Tariq Aftab · Jorge F.S. Ferreira
M. Masroor A. Khan · M. Naeem *Editors*

Artemisia annua - Pharmacology and Biotechnology

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Chapter 1
How Techniques of Herbal Drug Preparation Affect the Therapeutic Outcome: Reflections on Qinghao 青蒿 (Herba *Artemisiae annuae*) in the History of the Chinese Materia Medica

Elisabeth Hsu

Abstract This chapter summarises an earlier study that detailed in chronological order the translation of all the entries on *qinghao* 青蒿 (and its synonyms *caohao* 草蒿, *chouhao* 臭蒿, *huanghuahao* 黃花蒿, etc.) that Frederic Obringer and I could locate in the premodern Chinese *materia medica* (*bencao* 本草) in the time period between 168 BCE and 1596. The aim of that study (Hsu in Plants, health and healing: Berghahn, Oxford, pp 83–130, 2010) was threefold: it aimed to make a contribution to ethnobotany, the history of Chinese medicine and herbal medical practice. It underlined, first and foremost, that 'herbal' medications are not to be conceived of as 'natural' 'herbs' but as cultural artefacts: the entries on *qinghao* in the Chinese *materia medica* contained detailed information on the culture-specific transformation of plant parts into the drugs that the patient would then consume. This underlined that the so-called 'herbal' medical practice depends not only on plant classifications that are culture specific, but also on practical interventions that treat the plant as a thing. Accordingly, the study of *qinghao* involved not merely attending to the cultural acquisition of knowledges (epistemologies) but also to the techniques and practices of intervening with perceived realities (ontologies). Second, the study highlighted that the practical recommendations of how to use the plant and its various parts changed over time; it remains, to date, one of the first longitudinal studies on a specific item of the *materia medica* in the history of Chinese medicine. Finally, it evaluated the identification of *qinghao* and other *hao* 蒿 in terms of modern botanical taxonomies (as given in the *Zhongyao dacidian* 1986).

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1.1 Qinghao in the *materia medica* literature

This chapter begins with a table that lists the most important contents of all the entries on *qinghao* in the *materia medica (bencao)* literature dating from 168 BCE to CE 1596 that have been translated into English (see Table 1.1). This table evaluates the importance attributed to the disorders that *qinghao* was thought to treat by giving the first-named disorder in a *bencao* entry on *qinghao* a number 1, the second-named disorder a number 2, the third-named disorder a number 3 and so on. In a similar manner, it weights the mode of preparation that the text recommends: the first mode of preparation mentioned in a *bencao* entry on *qinghao* is numbered 1, the second 2 and so on. The simple method of tabling the paradigmatic reading of all the *bencao* texts translated in Hsu (2010), and of weighting the findings, by considering the first-mentioned disorders and preparation methods the most important ones, has yielded some interesting additional results:

1. The *bencao* literature underlined the use of *qinghao* for wound healing during the first millennium. It is the sole application recommended in 168 BCE and remains the first-mentioned one up to the seventh century.
2. The *bencao* literature started to mention *qinghao* as prime drug against fevers and, in particular, intermittent fevers only from the Song dynasty (960–1279) onwards; Su Song 蘇頌 was the first to do this in the *Bencao tujing* (Illustrated Canon) 本草圖經 of 1062 [as cited in the *Zhenglei* (Corrected and Arranged into Categories) *bencao* 證類本草].
3. The *bencao* literature consistently listed *qinghao*'s longevity-enhancing properties. These health-enhancing properties were emphasised particularly in dietetics [e.g. the *Shiliao* (Dietary Therapy) *bencao* 食療本草 of 721–739 by Meng Shen 孟詵], although they existed alongside the other recommendations from the earliest documented times onwards. Recommended as a fresh fragrant food supplement, they were mentioned already in the first extant *bencao,* namely Tao Hongjing's 陶弘景 *Bencao jing jizhu* (Notes to the Canon) 本草經集注 of CE 500.

These diverse usages of *qinghao* are certainly linked to the chemistry of the plant *Artemisia annua* L. and related species, but not merely. Socio-historical developments will have played a role too. One wonders, for instance, to what extent *qinghao's* usage for wound healing, and other herbal medications, as those recorded in Salazar (1999) and Harper (1998: 221–230), arise from an interdependent development of technologies of warfare and medicine in general. Furthermore, it would be interesting to explore how closely *qinghao*'s increased usage for treating intermittent fevers is linked to these intermittent fevers' soaring increase in the Song dynasty (Miyasita 1979; Obringer 2001) due to the newly developed agricultural technologies of wet flooded rice cultivation (Bray 1984: 597–615), which created ideal breeding grounds for the malaria mosquito vector.

Table 1.1 Recommended applications and usages of *qinghao* in the chronologically ordered *materia medica* (*bencao* 本草) literature until 1596

Title of the *materia medica (bencao)*	'Bone breaker', fevers	Wound healing	Enhances longevity	Daemonic *qi* convulsions	Soak in urine	Use it raw	Wring out juice	Food supplement	Description of the living kind	Name
(Mawangdui 馬王堆 'Wushier bing fang' 五十二病方, anon., of 168 BCE; manuscript text that is not actually a *bencao*)		1			x					*qinghao* 青蒿, *qin* 菦
Shennong bencao jing 神農本草 (Shennong's canon of the *materia medica*), anon., of first century CE, (L = lost and no longer extant)	2	1	3							*caohao* 草蒿, *qinghao* 青蒿, *fangkui* 方溃
Mingyi bielu 名醫別錄 (Informal Records of Famous Physicians), anon., of third century, (L)	2	1	3						(x)	*caohao* 草蒿, *qinghao* 青蒿, *fangkui* 方溃
Bencao jing jizhu 本草經集注 (Notes to Shennong's Canon of the *materia medica*) of 500 by Tao Hongjing 陶弘景 (fragments extant)	2	1	3					Fresh, mixed with vegetables	(x)	*caohao* 草蒿, *qinghao* 青蒿, *fangkui*方溃
Xinxiu bencao 新修本草(Newly Revised *materia medica*) of 657–659 by Su Jing蘇敬 (fragments extant)	2	1	3					Ditto	(x)	*caohao* 草蒿, *qinghao* 青蒿, *fangkui* 方溃
Shiliao bencao 食療本草 (*materia medica* for Successful Dietary Therapy) of 721–739 by Meng Shen 孟詵 (fragments extant)	3	2	1 and 5	4	x			Pickles in vinegar		*qinghao* 青蒿
Bencao shiyi 本草拾遺 (*materia medica:* Supplements) of eighth century by Chen Zangqi 陳藏器 (L)	(2?)	3		1	x	(x)	x			*hao* 蒿
Shu bencao 蜀本草 (*materia medica* from the Kingdom of Shu) of tenth century by Han Baosheng 韓保昇 (L)									x	*qinghao* 青蒿, *xin hao* 狃蒿

(continued)

Table 1.1 (continued)

Title of the *materia medica (bencao)*	'Bone breaker', fevers	Wound healing	Enhances longevity	Daemonic *qi* convulsions	Soak in urine	Use it raw	Wring out juice	Food supplement	Description of the living kind	Name
Rihuazi bencao 日華子本草 (*materia medica* of Master Sun Rays) of tenth century by Da Ming 大明 (L)	2	3	1	4	(b) x	(a) x			x	(a) *qinghao* 青蒿 leaves and stalks, (b) seeds, (c) *chouhao* 臭蒿seeds
Bencao tujing 本草圖經 (Illustrated Canon of *materia medica*), of 1062 by Su Song 蘇頌 (L)	1	2		3	x			Mix with vegetables	x	*caohao* 草蒿, *qinghao* 青蒿, *fangkui* 方溃
Zhenglei bencao 証類本草 (*materia medica* Corrected and Arranged into Categories) of ca 1082 by Tang Shenwei 唐慎微	2	1	3	4	x	x	x	Mix with vegetables, pickles in vinegar	x	*caohao* 草蒿, *qinghao* 青蒿, *fangkui* 方溃, *xinhao* 狃蒿*chouhao* 臭蒿
Bencao yanyi 本草衍義 (Dilations upon the *materia medica*) of 1116 by Kou Zongshi 寇宗奭								Use as a vegetable	x	*caohao* 草蒿, *qinghao* 青蒿, *xianghao* 香蒿, *changhao* 常蒿
Zhenzhu nang buyi yaoxing fu 珍珠囊補遺藥性賦 (Pearl Bag with Rhapsodies on the Properties of Drugs) of twelfth century by Li Gao 李杲	1	2			x					*caohao* 草蒿, *qinghao* 青蒿, also *xuanfuhua* 旋覆花, *jinfei* 金沸
Bencao pinhui jingyao 本草品匯精要 (Essentials of the *materia medica*, Classified by Grades) of 1505 by Liu Wentai 劉文泰	1	2	3	4	x	x			x	*caohao* 草蒿, *qinghao* 青蒿, *fangkui* 方溃, *xinhao* 狃蒿, *qin* 鼓, *haoqin* 蒿鼓
Bencao mengquan 本草蒙筌 (Enlightenment of the *materia medica*) of 1565 by Chen Jiamo 陳嘉謨	1	2	4	3	x	x	x		(x)	*caohao* 草蒿, *qinghao* 青蒿
Bencao gangmu 本草綱目 (Classified *materia medica*) of 1596 by Li Shizhen 李時珍	2	1	3	4	x	x	x	Mix with vegetables, pickles in vinegar	x	*qinghao* 青蒿, *caohao* 草蒿, *fangkui* 方溃, *qin* 鼓, *xinhao* 狃蒿, *xiang hao* 香蒿

All these entries in the *materia medica* literature are translated in their entirety in Hsu 2010; for *qinghao* formulas in the formula literature, see Wu, de Vries and Hsu forthcoming

1.2 What is in a Name?

In Chinese, the name for a medication (*yao* 葯) can refer to the raw materials, the living plant and the many stages of preparation that lead to the drug that is finally administered to patients. Several preparation methods were repeatedly mentioned in the *bencao* entries on *caohao* or *qinghao*. Among the most frequently mentioned ones belonged urine, which tended to be used as the watery medium within which the fresh plant materials were to be soaked. Furthermore, the recommendation to administer the drug in its fresh/raw state was sometimes very explicit. Finally, it needs to be borne in mind that in antiquity, the Chinese term *caohao* or *qinghao* did not necessarily refer to the plant as a living kind, although the texts spoke of seeds and leaves, roots and stems. It was only during the tenth century that the entry on *caohao* in a *bencao* text would refer to the plant's different stages of maturation and growth, as did Han Baosheng's 韓保昇 *Shu* (Kingdom of Shu) *bencao* 蜀本草 (cited in the *Zhenglei bencao*). From then on, the terms *caohao* and *qinghao* (and synonyms) were consistently used to refer not only to the cultural artefact that is a medicinal drug, but also to the living kind, namely the plant that grows out in the fields.

1.3 How Techniques of Drug Application Interrelate with the Drug's Therapeutic Efficacy

The techniques that turn natural 'herbs' into culture-specific 'drugs' determine and are simultaneously determined by the drugs' effects. At first sight, the shift that happened in the Song dynasty from *qinghao* as primarily a wound-healing drug to one for treating 'intermittent fevers' (瘧，寒熱) not all intermittent fevers are malarial, and malaria can present otherwise than as intermittent fever) would not appear to be related to technological changes in its application. However, if we take into account the observation that 'daemonic *qi*' can refer to the phenomenon of convulsions, and convulsions are sometimes caused by *Plasmodium falciparum* malarial fever bouts (Hsu 2009) techniques of drug preparation did matter.

The use of *qinghao* for treating 'daemonic *qi*' (*guiqi* 鬼氣) is mentioned for the first time in the same text that recommends soaking the presumably fresh plant materials in urine overnight in order to treat the feverish 'bone steaming' (see the *Shiliao bencao* in Table 1.1). Interestingly, 'daemonic *qi*' becomes soon thereafter the first-mentioned disorder in Chen Zangqi's 陳藏器 *Bencao shiyi* (Dilations) 本草拾遺 (cited in the *Zhenglei bencao*). Its application involves accordingly the ingestion of fresh plant materials: 'In autumn and winter use the seeds, in spring and summer use the sprouts, put them together, pound them into a juice and ingest' (updated and improved translation of Hsu 2010: 97).

Later *bencao* texts of the Song and Ming dynasty then also recommend the use of fresh/raw *qinghao*, sometimes after soaking it in urine, sometimes after pounding it into a juice. In those Song and Ming dynasty texts, intermittent fevers tend to be the first-mentioned disorder. Daemonic *qi* is also mentioned in those later texts but relegated to insignificance; it figures often only as number four (see Table 1.1).

So, in this roundabout way, one can indeed reach the conclusion that, contrary to first impressions, the technology of *qinghao*'s preparation was linked to its therapeutic effects and usage: in the history of the *materia medica* the application of *qinghao*, after pounding seeds and (fresh) sprouts into a juice, was recommended for the first time, in order to treat 'daemonic *qi*'. This application method, which involved the ingestion of fresh *qinghao* juice, remained important for its treating of what eventually became the disorder of primary consideration: intermittent fevers (*nüe* 瘧).

1.4 The Problem of Disseminating Specialised Scientific Knowledge

Meanwhile, the famous physician and alchemist Ge Hong 葛洪 had in the fourth century CE already noted that the soaking of the plant in water in order to subsequently wring it out and ingest the (wrung out) juice was effective in treating intermittent fevers (see Hsu 2010; Wright et al. 2010). Ge Hong had made this recommendation in a different genre, namely that of writing recipes and formularies (*fangji* 方劑), but the compilers of the *materia medica* (*bencao*) did not pay attention to it. It was only after 1,200 years that the observation that Ge Hong 葛洪 had made in the context of writing recipes started to be integrated into the *bencao* literature: Ge Hong's recipe is cited for the first time in Li Shizhen's 李時珍 *Bencao gangmu* (Classified Compendium) 本草綱目 1596.

This final observation provides also the motivation for why this chapter has been written for this volume: it is with the explicit aim to make connections between the different scientific genres that specialised research activity inevitably produces. The hope is that the reader may now turn to the literature that provides detailed information on the above problem in a context that provides a more grounded framework for understanding it.

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Chapter 2
Ethnopharmacology of *Artemisia annua* L.: A Review

Alia Sadiq, Muhammad Qasim Hayat and Muhammad Ashraf

Abstract *Artemisia annua* L. has been recognized as important ethanomedicinal herb since two millennia. It has been included in ancient pharmacopoeias of various Asian and European countries. World Health Organization has recommended *A. annua* as antimalarial drug. Its most common ethnobotanical practice involves the use of whole plant decoction for the treatment of malaria, cough, and cold. Diarrhea is also reported to be cured by taking its dry leaves powder. Whole flowering plant is known to be antihelminth, antipyretic, antiseptic, antispasmodic, carminative, stimulant, tonic, and stomachic. The tincture was formally used to treat nervous diseases and crushed plants in liniments. *A. annua* tea infusion has been used for the treatment of malaria in African countries. *A. annua* contains vital compound known as artemisinin that provide structural chemical base for combinatorial treatment therapy for world antimalarial program. Research studies also report that artemisinin is effective for killing human breast cancer cells. Therefore, isolation and characterization of artemisinin has increased the interest in *A. annua* worldwide. Several ethnobotanical uses in Africa claim that the *A. annua* tea is also effective against HIV. Recently, research investigations are more focused to evaluate its antiviral potential against HIV, as it is highly emerging disease throughout the world. Therefore, scientific validation can provide the support to the concept of "ethnopharmacology in overdrive".

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2.1 Introduction

Artemisia annua L is well-known medicinal plant (Bhakuni et al. 2001; Emadi 2013; Tayebe et al. 2012). *A. annua* is the only planta medica that has been recognized to research and developed as the standards of western medicine research by the WHO in China. It is a famous herb, known for its highest efficiency and lowest toxicity in treating ague (Wang et al. 2011). It is an aromatic annual herbaceous plant (Ellman 2010; Huang et al. 2010; Zanjani et al. 2012; Liu et al. 2013; Misra et al. 2013) belongs to genus *Artemisia* (Liu et al. 2013), family *Asteraceae* (Compositae) (Geldre et al. 2000; Mannan et al. 2010; Tayebe et al. 2012; Zanjani et al. 2012) and commonly known as sweet wormwood or Qinghao (Huang et al. 2010; Emadi 2013). It is the only member of genus Artemisia with an annual growth cycle (Willcox et al. 2004). Qing Hao is an ancient Chinese name for *A. annua,* which means "green herb." There are two different theories exist regarding the origin of name. According to first theory word, *Artemisia* is named after the name of Greek Goddess "Artemis" which literally mean "she who heals sickness." It was belief of local people that she heals the diseases and eliminates the evil. Second theory report that it is named after the Queen's name "Artemisia of Caria". She was the queen of Turkey (Ferreira 2004; Willcox et al. 2004). *A. annua* has remained the part of Chinese traditional medicine more than 2,000 years, and currently, it is endemic to China (Olliaro and Trigg 1995; Ferreira 2004; WHO Monographs 2006; Ellman 2010; Mannan et al. 2010). However, *A. annua* has been recognized all over the world since 1970s after the discovery of only natural phytomedicinal source for production (Huang et al. 2010) of the antimalarial lactone artemisinin. Presently, this important phytoconstituent and its derivatives have been widely explored for cure of drug-resistant malaria (Laughlin 2002; Liu et al. 2013; Emadi 2013; Misra et al. 2013). *A. annua* has been established as crop in agriculture after the statement of World Health Organization, as a valuable component of combinatorial therapy for malaria since 2001 (Ferreira 2007).

2.1.1 Origin

This plant is native of Asia and most appropriately originates in China particularly in Suiyuan and Chahar provinces. China has long history of cultivation of *A. annua* and skillful for its unique method of extraction of artemisinin, hence, it has become first country for isolation of artemisinin from plant extracts. In addition, China has also become the largest country on the global market as a supplier of raw material of *A. annua* (WHO Monographs 2006; Ferreira and Janick 2009; Huang et al. 2010; Sharma et al. 2011; Das 2012). There are very few studies that provide the evidence about its origin. Plant remains have been obtained from the Shengjindian cemetery about 2400–2000BP, Turpan, Xinjiang, China. These records provide a information

about its traditional use in ancient times, when People used stalks and inflorescence of *A. annua* to place in the corner of a tomb. Morphological examination of plant remains, ancient DNA extraction and further comparative analysis with modern specimens, provide the insight that these plant remains were belonging to *A. annua*. Further, it gives the rational insight toward its traditional use in the ancient times. This plant is strongly aromatic so local people used it with the purpose to eliminate the odor of the dead. This is the first evidence, based on the archeological studies. Several other ancient Chinese documented records also mention its numerous herbal uses. It is believed that it is not only indigenous to China but also found as native to Korea, Japan, Myanmar, Northern India, Vietnam, and Southern Siberia throughout Eastern Europe. Afterward, it spread to various other countries of North America and tropical areas (Willcox 2009; Liu et al. 2013). It wildly grows in Australia, Turkey, Iran, and Afghanistan. Now, it is commonly cultivated in Vietnam, Romania, Kenya,Tanzania (Bhakuni et al. 2001; Huang et al. 2010; Khosravi et al. 2011), Argentina, Bulgary, French, Hungary, Italy, Spain, United States, and Yugoslavia (Ferreira and Janick 2009; Lestari et al. 2011). Naturally, *A. annua* cover wide range of subtropical and temperate environments including northern hemisphere (mid to high latitudes). There are also very few representatives in the southern hemisphere (Ellman 2010; Das 2012). Cultivation on experimental basis in temperate and subtropical conditions has been started in India (Bhakuni et al. 2001). Breeding technique has been used to develop specific seed varieties for adapting lower latitudes, and it has been achieved successfully in various tropical countries including Congo, India, and Brazil (Willcox 2009).

Scientific names: *Artemisia annua* L.
Vernacular names
Chinese: Caohao, Cao Qinghao, Cao Haozi, Chouhao, Chou Qinghao, Haozi, Jiu Bingcao, Kuhao, San Gengcao, Xianghao, Xiang Qinghao, Xiang Sicao, Xiyehao
English: annual wormwood, sweet wormwood, sweet annie
French: armoise annuelle
Japanese: Kusoninjin
Korean: Chui-ho, Hwang-hwa-ho, Gae-tong-sook
Vietnamese: Thanh cao hoa vàng.

2.1.2 Pharmacognostical Studies

2.1.2.1 Macroscopic Characteristics

A. annua an aromatic annual herb with deeply grooved branches. Variation generally presents in the leaves and aerial parts. The leaves margins are not entire, but the base is asymmetrical. Leaf color varies from light green to dark green and arranged pinnately. Outer and inner surfaces are glabrous. Glandular and non-glandular trichomes are present on the both surfaces. Spongy parenchyma contains 4–6 layers of loosely arranged cells (Das 2012).

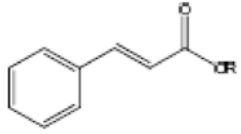
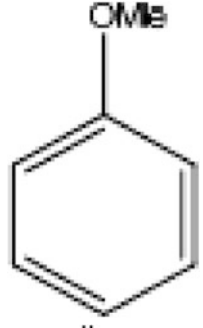
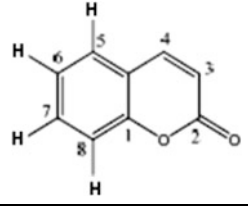
2.1.2.2 Microscopic Characteristics

Physiochemical analysis report average 9.2 w/w moisture content, 8.3 w/w total ash, 0.91 % acid insoluble ash, 6.2 w/w alcohol, and 3.8 v/w water content in *A. annua*. High percentage of protein, crude fat, and digestible fraction is also present in leaves and inflorescence. Plant tissue contains high amount of manganese and copper. Amino acid and vitamin profile are also very high, which increase nutritional value of this herb (Das 2012).

2.1.2.3 Chemical Constituents

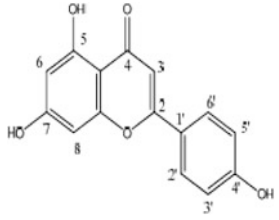
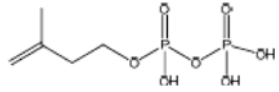
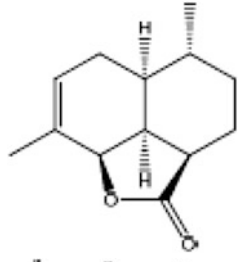
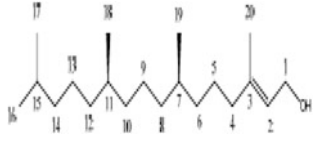
A. annua has become the subject of intensive phytochemical evaluation following the discovery of the antimalarial drug artemisinin (Wang et al. 2011). Phytochemical analysis has identified various compounds including steroids, coumarins, phenolics, flavonoids, purines, triterpenoids, lipids, and aliphatic compounds, monoterpenoids (Emadi 2013; Cafferata et al. 2010; Ferreira et al. 2010), essential oils, alkaloid, and glycoside (Zanjani et al. 2012). Major terpene derivatives such as artemisia ketone (Tellez et al. 1999), artemisinic alcohol, arteannuin B, and myrcene hydroperoxide have been identified. A few of them are also present in essential oil (Verdian-rizi et al. 2008; Brown 2010). Essential oils contain both nonvolatile and volatile constituents. The volatile components of essential oils are camphene, 1-camphor, isoartemisia ketone, β-camphene, β-caryophyllene, β-pinene, artemisia ketone, 1, 8-cineole, camphene hydrate, cuminal (WHO Monographs 2006; Willcox 2009; Das 2012), Artemisia ketone, 1.8-cineole camphor, germacrene D, camphene hydrate, and alpha-pinene, betacaryophyllene, myrcene, and artemisia alcohol (Liao et al. 2006; Ferreira and Janick 2009). The nonvolatile component of essential oil contains sesquiterpenoids (Brown 2010), flavonoids and coumarins, β-galactosidase, β-glucosidase, B-sitosterol, and stigmasterol (Willcox 2009; Cafferata et al. 2010; Das 2012). It also contains erythritol (50.30 %), camphor (7.25 %), pinocarveol (4.13 %), and diethoxyethane (2.18 %) (Haghighian et al. 2008). Scopoletin belongs to the group of coumarins that have been found in *A. annua* extracts (Tzeng et al. 2007). Scopoletin (coumarin), scopolin (comarin glycoside), domesticoside (phloroacetophenone), chrysosplenol-D (flavonoid), and norannuic acid (bisnor-cadinane) are vital phytoconstituents (Emadi 2013; Cafferata et al. 2010). First time, artemisinin (sesquiterpene lacton) isolated from *A. annua* in 1972 (Geldre et al. 2000; Ogwang et al. 2012). Artemisinin is a rare sesquiterpene lactone endoperoxide of the cadinane series (Laughlin 2002). Although there are approximately 400 species of artemisias (Ferreira 2004), artemisinin and essential oil levels in the leaves of *A. annua* ranged from 0.01 to 1.4 % and 0.04 to 1.9 %, respectively (Damtew et al. 2011). The leaves of *A. annua* are only natural source of artemisinin and other vital secondary metabolites (Table 2.1) which can be further used for the production of derivatives of pharmacological importance (Laughlin 2002; Willcox et al. 2004; Brown 2010; Cafferata et al. 2010).

Table 2.1 The phytochemical constituents of *Artemisia annua* L

S. No	Phytochemical groups	Phytoconstituents	Chemical structure	Alternative name (s)
1	Phenylpropanoids	Methyl cinnamate		3-phenyl-2-propenoic acid methyl ester
2	Phenols	Anisole		Phenyl methyl ether
3	Coumarins	2H-1-Benzopyran-2-one		2H-1-Benzopyran-2-one

(continued)

Table 2.1 (continued)

S. No	Phytochemical groups	Phytoconstituents	Chemical structure	Alternative name (s)
4	Flavones	Apigenin		4',5,7-Trihydroxyflavone
5	Monoterpenoids	Isopentenyl pyrophosphate		–
6	Sesquiterpenoids	Artemisinin		Artemisinin I
7	Terpenoids	Phytol		2-Phyten-1-ol (2E, 7R,11R)

Source Brown GD (2010) The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao). Molecules 15: 7603–7698

2.1.3 Ethnopharmacological History

Ethanopharmacological compilation named as "Fifty-two prescriptions" (dates back to 168 BC) mentioned *A. annua* (*qinghao*) as medicinal herb. This document describes it: as a remedy for hemorrhoids that resembles "cow lice" (possibly ticks). Traditional Chinese materia medica (*Shennong ben cao jing*), which was present in the first century AD, but now it has been lost, documented its use as food preservative, remedy for summer heat and for the treatment of "intermittent fevers". This *Handbook of Prescriptions for Emergency Treatment* (Zhouhou Beiji Fang) had been documented in 340 AD, describes number of preparations as traditional medicine (Willcox 2009). Traditionally, it has been used as flavoring agent. Based on this strong traditional use and characteristic fragrance later on, it becomes the potential source for essential oils for the perfume industry (Ferreira and Janick 2009; Huang et al. 2010; Liu et al. 2013). For over 2000 years, the Chinese have used *A. annua* as natural remedy to treat malaria (Geldre et al. 2000; Meier zu Biesen 2010). The *Pharmacopoeia of the People's Republic of China* also describes its use to cure consumptive fever and jaundice (WHO Monographs 2006; Castilho et al. 2008; Ogwang et al. 2012) wound healing and for the improvement of eye brightness (Liu et al. 2013). *A. annua* has also been used traditionally in Iran as medicinal plant for infants as an antispasmodic, carminative, or sedative/ hypnotic remedy (Emadi 2013; Sharma et al. 2011). *A. annua* decoction has been used as antihemorrhage to cure diarrhea (Mirdeilami et al. 2011). Effect of *A. annua* L. on hemostasis is well known in traditional medicine (Wang et al. 2011). Traditional medicinal practices involve usage of different plant parts of *A. annua* to cure different disease (Table 2.2).

2.1.4 Pharmacological Activities

2.1.4.1 Antihypertensive Activity

Antihypertensive potential of aqueous extracts of artemisia leaves of different species, have been assessed by using in vivo models of diabetic rats and rabbits that were administered with dose of 100–390 mg kg^{-1} for 2–4 weeks. Results revealed the significant effects of aqueous extracts by exhibiting the reduction in blood level. Consequently, this action prevents elevation of glycosylated hemoglobin level and produces hypoliposis effect. It also causes the protective effect against body weight loss in diabetic animals. Further, it caused significant inhibition of the phenylephrine-induced contraction, and simultaneously stimulates the endothelium-dependent relaxation of rat aortic rings (Das 2012).

Table 2.2 Medicinal uses of different plant parts of *Artemisia annua* L

S. No	Medicinal uses	Plant part	References
1	Antihemorrhage	Whole plant	Mirdeilami et al. (2011)
2	Diarrhea	Whole plant	
3	Anemia	Stem	Willcox et al. (2004)
4	Damp summer heat with nausea	Root	
5	Intense fever	Rhizome	
6	Stifling sensation in chest	Rhizome/seed powder	
7	Malaria	Leaf/whole Plant	Ogwang et al. (2012)
8	Asthma	Leaf	Anamed international (2011)
9	Eye infections	Leaf	
10	Bronchitis and sore throat	Leaf	
11	Cholera	Leaf	
12	Dengue fever	Leaf	
13	Lupus erythematosus	Whole plant	
14	Athlete's foot and eczema	Leaf	
15	Chagas disease	Leaf	Weathers et al. (2011)
16	Schistosomiasis	Leaf	Zanjani et al. (2012)
17	Viral hepatitis B	Leaf	
18	Chills and fever	Whole plant	Meier zu Biesen (2010)
19	Skin disease	Leaf	Sharma et al. (2011)
20	Parasitic disease including schistosomiasis and leishmaniasis	Leaf	Mannan et al. (2010)

2.1.4.2 Antimicrobial Activity

Research studies have been carried out to evaluate antimicrobial potential of the essential oils obtained from *A. annua*. Experiments revealed that essential oil showed antimicrobial potential against wide range of Gram-negative bacteria, Gram-positive bacteria, and fungi. Significant inhibitory activity of the oil was found against bacterial strains, including *Staphylococcus aureus, Escherichia coli,* and *Enterococcus hirae*. Whereas *Pseudomonas aeruginosa* showed no sensitivity for essential oil. *A. annua* extracts possess remarkable antibiotic potential against fungi particularly *Saccharomyces cerevisiae* and *Candida albicans* (Juteau et al. 2002; Das 2012). Furthermore, these investigations also revealed that essential oils showed more pronounced effects against fungal strains than against Gram bacterial strains (Verdian-rizi et al. 2008). Studies based on chemical evaluation of plant extracts evidenced that phytoconstituents are responsible for conferring this antimicrobial potential. Most vital compounds that have been studied for this bioactive potential are scopoletin (Tzeng et al. 2007), sesquiterpene lactone endoperoxide artemisinin and variety of other derivative compounds. Mechanism of action these Compounds at molecular level have been studied in *Escherichia coli*, *Mycobacterium smegmatis,* and *Mycobacterium tuberculosis*. It has been observed that Arteether acts at nuclear level and hampers the function of DNA-gyrase which is

resistant by quinolone (Kumar et al. 2003). Artemisinic acid is another well-known precursor compound used for semisynthesis of artemisinin, and it has also been studied for antibacterial activity (Bhakuni et al. 2001; Muzemil 2008; Huang et al. 2010).

2.1.4.3 Anti-inflammatory Activity

Anti-inflammatory activity of aqueous methanolic extract has been tested for acute and chronic inflammation by implying variety of inflammatory models. Aqueous extract exhibits anti-inflammatory effect in a dose-dependent manner and resulted in pronounced activity against edema. Phytochemical analysis reports the presence of number of important groups of compounds such as triterpenoids, flavonoid, polyphenols, and coumarin. Therefore, these compounds act additively and impart inhibitory potential against edema response in acute and chronic models (Das 2012). Some other research analysis also report more anti-inflammatory compounds named as scopoletin (a coumarin) (Muzemil 2008), artemisinin, dihydro artemisinin, and arteether. In vivo assays revealed that these compounds significantly inhibit the humeral responses at increased concentration. But some other studies suggest that pure compounds did not show significant efficacy in chronic hypersensitivity response (Bhakuni et al. 2001). Further experimental studies carried out on murine macrophage like RAW 264.7 cell showed the effect of scopoletin in a dose-dependent manner. Therefore, numerous research studies recommend scopoletin as a candidate for anti-inflammatory medicine (Tzeng et al. 2007).

2.1.4.4 Antioxidant Activity

A. annua is a good source of different nutritional constituents and antioxidants (Das 2012). Studies indicate that crude organic extracts of aerial parts have high antioxidant capacity which is most probably due to the fact that leaf contains high content and variety of flavonoids, including the newly reported C-glycosyl flavonoid as a possible component of the antioxidants. Flavonoids and essential oil content present in *A. annua* impart antioxidant potential. Therefore, these studies ranked *A. annua* among those medicinal plants which are at the top of list, based on their highest antioxidant potential (Juteau et al. 2002; Ferreira and Janick 2009). Major groups of hydroxylated and polymethoxylated flavonoids have been identified which further include chrysosplenol-D, cirsilineol, eupatin, chrysoplenetin, cirsilineol, casticin, and artemetin (Ferreira et al. 2010). Studies have identified respective five bioactive flavonoids and further subjected to structural analysis. These include 5-hydroxy-3,7,4′-trimethoxyflavone,5-hydroxy-6,7,3′,4′-tetramethoxyflavonol, blumeatin, 5,4′-dihydroxy-3,7,3′-trimethoxyflavone and quercetin (Yang et al. 2009).

2.1.4.5 Immunosuppressive Activity

A. annua has been evaluated for its immunosuppressive activity. Ethanolic extract of *A. annua* significantly suppressed concanavalin A (Con A) and lipopolysaccharide (LPS)-stimulated splenocyte proliferation in vitro and this activity increases with increase in dose. Results have also showed that ethanol extract of *A. annua* could suppress the cellular and humoral response (Das 2012). Immunosuppressive potential have been linked to flavonoids present in leaves which are capable to modulate the immune response (Ferreira et al. 2010).

2.1.4.6 Antiarthritis Activity

Experimental studies have revealed that artemisinin derivative SM905 (obtained from *A. annua*) suppresses the inflammatory and Th17 responses which cause the improvement in collagen-induced arthritis. These studies have been carried out on collagen-induced arthritis (CIA) by type II bovine collagen model (CII) in DBA/1 mice through oral administration of artemisinin derivative SM905. Incidence of disease and severity were observed regularly. Gene expression and T helper (Th) 17/Th1/Th2 type cytokine production level have also been examined. Observations of this study revealed that SM905 compound play key role as it delayed the onset of disease, hence reduce the incidence of arthritis. Furthermore, it also reduces the overexpression of variety of pro-inflammatory cytokines and chemokines (Das 2012).

2.1.4.7 Antimalarial Activity

Malaria is a global threat since long. In order to deal with this situation, it needs a coordinated approach consist of prevention strategies, therapeutic medicines, and curative treatment of patients. Therefore, extraction of artemisinins from *A. annua* has opened the way toward new and highly effective alternates (Ferreira 2004; Ridder et al. 2008; Ferreira and Janick 2009). *A. annua* L is now well recognized throughout the world (Liu et al. 2009; Willcox 2009), and currently, it is in use over 50 countries as a strong drug substitute against malaria, particularly chloroquine-resistant malaria (Ferreira et al. 2006). Studies have reported many other flavonoids (artemetin, casticin, chrysoplenetin, chrysoplenol-D, cirsilineol, and eupatorin) which possess antiplasmodial efficacy (El-feraly et al. 1989; Lubbe et al. 2012). Mechanism of methoxylated flavonoids is associated with activation of artemisinin, which explains the key role of methoxylated flavonoids, as it facilitates the interaction of artemisinin with plasmodial hemoglobin involving catabolic pathway that produces artemisinin peroxide. Furthermore, artemisinin peroxide inhibits the heme polymerization and ultimately confers the antimalarial effects against protozoan *Plasmodium* species: *falciparum vivax, malariae,* and *ovale*. Another mechanism of flavonoids suggests that it blocks the incorporation of hypoxanthine by *Plasmodium*

(Laughlin 2002; Muzemil 2008; Das 2012). Although artemisinin induce antiplasmodial effects through alkylation of malarial-specific proteins (Bhakuni et al. 2001), some flavonoids had no specific antiplasmodial activities but had capability to potentiate antiplasmodial activity of artemetin (Ferreira et al. 2010). In early 1970s, Chinese scientists have selected artemisinin, artemether, and sodium artesunate for clinical evaluation. There are studies in which malarial patients (more than 3000) were clinically subjected to the treatment by artemisinin and its derivatives. These results suggest more curative potential of artemisinin compounds particularly against drug-resistant *P. falciparum* (Mueller et al. 2000; Weathers and Towler 2012). Comparative clinical studies have been conducted to evaluate the efficacy of whole herb of *A. annua* and chloroquine. Organic extracts *A. annua* have been found more effective, faster, and less toxic than chloroquine in treating malaria (Huang et al. 2010; Tayebe et al. 2012). It significantly reduces parasitemia and improves the immune response by stimulating phagocytic activity of macrophages. Whole plant extract activity is more pronounced because of the presence of various phytoconstituents that impart synergistic antimalarial potential. Therefore, it is quite obvious that current combinatorial approach may be representing the formulations of phytoconstituents (and sometimes plant species) that confer synergistic effect, as they are present in the herbal prescriptions (Willcox 2009; Donno et al. 2012).

2.1.4.8 Antiparasitic Activity

Research studies suggest that artemisinin drugs have good antiparasitic potential for Leishmania, Trypanosoma *Babesia*, *Eimeria* or coccidiosis, trematodal blood fluke *Schistosoma* spp., and *Schistosoma japonicum*, *Schistosoma mansoni,* and *Schistosoma haematobium*. Therefore, currently, its use in livestock industry has been increasing (Kumar et al. 2003; Ferreira and Janick 2009). A study has been conducted against *Neospora canum*, which is a protozoal parasite of mammals. Cultured Vero cells or mouse peritoneal macrophages were infected with of Artemisinin for 14 days. All microscopic foci of *N. caninum* completely eliminated at 20 or 10 μg/ml after 11 days, and same results were obtained at concentration of 0.1 μg/ml. Therefore, artemisinin has potential to reduce the intracellular multiplication of *N. caninum* tachyzoites. In another study, the effect of artemether was tested against the larval stages of *Schistosoma mansoni*. It has been found that animals did not develop schistosomiasis after artemether treatment. Susceptibility of parasite was quite pronounced as compared to the nontreated controls (Das 2012). Recently, another research study reports that n-hexane extracts of *A. annua* leaves and seeds exhibit significant activity against *Leishmania donovani*. This antileishmanial activity includes morphological changes in promastigotes, apoptosis, and cell-cycle arrest at cellular level (Islamuddin et al. 2012).

2.1.4.9 Anticancer Activity

A. annua is well known by its pharmacological applications in the popular medicines, and currently it is a subject of research studies with the aim to find the treatment against cancer (Cafferata et al. 2010). Anticancer activity of various organic extracts of *A. annua* has been evaluated by determining their cytotoxic potential in *Trypanosoma b. brucei* (TC221 cells) and HeLa cancer cells. These evaluations showed that methanol extracts are more cytotoxic as compared to dichloromethane extracts (Efferth et al. 2011). Cytotoxicity studies of artemisinin and quercetagetin-6, 7, 3¢, 4¢-tetramethylether against various tumor cells including P-388, A-549, Ht-29, KB, and MCF-7 cells showed significant efficacy (Bhakuni et al. 2001; Muzemil 2008). In vitro and in vivo anticancer testing exhibits promising results of artemisinins, and further investigations reveal its mechanism of action, which provides an insight toward its constitutional property that is built in its structure. Artemisinin contains an endoperoxide group that imparts anticancer activities. Like some other compounds such as hydrogen peroxide, artemisinin reacts with ferrous iron and make free radical species. These free radicals trigger anticancer activities. Further extended research investigations report that these anticancer activities become more pronounced upon addition of iron complexes in cell culture. Artemisinin makes covalent conjugate with transferrin (an iron transport protein, found in human) so this artemisinin and transferrin conjugate actively transported inside the cancer cells by the involvement of transferrin receptor (TfR)-mediated endocytosis pathway and result in pronounced anticancer activity experimental cell cultures. This also explains the importance of iron metabolism that enhances the anticancer potential of artemisinin. In addition, artemisinin and its derivatives induce programmed cell death in cancer cells through activation of cytochrome C-mediated pathway which lead toward apoptosis (Ferreira et al. 2010). Therefore, several research investigations established artemisinin as s potent anticancer agent (Huang et al. 2010; Nadeem et al. 2013) and recommend it against cancer as drug therapy (Ferreira and Janick, 2009; Ferreira et al. 2010; Zanjani et al. 2012). Chemical and structural characteristics also recommend it as a lead compound, which can further become the basis of drug development (Bhakuni et al. 2001). Research studies have also identified some other vital compounds which possess antitumor activity such as scopoletin (Tzeng et al. 2007), artemisinin and its derivatives (Kumar et al. 2003).

2.1.4.10 Angiotensin Converting Enzyme Inhibitors

Studies have identified few flavonoid compounds from *A. annua* such as fisetin and patuletin-3, 7-dirhamnoside, which exhibit the potential for blocking nonpeptide angiotensin converting enzyme (Bhakuni et al. 2001; Muzemil 2008).

2.1.4.11 Antiviral Activity

Antiviral activity of *A. annua* tea infusions against HIV has been evaluated very first time through scientific investigation. Two independent cellular systems have been used for toxicity studies. The *A. annua* tea infusion exhibits highly significant activity at very low concentration (2.0 μg/mL). But artemisinin was found inactive at higher concentration (25 μg/mL). Similarly, no cellular cytotoxic effects were observed at higher concentration of tea infusion. Therefore, this in vitro study revealed that artemisnin plays limited role and may act synergistically against anti-HIV activity (Lubbe et al. 2012). Some other in vitro studies have claimed about inhibitory effects for hepatitis B virus (WHO Monographs 2006). Currently, artemisinin and its derivatives has become the subject of scientific studies to investigate their potential against number of viruses (Ferreira and Janick 2009) with the aim of advanced combination therapies of antivirals (Weathers and Towler 2012).

2.1.4.12 Plant Growth Regulatory Activity

Research studies report that *A. annua* contain series of vital compounds that have the potential to regulate the plant growth activities and some of them act as natural pesticides. These compounds have also been recommended as natural pesticide in agriculture. These compounds are bis (1-hydroxy-2-methylpropyl) phthalate, abscisic acid, and abscisic acid methyl ester, artemisinin, and its derivatives (Bhakuni et al. 2001).

2.1.4.13 Antifeedant Properties

Research studies have been conducted by implying various parameters of assessment of antifeedant activity for crude extracts of *A. annua*. Deterrency, growth regulatory effect and ovicidal potential strongly recommend it as a good antifeedant herb (Haghighian et al. 2008), as antihelminthes and anti-insecticidal agent (Khosravi et al. 2011; Vicidomini 2011). Some studies have reported that crude extracts of *A. annua* contain artemisinin and its derivatives which act as natural pesticide (WHO Monographs 2006; Huang et al. 2010; Weathers et al. 2011).

2.2 Conclusion

A. annua is ethanomedicinally important plant as its medicinal use has been well established in Chinese pharmacopeias since 168 BC *A. annua* has also obtained an important place among plant-based advanced therapeutics. Particularly against drug-resistant malaria, it has become a good hope for treatment, because it has

very low toxicity. Mefloquine is one of the antimalarial drug, but it is associated with multiple side effects. Recent several research studies have revealed that *A. annua* possess characteristic biological activities to cure various diseases. But their mechanisms of action at cellular and molecular level still need to be investigated. *A. annua* is a rich source of large number of biologically active phytoconstituents, and particularly, it is the only source of artemisinin. It possesses characteristic therapeutic potential against malaria, and besides antimalarial effects, it has various other biological activities such as anti-inflammatory, antibacterial, angiotensin converting enzyme inhibitory, cytokinin-like, and antitumor activities. Nowadays, there is increasing research focus toward investigation of its anticancer and antiviral effects particularly for HIV/AIDS. Therefore, mechanisms of action of the active phytoconstituents particularly artemisinin and their derivatives has become the emerging area of interest in the arena of scientific investigations. These research studies can validate the ethanomedicinal use of *A. annua* by local community on scientific bases. Therefore, *A. annua* is a strong alternate which can be widely explored and finally can lead toward drug development.

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Chapter 3
Artemisia annua: A Miraculous Herb to Cure Malaria

M. Naeem, Mohd Idrees, Minu Singh, M. Masroor A. Khan and Moinuddin

Abstract *Artemisia annua* L. (family Asteraceae) is the only source of a potent antimalarial drug artemisinin that has been used for centuries in Chinese traditional therapy for the treatment of fever and malaria. Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has been proved as an effective and safe alternative remedy against the highly adaptable malaria parasite *Plasmodium falciparum*, which has already become resistant to many other drugs. World Health Organization (WHO) recommended artemisinin-based combination therapy (ACT) as the most effective one against the drug-resistant malaria. As it is highly desirable, various scientific strategies have been applied to enhance the production of artemisinin. Malaria is a vector-borne disease usually caused by *Plasmodium* species. It is reported that malaria threatens more than one-third of the global population, killing approximately 2 million people annually. A multidimensional approach is needed to control the malaria, and one of them is increasing artemisinin production in the plant concerned. The present review covers the information regarding *A. annua* cultivation, medicinal uses, artemisinin production, causes, consequences and control of malaria including strategies and policies.

Keywords *Artemisia annua* · Artemisinin · ACTs · Malaria · Parasites

3.1 Introduction

Artemisia annua L. (family Asteraceae) is cultivated globally as the only plant source of the potent antimalarial drug, artemisinin. Its use in treating malaria has been known in China for over 2,000 years. The active ingredient of *A. Annua*,

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artemisinin, was isolated by Chinese scientists in 1972. *A. annua* L. is an antimalarial drug plant that has been used for centuries in Chinese traditional medicines for the treatment of fever and malaria (Klayman 1985; Zamiska and McKay 2007; and White 2008). Despite tremendous efforts regarding the control of malaria, the global morbidity and mortality figures have not significantly been changed for the last 50 years (WHO 2001). Artemisinin has proved effective against the highly adaptable malaria parasite, which has already become resistant to many other drugs. Artemisinin has been increasingly popular as an effective and safe alternative therapy against malaria (Abdin et al. 2003). The toxin-free, artemisinin-based combination therapy (ACT) is recommended by WHO as the most effective therapy against the drug-resistant malaria parasite (Shetty 2004 and Davis et al. 2005). Since the chemical synthesis of artemisinin is complicated and not economically feasible in view of the poor yield of the drug, the intact plant remains the only viable source of the artemisinin production. Hence, the enhanced production of artemisinin in the plants is highly desirable (Abdin et al. 2003; Aftab et al. 2010a, b, c; and Aftab et al. 2012). The main reason behind the poor artemisinin yield is the morphological damage to the plants due to excessive unfavourable conditions. Various approaches have been attempted to increase artemisinin production including chemical synthesis (Xu et al. 1986 and Avery et al. 1992) and genetic engineering of the pathway genes involved in artemisinin biosynthesis in *A. annua* (Chen et al. 2000; Xie et al. 2001; and Ro et al. 2006), but not much success has been achieved because of the high cost of chemical synthesis procedure or complex nature of regulation and expression of the genes responsible for artemisinin biosynthesis (Fig. 3.1).

3.2 *Artemisia annua*: An Antimalarial Herbal Drug

Artemisia annua, known with different names such as sweet wormwood, sweet annie, qinghao and annual wormwood, is a native shrub of China, where it has long been used both ornamentally and for medicinal purposes (Duke et al. 1987).

(A) Systematic Position

According to the system of classification of Arthur Cronquist (1919–1992), *Artemisia annua* occupies the following systematic position:

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	*Artemisia*
Species	*Annua*

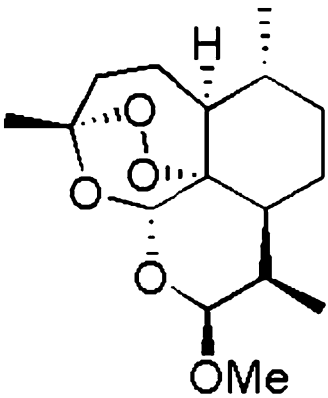

Fig. 3.1 Structural formula of artemisinin

A. annua is an annual weed reaching about 2 m in height with alternate branches. Leaves, with an aromatic odour, are deeply dissected, 2.5–5 cm in length and 1–3 cm in width. Flowers are tiny and yellow arranged in loose panicles with capitula 2–3 mm across. The seed pod consists of a single achene, which is faintly nerved and 1 mm long. Naturally, the plant is pollinated by insects and wind (Ferreira et al. 1995; WHO 2006).

(B) Production and Cultivation

Field production of *A. annua* is presently the only commercially viable process to produce artemisinin because the chemical synthesis of complex molecule of artemisinin is uneconomic. Currently used selections of *A. annua* reach the peak in artemisinin before flowering and at the end of vegetative growth, allowing maximal biomass accumulation of artemisinin before harvest (Ferreira et al. 1995). The most important management problems involve planting, the achievement of uniform stands, weed control and post-harvest drying of the crop. The plant is extremely vigorous and essentially disease and pest free. Most researchers transplant seedlings though direct seeding, and mechanical transplanters have been used in commercial production. *A. annua* is an annual crop cultivated in China and Vietnam as a source of artemisinin and in Romania as a source of essential oils.

Cultivation of *A. annua* has greatly expanded in China and Africa, mainly Kenya, Tanzania, and Nigeria to support new processing plants for the production of artemisinin. Cultivation of *A. annua* in Africa has started after the WHO recommended artemisinin combination therapies (ACTs) as a replacement of artemisinin monotherapy in the fight against multi-drug-resistant parasite of malaria, *Plasmodium falciparum* (Ferreira et al. 2005). The drug is stored in glandular trichomes of the leaves. *A. annua* is grown in parts of Asia within the temperate climate regions, particularly the northern parts of Chahar and Suiyuan provinces of China. However, wild populations have been established across the world,

including in the United States, Argentina and European countries. *A. annua* is cultivated on all six permanently inhabited continents, and the plant appears highly adaptive to non-temperate climates. Despite these advances, China and Vietnam remain by far the largest cultivators of this valuable medicinal plant (Ferreira et al. 2005). Farmers typically start its sowing using seeds, which are viable for up to 3 years if kept in cool and dry places. Cuttings have a high rooting rate (nearly 100 %), but the higher cost of this method makes it less feasible than sowing seeds. Young seedlings are highly vulnerable to weeds; so, early application of chemical herbicides in large-scale operations is typically recommended. The greatest biological threat to *A. annua* plant is a fungus, which affects <1 % of plants in certain cultivation areas (Ferreira et al. 2005). Ferreira (2007) explored the potential of Artemisia for crop cultivation under Appalachian ecosystems and for its potential use in animal health.

(C) Active Constituents

Artemisia annua contains sesquiterpene lactones, flavonoids and essential oils. The species of genus *Artemisia* that contain artemisinin include *A. annua*, *Artemisia apiacea* and *Artemisia lancea*. The highest content of artemisinin is found in *A. annua* wild plants before flowering, ranging from 0.01 to 0.50 % on dry weight basis (Klayman et al. 1984). Artemisinin yields of *A. annua* range from 0.01 to 0.60 % as reported from China (WHO 2006). A hybrid form of Artemisia, cultivated in Central Africa, contained 0.63–0.70 % artemisinin of which 40 % could be extracted by simple tea preparation (Karbwang et al. 1994). The production of 1 kg artemisinin requires 1,200 kg of dried leaves on three acres (1.2 ha) of land. Since *A. annua* plants, harvested from various sources, showed no sufficient yields, companies cultivate hybrids with higher artemisinin content (Ferreira et al. 1995). In Vietnam, farmers obtain 20 kg/ha as net yield of Artemisia (Hemskerk et al. 2006). Accompanying biochemical substances of artemisinin are dihydroartemisinin (dihydroqinghaosu), artemisinic acid (a potential precursor of artemisinin) and arteannuin A and B (Allen et al. 1997). The flavonoids of *A. annua* are artemetin, chrysosplenetin, eupatorin and casticin, special flavonoids with methylated character, which contribute to the pharmacological efficacy of artemisinin (Elford et al. 1987; Sanella et al. 2007 and Ferreira et al. 1997). The essential oil of Artemisia contains camphor (44 %), germacrene D (16 %), transpinocarveol (11 %), beta-selinen (9 %), beta-caryophyllene (9 %), artemisia ketone (3 %) and around 20 substances of which the content is less than 1 % (Juteau et al. 2002). Biseriate glandular trichomes of Artemisia are the source of highly aromatic volatile oils, including Artemisia ketone and 1.8-cineole camphor; germacrene D, camphene hydrate and alpha-pinene; and beta-caryophyllene, myrcene and artemisia alcohol.

(D) Medicinal Properties

The extract of *A. annua*, long used as an ornamental and medicinal shrub in traditional Chinese culture, was accidentally found to kill the malaria parasite, *P. falciparum*. Thereafter, the drug artemisinin (known in China as qinghaosu) was discovered and identified as the biochemical compound for curing malaria

(Enserink 2005; Duke and Ayensu 1985). Artemisinin-containing medicines, such as Coartem and artesunate, have only been available in South Africa for a few years. The Chinese have been using artemisinin for thousands of years as prophylaxis for malaria and as powerful immunity stimulant. Artemisinin is also used in contraception, relief of joint pains, deworming, haemorrhoids, immune boosting and cancer treatment. Many other effects of *A. annua* products (antiperiodic, antiseptic, digestive, febrifuge, etc.) have been described in various sources of the literature. An infusion of the leaves is used internally to treat fevers, colds, diarrhoea, etc. (Him-Che 1985). Leaf essential oil of *A. annua* is used as flavouring agent in spirits such as vermouth (Duke et al. 1987).

(E) Traditional Chinese Medicinal Uses

Artemisia plant has been shown to have antibacterial properties. Different species of *A. annua* have been used in Western and Chinese medicinal formulas to treat various ailments. Recent research has shown that it destroys malarial parasites, lowers fevers and checks bleeding. Artemisia is often used in the tropics as an affordable and effective antimalarial drug. An infusion of Artemisia leaves is used internally to treat fevers, colds and diarrhoea. Externally, Artemisia leaves are poulticed onto nose bleeds, boils and abscesses. The leaves are harvested in the summer, before the plant comes into flower, and are dried for later use. The seeds are also used in the treatment of flatulence, indigestion and night sweats. According to Clinical trials, artemisinin has been found to be 90 % effective and more successful than standard drugs for curing malaria (Mueller et al. 2000). Terpenoids and flavonoids derived from *A. annua* have cytotoxic activities in several human tumour cell lines. Two of the Artemisia components, artemisinin and artesunate, have proved as effective drugs in cancer treatment (Dondorp et al. 2011). Systematic reviews on artemisinin show that artemisinin is as effective as quinine in treating both uncomplicated and severe malaria. However, increased risk of relapse may limit its uses.

3.3 Symptoms, Causes and Consequences of Malaria

Malaria is a vector-borne disease usually caused by four species of *Plasmodium* (*P. falciparum, P. vivax*, *P. ovale* and *P. malariae*). Malaria is a major scourge of humankind, which still continues to confront medical science and technology. Malaria is a devastating global health crisis, with an estimated 300 million people succumbing to infection by the malaria parasite every year. The vast majority of cases occur in sub-Saharan Africa. Worldwide, between 500,000 and 2.7 million people die of malaria each year, of which children represent a disproportionately large fraction of the victims (Martens and Hall 2000 and Ferreira 2004). Most victims of malaria are children from the sub-Saharan Africa, but the number of victims is also rising all over the tropical world (April 1989). Although malaria has been eradicated from the USA and Europe, it still claims approximately 1,500 cases a year in the USA and a few others in Europe, mainly around international airports.

Malaria has the potential to cause outbreaks in the USA, where the mosquito vector is found in all 48 continental states. It has been slowly and steadily coming back. Hazardously, the malaria parasite is developing global resistance against two of the most common antimalarial drugs: chloroquine and the combination sulphadoxine/pyrimethamine (Ridley 2002 and Shetty 2005).

1. *P. falciparum*—A common and dangerous form, found in sub-Saharan Africa, New Guinea, the Amazon Basin and in the Indian subcontinent.
2. *P. vivax*—Persistent but not life-threatening, common in the Indian subcontinent.
3. *P. ovale*—Moderate form of malaria common in Africa.
4. *P. malariae*—Another moderate form of malaria that occurs everywhere, but rarely.

P. falciparum has its life cycle in the mosquito (vector) as well as in the human being (host). The '*Anopheles gambiae*' mosquito is the vector responsible for transmission of malaria. The prevalence of malaria is dependent on the abundance of the female *Anopheles* species, the tendency of the mosquito to bite, the longevity and rate of biting, and the rate of multiplication of the *Plasmodium* parasite inside the mosquito. Only *Anopheles* species of mosquitoes carry malaria parasite. They also transmit elephantiasis (filariasis) and encephalitis. These mosquitoes exploit human-exhaled carbon dioxide, body odours and temperature in order to recognize their victims. Only female *Anopheles* mosquitoes have the mouth parts necessary for sucking blood. While biting with their proboscis, they insert two micro-tubes into the skin, of which one injects an enzyme that is the inhibitor of blood clotting and the other micro-tube sucks the blood. Astonishingly, they do not use the blood as food. Instead, they use it as a source of protein for their eggs. For their own nourishment, both males and females eat nectar and other plant sugars. When female *Anopheles* mosquitoes bites and sucks the blood of a person (Fig. 3.2), she becomes infected with malaria parasites, and then she bites the next human host, thus transmitting the parasites to a healthy human being. Malaria parasite incubates in the human body for about 8–10 days.

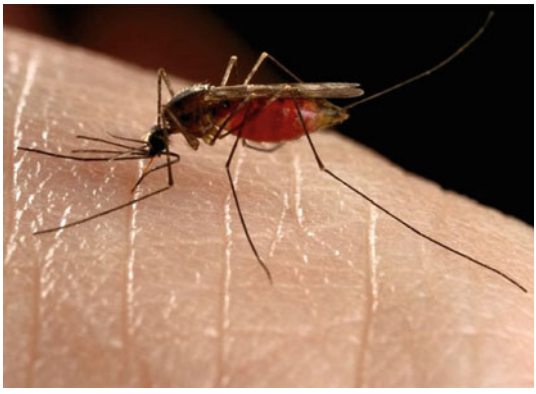

Fig. 3.2 Photograph by Darlyne A. Murawski (http://animals.nationalgeographic.co.in/animals/bugs/mosquito/)

The spread of malaria needs conditions favourable to the survival of the mosquito and the *Plasmodium* parasite. Increased rainfall and stagnant water pools or surface water provide generous breeding grounds for the mosquito. Malaria transmission is affected by their 'basic reproduction number'. In an entirely susceptible host (non-immune), the mosquitoe population after each primary malaria infection raises a varying number of secondary infections, referred to as the basic reproductive number. That number is directly proportional to the populations' risk for contracting malaria and can be increased by an increase in the abundance of mosquitoes relative to the human population, in the propensity of the mosquito to bite its human host, in the proportion of the infective mosquito bites, in the length of illness and in survival rates or longevity of the mosquito (Hamoudi and Sachs 1999).

The initial symptoms after infection with the parasite are chills and fever that last for several hours and recur every few days. Progression of the disease is marked by the onset of headaches, muscle cramping, diarrhoea and vomiting. If left untreated, enlargement of the liver and spleen occurs, followed by anaemia and jaundice. In late stages of the disease, clogging of the cerebral vessels leads to coma and death of the diseased. Because of the extent of the disease and the high costs of treatments, malaria also represents a significant economic cost to affected areas. It is estimated that malaria costs the combined GDP of the African continent approximately $12 billion every year (Ferreira et al. 2005).

(A) Life Cycle of *P. falciparum*

Malaria parasite is transmitted by the female *Anopheles* mosquito and transits through the liver and the blood of the mammalian host (Fig. 3.3). The symptoms

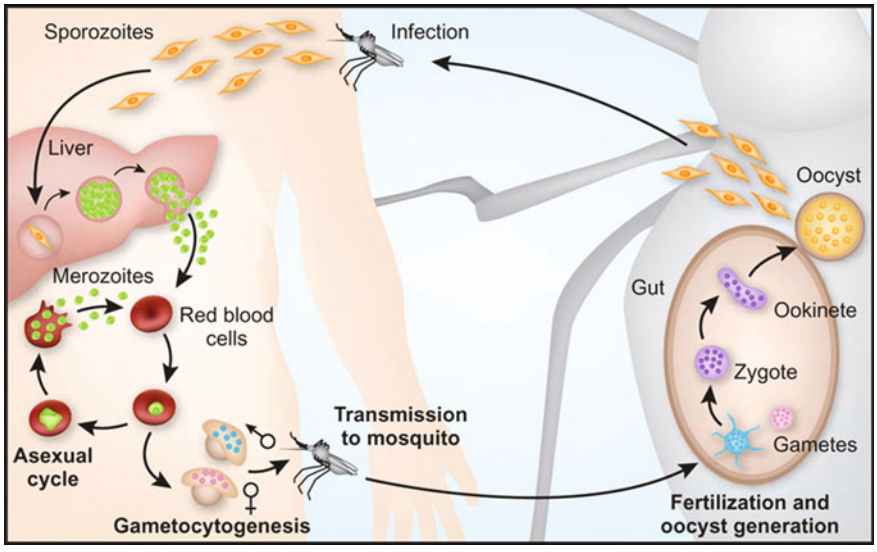

Fig. 3.3 Life Cycle of *Plasmodium falciparum*

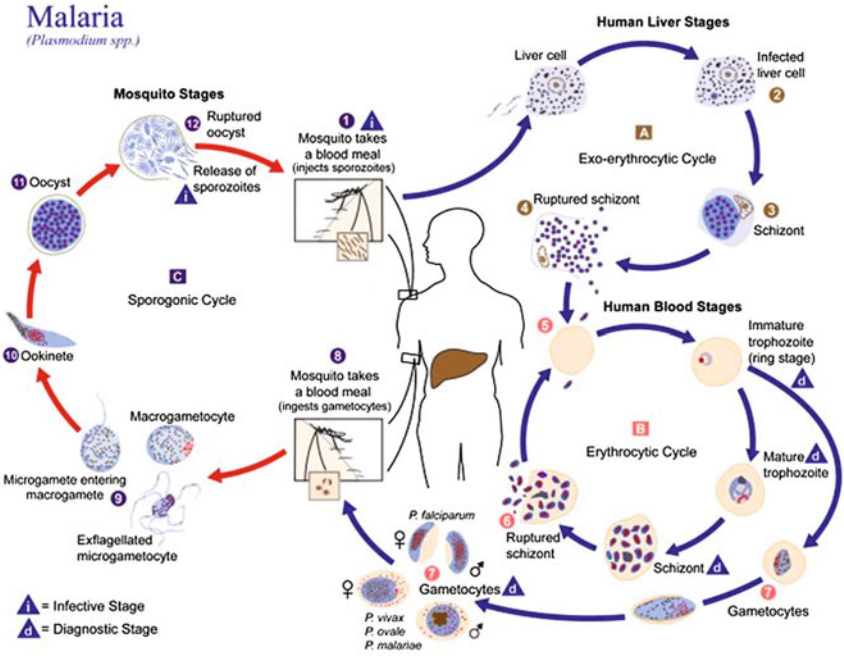

Fig. 3.4 Life cycle of *Plasmodium falciparum* showing schizogonic and sporogonic cycles

occur at the erythrocytic stage. The development of the parasite occurs in two cycles: an asexual one in human being and a sexual one in the mosquito (Fig. 3.4).

1. In Human: Asexual Cycle (Schizogonic Cycle)

When the mosquito bites a human being, hundreds of parasites (called sporozoites) mixed with its saliva pass into the blood of the person being bitten. The parasites migrate into the liver and enter the hepatic cells where they multiply, swell and at last cause the rupture of the cells. Then they (called merozoites) pass again in the blood and enter the red blood cells. Then, they are called trophozoites, the youngest stages with a ring form; they get bigger, deteriorate the haemoglobin of the red blood cells and multiplicate in the red blood cells. At that stage, they are called schizonts. When mature, the schizonts break out, releasing new merozoites, which invade intact red blood cells and continue the cycle. Duration of each cycle is about 48 h; rupture of red blood cells and formation of schizonts cause contemporary with feverish attack. Lastly, after several cycles, sexual forms of the parasite appear in the red blood cells, i.e. they develop into male and female gametocytes.

2. In Mosquito (*Anopheles* female): sexual cycle (sporogonic cycle)

After biting an infected human being, the mosquito absorbs schizonts, merozoites and gametocytes. Schizonts and merozoites are digested in the mosquito's stomach. The female gametocyte is inseminated by the male one, resulting in an egg

(oocyst) which go through the stomach wall and fix to it. Sporozoites develop in the oocyst and reach the salivary glands. Duration of sexual cycle in mosquito is 10–20 days. When the population is chronically exposed to a high risk of infection (as in sub-Saharan Africa), the more serious malaria attacks are observed in children. A partial clinical immunity is obtained during childhood, while adolescents and adults present only few malaria attacks, even if the parasite is chronically present in their blood.

(B) Mode of Action

Tests about uptake of radio-labelled substances like hypoxanthine in infected cells suggest that artemisinin diminishes the nucleic acid synthesis apart from primarily blocking the protein synthesis. In therapeutic concentrations of artemisinin, it alters polyamine metabolism of *P. falciparum* in vivo. Assays conducted in cultured endothelial cells indicated that artemisinin has little or no effect in preventing or reversing parasite adherence to endothelial cells. Artemisinin does not result in clumping of malaria parasite's haemin, but it inhibits clumping caused by subsequent exposure to chloroquine. Membrane changes may be associated with a decrease in cytochrome oxidase activity. Dihydroartemisinin in human erythrozoites infected with *P. falciparum* rapidly rose to the 300-fold concentration compared to uninfected cells which had only a duplication of the supplied substance. The steady state had been reached after 30 min. Dihydroartemisinin was concentrated mainly in the region of the parasite membrane. Some authors conclude that dihydroartemisinin is the main metabolic form of artemisinin (WHO 1998). Interaction between artemisinin with its analogues and haemin was investigated with high sensible physiochemical methods. Molecular docking simulations generated bioactive conformations between artemisinin derivatives and parasite's haemin (Cheng et al. 2002).

3.4 Malaria: Its Human Impact, Challenges and Control Strategies

Malaria is one of the most serious health problems facing the world today. The World Health Organization (WHO) estimates that over 300 million new cases of malaria arise a year, with approximately two to three million deaths resulting from contraction (Rathore et al. 2005 and Weathers et al. 2011). Malaria is the only vector-borne disease to be placed on WHO's disability-adjusted life year's (DALYs) list. Malaria is endemic in tropical Africa, with an estimated 90 % of the total malaria incidence and deaths occurring there, particularly among pregnant women and children. More specifically, malaria is causing various problems in Nigeria.

In the past, the fight against malaria was based on two strategies:

1. The extermination of the mosquito vector with pesticides such as DDT. This approach has undesirable side effects on the reproduction of animals and its persistence in food chains.

2. The large-scale use of quinine and chloroquine (isolated from bark of *Cinchona*) for the treatment of patients with malaria.

