* (Xia et al. 2004), while Bao et al. (2009) demonstrated that Andrographolide attenuates allergic airway inflammation by potentially inhibiting the NF-κB pathway at the level of inhibitory kB (IkB) kinase-b (IKKb) activation (Fig. 4.5). Andrographolide markedly suppressed the OVA-induced iNOS expression in the lungs, which may be due to the direct inhibition of NF-κB pathway (Fig. 4.5) (Bao et al. 2009).

Activated macrophages normally express inducible isoforms of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and produce excessive amounts of nitric oxide (NO) and prostaglandin E(2) (PGE(2)), which play key roles in the processes of inflammation. Andrographis and andrographolide significantly inhibit proinflammatory (NO, IL-1 beta, and IL-6), inflammatory (PGE_2 and TXB_2), and allergic (LTB_4, but not histamine) mediators in blood of human subjects (Chandrasekaran et al. 2010, 2011; Chao et al. 2009a, b).

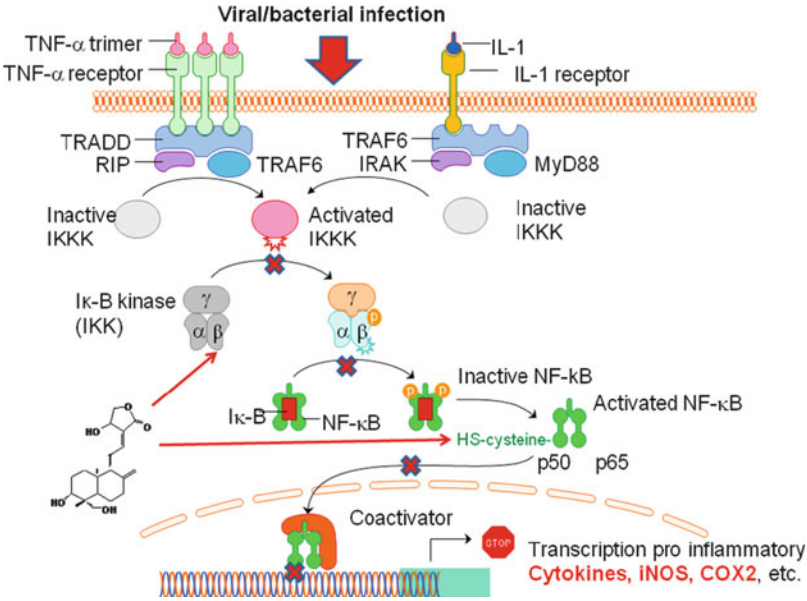


Fig. 4.5 Effects of andrographolide on NF-kB-mediated inhibition of iNOS, COX2, and proinflammatory cytokines. The subunits of p50 (NFkB1) and p65 (RelA) form transcriptionally active dimer. In resting cells, p50/p60 dimers are bound to an inhibitory protein IkBs and retained in an inactive form in the cytoplasm. Upon stimulation, IkB is phosphorylated mainly by the IkB kinases (IKKs), and degraded. The degradation of IkB proteins exposes the nuclear localisation signal, resulting in nuclear shuttling of the p50/p65 heterodimer for the transcription of multiple targeting genes, such as those for cytokines (IL-1, IL-2, IL-6, IL-8, and TNF-α), cell adhesion molecules (E-selectin, ICAM-1, and VCAM-1), cyclooxygenase II, inducible NO synthase, immunoreceptors, hematopoietic growth factors, growth factor receptors, and several cell survival genes. The specific recognition sequence (Arg, Tyr, Val, $Cys^{62\ position}$, Glu, Gly, Pro, Ser, His, Gly, Gly, Leu, Pro—amino acid residues 59–71) in p50 subunit is important in interacting with the kB site DNA. By binding to the kB site DNA, NF-κB (p50 subunit) can initiate transcription. Andrographolide covalently binds to the reduced cysteine 62 residue of the p50 subunit of NF-κB and therefore prevents NF-kB binding to DNA (Xia et al. 2004; Hidalgo et al. 2005). Additionally, andrographolide blocks tumour necrosis factor-a-induced phosphorylation of inhibitory kB kinase-b, downstream inhibitory kBa degradation, p65 subunit of NF-kB phosphorylation, p65 nuclear translocation, and DNA-binding activity. Similarly, andrographolide blocks p65 nuclear translocation and DNA-binding activity in the nuclear extracts from lung tissues of OVA-challenged mice (Bao et al. 2009)

It has been shown that anti-inflammatory and antiallergic effect of Andrographolide is not associated with the effect on metabolism of arachidonic acid into prostaglandins and leukotrienes in isolated human PMNL, stimulated by ionophore A23187 (Amroyan et al. 1999). In the same study it has been found that both andrographolide and *A. paniculata* SHA-10 extract, standardised for andrographolide content of 3.6 %, are PAF antagonists. *Platelet aggregation factor (PAF) is known as a potent mediator of inflammation and an important mediator of bronchocon striction. PAF antagonists have the potential for implication in acute*

inflammation and anaphylaxis (Zimmerman et al. 2002). It was shown that both SHR-10 and andrographolide inhibit PAF-induced human blood platelet aggregation in a dose-dependent manner ($IC_{50} \sim 5$ μM) (Amroyan et al. 1999). Pharmacokinetic studies of Kan Jang tablets in humans show that steady state plasma concentration of andrographolide for multiple doses of Kan Jang fixed combination (after the normal therapeutic dose regimen, 3 × 4 tablets/day, about 1 mg AND/kg/day) was approximately 660 ng/ml (approx. 1.9 μM) (Panossian et al. 2000). That is enough to reveal any anti-PAF effect, particularly after drug uptake, when the concentration of AND in blood is about 1,342 ng/ml (approx. 3.8 μM). It has been shown that 14-deoxyandrographolide (II) is a PAF antagonist as well, reducing PAF-induced calcium flux in isolated bovine neutrophils in a dose-dependent manner. In addition DAND (II) reduced the extracellular acidification rate, the intracellular alkalinisation, and the tyrosine phosphorylation of a 44 kDa protein corresponding to the MAPK (Burgos et al. 2005b).

Synergistic effect of different ingredients in Andrographis and the Kan Jang combination has been demonstrated (Panossian et al. 2002; Puri et al. 1993).

4.4 Clinical Efficacy of Andrographis in the Treatment of URT Infections

Two randomised clinical trials (Table 4.4) and one nonrandomised trial (Table 4.5), related to *A. paniculata* in individual preparations, were obtained in the literature search using MEDLINE, PubMed, Iowa Drug Information System, International Pharmaceutical Abstracts, the Cochrane Library Database, MICROMEDEX, and the Natural Medicines Comprehensive Database. The search also isolated ten randomised clinical trials and two nonrandomised trials study examining *Kan Jang fixed combination of A. paniculata* and *E. senticosus* (Table 4.6).

4.4.1 Indications and Endpoints

"Common cold" and "flu" are syndromes of familiar symptoms caused by viral infection of the upper respiratory tract. The common cold syndrome has been defined as a short and mild illness with early symptoms of headache, sneezing, chilliness, sore throat and later symptoms of nasal discharge (rhinorrhea), nasal obstruction (congestion), cough, and malaise. Generally the severity of symptoms increases rapidly, peaking 2–3 days after infection, with a mean duration of symptoms of 7–10 days. The *treatment* of common cold was investigated in six trials. Common cold was diagnosed using standard methods in five of these trials. Five of the trials were placebo controlled. Four of the trials show that Kan Jang significantly improves the symptoms of common cold. Two of the treatment trials

show effect on the following objective and semi-objective endpoints: "Days of sick leave after randomisation", "Subjects requiring additional treatment at termination", "Body temperature", and "Number of Lymphocytes".

The seventh study is a placebo-controlled trial showing a significantly better effect of Kan Jang in the *prevention* of common cold. The trial shows effect on two objective endpoints: "Incidence of common colds per month" and "No. subjects with common cold over 3 months".

4.4.2 Efficacy and Safety APE

Results of two randomised studies of *A. paniculata* extract are shown in Table 4.4, while summary on nonrandomised study is in Table 4.5.

A randomised, double-blind placebo-controlled clinical study of an extract of *A. paniculata* (200 mg/day, 60 mg of andrographolide for 5 days) in 223 patients with uncomplicated URT infections (Saxena et al. 2010). The self-assessment of cough, expectoration, nasal discharge, headache, fever, soar throat, earache, malaise/fatigue, and sleep disturbance has been performed by Visual Analogue Scale. Comparing mean between both groups, all symptoms at day 1 and day 3 were found to be the same, while at day 5 all symptoms, except earache, in APE-treated group improved significantly ($p < 0.05$) than placebo group. On between groups analysis, APE group showed significant reduction ($p < 0.05$) in overall symptom scores as compared to placebo group at the day 5. The comparison of overall efficacy of APE over placebo was found to be significant ($p < 0.05$) and it was 2.1 times (52.7 %) higher than placebo. The findings of this study revealed that APE was effective in reducing symptoms of upper respiratory tract infection (Saxena et al. 2010). Subjects self-evaluated adverse effects and were examined by a physician at the start of the study and on day 5. The *A. paniculata* group had total of six patients suffering from minor adverse effects, one patient each with vomiting, epistaxis, and urticaria and three with diarrhoea. Of the three with diarrhoea, in addition one each had nausea or lethargy. The placebo group had three patients with adverse effects, one each with diarrhoea, vomiting (both mild in severity), and moderate rigour. The adverse effects between two groups were found to be the same ($Z = 0.63$, $p < 0.05$). In eight patients the effects were mild and isolated, and in one patient the effect was moderate and isolated. Except for vomiting (patient in *A. paniculata* group) and urticaria, all other effects stopped spontaneously without any medical aid.

A randomised, double-blind, controlled trial was conducted in patients with pharyngotonsillitis and showed no difference between the highest *A. paniculata* dose (6 g dry herb/day, 360 mg/day of total andrographolides) and the active comparator (paracetamol), whereas the lower dosed *A. paniculata* (3 g dry herb/day, 180 mg/day of total andrographolides) was less efficacious. All participants were treated for 7 days (Thamlikitkul et al., 1991). Concomitant use of antibiotic, antihistamine, and/or decongestant was recorded in ~47 subjects from each

Table 4.4 Randomised clinical studies of *A. paniculata* extract, APE

Study [investigator, coordinating centre (s)]	Design	Number of subjects with age and sex	Diagnosis + criteria for inclusion	Duration of treatment	Test product Dosage regiment Route of administration	Reference therapy Dose regimen Route of administration	Criteria for evaluation	Results (efficacy)	Adverse reactions	Quality Poor 0–2 Good 3,4 Excellent 5
Saxena and 10 other investigators from four hospitals in India, 2010	Outpatient, double-blind, active-controlled, randomised, two-armed parallel group.	No pts: 223 Male 143 Female 80 Age (range: 18–60)	Symptoms of the common cold (cough, expectoration, running nose, headache, fever, sore throat, earache, malaise/fatigue, and sleep disturbance)	5 days	KalmCold capsules containing 100 mg of *Andrographis paniculata* leaves methanolic extract with andrographolide content of 31.3 % Dose: 62 mg of andrographolide (2 capsules) per day Mode: oral	Placebo capsules Mode: oral	Nine symptoms on a visual analogue scale on days 1, 3, and 5 of treatment	KalmCold was 52.7 % more effective (based on the effect size) than placebo at treating the common cold	Vomiting ($n = 1$), epistaxis (nosebleed, $n = 1$), urticaria (itchy rash, $n = 1$), and diarrhoea ($n = 3$). No difference between the three groups	5
Tanagkul 10 investigators six community hospitals, Thailand, 1991	Outpatient, double-blind, active-controlled, randomised, three-armed parallel group.	No pts: 152 Male 77 Female 75 Age: 28.9 (range: 12–69)	Pharyngotonsillitis Sore throat with history of fever and temp. $\geq$ 37.8 °C	7 days	2 different strengths: capsules containing 250/500 mg dried AP leaves, 6 % of andrographolides Dose: 180 and 360 mg of total andrographolides ($3 \times 4 = 12$ capsules) per day Mode: oral	Capsules containing 375 mg paracetamol Dose: 3 capsules 4 times per day = 12 capsules Mode: oral	1. Fever day 3 2. Fever day 7 3. Sore throat day 3 4. Sore throat day 7	1. 500 mg Ap and 375 mg paracetamol equal; 250 mg Ap worse ($p < 0.001$) 2. No difference ($p = 0.16$) 3. 500 mg Ap and 375 mg paracetamol equal; 250 mg Ap worse ($p < 0.001$) 4. No difference ($p = 0.49$)	No difference (23 % vs. 21 % vs. 18 %) between the three groups (500 mg Ap vs. 250 mg Ap vs. 375 mg paracetamol)	2

Table 4.5 Nonrandomised clinical studies of *A. paniculata* extract, APE

Study [investigator, coordinating centre(s)]	Design	Number of subjects with age and sex	Diagnosis + criteria for inclusion	Duration of treatment	Test product Dosage regiment Route of administration	Reference therapy Dose regimen Route of administration	Criteria for evaluation	Results (efficacy)	Adverse reactions	Quality Poor 0–2 Good 3,4 Excellent 5
Hancke Regional hospital Valdivia, Chile, 1999	Outpatient, double-blind, placebo-controlled, two-armed parallel group	No pts: 61 Male 32 Female 29 Age: 32.4 ± 1.0 vs. 31.9 ± 0.8	Common cold symptoms	3 days	KJ tablets (with 100 mg of standardised APE 70 % ethanolic extract, containing 4 mg of andrographolides Dose: 4 tablets × 3 times per day = 1200 mg APE extract corresponding to 48 mg of andrographolides Mode: oral	Placebo tablets In 28 patients Mode: oral	Sum of clinical symptom scores Tiredness Shivering Sore throat Muscular ache Rhinitis Sinus pains and headaches Lymphatic swellings	0.94 vs. 1.71 ($p < 0.001$) 1.67 vs. 1.15 ($p < 0.001$) 1.46 vs. 0.73 ($p < 0.01$) 1.25 vs. 0.76 ($p < 0.05$) 1.6 vs. 0.82 ($p < 0.001$) 1.6 vs. 1.32 ($p > 0.05$) 1.57 vs. 1.33 ($p > 0.05$) 1.21 vs. 0.88 ($p > 0.05$)	No adverse events	3

treatment group. Several adverse effects were experienced by participants. Eleven participants in 360 mg/day group, ten participants in the 180 mg/day group, and nine participants in the paracetamol treatment group reported adverse effects. These adverse effects consisted of nausea, vomiting, gastrointestinal discomfort, dizziness, drowsiness, and malaise (numbers not reported for each individual effect). Additionally, four participants in the 360 mg of andrographolides/day group, two participants in the 180 mg andrographolides/day group, and four participants in the paracetamol treatment group were lost to follow-up. There were no differences in the incidence of side effects across the treatment groups (~20 %) and events were described as mild and possibly related to concomitant use of antibiotics, antihistamines, or decongestants.

A nonrandomised, double-blind, placebo-controlled trial performed by Hancke and associates enrolled 59 participants of both sexes between the ages of 18 and 60 years old (Hancke et al. 1995). Thirty-three of these participants received 1,200 mg *A. paniculata* extract (greater than or equal to 48 mg/day of andrographolides) for 5 days. The authors assessed the treatment outcome using the CAS (Visual Analogue Score, total sum score). The clinical objective findings included rhinitis, sinus pain and headache, and inflammation of lymph nodes. In addition, symptoms recorded and evaluated by the patient included tiredness, strength of disease, sweating/shivering, sore throat, muscular pain, and headaches. The outcome was evaluated on day 3 or 4 after the start of treatment. Statistically significant reduction in five of the eight clinical common cold symptoms assessed (strength of disease, tiredness, shivering, sore throat, muscular aches) was seen in comparison with placebo at day 5. No difference between groups was observed for rhinitis, sinus pains, and headaches. There was no significant difference in lymphatic swellings. If the overall signs and symptoms were accumulated, the total sum showed a diminution of the symptoms which is more intense in the Andrographis group. Safety monitoring at baseline and end of study included analysis of the CBC; Kidney and liver function tests; and creatinine, alkaline phosphatase, SGOT (aspartate aminotransferase), SGPT (alanine aminotransferase), electrolytes (sodium, potassium, calcium), and urine analysis. No laboratory abnormalities or adverse effects were reported.

A recently published article in Chinese describes results of the multicenter randomised clinical trial of andrographolide—the active principle from Andrographis in 478 patients with URT infection (Chang et al. 2008). The efficacy of treatment was evaluated in accordance with "Guidance for Clinical Research on New Medicine of TCM" that defined the criteria for evaluating the treatment efficiency for acute upper respiratory disease. There were four levels for the treatment efficiency of Acute Upper Respiratory Disease:

- Completely healed—within 3 days of treatment, body temperature has returned to normal, Acute Respiratory Disease had disappeared, and symptoms have disappeared by greater than 95 %.

Table 4.6 Randomised clinical studies of Kan Jang

Study [investigator, coordinating centre(s)]	Design	Number of subjects with age and sex	Diagnosis + criteria for inclusion	Duration of treatment	Test product Dosage regiment Route of administration	Criteria for evaluation Primary outcome	Results (efficacy)	Adverse reactions	Quality Poor 0–2 Good 3,4 Excellent 5
Caceres Regional hospital Valdivia, Chile, 1999	Outpatient, double-blind, placebo-controlled, randomised, two-armed parallel group	No pts: 61 Male 32 Female 29 Age: 32.4 ± 1.0 vs. 31.9 ± 0.8	Common cold within the last 3 days	4 days	KJT with standardised Ap 70 % ethanolic extract Dose: 4 × 3 tablets (total—60 mg of andrographolides) per day Mode: oral	1. Sum of clinical symptom scores 2. Sum of patient's symptom scores 3. The same scores but evaluated individually	1. 0.9 vs. 1.5 ($p < 0.01$) 2. 1.2 vs. 1.4 ($p < 0.05$) 3. All eight symptoms in favour of verum. p-values from 0.001 to 0.22. Five were significantly different	No adverse events	4
Melchior Hallehälsan Center Ulricehamn, Sweden, 1992 (1996)	Single-centre, outpatient, double-blind, placebo-controlled, total randomised, two-armed parallel group	No pts: 50	Common cold within the last 3 days. Diagnosed according to Braunwald et al.	5 days	KJT with standardised Ap ethanolic extract and As extract Dose: 4 × 3 tablets (total—60 mg of andrographolides) per day Mode: oral	1. Days of sick leave after randomisation 2. Pt totally recovered 3. Symptom relief (disease easier than normal)	1. 0.21 vs. 0.96 ($p < 0.05$) 2. 68 % vs. 36 % ($p < 0.05$) 3. 55 % vs. 19 % ($p < 0.05$)	No adverse events reported	3
Caceres General praxis Pirque, Chile, 1997	Outpatient, double-blind, placebo-controlled, randomised, two-armed parallel group	No pts: 107 Male 57 Female 50 Age: 18.4	Healthy subjects without a common cold within the last 2 weeks	3 months 4 days	KJT with standardised Ap 70 % ethanolic extract Dose: 2 tablets per day, 5 days per week Mode: oral	1. Incidence of common colds per month (prevention) 2. No. subjects with common cold over the 3 months	1. Month 1: 9 % vs. 17 % ($p = 0.36$) Month 2: 13 % vs. 28 % ($p = 0.05$) Month 3: 13 % vs. 30 % ($p = 0.03$) 2. 30 % vs. 62 % ($p = 0.001$)	No adverse events reported	4 5

(continued)

Table 4.6 (continued)

Study [investigator, coordinating centre(s)]	Design	Number of subjects with age and sex	Diagnosis + criteria for inclusion	Duration of treatment	Test product Dosage regiment Route of administration	Criteria for evaluation Primary outcome	Results (efficacy)	Adverse reactions	Quality Poor 0–2 Good 3,4 Excellent 5
Caceres Regional hospital Valdivia, Chile, 1999	Outpatient, double-blind, placebo-controlled, randomised, two-armed parallel group	No pts: 158 Male 72 Female 85 Age: 25–50	Subjects with common cold		KJT tablets with standardised Ap 70 % ethanolic extract Dose: 4 × 3 tablets (total—60 mg of andrographolides) per day Mode: oral	1. Clinical symptom scores and patient's symptom scores evaluated individually	1. All eight symptom scores were in favour of verum on days 2 and 4. On day 2, four scores and on day 4, all scores were significantly different	No adverse events reported	
Melchior Hallehälsan Center Ulricehamn Sweden, 2000	Single-centre, outpatient, double-blind, placebo-controlled, total randomised, two-armed parallel group	No pts: 46 Males 17 Females 29 Age: 40.9 (range 30–52)	Uncomplicated upper respiratory tract infection	4–6 days	KJT tablets with standardised Ap 70 % ethanolic extract and As extract Dose: 4 × 3 tablets (total—60 mg of andrographolides) per day Mode: oral	1. Reduction in Patient's symptom score 2. Physicians diagnosis score 3. Symptom relief (nine symptoms)	1. 5.4 ± 3.7 vs. 3.8 ± 4.7 ($p = 0.08$) 2. Incomplete reporting of the measured values 3. Six directionally in favour of verum (one significantly $p = 0.03$), two tied, and one in favour of placebo.	No adverse events seen	5
Ostrovskij Interstam Moscow, Russia, 2000	Single-centre, outpatient, double-blind, placebo-controlled, randomised, two-armed parallel group	No pts: 180 of which 179 completed one drop-out	Uncomplicated upper respiratory tract infection started within the last 36 h	3 days	KJT tablets with standardised Ap extract and As extract Dose: 4 × 3 tablets (total—60 mg of andrographolides)	1. Reduction in Patient's symptom score 2. Physicians diagnosis score 3. Symptom relief (10 symptoms) 4. Subjects requiring	1. 6.3 ± 4.2 vs. 4.0 ± 4.4 ($p = 0.0006$) 2. 5.5 ± 4.1 vs. 3.6 ± 4.2 ($p = 0.003$) 3. Nine symptoms were directionally	1 person on verum received unpleasant sensations in the chest and intensified	5

					per day Mode: oral	additional treatment at termination 5. Blood parameters 6. Body temperature	favourable (four significantly so) for the verum. The last symptom showed no difference between treatments 4. 14 vs. 44 ($p = 0.01$) 5. Slight lymphocytosis (60 %) in verum group 6. Improvement ($p = 0.001$)	headache. The treatment was ended	
Shukurian "Ercebuni" Medical Center Yerevan, Armenia, 2002	Single-centre, outpatient, double-blind, placebo-controlled, total randomised, two-armed parallel group	No pts: 200 of which 185 completed the study Age: 15–64	Common cold, acute or chronic sinusitis	5 days	KJT tablets with standardised Ap extract and As extract Dose: 4 × 3 tablets (total—60 mg of andrographolides) per day Mode: oral	1. Reduction in complaint index for nine inflammatory symptoms (symptom ranged from 0 to 5 points) 2. Recovery	1. Statistically significant reduction for verum vs. placebo in total score ($p < 0.001$) 2. Intensified most in headache, nasal, and throat symptoms and general malaise ($p < 0.001$) moderate change in cough and eye symptoms ($p < 0.02$)	Few cases of minor adverse effects seen	3

(continued)

Table 4.6 (continued)

Study [investigator, coordinating centre(s)]	Design	Number of subjects with age and sex	Diagnosis + criteria for inclusion	Duration of treatment	Test product Dosage regiment Route of administration	Reference therapy Dose regimen Route of administration	Criteria for evaluation Primary outcome	Results (efficacy)	Adverse reactions	Quality Poor 0–2 Good 3,4 Excellent 5
Spasov Research Institute Pharmacology Medical Academy Volgograd, Russia, 2004	Single-centre, children—clinic, three-armed parallel group	No. pts: 129 completed, 133 included but 4 drop-outs Boys: 68 Girls: 65 Age: 6.9 (range: 6.7–7)	Uncomplicated upper respiratory tract (URT) infection started within the last 24 h	10 days	KJT tablets with standardised Ap extract and As extracts = SHA-10 Dose: 2 × 3 tabl. per day Mode: oral	Immunal drops Echinacea purpurea pressed juice IM 20 % ethanol Dose: 10 × 3 drops per day Mode: oral Traditional treatment: lavish warm drink, throat gargles, antiseptic nose drops, paracetamol 500 mg × 3 per day (only in case of fever and severe headache)	1. Reduction in patient's symptom score 2. Reduction in physician evaluation 3. 10 individual symptoms 4. Change in immuno-logical parameters IL-8, IgA, IgG, and secretory IgA in nasal secretion 5. Serum/blood parameters	1. KJT-treated pt recovered quicker than the ref. therapy both at second and third examination ($p < 0.001$) 2. KJT had an early, pronounced effect on symptoms in oral cavity and nasal symptoms 3. Patient score gave quicker symptom relief with KJT ($p < 0.001$) 4. Quicker mucociliary clearance with KJT. IL-8 and IgG increased in the other two treatments 5. KJT and IM were significantly different from trad. treatment on leukocyte and sediment	Well tolerated but 4 drop-outs 1 pt on KJT complication of URT symptoms 3 pt on reference therapy due to complication of URT symptoms and one type I allergy (urticaria)	2

Kulichenko outpatient clinic, Volgograd, Russia, 2003	Outpatient, randomised, two-armed parallel group	No pts: 66 female and male Age: 20–63 35 pt on KJT 31 pt on symptomatic control treatment	Subjects with influenza	5 days	KJT tablets with standardised AP extract and AS extract SHA-10 Dose: 3 × 3 tabl. per day Mode: oral	Paracetamol, 500 mg Dose: 2 × 3 tablets per day and for most patients an antiviral agent amantadine with 1 g ascorbic acid daily Mode: oral	Reduction in patient's symptom score day 1, 3, and 5 Patient self-evaluation: sick leave (days) body temp. (°C) general malaise (0–3) headache (0–3) rhinitis (nasal problem 0–3) pain in throat (0–3) cough (0–3) conjunctivitis (eye problem 0–3)	1. 600 mg AP and 2. 500 mg paracetamol: influenza A virus confirmed in 26 of 66 sick-leave duration KJT 7.2 days Control 9.8 days ($p < 0.001$) body temp. on day 3 KJT 36.9 °C Control 37.4 °C ($p < 0.001$) Headache on day 3 ($p < 0.001$) Pain in throat and general malaise on day 5 ($p < 0.001$) Cough, rhinitis on day 5 ($p < 0.01$)	The CRF asked about adverse and side effects, but no one was recorded	2

- Excellent—within 3 days of treatment, body temperature had returned to normal, Acute Upper Respiratory Disease had mostly disappeared, and symptoms have only disappeared by 70–95 %.
- Effective—within 3 days body temperature was lower than before, most of the Acute Upper Respiratory disease had disappeared, and symptoms had reduced by 30–70 %.
- Ineffective—within 3 days of treatment, body temperature had not lowered or has risen, Acute Upper Respiratory disease had not disappeared or has gotten worse, and symptoms have reduced by less than 30 %.

Both groups of patients were treated with andrographolide—in tablets and in drop pills (no placebo control) in the daily dose of 150 mg three times a day (450 mg/day). It is reported that efficacy of treatment was the same in both groups with a complete recovery ratio of 44.55 % and 42.57 % (Chang et al. 2008). Quality score of this study in Jadad scale is 2 of 5 (max). Actually it was not a blind study in respect of study medication, which was easy to distinguish—tablets containing 50 mg andrographolide (three tablet three times/day) and drop pills containing 150 mg of andrographolide (one pill three times/day). No placebo group was studied in order to evaluate the effect of andrographolide.

WHO recognises the use of *A. paniculata* in symptomatic treatment of upper respiratory infections, bronchitis, and pharyngotonsillitis (Herba Andrographidis 2002).

The available clinical studies of Andrographis demonstrate that the preparations are rather safe and well tolerated, when used in the dose of *60 mg of andrographolide for 3–8 consecutive days*.

Interactions of Andrographis with other drugs have never been studied clinically. No case reports on this matter are available as well. Evidence of possible inhibition of P450 activity in vitro models has been demonstrated at high concentrations for andrographis, but this has not been demonstrated with clinically relevant concentrations with the herbs in combination. Below, the results from non-clinical studies are discussed.

Pekthongetal.(2009) reported that aqueous soluble components of A.paniculata extract (APE, extraction solvent - 60% ethanol) and andrographolide (AND) significantly decreased CYP2C11 activity in isolated rat and human hepatic microsomes, after in vivo administration of APE at dose levels of 0.5 g/kg/day (i.e. 5mg/kg/day AND equivalents) and at 2.5 g/kg/day (i.e.25 mg/kg/day AND equivalents) and AND at dose levels of 5 and 25 mg/kg/day. In primary cultures of rat and human hepatocytes, treatment with AND 50 μM and APE-containing 50 μM AND also resulted in significant decreases in CYP2C expression and activity. In addition, in human hepatocytes, treatment with APE and AND 50 μM resulted in a decrease in CYP3A expression and activity. The authors conclude from their experiments that *APE at 50 μM (single dose tested) modulated 2C9 and 3A4 expression and activity*. That is about 45 times higher than the maximal concentration of andrographolide found in blood plasma of humans 393 ng/ml (approx. 1.12 μM) after administration of a single therapeutic dose, equal to 20 mg of

andrographolide (Panossian et al. 2000). This concentration is 25-fold higher than predicted steady state human plasma levels, while doses representative of therapeutic plasma levels (i.e. 2 μM AND) were not tested.

Jarukamjorn et al. (2006) administered aqueous and alcoholic extracts of *A. paniculata* (equivalent to 5 mg/kg/day andrographolide) to mice ($n = 3$–5), orally in distilled water daily for 7, 14, 21, and 30 days. Phenobarbital and methylcholanthrene were administered intraperitoneally and an untreated group was used as control. Total P450 content and ethoxyresorufin *O*-dealkylase (EROD), methoxyresorufin *O*-dealkylase (MROD), and pentoxyresorufin *O*-dealkylase (PROD) activities in hepatic microsomes were then determined.

Total CYP hepatic content was not significantly modified by andrographis treatment (regardless of duration), and *short-term (7 day) treatment with alcoholic andrographis extracts did not change P450 associated activity*. Aqueous extract on the other hand increased PROD activity over short-term treatment (but not other P450 associated activities). *Over longer duration of treatment*, both alcoholic and aqueous andrographis extracts significantly increased murine ethoxyresorufin *O*-dealkylase (EROD) and pentoxyresorufin *O*-dealkylase activities (PROD) (but not methoxyresorufin *O*-dealkylase activity), suggesting inhibitory effect on CYP1A and CYP2B.

It should be mentioned that the results of in vitro studies on isolated cells (which are valuable when the mechanisms behind an estimated pharmacological activity are studied) could not have any clinical significance. Direct pharmacokinetic studies of drugs sensitive to *CYP* enzymes provide more convincing evidence regarding possible drug–herb interaction, when they are applied concomitantly. Such a possibility of an interaction between warfarin and Kan Jang combination of *A. paniculata* and *E. senticosus* has been studied in rats (Hovhannisyan et al. 2006). The authors found that Kan Jang had no significant influence on the pharmacokinetics and pharmacodynamics of warfarin, indicating also that there should be no interactions of Kan Jang with other drugs sensitive to CYP1A2, CYP2C9, and CYP3A4 enzymes (CYP is predominantly used in metabolism of warfarin). Each day for 5 days two groups of rats ($n = 54$) were given an oral dose of Kan Jang (equivalent to 17 mg/kg andrographolide) or water. Sixty minutes after dosing the animals were administered 2 mg/kg warfarin and at 0, 2, 4, 6, 8, 12, 24, 30, and 48 h after six animals from each group were sacrificed, blood samples were taken, and the concentration of warfarin was measured by HPLC. The concentration of warfarin in the Kan Jang treatment group was slightly higher than that in the control group during the first 6–7 h following administration and attained its maximum value earlier. However, the mean C_{max} values, elimination half-life, and mean residence time between the two groups were not statistically significant. Prothrombin time measurements (mean PT_{max} and $AUC_{PT\ 0-\infty}$) were not statistically different between the two groups.

In a similar study extremely high doses of *A. paniculata* extract (1 and 2 g/kg of rats body weight) and its major component, andrographolide (77 and 154 mg/kg), on the pharmacokinetics of theophylline (a substrate of CYP 1A2 enzyme), were investigated (Chien et al. 2010). The results indicated that the clearance of

theophylline was significantly increased and the area under concentration–time curve (AUC) was reduced in both AG and APE pre-treated groups at low-dose theophylline administration (1 mg/kg). The authors suggest that patients who want to use CYP1A2-metabolised drugs such as caffeine and theophylline should be advised of the potential herb–drug interaction (Chien et al. 2010). However, this precaution is based on the assumption that patients have to take Andrographis in the doses 10–20 times higher than it is normally prescribed for the treatment of common cold.

4.4.3 Efficacy and Safety Kan Jang Fixed Combination

Table 4.6 includes the results of randomized clinical trials of Kan Jang fixed combination of *A. paniculata* SHA-10 extract (standardised to 5 mg of andrographolide and deoxyandrographolide) and an extract of *E. senticosus* containing total Eleutherosides B and E (ca. 2 %) has been studied in the daily dose equivalent to 60 mg of andrographolides. Clearly the overall goal is symptom relief and the clinical evidence accumulated indicates that Kan Jang has strong and significant effects on a variety of symptoms of the common cold. In general, significant improvements were seen in *nasal symptoms e.g. secretion (g/day) and congestion, frequency, throat symptoms (soreness), respiratory problems (incl. cough, frequency of cough), headache, general malaise, fatigue, earache, sleep disturbance, and the objective parameter body temperature*. Overall the quality of the six GCP trials (including those trials performed outside EU) corresponds to the level 1a evidence according to EMEA guidelines (Table 4.7).

As is apparent from these studies, Kan Jang (Table 4.6) is significantly more efficacious than the two active ingredients APE (Tables 4.4 and 4.5) and ESE alone (Table 4.1), has an excellent safety profile, and is well tolerated. In one study (Spasov et al. 2004), it was also shown to be superior to Echinacea, a commonly used herbal treatment for the common cold, with a long tradition of use in Europe.

The advantages of Kan Jang fixed combinations include an improvement of the benefit/risk ratio due to potentiation of therapeutic activities of the APE, which results in a level of efficacy above the one achievable by APE or ESE.

The results of three well-performed studies (Jadad's quality score 5)—one on the monodrug APE (Saxena et al. 2010) and two on Kan Jang fixed combination (Melchior et al. 2000; Cáceres et al. 1999)—are compared below (Fig. 4.6).

Normally, the severity of symptoms increases rapidly, reaching maximum 2–3 days after infection, with a mean duration of symptoms of 7–10 days, while, a significant improvements in Kan Jang group of patients was observed already on the 3rd day of the treatment with Kan Jang. Significant difference difference between Kan Jang and placebo groups is observed already on the third day of the treatment in development of *nasal secretion*, *soreness of throat, fatigue, and sleep disturbance* ("intent to treat" statistical analysis; Fig. 4.6). On the fourth day very

significant difference was observed for all tested symptoms including *cough*, *expectoration*, *headache*, *and earache* (Cáceres et al. 1999).

Significant difference between the treatment group and the placebo group in improvement of *sore throat*, *cough, rhinorrhea, muscle soreness and body temperature* was observed on day 3 of treatment in Melchior et al.'s pivotal study (2000).

On the other hand, no statistically significant difference between placebo and APE groups was observed on day 3 (Fig. 4.6; Saxena et al. 2010). Only on the fifth day of the treatment, significant difference between groups was observed (Saxena et al. 2010). Even longer onset in recovery of patients was observed in another study where *A. paniculata* leaves were used (Thamlikitkul et al. 1991).

Retrospective statistical analysis of the results, obtained from haematological study of blood samples, showed significant increase of the number of lymphocytes in Kan Jang group compared with placebo (Fig. 4.7) (Melchior et al. 2000).

This data is in line with the results of an in vitro study on whole blood cell culture where Kan Jang stimulates proliferation of lymphocytes and activates cytokine formation (Panossian et al. 2002). Moreover, this data corresponds with the number of publications where immune stimulating effect of Eleutherococcus activating cytokines in vivo and in vitro has been shown (Table 4.2).

Table 4.8 below shows the associations between various symptoms of common cold (Eccles 2005) and the mediators of inflammation involved in the activation of adaptive and innate immune systems in response to infectious challenge. This adaptive immune response is supported by Kan Jang apparently due to Eleutherococcus, which is known to stimulate immune system via different mechanisms (Table 4.2, Fig. 4.8), including activation of Hsp70 expression (data are not shown). Kan Jang activates adaptive and innate immune system, increases the level of heat shock proteins Hsp70 in blood resulting in accelerated reparation and disposal of damaged or defective proteins, and protects cells from further heat and infection-induced damages.

It can be suggested that the immunosupporting effect of Kan Jang accelerates the recovery of patients and relieves the inflammatory symptoms earlier than Andrographolide alone.

From this one can conclude that, Kan Jang is significantly more efficacious than Andrographis alone, has an excellent safety profile, and is better tolerated, presumably due to adaptogenic effects of Eleutherococcus (Brekhman 1982; Radix Eleutherococci 2002; ESCOP Monographs 2003; Radix et, Rhizoma seu Caulis Acanthopanacis Senticosi (Ciwaujia) 2005). Kan Jang has no adverse events of the conventional NSAID, e.g. liver toxicity of paracetamol or aspirin-induced stomach ulcerations. Moreover, it might be speculated that hepatoprotective and anti-ulcerogenic activity of *A. paniculata* can be beneficial in reduction of these adverse events if it will be used concomitantly with the conventional NSAID.

Table 4.7 Evidence level of efficacy of KJT, APE-10, APE, and ESE in URT infectious diseases accordance according to EMEA classification system

Degree of recommendation	Evidence level	Reference	Preparation	Evidence type
A	Ia	Poolsup et al. (2004)	KJ and APE[a]	Meta-analyses of randomised and controlled studies
	Ib	Cáceres et al. (1997); Cáceres et al. (1999); Kulichenko et al. (2003); Melchior et al., (1996/7); Melchior et al. (2000); Spasov et al. (2004); Shakhova et al. (2003); Saxena et al. (2010); Thamlikitkul et al. (1991)	KJ KJ KJ KJ KJ KJT APE + ESE APE APE	Evidence from at least one randomised study with control
B	IIa	Shadrin et al. (1986); Barkan et al. (1980); Hancke et al. (1995); Gabrielian et al. (2002)	ESE ESE APE-10 KJ	Evidence from at least one well-performed study with control group
	IIb	Kim (1992)	ESE	Evidence from at least one well-performed quasi-experimental study
	III	Gagarinova et al. (1995); Kalashnikov (1986); Kovalenko and Vereshchagin (1994); Kozlov et al., (1984)	ESE ESE ESE ESE	Evidence from well-performed non-experimental descriptive studies as well as comparative studies, correlation studies, and case studies
C	IV	Chang and But (1987); Farnsworth et al. (1985); Herba Andrographidis (2002); Coon and Ernst (2004); Kligler et al. (2006)	APE ESE APE KJ and APE KJ and APE	Evidence from Expert committee reports or appraisals and/or clinical experiences by prominent authorities

[a]Actually, the preparation used in Cáceres et al. (1997, 1999) and Melchior et al. (1996) studies is Kan Jang fixed combination, but not a monodrug (APE) as it can be concluded from the certificates of analysis of preparations used in these studies

4.4.4 Systematic Reviews and Meta-Analysis

Overall, three systematic reviews and meta-analysis of clinical trials of Andrographis and Kan Jang fixed combination of Andrographis and Eleutherococcus

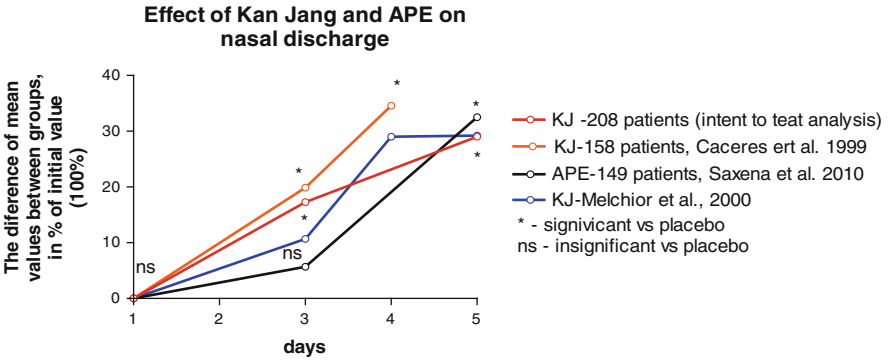


Fig. 4.6 Lymphocytes count in the peripheral blood of patients at the first and third day of common cold treatment with Kan Jang (KJ1—day 1, KJ3—day 3) or placebo (P1—day 1, P3—day 3). The difference in lymphocytes counts between days 1 and 3 in KanJang group (KJ1–KJ3) is significantly higher than in placebo group (P1–P3)

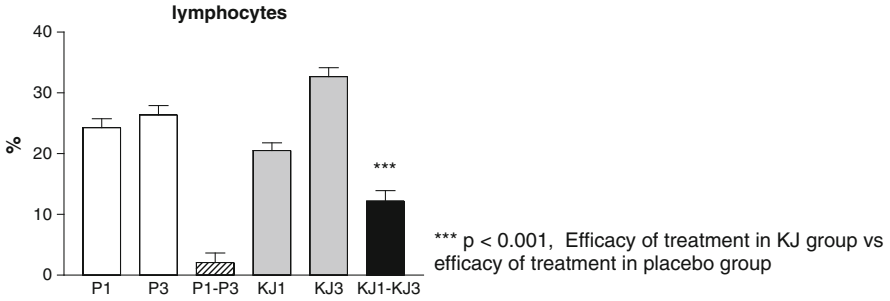


Fig. 4.7 Effect of Kan Jang and APE on nasal discharge; between group difference of mean values, in % to initial value (100 %)

in URT infectious diseases were published (Kligler et al. 2006; Poolsup et al. 2004; Coon and Ernst 2004). Seven double-blind, controlled trials ($n = 896$) were selected by Coon and Ernst, for evaluation of efficacy. All trials scored at least 3, out of a maximum of 5, for methodological quality on the Jadad scale. It was concluded that *A. paniculata* is superior to placebo in alleviating the subjective symptoms of uncomplicated upper respiratory tract infection (Coon and Ernst 2004).

A total of 433 patients reported in three trials were included in the meta-analysis of clinical trials of Kan Jang. Kan Jang was more effective than placebo. The mean difference was 2.13 points (95 % CI 1.00–3.26 points, $p = 0.0002$) on the symptom severity score. It was concluded that *A. paniculata* extract alone or in combination with *A. senticosus* extract may be more effective than placebo and may be an appropriate alternative treatment of uncomplicated acute upper respiratory tract infection (Poolsup et al. 2004).

Table 4.8 Mediators of inflammation involved in inflammatory symptoms mostly affected by KJ

Cell	Mediator	Target tissue, cells	Effect	Inflammatory symptoms	Effect of KJ on the days 3 and 4 Reference
Must cell, eosinophils	Histamine, PAF, leukotrienes	Vessels	Vascular permeability increases Vasodilation-bloodstream decreases Leukocyte extravasation increases	Sneezing, oedema, reddenings/warming	
		Neurons	Activation of nociceptors	Pain	Cáceres et al. (1997)
	NDA	Airway epithelium	Airway sensory nerve endings	Cough	Melchior et al. (2000), Cáceres et al. (1997)
Macrophages	IL-1, IL-6, TNF-α,	Brain		Fatigue, headache, malaise, anorexia, sleep disturbance	Cáceres et al. (1997)
		Hypothalamus	Interact with the vagus nerve endings to signal the temperature control centre	Fever	Melchior et al. (2000)
	Prostaglandins	Skin blood vessels	Vasoconstriction	Chilliness	
		Skeletal muscle	Effects on peripheral pain receptors	Muscle ache and pain	Melchior et al. (2000)
		Muscles	Catabolism	Weight loss	
	Leukotrienes	Fat tissue	Lipolysis		
		Neutrophils, monocytes	Chemotaxis, phagocytosis	Nasal discharge	Cáceres et al. (1997), Melchior et al. (2000)
	PAF	Bacteria, virus	Phagocytosis	Mucous secretion	
		Immune system	Immune defence	All symptoms	
	Prostaglandins, bradykinin	Nerve endings in the airway	Pain mediated by the cranial nerves supplying the nasopharynx and pharynx	Sore throat	Cáceres et al. (1997), Melchior et al. (2000)
Blood plasma globulins	Bradykinin	Large veins in the nasal epithelium (venous sinuses)	Vasodilation	Nasal congestion	

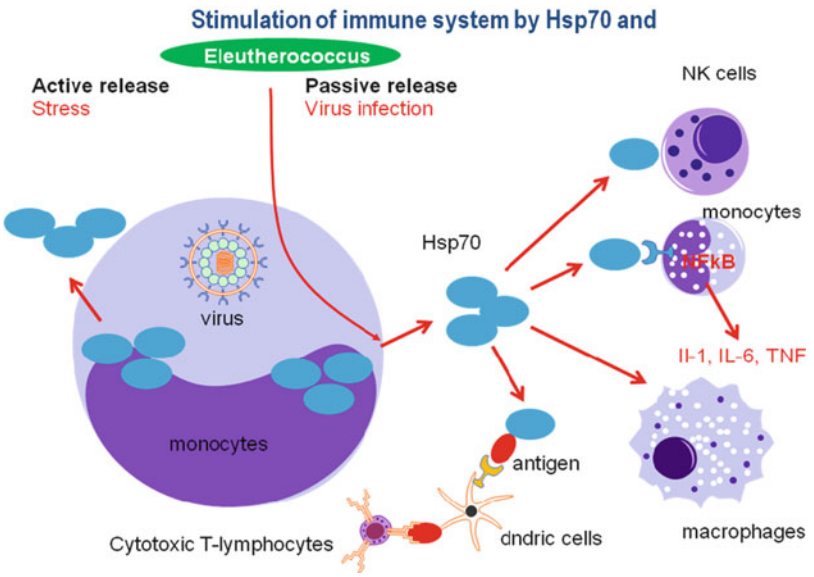


Fig. 4.8 Stimulation of immune system via upregulation of molecular chaperon Hsp70. Extracellular Hsp70 activates immune system of multicellular organism in order to kill virus/bacteria. One of the mechanism contributing in overall effect of Kan Jang, specifically of Eleutherococcus extract, is associated with stimulation of Hsp70, which is known to bind with high affinity and avidity to specific cells of the immune system including natural killer (NK) cells, dendritic cells (DC), macrophages, peripheral blood monocytes, and B cells. It activates specific signal transduction cascades, namely NF-κB (30 min), upregulates the expression of proinflammatory cytokines in human monocytes (2 h post challenge), and stimulates the hosts immune response including plethora of mediators such as TNF-α, IL-1β, IL-6, IL-12, GM-CSF, nitric oxide, chemokines including MIP-1, MCP-1, RANTES, etc. in an attempt to rid the host of infection (Tsan and Gao, 2009; Tsan and Gao 2004a; Asea 2005; Radons and Multhoff 2005; Asea and Pedersen 2010)

4.5 Conclusion

The large number of botanicals is currently used for the treatment of colds and flu. Basically, for colds and flu-like virus infections, *decongestants* (e.g. menthol, eucalyptus, camphor, the larch, the fir tree), broncholytics (theophylline, ephedrine), *expectorants* (including ipecacuanha, sage, ivy, Balm of Gilead, Tolu balsam, thyme, and senega), *demulcents* (e.g. Elder flower, Linden flower, Mallow flower, coltsfoot, marshmallow), *antivirals* (e.g. linden and elder flowers), and *immune system modulators* (e.g. Echinacea, astragalus) are popular and effective. Platelet-activating factor antagonists (e.g. the ginkgolides) have antiallergic effects, which can be useful as *cough suppressants*. Several herbal preparations, such as Echinacea (Echinacea spp.), Elderberry (*Sambucus nigra*), Garlic (*Allium sativa*), Panax quinquefolium, Isatis spp., and combination of *E. senticosus* and *Adhatoda vasica*, were found elective for influenza infections (Narimanian et al. 2005; Roxas and

Jurenka 2006). *Andrographis paniculata* indeed might be efficient in the treatment of respiratory tract infectious diseases providing "all (effects) in one (pill)".

It can be concluded that *A. paniculata*, as well as its fixed combination with *E. senticosus* (Kan Jang), significantly *reduce the symptoms of common cold*. It is possible, but not sufficiently documented yet, that Kan Jang tablets may have a preventive effect on common cold. Efficacy of Kan Jang is better documented. Apparently, Kan Jang is significantly more efficacious than Andrographis alone, has an excellent safety profile, and is well tolerated.

References

Abu-Ghefreh AA, Canatan H, Ezeamuzie CI (2009) In vitro and in vivo anti-inflammatory effects of andrographolide. Int Immunopharmacol 9(3):313–318

Akbar S (2011) *Andrographis paniculata*: a review of pharmacological activities and clinical effects. Altern Med Rev 16(1):66–77

Amaryan G, Astvatsatryan V, Gabrielian E, Panossian A, Panosyan V, Wikman G (2003) Double-blind, placebo-controlled, randomized, pilot clinical trial of Immuno-Guard® – a standardized fixed combination of *Andrographis paniculata* Nees, with *Eleutherococcus senticosus* Maxim, *Schizandra chinensis* Bail. and *Glycyrrhiza glabra* L. extracts in patients with Familial Mediterranean Fever. Phytomedicine 10(4):271–85

Amroyan E, Gabrielian E, Panossian A, Wikman G (1999) Inhibitory effect of andrographolide from *Andrographis paniculata* on PAF-induced platelet aggregation. Phytomedicine 6(1):27–31

Asea A (2005) Stress proteins and initiation of immune response: chaperokine activity of hsp72. Exerc Immunol Rev 11:34–45

Asea AAA, Pedersen BK (2010) Heat shock proteins and whole body physiology, vol 5. Springer, Dordrecht, pp 1–429

Bao Z, Guan S, Cheng C, Wu S, Wong SH, Kemeny DM, Leung BP, Wong WS (2009) A novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor-kappa B pathway. Am J Respir Crit Care Med 179(8):657–665

Barkan AI, Gaĭduchenia LI, Makarenko IA (1980) Effect of Eleutherococcus on respiratory viral infectious morbidity in children in organized collectives. Pediatriia 4:65–66

Basak A, Cooper S, Roberge AG, Banik UK, Chretien M, Seidah NG (1999) Inhibition of proprotein convertases-1, -7 and furin by diterpines of *Andrographis paniculata* and their succinoyl esters. Biochem J 338(Pt 1):107–113

Batkhuu J, Hattori K, Takano F, Fushiya S, Oshiman K, Fujimiya Y (2002) Suppression of NO production in activated macrophages in vitro and ex vivo by neoandrographolide isolated from *Andrographis paniculata*. Biol Pharm Bull 25(9):1169–1174

Brekhman II (1982) *On antitoxic action of eleutherococcus*, Moscow, Meditsina. p 37

Bohn B, Nebe CT, Birr C (1987) Flow-cytometric studies with eleutherococcus senticosus extract as an immunomodulatory agent. Arzneimittelforschung 37(10):1193–1196

Buckingham J (1993) Dictionary of natural products, 6th edn. Chapman & Hall/CRC, London http://www.ramex.com/title.asp?id=1795

Burgos RA, Hidalgo MA, Monsalve J, LaBranche TP, Eyre P, Hancke JL (2005a) 14-Deoxyandrographolide as a platelet activating factor antagonist in bovine neutrophils. Planta Med 71 (7):604–608

Burgos RA, Seguel K, Perez M, Meneses A, Ortega M, Guarda MI, Loaiza A, Hancke JL (2005b) Andrographolide inhibits IFN-gamma and IL-2 cytokine production and protects against cell apoptosis. Planta Med 71(5):429–434

Cáceres DD, Hancke JL, Burgos RA, Wikman GK (1997) Prevention of common colds with *Andrographis paniculata* dried extract. A pilot double blind study. Phytomedicine 4(2):101–104

Cáceres DD, Hancke JL, Burgos RA, Sandberg F, Wikman GK (1999) Use of visual analogue scale measurements (VAS) to assess the effectiveness of standardized *Andrographis paniculata* extract SHA-10 in reducing the symptoms of common cold. A randomized double blind-placebo study. Phytomedicine 6(4):217–223

Carr RR, Nahata MC (2006) Complementary and alternative medicine for upper-respiratory-tract infection in children. Am J Health Syst Pharm 63(1):33–39

Chandrasekaran CV, Gupta A, Agarwal A (2010) Effect of an extract of *Andrographis paniculata* leaves on inflammatory and allergic mediators in vitro. J Ethnopharmacol 129(2):203–207

Chandrasekaran CV, Thiyagarajan P, Deepak HB, Agarwal A (2011) In vitro modulation of LPS/calcimycin induced inflammatory and allergic mediators by pure compounds of *Andrographis paniculata* (King of bitters) extract. Int Immunopharmacol 11(1):79–84

Chang HM, But PP-H (eds) (1987) Pharmacology and applications of Chinese Materia Medica, Vol. 2 (trans: SC-S Yeung, SC Yao, L-L Wang). World Scientific Publishing Co., Pte. Ltd., pp 918–928

Chang J, Zhang RM, Zhang Y, Chen ZB, Zhang ZM, Xu Q, Yang YP, Long YY, Liu LL, Cai HY, Gao J, Lu N, Mao B, Wang L, Li TQ (2008) Andrographolide drop-pill in treatment of acute upper respiratory tract infection with external wind-heat syndrome: a multicenter and randomized controlled trial. Zhong Xi Yi Jie He Xue Bao 6(12):1238–1245, PubMed PMID: 19063836

Chao WW, Kuo YH, Hsieh SL, Lin BF (2009) Inhibitory effects of ethyl acetate extract of *Andrographis paniculata* on NF-{kappa}B trans-activation activity and LPS-induced acute inflammation in mice. Evid Based Complement Alternat Med 1–9

Chao WW, Kuo YH, Li WC, Lin BF (2009b) The production of nitric oxide and prostaglandin E2 in peritoneal macrophages is inhibited by *Andrographis paniculata*, *Angelica sinensis* and *Morus alba* ethyl acetate fractions. J Ethnopharmacol 122(1):68–75

Chao WW, Kuo YH, Lin BF (2010) Anti-inflammatory activity of new compounds from *Andrographis paniculata* by NF-kappaB transactivation inhibition. J Agric Food Chem 58 (4):2505–2512

Chao WW, Lin BF (2010) Isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). Chin Med 5(17):1–15

Chen JX, Xue HJ, Ye WC, Fang BH, Liu YH, Yuan SH, Yu P, Wang YQ (2009) Activity of andrographolide and its derivatives against influenza virus in vivo and in vitro. Biol Pharm Bull 32(8):1385–1391

Cheung HY, Cheung SH, Li J, Cheung CS, Lai WP, Fong WF, Leung FM (2005) Andrographolide isolated from *Andrographis paniculata* induces cell cycle arrest and mitochondrial-mediated apoptosis in human leukemic HL-60 cells. Planta Med 71(12):1106–1111

Chien CF, Wu YT, Lee WC, Lin LC, Tsai TH (2010) Herb-drug interaction of *Andrographis paniculata* extract and andrographolide on the pharmacokinetics of theophylline in rats. Chem Biol Interact 184(3):458–465

Chiou WF, Chen CF, Lin JJ (2000) Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells by andrographolide. Br J Pharmacol 129(8):1553–1560

Chiou WF, Lin JJ, Chen CF (1998) Andrographolide suppresses the expression of inducible nitric oxide synthase in macrophage and restores the vasoconstriction in rat aorta treated with lipopolysaccharide. Br J Pharmacol 125(2):327–334

Chuanxinlian Pian (Tabellae Andrographitis) (2010) Pharmacopoeia of the People's Republic of China 2010 (English edn), vol 1. China Medical Science, Beijing, China, p 599

Chubarev VN, Rubtsova ER, Filatova IV, Krendal' FP, Davydova ON (1989) Immunotropic effect of a tincture of the tissue culture biomass of ginseng cells and of an Eleutherococcus extract in mice. Farmakol Toksikol 52(2):55–59

Coon JT, Ernst E (2004) *Andrographis paniculata* in the treatment of upper respiratory tract infections: a systematic review of safety and efficacy. Planta Med 70(4):293–298

Deng WL, Nie R, Liu J (1982) Experimental research: pharmacological comparison of four kinds of Andrographolides. Chem Pharm Bull:195–198

Deng WL (1985) Pharmacological studies of thirteen kinds of injections of *Andrographis paniculata*. I. Antipyretic, anti-inflammatory effects and toxicity. Zhong Yao Tong Bao 10 (7):38–42

Denzler KL, Waters R, Jacobs BL, Rochon Y, Langland JO (2010) Regulation of inflammatory gene expression in PBMCs by immunostimulatory botanicals. PLoS One 5(9):e12561, pp 1–15

Drozd J, Sawicka T, Prosińska J (2002) Estimation of humoral activity of *Eleutherococcus senticosus*. Acta Pol Pharm 59(5):395–401

Eccles R (2005) Understanding the symptoms of the common cold and influenza. Lancet Infect Dis 5(11):718–725

EMEA/HMPC/244569/2006 (2007) Community herbal monograph on *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim., radix. Draft. London, 5 July, pp 1–6.

ESCOP Monographs (2003) Eleutherococci Radix *The Scientific Foundation of Herbal Medicinal Products*, 2nd edn. European Scientific Cooperative on Phytotherapy and Thieme, Exeter (UK). *European Scientific Cooperative Phytotherapy (ESCOP) Monographs*, 2nd edn. pp 142–149

Farnsworth NR, Kinghorn AD, Soejarto DD, Waller DP (1985) Siberian ginseng (*Eleuherococcus senticosus*): current status as an adaptogen. In: Wagner H, Hikino H, Farnsworth NR (eds) Economic and medicinal plant research, vol 1. Academic, London, pp 155–215

Gabrielian ES, Shukarian AK, Goukasova GI, Chandanian GL, Panossian AG, Wikman G, Wagner H (2002) A double blind, placebo-controlled study of *Andrographis paniculata* fixed combination Kan Jang in the treatment of acute upper respiratory tract infections including sinusitis. Phytomedicine 9(7):589–597

Gagarinova VM, Piskareva NA, Dekhtereva NI (1995) Mechanisms of health protection against acute respiratory diseases and corrective role of immunomodulators. International Collection of Scientific Papers, Issue 2, Vladivostok, "*Dal' nauka*", pp 126–133

Gladchun VP (1983) Effect of adaptogenes on the immunological reactivity of patients with a history of acute pneumonia. Vrach Delo 2:32–35

Glatthaar-Saalmüller B, Sacher F, Esperester A (2001) Antiviral activity of an extract derived from roots of *Eleutherococcus senticosus*. Antiviral Res 50:223–228

Hancke J, Burgos R, Cáceres D, Wikman G (1995) A double blind study with a new monodrug *Andrographis paniculata*: decrease of symptoms and improvement in the recovery from common colds. Phytother Res 9:559–562

Handa SS, Sharma A (1990) Hepatoprotective activity of andrographolide against galactosamine & paracetamol intoxication in rats. Indian J Med Res 92:284–292

Herba Andrographidis (2002) WHO monographs on selected medicinal plants, vol 2. WHO, Geneva, pp 12–24

Herba Andrographis (Chuanxinlian) (2010) Pharmacopoeia of the People's Republic of China 2010 (English edn), vol 1. China Medical Science, Beijing, China, pp 32–33

Hidalgo MA, Romero A, Figueroa J, Cortes P, Concha II, Hancke JL, Burgos RA (2005) Andrographolide interferes with binding of nuclear factor-kappaB to DNA in HL-60-derived neutrophilic cells. Br J Pharmacol 144(5):680–686

Hovhannisyan A, Abrahamyan H, Gabrielian E, Panossian A (2006) The effect of Kan Jan on the pharmacokinetics and pharmacodynamics of warfarin in rats. Phytomedicine 13:318–323

Hsu JH, Liou SS, Yu BC, Cheng JT, Wu YC (2004) Activation of alpha1A-adrenoceptor by andrographolide to increase glucose uptake in cultured myoblast C2C12 cells. Planta Med 70(12):1230–1233

Iruretagoyena MI, Tobar JA, Gonzalez PA, Sepulveda SE, Figueroa CA, Burgos RA, Hancke JL, Kalergis AM (2005) Andrographolide interferes with T cell activation and reduces experimental autoimmune encephalomyelitis in the mouse. J Pharmacol Exp Ther 312(1):366–372

Jarukamjorn K, Don-in K, Makejaruskul C, Laha T, Daodee S, Pearaksa P, Sripanidkulchai BO (2006) Impact of *Andrographis paniculata* crude extract on mouse hepatic cytochrome P450 enzymes. J Ethnopharmacol 105(3):464–467

Jeong HJ, Koo HN, Myung NI, Shin MK, Kim JW, Kim DK, Kim KS, Kim HM, Lee YM (2001) Inhibitory effects of mast cell-mediated allergic reactions by cell cultured Siberian Ginseng. Immunopharmacol Immunotoxicol 23(1):107–117

Ji LL, Wang Z, Dong F, Zhang WB, Wang ZT (2005) Andrograpanin, a compound isolated from anti-inflammatory traditional Chinese medicine *Andrographis paniculata*, enhances chemokine SDF-1alpha-induced leukocytes chemotaxis. J Cell Biochem 95(5):970–978

Jung CH, Jung H, Shin YC, Park JH, Jun CY, Kim HM, Yim HS, Shin MG, Bae HS, Kim SH, Ko SG (2007) Eleutherococcus senticosus extract attenuates LPS-induced iNOS expression through the inhibition of Akt and JNK pathways in murine macrophage. J Ethnopharmacol 113(1):183–187

Kalashnikov ZD (1986) The effect of long-term prophylactic administration of Eleutherococcus on morbidity among coal miners in the Far East. In: New data on Eleutherococcus. Proceedings of the second international symposium on Eleutherococcus, Moscow, 1984. Far East Science Center, USSR Acad. Sci., Vladivostok, USSR, pp 223–225

Kamdem RE, Sang S, Ho CT (2002) Mechanism of the superoxide scavenging activity of neoandrographolide – a natural product from *Andrographis paniculata* Nees. J Agric Food Chem 50(16):4662–4665

Kapil A, Koul IB, Banerjee SK, Gupta BD (1993) Antihepatotoxic effects of major diterpenoid constituents of *Andrographis paniculata*. Biochem Pharmacol 46(1):182–185

Kim AA (1992) Effect of eleutherococc extract and dibazole on general and intracellular adaptive reactions in children aged 4–14 years with acute pneumonia. Pediatriia 1:71–73

Kim TG, Hwi KK, Hung CS (2005) Morphological and biochemical changes of andrographolide-induced cell death in human prostatic adenocarcinoma PC-3 cells. In Vivo 19(3):551–557

Kimura Y, Sumiyoshi M (2004) Effects of various *Eleutherococcus senticosus* cortex on swimming time, natural killer activity and corticosterone level in forced swimming stressed mice. J Ethnopharmacol 95(2–3):447–453

Kligler B, Ulbricht C, Basch E, Kirkwood CD, Abrams TR, Miranda M, Singh Khalsa KP, Giles M, Boon H, Woods J (2006) *Andrographis paniculata* for the treatment of upper respiratory infection: a systematic review by the natural standard research collaboration. Explore (NY) 2 (1):25–29

Ko HC, Wei BL, Chiou WF (2006) The effect of medicinal plants used in Chinese folk medicine on RANTES secretion by virus-infected human epithelial cells. J Ethnopharmacol 107 (2):205–210

Kormosh N, Laktionov K, Antoshechkina M (2006) Effect of a combination of extract from several plants on cell-mediated and humoral immunity of patients with advanced ovarian cancer. Phytother Res 20(5):424–425

Kovalenko TI, Vereshchagin IA (1994) Treating meningo coccal infection in children – oral injection of eleutherococcus liquid extract from day 4 in addition to usual therapy. Don Med Inst Publication, Donetsk, pp 1–12

Kozlov VK (1986) Motivation of metabolic therapy using "energy complexes" and eleutherococcus preparation for improving adaptation disorders in children. In: New data on Eleutherococcus. Proceedings of the second international symposium on Eleutherococcus, Moscow, 1984. Far East Science Center, USSR Acad. Sci., Vladivostok, USSR, pp 204–207

Kulichenko LL, Kireyeva LV, Malyshkina EN, Wikman G (2003) A randomized, controlled study of Kan Jang versus amantadine in the treatment of influenza in Volgograd. J Herb Pharmacother 3(1):77–93

Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S (2004) Anticancer and immunostimulatory compounds from *Andrographis paniculata*. J Ethnopharmacol 92(2–3):291–295

Kunwar RM, Shrestha KP, Bussmann RW (2010) Traditional herbal medicine in far-west Nepal: a pharmacological appraisal. J Ethnobiol Ethnomed 6(35):1–18

Kupin VI, Polevaia EB (1986) Stimulation of the immunological reactivity of cancer patients by Eleutherococcus extract. Vopr Onkol 32(7):21–26

Liu J, Wang ZT, Ge BX (2008) Andrograpanin, isolated from *Andrographis paniculata*, exhibits anti-inflammatory property in lipopolysaccharide-induced macrophage cells through down-regulating the p38 MAPKs signaling pathways. Int Immunopharmacol 8(7):951–958

Liu J, Wang ZT, Ji LL, Ge BX (2007a) Inhibitory effects of neoandrographolide on nitric oxide and prostaglandin E2 production in LPS-stimulated murine macrophage. Mol Cell Biochem 298(1–2):49–57

Liu J, Wang ZT, Ji LL (2007b) In vivo and in vitro anti-inflammatory activities of neoandrographolide. Am J Chin Med 35(2):317–328

Madav S, Tripathi HC, Tandan SK, Mishra SK (1995) Analgesic, antipyretic and antiulcerogenic effects of andrographolide. Ind J Pharm Sci 57(3):121–125

Madav S, Tandan SK, Lal J, Tripathi HC (1996) Anti-inflammatory activity of andrographolide. Fitoterapia 67(5):452–458

Melchior J, Spasov AA, Ostrovskij OV, Bulanov AE, Wikman G (2000) Double-blind, placebo-controlled pilot and phase III study of activity of standardized *Andrographis paniculata* Herba Nees extract fixed combination (Kan Jang) in the treatment of uncomplicated upper-respiratory tract infection. Phytomedicine 7(5):341–350

Melchior J (1996/97) Controlled clinical study of standardized *Andrographis paniculata* extract (Kan-Jang tablet) in common cold a pilot trial. Phytomedicine 3(4):315–318

Mishra US, Mishra A, Kumari R, Murthy PN, Naik BS (2009) Antibacterial activity of ethanol extract of *Andrographis paniculata*. Ind J Pharm Sci 71(4):436–438

Misra H, Soni M, Mehta D, Mehta BK, Jain DC (2009) An improved HPTLC – UV method for rapid estimation ofandrographolide in *Andrographis paniculata* (Burm. f) Nees. InPharm Communique 2(2):51–54

Naclerio RM, Proud D, Lichtenstein LM, Kagey-Sobotka A, Hendley JO, Sorrentino J, Gwaltney JM (1988) Kinins are generated during experimental rhinovirus colds. J Infect Dis 157:133–142

Naik SR, Hule A (2009) Evaluation of immunomodulatory activity of an extract of andrographolides from *Andographis paniculata*. Planta Med 75(8):785–791

Narimanian M, Badalyan M, Panosyan V, Gabrielian E, Panossian A, Wikman G, Wagner H (2005) Clinical evidence of high efficacy of fixed combinations of herbal extracts with particular reference to *Adhatoda vasica*, in patients with non-complicated respiratory tract infections (bronchitis). Phytomedicine 12:539–547

Pande V, Ramos MJ (2005) NF-kB in human disease: current inhibitors and prospects for *de novo* structure based design of inhibitors. Curr Med Chem 12:357–374

Panossian AG, Sprygin VG, Dardimov IV (1982) Influence of Eleutherococcus and eleutherosides A, B, C, D and E on the arachidonic acid release and metabolism. Chem Pharm J 7:776–779

Panossian A, Hambartsumyan M, Hovanissian A, Gabrielian E, Wikman G (2007) The adaptogens rhodiola and schizandra modify the response to immobilization stress in rabbits by suppressing the increase of phosphorylated stress-activated protein kinase, nitric oxide and cortisol. Drug Targets Insights 1:39–54, http://www.la-press.com/the-adaptogens-rhodiola-and-schizandra-modify-the-response-to-immobili-a260

Panossian A, Oganessian A, Mamikonian G, Ambartsumian E, Gabrielian E, Wagner H, Wikman G (2000) Pharmacokinetic and oral bioavailability of Andrographolid from *Andrographis paniculata* extract SHA-10 and Andrographis fixed combination Kan-Jang in rats and humans. Phytomedicine 7(5):351–364

Panossian A, Davtyan T, Gukassyan N, Gukasova G, Mamikonyan G, Gabrielian E, Wikman G (2002) Effect of Andrographolide and Kan Jang – fixed combination of extract SHA-10 and extract SHE-3 – on proliferation of human lymphocytes, production of cytokines and immune activation markers in the whole blood cells culture. Phytomedicine 90:598–605

Panossian A, Wikman G (2010) Effects of adaptogens on the central nervous system and the molecular mechanisms associated with their stress—protective activity. Pharmaceuticals 3 (1):188–224, http://www.mdpi.com/1424-8247/3/1/188/pdf

Panossian A, Wikman G (2009) Evidence-based efficacy of adaptogens in fatigue, and molecular mechanisms related to their stress-protective activity. Curr Clin Pharmacol 4:198–219

Panossian A, Wikman G, Kaur P, Asea A (2009) Adaptogens exert a stress protective effect by modulation of expression of molecular chaperons. Phytomedicine 16(6–7):617–622

Panossian A, Wikman G, Kaur P, Asea A (2010) Molecular chaperones as mediators of stress protective effect of plant adaptogens. In: Asea AAA, Pedersen BK (eds) Heat shock proteins and whole body physiology, vol 5. Springer, Dordrecht, pp 351–364

Parichatikanond W, Suthisisang C, Dhepakson P, Herunsalee A (2010) Study of anti-inflammatory activities of the pure compounds from *Andrographis paniculata* (Burm. f.) Nees and their effects on gene expression. Int Immunopharmacol 10(11):1361–1373

Pekthong D, Blanchard N, Abadie C, Bonet A, Heyd B, Mantion G, Berthelot A, Richert L, Martin H (2009) Effects of *Andrographis paniculata* extract and Andrographolide on hepatic cytochrome P450 mRNA expression and monooxygenase activities after in vivo administration to rats and in vitro in rat and humanhepatocyte cultures. Chem Biol Interact 179(2–3):247–55

Peng GY, Zhou F, Ding RL, Li HD, Yao K (2002) Modulation of lianbizi injection (andrographolide) on some immune functions. Zhongguo Zhong Yao Za Zhi 27(2):147–150

Pinckard RN, Ludwig JC, McManus LM (1988) Platelet-activating factors. In: Gallin JI, Goldstein IM, Snyderman R (eds) Inflammation. Basic principles and clinical correlates. Raven, New York, pp 139–167

Poolsup N, Suthisisang C, Prathanturarug S, Asawamekin A, Chanchareon U (2004) *Andrographis paniculata* in the symptomatic treatment of uncomplicated upper respiratory tract infection: systematic review of randomized controlled trials. J Clin Pharm Ther 29(1):37–45

Protasova SF, Zykov MP (1984) Antiviral effect of Eleutherococcus in experimental influenza infection. In: New data on Eleutherococcus. Proceedings of the second international symposium on Eleutherococcus, Moscow, pp 170–174

Proud D, Gwaltney JM Jr, Hendley JO, Dinarello CO, Gillis S, Schleimer RP (1994) Increased levels of interleukin-1 are detected in nasal secretions of volunteers during experimental rhinovirus colds. J Infect Dis 169:1007–1013

Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS (1993) Immunostimulant agents from *Andrographis paniculata*. J Nat Prod 56(7):995–999

Qin LH, Kong L, Shi GJ, Wang ZT, Ge BX (2006) Andrographolide inhibits the production of TNF-alpha and interleukin-12 in lipopolysaccharide-stimulated macrophages: role of mitogen-activated protein kinases. Biol Pharm Bull 29(2):220–224

Radix Eleutherococci (2002) WHO monographs on selected medicinal plants, vol 2. WHO, Geneva, pp 83–96

Radix et, Rhizoma seu Caulis Acanthopanacis Senticosi (Ciwaujia) (2005) Pharmacopoeia of the People's Republic of China 2010 (English edn), vol 1. People's Medical Science, Beijing, China, p 121

Radons J, Multhoff G (2005) Immunostimulatory functions of membrane-bound and exported heat shock protein 70. Exerc Immunol Rev 11:17–33

Rajagopal S, Kumar RA, Deevi DS, Satyanarayana C, Rajagopalan R (2003) Andrographolide, a potential cancer therapeutic agent isolated from *Andrographis paniculata*. J Exp Ther Oncol 3 (3):147–158

Rogala E, Skopińska-Rózewska E, Sawicka T, Sommer E, Prosińska J, Drozd J (2003) The influence of *Eleuterococcus senticosus* on cellular and humoral immunological response of mice. Pol J Vet Sci 6(3):37–39

Roxas M, Jurenka J (2006) Colds and influenza: a review of diagnosis and conventional, botanical, and nutritional considerations. Altern Med Rev 11(2):102–113

Saxena RC, Singh R, Kumar P, Yadav SC, Negi MP, Saxena VS, Joshua AJ, Vijayabalaji V, Goudar KS, Venkateshwarlu K, Amit A (2010) A randomized double blind placebo controlled clinical evaluation of extract of *Andrographis paniculata* (KalmCold) in patients with uncomplicated upper respiratory tract infection. Phytomedicine 17(3–4):178–85

Schmolz MW, Sacher F, Aicher B (2001) The synthesis of rantes, G-CSF, IL-4, IL-5, IL-6, IL-12 and IL-13 in human whole-blood cultures is modulated by an extract from *Eleutherococcus senticosus* L. roots. Phytother Res 15:268–270

Shadrin AS, Kustikova YuG, Belogolovkina NA, Baranov NI, Oleinikova EV, Sigaeva VP, Ivlev AM, Romanov VL, Imkhanitskaya LI, Skripak SG, Ryazanova LA (1986) Estimation of prophylactic and immunostimulating effects of Eleutherococcus and Schizandra chinensis preparations. In: New data on Eleutherococcus. Proceedings of the second international symposium on Eleutherococcus, Moscow, 1984. Far East Science Center, USSR Acad. Sci., Vladivostok, USSR, pp 213–215

Shakhova EG, Spasov AA, Ostrovskiĭ OV, Konovalova IV, Chernikov MV, Mel'nikova GI (2003) Effectiveness of using the drug Kan-Yang in children with acute respiratory viral infection (clinico-functional data). Vestn Otorinolaringol 3:48–50

Sheeja K, Guruvayoorappan C, Kuttan G (2007) Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide. Int Immunopharmacol 7(2):211–221

Sheeja K, Kuttan G (2007a) Activation of cytotoxic T lymphocyte responses and attenuation of tumor growth in vivo by *Andrographis paniculata* extract and andrographolide. Immunopharmacol Immunotoxicol 29(1):81–93

Sheeja K, Kuttan G (2007b) Modulation of natural killer cell activity, antibody-dependent cellular cytotoxicity, and antibody-dependent complement-mediated cytotoxicity by andrographolide in normal and Ehrlich ascites carcinoma-bearing mice. Integr Cancer Ther 6(1):66–73

Sheeja K, Shihab PK, Kuttan G (2006) Antioxidant and anti-inflammatory activities of the plant *Andrographis paniculata* Nees. Immunopharmacol Immunotoxicol 28(1):129–140

Shen ML, Zhai SK, Chen HL, Luo YD, Tu GR, Ou DW (1991) Immunopharmacological effects of polysaccharides from *Eleutherococcus senticosus* on experimental animals. Int J Immunopharmacol 13(5):549–554

Shen Y-C, Chen C-F, Chiou W-F (2002) Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect. Br J Pharmacol 135:399–406

Shen YC, Chen CF, Chiou WF (2000) Suppression of rat neutrophil reactive oxygen species production and adhesion by the diterpenoid lactone andrographolide. Planta Med 66 (4):314–317

Shukla B, Visen PK, Patnaik GK, Dhawan BN (1992) Choleretic effect of andrographolide in rats and guinea pigs. Planta Med 58(2):146–149

Singha PK, Roy S, Dey S (2003) Antimicrobial activity of *Andrographis paniculata*. Fitoterapia 74(7–8):692–694

Spasov AA, Ostrovskij OV, Chernikov MV, Wikman G (2004) Comparative controlled study of *Andrographis paniculata* fixed combination, Kan Jang and an Echinacea preparation as adjuvant, in the treatment of uncomplicated respiratory disease in children. Phytother Res 18 (1):47–53

Steinmann GG, Esperester A, Joller P (2001) Immunopharmacological in vitro effects of *Eleutherococcus senticosus* extracts. Arzneim-Forsch/Drug Res 51(1):76–83

Suebsasana S, Pongnaratorn P, Sattayasai J, Arkaravichien T, Tiamkao S, Aromdee C (2009) Analgesic, antipyretic, anti-inflammatory and toxic effects of andrographolide derivatives in experimental animals. Arch Pharm Res 32(9):1191–1200

Sulaiman MR, Zakaria ZA, Abdul Rahman A, Mohamad AS, Desa MN, Stanslas J, Moin S, Israf DA (2010) Antinociceptive and antiedematogenic activities of andrographolide isolated from *Andrographis paniculata* in animal models. Biol Res Nurs 11(3):293–301

Tang W, Eisenbrand G (1992) Chinese drugs of plant origin: chemistry, pharmacology and use in traditional and modern medicine *Andrographis paniculata* (Burm. f.) Nees. Springer, Dordrecht, pp 97–103

Thamlikitkul V, Dechatiwongse T, Theerapong S, Chantrakul C, Boonroj P, Punkrut W, Ekpalakorn W, Boontaeng N, Taechaiya S, Petcharoen S, Riewpaiboon W, Riewpaiboon A,

Tenambergen ED (1991) Efficacy of *Andrographis paniculata*, Nees for pharyngotonsillitis in adults. J Med Assoc Thai 74(10):437–442

Thisoda P, Rangkadilok N, Pholphana N, Worasuttayangkurn L, Ruchirawat S, Satayavivad J (2006) Inhibitory effect of *Andrographis paniculata* extract and its active diterpenoids on platelet aggregation. Eur J Pharmacol 553(1–3):39–45

Tsai HR, Yang LM, Tsai WJ, Chiou WF (2004) Andrographolide acts through inhibition of ERK1/2 and Akt phosphorylation to suppress chemotactic migration. Eur J Pharmacol 498(1–3):45–52

Tsan MF, Gao B (2004a) Cytokine function of heat shock proteins. Am J Physiol Cell Physiol 286 (4):C739–744

Tsan MF, Gao B (2004b) Heat shock protein and innate immunity. Cell Mol Immunol 1 (4):274–279

Tsan MF, Gao B (2004c) Heat shock proteins and immune system. J Leukoc Biol 85(6):905–910

Tsan MF, Gao B (2009) Heat shock proteins and immune system. J Leukoc Biol 85(6):905–910

Vedavathy S, Rao KN (1991) Antipyretic activity of six indigenous medicinal plants of Tirumala Hills, Andhra Pradesh, India. J Ethnopharmacol 3(3):193–196

Visen PK, Shukla B, Patnaik GK, Dhawan BN (1993) Andrographolide protects rat hepatocytes against paracetamol-induced damage. J Ethnopharmacol 40(2):131–136

Wagner H, Bauer R, Melchart D, Pei-Gen X, Staudinger A (2011) Chromatographic fingerprint analysis of herbal medicines – thin layer and high performances liquid chromatography of Chinese drugs, vol 1. Springer, Wien, pp 273–280

Wagner H, Proksch A, Riess-Maurer I, Vollmar A, Odenthal S, Stuppner H, Jurcic K, Le Turdu M, Heur YH (1984) Immunostimulant action of polysaccharides (heteroglycans) from higher plants. Preliminary communication. Arzneimittelforschung 34(6):659–661

Wiart C, Kumar K, Yusof MY, Hamimah H, Fauzi ZM, Sulaiman M (2005) Antiviral properties of entlabdene diterpenes of *Andrographis paniculata* nees, inhibitors of herpes simplex virus type 1. Phytother Res 19(12):1069–1070

Xia YF, Ye BQ, Li YD, Wang JG, He XJ, Lin X, Yao X, Ma D, Slungaard A, Hebbel RP, Key NS, Geng JG (2004) Andrographolide attenuates inflammation by inhibition of NF-kappa B activation through covalent modification of reduced cysteine 62 of p50. J Immunol 173 (6):4207–4217

Xu Y, Chen A, Fry S, Barrow RA, Marshall RL, Mukkur TK (2007) Modulation of immune response in mice immunised with an inactivated Salmonella vaccine and gavaged with *Andrographis paniculata* extract or andrographolide. Int Immunopharmacol 7(4):515–523

Yamamoto Y, Gaynor RB (2001) Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 107(2):135–142

Yi JM, Hong SH, Kim JH, Kim HK, Song HJ, Kim HM (2002) Effect of *Acanthopanax senticosus* stem on mast cell-dependent anaphylaxis. J Ethnopharmacol 79(3):347–352

Yu CY, Kim SH, Lim JD, Kim MJ, Chung IM (2003) Intraspecific relationship analysis by DNA markers and in vitro cytotoxic and antioxidant activity in *Eleutherococcus senticosus*. Toxicol In Vitro 17(2):229–236

Zaidan MR, Noor Rain A, Badrul AR, Adlin A, Norazah A, Zakiah I (2005) In vitro screening of five local medicinal plants for antibacterial activity using disc diffusion method. Trop Biomed 22(2):165–170

Zhou J, Zhang S, Ong CN, Shen HM (2006) Critical role of pro-apoptotic Bcl-2 family members in andrographolide-induced apoptosis in human cancer cells. Biochem Pharmacol 72(2):132–144

Zhu Y-P, Nedichin BV (1998) Chinese materia medica: chemistry, pharmacology and applications. Harwood Academic Publishers, Amsterdam, pp 189–194

Zhu Z, Tang W, Gwaltney JM, Wu Y, Elias JA (1997) Rhinovirus stimulation of interleukin-8 in vivo and in vitro: role of NF-kB. Am J Physiol Lung Cell Mol Physiol 273:L814–L824

Zimmerman GA, McIntyre TM, Prescott SM, Stafforini DM (2002) The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. Crit Care Med 30(5):S294–S301

Chapter 5
From Traditional to Evidence-Based Use of *Hippophae rhamnoides* L.: Chemical Composition, Experimental, and Clinical Pharmacology of Sea Buckthorn Berries and Leaves Extracts

Alexander Panossian and Hildebert Wagner

5.1 Introduction

Sea buckthorn (*Hippophae* L., Elaeagnaceae) is a winter hardy, deciduous shrub with yellow or orange berries. The genus *Hippophae* includes seven species (*H. goniocarpa* Y.S. Lian & al. ex Swenson & Bartish, *H. gyantsensis* (Rousi) Lian, *H. litangensis* Y.S. Lian & X.L. Chen ex Swenson & Bartish, *H. neurocarpa* S.W. Liu & T.N. He, *H. rhamnoides* L.—common sea buckthorn, *H. salicifolia* D. Don, and *H. tibetana* Schlecht) and eight subspecies in the world (Rousi, 1965, 1971; Swenson and Bartish 2002; Sun et al. 2002). However, only *H. rhamnoides*[1] has an extremely wide distribution in the Eurasian continent (Bykov 2006; Sokolov 1988; Yao and Tigerstedt 1995). Isozyme analysis has shown large genetic diversity at the species, subspecies, and population levels (Yao and Tigerstedt 1993, 1994). The main factor influencing genetic differentiation is related to natural geographic barriers among populations (Chen et al. 2010).

[1] *Synonyms:* Elaeagnus rhamnoides (L.) A. Nelson, Hippophae angustifolia Lodd., Hippophae littoralis Salisb., Hippophae rhamnoideum Saint-Lager, Hippophae sibirica Lodd., Hippophae stourdziana Szabó, Osyris rhamnoides Scop., Rhamnoides hippophae Moench

Common names: The Chinese name for Sea Buckthorn is 沙棘, pinyin shājí sha1ji2, literally "sand thorn." Sea Buckthorn is called, Star-Bu or Dhar-Bu in Tibet, Dalechuk in Nepal; Yashildoo Chatsargana in Mongolia, Oblepikha in Russia, Tibet Sajee omm Japan, Seedorn in Germany, Argousier in France, Espino Amarillo in Spain, Finbär in Sweden, Tindved in Denmark, Rokitnik in Poland, Smiltserkskis in Latvia, Chichkhan in Armenia [Watanabe et al. 2005; Pakalns 2002; Natural Medicines Comprehensive Database 2011; Sea buckthorn (*Hippophae rhamnoides*) 2011]. Sea Buckthorn is also known as "Siberian Pineapple."

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Growing interest to this plant is mainly due to a high nutritional value of berries and potential health effects of leaves and bark.

Numerous sea buckthorn products are made from sea buckthorn leaves, berries, pulp, and seed residue. (Bernáth and Földesi 1992; Wolf and Wegert 1993; Morzewski and Bakowska 1960). Dried ripe fruit of *H. rhamnoides* is included in Pharmacopeia of the Peoples Republic of China (Hippophae Fructus 2010). Processed products include functional food (juice, alcoholic beverages, candies, and ice cream, tea, jam, biscuits, and food colors), food supplements (oil, extracts in capsule, tablet, or liquid forms), personal care products (facial anti-aging, sun-blocking and tan-enhancing creams, masks, lotions, etc.) (Niu 1991; Pashina 1993; Houghton and Parsons 1995; Li and Schroeder 1996) and pharmaceuticals (Olea Hippophae oil, Hiporamin tablets, Xindakang tablets, etc.) (Hippophae Fructus 2010; Sea buckthorn oil 1981; Hiporamin, Sea buckthorn leaves extract 2002). In this article, the results of chemical and pharmacological studies of the sea buckthorn medicinal products and food supplements are mainly reviewed with the aim to evaluate the level of scientific evidences supporting the health claims.

Oils (Oleum ex fructibus et foliis Hippopheae) and oil extracts are the mostly common sea buckthorn products produced in Russia. These oils are processed and sold as essential oils for various medicinal and therapeutic purposes (Mashkovskiy 2000).

More than ten different sea buckthorn-based drugs are available in different forms, such as liquids, powders, plasters, films, pastes, pills, liniments, suppositories, and aerosols.

Fixed combinations of sea buckthorn oil with levomycetin, anestesin, hydroboric acid (Olasolum—aerosol), collagen (Oblecolum—plates), methyluracyl, and other experiences (Hiposolum—aerosol) are used in Russia for the treatment of burns, wounds, trophic ulcers, eczema, dermatitis, colpitis, vulvitis, proctitis, colon ulcers, and other wounds (Mashkovskiy 2000).

Recently, a new antiviral product, sea buckthorn leaves extract (Hiporamin tablets, Hiporamin powder, and Hiporamin 0.5 % ointment) standardized for the content of total gallo elagic tannins has been developed from sea buckthorn leaves. This OTC in Russian Federation drug (Hiporamin, Sea buckthorn leaves extract 2002) is indicated mainly for prevention and treatment of acute respiratory viral infections (ARVI), such as common cold, flu, etc. (Fadeeva et al. 1988)

In Russia, the sea buckthorn industry has been growing since the 1940s. In 1950s Sea buckthorn oil was included in Russian Pharmacopeia and implemented as an anti-inflammatory aid in official medicine in USSR (Mashkovskij 1978). The Chinese industrial experience with sea buckthorn fruit production is more recent, although traditional uses date back many centuries. Research and large scale cultivation of sea buckthorn in China were initiated in the 1980s. Since 1982 over 300,000 ha of sea buckthorn have been planted in China. In addition, 150 processing factories have been established and are manufacturing over 200 products (Singh 2008).

Table 5.1 Uses supported by published clinical data

Use	Product name	Level of evidence[a]	References
Coronary artery disease with ventricular extra systoles	Xindakang tablets containing flavones extract of sea buckthorn fruit	C	Wu et al. (2004)
Anti-inflammatory and would healing herbal medicinal product for the treatment of burns, wounds, trophic ulcers, eczema, dermatitis, colpitis, vulvitis, proctitis, colon ulcers, and other wounds	Oleum Hippophae—oil from *H. rhamnoides* fruit	**C**	Sokolov (1988), Mashkovskiy (2000) Cimmerman and Mikhaylovskaya (1987)
Antiviral, antibacterial and immune-stimulating herbal medicinal product for the treatment and prevention of acute respiratory viral infections	Hiporamin: bookal tablets containing 20 mg of purified extract of sea buckthorn leaves	**C**	Fadeeva et al. (1988), Zamkovaya (2003)

[a]Grade of recommendation according to Natural Standards Evidence-Based Validated Grading Rationale

Grade A. Strong scientific evidence—Statistically significant evidence derived from (1) more than two properly conducted randomized controlled trials (RCT), or (2) one properly conducted randomized controlled trial and one properly conducted meta-analysis, or (3) multiple RCTs with a clear majority of the properly conducted trials and with supporting evidence in basic science, animal studies, or theory

Grade B. Good scientific evidence—Statistically significant evidence derived from (1) one or two properly conducted randomized trials, or (2) one or more properly conducted meta-analysis, or (3) more than one cohort/case control/nonrandomized trials and with supporting evidence in basic science, animal studies, or theory

Grade C. Unclear or conflicting scientific evidence—Evidence derived from (1) one or more small RCT without adequate size, power, statistical significance, or quality design by objective criteria, or (2) conflicting evidence from multiple RCTs without a clear majority of the properly conducted trials showing evidence of benefit or ineffectiveness, or (3) more than one cohort/case control/nonrandomized trial *and* without supporting evidence in basic science, animal studies, or theory *or* evidence of efficacy only from basic science, animal studies, or theory

5.2 Medicinal Uses

See Tables 5.1–5.3

Table 5.2 Uses described in pharmacopoeias and in traditional systems of medicine

Use[a]	Product name	Country
As a pain reliever, cough suppressant, expectorant, digestive tonic, and blood flow promoter in chest and heart pains, amenorrhea, abdominal dysfunctions, spleen deficiency, reduced food intake, blood-stasis amenorrhea, swelling, and stasis caused by injuries from falls	*Hippophae rhamnoides* L. leaf	China[a]
Anti-inflammatory and would healing herbal medicinal product for the treatment of burns, wounds, trophic ulcers, eczema, dermatitis, colpitis, vulvitis, proctitis, colon ulcers, and other wounds	Oleum hippophae—oil from *H. rhamnoides* fruit	Russia[b]
Antiviral, antibacterial, and immune-stimulating herbal medicinal product for the treatment and prevention of: • Influenza (A and B) • Para-grippe • Respiratory-syncytial, adenoviral, and other respiratory viral infections (RVI) • Angina and rhinitis, associated with RVI • Herpes simplex, genitally and extra genitally localized • Herpes zoster, variola • Cytomegalovirus infection	Hiporamin: – Bookal tablets containing 20 mg of purified extract of sea buckthorn leaves – Rectal and vaginal suppositories containing dry extract purified extract of sea buckthorn leaves – 0.5 % ointment	Russia[c]

[a]Hippophae Fructus (沙棘 Shaji) (2010)
[b]Mashkovskiy (2000)
[c]Fadeeva et al. (1988)

Table 5.3 Uses described in folk medicine, not supported by experimental or clinical data

Country	Use[a]	Plant parts used
China	Expectorant and demulcent (soothing agent)	Fruit
Tibet[b]	Pulmonary disorders, cough, colds, fever, inflammation, abscesses, toxicity, constipation, tumors, and gynecological diseases	Bark, fruit
India	To treat lung, gastrointestinal, heart, blood, liver, and metabolic disorders; topically—in numerous skin diseases, wounds, burns, etc.	Fruit
Russia	Eczema, psoriasis, frostbite, lupus, and cervical erosion and internally for blood clots as well as eye disorders	Fruit
Mongolia	Gastrointestinal (colitis, enterocolitis, diarrhea) and skin disorders, topically for rheumatoid arthritis	Leaves, stems
Nepal	To regulate the menstruation for woman, to dilute blood, as a tonic, appetizer, and in cough and cold	Fruit
Tajikistan	Topical application to improve the softness of skin	Flowers

[a]Sokolov (1988)
[b]Watanabe et al. (2005)

5.3 Uses as Dietary Supplements

Numerous preparations of sea buckthorn are used worldwide as a dietary supplement (Sea buckthorn (*H. rhamnoides*) Natural Standard Monograph (2011); Sea-Buckthorn—A Promising Multi-purpose Crop For Saskatchewan (2011)) The functional claims of sea buckthorn dietary supplements, currently mentioned in the consolidated list of Article 13 health claims of the European Food Safety Authority (EFSA), are formulated as following:

- Helps in case of dry skin, contributes to the mucosal function in dry eye, vagina, and mouth, and helps to maintain healthy skin from within
- Contributes to the natural defenses of the body—support of the body's defense
- Supports the immune system [Consolidated List of Article 13 Health Claims Database http://www.efsa.europa.eu/en/ndaclaims/ndaclaims13.htm]

5.4 Major Chemical Constituents

Sea buckthorn berries are among the most nutritious and vitamin-rich fruits in the plant kingdom (Plekhanova 1988; Pentegova 1983; Zeb 2004). The oil is the best source of vitamins, neutral lipids hydroxylated fatty acids, long chain alcohols and their esters, sterols, pentacyclic triterpenes, and volatile monoterpenes, while leaves contain numerous phenolic compounds. Below is a list of compounds isolated from various parts of *Hippophae rhamnoides*.

5.4.1 Vitamins

The primary vitamin in sea buckthorn berries is vitamin C, containing values of approximately 400 mg/100 g of berries for the European subspecies *rhamnoides* (Gutzeit et al. 2008a, b; Rousi and Aulin 1977; Plekhanova 1988; Yao et al. 1992) up to 2,500 mg/100 g of berries for the Chinese subspecies *sinensis* (Yang et al. 1988; Yao and Tigerstedt 1994; Kallio et al. 2002a, b). It is higher than in strawberry, kiwi, orange, tomato, carrot, and hawthorn (Centenaro et al. 1977; Haranovich 1981; Malena et al. 1984; Bernáth and Földesi 1992). During industrial juice production, the technological processing of the berries causes a loss of about 5 %–11 % of the total ascorbic acid (TAA) in the manufactured juice. The results of the experiments showed that storage of sea buckthorn juices for 7 days at cold temperature (6°C) already resulted in a degradation of TAA of about 11 %–12 % (Gutzeit et al. 2008a, b; Jeppsson and Gao 2000).

The carotene content ranges from 30 to 40 mg/100 g of berries (Sergeeva et al. 1979; Malena et al. 1984; Bernáth and Földesi 1992; Wolf and Wegert 1993; Bernáth and Földesi 1992). The total content of carotenoids may reach 0.373 %,

where xanthophyll may account for number >10 % of the total carotenoids, while the major portion is made up of carotenes (Lagazidze et al. 1984). Eighty percent of the oil carotenoids are epoxy-derivatives of β-carotene (Zhmyrko et al. 1984). Lutein, β-cryptoxanthin, and β-carotene were found to be quantitatively the most abundant pigments (Crapatureanu et al. 1996).

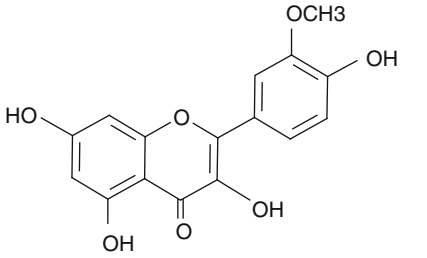
α-,β-,γ, and δ-tocopherols constituted 93–98 % of total tocopherols and tocotrienols in seeds, while α-tocopherol alone constituted 76–89 % in berries (Kallio et al. 2002a, b). The total contents of tocopherols and tocotrienols varied within the ranges of 84–318 and 56–140 mg /kg in seeds and whole berries, respectively (Kallio et al. 2002a). The fruit flesh of sinensis berries had contents of tocopherols and tocotrienols 2–3 times higher than those found in the other two subspecies (120 vs. 40 mg/kg in rhamnoides and 50 mg/kg in mongolica). The fresh whole berries of subsp. sinensis were clearly the best source of total tocopherols and tocotrienols (Kallio et al. 2002b). Vitamin E concentration can be up to 160 mg/100 g of berries (Dalgatov et al. 1985; Zhang et al. 1989a, b; Eliseev 1989; Lagazidze et al. 1984). The vitamin E content in sea buckthorn oil is higher than in wheat embryo, safflower, maize, and soybean, 9 times higher than corn oil and 35 times higher than in soybean oil. α-tocopherol (the most potent vitamin E form) is the major form of vitamin E in sea buckthorn oil (Huang and Xiao 1991, 1994; Fu et al. 1993; Bernáth and Földesi 1992). Levels of α-tocopherol are higher early in the ripening period, while at later dates, δ-tocopherol levels increased (Andersson et al. 2009).

Vitamins of group F were concentrated in the seed oil; approximately 60 % mixture of linoleic and linolenic acids from the total fatty acids isolated from oil in a 1.5:1 ratio. Sea buckthorn oils contain high concentrations of palmitoleic acid. This rare fatty acid is a component of skin fat and can support cell tissue and wound healing. The position of the ethylene bond in the C16:1 acid isolated from hydrolysis products of juice oil in the monoenic fraction was determined by chemical analysis. Its *cis*-configuration was determined by IR spectroscopy. The structure of this acid was confirmed as *cis*-9-hexadecaenic (palmitoleic). The unusually high content of C16:1 acid was characteristic only of juice oil (Zhmyrko et al. 1978; Bernáth and Földesi 1992; Crapatureanu et al. 1996). Oil from the juice and pulp is rich in palmitic (about 34 %), oleic (about 32 %), and palmitoleic acids (about 26 %), while the oil from the seed contains a higher quantity of unsaturated acids (around 86 %), a large part of these being essential fatty acids (linoleic 35 % and linolenic acid 26 %), and only unimportant concentrations of palmitic acid (about 10 %) (Undina et al. 1989). Similar results were obtained by other investigators (Franke and Mueller 1983; Hethelyi et al. 1989; Khusainov et al. 1988; Suleyman et al. 1998; Loskutova et al. 1989; Yang and Kallio 2001; Mamedov et al. 1981; Zham'yansan 1979).

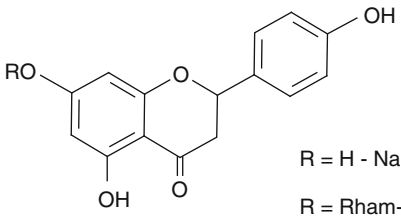
Content of vitamin K(1) in sea buckthorn berries ranges from 21 % up to 186 % (wet weight) depending on the storage time and temperature. During the industrial juice production, the technological processing of the berries caused a loss of about 36–54 % phylloquinone in the manufactured juice. The following processing steps, leading to the concentrated juice, result in the complete depletion of phylloquinone (Gutzeit et al. 2007a, b).

Sea buckthorn is rich in flavonoids; in total, 25 flavonol glycosides were isolated from different parts of the plant and identified by hydrolysis studies, ESI-MS(n), UV, HPLC–DAD–ESI-MS, and (1)H and (13)C NMR spectroscopy (Horhammer et al. 1966; Krolikowska 1972; Rasputina et al. 1975, 1976; Purve et al. 1979; Lachman et al. 1985; Zhang et al. 1988a, b, 1989a, b; Solonenko and Shishkina 1989; Fu et al. 1997; Wang et al. 1982; Beljanski 1989; Yoshida et al. 1991; Rösch et al. 2004a, b; Zu et al. 2006; Yang et al. 1998; Jeppsson and Gao 2000). Thus, most of the compounds identified were 7-rhamnosides of isorhamnetin, kaempferol, and quercetin, which exhibit different substitution patterns at the C-3 position, mainly glucosides, rutinosides, and sophorosides. In addition, numerous flavonol glycosides were detected lacking a sugar moiety at C-7. Eight flavonol derivatives were identified that are acylated by hydroxybenzoic or hydoxycinnamic acids, e.g., tiliroside (Yoshida et al. 1991). The occurrence of the major flavonol glycoside kaempferol 3-*O*-β-sophoroside-7-*O*-alpha-rhamnoside in sea buckthorn was described for the first time by Rösch et al. (2004a, b).

The contents of kaempferol and quercetin in sea buckthorn berries were measured at different maturation stages; quercetin decreased whereas kaempferol increased during maturation. Among three studied cultivars, the decrease in quercetin was significant (from 0.028 to 0.014 g/kg) in "Otradnaja," where as the increase (from 0.012 to 0.016 g/kg) in kaempferol was significant in the others two, "Prozratnaja" and "Gibrid Pertjik" (Jeppsson and Gao 2000).

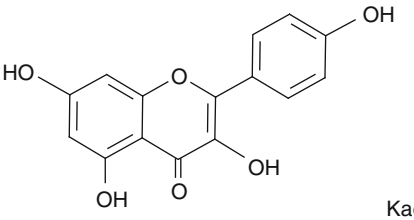


Isorhamnetin



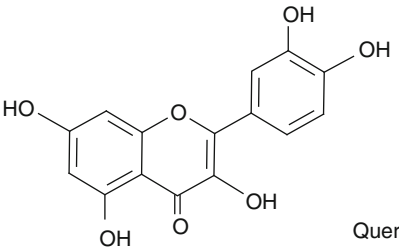
R = H - Naringenin

R = Rham-Glc - Naringin

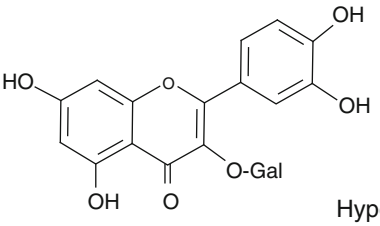


Kaempherol

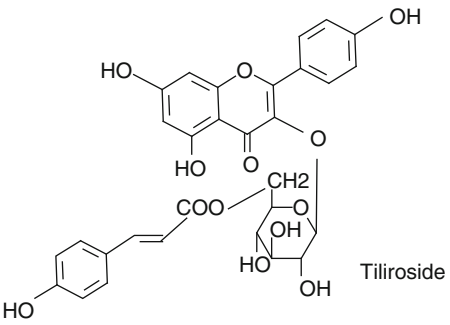
c



Quercetin



Hyperoside

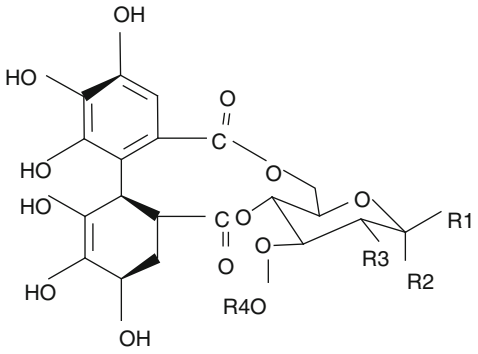


Tiliroside

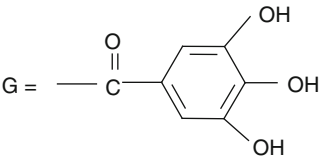
5.4.2 Polyphenolic Compounds

Numerous polyphenolic compounds (e.g., epicatechin, epigallocatechin, epigallocatechingallate) were found mainly in the leaves (ca. 12 %) of *H. rhamnoides* (Mukhamed'yarova and Chumbalov 1980; Novruzov et al. 1983; Kukina et al. 1991; Dembinska-Migas 1988, 1989; Yoshida et al. 1991; Sheichenko et al. 1987; Fadeeva et al. 1988). Only two polyphenols were also isolated from its fruits (Yoshida et al. 1991).

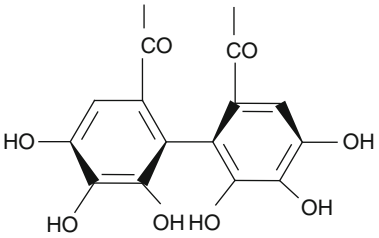
Along with flavonoids comprising about 4 %, 13 hydrolyzable gallo-, ellagi-, and galloellagitannins (hippophaenins A and B, 1,2,6-tri galloylglucose, peduncalagin, casuaricitin, strictinin, tellimagrandin I, isostrictinin, casuarinin, stachurin, castalagin, vescalagin, and hyporhamin) were identified in *H. rhamnoides* leaves (Sheichenko et al. 1987; Fadeeva et al. 1988; Yoshida et al. 1991). The structure of new ellagitannins—hyporhamnin (6-*O*-galloyl-1,3-*O*-hexahydroxydiphenoyl-β-D-glucose) (Sheichenko et al. 1987; Fadeeva et al. 1988), hippophaenins A and B (Yoshida et al. 1991), along with other hydrolyzable tannins isolated from sea buckthorn leaves is shown below.

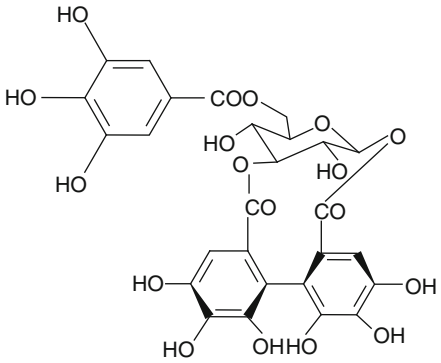


	R1	R2	R3	R4
Pundulculagin	H	OH	HHDP	
Casuarictin	OG	H	HHDP	
Strictinin	OG	H	H	H
Telinagrandin	H	OH	G	G

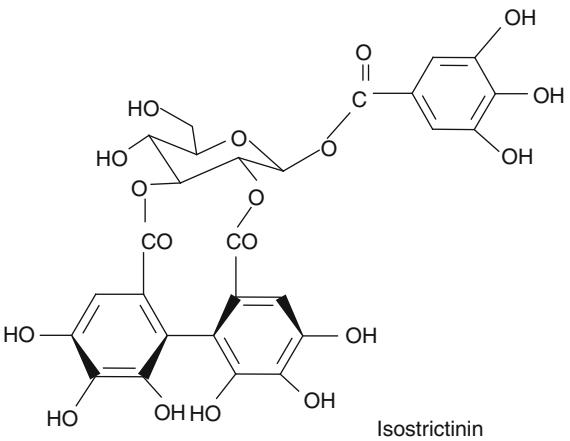


HHDP

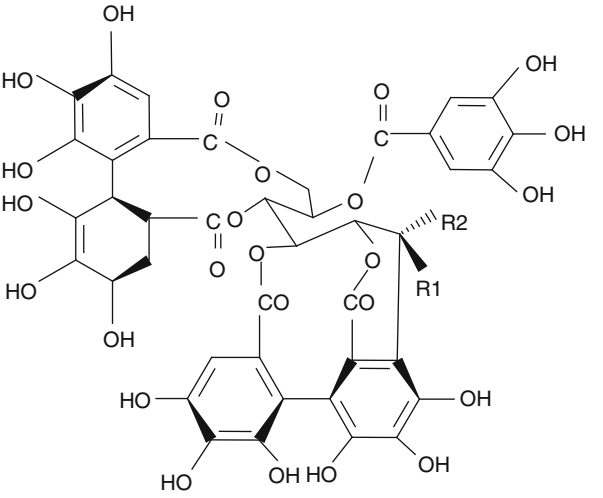




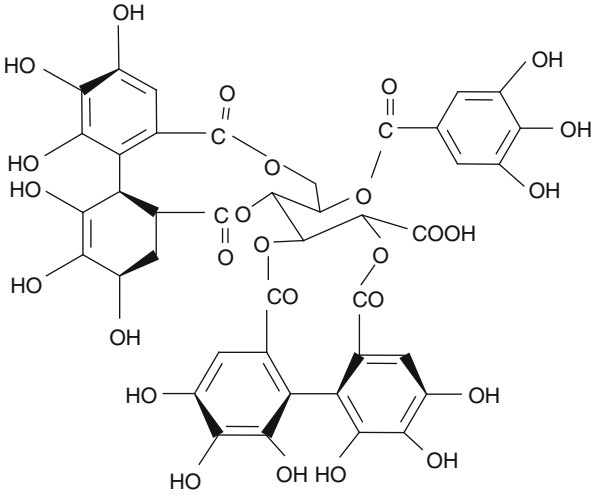
Hiporamnin



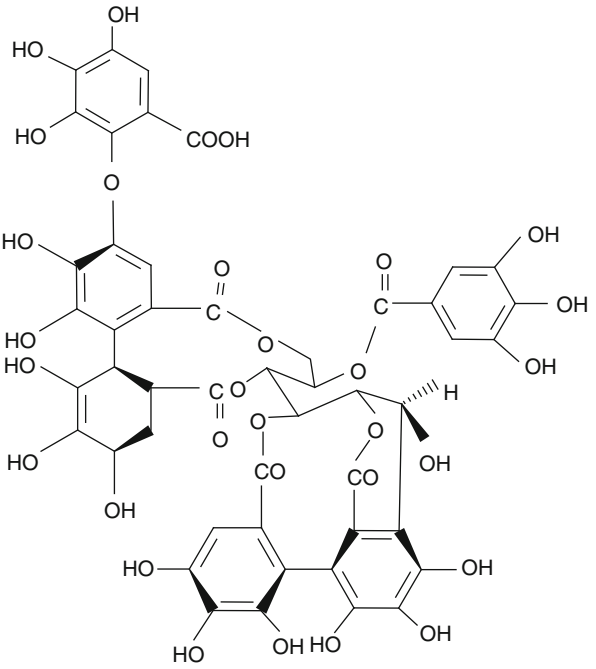
Isostrictinin



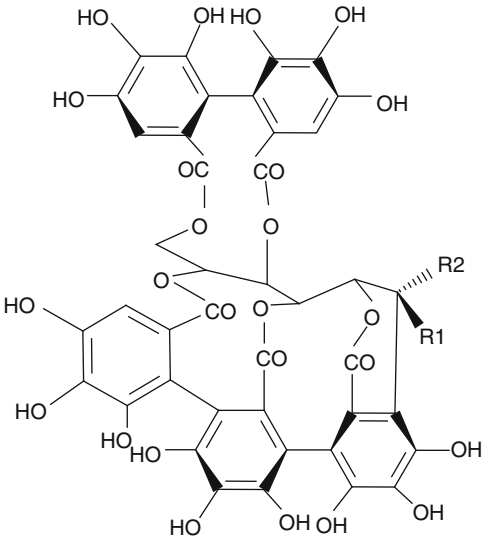
	R1	R2
Casuarinin	OH	H
Stachyurin	H	OH



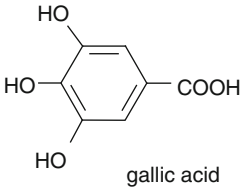
Hippophaenin A



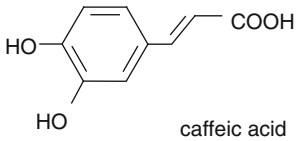
Hippophaenin B



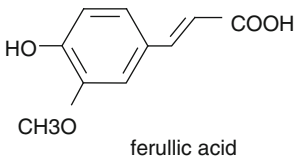
	R1	R2
Castalagin	OH	H
Vescalagin	H	OH



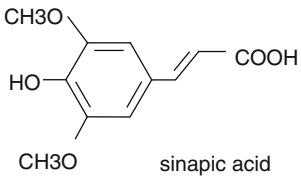
gallic acid



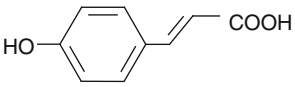
caffeic acid



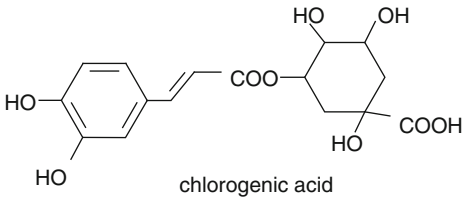
ferullic acid



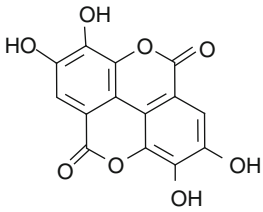
sinapic acid



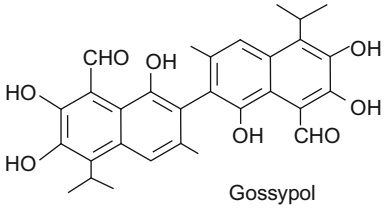
coumaric acid



chlorogenic acid



ellagic acid



Gossypol

The total polyphenol concentration in sea buckthorn leaves was found to vary from 7.62 % to 12.42 % of gallic acid equivalents (Panossian et al. unpublished data 2009), whereas in green tea, the polyphenol content ranged from 21.02 % to 14.32 % of GAE (Anesini et al. 2008).

It was found that the tannin fraction yields were from 34.3 % to 39.4 % in the polyphenol fraction, dependently the period of the plant vegetation. The yields fluctuations at 8-age- and 5-age-old plants were less noticeable and were 29.29–23.84 %. The tannin fraction yield is dependent on the shoots length (short cuts, up to 7 cm, yield 27.1–31.1 % of tannin fraction, medium cuts, to 14 cm, give 25.04–30.65 % of the key fraction, while long cuts, up to 21 cm, 23.62–28.35 %),

the period of harvesting, the ratio of leaves and stems in the vegetation mass, and age of shoots with the predominant accumulation of tannins in leaves. The highest content of the tannin fraction in the plant (on dry weight) was found in the first part of July (Sheichenko et al. 1987).

Determination of gossypol in *H. rhamnoides* seed oil has been described (Zhang et al. 1996).

The presence of phenolic acids - gallic, *p*-coumaric, sinapic, ellagic and chlorogenic, protocatechuic, gentisic, *p*-hydroxybenzoic, syringic, vanillic acid, salicylic acid, *p*-coumaric acid, cinnamic acid, caffeic acid and ferulic acid in sea buckthorn (Hippophaë rhamnoides) berries and leaves has been demonstrated (Dembinska-Migas 1988; Arimboor et al. 2008). Berry pulp contained a total of 1,068 mg/kg phenolic acids, of which 58.8 % was derived from phenolic glycosides. Free phenolic acids and phenolic acid esters constituted 20.0 % and 21.2 %, respectively, of total phenolic acids in SB berry pulp. The total phenolic acid content in seed kernel (5,741 mg/kg) was higher than that in berry pulp and seed coat. Gallic acid was the predominant phenolic acid both in free and bound forms in SB berry parts and leaves (Arimboor et al. 2008).

The oligomeric fraction accounted for 84 % of the total proanthocyanidins and 75 % of the total antioxidant activity of the sea buckthorn pomace extract. The mean degree of polymerization of the oligomeric proanthocyanidins was between 6 and 9 (Rösch et al. 2004a, b).

5.4.3 Neutral Lipids and Fatty Acids

The presence of more than 20 lipid fractions including alkanols, sterols, pentacyclic triterpenols and high molecular weight aliphatic alcohol ethers were identified in the oils of the fruit pulp and seeds of the sea buckthorn, *H. rhamnoides*. Eighty percent of the oil carotenoids are epoxy derivatives of β-carotene. Two unsaturated C20 and C25 isoprene homologs were found in the fruit pulp oil (Zhmyrko et al. 1984, 1987; Mironov et al. 1983; Salenko et al. 1982).

The main constituents of neutral lipids are waxes (fatty acids C20–C26 aliphatic alcohols esters) concentrated in skin of the fruit and in seed, C23–C29 hydrocarbons (most of the surface layer of shells) and "phytosterols" (70–100 % β-sitosterol, α- and β-amyrins, erythrodiol, and other constituents of the "unsaponifiable" lipid fraction of seeds) (Mironov et al. 1983, 1989).

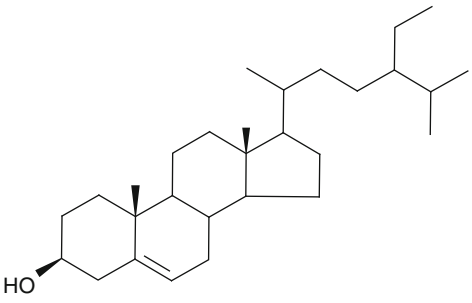
Twenty-five hydroxy and epoxy fatty acids were identified in *H. rhamnoides* seed oil by GC—mass spectrometry. Coriolic and dimorphecolic acids were the main hydroxyacids (Zhmurko et al. 1986, 1989). Carbon dioxide extract is rich in unsaturated aldehydes (15.23 %), tocopherols (10.46 %), five-ring triterpenes (amyrins 2.55 %), steroids, etc. The unsaturated fatty acids content are nearly 90 % of total fatty acids (Li and Zhou 1996). Typical GC-MS fingerprint is shown on Fig. 5.2. Unsaturated fatty acids are predominantly located at the carbon 2 of glycerol moiety of triacylglycerols isolated from *H. rhamnoides* seed oil

(Ozerina et al. 1987). *Comparison of* the oils from *H. rhamnoides* L fruit growing in Siberia, Central Asia, Baltic region, and Caucasus shows that the fatty acid composition and molecular types of TAG do not vary significantly (Ozerinina et al. 1997). 1, 3-Dicapryloyl-2-linoleoylglycerol, a novel triglyceride, was recently isolated from berries of *H. rhamnoides*. The metal-chelating-, superoxide ion-scavenging-, and radiation-induced lipid peroxidation inhibiting activity of the compound were estimated (Swaroop et al. 2005).

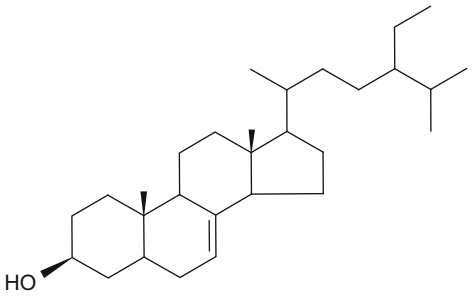
5.4.3.1 Sterols, Sterol-Glycosides, and Acylated Sterol Glycosides

The main components of the nonsaponified part of sea buckthorn fruit pulp extract are fatty alcohols, β-sitosterol, campesterol, 24-methylecycloartanol as well as β- and α-amyrins, erythrodiol, uvaol, citrostadienol, and 24-ethylcholest-7-en-3β-ol (Salenko et al. 1982). Acylated steryl glucosides were also isolated and identified from defatted seed (Jiang et al. 1988). It has been reported that sitosterol-β-D-glucoside in the dose of 12 mg/kg inhibits acetic acid-induced chronic gastric ulcer in rats and mice (Jiang et al. 1988) Both the glucoside and its aglycone showed antiulcer activity in chronic acetic acid-induced gastric ulcer models, and their effects were at least comparable to the effects of wishupin in combination with cimetidine (Xiao et al. 1992).

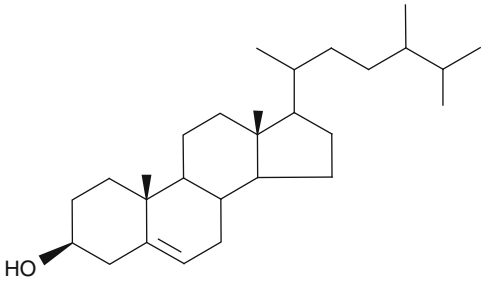
The maximal concentration of β-sitosterol in the carbon dioxide extract is about 0.5 % (Sajfrtová et al. 2010). The total sterol contents in the seeds, the fresh pulp/peel, and the whole berries were 1,200–1,800, 240–400, and 340–520 mg/kg, respectively, while sitosterol constituted 57–76 and 61–83 %, respectively, of the seed and pulp/peel sterols The sterol content and composition showed little variation between subspecies and collection sites. Different harvesting dates showed significant effects on the levels of some sterols both in the seeds and in the pulp/peel (Yang et al. 2001).



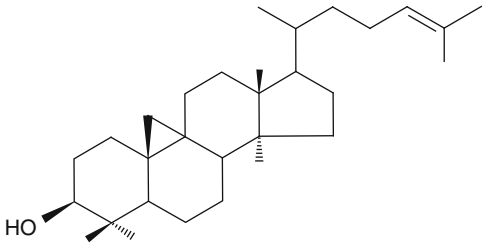
β-sitosterol



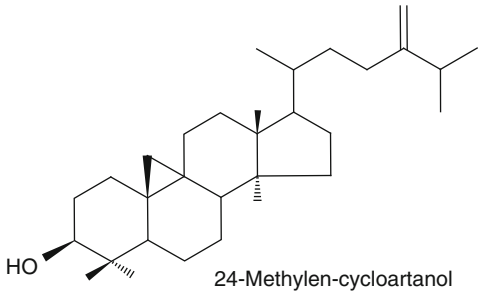
24-Ethyl-cholest-7-en-3β-ol



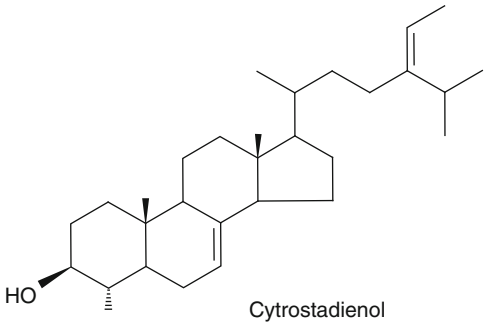
Campesterol



cycloartenol



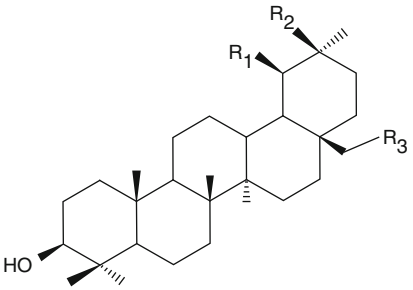
24-Methylen-cycloartanol



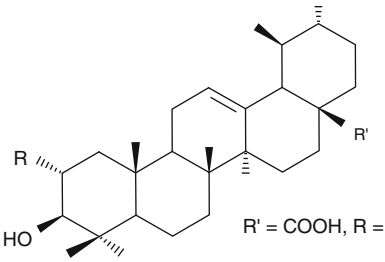
Cytrostadienol

5.4.4 Pentacyclic Terpenoids

First pentacyclic triterpene, identified in *H. rhamnoides*, was ursolic acid (Novruzov et al. 1980). Then, α- and β-amyrins, erythrodiol, and uvaol were identified in the nonsaponified part of sea buckthorn fruit pulp extract (Salenko et al. 1982). Further, 14 diterpene compounds and 11 fractions containing fatty aliphatic and polycyclic alcohols were identified using GLC, PMR, and chromatography mass spectrometry (Salenko et al. 1986). Finally, a new cycloartane terpenoid from the oils of sea buckthorn fruit was isolated (Glazunova et al. 1994) Maslinic and 2-β-hydroxyursolic acids from sea buckthorn leaves were also isolated (Kukina and Raldugin 1992).



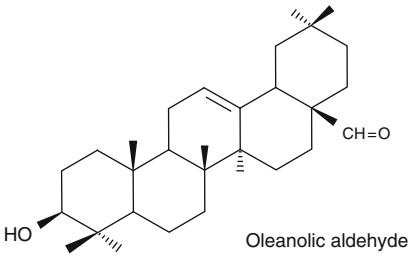
	R_1	R_2	R_3	
I	H	CH_3	H	β- amyrin
II	CH_3	H	H	α-amyrin
III	CH_3	H	OH	uvalol
IV	H	CH_3	OH	erythrodiol



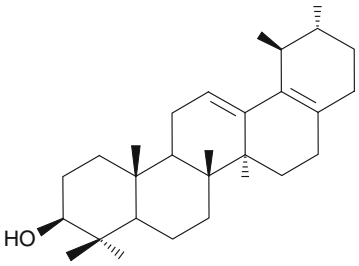
R' = COOH, R = H Ursolic acid

R' = COOH, R = OH 2-Hydroxy ursolic acid

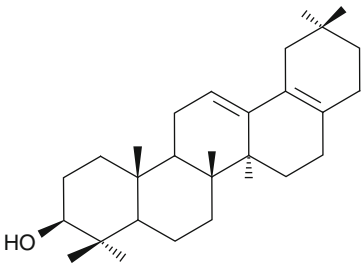
R' = CHO, R = H Ursolic aldehyde



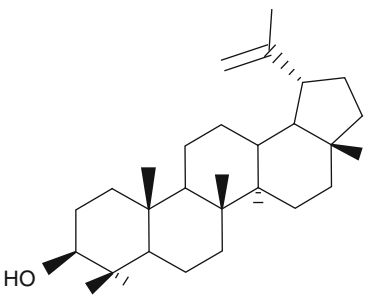
Oleanolic aldehyde



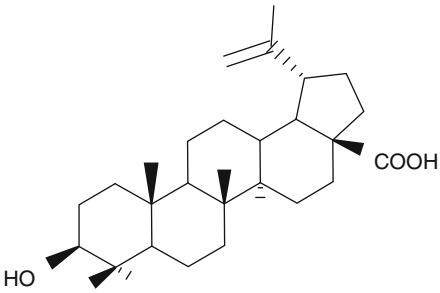
28-norurs-12,18(17)-dien-3β-ol



28-norolea-12,18(17)-dien-3β-ol



lupeol



betulinic acid

5.4.5 Phospholipids

Phospholipids content in seeds of *H. rhamnoides* is 0.2–0.5 %; among them inositol-containing phospholipids comprise 8.9 %, phosphatidylcholines 26.7 %, phosphatidylserines 5.8 %, and phosphatidylethanolamines 13.1 % (Isamukhamedov and Akramov 1983; Lagazidze et al. 1984).

5.4.6 Aromatic Volatile Oil

A total of 60 volatile compounds were identified in sea buckthorn fruit by GC-MS. The aroma of sea buckthorn fruit was characterized by the presence of several aliphatic esters such as ethyl, 3-methylbutyl, and *cis*-3-hexen-1-yl esters. The most

important compounds were ethyl hexanoate, 3-methylbutyl 3-methylbutanoate, 3-methylbutanoic acid, 3-methylbutyl hexanoate, 3-methylbutyl benzoate, and 3-methylbutyl octanoate (Hirvi and Honkanen 1984).

H. rhamnoides L. subsp. sinensis Rousi (Zhongguoshaji) contains aromatic volatile oil (0.036 %), which contains 95 individual compounds as detected by GC and GC-MS. The mixture comprised alkanes, alkenes, aromatic hydrocarbons, aldehydes, acetals, ketones, esters, terpenoids, and considerable free fatty acids (up to 30 %). 1,1-Diethoxy-*n*-tetradecane (myristic aldehyde acetal), one of the components, is a new natural organic compound (Yu et al. 1988).

5.4.7 Organic Acids

3.5–4.49 % organic acids, mainly malic and succinic acids were found in fruits of *H. rhamnoides* (Gao and Qiao 1985).

5.4.8 Amino Acids

Sea buckthorn is rich in proteins and free amino acids. A total of 18 amino acids have been found in sea buckthorn fruit (Zhang et al. 1989a, b; Mironov et al. 1989; Mirgaesiev 1992; Ji 1989).

5.4.9 Sugars, Inositols, and Polysaccharides

Alcohol insoluble substance (AIS), comprising about 4.70 % of the fresh berries, were fractionated into a water-soluble and a water-insoluble part. In these fractions Glc, Gal, and Xyl were the predominant neutral saccharide units. Ara, Rha, and Man were present in smaller amounts. A higher GalA content (in gm per 100 original AIS) was found in the soluble fractions. The composition of the AIS was determined as 9.25 % pectin, 34.8 % protein, and 38.9 % total polysaccharides (Dongowski 1996). One of water soluble neutral polysaccharide JS1 consisting of Ara, Xyl, Gal, Glc in the ratio of 1:6:12:4 was isolated in pure state by Wang et al. (1999) Similar composition of monosaccharides units Xyl, Ara, Glc, Gal, GaL A has SJ22 isolated from Hippophae rhamnoides leaves and scavenging superoxide radical and hydroxyl radical (Liu et al. 2006a, b). Sea buckthorn (*H. rhamnoides* L.) berries, especially of ssp. sinensis, contain significant quantities of unknown,

water-soluble compounds, evidently cyclitol derivatives. One of them was isolated in a pure state and identified as (−)-2-*O*-methyl-L-chiro-inositol (L-quebrachitol). In addition, methyl-myo-inositol, chiro-inositol, and myo-inositol existing in trace amounts were identified (Kallio et al. 2009).

5.4.10 Alkaloids

Two alkaloids, harman and harmaline, were identified in *H. rhamnoides* (Gill and Raszeja 1971).

5.4.11 Chemical Elements and Trace Minerals

There are at least 24 chemical elements present in sea buckthorn juice (Beveridge et al. 1999; Wolf and Wegert 1993; Zhang et al. 1989a, b; Tong et al. 1989). Potassium is the most abundant (140–360 ppm) of all the elements investigated in berries or juice (Sabir et al. 2005; Tong et al. 1989; Zhang et al. 1989a, b; Zeb 2004). Sea buckthorn contains 11 out of 14 essential trace minerals. Toxic metals content in different varieties of sea buckthorn was studied (Beveridge et al. 1999; Lobacheva and Letchamo 1998).

5.5 General Identity Tests

Along with macroscopic and microscopic examinations, commonly used TLC, HPLC, and GC fingerprints are normally employed for identification of sea buckthorn extracts, e.g., Figs. 5.1 and 5.2. Unusually high amount of palmitoleic acid in sea buckthorn oil is specifically used for the identification; Fig. 5.2.

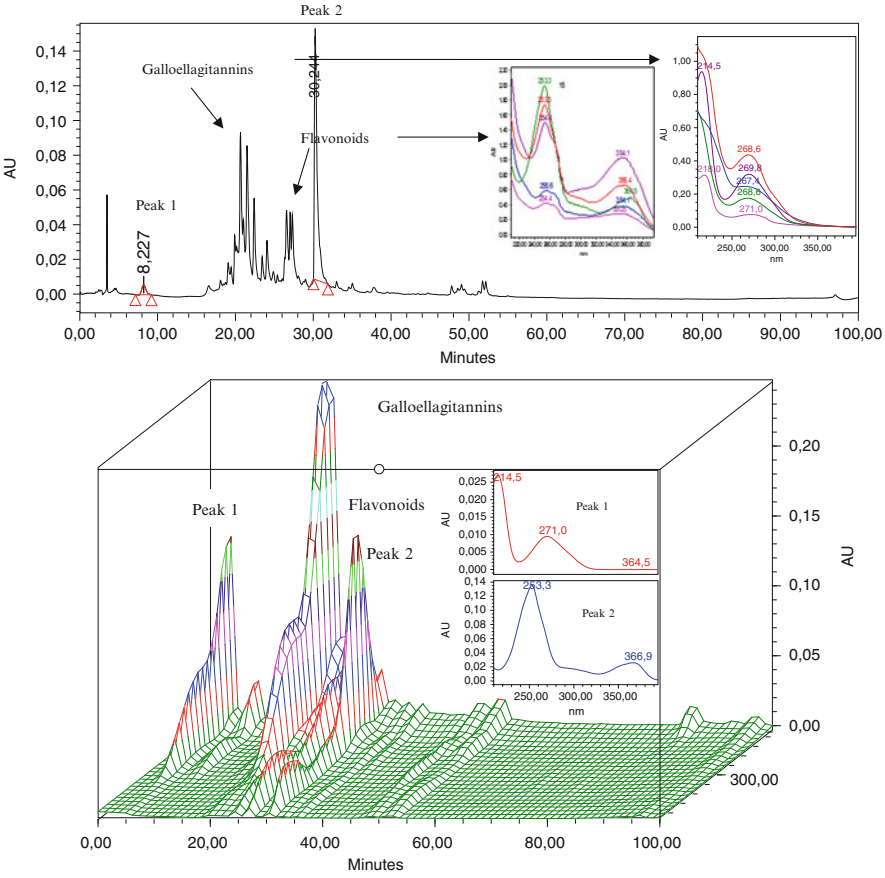


Fig. 5.1 Typical 3D HPLC-DAD fingerprint (*lower panel*) of *Hippophae rhamnoides* leaves water extract. *Peaks*: (1) gallic acid and (2) isorhamnetin 3-*O*-glucoside. *Upper panel*—detection at 254 nm. *Stationary phase*: SunFire™ C_{18}, packed with octadecyl silica (4.6 × 250 mm i.d., 5 μm particle size), No 01051430610 F16, Waters. *Mobile phase*: gradient of acetonitrile, containing 1 % (v/v) 0.1 N ortho phosphoric acid in 1 % (v/v) 0.1 N ortho phosphoric acid, from 0 5 % (5 min) to 95 % (90 min)

5.6 Chemical Assays

In accordance with the standards of Russian Federation (VFS 42-1741-87) total carotenoids content in fresh fruit must be not less than 90 mg% (calculated for dry herb); the content of tannins in leaves—not less than 15 % (calculated for casuarinin content).

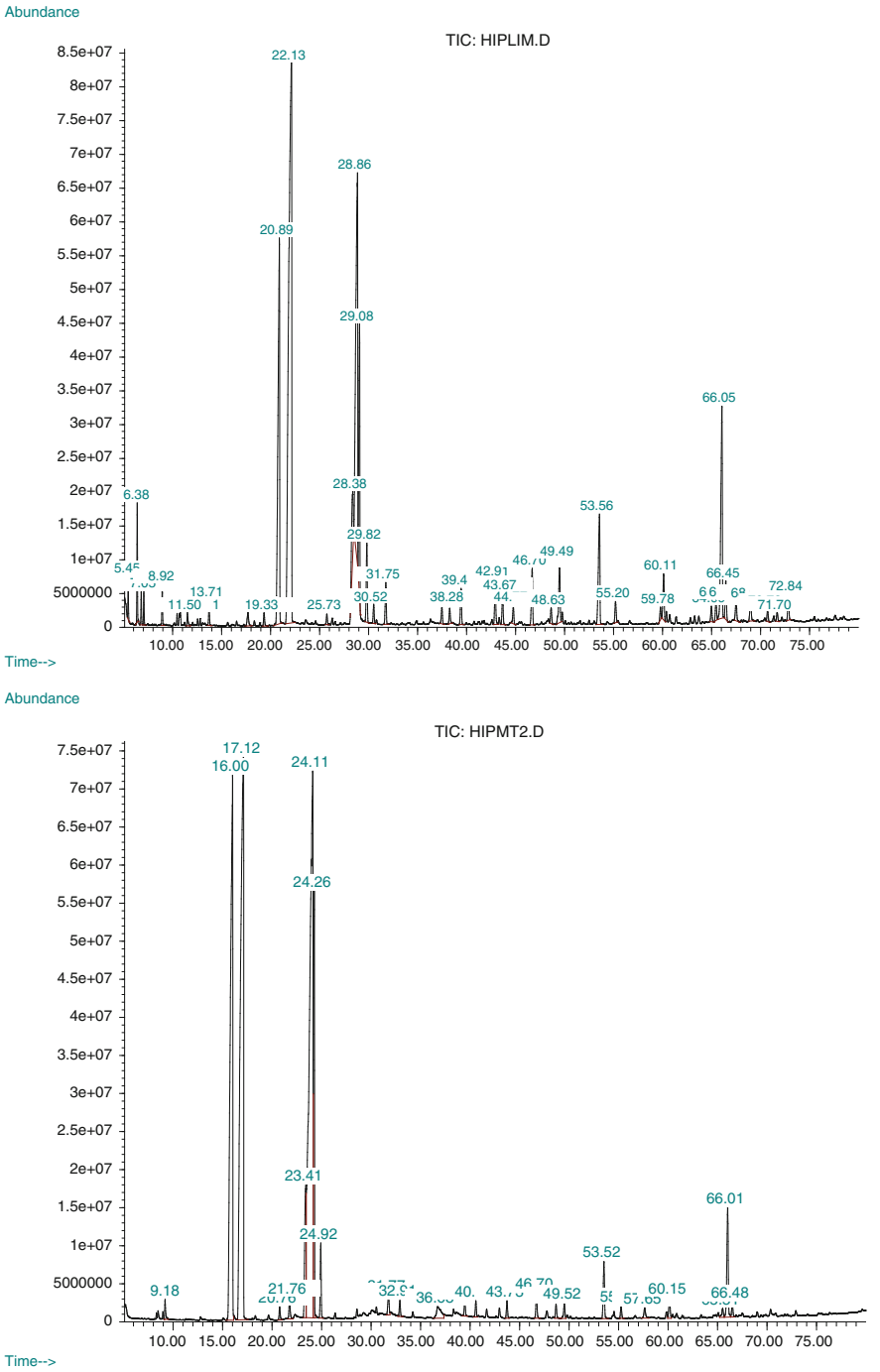


Fig. 5.2 (**a**) GC-MS fingerprint of Fructus hippophae carbon dioxide extract (TMS derivatives of free fatty acids (palmitoleic 20.89 min, hexadecanoic 22.13 min, 9,12-octadecadienoic acid 28.38 min, oleic acid 28.86 min, stearic acid 29.82 min), β-sitosterol (66.05 min), β-amyrin

Various new methods of analysis of active constituents in the extracts of herbal substance, herbal preparations, and biological fluids were developed during last decade (Guliyev et al. 2004; Pintea et al. 2005; Zhang et al. 1989a, b; Zu et al. 2006; Yue and Shi 2006; Tiitinen et al. 2005; Yang and Kallio 2006; Chen et al. 2007; Gorbatsova et al. 2007; Gutzeit et al. 2007a, b; Sharma et al. 2008; Arimboor et al. 2008).