In the 1960s, *P. falciparum* malaria started to show signs of resistance against quinine-derived drugs. This resistance was reported from places as far apart as Brazil, Colombia, Malaysia, Cambodia and Vietnam, making it harder to control the disease. In addition, mosquito species capable of transmitting the disease were found in many parts of Asia. In 1969, the Chinese army found that a diethyl ether extract of *Artemisia annua* L., or qinghao in Chinese, had an excellent effect against malaria, and in 1972, artemisinin was identified as the main active ingredient (Anonymous 1982). Artemisinin contains an endoperoxide bridge, which is rarely found in secondary metabolites and is responsible for the antimalarial and anticancer activity (Firestone and Sundar 2009). It is a promising drug, as it has a lack of cross-resistance with other antimalarial drugs, no known adverse effects to humans and the ability to clear the blood of parasites more rapidly than other available drugs (Meshnick et al. 2002). Complete chemical (*de novo*) synthesis of artemisinin was achieved by several research groups (Schmid and Hofheinz 1983; Xu et al. 1986; and Avery et al. 1992).

(A) The Burden of Malaria

Social and economic consequences are directly related to the severity of the malaria's increased morbidity and mortality. In highly endemic areas, the older population develops some collective immunity to malaria so the severity of malaria attacks is less than in children under five. *P. ovale* is less fatal than *P. falciparum*. Since *P. ovale* is more prevalent in non-endemic areas, in these areas, the burden of disease is less than in endemic areas where malaria is due to the fatal *P. falciparum*. Currently, studies show that any increase in the disease burden of malaria as expressed in terms of DALYs is an unsustainable development. The level of socio-economic development in a country usually affects how much is invested in health care, which in turn affects the health outcomes and severity of diseases like malaria. Like a vicious cycle, the health outcomes affect income and capital, which in turn affects the economic development of the country. These poor health outcomes are partially responsible for its low gross national income per capita (GNI) of US$260 (Carrington 2001). In the cause and effect relationship between malaria and economic growth, it is also possible that the severity of malaria leads to poor health outcomes which in turn lead to a low GNI and poor economic growth.

There is a high concern with deaths of children due to severe malaria. Its syndromes are cerebral malaria and severe anaemia, both due to acidosis, which lead to death within 24 h after hospitalized. Unfortunately, there has been a spread of resistant strains of the parasite to the actual therapeutics (especially chloroquine), in numerous regions such as South America, Africa and South-East Asia (Basco et al. 2002). A large number of collaborations are trying to develop a vaccine against malaria, as stated by the NIAID report (James and Miller 2001). These researches target either the blood stage or the liver stage of the parasite

(Woster 2003). As far as chemotherapy is concerned, the search goes on for (1) synthetic analogues of quinine and chloroquine, (2) artemisinin analogues, (3) febrifugine analogues, (4) inhibitors of fatty acid synthesis or of membrane synthesis (inhibitors of choline uptake) and (5) proteases inhibitors (Charles et al. 1990 and Egan 2004).

(B) Malaria in India

According to WHO report 2011–2012, South-East Asian region bears the second largest burden of malaria (13 %), only being next to African region (81 %). Among South-East Asia region, India shares two-thirds of the burden (66 %) followed by Myanmar (18 %) and Indonesia (10 %) (WHO 2010). The only country in South-East Asia region 'free of malaria' is Maldives, while Srilanka and the Democratic People's Republic of Korea are in the 'pre-elimination phase'. India, Bangladesh, Bhutan, the Democratic Republic of Timor-Leste, Indonesia, Myanmar, Nepal and Thailand are in the 'control phase' (WHO 2010 and 2012).

In India, the most common and deadliest species is *P. falciparum,* contributing to 52 % of the total malaria cases in 2010 which is followed by *P. vivax*. Other two species are *P. malariae* and *P. ovale* which contribute to <10 % of the burden with very few cases occurred due to ovale species. Recently, a new plasmodium species, *Plasmodium knowlesi* which usually infects macaques has been identified, and over the past few years, hundreds of human cases have been reported in South and South-East Asian countries especially Malaysia with increasing numbers in Europe as well (Singh et al. 2004). Malaria distribution in India depends much upon vector bionomics. In India, 58 *Anopheles* species have been described, six of which have been concerned to be main malaria vectors, namely *A. culicifacies*, *A. dirus*, *A. fluviatilis*, *A. minimus*, *A. sundaicus* and *A. stephensi*. Besides, some are of local importance, viz. *A. philippinensis-nivipes*, *A. varuna*, *A. annularis* and *A. Jeyporiensis* (Dash et al. 2007). Figure 3.5 shows the state-wise distribution of

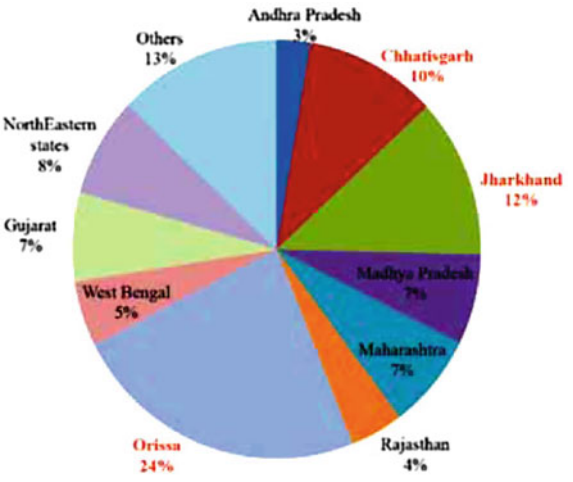

Fig. 3.5 State-wise distribution of malaria cases in India, 2011. *Source* NVBDCP 2011

malaria cases. Around 80 % of malaria burden is confined to 20 % of population residing in high-risk areas like Odisha, Jharkhand, Chhattisgarh, Madhya Pradesh, north-eastern states except Sikkim, Maharashtra and Rajasthan (Dua and Acharya 2013).

(C) Malaria Control Strategies in India

Drug-resistant malaria is common, and antimalarial drugs are becoming less effective as the plasmodium parasite develops resistance to affordable drugs. This poses a serious threat to clinical management and treatment of malaria. People cannot afford antimalarial drugs so they tend to self-medicate with local herbs. During the pre-independence era, malaria was considered as a major public health problem and the single largest cause of mortality in India. The highest incidence of malaria in India was reported in 1950s, with an estimated 75 million cases and 0.8 million deaths per year (Dua and Acharya 2013). Das et al. (2012) reported that confirmed cases of malaria have gradually decreased to 1.6 million cases and ~1,100 deaths in 2009 (Fig. 3.6).

With the start of 'National Malaria Control Programme' in India in 1953, the situation drastically improved within five years of implementation that led to change in its objective from 'Control to Eradication'. This led to the formulation of 'Modified Plan of Operation' in 1977, in which strategies differ in different regions according to the most commonly used parameter of annual parasite incidence (API). With the implementation of control strategy according to API, a decrease in overall number of cases was observed, and the total number of positive malaria cases being reported has remained almost constant over the last few years to around 1.5–2 million cases per year.

Scenario of malaria changed in 1990s as a result of number of factors that included the emergence of DDT resistance in vectors, emerging vectors with exophilic nature leading to failure of indoor residual spray (IRS) method and above all increasing urbanisation leading to increased migration of populations, thus changing the pattern of disease to a large extent (Fig. 3.7). Change in parasite occurrence with increasing proportion of falciparum from 10 % in 1970s to around

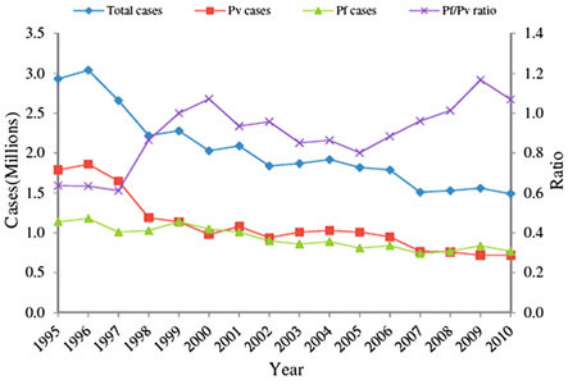

Fig. 3.6 Malaria cases and changing species pattern in India during the years 1995–2010. The total number of reported malaria cases has decreased since 1995, primarily through a reduction in the number of reported *P. vivax* cases. Currently, there is a 1:1 ration of *P. falciparum* to *P. vivax* cases. *Source* Das et al. 2012

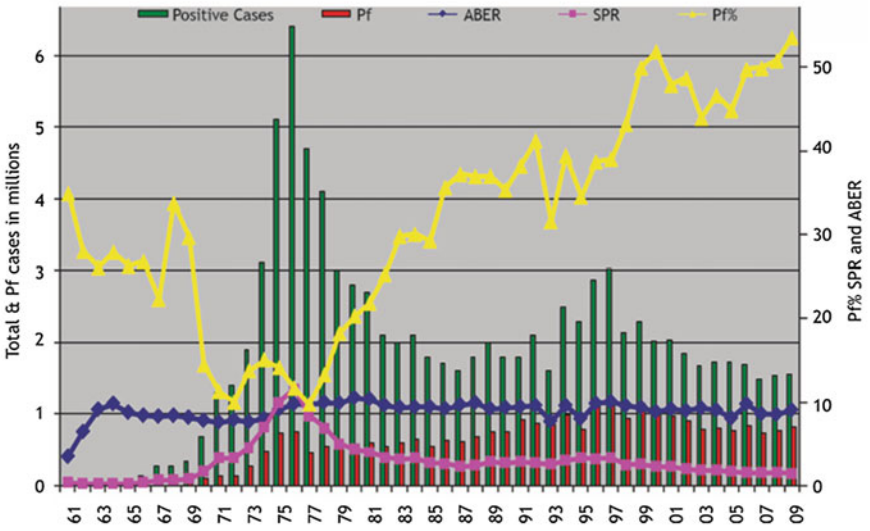

Fig. 3.7 Malaria trends in India from 1961 to 2009 (Pf—*Plasmodium falciparum*; ABER—Annual blood examination rate; SPR—Slide positivity rate; Pf %—*Plasmodium falciparum* percentage. Source Dua and Acharya 2013

50 % in 2010 is also one of the growing concerns of today. Emerging chloroquine resistance especially in the case of *P. falciparum* is considered as one of the important contributing factors responsible for an increase in its occurrence. Further, it has been observed that in subsequent years resistant *P. falciparum* will be soon replacing the susceptible *Plasmodium* population, leading to further worsening of the situation (Dua and Acharya 2013).

(D) Current Strategies under the Programme

The major strategies being pursued by the NVBDCP [9] for achievement of its objectives for control of malaria are as follows: (1) disease management through early case detection and complete treatment, (2) integrated vector management (IVM) to reduce the risk of vector-borne transmission and (3) supportive interventions which include communicating behaviour change, capacity building and monitoring and evaluation of programmes. Under the strategies, as far as diagnosis of the suspected case is concerned, rapid diagnostic kits were introduced in inaccessible areas or in areas with high API/high *P. falciparum* to facilitate easy and immediate diagnosis. Involvement of ASHA, peripheral health worker for diagnosis, is one of the newer initiatives introduced under National Rural Health Mission.

Now, as per the drug policy 2011 'presumptive treatment is no longer followed' (http://nvbdcp.gov.in/Round-9/Annexure-2%20%20Strategic%20action%20plan.pdf). The treatment schedule currently followed under the programme is as follows (www.mrcindia.org/Guidelines%20for%20Diagnosis2011.pdf).

- Uncomplicated *P. vivax*—Chloroquine for 3 days (Day 1:10 mg/kg + Day 2: 10 mg/kg + Day 3:5 mg/kg) plus Primaquine 0.25 mg/kg daily for 14 days
- Uncomplicated *P. falciparum*—Artesunate (4 mg/kg body weight) daily for 3 days and sulfadoxine–pyrimethamine (25 mg/kg + 1.25 mg/kg body weight) on Day 0 plus Primaquine 0.75 mg/kg body weight single dose on day 2
- Mixed Infections (*P. vivax* + *P. falciparum*)—Full course of ACT+Primaquine 0.25 mg/kg daily for 14 days.

3.5 Artemisinin-Based Combination Therapies: Significant Approach

ACTs are the most effective drugs for treating the disease. Increased funding for malaria treatments means that ACT demand is estimated to double from last year's figures to about 200 million treatments by 2012. Drugs, especially plant-based quinine, have played a key role for centuries. All have lost effectiveness over time due to the development of resistance. Currently, derivatives of artemisinin, an extract from the *Artemisia* plant, when combined with other drugs, provide the most effective treatment. They are known as artemisinin-based combination therapies or ACTs (Elfawal et al. 2012 and Dalrymple 2009). Artemisinin can also markedly reduce the mosquito infection rate in areas of low transmission (which are decreasing in Africa).

The emergence of chloroquine-resistant *P. falciparum* in South-East Asia in the 1960s (Peters 1987) 'brought the attention of the Chinese government to the seriousness of the malaria problem' (Dalrymple 2009). The antimalarial qualities of Artemisia were noted in 1971, and artemisinin was identified as the active ingredient and was isolated in 1972 (Klayman 1985; Anonymous 1992; Hein and White 1993; Zamiska and McKay 2007; White 2008; and Hsu 2006). In the period since 1972, the drug and several derivatives (artemether and artesunate) had been studied with regard to efficacy in laboratory malarial models, pharmacology and pharmacokinetics (PHC) and toxicology (TOX), and clinical trials had been conducted (Jiang et al. 1982 and Snow et al. 2005).

Drug combinations were not uncommon in China and had more generally been suggested for mefloquine in 1981 (WHO 1983). An antimalarial with the combined advantages of mefloquine and qinghaosu would be very valuable in the treatment of acute *falciparum* malaria, but the combination of a short-acting and a long-acting drug, although very effective therapeutically, does not avoid the potential problem of resistance developing against the long-acting component (Jiang et al. 1982). In the early 1990s, the Chinese developed an artemether–lumefantrine combination, one not previously tested. The rights to sell it outside of China were purchased by Ciba-Geigy, later to become Novartis (Zamiska and McKay 2007), which named it Coartem®. The subject was first discussed at the

international level in an 'Informal Consultation' on 'The Use of Antimalarial Drugs' at the WHO in Geneva in November 2000 (WHO 2001). Artemether–lumefantrine was therefore, the most valuable artemisinin combination treatment available in the market rated for the first time.

3.6 Clinical Efficacy

Joint efforts of research groups, policy-makers and funders will continue to be needed to prevent widespread ACT failure and to search for new antimalarial drugs (PATH 2011). These antimalarial drugs includes artemisinin, and its derivatives reduced the parasite biomass by 10,000-fold per asexual life cycle, compared to 100- to 1,000-fold for other antimalarials. They also decrease gametocyte carriage by 90 %, thus reducing transmission of malaria (White et al. 1999). When standard oral regimens of artemisinin monotherapy (2–4 mg/kg/day) are administered over seven consecutive days and adequate levels of drug are documented in plasma, the WHO confirms resistance if parasites are still present on Day 7 (i.e. at 168 h) or if parasites are present at 72 h and then recrudesce (i.e. initially clear but then reappear as late as day 42). A meta-analysis of clinical trials on 1919 patients has shown that artemether is at least as effective as quinine and is associated with fewer serious adverse effects (Artemether–Quinine Meta-analysis study group 2001). Chang and But (1986) reported that dilute alcohol extract of the herb, given as a total dose of 72 g of crude extract over 3 days in divided doses. The cure rate was said to be 100 % in 485 cases of *P. vivax* and 105 cases of *P. falciparum*. Hirt and Lindsey (2000) reported a 93 % parasite clearance rate in 254 patients who had taken a 7-day course of *A. annua* infusion in the Democratic Republic of Congo, mainly for *falciparum* malaria, although some had infections with other *Plasmodium* species. In a subset of 31 patients followed long term, the recrudescence rate was 13 % after 1 month (Hirt 2001).

Yao-De et al. (1992) report on a clinical trial of two types of gelatine capsules containing the herb *A. annua*, COEA and QHET (Table 3.1). One of these, called COEA, contained oil to enhance the absorption of the artemisinin. All the patients

Table 3.1 Clinical trial of *A. annua* in gelatine capsules

Drug	*N*[a]	Days of treatment	Total dose (g)	FCT[b] (h)	PCT[c] (h)	Recrudescence (%)
COEA	53	3	73.6	18.3	33.7	12/36 (33)
COEA	50	6	128.8	18.4	32.9	4/50 (8)
QHET	41	3	80.0	21.8	33.0	13/41 (32)
Chloroquine	20	3	1.2	23.7	49.9	0/20 (0)

[a] *N*= Number of subjects
[b] FCT= Fever clearance time
[c] PCT= Parasite clearance time
Source Yao-De et al. (1992)

were infected with *P. vivax*; 53 were treated with COEA for 3 days, and 50 were treated for 6 days. The dosage was 36.8 g on the first day, followed by 18.4 g on subsequent days. They reported parasite clearance times and fever clearance times were significantly faster for all the herbal preparations than for chloroquine. Patients were followed up for 30 days, with a blood film examined for parasitemia every 10 days. About one in three patients who had taken the capsules for 3 days only experienced a recrudescence, but this was reduced to only 8 % in those who had taken the capsules for 6 days. The larger clinical trials of different *A. annua* preparations are summarized in Table 3.2 (Wilcox et al. 2004a, b). Mueller et al. (2000) evaluated two preparations of *A. annua* for patients with *falciparum* malaria (Table 3.3). Although numbers were small, they found that both the infusion (1 L of boiling water added to 5 g of dried leaves and mixture left to cool for 1 min before filtering) and the decoction (5 g of dried leaves placed in 1 L of water, boiled for five minutes and then filtered) were effective. In each case, the dose was 250 mL four times a day, although the infusion group was treated for 5 days and the decoction group for 4 days. The results were good in both groups that the given decoction contained less artemisinin and was ineffective in mice. Unfortunately, there was no follow-up after day 7, so there are no data on recrudescence. Nevertheless, they all suggest that *A. annua* may be effective against malaria in humans. Yet, even in the Chinese studies, using relatively large amounts of *A. annua*, the dose of artemisinin would be less than half the WHO-recommended dose. There is an evident discrepancy between the reported clinical

Table 3.2 Clinical trial of *A. annua* preparations for patients and malaria

Efficacy	Recrudescence rate	N[a]	Species	Preparation	Total Dose (g)	Days	References
100 (%)	?	485	*Vivax*	Crude alcohol	72	3	Chang and But (1986)
100 (%)	?	105	*Falciparum*	Crude alcohol	72	3	Chang and But (1986)
100 (%)	8 %	50	*Vivax*	Oil based capsule	129	6	Yao-De et al. (1992)
100 (%)	?	5	*Falciparum*	Aqueous infusion	20	5	Mueller et al. (2000)
93 (%)	13 %	254	*Falciparum* + others	Aqueous infusion	35	7	Hirt and Lindsey (2000)
92 (%)	?	48	*Falciparum*	Aqueous decoction	20	4	Mueller et al. (2000)

[a] *N* = Number of subjects

Source Wilcox et al. 2004a, b

Table 3.3 Clinical data of 48 malaria patients before and after treatment with *A. annua* tea[a]

	Before treatment	After treatment
Parasitaemia (*P. falciparum*)	48 (100 %)	4 (8 %)
Subjective symptoms	48 (100 %)	11 (23 %)
Among these		
Headache	37	3
Fever	27	1
Chills	10	0
Arthralgia	14	4
Vertigo	6	1
Others	27	8

[a] Eighteen men, 30 women; mean age 38 years, range 18–67 years
Source Mueller et al. 2000

efficacy of traditional formulations of *A. annua* and their low artemisinin content. At present, it is not known whether slow clearance of artemisinin-treated parasites is associated with increases in gametocyte prevalence, density or infectivity to mosquitoes. Recently, however, one study from the Thailand–Myanmar border has reported that in patients treated with artesunate–mefloquine, delayed parasite clearance was associated with increased risk of developing gametocytemia (Carrara et al. 2009). If such increases are found to be attributable to the artemisinin component of ACTs, then post-treatment quantification of gametocytes could be useful in surveillance for artemisinin resistance and would suggest increased transmissibility of artemisinin-resistant parasites.

3.7 Management, Policies and Implications

The most effective, modern drugs for the treatment of malaria contain artemisinin, which the pharmaceutical industry extracts from plantations of *A. annua*, mostly in China and East Africa. Resistance has already occurred in some cases where artemisinin has been used alone, for example in drugs such as artesunate or Artenam. The World Health Organisation now recommends that drugs be produced which combine artemisinin with another antimalarial in what is known as 'artemisinin combination therapy' or ACT drugs. The best known such drug is named Coartem in Africa and Riamet in Europe and is a combination of artemisinin and lumefantrine (MMV 2012). The use of artemisia tea for the treatment of malaria is often the subject of criticism, because no authoritative clinical trials have been conducted. This is of course regrettable because artemisia tea is, in our opinion, the best possible means for enabling families, communities and hospitals to become almost self-sufficient in their treatment of malaria. Traditionally, chloroquine was a common treatment for Malaria. However, with the increase in chloroquine-resistant malaria, additional methods of control must be employed. A multidimensional approach should be used in the control strategy, such as good management of

clinical malaria, the use of insecticide-treated bed nets (ITBN), education and training programs in malaria prevention, vaccine research and the use of insecticide spraying such as DDT on breeding sites. It is also necessary to explore the use of indigenous natural mosquito-repellant plant species. Pharmaceutical companies should study local antimalarial herbs to determine their efficacy on malaria, and effective and safe dosages should be found. Policies pertaining to the use of impregnated (soaked in insecticide) bed nets would be doubly advantageous and economical in rural areas.

The following approach is key to avoiding malaria.

A awareness and advice
B bite prevention
C chemoprophylaxis
D diagnosis

In spite of considerable success in control of malaria, we lag far behind in the path to eliminate malaria in the near future. A number of challenges that need to be addressed along with suggested solutions are summarized in Table 3.4 (Dash et al. 2008).

New or updated WHO policies, operational manuals, guidelines and strategies for malaria control and elimination in 2012

New Policies:

- Seasonal Malaria Chemoprevention (SMC) for *P. falciparum* malaria control in highly seasonal transmission areas of the Sahel sub-region in Africa, March 2012.

Table 3.4 Challenges in malaria control

Challenges	Solution
Development of insecticide resistance	• Rotation policy to be used whereby use of unrelated compound are rotated every three months • Effective and continuous monitoring of resistance status in vectors using standardized bioassay technique
Development and spread of drug resistance	• Complete treatment to be given once diagnosis has been made • Systematic drug resistance studies to be carried out
Lack of information on true disease burden	• Systematic studies need to be carried out by effective utilization of resources
Communication gap between usage and availability of control measures	• Adequate IEC activities need to be carried to clear misconception of people • Adequate training of medical and paramedical staff needs to be carried out

Source Dash et al. 2008

Updated Policies:

- Intermittent Preventive Treatment of malaria in pregnancy using Sulfadoxine-Pyrimethamine (IPTp-SP), October 2012.
- Single dose primaquine as a gametocytocide in *P. falciparum* malaria, October 2012.

Position Statements:

- WHO interim position statement on larviciding in sub-Saharan Africa, March 2012.
- WHO position statement on effectiveness of non-pharmaceutical forms of *A. annua* against malaria, June 2012.

Operational Manuals, Handbooks and Guidelines:

- Disease surveillance for malaria control: an operational manual, April 2012.
- Disease surveillance for malaria elimination: an operational manual, April 2012.
- Guidelines for procuring public health pesticides, 2012.
- Management of severe malaria: A practical handbook. Third edition, December 2012.
- Seasonal Malaria Chemoprevention with sulfadoxine-pyrimethamine plus amodiaquine in children: a field guide, December 2012.
- Information note on recommended selection criteria for procurement of malaria rapid diagnostic tests (RDTs), April 2012.

Strategies, Action Plans and Initiatives:

- Global plan for insecticide resistance management in malaria vectors (GPIRM), May 2012.

3.8 Conclusions

A multidimensional approach is needed to control the malaria. Policy-makers need to immediately follow malaria control strategies because malaria infections are spreading across the developing countries. The responsible factors including the large-scale resettlement of people usually associated with ecological changes, increasing urbanization disproportionate to the infrastructure, drug-resistant malaria, insecticide-resistant mosquitoes, inadequate vector control operations and public health practices. Vector control is significant in the light of increasing drug-resistant malaria, as well as for cost-effective reasons. Insecticide-treated bed net trials are being conducted in some parts of Nigeria, and the results so far have been promising for the reduction in severity and prevalence of malaria in children.

A trusted method of controlling the mosquito is spraying breeding sites with insecticide such as DDT. Although some studies have reported the presence of

DDT-resistant mosquitoes, it is still one of the most effective and economical forms of insecticide in the control of malaria. The use of DDT was partly responsible for the reduction in malaria in areas where it is now mainly eradicated. Environmental laws are leading towards the total ban of the use of DDT. Due to its persistence in the environment and its effect on the ecosystem, it is regarded as a persistent organic pollutant. A total ban on the use of DDT, however, could prove disastrous to poor countries that still rely heavily on its use for malaria control. A more widely agreed-upon solution is that there should be mass campaigns for education training in malaria prevention. In addition, research for a vaccine for malaria would be a noble gift to Africa and other areas where malaria is endemic and should be intensified.

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Chapter 4
Whole Plant Approaches to Therapeutic Use of *Artemisia annua* L. (Asteraceae)

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Abstract Long used as a therapeutic tea by the Chinese to treat fever, *Artemisia annua* is more recently being studied and used for eventual treatment for not only malaria, but also many other diseases. This chapter describes studies using in vitro systems, animal models, and humans to evaluate use of not only combinations of pure compounds from the plant, but also tea infusions and the dried leaves of the plant.

4.1 Introduction

Indigenous to Asia, *Artemisia annua* L. is a generally regarded as safe (GRAS) herb (Duke 2001) that has been used >2,000 years in traditional Chinese medicine, usually as a tea infusion, to treat fevers, and febrile diseases, some currently associated with malaria. The main active compound is the sesquiterpene lactone

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artemisinin. Recent decreases in malaria deaths are mainly attributed to increased therapy, e.g., artemisinin, and prevention techniques including insecticide-treated bed nets. The threat of resistance to artemisinin monotherapy, however, is very real (Phyo et al. 2012), and high recrudescence rates associated with a short half-life of the drug (Giao et al. 2001) have brought about the need for costly combination therapies. To prevent and delay emergence of artemisinin drug resistance, the World Health Organization (WHO) now recommends artemisinin combination therapy (ACT) as the first-line treatment for malaria for two reasons: a second antimalarial drug, which has different biochemical modes of attack on parasites, would prove more effective, and the second drug should also kill parasites with developed artemisinin resistance (WHO 2010).

Parts of the world most affected by malaria—mainly sub-Saharan Africa—are desolate with poor infrastructure, making distribution of ACT antimalarial medicines difficult, even if 100 % of the demand were to be met. Additionally, the high cost of manufactured drugs deters treatment. Bed nets provide a preventive option to night-biting mosquitoes, but they may be difficult to obtain or afford (WHO 2013). A low-cost, local, effective, and reliably produced treatment may decrease mortality rates of malaria in these isolated regions; it could also spur economic development. Although artemisinin has the potential to treat a diversity of other diseases, here we review the results of *A. annua* therapeutic studies based on whole plant use.

4.2 *A. annua* Tea Infusion Therapy

4.2.1 Chemistry of Tea and its Preparation

To our knowledge, there have been few well-controlled studies examining the extraction recovery and stability of the many compounds in *A. annua* tea infusion. Recently, however, van der Kooy and Verpoorte (2011) performed a systematic study of different preparations of *A. annua* therapeutic tea infusion and showed that nearly 93 % of available artemisinin was extracted from dried *A. annua* leaves, but only under certain conditions. Ideal conditions were 9 g DW leaves L^{-1}, for 5 min at 100 °C. More importantly, they also showed that when stored at room temperature, the tea artemisinin concentration did not significantly decrease, which is important for people in developing countries where malaria is endemic and there is little or no access to refrigeration. Ideally, a liter of tea infusion would be prepared daily and consumed in equal aliquots of about 250 mL over 24 h (van der Kooy and Verpoorte 2011).

Carbonara et al. (2011) detected a wide variety of phenolics including 0.06 mg g^{-1} DW of the flavonoid, circilineol, in an *A. annua* tea infusion prepared as follows: 0.5 g in 13 mL boiling water, then leaving the infusion to cool for up to 48 h prior to extraction and measurement. The original starting artemisinin concentration in the leaves was not reported, thus preventing quantification of the

relative amount of constituents released into the tea. However, the artemisinin tea concentration remained constant during the 48-h room temperature infusion. Most of the measured phenolics also remained constant for 48 h. It is likely, however, that there was poor extraction of artemisinin mainly because the proportion of dried leaves to boiling water (38 g DW L^{-1}) was fourfold greater than that determined to be optimal (9 g DW L^{-1}) by van der Kooy and Verpoorte (2011). These authors showed data suggesting that increasing the ratio of dried leaves to water proportionately decreased the amount of extracted artemisinin; thus, at 40 g DW L^{-1}, only 43 % of the extractable artemisinin (93 %) appeared in the tea, a result also substantiated by Räth et al. (2004). Weathers and Towler (2012) later confirmed a high efficiency of extraction and 24-h stability of artemisinin retrieving about the same amount of artemisinin while using the same optimized tea protocol. However, several measured flavonoids, casticin and artemetin, were neither well extracted nor stable. Artemisinin solubility in water is about 50 mg L^{-1} (van der Kooy and Verpoorte 2011), so the amount of artemisinin retrieved via hot water tea infusions is reasonable. Clearly, if a tea infusion is to be a therapeutic option, however, it must be consistently and reliably prepared.

4.2.2 Tea Studies in Animals

To our knowledge, there is only one published study in animals using a tea infusion. Atemnkeng et al. (2009) compared parasite clearance in *Plasmodium chabaudi chabaudi*-infected mice ($n = 6$) treated twice daily for 6 days with either pure artemisinin or an *A. annua* tea infusion. Both treatments used an equal dose of artemisinin of 0.011 mg (0.275 mg kg^{-1}). This dosage was far lower than used in a third group of infected mice treated with WHO recommended doses of artemisinin of 0.4 mg (10 mg kg^{-1}) on day 1, followed by 0.2 mg (5 mg kg^{-1}) day^{-1} for days 2–7 (Table 4.1). After 6 days, only the WHO-dosed mice showed significant reduction in parasitemia; tea-treated mice had at best 50 % parasite clearance (Table 4.1).

4.2.3 Human Trials

Mueller et al. (2000) tested treatment of *A. annua* (cv. Artemis) tea on adults infected with uncomplicated *P. falciparum* malaria. The amount of artemisinin measured in the tea, although much lower than the usual dose of pure artemisinin and artemisinin derivatives, showed some success in treatment (Table 4.1). Later, Mueller et al. (2004) used doses of 5 and 9 g for *A. annua* tea preparations to treat *P. falciparum*-malaria-infected adults (Table 4.1). The *A. annua* (cv. Artemis), grown and dried in Germany, was delivered to the eastern Democratic Republic of Congo in prepackaged doses of dried leaves. Although the dried plant material was

Table 4.1 *A. annua* tea or dried whole-leaf effect on malaria-infected adult humans or animals

Delivery (drug, tea, or tablet) and days treated	AN-delivered dose (mg)	Control, dose (mg kg^{-1})	No. of Subjects	Leaf DW (g d^{-1})	Parasite clearance % (at day #)	Recrudescence % (at day #)	Reference
A. annua tea, 4d, 7d[i]	12	No control or placebo used	48	5	92 (4)	8 (4)	Mueller et al. (2000)
A. annua tea, 7d	11.75 (X4/d)	N/A	39	5	77 (7) 34 (35)[e]	43 (14) 62 (28) 66 (35)	Mueller et al. (2004)
	23.5 (X4/d)	N/A	33	9	70 (7) 30 (35)[f]	42 (14) 63 (28) 70 (35)	
Alternate drug	N/A	Quinine sulfate, 500	43	N/A	91 (7) 79 (35)[g]	10 (14) 14 (28) 21 (35)	
A. annua tea, 7d and placebo pill d1	<94/d	N/A	4	5	75 (7) 0 (28)	75 (14) 100 (28)	Blanke et al. (2008)
	<94/d	N/A	6	9	33.3 (7) 16.7 (28)	50 (14) 83.3 (28)	
Placebo tea, 7d & alternate drug d1	N/A	Sulfadoxine, 25 and Pyrimethamine, 1.25	9	N/A	77.8 (7) 37.5 (28)[h]	44.4 (14) 62.5 (28)[h]	
A. annua tea to *P. chabaudi chabaudi*-infected mice	0.0 0.011 0.011 0.4, d 1, 0.2, d 2–7	0.0 Dose in fed tea AN = to tea AN	6 6 6 6	N/A Leaves contained 1.15 % AN	100 (6) 72 (6) 50 (6) <1 (6)	N/D	Atemnkeng et al. (2009)

(continued)

Table 4.1 (continued)

Delivery (drug, tea, or tablet) and days treated	AN-delivered dose (mg)		Control, dose (mg kg^{-1})	No. of Subjects	Leaf DW (g d^{-1})		Parasite clearance % (at day #)	Recrudescence % (at day #)	Reference
Compressed crushed *A. annua* leaf tablets, given for 6d	Day 1	Day 2–6	No control or placebo used	12[b]	d 1	d 2–6	75 (28)[a,c]	25 (28)[a,c]	ICIPE (2005)
	7.4 × 2	3.7 × 2		12[d]	2		90.9 (28)[a,d]	9.1 (28)[a,d]	
	11.1 × 2	7.4 × 2		12[e]	3	1			
	14.8 × 2	11.1 × 2		12[g]	4	2			
	18.5 × 2	14.8 × 2			5	3		16.7 (28)[a,e,f]	
						4	83.3 (28)[a,e,f]	9.1 (28)[a,h]	
							90.9 (28)[a,h]		
A. annua (cv Sam) dried leaves vs. AN in mouse chow	0.6, in dried leaves		*P. chabaudi*-infected mice; 1st dose at ~8 % parasitemia; placebo control = AN in chow	6	0.04		100 (1.25)	100(4)	Elfawal et al. (2012)
				6	0.21			0(4)	
	3.0, in dried leaves			6	N/A		100 (1)	100 (4)	
	0.6, AN in chow			6	N/A		No clearance	~2 (4)	
	3.0, AN in chow						100 (1)		
Pure AN comparison	d 1, 500 × 2; d 2–7, 500 d^{-1}		Placebo used	227	N/A		76 (28)	24 (28)	Giao et al. (2001)

N/A not applicable
N/D not determined
[a] Based on Giemsa-stained blood smears counted against 200 WBC
[b] 1 subject migrated away after day 7
[c] 1 case recrudescence on day 14 (and a crudescence/reinfection? on day 28)
[d] 1 subject lost before completing course of treatment
[e] 1 subject not available by day 14
[f] 2 cases of early treatment failure
[g] 1 case of recrudescence on day 14
[h] 1 patient not available from day 3
[i] Treated 4 times daily
AN artemisinin

reported to have 1.4 % artemisinin, only 47 and 94 mg of artemisinin were extracted in a liter of tea prepared from 5 to 9 g of *A. annua*, respectively, which was ≤75 % of the original artemisinin in the dried leaves. Unfortunately, the study showed considerable recrudescence in the tea-treated group (Table 4.1). Because of the consistently lower rates of recrudescence in the quinine-treated control group (Table 4.1), it was inferred that most parasite reappearance in the tea-treated patients was the result of recrudescence, not reinfection (Mueller et al. 2004). Using 5 g dried leaves in 1 L, De Donno et al. (2012) showed that *A. annua* tea infusion was effective against both chloroquine (CQ)-sensitive (D10) and resistant (W2) strains of *P. falciparum* with IC50s of 7.08 and 5.60 nM, respectively.

In a more recent human tea trial (Blanke et al. 2008), a placebo tea with an alternate antimalarial drug was included on the first day of treatment in parallel with the test *A. annua* tea. Artemisinin concentration was at the same level as in Mueller et al. (2004), but tea concentration was at best 19 % (94 mg) of standard pure artemisinin treatments (500 mg) per person (Blanke et al. 2008). The *A. annua* used in this trial was also grown in Germany and sent to the test site in western Tanzania in dried, pre-dosed bags. At days 14 and 28, recrudescence of tea-treated patients was consistently greater than alternate drug treatments (Table 4.1).

Data from therapeutic tea trials in humans and in animals correlate well and unfortunately do not support the use of *A. annua* tea for treating malaria for the following reasons: animal and human data are comparably negative and compelling, artemisinin dose is not easily controlled, and other potentially useful components in the tea are not readily controlled or extractable. Nevertheless, use of the tea could play a role in malaria prophylaxis (Sect. 4.2.5) or in temporary control of malaria, mainly prevention of coma, until the infected person reaches a hospital or clinic stocked with ACT.

4.2.4 Comparative Pharmacokinetics of Dihydroartemisinin, Artemisinin, and Tea-Derived Artemisinin

Pharmacokinetic data for oral doses of artemisinin and dihydroartemisinin, the active form of artemisinin in the serum, were collected in 3 subjects by Zhao and Song (1993). When given orally or rectally, dihydroartemisinin showed higher bioavailability than artemisinin. Compared with artemisinin delivered orally, the results from Zhao and Song (1993) showed a higher serum dihydroartemisinin $C_{max,}$ 0.13–0.71 mg L^{-1}, in a shorter period of time with T_{max} ranging from 1.33–1.50 h (Table 4.2). Despite using a control of 15 mg kg^{-1} of pure artemisinin, C_{max} and T_{max} were 0.9 mg L^{-1} and 1.5 h; these results differed from other oral artemisinin studies (Table 4.2).

Alin et al. (1996) focused on the comparison of artemisinin and artemisinin–mefloquine combination therapy in oral delivery for the treatment for *P. falciparum* malaria. Pharmacokinetic parameters in infected and uninfected patients were

Table 4.2 Comparative pharmacokinetic parameters for oral artemisinin or *A. annua* infusion tea in humans

Drug delivery form (pure drug or tea)	AN dose	Subject	No. of subjects	C_{max} (mg/L)	T_{max} (h)	$T_{½}$ (h)	T_{lag} (h)	Reference
Oral dihydroartemisinin	1.1 mg kg^{-1}	Healthy adults	3	0.13 ± 0.03	1.33 ± 0.29	1.63 ± 0.68	–	Zhao and Song (1993)
	2.2 mg kg^{-1}			0.71 ± 0.30	1.33 ± 0.29	1.57 ± 0.34	–	
Oral AN	15 mg kg^{-1}			0.09 ± 0.01	1.50 ± 0.32	2.27 ± 0.22	–	
	250 mg (X2 d^{-1})[a]	Adults w/ fm	18	0.587 ± 0.385	2.5	2.2 ± 0.6	–	Alin et al. (1996)
	10.4-C	Healthy adults	6	0.483 ± 0.224	1.78 ± 1.23	2.61 ± 0.58	0.58 ± 0.30	Dien et al. (1997)
	10.4-F			0.623 ± 0.297	2.66 ± 1.50	2.51 ± 0.67	0.69 ± 0.37	
	250 mg	Healthy adult males	8	0.205 ± 0.127	2.8 ± 1.9	1.38 ± 0.40	1.5 ± 1.0	Ashton et al. (1998a)
	500 mg			0.450 ± 0.324	2.3 ± 0.9	2.00 ± 0.60	1.1 ± 0.4	
	1,000 mg			0.792 ± 0.498	2.8 ± 1.6	2.84 ± 1.08	1.0 ± 0.4	
Day 1	9.1 mg kg^{-1}		10	0.311 ± 0.232	–	3.0 ± 1.2	–	Ashton et al. (1998b)
Day 4			10	0.148 ± 0.093	–	3.8 ± 2.0	–	
Day 7			10	0.110 ± 0.104	–	4.8 ± 5.0	–	
Day 21 (after 14-d washout)			7	0.195 ± 0.126	–	2.7 ± 0.9	–	
Oral AN	10 mg kg^{-1}	Healthy adults	12	0.391	1.8	2.6	–	From review by Ilett and Batty (2005)
	6.8 mg kg^{-1}		4	0.150	–	2.3	–	
	6.2 mg kg^{-1}		6	0.360	1.7	$\alpha = 2.6$ $\beta = 4.3$	–	
	10 mg kg^{-1}	Pediatrics w/fm	23	–	–	1.8	–	
	10.8 mg kg^{-1}	Adults w/ fm	31	–	–	2.6	–	
	9.1 mg kg^{-1}		11	0.364	2.9	2.7	–	
Tea extract	94.5 mg	Healthy males	14	0.240	0.6	0.9	–	Räth et al. (2004)

C taken with food; *F* taken while fasting; fm *falciparum* malaria; AN. artemisinin

[a] Double loading dose

similar showing that, after a single dose, bioavailability of artemisinin was not altered. When comparing treatment failures with successes, however, pharmacokinetics were similar, suggesting that pharmacokinetic studies that only measure artemisinin were inadequate for predicting therapeutic success (Alin et al. 1996). Ilett and Batty (2005) also reported values for artemisinin pharmacokinetics in patients with falciparum malaria. The average weight of participants in Alin et al. (1996) was 53.8 kg, which gives an average dose of 9.3 mg kg^{-1} (close to the WHO recommended dose of 10 mg kg^{-1}). Ilett and Batty (2005) reported a dose of 9.1 mg kg^{-1}, which was comparable to that of Alin et al. (1996). Comparing these two reports, C_{max} did not differ much, only by 0.22 mg L^{-1} with T_{max} values differing by about 0.4 h (Table 4.2).

Ilett and Batty (2005) reviewed the pharmacokinetic parameters of artemisinin and its derivatives. For oral pure artemisinin doses ranging from about 6–11 mg kg L^{-1} in healthy people, C_{max} was also similar at 0.15–0.39 mg L^{-1} (Table 4.2). Dose appeared to have no major effect. In contrast, Ashton et al. (1998a) studied increasing artemisinin doses of 250, 500, and 1,000 mg per person and found apparently dose-dependent increases in C_{max} of 0.21, 0.45, and 0.79 mg L, respectively, but T_{max} remained relatively constant at 2.3–2.8 h (Table 4.2). However, $T_{1/2}$ values (1.38, 2.0, and 2.8 h, respectively) increased with dose (Table 4.2). It appears that the pharmacokinetics related to increasing artemisinin dose requires further study.

Diet is also very important for any orally delivered drug. For example, Dien et al. (1997) compared artemisinin oral doses delivered with and without food. The C_{max} values were similar between patients who fasted and those who did not, only differing by about 0.150 mg L^{-1} (Table 4.2). Intake of food with artemisinin did not seem to affect absorption of artemisinin. On the other hand, Weathers et al. (2011) found that when artemisinin was consumed as a component of *A. annua* dried leaf therapy, ~45-fold more entered the serum of mice than when orally administered as the pure drug (Table 4.3).

Interestingly, Ashton et al. (1998b) showed that when artemisinin at 9.1 mg kg^{-1} was given on days 1–7, and measurements taken on days 1, 4, 7, and 21, plasma C_{max} and $T_{1/2}$ were similar on day 1, and comparable to data from other studies using a similar dose (Table 4.2). Data collected on days 4 and 7, however, showed a decrease in C_{max} while $T_{1/2}$ increased. This trend showed that although artemisinin was delivered daily for 7 days, it was either not readily absorbed or degraded by CYP450 enzymes in the liver after the first dose. C_{max} fell from 0.31 to 0.11 mg L^{-1} after the third dose, and $T_{1/2}$ increased substantially from 3.0 to 4.8 h (Table 4.2). These results suggested that artemisinin either accumulated in the body or was degraded. In human liver microsomes, appearance of cytochrome P450 s, CYP2B6 in particular, correlates with decreasing artemisinin serum levels, suggesting that extended artemisinin dosing may not be beneficial (Svensson and Ashton 1999). Indeed, intermittent dosing, wherein the P450 levels are allowed to decline, was shown in the study by Ashton et al. (1998b) wherein by waiting 14 d to deliver another dose, C_{max} rose from 0.11 to 0.2 mg L^{-1}, and $T_{1/2}$ decreased from 4.8 to 2.7 h (Table 4.2). That being said, increased longevity of any

Table 4.3 Comparisons of oral artemisinin pharmacokinetics in rodent models

Delivery system (drug or whole plant)	Dose per individual (mg kg^{-1})	Subject	No. of subjects	C_{max} (mg/L)	T_{max} (h)	$T_{½}$ (h)	Reference
Intragastric dihydroartemisinin	10 or 0.035	Rats	4	0.77	0.25	0.24	Li et al. (1998)
Artemisinin (AN)	40		3	0.64 est[b]	1.0	0.25 est	Du et al. (2012)
A. annua dry leaves[a]	1.22 leaf	Mice	3	0.087	0.5	nd	Weathers et al. (2011)
vs. AN in mouse chow	1.22 AN in chow		3	nd	nd	nd	
	56 AN in chow		3	≥0.074	nd	nd	
A. annua dry leaves vs.	100 leaf (h[b])		6	4.3	1.0	0.86	Weathers et al. unpublished
AN or	100 leaf (i[b])		6	6.6, 4.7[d]	>2	nd	
AN in mouse chow[c]	100 AN		3 p[c,e]	nd[f]	nd	nd	
	100 AN in chow		3 p[c,e,f]	nd[f]	nd	nd	
	0		3 p[c,e]	nd[f]	nd	nd	

[a] Study ended at 60 min, so T_{max} and C_{max} not truly measured
[b] Abbreviations: est, estimated from figures; h, healthy; i, infected; p, serum pooled from 3 mice; nd, not detectable
[c] Study ended at 120 min, so T_{max} and C_{max} not truly measured
[d] Study response was biphasic, so data shown for 2 peaks at 15 and 120 min
[e] Three pooled mice; serum harvested 60 min post-gavage
[f] At 60 min, artemisinin serum level of 3 pooled mice was 2.44 mg mL^{-1} ; for other two pooled mouse treatments, no artemisinin was detectable

artemisinin treatment may reduce recrudescence. For the most part, the oral dosage data seemed consistent in that maximum concentration of artemisinin in the body increased with increasing doses. Generally, $T_{1/2}$ ranged from about 1.4–4.8 h for all trials using oral pure artemisinin.

With the exception of Räth et al. (2004), there are few reports on the pharmacokinetics of artemisinin delivered via tea to humans. In the Räth et al. (2004) study, peak concentrations of 0.24 mg artemisinin L^{-1} occurred at 0.6 h after tea intake. Table 4.2 compares the pharmacokinetic parameters for *A. annua* and artemisinin in humans. The tea extract containing 94.5 mg artemisinin showed a C_{max} plasma concentration equivalent to a dose of 250 mg pure artemisinin, but at a significantly shorter T_{max}, 0.6 h versus 2.8 h (Ashton et al. 1998a, 1998b, also see Table 4.2). Compared with pure artemisinin, the lower half-life of artemisinin in the tea extract (Table 4.2) may have resulted in the observed higher recrudescence. Pure artemisinin has a short half-life (Table 4.2). Although tea-delivered artemisinin seemed more bioavailable, it had a lower half-life of 0.9 h compared with about 2 h for pure artemisinin (Table 4.2), suggesting that more than two doses per day may be more beneficial; four doses a day were recommended.

Plasma concentrations, being almost 40 % lower than that of traditional doses (500 mg per person of 60 kg or 8.3 mg artemisinin kg^{-1}) of pure artemisinin, were concluded as cause for unacceptably high recrudescence rates in clinical tea trials (Table 4.2). Assuming that the average weight of subjects was 60 kg, the artemisinin dose/kg in the tea trial was estimated at about 1.5 mg kg^{-1}, close to the 1.1 mg kg^{-1} dose of pure artemisinin used by Zhao and Song (1993), way below the 8.3 mg/kg mentioned above as traditionally accepted as pharmacologically effective. Considering this, the C_{max} of artemisinin for the tea dose was nearly twice as much as that of pure artemisinin (Table 4.2). Notwithstanding, tea from *A. annua* showed potent antiplasmodial activity when tested against 40 field isolates of *P. falciparum* collected in Pikine, Senegal (mean IC50 0.095 μg mL^{-1}; Gueye et al. 2013).

4.2.5 Prophylactic Human Trials

In a randomized clinical trial in Uganda (Ogwang et al. 2011, 2012), artemisia tea was tested as a prophylaxis against malaria in 132 farm workers for 9 months, and any adverse clinical effects were tracked for 12 months. Tea consumed once a week at a 2.5 g adult infusion dose had an unadjusted protective efficacy of 37.5 % (Ogwang et al. 2012), which is better than that reported for vaccines RTS, S/AS01B and RTS, S/AS02A with protection efficacy of about 30 % in adults (Bojang et al. 2001; Polhemus et al. 2009). It was also superior to FMP1/AS02 vaccine that was reported to confer no protection and also to vaccines LSA-NRC/AS01 and LSA-NRC/AS02 that elicited antigen-specific antibody and CD4+ T cell responses, but with no protective immunity (Ogutu et al. 2009; Cummings et al. 2010). Tea protective effects also increased with duration of use. Unlike vaccines

such as RTS, S/AS02 whose protection wanes within a few weeks (Bojang et al. 2001), the increasing protection trend by artemisia tea suggested that curbing of malaria in a given population is a possibility. Persons who used artemisia tea also had 80 % fewer hospital visits due to fevers, with some individuals in the study community reporting use of the tea for >7 years with no incidence of malaria. More randomized clinical trials of *Artemisia* tea malaria prophylaxis need to be conducted in different populations and age groups.

A study carried out in Uganda among adults aged 18–60 years found that their immunity to malaria is greater than that in children or the very elderly. Although one might argue that weekly consumption of *A. annua* tea might lead to emergence of resistance, data soon to be submitted for publication from the Rich and Weathers laboratories in Massachusetts (USA) suggest otherwise.

4.3 *A. annua* Dried Leaf Consumption as Therapy

4.3.1 Animal Studies

Recently, Elfawal et al. (2012) measured parasitemia in mice infected with *P. chabaudi* that were fed two different doses (0.6 or 3.0 mg; 24 and 120 mg kg^{-1}, respectively) of pure artemisinin either in mouse chow or in dried leaves of *A. annua.* The dried leaves were at least five times more effective, and with a longer lasting response, than the pure drug in reducing parasitemia (Table 4.1). Interestingly, mice needed >45-fold more artemisinin (mixed with mouse chow) than artemisinin consumed via dried leaf in order for artemisinin to be detected in the serum (Weathers et al. 2011).

4.3.2 Human Trials

Except for the early tea trials of Mueller et al. (2000, 2004), in the Democratic Republic of Congo, clinical trials using dried leaf *A. annua* are scarce in the scientific literature. Although WHO does not encourage either whole plant or tea infusion clinical trials (WHO 2012), some African universities carried out their own trials (personal comm from C Kasongo to P Lutgen). Those involving a very limited number of patients were generally not published, and results were not assessed by polymerase chain reaction (PCR) as later done for clinical trials with ACTs. Their results are briefly described here, with best studies summarized in Table 4.1.

Compared with controls or even other antimalarial drugs, e.g., artesunate–amodiaquine, early unpublished trials mainly used *A. annua* decoctions and showed significantly greater sensitivity of the decoction with lower late therapeutic

failures. In the Democratic Republic of Congo, 54 volunteers suffering from malaria were treated for 10 days with capsules containing powdered leaves of *A. annua* at decreasing doses. The total amount of dried herb administered per patient was 15 g dried leaves containing 15 mg of artemisinin [0.1 % artemisinin leaf content; Tiruneh et al. (2010)]. All were free of fever after 2 days, and 51 were free of parasites after 10 days.

In an unusual study aimed at preventing severe postoperative malaria at Bangui, Central Africa, capsules containing powdered leaves of *A. annua* leaves were administered to 25 patients, 22 of them children aged 1–16 years, during surgical interventions for orthopedic disorders (Onimus et al. 2013). The duration of the treatment ranged from 3–4 days with a daily dose of 0.4–0.5 mg artemisinin delivered in 0.4–0.5 g of *A. annua* dried leaves, 0.1 % artemisinin leaf content. Despite the very low administered dose of artemisinin, average parasitemia in the patients dropped by 62 % with an added benefit of a strong antinociceptive response.

The most thorough study designed to assess clinical efficacy of whole-leaf *A. annua* was undertaken in a collaborative project between the International Centre of Insect Physiology and Ecology (ICIPE) and Kenya Medical Research Institute (KEMRI) (ICIPE 2005; Table 4.1). The study, conducted at ICIPE Mbita Field campus, Suba District, Western Kenya, was an open-label, non-randomized clinical trial primarily targeted to assess the efficacy, safety, and tolerance of increasing doses of whole-leaf *A. annua* in the form of tablets (Sawa et al., in preparation). The tablets were made from a hybrid of *A. annua* grown in the Tanzania highland (2,000–2,200 m altitude) by a Tanzania-based NGO, Natural Uwemba System for Health (NUSAG). Harvested leaves from 8-month-old plants (just before flowering) were dried for $\sim$3 weeks under shade, then crushed, finely powdered, homogenized, and pressed into 500 mg tablets under ambient temperature. Randomly selected batches of 100 tablets extracted with hexane, concentrated and analyzed by HPLC with diode array detector showed the artemisinin content of the tablets was highly consistent at 0.74 $\pm$ 0.06 % (i.e., $\sim$3.7 mg per tablet).

Forty-eight consenting patients aged 15–56 years (average 23.42), with *P. falciparum* malaria (parasitemia was 0.02–4 %, based on Giemsa-stained blood smears counted against 200 WBC) and hemoglobin levels $\geq$8 mg dL^{-1}, were recruited for the project. Patients were divided into four cohorts and treated with increasing levels of *A. annua* tablets, ranging from 2 to 5 tablets twice on day 1, followed by 1–4 tablets twice daily for the next 5 days. Although there were three cases of reappearance of parasites in blood smears scattered throughout different cohorts a week following the treatments, all doses were effective in clinical and parasitological regression of malaria with 9–20 % recrudescence at day 28. Patients also suffered no toxic affect; there was no significant change in the serum levels of urea, serum proteins, creatinine, γ-glutaryl transferase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, alkaline phosphatase levels, or hemoglobin; pre- and post-electrocardiograms were unchanged (ICIPE 2005).

Thus, despite the relatively low levels of artemisinin in the administered doses (14.8–37 mg on day 1 followed by between 7.4–29.6 mg on days 2–6) and

compared to daily doses of 60–90 mg day^{-1} of leaf artemisinin in a hot water extract for 5 days (Mueller et al. 2000; Räth et al. 2004), by day 28, 75–91 % cure was achieved (Table 4.1). This cure rate was also comparable to or exceeded other results: 500 mg day^{-1} of pure artemisinin in the form of tablets or capsules (Hien 1994; McIntosh and Olliaro 2010), and similar levels of dihydroartemisinin derivatives (artesunate, artemether, etc.) in the form of tablets or capsules (de Vries and Dien 1996). Furthermore, the positive therapeutic response seemed somewhat independent of the range of dose tested (Table 4.1; ICIPE 2005). Although the oral doses used in the ICIPE (2005) trials were far less than any tea studies, levels of recrudescence were much lower than tea and equivalent to studies using pure artemisinin (Giao et al. 2001; Table 4.1).

These results suggested that there is an important role for the phytochemical blend associated with powdered foliage of whole-leaf *A. annua* when orally administered as tablets. The results are also consistent with the study by Elfawal et al. (2012) and a study in China on mice infected with *P. berghei*, which compared the effects of pure artemisinin with crude *A. annua* extracts (Yao-De et al. 1992). The two products had comparable levels of artemisinin; however, crude preparations were at least 3.5 times more effective in reducing parasitemia than pure artemisinin, indicating a synergistic role played by non-artemisinin constituents in the extracts.

4.3.3 Pharmacokinetics of Whole-Plant-Delivered Artemisinin

Li et al. (1998) provided some of the earliest reported pharmacokinetics of intragastric delivery of dihydroartemisinin in rats. The pharmacokinetic parameters of artemisinin in animal models has also been used to study the delivery of artemisinin via whole plant *A. annua*, and the few known reports are summarized in Table 4.3 and compared to ingestion of pure artemisinin. Weathers et al. (2011) showed that when uninfected mice were orally gavaged either with artemisinin mixed in mouse chow or with dried leaves of *A. annua,* the serum C_{max} of artemisinin was about the same (Table 4.3). However, it was necessary to increase the amount of artemisinin in the chow >40-fold that in the leaves in order to achieve the same response. This result suggested higher bioavailability of plant-delivered artemisinin. More recently, pharmacokinetics was compared for healthy and *P. chabaudi*-infected mice treated with dried *A. annua* leaves; both C_{max} and T_{max} of artemisinin were greater in infected than healthy mice (Weathers et al. unpublished; Table 4.3). Interestingly, the infected mice showed biphasic artemisinin serum peaks at 15 min and 2 h with serum concentrations at 4.7 and 6.7 mg artemisinin L^{-1}, respectively. It is possible that the serum level increased further, but the study was terminated at 120 min. A liver metabolic product of artemisinin is deoxyartemisinin (Whirl-Carrillo et al. 2012). At the high (100 mg kg^{-1}) dose

used in the study, nearly equal amounts of deoxyartemisinin and artemisinin were measured in the serum, indicating that an excessive dose of artemisinin was used. These are, to our knowledge, the only known data available on pharmacokinetics for whole plant oral doses in animals or humans.

4.4 How Can We Explain the Enhanced Effect of Whole Plant Versus the Pure Drug: Synergism?

A. annua is rich in essential oils, polysaccharides, saponins, coumarins, acids, minerals, flavonoids, and polyphenols some of which were recently reviewed by Ferreira et al. (2010). This review is not intended to be exhaustive; rather, it intends to suggest that an in-depth study is overdue for the antimalarial properties of these molecules. Here, we highlight some of these potentially important constituents that could be providing the added benefits especially observed in studies of oral consumption of dried leaves of *A. annua*. These benefits could accrue not only as therapeutic activity, but also enhancing bioavailability of artemisinin.

4.4.1 Terpenes

α-Pinene is a volatile constituent of essential oil present in the plant regardless of origin at levels up to 0.05 % of dry weight (Bhakuni et al. 2001). In a South African study, which determined the antimalarial activity of 20 essential oils, α-pinene ranked second with an IC50 of 1.2 μM, similar to that of quinine at 0.29 μM (Seatholo 2007; van Zyl et al. 2006).

1,8-Cineole (eucalyptol) often comprises up to 30 % of the essential oil in *A. annua* or 0.24–0.42 % (V/DW) (Charles et al. 1990). The molecule is a strong inhibitor of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8 (Juergens et al. 2004). Growth and development of chloroquine-resistant and chloroquine-sensitive *Plasmodium* strains are affected and are stalled at the early trophozoite stage (Su et al. 2008). This volatile terpene is rapidly absorbed into the blood when delivered either orally or as an inhalant (Kovar et al. 1987; Stimpfl et al. 1995), reaching 15 μg mL^{-1} in 60 min. Indeed Kovar et al. (1987) suggested its possible use as an antimalarial inhalant. With an IC50 of 0.02 mg mL^{-1} and an LD50 of ~25 mg mL^{-1}, either inhalation or oral delivery is reasonable (Kengne 2010; Su et al. 2008).

Artemisia ketone is a major constituent of *A. annua*, but its role has barely been studied. It may, however, play a role in hemozoin formation. *Plasmodium* needs hemoglobin for its survival and multiplication in merozoites inside the red blood cell (Akkawi et al. 2012). Although this gives the parasite access to nitrogen, it leaves debris like heme, which is toxic. The parasite circumvents this by causing oxidation of Fe(II) in heme to Fe(III) forming hematin that polymerizes into an

insoluble product called β-hematin and hemozoin, which is non-toxic to the parasite and inhibits cell-mediated immunity against the parasite. Other ketones such as curcumin (Akhtar et al. 2012) were implicated as inhibitors of β-hematin synthesis, so it is possible that artemisia ketone plays a similar role.

Limonene is part of the so-called cineole cassette, which includes 1,8-cineole, limonene, myrcene, α-pinene, β-pinene, sabinene, and α-terpineol (Raguso et al. 2006), many of which affect particular stages of *Plasmodium* species. Limonene, for example, arrests isoprenoid biosynthesis in *Plasmodium* (Goulart et al. 2004) and development at the ring and trophozoite stages (Moura et al. 2001), while 1,8-cineole affects the trophozoite stage (Su et al. 2008). Limonene also inhibits protein isoprenylation in *P. falciparum*, arresting parasite development within 48 h of treatment (Moura et al. 2001). The in vitro IC50 against *Plasmodium* in these trials was 2.27 mM, significantly below 15.5 mM that was previously determined in vivo in patients with advanced cancer. The pharmacokinetics is favorable; limonene and its metabolites remain in the plasma for at least 48 h (Miller et al. 2010). This is important for the elimination of gametes and malaria transmission.

A combination of essential oils may enhance the antimalarial effect of artesunate and even reverse the resistance of *P. berghei* against artesunate (Liu et al. 2004). In *A. annual* The concentration of monoterpenes is higher in the pre-flowering phase (Yang et al. 2012), but is drastically reduced by high drying temperatures or drying in the sun (Khangholil and Rezaeinodehi 2008; Ferreira and Luthria 2010). The monoterpene limonene has a very favorable toxicity profile and is easily available at low prices. Limonene is also present in *A. annua* at concentrations up to 7 mg kg^{-1} (Bhakuni et al. 2001). So far, studies have concentrated on this particular monoterpene, but others such as eucalyptol, present in the essential oil of *Artemisia* plants, might have a similar detrimental action on the apicoplast, a non-photosynthetic plastid of most apicomplexan parasites, such as *Plasmodium*.

The sesquiterpene nerolidol, found in *Artemisia* species, arrests development of the intraerythrocytic stages of the parasite. It has an antiplasmodial IC50 of 0.99 μM compared to that of 533 μM for limonene (van Zyl et al. 2006). Indians of the Amazon Basin in Brazil use the vapors of the leaves of *Viola surinamensis* to treat malaria and the sesquiterpene nerolidol was identified as the active constituent leading to 100 % growth inhibition at the schizont stage (Lopes et al. 1999). Like limonene, nerolidol may affect the isoprenoid pathway in the apicoplast of *Plasmodium*. Nerolidol concentrations vary with the origin of *A. annua*. The highest value was found in *A. annua* from Ethiopia (Muzemil 2008). Nerolidol was also found to be higher in the stems than in the leaves of *A. annua* (Li et al. 2011).

4.4.2 Phenolic Acids

Rosmarinic and chlorogenic acids, recently identified in a wide variety of *A. annua* cultivars, are strong antioxidants (de Magalhães et al. 2012). In Caco-2 studies, these acids significantly reduced secretion of the inflammatory cytokines IL-6 and

IL-8. They also inhibited CYP3A4 activity and enhanced antimalarial activity while reducing inflammation.

4.4.3 Flavonoids

There are >40 flavonoids in *A. annua* (Ferreira et al. 2010), and at least 10, including artemetin, casticin, chrysoplenetin, chrysoplenol-D, circilineol, eupatorin, kaempferol, luteolin, myricetin, and quercetin, show some weak in vitro therapeutic efficacy against falciparum malaria (Liu et al. 1992; Elford et al. 1987; Lehane and Saliba 2008). When some of these flavonoids were tested in combination with artemisinin, the IC50 of artemisinin against *P. falciparum* in vitro improved by as much as 50 %, suggesting synergy with artemisinin (Liu et al. 1992). Interestingly, Elford et al. (1987) also showed that while casticin showed synergism with artemisinin, casticin did not synergize with chloroquine, suggesting a different interactive mechanism. Casticin and artemisinin, however, did inhibit parasite-mediated transport systems controlling influx of myoinositol and L-glutamine in malaria-infected erythrocytes. This apparent synergistic action between artemisinin and flavonoids suggests that flavonoids would likely be important for efficacious use of *A. annua* consumed either as dried leaves or as tea.

Some flavonoids have antiplasmodial effects and inhibit *P. falciparum* growth in liver cells in vitro as reported for dietary flavonoids (Lehane and Saliba 2008). Although data on pharmacokinetics of *A. annua* flavonoids are scant, some flavonoids generally have long plasma half-lives. For example, quercetin, a flavonoid also found in *A. annua* and most fruits, has a plasma half-life of 27 h (Manach and Donovan 2004). Lehane and Saliba (2008) demonstrated that quercetin found in garlic inhibits malaria parasite growth in liver cells. Considering the half-life of 27 h reported for quercetin, flavonoids can persist in the body for up to 5.83 days possibly enabling a once-a-week dose of artemisia tea to inhibit parasite growth and contribute to the prophylactic effect. According to Lehane and Saliba (2008), though dietary flavonoids inhibit malaria parasite growth in vitro, the amounts in the diets are insufficient to offer protection against malaria. This means that plants such as *A. annua* with high concentrations of flavonoids may prevent malaria when consumed regularly. Flavonoid content of plants is as high as 0.6 % of dry weight (Weathers, unpublished). In another study by Baraldi et al. (2008), the flavonoid content in *A. annua* varied with developmental growth stage, with highest amounts found during full bloom.

The flavone, luteolin, is found in *Artemisia* species comprising up to 0.0023 % of dry weight (Bhakuni et al. 2001). Ethnobotanical use of luteolin includes treatment for cough, diarrhea, dysentery, diabetes, cancer, and malaria. Compared with other flavones such as kaempferol, myricetin, quercetin, isoquercitrin, acacetin, apigenin, baicalein, and chrysin, luteolin was found to be the most active with IC50 values around 11 μM (Lehane and Saliba 2008). Luteolin also prevents progression of parasite growth beyond the young trophozoite stage, so they cannot

complete a full intraerythrocytic cycle. This strong antiplasmodial activity is eventually related to the inhibition of fatty acid biosynthesis by *P. falciparum*. These lipids are required for the detoxification of heme into hemozoin by the parasite (Tazedimir et al. 2006). Apicomplexan parasites use a fatty acid synthesis pathway, independent of the human host, and catalyzed by specific enzymes like FabG. These enzymes are a potential target of new antimalarials. Among 30 flavonoids studied, luteolin and quercetin had the lowest IC50 s for the inhibition of these enzymes (Tazedimir et al. 2006). In this same study, these two flavonoids also showed in vitro activity in the submicromolar range against multiple strains of *P. falciparum*.

4.4.4 Polysaccharides

So far, the presence of polysaccharides in *A. annua* has been barely covered in the scientific literature. The reason may be that they are only soluble in water and most *Artemisia* extracts for research are obtained using organic solvents. Polysaccharides are polymeric carbohydrates of high molecular weight. Although the presence of polysaccharides in other medicinal plants has been more extensively studied, they seem to have been overlooked in the research on *A. annua*. There are more polysaccharides in stems than in leaves, and their solubility is also higher for this part of the plant (Ahn and Jung 2011). The same authors found that polysaccharides extracted from *Artemisia* had hydroxyl radical scavenging activity 3 times stronger than glutathione or caffeic acid and the ROS inhibition 2 times stronger than ascorbic acid.

The complex of polysaccharides with lipophilic molecules may lead to a higher bioavailability of antimalarial constituents in *A. annua* and may explain the considerably lower therapeutic doses against malaria required for artemisinin, e.g., in whole plant studies (ICIPE 2005; Elfawal et al. 2012) than for pure artemisinin. Some sulfated polysaccharides inhibit the formation of rosettes between infected red blood cells (iRBC) and uninfected RBCs. More importantly, they inhibit the adhesion of iRBCs to placental chondroitin sulfate A (CSA), which is linked to severe disease outcome in pregnancy-associated malaria (Adams et al. 2006). Sulfated polysaccharides also interfere with the plasmodium merozoite surface protein and inhibit the invasion of merozoites into erythrocytes in vitro (Andrews et al. 2005; Xiao et al. 1996; Clark et al. 1997). Heparin and other sulfated polysaccharides have been shown to inhibit blood-stage growth of *P. falciparum* (Munir et al. 1980; Rampengan 1991). Ginseng polysaccharides show preventive and curative antimalarial activities and synergism with artesunate. This was confirmed in vivo in malaria-infected mice (Han 2008).