5.7 Pharmacology

5.7.1 Experimental Pharmacology

The pharmacological activity of sea buckthorn oil is associated with:

- Tissue regeneration promoting and wound healing effects
- Tissue reparative effects in experimental eye burns
- Cytoprotective, healing, and preventive effects in stomach, duodenal, and trophic ulcers radiation and burns induces injuries
- Hepato-protective action
- Stimulation of immune system
- Antitumor activity
- Antibacterial (bacteriostatic) activity
- Anti-inflammatory effect
- In antioxidant effects (superoxide dismutase activity in mice, lipid peroxidation, superoxide radicals scavenging)
- Hypolipidemic and antiatherogenic activity
- Hypoglycemic effect

It has been shown that flavonoids isolated from sea buckthorn fruits and leaves have multiple effects on the cardiovascular system including:

- Dilating coronary arteries, increasing myocardial blood flow, decreasing myocardial O_2 consumption, and improving myocardial microcirculation

Fig. 5.2 (continued) (65.45 min), and ursa-9(11),12-dien-3-one (70.73 min). (**b**) GC-MS fingerprint of total (free and bound in glycerolipids) fatty acids after methanolysis of Fructus hippophae carbon dioxide extract—methyl esters of palmitoleic 20.89 min, hexadecanoic 22.13 min, 9,12-octadecadienoic acid 28.38 min, oleic acid 28.86 min, and stearic acid 29.82 min), β-sitosterol (66.05 min), β-amyrin (65.45 min), and ursa-9(11),12-dien-3-one (70.73 min). Free fatty acids content—6.2 %, triacylglycerols—13.6 %, and sterols—0.7 % of total extract. Column: HP-5MS Cross-linked 5 % Methyl Siloxane, 30.0 m × 0.25 mm × 0.25 um film max. Temperature—320°C. Oven: 140°C(hold 5.0 min; to 305°C at 2°C/min; hold 10.0 min). Carrier gas: Helium, 42 cm/s, 15.2 psi at 140°C with EPC, Flow:1.2 mL/min. Injection: Pulsed Splitless, 2 μL, Inlet temperature of 250°C. Detector: Electron impact ionization, 70 eV, Total ion current mode, Detector temperature of 285°C

- Antiarrhythmic effects
- Relaxation of vascular smooth muscles
- Antagonistic effect on transmembrane Ca2+ channels
- Antagonizing inward flow of Ca2+ and accelerating the move of k+ outward
- Inhibition of Ca2+ influx and its interference with intracellular Ca2+ reservoir strengthening of cardiac pump function and myocardial contractility in canine with heart failure
- Improvement myocardial diastolic function and hemodynamic performance
- Decrease of the myocardia-used oxygen index and the total peripheral vessel resistance
- Ca-antagonizing action and inhibition of adenylate cyclase, with consequent reduction in the cAMP level
- Protective effect on myocardial ischemic reperfusion injury
- Increased PGI2 secretion and the PGI2/TXA2 ratio in plasma, inhibitory action on thrombosis

5.7.1.1 Antiviral Activity

Antiviral activity of sea buckthorn leaves is associated with galloellagotannins, comprising 60 % of Hiporamin—a dry purified extract of *H. rhamnoides* leaves (*H. rhamnoides* L., Elaeagnaceae), standardized for the content of total galloellagotannins calculated for casuarinin (Fadeeva et al. 1988). This medicinal product was developed in USSR and registered in Russian Federation since 1998 as an OTC antiviral drug [Hiporamin, Sea buckthorn leaves extract (2002)]. The tablets containing 20 mg of Hiporamin have been studied clinically, while the active galloelagotanins have been investigated in various bioassays (Table 5.6).

It has been found that Hiporaminum is active against various influenza A and B viruses adenoviruses, paramyxoviruses, simple herpes viruses, *Varicella zoster* (variola, herpes zoster), cytomegalovirus, respiratory-syncytial viruses (Zamkovaya 2003). The mechanism of action is associated with inhibition of viral neuraminidase. The inhibition of viral neuraminidase alters the synthesis of virion and consequently reproduction of viruses. Moreover, Hiporamin stimulates immune system, increasing interferon level in the blood of patients. Hiporamin inhibits growth of Gram-positive and Gram-negative bacteria, tuberculosis mycobacterium, and fungus (Candida) (Zamkovaya 2003).

It has been reported that *H. rhamnoides* leaf extract decreases TNF-alpha and increases IFN-gamma and the viability of Dengue-infected blood-derived human macrophages. Symptomatic dengue virus infection ranges from a self-limited febrile illness, dengue fever (DF), to a more severe disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). The anti-dengue treatment is severely hampered as no specific therapeutic agents are available. These observations suggest that the SBT leaf extract has a significant anti-dengue activity and has the potential for the treatment of dengue (Jain et al. 2008).

5.7.1.2 Cytoprotective Effect and Radioprotection

Cytoprotective, reparative, tissue regenerative activity of see buckthorn fruit, leaves, and bark extracts, associated with radiation, burn, trauma, bacterial infection-induced injuries has been documented in many in vivo and in vitro studies (Table 5.6).

Thus, oral administration of sea buckthorn fruit extract, before and after of a single or fractionated X-ray irradiation of rats at dose of 1 Gy, was accompanied by the increase of the animals' average life, the restoration of 11-oxicorticosteroids' level in blood, and of weight of isolated adrenals and also the normalization of their basal activity and response to ACTH in vitro conditions (Mizina and Sitnikova 1999). Similar radioprotective effect has been demonstrated in experiments in mice where *H. rhamnoides* berries extract RH-3 rendered 82 % survival as compare to no survival in irradiated control. Various hematological parameters also corroborated the radioprotective effect of RH-3 in a dose-dependent manner (Goel et al. 2002, 2003a; Agrawala and Goel 2002; Chen et al. 2003; Prakash et al. 2005). RH-3-inhibited radiation induced DNA strand breaks in a dose-dependent manner (Kumar et al. 2002; Goel et al. 2003b; Shukla et al. 2006). RH-3 treatment protected spermatogenesis by enhancing the spermatogonial proliferation, enhancing the stem cell survival, and reducing sperm abnormalities (Goel et al. 2006). RH-3 was observed to mitigate radiation-induced cellular and mitochondrial free radicals in isolated U 87 cells, suggesting that *H. rhamnoides* berries extracts act as an antioxidant, preventing cellular and mitochondrial free radical generation, that could contribute to its ability to inhibit radiation-induced apoptosis and cytotoxicity (Goel et al. 2002; Agrawala and Adhikari 2009; Chawla et al. 2007).

5.7.1.3 Antioxidant Effect

The antioxidant, cytoprotective, and antibacterial effects of aqueous and hydroalcoholic extracts of *H. rhamnoides* L. leaves were also investigated. Both extracts possess cytoprotective activity against hydrogen peroxide and hypoxanthine–xanthine oxidase induced damage to BHK-21 cell line. (Upadhyay et al. 2010). Similar antioxidant, cytoprotective effect of alcoholic leaf extract of sea buckthorn was observed in experiments in hypoxia-induced oxidative stress in C-6 glioma cells. Pretreatment of cells with alcoholic leaf extract of SBT significantly inhibited cytotoxicity, ROS production, and maintained antioxidant levels similar to that of control cells. Further, the leaf extract restored the mitochondrial integrity and prevented the DNA damage induced by hypoxia. (Narayanan et al. 2005). The butanol extract from sea buckthorn leaves along with quercetin 3-*O*-β-D-glucopyranoside had a higher antioxidant (DPPH radical-scavenging) activity. The butanol fraction contained the highest amount of phenolic compounds and the most powerful α-glucosidase inhibitory effect (86 %) (Kim et al. 2011). Sea buckthorn water–acetone and alcoholic extracts prevent lipid peroxidation due to the ability to annihilate free radicals and had a more

Table 5.4 Chemical composition and plant parts where these compounds were identified

Compounds	Plant parts	References
Vitamin A—carotenoids (β-carotene, zeaxanthin, lutein, β-cryptoxanthin, and other xanthophylls)	Fruit	Zhmyrko et al. (1978, 1984), Mironov et al. (1983, 1989), Lagazidze et al. (1984), Crapatureanu (1996), Sergeeva et al. (1979), Malena et al. (1984), Bernath and Foldesi (1992), Wolf and Wegert (1993), Bernath and Foldesi (1992), Andersson et al. (2009), Pintea et al. (2005)
Vitamin B5—pantothenic acid	Fruit	Gutzeit et al. (2007a, b)
Vitamin C—ascorbic acid	Fruit	Centenaro et al. (1978), Haranovich (1981), Malena et al. (1984), Bernath and Foldesi (1992), Lu (1992, 2005), Rousi and Aulin (1977), Plekhanova 1988; Yao et al. (1992), Yang et al. (1988), Yao and Tigerstedt (1994), Yao et al. (1992), Haranovich (1981), Jeppsson and Gao (2000), Kallio et al. (2002a, b), Gutzeit et al. (2008a, b)
Vitamin E—α-,β-, and γ-tocopherols	Fruit	Dalgatov et al. (1985), Zhang et al. (1989a, b), Eliseev (1989), Lagazidze et al. (1984), Huang and Xiao (1991), Huang and Xiao (1994), Fu et al. (1993), Bernáth and Földesi (1992), Kallio et al. (2002a, b), Andersson et al. (2009), Li and Zhou (1996)
Vitamin F—fatty acids (palmitic, oleic, palmitoleic, linoleic and linolenic, vaccenic)	Fruit, seed	Zheng et al. (2009), Gutzeit et al. (2007a, b), Undina et al. (1989), Franke and Mueller (1983), Bereznaya et al. (1988), Hethelyi et al. (1989), Loskutova et al. (1989), Khusainov et al. (1988), Suleyman et.al. (1998), Yang and Kallio (2001), Kallio et al. (2002a, b), Berezhnaya et al. (1988), Mamedov et al. (1981), Zham'yansan (1979), Lobanova (Lobanova et al. 1992), Li and Zhou (1996)
Vitamin K—phylloquinone	Fruit	Gutzeit et al. (2007a, b)
Vitamin P—flavonoids (isorhamnetin, quercetin, naringin, naringenin, kaempferol)	Fruit, leaf	Horhammer et al. (1966), Krolikowska (1972), Rasputina et al. (1975, 1976), Purve et al. (1979), Lachman et al. (1985), Zhang et al. (1988a, b, 1989a, b), Solonenko and Shishkina (1989), Fu et al. (1997), Wang et al. (1982), Beljanski (1989), Rösch et al. (2004a, b), Zu et al. (2006), Yang et al. (1998), Zhang et al. (1998), Jeppsson and Gao (2000), Yoshida et al. (1991)

(continued)

Table 5.4 (continued)

Compounds	Plant parts	References
Phenolic compounds (gossypol, condensed and hydrolyzable tannins, hippophaenins A and B, gallic, caffeic, *p*-coumaric, sinapic, ferulic, ellagic and chlorogenic, protocatechuic, gentisic, *p*-hydroxybenzoic, syringic acids, (+)-gallocatechin)	Leaf	Zhang et al. (1996), Mukhamed'yarova and Chumbalov (1980), Novruzov et al. (1983), Kukina et al. (1991), Dembinska-Migas (1988), Sheichenko et al. (1987), Fadeeva et al. (1988), Yoshida et al. (1991), Dembinska-Migas (1988), Arimboor et al. (2008), Rösch et al. (2004a, b)
Aliphatic hydrocarbons	Fruit, seed	Mironov et al. (1983, 1989)
Fatty acids asters of alkanols, pentacyclic triterpenols, sterols, ethanol, methanol	Fruit, seed	Mironov et al. (1983, 1989), Zhmyrko et al. (1984, 1987)
Triacylglycerols	Fruit, seed	Zhmyrko et al. (1984, 1987), Li and Zhou (1996), Salenko et al. (1986), Swaroop et al. (2005)
Hydroxyacyldiacylglycerols	Fruit seed	Zhmyrko et al. (1984, 1987)
Epoxyacylhydroxyacylmonoacylglycerols	Fruit, seed	Zhmyrko et al. (1984, 1987)
n-alkanols	Fruit, seed	Mironov et al. (1983, 1989), Salenko et al. (1982)
C20 and C25 isoprenols unsaturated	Fruit	Zhmyrko et al. (1984, 1987)
Unsaturated and 5-hydroxymethyl-2-furancarbox-aldehydes	Fruit	Zheng et al. (2009), Li and Zhou (1996)
Unsaturated isoprene homologs C20 and C25		
Hydroxy- and epoxy-fatty acids (coriolic and dimorphecolic, 11-hydroxy-9-tridecenic, 9-hydroxy-10,12-pentadecadienic, 13-hydroxy-9,11-hexadecadienic and 9,12-dihydroxy-15-nonanedecenic acids)	Fruit, seed	Zhmurko et al. (1986, 1989)
Phospholipids (inositol-containing phospholipids, phosphatidylcholines, phosphatidylserine, and phosphatidylethanolamine)	Fruit, seed	Lagazidze et al. (1984), Isamukhamedov and Akramov (1983), Pintea et al. (2001)
Cerebrosides	Fruit	Zheng et al. (2009)
Sterols (β-sitosterin, 24-methylecycloartanol, citrostadienol and 24-ethylcholest-7-en-3 β-ol), sterol-glycosides (β-sitosterol, β-D-glucoside), and acylated sterol glycosides	Fruit, seed	Mironov et al. (1983, 1989), Lagazidze et al. (1984), Li and Zhou (1996), Salenko et al. (1982), Jiang et al. (1988), Xiao et al. (1992), Sajfrtová et al. (2010), Yang et al. (2001)
Pentacyclic terpenoids (α- and β-amyrins, erythrodiol and uvaol, ursolic, 19-α-hydroxyursolic, oleanolic, maslinic, 2-hydroxyursolic, dulcioic acids, and cycloartranes)	Fruit, leaf, bark	Mironov et al. (1983, 1989), Zheng et al. (2009), Li and Zhou (1996), Novruzov et al. (1980), Glazunova et al. (1994), Kukina and Raldugin (1992), Glazunova et al. (1994), Yang et al. (2007)

(continued)

Table 5.4 (continued)

Compounds	Plant parts	References
Aromatic volatile oil (ethyl hexanoate, 3-methylbutyl 3-methylbutanoate, 3-methylbutanoic acid, 3-methylbutyl hexanoate, 3-methylbutyl benzoate, and 3-methylbutyl octanoate, alkanes, alkenes, aromatic hydrocarbons, aldehydes, acetals, ketones, esters, terpenoids, 1,1-diethoxy-*n*-tetradecane (myristic aldehyde acetal))	Fruit	Hirvi and Honkanen (1984), Yu et al. (1988)
Organic acids (malic, succinic, citric, tartaric)	Fruit	Gao and Qiao (1985)
Amino acids (a total of 18 amino acids)	Fruit	Zhang et al. (1989a, b), Mironov et al. (1989), Mirgaesiev (1992), Ji (1989)
Sugars (glucose, fructose, and xylose) and polysaccharides (consisted of Ara, Xyl, Gal, Glc)	Fruit	Dongowski (1996), Wang et al. (1999), Liu et al. (2006a, b)
Alkaloids (harman and harmaline)	Fruit	Gill and Raszeja (1971), Kondorskaya (1973), Pasich et al. (1984)
(−)-2-*O*-methyl-L-chiro-inositol (L-quebrachitol), chiro-inositol, Methyl-myo-inositol, and myo-inositol	Fruit	Kallio et al. (2009)

A number of compounds, e.g., 2-*O*-*trans*-*p*-coumaroyl maslinic acid, 2-*O*-caffeoyl maslinic acid, 1,3-Dicapryloyl-2-linoleoylglycerol, 1,1-diethoxy-*n*-tetradecane (myristic aldehyde acetal), hippophae cerebroside, 19-alpha-hydroxyursolic acid, dulcioic acid, 5-hydroxymethyl-2-furancarbox-aldehyde, cirsiumaldehyde, and 1-*O*-hexadecanolenin were obtained from the genus for the first time (Zheng et al. 2009; Swaroop et al. 2005; Yu et al. 1988; Yang et al. 2007)

Table 5.5 Main constituents of sea buckthorn oils from seed, fruit pulp (juice), and fruit residue after removing juice (Beveridge et al. 1999)

Concentration (mg/100 g)			
Ingredient	Seed oil	Pulp oil	Fruit residue oil
Vitamin E	207	171	300–600
Vitamin K	110–230	54–59	–
Carotenoids	30–250	300–870	1,280–1,860
Total acids	11	38	–
Total flavonoids	–	–	550
Total sterols	1,094	721	–
Unsaturated fatty acids	87 %	67 %	70 %
Saturated fatty acids	13 %	33 %	30 %

antioxidant capacity than BHT and BHA in different tests including scavenging of 1,1-diphenyl-2-picryl-hydrazil radical (DPPH), superoxide anion radical ($O_2^{\cdot -}$), and total antioxidant activity (Papuc et al. 2008).

Table 5.6 Pharmacological profile of *Hippophae rhamnoides* extracts

Pharmacological activity	Product/plant part	References
Cytoprotective and antioxidant effect, radioprotection	Oil, fruit juice, and extracts; aqueous and hydroalcoholic extracts of leaves	Goel et al. (2002, 2003a), Agrawala and Goel (2002), Chen et al. (2003), Prakash et al. (2005), Kumar et al. (2002), Goel et al. (2003b), Shukla et al. (2006), Goel et al. (2004, 2005, 2006), Agrawala and Adhikari (2009), Chawla et al. (2007), Kim et al.(2011), Geetha et al. (2009), Rösch et al. (2004a, b), Yang et al. (2007)
Reparative effects in experimental burns and wound healing	Fruit, seed, oil, leaf	Upadhyay et al. (2009), Wang et al. (2006), Oana et al. (1994), Neamtu and Cociu (1982), Ianev et al. (1995), Upadhyay et al. (2009), Wang et al. (2006), Ianev et al. (1995), Nikulin et al. (1992), Mironov et al. (1989), Upadhyay et al. (2009), Gupta and Flora (2005), Gupta et al. (2006), Fu et al. (2005)
Anti-ulcerogenic activity	Fruit, seed, oil	Loginov et al. (1983), Jiang et al. (1988), Zhu et al. (1997), Nuzov and Stadnikov (1994), Suleyman et al. (1998, 2001a, b), Xiao et al. (1992), Xiao and Wen (1996), Khizhazi (1998), Jiang et al. (1988), Xiao et al. (1992), Xiao and Wen (1996), Suleyman et al. (1998, 2001a, b), Nuzov and Stadnikov (1994), Xing et al. (2002), Li et al. (2005)
Hypolipidemic and antiatherogenic	Oil, fruit juice, seed	Olziikhutag (1968), Chou et al. (1986), Basu et al. (2007), Liu et al. (1980), Pang et al. (2008), Zhang et al. (2010)
Effects on the cardiovascular and blood coagulating systems	Fruit, oil, seed	Yu et al. (1990, 1992), Wu and Li (1990); Wu et al. (1994), (1997a, b), Liu et al. (1988), Wang et al. (1982), Wu et al. (1997a, b), Cheng et al. (1999), Xu and Chen (1991), Koyama et al. (2009), He et al. (2009), Purushothaman et al. (2008), Liu et al. (2008), Pang et al. (2008), Zhu et al. (2005), Cheng et al. (2003)
Hypoglycemic effect	Fruit, seed, water extract	Lehtonen et al. (2010), Pang et al. (2008), Zhang et al. (2010)
Antibacterial	Oil	Shustrova and Pashkin (1997)

(continued)

Table 5.6 (continued)

Pharmacological activity	Product/plant part	References
Hepato-protective	Fruit juice, seed, oil	Vengerovskii et al. (1994), Cheng et al. (1994), Cheng (1992), Wang et al. (1992a, b), Cheng et al. (1990), Mansurova et al. (1978), Hsu et al. (2009), Liu et al. (2006a, b)
Anti-inflammatory	Leaf	Sabynch et al. (1994); Ganju et al. (2005)
Antiviral	Leaf, Hiporamin	Zamkovaya (2003), Jain et al. (2008)
Effect on immune system	Fruit, juice, oil	Zhong et al. (1989), Xu et al. (1994), Spinu et al. (1996), Jain et al. (2008), Mishra et al. (2008), Ramasamy et al. (2010), Geetha et al. (2005)
Antitumor activity	Bark, seed, oil, fruit juice	Pukhalsskaia (1958), Sokoloff et al. (1961), Zhang et al. (1989a, b), Yu et al. (1993), Beljanski (1989), Abartiene and Malachovskis (1974), Spiridonov et al. (1997), Sauter and Wolfensberger (1989), Li and Liu (1989, 1991), Sun et al. (2003), Yasukawa et al. (2009), Teng et al. (2006), Zhang et al. (2005), Hibasami et al. (2005), Padmavathi et al. (2005)
Anti-mutagenic	Fruit, oil	Nersesyan et al. (1990)
Stress protective, antitoxic and adaptogenic activity	Oil, roots and shoots, bark and sprout extract, leaf	Son et al. (1997), Stakheeva and Zueva (1993), Krylova et al. (2000), Saggu et al. (2007), Saggu and Kumar (2007, 2008), Vijayaraghavan et al. (2006), Gupta and Flora (2005), Gupta et al. (2006), Xu et al. (2005), Cao et al. (2003), Geetha et al. (2003)

Antioxidant activity of various phenolic compounds isolated from sea buckthorn fruit juice was investigated. Flavonols iso-rhamnetin 3-*O*-glycosides (predominating polyphenols) were poor radical scavengers as shown by electron spin resonance spectroscopy, while minor (their concentration in sea buckthorn juice was small) phenolic compounds such as quercetin 3-*O*-glycosides, catechins, and hydroxybenzoic acids with a catechol structure exhibited good antioxidant capacities. Ascorbic acid was shown to be the major antioxidant in sea buckthorn juice. Because of its high concentration of 1.22 g/L, it contributes approximately 75 % to total antioxidant activity. The remaining difference can be attributed to higher molecular weight flavan-3-ols (proanthocyanidins), which were determined photometrically after acid depolymerization to colored anthocyanidins (Rösch et al. 2004a, b).

Triterpenoids, 2-*O*-*trans*-*p*-coumaroyl maslinic acid and 2-*O*-caffeoyl maslinic acid, oleanolic acid, 3-*O*-*trans*-*p*-coumaroyl oleanolic acid, 3-*O*-caffeoyl oleanolic acid, 6-methoxy-2*H*-1-benzopyran, and β-sitosterol isolated from the branch bark extract were found to inhibit of the production of NO in RAW 264 (Yang et al. 2007).

5.7.1.4 "Metabolic Syndrome" Associated Effects: Hypoglycemic, Hypolipidemic, and Antiatherogenic Activity

Apparently, antioxidant activity of sea buckthorn extracts is associated with their hypoglycemic, hypolipidemic, and hypotensive effects.

Thus, an aqueous extract of sea buckthorn seed (AESS) residues has been reported to possess hypoglycemic, hypolipidemic and antioxidant properties in normal mice and streptozotocin-induced diabetic rats. The administration of extract significantly lowered the serum glucose, triglyceride, and nitric oxide levels in *diabetic* rats. Moreover, ASSR treatment also increased serum superoxide dismutase activity and glutathione level markedly, suggesting that ASSR supplementation can be useful in preventing diabetic complications associated with hyperlipidemia and oxidative stress (Zhang et al. 2010).

It has been reported that total flavones extracted from seed residues of *H. rhamnoides* L. significantly suppressed the elevated blood pressure, hyperinsulinemia, and dyslipidemia in chronic sucrose-fed hypertensive rats. Furthermore, flavones extract (at the dose of 150 mg/kg/day) increased the circulatory blood angiotensin-II level as effectivie as angiotensin-II receptor blocker, suggesting that it might have potential use in the management of hyperinsulinemia in non-diabetic state with cardiovascular diseases (Pang et al. 2008).

It was found that polymethoxylated flavonoid pentamethylquercetin is an active constituent in sea buckthorn extracts increasing adipocyte-derived hormone adiponectin mRNA expressions in time- and concentration-dependent manners (Chen et al. 2011). Up-regulation of adiponectin expression is known to be beneficial for metabolic disorders, including type 2 diabetes, hyperlipidemia, etc.

In fact, these findings provide an additional "medicinal label" to *H. rhamnoides* and bring it very close to another widely used tea preparation, the Rooibos-tea (Aspalathus linearis), which is known has beneficial effects on vascular function and favorably modulates metabolic complications (Beltrán-Debón et al. 2011; Pantsi et al. 2011).

5.7.1.5 Reparative Effects in Experimental Burns and Wound Healing

The lipophilic carbon dioxide or alcohol extracts from sea buckthorn seed or berries was found to have significant healing effect in experimental burn wounds model in animals (Upadhyay et al. 2009; Wang et al. 2006; Oana et al. 1994; Neamtu and Cociu 1982; Ianev et al. 1995). Thus, sea buckthorn seed oil topically applied or

coadministered by two routes at a dose of 2.5 mL/kg body weight (p.o.) and 200 μL (topical) for 7–10 days on experimental burn wounds in rats significantly augmented the wound healing process compared to controls: the epithelization is more intensive and occurs earlier, and granulation tissue differentiation is quicker (Upadhyay et al. 2009; Wang et al. 2006; Ianev et al. 1995)

It has been shown that Hippophae oil has therapeutic effect on chemical burns of rabbit eyes. The effect was most significant in the phases of trophic disturbances and epithelization (Nikulin et al. 1992).

It has been reported that wound healing and antiulcer activity of sea buckthorn fruits is associated with neutral lipids (waxes) concentrated in the pulp and skins of fruits. They significantly contribute in the reparative effect of sea buckthorn in experiments on rodents (Mironov et al. 1989).

Flavones comprise the second group of burn wounds healing active compounds isolated from leaves of sea buckthorn (Upadhyay et al. 2009; Gupta and Flora 2005). The lyophilized aqueous leaf extract of sea buckthorn was applied topically, twice daily for 7 days. Treatment with silver sulfadiazine ointment was used as reference control. The most effective concentration of the extract was found to be 5.0 % (w/w). The *H. rhamnoides*-treated groups of rats showed faster reduction in wound area in comparison with control and silver sulfadiazine-treated groups. The topical application of *H. rhamnoides* increased collagen synthesis and stabilization at the wound site, as evidenced by increase in hydroxyproline, hexosamine levels, and up-regulated expression of collagen type-III. Furthermore, there was significant increase in levels of endogenous enzymatic and nonenzymatic antioxidants and decrease in lipid peroxide levels in *H. rhamnoides*-treated burn wound granulation tissue. The sea buckthorn also promoted angiogenesis (Upadhyay et al. 2009; Gupta and Flora 2005).

First evidence of positive effect of Hippophae berries on the healing of experimental stomach ulcers were demonstrated by Loginov et al. 1983. Later on antiulcer effect of *H. rhamnoides* was demonstrated in several publications (Jiang et al. 1988; Zhu et al. 1997; Nuzov and Stadnikov 1994; Suleyman et al. 1998, 2001a, b; Xiao et al. 1992; Xiao and Wen 1996; Khizhazi 1998). Both therapeutic and prophylactic anti-ulcerogenic action of sea buckthorn (Hippophae) oils was shown in neurogenic ulcerative lesions caused by immobilization, noise, and vibration (Khizhazi 1998).

It has been shown that β-sitosterol-β-D-glucoside is an effective antiulcer constituent of *H. rhamnoides* seeds. *H. rhamnoides* seed oil at doses of 2.5 mL/kg and ***β*-sitosterol** β-*D*-**glucoside** at relatively small doses of 12 mg/kg had a significant protective effect on acetic acid-induced chronic gastric ulcer in rats and mice (Jiang et al. 1988). Both the **α-sitosterol-α-*D*-glucoside and its aglycone** showed antiulcer activity in chronic acetic acid-induced gastric ulcer models, and their effects were at least comparable to the effects of wishupin in combination with cimetidine. The effect of aglycone appears better than the glucoside's. The glucoside was significantly active in cold stress-induced ulcers, while wishupin was not (Xiao et al. 1992; Xiao and Wen 1996).

Oil of the fruits showed a dose-dependent and significant inhibitory effect against **ethanol-induced ulcerogenesis in rats**. Through bioassay-guided fractionation using solvent extraction, **carotenoids-rich fraction** of the fixed oil showed significant anti-ulcerogenic activity, while that of glyceride fraction was found to be weak (Suleyman et al. 1998).

5.7.1.6 Antitumor Activity

Research in the late 1950s and early 1960s reported that 5-hydroxytryptamine (hippophan) isolated from sea buckthorn bark inhibited tumor growth (Pukhalsskaia 1958; Sokoloff et al. 1961). More recently, antitumor effects of fruit juice and seed oil of *H. rhamnoides* and their influences on immune function were studied (Zhang et al. 1989a, b).

The effect of *H. rhamnoides* L. juice (HRJ) on the immunologic function and the inhibition of tumor growth in mice were reported. Kunming mice were administered orally with HRJ for 7 days. The IL-2 produced by splenocytes and the reactivity of splenocytes to IL-2 were markedly stimulated (P lt 0.01). The activity of NK cells was also increased (P lt 0.05) and the growth of S180 tumor was inhibited. The DNA synthesis of tumor cells NS-1, HL-60, and YAC-1 was depressed by HRJ added in vitro ($p < 0.05$) (Yu et al. 1992).

Naringin and naringenin selectively inhibit the growth of cancer cells and are usable in the treatment of cancers resistant to chemo and radiotherapy. Experiments on 73 rats with induced trophic ulcers demonstrated naringin strongly inhibited DNA formation in vitro in human cancer (breast, colon, liver) tissue but had no such effect in normal human tissues (bone marrow, spleen) (Beljanski 1989).

Anticancer effect as well as antiviral and virus-enhancing properties of aqueous fruit extract of sea buckthorn was also investigated (Sauter and Wolfensberger 1989).

The formation of carcinomas of liver, lung, and kidney in rats by feeding aminopyrine (AP) plus sodium nitrite ($NaNO_2$) in diet was investigated. Seventy-two young Wister rats were divided into A, B, C, and D group. Group A was set as control receiving basal diet and tap water. Another three groups were given basal diet containing AP plus $NaNO_2$ (2 g/kg each). However, group B was given tap water, group C ascorbic acid solution, and group D sea buckthorn (*H. rhamnoides* L.) juice for drinking. After 38 weeks feeding, group B developed tumors in the livers (17/17), lungs (6/17), and kidneys, (4/17) and the average life span was 195 days; and group D delayed the development of tumors, and the average life span was 270 days, significantly longer than that of group B (195 days, $p < 0.01$) and group C (220 days, $p < 0.01$). The hepatocarcinoma bearing rates of the group C and D were 18/18 and 15/17, respectively. Furthermore, the livers of group D showed microscopically less foci of carcinogenesis than group B and C. The results suggested that sea buckthorn juice could block the synthesis of Nitroso compounds in rats in vivo more effectively than ascorbic acid, thereby could prevent the tumor formation (Li and Liu 1989).

The influence of sea buckthorn oil on cyclophosphamide, farmorubicin, and dioxadet mutagenicity was studied. The oil decreased the cytogenetic action of cyclophosphamide and farmorubicin, but not that of dioxadet. Possible mechanisms of the antimutagenic action of the oil are discussed (Nersesyan et al. 1990). Teratology of sea buckthorn leaves extract has been extensively studied (Kondorskaya 1973).

5.7.1.7 Adaptogenic Activity

It has been reported that sea buckthorn oil increases the cold tolerance (Son et al. 1997). The number of recent publications provides evidences on adaptogenic activity of polar water soluble compounds extracted from leaves, bark, roots, and shoots of sea buckthorn (Stakheeva and Zueva 1993; Krylova et al. 2000; Saggu and Kumar 2007, 2008; Saggu et al. 2007; Vijayaraghavan et al. 2006). For instance, ethanolic extract of leaf of *H. rhamnoides* and *H. rhamnoides* flavone from fruit can significantly reduce sulfur mustard-induced mortality and the body weight loss of rats, associated with the recovery in the blood levels of oxidative stress markers (Vijayaraghavan et al. 2006). Long-term administration of bark and sprout extracts normalize restraint stress-induced alterations of the neuro-endocrine system (disturbed ACTH, 11-deoxycortiysol, insulin, urea, and glucose levels) (Krylova et al. 2000). Restraint stress as well as cold and hypoxia (normally used as stress models in animal studies) suppresses the aerobic metabolism and hexose monophosphate pathway in muscles, liver, and blood of rats. It has been shown that single and repeated administration of sea buckthorn leaves' aqueous lyophilized extract prevents stress-induced decrease of activity of the key metabolic regulatory enzymes (blood hexokinase, citrate synthase, and glucose-6-phosphate dehydrogenase) in blood, liver, and muscle and tissue glycogen in rats. These results suggest that sea buckthorn treatment cause a trend for shifting anaerobic metabolism to aerobic during stress exposure and post-stress recovery (Saggu and Kumar 2007). Interestingly, aqueous and 70 % ethanol extracts of sea buckthorn dry leaves increase physical performance in rats in stressful conditions without any effect on cognitive functions (Saggu and Kumar 2008).

5.7.2 Clinical Pharmacology

Clinical efficacy of sea buckthorn oil, juice, or the extracts from oil, leaves, and bark have been studied in various diseases; Table 5.7.

5.7.2.1 Cardiovascular and Other Metabolic Diseases

A small-scale preliminary cross-over study in 12 healthy human subject shows that sea buckthorn berry oil can inhibit platelet aggregation (Johansson et al. 2000), suggesting that it might have beneficial effects on blood clotting in cardiovascular diseases. However, there were no significant changes in plasma total cholesterol, LDL-C, platelet aggregation, or plasma intercellular cell adhesion molecule 1 (ICAM-1) levels between treatment groups in 20 healthy male volunteers who were given either a placebo or sea buckthorn juice for 8 weeks (Eccleston et al. 2002). Adding sea buckthorn flavonols to oatmeal porridge does not seem to significantly lower CRP, homocysteine, or levels of oxidized low-density lipoprotein (LDL) cholesterol in humans (Suomela et al. 2006), while total flavones can strengthen myocardial contractility and pump function of the heart, reduce total peripheral vascular resistance, and increase vascular elasticity in normal subjects (Wang et al. 2001).

Preliminary clinical study shows that sea buckthorn flavonoids extract in the dose of 10 mg three times daily decreases cholesterol and platelet aggregation in patients with ischemic heart disease after for 6 weeks of the treatment (Zhang 1987).

Beneficial effect of sea buckthorn oil in patients with coronary arteriosclerosis was reported based on the results of a comparative study with sunflower oil (Olziikhutag 1968, 1969).

The effect of total *H. rhamnoides*, total flavones (TFH) on heart rate, blood pressure, and plasma catecholamines of the 88 patients with high blood pressure was compared to the effect of calcium antagonists nifedipine and verapamil after 8 weeks of the treatment. Isometric exercise may significantly increase the heart rate, blood pressure, and plasma catecholamine concentration in hypertensive patients. It was reported that TFH prevents exercise-induced increase of the heart rate, blood pressure, and plasma catecholamine concentration, while in nifedipine group these symptoms of hypertension were significantly increased after the exercise (Zhang et al. 2001).

The World Health Organization (WHO) has defined high insulin levels, an elevated fasting blood glucose, or an elevated post-meal glucose as a major symptom for the "metabolic syndrome," alone with at least two of the following criteria:

- Abdominal obesity as defined by a waist to hip ratio of greater than 0.9, a body mass index of at least 30 kg/m^2, or a waist measurement over 37 in.
- Cholesterol panel showing a triglyceride level of at least 150 mg/dL or an HDL cholesterol lower than 35 mg/dL.
- Blood pressure of 140/90 or above (or on treatment for high blood pressure).

Thus, type 2 diabetes symptoms were combined with the symptoms of obesity and atherosclerotic cardiovascular diseases under the term "metabolic syndrome" presumably because of various disorders in metabolic pathways of sugars and fats that is characteristic for these diseases. Consequently, the treatment of the symptoms

Table 5.7 Clinical studies of the sea buckthorn extracts and the grade[a] of recommendation

Condition	Product	Grade	References
Cardiovascular health factors in healthy human subjects	Flavonols extract	C	Suomela et al. (2006)
	Oil	C	Johansson et al. (2000)
	Juice	C	Eccleston et al. (2002)
	Fruit and leaf flavones extract	C	Wang et al. (1992a, b, 2001)
Atherosclerosis	Oil	C	Olziikhutag (1969)
Ischemic heart diseases	Flavonols extract	C	Jialiang et al. (1982), Zhang (1987)
Hypertension	Flavones extract	C	Zhang et al. (2001)
Chronic liver diseases (hepatitis, cirrhosis)	Undefined extract	C	Gao et al. (2003)
	Oil	C	Cheng et al. (1990), Huang and Xiao (1991)
Obesity	Berry	B	Lehtonen et al. (2011)
Healthy subjects	Berry	B	Lehtonen et al. (2010)
Duodenal and gastric ulcers	Oil	C	Gengquan and Xiang (1997) Nikitin et al. (1989), Okhotin et al. (1997)
Digestive tract infection	Berry	C	Larmo et al. (2008)
ARVI (common cold)	Berry	C	Larmo et al. (2008)
	Leaf derived tannins (Hiporamin)	C	Zamkovaya (2003), Fadeeva et al. (1988)
Dry eye	Oil	C	Larmo et al. (2010)
Chronic vaginal inflammation	Oil	C	Erkkola and Yang (2003)
Atopic dermatitis	Oil	C	Yang et al. (1999, 2000)
Skin burns	Oil	B	Vlasov (1970), Wang et al. (2006)
Cancer	Oil	C	Zhang et al. (1989a, b)
Radioprotective in cancer therapy	Oil	C	Gileva et al. (1994)

[a]Grade of recommendation according to natural standards evidence-based validated grading rationale

of one of these diseases is very often accompanied with the relief of the symptoms of other metabolic diseases. For instance, up-regulation of the expression and production of adipocytes-derived hormone adiponectin that plays a pivotal role in the regulation of lipid and glucose metabolism may benefit for all metabolic disorders, including type 2 diabetes, hyperlipidemia, etc.

Repeated postprandial hyperglycemia and subsequent mild, late hypoglycemia as well as high postprandial insulin response lead to metabolic events that may eventually develop into type 2 diabetes. It has been reported that sea buckthorn berries and two sea buckthorn extracts (lipophilic carbon dioxide extract and ethanol extract of CO_2-extraction residue) modulate the postprandial metabolism after a high-glucose meal in a small-scale pilot study of ten healthy normal-weight male volunteers who consumed four study breakfasts: one control (A) and three sea buckthorn meals on four distinct study days. Glucose, insulin, and tumor necrosis factor-α in their blood samples were analyzed before and during the 6-h study period. The ethanol-soluble components (presumably flavanones) showed

advantageous properties in both insulin and glucose responses, while carbon dioxide-soluble oil component from the berries did not show a significant change in the studied effects of the berries (Lehtonen et al. 2010).

This observation is in line with the results of the preclinical study of pentamethylquercetin, a polymethoxylated flavonoid, isolated from sea buckthorn in differentiated 3T3–L1 adiposities. It has been demonstrated that pentamethylquercetin upregulates adiponectin production (Chen et al. 2011).

In total, 110 overweight and obese women were recruited in a randomized crossover study of sea buckthorn berries (SB), their phenolic extract (SBe), and oil (SBo) supplemented for 33–35 consecutive days. Statistically significant decrease in waist circumference, vascular cell adhesion molecule, and intercellular adhesion molecule after SB and SBe after diet was observed. It was concluded that sea buckthorn berries and berry fractions have slightly positive effects on the associated variables of metabolic diseases (Lehtonen et al. 2011).

5.7.2.2 Atopic Dermatitis

Abnormal levels of fatty acids have been recognized in the plasma, skin, adipose tissue, and breast milk of atopic dermatitis (AD) patients compared to healthy controls. This abnormality is thought to be due to a deficiency in both incorporation and metabolism of ω-6 fatty acids in AD. It has been suggested that sea buckthorn can normalize fatty acid metabolism in AD patients; however, oil supplementations did not lead to any significant changes in the levels of the major fatty acids in skin glycerophospholipids of atopic dermatitis patients who took 5 g (10 capsules) of seed oil, pulp oil, or paraffin oil daily for 4 months (Yang et al. 1999, 2000). The results of these two clinical trials are in line with the results of another prospective, randomized, double blind, single center, placebo (miglyol cream)-controlled study where the effect of *H. rhamnoides*-containing cream on severity of atopic dermatitis (Scorad-Index), skin moisture, transepidermal water loss, and quality of life was evaluated in 53 patients of Caucasian skin type. No superior efficacy of the *H. rhamnoides*-containing preparations compared to placebo control has been observed in this study (Thumm et al. 2000).

5.7.2.3 Skin Burns

It has been reported that Hippophae oil is effective in the treatment of superficial skin burns (Vlasov 1970). This report is in consistency with the results of more recent study where 151 burned patients received the treatment with *H. rhamnoides* oil dressing applied on the burn wounds as an inner dressing and covered by disinfecting dressing, which obviously alleviated the swelling and effusion of the wounds and relieved the pains. Compared with the control patients (treated with vaseline gauze), patients receiving the dressing showed more obvious exudation

reduction, pain relief, and faster epithelial cell growth and wound healing, with statistically significant difference between the two groups (Wang et al. 2006).

5.7.2.4 Gastric and Duodenal Ulcers

Sea buckthorn is used orally for the treatment of gastric and duodenal ulcers (Nikitin et al. 1989; Okhotin et al. 1997; Gengquan and Xiang 1997).

It has been suggested that the route of administration of buckthorn oil, which is easily hydrolyzed in acidic conditions after oral administration, is rather important in the treatment of gastric and duodenal ulcers. The method of therapeutic endoscopy was applied in treatment of chronic gastroduodenal ulcers of 116 patients with gastric or duodenal ulcers. As a result, the period of the treatment was reduced 1.5–2 times with sufficiently high therapeutic efficacy (the results were positive in 93.7 % of cases) (Nikitin et al. 1989).

5.7.2.5 Acute Respiratory Viral Infections

Safety and antiviral efficacy of Hiporamin has been studied in the phase I (48 healthy volunteers) and two phase II clinical trials in 149 patients, 84 patients with uncomplicated flu, 65 patients with other ARVI complicated with bacterial infections—tonsillitis. Earlier prescribed at the 1–2 day of disease, Hiporamin significantly reduces symptoms of fever and intoxication and induces earlier recovery in inflamed tonsil (amygdala). Efficacy and tolerance of Hiporamin in 251 children (of 2 months–14 years old) including 120 patients with ARVI. It was claimed that "Hiporamin has strong stable antiviral effect and has also prophylactic action preventing complications particularly in immunodepressive therapy" (Zamkovaya 2003). Unfortunately, the results of these studies are not in compliance with CONSORT statement (Gagnier et al. 2006) and have rather low quality score in Jadad scale (Jadad et al. 1996).

In the contrary, consumption of sea buckthorn berries 28 g daily in frozen puree for 90 days has not significantly reduced the risk of developing the common cold or the duration of symptoms of the common cold in a double blind, placebo controlled, randomized clinical trial in 254 healthy volunteers. However, a significant reductive effect on CRP, a marker of inflammation, and a risk factor for cardiovascular diseases, was detected (Larmo et al. 2008).

5.7.2.6 Chronic Vaginal and Eyes Inflammation

A significant improvement in patients with chronic vaginal inflammation was observed in a small open-label pilot study of orally administered capsules containing a supercritical carbon dioxide extract of sea buckthorn seeds and berries (Omega 7 Sea Buckthorn Oil, Aromtech Ltd, Finland). Larger randomized

placebo-controlled clinical trials are justified (Erkkola and Yang 2003). It was reported that Omega 7 sea buckthorn oil reduces eye redness and burning and tear film osmolarity compared to placebo when applied in the dose of 1 g twice daily (Larmo et al. 2010).

5.7.2.7 Digestive Tract Infection

Preliminary clinical research shows that consumption of sea buckthorn berries 28 g daily in frozen puree for 90 days does not significantly reduce the risk of developing a digestive tract infection (Larmo et al. 2008).

5.7.2.8 Liver Cirrhosis

Preliminary clinical research suggests that taking sea buckthorn undefined extract might reduce liver enzymes and inflammatory markers (LN, HA, collagens types III and IV, cytokines IL-6 and TNFα, liver serum albumin, total bile acid, ALT, AST, and prothrombin time) in cirrhosis (Gao et al. 2003). Fifty cirrhotic patients of Child-Pugh grade A and B were randomly divided into two groups: Group A taking orally the sea buckthorn extract, 15 g 3 times a day for 6 months and control Group B taking vitamin B complex one tablet, 3 times a day for 6 months. It was reported that sea buckthorn could reduce the serum levels of laminin, hyaluronic acid, TBA, collagen types III and IV in patients with liver cirrhosis, suggesting that it may be a effective for prevention and the treatment of liver fibrosis (Gao et al. 2003).

5.7.2.9 Cancer

The assessment of clinical efficacy of sea buckthorn in cancer patients was initiated in Russia in 1950s (Gurevich 1956; Akulinin 1958). It has been reported that 5-hydroxytryptamine (hippophan) isolated from sea buckthorn bark inhibits tumor growth (Pukhalsskaia 1958; Sokoloff et al. 1961). The results of a clinical study of the antitumor activity of sea buckthorn oil conducted in China are in line with these data (Zhang et al. 1989a, b).

The efficacy of sea buckthorn extracts in the prevention of radiation-induced adverse effect in patients with larynx carcinoma was reported (Gileva et al. 1994).

5.8 Safety

Sea buckthorn fruit *is likely*[2] *safe*. . .when it is consumed as a food and *possibly safe* (See footnote 1) . . .when used orally and appropriately for medicinal purposes [Sea buckthorn. Natural Medicines Comprehensive Database (2011)]. Some clinical research suggests that sea buckthorn fruit or extract can be safely used for up to 90 days (Larmo et al. 2008; Gao et al. 2003; Zhang 1987; Larmo et al. 2010).

No significant changes were observed in organ weight/body weight ratios of any vital organ studied (except liver and kidney in 1 and 2 g/kg body weight doses, respectively), and biochemical and hematological parameters of the subacute drug-treated animals in comparison to control rats after subacute toxicity studies of sea buckthorn leaf aqueous extract on 10 and 20 times doses of maximal effective dose administered for 14 days (single oral dose of 1 and 2 g/kg once daily) and maximal effective dose administered for 30 days (single oral dose of 100 mg/kg once daily). In acute toxicity study, LD(50) of the extract was observed to be >10 g/kg when given orally (Saggu et al. 2007; Vijayaraghavan et al. 2006).

Safety of Hiporamin has been demonstrated in animals (Krepkova et al. 2009) and clinical studies in humans (Zamkovaya 2003).

5.9 Adverse Reactions

Clinical trials report few or no adverse reactions.

5.10 Drug Interactions

No published data available.

[2] Definitions from Natural Medicines Comprehensive Database
http://naturaldatabase.therapeuticresearch.com/home.aspx?cs=&s=ND

Likely safe: This product has a very high level of reliable clinical evidence showing its safe use when used appropriately. Products rated Likely Safe are generally considered appropriate to recommend.

Possibly safe: This product has some clinical evidence showing its safe use when used appropriately; however, the evidence is limited by quantity, quality, or contradictory findings. Products rated "Possibly Safe" appear to be safe but do not have enough high-quality evidence to recommend for most people.

5.11 Posology

Olei *H. rhamnoides* (Oleaum Hippopheae) for topical application in colpitis, endocervitis, cervical erosion, rectal and skin diseases. In stomach and duodenal ulcers, esophagus cancer, and atherosclerosis—one teaspoon 2–3 times per day, oral administration (Turova 1974; Bykov 2006; Mashkovskij 1978, 2000).

Hiporamin sublingual/bookal tablets containing 20 mg of Hiporamin for treatment and prevention of ARVI, one tablet 4–6 times a day for adults, for children older than 12 years—1 tablet 3–4 times a day, for children from 3 to 12 years: 1/2 tablets 2–4 times a day. Tablets must be fully absorbed in the mouth. The tablets provide sanitation mouth (suppression of viruses and pathogenic organisms). Duration of treatment depends on the severity of illness, normally of 3–5 days. In severe forms of the disease, course of treatment is for 2–3 weeks. For prevention of influenza—one tablet 2–4 times a day for adults, children older than 12 years: 1 tablet 2–3 times a day, for children from 3 to 12 years: 1/2 tablets 1–2 times a day depending on age (Zamkovaya 2003).

Various doses and forms have been studied, including teas, juices, seed oil capsules, and flavones, but there is not enough scientific evidence to support the safety or efficacy of any particular dose. For atopic dermatitis, 5 g (10 capsules) of sea buckthorn pulp oil has been used daily for 4 months. For liver disease (cirrhosis), 15 g of sea buckthorn extract has been taken by mouth three times daily for 6 months. Creams containing 10–20 % sea buckthorn have been applied on the skin for atopic dermatitis and burns.

5.12 Conclusions

1. Chemical composition of different parts of sea buckthorn has been extensively studied. Along with wide range and large amounts of well-known and valuable natural compounds, (vitamins, flavonoids triterpenes, polyphenols and their glycosides, etc.), a number earlier unknown compounds were isolated and identified.
2. Numerous in vitro and in vivo pharmacological studies of the extract and purified compounds support some of traditional uses of sea buckthorn, e.g., wound healing effects, etc.
3. Limited number of clinical trials in humans have been reported. Only few of them are randomized, double blind placebo controlled. The reports on these trials are not in accordance with consolidated standards of reporting of trials (CONSORT) statement. That makes difficult to assess the quality of these studies and compare the results between studies. A lack of independent replications and some of the primary studies were less than rigorous. More evidence is needed to rate sea buckthorn for these uses.

4. Several sea buckthorn-derived medicinal products are currently used in Russia and China, such as sea buckhorn berries and seed oils for the treatment of wounds, sea buckthorn leaves extract Hiporamin for the treatment of acute respiratory infectious diseases, and sea buckthorn berries flavones for reduction of symptoms of metabolic diseases (atherosclerosis, diabetes type 2, obesity). However, published articles have limitations mentioned above. That makes difficult to rate the quality of the studies and evaluate the level of evidences of their efficacy and safety. No major risks have been associated with *H. rhamnoides*.
5. More research on this promising herbal medicine seems warranted.

References

Abartiene D, Malachovskis A (1974) Combined action of cytostatic preparation and sea buckthorn oil on the growth of M-1 sarcoma in rats and their hematological and biochemical indexes 1 sarcolysine. Liet TSR Mokslu Akad Darb Ser C 3:181–186

Agrawala PK, Adhikari JS (2009) Modulation of radiation-induced cytotoxicity in U 87 cells by RH-3 (a preparation of Hippophae rhamnoides). Indian J Med Res 130(5):542–549

Agrawala PK, Goel HC (2002) Protective effect of RH-3 with special reference to radiation induced micronuclei in mouse bone marrow. Indian J Exp Biol 40(5):525–530

Akulinin IA (1958) Using sea-buckthorn oil in treating burns. Sov Med 11:137–138

Andersson SC, Olsson ME, Johansson E, Rumpunen K (2009) Carotenoids in sea buckthorn (Hippophae rhamnoides L.) berries during ripening and use of pheophytin a as a maturity marker. J Agric Food Chem 57(1):250–258

Anesini C, Ferraro GE, Filip R (2008) Total polyphenol content and antioxidant capacity of commercially available tea (*Camellia sinensis*) in Argentina. J Agric Food Chem 56(19):9225–9229

Arimboor R, Kumar KS, Arumughan C (2008) Simultaneous estimation of phenolic acids in sea buckthorn (Hippophaë rhamnoides) using RP-HPLC with DAD. J Pharm Biomed Anal 47(1):31–38

Basu M, Prasad R, Jayamurthy P, Pal K, Arumughan C, Sawhney RC (2007) Anti-atherogenic effects of seabuckthorn (Hippophaea rhamnoides) seed oil. Phytomedicine 14(11):770–777

Beljanski M (1989) Separation of naringin and naringenin from plant material as neoplasm inhibitors Eur Pat EP 352147. FR 2633182 A1 19891229 FR 1988–8434 US 5145839 DK 8903101 CA 1338325 JP 02111725

Beltrán-Debón R, Rull A, Rodríguez-Sanabria F, Iswaldi I, Herranz-López M, Aragonès G, Camps J, Alonso-Villaverde C, Menéndez JA, Micol V, Segura-Carretero A, Joven J (2011) Continuous administration of polyphenols from aqueous rooibos (Aspalathus linearis) extract ameliorates dietary-induced metabolic disturbances in hyperlipidemic mice. Phytomedicine 18(5):414–424

Bereznaya GA, Eliseev IP, Tsudendabaev BD, Vereshchagin AG (1988) Fatty acid composition and quantitative content of lipids in the sea buckthorn fruits. Prikl Biokhim Mikrobiol 24(4):568–573

Bernáth J, Földesi D (1992) Sea-buckthorn (Hippophae rhamnoides L.): a promising new medicinal and food crop. J Herbs Spices Med Plants 1(1/2):27–35

Beveridge T, Li TSC, Oomah BD, Smith A (1999) Sea buckthorn products: manufacture and composition. J Agric Food Chem 47(9):3480–3488

Bykov VA (2006) Atlas of medicinal plants of Russia. VILAR, Moscow, Russia, pp 252–253

Cao Q, Qu W, Deng Y, Zhang Z, Niu W, Pan Y (2003) Effect of flavonoids from the seed and fruit residue of Hippophae rhamnoides L. on glycometabolism in mice. Zhong Yao Cai 26(10):735–737

Centenaro G, Capietti GP, Pizzocaro F, Marchesini A (1977) A the fruit of the sea buckthorn Hippophae rhamnoides as a source of vitamin C. Atti della Societa Italiana di szienze Naturali di Milano, 118 (3–4): 371–378

Chawla R, Arora R, Singh S, Sagar RK, Sharma RK, Kumar R, Sharma A, Gupta ML, Singh S, Prasad J, Khan HA, Swaroop A, Sinha AK, Gupta AK, Tripathi RP, Ahuja PS (2007) Radioprotective and antioxidant activity of fractionated extracts of berries o Hippophae rhamnoides. J Med Food 10(1):101–109

Chen Y, Zhong X, Liu T, Ge Z (2003) The study on the effects of the oil from Hippophae rhamnoides in hematopoiesis. Zhong Yao Cai 26(8):572–575

Chen C, Zhang H, Xiao W, Yong ZP, Bai N (2007) High-performance liquid chromatographic fingerprint analysis for different origins of sea buckthorn berries. J Chromatogr A 1154 (1–2):250–259

Chen W, Su X, Zhang H, Sun K, Ma R, Chen X (2010) High genetic differentiation of Hippophae rhamnoides ssp.yunnanensis (Elaeagnaceae), a plant endemic to the Qinghai-Tibet Plateau. Biochem Genet 48(7–8):565–576

Chen L, He T, Han Y, Sheng JZ, Jin S, Jin MW (2011) Pentamethylquercetin improves adiponectin expression in differentiated 3 T3-L1 cells via a mechanism that implicates PPARγ together with TNF-α and IL-6. Molecules 16(7):5754–5768

Cheng TJ (1992) Protective action of seed oil of Hippophae rhamnoides L. (HR) against experimental liver injury in mice. Chin J Prevent Med (Chung Hua Yu Fang I Hsueh Tsa Chih) 26:227–229

Cheng T, Li T, Duan Z, Cao Z, Ma Z, Zhang P (1990) Acute toxicity of flesh oil of Hippophae rhamnoides L. and its protection against experimental hepatic injury. Zhongguo Zhong Yao Za Zhi 15(1):45–47, 64

Cheng T, Pu J, Wu L, Ma Z, Cao Z, Li T (1994) Preliminary study on hepatoprotective action of seed oil of Hippophae rhamnoides L. and mechanism of the action. Zhongguo Zhongyao Zazhi 19(6):367–370

Cheng J, Liu Q, Hou G, Gao N, Jia C, Pan L (1999) Effects of total flavones of Fructus Hippophae on PGI2 and TXA2 in rat plasma. Zhongguo Zhongyao Zazhi 24(7):433–434

Cheng J, Kondo K, Suzuki Y, Ikeda Y, Meng X, Umemura K (2003) Inhibitory effects of total flavones of Hippophae Rhamnoides L on thrombosis in mouse femoral artery and in vitro platelet aggregation. Life Sci 72(20):2263–2271

Chou S, Huang D, Jiang Z, Ye Z (1986) Chemistry and hypolipemic effect of Fructus hippophae oil. Zhongcaoyao 17(5):238–239

Cimmerman YS, Mikhaylovskaya LL (1987) Effect of sea buckthorn oil on some pathophysiological mechanism and ulcer disease. Clin Med 2:77–83

Consolidated List of Article 13 Health Claims Database. Consolidated list of article 13 health claimlist of references received by European Food Safety Authority (EFSA) Parma, Italy http://www.efsa.europa.eu/en/ndaclaims/ndaclaims13.htm. Accessed 21 Sep 2009, pp 1–235

Crapatureanu S, Rosca G, Neamtu G, Socaciu C, Britton G, Pfander H (1996) Carotenoids from seabuckthorn fruit determined by thin layer chromatography and mass spectrometry. Revue Roumaine de Biochimie 33:167–174

Dalgatov DD, Muratchaeva PM, Magomedmirzaev MM (1985) Correlations between some characters of the fruit of Hippophae rhamnoides and the contents of fatty oil and tocopherol in the fruit. Rastit resur 21(3):283–288

Dembinska-Migas W (1988) Phenolic aids in the plants from family elaeagnaceae. Herba Pol 34 (3):115–122

Dembinska-Migas W, Krauze M (1989) Polyphenolic compounds in leaves of plants of Elaeagnaceae Part 1. Analysis of tannin compounds. Herba Pol 35:9–15

Dongowski G (1996) Investigation on polysaccharide pectin and dietary fiber fractions of buckthornton berries. Zeitschrift fuer Lebensmittel-Untersuchung und –Forschung 203:302–308
Eccleston C, Baoru Y, Tahvonen R, Kallio H, Rimbach GH, Minihane AM (2002) Effects of an antioxidant-rich juice (sea buckthorn) on risk factors for coronary heart disease in humans. J Nutr Biochem 13(6):346–354
Eliseev IP (1989) Evolutionary genetic aspects in assessment of achievements and perspectives of sea-buckthorn selection in the USSR. In: Proceedings of Internatonal Symposium on Sea-buckthorn (*H. rhamnoides* L.), Xian, China, pp 184–193
Erkkola R, Yang B (2003) Sea buckthorn oils: towards healthy mucous membranes. AgroFood Industry Hi-Tech 3:53–57
Fadeeva II, Sheichenko OP, Tolkachev ON, Sheichenko VI, Semenova TS, Shipulina LD, Vichkanova SA, Simonova IY (1988). The method of isolation of 6-*O*-galloyl-1-3-*O*-hexahydroxydiphenoyl-beta-D-glucose. USSR Patent SU 1439107 A1, pp 1–8
Franke W, Mueller H (1983) Quantity and composition of fatty acids in the fat of the juicy fruit part and of the seed of fruits of Hippophae rhamnoides. Angew Bot 57(1–2):77–84
Fu Q, Yang QY, Yang GD, Shi J, Chang C, Chen HJ (1993) Analysis of alpha-tocopherol contents in sea-buckthorn oil by reversed phase-high performance liquid chromatography. Xi'an yike daxue xuebao 14(2):181–183
Fu G, Zhao S, Feng R, Xiao P (1997) Determination of flavonoids in the leaves of Hippophae L. by HPLC. Zhongguo Zhong Yao Za Zhi 22(5):299–300, 320
Fu SC, Hui CW, Li LC, Cheuk YC, Qin L, Gao J, Chan KM (2005) Total flavones of Hippophae rhamnoides promotes early restoration of ultimate stress of healing patellar tendon in a rat model. Med Eng Phys 27(4):313–321
Gagnier JJ, Boon H, Rochon P, Moher D, Barnes J, Bombardier C (2006) Reporting randomized, controlled trials of herbal interventions: an elaborated CONSORT statement. Ann Intern Med 144(5):364–367
Ganju L, Padwad Y, Singh R, Karan D, Chanda S, Chopra MK, Bhatnagar P, Kashyap R, Sawhney RC (2005) Anti-inflammatory activity of Seabuckthorn (Hippophae rhamnoides) leaves. Int Immunopharmacol 5(12):675–684
Gao J, Qiao TS (1985) Analysis of organic acids in fruits of Hippophae rhamnoides. Shipin kexue (Beijing) 71:39–41
Gao ZL, Gu XH, Cheng FT, Jiang FH (2003) Effect of sea buckthorn on liver fibrosis: a clinical study. World J Gastroenterol 9:1615–1617
Geetha S, Sai Ram M, Mongia SS, Singh V, Ilavazhagan G, Sawhney RC (2003) Evaluation of antioxidant activity of leaf extract of Seabuckthorn (Hippophae rhamnoides L) on chromium (VI) induced oxidative stress in albino rats. J Ethnopharmacol 87(2–3):247–251
Geetha S, Singh V, Ram MS, Ilavazhagan G, Banerjee PK, Sawhney RC (2005) Immunomodulatory effects of seabuckthorn (Hippophae rhamnoides L.) against chromium (VI) induced immunosuppression. Mol Cell Biochem 278(1–2):101–109
Geetha S, Ram MS, Sharma SK, Ilavazhagan G, Banerjee PK, Sawhney RC (2009) Cytoprotective and antioxidant activity of seabuckthorn (Hippophae rhamnoides L.) flavones against tert-butyl hydroperoxide-induced cytotoxicity in lymphocytes. J Med Food 12(1):151–158
Gengquan Q, Xiang Q (1997) A clinical report on the therapeutics of seabuckthorn oil softgels on peptic ulcer in 30 cases. Hippophae 10:39–41
Gileva TG, Lukin AV, Nyushkin AA, Agachev AR, Studentsova IA, Vizel AO (1994) Assessment of the efficacy of radioprotective compounds in radiation therapy of larynx carcinoma. Kazanskii Meditsinskii Zhurnal 75:374–375
Gill S, Raszeja W (1971) Chromatographic analysis of harman alkaloid derivatives in some plant raw materials. Gdansk Tow Nauk Rozpr Wydz 8:137–143
Glazunova EM, Mukhtarova ES, Zakharov KS, Gachechiladze ND (1994) New cycloartane terpenoid form the oil of sea buckthorn fruit. Chem Nat Comp 30(2):271–272

Goel HC, Prasad J, Singh S, Sagar RK, Kumar IP, Sinha AK (2002) Radioprotection by a herbal preparation of Hippophae rhamnoides, RH-3, against whole body lethal irradiation in mice. Phytomedicine 9(1):15–25

Goel HC, Kumar IP, Samanta N, Rana SV (2003a) Induction of DNA-protein cross-links by Hippophae rhamnoides: implications in radioprotection and cytotoxicity. Mol Cell Biochem 245(1–2):57–67

Goel HC, Salin CA, Prakash H (2003b) Protection of jejunal crypts by RH-3 (a preparation of Hippophae rhamnoides) against lethal whole body gamma irradiation. Phytother Res 17(3):222–226

Goel HC, Indraghanti P, Samanta N, Ranaz SV (2004) Induction of apoptosis in thymocytes by Hippophae rhamnoides: implications in radioprotection. J Environ Pathol Toxicol Oncol 23(2):123–137

Goel HC, Gupta D, Gupta S, Garg AP, Bala M (2005) Protection of mitochondrial system by Hippophae rhamnoides L. against radiation-induced oxidative damage in mice. J Pharm Pharmacol 57(1):135–143

Goel HC, Samanta N, Kannan K, Kumar IP, Bala M (2006) Protection of spermatogenesis in mice against gamma ray induced damage by Hippophae rhamnoides. Andrologia 38(6):199–207

Gorbatsova J, Lõugas T, Vokk R, Kaljurand M (2007) Comparison of the contents of various antioxidants of sea buckthorn berries using CE. Electrophoresis 28(22):4136–4142

Guliyev VB, Gul M, Yildirim A (2004) Hippophae rhamnoides L.: chromatographic methods to determine chemical composition, use in traditional medicine and pharmacological effects. J Chromatogr B Analyt Technol Biomed Life Sci 812(1–2):291–307

Gupta R, Flora SJ (2005) Therapeutic value of Hippophae rhamnoides L. against subchronic arsenic toxicity in mice. J Med Food 8(3):353–361

Gupta A, Kumar R, Pal K, Singh V, Banerjee PK, Sawhney RC (2006) Influence of sea buckthorn (Hippophae rhamnoides L.) flavone on dermal wound healing in rats. Mol Cell Biochem 290(1–2):193–198

Gurevich SK (1956) The application of sea-buckthorn oil in ophthalmology. Vesttn Oftalmologii 2:30–33

Gutzeit D, Baleanu G, Winterhalter P, Jerz G (2007a) Determination of processing effects and of storage stability on vitamin K1 (Phylloquinone) in Sea buckthorn berries (Hippophaë rhamnoides L. ssp. rhamnoides) and related products. J Food Sci 72(9):C491–C497

Gutzeit D, Winterhalter P, Jerz G (2007b) Application of preparative high-speed counter-current chromatography/electrospray ionization mass spectrometry for a fast screening and fractionation of polyphenols. J Chromatogr A 1172(1):40–46

Gutzeit D, Baleanu G, Winterhalter P, Jerz G (2008a) Vitamin C content in sea buckthorn berries (Hippophaë rhamnoides L) ssp. rhamnoides and related products: a kinetic study on storage stability and the determination of processing effects. J Food Sci 73:C615–C620

Gutzeit D, Winterhalter P, Jerz G (2008b) Nutritional assessment of processing effects on major and trace element content in sea buckthorn juice (Hippophaë rhamnoides L. ssp. rhamnoides). J Food Sci 73(6):H97–H102

Haranovich IM (1981) Ascorbic-acid content in leaves and fruit of the sea buckthorn Hippophae rhamnoides. Vyestsi akad navuk BSSR Syer Biyal Navuk 5:10–14

He J, Chen Y, Xiao HY, Chang BB, Ding QF, Zhang MS, Zeng Z, Zhang XJ (2009) Effect of total flavonoids of Hippophae rhamnoides L. on the expression of MCP-1 in aorta of spontaneously hypertensive rats. Sichuan Da Xue Xue Bao Yi Xue Ban 40(3):481–485

Hethelyi E, Tetenyi P, Zambo I, Kaposi P (1989) GC-MS Investigation of different fatty acids from seeds of some medicinal plants. Herba Hung 28(3):69–78

Hibasami H, Mitani A, Katsuzaki H, Imai K, Yoshioka K, Komiya T (2005) Isolation of five types of flavonol from seabuckthorn (Hippophae rhamnoides) and induction of apoptosis by some of the flavonols in human promyelotic leukemia HL-60 cells. Int J Mol Med 15(5):805–809

Hiporamin, Sea buckthorn leaves extract (2002) Pharmacopeial article FSP 42–0171295302. The Ministry of Health of Russian Federation, The State Register of Drugs, RF Ministry of Health

and Medical Industry Moscow, pp 1–7. http://grls.rosminzdrav.ru/GRLS.aspx, http://www.drugreg.ru/Soft/SoftCliphar.asp, http://www.piluli.ru/product/Giporamin, http://www.sanddorn.net/abstracts/SHEICHENKO.htm, http://www.sanddorn.net/abstracts/shipulina_1.htm, http://www.vidal.ru/poisk_preparatov/hiporhamin.htm, http://www.wiki-meds.ru/lekarstvennie-preparati/giporamin-10061.htm, http://www.rlsnet.ru/tn_index_id_10513.htm

Hippophae Fructus Shaji (2010) In: Pharmacopoeia of the People's Republic of China 2010 (English Ed), People's Medical Publishing House, Baijing, China, vol 1, pp 221–222

Hirvi T, Honkanen E (1984) The aroma of the fruit of sea buckthorn Hippophae rhamnoides. Z Lebensm Unters Forsch 179(5):387–388

Horhammer L, Wagner H, Khalil E (1966) The flavonol glycoside from fruits of Hippophae rhamnoides. Lloydia 29:225

Houghton PJ, Parsons R (1995) Sea buckthorn oil as a potential sunscreen agent. J Pharm Pharmacol 47(12B):1086

Hsu YW, Tsai CF, Chen WK, Lu FJ (2009) Protective effects of seabuckthorn (Hippophae rhamnoides L.) seed oil against carbon tetrachloride-induced hepatotoxicity in mice. Food Chem Toxicol 47(9):2281–2288

Huang YQ, Xiao YY (1991) Analysis of vitamin E contents in Hippophae rhamnoides. Zhongguo Zhongyao Zazhi 16(9):530–532

Huang YQ, Xiao YY (1994) Analytical studies on alpha-tocopherol and its isomers in seabuckthorn (Hippophae rhamnoides) oil by HPLC. Chung TS'AO Yao 25(3):130–131

Ianev E, Radev S, Balutsov M, Klouchek E, Popov A (1995) The effect of an extract of sea buckthorn (Hippophae rhamnoides L.) on the healing of experimental skin wounds in rats. Khirurgiia (Sofiia) 48(3):30–33

Isamukhamedov ASH, Akramov ST (1983) Phospholipids of the seeds of Hippophae rhamnoides. Chem Nat Compd (Engl Transl) 18(3):365–366

Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ (1996) Assessing the quality of reports of randomized clinical trials: is blinding necessary? Control Clin Trials 17(1):1–12

Jain M, Ganju L, Katiyal A, Padwad Y, Mishra KP, Chanda S, Karan D, Yogendra KM, Sawhney RC (2008) Effect of Hippophae rhamnoides leaf extract against Dengue virus infection in human blood-derived macrophages. Phytomedicine 15(10):793–799

Jeppsson N, Gao X (2000) Changes in the contents of kaempherol, quercetin and L-ascorbic acid in sea buckthorn berries during maturation. Agric Food Sci 9(1):17–22

Ji L-J (1989) Analysis of free amino acids and vitamin C in fruits of Hippophae rhamnoides L. Nitraria-tangutorum bobr and berberis-dasystachya maxin. Zhiwu Xuebao 31(6):487–488

Jialiang W, Maoshun Z, Zhiqi X (1982) Effect of flavonoids of Hippophae rhamnoides L. on ischemic heart disease. Acta Academiae Medicinae Sichuan 13(1):6–11

Jiang Z, Li G, Jiang H, Qian D, Sai Y (1988) Isolation and identification of active components of Hippophae rhamnoides L. seed against gastric ulcer. J Med Coll PLA 3(1):87–89

Johansson AK, Korte H, Yang B, Stanley JC, Kallio HP (2000) Sea buckthorn berry oil inhibits platelet aggregation. J Nutr Biochem 11:491–495

Kallio H, Yang B, Peippo P, Tahvonen R, Pan R (2002a) Triacylglycerols, glycerophospholipids, tocopherols, and tocotrienols in berries and seeds of two subspecies (ssp. sinensis and mongolica) of Sea Buckthorn (Hippophaë rhamnoides). J Agric Food Chem 50(10):3004–3009

Kallio H, Yang B, Peippo P (2002b) Effects of different origins and harvesting time on vitamin C tocopherols, and tocotrienols in sea buckthorn (Hippophae rhamnoides) berries. J Agric Food Chem 50:6136–6142

Kallio H, Lassila M, Järvenpää E, Haraldsson GG, Jonsdottir S, Yang B (2009) Inositols and methylinositols in sea buckthorn (Hippophaë rhamnoides) berries. J Chromatogr B Analyt Technol Biomed Life Sci 877(14–15):1426–1432

Khizhazi AA (1998) The therapeutic and prophylactic anti-ulcerogenic action of marigold (Tagetes patula L.) and sea buckthorn (Hippophae) oils in neurogenic ulcerative lesions caused by immobilization, noise and vibration. Lik Sprava 1:172–176

Khusainov KS, Mukhamadiev NK, Gulamova MT, Eremenko TV, Sultanovich YA, Kiseleva TV (1988) Gas-chromatographic analysis of the fatty acid composition of sea buckthorn oil from the Zarafhansk valley Dokl Akad Nauk UzSSR 9, pp 47–49

Kim JS, Kwon YS, Sa YJ, Kim MJ (2011) Isolation and identification of sea buckthorn (Hippophae rhamnoides) phenolics with antioxidant activity and α-glucosidase inhibitory effect. J Agric Food Chem 59(1):138–144

Kondorskaya VR (1973) Teratology of sea-buckthorn. Byull Mosk Ispit Prir Otd Prir Otd Biol 78(2):114–121

Koyama T, Taka A, Togashi H (2009) Effects of a herbal medicine, Hippophae rhamnoides, on cardiovascular functions and coronary microvessels in the spontaneously hypertensive stroke-prone rat. Clin Hemorheol Microcirc 41(1):17–26

Krepkova LV, Bortnikova VV, Dzhavakhyan MA, Sokol'skaya TA (2009) Toxicological characterization of new medicinal forms of hiporamin (gel and liniment). Pharmaceut Chem J 43(6):350–351, Translated from Khimiko-Farmatsevticheskii Zhurnal 43(6):44–45

Krolikowska M (1972) Flavonoid glycosides from common sea buckthorn berries Hippophae rhamnoides. Planta Med 22:418–427

Krylova SG, Konovalova ON, Zueva EP (2000) Common sea buckthorn bark and sprout extract improves the stress-disturbed hormonal-metabolic organism state in rats. Eksp Klin Farmakol 63(4):70–73

Kukina TP, Raldugin VA (1992) Maslinic and 2-alpha-hydroxyursolic acids from sea buckthorn leaves. Sib Khim ZH 2:92–93

Kukina TP, Demenkova LI, Raldugin VA, Maksimov BI, Chizhov OS, Veselovskii VV, Molseenkov AM (1991) Polyprenols and dolichols from sea buckthorn leaves. Sib Khim ZH 6:89–93

Kumar IP, Namita S, Goel HC (2002) Modulation of chromatin organization by RH-3, a preparation of Hippophae rhamnoides, a possible role in radioprotection. Mol Cell Biochem 238(1–2):1–9

Lachman J, Pivec V, Hubacek J, Rehakova V (1985) Flavonoid substances in the fruit of sea buckthorn (Hippophae rhamnoides L.). Sci Agric Bohemoslov 17(3):169–183

Lagazidze DS, Murav'eva DA, Bostoganashvili VS (1984) Content of pharmacologically active substances in the fattyoil of the fruit pulp of sea buckthorn Hippophae rhamnoides growing in the georgina-SSR USSR. Khim-Farm ZH 18(6):713–717

Larmo P, Alin J, Salminen E, Kallio H, Tahvonen R (2008) Effects of sea buckthorn berries on infections and inflammation: a double-blind randomized, placebo-controlled trial. Eur J Clin Nutr 62:1123–1130

Larmo PS, Jarvinen RL, Setala NL, Yang B, Viitanen MH, Engblom JR, Tahvonen RL, Kallio HP (2010) Oral sea buckthorn oil attenuates tear film osmolarity and symptoms in individuals with dry eye. J Nutr 140:1462–1468

Lehtonen HM, Järvinen R, Linderborg K, Viitanen M, Venojärvi M, Alanko H, Kallio H (2010) Postprandial hyperglycemia and insulin response are affected by sea buckthorn (Hippophaë rhamnoides ssp. turkestanica) berry and its ethanol-soluble metabolites. Eur J Clin Nutr 64(12):1465–1471

Lehtonen HM, Suomela JP, Tahvonen R, Yang B, Venojärvi M, Viikari J, Kallio H (2011) Different berries and berry fractions have various but slightly positive effects on the associated variables of metabolic diseases on overweight and obese women. Eur J Clin Nutr 65(3): 394–401

Li Y, Liu H (1989) Blocking effect of sea buckthorn juice on the formation of N-nitrosodimethylamine (NDMA) in vivo in rat. Acta Nutr Sin 11(1):47–53

Li Y, Liu H (1991) Prevention of tumour production in rats fed aminopyrine plus nitrite by sea buckthorn juice. IARC Sci Publ 105:568–570

Li TSC, Schroeder WR (1996) Sea buckthorn (Hippophae rhamnoides L.): a multipurpose plant. HortTechnology 6:370–380

Li J, Zhou Z (1996) Chemical analysis of seed oil from Hippophae rhamnoides by carbon dioxide supercritical fluid extraction (SFE). Chin Pharm J 31(1):19–20
Li Y, Xu C, Zhang Q, Liu JY, Tan RX (2005) In vitro anti-Helicobacter pylori action of 30 Chinese herbal medicines used to treat ulcer diseases. J Ethnopharmacol 98(3):329–333
Liu B, Wu Z, Liu W (1980) Preliminary observation on curing effects of sea-buckthorn fruit juice for high blood cholesterol and coronary heart disease. Acta Academiae Medicinae Sichuan 11(3):178–182
Liu F, Li Z, Shi S (1988) Effects of total flavones of Hippophae rhamnoides L. on cultured rat heart cells and on cAMP and adenylate cyclase in the myocardium. Zhongguo Yaoli Xuebao 9(6):539–542
Liu C, Xu J, Ye CQ, Huang C (2006a) Effects and comparison of seed oil and sarcocarp oil of Hippophae rhamnoides on rats with experimental hepatocirrhosis. Zhongguo Zhong Yao Za Zhi 31(13):1100–1102
Liu CL, Liu HQ, Deng YH, Liu ML (2006b) Study on the purification and scavenging free radical activity of water soluble polysaccharide of leave in Hippohae rhamnoides L. Zhong Yao Cai 29(2):151–154
Liu ZR, Zhang Q, Yang YM, Guan H, Liu FM (2008) Changes of proteomic spectra of total flavones of Hippophae rhamnoides on myocardial protection. Zhongguo Zhong Yao Za Zhi 33 (9):1060–1063
Lobanova AA, Dadochkin VM, Vinogradov AK, Pershin NS (1992) Oil from Hippophae rhamnoides L. leaves. Rastitel'nye Resursy 28(4):49–52
Lobacheva I, Letchamo W (1998) Toxic metals in different varieties of seabuckthorn Hippophae rhamnoides. (1998). vol. 33, No. 3, pp. 481. 95th annual international conference of the American society for horticultural science, charlotte, north Carolina, July 12–15, 1998. Hortscience
Loginov AS, Mironov VA, Amirov NS, Aruin LI, Vasil'ev GS (1983) Effect of preparations from Hippophae berries on the healing of experimental stomach ulcers. Patol Fiziol Eksp Ter 6:67–70
Loskutova GA, Baikov VG, Starkov AV, Medvedev FA (1989) Lipid fatty acid composition in fruits of Hippophae rhamnoides L. Rastit Resur 25(1):97–103
Malena TV, Lykova RV, Chigireva EA (1984) Content of biologically active compounds in common sea-buckthorn Hippophae rhamnoides fruits in its natural populations in issyk-kul oblast Kirgiz-SSR USSR. Khim-Farm ZH 18(10):1226–1228
Mamedov SS, Gigienova EI, Umarov AU, Aslanov SM (1981) Oil lipids in the fruits and leaves of Hippophae rhamnoides. Khim Prir Soedin (Tashk) 6:710–715
Mansurova ID, Linchevskaia AA, Molchagina RP, Bobodzhanova MB (1978) Oleum hippophae, a stabilizer of biological membranes. Farmakol Toksikol 41(1):105–109
Mashkovskij MD (1978) Medicines. Manual of pharmacotherapy for doctors, Part I, 8th edn. Meditsina, Moscow, p 133
Mashkovskiy MD (2000) Drugs. Manual for physicians, revised, corrected and supplemented, vol 2, 14th edn. Novaya Volna, Moscow, pp 105–107
Mirgaesiev M (1992) Essential amino acids in Hippophae rhamnoides L. fruits from the western Pamirs. Rastitel'nye Resursy 28(3):75–79
Mironov VA, Vasil'ev GS, Matrosov VS, Filippova TM, Zamurenko VA, Mishchenko VV, Marionovskii VG, Kas'yanenko II, Maksimova LM (1983) Physiologically active alcohols form sea buckthorn fruit. Khim-Farm ZH 17(10):1242–1247
Mironov VA, Guseva-Donskaya TN, Dubrovina Y, Osipova GA, Shabanova EA, Nikulin AA, Amirov NS, Trubitsina IG (1989) Chemical composition and biological activity of extracts form sea buckthorn fruit components. Khim-Farm ZH 23(11):1357–1364
Mishra KP, Chanda S, Karan D, Ganju L, Sawhney RC (2008) Effect of Seabuckthorn (Hippophae rhamnoides) flavone on immune system: an in-vitro approach. Phytother Res 22(11): 1490–1495

Mizina TY, Sitnikova SG (1999) Antiradiation activity of juice concentrate from Hippophae rhamnoides L fruits. Rastitel'nye Resursy 35:85–92
Morzewski S, Bakowska E (1960) The use of the fruit of the sea-buckthorn in food production. Przem Spoz 12:23–25
Mukhamed'yarova MM, Chumbalov TK (1980) Poly phenols of Hippophae rhamnoides. Chem Nat Compd (Engl Transl) 15(6):759
Narayanan S, Ruma D, Gitika B, Sharma SK, Pauline T, Ram MS, Ilavazhagan G, Sawhney RC, Kumar D, Banerjee PK (2005) Antioxidant activities of seabuckthorn (Hippophae rhamnoides) during hypoxia induced oxidative stress in glial cells. Mol Cell Biochem 278(1–2):9–14
Neamtu G, Cociu A (1982) Treatment of animal wounds with oil extracted from ripe fruits of Hippophae rhamnoides L. Stud Cercet Biochim 25(1):30–34
Nersesyan AK, Zil'fyan VN, Kumkumadzhian VA, Proshyan NV (1990) The antimutagenic action of sea-buckthorn oil. Genetika (Moscow) 26(2):378–380
Nikitin VA, Chistiakov AA, Bugaeva VI (1989) Therapeutic endoscopy in combined therapy of gastroduodenal ulcers. Khirurgiia (Mosk) 4:33–35
Nikulin AA, Yakusheva EN, Zakharova NM (1992) Comparative pharmacological estimation of Hippophae, Rosa, and plantain oils in experimental eye burns. Eksp Klin Farmakol 55(4):64–66
Niu M (1991) Developing Hippophae on a large scale for sharing the sorrows with the country and serving the well-being of the people. Hippophae 1:1–7
Novruzov EN, Aslanov SM, Imanova AA, Gasanova ZI (1980) Ursolic-acid from Hippophae rhamnoides. Khim Prir Soedin 6:868
Novruzov EN, Ismailov NM, Mamedov SS (1983) Phenol compounds in Hippophae rhamnoides leaves Azerbaijan SSR USSR Rastit. Resur 19(3):354–356
Nuzov BG, Stadnikov AA (1994) Effect of miliacin oil on healing of trophic ulcers. Patol Fiziol Eksp Ter 4:55–56
Oana L, Mates N, Roman R, Muste A, Ognean L, Neculoiu D, Oprea M, Ile G (1994) Researches on some plant extracts' stimulating effects in wound healing. Buletinul Universitatii de Stiinte Agricole Cluj-Napoca Seria Zootehnie si Medicina Veterinara 48:425–428
Okhotin IK, Lukanov AD, Zubeev PS (1997) Method of duodenum ulcer disease treatment. Patent RF 2082413. Izobreteniya, vol 18, pp 67
Olziikhutag A (1968) Influence of sea-buckthorn (Hippophae-rhamnoides) oil on the development of experimental atherosclerosis in rabbits. Sunflower-D Seed Oil. Cor Vasa, 10 (1): 59–67
Olziikhutag A (1969) Comparative study of the effect of hippophae and sunflower oil on patients with coronary arteriosclerosis. Kardiologiia 9(4):78–82
Ozerina OV, Bereznaya GA, Eliseev IP, Vereshagin AG (1987) Composition and structure of tryacylglycerols from Hyppophae rhamnoides seeds. Khim Prir Soedin (Tashk) 1:52–57
Ozerinina OV, Berezhnaya GA, Vereshchagin AG (1997) Triacylglycerol composition and structure of sea-buckthorn fruits grown in different regions. Fiziologiya Rastenii (Moscow) 44:74–82
Padmavathi B, Upreti M, Singh V, Rao AR, Singh RP, Rath PC (2005) Chemoprevention by Hippophae rhamnoides: effects on tumorigenesis, phase II and antioxidant enzymes, and IRF-1 transcription factor. Nutr Cancer 51(1):59–67
Pakalns D (2002) Lexicon plantarum medicinalium polyglotum. Tevans, Riga, Latvia, p 82
Pang X, Zhao J, Zhang W, Zhuang X, Wang J, Xu R, Xu Z, Qu W (2008) Antihypertensive effect of total flavones extracted from seed residues of Hippophae rhamnoides L. in sucrose-fed rats. J Ethnopharmacol 117(2):325–331
Pantsi WG, Marnewick JL, Esterhuyse AJ, Rautenbach F, van Rooyen J (2011) Rooibos offers cardiac protection against ischaemia/reperfusion in the isolated perfused rat heart. Phytomedicine 18(14):1220–1228
Papuc C, Diaconescu C, Nicorescu V (2008) Antioxidant activity of sea buckthorn (Hippophae Rhamnoides) extracts compared with common food additives. Rom Biotechnol Lett 13(6): 4049–4053

Pashina L (1993) Plants and cosmetics. Minsk, Byelorussia, 130
Pasich B, Juszko-Jjasinska M, Pasich J, Pilch M, Rompel H (1984) Harmaline and harman from Hippophae rhamnoides L. from the industrial part of oberschlesiens. Herba Pol 30:183–186
Pentegova VA (1983) Biology, chemistry and pharmacology of sea-buckthorn. Nauka Akad Nauk SSSR, Novosibirsk, p 124
Pintea A, Marpeau A, Faye M, Socaciu C, Gleizes M (2001a) Polar lipid and fatty acid distribution in carotenolipoprotein complexes extracted from sea buckthorn fruits. Phytochem Anal 12(5):293–298
Pintea A, Varga A, Stepnowski P, Socaciu C, Culea M, Diehl HA (2005) Chromatographic analysis of carotenol fatty acid esters in Physalis alkekengi and Hippophae rhamnoides. Phytochem Anal 16(3):188–195
Plekhanova MN (1988) Sea-buckthorn. Moscow. Publisher "Agromizdat", p 780
Prakash H, Bala M, Ali A, Goel HC (2005) Modification of gamma radiation induced response of peritoneal macrophages and splenocytes by Hippophae rhamnoides (RH-3) in mice. J Pharm Pharmacol 57(8):1065–1072
Pukhalsskaia EC (1958) The effect of extracts of Hippophae rhamnoides upon the development of tumor transplants in animals. Biul Efsptl Biol i Med 45:363–366
Purushothaman J, Suryakumar G, Shukla D, Malhotra AS, Kasiganesan H, Kumar R, Sawhney RC, Chami A (2008) Modulatory effects of seabuckthorn (Hippophae rhamnoides L.) in hypobaric hypoxia induced cerebral vascular injury. Brain Res Bull 77(5):246–252
Purve O, Zham'yansan YA, Malikov VM, Baldan T (1979) Flavonoids of Hippophae rhamnoides grooving in Mongalia. Chem Nat Compd (Engl Transl) 14(3):341–342
Ramasamy T, Varshneya C, Katoch VC (2010) Immunoprotective effect of Seabuckthorn (Hippophae rhamnoides) and Glucomannan on T-2 toxin-induced immunodepression in poultry. Vet Med Int 2010:1–6. doi:10.4061/2010/149373, Article ID 149373
Rasputina DB, Komissarenko NF, Tsybikova DT, Ash P (1975) Flavonoids from Hippophae rhamnoides leaves. Khim Prir Soedin 1:96–97
Rasputina DB, Komissarenko NF, Tsybikova DT (1976) Isorhaminetin and astragalin from the leaves of Hippophae rhamnoides. Chem Nat Comp 12(1):87
Rösch D, Krumbein A, Mügge C, Kroh LW (2004a) Structural investigations of flavonol glycosides from sea buckthorn (Hippophaë rhamnoides) pomace by NMR spectroscopy and HPLC-ESI-MS(n). J Agric Food Chem 52(13):4039–4046
Rösch D, Mügge C, Fogliano V, Kroh LW (2004b) Antioxidant oligomeric proanthocyanidins from sea buckthorn (Hippophae rhamnoides) Pomace. J Agric Food Chem 52(22):6712–6718
Rousi A (1965) Observation on the cytology and variation of European and Asiatic populations of Hippophae rhamnoides. Ann Bot Fenn 2:1–18
Rousi A (1971) The genus *Hippophae* L. A taxonomic study. Ann Bot Fennici 8:177–227
Rousi A, Aulin H (1977) Ascorbic acid content in relation to ripeness in fruit of six Hippophae rhamnoides clones from Pyhäranta. SW Finland Ann Agric Fenn 16:80–87
Sabir SM, Maqsood H, Hayat I, Khan MQ, Khaliq A (2005) Elemental and nutritional analysis of sea buckthorn (Hippophae rhamnoides ssp. turkestanica) Berries of Pakistani origin. J Med Food 8(4):518–522
Sabynch LV, Sibileva LA, Belova LS, Chuchalin VS, Sedykh IM, Rychkov AA, Saratikov AS, Prishchen TP (1994) Anti-inflammatory effect of thick extractions of the schrot of fruit of Hippophae rhamnoides L. Rastitel'nye Resursy 30:70–74
Saggu S, Kumar R (2007) Possible mechanism of adaptogenic activity of seabuckthorn (Hippophae rhamnoides) during exposure to cold, hypoxia and restraint (C-H-R) stress induced hypothermia and post stress recovery in rats. Food Chem Toxicol 45(12):2426–2433
Saggu S, Kumar R (2008) Effect of seabuckthorn (Hippophae rhamnoides) leaf aqueous and ethanol extracts on avoidance learning during stressful endurance performance of rats: a dose dependent study. Phytother Res 22(9):1183–1187

Saggu S, Divekar HM, Gupta V, Sawhney RC, Banerjee PK, Kumar R (2007) Adaptogenic and safety evaluation of seabuckthorn (Hippophae rhamnoides) leaf extract: a dose dependent study. Food Chem Toxicol 45(4):609–617

Sajfrtová M, Licková I, Wimmerová M, Sovová H, Wimmer Z (2010) Beta-sitosterol: supercritical carbon dioxide extraction from sea buckthorn (Hippophae rhamnoides L.) seeds. Int J Mol Sci 11(4):1842–1850

Salenko VL, Sidel'nikova VN, Troshkov ML, Raldugin VA, Pentegova VA (1982) Chemical study of Hippophae rhamnoides main components of the nonsaponified part of fruit pulp extract. Khim Prir Soedin (Tashk) 3:328–332

Salenko VL, Kukina TK, Karamyshev VN, Sidel'nikova VN, Pentegova VA (1986) Chemical study of Hippophae rhamnoides 2. Principal components of the neutral part of the saponification products of the extract of sea-bauckthorn leaves. Khim Prir Soedin (Tashk) 4:514–519

Sauter C, Wolfensberger C (1989) Aniticancer activities as well as antiviral and virus-enhancing properties of aqueous fruit extracts from fifty-six european plant species. Eur J Cancer Clin Oncol 25(6):987–990

Sea buckthorn (*Hippophae rhamnoides*) Natural standard monograph (2011) Natural Standard Inc. http://naturalstandard.com/index-abstract.asp?create-abstract=patient-seabuckthorn.asp&title=Sea%20buckthorn

Sea buckthorn oil (1981) Pharmacopoeial article FS 42-1730-81. The State Register of Drugs, RF Ministry of Health and Medical Industry Moscow, http://grls.rosminzdrav.ru/GRLS.aspx; http://www.drugreg.ru/Soft/SoftCliphar.asp

Sea buckthorn. Monograph. Natural Medicines Comprehensive Database (2011) http://naturaldatabase.therapeuticresearch.com/home.aspx?cs=&s=ND

Sea-buckthorn - a promising multi-purpose crop for Saskatchewan (2011) http://www4.agr.gc.ca/AAFC-AAC/display-afficher.do?id=1198782587774&lang=eng, http://www.mncgloble.com/other-files/seabuckthorn.pdf

Sergeeva NV, Shapiro DK, Bandyukova VA, Anikhimovskaya LV, Narizhnaya TI (1979) Carotenoids of the fruit of some varieties of Hippophae rhamnoides. Khim Prir Soedin 1:100–101

Sharma UK, Sharma K, Sharma N, Sharma A, Singh HP, Sinha AK (2008) Microwave-assisted efficient extraction of different parts of Hippophae rhamnoides for the comparative evaluation of antioxidant activity and quantification of its phenolic constituents by reverse-phase high-performance liquid chromatography (RP-HPLC). J Agric Food Chem 56(2):374–379

Sheichenko OP, Sheichenko VI, Fadeeva II, Zolotarev BM, Tolkachev ON (1987) Tannins from Hippophae rhamnoides L. Khim Prir Soedin (Tashk) 6:902–907, http://www.sanddorn.net/abstracts/SHEICHENKO.htm

Shukla SK, Chaudhary P, Kumar IP, Samanta N, Afrin F, Gupta ML, Sharma UK, Sinha AK, Sharma YK, Sharma RK (2006) Protection from radiation-induced mitochondrial and genomic DNA damage by an extract of Hippophae rhamnoides. Environ Mol Mutagen 47(9):647–656

Shustrova MV, Pashkin PI (1997) Method of treatment of basic demodecosis complicated with staphylococcal and fungal infections in dogs RU 2080117. Izobreteniya, Vol 15, p 57

Singh V (2008) Seabuckthorn: modern cultivation technologies. Daya, Delhi

Sokoloff BK, Funaoka M, Fujisawa CC, Saelhof E, Raniguchi LB, Miller C (1961) An oncostatic factor present in the bark of Hippophae rhamnoides. Growth 25(5):401–409

Sokolov PD (1988) Plant resourses of USSR. Flowering plants, their chemical composition, use: family Rutaceae–Elaeagnaceae, vol 4. Nauka, Leningrad, USSR, pp 202–207

Solonenko LP, Shishkina EE (1989). Electrophoretic and amino acid analysis of proteins in the sea-buckthorn fruit. In: Proceedings of International Symposium on Sea-buckthorn (H. rhamnoides L.), Xian, China. pp 91–95

Son Z, Zhang T, Gao Y (1997) Protective effects of sea buckthorn oil on experimental cold injury. Hebei Yike Daxue Xuebao 18(4):206–208

Spinu M, Vasiu C, Dumitru C, Brudasca F, Boldizsar E, Dobrean V, Lates C, Branzas P (1996) Effects of several Calendula officinalis and Hippophae rhamnoides extraction on non-specific

and specific cellular immune responses in different domestic species. 22nd Symposium of the Association of Romanian Veterinarians Cluj, Romania 7–8 Nov 1996. Association of Romanian Veterinarians, Romania, pp 167

Spiridonov NA, Arkhipov VV, Foigel' AG, Tolkachev ON, Sasov SA, Syrin AB, Tolkachev VN (1997) Cytotoxicity of Chamaenerium angustifolium (L) Scop. and Hippophae rhamnoides L tannins and their effect on mitochondrial respiration. Eksperimental'naya i Klinicheskaya Farmakologiya 60:60–63

Stakheeva MN, Zueva EP (1993) The effect of preparations of roots and shoots of Hippophae rhamnoides L on the state of the natural resistance system in mice. Rastitel'nye Resursy 29:91–95

Suleyman Z, Erdemoglu N, Kusmenoglu S, Gurbuz I, Yesilada E, Calis I (1998) Fatty acid composition and anti-ulcerogenic activity of Hippophae rhamnoides fruit oil. J Fac Pharm Gazi 15:85–91

Süleyman H, Demirezer LO, Büyükokuroglu ME, Akcay MF, Gepdiremen A, Banoglu ZN, Göçer F (2001a) Antiulcerogenic effect of Hippophae rhamnoides L. Phytother Res 15(7):625–627

Suleyman H, Buyukokuroglu ME, Koruk M, Akcay F, Kiziltunc A, Gepdiremen A (2001b) The effects of Hippophae rhamnoides L. extract on ethanol-induced gastric lesion and gastric tissue glutathione level in rats: a comparative study with melatonin and omeprazole. Ind J Pharmacol 33(2):77–81

Sun K, Chen X, Ma R (2002) Molecular phylogenetics of *Hippophae* L., (Elaeagnaceae) based on the internal transcribed spacer (ITS) sequences of nr DNA. Plant Syst Evol 235(1–4): 121–134

Sun B, Zhang P, Qu W, Zhang X, Zhuang X, Yang H (2003) Study on effect of flavonoids from oil-removed seeds of Hippophae rhamnoides on inducing apoptosis of human hepatoma cell. Zhong Yao Cai 26(12):875–877

Suomela JP, Ahotupa M, Yang B, Vasankari T, Kallio H (2006) Absorption of flavonols derived from sea buckthorn (Hippophaë rhamnoides L.) and their effect on emerging risk factors for cardiovascular disease in humans. J Agric Food Chem 54:7364–7369

Swaroop A, Sinha AK, Chawla R, Arora R, Sharma RK, Kumar JK (2005) Isolation and characterization of 1,3-dicapryloyl-2-linoleoylglycerol: a novel triglyceride from berries of Hippophae rhamnoides. Chem Pharm Bull 53(8):1021–1024

Swenson U, Bartish IV (2002) Taxonomic synopsis of *Hippophae* (Elaeagnaceae). Nord J Bot 22:369–374

Teng BS, Lu YH, Wang ZT, Tao XY, Wei DZ (2006) In vitro anti-tumor activity of isorhamnetin isolated from Hippophae rhamnoides L. against BEL-7402 cells. Pharmacol Res 54 (3):186–194

Thumm EJ, Stoss M, Bayerl C, Schurholz TH (2000) Uberprufung der Werksamkeit einer 20% igen and 10%igen Sanddornkernolcreme bei Patienten mit leichter bis mittelgradiger atopischer Dermatitis. Aktuelle Dermatologie 26:285–290

Tiitinen KM, Hakala MA, Kallio HP (2005) Quality components of sea buckthorn (Hippophae rhamnoides) varieties. J Agric Food Chem 53(5):1692–1699

Tong J, Zhang C, Zhao Z, Yang Y, TianK (1989). The determination of the physical-chemical constants and sixteen mineral elements in raw sea-buckthorn juice. Proceedings of International Symposium on Sea-buckthorn (H. rhamnoides L.), Xian, China. pp 132–137

Turova AD (1974) Medicinal plants of USSR and their use. Medicine, Moscow, USSR, pp 1–426

Undina S, Morar R, Neamtu G, Morar E (1989) Phytochemical research on Hippophae rhamnoides L. sea buckthorn. Fatty acid contents in certain morphological types of sea buckthorn. Bul Inst Agron Cluj-Napoca Ser Zooteh Med Vet 43:41–46

Upadhyay NK, Kumar R, Siddiqui MS, Gupta A (2009) Mechanism of wound-healing activity of Hippophae rhamnoides L. leaf extract in experimental burns. Evid Based Complement Alternat Med eCAM 200, Page 1 of 9. doi: 10.1093/ecam/nep189 Accessed 15 Jan 2011 http://ecam.oxfordjournals.org

Upadhyay NK, Kumar MS, Gupta A (2010) Antioxidant, cytoprotective and antibacterial effects of sea buckthorn (Hippophae rhamnoides L.) leaves. Food Chem Toxicol 48(12):3443–3448

Vengerovskii AI, Sedykh IM, Mizina TY, Plotnikov MB (1994) Hepato-protective effect of the concentrate of the juice of Hippophae rhamnoides L. under experimental CCl-4-hepatitis. Rastitel'nye Resursy 30:53–56

Vijayaraghavan R, Gautam A, Kumar O, Pant SC, Sharma M, Singh S, Kumar HT, Singh AK, Nivsarkar M, Kaushik MP, Sawhney RC, Chaurasia OP, Prasad GB (2006) Protective effect of ethanolic and water extracts of sea buckthorn (Hippophae rhamnoides L.) against the toxic effects of mustard gas. Indian J Exp Biol 44(10):821–831

Vlasov VV (1970) Hippophae oil in the treatment of superficial skin burns. Vestn Dermatol Venerol 44(6):69–72

Wang J, Zhang M, Xu Z, Zhang T, Cheng Y (1982) Effect of flavonoids of Hippophae rhamnoides on ishemic heart disease. Acta Acad Med Sichuan 13(1):6–11

Wang Y, Lu Y, Liu X (1992a) The protective effect of Hippophae rhamnoides L. on hyperlipidemic serum cultured smooth muscle cells in vitro. Chung Kuo Chung Yao Tsa Chih 17:601, 624–626

Wang Y, Lu Y, Liu X, Gou Z, Hu J (1992b) The protective effect of Hippophae rhamnoides L on hyperlipidemic serum cultured smooth muscle cells in vitro. Zhongguo Zhong Yao Za Zhi 17(10):601, 624–626

Wang GY, Liang ZY, Zhang LP (1999) Purification and characterization of water soluble polysaccharide JS1 from Hippophae rhamnoides. Chin Pharmaceut J 34:229–231

Wang B, Feng Y, Yu Y, Zhang H, Zhu R (2001) Effects of total flavones of Hippophae rhamnoides L. (sea buckthorn) on cardiac function and hemodynamics in healthy human subjects. Translation from the original Chinese provided by Rich Nature Nutroceutical Laboratories. Accessed 22 Jan 2008 http://www.richnature.com/products/herbal/articles/heart.pdf

Wang ZY, Luo XL, He CP (2006) Management of burn wounds with Hippophae rhamnoides oil. Nan Fang Yi Ke Da Xue Xue Bao 26(1):124–125

Watanabe T, Rajbhanddari KR, Malla KJ, Yahara S (2005) A handbook of medicinal plants of Nepal (Kobai Publishing Project. Foundation for Democracy and development studies, Bangkok, Thailand). AYUR SEED, Kanagawa, Japan, pp 106–107

Wolf D, Wegert F (1993) Experience gained in the cultivation, harvesting and utilization of sea-buckthorn. Cultivation and utilization of wild fruit crops. Bernhard Thalacker, Braunschweig, pp 23–29

Wu I, Li X (1990) The effect of total flavones of Hippophae rhamnoides L. on beating and electrical activity of cultured cardiac cell. Xi'an Yike Daxue Xuebao 11(4):301–303

Wu J, Yu XJ, Ma X, Li XG, Liu D (1994) Electrophysiologic effects of total flavones of Hippophae rhamnoides L on guinea pig papillary muscles and cultured rat myocardial cells. Zhongguo Yao Li Xue Bao 15(4):341–343

Wu Y, Wang Y, Wang B, Lei H, Yang Y (1997a) Effects of total flavones of fructus Hippophae (TFH) on cardiac function and hemodynamics of anesthetized open-chest dogs with acute heart failure. Zhongguo Zhong Yao Za Zhi 22(7):429–431, 448

Wu Y, Wang B, Wang Y, Kang J, Wang H (1997b) Protective effect of total flavones of Hippophae rhamnoides L. on myocardial ischemia and reperfusion injury in rats. Chin Pharmacol Bull 13:53–55

Wu J, Huang X, Zhang X (2004) Effects of Xindakang tablets on coronary artery disease with venticular extrasystoles in old patients. Sichuam Medical Journal, 2004–02. http://en.cnki.com.cn/Article_en/CJFDTOTAL-SCYX200402004.htm

Xiao R, Wen LP (1996) The seperation and assay of the antigastric ulcers constituents in the oil of Hippophae rhamnoides. Huaxi Yaoxue Zazhi 11:90–91

Xiao M, Yang Z, Jiu M, You J, Xiao R (1992) The antigastroulcerative activity of beta-sitosterol-beta-D-glucoside and its aglycone in rats. J West China Univ Med Sci 23(1):98–101

Xing J, Yang B, Dong Y, Wang B, Wang J, Kallio HP (2002) Effects of sea buckthorn (Hippophaë rhamnoides L.) seed and pulp oils on experimental models of gastric ulcer in rats. Fitoterapia 73:644–650

Xu Q, Chen C (1991) Effects of oil of Hippophae rhamnoides on experimental thrombus formation and blood coagulation system. Tianran Chanwu Yanjiu Yu Kaifa 3:70–73
Yang B, Kallio HP (2001) Fatty acid composition of lipids in sea buckthorn (Hippophae rhamnoides L.) berries of different origins. J Agric Food Chem 49:1939–1947
Yang B, Kallio H (2006) Analysis of triacylglycerols of seeds and berries of sea buckthorn (Hippophaë rhamnoides) of different origins by mass spectrometry and tandem mass spectrometry. Lipids 41(4):381–392
Yang H, Wang S, Su X (1988) A study on the dynamic variation of ascorbic acid in Hippophae. Hippophae 4:41–44
Yang F, Quan J, Zhang TY, Ito Y (1998) Multidimensional counter-current chromatographic system and its application. J Chromatogr A 803:298–301
Yang B, Kalimo KO, Mattila LM, Kallio SE, Katajisto JK, Peltola OJ, Kallio HP (1999) Effects of dietary supplementation with sea buckthorn (Hippophae rhamnoides) seed and pulp oils on atopic dermatitis. J Nutr Biochem 10:622–630
Yang B, Kalimo KO, Tahvonen RL, Mattila LM, Katajisto JK, Kallio HP (2000) Effect of dietary supplementation with sea buckthorn (Hippophae rhamnoides) seed and pulp oils on the fatty acid composition of skin glycerophospholipids of patients with atopic dermatitis. J Nutr Biochem 11:338–340
Yang B, Karlsson RM, Oksman PH, Kallio HP (2001) Phytosterols in sea buckthorn (Hippophaë rhamnoides L.) berries: identification and effects of different origins and harvesting times. J Agric Food Chem 49:5620–5629
Yang ZG, Li HR, Wang LY, Li YH, Lu SG, Wen XF, Wang J, Daikonya A, Kitanaka S (2007) Triterpenoids from Hippophae rhamnoides L. and their nitric oxide production-inhibitory and DPPH radical-scavenging activities. Chem Pharm Bull 55(1):15–18
Yao Y, Tigerstedt PMA (1993) Isozyme studies of genetic diversity and evolution in Hippophae. Genetic Res Crop Evol 42:153–164
Yao Y, Tigerstedt PMA (1994) Genetic diversity in Hippophae L. and its use in plant breeding. Euphytica 77:165–169
Yao Y, Tigerstedt PMA (1995) Geographic variation of growth rhythm, height and hardiness and their relations in Hippophae rhamnoides. J Am Soc Hort Sci 120(4):691–698
Yao Y, Tigerstedt PMA, Joy P (1992) Variation of vitamin C concentration and character correlation between and within natural sea buckthorn (Hippophae rhamnoides L.) populations. Acta Agriculturae Scandinavica Sec B Soil Plant Sci 42:12–17
Yasukawa K, Kitanaka S, Kawata K, Goto K (2009) Anti-tumor promoters phenolics and triterpenoid from Hippophae rhamnoides. Fitoterapia 80(3):164–167
Yoshida T, Tanakan K, Chen X-M, Okuda T (1991) Tannins from Hippophae rhamnoides. Phytochemistry 30(2):663–666
Yu JG, Cong PZ, Tan P, Luo XZ (1988) Studies on the chemical constituents in the volatile oil of Hippophae-rhamnoides -SSP-Sinensis rousi. Acta Pharm Sin 23(6):456–459
Yu X, Liu C, Li X, Zang W, Zhang S (1990) Effect of total flavones of Hippophae rhamnoides on isolated vascular smooth muscles. Xi'an Yike Daxue Xuebao 11(4):332–335
Yu X, Wu J, Zang W (1992) Effects of total flavones of Hippophae rhamnoides L. on electrical and mechanical activities in guinea pig papillary muscles. J Xi'an Medical Univ 13:343–345
Yue ME, Shi YP (2006) Application of 1-alkyl-3-methylimidazolium-based ionic liquids in separation of bioactive flavonoids by capillary zone electrophoresis. J Sep Sci 29:272–276
Zamkovaya EA (2003) Hyporamine as an agent for prophylaxis of acute virus infections of the respiratory tract. Rus Med J 11(4):170–174, http://www.rmj.ru/articles_533.htm
Zeb A (2004) Chemical and nutritional constituents of sea buckthorn. Pak J Nutr 3:99–106
Zham'yansan YA (1979) Oils of the seeds and of the fruit flesh of Hippophae rhamnoides. Chem Nat Compd (Engl Transl) 14(1):108
Zhang MS (1987) A control trial of flavonoids of Hippophae rhamnoides L. in treating ischemic heart disease. Zhonghua Xin Xue Guan Bing Za Zhi 15(2):97–99

Zhang TY, Hua X, Xiao R, Kong S (1988a) Seperation of flavonoids in crude extract from sea buckthorn by countercurrent chromatography with two types of coil planet centrifuge. J Liq Chromatogr 11(1):233–244

Zhang TY, Xiao R, Xiao ZY, Pannell LK, Ito Y (1988b) Rapid separation of flavonoids by analytical high-speed countercurrent chromatography. J Chromatogr 445(1):199–206

Zhang PX, Ding L. Mao L, Li D, Li L (1989a) Anti-tumor effects of fruit juice and seed oil of Hippophae rhamnoides and their influences on immune function. Proceedings of International Symposium on Sea-buckthorn (H. rhamnoides L.), Xian, China, pp 373–381

Zhang W, Yan J, Duo J, Ren B, Guo J (1989b) Preliminary study of biochemical constituents of sea-buckthorn berries growing in Shanxi Province and their changing trend. Proceedings of International Symposium on Sea-buckthorn (H. rhamnoides L.), Xian, China, pp 96–105

Zhang JW, Wang Y, Xu LM, Ma XL, Sun YX, Jiang WP (1996) Determination of gossypol in Hippophae rhamnoides L seed oil. Jilin daxue ziran kexue xuebao 3:105–106

Zhang X, Zhang M, Gao Z, Wang J, Wang Z (2001) Effect of total flavones of Hippophae rhamnoides L. on sympathetic activity in hypertension. Hua Xi Yi Ke Da Xue Xue Bao 32(4):547–550

Zhang P, Mao YC, Sun B, Qian M, Qu WJ (2005) Changes in apoptosis-related genes expression profile in human breast carcinoma cell line Bcap-37 induced by flavonoids from seed residues of Hippophae Rhamnoides L. Ai Zheng 24(4):454–460

Zhang W, Zhao J, Wang J, Pang X, Zhuang X, Zhu X, Qu W (2010) Hypoglycemic effect of aqueous extract of seabuckthorn (Hippophae rhamnoides L.) seed residues in streptozotocin-induced diabetic rats. Phytother Res 24(2):228–232

Zheng RX, Xu XD, Tian Z, Yang JS (2009) Chemical constituents from the fruits of Hippophae rhamnoides. Nat Prod Res 23(15):1451–1456

Zhmurko TG, Rashkes YV, Glushenkova AI (1986) Hydroxy acids from *Hippophae rhamnoides* seed oil. Khim Prir Soedin (Tashk) 2:161–168

Zhmurko TG, Rashkes YV, Plugar' VN, Ash I, Glushenkova AI (1989) Gas chromatographic - mass spectrometric analysis of hydroxy and epoxy acids from the seed oil of *Hippophae rhamnoides* Khim Prir. Soedin (Tashk) 5:626–634

Zhmyrko TG, Gigienova EI, Umarova AU (1978) Vitamins from the oils of *Hippophae rhamnoides* fruits. Khim Prir Soedin (Tashk) 3:313–317

Zhmyrko TG, Goncharova NP, Gigienova EI, Glushenkova AI (1984) Group composition of neutral lipids in the oil of *Hippophae rhamnoides* fruits. Khim Prir Soedin (Tashk) 3:300–305

Zhmyrko TG, Glushenkova AI, Zegel'man AB, Andronov VA (1987) Neutral lipids in freon extracts of *Hippophae rhamnoides* fruits. Khim Prir Soedin (Tashk) 2:231–234

Zhong F, Jiang Y, Wu F, Shu R, Cai X, Tan J (1989) Effect of the total flavone of *Hippophae rhamnoides* fruits on mouse cell immune function. Zhongcaoyao 20(7):331

Zhu M, Song JM, Cho CH (1997) Antiulcer effect of *Hippophae rhamnoides* in rats. J Gastroenterol Hepatol 12:A218

Zhu F, Huang B, Hu CY, Jiang QY, Lu ZG, Lu M, Wang MH, Gong M, Qiao CP, Chen W, Huang PH (2005) Effects of total flavonoids of *Hippophae rhamnoides* L. on intracellular free calcium in cultured vascular smooth muscle cells of spontaneously hypertensive rats and Wistar-Kyoto rats. Chin J Integr Med 11(4):287–292

Zu Y, Li C, Fu Y, Zhao C (2006) Simultaneous determination of catechin, rutin, quercetin kaempferol and isorhamnetin in the extract of sea buckthorn (*Hippophae rhamnoides* L.) leaves by RP-HPLC with DAD. J Pharm Biomed Anal 41(3):714–719

Chapter 6
New Results on the Pharmacology and Clinical Use of the TCM-Drug *Salvia miltiorrhiza*

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6.1 Introduction

The use of Chinese herbal medicines and natural products is becoming more popular in both Chinese communities and Western societies as an alternative treatment for diseases or as a health supplement. *Salvia miltiorrhiza* (chin. Danshen) is the dry root and rhizome of *Salvia miltiorrhiza* Bge, family Lamiaceae (Fig. 6.1). *Salvia miltiorrhiza* is officially listed in the Chinese Pharmacopoeia as a "blood-invigorating" herbal medicine which, in the Traditional Chinese Medicine concept, is non-toxic and slightly "cold" in nature. *Salvia miltiorrhiza* is believed to possess several functions. It can promote blood circulation, remove stasis, and is therefore used widely for the treatment of coronary heart disease, in particular angina pectoris and myocardial infarction. It can also be used in menstruation disorders, to relieve pain in acute arthritis and pathogen-induced inflammation, to relieve restlessness, and in the treatment of insomnia.

With advances in separation and analytical methodologies, a number of constituents of *Salvia miltiorrhiza* have been isolated and characterised. Chemical analysis methods including High Performance Liquid Chromatography (HPLC), Liquid Chromatography and Mass Spectrometry (LC-MS), and Gas chromatography and Mass Spectrometry (GC-MS) have enabled both authentication and quality control of *Salvia miltiorrhiza* root extracts (Ong and Len 2004; Li et al. 2005, 2007; Yuan et al. 2005; Zhang et al. 2005a; Liu et al. 2006a, b, 2010h; Yang et al. 2006, 2007; Zhi and Deng 2006; Zhou et al. 2006a; Ma et al. 2007; Gu et al. 2007; Wu et al. 2007b; Cao et al. 2008). More efficient extraction methodology also led to higher yields of the active ingredients such as salvianolic acids and tanshinones (Pan et al. 2001; Tian et al. 2002, 2011; Chen et al. 2006; Wang et al. 2007c, 2008a;

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Li et al. 2008a, b; Liu et al. 2008; Yang et al. 2008a; Kan et al. 2009; Wu et al. 2009a, 2010; Sun et al. 2011). Improvements in the sensitivity and specificity of the analytical methods have also enhanced the quality control in terms of identifying the active constituents present in *Salvia miltiorrhiza* roots as well as in different *Salvia miltiorrhiza* formulations, and fingerprinting of the products (Zhang et al. 2002; Feng et al. 2008; Liu et al. 2006b, 2007c; Xu et al. 2008a; Wang et al. 2010c). *Salvia miltiorrhiza* can be best separated using two different Thin-Layer Chromatography (TLC) and HPLC fingerprint analytical methods for the tanshinones and the phenol/caffeoyl carboxylic acids (Wagner et al. 2010, 2011). This is followed by extensive studies of the individual ingredients for their bioactivities and pharmacology. To date, *Salvia miltiorrhiza* and some of its active ingredients are among the most studied herbal medicines worldwide. This review summarises the most recent findings on the pharmacology of *Salvia miltiorrhiza* and its major bioactive ingredients.

6.2 Active Ingredients in *Salvia miltiorrhiza*

6.2.1 Chemical Constituents

The active components in *Salvia miltiorrhiza* can be divided into water-soluble and lipid-soluble compounds. Depending on the extraction methods and solvents used, the presence of the water-soluble and lipid-soluble compounds in different fractions and formulations varies greatly. The major water-soluble compounds isolated from aqueous extracts of *Salvia miltiorrhiza* include protocatechualdehyde, danshensu (3-(3,4-dihydroxyphenyl)lactic acid), lithospermic acid B, rosmarinic acid, salvianolic acid A, salvianolic acid B, and salvianolic acid C. About 40 diterpene quinones are originally isolated and identified in the lipid-soluble fraction of *Salvia miltiorrhiza* (Zhou 1993). The major lipid-soluble diterpene quinones include cryptotanshinone; dihydrotanshinone; 9-hydroxytanshinone II; tanshinone I, tanshinone IIA, and tanshinone IIB; isotanshinone I, isotanshinone IIA, and isotanshinone IB; isocryptotanshinone; and methyl tanshinone. Other minor lipid-soluble constituents include tanshindiol A, tanshindiol B, and tanshindiol C; danshenxinkum A, danshenxinkum B, danshenxinkum C, and danshenxinkum D; miltirone; salviol; and miltionone I and miltionone II. Both tanshinone IIA and salvianolic acid B are considered characteristic marker compounds for *Salvia miltiorrhiza* (Wagner et al. 2010; Wagner 2012). The chemical structures of some major water-soluble and lipid-soluble compounds from *Salvia miltiorrhiza* are summarised in Fig. 6.2 (Wang et al. 2010f).

The major active components of *Salvia miltiorrhiza* in commercially available crude extract and *Salvia miltiorrhiza*-containing compound preparations show great variations in composition and concentration. The variation may be a result of different growth conditions, the stage of growth at which the herb is harvested and the habitat where the medicinal plant is grown (Zhang et al. 2002, 2004a; Skala

Fig. 6.1 *Salvia miltiorrhiza* and its dried roots

and Wysokinska 2005; Yan et al. 2006; Tang et al. 2007b; Zhao et al. 2007; Li et al. 2008a, 2009a; Ran et al. 2008; Lee et al. 2008a; Qin et al. 2009; Sheng et al. 2009; Yuan et al. 2009; Zhong et al. 2009; Fu et al. 2010; Song et al. 2010; Guang et al. 2011; Kai et al. 2011; Liu et al. 2011a). For formulated oral or injectable preparations of *Salvia miltiorrhiza*, variation in the contents and components of active ingredients may be a result of different extraction and processing methods used during production, since the type of extraction method used plays a significant role in the extraction efficiency and thus the final contents of the extract (Yuan et al. 2005; He et al. 2007; Sun et al. 2009b; Tian et al. 2009). Salvianolic acid B, danshensu, rosmarinic acid, protocatechualdehyde, cryptotanshinone, and tanshinones I and IIA are optimised as markers for the evaluation of *Salvia miltiorrhiza* and *Salvia miltiorrhiza*-containing formulations (Hu et al. 2005a, b; Cao et al. 2008; Zhong et al. 2009). Salvianolic acid B is the major water-soluble constituent, and tanshinone IIA is the major lipid-soluble constituent in *Salvia miltiorrhiza* (Table 6.1)Yuan et al. 2005.

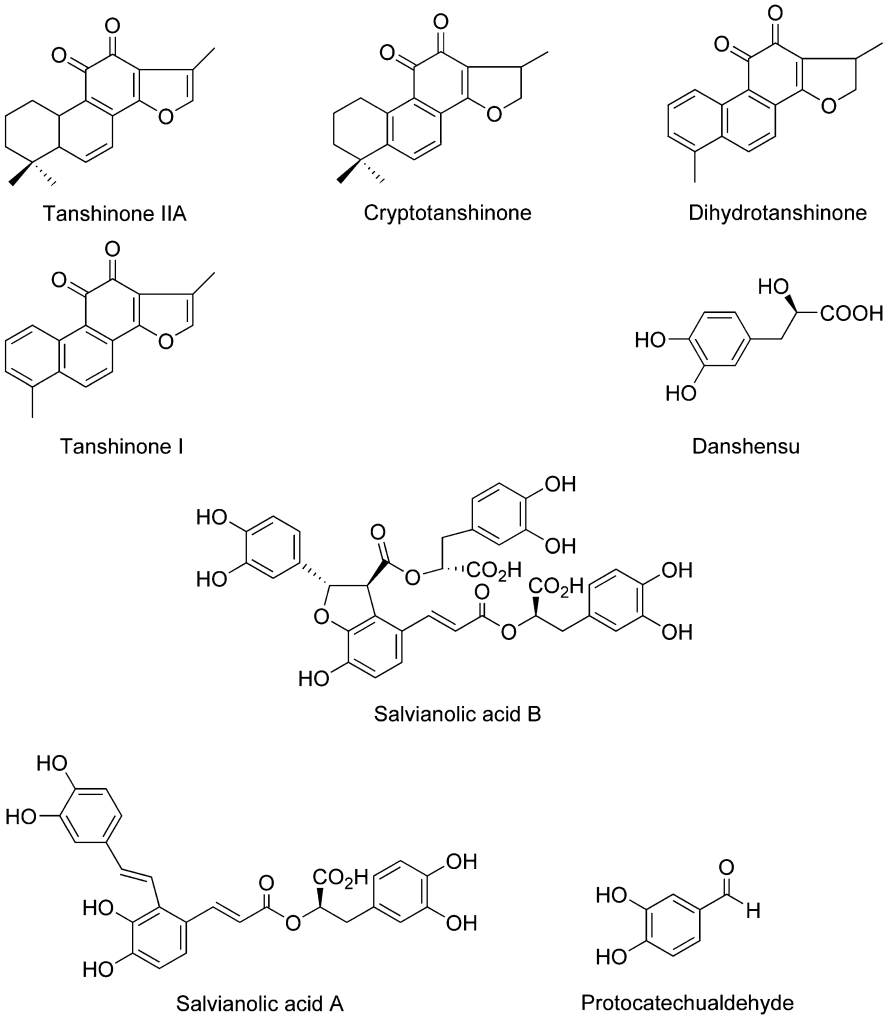

Fig. 6.2 Chemical structures of some major water-soluble and lipid-soluble compounds isolated from *Salvia miltiorrhiza*

6.2.1.1 Water-Soluble Ingredients

The major hydrophilic ingredients isolated from *Salvia miltiorrhiza* are phenolic acids, including polyphenolic acids (salvianolic acids) and compounds such as protocatechualdehyde and danshensu. Salvianolic acid B and danshensu are the major constituents in both the aqueous extract of dried root of the herb and various

Table 6.1 The contents of salvianolic acid B, danshensu, tanshinone IIA, tanshinone I, and crytotanshinone in *Salvia miltiorrhiza* from different regions of China (Yuan et al. 2005)

	Content (mg/g)				
Regions	SAB	DSS	Tan IIA	Tan I	CT
Zhongjuang, Sichuan	45.2	1.27	1.16	0.12	0.22
Laiwu, Shandong	43.4	0.29	4.59	1.07	3.8
Juxian, Shangdong	49.0	0.71	3.14	0.86	2.18
Taian, Shangdong	42.6	0.63	2.16	0.59	2.33
Wang yuanxiang, Zhejiang	63.3	0.91	0.86	0.24	0.3
Panan, Zhejiang	77.0	0.66	2.01	0.73	1.07
Shangluo, Shanxi	51.8	0.88	2.02	0.54	0.93
Anguo, Hebei	47.1	0.78	3.31	0.98	2.35
Xingtang, Heibei	48.9	1.04	0.56	0.25	0.36

Abbreviations: *SAB* salvianolic acid B; *DSS* danshensu; *Tan IIA* tanshinone IIA; *Tan I* tanshinone I; *CT* cryptotanshinone

Salvia miltiorrhiza-containing formulations. Salvianolic acid B and lithospermic acid B have identical structures, except for the configurational assignments of two stereocenters. Through chemical correlation, the absolute configuration of salvianolic acid B has been corrected to establish that salvianolic acid B and lithospermic acid B are in fact the same compound (Watzke et al. 2006). Salvianolic acid A is a minor phenolic acid that is recently shown to have effects similar to those of salvianolic acid B. Recent studies have focused on the pharmacological actions of water-soluble ingredients such as salvianolic acid A, salvianolic acid B, and danshensu. Salvianolic acid A and salvianolic acid B have been found to have potent antioxidative capabilities which are related to their polyphenolic structures as well as abilities to act as reactive oxygen species (ROS) scavengers, reduce leucocyte-endothelial adherence, inhibit inflammation and metalloproteinases expression from aortic smooth muscle cells, and indirectly regulate immune function (Ho and Hong 2011).

Danshensu

Danshensu has been shown to dilate blood vessels, including coronary arteries and small blood vessels, inhibit platelet aggregation, and prevent reperfusion injury of the ischaemic heart in animal studies. Danshensu inhibits calcium aggregation and prevents calcium overload by blocking calcium influx in cardiac muscle cells (Lam et al. 2007). Danshensu produces an endothelium-independent vasorelaxant effect in rat coronary artery by inhibition of calcium channels in vascular smooth muscle cells (Lam et al. 2007). Sodium danshensu produces biphasic effects on vessel tension in isolated rat aorta, producing small contractions through transient enhancement of calcium influx at low doses, but vasodilation at high doses through

opening of non-selective potassium ion channels and small-conductance calcium-sensitive potassium channels (Zhang et al. 2010b). Thus, derivatives of danshensu may be potential drug candidates for anti-myocardial ischaemia therapy and merit further investigation (Dong et al. 2009c).

Danshensu exerts a protective effect through its antioxidant and anti-inflammatory actions. Danshensu scavenges free radicals and protects endothelial cells against homocysteinemia (Chan et al. 2004) and hydrogen peroxide-induced endothelial cell damage (Yang et al. 2009). Danshensu lowers total homocysteine in rats with elevated total homocysteine acutely by increasing trans-sulphuration and chronically by upregulating trans-sulphuration enzyme activities (Cao et al. 2009). When combined with puerarin (constituent of *Pueraria lobata* (chin. *Ge Gen*), danshensu exerts a cardioprotective effect against acute ischaemic myocardial injury in rats, through antioxidant and anti-lipid peroxidation properties (Wu et al. 2007c). Danshensu and salvianolic acid B inhibit vascular smooth muscle cell proliferation by increasing the nitric oxide level, decreasing the endothelin-1 content, and maintaining cell integrity (Ding and Yuan 2007). Danshensu improves the proliferative and adhesive capacity of endothelial progenitor cells impaired by oxidised low-density lipoprotein (ox-LDL) by decreasing malondialdehyde and increasing superoxide dismutase activity (Chai et al. 2009). In rat vascular smooth muscle cells exposed to hydrogen peroxide, danshensu and cryptotanshinone increase cell viability and superoxide dismutase activity, and reduce the levels of malondialdehyde and hydroxyl free radicals (Wu et al. 2009b). Danshensu increases superoxide dismutase activity and inhibits hepatic stellate cell proliferation, reducing the degrees of liver fibrosis in rat liver (Li et al. 2008e; Yu et al. 2009a). Danshensu protects against hepatic injury induced by omethoate (organophosphorus insecticide) through its anti-inflammatory effect (Ren et al. 2010a). When combined with emodin, danshensu protects against early stages of experimental severe acute pancreatitis in rats by decreasing inflammatory response and oxidative stress (Wang et al. 2010a). Danshensu can protect human peritoneal mesothelial cells through inhibiting the expression of fibronectin and collagen-I induced by high glucose, which is related to the suppression of oxidative stress (Zhang et al. 2011a). Apart from these antioxidant, anti-inflammatory, and vasodilatory effects, danshensu downregulates protein expression of matrix metalloproteinase (MMP)-2, MMP-9 and vascular endothelial growth factor (VEGF), and inhibits angiogenesis and tumour cell invasion in melanoma cells (Zhang et al. 2010a). Danshensu is effective and safe in ameliorating the prognosis of maternal syndrome in a preeclampsia mouse model (Shen et al. 2010).

Protocatechualdehyde

Protocatechualdehyde shows diverse pharmacological properties. Protocatechualdehyde inhibits tyrosinase, which catalyses the rate-limiting step of melanin biosynthesis and is suggested to be a potential agent for treatment of pigmentation disorder (No et al. 2004). Protocatechualdehyde protects human umbilical vein

endothelial cells from oxidised low-density lipoprotein-induced injury (Han et al. 2007). Protocatechualdehyde inhibits mRNA expression of type I and III procollagen of fibroblasts from patients with systemic sclerosis, despite being less potent than tanshinone IIA in suppressing the proliferation (Lu et al. 2007). In streptozotocin-diabetic rats, protocatechualdehyde suppresses the development of lens opacity. In human lens epithelial cells, protocatechualdehyde inhibits the induction of the receptor for advanced glycation end product protein and mRNA expression by the receptor for advanced glycation end product-specific ligand S100b. Protocatechualdehyde may be of therapeutic interest in preventing diabetic complications such as diabetic cataracts (Kim et al. 2007b). Protocatechualdehyde inhibits hepatitis B virus in a hepatoma (HepG2 2.2.15) cell line and duck hepatitis B virus replication in ducklings *in vivo* (Zhou et al. 2007) and may have potential as a therapeutic agent for hepatitis B viral infections.

Salvianolic Acid A

Salvianolic acid A has diverse bioactivities, including protection against cerebral lesion, defence against oxidative damage, and improvement of memory loss. Salvianolic acid A inhibits human low-density lipoprotein oxidation by scavenging free radicals (Liu and Liu 2002). Salvianolic acid A protects against acute hepatic damage caused by carbon tetrachloride in rats through its antioxidative effect (Wang et al. 2007a; Wu et al. 2007d). In rats with acute myocardial infarction, salvianolic acid A decreases malondialdehyde levels and infarct size, improving left ventricular function and appearance of the myocardium and exerting preventive effects against myocardial remodelling after infarction (Jiang et al. 2009). Salvianolic acid A has antiplatelet and antithrombotic effects (Fan et al. 2010; Huang et al. 2010). Salvianolic acid A inhibits platelet-derived growth factor homodimer-activated rat hepatic stellate cell proliferation, partially through apoptosis induction (Lin et al. 2006c). The protective effects of salvianolic acid A on 1-methyl-4-phenylpyridinium ion-induced cytotoxicity may be related to its antioxidative and anti-apoptotic activities, suggesting that salvianolic acid A may provide a useful therapeutic strategy for the treatment of progressive neurodegenerative diseases (Wang and Xu 2005). Salvianolic acid A blocks nucleoside transport in cancer cells and potentiates the cytotoxicity of chemotherapeutic drugs. As an agent showing moderate antitumour effect, salvianolic acid A may be useful in combination cancer therapy (Zhang et al. 2004b). Salvianolic acid A protects bone from prednisone-induced bone marrow impairment by stimulating osteogenesis and depressing adipogenesis in bone marrow stromal cells (Cui et al. 2009), suggesting that salvianolic acid A may be a useful therapeutic strategy for the treatment of osteoporosis. Salvianolic acid A increased peripheral blood perfusion and vascular activities in streptozotocin (STZ)-induced type 2 diabetic rats which may be relevant to its possible use in diabetic foot treatment (Yang et al. 2011a).

Salvianolic Acid B

Salvianolic acid B protects against ischaemia–reperfusion injury and inhibits platelet aggregation through its superoxide radical scavenging activity (Zhang and Wang 2006; Zhao et al. 2008a). Salvianolic acid B ameliorates oxidative damage and eliminates reactive oxygen species accumulation in hepatocytes to attenuate hepatic stellate cell activation, conferring hepatoprotective and anti-fibrogenic effects (Lin et al. 2006d). Other effects of salvianolic acid B which may contribute to its beneficial cardiovascular effects include stimulation of nitric oxide production in endothelial cells and calcium channel blocking effects (Lam et al. 2006c), modulation of endothelial cell permeability (Ding et al. 2005a; Ding and Yuan 2007), inhibition of angiotensin-converting enzyme activity (Kang et al. 2003; Gao et al. 2004), increase in nitric oxide production via endothelial nitric oxide synthase (eNOS) phosphorylation and L-arginine uptake (Pan et al. 2011), and inhibition of reactive oxygen species (ROS) production (Yang et al. 2011b). Salvianolic acid B activates the opening of the BK(Ca) channels of the porcine coronary artery smooth muscle cells through the activation of guanylate cyclase without the involvement of the nitric oxide synthase activation (Lam et al. 2006a). Salvianolic acid B attenuates plasminogen activator inhibitor type 1 production in tumour necrosis factor (TNF)-alpha-treated human umbilical vein endothelial cells (Zhou et al. 2005) and inhibits hydrogen peroxide-induced endothelial cell apoptosis (Liu et al. 2007a).

Salvianolic acid B inhibits the malignant transformation of oral precancerous lesion through inhibition of angiogenesis (Zhou et al. 2006c) and inhibits growth of head and neck squamous cell carcinoma via cyclooxygenase-2 and apoptotic pathways (Hao et al. 2009). Salvianolic acid B inhibits platelet deposition from flowing, anticoagulated whole blood to immobilised collagen at both venous and arterial shear rate, inhibiting platelet adhesion to immobilised collagen by interfering with collagen receptor (Wu et al. 2008b). Salvianolic acid B inhibits high glucose-induced mesangial cell proliferation and extracellular matrix production, partially through modulating the cell cycle progress and MMP-2 and MMP-9 activities, suggesting that it may be a promising agent for treating diabetic nephropathy (Luo et al. 2008).

Salvianolic acid B possesses both antioxidative and cell protective properties. Salvianolic acid B ameliorates renal damage in rats with ischaemia–reperfusion-induced acute renal failure through its antioxidant activity against production of reactive oxygen species (Kang et al. 2004). Tubular epithelial cells can undergo epithelial-to-mesenchymal transition, which plays an important role in the pathogenesis of renal interstitial fibrosis. Salvianolic acid B can prevent tubular epithelial-to-mesenchymal transition in the fibrotic kidney induced by mercurial chloride (Wang et al. 2010d). Salvianolic acid B has potential protective effects against renal diseases. Salvianolic acid B alleviates *hydrocephalus* through improvement of energy metabolism in mice with acute cerebral ischaemia (Zhang et al. 2007), stimulates neurogenesis in both the sub-granular zone and the sub-ventricular zone after brain ischaemia, alleviates neural cell loss, and improves motor function recovery after brain ischaemia in rats (Zhong et al. 2007a, b). Salvianolic acid B

exerts a neuroprotective effect against 6-hydroxydopamine-induced cell death in human neuroblastoma SH-SY5Y cells by reducing generation of reactive oxygen species and preventing increases of intracellular calcium (Tian et al. 2008). Treatment of salvianolic acid B in rat pheochromocytoma PC12 cells significantly reversed the expression of brain–pancreas relative protein and cell viability while it decreased reactive oxygen species production and intracellular calcium (Lin et al. 2006b). The neuroprotective effects of salvianolic acid B against hydrogen peroxide-induced injury in PC12 cells are comparable to those of Vitamin E (Liu et al. 2007b), but more effective than *Ginkgo biloba* extract in inhibiting beta-amyloid peptide fibril formation (Liu et al. 2006c, d). Salvianolic acid B inhibits fibril aggregation and destabilises preformed amyloid beta-peptide fibril (Durairajan et al. 2008). Taken together, these studies showed that salvianolic acid B may be potentially important in treating neurodegenerative diseases associated with oxidative stress.

Salvianolic acid B has the potential to ameliorate bone healing by stimulating both the total metabolic activity and alkaline phosphatase activity of osteoblastic cells (Liu et al. 2007d). Danshensu and salvianolic acid B are both efficient radical scavengers and antioxidants, with salvianolic acid B being superior to danshensu (Zhao et al. 2008a).

6.2.1.2 Lipid-Soluble Ingredients

The major lipophilic ingredients of *Salvia miltiorrhiza* are the tanshinones, including cryptotanshinone, dihydrotanshinone, tanshinone I, and tanshinone IIA.

Cryptotanshinone

Cryptotanshinone possesses antibacterial, antioxidant, anti-inflammatory, and anticancer activities. Cryptotanshinone and dihydrotanshinone show antibacterial activity against a broad range of Gram-positive bacteria (Lee et al. 1999). Cryptotanshinone attenuates ischaemia- and reperfusion-induced microcirculatory disturbances by inhibition of proinflammatory cytokine production, by reduction of neutrophil infiltration, and possibly by inhibition of adhesion molecules during ischaemia and reperfusion (Jin et al. 2009). Cryptotanshinone inhibits cyclooxygenase-2 enzyme activity but not its expression (Jin et al. 2006b; Cao et al. 2010). Cryptotanshinone decreases mast cell degranulation to improve ischaemia- and reperfusion-induced vascular damage (Han et al. 2008) and exhibits anti-inflammatory activity against carrageenan-induced paw edema in rats by downregulating expression of proinflammatory molecules like cyclooxygenase-2 and inducible nitric oxide synthase (Jeon et al. 2008). Cryptotanshinone ameliorates the abnormal immunological functions in adjuvant-induced arthritis in rats by decreasing thymic T and splenic B lymphocyte proliferation (Zheng et al. 2009). Cryptotanshinone protects primary rat hepatocytes from tertiary-butylhydroperoxide or D-galactosamine-induced liver toxicity by decreasing lipid peroxidation and free radical generation (Park et al. 2009).

Cryptotanshinone inhibits growth and induces apoptosis in a number of human and animal cancer cell lines. It suppresses Mammalian target of rapamycin-mediated cyclin D1 expression and retinoblastoma protein (Rb) phosphorylation (Chen et al. 2010a), inhibits constitutive signal transducer and activator of transcription 3 (STAT3) function through blocking the dimerisation in DU145 prostate cancer cells (Shin et al. 2009), sensitises DU145 cells to Fas(APO1/CD95)-mediated apoptosis through suppressing Bcl-2 expression and mitogen-activated protein kinases (MAPK) regulation (Park et al. 2010), sensitises a number of tumour cells to a broad range of anticancer agents (Park et al. 2010), suppresses P-glycoprotein (Pgp)-mediated doxorubicin efflux in Pgp-overexpressed HepG2 subclone cells (Lee et al. 2010), and produces both apoptotic and radiosensitisation effects in the HeLa cell line of cervical cancer (Ye et al. 2010). Cryptotanshinone sensitised TNF-alpha-induced apoptosis in human myeloid leukaemia KBM-5 cells through ROS-dependent activation of caspase-8 and p38 MAPK (Kim et al. 2011). In melanoma cell lines with low/high-metastatic capacity (B16/B16BL6), cryptotanshinone induced G1 arrest with a concomitant increase in p21 expression in B16BL6 cells. However, cryptotanshinone induced the G2/M arrest through its induction of Cdc25c in B16 cells (Chen et al. 2011).

Cryptotanshinone and dihydrotanshinone are non-competitive inhibitors for human brain acetylcholinesterase and uncompetitive inhibitors for human butyrylcholinesterase (Ren et al. 2004; Kim et al. 2007a; Wong et al. 2010b). Cryptotanshinone protects primary rat cortical neurons from glutamate-induced neurotoxicity (Zhang et al. 2009a) and promotes amyloid precursor protein metabolism in rat cortical neuronal cells (Mei et al. 2009). Cryptotanshinone modulates amyloid precursor protein metabolism and attenuates beta-amyloid deposition to improve cognitive ability in Alzheimer's disease transgenic mice (Mei et al. 2010) and improves learning and memory in several pharmacological models of Alzheimer's disease (Wong et al. 2010a). Cryptotanshinone protects hepatocytes from lipopolysaccharide (LPS)- and ethanol-induced cell death, suppressing ethanol-induced lipid accumulation, which may indicate potential to treat alcoholic liver disease (Yin et al. 2009). Cryptotanshinone inhibits endothelin-1 expression and stimulates nitric oxide production in human vascular endothelial cells (Zhou et al. 2006b). Cryptotanshinone causes vasodilation in rat coronary artery (Lam et al. 2008a). Cryptotanshinone reduces the formation of TRAP-positive multinuclear osteoclasts and inhibits osteoclast differentiation (Lee et al. 2005). Cryptotanshinone inhibits angiogenesis *in vitro* by inhibiting basic fibroblast growth factor (bFGF)-induced angiogenesis without cytotoxicity (Hur et al. 2005).

Dihydrotanshinone I

Dihydrotanshinone I inhibits growth and induces apoptosis in a number of human and animal cancer cell lines. It induces cell growth arrest during the S phase and, subsequently, apoptosis in K562/ADR cells (Lee and Lee 2000); suppresses the expression of inducible nitric oxide synthase, interleukin-1beta, TNF-alpha, and TNF-alpha-converting enzyme in a mouse microglia cell line (BV-2) (Lee et al.

2006a); inhibits activation of hypoxia-inducible factor-1, which is essential to the adaptation and proliferation of cancer cells (Dat et al. 2007); inhibits proliferation of human breast cancer cell lines by inducing G1 phase arrest and apoptosis through activation of the caspase-3-dependent mitochondrial apoptosis pathways (Tsai et al. 2007); inhibits the growth of human breast cancer MDA-MB-231 cells *in vivo* in a nude mice xenograft experiment (Tsai et al. 2007); inhibits hypoxia-induced luciferase expression in a human gastric cell line (AGS cells); inhibits human umbilical vein endothelial cells by suppressing cell migration, invasion, and tube formation, and *in vivo* anti-angiogenic activity in chick embryo chorioallantoic membrane assay (Bian et al. 2008); induces activation of reactive oxygen species (ROS)-mediated p38 MAPK to cause apoptosis in HepG2 cells (Lee et al. 2009); and suppresses Pgp-mediated doxorubicin effluxin in a Pgp-overexpressed HepG2 subclone (Lee et al. 2010). Dihydrotanshinone I inhibited the proliferation of human prostate DU145 carcinoma cells and induced apoptosis through induction of endoplasmic reticular stress and/or inhibition of proteasome activity (Chuang et al. 2011).

Dihydrotanshinone I potently antagonises both mineralocorticoid and glucocorticoid receptors, and inhibits the expression of their target genes like sodium/potassium-ATPase, glucose 6-phosphatase, and phosphoenolpyruvate carboxykinase. Dihydrotanshinone I increases AMPKalpha phosphorylation and regulates its downstream pathways, including increasing acetyl-CoA carboxylase phosphorylation, inhibiting transducer of regulated CREB activity 2 (TORC2) translocation, and promoting glucose uptake (Liu et al. 2010c). Dihydrotanshinone I relaxes rat coronary artery by inhibition of calcium channels (Lam et al. 2008b). Dihydrotanshinone I exerts a potent antiplatelet activity via suppression of intracellular calcium mobilisation and arachidonic acid liberation (Park et al. 2008). Dihydrotanshinone I decreases mast cell degranulation and appeared to improve ischaemia- and reperfusion-induced vascular damage (Han et al. 2008). Similar to cryptotanshinone, dihydrotanshinone I inhibits acetylcholinesterases (Ren et al. 2004), being a mixed non-competitive inhibitor for human brain acetylcholinesterase and an uncompetitive inhibitor for human butyrylcholinesterase (Wong et al. 2010b). Like tanshinone I, tanshinone IIA, and cryptotanshinone, dihydrotanshinone can reverse scopolamine-induced cognitive impairments in mice (Kim et al. 2007a) and may be useful for the treatment of cognitive impairment. Dihydrotanshinone I reduces the formation of TRAP-positive multinuclear osteoclasts and inhibits osteoclast differentiation (Lee et al. 2005).

Dihydrotanshinone I, tanshinone IIA, and cryptotanshinone, but not tanshinone I, show significant inhibition of the lipopolysaccharide-induced nitric oxide production in mouse macrophage RAW 264.7 cells (Choi et al. 2004). Among the tanshinones, dihydrotanshinone I possesses the strongest inhibitory effects on mast cell degranulation (Choi and Kim 2004). Dihydrotanshinone also shows a pronounced inhibitory effect on human recombinant monoamine oxidase A (MAO A) and on inducible nitric oxide synthase (iNOS) induction by lipopolysaccharide in Raw 267.4 cells (Dittmann et al. 2004). Dihydrotanshinone I is more effective than tanshinone I or cryptotanshinone in inhibiting interleukin-12 production in mouse

macrophages and interferon-gamma production in lymph node cells, which may explain the anti-inflammatory actions of tanshinones and their potential use in the treatment of immunological diseases dominated by Th1-derived cytokine responses (Kang et al. 2000).

Tanshinone I

Tanshinone I has anti-inflammatory, antioxidant, and antitumour activities. Tanshinone I shows anti-inflammatory activity in rat carrageenan-induced paw oedema and adjuvant-induced arthritis (Kim et al. 2002). Tanshinone I inhibits prostaglandin E2 formation from lipopolysaccharide-induced (RAW 264.7) macrophages, with no effect on cyclooxygenase 2 activity and expression. A *Salvia miltiorrhiza* extract (PF2401-SF) enriched with tanshinone I (11.5 %), tanshinone IIA (41.0 %), and cryptotanshinone (19.1 %) protects primary cultured rat hepatocytes from bile acid-induced apoptosis by inhibiting the generation of intracellular reactive oxygen species (Park et al. 2007). Tanshinone I enhances learning and memory, and ameliorates memory impairment in mice via the extracellular signal-regulated kinase (ERK) signalling pathway (Kim et al. 2009a). The intracellular signalling kinase, ERK1/2, is required for new memory formation, suggesting that control of ERK signalling may be a target for the treatment of cognitive dysfunction. In models of learning and memory impairment induced by diazepam and MK-801, tanshinone I reverses learning and memory impairments detected by the passive avoidance test. Tanshinone I, IIA, and dihydrotanshinone I, but not cryptotanshinone, enhance the activity of insulin on the tyrosine phosphorylation of the insulin receptor as well as the activation of the downstream kinases Akt, ERK1/2, and GSK3beta in Chinese hamster ovary cells expressing human insulin receptors (CHO/IR cells) as well as in 3T3-L1 adipocytes (Jung et al. 2009).

Tanshinone I and tanshinone IIA are shown to be quite strongly cytotoxic against P388 lymphocytic leukaemia cells (Mosaddik 2003). Tanshinone I inhibits growth and induces apoptosis in a number of human and animal cancer cell lines: rat hepatic stellate cells transformed by simian virus 40 (T-HSC/Cl-6), by apoptosis through caspase activation, cytochrome *c* release, and loss of mitochondrial membrane potential (Kim et al. 2003); monocytic leukaemia cells (U937, THP-1, and SHI 1), by downregulating telomerase activity and activating caspase-3 (Liu et al. 2010a, f); human lung adenocarcinoma (CL1-5) cells, by inhibition of migration and invasion; human colon cancer (Colo 205) cells, through mitochondrial-mediated intrinsic cell death pathways and p21-mediated G0/G1 phase cell cycle arrest (Su et al. 2008b); and human oestrogen receptor-negative breast cancer cells, MDA-MB-231, through regulation of adhesion intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Jing et al. 2007; Nizamutdinova et al. 2008a, b).

Tanshinone IIA

Tanshinone IIA is the most abundant lipid-soluble active ingredient in *Salvia miltiorrhiza* roots and its ethanol and ethyl acetate extracts. Tanshinone IIA is one of the characteristic marker compounds for *Salvia miltiorrhiza*, along with salvianolic acid B (Wagner et al. 2010; Wagner 2012). Tanshinone IIA is the most studied tanshinones isolated from *Salvia miltiorrhiza*. Like the other tanshinones, tanshinone IIA possesses anticoagulant, vasodilatory, anti-inflammatory, antioxidant, and antitumour activities. A number of tanshinone IIA derivatives have been synthesised for further research into their pharmacological and therapeutic potential (Bi et al. 2010).

The antioxidative activities of tanshinone IIA reduce the severity of brain injury or neurotoxicity with different underlying pathophysiology. Tanshinone IIA increases the ipsilateral brain weight and neuron density in a neonatal rat model of hypoxia–ischaemia brain damage (Xia et al. 2005). Tanshinone IIA protects against ethanol-induced neurotoxicity in PC12 cells, accompanied by downregulating pro-apoptotic p53 protein expression (Meng et al. 2006), suggesting that it may be useful for ethanol-induced neurological disorders. Tanshinone IIA protects mouse brain subjected to permanent middle cerebral artery occlusion from ischaemic injury by suppressing the oxidative stress and free radical-mediated inflammatory insult (Dong et al. 2009a). Tanshinone IIA exerts neuroprotective effects on amyloid beta (Aβ)-peptide-induced toxicity in cultured rat cortical neurons by reducing the Aβ-peptide-induced increase of caspase-3 activity, and cytochrome *c* translocation into the cytosol from mitochondria, and ameliorating the Aβ-peptide-induced Bcl-2/Bax ratio reduction in cortical neurons (Liu et al. 2010d). Tanshinone IIA reduces the hypoxic ischaemic brain damage-related downregulation of phospho-NR1 S897 and the HIBD-caused intracellular calcium elevation in the cortex. The neuroprotective effect may be related to influencing excitatory amino acid (NMDA) receptor expression and decreasing intracellular free calcium aggregation (Hei et al. 2010). Tanshinone IIA pretreatment reduces the expression of the inflammatory mediator interleukin 1 and Re1A mRNA expression in rats with focal cerebral ischaemia (Chen et al. 2010b). Tanshinone IIA inhibits the calcium current amplification induced by Aβ-peptide in neurons of nucleus basalis of Meynert and protected the neurons against toxicity (Zhu et al. 2010b). The neuroprotective effects of tanshinone IIA are associated with induced nuclear translocation of transducer of regulated CREB activity 1 (TORC1) and upregulated expression of TORC1, phosphorylated cAMP response element binding protein, and brain-derived neurotrophic factor in the acute stage of ischaemic stroke (Liu et al. 2010b). Tanshinone IIA alleviates oxidative damage and excitotoxicity effects of glutamate on human neuroblastoma SH-SY5Y cells through its antioxidant effects (Sun et al. 2010). Tanshinone IIA protects the human blood–brain barrier model from leucocyte-associated hypoxia–reoxygenation injury in primary human brain microvascular endothelial cells (Zhang et al. 2010c). Thus, tanshinone IIA may be beneficial for the treatment of cerebral ischaemia–reperfusion injury.

Tanshinone IIA protects neonatal rat cardiomyocytes from doxorubicin-induced apoptosis, by decreasing reactive oxygen species production (Gao et al. 2008). Tanshinone IIA prevents left ventricular hypertrophy of hypertensive rats with abdominal aorta constriction by downregulating expression of AT1R mRNA and Smad-3 gene, increasing production of Smad-7 protein, and blocking TGF beta1/Smads signal pathway in local myocardium (Li et al. 2009d). Tanshinone IIA protects against sudden cardiac death induced by lethal arrhythmias in a rat model of myocardial infarction by downregulation of the miR-1 gene and consequent recovery of Kir2.1 protein (Shan et al. 2009). Tanshinone IIA elicits a cardioprotective effect by improving heart function, reducing infarct size, increasing survival rate in a rat model of myocardial infarction, promoting angiogenesis, and upregulating vascular endothelial growth factor expression (Xu et al. 2009b), as well as through nitric oxide production, such as eNOS phosphorylation and L-arginine uptake (Pan et al. 2011). Tanshinone IIA pretreatment protects myocardium against ischaemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway in diabetic rats, reducing infarct size and improving cardiac dysfunction in streptozocin-induced diabetic rats (Zhang et al. 2010e). Tanshinone IIA modulates pulmonary vascular response to agonist and hypoxia primarily via inhibiting calcium ion influx and release in normal and hypoxic pulmonary hypertension rats (Wang et al. 2010b). Tanshinone IIA attenuates inflammatory responses in rats with myocardial infarction by reducing MCP-1 expression (Ren et al. 2010b) and protects against immune-mediated liver injury by activating T-cell subsets and cytokine regulation (Qin et al. 2010).

Tanshinone IIA decreases the serum levels of interleukin-1-beta, TNF-alpha, and platelet number, with the efficacy comparable to aspirin in a rabbit model of immune vasculitis, suggesting that it may diminish the inflammation damage of vessels in patients with immune vasculitis (Li et al. 2009c). The inhibitory effect of tanshinone IIA on renal interstitial fibrosis may be related to its blocking effect on transforming growth factor beta1 (TGFbeta1)–Smads signal pathway in rat renal interstitial fibroblasts (Tang et al. 2008). Tanshinone IIA inhibits vascular smooth muscle cell proliferation and reduces hyperplasia in the rat carotid balloon-injured model through inhibition of mitogen-activated protein kinase (MAPK) signalling pathway and downregulation of c-fos expression (Li et al. 2010a). Tanshinone IIA increases the serum total antioxidant capability, improves the activities of sodium/potassium-ATPase, increases the levels of superoxide dismutase and catalase, and reduces the malondialdehyde level in sciatic nerves in diabetic rats (Liu et al. 2010g). Tanshinone IIA may be a useful therapeutic agent for diabetic neuropathy. Tanshinone IIA protects against immune-mediated liver injury through activation of T-cell subsets and regulation of cytokines (Qin et al. 2010). Tanshinone IIA protects rat primary hepatocytes against carbon tetrachloride toxicity via inhibiting mitochondria permeability transition (Zhu et al. 2010a).

Tanshinone IIA may have potential anticancer activity in both oestrogen receptor-positive and -negative breast cancers, which could be attributed in part to its inhibition of proliferation and apoptosis induction in cancer cells through upregulation and downregulation of multiple genes involved in cell cycle regulation, cell proliferation, apoptosis, signal transduction, transcriptional regulation,

angiogenesis, invasive potential and metastatic potential of cancer cells (Wang et al. 2005b, 2007b, 2009; Su et al. 2008a; Su and Lin 2008a; Zhou et al. 2008; Liu et al. 2009b; Yuxian et al. 2009). Tanshinone IIA decreases the expression of p53 and bcl-2, but not of cerbB-2, in oestrogen receptor-positive and -negative xenografted nude mice and decreased the proliferation of oestrogen receptor-positive and -negative cancer cell lines (Lu et al. 2009). Tanshinone IIA exhibits induction of apoptosis by activation of caspase-3, downregulation of anti-apoptotic protein bcl-2 and bcl-xl, and upregulation of pro-apoptotic protein bax, as well as disruption of the mitochondrial membrane potential (Liu et al. 2006e). Tanshinone IIA treatment regulates the expressions of proteins involved in apoptotic processes, spindle assembly, and p53 activation, including vimentin, Maspin, alpha- and beta-tubulin, and GRP75. Tanshinone IIA interacts with DNA by minor groove-binding and does not act as an intercalator of DNA (Zhang et al. 2008, 2009e). Tanshinone IIA induces cell cycle arrest in mitosis by disrupting the mitotic spindle and subsequent apoptotic cell death through the mitochondria-dependent apoptotic pathway, destroying only the mitotic spindle during M phase but not the microtubule structure in interphase cells (Zhou et al. 2008). Tanshinone IIA effectively inhibits invasion and metastasis of human hepatocellular carcinoma cells *in vitro* and *in vivo*, partly by inhibiting the activity of metalloproteinases MMP-2 and MMP-9, and partly via the Nuclear factor (NF)-kappa B signal transduction pathway. Tanshinone IIA inhibits the growth of a number of cancer cells: human leukaemia cell lines HL-60 and K562, by inhibiting telomerase activities and proliferation (Song et al. 2005), decreasing the level of RNA polymerase II, and altering DNA structure (Guo et al. 2008); human glioma cells, through induction of apoptosis and inhibition of colony formation and BrdU incorporation (Wang et al. 2007b); human hepatoma cell line HepG2, through induction of apoptosis (Zhong et al. 2007b), inhibition of microsomal triglyceride transfer protein expression and atherogenic risk factor apolipoprotein B100 secretion (Kang et al. 2008), and blocking of the adhesive ability to endothelial cells (Qian et al. 2010); human hepatoma BEL-7402 cells, through apoptosis induction (Tang et al. 2003); human hepatocellular carcinoma (HepJ5) cells, by increasing calreticulin, caspase-12, and GADD153 protein expression and arresting cell growth in the G2/M phase (Cheng and Su 2010); human breast cancer MDA-MB-231 cells, through upregulating the expression of Bax but downregulating Bcl-2 expression and inducing apoptosis (Su and Lin 2008a); human colon cancer Colo-205 cells in the sub-G1 fraction, by increasing the expression of p53 and p21 and mitochondrial cytochrome *c* release (Su and Lin 2008b), downregulating the protein expression of ErbB-2, and upregulating TNF-alpha and caspase-3 (Su and Lin 2008b) *in vitro* and *in vivo* in a Colo 205 xenograft model; leukaemia THP-1 cell by induction of apoptosis, by activation of caspase-3, by downregulation of anti-apoptotic protein Bcl-2 and survivin, and by upregulation of pro-apoptotic protein Bax (Liu et al. 2009b); human lung cancer A549 cells, through induction of apoptosis by induction of reactive oxygen species, decrease in the mitochondrial membrane potential, and induction of a higher ratio of Bax/Bcl-2 (Chiu and Su 2010); human cervical cancer (HeLa) cells, through interfering in the process of microtubule assembly, leading to G(2)/M phase arrest and subsequent apoptosis (Pan et al. 2010);

human lung adenocarcinoma cell line (SPC-A-1), by inhibiting DNA synthesis by upregulating gene p53, Fas, and Bax and downregulating gene Bcl-2 (Ji et al. 2008). Tanshinone IIA inhibits constitutive signal transducer and activator of transcription 3 (STAT3) activation, suppresses proliferation, and induces apoptosis in rat C6 glioma cells (Tang et al. 2010). Tanshinone IIA induced apoptosis as demonstrated by DNA fragmentation, poly(ADP-ribose) polymerase and caspase-3 cleavage, increased Bax/Bcl-2 protein ratio, and depolarisation of mitochondrial membranes to facilitate cytochrome *c* release into the cytosol and S phase cell cycle arrest in activated rat hepatic stellate cells (Che et al. 2010) which may be beneficial for treating chronic hepatitis and hepatic fibrosis.

Tanshinone IIA protects human umbilical vein endothelial cell line ECV-304 damage induced by hydrogen peroxide through its antioxidant effect (Lin et al. 2006a). Tanshinone IIA attenuates TNF-alpha, angiotensin II, and hydrogen peroxide-mediated reactive oxygen species (ROS) production (Zhang and Wang 2007). Tanshinone I, tanshinone IIA, and cryptotanshinon all inhibit lactate dehydrogenase leakage, glutathione depletion, lipid peroxidation, and free radical generation in rat hepatocytes (Park et al. 2009). Tanshinone IIA inhibits trinitrobenzene sulfonic acid (TNBS)-induced murine colitis (Bai et al. 2008) by downregulating the production of proinflammatory cytokines, such as TNF-alpha and interleukin-1beta, and attenuating oxidative stress with a higher level of glutathione in colonic tissue, which suggested that tanshinone IIA may be a potential agent for treatment of inflammatory bowel diseases. Tanshinone IIA decreases the radiation-induced release of proinflammatory cytokines in microglia BV-2 cells and exerts anti-inflammatory properties by suppressing the transcription of proinflammatory cytokine genes that may be associated with the NF-kappabeta signalling pathway (Dong et al. 2009b). Tanshinone IIA reduces macrophage death induced by hydrogen peroxide by upregulating glutathione peroxidase gene expression and enzyme activity (Li et al. 2008f). Tanshinone IIA may have potential to inhibit alcoholic liver disease by reducing lipopolysaccharide- and ethanol-induced Kupffer cell sensitisation, inhibiting synthesis of reactive oxygen/nitrogen species, inhibiting fatty acid synthesis, and stimulating fatty acid oxidation (Yin et al. 2008). In lipopolysaccharide-induced RAW 264.7 cells, Tanshinone IIA exerts anti-inflammatory effects through inhibition of inducible nitric oxide synthase (iNOS) gene expression and nitric oxide production, as well as inhibition of inflammatory cytokine (IL-1beta, IL-6, and TNF-alpha) expression via oestrogen receptor-dependent pathway (Jang et al. 2006; Fan et al. 2009). Tanshinone IIA downregulates the expression of matrix metalloproteinase-12 (MMP-12) and tissue factor in RAW 264.7 cells stimulated by ox-LDL (Wang et al. 2009).

Tanshinone IIA inhibits atherosclerotic plaque formation by downregulating MMP-2 and MMP-9 expression and activities, and cluster of differentiation 40 (CD40) expression in rabbits fed a high-fat diet (Fang et al. 2007, 2008). Tanshinone IIA had protective effect on the early stage of streptozotocin-induced diabetic nephropathy in rats, ameliorating renal hypertrophy and 24-h urinary protein excretion and reducing advanced glycation end products, angiotensin II,

transforming growth factor beta1, type IV collagen, and monocyte/macrophage in either the serum or kidney (Kim et al. 2009b). Tanshinone IIA treatment reduces adipose mass and body weight, improves glucose tolerance, and decreases the low-density lipoprotein to high-density lipoprotein ratio without changing the food intake in a high-fat diet-induced obese animal model (Gong et al. 2009). Tanshinone IIA inhibits FBS-induced proliferation of cultured rat vascular smooth muscle cells in G0/G1 phase via by inhibiting ERK1/2 activity and decreasing the expression level of c-fos (Li et al. 2008d). Tanshinone IIA inhibits human aortic smooth muscle cell migration and MMP-9 activity through AKT signalling pathway (Jin et al. 2008). Tanshinone IIA inhibits TNF-alpha-induced ERK and c-jun phosphorylation, but not other MAPKs such as JNK and p38. Tanshinone IIA also inhibits NF-kappaB and AP-1 DNA binding.

Tanshinone IIA activates human cardiac KCNQ1/KCNE1 potassium channels (I(Ks)) in HEK 293 cell by affecting the channels' kinetics (Sun et al. 2008). Tanshinone IIA inhibits endothelin-1 production in TNF-alpha-induced rat brain microvascular endothelial cells through suppression of endothelin-converting enzyme-1 synthesis (Tang et al. 2007a). Tanshinone IIA protects cardiac myocytes against oxidative stress-triggered damage and apoptosis induced by hydrogen peroxide *in vitro* in neonatal rat ventricular myocytes and *in vivo* after occlusion/reperfusion of the left anterior descending coronary artery in adult rats (Fu et al. 2007). This protection is attributed to elevated serum antioxidant, reduced lipid peroxidation, and upregulated Bcl-2/Bax ratio in the cardiac myocytes.

Tanshinone IIA inhibits inducible nitric oxide synthase expression and production of TNF-alpha, IL-1beta, and IL-6 in LPS-stimulated RAW 264.7 cells (Jang et al. 2003). This effect is partially through the regulation of NF-kappaB-inducing kinase-IkappaB alpha kinase, ERK1/2, p38 MAPK and c-Jun N-terminal kinase pathways (Jang et al. 2006). Tanshinone IIA inhibits COX-2 and iNOS expression in LPS-activated RAW 264.7 macrophages (Chen et al. 2007). Tanshinone IIA induces nitric oxide production from human vascular endothelial cells (Huang et al. 2007).

Tanshinone IIA may be a therapeutic agent for the treatment of bone disease such as osteoporosis. The bone resorptive activity of differentiated osteoclasts, accompanied with the disruption of the actin ring, is inhibited by tanshinone IIA. Tanshinone IIA reduces both the number and activity of osteoclasts (Kim et al. 2004) and inhibits osteoclast differentiation by suppressing the expression levels of c-Fos and NFATc1 induced by receptor activator of nuclear factor-kappaB ligand (Kwak et al. 2006). Addition of tanshinone IIA to osteoclast precursor culture causes a significant decrease in the level of calcitonin receptor, c-Src, and integrin beta3 mRNA, which are normally upregulated during the osteoclast differentiation. Tanshinone IIA suppresses inflammation-mediated osteoclastic bone resorption by inhibiting the synthesis of prostaglandin E2 in cocultures of bone marrow cells and calvarial osteoblasts (Kwak et al. 2008) and enhances bone morphogenetic protein-stimulated commitment of bi-potential mesenchymal precursor C2C12 cells into osteoblasts via p38 MAPK activation (Kim and Kim 2010).

6.2.2 *Pharmacological Effects of* Salvia miltiorrhiza

Salvia miltiorrhiza is one of the most versatile Chinese herbal medicines that have been used for hundreds of years in the treatment of numerous ailments, due to its activities in improving microcirculation, causing coronary vasodilatation, suppressing the formation of thromboxane, inhibiting platelet adhesion and aggregation, and protecting against myocardial ischaemia (Cheng 2007). Prevention and treatment of cerebral infarction by *Salvia miltiorrhiza* may involve multiple pathways, including anti-atherosclerosis, antihypertension, antiplatelet aggregation, anti-inflammatory, and antioxidative effects (Ling et al. 2008a; Lin and Hsieh 2010), all contributing to the clinical benefit in patients with coronary artery disease.

6.2.2.1 Antioxidant Effects

Salvianolic acids (salvianolic acids A and B) and tanshinones exhibit free radical scavenging and antioxidant effects. Therefore, the water, ethanol, or ethyl acetate extracts of *Salvia miltiorrhiza* are expected to possess free radical scavenging and antioxidant activities. In addition, *Salvia miltiorrhiza* enhances endogenous antioxidative enzyme activities such as the expression of endothelial nitric oxide synthase. *Salvia miltiorrhiza* and its active ingredients are effective at eliminating factors that contribute to the rise in cellular phosphorylation and help to maintain the integrity of endothelial junction structure (Ding and Yuan 2007). *Salvia miltiorrhiza* root aqueous extract and salvianolic acid B exert their protective effect through circulating reactive oxygen species suppression and subsequent modulation of protein carbonylation in rat aortic smooth muscle cells (Hung et al. 2009). An extract of *Salvia miltiorrhiza* abolishes triol-induced endothelial cell apoptosis to protect endothelial integrity and prevent endothelial damage (Nakazawa et al. 2005). Apart from the root extracts, phenolic acids isolated from leaf extracts of *Salvia miltiorrhiza* also possess antioxidant activities (Zhang et al. 2010d). *Salvia miltiorrhiza* may enhance endogenous antioxidative enzyme activities such as the expression of endothelial nitric oxide synthase and may scavenge oxygen free radicals (Li et al. 2010b).

6.2.2.2 Cardiovascular Effects

The vasorelaxant actions of *Salvia miltiorrhiza* aqueous extract and danshensu are mediated through inhibition of calcium ion influx in the vascular smooth muscle cells and opening of potassium ion channels (Lam et al. 2005, 2006b). The opening of potassium channels makes only a minor contribution to the response of *Salvia miltiorrhiza*, with no involvement of endothelium-dependent mechanisms (Lam et al. 2007). Magnesium tanshinoate B is one of the components responsible for the

cardiovascular effects of *Salvia miltiorrhiza*, and that the beneficial cardiovascular effect of the extract is more prominent under conditions of elevated blood pressure (Leung et al. 2010). Chronic treatment of *Salvia miltiorrhiza* inhibits and reverses the development of left ventricular hypertrophy in spontaneously hypertensive rats, independent of blood pressure (Sun and Zheng 2007). An aqueous extract of *Salvia miltiorrhiza* containing danshensu and salvianolic acid B attenuates TNF-alpha-induced increase in endothelial permeability (Ding et al. 2005b) and is beneficial to ischaemic–reperfusion injury and atherosclerosis. Lipophilic compounds of *Salvia miltiorrhiza* also prevent the development of vascular damage. Thus, both the water-soluble and lipophilic compounds of *Salvia miltiorrhiza* appear to improve the ischaemia/reperfusion-induced vascular damage multifactorially and synergically (Han et al. 2008).

Treatment of a human endothelial cell monolayer with a preparation of Danshen and Gegen resulted in a dose-related suppression of acetylated-LDL uptake by human macrophages and an increase in the level of ICAM-1 expression and adhesion of monocytes to endothelial cells. These herbs therefore show the ability to modulate key early events in atherosclerosis (Sieveking et al. 2005). The vasorelaxant effect of a Danshen and Gegen formulation (ratio 7:3) on rat basilar artery is independent of endothelium-derived mediators, whereas inhibition of calcium ion influx in the vascular smooth muscle cells is important, and a minor component is mediated by the opening of potassium (ATP) channels. Danshen and Gegen formulation could be a useful cerebroprotective agent in some patients with occlusive cerebrovascular disease (Lam et al. 2010).

The lipophilic extract of *Salvia miltiorrhiza* exerts dual effects on catecholamine secretion in cultured bovine adrenal medullary cells. Lipophilic extract (ethyl acetate) of *Salvia miltiorrhiza* exerts antagonistic effects on voltage-dependent sodium and calcium channels, whereas it is an agonist of L-type calcium channel when used alone. *Salvia miltiorrhiza* aqueous extract (water decoction) significantly inhibited (>60 %) the growth of a rat smooth muscle cell line (A10) under homocysteine stimulation and the intracellular reactive oxygen species (ROS) concentration obviously decreased after *Salvia miltiorrhiza* aqueous extract treatment in terms of reducing p47(phox) translocation and increasing catalase activity (Hung et al. 2010). In human umbilical vein endothelial cells, *Salvia miltiorrhiza* inhibits adhesion molecule expression and protects endothelial function and inhibits atherogenesis, while their actions to inhibit DNA synthesis and cell growth may weaken the ability of endothelial repair (Ling et al. 2008b).

The extract of *Salvia miltiorrhiza* and its major ingredients, danshensu and salvianolic acid B, inhibit tumour necrosis factor (TNF-alpha)-induced endothelial permeability (Ding et al. 2005a). When preincubated with *Salvia miltiorrhiza* aqueous extract, the adhesion of HL-60 cells to TNF-alpha-induced endothelial cells is significantly decreased, with downregulation of adhesion molecules, intracellular cell adhesion molecule-1, and vascular cell adhesion molecule-1 (Ding et al. 2005b).

6.2.2.3 Effects on Liver and Liver Fibrosis

Salvia miltiorrhiza ameliorates cirrhosis and portal hypertension in rats by inhibiting nitric oxide synthase type II, nitric oxide production, and expression of inducible nitric oxide synthase mRNA (Wang et al. 2003). *Salvia miltiorrhiza* partially restores intestinal microflora balance, improves intestinal mucosal integrity, and reduces bacterial translocation and plasma endotoxin in rats with hepatic ischaemia/reperfusion injury (Xing et al. 2005). *Salvia miltiorrhiza* protects against chronic alcoholic liver injury in mice by alleviating fatty degeneration and adiponecrosis of hepatic cells, downregulating the expressions of toll-like receptor-4 mRNA and hemeoxygenase-1 mRNA, and decreasing the number of toll-like receptor-4-positive cells (Xiong et al. 2005). *Salvia miltiorrhiza* protects against doxorubicin-induced cardiac and hepatic toxicity in rats (You et al. 2007); hepatocytes against cold preservation and reperfusion-induced apoptosis, in dimethylnitrosamine-induced fibrosis in rats (Hsu et al. 2005); and ischaemia–reperfusion injury in rat liver graft after orthotopic transplantation (Wang et al. 2008a, b). Tanshinone IIA may have the potential to inhibit alcoholic liver disease by reducing LPS- and ethanol-induced Kupffer cell sensitisation, inhibiting synthesis of reactive oxygen/nitrogen species and fatty acid, and stimulating fatty acid oxidation (Yin et al. 2008). The water-soluble extract of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats (Lee et al. 2006b). *Salvia miltiorrhiza* polysaccharides have hepatoprotective effects in immunological liver injury induced by Bacille-Calmette-Guerin (BCG) and LPS in mice, improving the liver index, spleen index, and thymus index, reducing the serum levels of alanine aminotransferase, aspartate aminotransferase, and nitric oxide, and restoring liver homogenate contents of TNF-alpha and interleukin-1beta (Song et al. 2008). *Salvia miltiorrhiza* reduces the levels of plasma endotoxin and inhibits effectively the expressions of TLR4 protein in the liver of rats with severe acute pancreatitis and obstructive jaundice, decreasing inflammation and exerting protective effect on liver function (Zhang et al. 2009b, c). A purified extract isolated from *Salvia miltiorrhiza* enriched with tanshinone I, tanshinone IIA, and cryptotanshinone also protects hepatocyte injury *in vitro* and *in vivo* (Park et al. 2009). *Salvia miltiorrhiza* improves the liver function of severe acute pancreatitis or obstructive jaundice rats, suppresses the expression of NF-kappaB p65 protein in the liver of severe acute pancreatitis rats, and inhibits apoptosis in obstructive jaundice rats, thereby showing some protective effects on the liver of these rats (Zhang et al. 2009c).

Hepatic stellate cells (HSC) play a central role in hepatic fibrosis, and compounds that promote apoptosis in hepatic stellate cells may have anti-fibrotic potentials. Salvianolic acid A demonstrates both anti-proliferative and pro-apoptotic activities in hepatic stellate cells HSC-T6 (Liu et al. 2000; Chor et al. 2005), decreasing intracellular calcium in activated hepatic stellate cells (Wang et al. 2005a). Chronic administration of *Salvia miltiorrhiza* in rats ameliorates carbon tetrachloride-induced hepatic injury through reduced oxidant stress and hepatic fibrosis (Lee et al. 2003).

6.2.2.4 Effects on Platelet Aggregation

Salvia miltiorrhiza shows inhibition of platelet aggregation, platelet activation, platelet–leucocyte conjugate formation, and leucocyte activation in response to all the agonists (Han et al. 2008; Zhao et al. 2008b). *Salvia miltiorrhiza* may reduce or prolong the development of atherosclerosis and may have antihypertensive and antiplatelet aggregation effects, which prevent cerebral infarction (Lin and Hsieh 2010). Salvianolic acid A inhibits platelet activation and arterial thrombosis via inhibition of phosphoinositide 3-kinase (Huang et al. 2010; Fan et al. 2010). Dihydrotanshinone I inhibits rabbit platelet aggregation by suppressing intracellular calcium mobilisation (Park et al. 2008). Rosmarinic acid, a water-soluble component, also possesses antithrombotic and antiplatelet effects (Zou et al. 1993).

6.2.2.5 Effects on Tumours

Salvia miltiorrhiza contains a variety of antitumour active ingredients, such as the water-soluble components (salvianolic acid A, salvianolic acid B, and salvinal) and lipid-soluble constituents (tanshinone I, tanshinone IIA, dihydrotanshinone I, miltirone, cryptotanshinone, ailantholide, neo-tanshinlactone, and nitrogen-containing compounds). These antitumour components play important roles in the different stages of tumour evolution, progression, and metastasis. Discovery of new antitumour active ingredients may benefit the application of *Salvia miltiorrhiza* for clinical tumour treatment (Zhang and Lu 2010). The antihepatocellular carcinoma effects of chi-shen extract (CSE) from the water-soluble compounds of *Salvia miltiorrhiza* and *Paeoniae radix* are investigated in HepG2 cells (hepatocellular carcinoma cell line) and the anticancer effects of chi-shen extract are related to the Bcl-2 family pathway and the activation of caspase-3 and -9 in HepG2 cells (Hu et al. 2007; Lee et al. 2008b). In doxorubicin-resistant HepG2 cells, cryptotanshinone suppresses doxorubicin efflux, while tanshinone IIA provides the best synergism with doxorubicin despite its comparatively moderate cytostatic effects with HepG2 cells (Lee et al. 2010). As shown in Fig. 6.3, the importance of mitochondrial ROS level in regulating the cellular response of the tanshinones is illustrated. Oxidative stress may account for the difference in the apoptotic effects of dihydrotanshinone and tanshinone IIA in HepG2 cells. Dihydrotanshinone is more potent than tanshinone IIA in decreasing the mitochondrial membrane potential and in causing mitochondrial ROS depletion. The protective effect of N-acetyl cysteine on dihydrotanshinone-induced apoptosis observed may be related to preservation of mitochondrial ROS. A compound *Astragalus* and *Salvia miltiorrhiza* extract inhibits cell invasion by modulating transforming growth factor-beta/Smad in HepG2 cell (Liu et al. 2010e). Herbal extract "Songyou Yin" (containing *Salvia miltiorrhiza* and other four herbs) inhibits tumour growth and prolongs survival in nude mice bearing human hepatocellular carcinoma (HCC) xenograft, via inducing apoptosis and downregulation of MMP-2 and vascular endothelial growth factor, which indicated its potential use in patients with advanced HCC (Huang et al. 2009). *Salvia miltiorrhiza* treatment induces the downregulation of Akt phosphorylation and an increase in p27 in breast cancer (MCF-7 vec and MCF-7 HER2) cells (Yang et al. 2010).

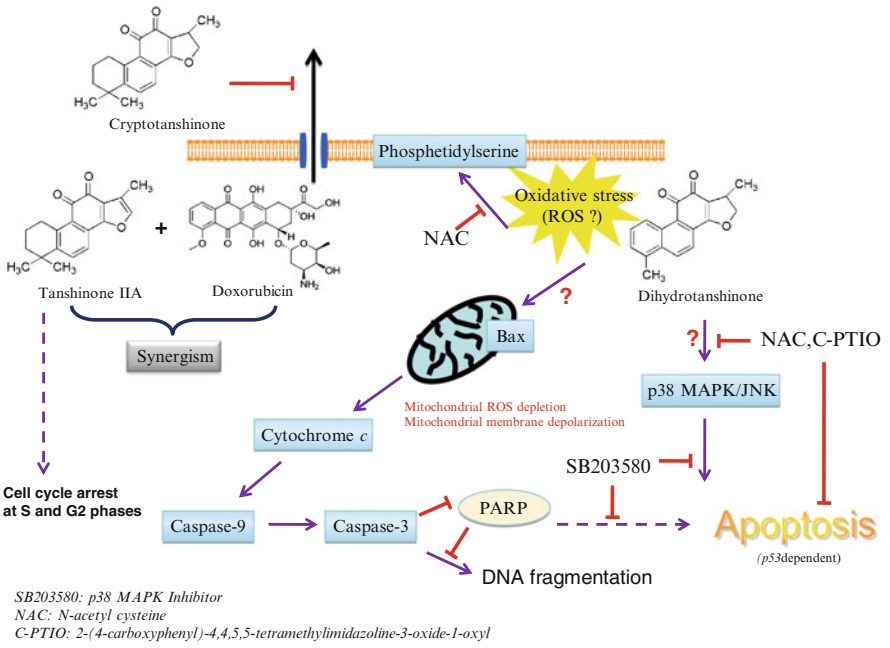

Fig. 6.3 Proposed mechanisms of apoptotic effect of tanshinones and synergism with doxorubicin in hepatocellular carcinoma cell line (HepG2)

6.2.2.6 Miscellaneous Effects

Salvia miltiorrhiza extract in collagen matrix increases new bone formation locally and can be used for bone grafting, especially in cases of compromised vascular responses (Wong and Rabie 2008). *Salvia miltiorrhiza* has anti-hepatitis B virus activity (Cui et al. 2010). *Salvia miltiorrhiza* was able to block the lethal toxicity of LPS in mice via suppression of TNF-alpha release and protection on liver injury. The ability of *Salvia miltiorrhiza* to suppress LPS-induced TNF-alpha release is further confirmed by *in vitro* experiments conducted on human peripheral blood leucocytes (PBL) and the RAW 264.7 macrophage cell line (Wan et al. 2006). The ethyl acetate extract and water extract of *Salvia miltiorrhiza* neutralised the entero-virus 71-induced cytopathic effect in Vero, rhabdomyosarcoma, and MRC-5 cells (Wu et al. 2007a). Antiviral activity is more efficient in cultures treated with *Salvia miltiorrhiza* extracts during viral infection compared to the cultures treated before or after infection, suggesting that danshen extracts could interfere with viral entry. *Salvia miltiorrhiza* reduces the serum levels of interleukin-6 (IL-6), interleukin-8 (IL-8), and TNF-alpha in patients with severe acute pancreatitis (Peng and Zhang 2007). *Salvia miltiorrhizae* injection reduces the contents of inflammatory mediators in the blood of obstructive jaundice rats and exert some protective effects on multiple organs of these rats (Ling et al. 2009b). Purified *Salvia miltiorrhiza*

extract as a farnesoid X receptor/liver X receptor alpha coagonist largely improves the lipid profiles in the hyperlipidemic rats (Ji and Gong 2008). The total *Salvia miltiorrhizae* extract and tanshinone I, tanshinone IIA, and dihydrotanshinone I (except cryptotanshinone) enhances the activity of insulin on the tyrosine phosphorylation of the insulin receptor as well as the activation of the downstream kinases Akt, ERK1/2, and GSK3beta (Jung et al. 2009). These tanshinones may be useful antidiabetic agents through their insulin-sensitising activities.

6.3 Different Formulations of *Salvia miltiorrhiza*

According to the Cochrane Library's Cochrane Central Register of Controlled Trials, *Salvia miltiorrhiza* (Danshen) is widely used in China for the treatment of several diseases, including acute myocardial infarction. Evidence from Register of Controlled Trials is insufficient and has so far been of low quality. The safety of *Salvia miltiorrhiza* preparations is unproven, although some adverse events have been reported. More evidence from high-quality trials is needed to support the clinical use of *Salvia miltiorrhiza* preparations (Wu et al. 2008a). One hundred and fifty (150) *Salviae Miltiorrhizae* randomised controlled trials were identified. The mean (standard deviation) score of 150 Danshen randomised controlled trials assessed by CONSORT for TCM and the Jadad scale is 23.87 (3.68) and 1.94 (0.82), respectively. Only 6.7 % (10/150) of randomised controlled trials are identified with high quality (Jadad score $\geq$ 4). The quality of Danshen Register of Controlled Trials in mainland China has not been improved significantly over recent years, and the overall quality of Danshen randomised controlled trials is still poor (Yu et al. 2009c). However, despite the deficiency of the clinical trials, *Salvia miltiorrhiza* and other formulations containing *Salvia miltiorrhiza* extract are extensively used in China and other parts of the world.(Adams et al. 2006).

6.3.1 Pure Compounds

Pure compounds such as sodium tanshinone IIA sulfonate, tanshinone IIA, and cryptotanshinone are registered with State Food and Drug Administration, People's Republic of China. Formulated pure compounds of *Salvia miltiorrhiza* include a Salvianolate injection (containing >80 % Salvianolic acid B) and a Tanshinone capsule (containing tanshinone IIA and cryptotanshinone). The isolated components of *Salvia miltiorrhiza* including salvianolic acids, danshensu, and the tanshinones are currently investigated for their cardiovascular, anticancer, and antioxidant activities and therapeutic potential. Sodium tanshinone IIA sulfonate, a more hydrophilic derivative of tanshinone IIA, protects cardiomyocytes against oxidative stress-mediated apoptosis (Yang et al. 2008b), activates high conductance calcium-activated potassium ion channels in porcine coronary artery smooth muscle cells (Yang et al. 2008c), and protects immune-mediated liver injury in mice. The protection is associated with its suppressive effect on the production of

important inflammatory mediators (Xu et al. 2008b). Sulfotanshinone Sodium Injection decreases fibrinogen level and improves clinical outcomes in patients with unstable angina pectoris (Yan et al. 2009). Novel polylactic acid nanoparticles containing tanshinone IIA are synthesised for testing on human liver cancer cells and in mice with hepatoma (Li et al. 2008c). Individual tanshinones such as cryptotanshinone, dihydrotanshinone, tanshinone I, and tanshinone IIA are being tested for their apoptotic effects of cancer cells. Danshensu possesses antioxidant activities and is relatively non-toxic in laboratory animals (Gao et al. 2009; Li et al. 2009b) and danshensu derivatives may be good drug candidates for anti-myocardial ischaemia therapy (Dong et al. 2009a).

6.3.2 Salvia miltiorrhiza *Extracts*

Salviae miltiorrhiza can effectively reduce the mortality and complications of acute pancreatitis through improvement of microcirculatory disturbances, elimination of oxygen free radicals, modulation of the metabolism of lipid inflammatory mediator, and blocking of calcium inflow and prevention of calcium overload (Zhang et al. 2006). Water-soluble fractions of *Salvia miltiorrhiza* root extract scavenge peroxides and inhibit the expression of adhesion molecules in vascular endothelium and leucocytes. Lipophilic compounds of *Salvia miltiorrhiza* root extracts prevent the development of vascular damage, platelet aggregation, and mast cell degranulation. Thus, both the water-soluble and lipophilic compounds of *Salvia Miltiorrhiza* root extract appear to improve the infarction/reperfusion-induced vascular damage multifactorially and synergically (Han et al. 2008). The efficacy of a Danshen (*Salvia miltiorrhiza*) Dripping Pill (DDP) for secondary stroke prevention was evaluated in patients with ischaemic cerebrovascular disease and may reduce the risk for stroke via its anti-inflammatory effects (Xu et al. 2009a).

Given that both the water-soluble (Salvianolic acids and danshensu) and lipophilic compounds (tanshinones) possess diverse pharmacological effects, another important issue would be the ways in which *Salvia miltiorrhiza* extracts are prepared. The composition of the water-soluble and lipophilic compounds present in different extracts of *Salvia miltiorrhiza* would be expected to differ in water, alcohol, or alcohol/water extracts. Salvianolic acid B and danshensu are the major ingredients in water extracts of *Salvia miltiorrhiza* while tanshinone IIA is among the most abundant of the major tanshinones present in the alcohol extract. With the new evidence that the tanshinones may have anticancer potential, it is important to investigate water and alcoholic extracts of *Salvia miltiorrhiza* separately. In addition, the conditions of the extraction procedures would need to be verified and standardised as it is apparent that some of the active ingredients of *Salvia miltiorrhiza* may undergo degradation in severe conditions (Zhou et al. 2011).

6.3.2.1 Single Use

Differently formulated *Salvia miltiorrhiza* (Danshen) extracts including Danshen tablet, granule, and capsule, Danshen Dripping Pill, and Danshen Injection are

registered with the State Food and Drug Administration, People's Republic of China. Danshen Injection reduces endothelium-dependent vasodilation and decreases reserve of tissue plasminogen activator and nitric oxide in endothelium in diabetic patients (Zhang et al. 2005b). Danshen Injection is also effective in improving liver function and inhibiting liver fibrosis in patients with chronic hepatitis B (Jin et al. 2006a) and in patients with liver cirrhosis (Ye et al. 2005). Danshen Injection improves blood microcirculation and decreases the incidence of renal function recovery retardation, effects which are helpful for recovery of renal function after renal transplantation. Danshen protects endothelial progenitor cells from oxidised low-density lipoprotein-induced impairment on both endothelial progenitor cells in patients with hypercholesterolemia and endothelial progenitor cells of healthy volunteers *in vitro* (Ji et al. 2010).

6.3.2.2 Compound Formulae

Different compound formulae of *Salvia miltiorrhiza* (Danshen) extracts are registered with State Food and Drug Administration, People's Republic of China. Compound Danshen tablet (Fufang Danshen Pian) contains *Salvia miltiorrhiza*, *Panax pseudoginseng*, and *Borneolum Syntheticum*; Compound Danshen Injection contains *Salvia miltiorrhiza* and *Lignum dalbergiae odoriferae*; Danshen Pill contains *Salvia miltiorrhiza*, *Panax pseudoginseng*, and *Lignum dalbergiae odoriferae*. The 'Cardiotonic Pill' (CP) is a pharmaceutical product derived from *Salvia miltiorrhiza bunge* and recently widely used in Chinese hospitals for the prevention and management of ischaemic cardiovascular diseases (Ling et al. 2005). The 'Cardiotonic Pill' inhibits expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in cultured human vascular endothelial cells and proliferation of vascular smooth muscle cells in a manner that has potentially beneficial therapeutic effects. The actions by Cardiotonic Pill to reduce apoptotic damage in myocytes and collagen synthesis in fibroblasts may help to preserve the heart function and reduce heart failure risk (Ling et al. 2009a). In an *in vivo* model using apolipoprotein E-deficient (ApoE–/–) mice fed with an atherogenic (high fat) diet, the Cardiotonic Pill reduces whole atherosclerotic lesions and fibrous plaques in the artery. The effect of compound Danshen Dripping Pill (DSP) on carotid arterial intima-media thickness (IMT) in patients with type 2 diabetes mellitus (T2DM) was tested and Danshen Dripping Pill may delay the occurrence and development of diabetic macrovascular disease (Ma et al. 2010). The major active ingredient of *Salvia miltiorrhiza* present in Danshen Dripping Pill is Danshensu. An oral herbal medicine, including Danshen (*Salvia miltiorrhiza*), *Panax notoginseng*, and *Dyroblanops aromatica gaertn*., has been clinically used for coronary diseases and cerebral infarction in Japan (Horie et al. 2009). Danshen (*Salvia miltiorrhiza*) and Gegen (*Pueraria lobata*) have long been used in the treatment of angina and other cardiac symptoms in Chinese materia medica. Recent pharmacological studies on the therapeutic values of Danshen (*Salvia miltiorrhiza*) and Gegen (*Pueraria lobata*) suggest that their adjunctive treatment in coronary patients is well tolerated and effective in improving vascular function (Tam et al. 2009). Danhong

Injection, a Chinese Materia Medica standardised product extracted from *Radix Salviae miltiorrhizae* and *Flos Carthami tinctorii*, is effective in the recovery of patients with traumatic intracranial hematoma (Sun et al. 2009a).

Note. Danshen Dripping Pill is a formulated product available in China for sublingual or oral administration. Preparation of Dripping pill: Herbal extracts are heated and mixed with hydrophilic or hydrophobic matrix such as PEG6000, gelatin, and stearic acid, dripped into chilled condensing agents that are insoluble in the matrix, and then cooled down and shrunk into small balls of same sizes (Chinese Pharmacopoeia). Compound Danshen Dripping Pill is composed of Danshen (Radix Salviae Miltiorrhizae), Sanqi (Radix Notoginseng), and Borneol (Chinese Pharmacopoeia).

6.4 Safety of *Salvia miltiorrhiza*

6.4.1 Side Effects of Salvia miltiorrhiza

Danshensu does not cause toxicity in laboratory animals after acute and chronic treatment in mice and rats (Gao et al. 2009) and in beagle dogs (Li et al. 2009b). In a randomised control trial in women with oligohydramnios, *Salvia miltiorrhiza* improves the amniotic fluid volume in pre-term oligohydramnios by improving uteroplacental circulation without showing any side effects (Chu and Shen 2008). Tanshinone IIA used during the last third of gestation does not cause the biochemical changes related to renal, liver and cardiac functions in both the mother and fetus. This provides new information to guide the use of herbal medicine during pregnancy (Mao et al. 2009a; Zhang et al. 2009d). *Salvia miltiorrhiza* is well tolerated in adjunctive treatment for secondary prevention in patients with coronary heart failure (Tam et al. 2009).

6.4.2 Herb–Drug Interaction Potentials

6.4.2.1 Pharmacodynamic Interactions

Salvia miltiorrhiza can affect hemostasis in several ways, including inhibition of platelet aggregation, interference with the extrinsic blood coagulation, antithrombin III-like activity, and promotion of fibrinolytic activity. Recent studies with salvianolic acid B, danshensu, and aqueous extract of *Salvia miltiorrhiza* containing these two compounds exert vasorelaxant actions by inhibition of calcium channels, and a minor component mediated by the opening of potassium channels (Lam et al. 2006b, c, 2007, 2010). The lipid-soluble ingredients of *Salvia miltiorrhiza* such as cryptotanshinone and dihydrotanshinone are also calcium channel blockers (Lam et al. 2008a, b). These effects of *Salvia miltiorrhiza* and its major ingredients may be relevant to potential herb–drug interaction with cardiovascular agents. *Salvia miltiorrhiza* and its ingredients, such as salvianolic acids, rosmarinic acid, and

tanshinones, inhibit platelet aggregation and possess antithrombotic and antiplatelet effects (Zou et al. 1993; Han et al. 2008; Park et al. 2008; Zhao et al. 2008b; Fan et al. 2010; Lin and Hsieh 2010; Huang et al. 2010). These effects may be relevant to the clinically significant herb–drug interaction with warfarin, which may be mediated via both pharmacodynamic and pharmacokinetic mechanisms (Lo et al., 1992; Chan 2001).

6.4.2.2 Pharmacokinetic Interactions

Single-dose and steady-state studies in rats indicated that *Salvia miltiorrhiza* increased the absorption rate constants, area under the curves (AUC), maximum concentrations, and elimination half-lives, but decreased the clearances and apparent volume of distribution of both R- and S-warfarin (Yu et al. 1997; Chan 2001). Tanshinones inhibit CYP1A1-, CYP2C6-, and CYP2C11-mediated warfarin metabolism both *in vitro* and *in vivo* in the rat. The timing of *Salvia miltiorrhiza* intake relative to warfarin contributes to different pharmacokinetics of the free warfarin concentration (Wu and Yeung 2010). Tanshinone IIA is metabolised by rat CYP2C, CYP3A, and CYP2D, as ticlopidine, ketoconazole, and quinidine all inhibit tanshinone IIA metabolism in rat liver microsomes (Bi et al. 2008). Tanshinone IIA selectively inhibits mouse and human CYP1A2 activity (Ueng et al. 2003) and shows induction of CYP1A in C57BL/6J but not in DBA/2J mice with elevation of cyp1a mRNA and protein expression (Ueng et al. 2004). The study with a chemical selective inhibitor, cDNA-expressed human cytochrome P450s, correlation assay, and kinetics study demonstrates that CYP2A6 is the specific isozyme responsible for the hydroxyl metabolism of tanshinone IIA in human liver microsomes (Liu et al. 2009a). Mouse CYP1A-, CYP2C-, and CYP3A-inducing agents are thought to be present in the ethyl acetate extract, but not in the aqueous extract of *Salvia miltiorrhiza* (Kuo et al. 2006). CYP induction by the ethyl acetate extract and pharmaceutical product containing *Salvia miltiorrhiza* suggests possible drug interactions between *Salvia miltiorrhiza* and CYP substrates. In healthy volunteers, *Salvia miltiorrhiza* extract has no effect on the metabolism of theophylline, a model CYP1A2 substrate (Qiu et al. 2008), but an aqueous extract affects the metabolism of CYP1A2 substrates through competitive inhibition and alters their clearance in the rat *in vivo* and in humans and rats *in vitro* (Wang and Yeung 2010). Major tanshinones isolated from *Salvia miltiorrhiza* competitively inhibit the metabolism of model CYP1A2 probe substrates without affecting CYP1A2 expression (Wang et al. 2009). In human HepG2 hepatoma cell line, tanshinone IIA, cryptotanshinone, tanshinone I, and dihydrotanshinone induce CYP1A1 and CYP1A2 expression through transcriptional activation mechanism (Zhang et al. 2011a, b). A formulated Danshen pill (containing mainly danshensu, salvianolic acid B, and the tanshinones) upregulated CYP1A2 protein expression and enzyme activity while danshensu and salvianolic acid B, when used individually, do not alter CYP1A2 expression (Lee et al. 2011a). The significance of these findings remains to be established.

Salvia miltiorrhiza decreases hepatic CYP3A protein expression but has no enzyme-inducing effects on rat CYP3A (Wang et al. 2010g), and did not affect the pharmacokinetics of Pgp and CYP3A substrate in the rat (Lee et al. 2011b). The structural difference between dihydrotanshinone and tanshinone I at the C-15 position of furan ring may be related to the different modes of inhibition of rat CYP3A and human CYP3A4 activity (Wang and Yeung 2011a, b, c). Despite competitive inhibition of rat CYP2C11 *in vitro* and *in vivo*, *Salvia miltiorrhiza* and its active components (tanshinone I, tanshinone IIA, dihydrotanshinone, and cryptotanshinone) decrease tolbutamide 4-hydroxylation, with minor changes in tolbutamide pharmacokinetics *in vivo* (Wang et al. 2010f). Tanshinones inhibit the metabolism of various CYP probe substrates in human liver microsomes and specific human CYP isoforms *in vitro* (Wang et al. 2010a) and both cryptotanshinone and tanshinone IIA can activate transcription of CYP3A4 (Yu et al. 2009b). A molecular docking study shows that salvianolic acid B, danshensu, protocatechuic aldehyde, cryptotanshinone, and tanshinone IIA significantly transactivated the CYP3A4 reporter gene construct in either HepG2 or Huh7 cells (Liu et al. 2011b). Given that CYP1A2, 2C9, 2E1, and 3A4 are responsible for the metabolism and disposition of a large number of drugs currently used clinically, the potential herb–drug interactions of *Salvia miltiorrhiza* preparations containing the major tanshinones with drugs which are substrates of these CYPs may be important (Wang et al. 2010e) and merit further investigations and careful monitoring.

6.5 Conclusions

Salvia miltiorrhiza and formulated products containing *Salvia miltiorrhiza* or its active ingredients have been widely used for the treatment of cardiovascular diseases in China. Randomised clinical trials and clinical experience in China suggest that *Salvia miltiorrhiza* and its related products are safe, with a low side-effect profile, the results of which need to be confirmed by more well-designed clinical trials. Recent studies with individual components of *Salvia miltiorrhiza* have identified diverse pharmacological actions and thus other therapeutic potential of the herb and its active ingredients.

References

Adams JD, Wang R, Yang J, Lien EJ (2006) Preclinical and clinical examinations of *Salvia miltiorrhiza* and its tanshinones in ischemic conditions. Chinesische Medizin 1:3

Bai A, Lu N, Guo Y, Fan X (2008) Tanshinone IIA ameliorates trinitrobenzene sulfonic acid (TNBS)-induced murine colitis. Dig Dis Sci 53:421–428

Bi HC, Zuo Z, Chen X, Xu CS, Wen YY, Sun HY, Zhao LZ, Pan Y, Deng Y, Liu PQ, Gu LQ, Huang ZY, Zhou SF, Huang M (2008) Preclinical factors affecting the pharmacokinetic behaviour of tanshinone IIA, an investigational new drug isolated from *Salvia miltiorrhiza* for the treatment of ischaemic heart diseases. Xenobiotica 38:185–222

Bi YF, Xu HW, Liu XQ, Zhang XJ, Wang ZJ, Liu HM (2010) Synthesis and vasodilative activity of tanshinone IIA derivatives. Bioorg Med Chem Lett 20:4892–4489

Bian W, Chen F, Bai L, Zhang P, Qin W (2008) Dihydrotanshinone I inhibits angiogenesis both in vitro and in vivo. Acta Biochim Biophys Sin 40:1–6

Cao H, Yu R, Choi Y, Ma ZZ, Zhang H, Xiang W, Lee DY, Berman BM, Moudgil KD, Fong HH, van Breemen RB (2010) Discovery of cyclooxygenase inhibitors from medicinal plants used to treat inflammation. Pharmacol Res 61:519–524

Cao J, Wei YJ, Qi LW, Li P, Qian ZM, Luo HW, Chen J, Zhao J (2008) Determination of fifteen bioactive components in Radix et Rhizoma Salviae Miltiorrhizae by high-performance liquid chromatography with ultraviolet and mass spectrometric detection. Biomed Chromatogr 22:164–172

Cao Y, Chai JG, Chen YC, Zhao J, Zhou J, Shao JP, Ma C, Liu XD, Liu XQ (2009) Beneficial effects of danshensu, an active component of *Salvia miltiorrhiza*, on homocysteine metabolism via the trans-sulphuration pathway in rats. Br J Pharmacol 157:482–490

Chai J, Ji K, Lin J, Ye R, Tang J, Yang P (2009) Effects of danshensu on function of EPCs which were damaged by Ox-LDL and study its possible mechanism (in Chinese). China J Chin Mater Med 34:904–907, Zhongguo Zhong Yao Za Zhi/Zhongguo Zhongyao Zazhi

Chan K, Chui SH, Wong DY, Ha WY, Chan CL, Wong RN (2004) Protective effects of danshensu from the aqueous extract of *Salvia miltiorrhiza* (Danshen) against homocysteine-induced endothelial dysfunction. Life Sci 75:3157–3171

Chen L, Zheng SZ, Sun ZG, Wang AY, Huang CH, Punchard NA, Huang SL, Gao X, Lu Y (2011) Cryptotanshinone has diverse effects on cell cycle events in melanoma cell lines with different metastatic capacity. Cancer Chemother Pharmacol 68:17–27

Chan TY (2001) Interaction between warfarin and danshen (*Salvia miltiorrhiza*). Ann Pharmacother 35:501–504

Che XH, Park EJ, Zhao YZ, Kim WH, Sohn DH (2010) Tanshinone IIA induces apoptosis and S phase cell cycle arrest in activated rat hepatic stellate cells. Basic Clin Pharmacol Toxicol 106:30–37

Chen J, Wang F, Lee FS, Wang X, Xie M (2006) Separation and identification of water-soluble salvianolic acids from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography and ESI-MS analysis. Talanta 69:172–179

Chen TH, Hsu YT, Chen CH, Kao SH, Lee HM (2007) Tanshinone IIA from *Salvia miltiorrhiza* induces heme oxygenase-1 expression and inhibits lipopolysaccharide-induced nitric oxide expression in RAW 264.7 cells. Mitochondrion 7:101–105

Chen W, Luo Y, Liu L, Zhou H, Xu B, Han X, Shen T, Liu Z, Lu Y, Huang S (2010a) Cryptotanshinone inhibits cancer cell proliferation by suppressing Mammalian target of rapamycin-mediated cyclin d1 expression and rb phosphorylation. Cancer Prevent Res 3:1015–1025

Chen WY, Sun CM, Wang HM, Huang M, Zhu GX, Zhu BD, Liu FY (2010b) Effect of tanshinone IIA pretreatment on IL-1 and Re1A mRNA expression in rats with focal cerebral ischemia (in Chinese). J Southern Med Univ 30:2115–2118, Nan Fang Yi Ke Da Xue Xue Bao

Cheng CY, Su CC (2010) Tanshinone IIA inhibits Hep-J5 cells by increasing calreticulin, caspase 12 and GADD153 protein expression. Int J Mol Med 26:379–385

Cheng TO (2007) Cardiovascular effects of Danshen. Int J Cardiol 121:9–22

Chiu TL, Su CC (2010) Tanshinone IIA induces apoptosis in human lung cancer A549 cells through the induction of reactive oxygen species and decreasing the mitochondrial membrane potential. Int J Mol Med 25:231–236

Choi HS, Cho DI, Choi HK, Im SY, Ryu SY, Kim KM (2004) Molecular mechanisms of inhibitory activities of tanshinones on lipopolysaccharide-induced nitric oxide generation in RAW 264.7 cells. Arch Pharm Res 27:1233–1237

Choi HS, Kim KM (2004) Tanshinones inhibit mast cell degranulation by interfering with IgE receptor-mediated tyrosine phosphorylation of PLCgamma2 and MAPK. Planta Med 70:178–180

Chor SY, Hui AY, To KF, Chan KK, Go YY, Chan HL, Leung WK, Sung JJ (2005) Anti-proliferative and pro-apoptotic effects of herbal medicine on hepatic stellate cell. J Ethnopharmacol 100:180–186

Chu HN, Shen MJ (2008) Treating oligohydramnios with extract of *Salvia miltiorrhiza*: a randomized control trial. Ther Clin Risk Manag 4:287–290

Chuang MT, Ho FM, Wu CC, Zhuang SY, Lin SY, Suk FM, Liang YC (2011) 15,16-Dihydrotanshinone I, a compound of *Salvia miltiorrhiza bunge*, induces apoptosis through inducing endoplasmic reticular stress in human prostate carcinoma cells. Evid Based Complement Alternat Med 2011:865435

Cui L, Liu YY, Wu T, Ai CM, Chen HQ (2009) Osteogenic effects of D+beta-3,4-dihydroxyphenyl lactic acid (salvianic acid A, SAA) on osteoblasts and bone marrow stromal cells of intact and prednisone-treated rats. Acta Pharmacol Sin 30:321–332, Zhongguo Yao Li Xue Bao

Cui X, Wang Y, Kokudo N, Fang D, Tang W (2010) Traditional Chinese medicine and related active compounds against hepatitis B virus infection. Biosci Trends 4:39–47

Dat NT, Jin X, Lee JH, Lee D, Hong YS, Lee K, Kim YH, Lee JJ (2007) Abietane diterpenes from *Salvia miltiorrhiza* inhibit the activation of hypoxia-inducible factor-1. J Nat Prod 70:1093–1097

Ding M, Ye TX, Zhao GR, Yuan YJ, Guo ZX (2005a) Aqueous extract of *Salvia miltiorrhiza* attenuates increased endothelial permeability induced by tumor necrosis factor-alpha. Int Immunopharmacol 5:1641–1651

Ding M, Yuan YJ (2007) Study on the mechanisms of an extract of *Salvia miltiorrhiza* on the regulation of permeability of endothelial cells exposed to tumour necrosis factor-alpha. J Pharm Pharmacol 59:1027–1033

Ding M, Zhao GR, Yuan YJ, Guo ZX (2005b) Aqueous extract of Salvia miltiorrhoza regulates adhesion molecule expression of tumor necrosis factor alpha-induced endothelial cells by blocking activation of nuclear factor kappaB. J Cardiovasc Pharmacol 45:516–524

Dittmann K, Gerhuser C, Klimo K, Hamburger M (2004) HPLC-based activity profiling of *Salvia miltiorrhiza* for MAO A and iNOS inhibitory activities. Planta Med 70:909–913

Dong K, Xu W, Yang J, Qiao H, Wu L (2009a) Neuroprotective effects of Tanshinone IIA on permanent focal cerebral ischemia in mice. Phytother Res 23:608–613

Dong X, Dong J, Zhang R, Fan L, Liu L, Wu G (2009b) Anti-inflammatory effects of tanshinone IIA on radiation-induced microglia BV-2 cells inflammatory response. Cancer Biother Radiopharm 24:681–687

Dong C, Wang Y, Zhu YZ (2009c) Asymmetric synthesis and biological evaluation of Danshensu derivatives as anti-myocardial ischemia drug candidates. Bioorg Med Chem 17:3499–507

Durairajan SS, Yuan Q, Xie L, Chan WS, Kum WF, Koo I, Liu C, Song Y, Huang JD, Klein WL, Li M (2008) Salvianolic acid B inhibits Abeta fibril formation and disaggregates preformed fibrils and protects against Abeta-induced cytotoxicty. Neurochem Int 52:741–750

Fan GW, Gao XM, Wang H, Zhu Y, Zhang J, Hu LM, Su YF, Kang LY, Zhang BL (2009) The anti-inflammatory activities of Tanshinone IIA, an active component of TCM, are mediated by estrogen receptor activation and inhibition of iNOS. J Steroid Biochem Mol Biol 113:275–280

Fan HY, Fu FH, Yang MY, Xu H, Zhang AH, Liu K (2010) Antiplatelet and antithrombotic activities of salvianolic acid A. Thromb Res 126:e17–22

Fang ZY, Lin R, Yuan BX, Liu Y, Zhang H (2007) Tanshinone IIA inhibits atherosclerotic plaque formation by down-regulating MMP-2 and MMP-9 expression in rabbits fed a high-fat diet. Life Sci 81:1339–1345

Fang ZY, Lin R, Yuan BX, Yang GD, Liu Y, Zhang H (2008) Tanshinone IIA downregulates the CD40 expression and decreases MMP-2 activity on atherosclerosis induced by high fatty diet in rabbit. J Ethnopharmacol 115:217–222

Feng J, Jin Y, Wang J, Xiao Y, Liang X (2008) Development of full-quantified fingerprint for quality control of traditional Chinese medicines (in Chinese). Chin J Chromatogr 26:180–185

Fu G, Feng X, Ge X, He X, Wu Z, Yang J, Wang J, Li X, Yuan Y (2010) Differences in shapes and properties and microscopic frameworks of wild and cultivated Radix Salviae Miltiorrhizae from different regions (in Chinese). China J Chin Materia Med 35:1235–1238, Zhongguo Zhong Yao Za Zhi/Zhongguo Zhongyao Zazhi

Fu J, Huang H, Liu J, Pi R, Chen J, Liu P (2007) Tanshinone IIA protects cardiac myocytes against oxidative stress-triggered damage and apoptosis. Eur J Pharmacol 568:213–221

Gao J, Yang G, Pi R, Li R, Wang P, Zhang H, Le K, Chen S, Liu P (2008) Tanshinone IIA protects neonatal rat cardiomyocytes from adriamycin-induced apoptosis. Translational research. J Lab Clin Med 151:79–87

Gao XP, Xu DY, Deng YL, Zhang Y (2004) Screening of angiotensin converting enzyme inhibitors from *Salvia miltiorrhiza*e (in Chinese). China J Chin Materia Med 29:359–362, Zhongguo Zhong Yao Za Zhi/Zhongguo Zhongyao Zazhi

Gao Y, Liu Z, Li G, Li C, Li M, Li B (2009) Acute and subchronic toxicity of danshensu in mice and rats. Toxicol Mech Methods 19:363–368

Gong Z, Huang C, Sheng X, Zhang Y, Li Q, Wang MW, Peng L, Zang YQ (2009) The role of tanshinone IIA in the treatment of obesity through peroxisome proliferator-activated receptor gamma antagonism. Endocrinology 150:104–113

Gu M, Wang X, Su Z, Ouyang F (2007) One-step separation and purification of 3,4-dihydroxyphenyllactic acid, salvianolic acid B and protocatechualdehyde from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography. J Chromatogr A 1140:107–111

Guang LC, Jun SS, Pang EC, May B, Chang Li XC (2011) Plant density-dependent variations in bioactive markers and root yield in Australian-grown *Salvia miltiorrhiza* Bunge. Chem Biodivers 8:699–709

Guo L, Lei CK, Shan M (2008) Study of growth inhibitory effect and apoptosis induced by different matches of Tanshinone IIA and salvianolic acid B on acute promyelocytic leukemia cells (HL-60) (in Chinese). Zhong Yao Cai 31:1512–1514

Han CJ, Lin R, Liu JT, Liu Y, Zhang H (2007) Protection of vascular endothelial cells from ox-LDL induced injury by protocatechualdehyde (in Chinese). Zhong Yao Cai 30:1541–1544

Han JY, Fan JY, Horie Y, Miura S, Cui DH, Ishii H, Hibi T, Tsuneki H, Kimura I (2008) Ameliorating effects of compounds derived from *Salvia miltiorrhiza* root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion. Pharmacol Ther 117:280–295

Hao Y, Xie T, Korotcov A, Zhou Y, Pang X, Shan L, Ji H, Sridhar R, Wang P, Califano J, Gu X (2009) Salvianolic acid B inhibits growth of head and neck squamous cell carcinoma in vitro and in vivo via cyclooxygenase-2 and apoptotic pathways. Int J Cancer 24:2200–2209

He W, Li Y, Shen XM, Sun LM (2007) Optimization of preparative separation and purification of water-soluble substance from *Salvia miltiorrhiza* by macroporous resins (in Chinese). Zhong Yao Cai 30:1308–1310

Hei M, Liu F, Luo Y (2010) Effect of Tanshinone IIA on phosphorylated NMDA receptor 1 expression and intracellular free calcium concentration in neonatal SD rats with hypoxic ischemic brain damage (in Chinese). J Central South Univ Med Sci 35:940–946, Zhong Nan da Xue Xue Bao. Yi Xue Ban

Ho JH, Hong CY (2011) Salvianolic acids: small compounds with multiple mechanisms for cardiovascular protection. J Biomed Sci 18:30

Horie Y, Kikuchi M, Yamagishi Y, Umeda R, Ebinuma H, Saito H, Kato S, Ishii H, Hibi T, Han JY (2009) Effect of a herbal medicine on fatty liver in rats fed ethanol chronically. Nihon Arukoru Yakubutsu Igakkai Zasshi 44:636–648

Hsu YC, Lin YL, Chiu YT, Shiao MS, Lee CY, Huang YT (2005) Antifibrotic effects of *Salvia miltiorrhiza* on dimethylnitrosamine-intoxicated rats. J Biomed Sci 12:185–195

Hu P, Luo GA, Zhao Z, Jiang ZH (2005a) Quality assessment of radix salviae miltiorrhizae. Chem Pharm Bull 53:481–486

Hu P, Luo GA, Zhao ZZ, Jiang ZH (2005b) Quantitative determination of four diterpenoids in Radix Salviae Miltiorrhizae using LC-MS-MS. Chem Pharm Bull 53:705–709

Hu S, Chen SM, Li XK, Qin R, Mei ZN (2007) Antitumor effects of chi-shen extract from *Salvia miltiorrhiza* and *Paeoniae radix* on human hepatocellular carcinoma cells. Acta Pharmacol Sin 28:1215–1223

Huang KJ, Wang H, Xie WZ, Zhang HS (2007) Investigation of the effect of tanshinone IIA on nitric oxide production in human vascular endothelial cells by fluorescence imaging. Spectrochimica Acta. Part A. Mol Biomol Spectrosc 68:1180–1186

Huang XY, Wang L, Huang ZL, Zheng Q, Li QS, Tang ZY (2009) Herbal extract "Songyou Yin"; inhibits tumor growth and prolongs survival in nude mice bearing human hepatocellular carcinoma xenograft with high metastatic potential. J Cancer Res Clin Oncol 135:1245–1255

Huang ZS, Zeng CL, Zhu LJ, Jiang L, Li N, Hu H (2010) Salvianolic acid A inhibits platelet activation and arterial thrombosis via inhibition of phosphoinositide 3-kinase. J Thromb Haemost 8:1383–1393

Hung YC, Wang PW, Pan TL (2010) Functional proteomics reveal the effect of *Salvia miltiorrhiza* aqueous extract against vascular atherosclerotic lesions. Biochim Biophys Acta 1804:1310–1321

Hung YC, Wang PW, Pan TL, Bazylak G, Leu YL (2009) Proteomic screening of antioxidant effects exhibited by radix *Salvia miltiorrhiza* aqueous extract in cultured rat aortic smooth muscle cells under homocysteine treatment. J Ethnopharmacol 124:463–474

Hur JM, Shim JS, Jung HJ, Kwon HJ (2005) Cryptotanshinone but not tanshinone IIA inhibits angiogenesis in vitro. Exp Mol Med 37:133–137

Jang SI, Jeong SI, Kim KJ, Kim HJ, Yu HH, Park R, Kim HM, You YO (2003) Tanshinone IIA from *Salvia miltiorrhiza* inhibits inducible nitric oxide synthase expression and production of TNF-alpha, IL-1beta and IL-6 in activated RAW 264.7 cells. Planta Med 69:1057–1059

Jang SI, Kim HJ, Kim YJ, Jeong SI, You YO (2006) Tanshinone IIA inhibits LPS-induced NF-kappaB activation in RAW 264.7 cells: possible involvement of the NIK-IKK, ERK1/2, p38 and JNK pathways. Eur J Pharmacol 542:1–7

Jeon SJ, Son KH, Kim YS, Choi YH, Kim HP (2008) Inhibition of prostaglandin and nitric oxide production in lipopolysaccharide-treated RAW 264.7 cells by tanshinones from the roots of *Salvia miltiorrhiza bunge*. Arch Pharmacol Res 31:758–763

Ji KT, Chai JD, Xing C, Nan JL, Yang PL, Tang JF (2010) Danshen protects endothelial progenitor cells from oxidized low-density lipoprotein induced impairment. J Zhejiang Univ Sci B 11:618–626

Ji W, Gong BQ (2008) Hypolipidemic activity and mechanism of purified herbal extract of *Salvia miltiorrhiza* in hyperlipidemic rats. J Ethnopharmacol 119:291–298

Ji Y, He J, Zhou Q, Chen J, Li Q, Zhu J, Liu H (2008) The inhibiting effect and its molecular mechanism of Tanshinone on human lung cancer cell line in vitro (in Chinese). Chin J Lung Cancer 11:202–205

Jiang B, Wu W, Li M, Xu L, Sun K, Yang M, Guan S, Liu X, Guo DA (2009) Cardioprotection and matrix metalloproteinase-9 regulation of salvianolic acids on myocardial infarction in rats. Planta Med 75:1286–1292

Jin CX, Yang J, Sun HF (2006a) Comparative study of the clinical effects of *salvia miltiorrhiza* injection and shengmai injection on chronic hepatitis B (in Chinese). Chin J Integ Trad West Med 26:936–938, Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi

Jin DZ, Yin LL, Ji XQ, Zhu XZ (2006b) Cryptotanshinone inhibits cyclooxygenase-2 enzyme activity but not its expression. Eur J Pharmacol 549:166–172

Jin UH, Suh SJ, Chang HW, Son JK, Lee SH, Son KH, Chang YC, Kim CH (2008) Tanshinone IIA from *Salvia miltiorrhiza* BUNGE inhibits human aortic smooth muscle cell migration and MMP-9 activity through AKT signaling pathway. J Cell Biochem 104:15–26

Jin YC, Kim CW, Kim YM, Nizamutdinova IT, Ha YM, Kim HJ, Seo HG, Son KH, Jeon SJ, Kang SS, Kim YS, Kam SC, Lee JH, Chang KC (2009) Cryptotanshinone, a lipophilic compound of Salvia miltiorrriza root, inhibits TNF-alpha-induced expression of adhesion molecules in HUVEC and attenuates rat myocardial ischemia/reperfusion injury in vivo. Eur J Pharmacol 614:91–97

Jing J, Zheng H, Wang J, Lin P, Zhang J, Xiong ZJ, Wu YY, Ren JJ, Yang HL, Wang XJ (2007) Growth inhibition and multidrug resistance-reversing effect of tanshinone I A on human breast cancer cell with estrogen receptor negative (in Chinese). J Sichuan Univ Med Sci 38:391–395, Sichuan da Xue Xue Bao. Yi Xue Ban

Jung SH, Seol HJ, Jeon SJ, Son KH, Lee JR (2009) Insulin-sensitizing activities of tanshinones, diterpene compounds of the root of *Salvia miltiorrhiza* Bunge. Phytomedicine 16:327–335

Kan S, Li J, Huang W, Shao L, Chen D (2009) Microsphere resin chromatography combined with microbial biotransformation for the separation and purification of salvianolic acid B in aqueous extract of roots of *Salvia multiorrihza* Bunge. J Chromatogr A 1216:3881–3886

Kai G, Xu H, Zhou C, Liao P, Xiao J, Luo X, You L, Zhang L (2011) Metabolic engineering tanshinone biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures. Metab Eng 13:319–327

Kang BY, Chung SW, Kim SH, Ryu SY, Kim TS (2000) Inhibition of interleukin-12 and interferon-gamma production in immune cells by tanshinones from *Salvia miltiorrhiza*. Immunopharmacology 49:355–361

Kang DG, Oh H, Chung HT, Lee HS (2003) Inhibition of angiotensin converting enzyme by lithospermic acid B isolated from Radix Salviae miltiorrhiza Bunge. Phytother Res 17:917–920

Kang DG, Oh H, Sohn EJ, Hur TY, Lee KC, Kim KJ, Kim TY, Lee HS (2004) Lithospermic acid B isolated from *Salvia miltiorrhiza* ameliorates ischemia/reperfusion-induced renal injury in rats. Life Sci 75:1801–1816

Kang YJ, Jin UH, Chang HW, Son JK, Lee SH, Son KH, Chang YC, Lee YC, Kim CH (2008) Inhibition of microsomal triglyceride transfer protein expression and atherogenic risk factor apolipoprotein B100 secretion by tanshinone IIA in HepG2 cells. Phytother Res 22:1640–1645

Kim DH, Jeon SJ, Jung JW, Lee S, Yoon BH, Shin BY, Son KH, Cheong JH, Kim YS, Kang SS, Ko KH, Ryu JH (2007a) Tanshinone congeners improve memory impairments induced by scopolamine on passive avoidance tasks in mice. Eur J Pharmacol 574:140–147

Kim DH, Kim S, Jeon SJ, Son KH, Lee S, Yoon BH, Cheong JH, Ko KH, Ryu JH (2009a) Tanshinone I enhances learning and memory, and ameliorates memory impairment in mice via the extracellular signal-regulated kinase signalling pathway. Br J Pharmacol 158:1131–1142

Kim HH, Kim JH, Kwak HB, Huang H, Han SH, Ha H, Lee SW, Woo ER, Lee ZH (2004) Inhibition of osteoclast differentiation and bone resorption by tanshinone IIA isolated from *Salvia miltiorrhiza* Bunge. Biochem Pharmacol 67:1647–1656

Kim HJ, Kim SH (2010) Tanshinone IIA enhances BMP-2-stimulated commitment of C2C12 cells into osteoblasts via p38 activation. Amino Acids 39:1217–1226

Kim JH, Jeong SJ, Kwon TR, Yun SM, Jung JH, Kim M, Lee HJ, Lee MH, Ko SG, Chen CY, Kim SH (2011) Cryptotanshinone enhances TNF-alpha-induced apoptosis in chronic myeloid leukemia KBM-5 cells. Apoptosis 16:696–707

Kim JY, Kim KM, Nan JX, Zhao YZ, Park PH, Lee SJ, Sohn DH (2003) Induction of apoptosis by tanshinone I via cytochrome c release in activated hepatic stellate cells. Pharmacol Toxicol 92:195–200

Kim SK, Jung KH, Lee BC (2009b) Protective effect of Tanshinone IIA on the early stage of experimental diabetic nephropathy. Biol Pharm Bull 32:220–224

Kim SY, Moon TC, Chang HW, Son KH, Kang SS, Kim HP (2002) Effects of tanshinone I isolated from *Salvia miltiorrhiza bunge* on arachidonic acid metabolism and in vivo inflammatory responses. Phytother Res 16:616–620

Kim YS, Kim NH, Lee SW, Lee YM, Jang DS, Kim JS (2007b) Effect of protocatechualdehyde on receptor for advanced glycation end products and TGF-beta1 expression in human lens epithelial cells cultured under diabetic conditions and on lens opacity in streptozotocin-diabetic rats. Eur J Pharmacol 569:171–179

Kuo YH, Lin YL, Don MJ, Chen RM, Ueng YF (2006) Induction of cytochrome P450-dependent monooxygenase by extracts of the medicinal herb *Salvia miltiorrhiza*. J Pharm Pharmacol 58:521–527

Kwak HB, Sun HM, Ha H, Kim HN, Lee JH, Kim HH, Shin HI, Lee ZH (2008) Tanshinone IIA suppresses inflammatory bone loss by inhibiting the synthesis of prostaglandin E2 in osteoblasts. Eur J Pharmacol 601:30–37

Kwak HB, Yang D, Ha H, Lee JH, Kim HN, Woo ER, Lee S, Kim HH, Lee ZH (2006) Tanshinone IIA inhibits osteoclast differentiation through down-regulation of c-Fos and NFATc1. Exp Mol Med 38:256–264

Lam FF, Deng SY, Ng ES, Yeung JH, Kwan YW, Lau CB, Koon JC, Zhou L, Zuo Z, Leung PC, Fung KP (2010) Mechanisms of the relaxant effect of a danshen and gegen formulation on rat isolated cerebral basilar artery. J Ethnopharmacol 132:186–192

Lam FF, Seto SW, Kwan YW, Yeung JH, Chan P (2006a) Activation of the iberiotoxin-sensitive BKCa channels by salvianolic acid B of the porcine coronary artery smooth muscle cells. Eur J Pharmacol 546:28–35

Lam FF, Yeung JH, Chan KM, Or PM (2008a) Mechanisms of the dilator action of cryptotanshinone on rat coronary artery. Eur J Pharmacol 578:253–260

Lam FF, Yeung JH, Chan KM, Or PM (2008b) Dihydrotanshinone, a lipophilic component of *Salvia miltiorrhiza* (danshen), relaxes rat coronary artery by inhibition of calcium channels. J Ethnopharmacol 119:318–321

Lam FF, Yeung JH, Chan KM, Or PM (2007) Relaxant effects of danshen aqueous extract and its constituent danshensu on rat coronary artery are mediated by inhibition of calcium channels. Vasc Pharmacol 46:271–277

Lam FF, Yeung JH, Cheung JH (2005) Mechanisms of the dilator action of Danshen (*Salvia miltiorrhiza*) on rat isolated femoral artery. J Cardiovasc Pharmacol 46:361–368

Lam FF, Yeung JH, Cheung JH, Or PM (2006b) Pharmacological evidence for calcium channel inhibition by danshen (*Salvia miltiorrhiza*) on rat isolated femoral artery. J Cardiovasc Pharmacol 47:139–145

Lam FF, Yeung JH, Kwan YW, Chan KM, Or PM (2006c) Salvianolic acid B, an aqueous component of danshen (*Salvia miltiorrhiza*), relaxes rat coronary artery by inhibition of calcium channels. Eur J Pharmacol 553:240–245

Lee CY, Agrawal DC, Wang CS, Yu SM, Chen JJ, Tsay HS (2008a) T-DNA activation tagging as a tool to isolate *Salvia miltiorrhiza* transgenic lines for higher yields of tanshinones. Planta Med 74:780–786

Lee DS, Lee SH (2000) Biological activity of dihydrotanshinone I: effect on apoptosis. J Biosci Bioeng 89:292–293

Lee DS, Lee SH, Noh JG, Hong SD (1999) Antibacterial activities of cryptotanshinone and dihydrotanshinone I from a medicinal herb, *Salvia miltiorrhiza* Bunge. Biosci Biotech Biochem 63:2236–2239

Lee JH, Shin YJ, Kim HJ, Oh JH, Jang YP, Lee YJ (2011a) Danshen extract does not alter pharmacokinetics of docetaxel and clopidogrel, reflecting its negligible potential in P-glycoprotein- and cytochrome P4503A-mediated herb-drug interactions. Int J Pharm 410:68–74

Lee P, Hur J, Lee J, Kim J, Jeong J, Kang I, Kim SY, Kim H (2006a) 15,16-dihydrotanshinone I suppresses the activation of BV-2 cell, a murine microglia cell line, by lipopolysaccharide. Neurochem Int 48:60–66

Lee SY, Choi DY, Woo ER (2005) Inhibition of osteoclast differentiation by tanshinones from the root of *Salvia miltiorrhiza bunge*. Arch Pharmacol Res 28:909–913

Lee TY, Chang HH, Wang GJ, Chiu JH, Yang YY, Lin HC (2006b) Water-soluble extract of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats. J Pharm Pharmacol 58:659–665

Lee TY, Wang GJ, Chiu JH, Lin HC (2003) Long-term administration of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-induced hepatic fibrosis in rats. J Pharm Pharmacol 55:1561–1568

Lee WY, Cheung CC, Liu KW, Fung KP, Wong J, Lai PB, Yeung JH (2010) Cytotoxic effects of tanshinones from *Salvia miltiorrhiza* on doxorubicin-resistant human liver cancer cells. J Nat Prod 73:854–859

Lee WY, Chiu LC, Yeung JH (2008b) Cytotoxicity of major tanshinones isolated from Danshen (*Salvia miltiorrhiza*) on HepG2 cells in relation to glutathione perturbation. Food Chem Toxicol 46:328–338

Lee WY, Liu KW, Yeung JH (2009) Reactive oxygen species-mediated kinase activation by dihydrotanshinone in tanshinones-induced apoptosis in HepG2 cells. Cancer Lett 285:46–57

Lee WY, Zhou, XL, Or PM, Kwan YW, Yeung JH (2011) Tanshinone I increases CYP1A2 protein expression and enzyme activity in primary rat hepatocytes. Phytomedicine. doi:10.1016/j.phymed.2011.07.006

Leung SW, Zhu DY, Man RY (2010) Effects of the aqueous extract of *Salvia Miltiorrhiza* (danshen) and its magnesium tanshinoate B-enriched form on blood pressure. Phytother Res 24:769–774

Li CG, Sheng SJ, Pang EC, Marriott P, May B, Zhou S, Story D, Xue CC (2009a) Cultivar variations of Australian-grown Danshen (*Salvia miltiorrhiza*): bioactive markers and root yields. Chem Biodivers 6:170–181

Li G, Gao Y, Li S, Li C, Zhu X, Li M, Liu Z (2009b) Study on toxicity of danshensu in beagle dogs after 3-month continuous intravenous infusion. Toxicol Mech Methods 19:441–446

Li JT, Dong JE, Liang ZS, Shu ZM, Wan GW (2008a) Distributional difference of fat-soluble compounds in the roots, stems and leaves of four Salvia plants (in Chinese). J Mol Cell Biol 41:44–52, Fen Zi Xi Bao Sheng Wu Xue Bao

Li P, Li SP, Yang FQ, Wang YT (2007) Simultaneous determination of four tanshinones in *Salvia miltiorrhiza* by pressurized liquid extraction and capillary electrochromatography. J Sep Sci 30:900–905

Li P, Xu G, Li SP, Wang YT, Fan TP, Zhao QS, Zhang QW (2008b) Optimizing ultraperformance liquid chromatographic analysis of 10 diterpenoid compounds in *Salvia miltiorrhiza* using central composite design. J Agric Food Chem 56:1164–1171

Li Q, Wang Y, Feng N, Fan Z, Sun J, Nan Y (2008c) Novel polymeric nanoparticles containing tanshinone IIA for the treatment of hepatoma. J Drug Target 16:725–732

Li X, Du JR, Bai B, Yu Y, Zheng XY, Yang F, Zheng H (2008d) Inhibitory effects and mechanism of tanshinone IIA on proliferation of rat aortic smooth muscle cells (in Chinese). China J Chin Materia Med 33:2146–2150, Zhongguo Zhong Yao Za Zhi/Zhongguo Zhongyao Zazhi

Li X, Du JR, Yu Y, Bai B, Zheng XY (2010a) Tanshinone IIA inhibits smooth muscle proliferation and intimal hyperplasia in the rat carotid balloon-injured model through inhibition of MAPK signaling pathway. J Ethnopharmacol 129:273–279

Li X, Yu C, Cai Y, Liu G, Jia J, Wang Y (2005) Simultaneous determination of six phenolic constituents of danshen in human serum using liquid chromatography/tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 820:41–47

Li X, Peng XD, Zhang WL, Dai LL (2008e) Inhibiting effects of denshensu, baicalin, astragalus and Panax notoginseng saponins on hepatic fibrosis and their possible mechanisms (in Chinese). Chung Hua Kan Tsang Ping Tsa Chih 16:193–197

Li XJ, Zhou M, Li XH, Xu YH, Liu H, Yang M (2009c) Effects of Tanshinone IIa on cytokines and platelets in immune vasculitis and its mechanism (in Chinese). Zhongguo Shi Yan Xue Ye Xue Za Zhi 17:188–192

Li Y, Yang Y, Yu D, Liang Q (2009d) The effect of tanshinone IIA upon the TGF-beta1/Smads signaling pathway in hypertrophic myocardium of hypertensive rats. J Huazhong Univ Sci Technol Med Sci 29:476–480

Li YI, Elmer G, Leboeuf RC (2008f) Tanshinone IIA reduces macrophage death induced by hydrogen peroxide by upregulating glutathione peroxidase. Life Sci 83:557–562

Li YJ, Duan CL, Liu JX, Xu YG (2010b) Pro-angiogenic actions of Salvianolic acids on in vitro cultured endothelial progenitor cells and chick embryo chorioallantoic membrane model. J Ethnopharmacol 131:562–566

Liang Q, Yang L, Wang Z, Huang S, Li S, Yang G (2009) Tanshinone IIA selectively enhances hyperpolarization-activated cyclic nucleotide-modulated (HCN) channel instantaneous current. J Pharmacol Sci 110:381–388

Lin R, Wang WR, Liu JT, Yang GD, Han CJ (2006a) Protective effect of tanshinone IIA on human umbilical vein endothelial cell injured by hydrogen peroxide and its mechanism. J Ethnopharmacol 108:217–22

Lin TH, Hsieh CL (2010) Pharmacological effects of *Salvia miltiorrhiza* (Danshen) on cerebral infarction. Chinesische Medizin 5:22

Lin YH, Liu AH, Wu HL, Westenbroek C, Song QL, Yu HM, Ter Horst GJ, Li XJ (2006b) Salvianolic acid B, an antioxidant from *Salvia miltiorrhiza*, prevents Abeta(25–35)-induced reduction in BPRP in PC12 cells. Biochem Biophys Res Commun 348:593–599

Lin YL, Lee TF, Huang YJ, Huang YT (2006c) Antiproliferative effect of salvianolic acid A on rat hepatic stellate cells. J Pharm Pharmacol 58:933–939

Lin YL, Wu CH, Luo MH, Huang YJ, Wang CN, Shiao MS, Huang YT (2006d) In vitro protective effects of salvianolic acid B on primary hepatocytes and hepatic stellate cells. J Ethnopharmacol 105:215–222

Ling S, Dai A, Guo Z, Komesaroff PA (2008a) A preparation of herbal medicine *Salvia miltiorrhiza* reduces expression of intercellular adhesion molecule-1 and development of atherosclerosis in apolipoprotein E-deficient mice. J Cardiovasc Pharmacol 51:38–44

Ling S, Dai A, Guo Z, Yan X, Komesaroff PA (2005) Effects of a Chinese herbal preparation on vascular cells in culture: mechanisms of cardiovascular protection. Clin Exp Pharmacol Physiol 32:571–578

Ling S, Luo R, Dai A, Guo Z, Guo R, Komesaroff PA (2009a) A pharmaceutical preparation of *Salvia miltiorrhiza* protects cardiac myocytes from tumor necrosis factor-induced apoptosis and reduces angiotensin II-stimulated collagen synthesis in fibroblasts. Phytomedicine 16:56–64

Ling S, Nheu L, Dai A, Guo Z, Komesaroff P (2008b) Effects of four medicinal herbs on human vascular endothelial cells in culture. Int J Cardiol 128:350–358

Ling Z, Xiping Z, Fengmei Q, Ping Y, Qihui C (2009b) Protective effects of *Salvia miltiorrhiza*e on multiple organs of rats with obstructive jaundice. Mediators Inflamm 2009:602935

Liu AH, Li L, Xu M, Lin YH, Guo HZ, Guo DA (2006a) Simultaneous quantification of six major phenolic acids in the roots of *Salvia miltiorrhiza* and four related traditional Chinese medicinal preparations by HPLC-DAD method. J Pharm Biomed Anal 41:48–56

Liu AH, Lin YH, Yang M, Sun JH, Guo H, Guo DA (2006b) High-performance liquid chromatographic determination of tanshinones in the roots of *Salvia miltiorrhiza* and related traditional chinese medicinal preparations. J Pharm Pharm Sci 9:1–9

Liu CH, Liu P, Hu YY, Xu LM, Tan YZ, Wang ZN, Liu C (2000) Effects of salvianolic acid-A on rat hepatic stellate cell proliferation and collagen production in culture. Acta Pharmacol Sin 21:721–726

Liu CL, Xie LX, Li M, Durairajan SS, Goto S, Huang JD (2007a) Salvianolic acid B inhibits hydrogen peroxide-induced endothelial cell apoptosis through regulating PI3K/Akt signaling. PLoS One [Electronic Resource] 2(12):e1321

Liu CS, Chen NH, Zhang JT (2007b) Protection of PC12 cells from hydrogen peroxide-induced cytotoxicity by salvianolic acid B, a new compound isolated from Radix Salviae miltiorrhizae. Phytomedicine 14:492–497

Liu CS, Cheng Y, Hu JF, Zhang W, Chen NH, Zhang JT (2006c) Comparison of antioxidant activities between salvianolic acid B and Ginkgo biloba extract (EGb 761). Acta Pharmacol Sin 27:1137–1145

Liu CS, Hu JF, Chen NH, Zhang JT (2006d) Comparison of the inhibitory activities of salvianolic acid B and Ginkgo biloba extract EGb 761 on neurotoxicity of beta-amyloid peptide (in Chinese). Acta Pharm Sin 41:706–711

Liu D, Guo L, Huang L, Jin H, Wu L, Zeng Y, Zhang J, Yang Y (2011a) Effects of soil water content on seedlings growth and active ingredients of *Salvia miltiorrhiza* (Chinese). China J Chin Materia Med 36:321–325

Liu HX, Hu Y, Liu Y, He YQ, Li W, Yang L (2009a) Hydroxylation of tanshinone IIa in human liver microsomes is specifically catalysed by cytochrome P4502A6. Xenobiotica 39:382–390

Liu JJ, Lin DJ, Liu PQ, Huang M, Li XD, Huang RW (2006e) Induction of apoptosis and inhibition of cell adhesive and invasive effects by tanshinone IIA in acute promyelocytic leukemia cells in vitro. J Biomed Sci 13:813–823

Liu JJ, Liu WD, Yang HZ, Zhang Y, Fang ZG, Liu PQ, Lin DJ, Xiao RZ, Hu Y, Wang CZ, Li XD, He Y, Huang RW (2010a) Inactivation of PI3k/Akt signaling pathway and activation of caspase-3 are involved in tanshinone I-induced apoptosis in myeloid leukemia cells in vitro. Annal Hematol 89:1089–1097

Liu JJ, Zhang Y, Lin DJ, Xiao RZ (2009b) Tanshinone IIA inhibits leukemia THP-1 cell growth by induction of apoptosis. Oncol Rep 21:1075–1081

Liu JR, Chen GF, Shih HN, Kuo PC (2008) Enhanced antioxidant bioactivity of *Salvia miltiorrhiza* (Danshen) products prepared using nanotechnology. Phytomedicine 15:23–30

Liu L, Zhang X, Wang L, Yang R, Cui L, Li M, Du W, Wang S (2010b) The neuroprotective effects of Tanshinone IIA are associated with induced nuclear translocation of TORC1 and upregulated expression of TORC1, pCREB and BDNF in the acute stage of ischemic stroke. Brain Res Bull 82:228–233

Liu M, Li YG, Zhang F, Yang L, Chou GX, Wang ZT, Hu ZB (2007c) Chromatographic fingerprinting analysis of Danshen root (*Salvia miltiorrhiza* Radix et Rhizoma) and its preparations using high performance liquid chromatography with diode array detection and electrospray mass spectrometry (HPLC-DAD-ESI/MS). J Sep Sci 30:2256–2267

Liu Q, Zhang Y, Lin Z, Shen H, Chen L, Hu L, Jiang H, Shen X (2010c) Danshen extract 15,16-dihydrotanshinone I functions as a potential modulator against metabolic syndrome through multi-target pathways. J Steroid Biochem Mol Biol 120:155–163

Liu T, Jin H, Sun QR, Xu JH, Hu HT (2010d) The neuroprotective effects of tanshinone IIA on amyloid-induced toxicity in rat cortical neurons. Neuropharmacology 59:595–604

Liu X, Yang Y, Zhang X, Xu S, He S, Huang W, Roberts MS (2010e) Compound Astragalus and *Salvia miltiorrhiza* extract inhibits cell invasion by modulating transforming growth factor-beta/Smad in HepG2 cell. J Gastroenterol Hepatol 25:420–426

Liu XD, Fan RF, Zhang Y, Yang HZ, Fang ZG, Guan WB, Lin DJ, Xiao RZ, Huang RW, Huang HQ, Liu PQ, Liu JJ (2010f) Down-regulation of telomerase activity and activation of caspase-3 are responsible for tanshinone I-induced apoptosis in monocyte leukemia cells in vitro. Int J Mol Sci 11:2267–2280

Liu Y, Wang L, Li X, Lv C, Feng D, Luo Z (2010g) Tanshinone IIA improves impaired nerve functions in experimental diabetic rats. Biochem Biophys Res Commun 399:49–54

Liu Y, Li X, Li Y, Wang L, Xue M (2010h) Simultaneous determination of danshensu, rosmarinic acid, cryptotanshinone, tanshinone IIA, tanshinone I and dihydrotanshinone I by liquid chromatographic-mass spectrometry and the application to pharmacokinetics in rats. J Pharm Biomed Anal 53:698–704

Liu YH, Mo SL, Bi HC, Hu BF, Li CG, Wang YT, Huang L, Huang M, Duan W, Liu JP, Wei MQ, Zhou SF (2011b) Regulation of human pregnane X receptor and its target gene cytochrome P450 3A4 by Chinese herbal compounds and a molecular docking study. Xenobiotica 41:259–280

Liu YL, Liu GT (2002) Inhibition of human low-density lipoprotein oxidation by salvianolic acid-A (in Chinese). Acta Pharm Sin 37:81–85

Liu YR, Qu SX, Maitz MF, Tan R, Weng J (2007d) The effect of the major components of *Salvia Miltiorrhiza* Bunge on bone marrow cells. J Ethnopharmacol 111:573–583

Lo AC, Chan K, Yeung JH, Woo KS (1992) The effects of Danshen (Salvia miltiorrhiza) on pharmacokinetics and pharmacodynamics of warfarin in rats. Eur J Drug Metab Pharmacokinet 17:257–262

Lu Q, Zhang P, Zhang X, Chen J (2009) Experimental study of the anti-cancer mechanism of tanshinone IIA against human breast cancer. Int J Mol Med 24:773–780

Lu XY, Li M, Weng MW (2007) Inhibition effects of constituents of Radix Salviae Miltiorrhizae on proliferation and procollagen transcription of dermal fibroblasts in systemic sclerosis (in Chinese). Chinese Med J 87:2426–2428

Luo P, Tan Z, Zhang Z, Li H, Mo Z (2008) Inhibitory effects of salvianolic acid B on the high glucose-induced mesangial proliferation via NF-kappaB-dependent pathway. Biol Pharm Bull 31:1381–1386

Ma HL, Qin MJ, Qi LW, Wu G, Shu P (2007) Improved quality evaluation of Radix *Salvia miltiorrhiza* through simultaneous quantification of seven major active components by high-performance liquid chromatography and principal component analysis. Biomed Chromatogr 21:931–939

Ma RW, Zou DJ, Wang QJ (2010) Effect of compound Danshen Dripping Pill on carotid arterial intima-media in patients with type 2 diabetes mellitus (in Chinese). Chin J Integ Trad West Med 30:833–837

Mao C, Zhang Y, Zhang Y, Cao L, Shao H, Wang L, Zhu L, Xu Z (2009a) The effect of tanshinone IIA on the cardiovascular system in ovine fetus in utero. Am J Chin Med 37:1031–1044

Mei Z, Situ B, Tan X, Zheng S, Zhang F, Yan P, Liu P (2010) Cryptotanshinione upregulates alpha-secretase by activation PI3K pathway in cortical neurons. Brain Res 1348:165–173

Mei Z, Zhang F, Tao L, Zheng W, Cao Y, Wang Z, Tang S, Le K, Chen S, Pi R, Liu P (2009) Cryptotanshinone, a compound from *Salvia miltiorrhiza* modulates amyloid precursor protein metabolism and attenuates beta-amyloid deposition through upregulating alpha-secretase in vivo and in vitro. Neurosci Lett 452:90–95

Meng XF, Zou XJ, Peng B, Shi J, Guan XM, Zhang C (2006) Inhibition of ethanol-induced toxicity by tanshinone IIA in PC12 cells. Acta Pharmacol Sin 27:659–664

Mosaddik MA (2003) In vitro cytotoxicity of tanshinones isolated from *Salvia miltiorrhiza* Bunge against P388 lymphocytic leukemia cells. Phytomedicine 10:682–685

Nakazawa T, Xui N, Hesong Z, Kinoshita M, Chiba T, Kaneko E, Yui K, Shimokado K (2005) Danshen inhibits oxysterol-induced endothelial cell apoptosis in vivo. J Atheroscler Thromb 12:132–137

Nizamutdinova IT, Lee GW, Lee JS, Cho MK, Son KH, Jeon SJ, Kang SS, Kim YS, Lee JH, Seo HG, Chang KC, Kim HJ (2008a) Tanshinone I suppresses growth and invasion of human breast cancer cells, MDA-MB-231, through regulation of adhesion molecules. Carcinogenesis 29:1885–1892

Nizamutdinova IT, Lee GW, Son KH, Jeon SJ, Kang SS, Kim YS, Lee JH, Seo HG, Chang KC, Kim HJ (2008b) Tanshinone I effectively induces apoptosis in estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells. Int J Oncol 33:485–491

No JK, Kim MS, Kim YJ, Bae SJ, Choi JS, Chung HY (2004) Inhibition of tyrosinase by protocatechuic aldehyde. Am J Chin Med 32:97–103

Ong ES, Len SM (2004) Evaluation of pressurized liquid extraction and pressurized hot water extraction for tanshinone I and IIA in *Salvia miltiorrhiza* using LC and LC-ESI-MS. J Chromatogr Sci 42:211–216

Pan C, Lou L, Huo Y, Singh G, Chen M, Zhang D, Wu A, Zhao M, Wang S, Li J (2011) Salvianolic acid B and Tanshinone IIA attenuate myocardial ischemia injury in mice by NO production through multiple pathways. Ther Adv Cardiovasc Dis 5:99–111

Pan TL, Hung YC, Wang PW, Chen ST, Hsu TK, Sintupisut N, Cheng CS, Lyu PC (2010) Functional proteomic and structural insights into molecular targets related to the growth inhibitory effect of tanshinone IIA on HeLa cells. Proteomics 10:914–929

Pan X, Niu G, Liu H (2001) Microwave-assisted extraction of tanshinones from *Salvia miltiorrhiza bunge* with analysis by high-performance liquid chromatography. J Chromatogr A 922:371–375

Park EJ, Zhao YZ, Kim YC, Sohn DH (2007) PF2401-SF, standardized fraction of *Salvia miltiorrhiza* and its constituents, tanshinone I, tanshinone IIA, and cryptotanshinone, protect primary cultured rat hepatocytes from bile acid-induced apoptosis by inhibiting JNK phosphorylation. Food Chem Toxicol 45:1891–1898

Park EJ, Zhao YZ, Kim YC, Sohn DH (2009) Preventive effects of a purified extract isolated from *Salvia miltiorrhiza* enriched with tanshinone I, tanshinone IIA and cryptotanshinone on hepatocyte injury in vitro and in vivo. Food Chem Toxicol 47:2742–2748

Park IJ, Kim MJ, Park OJ, Park MG, Choe W, Kang I, Kim SS, Ha J (2010) Cryptotanshinone sensitizes DU145 prostate cancer cells to Fas(APO1/CD95)-mediated apoptosis through Bcl-2 and MAPK regulation. Cancer Lett 298:88–98

Park JW, Lee SH, Yang MK, Lee JJ, Song MJ, Ryu SY, Chung HJ, Won HS, Lee CS, Kwon SH, Yun YP, Choi WS, Shin HS (2008) 15,16-dihydrotanshinone I, a major component from *Salvia miltiorrhiza* Bunge (Dansham), inhibits rabbit platelet aggregation by suppressing intracellular calcium mobilization. Arch Pharmacol Res 31:47–53

Peng GL, Zhang XY (2007) Effects of *Salvia miltiorrhiza* on serum levels of inflammatory cytokines in patients with severe acute pancreatitis (in Chinese). J Chin Integr Med 5:28–31

Qian SK, Chen D, Li Y, Yang XW, Deng WJ, Li Q, Lin H, Hu H, Xiao JQ, Chen SW (2010) Effects of E-selectin and their legends on the adhesive metastasis of hepatocellular carcinoma (in Chinese). Chung Hua Kan Tsang Ping Tsa Chih 18:440–444

Qin HY, Suo ZR, Liu WZ (2009) Dynamic changes of content of salvianolic acid in vegetative organs of *Salvia miltiorrhiza* (in Chinese). Zhong Yao Cai 32:1199–201

Qin XY, Li T, Yan L, Liu QS, Tian Y (2010) Tanshinone IIA protects against immune-mediated liver injury through activation of T-cell subsets and regulation of cytokines. Immunopharmacol Immunotoxicol 32:51–55

Qiu F, Wang G, Zhao Y, Sun H, Mao G, J A, Sun J (2008) Effect of danshen extract on pharmacokinetics of theophylline in healthy volunteers. Br J Clin Pharmacol 65:270–274

Ran R, Zhou FQ, Wang T, Kong QY (2008) Comparison of tanshinone IIA in Radix Salviae Miltorrhizae from Shandong (in Chinese). Zhong Yao Cai 31:331–333

Ren R, Wang T, Jiang N, Liu T, Du Y, Li C, Zhang L, Fu F (2010a) Protective effects of Danshensu on liver injury induced by omethoate in rats. Toxicol Mech Methods 20:510–514

Ren Y, Houghton PJ, Hider RC, Howes MJ (2004) Novel diterpenoid acetylcholinesterase inhibitors from *Salvia miltiorhiza*. Planta Med 70:201–204

Ren ZH, Tong YH, Xu W, Ma J, Chen Y (2010b) Tanshinone II A attenuates inflammatory responses of rats with myocardial infarction by reducing MCP-1 expression. Phytomedicine 17:212–218

Shan H, Li X, Pan Z, Zhang L, Cai B, Zhang Y, Xu C, Chu W, Qiao G, Li B, Lu Y, Yang B (2009) Tanshinone IIA protects against sudden cardiac death induced by lethal arrhythmias via repression of microRNA-1. Br J Pharmacol 158:1227–1235

Shen Y, Hu YL, Zhang Y, Wang JM (2010) Effects of Danshensu on maternal syndrome in phosphatidylserine/phosphatidylcholine microvesicle induced-mouse model: is it a candidate for preeclampsia remedy? Chin Med J 123:895–900

Sheng SJ, Pang EC, Xue CC, Li CG (2009) Seasonal variations in bioactive marker contents in Australian-grown *Salvia miltiorrhiza* roots. Chem Biodivers 6:551–560

Shin DS, Kim HN, Shin KD, Yoon YJ, Kim SJ, Han DC, Kwon BM (2009) Cryptotanshinone inhibits constitutive signal transducer and activator of transcription 3 function through blocking the dimerization in DU145 prostate cancer cells. Cancer Res 69:193–202

Sieveking DP, Woo KS, Fung KP, Lundman P, Nakhla S, Celermajer DS (2005) Chinese herbs Danshen and Gegen modulate key early atherogenic events in vitro. Int J Cardiol 105:40–45

Skala E, Wysokinska H (2005) Tanshinone production in roots of micropropagated Salvia przewalskii Maxim. J Biosci 60:583–586, Zeitschrift fur Naturforschung. Section C

Song Y, Yuan SL, Yang YM, Wang XJ, Huang GQ (2005) Alteration of activities of telomerase in tanshinone IIA inducing apoptosis of the leukemia cells (in Chinese). China J Chin Materia Med 30:207–211

Song YH, Liu Q, Lv ZP, Chen YY, Zhou YC, Sun XG (2008) Protection of a polysaccharide from *Salvia miltiorrhiza*, a Chinese medicinal herb, against immunological liver injury in mice. Int J Biol Macromol 43:170–175

Song Z, Li X, Wang H, Wang J (2010) Genetic diversity and population structure of *Salvia miltiorrhiza* Bage in China revealed by ISSR and SRAP. Genetica 138:241–249

Su CC, Chen GW, Kang JC, Chan MH (2008a) Growth inhibition and apoptosis induction by tanshinone IIA in human colon adenocarcinoma cells. Planta Med 74:1357–1362

Su CC, Chen GW, Lin JG (2008b) Growth inhibition and apoptosis induction by tanshinone I in human colon cancer Colo 205 cells. Int J Mol Med 22:613–618

Su CC, Lin YH (2008a) Tanshinone IIA inhibits human breast cancer cells through increased Bax to Bcl-xL ratios. Int J Mol Med 22:357–61

Su CC, Lin YH (2008b) Tanshinone IIA down-regulates the protein expression of ErbB-2 and up-regulates TNF-alpha in colon cancer cells in vitro and in vivo. Int J Mol Med 22:847–851

Sun DD, Wang HC, Wang XB, Luo Y, Jin ZX, Li ZC, Li GR, Dong MQ (2008) Tanshinone IIA: a new activator of human cardiac KCNQ1/KCNE1 (I(Ks)) potassium channels. Eur J Pharmacol 590:317–321

Sun M, Zhang JJ, Shan JZ, Zhang H, Jin CY, Xu S, Wang YL (2009a) Clinical observation of Danhong Injection (herbal TCM product from Radix Salviae miltiorrhizae and Flos Carthami tinctorii) in the treatment of traumatic intracranial hematoma. Phytomedicine 16:683–689

Sun L, Zheng Z (2007) Effect of *salvia miltiorrhiza* Bge on left ventricular hypertrophy and the expression of tumor necrosis factor-alpha in spontaneously hypertensive rats. J Huazhong Univ Sci Technol Med Sci 27:245–247

Sun Y, Zhu H, Wang J, Liu Z, Bi J (2009b) Isolation and purification of salvianolic acid A and salvianolic acid B from *Salvia miltiorrhiza* by high-speed counter-current chromatography and comparison of their antioxidant activity. J Chromatogr B Analyt Technol Biomed Life Sci 877:733–737

Sun ZW, Zhang L, Zhu SJ, Chen WC, Mei B (2010) Excitotoxicity effects of glutamate on human neuroblastoma SH-SY5Y cells via oxidative damage. Neurosci Bull 26:8–16

Sun A, Zhang Y, Li A, Meng Z, Liu R (2011) Extraction and preparative purification of tanshinones from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 879:1899–1904

Tam WY, Chook P, Qiao M, Chan LT, Chan TY, Poon YK, Fung KP, Leung PC, Woo KS (2009) The efficacy and tolerability of adjunctive alternative herbal medicine (*Salvia miltiorrhiza* and *Pueraria lobata*) on vascular function and structure in coronary patients. J Alt Comp Med 15:415–421

Tang C, Wu AH, Xue HL, Wang YJ (2007a) Tanshinone IIA inhibits endothelin-1 production in TNF-alpha-induced brain microvascular endothelial cells through suppression of endothelin-converting enzyme-1 synthesis. Acta Pharmacol Sin 28:1116–1122

Tang C, Xue HL, Huang HB, Wang XG (2010) Tanshinone IIA inhibits constitutive STAT3 activation, suppresses proliferation, and induces apoptosis in rat C6 glioma cells. Neurosci Lett 470:126–129

Tang J, Zhan C, Zhou J (2008) Effects of tanshinone IIA on transforming growth factor beta1-Smads signal pathway in renal interstitial fibroblasts of rats. J Huazhong Univ Sci Technol Med Sci 28:539–542

Tang XQ, Wang KC, Chen X, Yu BY (2007b) Analysis on biomasses and contents of active components in roots of *Salvia miltiorrhiza* from different populations (in Chinese). China J Chin Materia Med 32:2485–2488

Tang ZZ, Tang Y, Fu LB (2003) Effect of tanshinone IIA on the growth behavior of human hepatoma cell line BEL-7402 in vitro and its mechanism (in Chinese). Di Yi Junyi Daxue Xuebao 23:595–597

Tian G, Zhang T, Zhang Y, Ito Y (2002) Separation of tanshinones from *Salvia miltiorrhiza bunge* by multidimensional counter-current chromatography. J Chromatogr A 945:281–285

Tian LL, Wang XJ, Sun YN, Li CR, Xing YL, Zhao HB, Duan M, Zhou Z, Wang SQ (2008) Salvianolic acid B, an antioxidant from *Salvia miltiorrhiza*, prevents 6-hydroxydopamine induced apoptosis in SH-SY5Y cells. Int J Biochem Cell Biol 40:409–422

Tian M, Yan H, Row KH (2009) Solid-phase extraction of tanshinones from *Salvia Miltiorrhiza* Bunge using ionic liquid-modified silica sorbents. J Chromatogr B Analyt Technol Biomed Life Sci 877:738–742

Tian M, Bi W, Row KH (2011) Molecular imprinting in ionic liquid-modified porous polymer for recognitive separation of three tanshinones from *Salvia miltiorrhiza* Bunge. Anal Bioanal Chem 399:2495–2502

Tsai SL, Suk FM, Wang CI, Liu DZ, Hou WC, Lin PJ, Hung LF, Liang YC (2007) Anti-tumor potential of 15,16-dihydrotanshinone I against breast adenocarcinoma through inducing G1 arrest and apoptosis. Biochem Pharmacol 74:1575–1586

Ueng YF, Kuo YH, Peng HC, Chen TL, Jan WC, Guengerich PF, Lin YL (2003) Diterpene quinone tanshinone IIA selectively inhibits mouse and human cytochrome p4501A2. Xenobiotica 33:603–613

Ueng YF, Kuo YH, Wang SY, Lin YL, Chen CF (2004) Induction of CYP1A by a diterpene quinone tanshinone IIA isolated from a medicinal herb *Salvia miltiorrhiza* in C57BL/6J but not in DBA/2J mice. Life Sci 74:885–896

Wagner H (2012) New analytical monographs of TCM-drugs. In: Wagner H, Ulrich Merzenich G (eds) Evidence and rational based research on Chinese drugs, Chapter 1. Springer Publication Comp, Wien

Wagner H, Bauer R, Melchart D, Xiao PG, Staudinger A (2011) Radix et Rhizoma Salviae miltiorrhizae (Danshen). In: Wagner H, Bauer R, Melchart D, Xiao P-G, Staudinger A (eds) Chromatographic fingerprint analysis of herbal medicines – thin-layer and high performance liquid chromatography of Chinese drugs, vol 2, Monograph 74, 2nd edn. Springer, Wien

Wagner H, Bauer, R, Xiao PG, Marotel D, Bacher S (2010) Radix et Rhizoma Salviae miltiorrhizae – Danshen. In: Chinese drug monographs and analysis, vol 10(74). Verlag fur Ganzheitliche Madizin, pp 1–16

Wan JM, Sit WH, Lee CL, Fu KH, Chan DK (2006) Protection of lethal toxicity of endotoxin by *Salvia miltiorrhiza* BUNGE is via reduction in tumor necrosis factor alpha release and liver injury. Int Immunopharmacol 6:750–758

Wang H, Chen XP, Qiu FZ (2003) Salviae miltiorrhizae ameliorates cirrhosis and portal hypertension by inhibiting nitric oxide in cirrhotic rats. Hepatobiliary Pancreat Dis Int 2:391–396

Wang CY, Ma FL, Liu JT, Tian JW, Fu FH (2007a) Protective effect of salvianic acid a on acute liver injury induced by carbon tetrachloride in rats. Biol Pharm Bull 30:44–47

Wang G, Sun B, Zhu H, Gao Y, Li X, Xue D, Jiang H (2010a) Protective effects of emodin combined with danshensu on experimental severe acute pancreatitis. Inflamm Res 59:479–488

Wang J, Dong MQ, Liu ML, Xu DQ, Luo Y, Zhang B, Liu LL, Xu M, Zhao PT, Gao YQ, Li ZC (2010b) Tanshinone IIA modulates pulmonary vascular response to agonist and hypoxia primarily via inhibiting Ca2+ influx and release in normal and hypoxic pulmonary hypertension rats. Eur J Pharmacol 640:129–138

Wang J, Wang X, Jiang S, Yuan S, Lin P, Zhang J, Lu Y, Wang Q, Xiong Z, Wu Y, Ren J, Yang H (2007b) Growth inhibition and induction of apoptosis and differentiation of tanshinone IIA in human glioma cells. J Neuro-Oncol 82:11–21

Wang JM, Yao XX, Li XT, Yang SL, Su Z (2005a) Effects of Radix Salviae Miltiorrhizae on Ca2+ in hepatic stellate cells (in Chinese). J Sichuan Univ Med Sci 36:221–224

Wang L, Bao Y, Yang Y, Wu Y, Chen X, Si S, Hong B (2010c) Discovery of antagonists for human scavenger receptor CD36 via an ELISA-like high-throughput screening assay. J Biomol Screen 15:239–250

Wang L, Song Y, Cheng Y, Liu X (2008a) Orthogonal array design for the optimization of supercritical fluid extraction of tanshinones from Danshen. J Sep Sci 31:321–328

Wang QL, Tao YY, Yuan JL, Shen L, Liu CH (2010d) Salvianolic acid B prevents epithelial-to-mesenchymal transition through the TGF-beta1 signal transduction pathway in vivo and in vitro. BMC Cell Biol 11:31

Wang X, Cheung CM, Lee WY, Or PM, Yeung JH (2010e) Major tanshinones of Danshen (*Salvia miltiorrhiza*) exhibit different modes of inhibition on human CYP1A2, CYP2C9, CYP2E1 and CYP3A4 activities in vitro. Phytomedicine 17:868–875

Wang X, Geng Y, Li F, Liu J (2007c) Large-scale separation of salvianolic acid B from the Chinese medicinal plant *Salvia miltiorrhiza* by pH-zone-refining countercurrent chromatography. J Sep Sci 30:3214–3217

Wang X, Lee WY, Or PM, Yeung JH (2009) Effects of major tanshinones isolated from Danshen (*Salvia miltiorrhiza*) on rat CYP1A2 expression and metabolism of model CYP1A2 probe substrates. Phytomedicine 16:712–725

Wang X, Lee WY, Or PM, Yeung JH (2010f) Pharmacokinetic interaction studies of tanshinones with tolbutamide, a model CYP2C11 probe substrate, using liver microsomes, primary hepatocytes and in vivo in the rat. Phytomedicine 17:203–211

Wang X, Lee WY, Zhou X, Or PM, Yeung JH (2010g) A pharmacodynamic-pharmacokinetic (PD-PK) study on the effects of Danshen (*Salvia miltiorrhiza*) on midazolam, a model CYP3A probe substrate, in the rat. Phytomedicine 17:876–883

Wang X, Wei Y, Yuan S, Liu G, Lu Y, Zhang J, Wang W (2005b) Potential anticancer activity of tanshinone IIA against human breast cancer. Int J Cancer 116:799–807

Wang X, Yeung JH (2010) Effects of the aqueous extract from *Salvia miltiorrhiza* Bunge on caffeine pharmacokinetics and liver microsomal CYP1A2 activity in humans and rats. J Pharm Pharmacol 62:1077–1083

Wang X, Yeung JH (2011a) Inhibitory effect of tanshinones on rat CYP3A2 and CYP2C11 activity and its structure-activity relationship. Fitoterapia 82:539–545

Wang X, Yeung JH (2011b) Effects of *Salvia miltiorrhiza* extract on the liver CYP3A activity in humans and rats. Phytother Res 25:1653–1659

Wang X, Yeung JH (2011c) Investigation of cytochrome P450 1A2 and 3A inhibitory properties of Danshen tincture. Phytomedicine. doi:10.1016/j.phymed.2011.09.075

Wang XJ, Xu JX (2005) Salvianic acid A protects human neuroblastoma SH-SY5Y cells against MPP+−induced cytotoxicity. Neurosci Res 51:129–138

Wang Y, Zhou J, Jiang XQ (2008b) Effects of Radix salviae miltiorrhizae on reperfusion injury and hepatocyte apoptosis in donor liver after orthotopic liver transplantation in rats (in Chinese). J Southern Med Univ 28:870–873

Watzke A, O'Malley SJ, Bergman RG, Ellman JA (2006) Reassignment of the configuration of salvianolic acid B and establishment of its identity with lithospermic acid B. J Nat Prod 69:1231–1233

Wong KK, Ho MT, Lin HQ, Lau KF, Rudd JA, Chung RC, Fung KP, Shaw PC, Wan DC (2010a) Cryptotanshinone, an acetylcholinesterase inhibitor from *Salvia miltiorrhiza*, ameliorates scopolamine-induced amnesia in Morris water maze task. Planta Med 76:228–234

Wong KK, Ngo JC, Liu S, Lin HQ, Hu C, Shaw PC, Wan DC (2010b) Interaction study of two diterpenes, cryptotanshinone and dihydrotanshinone, to human acetylcholinesterase and butyrylcholinesterase by molecular docking and kinetic analysis. Chem Biol Interact 187:335–339

Wong RW, Rabie AB (2008) Effect of *Salvia miltiorrhiza* extract on bone formation. J Biomed Mater Res A 85:506–512

Wu BW, Pan TL, Leu YL, Chang YK, Tai PJ, Lin KH, Horng JT (2007a) Antiviral effects of *Salvia miltiorrhiza* (Danshen) against enterovirus 71. Am J Chin Med 35:153–168

Wu D, Jiang X, Wu S (2010) Direct purification of tanshinones from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography without presaturation of the two-phase solvent mixture. J Sep Sci 33:67–73

Wu K, Zhang Q, Liu Q, Tang F, Long Y, Yao S (2009a) Ionic liquid surfactant-mediated ultrasonic-assisted extraction coupled to HPLC: application to analysis of tanshinones in *Salvia miltiorrhiza bunge*. J Sep Sci 32:4220–4226

Wu JL, Yee LP, Jiang ZH, Cai Z (2007b) One single LC-MS/MS analysis for both phenolic components and tanshinones in Radix Salviae Miltiorrihizae and its medicinal products. Talanta 73:656–661

Wu L, Li X, Li Y, Wang L, Tang Y, Xue M (2009b) Proliferative inhibition of danxiongfang and its active ingredients on rat vascular smooth muscle cell and protective effect on the VSMC damage induced by hydrogen peroxide. J Ethnopharmacol 126:197–206

Wu L, Qiao H, Li Y, Li L (2007c) Protective roles of puerarin and Danshensu on acute ischemic myocardial injury in rats. Phytomedicine 14:652–658

Wu T, Ni J, Wu J (2008a) Danshen (Chinese medicinal herb) preparations for acute myocardial infarction. Cochrane Database Syst Rev 2:CD004465

Wu WW, Yeung JH (2010) Inhibition of warfarin hydroxylation by major tanshinones of Danshen (*Salvia miltiorrhiza*) in the rat in vitro and in vivo. Phytomedicine 17:219–226

Wu YP, Zhao XM, Pan SD, de Guo A, Wei R, Han JJ, Kainoh M, Xia ZL, de Groot PG, Lisman T (2008b) Salvianolic acid B inhibits platelet adhesion under conditions of flow by a mechanism involving the collagen receptor alpha2beta1. Thromb Res 123:298–305

Wu ZM, Wen T, Tan YF, Liu Y, Ren F, Wu H (2007d) Effects of salvianolic acid a on oxidative stress and liver injury induced by carbon tetrachloride in rats. Basic Clin Pharmacol Toxicol 100:115–120

Xia WJ, Yang M, Fok TF, Li K, Chan WY, Ng PC, Ng HK, Chik KW, Wang CC, Gu GJ, Woo KS, Fung KP (2005) Partial neuroprotective effect of pretreatment with tanshinone IIA on neonatal hypoxia-ischemia brain damage. Pediatr Res 58:784–790

Xing HC, Li LJ, Xu KJ, Shen T, Chen YB, Chen Y, Fu SZ, Sheng JF, Chen CL, Wang JG, Yan D, Dai FW, Sha XY (2005) Effects of *Salvia miltiorrhiza* on intestinal microflora in rats with ischemia/reperfusion liver injury. Hepatobil Pancreat Dis Int 4:274–280

Xiong ZB, Wu P, Huang YF (2005) Protective mechanisms of radix salviae miltiorrhizae against chronic alcoholic liver injury in mice (in Chinese). Chinese J Integ Trad West Med 25:425–428

Xu JZ, Shen J, Cheng YY, Qu HB (2008a) Simultaneous detection of seven phenolic acids in Danshen injection using HPLC with ultraviolet detector. J Zhejiang Univ Sci B 9:728–733

Xu G, Zhao W, Zhou Z, Zhang R, Zhu W, Liu X (2009a) Danshen extracts decrease blood C reactive protein and prevent ischemic stroke recurrence: a controlled pilot study. Phytother Res 23:1721–1725

Xu W, Yang J, Wu LM (2009b) Cardioprotective effects of tanshinone IIA on myocardial ischemia injury in rats. Pharmazie 64:332–336

Xu Y, Feng D, Wang Y, Lin S, Xu L (2008b) Sodium tanshinone IIA sulfonate protects mice from ConA-induced hepatitis via inhibiting NF-kappaB and IFN-gamma/STAT1 pathways. J Clin Immunol 28:512–519

Yan FF, Liu YF, Liu Y, Zhao YX (2009) Sulfotanshinone Sodium Injection could decrease fibrinogen level and improve clinical outcomes in patients with unstable angina pectoris. Int J Cardiol 135:254–255

Yan Q, Hu ZD, Wu JY (2006) Synergistic effects of biotic and abiotic elicitors on the production of tanshinones in *Salvia miltiorrhiza* hairy root culture (in Chinese). China J Chin Materia Med 31:188–191

Yang GD, Zhang H, Lin R, Wang WR, Shi XL, Liu Y, Ji QL (2009) Down-regulation of CD40 gene expression and inhibition of apoptosis with Danshensu in endothelial cells. Basic Clin Pharmacol Toxicol 104:87–92

Yang H, Deng M, Yi H (2008a) Experiment of extracting Salviae miltiorrhizae on using O/W microemulsion (in Chinese). China J Chin Materia Med 33:2617–2622

Yang M, Liu A, Guan S, Sun J, Xu M, Guo D (2006) Characterization of tanshinones in the roots of *Salvia miltiorrhiza* (Dan-shen) by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 20:1266–1280

Yang Q, Zhang XL, Li XY, Tang WK, Zhang JX, Fang CX, Zheng CY (2007) Coupling continuous ultrasound-assisted extraction with ultrasonic probe, solid-phase extraction and high-performance liquid chromatography for the determination of sodium Danshensu and four tanshinones in *Salvia miltiorrhiza bunge*. Anal Chim Acta 589:231–238

Yang R, Liu A, Ma X, Li L, Su D, Liu J (2008b) Sodium tanshinone IIA sulfonate protects cardiomyocytes against oxidative stress-mediated apoptosis through inhibiting JNK activation. J Cardiovasc Pharmacol 51:396–401

Yang TL, Lin FY, Chen YH, Chiu JJ, Shiao MS, Tsai CS, Lin SJ, Chen YL (2011a) Salvianolic acid B inhibits low-density lipoprotein oxidation and neointimal hyperplasia in endothelium-denuded hypercholesterolaemic rabbits. J Sci Food Agric 91:134–141

Yang W, Ju JH, Jeon MJ, Han X, Shin I (2010) Danshen (*Salvia miltiorrhiza*) extract inhibits proliferation of breast cancer cells via modulation of Akt activity and p27 level. Phytother Res 24:198–204

Yang XY, Sun L, Xu P, Gong LL, Qiang GF, Zhang L, Du GH (2011b) Effects of salvianolic scid A on plantar microcirculation and peripheral nerve function in diabetic rats. Eur J Pharmacol 665:40–46

Yang Y, Cai F, Li PY, Li ML, Chen J, Chen GL, Liu ZF, Zeng XR (2008c) Activation of high conductance Ca(2+)-activated K(+) channels by sodium tanshinoneII-A sulfonate (DS-201) in porcine coronary artery smooth muscle cells. Eur J Pharmacol 598:9–15

Ye F, Liu Y, Qiu G, Zhao Y, Liu M (2005) Clinical study on treatment of cirrhosis by different dosages of salvia injection (in Chinese). Zhong Yao Cai 28:850–854
Ye Y, Xu W, Zhong W (2010) Effects of cryptotanshinone on proliferation and apoptosis of Hela cell line of cervical cancer (in Chinese). China J Chin Materia Med 35:118–121
Yin HQ, Choi YJ, Kim YC, Sohn DH, Ryu SY, Lee BH (2009) *Salvia miltiorrhiza* Bunge and its active component cryptotanshinone protects primary cultured rat hepatocytes from acute ethanol-induced cytotoxicity and fatty infiltration. Food Chem Toxicol 47:98–103
Yin HQ, Kim YS, Choi YJ, Kim YC, Sohn DH, Ryu SY, Lee BH (2008) Effects of tanshinone IIA on the hepatotoxicity and gene expression involved in alcoholic liver disease. Arch Pharm Res 31:659–665
You JS, Pan TL, Lee YS (2007) Protective effects of Danshen (*Salvia miltiorrhiza*) on adriamycin-induced cardiac and hepatic toxicity in rats. Phytother Res 21:1146–1152
Yu BB, Dai LL, Li X, Li D, Jiang ZH (2009a) Effect of danshensu on activation of JNK pathway in hepatic stellate cells (HSCs) induced by IL-1 beta (in Chinese). Chung Hua Kan Tsang Ping Tsa Chih 17:451–454
Yu C, Ye S, Sun H, Liu Y, Gao L, Shen C, Chen S, Zeng S (2009b) PXR-mediated transcriptional activation of CYP3A4 by cryptotanshinone and tanshinone IIA. Chem Biol Interact 177:58–64
Yu CM, Chan JC, Sanderson JE (1997) Chinese herbs and warfarin potentiation by 'danshen'. J Intern Med 241:337–339
Yu S, Zhong B, Zheng M, Xiao F, Dong Z, Zhang H (2009c) The quality of randomized controlled trials on DanShen in the treatment of ischemic vascular disease. J Alt Complem Med 15:557–565
Yuan D, Pan YN, Fu WW, Makino T, Kano Y (2005) Quantitative analysis of the marker compounds in *Salvia miltiorrihiza* root and its phytomedicinal preparations. Chem Pharm Bull 53:508–514
Yuan Y, Liu Y, Lu D, Huang L, Liang R, Yang Z, Chen S (2009) Genetic stability, active constituent, and pharmacoactivity of *Salvia miltiorrhiza* hairy roots and wild plant. J Biosci 64:557–563, Zeitschrift fur Naturforschung. Section C
Yuxian X, Feng T, Ren L, Zhengcai L (2009) Tanshinone II-A inhibits invasion and metastasis of human hepatocellular carcinoma cells in vitro and in vivo. Tumori 95:789–795
Zhang C, Yan Q, Cheuk WK, Wu J (2004a) Enhancement of tanshinone production in *Salvia miltiorrhiza* hairy root culture by Ag+ elicitation and nutrient feeding. Planta Med 70:147–151
Zhang F, Zheng W, Pi R, Mei Z, Bao Y, Gao J, Tang W, Chen S, Liu P (2009a) Cryptotanshinone protects primary rat cortical neurons from glutamate-induced neurotoxicity via the activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. Exp Brain Res 193:109–118
Zhang H, Xu Y, Wang J, Zhang K, Yi B, Liu Y, Cai X (2011a) Effect of Danshensu on fibronectin and collagen-1 secretion induced by high glucose in human peritoneal mesothelial cells (in Chinese). J Central South Univ Med Sci 36:44–50
Zhang H, Yu C, Jia JY, Leung SW, Siow YL, Man RY, ZHU DY (2002) Contents of four active components in different commercial crude drugs and preparations of danshen (*salvia miltiorrhiza*). Acta Pharm Sin 23:1163–1168
Zhang HS, Wang SQ (2006) Salvianolic acid B from *Salvia miltiorrhiza* inhibits tumor necrosis factor-alpha (TNF-alpha)-induced MMP-2 upregulation in human aortic smooth muscle cells via suppression of NAD(P)H oxidase-derived reactive oxygen species. J Mol Cell Cardiol 41:138–148
Zhang HS, Wang SQ (2007) Nrf2 is involved in the effect of tanshinone IIA on intracellular redox status in human aortic smooth muscle cells. Biochem Pharmacol 73:1358–66
Zhang JL, Cui M, He Y, Yu HL, Guo DA (2005a) Chemical fingerprint and metabolic fingerprint analysis of Danshen injection by HPLC-UV and HPLC-MS methods. J Pharm Biomed Anal 36:1029–1035
Zhang LJ, Chen L, Lu Y, Wu JM, Xu B, Sun ZG, Zheng SZ, Wang AY (2010a) Danshensu has anti-tumor activity in B16F10 melanoma by inhibiting angiogenesis and tumor cell invasion. Eur J Pharmacol 643:195–201

Zhang M, Li X, Qin G, Liu Y, Zhao X (2005b) Effects of Danshen injection on the reserve of tissue plasminogen activator and nitric oxide in endothelium and vasodilatation in diabetic patients (in Chinese). Zhong Yao Cai 28:529–532

Zhang N, Zou H, Jin L, Wang J, Zhong MF, Huang P, Gu BQ, Mao SL, Zhang C, Chen H (2010b) Biphasic effects of sodium danshensu on vessel function in isolated rat aorta. Acta Pharm Sin 31:421–428

Zhang R, Sun J, Ma L, Wu X, Pan G, Hao H, Zhou FAJ, Liu C, Ai H, Shang L, Gao H, Peng Y, Wan P, Wu H, Wang G (2011b) Induction of cytochromes P450 1A1 and 1A2 by tanshinones in human HepG2 hepatoma cell line. Toxicol App Pharmacol 252:18–27

Zhang SH, Su J, Zhen YS (2004b) Salvianolic acid A inhibits nucleoside transport and potentiates the antitumor activity of chemotherapeutic drugs (in Chinese). Acta Pharm Sin 39:496–499

Zhang W, Lu Y (2010) Advances in studies on antitumor activities of compounds in *Salvia miltiorrhiza* (in Chinese). China J Chin Materia Med 35:389–392

Zhang WJ, Feng J, Zhou R, Ye LY, Liu HL, Peng L, Lou JN, Li CH (2010c) Tanshinone IIA protects the human blood–brain barrier model from leukocyte-associated hypoxia-reoxygenation injury. Eur J Pharmacol 648:146–152

Zhang X, Chen L, Zhang J, Tian H, Zhang X, Zhou Y, Wang Z, Wang K (2009b) Effect of *salvia miltiorrhiza*e on apoptosis and NF-kappaB p65 expression in the liver of rats with severe acute pancreatitis or obstructive jaundice. J Gastroenterol Hepatol 24:841–852

Zhang X, Liu D, Wu D, Zhu C, Ye J, Wang K, Peng L, Zhuo G (2009c) Effect of *salvia miltiorrhiza*e on the expressions of TLR4 protein in the liver of rats with SAP or OJ. Inflammation 32:151–162

Zhang XP, Li ZJ, Liu DR (2006) Progress in research into the mechanism of Radix salviae miltiorrhizae in treatment of acute pancreatitis. Hepatobil Pancreat Dis Int 5:501–504

Zhang Y, Can R, Mao C, Zhang Y, Zhu L, Shao H, Wang L, Wang A, Xu Z (2009d) The effect of tanshinone IIA on renal and liver functions in ovine fetuses in utero. Drug Chem Toxicol 32:362–371

Zhang Y, Jiang YF, Liu ZQ, Ren LW, Wang Q, Wang WR, Wang XM, Huang QF (2007) Effect of salvianolic acid B on brain energy metabolism and hydrocephalus of cerebral ischemia in mice at different time (in Chinese). Acta Pharm Sin 42:1250–1253

Zhang Y, Li X, Wang Z (2010d) Antioxidant activities of leaf extract of *Salvia miltiorrhiza* Bunge and related phenolic constituents. Food Chem Toxicol 48:2656–2662

Zhang Y, Wei L, Sun D, Cao F, Gao H, Zhao L, Du J, Li Y, Wang H (2010e) Tanshinone IIA pretreatment protects myocardium against ischaemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway in diabetic rats. Diabetes Obes Metab 12:316–322

Zhang Z, Gao J, Wang Y, Song T, Zhang J, Wu G, Zhang T, Du G (2009e) Tanshinone IIA triggers p53 responses and apoptosis by RNA polymerase II upon DNA minor groove binding. Biochem Pharmacol 78:1316–1322

Zhang Z, Zhang J, Jin L, Song T, Wu G, Gao J (2008) Tanshinone IIA interacts with DNA by minor groove-binding. Biol Pharm Bull 31:2342–2345

Zhao GR, Zhang HM, Ye TX, Xiang ZJ, Yuan YJ, Guo ZX, Zhao LB (2008a) Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B. Food Chem Toxicol 46:73–81

Zhao L, Gaudry L, Dunkley S, Brighton T, Guo ZX, Ye ZL, Luo RZ, Chesterman CN (2008b) Modulation of platelet and leucocyte function by a Chinese herbal formulation as compared with conventional antiplatelet agents. Platelets 19:24–31

Zhao LL, Xie K, Zhu SS, Jiang HY, Huo WZ (2007) Quality analysis of Radix Salviae Miltiorrhizae from different habitats by HPLC and TLC (in Chinese). Zhong Yao Cai 30:646–648

Zheng FL, Chang Y, Jia XY, Wei W (2009) Effects of cryptotanshinone on immune functions in rats with adjuvant arthritis. Chinese Med J 122:3039–3042

Zhi W, Deng Q (2006) Purification of salvianolic acid B from the crude extract of *Salvia miltiorrhiza* with hydrophilic organic/salt-containing aqueous two-phase system by counter-current chromatography. J Chromatogr A 1116:149–152

Zhong GX, Li P, Zeng LJ, Guan J, Li DQ, Li SP (2009) Chemical characteristics of *Salvia miltiorrhiza* (Danshen) collected from different locations in China. J Agric Food Chem 57:6879–6887

Zhong J, Tang MK, Zhang Y, Xu QP, Zhang JT (2007a) Effect of salvianolic acid B on neural cells damage and neurogenesis after brain ischemia-reperfusion in rats (in Chinese). Acta Pharm Sin 42:716–721

Zhong ZH, Chen WG, Liu YH, Li QX, Qiu Y (2007b) Inhibition of cell growth and induction of apoptosis in human hepatoma cell line HepG2 by tanshione IIA (in Chinese). J Central South Univ Med Sci 32:99–103

Zhou L, Chan WK, Xu N, Xiao K, Luo H, Luo KQ, Chang DC (2008) Tanshinone IIA, an isolated compound from *Salvia miltiorrhiza* Bunge, induces apoptosis in HeLa cells through mitotic arrest. Life Sci 83:394–403

Zhou LN, Zhang X, Xu WZ, Ma XN, Jia Z, Zheng YM, You S (2011) Studies on the stability of salvianolic acid B as potential drug material. Phytochem Anal 22:378–384

Zhou L, Chow M, Zuo Z (2006a) Improved quality control method for Danshen products–consideration of both hydrophilic and lipophilic active components. J Pharm Biomed Anal 41:744–750

Zhou RH (1993) Resource science of Chinese medicinal materials. Medical and Pharmaceutical Science Press, Beijing, China

Zhou Z, Liu Y, Miao AD, Wang SQ (2005) Salvianolic acid B attenuates plasminogen activator inhibitor type 1 production in TNF-alpha treated human umbilical vein endothelial cells. J Cell Biochem 96:109–116

Zhou Z, Wang SQ, Liu Y, Miao AD (2006b) Cryptotanshinone inhibits endothelin-1 expression and stimulates nitric oxide production in human vascular endothelial cells. Biochim Biophys Acta 1760:1–9

Zhou Z, Zhang Y, Ding XR, Chen SH, Yang J, Wang XJ, Jia GL, Chen HS, Bo XC, Wang SQ (2007) Protocatechuic aldehyde inhibits hepatitis B virus replication both in vitro and in vivo. Antiviral Res 74:59–64

Zhou ZT, Yang Y, Ge JP (2006c) The preventive effect of salvianolic acid B on malignant transformation of DMBA-induced oral premalignant lesion in hamsters. Carcinogenesis 27:826–832

Zhu B, Zhai Q, Yu B (2010a) Tanshinone IIA protects rat primary hepatocytes against carbon tetrachloride toxicity via inhibiting mitochondria permeability transition. Pharm Biol 48:484–487

Zhu S, Qian Y, Shi L, Yang W, Feng X, Li C, Liu Y (2010b) Effect of tanshinone IIA on the change of calcium current induced by beta-amyloid protein 25–35 in neurons of nucleus basalis of Meynert (in Chinese). J Central South Univ Med Sci 35:840–846

Zou ZW, Xu LN, Tian JY (1993) Antithrombotic and antiplatelet effects of rosmarinic acid, a water-soluble component isolated from radix Salviae miltiorrhizae (danshen) (in Chinese). Acta Pharm Sin 28:241–245

Chapter 7
Inhibition of ATP-Binding Cassette Transporters by Chinese Herbs and Phytochemicals

Thomas Efferth

7.1 Introduction

Traditional Chinese medicine looks back on a millennia-old history and the pool of Chinese herbal drugs is the largest worldwide. For this reason, traditional Chinese medicine is a treasure box for the identification of novel bioactive phytochemicals. It comes as no surprise that pharmaceutical companies in the western world have recognized the considerable potential for the development of new drugs derived from Chinese plants. Drugs such as the camptothecin derivatives, topothecan, and irinothecan, for cancer therapy, or the anti-malarial artemisinin-derivatives, artesunate, and artemether are success stories providing evidence for the attractiveness of drug development based on phytochemicals derived from Chinese herbs. However, drug development frequently takes one or two decades to bring a promising candidate compound to the market. Therefore, the concept of target-based therapy has been developed with the hope that rational drug design based on modern molecular biological and bioinformatical techniques would speed up the drug developmental process.