4.4.5 Saponins

Saponins are common in a large number of plants, and they have an important role in human and animal nutrition. They are reportedly present in *A. annua*, albeit only as measured by the non-quantitative foaming test of alcoholic extracts (Ashok and Upadhyaya 2013; Massiha et al. 2013; Weathers, unpublished). Saponins are soap-like amphiphilic (lyophilic and hydrophilic) bioactive compounds produced mainly by plants. Recently, there has been unforeseen interest in the clinical use of saponins as chemotherapeutic agents (Podolak et al. 2010). They are efficient at very low doses, have hemolytic properties, and produce 40–50 Å pores in erythrocyte membranes. Saponins are used as adjuvants for vaccines (Song et al. 2009). Saponins also modulate the sodium pump and ATPase (Haruna et al. 1995). They inhibit the intestinal permeability of glucose and may consequently inhibit the growth of *P. falciparum,* which needs glucose to grow. Saponins also have a hypoglycemic effect mainly by inhibiting intestinal absorption of glucose (Francis et al. 2002). Further quantitation and investigation into the role of saponins in *A. annua* whole plant therapeutic effects, it seems, are warranted.

4.4.6 Coumarins

Most *Artemisia* species contain the coumarin, scopoletin. The concentration of scopoletin in several plant samples of *A. annua* as measured at Luxembourg is around 0.2 % (W/W). Scopoletin is known for its antioxidant, hepatoprotective, and anti-inflammatory activities (Malik et al. 2011). Scavenging capacity for hydroxyl radical, DPPH, superoxide anion, hydrogen peroxide, and Fe^{2+} chelating activity are almost at the level of α-tocopherol (Vitamin E) (Malik et al. 2011). At mM concentrations, scopoletin inhibits TNF-α, IL-6, and IL-8 and is thus likely one of the major anti-inflammatory and antipyretic constituents of *A. annua* (Moon et al. 2007). Scopoletin is also known for its antinociceptive properties (Meotti et al. 2006; Chang et al. 2012).

Coumarins are capable of activating lymphocytes, thus stimulating immunological functions. Moon et al. (2007) showed that scopoletin has an immunomodulatory effect and induces cell proliferation on normal lymphocytes. A significant hormetic effect was also noticed: stimulation is higher at 10 μg mL^{-1} than at 1 or at 100 μg mL^{-1}.

Scopoletin significantly stimulates RBC membrane ATPases at 0.1 μM in particular Na–K-ATPase versus Ca-ATPase or Mg-ATPase (Ezeokonkwo and Obidoa 2001), which may affect malaria infection. In uninfected erythrocytes, the internal Na concentration is much lower than outside the cell, but the *K* concentration is higher. However, in infected blood cells, this situation is drastically reversed (Surono et al. 2008). Scopoletin also inhibits ADP-platelet aggregation at a range of 0.1 to 5 μM and improves blood rheology (Dunn 1969).

Scopoletin may also affect the interaction between uric acid and malaria. Malaria is characterized by cyclical fevers and high levels of inflammation, and while an early inflammatory response contributes to parasite clearance, excessive and persistent inflammation can lead to severe forms of the disease (Clark et al. 2004). *P. falciparum*-infected erythrocytes contain uric acid precipitates in the cytoplasm of the parasitophorous vacuole, which are released when erythrocytes rupture. Uric acid precipitates are highly inflammatory mediators for inflammatory cytokines IL-6, IL-8; they are considered a danger signal for innate immunity and are the causative agent in gout. These precipitates could offer a novel molecular target for anti-inflammatory therapies in malaria. Scopoletin exhibits an immediate and dose-dependent hypouremic effect and inhibits the activity of xanthine oxidase in hyperuricemic mice after peritoneal administration (Ding et al. 2005).

4.5 Conclusions and Future Prospects

Alternative methods for delivering artemisinin to patients have been considered through use of tea or oral ingestion of dried leaves of *A. annua.* Unfortunately, data indicate that delivery via tea is therapeutically not very efficacious. Furthermore, the extraction of artemisinin during tea preparation is variable unless carefully controlled. Although pharmacokinetic data for *A. annua* tea show some similarities with oral consumption of pure artemisinin, T_{max} and $T_{1/2}$ are lower for tea delivery, which may be the reason underlying greater recrudescence rates for tea treatments. Most studies equate treatment failure with low artemisinin in tea, in turn associating recrudescence with low artemisinin content in plant material, but despite the variations in plant material, serum peak concentrations of artemisinin are still similar. The only differences are the C_{max} and $T_{1/2}$, which appear to be similar across pure artemisinin doses. Mice fed dried *A. annua* leaves showed better therapeutic results than the tea. Results of pharmacokinetic studies using dried leaf delivery in mice are also consistent with the antimalarial success of the human trial. More structured studies in assessing the dosage of *A. annua* delivered as dried leaf tablets, in capsules, or mixed with food needs to be explored in greater depth in pharmacokinetic and clinical trials. Moreover, in order to prevent recrudescence, it is important to evaluate other treatment protocols, such as a two-tiered therapeutic regimen separated by 5–7 days. This is based on the assumption that recrudescence largely occurs because of sequential infections with a still emerging liver-stage malaria. The promising results of the studies using oral consumption of dried leaf *A. annua* may offer a more sustainable treatment for malaria, especially in low-income developing countries. This will eventually lead to improved understanding of how the whole plant therapy works better than the pure drug. This report will hopefully guide investigators toward what seem to be the more likely chemical targets.

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Chapter 5
Pharmacological Potentials of Artemisinin and Related Sesquiterpene Lactones: Recent Advances and Trends

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Abstract Natural products represent a goldmine of innovative therapeutic molecules to prevent and/or treat human diseases. For over 1,000 years, malaria has been one of the major causes of suffering and death for mankind. Artemisinin is an unusual sesquiterpene lactone (SL) endoperoxide that has been isolated as the active principle of the Chinese antimalarial herb *Artemisia annua* L. Since artemisinin was discovered to be the active component of *A. annua* in the early 1970s, hundred of papers have focused on the antiparasitic effects of artemisinin and its semi-synthetic analogues. Nowadays, artemisinin and its derivatives have become essential components of antimalarial treatment and are recommended by the World Health Organization (WHO) to treat especially multidrug-resistant forms of malaria. These features have prompted various scientists around the world to evaluate the potential of artemisinin and derivatives to control other human diseases. This review will centre on the significant achievements in recent years (2000 to date) with regard the chemistry and biological properties of SL from *A. annua*, with particular attention on artemisinin and related compounds. The discussion will also focus on the understanding of its mechanism of action and structure/activity relationships.

Abbreviations

ART	Antiretroviral therapy
AL	Artemether-Lumefantrine
ACTs	Artemisinin-based combinatory therapies
CHS	Contact hypersensitivity
CDK	Cyclin-dependent kinase
COX-2	Cyclooxygenase-2
CYP450	Cytochrome P450

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CMV	Cytomegalovirus
DTH	Delayed-type hypersensitivity
DNA	Deoxyribonucleic Acid
DHA	Dihydroartemisinin
EBV	Epstein–Barr virus
ERK	Extracellular signal-regulated kinase
HSV-1	Herpes simplex virus type 1
HIV	Human immunodeficiency virus
Igs	Immunoglobulins
iNOS	Inducible nitric oxide synthase
IκB	Inhibitor of NF-kappaB
IC_{50}	50 % Inhibitory concentration
ILs	Interleukins
JNK	Jun N-terminal kinase
LC_{50}	Lethal concentration for 50 % mortality
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MAPK	Mitogen-activated protein kinase
NO	Nitric oxide
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NF-κB	Nuclear factor-kappaB
PMA	Phorbol myristate acetate
pFAK	Phosphorylated focal adhesion kinase
PGE_2	Prostaglandin E_2
PIs	Protease inhibitors
ROS	Reactive oxygen species
PfATPase	Sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphate
SL	Sesquiterpene lactone
TGF-β1	Transforming growth factor-β1
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WHO	World Health Organization

5.1 Introduction

In recent years, research on medicinal plants has attracted considerable attention globally. A large body of evidence has accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary and

alternative systems of treatment for human diseases (Cordell and Colvard 2012; Newman and Cragg 2012).

Malaria affects 350–500 million people per year worldwide and is responsible for 1.1 million deaths per year. Malaria is a parasitic disease caused by the *Plasmodium* species transmitted from the blood of an infected person and passed to a healthy human by a female *Anopheles* mosquito. Malaria, caused by a parasitic infection of the red blood cells and liver, is among the diseases that have had the most widespread impact in the last 100 years. The life cycle, immunological defence mechanisms and clinical development of malaria in humans is a complex process (Kumar et al. 2002; Miller et al. 2013). Clinical malaria is characterized by periodic fever following the lysis of infected erythrocytes, caused mainly by the induction of cytokines such as interleukins (ILs) and tumour necrosis factor-α (TNF-α). *Plasmodium falciparum* infection, the main causative agent of malaria, can have serious effects including anaemia, cerebral complications (from coma to convulsions), hypoglycaemia and glomerulonephritis. This disease is most serious in non-immune individuals, including children, pregnant women and tourists. In many parts of the world, the parasites have developed resistance to a number of antimalarials such as chloroquine and derivatives—the most widely used treatment for malaria—quinine and other clinically used agents. Additionally, mosquitoes that carry malaria parasites have become resistant to insecticides. Due to the widespread incidence of malaria in certain parts of the world and to increasing parasite resistance to standard antimalarial agents, there is an urgent need to discover new compounds with an original mode of action.

Plants commonly used in traditional medicine are a source of active new compounds, including antimalarials (Taylor and Berridge 2006; Batista et al. 2009; Bero et al. 2009; Kaur et al. 2009; Sher 2009; Alves et al. 2012; Thiengsusuk et al. 2013). For example, artemisinin (Fig. 5.1) isolated from *Artemisia annua* L. (Asteraceae) and used in China to treat malaria is a sesquiterpene lactone (SL) prescribed in combination therapies to fight chloroquine-resistant *P. falciparum* (Hsu 2006; Efferth 2007; de Ridder et al. 2008; Kuhn and Wang 2008; Cui and Su

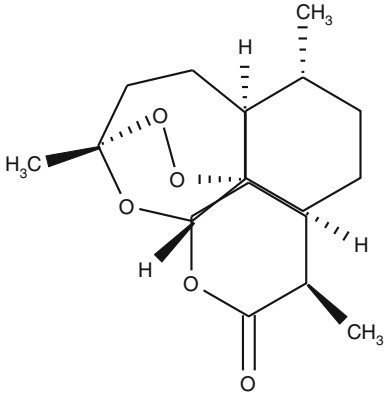

Fig. 5.1 Structure of artemisinin

2009; Brown 2010). The natural product artemisinin was isolated from the sweet wormwood plant *A. annua* in 1972 during a programme started by the Chinese army in the 1960s. *A. annua* is an annual herb endemic to the northern parts of Chahar and Suiyuan provinces in China, where it is known as "quinghao", and it has been used to treat chills and fever for more than 2,000 years.

The remarkable story of the discovery of artemisinin and the detection of its antimalarial activity by Chinese scientists represents one of the great medical breakthroughs of the latter half of the twentieth century. Through a collaborative effort, collectively referred to as "Project 523", the Chinese prepared dihydroartemisinin (DHA) (Fig. 5.2), artemether (Fig. 5.3) and artesunate (Fig. 5.4) in the 1970s. It is these derivatives, with others including artemisone, arteether and artelinic acid, generally known as "artemisinins", that are now making a crucial contribution to the management of malaria, one of our most serious infections (Haynes 2006; Krishna et al. 2008; White 2008). Nowadays, artemisinin and derivatives have become essential components of antimalarial treatment, and the artemisinin-based combinatory therapies (ACTs) are recommended by the WHO

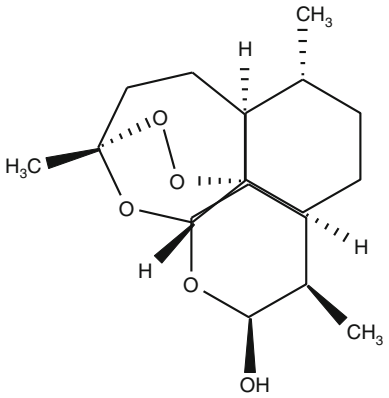

Fig. 5.2 Structure of dihydroartemisinin

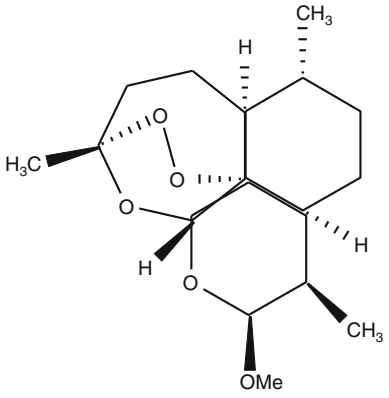

Fig. 5.3 Structure of artemether

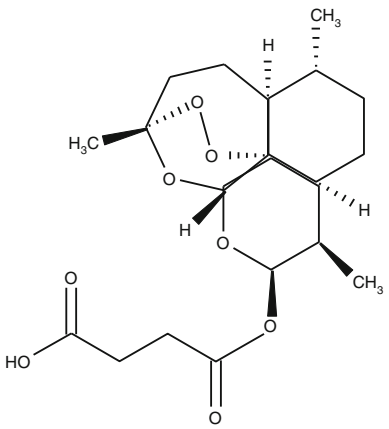

Fig. 5.4 Structure of artesunate

to treat especially multidrug-resistant forms of malaria. Other studies also point out that despite their huge potential to reduce or block malaria transmission, ACTs cannot be relied on to prevent it altogether. New approaches have been proposed, including co-administration of ACTs with other antimalarial drugs such as primaquine, ferroquine (Mustfa et al. 2011; Chaudhari et al. 2013; Jelinek 2013; Ogutu 2013; Pareek et al. 2013: Price 2013; Wells et al. 2013).

These features have prompted various scientists around the world to evaluate the potential of artemisinin and its derivatives to control other human diseases. In addition to its antimalarial properties, many researchers have confirmed that artemisinin and its related compounds have many other important pharmacological effects, including its antitumour, antimicrobial, immune function regulation and antiallergic properties, and in combating both neurodegenerative disorders and trauma-induced neuronal injuries (Table 5.1) (Yao et al. 2012; Sullivan 2013). This review will centre on the significant achievements in recent years (2000 to date) with regard the chemistry and biological properties of SLs from *A. annua*, with particular attention to artemisinin and related compounds.

5.2 Antimalarial Activity

Artemisinin kills nearly all of the asexual stages of parasite development in the blood and also affects the sexual stages of *P. falciparum* (gametocytes), which transmit the infection to others, but they do not affect pre-erythrocytic development or the latent stages of *Plasmodium vivax* and *Plasmodium ovale* (hypnozoites). The mechanism of action of artemisinins remains uncertain. The integrity of the endoperoxide bridge is necessary (but not sufficient) for antimalarial activity. Artemisinin's broad-stage specificity of antimalarial action has two therapeutic consequences. Killing young circulating ring-stage parasites in *P. falciparum*

Table 5.1 Reported pharmacological activities of artemisinin

Activity	Effect	Dose tested	References
Anti-infective	Mortality of the gametocytes of *Plasmodium falciparum*	1 μM	Eckstein-Ludwig et al. (2003); Haynes et al. (2007)
	Antiviral activity against the bovine viral diarrhoea virus	0.2 %	Romero et al. (2006)
	Antiviral activity against human cytomegalovirus and other members of Herpesviridae family	500 mg	Efferth et al. (2008)
	Antipromastigote activity against *Leishmania* spp.	10 and 25 mg/Kg	Sen et al. (2010)
	Moderate antihistomonal effect in vitro	100–2,600 mg/Kg	Thofner et al. (2012)
	Moderate antifeedant effect against *Epilachna paenulata* and *Spodophtera eridania*	0.03–0.07 mg/cm^2	Maggi et al. (2005)
	Inhibition of *Culex quinquefasciatus* and *Culex tritaeniorhynchus*	1 μM	Sharma et al. (2012)
	Anticoccidial effect against *Eimeria tenella*	10 and 17 ppm	del Cacho et al. (2010)
	Strong anti-*Helicobacter pylori* activity	50 mg/Kg	Goswani et al. (2012)
	Allelopathic effect on rhizobium	48 mg/l	Li et al. (2011)
Antitumour	Growth inhibition of prostate cancer cells	25 μM	Willoughby et al. (2009)
	Inhibition of proliferation of human breast cancer cells	25 μM	Tin et al. (2012)
	Reduction in human melanoma cell migration	1 mM	Buommino et al. (2009)
Anti-inflammatory and immunomodulatory	Suppression of contact hypersensitivity in mice	2–8 %	Li et al. (2012)
	Regulation of transcriptional factors in lupus nephritis mice	10^{-5} M	Wu et al. (2012b)
	Inhibition of the calmodulin-mediated activation of phosphodiesterase	10^{-6}–9.10^{-6} M	Noori et al. (2008)
	Suppression of delayed-type hypersensitivity in Balb/c mice	10^{-5} M	Noori et al. (2004)
	Inhibition of Jun N-terminal kinase activation	0.5–5 μg/ml	Cho et al. (2012)
Neuroprotective	Extenuation amyloidogenesis and neuroinflammation in APPswe/PS1dE9 transgenic mice	40 mg/Kg	Shi et al. (2013)

infections results in a more rapid reduction in parasitaemia compared to other antimalarials and considerably reduces the number of parasites that mature to sequester in and block capillaries and venules. This explains the rapidity of clinical responses and the life-saving benefit in severe malaria compared with quinine (which does not stop sequestration because it kills parasites only after they have matured and adhered to vascular endothelium).

Artemisinin probably exerts antimalarial activity through activation by haem. Ion-dependent alkylation (principally by Fe^{2+}) is a likely mode of action, and the sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphate (PfATPase) has been proposed as the primary target (Eckstein-Ludwig et al. 2003; Haynes et al. 2007). The most widely accepted mechanism of artemisinin action is that it structure (an endoperoxide SL with a polycyclic ring) is modified by Fe^{2+} present in abundance in the intracellular environment of the parasite, to structures containing carbon-centred free radicals. These free radicals—akin to "dirty bomb"—are thought to be responsible for its antimalarial action. Since the haemolysis in malaria results in elevated levels of plasma haem, Tangnitipong et al. (2012) hypothesized that the extracellular haem would boost the antimalarial activity of artemisinin. These results demonstrated that hemic (Fe^{3+}) at pathologic concentrations enhanced the activity of artemisinin against *P. falciparum* in vitro, as a result of the increased oxidative effect of hemin.

Despite the marked activity of artemisinin in *P. falciparum* parasites, 3–50 % of non-immune patients fail treatment if artemisinin is given as a monotherapy. This wide range of treatment failure rates is mainly due to the duration of treatment, where higher failure rates were observed after 3-day treatments, decreasing for 5–7-day treatment. High parasite densities prior to treatment also contribute to increased treatment failure (Klonis et al. 2013). It has been proposed that because artemisinin compounds have a short elimination half-life, they are unable to eliminate all parasites during the treatment, resulting in recrudescence. However, increasing the treatment duration from 3–5 days to 5–7 days only reduced, but did not eliminate, recrudescence.

One of the major threats to the efficacy and useful life of ACTs is the development of parasite resistance to artemisinin and its related compounds (Cheng et al. 2012; Wongsrichanalai 2013). Recent reports of parasites in Cambodia and Myanmar with decreased in vivo sensitivity to artesunate, an artemisinin derivative, raise serious concerns about the development of artemisinin resistance (Noedl et al. 2008; Dondorp et al. 2009; Carrara et al. 2013; Kyaw et al. 2013). However, several in vitro studies showed that artemisinin, when given in combined therapy, may have a synergistic effect on ACT-resistant parasites. For example, Mishra et al. (2009) demonstrated that interaction between two phytochemicals, artemisinin and licochalcone A in combination, shows synergistic antiplasmodial activity against synchronized erythrocyte stages of chloroquine-sensitive 3D7 and chloroquine-resistant RKL 303 strains of *P. falciparum*.

5.3 Other Anti-Infective Activities

In recent years, a large number of studies have been conducted on other anti-infective properties of artemisinin and related compounds. The bioactivity of artemisinin and its semi-synthetic derivatives is even broader and includes the inhibition of certain viruses such as human cytomegalovirus (CMV) and other members of the Herpesviridae family (e.g. herpes simplex virus type 1, HSV-1; Epstein–Barr virus, EBV), hepatitis B and C viruses, and bovine viral diarrhoea virus (Romero et al. 2006; Efferth et al. 2008).

Treatment for malaria in human immunodeficiency virus (HIV)-infected individuals receiving antiretroviral therapy (ART) posses significant challenges and has potential for drug interactions. Artemether–lumefantrine (AL) is one of the ACTs recommended for the treatment for malaria. The drug combination is highly efficacious against sensitive and multidrug-resistant *P. falciparum*. Both artemether and lumefantrine are metabolized by hepatic cytochrome P450 (CYP450) enzymes which metabolize the protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) used for HIV-1 treatment (Byakika-Kibwika et al. 2011). Byakika-Kibwika et al. (2012) demonstrated that co-administration of AL with lopinavir/ritonavir, two PIs, significantly increases lumefantrine exposure, but decreases artemether exposure. Population pharmacokinetic and pharmacodynamic trials will be highly valuable in evaluating the clinical significance of this interaction and determining whether dosage modifications are indicated. Similarly, co-administration of AL with efavirenz or nevirapine, two NNRTIs, resulted in a reduction in artemether, DHA, lumefantrine and nevirapine exposure. These drug interactions may increase the risk of malaria treatment failure and development of resistance to AL and nevirapine.

Visceral leishmaniasis, caused by the protozoan *Leishmania* spp., affects 500,000 people annually, with the Indian subcontinent contributing a significant proportion of these cases. Emerging refractoriness to conventional antimony therapy has emphasized the need for safer yet effective antileishmanial drugs. Artemisinin demonstrated antipromastigote activity, and the 50 % inhibitory concentration (IC50) ranged from 100 to 120 μM irrespective of *Leishmania* species studied (Sen et al. 2010). *Leishmania donovani*-infected macrophages demonstrated decreased production of nitrite as well as messenger ribonucleic acid (mRNA) expression of inducible nitric oxide synthase (iNOS), which was normalized by artemisinin, indicating that it exerted both a direct parasiticidal activity and a host-protective response. Artemisinin was also effective against histomanosis, a parasitic disease on gallinaceous birds, primarily affecting turkeys and chickens (Thofner et al. 2012), and against *Microcystis aeruginosa* (Ni et al. 2012a, b). The results showed that the decrease in photosynthesis in exposure to excess artemisinin may be a result of the inactivation of per photosystem II reaction centres and the inhibition of electron transport in the acceptor side.

Artesunate, a water-soluble semi-synthetic drug derived from artemisinin, has also been proved to be effective against trematodes like *Schistosoma japonicum*,

Schistosoma mansoni, *Fasciola hepatica* and *Clonorchis sinensis* (Fathy 2011). Results showed that artesunate was effective in the treatment for experimental heterophydiasis as proved by a 100 % reduction in the intestinal adult worm count, at a dose regimen of 200 mg/Kg/day given for 3 successive days. More recently, El-Beshbishi et al. (2013) demonstrated the effect of single-dose therapy with an artemisinin–naphthoquinone phosphate combination in a mouse model of *Schistosoma mansoni* infection. The study shows that the oral administration of this combination in a single dose of 400 mg/Kg on day 7 resulted in a significant worm burden reduction of 95 %. Artemisinin–naphthoquinone phosphate combination also induced significant alterations in the oogram pattern with elevated levels of dead eggs.

Artemisinin showed antifeedant activity against the insects *Epilachna paenulata* and *Spodoptera eridania* (Maggi et al. 2005). More recently, Sharma et al. (2012) evaluated different extracts of *A. annua* as well as pure artemisinin against larvae of *Culex quinquefasciatus* and *Culex tritaeniorhynchus*. The lethal concentration of artemisinin for 50 % mortality (LC50) was significantly higher for *Culex quinquefasciatus* than for *Culex tritaeniorhynchus* (14.31 and 4.13 ppm, respectively).

Artemisinin was also active against the parasite *Eimeria tenella* (del Cacho et al. 2010), against *Helicobacter pylori*, the pathogen responsible for peptic ulcer diseases (Goswani et al. 2012), and showed allelopathic effect on rhizobium (Li et al. 2011; Bharati et al. 2012).

5.4 Antitumour Activity

In recent years, a large number of studies have been conducted on the antitumour activity of artemisinin and its related compounds. The endoperoxide moiety in the chemical structure of artemisinin is thought to be responsible for its bioactivity. Artemisinin and its bioactive derivatives exhibit potent anticancer effects in a variety of human cancer cell model systems, although the mechanisms of its anticancer signalling are not well understood (Firestone and Sundar 2009). The pleiotropic response in cancer cells includes growth inhibition by cell cycle arrest, apoptosis, inhibition of angiogenesis, disruption of cell migration and modulation of nuclear receptor responsiveness. These effects of artemisinin and its derivatives result from disturbances in numerous cell signalling pathways. Willoughby et al. (2009) demonstrated that 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. Taken together, these results demonstrate that a key event in artemisinin antiproliferative effects in prostate cancer cells is the transcriptional down-regulation of CDK4 expression by disruption of Sp1 interactions with the CDK4 promoter.

Artemisinin also inhibited proliferation and induced a strong G1 cell cycle arrest of cultured MCF7 cells, an oestrogen-responsive human breast cancer cell line that represents an early-stage cancer phenotype, and effectively inhibited the in vivo

growth of MCF7 cell-derived tumours from xenografts in athymic nude mice (Tin et al. 2012). Artemisinin also induced a growth arrest of tumorigenic human breast cancer cell lines with preneoplastic and late-stage cancer phenotypes, but failed to arrest the growth of a non-tumorigenic human mammary cell line. Taken together, these results demonstrate that the artemisinin disruption of E2F1 transcription factor expression mediates the cell cycle arrest of human breast cancer cells and represents a critical transcriptional pathway by which artemisinin controls human reproductive cancer cell growth. Artemisinin also reduces human melanoma cell migration by down-regulating $\alpha V\beta$-integrin and reducing matrix metalloproteinase-2 (MMP2) production (Buommino et al. 2009). These findings suggest a potential for artemisinin as a chemotherapeutic agent in melanoma treatment.

Previously, Nam et al. (2007) reported that artemisinin and its derivatives inhibited the growth and apoptosis of oral cancer cells. Dimers and trimers were found to have more potent antitumour activity than monomers. More recently, novel artemisinin–glycolipid hybrids were synthesized directly (Ricci et al. 2011). These hybrids exhibited exceptional in vitro anticancer activity, particularly against oral carcinoma cell lines. The artemisinin–glycolipid hybrids, with IC_{50} under 20 μM, demonstrated better anticancer activity than either artemisinin or glycolipid alone and showed five times more antioral cancer activity than either cisplatin or paclitaxel. Galactosylated artemisinin also exhibits high antitumour activities in HeLa cells, possibly related to the fact that it triggers the CYP450 apoptotic pathway mediated by BCL-2 family (Ren 2012).

DHA and artesunate, two semi-synthetic derivatives of artemisinin, also showed antitumour activity. Calcium and survivin are involved in the induction of apoptosis by DHA in human lung cancer SPC-A-1 cells (Mu et al. 2007). DHA also induces caspase-3-dependent apoptosis in human lung adenocarcinoma ASTC-A-1 cells (Lu et al. 2009). More recently, Lu et al. (2010) demonstrated for the first time that DHA induces cell apoptosis by triggering reactive oxygen species (ROS)-mediated caspase-8 activation and the mitochondrial pathway. These results provide some novel insights into the application of DHA as a potential anticancer drug and a new therapeutic strategy by targeting ROS signalling in lung adenocarcinoma therapy for the future. Additionally, the combined treatment of cisplatin and DHA exerts a strong and synergistic antiproliferative effect in human lung carcinoma cells (Zhang et al. 2013). The level of apoptosis in the tumour cells increased with the combined treatment of DHA and cisplatin. DHA also inhibited the proliferation of human hepatocarcinoma BEL-7402 cells in a concentration-dependent manner (Lu et al. 2012). These results indicate that the imbalance of energy metabolism induced by DHA may contribute, at least in part, to its anticancer potential in BEL-7402 cells.

These results were also confirmed in vivo. Zhang et al. (2012) demonstrated that DHA significantly inhibited hepatocellular carcinoma cell growth in vitro and in vivo inducing G2/M cell cycle arrest and apoptosis. The induction of p21 and the inhibition of cyclin B and CDC25C contributed to DHA-induced G2/M arrest. In a hepatocellular carcinoma xenograft mouse model, the intraperitoneal injection of DHA resulted in significant inhibition of xenograft tumours.

DHA also inhibits the growth and metastasis of epithelial ovarian cancer (Wu et al. 2012a). DHA inhibits ovarian cancer cell proliferation, adhesion and migration in vitro in a dose-dependent manner, consistent with decreased expression of phosphorylated focal adhesion kinase (pFAK) and MMP2, but not MMP9. DHA inhibited metastasis significantly in vivo, associated with reduced von Willebrand factor (vWF) expression and macrophage infiltration. In conclusion, DHA inhibits the development of ovarian cancer, in part via down-regulating pFAK, MMP2, vWF and macrophage infiltration.

Another semi-synthetic derivative of artemisinin is artesunate. Several reports suggest that artesunate is active against cancer cells by inducing deoxyribonucleic acid (DNA) damage (Li et al. 2008). Artesunate induces oxidative DNA damage and sustained DNA double-strand breaks (Berdelle et al. 2011). Artesunate also inhibits angiogenesis and down-regulates vascular endothelial growth factor (VEGF) expression in chronic myeloid leukaemia K562 cells (Zhou et al. 2007). The antiangiogenic effect of artesunate was further evaluated in vivo in chicken chorioallantoic membrane neovascularization model. The results indicated that the stimulating angiogenic activity decreased in response to the K562 cells treated with artesunate or the conditioned media, from K562 cells pretreated with artesunate in a dose-dependent manner (3–12 μM/l). Artesunate reduces chicken chorioallantoic membrane neovascularization and exhibits antiangiogenic and apoptotic activity on human microvascular dermal endothelial cells (Huan-Huan et al. 2004). More recently, Wang et al. (2012) demonstrated that artesunate significantly inhibited proliferation of cultured primary rat hepatic stellate cells in a dose- and time-dependent manner. Artesunate significantly inhibited the transforming growth factor-β1 (TGF-β1) mRNA expression in these cells and inhibited the expression of intracellular and secreted TGF-β1 protein.

Examples of other antiproliferative derivatives of artemisinin also included artemisitene, arteannuin B and arteether (Efferth et al. 2011; Azimi-Mohamadabadi et al. 2013). The last compound, arteether, is an oil-soluble derivative of artemisinin, which reveals certain antitumour properties that may aid in the development of more effective antitumour agents.

5.5 Anti-Inflammatory and Immunomodulatory Activities

In recent years, a large number of studies have been conducted on the anti-inflammatory and immunomodulatory properties of artemisinin and its related compounds. Konkimalla et al. (2008) investigated the cytotoxicity and the ability of artemisinin, five of its derivatives and two other endoperoxides, to inhibit generation of nitric oxide (NO). In the RAW 264.7 mouse macrophage cell line, artesunate revealed the highest ability to inhibit NO production among all compounds tested.

In vivo, artesunate significantly ameliorated dextran sulphate sodium salt colitis and 2,4,6-trinitrobenzene sulphonic acid (TNBS) colitis (but not oxazolone

colitis), including reduced weight loss and disease activity as well as macroscopic and microscopic colonic injury (Yang et al. 2012). The expression of nuclear factor-kappaB (NF-κB) p65 and the subunit α of the inhibitor of NF-κB (IκB) were reduced in artesunate-treated TNBS colitis compared to non-treated. Furthermore, in vitro artesunate treatment significantly inhibited TNF-α production by lipopolysaccharide (LPS)-activated macrophages. Artesunate suppresses TNF-α expression in vitro and in vivo as well as T-helper responses in TNBS colitis model. These data suggest a novel clinical application of artesunate as a potential therapy for Crohn's disease.

Li et al. (2013) evaluated the in vivo immunomodulatory effect of artesunate in a mouse model of delayed-type hypersensitivity (DTH) reaction, which was based on a T-cell-mediated immune response. The data revealed that artesunate had a relatively high immunosuppressive activity with low toxicity and could inhibit T-lymphocyte proliferation induced by mitogen and alloantigen. Artesunate prevented immunoglobulin E (IgE)-mediated cutaneous vascular hyperpermeability, hypothermia, elevation in plasma histamine level and tracheal tissue mast cell degranulation in mice in a dose-dependent manner (Cheng et al. 2013). In addition, artesunate suppressed ovalbumin-mediated guinea pig bronchial smooth muscle contraction. Artesunate also concentration-dependently blocked IgE-mediated degranulation of RBL-2H3 mast cells and human culture mast cells.

Artemisinin was also active in a mouse model of contact hypersensitivity (CHS), a T-cell-mediated cutaneous inflammatory reaction and DTH (Noori et al. 2004; Li et al. 2012). The data showed that topical administration of artemisinin could effectively suppress CHS response and concanavalin-A-induced T-cell proliferation and also that artemisinin was able to successfully block CHS response in mice by inducing the generation of regulatory T cells and suppressing the development of T-helper 17. These studies indicate the potential of artemisinin for application as an effective therapeutic agent for treating immune-related diseases.

Artemisinin was also active in vivo in lupus nephritis mice (Wu et al. 2012b). The underlying therapeutic mechanism may be correlated with the regulation of the expression of transcriptional factor proteins in renal tissue.

Examples of other anti-inflammatory and immunomodulatory compounds related to artemisinin also include DHA and artemisinic acid, the biogenetic precursor of artemisinin. Artemisinic acid, which was isolated from *A. annua*, inhibited adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells (Lee et al. 2012). Artemisinic acid reduced expression of the enhancer-binding protein δ gene without impacting β. In addition, attempts to elucidate a possible mechanism underlying the artemisinic acid-mediated effects revealed that reduced expression of the enhancer-binding protein δ gene was mediated by inhibiting Jun N-terminal kinase (JNK). Additionally, artemisinic acid also reduced the expression of the adipogenesis-associated genes glucose transporter-4 and VEGF. These results suggest that artemisinic acid may be used as a complementary treatment option for obesity associated with metabolic syndrome. Artemisinin also induces the production of IL-12 in LPS-stimulated macrophages RAW 264.7 cells by inhibiting JNK activity (Cho et al. 2012).

DHA ameliorates inflammatory diseases by its reciprocal effect on T-helper and regulatory T-cell function by modulating the mammalian target of rapamycin pathway (Zhao et al. 2012). More recently, Kim et al. (2013) investigated the effect of cyclooxygenase-2 (COX-2) and molecular mechanisms by DHA in phorbol myristate acetate (PMA)-stimulated RAW 264.7 cells. DHA dose-dependently decreased PMA-induced COX-2 expression and prostaglandin E_2 (PGE_2) production, as well as COX-2 promoter-driven luciferase activity. Additionally, DHA decreased luciferase activity of COX-2 regulation-related transcription factors including NF-κB.

5.6 Neuroprotective Activity

The discovery of new compounds that can stimulate regeneration and differentiation of neurons could be of great importance for developing new therapeutics against both neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease and trauma-induced neuronal injuries such as cerebral ischaemia and spinal cord injury. Growth of neurite processes from the cell body is a critical step in neuronal development, regeneration, differentiation and response to injury. The discovery of compounds that can stimulate neurite formation would be important for developing new therapeutics against these diseases.

Artemisinins containing an endoperoxide bridge, such artemisinin and DHA, induced growth of neurite processes at concentrations that were slightly cytotoxic; artemisinin had the most potent effect among them (Sarina et al. 2013). Deoxyartemisinin (Fig. 5.5), which lacks the endoperoxide bridge, was ineffective. Artemisinin up-regulated phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), critical signalling molecules in neuronal differentiation.

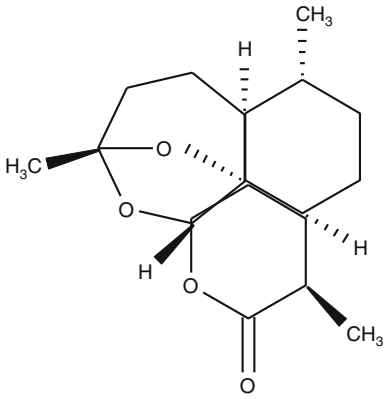

Fig. 5.5 Structure of deoxyartemisinin

The activation of NF-κB and NLRP3 inflammasome is implicated in neuroinflammation, which is closely linked to Alzheimer's disease. Shi et al. (2013) investigated the effect of artemisinin on Alzheimer's disease in 5-month-old APPswe/Ps1E9 transgenic mice. These results revealed that artemisinin treatment decreased neuritic plaque burden, did not alter β-amilod transport across the blood–brain barrier, inhibited β-secretase activity and inhibited NF-κB activity and NLRP3 inflammasome activation in transgenic mice. These studies suggest that targeting NF-κB activity and NLRP3 inflammasome activation offers a valuable intervention for Alzheimer's disease.

5.7 Conclusions

Artemisinins are firmly established in combination therapies to treat drug-resistant malaria. Their true potential now lies in broader antidisease and other applications, particularly in addressing the difficult challenge posed by advanced cancers for which expensive treatments are providing, at best, incremental gains in outcome. Questions about dosing regimens, safety of long-term use and possible interactions (either positive or negative) with existing therapies and toxicities that might be related to the treatment should be answered by appropriate clinical studies.

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Chapter 6
Taxonomic Implications of *Artemisia annua* L.

Sadia Malik, Muhammad Qasim Hayat and Muhammad Ashraf

Abstract *Artemisia annua* L. (sweet sagewort) belongs to genus *Artemisia* L. of family Asteraceae and tribe Anthemideae. *Artemisia* L. approximately comprises of about 500 taxa and considered as largest genus of Asteraceae. *A. annua,* which are now widely and naturally distributed in various regions of the world (temperate, subtropical zones, Asia), have originated from China and are introduced and naturalized in Canada and USA. later on. On the basis of fossil records and phytogeographic data, it has been discovered that the Quaternary was the main time period for the variegation and dispersal of the taxa worldwide. Globally, many countries are currently cultivating *A. annua* on a large industrial scale, as it is a key source of a potent antimalarial drug, artemisinin. In this chapter, we will set out its origin, geological distribution, and phytography emphasizing on important characters, for instance pollen as pollen morphology is considered as worthy systematic marker for genus *Artemisia*. Furthermore, special protuberant structures viz. glandular trichomes and their role in artemisinin and various other phytotoxic secondary metabolites will be addressed. The second part of the chapter will draw attention to the possibilities and methods used to solve mysteries of *Artemisia* systematics on subtribal, generic, and subgeneric level over time. To date, for systematic studies at all taxonomic levels, none of the techniques alone is considered sufficient. In conclusion, the perspectives and issues for the further study of the plant and genus will be raised.

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6.1 Origin and Geological Distribution

Artemisia annua along with its related taxa is thought to have originated from Asia in the late Eocene, spread west and east in the Oligocene became common in the Miocene and ever since the Pliocene particularly in Pleistocene *Artemisia* distribution was very much comparable to its current dissemination (Wang 2004; Miao et al. 2011). The origin of the subtribe *Artemisiinae* is reported from Asia at the Late Oligocene and most closely related genera in the Early Miocene (Sanz et al. 2008, 2011). Overall, the origin on the basis of fossil records and phytogeographic data have discovered that the origin, consequent evolution and variation of the *Artemisia* in most part of the world have taken place during three main stages: appearance Paleocene, low representation in Miocene and speciation in Pliocene. So Quaternary was the most important time period for worldwide dispersal and diversification of the genus. Ling (1991a, b, 1994) assumed a putative ancestor of *Artemisia* in Northern Asia and dispersed throughout the world via three major directions: (1) toward east into Siberia and into western North America (North America in the Late Miocene (Tkach et al. 2008a); and in North West America in Miocene (Davis and Ellis 2010), (2) toward west into Europe, Asia and the Mediterranean Basin and Africa and lastly toward South Asia. Naturally, wide range of ecological regions are being covered by *A. annua* including predominantly temperate environments and subtropical areas, mid- to high-latitude temperate areas of the northern hemisphere, arid and semiarid environments. However, few populations are recognized in the southern hemisphere (Pellicer et al. 2010; Das 2012; Ellman 2010). A number of *Artemisia* species (*A. annua*, *A. apiacea,* and *A. lancea*) have been used as antimalarial in China since ancient times (Hsu 2006; Willkox 2009). *A. annua* is component of flora in the Northern China (Chahar and Suiyuan provinces) at 1,000–1,500 m beyond sea level (Wang 1961; Ferreira et al. 1997). The old Chinese name of *A. annua* and a few other species, i.e., "qinghao" is mentioned in the classical *Materia medica*, compiled in the first century, and their earliest aver as antimalarial was recorded by Ge Hong in the fourth century (Willkox 2009). A recent study on archeological evidence in China has shown that *A. annua* have also been in use to inhibit stinking of the departed, not merely for therapeutic purposes (Liu 2013). The plant grows in numerous countries, for instance Argentina, Bulgaria, France, Hungary, Italy, Romania, Spain, USA, and Yugoslav state (Klayman 1993). Central speciation and diversification hub of the genus *Artemisia* is Central Asia, and according to Tkach et al. (2008a, b), more migrations may have occurred starting from Asia into Northern America than from North America toward Asia. On the basis of fossil pollen, palaeo historical information, and the molecular phylogenetic studies Riggins (2012) inferred the contemporary dispersion and segregation of North America and Eurasia taxa may be due to multifarious reasons, including precedent glaciations, geographic barriers, migration and to an extent long-distance dispersal. The herb is cultivated on small and large scale especially in tropical countries where incidence of malaria is high. Both breeding programs and

hybridization have been carried out successfully as a result of cultivars such as "Anamed A3" and "Artemis" (Bartlet 2010; Graham et al. 2010; Reale et al. 2011 and references in that) are produced. Transgenic plants have also been developed, and studies concerning increased production artemisnin in vivo and in vitro are being performed, which are discussed later in this chapter.

6.2 Phytography and Morphology

The morphology of the genus *Artemisia* is intricate and mystifying as it is largely affected by ecological conditions and vegetative stages thus show great biodiversity. As far as *A. annua* is concerned, it is a short day plant (Ferreira and Janick 2009), blossom time begins in mid-August (Muller and Brandes 1997) and requires 13.5 h of critical photoperiod (Ferreira et al. 1995). This herb is annual, generally weedy (Hall and Clements 1923), single stemmed, approximately 2 m tall, reddish in color, alternately branched, sparingly hairy and with strong aromatic odor (Ferreira and Janick 2009; Das 2012; Sharma et al. 2011). The illustration of *A. annua* is given in Fig. 6.1. Leaves are petiolate, 1.5–6.5 cm long,

Fig. 6.1 A wild *A. annua* plant growing in Western Himalayan region of Pakistan (*Image* Muhammad Qasim Hayat)

punctate, and pinnatipartite. Lobules are deeply denticulate with triangular teeth. Mid-vein is prominent adaxially.

Little yellow, globose flowers (capitula) are having paniculate arrangement containing bisexual central (disk) florets each engender one achene and pistillate (ray) florets produce numerous achenes. The involucre is imbricate with numerous layers of bracts. Ovaries are inferior as well as unilocular. Fruits are ovoid, pappus-lacking and are known as achenes and cypselas (Shishkin and Bobrov 1995; Ferreira and Janick 1996; Muller and Brandes 1997; Hayat 2009e; WHO 2006). The plant is entomophilic as well as anemophilic too, which is an extraordinary in the Asteraceae (McVaugh 1984).

6.3 Palynology

Exploration of pollen morphology of *Artemisia* has been started from the time of Wodehouse (1926). The following workers elaborated different perspectives of *Artemisia* pollen characteristics including the structural organization, size and shape of pollen, sculptural range in exine, aperture magnitudes, etc., along with their taxonomic applications (Stix 1960; Singh and Joshi 1969; Dimon 1971; Valles et al. 1987; Martin et al. 2001, 2003; Jiang 2005). According to Stix (1960), the pollen is tricolpate, smooth, and archetypal of anemophilous species and has rudimentary or no spines. The pollen contains an internal, compound, columellae tecta design in the exine, which is characteristic feature of the entire taxa of the tribe Anthemideae in contrast to two to three layers in *A. annua* (Skvarla and Larson 1965). Pollen grains of this genus are comparatively unique and straight forwardly identifiable and are marked by tiny spines or without spines (Bremer and Humphries 1993).They are minute in size but bulky in amount (Jiang 2005). A palynological investigation carried out by Martin et al. (2001, 2003) on the Anthemideae together with *Artemisia*, its allies in addition to set apart genera showed embellishments with tiny spinules. Wang (2004) also point out that *Artemisia* type pollen with diminutive spinules evolved from taxa having long spines (*Anthemis* type) on the basis of order of prevalence in the geographical history. Eight micromorphological properties (pollen shape, spinules arrangement, exine sculpture, spinules base, polar length, equatorial width, exine thickness, and colpus width) of pollen grains of *Artemisia* have been determined and stated as fine systematic markers that can be employed as marker in systematic studies of genus *Artemisia* at infrageneric level (Hayat et al. 2010b). Initially, characterization has been performed by considering pollen grains as systematic marker as stated earlier (Wodhouse 1926; Singh and Joshi 1969; Skvarla et al. 1977; Martin et al. 2001, 2003; Jiang et al. 2005; Sanz et al. 2008; Pellicer et al. 2009; Hayat et al. 2009a). These studies considered the pollen as indistinguishable from each other along with the reduction of granules or even no granules on exine.

Palynological studies on the of the genus also supports the coexistence of two pollen morphological forms that is taxa with petite *Artemisia* type spinules and the other with lengthy *Anthemis* type spines (Martin et al. 2001, 2003). In accordance with studies, the Anthemis pollen type symbolizes the primeval character within subtribe *Artemisiinae* and all the taxa in the *Artemisia* share the petite spinules pollen type (Sanz et al. 2008). By studying chronological and spatial fluctuations in *Artemisia* pollen calculation, it can be concluded that *Artemisia* pollen seasons are greatly dependent on temperatures during summers (June and July) and the ecological position or increasing urbanization did not impinge on the pollen indicator (Grewling 2012).

6.4 Glandular Trichomes: Site for Artemisnin Biosynthesis

Artemisinin is a cadinane-type sesquiterpene lactone with a 4, 6-endoperoxide function is a natural plant product formed by *A. annua* along with various other active constituents used for the cure for malaria (Graham et al. 2010). Artemisinin (known as qinghaosu, derived from qinghao in China) was initially isolated in China in 1970s (Klayman 1985; Liao 2009) and showed strong antimalarial properties with little or no side effects (Efferth 2007). This molecule is called as artemisnin and its derived phytoconstituents became the foundation as ethnobotanical elucidation to modern drugs treat malaria. The site of artemisinin and numerous additional terpenoids syntheses is the secretory cells of the glandular secretory trichomes. The glandular trichomes are found on aerial parts of the plant and in 10-cell arrangement in the group of three pairs of secretory cells (Duke 1993). Extensive studies have been carried out on the glandular and nonglandular trichomes of *A. Annua*. Ferreira and Janick in 1995 reported the presence of T-shape trichomes in *A. annua*. Hayat (2009b; 2009d plus references there in) reported 16 main types of the trichomes and range of the capitate glands, differences in T-shaped hairs and existence of supplementary types of foliar trichomes in *Artemisia*. Artemisinin content in *A. annua* varies between 0.01 to 0.4 % depending on the genotype, variety, season, cultivation condition, and plant developmental stage (Lommen et al. 2007; Delabays et al. 2001; Yang et al. 2009; Davies et al. 2009) and a few clones have shown production >1 % (Delabays et al. 2001). Selection and cross breeding of wild genotypes with high artemisinin content resulted in hybrid lines containing approx. 1.4 % artemisinin (on dry leaves basis). The inconsistency in distribution of artemisinin and the derivatives throughout the plant is also reported. The most valuable parts are the leaves and the flowers where artemisinin is concentrated (Ferreira and Janick 1995). According to studies done by Lommen et al. (2006), Biswas et al. (2009) and Kjær et al. (2012), the artemisnin concentration increase with increase in abundance of trichomes on leaf, higher capacity, and size per trichome. As in some cases, artemisnin levels continue to increase irrespective of trichome numbers which may be due to mature higher capacity capitate trichomes (Lommen et al. 2006).

Currently, in the treatment of malaria, mostly in artemisnin and its derivative are used as combination therapies (Haynes 2006). Artemisinin biosynthetic pathway is well studied (Weathers et al. 2006 and references within). The transcriptome mining, investigation on regulation of fundamental pathways for artemisinin production (Wang et al. 2009; Dai et al. 2010), QTL analysis (Graham et al. 2010) and regulation of artemisinin synthesis have been studied (Maes et al. 2011). Studies have shown that genes [amorpha-4, 11-diene synthase (ADS), amorphadiene-12-hydroxylase, artemisinic aldehyde reductase, and aldehyde dehydrogenase] of the artemisinin biosynthetic pathway show amazingly elevated expression in these particular trichomes present in buds of flowers and young leaves in contrast to tissues such as mature leaves, other vegetative parts of the plant and their cultures (Zhang et al. 2008; Olofsson et al. 2011). The genes of the artemisinin pathway are expressed in every cell of secretory trichomes (Olofsson et al. 2012).

Various transgenic *A. annua* plants have been produced to increase the yield of artemisinin. These attempts include down-regulation of squalene synthase reported by Zhang et al. (2009) and Feng et al. (2009) or up-regulation of farnesyl diphosphate synthase (Han et al. 2006) and investigation of trichome-specific regulation of ADS and artemisinin synthesis encoding *cyp71av1* gene in *A. annua* (Wang et al. 2011, 2013). Also, study has been performed on *AaORA*, a trichome-specific AP2/ERF transcription factor of *A. annua* which is a positive regulator in the artemisinin biosynthetic pathway (Lu et al. 2013). Ro et al. (2006), Chang et al. (2007) and Zeng et al. (2008) have reported the use microbes systems to produce precursors of artemisinin. However, agricultural production is expected to remain the key resource (Olofsson et al. 2011). Glandular trichomes are cellular factories, hence can be used as targets for breeding, production of organic compounds for pharmaceutical industry, explication of the biosynthetic pathways, pharmaceutical constituents and proteins (Tissier 2012; Wu et al. 2012).

6.5 Molecular Identification, Classification, and Phylogeny

Various studies have been reported on *Artemisia* globally including basic and applied researches (Cronquist 1977; Valles et al. 1987, 2003; Torrell and Valles 2001; Ghafoor 2002; Valles and Garnatje 2005; Hayat et al. 2008, 2009a, b, c; Graham et al. 2010; Brown 2010; Pellicer et al. 2011; Nazar and Mahmood 2011; Badr et al. 2012; Tabur et al. 2012). Phylogenetic studies of *Artemisia* have been in limelight and taxon reshuffling has been done several times on the basis of classical as well as modern molecular characters. The accurate and complete classification of the taxa has not yet been achieved because of confusing similarities and missing links (Noorbakhsh et al. 2008; Hayat et al. 2010a, b, 2009d). It is the need of the hour to take insight into several other genome sequences and tools to reveal ambiguities in *Artemisia* at all taxonomic levels. Now trend has been shifted from classical systematics to the use of modern molecular techniques. Important questions arisen on the way include concerning origin of *Artemisia*, its

biogeography, placement within Asteraceae, intra- and infrageneric structuring and questions regarding its monophyly/paraphyly.

Earlier *Artemisia* was divided into three taxa (genus *Artemisia*, genus *Absinthium* DC. and genus *Abrotanum* Besser) by Tournefort (1700). After Linnaeus (1735), many scientists have put all three in one large genus *Artemisia* L. having sections followed by ranking up to the level of subgenus (Rouy 1903; Rydberg 1916; McArthur et al. 1981). Based on evolution of floral and pollen characteristics, authors have treated it as subgenera or section (*Artemisia, Absinthium, Dracunculus* and *Seriphidium*) (Hall and Clements 1923; Watson et al. 2002; Martin et al. 2001, 2003). By taking into account floral characteristics, the known five subgenera are: *Absinthium, Artemisia, Dracunculus, Seriphidium,* and *Tridentatae*. Rydberg (1916) reported the independent origin of American woody sagebrushes from the woody Asian species (*Seriphidium*) and documented these in section *Tridentatae* which is native to Northern America. This segregation was also sustained by a number of later authors (McArthur et al. 1981; Watson et al. 2002) and recognized *Seriphidium* as separate genus (Bohm and Stuessy 2001; Bremer and Humphries 1993; Bremer 1994; Dobignard 1997; Ling 1991a, b, 1994, 1995a, b). Ling (1992) supported that subgenera *Absinthium* taxa are considered primeval in the genus *Artemisia*. Nevertheless, molecular researches based on plastid genome, restriction analysis and nuclear ribosomal DNA (Kornkven et al. 1998; Torrell et al. 1999; Watson et al. 2002; Valles et al. 2003) have invalidated the severance of taxa (Pellicer et al. 2007). Figure 6.2 depicts the relationship of *Artemisia* with some other members of *Artemisinae* based on nrDNA inferred using Neighbor-Joining consensus tree.

After the comprehensive studies carried out by Funk et al. (2009) and Kubitzki (2007) based on morphological and molecular data, *Seriphidium* was been fit in back to its parent genus and not as independent. On the whole taxa, significant studies performed on *Artemisia* L. classification based on DNA include (Kornkven et al. 1998, 1999; Torrell et al. 1999; Watson et al. 2002; Valles et al. 2003; Riggins 2008; Sanz et al. 2008; Hayat et al. 2009c; Garcia et al. 2011a; Pellicer et al. 2011). According to a study based on nrDNA, *Artemisia* seems as polyphyletic and section Absinthium is submerged in section *Artemisia* while *Dracunculus* and *Seriphidium* form distinct clades (Hayat 2011) (Fig. 6.3).

Up to date phylogenetic analyses (Tkach et al. 2008a; Garcia et al. 2008, 2011a; Pellicer et al. 2011) have exposed numerous pattern of reshuffling of morphological characters used to characterize infra- and intergeneric classification. Hayat (2011) reported the infrageneric classification of *Artemisia* based on morphological characters (Fig. 6.4).

Studies on morphological characters with the molecular phylogenies show evident evolutionary suppleness and homoplasy in floral characters (Sanz et al. 2008; Watson et al. 2002; Zhao et al. 2010). The molecular phylogeny perfectly solves the independence of subgenera *Seriphidium* and *Tridentatae* (Garcia et al. 2011a). *Tridentatae* are evidently alienated from the *Seriphidium* of Old World (Riggin and Seigler 2012) in contradiction to findings of Bremer and Humphries (1993) and Ling (1995b). Classical morphological characters that are being

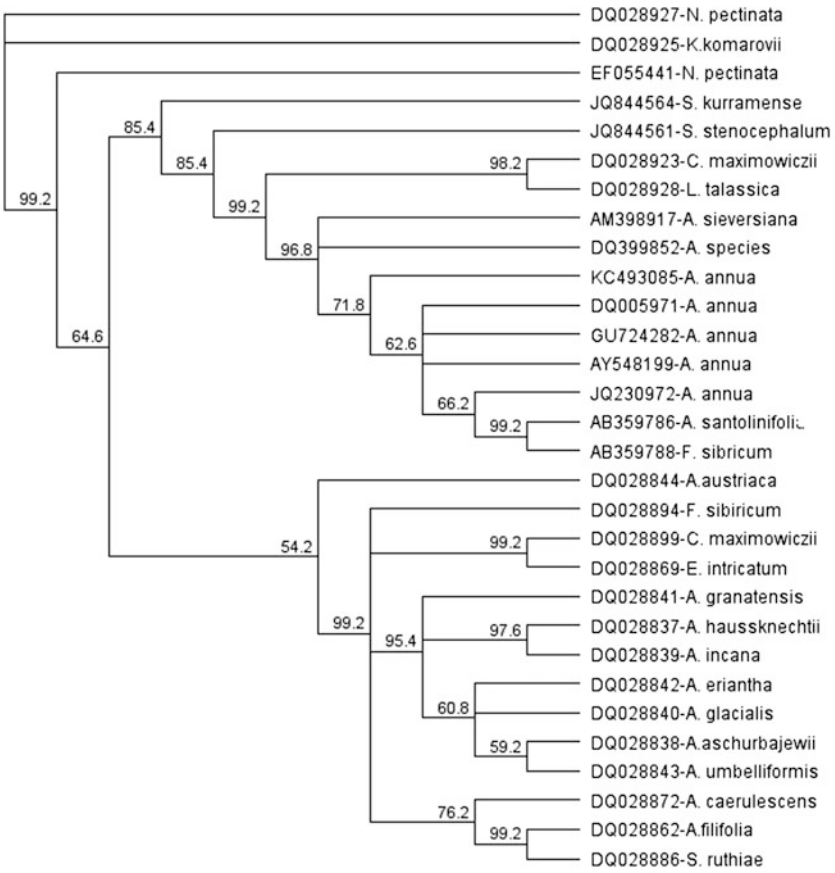

Fig. 6.2 Phylogenetic relationship of *Artemisia* with some other members of *Artemisinae* from different parts of the world based on nrDNA (ITS, ETS) inferred using neighbor-joining consensus tree. Bootstrap values based on 500 replicates are shown on the branches. The analysis involved 30 sequences taken from GenBank

traditionally used may not characterize and classify accurate synapomorphies (Riggins and Seigler 2012). Riggins and Seigler (2012) proposed that most primitive deviating lineages of *Artemisia* may originate inside subgenus *Dracunculus* and the *Heterophyllae/Norvegicae* clusters.

The wobbly taxonomy of many taxa has generated many names that make the nomenclature problematic (such as *Hippolytia megacephala* described as *A. megacephala* and *Lepidolopsis turkestanica* described as *Crossostephium turkestanicum*. The molecular phylogenetic studies, including *Artemisia* and related genera, have solved the query and placed them or revert them in *Artemisia* (Watson et al. 2000, 2002; Valles et al. 2003; Sanz et al. 2008; Tkach et al. 2008b; Zhao et al. 2010; Garcia et al. 2009, 2011a, b; Pellicer et al. 2011).

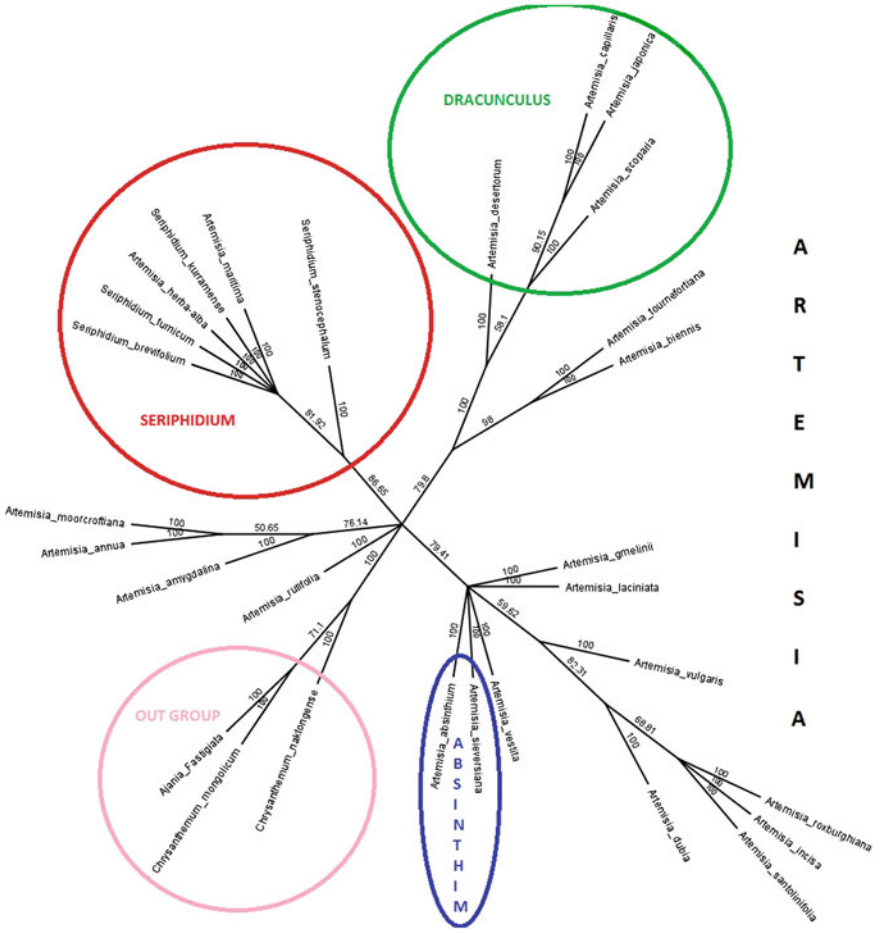

Fig. 6.3 Phylogenetic tree of Pakistani *Artemisia* based on ITS and ETS sequences of nrDNA inferred by using maximum parsimony method (Hayat 2011)

Molecular studies associated with morphological and cytogenetic ones may make it promising to explain the position of controversial taxa. In addition to that, numerous works have been performed on the genetic diversity/variability assessment (isoenzymes, AFLP, RAPD, and microsatellites) (McArthur et al. 1998a, b; Torrell et al. 1999; Huang et al. 2009; Nazar and Mahmood 2011; Badr et al. 2012), on genus in molecular cytogenetics (chromosome banding, fluorescence in situ hybridization) (Garcia et al. 2009, Pellicer et al. 2013 along with references within), chromosome number and genome size of *A. annua* diploid plant $2n = 2x = 18$ and for tetraploid $2n = 4x = 36$ has been reported. Genome size of *A. annua* is estimated as 1.75 pg where 1 pg = 978 Mbp (Torrell and Valles 2001). According to the recent studies, *Absinthium* and *Artemisia* are more closely

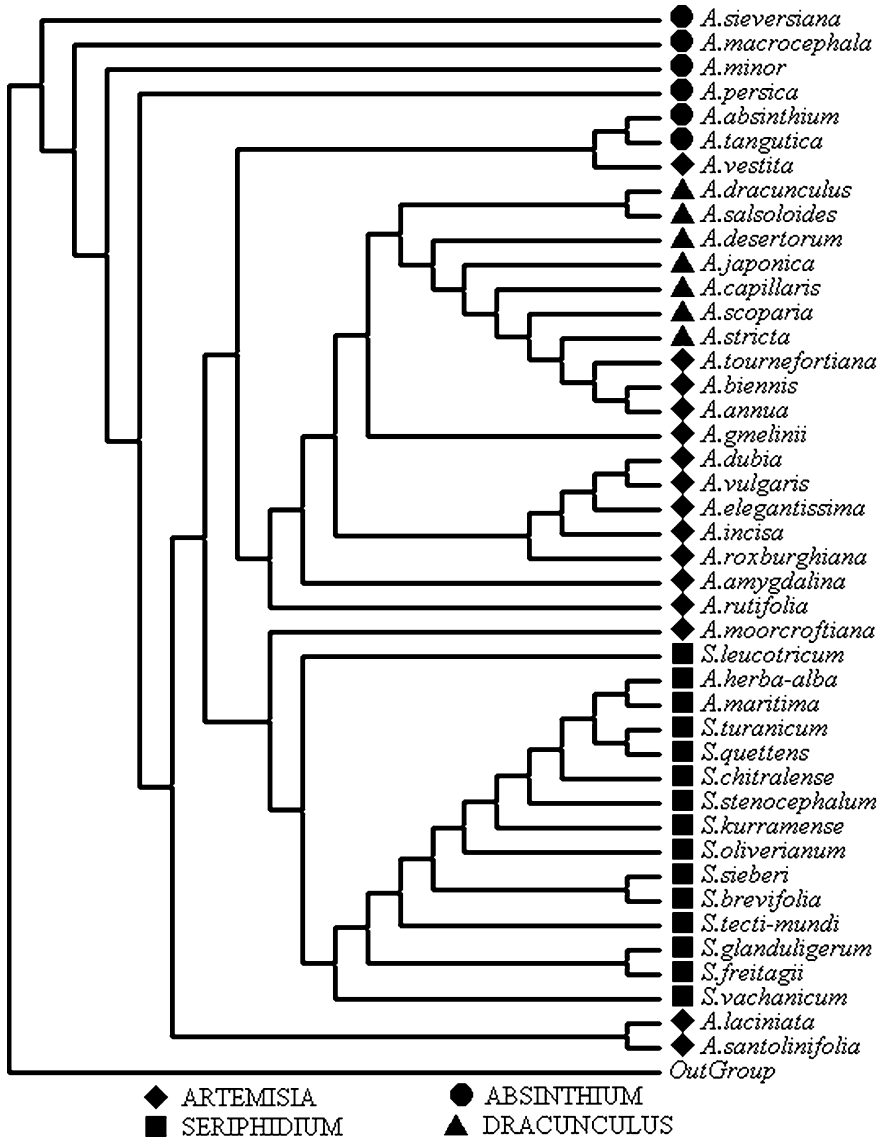

Fig. 6.4 Strict consensus tree of the genus *Artemisia* (found in Pakistan) based on morphological characters using maximum parsimony method. Geometrical shapes correspond to the conventional infrageneric classification of *Artemisia* (Hayat 2011)

related, and the other three subgenera *Dracunculus*, *Seriphidium,* and *Tridentatae* show more homology in their genome size. However, there is variation in genome sizes throughout the genus ranging from smallest *A. annua* to other species

$2n = 16x = 144$ where x can be either 7, 8, or 9. So polyploidy exist throughout the genus.

Plant barcoding is much highlighted tool today having numerous applications. Various studies have reported its utilization in case of medicinal plants as well. Sequences of nuclear ribosomal (nr) DNA regions (ITS1 and ITS2) and *trn*H-*psb*A spacer is frequently used and suggested for medicinal plants (Chen et al. 2010; Gao et al. 2010; Fu et al. 2011; Li et al. 2011; He et al. 2012). ITS2 is regarded as best candidate barcode for Asteraceae (Gao et al. 2010). Still taxonomists have also shown the limitations in resolution power for the use of ITS in the phylogeny of *Artemisiinae* (Torrell et al. 1999; Valles et al. 2003).

6.6 Conclusion

Ethnobotanical knowledge about the whole genus is well documented. The entire genus is ethnobotanically rich. Various members are used at many localities of the world and are in common use as seasoning and spices such as tarragon in food, as medicine, forage, feed for animals, in making brooms and to smoke, etc. Apart from these uses, the members of the genus are also utilized in industries, namely pharmaceutical, brewery, liqueur preparation, perfumery, and for ornamental purposes. *A. annua* is most surely the best studied and known *Artemisia* species, basically due to its powerful antimalarial artemisinin. Despite this the plant is also documented to be insect repellant and for the removal of smell in cemeteries since ancient times particularly in Asia. In addition, there is sign of extinction of ethnobotanical knowledge from generations to generations. *A. annua* has been evidently reported to be originated from China and migrated in Quarternary period along with other members from there to other regions later on by taking various routes. Some species such as *A. annua* have restricted ecological amplitude and are abundantly harvested from wild leading to vulnerability of next to extinction at various regions in the world. However, many countries of United States, Europe, Asia, and Africa are now cultivating *A. annua* mostly or medicinal purposes.

Identification of *Artemisia* species seems problematic and the species resemble morphologically that lead to wrong identification. Furthermore, most species acquire different morphological forms during their life cycle leading to intricacy in the accurate species credentials.