One of the target proteins, which have been discussed in this context is the ATP-binding cassette transporter, P-glycoprotein. The members of the ABC transporter gene family are evolutionary old and highly conserved genes. They are ubiquitously found from bacteria (Nikaido 1994; Goffeau et al. 1997; Gottesman and Pastan 1993; Allen et al. 1995). The human ABC transporter gene family comprises 49 members, which belong to seven subfamilies (ABCA–ABCG).

These transporters are transmembrane proteins with certain structural features in common (Michaelis and Berkower 1995; Cole and Deeley 1998) and translocate a wide array of compounds, including organic and inorganic ions, peptides and

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proteins, heavy metals, steroids, antibiotic and anticancer drugs, etc. (Saurin et al. 1999; Dean and Allikmets 1995). ABC transporters have been well investigated to mediate multidrug resistance in cancer and also in bacteria.

A strategy to modulate multidrug resistance and to improve cancer therapy and antibacterial therapy is to inhibit ABC transporters and to prevent the extrusion of chemotherapeutic drugs out of cells. Increasing lethal concentrations of chemotherapeutics by ABC transporter inhibitors may increase therapeutic success. Another option is that inhibition of ABC transporters may lower the concentrations required to kill cancer cells and, thereby, to decrease the severe side effects of many current chemotherapy regimens in oncology. Considering the fact that ABC transporters are not only expressed in tumors but also in several normal organs such as liver, kidney, gastrointestinal tract, blood brain barrier, blood placenta barrier, etc., ABC transporters have a tremendous relevance for bioavailability and pharmacokinetics of many drugs used for diverse diseases. Dose reduction of those drugs by co-application of inhibitors of ABC transporters might be an option to lower the costs in health system.

This chapter focuses on the inhibition of ABC transporters by Chinese herbs and phytochemicals derived thereof. The good tolerability of many compounds derived from medicinal herbs may be an attractive feature fostering the development of phytochemical-based inhibitors of ABC transporters in cancer therapy as well as in general pharmacology.

7.2 The Biology of ATP-Binding Cassette Transporters

7.2.1 ABCA Subfamily

Amplification and overexpression of the *ABCA2* gene contribute to estramustine resistance (Vulevic et al. 2001; Laing et al. 1998). Furthermore, mitoxantrone resistance was also associated with overexpression of the *ABCA2* gene (Boonstra et al. 2004).

The ABCA2 protein is localized in lysosomes and facilitates the translocation cytotoxic compounds from the cytoplasm to the lysosomal compartment for detoxification. The ABCA3 protein is located at intracellular membranes (Yamano et al. 2001). It does not confer a "classical" outer cell membrane-associated drug efflux. Rather it is involved in intracellular sequestration and vesicular transport of its physiological substrates as well as chemotherapeutic agents such as daunorubicin and mitoxantrone (Hirschmann-Jax et al. 2004; Wulf et al. 2004).

Several studies have highlighted the expression of this gene in clinical samples (Yasui et al. 2004; Zhang et al. 2004a). Norwood et al. (2004) described the expression of ABCA3 in an in vivo propagated human AML cell line. Efferth et al. (2006) observed that the *ABCA2* and *ABCA3* genes were significantly expressed in 14 of 21 childhood T-cell acute lymphoblastic leukemia (T-ALL).

In an effort to elucidate the role of these genes for multidrug resistance, the authors treated the T-ALL cell lines, CCRF-CEM, and Jurkat, with methotrexate, vinblastine, or doxorubicin and observed an increase of *ABCA3* mRNA expression in both cell lines, whereas a significant increase in *ABCA2* mRNA expression was only found in Jurkat cells. The induction of *ABCA2* and *ABCA3* mRNA expression upon drug treatment indicates that these two transporters may contribute to resistance of T-ALL patients to chemotherapy. An unexpected observation was that co-treatment of siRNA directed against the *ABCA2* gene combined with methotrexate and vinblastine led to an upregulation of *ABCA3* mRNA. Vice versa, siRNA directed against *ABCA3* plus these two cytostatic drugs increased the expression of *ABCA2* mRNA. This data can be explained by a presumbable compensatory mechanism among these ABC transporters. Since both transporters have an overlapping spectrum of cytotoxic compounds that are transported, functional loss of one transporter was compensated by upregulation of the other one. Such a mechanism is an efficient way for cell to cope with cytotoxic challenge. Redundancy represents a very important principle in biology, if an organism has to secure physiological homeostasis. The results of Efferth et al. (2006) enlarge this concept for at least partially redundant functional redundancy of ABC transporters.

A second study led by the same group and performed on childhood acute myeloic leukemia patients showed that the *ABCA3* gene was highly expressed in patients with a poor response to chemotherapy (Steinbach et al. 2006). The data indicated that *ABCA3* was an independent factor of poor response to chemotherapy and not just co-expressed with other drug resistance genes. Furthermore, there was a significant effect on cell viability after combination treatment of siRNA-mediated suppression of *ABCA3* gene expression and doxorubicin (Steinbach et al. 2006).

The expression of the ABCA3 gene was associated with reduced clinical response to therapy (Steinbach et al. 2006; Wulf et al. 2004).

These two genes were also found to be highly expressed in most breast cancers, indicating intrinsic resistance of these tumors to anticancer drugs (Gillet et al. 2006).

7.2.2 ABCB Subfamily

7.2.2.1 ABCB1

P-glycoprotein was the first ABC transporter described to exert a multidrug resistance phenotype in cancer, and P-glycoprotein/ABCB1/MDR1 is the most investigated human ABC transporter as yet. Juliano and Ling (1976) were the first to describe P-glycoprotein in drug-resistant cells with a defined pattern of "multidrug resistance" including anthracyclines, anthracendiones, Vinca alkaloids. The name P-glycoprotein was chosen, as it was assumed to play a role in drug permeability (P for permeability) (Goda et al. 2009). P-glycoprotein confers drug resistance by lowering the intracellular drug concentrations to sublethal levels.

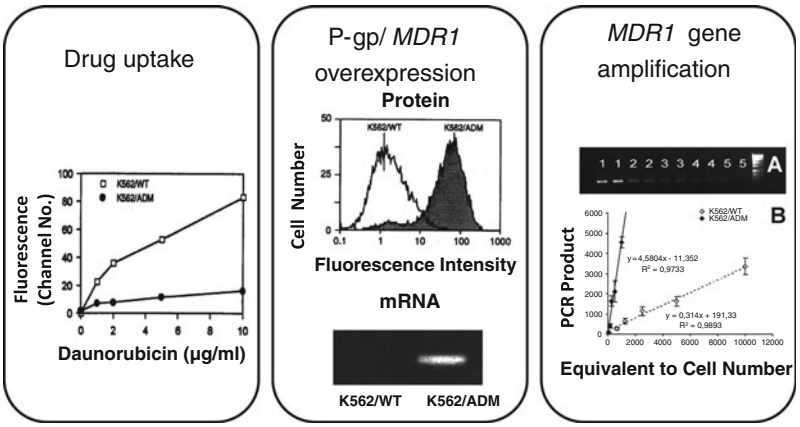

Fig. 7.1 Molecular biology of P-glycoprotein/*ABCB1*/*MDR1* (Efferth 1997). *Left panel*: Accumulation of daunorubicin in sensitive and doxorubicin-resistant K562 leukemia cells as measured by flow cytometry. *Middle panel*: Expression of P-glycoprotein in sensitive and doxorubicin-resistant K562 leukemia cells as measured by monoclonal antibody MRK16 and flow cytometry and expression of *ABCB1*/*MDR1* mRNA as measured by RT-PCR. *Right panel*: Amplification of the *ABCB1*/*MDR1* gene in sensitive and doxorubicin-resistant K562 leukemia cells as measured by PCR

Topoisomerase I poisons, antimetabolites, alkylating agents, or platinum compounds are not involved in this type of multidrug resistance. The gene coding for P-glycoprotein (ABCB1/MDR1) was the very first identified human ABC transporter gene (Chen et al. 1986). A large body of evidence has been acquired confirming that this transporter is responsible for drug efflux and resistance to many unrelated drugs used in cancer chemotherapy (Juliano and Ling 1976; Ueda et al. 1987; Scala et al. 1997).

It consists of 1,280 amino acids and has a molecular weight of about 170 kDa (Murakami and Takano 2008). Sometimes, P-glycoprotein was also termed P-170. Roninson et al. (1986) found that multidrug-resistant cells overexpress and amplify the ABCB1/MDR1 gene. Amplified MDR1 genes are cytogenetically visible as homogeneously staining regions at chromosomal locus 7q21 or as extrachromosomal elements like double minutes or episomes (Slovak et al. 1987; Ruiz et al. 1989). Beside gene amplification, the gene is also activated in response to demethylation of the MDR1 promotor (Nakayama et al. 1998) or chromosomal translocations, e.g., t(4q;7q) (Mickley et al. 1997). While gene amplification was frequently observed in cell lines selected in vitro for high-level drug resistance, the latter activation modes are clinically more relevant, where low degrees of drug resistance are likely to occur. An overview of the molecular biology of P-glycoprotein and its coding ABCB1/MDR1 gene is depicted in Fig. 7.1. Although specific point mutations have been found in multidrug-resistant human cell lines (Choi et al. 1988), clinical correlates are still missing as of yet. Radiation as well as chemical or viral carcinogens induce resistance towards cytostatic drugs (Holland et al. 1980; Carr 1987; Osmak and Perovic 1989; Sklar 1988; Chapman et al. 1994) and the expression of drug resistance

genes (Fairchild et al. 1987; Hill et al. 1990; Volm et al. 1990; Keith and Brown 1991; Efferth and Grassmann 2000). This led to the hypothesis that carcinogenic events not only cause cancer but also decrease chances for successful chemotherapy. While it has been demonstrated that ABCB1 was expressed in many human cancers, it was found that in some cancers this gene was rarely expressed (Goldstein et al. 1989).

Up to now, no X-ray structure is available of the human P-glycoprotein, but Aller et al. (2009) recently determined the X-ray structure of P-glycoprotein from *Mus musculus*, which has a sequence identity of 87 % compared to the human P-glycoprotein. This structure is shown in Fig. 7.2 and has a resolution of 3.8 Å. P-glycoprotein consists of two halves with a nucleotide free inward-facing conformation, when no ATP is bound. It has a total of 12 transmembrane (two bundles with six domains each) domains leading to a large cavity that is open to the inner leaflet as well as to the cytoplasm. This cavity allows hydrophobic substances to reach the cavity directly from the membrane. The cavity's size within the lipid bilayer is about 6,000 Å large, making it possible to provide enough space for two compounds (Loo et al. 2004). The presumptive drug-binding pocket mainly consists of aromatic and hydrophobic residues and seems to be highly conserved (Aller et al. 2009). As the structure is inward facing and open to the cytoplasm, access is possible neither from the extracellular space nor the outer membrane leaflet. Although most substrates are hydrophobic, P-glycoprotein has an unusually broad substrate specificity. It is capable of recognizing several hundreds of compounds that can reach from 330 to 4,000 Da size (Gottesman 1993). Typical substrates for P-gp are alkaloids, steroids, cyclic, and linear peptides. This includes many chemotherapeutic drugs.

Drug transport usually occurs from the inner leaflet to the outer membrane (Goda et al. 2009). There are two different models for possible drug export mechanism. One model states that P-gp acts as a flippase and is flipping its substrates from the inner leaflet only to the outer membrane (Higgins and Gottesman 1992). According to the second model of P-glycoprotein as a "hydrophobic vacuum cleaner," drugs enter the drug-binding pocket from the inner membrane and are then actively transported into extracellular space (Goda et al. 2009).

Binding and cleavage of ATP provides the energy for the transport process. It is believed that the binding of ATP acts as a power stroke for transporting the bound drug and ATP hydrolysis is necessary to reset the conformation so that another drug is able to bind (ATP switch model) (Higgins and Linton 2004). Both nucleotide-binding sites need to be loaded with ATP causing a conformational change (from inward- to outward-facing conformation) that brings both NBDs close together thereby pushing the substrate into extracellular space. Afterwards, one ATP is hydrolyzed and the open conformation is reached again. Due to the fact that hydrophobic substances are preferred binding partners, even drugs at very low cellular concentration can be transported outward. According to the vacuum cleaner model, the main entry for drugs is the lipid bilayer. Hydrophobic drugs may accumulate within the membrane leading to higher local concentrations than in the aqueous phase allowing drug binding to P-glycoprotein even at lower binding affinities (Goda et al. 2009). An interesting fact is that the activity of P-glycoprotein

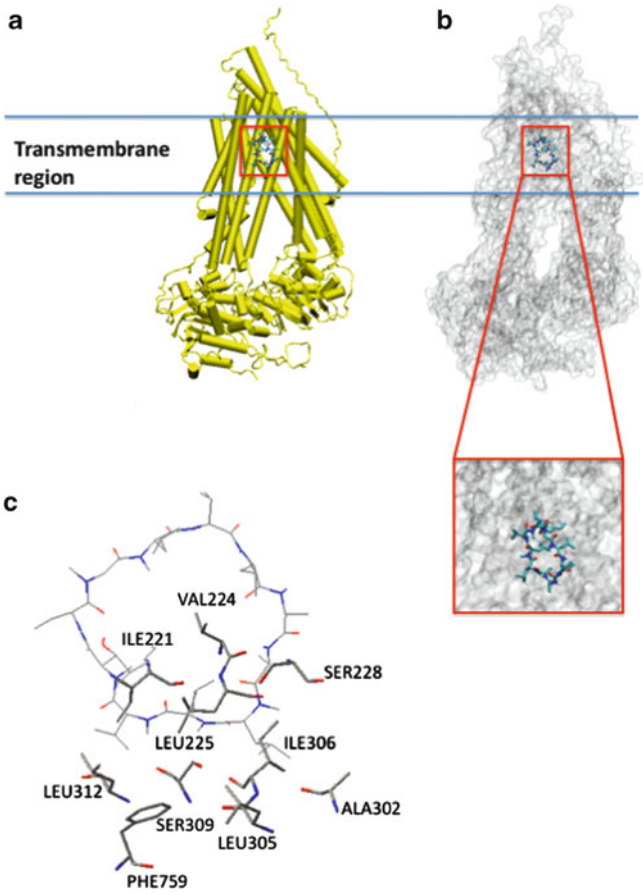

Fig. 7.2 Graphical representation of the binding of cyclosporin A to human P-glycoprotein (P-gp). Cyclosporin A was used as control drug, since its P-glycoprotein modulating activity is well known. Human P-gp was homology-modeled using MODELLER software (Eswar et al. 2006) with mouse P-gp crystal structure (PDB-ID 3G5U at http://www.pdb.org) as template. The docking was performed using AutoDock version 4.2 (http://autodock.scripps.edu/). The best docked position of verapamil is illustrated in different styles. (**a**) P-gp is drawn in cartoon mode for orientation by secondary structures, verapamil is highlighted by the *box*. (**b**) The P-gp surface was calculated and drawn as *black shadow* (ghost mode) for better contrast to the drug. In addition, for better perception of the cavity, which verapamil was docked to, we zoomed into the molecule (highlighted in the *close-up box*). (**c**) Amino acids in close neighborhood of docked verapamil constituting the binding cavity for verapamil. This figure has been generated by Dr. Tolga Eichhorn (Department of Pharmaceutical Biology, University of Mainz, Germany)

is increased if a drug is cotransported with a phospholipid. This may be a hint for possible ligand–ligand interactions and influences of the surrounding membrane (Goda et al. 2009). Taking together, the exact mechanisms of drug binding are still unclear despite huge efforts in the past years.

7.2.2.2 Other Members of the ABCB Subfamily

The ABCB2/MDR2 gene is co-amplified along with the MDR1 gene in multidrug-resistant cancer cell lines and reveals a close amino acid homology with ABCB1/MDR1 (van der Bliek et al. 1987). The ABCB4/MDR2 gene product is involved in phosphatidylcholine translocation into the bile (Smit et al. 1993). ABCB4/MDR2 reveals a flippase function (Ruetz et al. 1994) and mediates the efflux of several ABCB1 substrates such as daunorubicin, doxorubicin, vincristine, etoposide, mitoxantrone paclitaxel, ivermectine, and vinblastine (Smith et al. 2000; Johnsson et al. 2005). Moreover, ABCB4 expression is a prognostic factor for negative clinical outcome (Herweijer et al. 1990; Arai et al. 1997). *ABCB5* gene expression was strongly correlated with resistance towards 7-Cl camptothecin (Huang et al. 2004). Melanoma cells transfected with siRNA directed against *ABCB5* were more sensitive towards camptothecin, 10-hyhdroxycamptothecin, and 5-fluorouracil than control cells transfected with mock siRNA (Huang et al. 2004). Chen et al. (2006) proposed ABCB5-mediated resistance due to a mechanism of sequestration . Inhibition of ABCB5 increased doxorubicin accumulation in ABCB5-expressing tumor cells, demonstrating that ABCB5 acts as a doxorubicin efflux transporter (Frank et al. 2005).

The ABC transporters ABCB6 and ABCB7 are involved in iron homeostasis. They are located in the mitochondria and transport heme and protoporphyrins into these organelles (Higgins 1995). A significant correlation was found between the expression of microarray-based *ABCB6*, but not *ABCB7* expression and IC50 values for artesunate and 55 tumor cell lines (Kelter et al. 2007).

The human orthologue of the rat "sister of Pglycoprotein" (Spgp) (Gerloff et al. 1998) is a bile salt export pump (BSEP) in the liver canalicular membrane. ABCB11 is closely related to ABCB1(Childs et al. 1995). ABCB11 conferred resistance towards paclitaxel (Childs et al. 1998).

7.2.3 ABCC Subfamily

7.2.3.1 ABCC1

The ABCC1/MRP1 gene has been cloned from a multidrug-resistant, but P-glycoprotein-negative lung cancer cell line (Cole et al. 1992). This ABC transporter also acts as a drug efflux pump rendering cancer cells resistant to cytostatic drugs (Zaman et al. 1994). Evidence for a causative contribution of ABCC1/MRP1 to drug resistance came from transfection experiments (Grant et al. 1994; Cole et al. 1994). Functional ABCC1/MRP1 is expressed in the outer plasma membrane as well as in intracellular vesicles and the Golgi apparatus. This indicates that ABCC1/MRP1 exports drugs out of the cell and sequestrates drugs into vesicles. ABCC1/MRP1 knockout mice are hypersensitive to

etoposide, especially in bone marrow, testis, and kidney (Wijnholds et al. 1998). MRP1 has various functions:

1. Transport of exogenous xenobiotic compounds such as
   - Anticancer drugs such as doxorubicin, etoposide, or vincristine (Jedlitschky et al. 1996; Loe et al. 1998; Priebe et al. 1998)
   - Organic anions derived from phases I and II metabolism of xenobiotics, e.g., aflatoxin B1 (Ishikawa 1992; Loe et al. 1997)
   - Cysteinyl leukotriene LTC4, a mediator of inflammatory responses and regulator of vascular permeability and smooth muscle contraction (Ishikawa et al. 1990)
2. Transport of endogenous compounds such as bilirubin-glucuronides, sulfate, conjugated bile salts, glutathione disulfide, prostaglandin A2, estradiol-gluconuride, and others (Jedlitschky et al. 1996, 1997; Evers et al. 1997; Heijn et al. 1997).

MRP1 translocates its substrates as GSH-conjugates (Jedlitschky et al. 1996; Priebe et al. 1998; Loe et al. 1997), indicating that MRP1 may be part of the cellular machinery coping with oxidative stress. MRP1 also cotransports unmodified drugs along with GSH, e.g., vincristine (Loe et al. 1998). GSH may stimulate drug binding and transport. The transport energy is delivered by ATP hydrolysis (Loe et al. 1996). MRP1 protein has one drug-binding site with high affinity for GSH and low affinity for drugs (G-site) and another one with low affinity for GSH and high affinity for drugs (D-site). In the absence of drugs, both sites reveal a low constitutive GSH extrusion activity. Low-drug concentrations cause occupation of the D-site with cotransport of GSH and drug. At high drug concentrations, both drug-binding sites translocate drug molecules without GSH (Borst et al. 1999).

A phase I clinical and pharmacokinetic study of the ABCC1 inhibitor sulindac in combination with epirubicin in patients with advanced cancer has been reported (Girodon et al. 1997).

7.2.3.2 ABCC2

The ABCC2/MRP2 gene has been cloned by Büchler et al. (1996) and Taniguchi et al. (1996). The gene is expressed in the basolateral and apical parts of hepatocytes (Mayer et al. 1995) and apical membrane of kidney proximal tubule epithelia (Schaub et al. 1997). It mediates the excretion of biliary organic anions. In comparable manner as MRP1, MRP2 exports oxidized GSH (Leier et al. 1996) and is an antioxidant defense mechanism. MRP2 translocates a similar spectrum of compounds as MRP1 does (Fernandez-Checa et al. 1992; Kobayashi et al. 1988), though the substrate-binding affinities differ (Ishikawa et al. 1990; Kobayashi et al. 1988; Cui et al. 1999). MRP2 is involved in the development of multidrug resistance as well as in resistance to cisplatin or methotrexate resistance, both of which are not involved in the classical multidrug resistance phenotype

(Taniguchi et al. 1996; Kool et al. 1997; Evers et al. 1998; Minemura et al. 1999). Evidence for a causative role in cancer drug resistance has been presented in transfection experiments using MRP2 cDNA or antisense constructs (Cui et al. 1999; Koike et al. 1997).

7.2.3.3 Other Members of the ABCC Subfamily

ABCC3 protein mediates the transport of fewer substrates than ABCC2. Known substrates are epipodophyllotoxins (etoposide and teniposide) and methotrexate (Zelcer et al. 2001; Zeng et al. 2001). The low-basal level expression is strongly increased, when the canalicular secretion of MRP2 substrates is impaired (Hirohashi et al. 1999).

7.2.3.4 ABCC4 and ABCC5

ABCC4 and ABCC5 confer resistance to 6-mercaptopurine and 6-thioguanine, two anticancer purine analogues (Chen et al. 2001; Tian et al. 2005, 2006; Pratt et al. 2005).

The *ABCC6* gene was found in cancer cells selected for epirubicin resistance (Longhurst et al. 1996). The ABCC6 protein mediates low levels of resistance to standard anticancer agents, including etoposide, doxorubicin, daunorubicin, or actinomycin D (Belinsky et al. 2002). Concerning its ability to confer resistance to natural product anticancer agents, ABCC6 is similar to ABCC1, ABCC2, and ABCC3. This gene is also expressed in normal kidney, liver, primitive hematopoietic precursor cells, and to a lesser extent in skin, retina, and vascular tissues (Kuss et al. 1998; Bergen et al. 2000). The ABCC6 protein detoxifies compounds from the kidney and liver and contributes to extracellular matrix deposition or turnover of connective tissue (Bergen et al. 2000, Kool et al. 1999; Kruh et al. 2001; Zeng et al. 1999).

ABCC10/MRP7 confers resistance to taxanes (docetaxel, paclitaxel), *Vinca*-alkaloids (vinblastine, vincristine), and anthracyclines (doxorubicin) (Hopper-Borge et al. 2004). Naramoto et al. (2007) observed the ABCC10 expression in refractory head and neck cancers.

Using a computer-based screening approach to generate ESTs, two further genes of the ABCC subfamily have been identified: ABCC11 and ABCC12 (Tammur et al. 2001). The ABCC12 gene encodes two different mRNA transcripts that are differentially expressed in different tissues (Bera et al. 2002). Both genes are highly expressed in many breast cancer samples, with less expression in normal breast tissue (Bera et al. 2001, 2002; Bieche et al. 2004; Park et al. 2006). ABCC11 expression conferred resistance to nucleotide analogs (Guo et al. 2003; Oguri et al. 2007).

7.2.4 ABCG Subfamily

The ABCG2/BCRP gene has been first identified in multidrug-resistant MCF-7 mamma carcinoma cells (Allikmets et al. 1998a, b; Doyle et al. 1998) and was, therefore, termed breast cancer resistance gene. Shortly afterwards it became clear that BCRP expressed is not restricted to breast tissue. Two other groups reported an identical gene expressed in the placenta and in a mitoxantrone-selected colon carcinoma cell line (Allikmets et al. 1998a, b; Miyake et al. 1999). Transfection of the cDNA confers resistance towards various anti-cancer drugs (mitoxantrone, doxorubicin, daunorubicin, and topotecan) (Allen et al. 1999; Yang et al. 2000; Bates et al. 2001; Doyle and Ross 2003; Kawabata et al. 2001; Ma et al. 1998; Maliepaard et al. 1999; Ross et al. 1999). Bcrp expression can also be found in cancer stem cell side populations.

7.3 Inhibition of ABC Transporters

7.3.1 Inhibition of P-Glycoprotein/ABCB1

A selective inhibition of P-glycoprotein may reduce drug efflux and increase concentrations of pharmacologically effective levels in tumors (Nobili et al. 2006; Takara et al. 2006). Blocking of P-glycoprotein can be reached by functional inhibition or downregulation of P-glycoprotein/MDR1 expression. Despite huge efforts in academia and industry, no chemical P-glycoprotein inhibitor has clinically showed satisfying results and reached the pharmaceutical market yet (Efferth et al. 1991, 1993; Efferth and Volm 1993b; Ford and Hait 1993; Majumder et al. 2006; Tiwari et al. 2011). First-generation compounds (verapamil, cyclosporin A; Fig. 7.2) have initially been developed for other indications. Thus, high adverse effects come as no surprise, e.g., verapamil's cardiac toxicity (Sikic 1993).

P-glycoprotein inhibitors inhibit drug efflux according to their P-glycoprotein content, independent of whether the cells are of cancerous or of noncancerous origin (Volm et al. 1991). *Mdr1a/1b* (−/−) knockout mice were viable, suggesting that P-glycoprotein inhibition is not life-threatening (Schinkel et al. 1997). Therefore, blocking of P-glycoprotein by chemical compounds might also not lead to life-threatening side effects. Therefore, the search for novel P-glycoprotein inhibitors with less unwanted effects on other targets (e.g., cardiac toxicity) is justified.

Second generation modulators (dexverapamil or valspodar/PSC-833) were not only active in animal experiments (Fellner et al. 2002) but also failed in clinical trials due to side effects and concomitant inhibition of cytochrome P-450 monooxygenases (CYPs) (Fischer et al. 1998). Clinical trials with third-generation drugs such as elacridar (GF120918) (Kruijtzer et al. 2002), biricodar, and zosuquidar (Fracasso et al. 2004) are ongoing, but do not seem to be very promising yet. Hence, there is

Table 7.1 Inhibition f P-glycoprotein (P-gp/MDR1) function and/or expression by herbs or natural products derived from traditional Chinese medicine (Eichhorn and Efferth, 2012).

Plant	Compound	Test model	Remarks	References
Coptis chinensis	Berberine	Human and murine hepatoma cells	Increase in P-gp expression, reduction in rhodamine 123 accumulation	Lin et al. (1999a, b)
Coptis chinensis	Berberine	Oral (KB, OC2), gastric (SC-MN1, NKGC-3), colon (CoLo205, CT26) cancer cells	Increase of P-gp expression. Decrease of rhodamine 123 retention. Increased resistance to paclitaxel	Lin et al. (1999a, b)
Coptis chinensis	Berberine	Rat	The hepatobiliary excretion of berberine is increased by the P-gp inhibitors, cyclosporin A or quinidine	Tsai and Tsai (2004a, b)
Coptis chinensis, Rhizoma	Berberine, canadine	Hep G2 liver cancer cells	*MDR1* expressing cells are cross-resistant to berberine, but not to canadine	Abidi et al. (2006)
Coptis chinensis, Rhizoma	Berberine	Rat primary cultured cortical neurons	The metabolic inhibitor KCN or verapamil inhibited P-gp mediated berberine transport in neurons	Chen et al. (2008a, b)
Coptis chinensis	Berberine	Rat	Dose-dependent increased bioavailability of digoxin and cyclosporin by inhibition of intestinal P-gp	Qiu et al. (2009)
Coptis chinensis, Rhizoma	Protoberberine alkaloids (berberine, palmatine, coptisine, epiberberine, jatrorrhizine)	Streptotozin-induced diabetic rats	Diabetic rats showed impaired function and expression of P-gp compared to control rats after exposure to Coptidis Rhizoma extract	Yu et al. (2010)
Coptis chinensis	Coptisine, berberine, plamatine	A10 rat vascular smooth muscle cells	Coptisine, in contrast to berberine and palmatine upregulated *mdr1a* and *mdr1b* mRNA expression induced	Suzuki et al. (2010)

(continued)

Table 7.1 (continued)

Plant	Compound	Test model	Remarks	References
			P-gp expression and enhanced rhodamine 123 efflux	
Coptis chinensis, Rhizoma	Protoberberine alkaloids (berberine, palmatine, coptisine, epiberberine, jatrorrhizine)	Streptotozin-induced diabetic rats	Diabetic rats showed impaired function and expression of P-gp compared to control rats after exposure to Coptidis Rhizoma extract	Yu et al. (2010)
Coptis japonica Makino	Protoberberine alkaloids	5 tumor cell lines	8-oxocoptisine inhibited P-gp activity	Min et al. (2006)
Camelia sinensis	Tea polyphenols	MCF-7/ADR breast cancer	Increase doxorubicin sensitivity, increase ^{99m}Tc-tetrofosmon uptake	Zhu et al. (2001)
Camelia sinensis	Catechins		Some catechins inhibit P-gp efflux function	Wang et al. (2002)
Camelia sinensis	(-Epicatechin-3-gallate	CaCo-2 intestinal cells	P-gp mediated efflux	Vaidyanathan and Walle (2003)
Camelia sinensis	Polyphenols	Nasopharyngeal KB-A-1 in vitro and in vivo	Polyphenols and (−)-epigallocatechin gallate reversed multidrug resistance by down-regulation of *MDR1* expression	Mei et al. (2004)
Camelia sinensis	(−)Epigallo-catechin, epi-catechin gallate, (−) epigallocatechin gallate	Nasopharyngeal KB-C2 cancer cells	Catechins inhibited efflux of P-gp substrates galloyl moiety on C-ring and trihydric pyrogallol group as B-ring increased inhibitory activity	Kitagawa et al. (2004)
Camelia sinensis	Epicatechin gallate epigallocathecin gallase	BEL-7404/DOX liver cancer in vitro and in vivo	Increase of doxorubicin efficacy; enhancement of doxorubicin accumulation and inhibition of doxorubicin efflux	Liang et al. (2010)
Camelia sinensis	(−)Epigallo-catechin-3-gallate	Tamoxiflen-resistant MCR-7 Tam breast cancer cells	100 μg/ml decreased P-gp expression, but not *MDR1* mRNA expression	Farabegoli et al. (2010)
Salvia miltiorrhiza	Salvinal	Nasopharyngeal KB vin and KB taxol 50 cells	Poor substrate of P-gp	Chang et al. (2004)

Salvia miltiorrhiza	Cryptotanshinone	CaCo-2 intestinal cells, MDCKII cells, rat intestinal perfusion	Cryptotanshinone is a P-gp substrate, P-gp pumps cryptotanshinone into the luminal side of the intestine	Zhang et al. (2006)
	Danshensu	Ca-Co-2 intestinal cells	Danshensu is a P-gp substrate	Zhu et al. (2006)
Salvia miltiorrhiza, Radix	Tanshinone B	Rat brain microvessel endothelial cells in vitro	Tanshinone B is transported by P-gp across the blood brain barrier. The neurotoxin, quinolinic acid inhibited this transport	Zhou et al. (2007)
Salvia miltiorrhiza	Cryptotanshinone	Rats, rat brain microvessel endothelial cells in vitro	Crypthothanshinone is a substrate of P-gp at the blood brain barrier	Yu et al. (2007a)
Salvia miltiorrhiza, Radix	Tanshinone II A	Rats, CaCo-2 cells	Tanshinone II A is a substrate and reversing agent of P-gp in the gut explaining low oral bioavailability by first-pass metabolism	Yu et al. (2007b)
Salvia milthiorrhiza	Tanshinone IIB	CaCo-2 intestinal cells, rats	Substrate of P-gp; efflux can be inhibited by P-gp inhibitors	Yu et al. (2007a, b, c, d)
Ginkgo biloba	Quercetin	Rats	Decrease of cyclosporin A bioavailability	Yang et al. (2008a, b)
Ginkgo biloba extract		Pregnane X receptor-transfected HepG2 liver cancer cells	Activation of the transcription factor, PxR and increase of *MDR1* mRNA expression	Yang et al. (2008a, b)
Gingko biloba, extract		10 healthy male volunteers	Repeated extract ingestion increased talinolol maximum plasma concentration and AUC. Talinolol is a P-gp substrate	Fan et al. (2009a)
Ginkgo biloba	Kaempferol	Liver of mice, in vivo; mouse hepatoma Hepa-1c1c7 cells	Inhibition of P-gp by verpamil increased kaempferol uptake	Mukai et al. (2009)

(continued)

Table 7.1 (continued)

Plant	Compound	Test model	Remarks	References
Ginkgo biloba	EGb761, ginkolide A, ginkolide B, bilobalide, flavonoids (quercetin, kaempferol, tamarixetin)	Human primary hepatoxytes, HepF2 liver cancer cells	Induction of *MDR1* expression by Egb 761, ginkolide A, ginkolide B, but not bilobalide and flavonoids	Li et al. (2009a, b)
Stephania tetandra	Tetrandrine, fangchinoline	P-gp positive HCT15 colon cancer cells	Enhancement of cytotoxicity of MDR-related drugs via P-gp modulation	Choi et al. (1998)
Stephania tetandra	Tetrandrine	Drug-resistant S180 mouse sarcoma	Reduction of P-gp expression	Li et al. (2010)
Stephania tetandra	Tetrandrine	Doxorubicin-pretreated K562 leukemia	Inhibition of *MDR1*/P-gp and increased doxorubicin retention, inhibition of doxorubicin-induced NFκB activity	Shen et al. (2010)
	Fang choline	MDR1-MDCK II cells	Decreased efflux of paclitaxel	He et al. (2010)
Schisandra chinensis	Gomisin A, gomisin N schisandrin C	HepG2-DR liver cancer	Gomisin A alters P-gp-substrate interaction, but itself is neither a P-gp substrate nor competitive inhibitor	Wan et al. (2006)
Schisandra chinensis, Fructus	Schisandrol A	HepG2-DR liver cancer	Reversion of P-gp-mediated multidrug resistance by affecting P-gp substrate complexes	Fong et al. (2007a, b)
Schisandra chinensis	Gomisin A, gomisin N schisandrin C	CaCo-2 intestinal cells	Gomisin N is a P-gp substrate	Madgula et al. (2008)
Fructus Schisandrae	5 schisandrins	KBV200, MCR-7/DOX Bel 7402	Schisandrin A most potentially reversed vincristine paclitaxel and doxorubicin resistance, increase of doxorubicin accumulation, downregulation of P-gp and MDR1	Huang et al. (2008a, b)
Schisandra chinensis extracts, *Gingko biloba* extracts		12 healthy male volunteers	300 mg s.c. extract twice daily for 14 days or 120 mg G.b. extract three times daily for 14 days incrased Cmax and AUC of talinolol	Fan et al. (2009b)

Schisandra sphenanthera extract		Rats	Inhibition of P-gp-mediated efflux of tacrolimus and reduction of intestinal first pass effect, increased oral bioavailability of Tacrolimus	Qin et al. (2010)
Glycorrhiza glabra	Glabridin	CaCo-2 intestinal cells, MDCKII cells, rats	Glabridin is a substrate of P-gp glabridin inhibits P-gp mediated digoxin transport. The P-gp inhibitor verpamil increases systemic bioavailability of glabridin, in vivo	Cao et al. (2007)
Glycyrrhiza glabra decoction		Rats	Oral administration (10 g/kg, twice daily for a week) increased the absorption of rhodamine 123 and enhanced secretion across the jejunum mucosa	Yao et al. (2009)
Glycyrrhiza inflata, Daphne genkwa		Rat jejunum membranes in vitro	*G.i.* slightly and *D.g.* strongly inhibited P-gp function as measured by rhodamine 123	Huang et al. (2008a, b)
Panax ginseng	Protopanaxatriol ginsenosides	AML-2ID100 leukemia	Reversion of daunorubicin resistance by interaction with the azidopine-binding size of P-gp	Choi et al. (2003)
Panax ginseng		Sprague–Dawley rats	Decreased bioavalability of fexofenadine by long-term feeding with 150 mg/kg day due to induction of intestinal and brain endothelium P-gp expression	Zhang et al. (2009)

(continued)

Table 7.1 (continued)

Plant	Compound	Test model	Remarks	References
Panax ginseng	20(S)-ginsenoside Rh2	In vitro, in situ, and in vivo models (rats)	Decrease of efflux of digoxin, fexotenadine and etoposide; enhance rhodamine 123 retention; no P-gp substrate, but non-competitive inhibitor; increase of absorption of P-gp substrates without long-term induction of P-gp expression in rats.	Zhang et al. (2009)
Curcuma longa	Curcumin, demethoxy curcumin, bisdemethoxy curcumin	CaCo-2 intestinal cells LLC-GA5-COL300	Curcumin and demethoxy curcumin, but not bisdemethoxy curcumin inhibit P-gp	Wang et al. (2010a, b)
Curcuma longa	Ramified curcumin hydrolyzed	K562/A02 leukemia	Downregulation of P-gp and *MDR1* expression, increased sensititvity to anticancer drugs	Huang et al. (2010)
Scutellaria baicalensis	Baicalin	Rats	Involvement of P-gp in active baicalin efflux into bile. P-gp inhibitors cyclosporin a and quinidine promoted active baicalin transport into bile and reduced blood levels, but had not effect on blood brain barrier passage of baicalin	Tsai and Tsai (2004a, b)
Scutellaria baicalensis	Baicalin	Rats	Involvement of P-gp in active baicalin eflux into bile. P-gp inhibitors cyclosporin a and quinidine promoted active baicalin transport into bile and reduced blood levels, but had not effect on blood brain barrier passage of baicalin	Tsai and Tsai (2004a, b)

	Alkylated baicalein dervatives	Nasopharyngeal KB/MDR cancer cells	Alkylation of R6 or R7 increased vinblastine accumulation. The optimal functionality was a propyl side chain. The best compound was 5-methoxy-6,7-dipropyloxyflavone. Benzylated compounds are inhibitors, but not substrates of P-gp	Lee et al. (2004)
	Baicalin, baicalein, chlorogenic acid, ginsenoside Rf	HepG2 liver cancer	Bacalein induced *MDR1* expression by activating pregnance X receptor and constitutive androstane	Li et al. (2010)
Cephalotaxus harringtonia	Homoharringtonine	Cancer cells	Multidrug-resistant, P-gp expressing cancer cells are cross-resistant to homoharringtonine	Zhou et al. (1995)
	Tetrandrine, ditetrahydropalmatine, dauricine, berbamine, daurisoline, berberine, tetramethylpyrazine	Bovine brain capillary endothelial cells	Increase of rhodamine 123 accumulation	He and Liu (2002)
Phellodendron wilsonii Hayata et Kanehira	Phellamurin	Rats	Inhibition of intestinal P-gp; increase of Cmax and AUC of cyclosporin	Chen et al. (2002)
Soybean	Lecithin	Rats	Modification of P-gp-mediated plasma lipid transport in liver	LeBlanc et al. (2003)
Sinomonium acutum	Sinomenine	Sprague–Dawley rats	Hepatobiliary elimination regulated by P-gp	Tsai and Wu (2003)
	Acetogenin	KBV200 xenografts in nude mice	No cross-resistance, decrease of P-gp function	Fu et al. (2003)
Peucadanum praeruptorum Dunn, Radix	Pyranocoumarins	KB-V1 cells	Collateral sensitivity in MDR cells, synergistic interaction with anticancer drugs; down-regulation of P-gp expression; increase in Doxorubicin accumulation	Wu et al. (2003)

(continued)

Table 7.1 (continued)

Plant	Compound	Test model	Remarks	References
Rhinacanthus nasutus (L.) Kurz, Radix, Folia	Rhinacanthin C	HeLa, P-gp expressing Hcvr100-6, cancer cell lines	No cross-resistance of P-gp expressing cells towards rhinacanthin C	Gotoh et al. (2004)
Melaleuca alternifolia	Tea tree oil, terpinen-4-ol	Adriamycin-resistant M14 melanoma cells	Collateral sensitivity in resistant cells	Calcabrini et al. (2004)
Alisma oriental is, Rhizoma	Alisol B 23-acetate	HepG2-DR liver cancer K562-DR leukemia	Resensitzization to anticancer drugs; decrease of rhodamine 123 efflux	Wang et al. (2004)
Radix Astragali	Astragaloside IV	Perfused rat intestinal model, Ca Co-2 cells, rats in vivo	No clear relation to P-gp function	Gu et al. (2004)
Sho-saiko-to (Xiao-Chi-Hu-Tang)		CaCo-2 intestinal cells, rat jejunum in situ	Increase of membrane permeability of tolbutamine. Inhibition of P-gp mediated efflux. Decrease of *MDR1* expression by Glycorrhiza radix, glycyrrhizic acid and liquiritin	Nishimura (2005)
Pseudolarix kaempferi, root bark	Pseudolaric acid B	Several cell lines	Activity towards P-gp overexpressing cells	Wong et al. (2005)
	Honokiol	MCF-7/ADR breast cancer	Down-regulation of P-gp and increase of vincristine transport	Xu et al. (2006)
Paeonia lactiflora Pall. Sinomenium acutum Rehder & Wilson	Paeoniflorin, Sinomenine	Rat gut sac model in vitro	Sinomenine improves bioavailability of paeoniflorin in rats sinomenine decreases paeoniflorin efflux by P-gp	Chan et al. (2006)
Stemona tuberosa Lour	Neotuberostemonine, Neostenine	CaCo-2 intestinal cells	Substrates of P-gp. The P-gp inhibitor cyclosporin A inhibits efflux of both compounds	Leung et al. (2006)

Peucedanum praeruptorum Dunn	Praruptorin A (+/−)-3′-0,4′-dicynnamoyl-*cis*-khellactone	Multidrug-resistant cells	Resensitization of multidrug-resistant cells to anticancer drugs	Shen et al. (2006)
Evodia rutaecarpa	Quinolones, indoloquinazoline alkaloids	CEM/ADR5000 leukemia, porcine brain capillary endothelial cells	Weak to moderate modulators of P-gp activity	Adams et al. (2007a, b)
		Rats, rat brain microvessel endothelial cells in vitro	Increase of nimodipine transport across the blood brain barrier, decreased ninodipine accumulation and P-gp expression in vitro	Zhang et al. (2007)
Huanglian Jiedu Tang		S180 MDR mouse sarcoma	Down-regulation of *MDR1* expression	Li et al. (2007a, b)
Shongde powder		SGC-7901/VCR gastric carcinoma	Down-regulation of P-gp and increase of vincristine transport	Wang et al. (2007)
Alisma orientalis (Sam) Juzep, rhizoma		HepG2-DR liver cancer, K562-DR leukemia	Synergistic growth inhibition in combination with P-gp substrate drugs by inhibition of P-gp	Fong et al. (2007a, b)
	Coraria lactone	Astrocytes fro natal Sprague–Dawley rats in vitro	Induction of P-gp expression	Geng and Zhou (2008)
Xiaochaihu-tang	Flavonoids (baicalin, wogonoside, oroxylin-A-7-o-beta-D-glucopyranosid-uronide, liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside bacalein, wogonin, oroxylin-A, liquiritigenin, isoliquiritigenin	Intestinal CaCo2 cell monolayers	Aglycones, but not glucuronidated flavonoids exhibited favorable membrane permeability	Dai et al. (2008)
Angelica sinensis (Oliv) Diel	Vauqueline, ephedrine, strychnine)	K562/A02 leukemia	Vauqueline and ephedrine, but not strychnine down-regulated *MDR1* and P-gp expression	Gao et al. (2008)

(continued)

Table 7.1 (continued)

Plant	Compound	Test model	Remarks	References
Caulis mahoniae	Isotetrandrine	MCF-7/DOX breast carcinoma	Reversibel functional inhibition of P-gp; no effect on P-gp expression	Wang and Yang (2008)
Marsdenia tenacissima	Tenacissimoside A, 11-alpha-O-benzoyl-12-beta-O-acetyl-tenacigenin B	HepG2/DOX liver cancer	Inhibition of P-gp function	Hu et al. (2008)
Hippophae rhamnoides L.	Quercetin, isorhamnetin	MDR-transfected MDCKII cells, CaCo-2 intestinal cells, rats.	Isorhametin is effluxed by P-gp. Coadministration increased efflux	Lan et al. (2008)
	Quercetin	CaCo-2 intestinal cells, female Wistar rats	Enhancement of absorptive permeability of P-gp interaction; enhancement of bioavailability and reduction of toxicity of irinotecan	Bansal et al. (2008)
Antrodia camphorata		HepG2 liver cancer	Down-regulation of *MDR1* expression	Li et al. (2009a, b)
Pinus massoniana Lamb	Procyanidine	Rat brain microvessel endothelial cells in vitro	Inhibition of blood brain barrier	He et al. (2009)
Paeonia alba, Radix	Paeoniflorin		Paeoniflorin is a P-gp substrate, Radix Paeoniae Alba induces intestinal P-gp expression	Dong et al. (2009)
	Procyanidine	Rat brain microvessel endothelial cells invitro, xenograft tumors in nude mice	Inhibition of P-gp at the blood–brain barrier	He et al. (2009)

Ligusticum Chuanxiong Hort	Tetramethylpyrazine	BEL-7402/ADM liver cancer	Reversion of multidrug resistance by down-regulation of *MDR1*	Wang et al. (2010a, b)
Rhei Rhizoma		Rat everted intestine ex vivo and in vivo	300 μg/ml suppressed P-gp mediated rhodamine 123 efflux	Yokooji et al. (2010)
	Apigenin, fisetin, honokiol	MES-SA/Dx5 sarcoma cells	Increase of doxorubicin accumulation, increase of doxorubicin cytotoxicity	Angelini et al. (2010)
Acanthopanax senticosus Harms		CaCo-2 intestinal cells	Increase of rhodamine 123 uptake	Takahashi et al. (2010)
Triterygum wilfordii	Triptolide	Nasopharyngeal KB-tax cells in vitro and in vivo	Down-regulation of *MDR1* expression	Chen et al. (2010)
Epimedii Herba	Icariin	Rats	P-gp contributes to the biliary excretion	Wu et al. (2010)
Zilongjin (ZLJ)		MCF-7/DOX breast cancer cells, KBv200 nasopharyngeal carcinoma cells	Multidrug-resistant cells reveal no cross-resistance. Down-regulation of P-gp and increase of doxorubicin or vincristine cytotoxicity	Zou et al. (2010)

Taken from Eichhorn and Efferth (2011)

a continued requirement for more suitable P-glycoprotein inhibitors. Natural products may be promising because of low side effects and good tolerability.

P-glycoprotein detoxifies xenobiotic compounds in normal tissues taken up with food. Therefore, many herbal compounds should be substrates of P-glycoprotein. Indeed, there is a large body of evidence that natural compounds are transported by P-glycoprotein. During evolution of life, P-glycoprotein substrates and inhibitors have been frequently codeveloped in the same plant species. If herbivores detoxify harmful phytochemicals by P-glycoprotein, plants need inhibitors for self-defense. Hence, it can be expected that many P-glycoprotein inhibitors can be found in plants (Molnár et al. 2010). Indeed, focusing on plants derived from TCM confirmed this point of view. TCM herbs or natural products derived from TCM can be separated in two major categories. They either functionally inhibit P-glycoprotein by interference with efflux activity of the drug pump or they downregulate P-glycoprotein/*MDR1* expression, thereby resensitizing multidrug-resistant cells (Table 7.1).

More than a decade ago, we initiated a research program on molecular pharmacology and pharmacogenomics of natural products derived from TCM. An entire panel of natural products with activity towards tumor cells and viruses were identified (Efferth et al. 2007, 2008). Based on this rationale, we started a systematic investigation of P-glycoprotein inhibitors derived from TCM.

In a pilot study, we tested 22 natural products for their cytotoxic and ABC-transporter-inhibiting activities: artesunate, artemisinin, baicalein, baicalin, berberine, bufalin, cantharidin, cephalotaxine, curcumin, daidzein, daidzin, diallyl disulfide, ginsenoside Rh2, glycyrrhizic acid, isonardosinon, homoharringtonine, nardosinon, nardofuran, puerarin, quercetin, tannic acid, and tetrahydronardosinon (Efferth et al. 2002). We used CCRF-CEM parental cells and doxorubicin-selected P-glycoprotein (P-gp)/*MDR1*-expressing CEM/ADR5000, vinblastine-selected P-gp/*MDR1*-expressing CEM/VBL_{1000}, and epirubicin-selected multidrug resistance-related protein 1 (*MRP1*)-expressing CEM/E1000 sublines thereof. While CEM/ADR5000, CEM/VBL_{1000}, and CEM/E1000 cells were highly resistant to the corresponding selecting agents, no or only minimal degrees of cross-resistance were observed to TCM drugs in both growth inhibition assay and MTT assay (range from 0.4- to 8-fold). Homoharringtonine, artesunate, and bufalin were most active among this panel of compounds. Artesunate significantly increased daunorubicin accumulation in CEM/E1000 cells, but not in CEM/VLB(100) or CCRF-CEM parental cells. Bufalin caused a small, but significant increase in daunorubicin accumulation in CEM/VLB(100) and CEM/E1000 cells. As artesunate and bufalin showed both antileukemic activity if applied alone and modulation activity in combination with daunorubicin in multidrug-resistant cells, these two drugs may be suitable for novel combination treatment regimens to improve leukemia cell killing.

The antimycobacterial quinolones 1-methyl-2-undecyl-4-quinolone, dihydroevocarpine and evocarpine as well as the indoloquinazoline alkaloids rutaecarpine and evodiamine—all from the Chinese medicinal herb *Evodia rutaecarpa*—were tested in two in vitro assays, for cytotoxicity and interaction with P-glycoprotein. Cytotoxicity was measured in sensitive CCRF–CEM and multidrug-resistant

CEM/ADR5000 cells. An assay monitoring P-glycoprotein-dependent accumulation of the dye calcein in porcine brain capillary endothelial cells (PBCECs) was used to study interactions of the test substances with this efflux pump. Rutaecarpine and evodiamine showed a high toxicity (IC_{50} values from 2.64 to 4.53 μM) and were weak modulators of P-glycoprotein activity. The degrees of resistance in CEM/ADR5000 towards the saturated quinolones 1-methyl-2-undecyl-4-quinolone and dihydroevocarpine were between 3 and 4. In the calcein assay, these two quinolones were shown to be modulators of P-glycoprotein activity (Adams et al. 2007a).

Furthermore, four antimycobacterial geranylated furocoumarins, from the fruits of *Tetradium daniellii* (Rutaceae), showed considerable cell proliferation inhibition with IC_{50} values ranging from 1.72 to 1.02 μM against CCRF-CEM and 2.09 to 13.56 μM against CEM/ADR5000, respectively. The calcein assay to monitor P-glycoprotein function showed that all four compounds are modulators of P-glycoprotein (Adams et al. 2007b).

In a recent project, we assessed 57 chemically defined compounds derived from medicinal plants used in TCM for their potential to inhibit P-glycoprotein (Mahringer et al. 2010). For illustration, the binding of a well-known P-glycoprotein inhibitor, diosmetin, to human P-glycoprotein is shown in Fig. 7.3. Nine phytochemicals inhibited P-glycoprotein in multidrug-resistant CEM/ADR5000 cells as shown by a calcein fluorescence assay. The cytotoxicity of the 57 phytochemicals was measured by a growth inhibition assay. Seven compounds inhibiting P-glycoprotein at lower doses were cytotoxic to drug-sensitive parental CCRF-CEM cells at higher doses. Of them, five were not cross-resistant to CEM/ADR5000 cells (baicalein, bufalin, glybomine B, deoxyserofendic acid, and shogaol). Two compounds were not or were only weakly cytotoxic but inhibited P-glycoprotein (ent-16-atisen-19-oic acid and 4-methoxy[2,3-b]quinoline). Interestingly, P-glycoprotein-expressing multidrug-resistant CEM/ADR5000 cells were not cross-resistant, but were collateral sensitive towards scillarenin, and, thirdly, this compound was a strong inhibitor of P-glycoprotein in multidrug-resistant cancer cells and at the BBB. Hence, scillarenin may represent a novel candidate for improving the efficacy of cancer combination therapy regimens. Further analyses are warranted to characterize this compound in more detail. Collateral sensitivity is a frequently observed phenomenon in multidrug-resistant cells and represents hypersensitivity of multidrug-resistant cells to certain drugs (Hall et al. 2009). Hypersensitive drugs may be exquisitely suited to treat otherwise multidrug-resistant tumors.

Finally, it has also to be taken into account that modulators of multidrug resistance are not acting by inhibition of P-glycoprotein, but by altering membrane fluidity (Ramu et al. 1989). Further investigations to dissect drug-modulatory effects caused by inhibition of P-glycoprotein and by altering the membrane fluidity or integrity are warranted.

Another question is how to deal with the problem that P-glycoprotein in other normal tissues may also be affected by P-glycoprotein inhibitors (Volm et al. 1991; Efferth and Volm 1993a). Efforts to improve cancer chemotherapy by MDR inhibitors such as verapamil or PSC-833 were not successful in clinical phase III studies (Dalton et al. 1995; Baer et al. 2002) due to increased neurotoxic side effects.

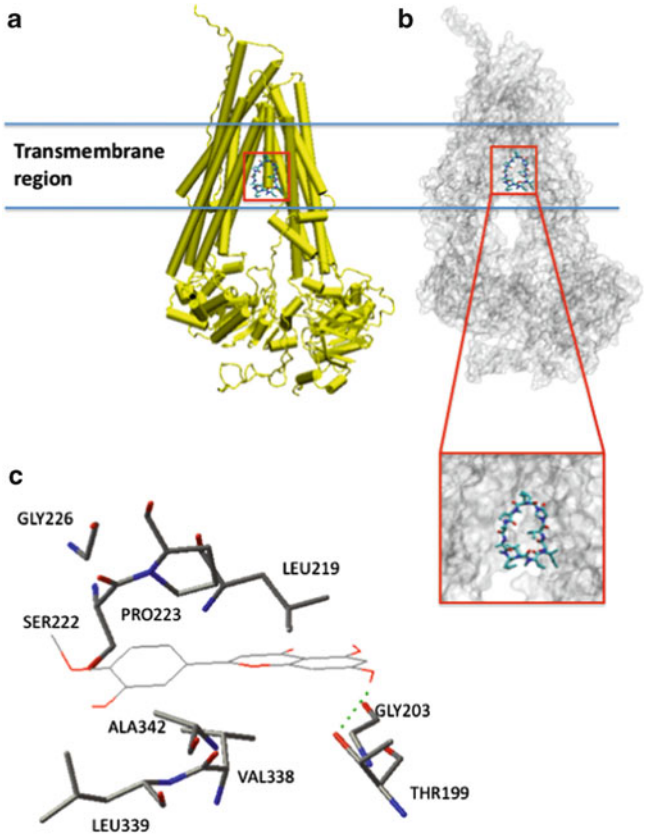

Fig. 7.3 Graphical representation of the binding of diosmetin to human P-glycoprotein. Details see Fig. 7.1. Diosmetin was used as an example for a natural product inhibiting P-glycoprotein. This figure has been generated by Dr. Tolga Eichhorn (Department of Pharmaceutical Biology, University of Mainz, Germany)

Novel MDR inhibitors may overcome these problems. Since herbal mixtures have been used in TCM for thousands of years, much is known about toxic effects. Therefore, it is not beyond the scope of expectations to search for safe P-glycoprotein inhibitors with fewer side effects towards normal tissues than synthetic drugs.

7.3.2 Inhibition of ABCC Transporters

Considering that cancer multidrug resistance (MDR) is multifactorial, agents with broad activities are preferable to the use of combination of several specific modulators to prevent drug–drug interaction and cumulative toxicity.

Vaidyanathan and Walle (2001) suggest a role for the multispecific organic anion transporter MRP2 in the bioavailability of (−)-epicatechin and possibly other tea flavonoids. The authors found a reduction in the efflux of (−)-epicatechin in the presence of MK-571, a competitive inhibitor of the MRP2 transporter expressed in the apical membrane of Caco-2 cells.

Theanine is a major amino acid in green tea. Sadzuka et al. (2002) examined its effects on the antitumor activity of cisplatin and irinotecan (CPT-11), which is known to be transported by an MRP-related efflux system. The combination of theanine with cisplatin decreased the volume of M5076 tumors in mice compared with the cisplatin-alone group. Tumor volume in the CPT-11-alone group did not show a decrease, but the combination of theanine with CPT-11 significantly reduced tumor volume. The concentration of cisplatin in the tumor was significantly increased by combination with theanine. Changes in drug concentrations with theanine were not significant.

The green tea components (−)-epigallocatechin gallate, (−)-epigallocatechin, theanine, or caffeine, each in corresponding concentrations to the respective concentration of green tea extracts, did not show any effect on MRP2 function (Netsch et al. 2005). The mRNA expression patterns of P-gp and MRP2 in LS-180 cells are not altered by green tea extracts. However, MRP2 function was inhibited by green tea extracts, whereas none of the green tea components were responsible for this effect.

ECG or EGCG at higher doses had a slight inhibitory effect on cell proliferation in the resistant human hepatocellular carcinoma cell line BEL-7404/DOX in vitro and in vivo, whereas the administration of DOX with these compounds at lower doses significantly inhibited HCC cell proliferation in vitro and hepatoma growth in a xenograft mouse model, compared with treatment with either agent alone at the same dose (Liang et al. 2010). Furthermore, the administration of DOX in combination with ECG or EGCG markedly enhanced intracellular DOX accumulation and the level of P-gp was decreased in cells concurrently treated with DOX and ECG or EGCG, whereas MRP1 expression was not changed significantly (Liang et al. 2010).

The roots of *Salvia miltiorrhiza* (Danshen) are widely used in the treatment of coronary heart disease, stroke, and less commonly Alzheimer's disease. Tanshinone IIB is a major constituent of Salvia miltiorrhiza. The uptake and efflux of tanshinone IIB in rat primary microvascular endothelial cells (RBMVEC) were ATP-dependent and significantly altered in the presence of a P-glycoprotein (P-gp) or multidrug resistance-associated protein (Mrp1/2) inhibitor (Zhou et al. 2007). A polarized transport of TSB was found in RBMVEC monolayers with facilitated efflux from the abluminal to luminal side. Addition of a P-gp inhibitor (e.g., verapamil) in both abluminal and luminal sides attenuated the polarized transport. In an in situ rat brain perfusion model, tanshinone IIB crossed the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier, and the brain penetration was increased in the presence of a P-gp or Mrp1/2 inhibitor. Furthermore, the brain levels of tanshinone IIB in mdr1a(−/−) and mrp1(−/−) mice were higher than those in the wild-type mice (Zhou et al. 2007).

The uptake and efflux of tanshinone IIB in Caco-2 cells were also significantly altered in the presence of an inhibitor for P-glycoprotein (PgP) or for multi-drug resistance-related protein (MRP1/2). Tanshinone IIB significantly inhibited the uptake of digoxin and vinblastine in membrane vesicles containing PgP or MRP1. Therefore, tanshinone IIB is a substrate for PgP and MRP1 (Yu et al. 2007c).

Cryptotanshinone (CTS) is another major constituent from the roots of Salvia miltiorrhiza (Danshen). Addition of a PgP (e.g., verapamil and quinidine) or multi-drug resistance protein 1/2 (MRP1/2) inhibitor (e.g., probenecid and MK-571) in both luminal and abluminal sides attenuated the polarized transport. In a bilateral in situ brain perfusion model, MRP1/2 inhibitors (e.g., probenecid) significantly increased the brain distribution of cryptotanshinone. The brain levels in mdr1a(−/−) and mrp1 (−/−) mice were higher than those in the wild-type mice, respectively, indicating that PgP and Mrp1 limit the brain penetration of cryptotanshinone (Yu et al. 2007b).

Chrysin (5,7-dihydroxyflavone) was examined using the human colonic cell line Caco-2 as a model of human intestinal absorption (Walle et al. 1999). The addition of the anion transport inhibitor MK-571 on the apical side inhibited the efflux of the metabolites chrysin glucuronide and chrysin sulfate, suggesting the involvement of the multidrug resistance protein MRP2 pump. Indeed, using specific antibodies, MRP2 was in fact detected by Western blotting in Caco-2 plasma membranes.

Plant flavonoids are polyphenolic compounds, commonly found in vegetables, fruits, and many food sources. Wu et al. (2005) investigated the interactions of six common polyphenols, quercetin, silymarin, resveratrol, naringenin, daidzein, and hesperetin, with the multidrug-resistance-associated proteins, MRP1, MRP4, and MRP5. At nontoxic concentrations, several of the polyphenols were able to modulate MRP1-, MRP4-, and MRP5-mediated drug resistances. The polyphenols also reversed resistance to NSC251820, a compound that appears to be a good substrate for MRP4, as predicted by data-mining studies. Furthermore, most of the polyphenols showed direct inhibition of MRP1-mediated [3 H]dinitrophenyl S-glutathione and MRP4-mediated [3 H]cGMP transport in inside-out vesicles prepared from human erythrocytes. Also, both quercetin and silymarin were found to inhibit MRP1-, MRP4-, and MRP5-mediated transport from intact cells with high affinity. They also had significant effects on the ATPase activity of MRP1 and MRP4 without having any effect on [32P]8-azidoATP[alphaP] binding to these proteins. These results suggest that dietary flavonoids such as quercetin and silymarin can modulate transport activities of MRP1, -4, and -5.

O'Leary et al. (2003) reported that the efflux of quercetin metabolites from HepG2 cells (methylated glucuronide and sulfate conjugates) was not altered by verapamil as P-glycoprotein inhibitor, but efflux was competitively inhibited by MK-571 as MRP inhibitor, indicating a role for multidrug resistant protein in the efflux of quercetin conjugates from HepG2 cells.

The influence of the two pairs of isoflavones: formononetin/daidzein and biochanin A/genistein on the efflux of fluorescent substrate of MRP1-like protein from erythrocytes and biophysical properties of lipid membranes has been compared by Łania-Pietrzak et al. (2005a). Compounds in each pair differ by the substituent in position 4′ of B ring of isoflavone molecule. In the process of

O-demethylation, CH_3-group (present in formonetin and biochanin A) is replaced by hydrogen (daidzein, genistein). Inhibition of MRP1-like protein transport activity by methylated and demethylated isoflavones was very similar. Their influence on lipid thermotropic properties and fluidity of lipid bilayer was not also significantly different.

Łania-Pietrzak et al. (2005b) investigated the influence of novel synthetic and plant origin flavonoids on activity of multidrug resistance-associated protein (MRP1) in human erythrocytes used as a cell model expressing MRP1 in plasma membrane. The fluorescent probe, BCPCF (2′, 7′-bis-(3-carboxy-propyl)-5-(and-6)-carboxyfluorescein), was applied as a substrate for MRP1 multidrug resistance transporter. The effect of natural flavonoids (flavone, flavonol, isoflavones, and flavanolignan) was compared with action of new synthetic derivatives of genistein. Most of the flavonoids showed strong or moderate ability to inhibit transport carried out by MRP1. Inhibitory properties of flavonoids were compared to the effects of indomethacin, probenecid, and MK-571 known as MRP1 inhibitors. Studying the influence of new synthetic genistein derivatives on BCPCF transport, the presence of hydrophobic groups substituting hydrogen of hydroxyl group at the position 4′ in ring B of isoflavone is more important for inhibitory properties than hydrophobic substitution at the position 7 in ring A. In case of naturally occurring isoflavones, the replacement of hydrogen at position 4′ by hydrophobic ring structure seems also to be favorable for inhibition potency.

Xanthohumol decreased the mRNA levels of ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCC3 (MRP3) (Lee et al. 2007). These results suggest that xanthohumol might be used in conjunction with other anticancer chemotherapeutic agents to reduce the drug resistance inhibiting the efflux drug transporters.

Glabridin is a major active constituent of *Glycyrrhiza glabra*. The uptake and efflux of glabridin in cultured RBMVECs were ATP-dependent and significantly altered in the presence of a PgP or multi-drug resistance protein (Mrp1/2) inhibitor (e.g., verapamil or MK-571) (Yu et al. 2007a). In an in situ brain perfusion model, co-perfusion of a PgP or Mrp1/2 inhibitor significantly increased the brain distribution of glabridin The area under the brain concentration–time curve (AUC) of glabridin in mdr1a(−/−) mice was higher than the wild-type mice, indicating that PgP limits the brain penetration of glabridin through the BBB (Yu et al. 2007a).

Patanasethanont et al. (2007) examined extracts and flavone derivatives from the rhizome of *Kaempferia parviflora* on multidrug resistance-associated proteins (MRPs)-mediated transport in A549 cells expressing MRP1 and MRP2, but not P-glycoprotein. The cellular accumulation of calcein, an MRP substrate, was significantly increased by various MRP inhibitors without being affected by verapamil, a typical P-glycoprotein inhibitor. The inhibitory potency of the ethanol extract for MRP function was greater than that of the aqueous extract. Among six flavone derivatives isolated from K. parviflora rhizome, 5,7-dimethoxyflavone exhibited a maximal stimulatory effect on the accumulation of doxorubicin in A549 cells. The accumulation of doxorubicin was increased by four flavone derivatives without 5-hydroxy group but not by the other two flavone derivatives with 5-hydroxy group.

Efferth et al. (2002) used CCRF-CEM parental cells and doxorubicin-selected P-glycoprotein (P-gp)/MDR1-expressing CEM/ADR5000, vinblastine-selected P-gp/MDR1-expressing CEM/VLB(100), and epirubicin-selected multidrug resistance-related protein 1 (MRP1)-expressing CEM/E1000 sublines thereof. As shown by flow cytometry, artesunate significantly increased daunorubicin accumulation in CEM/E1000 cells but not in CEM/VLB(100) or CCRF-CEM parental cells. Bufalin caused a small, but significant increase in daunorubicin accumulation in CEM/VLB(100) and CEM/E1000 cells. As artesunate and bufalin showed both antileukemic activity, if applied alone and modulation activity in combination with daunorubicin in multidrug-resistant (MDR) cells, these two drugs may be suitable for novel combination treatment regimens to improve leukemia cell killing.

Hwang-Ryun-Hae-Dok-Tang (HT; a standardized herbal formula consisting of extracts from Coptidis Rhizoma, Scutellariae Radix, Phellodendri Cortex, and Gardeniae Fructus). Yi et al. (2010) reported that HT was not affected by Mrp2-mediated herb–drug interaction in vivo.

A curcumin mixture and three major curcuminoids purified from turmeric (curcumin I, II, and III) were tested for their ability to modulate the function of MRP1 using HEK293 cells stably transfected with MRP1-pcDNA3.1 and pcDNA3.1 vector alone (Chearwae et al. 2006). Upon treating the cells with etoposide in the presence of curcuminoids, the sensitivity of etoposide was increased by several folds only in MRP1 expressing and not in pcDNA3.1-HEK 293 cells. Western blot analysis showed that the total cellular level of MRP1 protein level was not affected by treatment with curcuminoids for three days. The modulatory effect of curcuminoids on MRP1 function was confirmed by the inhibition of efflux of two fluorescent substrates, calcein-AM and fluo4-AM. Although all three curcuminoids increased the accumulation of fluorescent substrates in a concentration-dependent manner, curcumin I was the most effective inhibitor. In addition, curcuminoids did not affect 8-azido[alpha-(32)P]ATP binding; however, they did stimulate the basal ATPase activity and inhibited the quercetin-stimulated ATP hydrolysis of MRP1 indicating that these bioflavonoids interact most likely at the substrate-binding site(s).

Schisandrin B was an effective dual inhibitor of P-glycoprotein and multidrug resistance-associated protein 1 (MRP1) using HL60/ADR and HL60/MRP, the human promyelocytic leukemia cell lines with the overexpression of MRP1 but not P-gp. Schisandrin B resumed daunorubicin and carboxyfluorescein diacetate (CFDA, a specific substrate for MRP1) accumulation and retention. At the equimolar concentration, Sch B demonstrated significantly stronger potency than probenecid, a MRP1 inhibitor (Sun et al. 2007).

Li et al. (2007a, b) demonstrated that dibenzocyclooctadiene lignans were effective inhibitors of multidrug resistance-associated protein 1 (MRP1). The activities of schisandrin A, schisandrin B, schisantherin A, schisandrol A, and schisandrol B to reverse MRP1-mediated drug resistance were tested in HL60/Adriamycin (ADR) and HL60/Multidrug resistance-associated protein (MRP), two human promyelocytic leukemia cell lines with overexpression of MRP1 but not

P-gp. The five lignans could effectively reverse drug resistance of the two cell lines to vincristine, daunorubicin, and VP-16.

Cannabinoids enhanced the intracellular accumulation of two ABCC1 substrates, Fluo3 and vincristine, in ovarian carcinoma cells overexpressing ABCC1 (2008/MRP1) with a rank order of potency: cannabidiol > cannabinol > Delta(9)-tetrahydrocannabinol (Holland et al. 2008). Cannabinoid inhibition of ABCC1 was confirmed using insect cell membrane MRP1 ATPase assays.

Nabekura et al. (2008) investigated the effects of dietary phytochemicals on the functions of P-glycoprotein and MRP1. The effects of dietary phytochemicals on the functions of P-glycoprotein and MRP1 were investigated using P-glycoprotein-overexpressing human carcinoma KB-C2 cells and human MRP1 gene-transfected KB/MRP cells. The effects of natural compounds found in dietary supplements, herbs, and foods such as sesame, ginkgo, soybean, and licorice were evaluated. The accumulation of daunorubicin, a fluorescent substrate of P-glycoprotein, increased in the presence of sesamin, ginkgolic acid, matairesinol, glycyrrhetinic acid, glabridin, and phyllodulcin in KB-C2 cells. Glycyrrhetinic acid and matairesinol also increased the accumulation of calcein, a fluorescent substrate of MRP1, in KB/MRP cells. KB-C2 and KB/MRP cells were sensitized to anticancer drugs by glycyrrhetinic acid, showing that glycyrrhetinic acid reverses multidrug resistance. The verapamil-stimulated P-glycoprotein ATPase activity was inhibited by glycyrrhetinic acid. Glycyrrhetinic acid stimulated the ATPase activity of MRP1. These results suggest that glycyrrhetinic acid has dual inhibitory effects on P-glycoprotein and MRP1.

The absorption and transepithelial transport of six coumarins isolated from the roots of *Angelica pubescens f. biserrata* has been studied in the human Caco-2 cell monolayer model (Zhong et al. 2008). Umbelliferone, osthole, angelol-A, and angelol-B were highly absorbed compounds, and columbianadin and columbianetin acetate were moderately absorbed. Osthol and columbianadin accumulated in Caco-2 cells, and columbianetin acetate may be metabolized. The absorption and transport of angelol-B were not influenced by the change of pH and the presence of iodoacetamide or MK571, indicating no involvement of MRP1-mediated transport.

Gallbladder carcinoma represents a drug-resistant tumor type and novel strategies for cancer therapy are required. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a reactive oxygen species (ROS) generator. Co-treatment with emodin remarkably enhanced chemosensitivity of SGC996 gallbladder carcinoma cells in comparison with cisplatin, carboplatin, or oxaliplatin treatment alone (Wang et al. 2010a, b). The mechanisms may be attributed to reduction of glutathione and downregulation of multidrug resistance-related protein 1 (MRP1) expression in SGC996 cells. Co-treatment also inhibited tumor growth in vivo via increasing tumor cell apoptosis and downregulating MRP1 expression (Wang et al. 2010a, b).

Tetramethylpyrazine is a bioactive constituent isolated from the root of *Ligusticum chuanxiong* Hort, a Chinese herb. The mRNA level of multidrug-resistant gene MDR1, MRP2, MRP3, and MRP5 and the level of the proteins they encode were decreased after treatment with tetramethylpyrazine, indicating

this compound effectively reversed MDR in BEL-7402/ADM cells, and its activity mechanism may be correlated with the downregulation of expression in these transporters (Wang et al. 2010a, b).

7.3.3 *Inhibitors of BCRP/ABCG2*

Zhang et al. (2004a, b) determined the effective concentration 50 % (EC_{50}) of flavonoids (apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin) for breast cancer resistance protein (BCRP) inhibition when used alone and to evaluate their potential interactions (additive, synergistic, or antagonistic) with regard to BCRP inhibition in multiple-flavonoid combinations in MCF-7 MX100 cells overexpressing BCRP. Quantitative analysis of the combined effects of multiple flavonoids on mitoxantrone accumulation indicated that these flavonoids act additively in inhibiting BCRP when given as 2-, 3-, 5-, or 8-flavonoid combinations with equimolar concentrations of all constituents. The additive effects of multiple flavonoids provide a rationale for using "flavonoid cocktails" as a potential approach for multidrug resistance reversal in cancer treatment.

Zhang et al. (2005a, b) studied structure–activity relationships (SAR) and derived a quantitative SAR (QSAR) model for flavonoid–BCRP interaction. Testing of 25 flavonoids from five flavonoid subclasses revealed that the presence of a 2,3-double bond in ring C, ring B attached at position 2, hydroxylation at position 5, lack of hydroxylation at position 3, and hydrophobic substitution at positions 6, 7, 8, or 4′ were important structural properties important for potent flavonoid–BCRP interaction. These structural requirements were similar but not identical to those for a flavonoid-–nucleotide-binding domain of P-glycoprotein interaction, indicating that inhibition of BCRP by flavonoids may involve, in part, the binding of flavonoids with the nucleotide-binding domain of BCRP. In addition, a QSAR model consisting of three structural descriptors was constructed and validated. These findings should be useful for predicting BCRP inhibition activity of other untested flavonoids and for guiding the synthesis of potent BCRP inhibitors for potential clinical application.

Chrysin and 7,8-benzoflavone significantly inhibited the BCRP-mediated transport of topotecan in BCRP-overexpressing MCF-7 MX100 cells (MCF-7 cells selected with mitoxantrone) to a level comparable to that observed with the control BCRP inhibitor, fumitremorgin C. However, neither chrysin nor 7,8-benzoflavone significantly altered topotecan pharmacokinetics in rats or in mdr1a/1b (−/−) mice after oral coadministration (Zhang et al. 2005a, b). The reason for this lack of in vitro–in vivo association may be the lack of potent inhibition activity of the flavonoids against mouse or rat BCRP, as evidenced by our observation that these flavonoids have only weak, if any, inhibition activity against mouse Bcrp1-mediated transport of topotecan in MDCK-Bcrp1 cells.

Since numerous plant-derived anticarcinogens with aryl hydrocarbon receptor (AhR)-agonistic activity are known, Ebert et al. (2007) investigated the effects of

naturally occurring dietary compounds on BCRP expression. In Caco-2 cells, the most pronounced induction of BCRP expression was observed after treatment with dibenzoylmethane and quercetin, while green tea component (−)-epicatechin decreased BCRP expression. On mRNA level, quercetin, chrysin, flavone, and indole-3-carbinol showed a strong inducing effect, while genistein had no effect on BCRP mRNA expression. Curcumin and resveratrol showed a strong effect on BCRP induction in MCF-7 wild-type cells, but no response in AhR-deficient MCF-7AHR200 cells, supporting the hypothesis that BCRP is regulated via AhR-dependent signaling pathways. Antioxidant responsive element activators, sulforaphane and diethylmaleate, had no inducing effect on BCRP mRNA expression. Caco-2 cells pretreated with quercetin or diethylmaleate showed an enhancement of apically transported benzo[a]pyrene-3-sulfate, indicating that induced BCRP was functionally active. The authors concluded that the induction of BCRP by dietary constituents may contribute to the detoxification of food-derived procarcinogens.

Wang and Morris (2007) investigated the potential pharmacokinetic interactions between chrysin and nitrofurantoin (a specific BCRP substrate) in rats. Chrysin significantly inhibited nitrofurantoin transport mediated by human BCRP and murine Bcrp1 in vitro and in vivo. Bcrp1 inhibition by chrysin is likely one potential mechanism for the observed chrysin–nitrofurantoin pharmacokinetic interactions in rats.

Human ER negative breast cancer cells (MDA-MB-231) were tested in vitro for their response to tanshinone 1 A treatment (Jing et al. 2007). In addition to strong cytotoxic effects and the differential regulation of several biomarkers, BCRP/ABCG2 mRNA expression was downregulated by tanshinone I A.

Cannabinoids are used therapeutically for the palliation of the adverse side effects associated with cancer chemotherapy. Cannabinol, cannabidiol, and delta 9-tetrahydrocannabinol (THC) increased the intracellular accumulation of the BCRP substrate, mitoxantrone, in a BCRP-overexpressing cell line (Holland et al. 2007). The THC metabolite, (−)-11-nor-9-carboxy-delta 9-THC, was much less potent. The cannabinoids inhibited both basal and substrate-stimulated ATPase activity of BCRP. The cannabinoid were only cytotoxic at concentrations higher than those required for BCRP inhibition. Subtoxic concentrations of the cannabinoids resensitized the overexpressing cell line to the cytotoxic effect of BCRP substrates, mitoxantrone, and topotecan. This occurred in the absence of any effect on ABCG2 expression.

Marketed red clover (*Trifoleum pratense*) products use a wide variety of labels and the isoflavone content is frequently unclear. Wang et al. (2008) analyzed the content of various isoflavone products, and determined the content and how the sample matrix of red clover products affects the intestinal disposition of main isoflavones in Caco-2 cells. The isoflavone content varied significantly between the chosen products. Consequently, rates of isoflavone absorption across the Caco-2 cell monolayers also varied greatly. Permeabilities of biochanin A and formononetin were significantly affected by the product matrix. Biochanin A was the only isoflavone with noticeable metabolite peaks in both the apical and

basolateral sides. Rates of metabolism and the polarity of the glucuronidated biochanin A excretion were also altered by the product matrix. Studies using the BCRP inhibitor, dipyridamole, showed that both the apical and basolateral excretion of biochanin A glucuronides were reduced by dipyridamole. This provides evidence that BCRP is the main transporter responsible for the apical efflux of isoflavone glucuronides. The authors conclude that isoflavone contents of marketed red clover products were highly variable, and the product matrix significantly affected the intestinal disposition of red clover isoflavones by altering their absorption rates, permeabilities, biochanin A glucuronide excretion rates, and the polarity of biochanin A glucuronide excretion. This research provides scientific evidence to support the standardization effort, so that consumers can make intelligent product choices.

Brand et al. (2008) studied metabolism and transport of the flavonoid, hesperetin, and its aglycone using a two-compartment transwell Caco-2 cell monolayer system, simulating the intestinal barrier. Apically applied hesperetin (10 μM) was metabolized into hesperetin 7-O-glucuronide and hesperetin 7-O-sulfate. Hesperetin aglycone also permeated to the basolateral side, and this process was unaffected by several inhibitors of ABC transporters, possibly implying a passive diffusion process. Inhibition studies, however, showed that efflux of hesperetin conjugates to the apical side involved active transport, which from the pattern of inhibition appeared to involve mainly BCRP. Upon inhibition by the BCRP inhibitor Ko143, the apical efflux of hesperetin conjugates was reduced and transport to the basolateral side was increased. These findings show that BCRP-mediated transport could be a limiting step for hesperetin bioavailability.

Chen et al. (2008a, b) determined the absorption mechanism of five bioactive prenylated flavonoids (baohuoside I, icariin, epimedine A, B, and C) present in heat-processed *Epimedium koreanum* Nakai (Yin Yanghuo). In the perfused rat intestinal model, prenylated flavonoids with a monoglucosidic bond (e.g., icariin) were rapidly hydrolyzed into corresponding metabolites (e.g., baohuoside I). In the Caco-2 model, apical to basolateral permeability of a monoglycoside baohuoside I was greater than four prenylated flavonoids with 2 or more sugar moieties. The slow apical to basolateral transport of baohuoside I was the result of efflux. Efflux of baohuoside I was significantly suppressed by inhibitors of BCRP and MRP2, whereas efflux of icariin was significantly inhibited only by P-glycoprotein inhibitors. Because YHH is often heat-processed for better efficacy, Chen and colleagues determined the optimal condition for increasing contents of more bioavailable flavonoids (i.e., baohuoside I) to be 160–170°C for 5–7 min.

A total of 34 of compounds derived from naturally occurring flavonoids and synthetic analogs have been evaluated on cell lines overexpressing BCRP by Nicolle et al. (2009). By constructing a 3-day linear solvation energy, QSAR shape parameters and hydrophobicity were revealed to be major physicochemical parameters responsible for the inhibition activity of flavonoid derivatives and synthetic analogs towards ABCG2, whereas hydrogen-bond donor capacity appeared highly unfavorable.

Bio-guided fractionation of the roots of *Paris polyphylla* (Trilliaceae), based on inhibition of P-glycoprotein-mediated daunorubicin efflux in K562/R7 cell line, led to isolation of three saponins, 3-O-Rha(1 → 2)[Ara(1 → 4)]Glc-pennogenine, gracillin, and polyphyllin D, and two ecdysteroids, 20-hydroxyecdysone and pinnatasterone (Nguyen et al. 2009). These compounds were tested for multidrug reversion on P-glycoprotein and BCRP. In contrast to a weak efficiency on BCRP, the three saponins displayed significant inhibitory effects towards P-glycoprotein-mediated drug efflux.

Absorption of scutellarin is difficult in Caco-2 monolayer cells, which contributes to its low bioavailability. As reported by You et al. (2010) scutellarein absorption is better than scutellarin absorption. Scutellarein transepithelial transport was passive diffusion. P-gp inhibitors improved scutellarin and scutellarein transportation. Inhibitors of MRP2 and BCRP promoted transportation of scutellarin. MRP2 inhibitors promoted efflux of scutellarein. Hence, multidrug resistance-associated protein 2 may be a reason for low bioavailability f scutellarin.

Farabegoli et al. (2010) investigated the anticancer effect of (−)-Epigallocatechin-3-gallate (EGCG) treatment on a breast carcinoma cell line resistant to tamoxifen (MCF-7Tam cells). EGCG treatment caused cell growth inhibition and dose-dependent apoptosis. EGCG did not affect MRP1, but decreased P-gp expression. EGCG treatment also inhibited BCRP activity, but mRNA transcription and protein level did not change after treatment. The authors concluded that the potential use of EGCG in drug-resistant diseases should be considered.

The inhibitory effects of 9 herbal extracts and 23 isoflavonoids, including soybean-derived isoflavones, on BCRP-mediated methotrexate (MTX) transport were evaluated using BCRP-expressing membrane vesicles by Tamaki et al. (2010). Extracts of soybean, *Gymnema sylvestre*, black cohosh, passion flower, and rutin strongly inhibited BCRP-mediated transport of MTX, while inhibition by chlorella, milk thistle, and Siberian ginseng extracts was weak. Among the 23 isoflavonoids examined, all of which inhibited BCRP-mediated transport, coumestrol showed the most potent inhibition. The inhibitory potencies of six isoflavonoid glucosides were lower than those of the corresponding aglycones. The addition of a 5-hydroxyl or 6-methoxyl moiety tended to potentiate the inhibition. The inhibitory potency of daidzein was decreased by 7-glucuronidation but was virtually unaffected by 4′-sulfation. The authors conclude that some herbal and dietary supplements and isoflavonoids may increase the systemic availability of BCRP substrates when concomitantly given orally.

7.4 Conclusion and Perspectives

As described above, a huge number of natural products have been described to inhibit ABC transporters. Given the fact that also many synthetic compounds have been identified inhibiting P-glycoprotein or other ABC transporters the question arises, why synthetic inhibitors are not already established in clinical routine. The

answer is that clinical trials with synthetic inhibitors of ABC transporters have not been successful so far because of non-tolerable adverse effects. The toxicity of synthetic ABC transporter inhibitors might be a chance for natural products, many of which are well tolerable.

Another aspect concerns the fact that several ABC transporters are simultaneously expressed in tumors or certain organs. Therefore, the inhibition of one ABC transporter by a mono-specific synthetic compound may not result in considerably improved therapeutic effects, since drug transport may be taken over by other still functioning ABC transporters. The proof of principle of this hypothesis has been shown by us in a previous investigation (Efferth et al. 2006). Therefore, it may be favorable to inhibit several ABC transporters by one and the same inhibitor. Again, phytochemicals may be superior in this respect compared to synthetic compounds, since many natural compounds are multi-specific in nature. This means that they do not bind to a single target but to several targets. Exploiting this common feature of natural products might lead to the development of inhibitors, which specifically inhibit several ABC transporters at the same time leading to a more efficient drug treatment. Polyphenols may be interesting in this context, because they are well known for their bioactivity on the one hand and their multi-specificity in binding to proteins.

It can be envisaged that polyphenolic drugs are developed by selective derivatization with improved bioavailability or by galenic techniques, which fulfill the requirements of effective, safe, and multispecific ABC transporters.

References

Abidi P, Chen W, Kraemer FB, Li H, Liu J (2006) The medicinal plant goldenseal is a natural LDL-lowering agent with multiple bioactive components and new action mechanisms. J Lipid Res 47:2134–2147

Adams M, Mahringer A, Kunert O, Fricker G, Efferth T, Bauer R (2007a) Cytotoxicity and p-glycoprotein modulating effects of quinolones and indoloquinazolines from the Chinese herb Evodia rutaecarpa. Planta Med 73:1554–1557

Adams M, Mahringer A, Bauer R, Fricker G, Efferth T (2007b) In vitro cytotoxicity and P-glycoprotein modulating effects of geranylated furocoumarins from *Tetradium daniellii*. Planta Med 73:1475–1478

Allen SS, Cutting GR, Guggino WB (1995) CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. Cell 81:1063–1073

Allen JD, Brinkhuis RF, Wijnholds J, Schinkel AH (1999) The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. Cancer Res 59:4237–4241

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323:1718–1722

Allikmets R, Schrime LM, Hutchinson A, Romano-Spica V, Dean M (1998a) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 58:5337–5339

Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M (1998b) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 58:5337–5339

Angelini A, Di Ilio C, Castellani ML, Conti P, Cuccurullo F (2010) Modulation of multidrug resistance p-glycoprotein activity by flavonoids and honokiol in human doxorubicin- resistant sarcoma cells (MES-SA/DX-5): implications for natural sedatives as chemosensitizing agents in cancer therapy. J Biol Regul Homeost Agents 24:197–205

Arai Y, Masuda M, Sugawara I, Arai T, Motoji T, Tsuruo T, Oshimi K, Mizoguchi HY (1997) Expression of the MDR1 and MDR3 gene products in acute and chronic leukemias. Leuk Res 21:313–319

Baer MR, George SL, Dodge RK (2002) Phase 3 study of the multidrug-resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood 100:1224–1232

Bansal T, Awasthi A, Jaggi M, Khar RK, Talegaonkar S (2008) Pre-clinical evidence for altered absorption and biliary excretion of irinotecan (CPT-11) in combination with quercetin: possible contribution of P-glycoprotein. Life Sci 83:250–259

Bates SE, Robey R, Miyake K, Rao K, Ross DD, Litman T (2001) The role of half-transporters in multidrug resistance. J Bioenerg Biomembr 33:503–511

Belinsky MG, Chen ZS, Shchaveleva I, Zeng H, Kruh GD (2002) Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). Cancer Res 62:6172–6177

Bera TK, Lee S, Salvatore G, Lee B, Pastan I (2001) MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. Mol Med 7:509–516

Bera TK, Iavarone C, Kumar V, Lee S, Lee B, Pastan I (2002) MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proc Natl Acad Sci USA 99:6997–7002

Bergen AAB, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, de Jong PT (2000) Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 25:228–231

Bieche I, Girault I, Urbain E, Tozlu S, Lidereau R (2004) Relationship between intratumoral expression of genes coding for xenobioticmetabolizing enzymes and benefit from adjuvant tamoxifen in estrogen receptor alpha-positive postmenopausal breast carcinoma. Breast Cancer Res 6:R252–R263

Boonstra R, Timmer-Bosscha H, van Echten-Arends J, van der Kolk DM, van den Berg A, de Jong B, Tew KD, Poppema S, de Vries EG (2004) Mitoxantrone resistance in a small cell lung cancer cell line is associated with ABCA2 upregulation. Br J Cancer 90:2411–2417

Borst P, Evers R, Kool M, Wijnholds J (1999) The multidrug resistance protein family. Biochim Biophys Acta 1461:347–357

Brand W, van der Wel PA, Rein MJ, Barron D, Williamson G, van Bladeren PJ, Rietjens IM (2008) Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers. Drug Metab Dispos 36:1794–1802

Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 271:15091–15098

Calcabrini A, Stringaro A, Toccacieli L, Meschini S, Marra M, Colone M, Salvatore G, Mondello F, Arancia G, Molinari A (2004) Terpinen-4-ol, the main component of Melaleuca alternifolia (tea tree) oil inhibits the in vitro growth of human melanoma cells. J Invest Dermatol 122:349–360

Cao J, Chen X, Liang J, Yu XQ, Xu AL, Chan E, Wei D, Huang M, Wen JY, Yu XY, Li XT, Sheu FS, Zhou SF (2007) Role of P-glycoprotein in the intestinal absorption of glabridin, an active flavonoid from the root of Glycyrrhiza glabra. Drug Metab Dispos 35:539–553

Carr IB (1987) Pleiotropic drug resistance in hepatocytes induced by carcinogens administered to rats. Cancer Res 47:5577–5583

Chan K, Liu ZQ, Jiang ZH, Zhou H, Wong YF, Xu HX, Liu L (2006) The effects of sinomenine on intestinal absorption of paeoniflorin by the everted rat gut sac model. J Ethnopharmacol 103:425–432

Chang JY, Chang CY, Kuo CC, Chen LT, Wein YS, Kuo YH (2004) Salvinal, a novel microtubule inhibitor isolated from Salvia miltiorrhizae Bunge (Danshen), with antimitotic activity in multidrug-sensitive and -resistant human tumor cells. Mol Pharmacol 65:77–84

Chapman RS, Whetton AD, Dive C (1994) The suppression of drug-induced apoptosis by activation of v-ABL protein tyrosine kinase. Cancer Res 54:5131–5137

Chearwae W, Wu CP, Chu HY, Lee TR, Ambudkar SV, Limtrakul P (2006) Curcuminoids purified from turmeric powder modulate the function of human multidrug resistance protein 1 (ABCC1). Cancer Chemother Pharmacol 57:376–388

Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47:381–389

Chen ZS, Lee K, Kruh GD (2001) Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. J Biol Chem 276:33747–33754

Chen HY, Wu TS, Su SF, Kuo SC, Chao PD (2002) Marked decrease of cyclosporin absorption caused by phellamurin in rats. Planta Med 68:138–141

Chen KG, Valencia JC, Lai B, Zhang G, Paterson JK, Rouzaud F, Berens W, Wincovitch SM, Garfield SH, Leapman RD, Hearing VJ, Gottesman MM (2006) Melanosomal sequestration of cytotoxic drugs contributes to the intractability of malignant melanomas. Proc Natl Acad Sci USA 103:9903–9907

Chen Y, Wang X, Sun H, Xing D, Hu J, Wai Z, Du L (2008a) Characterization of the transportation of berberine in Coptidis rhizoma extract through rat primary cultured cortical neurons. Biomed Chromatogr 22:28–33

Chen Y, Zhao YH, Jia XB, Hu M (2008b) Intestinal absorption mechanisms of prenylated flavonoids present in the heat-processed Epimedium koreanum Nakai (Yin Yanghuo). Pharm Res 25:2190–2199

Chen YW, Lin GJ, Chuang YP, Chia WT, Hueng DY, Lin CK, Nieh S, Sytwu HK (2010) Triptolide circumvents drug-resistant effect and enhances 5-fluorouracil antitumor effect on KB cells. Anticancer Drugs 21:502–513

Childs S, Yeh RL, Georges E, Ling VS, Childs RL, Yeh E, Georges VL (1995) Identification of a sister gene to P-glycoprotein. Cancer Res 55:2029–2034

Childs S, Yeh RL, Hui D, Ling V (1998) Taxol resistance mediated by transfection of the liver-specific sister gene of P-glycoprotein. Cancer Res 58:4160–4167

Choi KH, Chen CJ, Kriegler M, Roninson IB (1988) An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the mdr1 (P-glycoprotein) gene. Cell 53:519–529

Choi SU, Park SH, Kim KH, Choi EJ, Kim S, Park WK, Zhang YH, Kim HS, Jung NP, Lee CO (1998) The bisbenzylisoquinoline alkaloids, tetrandine and fangchinoline, enhance the cytotoxicity of multidrug resistance-related drugs via modulation of P-glycoprotein. Anticancer Drugs 9:255–261

Choi CH, Kang G, Min YD (2003) Reversal of P-glycoprotein-mediated multidrug resistance by protopanaxatriol ginsenosides from Korean red ginseng. Planta Med 69:235–240

Cole SP, Deeley RG (1998) Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. Bioessays 20:931–940

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650–1654

Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley DR (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 54:5902–5910

Cui Y, König J, Buchholz U, Spring H, Leier I, Keppler D (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 55:929–937

Dai JY, Yang JL, Li C (2008) Transport and metabolism of flavonoids from Chinese herbal remedy Xiaochaihu- tang across human intestinal Caco-2 cell monolayers. Acta Pharmacol Sin 29:1086–1093

Dalton WS, Crowley JJ, Salmon SS, Grogan TM, Laufman LR, Weiss GR, Bonnet JD (1995) A phase II randomized study of oral verapamil as a chemosensitizer to reverse drug resistance in patients with refractory myeloma. A Southwest Oncology Group study. Cancer 75:815–820

Dean M, Allikmets R (1995) Evolution of ATP-binding cassette transporter genes. Curr Opin Genet Dev 5:779–785

Dong Y, Zhang Y, Yang Q, Li Y, Zhu X (2009) Absorption of extractive Radix Paeoniae Alba in rat everted gut sacs and its interaction with P-glycoprotein [Article in Chinese]. Zhongguo Zhong Yao Za Zhi 34:884–888

Doyle LA, Ross DD (2003) Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 22:7340–7358

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA 95:15665–15670

Ebert B, Seidel A, Lampen A (2007) Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. Toxicol Sci 96:227–236

Efferth T (1997) Die Zellbiologie der Tumorresistenz: Multidrug-Resistenz, genspezifische DNS-Schädigung und Apoptose. Habilitationsschrift, Aachen

Efferth T, Grassmann R (2000) Impact of viral oncogenesis on responses to anti-cancer drugs and irradiation. Crit Rev Oncog 11:165–187

Efferth T, Volm M (1993a) Modulation of P-glycoprotein-mediated multidrug resistance by monoclonal antibodies, immunotoxins or antisense oligodeoxynucleotides in kidney carcinoma and normal kidney cells. Oncology 50:303–308

Efferth T, Volm M (1993b) Reversal of doxorubicin-resistance in sarcoma 180 tumor cells by inhibition of different resistance mechanisms. Cancer Lett 70:197–202

Efferth T, Klett T, Mattern J, Osswald H, Pommerenke EW, Stöhr M, Volm M (1991) Reversing multidrug resistance in L1210 tumor cells by hycanthone or chlorophenoxamine in vitro and in vivo. Anticancer Res 11:1275–1279

Efferth T, Dunn TA, Berlion M, Langenbahn H, Pommerenke EW, Volm M (1993) Reversal of inherent multidrug-resistance in primary human renal cell carcinoma cell cultures by S 9788. Anticancer Res 13:905–908

Efferth T, Davey M, Olbrich A, Rücker G, Gebhart E, Davey R (2002) Activity of drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. Blood Cells Mol Dis 28:160–168

Efferth T, Gillet JP, Sauerbrey A, Zintl F, Bertholet V, de Longueville F, Remacle J, Steinbach D (2006) Expression profiling of ATP binding cassette transporters in childhood T-cell acute lymphoblastic leukemia. Mol Cancer Ther 5:1986–1994

Efferth T, Li PC, Konkimalla VS, Kaina B (2007) From traditional Chinese medicine to rational cancer therapy. Trends Mol Med 13:353–361

Efferth T, Konkimalla VB, Wang YF, Sauerbrey A, Meinhardt S, Zintl F, Mattern J, Volm M (2008) Prediction of broad spectrum resistance of tumors towards anticancer drugs. Clin Cancer Res 14:2405–2412

Eichhorn T, Efferth T (2012) P-glycoprotein and its inhibition in tumors by phytochemicals derived from Chinese herbs. J Ethnopharmacol 141:557–70

Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, Sali A (2006) Comparative protein structure modeling using modeller. Curr Protoc Bioinformatics Chapter 5: Unit 5.6

Evers R, Cnubben NHP, Wijnholds J, van Deemter L, van Bladeren PJ, Borst P (1997) Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. FEBS Lett 419:112–116

Evers R, Kool M, van Deemter L, Janssen H, Caolafat J, Oomen LC, Paulusma CC, Oude Elferink RP, Baas F, Schinkel AH, Borst P (1998) Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. J Clin Invest 101:1310–1319

Fairchild CR, Ivy SP, Rushmore T, Lee G, Koo P, Goldsmith M, Myers CE, Farber E, Cowan KH (1987) Carcinogen-induced mdr overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinomas. Proc Natl Acad Sci USA 84:7701–7705

Fan L, Tao GY, Wang G, Chen Y, Zhang W, He YJ, Li Q, Lei HP, Jiang F, Hu DL, Huang YF, Zhou HH (2009a) Effects of Ginkgo biloba extract ingestion on the pharmacokinetics of talinolol in healthy Chinese volunteers. Ann Pharmacother 43:944–949

Fan L, Mao XQ, Tao GY, Wang G, Jiang F, Chen Y, Li Q, Zhang W, Lei HP, Hu DL, Huang YF, Wang D, Zhou HH (2009b) Effect of Schisandra chinensis extract and Ginkgo biloba extract on the pharmacokinetics of talinolol in healthy volunteers. Xenobiotica 39:249–254

Farabegoli F, Papi A, Bartolini G, Ostan R, Orlandi M (2010) (−)-Epigallocatechin-3-gallate downregulates Pg-P and BCRP in a tamoxifen resistant MCF-7 cell line. Phytomedicine 17:356–362

Fellner S, Bauer B, Miller DS, Schaffrik M, Fankhanel M, Spruss T, Bernhardt G, Graeff C, Farber L, Gschaidmeier H, Buschauer A, Fricker G (2002) Transport of paclitaxel (Taxol) across the blood–brain barrier in vitro and in vivo. J Clin Invest 110:1309–1318

Fernandez-Checa JC, Takikawa H, Horie T, Ookhtens M, Kaplowitz N (1992) Canalicular transport of reduced glutathione in normal and mutant Eisai hyperbilirubinemic rats. J Biol Chem 267:1667–1673

Fischer V, Rodriguez-Gascon A, Heitz F, Tynes R, Hauck C, Cohen D, Vickers AE (1998) The multidrug resistance modulator valspodar (PSC 833) is metabolized by human cytochrome P450 3A. Implications for drug–drug interactions and pharmacological activity of the main metabolite. Drug Metab Dispos 26:802–811

Fong WF, Wan CK, Zhu GY, Chattopadhyay A, Dey S, Zhao Z, Shen XL (2007a) Schisandrol A from Schisandra chinensis reverses P-glycoprotein-mediated multidrug resistance by affecting Pgp-substrate complexes. Planta Med 73:212–220

Fong WF, Wang C, Zhu GY, Leung CH, Yang MS, Cheung HY (2007b) Reversal of multidrug resistance in cancer cells by Rhizoma Alismatis extract. Phytomedicine 14:160–165

Ford JM, Hait WN (1993) Pharmacologic circumvention of multidrug resistance. Cytotechnology 12:171–212

Fracasso PM, Goldstein LJ, de Alwis DP, Rader JS, Arquette MA, Goodner SA, Wright LP, Fears CL, Gazak RJ, Andre VA, Burgess MF, Slapak CA, Schellens JH (2004) Phase I study of docetaxel in combination with the P-glycoprotein inhibitor, zosuquidar, in resistant malignancies. Clin Cancer Res 10:7220–7228

Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, Sayegh MH, Sadee W, Frank MH (2005) ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. Cancer Res 65:4320–4333

Fu LW, He LR, Liang YJ, Chen LM, Xiong HY, Yang XP, Pan QC (2003) Experimental chemotherapy against xenografts derived from multidrug resistant KBv200 cells and parental drug-sensitive KB cells in nude mice by annonaceous acetogenin 89–2. Yao Xue Xue Bao 38:565–570

Gao N, Zhang Y, Mao JQ, Li GQ, Zhou W, Gao B, Gu J (2008) Effects of some traditional Chinese drugs on Mdr1 gene and its expression product in K562/A02 cells [Article in Chinese]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 16:785–789

Geng J, Zhou D (2008) Coriaria lactone induces in vitro the overexpression of multidrug resistance P-glycoprotein in astrocyte from rat brain [Article in Chinese]. Sichuan Da Xue Xue Bao Yi Xue Ban 39:80–83

Gerloff T, Steiger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 273:10046–10050

Gillet JP, Schneider J, Bertholet V, de Longueville F, Remacle J, Efferth T (2006) Microarray expression profiling of ABC transporters in human breast cancer. Cancer Genomics Proteomics 3:97–106

Girodon E, Cazeneuve C, Lebargy F, Chinet T, Costes B, Ghanem N, Martin J, Lemay S, Scheid P, Housset B, Bignon J, Goossens M (1997) CFTR gene mutations in adults with disseminated bronchiectasis. Eur J Hum Genet 5:149–155

Goda K, Bacsó Z, Szabó G (2009) Multidrug resistance through the spectacle of P-glycoprotein. Curr Cancer Drug Targets 9:281–297

Goffeau A, Park J, Paulsen IT, Jonniaux JL, Dinh T, Mordant P, Saier MH Jr (1997) Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames with the major facilitator superfamily. Yeast 13:43–54

Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM (1989) Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst 81:116–124

Gotoh A, Sakaeda T, Kimura T, Shirakawa T, Wada Y, Wada A, Kimachi T, Takemoto Y, Iida A, Iwakawa S, Hirai M, Tomita H, Okamura N, Nakamura T, Okumura K (2004) Antiproliferative activity of Rhinacanthus nasutus (L.) Kurz extracts and the active moiety, Rhinacanthin C. Biol Pharm Bull 27:1070–1074

Gottesman MM (1993) How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res 53:747–754

Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385–427

Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley DR (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Res 54:357–361

Gu Y, Wang G, Pan G, Fawcett JP, A J, Sun J (2004) Transport and bioavailability studies of astragaloside IV, an active ingredient in Radix Astragali. Basic Clin Pharmacol Toxicol 95:295–298

Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG, Kruh GD (2003) MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. J Biol Chem 278:29509–29514

Hall MD, Handley MD, Gottesman MM (2009) Is resistance useless? Multidrug resistance and collateral sensitivity. Trends Pharmacol Sci 30:546–556

He L, Liu GQ (2002) Effects of various principles from Chinese herbal medicine on rhodamine123 accumulation in brain capillary endothelial cells. Acta Pharmacol Sin 23:591–596

He L, Zhao C, Yan M, Zhang LY, Xia YZ (2009) Inhibition of P-glycoprotein function by procyanidine on blood–brain barrier. Phytother Res 23:933–937

He L, Yang J, Hu L (2010) Transmembrane transport activity of paclitaxel regulated by fangchinoline in MDR1-mDCK II cells [Article in Chinese]. Zhongguo Zhong Yao Za Zhi 35:1478–1481

Heijn M, Hooijberg JH, Scheffer GL, Szabo G, Westerhoff HV, Lankelma J (1997) Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. Biochim Biophys Acta 1326:12–22

Herweijer H, Sonneveld P, Baas F, Nooter KH, Herweijer P, Sonneveld F, Baas KN (1990) Expression of mdr1 and mdr3 multidrug-resistance genes in human acute and chronic leukemias and association with stimulation of drug accumulation by cyclosporine. J Natl Cancer Inst 82:1133–1140

Higgins CF (1995) The ABC of channel regulation. Cell 82:693–696

Higgins CF, Gottesman MM (1992) Is the multidrug transporter a flippase? Trends Biochem Sci 17:18–21

Higgins CF, Linton KJ (2004) The ATP switch model for ABC transporters. Nat Struct Mol Biol 11:918–926

Hill BT, Deuchars K, Hosking LK, Ling V, Whelan RDH (1990) Overexpression of P-glycoprotein in mammalian tumor cell lines after fractionated X irradiation in vitro. J Natl Cancer Inst 82:607–612

Hirohashi T, Suzuki H, Sugiyama Y (1999) ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). J Biol Chem 274:15181–15185

Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA, Brenner MK, Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA, Brenner MK (2004) A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101:14228–14233

Holland JK, Glidewell O, Cooper RG (1980) Adverse effect of radiotherapy on adjuvant chemotherapy for carcinoma of the breast. Surg Gynecol Obstet 150:817–821

Holland ML, Lau DT, Allen JD, Arnold JC (2007) The multidrug transporter ABCG2 (BCRP) is inhibited by plant-derived cannabinoids. Br J Pharmacol 152:815–824

Holland ML, Allen JD, Arnold JC (2008) Interaction of plant cannabinoids with the multidrug transporter ABCC1 (MRP1). Eur J Pharmacol 591:128–131

Hopper-Borge E, Chen ZS, Shchaveleva I, Belinsky MG, Kruh GD (2004) Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. Cancer Res 64:4927–4930

Hu YJ, Shen XL, Lu HL, Zhang YH, Huang XA, Fu LC, Fong WF (2008) Tenacigenin B derivatives reverse P-glycoprotein-mediated multidrug resistance inHepG2/Dox cells. J Nat Prod 71:1049–1051

Huang Y, Anderle P, Bussey KJ, Barbacioru C, Shankavaram U, Dai Z, Reinhold WC, Papp A, Weinstein JN, Sadée W (2004) Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance. Cancer Res 64:4294–4301

Huang M, Jin J, Sun H, Liu GT (2008a) Reversal of P-glycoprotein-mediated multidrug resistance of cancer cells by five schizandrins isolated from the Chinese herb Fructus Schizandrae. Cancer Chemother Pharmacol 62:1015–1026

Huang BB, Li GF, Ren F, Tang ZK, Ma HF, Sun YB, Chen LJ, Yang L (2008b) Effect of Glycyrrhiza inflata and Daphne genkwa on permeabilities of rhodamine 123, a P-glycoprotein substrate across rat jejunum membranes in vitro [Article in Chinese]. Zhongguo Zhong Yao Za Zhi 33:2521–2526

Huang Y, Dong G, Hong X, Chai H, Yuan X (2010) Studies on reversing MDR of K562/A02 by ramification of curcumin hydrolyzed [Article in Chinese]. Zhongguo Zhong Yao Za Zhi 35:2460–2463

Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci 17:463–468

Ishikawa T, Müller M, Klunemann C, Schaub T, Keppler D (1990) ATP-dependent primary active transport of cysteinyl leukotrienes across liver canalicular membrane Role of the ATP-dependent transport system for glutathione S-conjugates. J Biol Chem 265:19279–19286

Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D (1996) Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res 56:988–994

Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, Keppler D (1997) ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. Biochem J 327:305–310

Jing J, Zheng H, Wang J, Lin P, Zhang J, Xiong ZJ, Wu YY, Ren JJ, Yang HL, Wang XJ (2007) Growth inhibition and multidrug resistance-reversing effect of tanshinone I A on human breast cancer cell with estrogen receptor negative [Article in Chinese]. Sichuan Da Xue Xue Bao Yi Xue Ban 38:391–395

Johnsson A, Vallon-Christensson J, Strand C, Litman T, Eriksen J (2005) Gene expression profiling in chemoresistant variants of three cell lines of different origin. Anticancer Res 25:2661–2668

Juliano RL, Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455:152–162

Kawabata S, Oka M, Shiozawa K, Tsukamoto K, Nakatomi K, Soda H, Fukuda M, Ikegami Y, Sugahara K, Yamada Y, Kamihira S, Doyle LA, Ross DD, Kohno S (2001) Breast cancer resistance protein directly confers SN-38 resistance of lung cancer cells. Biochem Biophys Res Commun 280:1216–1223

Keith WN, Brown R (1991) Carcinogenesis and the response of tumours to anticancer drugs. Anticancer Res 11:1739–1743

Kelter G, Steinbach D, Konkimalla VB, Tahara Tahara T, Taketani S, Fiebig HH, Efferth T (2007) Role of transferrin receptor and the ABC transporters ABCB6 and ABCB7 for resistance and differentiation of tumor cells towards artesunate. PLoS One 2:e798

Kitagawa S, Nabekura T, Kamiyama S (2004) Inhibition of P-glycoprotein function by tea catechins in KB-C2 cells. J Pharm Pharmacol 56:1001–1005

Kobayashi K, Sogame Y, Hayashi K, Nicotera P, Orenius S (1988) ATP stimulates the uptake of S-dinitrophenylglutathione by rat liver plasma membrane vesicles. FEBS Lett 240:55–58

Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M, Kuwano M (1997) A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res 57:5475–5479

Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 57:3537–3547

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, Borst PM (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci USA 96:6914–6919

Kruh GD, Zeng H, Rea PA, Liu G, Chen ZS, Lee K, Belinsky MG (2001) MRP subfamily transporters and resistance to anticancer agents. J Bioenerg Biomembr 33:493–501

Kruijtzer CM, Beijnen JH, Rosing H, ten Bokkel Huinink WW, Schot M, Jewell RC, Paul EM, Schellens JH (2002) Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J Clin Oncol 20:2943–2950

Kuss BJ, O'Neill GM, Eyre H, Doggett NA, Callen DF, Davey RA (1998) ARA, a novel ABC transporter, is located at 16p13.1, is deleted in inv(16) leukemias, and is shown to be expressed in primitive hematopoietic precursors. Genomics 51:455–458

Laing NM, Belinsky MG, Kruh GD, Bell DW, Boyd JT, Barone L, Testa JR, Tew KD (1998) Amplification of the ATP-binding cassette 2 transporter gene is functionally linked with enhanced efflux of estramustine in ovarian carcinoma cells. Cancer Res 58:1332–1337

Lan K, He JL, Tian Y, Tan F, Jiang XH, Wang L, Ye LM (2008) Intra-herb pharmacokinetics interaction between quercetin and isorhamentin. Acta Pharmacol Sin 29:1376–1382

Łania-Pietrzak B, Hendrich AB, Zugaj J, Michalak K (2005a) Metabolic O-demethylation does not alter the influence of isoflavones on the biophysical properties of membranes and MRP1-like protein transport activity. Arch Biochem Biophys 433:428–434

Łania-Pietrzak B, Michalak K, Hendrich AB, Mosiadz D, Grynkiewicz G, Motohashi N, Shirataki Y (2005b) Modulation of MRP1 protein transport by plant, and synthetically modified flavonoids. Life Sci 77:1879–1891

LeBlanc MJ, Brunet S, Bouchard G, Lamireau T, Yousef IM, Gavino V, Lévy E, Tuchweber B (2003) Effects of dietary soybean lecithin on plasma lipid transport and hepatic cholesterol metabolism in rats. J Nutr Biochem 14:40–48

Lee Y, Yeo H, Liu SH, Jiang Z, Savizky RM, Austin DJ, Cheng YC (2004) Increased anti-P-glycoprotein activity of baicalein by alkylation on the A ring. J Med Chem 47:5555–5566

Lee SH, Kim HJ, Lee JS, Lee IS, Kang BY (2007) Inhibition of topoisomerase I activity and efflux drug transporters' expression by xanthohumol. from hops. Arch Pharm Res 30:1435–1439

Leier I, Jedlitschky G, Buchholz U, Center M, Cole SPC, Deeley RG, Keppler D (1996) ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. Biochem J 314:433–437

Leung PH, Zhang L, Zuo Z, Lin G (2006) Intestinal absorption of Stemona alkaloids in a Caco-2 cell model. Planta Med 72:211–216

Li GH, Sun FJ, Chen F, Yang SB, Zhang J (2007a) Effect on mouse S180 MDR tumour cell expression correlated factorial matter by 70 % ethanol with Huanglian Jiedu Tang [Article in Chinese]. Zhongguo Zhong Yao Za Zhi 32:1906–1908

Li L, Pan Q, Sun M, Lu Q, Hu X (2007b) Dibenzocyclooctadiene lignans: a class of novel inhibitors of multidrug resistance-associated protein 1. Life Sci 80:741–748

Li L, Stanton JD, Tolson AH, Luo Y, Wang H (2009a) Bioactive terpenoids and flavonoids from Ginkgo biloba extract induce the expression of hepatic drug-metabolizing enzymes through pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor-mediated pathways. Pharm Res 26:872–882

Li SL, Huang ZN, Hsieh HH, Yu WC, Tzeng WY, Lee GY, Chen YP, Chang CY, Chuu JJ (2009b) The augmented anti-tumor effects of Antrodia camphorata co-fermented with Chinese medicinal herb in human hepatoma cells. Am J Chin Med 37:771–783

Li Y, Wang Q, Yao X, Li Y (2010) Induction of CYP3A4 and MDR1 gene expression by baicalin, baicalein, chlorogenic acid, and ginsenoside Rf through constitutive androstane receptor- and pregnane X receptor-mediated pathways. Eur J Pharmacol 640:46–54

Liang G, Tang A, Lin X, Li L, Zhang S, Huang Z, Tang H, Li QQ (2010) Green tea catechins augment the antitumor activity of doxorubicin in an in vivo mouse model for chemoresistant liver cancer. Int J Oncol 37:111–123

Lin HL, Liu TY, Lui WY, Chi CW (1999a) Up-regulation of multidrug resistance transporter expression by berberine in human and murine hepatoma cells. Cancer 85:1937–1942

Lin HL, Liu TY, Wu CW, Chi CW (1999b) Berberine modulates expression of mdr1 gene product and the responses of digestive track cancer cells to Paclitaxel. Br J Cancer 81:416–422

Loe DW, Almquist KC, Ceeley RG, Cole SPC (1996) Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. J Biol Chem 271:9675–9682

Loe DW, Stewart RK, Massey TE, Deeley RG, Cole SPC (1997) ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. Mol Pharmacol 51:1034–1041

Loe DW, Deeley RG, Cole SPC (1998) Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res 58:5130–5136

Longhurst TJ, O'Neill GM, Harvie RM, Davey RA (1996) The anthracycline resistance-associated (ara) gene, a novel gene associated with multidrug resistance in a human leukaemia cell line. Br J Cancer 74:1331–1335

Loo TW, Bartlett MC, Clarke DM (2004) Disulfide cross-linking analysis shows that transmembrane segments 5 and 8 of human P-glycoprotein are close together on the cytoplasmic side of the membrane. J Biol Chem 279:7692–7697

Ma J, Maliepaard M, Nooter K, Loos WJ, Kolker HJ, Verweij J, Stoter G, Schellens JH (1998) Reduced cellular accumulation of topotecan: a novel mechanism of resistance in a human ovarian cancer cell line. Br J Cancer 77:1645–1652

Madgula VL, Avula B, Choi YW, Pullela SV, Khan IA, Walker LA, Khan SI (2008) Transport of Schisandra chinensis extract and its biologically-active constituents across Caco-2 cell monolayers - an in-vitro model of intestinal transport. J Pharm Pharmacol 60:363–370

Mahringer A, Karamustafa S, Klotz D, Kahl S, Konkimala VB, Wang YF, Wang JS, Liu H-Y, Boechzelt H, Hao XJ, Bauer R, Fricker G, Efferth T (2010) Inhibition of P-glycoprotein at the Blood–Brain Barrier by Phytochemicals Derived from Traditional Chinese Medicine. Cancer Genomics Proteomics 7:191–206

Majumder S, Dutta P, Mukherjee P, Datta ER, Efferth T, Bhattacharya S, Choudhuri SK (2006) Reversal of drug resistance in P-glycoprotein-expressing T-cell acute lymphoblastic CEM leukemia cells by copper N-(2-hydroxy acetophenone) glycinate and oxalyl bis (N-phenyl) hydroxamic acid. Cancer Lett 244:16–23

Maliepaard M, van Gastelen MA, de Jong LA, Pluim D, van Waardenburg RC, Ruevekamp-Helmers MC, Floot BG, Schellens JH (1999) Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. Cancer Res 59:4559–4563

Mayer R, Kartenbeck J, Büchler M, Jedlitschky G, Leier I, Keppler D (1995) Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. J Cell Biol 131:137–150

Mei Y, Qian F, Wei D, Liu J (2004) Reversal of cancer multidrug resistance by green tea polyphenols. J Pharm Pharmacol 56:1307–1314

Michaelis S, Berkower C (1995) Sequence comparison of yeast ATP-binding cassette proteins. Cold Spring Harb Symp Quant Biol 60:291–307

Mickley LA, Spengler BA, Knutsen TA, Biedler JL, Fojo AT (1997) Gene rearrangement: a novel mechanism for MDR-1 gene activation. J Clin Invest 99:1947–1957

Min YD, Yang MC, Lee KH, Kim KR, Choi SU, Lee KR (2006) Protoberberine alkaloids and their reversal activity of P-gp expressed multidrug resistance (MDR) from the rhizome of Coptis japonica Makino. Arch Pharm Res 29:757–761

Minemura M, Tanimura H, Tabor E (1999) Overexpression of multidrug resistance genes MDR1 and cMOAT in human hepatocellular carcinoma and hepatoblastoma cell lines. Int J Oncol 15:559–563

Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, Brangi M, Greenberger L, Dean M, Fojo T, Bates SE (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res 59:8–13

Molnár J, Engi H, Hohmann J, Molnár P, Deli J, Wesolowska O, Michalak K, Wang Q (2010) Reversal of multidrug resitance by natural substances from plants. Curr Top Med Chem 10:1757–1768

Mukai R, Satsu H, Shimizu M, Ashida H (2009) Inhibition of P-glycoprotein enhances the suppressive effect of kaempferol on transformation of the aryl hydrocarbon receptor. Biosci Biotechnol Biochem 73:1635–1639

Murakami T, Takano M (2008) Intestinal efflux transporters and drug absorption. Expert Opin Drug Metab Toxicol 4:923–939

Nabekura T, Yamaki T, Ueno K, Kitagawa S (2008) Inhibition of P-glycoprotein and multidrug resistance protein 1 by dietary phytochemicals. Cancer Chemother Pharmacol 62:867–873

Nakayama M, Wada M, Harada T, Nagayama J, Kusaba H, Ohshima K, Kozuru M, Komatsu H, Ueda R, Kuwano M (1998) Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. Blood 92:4296–4307

Naramoto H, Uematsu T, Uchihashi T, Doto R, Matsuura T, Usui Y, Uematsu S, Li X, Takahashi M, Yamaoka M, Furusawa K (2007) Multidrug resistance-associated protein 7 expression is involved in cross-resistance to docetaxel in salivary gland adenocarcinoma cell lines. Int J Oncol 30:393–401

Netsch MI, Gutmann H, Luescher S, Brill S, Schmidlin CB, Kreuter MH, Drewe J (2005) Inhibitory activity of a green tea extract and some of its constituents on multidrug resistance-associated protein 2 functionality. Planta Med 71:135–141

Nguyen VT, Darbour N, Bayet C, Doreau A, Raad I, Phung BH, Dumontet C, Di Pietro A, Dijoux-Franca MG, Guilet D (2009) Selective modulation of P-glycoprotein activity by steroidal saponines from *Paris polyphylla*. Fitoterapia 80:39–42

Nicolle E, Boccard J, Guilet D, Dijoux-Franca MG, Zelefac F, Macalou S, Grosselin J, Schmidt J, Carrupt PA, Di Pietro A, Boumendjel A (2009) Breast cancer resistance protein (BCRP/ABCG2): new inhibitors and QSAR studies by a 3D linear solvation energy approach. Eur J Pharm Sci 38:39–46

Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264:382–388, Review

Nishimura N (2005) Effects of Chinese herbal medicines on intestinal drug absorption [Article in Japanese]. Yakugaku Zasshi 125:363–369

Nobili S, Landini I, Giglioni B, Mini E (2006) Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets 7:861–879

Norwood K, Wang RY, Hirschmann-Jax C, Andreeff M, Brenner MK, Goodell MA, Wulf GG (2004) An in vivo propagated human acute myeloid leukemia expressing ABCA3. Leuk Res 28:295–299

O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G (2003) Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol 65:479–491

Oguri T, Bessho Y, Achiwa H, Ozasa H, Maeno K, Maeda H, Sato S, Ueda R, Oguri T, Bessho Y, Achiwa H, Ozasa H, Maeno K, Maeda H, Sato S, Ueda R (2007) MRP8/ABCC11 directly confers resistance to 5-fluorouracil. Mol Cancer Ther 6:122–127

Osmak M, Perovic S (1989) Multiple fractions of gamma rays induced resistance to cis-dichlorodiammineplatinum (II) and methotrexate in human HeLa cells. Int J Radiat Oncol Biol Phys 16:1537–1541

Park S, Shimizu C, Shimoyama T, Takeda M, Ando M, Kohno T, Katsumata N, Kang YK, Nishio K, Fujiwara Y (2006) Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients. Breast Cancer Res Treat 99:9–17

Patanasethanont D, Nagai J, Matsuura C, Fukui K, Sutthanut K, Sripanidkulchai BO, Yumoto R, Takano M (2007) Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. Eur J Pharmacol 566:67–74

Pratt S, Shepard RL, Kandasamy RA, Johnston PA, Perry W 3rd, Dantzig AH (2005) The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. Mol Cancer Ther 4:855–863

Priebe W, Krawczyk M, Kuo MT, Yamane Y, Savaraj N, Ishikawa T (1998) Doxorubicin- and daunorubicin-glutathione conjugates, but not unconjugated drugs, competitively inhibit leukotriene C4 transport mediated by MRP/GS-X pump. Biochem Biophys Res Commun 247:859–863

Qin XL, Bi HC, Wang XD, Li JL, Wang Y, Xue XP, Chen X, Wang CX, le Xu J, Wang YT, Huang M (2010) Mechanistic understanding of the different effects of Wuzhi Tablet (Schisandra sphenanthera extract) on the absorption and first-pass intestinal and hepatic metabolism of Tacrolimus (FK506). Int J Pharm 389:114–121

Qiu W, Jiang XH, Liu CX, Ju Y, Jin JX (2009) Effect of berberine on the pharmacokinetics of substrates of CYP3A and P-gp. Phytother Res 23:1553–1558

Ramu A, Pollard HB, Rosario LM (1989) Doxorubicin resistance in P388 leukemia–evidence for reduced drug influx. Int J Cancer 44:539–547

Roninson IB, Chin JE, Choi K, Gros P, Housman DE, Fojo A, Shen D, Gottesman MM, Pastan I (1986) Proc Natl Acad Sci USA 83:4538–4542

Ross DD, Yang W, Abruzzo LV, Dalton WS, Schneider E, Lage H, Dietel M, Greenberger L, Cole SP, Doyle LA (1999) Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J Natl Cancer Inst 91:429–433

Ruetz S, Gros P, Ruetz S, Gros P (1994) Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell 77:1071–1081

Ruiz JC, Choi K, von Hoff DD, Roninson IB, Wahl GM (1989) Autonomously replicating episomes contain mdr1 genes in a multidrug-resistant human cell line. Mol Cell Biol 9:109–115

Sadzuka Y, Yamashita Y, Kishimoto S, Fukushima S, Takeuchi Y, Sonobe T (2002) Glutamate transporter mediated increase of antitumor activity by theanine, an amino acid in green tea [Article in Japanese]. Yakugaku Zasshi 122:995–999

Saurin W, Hofnung M, Dassa E (1999) Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J Mol Evol 48:22–41

Scala S, Akhmed N, Rao US, Paull K, Lan LB, Dickstein B, Lee JS, Elgemeie GH, Stein WD, Bates SE (1997) P-glycoprotein substrates and antagonists cluster into two distinct groups. Mol Pharmacol 51:1024–1033

Schaub TP, Kartenbeck J, König J, Vogel O, Witzgall R, Kriz W, Keppler D (1997) Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules. J Am Soc Nephrol 8:1213–1221

Schinkel AH, Mayer U, Wagenaar E, Mol CAAM, van Deemter L (1997) Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc Natl Acad Sci USA 94:4028–4032

Shen X, Chen G, Zhu G, Fong WF (2006) (+/−)-3'-O, 4'-O-dicynnamoyl-cis-khellactone, a derivative of (+/−)-praeruptorin A, reverses P-glycoprotein mediated multidrug resistance in cancer cells. Bioorg Med Chem 14:7138–7145

Shen H, Xu W, Chen Q, Wu Z, Tang H, Wang F (2010) Tetrandrine prevents acquired drug resistance of K562 cells through inhibition of mdr1 gene transcription. J Cancer Res Clin Oncol 136:659–665

Sikic BI (1993) Modulation of multidrug resistance: at the threshold. J Clin Oncol 11:1629–1635

Sklar MD (1988) Increased resistance to cis-diamminedichloroplatinum(II) in NIH 3 T3 cells transformed by ras oncogenes. Cancer Res 48:793–797

Slovak ML, Hoeltge GA, Trent JM (1987) Cytogenetic alterations associated with the acquisition of doxorubicin resistance: possible significance of chromosome 7 alterations. Cancer Res 47:6646–6652

Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 75:451–462

Smith AJ, van Helvoort A, van Meer G, Szabo K, Welker E, Szakacs G, Varadi A, Sarkadi B, Borst P (2000) MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. J Biol Chem 275:23530–23539

Steinbach D, Gillet JP, Sauerbrey A, Gruhn B, Dawczynski K, Bertholet V, de Longueville F, Zintl F, Remacle J, Efferth T (2006) ABCA3 as a possible cause of drug resistance in childhood acute myeloid leukemia. Clin Cancer Res 12:4357–4363

Sun M, Xu X, Lu Q, Pan Q, Hu X (2007) Schisandrin B: a dual inhibitor of P-glycoprotein and multidrug resistance-associated protein 1. Cancer Lett 246:300–307

Suzuki H, Tanabe H, Mizukami H, Inoue M (2010) Selective regulation of multidrug resistance protein in vascular smooth muscle cells by the isoquinoline alkaloid coptisine. Biol Pharm Bull 33:677–682

Takahashi T, Kaku T, Sato T, Watanabe K, Sato J (2010) Effects of Acanthopanax senticosus HARMS extract on drug transport in human intestinal cell line Caco-2. J Nat Med 64:55–62

Takara K, Sakaeda T, Okumura K (2006) An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. Curr Pharm Des 12:273–286

Tamaki H, Satoh H, Hori S, Ohtani H, Sawada Y (2010) Inhibitory effects of herbal extracts on breast cancer resistance protein (BCRP) and structure-inhibitory potency relationship of isoflavonoids. Drug Metab Pharmacokinet 25:170–179

Tammur J, Prades C, Arnould I, Rzhetsky A, Hutchinson A, Adachi M, Schuetz JD, Swoboda KJ, Ptácek LJ, Rosier M, Dean M, Allikmets R (2001) Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. Gene 273:89–96

Taniguchi K, Wada M, Kohno K, Nakamura T, Kawabe T, Kawakami M, Kagotani K, Okumura K, Akiyama S, Kuwano M (1996) A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res 56:4124–4129

Tian Q, Zhang J, Tan TM, Chan E, Duan W, Chan SY, Boelsterli UA, Ho PC, Yang H, Bian S, Huang M, Zhu YZ, Xiong W, Li X, Zhou S (2005) Human multidrug resistance associated protein 4 confers resistance to camptothecins. Pharm Res 22:1837–1853

Tian Q, Zhang J, Chan SY, Tan TM, Duan W, Huang M, Zhu YZ, Chan E, Yu Q, Nie YQ, Ho PC, Li Q, Ng KY, Yang HY, Wei H, Bian JS, Zhou SF (2006) Topotecan is a substrate for multidrug resistance associated protein 4. Curr Drug Metab 7:105–118

Tiwari AK, Sodani K, Dai CL, Ashby CR Jr, Chen ZS (2011) Revisiting the ABCs of multidrug resistance in cancer chemotherapy. Curr Pharm Biotechnol 12(4):570–594

Tsai PL, Tsai TH (2004a) Hepatobiliary excretion of berberine. Drug Metab Dispos 32:405–412

Tsai PL, Tsai TH (2004b) Pharmacokinetics of baicalin in rats and its interactions with cyclosporin A, quinidine and SKF-525A: a microdialysis study. Planta Med 70:1069–1074

Tsai TH, Wu JW (2003) Regulation of hepatobiliary excretion of sinomenine by P-glycoprotein in Sprague–Dawley rats. Life Sci 72:2413–2426

Ueda K, Cardarelli C, Gottesman MM, Pastan I (1987) Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. Proc Natl Acad Sci USA 84:3004–3008

Vaidyanathan JB, Walle T (2001) Transport and metabolism of the tea flavonoid (−)-epicatechin by the human intestinal cell line Caco-2. Pharm Res 18:1420–1425

Vaidyanathan JB, Walle T (2003) Cellular uptake and efflux of the tea flavonoid (−)epicatechin-3-gallate in the human intestinal cell line Caco-2. J Pharmacol Exp Ther 307:745–752

van der Bliek AM, Baas F, Houte T, de Lange T, Koolman PM, van der Velde-Koerts T, Borst P (1987) The human mdr3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver. EMBO J 6:3325–3331

Volm M, Zerban H, Mattern J, Efferth T (1990) Overexpression of P-glycoprotein in rat hepatocellular carcinomas induced with N-nitrosomorpholine. Carcinogenesis 11:169–172

Volm M, Wagner-Pommerenke EW, Efferth T, Löhrke H, Mattern J (1991) Circumvention of multi-drug resistance in human kidney and kidney carcinoma in vitro. Cancer 67:2484–2489

Vulevic B, Chen Z, Boyd JT, Davis W Jr, Walsh ES, Belinsky MG, Tew KD (2001) Cloning and characterization of human adenosine 5Vtriphosphate-binding cassette, sub-family A, transporter 2 (ABCA2). Cancer Res 61:3339–3347

Walle UK, Galijatovic A, Walle T (1999) Transport of the flavonoid chrysin and its conjugated metabolites by the human intestinal cell line Caco-2. Biochem Pharmacol 58:431–438

Wan CK, Zhu GY, Shen XL, Chattopadhyay A, Dey S, Fong WF (2006) Gomisin A alters substrate interaction and reverses P-glycoprotein-mediated multidrug resistance in HepG2-DR cells. Biochem Pharmacol 72:824–837

Wang X, Morris ME (2007) Effects of the flavonoid chrysin on nitrofurantoin pharmacokinetics in rats: potential involvement of ABCG2. Drug Metab Dispos 35:268–274
Wang TX, Yang XH (2008) Reversal effect of isotetrandrine, an isoquinoline alkaloid extracted from Caulis Mahoniae, on P-glycoprotein-mediated doxorubicin-resistance in human breast cancer (MCF-7/DOX) cells. Yao Xue Xue Bao 43:461–466
Wang EJ, Barecki-Roach M, Johnson WW (2002) Elevation of P-glycoprotein function by a catechin in green tea. Biochem Biophys Res Commun 297:412–418
Wang C, Zhang JX, Shen XL, Wan CK, Tse AK, Fong WF (2004) Reversal of P-glycoprotein-mediated multidrug resistance by Alisol B 23-acetate. Biochem Pharmacol 68:843–855
Wang J, Xia Y, Wang H, Hou Z (2007) Chinese herbs of Shenghe Powder reverse multidrug resistance of gastric carcinoma SGC-7901. Integr Cancer Ther 6:400–404
Wang SW, Chen Y, Joseph T, Hu M (2008) Variable isoflavone content of red clover products affects intestinal disposition of biochanin A, formononetin, genistein, and daidzein. J Altern Complement Med 14:287–297
Wang W, Sun YP, Huang XZ, He M, Chen YY, Shi GY, Li H, Yi J, Wang J (2010a) Emodin enhances sensitivity of gallbladder cancer cells to platinum drugs via glutathion depletion and MRP1 downregulation. Biochem Pharmacol 79:1134–1140
Wang XB, Wang SS, Zhang QF, Liu M, Li HL, Liu Y, Wang JN, Zheng F, Guo LY, Xiang JZ (2010b) Inhibition of tetramethylpyrazine on P-gp, MRP2, MRP3 and MRP5 in multidrug resistant human hepatocellular carcinoma cells. Oncol Rep 23:211–215
Wijnholds J, Scheffer GL, van der Valk M, Beijnen JH, Scheper RJ, Borst P (1998) Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. J Exp Med 188:797–808
Wong VK, Chiu P, Chung SS, Chow LM, Zhao YZ, Yang BB, Ko BC (2005) Pseudolaric acid B, a novel microtubule-destabilizing agent that circumvents multidrug resistance phenotype and exhibits antitumor activity in vivo. Clin Cancer Res 11:6002–6011
Wu JY, Fong WF, Zhang JX, Leung CH, Kwong HL, Yang MS, Li D, Cheung HY (2003) Reversal of multidrug resistance in cancer cells by pyranocoumarins isolated from Radix Peucedani. Eur J Pharmacol 473:9–17
Wu CP, Calcagno AM, Hladky SB, Ambudkar SV, Barrand MA (2005) Modulatory effects of plant phenols on human multidrug-resistance proteins 1, 4 and 5 (ABCC1, 4 and 5). FEBS J 272:4725–4740
Wu YT, Lin CW, Lin LC, Chiu AW, Chen KK, Tsai TH (2010) Analysis of biliary excretion of icariin in rats. J Agric Food Chem 58:9905–9911
Wulf GG, Modlich S, Inagaki N, Reinhardt D, Schroers R, Griesinger F, Trümper L (2004) ABC transporter ABCA3 is expressed in acute myeloid leukemia blast cells and participates in vesicular transport. Haematologica 89:1395–1397
Xu D, Lu Q, Hu X (2006) Down-regulation of P-glycoprotein expression in MDR breast cancer cell MCF-7/ADR by honokiol. Cancer Lett 243:274–280
Yamano G, Funahashi H, Kawanami O, Zhao LX, Ban N, Uchida Y, Morohoshi T, Ogawa J, Shioda S, Inagaki N (2001) ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells. FEBS Lett 508:221–225
Yang CH, Schneider E, Kuo ML, Volk EL, Rocchi EM, Chen YC (2000) BCRP/MXR/ABCP expression in topotecan-resistant human breast carcinoma cells. Biochem Pharmacol 60:831–837
Yang XW, Guo QM, Wang Y (2008) Absorption and transport of 6 coumarins isolated from the roots of Angelica pubescens f. biserrata in human Caco-2 cell monolayer model (Article in Chinese). Zhong Xi Yi Jie He Xue Bao. 6(4):392–8
Yang CY, Chao PD, Hou YC, Tsai SY, Wen KC, Hsiu SL (2008a) Marked decrease of cyclosporin bioavailability caused by coadministration of ginkgo and onion in rats. Food Chem Toxicol 44:1572–1578

Yang XW, Guo QM, Wang Y (2008b) Absorption and transport of 6 coumarins isolated from the roots of Angelica pubescens f. biserrata in human Caco-2 cell monolayer model. [Article in Chinese]. Zhong Xi Yi Jie He Xue Bao 6:392–398

Yao HW, Fu XY, Xie QD, Huang BB, Sun YB, Li GF (2009) Effect of liquorice decoction on rat intestinal P-glycoprotein [Article in Chinese]. Nan Fang Yi Ke Da Xue Xue Bao 29:1571–1573

Yasui K, Mihara S, Zhao C, Okamoto H, Saito-Ohara F, Tomida A, Funato T, Yokomizo A, Naito S, Imoto I, Tsuruo T, Inazawa J (2004) Alteration in copy numbers of genes as a mechanism for acquired drug resistance. Cancer Res 64:1403–1410

Yi HJ, Oh JH, Lee YJ (2010) Absence of drug interaction between Hwang-Ryun-Hae-Dok-Tang and phenolsulfonphthalein. Arch Pharm Res 33:2025–2031

Yokooji T, Kida M, Mori M, Akashi H, Mori N, Yoshihara S, Murakami T (2010) Interaction of Rhei Rhizoma extract with cytochrome P450 3A and efflux transporters in rats. Pharmazie 65:367–374

You HS, Zhang HF, Dong YL, Chen SY, Wang MY, Dong WH, Xing JF (2010) Absorption and transportation characteristics of scutellarin and scutellarein across Caco-2 monolayer model. [Article in Chinese]. Zhong Xi Yi Jie He Xue Bao 8:863–869

Yu XY, Lin SG, Chen X, Zhou ZW, Liang J, Duan W, Chowbay B, Wen JY, Chan E, Cao J, Li CG, Zhou SF (2007a) Transport of cryptotanshinone, a major active triterpenoid in Salvia miltiorrhiza Bunge widely used in the treatment of stroke and Alzheimer's disease, across the blood–brain barrier. Curr Drug Metab 8:365–378

Yu XY, Lin SG, Zhou ZW, Chen X, Liang J, Yu XQ, Chowbay B, Wen JY, Duan W, Chan E, Li XT, Cao J, Li CG, Xue CC, Zhou SF (2007b) Role of P-glycoprotein in limiting the brain penetration of glabridin, an active isoflavan from the root of Glycyrrhiza glabra. Pharm Res 24:1668–1690

Yu XY, Lin SG, Zhou ZW, Chen X, Liang J, Liu PQ, Duan W, Chowbay B, Wen JY, Li CG, Zhou SF (2007c) Role of P-glycoprotein in the intestinal absorption of tanshinone IIA, a major active ingredient in the root of Salvia miltiorrhiza Bunge. Curr Drug Metab 8:325–340

Yu XY, Zhou ZW, Lin SG, Chen X, Yu XQ, Liang J, Duan W, Wen JY, Li XT, Zhou SF (2007d) Role of ATP-binding cassette drug transporters in the intestinal absorption of tanshinone IIB, one of the major active diterpenoids from the root of Salvia miltiorrhiza. Xenobiotica 37:375–415

Yu S, Yu Y, Liu L, Wang X, Lu S, Liang Y, Liu X, Xie L, Wang G (2010) Increased plasma exposures of five protoberberine alkaloids from Coptidis Rhizoma in streptozotocin-induced diabetic rats: is P-GP involved? Planta Med 76:876–881

Zaman GJR, Flens MJ, van Leusden MR, de Haas M, Mülder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 91:8822–8826

Zelcer N, Saeki T, Reid G, Beijnen JH, Borst P, Zelcer N, Saeki T, Reid G, Beijnen JH, Borst P (2001) Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). J Biol Chem 276:46400–46407

Zeng H, Bain LJ, Belinsky MG, Kruh GD (1999) Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. Cancer Res 59:5964–5967

Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD, Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD (2001) Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. Cancer Res 61:7225–7232

Zhang JP, Ying K, Xiao ZY, Zhou B, Huang QS, Wu HM, Yin M, Xie Y, Mao YM, Rui YC (2004a) Analysis of gene expression profiles in human HL-60 cell exposed to cantharidin using cDNA microarray. Int J Cancer 108:212–218

Zhang S, Yang X, Morris ME (2004b) Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. Pharm Res 21:1263–1273

Zhang S, Wang X, Sagawa K, Morris ME (2005a) Flavonoids chrysin and benzoflavone, potent breast cancer resistance protein inhibitors, have no significant effect on topotecan pharmacokinetics in rats or mdr1a/1b (−/−) mice. Drug Metab Dispos 33:341–348

Zhang S, Yang X, Coburn RA, Morris ME (2005b) Structure activity relationships and quantitative structure activity relationships for the flavonoid-mediated inhibition of breast cancer resistance protein. Biochem Pharmacol 70:627–639

Zhang J, Huang M, Guan S, Bi HC, Pan Y, Duan W, Chan SY, Chen X, Hong YH, Bian JS, Yang HY, Zhou S (2006) A mechanistic study of the intestinal absorption of cryptotanshinone, the major active constituent of Salvia miltiorrhiza. J Pharmacol Exp Ther 317:1285–1294

Zhang DM, He ZW, Liu XD, Li Y, Xie L, Wang GJ, Liu L (2007) In-vivo and in-vitro studies on the effect of Huang-Lian-Jie-Du-Tang on nimodipine transport across rat blood–brain barrier. J Pharm Pharmacol 59:1733–1738

Zhang R, Jie J, Zhou Y, Cao Z, Li W (2009) Long-term effects of Panax ginseng on disposition of fexofenadine in rats in vivo. Am J Chin Med 37:657–667

Zhou DC, Zittoun R, Marie JP (1995) Homoharringtonine: an effective new natural product in cancer chemotherapy. Bull Cancer 82:987–995

Zhou ZW, Chen X, Liang J, Yu XY, Wen JY, Zhou SF (2007) Involvement of P-glycoprotein and multidrug resistance associated protein 1 in the transport of tanshinone IIB, a primary active diterpenoid quinone from the roots of Salvia miltiorrhiza, across the blood–brain barrier. Drug Metab Lett 1:205–217

Zhu A, Wang X, Guo Z (2001) Study of tea polyphenol as a reversal agent for carcinoma cell lines' multidrug resistance (study of TP as a MDR reversal agent). Nucl Med Biol 28:735–740

Zhu DF, Zhao XP, Cheng YY (2006) Transport mechanism of danshensu across Caco-2 monolayer model [Article in Chinese]. Zhongguo Zhong Yao Za Zhi 31:1517–1521

Zou SS, Xu R, He QY (2010) Mechanism of Chinese herbal compound Zilongjin for antagonizing multi-drug resistance of tumor cells [Article in Chinese]. Zhongguo Zhong Xi Yi Jie He Za Zhi 30:601–606

Chapter 8
Activity of Artemisinin-Type Compounds Against Cancer Cells

Serkan Sertel, Peter K. Plinkert, and Thomas Efferth

8.1 Introduction

8.1.1 Botany and Geographical Distribution

Artemisia annua L. (Chinese: 青蒿; pinyin: qīnghāo), also known as sweet annie, sweet sagewort, or armoise annuelle, belongs to the medicinal plants derived from Traditional Chinese Medicine (TCM). This annual plant belongs to the family of *Asteraceae*. It is native to China and grows naturally as a part of steppe vegetation in northern parts of China at 1,000–1,500 m above the sea level (Wang 1961). It has a single stem of 50–200 cm in height with fern-like leaves, bright yellow blossoms, and a camphor-like scent. The reproduction occurs through cross-pollination by insect or wind distribution. The plant represents a typical neophyte in lowlands and hill countries in Asia and Europe continental to subcontinental climate.