To solve the problems of apparent similarity in species various techniques and tools are being utilized including leaf anatomy, stomata types, pollen morphology and characteristic traits of glandular and nonglandular trichomes present on different parts of the plants. Pollen traits for instance size and exine characters are in consideration of taxonomists for decades. These are served as noteworthy taxonomic markers in studies of systematics and evolution. Additionally, during evolution, modifications have occurred in the pollen characteristics for example from having long spines to short protuberances on surface which might have also caused the presence of both pollination modes that are entomophily and anemophily in *A. annua*

and in other taxa. Changes in global environment also affected various morphological traits. Palynology is being used to solve issues both at infra- and intrageneric levels.

The presence of glandular and nonglandular trichomes is evident in various *Artemisia* species and possesses notable variation and diversity among taxa that are reportedly attributed to changes in ecological conditions over time. Also trend from complex to simple morphological characters during evolution have been reported. Previously, the morphological traits of trichomes were studied via microscopy such as Light Microscopy, but now Scanning Electron Microscopy has also being used along with latest use of pyrosequencing to study genes and so metabolic pathways as well. Secretory glandular trichomes are renowned for the production of artemisnin, an accredited antimalarial drug. *A. annua* is natural source of this valuable drug. *Artemisnin* concentrations are also found in other *Artemisia* species additionally, but quantities are not found significant. Various modern biotechnological techniques viz. transformation and microorganism systems are also being employed for enhanced production of artemisnin and its precursors. Numerous studies have also been performed on understanding enzymology and regulation of metabolic pathways involved in it.

Systematics of *Artemisia* as whole has been studied extensively, but some taxa are over emphasized as compare to others. That may be a reason of gaps in elucidation of true phylogeny at various levels. DNA profiling and barcoding is also being done. As stated previously, *A. annua* is most extensively studied species of *Artemisia*, its genome is not yet fully sequenced, but its small size makes it suitable candidate, and also its phylogeny, biogeography, biochemistry is well studied. Its chromosome number, banding, and ribosomal DNA-FISH patterns, genome size, genetic map, and molecular phylogenetic position are now comprehensively studied. But ample work is still required to make it a common crop as it is the only source of expensive artemisnin. In a nut shell, even though *A. annua* is among the most studied plant taxa still there is a need of advance research not only in the field of basic but also in applied research.

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Chapter 7
Trichomes in *Artemisia annua*: Initiation, Development, Maturation and the Possibilities to Influence these Factors

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Abstract The glandular trichomes (GT) of *Artemisia annua* (*A. annua*) synthesize and store the antimalarial compound artemisinin (AN) and several hundreds of other secondary metabolites (SM), and from an economical viewpoint, the GT are the most important structures of the plants. This chapter will give an overview of the contemporary knowledge on the morphology of the GT, the mechanisms of initiation of GT, the development and maturation processes of GT and an overview of the current research on elucidating the effect of the application or change in external factors on the density and size of GT in *A. annua*.

Abbreviations

NGT	Non-glandular trichomes
GT	Glandular trichomes
SM	Secondary metabolites
AN	Artemisinin
A. annua	*Artemisia annua*

7.1 Introduction to Trichomes in Plants

Plant trichomes have been defined as protuberances which have relatively well-defined size parameters and a morphogenetic origin in epidermal cells (Werker 2000; Wagner et al. 2004). Trichomes can have a multitude of appearances and can roughly be separated into two groups, non-glandular (NGT) and glandular trichomes (GT). NGT are characterized by not having any storage capacity, but apart

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from that, no common feature can characterize the diversity of the structure. NGT can appear as hairs, hooks, needles, plates, umbrellas, etc. and have very diverse functions, including prevention of desiccation or soaking, assistance to seed dispersal, cold and heat insulation, herbivore deterrence. Probably the most important NGT to humans are the hair-like trichomes on the seeds of cotton (Levin 1973; Wagner et al. 2004). GT, on the other hand, have the ability to store and sometimes synthesize and sequester different compounds, which are either unwanted or autotoxic to the plants in secluded organs, and 20–30 % of all vascular plants invest considerable resources in building, maintaining and filling GT on the aerial surfaces of the plants (Levin 1973; Wagner 1991; Agren and Schemske 1993; Duke 1994).

7.2 Glandular Trichomes in *Artemisia annua*

Though *A. annua* plants possess hair-like NGT, these are only peripherally dealt with here. In relation to the production of artemisinin (AN) and other secondary metabolites (SM), the GT are much more interesting and will here receive the main focus. The GT of *A. annua* are composed of 10 cells combined into 5 cell pairs in a ladder-like formation, and therefore termed biseriate (Ferreira and Jannick 1995). In *A. annua*, GT are primarily thought to contribute to the stress protection (Hu et al. 1993; Duke and Paul 1993; Duke 1994). Comparative studies between a glanded and a glandless biotype of *A. annua* showed that the glanded cultivar contained a much higher cumulative amount of SM than the glandless, and that the SM production in the glandless biotype was restricted to a few sesquiterpene compounds, not including AN (Duke et al. 1994; Tellez et al. 1999). This indicates that the GT are the primary, but not the sole site of the overall production of SM. Delicate laser dissection studies of GT of *A. annua* (Olsson et al. 2009; Olofsson et al. 2012) showed that key enzymes of AN production were exclusively expressed in the GT. This makes the understanding of the development and function of GT essential for the overall understanding of SM production in *A. annua*.

7.3 Initiation and Ontogeny of Glandular Trichomes

The understanding of how, and especially when, the initiation of GT takes place during the development of the leaves is critical for the present chapter, as part of it deals with the possibility of influencing the number of GT by applying external stress and other environmental factors. It is thus important to know whether it is possible to initiate new GT in existing leaves, or if it is only possible to influence the initiation of GT on leaves produced later in the development of the plant. Despite the fact that the initiation of GT creates the starting point for all further production of AN in *A. annua*, this subject has been dedicated surprisingly little scientific focus.

In the 1970s, 1980s and 1990s, a wave of microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies and reviews elucidated many aspects of GT of plants in general (Werker 2000; Duke et al. 2000), in the genus *Artemisia* as a whole [Kelsey and Shafizadeh 1980 (*A. nova*); Corsi and Nencioni 1995 (*A. nitida*); Ascensão and Pais 1987 (*A. campestris*); Werker et al. 1994 (*A. dracunculus*)], and in *A. annua* in particular (Duke and Paul 1993; Hu et al. 1993; Duke et al. 1994; Ferreira and Jannick 1995).

A consensus was created that the ontogeny of GT in the genus *Artemisia* is acropetal and begins with the enlargement of a single epidermal cell, which divides anticlinally, after considerable expansion above the leaf plane. The two resulting cells divide periclinally to form two new cells, which in turn divide periclinally. This process is continued until the GT consists of five cell pairs (Duke and Paul 1993). This phase in the development of the GT has been termed pre-secretive (Werker 2000). The initiation of GT in plants in general has been stated to commence at an early stage of leaf development often prior to stomatal development, sometimes even before the leaf primordium can be distinguished (Werker 2000). This was confirmed for *A. annua* by Duke and Paul (1993) and for *A. campestris* by Ascensão and Pais (1987), who also stated that the final number of GT in the leaves was defined at an early developmental stage of the leaves. Consequently, the current consensus is that the number of GT of the fully developed leaf in *A. annua* is predetermined already at an early developmental stage of the leaves. Since this wave of microscopy studies, the direct study of the initiation and ontogeny of GT in *A. annua* has been very limited, and the interest in GT has moved on to other levels of information.

7.4 Morphology, Development and Maturation of Glandular Trichomes

The two lower cell pairs of the GT primarily function as stalk cells, carrying two subapical cell pairs, which in turn carry the apical cell pair (Duke et al. 1994) (Fig. 7.1). The stalk cells contain chloroplast and the organelle structure resembles a normal epidermal cell structure, the subapical cells contain modified chloroplasts, and the apical cells contain no chloroplasts. All GT cells contain high amounts of smooth endoplasmic reticulum, which is associated with synthesis of lipids, including oils, phospholipids and steroids, and metabolism of carbohydrates. Particularly, the areas of reticulated ingrowth in the cell wall of the apical cells, which facilitate the SM export to a subcuticular sac, contain high amounts of endoplasmic reticulum (Duke and Paul 1993). Shortly after the differentiation of the 10 cells, the GT go into the secreting phase (Werker 2000), and the 6 apical and subapical cells (secreting cells) start synthesizing SM and secrete these into a subcuticular sac, which only borders with the secreting cells and not the stalk cells (Duke and Paul 1993). The subcuticular sac is gradually filled with SM, and the

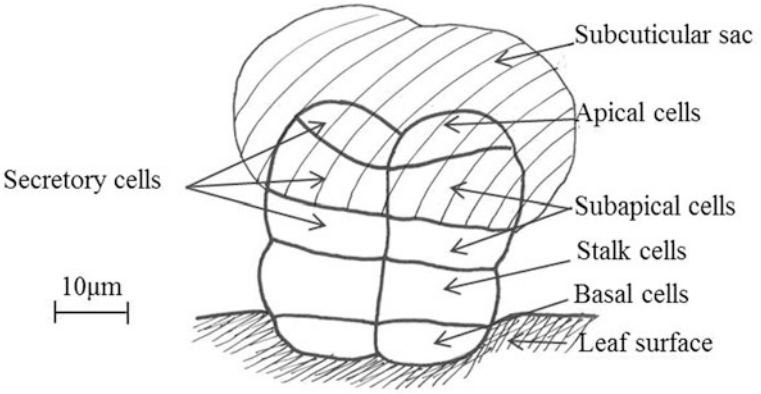

Fig. 7.1 Schematic diagram of glandular trichome *in Artemisia annua* with subcuticular sac fully expanded by secondary metabolites

sac separates from the cell walls of the secreting cells, forming a bilobed sac (Duke and Paul 1993). The subsequent detachment of the cuticular wall from the apical cells is considered to be associated with the termination of secretory activity (Ferreira et al. 1997). No research has directly investigated the speed by which the subcuticular sac is filled with SM. However, Duke and Paul (1993) stated that it was "rapid", and Kjaer et al. (2012) demonstrated that, in leaves, which had unfolded only one or two weeks before, the GT were already similar in size to GT of leaves, which were more than two month old.

The GT are proposed to go through a process of maturation and later of senescence and thus enter a post-secretive phase (Ascensão and Pais 1987; Werker 2000; Kjaer et al. 2013). Duke and Paul (1993) reported that during the maturation process, the amount of endoplasmic reticulum increases in the secreting cells, until a point where the cell membranes begin to break, resulting in loss of organellar and cellular integrity of secretive cells. In other species of GT-bearing plants, the stalk cells are shown to become more active and are suggested to contribute to the terpene catabolism (Werker 2000). The exact progress of the maturation and timing of the senescence of GT in *A. annua* are still largely unresolved.

7.5 Excretion of Secondary Metabolites

Anyone who has been near an *A. annua* plant will have noticed the strong odour of volatiles emanating from the plant, but the intent observer will notice that younger plants left undisturbed do not smell until touched, whereas older plants have a more constant "background" smell, which can be intensified by touching the plants. This is due to the mechanism of excretion of SM from the plant. Stored SM in GT of plants in general can be released to the surroundings by either a

continuous leaking of SM, or by a single event of rupturing the storage organ and thus releasing all the stored SM. The SM of *A. annua* are by many thought only to be released by the rupture of GT (Duke and Paul 1993; Ferreira et al. 1997; Lommen et al. 2006; Zhang et al. 2006; Lommen et al. 2007; Arsenault et al. 2010; Kjaer et al. 2012). This can happen either by forceful breaking of the subcuticular sac (e.g. by touching the leaves), or by auto-rupture of mature and senesced GT.

The mechanisms of maturation and auto-rupture of individual GT are difficult to investigate, as it would essentially require following the development of the exact same GT throughout their lifetime, and presently, no research has focused on setting up such an experiment. But research has provided some indications of the functionality of the mechanism. Duke and Paul (1993) reported that the subcuticular sac would start splitting in the central area of the bilobed structure. Zhang et al. (2006) demonstrated that 4 % of GT in upper leaves were split open, 12 % were split in middle leaves and 25 % were split in lower leaves of large *A. annua* plants, indicating that older and more mature GT had a higher risk of rupturing. Lommen et al. (2006) followed the development of several sets of comparable leaves and demonstrated a gradual decline in detectable GT after the leaves reached a certain age, coinciding with a maximum in the size of the leaves. Arsenault et al. (2010) found that the number of GT in relation to epidermal cells was at a maximum when leaves reached full size and decreased thereafter. Ferreira et al. (1997) stated that intact GT were observed in inflorescences during their development, but seldom after anthesis. This disappearance of GT possibly indicates that the GT ruptured more frequently with increasing leaf age. Kjaer et al. (2013) further demonstrated that several types of applied stress apparently accelerated the maturation of the GT and in older leaves caused a higher proportion of the GT to rupture in treated leaves than in untreated leaves. Ferreria and Janick (1995) focused on the floral morphology of *A. annua* and stated that physiological maturity of GT in the inflorescence coincided with full bloom. Very little is, however, currently published on the mechanisms of rupture of GT, and the possible roles of temperature, wind, turgor pressure, touching, internal signalling, etc. on the susceptibility of the GT to rupture are largely undocumented.

7.6 Glandular Trichomes as a Protection Strategy

A. annua apparently relies on a chemical protection strategy, as it has no external structures such as thorns to deter herbivores, and the hair-like trichomes do not appear to act as protection to the plants. Chemical protection may consist of deterring from a distance by smell or irritants, deterring close-up by touch-provoked smell or irritant release, and deterring by ingestion of bad tasting or poisonous compounds (Levin 1973; Wagner 1991). *A. annua* likely utilize all three strategies. Most of the chemical protection compounds in the aerial parts of *A. annua* are produced and stored in GT (Brown 2010). Here, they stay until the GT rupture and release the SM. In young undisturbed plants, this happens only

when the plants are touched, whereas in lower parts of older plants, it happens continuously, and particularly when touched (Pers. obs.). This provides the plants with a cover of smell, which deters predators from a distance and particularly if they get close enough to touch the plants. If they nevertheless should start eating the plants, they get a bitter taste and possibly poisonous effects from AN and other compounds.

Observing the plants during growth gives some clues to how the plants prioritize their protection throughout the development. In the first weeks after the plants have emerged from the seeds, the first leaves have very few GT and only have a very weak bitter taste, indicating that the plants are allocating most resources to growth rather than protection. Later in the plant development, the density of GT in newly developed leaves becomes increasingly higher, and the bitter taste is strongly intensified (Pers. obs.). *A. annua* seems to hold a particular interest in protecting the inflorescences, as these are densely covered with faster maturing GT, which rupture and release their content during anthesis to the point where almost all GT have ruptured at the end of anthesis (Duke and Paul 1993; Ferreria and Janick 1995) and the leaf content of AN and flavonoids declined (Baraldi et al. 2008).

7.7 Density and Dimensions of Glandular Trichomes

The GT of *A. annua* are impossible to see by the naked eye, and historically, several types of microscopy have been applied to observe and quantify the GT. In recent years, fluorescent microscopy has become popular, because the secondary metabolites of the GT will auto-fluoresce at different wavelength than the chlorophyll, which makes it possible to distinguish and measure the GT automatically by computer recognition (Graham et al. 2010; Kjaer et al. 2012) (Fig. 7.2).

Generally GT densities have been measured to range from 10 to 75 GT mm^{-2} (Lommen et al. 2006; Arsenault et al. 2010; Graham et al. 2010; Maes et al. 2011; Kjaer et al. 2012; Nguyen et al. 2013). Other studies have reported relatively low or high GT densities compared to this, including Kapoor et al. (2007) reporting 80–140 GT cm^{-2} (corresponding to 0.8–1.4 GT mm^{-2}) and Hu et al. (1993) reporting 190–225 GT mm^{-2}. Mean values of the GT length and width were reported by Kjaer et al. (2012) to be in the ranges of 60–65 and 30–33 μm, respectively, and Hu et al. (1993) reported results of 45–48 and 27–30 μm, respectively. Mean values of projected GT areas were measured to be 1,700–2,300 μm^2 by Kjaer et al. (2012), 1,300–2,100 μm^2 by Nguyen et al. (2013) and 400–1,100 μm^2 by Maes et al. (2011). However, GT densities and sizes are probably highly dependent on leaf age and expansion, growth conditions, *A. annua* cultivar, etc., so comparisons should always be drawn cautiously.

The GT are found on both sides of the leaves and are relatively evenly distributed across the blade. Comparing densities of GT from the adaxial and abaxial sides of the leaf have shown varying results as Kjaer et al. (2012) demonstrated significantly higher densities of GT on the abaxial than the adaxial side, whereas

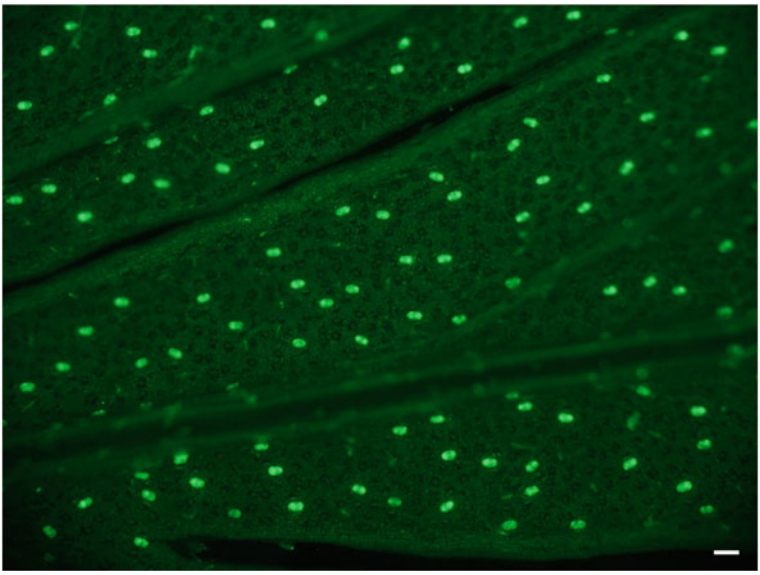

Fig. 7.2 Glandular trichomes of *Artemisia annua* contain autofluorescing secondary metabolites which makes them highly visible at specific wavelengths of light. *White bar* in *lower right* corner: 100 μm

Hu et al. (1993) found the opposite trend, and Arsenault et al. (2010) found no difference between the two sides of vegetative leaves. Kjaer et al. (2012) demonstrated that the length of the GT did not differ significantly from the abaxial to the adaxial side of the leaf, irrespective of leaf age.

7.8 Environmental and Stress Effects on Densities and Sizes of GT

If stress is defined as conditions, that are different from normal conditions, research has demonstrated that stress can affect the formation of GT on leaves. Liersch et al. (1986) concluded that the growth regulators diaminozide and chlormequat *may* have had an influence on the formation of GT, and Kapoor et al. (2007) demonstrated that some arbuscular mycorrhizal fungi and micronutrients increased GT densities. Liu et al. (2009) showed that application of jasmonic acid increased GT densities. Maes et al. (2011) demonstrated that application of jasmonic acid (JA) and gibberellic acid (GA), but not cytokinin (BAP), increased the density of GT and further showed that a cultivar with a low AN content had a greater plasticity for changes in the GT density than a cultivar with higher AN content. This finding suggested a mechanism whereby the plant has a maximum

capacity for GT on the leaves. Kjaer et al. (2012) applied several types of hormonal, chemical and physical stress to large *A. annua* plants and found that in upper leaves, developed under the influence of the treatments, the GT densities had only a weak or no tendency to increase, and it was deducted that larger plants are less sensitive to stress treatments than the younger plants used in most other experiments. Kjaer et al. (2012) further showed that in lower leaves, developed before the application of treatments, the GT densities had a tendency to decline, as compared to untreated controls, and deducted that the stress treatments accelerated the maturation of the GT, causing more of these to auto-rupture. Nguyen et al. (2013) found that the presence/absence of roots did not affect the GT densities and further demonstrated that the cytokinin hormone BAP significantly increased the GT densities in young leaves, whereas the auxinn aphthaleneatic acid (NAA) had no effect on the densities. Arsenault et al. (2010) determined that the GT distribution on leaves differed when plants were in a vegetative versus a reproductive stage and further found strong relations between content of AN and GT densities regardless of leaf types. This strong relation between GT densities, AN and key precursors was confirmed by Graham et al. (2010). Lommen et al. (2006) have given an extensive insight into the densities of GT on individual leaves during development and maturation of the leaves, and Nguyen et al. (2011) presented a review of the overall knowledge on the influence of external factors on the production of GT and AN in *A. annua.*

Nguyen et al. (2013) demonstrated that cultured shoots of *A. annua* produced significantly larger GT on young leaves, if the plants were rooted than if they were kept unrooted. Nguyen et al. (2013) further demonstrated that the hormones BAP and NAA had no effect on GT size if applied separately, but caused a significant decline in GT sizes of older leaves if applied together. Maes et al. (2011) observed a significant increase in the area of GT following treatment with JA and gibberellic acid, and a significant decrease in the GT area in cytokinin treated leaves of young individuals of a low AN producing *A. annua* cultivar. They interpreted the increase in GT area to be linked with advanced maturation of the GT and/or increased biosynthetic activity, and the decreased areas linked with cytokinin keeping cells in a division state, rather than proceeding towards maturity. Kjaer et al. (2012) demonstrated that several stress types decreased GT sizes on both younger and older leaves as compared to untreated controls, and in contrast to Maes et al. (2011), the smaller GT were interpreted as an indication of more mature GT on the stress-treated plants than on the control plants.

The mechanisms of initiation, development and rupturing of GT in *A. annua* appear to be highly complex, and the overall understanding of the effect of externally applied conditions on the GT still remains fragmented, and more research is needed to reveal the bigger picture. Generally, the effect of different environmental conditions, including nutrient availability, temperature, light, wind, UV-light on the GT formation, distribution and development remains largely undocumented. Particularly, the role of the environmentally controlled degree and rate of leaf expansion on the densities and sizes of GT apparently remains to be demonstrated.

7.9 Conclusions

Seen from a production view point, the microscopic GT are the most important structures of the *A. annua* plants, as these are the site of production and storage of the economically important and life-saving antimalarial compound artemisinin. As reviewed above, many details of the morphology, ontogeny, distribution and sensitivity of GT to various stress types have been researched in several experiments, and many interesting findings and hypotheses have been published. Many pieces for the overall jigsaw puzzle of the full understanding of the initiation, development, maturation and eventual rupture of the GT are now on the table, but contrasting findings, different experimental set-ups, different varieties and blanks in the published knowledge still make it difficult to put all the pieces together in a coherent model of fully understanding the life of an individual GT.

Many aspects of the GT of *A. annua* are still open for investigation. Considering the importance of attempting to eliminate loss of GT and AN content in leaves during cultivation, the present authors find it particularly interesting to create a deeper understanding of the maturation process of the GT. How does the maturation process proceed, and can the speed of maturation be manipulated by external factors, i.e., stress? How do the GT rupture, and which external factors will affect the rupturing process? And, can loss of AN during cropping and harvest be minimized by preventing the rupture of GT, i.e., by selecting for stronger GT?

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Chapter 8
Potential Methods to Improve the Efficiency of Artemisinin Extraction from *Artemisia annua*

Rhianna Briars and Larysa Paniwnyk

Abstract Recently, there has been a move to improve the extraction of artemisinin in terms of energy, solvent and purification costs and so, as a result, many innovative processes, such as supercritical fluid extraction (SFE), pressurised solvent extraction (PSE), microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE), are currently being explored. The most basic industrial-scale method for extracting artemisinin involves high volumes of heated organic solvents, such as petroleum ether and hexane, coupled with extended extraction time periods in order to achieve reasonable quantities of extract. Solvent considerations are not only underpinned by the solubility of artemisinin but also that of other phytological compounds which can complicate the purification stages. This has lead to some of the obvious alternative solvent choices, such as ethanol, being considered as unviable. Therefore, some of the more modern technologies have largely maintained the use of the traditional solvents but altered the parameters with which they are used, such as increasing pressures and temperatures of reactants in order to speed up extraction. This chapter will include an evaluation of the aforementioned alternate extraction techniques (SFE, PSE, MAE and UAE), including consideration of artemisinin yield, extract purity and industrial feasibility, in addition to examining the advantages and disadvantages of a range of solvent polarities.

8.1 Conventional Industrial Extraction

The most implemented technique to extract artemisinin is conducted simply through multiple soakings of dried and crushed *Artemisia annua* leaves in boiling organic solvents, for between 10 and 48 h each time. The solvents most often employed for

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this extraction are hexane or petroleum ether (Elsohly et al. 1990; Christen and Veuthey 2001; Cutler et al. 2006; Lapkin et al. 2006; Kuhn and Wang 2007; Liu et al. 2011). Soxhlet extractors are the easiest way to achieve this as the leaf material can have a constant infusion of boiling solvent (Ormeño et al. 2011) (Fig. 8.1).

The time frame for extraction can be reduced by using flow or percolated systems; these methods still require a series of solvent steeps but each one lasts only 120 min (Cutler et al. 2006; Sticher 2008) (Fig. 8.2).

This technology is simple and the investment cost is low but due to the protracted and arduous extraction times of 6 h, minimum, there is a great consumption of energy; and yet the yields are still quite low with only around 60–70 % overall extraction efficiency and recovery (in regard to artemisinin content in dried leaf) and co-extracted impurities are present, such as waxy lipids, essential oils, terpenes, chlorophylls, sugars and artemisinin derivatives (Chemat et al. 2012; Cutler et al. 2006; Lapkin et al. 2006, 2010; Li et al. 2011; Liu et al. 2011; Ormeño et al. 2011; Sticher 2008). There is also the worry of artemisinin destruction from the heat of extraction (Neill 2011; Ormeño et al. 2011; Sticher 2008), and as a result, the organic solvents most often used for extraction are ones with low boiling points (such as hexane, toluene, petroleum ether and chloroform) in order to try and avoid this problem (Cutler et al. 2006; Haynes and Vonwiller 1994; Lapkin et al. 2006, 2010; Liu et al. 2011; Neill 2011). Therefore, finding more cost-effective and faster techniques, while maintaining maximal yields with minimal impurities, is a high priority.

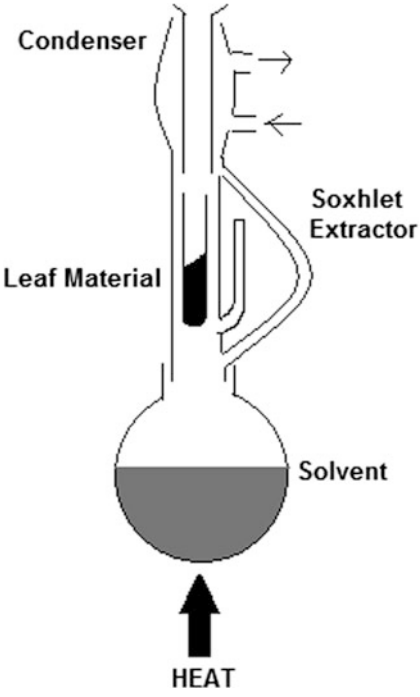

Fig. 8.1 A diagram of the basic set-up of a soxhlet extractor

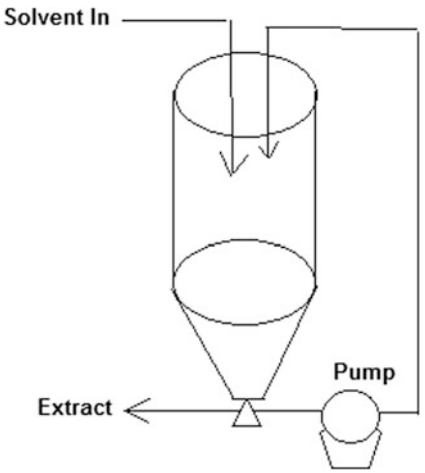

Fig. 8.2 A diagram showing how a percolated extraction system is achieved

8.2 Solvent Considerations

A simple way of maximising artemisinin extraction is through achieving optimum solubility of artemisinin into a solvent. Artemisinin is a molecule with low polarity, and so, the well-documented principle of "like dissolves like" would suggest that medium to low-polarity solvents would be good for extraction (Cutler et al. 2006; Lapkin et al. 2006; Liu et al. 2009; Nti-Gyabaah et al. 2010). There is not a vast amount of data on the solubility of artemisinin in specific different solvents but one study by Nti-Gyabaah et al. (2010) stated that toluene, ethyl acetate and acetone were the best solvents, that they studied, to dissolve artemisinin, as ascertained by high performance liquid chromatography (HPLC). Ethanol, acetonitrile, methanol and hexane, in that order, were the next best solvents. The polarities of these solvents are displayed in the Table 8.1; with this information, it can be seen that, generally, as the reported suitability declines the polarity increases, apart from hexane which is non-polar. This confirms that a medium level of polarity is a desired quality for artemisinin extraction solvent (Liu et al. 2009; Nti-Gyabaah et al. 2010).

Table 8.1 Polarity indexes of solvents studied for artemisinin solubility

Solvent	Polarity index	Artemisinin mole fraction solubility at 50 °C
Toluene	2.4	130.85
Ethyl Acetate	4.4	64.70
Acetone	5.1	28.42
Ethanol	5.2	14.87
Acetonitrile	5.8	6.61
Methanol	5.1	6.39
Hexane	Non-polar	6.81×10^{-1}

It is curious that hexane is such a widely used solvent, for the extraction of artemisinin, but seems to be outperformed in terms of solubility by so many other solvents, but it seems that hexane is chosen due to its low cost (Cutler et al. 2006; Nti-Gyabaah et al. 2010).

Specificity is a universal problem with artemisinin extraction, as there is an issue with co-extracted impurities. With the non-polar solvents, such as hexane, the most abundant of these impurities are the waxy long-chain hydrocarbon lipids from the leaves' protective layers (Cutler et al. 2006; Kuhn and Wang 2007; Lapkin et al. 2010; Nti-Gyabaah et al. 2010). The presence of these waxy compounds has been held responsible for reductions in the total recovery of artemisinin as its solubility with the waxes is high, therefore possibly complicating separation. But because of this high level of solubility, these co-extracted waxy impurities could also augment the actual extraction of artemisinin thereby providing a potential benefit for continuing the use of hexane for artemisinin extraction (Lapkin et al. 2010; Nti-Gyabaah et al. 2010).

Another reason why hexane is actually beneficial over the medium polarity solvents could be because the more polar a solvent is, the more ionic compounds from the plant will also be co-extracted which would result in a more intensive purification process (Nti-Gyabaah et al. 2010). Ethanol, a popular possible alternative, has been noted to be particularly problematic with the co-extraction of a broader range of impurities than hexane. These impurities include pigments and sugars which pose issues for the purification process, particularly crystallisation, as detailed in the section below (Kuhn and Wang 2007; Liu et al. 2011; Sticher 2008).

8.3 Purification of Extracts

There are four main routes for artemisinin purification which can be used in combination to enhance the purity of the artemisinin extracted. These processes entail crystallisation cycles, activated carbon adsorption, partitioning with solvents and silica gel column chromatography (Elsohly et al. 1990; Haynes and Vonwiller 1994; Christen and Veuthey 2001; Cutler et al. 2006; Cutler and Lapkin 2007; Kuhn and Wang 2007; Liu et al. 2007, 2011; Sticher 2008; Stringham et al. 2009). Each method involves its own issues. Crystallisation has been noted as unsuitable for removing sugary impurities as they inhibit the drying of crude extracts. Using an exact concentration of activated carbon is fundamental to its success because excessive activated carbon can actually remove some of the extracted artemisinin as well as impurities. Large solvent volumes lead to high costs for partitioning, and silica gel column chromatography is costly and laborious, due to the requirement for a high solute to silica gel ratio of 1:44 (Kuhn and Wang 2007; Liu et al. 2011). Plus the more impurities that are present within crude extracts the more extensive, and therefore expensive, purification becomes.

The focus of the last two decades, for artemisinin extraction, has been looking at finding straightforward, quick and highly precise extraction techniques to

replace these basic solvent extractions (Christen and Veuthey 2001). The overall aim has been to reduce the use and volumes of solvents, to in turn dampen the impact of the inevitable environmental problems which come with organic solvents, and to try to achieve cleaner and more specific extractions. These aims have been addressed through focussing on reducing extraction times (Cutler et al. 2006; Lapkin et al. 2006, 2010). Four of the potential techniques to accomplish this are supercritical fluid extraction (SFE), pressurised solvent extraction (PSE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (USE).

8.4 Supercritical Fluid Extraction

SFE has been in use since the 1970s with particular usage in the late 1990s (Sticher 2008). The technique, usually utilising supercritical carbon dioxide, has been used to extract a number of hydrophobic to medium polarity plant-based compounds including removing the caffeine from coffee beans and piperines from pepper (Asfaw et al. 2005; Pourmortazavi and Hajimirsadeghi 2007; Sticher 2008; Lapkin et al. 2010; Martín et al. 2011; Ormeño et al. 2011).

Subjecting CO_2 to high pressures results in it being constrained into a liquid state even at higher temperatures which would ordinarily produce gaseous CO_2; this state is what is termed supercritical as the compound is maintained as a liquid beyond its critical point (Ormeño et al. 2011). In this state, carbon dioxide has a similar polarity to liquid pentane, and therefore, it becomes a fantastic solvent for compounds with a degree of hydrophobicity including lactones such as artemisinin (Pourmortazavi and Hajimirsadeghi 2007; Sticher 2008). The main reasons that carbon dioxide is normally chosen as the supercritical extraction solvent are that it is comparatively inexpensive, common, non-toxic, safe and inert, plus its critical temperature is the low temperature of 31.1 °C (Pourmortazavi and Hajimirsadeghi 2007; Sticher 2008; Martín et al. 2011; Ormeño et al. 2011). This low temperature allows compounds such as artemisinin to be extracted without any risk of damage through exposure to high temperatures (Kohler et al. 1997; Sticher 2008; Tang et al. 2009; Ormeño et al. 2011). Carbon dioxide solvent is also easily removed from the extract because as it returns to normal atmospheric pressure, it simply turns back into its gaseous state leaving behind the extracted material without any contamination from the extraction solvents (Kohler et al. 1997; Corr 2002; Sticher 2008; Tang et al. 2009; Lapkin et al. 2010; Ormeño et al. 2011; Chemat et al. 2012).

The carbon dioxide which is used in the extraction can also be recycled due to the way the three-stage extraction process is conducted. Firstly, the CO_2 is subjected to high pressures and temperatures, and this supercritical fluid is driven into the tank containing the plant material where the extraction of the phytochemicals can take place. The extraction mixture can then be conveyed into a further vessel where the CO_2 can be collected as a gas once it has been depressurised and therefore evaporates. The extracted components are then left behind ready to be

separated out and the carbon dioxide can be used again in future extractions (Kohler et al. 1997; Sticher 2008; Ormeño et al. 2011; Chemat et al. 2012).

Despite the solvent phase being replaced by supercritical CO_2, a small amount of an organic solvent is required as a polar modifier to aid the solubility of the particular compounds of interest (Pourmortazavi and Hajimirsadeghi 2007; Sticher 2008). The type of solvent used as a polar modifier is one of the important parameters which require refining to achieve optimal extraction; for the case of artemisinin, a hydrophilic solvent such as ethanol, methanol or acetonitrile is used to create an overall medium level polarity solvent, due to the aforementioned knowledge of solubility (Kohler et al. 1997; Cutler et al. 2006; Lapkin et al. 2006; Sticher 2008). The amount of polar modifier, the temperature, pressure, flow rate and extraction time are also key factors to be considered (Kohler et al. 1997; Tzeng et al. 2007). The optimal parameters for using SFE to extract artemisinin have been ascertained and are listed as conducting the 3–6 h extraction at a pressure of 15 Mpa and a temperature of 40–50 °C with 3 % methanol or ethanol used as polar modifier and a CO_2 flow rate of 2 ml/min (Kohler et al. 1997; Cutler et al. 2006; Tzeng et al. 2007).

It has been noted that SFE has a particular affinity for, and better suited than conventional techniques at, extracting higher molecular weight sesquiterpenes, and therefore, this could indicate its potential benefit for extracting artemisinin (Asfaw et al. 2005; Ormeño et al. 2011). A number of research groups have shown its effectiveness in comparison over more conventional protocols for artemisinin extraction; the extraction yield using SFE is high with one group displaying a yield of 1.49 %, which is a 3-fold increase in the artemisinin extracted with a hexane soxhlet extraction, and also a higher degree of extract purity (Lin et al. 2006). Other papers state that the yields are at least comparable to those seen with soxhlet but are much higher than the yields achievable with hydrodistillation with up to 6 times more artemisinin present in SFE extracts (Quispe-Condori et al. 2005; Lin et al. 2006; Tzeng et al. 2007; Martín et al. 2011; Ormeño et al. 2011).

But this discrepancy in the yields produced through SFE in comparison to a hexane soxhlet points to one of the disadvantages that reviewers have deemed to be an issue with the use of SFE. Cutler et al. (2006) attribute this to the parameters needing to be precise, particularly in regard to the polar modifier; without meticulous attention to this, the efficiency is inconsistent (Lapkin et al. 2006). Other disadvantages that have been observed are in relation to the need for the purification of extracts still being present. SFE has shown to co-extract impurities including waxes when used in other natural product extractions, and therefore, the recovery has been shown to be low (Tzeng et al. 2007; Liu et al. 2009; Martín et al. 2011; Ormeño et al. 2011; Grevenstuk et al. 2012). Another disadvantage that is pinned to SFE is the high level of investment needed to achieve and sustain the operating temperatures and pressures despite a reduction in energy costs due to supercritical CO_2 having a lower working temperature. Therefore, it is reported that SFE is a choice more suited to larger laboratories (Christen and Veuthey 2001; Corr 2002; Cutler et al. 2006; Lapkin et al. 2006, 2010; Liu et al. 2008; Chemat et al. 2012).

8.5 Pressurised Solvent Extraction

In use since the late 1990s, PSE, as the name suggests, uses solvents under high pressure to allow high temperatures to be utilised without the solvent reaching boiling point. The high temperatures, ranging from 50 to 200 °C, allow the solvent to penetrate plant material and augment the solubility and transport of compounds in the solvent, which in turn accelerates extraction (Sticher 2008; Tang et al. 2009; Ormeño et al. 2011). The pressures required for the technology are between 10 and 15 MPa (Sticher 2008). For artemisinin extraction, it was discovered that changing the pressure did not have an overt effect but the higher temperatures employed still enhanced extraction as shown in the Table 8.2 (Christen and Veuthey 2001). The technique still requires organic solvents, but it is possible to operate with a lower consumption of solvent (Sticher 2008; Ormeño et al. 2011).

The theory is similar to that of SFE with the temperatures and pressures implemented having the ability to change the properties of liquids, thereby enhancing the breaking of molecular bonds, allowing a rapid dissolvability and dispersion of compounds (Christen and Veuthey 2001; Sticher 2008; Miron et al. 2010; Ormeño et al. 2011). Despite the high temperature of 80 °C, PSE is not seen to degrade artemisinin or related compounds (such as artemisinic acid) unlike refluxing at 100 °C; as artemisinic acid is not present in the studied conventional conditions but is in PSE extracts and the concentration of artemisinin is doubled with PSE (Christen and Veuthey 2001; Sticher 2008).

The major observed trend in favour of the use of PSE is the rapidity of extraction time with the possibility of between only 15–180 min, thereby being more environmentally friendly in terms of energy consumption (Christen and Veuthey 2001; Sticher 2008; Paniwnyk et al. 2009; Ormeño et al. 2011). This allows PSE to be well suited for laboratories needing to process many samples in a short space of time (Christen and Veuthey 2001).

There are still a few flaws in the use of PSE including the need for specialised machinery, including a series of pumps and valves, resulting in a requirement for a high level of capital for investment (Christen and Veuthey 2001; Liu et al. 2008; Sticher 2008). There are also continued issues with the co-extraction of compounds, seen with high temperature solvents; therefore, the specificity of the technique is still in question (Ormeño et al. 2011).

Table 8.2 A table showing how changing the parameters of PSE, in particular temperature, alters the extraction of artemisinin

Temperature (°C)	Pressure (MPa)	Artemisinin (% dry wt.)	
		Extracted in water	Extracted in ethanol
30	5	0.33	0.45
	35	0.28	0.45
80	5	0.57	0.50
	35	0.46	0.49

8.6 Microwave-Assisted Extraction

Microwaves have been used in extraction since 1992 where they were used to extract fragrance compounds from lemons (Guo et al. 2001). Since then, they have been used, usually with a wave frequency of 2,450 MHz, to extract a variety of phytochemicals including hesperidin from orange peel, saponins from chickpeas and trimyristin from nutmeg (Sticher 2008).

The use of microwave energy allows uniform heating of the entire solution to be immediate, thereby speeding the rate of extraction as the heat does not have to be transferred through the reaction vessel (Sticher 2008; Tang et al. 2009; Li et al. 2011; Ormeño et al. 2011). Moisture within the plant cells evaporates upon heating producing high pressures which, when inflicted on the cell walls, can cause breaks thereby allowing the phytoconstituents to leach out into the extraction solvent (Christen and Veuthey 2001; Khalil et al. 2011; Li et al. 2011).

Extraction, therefore, is very quick with the possibility of being complete in mere minutes (Guo et al. 2001; Asfaw et al. 2005; Sticher 2008; Li et al. 2011). In the case of polyphenols from grape seeds, more than 90 % were extracted by microwaves in only a few minutes, and greater yields of desired compounds from *Paris polyphylla* were seen with MAE than normal reflux in less than 10 % of the time (Guo et al. 2001; Li et al. 2011).

The parameters that need to be refined include temperature, pressure, power, volume of extract components and the type of solvent (Li et al. 2011). As expected, organic solvents are chosen to extract organic compounds, and metallic or inorganic compounds are extracted using aqueous-based solvents (Guo et al. 2001).

When describing the use of MAE technology for artemisinin extraction, Christen and Veuthey (2001) focus on the effects of altering the solvent to see whether specificity can be increased. They comment on the use of water, ethanol, toluene and hexane as these cover a range of polarity. Hexane is noted as having the highest selectivity for artemisinin with 0.8 % extracted, which was also achieved at the lower temperature of 35 °C (as opposed to 60 °C for the other solvents), whereas water extraction was unsuccessful as it caused the plant to degrade. Ethanol seems to display the next lowest yield with a relatively high extraction of a related component (Artemisinic acid at 0.072 %) which suggests it is not a very selective solvent for artemisinin.

The benefit of microwave extraction, beside the sheer speed of extraction, is that degradation through high temperatures is not observed (Sticher 2008; Ormeño et al. 2011). But this is paralleled with a disadvantage, alluded to by some papers, that microwave energies themselves could potentially be damaging to compounds as isomerisation can occur under high powered microwaves (Ormeño et al. 2011).

It has also been recorded that MAE artemisinin extracts, conducted in hexane, contain high amounts of large compounds such as chlorophylls (Khalil et al. 2011). This presence of co-extracted impurities and the report that the technique is particularly effective at extracting polar compounds but not higher molecular weight sesquiterpenes may prove to be disadvantageous for use in artemisinin extraction

(Guo et al. 2001; Asfaw et al. 2005). Another drawback in the use of MAE is that, unfortunately, it is still associated with the requirement for technical machinery, including a magnetron to apply the microwaves to samples (Christen and Veuthey 2001; Liu et al. 2008).

8.7 Ultrasonic-Assisted Extraction

Ultrasound can be defined as sound waves at frequencies above the audible limit for humans, classically set at higher than 20 kHz (Mason 2007; Asakura et al. 2008; Leonelli and Mason 2010). It has the ability to partake in natural product extraction due to the kinetics of the alternating compression and rarefaction periods of sound waves through aqueous media (Leonelli and Mason 2010; Sutkar et al. 2010; Veillet et al. 2010). During the rarefaction stage, miniature bubbles, designated "cavitation bubbles", are formed under the radiated force (Keil and Dahnke 1997; Mason 1999, 2007; Birkin et al. 2001; Han 2007; Leighton 2007; Asakura et al. 2008; Leonelli and Mason 2010; Sutkar et al. 2010; Veillet et al. 2010; Sun et al. 2011). A critical moment, during the inflation of these bubbles, is reached under the tension of the compressing portions of the waves, where they undergo intense and powerful collapse generating excessive conditions, comprising high temperatures (can reach up to 5,000 °C) and pressures, within the immediate environment of the collapsed bubble (Keil and Dahnke 1997; Birkin et al. 2001; Gogate and Pandit 2004; Han 2007; Leighton 2007; Ai et al. 2010; Leonelli and Mason 2010; Sutkar et al. 2010; Veillet et al. 2010; Sun et al. 2011).

Surfaces and objects in the extraction media produce a platform for the phenomena of "acoustic streaming" and "micro-jets". Acoustic streaming is where sound waves suffer energy reduction when solid interfaces halt natural progression and impart turbulence to the liquid and micro-jets are produced through cavitation bubbles collapsing abnormally (Keil and Dahnke 1997; Dahlem et al. 1999; Birkin et al. 2001; Gogate and Pandit 2004; Han 2007; Kumar et al. 2007; Leighton 2007; Leonelli and Mason 2010; Sutkar et al. 2010; Veillet et al. 2010). These forces have the ability to form fissions in cell walls, and materials of a biological nature can be disturbed aiding solvent travel and the release of components from the plant cells, including the trichome glands, which enclose artemisinin (Birkin et al. 2001; Albu et al. 2004; Asfaw et al. 2005; Wang and Deng 2006; Leighton 2007; Mason 1999; Paniwnyk et al. 2009; Leonelli and Mason 2010; Veillet et al. 2010; Sun et al. 2011; Glisic et al. 2011; Xia et al. 2011) (Fig. 8.3).

Ultrasound has been used to extract a variety of plant-based compounds including anti-oxidants from sage and rosemary, vanillin from vanilla pods, all-trans-β-carotenes from citrus peels, proteins from soybeans and medicaments from other traditional Chinese medicines (Albu et al. 2004, Jadhav et al. 2009; Glisic et al. 2011; Sun et al. 2011; Wang et al. 2011; Xia et al. 2011). Extraction yields have been shown to be heightened, as the effects of acoustic streaming and

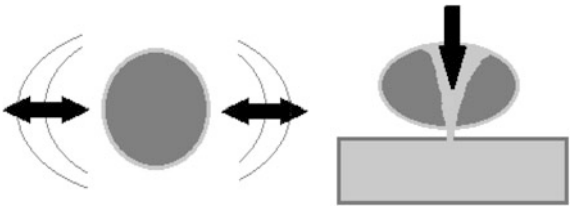

Fig. 8.3 Diagrams displaying the acoustic streaming and micro-jets which occur with cavitation bubbles created by ultrasound

micro-jets enhance the accessibility of some targets therefore making the products much easier to obtain (Leonelli and Mason 2010).

Approximately 15 mg/g of carnosic acid is extracted from rosemary in only 15 min which is the same amount extracted in 3 h by the conventional techniques (Albu et al. 2004). A seven-hour reduction in extraction time can be seen when ultrasound is used to extract 140 ppm of vanillin at room temperature instead of the 95 °C used in conventional extraction, and in 60 min, ultrasound extracts 82.6 % of phillyrin from *Forsythia suspensa* which is much greater than the yields extracted through the traditional methods (20.34–57.89 %) (Jadhav et al. 2009; Xia et al. 2011).

It has been observed that the efficacy of UAE is best seen when extraction is conducted at low temperatures. The processes are therefore said to be more environmentally friendly as energy costs are diminished due to the ability to operate at ambient temperatures and with quicker extraction times. Consequently, the release of carbon dioxide and other pollutants is condensed (Asfaw et al. 2005; Schilmiller et al. 2008; Veillet et al. 2010; Sun et al. 2011; Xia et al. 2011; Briars and Paniwnyk 2013). One benefit for the use of ultrasound for artemisinin extraction is the potential for extracts to be "cleaner" with extracts being more concentrated with artemisinin and therefore the extraction having greater specificity. When UAE was applied to a member of the same family as *Artemisia annua*, *A. afra*, the concentration of high molecular weight sesquiterpenes, like artemisinin, is high but the co-extracted oils are reduced (Asfaw et al. 2005; Schilmiller et al. 2008; Briars and Paniwnyk 2013).

Despite the benefits, some chemists have been reluctant to embrace UAE as a standard protocol due to two reasons; a suspicion of the efficacy of sound energy and, more significantly, a lack of tools for larger-scale application (Mason 1999; Wang and Deng 2006; Sutkar et al. 2010). Another potential disadvantage of UAE is the production of free radicals, particularly at higher frequencies, such as 850 kHz which is used in the breakdown of azo dyes, which could cause destruction of extracted compounds through the inducement of oxidative and radical chemical reactions (Dahlem et al. 1999; Gogate and Pandit 2004; Kumar et al. 2007; Mason et al. 2011; Sun et al. 2011).

8.8 Conclusions

The four techniques that have been explored in this chapter are all feasible methods for artemisinin extraction. They all require a high level of monetary investment to enable large-scale use of the technologies; but the possibilities of higher and more specific yields, with reduced temperatures or solvent consumption, could overcome any reservations in this regard. With further research and refinement, these techniques could become cost-effective standard protocols for artemisinin extraction from *Artemisia annua*.

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Chapter 9
Extraction, Purification, and Quantification of Artemisinin and its Analogs from *Artemisia annua* L.

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Abstract Malaria is one of the most important parasitic diseases, affecting at least 300 million people a year globally, and resulting in more than 1 million deaths. Artemisinin, an endoperoxide-containing sesquiterpene lactone isolated from the aerial parts of *Artemisia annua* L., which is an herb of the Asteraceae family that has been used for centuries for the treatment of fever and chills in China, is currently the best therapy against malaria. In addition, artemisinin proved effective against hepatitis B, schistosomiasis, several blood parasitic protozoans, and against a variety of cancer cell lines including breast cancer, human leukemia, colon, small-cell lung carcinomas, and drug-resistant cancers. Artemisinin demand has increased sharply since the World Health Organization recommended its use as part of the artemisinin combination therapies. Since *A. annua* is currently the only practical source of artemisinin, the area for the crop cultivation has expanded in Africa and Asia and the number of manufacture focusing on artemisinin production is increasing. Therefore, extraction of artemisinin from *A. annua* and quality control on plant crop and final products have become more and more important. Various novel methods have been developed for production and quantification of artemisinin in *A. annua*. This chapter reviewed the most wildly used extraction and determination methods for artemisinin and mainly introduced the artemisinin extraction under room temperature, the isolation of artemisinin, and its analogs by preparative high-performance liquid chromatography, and the quantification of density of glandular trichomes (GTs) on surface of *A. annua* leaves by light microscope, analysis of artemisinin in fresh and dried material by gas chromatography coupled with electron capture detector (ECD).

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9.1 Introduction

Artemisia annua L., known as Huanghuahao in China, is an annual herb native of Asia, which has been used for many centuries in traditional Chinese medicine for the treatment of fever and malaria. Malaria is one of the world's most important parasitic diseases which cause approximately 300–500 million people worldwide affected and more than 1 million deaths every year (Newman 2012). Artemisinin, an endoperoxide-containing sesquiterpene lactone isolated from the aerial parts of *A. annua*, have proven efficacy in killing *Plasmodium falciparum* parasites in severe as well as uncomplicated malaria (Dhingra et al. 1999). Owing to the increasing resistance to traditional antimalarial drugs like chloroquine and sulfadoxine-pyrimethamine, the demand for artemisinin is increasing enormously (Krishna et al. 2008). Moreover, recent study revealed that artemisinin and its analogs, including dihydroartemisinic acid, and artemisinic acid, proved effective against hepatitis B, schistosomiasis, several blood parasitic protozoans, and against a variety of cancer cell lines including breast cancer, human leukemia, colon, small-cell lung carcinomas, and drug-resistant cancers (Chaturvedi et al. 2010). Consequently, the worldwide demand of artemisinin and its precursors is continuously increasing rapidly resulting in more and more attention to the production of artemisinin and its precursors (Krishna et al. 2008). The complete organic synthesis of artemisinin has been established, but it is not yet economically feasible because of its complexity and low yield (Ferreira and Luthria 2010). Recently, synthetic biology has been used to try to develop a heterogenous production of artemisinin. Martin et al. were the first to report the generation of an artemisinin precursor in a microbial system (Martin et al. 2003). They engineered Escherichia coli with a synthetic mevalonate pathway from *Saccharomyces cerevisiae* and expressed amorphadiene synthase (ADS) from *A. annua* in this strain, resulting in production of amorpha-4,11-diene, the sesquiterpene olefin precursor to artemisinin. Ro et al. (2006) first reported the engineering of *S. cerevisiae* to produce artemisinic acid using an engineered mevalonate pathway, ADS, and a novel cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* that transformed amorpha-4,11-diene to artemisinic acid through three steps of oxidation. Afterward, extensive efforts have been invested aimed to enhance the amount of artemisinic acid or dihydroartemisic acid in engineered yeast. And until more recently, Paddon et al. (2013) demonstrated an efficient biosynthetic route to artemisinic acid, with fermentation titers of 25 g/L of artemisinic acid by introduced a plant dehydrogenase and a second cytochrome into engineered *S. cerevisiae*. Attempts to produce the artemisinin precursors, artemisinic and dihydroartemisinic acids in heterologous plants have been extensively performed recently, but did not lead to their accumulation due to internal glycosylation and insufficient oxidation toward the acids. Farhi et al. (2011) reported the metabolic engineering of tobacco to produce artemisinin, by generating transgenic plants that express five plant—and yeast-derived genes involved in the mevalonate and artemisinin pathways, all expressed from a single vector. Their experiments demonstrated that artemisinin

can be fully biosynthesized in a heterologous plant system, such as tobacco. However, the artemisinin levels generated in transgenic tobacco are currently lower than those in *A. annua*. Therefore, *A. annua* is currently the only practical source of artemisinin. How to efficiently produce and quantify the artemisinin and its analogs in *A. annua* is very important.

9.2 Extraction of Artemisinin and its Analogs

Artemisinin and its precursor artemisinic acid have been predominately produced in the glandular trichomes (GTs) on the aerial part of the *A. annua* plant. The extraction method is very important not only for production of artemisinin, but also for the quality control of products. Since artemisinin is of poor solubility in water and good solubility in organic solvents, it is usually extracted with non-polar solvents and then the essential oils, chlorophylls, and waxes were simultaneously extracted. Several methods, such as reflux extraction, microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), were used to extract artemisinin from plant.

9.2.1 Reflux Extraction

Reflux extraction is one of the most widely used technologies to extract active compounds from plant, which allows the biomass to be heated constantly without loss of material through a reflux vessel attached to a water-cooled condenser which prevents vapors from escaping during the heating process. Reflux extraction has been widely applied on artemisinin production. Liquid solvent, such as toluene, n-hexane, chloroform or petroleum ether, is the most currently applied for artemisinin extraction. No significant differences were found among the different solvents, such as *n*-hexane, toluene, and petroleum ether. A small amount of co-solvent ethyl acetate (EtOAc) can significantly improve the efficiency of extraction due to the increase in artemisinin solubility in the solvent mixture by about two orders of magnitude (Lapkin et al. 2006). Ethanol is potentially an attractive solvent due to its wide spread availability from renewable feedstocks, and it is used to extract artemisinin from *A. annua* under a reflux extraction way (Lapkin et al. 2006). However, ethanol technology has an even lower extraction efficiency, compared to non-polar hydrocarbon solvent.

9.2.2 Supercritical Fluid Extraction

A supercritical fluid is any substance at a temperature and pressure above its critical point, where distinct liquid and gas phases do not exist, which can effuse through solids like a gas and dissolve materials like a liquid. Small changes in

pressure or temperature for supercritical fluid, result in large changes in density, which allows the properties of supercritical fluid can be finely tuned. Carbon dioxide is the most commonly used supercritical fluids. Supercritical fluids can be used to extract active compounds from plants. The main advantages of using supercritical fluids for extractions are that they are contaminant free, and easy to dispose. A supercritical fluid extractor composes of a tank of the mobile phase, a pump to pressurize the gas, an oven containing the extraction vessel, a restrictor to maintain a high pressure in the extraction line, and a trapping vessel. Extract is trapped by decompressing the solute contained supercritical fluid into an empty vial. SFE are done in static, dynamic, or combination modes. For static mode, the supercritical fluid circulates in a loop containing the extraction vessel for some period of time before being released through the restrictor to the trapping vessel. For a dynamic extraction, the supercritical fluid continuously flows through the sample in the extraction vessel and out the restrictor to the trapping vessel. In the combination mode, a static extraction is performed for some period of time, followed by a dynamic extraction.

SFE has been applied in food and flavor industry, such as decaffeinication of tea and coffee and extraction of essential oils and aroma materials from spices, since the residual solvent could be easily removed from the product. Nowadays, SFE has wildly used to isolate active ingredients from plants in order to avoid thermo- or chemical degradation and eliminate residual solvents in the products.

Extraction of artemisinin by SFE in a analytical scale using the method of Kohler et al. (1997). With optimized operating conditions, artemisinin and artemisinic acid were quantitatively extracted at a flow rate of 2 ml/min within 20 min. The supercritical fluid used in their SFE was composed of carbon dioxide and 3 % methanol with temperature and pressure set at 50 °C and 15 MPa, respectively. The measurement and correlation of the solubility of the antimalarial artemisinin in supercritical carbon dioxide (SC-CO_2) was investigated by Coimbra et al. and good correlation between the calculated and the experimental solubility to all fitted models were obtained, suggesting that the feasibility of artemisinin extraction using supercritical fluid technologies in a large scale (Coimbra et al. 2006). Quispe-Condori et al. compared SFE, hydrodistillation, and hexane soxhlet on artemisinin extraction, and the maximum artemisinin global yields were obtained by using SFE at 50 °C and 300 bar. The extract obtained by hydrodistillation presented no artemisinin, and the main compounds presented were camphor, β-caryophyllene, borneol, and 1,8-cineol. The artemisinin yield extracted by hexane soxhlet was 0.77 % in dry basis (Quispe-Condori et al. 2005). Lin et al. reported that carbon dioxide added with 16.25 wt % of *n*-hexane, extracted at 18.72 Mpa and 33 °C, and for a period of 1.5 h, could obtain an optimal value of quick recovery and high purity of artemisinin (Lin et al. 2006). Tzeng et al. (2007) obtained scopoletin and artemisinin from *A. annua* by the SC-CO_2 extractions with adding 16.25 % ethyl alcohol as a co-solvent. And they reported that 2-h ethanol-modified SC-CO_2 extractions were superior to 16-h soxhlet n-hexane extractions in producing more pure artemisinin and scopoletin and the amount of the extracts increased with the density of SC-CO_2 (Tzeng et al. 2007).

9.2.3 Microwave-Assisted Extraction

MAE is a new extraction technology that combines microwave and traditional solvent extraction. Application of microwave energy, which causes molecular turbulence by ion migration and dipole rotation, for heating the solvents and plant tissues in extraction process, increases the kinetic of extraction. Unlike traditional conductive heating systems, microwaves directly heat the whole sample but not the vessel, which is more efficient and thus homogeneity and reproducibility greatly improve. Therefore, MAE has a number of advantages, compared to classical reflux extraction technology. The boiling point of the solution is reached very quickly by using microwave energy, which leads to shorter extraction time, less solvent, higher extraction rate, and lower cost. The first application of MAE started in the late 1970s, and it has now been developed one of the most cost-effective extraction technologies.

The microwave extractors are consisted of a microwave generator, a waveguide, an extraction vessel, and a circulator. The hazards produced by heating highly flammable solvents in microwave system are overcome through the use of recent security techniques, such as high capacity exhaust fans, solvent vapor detectors, or pressure-burst safety membranes placed on each vessel. Normally, two types of microwave systems are applied in MAE, which are a closed system and an open system. A closed system is composed mainly of extraction vessels, a turntable, a closed chamber, reflectors, and microwave source, which is performed under controlled pressure and temperature. On the other hand, an open system comprises primarily a focused microwave source and extraction vessel with solvent, which is performed at atmospheric pressure and an additional cooling system is required. There is no significant loss of volatile substances in a closed system and less volume of solvent is required. Moreover, multiple samples can be easily extracted simultaneously in a closed system. Among the disadvantages of the closed microwave extraction system, the risk associated with the use of high pressure is the major one. The open systems are much safer than the closed systems as they can be operated at atmospheric pressure and the reagents can be added at any time during the treatment. However, the open systems cannot process multiple samples simultaneously. Closed systems are generally recommended for extractions under drastic conditions, since the solvents may be heated up to about 100 °C above their atmospheric boiling point, in which both extraction speed and efficiency are enhanced.

Different extraction methods for artemisinin extraction from *A. annua* were compared by Hao et al. (2002). The time used in MAE is only 12 min with 92.1 % extraction rate, while soxhlet method and normal stirring extraction need several hours with only about 60 % extraction rate. SC-CO_2 extraction gives the lightest color of extractive but lowest extraction rate (Hao et al. 2002). A simple, rapid, and precise MAE process was developed by Liu and his co-workers (Liu et al. 2007) for fast sample preparation for the quantitative determination of artemisinin from dried leaves of *A. annua*. Several solvents were tested in MAE by Hao et al.

(2002) such as ethanol, trichloromethane, cyclohexane, n-hexane, petroleum ether (30–60 °C), petroleum ether (60–90 °C), No. 120 solvent oil, and No. 6 extraction solvent oil. The results show that No. 6 extraction solvent oil is the best solvent in the artemisinin extraction using MAE (Hao et al. 2002). Optimal conditions of MAE of artemisinin were obtained by Hao and his collaborates (Hao et al. 2002), which indicated that the duration of microwave radiation, the diameter of raw materials and the solvent to material ratio were 12 min, less than 0.125 mm, and more than 11.3, respectively. Recently, Misra et al. (2013) designed a simple experiment for the optimization of the appropriate solvent under same extraction conditions. And the most favorable conditions were obtained by using plant material of 25 mesh (particle size) extracted with acetone for 120 s at 160 W (Misra et al. 2013).

9.2.4 Room Temperature Extraction

Duke et al. described that 97 % of the artemisinin and 100 % of artemisitene were obtained by a 5-s dip of fresh *A. annua* leaves in chloroform at room temperature (Duke et al. 1994). Light microscopy and transmission electron microscopy revealed that the 5-s dip resulted in collapse of the subcuticular cavity of the glands on the leaf surface but did not disrupt cell membranes (Duke et al. 1994). Woerdenbag found extraction of artemisinin from 0.2-mg fresh leaf homogenates in 2 mL tobulene for 2 min was exhaustive (Woerdenbag et al. 1991). Our previous results indicated that artemisinin and its bioprecursors can be rapidly extracted from the fresh leaves of *A. annua* by suitable organic solvent at room temperature in an analytical scale (Liu et al. 2009b). Recently, we established a large-scale extraction of artemisinin and its analogs from fresh *A. annua* leaves by using EtOAc, chloroform or dichloromethane with a low cost (as shown in Fig. 9.1). The whole extraction process could be finished within 30 min under room temperature and ambient pressure. The obtained extract was concentrated to 1/10 of its initial volume and decolorized by using activated carbon adsorbent. Then, the crude artemisinin product was obtained, and it was purified by crystallization and re-crystallization with hexane or petroleum ether to yield highly pure artemisinin product (as shown in Fig. 9.2).

We figured out the possible reasons why the high-extraction effectiveness was obtained by using fresh materials instead of dried material. Firstly, artemisinin and its precursors is presented in foliar tissue are localized entirely in the subcuticular space of GTs (Duke et al. 1994). Secondly, the trichomes in fresh leaves bear highest areas due to their protuberant epidermal structures on the leaves of *A. annua*, so that there is a sufficient contact between trichomes and extract solvent. Thirdly, the artemisinin and its analogs are in liquid status in the fresh trichomes, therefore they are easy to be dissolved into extract solvent. On the contrast, when the trichomes are dried, their surface area is decreased, and the ingredients in the trichomes are in solid status, which add the declined the extraction efficiency.

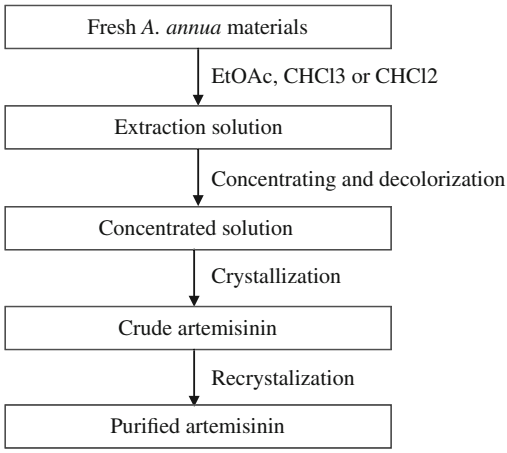

Fig. 9.1 The production flowchart of artemisinin extraction by using fresh materials

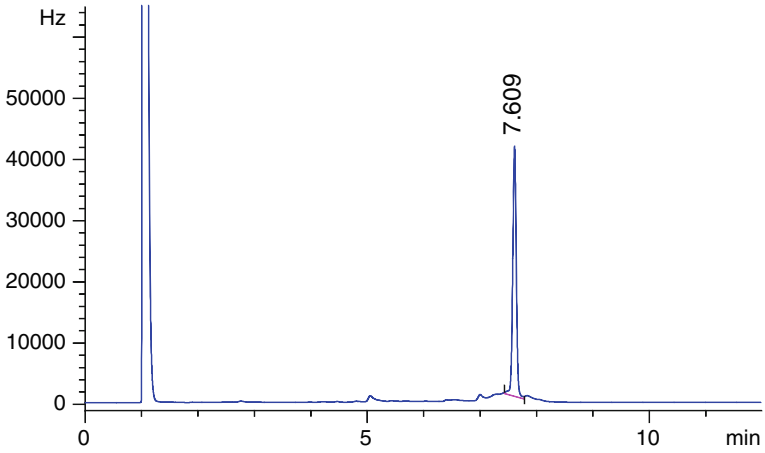

Fig. 9.2 GC-ECD quantification of products showed that the content of artemisinin in the products was more than 98 %. GC-ECD analysis was performed according to Liu et al. (2009b)

9.3 Purification of Artemisinin and its Analogs

Various methods for purification of artemisinin from raw extracts have been developed (Liu et al. 2009b). Klayman et al. (1984) established a silica gel column chromatography (CC) system with a elution system composed of a chloroform–EtOAc mixture for purification artemisinin from *A. annua* air-dried leaves extract by petroleum ether. Elsohly and his colleagues (ElSohly et al. 1990) changed Klayman's extraction process by using hexane as extraction solvent and partitioning the hexane solution with acetonitrile, resulting in a significant reduction of the volume of extract solution and cleanly separation of artemisinic acid.

Complete separation of the compounds from the crude extracts was achieved by CC in both of above methods, using a sufficiently long column, or by selecting appropriate stationary and mobile phases. In practice, many of the extraction plants located in the traditional cultivation and processing areas of *A. annua*, such as China and Vietnam, have excluded the chromatographic purification step and solely use crystallization for separation of artemisinin. In order to produce a high-purity product, artemisinin has to be re-crystallized several times, which leads to a significant loss of the product. Qu et al. (2010) proposed chromatography–crystallization hybrid process for artemisinin purification from *A. annua* by using a flash CC and a two-step anti-solvent crystallization process. This method combined the advantages and overcame the disadvantages of CC and crystallization to improve efficiency of the isolation of artemisinin from *A. annua*.