8.1.2 Phytochemistry

Artemisinin is a sesquiterpene lactone with an internal peroxide bridge (Fig. 8.1) essential for its antiparasitic effect (Klayman 1985). Its systematical nomenclature is [3R-(3α, 5a β, 6 β, 9 α, 12 β, 12aR*)]-octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-j]-1,2 benzodioxepin-10(3*H*)-one.

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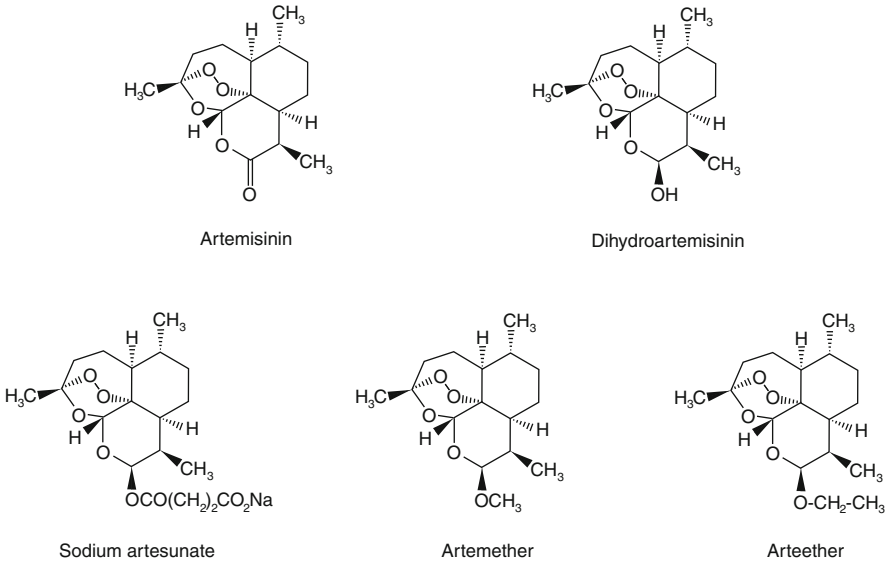

Fig. 8.1 Chemical structures of artemisinin and its semi-synthetic derivatives, artesunate, artemether, arteether, and dihydroartemisinic acid

Artemisinin is the lead compound of a novel class of antimalarial drugs of importance in the treatment of malaria in areas with multidrug-resistant *Plasmodium falciparum*. Relevant semi-synthetic derivatives of artemisinin with actual therapeutic application in malaria treatment are artesunate (ART), artemether (ARM), and arteether (ARE) (Fig. 8.1).

8.1.3 History

Qīnghāo has been used as a medicinal plant for at least 2,000 years in China. The earliest written record in silk so far discovered is the "Recipes for 52 kinds of diseases," which was found in the Mawangdui tomb of the West Han Dynasty (168 BC) in Changsha, Hunan Province (van Agtmael et al. 1999). The first record of qīnghāo for the treatment of fever and chills was "The Handbook of Prescriptions for Emergency Treatments" by Ge Hong (281–340 BC). The next historical source stems from the year 1,086 written by Shen Gua. Afterwards, a series of Chinese medicine books including the most famous book "The Compendium of Medical Herbs" (Bencao Gangmu) by Li Shizen in 1596 referred to qīnghāo.

8.1.4 Renaissance

The discovery of artemisinin goes back to the Vietnam War, in which Vietnam asked China for support, as more Vietnamese soldiers were dying from malaria than from armed conflicts. The Chinese government launched an antimalarial research program

to systematically search for antimalarial TCM plants to support the Vietnamese army. In 1972, Tu Youyou, a scientist from the Chinese Academy of TCM (Beijing, China), identified artemisinin (qīnghāosu) as the active antimalarial constituent of *A. annua* L. (Klayman 1985; Tu 1999; Li and Wu 1998). Today, artemisinin is used worldwide to combat otherwise drug-resistant *Plasmodium* strains, cerebral malaria, and malaria in children (Yeung et al. 2004). While *A. annua* and artemisinin were regarded by the World Health Organization (WHO) with much reluctance for a long time, the full potential was recently recognized. In the meantime, the WHO officially recommends artemisinin and its derivatives for the treatment of malaria, particularly as a part of combination therapies with other antimalarial drugs, called artemisinin-based combination therapies (ACTs).

Since the 1990s we focused our research on the sesquiterpene lactones of the artemisinin type from *A. annua* L. (Efferth et al. 1996). The *Artemisia* genus is known to contain many bioactive compounds (Tan et al. 1998). In previous studies, we have already shown the effects of artemisinin-type drugs against cancer cells lines in vitro and in vivo. In the present review, we have screened literature in order to illuminate scientific evidences and possible clinical indications for application in cancer therapy.

8.2 Molecular Pharmacology

8.2.1 Mechanisms of Action in Cancer Cells

Sensitivity or resistance to anticancer drugs is determined in a complex and multifactorial manner (Efferth et al. 1992; Efferth and Volm 1993; Volm et al. 1993, 2002a, b). Despite their diversity regarding chemical and physical features and cellular and molecular actions, a synopsis of the relevant mechanisms influencing drug effects allows their categorization into (1) those acting upstream of the actual drug target, (2) those acting at critical target sites, or (3) those acting downstream of them (Efferth and Grassmann 2000; Efferth and Volm 2005a).

Upstream mechanisms acting upstream include transporter proteins for uptake or excretion (i.e., ATP-binding cassette transporters (ABC-Transporter), reduced folate carriers, and nucleoside transporters) and drug-metabolizing enzymes that activate, inactivate, or detoxify drugs (i.e., phase I/II enzymes). Metabolizing enzymes and transporter molecules often do not exhibit high substrate specificity. Rather they are operative towards a wide array of divergent xenobiotic compounds and drugs. Drug-metabolizing enzymes affect pharmacokinetics and -dynamics. Drug target sites for alkylating agents and platinum drugs are DNA (and DNA repair mechanisms), RNA (RNA synthesis inhibitors, i.e., actinomycin D), and specific proteins such as DNA topoisomerases I/II (camptothecins, anthracyclines, and epipodophyllotoxins), tubulins (*Vinca* alkaloids and taxanes), or enzymes of DNA biosynthesis (antimetabolites).

Mechanisms downstream of the actual drug targets and at distinct intracellular locations are operative after injury by drugs has been taken place. The most important downstream mechanisms are the diverse apoptosis pathways. Disregulated apoptotic cell death contributes to drug resistance and survival of cancer cells, even if the appropriate drug targets have successfully been attacked (Efferth et al. 1997; Pommier et al. 2004). Apoptosis is not only regulated by the proteins directly involved in the apoptotic cascade but also by external factors, i.e., by chemokines that act as "survival factors" involved in prevention of apoptosis and, hence, contributing to survival and drug resistance of tumor cells after chemotherapeutic insult (Lotem and Sachs 1996; Efferth et al. 2002a).

It is, therefore, reasonable to propose that the same is true for cytotoxic compounds from TCM such as artemisinin and its derivatives. In order to get insight into the relevant mechanisms of this class of drugs, we applied pharmacogenomic approaches (Efferth et al. 2002b, 2003a; Efferth 2005, 2006). The anticancer activity of ART, ARE, ARM (Woerdenbag et al. 1993; Efferth et al. 2001, 2002a; Sertel et al. 2010a, b) is associated with the basal mRNA expression of genes, which most probably affect the proliferation of cells (cell cycle regulating genes, growth factors and their receptors, oncogenes, and tumor suppressor genes) (Efferth et al. 2002b). By microarray and hierarchical cluster analyses, a set of apoptosis-regulating genes was identified whose mRNA expression correlated significantly with the IC_{50} values for ART in 55 NCI cell lines. Furthermore, ART acts via p53-dependent and -independent pathways in isogenic p53+/+p21$^{WAF1/CIP1}$+/+, p53−/−p21$^{WAF1/CIP1}$+/+, and p53+/+p21$^{WAF1/CIP1}$−/− colon carcinoma cells (Efferth et al. 2003a).

DHA is the first metabolite of ART, ARM, or ARE and reveals considerable cytotoxicity towards cancer cells. DHA has exhibited the strongest anticancer activity among the derivatives of artemisinin. A number of studies have investigated the use of DHA to inhibit growth and/or to induce apoptosis of cells of breast cancer (Singh and Lai 2001), cervical cancer, uterus chorion cancer, embryo transversal cancer, ovarian cancer (Chen et al. 2003, 2008; Jiao et al. 2007), glioma (Huang et al. 2007), lung cancer (Mu et al. 2007, 2008), leukemia (Singh and Lai 2005; Lee et al. 2006), fibrosarcoma (Singh and Lai 2004), osteosarcoma (Fujita et al. 2008), and oral cancer (Nam et al. 2007). These compounds have also been used in vitro for enhancing radiosensitivity of glioma cells (Kim et al. 2006), cytotoxicity of pirarubicin and doxorubicin in leukemia and lung cancer cells (Reungpatthanaphong and Mankhetkorn 2002), cytotoxicity of sodium butyrate in leukemia cells (Singh and Lai 2005), and cytotoxicity of temozolomide for glioma cells (Huang et al. 2008). More recently, DHA has displayed significant cytotoxic effects towards human hepatoma cells with minimal effects on normal cells (Hou et al. 2008). Mechanisms that might explain the cytotoxic activity of DHA include its ability to induce apoptosis of lymphatic endothelial cells by regulating apoptosis-related proteins and downregulating VEGF-3, thus inhibiting lymphangiogenesis (Wang et al. 2007a). In lung cancer cells, an activation of P38 MAPK and increase of intracellular Ca^{2+} (Mu et al. 2008) or downregulating survivin expression was observed (Mu et al. 2007). Ovarian cancer cells have been reported to be regulated by the apoptosis-related proteins of the Bcl-2 family

(Jiao et al. 2007). DNA fragmentation in U2OS osteosarcoma cells by interfering with fortilin (Fujita et al. 2008) was found. Growth inhibition of C6 glioma cells was associated with an increase of reactive oxygen species (ROS) and inhibition activation of HIF1α (Huang et al. 2007). Other investigations point to the inhibition of angiogenesis by reducing extracellular signal-regulated kinase1/2 activation (Wu et al. 2006), downregulation of VEGF expression (Lee et al. 2006) and inhibition of proliferation, migration, and tube formation of vascular endothelial cells (Chen et al. 2003). More importantly, DHA revealed selective toxicity on breast cancer cells, but not on normal human breast cells (Singh and Lai 2001), and exerted potent cytotoxicity on ovarian carcinoma cells but had minimal effects on nontumorigenic human ovarian surface epithelial cells (Chen et al. 2008). This suggests that DHA might be well tolerated in a clinical setting and represents a potent promising therapeutic agent to treat cancers.

8.2.2 Angiogenesis Inhibition

The supply of tumor cells with oxygen and nutrients is crucial for tumor growth. Therefore, tumor neoangiogenesis represents a key step in cancer progression (Folkman 1992). Angiogenesis is promoted by numerous factors including cytokines, VEGF, bFGF, PDGF, etc., and negatively regulated by angiostatin, endostatin, thrombospondin, TIMP, and others. These factors are generated in tumor tissue and tumor-neighboured microenvironment. They act in a balance to promote either pro-angiogenic or anti-angiogenic processes (Relf et al. 1997). Inhibitors of angiogenesis that block angiogenic signals have been developed, and anti-angiogenic therapy strategies play clinically an important role as valuable adjuncts to cytostatic and cytotoxic chemotherapy (Kerbel and Folkman 2002; Broxterman et al. 2003; Shimizu and Oku 2004).

Artemisinin and DHA inhibited tumor angiogenesis as demonstrated by measurement of proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVEC) (Chen et al. 2003). DHA reduced VEGF binding to its receptors on the surface of HUVEC and reduced the expression levels of two major VEGF receptors, Flt-1 and KDR/flk-1, on HUVEC. Chicken chorioallantoic membrane (CAM) neovascularization was significantly inhibited by DHA (Chen et al. 2004a). The inhibitory effect of artemisinin on HUVEC proliferation was stronger than that on HeLa, JAR, HO-8910 cancer cells, NIH-3T3 fibroblast cells, and human endometrial cells (Chen et al. 2004b).

VEGF has an important role for tumor neo-vascularization. It binds to endothelial cell surface receptors and activates the growth of endothelial cells for vessel formation. One of the major VEGF receptors on vascular endothelial cells is KDR (kinase-insert-containing receptor)/Flk-1. ART also acts in an anti-angiogenic manner. It inhibited chicken CAM angiogenesis, as well as proliferation and differentiation of human microvascular dermal endothelial cells, and downregulated Flt-1 and KDR/flk-1 expression (Huan-huan et al. 2004). Furthermore, ART reduced

angiogenesis in vivo as determined by vascularization of Matrigel plugs injected *s.c.* into syngenic mice (Dell'Eva et al. 2004). ART also retarded growth of human ovarian cancer HO-8910 xenografts in nude mice. Microvessel density was less after ART exposure without apparent toxicity to the animals. ART also downregulated VEGF expression in tumor cells and KDR/flk-1 expression in both endothelial and tumor cells (Chen et al. 2004b). ART inhibited VEGF expression, a result, which correlated well with secreted VEGF levels in conditioned media (Zhou et al. 2007). The microarray-based mRNA expression of 30 out of 89 angiogenesis-related genes correlated with the cellular response to several artemisinins. Among this panel were many fundamental angiogenic regulators such as vascular endothelial growth factor C (VEGFC), fibroblast growth factor-2 (FGF2), matrix metalloproteinase 9 (MMP9), thrombospondin-1 (THBS1), hypoxia-inducing factor-α (HIF1A), angiogenin (ANG), and others. By means of hierarchical cluster analysis, expression profiles were identified that determined the cellular response to ART, ARE, ARM, and dihydroartemisinylester stereoisomer 1. A borderline significance ($0.05 < p < 0.1$) was observed to dihydroartemisinylester stereoisomer 2 and artemisinin (Anfosso et al. 2006). Sensitivity and resistance of tumor cells could be predicted by the mRNA expression profile of angiogenesis-related genes indicating that artemisinins reveal their antitumor effects at least in part by inhibition of tumor angiogenesis. Thioacetal artemisinin derivatives also inhibited HUVEC tube formation and exhibited anti-angiogenic effects (Oh et al. 2004). Endothelial cell proliferation and vessel-like formation were inhibited in a dose-dependent fashion by both DHA and artemisone. The effect of artemisone was less visible than that of DHA (D'Alessandro et al. 2007).

Tumor hypoxia activates the transcription factor hypoxia-inducible factor-1α (HIF1α) to increase tumor angiogenesis and to support the survival of poorly nourished cancer cells. Unfortunately, hypoxic tumors are resistant to radiation and many anticancer agents (Yu et al. 2002; Wouters et al. 2004). HIF-1α is activated during angiostatic therapy and upregulates transferrin receptor expression (McCarty 2003). Since artemisinin is selectively toxic to iron-loaded cells, radio-, and drug-resistant tumors, it is reasonable to speculate that this drug may be selectively susceptible to attack by iron-loading/artemisinin strategies.

Artemisinin dose-dependently inhibited angiogenesis in mouse embryoid stem cell-derived embryoid bodies through inhibiting HIF-1α and VEGF and raising the level of intracellular ROS. Furthermore, Artemisinin increases cell permeability by interfering organization of the extracellular matrix component laminin and varying expression patterns of MMP 1, 2, and 9 were observed during the time–course of embryoid body differentiation (Wartenberg et al. 2003). Inhibition of angiogenesis and increasing cell permeability for chemotherapeutics are both valuable features of artemisinin that qualify for usage in clinical oncology.

It can be concluded that inhibition of tumor angiogenesis represents an important determinant of artemisinin and its derivatives towards cancer cells in vitro and in vivo.

8.2.3 Metastasis

Most malignant tumors metastasize, although in varying degrees (e.g., glioma and basal cell carcinoma rarely metastasize). Lymph node involvement represents a clinical key factor for staging of cancers and is considered as an important prognostic indicator in a variety of human cancers (Tuttle 2004). Tumor-induced lymphangiogenesis can promote metastatic spread of cancer cells and influence prognosis and overall survival of cancer patients (McColl et al. 2005). Various lymphangiogenic molecules have been described, among which VEGF-C and VEGF-D are the most important lymphangiogenic growth factors. Both are able to stimulate growth, migration, and tube-like formation of lymphatic endothelial cells (LECs) and induce lymphangiogenesis by activating VEGF receptor 3 tyrosine kinase signals (Joukov et al. 1996; Achen et al. 1998).

DHA inhibited lymphangiogenesis by induction of apoptosis, inhibition of migration, and formation of tube-like structures in LECs. These effects were mediated by downregulation of VEGFR-3/Flt-4 (Wang et al. 2007a). Furthermore, artemisinin inhibited lymph node and lung metastasis by downregulating VEGF-C and reducing tumor lymphangiogenesis (Wang et al. 2008).

In human fibrosarcoma HT-1080 cells, DHA reduced PMA-induced activation of MMP-9 and MMP-2 and further inhibited cell invasion and migration (Hwang et al. 2010). DHA suppressed PMA-enhanced expression of MMP-9 protein, mRNA, and transcriptional activity through suppressing NF-kappaB and AP-1 activation without changing the level of tissue inhibitor of metalloproteinase (TIMP)-1. DHA also reduced PMA-enhanced MMP-2 expression by suppressing membrane-type 1 MMP (MT1-MMP) but did not alter TIMP-2 levels. DHA inhibited PMA-induced NF-kappaB and c-Jun nuclear translocation, which are upstream of PMA-induced MMP-9 expression and invasion. Furthermore, DHA strongly repressed the PMA-induced phosphorylation of Raf/ERK and JNK, which are dependent on the PKCalpha pathway. In conclusion, we demonstrated that the anti-invasive effects of DHA may occur through inhibition of PKCalpha/Raf/ERK and JNK phosphorylation and reduction of NF-kappaB and AP-1 activation, leading to downregulation of MMP-9 expression.

In colorectal tumor xenografts, ART not only decreased tumor growth but also delayed spontaneous liver metastasis. This was induced by membranous translocation of β-catenin and inhibition of the Wnt/β-catenin pathway (Li et al. 2007). Other evidence for the relevance of the Wnt/β-catenin pathway comes from microarray-based mRNA expression profiling (Konkimalla et al. 2008). Interestingly, this pathway plays an important role in colon carcinogenesis (Segditsas and Tomlinson 2006), and colon cancer cell lines were most sensitive towards ART among all solid tumor types tested (Efferth et al. 2001).

Artemisinin significantly inhibited the in vivo metastatic abilities of the HepG2 HCC cell line (Weifeng et al. 2011). The drug inhibited the invasion and metastasis of HCC cells both in vitro and in vivo by reducing the level of the MMP2 metalloproteinase and by inducing the TIMP2 protein. Artemisinin activated

Cdc42, which enhanced E-cadherin activity, resulting in greater cell–cell adhesion and significantly reduced metastasis.

8.2.4 Transferrin Receptor

Cancer cells require and uptake a large amount of iron to proliferate. Iron is an essential micronutrient for cell growth that plays an important role in energy metabolism and DNA synthesis, and iron levels are much higher in cancer cells compared with normal cells (Reizenstein 1991).

Artemisinin contains an endoperoxide group that can be activated by intracellular iron to generate toxic radical species and radical molecules. Oxidative stress induced by artemisinin-type drugs provoked oxidative stress response gene expression in cancer cells (Efferth and Volm 2005a; Efferth et al. 2003b; Efferth and Oesch 2004). Oxidative stress-mediated DNA damage may explain the cytotoxicity of this type of compounds towards cancer cells (Li et al. 2008; Mercer et al. 2011; Cabello et al. 2012).

TfR is involved in iron uptake by internalization of transferrin and is overexpressed in rapidly growing tumors. The tumor cell-specific cytotoxic effect of artemisinin can be attributed to the high concentrations of transferrin receptors (TfR) on cell surface and high intracellular iron contents as compared to normal cells. The susceptibility of tumor cells to artemisinins can be further enhanced by the addition of transferrin or ferrous iron (Moore et al. 1995; Efferth et al. 2004a).

In addition to TfR, iron is transported by ABC transporters ABCB6 and ABCB7. ABCB6 is involved in the biosynthesis of heme via interaction with ferrochelatase, which is regulated by iron (Taketani et al. 2003). Microarray-based mRNA expression of ABCB6, but not of ABCB7, correlated with IC_{50} values for ART in the NCI cell line panel. ART treatment induces ABCB6 but downregulates ABCB7 expression in MCF7 and CCRF-CEM cells. Consequently, ABCB6 may have a role in determining sensitivity to ART (Kelter et al. 2007).

TfR play another important role in tumor biology, as cancer cells express a large concentration of cell surface TfR that facilitate uptake of the plasma iron-carrying protein transferrin via endocytosis. By covalently tagging artemisinin to transferrin, artemisinin is selectively picked up and concentrated by cancer cells. Furthermore, both artemisinin and iron are transported into the cell in one package. Once an artemisinin-tagged transferrin molecule is endocytosed, iron is released and reacts with artemisinin moieties tagged to transferrin. Formation of free radicals kills the cancer cell. Artemisinin-tagged transferrin is highly selective and potent in killing cancer cells. Thus, artemisinin and artemisinin-tagged iron-carrying compounds could be developed into powerful anticancer drugs (Lai et al. 2005).

Artemisinin tagged to transferrin via carbohydrate chain revealed a high potency and specificity towards cancer cells. This drug conjugate enabled targeted delivery of artemisinin to cancer cells (Nakase et al. 2008). Artemisinin-tagged transferrins

exerted cytotoxic activity towards DU 145 prostate carcinoma cells by the mitochondrial pathway of apoptosis (Nakase et al. 2009).

Artemisinin can be enabled to cointernalize with receptor-bound transferrin by covalently conjugating it to HAIYPRH, a TfR-targeting peptide, that binds to a cavity on the surface of transferrin receptor. The iron released from transferrin can activate artemisinin to generate toxic radical species killing cells. The artemisinin-peptide conjugates showed potent anticancer activity against Molt-4 leukemia cells with a significantly improved cancer/normal cells selectivity (Oh et al. 2009).

DHA enhances cytotoxicity towards myeloid leukemia K562 cells growth by iron. In contrast, DHA downregulates TfR and VEGF expression (Wang et al. 2008). Furthermore, DHA induced HL-60 leukemia cell apoptosis by downregulation of TfR (Zhou et al. 2008) indicating a potential novel anti-leukaemic strategy.

8.2.5 Estrogen Receptor

Estrogen receptor α (ERα) overexpression in relation to ERβ is a typical feature of many breast cancer cells compared to normal breast tissues. Artemisinin selectively downregulated ERα expression without altering ERβ levels and disrupted ERα-responsive growth and gene expression. Artemisinin turned highly proliferative human breast cancer cells from expressing a high ERα to ERβ ratio to a growth-arrested state in which expression of ERβ was greater than that of ERα. This paralleled to the antiproliferative physiological state both in normal mammary epithelium and in breast cancer (Sundar et al. 2008).

Artemisinin may be potentially useful in adjuvant treatment settings in combination with well-established antiestrogens. In this regard, tamoxifen, a selective ER modulator (Gallo and Kaufman 1997), was currently used for the treatment of both early and advanced ER + (estrogen receptor positive) breast cancer. Patients may also benefit from lowering the systemic exposure to antiestrogens and minimizing undesirable side effects due to artemisinin–antiestrogen interactions.

8.2.6 Signal Transduction

Protein kinases (PK) are fundamental in many cellular processes such as proliferation, apoptosis, and differentiation (Grant et al. 2002; Shaul and Seger 2007). Further, PKs are also involved in signal transduction related to resistance towards established anticancer drugs (Navolanic et al. 2003; McCubrey et al. 2007). Previous findings show that EGFR confers resistance to ART (Efferth et al. 2003a); the role of PKs for ART's cytotoxic activity towards cancer cells was investigated (Konkimalla et al. 2009). AKT1 as a key molecule in the EGFR signaling was involved in ART resistance. There was also a significant relationship between MYC expression and ART response. MYC represents an important transcription

factor and oncogene, which is a downstream element in the EGFR signaling route and which regulates the cell cycle machinery also affecting cytotoxic cancer therapy. In contrast, no correlation was found between CRK, ABL1, and ART resistance. The AKT1 and MAPK pathways seem to be the most relevant ones associated with resistance of cancer cells to ART.

Exposure of dihydroartemisinin resulted in a pronounced increase in apoptosis in both transformed and primary human leukemia cells but not in normal peripheral blood mononuclear cells (Gao et al. 2011). Furthermore, DHA-mediated inhibition of tumor growth of mouse U937 xenograft was associated with induction of apoptosis and inactivation of ERK signaling.

In human prostate cancer cells, dihydroartemisinin suppressed the PI3-K/Akt and ERK cell survival pathways and triggered the induction of death receptor DR5 and activation of extrinsic and intrinsic cell death signaling (He et al. 2010).

8.2.7 Cell Cycle Effects

Cell cycle analyses performed by means of flow cytometry showed that 5-fluoro-1*H*-pyrimidine-2,4-dione (5-FU)-treated human gingival epithelial cells arrested in the S-phase (45 % vs. only 21 % of DHA-treated cells). This may explain the lower cytotoxicity and apoptosis induction by DHA in comparison to the highly cytotoxic effects of 5-FU (Yamachika et al. 2004).

Major players of the cell cycle machinery are the cyclin-dependent kinases (CDKs), their activating binding partners called cyclins, and a variety of cyclin-dependent kinase inhibitors (CKIs). CDKs bind to specific cyclin subunits to achieve the kinase activity necessary for the phosphorylation of substrates, which is required for cell cycle progression. Artemisinin signaling pathways inhibit prostate cancer cell growth in part by targeting the transcription of CDK4 and CDK2. This induces a G1 phase arrest. Artemisinin transcriptionally downregulated CDK4 expression by disruption of Sp1 interactions with the CDK4 promoter and, thereby, inhibited proliferation of prostate cancer cells (Willoughby et al. 2009).

Concurrent with the cell cycle arrest of MCF7 cells in the G1 phase, artemisinin selectively downregulated the transcript and protein levels of the CDK2 and CDK4 cyclin-dependent kinases, cyclin E, cyclin D1, and the E2F1 transcription factor (Tin et al. 2012). Analysis of CDK2 promoter–luciferase reporter constructs showed that the artemisinin ablation of CDK2 gene expression was accounted for by the loss of CDK2 promoter activity. Chromatin immunoprecipitation revealed that artemisinin inhibited E2F1 interactions with the endogenous MCF7 cell CDK2 and cyclin E promoters. Moreover, constitutive expression of exogenous E2F1 prevented the artemisinin-induced cell cycle arrest and downregulation of CDK2 and cyclin E gene expression. These results demonstrated that the artemisinin disruption of E2F1 transcription factor expression mediates the cell cycle arrest of human breast cancer cells.

Moloney murine leukemia virus insertion site 1 (BMI-1) has been shown to regulate proliferation by inhibiting p16(ink4a) transcription. It is well known that BMI-1 overexpression was found in nasopharyngeal carcinoma cell lines and correlated with advanced invasive stage of the tumor progression and poor prognosis. Wu et al. (2011) analyzed the inhibitory effects of artemisinin on proliferation of nasopharyngeal carcinoma cell lines and demonstrated that artemisinin induced G1 cell cycle arrest. Artemisinin inhibited BMI-1 both in protein and transcript levels. BMI-1 knockdown made the cells more sensitive to artemisinin with an increase in G1 phase, but overexpression of BMI-1 partially reversed the artemisinin-induced G1 cell cycle arrest. Depletion of BMI-1 was able to intensify the increment of p16 and the reduction of CDK4 induced by artemisinin. In addition, overexpression of BMI-1 was capable of attenuating the increasing p16 and decreasing CDK4 in cells treated with artemisinin.

To elucidate the genes mediating the effect of artesunate in the mitotic spindle checkpoint, knockout mutants of *Saccharomyces cerevisiae* were generated by Steinrück et al. (2010), since yeast knockouts are easier to generate than knockout strains of mammalian cells. Four out of the seven tested cell lines showed a G_2/M arrest upon artesunate exposure. Cells residing in the G_2/M arrest revealed multiple centrosomes, small multiple spindles, and multinucleated cells, suggesting a defect in cytokinesis. The mitotic spindle checkpoint genes bub1, bub2, bub3, mad1, mad2, and mad3 were individually deleted and the sensitivity of these mutants towards artesunate was determined by monitoring the cell growth. The Δbub3 and Δmad3 mutants showed an increased sensitivity and the Δmad2 mutant a slightly decreased sensitivity to artesunate in comparison to the respective wild type. Bub3, Mad3, and Mad2 are the main regulators of the mitotic spindle checkpoint, suggesting that artesunate may interfere with this control mechanism.

8.2.8 Apoptosis

ART and DHA were also cytotoxic towards human hepatoma cells, regardless of p53 status, with minimal effects on normal liver cells. Both compounds inhibited cell proliferation, induced G1 phase arrest, decreased cyclin D1, cyclin E, cyclin-dependent kinase 2, cyclin-dependent kinase 4, and E2F1 levels. In addition, Cip1/p21 and Kip1/p27 expression increased. ART and DHA induced apoptosis, activated caspase-3, increased the Bax/Bcl-2 ratio and poly (ADP-ribose) polymerase activity, and downregulated MDM2. Furthermore, DHA synergized the efficacy of the chemotherapeutic agent gemcitabine (Hou et al. 2008).

Artemisinin and its derivatives also exhibit potent immunosuppressive activity. ARM shows immunosuppressive effects directed towards T-cells both in vitro and in vivo by inhibiting the activation of the Ras–Raf1–ERK1/2 protein kinase cascade in T cells (Wang et al. 2007b). SM905, a new water-soluble artemisinin derivative suppresses T-cell activation both in vitro and in vivo associated with the inhibition of MAP kinases and Ras activation. It remains to be further analyzed, whether

artemisinin-type compounds represent a novel option for treating T-cell-mediated immune disorders (Wang et al. 2007c).

Treatment of Jurkat T-lymphoma cells with DHA induced a breakdown of the mitochondrial transmembrane potential, release of cytochrome c, activation of caspases, and DNA fragmentation indicative of apoptosis induction (Handrick et al. 2010). Although the absence of FADD or caspase-8 did not alter apoptosis rates in Jurkat cells, overexpression of dominant-negative caspase-9 or of anti-apoptotic Bcl-xL or Bcl-2 largely decreased the cytotoxicity of DHA, demonstrating a role of the intrinsic death pathway. The proapoptotic Bcl-2 effector protein Bak and the Bcl-2 homology domain 3-only protein NOXA turned out to be important mediators of DHA-induced apoptosis in Jurkat cells. DHA treatment triggered the expression of NOXA and the activation of Bak. Furthermore, DHA-induced apoptosis was completely abrogated by loss of Bak and largely reduced in cells with siRNA-mediated downregulation of Bak or NOXA. Proapoptotic signaling of DHA also involved the formation of ROS and membrane oxidation. Pretreatment with the lipophilic radical scavenger vitamin E or the hydrophilic radical scavengers glutathione and *N*-acetylcysteine reduced DHA-induced membrane oxidation and apoptosis, respectively. Oxidative changes also occurred in cells with disruption of the mitochondrial death pathway, suggesting a role of ROS and oxidative membrane changes in death signaling upstream of the mitochondria.

8.3 Metabolism

Hepatic metabolism plays a major role in processing and degradation of drugs and toxins. Hepatic enzymes, as predominantly the cytochrome P450 superfamily, presumably determine bioactivity of artemisinin and its derivatives.

After absorption, artemisinin derivatives such as ART are metabolized in the liver by phase II enzymes, cytochrome P450 monoxygenases, to DHA retaining its bioactivity (Haynes 2001; Woodrow et al. 2005). The metabolism of artemisinin in human liver microsomes is primarily mediated by cytochrome P-450 monooxygenase enzyme (CYP) 2B6, with a secondary contribution by CYP3A4 in individuals with low CYP2B6 expression. The contribution of CYP2A6 to artemisinin metabolism is likely of minor importance (Svensson and Ashton 1999). On the other hand, artemisinin can influence CYP activity, which could result in drug–drug interactions (Sukhija et al. 2006). An induction of activity by artemisinin was reported for CYP2A5, CYP2A6, CYP2B1, CYP2B6, CYP2B10, CYP2C19, and CYP3A4 (Svensson et al. 2003; Burk et al. 2005; Simonsson et al. 2006; Asimus et al. 2007, 2008; Elsherbiny et al. 2008; Bapiro et al. 2002, 2005). Induction of CYP2B6 was reported for artemisinin, DHA, ARE, ARM, and ART. Supplementary ARE and ARM also induced activity of CYP2C19 (Asimus et al. 2007). Upregulation of CYP2B6 and CYP3A4 might be explained by activation of the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) through artemisinin (Elsherbiny et al. 2008). In another investigation, artemisinin was an activator of

CAR, but not of PXR that resulted in upregulation of CYP2B (Simonsson et al. 2006). The data regarding CYP1A2 are contradictory (Bapiro et al. 2002, 2005; Asimus et al. 2007; He et al. 2007), whereas artemisinin inhibited CYP2D6 (Asimus et al. 2007). Artemisinin lead to auto-induction of drug metabolism, which reduced its own bioavailability (Gordi et al. 2005; Efferth et al. 2008). More research on this topic will be beneficial to gain insight in artemisinins bioavailability.

8.4 In Vivo Studies

In vivo studies pave the way for evaluation of biocompounds' suitability to inhibit tumor cell growth in patients. Nevertheless, in vivo studies with artemisinin are still sparse in literature.

ART inhibited Kaposi's sarcoma xenograft growth in vivo with growth retardation in endothelial cells that accounts for the anti-angiogenic effect (Dell'Eva et al. 2004).

ART suppressed cell growth of human colorectal carcinoma cell line CLY, established from liver metastasis of a 64-year-old patient with colon adenocarcinoma. In vitro, ART strongly inhibited the hyperactive Wnt/β-catenin pathway and promoted the apoptosis of CLY cells. In vivo, ART not only inhibited the volumetric development of tumor xenografts but also delayed spontaneous liver metastasis (Li et al. 2007).

DHA inhibits human ovarian cancer cell growth in vivo when administered alone or in combination with carboplatin, presumably through the death receptor- and mitochondrion-mediated caspase-dependent apoptotic pathway (Chen et al. 2008).

In human pancreatic cancer cells, DHA inhibited cell viability through downregulating the expression of proliferating cell nuclear antigen and cyclin D1 and upregulating p21 (WAF1/CIP1). Furthermore, DHA induced apoptosis by reducing the ratio of Bcl-2/Bax and increasing the activation of caspase-9 in a dose-dependent manner (Chen et al. 2009).

DHA and ART showed considerable growth inhibitory activity towards HPV-immortalized and HPV-transformed cervical cells in vitro through activation of the mitochondrial pathway of apoptosis. Topical application of DHA inhibited virus-induced tumor formation in vivo without preventing canine oral papilloma virus infection or replication in oral mucosa (Disbrow et al. 2005).

8.5 Toxicity

A discrepancy seems to prevail with regard to the toxicity and safety of the artemisinin family of antimalarials. While these compounds have been found to be virtually devoid of any serious side effects in humans, their neurotoxicity in animal models has raised concerns about their use. Mild and reversible hematological and electrocardiographic abnormalities, such as neutropenia and first-degree

heart block have been infrequently observed (Toovey 2006). Various neurotoxic side effects represent the main aspects of toxicity of artemisinin and its analogues in animal, in vitro, and human clinical studies. A specific and consistent pattern of brainstem injuries that includes auditory processing centers have been reported from all laboratory animals studied. Neurotoxicity appears to be mediated in part through artemisinin-induced oxidative stress in exposed brain stems. In vitro studies suggested that artemisinins' neurotoxicity does not manifest immediately upon exposure, but that once commenced, it is inevitable and irreversible. Extrapolation from in vitro data suggests that 14 days may possibly be required for full development, casting doubt upon some animal safety studies and human necropsy studies. Uncertainty remains over the neurotoxicity of currently deployed artemisinins and their safety profile should be reviewed, especially in pediatric use (Toovey 2006).

In laboratory studies, artemisinins can produce brainstem neurotoxicity. Selected nuclei in the medulla, pons, and mesencephalon are usually found to be most vulnerable. Species-specific differences in the vulnerability of nuclei may also exist. While not yet completely understood, occurrence of the lesion seems to be dependent upon sustained rather than peak levels of circulating drug or metabolite. With daily administrations, the onset of signs of brain stem neurotoxicity frequently develops abruptly and sometimes is observable only at the end of, or after, a regimen of administration. Behavioral correlates of brain stem neurotoxicity in laboratory animals include ataxic symptoms such as tremor, gait impairment, and balance disturbance (Genovese and Newman 2008).

In rats, dogs, and monkeys ARM was associated with an unusual toxicity pattern in specific brain nuclei involving the auditory and vestibular pathways (Nontprasert et al. 2002; Brewer et al. 1994a; Petras et al. 1997). Although artemisinin and its derivatives are tolerated well by malaria patients (Ribeiro and Olliaro 1998; Gordi and Lepist 2004; Adjuik et al. 2004), reports of toxicity studies are controversial. A report from Mozambique described a small but significant and irreversible hearing loss in patients exposed to ARM-lumefantrine (Toovey and Jamieson 2004). In contrast, in a case–control study from Thailand no irreversible and clinically significant neurophysiologic evidence of auditory brainstem toxicity could be attributed to ARM-lumefantrine in humans (Hutagalung et al. 2006). A recent prospective study came to the same result, in which neither audiometric nor auditory brainstem responses tests showed clinical evidence of auditory toxicity seven days after receiving oral ART and mefloquine (Carrara et al. 2008).

Clinical neurological defects have been attributed to toxicity in specific regions of the brain stem, e.g., the reticular system, the vestibular system, the auditory system (trapezoid nucleus), and the red nucleus (Brewer et al. 1994a, b; Petras et al. 1997; Genovese et al. 1998a, b; Kamchonwongpaisan et al. 1997; Panossian et al. 2005; Petras et al. 2000).

The main cause of the observed toxicity in animal studies seems to be the prolonged presence of artemisinins upon slow release from oil-based intramuscular formulations. Neurotoxicity increases with longer exposure time to a lower peak blood concentration of artemisinin compounds compared to a shorter duration of

exposure and a higher peak blood concentration (Li et al. 2002). In contrast, oral intake of these compounds, which is by far the most common formulation used for treatment of malaria patients, results in rapid clearance of these drugs and is, thus, unlikely to cause any toxicity in human subjects. Another plausible factor may be the relatively high doses of artemisinin compounds used in animal studies. In conclusion, the observation of the toxicity of artemisinin compounds in animals, but not in humans, is most likely due to different pharmacokinetic profiles after different routes of administrations (Gordi and Lepist 2004). In accord with these findings, artemisinin compounds show no significant neurotoxicity in a clinical safety review of 108 clinical studies with 9,241 malaria patients (Ribeiro and Olliaro 1998). Contrary, in a few clinical cases, artemisinin has caused ataxia, slurred speech, and hearing (Davis et al. 2005). Intramuscular ARM treatment of children with malaria has caused delayed coma recovery times in contrast to intravenous treatment with quinine (van Hensbroek et al. 1996). This unfavorable side effect has been disproved with a meta-analysis of 7 studies involving 1,919 malaria patients (Stepniewska et al. 2001). No significant differences in coma recovery time and neurological sequelae have been found between patients treated with ARM and quinine. This result has been reconfirmed in another study, where patients with malaria were treated with either ART or quinine (Dondorp et al. 2005). Again, no significant differences in neurotoxic symptoms have been found between treatment groups with no neurological sequelae after treatment. Interestingly, patients with malaria, who developed late onset hypoglycemia had a higher incidence of death than did patients treated with ART, who did not have hypoglycemia. This may be an issue that deserves additional investigation (Efferth et al. 2008).

In a clinical study, 60 out of 120 patients suffering from advanced non-small cell lung cancer (NSCLC) were treated with ART combined with a standard chemotherapy regimen of vinorelbine and cisplatin versus standard chemotherapy alone. Toxicity was observed including myelosuppression and digestion reactions without differences between ART-containing and noncontaining treatment arms (Zhang et al. 2008). This indicates that ART did not further contribute to side effects other than those provoked by vinorelbine and cisplatin.

All in all, human based clinical studies with artemisinin and its derivatives show advantageous effects in malaria treatment with less adverse reactions (Efferth and Kaina 2010). Therefore, artemisinin-type drugs seem also eligible for adjuvant therapy against cancer. The development of non-neurotoxic artemisinin-type drugs is possible and should be encouraged. However, phase I studies need to be conducted to pave the way for broader clinical implementation of these novel drugs.

8.6 Clinical Oncology

Application of artemisinin and its derivatives in clinical oncology is still not common, although the WHO officially recommends artemisinin and its derivatives for the treatment of malaria in combination with other antimalarial drugs (ACTs).

Otolaryngology was the first medical discipline, which applied ART the first time clinically in 2002. A 71-year-old male from India with laryngeal squamous cell carcinoma (T2 N1 M0) was treated with ART over a period of 9 months (60 mg ART *i.m.* per day for 16 days and 50 mg ART *p.o.* per day from day 16 onward). The tumor decreased by 70 % to its original size after 2 months of treatment (Singh and Verma 2002).

Two patients with metastatic uveal melanoma were treated with 100 mg ART *p.o.* per day on a compassionate use basis in combination with standard chemotherapy, after standard chemotherapy alone was ineffective in stopping tumor growth. One patient experienced a temporary response after the addition of ART to fotemustine while the disease was progressing under therapy with fotemustine alone. The second patient first experienced a stabilization of the disease after the addition of ART to dacarbazine, followed by objective regressions of splenic and lung metastases. This patient was still alive 47 months after first diagnosis of stage IV uveal melanoma, a situation with a median survival of 2–5 months (Berger et al. 2005).

A 75-year-old male patient with pituitary macroadenoma was treated with ARM over a period of 12 months. A 75-year-old male patient presented with vision, hearing, and locomotion-related problems. ARM was administered *p.o.* to the patient over a period of 12 months. Although the tumor remained consistent in size, CT scan showed a reduction in its density, and clinically, the related symptoms such as vision, hearing, and locomotion impairment considerably resolved. Overall, the ARM treatment was beneficial in improving the patient's quality of life (Singh and Panwar 2006).

In a Chinese clinical study, ART was applied in the treatment of 60 patients with advanced NSCLC. ART (120 mg *i.v.* per day, from the first day to eighth day, for 8 days) combined with a chemotherapy regimen of vinorelbine and cisplatin elevated the short-term survival rate and prolonged the time to progression of patients compared to chemotherapy treatment alone (Zhang et al. 2008).

ART has the potential of augmenting the activity of established chemotherapies. More applications of artemisinin and its derivatives in clinical oncology are to be expected in the next years.

8.7 Adjuvant Therapies

Therapy-resistant tumors require new treatment approaches with new drugs and adjuvant treatments. Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults, but the efficacy of chemotherapy is limited. The combination of ART and the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib in GBM cell lines resulted in an increased growth inhibition of GBM cell lines as compared to each drug alone. In addition, a profile of genomic imbalances was detected that predicted cellular response to ART and erlotinib (Efferth et al. 2004b).

Radiation in combination with chemotherapy is a common therapeutic strategy in clinical oncology. Hence possible radiosensitizing effects of drugs are an important factor improving response to treatment. DHA has a therapeutically relevant radiosensitizing effect on human glioma cell lines by triggering the production of ROS and inhibiting GST activity (Kim et al. 2006). Artesunate enhanced radiosensitivity of A549 cells in vitro and artesunate combined with local radiotherapy retarded the tumor growth in nude tumor xenografts in vivo (Zhao et al. 2011). In addition, DHA is able to increase temozolomide-induced apoptosis and necrosis via generating ROS in rat glioma C6 cells, suggesting a beneficial combination for the chemotherapy of gliomas (Huang et al. 2008).

Multidrug resistance (MDR) considerably hampers the success of anticancer therapy. Artemisinin, ART, and DHA increase cytotoxicity of pirarubicin and doxorubicin without decreasing the function of P-glycoprotein (Pgp) suggesting a mechanism by which the drugs reverse MDR at the mitochondrial level. Artemisinin and its derivatives qualify as compounds that can be used in combination with anticancer drugs to overcome MDR (Reungpatthanaphong and Mankhetkorn 2002). Another strategy to overcome MDR in cancer cells is the combination of a chemotherapeutic and a chemomodulator that inhibits the activity of the resistance-causing protein. Glutathione S-transferases (GSTs) have been suggested to be necessary for the efflux of anticancer drugs from tumor cells (Ishikawa 1996). Artemisinin was found to inhibit human GSTs (Mukanganyama et al. 2002; Efferth and Volm 2005b). The ubiquitous expression of GSTs in different malignancies suggests that artemisinin, as a chemomodulator during chemotherapy could involve targeting GSTs and, thereby, enhancing the efficacy of a variety of alkylating agents (Mukanganyama et al. 2002). Moreover, it seems reasonable to hypothesize that glutathione-related enzymes contribute to resistance of tumor cells to ART (Efferth and Volm 2005b).

There is considerable interest among basic and clinical researchers in novel drugs with activity against leukemia. ART and bufalin show anti-leukemic activity if applied alone. In addition, both show modulation activity in combination with daunorubicin in MDR cells. For this reason, these two drugs may be suitable for a novel combination treatment of leukemia (Efferth et al. 2002c).

DHA is discussed as a promising therapeutic agent for ovarian cancer either alone or in combination with conventional chemotherapy, as it inhibits human ovarian cancer cell growth in vitro and in vivo when administered alone or in combination with carboplatin (Chen et al. 2008).

Artemisinin is proposed to be used in combinational therapies with fulvestrant, a steroidal antiestrogen, that cooperate in decreasing ER protein levels, leading to attenuation of estrogen-mediated proliferative signaling in breast cancer cells. Thus, artemisinin qualifies as a candidate for adjuvant therapy with fulvestrant and could be extended to other breast cancer therapies such as tamoxifen (Sundar et al. 2008).

MDR is a limiting factor in chemotherapy of non-Hodgkin's lymphoma. The monoclonal antibody rituximab specifically targets the CD20 antigen and sensitizes B-cell lymphoma cells to standard anticancer drugs. The combination of rituximab

and ART act in a complementary manner and synergize in tumor cell killing (Sieber et al. 2009).

Artemisinin and its derivatives excel as pleiotropic in their antitumor effects (Efferth 2006; Nakase et al. 2008). ART induces apoptosis in a doxorubicin-resistant leukemia T-cell line mainly through the mitochondrial pathway via generation of ROS, a mechanism different from doxorubicin. In addition, the combination of ART and doxorubicin enhances apoptosis in leukemic T cells. This synergistic effect can be explained by the fact that ART and doxorubicin act through different killing pathways or mechanisms (Efferth et al. 2007). Interestingly, artemisinin and its structural homologue parthenolide in combination with doxorubicin have a converse effect in colon cancer cells, as both induce resistant cancer cells. This drug resistance is induced by (1) RhoA/Rho kinase activation (Riganti et al. 2008) and (2) inhibition of sarcoplasmic/endoplasmic reticulum Ca^{++}-ATPase (SERCA) via the CaMKII-dependent activation of HIF-1α and the induction of Pgp (Riganti et al. 2009). Contrary, ART is similarly active towards drug-sensitive and multidrug-resistant cell lines which overexpress Pgp (Efferth 2006). Parthenolide has antiproliferative and anti-angiogenic properties and therefore may qualify as an adjuvant drug in chemotherapy (Sweeney et al. 2005). A phase I clinical trial has shown the safety of orally administered parthenolide in cancer patients (Curry et al. 2004).

Butyric acid is a known short chain fatty acid with apoptotic effect on colon cancer cells. A combined treatment of DHA and butyric acid acted synergistically at low doses in killing human lymphoblastoid leukemia cells (Singh and Lai 2005).

Adjuvant therapies of conventional chemotherapeutics in combination with artemisinin, as a chemomodulator, seem to provide less toxic, inexpensive, and effective cancer chemotherapies in future.

8.8 Biotechnology

The worldwide demand of artemisinin has exponentially increased since the WHO has officially recommended artemisinin and its derivatives for the treatment of malaria, especially in ACT. As the raw material is extracted from plants with long growing seasons, artemisinin is often in short supply. Chemical synthesis of artemisinin is not practical due to its complexity and low yield (White 2008). Other possibilities for meeting the high demand for artemisinin are found in the natural production of artemisinin by phytotherapeutic and agricultural approaches and in biotechnological approaches.

The yield of artemisinin in wild populations of *Artemisia annua* is low (0.01–0.8 % dry weight). Therefore, there is a considerable limitation to commercialization of the drug (Van Geldre et al. 1997; Abdin et al. 2003). Total synthesis of the product is feasible but time-consuming and expensive. Several synthesis routes with (−)-isopulegol, (+)-isolimenene, or (R)-(+)-pulegone as starting molecules have been described (Efferth 2007). The semi-synthetic production of artemisinin

from its precursor artemisinic acid has also been shown. Artemisinic acid is present in tenfold excess in the plants. Hence, the semi-synthetic artemisinin yield is considerably higher than the isolation of artemisinin from plants. To preserve the natural resources of *A. annua* plants, artemisinin-like endoperoxides, e.g., arteflene, have been synthesized chemically (Hofheinz et al. 1994).

Other possibilities for meeting the high demand for artemisinin are agricultural and biotechnological approaches. Conventional agricultural approaches allow the cultivation of wild-type plants in fields and greenhouses or the breeding of high-yield cultivars. Breeding techniques can optimize harvest by crossing high yield clones or creating synthetic variants of *A. annua*. In addition, transgenic plants deliver considerably higher amounts of artemisinin than wild-type plants (Laughlin 1994; Delabays et al. 2001).

Commercial large-scale production of artemisinin is mainly achieved with biotechnological approaches. Infecting roots of *A. annua* with *Agrobacterium rhizogens*, a gram negative soil bacterium, generated hairy root cultures, which grew more rapid, reached higher densities, and produced significant amounts of secondary metabolites such as artemisinin (De Jesus-Gonzalez and Weathers 2003; Souret et al. 2003).

Synthesis of artemisinin is also accomplished with tissue cultures of *A. annua* (Nair et al. 1986). The expression of the biosynthetic pathway for artemisinin or related metabolites in genetically modified organisms, i.e., *Escherichia coli* and *Aspergillus flavipes* (Elmarakby et al. 1987; Martin et al. 2003; Hampton 2005) or *Saccharomyces cerevisiae* (Ro et al. 2006) has been reported. It is a prerequisite that the biosynthetic pathways for artemisinins in *A. annua* are known. The biosynthesis of artemisinin has been elucidated, and the corresponding genes have been cloned. In brief, starting from the cytosolic MVA pathway (3R-mevalonic acid) and 3-acetyl-CoA on one side and from the plasticidal DXP pathway (1-deoxy-D-xylulose 5-phosphate), pyruvate and glyceraldehyde 3-phosphate as starting molecules on the other side, several enzymatic steps lead to the synthesis of farnesyl diphosphate. Several further enzymatic reactions result in the generation of dihydroartemisinic acid and artemisinin [for a detailed representation of the biosynthesis of artemisinin, see reviews (Bertea et al. 2005; Liu et al. 2006)]. If coding genes of these enzymes are transferred to microorganisms such as bacteria or yeast, it should be possible to reconstruct the biosynthetic pathway of artemisinin in these organisms.

Biotechnological approaches for the large-scale production of artemisinin represent a technical challenge. The obtainable yields should exceed the ones obtained by classical breeding methods. The artemisinin yield of one ton dry leaves of wild-type *A. annua* is 6 kg/ha. Time to grow is 100 ± 120 days allowing three harvests per year under optimal conditions 18 kg artemisinin/ha/year. With the use of genetically engineered organisms, it should be possible to produce 25 kg artemisinin within an 8-h working day. This calculation is based on the assumption that engineered yeast will produce 100 ± 150 mg artemisinin per liter culture medium or 100 ± 150 g/1,000 L in an industrial set-up. The doubling time of yeast is about 1 h; hence, starting with 100 g artemisinin at time point 0 will result in 25.6 kg artemisinin after 8 h.

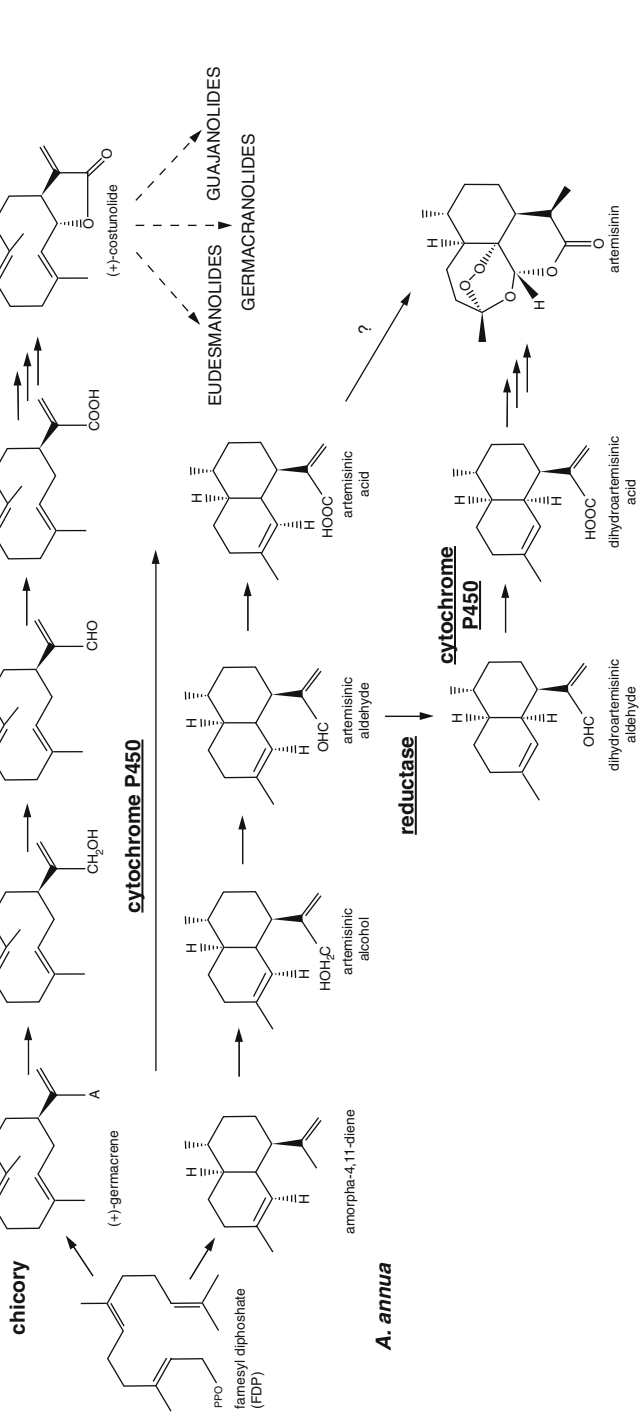

Fig. 8.2 Biosynthetic routes of sesquiterpene lactones in chicory and artemisinin in *Artemisia annua* (with permission of H. Jansen, Dafra, Turnhout, Belgium)

An alternative to total chemical synthesis of artemisinin is the reconstruction of its biosynthetic pathway in microbes leading to the production of precursor molecules that can be converted to artemisinin with relatively few chemical manipulations. Development of a semi-synthetic microbial process for the production of artemisinin would allow for a consistent, second source of the drug to supplement cultivation of *A. annua*. Heterologous production of artemisinin precursors by fermentation is of active research interest, to ensure a consistent no-season supply of artemisinin for ACT, the current WHO recommended treatment for malaria (Sect. 8.1.3). Biosynthesis of amorpha-4,11-diene, the precursor of artemisinic acid has reached 0.5 g/L in *E. coli* (Newman et al. 2006) and 150–600 mg/L in the yeast *S. cerevisiae* (Shiba et al. 2007; Lindahl et al. 2006). Production of artemisinic acid in *S. cerevisiae* has been reported at 100 mg/L (Ro et al. 2006). Artemisinic acid production was increased dramatically to 25-fold from a 100 mg/L flask process to a 2.5 g/L process in bioreactors by developing a high-density fed-batch fermentation process with a DO-stat algorithm that controlled carbon delivery and agitation simultaneously (Lenihan et al. 2008).

A very new and auspicious method of biotechnological production of artemisinin is the utilization of chicory roots (*Cichorium intybus*), which are waste products, produced at the harvest of the edible chicory sprouts. They contain a number of bitter compounds, e.g., lactucin, which all belong to the class of sesquiterpene lactones (Fig. 8.2). Chicory enzymes are involved in the biosynthesis of the bitter sesquiterpene lactones and are applicable for the biosynthesis of DHA (Bertea et al. 2005; De Kraker 2003). The advantage of this new method is the large-scale production of artemisinin and consequently cheaper production cost of ACTs.

With the implementation of sophisticated biotechnological production techniques, it will be possible to meet the high demand for artemisinin for malaria treatment and hopefully in the future for cancer chemotherapy as well.

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References

Abdin MZ, Israr M, Rehman RU, Jain SK (2003) Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Med 69:289–299

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 95:548–553

Adjuik M, Babiker A, Garner P, Olliaro P, Taylor W, White N (2004) Artesunate combinations for treatment of malaria: meta-analysis. Lancet 363:9–17

Anfosso L, Efferth T, Albini A, Pfeffer U (2006) Microarray expression profiles of angiogenesis-related genes predict tumor cell response to artemisinins. Pharmacogenomics J 6:269–278

Asimus S, Elsherbiny D, Hai TN, Jansson B, Huong NV, Petzold MG, Simonsson US, Ashton M (2007) Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects. Fundam Clin Pharmacol 21:307–316

Asimus S, Hai TN, Van Huong N, Ashton M (2008) Artemisinin and CYP2A6 activity in healthy subjects. Eur J Clin Pharmacol 64:283–292

Bapiro TE, Andersson TB, Otter C, Hasler JA, Masimirembwa CM (2002) Cytochrome P450 1A1/2 induction by antiparasitic drugs: dose-dependent increase in ethoxyresorufin O-deethylase activity and mRNA caused by quinine, primaquine and albendazole in HepG2 cells. Eur J Clin Pharmacol 58:537–542

Bapiro TE, Sayi J, Hasler JA, Jande M, Rimoy G, Masselle A, Masimirembwa CM (2005) Artemisinin and thiabendazole are potent inhibitors of cytochrome P450 1A2 (CYP1A2) activity in humans. Eur J Clin Pharmacol 61:755–761

Berger TG, Dieckmann D, Efferth T, Schultz ES, Funk JO, Baur A, Schuler G (2005) Artesunate in the treatment of metastatic uveal melanoma–first experiences. Oncol Rep 14:1599–1603

Bertea CM, Freije JR, van der Woude H, Verstappen FW, Perk L, Marquez V, De Kraker JW, Posthumus MA, Jansen BJ, de Groot A, Franssen MC, Bouwmeester HJ (2005) Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in Artemisia annua. Planta Med 71:40–47

Brewer TG, Peggins JO, Grate SJ, Petras JM, Levine BS, Weina PJ, Swearengen J, Heiffer MH, Schuster BG (1994a) Neurotoxicity in animals due to arteether and artemether. Trans R Soc Trop Med Hyg 88(Suppl 1):S33–S36

Brewer TG, Grate SJ, Peggins JO, Weina PJ, Petras JM, Levine BS, Heiffer MH, Schuster BG (1994b) Fatal neurotoxicity of arteether and artemether. Am J Trop Med Hyg 51:251–259

Broxterman HJ, Lankelma J, Hoekman K (2003) Resistance to cytotoxic and anti-angiogenic anticancer agents: similarities and differences. Drug Resist Updat 6:111–127

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF, Eichelbaum M (2005) Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. Mol Pharmacol 67:1954–1965

Cabello CM, Lamore SD, Bair WB 3rd, Qiao S, Azimian S, Lesson JL, Wondrak GT (2012) The redox antimalarial dihydroartemisinin targets human metastatic melanoma cells but not primary melanocytes with induction of NOXA-dependent apoptosis. Invest New Drugs 30(4):1289–1301

Carrara VI, Phyo AP, Nwee P, Soe M, Htoo H, Arunkamomkiri J, Singhasivanon P, Nosten F (2008) Auditory assessment of patients with acute uncomplicated plasmodium falciparum malaria treated with three-day mefloquine-artesunate on the north-western border of Thailand. Malar J 7:233

Chen HH, Zhou HJ, Fang X (2003) Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives in vitro. Pharmacol Res 48:231–236

Chen HH, Zhou HJ, Wang WQ, Wu GD (2004a) Antimalarial dihydroartemisinin also inhibits angiogenesis. Cancer Chemother Pharmacol 53:423–432

Chen HH, Zhou HJ, Wu GD, Lou XE (2004b) Inhibitory effects of artesunate on angiogenesis and on expressions of vascular endothelial growth factor and VEGF receptor KDR/flk-1. Pharmacology 71:1–9

Chen T, Li M, Zhang R, Wang H (2008) Dihydroartemisinin induces apoptosis and sensitizes human ovarian cancer cells to carboplatin therapy. J Cell Mol Med 13(7):1358–1370

Chen H, Sun B, Pan S, Jiang H, Sun X (2009) Dihydroartemisinin inhibits growth of pancreatic cancer cells in vitro and in vivo. Anticancer Drugs 20:131–140

Curry EA 3rd, Murry DJ, Yoder C, Fife K, Armstrong V, Nakshatri H, O'Connell M, Sweeney CJ (2004) Phase I dose escalation trial of feverfew with standardized doses of parthenolide in patients with cancer. Invest New Drugs 22:299–305

D'Alessandro S, Gelati M, Basilico N, Parati EA, Haynes RK, Taramelli D (2007) Differential effects on angiogenesis of two antimalarial compounds, dihydroartemisinin and artemisone: implications for embryotoxicity. Toxicology 241:66–74

Davis TM, Karunajeewa HA, Ilett KF (2005) Artemisinin-based combination therapies for uncomplicated malaria. Med J Aust 182:181–185

De Jesus-Gonzalez L, Weathers PJ (2003) Tetraploid Artemisia annua hairy roots produce more artemisinin than diploids. Plant Cell Rep 21:809–813
De Kraker JW (2003) Hydroxylation of sesquiterpenes by enzymes from chicory (Cichorium intybus L.) roots. Tetrahedron 59:409–418
Delabays N, Simonnet X, Gaudin M (2001) The genetics of artemisinin content in Artemisia annua L. and the breeding of high yielding cultivars. Curr Med Chem 8:1795–1801
Dell'Eva R, Pfeffer U, Vene R, Anfosso L, Forlani A, Albini A, Efferth T (2004) Inhibition of angiogenesis in vivo and growth of Kaposi's sarcoma xenograft tumors by the anti-malarial artesunate. Biochem Pharmacol 68:2359–2366
Disbrow GL, Baege AC, Kierpiec KA, Yuan H, Centeno JA, Thibodeaux CA, Hartmann D, Schlegel R (2005) Dihydroartemisinin is cytotoxic to papillomavirus-expressing epithelial cells in vitro and in vivo. Cancer Res 65:10854–10861
Dondorp A, Nosten F, Stepniewska K, Day N, White N (2005) Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. Lancet 366:717–725
Efferth T (2005) Mechanistic perspectives for 1,2,4-trioxanes in anti-cancer therapy. Drug Resist Updat 8:85–97
Efferth T (2006) Molecular pharmacology and pharmacogenomics of artemisinin and its derivatives in cancer cells. Curr Drug Targets 7:407–421
Efferth T (2007) Willmar Schwabe Award 2006: antiplasmodial and antitumor activity of artemisinin–from bench to bedside. Planta Med 73:299–309
Efferth T, Grassmann R (2000) Impact of viral oncogenesis on responses to anti-cancer drugs and irradiation. Crit Rev Oncog 11:165–187
Efferth T, Kaina B (2010) Toxicity of the antimalarial artemisinin and its derivatives. Crit Rev Toxicol 40:405–421
Efferth T, Oesch F (2004) Oxidative stress response of tumor cells: microarray-based comparison between artemisinins and anthracyclines. Biochem Pharmacol 68:3–10
Efferth T, Volm M (1993) Reversal of doxorubicin-resistance in sarcoma 180 tumor cells by inhibition of different resistance mechanisms. Cancer Lett 70:197–202
Efferth T, Volm M (2005a) Pharmacogenetics for individualized cancer chemotherapy. Pharmacol Ther 107:155–176
Efferth T, Volm M (2005b) Glutathione-related enzymes contribute to resistance of tumor cells and low toxicity in normal organs to artesunate. In Vivo 19:225–232
Efferth T, Mattern J, Volm M (1992) Immunohistochemical detection of P glycoprotein, glutathione S transferase and DNA topoisomerase II in human tumors. Oncology 49:368–375
Efferth T, Rucker G, Falkenberg M, Manns D, Olbrich A, Fabry U, Osieka R (1996) Detection of apoptosis in KG-1a leukemic cells treated with investigational drugs. Arzneimittelforschung 46:196–200
Efferth T, Fabry U, Osieka R (1997) Apoptosis and resistance to daunorubicin in human leukemic cells. Leukemia 11:1180–1186
Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR (2001) The anti-malarial artesunate is also active against cancer. Int J Oncol 18:767–773
Efferth T, Fabry U, Osieka R (2002a) Interleukin-6 affects melphalan-induced DNA damage and repair in human multiple myeloma cells. Anticancer Res 22:231–234
Efferth T, Olbrich A, Bauer R (2002b) mRNA expression profiles for the response of human tumor cell lines to the antimalarial drugs artesunate, arteether, and artemether. Biochem Pharmacol 64:617–623
Efferth T, Davey M, Olbrich A, Rucker G, Gebhart E, Davey R (2002c) Activity of drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. Blood Cells Mol Dis 28:160–168
Efferth T, Sauerbrey A, Olbrich A, Gebhart E, Rauch P, Weber HO, Hengstler JG, Halatsch ME, Volm M, Tew KD, Ross DD, Funk JO (2003a) Molecular modes of action of artesunate in tumor cell lines. Mol Pharmacol 64:382–394
Efferth T, Briehl MM, Tome ME (2003b) Role of antioxidant genes for the activity of artesunate against tumor cells. Int J Oncol 23:1231–1235

Efferth T, Benakis A, Romero MR, Tomicic M, Rauh R, Steinbach D, Hafer R, Stamminger T, Oesch F, Kaina B, Marschall M (2004a) Enhancement of cytotoxicity of artemisinins toward cancer cells by ferrous iron. Free Radic Biol Med 37:998–1009

Efferth T, Ramirez T, Gebhart E, Halatsch ME (2004b) Combination treatment of glioblastoma multiforme cell lines with the anti-malarial artesunate and the epidermal growth factor receptor tyrosine kinase inhibitor OSI-774. Biochem Pharmacol 67:1689–1700

Efferth T, Giaisi M, Merling A, Krammer PH, Li-Weber M (2007) Artesunate induces ROS-mediated apoptosis in doxorubicin-resistant T leukemia cells. PLoS One 2:e693

Efferth T, Romero MR, Wolf DG, Stamminger T, Marin JJ, Marschall M (2008) The antiviral activities of artemisinin and artesunate. Clin Infect Dis 47:804–811

Elmarakby SA, El-Feraly FS, Elsohly HN, Croom EM, Hufford CD (1987) Microbial transformation studies on arteannuin B. J Nat Prod 50:903–909

Elsherbiny DA, Asimus SA, Karlsson MO, Ashton M, Simonsson US (2008) A model based assessment of the CYP2B6 and CYP2C19 inductive properties by artemisinin antimalarials: implications for combination regimens. J Pharmacokinet Pharmacodyn 35:203–217

Folkman J (1992) The role of angiogenesis in tumor growth. Semin Cancer Biol 3:65–71

Fujita T, Felix K, Pinkaew D, Hutadilok-Towatana N, Liu Z, Fujise K (2008) Human fortilin is a molecular target of dihydroartemisinin. FEBS Lett 582:1055–1060

Gallo MA, Kaufman D (1997) Antagonistic and agonistic effects of tamoxifen: significance in human cancer. Semin Oncol 24:S1-71–S1-80

Gao N, Budhraja A, Cheng S, Liu EH, Huang C, Chen J, Yang Z, Chen D, Zhang Z, Shi X (2011) Interruption of the MEK/ERK signaling cascade promotes dihydroartemisinin-induced apoptosis in vitro and in vivo. Apoptosis 16:511–523

Genovese RF, Newman DB (2008) Understanding artemisinin-induced brainstem neurotoxicity. Arch Toxicol 82:379–385

Genovese RF, Newman DB, Li Q, Peggins JO, Brewer TG (1998a) Dose-dependent brainstem neuropathology following repeated arteether administration in rats. Brain Res Bull 45:199–202

Genovese RF, Newman DB, Petras JM, Brewer TG (1998b) Behavioral and neural toxicity of arteether in rats. Pharmacol Biochem Behav 60:449–458

Gordi T, Lepist EI (2004) Artemisinin derivatives: toxic for laboratory animals, safe for humans? Toxicol Lett 147:99–107

Gordi T, Xie R, Huong NV, Huong DX, Karlsson MO, Ashton M (2005) A semiphysiological pharmacokinetic model for artemisinin in healthy subjects incorporating autoinduction of metabolism and saturable first-pass hepatic extraction. Br J Clin Pharmacol 59:189–198

Grant S, Qiao L, Dent P (2002) Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. Front Biosci 7:d376–d389

Hampton T (2005) Collaboration hopes microbe factories can supply key antimalaria drug. JAMA 293:785–787

Handrick R, Ontikatze T, Bauer KD, Freier F, Rübel A, Dürig J, Belka C, Jendrossek V (2010) Dihydroartemisinin induces apoptosis by a Bak-dependent intrinsic pathway. Mol Cancer Ther 9:2497–2510

Haynes RK (2001) Artemisinin and derivatives: the future for malaria treatment? Curr Opin Infect Dis 14:719–726

He F, Bi HC, Xie ZY, Zuo Z, Li JK, Li X, Zhao LZ, Chen X, Huang M (2007) Rapid determination of six metabolites from multiple cytochrome P450 probe substrates in human liver microsome by liquid chromatography/mass spectrometry: application to high-throughput inhibition screening of terpenoids. Rapid Commun Mass Spectrom 21:635–643

He Q, Shi J, Shen XL, An J, Sun H, Wang L, Hu YJ, Sun Q, Fu LC, Sheikh MS, Huang Y (2010) Dihydroartemisinin upregulates death receptor 5 expression and cooperates with TRAIL to induce apoptosis in human prostate cancer cells. Cancer Biol Ther 9:819–824

Hofheinz W, Burgin H, Gocke E, Jaquet C, Masciadri R, Schmid G, Stohler H, Urwyler H (1994) Ro 42–1611 (arteflene), a new effective antimalarial: chemical structure and biological activity. Trop Med Parasitol 45:261–265

Hou J, Wang D, Zhang R, Wang H (2008) Experimental therapy of hepatoma with artemisinin and its derivatives: in vitro and in vivo activity, chemosensitization, and mechanisms of action. Clin Cancer Res 14:5519–5530

Huang XJ, Ma ZQ, Zhang WP, Lu YB, Wei EQ (2007) Dihydroartemisinin exerts cytotoxic effects and inhibits hypoxia inducible factor-1alpha activation in C6 glioma cells. J Pharm Pharmacol 59:849–856

Huang XJ, Li CT, Zhang WP, Lu YB, Fang SH, Wei EQ (2008) Dihydroartemisinin potentiates the cytotoxic effect of temozolomide in rat C6 glioma cells. Pharmacology 82:1–9

Huan-huan C, Li-Li Y, Shang-Bin L (2004) Artesunate reduces chicken chorioallantoic membrane neovascularisation and exhibits antiangiogenic and apoptotic activity on human microvascular dermal endothelial cell. Cancer Lett 211:163–173

Hutagalung R, Htoo H, Nwee P, Arunkamomkiri J, Zwang J, Carrara VI, Ashley E, Singhasivanon P, White NJ, Nosten F (2006) A case–control auditory evaluation of patients treated with artemether-lumefantrine. Am J Trop Med Hyg 74:211–214

Hwang YP, Yun HJ, Kim HG, Han EH, Lee GW, Jeong HG (2010) Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKCalpha/Raf/MAPKs and NF-kappaB/AP-1-dependent mechanisms. Biochem Pharmacol 79:1714–1726

Ishikawa TAK (1996) Transport of glutathione S-conjugates from cancer cells: function and structure of GS-X pump. Taylor and Francis, London, pp 199–211

Jiao Y, Ge CM, Meng QH, Cao JP, Tong J, Fan SJ (2007) Dihydroartemisinin is an inhibitor of ovarian cancer cell growth. Acta Pharmacol Sin 28:1045–1056

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:290–298

Kamchonwongpaisan S, McKeever P, Hossler P, Ziffer H, Meshnick SR (1997) Artemisinin neurotoxicity: neuropathology in rats and mechanistic studies in vitro. Am J Trop Med Hyg 56:7–12

Kelter G, Steinbach D, Konkimalla VB, Tahara T, Taketani S, Fiebig HH, Efferth T (2007) Role of transferrin receptor and the ABC transporters ABCB6 and ABCB7 for resistance and differentiation of tumor cells towards artesunate. PLoS One 2:e798

Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2:727–739

Kim SJ, Kim MS, Lee JW, Lee CH, Yoo H, Shin SH, Park MJ, Lee SH (2006) Dihydroartemisinin enhances radiosensitivity of human glioma cells in vitro. J Cancer Res Clin Oncol 132:129–135

Klayman DL (1985) Qinghaosu (artemisinin): an antimalarial drug from China. Science 228:1049–1055

Konkimalla VB, Blunder M, Korn B, Soomro SA, Jansen H, Chang W, Posner GH, Bauer R, Efferth T (2008) Effect of artemisinins and other endoperoxides on nitric oxide-related signaling pathway in RAW 264.7 mouse macrophage cells. Nitric Oxide 19:184–191

Konkimalla VB, McCubrey JA, Efferth T (2009) The role of downstream signaling pathways of the epidermal growth factor receptor for Artesunate's activity in cancer cells. Curr Cancer Drug Targets 9:72–80

Lai H, Sasaki T, Singh NP (2005) Targeted treatment of cancer with artemisinin and artemisinin-tagged iron-carrying compounds. Expert Opin Ther Targets 9:995–1007

Laughlin JC (1994) Agricultural production of artemisinin–a review. Trans R Soc Trop Med Hyg 88(Suppl 1):S21–S22

Lee J, Zhou HJ, Wu XH (2006) Dihydroartemisinin downregulates vascular endothelial growth factor expression and induces apoptosis in chronic myeloid leukemia K562 cells. Cancer Chemother Pharmacol 57:213–220

Lenihan JR, Tsuruta H, Diola D, Renninger NS, Regentin R (2008) Developing an industrial artemisinic acid fermentation process to support the cost-effective production of antimalarial artemisinin-based combination therapies. Biotechnol Prog 24:1026–1032

Li Y, Wu YL (1998) How Chinese scientists discovered qinghaosu (artemisinin) and developed its derivatives? what are the future perspectives? Med Trop (Mars) 58:9–12

Li QG, Mog SR, Si YZ, Kyle DE, Gettayacamin M, Milhous WK (2002) Neurotoxicity and efficacy of arteether related to its exposure times and exposure levels in rodents. Am J Trop Med Hyg 66:516–525

Li LN, Zhang HD, Yuan SJ, Tian ZY, Wang L, Sun ZX (2007) Artesunate attenuates the growth of human colorectal carcinoma and inhibits hyperactive Wnt/beta-catenin pathway. Int J Cancer 121:1360–1365

Li H, van Berlo D, Shi T, Speit G, Knaapen AM, Borm PJ, Albrecht C, Schins RP (2008) Curcumin protects against cytotoxic and inflammatory effects of quartz particles but causes oxidative DNA damage in a rat lung epithelial cell line. Toxicol Appl Pharmacol 227:115–124

Lindahl AL, Olsson ME, Mercke P, Tollbom O, Schelin J, Brodelius M, Brodelius PE (2006) Production of the artemisinin precursor amorpha-4,11-diene by engineered Saccharomyces cerevisiae. Biotechnol Lett 28:571–580

Liu C, Zhao Y, Wang Y (2006) Artemisinin: current state and perspectives for biotechnological production of an antimalarial drug. Appl Microbiol Biotechnol 72:11–20

Lotem J, Sachs L (1996) Control of apoptosis in hematopoiesis and leukemia by cytokines, tumor suppressor and oncogenes. Leukemia 10:925–931

Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21:796–802

McCarty MF (2003) Turning an 'Achilles' Heel' into an asset–activation of HIF-1alpha during angiostatic therapy will increase tumor sensitivity to iron-catalyzed oxidative damage. Med Hypotheses 61:509–511

McColl BK, Loughran SJ, Davydova N, Stacker SA, Achen MG (2005) Mechanisms of lymphangiogenesis: targets for blocking the metastatic spread of cancer. Curr Cancer Drug Targets 5:561–571

McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773:1263–1284

Mercer AE, Copple IM, Maggs JL, O'Neill PM, Park BK (2011) The role of heme and the mitochondrion in the chemical and molecular mechanisms of mammalian cell death induced by the artemisinin antimalarials. J Biol Chem 286:987–996

Moore JC, Lai H, Li JR, Ren RL, McDougall JA, Singh NP, Chou CK (1995) Oral administration of dihydroartemisinin and ferrous sulfate retarded implanted fibrosarcoma growth in the rat. Cancer Lett 98:83–87

Mu D, Chen W, Yu B, Zhang C, Zhang Y, Qi H (2007) Calcium and survivin are involved in the induction of apoptosis by dihydroartemisinin in human lung cancer SPC-A-1 cells. Methods Find Exp Clin Pharmacol 29:33–38

Mu D, Zhang W, Chu D, Liu T, Xie Y, Fu E, Jin F (2008) The role of calcium, P38 MAPK in dihydroartemisinin-induced apoptosis of lung cancer PC-14 cells. Cancer Chemother Pharmacol 61:639–645

Mukanganyama S, Widersten M, Naik YS, Mannervik B, Hasler JA (2002) Inhibition of glutathione S-transferases by antimalarial drugs possible implications for circumventing anticancer drug resistance. Int J Cancer 97:700–705

Nair MS, Acton N, Klayman DL, Kendrick K, Basile DV, Mante S (1986) Production of artemisinin in tissue cultures of Artemisia annua. J Nat Prod 49:504–507

Nakase I, Lai H, Singh NP, Sasaki T (2008) Anticancer properties of artemisinin derivatives and their targeted delivery by transferrin conjugation. Int J Pharm 354:28–33

Nakase I, Gallis B, Takatani-Nakase T, Oh S, Lacoste E, Singh NP, Goodlett DR, Tanaka S, Futaki S, Lai H, Sasaki T (2009) Transferrin receptor-dependent cytotoxicity of artemisinin-transferrin conjugates on prostate cancer cells and induction of apoptosis. Cancer Lett 274:290–298

Nam W, Tak J, Ryu JK, Jung M, Yook JI, Kim HJ, Cha IH (2007) Effects of artemisinin and its derivatives on growth inhibition and apoptosis of oral cancer cells. Head Neck 29:335–340

Navolanic PM, Steelman LS, McCubrey JA (2003) EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (review). Int J Oncol 22 :237–252

Newman JD, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman KL, Keasling JD (2006) High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol Bioeng 95:684–691

Nontprasert A, Pukrittayakamee S, Dondorp AM, Clemens R, Looareesuwan S, White NJ (2002) Neuropathologic toxicity of artemisinin derivatives in a mouse model. Am J Trop Med Hyg 67:423–429

Oh S, Jeong IH, Ahn CM, Shin WS, Lee S (2004) Synthesis and antiangiogenic activity of thioacetal artemisinin derivatives. Bioorg Med Chem 12:3783–3790

Oh S, Kim BJ, Singh NP, Lai H, Sasaki T (2009) Synthesis and anti-cancer activity of covalent conjugates of artemisinin and a transferrin-receptor targeting peptide. Cancer Lett 274:33–39

Panossian LA, Garga NI, Pelletier D (2005) Toxic brainstem encephalopathy after artemisinin treatment for breast cancer. Ann Neurol 58:812–813

Petras JM, Kyle DE, Gettayacamin M, Young GD, Bauman RA, Webster HK, Corcoran KD, Peggins JO, Vane MA, Brewer TG (1997) Arteether: risks of two-week administration in Macaca mulatta. Am J Trop Med Hyg 56:390–396

Petras JM, Young GD, Bauman RA, Kyle DE, Gettayacamin M, Webster HK, Corcoran KD, Peggins JO, Vane MA, Brewer TG (2000) Arteether-induced brain injury in Macaca mulatta. I. The precerebellar nuclei: the lateral reticular nuclei, paramedian reticular nuclei, and perihypoglossal nuclei. Anat Embryol 201:383–397

Pommier Y, Sordet O, Antony S, Hayward RL, Kohn KW (2004) Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. Oncogene 23:2934–2949

Reizenstein P (1991) Iron, free radicals and cancer. Med Oncol Tumor Pharmacother 8:229–233

Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R, Moghaddam A, Whitehouse R, Bicknell R, Harris AL (1997) Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 57:963–969

Reungpatthanaphong P, Mankhetkorn S (2002) Modulation of multidrug resistance by artemisinin, artesunate and dihydroartemisinin in K562/adr and GLC4/adr resistant cell lines. Biol Pharm Bull 25:1555–1561

Ribeiro IR, Olliaro P (1998) Safety of artemisinin and its derivatives. A review of published and unpublished clinical trials. Med Trop (Mars) 58:50–53

Riganti C, Doublier S, Costamagna C, Aldieri E, Pescarmona G, Ghigo D, Bosia A (2008) Activation of nuclear factor-kappa B pathway by simvastatin and RhoA silencing increases doxorubicin cytotoxicity in human colon cancer HT29 cells. Mol Pharmacol 74:476–484

Riganti C, Doublier S, Viarisio D, Miraglia E, Pescarmona G, Ghigo D, Bosia A (2009) Artemisinin induces doxorubicin resistance in human colon cancer cells via calcium-dependent activation of HIF-1alpha and P-glycoprotein overexpression. Br J Pharmacol 156:1054–1066

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943

Segditsas S, Tomlinson I (2006) Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene 25:7531–7537

Sertel S, Eichhorn T, Simon CH, Plinkert PK, Johnson SW, Efferth T (2010a) Pharmacogenomic identification of c-Myc/Max-regulated genes associated with cytotoxicity of artesunate towards human colon, ovarian and lung cancer cell lines. Molecules 15:2886–2910

Sertel S, Eichhorn T, Sieber S, Sauer A, Weiss J, Plinkert PK, Efferth T (2010b) Factors determining sensitivity or resistance of tumor cell lines towards artesunate. Chem Biol Interact 185:42–52

Shaul YD, Seger R (2007) The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim Biophys Acta 1773:1213–1226

Shiba Y, Paradise EM, Kirby J, Ro DK, Keasling JD (2007) Engineering of the pyruvate dehydrogenase bypass in Saccharomyces cerevisiae for high-level production of isoprenoids. Metab Eng 9:160–168

Shimizu K, Oku N (2004) Cancer anti-angiogenic therapy. Biol Pharm Bull 27:599–605

Sieber S, Gdynia G, Roth W, Bonavida B, Efferth T (2009) Combination treatment of malignant B cells using the anti-CD20 antibody rituximab and the anti-malarial artesunate. Int J Oncol 35:149–158

Simonsson US, Lindell M, Raffalli-Mathieu F, Lannerbro A, Honkakoski P, Lang MA (2006) In vivo and mechanistic evidence of nuclear receptor CAR induction by artemisinin. Eur J Clin Invest 36:647–653

Singh NP, Lai H (2001) Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. Life Sci 70:49–56

Singh NP, Lai HC (2004) Artemisinin induces apoptosis in human cancer cells. Anticancer Res 24:2277–2280

Singh NP, Lai HC (2005) Synergistic cytotoxicity of artemisinin and sodium butyrate on human cancer cells. Anticancer Res 25:4325–4331

Singh NP, Panwar VK (2006) Case report of a pituitary macroadenoma treated with artemether. Integr Cancer Ther 5:391–394

Singh NP, Verma KB (2002) Case report of a laryngeal squamous cell carcinoma treated with artesunate. Arch Oncol 10:279–280

Souret FF, Kim Y, Wyslouzil BE, Wobbe KK, Weathers PJ (2003) Scale-up of Artemisia annua L. hairy root cultures produces complex patterns of terpenoid gene expression. Biotechnol Bioeng 83:653–667

Steinbrück L, Pereira G, Efferth T (2010) Effects of artesunate on cytokinesis and G_2/M cell cycle progression of tumour cells and budding yeast. Cancer Genomics Proteomics 7:337–346

Stepniewska K, Day N, Babiker A, Lalloo D, Warrell D, Olliaro P, White N (2001) A meta-analysis using individual patient data of trials comparing artemether with quinine in the treatment of severe falciparum malaria. Trans R Soc Trop Med Hyg 95:637–650

Sukhija M, Medhi B, Pandhi P (2006) Effects of artemisinin, artemether, arteether on the pharmacokinetics of carbamazepine. Pharmacology 76:110–116

Sundar SN, Marconett CN, Doan VB, Willoughby JA Sr, Firestone GL (2008) Artemisinin selectively decreases functional levels of estrogen receptor-alpha and ablates estrogen-induced proliferation in human breast cancer cells. Carcinogenesis 29:2252–2258

Svensson US, Ashton M (1999) Identification of the human cytochrome P450 enzymes involved in the in vitro metabolism of artemisinin. Br J Clin Pharmacol 48:528–535

Svensson US, Maki-Jouppila M, Hoffmann KJ, Ashton M (2003) Characterisation of the human liver in vitro metabolic pattern of artemisinin and auto-induction in the rat by use of nonlinear mixed effects modelling. Biopharm Drug Dispos 24:71–85

Sweeney CJ, Mehrotra S, Sadaria MR, Kumar S, Shortle NH, Roman Y, Sheridan C, Campbell RA, Murry DJ, Badve S, Nakshatri H (2005) The sesquiterpene lactone parthenolide in combination with docetaxel reduces metastasis and improves survival in a xenograft model of breast cancer. Mol Cancer Ther 4:1004–1012

Taketani S, Kakimoto K, Ueta H, Masaki R, Furukawa T (2003) Involvement of ABC7 in the biosynthesis of heme in erythroid cells: interaction of ABC7 with ferrochelatase. Blood 101:3274–3280

Tan RX, Zheng WF, Tang HQ (1998) Biologically active substances from the genus Artemisia. Planta Med 64:295–302

Tin AS, Sundar SN, Tran KQ, Park AH, Poindexter KM, Firestone GL (2012) Antiproliferative effects of artemisinin on human breast cancer cells requires the downregulated expression of the E2F1 transcription factor and loss of E2F1-target cell cycle genes. Anticancer Drugs 23(4):370–379

Toovey S (2006) Are currently deployed artemisinins neurotoxic? Toxicol Lett 166:95–104

Toovey S, Jamieson A (2004) Audiometric changes associated with the treatment of uncomplicated falciparum malaria with co-artemether. Trans R Soc Trop Med Hyg 98:261–267, discussion 268–269

Tu Y (1999) The development of new antimalarial drugs: qinghaosu and dihydro-qinghaosu. Chin Med J (Engl) 112:976–977

Tuttle TM (2004) Technical advances in sentinel lymph node biopsy for breast cancer. Am Surg 70:407–413

van Agtmael MA, Eggelte TA, van Boxtel CJ (1999) Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends Pharmacol Sci 20:199–205

Van Geldre E, Vergauwe A, Van den Eeckhout E (1997) State of the art of the production of the antimalarial compound artemisinin in plants. Plant Mol Biol 33:199–209

van Hensbroek MB, Onyiorah E, Jaffar S, Schneider G, Palmer A, Frenkel J, Enwere G, Forck S, Nusmeijer A, Bennett S, Greenwood B, Kwiatkowski D (1996) A trial of artemether or quinine in children with cerebral malaria. N Engl J Med 335:69–75

Volm M, Kastel M, Mattern J, Efferth T (1993) Expression of resistance factors (P-glycoprotein, glutathione S-transferase-pi, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas. Cancer 71:3981–3987

Volm M, Koomagi R, Mattern J, Efferth T (2002a) Expression profile of genes in non-small cell lung carcinomas from long-term surviving patients. Clin Cancer Res 8:1843–1848

Volm M, Koomagi R, Mattern J, Efferth T (2002b) Protein expression profiles indicative for drug resistance of non-small cell lung cancer. Br J Cancer 87:251–257

Wang CW (1961) The forests of China, with a survey of grassland and desert vegetation, vol 5. Harvard University, Maria Moors Cabot Foundation, Cambridge, MA, pp 171–187

Wang J, Guo Y, Zhang BC, Chen ZT, Gao JF (2007a) Induction of apoptosis and inhibition of cell migration and tube-like formation by dihydroartemisinin in murine lymphatic endothelial cells. Pharmacology 80:207–218

Wang JX, Tang W, Yang ZS, Wan J, Shi LP, Zhang Y, Zhou R, Ni J, Hou LF, Zhou Y, He PL, Yang YF, Li Y, Zuo JP (2007b) Suppressive effect of a novel water-soluble artemisinin derivative SM905 on T cell activation and proliferation in vitro and in vivo. Eur J Pharmacol 564:211–218

Wang JX, Tang W, Shi LP, Wan J, Zhou R, Ni J, Fu YF, Yang YF, Li Y, Zuo JP (2007c) Investigation of the immunosuppressive activity of artemether on T-cell activation and proliferation. Br J Pharmacol 150:652–661

Wang J, Zhang B, Guo Y, Li G, Xie Q, Zhu B, Gao J, Chen Z (2008) Artemisinin inhibits tumor lymphangiogenesis by suppression of vascular endothelial growth factor C. Pharmacology 82:148–155

Wartenberg M, Wolf S, Budde P, Grunheck F, Acker H, Hescheler J, Wartenberg G, Sauer H (2003) The antimalaria agent artemisinin exerts antiangiogenic effects in mouse embryonic stem cell-derived embryoid bodies. Lab Invest 83:1647–1655

Weifeng T, Feng S, Xiangji L, Changqing S, Zhiquan Q, Huazhong Z, Peining Y, Yong Y, Mengchao W, Xiaoqing J, Wan-Yee L (2011) Artemisinin inhibits in vitro and in vivo invasion and metastasis of human hepatocellular carcinoma cells. Phytomedicine 18:58–62

White NJ (2008) Qinghaosu (artemisinin): the price of success. Science 320:330–334

Willoughby JA Sr, Sundar SN, Cheung M, Tin AS, Modiano J, Firestone GL (2009) Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with

the cyclin-dependent kinase-4 (CDK4) promoter and inhibiting CDK4 gene expression. J Biol Chem 284:2203–2213

Woerdenbag HJ, Moskal TA, Pras N, Malingre TM, el-Feraly FS, Kampinga HH, Konings AW (1993) Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumor cells. J Nat Prod 56:849–856

Woodrow CJ, Haynes RK, Krishna S (2005) Artemisinins. Postgrad Med J 81:71–78

Wouters BG, van den Beucken T, Magagnin MG, Lambin P, Koumenis C (2004) Targeting hypoxia tolerance in cancer. Drug Resist Updat 7:25–40

Wu XH, Zhou HJ, Lee J (2006) Dihydroartemisinin inhibits angiogenesis induced by multiple myeloma RPMI8226 cells under hypoxic conditions via downregulation of vascular endothelial growth factor expression and suppression of vascular endothelial growth factor secretion. Anticancer Drugs 17:839–848

Wu J, Hu D, Yang G, Zhou J, Yang C, Gao Y, Zhu Z (2011) Down-regulation of BMI-1 cooperates with artemisinin on growth inhibition of nasopharyngeal carcinoma cells. J Cell Biochem 112:1938–1948

Yamachika E, Habte T, Oda D (2004) Artemisinin: an alternative treatment for oral squamous cell carcinoma. Anticancer Res 24:2153–2160

Yeung S, Pongtavornpinyo W, Hastings IM, Mills AJ, White NJ (2004) Antimalarial drug resistance, artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. Am J Trop Med Hyg 71:179–186

Yu JL, Coomber BL, Kerbel RS (2002) A paradigm for therapy-induced microenvironmental changes in solid tumors leading to drug resistance. Differentiation 70:599–609

Zhang ZY, Yu SQ, Miao LY, Huang XY, Zhang XP, Zhu YP, Xia XH, Li DQ (2008) Artesunate combined with vinorelbine plus cisplatin in treatment of advanced non-small cell lung cancer: a randomized controlled trial. Zhong Xi Yi Jie He Xue Bao 6:134–138

Zhao Y, Jiang W, Li B, Yao Q, Dong J, Cen Y, Pan X, Li J, Zheng J, Pang X, Zhou H (2011) Artesunate enhances radiosensitivity of human non-small cell lung cancer A549 cells via increasing NO production to induce cell cycle arrest at G(2)/M phase. Int Immunopharmacol 11:2039–2046

Zhou HJ, Wang WQ, Wu GD, Lee J, Li A (2007) Artesunate inhibits angiogenesis and downregulates vascular endothelial growth factor expression in chronic myeloid leukemia K562 cells. Vascul Pharmacol 47:131–138

Zhou HJ, Wang Z, Li A (2008) Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression. Anticancer Drugs 19:247–255

Chapter 9
Chinese Herbal Medicines for Neuroprotection in Ischemic Stroke: Promise and Reality

Nikolaus J. Sucher and Maria C. Carles

9.1 Introduction

Stroke is not only the third leading cause of death worldwide responsible for almost 10 % of all deaths but also the leading cause of long-term disability among adults. According to data collected by the World Health Organization (WHO), more than 15 million people experience a stroke each year of which 5 million die and as many remain permanently disabled (Wolfe and Rudd 2007). Both individual suffering and the economic burden associated with stroke are immense (Di Carlo 2009). The WHO predicts that by 2020 up to 51 million disability-adjusted life years (DALYs) will be lost to stroke, up from 38 million presently (one DALY corresponds the loss of 1 year of full health). In addition, the costs of caring for stroke victims consume as much as 2–4 % of total healthcare expenditure in developed countries and total and indirect stroke-related healthcare costs in 2006 alone have been estimated at approximately €25 billion in Europe and US$57.9 billion in the USA. Like heart disease, with which it shares most of the risk factors, stroke is rare during the first five decades of life but increasingly common in older people. Overall, the lifetime risk of stroke in men is 25 % and 20 % for women. Thus globally, with more individuals reaching their sixth decade and beyond, it is likely that the incidence of stroke will increase significantly in the near future. At the same time, however, only one effective treatment is available: intravenous thrombolysis with recombinant tissue plasminogen activator (rt-PA, Alteplase®). This has been proven effective in improving the clinical outcome when tested rigorously in a randomized controlled

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trial (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group 1995), the often cited "gold standard" of evidence in medicine. Unfortunately, rt-PA only benefits patients when it is administered within 4.5 h (Davis and Donnan 2009) whereas most stroke victims (between 70 % and 98 % depending on location) do not reach the hospital within this very narrow time frame. Nonetheless, the rt-PA trial proved transformative, as it demonstrated for the first time that acute stroke was treatable and hence provided the stimulus for intense research activity directed at developing additional stroke treatments (Beresford et al. 2003).

The search for new treatments for stroke has been underpinned by unprecedented progress in basic molecular neuroscience research over the last 30 years or so. This research has not only led to the elucidation of the molecular mechanisms involved in the cascade of events leading to neuronal injury and death but also demonstrated that pharmacological or genetic manipulation of these pathways can protect neurons in the face of a variety of otherwise deadly insults (Bredesen et al. 2006; Lipton 1999; Lipton and Rosenberg 1994). The strategy aimed at antagonizing, interrupting, or slowing of the molecular events leading to irreversible injury and death of neurons is commonly referred to as neuroprotection (Ginsberg 2008; Neuroprotection as initial therapy in acute stroke. Third Report of an Ad Hoc Consensus Group Meeting. The European Ad Hoc Consensus Group 1998).

Alas, where there was great hope, there is now great despair as more than 100 experimental drugs tested clinically for acute stroke based on the neuroprotection hypothesis have failed (O'Collins et al. 2006). The future does not look much better either as the pipelines of novel drugs in many pharmaceutical companies are reported to have dried up (Martinez and Goldstein 2007). Although a number of factors have contributed to the apparent drug development crisis, the pharmaceutical industry's emphasis on the role of combinatorial chemistry instead of natural products in drug discovery has been singled out repeatedly as one of the main causes (Rouhi 2003). Natural products or derivatives of natural products form "*the backbone of modern pharmacopoeias*" (Koehn and Carter 2005) and not only constitute the majority of all approved cancer and antimicrobial agents but include the cholesterol-lowering medicines known as statins, which have become so-called blockbuster drugs with cumulative sales of tens of billions of dollars (Newman and Cragg 2007; Newman et al. 2000, 2003).

With the historical predominance of natural products in drug development and the apparent deficiencies of purely synthetic approaches as a backdrop, there has been renewed interest in natural products as sources of drug discovery (Rouhi 2003). Along these lines, we proposed more than 10 years ago that traditional Chinese herbal medicines might provide a source of more structurally diverse compounds that could serve as leads for the development of "Western-style" drugs (Gong and Sucher 1999). By "Western-style" drugs we mean the development of one drug for one target, which has been at the heart of drug development in the West for the last 100 years (Heath and Colburn 2000).

China's efforts in promoting traditional Chinese medicine in Western countries on the background of the lack of promising drug candidates in the pipeline of the

pharmaceutical industry have contributed to an explosive growth in the last decade of the scientific literature devoted to both studies aimed at identifying lead compounds from herbal extracts as well as the characterization of neuroprotective effects of single herbs and complex formulations (Li et al. 2009a). There is also a growing literature on the treatment of stroke with traditional Chinese medicine (Shen et al. 2005) and clinical studies with Chinese patent medicines (Wu et al. 2007a). Underpinning all of this work is the hope that it will lead to the development of effective stroke treatments. How close are we to this goal? Is it even realistic? What can we learn from the apparent failure of the neuroprotective drug development? What is the best way forward? This chapter provides the background necessary in an attempt to answer these questions.

9.2 The Quest for Effective Stroke Treatments

9.2.1 Definition and Current Treatment of Stroke

The WHO defines stroke as a neurological deficit of cerebrovascular cause that persists beyond 24 h or is interrupted by death within 24 h. Stroke is commonly classified into two groups: (1) ischemic stroke, which is due to the sudden and complete blockage of the blood supply to all or part of the brain and (2) hemorrhagic stroke, which is caused by bleeding from a ruptured blood vessel into the brain (intracerebral hemorrhage) or into the space between the brain and the skull (subarachnoid hemorrhage). Ischemic stroke is the most common type of stroke (85 % vs. 15 % for hemorrhagic stroke) in Western countries, but up to 50 % of stroke victims in China experience a hemorrhagic stroke linked to chronic hypertension (Shi et al. 1989). The most common direct causes of ischemic stroke are: (1) obstruction of a blood vessel by a blood clot forming locally in an atherosclerotic vessel leading to or in the brain (thrombosis), (2) obstruction due to a blood clot that was formed and then dislodged from elsewhere in the body (embolism), and (3) the general decrease in blood supply such as during shock, for example. Thus, stroke is primarily a cardiovascular disease and in fact, the risk factors for stroke are virtually identical with those of heart disease (e.g., hypertension, hypercholesterolemia, smoking). The consequences of stroke are, however, of a neurological nature, resulting from neuronal cell death (e.g., facial hemiparalysis and paralysis of the upper and lower limbs on the contralateral side of the body, loss of speech, depending on the extent and location of the lesion in the brain). In the part of the brain where the blood supply is completely interrupted, the core of the stroke, nerve cells are lethally injured within less than 2 min, because their functional and structural integrity can only be maintained through continuous supply of oxygen and nutrients (Dirnagl et al. 1999; Lipton 1999). Surrounding this irreversibly injured core is the ischemic penumbra, where brain tissue is not yet irreversibly

injured and is thus potentially salvageable (Fisher 2004; Savitz and Fisher 2007; Dirnagl et al. 1999).

The practical demonstration that tissue could indeed be saved and the fate of stroke patients could be influenced by treatment came in 1995, when the results of a clinical trial involving 624 patients were published in the New England Journal of Medicine (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group 1995). This study clearly demonstrated that if intravenous rt-PA was administered within 3 h of the onset of ischemic stroke there was an improved clinical outcome at 3 months (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group 1995).

The trial was based on the observation that some 80 % of stroke patients undergoing cerebral angiography exhibited arterial occlusions, i.e., thrombotic stroke, which suggested that reopening of clogged brain arteries using thrombolytic therapy might reduce stroke-related brain injury if it could be administered as soon as possible after the occurrence of the thrombotic event and before onset of widespread infarction in the penumbra. At the same time, however, clinicians were acutely aware that any thrombolytic therapy was inherently dangerous as it often led to intracerebral hemorrhage, turning ischemic into hemorrhagic stroke. Indeed, an increased incidence of symptomatic intracerebral hemorrhage was observed in the trial, but the patients in the treatment group fared overall better than the control group. These results have since been confirmed and the therapeutic window for rt-PA treatment has recently been extended to 4.5 h (Davis and Donnan 2009; Hacke et al. 2008) but at the price of potentially higher risk of intracerebral hemorrhage (Shobha et al. 2010).

To date, thrombolytic therapy with rt-PA remains the only treatment that has been shown to improve the outcome after stroke, but unfortunately most stroke victims (between 70 % and 98 % depending on location) do not reach the hospital within the 4.5 h after the onset of stroke to be eligible for treatment. Nonetheless, the rt-PA trial showed that ischemic stroke was treatable and no doubt stimulated efforts directed at developing additional treatment strategies. Specifically, these strategies have been based on the concept of neuroprotection, which has been defined operationally as "*as any strategy, or combination of strategies, that antagonizes, interrupts, or slows the sequence of injurious biochemical and molecular events that, if left unchecked, would eventuate in irreversible ischemic injury*" (Ginsberg 2008).

9.2.2 The Promise and Apparent Failure of Neuroprotection in Ischemic Stroke

Basic neuroscience research both in vitro and in vivo over the last 30 years or so has led to identification of five key mechanisms that lead to neuronal injury and death following the interruption of blood flow at the onset of stroke (Dirnagl et al. 1999): (1) release of glutamate and activation of receptors for excitatory amino acids,

(2) calcium influx, (3) generation of oxidants (reactive oxygen species such as superoxide, hydroxyl radical, and reactive nitrogen species such as nitric oxide, peroxynitrite), (4) cell suicide (apoptosis), and (5) inflammation. The extracellular accumulation of the amino acid glutamate, the predominant excitatory neurotransmitter in the brain, and concomitant activation of ionotropic glutamate receptors (iGluRs) set in motion a cascade of events leading to neuronal death (Lau and Tymianski 2010). Glutamate receptor mediated neuronal injury and death is referred to as excitotoxicity and is predominately due to activation of the *N*-methyl-D-aspartate receptor (NMDAR) subtype of iGluRs. Excitotoxicity is thought to underlie degeneration of neurons in a number of acute and chronic neurodegenerative diseases such as traumatic brain injury, epilepsy, Huntington's disease, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Lipton and Rosenberg 1994).

Pharmacologic or genetic manipulation of any of these five key mechanisms has been shown to afford neuroprotection in controlled in vitro experiments and laboratory animals subjected to focal or global brain ischemia. To date, however, despite more than 1,000 experimental studies including more than 100 clinical trials no neuroprotective treatment has been shown to be both effective and safe in patients (O'Collins et al. 2006).

The discrepancy between the effectiveness of neuroprotective agents in preclinical animal studies and their failure in humans led to the establishment of the Stroke Therapy Academic Industry Roundtable (STAIR). STAIR brings together academic scientists, clinicians, industry representatives, and regulators to discuss issues related to the development of novel, effective stroke therapies (Stroke Therapy Academic Industry Roundtable II (STAIR-II) 2001; Saver et al. 2009; STAIR 1999). Based on the observation that many clinical studies were conducted with drugs that had shown only limited or even inconsistent preclinical evidence, the first STAIR meeting resulted in the release of guidelines for the preclinical evaluation of neuroprotective drugs and recommendations to clinical investigators regarding what data should be reviewed to increase the chance that a particular neuroprotective drug will succeed in a clinical trial (STAIR 1999). Subsequent meetings have resulted in additional recommendations for preclinical work and clinical trials (Stroke Therapy Academic Industry Roundtable II (STAIR-II) 2001; Saver et al. 2009; Fisher et al. 2009). The initial and updated preclinical STAIR recommendations as listed by Fisher et al. (2009) are shown in Table 9.1.

As has been pointed out recently, however, there is a *"... likelihood that none of the prior neuroprotection drug development efforts was performed in a manner that maximized the chances for success*" (Fisher 2010). This statement also includes the by now famous case of NXY-059, a free radical trapping agent that was advanced to the clinical trial stage specifically because it was claimed to have met the STAIR criteria (Lees et al. 2006). The results of this trial referred to by the acronym SAINT I (Stroke-Acute-Ischaemic-NYX-Treatment) indicated that administration of NXY-059 within 6 h after the onset of ischemic stroke significantly reduced disability at 90 days, but did not significantly improve neurologic functioning as measured by the NIHSS score (Lees et al. 2006). SAINT II, a second, larger trial led to the final conclusion that "*NXY-059 is ineffective for the treatment of acute*

Table 9.1 Initial and updated STAIR preclinical recommendations (Fisher 2010)

1. Adequate dose–response curve defined
2. Document that the drug accesses the target organ, the brain
3. Define the therapeutic time window in well-characterized animal stroke models
4. All animal treatment experiments should be done in a blinded, randomized manner with control of physiological variables with predefined inclusion/exclusion criteria using an adequate sample size based on an appropriate sample size estimate
5. Both histological and functional outcomes should be assessed acutely and long term
6. Efficacy studies should be performed initially in young healthy male animals using permanent occlusion modeling in most cases
7. Initial studies should be performed in rodents and then studies in gyrencephalic species should be considered
8. Additional studies with promising agents should be performed in female animals, aged animals, and animals with comorbid conditions such as hypertension, diabetes, and hypercholesterolemia
9. Relevant biomarker endpoints such as diffusion/perfusion MRI and serum tissue injury markers should be considered
10. Interaction studies with commonly used medications should be performed

Fisher (2010) New Approaches to Neuroprotective Drug Development. Stroke:STROKEAHA. 110.592394. doi:10.1161/strokeaha.110.592394

ischemic stroke within 6 h after the onset of symptoms" (Shuaib et al. 2007) and the drug has since been withdrawn from further development (Shuaib and Hussain 2008). While some have pointed to apparent shortcomings of both the quality of some preclinical studies (Macleod et al. 2008) as well as the drug itself as possible reasons for its failure and maintain hope that neuroprotection can be successfully achieved in humans (Ginsberg 2008; Savitz and Fisher 2007), others have questioned the validity of the neuroprotection hypothesis per se (Röther 2008) and viability of neuroprotection-related drug development by the pharmaceutical industry (Green 2008). For many, great optimism has given way to pessimism, as "*a common perception of neuroprotection is that everything works in animals but nothing works in people*" (O'Collins et al. 2006) but at the same time novel approaches to neuroprotective drug development are being proposed (Chavez et al. 2009; Fisher 2010; Hussain and Shuaib 2008; Tymianski 2010). Thus, the jury is still out and no consensus on the final verdict regarding neuroprotection in stroke has been reached.

9.2.3 Chinese Herbal Medicine as Source of Novel Drugs for Ischemic Stroke

As a coherent system of medical thought and practice that has been in continuous development and use for thousands of years, Chinese medicine easily captures the

interest of anyone who is prepared to take a close look at its theory and role in everyday life in the world's most populous country and beyond. The oldest and most commonly practiced form of therapy in Chinese medicine is pharmacotherapy with drugs derived from plant, animal, and mineral sources (Unschuld 1986). Its origins can be traced back to the beginnings of the Chinese civilization. Transmitted orally at first, Chinese medicine has been documented in written form since about 2,500 years ago (Unschuld 1986). Chinese medicine is an integral part of Chinese culture and the provision of health care. For example, 66 % of doctors in China routinely use herbal medicines during the first 48 h after the insult for the treatment of stroke patients (Chen et al. 1997). Both the theory and practice of Chinese medicine are alive and continue to be developed and adapted to current trends and circumstances today just as they have been in the past (Unschuld 1985, 1986; Goldschmidt 2009; Shen et al. 2005).

In recent years, a drive to "modernize" this ancient form of medicine in China has been gaining momentum (Chen et al. 2004; Fruehauf 1999, 2011). In this context, it is now promoted worldwide as traditional Chinese medicine and commonly referred to using the acronym TCM. It is commonly acknowledged that TCM holds the promise of considerable economic rewards if it can gain recognition as an officially sanctioned complementary and alternative mode of therapy in the industrialized nations. Along these lines, the official policy of the Chinese government has been to conduct research of Chinese medicines according to the standards of Western pharmaceutical research and has made it the basis for the regulatory framework for so-called traditional Chinese patent medicines of which more than 5,000 have been approved by the Chinese State Food and Drug Administration (Wang et al. 2011).

While many advocates of the scientific development of herbal medicine view this approach as the only one that will guarantee the future of herbal medicine and its acceptance by the Western medicine oriented medical establishment, an increasing number of practitioners of Chinese medicine have described and deplored this approach as "*a process that involves gutting the indigenous art of its spirit and essence, and subsequently appropriating its material hull (*i.e. *herbs and techniques) into the realm of a medicine that declares itself scientifically superior*" (Fruehauf 2011). Acknowledging this point of view, we have recently reviewed evidence indicating that herbal medicines used for the treatment of cardiovascular diseases possess biological activity that parallels that of the major classes of drugs used in orthodox pharmacotherapy of cardiovascular diseases (Lee et al. 2008). At the same time, we cautioned that direct evidence linking clinical outcomes to these observed mechanistic actions was often lacking. We suggested that these data provided a starting point for hypothesis-guided basic and clinical research into the use of herbal medicines for effective and affordable pharmacotherapy of cardiovascular disease.

The basic hypothesis underlying one line of our research was that Chinese herbal stroke medicines might contain natural products that exhibited neuroprotective properties and could be used as effective and safe neuroprotective drugs or serve as leads for the development of such drugs (Gong and Sucher 1999). Thus, we

approached the Chinese materia medica as a source of natural products for the development of Western-style drugs. At the same time, however, this line of research will also help to understand the apparent or purported effects of Chinese herbal medicines in modern ("Western") scientific terms and contribute to the further development of Chinese herbal medicines along the lines of Western pharmaceutical standards promoted by the Chinese regulatory bodies. Most importantly, however, the identification of active compounds and their molecular mode of action can guide quality control measures in the preparation of herbal medicines and their hypothesis-driven evaluation in clinical trials (Huang et al. 2004; Yuan and Lin 2000).

While little was published on the neuroprotective effects of herbal medicines in the last century, a great number of studies have been published in the last 10 years trailing by only a few years a general surge of publications on the topic of neuroprotection for ischemic brain injury from the 1990s noted previously (Ginsberg 2008; O'Collins et al. 2006). This includes an increasing number of systematic reviews of the rather extensive mostly Chinese literature reporting the results from clinical trials of Chinese herbal medicines, herbal medicine derived natural products, and Chinese patent medicines (Chen et al. 2009; Zhuo et al. 2008; Cao et al. 2008; Yuan et al. 2008; Yang et al. 2009a; Li et al. 2009b; Zeng et al. 2005; Wu et al. 2007a, b; Tan et al. 2008; Sze et al. 2005). Unfortunately, the common conclusion from the systematic reviews is that the overwhelming majority of studies are of low quality without providing evidence for a beneficial therapeutic effect of any of the Chinese medicines subjected to evaluation in clinical trials. As the reviewers found some clinical observations indicating that there might at times have been some ameliorative effect in regard to the stroke-induced neurological deficit, they invariably end their reviews with the suggestion for more research to be performed.

In order to get an overview of the existing literature on the preclinical research in this regard, we performed a MEDLINE search during the first week of February 2011 using the following compound search expression *"(focal OR global) AND (ischemia OR ischaemia) AND (natural product OR Chinese medicine OR drug)"* and then selected manually those studies that reported the use of single naturally occurring compounds isolated from various Chinese herbal medicines in animal models of transient or permanent focal ischemia of the brain. We excluded studies that used crude extracts, mixtures of herbal extracts, or patent medicines. While we made every effort to be as comprehensive as possible, it is very likely that we have missed some studies, which should have been included. Overall, however, we feel that the studies listed in Table 9.2 are representative of work in the field. Table 9.2 lists 63 studies using 48 different compounds that were published between 2002 and the beginning of February 2011.

The average level of protection (reduction of infarct size compared to vehicle control) was 44.6 ± 17.3 % ($n = 60$). This value is significantly higher than the average values of neuroprotection in focal models of ischemia with either purely experimental agents (24.4 ± 32.9 %, $n = 351$, $p < 0.0001$) or clinically tested drugs (31.35 ± 16.7 %, $n = 66$, $p < 0.05$) that were calculated by O'Collins et al.

Table 9.2 Effects of Chinese medicine derived natural products in animal models of cerebral ischemia

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
20(*S*)-Ginsenoside Rg_3	*Panax ginseng*	Permanent middle cerebral artery occlusion (pMCAO)	Male Wistar–Kyoto rats	30 min after the onset of ischemia	2.5, 5, 10 mg/kg intravenously	Significant reduction of neurological deficit 7 days after MCAO at 5 and 10 mg/kg	Reduction of infarct area by 25 % and 60 % at 5 and 10 mg/kg at 24 h after MCAO	Tian et al. (2005)
4-hydroxybenzyl alcohol	*Gastrodia elata*	tMCAO for 1 h	Adult male Sprague–Dawley rats	30 min before MCAO	25, 50 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 25 and 50 mg/kg	Reduction of 44 % at 50 mg/kg compared to control	Yu et al. (2010)
		tMCAO for 1 h	C57BL/6 mice	After 60 min of MCAO (at the beginning of reperfusion)	25 mg/kg intravenously	Not assessed	Reduction of 41 % compared to control	Descamps et al. (2009)
Apigenin	Fruits, vegetables	tMCAO for 1.5 h	Adult male ICR mice	30 min after MCAO	20 mg/kg orally	Not assessed	Reduction of 20 %	Ha et al. (2008)
Asiatic acid	*Centella asiatica*	pMCAO	Adult male C57BL/6 mice	1 h before and 3, 10, and 20 h after the induction of ischemia. In a second paradigm, 75 mg/kg was administered at 1, 3, 10, and 20 h post-pMCAO	30, 75, or 165 mg/kg by oral gavage	Significant reduction of neurological deficit 24 h but not 7 days after occlusion	Reduction of 54 % at 75 mg/kg compared to control at 24 h after MCAO and reduction of 26.5 % at 7 days after MCAO with treatment before and after MCAO. Reduction of 60 % upon treatment after MCAO, only.	Krishnamurthy et al. (2009)

(continued)

Table 9.2 (continued)

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
Astragaloside	*Astragalus membranaceus*	Transient focal ischemia by middle cerebral artery occlusion (tMCAO) for 2 h	Adult male Sprague–Dawley rats or male Wistar rats	At 0, 8, 24 h, then once a day up to 14 days after reperfusion	40 mg/kg by gavage	Significant reduction of neurological deficit 3 days after occlusion	Reduction of 17 %, 18 %, and 21 % compared to control 3, 7, and 14 days after occlusion	Yin et al. (2010)
Astragaloside IV	*Astragalus membranaceus*	tMCAO for 1.5 h	Adult male C57/B mice	Immediately after MCAO, and then 24 and 48 h after MCAO	20 and 40 mg/kg intraperitoneally	Not assessed	Reduction of 30 % and 32 % compared to control at 20 and 40 mg/kg, respectively	Luo et al. (2004)
Baicalein	*Scutellaria baicalensis*	pMCAO	Adult male Sprague–Dawley rats	After pMCAO	30 mg/kg intravenously	Significant reduction of neurological deficit at 24 h after occlusion	Reduction of 16 % compared to control	Cui et al. (2010)
Baicalin	*Scutellaria baicalensis*	pMCAO	Adult male Sprague–Dawley rats	2 h after MCAO	40 mg/kg per os	Not assessed	Reduction of 40 % compared to control	Zhang et al. (2005)
		tMCAO for 1.5 h	Male Kunming mice	Immediately before reperfusion	20 mg/kg intravenously	Not assessed	Reduction of 40 % compared to control	Zhang et al. (2009b)
		pMCAO	Adult male Sprague–Dawley rats	Two and 12 h after the onset of ischemia	10, 30, and 100 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 30 and 100 mg/kg	Reduction of 40 % and 50 % at 30 and 100 mg/kg compared to control	Tu et al. (2009)

		tMCAO for 2 h	Adult male Wistar rats	After the onset of ischemia	50, 100, and 200 mg/kg intravenously	Significant reduction of neurological deficit at 24 h after occlusion with 100 and 200 mg/kg	Reduction of 27 %, 54 %, and 67 % at 50, 100, and 200 mg/kg compared to control	Xue et al. (2010)
Baicalin, jasminoidin	*Scutellaria baicalensis*	tMCAO for 1.5 h	Adult male Sprague–Dawley rats	Just before reperfusion	15 mg/kg of each baicalin or jasminoidin and 15 mg/kg of baicalin and jasminoidin intravenously	Significant reduction of neurological deficit at 24 h after occlusion only after combined treatment with baicalin and jasminoidin	Reduction of 36 % compared to control for either baicalin or jasminoidin and 48 % for the combination	Zhang et al. (2006)
Brazilein	*Caesalpinia sappan*	tMCAO for 1.5 h	Male Wistar rats	90 min after the onset of MCAO	2.5, 5, and 10 mg/kg intravenously	Significant reduction of neurological deficit at 24 h at 5 and 10 mg/kg.	Reduction of 12 % to 13 %, and 17 % at 2.5, 5 and 10 mg/kg, respectively, compared to control.	Shen et al. (2007)
Breviscapine	*Erigeron breviscapus*	tMCAO for 2 h	Rats	For 7 days before MCAO	50 or 100 mg/kg/day	Not assessed	Significant reduction of apoptotic cells, marked inhibition of caspase-3 and upregulation of bcl-2	Yiming et al. (2008)
Caffeic acid	Vegetables, fruits, coffee, and tea	tMCAO for 30 min	Male Sprague–Dawley rats	30 min before MCAO and 0, 1, 2 h after reperfusion on the first day, and twice daily on the 2nd to 5th day	10 and 50 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h 50 mg/kg.	Reduction of 30 % at 24 h after MCAO, and 50 % after 14 days at 50 mg/kg compared to control	Zhou et al. (2006)

(continued)

Table 9.2 (continued)

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
Cinnamophilin	*Cinnamomum philippinense*	tMCAO for 1 h	Adult male C57BL/B6mice	15 min before or 2 h after MCAO	20, 40, or 80 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion upon pretreatment with 20, 40, or 80 mg/kg and posttreatment with 80 mg/kg	Reduction of 33 %, 46 %, and 46 % in the groups pretreated with CINN at 20, 40, and 80 mg/kg; reduction of 43 % compared to control upon posttreatment with 80 mg/kg	Lee et al. (2005)
Curcumin	*Curcuma longa*	tMCAO for 2 h	Adult male Wistar albino rats	Rats were pretreated with curcumin for 5 days before MCAO and for another 3 days after MCAO	100 mg/kg orally	Significant reduction of neurological deficit at 24 h after occlusion	Reduction of 23 % compared to control	Shukla et al. (2008)
		tMCAO for 2 h	Adult male Sprague–Dawley rats	30 min after MCAO	30, 100, and 300 mg/kg intraperitoneally	No significant reduction of neurological deficit	Reduction of 37 % and 46 % in infarct volume at 100 and 300 mg/kg, respectively, as compared to control.	Thiyagarajan and Sharma (2004)
		tMCAO for 1 h	Adult male Sprague–Dawley rats	After 60 min of MCAO (at the beginning of reperfusion)	100, 300, and 500 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 and 72 h after occlusion at 300 mg/kg	Reduction of 32 %, 47%, and 42 % at 100, 300, and 500 mg/kg compared to control at 24 h after MCAO	Zhao et al. (2010b)

		pMCAO	Adult male Sprague–Dawley rats	15 min after MCAO	50 and 100 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 after occlusion at 100 mg/kg	Reduction of 37 %, at 100 mg/kg compared to control at 24 h after MCAO	Yang et al. (2009b)
		Focal embolic model of MCAO	Male Sprague–Dawley rats	4 h post-ischemia	100, 200, and 300 mg/kg body weight	Significant reduction of neurological at all doses	Reduction of 11 %, 33 %, and 54 % at 100, 200, and 300 mg/kg compared to control	Dohare et al. (2008)
Emodin-8-O-β-D-glucoside	*Polygonum cuspidatum*	tMCAO for 2 h	Adult male Wistar rats	15 min after onset of MCAO	2.5, 5, or 10 mg/kg intravenously	Significant reduction of neurological deficit at 72 h after MCAO	Reduction of 20 % at 5 mg/kg and 27 % at 10 mg/kg compared to control	Wang et al. (2007b)
Ferulic acid	*Angelica sinensis, Ligusticum chuanxiong*	tMCAO for 1.5 h	Adult male Sprague–Dawley rats	At the beginning of MCAO or 30 min after MCAO	60, 80, and 100 mg/kg intravenously	Significant reduction of neurological deficit at 24 h after occlusion at 80 and 100 mg/kg	Reduction of 50 % at 80 mg/kg and 57 % at 100 mg/kg compared to control	Cheng et al. (2008)
Gardenin	Qing Kai Ling injection (QKLI) is a modified preparation of the "An Gong Niu Huang" pill	pMCAO	Adult male Sprague–Dawley rats	2 h after MCAO	40 mg/kg per os	Not assessed	Reduction of 38 % compared to control	Zhang et al. (2005)
Gastrodin	*Gastrodia elata*	tMCAO for 50 min	Adult male Sprague–Dawley rats	At the onset of MCAO	50 or 100 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 100 mg/kg	Reduction of 16 % at 100 mg/kg compared to control	Zeng et al. (2006)

(continued)

Table 9.2 (continued)

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
Glabridin	*Glycyrrhiza glabra*	tMCAO for 2 h	Male Sprague–Dawley rats	Daily for 7 days before and 7 days after MCAO	5 and 25 mg/kg intraperitoneally	No significant improvement of neurological deficit	Reduction of 12 % compared to control at 25 mg/kg	Liang et al. (2008)
Honokiol	*Magnolia officinalis*	tMCAO for 1 h	Male Long-Evans rats	Either 15 min before MCAO or 60 min after MCAO immediately before reperfusion	0.01, 0.1, or 1 μg/kg intravenously	Not assessed	Reduction of 56 % with pretreatment of 0.1 and 1 μg/kg; 65 % and 70 % with posttreatment of 0.1 and 1 μg/kg	Liou et al. (2003b)
		tMCAO for 1 h (right MCA and both common carotid arteries were occluded)	Male Long-Evans rats	Either 15 min before MCAO or 60 min after MCAO immediately before reperfusion	0.01, 0.1, or 1 μg/kg intravenously	Not assessed	Reduction of 61 % and 53 % with pretreatment of 0.1 and 1 μg/kg; 15 %, 58 %, and 68 % with posttreatment of 0.01, 0.1, and 1 μg/kg	Liou et al. (2003a)
Huperzine A	*Huperzia serrata*	tMCAO for 45 min	Male Sprague–Dawley rats	At the onset of occlusion and 6 h later, followed by daily injection for 14 days	0.1 mg/kg intraperitoneally together with 5 mg/kg mecamylamine	Significantly reduced neurological deficit 1, 3, 7, and 14 days after MCAO	Reduction of 70 % compared to control	Wang et al. (2008)
Hydroxysafflor yellow A	*Carthamus tinctorius*	pMCAO	Adult male Wistar–Kyoto	30 min after the onset of ischemia	1.5, 3, 6 mg/kg intravenously	Significantly reduced neurological deficit at 3.0 and 6.0 mg/kg	60 % and 85 % reduction of infarct area at 3.0 and 6.0 mg/kg, respectively, compared control	Zhu et al. (2003)

Isoliquiritigenin	*Glycyrrhiza glabra*	tMCAO for 2 h	Male Sprague–Dawley rats	Once a day, for 7 days prior to ischemia	5, 10, 20 mg/kg intragastrically	Significantly reduced neurological deficit	Reduction of 32 %, 39 %, and 41 % compared to control	Zhan and Yang (2006)
Liquiritin	*Glycyrrhiza uralensis*	tMCAO for 2 h	Male ICR mice	Once a day for 3 days before ischemia	10, 20, and 40 mg/kg intragastrically	Significantly reduced neurological deficit at 40 mg/kg	Reduction of 19 % and 31 % at 20 and 40 mg/kg compared to control	Sun et al. (2010)
Luteolin (liposome-encapsulated)	*Perilla frutescens*	fMCAO for 40 min	Adult female Sprague–Dawley rats	At 6 h after reperfusion, animals were subject to a 13-day treatment of a once-daily injection	5 and 20 mg/kg intraperitoneally	Significantly reduced neurological deficit 7 and 14 days after occlusion at 20 mg/kg	Reduction of 27 % and 45 % at 5 and 20 mg/kg	Zhao et al. (2010a)
Morroniside	Cornus officinalis	tMCAO for 30 min	Adult male Wistar rats	3 h after onset of MCAO	30 mg/kg/day, 90 mg/kg/day, 270 mg/kg/day intragastrically	Significant reduction of neurological deficit 3 days after occlusion at 90 mg/kg, 270 mg/kg	Reduction of 16 % at 90 mg/kg and 62 % at 270 mg/kg compared to control	Wang et al. (2010b)
Morronoside (67 %), loganin (33 %)	Cornus officinalis	tMCAO for 90 min	Male Sprague–Dawley rats	3 h after MCAO	Once a day at 20, 60, and 180 mg/kg, intragastrically	Significant reduction of neurological deficit 7, 14, and 28 days after occlusion at 60 mg/kg, 180 mg/kg	Not assessed	Yao et al. (2009)
Nicotiflorin (kaempferol-3-β-rutinoside)	Flos Carthami	pMCAO	Adult male Sprague–Dawley rats	At the onset of MCAO	2.5, 5 or 10 mg/kg intravenously	Significant reduction of neurological deficit 24 h after occlusion	Reduction of 33 % at 2.5 mg/kg, 50 % at 5 mg/kg, and 61 % at 10 mg/kg compared to control	Li et al. (2006)

(continued)

Table 9.2 (continued)

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
Paeoniflorin	Radix Paeoniae	tMCAO for 1.5 h	Adult male Sprague–Dawley rats	24 h, 48 h, 5, or 7 days before MCAO	10, 20, or 40 mg/kg intraperitoneally	Significant reduction of neurological deficit 24 h after occlusion upon drug application (20 mg/kg) 24 h, 48 h, or 5 days before MCAO	Reduction of 40 % and 60 % compared to control upon drug application at 24 and 48 h before MCAO, respectively	Chen et al. (2006)
Paeonol	*Paeonia suffruticosa*	Transient ischemia occlusion of both carotid arteries and the right middle cerebral artery for 1.5 h	Male Sprague–Dawley rats	20 min before occlusion or 30 min after occlusion (20 mg/kg only)	10, 15, and 20 mg/kg intravenously	Significant reduction of neurological deficit 24 h after occlusion upon pretreatment with15 and 20 mg/kg or posttreatment with 20 mg/kg	Reduction of 66 % of infarct area at 24 h compared to control upon pretreatment with 15 or 20 mg/kg; 50 % reduction upon posttreatment with 20 mg/kg	Hsieh et al. (2006)
Pinocembrin	Propolis	pMCAO	Male Sprague–Dawley rats	0, 8, and 16 h after MCAO	3, 10, and 30 mg/kg intravenously	Significant reduction of neurological deficit 24 h after occlusion	Reduction of 47, 39, and 37 % compared to control at 24 h after MCAO	Gao et al. (2008)
Protopine	*Corydalis ambailis migo*	pMCAO	Male Sprague–Dawley rats	Once a day for 3 days prior to the ischemia	0.98, 1.96, and 3.92 mg/kg intraperitoneally	Significant reduction of neurological deficit 8 h after occlusion at 1.96 and 3.92 mg/kg	Reduction of 28 and 35 % compared to control at 24 h after MCAO at 1.96 and 3.92 mg/kg	Xiao et al. (2007)

Puerarin	Radix puerariae	tMCAO for 1 h	Adult male Wistar rats	10 min before onset of MCAO	20 mg/kg, 50 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 50 mg/kg	Reduction of 13 % at 20 mg/kg and 60 % at 50 mg/kg compared to control	Chang et al. (2009)
		tMCAO for 2 h	Rats	10 min before onset of MCAO	100, 200, and 400 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 400 mg/kg	Reduction at 200 mg/kg and 400 mg/kg compared to control	Gao et al. (2009)
Quercetin (liposomal preparation with lecithin)	Fruits, vegetables	pMCAO	Male Sprague–Dawley rats	30 min or 1 and 4 h after pMCAO	30 mg/kg intraperitoneally	Significant reduction of neurological deficit for animals treated at 30 min after MCAO	Reduced cerebral tissue damage, as shown by the number of neurons and the edema in animals treated at 30 min after MCAO	Rivera et al. (2008)
Resveratrol	*Polygonum cuspidatum*	tMCAO for 2 h	Three months of male Sprague–Dawley rats	Drug was given between 10 and 11 a.m. every day for 6 days; on the 7th day, the last administration was performed 1 h before surgery and tMCAO.	30 mg/kg intraperitoneally	Significant reduction of neurological deficit 7 days after MCAO	Reduction of 65 % compared to control 7 days after MCAO	Li et al. (2011)
		tMCAO for 2 h	Adult male Wistar rats	Drug was administered between 10 and 11 a.m. every day for 21 days before tMCAO on the 22 s day	20 mg/kg intraperitoneally	Significantly improved neurological performance	Reduction of 65 % compared to control 7 days after MCAO	Sinha et al. (2002)

(continued)

Table 9.2 (continued)

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
Safflor yellow B	*Carthamus tinctorius*	pMCAO	Male Wistar–Kyoto (WKY) rats	30 min after MCAO	1.5, 3, and 6 mg/kg intravenously	Significant reduction of neurological deficit at 24 h after occlusion at 3 mg/kg and 6 mg/kg	Reduction of 13 % at 20 mg/kg and 60 % at 50 mg/kg compared to control	Wang et al. (2007a)
Scutellarin	*Erigeron breviscapus*	tMCAO for 2 h	Adult Male Sprague–Dawley rats	For 7 days before ischemia	25, 50, 75 mg/kg intragastrically	Significant reduction of neurological deficit at 24 h after occlusion at 50 and 75 mg/kg	Reduction of 30 % at 50 mg/kg and 56 % at 75 mg/kg compared to control	Zhang et al. (2009a)
Sophocarpine	*Sophora pachycarpa*	tMCAO for 1 h	Adult male Sprague–Dawley rats	30 min before MCAO	5, 10, or 20 mg/kg subcutaneously	Significant reduction of neurological deficit at 24 h after occlusion at 10 and 20 mg/kg	Reduction of 71 % at 10 mg/kg, 86 % at 20 mg/kg compared to control	Yifeng et al. (2011)
Sulforaphane	Cruciferous vegetables (e.g., broccoli)	Transient occlusion of the MCA and common carotid artery for 3 h	Male Long-Evans rats	15 min after onset of ischemia	5 mg/kg intraperitoneally	Not assessed	Reduction of 34 % at 3 days after occlusion and reperfusion	Zhao et al. (2006)
Tanshinone IIA	*Salvia miltiorrhiza*	pMCAO	Adult male ICR mice	One day and 30 min prior occlusion and then again 4 h after occlusion	5 mg/kg, 10 mg/kg, 20 mg/kg intraperitoneally	Significant reduction of neurological deficit at 8 h after occlusion at 20 mg/kg	Reduction of 20 % at 5 mg/kg, 29 % at 10 mg/kg 43 % at 20 mg/kg compared to control	Dong et al. (2009)

		Carotid artery ligation followed by 2 h of hypoxia	Postnatal day 7 rat pubs	From 2 days prior to surgery up to 16 days after surgery	10 mg/kg/day intraperitoneally	Significantly improved postural reflex	"Remarkable reduction in the severity of injury"	Xia et al. (2005)
		pMCAO	Adult male Sprague–Dawley rats	Immediately after pMCAO	10 mg/kg, 20 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 20 mg/kg	Reduction of 40 % at 20 mg/kg compared to control	Wang et al. (2010a)
		pMCAO	Adult male Sprague–Dawley rats	Immediately after pMCAO	10 mg/kg, 20 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion at 20 mg/kg	Reduction of 20 % at 20 mg/kg compared to control	Liu et al. (2010)
		tMCAO for 2 h	Adult male C57BL/6 N x CBA F1 mice	5 min after onset of ischemia	10 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion	Reduction of 29 % compared to control	Lam et al. (2003)
Tanshinone IIB	*Salvia miltiorrhiza*	tMCAO for 2 h	Adult male C57BL/6 N x CBA F1 mice	5 min after onset of ischemia	10 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion	Reduction of 37 % compared to control	Lam et al. (2003)
		tMCAO for 2 h	Adult male Sprague–Dawley rats	One week pretreatment	5 mg/kg, 25 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h after occlusion	Reduction of 13 % at 5 mg/kg, 21 % at 25 mg/kg compared to control	Yu et al. (2007)

(continued)

Table 9.2 (continued)

Compound	Source	Ischemia model	Animal species	Time of drug administration	Dose and route of administration	Neurological assessment	Effect on infarct size	References
Tetramethylpyrazine	*Ligusticum wallichii*	tMCAO for 1.5 h	Adult male Sprague–Dawley rats	60 min before occlusion or 30 min after occlusion or 30 min after occlusion and 60 min after reperfusion	10 mg/kg, 40 mg/kg intraperitoneally	Significant reduction of neurological deficit 3 days after occlusion upon pretreatment with 40 mg/kg	Reduction of 62 % compared to control upon pretreatment with 40 mg/kg and 33 % upon treatment at 30 min after occlusion and 60 min after reperfusion	Kao et al. (2006), Liao et al. (2004)
		tMCAO for 2 h	Adult male Sprague–Dawley rats	One, 2, 4, and 6 h after the start of reperfusion and then every 24 h for 3 days	20 mg/kg intraperitoneally	Signixficant reduction of neurological deficit for drug injection at 1, 2, and 4 but not 6 h after MCAO	Reduction of 43 %, 38 %, and 34 % for treatment at 1, 2, or 4 h after MCAO. There was not significant difference compared to control upon pretreatment 6 h after MCAO	Zhu et al. (2009)
Theaflavin	Black tea	tMCAO for 2 h	Male Sprague-Dawley rats	Immediately before reperfusion	5, 10, 20 mg/kg intravenously	Not assessed	Reduction of 40 % and 52 % in infarct volume at 10 and 20 mg/kg, respectively	Cai et al. (2006)

Tyrosol	Olive oil, wine	tMCAO for 2 h	Male Sprague–Dawley rats	At 0 and 120 min after ischemia	3, 10, and 30 mg/kg intraperitoneally	Significant improvement in sensory motor dysfunction at 10 and 30 mg/kg	Reduction of 24, 32 %, and 64 % at a dose of 3, 10, and 30 mg/kg, respectively	Bu et al. (2007)
Wogonin	*Scutellaria baicalensis*	tMCAO for 2 h	Male Sprague–Dawley rats	At the end of MCAO	50 mg/kg intraperitoneally	Not assessed	Reduction of 44 % at 24 h after onset of MCAO	Piao et al. (2004)
		pMCAO	Male Sprague–Dawley rats	30 min before and 4 h after surgery	20 mg/kg intraperitoneally	Significant reduction of neurological deficit at 24 h	Reduction of 40 % in infarct	Cho and Lee (2004)
Z-Ligustilide	*Angelica sinensis*	pMCAO	Male Sprague–Dawley rats	2 h after onset of ischemia	20 and 80 mg/kg per os	Significant reduction of neurological deficit at 24 h	Reduction of 48 % and 84 % at 20 and 80 mg/kg, respectively, compared to control group	Peng et al. (2007)

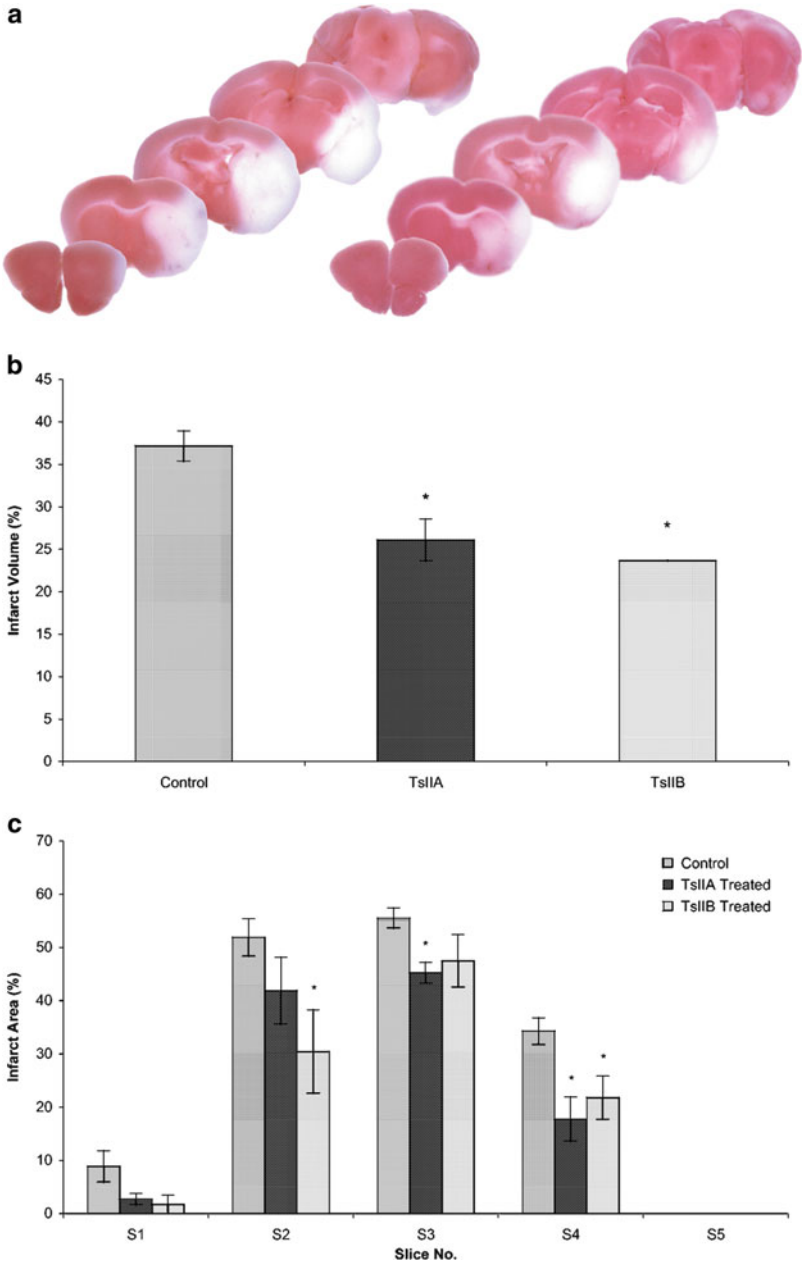

Fig. 9.1 (**a**) Coronal brain slices from mice subjected to 24 h after middle cerebral artery occlusion (MCAo). The slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) to show the area of infarct (*white*). Treatment with tanshinone IIA (right) resulted in a significant decrease in infarct when compared with control (left). (**b**) Effects of TsIIA and tanshinone IIB (TsIIB) treatments on the infarct volume (%) 24 h after MCAo. (**c**) Effects of TsIIA and TsIIB treatment on the area of infarct (%) in each coronal slice 24 h after MCAo. Values are shown in mean ± S.E.M., with $n = 11$ for control, $n = 10$ for TsIIA, and $n = 6$ for TsIIB. * Significantly different from control. Reproduced with permission from Lam et al. (2003)

(2006). Exemplary original data from one of the studies (Lam et al. 2003) included in Table 9.2 is illustrated in Fig. 9.1.

Using the scoring system for the quality of evidence (0–10 points from low to high) used by these authors (O'Collins et al. 2006), baicalin is scored 7, followed by curcumin and tanshinone IIA both with a score of 6, while the majority of studies, which were performed in only a single lab and only in a single species, are scored 0.

All three compounds have been reported to exhibit a variety of pharmacological activities including antioxidant and anti-inflammatory effects (Wang et al. 2007a, b, c; Epstein et al. 2010; Srinivas 2010). Hundreds of published reports have described a similarly large number of molecular effects of these compounds in both in vitro as well as in vivo studies in a number of different assays and experimental systems. Intriguingly, however, all three compounds have been reported to influence the activity of the transcription factor NF-κB (Xue et al. 2010a, b; Jang et al. 2006; Dong et al. 2009; Singh and Aggarwal 1995). NF-κB is known to regulate the expression of a large number of genes involved in cell survival and inflammation and to be activated in neurons in cerebral ischemia (Ridder and Schwaninger 2009), where it can exert either pro- or antiapoptotic effects (Sarnico et al. 2009). Thus, it is tempting to speculate that the observed neuroprotective effects might at least in part be related to the effect of baicalin, curcumin, and tanshinone on the activity of NF-κB. Accordingly, detailed comparative studies of the molecular mechanism underlying the direct and/or indirect effects of these compounds on this transcription factor are warranted.

It is noteworthy that all three drugs have been detected in the brain following intravenous or intraperitoneal administration at concentrations similar to those used in the ischemia experiments (Anand and Newman 2007; Huang et al. 2008; Lam et al. 2003). Curcumin, however, exhibits only very low bioavailability and the doses used in the experiments listed in Table 9.2 were accordingly very high corresponding to amounts of 2.1–35 g for a human patient weighing 70 kg!

Several of the compounds listed in Table 9.2 are found in the same medicinal plant. Baicalin (baicalein is the aglycone of baicalin) and wogonin [the aglycone of wogonoside are constituents of *Scutellaria baicalensis*, the Chinese herbal medicine Radix scutellariae (huangqin in Chinese)]. Oroxylin A is another flavonoid found in this plant that has been reported to have neuroprotective effects (Kim et al. 2006). Tanshinone IIA and IIB are both from *Salvia miltiorrhiza*, the Chinese herbal Radix salviae miltiorrhizae (danshen in Chinese) that is commonly used in the treatment of acute stroke in China, although any beneficial therapeutic effects in stroke patients remain to be established (Zhou et al. 2005; Sze et al. 2005; Wu et al. 2007b).

The studies summarized in Table 9.2 provide clear experimental evidence for neuroprotective effects of single compounds that have been isolated from Chinese herbal medicines. Statistical comparison of the results indicates a significantly higher level of neuroprotection compared to drugs that previously failed in clinical trials. At the same time, however, there is no definitive evidence to date for beneficial therapeutic effects of any Chinese herbal medicine in stroke patients. Thus, it remains to be established whether or not these or other compounds will

prove effective in clinical neuroprotection stroke trials, where more than 100 drugs have failed previously (O'Collins et al. 2006).

In several studies listed in Table 9.2, some drugs were administered for several days before induction of ischemia (Yiming et al. 2008; Zhan and Yang 2006; Sun et al. 2010; Chen et al. 2006; Xiao et al. 2007; Li et al. 2011; Zhang et al. 2009a, b; Yu et al. 2007). It will be interesting to investigate if these (or other) compounds will similarly exhibit neuroprotective effects upon long-term administration (weeks, months, or even years). If that is the case and there are no adverse effects, then prophylactic use of such drugs in persons at risk of stroke (e.g., those experiencing transitory ischemic attacks) might offer a novel neuroprotective approach in stroke and should be tested in clinical trials.

Overall, however, future work involving both single plant-derived compounds as well as complex Chinese herbal (patent) medicines will have to be planned and executed with careful consideration of the lessons learned from the earlier failures of neuroprotective drug development efforts.

9.3 The Way Forward: Social Network Driven Drug Development?

Drug development is clearly a failure prone and thus risky business as the dramatic and to date complete failure in approving new drugs for treatment of ischemic stroke has once more been highlighted. At the same time, the world is facing a shift from an acute to a chronic disease burden and an unsustainable rise in healthcare spending, a situation that has been likened to a "perfect storm" (Helmers et al. 2010) and given rise to the concepts (or has it already become a "movement?") of translational medicine (Marincola 2003; Nussenblatt et al. 2010; Wehling 2008) and translational research (Rubio et al. 2010). While the purpose of translational research was early on viewed as "*to test, in humans, novel therapeutic strategies developed through experimentation*" (Marincola 2003), others have come to see it merely as "*a fashionable term used to describe the wish of biomedical researchers to ultimately help patients by others*." (Wehling 2008). The pharmaceutical industry considers the mission of translational medicine "*simply to improve predictability of the potential success of compounds as they transition through the different stages of drug development toward fulfilling a medical benefit*" (Feuerstein and Chavez 2009).

Selection of the right candidate molecules and early elimination of those that are likely to fail later on is critical. In addition to exhibiting the desired bioactivity (have the right pharmacodynamic properties), drug candidates also need to be able to reach their target (have the right pharmacokinetic properties) and be safe. The fact that a chemical was isolated from an herbal medicine does not automatically guarantee that all of these criteria are fulfilled. In fact, many compounds that have been described as having pharmacologically interesting effects in vitro have very poor bioavailability,

which makes it important to ensure that effective concentrations observed in vitro are achievable in vivo.

The cost of developing a new drug has been estimated at US$800 million in 2003, of which some 60 % are associated with clinical trials. The increasing failure rate of initially promising drug candidates in the clinical trials has recently led some to revise the number considerably upward to as high as US$1.7 billion (Singh 2006). The numbers make plainly clear why the for-profit pharmaceutical industry cannot afford to invest in the development of drugs based on natural products that it cannot protect by patents. At the same time, drug development requires the concerted effort of researchers with widely differing expertise as well as clinicians. Along these lines, some have called for a consortium approach including academia, government, and pharmaceutical industry partnerships and suggested that that more attention be given to the discipline of Translational Medicine (Feuerstein et al. 2008).

We would like to propose to establish a similar worldwide consortium in the field of Chinese herbal medicine through the formation of a World Wide Web based social network. To this end, a website might be set up, where published research data can be publicly discussed and matched against the STAIR criteria so that gaps can be identified. Investigators can then register their interest in working on filling the gaps and national and international funding agencies can announce calls for grant applications to fund the work aimed at filling the gaps. Results (both positive and negative) from this work can be published in peer-reviewed journals and updated on the web site so that progress can be monitored and researchers with appropriate expertise and interest can join in when they feel that prerequisites enabling their work have been provided. Thus, we imagine a social network as a basis for community-driven drug development. This effort does not need to be limited to single chemical compounds but could include combinations of selected compounds, complex herbal extracts, or combinations of herbs that are prepared as tablets, capsules, or injections.

The development of drugs combining multiple active compounds may be more palatable to Western tastes as drug combinations and combination drugs are increasingly used successfully in Western medicine. Combinations of single identified compounds will also be easier to chaperone through existing drug laws and regulations. Individual compounds could be chosen based on their presence in "proven" traditional prescriptions, clearly established molecular targets, and mechanisms of action. Combinations would then be developed from the perspective of a network and systems oriented understanding of the underlying pathology.

On the other hand, the clinical testing of Chinese patent medicines, which are marketed in modern forms of preparation, might be seen as conserving the holistic aspect of traditional herbal Chinese medicine while satisfying requirements for standardization, and quality control. Use of Chinese medicines in standard preparations may pave the way for their use in large-scale clinical trials. Specifically, the potential benefits or adverse effects of integration of the holistic,

traditional medicine based treatment approaches with standard orthodox treatments (e.g., thrombolysis) should be explored.

The community of interested scientists and clinicians, industry partners, and governmental regulators could develop additional model guidelines describing the minimum requirements for the preparation and analytical characterization of complex mixtures of compounds (such as herbal extracts and patent medicines) similar to the STAIR criteria.

The benefits arising from a concerted effort by the international scientific community are potentially huge. Considering the generally acknowledged low quality of many clinical studies that have been performed to date with Chinese herbal medicines or natural products from those medicines, it appears that potentially large sums of money could find a much better use for internationally and publicly reviewed and agreed-on research priorities in the field.

References

Anand K, Newman A (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4(6):807–818. doi:10.1021/mp700113r

Beresford I, Parsons A, Hunter A (2003) Treatments for stroke. Exp Opin Emerg Drugs 8(1):103–122

Bredesen DE, Rao RV, Mehlen P (2006) Cell death in the nervous system. Nature 443(7113):796–802. doi:nature05293[pii]10.1038/nature05293

Bu Y, Rho S, Kim J, Kim MY, Lee DH, Kim SY, Choi H, Kim H (2007) Neuroprotective effect of tyrosol on transient focal cerebral ischemia in rats. Neurosci Lett 414(3):218–221. doi:10.1016/j.neulet.2006.08.094

Cai F, Li CR, Wu JL, Chen JG, Liu C, Min Q, Yu W, Ouyang CH, Chen JH (2006) Theaflavin ameliorates cerebral ischemia-reperfusion injury in rats through its anti-inflammatory effect and modulation of STAT-1. Mediators Inflamm 2006(5):30490. doi:S0962935106304905 [pii] 10.1155/MI/2006/30490

Cao W, Liu W, Wu T, Zhong D, Liu G (2008) Dengzhanhua preparations for acute cerebral infarction. Cochrane Database Syst Rev 4:CD005568. doi:10.1002/14651858.CD005568.pub2

Chang Y, Hsieh C-Y, Peng Z-A, Yen T-L, Hsiao G, Chou D-S, Chen C-M, Sheu J-R (2009) Neuroprotective mechanisms of puerarin in middle cerebral artery occlusion-induced brain infarction in rats. J Biomed Sci 16:9. doi:10.1186/1423-0127-16-9

Chavez JC, Hurko O, Barone FC, Feuerstein GZ (2009) Pharmacologic Interventions for Stroke: Looking Beyond the Thrombolysis Time Window Into the Penumbra With Biomarkers, Not a Stopwatch. Stroke 40(10):e558–e563. doi:10.1161/STROKEAHA.109.559914

Chen Z, Sandercock P, Xie JX, Peto R, Collins R, Liu LS (1997) Hospital management of acute ischemic stroke in China. J Stroke Cerebrovasc Dis 6(5):361–367

Chen CF, Shum YC, Yang SP (2004) The modernization of traditional Chinese medicine in Taiwan–past, present and future. Adv Exp Med Biol 546:35–42

Chen D-M, Xiao L, Cai X, Zeng R, Zhu X-Z (2006) Involvement of multitargets in paeoniflorin-induced preconditioning. J Pharmacol Exp Ther 319(1):165–180. doi:10.1124/jpet.106.104380

Chen X, Zhou M, Li Q, Yang J, Zhang Y, Zhang D, Kong S, Zhou D, He L (2009) Sanchi for acute ischemic stroke. Stroke 40(5):e394–e395. doi:10.1161/STROKEAHA.108.536102

Cheng C-Y, Ho T-Y, Lee E-J, Su S-Y, Tang N-Y, Hsieh C-L (2008) Ferulic acid reduces cerebral infarct through its antioxidative and anti-inflammatory effects following transient focal cerebral ischemia in rats. Am J Chin Med 36(6):1105–1119

Cho J, Lee HK (2004) Wogonin inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion. Biol Pharm Bull 27(10):1561–1564. doi:JST.JSTAGE/bpb/27.1561 [pii]

Cui L, Zhang X, Yang R, Liu L, Wang L, Li M, Du W (2010) Baicalein is neuroprotective in rat MCAO model: role of 12/15-lipoxygenase, mitogen-activated protein kinase and cytosolic phospholipase A2. Pharmacol Biochem Behav 96(4):469–475. doi:10.1016/j.pbb.2010.07.007

Davis SM, Donnan GA (2009) 4.5 hours: the new time window for tissue plasminogen activator in stroke. Stroke 40(6):2266–2267. doi:10.1161/strokeaha.108.544171

Descamps E, Petrault-Laprais M, Maurois P, Pages N, Bac P, Bordet R, Vamecq J (2009) Experimental stroke protection induced by 4-hydroxybenzyl alcohol is cancelled by bacitracin. Neurosci Res 64(2):137–142. doi:S0168-0102(09)00059-5 [pii]10.1016/j.neures.2009.02.005

Di Carlo A (2009) Human and economic burden of stroke. Age Ageing 38(1):4–5. doi:afn282[pii] 10.1093/ageing/afn282

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22(9):391–397

Dohare P, Garg P, Jain V, Nath C, Ray M (2008) Dose dependence and therapeutic window for the neuroprotective effects of curcumin in thromboembolic model of rat. Behav Brain Res 193 (2):289–297. doi:S0166-4328(08)00313-6 [pii]10.1016/j.bbr.2008.06.012

Dong K, Xu W, Yang J, Qiao H, Wu L (2009) Neuroprotective effects of Tanshinone IIA on permanent focal cerebral ischemia in mice. Phytother Res 23(5):608–613. doi:10.1002/ptr.2615

Epstein J, Sanderson IR, Macdonald TT (2010) Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. Br J Nutr 103(11):1545–1557. doi:S0007114509993667[pii] 10.1017/S0007114509993667

Feuerstein GZ, Chavez J (2009) Translational medicine for stroke drug discovery: the pharmaceutical industry perspective. Stroke 40(3 Suppl):S121–125. doi:10.1161/STROKEAHA.108.535104

Feuerstein GZ, Zaleska MM, Krams M, Wang X, Day M, Rutkowski JL, Finklestein SP, Pangalos MN, Poole M, Stiles GL, Ruffolo RR, Walsh FL (2008) Missing steps in the STAIR case: a Translational Medicine perspective on the development of NXY-059 for treatment of acute ischemic stroke. J Cereb Blood Flow Metab 28(1):217–219. doi:9600516[pii]10.1038/sj.jcbfm.9600516

Fisher M (2004) Current concepts of the ischemic penumbra: introduction. Stroke 35(11_suppl_1): 2657–2658. doi:10.1161/01.STR.0000143217.53892.18

Fisher M (2010) New approaches to neuroprotective drug development. Stroke: STROKEAHA.110.592394. doi:10.1161/strokeaha.110.592394

Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, Lo EH (2009) Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 40(6):2244–2250. doi:STROKEAHA.108.541128[pii]10.1161/STROKEAHA.108.541128

Fruehauf H (1999) Science, politics, and the making of "TCM". Chinese medicine in crisis. J Chinese Med 61:1–9

Fruehauf H (2011) Chinese Medicine In Crisis: Science, Politics, And The Making Of "TCM". http://www.classicalchinesemedicine.org/2009/04/chinese-medicine-in-crisis-1/.

Gao M, Liu R, Zhu SY, Du GH (2008) Acute neurovascular unit protective action of pinocembrin against permanent cerebral ischemia in rats. J Asian Nat Prod Res 10(5-6):551–558. doi:793082119 [pii]10.1080/10286020801966955

Gao L, Ji X, Song J, Liu P, Yan F, Gong W, Dang S, Luo Y (2009) Puerarin protects against ischemic brain injury in a rat model of transient focal ischemia. Neurol Res 31(4):402–406. doi:10.1179/174313209X444017

Ginsberg MD (2008) Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology 55(3):363–389. doi:10.1016/j.neuropharm.2007.12.007

Goldschmidt AM (2009) The evolution of Chinese medicine: Song dynasty, 960–1200. Needham Research Institute series. Routledge, London

Gong X, Sucher NJ (1999) Stroke therapy in traditional Chinese medicine (TCM): prospects for drug discovery and development. Trends Pharmacol Sci 20(5):191–196

Green AR (2008) Pharmacological approaches to acute ischaemic stroke: reperfusion certainly, neuroprotection possibly. Br J Pharmacol 153(Suppl 1):S325–338. doi:10.1038/sj.bjp.0707594

Ha SK, Lee P, Park JA, Oh HR, Lee SY, Park JH, Lee EH, Ryu JH, Lee KR, Kim SY (2008) Apigenin inhibits the production of NO and PGE2 in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model. Neurochem Int 52 (4-5):878–886. doi:S0197-0186(07)00292-6 [pii]10.1016/j.neuint.2007.10.005

Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, Larrue V, Lees KR, Medeghri Z, Machnig T, Schneider D, von Kummer R, Wahlgren N, Toni D (2008) Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med 359(13):1317–1329. doi:359/13/1317[pii]10.1056/NEJMoa0804656

Heath G, Colburn WA (2000) An evolution of drug development and clinical pharmacology during the 20th century. J Clin Pharmacol 40(9):918–929

Helmers SL, Phillips VL, Esper GJ (2010) Translational medicine in neurology: the time is right. Arch Neurol 67(10):1263–1266. doi:10.1001/archneurol.2010.253

Hsieh CL, Cheng CY, Tsai TH, Lin IH, Liu CH, Chiang SY, Lin JG, Lao CJ, Tang NY (2006) Paeonol reduced cerebral infarction involving the superoxide anion and microglia activation in ischemia-reperfusion injured rats. J Ethnopharmacol 106(2):208–215. doi:S0378-8741(05)00846-9 [pii]10.1016/j.jep.2005.12.027

Huang X, Kong L, Li X, Chen X, Guo M, Zou H (2004) Strategy for analysis and screening of bioactive compounds in traditional Chinese medicines. J Chromatogr B Analyt Technol Biomed Life Sci 812(1–2):71–84

Huang H, Zhang Y, Yang R, Tang X (2008) Determination of baicalin in rat cerebrospinal fluid and blood using microdialysis coupled with ultra-performance liquid chromatography-tandem mass spectrometry. J Chromatogr B 874(1–2):77–83. doi:10.1016/j.jchromb.2008.09.005

Hussain MS, Shuaib A (2008) Research into neuroprotection must continue . . . but with a different approach. Stroke 39(2):521–522. doi:10.1161/STROKEAHA.107.494781

Jang SI, Kim HJ, Kim YJ, Jeong SI, You YO (2006) Tanshinone IIA inhibits LPS-induced NF-kappaB activation in RAW 264.7 cells: possible involvement of the NIK-IKK, ERK1/2, p38 and JNK pathways. Eur J Pharmacol 542(1–3):1–7. doi:S0014-2999(06)00464-X[pii]10.1016/j.ejphar.2006.04.044

Jia J, Zhang X, Hu YS, Wu Y, Wang QZ, Li NN, Wu CQ, Yu HX, Guo QC (2009) Protective effect of tetraethyl pyrazine against focal cerebral ischemia/reperfusion injury in rats: therapeutic time window and its mechanism. Thromb Res 123(5):727–730. doi:S0049-3848(08)00535-5[pii]10.1016/j.thromres.2008.11.004

Kao TK, Ou YC, Kuo JS, Chen WY, Liao SL, Wu CW, Chen CJ, Ling NN, Zhang YH, Peng WH (2006) Neuroprotection by tetramethylpyrazine against ischemic brain injury in rats. Neurochem Int 48(3):166–176. doi:S0197-0186(05)00251-2 [pii]10.1016/j.neuint.2005.10.008

Kim DH, Jeon SJ, Son KH, Jung JW, Lee S, Yoon BH, Choi JW, Cheong JH, Ko KH, Ryu JH (2006) Effect of the flavonoid, oroxylin A, on transient cerebral hypoperfusion-induced memory impairment in mice. Pharmacol Biochem Behav 85(3):658–668. doi:S0091-3057(06)00357-1[pii]10.1016/j.pbb.2006.10.025

Koehn FE, Carter GT (2005) The evolving role of natural products in drug discovery. Nat Rev Drug Discov 4(3):206–220

Krishnamurthy RG, Senut MC, Zemke D, Min J, Frenkel MB, Greenberg EJ, Yu SW, Ahn N, Goudreau J, Kassab M, Panickar KS, Majid A (2009) Asiatic acid, a pentacyclic triterpene from Centella asiatica, is neuroprotective in a mouse model of focal cerebral ischemia. J Neurosci Res 87(11):2541–2550. doi:10.1002/jnr.22071

Lam BYH, Lo ACY, Sun X, Luo HW, Chung SK, Sucher NJ (2003) Neuroprotective effects of tanshinones in transient focal cerebral ischemia in mice. Phytomedicine 10(4):286–291

Lau A, Tymianski M (2010) Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch – Eur J Physiol 460(2):525–542. doi:10.1007/s00424-010-0809-1

Lee EJ, Chen HY, Lee MY, Chen TY, Hsu YS, Hu YL, Chang GL, Wu TS (2005) Cinnamophilin reduces oxidative damage and protects against transient focal cerebral ischemia in mice. Free Radic Biol Med 39(4):495–510. doi:S0891-5849(05)00196-6 [pii]10.1016/j.freeradbiomed.2005.04.004

Lee BJ, Carles MC, Sucher NJ (2008) Traditional Chinese herbal medicines for the treatment of cardiovascular diseases. In: Watson RR, Preedy VR (eds) Botanical medicine in clinical practice. CABI, Wallingford, UK

Lees KR, Zivin JA, Ashwood T, Davalos A, Davis SM, Diener HC, Grotta J, Lyden P, Shuaib A, Hardemark HG, Wasiewski WW (2006) NXY-059 for acute ischemic stroke. N Engl J Med 354(6):588–600. doi:354/6/588[pii]10.1056/NEJMoa052980

Li R, Guo M, Zhang G, Xu X, Li Q (2006) Neuroprotection of nicotiflorin in permanent focal cerebral ischemia and in neuronal cultures. Biol Pharm Bull 29(9):1868–1872. doi:JST.JSTAGE/bpb/29.1868 [pii]

Li HY, Cui L, Cui M (2009a) Hot topics in Chinese herbal drugs research documented in PubMed/MEDLINE by authors inside China and outside of China in the past 10 years: based on co-word cluster analysis. J Altern Complement Med 15(7):779–785. doi:10.1089/acm.2008.0594

Li W, Liu M, Feng S, Wu B, Zhang S, Yang W, Liu GJ (2009b) Acanthopanax for acute ischaemic stroke. Cochrane Database Syst Rev (3):CD007032. doi:10.1002/14651858.CD007032.pub2

Li H, Yan Z, Zhu J, Yang J, He J (2011) Neuroprotective effects of resveratrol on ischemic injury mediated by improving brain energy metabolism and alleviating oxidative stress in rats. Neuropharmacology 60(2–3):252–258. doi:S0028-3908(10)00226-1[pii]10.1016/j.neuropharm.2010.09.005

Liang HW, Qiu SF, Shen J, Sun LN, Wang JY, Bruce IC, Xia Q (2008) Genistein attenuates oxidative stress and neuronal damage following transient global cerebral ischemia in rat hippocampus. Neurosci Lett 438(1):116–120. doi:S0304-3940(08)00483-7 [pii]10.1016/j.neulet.2008.04.058

Liao SL, Kao TK, Chen WY, Lin YS, Chen SY, Raung SL, Wu CW, Lu HC, Chen CJ (2004) Tetramethylpyrazine reduces ischemic brain injury in rats. Neurosci Lett 372(1–2):40–45. doi:S0304-3940(04)01138-3 [pii]10.1016/j.neulet.2004.09.013

Liou KT, Lin SM, Huang SS, Chih CL, Tsai SK (2003a) Honokiol ameliorates cerebral infarction from ischemia-reperfusion injury in rats. Planta Med 69(2):130–134. doi:10.1055/s-2003-37707

Liou KT, Shen YC, Chen CF, Tsao CM, Tsai SK (2003b) Honokiol protects rat brain from focal cerebral ischemia-reperfusion injury by inhibiting neutrophil infiltration and reactive oxygen species production. Brain Res 992(2):159–166. doi:S0006899303036023 [pii]

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 79(4):1431–1568

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330(9):613–622. doi:10.1056/NEJM199403033300907

Liu L, Zhang X, Wang L, Yang R, Cui L, Li M, Du W, Wang S (2010) The neuroprotective effects of Tanshinone IIA are associated with induced nuclear translocation of TORC1 and upregulated expression of TORC1, pCREB and BDNF in the acute stage of ischemic stroke. Brain Res Bull 82(3–4):228–233. doi:S0361-9230(10)00086-9 [pii]10.1016/j.brainresbull.2010.04.005

Luo Y, Qin Z, Hong Z, Zhang X, Ding D, Fu JH, Zhang WD, Chen J (2004) Astragaloside IV protects against ischemic brain injury in a murine model of transient focal ischemia. Neurosci Lett 363(3):218–223. doi:10.1016/j.neulet.2004.03.036S0304394004003362 [pii]

Macleod MR, van der Worp HB, Sena ES, Howells DW, Dirnagl U, Donnan GA (2008) Evidence for the efficacy of NXY-059 in experimental focal cerebral ischaemia is confounded by study quality. Stroke 39(10):2824–2829. doi:STROKEAHA.108.515957[pii]10.1161/STROKEAHA.108.515957

Marincola FM (2003) Translational Medicine: A two-way road. J Transl Med 1(1):1. doi:10.1186/1479-5876-1-1

Martinez B, Goldstein J (2007) Big Pharma Faces Grim Prognosis. The Wall Street Journal (December 6, 2007):A1

Neuroprotection as initial therapy in acute stroke. Third Report of an Ad Hoc Consensus Group Meeting. The European Ad Hoc Consensus Group (1998). Cerebrovasc Dis 8 (1):59–72

Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. J Nat Prod 70(3):461–477

Newman DJ, Cragg GM, Snader KM (2000) The influence of natural products upon drug discovery. Nat Prod Rep 17(3):215–234

Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 66(7):1022–1037

Nussenblatt RB, Marincola FM, Schechter AN (2010) Translational medicine–doing it backwards. J Transl Med 8:12. doi:10.1186/1479-5876-8-12

O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW (2006) 1,026 experimental treatments in acute stroke. Ann Neurol 59(3):467–477. doi:10.1002/ana.20741

Peng HY, Du JR, Zhang GY, Kuang X, Liu YX, Qian ZM, Wang CY (2007) Neuroprotective effect of Z-ligustilide against permanent focal ischemic damage in rats. Biol Pharm Bull 30 (2):309–312. doi:JST.JSTAGE/bpb/30.309 [pii]

Piao HZ, Jin SA, Chun HS, Lee JC, Kim WK (2004) Neuroprotective effect of wogonin: potential roles of inflammatory cytokines. Arch Pharm Res 27(9):930–936

Ridder DA, Schwaninger M (2009) NF-kappaB signaling in cerebral ischemia. Neuroscience 158(3):995–1006. doi:S0306-4522(08)01027-0[pii]10.1016/j.neuroscience.2008.07.007

Rivera F, Costa G, Abin A, Urbanavicius J, Arruti C, Casanova G, Dajas F (2008) Reduction of ischemic brain damage and increase of glutathione by a liposomal preparation of quercetin in permanent focal ischemia in rats. Neurotox Res 13(2):105–114

Röther J (2008) Neuroprotection Does Not Work! Stroke 39(2):523–524. doi:10.1161/STROKEAHA.107.494799

Rouhi AM (2003) Rediscovering natural products. Chem Eng News 81(41):77–78

Rubio DM, Schoenbaum EE, Lee LS, Schteingart DE, Marantz PR, Anderson KE, Platt LD, Baez A, Esposito K (2010) Defining translational research: implications for training. Acad Med 85 (3):470–475. doi:10.1097/ACM.0b013e3181ccd6100001888-201003000-00021[pii]

Sarnico I, Lanzillotta A, Benarese M, Alghisi M, Baiguera C, Battistin L, Spano P, Pizzi M (2009) NF-kappaB dimers in the regulation of neuronal survival. Int Rev Neurobiol 85:351–362. doi:S0074-7742(09)85024-1[pii]10.1016/S0074-7742(09)85024-1

Saver JL, Albers GW, Dunn B, Johnston KC, Fisher M, Consortium SV (2009) Stroke Therapy Academic Industry Roundtable (STAIR) recommendations for extended window acute stroke therapy trials. Stroke 40(7):2594–2600. doi:10.1161/STROKEAHA.109.552554

Savitz SI, Fisher M (2007) Future of neuroprotection for acute stroke: in the aftermath of the SAINT trials. Ann Neurol 61(5):396–402. doi:10.1002/ana.21127

Shen SY, Fu XD, Fei ZY (2005) [Assessment and explorations on the mechanism of neuroprotection of patients in ischemic stroke by traditional Chinese medicine]. Chin J Integr Med 11(3):237–240

Shen J, Zhang H, Lin H, Su H, Xing D, Du L (2007) Brazilein protects the brain against focal cerebral ischemia reperfusion injury correlating to inflammatory response suppression. Eur J Pharmacol 558(1–3):88–95. doi:S0014-2999(06)01319-7 [pii]10.1016/j.ejphar.2006.11.059

Shi FL, Hart RG, Sherman DG, Tegeler CH (1989) Stroke in the People's Republic of China. Stroke 20(11):1581–1585

Shobha N, Buchan AM, Hill MD (2010) Thrombolysis at 3–4.5 hours after acute ischemic stroke onset—evidence from the Canadian Alteplase for Stroke Effectiveness Study (CASES) Registry. Cerebrovasc Dis 31(3):223–228. doi:000321893[pii]10.1159/000321893

Shuaib A, Hussain MS (2008) The past and future of neuroprotection in cerebral ischaemic stroke. Eur Neurol 59(1–2):4–14. doi:10.1159/000109254

Shuaib A, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, Diener HC, Ashwood T, Wasiewski WW, Emeribe U (2007) NXY-059 for the treatment of acute ischemic stroke. N Engl J Med 357(6):562–571. doi:357/6/562[pii]10.1056/NEJMoa070240

Shukla PK, Khanna VK, Ali MM, Khan MY, Srimal RC (2008) Anti-ischemic effect of curcumin in rat brain. Neurochem Res 33(6):1036–1043. doi:10.1007/s11064-007-9547-y

Singh R (2006) Clinical research in China and India: a paradigm shift in drug development. Drug Discov Today 11(15–16):675–676. doi:10.1016/j.drudis.2006.06.009

Singh S, Aggarwal BB (1995) Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem 270(42):24995–25000

Sinha K, Chaudhary G, Gupta YK (2002) Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. Life Sci 71(6):655–665. doi:S0024320502016910 [pii]

Srinivas NR (2010) Baicalin, an emerging multi-therapeutic agent: pharmacodynamics, pharmacokinetics, and considerations from drug development perspectives. Xenobiotica 40(5):357–367. doi:10.3109/00498251003663724

STAIR (1999) Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 30(12):2752–2758

Stroke Therapy Academic Industry Roundtable II (STAIR-II) (2001) Recommendations for clinical trial evaluation of acute stroke therapies. Stroke 32(7):1598–1606

Sun YX, Tang Y, Wu AL, Liu T, Dai XL, Zheng QS, Wang ZB (2010) Neuroprotective effect of liquiritin against focal cerebral ischemia/reperfusion in mice via its antioxidant and antiapoptosis properties. J Asian Nat Prod Res 12(12):1051–1060. doi:930404879[pii]10.1080/10286020.2010.535520

Sze FK, Yeung FF, Wong E, Lau J (2005) Does Danshen improve disability after acute ischaemic stroke? Acta Neurol Scand 111(2):118–125

Tan Y, Liu M, Wu B (2008) Puerarin for acute ischaemic stroke. Cochrane Database Syst Rev (1): CD004955. doi:10.1002/14651858.CD004955.pub2

The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (1995) Tissue plasminogen activator for acute ischemic stroke. N Engl J Med 333(24):1581–1587. doi:10.1056/NEJM199512143332401

Thiyagarajan M, Sharma SS (2004) Neuroprotective effect of curcumin in middle cerebral artery occlusion induced focal cerebral ischemia in rats. Life Sci 74(8):969–985. doi:S0024320503009731 [pii]

Tian J, Fu F, Geng M, Jiang Y, Yang J, Jiang W, Wang C, Liu K (2005) Neuroprotective effect of 20(S)-ginsenoside Rg3 on cerebral ischemia in rats. Neurosci Lett 374(2):92–97. doi:S0304-3940(04)01293-5 [pii]10.1016/j.neulet.2004.10.030

Tu XK, Yang WZ, Shi SS, Wang CH, Chen CM (2009) Neuroprotective effect of baicalin in a rat model of permanent focal cerebral ischemia. Neurochem Res 34(9):1626–1634. doi:doi:10.1007/s11064-009-9953-4

Tymianski M (2010) Can molecular and cellular neuroprotection be translated into therapies for patients?: Yes, but not the way we tried it before. Stroke 41(10, Suppl 1):S87–S90, 10.1161/STROKEAHA.110.595496

Unschuld PU (1985) Medicine in China: a history of ideas. Comparative studies of health systems and medical care. University of California Press, Berkeley

Unschuld PU (1986) Medicine in China: a history of pharmaceutics. Comparative studies of health systems and medical care. University of California Press, Berkeley

Wang X, Morris-Natschke SL, Lee KH (2007a) New developments in the chemistry and biology of the bioactive constituents of Tanshen. Med Res Rev 27(1):133–148. doi:10.1002/med.20077

Wang C, Zhang D, Li G, Liu J, Tian J, Fu F, Liu K (2007a) Neuroprotective effects of safflor yellow B on brain ischemic injury. Exp Brain Res 177(4):533–539. doi:10.1007/s00221-006-0705-2

Wang C, Zhang D, Ma H, Liu J (2007b) Neuroprotective effects of emodin-8-O-beta-D-glucoside in vivo and in vitro. Eur J Pharmacol 577(1-3):58–63. doi:S0014-2999(07)00974-0 [pii]10.1016/j.ejphar.2007.08.033

Wang C, Zhang D, Li G, Liu J, Tian J, Fu F, Liu K (2007c) Exp Brain Res 177:533–539

Wang ZF, Wang J, Zhang HY, Tang XC (2008) J Neurochem 106:1594–1603
Wang L, Zhang X, Liu L, Cui L, Yang R, Li M, Du W (2010a) Tanshinone II A down-regulates HMGB1, RAGE, TLR4, NF-kappaB expression, ameliorates BBB permeability and endothelial cell function, and protects rat brains against focal ischemia. Brain Res 1321:143–151. doi:S0006-8993(09)02715-2 [pii]10.1016/j.brainres.2009.12.046
Wang W, Xu J, Li L, Wang P, Ji X, Ai H, Zhang L, Li L (2010b) Neuroprotective effect of morroniside on focal cerebral ischemia in rats. Brain Res Bull 83(5):196–201. doi:10.1016/j.brainresbull.2010.07.003
Wang Z, Liu J, Cheng Y, Wang Y (2011) Fangjiomics: In Search of Effective and Safe Combination Therapies. J Clin Pharmacol:1–21. doi:10.1177/0091270010382913
Wehling M (2008) Translational medicine: science or wishful thinking? J Transl Med 6:31. doi:10.1186/1479-5876-6-31
Wolfe C, Rudd T (2007) The burden of stroke. The stroke association, 240 City Road, London EC1V 2PR, London
Wu B, Liu M, Liu H, Li W, Tan S, Zhang S, Fang Y (2007a) Meta-analysis of traditional Chinese patent medicine for ischemic stroke. Stroke 38(6):1973–1979. doi:10.1161/STROKEAHA.106.473165
Wu B, Liu M, Zhang S (2007b) Dan Shen agents for acute ischaemic stroke. Cochrane Database Syst Rev (2):CD004295. doi:10.1002/14651858.CD004295.pub3
Xia WJ, Yang M, Fok TF, Li K, Chan WY, Ng PC, Ng HK, Chik KW, Wang CC, Gu GJ, Woo KS, Fung KP (2005) Partial neuroprotective effect of pretreatment with tanshinone IIA on neonatal hypoxia-ischemia brain damage. Pediatr Res 58(4):784–790. doi:58/4/784 [pii]10.1203/01.PDR.0000180550.99162.BC
Xiao X, Liu J, Hu J, Li T, Zhang Y (2007) Protective effect of protopine on the focal cerebral ischaemic injury in rats. Basic Clin Pharmacol Toxicol 101(2):85–89. doi:PTO075[pii]10.1111/j.1742-7843.2007.00075.x
Xue X, Qu XJ, Yang Y, Sheng XH, Cheng F, Jiang EN, Wang JH, Bu W, Liu ZP (2010a) Baicalin attenuates focal cerebral ischemic reperfusion injury through inhibition of nuclear factor kappaB p65 activation. Biochem Biophys Res Commun 403(3–4):398–404. doi:S0006-291X(10)02101-7[pii]10.1016/j.bbrc.2010.11.042
Xue X, Qu XJ, Yang Y, Sheng XH, Cheng F, Jiang EN, Wang JH, Bu W, Liu ZP (2010b) Biochem Biophys Res Commun 403:398–404
Yang W, Hao Z, Zhang S, Dong W, Wu T, Liu GJ, Liu M (2009a) Mailuoning for acute ischaemic stroke. Cochrane Database Syst Rev (2):CD007028. doi:10.1002/14651858.CD007028.pub2
Yang C, Zhang X, Fan H, Liu Y (2009b) Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. Brain Res 1282:133–141. doi:S0006-8993(09)00929-9 [pii]10.1016/j.brainres.2009.05.009
Yao R-Q, Zhang L, Wang W, Li L (2009) Cornel iridoid glycoside promotes neurogenesis and angiogenesis and improves neurological function after focal cerebral ischemia in rats. Brain Res Bull 79(1):69–76. doi:10.1016/j.brainresbull.2008.12.010
Yifeng M, Bin W, Weiqiao Z, Yongming Q, Bing L, Xiaojie L (2011) Neuroprotective effect of sophocarpine against transient focal cerebral ischemia via down-regulation of the acid-sensing ion channel 1 in rats. Brain Res. doi:S0006-8993(11)00014-X [pii]10.1016/j.brainres.2011.01.004
Yiming L, Wei H, Aihua L, Fandian Z (2008) Neuroprotective effects of breviscapine against apoptosis induced by transient focal cerebral ischaemia in rats. J Pharm Pharmacol 60(3):349–355. doi:10.1211/jpp.60.3.0010
Yin Y-Y, Li W-P, Gong H-L, Zhu F-F, Li W-Z, Wu G-C (2010) Protective effect of astragaloside on focal cerebral ischemia/reperfusion injury in rats. Am J Chin Med 38(3):517–527
Yu X-Y, Lin S-G, Zhou Z-W, Chen X, Liang J, Duan W, Yu X-Q, Wen J-Y, Chowbay B, Li C-G, Sheu F-S, Chan E, Zhou S-F (2007) Tanshinone IIB, a primary active constituent from Salvia miltiorrhza, exhibits neuro-protective activity in experimentally stroked rats. Neurosci Lett 417(3):261–265. doi:10.1016/j.neulet.2007.02.079

Yu S-S, Zhao J, Zheng W-P, Zhao Y (2010) Neuroprotective effect of 4-hydroxybenzyl alcohol against transient focal cerebral ischemia via anti-apoptosis in rats. Brain Res 1308:167–175. doi:10.1016/j.brainres.2009.10.037

Yuan R, Lin Y (2000) Traditional Chinese medicine: an approach to scientific proof and clinical validation. Pharmacol Ther 86(2):191–198

Yuan Y, Zeng X, Luo Y, Li Z, Wu T (2008) Chuanxiong-type preparations for acute ischemic stroke. Cochrane Database Syst Rev (4):CD005569. doi:10.1002/14651858.CD005569.pub2

Zeng X, Liu M, Yang Y, Li Y, Asplund K (2005) Ginkgo biloba for acute ischaemic stroke. Cochrane Database Syst Rev (4):CD003691. doi:10.1002/14651858.CD003691.pub2

Zeng X, Zhang S, Zhang L, Zhang K, Zheng X (2006) A study of the neuroprotective effect of the phenolic glucoside gastrodin during cerebral ischemia in vivo and in vitro. Planta Med 72 (15):1359–1365. doi:10.1055/s-2006-951709

Zhan C, Yang J (2006) Protective effects of isoliquiritigenin in transient middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Pharmacol Res 53(3):303–309. doi:S1043-6618(06)00004-1[pii]10.1016/j.phrs.2005.12.008

Zhang ZJ, Wang Z, Zhang XY, Ying K, Liu JX, Wang YY (2005) Gene expression profile induced by oral administration of baicalin and gardenin after focal brain ischemia in rats. Acta Pharmacol Sin 26(3):307–314. doi:10.1111/j.1745-7254.2005.00051.x

Zhang Z-J, Li P, Wang Z, Li P-T, Zhang W-S, Sun Z-H, Zhang X-J, Wang Y-Y (2006) A comparative study on the individual and combined effects of baicalin and jasminoidin on focal cerebral ischemia-reperfusion injury. Brain Res 1123(1):188–195. doi:10.1016/j.brainres.2006.09.063

Zhang H-F, Hu X-M, Wang L-X, Xu S-Q, Zeng F-D (2009a) Protective effects of scutellarin against cerebral ischemia in rats: evidence for inhibition of the apoptosis-inducing factor pathway. Planta Med 75(2):121–126. doi:10.1055/s-0028-1088368

Zhang Z, Wu R, Li P, Liu F, Zhang W, Zhang P, Li P, Wang Y (2009b) Baicalin administration is effective in positive regulation of twenty-four ischemia/reperfusion-related proteins identified by a proteomic study. Neurochem Int 54(8):488–496. doi:10.1016/j.neuint.2009.02.005

Zhao J, Kobori N, Aronowski J, Dash PK (2006) Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. Neurosci Lett 393(2–3):108–112. doi:S0304-3940(05)01114-6 [pii]10.1016/j.neulet.2005.09.065

Zhao J, Yu S, Zheng W, Feng G, Luo G, Wang L, Zhao Y (2010b) Curcumin improves outcomes and attenuates focal cerebral ischemic injury via antiapoptotic mechanisms in rats. Neurochem Res 35(3):374–379. doi:10.1007/s11064-009-0065-y

Zhao G, Zang SY, Jiang ZH, Chen YY, Ji XH, Lu BF, Wu JH, Qin GW, Guo LH (2010a) Postischemic administration of liposome-encapsulated luteolin prevents against ischemia-reperfusion injury in a rat middle cerebral artery occlusion model. J Nutr Biochem. doi:S0955-2863(10)00195-6 [pii]10.1016/j.jnutbio.2010.07.014

Zhou L, Zuo Z, Chow MSS (2005) Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. J Clin Pharmacol 45(12):1345–1359. doi:10.1177/0091270005282630

Zhou Y, Fang SH, Ye YL, Chu LS, Zhang WP, Wang ML, Wei EQ (2006) Caffeic acid ameliorates early and delayed brain injuries after focal cerebral ischemia in rats. Acta Pharmacol Sin 27(9):1103–1110. doi:10.1111/j.1745-7254.2006.00406.x

Zhu H, Wang Z, Ma C, Tian J, Fu F, Li C, Guo D, Roeder E, Liu K (2003) Neuroprotective effects of hydroxysafflor yellow A: in vivo and in vitro studies. Planta Med 69(5):429–433. doi:10.1055/s-2003-39714

Zhu XL, Xiong LZ, Wang Q, Liu ZG, Ma X, Zhu ZH, Hu S, Gong G, Chen SY (2009) Therapeutic time window and mechanism of tetramethylpyrazine on transient focal cerebral ischemia/reperfusion injury in rats. Neurosci Lett 449(1):24–27. doi:S0304-3940(08)01235-4 [pii]10.1016/j.neulet.2008.09.007

Zhuo Q, Yang X, Wu T, Liu G, Zhou L (2008) Tongxinluo capsule for acute stroke. Cochrane Database Syst Rev (4):CD004584. doi:10.1002/14651858.CD004584.pub2

Chapter 10
Complementary and Traditional Chinese Medicine Methods in the Treatment of Gynecological Diseases

Wolfgang Wuttke and Dana Seidlova-Wuttke

10.1 Introduction

Many women experience menopausal symptoms and most find them distressing. This has led to efforts to find alternatives to classical hormone replacement therapy (HRT). Basic and clinical researchers, often supported by plant and food additive producing companies seek for such alternatives. This specifically includes the integration of traditional Chinese Medicine (TCM) or of complementary and alternative medicines (CAM) into treatment protocols tailored to women's individual needs.

Generally TCM attributes problems of the menopause to kidney deficiency (shen xu). In TCM the kidneys (shen) are a functional visceral system (zang) which regulates and sustains growth, maturation, and aging thus harmonizing all metabolic processes. According to TCM beliefs, kidney disease may lead to dysfunction of other visceral systems. Therefore, the TCM claims that kidney deficiency is "always at the root of menopausal problems." "Chinese medicine works by gently tonifying the Kidneys and the Kidney-Essence to help the woman in this transitional time of life".

TCM is provided by a professional class of physicians with its own diagnostic system. In a recent review (Scheid et al. 2010) it is stated that the diagnostic textbook descriptions of diseases are not always founded in clinical experience even if the TCM textbooks make such claims. According to these authors this raises questions about the diagnostic procedures proposed by textbooks to clinical practice and therefore about the validity of clinical research based on TCM textbooks. This textbook knowledge is accumulated over thousands of years and is therefore claimed to be efficient. Many clinical studies seem to support this

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notion. Upon a closer look, however, very few clinical studies meet the criteria of evidence-based medicine, i.e., they are often not placebo-controlled.

Hence, all TCM preparations should be standardized; preferentially active compounds should be identified and used for standardization. According to Western criteria, most TCM preparations can currently not be considered to be clinically proven and therefore the term complementary medicine should not be used for most TCM products.

10.2 CAM, TCM, and Herbal Extracts

Nowadays legal authorities, physicians, and patients demand proof of efficacy in clinical studies and many studies were conducted and published in the past. However, most of them do not fulfill the criteria of evidence-based medicine which may be deleterious for survival of TCM in the Western world.

It is surprising that many TCM preparations used for the treatment of elderly women contain mixtures of up to dozens of herb extracts and many of them use herbs containing phytoestrogens. In many published articles it is admitted that robust evidence for the efficacy is lacking but that existing evidence warrants further research.

Similarly, the weakness of many clinical studies using CAM preparations is enforced by the following: The literature about alternative methods in the treatment of gynecological diseases is plentiful and often ends with statements such as "more clinical studies with a larger number of patients need to be performed before final conclusions can be drawn."

A large number of solid placebo-controlled studies on climacteric complaints and postmenopausal development of osteoporosis in females have been published. Preparations used in Western world utilizing CAM or TCM which are commonly prescribed for postmenopausal symptoms or osteoporosis often consist of phytoestrogen, primarily isoflavone-containing plant extracts which may provide a scientific basis for their action.

In addition, many TCM preparations contain poorly characterized extracts of a variety of plants. Cell biological and animal experiments studying effects of phytoestrogen-containing Asian plants are almost innumerous. However, due to different mixtures, durations, and endpoints, most results obtained with TCM preparations are not comparable to other studies, primarily those performed in the Western world. Most solid clinical studies utilizing phytoestrogens-containing preparations were done in Western countries.

Other widely used plants are *Vitex agnus-castus* and Cimicifuga species for which some solid placebo-controlled studies are available (for details see later).

10.2.1 CAM and TCM and Gynecological Diseases

In the life of women five global types of diseases may occur:

1. Life threatening are carcinomas, particularly breast cancer.
2. Peri- and postmenopausal women often develop psychosomatic symptoms of which most troublesome are climacteric complaints such as hot flashes.
3. As a result of the postmenopause, women often develop osteoporosis. Particularly, a slim postmenopausal woman is prone to develop this disease because the fat cells of the female type of fat distribution around the gluteal and thigh area (the so-called pear fat distribution) express aromatases which are able to aromatize circulating androgens into estrogens which partially prevent osteoporosis and which are not produced in slim postmenopausal women.
4. Obesity: this has almost reached pandemic properties. In the United States more than 50 % of the population is obese. In Europe estimations go up to 30 % and even in countries considered to be poor obesity is a major health threatening condition. Obesity is often related to poverty because the socio-economic situation of poorer people is frequently associated with a lower education and with a high intake of high caloric fast food. This leads to other threatening effects in the aging population and the development of cardiovascular diseases which are often associated with obesity.
5. Infertility: estimations report that 30–40 % of infertility is associated with obesity of the male type (i.e., the apple type with large amounts of visceral fat) and the thereof resulting hyperandrogenemia in females.

10.2.2 Life Style

There is considerable evidence that life style; particularly, nutrition may prevent many of the above described diseases. Nutrition of Asian people utilizes protein sources which stem primarily from soy beans. Also fish which contains healthy omega-3-fatty acids is commonly eaten. Pork and beef meat, which often contains high amounts of cholesterol and triglycerides, is avoided in the kitchen of most Asian populations. While Indian Ayurveda and Japanese medicine educates people to remain slim, TCM does not include healthy life style.

In this chapter we will address the proven potential of complementary alternative medicine (CAM) including traditional Chinese medical (TCM) procedures for the treatment of menopausal symptoms, infertility, and for the prevention of diseases such as osteoporosis. Also the preventive effects of CAM for mammary cancers and possible adverse effects in the mammary gland and uterus will be highlighted.

10.3 Plant Extracts: Safety Aspects

In recent years it became evident that hormone replacement therapy may have severe side effects. The incidence of mammary cancer is slightly increased (Rossouw et al. 2002; Beral et al. 2011). An estrogen treatment which is not opposed by progestins also increases the risk of endometrial cancer and therefore, a special attention must be paid to avoid such effects by alternative treatments.

10.3.1 Soy and Other Isoflavone-Containing Plants

In view of the possible health benefits of phytoestrogens, many physicians, pharmacists, and patients focus on the effects of isoflavones.

Isoflavones are common in many plants and high concentrations are present in soy and red clover. They belong to the class of isoflavonoids which in turn belong to the superfamily of phytoestrogens (Fig. 10.1) which is based on a classification published recently (Murkies et al. 1998).

The daily uptake of isoflavones of women in Far East Asian countries averages 40–60 mg; that of US American women 5 mg/day (Messina et al. 2006b). Profiles of phytoestrogens in the urine from several Asian countries clearly indicated that isoflavone intake is markedly higher in these Asian countries in comparison to the United States. Interestingly regional differences existed also within these Asian countries (Kunisue et al. 2010). These data stem from women in which the isoflavone uptake was estimated on the basis of the natural food intake.

For reasons that will be explained later, it is important to note that the intake of phytoestrogens in East Asian populations begins early in life and lasts life long, i.e., also Asian children and adolescent females have an average supply of 0.1–1 mg of isoflavones per kg bodyweight. Many isoflavone enriched soy extracts are commercially available in the Western world. Some contain high amounts of proteins; others are almost devoid of the proteins that are normally present in soy. Isoflavone free soy extracts were also proposed to have health promoting effects.

Since not too long ago it is known that 2 estrogen receptors (ERs) exist. The first described "classic" ER is now the ERα and the recently cloned is the ERß (Kuiper et al. 1996). Isoflavones have structural similarities to estradiol-17ß (Fig. 10.2) and fit therefore into the pouch of both ERα and ERß, but they bind less potently than estradiol-17ß (E2) to both receptor subtypes (Fig. 10.3) (Kuiper et al. 1996; Mueller et al. 2004; Messina et al. 2006a). Also Equol which is a metabolite of Daidzein binds to both estrogen receptors (Fig. 10.3). Equol was proposed as an especially beneficial compound that is produced by individuals possessing Equol producing intestinal bacteria. It was hypothesized that such Equol producers are more likely to benefit from soy food consumption than those who did not (Setchell et al. 2002; Messina 2010). Since Equol producers cannot be easily detected, it is impossible to predict whether a person would benefit from

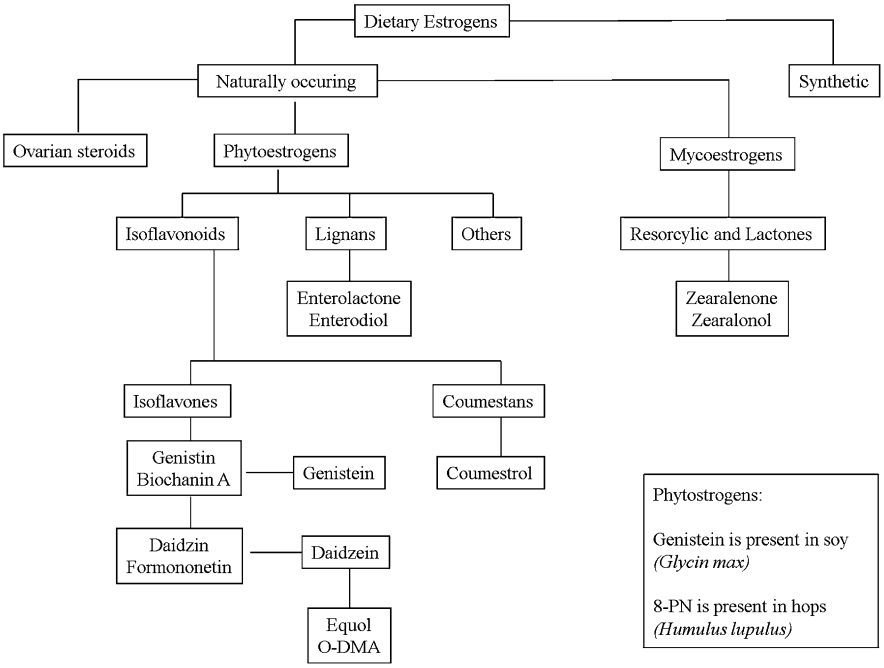
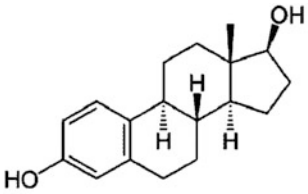


Fig. 10.1 Classification of dietary estrogens. Synthetic estrogens are heavily utilized in oral contraceptives and in HRT preparations. The naturally occurring estrogens can be subdivided in several groups of which CAM and TCM utilize primarily the phytoestrogens. The most commonly used plants containing phytoestrogens are soy and red clover and hops. Equol (Eq) is not a plant-derived substance but can be formed out of Daidzein (Daid) in the gut flora of about 30 % of human beings

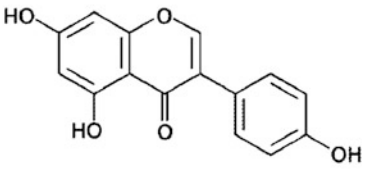
soy consumption. Furthermore, antibiotics which often interfere with gut flora may have profound effects on Equol production.

Controversial data exist concerning the safety of phytoestrogens, particularly of the widely used soy or red clover products. At a daily uptake of 50 mg/day no adverse effects appear to occur. A gray zone represents an uptake between 50 and 100 mg/day. At a high uptake of above 1 mg/day it is highly likely that isoflavones exert estrogenic effects not only in organs in which estrogenic effects are desired but also in uterine and mammary gland epithelium. Indeed in numerous animal experimental studies, a mild estrogenic effect to stimulate uterine weights of ovariectomized rats or mice was documented and also mammary gland tissue that atrophies following ovx showed clear estrogenic effects (Wuttke et al. 2003a; Hertrampf et al. 2006; Rimoldi et al. 2007); hence, most isoflavones studied so far exert mild estrogenicity undesired effects in the uterus and mammary glands.

In earlier reports it was suggested that isoflavones have a much higher affinity to ERß than to ERα. Most adverse effects of estrogens in the uterus and mammary glands are primarily exerted via the ERα. Therefore, food additive producing companies selling isoflavone-containing preparations suggested that the isoflavones



Estradiol-17ß



Genistein
(4',5,7-Trihydroxy-isoflavone)

Fig. 10.2 The structural similarity between estradiol-17ß and the three main isoflavones as examples for phytoestrogens, explains the capability of the phytoestrogens to bind to estrogen receptor α and ß

exert only positive estrogenic effects and are devoid of utero- and mammotrophic effects. Fact is that the major isoflavones, Genistein, Daidzein, and its metabolite Equol have only a slightly higher affinity to ERß than to ERα (Fig. 10.3) and also the transactivational activity of isoflavones including Genistein is almost identical in ERα or ERß-transfected cells (Mueller et al. 2004). At higher concentrations of $>10^6$ M, isoflavones are known to inhibit tyrosine kinases which may explain some data indicating that isoflavones may inhibit mammary cancer cell growth (Akiyama et al. 1987; Klein and King 2007).

There is also some evidence that isoflavones have antioxidant effects (Bartlett and Eperjesi 2008; Armstrong et al. 2010; Siow and Mann 2010).

10.3.1.1 Isoflavones and Breast Cancer

It is nowadays almost a dogma that the longer the exposure to estrogens, the higher the risk to develop a mammary cancer. On the other hand it is well accepted that Far East Asian postmenopausal women develop less mammary cancers and this was attributed to their isoflavone-containing soy intake (Adlercreutz et al. 1986; Adlercreutz and Mazur 1997; Messina et al. 2006b). This argument was strengthened by the observation that when Far East Asian women migrate into the United States, their breast cancer incidence increases within one to three generations to that of the Caucasian US population indicating that the life style and not genetic

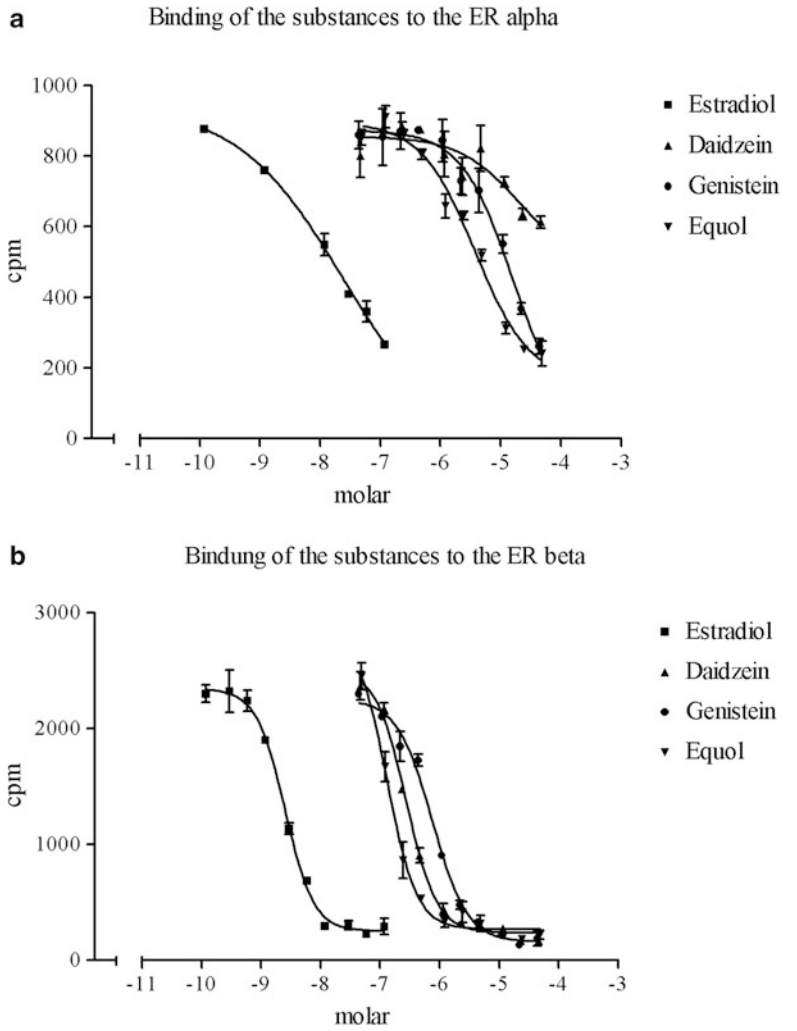


Fig. 10.3 (**a**) Binding of Daidzein, Genistein, and Equol to the ERα. (**b**) Binding of the substances to the ERß. Note the shift of the displacement curves by a factor 3 to the right which indicates an about 1,000 × lower affinity for both receptor subtypes. Note also that the phytoestrogens bind with an almost identical affinity to ERα and ERß

factors are responsible for this phenomenon. This "Knowledge" of beneficial effects of soy isoflavones has resulted in a marketing strategy for food additives claiming that isoflavone containing soy or red clover capsules prevent the development of mammary cancers. This boosted the sales of CAM and TCM products markedly particularly because their advertisements suggested that isoflavone intake may prevent mammary cancers when taken at the peri- and postmenopausal time.

In highly emotional "scientific" discussions the putatively positive effects of isoflavones in preventing mammary cancers are contrasted by adverse effects of phytoestrogens in the mammary gland. The latter view is primarily based on cell biological and animal experimental data. Estrogen receptive mammary cancer cells such as MCF-7 cells are stimulated by physiologic concentrations of phytoestrogens. Transplantation of MCF-7 cells into immune deficient nu/nu mice and carcinogens result in mammary cancers which are stimulated by isoflavones (Ju et al. 2006a, b). Estrogenic effects in mammary glands of ovariectomized (ovx) animals have been reported repeatedly (Wuttke et al. 2003a; Hertrampf et al. 2006; Rimoldi et al. 2007). Others argue that cell biologic and animal experimental data are not significant for the human data and state that Far East Asian females develop significantly less mammary cancers and that this is due to their higher isoflavone intake (Adlercreut et al. 2000; Adlercreutz 2002; Adlercreutz et al. 2004; Messina et al. 2006b). In a recent clinical study performed in Sweden, no positive correlation between isoflavone intake and incidence of breast cancers could be demonstrated (Hedelin et al. 2008).

In a randomized, placebo-controlled study, daily administration of 80 or 120 mg isoflavones did not alter mammographic density (Maskarinec et al. 2009). A high density is a risk factor for the development of breast cancer (Boyd et al. 1998). In this study the majority of the postmenopausal women were of Caucasian nature, hence it can be assumed that they had low isoflavone intake before entering the trial. This is important because in pioneering animal experiments it was shown that soy isoflavones may indeed have protective effects in the mammary gland but only when they are taken up peripubertally (Peng et al. 2010 ; Lamartiniere et al. 1995a). This concept was recently confirmed in further animal experimental and in an epidemiologic and recently in a population-based control study. In the animal experiments, it was shown that carcinogen induced mammary cancers in Sprague Dawley rats can be largely prevented by pre- and peripubertal treatment with Genistein, the major isoflavone in soy. Several mechanisms of action were elaborated. The growth promoting expression of EGF receptors in the mammary gland were reduced lifelong in the animals which were peripubertally treated with Genistein (Lamartiniere et al. 1995a, b, 2000). The epidermal growth factor receptor erbB2/Akt, one of the most important oncogene, was markedly down-regulated by pre-/peripubertal treatment with Genistein (Peng et al. 2010). Furthermore, the tumor repressor gene BRCA-1 was largely stimulated by such treatment (Fan et al. 2006). Consequently, it was suggested that isoflavones may indeed prevent development of mammary cancers also in women but only when taken early in life, i.e., prior and during puberty at the time when mammary gland tissue develops (Lamartiniere 2002; Whitsett and Lamartiniere 2006).

Lately, epidemiologic data appear to support the concept that exposure to isoflavones at the time of development of breast tissue decreases the development of breast cancer of women later in life. Breast cancer risk was significantly reduced by approximately 50 % in women with a high soy or phytoestrogen intake during childhood (Shu et al. 2001; Korde et al. 2009). In the only so far published population-based case control study, authors came to the conclusion that soy intake early in life may be especially relevant to carcinogenesis of the mammary gland

as women with high soy intake during childhood have less breast cancer later in life (Thanos et al. 2006). Hence, isoflavones may indeed exert estrogen receptor-mediated effects to prevent the development of mammary cancer when the developing breast tissue is exposed to these phytoestrogens. This view is now generally accepted even by those who advocated earlier that soy (isoflavones) may prevent breast cancer when taken peri-/postmenopausally (Messina et al. 2006b).

If it is the peripubertal exposure of mammary gland tissue which prevents later the development of breast cancer, the question remains open whether the estrogenic properties of isoflavones may be harmful for women in the Western world who begin isoflavone intake at the time when they experience climacteric complaints or when they want to protect themselves against the development of osteoporosis. Here the experimental data have been outlined already. Clinical data are controversial. Earlier views (Messina and Loprinzi 2001; Messina 2008) claim that existing mammary cancers in postmenopausal women are not affected by isoflavones. This is supported by clinical studies. The Shanghai Breast Cancer Survival Study, a large population-based cohort study involved 5,045 female breast cancers survivors in which it was shown that the higher the soy intake, the lower was the mortality (Shu et al. 2009). In another trial, soy isoflavone intake was also inversely correlated with survival of breast cancer patients (Guha et al. 2009). In yet another study in breast cancer patients, the isoflavone-treated women had a better prognosis concerning survival of their breast cancer (Zhang et al. 2010). Study subjects in the Shanghai Breast Cancer survival Study and the Australian study were women with a Chinese background. Hence, from these studies it appears that a moderate daily intake of isoflavones is not dangerous for breast cancer patients. This conclusion, however, has a severe drawback. All of these trials were done in mammary cancer patients with a Chinese background. As indicated earlier, a peripubertal intake of isoflavones decreases the occurrence of breast cancers. Nevertheless, a number of women will develop breast cancer which, however, may be less malignant due to the peripubertal exposure to isoflavones.

There is also one contrasting published study which demonstrated a higher mortality in women with a metastasized breast cancer who ingested higher amounts of isoflavones in comparison to the low intake control groups (Martinez-Montemayor et al. 2010).

Before safety of a high isoflavone uptake in isoflavone "inexperienced" women, i.e., in most women living in the Western hemisphere, is proven, preparations containing isoflavones should not be recommended to patients with breast cancers.

10.3.1.2 Isoflavones and the Uterine Endometrium

Another organ of concern is the uterine endometrium in which a progestin-unopposed estrogen exposure was shown to increase the incidence of endometrial cancer. In ovarectomized rats however an intermediate dose of Genistein and Equol stimulated uterine weights (Fig. 10.4). In postmenpausal women a low dose(<50 mg/day) isoflavones appeared to be safe. At the daily recommended dose of around 50 mg/day, Genistein appears to be devoid of estrogenic effects in premenopausal women

(Bitto et al. 2009). In this double-blind, placebo-controlled study, a simple endometrial hyperplasia that often occurs premenopausally due to unovulatory cycles was improved after a 6-month treatment with Genistein. In an open-label study, 197 postmenopausal women were treated with 70 mg of a standardized soy isoflavone extract and this had also no adverse effects in the endometrium (Palacios et al. 2010). Similar data were published by others and indicated that doses around 50 mg of isoflavones/day may not be harmful for the endometrium. At a daily dose of 150 mg taken over 3 years, however, an increased incidence of endometrial hyperplasia was reported (Unfer et al. 2004) which endangers the endometrium to become malign.

10.3.2 Cimicifuga racemosa

Cimicifuga racemosa is traditionally used by indigenous American Indians for the treatment of a variety of women's diseases.

There is ample evidence that extracts of *Cimicifuga racemosa* do not contain estrogenic compounds as it does neither bind to ERα nor to ERß (Fig. 10.3) and also uterine weights and estrogen-regulated genes in the uterus remain unaffected. Furthermore, CR extracts do not have mammotropic effects but appear to have anti-inflammatory effects (Schmid et al. 2009; Yang et al. 2009a, b).

10.3.2.1 *Cimicifuga racemosa* and Breast Cancer

In the last 20 years, evidence has accumulated that extracts of *Cimicifuga racemosa* do not contain estrogenic compounds. Therefore, adverse effects in the mammary glands are highly unlikely. Estrogenic substances would stimulate proliferation of estrogen receptive human mammary carcinoma cells. The world wide most widely used cell lines with these properties are MCF-7 cells of which the proliferation is inhibited rather than stimulated by extracts of *Cimicifuga racemosa* (Bodinet and Freudenstein 2002; Stromeier et al. 2005; Einbond et al. 2008). Mammary gland tissue and MCF-7 cells express aromatases which can increase the availability of estradiol in mammary glands by aromatizing androgens into estrogens. This conversion is profoundly inhibited by a *Cimicifuga racemosa* extract (Rice et al. 2007) which would argue for a protective effect. When Sprague–Dawley rats are treated with dimethylbenzanthracene (DMBA), they develop typical mammary tumor structures which are profoundly inhibited by oral administration of a special Cimicifuga extract (Freudenstein et al. 2002). In experiments with ovx rats, a Cimicifuga extract was shown not to affect the lobulo alveolar and ductus apparatus of ovx rats (Rimoldi et al. 2007; Wuttke et al. 2007). In a large 6 months lasting study, the investigated *Cimicifuga racemosa* extract did neither affect mammary gland density as determined by mammography nor was endometrial thickness altered (Raus et al. 2006). Furthermore, a prospective observational study was carried out in 50 breast cancer patients under Tamoxifen treatment. In this trial, a

6 months lasting therapy with a *Cimicifuga racemosa* extract reduced psycho-vegetative symptoms as measured by the menopause rating scale too significantly (Rostock et al. 2011).

A recently published case control study involving 949 breast cancer patients demonstrated that the use of Black cohosh had a significant breast cancer protective effect (Rebbeck et al. 2007).

In a 12-week lasting clinical trial effects of an extract containing 2.5 % tri-terpenes deriving from a *Cimicifuga racemosa* extracts were compared to an extract containing trace amounts of tri-terpenes and in this study no breast specific estrogenic effects were observed (Ruhlen et al. 2007).

Hence, there is convincing evidence that *Cimicifuga racemosa* extracts are not harmful but may actually protect the mammary gland.

10.3.2.2 *Cimicifuga racemosa* and the Endometrium

Due to the lack of estrogenic substances in *Cimicifuga racemosa*, it is not surprising that uterine, particularly, endometrial parameters remained unchanged in both animal experimental and clinical studies. A number of experiments were performed in ovariectomized rats. In none of them a *Cimicifuga racemosa* extract exerted estrogenic effects. Neither uterine weights nor uterine histology were affected (Seidlova-Wuttke et al. 2003b; Raus et al. 2006).

In a placebo-controlled clinical study the tested Cimicifuga extract had no effect in the endometrium whereas an estrogen preparation stimulated the thickness of the endometrium as measured by vaginal ultrasound (Wuttke et al. 2003c). In the longest so far conducted clinical study, a *Cimicifuga racemosa* preparation was tested for 1 year and endometrial safety was assessed by histological evaluation of the tissue. There was no case of endometrial abnormality present in the more than 300 tested patients (Raus et al. 2006). Hence, it can be concluded that *Cimicifuga racemosa* extracts have clearly no estrogenic effects in the endometrium and, therefore, bare no risk for the uterus.

10.3.3 Vitex agnus-castus

Several Vitex species exist. *Vitex trifolia* and *Vitex rotundifolia* are widely used in TCM. However, experimental and clinical data about their efficacy in gynecological disorders is scarce. Another Vitex species is *Vitex agnus-castus* (Chastetree) for which some data are available concerning its efficacy in the treatment of climacteric symptoms.

The dried fruits (Chasteberries) of chaste trees, also called monk's pepper belong to the families of Lamiaceae of which several species exist which are widely used in CAM and TCM. Dried fruits were believed to reduce libido and therefore

used in the middle age by monks. At this time it served also as a substitute for pepper which was very expensive.

A number of compounds were isolated from the dried fruits of Chasteberry of which only linolenic acid proved to have very mild estrogenic effects by its binding properties to both estrogen receptors (Li et al. 2004). We demonstrated that Agnus castus extracts contain dopaminergic compounds which inhibited pituitary prolactin release (Jarry et al. 1994) which were later identified as di-terpenes (Wuttke et al. 2003b; Jarry et al. 2006).

10.3.3.1 *Vitex agnus-castus* and the Breast

Many compounds in *Vitex agnus-castus* are dopaminergic in nature and have inhibitory effects on pituitary prolactin release (Jarry et al. 2003b, 2006). Premenstrually many women suffer from mastodynia. This premenstrual mastodynia is always benign but is often frightening to the patients. Many women with premenstrual mastodynia have a latent hyperprolactinemia, i.e., they release nocturnally and in response to stress high amounts of prolactin, which is particularly evident during the premenstrual period (Wuttke et al. 2003b). This exaggerated prolactin release stimulated the proliferation of mammary epithelial cells and Agnus castus extracts are able to suppress this exaggerated prolactin release (Wuttke et al. 1997; Halaska et al. 1999). Thereby the premenstrual mastodynia is ameliorated. Also cystic mastopathy has roots in high prolactin action in the mammary gland (Lopez Rosales et al. 1991; Castillo et al. 2006) and can therefore be beneficially influenced by Agnus castus extracts.

10.3.3.2 *Vitex agnus-castus* and the Endometrium

In ligand binding assays, extracts of *Vitex agnus-castus* bind to ERß preparations (Jarry et al. 2003b). In animals experiments, *Vitex agnus-castus* extracts were devoid of uterotrophic effects (Wuttke et al. 2003b).

10.4 Specified Diseases

TCM as well as CAM preparations are often proposed for the treatment of climacteric complaints and postmenopausal diseases such as osteoporosis. Not only aged people but also younger women and men often develop obesity, and as a consequence, the metabolic syndrome which nowadays has pandemic properties. About 14 % of couples in the Western hemisphere suffer from infertility. For all of these diseases, TCM and CAM preparations are commercially available. Their value will be commented in the following paragraphs.

10.4.1 Climacteric Complaints

Fifty to eighty percent of women develop climacteric complaints of which the hot flushes are most distressing. Therefore, many clinical studies are available testing TCM and CAM preparations to reduce climacteric complaints. Particularly, the number and severity of hot flushes were studied extensively.

Ideal animal models to study climacteric complaints are not available. Attempts have been made to measure tail skin temperature of ovx rats as a means to allow conclusions as to whether a substance might have beneficial effects on hot flashes (Dacks and Rance 2010; Williams et al. 2010; Simpkins et al. 1983; Berendsen et al. 2001). Data stemming from this animal model, however, are inconsistent due to the fact that under ambient room temperature, a large diurnal rhythmicity exists in the presence of estrogens which are not present in ovx rats (Simpkins 1995). In addition, both estrogens and sham-treated ovariectomized rats show extremely large fluctuations in tail skin temperature which barely resemble hot flashes.

Nevertheless, this model was used to study the effects of isoflavones. In one study, the calcium channel blocker nifedipine augmented ovx induced hot flushes and these fluctuations in tail skin temperature were reduced by isoflavones.

Many clinical data stem from a large number of studies, many of them being conducted without appropriate placebo controls. Since psychosomatic symptoms such as climacteric complaints including hot flashes are subject to a large placebo effect, non-placebo controlled studies must be disregarded.

10.4.1.1 Soy and Other Isoflavone-Containing Plants

Meta-analyses were made to work up the considerable number of placebo-controlled studies utilizing soy or red clover extracts enriched with isoflavones. The data are highly inconsistent and most meta-analysis came to the conclusion that isoflavone-containing products have a little, if any, effect on climacteric complaints (Lethaby et al. 2007; Wuttke et al. 2007).

Treatment of climacteric and postmenopausal symptoms often utilizes a mixture of herbal extracts.

In many studies TCM preparations intended to alleviate climacteric complaints. The plants present in the respective preparations were acknowledged. However, active compounds and amounts of phytoestrogens are often not mentioned.

In a recent study, the amount of such herbal extract mixture was analyzed for its isoflavone content. This study came to the conclusion that the TCM product most widely used to treat climacteric or postmenopausal symptoms or diseases contain similarly high amounts of isoflavones as soy products used in Western medicine (Miller-Martini et al. 2001; Li et al. 2004; Zhao et al. 2007).

The most frequently used TCM formula for the treatment of climacteric complaints in Taiwan is Jia-wei-xiao-yao-san (Yang et al. 2009a, b). It consists of at least nine different plants of which at least two (Paeoniae radix, Blupeuri radix)

contain strong phytoestrogens (Miller-Martini et al. 2001). Another TCM preparation commercially developed for the management of menopausal symptoms is Menoprogen (Liu et al. 2009a, b). It consists of four plants and in animal experiments it had slight uterotrophic properties. Interestingly, however, it stimulated the serum estradiol levels of ovx animals almost to an extent which was present in E2-treated animals which clearly had large uteri. Similarly, *Erxian tang* was reported to relieve not only the climacteric complaints but also many other chronic diseases (Li et al. 2007). In animal experiments, it also stimulated serum estradiol levels (Sze et al. 2009). In both publications reporting estrogenic effects, no attempts were made to test whether any of the plant extracts contain estrogenic substances which might interfere with the employed estradiol assay.

In a small clinical trial, an extract of *Pueraria lobata* containing 100 mg isoflavones primarily Puerarin was tested on menopausal symptoms and a quality of life parameters. No significant effects were observed on vasomotor symptoms or on any other quality of life parameters. *Pueraria lobata* contains primarily Puerarin which can be converted in Daidzein and Equol by the gut flora of patients (Woo et al. 2003).

10.4.1.2 *Cimicifuga racemosa*

There are other plant derived alternatives for the treatment of climacteric complaints. Black cohosh (*Cimicifuga racemosa*) grew originally in the moderate climates of the United States and was used by Indian tribes for the treatment of a number of diseases.

Recently the effectiveness of the special *Cimicifuga racemosa* extract BNO 1055 to prevent hot flushes in ovariectomized rats was shown (Kapur et al. 2010). It is a relatively new scientific experience that rats have hot flushes (Dacks and Rance 2010). In this animal model, it was shown that a variety of serotoninergic drugs or serotonin reuptake inhibitors are able to inhibit hot flushes and consequently it was shown that a widely used plant extract to ameliorate climacteric complaints namely *Cimicifuga racemosa* contains 5-methyl-serotonin which mimics the effects of serotonin (Powell et al. 2008).

In the last 50 years, it was demonstrated in studies performed in German-speaking countries that Black cohosh is equally potent to alleviate climacteric complaints as conjugated estrogens (Wuttke et al. 2003c, 2007; Osmers et al. 2005; Raus et al. 2006).

Hence, *Cimicifuga racemosa* extracts have now a longstanding history for its efficacy to ameliorate climacteric complaints. In the few placebo-controlled studies utilizing Black cohosh preparations, a significant reduction of climacteric complaints primarily of hot flashes was observed. In most studies the Kupperman index was evaluated which rates primarily hot flashes and underrates other climacteric complaints. For this reason, a new instrument, the menopause rating scale (Schneider et al. 2000a, b) was created and utilized in a double-blind, placebo-controlled study (Wuttke et al. 2003c). In this study the major climacteric complaints were significantly reduced. In another randomized trial, a Cimicifuga

extract proved to be similarly effective to ameliorate climacteric complaints as a transdermal estrogen therapy (Nappi et al. 2005). At this point it should be stated that quite some knowledge has accumulated about the mechanisms of action by which CR ameliorates climacteric complaints. It should also be mentioned that all knowledge of active ingredients stems from analyses done with field grown CR extracts and may therefore not be valid for CR extracts not grown under rigidly controlled conditions and also not for other Cimicifuga species.

It was mentioned earlier that *Cimicifuga racemosa* does not contain estrogenic activities. Neither binding to estrogen receptors nor an uterotrophic effect was ever observed (Jarry et al. 2003a). Yet it acts in the hypothalamus to ameliorate hot flashes.

What do we know about the generation of hot flashes?

They are due to lack of estrogenic actions in the hypothalamus and therefore neurotransmitters in the hypothalamus try to counteract this lack of estrogens. These neurotransmitters are primarily norepinephrine, dopamine, serotonin, and gamma amino butyric acid (GABA). Clinically it is now well established that GABAergic drugs such as GABApentin, serotoninergic compounds such as the 5-HT reuptake inhibitor fluoxetine are able to ameliorate hot flashes (Sideras and Loprinzi 2010).

Therefore, it was tempting to analyze CR extracts for the presence of such neurotransmitter like activities. Indeed, we did demonstrate activities in the investigated CR extract BNO 1055 that inhibited the secretion of prolactin by pituitary cells. Since this effect was prevented by haloperidol, a dopamine receptor blocker, it was concluded that one or more substances in CR BNO 1055 exerted the dopaminergic activity (Jarry et al. 2003a). The presence of 5-methyl-serotonin was demonstrated in a CR extract (Powell et al. 2008) and we confirmed the presence of this compound in the field grown CR BNO 1055 extract. As mentioned above, serotoninergic and dopaminergic compounds ameliorate hot flashes, hence it is likely that substances with such neurotransmitter-like properties are involved in the effects of CR extracts to prevent climacteric complaints.

Recently we investigated 2 CR fractions, one containing the unpolar saponins (S-fraction) and the other more polar substances (R-fraction). These fractions were tested whether they ameliorate hot flashes in ovx animals. Indeed, both, the S- and the R-fraction prevented occurrence of hot flashes

10.4.1.3 *Vitex agnus-castus*

Rigorously controlled studies about the effects of Vitex species are missing. Due to the isolated dopaminergic substances in extracts of *Vitex agnus castus* (Wuttke et al. 2003b) further experimental and clinical studies may be appropriate (van Die et al. 2009a).

10.4.2 Osteoporosis

Osteoporosis may develop as a consequence of many situations. In women, the most common reason is the menopause. The lack of estrogens causes a hyperactivity of osteoclasts and osteoblasts with higher activity of the bone resorptive osteoclasts (Turner et al. 2001; Seidlova-Wuttke et al. 2003a). In women, this increased bone metabolism lasts during the first 5–15 years following the menopause which results in demineralization of the bone. Interestingly in different women, different bones may demineralize. In most women, it is primarily the trabecular apparatus that is being resorbed with hip and vertebral spongeous structures being the primary sites that develop osteoporosis; in other women, it is the wrist area. In each case the osteoporotic spongeous part of the bone becomes fragile. This may result in spontaneous compression fractures of vertebral bodies which in turn cause forward bending of the upper trunk and formation the so-called "widow's hump." Another typical sign of vertebral compression fractures is shortening of the body length.

10.4.2.1 Osteoporosis and Isoflavones

Numerous cell biological and animal studies have been performed to validate the efficiency of phytoestrogens, particularly of isoflavones to prevent the development of osteoporosis. There are many osteoblast and osteoclast-derived cell lines available and most data stemming from such experiments tend to prove that isoflavones may be useful to prevent osteoporosis.

The most widely used animal model for the study of osteoporosis is the ovariectomized rat and in this model, the metaphysis of the tibia reacts extraordinarily sensitive to estrogen withdrawal and therefore, this part of the skeleton has been widely studied (Garner et al. 1991; Wronski 1991; Seidlova-Wuttke et al. 2003a). In most investigations, Genistein, the major isoflavone in soy, had a mild antiosteoporotic effect. More promising data were published utilizing Daidzein another component present in soy. As mentioned earlier, Daidzein is an interesting substance as it can be reduced by the intestinal flora to Equol by about 25–30 % of human beings but by most rodents (Setchell et al. 1984; Setchell 1985). It was proposed that Equol producers benefit more from soy food consumption than those who could not produce Equol. Effects of these isoflavones to prevent osteoporosis are weak (Fig. 10.4).

The bone is an exclusively sensitive organ for estrogens. Therefore, the possibility exists that doses of isoflavones which do not stimulate uterine or mammary gland tissue prevent osteoporosis. We tested this in ovariectomized animals which developed severe osteoporosis within 3 months following castration. Particularly, the trabecular apparatus of the metaphysis of the tibia looses more than 50 % of its mineral density within this time and is therefore the preferred subject for osteoporosis studies (Garner et al. 1991; Wronski 1991; Seidlova-Wuttke et al. 2003a, b). Within 3 months following ovx more than 50 % of trabecular density as measured

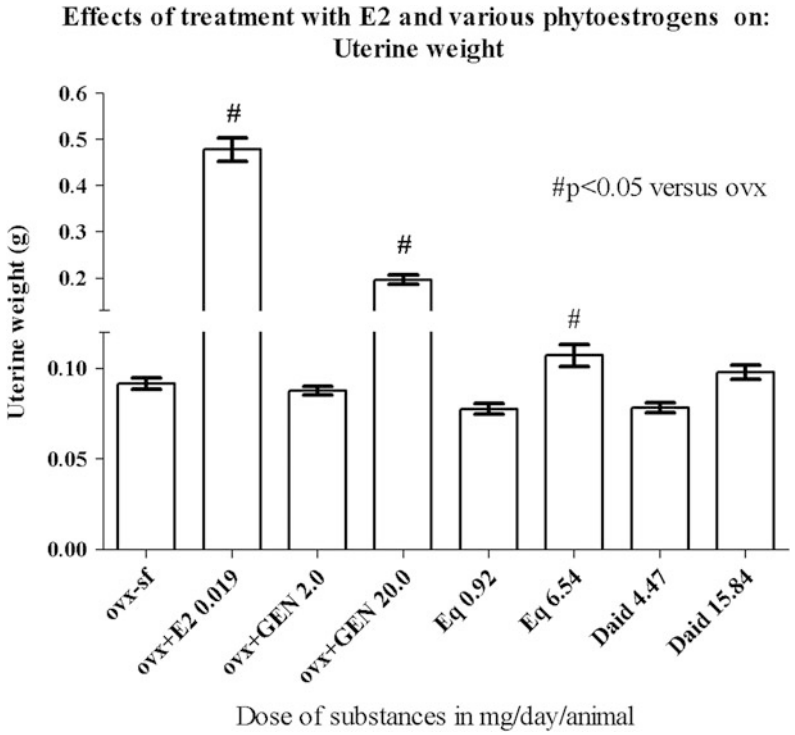


Fig. 10.4 Uterine weights were low 3 months after ovx and high in E2-treated animals. At the higher doses, the isoflavones Genistein (Gen), Equol (Eq), and Daidzein (Daid) were stimulatory to uterine weights

by quantitative computer tomography (qCT) was lost. When the animals were substituted with estradiol-17ß (given with the pelleted food and resulting in around 50 pg/ml serum), the bone loss was almost totally prevented but not by a low dose of 2 mg Genistein/day resulting in serum concentrations below 10^{-7} M. The high dose of 20 mg/day, which resulted in a concentration of 0.4×10^{-6}, was slightly effective to improve bone quality (Fig. 10.5a). Less effective were Daidzein and Equol.

In one large 2-year-lasting study, the daily supplementation of 80–120 mg of soy hypocothyl aglycone isoflavones plus calcium and vitamin D were studied in 403 postmenopausal women. They were tested annually for changes in whole body and regional bone mineral density and a reduced whole body bone loss was noted but the bone loss at common fracture sites was not affected by this treatment (Wong et al. 2009a, b).

So far, no studies are available to indicate that bone fragility is reduced in postmenopausal women taking isoflavones. The fracture risk of the femoral neck, however, in Chinese women who consume regularly isoflavone-containing soy food was reduced in comparison to Caucasian women (Yu et al. 2010). On the other hand, the risk for vertebral fractures in postmenopausal Japanese women,

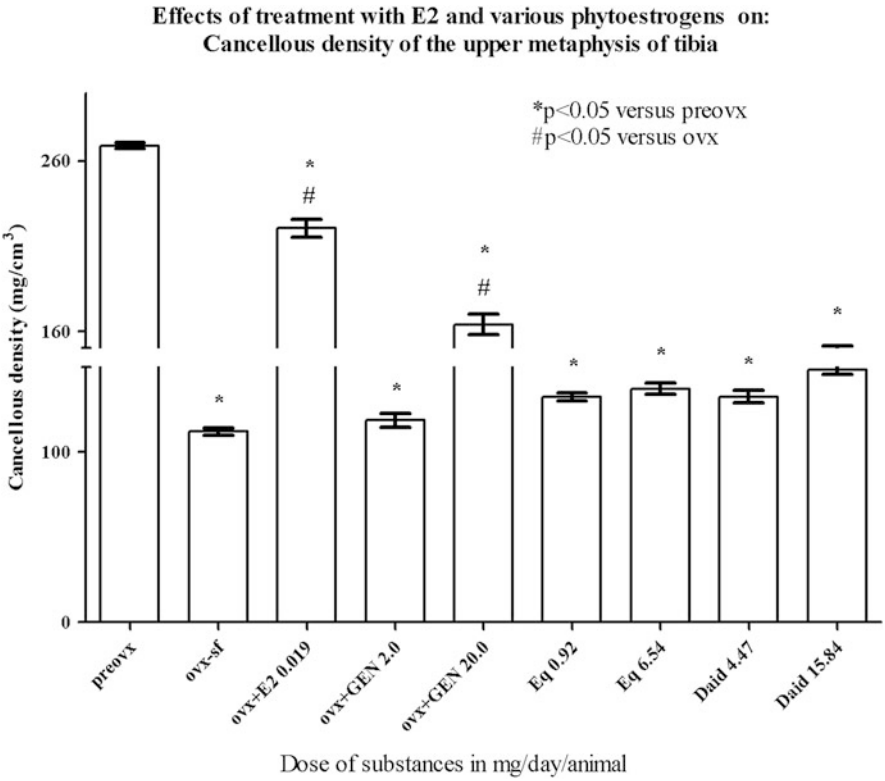


Fig. 10.5 The cancellous density in the metaphysis of the tibia of rats reflects largely the density of the trabecular apparatus. Following ovariectomy rats loose more than 50 % of cancellous density within 3 months and this can be largely prevented by E2 and to a lesser degree by Genistein, Daidzein, or Equol

who are also likely to consume isoflavones regularly did not differ significantly from that of women living in the Western world (Kadowaki et al. 2010).

In clinical studies isoflavones were slightly effective to positively influence surrogate parameters of osteoblast and osteoclast activities. A recent meta-analysis included ten clinical trials enrolling a few thousand patients and stated serum alkaline phosphatase, a marker for osteoblast activity was slightly (insignificantly) increased whereas the osteoclast marker, urine deoxypyridinoline was significantly decreased indicating an antiresorptive effect of the different isoflavone preparations (Taku et al. 2010a, b). In another meta-analysis authors evaluated the effects of soy isoflavones on bone mineral density and concluded that in nine evaluable studies few significant antiosteoporotic effects were uncovered and state "soy isoflavone supplementation is unlikely to have significantly favorable effects on BMD at the lumbar spine in hip in women" (Liu et al. 2009a, b).

These findings are in line with the view that isoflavones have mild estrogenic effects which are similar, though much weaker than those of estradiol-17ß which has also antiresorptive and little if any bone anabolic effect.

Many TCM preparations contain a large variety of plant extracts containing phytoestrogens which may explain their often proposed but seldomly-approved efficacy to reduce climacteric complaints and postmenopausal diseases such as osteoporosis. In view of the above described possible adverse effects of phytoestrogens in the uterus and in the mammary gland, more safety aspects of TCM preparations for the management of climacteric and postmenopausal complaints and diseases have to be studied.

In Asian countries, extracts of *Pueraria lobata*, the Japanese Kudzu, another soy-related leguminosis, were shown to contain large amounts of Puerarin which can be converted to Daidzein and consequently to Equol and also Puerarin had slight bone protective effects (Wang et al. 2003). Evidence that Puerarin exerts estrogenic effects in the uterus and mammary gland has been published (Xue et al. 2009) and therefore, precautions should be taken when consumed in large amounts by mammary cancer patients.

Epimedium belongs to the family of berberidaceae and some extracts are used in TCM. A less well-studied phytoestrogens such as Icariin present in *Epimedii herbae* which is one of the most frequently used in formulas prescribed for the treatment of osteoporosis in China were shown in in vitro experiments to have osteogenic effects (Mok et al. 2010; Wong et al. 2009a, b). In all biological experiments it had inhibitory effects on osteoclastogenesis (Meng et al. 2005; Zhang et al. 2008; Choi et al. 2010; Hsieh et al. 2010, 2011; Ma et al. 2011).

In a controlled 24 months lasting clinical trial, epimedium-derived phytoestrogen flavonoids had bone protective effects in late postmenopausal women (Zhang et al. 2007). The preparations used contained primarily Icariin, a bit of Daidzein and Genistein and about 30 % of the daily recommended dose of calcium which was also given to the placebo-treated patients. These doses of flavonoids had no effects in the endometrium of the verum-treated women.

10.4.2.2 Osteoporosis and *Cimicifuga racemosa*

Prevention of osteoporosis by compounds in *Cimicifuga racemosa* was first described in animal experiments. Attempts were made to establish whether the substances which prevent osteoporosis are different to those that ameliorate climacteric complaints. As mentioned earlier, the ovx rat is also an excellent model to study development and prevention of osteoporosis. Bone mineral density of the metaphysis of the tibia was analyzed by quantitative computer tomography (qCT) prior to ovx. Animals were then ovx and received an oral treatment with the mother extract CR BNO 1055 or with the S- or R-fraction. After a 4-week-lasting treatment period, qCTs were taken again and knee joint specimen of the lower femur/knee joint/upper tibia were collected and processed for histomorphometric analysis (Fig. 10.6a, b).

The histomorphometric workup of the metaphysis of the tibia demonstrated a better trabecular ultrastructure in the treated in comparison to the ovx control animals (Fig. 10.7a–c) (Seidlova-Wuttke et al. 2010) Hence, osteoprotective effects

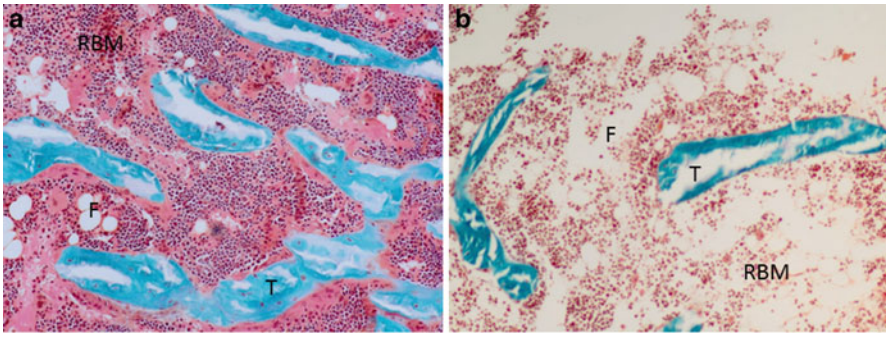


Fig. 10.6 Histology of intraosteal areas of the metaphysis of tibia, (**a**) animal treated with *Cimicifuga racemosa* (**b**) control animal. Note fewer fat cells (F), more trabecles (T), and more red bone marrow (RBM) in *Cimicifuga racemosa*-treated animals than in the controls

were confirmed for the mother extract and for both, the S- and the R-fraction. Identical results were obtained when q CT analyses were applied.

Histologic preparations of all lower femur/knee joint/upper tibia (Fig. 10.8) were histomorphometrically analyzed.

Analysis of the size of cartilage tissue in the knee joint yielded positive effects of CR BNO 1055 and its S- and R-fraction (Fig. 10.9) (Puri et al. 2012).

Further subfractions of the S- and R-fractions are currently investigated and should finally lead to chemical identification of active compounds. In the treated ovx animals, no uterotophic effect was observed. Hence, the beneficial effects of CR BNO 1055 and its fractions in the bone and joints cannot be explained by estrogenic effects. But other explanations are available.

What do we know about mechanism of action of Cimicifuga compounds? We showed repeatedly that CR BNO 1055 prevents the development of obesity following ovx of rats. Obese humans as well as obese rats develop often a metabolic syndrome. Large visceral fat depots produce large amounts of a variety of cyto- and adipokines which cause a chronic inflammatory state with a high load of oxidative stress (Koh et al. 2009; Rizvi 2009). This resulted in arteriosclerosis, hypertension, and consequently in an increased incidence of heart attacks and strokes. Obese rats have also a higher load of fat tissue in the bone marrow and joint fat pads are larger than in non-obese animals (Koh et al. 2009; Rizvi 2009). Lately evidence accumulated that the fat cells in these structures share the properties of visceral adipocytes because they secrete pro-inflammatory cytokines (Reaven 1988). These cytokines inhibit osteoblasts, the bone forming cells and stimulate osteoclasts, the bone digesting cells, which causes osteoporosis (Halade et al. 2011). In joints, the cytokines cause irritation of joint cartilage and thereby promote the development of osteoarthritis (Huang et al. 2011; Kapoor et al. 2011).

All of these diseases can be prevented or cured by a reduction of fat load. The thereof resulting reduction of cytokine production and consequently of the oxidative stress reduces the risk to develop arteriosclerosis and osteoporosis

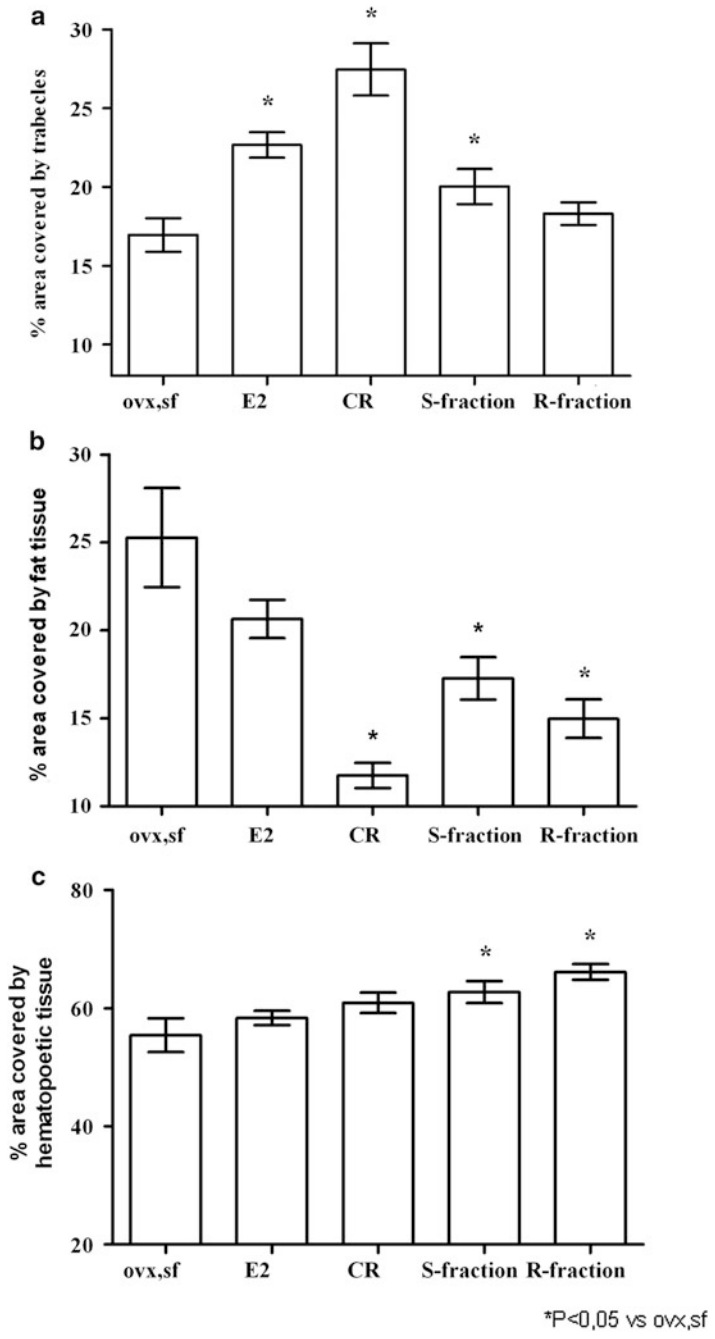


Fig. 10.7 E2 and CR prevented reduction of trabecular surface and also the S- and R-fraction were active (**a**); Treatment with E2 and more so with CR resulted in a large reduction of fat tissue in the bone marrow, an effect shared by the two subfractions (**b**); the two subfractions had positive effects on hematopoietic tissue in the bone marrow (**c**)

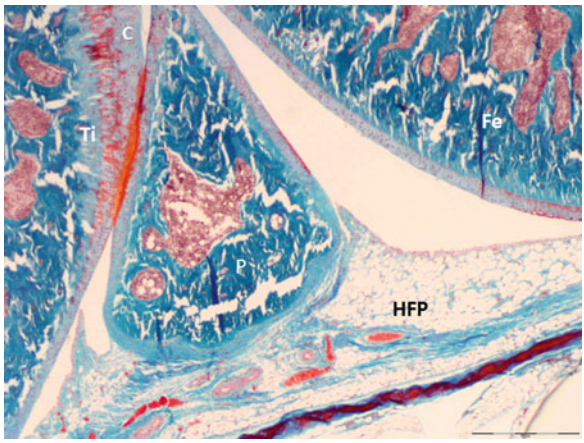


Fig. 10.8 Histologic preparation of a rat knee joint in which the size of the tibial cartilage layer (C) and the size of Hoffa's fat pad (HFP) were histomorphometrically quantified.P=Patella, Fe=femur, Ti=tibia

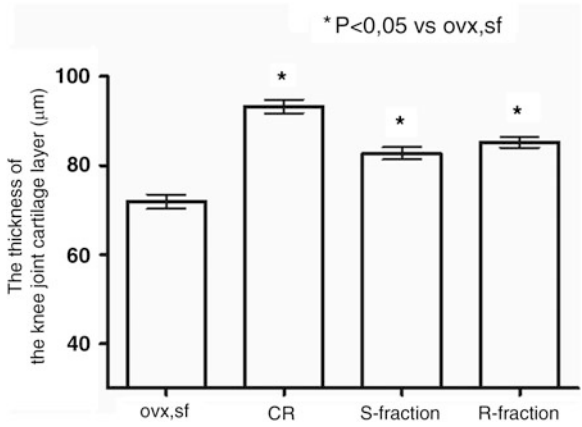


Fig. 10.9 Treatment with CR and its S- and R-fraction resulted in significantly higher cartilage layer in the knee joint

(Koh et al. 2009; Rizvi 2009; Shen et al. 2010). This can be achieved by less intake or higher utilization of calories which requires less eating or more bodily exercise. In many persons the will is often not strong enough to fulfill these tasks. Therefore a number of drugs have been developed, many of which have severe side effects. Hence, plant extracts with obesity preventing effects would be desirable. As mentioned above, a special extract of the rhizome of *Cimicifuga racemosa* inhibited ovx-induced obesity including adipocyte accumulation in the bone marrow and joints and both, the lipophilic saponin and the hydrophilic rest fraction share these properties (Fig. 10.7) (Seidlova-Wuttke et al. 2012). This indicates that two or more substances present in the extract have each other synergizing effects.

Whether other Cimicifuga species used in Chinese medicine exert similar effects as the field-grown American *Cimicifuga racemosa* needs to be tested.

Also in postmenopausal women, bone metabolism was positively influenced by a *Cimicifuga racemosa* extract. Serum osteocalcin and serum alkaline phosphatase were both increased after a 3 months therapy with a well-defined Black cohosh extract (Seidlova-Wuttke et al. 2003a, b; Raus et al. 2006).

These two substances are osteoblast products and therefore, reflect the activity of a total osteoblast population of the skeleton.

In each case, these experiments show convincingly three points:

1. Many plant extracts—here exemplified for *Cimicifuga racemosa*—contain more than one substance of which the effects synergize each other. This proves effectiveness of the extract to treat diseases of the metabolic syndrome.
2. Many extracts used in Chinese medicine may have similar effects, but this need to be proven in a similar way as outlined above.
3. Fractionation of plant extracts may lead to purification of active principles allowing development of drugs which may be synthetically produced.

10.4.2.3 Osteoporosis and *Vitex agnus-castus*

In an animal experiment utilizing the ovx rat model, a *Vitex agnus-castus* extract had mild osteoprotective effects (Sehmisch et al. 2009). No clinical studies are available to confirm such efficacy.

10.4.3 Fertility

Recently evidence has accumulated that early exposure to endocrine disruptors, including phytoestrogens, at the time of fetal and early postnatal development may decrease fertility later in adulthood (Bourguignon and Parent 2010; Ma 2009). These effects are nowadays considered to be epigenetic in nature, being due to decreased or increased methylation of nuclear chromatin thereby affecting genes that are important to reproduction. Obviously, during their development, not only the gonads but also the mammary glands are highly susceptible to such epigenetic effects (Graff et al. 2010; Delclos and Newbold 2007; Godmann et al. 2009).

10.4.3.1 Fertility and Isoflavones

Effects of isoflavones came to the attention of scientist when Australian sheep grazing on red clover fields rendered infertile. This red clover produced large amounts of isoflavones (Bennetts et al. 1946). There is little evidence that this may also happen in women but points to the estrogenicity of the isoflavones present in red clover.

10.4.3.2 Fertility and *Cimicifuga racemosa*

Neither animal nor clinical studies exist which would show improvement or deleterious effects of Black cohosh on fertility.

10.4.3.3 Fertility and *Vitex agnus-castus*

A variety of plant extracts have been reported to improve fertility. Most of the clinical studies, however, have been uncontrolled. The few randomized controlled trials included the use of extracts of the dried fruit of chasteberry (*Vitex agnus-castus*), Black cohosh (*Cimicifuga racemosa*), Vitamin B and E, and calcium. With the exception of chasteberry there have been too few trials involving herbal supplements in infertility to warrant a solid recommendation (Wuttke et al. 2001; Dennehy 2006). The most promising treatment of infertility appears to be with extracts of dried chasteberries. In in vitro and animal studies, these extracts were shown to contain mild dopaminergic substances which inhibited pituitary prolactin release in cultivated pituitary cells as well as in rats and humans (Wuttke et al. 2003b). A number of infertile women suffer from latent hyperprolactinemia, i.e., they release high prolactin levels in response to daily stresses as well as nocturnally (Wuttke et al. 2003b). These unphysiologically high prolactin levels recur several times a day and have obviously deleterious effects on the function of corpora lutea. Many women with such latent hyperprolactinemia have low serum progesterone levels, i.e., they suffer from corpus luteum insufficiency which may render them infertile. There is an association between hyperprolactinemia and infertility. Through its dopaminergic components *Agnus castus* inhibits pituitary prolactin release which may explain the beneficial effects on corpus luteum function and thereby on fertility (Wuttke et al. 2001). Nowadays extracts of dried Agnus castus fruits are used to ameliorate premenstrual symptoms. There is also some clinical data available to indicate that it can ameliorate climacteric complaints (van Die et al. 2009b).

10.4.4 The Metabolic Syndrome

Many postmenopausal women develop obesity of the male type, i.e., their visceral fat tissue increases. The fat cells of this male fat type secrete cytotoxic cytokines such as tumor necrosis factor alpha (TNFα) and other inflammatory cytokines which cause a chronic inflammatory state in the whole body with the result of increased cholesterol and triglyceride levels resulting in arteriosclerosis, hypertension, and consequently in a higher incidence of heart attacks and strokes (Piche et al. 2005; Lobo 2008; Gaspard 2009). Furthermore, insulin receptors desensitize which causes insulin-independent diabetes, the so-called type II diabetes. Also in

the bone marrow "bad" fat type cells increase in the absence of estrogens (Engdahl et al. 2010; Kim et al. 2010; Pacifici 2010) which inhibit osteoblast and stimulate osteoclast activity. This adds to the direct estrogen deficiency-mediated effects to cause a rapid deterioration of spongeous bones. The metabolic syndrome of postmenopausal women develops in approximately 14 % of American women who were not overweight prior to the menopause (Janssen et al. 2008) and slightly less in European postmenopausal females. Particularly, in the United States, obesity and the metabolic syndrome develop even earlier in life. Many children, adolescent, and women in their fertile age are overweight (Ryan 2009). Some experts talk about epidemic dimensions of obesity and the metabolic syndrome. Hence, this condition may cause several diseases and as mentioned, estrogen withdrawal appears to augment the metabolic syndrome. The fact that many Japanese women do not develop obesity as often as US American or European postmenopausal women is suggestive that habits in the Western world are responsible for this phenomenon. This is indeed the case. The large amounts of fast food and carbohydrate-rich drinks in addition to little exercise and high stress are factors that further obesity. The major protein source in Japanese women is soy whereas in the western world proteins are primarily covered by meat which contains often high amounts of triglycerides. In addition the Japanese population consumes more fish which contains high amounts of free fatty acids. They are, however, of the healthy type, i.e., fish contains large amounts of omega-3-fatty acids.

10.4.4.1 Metabolic Syndrome and Isoflavones

There have been attempts to study the effects of isoflavones on the development of obesity in ovariectomized rats which however failed. High amounts of isoflavones were unable to prevent ovariectomy-induced accumulation of fat tissue and these amounts of isoflavones stimulated uterine weight as well as epithelial proliferation in the mammary glands (Rimoldi et al. 2007). There is, however, convincing evidence that soybean isoflavones have beneficial effects in animals as well as in the human on a variety of metabolic parameters. Soy protein isolates containing isoflavones attenuated symptoms of the metabolic syndrome in weaning rats fed with a "Western-type" diet (Ronis et al. 2009). Final body weights and body fat mass were decreased whereas lean body mass increased in the isoflavone-treated animals in comparison to animals with a low isoflavone intake after a 2-week-lasting test period. Following ovx, spontaneously hypertensive, obese rats develop not only hypertension but also insulin resistance and hyperlipidemia which were partially prevented by Genistein (Bitto et al. 2009). Similar effects were seen in stroke-prone hypertensive rats treated with Kudzu (*Pueraria lobata*)-containing extracts (Peng et al. 2009).

In postmenopausal women, some studies revealed that a phytoestrogen-rich diet was associated with favorable metabolic effects on the cardiovascular risk factor triglycerides whereas serum cholesterol, LDL, and HDL remained unaffected (de Kleijn et al. 2002). A meta-analysis of the effects of soybean food on serum

lipids in patients suffering from the metabolic syndrome analyzed positive effects on plasma total cholesterol (10–19 %), LDL (14–20 %), and triglycerides (8–14 %) (Merritt 2004). Hence, isoflavones may have interesting effects in patients suffering from a metabolic syndrome. In 2005 the Agency for Healthcare Research and Quality, Department of Health and Human Services US published a report "The effects of soy on health outcome" (Balk et al. 2005). In this study, 200 clinical trials were examined and the report revealed that the average amount of 450 g of tofu resulted in a much smaller reduction of LDL (3 %) and triglyceride levels (6 %) (Balk et al. 2005). These findings were confirmed in a recent meta-analysis (Taku et al. 2010a, b). Authors also demonstrated that soy extracts reduced systolic blood pressure by 2 mmHg. Hence, the effects were not large.

10.4.4.2 The Metabolic Syndrome and *Cimicifuga racemosa*

In animal experiments, extracts of *Cimicifuga racemosa* decreased fat load of ovx animals and serum cholesterol levels (Rachon et al. 2008). Clinical studies about beneficial effects of *Cimicifuga racemosa* extracts are not available; one report stated no effects of Black cohosh on lipids in peri- or postmenopausal women (Spangler et al. 2007).

10.4.4.3 Metabolic Syndrome and *Vitex agnus-castus*

Neither animal experimental nor clinical studies are available.

10.5 Other Alternatives

Recently, we demonstrated that extracts of *Tinospora cordifolia* which (in Ayurveda medicine called Guduchi) exert osteoprotective effects in ovariectomized rats without stimulating uterine weights (Kapur et al. 2010; Seidlova-Wuttke et al. 2010). In these extracts we identified as putatively active component 20-OH ecdysone (beta-ecdysone = ß-Ecd) and consequently we studied highly purified ß-Ecd in the ovariectomized rat model which develops severe osteoporosis within a relatively short time following castration (Turner et al. 2001; Seidlova-Wuttke et al. 2003a, b). In this animal model, we demonstrated profound osteoprotective effects of ß-Ecd without estrogenic effects in the uterus or mammary glands. In addition, ligand binding assays utilizing recombinant human estrogen receptor alpha or beta preparations yielded the information that ß-Ecd does not bind to these two estrogen receptors. Hence, the osteoprotective effects must be exerted via different non-estrogenic effects which await further elucidation.

There are older not placebo-controlled studies available that indicate a potent musculotropic effect of Ecd and this is the reason why it is advertised through the internet for bodybuilders who often consume gram quantities.

10.6 Conclusions

The use of traditional Chinese medicines became fashionable in the Western world but solid clinical data are missing for indications in gynecological diseases. Undoubtedly the vast amount of TCM and other Far East Asian medicinal products provide a pool of interesting plant extracts which certainly deserve thorough analytical, cell biological, animal experimental, and clinical studies. Many TCM products provide phytoestrogen-containing plant extracts for which solid placebo-controlled studies are available, many of which were performed in the western world. They give proof to a mild estrogenic effect of isoflavones not only in the bone, possibly on climacteric complaints, but also in the mammary gland.

Cimicifuga racemosa extracts are traditionally used to ease climacteric complaints and several double-blind placebo-controlled studies demonstrated this effect. They suggest also a mild osteoprotective effect of Black cohosh extracts. Most importantly, *Cimicifuga racemosa* extracts do not contain estrogenic compounds.

Fertility products are available but relatively solid data are only available for extracts of the fruit of *Vitex agnus-castus* (Chasteberries).

Hence, TCM must be modernized since TCM uses primarily plant extracts. They must be standardized and active compounds should be identified and used for standardization. Finally preclinical and clinical toxicology data should be made available and double-blind placebo-controlled studies should give final proof safety and efficacy of the TCM products.