We described a simultaneous production of artemisinin, dihydroartemisinic acid and artemisinic acid from *A. annua* by preparative RP-HPLC. High-performance liquid chromatography (HPLC) is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying, or purifying the individual components of the mixture, which relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with a sorbent, leading to the separation of the sample components. HPLC is distinguished from traditional CC because operational pressures are significantly higher (50–350 bar), while ordinary CC typically relies on the force of gravity to pass the mobile phase through the column. Also HPLC columns are filled with smaller sorbent particles, which gives HPLC superior resolving power when separating mixtures, which is why it is a popular chromatographic technique. HPLC is widely used for quality analysis and sample preparation. Preparative HPLC, with high level of automation, has been widely used for preparation of pure samples, and especially for the isolation of active compounds from natural plants. Preparative HPLC systems (including semi preparative and narrow-bore) accomplish high flow rates of up to 100 ml/min and at pressures of up to 50 bar, with high volumetric linearity. Moreover, a preparative HPLC system typically employs a UV, RID, and/or IR detector for highly accurate separation analysis.

Ethanol, methanol, acetone, acetonitrile, *N*,*N*-dimethyl formamide, and tetrahydrofuran were tested in order to select a suitable solvent to dissolve crude extract of *A. annua*, and the result showed that the sample was easily dissolved in acetone and acetonitrile. However, the separation was not ideal when the acetone solution was injected into HPLC. Therefore, the acetonitrile was selected as the solvent used to resuspend *A. annua* crude extract.

Sample concentration is also a very important factor that affects the separation efficiency of preparative HPLC. The HPLC separation will be better if lower concentration of sample was applied. While as it will increase the cost of separation. On the contrary, more concentrated sample leads to reduced cost and separation efficiency. Various concentrations of sample were tested to obtain an ideal concentration for sample preparation, and the result indicated that the separation was acceptable when the sample concentration was set as about 4 g/mL.

The chromatographic separation HPLC was performed on Shimadzu (Kyoto, Japan) chromatographic system, consisting of LC-8A pump and SPD-20A photodiode array detector, by using of a Shimadzu PRC-ODS column (250 × 20 mm, 5 μm). The preparative HPLC conditions were set as follows: mobile phase composed by acetonitrile and water (60:40), flow rate at 15 mL/min, detection wavelength at 192 nm, and loading amount of 400 μL. Fractions were collected according to the on-line chromatogram. Fraction 1 was crystallized directly. Fractions 2 and 3 were concentrated under vacuum till the organic solvent was completely removed and filtrated and then were crystallized by using EtOAc (as shown in Fig. 9.3). The purity was assessed by GC and identified by MS. The results indicated that the preparative HPLC could provide effective and automatical purification, high recovery, and sustainable utilization of medicinal plants in a fast and cost-effective manner.

9.4 Quantification of Artemisinin and its Analogs

Various new analytical methods for the quantification of artemisinin and its analogs have been established during the last decade (Christen and Veuthey 2001), including thin-layer chromatography (TLC) (Bhandari et al. 2005), HPLC with UV detection via pre (Zhao and Zeng 1985) or post-column (ElSohly et al. 1987) derivation, HPLC with electrochemical detection (HPLC-EC) (Acton et al. 1985), HPLC with diode array detection (HPLC-DAD) (Bilia et al. 2006), HPLC with evaporative light-scattering detection (ELSD) (Peng et al. 2006), HPLC with mass spectrometric detection (HPLC-MS) or MS/MS (Van Nieuwerburgh et al. 2006), gas chromatography with flame ionization detection (GC-FID) directly or after

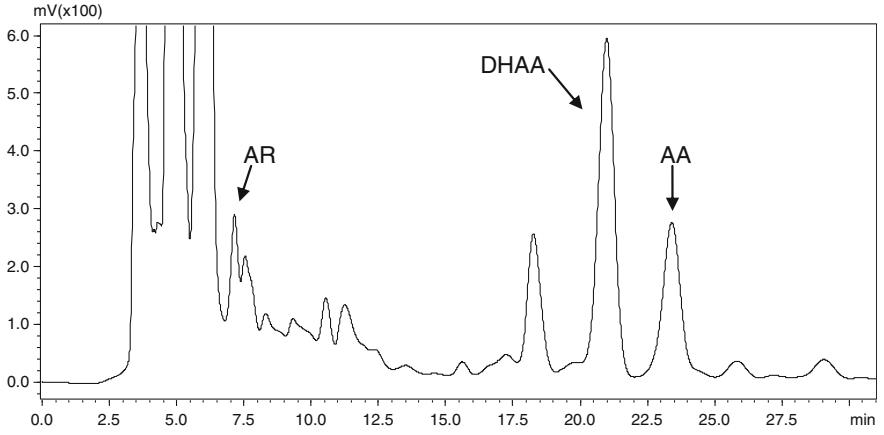

Fig. 9.3 Preparative HPLC chromatography of *A. annua* extract. *AR* artemisinin, *DHAA* dihydroartemisic acid, *AA* artemisinic acid (Tian et al. 2012)

derivation (Peng et al. 2006), GC with MS (GC-MS) (Woerdenbag et al. 1991), and supercritical fluid chromatography with FID (SFC-FID) (Kohler et al. 1997). TLC is not a reliable technique to quantify artemisinin due to the poor staining characteristics of artemisinin and interference of other constituents in the plant matrix. Quantitative analysis of artemisinin by HPLC with different detectors is either time-consuming or expensive or low sensitive. Mass spectrometry has high sensitivity compared with other detectors, and the benefit of molecule confirmation through its major ions, but requires great investment and expertise. Detection by SFC requires sophisticated equipment which cannot be applied in routine analysis.

9.4.1 Determination of Glandular Trichome Density

GTs, in general, have been shown to accumulate large quantities of potentially toxic, secondary metabolites. Trichomes accumulate and store these compounds, in specific cellular locations, outside metabolically active compartments of the plant; the diverse nature of their chemistry fuels a multifunctional understanding of plant defense options. It is clear that these glands contain cells with metabolically functional chloroplasts containing starch, which in some specific cases are shown to be involved in terpenoid production. This is supported by more recent evidence of terpenoid biosynthetic in situ gland enzyme activity, which has developed our understanding of metabolite accumulation in relation to gene transcript levels of various terpene synthases. Secreting GTs are a major site for biosynthesis and accumulation of a wide range of plant natural products. These plant natural products often function to protect the plants against insect predation and contribute to the flavor and aroma of plants. Many of the natural products also have pharmacological effects, such as the analgesic drug morphine, the anticancer compound taxol, and the antimalarial drug artemisinin. Artemisinin, a sesquiterpene lactone, is currently recognized as one of the most prominent anti-malarial treatment.

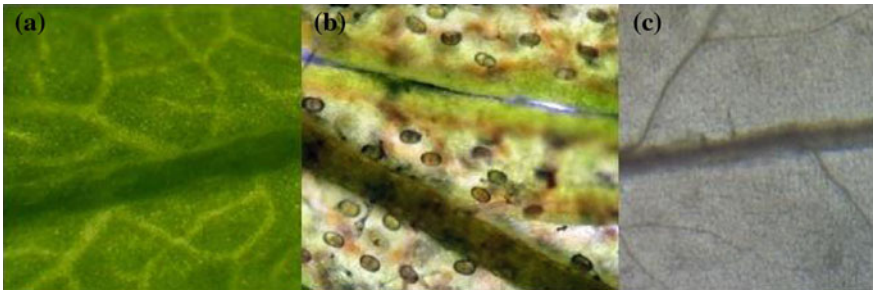

Fig. 9.4 The light microscope pictures of glandular (**d**, **g** and **h**) and no-glandular (**c**, **e** and **f**) trichomes on upper surface of *A. annua* leaves by using a 10× eyepiece coulpled with a 4× (**a**), 10× (**b**), 40× (**c** and **d**) and 100× (**e**–**h**) objective lens, respectively. *Bar* 200 μm

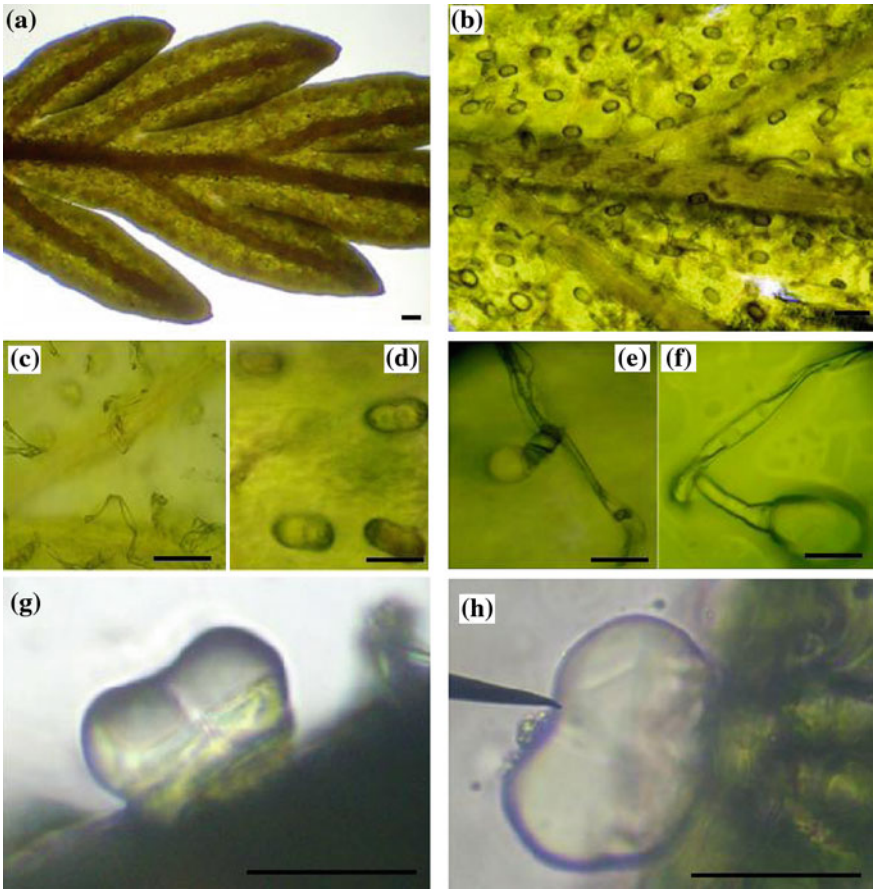

Fig. 9.5 Micrograph of *A. annua* glandular trichomes present on a leaf at 5 × 10 min and 4 h after treated by ideal reagent respectively

Ferreira and Janick (1995) reported the observation of trichomes on *A. annua* leaves by using scanning electron microscopy. Graham et al. (2010) quantified the GTs on surface of *A. annua* leaves by using an fluorescence microscope. A light microscope method for quantifying trichome density on *A. annua* leaves was described by Lommen et al. (2006). Trichome density was quantified on the abaxial surface of the terminal leaflets of leaves. The abaxial side of these pieces was glued by self-adhesive tape to paper. Thereafter, pieces were incubated for 2 days in the dark at 6, before counting using a binocular microscope at 310 magnifications. The gluing and incubation facilitated trichome counting by leveling the leaf surface and slightly reducing the cell turgor. We have developed a simple method for observation of *A. annua* trichomes and determination of trichome density by using a light microscope (Liu et al. 2009b). Prior to

observation on light microscope (Fig. 9.4), the *A. annua* leaves were incubated in 0.5–1 % acetic acid in petroleum ether for 5–20 min, depending on the age of leaves. We compared the micrograph of trichomes under light microscope treated with different times, and the results showed the trichomes can be observed very clearly in light microscope after treated by 0.5–1 % acetic acid in petroleum ether for 10 min, while they cannot be observed when the incubation time was less than 5 min or more than 4 h (Fig. 9.5).

9.4.2 GC-ECD Quantification of Artemisinin

The electron capture detector (ECD) uses a radioactive beta particle emitter, which typically consists of a metal foil holding 10 millicuries (370 MBq) of the radionuclide 63Ni, in conjunction with a makeup gas flowing through the detector chamber. Usually, nitrogen is used as makeup gas, because it exhibits a low excitation energy, so it is easy to remove an electron from a nitrogen molecule. The electrons emitted from the electron emitter collide with the molecules of the makeup gas, resulting in many more free electrons. The electrons are accelerated toward a positively charged anode, generating a current. Therefore, there is always a background signal present in the chromatogram. As the sample is carried into the detector by the carrier gas, electron-absorbing analyte molecules capture electrons and thereby reduce the current between the collector anode and a cathode. The analyte concentration is thus proportional to the degree of electron capture.

The ECD is a highly sensitive and selective detector capable of detecting picogram amounts of specific types of compounds, particularly sensitive to organic molecules that contain electronegative functional groups, such as halogens, organometallic compounds, nitriles, or nitro compounds. Depending on the analyte, an ECD can be 10–1,000 times more sensitive than a flame ionization detector (FID), and 1 million times more sensitive than a thermal conductivity detector (TCD). An ECD has a limited dynamic range and finds its greatest application in analysis of halogenated compounds. The detection limit for ECDs is 5 fg/s, and the detector commonly exhibits a 10,000-fold linear range. This made it possible to detect halogenated compounds such as pesticides and CFCs, even at levels of only 1 ppt, thus revolutionizing our understanding of the atmosphere and pollutants. In addition, this detector is a commonly used and a cheap one.

Since there is a peroxide group in artemisinin molecule (as shown in Fig. 9.6), we propose the hypothesis that ECD could be applied in quantification of artemisinin. And then, we developed a GC-CED quantification of artemisinin in fresh and dried *A. annua* (Liu et al. 2008, 2009b).

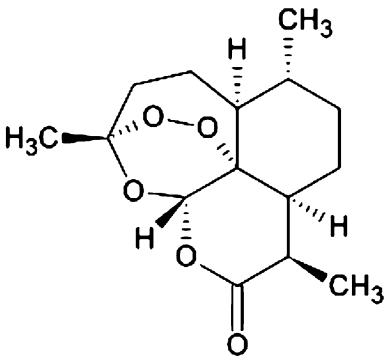

Fig. 9.6 Molecular structure of artemisnin

9.4.2.1 Quantification of Artemisinin in Dried Material

Sample Preparation

Because n-hexane is effective in extracting artemisinin, arteannuin B, and artemisinic acid, it was selected as the extraction solvent for artemisinin. Artemisinin was directly analyzed by GC-ECD from the extraction solution without any additional separation or evaporating steps. Artemisinin extraction was performed by immersing 0.1 g of sieved dry leaves to 10 mL of n-hexane, resulting in a plant material to solvent ratio of 1:100 to prevent solvent saturation. The extraction was performed at 69 °C for 1 h. The extract solution was cooled to room temperature, then was filtered through 0.2–μm-size nylon Millex-GN filters (Millipore, Bedford, MA, USA), pre-wetted with n-hexane, and attached to disposable 3-mL syringes. Filtered aliquots from the samples were transferred to GC flasks and were analyzed in the same day.

GC Conditions

Analysis of artemisinin was performed by GC using an Agilent (Santa Clara, CA, USA) GC-6890N system with a micro-ECD system, a FID system, an autosampler, and an Agilent data collection system (Rev. A. 09.01). Nitrogen was the carrier gas with a column flow rate of 2 mL/min, a split ratio of 3:1. The column was a HP-5 crossbond 95 % dimethyl polysiloxane (Agilent), (30 m × 0.32 mm ID, 0.25 μm film thickness). Injector temperature was set at 240 °C, and detector temperature set at 300 °C. Oven temperature was programmed to start at 180 °C (1 min), increasing 0.8 °C/min to 198 °C, then increasing 30 °C/min to 280 °C, then holding at that temperature for 10 min. The injection volume was set at 1.0 μL. Possible problems, including malfunctioning autosampler or polluted liner, were monitored before and throughout the analysis by running an artemisinin standard of known concentration (normally 0.1 mg/ml) twice in the beginning, once every 10 samples, and at the end to check whether changes in detector sensitivity and response would occur.

9.4.2.2 Quantification of Artemisinin in Fresh Material

Sample Preparation

One piece of completely unfold fresh leaf of *A. annua* was picked and accurately weighed (wet weight) and extracted with 1 mL EtOAc in 2-mL centrifuge tube by vortex for 2 min at room temperature. Then, the sample was centrifuged at 10,000 × *g* for 2 min and the supernatant was transferred into a fresh 2-mL centrifuge tube and was evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted in 1 mL acetonitrile and then was filtered through 0.2-μm-size nylon Millex-GN filters (Millipore, Bedford, MA, USA), pre-wetted with acetonitrile, and attached to disposable 3-mL syringes. Filtered aliquots from the samples were transferred to GC flasks and 1 μL was analyzed in the same day.

GC Condition

Analysis of artemisinin was performed by GC using a 6890 N GC system (Agilent, Santa Clara, CA, USA) coupled with a micro-ECD, a FID, an autosampler, and an Agilent data collection system (Rev. A. 09.01). Nitrogen was used as the carrier gas with a column flow rate of 3 mL/min, a split ratio of 1:1. The column was a HP-5 crossbond 95 % dimethyl polysiloxane (Agilent), (30 m × 0.32 mm ID, 0.25 μm film thickness). Injector temperature was set at 235 °C, and detector temperature set at 285 °C. Oven temperature was programmed to start at 180 °C, increasing 6 °C/min to 220 °C, holding at that temperature for 3 min, then increasing 30 °C/min to 280 °C, holding for 10 min. The injection volume was set at 1.0 μL. Without suitable interstandard available, possible problems (including malfunctioning autosampler or polluted liner) were monitored before and throughout the analysis by running an artemisinin standard of known concentration (normally 0.1 mg/mL) twice in the beginning, once every 10 samples, and at the end to check whether changes in detector sensitivity and response would occur.

9.5 Conclusions

Various new methods for the production and determination of artemisinin and derivatives have developed during the last decade. This chapter reviewed the most wildly used extraction and determination methods for artemisinin and mainly introduced the artemisinin extraction under room temperature, the isolation of artemisinin and its analogs by preparative high-performance liquid chromatography, and the quantification of density of GTs on surface of *A. annua* leaves by light microscope, analysis of artemisinin in fresh and dried material by gas chromatography coupled with ECD.

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Chapter 10
Effect of Mineral Nutrition, Growth Regulators and Environmental Stresses on Biomass Production and Artemisinin Concentration of *Artemisia annua* L.

Tariq Aftab, M. Masroor A. Khan and J. F. S. Ferreira

Abstract Malaria is a mosquito-borne disease caused by different species of *Plasmodium*. It is the world's most severe parasitic infection and kills almost two million people a year, afflicting more than one-third of the global population. The burden of malaria has increased by the worldwide spread of multi-drug-resistant *Plasmodium falciparum*. *Artemisia annua* L. has been used for centuries in Chinese traditional medicine for the treatment for fever and malaria and is the only commercial source of artemisinin, a rare sesquiterpene lactone that is the only safe alternative therapy against multi-drug-resistant malaria. Because the chemical synthesis of artemisinin is very costly, the plant remains the only viable source of artemisinin for pharmacological use. Therefore, the enhanced production of artemisinin by the whole plant is highly desirable. Although artemisinin production a (*in planta*) is controlled mostly by genetic factors, the plant reacts to certain abiotic stresses by increasing artemisinin concentration. In the past 15 years, selection has increased artemisinin concentration in the plant from 0.3–0.5 % (g/100 g) to 1.0–1.8 %. However, artemisinin increase is still possible by applying selected stresses to the plant. In the present chapter, we are reviewing the various factors that affect biomass and artemisinin production of *A. annua*.

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10.1 Introduction

The most imperative concern of today is the success in achieving the highest possible level of human health and keeping diseases at bay in the global context. Of the several diseases of global concern, such as SARS, tuberculosis, influenza, HIV/AIDS, malaria is the one that causes the most losses in human lives. Malaria is an infectious disease caused by *Plasmodium falciparum*, a protozoan organism, which is carried by mosquitoes of the genus *Anopheles*. Worldwide, the most severe form of malaria is responsible for the incidence of 300–500 million clinical cases every year. Malaria is estimated to cause between 1.5 and 3 million deaths per year, mainly of African children under the age of 5 (Butler et al. 1997; Rinaldi 2004). In the past, the fight against malaria was based on two strategies:

1. Extermination of disease vector (mosquito) with DDT. This approach has undesirable side effects on the reproduction of birds and results in the persistent presence of pesticides in food chains. Also, although very effective when started, it also leads to changing behaviour of the vector to avoid surfaces treated with DDT.
2. Large-scale use of malaria drugs based on quinine (isolated from bark of *Cinchona*) and its cheap derivative chloroquine for the treatment for malaria patients. Unfortunately, *Plasmodium* spp. developed resistance to these drugs.

In 1960s, *P. falciparum* started showing signs of resistance against quinine-derived drugs. Such a resistance was reported from places as far apart as Brazil, Colombia, Malaysia, Cambodia and Vietnam, making it harder to control the disease. In addition, the mosquito species capable of transmitting the disease are found in many parts of the world (Fig. 10.1).

In 1969, the Chinese army found that the diethyl ether extract of *Artemisia annua* L., called "qinghao" in Chinese, had an excellent effect against malaria; in 1972, artemisinin (the bioactive sesquiterpene extracted from *A. annua* L.) was identified as the most active plant metabolite for malaria drugs (Klayman 1985). Artemisinin, responsible for antimalarial and anticancer activities, contains an endoperoxide bridge, which is rarely found in other secondary metabolites. It is a promising drug as it lacks cross-resistance with other antimalarial drugs and is known to have no adverse effects on humans, while providing fast clearance of blood parasites (24–48 h), compared to other available antimalarial drugs of malaria (Meshnick 2002). Complete chemical (*de novo*) synthesis of artemisinin has, so far, been achieved by several research groups (Ravindranathan et al. 1990; Avery et al. 1992). The procedure requires several steps and can start with different raw materials. However, because of low yield, complexity and high manufacturing cost, the isolation of artemisinin from *A. annua* is the most feasible and economic method for its commercial production. Since 2001, artemisinin-based combination therapies (ACTs) have been recommended by the World Health Organization (WHO 2010). This resulted in the enhanced demand for artemisinin that ultimately led to its supply shortages in the pharmaceutical markets (Cyranoski 2004).

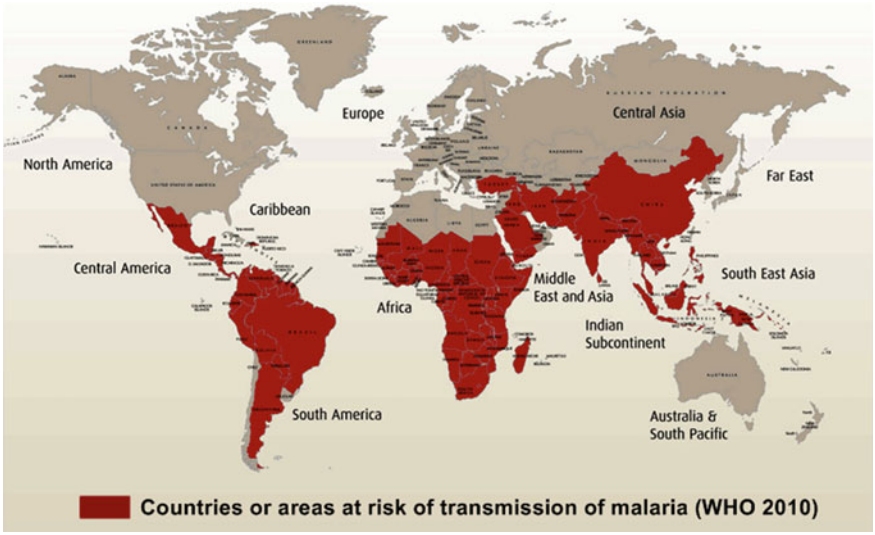

Fig. 10.1 Countries at risk of malaria

Although the situation has recently been reported to be under control (Roll Back Malaria 2004), it is still highly desirable to explore scientific methods to enhance the productivity of *A. annua* L. in conjunction with cutting short the manufacturing cost of artemisinin.

10.1.1 Artemisia annua

A. annua is a qualitative short-day plant (Ferreira et al. 1995a). The mature plant with a single stem can reach about 2 m in height. Aromatic leaves are about 2.5–5.0 cm long, deeply dissected and alternately branched around the stem (Fig. 10.2). At least 40 volatile compounds and several non-volatile compounds have been extracted from *A. annua* and identified. Artemisinin (Fig. 10.3) and other artemisinic compounds are the most important compounds isolated from this plant due to their pharmaceutical value (Ferreira and Janick 1996b). Artemisinin is stored in the glandular trichomes of *A. annua* and the glands of old leaves normally rupture open and release their stored materials, and thus, the artemisinin level in older leaves on whole plants is lower (Duke et al. 1994; Ferreira and Janick 1996b).

The major recommended use of artemisinin is for the production of artemisinin-based combination therapies (ACTs) in the treatment of malaria. ACTs have been shown to have rapid resolution to fever and parasitaemia and low toxicity and are well tolerated. The artemisinin compounds are effective against *P. falciparum* and *P. vivax*, including multi-drug-resistant strains. It has been recommended for use

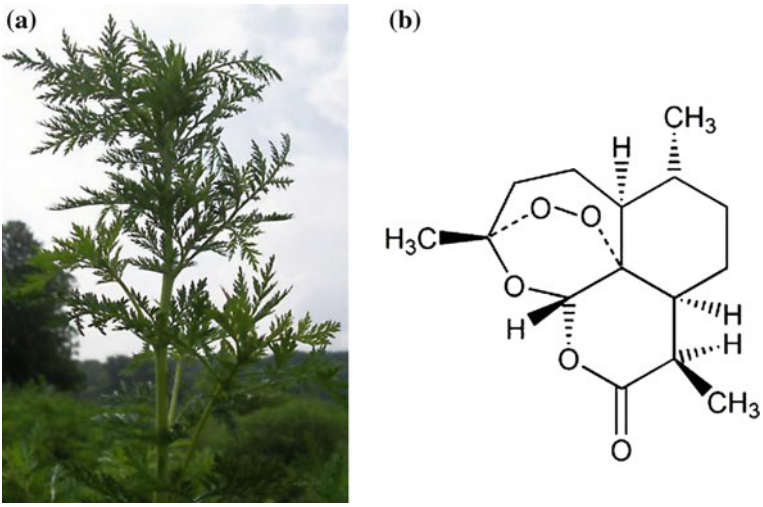

Fig. 10.2 **a** Vegetative top of *Artemisia annua* L. (*source* http://it.wikipedia.org/wiki/File:Artemisia_annua.jpg). **b** Chemical structure of artemisinin

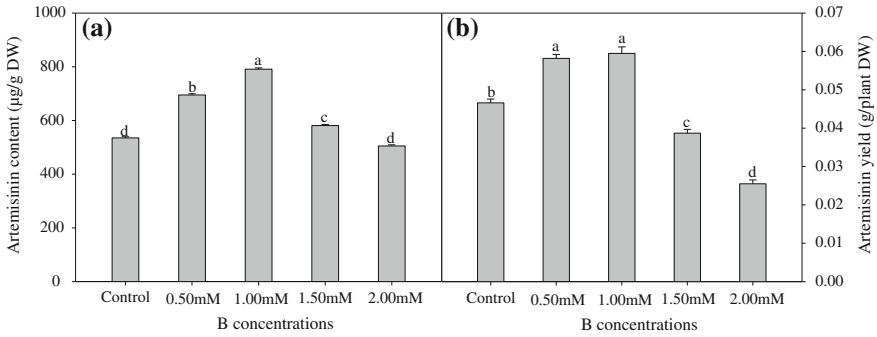

Fig. 10.3 Effect of different concentration of B on artemisinin content (**a**) and artemisinin yield (**b**) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. *Error bars* (⊤) show SE

in herbal tea infusions for treatment of malaria. However, based on current knowledge, the recommendation of artemisinin teas is not acceptable by WHO as a replacement of ACTs.

Artemisia annua has several anecdotal uses that include contraception, relief of joint pains, deworming, haemorrhoids, antiperiodic, antiseptic, digestive and febrifuge; an infusion of the leaves is used internally to treat fevers, colds, diarrhoea, etc. These uses, however, could be explained by current research showing artemisinin and flavonoid effects as anti-inflammatory, immune modulation and anticancer effects in vitro and in dogs (Dr. Tomi Sazaki, pers. comm.). So far,

apart from the antimalarial effect, the only use in humans supported by controlled clinical trials is the effect on schistosomiasis (Utzinger et al. 2002). An essential oil in the leaves is used as flavouring in spirits such as vermouth (Duke et al. 1994).

10.2 Various Factors Affecting Biomass and Artemisinin Yield

Although biomass and artemisinin production in *A. annua* are mostly regulated by genetic factors, it is still unknown if artemisinin follows a Mendelian inheritance or if the genetic control of artemisinin is multi-allelic. The plants referred to as "hybrids" are not true hybrids because the parents are not proved to be homozygous for artemisinin production. It is known, however, that artemisinin is mostly controlled by genetic factors, as indicated by broad-sense (Ferreira et al. 1995b) and narrow-sense (Delabays et al. 2001) heritability studies. However, there are several environmental conditions (e.g. abiotic stresses) that affect plant biomass and artemisinin yield. Currently, we do not know the direct effects of drought, mineral deficiency, growth hormones or salinity (among others) on artemisinin biosynthesis and why abiotic stresses have been more successful in increasing artemisinin than biotic stresses. It is currently accepted that the artemisinin precursor dihydroartemisinic acid (DHAA) may act as a scavenger of reactive oxygen species (ROS) in the plant, then getting transformed into artemisinin. However, artemisinin is only one of 16 molecules that are formed from DHAA (Brown 2010), indicating that scavenging for reactive oxygen species is not the main function of DHAA. However, abiotic stresses have reported to increase artemisinin in the plant, and we provide detailed description of individual stresses that affect this valuable plant, as follows.

10.3 Effect of Mineral Nutrients

Singh (2000) conducted a field experiment to study the effect of levels of nitrogen, phosphorus and potassium on herb, oil and artemisinin yield of *A. annua*. Herbage, essential oil and artemisinin yields increased significantly with application of 50 kg N ha^{-1} compared to control (0 kg N ha^{-1}), but were statistically similar at 100 kg N ha^{-1}. Application of 50 and 100 kg N ha^{-1} increased herb, oil and artemisinin yields by 26.2 and 40.1 % compared with control.

Kapoor et al. (2007) studied the effects of inoculation by two arbuscular mycorrhizal (AM) fungi, *Glomus macrocarpum* and *Glomus fasciculatum*, either alone or supplemented with P-fertilizer, on artemisinin concentration in *A. annua*. Although there was significant increase in concentration of artemisinin in non-mycorrhizal P-fertilized plants as compared to control, the increase was less compared to mycorrhizal plants grown with or without P-fertilization. They suggested that the

increase in artemisinin concentration may not be entirely attributed to enhanced P-nutrition and improved growth. The plants supplied with AM fungi and P-fertilizer produced up to tenfold more shoot biomass than control plant. Also, AM fungi and P-fertilizer combined resulted in significant increases in concentrations of chlorophyll-*a*, chlorophyll-*b* and carotenoids compared to their respective controls. Artemisinin content was also maximum in the plants fertilized with P, and inoculated with AM fungi.

Özgüven et al. (2008) evaluated yield, yield components and artemisinin content of *A. annua* grown under four nitrogen applications (0, 40, 80 and 120 kg ha^{-1}) for two successive years. In their study, nitrogen doses had no significant effect on plant height, number of branches, fresh herbage yield, dry herbage yield, fresh leaf yield, dry leaf yield or essential oil content. However, artemisinin concentration of the dried leaves were significantly increased by nitrogen applications and ranged from 6.3 to 27.5 mg 100 g^{-1} among the treatments. Peyvandi et al. (2009) investigated the effects of different nitrogen and phosphorus on the plant growth parameters, yield and essential oil composition of *A. annua*. They observed that differences between the average height, number of branches and dry weight were significantly increased by the treatments. The maximum number of branches and plant height increased in $N_{80}P_{40}$ treatment. Increasing P-fertilizer more than 40 kg ha^{-1} decreased the growth parameters significantly. They did not observe the changes in artemisinin content due to the treatments. Davies et al. (2009) examined the effect of various concentrations of nitrogen and potassium on artemisinin concentration and yield responses of *A. annua*. The nutrients were supplied in irrigation water to plants in pots, and after a growth period, biomass production and leaf artemisinin concentration were measured. Nitrogen nutrition enhanced plant nitrogen concentration and biomass production successively up to 106 mg N L^{-1} for biomass and 206 mg N L^{-1} for leaf nitrogen; further increases in nitrogen had no influence on biomass. Artemisinin concentration in dried leaf material was maximum at a nitrogen application of 106 mg L^{-1}, but declined at higher concentrations. Increasing potassium application from 51 to 153 mg L^{-1} increased total plant biomass, but not at higher applications. Potassium application enhanced leaf potassium concentration, but there was no effect on leaf artemisinin concentration or leaf artemisinin yield. They suggested that maximization of artemisinin yield (amount per plant) requires optimization of plant biomass via control of nitrogen nutrition.

Aftab et al. (2011a) evaluated varying levels of soil-applied nitrogen with foliar GA_3. Application of GA_3 proved effective in increasing growth, photosynthesis and enzyme activities of *A. annua*. However, N levels combined with GA_3 led to further improvement in shoot lengths and dry weights, and photosynthetic rate. Furthermore, N combined (80 mg kg^{-1} soil) with GA_3 augmented the content and yield in the treated plants over the control (soil had 47.46 mg N kg^{-1}). Jha et al. (2011) assessed the effect of organic manure and chemical fertilizers on the accumulation of artemisinin and biomass in various plant parts through the developmental stages of *A. annua*. Phosphorus and potassium fertilizers (40 kg ha^{-1} each) were applied at the time of transplantation, while nitrogen and sulphur fertilizers at the rate of

80 and 30 kg ha^{-1} were applied in two equal splits, one at the time of transplantation and second at bolting stage, respectively. They found that artemisinin content and artemisinin yield of dried leaves increased significantly (27.3 and 53.6 %, respectively) at pre-flowering stage in the plants treated with NPKS and NPK (18.2 and 33.5 %, respectively) when compared with control. Maximum dry leaf yield ranged from 2,596 to 3,141 kg ha^{-1} at pre-flowering stage with various treatments.

The work of Davies et al. (2011) aimed to determine the response of *A. annua* through dry matter and artemisinin concentration in response to different levels of phosphorus (P) and boron (B). Mineral nutrients were supplied in irrigation water to potted plants, and after a period of growth, dry matter production and leaf artemisinin concentration were determined. Increases in P application enhanced plant growth and total dry matter production up to 30 mg P L^{-1}. Although P applications had no influence on leaf artemisinin concentration, optimal yields of artemisinin per plant were achieved at P rates from 30 to 60 mg L^{-1}, reflecting the increase in biomass caused by P applications. Increasing B application rate had no significant effect on dry matter production. Leaf artemisinin concentration significantly increased by 20 % (0.65–0.78 %) with B increases from 0.1 to 0.6 mg B L^{-1}. This increase in B also increased artemisinin from 0.36 to 0.46 g $plant^{-1}$ (28 %). Increasing B concentration to 0.9 mg L^{-1} had no further effect on artemisinin concentration or yield.

Thus, to our knowledge, little to no increase in artemisinin has been achieved by providing the plant with ideal (or surplus) rates of macro- and micronutrients. Ideally, a primary experiment should be done to establish the ideal mineral requirement for vegetative growth of *A. annua* in a certain soil. Then, the effect of providing additional nutrients could be evaluated against the ideal mineral fertilization previously established. That would establish if the increase in yield was indeed a result of additional fertilization instead of a response caused by feeding a starved plant with proper minerals. Next, an economical evaluation could establish the cost-effectiveness of the investment in extra fertilization versus the additional benefit of the achieved artemisinin yield increase. To date, we could find no such economic studies.

10.4 Effect of Plant Growth Regulators

Shukla et al. (1992) determined artemisinin and herbage yield of *A. annua* plants after application of triacontanol (Tria) and 2-chloroethyl trimethyl ammonium chloride (chlormequat). Tria produced a statistically significant increase on artemisinin concentration as well as on plant height, leaf and herbage yield. Chlormequat also increased artemisinin level, decreased the plant height at higher concentrations and increased the leaf and herbage yield at lower concentrations. They suggested that the effect of Tria on artemisinin yield seems to be mediated through GA- and ABA-like activities on plant growth. Smith et al. (1997) explored the effect of gibberellic acid (GA_3) on the growth and artemisinin production of

hairy roots of *A. annua*. They used six different concentrations of GA_3 to determine the optimum concentration. GA_3 levels of 0.01–0.001 mg L^{-1} (28.9–2.89 μM) provided the most significant increase in biomass and 0.01 mg L^{-1} (28.9 μM) produced the highest amount of artemisinin. They also studied growth kinetics and found that the use of GA_3 at 0.01 mg L^{-1} (28.9 μM) increased the growth rate of hairy roots of *A. annua* by 24.9 %. Wang et al. (2002) focused their research on artemisinin accumulation in hairy root cultures of *A. annua* by (22S, 23S)-homobrassinolide (SSHB). They observed that when 1 μg L^{-1} of SSHB was added to the hairy root cultures, the production of artemisinin reached 14 mg L^{-1}, an increment of 57 % over the control. Furthermore, artemisinin accumulation in hairy roots was found to be dose dependent as well in the treatment with SSHB. They also found that the SSHB treatments at 0.1–10 μg L^{-1} increased the root biomass up to 12–15 g L^{-1} from that of the control. However, if SSHB concentrations were higher than 100 μg L^{-1}, a decline in growth was detected with some root browning, an indication of the cell necrosis.

Studying root cultures, Weathers et al. (2005) focused on the effect of a broad range of phytohormones on growth and secondary metabolism of *A. annua*. They measured growth, development and production of the antimalarial drug, artemisinin, in *A. annua* hairy roots in response to the five main hormones: auxins, cytokinins, ethylene, gibberellins (GA) and abscisic acid (ABA). Single roots grown in six-well plates in medium B5 with 0.01 mg L^{-1} GA_3 produced the highest values overall in terms of the number of lateral roots, length of the primary root, lateral root tip density, total lateral root length and total root length. When the total root lengths were compared, the best conditions for stimulating root elongation was with 0.01 mg L^{-1} GA. Bulk yields of biomass were inversely proportional to the concentration of each hormone tested in their study. All root cultures provided with ABA yielded the highest amount of biomass. Both 6-benzylaminopurine and 2-isopentenyladenine inhibited root growth, however, only 2-isopentenyladenine stimulated artemisinin production, more than twice to that of the B5 controls, and more than any other hormone studied.

Regarding shoot cultures, Ferreira and Janick (1996a) used the growth regulators benzyladenine (BA), kinetin, chlormequat (CCC) and daminozide to induce shoot development. Shoot proliferation was increased by BA at 0.5 and 5.0 μM, but decreased root production at all concentrations. A highly significant correlation was observed between shoot artemisinin concentration and the number of roots ($r = 0.775^{**}$), while shoot number and artemisinin were unrelated ($r = -0.198$). These authors reported that the highest levels of shoot artemisinin (0.29 %) in shoot cultures were obtained with hormone-free medium (control), when root production was maximized. In the same study, removal of roots from shoot cultures grown in hormone-free medium reduced shoot artemisinin in 53 % and arteannuin B by 60 %, confirming the pivotal role of roots in artemisinin biosynthesis. The use of BA in shoot cultures, even at 0.5 μM, can cause vitrification and is not needed because shoot cultures grown in hormone-free medium produced artemisinin shoot concentrations at similar levels found in greenhouse and field-grown clones (Ferreira et al. 1995b).

The research of Pu et al. (2009) provided evidence that salicylic acid (SA) can activate artemisinin biosynthesis in *A. annua*. They observed that exogenous application of SA to *A. annua* leaves was followed by a burst of reactive oxygen species (ROS) and the conversion of dihydroartemisinic acid into artemisinin. Within 24 h from application, SA led to a gradual increase in the expression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) gene and a temporary peak in the expression of the amorpha-4, 11-diene synthase (*ADS*) gene. However, the expression of the farnesyl diphosphate synthase (*FDS*) gene and that of the cytochrome P450 monooxygenase (*CYP71AV1*) gene changed little. At 96 h after SA (1.0 mM) treatment, the concentration of artemisinin, artemisinic acid and dihydroartemisinic acid were 54, 127 and 72 % higher than that of the control, respectively. On the basis of their results, they suggested that SA induces artemisinin biosynthesis in at least two ways: by increasing the conversion of dihydroartemisinic acid into artemisinin caused by a burst in ROS and by upregulating the expression of genes involved in artemisinin biosynthesis.

Jing et al. (2009) evaluated the effect of different concentrations of abscisic acid (ABA) on artemisinin concentration in *A. annua*, under tissue culture conditions. Artemisinin content in plants treated with 10 μM ABA was 65 % higher than that in control plants (1.1 % on a dry weight basis) and ranged from 1.5 % to 1.84 %. They also studied gene expression analysis and showed that in both ABA-treated plants and cell suspension cultures, the important genes in the artemisinin biosynthetic pathway, such as *HMGR*, *FPS*, *CYP71AV1* and *CPR* (cytochrome P450 reductase), were significantly induced. While only a slight increase in ADS expression was observed in ABA-treated plants, no expression of ADS was detected in cell suspension cultures. They suggested that there is probably a crosstalk between the ABA signalling pathway and artemisinin biosynthetic pathway and that *CYP71AV1*, which was induced most significantly, may play a key regulatory role in the artemisinin biosynthetic pathway. Aftab et al. (2010a) investigated the effects of foliar sprays of triacontanol (Tria) alone and in combination with gibberellic acid (GA_3) on growth attributes, photosynthesis, enzymatic activities, essential oil and artemisinin content and yield of *A. annua*. The results indicated that combination of Tria and GA_3 significantly increased activities of nitrate reductase and carbonic anhydrase by 25.9 and 21.5 %, and net photosynthetic rate, stomatal conductance and internal CO_2 by 25.4, 14.1 and 15.4 %, respectively, when compared to unsprayed plants. Combination of Tria and GA_3 also significantly enhanced artemisinin content and yield.

In a study by Banyai et al. (2011) involving cloned plants grown in mixed soil potting medium in a growth chamber, the production of artemisinin and leaf biomass in *A. annua* (varieties 007 and 253-2) was significantly increased by exogenous GA_3 applied to the soil. They also worked out the effects of GA_3 application on expression of key enzymes involved in artemisinin biosynthesis. They postulated that the increased artemisinin content (close to 1 % DW) from exogenous GA_3 treatment was associated with increased expression of key enzymes in the artemisinin biosynthesis pathway. Interestingly, exogenous GA_3 continuously enhanced artemisinin content from the vegetative stage to flower

initiation in both plant lines involved and gave significantly higher leaf biomass than in control plants. Consequently, the artemisinin yield in GA_3-treated plants was much higher than in control plants. In their work, although the maximum artemisinin content was found at the full blooming stage, the highest artemisinin yield in GA_3-treated plants was obtained during the flower initiation stage. This was 26.3 and 27.8 % higher, respectively, than in non-treated plants 007 and 253-2.

Wang et al. (2010) reported the effects of exogenous foliar methyl jasmonate (MJ) on artemisinin biosynthesis and secondary metabolites in *A. annua* under greenhouse conditions. They found a 49 % increase in artemisinin concentration on day 8 after treatment with MJ, associated with an 80 % increase in artemisinic acid and 28 % in dihydroartemisinic acid, the latter currently accepted as being the main precursor of artemisinin. In addition, they also worked out some other secondary metabolites using metabolite profiling after exogenous methyl jasmonate treatment. Their content also changed significantly after MJ treatment, including a 50 % increase in methyl artemisinic acid, a 67 % increase in squalene and a 60 % increase in an unidentified sesquiterpenoid. They argued that these compounds may be promising targets for further studies on artemisinin biosynthesis.

Aftab et al. (2010b) showed that salicylic acid (SA) acts as a potential plant growth promoter and plays an important role in regulating a number of plant physiological and biochemical processes under field conditions. Four levels of SA (0, 0.25, 0.50 and 1.00 mM SA) were applied in the form of diluted aqueous sprays on the aboveground plant parts in that study. Plant height, dry weight, chlorophyll and carotenoid contents were improved significantly as the level of SA increased. Furthermore, significant enhancement in net photosynthetic rate (31.7 %), the activity of nitrate reductase (17.2 %) and carbonic anhydrase (10.9 %) was noticed as the level of SA application was increased from 0 to 1.00 mM SA. Most importantly, the content and yield of artemisinin were increased by 25.8 and 50.0 %, respectively, after treatment with SA.

10.5 Effect of Environmental Stresses

Prasad et al. (1998) observed the effect of soil salinity (mixture of cations and anions) on the growth, yield, mineral composition and artemisinin concentration of *A. annua* cultivar from Kew, England. They noticed that plant height decreased as salinity stress increased, and the leaf-to-stem ratio was generally increased in salinized plants as compared with control plants not subjected to salinity stress. The vegetative yield of shoots increased significantly with increasing salinity stress up to 6.0 dS/m, but further increases in salinity decreased shoot yield. Artemisinin content in vegetative tissue was around 0.01 % (g/100 g dry weight) and was not influenced by salinity levels from 0.9 to 10.4 dS/m, but decreased to 0.006 % at 14 dS/m. The concentration of nitrogen was significantly higher, and the concentrations of phosphorus and calcium were lower in plants subjected to salinity stress. Potassium concentration and the potassium-to-sodium ratio in

shoots decreased, while the sodium and magnesium concentrations increased with salinity stress to 6.0 dS/m. The potassium-to-sodium ratio was significantly and negatively correlated with the dry weight of shoot.

Qureshi et al. (2005) reported the effects of NaCl (0–160 mM) and lead acetate (0–500 μM) on 90 (S1 treatment)- and 120-day-old (S2 treatment) *A. annua* plants. Treated plants were evaluated for lipid peroxidation rate, photosynthetic rate (Pn), chlorophyll content, artemisinin concentration and artemisinin yield, and for total biomass accumulation, through leaf samples, at 100, 130 and 160 days after sowing (DAS) in S1, and at 130 and 160 DAS in S2 treatments. Treatments enhanced lipid peroxidation at all stages of plant growth and increased the concentration and yield of artemisinin at 100 and 130 DAS in S1 and S2, respectively, while other parameters declined at all growth stages. The magnitude of changes was greater in lead-treated than in salt-treated plants. Both treatments induced oxidative stress, which might have damaged the photosynthetic apparatus resulting in a loss of chlorophyll content and a decline in photosynthetic rate, biomass accumulation and artemisinin production. They postulated that increase in artemisinin content, observed during the early phase of plant growth, might be due to a sudden conversion of artemisinin precursors into artemisinin by activated oxygen species (ROS) under oxidative stress. However, no work so far has quantified ROS in *A. annua* or directly correlated artemisinin increase with increased ROS in response to abiotic stress.

Ferreira (2007) reported that a cloned, greenhouse-grown *A. annua* (Artemis) subjected to an acidic soil and macronutrient deficit was evaluated for artemisinin production. Lack of lime (L) and macronutrients (N, P and K) reduced leaf biomass accumulation. When L was provided, the highest average leaf biomass was achieved with the "complete" (+N, +P, +K and +L) treatment, and the least biomass was achieved with the untreated (−N, −P, −K and −L) treatment. The macronutrient least required for biomass accumulation per plant was K (49.0 g), followed by P (36.5 g) and N (14.3 g). The artemisinin concentration (g/100 g) was significantly higher (75.5 %) in −K plants when compared to plants under the complete treatment. Although the artemisinin total yield was 21 % higher in −K plants, it was not significantly different from plants under the complete treatment, due to the lower biomass accumulation caused by deficiency of K. There were no marked treatment effects for concentration or yield of both dihydroartemisinic acid and artemisinic acid, although higher levels were achieved in plants under the complete or −K treatments. There was a positive and significant correlation between artemisinin and both artemisinic acid and dihydroartemisinic acid, in g/100 g and g/plant. This is the first report where potassium deficiency significantly increases the concentration (%) of artemisinin. These results were confirmed with a different set of cloned Artemis plants under the same GH conditions, same macronutrient deficiencies and same soil, but with the addition, or lack, of tannic acid to soils. Potassium deficiency (regardless the addition or lack of tannic acid) significantly increased both artemisinin concentration and yield (Ritchey and Ferreira unpublished).

A. annua plants were cultivated by Marchese et al. (2010) in growth chambers and submitted to five water deficit treatments. Water deficits of 38 and 62 h increased leaf artemisinin content, but only 38 h led to a significant increase in both leaf and plant artemisinin, with no detriment to plant biomass production. The other treatments had no effect on, or decreased artemisinin accumulation. *A. annua* plants tolerated well water deficit treatments, including the most severe water deficit applied and recovered their turgor pressure after rehydration. They concluded that moderate water deficit prior to harvesting the crop may not only reduce time and costs in drying the crop, but can also induce artemisinin accumulation, both of which increase crop profit margins.

Aftab et al. (2010c) studied the effect of increasing levels of boron (B) on oxidative stress, antioxidant defence response and changes in artemisinin content in *A. annua*. Toxicity caused by B reduced growth parameters such as stem height, fresh weight and dry weight. Treatments induced oxidative stress resulting in lower net photosynthetic rate, stomatal conductance, internal CO_2 and total chlorophyll content. The increased activities of antioxidant enzymes like CAT (catalase), POX (peroxidase) and SOD (superoxide dismutase) were also noted in response to increasing levels of B stress. However, H_2O_2 and artemisinin content were found to be high up to 1.00 mM concentration of boron compared to control, and on applying higher doses, further reduced contents were obtained. Their results suggest that a mild stress of B can be utilized to enhance artemisinin production.

The role of salicylic acid (SA) in inducing salinity tolerance was studied in *A. annua* L., by Aftab et al. (2011b). When applied to leaves at 1.0 mM, SA provided considerable protection against salt stress imposed by 50, 100 or 200 mM NaCl to soil. Salt stress negatively affected plant growth as assessed by length and dry weight of shoots and roots. Salinity also reduced the values of photosynthetic attributes and total chlorophyll content and inhibited the activities of nitrate reductase and carbonic anhydrase. Furthermore, salt stress significantly increased electrolyte leakage and proline content. Salt stress also induced oxidative stress as indicated by the elevated levels of lipid peroxidation compared to the control. A foliar spray of SA at 1.0 mM promoted the growth of plants, independent of salinity level. The activity of antioxidant enzymes, namely CAT, POX and SOD, was upregulated by salt stress and was further enhanced by SA treatment. Artemisinin concentration increased at 50 and 100 mM NaCl but decreased at 200 mM NaCl. The application of SA further enhanced artemisinin concentration when applied with 50 and 100 mM NaCl by 18.3 and 52.4 %, respectively. Their results indicate that moderate saline conditions can be exploited to obtain higher artemisinin content in *A. annua* plants, whereas the application of SA can be used to maintain plant growth and induce its antioxidant defence system under salt stress (Fig. 10.4).

Aftab et al. (2011b) conducted a study to determine whether the exogenous application of methyl jasmonate (MJ) on *A. annua* could counteract the ill effects of excessive B present in the soil. According to the results obtained, B toxicity induced oxidative stress and reduced stem height, as well as fresh and dry masses of the plant significantly. The excessive amounts of soil B also lowered the net

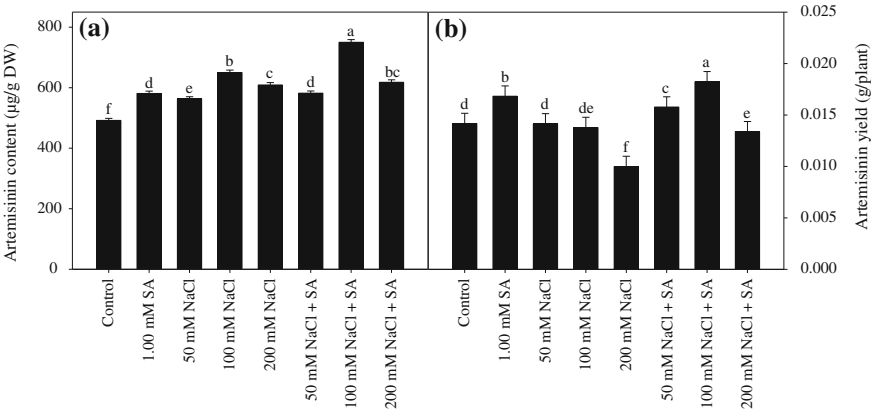

Fig. 10.4 Effect of SA and different concentrations of salinity on artemisinin content (**a**) and artemisinin yield (**b**) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. *Error bars* (⊤) show SE

photosynthetic rate, stomatal conductance, internal CO_2 concentration and total chlorophyll content in the leaves. In contrast, the foliar application of MJ enhanced the growth and photosynthetic efficiency in both the stressed and non-stressed plants. The excessive B levels also increased the activities of antioxidant enzymes, such as CAT, POX and SOD. Endogenous H_2O_2 and O_2^- levels were also high in stressed plants. However, MJ application on stressed plants reduced the amount of lipid peroxidation and stimulated the synthesis of antioxidant enzymes, enhancing the concentration and yield of artemisinin as well. They concluded that MJ might be utilized in mitigating B toxicity and improving both concentration and yield of artemisinin in *A. annua*.

Aftab et al. (2012) reported the effects of B and aluminium (Al) contamination in soil, carried out with or without application of exogenous sodium nitroprusside or SNP (a nitric oxide donor), on various plant processes in *A. annua*, including changes in artemisinin content. The addition of B or Al to soil significantly reduced the yield and growth of plants and lowered the values of net photosynthetic rate, stomatal conductance, internal CO_2 concentration and total chlorophyll content. The follow-up treatment of NO donor favoured growth and improved the photosynthetic efficiency in stressed and non-stressed plants. Artemisinin content was enhanced by 24.6 and 43.8 % at 1.0 mol of soil-applied B or Al. When SNP was applied at 2.0 mol concentration with either 1.0 mol of B and/or Al, it further stimulated artemisinin biosynthesis compared to the control. Application of B + Al + SNP proved to be the best treatment combination to increase artemisinin concentration in *A. annua* leaves.

Although we cannot safely conclude that *A. annua* responds to stress by using dihydroartemisinic acid as a scavenger of ROS, the response of the plant to SA, MJ, GA_3, B, K deficiency, water stress, etc., all seem to cause slight to significant

increases in artemisinin biosynthesis. These responses, associated with the reports that rootless shoot cultures have traces of artemisinin and that well-fed crops increase biomass but no artemisinin indicate that, although roots do not seem to be a site of artemisinin accumulation (as glandular trichomes are), artemisinin could be part of *A. annua* chemical system of response abiotic stresses sensed by the roots. Although we found one study that reports artemisinin increase after frost, there are no other studies confirming such effect of low temperatures, what would obfuscate the idea that roots are an important organ that senses stress and triggers increased artemisinin biosynthesis.

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Chapter 11
Recent Advances to Enhance Yield of Artemisinin: A Novel Antimalarial Compound, in *Artemisia annua* L. Plants

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Abstract Malaria is probably as old as mankind and continues to affect millions of people throughout the world. Today, some 500 million people in Africa, India, Southeast Asia, and South America are exposed to endemic malaria, and it is estimated to cause one and half million deaths annually, one million of which are children. As a consequence, effective therapeutic agents against malaria are continuously being sought, especially against those strains of *Plasmodium falciparum*, which have become resistant to nearly all antimalarial drugs, including chloroquine and quinine. In the absence of reports of artemisinin resistance in malaria parasite, WHO now recommends the use of artemisinin-based combination therapies (ACTs) with formulations containing an artemisinin derivative. Artemisinin, a sesquiterpene endoperoxide lactone, is isolated from the shoots of *Artemisia annua* L. plants. Apart from a novel and potent antimalarial drug, artemisinin and its derivatives are also used in therapies against hepatitis, leishmaniasis, and schistosomiasis. Artemisinin also possess lethal activities against cancerous cells, fungi, and bacteria. It has also shown to be immune-suppressant in mammals and a potent herbicide. Despite of its immense commercial value, the production of artemisinin is not cost-effective because of its low concentration (0.01–1.1 %) in the plant. Moreover, its de novo synthesis is complex, uneconomical and gives low yields. Further, classical breeding and selection techniques have failed to develop high-yielding strains of *A. annua* L. plants. Efforts are therefore, needed to elucidate the complex pathway of artemisinin biosynthesis and its biochemical and molecular regulation. Non-conventional approaches have to be developed to

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evolve novel strains of the plant to optimize and scale up the production of artemisinin in bulk and make it available to ACT manufacturers at a price much lower than their current cost in turn making an important contribution toward attaining the goals of global malaria eradication programs. The details of past and current status of both conventional and non-conventional approaches for enhancing artemisinin content in *A. annua* L. plants and its yield have been discussed in this chapter.

11.1 Introduction

Being the world's most severe parasitic infection, malaria caused more than a million deaths and 500 million cased annually. Despite tremendous efforts for the control of malaria, the global morbidity and mortality have not been significantly changed in the 50 years (Reley 1995). The key problem is the failure to find effective medicines against malaria. Obtained from a Chinese medicinal plant *Artemisia annua* L., artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has become increasingly popular as an effective and safe alternative therapy against malaria (Luo and Shen 1987). Artemisinin and its derivative are effective against multidrug-resistant *Plasmodium falciparum* strain mainly in Southeast Asia and more recently in Africa, without any reported cases of resistance (Mohapatra et al. 1996; Krishana et al. 2004).

A. annua is a cosmopolitan species, growing wild in many countries, e.g., in China and Vietnam, the Balkan, the former Soviet Union, Argentina, and southern Europe (Van Geldre et al. 1997), and large differences exist in artemisinin content between different varieties of *A. annua* (Delabays et al. 1993; Woerdenbag et al. 1993). A substantial increase in the content of artemisinin would be required to make artemisinin available on a large scale also to the people in the Third World. Selection for high-producing lines and traditional breeding, and research on the effects of environmental conditions and cultural practices could perhaps lead to an improvement of artemisinin content (Delabays et al. 1993; Ferreira et al. 1995; Gupta et al. 1996).

Artemisinin is a sesquiterpene-lactone isolated from the aerial parts of *A. annua* L. plants. Besides being currently the best therapeutic agent against both drug-resistant and cerebral malaria causing strains of *Plasmodium* sp., (Newton and White 1999), it is also effective against other infectious diseases such as schistosomiasis, hepatitis B and leishmaniasis (Borrmann et al. 2001; Utzinger et al. 2001; Romero et al. 2005; Sen et al. 2007). More recently, it has also been shown to be effective against a variety of cancer cell lines including breast cancer, human leukemia, colon cancer, and small cell-lung carcinomas (Efferth et al. 2001; Singh and Lai 2001). Due to its current use in artemisinin-based combination therapy (ACT), its global demand continuously is increasing. The relatively low yield of artemisinin in *A. annua* L. plant leaves (0.01–1.1 %), however, a serious

limitation to the commercialization of the drug Laughlin (1994; Van Agtmael et al. 1999; Abdin et al. 2003).

Artemisinin and its semisynthetic analogues have undergone clinical trials as new lifesaving antimalarials under the auspices of the World Health Organization and have been intensively studied due to their unique structure, with an endoperoxide (1,2,4-trioxane) linkage, their novel mechanism of action as the first nonnitrogenous antimalarial, and the worldwide resurgence of drug-resistant falciparum infections. The state-of-the-art of production of artemisinin by chemical and biotechnological methods and analytical aspects has recently been reviewed. Five various approaches have been employed to increase artemisinin content in the plant including conventional breeding, biochemical, physiological and molecular approaches, hairy root culture techniques, for the artemisinin biosynthetic pathway in *A. annua* L. (Smith et al. 1997; Liu et al. 1999; Chang et al. 2000; Wallaart et al. 2001; Wang et al. 2002; Abdin et al. 2003; Martin et al. 2003; Weathers et al. 2005; Picaud et al. 2005; Newman et al. 2006; Ro et al. 2006; Zeng et al. 2007). Chemical synthesis has also been attempted (Xu et al. 1986; Avery et al. 1992), but the yield of artemisinin is very low. Thus, it is economically not viable for the large-scale production of artemisinin. Four genes of artemisinin biosynthesis pathway (HMG-CoA reductase, fernesyl diphosphate synthase, amorpha-4,11-diene synthase (ADS), and CYP71AV1) were overexpressed (Han et al. 2006; Ping et al. 2008; Teoh et al. 2006; Aquil et al. 2009) and artemisinin content was increased by 34.4 %.

11.2 Biosynthetic Pathway of Artemisinin

The biosynthetic pathway of artemisinin belongs to the isoprenoid metabolite pathway (Fig. 11.1). Based on the experimental evidences related to its biosynthesis, artemisinin is suggested to be derived from two common precursors, isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). It has been established that higher plants have two independent biosynthetic pathways leading to the formation of IPP: the cytosolic mevalonate pathway and the plastid-localized mevalonate-independent (MEP/Rohmer) pathway (Liu et al. 2005). As a result, mevalonate pathway has no more been considered as the sole route to the synthesis of artemisinin in *A. annua* L. It was further supported by isolation of two clones encoding deoxy-D-xylulose-5-phosphate synthase (DXPS) and deoxy-D-xylulose-5-phosphate reductoisomerase (DXPR) from transformed hairy roots of *A. annua* L. (Souret et al. 2002; Krushkal et al. 2003). The partial carbon supply to the synthesis of artemisinin was reported to be made by MEP pathway operating in plastids and DXR catalyzing the rate-limiting step (Towler and Weathers 2007). Recently, the relative contribution of these pathways toward carbon supply in artemisinin production was evaluated by

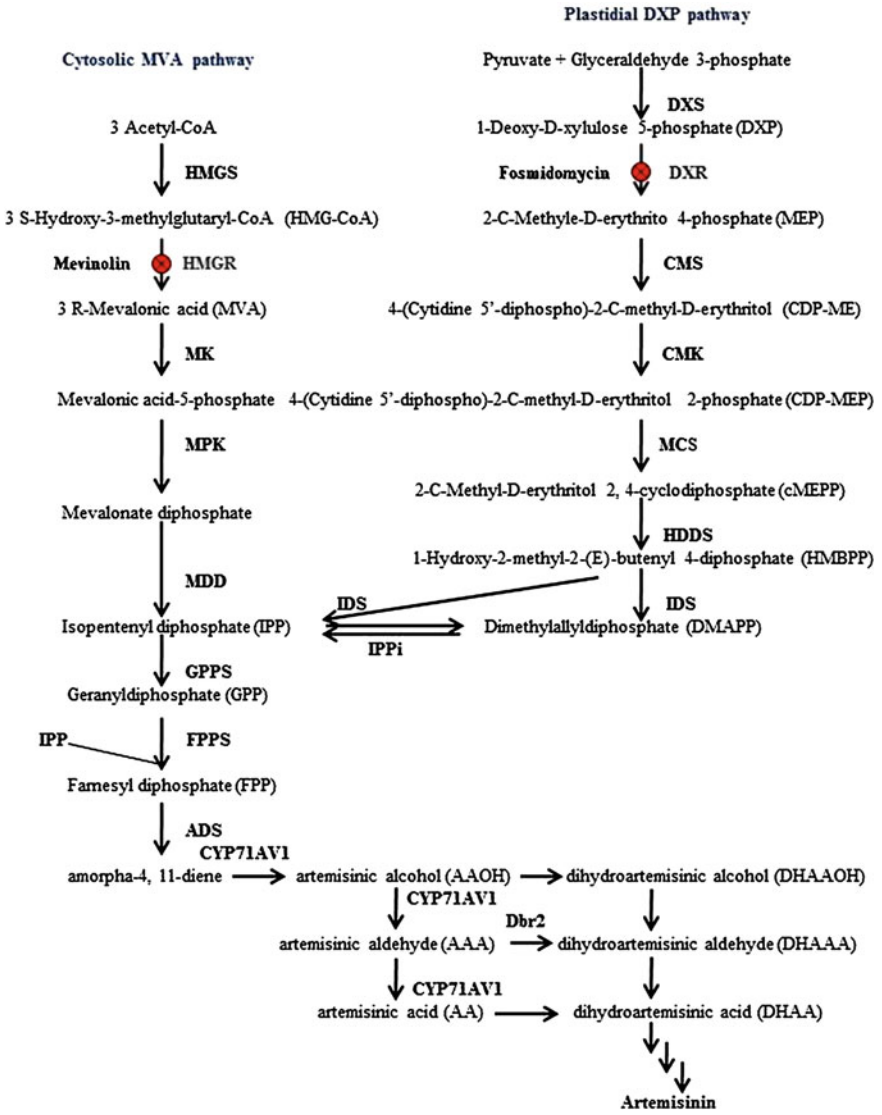

Fig. 11.1 Proposed artemisinin biosynthesis pathway in *A. annua* L.CMK 4-(Cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase, CMS 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase, DXR 1-deoxy-D-xylulose 5-phosphate reductoisomerase, DXS 1-deoxy-D-xylulose 5-phosphate synthase, FPPS farnesyl diphosphate synthetase, GPPS geranyl diphosphate synthase, HMGR 3-hydroxy-3-methylglutaryl coenzyme A(HMGCoA) reductase; HMGS HMG-CoA synthase; IDS isopentenyl diphosphate synthase, MCS 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, MDD mevalonate diphosphate decarboxylase, MK mevalonate kinase, MPK mevalonate-5-phosphate kinase, SES sesquiterpene synthase, CYP71AV1, cytochrome P450 monooxygenase; Dbr2, artemisinic aldehyde reductase. Adapted from Liu et al. (2006)

Ram et al. (2010). They demonstrated that mevalonate pathway is the major contributor of carbon and supplies 80 % of the carbon to artemisinin biosynthesis, whereas MEP pathway supplies only 20 % of the carbon.

In mevalonate pathway, three molecules of acetyl-coenzyme A condensed together to yield 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), which is subsequently reduced by the enzyme HMG-CoA reductase (HMGR) to yield mevalonic acid (MVA). Then, under the catalysis of mevalonate kinase, mevalonate 5-diphosphate is formed which is subsequently decarboxylated to yield isopentenyl pyrophosphate (IPP) (Newman and Chappell 1999). The synthesis of IPP and DMAPP by either MVA or DXP pathways is followed by chain elongation. The carbonium ion is a potent alkylating agent that reacts with IPP, giving geranyl diphosphate (GPP). GPP has the active allylic phosphate group and further react with IPP to produce farnesyl pyrophosphate (FPP). FPP takes part in a cyclization reaction catalyzed by cyclases to produce various final products of isoprenoids including artemisinin (Barkovich and Liao 2001).

All the steps of mevalonate and MEP pathway have been fully characterized, but post-FPP production of artemisinin is not yet completely elucidated. Several enzymes involved in the early steps of artemisinin biosynthesis have been discovered which include HMGR, farnesyl pyrophosphate synthase (FPS), ADS, DXS, DXR, squalene synthase (SQS), and CYP71AV1. (Table 11.1).

The formation of the sesquiterpene carbon skeleton, amorpha-4, 11-diene is catalyzed by ADS (Bouwmeester et al. 1999) for which corresponding cDNAs have been cloned (Chang et al. 2000; Mercke et al. 2000; Wallaart et al. 2001). The non-descript arrangement of the amorphadiene product belies the unique structural features that ultimately allow for the formation of the 1, 2, 4-trioxane moiety (Sy and Brown 2002) (Fig. 11.2). Expression analysis of CYP71AV1 in *A. annua* L. tissues indicates that it is most highly expressed in secretory glandular trichomes (GSTs) (Teoh et al. 2006). The moderate expression observed in flower buds presumably reflects their high density of GSTs. Low but detectable levels of RT-PCR products could be observed in leaves. The role of CYP71AV1 in the hydroxylation of amorpha-4, 11-diene is undoubtedly important in artemisinin biosynthesis. The subsequent route to artemisinin is less clear. Most evidence implicates dihydroartemisinic acid as a late precursor to artemisinin biosynthesis, which is derived from artemisinic alcohol by oxidation at C12 and reduction in the C11–C13 double bond. This is based on in vitro biochemical evidence (Bertea et al. 2005), as well as the conversion of dihydroartemisinic acid to artemisinin both in vivo (Brown and Sy 2004) and in vitro in an oxygen-dependent non-enzymatic fashion (Sy and Brown 2002).