Taken all together, data on CAM and TCM preparations in gynecological diseases are not very solid and therefore more clinical studies need to be performed. This last sentence is incidentally the end of many studies reporting data on TCM or CAM preparations.

References

Adlercreut H, Mazur W, Stumpf K, Kilkkinen A, Pietinen P, Hulten K, Hallmans G (2000) Food containing phytoestrogens, and breast cancer. Biofactors 12:89–93

Adlercreutz H (2002) Phytoestrogens and breast cancer. J Steroid Biochem Mol Biol 83:113–118

Adlercreutz H, Mazur W (1997) Phyto-oestrogens and Western diseases. Ann Med 29:95–120

Adlercreutz H, Fotsis T, Bannwart C, Wahala K, Makela T, Brunow G, Hase T (1986) Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. J Steroid Biochem 25:791–797

Adlercreutz H, Heinonen SM, Penalvo-Garcia J (2004) Phytoestrogens, cancer and coronary heart disease. Biofactors 22:229–236

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595

Armstrong ME, Spencer EA, Cairns BJ, Banks E, Pirie K, Green J, Wright FL, Reeves GK, Beral V (2011) Body mass index and physical activity in relation to the incidence of hip fracture in postmenopausal women. J Bone Miner Res 26:1330–1338

Balk E, Chung M, Chew P, Ip S, Raman G, Kupelnick B, Tatsioni A, Sun Y, Devine D, Lau J (2005) Effects of soy on health outcomes. Evid Rep Technol Assess (Summ) 126:1–8

Bartlett HE, Eperjesi F (2008) Nutritional supplementation for type 2 diabetes: a systematic review. Ophthalmic Physiol Opt 28:503–523

Bennetts HW, Underwood EJ, Shier FL (1946) A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Br Vet J 102:348–352

Beral V, Reeves G, Bull D, Green J (2011) Breast cancer risk in relation to the interval between menopause and starting hormone therapy. J Natl Cancer Inst 103:296–305

Berendsen HH, Weekers AH, Kloosterboer HJ (2001) Effect of tibolone and raloxifene on the tail temperature of oestrogen-deficient rats. Eur J Pharmacol 419:47–54

Bitto A, Altavilla D, Bonaiuto A, Polito F, Minutoli L, Di Stefano V, Giuliani D, Guarini S, Arcoraci V, Squadrito F (2009) Effects of aglycone genistein in a rat experimental model of postmenopausal metabolic syndrome. J Endocrinol 200:367–376

Bodinet C, Freudenstein J (2002) Influence of *Cimicifuga racemosa* on the proliferation of estrogen receptor-positive human breast cancer cells. Breast Cancer Res Treat 76:1–10

Bourguignon JP, Parent AS (2010) Early homeostatic disturbances of human growth and maturation by endocrine disrupters. Curr Opin Pediatr 22:470–477

Boyd NF, Lockwood GA, Martin LJ, Knight JA, Byng JW, Yaffe MJ, Tritchler DL (1998) Mammographic densities and breast cancer risk. Breast Dis 10:113–126

Castillo E, Garibay M, Mirabent F (2006) Effect of alpha dihydroergocryptine in patients with fibrocystic breast disease. Ginecol Obstet Mex 74:580–584

Choi HJ, Park YR, Nepal M, Choi BY, Cho NP, Choi SH, Heo SR, Kim HS, Yang MS, Soh Y (2010) Inhibition of osteoclastogenic differentiation by Ikarisoside A in RAW 264.7 cells via JNK and NF-kappaB signaling pathways. Eur J Pharmacol 636:28–35

Dacks PA, Rance NE (2010) Effects of estradiol on the thermoneutral zone and core temperature in ovariectomized rats. Endocrinology 151:1187–1193

de Kleijn MJ, van der Schouw YT, Wilson PW, Grobbee DE, Jacques PF (2002) Dietary intake of phytoestrogens is associated with a favorable metabolic cardiovascular risk profile in postmenopausal U.S. women: the Framingham study. J Nutr 132:276–282

Delclos KB, Newbold R (2007) NTP toxicity report of reproductive dose range-finding Study of Genistein (CAS No. 446-72-0) administered in feed to Sprague–Dawley rats. Toxic Rep Ser 79:1–C2

Dennehy CE (2006) The use of herbs and dietary supplements in gynecology: an evidence-based review. J Midwifery Womens Health 51:402–409

Einbond LS, Wen-Cai Y, He K, Wu HA, Cruz E, Roller M, Kronenberg F (2008) Growth inhibitory activity of extracts and compounds from Cimicifuga species on human breast cancer cells. Phytomedicine 15:504–511

Engdahl C, Jochems C, Windahl SH, Borjesson AE, Ohlsson C, Carlsten H, Lagerquist MK (2010) Amelioration of collagen-induced arthritis and immune-associated bone loss through signaling via estrogen receptor alpha, and not estrogen receptor beta or G protein-coupled receptor 30. Arthritis Rheum 62:524–533

Fan S, Meng Q, Auborn K, Carter T, Rosen EM (2006) BRCA1 and BRCA2 as molecular targets for phytochemicals indole-3-carbinol and genistein in breast and prostate cancer cells. Br J Cancer 94:407–426

Freudenstein J, Dasenbrock C, Nisslein T (2002) Lack of promotion of estrogen-dependent mammary gland tumors in vivo by an isopropanolic *Cimicifuga racemosa* extract. Cancer Res 62:3448–3452

Garner SC, Anderson JJ, Mar MH, Parikh I (1991) Estrogens reduce bone loss in the ovariectomized, lactating rat model. Bone Miner 15:19–31

Gaspard U (2009) Hyperinsulinaemia, a key factor of the metabolic syndrome in postmenopausal women. Maturitas 62:362–365

Godmann M, Lambrot R, Kimmins S (2009) The dynamic epigenetic program in male germ cells: its role in spermatogenesis, testis cancer, and its response to the environment. Microsc Res Tech 72:603–619

Graff M, Yount KM, Ramakrishnan U, Martorell R, Stein AD (2010) Childhood nutrition and later fertility: pathways through education and pre-pregnant nutritional status. Demography 47:125–144

Guha N, Kwan ML, Quesenberry CP Jr, Weltzien EK, Castillo AL, Caan BJ (2009) Soy isoflavones and risk of cancer recurrence in a cohort of breast cancer survivors: the life After Cancer Epidemiology study. Breast Cancer Res Treat 118:395–405

Halade GV, El Jamali A, Williams PJ, Fajardo RJ, Fernandes G (2011) Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice. Exp Gerontol 46:43–52

Halaska M, Beles P, Gorkow C, Sieder C (1999) Treatment of cyclical mastalgia with a solution containing a *Vitex agnus castus* extract: results of a placebo-controlled double-blind study. Breast 8:175–181

Hedelin M, Lof M, Olsson M, Adlercreutz H, Sandin S, Weiderpass E (2008) Dietary phytoestrogens are not associated with risk of overall breast cancer but diets rich in coumestrol are inversely associated with risk of estrogen receptor and progesterone receptor negative breast tumors in Swedish women. J Nutr 138:938–945

Hertrampf T, Schmidt S, Seibel J, Laudenbach-Leschowsky U, Degen GH, Diel P (2006) Effects of genistein on the mammary gland proliferation of adult ovariectomised Wistar rats. Planta Med 72:304–310

Hsieh TP, Sheu SY, Sun JS, Chen MH, Liu MH (2010) Icariin isolated from Epimedium pubescens regulates osteoblasts anabolism through BMP-2, SMAD4, and Cbfa1 expression. Phytomedicine 17:414–423

Hsieh TP, Sheu SY, Sun JS, Chen MH (2011) Icariin inhibits osteoclast differentiation and bone resorption by suppression of MAPKs/NF-kappaB regulated HIF-1alpha and PGE(2) synthesis. Phytomedicine 18:176–185

Huang W, Mabrouk ME, Sylvester J, Dehnade F, Zafarullah M (2011) Enhanced expression of tissue inhibitor of metalloproteinases-4 gene in human osteoarthritic synovial membranes and its differential regulation by cytokines in chondrocytes. Open Rheumatol J 5:81–87

Janssen I, Powell LH, Crawford S, Lasley B, Sutton-Tyrrell K (2008) Menopause and the metabolic syndrome: the Study of Women's Health across the nation. Arch Intern Med 168:1568–1575

Jarry H, Leonhardt S, Gorkow C, Wuttke W (1994) In vitro prolactin but not LH and FSH release is inhibited by compounds in extracts Of Agnus castus: direct evidence for a dopaminergic principle by the dopamine receptor assay. Exp Clin Endocrinol 102:448–454

Jarry H, Metten M, Spengler B, Christoffel V, Wuttke W (2003a) In vitro effects of the *Cimicifuga racemosa* extract BNO 1055. Maturitas 44(Suppl 1):S31–S38

Jarry H, Spengler B, Porzel A, Schmidt J, Wuttke W, Christoffel V (2003b) Evidence for estrogen receptor beta-selective activity of *Vitex agnus-castus* and isolated flavones. Planta Med 69:945–947

Jarry H, Spengler B, Wuttke W, Christoffel V (2006) In vitro assays for bioactivity-guided isolations of endocrine active compounds in *Vitex agnus castus*. Maturitas 55:S26–S36

Ju YH, Allred KF, Allred CD, Helferich WG (2006a) Genistein stimulates growth of human breast cancer cells in a novel, postmenopausal animal model, with low plasma estradiol concentrations. Carcinogenesis 27:1292–1299

Ju YH, Fultz J, Allred KF, Doerge DR, Helferich WG (2006b) Effects of dietary daidzein and its metabolite, equol, at physiological concentrations on the growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in ovariectomized athymic mice. Carcinogenesis 27:856–863

Kadowaki E, Tamaki J, Iki M, Sato Y, Chiba Y, Kajita E, Kagamimori S, Kagawa Y, Yoneshima H (2010) Prevalent vertebral deformity independently increases incident vertebral fracture risk in middle-aged and elderly Japanese women: the Japanese Population-based Osteoporosis (JPOS) Cohort Study. Osteoporos Int 21:1513–1522

Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H (2011) Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 7:33–42

Kapur P, Wuttke W, Jarry H, Seidlova-Wuttke D (2010) Beneficial effects of beta-Ecdysone on the joint, epiphyseal cartilage tissue and trabecular bone in ovariectomized rats. Phytomedicine 17:350–355

Kim YY, Kim SH, Oh S, Sul OJ, Lee HY, Kim HJ, Kim SY, Choi HS (2010) Increased fat due to estrogen deficiency induces bone loss by elevating monocyte chemoattractant protein-1 (MCP-1) production. Mol Cells 29:277–282

Klein CB, King AA (2007) Genistein genotoxicity: critical considerations of in vitro exposure dose. Toxicol Appl Pharmacol 224:1–11

Koh KK, Oh PC, Quon MJ (2009) Does reversal of oxidative stress and inflammation provide vascular protection? Cardiovasc Res 81:649–659

Korde LA, Wu AH, Fears T, Nomura AM, West DW, Kolonel LN, Pike MC, Hoover RN, Ziegler RG (2009) Childhood soy intake and breast cancer risk in Asian American women. Cancer Epidemiol Biomarkers Prev 18:1050–1059

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930

Kunisue T, Tanabe S, Isobe T, Aldous KM, Kannan K (2010) Profiles of phytoestrogens in human urine from several Asian countries. J Agric Food Chem 58:9838–9846

Lamartiniere CA (2002) Timing of exposure and mammary cancer risk. J Mammary Gland Biol Neoplasia 7:67–76

Lamartiniere CA, Moore J, Holland M, Barnes S (1995a) Neonatal genistein chemoprevents mammary cancer. Proc Soc Exp Biol Med 208:120–123

Lamartiniere CA, Moore JB, Brown NM, Thompson R, Hardin MJ, Barnes S (1995b) Genistein suppresses mammary cancer in rats. Carcinogenesis 16:2833–2840

Lamartiniere C, Zhao Y, Fritz W (2000) Genistein: mammary cancer chemoprevention, in vivo mechanisms of acrion, potential for toxicity and bioavailability in rats. J Women's Cancer 2:11–19

Lethaby AE, Brown J, Marjoribanks J, Kronenberg F, Roberts H, Eden J (2007) Phytoestrogens for vasomotor menopausal symptoms. Cochrane Database Syst Rev CD001395

Li M, Poon P, Woo J (2004) A pilot study of phytoestrogen content of soy foods and traditional Chinese medicines for women's health in Hong Kong. Int J Food Sci Nutr 55:201–205

Li JJ, Li JT, Fu JP (2007) *Erxian tang*–introduction of a Chinese herbal formula, clinical practice, and experimental studies. Chin J Integr Med 13:67–73

Liu D, Lu Y, Ma H, Wei RC, Li J, Fang J, Mahady GB (2009a) A pilot observational study to assess the safety and efficacy of Menoprogen for the management of menopausal symptoms in Chinese women. J Altern Complement Med 15:79–85

Liu J, Ho SC, Su YX, Chen WQ, Zhang CX, Chen YM (2009b) Effect of long-term intervention of soy isoflavones on bone mineral density in women: a meta-analysis of randomized controlled trials. Bone 44:948–953

Lobo RA (2008) Metabolic syndrome after menopause and the role of hormones. Maturitas 60:10–18

Lopez Rosales C, Romero Espinosa RE, Juarez Vazquez J (1991) Treatment of fibrocystic breast disease with lisuride. Ginecol Obstet Mex 59:358–361

Ma L (2009) Endocrine disruptors in female reproductive tract development and carcinogenesis. Trends Endocrinol Metab 20:357–363

Ma HP,Ming LG,Ge BF,Zhai YK,Song P,Xian CJ,Chen KM (2011) Icariin is more potent than genistein in promoting osteoblast differentiation and mineralization in vitro. J Cell Biochem 112:916–923

Maciocia G,Livingstone C (1998) Obstetrics and gynacology in Chinese medicine

Martinez-Montemayor MM, Otero-Franqui E, Martinez J, De La Mota-Peynado A, Cubano LA, Dharmawardhane S (2010) Individual and combined soy isoflavones exert differential effects on metastatic cancer progression. Clin Exp Metastasis 27:465–480

Maskarinec G, Verheus M, Steinberg FM, Amato P, Cramer MK, Lewis RD, Murray MJ, Young RL, Wong WW (2009) Various doses of soy isoflavones do not modify mammographic density in postmenopausal women. J Nutr 139:981–986

Meng FH, Li YB, Xiong ZL, Jiang ZM, Li FM (2005) Osteoblastic proliferative activity of Epimedium brevicornum Maxim. Phytomedicine 12:189–193

Merritt JC (2004) Metabolic syndrome: soybean foods and serum lipids. J Natl Med Assoc 96:1032–1041

Messina M (2008) Conclusion that isoflavones exert estrogenic effects on breast tissue and may raise breast cancer risk unfounded. Mol Nutr Food Res 52:299–300

Messina M (2010) A brief historical overview of the past two decades of soy and isoflavone research. J Nutr 140:1350S–1354S

Messina MJ, Loprinzi CL (2001) Soy for breast cancer survivors: a critical review of the literature. J Nutr 131:3095S–3108S

Messina M, McCaskill-Stevens W, Lampe JW (2006a) Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. J Natl Cancer Inst 98:1275–1284

Messina M, Nagata C, Wu AH (2006b) Estimated Asian adult soy protein and isoflavone intakes. Nutr Cancer 55:1–12

Miller-Martini DM, Chan RY, Ip NY, Sheu SJ, Wong YH (2001) A reporter gene assay for the detection of phytoestrogens in traditional Chinese medicine. Phytother Res 15:487–492

Mok SK, Chen WF, Lai WP, Leung PC, Wang XL, Yao XS, Wong MS (2010) Icariin protects against bone loss induced by oestrogen deficiency and activates oestrogen receptor-dependent osteoblastic functions in UMR 106 cells. Br J Pharmacol 159:939–949

Mueller SO, Simon S, Chae K, Metzler M, Korach KS (2004) Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. Toxicol Sci 80:14–25

Murkies AL, Wilcox G, Davis SR (1998) Clinical review 92: Phytoestrogens. J Clin Endocrinol Metab 83:297–303

Nappi RE, Malavasi B, Brundu B, Facchinetti F (2005) Efficacy of *Cimicifuga racemosa* on climacteric complaints: a randomized study versus low-dose transdermal estradiol. Gynecol Endocrinol 20:30–35

Osmers R, Friede M, Liske E, Schnitker J, Freudenstein J, Henneicke-von Zepelin HH (2005) Efficacy and safety of isopropanolic black cohosh extract for climacteric symptoms. Obstet Gynecol 105:1074–1083

Palacios S, Pornel B, Vazquez F, Aubert L, Chantre P, Mares P (2010) Long-term endometrial and breast safety of a specific, standardized soy extract. Climacteric 13:368–75

Pacifici R (2010) The immune system and bone. Arch Biochem Biophys 503:41–53

Peng N, Prasain JK, Dai Y, Moore R, Arabshahi A, Barnes S, Carlson S, Wyss JM (2009) Chronic dietary kudzu isoflavones improve components of metabolic syndrome in stroke-prone spontaneously hypertensive rats. J Agric Food Chem 57:7268–7273

Peng JH, Zhu JD, Mi MT, Li FJ, Cai L, Dong JZ, Zhang HX, Zhao Y, Xue RL (2010) Prepubertal genistein exposure affects erbB2/Akt signal and reduces rat mammary tumorigenesis. Eur J Cancer Prev 19:110–119

Piche ME, Lemieux S, Weisnagel SJ, Corneau L, Nadeau A, Bergeron J (2005) Relation of high-sensitivity C-reactive protein, interleukin-6, tumor necrosis factor-alpha, and fibrinogen to abdominal adipose tissue, blood pressure, and cholesterol and triglyceride levels in healthy postmenopausal women. Am J Cardiol 96:92–97

Powell SL, Godecke T, Nikolic D, Chen SN, Ahn S, Dietz B, Farnsworth NR, van Breemen RB, Lankin DC, Pauli GF, Bolton JL (2008) In vitro serotonergic activity of black cohosh and identification of N(omega)-methylserotonin as a potential Active constituent. J Agric Food Chem 56:11718–11726

Puri P, Wuttke W, Seidlova-Wuttke D (2012) 20-OH-ecdysone prevents hot flushes in ovariectomized rats. Planta Med 78:109–114

Rachon D, Vortherms T, Seidlova-Wuttke D, Wuttke W (2008) Effects of black cohosh extract on body weight gain, intra-abdominal fat accumulation, plasma lipids and glucose tolerance in ovariectomized Sprague–Dawley rats. Maturitas 60:209–215

Raus K, Brucker C, Gorkow C, Wuttke W (2006) First-time proof of endometrial safety of the special black cohosh extract (Actaea or *Cimicifuga racemosa* extract) CR BNO 1055. Menopause 13:678–691

Reaven GM (1988) Banting lecture 1988. Role of insulin resistance in human disease. Diabetes 37:1595–1607

Rebbeck TR, Troxel AB, Norman S, Bunin GR, DeMichele A, Baumgarten M, Berlin M, Schinnar R, Strom BL (2007) A retrospective case–control study of the use of hormone-related supplements and association with breast cancer. Int J Cancer 120:1523–1528

Rice S, Amon A, Whitehead SA (2007) Ethanolic extracts of black cohosh (Actaea racemosa) inhibit growth and oestradiol synthesis from oestrone sulphate in breast cancer cells. Maturitas 56:359–367

Rimoldi G, Christoffel J, Seidlova-Wuttke D, Jarry H, Wuttke W (2007) Effects of chronic genistein treatment in mammary gland, uterus, and vagina. Environ Health Perspect 115 (Suppl 1):62–68

Rizvi AA (2009) Cytokine biomarkers, endothelial inflammation, and atherosclerosis in the metabolic syndrome: emerging concepts. Am J Med Sci 338:310–318

Ronis MJ, Chen Y, Badeaux J, Badger TM (2009) Dietary soy protein isolate attenuates metabolic syndrome in rats via effects on PPAR, LXR, and SREBP signaling. J Nutr 139:1431–1438

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. J Am Med Assoc 288:321–333

Rostock M, Fischer J, Mumm A, Stammwitz U, Saller R, Bartsch HH (2011) Black cohosh (*Cimicifuga racemosa*) in tamoxifen-treated breast cancer patients with climacteric complaints - a prospective observational study. Gynecol Endocrinol 10:844–848

Ruhlen RL, Haubner J, Tracy JK, Zhu W, Ehya H, Lamberson WR, Rottinghaus GE, Sauter ER (2007) Black cohosh does not exert an estrogenic effect on the breast. Nutr Cancer 59:269–277

Ryan JG (2009) Cost and policy implications from the increasing prevalence of obesity and diabetes mellitus. Gend Med 6(Suppl 1):86–108

Scheid V, Ward T, Cha WS, Watanabe K, Liao X (2010) The treatment of menopausal symptoms by traditional East Asian medicines: review and perspectives. Maturitas 66:111–130

Schmid D, Woehs F, Svoboda M, Thalhammer T, Chiba P, Moeslinger T (2009) Aqueous extracts of *Cimicifuga racemosa* and phenolcarboxylic constituents inhibit production of proinflammatory cytokines in LPS-stimulated human whole blood. Can J Physiol Pharmacol 87:963–972

Schneider HP, Heinemann LA, Rosemeier HP, Potthoff P, Behre HM (2000a) The Menopause Rating Scale (MRS): reliability of scores of menopausal complaints. Climacteric 3:59–64

Schneider HP, Heinemann LA, Rosemeier HP, Potthoff P, Behre HM (2000b) The Menopause Rating Scale (MRS): comparison with Kupperman index and quality-of-life scale SF-36. Climacteric 3:50–58

Sehmisch S, Boeckhoff J, Wille J, Seidlova-Wuttke D, Rack T, Tezval M, Wuttke W, Stuermer KM, Stuermer EK (2009) *Vitex agnus castus* as prophylaxis for osteopenia after orchidectomy in rats compared with estradiol and testosterone supplementation. Phytother Res 23:851–858

Seidlova-Wuttke D, Hesse O, Jarry H, Christoffel V, Spengler B, Becker T, Wuttke W (2003a) Evidence for selective estrogen receptor modulator activity in a black cohosh (*Cimicifuga racemosa*) extract: comparison with estradiol-17beta. Eur J Endocrinol 149:351–362

Seidlova-Wuttke D, Jarry H, Becker T, Christoffel V, Wuttke W (2003b) Pharmacology of *Cimicifuga racemosa* extract BNO 1055 in rats: bone, fat and uterus. Maturitas 44(Suppl 1): S39–S50

Seidlova-Wuttke D, Ehrhardt C, Wuttke W (2010) Metabolic effects of 20-OH-ecdysone in ovariectomized rats. J Steroid Biochem Mol Biol 119:121–126

Seidlova-Wuttke D, Stecher G, Kammann M, Haunschild J, Eder N, Stahnke V, Wessels J, Wuttke W (2012) Osteoprotective effects of Cimicifuga racemosa and its triterpene-saponins are responsible for reduction of bone marrow. Phytomedicine 19:855–860

Setchell K (1985) Naturally, non-steroidal estrogens of dietary origin. In: McLachlan J (ed) Estrogens in the environment. Elsevier Science publishing Co, New York, pp 69–86

Setchell KD, Borriello SP, Hulme P, Kirk DN, Axelson M (1984) Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am J Clin Nutr 40:569–578

Setchell KD, Brown NM, Lydeking-Olsen E (2002) The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. J Nutr 132:3577–3584

Shen CL, Yeh JK, Cao JJ, Tatum OL, Dagda RY, Wang JS (2010) Green tea polyphenols mitigate bone loss of female rats in a chronic inflammation-induced bone loss model. J Nutr Biochem 21:968–974

Shu XO, Jin F, Dai Q, Wen W, Potter JD, Kushi LH, Ruan Z, Gao YT, Zheng W (2001) Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. Cancer Epidemiol Biomarkers Prev 10:483–488

Shu XO, Zheng Y, Cai H, Gu K, Chen Z, Zheng W, Lu W (2009) Soy food intake and breast cancer survival. J Am Med Assoc 302:2437–2443

Sideras K, Loprinzi CL (2010) Nonhormonal management of hot flashes for women on risk reduction therapy. J Natl Compr Canc Netw 8:1171–1179

Simpkins JW (1995) Effects of age, reproductive status and ambient temperature on skin temperature regulation in the female rat. Maturitas 21:97–102

Simpkins JW, Katovich MJ, Song IC (1983) Similarities between morphine withdrawal in the rat and the menopausal hot flush. Life Sci 32:1957–1966

Siow RC, Mann GE (2010) Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? Mol Aspects Med 31:468–477

Spangler L, Newton KM, Grothaus LC, Reed SD, Ehrlich K, LaCroix AZ (2007) The effects of black cohosh therapies on lipids, fibrinogen, glucose and insulin. Maturitas 57:195–204

Stromeier S, Petereit F, Nahrstedt A (2005) Phenolic esters from the rhizomes of *Cimicifuga racemosa* do not cause proliferation effects in MCF-7 cells. Planta Med 71:495–500

Sze SC, Tong Y, Zhang YB, Zhang ZJ, Lau AS, Wong HK, Tsang KW, Ng TB (2009) A novel mechanism: Erxian Decoction, a Chinese medicine formula, for relieving menopausal syndrome. J Ethnopharmacol 123:27–33

Taku K, Lin N, Cai D, Hu J, Zhao X, Zhang Y, Wang P, Melby MK, Hooper L, Kurzer MS, Mizuno S, Ishimi Y, Watanabe S (2010a) Effects of soy isoflavone extract supplements on blood pressure in adult humans: systematic review and meta-analysis of randomized placebo-controlled trials. J Hypertens 28:1971–1982

Taku K, Melby MK, Kurzer MS, Mizuno S, Watanabe S, Ishimi Y (2010b) Effects of soy isoflavone supplements on bone turnover markers in menopausal women: systematic review and meta-analysis of randomized controlled trials. Bone 47:413–423

Thanos J, Cotterchio M, Boucher BA, Kreiger N, Thompson LU (2006) Adolescent dietary phytoestrogen intake and breast cancer risk (Canada). Cancer Causes Control 17:1253–1261

Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD (2001) Animal models for osteoporosis. Rev Endocr Metab Disord 2:117–127

Unfer V, Casini ML, Costabile L, Mignosa M, Gerli S, Di Renzo GC (2004) Endometrial effects of long-term treatment with phytoestrogens: a randomized, double-blind, placebo-controlled study. Fertil Steril 82:145–148, quiz 265

van Die MD, Bone KM, Burger HG, Reece JE, Teede HJ (2009a) Effects of a combination of Hypericum perforatum and *Vitex agnus-castus* on PMS-like symptoms in late-perimenopausal women: findings from a subpopulation analysis. J Altern Complement Med 15:1045–1048

van Die MD, Burger HG, Teede HJ, Bone KM (2009b) *Vitex agnus-castus* (Chaste-Tree/Berry) in the treatment of menopause-related complaints. J Altern Complement Med 15:853–862

Wang X, Wu J, Chiba H, Umegaki K, Yamada K, Ishimi Y (2003) Puerariae radix prevents bone loss in ovariectomized mice. J Bone Miner Metab 21:268–275

Whitsett TG Jr, Lamartiniere CA (2006) Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat. Expert Rev Anticancer Ther 6:1699–1706

Williams H, Dacks PA, Rance NE (2010) An improved method for recording tail skin temperature in the rat reveals changes during the estrous cycle and effects of ovarian steroids. Endocrinology 151:5389–5394

Wong SP, Shen P, Lee L, Li J, Yong EL (2009a) Pharmacokinetics of prenylflavonoids and correlations with the dynamics of estrogen action in sera following ingestion of a standardized Epimedium extract. J Pharm Biomed Anal 50:216–223

Wong WW, Lewis RD, Steinberg FM, Murray MJ, Cramer MA, Amato P, Young RL, Barnes S, Ellis KJ, Shypailo RJ, Fraley JK, Konzelmann KL, Fischer JG, Smith EO (2009b) Soy isoflavone supplementation and bone mineral density in menopausal women: a 2-y multicenter clinical trial. Am J Clin Nutr 90:1433–1439

Woo J, Lau E, Ho SC, Cheng F, Chan C, Chan AS, Haines CJ, Chan TY, Li M, Sham A (2003) Comparison of *Pueraria lobata* with hormone replacement therapy in treating the adverse health consequences of menopause. Menopause 10:352–361

Wronski TJYC (1991) The ovariectomized rat as an animal model for postmenopausal bone loss. Cells Mater 1991(Supp 1):69–74

Wuttke W, Splitt G, Gorkow C, Sieder C (1997) Behandlung zyklusabhängiger Brustschmerzen mit einem Agnus castus-haltigen Arzneimittel - Ergebnisse einer randomisierten, plazebokontrollierten Doppelblindstudie. Geburtsh u Frauenheilk 57:569–574

Wuttke W, Pitzel L, Seidlova-Wuttke D, Hinney B (2001) LH pulses and the corpus luteum: the luteal phase deficiency LPD. Vitam Horm 63:131–158

Wuttke W, Jarry H, Becker T, Schultens A, Christoffel V, Gorkow C, Seidlova-Wuttke D (2003a) Phytoestrogens: endocrine disrupters or replacement for hormone replacement therapy? Maturitas 44(Suppl 1):S9–S20

Wuttke W, Jarry H, Christoffel V, Spengler B, Seidlova-Wuttke D (2003b) Chaste tree (*Vitex agnus-castus*)–pharmacology and clinical indications. Phytomedicine 10:348–357

Wuttke W, Seidlova-Wuttke D, Gorkow C (2003c) The Cimicifuga preparation BNO 1055 vs. conjugated estrogens in a double-blind placebo-controlled study: effects on menopause symptoms and bone markers. Maturitas 44(Suppl 1):S67–S77

Wuttke W, Jarry H, Seidlova-Wuttke D (2007) Isoflavones–safe food additives or dangerous drugs? Ageing Res Rev 6:150–188

Xue CC, Zhang AL, Yang AW, Zhang CS, Story DF (2009) Recent developments of acupuncture in Australia and the way forward. Chin Med 4:7

Yang CL, Chik SC, Li JC, Cheung BK, Lau AS (2009a) Identification of the bioactive constituent and its mechanisms of action in mediating the anti-inflammatory effects of black cohosh and related Cimicifuga species on human primary blood macrophages. J Med Chem 52: 6707–6715

Yang YH, Chen PC, Wang JD, Lee CH, Lai JN (2009b) Prescription pattern of traditional Chinese medicine for climacteric women in Taiwan. Climacteric 12(6):541–547

Yu N, Liu YJ, Pei Y, Zhang L, Lei S, Kothari NR, Li DY, Papasian CJ, Hamilton J, Cai JQ, Deng HW (2010) Evaluation of compressive strength index of the femoral neck in Caucasians and chinese. Calcif Tissue Int 87:324–332

Zhang G, Qin L, Shi Y (2007) Epimedium-derived phytoestrogen flavonoids exert beneficial effect on preventing bone loss in late postmenopausal women: a 24-month randomized, double-blind and placebo-controlled trial. J Bone Miner Res 22:1072–1079

Zhang DW, Cheng Y, Wang NL, Zhang JC, Yang MS, Yao XS (2008) Effects of total flavonoids and flavonol glycosides from Epimedium koreanum Nakai on the proliferation and differentiation of primary osteoblasts. Phytomedicine 15:55–61

Zhang M, Liu X, Holman CD (2010) Effect of dietary intake of isoflavones on the estrogen and progesterone receptor status of breast cancer. Nutr Cancer 62:765–773

Zhao PW, Wang DW, Niu JZ, Wang JF, Wang LQ (2007) Evaluation on phytoestrogen effects of ten kinds of Chinese medicine including flos carthami. Zhongguo Zhong Yao Za Zhi 32:436–439

Chapter 11
Ginkgo biloba Extract EGb 761®: From an Ancient Asian Plant to a Modern European Herbal Medicinal Product

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11.1 Introduction

This chapter differs from the other chapters in the book in a number of aspects. First of all, it is not about traditional Chinese medicine (TCM) and hence does not describe internationally recognized standards which need to be established in the future to scientifically validate the traditional expertise on TCM.

Rather, this chapter outlines the development process and present knowledge on EGb 761®, a special extract from leaves of *Ginkgo biloba* L. Although EGb 761® is produced from a plant which is native to China and other Asian countries; the extract was developed as a Western medicine. The early research program—which started almost 50 years ago—and the subsequent development into a modern herbal medicinal product (HMP) took place exclusively in Europe, mainly in Germany but also in France. The researchers involved in this process did not take TCM into consideration, even though this is sometimes erroneously assumed today. Rational pharmaceutical, pharmacological, and clinical procedures of conventional medicine were used instead. Although such standards are commonly employed in the development of chemically defined pharmaceutical substances, such procedures were not generally applied for HMP at that time. Under this perspective, however, the experience gathered during the development of EGb 761® may provide helpful hints for the advancement of TCM preparations to modern pharmaceuticals.

Today, EGb 761® is registered and marketed in more than 70 countries worldwide. It is a well-established active pharmaceutical ingredient with an economic turnover of several hundred millions of €. The success of products containing EGb 761® is certainly based on the therapeutic effects felt directly by patients, which already emerged in the early years. The evaluation of the underlying clinically relevant mechanisms of action still continues today. The documentation of clinical efficacy

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and safety has developed over decades in parallel to the development of scientific and regulatory standards and guidelines.

Over the last 40 years, numerous publications, excellent reviews, and books concerning the pharmacy and chemistry as well as the pharmacological and clinical aspects of EGb 761® have been published. It is not the intention of this chapter to review all of the available literature but to give an overview of the development process from the point of view of the original manufacturer.

11.2 General and Pharmaceutical Aspects

Much of the following information has been taken from the excellent overviews prepared by De Feudis (2003), van Beek (2000), and Upton (2003) and the *Ginkgo biloba* monograph by Spiess and Juretzek (2004) contained in Hagers Handbuch für Drogen und Arzneistoffe.

11.2.1 The Ginkgo Tree

Ginkgo biloba L., the maidenhair tree, is native to China and some other Asian countries (Japan, Korea). In the Western world, for example in Holland, the botany of the Ginkgo tree was already investigated at the end of the seventeenth century.

Ginkgo biloba is believed to be the oldest living tree on earth. Sometimes the expression "living fossil" is used. The Ginkgo tree is remarkably resistant to plant diseases and seems to have inherent insecticidal and fungicidal properties.

Ginkgo trees are dioecious. This means that male and female flowers are borne by different plants. Female trees bear seeds, or "fruits". Ginkgo trees grow best in full sun on well-drained soils rich in humus. They were cultivated in Asia mainly for their edible nutlike seeds and for ornamental reasons but not for the medicinal properties of the leaves. The roasted seeds are traditionally consumed in China and other Asian countries.

Only very few historical records on the use of Ginkgo leaves in TCM or as foodstuff exist. Consequently, only some scattered reports on the topical use of Ginkgo leaves to treat freckles and the internal administration to treat dysentery and diarrhea are available from the fifteenth and sixteenth century (Upton 2003).

Ginkgo leaf became official in China for the first time in the Pharmacopoeia of the People's Republic of China in 1977 in connection with dysfunctions of the heart and lungs. Following the use of Ginkgo leaves in the support of the circulatory system and the improvement of mental acuity in Western medicine, they are currently also used in Chinese medicine to calm wheezing, as an analgesic, and for the treatment of hypercholesterolemia, hypertension, coronary artery disease, angina pectoris, and cerebrovascular disorders, amongst others (Upton 2003).

There is, however, no scientific basis for the use in these indications. They were often only passed on by word of mouth and may also change. All in all, the traditional medicinal use of Ginkgo leaves has not been very common in China.

More recently, Ginkgo therapy in China has increasingly been associated with the use of modern European medicinal products containing EGb 761®. Owing to this interweaving with Chinese culture, it has often been claimed that these modern Ginkgo preparations should also be assigned to TCM. However, this is unsupported by the historical use and the development of EGb 761® as a modern HMP.

11.2.2 The History and Development of EGb 761®

Dr. Willmar Schwabe III (1907–1983), the grandson of the Schwabe company founder, was a German doctor and pharmacist (Fig. 11.1). He was also an exceptionally gifted herbalist and authority on plants. During his travels and botanical expeditions, he was keenly interested in non-European medicinal plants. In his company these plants were also occasionally used as starting material for new drugs. These included, e.g., *Cardiospermum halicacabum* (Belgian Congo), *Hamamelis virginiana* (North America), *Haronga madagascariensis* (Madagascar), and *Piper methysticum*/kava kava (Micronesia).

Willmar Schwabe III was never in China, but through his general interest in medicinal plants he came across Ginkgo, and Ginkgo leaves in particular. As others before him, he was also fascinated by the elegant shape of the leaves. The first work and publications of a research team within the company he directed were concerned with cerebral and peripheral circulatory disorders and the efficacy of simple Ginkgo leaf extracts. In this context, the team also experimented with free aglycones such as quercetin, kaempferol, and other flavonoids. The extract EGb 761®, which was developed later, was not used at the time.

During this period, it was still possible to place pharmaceutical preparations on the market solely based on theoretical assumptions and pharmacological investigations but without clinical trials. In 1965, an extract from *Ginkgo biloba* leaves was registered by Dr. Willmar Schwabe GmbH and Co. KG and launched under the trade name Tebonin® as drops and coated tablets.

During the course of further pharmacological experiments, the flavonoids (mostly flavonolglycosides) contained in the extract were found to be the analytically most conspicuous and pharmacologically most interesting constituents. Schwabe and his team therefore developed an extract with higher concentrations of flavonolglycosides, which was also designed to be suitable for parenteral injection. During development it became clear that in addition to increasing the concentration of flavonolglycosides it was also necessary to deplete other substances, such as those that caused pharmaceutical problems (e.g., clouding, precipitation, and other solubility problems). This resulted in the development of an "ampoule extract" which was then placed on the market as Tebonin® Ampoules.

Fig. 11.1 Dr. Willmar Schwabe III (1907–1983)

In collaboration with the French pharmaceutical group Beaufour-IPSEN, a corresponding extract for oral administration at a dosage of 40 mg was developed. Earlier formulations contained much lower amounts of Ginkgo extract. Corresponding preparations came onto the market, such as "Rökan®" (1978) and "Tebonin® forte" (1982) in Germany and "Tanakan®" in France.

The cooperation with the French company was very stimulating during this period. The French partner was largely developing chemically defined active drugs and initiated numerous toxicological and clinical studies at an early stage of extract development. Together with the broad pharmacological studies of the German group, this led to a high level of acceptance in the German and French medical community in the subsequent years. In view of the substantial investment required by these studies, this special Ginkgo extract was designated the codename "EGb 761®". The idea was to emphasize that the study results were clearly specific to products containing this Ginkgo extract with its special composition. EGb stands for Extractum *Ginkgo biloba* and 761 was the patent code number. This coding of plant extracts set precedence later and is still used by research groups who are interested in showing the efficacy of plant extracts and who want to make original status and authorship absolutely clear.

To wit, extracts from one and the same plant can easily have completely different compositions, depending on the solvent and manufacturing procedure used. As a consequence, different pharmacological profiles may result, which are typical for a particular extract and cannot be assigned without further ado to other extracts.

11.2.3 Quality of Ginkgo Leaves

In the early stages of development and marketing of preparations containing EGb 761®, the Ginkgo leaves were collected in the wild in Asia. This was adequate to cover the small amounts required at that time. However, problems with the distributors and their delivery capacity soon emerged. It became increasingly difficult to obtain high-quality raw materials from collections in Japan, Korea, and China. For this reason, cultivation of approx. two million small Ginkgo trees grown from seed was started on a plantation near Karlsruhe (Germany) in 1978. It quite soon became apparent, however, that the growing conditions were not optimal owing to the unsuitable climate in Germany. Consequently, plantations in France (Bordeaux region) and the USA (South Carolina) were started. This resulted in stands of sometimes up to approx. 25 million trees from 1982. Two-year-old Ginkgo trees grown from seed and approx. 30 cm tall are used for planting and replanting. The Ginkgo trees are shaped into bushes by winter pruning and do not exceed a height of around 2 m.

The Ginkgo leaves are harvested with specially constructed harvesting machines in late summer/early autumn shortly prior to turning yellow (Fig. 11.2). At this point in time the concentrations of the valuable constituents, flavonolglycosides and terpene lactones, are fortunately still high and harvesting is easy as the leaf stalks are already loose.

The leaves are dried quickly and as gently as possible with warm air. The dried leaves are then sent in bales to the extraction plants, which were established at Karlsruhe/Germany and Cork/Ireland. The dried leaves from different plantations and collections are examined with regard to the most important constituents, flavonolglycosides and terpene lactones, and blended prior to extraction so that the desired final content is adhered to as closely as possible. This procedure is called "crude herbal material standardization".

The standardization process is complex and is similar to certain procedures in the foodstuffs industry. There too, certain products, such as sparkling wine/champagne or chocolate, are sometimes blended to obtain a suitable quality. The quality adjustment leads to a particularly consistent product. As EGb 761® is an active pharmaceutical substance, this comparison is not entirely appropriate but definitely helps to explain the process which results in consistent composition and defined pharmaceutical quality.

11.2.4 Manufacture of EGb 761®

The manufacturing process for EGb 761® was patented worldwide. It mainly aims at concentrating the substance groups that are most important for the pharmacological and clinical effects, i.e., Ginkgo flavonolglycosides and terpene lactones. Secondly, pharmaceutically problematic constituents and especially constituents

Fig. 11.2 Harvesting at a Ginkgo plantation

with potential side effects are removed or largely depleted during the extraction process. Adverse effects have been ascribed to ginkgolic acids in particular. Ginkgolic acids and related compounds should, therefore, be removed by extractive and absorptive methods as far as technically possible.

Initially, a primary extract is produced with acetone:water (60:40). This primary extract is subsequently concentrated and at the same time purified by means of a number of further technological stages. As far as possible, uniform and standardized leaf material, which has been previously analyzed, is fed in during this process. Numerous analytical in-process controls are necessary in order to regulate the process and produce a well-defined final extract.

In order to adjust for small differences during the course of the manufacturing process, portions of the extract can also be mixed/blended after corresponding analysis. The quantification (formerly described as standardization) of two groups of constituents leads to high consistency of most extract constituents.

The complex purification stages used during manufacturing result in a relatively low extract yield of approx. 1.5–2.5 % (drug:extract ratio [DER] ca. 35–67:1) related to the dried leaf material. For the manufacture of EGb 761® alone, more than 2.500 t dried Ginkgo leaves are needed each year. This demonstrates the continued therapeutic significance of this herbal preparation worldwide. Because of this importance, EGb 761® was protected by a whole series of patents. In contrast to this, traditional medicinal products and TCM can barely be protected owing to the lack of novelty.

11.2.5 Modern Phytopharmaceutical Formulations

The composition and quality of EGb 761® is certainly of paramount importance for its efficacy and safety. The extract as a whole is to be considered as the active pharmaceutical ingredient (API). This is universally true for all plant preparations.

Alongside this, the pharmaceutical formulation is also very important and defines the proprietary medicinal product. The original dosage forms of Tebonin® were drops or coated tablets and contained EGb 761® in relatively low doses. During further development accompanied by corresponding studies, dosages were increased and were recently raised to 240 mg/day. This requires modern and sophisticated formulations developed under biopharmaceutical considerations. Current modern solid dosage forms of EGb 761® are well characterized with regard to their release characteristics. This is complemented by pharmacokinetic investigations on the bioavailability of the constituents that are important for efficacy.

The stability of the constituents must also be assured. The ICH guidelines on stability testing are also valid for HMP. The stability over the shelf life of the proprietary medicinal products must be guaranteed using tests on a multitude of constituents of EGb 761®.

11.2.6 Ginkgo and EGb 761® in Pharmacopoeias and Other Monographs

For many years, no generally valid pharmacopoeial monographs or similar monographs in alternative standard publications were issued. This changed with the so-called Commission E Monographs in Germany in 1994 that were also translated into English (Blumenthal et al. 1998). Therapeutic indications for medicinal plants together with dosages and basic quality standards were laid down in these monographs as a basis for market authorization in Germany. The Commission E monograph on *Ginkgo biloba* leaf extract also served as a model for the efficacy monograph of the European Scientific Cooperative on Phytotherapy" (ESCOP 2003).

Interestingly, a monograph for a purified and quantified Ginkgo extract did not appear in the German Pharmacopoeia until 2000. Finally, this monograph described some framework specifications and associated analytical procedures for pharmaceutically applied Ginkgo extracts. The monograph was principally based on the data for EGb 761®. Up to this time there were only monographs on medicinal plants, and some traditional preparations such as tinctures, in the European Pharmacopoeia, but no monographs on plant extracts. A first basic monograph on Ginkgo leaves was included in the European Pharmacopoeia in 2002. The monograph on Ginkgo extract, refined and quantified, was finally included in the European Pharmacopoeia in 2008.

The American Pharmacopeia (USP)/National Formulary (NF) anticipated this development. In the USA, Ginkgo extracts are treated and marketed as dietary supplements and not as pharmaceuticals. Thus, monographs for Ginkgo leaves, dry extract, and proprietary dietary supplement products were included in the USP/NF. The specifications are similar to those in the later European monographs.

Within the Chinese Pharmacopoeia, modern monographs on Ginkgo leaves, Ginkgo leaf extracts, and Ginkgo tablets appeared after 2005. The Chinese

specifications for *Ginkgo biloba* leaf extract largely correspond to those of the European Pharmacopoeia with regard to flavonolglycosides and terpene lactones. However, the limit for ginkgolic acids (10 ppm) is higher than that in the European Pharmacopoeia (5 ppm). In contrast to other pharmacopoeias, therapeutical indications are also described in the Chinese Pharmacopoeia. These are chest impediment, heart pain, stroke, hemiplegia, and dysphasia due to blockage of meridians by stagnated blood; angina pectoris of the stable type in coronary heart disease; and cerebral infarction with above symptoms. For the most part, these indications are not supported by systematic clinical studies. For tablets, dosages/strengths up to 19.2 mg flavonolglycosides and 4.8 mg ginkgolides are described. This corresponds to an extract content of approx. 100 mg and is lower than the daily dosage of up to 240 mg usual for Ginkgo extracts.

11.2.7 Constituents of EGb 761®

11.2.7.1 Flavonoids

In the early 1960s, the flavonoids, which may be easily detected by spectrophotometry, thin layer chromatography, and high-performance liquid chromatography (HPLC), which came later, were investigated in detail by the Dr. W. Schwabe Pharmaceuticals' research team as analytical and pharmacological marker substances. It soon became clear that the Ginkgo flavonoids were mainly represented by flavonol-O-glycosides, structurally based on quercetin, kaempferol, and isorhamnetin as aglycones and glucose, rhamnose, and glucorhamnose (rutinose) as sugar units. In the 1970s, the introduction of HPLC also became established in pharmaceutical analysis. The Schwabe analysts were soon able to separate and identify this multitude of Ginkgo flavonolglycosides using HPLC gradient techniques. For peak identification, practically all flavonolglycosides were isolated and structurally analyzed. Figure 11.3 shows a HPLC chromatogram of the typical pattern of flavonolglycosides.

The flavonol mono-, di-, and triglycosides and especially the coumaric acid esters of quercetin- and kaempferolglucorhamnosides were recognized as typical for Ginkgo, and have not yet been identified in any other plant. They are quantitatively the most important compounds in the Ginkgo flavone glycoside group (Fig. 11.4).

For reason of industrial property protection, the Schwabe team was reluctant in publishing their results at that time. Thus, pertinent publications during this period concerning constituents originated, if anything, from other (university) groups, e.g., Meier et al. (1992), Sticher (1992), van Beek (2000), and more recently van Beek (2009).

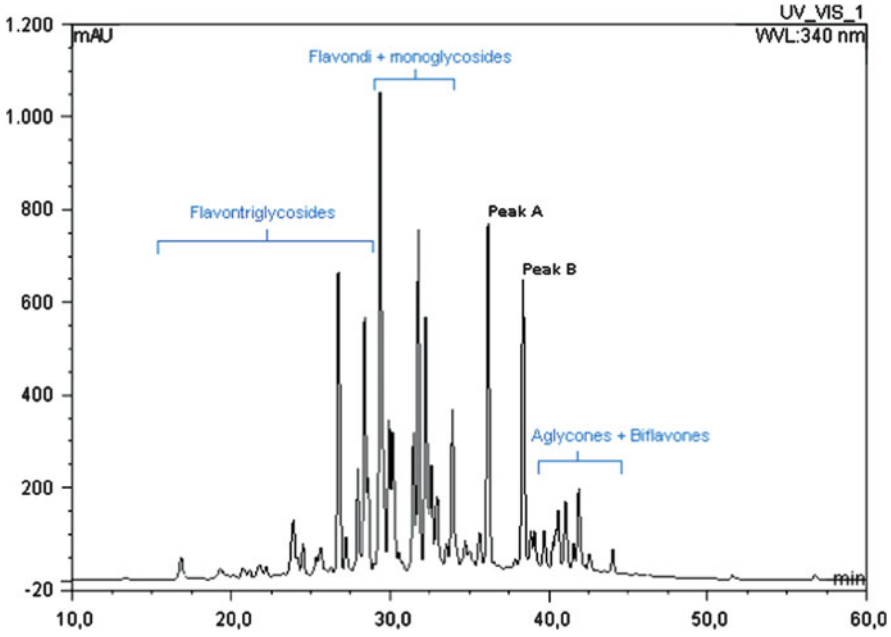


Fig. 11.3 HPLC chromatogram of flavonolglycosides of EGb 761®; Peak A: Quercetin-3-O-[4-Hydroxy-E-cinnamoyl-(6)-beta-D-glucopyranosyl-(1→2)-alpha-L-rhamnopyranoside; Peak B: Kaempferol-3-O-[4-Hydroxy-E-cinnamoyl-(6)-beta-D-glucopyranosyl-(1→2)-alpha-L-rhamnopyranoside

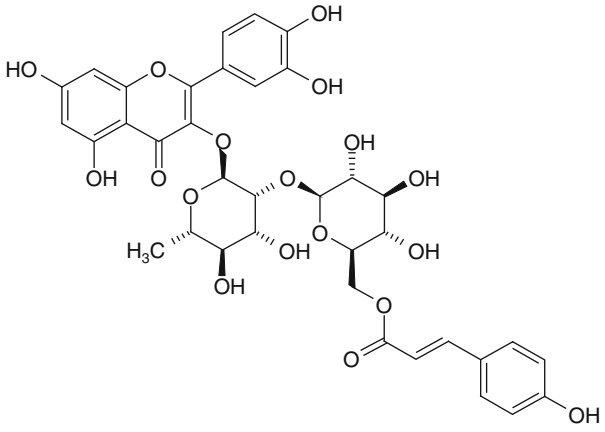


Fig. 11.4 Structure of a typical Ginkgo flavone glycoside: Quercetin-3-O-[4-Hydroxy-E-cinnamoyl-(6)-beta-D-glucopyranosyl-(1→2)-alpha-L-rhamnopyranoside

HPLC procedures were developed for assaying the main aglycones quercetin and kaempferol/isorhamnetin after hydrolysis of the flavonolglycosides. These two- or three-peak HPLC procedures were much simpler and quicker than the

protracted and multi-peak assays of all glycosides using gradient HPLC. A rapid HPLC procedure was the basis for the analysis and blending of thousands of Ginkgo crude drug batches and corresponding batches of extracts. The HPLC procedure was later introduced in the pharmacopoeias practically without modification.

According to the current definition, pharmacopoeia-conforming Ginkgo extracts such as EGb 761® contain 22–27 % flavonolglycosides determined as quercetin and calculated using a molecular weight factor of 2.52.

11.2.7.2 Terpene Lactones

Whilst initially the flavonolglycosides were at the focus of research owing to their analytical conspicuousness and relatively high content in EGb 761®, the terpene lactones moved into the limelight later.

Ginkgolides and bilobalide are largely responsible for the extremely bitter flavor of EGb 761® and are typical constituents unique to *Ginkgo biloba*. They are terpenoid compounds with several lactone groups and an interesting tertiary butyl group (Figs. 11.5 and 11.6).

In the early years of development, the terpene lactones were not easy to analyze as they lack a UV chromophore. Reliable assay and quantification first became possible with HPLC and using refractometry or light-scattering detection (Chromatogram see Fig. 11.7). Later, HPLC/MS or GC/MS (following derivatization of the nonvolatile terpene lactones) also became possible. In addition to bilobalide and ginkgolides A, B, and C, ginkgolide J was identified and structurally elucidated.

In EGb 761® both the terpene lactones and the flavonolglycosides are adjusted. Such a quality adjustment of 30 % of the constituents of a plant extract is extremely difficult and complex. The blending process is only possible if a certain spectrum of crude plant material qualities is in stock and a correspondingly large number of batches is available. The blending strategy is complex as it is accompanied by analytical procedures and the blending ratios are controlled taking into account the analytical results and the stocks.

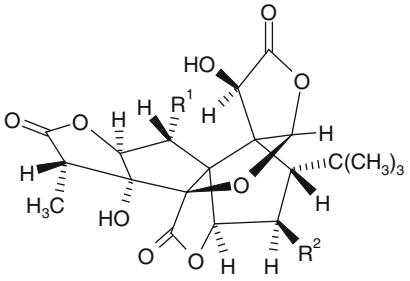
Beyond this the extensive elimination of constituents that might cause adverse or side effects is particularly important.

11.2.8 Ginkgolic Acids

Ginkgolic acids are prominent constituents of Ginkgo seeds and also of Ginkgo leaves (structure see Fig. 11.8). The concentrations contained in dried leaves may be up to 2 % and in seeds the concentrations may be even higher (Jaggy and Koch 1997).

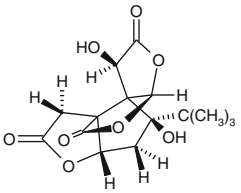
Crude and unrefined extracts from Ginkgo leaves consequently contain very high concentrations of ginkgolic acids up to several percent.

Fig. 11.5 Structures of ginkgolides



	R^1	R^2
Ginkgolid A	H	H
Ginkgolid B	OH	H
Ginkgolid C	OH	OH
Ginkgolid J	H	OH

Fig. 11.6 Structure of bilobalide



Since ginkgolic acids have been described to possess an allergic, cytotoxic, and genotoxic potential (Liu et al. 2007), their content is limited to less than 5 ppm (0.0005 %) in EGb 761®. This dramatic reduction may be achieved by cold precipitation and further solvent extraction/partition steps during the manufacturing process.

Ginkgolic acids may be analyzed by sensitive procedures such as HPLC-UV detection or other chromatographic techniques. Ginkgolic acids may be regarded as analytical markers for the removal of related long chain alkyl phenols, such as cardanols, cardols, and urushiols. Urushiols, a group of structurally related compounds responsible for the high allergenic potential of poison ivy, have also been reported to be present in small amounts in Ginkgo leaves (Schötz 2002). According to the guideline of the European Medicines Agency regarding impurities with genotoxic potential, such constituents have to be completely removed or at least eliminated as far as is technically possible (EMEA 2007).

During the last years more and more tea mixtures appear on the food market that contain Ginkgo leaves. Despite their limited solubility, ginkgolic acids are partially extracted from the tea leaves with hot water and the amounts of intake by the tea consumer may be higher than via pharmaceutical preparations (Krzywon et al. 2008).

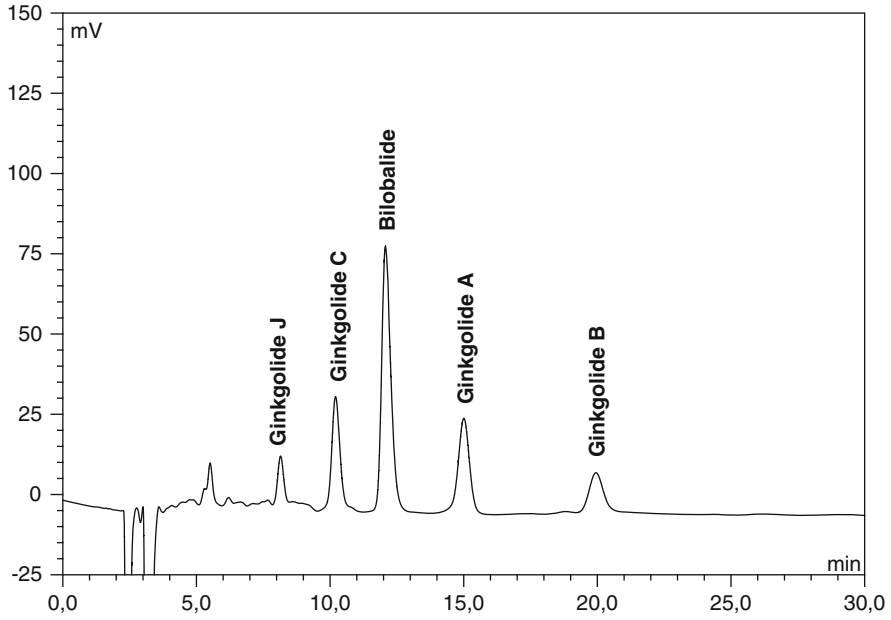


Fig. 11.7 HPLC chromatogram of terpene lactones with RI detection

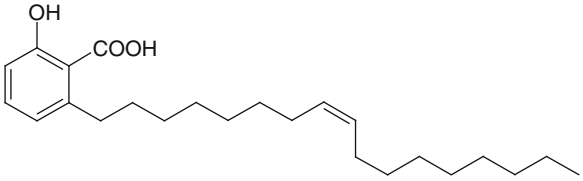


Fig. 11.8 Structure of a typical ginkgolic acid: 2-(8-Heptadecenyl)-6-hydroxybenzoic acid

11.2.9 4-O-Methylpyridoxine

More recently, 4-O-methylpyridoxine (sometimes called ginkgotoxin, MPN) has also been discussed as a further potentially toxicological relevant constituent of Ginkgo leaves (structure see Fig. 11.9). 4-O-methylpyridoxine is contained in Ginkgo seeds and leaves. In seeds, quantities of 85 µg/seed have been reported (Arenz et al. 1996a). In fresh Ginkgo leaves, MPN is found in concentrations of around 7 µg/g. This corresponds to an approx. fivefold higher quantity (35 µg/g) in the dried leaves.

Intake of large doses of MPN (much higher than contained in 240 mg Ginkgo extract/day) can cause intoxications with symptoms such as vomiting, tonic-clonic seizures, paralysis of the extremities, and loss of consciousness. In animal experiments a drop of GABA levels in the brain was observed after oral administration of MPN. This has led to the hypothesis that MPN directly inhibits vitamin B_6-dependent enzymes (e.g., glutamate decarboxylase) or indirectly impedes the cellular supply of vitamin B_6. Indeed, MPN poisoning, which is accompanied by a

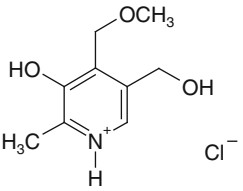


Fig. 11.9 Structure of 4-O-methylpyridoxine (hydrochloride)

high rate of lethality, can be cured by the timely injection of vitamin B_6. Recently, it has been reported that MPN is phosphorylated by the enzyme pyridoxal kinase (PK) with a significantly lower K_m value than the natural substrates, thereby competitively inhibiting the formation of active vitamin B_6 (pyridoxal 5′-phosphate) (Leistner and Drewke 2010a, b).

Sensitive detection of MPN can be carried out using different chromatographic techniques, HPLC and fluorescence detection, in particular. Owing to its ionic properties, MPN is readily soluble in water and alcohol/water mixtures. The compound is chemically very stable. It is partially removed during the EGb 761® manufacturing process so that EGb 761® contains MPN in concentrations of less than 200 ppm. With a daily dosage of 240 mg, this corresponds to approx. 48 µg of ginkgotoxin. According to Arenz et al. (1996b) medicinal dosages based on European pharmacopoeia-conforming Ginkgo extracts contain approx. 11–59 µg of MPN/day, i.e., of the same order of magnitude.

11.2.10 Ginkgo Extracts: Herbal Medicinal Products (HMPs) or Dietary Supplements?

EGb 761® has been developed as a pharmaceutical active ingredient and is used for many years predominantly in the pharmaceutical–medical sector.

In recent years, more and more Ginkgo preparations of questionable quality and variable composition appear on the market. The Ginkgo leaf with its magical radiance is speculatively exploited for wellness teas, energy drinks, sweets, cereals, and numerous dietary supplements and even for cosmetics. Quality and composition of such products have been investigated in several studies (Gawron-Gzella et al. 2010; Tawab et al. 2010).

The composition of these products varied widely; the content of flavonol-glycosides and terpene lactones was in general considerably lower than in the quantified Ginkgo leaf extract EGb 761®. High contents of ginkgolic acids have repeatedly been reported. Because of the lower dose and poorer pharmaceutical quality neither the health benefits nor the safety profile demonstrated for EGb 761® can be extrapolated to these non-pharmaceutical Ginkgo preparations.

11.2.11 Adulterations of Ginkgo Extracts

Ginkgo leaves of acceptable quality are short in supply and the price is high. The manufacture of high-quality Ginkgo extracts such as EGb 761® is elaborate and expensive. Observations in the USA and Australia suggest that certain manufacturers employ "alternative" extracts for their Ginkgo dietary supplements (Myers 2007). Obviously, they use adulterated extracts, e.g., by increasing the flavone glycoside content. Addition of pure flavone aglycones (such as quercetin or kaempferol) from other sources can increase the calculated flavonoid content significantly as the analytical assay uses a factor of about 2.5 in order to estimate for the molecular mass of flavonolglycosides. Sometimes pure rutin seems to be used also.

Such observations have also been made in Europe. In a systematic investigation of dietary supplements containing Ginkgo extracts, Tawab et al. (2010) discovered a manipulation of the flavone glycoside composition and content in several preparations. Like native Ginkgo leaves, genuine EGb 761® contains only flavonol-glycosides and practically no free aglycones. The natural ratio of aglycones following analytical hydrolysis is around 0.8–1.2 for quercetin/kaempferol (including isorhamnetin) (this ratio is specified in USP 31-NF26).

In many of the dietary supplements on the German market that were investigated, the flavonoid content was artificially increased by addition of quercetin, rutin, or extracts from the Japanese pagoda tree (Sophora japonica). This may be detected by a nonnatural Q/K ratio, HPLC of the intact flavonolglycosides, or TLC according Pharm. Eur. Monograph for Ginkgo b. extract.

Such adulterations of "Ginkgo" supplements marketed as nutraceuticals highlight the shortcomings of regulations, surveillance, and control in the health food sector. Quality, as the basis of efficacy and safety of complex herbal preparations, such as *Ginkgo biloba* leaf dry extracts, can only be safeguarded in the pharmaceutical sector with its strict regulation of good agricultural practice as well as manufacturing and quality control.

11.3 Pharmacology

11.3.1 Effects on the Cardiovascular System

The introduction of EGb 761® in the therapy of peripheral artery occlusive disease in 1965 was based on the observation that the extract increased blood flow in the isolated guinea pig hind leg (Peter et al. 1966). Later on it was demonstrated that the extract has particularly strong effects on the cerebral blood flow increasing the perfusion in almost all brain regions by 50–100 % (Krieglstein et al. 1986). This effect was found to be mediated by the non-flavone fraction of EGb 761® (Ahlemeyer and Kriegelstein 2003). Beneficial cerebrovascular activity of *Ginkgo biloba* extract

and some of its constituents was also observed in experimental animal models of focal or global ischemia (Le Poncin-Lafitte et al. 1980; Krieglstein et al. 1995).

The favorable vascular effects of *Ginkgo biloba* extract, which are preferentially seen in ischemic tissues, have largely been explained by a direct impact on both arteries and veins with the adrenergic vasoregulatory system and the vascular endothelium as the preferential targets on the arterial side. The extract reinforces the physiological vasoregulation of the sympathetic nervous system by directly acting on neuromediator release, and indirectly by inhibiting their extraneuronal degradation by catechol-O-methyltransferase (COMT). On the venous system the extract has been shown to have mainly a vasoconstrictor component that maintains the vessel tonus. In the arterial endothelium EGb 761® stimulates the release of endogenous relaxing factors, such as nitric oxide (NO) and possibly prostacyclin (Auguet et al. 1986). The effect on NO synthesis has in the meanwhile been investigated in more detail. Thus, incubation of endothelial cells with EGb 761® for 48 h demonstrated that the enhanced NO production is due to an increasing endothelial nitric oxide synthase (eNOS) promoter activity and eNOS expression. Phosphorylation of eNOS at a site typical for Akt (Ser 1177) was acutely enhanced by treatment with EGb 761® as was Akt phosphorylation at Ser 478. Furthermore, the extract caused acute relaxation of isolated aortic rings and NO-dependent reduction of blood pressure in vivo in rats. These influences on eNOS represent a putative molecular basis for the protective cardiovascular properties of EGb 761® (Koltermann 2007).

Besides regulation of the blood vessel tone, the pharmacological impact of EGb 761® on the cardiovascular system encompasses other elements of this system, including influences on vascular permeability, blood rheology as well as effects on cellular elements such as thrombocytes, and leukocytes (Koch and Chatterjee 1993).

Some of the hemorheological effects of EGb 761® have been explained by the antagonistic effect of ginkgolides on the platelet-activating factor (PAF) receptor (Braquet 1987). However, as there is a large difference between the PAF inhibitory activity observed in vitro and the plasma concentrations determined after intake of EGb 761® at therapeutic doses, it appears extremely unlikely that an inhibition of platelet aggregation can be achieved in vivo (Koch 2005) (Fig. 11.10). In humans, EGb 761® at therapeutic doses does not inhibit platelet aggregation nor blood coagulation (Kellermann and Kloft 2011).

11.3.2 Antioxidative Activity

Because of the complex vascular effects observed during the early phase of development, it very soon became evident that the clinical activity profile of EGb 761® could not be entirely explained only on the basis of vasoregulatory properties. In particular, pharmacological studies in the Schwabe laboratories demonstrated that the vascular effects of the extract could not be defined in terms of classical vasodilation, as it did not relax isolated smooth muscle preparations. Instead the

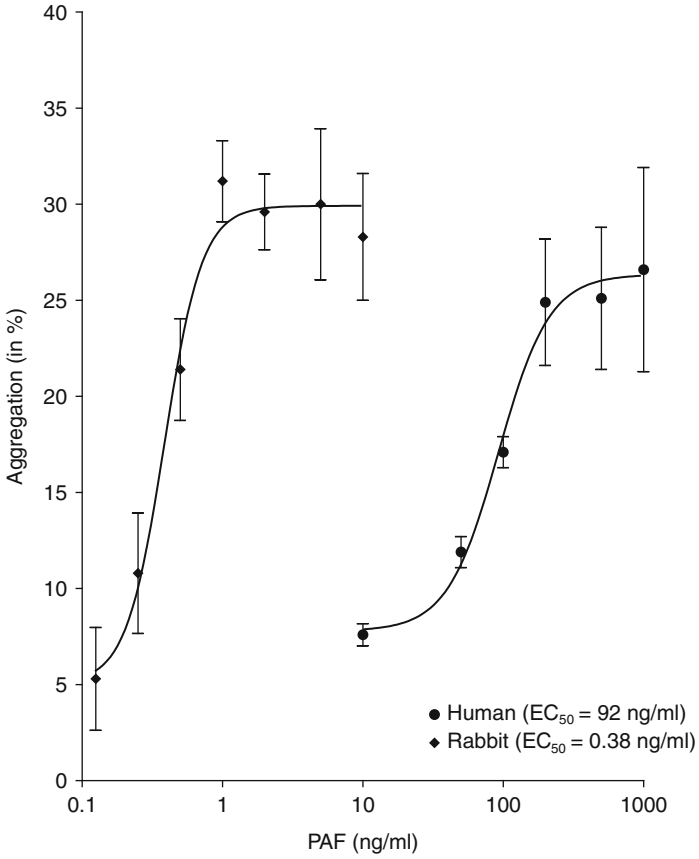


Fig. 11.10 PAF-induced aggregation of rabbit and human platelets in platelet-rich plasma. Given are the means ± SD with samples from four rabbits and two each female and male human volunteers, respectively. The concentration needed to induce a half-maximal aggregation (EC_{50}) of human platelets (92 ng/ml) was more than 200 times higher when compared to rabbit thrombocytes (0.38 ng/ml), demonstrating a low reactivity of human thrombocytes to PAF. PAF-mediated aggregation of human platelets was half-maximally inhibited by ginkgolide B, A, C, and J at concentrations of 2.5, 15.8, 29.8, and 43.5 mg/ml, respectively. These concentrations are generally more than 100 times higher as the peak plasma values measured after oral intake of EGb 761® at recommended doses between 120 and 240 mg

findings suggested that the vascular effects may be due to some metabolic activity (Chatterjee 1985).

At that time it was realized that the extract, unlike many other known vasodilators, was able to prolong the survival time of animals under hypoxia (see below). Efforts to clarify the mechanism of this antihypoxic effect led to the discovery of antioxidative activity. Initially this conclusion was reached by comparison with other substances known to prolong survival time of animals under hypoxic conditions which all possess radical scavenging properties (Chatterjee and Gabard 1981). The antioxidant effects of EGb 761® have been demonstrated in a variety of in vitro investigations on

subcellular and cellular structures, but also in ex vivo and in vivo studies. By now this activity is possibly the most well-established mode of action of EGb 761® which can explain many of its pharmacological and therapeutic effects.

A concentration-dependent inhibition of lipid peroxidation in rat brain homogenates by EGb 761® was already reported in 1982 (Chatterjee and Gabard 1982a). This observation was confirmed by other investigators after inducing lipid peroxidation by applying a NADPH/Fe^{3+}/ADP system, cyclosporine, or UV radiation (Pincemail and Deby 1986; Barth et al. 1991; Dumont et al. 1992).

Radical scavenging activity has also been proven in vivo. After induction of oxidative stress in mice or rats by administration of alloxan (Chatterjee and Gabard 1981), doxorubicin (Chatterjee and Gabard 1982b), triethyltin (Boulie et al. 1988), or bromethalin (Dorman et al. 1992) protective effects such as inhibition of hyperglycemia, reduced concentration of thiobarbituric acid reactive substances in the brain, or an increased survival rate were observed. In old rats lipid peroxidation in the cerebral cortex was suppressed by oral treatment with EGb 761® (Sram and Binkova 1993). Over the years the antioxidative activity of EGb 761® has intensively been investigated in numerous other experimental studies applying a broad range of animal models (Fitzl et al. 2000; Rojas et al. 2000, 2001; Schindowski et al. 2001; Uríková et al. 2006; Kampkötter et al. 2007; Lin et al. 2007; Keles et al. 2008; Rojas et al. 2008; Yeh et al. 2009; Martin et al. 2010).

The antioxidative properties appear to be mediated by two mechanisms, directly by scavenging free radicals and indirectly by inhibiting the formation of radical species. EGb 761® can scavenge different reactive oxygen species (e.g., hydroxyl radical, peroxyl radical, superoxide anion, nitric oxide radical, hydrogen peroxide), and ferryl ion species (De Feudis 2003; Mahadevan and Park 2008). Both main groups of constituents, the flavonoids (Emerit et al. 1995) and the terpene lactones (Scholtyssek et al. 1997) have been found to contribute to this activity. The extract contributes to the antioxidative defense of the body by enhancing the expression of antioxidative enzymes such as superoxide dismutase, glutathione peroxidase, catalase, and hemeoxygenase and activity of systems (Mahadevan and Park 2008; Saleem et al. 2008).

11.3.3 Effects on Cerebral Energy Metabolism and Mitochondrial Function

Already during the early 1980s it became clear that some of the clinically important properties of EGb 761® cannot solely be explained by its antioxidative activity (Chatterjee 1985). At that time it was observed that the antihypoxic action was associated with an improvement of cerebral energy metabolism. Rats treated with EGb 761® survived hypobaric hypoxia for a much longer period than control animal. The brain glucose levels in treated rats were elevated while the lactate concentrations were slightly lower. The lowering of lactate/pyruvate ratio was due

to a decreased level of lactate and an enhanced concentration of pyruvate as well. The data suggest that changes in brain energy metabolism and blood flow contribute to the protective effect of EGb 761® against hypoxia (Karcher et al. 1984). These findings were confirmed some years later in mice. EGb 761® as well as its non-flavone fraction considerably prolonged the survival time of mice under lethal hypoxia. The extract and the non-flavone fraction retarded the breakdown of brain energy metabolism (Oberpichler-Schwenk et al. 1988). More precise information on the ingredients responsible for these effects of Ginkgo extract was obtained in a mouse model of focal cerebral ischemia. Bilobalide and the ginkgolides A and B, but not C, significantly reduced the infarct size after occlusion of the middle cerebral artery (Ahlemeyer and Kriegelstein 2003).

Further evidence for a positive influence of EGb 761® on cerebral energy metabolism was obtained in a rat model of brain edema induced by triethyltin (TET) which could be cured by oral treatment with the extract or bilobalide but not a flavonoid fraction (Chatterjee 1985; Chatterjee and Gabard 1984; Sanceario and Kreutzberg 1986; Otani et al. 1986). Since it is known that TET is an uncoupler of oxidative phosphorylation (Aldridge et al. 1977). It has been speculated that the extract and some of its constituents possess mitochondria protective effects (De Feudis 2003). Indeed, by now most of the effects of EGb 761® on energy metabolism and protection against hypoxia are explained by a stabilization of mitochondrial function (Müller et al. 2009).

Direct evidence for an improvement of mitochondrial function was provided by Janssens et al. (1995). These authors demonstrated that EGb 761®, as well as bilobalide, protects endothelial cells against a hypoxia-induced ATP decrease. In addition, both compounds were shown to increase the respiratory control ratio of mitochondria isolated from liver of orally treated rats. The protection of ATP content and the delay in glycolysis activation are explained by a protection of mitochondrial respiratory activity. Both products appear to improve the ability to form ATP and reduced the requirement for cellular glycolysis by preservation of ATP regeneration in mitochondria. Further investigations provided evidence that bilobalide allows mitochondria to maintain their respiratory activity in ischemic conditions by protecting complex I and probably complex III activities (Janssens et al. 1999, 2000).

Using isolated mitochondria, PC12 cells, or dissociated mouse brain cells, mitochondrial abnormalities occurring during aging were mimicked by applying external factors (e.g., nitrosative stress, serum deprivation, and complex inhibitors). EGb 761® was found to prevent mitochondrial dysfunction at low concentrations (10 μg/ml) as determined by the measurement of ATP levels and mitochondrial membrane potential (Eckert et al. 2005; Abdel-Kader et al. 2007). After treating young (2–3 months) and old (15–16 months) mice with EGb 761® (100 mg/kg body weight for 14 days) beneficial effects on complexes I, IV, and V of the mitochondrial respiratory chain against NO-induced damage were observed in aged mice, indicating a higher efficacy during aging. Since protection of mitochondrial membrane potential was seen for different extract components the versatile actions of

EGb 761® seem to depend on the complementary action of these constituents (Abdel-Kader et al. 2007).

11.3.4 Antiapoptotic and Neuroprotective Effects

Besides their primary function in energy metabolism, mitochondria play an important role in the regulation of cell death. The release of cytochrome *c* and other pro-apoptotic proteins from mitochondria following opening of pores such as the permeability transition pore is a key step of apoptosis. Other pores may form after insertion of pro-apoptotic members of the bcl-2 family in the outer mitochondrial membrane, which in turn are activated by apoptotic signals such as cell stress, free radical damage, or growth factor deprivation (De Marchi et al. 2004; Niizuma et al. 2010).

In accordance with the beneficial effects of EGb 761® on mitochondrial function, the extract has been shown in a large number of publications to protect cells against apoptosis induced by various noxious insults. For instance, the extract was found to suppress apoptotic neuronal cell death caused by glutamate, nitric oxide, hydrogen peroxide, simvastastin, staurosporin, MPP+, olfactory nerve sectioning, or middle cerebral artery occlusion (Ahlemeyer and Kriegelstein 2003; Christen 2010). Prevention of apoptosis was also demonstrated for other cell types such as lymphocytes (Schindowski et al. 2001), endothelial cells (Hsu et al. 2009), testicular tissue (Yeh et al. 2009), or cardiomyocytes (Shen et al. 2011). As possible mechanisms for the prevention of apoptosis inhibition of caspase-3 (Luo et al. 2002) a transcriptional upregulation of HO-1 via the MAPKs/Nrf2 pathway (Hsu et al. 2009), a reduced increase of Bcl-2 family proteins (Koh 2009), or activation of the PI3K/Akt pathway (Shi et al. 2010) has been discussed.

In general, two mechanisms are considered to be involved in the pathogenesis of neurodegenerative disorders. Besides accumulation of cellular damage by repeated exposure to oxidative stress during senescence, the abnormal aggregation and deposition of proteins is associated with some specific neurological diseases, for example in Alzheimer's dementia (AD) or Parkinson's disease (Christen 2004). The main component of senile plaques in AD is Aβ, a 40–42 amino acid peptide formed from the amyloid precursor protein (APP) by the action of β- and γ-secretases, while α-secretase cleaves APP within the Aβ sequence. It is now believed that not large aggregates of Aβ, but small intracellular oligomers already very early induce a disturbance of mitochondrial and synaptic function which over a long time period spreads and causes a massive loss of neurons with the accompanying clinical symptomatic (Müller et al. 2009; Christen 2010).

EGb 761® has been shown to interfere with this deleterious process at various levels. For example, the extracts have been found to protect neuronal cells against Aβ toxicity (Bastianetto et al. 2000; Longpre et al. 2006), to inhibit Aβ oligomerization (Luo et al. 2002; Wu et al. 2006), to regulate APP processing towards the non-amyloidogenic α-secretase pathway (Colciaghi et al. 2004), and to improve

Aβ clearance (Yan et al. 2008). Investigating the impact of dietary EGb 761® (300 mg/kg) for 1 or 16 months on APP metabolism in mice transgenic for human APP (Tg2576), it was observed that long-term but not short-term treatment significantly lowered APP protein levels by approximately 50 % as compared to controls in the cortex but not in the hippocampus (Augustin et al. 2009). In a double transgenic mouse model (TgAPP/PS1), the extract significantly increases cell proliferation in the hippocampus of both young (6 months) and old (22 months) mice, reduced Aβ oligomers, and restored cyclic-AMP response element binding protein (CREB) phosphorylation. The stimulation of neurogenesis may contribute to improved cognitive functions in the animal model of AD and to its advantageous effects in AD patients (Tchantchou et al. 2007). Using the same mouse model, oral treatment with EGb 761® led to a progressive reversal of the structural changes in dystrophic neurites associated with senile plaques. These results suggest a causal relationship between plaque-associated oxidative stress and neuritic alterations and demonstrate that the focal neurotoxicity associated with the senile plaques of AD is partially reversible by treatment with Ginkgo extract (Garcia-Alloza et al. 2006). After all, prevention of age-related spatial memory deficits has been demonstrated in a transgenic mouse model of AD by chronic treatment with the extract (Stackman et al. 2003).

The influence of a 7-day oral treatment with EGb 761® or bilobalide on global ischemia and glutamate-induced excitotoxicity was examined in Mongolian gerbils. It was shown that the extract and bilobalide dose-dependently protect hippocampal CA1 neurons against ischemia-induced neuronal death (Chandrasekaran et al. 2002). In rat hippocampal slices it could also be shown that bilobalide inhibits hypoxia-induced release of choline from lipid membranes, probably by inhibiting the activity of phospholipase 2 (Klein et al. 1995, 1997). In a recent study, mice were subjected to permanent distal middle cerebral artery occlusion and treated 4 h later with 100 mg/kg EGb 761®, 6 mg/kg bilobalide, ginkgolides A, ginkgolide B, or 10 mg/kg of a terpene lactone-free extract fraction. All treatments significantly lowered infarct volumes in comparison to vehicle-treated mice. Similarly, neurologic deficit scores were lower in the treated groups as compared with controls. Interestingly, the protective effect of EGb 761® was essentially lost when heme oxygenase (HO)-1 knockout mice were treated with EGb 761®. The results suggest that HO-1 plays, at least in part, an important role in the neuroprotective mechanism of EGb 761® and in delayed ischemia (Shah et al. 2011). Neuroprotective effects of EGb 761® have also been shown with respect to age-dependent structural changes in the hippocampi of inbred mice (Barkats et al. 1995) or in HIV-associated neurological diseases (Zou et al. 2007).

11.3.5 Gene Regulating Effects

Considering the above findings, it seems that the neuroprotective effect of EGb 761® is due to the interplay of various activities, e.g., vascular and hemorheological

effects, antioxidative and antiapoptotic action, improvement of mitochondrial function, and reduced ischemic or toxic brain damage. There is now ample evidence that gene regulatory activity of Ginkgo extract may also contribute to neuroprotection.

In order to gain a deeper insight into the complex pharmacological action of EGb 761®, changes of gene expression in the brains of mice were analyzed after 4 weeks treatment using oligonucleotide microarrays. Of the 12,000 combined genes and expressed sequence tags on the array, only 10 changed in expression by a factor of three or more whereupon all were upregulated (Watanabe et al. 2001). The study demonstrated differential effects of the extract in separate brain regions. In the cortex, mRNA for neuronal tyrosine/threonine phosphatase 1 and microtubule-associated tau were significantly enhanced. Both proteins are involved in the formation and breakdown of intracellular neurofibrillary tangles, which represent a hallmark lesion of AD. In addition, the expression of AMPA-2, calcium and chloride channels, prolactin, and growth hormone—which are all associated with brain functions—was upregulated. In the hippocampus, only the expression of transthyretin mRNA was increased. Transthyretin is of particular interest as it plays a role in transport of thyroxine and the retinol-binding protein. Thyroid hormones regulate neuronal proliferation as well as differentiation. Transthyretin has also been shown to sequester Aβ and to prevent its aggregation. Most interestingly, transthyretin levels have been found to be markedly decreased in cerebrospinal fluid of AD patients. Thus, EGb 761® may exert important neurological effects by the modulation of synthesis of transthyretin and other proteins in the brain (Rimbach et al. 2004).

Oral administration of EGb 761® or bilobalide for 7 days before global brain ischemia in gerbils protected hippocampal CA1 neurons against death which may be due to enhanced levels of mitochondrial DNA-encoded cytochrome oxidase (COX) subunit III mRNA (Chandrasekaran et al. 2002). In a cellular model an enhanced gene expression has also been reported for mitochondrial NADH dehydrogenase subunit I (Tendi et al. 2002).

Altered transcriptional patterns have been observed in other studies. Already in 1996, Mizuno et al. reported that pretreatment of Jurkat T cells with 10 μg/ml EGb 761® suppresses AP-1 DNA activation and c-fos mRNA expression. The results suggest that the inhibition of the AP-1 signal transduction pathway is caused by an upstream downregulation of c-fos mRNA expression. In PC12 cells treated with the extract the expression of the anti-apoptotic Bcl-2 protein was increased and those of the pro-apoptotic caspase-12 was downregulated (Smith et al. 2002). In primary neuronal cell cultures, the hypothesis was tested that the neuroprotective action of EGb 761® could be due to an induction of heme oxygenase-1 (HO-1). HO-1 acts as an antioxidant enzyme by degrading heme into iron, carbon monoxide, and biliverdin. Indeed, it has been shown that the extract induced HO-1 in a concentration- and time-dependent manner with a maximal induction at 8 h (Zhuang et al. 2002). cDNA microarrays were used by Soulie et al. (2002) to define the transcriptional effects of EGb 761® on genes implicated in the antioxidant and stress responses in human hNT neurons. Seven genes were identified whose expression was strongly modified by the treatment. Three groups could be distinguished: genes encoding

transcription factors (increase of NF-kappaB p65 subunit and zinc finger protein 91 mRNAs, and decrease of c-myc transcripts), genes involved in antioxidant defenses (increase of the CuZn SOD mRNAs, and decrease of glutathione reductase and glutathione *S*-transferase pi mRNAs), and genes involved in stress responses (upregulation of HSP70 transcripts). In summary, the above results support the idea that modulation of target genes and transcription factors may be involved in the neuroprotective action of EGb 761®.

Using transcriptomic analysis it has recently been demonstrated that EGb 761® not only exhibits neuroprotective effects on the gene expression level but also regulates neuroactive receptor pathways. Treatment with the extract significantly altered neuroactive ligand–receptor interaction pathways in the frontal cortex but not in striatum and kidney of mice. In particular, a strong upregulation of dopamine receptors, especially dopamine receptor 1a (Drd1a), was observed (Su et al. 2009). This mode of action may contribute to the positive cognitive effects of EGb 761® (see below).

11.3.6 Cognitive and Behavioral Effects

Besides the above-described neuroprotective properties of EGb 761® already very early during the development process an influence on animal behavior, learning, and memory was observed. Continella and Drago reported in 1984 effects of EGb 761® in models of passive and active avoidance. In behavioral tests an influence on some specific central cholinergic systems was noticed that could be inhibited by scopolamine (Chatterjee and Noeldner 1989). Since a loss of cholinergic neurons in the forebrain occurs early in AD, such cholinergic effects provide a useful therapeutic mechanism in dementia. In the following years, numerous studies were conducted to evaluate the influence on cognitive functions applying various techniques to induce mental deficiencies. In these studies both protective as well as curative activities were seen. Beneficial effects on memory and learning have been reported in a series of experiments with rodents (Winter 1991; Stoll et al. 1996; Walesiuk et al. 2005). Interestingly, the strongest influence on cognitive performance was usually observed in aged animals (Continella and Drago 1984; Cohen-Salmon et al. 1997; Winter 1998; Wirth et al. 2000; Blecharz-Klin et al. 2009). In newly hatched chickens, the extract significantly improved long-term memory already at very small doses (Rickard et al. 2001).

Hoyer et al. (1999) investigated the effect of treatment with EGb 761® on behavioral and metabolic brain parameters in an experimental model of disturbed neuronal energy metabolism induced by the intracerebroventricular administration of streptozotocin (STZ). The deterioration in behavior and in cerebral energy metabolism occurring after the STZ injection was significantly slowed down by EGb 761® treatment. Over the 12-week investigation period the deficits in learning, memory, and cognition were partially compensated (Fig. 11.11), and the disturbances in cerebral energy metabolism returned to almost normal values. Recently it has been

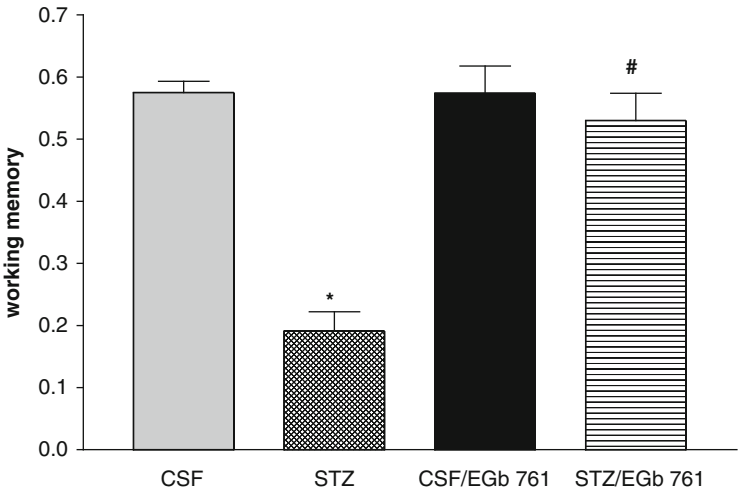


Fig. 11.11 Holeboard test in 1-year-old male rats. Mean values (±SD) of working memory on retest day 40. After a habituation (day 1–7) and training period (day 8–14) animals were treated intracerebroventricularly (icv) with streptozotocin (STZ) or with artificial cerebral spinal fluid (CSF) on experimental days 15, 17, and 33. EGb 761® treatment started after the first injection of icv STZ or CFS. Working memory ratio was defined as (number of food rewarded visits)/(number of visits and revisits to the baited set of holes); it refers to the short-term memory. $*p < 0.05$ between CSF and STZ; $\#p < 0.05$ between STZ and STZ/EGb 761®

shown that EGb 761® also improves auditory discrimination learning in Mongolian gerbils. Since the extract increases the extracellular concentration of dopamine in the prefrontal cortex of rats (see below) and the dopaminergic system plays a major role in this learning paradigm, it is supposed that this may contribute to the enhanced learning performance and could as well explain the beneficial therapeutic effect of EGb 761® in the treatment of tinnitus (Moeller et al. 2009).

Besides effects on learning and memory, Continella and Drago already in 1984 reported effects of EGb 761® in a behavioral despair model which point to therapeutic effects in depression. Applying various behavioral paradigms, the effect of stress in rodents was investigated (Porsolt et al. 1990). The results obtained indicated that EGb 761® reduces the consequences of stress in some experimental situations, but the effect cannot be explained by classical anxiolytic and antidepressant activity even though weak anxiolytic and antidepressant effects of EGb 761® and bilobalide were reported by Noeldner and Chatterjee (1994). In more recent studies, an inhibition of the immobilization time in the behavior despair and in the tail suspension test was shown supporting an antidepressant action (Sakakibara et al. 2006).

11.3.7 Effect on Neurotransmitter Systems

EGb 761® has been observed in many studies to influence different neurotransmitter systems which may contribute to its neuroprotective as well as cognitive and affective effects. Thus, the extract has been shown to influence cholinergic, noradrenergic, dopaminergic, and serotonergic neurotransmission, as it for example improves choline uptake in the hippocampus of old rats (Kristofikova et al. 1992), reactivates the noradrenergic system during aging (Huguet and Tarrade 1992), reverses the decreased 5-HT1A receptor density in the cerebral cortex of aged rats (Huguet et al. 1994), and regulates serotonergic neurotransmission as well as monoamine oxidase activity in aged or stressed mice (Pardon et al. 2000).

In vitro and in vivo EGb 761® has been shown to suppress hypoxia-induced membrane breakdown and choline release in the brain. The effect was also found for bilobalide but not for the ginkgolides (Klein et al. 1997). The same research group subsequently reported that bilobalide inhibits NMDA-induced phospholipase A2 activation and phospholipid breakdown in rat hippocampus It is concluded that the prevention of glutamatergic excitotoxic membrane breakdown may be beneficial in the treatment of brain hypoxia and/or neuronal hyperactivity. Interestingly, convulsions which were observed in the NMDA-treated control rats were almost totally suppressed by bilobalide (Weichel et al. 1999). Obviously, bilobalide does not interfere with NMDA-induced calcium influx but appears to inhibit NMDA-induced fluxes of chloride ions through glycine/GABA-operated chloride channels (Klein et al. 2003). For bilobalide antagonistic activity has also been described on recombinant GABAA receptors (Huang et al. 2003). Similarly, inhibition of GABAA and glycine receptors has been reported for ginkgolides (Ivic et al. 2003). It has been suggested that inhibitory effect on ligand-operated chloride channels may contribute to "stimulant-like" effects of EGb 761® as well as an enhancement of long-term potentiation in aged mice (Christen 2004).

Applying microdialysis techniques, it has recently been shown that treatment with EGb 761® for 2 weeks leads to a marked and highly significant increase in the extracellular concentration of dopamine in the prefrontal cortex of rats. The concentration of norepinephrine was only slightly enhanced while no effect on the serotonin level was observed. This effect of the extract is mediated primarily by the flavonoids and to a lesser extent by the ginkgolides (Yoshitake et al. 2010). Since functions of the working memory, such as attention and concentration, are controlled by the dopaminergic system in the prefrontal cortex, this activity of the extract could explain its cognition-enhancing effects (Müller et al. 2009). While the mode of action is not yet established, it has been reported that EGb 761® influences monoaminergic neurotransmission via inhibition of noradrenalin uptake. As synaptic dopamine clearance in the frontal cortex is mediated by the noradrenalin but not the dopamine transporter, these findings may explain the enhancement of dopaminergic neurotransmission as well as positive effects on cognition and attention (Fehske et al. 2009).

11.3.8 Pharmacokinetics

Like other extracts prepared from medicinal plants, EGb 761® contains a variety of bioactive substances which contribute to the clinical efficacy of the total extract. The main problem performing pharmacokinetic studies with plant extracts is that in contrast to single components, different constituents need to be analyzed and quantified.

First studies on the pharmacokinetics of EGb 761® were performed by Moreau et al. (1986). They treated growing Ginkgo trees with radiolabeled substrates and after incorporation of radioactivity into the leaves an extract was prepared. The labeled extract was applied orally to rats and the radioactivity excreted in urine and feces and by respiration was determined. The obtained pharmacokinetic profile of [^{14}C] EGb 761® showed a typical pattern for a two compartment model (Schennen 1988).

The analytical detection of the flavonolglycosides in general is difficult since the intestinal microorganisms completely cleave the glycosidic bonds and thus only the corresponding aglycones and their metabolites are detectable in the blood.

Pietta et al. (1995) could demonstrate that after oral administration of relatively high dosages of EGb 761® to rats metabolites of the flavonoids are present in plasma.

In contrast to flavonoids the terpene lactones are very good pharmacokinetic marker substances. They are not as extensively metabolized as the flavonolglycosides and can be easily detected in blood plasma using GC/MS after derivatization or HPLC/MS procedures directly without derivatization.

In plasma obtained during clinical studies, it could be shown that the terpenoids bilobalide and the ginkgolides A and B are almost completely absorbed, while ginkgolide C is only slightly bioavailable in men (Fourtillan et al. 1995).

In the following years, the bioavailability of the terpene lactones bilobalide and ginkgolides A and B could be confirmed by different authors (Biber and Koch 1999; Biber 2003).

Two recent studies, performed on rats, have demonstrated for the first time that the flavonoids kaempferol, quercetin, and isorhamnetin/tamaraxetin as well as the terpene lactones bilobalide and ginkgolides are detectable in relevant concentrations not only in plasma but also in the brain after oral administration of EGb 761® (Rangel-Ordóñez et al. 2010; Ude et al. 2011).

11.3.9 Toxicology

Subchronic toxicity studies were performed in rats (15–100 mg/kg/day i.p.) for 12 weeks and in dogs (7.5–30 mg/kg/day i.v. or 5 mg/kg/day i.m.) for 8 weeks, while chronic toxicity was tested in rats and dogs over a period of 6 months. The animals were treated daily with oral doses of 20 mg/kg and 100 mg/kg, increasing

up to 500 mg/kg in rats and up to 400 mg/kg in dogs, respectively. Observational, biochemical, hematological, and histological investigations did not indicate any toxic effects; in particular no disturbance of liver and kidney function was observed (Spiess and Juretzek 2004).

In reproductive toxicity studies doses up to 1,600 mg/kg in rats and 900 mg/kg in rabbits were administered orally. No evidence for teratogenic nor embryotoxic effects was obtained and no influence on fertility and reproducitve performance of male and female animals was seen (Spiess and Juretzek 2004).

In genotoxicity tests (Ames test, host-mediated-Assay, micronucleus test, chromosomes aberration test) and a carcinogenicity study in rats no mutagenic and carcinogenic potential was observed for EGb 761® (Hager 2009).

11.4 Clinical Development

The clinical development of *Ginkgo biloba* extract EGb 761® closely mirrors the progress of scientific insight into the pathology of aging-associated cognitive decline and dementia.

11.4.1 The Starting Point

During the 1960s and 1970s, when Western Europe had recovered from World War II, economy was prospering, food was available in abundance, and luxury articles such as cigarettes were generally affordable. However, at the same time hypertension, overweight, and cardiovascular disorders became more and more prevalent. At that time, impaired cerebral perfusion was considered to be the main cause of memory loss and dementia in old age, and vasodilation was thought to be the therapeutic principle of choice. With findings from animal studies pointing towards a perfusion-enhancing effect, this was the starting point of the clinical development of Schwabe's *Ginkgo biloba* extract EGb 761® for the treatment of vascular disorders. In 1968, using a variety of methods then available, Mußgnug and Alemany (1968) were able to demonstrate an enhancement of peripheral blood flow following both acute intravenous and chronic oral administration of the extract. Three years later, Saponaro et al. (1971) reported an increased cranial blood flow after intravenous injection of the drug.

Later, Heiss and Zeiler (1978) measured the effects of several purportedly vasodilating and perfusion-enhancing drugs on global and regional cerebral blood flow using the xenon clearance method. Although not immediately perceived as such, this study was a breakthrough regarding the mechanism of action of EGb 761®. The drug increased not only global cerebral blood flow but also regional blood flow in ischaemic areas. From this experiment it became clear that EGb 761® was not a mere vasodilator.

Hardened atherosclerotic vessels do not react to vasodilators. As a consequence, overall vasodilation would have caused a steal effect, i.e., a diversion of blood from the ischaemic areas. This was not the case with the Ginkgo extract, however. It was later discovered that EGb 761® enhances the deformability of red blood cells and decreases blood viscosity, thus enhancing blood flow in the small vessels and capillaries (Költringer et al. 1995; Erdinçler et al. 1996). This explains why it is possible to enhance blood flow by EGb 761® treatment even in atherosclerotic vessels.

11.4.2 Peripheral Arterial Disease

Confirmation of peripheral perfusion-enhancing effects in humans and of clinical improvement observed in the first exploratory, uncontrolled studies stimulated a series of controlled clinical trials with EGb 761® to prove its efficacy in peripheral arterial occlusive disease (PAOD). Findings from nine randomized, placebo-controlled trials were summarized by Horsch and Walther (2004). This successful research program led to the registration of the extract for the treatment of PAOD in Germany and numerous other countries. Of note, when administered in addition to exercise therapy, EGb 761® further increased the pain-free and total walking distance (Bulling and von Bary 1991; Blume et al. 1996).

11.4.3 Aging-Associated Cognitive Decline and Dementia

In view of the general opinion about the causes and pathogenesis of aging-associated cognitive decline, which was held until the 1970s, and the first known pharmacodynamic actions of EGb 761®, the early studies in this field enrolled subjects with aging-associated cognitive problems related to evident vascular disease. This was often referred to as cerebrovascular insufficiency (Moreau 1975; Taillandier et al. 1986; Halama et al. 1988; Hofferberth 1991). As expected, the drug improved cognitive performance, but it also improved overall mental functioning and behavioral problems. When, during the 1980s, further research into brain pathology brought to light that Alzheimer-type pathology (amyloid plaques and neurofibrillary tangles) was more prominent than vascular pathology in a large proportion of patients who experienced severe cognitive decline, the question of whether EGb 761® might also improve cognitive impairment in patients with nonvascular brain pathology arose. Further trials therefore addressed this question and found the drug to be effective, irrespective of the underlying cause (Wesnes et al. 1987; Israël et al. 1987; Hofferberth 1989).

While in the USA the modern concept of dementia was shaped and defined by diagnostic criteria, Europeans were hesitant to use a term with such a negative connotation until the 1990s. In Germany, the less stigmatizing term "Hirnleistungsstörung", i.e., impairment of cerebral function, was mainly used as a

diagnostic label for the mildest to the severest stages of cognitive decline. Only one study of EGb 761® in the mid-1980s explicitly enrolled patients with primary degenerative dementia (i.e., Alzheimer-type dementia). The drug was found to be significantly superior to placebo in neuropsychological, neurophysiological, and clinical outcome measures (Weitbrecht and Jansen 1986). In the second half of the decade, it was clear that EGb 761® does not just act by enhancing blood flow and that it is not only patients with vascular disorders who benefit from treatment.

With increasing acceptance of the dementia concept in Europe during the 1990s, the dementia syndrome came into the focus of EGb 761® research. Taking into account the evident effects on vascular and nonvascular types of neurocognitive disorders, most of these studies enrolled patients with both types of dementia: Alzheimer's disease and vascular dementia. This was not generally accepted in the field, but accumulating data from neuropathology studies in the late 1990s and 2000s now strongly endorse this approach. A closer look at the neuropathology revealed that most patients with dementia have mixed pathology, above all Alzheimer's pathology associated with cerebrovascular disease (Snowdon et al. 1997; Neuropathology Group of the MRC CFAS 2001; Schneider et al. 2007). As a consequence of the clinical concept of dementia, which requires clear-cut impairment in memory and at least one further domain of cognition together with a significant impact on the abilities to cope with the demands of daily living and that usually encompasses neuropsychiatric symptoms, such as apathy, depression, anxiety, restlessness, and aberrant nighttime behavior, clinical trials with EGb 761® addressed a broad range of symptoms and ailments.

Today, the evidence for the efficacy of EGb 761® in the treatment of dementia syndromes has accumulated from a series of randomized, placebo-controlled, double-blind studies (e.g., Kanowski et al. 1996; Le Bars et al. 1997; Napryeyenko et al. 2007; Ihl et al. 2011; Herrschaft et al. 2012). Systematic reviews and meta-analyses corroborated the benefits patients experience from EGb 761® treatment (IQWiG 2008; Kasper and Schubert 2009; Weinmann et al. 2010; Wang et al. 2010). EGb 761® not only improves memory and concentration but also enables patients with dementia to cope better with the demands of everyday life and alleviates neuropsychiatric symptoms (Fig. 11.12). The latest studies also addressed quality of life, which, in fact, was improved by EGb 761® (Ihl et al. 2011; Herrschaft et al. 2012). Furthermore, caregivers of EGb 761®-treated patients felt significant relief from the distress that had been caused by the patients' aberrant behavior.

After many years of research it is evident now that, due to advanced neurodegeneration, it is impossible to cure manifest dementia and treatment effects remain limited. As a consequence, scientists are now turning to the early and preclinical stages of dementing illnesses. To prevent or at least delay the onset of dementia is the goal of future research efforts. In our current understanding, age-associated decline of cognitive abilities, foremost episodic memory, working memory, and speed-related functions like choice reaction time, reflect pathologic changes of brain structure and function (Wilson et al. 2010). These alterations slowly progress over decades, starting from the high-performance mid-twenties and eventually lead to dementia (Jack et al. 2010). Subjective memory impairment and

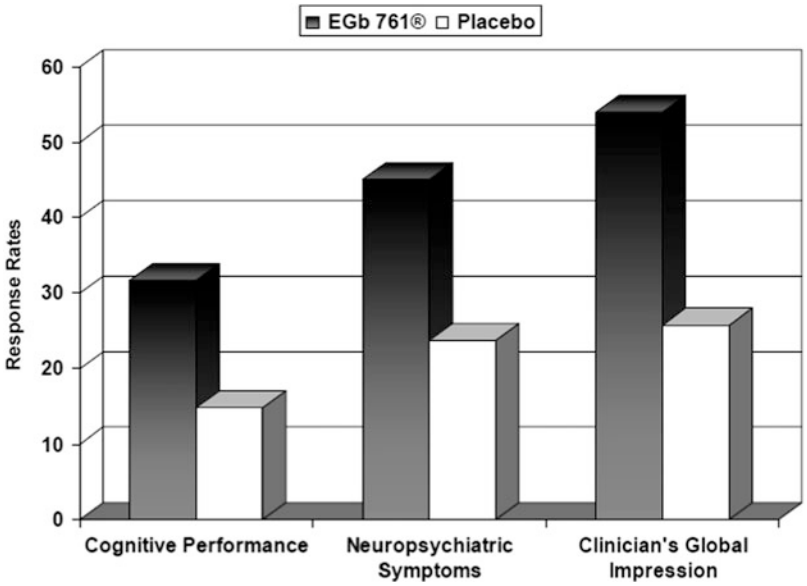


Fig. 11.12 Rates of clinically relevant improvements found in a clinical trial in patients with mild to moderate dementia (Ihl et al. 2011); clinically relevant response was defined as improvement in cognitive performance by at least 3 points in the SKT cognitive battery, improvement in neuropsychiatric symptoms by at least 4 points on the Neuropsychiatric Inventory, and any improvement in the Clinician's Global Impression of Change

mild cognitive decline have been identified as important indicators of an incipient dementia process (Jessen et al. 2010; Bennett et al. 2002). Therefore treatments that alleviate mild cognitive impairment can provide significant benefits to affected individuals and a major research effort is directed to interventions that slow cognitive decline. In a randomized, placebo-controlled trial, EGb 761® improved cognitive function, including attention and memory, in patients with mild cognitive impairment, with benefits visible even in those with very mild impairment (Grass-Kapanke et al. 2011). In middle-aged subjects who spent the majority of their office hours on computer work, performance in a computerized test of continuous attention was improved while the perceived stress level was decreased by EGb 761® treatment (Kaschel et al. 2007). While trial designs feasible to assess drug effects in the prevention of dementia are still under development (Aisen et al. 2011), encouraging findings from a study suggesting that EGb 761® may prevent or delay dementia in a proportion of people at risk after long-term treatment were presented recently (Vellas et al. 2010).

Based on scientific evidence from methodologically sound clinical trials, EGb 761® is now registered in many countries for the treatment of dementia or milder forms of aging-associated cognitive impairment.

11.4.4 Neurosensory Symptoms

Neurosensory problems, such as vertigo and tinnitus, are often associated with decreased perfusion of the inner ear or vestibular and auditory tracts and areas of the brain. In five randomized, placebo-controlled, double-blind trials recently reviewed by Hamann (2007), EGb 761® treatment was found effective in vestibular as well as in non-vestibular vertigo. The Ginkgo extract not only alleviated the severity of vertigo symptoms but also decreased the lateral sway amplitude in tests of equilibrium control. Likewise, EGb 761® attenuated the severity of dizziness associated with neurodegeneration in dementia disorders (Napryeyenko et al. 2007; Ihl et al. 2011). A decrease in tinnitus intensity could be demonstrated by three randomized, placebo-controlled, double-blind trials of EGb 761® (Meyer 1986; Morgenstern and Biermann 1997, 2002) in patients with tinnitus as a major complaint. Likewise, symptoms of tinnitus associated with dementia (Napryeyenko et al. 2007; Ihl et al. 2011) or aging-associated cognitive decline (Halama et al. 1988) were alleviated by the same extract. In Germany and numerous other countries, EGb 761® has therefore been registered for the treatment of vertigo and tinnitus.

11.4.5 Safety and Tolerability

Placebo-controlled clinical trials in thousands of patients as well as abundant use in daily practice for decades have yielded an extensive and excellent safety record for EGb 761®. In large trials, adverse events were not more frequent in subjects treated with EGb 761®, even at the high dose of 240 mg/day, than in those receiving placebo (e.g., Schneider et al. 2005; Napryeyenko et al. 2007; De Kosky et al. 2008; Ihl et al. 2011). Mild gastrointestinal symptoms, allergic skin reactions or headache may occur occasionally in the context of EGb 761® treatment. Bleeding from single organs has been reported in temporal relationship with the intake of Ginkgo preparations, mostly products of unknown quality or multi-ingredient products, and often during concomitant treatment with aspirin or anticoagulants. In specific trials of the high-quality extract EGb 761®, neither an influence on blood coagulation or platelet function (Kellermann and Kloft 2011) nor an enhancement of the activity of aspirin or anticoagulants could be detected. This is in line with findings from large placebo-controlled trials in which events of bleeding were no more frequent in EGb 761®-treated subjects than in those taking placebo (Napryeyenko et al. 2007; De Kosky et al. 2008).

Caution is however warranted when it comes to Ginkgo products that are of unknown quality and extracts that are not produced in accordance with international quality standards of drug manufacturing. Such extracts, which may be used for uncontrolled dietary supplements, may contain harmful substances which are present in Ginkgo leaves, but which are removed during the particular multistage extraction

process that results in the special extract EGb 761®. Since extracts produced by different manufacturing processes may differ considerably with respect to their chemical constituents, the efficacy and safety cannot be assumed to be consistent for all extracts, but must be demonstrated for each individual extract separately.

11.5 Overall Conclusion

In summary, the example of EGb 761® shows how a plant species that has played a certain role in Chinese culture and TCM can become the source material for the science-based development of a Western medicine.

With the move to the Western world, *Ginkgo biloba* started its career outside the healing system of TCM. Accruing knowledge from scientific research on the leaf extract EGb 761® opened formerly unknown areas of medicinal use. The example of this unique extract demonstrates how a HMP—which has its roots in TCM—may be developed to meet highest pharmaceutical quality standards, to be subjected to preclinical and clinical research in accordance with Western rules and guidelines, and to meet the requirements for regulatory approval as a drug. The example of EGb 761® may thus encourage manufacturers and researchers to embark on developing further agents that are used successfully in TCM along a similar scientifically based drug development process.

References

Abdel-Kader RM, Hauptmann S, Keil U, Scherping I, Leuner K, Eckert A, Mueller WE (2007) Stabilization of mitochondrial function by Ginkgo biloba extract (EGb 761). Pharmacol Res 56(6):493–502

Aisen PS, Andrieu S, Sampaio C, Carrillo M, Khatchaturian ZS, Dubois B, Feldman HH, Petersen RC, Siemers E, Doody RS, Hendrix SB, Grundman M, Schneider LS, Schindler RJ, Salmon E, Potter WZ, Thomas RG, Salmon D, Donohue M, Bednar MM, Touchon J, Vellas B (2011) Report of the task force on designing clinical trials in early (predementia) AD. Neurology 76:280–286

Aldridge WN, Street BW, Skilleter DN (1977) Oxidative phosphorylation. Halide-dependent and halide-independent effects of triorganotin and trioganolead compounds on mitochondrial functions. Biochem J 168:353–64

Arenz A, Klein M, Fiehe K, Gross J, Drewke C, Hemscheidt T, Leistner E (1996) Occurrence and biosynthesis of neurotoxic 4'-O-methylpyridoxine in *Ginkgo biloba* [Abstr. SL 20], 44th Annu. Congr. Med. Plant Res., Prague, CZ, 3.-7.9.1996, p 25

Arenz A, Klein M, Fiehe K, Gross J, Drewke C, Hemscheidt T, Eckhard L (1996b) Occurrence of neurotoxic 4'-O-methylpyridoxine in *Ginkgo biloba* leaves, Ginkgo medications and Japanese Ginkgo food. Planta Med 62(6):548–551

Auguet M, Clostre F, De Feudis FV (1986) Effects of antidepressants on receptor-activated and Ca2+-activated contractions of rabbit isolated aorta. Gen Pharmacol 17:607–610

Augustin S, Rimbach G, Augustin K, Schliebs R, Wolffram S, Cermak R (2009) Effect of a short- and long-treatment with *Ginkgo biloba* extract on amyloid precursor protein levels in a transgenic mouse model relevant to Alzheimer's disease. Arch Biochem Biophys 481:177–182

Ahlemeyer B, Krieglstein J (2003) Pharmacological studies supporting the therapeutic use of *Ginkgo biloba* extract for Alzheimer's disease. Pharmacopsychiatry 36(Suppl. 1): S8–S14

Barkats M, Venault P, Christen Y, Cohen-Salmon C (1995) Effect of long-term treatment with EGb 761® on age-dependent structural changes in the hippocampi of three inbred mouse strains. Life Sci 56:213–222

Barth SA, Inselmann G, Engemann R, Heidemann HT (1991) Influences of *Ginkgo biloba* on cyclosporin A induced lipid peroxidation in human liver microsomes comparison to vitamin E, glutathione and N-acetylcysteine. Biochem Pharmacol 41:1521–1526

Bastianetto S, Ramassamy C, Doré S, Christen Y, Poirier J, Quirion R (2000) The *Ginkgo biloba* extract (EGb 761®) protects hippocampal neurons against cell death induced by beta-amyloid. Eur J Neurosci 12:1882–90

Bennett DA, Wilson RS, Schneider JA, Evans DA, Beckett LA, Aggarwal NT, Barnes LL, Fox JH, Bach J (2002) Natural history of mild cognitive impairment in older persons. Neurology 59:198–205

Biber A, Koch E (1999) Bioavailability of ginkgolides and bilobalide from extracts of *Ginkgo biloba* using GC/MS. Planta Med 65:192–193

Biber A (2003) Pharmacokinetics of *Ginkgo biloba* extracts. Pharmacopsychiatry 36(Suppl. 1): S32–S37

Blecharz-Klin K, Piechal A, Joniec I, Pyrzanowska J, Widy-Tyszkiewicz E (2009) Pharmacological and biochemical effects of *Ginkgo biloba* extract on learning, memory consolidation and motor activity in old rats. Acta Neurobiol Exp 69:217–231

Blume J, Kieser M, Hölscher U (1996) Placebo-controlled double blind study on the efficacy of *Ginkgo biloba* special extract EGb 761® in maximum level trained patients with intermittent claudication (in German). Vasa 25:265–274

Blumenthal M, Busse WR, Goldberg A, Gruenwald J, Hall T, Riggins CW, Rister RS, Klein S (1998) The complete German commission E monographs therapeutic guide to herbal medicines developed by a special expert Committee of the German Federal Institute for Drugs and Medical Devices. Am. Bot. Counc., Austin, TX, USA

Boulie R, Munoz JF, Macovshi Panes O, Pacheco H (1988) Implication de la peroxidation lipidique dans l'intoxication par le triethyletain chez le rat. C R Seances Soc Biol Ses Fil 182:196–201

Braquet P (1987) The ginkgolides: potent platelet-activating factor antagonists' isolated from *Ginkgo biloba* L.: chemistry, pharmacology and clinical applications. Drugs Fut 12:643–699

Bulling B, von Bary S (1991) The treatment of chronic peripheral arterial occlusion disease with physical exercise and *Ginkgo biloba* extract 761 (in German). Medwelt 42:702–708

Chandrasekaran K, Mehrabian Z, Spinnewyn B, Chinopoulos C, Drieu K, Fiskum G (2002) Bilobalide, a component of the *Ginkgo biloba* extract (EGb 761®), protects against neuronal death in global brain ischemia and in glutamate-induced excitotoxicity. Cell Mol Biol 48:663–669

Chatterjee SS (1985) Effect of Ginkgo biloba extract on cerebral metabolic processes. In: Agnoli A, Rapin JR, Scapagnini V, Weibrecht WV (eds) Effect of *Ginkgo biloba* extract on organic cerebral impairment. John Libbey Eurotext, London, pp 5–15

Chatterjee SS, Gabard BL (1981) Protective effect of an extract of *Ginkgo biloba* and other hydroxyl radical scavengers against hypoxia [Abstr. 866]. IUPHAR 8th Int. Congr. Pharmacol. Tokyo, JP 19.7.1981–24.7.1981, p 483

Chatterjee SS, Gabard BL (1982a) Studies on the mechanism of action of an extract of *Ginkgo biloba*, a drug used for treatment of ischemic vascular diseases [Abstr. 207]. Naunyn Schmiedeberg's Arch Pharmacol 321(suppl):R52

Chatterjee SS, Gabard BL (1982b) Protection of doxorubicin toxicity by an extract of *Ginkgo biloba* [Abstr. 57]. Naunyn Schmiedeberg's Arch Pharmacol 319(suppl):R15

Chatterjee SS, Gabard BL (1984) Effect of an extract of *Ginkgo biloba* on experimental neurotoxicities [Abstr. 327]. Naunyn Schmiedeberg's Arch Pharmacol 325(suppl):R82

Chatterjee SS, Noeldner M (1989) Behavioural observations demonstrating influences of the extract of *Ginkgo biloba* (EGb 761®) on some specific central cholinergic systems [Abstr. 425]. Naunyn Schmiedeberg's Arch Pharmacol 339(suppl):R107

Chatterjee SS, Cees J, Doelman A, Nöldner M, Biber A, Koch E (2005) Influence of the Ginkgo extract EGb 761® on rat liver cytochrome P450 and metabolism and excretion in rats and man. J Pharm Pharmacol 57:1–10

Christen Y (2004) *Ginkgo biloba* and neurodegenerative disorders. Front Biosci 9:3091–3104

Christen Y (2010) Does *Ginkgo biloba* extract exert an effect on Alzheimer's disease progression? [Chap. 17]. In: Micronutrients and brain health. Taylor & Francis, Boca Raton, FL, USA, Oxid Stress Dis 26:243–254

Cohen-Salmon C, Venault P, Martin B, Raffalli-Sébille MJ, Barkats M, Clostre F, Pardon MC, Christen Y, Chapouthier G (1997) Effects of *Ginkgo biloba* extract (EGb 761®) on learning and possible actions on aging. J Physiol Paris 91:291–300

Colciaghi F, Borroni B, Zimmermann M, Bellone C, Longhi A, Padovani A, Cattabeni F, Christen Y, Di Luca M (2004) Amyloid precursor protein metabolism is regulated toward alpha-secretase pathway by *Ginkgo biloba* extracts. Neurobiol Dis 16:454–460

Continella G, Drago F (1984) Behavioral effect of *Ginkgo biloba* extract. In: Agnoli A, Rapin JR, Scapagnini V, Weitbrecht WV (eds) Effects of *Ginkgo biloba*-extract on organic cerebral impairment. John Libbey, London, pp 35–42

De Feudis FV (2003) Ginkgo biloba extract, from chemistry to the clinic. Urban & Fischer, Munich

De Kosky ST, Williamson J, Fitzpatrick A, Kronmal RA, Ives DG, Saxton J, Lopez O, Burke G, Carlson M, Fried L, Kuller LH, Robbins J, Tracy RP, Woolard NF, Dunn L, Snitz BE, Nahin RL, Furberg CD, for the GEM Study Investigators (2008) *Ginkgo biloba* for primary prevention of dementia: results of the Ginkgo evaluation of memory (GEM) study. JAMA 300:2253–2262

De Marchi U, Campello S, Szabò I, Tombola F, Martinou JC, Zoratti M (2004) Bax does not directly participate in the Ca(2+)-induced permeability transition of isolated mitochondira. J Biol Chem 279:37415–22

Dorman DC, Cote LM, Buck WB (1992) Effects of an extract of *Ginkgo biloba* on bromethalin-induced cerebral lipid peroxidation and edema in rats. Am J Vet Res 53:138–142

Dumont E, Petit E, Tarrade T, Nouvelot A (1992) UV-C irradiation-induced peroxidative degradation of microsomal fatty acids and proteins: protection by an extract of *Ginkgo biloba* (EGb 761®). Free Radic Biol Med 13:197–203

Eckert A, Keil U, Scherping I, Hauptmann S, Mueller WE (2005) Stabilization of mitochondrial membrane potential and improvement of neuronal energy metabolism by *Ginkgo biloba* extract EGb 761®. Ann N Y Acad Sci 1056:474–485

EMEA/CHMP/SWP/431994 (2007) Guideline on the limits of genotoxic impurities

Emerit I, Arutyunyan R, Oganesian N, Levy A, Cernjavsky L, Sarkisian T, Pogossian A, Asrian K (1995) Radiation-induced clastogenic factors: anticlastogenic effect of *Ginkgo biloba* extract. Free Radic Biol Med 18:985–991

Erdinçler DS, Karakoç Y, Toplan S, Önen S, Sukyasyan A, Beğer T, Demiroğlu Ç (1996) The effect of *Ginkgo biloba* glycoside on the blood viscosity and erythrocyte deformability. Clin Hemorheol 16:271–276

ESCOP (2003) *Ginkgo biloba*, Euro. Sci. Cooperative Phytother. (ESCOP). Thieme, Stuttgart

Fehske CJ, Leuner K, Müller WE (2009) *Ginkgo biloba* extract (EGb 761®) influences monoaminergic neurotransmission via inhibition of NE uptake, but not MAO activity after chronic treatment. Pharmacol Res 60:68–73

Fitzl G, Welt K, Martin R, Dettmer D, Hermsdorf T, Clemens N, Konig S (2000) The influence of hypoxia on the myocardium of experimentally diabetic rats with and without protection by

Ginkgo biloba extract. I. Ultrastructural and biochemical investigations on cardiomyocytes. Exp Toxicol Pathol 52:419–30

Fourtillan JB, Brisson AM, Girault J, Ingrand I, Decourt JP, Drieu K, Jouenne P, Biber A (1995) Proprietes pharmacocinetiques du Bilobalide et des Ginkgolides A et B chez le sujet sain apres administrations intraveneuses et orals d'extrait de Ginkgo biloba (EGb-761®) [Pharmacokinetics of bilobalide, Ginkgolide A and Ginkgolide B in healthy volunteers following oral and intravenous administrations of *Ginkgo biloba* extract (EGb 761®)]. Therapie 50(2):137–144

Fransen HP (2010) Assessment of health claims, content, and safety of herbal supplements containing *Ginkgo biloba*. Food Nutr Res 54:5221

Garcia-Alloza M, Dodwell SA, Meyer-Luehmann M, Hyman BT, Bacskai BJ (2006) Plaque-derived oxidative stress mediates distorted neurite trajectories in the Alzheimer mouse model. J Neuropathol Exp Neurol 65:1082–9

Gawron-Gzella A, Marek P, Chanay J, Matlawska I (2010) Comparative analysis of aharmaceuticals and dietary supplements containing extracts from the leaves of *Ginkgo biloba* L. Acta Pol Pharm 67(4):335–43

Grass-Kapanke B, Busmane A, Lasmanis A, Hoerr R, Kaschel R (2011) Effects of *Ginkgo biloba* special extract EGb 761® in very mild cognitive impairment (vMCI). Neurosci Med 2:48–56

Halama P, Bartsch G, Meng G (1988) Cerebrovascular insufficiency – placebo-controlled, randomised, doubleblind trial on the effect of *Ginkgo biloba* extract (in German). Fortschr Med 106:408–412

Hamann KF (2007) Ginkgo special extract EGb 761® in vertigo: a systematic review of randomised, double-blind, placebo-controlled clinical trials. Internet J Otorhinolaryngol 6(2)

Heiss WD, Zeiler K (1978) Medikamentöse Beeinflussung der Hirndurchblutung. Pharmakotherapie 1:137–144

Herrschaft H, Nacu A, Likhachev S, Sholomov I, Hoerr R, Schlaefke S, on behalf of the GOT-IT! Study Group (2012) *Ginkgo biloba* extract EGb $761^{(R)}$ in dementia with neuropsychiatric features: a randomised, placebo-controlled trial to confirm the efficacy and safety of a daily dose of 240 mg. J Psychiatr Res 46:716–723

Hofferberth B (1989) Effect of *Ginkgo biloba* extract on neurophysiological and psychometric measurement results in patients with cerebro-organic syndrome – a double-blind study versus placebo (in German). Arzneimittelforschung 39:918–922

Hofferberth B (1991) Simultanerfassung elektrophysiologischer, psychometrischer und rheologischer Parameter bei Patienten mit hirnorganischem Psychosyndrom und erhöhtem Gefäßrisiko – Eine placebokontrollierte Doppelblindstudie mit Ginkgo-biloba-Extrakt EGb 761®. In: Stodtmeister R, Pillunat LE (eds) Mikrozirkulation in Gehirn und Sinnesorganen. Enke, Stuttgart, pp 64–74

Horsch S, Walther C (2004) *Ginkgo biloba* special extract EGb 761® in the treatment of peripheral arterial occlusive disease (PAOD) – a review based on randomized, controlled studies. Int J Clin Pharmacol Ther 42:63–72

Hoyer S, Lannert H, Noeldner M, Chatterjee SS (1999) Damaged neuronal energy metabolism and behavior are improved by *Ginkgo biloba* extract (EGb 761®). J Neural Transm 106:1171–1188

Hsu CL, Wu YL, Tang GJ, Lee TS, Kou YR (2009) *Ginkgo biloba* extract confers protection from cigarette smoke extract-induced apoptosis in human lung endothelial cells: role of heme oxygenase-1. Pulm Pharmacol Ther 22:286–296

Huang SH, Duke RK, Chebib M, Sasaki K, Wada K, Johnston GA (2003) Bilobalide, a sesquiterpene trilactone from *Ginkgo biloba*, is an antagonist at recombinant alpha1beta2gamma2L GABA(A) receptors. Eur J Pharmacol 464:1–8

Huguet F, Tarrade T (1992) Alpha2-adrenoceptor changes during cerebral ageing: the effect of *Ginkgo biloba* extract. J Pharm Pharmacol 44:24–27

Huguet F, Drieu K, Piriou A (1994) Decreased cerebral 5-HT1A receptors during ageing: reversal by *Ginkgo biloba* extract EGb 761®). J Pharm Pharmacol 46:316–318

Ihl R, Bachinskaya N, Korczyn AD, Vakhapova V, Tribanek M, Hoerr R, Napryeyenko O, on behalf of the GOTADAY Study Group (2011) Efficacy and safety of a once-daily formulation

of *Ginkgo biloba* extract EGb 761® in dementia with neuropsychiatric features – a randomized controlled trial. Int J Geriatr Psychiatry 26:1186–1194

IQWiG Institut für Qualität und Wirtschaftlichkeit im Gesundheitswesen (2008) Ginkgohaltige Präparate bei Alzheimer Demenz – Abschlussbericht A05–19B. IQWiG, Köln

Israël L, Dell'Accio E, Martin G, Hugonot R (1987) *Ginkgo biloba* extract and memory training programs – comparative assessment on elderly outpatients living in the community (in French). Psychol Med (Paris) 19:1431–1439

Ivic L, Sands TT, Fishkin N, Nakanishi K, Kriegstein AR, Strømgaard K (2003) Terpene trilactones from *Ginkgo biloba* are antagonists of cortical glycine and GABA(A) receptors. J Biol Chem 278:49279–85

Jack CR, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ (2010) Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet Neurol 9:119–128

Jaggy H, Koch E (1997) Chemistry and biology of alkylphenols from *Ginkgo biloba* L. Pharmazie 52(10):735–738

Janssens D, Michiels C, Delaive E, Eliaers F, Drieu K, Remacle J (1995) Protection of hypoxia-induced ATP decrease in endothelial cells by *Ginkgo biloba* extract and Bilobalide. Biochem Pharmacol 50:991–999

Janssens D, Remacle J, Drieu K, Michiels C (1999) Protection of mitochondrial respiration activity by Bilobalide. Biochem Pharmacol 58:109–119

Janssens D, Delaive E, Remacle J, Michiels C (2000) Protection by bilobalide of the ischaemia-induced alterations of the mitochondrial respiratory activity. Fundam Clin Pharmacol 14:193–201

Jessen F, Wiese B, Bachmann C, Eifflaender-Gorfer S, Haller F, Kölsch H, Luck T, Mösch E, van den Bussche H, Wagner M, Wollny A, Zimmermann T, Pentzek M, Riedel-Heller SG, Romberg HP, Weyerer S, Kaduszkiewicz H, Maier W, Bickel H, for the German Study on Aging, Cognition and Dementia in Primary Care Patients Study Group (2010) Prediction of dementia by subjective memory impairment. Arch Gen Psychiatry 67:414–422

Kampkötter A, Pielarski T, Rohrig R, Timpel C, Chovolou Y, Wätjen W, Kahl R (2007) The *Ginkgo biloba* extract EGb 761® reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*. Pharmacol Res 55:139–47

Kanowski S, Herrmann WM, Stephan K, Wierich W, Hörr R (1996) Proof of efficacy of the *Ginkgo biloba* special extract EGb 761® in outpatients suffering from mild to moderate primary degenerative dementia of the Alzheimer type or multi-infarct dementia. Pharmacopsychiatry 29:47–56

Karcher L, Zagrmann P, Krieglstein J (1984) Effect of an extract of *Ginkgo biloba* on rat brain energy metabolism in hypoxia. Naunyn-Schmiedeberg's Arch Pharmacol 327:31–35

Kaschel R, Hörr R, Kresimon J, Rychlik R (2007) Einfluss von Ginkgo-Spezialextrakt EGb 761® auf die Leistungsfähigkeit bei gesunden Probanden am Bildschirmarbeitsplatz – Offene klinische Studie im Prä-Post-Design mit Kontrollgruppe. Perfusion 16:3–9

Kasper S, Schubert H (2009) *Ginkgo biloba* extract EGb 761® in the treatment of dementia: evidence of efficacy and tolerability (in German). Fortschr Neurol Psychiatr 77:494–506

Keles MS, Demirci N, Yildirim A, Atamanalp SS, Altinkaynak K (2008) Protective effects of N-acetylcysteine and *Ginkgo biloba* extract on ischaemia-reperfusion-induced hepatic DNA damage in rats. Clin Exp Med 8:193–8

Kellermann AJ, Kloft C (2011) Is there a risk of bleeding associated with standardized *Ginkgo biloba* extract therapy? A systematic review and meta-analysis. Pharmacotherapy 31:490–502

Klein J, Chatterjee SS, Loeffelholz K (1995) Hypoxia-induced release of choline from rat brain: potent inhibition by bilobalide. Biol Chem Hoppe-Seyler 376(suppl):S116

Klein J, Chatterjee SS, Loeffelholz K (1997) Phospholipid breakdown and choline release under hypoxic conditions: inhibition by bilobalide, a constituent of *Ginkgo biloba*. Brain Res 755:347–350

Klein J, Weichel O, Hilgert M, Rupp J, Chatterjee SS, Nawrath H (2003) Excitotoxic hippocampal membrane breakdown and its inhibition by bilobalide: role of chloride fluxes. Pharmacopsychiatry 36(suppl 1):78–83

Koch E (2005) Inhibition of platelet activating factor (PAF)-induced aggregation of human thrombocytes by ginkgolides: considerations on possible bleeding complications after oral intake of *Ginkgo biloba* extracts. Phytomedicine 12:10–16

Koch E, Chatterjee SS (1993) Experimentelle Grundlagen fuer die therapeutische Anwendung von Ginkgoextrakt EGb 761® bei der Behandlung vaskulaerer Erkrankungen. Haemostaseologie 13:11–27

Koh PO (2009) *Ginkgo biloba* extract (EGb 761®) prevents increase of Bad-Bcl-X(L) interaction following cerebral ischemia. Am J Chin Med 37:867–876

Koltermann A (2007) Influence of *Ginkgo biloba* extract EGb 761® on signaling pathways in endothelial cells. Dissertation, Pharm Biol Pharm, Ludwig-Maximilians-Universitaet, Muenchen DE. p 155

Költringer P, Langsteger W, Eber O (1995) Dose-dependent hemorheological effects and microcirulatory modifications following intravenous administration of *Ginkgo biloba* special extract EGb 761®. Clin Hemorheol 15:649–656

Krieglstein J, Beck T, Seibert A (1986) Influence of an extract of *Ginkgo biloba* on cerebral blood flow and metabolism. Life Sci 39:2327–34

Krieglstein J, Ausmeier F, El-Abhar H, Lippert K, Welsch M, Rupalla K, Henrich-Noack P (1995) Neuroprotective effects of *Ginkgo biloba* constituents. Eur J Pharm Sci 3:39–48

Kristofikova Z, Benesova O, Tejkalova H (1992) Changes of high-affinity choline uptake in the hippocampus of old rats after long-term administration of two nootropic drugs (Tacrine and *Ginkgo biloba* extract). Dementia 3:304–307

Krzywon M, Tawab M, Schubert-Zsilavecz M (2008) Ginkgohaltige Teeprodukte nicht ohne Risiko. Pharm Ztg 153(13):4474–4479

Le Bars PL, Katz MM, Berman N, Itil TM, Freedman AM, Schatzberg AF, for the North American EGb Study Group (1997) A placebo-controlled, double-blind randomized trial of an extract of *Ginkgo biloba* for dementia. JAMA 278:1327–1332

Le Poncin-Lafitte M, Rapin J, Rapin JR (1980) Effects of *Ginkgo Biloba* on changes induced by quantitative cerebral microembolization in rats. Arch Int Pharmacodyn Ther 243:236–44

Leistner E, Drewke C (2010a) *Ginkgo biloba* and ginkgotoxin. J Nat Prod 73:86–92

Leistner E, Drewke C (2010b) Arznei- und Nahrungsmittel aus Ginkgo biloba Potenziell toxische Inhaltsstoffe und ihre Wirkmechanismen [Drug and nutrition aspects of *Ginkgo biloba*] Dtsch. Apoth Ztg 150(17):1930–1935

Lin FY, Chen YH, Chen YL, Wu TC, Li CY, Chen JW, Lin SJ (2007) *Ginkgo biloba* extract inhibits endotoxin-induced human aortic smooth muscle cell proliferation via suppression of toll-like receptor 4 expression and NADPH oxidase activation. J Agric Food Chem 55:1977–1984

Liu KX, Wu WK, He W, Liu CL (2007) *Ginkgo biloba* extract (EGb 761®) attenuates lung injury induced by intestinal ischemia/reperfusion in rats: roles of oxidative stress and nitric oxide. World J Gastroenterol 14:299–305

Longpre F, Garneau P, Christen Y, Ramassamy C (2006) Protection by EGb 761® against beta-amyloid-induced neurotoxicity: involvement of NF-kappa B, SIRT1, and MAPKs pathways and inhibition of amyloid fibril formation. Free Radic Biol Med 41:1781–1794

Luo Y, Smith JV, Paramasivam V, Burdick A, Curry KJ, Buford JP, Khan I, Netzer WJ, Xu H, Butko P (2002) Inhibition of amyloid-beta aggregation and caspase-3 activation by the *Ginkgo biloba* extract EGb761. Proc Natl Acad Sci USA 99:12197–202

Mahadevan S, Park Y (2008) Multifaceted therapeutic benefits of *Ginkgo biloba* L.: chemistry, efficacy, safety, and uses. J Food Sci 73:R14–R19

Martin R, Mozet C, Martin H, Welt K, Engel C, Fitzl G. (2010) Effect of Ginkgo biloba extract (EGb 761®) on parameters of oxidative stress in different regions of aging rat brain after acute hypoxia. Aging Clin. Exp. Res. [Epub ahead of print], DOI: 10.3275/7229

Meier B, Hasler A, Sticher O (1992) HPLC analyses of the main Ginkgo acylflavonol glycosides. Planta Med 58(suppl 7):A688–A689

Meyer B (1986) A mulitcentre, randomized, double-blind drug versus placebo study of *Ginkgo biloba* extract in the treatment of tinnitus (in French). Presse Med 15:1562–1564

Mizuno M, Droy-Lefaix MT, Packer L (1996) *Ginkgo biloba* extract EGb 761® is a suppressor of AP-1 transcription factor stimulated by phorbol 12-myristate 13-acetate. Biochem Mol Biol Int 39:395–401

Moeller CK, Kurt S, Scheich H, Schulze H (2009) Improvement of auditory discrimination learning by *Ginkgo biloba* extract EGb 761® (R). Neurosci Lett 463:219–222

Moreau P (1975) Un nouveau stimulant circulatoire cérébral. Nouv Presse Med 4:2401–2402

Moreau J-P, Bommelaer G, Buscail L, Benque A, Jacob CO, Galleyrand J, Ribet (1986) A preliminary results of a multicentric double blind trial of somatostatin [s] vs placebo [p] in akute pancreatitis [ap] [Abstr. 85], 8th Congr. Gastroenterol. (OMGE), 6th Congr. Dig. Endosc. (OMED), 3rd. Congr. Colo-Proctol., Sao Paolo BR, 7.-12.9.1986, Dig Dis Sci 31(10, Suppl.)