Bertea et al. (2005) showed that *A. annua* L. leaf microsomes convert amorphadiene to artemisinic alcohol in the presence of NADPH. The route from artemisinic alcohol to artemisinin is still not entirely clear, which is evident from the published data reviewed by Li et al. (2006). In this regard, it is useful to consider the possible route(s) to artemisinin among the pathways shown in Fig. 11.2. These pathways are based on a few conversions whose order may vary. These conversions include the oxidation of C12 from alcohol to aldehyde as well

Table 11.1 Genes related to artemisinin biosynthesis in *A. annua* L

Enzyme	Gene	Function	Location	Gene Bank (Accession no.)	Reference
Deoxyxylulose synthase	dxs	l-Deoxy-D-xylulose-5-phosphate synthase activity	Plastid	AF182286	Souret et al. (2002)
Deoxyxylulose reductase	dxr	Isomerase and oxidoreductase activity	Plastid	AF182287	Souret et al. (2002)
HMG-Co A reductase	hmgr	Catalyze the two step reduction of S-HMG-CoA into MVA Cytosol	Cytosol	AF142473	Souret et al. (2002)
Farnesyl diphosphate synthase	fps	Synthesis of FDP	Cytosol	AF112881	Chen et al. (2000)
Sesquiterpene cyclases		Catalyzes cyclization of FDP to:	All likely in cytosol		
(a) Epicedrol synthase	eps	8-Epicedrol		AJ001539	Mercke et al. (1999)
(b) Amorphadiene synthase	ads	Amorpha-4,11 diene		AJ251751	Mercke et al. (2000)
(c) *β*-caryophyllene synthase	cs(qhsl)	*β*-Catyophyllene		AF472361	Cai et al. (2002)
(d) *β*-farnesene synthase	fs	*β*-Farnesene		AY835398	Picaud et al. (2005)
Putative sesquiterpene cyclases	casc125	Isoprenoid biosynthesis and lyase activity	Isolated from: flowers	AJ271792	Van geldre (2000)
	casc34	Isoprenoid biosynthesis and lyase activity	leaves and flowers	AJ271793	Van geldre (2000)
	ses	Reduction product not determined	Young leaves	AAD39832	Liu et al. (2002)
Squalene synthase	aasqs	Farnesyl diphosphate farnesyltransferase activity	Endoplasmic reticulum	AY445506	Liu et al. (2003)
Squalene synthase fragment	sqsl	Transferase activity	Cytosol	AF1S2286	Souret et al. (2003)
CYP71AV1	na	Catalyzes 3 steps post-ADS	Trichomes	DQ315671	Teoh et al. (2006)
PsbA (Fragment)	psba	Act as barcode for flowering plants	Chloroplast	DQ006143	Kress et al. (2005)
Ribulose-1,5-biphosphate (CO)	rbcl	Carbon dioxide fixation; bar coding	Chloroplast	DQ006057	Kress et al. (2005)
Peroxidase 1	podl	Favored the bioconversion of artemisinic acid to artemisinin	Root, stems and leaves	AY208699	Zhang et al. (2004)
Beta-pinene synthase	gh6	Circadian pattern of expression	Juvenile leaves	AF276072	Lu et al. (2002)
(3R)-linalool synthase	gh1	Lyase activity	Leaves and flowers	AF154125	Jia et al. (1999)
Isopentenyl transferase	ipt	Biosynthesis of cytokinins and phytohormones	Transferred into *A. annua L.*	M91610	Sa et al. (2001)

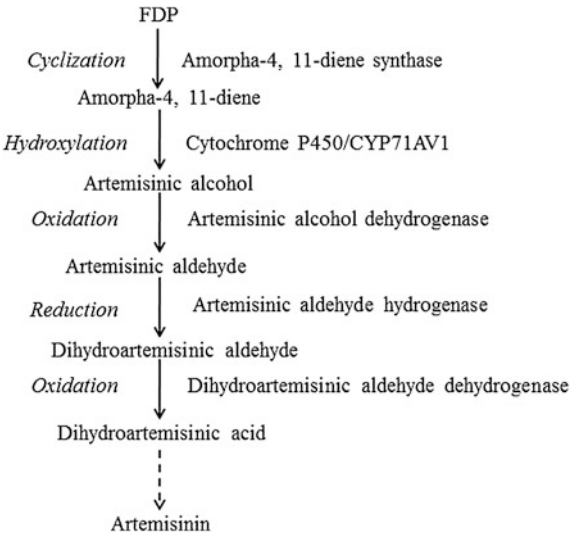

Fig. 11.2 Proposed biosynthetic pathway of artemisinin starting from farnesyl diphosphate. *On the left* is the type of reaction; *on the right* is the enzyme for each known enzymatic action. *Broken arrow* indicates multiple steps. Adapted from Bertea et al. (2005)

as aldehyde to acid, the reduction of the double bond at C11, C13, and the formation of the 1,2,4-trioxane moiety. The later steps in artemisinin biosynthesis remain controversial and theories differ mainly in the identification of either artemisinic acid or dihydroartemisinic acid as the later precursor. The evidence for artemisinic acid has been reviewed by Li et al. (2006). This includes the suggestion that C11, C13 double-bond reductions occur at the level of an intermediate beyond artemisinic acid, such as arteannuin B or artemisitene. On the other hand, the co-occurrence of dihydroartemisinic acid with high artemisinin levels suggests that even if double-bond reduction could occur at a very late step, it also occurs in less oxidized precursors. The double-bond reduction at C11, C13 is of general interest biochemically, given the relative rarity of enzymes catalyzing double-bond reductions (Kasahara et al. 2006). The dihydroartemisinic acid is also being considered as a late precursor of artemisinin biosynthesis. Labeled dihydroartemisinic acid is incorporated into artemisinin in vivo, a sequence which can occur in the absence of enzymes (Brown and Sy 2004; Haynes et al. 2006; Sy and Brown 2002). Upstream of dihydroartemisinic acid, the order of oxidations, and reduction of artemisinic alcohol *en route* to dihydroartemisinic acid are still not settled. Bertea et al. (2005) provided biochemical evidence for the fate of artemisinic alcohol in *A. annua* L. using GST cell-free extracts.

11.3 Agrotechnology

A. annua (family Asteraceae), also known as qinghao (Chinese), annual or sweet wormwood, or sweet Annie, is an annual herb native to Asia, most probably China (McVaugh 1984). *A. annua* occurs naturally as part of the steppe vegetation in the

northern parts of Chahar and Suiyuan provinces (408°N, 1098°E) in northern China (now incorporated into Inner Mongolia), at 1,000–1,500 m above sea level (Wang 1961). The plant now grows wild in many countries, such as Argentina, Bulgaria, France, Hungary, Romania (cultivated for its essential oil), Italy, Spain, USA, and former Yugoslavia (Klayman 1989; Klayman et al. 1993). In addition, it has been introduced into experimental cultivation in India (Singh et al. 1986), Vietnam, Thailand, Myanmar, Madagascar, Malaysia, USA, Brazil, Australia (Tasmania) and in Europe into the Netherlands, Switzerland, France, and as far north as Finland (Laughlin 2002).

A. annua is a xeromorphic temperate plant belonging to Asteraceae family that contains promising antimalarial drugs, the sesquiterpene lactone artemisinin and derivatives of this compound. An examination of the growth and flowering behavior of *A. annua* in the subtropical climate region of India demonstrated the plant grew normally and flowered profusely in the winter cropping season, late October to late April, at Lucknow, India. Considerable inter-plant variation was observed, however, in growth habit and flowering time. Plants could be grouped into four classes: early-maturing dwarf, early-maturing tall, late-maturing dwarf, and late-maturing tall. Early-maturing plants which flowered in February and March produced fertile achenes, completing the life cycle in 7–8 months. Late-flowering plants that flowered in May and June, when the maximum day temperature was over 40 °C, produced florets without seeds. The high-temperature conditions to which the late-flowering plants were exposed appeared to prematurely dry the stigma. Late-flowering plants sprouted branches from the vegetative and flowering parts of the plant during the rainy season.

A. annua appears to be the only *Artemisia* species that contain appreciable amounts of artemisinin. Chinese scientists have reported that extracts from 30 other different species of *Artemisia* did not show antimalarial activity (UNDP/World Bank/WHO special programme for Research and Training in Tropical Diseases *Artemisia* species, 1981), American scientists have failed to detect artemisinin in various species endemic to America (Klayman et al. 1984). In India, (Balachandran et al. 1987) also did not find artemisinin in various *Artemisia* species of Indian origin. Considering the importance of artemisinin which is tedious and difficult to synthesize chemically, an all out program was undertaken to develop *A. annua* plant varieties with high artemisinin content starting with development of agrotechnology for increase yield of these compounds, followed by improved extraction procedure. More particularly, the program focused on agrotechnology involving method of optimizing the planting time, transplanting scheduling, population density, number of harvests and harvesting schedule leading to enhanced yields of artemisinin and related metabolites which have pharmaceutical value of anti-infectives, particularly as antimalarial drug. In this direction, the inventors were successful in developing and releasing a variety named 'Jeevan Raksha' from an isolated population containing high artemisinin in the foliage (0.5–1.0 %) (Kumar et al. 1999). This plant 'Jeevan Raksha' not only produces high artemisinin but also maintains the synchronized conversion to higher level of artemisinin during May to October. As the content of artemisinin

fluctuates from zero level at the time of planting to more than 0.4–1.00 % during May and June with subsequent functions of increase till October, it was necessary to scientifically develop cultivation methodology for the crop to maximize the vigor of the foliage and biosynthesis of artemisinin by systematic scheduling. For this purpose, the inventors carried out planned experiments with variation in planting times, population density, and number of harvest from the crop to increase the yield from limited area within optimum span of time. Until now, artemisinin production has depended on extraction from *A. annua* L. plants grown outdoors. There are two methods to enhance artemisinin production in intact *A. annua* plants. One method is to define the appropriate developmental stage at which to harvest the leaves of the plants. At this developmental stage, both the highest artemisinin content and leaves yielding can be obtained. The other method is to breed high-artemisinin-yielding strains.

11.4 Biochemical and Physiological Approach

Although artemisinin is an effective medicine for treating malaria, the application of this medicine is limited by the availability of the source. The artemisinin content in the leaves or florets of *A. annua* is very low, and the chemical method for the synthesis of this compound is difficult. These factors make the medicine expensive and hardly available on a global scale for patients (Van Geldre et al. 1997; Abdin et al. 2003). Despite the commercial value of artemisinin, exact biosynthetic pathway of artemisinin in *A. annua*, particularly about the early enzymatic steps leading to (dihydro) artemisinic acid are still unclear. Several authors have demonstrated that *A. annua* converts artemisinic acid and dihydroartemisinic acid to artemisinin (Sangwan et al. 1993; Wallaart et al. 1999b). Akhila et al. (1990) hypothesized a pathway in which the formation from farnesyl diphosphate (FDP) of an unidentified enzyme-bound sesquiterpene-like intermediate represents the first committed step in the biosynthesis of artemisinin. In addition, many authors have analyzed extracts of *A. annua* to search for possible intermediates in the biosynthesis of artemisinin. Artemisinic and dihydroartemisinic acid were reported by many authors, as well as many olefinic mono- and sesquiterpenes and putative intermediates *en route* from dihydroartemisinic acid to artemisinin (Brown 1994; Jung et al. 1990; Ranasinghe et al. 1993; Wallaart et al. 1999b; Woerdenbag et al. 1993). However, none of the reported olefinic sesquiterpenes seemed to fit in the biosynthetic pathway, nor was a possible intermediate between the sesquiterpene olefin and artemisinic acid ever detected, with the exception of artemisinic alcohol, which was tentatively identified in the roots of *A. annua* (Woerdenbag et al. 1993). It has been shown by several groups that the cyclization of the ubiquitous precursors GPP, FDP, and GPP to the respective olefinic mono-, sesqui- and diterpene skeletons represents the regulatory step in the biosynthesis of terpenoids (Gershenzon and Croteau 1990; Mc garry and Croteau 1995). The accumulation of artemisinic acid and dihydroartemisinic acid and the absence of any intermediates en route

from FDP to these two compounds support that the first step(s) in the biosynthetic pathway of artemisinin [and again some step(s) from (dihydro) artemisinic acid to artemisinin] are indeed regulatory/rate limiting. Here, we describe the elucidation of the unknown four intermediates and the corresponding five enzymatic steps that constitute the first part of the artemisinin biosynthetic pathway. The implications for strategies to improve artemisinin production are discussed. Chemical synthesis of artemisinin is an expensive multistep process; the plant remains the only commercial source of the drug.

In our biosynthetic studies on artemisinin toward purification of endoperoxidase enzyme from *A. annua*, and determination of the source of the endoperoxide Oxygen Bridge by ^{18}O-isotope labeling in plant cell-free and tissue culture, we required an experimental method suitable for direct detection, quantitation, and isotopomeric analysis since artemisinin is unstable and lacks a chromophore for UV detection in HPLC.

Alternatively, the C11-C13 double-bond reduction may occur in artemisinic alcohol or artemisinic aldehyde, yielding dihydroartemisinic alcohol or dihydroartemisinic aldehyde as intermediates, respectively. To study this unknown part of the pathway, we analyzed the presence of putative intermediates and enzymes involved in the conversion of these intermediates in leaves and glandular trichome extracts of *A. annua*. Hereto, first a number of reference compounds such as artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde, and dihydroartemisinic aldehyde were synthesized using artemisinic acid and dihydroartemisinic acid as starting materials or isolated (artemisinic aldehyde). The structures of all isolated or synthesized compounds were confirmed using NMR and MS (Bertea et al. 2005). Subsequently, we looked for these compounds in extracts of *A. annua* leaves as well as in extracts of isolated trichomes. The chromatograms obtained with these two extracts were very similar, indicating that most (if not all) of *A. annua* terpenoids are present in the trichomes. In both cases, artemisinic alcohol, artemisinic aldehyde, artemisinic acid, dihydroartemisinic alcohol, dihydroartemisinic aldehyde, dihydroartemisinic acid, and a series of olefinic terpenes were detected (Bertea et al. 2005). Artemisinic acid, dihydroartemisinic acid, and the sesquiterpene olefins have been reported before as constituents of *A. annua* (Boumeester et al. 1999; Wallaart et al.1999b) but this was the first time artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic alcohol, and dihydroartemisinic aldehyde have been identified in *A. annua*. In enzyme assays with microsomal pellets of *A. annua* leaf extracts, using amorpha-4, 11-diene as a substrate and in the presence of NADPH, we found a small, but consistent amorpha-4, 11-diene hydroxylase activity which was absent in the soluble protein fraction (150,000 g supernatant), confirming that a cytochrome-P450 enzyme catalyzes the formation of artemisinic alcohol from amorpha-4, 11-diene. The next putative enzymatic step was assayed by incubating a mixture of artemisinic alcohol and dihydroartemisinic alcohol with the 150,000 g young-leaf supernatant in the presence of $NAD^+/NADP^+$ at pH 9.0. In the presence of cofactors, the ratio between artemisinic alcohol and dihydroartemisinic alcohol strongly decreased showing that artemisinic alcohol was converted to artemisinic

aldehyde, dihydroartemisinic aldehyde, and dihydroartemisinic acid (Bertea et al. 2005). None of these intermediates were formed in the absence of cofactors. Artemisinic acid was not detected in any of these experiments. To test whether the conversion of dihydroartemisinic aldehyde to dihydroartemisinic acid that was observed in leaf extracts also occurred in trichomes, we incubated the 150,000 g supernatant of the GSTs with dihydroartemisinic aldehyde in the presence of $NAD^+/NADP^+$. Under these conditions, we detected conversion of dihydroartemisinic aldehyde into dihydroartemisinic acid, whereas conversion did not occur in the absence of cofactors.

Artemisinin was first isolated from the aerial parts of *A. annua* by the Chinese scientists and later characterized by others. But, the details of isolation procedure were not published for long time (reviewed by Klayman 1985). The researchers at the Walter Reed Army Institute of Research, USA, spotted some *A.annua* growing in the neighborhood of Washington D.C. and extracted its various air-dried parts with a number of apolar organic solvents. The petroleum ether extraction proved most satisfactory for the isolation of artemisinin and its derivatives (Klayman 1985).

Artemisinin has been reported to accumulate in leaves, small green stems, buds, flowers, and seeds (Acton and Roth 1985; Ferreira et al. 1995, Liersch et al. 1986; Martinez and Staba 1988). Its content was found more in leaves and inflorescence, but neither artemisinin nor its precursors were detected in roots (Trigg 1990; Charles et al. 1991). Duke and Paul (1994) showed that artemisinin is sequestered in glandular trichome of *A. annua.* Artemisinin content in full-bloomed flowers was 4–5 times higher than in leaves (Ferreira et al. 1995). The artemisinin yield estimated at different steps of development reveals a possible correlation between plant age and artemisinin content. This is assumed to be due to both an increase in leaf yield and artemisinin content with the progressive increase in plant growth (Singh et al. 1988). Our own observations have revealed that the artemisinin content was highest at full vegetative stage. Some researchers reported that artemisinin content is highest just prior to flowering (Acton and Klayman 1985; ElSohly 1990; Liersch et al. 1986; Woerdenbag et al. 1991, 1993); others found an artemisinin peak at full-flowering stage (Morales et al. 1993; Pras et al. 1991; Singh et al. 1986).

Artemisinin yields reported from plants in China range from 0.01 to 0.5 % (w/w), varieties growing in Siachuan Province showing the highest content. Klayman et al. (1984) reported 0.06 % (W/W) yield from *A. annua* growing wild in Washington D.C. Other reports claim the yield to be 0.09–0.17 % (Liersch et al. 1986; Singh et al. 1986, UNDP/World bank/WHO special program for research/Training in Tropical Diseases 1986). The yields of the related sesquiterpenes, i.e., artemisinic acid and arteannuin B also show variation in their contents. In USA, artemisinic acid content is 8–10 times more than the artemisinin (Jung et al. 1990; Roth and Acton 1987) followed by arteannuin B (Klayman 1993). In India, the yield of arteannuin B (0.27 %) is relatively higher than the other two sesquiterpenes (Singh et al. 1986; Gulati et al. 1996).

A study on effect of levels of nitrogen (0, 50 and 100 kg ha^{-1}) phosphorus (0 and 50 kg ha^{-1}), and potassium (0 and 50 kg ha^{-1}) on growth, oil and artemisinin yield revealed that application of 50 and 100 kgN/ha increased herbage, oil and artemisinin yield by 26.2 and 40.1 %, respectively, compared with control (no nitrogen) (Singh 2000; Jha et al. 2011). The influence of micro-nutrient imbalance on growth and artemisinin contents shows that *A. annua* was very sensitive to boron (B) deficiency. Boron-deficient plants did not show flowering and there was approximately 50 % reduction in artemisinin content. Similarly, artemisinin content declined by 25–30 % in Fe-, Mn-, Zn-, and Cu-deficient plants (Srivastava and Sharma 1990). Effect of plant growth regulators on yield, oil composition, and artemisinin content of *A. annua* under temperate condition was studied in 1998 by Yaseen. Foliar application of IAA at 100 ppm produced significantly higher herb and oil yields than the control, due to increase in plant height, leaf/stem ratio and oil content, and delayed leaf senescence. Although the artemisia ketone in the oil was highest following application of GA_3 and IAA at 150 ppm, the artemisinin content was higher in the plants treated with six ppm triacontanol. Effect of bioregulators, chlormequat and triacontanol, was studied for artemisinin content, growth parameters, and leaf yield. Plants treated with chlormequat were found to have more herbage yield, but the effect of higher dose was not statistically significant (Shukla et al. 1992). Level of ABA in chlormequat-treated plants was higher than in control plants, whereas treatment with triacontanol lowered the abscisic acid level. On the contrary, application of triacontanol increased the level of endogenous GA_3-like components while chlormequat caused reduction in their concentration (CIMAP 1988–1989). According to (Liersch et al. 1986), chlormequat was able to increase the artemisinin contents by 30 %. Local climatic conditions, season of harvesting as well as the post-harvest handling seems to play an important role in the levels of artemisinin content (Chen et al. 1987; Ferreira et al. 1995; Martinez and Staba 1988; Singh et al. 1986). The time of planting seems to play an important role on the yield of essential oils and artemisinin in *A. annua.* Plants planted between September and December produced significantly higher herbage yields [on the basis of fresh weight and dry weight (DW)] as compared to that of plants planted in February. Plants planted in September produced the highest amount of artemisinin. Plants established during pre-winter (August–September) and allowed to grow through the entire winter synthesized and accumulated more artemisinin than plants established during early (October–November) and late (February) winter periods. It was concluded that the artemisinin content was dependent on the weather conditions (Ram et al. 1997). Environmental stress, such as light, temperature, water, and salt significantly alter artemisinin yields (Weathers et al. 1994; Wallaart et al. 2000).

Genetic studies on *A. annua* have confirmed that the diploid plants are $2n = 18$ (CIMAP India 1986–1987). The average artemisinin level in tetraploids was 38 % higher than that of the wild type (diploid) as measured over the whole vegetation period (Wallaart et al. 1999a). A hybrid form of *A. annua* was successfully cultivated in Central Africa. The aerial parts of the plants contained 0.63–0.7 % artemisinin on DW basis (Mueller et al. 2000).

11.4.1 *Effect of Endophytic Bacteria and Fungus on the Growth of A. annua and Artemisinin Production*

Plants constitute a vast and diverse niche for endophytic organisms resulting in the development of closer biological associations between the plant and the endophytes than epiphytes or other soil organisms (Strobel 2003). Evidence has also accumulated suggesting that secondary metabolites in plants actively take part in plant–microbe interactions. Endophytes colonize the internal tissue of the plant and are capable of triggering physiological plant responses (Hardoim et al. 2008; Van Wees et al. 2008) and influencing the production of secondary metabolites in the host plant (Yuan et al. 2007; Satheesan et al. 2012). Wang et al. (2001) purified a chemical elicitor from the extract of the endophytic fungus *Colletotrichum* sp., which could be used to stimulate the accumulation of artemisinin in *A. annua* hairy roots. Artemisinin biosynthesis has also been shown to be enhanced in *A. annua* after treatment with *Piriformospora indica* or arbuscular mycorrhizal fungi (Chaudhary et al. 2008; Kapoor et al. 2007; Varma et al. 2012). A large number of endophytic actinomycetes have been isolated from *A. annua* (Li et al. 2012a, b). However, *A. annua*-endophytic actinomycete interactions have only been rarely documented.

In a preliminary assay, 6-day-old geminated *A. annua* seedlings were inoculated with the endophytic strains YIM 63654, YIM 63673, YIM 63342 YIM 63538, and YIM 63111. The plants were incubated for 66 days, and all treated and untreated seedlings were harvested to determine the plant height and artemisinin content. The height of the seedlings that were inoculated with strains YIM 63654, YIM 63673, YIM 63342, and YIM 63538 were similar to the non-inoculated control plants, whereas the growth of *A. annua* seedlings inoculated with strain YIM 63111 was significantly inhibited when compared with the control plants. However, artemisinin production was significantly elevated upon inoculation with strain YIM 63111 (P, 0.05). The artemisinin content was 2.5760.18 mg g^1 DW compared to 1.9960.09 mg g^1 DW that was observed in the control samples (Li et al. 2012).

11.5 Biotechnological Production of Artemisinin

The commercial sources of most artemisinin are from field grown leaves and flowering tops of *A. annua*, which are subjected to seasonal and somatic variation and infestation of bacteria, fungi, and insects that can affect the functional medicinal content of this plant (Klayman 1985; Luo and Shen 1987). The total organic synthesis is very complicated with low yields, and economically unattractive (Avery et al. 1992; Xu et al. 1986). In view of these problems, artemisinin production from in vitro plant tissue culture has been considered as an attractive

alternative. The biosynthesis of artemisinin was studied in the calli, suspension cells, shoots, and hairy roots of *A. annua* during their cultivation in vitro (He et al. 1983; Tawfiq et al. 1989; Weathers et al. 1994; Paniego and Giulietti 1996; Liu et al. 1997; Nair et al. 1986; Teo et al. 1995). A certain degree of differentiation of *A. annua* tissue cultures is a prerequisite for the synthesis of artemisinin. Paniego and Giulietti (1994) reported that no artemisinin was found in cell suspension cultures of *A. annua*, whereas trace amounts were found in the multiple shoot cultures. Woerdenbag et al. (1993) reported a high percentage of artemisinin content in *A. annua* shoots cultured on 1/2-MS medium supplemented with 0.05 mg/l naphthaleneacetic acid, 0.2 mg/l benzyladenine (BA), and 2 % sucrose. The flowering of *A. annua* was observed in vitro by supplementing with gibberellic acid (GA_3) where artemisinin content reached 0.1 % in *A. annua* plantlets, and the highest artemisinin content in the plantlets was observed in full bloom (Gulati et al. 1996). Most groups did not find artemisinin in root part of *A. annua* plant. However, artemisinin content in the shoot part of cultured plantlet was higher than that in the cultured shoots without roots (Ferreira and Janick 1996; Martinez and Staba 1988). Attempts were also made to improve the artemisinin production by optimizing chemical and physical environmental factors. Wang and Tan (2002) reported the influence of the ratio of NO_3/NH_4 and total initial nitrogen concentration on the artemisinin yield in hairy roots. With the ratio of NO_3/NH_4 at 5:1(w/w), the optimum concentration of total nitrogen for artemisinin production was 20 mM. Under this concentration, artemisinin production was 57 % higher than that in the standard MS medium. Weathers' research group investigated the effects of media sterilization method and types of sugar on growth and artemisinin accumulation of *A. annua* hairy roots. They found that biomass from filter-sterilized medium was greater than that from autoclaved medium, but artemisinin accumulation from filter-sterilized medium was less than that from autoclaved medium. Growth of hairy roots in the medium with sucrose (3.99 g DW/l) was equivalent to the growth in the medium with fructose (3.75 g DW/l) and significantly better than in the medium with glucose (2.16 g DW/l), while the roots that grew in glucose showed a dramatic stimulation in artemisinin content which is three- and twofold higher than that in medium with sucrose and fructose (Weathers et al. 2004). Casein hydrolysate, a source of amino acids and oligopeptides, at low concentration enhances artemisinin production in *A. annua* shoot cultures (Woerdenbag et al. 1993). A combination of BA and kinetin increased the yields of artemisinin in cultured shoots by 3.6- and 2.6-fold (Whipkey et al. 1992). GA3, a plant hormone that can induce blooming, has been reported to improve growth and artemisinin biosynthesis in shoot cultures, root cultures, and plantlets of *A. annua* (Fulzele et al. 1995; Charles et al. 1990; Smith et al. 1997; Weathers et al. 2005). The effects of light irradiation on growth and production of artemisinin were studied in hairy root cultures of *A. annua* L. by Liu et al. (2002). They found that when the hairy roots were cultured under illumination of 3,000 lx for 16 h using several cool-white fluorescent lamps, the DW and artemisinin concentration reached 13.8 g/l and 244.5 mg/l, respectively (Liu et al. 2002). Wang et al. (2001) investigated the dependence of biomass of hairy roots and artemisinin content on

the light spectrum. They found that the highest biomass (5.73 g DW/l) and artemisinin content (31 mg/g) were obtained under red light at 660 nm which were 17 and 67 % higher than those obtained under white light, respectively. Temperature in the range of 15–35 °C also affected growth and artemisinin biosynthesis in the cultured *A. annua* hairy roots. The maximum hairy root growth was found at 25 °C. However, the highest artemisinin content in the root cultures was observed at 30 °C (Guo et al. 2004). Enhancing the artemisinin production by precursor feeding was also investigated. Addition of artemisinin precursors to the medium used for tissue cultures of *A. annua* resulted in a fourfold increase in artemisinin in the tissue and an 11-fold increase in artemisinin in the spent medium (Weathers et al. 1994). The feeding of MVA alone, however, did not induce an enhancement of artemisinin production (Woerdenbag et al. 1993). But the addition of some compounds such as naphtiphine (an inhibitor of the enzyme squalene epoxidase) to the medium improved the artemisinin production. Other additions, such as 5-azacytidine (a gene regulator), colchicine (a gene regulator), miconazole (an inhibitor of sterol demethylase), and terbinaphine (an inhibitor of the enzyme squalene epoxidase), were too toxic for the cultures to induce an enhancement of the artemisinin production (Woerdenbag et al. 1993). Kudakasseril et al. (1987), however, reported a concentration-dependent increase in the levels of artemisinin and growth of shoot cultures with miconazole. Other sterol inhibitors, such as chlorocholine chloride, 2-isopropyl-4-(trimethylammonium chloride)-5-methylphenylpiperidinecarboxylate, and 4-chloro-2-(2-diethylaminoethoxyphenyl)-2-(4-methyl-phenyl)-benzeneethanol, increased both the incorporation of 14C-IPP into artemisinin by cell-free extracts and the production of artemisinin in shoot culture of *A. annua*. Sterol inhibitors inhibited the enzyme in the mevalonate pathway, resulting in increased terpenoid production rather than sterol production (Fig. 11.3; Zhang et al. 2009). To develop more potent antimalarial agents with improved in vivo stability, tremendous efforts have been made toward structure modification of artemisinin and analogue synthesis. Due to the difficulties of structural modification by conventional chemical methods, microbial transformation serves as a valuable tool that comes to play an important role in the modification. To date, a number of oxidating products of artemisinin at different positions of artemisinin structure have been reported. These transformations include conversion to 3α-hydroxydeoxyartemisinin and deoxyartemisinin, conversion to 9β-hydroxy-artemisinin and 3α-hydroxy-artemisinin, and conversion to 10-hydroxy-artemisinin and 9β-hydroxy-11α-artemisinin. In addition, microbial transformations on some artemisinin analogue, such as artemether, arteether, artemisitene, and 12-deoxoartemisinin, have been reported to produce oxidative products by different microorganisms (Liu et al. 2006).

Many studies have been performed trying to enhance artemisinin content using bioengineering methods. Because *A. annua* is easily propagated in vitro, the production of artemisinin by cultures of cells or tissues (Nair et al. 1986; Kudakasseril et al. 1987; Martinez and Staba 1988; Tawfiq et al. 1989; Paniego et al. 1994), transformed hairy root (Qin et al. 1994; Jaziri et al. 1995; Wang et al. 2000; Xie et al. 2000; Kim et al. 2002, 2003; Souret et al. 2003), and shoot cluster

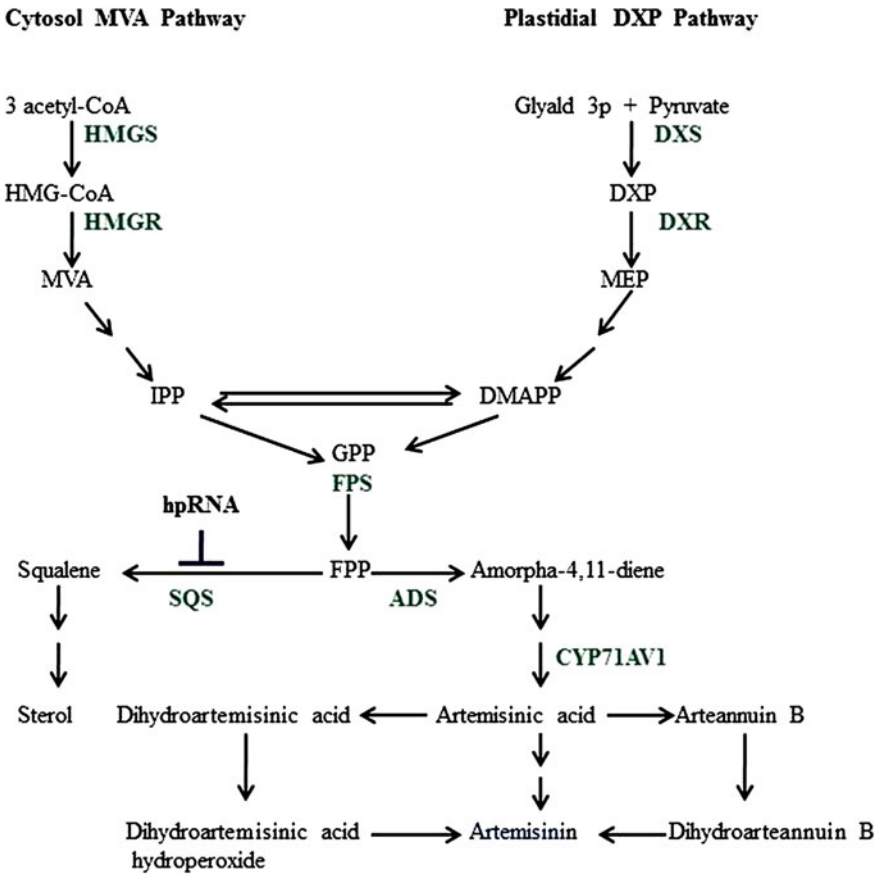

Fig. 11.3 RNAi-mediated suppression of SQS gene (Zhang et al. 2009)

(He et al. 1983; Woerdenbag et al. 1993) has been investigated widely. However, the yield of artemisinin remained low and undifferentiated cell or callus cultures, in particular, contained null or trace amounts of artemisinin. The production of artemisinin in shoot clusters and transformed hairy root is still disappointing at present.

It is envisaged to produce high-artemisinin-yielding transgenic strains of *A. annua* L. plants, which will ensure a constant high production of artemisinin by overexpressing the key enzymes in the terpene and artemisinin biosynthetic pathways, or by inhibiting enzyme(s) of another pathway competing for artemisinin precursors (Fig. 11.3).

In recent years, remarkable progress has been made in the understanding of molecular biology of artemisinin biosynthesis and its regulation (Bouwmeester et al. 1999; Weathers et al. 2006). The genes of the key enzymes involved in the biosynthesis of artemisinin, such as HMG-CoA reductase, FPS, ADS, and the

genes of the enzymes involved in the pathway competing for artemisinin precursors, such as SQS involved in sterol biosynthesis, have been cloned from *A. annua* L. (Matsushita et al. 1996; Mercke et al. 2000; Wallaart et al. 2001; Liu et al. 2003; Abdin et al. 2003). On the other hand, (Weathers et al. 1994; Qin et al. 1994) induced hairy roots in *A. annua* L. employing *Agrobacterium rhizogenes*. Further, the factors influencing transformation efficiency of *A. rhizogenes* were explored to optimize the transformation system by Liu et al. (1998). Xie et al. (2001) induced hairy root in *A. annua* L. leaf blade pieces and petiole segments infected with *A. rhizogenes* strain 1,601 and obtained a clone with high content of artemisinin (1.195 mg/g DW).

To develop transgenic *A. annua* L. strains with high content of artemisinin by modulating the expression of above-mentioned genes, an efficient system of genetic transformation as well as regeneration of explants of *A. annua* L. should be in place. Vergauwe et al. (1996) developed an *Agrobacterium tumefaciens*-mediated transformation system for *A. annua* L. plants with high transformation rates (75 % regenerants harboring foreign gene). Artemisinin content in the leaves of regenerated plants was 0.17 %, a little bit higher than that present in the leaves of normally cultured plants (0.11 % DW). They further investigated the factors, viz. the age of explants, *A. tumefaciens* strain, and plant genotype influencing the transformation efficiency (Vergauwe et al. 1998). Later, (Han et al. 2005) established a high-efficiency genetic transformation and regeneration system for *A. annua* L. via *A. tumefaciens*.

Artemisinic acid is one of the precursors of biosynthesis of artemisinin, which has the cadinene structure. Chen et al. (1998) transformed a cotton cadinene synthase cDNA into the leaf explants of *A. annua* L. using *A. rhizogenes*. In the isoprenoid biosynthesis pathway, *FPS* catalyzes the two sequential 1-4 condensations of IPP with DMAPP to produce GPP and with GPP to give FPP, which is then utilized by isoprenoid pathway and artemisinin biosynthetic pathway to produce isoprenoids and artemisinin, respectively (Cane 1990). The cDNAs encoding *FPS* have been isolated from a number of plant species, including *Arabidopsis thaliana* (Delourme et al. 1994) and *Lupinus albus* (Attucci et al. 1995). Since 15-carbon FPP can be catalyzed by sesquiterpene cyclases, such as, *ADS* to form cyclic sesquiterpenoids (amorpha-4, 11-diene in *A. annua* L.), overexpressing foreign *FPS* gene into *A. annua* L. plants holds the possibility of affecting accumulation of artemisinin. A cDNA encoding cotton FPPS placed under a CaMV 35S promoter was, hence, transferred into *A. annua* L. Plants via *A. tumefaciens* strain LBA 4404 or *A. rhizogenes* strain ATCC 15834 mediated genetic transformation (Chen et al. 1999, 2000). In the transgenic plants, the concentration of artemisinin was approximately 8–10 mg/g DW, which was 2- to 3-fold higher than that in the control plants. Han et al. (2006) achieved about 34.4 % increase in artemisinin content by overexpressing FPS. We have overexpressed one of the key regulatory enzymes of MVA pathway (HMGR) in *A. annua* L. plants via *A. tumefaciens*-mediated transformation and achieved 39 % enhancement in artemisinin contents as compared to control plants (Tazyeen et al. 2010). Jing et al. (2008) simultaneously overexpressed cyp71av1 and cpr genes in

A. annua L. and recorded 2.4-fold enhancement in artemisinin content. The cytokinin biosynthetic gene codes for the enzyme isopentenyl transferase (ipt), which catalyzes the condensation of isopentenyl pyrophosphate and adenosine monophosphate (AMP) to yield isopentenyl AMP, are believed to represent the rate-limiting step in cytokinin biosynthesis in tumorous plant tissue (Akiyoshi et al. 1983, 1984). The influence of overexpression of isopentenyl transferase gene on the physiological and biochemical characteristics of *A. annua* L. plant was studied by Geng et al. (2001). The transgenic *A. annua* L. plants were found to accumulate more cytokinins (2–3-fold), chlorophyll (20–60 %), and artemisinin (30–70 %), when compared with control plants (Geng et al. 2001). Previous studies indicated that capitate glands on the leaf surface (Duke and Paul 1994) and specialized chloroplasts of the capitate gland appeared to play very important role in artemisinin biosynthesis (Duke and Paul 1993). Light affects to terpene biosynthesis in general and artemisinin biosynthesis in particular by modulating carbon flux through regulation of HMG-CoA reductase, a key regulatory enzyme in mevalonate pathway. In case of potato, it has been reported that light regulates *HMGR* at both transcriptional and translational level (Korth et al. 2000). In *A. annua* L., β-pinene synthase was found to have a circadian pattern of gene expression, accompanied by a similar temporal pattern of β-pinene emission under light exerting a stimulatory effect (Lu et al. 2002). Analysis of root cultures of *A. annua* L. suggested that light also positively regulates artemisinin biosynthesis because the root cultures exhibited a substantial decrease in artemisinin content when moved from light to dark (Liu et al. 1997, 2002; Guo et al. 2004). Hong et al. (2009), hence, overexpressed *Arabidopsis* blue light receptor CRY1 in *A. annua* L. to evaluate its effect on artemisinin synthesis and accumulation. They found that overexpression of *CYP1* gene had resulted in increased accumulation of both artemisinin (30–40 %) and anthocyanins (2-fold) as compared to control plants.

11.5.1 Combinatorial Biosynthesis of Artemisinin

Naturally occurring terpenoids are produced in small quantities, and thus, their purification results in low yields. Further, the complex structure of these molecules makes chemical synthesis challenging and often uneconomical due to poor yields. Metabolic engineering of these pathways in a common industrial biological host (*Escherichia coli*) offers an attractive alternative to extractions from plants or chemical syntheses for producing large quantities of these complex molecules. To accomplish this goal may require altering the MVA and the MEP pathways along with addition of very specialized enzymes, e.g., ADS. Based on preliminary work by others who described engineering of the MEP pathway to increase isoprenoid precursors for high-level production of carotenoids (Kajiwara et al. 1997; Farmer and Liao 2001; Kim and Keasling 2001; Abdin et al. 2003), Keasling's group further developed a base technology for production of amorphadiene in *E. coli* (Martin et al. 2003). Bacteria already contain the MEP pathway for production of

IPP/DMAPP, but they lack the MVA pathway. Keasling's group posited that the MEP pathway is likely linked to unknown control elements in bacteria and that direct alteration might impair growth. Instead, they added a truncated MVA pathway from *Saccharomyces cerevisiae* that was coupled to ADS in *E. Coli* resulting in good bacterial growth and high-level production of amorphadiene estimated at 100 mg l 21 in 12 h. Keasling's work is important because these engineered *E. coli* strains can serve as platform hosts for the production of essentially any terpenoid for which the biosynthetic genes are available because IPP and DMAPP produced by either arm of the terpenoid pathway are universal precursors to all terpenoids. More recently, (Teoh et al. 2006) have isolated the next enzyme in the artemisinin pathway, a cytochrome P450 enzyme (CYP71AV1); this enzyme appears to catalyze the next three steps in artemisinin biosynthesis, an enzymatic function also confirmed by Keasling's group (J. Keasling, personal communication). Once cloned into a bacterial host and after optimization of the culture conditions, it should be possible to produce very large quantities of a close precursor to artemisinin in *E. coli*, thus making this important drug readily available in much larger quantities than previously thought possible. The concept of *E. coli* as a host cell producing sesquiterpenoids out of the endogenous pool of FDP has been investigated (Martin et al. 2001). This work resulted in the production of 10.3 μg of (+)-δ-cadinene, 0.24 μg of 5-epi-aristolochene, or 6.4 μg vetispiradiene per liter of bacterial culture. Furthermore, the authors concluded that the poor expression of the plant terpene cyclases was limiting for the synthesis of sesquiterpenes and not the endogenous supply of FDP. This has been confirmed in their further work by coexpressing the *E. coli dxs* gene, which did not result in an increase in sesquiterpenoids produced where it did result in an increase in lycopene production in *E. coli* (Harker and Bramley 1999; Kim and Keasling 2001). To overcome the low enzyme levels, the expression of amorphadiene synthase has been optimized by constructing a synthetic amorphadiene synthase gene completely optimized for the expression in the bacterial host. This strategy has been combined with engineering of genes from the mevalonate-dependent isoprenoid pathway (Fig. 11.4), which resulted in an *E. coli* strain producing 24 μg/ml amorpha-4,11-diene (calculated as caryophyllene equivalent) from acetyl-CoA after supplementation of 0.8 % glycerol (Martin et al. 2003).

Recently, attempts to use *S. cerevisiae* for the production of artemisinin precursors have been described. The expression of the amorphadiene synthase gene in yeast using plasmids and chromosomal integration led to the production of, respectively, 600 and 100 μg/amorpha-4, 11-diene after 16 days batch cultivation (Lindahl et al. 2006). Using a *S. cerevisiae* strain containing an engineered MVA pathway coupled with the genes encoding amorphadiene synthase and CYP71AV1, the production of artemisinic acid up to 100 mg/l has been reported (Ro et al. 2006). This strain transported the artemisinin precursor outside the yeast cell, which makes purification of the product less complex. Artemisinic acid can be used for the semisynthesis of artemisinin, but to lower the costs for production of the drug, bioprocessing must be optimized (Liu et al. 1998).

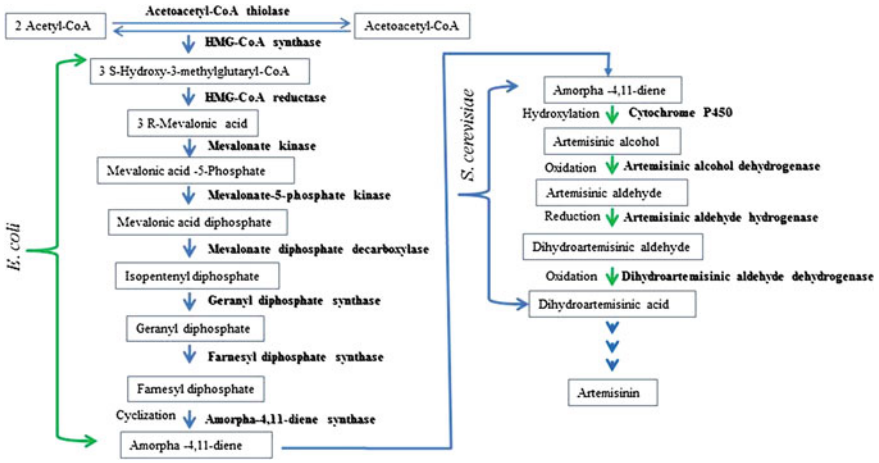

Fig. 11.4 Combinatorial biosynthesis of artemisinin starting from acetyl coenzyme A

Dafra Pharma International NV and Plant Research International (PRI) have initiated new research to produce artemisinin via genetically modified chicory plants. In studies carried out at Wageningen, the complete biosynthetic pathway of artemisinin was resolved (de Kraker et al. 2003; Bertea et al. 2005; Fig. 11.5).

In addition, the Wageningen group, headed by Prof. Harro Bouwmeester and Dr. Maurice Franssen, demonstrated that chicory enzyme(s) normally involved in the biosynthesis of the bitter sesquiterpene lactones in chicory, were capable of performing reactions required for the biosynthesis of artemisinin (de Kraker et al. 2003). The group of Prof. Bouwmeester has tried to produce the chemical

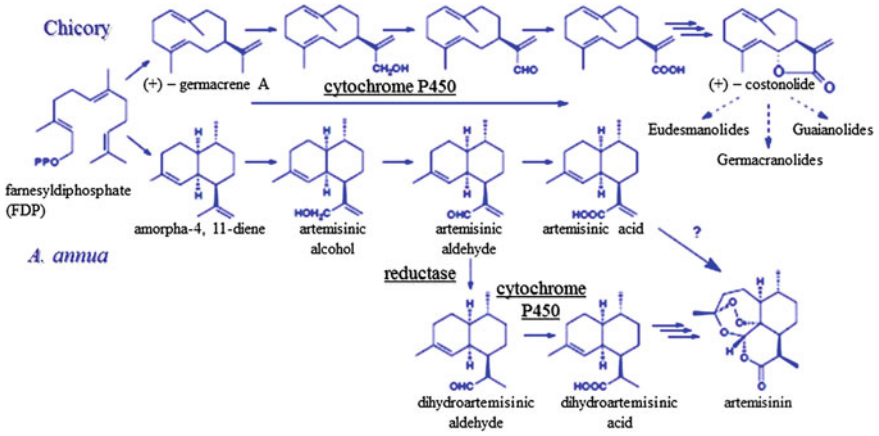

Fig. 11.5 Biosynthetic routes of bitter sesquiterpene lactones in chicory and artemisinin in *Artemisia annua* (Bertea et al. 2005; de Kraker et al. 2003)

precursor for artemisinin (dihydroartemisinic acid) in the roots of chicory via a diversion of the biosynthesis of bitter compounds. On the other hand, the group of Prof. Bouwmeester has shown in a wide range of plant species that diversion of the biosynthesis of terpenes can be carried out very efficiently (Kappers et al. 2005). Moreover, they also demonstrated that up to 40 kg ha^{-1} dihydroartemisinic acid can be produced using genetically modified chicory.

11.5.2 Breeding and Marker-Assisted Breeding

Because the *A. annua* is a hybrid species, these transformed strains can only be preserved in flasks in the laboratory and the characteristic of high artemisinin content will be lost through sexual propagation. Although the transgenic strains can be multiplied on a large scale using micropropagation methods, the cost is high.

By bringing herbs into cultivation, traditional and biotechnological plant-breeding techniques can be applied at the genetic level to improve yield and uniformity, and to modify potency or toxicity. The high heritability and useful range of variation for artemisinin suggests that the development of molecular tags for the trait and their exploitation in a marker-assisted breeding program are feasible (Delabays et al. 2001). Although the impact to date has been minimal, it is certain that the '-omics' revolution, as it spreads out from model species to those with more complex genomes (so-called muddle species) will influence research and exploitation of medicinal species as it will plant in general.

Increasing the production of active phytochemical constituents is a well-established target for genetic manipulation but presents some severe challenges. In particular, the metabolic pathways by which active compounds are biosynthesized are mostly poorly understood, and relatively few genes for key enzymatic or regulatory steps have been isolated. Nevertheless, there are examples of pathway engineering leading to improvements of potential value in the breeding of medicinal plants (Ferreira and Duke 1997; Charlwood and Pletsch 2002).

The artemisinin content is distinctly different in *A. annua* of diverse origins. Although the artemisinin content in leaves is influenced by environmental factors and the developmental situation of the herb, the primary factor contributing to variations in the artemisinin content is genetic (Delabays et al. 2001). So, it is important to analyze genetic variations of different strains of *annua* that have different artemisinin contents. To evaluate the availability of the genetic variability of strains of *A. annua*, (Zhang et al. 2006) performed RAPD analysis of selected chemotypes. The data clearly supported the conclusion of distinct variation of heredity among these chemotypes (Sangwan et al. 1999). Studying the variation of heredity between high- and low-yielding strains by RAPD techniques is more accessible for the selection and breeding of high-yielding strains of *A. annua*. In the present study, the OPA151000 band could be used as a marker to predict the strains with high artemisinin content.

A recent project in our department includes the study of the production of an antimalarial compound, artemisinin in *A. annua*. Artemisinin is currently still extracted from the plant itself, but due to the low amount of artemisinin in the plant, there is a shortage in the production (Cyranoski 2004). In the case of *A. annua*, it has been shown that the genetic variability linked to artemisinin content can be used to generate improved high-yielding varieties (Delabays et al. 2001); thus, it is likely that genetic factors will be identified that are involved in the low or high artemisinin trait. A biosynthetic pathway of artemisinin has been proposed, but most of the genes underlying the synthesis and the control of it, are not yet identified (Bertea et al. 2005; (Bouwmeester et al. 1999) in press). In our department, the biosynthesis of artemisinin will be studied using cDNA–AFLP gene profiling. The knowledge of genes activated upon artemisinin biosynthesis, combined with metabolome data from the same time points as used for the transcript profiling, will allow us to identify key genes, encoding enzymes and/or transcription factors involved in the biosynthesis of artemisinin and the regulation of this pathway. This information will be the basis for a more detailed study, which will ideally give the information needed to engineer a high-artemisinin-producing plant through metabolic engineering.

11.5.3 Current Status and Future Prospective

Knowledge of the exact biosynthesis of artemisinin should enable us to influence its formation in a direct way, for example by metabolic engineering. As an alternative to targeting an individual rate-limiting enzyme reaction, exploiting transcription factors that turn whole secondary pathways on or off shows great promise as a metabolic engineering strategy. New genomic approaches and efficient gene isolation methods applied to difficult secondary pathways in medicinal plant metabolism will undoubtedly expand the range and precision of manipulations via transgenesis, providing potentially superior material for the breeder. Plant natural products have been a very productive source in drug development. The study of plant secondary metabolism is a fully expanding and challenging field in molecular biology and biotechnology, with many opportunities ahead. New tools of functional genomics combined with metabolomics and proteomics will revolutionize our knowledge on the pathways and enzymes involved in the synthesis of natural products and thus allow a more focused approach for their production. With the increasing need for novel drugs for newly identified molecular targets, this field will likely become increasingly relevant. The appealing economic aspects of large-scale production of pharmaceuticals in plants could attract increasing investments and create new opportunities in this promising research field. It would be interesting to develop transgenic plants of *A. annua* to ensure a constant high production of artemisinin after the introduction of genes encoding enzyme(s) regulating the biosynthesis pathway of artemisinin.

11.5.4 Conclusion and Summary

A. annua is the main source of artemisinin, the potent and efficacious antimalarial after quinine. Recently, artemisinin has also been proved to be a selective anticancer drug (Moore et al. 1995; Efferth et al. 2001). Currently, the limited availability of artemisinin and the lack of real competition among producers of raw material seem to be major barriers to scaling-up production and are partially responsible for its high price (World Bank 2003). Also, the lack of affordable certified seeds hampers the extension of *A. annua* cultivation around the world. Breeding high-yielding, late-flowering cultivars of *A. annual* adapted to the tropics, where malaria is endemic, is a desirable approach that needs to be pursued. Scientists are trying to understand the intricate and self-regulated biosynthetic pathway of artemisinin, its potential increase by the manipulation of terpene cyclase genes, although commercially feasible results are still to be seen. Currently, the hope to curb malaria rests on hampering the spread of the disease by mosquito vectors, on the availability of an effective and affordable vaccine, on the widespread use of insecticide-treated nets, on new antimalarial drugs effective against multidrug-resistant Plasmodium, and on meeting the world demand for artemisinin-combination treatments. Of course this last factor depends on a steady production of artemisinin, at affordable prices, to meet global demand. Although field production of *A. annua* is presently the most commercially feasible approach to produce artemisinin and related compounds, farmers must have access to good-quality seed generated from high-artemisinin parents. Although these seeds do not constitute 'true hybrids' because the parents are not homozygous, artemisinin content found currently in seeds available for research is approximately twice as high as it was 10 years ago (1.0 % compared to less than 0.5 %). Also, the agricultural aspects of artemisinin production such as soil fertility and pH, plant density, water availability, latitude and altitude, hormones, harvesting and drying protocols must be fine-tuned for each geographic area where artemisinin is to be produced as a raw material.

In addition, factors that affect temporal (when artemisinin reaches its maximum) or spatial (tissue localization) accumulation must not be ignored when evaluating the commercial potential of *A. annua* as a new crop for tropical or temperate regions. Artemisinin-based combination therapies (ACTs) have been long considered more effective than the existing drugs. ACTs are much more expensive than other drugs because of the relatively low yields of artemisinin in *A. annua*. Therefore, there have been many efforts to enhance the production of artemisinin in vivo and in vitro by biotechnology. Even though viable methods of increasing artemisinin content, e.g., *A. annua* organ culture, hormone medium, and metabolic manipulation, have been investigated and show potential for future development, the improvements delivered by these methods have not yet met the demand. To increase the yield of artemisinin by biotechnology, it is necessary to study the enzymatic pathway. Enzymes and precursors involved in the artemisinin biosynthesis have to be isolated and characterized. In recent years, many

researchers have focused their efforts on investigating the molecular regulation of artemisinin biosynthesis and the genes coding for the key enzymes involved in the artemisinin biosynthesis. The high efficiency of genetic transformation and regeneration procedure developed by (Han et al. 2006) allows the manipulation of artemisinin biosynthesis by genetic methods. By genetic engineering, we can overexpress the key enzymes involved in biosynthesis of artemisinin or inhibit the enzymes involved in other pathways competing for its precursors to obtain transgenic high-yielding. *A. annua*. Although greatly improved yields were obtained by combining the expression of a synthetic sesquiterpene synthase with a recombinant mevalonate pathway, the data suggest that a maximum yield was not attained. Furthermore, in vitro evolution and combinatorial biosynthesis of sesquiterpene biochemical pathways in microbes may lead to artemisinin derivatives or even new sesquiterpene compounds. Efforts, therefore, are being made to enhance the production of artemisinin both in vivo and in vitro. Chemical synthesis of artemisinin is very complex and uneconomical. Breeding of high-artemisinin-yielding plants as well as the manipulation of culture conditions, growth media, and hormone levels to increase the yield of artemisinin in tissue and cell culture have not been successful. It is, therefore, essential to look for non-conventional, alternate strategies, which are economically viable for commercial production of artemisinin. Two approaches can be used to achieve this goal. The first approach could be the use of a semisynthetic route for the synthesis of artemisinin from its simple precursors such as artemisinic acid and arteannuin B. The second approach could involve the use of genetic engineering to overexpress enzyme(s) catalyzing the rate-limiting steps of artemisinin biosynthesis or by using antisense RNA technology to inhibit the enzyme(s) of other pathway competing for its precursors.

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Chapter 12
Artemisinin in Cancer Therapy

Bianca Ivanescu and Andreia Corciova

12.1 Overview

Cancer is one of the leading causes of death worldwide, with approximately 12.7 million new cases and 7.6 million deaths annually (GLOBOCAN 2008, http://globocan.iarc.fr/factsheets/cancers/all.asp). Conventional cancer therapies, such as chemotherapy, surgery and radiotherapy, have limited efficiency, high toxicity and are often associated with the development of drug resistance. In this context, plants remain an important source of anti-cancer drugs, as exemplified by paclitaxel, vincristine, vinorelbine, teniposide or camptothecin. In effect, 42 % of all approved cancer drugs are natural products or agents derived from natural products and semisynthesis (Newman and Cragg 2007). The anti-malarial artemisinin and its derivatives are new promising anti-cancer compounds. Artemisinin is a potent anti-cancer drug, active on drug- or radiation-resistant cell lines, with virtually no side effects on normal cells. Artemisinin is highly selective in killing cancer cells and has a broad spectrum of action, being effective against many types of cancers (Woerdenbag et al. 1993; Efferth et al. 2003b).

Artemisinin is a naturally occurring sesquiterpene lactone with a 1,2,4-trioxane ring system. The cleavage of its endoperoxide bridge and formation of free radicals are essential events for anti-malarial action, as well as for anti-cancer activity. Some of the shortcomings of artemisinin, such as low solubility, short plasma half-life and poor bioavailability (Ashton et al. 1998; Li et al. 2007), were surpassed by the semisynthetic or fully synthetic artemisinin derivatives (Haynes 2006; Jefford 2007). In order to increase the cytotoxicity towards cancer cells, artemisinins can be tagged to cancer cell–targeting molecules (Lai et al. 2013).

Although in recent years, many studies focused on the anti-tumour mode of action of artemisinin, the underlying molecular mechanism is not fully understood.

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Artemisinins exert their anti-cancer activity by disrupting many cellular signalling pathways. They manifest anti-proliferative, proapoptotic, anti-metastatic and anti-angiogenic activity (Crespo-Ortiz and Wei 2012). This multi-modal mechanism of action could explain the effectiveness of artemisinin compounds in multi-drug-resistant types of cancer. Another advantage of artemisinins in cancer therapy is the possibility to be used in synergic combinations with traditional chemotherapeutic drugs, thus reducing the dosage and the side effects of these drugs (Crespo-Ortiz and Wei 2012; Lai et al. 2013).

12.2 Generation of Free Radicals: Determining Factor of Cytotoxicity

Artemisinins cytotoxicity was first reported by Woerdenbag et al. (1993). Artemisinin and its derivatives exhibited potent activity against Ehrlich ascites tumour cells, with half maximal inhibitory concentration (IC_{50}) values ranging from 1.4 to 29.8 μM (Woerdenbag et al. 1993). These drugs mainly induced growth inhibition, the presence of the endoperoxide moiety being crucial for the cytotoxic effect (Beekman et al. 1996, 1998). Artesunate, the sodium salt of the hemisuccinate ester of artemisinin, is active against leukaemia, colon, melanoma, breast, ovarian, prostate, central nervous system and renal cancer cells (Efferth et al. 2001). Dihydroartemisinin (DHA), the main active metabolite of the artemisinin derivatives, shows prominent activity against pancreatic, leukaemic, osteosarcoma, ovarian and lung cancer cells (Jiao et al. 2007; Chen et al. 2009a; Lu et al. 2009; Zhou et al. 2008; Ji et al. 2011). Artemisone, a new derivative with improved pharmacokinetic properties, has manifested better activity than artemisinin against breast, colon, melanoma and pancreas cancer cells and significant synergistic interactions with other anti-cancer drugs (Gravett et al. 2011).

The cytotoxicity of artemisinins can be chiefly attributed to the endoperoxide bond in their molecules. Derivatives lacking the endoperoxide moiety have considerably reduced anti-cancer activity (Galal et al. 2002). The anti-cancer mode of action is based on the iron-mediated cleavage of the endoperoxide bridge, resulting in the formation of reactive oxygen species (ROS) (Efferth et al. 2004; Efferth 2006) and/or highly alkylating carbon-centred radicals that destroy tumour cells (Mercer et al. 2007, 2011).

The mechanism by which the bioreductive activation of artemisinin in tumour cells takes place is still unclear, although iron and haem or haem-bound proteins are involved in artemisinins cytotoxicity (Zhang and Gerhard 2009; Hamacher-Brady et al. 2011). Thus, preloading cancer cells with iron or iron-saturated holotransferrin dramatically increased cytotoxicity of artemisinins (Lai and Singh 1995; Lu et al. 2010b) and inhibited tumour growth (Lai et al. 2009; Moore et al. 1995). Also, cytotoxicity of dihydroartemisinin is significantly reduced by the iron chelator desferoxamine (Huang et al. 2007). Haem is a mediator of artemisinin

cytotoxicity as shown by studies using promoters or inhibitors of haem synthesis in tumour cells; the first ones increased, whereas the latter decreased the cytotoxicity of artemisinin compounds (Stockwin et al. 2009; Zhang and Gerhard 2009; Mercer et al. 2011).

The higher iron influx rates of cancer cells compared to normal cells can explain the high selectivity of artemisinin and its derivatives against tumour cells. The same property can account for artemisinins low toxicity and efficiency in many different types of cancers. For instance, dihydroartemisinin is 100 times more toxic to human leukaemia cells than to normal lymphocytes (Lai and Singh 1995). Cancer cells require a large amount of iron to sustain continuous proliferation (Reizenstein 1991). Iron cellular uptake involves the serum iron-binding protein transferrin and the cell surface transferrin receptors. The iron-bound transferrin (holo-transferrin) is engulfed and internalize through receptor-mediated endocytosis (Lee et al. 2001; Gomme et al. 2005). Due to their higher iron metabolism, cancer cells express a great concentration of transferrin receptors on cell surface (Qian et al. 2002; Gomme et al. 2005). For example, breast cancer cells display 5–15 times more transferrin receptors on their cell surface than normal breast cells (Reizenstein 1991).

Iron-activated artemisinin induces formation of free radicals that in turn produce cell alterations such as apoptosis, deoxyribonucleic acid (DNA) damage, arrest of growth, inhibition of angiogenesis, inhibition of tumour invasion, migration and metastasis (Crespo-Ortiz and Wei 2012). For instance, artesunate caused DNA fragmentation and membrane damage through ROS-mediated mechanism in pancreatic cells. Low concentration of artesunate induced oncosis-like cell death, while higher doses induced apoptosis (Du et al. 2010). Anti-cancer activity of artemisinin compounds has been directly correlated with ROS levels (Michaelis et al. 2010). On the other hand, the use of anti-oxidant agents reverted artemisinins activity in several experiments (Kim et al. 2006; Michaelis et al. 2010; Stockwin et al. 2009). Although many studies confirm that ROS-mediated damage (Hamacher-Brady et al. 2011; Lu et al. 2010a, b) is involved in anti-cancer mechanism of artemisinins, cell damage has been observed independent from oxidative stress. For example, some artemisinin dimers manifest anti-tumour activity without the formation of ROS (Beekman et al. 1998), and it is possible that necrosis induced by artemisinin is also independent of ROS generation (Mercer et al. 2011).

The production of ROS may concur to the selectivity of artemisinin compound towards cancer cell, considering that tumour cells have lower expression of anti-oxidant enzymes and are more vulnerable to oxidative stress (Bostwick et al. 2000; Das 2002). Efferth T. demonstrated the existence of an inverse correlation between activity of artesunate and the baseline anti-oxidant mRNA gene expression in the US National Cancer Institute (NCI) cell line panel (Efferth et al. 2003a; Efferth and Oesch 2004; Efferth and Volm 2005). Over-expression of enzymes associated with oxidative stress, such as catalase, thioredoxin reductase, superoxide dismutase and glutathione S-transferase, correlated significantly with resistance of tumour cells to artemisinins (Efferth et al. 2003b; Efferth 2006).

12.3 Molecular Mechanism of Anti-cancer Action

Unlike the anti-malarial activity, where protein alkylation in parasites is the established mode of action, a definitive molecular target is yet to be uncovered in the anti-cancer mechanism of artemisinins. The sarcoendoplasmic Ca^{2+} ATPase (SERCA), the translationally controlled tumour protein (TCTP) and haem are potential molecular targets of artemisinins cytotoxicity. Artemisinin compounds have the ability to kill cancer cells through multiple molecular events. The anti-cancer activity of artemisinins is influenced by endoplasmic reticulum (ER) stress (Lu et al. 2010a, 2011), calcium metabolism (Mu et al. 2007, 2008; Noori et al. 2010a) and the expression of TCTP (Efferth 2007).

Given the fact that SERCA is a target of artemisinin in *Plasmodium* parasites, the role of SERCA in artemisinin cytotoxicity has also been explored. Early research states that artemisinins does probably not alkylate human SERCA (Uhlemann et al. 2005). Another study reports that artemisinin rapidly decreased the activity of SERCA in intestinal cancer cells, leading to increased calcium levels (Riganti et al. 2009). Cancer cells treated with two highly active artemisinin dimers showed DNA damage, iron/haem and cysteine/methionine metabolism alterations, anti-oxidant response and ER stress (Stockwin et al. 2009). Both dimers possess the same SERCA inhibitory activity that leads to ER stress, although the deoxy-derivative has negligible anti-cancer activity, meaning that inhibition of SERCA plays a minimal role in ER stress induction and overall activity (Stockwin et al. 2009).

TCTP represents a proliferation-related Ca^{2+}-binding protein, which associates transiently with microtubules during the cell cycle and can be a common target of both parasites and cancer cells (Gachet et al. 1999). TCTP has been implicated in cell growth, cell cycle progression and malignant transformation and in the protection of cells against various stress conditions and apoptosis (Bommer and Thiele 2004). Tumour cell lines with high TCTP expression were sensitive to artesunate, while a low TCTP expression was associated with resistance to artesunate (Efferth 2005).

Intracellular haem, Fe^{2+} protoporphyrin IX, is the mediator of the cytotoxic effects of artemisinins. Increasing intracellular haem synthesis enhanced the cytotoxicity of dihydroartemisinin, while decreasing haem synthesis reduced its action. The cytotoxicity of artemisinin was found to depend on the activity of synthetic haem (Zhang and Gerhard 2009), which indicates that cellular haem may also serve as a general target of artemisinins for the development of anti-malarial and anti-cancer activity.

It is possible that growth inhibition effects of artemisinins on tumour cells may be due to alkylation of many different protein species, rather than a target protein. For instance, nitric oxide synthase may represent a major cellular target of artesunate in killing tumour cells. Artesunate may exert the anti-tumour activity by alkylating haem-harbouring nitric oxide synthase, as demonstrated by a recent study in a hepatoma cell line, HepG2 (Zeng and Zhang 2011).

Furthermore, artemisinins may alkylate other molecules in cancer cells, such as DNA. Although previous studies suggested that the anti-cancer effect of artemisinin may not be attributed to genotoxicity (Efferth et al. 2002), it has been shown that artesunate induces dose-dependent DNA damage, specifically double-strand breaks, in a hamster lung fibroblast cell line (Li et al. 2008). Furthermore, DHA acts by generating ROS and possible genotoxic damage, as observed in rat glioma cells by employing ROS scavengers (Huang et al. 2008). Also, artesunate was identified as a novel topoisomerase inhibitor, as shown by gene expression profiling research on pancreatic cancer cells (Youns et al. 2009). Topoisomerase inhibitors block the ligation step of the cell cycle, generating single- and double-stranded breaks that induce genotoxicity. The introduction of these break subsequently lead to apoptosis and cell death (O'Neill et al. 2010).

Artemisinin and its derivatives exhibit potent anti-cancer activity through pleiotropic effects in a variety of human cancer cell model systems. These effects include growth inhibition by cell cycle arrest, apoptosis, inhibition of angiogenesis and metastasis, and modulation of nuclear receptor responsiveness (Firestone and Sundar 2009).

12.3.1 Anti-proliferative Effects of Artemisinins

Uncontrolled proliferation in cancer cells is the result of mutations inducing amplification of growth signals, deregulation of checkpoints and loss of sensitivity to growth inhibitors (Vogelstein and Kinzler 2004). Artemisinin and its derivatives induce cell growth arrest in cancer lines that involves selective changes in the expression and activity of cell cycle components. These modifications are different depending on the tissue origin and type of cancer (Firestone and Sundar 2009).

Artemisinins induce growth arrest at all cell cycle phases, but most commonly at G0/G1 to S transition seems (Efferth et al. 2003b; Li et al. 2009; Lijuan 2010). Disruption of the cell cycle at G2/M was also noticed after dihydroartemisinin (Jiao et al. 2007; Zhou et al. 2007; Ji et al. 2011) or artesunate treatment (Efferth et al. 2003b). The growth arrest mechanisms include alterations in the expression and activity of regulatory enzymes of the cell cycle (Firestone and Sundar 2009).

12.3.2 Artemisinins Induce Apoptosis in Cancer Cells

Apoptosis, the process of programmed cell death, depends on the ratio between proapoptotic and anti-apoptotic genes and their effects on mitochondria. An increase in this ratio induces the release of cytochrome c followed by sequential activation of caspases and cell death (Elmore 2007). Artemisinin and its derivatives induce apoptosis via intrinsic pathway, by modulating the level of expression of

anti-apoptotic (Bcl2) and proapoptotic (Bax) genes in cancer cells (Efferth et al. 2007; Morissey et al. 2010; Karnak and Xu 2010; Lu et al. 2010b; Aung et al. 2011).

DHA and artesunate caused cytochrome c release, Bax over-expression, increase in Bax/Bcl2 ratio and activation of caspases 3 and 9 in a panel of osteosarcoma cells. Furthermore, artesunate depletes survivin (an inhibitor of apoptosis), and DHA activates caspase 8 and decreases the levels of cyclin B1, NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B cells) and CDC25B (a dual specific phosphatase involved in the activation of cyclin-dependent kinases) (Ji et al. 2011; Xu et al. 2011). The apoptotic effect of DHA has also been associated with upregulation of NOXA—a proapoptotic protein (Cabello et al. 2012), increased calcium concentration and activation of p38 mitogen-activated protein kinase (Mu et al. 2008). Survivin has also been involved in the apoptotic response of DHA in lung cancer cells (Mu et al. 2007).

12.3.3 Anti-metastatic Effect of Artemisinins

Metastasis is a complex process that involves the spread of a tumour to distant parts of the body from its original site, and it is associated with elevated mortality and morbidity in patients with cancer. One of the advantages of artemisinin compounds is their potent activity against highly aggressive and invasive types of cancer (Mu et al. 2007; Rasheed et al. 2010; Weifeng et al. 2011). This activity has been correlated with modified expression of the matrix metalloproteinase gene family involved in metastasis (Buommino et al. 2009; Hwang et al. 2010). In addition, artemisinin inhibits metastasis by enhancing E-cadherin activity, a calcium-binding molecule involved in cell adhesion (Weifeng et al. 2011). Also, artemisinin retarded lymphoid node metastasis and lymphangiogenesis by inhibition of vascular endothelial growth factor C, in mouse lung Lewis cancer (Wang et al. 2008a).

12.3.4 Artemisinins Inhibit Angiogenesis

Cancer cells induce neovascularization in order to provide the extra blood supply required by the growing metastases and solid tumours. Artemisinin and its derivatives inhibit neovascularization by downregulating growth factors and upregulating angiogenesis inhibitors (Anfosso et al. 2006). Different studies showed that the anti-angiogenic effect of artemisinins is mediated by downregulation of vascular endothelial growth factor, fibroblast growth factor (Chen et al. 2004; Zhou et al. 2007), hypoxia-inducible factor 1 alpha (He et al. 2011 b; Huang et al. 2007), new vessel mediator angiogenin, the cysteine-rich angiogenic inducer, some metalloproteinases (MMP9, MMP11 and BMP1) and collagens (Anfosso et al. 2006). In pancreatic cancer, dihydroartemisinin inhibits angiogenesis by targeting the NF-κB pathway (Wang et al. 2011a), reduced levels of NF-κB being

previously associated with proliferation and metastasis inhibition (Li et al. 2009; Hwang et al. 2010; Wang et al. 2010).

12.3.5 Anti-inflammatory and Immunomodulatory Activity of Artemisinins

Artemisinin compounds also exhibit anti-inflammatory and immunomodulatory actions, as reported in numerous studies. These properties can be beneficial in tumour treatment and can enhance the success of cancer therapy.

It has been shown that artemisinin exerts an anti-inflammatory effect on phorbol myristate acetate (PMA)-induced THP-1 human monocytes, by inhibiting the secretion of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in a dose-dependent manner (Wang et al. 2011b). Topical administration of artemisinin demonstrated anti-inflammatory and immunomodulatory effects in a mouse model of contact hypersensitivity, a T-cell-mediated cutaneous inflammatory reaction (Li et al. 2012).

Artesunate inhibits production of IL-1β, IL-6 and IL-8 in human rheumatoid arthritis through inhibition of NF-κB-signalling pathway (Xu et al. 2007). Moreover, artesunate is therapeutically relevant to inflammatory responses of microglial cells (Lee et al. 2012).

Oral treatment with a novel artemisinin derivative (SM905), in collagen-induced arthritis in mice, suppressed the enhanced expression of pro-inflammatory cytokines, chemokines and chemokine receptors in draining lymph nodes, delaying disease onset and reducing its severity (Wang et al. 2008b). Another artemisinin analogue (SM934) had therapeutic effects on lupus-prone mice by inhibiting the pathogenic helper T-cell development and enhancing anti-inflammatory cytokine IL-10 production (Hou et al. 2012).

Both artemissinin and DHA are immunosupressive agents, as demonstrated by the fact that they suppress delayed hypersensitivity to sheep blood cells in mice (Noori et al. 2004). DHA also reduced growth of ductal carcinoma in mice and decreased the levels of IL-4 (Noori and Hassan 2011). Furthermore, artemisinin exhibits an immunomodulatory effect in murine breast cancer model by reducing the number of regulatory T cells whose accumulation correlates with tumour progression (Langroudi et al. 2010).

12.4 Artemisinins Resistance and Toxicity

One of the important benefits of artemisinin is the ability to delay the emergence of resistance in malignant cells due to its pleiotropic action. Hence, only few cancer cell lines have shown low sensitivity or no response to artemisinin compounds.

Artemisinin seems to be less active in breast cancer cells (MCF-7) and gastric cancer (MKN) (Buommino et al. 2009). A low response to artemisinin has also been observed in highly metastatic nasopharyngeal cancer cell lines (CNE-1 and CNE-2) (Wu et al. 2011). In addition, some cross resistance to artesunate and DHA was reported in a unique cisplatin chemo-resistant cell line (Michaelis et al. 2010). Moreover, in vitro resistance has been developed: pre-incubation with artesunate induced resistance in highly metastatic breast cancer cells (Bachmeier et al. 2011).

Another advantage of artemisinins is the insignificant level of toxicity in humans, even after 30 years of use as anti-malarial. Clinical doses of artemisinin used in malaria treatment reach plasma concentrations up to three orders of magnitude higher than those needed for anti-tumour activity (Efferth et al. 2003b). The toxicity of artemisinin compounds has been associated with long-term availability, whereas short-term peak concentrations are not toxic (Efferth and Kaina 2010). No relevant side effects have been reported after long-term treatment with artemisinin up to 12 months (Singh and Verma 2002; Berger et al. 2005; Singh and Panwar 2006). However, brainstem neurotoxicity has been reported in animal studies after long-term, high-dose treatment (Schmuck et al. 2002) and also in a patient treated for two weeks with an herbal/artemisinin combination (Panossian et al. 2005). DHA is the most neurotoxic artemisinin compound, in a dose- and time-dependent manner (McLean and Ward 1998).

Due to their low toxicity to normal cells, artemisinin and its derivatives can be used in combination with classic chemotherapeutic agents with no additional side effects and can also enhance potency and reduce doses of more toxic anti-cancer partners.

12.5 Clinical Trials and Case Reports

In spite of the numerous in vitro and in vivo studies documenting the anti-cancer activity of artemisinins, only a small number of clinical trials and individual clinical cases were reported to date. Artesunate, artemether and dihydroartemisinin were used in cancer therapy with good results and lack of side effects.

In a seventy-two-year-old male patient with laryngeal squamous cell carcinoma, artesunate injections and tablets were administered over a period of nine months. The tumour was reduced by 70 % after two months of treatment. Overall, the artesunate treatment of the patient was beneficial in prolonging and improving the quality of life (Singh and Verma 2002). In addition, artesunate was efficient in increasing survival in two patients with metastatic uveal melanoma in combination with standard chemotherapy. These patients were treated on a compassionate-use basis, after failure of classic chemotherapy, without having additional side effects. One patient experienced a temporary response after the addition of artesunate to fotemustine and the second patient experienced stabilization after the addition of artesunate to dacarbazine, followed by a substantial metastasis reduction. This

patient was still alive 47 months after first diagnosis of stage IV uveal melanoma (Berger et al. 2005).

Artemether oral treatment over a period of 12 months significantly improved the quality of life of a 75-year-old male patient with pituitary macroadenoma. Although the tumour remained consistent in size, it showed a reduction in its density, and the related symptoms and signs resolved significantly. Artemether was chosen for the treatment because it easily crosses the blood–brain barrier and has longer half-life (Singh and Panwar 2006).

A clinical trial in 120 patients with advanced non-small cell lung cancer has shown significant improvement in disease control and time to progression, after treatment with artesunate in combination with traditional chemotherapeutic drugs. Artesunate combined with vinorelbine and cisplatin elevated one-year survival rate by 13 % compared with the standard vinorelbine/cisplatin therapy, without additional artesunate-related side effects (Zhang et al. 2008).

A pilot study in ten patients with advanced cervix carcinoma provides evidence on the improvement of the clinical symptoms and the good tolerability of Artenimol (dihydroartemisinin). Orally administered Artenimol treatment induced clinical remission with seven days—median time for the disappearance of the symptoms (Jansen et al. 2011).

There is an ongoing trial in Germany that will assess efficacy and tolerability of artesunate combination therapy in advanced breast cancer (http://clinicaltrials.gov/show/NCT00764036). Another trial, completed in United Kingdom, but not published yet, evaluated the anti-cancer activity and tolerability of artesunate in colorectal adenocarcinoma (http://www.controlled-trials.com/ISRCTN05203252).

12.6 Artemisinin Combinations

Numerous studies have investigated the benefits of combining artemisinins with traditional chemotherapeutic agents or with ionizing radiation, showing that multifactorial anti-neoplastic mechanism of artemisinin may improve overall activity. In such combinations, artemisinin compounds may enhance anti-tumour activity with no additional side effects.

DHA and artesunate exhibited a strong chemosensitizing effect on resistant cancer cell lines, when administered with conventional chemotherapeutic agents (Singh and Lai 2005; Reungpatthanaphong and Mankhetkorn 2002). DHA in combination with cyclophosphamide increased apoptosis in Lewis lung carcinoma and in combination with cisplatin decreased metastasis in human non-small cell lung cancer (A549) (Zhou et al. 2010). Furthermore, DHA combined with carboplatin increased growth inhibition in ovarian cancer cells (Chen et al. 2009b). In vivo and in vitro analysis in pancreatic cancer cells that develop resistance to gemcitabine over time, demonstrated that DHA significantly improves the anticancer effect of gemcitabine (Wang et al. 2010). DHA reduces tumour growth by

45 % in hepatoma cancer cell lines when used in combination with gemcitabine, while artemisinin only induces additive effects (Hou et al. 2008).

Consistent with this observation, it has been shown that artemisone has only additive effects when combined with gemcitabine or oxaliplatin in colon and breast cancer cells, whereas artemisinin exhibited antagonistic properties (Gravett et al. 2011). In cancer colon cells, artemisinin impairs doxorubicin activity by SERCA inhibition with subsequent accumulation of calcium, over-expression of P-glycoprotein and generation of doxorubicin resistant cells (Riganti et al. 2009).

On the other hand, artesunate and DHA remarkably potentiate the anti-tumour activity of doxorubicin and pirarubicin in leukaemia and human-small-cell cancer-resistant cell lines, without improving the activity in sensitive parent cell lines (Reungpatthanaphong and Mankhetkorn 2002). In rat C6 glioma cells, addition of DHA promotes apoptotic and necrotic activity of temozolomide, a DNA-alkylating agent used in the treatment of brain cancer (Huang et al. 2008).

Combination treatment of artesunate with TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) enhances TRAIL-mediated cytotoxicity and has the ability to overcome TRAIL resistance (Thanaketpaisarn et al. 2011). TRAIL selectively induces apoptosis and kills cancer cells with little or no adverse effects on normal cells, but many cancer cells manifest TRAIL resistance. A previous study reported increase apoptosis in prostate cancer cell lines (DU145, PC-3, LNCaP) after treatment with DHA and TRAIL (He et al. 2010).

Artesunate combined with the immunomodulatory drug lenalidomide improves anti-tumour activity in lung (A549) and breast (MCF-7) cancer (Liu et al. 2011). Recently, it has been reported that artesunate has a radiosensitizing effect in glioblastoma cells by selectively downregulating survivin (Reichert et al. 2012). In addition, artesunate enhances the radiosensitivity of non-small cell lung cancer (A549) cells in vitro and retards the tumour growth in nude tumour xenografts when combined with local radiotherapy (Zhao et al. 2011).

Treatment of human cancer cervical cells (HeLa and SiHa) with artemisinin and radiation significantly improved cytotoxicity (Gong et al. 2012). Artemisinin also exhibits an adjuvant effect in hyperbaric oxygen treatment of Molt-4 human leukaemia cells (Ohgami et al. 2010). DHA treatment improves the cytotoxicity of radiation therapy on glioma cells U373MG (Kim et al. 2006).

12.7 Artemisinin Derivatives

Considering the fact that artemisinin and its monomer analogues are generally not potent enough to ensure cancer cure due to their relatively low toxicity towards cancer cells and short half-lives, efforts are made to increase the efficacy of artemisinin compounds. Thus, new compounds such as artemisinin dimers, tetraoxanes and hybrids have been developed (Lai et al. 2013).

12.7.1 Monomers and Artemisinin-like Compounds

Deoxoartemisinin and carboxypropyldeoxoartemisinin manifested different profiles of toxicity on different cancer cell lines (Lee et al. 2000; Jeyadevan et al. 2004; Posner et al. 2002). Deoxoartemisinins compounds were found to be more potent anti-malarials than artemisinin (Jung et al. 1990). A series of deoxoartemisinin monomers, dimers and, particularly, trimers were more active in killing cancer cells than adriamycin, mitomycin and taxol (Jung et al. 2003).

The 10-substituted triazolyl artemisinin compounds exhibited potent anti-cancer activity in human colorectal adenocarcinoma, human glioma, human cervical carcinoma and mouse melanoma cell lines (Cho et al. 2009). The dimer of 12-(2′-hydroxyethyl) deoxoartemisinin showed potent anti-proliferative effect on oral cancer cells and the trimer was more potent than paclitaxel (Nam et al. 2007). C-12 non-acetal deoxoartemisinins were significantly less potent than doxorubicin on various cancer cell lines (Jung et al. 2009).

A high anti-angiogenic activity, uncorrelated with anti-cancer activity, was reported for a thioacetal artemisinin derivative (Oh et al. 2004) and a non-acetal deoxoartemisinin (Jung et al. 2006). Similarly, an elevated anti-angiogenic activity, comparable with fumagillin and thalidomide, was reported in the case of artemisinin/glycolipid hybrids (Ricci et al. 2010). Besides, artemisinin/glycolipid hybrids were shown to possess higher anti-cancer activity than that of artemisinin or glycolipid alone on several cancer cell lines, notably on oral cancer cells (Ricci et al. 2011).

Artemisinin/chalcone hybrids are cytotoxic to human HT-29 colon and HeLa cervical cancer cells (Xie et al. 2011a) and 15 dihydroartemisinin/chalcone hybrids manifest higher toxicity towards human HL-60 leukaemia and mouse P388 lymphoma cells than dihydroartemisinin alone (Yang et al. 2009). In order to make the molecule more water-soluble, artemisinin/guanidine hybrids were synthesized and tested on cancer cells. They proved to be more potent than dihydroartemisinin on human non-small cell lung cancer, colon cancer and breast cancer cells (Xie et al. 2011b). Artemisinin derivatives containing lipophilic alkyl carbon chains have been shown to be up to 200 times more cytotoxic towards cancer cells than artemisinin (Liu et al. 2005). However, toxicity of these more lipophilic artemisinin compounds on normal cells was not tested.

The newly developed artemisone has a more potent anti-proliferative effect than artemisinin, and it acts synergistically with oxaliplatin and gemcitabine (Gravett et al. 2011). However, artemisone is less anti-angiogenic than dihydroartemisinin (D'Alessandro et al. 2007) and has significant embryo- and fetotoxic effects (Schmuck et al. 2009).

Tehranolide is a sesquiterpene lactone with an endoperoxide moiety, isolated from *Artemisia diffusa*. Apart from its selective toxicity to cancer cells, tehranolide also modifies the immune responses and enhances anti-tumour immunity (Noori et al. 2009, 2010b). Tehranolide inhibited proliferation of breast cancer cells by induction of G0/G1 cell cycle arrest and apoptosis (Noori and Hassan 2012).

12.7.2 Dimers and Trimers

Artemisinin dimers are effective in retarding the growth or causing apoptosis in many different cancer cell lines (Alagbala et al. 2006; Stockwin et al. 2009). The cytotoxicity of dimers is 10-200-fold greater than that of the monomers (Paik et al. 2006; Horwedel et al. 2010; He et al. 2011a), although it is not clear why. One possible explanation would be that dimers, with two active groups, after bioactivation, can form cross-links between biological molecules, which could cause a more devastating effect on cellular functions (Lai et al. 2013).

Some dimers are even more potent than some chemotherapeutic agents, such as doxorubicin (Slade et al. 2009). Furthermore, dimers are much less toxic to normal cells than cancer cells (Rosenthal et al. 2009; Posner et al. 2004) and this selectivity makes them an attractive option in cancer treatment. In vivo studies of artemisinin dimers showed retardation of tumour growth in HL-60 human leukaemia xenografts in the mouse (Galal et al. 2009) and in rat mammary adenocarcinoma (Singh et al. 2011).

Regarding the structure–activity relation, it was observed that the presence of two endoperoxides in one molecule would not guarantee dimer effectiveness towards cancer cells. The spatial positions of the active groups seems to play an important role in dimer potency, as suggested by Beekman et al. (1997) after the observation that non-symmetric DHA dimers are more potent than symmetric dimers in killing cancer cells. The nature (Jeyadevan et al. 2004) and the size (Jung et al. 2003) of the linker in the dimers affects their anti-proliferative effect on cancer and may be involved in overcoming cross-resistance in drug-resistant cancer cells (Reiter et al. 2012). Different artemisinin dimers have different potencies towards different cancer cell lines, so they cannot be considered as a group of compounds with similar properties.

12.7.3 Tetraoxanes

Although structurally different from artemisinin-like compounds, tetraoxanes also contain endoperoxide moiety that can react with iron to form free radicals. A series of tetraoxanes manifested cytotoxicity on neuroblastoma cells (Vennerstrom et al. 2000), non-small cell lung cancer, colon cancer, brain cancer, melanoma, ovarian and renal cancer (Opsenica et al. 2003). Some tetraoxanes manifested a more potent activity against melanoma (Terzic et al. 2007) and ovarian cancer cells (Opsenica et al. 2008).

Generally, tetraoxanes have a higher cytotoxicity towards cancer cells compared with artemisinin compounds. This effect is probably due to the different free radical species produced by the two groups (Kumura et al. 2009). Yet, not all tetraoxanes have significant cytotoxic effects on cancer cells (Kumar et al. 2011).

It seems that hydrophobicity and H-bond donor properties are the main factors affecting the anti-cancer activity of tetraoxane compounds (Cvijetic et al. 2010).

12.8 Targeted Drug Delivery

Cancer cells need a higher iron uptake by the transferrin-mediated mechanism to maintain their continual proliferation, and transferrin receptors are highly expressed on cancer cell surface. Therefore, transferrin can be used as a drug carrier to target cancer cells (Li and Qian 2002; Qian et al. 2002). Transferrin is a glycoprotein with two carbohydrate chains that are not involved in receptor binding of transferrin (Mason et al. 1993), so they are ideal places to attach anti-cancer drugs to holo-transferrin (Nakase et al. 2008).

Covalent tagging of artemisinin analogues to the N-glycoside moiety of holo-transferrin dramatically increased the anti-cancer activity of these compounds (Lai et al. 2005a, b). The artelinic acid–tagged transferrin was 34,000 times more potent in killing leukaemia Molt-4 cells than normal human lymphocytes, while DHA was only 36 times more potent. However, holo-transferrin with artelinic acid tagged to lysine residues of the protein was less effective than holo-transferrin with artelinic acid tagged to carbohydrate chains, probably because it hindered its ability to bind to transferrin receptors. The artelinic acid–tagged transferrin was two times more potent than DHA in killing cancer cells, so the enhanced selectivity of the tagged compound is due to the fact that it is much less toxic to normal cells compared with DHA (Lai et al. 2005a).

This fact was confirmed by a later study that assessed the effect of a DHA analogue tagged to transferrin on human breast cancer cells (MCF-7) and normal breast cells. The results showed that 4-(12-dihydroartemisininoxy) benzoic acid hydrazide tagged to holo-transferrin was 286 times more potent in killing MCF-7 cells than normal cells (Xie et al. 2009). Furthermore, this compound is 172 times more potent than DHA in killing MCF-7 breast cancer cells and induces different modifications in the ultrastructure of plasma membrane of breast cancer cells: large holes were observed after treatment with DHA analogue tagged to holo-transferrin, whereas small irregular shaped holes were observed after DHA treatment (Xie et al. 2010).

Nakase et al. (2009) have optimized the tagging reaction, with the possibility to control the average number of artemisinin moieties per protein molecule. The transferrin bearing an average of 16 molecules of artemisinin retained the functionality of both transferrin and artemisinin and killed effectively prostate cancer cells (DU-145) through receptor-mediated endocytosis (Nakase et al. 2009). Recently, transferrin conjugates of monomeric artemisinin and artemisinin dimer were tested on breast cancer cells. Both transferrin conjugates strongly inhibited the growth of BT474 breast cancer cells in the same concentration range, while showing essentially no toxicity towards MCF10A normal breast cells (Gong et al. 2013). In vivo anti-tumour activity of transferrin conjugates of artelinic acid was

evaluated on an animal model of breast cancer. The tagged compound considerably retarded the growth of breast tumour in the rat, and it was significantly more effective than DHA (Lai et al. 2009).

The results of these studies suggest that by tagging artemisinin to transferrin, it is selectively picked up and concentrated by cancer cells. Furthermore, both artemisinin and iron—its activator—are transported into the cell in one package. This strategy provides a very effective mean of targeted delivery of drugs into cancer cells (Nakase et al. 2008).

Except for transferrin, other molecules can be used as potential drug carriers for targeted delivery of artemisinin compounds to cancer cells. A transferrin-receptor-targeting peptide, HAIYPRH, was covalently conjugated to one or two artemisinin moieties. The peptide binds to a cavity on the surface of transferrin receptor, enabling artemisinin to be co-internalized with receptor-bound transferrin. The artemisinin/peptide conjugates showed potent anti-cancer activity against Molt-4 leukaemia cells with a significant selectivity in killing leukaemia cells. They were virtually non-toxic to normal leucocytes. The peptide conjugate with two artemisinin moieties was markedly more potent than the conjugate with one artemisinin moiety (Oh et al. 2009).

In addition, artemisinins conjugated with iron-carrying molecules can be used to treat bacterial infections. In a recent study, artemisinin-tagged mycobactin, a microbial iron chelator, manifested selective and potent activity against drug-resistant strains of *Mycobacterium tuberculosis* (Miller et al. 2011).

Anti-cancer drug delivery systems based on liposomes and/or polymers were approved recently for clinical use and already favourably impact cancer treatments by delivering a high dose of drugs to tumour sites while minimizing side effects. For instance, artemisinin loaded on multi-walled carbon nanotubes is more effective in inhibiting cancer cell lines growth (Rezaei et al. 2011). Liposomes loaded with artemisinins could be efficacious therapeutic nanocarriers to treat tumours by increasing the bioavailability of the anti-cancer drug (Isacchi et al. 2011; Righeschi et al. 2011). Artemisinin associated into polymeric micelles conjugated with tumour- and tumour lymphatics-homing peptide enhanced specific delivery to highly metastatic tumour and its lymphatics (Wang et al. 2012).

12.9 Conclusion

In conclusion, the extensive research in the anti-tumour activity of artemisinins supports the introduction of these compounds in cancer therapy. Artemisinins have highly selective cytotoxicity on cancer cells and have no significant toxicity in normal cells, as documented by the extensive use as anti-malarial for the last three decades. Moreover, they have a broad spectrum of action and may develop into an effective low-cost therapeutic alternative in highly metastatic and aggressive cancers. Their multi-modal anti-cancer activity prevents the emergence of resistance in cancer cells. In addition, artemisinin and its derivatives manifest other

properties that can enhance the efficacy of cancer therapy, such as anti-angiogenic, anti-metastatic, anti-inflammatory and immunological effects.

Yet, artemisinin compounds have some drawbacks: monomers alone are not effective enough for use in cancer treatment; some compounds have short plasma half-life and poor bioavailability; different artemisinin compounds have different potency on different types of cancer cells; the isolation of parent compound relies on limited natural product resources. Some of these shortcomings may be overcome by development of new artemisinin-like compounds, the use of synergic combination with conventional cancer therapy and the formation of conjugates with different drug carriers, especially molecules involved in cellular iron transport, for targeted delivery of artemisinin compounds to cancer cells.

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Chapter 13
Recent Developments in Controlling Insect, Acari, Nematode, and Plant Pathogens of Agricultural and Medical Importance by *Artemisia annua* L. (Asteraceae)

Jalal Jalali Sendi and Roya Khosravi

Abstract One key problem in agriculture is the development of resistance in pests against synthetic insecticides. Harmful effect of chemical pesticides on non-target organisms, farm workers, and consumer of agriculture products is considerable. In order to resolve the replacement of chemical pesticides, essential oils obtained from aromatic plants have been highly appreciated. *Artemisia annua* L. belongs to Asteraceae family, known as sweet wormwood, grows wild in Europe and America and is planted widely in some parts of the world. Extracts of *A. annua* have many remarkable properties which make it a very extensive front of potential applications in agroecological and biomedical fields. Moreover, many studies showed that these phyto-extracts have insecticides, acaricides, fungicide, and nematocide properties. Today, we know malaria is the most important parasitic disease of mankind. Malaria is a vector-borne infectious disease caused by the protozoan Plasmodium parasites. *A. annua* is also the only source of artemisinin, an important natural endoperoxide sesquiterpene lactone with antimalarial effect against susceptible and multidrug-resistant *Plasmodium* spp. The finding and improvement of ART from *A. annua* L. have provided a new class of highly effective antimalarials and have already influenced the therapy of malaria. Essential oils and extracts of *A. annua* showed very strong effect on pests by several ways, such as repellent, fumigant, and antifeedant actions, interference in enzyme activity, and changes in insect growth. Artemisia ketone, camphor, germacrene-D, and 1, 8-cineole are usually the main components. Several isolated compounds from this species have shown antimalarial, antibacterial, anti-inflammatory, plant growth regulatory, and cytotoxicity activities. Antimicrobial activities of essential oils of *A. annua* from different countries have been previously reported against a variety of food-borne and human

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pathogens. In the present study, the latest developments in *Artemisia* research will be reviewed and prospects of future research for this magnificent plant species will be provided.

Keywords *Artemisia annua* · Insect · Acari · Nematode · Plant pathogens

13.1 Introduction

The primary means of control of arthropod pests in agriculture is through the application of broad-spectrum insecticides. While such involvements are capable of rapidly killing a range of pests, over-reliance on chemical pesticides has generated problems including dangers of acute and chronic toxicity for sellers, farm workers, and consumers of agriculture products, outbreaks of secondary pests normally held in check by natural enemies, environmental contamination, decrease in biodiversity, and insecticide resistance (Lacey et al. 2008).

To protect different cultural plants from harmful activity of pests and diseases, more attention should be paid to developing and establishment of environment-friendly regulation actions. The use of plant extracts in agroecosystems is now emerging as one of the prime means to protect crop products and the environment from synthetic pesticide pollution. In general, botanical pesticides have low mammalian toxicity, less impact on non-target organisms and are easily available and less expensive than their synthetic counterparts (Prakash and Rao 1997; Schmutterer 1995).

In addition, more than 2000 years is known the use of botanical active ingredients such as insecticides and/or entomo-repellents in China, India, Europe, and Egypt (Isman 2006). The number of substances extracted from the present known Asteraceae amounts to more than 140 different molecules (number to be considered provisional and updated in mid-2007) that can be classified as follows (Bhakuni et al. 2001; Brown et al. 2003; Li and Wu 2003; VanderMersh 2005):

- Alkaloids (purines, zeatin, protein-alkaloids);
- Benzenoids;
- Heterocyclic compounds;
- Coumarins;
- Phytosteroids;
- Flavonoids;
- Hydrocarbons (alkanes, alcheneini, phenylpropanoid);
- Terpenoids (oxy-sesquiterpenes, sesquiterpenes, triterpenes, diterpenes, monoterpenes).

The research, however, is advancing all the time, and several new substances are added to the list of secondary metabolites not only *A. annua* but of *Artemisia* in general (Kalemba 1999; Pujar et al. 2000; Song et al. 2006).

Among the plant families studied, the Meliaceae, Asteraceae, Labiatae, Piperaceae, and Annonaceae are the most promising (Isman 2006). The genus *Artemisia* (commonly wormwood or sagebrush) is one of the largest and most widely distributed genera of the family Asteraceae. It consists of around 300–400 species of herbs and shrubs well known for their volatile oil that is extensively used in food and pharmaceutical industry.

A. annua is commonly known as qinghao, annual wormwood, and sweet wormwood. It is annual herb native to Asia, most probably China. The plant also grows wild in Europe and America and is planted widely in China, Turkey, Vietnam, Afghanistan and Australia. It is a herbaceous and annual weed propagated by seeds. They can remain viable for three years under suitable conditions (Goel et al. 2007).

A. annua is an important traditional Chinese medicinal plant, and it is well known for its antimalarial activity, attributed to the presence of artemisinin. In the agricultural field, there were also some applications. *A. annua* contains artemisinin, artemisia A, B, C, D, and E, artemisinic acid, methyl wormwood, artemisinic alcohol, and the volatile oil, mainly including eucalyptol, artemisia ketone, camphor, caryophyllene, oxidation caryophyllene. Volatile oil has antibacterial, antiviral, antiparasitic, regulation of immune function, antipyretic and antitumor and other roles (Wei et al. 2004).

13.2 Insecticidal and Acaricidal Effects of *Artemisia annua*

The insecticidal activity of sweet wormwood *A. annua* has been described in several studies. Sharma et al. (2006) reported that petroleum ether (Pee) extract of *A. annua* against *Culex quinquefasciatus* Say larvae exhibited minimum LC_{50} value 78.2 and 74.2 ppm after 24 and 48 h of treatment, respectively. It was followed by methanol extract (Mee) of *A. annua* with LC_{50} value 360.1 and 159 ppm after 24 and 48 h of exposure, respectively. Pee of *A. annua* was the most potent extract.

Tonk et al. (2006) studied effective method for the extraction of larvicidal component from leaves of *A. annua*. Mortality of *Anopheles stephensi* Liston larvae by using *A. annua* crude ranged from 10 to 100 % after 72 h in extracts obtained by different methods as compared to control. In case of *A. annua*, although both soxhlet and reflux extraction methods showed 100 % mortality at 200 ppm after 72 h; however, LC_{50} value of crude obtained by soxhlet extraction showed higher (19.9 ppm) larval mortality than reflux extraction (35 ppm), indicating soxhlet method to be more effective for the extraction of larvicidal components.

The biological effects of medicinal plant, *A. annua* L., were studied on small white *Pieris rapae* L, a deleterious pest of cruciferous plants under controlled conditions. The LC_{50} and LC_{25} values were 9.387 and 3.645 % for *A. annua* L., and 4.19 and 1.69 % for *A. millefolium* L., respectively (Hasheminia et al. 2011).

Zibaee et al. (2010) showed that methanolic extract of *A. annua* was effective against *Hyphantria cunea* Drury larvae with LC_{50} value 0.42 % on mulberry

leaves. Shekari et al. (2008) investigated toxic effects of *A. annua* methanolic extract on elm leaf beetle *Xanthogaleruca luteola* Mull. The LC_{50} values for third instar larvae were 48 and 43.77 % at 24 and 48 h, respectively. The LC_{50} values measured for adults after 24 and 48 h were 19.14 and 15.43 %. The toxicity tests indicate that insecticidal effect was 2.4-fold higher in third instar larvae than in adults.

Zibaee and Bandani (2009) evaluated insecticidal activities of methanolic extract of *A. annua* against the sunn pest *Eurygaster integriceps* Puton. The LC_{50} values for adults were 25 and 11.67 % for 24 and 48 h after treatment, respectively. The mortality of adults, due to using different concentrations of plant extract, varied between 4 and 100 %, which showed a dose- and time-dependent relationship.

Khosravi et al. (2011) reported insecticidal property of methanolic extract of *A. annua* on *Glyphodes pyloalis* Walker larvae. The LC_{50} and LC_{20} values on fourth instar larvae were calculated as 0.33 and 0.22 gram leaf equivalent/ml, respectively. Complete mortality in *Epilachna paenulata* Germar and 50 % mortality in *Spodoptera eridania* Stoll were observed with extract ethanolic extract of aerial parts of *Artemisia annua* at 1.5 mg/cm^2 (Maggi et al. 2005).

Jalali Sendi et al. (2003) studied the effect of *A. annua* L. against confused flour beetle *Tribolium confusum* Duv. The results indicated that all concentrations of the plant extract were effective. LC_{50} in *Artemisia annua* ether extracts was determined 3.24 %. The mortality rate in males was higher than that in female beetles.

Goel et al. (2007) showed that the essential oil of the root of *A. annua* demonstrated toxicity against the coleopteran beetles *T. castaneum*. About 80 % of the adult beetles died upon fumigation with 10 μg of oil in a volume of 1 L for 24 h. The beetles were repelled by the oil; a non-lethal 3.3-fold lower concentration of the oil repelled ≥80 % of the beetles.

A methanolic extract of *A. annua* was obtained to evaluate its insecticidal activities against the sunn pest, *E. integriceps*. Topical application of plant extract on adults showed that the mortality was dose dependent, i.e., with increasing plant extract concentrations, more mortality was achieved (Zibaee et al. 2010).

Mites, particularly phytophagous pest mites, are one of the important harmful organisms in fruit trees, forest, vegetables, flowers, crops, and so on. Because of small individual size, short generation cycle, and high mutation rate, mites were thought as a kind of pest which was more difficult to be controlled. Now, mite's resistance to many of chemical pesticides is reported due to frequent and irrational drug use. Developing green, safe, and environmental pesticides is an effective way for solving the lack of excellent acaricides.

Pirali-Kheirabadi and Teixeira da Silva (2011) reported acaricidal effect of *A. annua* essential oils on *Rhipicephalus annualtus* Say. The mortality rate caused by different dilutions of *A. annua* essential oil was 33.2–100 % (using 20 and 80 μL/cm^3, respectively) by the end of the experiment (36 hpi). The LC_{50} was 3.73 μL/cm^3 for *Artemisia*, indicating that this plant had a considerable acaricidal effect against engorged *R. annulatus* in vitro.

The median lethal concentrations (LC_{50}) against *Tetranychus cinnabarinus* (Boisduval) of acetone parallel extracts of *A. annua* leaves in September, July, June, May, and April were 0.5986, 0.4341, 0.8376, 0.9443, and 1.3817 mg mL^{-1},

respectively, treated after 48 h. The LC_{50} of *A. annua* different parallel leaf acetone extracts varied with the acquisition season (Table 13.1). The results indicated that the acaricidal activity increased with the development of *A. annua* plant individual. However, the most effective extracts in five months were all acetone parallel extract of *A. annua* leaf, and the corrected mortalities treated after 48 h ranged from 74 to 100 % (Yong-qiang et al. 2008).

Acetone extract from leaf of *A. annua* L. has strong acaricidal activity. For practical application and extensive popularization, WenMing (2009) tried to develop acetone extract from *A. annua* to a commercial formulation. The results of this study showed that 5 % extract of *A. annua* ME had better contact toxicity against *Tetranychus cinnabarinus* (Boisduval), *Panonychus citri* (Mc Gregor), and *Petrobia harti* (Ewing). The LC_{50} values (48 h) were 117.095, 153.303, and 82.170 mg/L, respectively, to adults of three kinds of phytophagous mites. The LC_{50} (48 h) was 102.209 mg/L to nymphs of *T. cinnabarinus*. Meanwhile, it was found that egg hatch of *T. cinnabarinus* was delayed, but the formulation had a little influence on hatching rate finally. The result of field trials showed that the *T. cinnabarinus* could be effectively controlled by spraying with a 400-fold 5 % extract of *A. annua* ME, and the control effect was 88.38 % in 14 d. The control effect of the mixture which was 5 % extract of *Artemisia annua* ME diluting 1,000-fold and 24 % spirodiclofen SC diluting 8,000-fold was 79.54 % in 30 d, and it can be a way to control *Panonychus citri* in citrus orchard.

13.3 Insecticidal Effects of Constituents of *A. annua* Essential Oil

The deleterious effects of plant extracts or pure compounds on insects can be manifested in several manners including toxicity, mortality, antifeedant growth inhibitor, suppression of reproductive behavior and reduction in fecundity and fertility.

The identified compounds in roots of *A. annua* were mostly sesquiterpenes (72.7 %). Mono- and diterpenoids were found in low concentrations (0.5 %). The major components (those present at concentrations of more than 5 %) were as

Table 13.1 The toxicity analysis of leaves acetone extracts of *Artemisia annua* against *Tetranychus cinnabarinus* (48 h) (Yong-qiang et al. 2008)

Materials	Toxicity regression linear	Correlation coefficient	LC_{50} and its 95 % confidence interval (mg mL^{-1})
April acetone extract	$y = 1.3732 + 1.1549x$	0.9919	1.3817 (1.0120–1.8863)
May acetone extract	$y = 0.9444 + 1.3632x$	0.9624	0.9443 (0.7141–1.2486)
June acetone extract	$y = 0.1146 + 1.6743x$	0.9883	0.8376 (0.6508–1.0524)
July acetone extract	$y = 1.8462 + 1.1957x$	0.9800	0.4341 (0.2708–0.6957)
September acetone extract	$y = 1.5818 + 1.2308x$	0.9951	0.5986 (0.4201–0.8530)

follows: cis-arteannuic alcohol (25.9 %), (E)-b-farnesene (6.7 %), b-maaliene (6.3 %), and b-caryophyllene (5.5 %). The essential oil of the root of *A. annua* demonstrated toxicity against the coleopteran beetles *T. castaneum*. About 80 % of the adult beetles died upon fumigation with 10 μg of oil in a volume of 1 L for 24 h. The beetles were repelled by the oil; a non-lethal 3.3-fold lower concentration of the oil repelled ≥80 % of the beetles (Goel et al. 2007).

Based on the report of Rabie et al. (2003) artemisia ketone (14.31 %), 1.8-cineole (9.78 %), pinocarvone (9.07 %), camphor (8.11 %), and *trans*-pinocarveol were the main constituents of the aromatic parts of *A. annua*.

1, 8-cineole isolated from *A. annua* is also a potential insecticidal allelochemical that could reduce the growth rate, food consumption, and food utilization in some post-harvest pests and household insects (Jacobson and Halber 1947; Klocke et al. 1989; Obeng-ofori and Reichmuth 1997).

1, 8-cineole, one of the components of the essential oil of *A. annua*, was evaluated for repellency and toxicity against three stored product coleopterans *Callosobruchus maculatus* F. (Coleoptera: Bruchidae), *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae), and *Sitophilus oryzae* L. (Coleoptera: Curculionidae). It was found to be moderately repellent to all three species, with a mean repellency in the range of 65–74 % at the highest dose tested (4.0 ml/ml) within 1 h. A contact toxicity assay revealed that direct topical application was more effective than using impregnated filter paper. The compound was more effective as a fumigant and gave 93–100 % mortality against the entire three pest species at the dose of 1.0 ml/l air under empty jar conditions as compared to treatment of jars filled with grain (11–26 % mortality). The lethal dose and lethal concentration required to kill 50 % of the beetles (LD_{50} and LC_{50}, respectively) varied with the toxicity assay method. LD_{50} values of 0.03, 0.04, and 0.04 ml/insect against *C. maculatus, R. dominica* and *S. oryzae*, respectively, were found in the topical application assay, while the LC_{50} values in the fumigant assay were 0.28, 0.33, and 0.46 ml/l against *C. maculatus, R. dominica*, and *S. oryzae*, respectively (Aggarwal et al. 2001).

1, 8-cineole produced 100 % contact toxicity in *C. maculatus, R. dominica,* and *S. oryzae* adults at the highest dose of 0.1 ml/insect. The LD_{50} values in the topical application assay were 0.03, 0.04, and 0.04 ml/insect for *C. maculatus, R. dominica,* and *S. oryzae*, respectively (Aggarwal et al. 2001). Rao et al. (1999) reported similar toxicity from topical application of *A. annua* oil (which contains 1, 8-cineole) against *Dysdercus koeningii* Fabr with a LD_{50} value of 0.48 ml/insect. Prates et al. (1998) demonstrated insecticidal effects of 1, 8-cineole against adult beetles of *R. dominica* and *T. castaneum* in contact toxicity assays. Higher doses of 1, 8-cineole were required to achieve 100 % kill when using filter-paper disks than in topical application. This may be because impregnated filter paper provides less direct contact. *Callosobruchus maculatus* was less susceptible to the toxic effects of 1, 8-cineole than the other two species in the filter-paper contact toxicity test. This might arise from morphological and behavioral features of this insect, in that only the tips of its legs come into contact with the chemical, while in *R. dominica* and *S. oryzae*, the whole abdominal surface makes contact.

Comparison of the results of the fumigation tests with 1, 8-cineole revealed that it was more effective in killing the beetles under empty jar conditions than when treated jars were filled with grain. Obeng-Ofori et al. (1997) also reported 100 % mortality caused by 1, 8-cineole at the dose of 0.5 ml/kg to adult *S. oryzae, T. castaneum, Lasioderma serricorne* Fabr, and *Stegobium paniceum* L.

13.4 Effect on Insect Development

Sharma et al. (2006) selected 78.2 ppm of petroleum extract of *A. annua* to study its influence on the development and metamorphosis of the culicine mosquito. The extract significantly affected the hatching, larval development, and pupal transformation and also lengthened the larval and pupal periods. Growth index was remarkably reduced. Treated culicine eggs, larvae, and pupae showed deformities, including disruption of the body wall, distorted alimentary canal, damaged tracheal network, and arrested histogenesis. The extract has remarkable effect on the metamorphosis and high larvicidal potential and hence can be used as an effective alternative to the existing synthetic pesticides for the control of *Culex quinquefasciatus* Say. Successful pupation followed deformed and distorted progress in most of the cases, with reduction in average body size and damaged body wall, which may again be attributed to interruption in chitin synthesis. Histogenesis was destructed indicating the interrupting role of the extract in normal protein synthesis during the process.

Pirali-Kheirabadi and Teixeira da Silva (2011) demonstrated that the *Artemisia annua* essential oils applied at more than 20 and 60 μL/cm^3 caused 100 % egg-laying failure in engorged female ticks, while no failure was observed for the non-treated control group.

The methanol extract of *A. annua* caused discrepancy in the development of treated fourth instar larvae under sublethal doses. The larval duration was higher in fourth instar larvae compared with the control in all tested concentrations. The fifth instar larval and pupal duration was also increased in LC_{50} treatment (Khosravi et al. 2011).

Methanolic extract of *A. annua* L., was investigated for its toxic effects on feeding, growth, fecundity, fertility including the biochemical characteristics of elm leaf beetle *Xanthogaleruca luteola* Mull. Larval duration significantly increased at higher concentrations. Larval treatment with 10 % methanolic extract resulted in deformed adults (adultoids) with drastic changes in gross morphology of female reproductive system. Larval duration and adult fecundity and fertility were also significantly reduced (Shekari et al. 2008). Growth regulatory effect such as a deformed wings and growth retardation of ovarian development occurred at higher concentrations of *A. annua* extract (Fig. 13.1).

Fig. 13.1 Note the mature oocytes in control (**a**) and undeveloped ovary in treated insect with methanolic extract of *Artemisia annua* (**b**) (Shekari et al. 2008)

13.5 Effect on Insect Feeding

Antifeedant materials may be defined as being deterrent from feeding once contact has been made with insects. Ethanolic extract of aerial parts of *A. annua* and artemisinin were evaluated as anti-insect products. In a feeding deterrence assay on *E. paenulata* larvae, complete feeding rejection was observed at an extract concentration of 1.5 mg/cm^2 on pumpkin leaf tissue. The same concentration produced a feeding inhibition of 87 % in *S. eridania* (Table 13.2). Artemisinin exhibited a moderate antifeedant effect on *E. paenulata* and *S. eridania* at 0.03–0.375 mg/cm^2. However, a strong effect on survival and body weight was observed when *E. paenulata* larvae were forced to feed on leaves treated at 0.03

Table 13.2 Feeding deterrence effects of *Artemisia annua* aerial parts of ethanolic extract on *Epilachna paenulata* and *Spodoptera eridania* (Maggi et al. 2005)

Extract dosage (mg/cm^2)	Leaf area eaten (%) ± SE		AI %[a]
	Treated control		
Epilachna paenulata			
0.15	7 ± 6.8**	33 ± 10.3	78.8
1.5	0.5 ± 1.6**	31.3 ± 15.7	98.5
Spodoptera eridania			
0.15	15.3 ± 8.3	16.3 ± 12	6.1
1.5	2.5 ± 4.3*	19.5 ± 22.7	87.1

[a] Results observed at 24 h. Antifeedant index (AI) = $(1 - T/C) \times 100$. Means of ten replications are presented

and 0.075 mg/cm^2 (Maggi et al. 2005). Shekari et al. (2008) also reported on *A. annua* extract deterrency against the elm leaf beetle *Xanthogaleruca luteola*. Hasheminia et al. (2011) measured a 29.826 % deterrence in lowest incorporated dosage (0.625%) of *A. annua* on third instar larvae of *P. rapae*. Also, methanolic extract of *A. annua* decreased relative consumption rate and relative growth rate of treated larvae. Some researchers believe that the deterrence may be due to the presence of a number of chemical compounds like, flavonoids, terpenes, tannins, and sterols (Salama and Sharaby 1988).

Several studies demonstrated the effect of botanical insecticides on feeding parameters of insects by demonstrating food consumption [CR = I/DT], approximate digestibility of consumed food [%AD = 100(I − F)/I], efficiency of converting the ingested food to body substance [%ECI = 100 G/I], efficiency of converting digested food to body substance [%ECD = 100G/(I − F)], and consumption index [CI = I/W]. These indices demonstrate the digestion efficiency or utilization of diet or diet ingredients and in fact illustrate the conversion of food to the biomass of insects (Waldbauer 1968).

Khosravi et al. (2010) found that the feeding efficiency of fifth instar larvae of *G. pyloalis* feeding on *A. annua* extract-treated leaves is intensely affected. It was shown that *A. annua* extract had a strong effect on feeding behavior and growth of this pest. By using the plant extract, RGR, ECI, and ECD were reduced. Relative growth rate (RGR) that follows weight gain in control larvae is significantly higher than treated larvae. The lower RGR could be due to irreparable damages made to midgut lumen cellular surfaces (Jansen and Groot 2004). Unsuitable food ingredients and the food regiments lacking essential components for growth lead to a higher digestibility and consumption index but reduced RGR and ECI (Cohen 2001). Lower RGR, ECI, and ECD probably lead to delay in larval growth and formation of smaller pupa, which have a direct relation to fecundity and longevity of the adult insect and make them susceptible to diseases and natural enemies.

Shekari et al. (2008) reported methanolic extract of *A. annua* affect on nutritional physiology of elm leaf beetle. ECD, ECI, and AD were significantly reduced at all treated concentrations. Their results indicate that reduction in AD and ECI in adults by *A. annua* extract causes the insect to use the food to detoxify the extract and thus only small amount is used for growth causing reduced ECI and ECD.

Hasheminia et al. (2011) reported increase in larval duration treated with methanolic extracts of *A. annua* at 2.5 and 1.25 % concentrations. No adult emergence from treated larvae of *P. rapae*.

13.6 Effects on Enzymatic Activity and Biochemical Compounds

Plant defenses against insect herbivores are mediated, in part, by enzymes that impair digestive processes in the insect gut. Digestive enzymes play a major role in the body of insects by converting complex food materials into smaller molecules

necessary to provide energy and metabolites. Any disruption in their activity disables insects to provide their nutrients for biological requirements.

The activity of α-amylase and protease in *G. pyloalis* larvae 48 h post-treatment with methanolic extract of *A. annua* was significantly reduced compared with the control (Khosravi et al. 2011).

The biological effects of medicinal plant, *A. annua* L., were studied on small white *P. rapae* L., a deleterious pest of cruciferous plants under controlled conditions. The activity level of alkaline phosphatase increased sharply, while alanine and aspartate aminotransferases showed a sharp decrease. For non-enzymatic compounds, the amount of glucose and uric acid increased, but total protein and cholesterol decreased. These results indicate that these two medicinal plants might possess potential secondary metabolites that may be useful for controlling potential insect pests (Hasheminia et al. 2011).

Laboratory assays were carried out to evaluate the effect of methanolic extract of *A. annua* L. on some biochemical compounds in the hemolymph of *H. cunea* Drury (Lepidoptera: Arctiidae). It was found that the activity levels of two aminotransferases and phosphatases increased after extract exposure. Activity of lactate dehydrogenase, as enzyme showing tissue damage, significantly increased in all time intervals. Non-enzymatic parameters including trehalose, protein, and urea decreased after treatment by plant extract, but the amount of total lipid showed no significant differences. These results indicate that *A. annua* L. extract contains inhibitors of key metabolic pathways that may be useful in future control of the fall webworm (Zibaee et al. 2011).

Twenty-four hours after treating third instar larva with the extract, the levels of glucose, protein, urea, uric acid, a-amylase, alkaline phosphatase, alanine amino transferase, and aspartate amino transferase significantly changed. However, at 48 h, the extract lost its potency. The extract did not affect the level of cholesterol in treated larva (Shekari et al. 2008).

Insects use detoxifying enzymes such as cytochrome P450, glutathione S-transferase, and esterase to metabolize toxic plant secondary metabolites. Therefore, the metabolic enzymes are related to the mode of action of insecticides and the immunity of insects (Ramsey et al. 2010).

The responses of general esterase (EST), glutathione *S*-transferase (GST), alkaline phosphatase (ALP), acid phosphatase (ACP), and acetylcholinesterase (AChE) to the methanolic extract of *A. annua* were investigated. Esterase and GST activities were increased in the first 24 h post-treatment. However, the enzymes activities were decreased after 24 until 72 h. The activities of ALP, ACP, and AChE in insect body decreased significantly, and inhibition was higher along with increasing concentrations of plant extract. Isozyme electrophoresis profiles indicated that responses of isozymes (EST and GST) to plant extract were decreased after 48-h exposure to extract so that some enzyme bands disappeared (Table 13.3). The results indicated that the highest concentration of *A. annua* extract was the most toxic among the four extracts. The decline of the detoxification ability in insects' tissues might be the main reason for the insecticidal activities (Zibaee and Bandani 2010).

Table 13.3 Effect of *Artemisia annua* extract on detoxifying enzyme of *Eurygaster integriceps* hemolymph after 24 h (Zibaee and Bandani 2010)

Treatment (%)	Esterase (α-naphthyl substrate)	Glutathione S-transferase (CDNB substrate)	Acetylcholinesterase	Alkaline phosphatase	Acid phosphatase
Control	3.76 ± 0.062 a	2.82 ± 0.036 a	7.56 ± 0.027 a	4.92 ± 0.024 a	3.93 ± 0.018 a
10	4.11 ± 0.021 b	2.85 ± 0.022 b	7.32 ± 0.020 b	4.91 ± 0.020 b	3.88 ± 0.046 b
15	4.27 ± 0.083 b	3.17 ± 0.031 bc	6.01 ± 0.052 c	5.11 ± 0.034 c	4.15 ± 0.026 c
25	4.75 ± 0.095 c	3.49 ± 0.025 c	5.31 ± 0.031 d	5.46 ± 0.027 c	4.35 ± 0.021 c

*means (±SEM) followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey's test. CDNB-1-chloro-2,4-dinitrobenzene

Methanolic extract of *A. annua* significantly decreased the mean total protein carbohydrate and lipid contents in all treatments compared with that of the control (Khosravi et al. 2010).

13.7 Nematicidal Effects of *A. annua*

Plant parasitic nematodes are major pests in many countries, particularly in the tropics and subtropics, where they are recognized as the cause of serious yield losses on a wide range of crops (Sasser and Freckman 1987). A number of genera and species of nematodes are highly damaging to a great range of hosts, including foliage plants, agronomic and vegetable crops, fruit and nut trees, turf grass, and forest trees. Plant parasitic nematodes may attack the roots, stem, foliage, and flower of plants. Nematodes are simple, multicellular animals, typically containing 1,000 cells or less. They are worm like in appearance but are taxonomically distinct from earthworms, wireworms, or flatworms (Ramana and Eapen 1995).

In vitro toxicity of *A. annua* was evaluated against J_2 of *Meloidogyne incognita* Chitwood and preadults of *Rotylenchulus reniformis* Linford and Oliveira. 100 % mortality of both the nematode species was found in 500 and 250 ppm concentrations, which gradually decreased with the lower concentrations. ED_{50} was calculated to be 53.96 and 54.83 at 24 h of *M. incognita* and *R. reniformis*, respectively. When treated juveniles of *M. incognita* were pooled and inoculated on cowpea seedlings, the penetration was hampered with higher concentrations than lower ones; it was more than 3 times less penetration in 500 and 250 ppm as compared to control (Shakil et al. 2004).

Nematicidal activities of some native plants of Iran have been investigated against cucumber root-knot nematode, *M. incognita* in laboratory and greenhouse. For this purpose, the effects of alcoholic extract of the leaf of sweet wormwood with concentrations of 0, 50, 100, 200, 300, 400, 500, and 1,000 ppm on the percentage of immobility of second-stage juveniles and hatching of eggs were evaluated. After proving the antinematode activity of treatments, the investigation was held in greenhouse by two methods. In the first method, extract of plants was added into pots having cucumber seedlings infested with nematode, and in the second method, seedlings were dipped in alcoholic extract and then transplanted into nematode-infested pots. Overall alcoholic extracts of sweet wormwood with 32.69 % had higher efficacy on unhatched eggs of nematode in the laboratory conditions (Katooli et al. 2011).

Zahabi Asl (2011) investigated the effect of aqueous extract of *A. annua* on tea root lesion nematode *Pratylenchus loosi* Loof. Aqueous extract 20 % w/v of whole plant causes 34 % aerial parts wet weight of tea to increase. Also, 61 and 40 % cause population of *P. loosi* at soil and root to decrease, respectively.

13.8 Fungicidal and Bactericidal Activities of *A. annua*

The results of bioassay of primary extracts from *A. annua* compared against four fungi *Bipolaris maydis* Shoem, *Rhizoctonia cerelis* Vander Hoeven, *Altenaria alternate* Keissler, *Fusarium oxysporum* f.sp Snyder and Hansen. The bioassay test indicated that acetone extracts from the leaf of *A. annua* had 83.67 % and 89.55 % on mycelium growth at the concentration of 3 g/L (72 h), and the EC_{50} values were 0.2056 g/L and 0.1828 g/L (72 h) against *B. maydis* and *R. cerelis*, respectively. Comparing inhibition ratio of primary extracts from different parts of *A. annua* (the leaf, the stem, the root) compared against four fungi. The inhibition ratio of parallel extracts was better than that of sequence extracts. The inhibition ratio of acetone primary extracts was the highest one among solvents' primary extracts against four fungi (Jing 2008).

The results of bioassay test indicated that the water extracts had obvious restraining effect on tobacco bacterial wilt bactericides with the bacteriostasis diameters was 1.5 cm by 3 g/L in 48 h. The bioassay results of bacteriostasis on field showed that the water extracts had inhibition with growing concentration on the bourgeon velocity of the seed; the growth of the roots of seedling and the inhibition rate of 1,000 mg/L solution were above 45 %. The comminuting of *A. annua* L had slight restrain, and the residue of *A. annua* L got rid of artemisinin had no restrain on tobacco bacterial wilt bactericides, respectively (Jing 2008).

The in vitro antifungal activity of the essential oil was evaluated against economically important foliar and soil-borne fungal pathogens of tomato, including *Sclerotinia sclerotiorum, Botrytis cinerea, Phytophthora infestans,* and *Verticillium dahlia*. Both volatile and contact phases of essential oil were found to inhibit the growth of fungal isolates used in the experiment in a dose-dependent manner. The volatile phase of essential oil was consistently found to be more effective on fungal growth than the contact phase (Fig. 13.2) (Soylu et al. 2005).

13.9 Medical Importance

Malaria is among the most prevalent infectious diseases in the developing world, imposing a vast burden of mortality and perpetuating cycles of poverty. In 2009, the World Health Organization (WHO) estimated that 225 million cases of malaria occurred, with >780,000 deaths. The discovery and development of artemisinin from *A. annua* L. have provided a new class of highly effective antimalarials and have already influenced the therapy of malaria (Sriram et al. 2004; Haynes 2006; Hsu 2006; Bosman and Mendis 2007).

During the Vietnam War (1965–1975), the Chinese government started an antimalarial research program to search for traditional Chinese medicinal plants to support the Vietnamese army. As a result, artemisinin (*qinghaosu*) was identified in 1972 as the active antimalarial constituent of *A. annua*. Artemisinin (or its

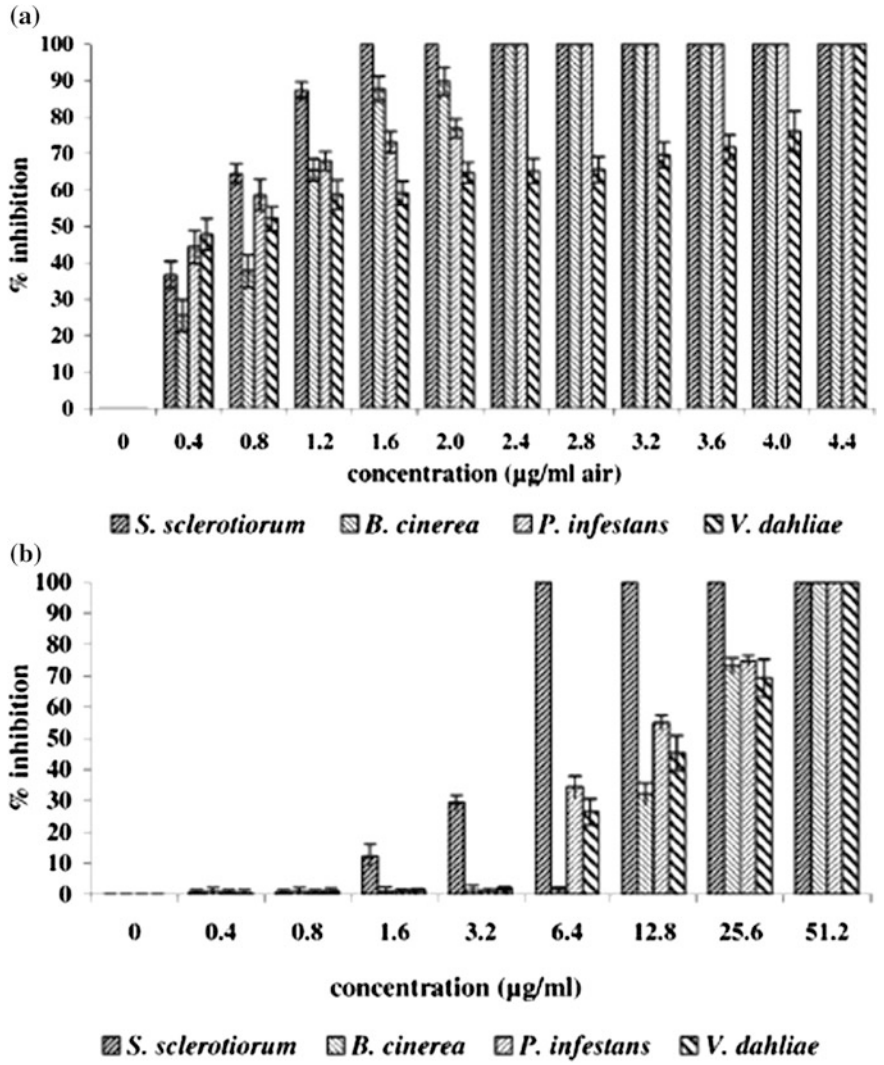

Fig. 13.2 The effects of different concentrations of volatile (**a**) and contact (**b**) phases of essential oils of *Artemisia annua* on the mycelial growth of *Sclerotinia sclerotiorum, Botrytis cinerea, Phytophthora infestans,* and *Verticillium dahliae.* Bars, ±SD of the means (Soylu et al. 2005)

derivatives) is now acknowledged as an important ingredient for new drug combinations to treat malaria when drug resistance to monotherapy is experienced (Shou-zhong 1997). Besides treatment for malaria, current research also shows that ART is also used to treat infections of Leishmania (Yang and Liew 1993; Sen et al. 2007), Trypanosoma (Mishina et al. 2007), *Schistosoma* spp.,

Pneumocystis carinii, *Toxoplasma gondii*, human cytomegaloviruses, *Herpes simplex* viruses, and hepatitis B and C viruses. Artemisinin has also been shown to have in vitro activities against certain tumor cells and also has an effect on gastrointestinal nematodes by decreasing the number of eggs per gram of feces (EPG) in small ruminants (Efferth 2007).

Furthermore, *A. annua* has a high content of flavonoid compounds, which are responsible for its high antioxidant activity. Also, the polymethoxy flavonoids are indicated as important compounds with potential anticancer activity. Cancer cell lines show a differential sensitivity as well as resistance to this group of compounds. Like hydrogen peroxide, H_2O_2, artemisinin reacts with ferrous iron, Fe^{2+}, to generate radical species (Ferreira et al. 2010). Artemisinin derivatives induce programmed cell death of cancer cells by activating the intrinsic or the cytochrome C-mediated pathway for apoptosis, although the initial protein targets of artemisinin derivatives for apoptosis in human cancer cells have not yet been identified (Nakase et al. 2009). AMDT is a sesquiterpene found in the hairy roots of *A. annua*. It has been demonstrated that it induces apoptosis through the mitochondrial-dependent pathway in human lung 95-D cells. Cytotoxicity of this compound was also found in ovary, liver, and cervix cancer cells (Zhai et al. 2010).

There are potential uses of the *A. annua* plant extracts for humans and livestock based on the synergistic effects of flavonoids, artemisinin precursors, etc., including antimalarial effects reported for the *A. annua* traditional tea (Blanke et al. 2008). Despite the use of traditional tea preparations not being recommended as a replacement for the WHO's recommendation use of artemisinin-based combination therapies (ACT), it deserves further investigation on its combined use with other non-artemisinin drugs commonly used in ACTs. In addition, *A. annua* leaves (Zheng and Wang 2001) and crude extracts have been reported to be a good source of antioxidants (Cai et al. 2004), being among the four medicinal plants with the highest ORAC (oxygen radical absorbance capacity) level among other medicinals (Zheng and Wang 2001). This high antioxidant capacity is probably due to the high content (Bilia et al. 2006) and diversity of its leaf flavonoids, including the newly reported C-glycosyl flavonoids as a possible component of the antioxidant and antiviral activity (Han et al. 2008). Emami et al. (2010) reported that strong and dose-dependent inhibition of cancer cell growth by methanol extract of *A. annua*. This extract caused a significant decrease in proliferation of tested cancer cell lines and had less toxicity on normal cells.

13.10 Conclusion

Although botanicals face great competitions from reduced risk synthetic insecticides such as the neonicotinoids in industrialized countries (Isman 2006), but they have great potentials in developing and underdeveloped countries. However, it is not hard to conclude that the best role for botanicals in the wealthier countries is in public health (mosquito, cockroach abatement) and for consumer (home and garden) use

(Isman 2006). The real benefits of botanical insecticides can be best realized in developing countries, where farmers may not be able to afford synthetic insecticides and the traditional use of plants and plant derivatives for the protection of stored products is long established. Even where synthetic insecticides are affordable to growers (e.g., through government subsidies), limited literacy and a lack of protective equipment result in thousands of accidental poisonings annually (Forget et al. 1993). In other ways, the use of natural products available in the tropics and subtropics could help to reduce the need for imported pesticides and thereby gradually increase the developing countries' self-efficiency. One example of such natural product is the neem tree (*Azadirachta indica*), a multipurpose plant, that leads to several commercial formulations for use in agriculture and other areas (Schmutterer 1987).

Essential oils and extract of *A. annua* have the potential for further studies and application on several insect pests by way of toxicity and even causing reduced growth rate, lower food efficiency, and reduced key metabolic components as has been shown in our several studies. Moreover, compounds present in the extract of *A. annua* affect the activity of digestive and detoxification enzymes. Traditionally, this plant was used as a repellent to ants attacking the juvenile stages of silkworm in silkworm cottages in rural areas of Gilan Province (north of Iran). They used freshly cut plants and used them as fences around the cottage used for silkworm rearing. Therefore, we should further the research on this plant and exploit compounds present as alternative pesticides or as templates for new synthetic pesticides. Physiological analysis would be particularly informative to gain insight into the efficiency of a safe management process. This extract may thus serve as an alternative to conventional insecticide for the control of pests.

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Chapter 14
Reverse Pharmacology and Drug Discovery: *Artemisia annua* and Its Anti-HIV Activity

Frank van der Kooy

14.1 Introduction

14.1.1 Drug Discovery and Reverse Pharmacology

There are various ways in which new drugs can be developed. One approach is *in silico* drug design based on our existing knowledge of the biology of a specific disease and the specific target site binding chemistry. Based on this knowledge, a range of molecules will be designed and synthesised after which they will be tested in in vitro bioassays for activity and toxicity. The best candidates, called lead compounds, will then be "fine-tuned" by chemical derivatisation in order to improve their activity and/or to reduce their toxicity. Lead compounds are then tested in various animal models before entering clinical trials in people. Another approach is to screen a large number of biological samples (plants, bacteria and fungi) for activity against a specific disease. Any active extract, consisting of many compounds, will be fractionated by chromatographic techniques, and each fraction will be tested for in vitro activity. Active fractions will again be fractionated until the active compound is identified. This process, also called bioguided fractionation, can go through a number of fractionation cycles before the active compound is identified. The active compound will be chemically derivatised in order to improve its properties before in vivo animal studies will be conducted. Based on these test results, the most promising lead compounds will then be tested in clinical trials in people. There are however a number of shortcomings with both approaches. It is expensive, time consuming, makes use of in vitro bioassays and it suffers from a very low success rate. Due to these shortcomings, it is currently estimated that the development of one new drug costs around $1–1.5 billion, simply because so many lead compounds fail during clinical trials. Keeping these high costs in mind, one would think that all

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registered drugs are effective and importantly non-toxic. Unfortunately, this is not the case, as there are a number of drugs currently on the market that are causing severe side effects and whose efficacy should be questioned. This holds true particularly for cancer chemotherapeutics. It was estimated that cancer chemotherapy improves the average 5-year survival rate of patients (for all cancer types) by only 2 % (Morgan et al. 2004). Another relatively unknown fact is that each year, 200,000 people die in the EU due to adverse drug reactions (all types of drugs), highlighting the severe shortcomings of the drug development and drug licensing pipelines (Archibald and Coleman 2012). To put this into perspective, there are a large number of drugs that work perfectly well and are safe to use, but we have to concede that our approach to drug discovery and our overall approach to health care suffers from some major problems.