Morgenstern C, Biermann E (1997) Ginkgo special extract (EGb 761®) in the treatment of tinnitus aurium – results of a randomized, double-blind, placebo-controlled study (in German). Fortschr Med Orig 115:7–11

Morgenstern C, Biermann E (2002) The efficacy of Ginkgo special extract EGb 761® in patients with tinnitus. Int J Clin Pharmacol Ther 40:188–197

Müller WE, Abdel-Kader RM, Fehske CJ, Leuner K (2009) Grundlagen der therapeutischen Anwendung von EGb 761® (R). Wirkung auf die Kraftwerke der Zellen. Pharm Unserer Zeit 38:408–416

Mußgnug G, Alemany J (1968) Studies on disorders of peripheral arterial circulation. Part XV: problems of conservative therapy of obliterating disorders of peripheral circulation demonstrated on tincture and extract of *Ginkgo biloba* (in German). Arzneimittelforschung 18:453–550

Myers S (2007) The complexities of botanical quality, natural products insider, http://www.naturalproductsinsider.com. Accessed 05 July 2011, Article not accessible)

Napryeyenko O, Borzenko I, for the GINDEM-NP Study Group (2007) *Ginkgo biloba* special extract in dementia with neuropsychiatric features – a randomized, placebo-controlled, double-blind clinical trial. Arzneimittelforschung 57:4–11

Neuropathology Group of the Medical Research Council Cognitive Function and Ageing Study (2001) Pathological correlates of late-onset dementia in a multicentre, community-based population in England and Wales. Lancet 357:169–175

Niizuma K, Yoshioka H, Chen H, Kim GS, Jung JE, Katsu M, Okami N, Chan PH (2010) Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. Biochim Biophys Acta 1802:92–9

Noeldner M, Chatterjee SS (1994) Anxiolytic-like activity of bilobalide [Abstr. 388]. Naunyn Schmiedeberg's Arch Pharmacol 349(suppl):R97

Oberpichler-Schwenk H, Beck T, Abdel-Rahman MM, Bielenberg GW, Krieglstein J (1988) Effects of *Ginkgo biloba* constituents related to protection against brain damage caused by hypoxia. Pharmacol Res Commun 20:349–368

Otani M, Chatterjee SS, Gabard BL, Kreutzberg GW (1986) Effect of an extract of *Ginkgo biloba* on Triethyltin-induced cerebral edema. Acta Neuropathol 69:54–65

Pardon MC, Joubert C, Perez-Diaz F, Christen Y, Launay JM, Cohen-Salmon C (2000) In vivo regulation of cerebral monoamine oxidase activity in senescent controls and chronically stressed mice by long-term treatment with *Ginkgo biloba* extract (EGb 761®). Mech Ageing Dev 113:157–68

Peter H, Fisel J, Weisser W (1966) Zur Pharmakologie der Wirkstoffe aus *Ginkgo biloba*. Arzneimittelforschung 16:719–25

Pietta PG, Gardana C, Mauri PL, Maffei FR, Carini M (1995) Identification of flavonoid metabolites after oral administration to rats of a *Ginkgo biloba* extract. J Chromatogr B Biomed Appl 673(1):75–80

Pincemail J, Deby C (1986) The antiradical properties of *Ginkgo biloba* extract. Presse Med 15(31):1475–1479

Porsolt RD, Martin P, Lenegre A, Fromage S, Drieu K (1990) Effects of an extract of *Ginkgo biloba* (EGb 761® [TANAKAN]) on "learned helplessness" and other models of stress in rodents. Pharmacol Biochem Behav 36:963–971

Rangel-Ordóñez L, Noeldner M, Schubert-Zsilavecz M, Wurglics M (2010) Plasma levels and distribution of flavonoids in rat brain after single and repeated doses of standardized *Ginkgo biloba* extract EGb 761® (R). Planta Med 76(15):1683–1690

Rickard NS, Kowadlo N, Gibbs ME (2001) Effect of the *Ginkgo biloba* extract, EGb 761®, on memory formation in day-old chicks. Pharmacol Biochem Behav 69:351–358

Rimbach G, Cermak R, Wolffram S (2004) Gene regulatory activity of *Ginkgo biloba* L. (Chap. 5). In: Bao Y, Fenwick R (eds) Phytochemicals in health and disease. Marcel Dekker, New York, pp 105–119

Rojas P, Rojas-Castañeda J, Vigueras RM, Rojas C (2000) *Ginkgo biloba* pretreatment partially protects from the dopaminergic neurotoxicity of 1-methyl-4-phenylpyridinium. Proc West Pharmacol Soc 43:87–8

Rojas P, Garduño B, Rojas C, Vigueras RM, Rojas-Castañeda J, Rios C, Serrano-Garcia N (2001) EGb761 blocks MPP+-induced lipid peroxidation in mouse corpus striatum. Neurochem Res 26:1245–51

Rojas P, Serrano-García N, Mares-Sámano JJ, Medina-Campos ON, Pedraza-Chaverri J, Ogren SO (2008) EGb761 protects against nigrostriatal dopaminergic neurotoxicity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinsonism in mice: role of oxidative stress. Eur J Neurosci 28:41–50

Sakakibara H, Ishida K, Grundmann O, Nakajima JI, Seo S, Butterweck V, Minami Y, Saito S, Kawai Y, Nakaya Y, Terao J (2006) Antidepressant effect of extracts from *Ginkgo biloba* leaves in behavioral models. Biol Pharm Bull 29:1767–1770

Saleem S, Zhuang H, Biswal S, Christen Y, Dore S (2008) *Ginkgo Biloba* extract neuroprotective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury. Stroke 39:3389–3396

Sanceario G, Kreutzberg GW (1986) Stimulation of astrocytes affects cytotoxic brain edema. Acta Neuropathol 72:3–14

Saponaro A, Gambelli G, Cosentino F (1971) First results from a clinical and instrumental investigation of the effects of a new phytotherapeutic substance on the cerebral and peripheral arterial circulation (in Italian). Minerva Med 62:4838–4849

Schennen A (1988) Neue Inhaltsstoffe aus den Blaettern von *Ginkgo biloba*. l. sowie Praeparation 14c-markierter Ginkgo-flavonoidepharm Biol. Dissertation, Univ. Marburg

Schindowski K, Leutner S, Kressmann S, Eckert A, Mueller WE (2001) Age-related increase of oxidative stress-induced apoptosis in mice: prevention by *Ginkgo biloba* extract (EGb 761®). J Neural Transm 108:969–978

Schneider LS, DeKosky ST, Farlow MR, Tariot PN, Hoerr R, Kieser M (2005) A randomized, double-blind, placebo-controlled trial of two doses of *Ginkgo biloba* extract in dementia of the Alzheimer's type. Curr Alzheimer Res 2:541–551

Schneider JA, Arvanitakis Z, Bang W, Bennett DA (2007) Mixed brain pathologies account for most dementia cases in community-dwelling older persons. Neurology 69:2197–2204

Scholtyssek H, Damerau W, Wessel R, Schimke I (1997) Antioxidative activity of ginkgolides against superoxide in an aprotic environment. Chem Biol Interact 106:183–190

Schötz K (2002) Detection of allergenic urushiols in *Ginkgo biloba* leaves. Pharmazie 57 (7):508–510

Shah ZA, Nada SE, Doré S (2011) Heme oxygenase 1, beneficial role in permanent ischemic stroke and in Gingko biloba (EGb 761®) neuroprotection. Neuroscience 180:248–255

Shen J, Lee W, Gu Y, Tong Y, Fung PC, Tong L (2011) *Ginkgo biloba* extract EGb 761®) inhibits mitochondria-dependent caspase pathway and prevents apoptosis in hypoxia-reoxygenated cardiomyocytes. Chin Med 6:8

Shi C, Wu F, Yew DT, Xu J, Zhu Y (2010) Bilobalide prevents apoptosis through activation of the PI3K/Akt pathway in SH-SY5Y cells. Apoptosis 15:715–727

Smith JV, Burdick AJ, Golik P, Khan I, Wallace D, Luo Y (2002) Anti-apoptotic properties of *Ginkgo biloba* extract EGb 761® in differentiated PC12 cells. Cell Mol Biol 48:699–707

Snowdon DA, Greiner LH, Mortimer JA, Riley KP, Greiner PA, Markesbery WR (1997) Brain infarction and the clinical expression of Alzheimer disease – The Nun Study. JAMA 277:813–817

Soulie C, Nicole A, Christen Y, Ceballos-Picot I (2002) The *Ginkgo biloba* extract EGb 761® increases viability of hnt human neurons in culture and affects the expression of genes implicated in the stress response. Cell Mol Biol 48:641–646

Spiess E, Juretzek W (2004) Hagers Handbuch der Pharmazeutischen Praxis, CD-Ausg. Hager ROM, Springer, Berlin, Heidelberg *Ginkgo biloba*, HN 2032700

Sram RJ, Binkova B (1993) Side-effects of psychotropic therapy. In: Packer L, Prilipko L, Christen Y (eds) Free radicals in the brain – aging, neurological and mental disorders; Joint Meeting World Health Organization (WHO), Found. IPSEN. Springer, Berlin, pp 153–166

Stackman RW, Eckenstein F, Frei B, Kulhanek D, Nowlin J, Quinn JF (2003) Prevention of age-related spatial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment. Exp Neurol 184:510–20

Sticher O (1992) *Ginkgo biloba* – Analytik und Zubereitungsformen. Pharm Unserer Zeit 21(6):253–265

Stoll S, Scheuer K, Pohl O, Mueller WE (1996) *Ginkgo biloba* extract (EGb-761) independently improves changes in passive avoidance learning and brain membrane fluidity in the aging mouse. Pharmacopsychiatry 29:144–149

Su SY, Hsieh CL, Wu SL, Cheng WY, Li CC, Lo HY, Ho TY, Hsiang CY (2009) Transcriptomic analysis of EGb 761®-regulated neuroactive receptor pathway in vivo. J Ethnopharmacol 123:68–73

Taillandier J, Ammar A, Rabourdin JP, Ribeyre JP, Pichon J, Niddam S, Pierart H (1986) *Ginkgo biloba* extract in the treatment of cerebral disorders due to ageing – a longitudinal, multicentric, double-blind, drug versus placebo trial (in French). Presse Med 15:1583–1587

Tawab M, Krzywon M, Schubert-Zsilavecz M (2010) Nahrungsergänzungs-mittel mit Ginkgo unter der Lupe. Pharm Ztg 155(20):1994–1999

Tchantchou F, Xu Y, Wu Y, Christen Y, Luo Y (2007) EGb 761® enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer's disease. FASEB J 21:2400–2408

Tendi EA, Bosetti F, Dasgupta SF, Stella AM, Drieu K, Rapoport SI (2002) *Ginkgo biloba* extracts EGb 761® and bilobalide increase NADH dehydrogenase mRNA level and mitochondrial respiratory control ratio in PC12 cells. Neurochem Res 27:319–323

Ude C, Paulke A, Noeldner M, Schubert-Zsilavecz M, Wurglics M (2011) Plasma and brain levels of terpene trilactones in rats after an oral single dose of standardized *Ginkgo biloba* extract EGb 761® (R). Planta Med 77(3):259–264

Upton R, (2003) Ginkgo leaf Ginkgo leaf dry extract *Ginkog biloba* L: standards of analysis, quality control and therapeutics. American Herbal Pharmacopoeia and Therapeutic Compendium, Santa Cruz, CA, USA

Uríková A, Babusíková E, Dobrota D, Drgová A, Kaplán P, Tatarková Z, Lehotský J (2006) Impact of *Ginkgo biloba* Extract EGb 761® on ischemia/reperfusion-induced oxidative stress products formation in rat forebrain. Cell Mol Neurobiol 26:1343–53

van Beek TA (ed) (2000) *Ginkgo biloba*. Harwood Academic Publishers, Amsterdam

van Beek TA, Montoro P (2009) Chemical analysis and Quality control of *Ginkgo biloba* leaves, extracts and phytopharmaceuticals. J Chromatogr A 1216(11):2002–2032

Vellas B, Coley N, Ousset PJ, Berrut G, Dartigues JF, Dubois B, Grandjean H, Pasquier F, Piette F, Robert P, Touchon J, Garnier P, Mathiex-Fortunet H, Andrieu S, for the GuidAge Study Group (2010) Results of GuidAge – a 5-year placebo-controlled study of the efficacy of EGb 761® 120 mg to prevent or delay Alzheimer's dementia onset in elderly subjects with memory complaint. J Nutr Health Aging 14(suppl 2):S23

Walesiuk A, Trofimiuk E, Braszko JJ (2005) *Gingko biloba* extract diminishes stress-induced memory deficits in rats. Pharmacol Rep 57:176–87

Watanabe CMH, Wolffram S, Ader P, Rimbach G, Packer L, Maguire JJ, Schultz PG, Gohil K (2001) The in vivo neuromodulatory effects of the herbal medicine *Ginkgo biloba*. Proc Natl Acad Sci USA 98:6577–6580

Wang BS, Wang H, Song YY, Qi H, Rong ZX, Wang BS, Zhang L, Chen HZ (2010) Effectiveness of standardized *Ginkgo biloba* extract on cognitive symptoms of dementia with a six-month treqatment: a bivariate random effect meta-analysis. Pharmacopsychiatry 43:86–91

Weichel O, Hilgert M, Chatterjee SS, Lehr M, Klein J (1999) Bilobalide, a constituent of *Ginkgo biloba*, inhibits NMDA-induced phospholipase A2 activation and phospholipid breakdown in rat hippocampus. Naunyn Schmiedebergs Arch Pharmacol 360:609–15

Weinmann S, Roll S, Schwarzbach C (2010) Effects of *Ginkgo biloba* in dementia: systematic review and meta-analysis. BMC Geriatr 10(14)

Weitbrecht WU, Jansen W (1986) *Ginkgo biloba* extract in the treatment of primary degenerative dementia – placebo-controlled, double-blind and comparative study (in German). Fortschr Med 104:199–202

Wesnes K, Simmons D, Rook M, Simpson P (1987) A double-blind placebo-controlled trial of Tanakan in the treatment of idiopathic cognitive impairment in the elderly. Hum Psychopharmacol 2:159–169

Wilson RS, Leurgans SE, Boyle PA, Schneider JA, Bennett DA (2010) Neurodegenerative basis of age-related cognitive decline. Neurology 75:1070–1078

Winter E (1991) Effects of an extract of *Ginkgo biloba* on learning and memory in mice. Pharmacol Biochem Behav 38:109–14

Winter JC (1998) The effects of an extract of *Ginkgo biloba*, EGb 761®, on cognitive behavior and longevity in the rat. Physiol Behav 63:425–33

Wirth S, Stemmelin J, Will B, Christen Y, Di Scala G (2000) Facilitative effects of EGb 761® on olfactory recognition in young and aged rats. Pharmacol Biochem Behav 65:321–6

Wu Y, Wu Z, Butko P, Christen Y, Lambert MP, Klein WL, Link CD, Luo Y (2006) Amyloid-beta-induced pathological behaviors are suppressed by *Ginkgo biloba* extract EGb 761® and ginkgolides in transgenic *Caenorhabditis elegans*. J Neurosci 26:13102–13113

Yan FL, Zheng Y, Zhao FD (2008) Effects of *Ginkgo biloba* extract EGb-761 on expression of RAGE and LRP-1 in cerebral microvascular endothelial cells under chronic hypoxia and hypoglycemia. Acta Neuropathol 116:529–535

Yeh YC, Liu TJ, Wang LC, Lee HW, Ting CT, Lee WL, Hung CJ, Wang KY, Lai HC, Lai HC (2009) A standardized extract of *Ginkgo biloba* suppresses doxorubicin-induced oxidative stress and p53-mediated mitochondrial apoptosis in rat testes. Br J Pharmacol 156:48–61

Yoshitake T, Yoshitake S, Kehr J (2010) The *Ginkgo biloba* extract EGb 761® and its main constituent flavonoids and ginkgolides increase extracellular dopamine levels in the rat prefrontal cortex. Br J Pharmacol 159:659–68

Zhuang H, Pin S, Christen Y, Dore S (2002) Induction of heme oxygenase 1 by *Ginkgo biloba* in neuronal cultures and potential implications in ischemia. Cell Mol Biol 48:647–653

Zou W, Kim BO, Zhou BY, Liu Y, Messing A, He JJ (2007) Protection against human immunodeficiency virus type 1 Tat neurotoxicity by *Ginkgo biloba* extract EGb 761® involving glial fibrillary acidic protein. Am J Pathol 171:1923–35

Chapter 12
Ginkgolides and Their Derivatives: Synthetic and Bioorganic Studies

Sergei V. Dzyuba, Laramie P. Jameson, and Koji Nakanishi

12.1 Introduction

The effect of the extract from *Ginkgo biloba* tree has been investigated in relation to an extended range of disorders and diseases. Commercial preparations of the extract, over-the-counter dietary supplements, or prescription products worldwide have long been advocated for their ability to improve various cognitive functions. Several extensive reviews on the chemistry, therapeutic effect, and the neuropsychological efficacy of the *Ginkgo biloba* extract have been published in recent years (Abad et al. 2010; Mahadevan and Park 2008; Crews et al. 2005) and they will not be discussed here. Although the exact molecular mechanisms of *Ginkgo biloba* extracts' action remain unclear, the neuromodulatory activity has been attributed to the so-called terpene trilactone fractions. The synthetic and biological aspects of the compounds that comprise the terpene trilactone fraction will be presented here.

12.2 Structure of Ginkgolides and Their Isolation from the *Ginkgo biloba* Extract

Ginkgo biloba extract contains a variety of phytochemicals, with the main components being flavonoids and terpernoids, primarily terpene trilactones. The terpene trilactone fraction can be further divided into ginkgolides and bilobalide. Ginkgolides are diterpenes with a cage-like skeleton consisting of six 5-membered

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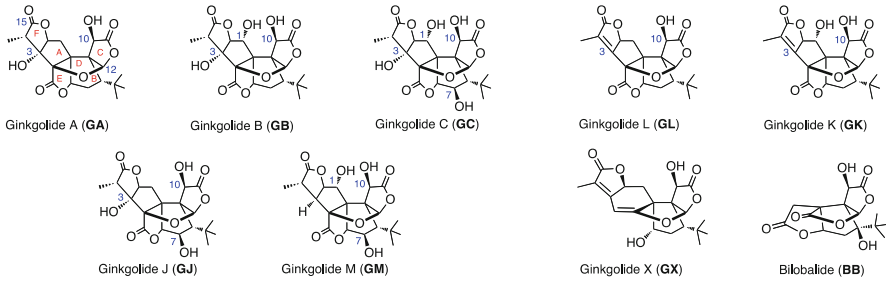


Fig. 12.1 Structures of ginkgolides and bilobalide from *Ginkgo biloba* L.

rings, including a spiro[4.4]nonane carbocyclic ring, three lactones, and a tetrahydrofuran moiety (Fig. 12.1). The number and position of hydroxy groups around the skeleton provide the differentiation among ginkgolides.

All ginkgolides have a hydroxy group at C-10 (Fig. 12.1). Ginkgolide A (**GA**) has only two OH functionalities that are located at C-3 and C-10. In ginkgolide B (**GB**) an additional OH group at C-1 is present. Ginkgolide C (**GC**) is the most oxygenated ginkgolide with hydroxy groups at 1, 3, 7, and 10 positions. Ginkgolide J (**GJ**) is isomeric to **GB**. Ginkgolide M (**GM**) lacks the tertiary hydroxy group at the C-3 position. Ginkgolide L (**GL**) and ginkgolide K (**GK**) can be viewed as dehydrated versions of **GA** and **GB**, respectively. **GX**, which was first reported in 2002, is the most distinct compound in the set of ginkgolides, and it was isolated from large waste accumulation during the production of the *Ginkgo biloba* extract (Jensen et al. 2010). Unlike other ginkgolides, **GX** only contains two lactone groups and features an unsaturated six-membered ring. The lack of hydroxy groups in the 1 and 7 positions might indicate a relationship of **GX** to **GA**.

GA, **GB**, **GC**, and **GM** were initially isolated and separated from the root bark of five *Ginkgo biloba* trees, which were damaged by a typhoon in 1967 (Nakanishi 2005). Their structures were deduced by a combination of chemical and spectroscopic approaches (Maruyama et al. 1967a, b, c, d; Woods et al. 1967). **GJ** was isolated in 1987 from the *Ginkgo biloba* leaves (Weinges et al. 1987a).

The sesquiterpenoid bilobalide (**BB**) is related to ginkgolides by the presence of three lactone rings, one of which contains an α-OH, and the tert-butyl group (Fig. 12.1). Distinctions are made by the absence of the rings A and F, and the tetrahydrofuran ring found on the ginkgolide skeleton is replaced by a lactone. The tert-butyl group found in **BB** (and in ginkgolides) is very rare for terrestrial natural products, but it is more common in marine natural products (Bisel et al. 2008). Although **BB** exhibits a spectrum of biological activities (Strømgaard and Nakanishi 2004), from the chemical point of view, **BB** is much more labile than the ginkgolides, and as a result very few derivatives have been prepared (Nakanishi et al. 1971; Weinges and Bäehr 1972; Weinges et al. 1987b). Therefore, the synthetic and bioorganic aspects of **BB** will not be discussed here.

Several spectroscopic (van Beek et al. 1993; Li et al. 2004), spectrometric (Lang and Wai 1999), and chromatographic (van Beek 2005) methods for quantification

of terpene trilactones in the extract have been developed. However, for the evaluation of the biological activities of ginkgolides and for the preparation of the required derivatives, an access to large quantities of pure compounds is required. Typically, commercially available leaf extracts, e.g., BioGinkgo™ from Pharmanex, are used as a starting point. **BB** and the main ginkgolides, i.e., **GA**, **GB**, and **GC**, constitute up to 7 wt.% of the whole *Ginkgo biloba* extract. Several procedures have been reported that allowed for a fast and facile separation of the terpene trilactone fraction from the rest of the extracts' components. Specifically, boiling the extract in dilute hydrogen peroxide solution was noted to prevent the formation of stable emulsions that usually hamper the subsequent extraction and purification steps (Lichtblau et al. 2002). Additionally, direct solid/liquid extraction of the solid BioGinkgo™ with ethyl acetate quantitatively, and almost selectively, led to isolation of all ginkgolides and **BB** from the extract (Nakanishi et al. 2005).

Similarities in solubilities and chromatographic behaviors might be considered among the main challenges associated with the separation of ginkgolides. In fact, only **BB** can be easily separated from the ginkgolides using chromatography. Typically the difficulties arise during separation of **GA/GB** and **GC/CJ** pairs, which cannot be separated by routine chromatographic techniques (Weinges and Bäehr 1972; Lobstein-Guth et al. 1983). In **GB**, for example, separation difficulties are attributed to strong hydrogen bonding between the 1-OH and 10-OH, which makes the polarity of **GB** very similar to that of **GA**. However, for the **GC/GJ** pair, the presence of multiple hydroxy groups in each ginkgolide negates the difference in polarities. The use of NaOAc impregnated silica gel allowed to obtain individual ginkgolides in high purity using medium-pressure liquid chromatography (van Beek and Lelyveld 1997). Derivatization of a **GA/GB** mixture with an ether functionality to alter polarities, followed by a facile separation and subsequent conversion of the derivatives to pure ginkgolides, was successfully accomplished in the early 1900s. This proved to be an efficient route for separation of larger quantities of ginkgolide mixtures (Corey et al. 1992). Subsequently, a convenient method that involved benzylation, column chromatography separation and debenzylation allowed for a gram-scale access to all individual ginkgolides (Jaracz et al. 2004a).

12.3 Synthetic Studies on Ginkgolides

12.3.1 Modification of the OH Functionalities

Distinct reactivities of the OH groups around the ginkgolide skeleton present an opportunity for a straightforward and selective functionalization of these compounds. It has been demonstrated that selective silylation at 1-OH can be achieved by reaction of ginkgolides with bulky silylating agents, such as t-butyl (chloro)diphenylsilane, in the presence of imidazole as a base (Weinges and Schick

1991). To date, this remains virtually the only route for direct functionalization of the 1-OH group.

Transformations such as alkylation, benzylation, allylation, propargylation, etc., occur almost exclusively at C-10. Furthermore, it was recognized early on that deprotonation of the 10-OH group of **GB** and **GC** is much more facile than deprotonation of the 10-OH of **GA** (Corey et al. 1992). This phenomenon was attributed to the neighboring group participation from 1-OH, and the resulting alkoxides are apparently stabilized by the intramolecular hydrogen bonding. It should be pointed out that alkylations at **GA**'s 10-OH are still possible, albeit they require an excess of stronger bases, such as NaH or KH.

Acetylation of the hydroxy groups can be tuned by the proper choice of a base (Jaracz et al. 2002). Specifically, acetylation of all 1-, 7-, and 10-OH groups is possible when **GC** is treated with Ac_2O/pyridine, whereas the acetylation at 10-OH or 7-OH takes place when **GC** is treated with Ac_2O/HOBr/pyridine or AcOH/H_2SO_4, respectively. Significantly, acetylation of **GA** using Ac_2O/NaOAc induces the elimination of the 3-OH group in addition to the acetylation of 10-OH.

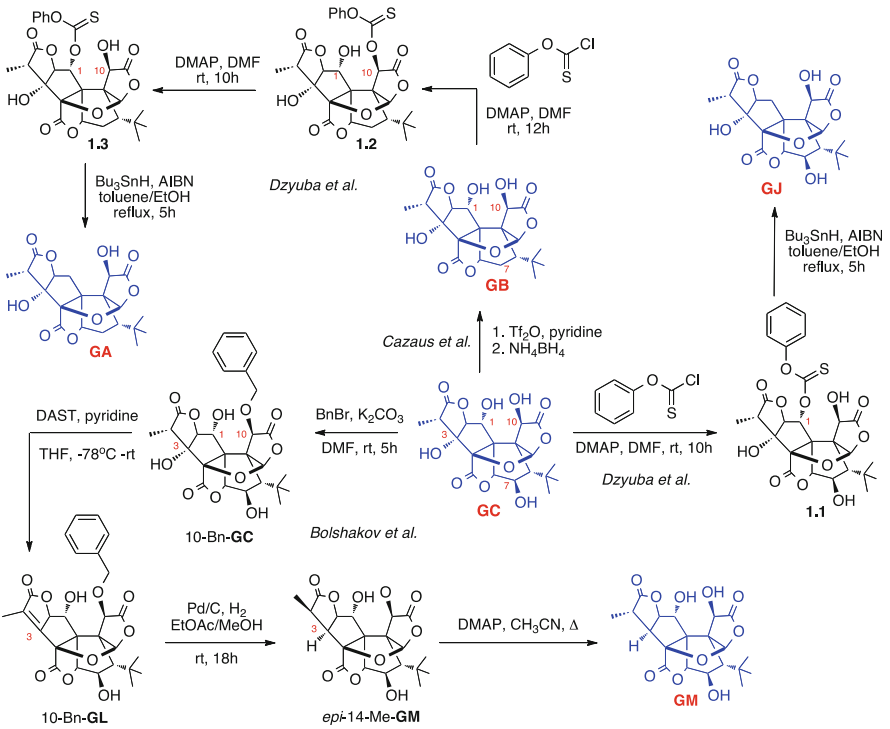
Exclusive derivatization of 7-OH was also realized when **GC** was reacted with triflic anhydride in the presence of pyridine to prepare 7-OTf-**GC** (Cazaux et al. 1995). The OTf group provides a convenient entry for the preparation of a diverse range of 7-substituted ginkgolides (Vogensen et al. 2003).

12.3.2 Ginkgolide Interconversion

The isolation of ginkgolides from *Ginkgo biloba* extracts represents the main source of these trilactones for all synthetic, structural, and biological studies. However, some ginkgolides are more abundant in the extracts than others. In addition, the leaf extract contains only **GA**, **GB**, **GC**, and **GJ**, with **GJ** being the minor component, whereas **GM** is only present in the root bark of the *Ginkgo* trees. In this light, ginkgolide interconversion becomes an attractive way to access minor ginkgolides. Although it would be most appealing to start with the most abundant **GA** and introduce C–O functionality via selective C–H activation, for example, on a scaffold as complex as the ginkgolides', it still presents a synthetic challenge. Therefore, a number of accounts have addressed the selective removal of the OH groups from the most oxygenated **GC** *en route* to other ginkgolides.

The groups of Weinges (Weinges and Schick 1991), Corey (Corey et al. 1992), and Teng (Cazaux et al. 1995) took advantage of selective silylation at 1-OH, alkylation at 10-OH, and sulfonylation at 7-OH, respectively, to install suitable moieties which aided in the subsequent conversion of **GC** into **GB** (Scheme 12.1). In addition, **GB** was converted to **GA** via protecting the 10-OH, and subjecting the 1-OH to xanthane formation, following the reduction with tri-*n*-butyltin hydride and deprotection (Corey and Ghosh 1988).

Later it was shown that selective functionalization of the 1-OH of **GC** via formation of phenylthiocarbonate (Scheme 12.1), followed by the deoxygenation



Scheme 12.1 Interconversions of ginkgolides

under Barton–McCombie conditions could lead to an efficient conversion of **GC** to **GJ** in just two steps via the formation of **1.1** (Dzyuba et al. 2005). The selectivity of the thiocarbonation step was solvent controlled, and functionalization of the 10-OH was possible by using CH_3CN as the solvent. This derivative was deoxygenated as well to produce a **GC** analogue that lacked the 10-OH group. In DMF, the thiocarbonation of the 1-OH of **GB** was achieved via a migration from the position 10 (**1.2**) to the position 1 (**1.3**) in the presence of DMAP; subsequent Barton–McCombie deoxygenation yielded **GA** in a moderate yield.

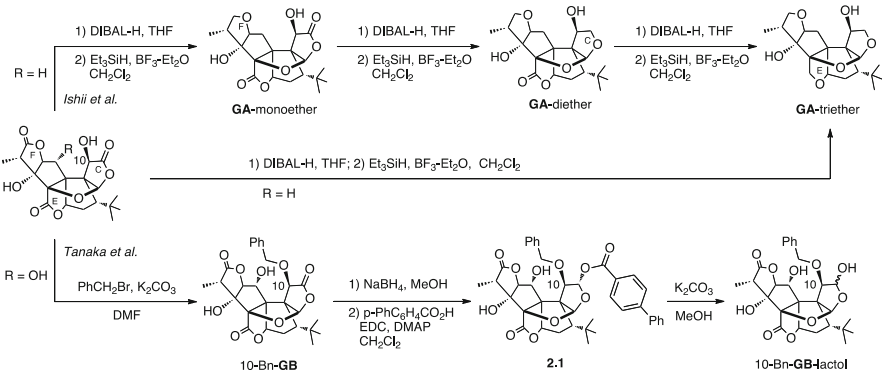
Access to the rare **GM** was reported in a few accounts. The first synthesis of **GM** was accomplished in 1993 via silylation of the 1-OH of **GC**, followed by a nonselective acetylation, which required a chromatographic isolation of the 7-OAc derivative. Subsequent dehydration, hydrogenation, deacetylation, and DMAP-promoted epimerization afforded **GM** (Weinges et al. 1993a, b). A more recent example relied on the selective dehydration of the easily accessible 10-Bn-**GC**, i.e., an intermediate in the isolation of ginkgolides from the extract (Jaracz et al. 2004a) as the starting material (Scheme 12.1). 10-Bn-**GC** was successfully dehydrated to 10-Bn-**GL**, and subsequent hydrogenation and DMAP-promoted epimerization of the methyl group in position 14 furnished **GM** in a moderate overall yield (Bolshakov et al. 2006).

GL is a dehydrated version of **GA**, and is likely to be produced as an impurity during the isolation of terpene trilacones fraction from the *Ginkgo biloba* extract. Several protocols demonstrated that **GA** can be converted to **GL** directly upon treatment with either pyridine/$POCl_3$ (Weinges et al. 1993a, b) or DAST (Bolshakov et al. 2006) The DAST-mediated dehydration turned out to be generally applicable for the ginkgolides as a clean, high yielded elimination of the 3-OH group was observed upon reaction with **GB** and **GC** derivatives.

In 1992, Corey's group suggested that some of the methodology developed for ginkgolide interconversion could and should be used for introduction of isotopes onto the ginkgolide skeleton (Corey et al. 1992). These isotopically labeled ginkgolides are of interest since they enable metabolic and pharmacological studies. Specifically, the introduction of ^{3}H and ^{18}F isotopes into position 7 was disclosed in the early 2000s via several two-step procedures using **GC** as a starting material (Strømgaard et al. 2004; Suehiro et al. 2004). Notably, these labeled **GB** were used for some in vivo studies to map bio-distribution of ginkgolides in various tissues (Suehiro et al. 2005). In addition, the methodology for installing a ^{14}C label on **GA** was developed (Weinges et al. 2000).

12.3.3 Core-Modified Ginkgolides

Although a great number of accounts on the synthetic transformations of ginkgolides relied on the modification of the OH functionalities, it might be argued that functionalization of the hydroxy groups produces ligands that are drastically larger than the original ginkgolides, and thus would be more likely to alter the mode of interaction with a receptor. In this light, modifications of the ginkgolide skeleton might be appealing. The first set studies on the modification of the ginkgolide core were performed during the structure elucidation in the mid/late 1960s. The majority of efforts focused on using **GA** as a starting point, in view of its higher abundance in the extract. It was then established that the treatment of **GA** with $LiAlH_4$, followed by an acidic work-up and subsequent sublimation and/or extensive vacuum drying produced **GA**-triether (Maruyama et al. 1967b; Woods et al. 1967). This compound was crucial for determining the overall structure of ginkgolides. A more direct and facile synthesis of **GA**-triether was disclosed in the mid-2000s (Scheme 12.2), when all of **GA**'s lactone moieties were converted into the corresponding ethers in a stepwise manner (Ishii et al. 2005). The first reduction to the lactol was shown to occur at ring F, followed by the reduction at ring C, with the final reduction taking place at ring E. Except for the reduction of lactol C, mixtures of epimers were obtained, while a single epimer was obtained upon the reduction of the lactone C, which is consistent with the presence of the 10-OH group. The **GA**-triether was also prepared directly from **GA** in just two steps using the excess of the reagents. This methodology, however, appeared to be of limited use, as its application to the delactonization of **GB** and **GC** proved fairly inefficient. Only **GB**-monoether was



Scheme 12.2 Reduction of lactones of **GA** and **GB**

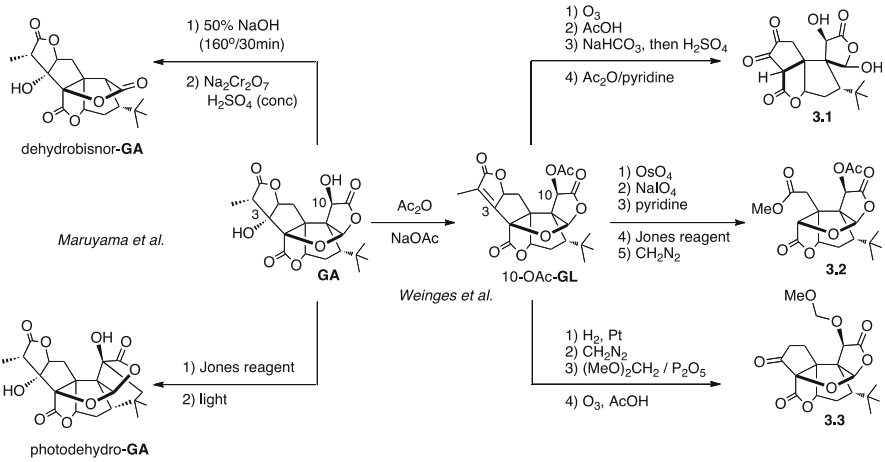
obtained in a moderate yield, and deoxygentation of both lactol of **GB**-monoether and lactol of **GC** failed completely.

Notably, the modification of the lactone moieties of 10-Bn-**GB** derivatives (Scheme 12.2) was also accomplished upon treatment with $NaBH_4$ and subsequent functionalization of the lactol to give **2.1** after chromatographic separation (Tanaka et al. 2005a). The lactol functionality could be liberated upon treatment with K_2CO_3 in MeOH to give 10Bn-**GB**-lactol. The lactone C was reduced preferentially due to the neighboring group participation of the 10-O (Scheme 12.2). However, the lactone of ring E was also reduced to the corresponding lactol, albeit at a slower rate. This reduction might be attributed to the close proximity of the 3-OH, since the lactone of ring F, which does not have any neighboring OH groups that were proposed to aid in the coordination of the reducing agent, was not reduced at all. No subsequent deoxygenations or other modifications of the lactol functionalities were carried out.

Additionally, it was demonstrated that ring C of **GA** is susceptible to various modifications (Scheme 12.3). Oxidation of **GA** was shown to produce 10-oxo-**GA**, which upon exposure to UV light produced photodehydro-**GA** that features a seven five-membered ring structure (Maruyama et al. 1967d). This compound was also obtained as a very minor product upon treating **GA** with the Jones reagent (Hu et al. 2001). The 10-oxo-**GA** was also shown to undergo a ring opening of the lactone C upon treatment with various amines to produce α-oxo-amide derivatives (Hu et al. 2001). Furthermore, submitting **GA** to alkali fusion under elevated temperatures led to the removal of ring C, yielding bisnor **GA**, while the subsequent oxidation produced an unnatural trilactone, i.e., dehydrobisnor-**GA** (Maruyama et al. 1967d).

It is of interest to note that exposing **GA** to Ac_2O/NaOAc produces an unsaturated, acetylated derivative (Scheme 12.3), whose reactivity was explored in a series of accounts from Weinges' group (Weinges et al. 1986, 1997a, b). Interesting and unique structures, i.e., **3.1**, **3.2**, and **3.3**, with partially removed and/or modified rings F and D were obtained in several steps (Scheme 12.3).

Ring F of **GA** could also be easily opened by dehydration of the 3-OH, using $POCl_3$/pyridine which converted **GA** into **GL**. The conversion was followed by the



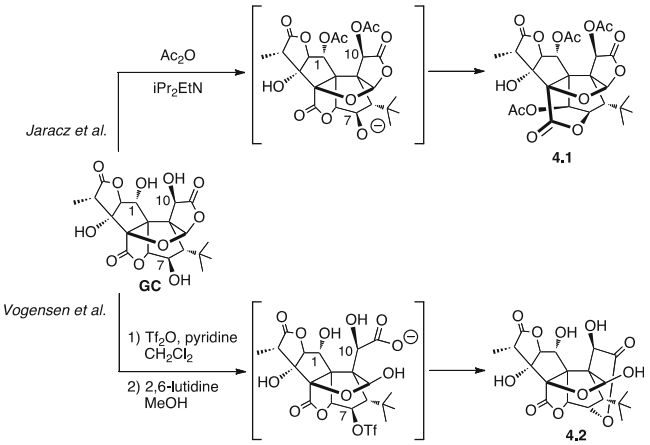
Scheme 12.3 Transformations of **GA**

oxidation of the alkene functionality into the diol with $OsO_4/NaClO_3$, and subsequent oxidation with $NaIO_4$ to produce F-nor-**GA** (Hu et al. 1999). Similar transformations were also reported for **GB**.

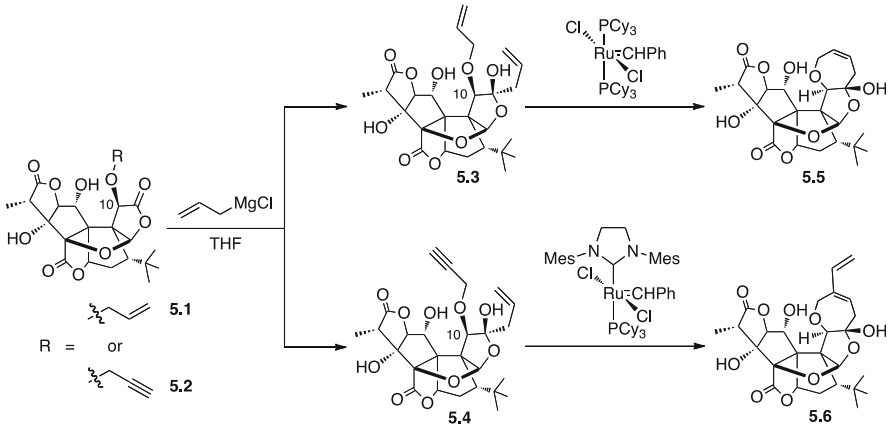
During the ginkgolide acetylation studies in the early 2000s (Scheme 12.4), it was noted that in the presence of iPr_2NEt an efficient translactonization of ring E produced a novel ginkgolide skeleton **4.1** (Jaracz et al. 2002). The availability of the X-ray structure helped in the rationalization of the mechanism for this interesting transformation. It was proposed that the intramolecular ring opening of the E lactone by the deprotonated 7-OH and the capture of the intermediate with acetic anhydride were responsible for the observed formation of the *iso*-ginkgolide. Further evidence for the observed mechanism was obtained by treating 7-OAc-**GC** with either iPr_2EtN or Ac_2O/iPr_2EtN, and observing no rearrangement products.

In addition, when reacted with 2,6-lutidine/MeOH, 7-OTf-**GC** was shown to undergo a unique ring opening of lactone C (Scheme 12.4), followed by intramolecular substitution of the triflate group to produce a relactonized product **4.2** (Vogensen et al. 2003). This relatively thermodynamically unstable ginkgolide derivative was cleanly transformed into *epi*-7-OH-**GC** under basic conditions.

The special reactivity of the C lactone ring, i.e., α-hydroxy-lactone group (Scheme 12.5), was also explored as a key step *en route* to some elaborated ginkgolide scaffolds (Tanaka et al. 2005b). Allylation and propargylation of **GB** produced the corresponding 10-allyl (**5.1**) or 10-propargyl (**5.2**) derivatives of **GB**, which were treated with the allyl Grignard reagent to yeild **5.3** and **5.4**, respectively. Subsequently, either alkene or alkene/alkyne metathesis using Grubbs' second generation catalysts afforded seven-membered, bowl-like structures **5.5** and **5.6** (Scheme 12.5). The newly installed ene/diene moieties may potentially be used as a handle for the subsequent functionalization.



Scheme 12.4 Translactonizations of **GC** derivatives



Scheme 12.5 Synthesis of 7-membered ginkgolides via olefin metatheses

12.4 Bioorganic Studies on Ginkgolides and Their Derivatives

The extracts from *Ginkgo biloba* trees demonstrate a diverse range of activities, albeit with various levels of efficiencies. Arguably, this could be attributed to the heterogeneity of terpene trilactone fractions in various batches. Therefore, studies with ginkgolides might prove to be more viable for establishing and expanding the biological mode of action.

12.4.1 Interactions with Platelet-Activating Factor Receptor

In the mid-1980s, **GB** was identified as a potent antagonist of the platelet-activating factor receptor (PAFR) in vitro (Braquet 1987; Braquet et al. 1991). PAFR is a member of the G protein-coupled receptor family and its mRNA was detected among several neuronal tissues including hypothalamic, cerebellar, hippocampal, and cortical. The expression of PAFR was noted in both neuronal and glial cells (Maclennan et al. 2002). PAF is a phospholipid (1-*O*-alkyl-2-acetyl-*sm*-glycero-3-phosphocholine, with hexadecyl to octadecyl alkyl chain lengths) that is produced in a variety of tissues and is a very potent PAFR agonist that can exert its effects at picomolar concentrations. Binding of PAF to PAFRs is believed to trigger a number of events in the CNS, including Ca^{2+} mobilization, long-term potentiation, and apoptosis. However, the specific details of PAF and PAFR modulation on neuronal function still remain to be elucidated.

Compared to **GB**, other ginkgolides either are inactive or exhibit significantly lower levels of activity towards PAFR. From the structure–activity point of view, it is of interest to note that a very fine balance exists between potency and the number of hydroxy groups around the ginkgolide skeleton (Strømgaard and Nakanishi 2004). Specifically, as an antagonist, **GA** (a ginkgolide that lacks the 1-OH group) is several fold less efficient as compared to **GB**. **GC**, which has an OH-group at position 7, is an order of magnitude less potent inhibitor than **GB**. The importance of the t-Bu group was also established (Corey and Rao 1991).

The majority of studies on ginkgolide–PAFR interactions were done using radioactive ligand displacement assays. However, the nonradioactive microphysiometry assay was developed and proved to be effective in determining the functional activity of native ginkgolides, their derivatives, and the *Ginkgo biloba* extract (Krane et al. 2003).

Following the initial reports on the antagonistic activity of **GB**, a variety of synthetic studies that aimed at improving the potency of **GB** followed. It was established that the introduction of substitutents in positions 1 and 10 led to potent PAFR antagonists. Hence, it was suggested that the incorporation of photoaffinity labels in position 10 might produce potent antagonists, which would allow mapping of the site-specific interactions between ginkgolides and PAFR. Initial studies demonstrated that the presence of benzophenone, trifluoromethyldiazirine, and tetraphenylazide photoactivatable moieties produced potent antagonists (Strømgaard et al. 2002). It was also shown that 10-substituted **GB** derivatives were several fold more active than the corresponding 10-substituted **GC** derivatives, which is in accord with the higher potency of **GB** over **GC**. In addition, it was demonstrated that the introduction of a fluorescent group, which would aid in the fragment characterization steps such as dansyl, at the 1-OH did not reduce the activity. Photolabeling studies using the synthesized ginkgolide probes have not been reported to date.

A recent account examined the synthesis of photoaffinity biotin containing chimeras of **GA** and **GB** (Kato et al. 2007), which could potentially simplify the post-photoaffinity isolation and identification steps significantly. The strategies relied

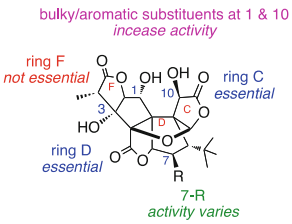


Fig. 12.2 Ginkgolide–PAFR structure–activity relationships

on the incorporation of the benzophenone moiety at C-10, while incorporating biotin via a linker at position 15 of ring F, since modifications of this part of the molecule are known to have no affect on the activity towards PAFR. The activity of these benzophenone–biotin chimeric compounds towards PAFR was not evaluated.

The effect of the nature of the substituent at position 7 and its stereochemistry was investigated in some detail, but proved to be particularly involved (Vogensen et al. 2003). As compared to **GB** (K_i = 0.88 μM), the binding of the 7-NHR derivatives depended strongly on the nature of the R-group. 7-NH_2 compound (R = H) showed a drastic decrease in affinity (K_i = 8.64 μM) while the alkyl versions, R = Me and Et, appeared to be somewhat equipotent to **GB** with a K_i of 0.61 and 1.62 μM, respectively. Significantly, introduction of chlorine at the 7-position produced a ginkgolide congener with drastically improved binding ability to PARF (K_i = 0.11 μM).

Acetylation of some or all hydroxy groups of ginkgolides **GA**, **GB**, and **GC** appeared to produce compounds with similar or reduced antagonistic activities towards PAFR as compared to the native ginkgolides (Jaracz et al. 2002). The acetylated version of the newly synthesized *iso*-**GC** (Scheme 12.4) was shown to be an equally inefficient antagonist.

A variety of synthetic derivatives that lacked ring F, for example, were also prepared and proved to be efficient in their ability to inhibit platelet aggregation (Corey and Gavai 1989). Some of the derivatives were used for several theoretical SAR studies on ginkgolide–PAFR interactions. A three-dimensional quantitative SAR investigated the effect of ginkgolides and their analogues (Chen et al. 1998; Zhu et al. 2005). In general, very good correlations between experimental and calculated activity values were observed. Specifically, the calculations demonstrated that bulky, hydrophobic substitutents at 1-OH and 10-OH would lead to more potent analogues. In agreement, the synthesized derivatives of **GB** with benzyl and BOM moieties at those positions exhibited IC_{50} values several fold lower than **GB** itself. Collectively, the aforementioned structure–activity studies are summarized in Fig. 12.2.

12.4.2 Interactions with Glycine Receptors

Glycine gated chloride channels (GlyRs) are one of the major inhibitory receptors in the CNS. These ligand-gated ion channels are found in the hippocampus, cortex,

spinal cord, and brainstem. GlyRs are found as either homomeric receptors, which consist of four identical α subunits (α1–α4), or heteromeric ones, which are composed of α and β subunits.

In 2002, **GB** was demonstrated to be an efficient blocker of GlyRs in pyramidal hippocampal neurons (Kondratskaya et al. 2002). Based on the whole-cell voltage-clamp and concentration-clamp recording techniques, using glycine as an agonist and strychnine as an antagonist of GlyR, it was suggested that **GB** inhibited glycine-mediated currents by interacting at the pore region of the GlyR. Subsequent studies that introduced a mutation in the pore region of the α1 subunit proved this mode of action (Kondratskaya et al. 2004). In addition, investigations using recombinant GlyRs demonstrated a high affinity of **GB** toward the β subunit (Kondratskaya et al. 2005). It was also indicated that **GB** could be used as a probe for discrimination between synaptic and extrasynaptic glycine currents.

The antagonistic effect of **GB** on GlyRs was further confirmed using embryonic cortical neurons in a series of whole-cell patch-clamp measurements (Ivic et al. 2003). This account also investigated the ability of other ginkgolides to interact with GlyRs. It was determined that **GB**, **GC**, and **GM** were more potent antagonists than **GA** and **GJ**, thus indicating the significance of the 1-OH group.

An extensive structure–activity study on the interaction of 49 **GC** derivatives, including ether, ester, and carbamoyl functionalities with homomeric α1 GlyRs revealed that hydroxy groups were required for the GlyR inhibition as virtually any modification of the hydroxy functionalities around the ginkgolide skeleton produced less potent compounds (Jaracz et al. 2004b). A follow-up study focused on more subtle changes of the ginkgolides' structure to gage further requirements about the biological activity towards α1, α2, and αβ GlyR using the FLIPR membrane potential assay as well as a patch-clamp technique (Jensen et al. 2007). In addition to native ginkgolides, i.e., **GA**, **GB**, **GC**, **GJ**, and **GM**, 29 ginkgolide derivatives were analyzed using in vitro and in silico screenings. Among native ginkgolides, **GM** was shown to be the most potent antagonist, whereas virtually every synthetic derivative turned out to be less potent than the native compounds. The rigidity of the ginkgolide core was identified as the main determinant of the antagonistic properties towards GlyR. Overall, increasing the activity of ginkgolides toward GlyRs appears to be much more challenging as compared to the PAFR.

A recent account reported on the ability of **GX** to act as a potent antagonist of GlyR (Jensen et al. 2010). Unlike other ginkgolides, **GX** exhibited complete selectivity for the homomeric GlyRs over the heteromeric GlyRs. Based on docking studies, different modes of interactions of **GX** with GlyRs, as compared to other ginkgolides, were proposed. The full potential of this ginkgolide is yet to be elucidated, but its restricted availability might prevent extensive biological studies.

12.4.3 Interactions with Amyloid Peptides and Relations to Alzheimer's Disease

Alzheimer's disease is an age-related pathology, which is responsible for the deterioration of cognitive function. The underlying causes of this disease are extremely complex, and are yet to be fully understood. However, it is largely accepted that conformational changes and aggregation of so-called amyloid peptides, i.e., Aβ, are responsible for the occurrence and progression of the disease (Hardy and Selkoe 2002). Typically, full-length amyloid peptides that are composed of 40–42 amino acids, commonly referred to as Aβ1-40 or Aβ1-42, are used for various in vitro and in vivo studies. In view of the difficulties associated with handling these full-length peptides and the reproducibility of the results, a number of truncated versions, such as Aβ25-35, for example, have been used as other suitable, easy-to-handle models.

Studies on developing inhibitors of Aβ aggregation have attracted enormous attention from academic and industrial research laboratories. Ginkgolides have been used in several in vitro and in vivo studies to test their effects on amyloid-induced damage. Initial studies demonstrated that among the main native ginkgolides, **GJ** was the most efficient compound in suppressing the aggregation of Aβ1-40 peptide in vitro by using the thioflavin T dye binding method (Luo et al. 2002). Subsequently, the neuroprotective effect of **GJ** was confirmed by preventing Aβ1-42-induced inhibition of long-term potentiation in the CA1 region of mouse hippocampal slices (Vitolo et al. 2009). This ginkgolide was also demonstrated to be efficient in inhibiting cell death of rodent hippocampal neurons caused by the Aβ1-42 oligomers. Other ginkgolides, such as **GA** and **GB** as well as the synthetic derivative, **GA**-triether (Scheme 12.2), were also able to partially block Aβ1-42-induced damage to synaptic plasticity.

Spectroscopic studies indicated that the effect of ginkgolides in modulating the aggregation of Aβ25–35 peptide was relatively small (He et al. 2008). The absence of a specific interaction between ginkgolides, which was established with the aid of some lactone-free derivatives, such as **GA**-monoether and **GA**-diether (Scheme 12.2), was attributed to the inability to affect the aggregation of amyloid peptides. Thus, it could be suggested that ginkgolides might not be suitable inhibitors of amyloid aggregation per se and that a direct interaction of ginkgolides with Aβ peptides is an unlikely mode of their neuroprotective actions.

Using neuroblastoma cell lines and primary cortical neurons, it was demonstrated that both **GA** and **GB**, at nano- to micromolar concentrations, could inhibit Aβ1-42-induced cell death (Bate et al. 2004). It was also established that **GB** is somewhat more efficient than **GA**. The protective effects of ginkgolides were suggested to correlate with reduced caspase-3 responses, a known apoptosis marker, as well as with the ability to suppress PAF-induced effects. In a related study, the effect of **GA** and **GB** on Aβ1-42 and PAF-induced reduction of the presynaptic membrane protein, synaptophysin, was investigated (Bate et al. 2008). Although ginkgolides did not alter the binding of Aβ1-42 into neurons, they were able to increase the neuronal survival in the presence of high micromolar concentrations of amyloid peptide.

The effect of **GB** and **GA** was also investigated in relation to the Aβ25-35-induced acetylcholine release from hippocampal brain slices (Li et al. 2004). It was demonstrated that while **GB** could effectively restore the acetylcholine release from rat hippocampal slices to their control levels in a dose-dependent manner, **GA** was completely inactive. It was proposed that the direct interaction of **GB** on the cholinergic nerve terminals was responsible for the observed effects.

Recently, Aβ25–35-induced apoptosis was studied in hippocampal neurons (Xiao et al. 2010). The changes in cell viability, morphology, caspase-3 activity, expression of brain-derived neurotrophic factor mRNA, and protein synthesis were detected due to the presence of Aβ25–35 peptides. It was found out that **GB** could reduce the amyloid-induced insults.

12.5 Conclusions

As the knowledge of ginkgolide–receptor interactions continues to improve and expand, new insights into the molecular mode of action of these compounds and the discovery of novel biological targets should be anticipated. Although ginkgolides were examined in a great wealth of pharmacological assays, it is presently unclear whether any drugs could arise from them. However, it is plausible that the combination of their unique and interesting structure and the great history of these natural products will continue to be the source of inspiration for organic synthesis, bioorganic chemistry, pharmacology, and chemical biology.

References

Abad MJ, Bedoya LM, Bemejo P (2010) An update on drug interactions with the herbal medicine *Ginkgo biloba*. Curr Drug Metab 11:171–181

Bate C, Salmona M, Williams A (2004) Ginkgolide B inhibits the neurotoxicity of prions or amyloid-β1-42. J Neuoroinflammation. http://www.jneuroinflammation.com/content/1/1/4

Bate C, Tayebi M, Williams A (2008) Ginkgolides protect against amyloid-β_{1-42}-mediated synapse damage in vitro. Mol Neurodegeneration 3:1. http://www.molecularneurodegeneration.com/content/3/1/1

Bisel P, Al-Momani L, Muller M (2008) The tert-butyl group in chemistry and biology. Org Biomol Chem 6:2655–2665

Bolshakov S, Dzyuba SV, Nakanishi K (2006) A concise synthesis of ginkgolide M, a minor component of a terpene trilactone fraction from *Ginkgo biloba* roots. J Nat Prod 69:429–431

Braquet P (1987) The ginkgolides: potent platelet-activating factor antaginists isolated from *Ginkgo biloba* L.: chemistry, pharmacology and clinical applications. Drug of the Future 12:643–699

Braquet P, Esanu A, Buisine E, Hosford D, Broquet C, Koltai M (1991) Recent progress in ginkgolide research. Med Res Rev 11:295–355

Cazaux J-B, Dafniet M, Rebollo J, Teng B-p (1995) Conversion of ginkgolide C to ginkgolide B. Brit UK Pat Appl GB 2288599 A 19951025

Chen JZ, Hu LH, Jiang HL, Gu JD, Zhu WL, Chen ZL, Chen KX, Ji RY (1998) A 3D-QSAR study on ginkgolides and their analogues with comparative molecular field analysis. Bioorg Med Chem Lett 8:1291–1296

Corey EJ, Gavai AV (1989) Simple analogues of ginkgolide B which are highly active antagonosts of platelet activating factor. Tetrahedron Lett 30:6959–6962

Corey EJ, Ghosh AK (1988) Total synthesis of ginkgolide A. Tetrahedron Lett 29:3205–3206

Corey EJ, Rao KS (1991) Enantioselective total synthesis of ginkgolide derivatives lacking the tert-butyl group, an essential structural subunit for antagonism of platelet activating factor. Tetrahedron Lett 32:4623–4626

Corey EJ, Rao KS, Ghosh AK (1992) Intramolecular and intermolecular hydroxyl reactivity differences in ginkgolides A, B and C and their chemical applications. Tetrahedron Lett 33:6955–6958

Crews WD, Harrison DW, Griffin ML, Falwell KD, Crist T, Longest L, Hehemann L, Rey ST (2005) The neurophyscological efficacy of Ginkgo preparations in healthy and congnitivily intact adults. HerbalGram 67:43–62

Dzyuba SV, Bolshakov S, Nakanishi K (2005) Regioselective thionocarbonation of ginkgolides: facile preparation of ginkgolide J. Tetrahedron Lett 46:7797–7799

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356

He J, Petrovic AG, Dzyuba SV, Berova N, Nakanishi K, Polavarapy PL (2008) Spectrosopic investigation of *Ginkgo biloba* trialactones and their interaction with amyloid peptide Aβ (25–35). Spectrochim Acta A 69:1213–1222

Hu L, Chen Z, Cheng Z, Xie Y (1999) Chemistry of ginkgolides: structure–activity relationship as PAF antagonists. Pure Appl Chem 71:1153–1156

Hu LH, Chen ZL, Xie YY (2001) Synthesis and biological activity of amide derivatives of ginkgolide A. J Asian Nat Prod Res 3:219–227

Ishii H, Dzyuba SV, Nakanishi K (2005) Lactone-free ginkgolides via regioselective DIBAL-H reduction. Org Biomol Chem 3:3471–3472

Ivic L, Sands TTJ, Fishkin N, Nakanishi K, Kriegstein AR, Strømgaard K (2003) Terpene trialctones from *Ginkgo biloba* are antagonists of cortical glycine and $GABA_A$ receptors. J Biol Chem 278:49279–49285

Jaracz S, Strømgaard K, Nakanishi K (2002) Ginkgolides: selective acetylations, translactonization, and biological evaluation. J Org Chem 67:4623–4626

Jaracz S, Malik S, Nakanishi K (2004a) Isolation of ginkgolides A, B, C, J and biloblide from *G. biloba* extracts. Phytochemistry 65:2897–2902

Jaracz S, Nakanishi K, Jensen AA, Strømgaard K (2004b) Ginkgolides and glycine receptors: a structure–activity relationship study. Chemistry 10:1507–1518

Jensen AA, Begum N, Vogensen SB, Knapp KM, Gundertoffe L, Dzyuba SV, Ishii H, Nakanishi K, Kristiansen U, Strømgaard K (2007) Probing the pharmacophore of ginkgolides as glycine receptor antagonists. J Med Chem 50:1610–1617

Jensen AA, Bergmann ML, Sander T, Balle T (2010) Ginkgolide X is a potent antagonist of anionic cys-loop receptors with a unique selectivity profile at glycine receptors. J Biol Chem 285:10141–10153

Kato E, Howitt R, Dzyuba SV, Nakanishi K (2007) Synthesis of novel ginkgolide photoaffinity–biotin probes. Org Biomol Chem 5:3758–3761

Kondratskaya EL, Lishko PV, Krishtal OA, Chatterjee SS (2002) BN52021, A platelet activating factor antagonist, is a selective blocker of glycine-gated chloride channel. Neurochem Int 40:647–653

Kondratskaya EL, Firsunov AI, Chatterjee SS, Kristal OA (2004) Ginkoglide B preferentially blocks chloride channels formed by heterometic glycine receptors in hippocampal pyramidal neurons of rat. Brain Res Bull 63:309–314

Kondratskaya EL, Betz H, Krishtal OA, Laube B (2005) The β subunit increases the ginkgolide B sensitivity of inhibitory glycine receptors. Neuropharmacology 49:945–951

Krane S, Kim SR, Abrell LM, Nakanishi K (2003) Microphysiometric measurement of PAF receptor responses to ginkgolides. Helv Chim Acta 86:3776–3786

Lang Q, Wai CM (1999) An extraction method for determination of ginkgolides and bilobalide in Ginkgo leaf extracts. Anal Chem 71:2929–2933

Li C-Y, Lin C-H, Wu C-C, Lee K-H, Wu T-S (2004) Efficient 1H nuclear magnetic resonance method for improved quality control analyses of ginkgo constituents. J Agric Food Chem 52:3721–3725

Lichtblau D, Berger JM, Nakanishi K (2002) Efficient extraction of ginkgolides and bilobalide from *Ginkgo biloba* leaves. J Nat Prod 65:1501–1504

Lobstein-Guth A, Briançon-Scheid F, Anton R (1983) Analysis of terpenes from *Ginkgo biloba* L. by high-performance liquid chromatography. J Chromatogr 267:431–438

Luo Y, Smith JV, Paramasivam V, Burdick A, Curry KJ, Buford JP, Khan I, Netzer WJ, Xu H, Butko P (2002) Inhibition of amyloid-β aggregation and caspase-3 activation by the *Ginkgo biloba* extract EGb761. Proc Natl Acad Sci USA 99:12197–12202

Maclennan KM, Darlington CL, Smith PF (2002) The CNS effects of *Ginkgo biloba* extracts and ginkgolide B. Prog Neurobiol 67:235–257

Mahadevan S, Park Y (2008) Multifaceted therapeutic benefits of *Ginkgo biloba* L.: chemistry, efficacy, safety, and uses. J Food Sci 73:R14–R19

Maruyama M, Terahara A, Itagaki Y, Nakanishi K (1967a) The ginkgolides. I. Isolation and characterization of the various groups. Tetrahedron Lett 4:299–302

Maruyama M, Terahara A, Itagaki Y, Nakanishi K (1967b) The ginkgolides. II. Derivations of partial structures. Tetrahedron Lett 4:303–308

Maruyama M, Terahara A, Nakadaira Y, Woods MC, Nakanishi K (1967c) The ginkgolides. III. The structure of the ginkgolides. Tetrahedron Lett 4:309–313

Maruyama M, Terahara A, Woods MC, Tagaki Y, Nakanishi K (1967d) The ginkgolides. IV. Stereochemistry of the ginkgolides. Tetrahedron Lett 4:315–319

Nakanishi K (2005) Terpene trilactones from *Ginkgo biloba*: from ancient times to the 21st century. Bioorg Med Chem 13:4987–5000

Nakanishi K, Habaguchi K, Nakadaira Y, Woods MC, Maruyama M, Major RT, Alauddin M, Patel AR, Weinges K, Baehr W (1971) Structure of bilobalide, a rare tert-butyl containing sesquiterpenoid related to the C20-ginkgolides. J Am Chem Soc 93:3544–3546

Nakanishi K, Jaracz S, Malik S, Ishii H, Dzyuba SV (2005) Separation of ginkgolides and bilobalide from G. biloba by column chromatography. PCT Int Appl WO 20005046829

Strømgaard K, Nakanishi K (2004) Chemistry and biology of terpene trilactones from *Ginkgo biloba*. Angew Chem Int Ed 43:1640–1658

Strømgaard K, Saito DR, Shindou H, Ishii S, Shimizu T, Nakanishi K (2002) Ginkgolide derivatives for photolabeling studies: preparation and pharmacological evaluation. J Med Chem 45:4038–4046

Strømgaard K, Suehiro M, Nakanishi K (2004) Preparation of a tritiated ginkgolide. Bioorg Med Chem Lett 14:5673–5675

Suehiro M, Simpson N, van Heertum R (2004) Radiolabeling of ginkgolide B with ^{18}F. J Label Compd Radiopharm 47:485–491

Suehiro M, Simpson NR, Underwood CJ, Nakanishi K, van Heertum R (2005) In vivo biodistribution of ginkgolide B, a constituent of *Ginkgo biloba*, visualized by microPET. Planta Med 71:622–627

Tanaka K, Kester KD, Berova N, Nakanishi K (2005a) Preparation of ginkogolide and F-seco-ginkgolide lactols: the unique reactivity of α-hydroxy lactones towards $NaBH_4$. Tetrahedron Lett 46:531–534

Tanaka K, Pimentel M, Berova NK (2005b) Unique reactivity of α-alkoxy ginkgolide lactones to nucleophilic reagents: preparation of new lactol derivatives. Bull Chem Soc Jpn 78:1843–1850

T-f L, C-f C, Wang LCH (2004) Effect of ginkgolides on β-amyloid-supressed acetylcholine release from rat hippocampal slices. Phytother Res 18:556–560

van Beek TA (2005) Ginkgolides and bilobalide: their physical, chromatographic and spectroscopic properties. Bioorg Med Chem 13:5001–5012

van Beek TA, Lelyveld GP (1997) Preparative isolation and separation procedure for ginkgolide A, B, C, and J and bilobalide. J Nat Prod 60:735–738

van Beek TA, van Veldhuizen A, Lelyveld GP, Pron I, Lankhorst PP (1993) Quantification of bilobalide and ginkgolides A, B, C and J by means of nuclear magnetic resonance spectroscopy. Phytochem Anal 4:261–268

Vitolo O, Gong CZ, Ishii H, Jaracz S, Nakanishi K, Arancio O, Dzyuba SV, Lefort R, Shelanski M (2009) Protection against β-amyloid induced abnormal synaptic function and cell death by ginkgolide J. Neurobiol Aging 30:257–265

Vogensen SB, Strømgaard K, Shindou H, Jaracz S, Suehiro M, Ishii S, Shimizu T, Nakanishi K (2003) Preparation of 7-substituted ginkgolide derivatives: potent platelet activating factor (PAF) receptor antagonists. J Med Chem 46:601–608

Weinges K, Bäehr W (1972) Natural products from medicinal plants. XVI. Comparison of the NMR and mass spectra of bilobalide C15H18O8 and of the ginkgolides C20H24O9-11 (in German). Leibigs Ann Chem 759:158–172

Weinges K, Schick H (1991) Chemistry of ginkgolides. IV. Synthesis of ginkgolide B from ginkgolide C (in German) Leibigs Ann Chem 81–83

Weinges K, Hepp M, Huber-Patz U, Rodewald H, Irngartinger H (1986) Chemistry of the ginkgolides. I. 10-Acetyl-1-(methoxycarbonyl)-3,14,15,16-tetranorginkgolide A, an intermediate for the synthesis of bilobalide (in German). Leibigs Ann Chem 1057–1066

Weinges K, Hepp M, Jaggy H (1987a) Chemistry of ginkgolides. II. Isolation and structural elucidation of a new ginkgolide (in German) Leibigs Ann Chem 521–526

Weinges K, Hepp M, Huber-Patz U, Irngartinger H (1987b) Chemistry of ginkgolides. III. Bilobalide/isobilobalide. Structure determination by X-ray analysis (in German). Leibigs Ann Chem 1079–1085

Weinges K, Rümmler M, Schick H, Schilling G (1993a) Chemistry of the ginkgolides. V. On the preparation of the ginkgolide skeleton. Leibigs Ann Chem 287–291

Weinges K, Rümmler M, Schick H (1993b) Chemistry of the ginkgolides. VI. Preparation of 1,10-dihydroxy- and 1,7,10-trihydroxyginkgolide from 1,3,7,10-tetrahydroxyginkgolide (in German). Leibigs Ann Chem 1023–1027

Weinges K, Schick H, Pritzkow H (1997a) Chemistry of ginkgolides. VII. Preparation and crystal structure analysis of a secondary ozonide from ginkgolide A. Leibigs Ann Chem 1607–1609

Weinges K, Schick H, Irngartinger H, Oeser T (1997b) Chemistry of the ginkgolides. IX. Ozonolysis of (10R)-10-acetoxy-3,14-didehydroginkgolide. Leibigs Ann Chem 1755–1756

Weinges K, Schick H, Irngartinger H, Oeser T (2000) Chemistry of the ginkgolides. Part 10: access to ^{14}C-labelled ginkgolide A. Tetrahedron 56:3173–3176

Woods MC, Miura I, Nakdaira Y, Terahara A, Maruyama M, Nakanishi K (1967) The ginkgolides. V. Some aspects of their NMR spectra. Tetrahedron Lett 4:321–326

Xiao Q, Wang C, Li J, Hou Q, Li J, Ma J, Wang W, Wang Z (2010) Ginkgolide B protects hippocampal neurons from apoptosis induced by beta-amyloid 25–35 partly via up-regulation of brain-derived neurophilic factor. Eur J Pharmacol 647:48–54

Zhu W, Chen C, Hu L, Luo X, Gui C, Luo C, Puah CM, Chen K, Jiang H (2005) QSAR analyses on ginkgolides and their analogues using CoMFA, CoMSIA, and HQSAR. Bioorg Med Chem 13:313–322

Chapter 13
Towards a Contemporary and Evidence-Based Development of TCM

Hildebert Wagner and Gudrun Ulrich-Merzenich

13.1 Retrospective and Outlook of TCM

Part 1: H. Wagner

- The preceding 12 chapters of this book address basic research on TCM drugs and their applicability. They feature chemical analysis, botanical authentication, and identification of the chemical constituents, pharmacological evaluation, and modern molecular–biological investigations of particular TCM drugs. Only one chapter provides an overview of the development of a new herbal medicinal product which originates from Traditional Chinese Medicine (see Chap. 11).
- Despite promising developments in TCM research in China and Europe over the past 15 years, TCM drugs are far from being integrated into Western systems of medicine. Multiple reasons in the areas of basic research as well as in the application of TCM preparations cannot be ignored and need to be discussed.

  1. China possesses one of the world's largest resources of potential medicinal plants, the majority of which have received neither complete taxonomic identification nor comprehensive chemical, pharmacological, and molecular–biological investigation. Experts estimate that only about half of the medicinal plants used in China have been systematically investigated clinically. Therefore, the potential for medicinal use can be regarded far from being fully exploited. The task to achieve a full coverage is immense and of a great challenge.
  2. Another reason for the existing backlog in research is the late establishment of a Chinese pharmaceutical industry that is focused specifically on research on Chinese drugs and the development of new phytopharmaceuticals, despite

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the broad use of Chinese products in the pharmaceutical industry elsewhere. Two examples may illustrate this:
As early as 40 years ago, the bark of the *Camptotheca acuminata* tree, indigenous to China, and the plant *Artemisia annua* (see Chaps. 7 and 8) were the objects of intensive chemical investigations. The new compounds isolated from their plant materials formed the basis for the synthesis of highly effective "Blockbuster" drugs (Topotecan, Artesunate) which were developed in the USA and are now used successfully as drugs for the treatment of cancer and malaria, respectively. Another example is the *Ginkgo* tree. The leaves and seeds, which in former times were of only minor medicinal importance in TCM, were the originating material for basic research conducted outside of China, particularly in the USA and Germany, which resulted in a successfully standardized plant extract that is now available on the drug market for the treatment of dementia and Alzheimer's disease (see Chap. 11).
Because molecular–biological research over the last decade has led to a revival of interest in the potential of medicinal plants, we can expect the discovery of further new promising drugs developed in Chinese and western laboratories in the next few years.

3. The continued lack of modern and reliable standardization methods for TCM drugs was a further major obstacle to a rapid expansion of the use of TCM drugs in European countries. The unsatisfactory methods for the authentication of TCM drugs and the assessment of their safety, which did not meet the high standards of the European drug regulatory authorities, have stimulated the development of new Analytical Monographs as supplementation to the Chinese Pharmacopoeias.
4. A not insignificant cause for the still slow introduction of TCM in western medicine and the remaining skepticism of western physicians regarding the use of TCM drug preparations is, without doubt, the fact that the therapeutic concept of TCM is very different from that of western medicine. It had first to be understood through training provided by Chinese practitioners in China or in TCM clinics established in western countries, including the first TCM clinic established 20 years ago in Bad Kötzting, Germany. Although the last 10 years have yielded reports of "controlled clinical studies" performed in China on Chinese drugs and formulas, which were described as unsatisfactory even by Chinese clinicians. Not only one review identified "inadequate standards of planning and implementation" (Jiang et al. 2010) as the primary reason for these critical evaluations.

- In this context it is difficult for experts in the pharmaceutical and biological sciences to understand why Chinese clinicians are obviously not aware of the substantial progress in basic research of TCM drugs and believe that the unsatisfactory results of controlled studies are only or primarily due to imperfect or insufficient standards in the planning and performance of the clinical trials. Chinese experts in the field of pharmacy and biology agree with their western colleagues that the unsatisfactory results of the controlled studies with Chinese drug formulas are, if not primarily caused, at least linked, to the same extent to the imperfect and suboptimal manufacturing process of the herbal drug preparations

and the unknown chemical and pharmacological requirements which, finally, determine therapeutic efficacy.

But why do we have this lack of knowledge about the drug material, used by physicians for their therapy of patients in spite of an international consensus that *the chemical composition and pharmacological effects of a drug determine its therapeutic efficacy*, provided other treatments such as acupuncture or tuina massage (as part of the holistic TCM approach) are not used simultaneously. The reason may be explained as follows:

- This gap of knowledge appears at first glance as ignorance, but it is not. It is a phenomenon not limited to physicians practicing TCM, but can be observed all over the world among physicians applying phytomedicine. It may relate in Europe to developments in the twelfth century. At that time a division of responsibilities for health care has taken place. In a contractual edict of Salerno (Italy) in 1241, a separation was established between "pharmacists," who should be responsible for the manufacturing of drugs, and the "physicians," who should have to carry out diagnosis and therapy. This led to some degree of alienation between the two professions which resulted in the development and establishment of separate training centers, university departments, symposia, congresses, and workshops. In Europe and in the western world, pharmaceutical companies have taken over the development and production of drugs and the university institutes should be responsible for research, so that new therapeutic drugs could be provided to the physicians in clinical research and practice.
- With respect to an integration of both disciplines, a significant change has taken place over the past years.

In the area of TCM, intensive exchanges in the form of symposia and workshops have been initiated e.g., between Chinese and German physicians experts in pharmacognosy and pharmaceutical biology, which are supported by the Chinese government and the German Research Foundation as well as further Chinese and German institutions. With support of the European community, an interdisciplinary consortium on TCM research was established in 2009.

It would be desirable to extend these collaborations in the future, with an increased scientific transfer between physicians and pharmacists interested in basic TCM research. Symposia and workshops are efficient tools for accelerating the modernization of the TCM in all areas, but especially in the development of new drugs from TCM. An excellent example is the Sino-European Forum, which was established in 2011 by a collaboration between the university of Beijing and the Institute for Complementary Medicine of the Technical University in Munich and the TCM clinic in Bad Kötzting, Germany. Similarly, a Sino-American collaboration is developing a library of authenticated Traditional Chinese Medicinal (TCM) plants for systemic biological evaluation (Eisenberg et al. 2011)

Following the chapters on recent research on TCM and an effort to elucidate the present status quo of TCM, the editors of this book propose a catalogue of recommendations for the contemporary further development of TCM. This includes

important premises for an evidence-based development and optimization of Chinese drug preparations for therapeutic use and, thus for the modernization of TCM.

13.2 Recommendations for the Modernization of TCM–Herbal Drug Preparations

13.2.1 Quality and Authenticity Proofs of TCM Drugs

Chapter 1 of this book deals with the most important analytical methods for the authenticity proof of TCM drugs. This examination for the purpose of quality assurance should be performed before each preclinical study. TLC, GC, and HPLC analysis, the latter coupled with UV diode array technique, were used for the purpose of the newly developed analytical monographs (see Chap. 1). These methods meet both the requirements of science-based authenticity proofs and the high standards of the European drug regulatory authorities. They also enable the researchers, for the first time, to detect the complex entities of all main low molecular weight constituents of plant drugs with the advantage that the single constituents can be made visible in colored TLC photographs and in quantifiable HPLC peaks. For safety reasons, this technique can also be used to exclude possible falsifications and adulterations of herbal drugs. The methods are not sophisticated and are feasible and applicable in all laboratories possessing common contemporary equipment and documentation techniques. Admittedly, these identity proofs have their limitations in the cases of drugs with very similar chemical and botanical characteristics. In the near future, however, the barcode fingerprint DNA analysis will be available in order to supplement and correlate the chromatographic analyses with the DNA fingerprint analyses and thereby to optimize the quality proofs of the drugs (see Chap. 2).

The main challenge that has, however, not yet been satisfactorily solved is, that considering the existence of the great botanical diversity, the Chinese pharmacopoeias rarely, if at all, distinguished among the different plant subspecies and subvarieties and herewith among the identical or deviating pattern of chemical constituents they possess. If these constituents of the various species are not exactly identical, no pharmacological and therapeutic equivalence can be expected. In such a case both species are not interchangeable with one another and cannot be designated as “synonyms.”

One should also bear in mind the fact that the Linnean binomial identification system for plants does not match exactly with the Chinese system and this has led in more than one case, to falsifications and adulteration of individual drugs and also cases of poisoning. Therefore the adoption of the internationally accepted Linnean binomial system for species, subspecies, and varieties contained in the Chinese Pharmacopoeias is essential.

13.2.1.1 Conclusion

Without a definite examination of its authenticity, a herbal drug should not be included in a pharmacopoeia. This also applies to any herbal drug combination developed by mixing several individual drugs. In such cases, microscopic, TLC, and/or HPLC analysis certificates should be presented for individual drugs in order to confirm their authenticity unambiguously.

13.2.1.2 Literature

Bauer and Franz (2010), Chan et al. (2012), Efferth (2011), Heubl (2012), Liang et al. (2010), Vlietinck et al. (2009), Wagner (2012), Wagner et al. (2011), Wu et al. (2007b), Yuan et al. (2011).

13.2.2 Processing (Preparation of TCM Drugs)

- Apart from cutting and cleaning of the raw drugs, the Chinese Pharmacopoeia describes many other methods of pretreatment or "processing" unknown to western Pharmacopoeias. In the Chinese Pharmacopoeia, "processing" is associated individually with the creation of a "drug," whatever that may mean in each individual case. The purpose of this processing is explained as altering the appearance, the physical characteristics, and/or the chemical composition of a herbal drug. In none of the monographs, except for the drugs containing toxic constituents, the necessity of the various processing is rationalized and clearly substantiated. The following methods are used and described: roasting and boiling, scalding, calcinating, carbonizing, steaming, boiling, stirring, processing with wine, vinegar, or salt water, or using different kinds of stir boiling.
  Investigating the unprocessed and processed root of the alkaloid herbal drug *Aconitum kusnezoffii* by HPLC, we could show in the processed drug pretreated with boiling water, that the strongly toxic Aconitine and its concomitant Mesaconitine were degraded substantially and appeared in the HPLC only as small peaks (see New Chinese Analytical Monographs Vol. II Springer 2011 and Chap. 1). However, this description is insufficient without exact determination of the aconitine and mesacontine content after the processing. Here the toxicologist must decide and stipulate which alkaloid content can be tolerated and accordingly to which concentration the alkaloids have to be degraded.
  These and other examples of processed herbal drugs indicate that the processing methods do not correspond to the current state-of-the art of the chemical or physical methods. They should be thoroughly reassessed with the aim to replace

them with more controllable methods, with a higher rate of efficiency. In this process it will be important for which purpose the pretreatments are undertaken.
- In this context, the approach of various companies to promote granules of TCM drugs and their formulas instead of decoctions requires closer scrutiny.

The main objection to the TCM granules is that the ingredients can be altered by preprocessing with heat, solvents, and additions of foreign substances. These possibilities have never been considered and investigated. It would also be difficult to determine analytically the main bioactive compounds and their quantities within the granules, a task which will be hardly possible in the case of drug mixtures. A recent meta-analysis based on the evaluation of 56 clinical studies (Luo et al. 2012) addresses the question of equivalence of granules and decoctions. Authors concluded based on the quality of the methodology of the clinical trial that it is presently not possible to reach a definitive conclusion whether both Chinese herbal medicine granules and decoctions have the same degree of effectiveness and safety in clinical practice. The preliminary evidence seems to support the continued use of granules in clinical practice and research. Standardization of granules and further more rigorous pharmacological, toxicological, and clinical studies are needed (Luo et al. 2012). Detailed chemical analyses of both products till now have so far not been provided.

13.2.2.1 Conclusion

"Traditional" processing of the TCM drugs should be systematically reassessed with the aim to replace them with more controllable and efficient methods. The purpose for processing should be comprehensibly provided. For granules, as upcoming new form of preparation, chemical standardization from the manufacturing point of view should have first priority. As yet, they cannot be taken as an equivalent or alternative to other standardized extract preparations.

13.2.2.2 Literature

Bauer and Franz (2010), Butterweck and Nahrstedt (2012), Duan et al. (2012), Pharmacopeia of the People's Republic of China (2010), Singhuber et al. (2009), Zhao et al. (2010).

13.2.3 TCM Drug Extraction Procedure

Hot water decoction remains the most common form of preparation in TCM for the manufacture of individual and multiple drug extracts.

- The procedures are subject to different cooking times, for which different methods of single boiling and multiple extractions are described.
- For some of the drugs containing high contents of mucilage polysaccharides, a cold maceration process is recommended.
- The water decoctions are not "durable" and must be used within a few days.
- In order to ensure the quality of drugs, the Chinese Pharmacopoeias also prescribe extraction of the raw herbal drugs with alcohol. The active substances to be found in the alcohol extracts, however, correspond not at all or only partially to the ones in the later produced decoctions. This means that the therapeutic effects of the decoctions cannot be identical to those of alcoholic extracts.
- The current decoction manufacturing process does not consider the fact that TCM drugs contain not only water-soluble constituents but also highly lipophilic active substances, which are not soluble in hot water and therewith at best only to a small extent in the decoctions. This applies to the volatile active substances, including, for example, essential oils from chamomile, peppermint, or eucalyptus which contain a multitude of bioactive compounds that possess a great number of multivalent pharmacological activities. Why does the Chinese Pharmacopoeia suggest the determination of the content of essential oils as part of a drug's qualities proof, if the high volatility of essential oils means that, after decoction, they are present in the teas not at all or only in traces?
- Another, even more striking example is one of the best known TCM drug, prepared from the root of *Salvia miltiorrhiza*, commonly used in the treatment of cardiovascular diseases. Pharmacological and clinical studies have shown that it possesses antihypertonic, antiplatelet, antiarrythmic, antiischemic, and antioxidative effects. Two different groups of active substances are responsible for these effects (see Chap. 6).

The first group of active substances is composed of approx. 4–5 diterpenquinone compounds, the so-called tanshinones. These are highly lipophilic and are soluble in ether and alcohol, but not in water.

The second, extremely water-soluble, group of constituents, the phenolcarboxylic acids of the salvianolic acid type, are also soluble in alcohol. They are present primarily in the decoction, to the extent that the heat-sensitive phenolic compounds are not partially degraded.

In order to include both groups of active substances in one extract, alcoholic or alcohol–water extracts should be manufactured. If both groups of pharmacological substances are obtained in one extract synergistic pharmacological effects can be expected, as recently was reported for an ethyl alcohol extract of *Salvia miltiorrhiza*. Several similar examples support the assertion that improvements in effectiveness can be achieved by using the right solvent for extraction, adapted to the appropriate solubility of the main bioactive substances of the herbal drug.

13.2.3.1 Conclusion

The today still most common prepared decoctions do not contain also extremely lipophilic bioactive plant substances. For a rational drug development the use of water decoctions should be extended to or eventually replaced by appropriate, standardized, and optimized new extraction procedures to make use of the full potential of the herbal drugs. In single selected medicinal applications there is also the possibility of manufacturing fixed extract combinations.

13.3 Are TCM Multidrug Formulations Optimal for Therapeutic Application and Efficacy?

A common characteristic of most TCM drug preparations is their herbal multidrug composition. Most of the fixed formulas, composed centuries ago contain between 4 and 8 drugs. Those composed of only 2–3 drugs are rare. This multiplicity of plants in phyto-preparations is not unique to TCM. Many preparations in traditional medicine of other countries also consist of fixed complex formulas. Often they may also include minerals and animal products in addition to plant constituents. Many of the Chinese fixed herbal drug formulas used today are mostly unchanged in their composition since the times of the Han-, Jin- or Qin-, and Tang dynasties (1000–1700 AD).

We assume that the drug combinations were developed by former TCM physicians only on the basis of their observed better efficacy over the one of a single herbal drug. This was also probably the origin of the continuously advocated "holistic therapy." Even the recently initiated "Synergy Research" in phytomedicine is connected to this concept. Its aim is to elucidate which chemical and pharmacological requirements need to be fulfilled for a herbal drug mixture to achieve a synergistic, i.e., overadditive or potentiated, pharmacological effect and a higher therapeutic efficiency compared to the one of a single herbal drug.

The synergy approach in phytomedicine is presently paralleled also in allopathic medicine. After a long period of vehement propagation of the monodrug therapy, now a turn towards multidrug or multitarget therapies in chronic or complex disease traits, e.g., in the treatment of cancer, rheumatic, and cardiovascular diseases, can be observed.

However, an equation between the multitarget concept in chemotherapy and the multitarget approach of Chinese formula therapy is not possible. In chemotherapy, several chemically known and pharmacologically tested pure substances are used. In phytotherapy, even one drug extract contains already at least 10–20 main bioavailable chemical compounds. Formulas containing 4–8 herbal drugs may include up to 100–150 defined compounds. All could exhibit their own pharmacological effects—quite apart from additional possible interactions between the single compounds in the drug mixture. At present, the allocation of the different

pharmacological effects to the complex drug formula is challenging and has to be the task of future molecular, biological, and particularly of genomic and metabolomic investigations in order to rationalize complex phytomedical treatments.

At this time one strategy for the advancement of phytomedicine in the area of drug formulas is to determine the exact quantity of the so far known main bioactive substances of a fixed drug formula, using one or 3D high pressure liquid chromatography and to test their effectiveness in animal trials. After the establishment of the safety and nontoxicity of the standardized drug formula, clinical studies can follow according to international standards. In order to achieve the optimal therapeutic effect, the selection of suitable drugs should be *based on a chemical and pharmacological approach*.

The validity of this strategy has also been confirmed recently by a Chinese research group's chemical and pharmacological investigation of the ancient Chinese herbal formula Danggui Buxue Tang (DBT). The herbal composition of this formula has undergone three modifications over the course of its historical use. The formula originating from the Jin dynasty (DBT 1247) showed the best pharmacological activity and is today the formula prescribed preferentially in herbal clinics (Zhang et al. 2012b). The authors came to the conclusion that "for the first time, the chemical and pharmacological approach was used here for the valuation of an ancient Chinese herbal formula and the current results explain the reason of DBT 1247 popularity as compared to DBT 1155 and DBT1687."

Recent studies to select drugs for fixed combination thereby reducing the number of drugs in complex formulas, for example to a three-drug combination, demonstrated that this goal can be achieved without an essential loss of effectiveness. It can even lead to an improvement of the effectiveness. Further advantages of this strategy are the reduction of expenditures for an analytic standardization of the extract formula and, secondly, the likelihood to improve the reproducibility of the effectiveness of such preparations, which represents the primary aim of the "Modernization of the Traditional Chinese Medicine."

13.3.1 Conclusion

The optimization of the therapeutic effectiveness of TCM formula preparations implies that the individual drugs are subjected to a chemical and botanical authenticity proof according to the methods described. The main constituents must be proven pharmacologically and toxicologically. The extracts should be manufactured in such a way that the active substances, which determine the effectiveness of the formula, are made available optimally. The fixed multidrug preparations should be composed according to the chemical–pharmacological and molecular–biological criteria described before. All herbal drug combinations used over the past centuries should be reassessed to determine whether they still conform to modern medical standards or should be adapted to improve their efficacy and safety.

13.3.2 Literature

Li et al. (2011), Tan et al. (2011), Xu et al. (2011), Zeng et al. (2012); Zhang et al. (2012a, 2012b).

13.4 Strategies for the Selection of Herbs for Drug Development

Part 2: Gudrun Ulrich-Merzenich

13.4.1 Computational Strategy for the Collection of Clinical Data and Decision Support on Herbal Drug Development

The history and future of TCM is based on a tremendous treasure of plants and formulas for drug development. Computational strategies are increasingly used for screening purposes. This may be exemplified in a recent work of Zhou et al. (2010). Authors proposed the development of a TCM clinical data "warehouse platform" for medical knowledge discovery and decision-making. Authors argue that the TCM knowledge is gained in the daily clinical experience and that their proposed project aims at extracting knowledge from daily practice as a first step to identify promising TCM drugs (combinations) for various kinds of diseases.

Research was undertaken to develop a program that is flexible enough to integrate data derived from case reports and individual prescriptions up to meta-analyses into one common information platform to extract basic information, which disease or symptom complex is treated with which herbal preparation(s) or other TCM treatment approaches. This compilation of key information is based on the assumption that there are mainly two types of empirical knowledge in the TCM clinical data: One is about how to perform a TCM diagnosis, and the other is about how to undertake TCM prescription.

The authors used their "clinical data warehouse" (CDW) already for the data collection and the organization of data. Their newly developed "Complex network analysis" (CAN) methods were applied to extract clinical knowledge. 20,000 TCM inpatient and 20,000 outpatient data sets, which contained manifestations (e.g., symptoms, physical examinations, and laboratory test results), diagnoses and prescriptions as the main information components were analyzed, e.g., to identify herb combination patterns from the clinical prescriptions.

The frequent formulas of herbs, the properties of herbs, and the therapeutic methods for a specific disease were explored. Results of a model analysis on the clinical diagnosis of patients are shown in Table 13.1, demonstrating the diagnostic challenge for an integration of TCM and Western medicine.

In an effort to identify herbs for the treatment of the *metabolic syndrome*, the same group of researchers selected 188 inpatient cases of Type 2 Diabetes mellitus

Table 13.1 Data on the frequency of diagnoses of patients classified according to western and Chinese diagnostic criteria

Modern disease: top 10 of 151		TCM disease: top 10 of 91		TCM syndrome: top 10 of 216	
Disease	*n*	Disease	*n*	Syndrome	*n*
Chronic gastritis	104	Thoraic obstruction of Qi	170	Qui Deficiency	215
Coronary heart diseases	66	Vertigo	130	Spleen deficiency	202
Hypertension	66	Stomach pain	105	Stagnation of liver Qi	154
Rheumatism	51	Palpitation	74	Dampness–heat blocking	126
Arrhythmia	49	Arthralgia	63	Yin deficiency	124
Urinary tract infections	42	Insomnia	37	Blood deficiency	123
Insomnia	36	Distention and fullness	33	Kidney deficiency	115
Cervical spondylosis	30	Common cold	32	Stomach disharmony	91
Sjögren's syndrome	26	Goiter	24	Qi stagnation	81
Cerebral circulation insufficiency	26	Headache	22	Dampness–turbid blocking	79

Diagnostic frequency in 1,135 Patients (according to Zhou et al. 2010)

affiliated Metabolic syndrome (DAMS), who had been treated with herb prescriptions, from the over 5,000 diabetes inpatient data according to the WHO standard. The data set showed a total of 752 different herb treatments which had been prescribed in the different encounters. The herb prescriptions used in total 320 different herbs (Zhou et al. 2010). The core herb combinations contained herbs such as *Chinese angelica* (Angelica sinensis (Oliv.) DIELS), *dwarf lilyturf tuber* (Ophiopogon japonicus (Thunb.) Ker-Gawl.), *milkvetch root* (Astralagus membranaceus (Fisch.) Bge.), *Chinese magnoliavine fruit* (Schisandra chinensis (Turcz.) Baill.; Schisandra sphenanthera (Rehd & Wils), and *figwort root* (Scophularia ningpoensis (Hemsl.)) Authors propose that this CDW platform could be a promising tool to make full use of the TCM clinical data for scientific hypothesis generation and to promote further the development of TCM from an individualized empirical knowledge to a large-scale evidence-based medicine.

They, however, also acknowledge that privacy and security issues are main challenges in clinical data sharing and data mining and will address the information content protection of both physicians and patients in future (Zhou et al. 2010).

Another challenge which can be derived from Table 13.1 has been addressed by Sun and Sun (2012). They analyzed clinical data sets of patients to evaluate the number of syndromes commonly involved in the diagnosis of one patient. Based on the analysis of 875 cases, more than 3 syndromes were involved in at least 94 % of the patients. Thus, computational approaches need to consider that prescriptions of herbs to patients are mostly developed for several simultaneous occurring syndromes.

Another computational approach, directly oriented towards the chemical structure and biological activity of drugs, is the pharmacophore-based virtual screening to support drug discovery and development. It is a well-established approach and increasingly used in natural product research for the identification of bioactive natural products including TCM drugs (Li et al. 2010; Rollinger 2009). Here it may represent a fast and cost-effective prescreening tool for drug development.

13.4.1.1 Conclusion

Computational approaches are presently used in clinical research and appear to be highly promising. However, they still need to be further developed in order to reflect the complexity of the TCM practice. Their practical utility still needs to be established. A combination with virtual screening approaches to identify after the herb (combination) selection also the most promising chemical structure with the desired pharmacological activity may in future support a time and cost-saving drug development in TCM.

13.4.1.2 Literature

Li et al. (2010), Rollinger et al. (2009), Yu and Liu (2010), Zhou et al. (2010), Sun and Sun (2012).

13.4.2 Meta-analyses of Clinical Data for Decision Support on Herbal Treatments

Summarizing results of clinical trials in the form of meta-analyses is another strategy to identify herbal drugs or drug combinations suitable for a future development. The primary approach for summarizing clinical data has been an indication-oriented one. However, trials can also be undertaken with the question "Which plant material/preparation is used successfully in which disease entity?"

The types of interventions commonly included in a meta-analysis of TCM drugs may be best demonstrated by the following common description:

> Traditional Chinese medicinal herbs (TCMHs) are defined as preparations derived from plants, or parts of plants, including single herbs or mixtures of different herbs. We included any types of preparation, such as decoction, oral liquid, tablet, capsule or powder. We also included single chemicals extracted from a plant, or synthetic chemicals based on plant constituents. Some of the studies were undertaken with conventional drugs as reference others were run versus placebos.

Table 13.2 shows recent examples of meta-analyses which have been published between 2008 and 2012. They cover more or less all organ systems and include the major world-wide disease challenges. We documented whether information on the herbal preparations was mentioned (QM: quality management of herbs) or was considered as parameter for scoring the quality of the RCTs. Even though more or less all studies were judged by their authors as methodologically doubtful, or as too heterogenous with no proper or no placebo control leading to the general recommendation that more controlled clinical trials are necessary and some authors even went to the extent to state that no robust evidence was provided for the claim that

Table 13.2 Examples of recent meta-analyses (2008-2012)

Authors/disease	Disease Spec.	Trials (*n*)	Patients (*n*)	Medication[c]	Outcome measures	AEs	QM herbs	Conclusions + trial quality as judged by authors
Alzheimer		9						
Fu and Li (2011)		6 RCT	976	7 herbs: 1.Gingko biloba (EGB 761), 2.Ginseng, 3.Huperzin(H. serrata) + others	Various (SKT, CT, MRI, ADAS-cog, ADCA-CGIC)	Yes	Yes	[a]1.-3.: limited positive evidence, current evidence inconclusive or inadequate[b] Recommendation: high quality studies (RCTs)
Cancer								
Meng et al. (2008)	UHCC	12 RT	1.008	TACE	Immune response	No	NA	Co-medication positive
				TACE + herbs	(CD3+, CD4+, Nk-cells)			Recommendation:
		9		TACE + Panax ginseng				Additional RTCs are needed
		10		TACE + Astragalus spec.				
Chen et al. (2010)	NSCLC	15	862	Chemotherapy	Short + long term	Yes	NA	NS
				Chemotherapy + herbs	Survival, quality of life			Co-medication positive
				(R.Ophiopogonis, R.Curcumae zedoariae, H.Oldenlandia diffusa)	+ others			Recommendation: More rigorous methodology trials are required
Dong et al. (2010)	NSCLC	29		Shenji fuzheng	Tumor response	Yes	C-FDA	Efficacy ↑, Toxicity ↓
		10 of HQ		(Injection) +	Toxicity AEs			Recommendation:
				Platinum-based Chemotherapy				Higher quality studies
Diabetes								
Grant et al. (2009)		CR: 16 trials	1.391	15 different herbal preparations	Blood glucose	Yes	No	Lack of uniform herbal interventions
				CHM vs. placebo, lifestyle, Metformin, Ascarbose,	Glucose tolerance			Lack of reporting of co-medication
				CHM + lifestyle vs. various interventions				General poor reporting Positive with above limitations
Diabetic neuropathy								
Chen et al. (2011)		39 RCTs	2.890	4 single herbs 8 patent medicines	Nerve conduction	NA	NA	Low methodological quality

(continued)

Table 13.2 (continued)

Authors/disease	Disease Spec.	Trials (*n*)	Patients (*n*)	Medication[c]	Outcome measures	AEs	QM herbs	Conclusions + trial quality as judged by authors
		18 self-controlled		Compound M				
Epilepsy								
Li et al. (2011)		5 short-term trials	1.125	1 formula of TCMH vs. no interventions 1 formula of TCMH vs. placebo 1 formula of TCMH vs. single western medication	Frequency and duration of seizure	Yes	Partially, not uniform	Insufficient evidence Recommendation: Much larger trials needed
Hepatitis B								
Tao et al.(2011)		138 RCTs	16.393	TCPMs ($n = 62$) 5 patent drugs	Antiviral Liver function Liver fibrosis	Yes (mild)	NA	64,30 % ↑ 79,10 % ↑ 29,99 % ↑ General criticism: Poor quality of trials
Qin et al. (2009)		51 RTs 6 of good quality	1.057	Herbal paste Bathing Fuming	Kidney function	NA	NA	Kidney function ↑, Poor design and reporting, RCTs should confirm results
Hypercholesterolaemia								
Liu et al. (2011)		22 RCTs	2.130	5 herbal preparations Xuezhikang (positive)	LDL-cholesterol	Yes	NA	Recommendation: further and higher quality as well as rigorously performed studies
Infertility (f)								
Ried and Stuart (2011)		8 RCTs	1.005	CHM vs. western medical drug treatments of IVF	Pregnancy	NA	NA	Positive for CHM Quality sufficient
Insomnia								
Yeung et al. (2012)		Evaluation: 8 of 217 RCTs	702	Formula: Gui Pi Tang Single herb: Zizibus vinjabu		Yes	NA	Number of trials (8): too low, Insufficient evidence Recommendation: Double-blind placebo controlled Studies are required

Kidney								
Zhang et al. (2012a)		23 trials	1.057	Herbal paste Bathing Fuming	Kidney function	NA	NA	Kidney function ↑ Low quality of trials and reporting No definite conclusion
Respiratory tract								
Wu et al. (2008)	ARTIs	6 Cochran-reviews		Mixed	Various	NA	NA	Recommendation: Repeat studies according to international standards
Li and Brown (2009)	Asthma	5 RCTs[a]		Multi-herbal formulas (3 to 10 Herbs): ASHMI, FAHF-2 Common: Radix glycyrrhizae	Various parameters of disease activity	Yes	Yes +++	Positive: ASHMI and FAHF-2 enter clinical studies in the USA
Stroke								
Li et al. (2009)		CR: 14 RCTs	962	Acanthopanax	Death, dependency	Yes	Injections + various drugs	Poor quality of trials Recommendation: Higher quality of trials required
Yang et al. (2009)		CR: 15 RCTs	1.280	Mailuoning	Death, dependency, Barthel Index	Yes	Injections, oral Liquid	No convincing evidence Recommendation Higher quality and large-scale RCTs required
Vascular dementia								
Hao et al. (2009)		1	14	Huperzine A	Cognitive function: MMSE	Yes	Yes	High quality evidence by RCTs required for conclusion

[a]Randomized controlled double blinded trial

[b]Jadad scale, [c]For details see original references.

Examples of recently published Meta-Analyses (2008–2012) on CHM covering a broad spectrum of diseases.

Abbreviations: *AD* Alzheimer's disease, *ADAS* AD-assessment scale, *AEs* adverse events, *ARTIs* acute respiratory tract infections; *C-FDA* "Chinese Food and Drug Administration", *CGIC* clinical global expression, *CHM* Chinese herbal medicine, *CR* cochrane review, *CT* computer tomography; *f* female, *I* intervention, *LDL* low density lipoprotein, *MMSE* mini-mental state examination, *NA* not available, *NSCLC* non small cell lung cancer, *QM* quality management *RCT* randomized controlled trial, *T* trial, *TACE* transcatheter arterial chemoembolization, *TCHMH* traditional Chinese medicinal herbs, *TCPM* traditional Chinese patent medicine, *UHCC* unresectable hepatocellular carcinoma, *SKT* Syndrome Kurz-Test, *VS* Virusstatika.+++ HPLC fingerprinting, Mass spectrometry, Quantitative analysis

TCM was effective for any indication (Jiang et al. 2010), the quality management (QM) of herbs was not a criteria considered for judging the quality of the trials.

Many authors used the Jadad Score (Jadad et al. 1996) as one of the most common tools for a fast quality assessment of a clinical trial. This score considers the following three questions:

1. Was the study described as randomized?
2. Was the study described as double blind?
3. Was there a description of withdrawals and dropouts? This score, however, was not developed for the specific challenges of trials in the field of phytopharmaceuticals.

As already mentioned by other authors (Wu et al. 2008; Yeung et al. 2012; Grant et al. 2009), any meaningful scientific evaluation of clinical data without thoroughly considering the prescribed herbal material (the variability in individual components of herbal preparations, the variability between different preparations of combination products, their identification both by the specific TCM names as well as the international recognized taxonomic names) is difficult to impossible. The FDA frequently relies on a combination of tests for phytopharmaceuticals when the active chemicals are not well defined compared to synthetic drugs. HPLC fingerprints, assays of characteristic markers, and biological assays are accepted methods to ensure the quality, potency, and consistency of botanical drugs. The FDA guidelines, make provision for sufficient quality and safety data at three levels—raw herbs, extracts (substance in FDA terminology) are made (Li and Brown 2009). Highly interesting may in this context be recent studies described by Li and Brown (2009) e.g., on ASHMI. ASHMI was developed from an original formula containing 14 herbs to a preparation which contains today only 3 plants (*Ganoderma lucidum*, *Sophora flavescens*, and *Glycyrrhiza uralensis*). It is used for the treatment of allergic asthma (Kelly-Pieper et al. 2009) and has been approved by the FDA for Phase II clinical trials.

Besides the detailed description of the plant material, further recommendations addressing the clinical trial design or its stakeholders have been made by Yeung et al. (2012) and others:

1. Investigators conducting RCTs should have formal training about clinical trial design
2. Registration of clinical trials and publishing their protocols in recognized trial registration platforms
3. Collaboration between researchers in different fields
4. Development and implementation of good agricultural practice, good manufacturing practice, and good clinical practice in CHM research
5. A revised CONSORT checklist should be used in reporting RCTs of CHM
6. Standardized rating scales for the documentation of adverse events should be used

7. The long-term safety of Chinese herbs, the effects of overdose, and their use in pregnancy and during lactation need to be recorded
8. More pharmacodynamic and pharmacokinetic studies of the different CHM interventions are needed

13.4.2.1 Conclusion

Critical judgments regarding the present value of meta-analyses for decision-making supports are obviously endorsed by many Chinese authors as well as authors of other nationalities. It is highly recommended to include "quality standards for the description of the applied plants or their preparations" into the quality criteria for clinical studies investigating CHM. These quality requirements have, e.g., already been formulated by the scientific community dealing with research in plants in the form of authors' guidelines for publications in international scientific journals (e.g., Phytomedicine, Journal of Ethnopharmacology, Planta medica, Journal of Natural product research, Fitotherapia, Phytotherapy research). They have also been formulated by regulatory agencies (EMEA, FDA) to a variable extent. Recently "guidelines for clinical trials for CHM" as part of an (FP7) EU program to promote the integration of Chinese medicine into Western medicine have been published (Flower et al. 2012). When such standards are implemented, RCTs and meta-analyses of clinical trials will have a much higher predictability and scientific value.

13.4.2.2 Literature

Bian et al. (2006a, b, c), Cheng et al. (2008), Grant et al. (2009), Flower et al. (2012), Jadad et al. (1996), Jiang et al. (2010), Kelly-Pieper et al. (2009), Leung et al. (2006), Li and Brown (2009), Manheimer et al. (2009), Paolino (2007), Wu et al. (2007a, c), Wu et al. (2008), Yeung et al. (2011).

13.4.3 Design of a "Placebo" Control for Randomized Controlled Trials in TCM

Further studies are required to determine and assure appropriate types of control groups. This leads to another major issue:

One of the challenges of research in TCM is the design of a placebo control for a herbal formulation especially if information from the traditional application of a decoction should be collected. A highly interesting study has been undertaken by Flower et al. (2011) in a clinical trial for endometriosis. Herbal formulae (CHM or placebos) were precooked (industrially) and dispensed as individual doses in sealed plastic sachets. This permitted the development and testing of a plausible placebo decoction. The production of an inert, strong tasting, plausible herbal placebo was

challenging and mounted in the following composition for this trial: Chicory (*Cichorium intybus*), Lemon verbena (*Aloysia triphylla*), Coriander (*Coriandrum sativum*), Cabbage (*Brassica oleracea*), Sweet corn (*Zea mays* var. *rugosa*) replaced by puy lentils (*Lens culinaris*),Turnip (*Brassica rapa* var. *rapa*), Peas (*Pisum sativum*), and Leek (*Allium ampeloprasum* var. *porrum*).

Even though "food items" are criticized for not being a real placebo, such studies will have the advantage of establishing a better understanding which of the TCM herbal formulations are true medical interventions and which can be justifiably termed food items at a very early stage of drug development.

In addition, this method may allow a better comparison of treatment effects obtained from decoctions to effects derived from other methods of administering Chinese herbal medicine such as encapsulated herbal powders. The aim will be to find the optimum method of TCM drug delivery for each composition including dosage-related issues.

13.4.4 Literature

Fai et al. (2011), Flower et al. (2011), Lewith et al. (2009), Qi et al. (2008).

13.4.5 New Drug Delivery Methods

13.4.5.1 Injections

Another rather new development is the preparation of herbal injections. A number of clinical studies on four kinds of herbal injections can be found in the international literature. These are *Mailuoning, Acanthopanax, β-Elemene*, and *Shenque Fuzheng*. They will be discussed more in detail since so far they are internationally hardly known.

13.4.5.2 Mailuoning

Mailuoning is a compound prepared from a traditional formula of Chinese medicines and widely used as an antithrombotic agent. It is in clinical practice since 1985 (Wang and Lai 1997) and was identified as a Chinese emergency medicine by the State Administration of Traditional Chinese Medicine in 1993 (Zhao 1993). It is produced by extracting Dendrobium spec., Radix Scrophulariae (Scrophularia ningpoenis), Flos Lonicerae (Lonicera (L.) hypoglauca, L. confusa or L. macranthoides), and Radix Achyranthis Bidentatae. Scoparone and Ayapin are regarded the major effective components (Zhu et al. 1992). According to the manufacturer 10 or 20 ml at a time are to be added into 250 ml of 5 % or 10 % glucose injection liquid or 0.9 % sodium chloride injection liquid for vein

drop injection, once a day, and shall be given 10–14 days to adults. A recent meta-analysis documented the treatment of cases of ischemic stroke with Mailuoning *injections or oral liquids*. Fifteen trials were evaluated with no convincing evidence and authors recommended that larger and more high-quality RCTs are required (Yang et al. 2009).

As another application, *peridural injections* of Mailuoning Compound Liquor (MCL), were given to 100 cases of prolapse of lumbar intervertebral disc PLID—once a week with four sessions constituting a therapeutic course (Zhi et al. 2009).

Besides efficacy trials, the number of clinical trials in humans to identify plasma markers and to determine the pharmacokinetics for "Mailuoning" is increasing (Yu et al. 2011, Zhang et al. 2010a, b).

13.4.5.3 Acanthopanax

Acanthopanax is one of the most widely used Chinese herbal medicines. The official drug is Acanthopanax (Eleutherococcus) senticosus (Rupr. et Maxim.) Harms, commonly known as "Siberian ginseng." The alcoholic extracts of the dried roots are used as drug. The main constituents are lignans (eleutherosides and their glycosides) and phenylpropan derivates (Wagner et al. 2011). Eleutheroside B (syringin) and eleutheroside E are two major glycosides (Deyama et al. 2001; Fan et al. 2003; Wang et al. 2003).

Like Maiuoling, Acanthopanax injections are used for the treatment of stroke. A recent meta-analysis (Cochrane review) (Li et al. 2009a, b) has analyzed its efficacy. In total 13 trials were analyzed. In all trials acanthopanax in combination with another treatment was compared with the other treatment alone. Acanthopanax injections ranged from 30 to 250 ml per day. The course of treatment ranged from 10 to 30 days. Authors judged the risk of bias in all included trials as too high to draw reliable conclusions about the efficacy of acanthopanax in the treatment of stroke. Much larger trials of more stringent methodological quality were requested.

Nevertheless, also several studies on the safety of Acanthopanax injections have been carried out. Fu Zhuang reported from the Hainan Sanya municipal Hospital of traditional Chinese medicine a total of 48 adverse drug reaction (ADR) cases induced by acanthopanax injections which had been reported from clinical departments of internal medicine, surgery, and gynecology obstetrics since 2002. The ADRs were characterized by drug fever, anaphylactic shock, drug eruption, etc. The author concluded that the ADRs of acanthopanax injections vary and can even result in severe ADRs; yet the related reports are few (in international literature). Clinicians and the nurses should attach great importance to the potential ADRs of acanthopanax injections (Zhong 2009).

13.4.5.4 β-Elemene (Curcuma wenyujin)

Herbal injections of β-Elemene, a natural plant drug extracted from Curcuma wenyujin, have shown a strong antiglioblastoma effect (Zhu et al. 2011) and are used to treat solid tumors. Elemene injections with h-elemene as the main ingredient have been manufactured by DaLian JinGang Pharmacy Ltd. of China since 1995 after the State Administration of Pharmacy of China and Ministry of Health People's Republic of China approved it as a medical treatment in clinical practice.

Since Elemene injection was introduced in the middle of the 1990s, a large number of clinical trials of Elemene injection, including some reports of randomized controlled trials (RCTs), were published in many Chinese academic publications (Peng et al. 2006). A meta-analysis (cochrane review) of 127 trials has been performed by Peng et al. (2006). Authors judged the quality of the RTCs according to the CONSORT guideline and the Jadad scale and concluded that the overall quality of clinical trials still lags behind the requirements of GCP development in China in 1998. The study shows that the methodological quality of RCTs of Elemene injection against malignant tumors was low. There is obviously a need to supervise and urge researchers to conform to GCP (http://www.sdatc.com/wlpx.htm) in clinical trials and to CONSORT statement (http://www.consort-statement.org) while reporting (Peng et al. 2006).

13.4.5.5 Shenqi Fuzheng

Platinum-based chemotherapy has been a standard therapy for advanced non-small cell lung cancer (NSCLC), but it possesses a high toxicity (Dong et al. 2010). In China, Shenqi Fuzheng (SF) a newly developed injection concocted from Chinese medicinal herbs has been reported to increase efficacy and reduce toxicity when combined with platinum-based chemotherapy, but little is known about it outside China (Dong et al. 2010). Shenqi Fuzheng is prepared from two kinds of Chinese medicinal plants: Radix Astragali (root of Astragalus membranaceus; Chinese name: huangqi) and Radix Codonopsis (root of Codonopsis pilosula; Chinese name: dangshen) (Yang et al. 2009; Lu et al. 2005), approved by the State Food and Drug Administration of the People's Republic of China in 1999 primarily as an antitumor injection to be manufactured and marketed in China (Pan 2009; Zhong 2009). A recent meta-analysis was carried out on 29 RCTs by Dong et al. (2010). As with most other meta-analyses, the quality of studies was assessed by the modified Jadad's scale. Authors concluded that SF intervention appears to be useful to increase efficacy and reduce toxicity when combined with platinum-based chemotherapy for advanced NSCLC, although this result needs to be further verified by more rigorous studies.

13.4.5.6 Conclusion

Herbal injections are manufactured and used, solely in China, primarily in the treatment of stroke and certain cancers. Outside of China nothing is known about the rationale behind this form of application. None of the meta-analyses published in international journals reported any convincing evidence for an effectiveness of this application form. On the contrary, the number of studies reporting severe side effects, including anaphylactic shocks, are increasing. Considering the non-sophisticated description of the requirements for herbal injections in the Chinese pharmacopeias, this is not astonishing. To name just two issues pertaining to plant extracts: safety evaluation criteria regarding the removal of proteins or the removal of known toxic plant ingredients are missing. Authors urge researchers to conform to GMP, GCP and to the CONSORT statement as also demanded by several Chinese researchers.

13.4.5.7 Literature

Chen et al. (2012), Deyama (2001), Dong et al. (2010), Fan (2003), Fei et al. (2008a, b), Fu Zhuang (2009), Lu and Lu (2006), Jadad et al. (1996), Pach et al. (2002), Pan (2009), Peng et al. (2005), Yang and Xu (2004), Wang (1997, 2003), Wang et al. (2012), Zhao (1993), Zhi et al. (2009), Zhong (2009), Zhu (1992), Zhu et al. (1999, 2011).

13.5 "Omic" Technologies in Contemporary TCM

The major strategy to arrive at a contemporary TCM from the pharmaceutical point of view has so far been bioassay-guided plant screenings. However, looking at the mass bioprospecting program of the national cancer institute of the United States, which screened not only Chinese plants but also approximately 114,000 extracts from an estimated 35,000 plant samples against a number of tumor systems, the yield regarding the identification of single bioactive compounds appears to be rather moderate with so far only five (taxol, topotecan, CPT-11, and derivates of camptothecin) clinically significant chemotherapeutic agents (Ulrich-Merzenich et al. 2007). The more recent development of the "omic" technologies allows, however, the so-called "system biology" approach which is more adequate for the investigation of a complex medical system like TCM.

The technology platforms of genomics, proteomics, metabolomics, and metabonomics are high throughput technologies. These technologies increase substantially the number of genes, proteins, metabolites, and small metabolites that can be detected and examined simultaneously and have the potential to relate complex mixtures to complex effects in the form of gene/protein expression profiles. They will be useful

for the chemical and pharmacological standardization and the proof of the toxicological potential of plant extracts. Over a long-term perspective they may economize the proof of efficacy as well as the determination of the mode of action of phytomedicine in general and specifically for TCM and allow even to investigate herbal extracts without prominent active principles. It can be expected that the combination with reproducible gene and protein expression profiles will support the development of causality-based phytotherapy and thus also TCM.

Several recent reviews are dedicated to the potential and recent achievements of these methodologies for the research and understanding of TCM drugs (e.g., Buriani et al. 2012; Zhang et al. 2010a, b). A systematic barcoding of plant species for plant authentication (Heubl 2010, Chap. 2) is one of the first outcomes. Several new biomarkers have been identified. With respect to the mode of action of TCM drugs, an increasing number of investigations, mainly by in vitro models like cell cultures, are undertaken and yield new insights into the (potential) mode of action of various formulas (see reviews for detailed results)—including a better understanding of synergy effects of the different components of herbal formulas in the context of a network pharmacology.

However, the reproducibility of the new technologies—e.g., gene and protein arrays considering extract variations due to seasonal or batch-related fluctuations—still needs to be systematically evaluated. Those data, including the use of the methodology in toxicology screening, will decide how easy and how soon we can implement these technologies in the routine standardization process and how far legislation can provide appropriate framework conditions for these new developments (Ulrich-Merzenich et al. 2007, 2009).

13.5.1 Conclusion

The "omic"-technologies are a highly promising tool to understand the mode of action of complex mixtures with their different targets and feedback loops. Nevertheless, presently we are (only) far advanced with the data collection, but still without a thorough conceptual framework for the integration of the complexity of these data. Methodological challenges include the establishment of the reproducibility of results obtained by different methodological standards, the target selection, its validation, and the scale of precision. Integration of the profiles of single plant components and complex extract mixtures into the international freely accessible data banks of genetic, proteomic, and metabolomic screens will be essential for a systematic and sustainable development to further advance Chinese herbal medicine and to thereby enrich modern pharmacotherapy.

13.6 Final Comments

As already stated in the introduction, there has been a tremendous amount of promising research undertaken over the past 10 years to reach the goal of an evidence-based and contemporary TCM. Due to the complexity of the task, the present status may be described as "growing awareness of the challenges" and an increasingly detailed description of the future scope of work. However, already now TCM along with the other formerly called "alternative systems" of medicine has influenced and challenged "conventional" medicine with its concepts of an individualized treatment with its herbal medicine consisting of complex multicomponent mixtures and their multitargeting potentials as well as with first evidences that the combination of CHM with Western medication may be an option to reduce the frequency of side effects in defined indications. A prerequisite to achieve a further and true cooperation and integration of both medical systems will be the further preclinical and clinical cooperation in multidisciplinary and multinational teams from China and the West supported by appropriate resources on all levels. This will make medicine richer and will deliver deeper insights into fundamental questions of life.

References

Bauer R, Franz G (2010) Modern European monographs for quality control of Chinese herbs. Planta Med 76:2004–2011

Bian ZX, Li YP, Moher D, Dagenais S, Liu L, Wu TX, Miao JX, Kwan AK, Song L (2006a) Improving the quality of randomized controlled trials in Chinese herbal medicine, part I: clinical trial design and methodology. Zhong Xi Yi Jie He Xue Bao 4:120–129

Bian ZX, Moher D, Dagenais S, Li YP, Liu L, Wu TX, Miao JX (2006b) Improving the quality of randomized controlled trials in Chinese herbal medicine, part II: control group design. Zhong Xi Yi Jie He Xue Bao 4:130–136

Bian ZX, Moher D, Dagenais S, Li YP, Wu TX, Liu L, Miao JX, Song L, Zhang HM (2006c) Improving the quality of randomized controlled trials in Chinese herbal medicine, part IV: applying a revised CONSORT checklist to measure reporting quality. Zhong Xi Yi Jie He Xue Bao 4:233–242

Buriani A, Garcia-Bermejo ML, Bosisio E, Xu Q, Li H, Dong X, Simmonds MS, Carrara M, Tejedor N, Lucio-Cazana J, Hylands PJ (2012) Omic techniques in systems biology approaches to traditional Chinese medicine research: present and future. J Ethnopharmacol 140:535–44

Butterweck V, Nahrstedt A (2012) What is the best strategy for preclinical testing of botanicals? A critical perspective. Planta Med 78:747–754

Chan K, Shaw D, Simmonds MS, Leon CJ, Xu Q, Lu A, Sutherland I, Ignatova S, Zhu Y-P, Verpoorte R, Williamson EM, Duez P (2012) Good practice in reviewing and publishing studies on herbal medicine, with special emphasis on traditional Chinese medicine and Chinese materia medica. J Ethnopharmacol 140:469–475

Chen S, Flower A, Ritchie A, Liu J Molassiotis A, Yu H, Lewith G (2010) Lung Cancer 68: 137–145

Chen W, Luo YF, Liu JP (2011) Topical herbal medicine for treatment of diabetic peripheral neuropathy: a systematic review of randomized controlled trials. Forsch Komplementmed 18:134–145

Chen L, Titch T, Luo Z, Xu Y, Li X, Huang F, Liu L, Yiao W, Wu H, Wang Z (2012) Confirmation of a proarrhythmic risk underlying the clinical use of common Chiniese herbal intravenous injections. J Ethnopharmacol 142:829–835

Cheng CW, Bian ZX, Li YP, Moher D, Wu TX, Dagenais S, Li J, Li TQ (2008) Transparently reporting adverse effects of traditional Chinese medicine interventions in randomized controlled trials. Zhong Xi Yi Jie He Xue Bao 6:881–886

Cheng CW, Bian ZX, Zhu LX, Wu JC, Sung JJ (2011) Efficacy of a Chinese herbal proprietary medicine (Hemp Seed Pill) for functional constipation. Am J Gastroenterol 106:120–129

Deyama T, Nishibe S, Nakazawa Y (2001) Constituents and pharmacological effects of Eucommia and Siberian ginseng. Acta Pharmacol Sin 22:1057–1070

Dong J, Su SY, Wang MY, Zhan Z (2010) Shenqi fuzheng, an injection concocted from Chinese medicinal herbs, combined with platinum-based chemotherapy for advanced non-small cell lung cancer: a systematic review. J Exp Clin Cancer Res 29:137

Duan B, Huang L, Chen S (2012) Study on the destructive effect to inherent quality of Fritillaria thunbergii Miq. (Zhebeimu) by sulfur-fumigated process using chromatographic fingerprinting analysis. Phytomedicine 19:562–568

Efferth T (2011) Perspectives for globalized natural medicines. Chin J Nat Med 9(1):1–6

Eisenberg DM, Harris ES, Littlefield BA, Cao S, Craycroft JA, Scholten R, Bayliss P, Fu Y, Wang W, Qiao Y, Zhao Z, Chen H, Liu Y, Kaptchuk T, Hahn WC, Wang X, Roberts T, Shamu CE, Clardy J (2011) Developing a library of authenticated Traditional Chinese Medicinal (TCM) plants for systematic biological evaluation - Rationale, methods and preliminary results from a Sino-American collaboration. Fitoterapia 82:17–33

Fai CK, Qi GD, Wei DA, Chung LP (2011) Placebo preparation for the proper clinical trial of herbal medicine-requirements, verification and quality control. Recent Pat Inflamm Allerg Drug Discov 5:169–174

Fan L, Jiang X, Yao G (2003) Studies of acanthopanax injection. Chin Traditional Patent Med 25:58–60

Fei YT, Liu JP (2008a) Herbal CONSORT statement and standardization of reporting traditional Chinese drug trials. Zhongguo Zhong Yao Za Zhi 33:89–94

Fei YT, Liu JP (2008b) Improving the quality of reporting Chinese herbal medicine trials: an elaborated checklist. Zhong Xi Yi Jie He Xue Bao 6:233–238

Flower A, Liu JP, Chen S, Lewith G, Little P (2009) Chinese herbal medicine for endometriosis. Cochrane Database Syst Rev CD006568

Flower A, Lewith G, Little P (2011) Combining rigour with relevance: A novel methodology for testing Chinese herbal medicine. J Ethnopharmacol 134:373–378

Flower A, Witt C, Liu JP, Ulrich-Merzenich G, Muir K, Yu H, Prude M, Lewith G (2012) GP-TCM Guidelines for randomized controlled trials investigating Chinese herbal medicine (CHM). J Ethnopharmacol 140:550–554

Fu Zhuang (2009) (Dept. of Pharmaceuticals & Machinery, Hainan Sanya Municipal Hospital of Traditional Chinese Medicine, Sanya 572000, China)

Fu LM, Li JT (2011) A systematic review of single Chinese herbs for Alzheimer's disease treatment. Evid Based Complement Alternat Med 2011:640284

Grant SG, Bensoussan A, Chang D, Kiat H, Klupp NL, Liu JP, Li X (2009) Chinese herbal medicines for people with impaired glucose tolerance or impaired fasting blood glucose, Cochrane Database Syst Rev. 4: CD006690, doi:10.1002/14651858.CD006690.pub2

Hao Z, Liu M, Liu Z, Lv D (2009) Huperzine A for vascular dementia, Cochrane Database Syst Rev. 2: CD007365

Heubl G (2010) New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. Planta Med 76:1963–1975

Heubl G (2012), Chapter 2 in Wagner H, Ulrich-Merzenich G (Eds) Evidence and rational based research on Chinese drugs, Springer Wien New York

Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJM, Gavaghan DJ, McQuay HJ (1996) Assessing the quality of reports of randomized clinical trials: Is blinding necessary? Control Clin Trials 17:1–12

Jiang M, Yang J, Zhang C, Liu B, Chan K, Cao H, Lu A (2010) Clinical studies with traditional Chinese medicine in the past decade and future research and development. Planta Med 76:2048–2064

Kelly-Pieper K, Patil SP, Busse P, Yang N, Sampson H, Li XM, Wisnivesky JP, Kattan M (2009) Safety and Tolerability of an Antiasthma Herbal Formula (ASHMI) in Adult Subjects with Asthma: A Randomized, Double-Blinded, Placebo-Controlled, Dose-Escalation Phase I Study. J Altern Complement Med 15:735–743

Leung KS, Bian ZX, Moher D, Dagenais S, Li YP, Liu L, Wu TX, Miao JX (2006) Improving the quality of randomized controlled trials in Chinese herbal medicine, part III: quality control of Chinese herbal medicine used in randomized controlled trials. Zhong Xi Yi Jie He Xue Bao 4:225–232

Lewith G, Barlow F, Eyles C, Flower A, Hall S, Hopwood V, Walker J (2009) The context and meaning of placebos for complementary medicine. Forsch Komplementmed 16:404–412

Li M, Brown LV (2009) Efficacy and mechanisms of action of traditional Chinese medicines for treating asthma and allergy. J Allergy Clin Immunol 123:297–306

Li HY, Cui L, Cui M (2009a) Hot topics in Chinese herbal drugs research documented in PubMed/MEDLINE by authors inside China and outside of China in the past 10 years: based on co-word cluster analysis. J Altern Complement Med 15:779–785

Li W, Liu M, Feng S, Wu B, Zhang S, Yang W, Liu GJ (2009) Acanthopanax for acute ischaemic stroke, Cochrane Database Syst Rev 3: CD007032, DOI:10.1002/14651858.CD007032.pub2

Li XJ, Kong DX, Zhang HY (2010) Chemoinformatics approaches for traditional Chinese medicine research and case application in anticancer drug discovery. Curr Drug Discov Technol 9:22–31

Li JC, Shen XF, Meng XL, Zhang Y, Lai XR (2011) Analgesic effect and mechanism of the three TCM-herbal drug-combination Tou Feng Yu pill on treatment of migraine. Phytomedicine 18:788–794

Liang YZ, Xie PS, Chan K (2010) Perspective of chemical fingerprinting of Chinese herbs. Planta Med 76:1997–2003

Liu ZL, Liu JP, Zhang AL, Wu Q, Ruan Y, Lewith G, Visconte D (2011) Chinese herbal medicines for hypercholesterolemia. Cochrane Database Syst Rev 7:CD008305

Lu Y, Lu YX (2006) Clinical application and pharmacology function of Shenqi fuzheng injection. Li Shi Zhen Medicine and material medica research 17:2083–2085

Lu JG, Pan YB, Cao YQ, Yang W, Zhang XL (2005) Discussion on design and quality control of clinical trials of traditional Chinese medicine on the basis of Fuhuang Tablets clinical study. Zhong Xi Yi Jie He Xue Bao 3:191–194

Luo H, Li Q, Flower A, Lewith G, Liu J (2012) Comparison of effectiveness and safety between granules and decoction of Chinese herbal medicine: a systematic review of randomized clinical trials. J Ethnopharmacol 140:555–567

Manheimer E, Wieland S, Kimbrough E, Cheng K, Berman BM (2009) Evidence from the Cochrane Collaboration for Traditional Chinese Medicine therapies. J Altern Complement Med 15:1001–1014

Meng MB, Cui YL, Guan YS, Ying Z, Zheng MH, Yuan CK, Zhang RM (2008) Traditional Chinese medicine plus transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma. J Altern Complement Med 14:1027–1042

Pach D, Willich SN, Becker-Witt C (2002) Availability of research results on traditional Chinese pharmacotherapy. Forsch Komplementaermed Klass Naturheilkd 9:352–358

Pan L (2009) Practical road of "the numeral turn Chinese herbal medicine" for Shenqi fuzheng injection. J China Prescription Drug 1:37–39

Paolino A (2007) Validity of Chinese herbal medicine called into question. Lancet Oncol 8:196

Peng X, Zhao Y, Liang X, Wu L, Cui S, Guo A, Wang W (2006) Assessing the quality of RCTs on the effect of beta-element, one ingredient of a Chinese herb, against malignant tumors. Contemp Clin Trials 27:70–82

Pharmacopoeia of the People's Republic of China, English Edition Vol. I, 2005, Appendix II p. A-24 – A-25; 2010 Appendix II p. A-25 – A-27, People's Medical Publishing House, Beijing

Qi GD, We DA, Chung LP, Fai CK (2008) Placebos used in clinical trials for Chinese herbal medicine. Recent Pat Inflamm Allergy Drug Discov 2:123–127

Qin XK, Han M, Liu JP (2009). Compound Chinese herbal medicines, Chinese herbal drugs and their active extracts for treatment of chronic hepatitis C: a systematic review and meta-analysis of randomized clinical trials. J Chin Integr Med 7(10):913–928.

Ried K, Stuart K (2011) Efficacy of traditional Chinese herbal medicine in the management of female infertility: a systematic review. Complement Ther Med 16(6):319–31

Rollinger JM (2009) Accessing target information by virtual parallel screening – The impact an natural product research. Phytochem Lett 2:53–58

Singhuber J, Zhu M, Prinz S, Kopp B (2009) Aconitum in traditional Chinese medicine: a valuable drug or an unpredictable risk? J Ethnopharmacol 126:18–30

Sun X, Sun B (2012) TCM clinical Syndromes and the TCM theoretical system mathematic basics it reflects. 2nd Forum on Sino-European Cooperation and Development of TCM; Munich 2012

Tan Y, Kamal MA, Wang ZZ, Xiao W, Seale JP, Qu X (2011) Chinese herbal extracts (SK0506) as a potential candidate for the therapy of the metabolic syndrome. Clin Sci (Lond) 120:297–305

Ulrich-Merzenich G, Zeitler H, Jobst D, Panek D, Vetter H, Wagner H (2007) Application of the "Omic"-technologies in phytomedicine. Phytomedicine 14:70–82

Ulrich-Merzenich G, Zeitler H, Panek D, Wagner H, Vetter H (2009) New perspectives for synergy research with the "omic"-technologies, Part II. Phytomedicine 16:495–508

Vlietinck A, Pieters L, Apers S (2009) Legal requirements for the quality of herbal substances and herbal preparations for the manufacturing of herbal medicinal products in the European Union. Planta Med 74:683–688

Wagner H (2012) Chapter 1 in Wagner H, Ulrich-Merzenich G (eds) Evidence and rational based research on Chinese drugs. Springer, Wien New York

Wang M, Liu J, Zhou B, Yu R, Tao L, Ji M, Zhu L, Jiang J, Shen J, Gui X, Gu L, Bai W, Sun W, Cheng J. (2012) Acute and sub-chronic toxiity studies of Danshen injection in Sprague-Dawley rats. J Ethnopharmacol 141:96–103

Wagner H, Bauer R, Melchart D, Xiao pei-gen, Staudinger A (2011) Chromatographic fingerprint analysis of herbal medicines – thin-layer and high performance liquid chromatography of Chinese drugs, Vol. I 1–461; Vol. II 467–1019, Springer Wien New York

Wang DJ, Lai RJ (1997) Progressing in pharmacological and clinical research of mailuoning. J Chin Microcirculation 1:48–50

Wang Z, Lin J, Zhang Z (2003) Studies on constituents and pharmacological effects of acanthopanax senticosus. J Chin Med Mater 26:603–606

Wu B, Liu M, Liu H, Li W, Tan S, Zhang S, Fang Y (2007a) Meta-analysis of traditional Chinese patent medicine for ischemic stroke. Stroke 38:1973–1979

Wu KM, Farelly JG, Upton R, Chen J (2007b) Complexities of the herbal nomenclature system in traditional Chinese medicine (TCM): Lessons learned from the misuse of Aristolochia-related species and the importance of the pharmaceutical name during botanical drug product development. Phytomedicine 14:273–279

Wu T, Zhang J, Qiu Y, Xie L, Liu GJ (2007) Chinese medicinal herbs for the common cold, Cochrane Database Syst Rev 1: CD004782

Wu T, Yang X, Zeng X, Poole A (2008) Traditional Chinese medicine in the treatment of acute respiratory tract infections. Respir Med 102:1093–1098

Xu F, Peng D, Tao C, Yin D, Kou J, Zhu D, Yu B (2011) Anti-depression effects of Danggui-Shaoyao-San, a fixed combination of Traditional Chinese Medicine, on depression model in mice and rats. Phytomedicine 18:1130–1136

Yang LR, Xu XY (2004) Clinical application and curative effect evaluation of Shenqi fuzheng injection. Heilongjiang J Traditional Chin Med 4:46–48

Yang W, Hao Z, Zhang S, Dong W, Wu T, Liu GJ, Liu M (2009) Mailuoning for acute ischaemic stroke, Cochrane Database Syst Rev 2: CD007028

Yeung WF, Chung KF, Man-Ki Poon M, Yan-Yee Ho F, Zhang SP, Zhang ZJ, Tat-Chi Ziea E, Wong VT (2012) Chinese herbal medicine for insomnia: A systematic review of randomized controlled trials, Sleep Med Rev (ahead of print) doi:10.1016/j.smrv.2011.12.005

Yu H, Liu JP (2010) A qualitative study of exploring the therapeutic components of traditional Chinese medicine as a complex intervention through grounded theory. Zhong Xi Yi Jie He Xue Bao 8:928–943

Yu L, Li Y, Fan H, Duan J, Zhu Q, Li S (2011) Analysis of marker compounds with anti-platelet aggregation effects in Mailuoning injection using platelet binding assay combined with HPLC-DAD-ESI-MS and solid-phase extraction technique. Phytochem Anal 22:87–93

Yuan HL, Zhang TT, Xiao XW (2011) Quality control approaches for Chinese herbal medicines. Chin Herbal Med 3:17–22

Zeng KW, Zhang T, Fu H, Liu GX, Wang XM (2012) Modified Wu-Zi-Yan-Zong prescription, a traditional Chinese polyherbal formula, suppresses lipopolysaccharide-induced neuroinflammatory processes in rat astrocytes via NF-κB and JNK/p38 MAPK signaling pathways. Phytomedicine 19:122–129

Zhang J, Chen W, Ju W, Liu F, Tan H (2010a) Determination of plasma concentration of cinnamic acid by LC-MS-MS and study of pharmacokinetics in healthy volunteers after mailuoning injection. Zhongguo Zhong Yao Za Zhi 35:1887–1891

Zhang T, Srivastava K, Wen MC, Yang N, Cao J, Busse P, Birmingham N, Goldfarb J, Li XM (2010b) Pharmacology and immunological actions of a herbal medicine ASHMI on allergic asthma. Phytother Res 24:1047–1055

Zhang H, Ho YF, Che CT, Lin ZX, Leung C, Chan LS (2012a) Topical herbal application as an adjuvant treatment for chronic kidney disease - a systematic review of randomized controlled clinical trials. J Adv Nurs 68:1679–1691

Zhang WL, Zheng KY-Z, Zhu KY, Zhan JY-X, Bi CW-C, Chen JP, Du CY-Q., Zhao K-J, Lau DT-W, Dong TT-X, Tsim K (2012b) Chemical and biological assessment of Angelica herbal decoction: Comparison of different preparations during historical applications. Phytomedicine 19:1042–1048

Zhao LX (1993) The first batch emergency medicine identified by State Administration of Traditional Chinese Medicine. J Chin Clin 4:53

Zhao Z, Liang Z, Chan K, Lu G, Lee EL, Chen H, Li L (2010) A unique issue in the standardization of Chinese materia medica: processing. Planta Med 76:1975–1986

Zhao S, Liu E, Wei K, Lu S, Chu Y, Li Y, Wang Y, Huang B, Chen Y, Yang P (2011) Interferon plus Chinese herbs are associated with higher sustained virological response than interferon alone in chronic Hepatitis C: a meta-analysis of randomised trials. Antiviral Res 89:156–164

Zhi MX, Zhang GB, Hou JC, Yang YH, Wan ZZ (2009) Peridural injection of Mailuoning compound liquor for treatment of prolapse of lumbar intervertebral disc in 100 cases. J Tradit Chin Med 29:6–8

Zhong ZH (2009) Dictate history: A national new medicine that come from experiential prescription. J China Prescript Drug 1:33–36

Zhou X, Chen S, Liu B, Zhang R, Wang Y, Li P, Guo Y, Zhang H, Gao Z, Yan X (2010) Development of traditional Chinese medicine clinical data warehouse for medical knowledge discovery and decision support. Artif Intell Med 48:139–152

Zhu RZ, Jia XB, Gao JS (1992) Research on chemical components of mailuoning injection. Chin Traditional Patent Med 14:35

Zhu Y, Cai W, He Y, Xu J (1999) Observation on curative effects of acanthopanax root injection on treating acutely deteriorative phase of cor pulmonale. Chin J Integr Traditional Western Med Intensive Criti Care 6:502–504

Zhu T, Zhao Y, Zhang J, Li L, Zou L, Yao Y, Xu Y (2011) β-Elemene inhibits proliferation of human glioblastoma cells and causes cell-cycle G0/G1 arrest via mutually compensatory activation of MKK3 and MKK6. Int J Oncol 38:419–426

Zhuo Q, Yang X, Wu T, Liu G, Zhou L (2008) Tongxinluo capsule for acute stroke, Cochrane Database Syst Rev 4: CD004584

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