There are alternative approaches in which these problems can partly be sidestepped. One such approach can be called reverse pharmacology. This relatively simple approach identifies traditional medicines (e.g. predominantly medicinal plants) that are known to be non-toxic in vivo. This is an extremely important aspect as most lead compounds identified during the normal drug discovery processes fail during clinical trials due to in vivo toxicity. If we identify medicinal plants that have been used and are still being used by thousands of people without any reports of toxicity, we have practically sidestepped this major problem. The second step in reverse pharmacology is to work our way back to the active compound(s) with the use of in vitro bioassays. We therefore start with the in vivo uncontrolled clinical trial followed by the in vitro identification of the active compound(s). One notable difference with the normal drug discovery process is that this system does not necessarily need in vivo animal studies to be conducted (however, the drug registration authority demands animal studies to be conducted before registration). The reverse pharmacology approach is however not without its shortcomings either. The main problem here is that in vitro bioassays are being used, leading to the same set off problems experienced with the normal drug discovery pipeline at this stage of the process. Some of the main problems with in vitro bioassays are as follows:

- They exclude the influence of human metabolism on the active compounds and will therefore not detect the so-called prodrugs. Prodrugs are compounds that are inactive in their natural form but are chemically altered during normal metabolism to become active.
- Bioassays are designed to identify compounds with a specific mechanism of action. It can for instance consist of a single enzyme (e.g. HIV integrase) or a specific life cycle stage of a parasite. Any active compound that does not bind to this enzyme or does not act against a specific life cycle stage might therefore be deemed inactive—A so-called false negative.
- Bioassays do not test for important pharmacokinetic and dynamic aspects such as bioavailability. If the compound is not bioavailable in vivo, then the in vitro activity does not matter much (bioavailability can however be improved by encapsulation techniques).

- Bioassays normally make use of "foreign" organic chemicals or media that can react and thereby deactivate active compounds, leading to a large number of false negatives. Common compounds found in many plants can also react with these bioassay media constituents, making them active, leading to a large number of false positives.

The main issue is however the problem of false positives and false negatives. The false positives are compounds that are deemed "active" in a specific bioassay after which a large amount of time and effort will be spent on their identification. Fortunately, false positives eventually always reveal themselves but, unfortunately, at a cost of time and funds. False negatives are a far more serious problem in that they do not react with a specific enzyme in the bioassay used and are therefore deemed inactive. They may even degrade in the "foreign" media used in the bioassay. The main problem here is that false negatives never reveal itself. For example, if it is published that medicinal plant X show no activity against disease X, very few people will go and reinvestigate, if any. Unfortunately, this problem is also important when using in vitro bioassays to test for the toxicity of lead compounds or active extracts. Here, again we find the occurrence of false positives and negatives. False positives are again compounds reacting with specific cells, leading to cell death and the conclusion is made that this compound or extract is toxic. A good example of this was that we recently tested English tea (*Camellia sinensis*) as negative control on a specific cell line. Due to toxicity in that specific cell line, our experiments could not continue, and we could not use normal tea as a negative control (unpublished results). Many millions of people are drinking tea every day without any short- or long-term toxicity reported thus far. To put this into perspective, if this was a medicinal plant, we would have concluded that it is far too toxic and we would not have continued our investigations. A false negative in toxicity bioassays speaks for itself. There are countless examples of lead compounds that were deemed safe after in vitro and even in vivo animal studies that turned out to be toxic in people.

There are many problems associated with in vitro bioassays, and it will remain a problem for the normal drug discovery pipeline as well as for the reverse pharmacology approach. The only advice is to interpret in vitro results very carefully.

14.1.2 Reverse Pharmacology: Economic and Political Considerations

These two approaches to drug discovery that we can call the "normal" and "reverse" approaches are, unfortunately, at the moment exclusive. The main reason for this is not only scientific but economic and political, and that is why this aspect should shortly be discussed here. We live in a segregated world with on the one side the "rich" Western countries with their own unsustainable complex

economic system (capitalism) and, on the other side, the "poorer" non-Western countries with their more sustainable simpler economic systems. In Western countries, scientific research should lead to the creation of financial profits in the short term in order to sustain their complex economic system. In practice, this means that funding for research into (new) medicines will be made available only if research will lead to the development of a new drug that can be sold for a profit in order to create or sustain a company and keep people employed. In short, to keep the economy growing, profits need to be made. As such, there is nothing wrong with this approach, but it does however focus on the creation of wealth and employment on the short term, and not on the health of people. One advantage of this economic system on a scientific level is that these countries have extremely well-equipped laboratories to perform in-depth scientific research into new medicines, but as long as potential profits take centre stage. Because of this, research into medicinal plants has basically been stopped for the simple reason that a medicinal plant cannot be produced and sold in order to create wealth—it is just too inexpensive and will therefore not be able to financially sustain anyone or any company. Identifying a new lead compound from a medicinal plant will be supported to some degree, but only if the possibility of patenting and eventual profits exist. The harsh reality is that if a medicinal plant works or not (the science behind it) does not really matter at all—it just does not fit into the economic model. In non-Western countries, the focus is still primarily on the health of people and not on short-term direct profits. Here, medicinal plants have always played a role and will continue to do so as long as the main objective is the health of their citizens and not direct short-term profits. That people sell medicinal plants for a profit in these countries does of course take place, but this is not on a comparable scale to the cost of drugs in Western countries.

Is there any possibility to reconcile these two worlds? Can and should research into medicinal plants take place in Western countries in order to use it as such? The way we see it is that we do not have a choice anymore. The health care systems in many Western countries are on the verge of collapse, and it appears that the capitalistic model and health care are not a good mix after all. A fast growing lack of trust and the extremely high costs involved are probably the main reasons. It has become too expensive, proven by the fact that many people in Western countries are already excluded from primary health care, simply because they cannot afford it. We have to find a way to reduce the cost of health care in Western countries. Expensive medicine is of course only one part of the overall cost of the health care system, but it is a good starting point. What needs to be done is to study the health care systems in non-Western countries and to apply our vast resources in Western countries into studying specifically the types of medicine that they use in order to provide and create an evidence-based, quality-controlled, inexpensive and effective medicine. We have to be clear that not all of the traditional medicines used in non-Western countries will work; there will be many failures, but there will (or might) also be big successes. One medicinal plant that should be studied in detail is *Artemisia annua* L. (Asteraceae) (Fig. 14.1). This medicinal plant has the potential to satisfy the current needs of both worlds but also has the potential to

Fig. 14.1 In the foreground, the *Artemisia annua* plant and in the background the users of this plant as a tea infusion for the treatment of various diseases (Photo with permission from Anamed)

bring these two worlds closer together. It can yield single compounds that can be further developed into a drug—which it already did. It can also be used in the form of a medicinal plant as an inexpensive, effective treatment for life-threatening diseases in non-Western countries—which it already did. Our sincere hope is that this plant will one day even be used in Western countries as soon as the scientific evidence for its efficacy is provided. All we need to do is some in-depth scientific research, which at the moment is unfortunately not being supported in Western countries due to the aforementioned economic reasons. To address this, we have recently published a review paper dealing with the scientific complexities we face in studying this plant and recommended the logical next steps that should be taken (van der Kooy and Sullivan 2013). This chapter will therefore describe another important aspect of *A. annua* (Fig. 14.1) and the discovery of the remarkable anti-HIV activity of this plant. We will give a short introduction on the history of *A. annua*, the biological activities associated with this plant, the reported antiviral activities and more specifically the anti-HIV activities of all compounds identified in the traditionally prepared *A. annua* formulation. The potential impact of this discovery and the need to continue with this research will also be discussed.

14.1.3 Background of Artemisia annua and Artemisinin

A. annua has been used for hundreds of years to treat various ailments, specifically those related to the treatment of intermittent fevers. This traditional use has been linked to malarial infections caused by the *Plasmodium falciparum* parasite. From *A. annua,* one active compound, a sesquiterpene lactone endoperoxide called artemisinin has been identified and is currently being used, as artemisinin derivatives, as a first-line treatment for malaria (De Ridder et al. 2008). The traditional use of *A. annua* in the form of a tea infusion prepared from the dried leaves

continues to be used in China and due to its apparent effectiveness has rapidly spread across the globe in recent times. Currently, it is being used all over the world as an inexpensive treatment for malaria, as opposed to the WHO-recommended artemisinin derivatives. The traditional use of *A. annua* has caused a large amount of controversy, and it is feared that resistance against artemisinin might develop because of the traditional use of the plant. There is however an opposing view that states *A. annua* has been used for hundreds of years without resistance developing and only now since a single compound from this plant is being used, resistance has developed. The use of *A. annua* can however have important implications specifically for antimalarial treatments in developing countries, where the high cost of commercial drugs makes it practically inaccessible to many. It can also have important implications for Western countries where the general high cost of health care has already excluded many from receiving adequate treatment for disease. The common view on the use of *A. annua* supported by the WHO is relatively simplistic. In order to reach the prescribed doses of the main active compound artemisinin, they have concluded that artemisinin combination therapies (ACTs) are the only way to approach the treatment of malaria (Mueller et al. 2004; WHO 2012). This clash between Western-based drugs (e.g. artemisinin) and traditional medicines (medicinal plants e.g. *A. annua*) has culminated in the publication of a Position Statement by the WHO on the use of *A. annua* for the treatment of malaria. The main recommendation in this statement was not to use *A. annua* in any form including a tea infusion due to the low natural abundance of artemisinin in *A. annua* (Liu et al. 2006 and WHO, 2012). For a full discussion on the traditional use of *A. annua,* Hsu (2006) should be consulted, and for a full discussion on the controversy surrounding the use of *A. annua* versus the WHO-recommended use of artemisinin derivatives, van der Kooy and Sullivan (2013) should be consulted.

There are however some problems relating to the use of artemisinin. The main problem is that it is a very fast-acting drug with a very short half-life. It therefore needs to be combined with a slower-acting drug in order to assure the effective elimination of all the parasitaemia. Another problem is the assurance of an adequate supply of artemisinin. Due to its complex chemical structure, the synthesis and/or semi-synthesis remains to be difficult, and therefore the main source of artemisinin remains to be isolation and purification from the *A. annua* plant. Due to environmental and economic influences, the annual production of *A. annua* suffers from a boom and bust cycle with overproduction and low prices the one year to underproduction and high prices the next year. The cost of this Western-style treatment is also far too high for where it is used—predominantly in non-Western countries. In order to stabilise the global production of *A. annua* and the price of artemisinin, various approaches have been taken. To increase the yield of artemisinin in *A. annua,* classical plant breeding techniques have been employed (Bartlet 2010); the isolation and purification techniques are continuously being improved (Liu et al. 2011a), and the chemical synthesis of artemisinin has been successful to some degree (Dietrich et al. 2009). It is of note that all of this research has taken place in Western countries and that the focus is on the single compound artemisinin. Another novel approach would be to identify a second

active pharmaceutical ingredient (API) in order to reduce the overall production cost of artemisinin (Lubbe et al. 2012). In order to do this, the traditional and current use of *A. annua* for diseases other than malaria was investigated.

14.2 Anti-HIV Activity of *Artemisia annua*

14.2.1 The Antiviral Activity of Artemisia annua and Artemisinin

A. annua is currently mainly being used for the treatment of malaria but also for the treatment of skin and digestion ailments, HIV-AIDS, bronchitis, cancer and haemorrhoids. These alternative uses for this medicinal plant is recommended by NGOs such as IFBV-BELHERB and Anamed (2012). This section will however only deal with the antiviral activities associated with *Artemisia* spp., and artemisinin.

Reports of other *Artemisia* species showing anti-HIV activity are limited to *A. caruifolia* and *A. capillaris.* Ma et al. (2001) identified four compounds from methanol extracts of *A. caruifolia,* namely *N*1, *N*5, *N*10-tri-*p*-coumaroylspermidine derivatives which showed around 70 % inhibition of HIV-1 protease at a concentration of 100 μg/mL. The three dicaffeoylquinic acid derivatives also isolated during this study did not show any appreciable activity against HIV-1 protease. However, Cos et al. (2008) reported that 3,5-dicaffeoylquinic acid did indeed show good activity against HIV integrase, although controversy remains around its potency and activity in vivo. In a metabolomic investigation of *A. annua* and *A. afra*, coumaroylspermidine derivatives were not detected in either species tested (Liu et al. 2010). No other reports could be found that these compounds have been identified in *A. annua*. Wu et al. (2001) concluded that the active anti-HIV components present in *A. capilaris* were the flavonoids isorhamnetin and arcapillin, as well as the coumarin derivative aesculetin.

A. annua has been shown to possess a wide variety of antiviral effects, notably against herpes simplex virus, Type 1 (HSV1). Karamoddini (2011) conducted in vitro tests on methanolic extracts of homogenised plant material of various *Artemisia* species, where *A. annua* showed the highest antiherpetic activity. *A. annua* was also tested with the common antiviral drug acyclovir, where it showed higher anti-HSV1 activity than the commercial drug (Karamoddini 2011). It is possible that the noted antiherpetic effects could be caused by artemisinin; however, synergism or other active compounds has yet to be tested in relation to HSV1. *A. annua* has been shown to be active against the severe acute respiratory syndrome-associated coronavirus, a highly deadly and contagious disease (Li et al. 2005). Li et al. (2005) tested ethanolic extracts of *A. annua* and three other plant species against the virus, determining *A. annua* to have an EC_{50} of 34.5 ± 2.6 μg/mL. Abid Ali Khan et al. (1991) studied the antiviral activity of *A. annua* against the

tobamoviruses, where plant samples were extracted with *n*-hexane and evaluated against tobamovirus virus cultures, with 75 % inhibition noted. Abid Ali Khan et al. (1991) then fractionated the extract, collecting the active fraction and determined the active compound/s to be sterols. The outcome of this study is important to note as artemisinin was not identified as the virus inhibitor.

The pure compound artemisinin also showed significant activity towards the bovine viral diarrhoea virus (BVDV) (Romero et al. 2006). BVDV is classed as a pestivirus, under the *flaviviridae* family, and in this study, bovine epithelial cells were infected with the BVDV virus and treated with artemisinin. It was found that artemisinin is an inhibitor of *flaviviridae* viruses, with a possible application to the treatment of the hepatitis C virus (HCV). HCV is very similar to HIV in that they are both RNA viruses; however, HIV is a retrovirus and integrates itself into the hosts DNA. Paeshuyse et al. (2006) confirmed this by testing artemisinin against HCV and found it to be active at levels of 78 ± 21 μM. This activity was potentiated by hemin at concentrations that had no effect on the host cells, indicating a pronounced synergistic antiviral activity when artemisinin and hemin were combined. A further understanding of how *A. annua* extracts, and not just artemisinin, interact and inhibit viruses could enable future investigations to discover and develop practical and inexpensive treatments for viruses of a similar nature, like that of HIV. Romero et al. (2005) studied the activity of artemisinin against hepatitis B virus (HBV), noting IC_{50} values of 55 and >100 μM for the inhibition of hepatitis B envelope protein surface antigen and HBV-DNA release, respectively. Interestingly, artemisinin proved to be a more effective antiviral than its commonly used derivative, artesunate, with IC_{50} values of 2.3 and 0.5 μM, respectively.

14.2.2 The Anti-HIV Activity of Artemisia annua and Artemisinin

In vivo activity: A 2011 survey completed by Willcox et al. (2011) in Kenya and Uganda reported that 51 % ($n = 20$) of respondents were using *A. annua* infusions for diseases other than malaria, where 28 % ($n = 11$) were using the infusions for the treatment of HIV. Furthermore, a survey completed by Noumi and Manga (2011) in Cameroon also noted that 13.8 % of respondents were using *A. annua* in relation to HIV/AIDS. Abu-Raddad et al. (2006) hypothesised that the spread of the malaria parasite may be enabling the spread of the HIV virus throughout Africa, due to tainted blood used for transfusions. This hypothesis gives reason to the further study of current malaria treatments in relation to HIV, like that of *A. annua* and other commonly used medicinal plants.

In vitro activity: Information regarding the in vitro anti-HIV activity of *A. annua* and artemisinin is rather limited. Chang and Woo (2003) tested the methanolic extracts of 80 commonly used Korean medicinal plants against HIV in relation to virus—cell fusion inhibition. They used the syncytium inhibition assay,

which is based on the interaction between the HIV-1 envelope and the cellular membrane protein CD4 on T-lymphocytes. Some inhibition was seen at the concentration tested (15.8 % ± 5.9), even though it was relatively low compared to some of the other 80 plants in the test series and therefore no other solvent extracts or fractions of *A. annua* were tested during this study. In reality, organic extracts are not used, but rather a tea infusion is brewed and administered. Various patents exist that cover the broad biological activity including the antiviral activity of *A. annua* in combination with other medicinal herbs (Zhang and Zhang 2010; Chen 2010; Nagaura 2009; Xue 2008a, b; Zhang 2003; and Chen 2007).

The first report on artemisinin derivatives being used as anti-HIV agents was published by Jung and Schinazi (1994) who embarked on a study of artemisinin trioxane derivatives. They concluded that anti-HIV activity was common in artemisinin trioxane derivatives and, given their results, further evaluation was needed for a potential anti-HIV treatment to be produced. Lubbe et al. (2012) investigated the traditional infusion of both *A. annua* and *A. afra*, compared to pure artemisinin, in relation to anti-HIV activity, noting that both infusions exhibited potent inhibition with *A. annua* having an IC_{50} of 2.0 μg/mL, whereas pure artemisinin showed no inhibition even at 25 μg/mL. *Artemisia afra* which does not contain artemisinin showed a similar level of activity against HIV, indicating that the active compounds are probably not artemisinin. Given the surveys completed by Willcox et al. (2011) and Noumi and Manga (2011), a clear relationship between plant and disorder can be seen. The implementation of reverse pharmacology in cases like this would enable researchers to possibly develop effective treatments for various viruses (specifically HIV) and diseases, given their socio-historical validation.

14.2.3 The Discovery of the Anti-HIV Activity of the Artemisia annua Tea Infusion

Keeping in mind the economic and political aspects surrounding the discovery and development of new drugs, we embarked on a non-funded project in order to study the possibility of reducing the price and the accompanying price fluctuations of artemisinin by identifying a second API in *A. annua*. A reverse pharmacology approach was taken, which means that we had to find evidence that *A. annua* is being used by thousands of people without any reports of toxicity. In many parts of the world, specifically in non-Western countries, people rely on traditional medicines as primary source for their health care needs (Farnsworth 1985). For a relatively new disease such as HIV, the identification of medicinal plants that work against these new diseases will therefore still undergo the process of trial and error. Nevertheless, the small-scale surveys conducted by Wilcox et al. (2011) and Noumi and Manga (2011) revealed that HIV-infected patients had started to use *A. annua* infusions for the treatment of HIV although the original treatment was intended for malaria. In both these surveys, the patients prepared a tea infusion

from *A. annua* either alone or with other plant species. Feedback from the non-governmental organisation Anamed also claimed that the herb is often used by HIV patients. This information is important in that we know that *A. annua* is being used by thousands of people for the treatment of malaria without any toxicity reported thus far. In effect, a large uncontrolled clinical trial has been conducted exactly where it should take place, where the highest burden of HIV occurs. This has given us two important outcomes: The *A. annua* tea infusion appears to be non-toxic and exhibits claimed in vivo anti-HIV activity. This is the first important step as toxicity is the biggest problem experienced in the drug discovery process. The next step in reverse pharmacology is to provide in vitro evidence that the traditional tea infusion does indeed have activity against HIV.

Two questions needed to be answered. Does the *A. annua* tea infusion exhibit any in vitro anti-HIV activity? And if so, is this activity caused by artemisinin? In order to test if artemisinin was responsible for the reported in vivo anti-HIV activity, we therefore had to include a chemically similar plant which does not contain any artemisinin. Previous work conducted on *Artemisia afra* Jacq. ex Wild. (Asteraceae) revealed it to be chemically similar to *A. annua* but, importantly, it does not contain artemisinin (Liu et al. 2010; van der Kooy et al. 2008). The inclusion of *A. afra* was done in order to determine whether artemisinin was responsible for any observed anti-HIV activity, or whether a combination of artemisinin and other components can explain any observed activity (synergism). Furthermore, we could not find any literature reports that *A. afra* was tested against HIV (Liu et al. 2009) except one report where HIV-infected patients were given *A. afra* together with standard HIV treatments in order to boost their immune systems (Mulholland and Drewes, 2004).

We prepared tea infusions from nine *A. annua* and one *A. afra* sample according to van der Kooy and Verpoorte (2011) and tested them against HIV according to Lubbe et al. (2012). The plant material was collected from different parts of the world and in different years. The results indicated that the *A. annua* tea infusions had IC_{50} values of between 0.6 and 3.7 μg/mL and for the *A. afra* infusion an IC_{50} value of 1 μg/mL (values corrected for variation in the positive control—Lubbe et al. 2012) (Table 14.1). These results by themselves are quite remarkable in that Cos et al. (2008) reported that any single compound with an activity of below 25 μg/mL should be considered to have significant antiviral activity. We know that the tea infusion prepared from *A. annua* consists of many compounds (Mouton et al. 2013), making this chemically complex "extract" highly active against HIV. Furthermore, the activities of all the samples appeared to be relatively similar, indicating that the concentration of the active compound in the samples is probably very similar. Furthermore, the active compound appears to be stable for a number of years as the oldest plant sample tested was about 10 years old. These results answered our first question in that the *A. annua* tea infusion does indeed exhibit good in vitro activity against HIV. We have to be careful in interpreting these results, and we have to keep in mind that in vitro bioassays suffer from some severe shortcomings as was discussed in the

Table 14.1 The *Artemisia annua* and *Artemisia afra* samples tested against HIV.

Sample	Country of cultivation	Harvest period	Plant parts	Artemisinin content	Corrected IC_{50} low (μg/mL)
Artemisia annua					
1	South Africa	1999	Leaves/flowers	0.36	1.0
2	South Africa	2002	Leaves/flowers	0.30	2.3
3	Tanzania	2005	Leaves	0.49	1.4
4	South Africa	2006	Leaves	0.74	1.2
5	Tanzania	2006	Leaves	0.46	1.3
6	Cameroon	2007	Leaves	0.56	2.6
7	Germany	2007	Leaves	0.58	4.5
8	Mozambique	2007	Leaves	0.40	0.6
9	Germany	2009	Leaves	0.80	3.7
Artemisia afra					
10	South Africa	>2008	Leaves	nd	1.0

nd = not detected

The country of cultivation, year of harvest, plant parts used and the artemisinin content of all the samples are given. The IC_{50} value against HIV has been corrected to compensate for the variation experienced with the positive control used in this bioassay according to Lubbe et al. (2012)

introduction of this chapter. The possibility exists that this result can be nothing more than a false positive. Unfortunately, only time will tell.

Another possible explanation for the anti-HIV activity is synergism between artemisinin and other compounds in the extract. With the inclusion of *A. afra* (not containing artemisinin) and the observed activity of this sample, it appears that artemisinin had a very limited role to play, if any. We have shown (van der Kooy et al. 2008 and Liu et al. 2010) that these two species are chemically closely related with the major exception that artemisinin has not yet been detected in any *A. afra* specimen. This was also one of the first reports that *A. afra* possesses significant in vitro anti-HIV activity (Lubbe et al. 2012) and provides scientific evidence to the reports of Mulholland and Drewes (2004) that patients given *A. afra* in combination with standard HIV treatment reported improvement of symptoms compared to patients taking only standard HIV treatments. *Artemisia afra* might therefore not only boost the immune system but may also have a direct activity against HIV.

The first step in the reverse pharmacology approach is relatively simple. We have identified a medicinal plant that has been used by thousands of people all around the world without any reports of toxicity. The second step is somewhat more difficult in that we now have to use in vitro bioassays in order to identify which compounds are responsible for the activity. As it stands, we do not yet know which compound(s) in *A. annua* or *A. afra* is responsible for its observed in vitro anti-HIV activity. Due to the reasons discussed in the introduction, research into the chemistry of the *A. annua* tea infusion will not be supported in any Western country. It is therefore interesting to note that very little chemical work has been done on the traditional tea infusion prepared from this plant. Due to this limited data, we can only report on what is currently known on the few identified compounds in the *A. annua* tea infusion regarding their possible anti-HIV activity.

14.3 The Chemistry of *Artemisia annua* Tea Infusions

There are a limited amount of chemical studies that have been performed on the *A. annua* tea infusion. Most of these studies have also focussed only on the extraction and quantification of artemisinin without conducting a complete chemical profile on the *A. annua* formulations.

Based on this limited data, the main conclusion was drawn that the tea infusion is ineffective and should not be used (Mueller et al. 2000, 2004; Rath et al. 2004; Jansen 2006; and van der Kooy and Sullivan 2013). This view is unfortunately too simplistic. If we take a closer look at traditional medicines, we have to realise that the chemistry of any traditional formulation consists of two parts (1) the chemistry of the medicinal plant and (2) the chemical influence that the preparation method has on the final formulation. The first aspect has been studied in some detail, and over the years, more than 600 secondary metabolites have been identified and reported for *A. annua* (Brown 2010). In contrast to this, only 37 compounds have been identified in tea infusions or cold water extracts, mainly consisting of caffeic acid derivatives, flavonoids, coumarins and artemisinin (van der Kooy and Sullivan 2013). The influence of the preparation method on the chemistry of the final formulation has not been studied at all. To put this into perspective, we all know that a raw potato tastes different than a cooked potato, indicating that the preparation method has changed the chemistry. What effect will the preparation method have on the chemistry of the final formulation of the *A. annua* tea infusion? The chemical changes that can occur are not only due to the high temperatures involved but also due to water chemistry. Under field conditions, people will use any water available to them to prepare their tea infusions, and we know that water contains a large and variable amount of salts and elements. Any of these salts or elements can react to any organic molecule extracted from *A. annua*. The implications of this have been discussed by Van der Kooy and Sullivan (2013). These chemical changes can possibly have a large effect on the overall activity of the tea infusion. Here, we also have to clearly state that it can potentially have the opposite effect as well—the infusion might become less effective.

In our lab, we recently identified two new compounds in the tea infusion, *cis*- and *trans*-melilotoside, which was the first report of these compounds in any *Artemisia* spp. (Mouton et al. 2013). Figure 14.2 illustrates the chemical structures of some of the compounds identified in the *A. annua* tea infusion. Due to the limited chemical data, we do not yet know which compounds in the *A. annua* tea infusion are responsible for the anti-HIV activity, and the possibility that it might be one of the 37 compounds already identified will be further discussed. We have to keep in mind that we are using in vitro bioassays, which will exclude any prodrugs being identified or any compound whose mechanism of action is different to that targeted in the bioassay. In this section, we will describe what is currently known on the anti-HIV activity of all the identified compounds to date.

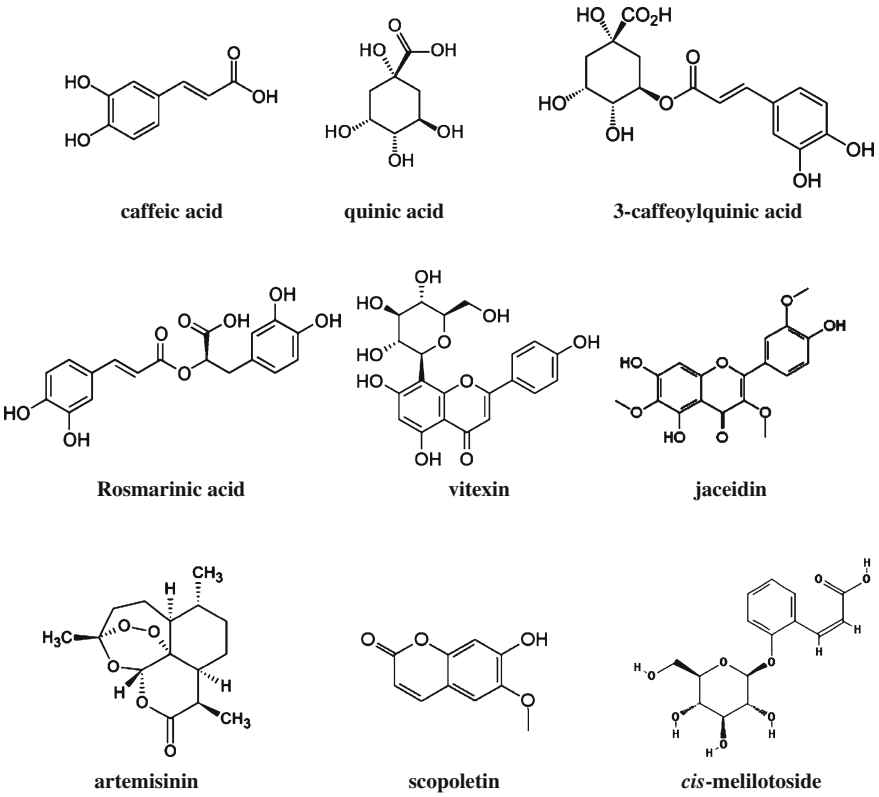

Fig. 14.2 The chemical structures of selected compounds identified in the *Artemisia annua* tea infusion, illustrating the chemical diversity in the tea infusion

14.3.1 Anti-HIV activity of compounds identified in the Artemisia annua tea infusion

Chlorogenic acid derivatives: This group of compounds are widely distributed in the plant kingdom. This fact also makes their biological activity and especially their anti-HIV activity controversial. Almost all plants contain chlorogenic acid and its derivatives, and it would therefore be odd if these compounds are also the main active constituents. Or is this maybe proof for "you are what you eat", indicating that a good balanced diet consisting of fruits and vegetables will fend off disease? Nevertheless, this class of compounds are thought to exhibit good anti-HIV activity with patents covering their use as anti-HIV treatments (Harding et al. 2011; Sun et al. 2008). Cos et al. (2004) have also reported that 3,5-dicaffeoyl-quinic acid does indeed show good activity against HIV integrase, although controversy remains around its potency and activity in vivo. However, our latest finding based on the positive reports from African communities using the

A. annua tea infusion for the treatment of HIV indicates that there is indeed strong anti-HIV activity in the *A. annua* tea infusion (Lubbe et al. 2012) and that this activity can therefore partly be ascribed to the chlorogenic acid class of compounds.

Coumarins: Both coumarins identified in the *A. annua* tea infusion were tested by Wu et al. (2001) against HIV and were found to be inactive. Coumarins are however known for their anticoagulation properties with warfarin being the best example. This possible influence on blood coagulation properties may have an indirect effect on the anti-HIV compounds, although this remains to be shown.

Melilotosides: These relatively rare compounds did show good activity against the diarrhoea-causing pathogens *Entamoeba histolytica* and *Giardia lamblia* (IC_{50} = 12.5 and 16.8 μg/mL, respectively), indicating that the traditional use of *A. annua* for the treatment of diarrhoea might be effective (Calzada et al. 2003). No reports could be found on their activity against HIV, although these compounds will be hydrolysed into their respective *o*-coumaric acids in the digestive tract. The *o*-coumaric acids have not yet been tested against HIV, but Zhuang et al. (2009) have reported that they exhibit some activity against the SARS virus.

Flavonoids: Vitexin, chrysoeriol and cirsilineol were tested by Wu et al. (2001) against HIV replication and were found to be inactive. Piccinelli et al. (2005) also tested vitixin and isovitixin against HIV and reported weak activity. Patuletin glycoside has not yet been studied against HIV, while luteolin-7-O-glucoside and rutin were tested by Lee-Huang et al. (2003) and were found to be inactive. Jaceidin, chrysosplenol D and chrysosplenetin have also not yet been tested against HIV. None off the flavonoids identified thus far in the *A. annua* tea infusion show strong activity against HIV in any in vitro HIV bioassay used. We have to keep in mind that in vitro bioassays will exclude identification of any prodrugs. To put this into perspective, Tao et al. (2007) reported on the strong anti-HIV activity of the sodium–rutin–sulphate complex. This highlights the possibility that salts and elements in water can "activate" common compounds which might otherwise be inactive.

Terpenoids: Artemisinin has been tested against various viruses. Efferth (2008) reported on the broad antiviral activity of artemisinin and its semi-synthetic derivative artesunate. Artesunate showed inhibition of HIV at levels of 600 nM, but no reports on the activity of artemisinin against HIV was given. Jung and Schinazi (1994) reported on the anti-HIV activity of artemisinin, with EC_{50} and IC_{50} greater than 100 μM. Benedikt et al. (2005) have patented artemisinin and some of its derivatives against various viruses. In the patent description, the activity of artemisinin was given to be insignificant against HIV-1 and HIV-2. Lubbe et al. (2012) found artemisinin to be inactive against HIV at a concentration of 25 μg/mL. We therefore have some conflicting reports on the activity of artemisinin against HIV probably caused by the different bioassays used.

14.4 Discussion and Conclusions

From the above discussion on the available literature associated with the identified compounds, we can conclude that it remains unclear which compounds are responsible for the observed in vitro activity and the claimed in vivo activity. The chlorogenic acid family does have some potential role to play in this observed activity, while the coumarins appear to have no direct role to play. The melilotosides have not yet been tested, while some of the flavonoids were tested but did not show a high level of activity against HIV. The concentration of the flavonoids in the tea infusion was also found to be rather low, which furthermore decreases the possibility that this class of compounds are responsible for the activity. If the flavonoids have any role to play, it will be in the form of a prodrug, as Tao et al. (2007) reported on the strong anti-HIV activity of the sodium–rutin–sulphate complex. Artemisinin has been tested against HIV using various bioassays, and the reported activity is somewhat contradictory. It therefore appears that none of the compounds identified thus far can be seen as the main anti-HIV compound. The logical next steps to take would be to perform a full chemical investigation into the tea infusion including the chemical reactions that can take place during the preparation of the tea with various chemicals present in water. It will also be important that the in vitro anti-HIV activity of the tea infusion be confirmed by an independent laboratory in order to decrease the possibility that this result might be a false positive. If the activity can be confirmed, the active compounds should be identified with the normal bioguided fractionation processes. Here, we have to keep in mind that fractionation can lead to the loss of activity and this effect, combined with the difficulties we face with in vitro bioassays, can make this task more difficult than what it appears (Schmidt et al. 2007; Wagner and Ulrich-Merzenich 2009; and Gertsch 2011). To give us a potential lead and to narrow down the possibilities, we can again take a reverse approach. If we look at the chemical structures of existing HIV drugs that are currently being developed or used, is there any similar structure to those compounds identified in the *A. annua* tea infusion? There is one HIV drug, Bevirimat, based on the structure of betulinic acid, a triterpenoid. The Asteraceae family of plants is known to contain a wide variety of terpenoids with various biological activities associated with this group of compounds. *A. annua* has been reported to contain oleanolic acid and other tritepenoids which is structurally similar to Bevirimat. As of yet, none of these compounds have been identified in the tea infusion, but it appears that focussing on the identification of terpenoids in the tea infusion might be a good starting point.

In this chapter, we have highlighted some aspects regarding the drug discovery process and the severe shortcomings with our current approaches. This chapter is not about *A. annua* but rather uses this medicinal plant as an example to illustrate some rather philosophical aspects in our approach to the general health care system in Western countries. The reverse pharmacology approach does indeed hold a key to a faster and more inexpensive development of new medicines, but in order for this approach to be fully developed and exploited, it will unfortunately

take a lot of persuasion. The best way to persuade the powers that be is to provide the scientific evidence of the effectiveness of this approach. And this is just where the problem lies. It will only be supported once the evidence is provided, and in order to provide the evidence, it must first be supported—a classical catch 22.

The cost of medicine plays only a modest role in the overall cost of health care in Western countries. The capitalistic approach to health care has already unfortunately resulted in the exclusion of a large number of citizens in these countries, and it will be interesting to see how governments will deal with this growing problem. Will they shift their focus from sustaining a too expensive health care system or will they start to investigate any and all possible methods to improve the health of their citizens and reduce the cost, even if this means using inexpensive medicinal plants. In this chapter, we used *A. annua* as an example of how quick a potential lead can be found with the use of the reverse pharmacology approach. The scientific investigation into the medicinal properties of this remarkable plant is unfortunately very limited for the reasons stated above. We do, however, know that this plant has been used for hundreds of years for the treatment of various ailments without any reports of serious side effects or toxicity. This plant is currently being used for the treatment of malaria (approved by the WHO) and HIV (ethnobotanical use), and there are also reports that people have started to use *A. annua* for the treatment of various forms of cancer (Anamed). Lui et al. (2011b) have recently shown that artemisinin does indeed show selective action against breast cancer cell lines, and this selectivity should be more than enough reason to study this plant in more detail. We can conclude that the development of new drugs is prohibitively expensive, and that many lead compounds fail during clinical trials. With *A. annua,* we have an uncontrolled clinical trial happening in exactly the right place—in countries suffering the most under the burden of HIV. It is therefore important to put health before short-term profits as this will lead to long-term profits. We are therefore calling for performing an in-depth scientific study into the traditional formulation consisting of *A. annua* for the treatment of HIV, malaria and also certain forms of cancer. This medicinal plant definitely has the potential to have a global impact on our approach to health care, but importantly we also have to be honest. After scientific scrutiny of *A. annua,* it might turn out not to work in the way we expect—only time will tell.

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Chapter 15
Production of Artemisinin *In Planta* and in Microbial Systems Need Not Be Mutually Exclusive

Ebiamadon Andi Brisibe and Peter Nkachukwu Chukwurah

Abstract *Artemisia annua* L. is an innocuous medicinal plant that is suddenly found at the forefront of global efforts aimed at the eradication of malaria. The plant is also seen as an effective treatment against several other infectious diseases and human cancer cell lines, and this has been correlated with its richness in several bioactive compounds including artemisinin, other sesquiterpenes, and antioxidants. Undoubtedly, this is a development that has drastically increased artemisinin demand worldwide. Up until now, *A. annua* L. remains the only commercial source for the supply of this vital antimalarial drug to the international market. Recent advances in biotechnology, however, such as have been demonstrated in the production of isoprenoid precursors of artemisinin in yeast, and bacteria are feasible complementary strategies that would help reduce artemisinin cost in the future. The key genes encoding for enzymes regulating the biosynthesis of artemisinin *in planta* are fully understood to enable metabolic engineering of the pathway, and results from pilot genetic engineering studies in microbial strains thus far are very inspiring. This current treatise, therefore, explores the status of artemisinin and other plant metabolites for use in both human and animal healthcare and highlights the implications of *in planta* production of artemisinin in comparison with that from synthetic biology. Overall, these two methods need not be mutually exclusive and can be made complementary to each other depending on the location of production. Aside from artemisinin, required for saving the lives of countless patients in malaria-stricken societies, the plant also contains several other secondary metabolites with enormous benefits in the promotion of human and animal health. Consequently, keeping *in planta* production of artemisinin would play a pivotal role in providing artemisinin for ACTs as well as maintaining

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profit margins to local and regional economies in countries where malaria is endemic, especially in Africa where *A. annua* has been cultivated for the past 10 years.

15.1 Introduction

Malaria is the most deadly parasitic disease known to man today, especially in the developing world where it imposes an enormous burden of morbidity and mortality. It is caused by protozoan parasites, notably *Plasmodium falciparum*, which is spread following the bite of infected female *Anopheles* mosquitoes. At the moment, over 40 % of the world population is under some risk of contracting malaria, with recent estimates suggesting several hundred million cases of clinical episodes and about 800,000 deaths per year (Kappe et al. 2010). Unfortunately, about 90 % of malaria-related mortality usually occurs among children less than five years of age and pregnant women in sub-Saharan Africa (Rinaldi 2004), where a large proportion of the entire population has no access to proper healthcare services. In addition, the drain on local African economies is inconceivable, often estimated to be more than US$12 billion each year in lost gross domestic product (WHO 2002). Threat of malaria is also seen as a deterrent to tourism and internal trade, further constituting a serious obstacle to socio-economic development that perpetuates a cycle of poverty in the continent.

In the past, quinoline-based drugs were the main choice for the prevention and treatment for malaria. Unfortunately, the emergence, through mutation, of drug-resistant *Plasmodium* species in many parts of the world, has rendered these traditional and low-cost antimalarial medicines, such as chloroquine, ineffective. Presently, the best hope for a replacement treatment lies with drugs based on artemisinin, to which *Plasmodium* parasites have not yet developed resistance (Gordi et al. 2002; Xu et al. 1986; Schmid and Hofheinz 1983), except in an area in western Cambodia (Cheeseman et al. 2012). Thus, artemisinin-based drugs remain the primary weapon for reducing the burden of disease in individuals and general populations in malaria-endemic societies.

Artemisinin is a cadinene-type sesquiterpene lactone with a crucial endoperoxide bridge. It is produced and sequestered in glandular trichomes that are found on leaves, floral buds, and flowers (Ferreira and Janick 1995; Tellez et al. 1999) of a weedy plant called annual wormwood (*Artemisia annua* L). Commercial production of this sesquiterpenoid compound, which is highly potent and effective against all *Plasmodium* species, including multidrug-resistant strains, requires that it be extracted from the aerial parts of this herb.

With its semi-synthetic derivatives such as dihydroartemisinin, artesunate, and artemether, artemisinin has displayed unique pharmacological activities against a wide range of other parasitic organisms including *Schistosoma* species (Mishina et al. 2007; Xiao et al. 2001; Utzinger et al. 2001), *Leishmania donovani* (Yang

and Liew 1993; Ma et al. 2004), *Toxoplasma gondii* (Jones-Brando et al. 2006), *Pneumocystis carinii* (Merali and Meshnick 1991), and the pathogens responsible several neglected diseases including for cryptosporidiosis, amoebiasis, giardiasis, clonorchiasis, and leishmaniasis (Ma et al. 2004; Yang and Liew 1993). Artemisinin has also been recently indicated as having antiviral activities (Romero et al. 2006) and has the potential to be used in the treatment of hepatitis B, C, and others (Efferth et al. 2008). More compelling and of greater pharmacological significance, even to those in industrially developed societies, is the fact that artemisinin or its semi-synthetic derivatives have been demonstrated to be novel antitumour agents for some of the deadliest cancers known to man. For example, artemisinin derivatives have been shown to be very effective against radiation-resistant breast cancer cells in vitro (Singh and Lai 2001), drug-resistant small-cell lung carcinoma cells (Sadava et al. 2002), human leukaemia cell lines (Lai and Singh 1995), colon cancer, and active melanomas (Efferth et al. 2001). Collectively, these parasitic diseases and cancers appear to afflict over a billion people each year in different parts of the world and can be successfully treated with artemisinin or its semi-synthetic derivatives, where an adequate source of the drug is guaranteed and at an affordable cost. Invariably, these developments have attracted a very high degree of attention; a situation that has led to the source plant of the drug to be rated as one of the top ten industrial crops of the modern world (Sangwan et al. 1998).

In spite of the enormous pharmacological importance of artemisinin in both human and animal healthcare (Efferth et al. 2011; Brisibe et al. 2008a, b; Turner and Ferreira 2005; Ferreira 2009; Ferreira et al. 2005), its availability, especially as a key active ingredient in the production of the world's most effective antimalarial drugs, artemisinin-based combination therapies (ACTs), is limited not only by low yield in *A. annua* but even more so by the uncertainty of farmers and producers in the current debate, in which plant-derived artemisinin stands to be replaced by its bioengineered counterpart (http://www.malariaworld.org/poll/semi-synthetic-artemisinin-production-through-bioengineered-yeast-great-step-forward-cover#comment-1244). Not surprisingly, this situation can lead to a more unstable supply of plant-derived artemisinin, resulting in shortages and price fluctuations that will further complicate production planning by ACT manufacturers. Coupled with the added cost of the secondary antimalarial drug partner(s) in the ACT, this has become a major hindrance to the availability of affordable ACTs to patients with the need, especially in countries where the use of ACTs has been encouraged (WHO 2006), further fuelling an increased demand within the past 10 years. Consequently, there is the need for a stable source of affordable artemisinin that will be sufficient to meet the current market demand. For example, artemisinin demand for 2013, as reported by the UNITAID-supported and BCG-managed ACT forecasting consortium, is between 101 and 119 metric tons, depending on the different scenarios (http://www.a2s2.org/upload/4.NewsandEvents/Newsletter3Oct2012/A2S2MarketUpdateOctober2012.pdf).

15.2 Production of Artemisinin *In Planta*

Although *de novo* chemical synthesis of artemisinin is possible (Zhu and Cook 2012), the process is very complex with many reaction steps, resulting in low yields. The chemical analogues produced are thus not economically competitive with that synthesized *in planta* in *A. annua* (Ferreira et al. 2005; Xu et al. 1986; Schmid and Holheinz 1983), which means its solvent extraction from the leafy biomass of the plant invariably appears to be the most viable option for producing cheap and large quantities of the drug. At the moment, increased cultivation of the crop in smallholder fields in Asia and Africa, which are usually less than 1 ha, and the improvement in extraction methods are the most effective strategies for producing artemisinin. However, one of the major shortcomings on the production of sesquiterpenoid compounds via *A. annua*, especially in tropical countries, is the relatively lengthy growing cycle required to obtain appreciable yields (g/100 g dry weight). So far, the best commercial varieties, when harvested multiple times in the same year, are estimated to produce 70 kg of artemisinin/ha (Kumar et al. 2004). Usually, the period from time of planting to artemisinin extraction from the plant is approximately 5–8 months. Not surprisingly, the yields derived from dried leafy biomass after such a lengthy period are considered low for commercial production, where a full ton of plant materials can produce between 6 and 18 kg of purified artemisinin (Brisibe et al. 2012). This low yield thus appears to be one of the most intractable problems related to *in planta* production and use of artemisinin-derived drugs against malaria, especially in Africa where the cultivation of the crop is found in only about five countries.

Presently, about 95 % of artemisinin used in the formulation of ACTs for malaria treatment is produced in China and Vietnam, while the reminder comes from Africa and India. However, in many regions of sub-Saharan Africa with a high incidence of malaria, local populations, against the advice of WHO, continue to drink extracts of *A. annua* leaves as a tea or take the fresh leaves of the plant directly not only in the treatment for malaria fever but also other ailments such as hyperglycaemia (Brisibe et al. 2011a) and HIV (Lubbe et al. 2012). Undeniably, both hot water extracts and the fresh plant material consumed would contain not only artemisinin but other bioactive compounds, including polymethoxylated flavonoids such as artemetin, casticin, chrysosplenetin, chrysosplenol D, cirsilineol, and eupatorin and more than a dozen other sesquiterpenes that abound in the leaves, which have been indicated as important compounds with antimalarial (Elfawal et al. 2012; Willcox 2009) and potential anticancer activities. Synergistic benefits may also be derived from the presence of other antimalarial compounds such as dehydrosilibin and dimethylallyl campferide. Aside from this, it has been reported that the traditional Artemisia tea therapy contained artemisinin as well as some antioxidant compounds mostly flavonoids (Rath et al. 2004; Willcox et al. 2007). In addition to their bioavailability, these compounds such as phenols, saponins, flavonoids, alkaloids, and tannins act to reduce parasitaemia independent of artemisinin (Liu et al. 1992). The presence of other compounds in *A. annua*

leaves has thus raised suspicion as to the possibility of their synergistic role with artemisinin in malaria and cancer treatment (Ferreira et al. 2010). These *in planta* constituents potentiate and enhance the overall activity of artemisinin (Elford et al. 1987), the reason given for the long-term use of the plant as a tea in China even before the discovery of artemisinin (Ferreira et al. 2010). Consequently, given the complex nature of *A. annua* and the many bioactive components and nutrients present in its tissues (Bhakuni et al. 2001; Brisibe et al. 2009), it would be simplistic to consider the consumption of either the traditional tea or whole-plant material essentially as a monotherapy, an understandable fear expressed by many people. However, this worry appears to be misplaced. Some studies have actually shown that there may be less chance of resistance occurring from the combined use of numerous plant constituents, which enhances the overall activity of artemisinin and can prevent *Plasmodium* or any other microbial parasite from developing resistance to the compound.

Now, considering that some plant secondary metabolites appear to have a more synergistic effect when provided *in planta* than in a purified form (Gilbert and Alves, 2003), an edible form of Artemisia leaf biomass via a compacted capsule in combination with an ACT partner has also been offered as a reliable, safe, and inexpensive mode to deliver the drug (Elfawal et al. 2012). In fact, it would be very tempting to consider the whole-plant treatment as an alternative delivery mechanism for artemisinin. This is supported by the results of Weathers et al. (2011) and Elfawal et al. (2012), which have provided strong evidence to suggest that the parasite-killing substances present in the whole-plant material may be acting through their potentiation of artemisinin that renders whole-plant consumption as an innovative plant-based artemisinin combination therapy (pACT). In one of their recent studies, Weathers et al. (2011) actually demonstrated that mice fed with dried whole-plant material of *A. annua* had about 40 times more artemisinin in their bloodstream than those fed with a corresponding amount of the pure drug. This amount exceeded by eightfold the minimum concentration of serum artemisinin (10 μg/l) required against *P. falciparum* (Alin and Bjorkman 1994), which suggests that the active ingredients contained in the whole-plant material were delivered faster and in greater quantity than those from pure drug treatments.

Though plant-based supply of active pharmaceutical ingredients (in this case, artemisinin) is not in agreement with the preference of modern pharmaceutical industry for single-ingredient drugs; nonetheless, this method would dramatically reduce the cost of healthcare not only in developing countries, but perhaps also in more developed nations where a holistic approach to disease treatment with herbal products has recently become fashionable. There are several examples that illustrate the synergistic benefits of drug delivery using complex botanical materials in preference to that in an isolated form (Raskin et al. 2002; Gilbert and Alves 2003). We, therefore, completely agree with the proposal of Weathers et al. (2011) that loading of capsules with compacted *A. annua* leaf powder of a known dosage artemisinin to which the ACT drug partner can be added or administered separately could be another cost-effective, inexpensive, and reliable method of artemisinin delivery in resource-poor settings, especially in Africa where the scourge of malaria

Table 15.1 in vitro activity of fractions and compounds derived from locally grown *Artemisia* species in Nigeria against chloroquine-resistant (KI) strain of *Plasmodium falciparum*

Fraction/Compound	Activity against *Plasmodium falciparum* [IC_{50} (μg/ml)[a] ± SD (μM)][b]
Ethanol fraction from *A. annua*	10.531 ± 0.2175
Artemisinin extracted from *A. annua*	0.0134 ± 0.00197
Methanol fraction from *A. maciverae*	72.293 ± 1.05528
Ethanol fraction from *A. maciverae*	39.761 ± 0.55655
Hexane fraction from *A. maciverae*	54.607 ± 1.45298
Chloroquine[c]	0.3179 ± 0.21145
Artemisinin[c]	0.0248 ± 0.00972

[a] Concentration that kills 50 % of *Plasmodium falciparum*
[b] Values obtained from the means of three experiments or determinations
[c] Positive control

has its highest toll of mortality. The processing facility for such inexpensive artemisinin delivery route could be centred within an area where local farmers currently grow the plant such that the entire process could be self-sustaining.

This proposition will not only strengthen local health, as confirmed by the efficacy of the plant-derived artemisinin and the ethanol extract from locally cultivated plants in in vitro evaluation studies, but also the local economy. For example, Table 15.1 shows the IC_{50} of ethanol, methanol, and hexane fractions of two *Artemisia* species as well as pure artemisinin extracted from the locally cultivated *A. annua* plants in Nigeria in comparison with the values for chloroquine and artemisinin purchased from a commercial source in China. It is obvious that the in vitro activity against chloroquine-resistant (KI) strain of *Plasmodium falciparum* from artemisinin locally extracted was also very high. Besides, the fact that the ethanol extract from the locally cultivated *A. annua* plants was very efficacious against the KI strain of the parasite in the study suggests the presence of artemisinin and other compounds, further confirming the use of plant material as an effective alternative mode of delivery of artemisinin (Weathers et al. 2011).

Taken together, these observations strong support that apart from the use of WHO-recommended ACTs, some researchers have vigorously campaigned in favour of either re-establishing the use of traditional *Artemisia* tea (Van der Koov and Verpoorte 2011; De Ridder et al. 2008; Hsu 2006) or using fresh whole leaves (Brisibe and Daniel 2013) or encapsulated dried leaves (Elfawal et al. 2012; Weather et al. 2011), with the caveat that the plant material used has high or clinical levels of artemisinin in remote areas where malaria is endemic. Considering that the onset of cerebral malaria and malaria-induced coma is fast and the nearest hospital or clinic may be 2–3 days away, the use of the plant material (in whole or as tea) should be investigated seriously and, hopefully, permitted to sustain a malaria patient to reach a health centre stocked with antimalarial drugs (Ferreira et al. 2010). 'Plant materials (pACTs) may not be as perfect as the ideal doses administered in active pharmaceutical formulations, however they may be better than no treatment at all. These treatment methods will not only save

precious lives but also have several advantages. Firstly, they are inexpensive. Secondly, they are in forms that most resource-poor societies can rely on. It can be construed from the above that continuous cultivation of *A. annua*, not solely for the purpose of artemisinin extraction, but also for the significance of the plant in its multipurpose therapeutic potential and holistic treatment for malaria and a variety of other diseases and neglected parasitic ailments, must be encouraged.

15.3 *Artemisia annua* and Some of Its Pharmacological Activities

A. annua is an annual weed with an aggressive and vigorous growth habit. It is considered to have originated, and occurs naturally, as part of the steppe vegetation in Northern China (Ferreira et al. 2005). However, it now grows effectively in other climatic conditions. In Asia, for example, it is well distributed and extends as a native into southern Siberia, Vietnam, and northern India. Outside of Asia, the plant has adapted ubiquitously to different growth conditions as seen in many parts of Europe, USA, Australia, and Argentina (Ferreira et al. 2005). In Africa, it has been introduced into commercial-scale cultivation in Tanzania, Kenya, Uganda, and Madagascar within the past 10 years and more recently in Nigeria (Brisibe et al. 2012), where evaluation of optimal agronomic practices and mass selection for late-flowering and high artemisinin-yielding lines were evaluated with interesting results. For these studies, seeds were obtained from six different countries—Brazil, China, Vietnam, India, Germany, and USA. Some of these, especially the hybrid populations from Brazil, originated plants that had a growth span of about 192 days before flowering and were up to 2.84 m in height (Fig. 15.1) with an average leaf biomass yield of 324 g/plant and artemisinin concentrations as high as 1.0975 % (on a g/100 g dry weight basis) under humid lowland tropical conditions (Brisibe et al. 2012).

As a crop with a rich ethnopharmacological significance, it is surprising that *A. annua* has been relatively undeveloped over several millennia, despite its depiction in the Chinese *Materia Medica* as a therapeutic tea for malaria and fever (Hsu 2006) and its uses, a non-trivial matter considering its bitter taste, as a condiment by various Asian cultures (Weathers et al. 2011). It has been documented that the artemisinin content in the dry leaf of varieties from different geographical origins varies considerably, ranging from 0.01 to 1.9 % (Brisibe et al. 2012; Delabays et al. 2001; Ferreira and Gonzales 2009). To a large extent, such variations can be attributed to differences in field management practices that must be adapted to specific environments and local costs, level of intensification, and interactions between methods of cultivation and variety. Other factors that are equally known to affect artemisinin content in the plant include periods of harvest and environmental components such as temperature and nutrient availability (Ferreira 2007; Delabays et al. 2002; Delabays et al. 2001). Aside from these, artemisinin content has also been shown to be highly heritable, indicating that a strong genetic

Fig. 15.1 Plants from hybrid populations such as these from Brazil have become locally adapted to humid tropical conditions and boosted leafy biomass production in Nigeria from which artemisinin was extracted

component contributes to the variation seen in the cultivated crop (Delabays et al. 2001; Graham et al. 2010). Such genetic components and their interactions with environmental factors have been exploited for breeding purposes to produce improved hybrid lines (Townsend et al. 2013), which can boost artemisinin supplies from smallholders in Asia and Africa.

In recent years, there has been an increase in the number of scientific investigations that have validated the potential of *A. annua* and its extracts both as dietary feed supplement and to treat a variety of ailments afflicting both humans and livestock (Almeida et al. 2012; Brisibe et al. 2008a, 2011a; Cherian et al. 2013; Drăgan et al. 2010; Ferreira 2009). In addition to artemisinin, the plant is a storehouse of many biologically active compounds including more than 40 different flavonoids, phenolics, purines, lipids, aliphatic compounds, antioxidants, and others (Brisibe et al. 2009; Ferreira et al. 2010). The relatively high amino acid and vitamin profiles coupled with the very low and often negligible levels of

inherent antinutritive factors, especially in the leaves, which are far below levels considered toxic, establish *A. annua* also as a good reservoir of nutrients and antioxidants that favour its use as an important supplementary or phytogenic feed additive (Brisibe et al. 2008a; Cherian et al. 2013) for livestock production systems and a potential herbal tonic for humans (Brisibe et al. 2009).

Aside from these, recent research works have continued to buttress the need for further investigations on the use of *A. annua* and its extracts. For example, ethanol extract of the plant showed immunosuppressive effect on autoimmune diseases such as lupus erythematosus and rheumatoid arthritis (Zhang and Sun 2009), while SM905, a water-soluble artemisinin derivative, also obtained from *A. annua*, ameliorates collagen-induced arthritis by the suppression of inflammatory and Th17 responses. Oral treatment with SM905 not only delayed disease onset, reduced arthritis incidence and severity, but also suppressed the enhanced expression of pro-inflammatory cytokines, chemokines, and chemokine receptors in draining lymph nodes. In established arthritis, SM905 profoundly inhibited disease progression, reduced IL-17A, and RORgt mRNA expression and suppressed pro-inflammatory mediator expression in arthritic joints (Wang et al. 2008). Similarly, as the incidence of HIV/AIDS becomes more prevalent in different parts of the world with varying consequences, a lot of new drugs (both natural and synthetic) are presently evaluated as lead molecules in the fight against HIV/AIDS. So far, *A. annua* has been identified as one of the few medicinal plants to show great promise in this regard (Lubbe et al. 2012). Intuitively, this will be a major pharmacological novelty once the anti-HIV effects of *A. annua* are confirmed in humans.

Now apart from its commonly known traditional uses, several research groups and stakeholders are presently seeking for alternative uses and therapies for *Artemisia* that are efficient, affordable, accessible, and widely available. Some of those highlighted so far include its use for immune boosting, production of scopoletin (by extraction), insect repellents, perfumes from essential oils, and flavouring for alcoholic beverages. The plant also has an important role in agriculture, where its anticoccidial, anthelminthic, allelopathic, antifungal, and insecticidal activities have been identified in livestock (Almeida et al. 2012; Brisibe et al. 2008b; Ferreira 2009; Hart et al. 2007), fishery (Ekanem and Brisibe 2010) and crop production (Tang et al. 2000), respectively. It is also a highly effective plant growth inhibitor with great potential as an organic herbicide (Abate et al. 2011) or pesticide in stored grains (Brisibe et al. 2011b). All of these are capacities conferred on the plant due to its numerous bioactive compounds. This, in itself, is not surprising as many natural products in plants are multifunctional molecules that protect them from bacterial, viral, and other microbial infections, or even from herbivores such as insects and worms. Against this backdrop, therefore, there should be no single usage intended for *A. annua*, but a range of treatment possibilities provided by the plant ingredients. These are further indications that highlight the need for expanding the scope of cultivating the plant and thereby potentially increasing artemisinin supply and reducing its cost of production.

15.4 Strategies for Increased Production of Artemisinin

In 2012, with an increased *A. annua* planting and good weather conditions during the growing season, the global production of artemisinin increased considerably and was predicted to be sufficient to meet the most optimistic ACT forecasts for the 2012/2013 production season. However, there was considerable uncertainty throughout 2012 as to the future of the Affordable Medicines Facility—malaria (AMFm) programme, and therefore, the earlier demand for a timely ACT forecast has not been met (Malcolm Cuttler, Kenya Artemisinin Conference, Jan 2013). Considering that the drug must be produced cheaply, and in much greater quantities than currently available to meet this short- and medium-term demand, several strategies must be adopted for improving its supply. Some of these include an improvement in the agronomic practices that increase leaf biomass yield as well as an increase in the land area devoted to the cultivation of *A. annua*, following a genetic approach as it has been demonstrated previously (Debruner et al. 1996; Delabays 1994). Recently, this approach has been supported by the identification of the loci associated with artemisinin production (Graham et al. 2010). Other strategies include the induced production of artemisinic acid in *Nicotiana benthamiana* (van Harpen et al. 2010), production of artemisinin precursors in heterologous systems such as microorganisms (Paddon et al. 2013; Ro et al. 2006; Teoh et al. 2006), and the semi-synthesis of artemisinin from two of its precursors, artemisinic acid and dihydroartemisinic acid, which are usually discarded in the extraction process (Brisibe et al. 2008b). Some of these strategies are highlighted below.

15.4.1 Optimization of Agro-technologies for Enhanced Production of Artemisinin

The availability and cost of artemisinin are largely functions of its yield in *A. annua* cultivars, which has significant effect on the dynamics of supply that is currently a key cost driver for the production of ACTs. However, global supply of plant-derived (or natural) artemisinin has lately experienced boom-and-bust cycles that has led to ACT drugs being priced out of reach for poor people. It is not surprising, therefore, that there is a current surge in the cultivation of the plant around the world, most notably in Africa, where farmers have pioneered the commercial cultivation of *A. annua* since the late 1990s with high artemisinin-yielding lines. However, African regions mostly afflicted by malaria are within the tropics, where day lengths are short, thus likely to induce most cultivars which are not adapted to the tropics to flower early without the accumulation of sufficient leafy biomass (Ferreira et al. 2005). Ostensibly, there are currently genotypes that have been developed by Mediplant in Switzerland (Delabays 1994) and by the Chemical, Biological and Agricultural Research Centre (Debrunner et al. 1996),

Table 15.2 Mean values of growth and yield parameters in six *A. annua* accessions derived from different countries cultivated under lowland humid tropical conditions (culled from Brisibe et al. 2012)

Accession number and seed source[a]	Number of days to flowering	Plant height at flowering (cm)	Fresh herbage biomass	Dry leaf biomass yield (tonnes/ha)[b]	Artemisinin content (g/100 g DW)
1. (China)	126.65 (0.87)	87.82 (0.78)	15.609 (4.01)	1.474 (0.59)	0.450 (0.01)
2. (China)	134.56 (0.32)	102.12 (1.24)	16.168 (4.17)	1.481 (1.12)	0.454 (2.12)
3. (India)	152.35 (0.56)	164.10 (1.16)	23.185 (4.26)	2.097 (1.35)	0.564 (0.01)
4. (USA)	149.95 (0.40)	172.45 (0.70)	23.886 (4.25)	2.232 (1.37)	0.563 (0.02)
5. (Brazil)	189.85 (0.71)	284.78 (0.76)	32.616 (6.35)	3.126 (1.34)	1.0975 (0.04)
6. (Brazil)	201.22 (1.42)	261.98 (0.99)	32.804 (5.66)	3.164 (1.29)	1.0826 (0.02)

Values in parentheses represent the standard error (*SE*) of the respective means

[a] Five of the six seed lines evaluated were obtained courtesy of Prof. Li Longyuan (Chongqing Academy of Traditional Chinese Medicine, Chongqing, China), Dr. Dai Yong (Guangzhou Pharmaceutical Company Limited, Guangzhou, China), Dr. Pedro de Magalhães (CPQBA, University of Campinas, Campinas, Brazil), Col. Bhuwan Pandey (through a private source in Lucknow, India). The seeds from the USA were purchased from Elixir Farm Botanicals, Missouri

[b] The fresh herbage (leafy biomass) yield in tonnes/ha was calculated from a population of 20,000 plants

University of Campinas, Campinas, Brazil (in collaboration with Mediplant), which are late-flowering and produce sufficient leafy biomass that appear most suitable for cultivation in the tropics. The interpretation that these varieties, especially those from Brazil, can perform well within the tropics has support from our recent studies in Nigeria (Fig. 15.1), which showed that these populations can produce on average 1.0975 % artemisinin (Table 15.2) and can be selected further for adaptation to lower latitudes quite close to the equator (Brisibe et al. 2012). Plants from this Brazilian selection have produced as high as 1.5 % artemisinin when tested in West Virginia, USA (Jorge Ferreira, pers. comm.).

Artemisia is well suited to both smallholder and plantation agriculture. However, the most significant bottleneck for feasible commercial production of artemisinin anywhere in the world presently is the availability of seed stocks of lines suitable for the local conditions which can produce high leafy biomass and artemisinin yields. Once the problem associated with seed production has been conquered, other agronomic practices that seek to maximize the yield of leaf biomass and artemisinin per unit area of land need to be optimized. One of such factors is discussed here.

15.4.1.1 Selective Breeding and Cultivation of Genetically Superior and High-Yielding Hybrid Lines

Leafy biomass yields could be vastly improved by cultivating new strains of the crop where, on average, one kilogram of its dried leaves produces about 8 g of crystalline artemisinin. Lately, however, researchers in the United Kingdom have used selective breeding to create hybrid populations that produce up to 24 g. These plants are now being grown and harvested commercially in Madagascar, while trials are currently ongoing in South Africa, Uganda, Zimbabwe, the United States, and Britain.

In an alternative approach, Graham and colleagues at the University of York, United Kingdom, identified key genes that could be manipulated to optimize agricultural yields, robustness, or other desirable traits when Artemisia is grown in different regions of the world (Graham et al. 2010; Bowles et al. 2008). The work has helped to create plants that produce up to 50 % more artemisinin per kilogram of leaf biomass than the best commercial varieties presently in cultivation. This is a major breakthrough as the interaction between improved genetic material and good field management practices in the right environment with adequate soil moisture and nutrients leads to the production of an *A. annua* crop with high leafy biomass and artemisinin content, as we have demonstrated in Nigeria (Brisibe et al. 2012; Brisibe 2006).

Generally, Artemisia seeds are very small, and usually, commercial cultivation involves transplanting of vigorous nursery-grown seedlings to the field at the 3–5 leaf stage when they are about 10–15 cm in height. However, in localities where labour is scarce or expensive, seedlings can be raised directly in the field after the preparation of a fine seedbed. We observed in several trials that transplanting was clearly inferior in terms of agronomic performance and artemisinin yield of plants when raising seedlings in a nursery prior to field cultivation was compared with direct seeding in the field (Brisibe et al. 2012; Ferreira et al. 2005). However, irrespective of the method of establishment, it is always preferable to plant after the rains have started. This would mean that the soil has high moisture content since any moisture stress in the early- and mid-vegetative growth stages of the plant tends to induce premature flowering or leaf atrophy (Brisibe et al. 2012). Our preliminary studies in Nigeria have also demonstrated that leaf biomass yield and artemisinin production have a wider variation in plants generated from seeds than in those generated from asexual propagation methods such as cuttings or in vitro culture. Although this has not been evaluated on a large scale, however, a recent study using plants generated by cloning plants donated by Mediplant has proven that 0.9 % artemisinin and an average of 450 g dry leafy biomass per plant can be obtained with low variability when compared to a Chinese seed-generated crop that produced an average of 0.6 % artemisinin (Ferreira, unpublished). The same clone from Mediplant, tested on potassium-deficient soil maintained the average concentration of 0.9 % artemisinin when potassium was supplied, but produced 1.6 % artemisinin when potassium was withheld (Ferreira 2007). Against this backdrop, crop establishment from cloned plants looks like an attractive option if

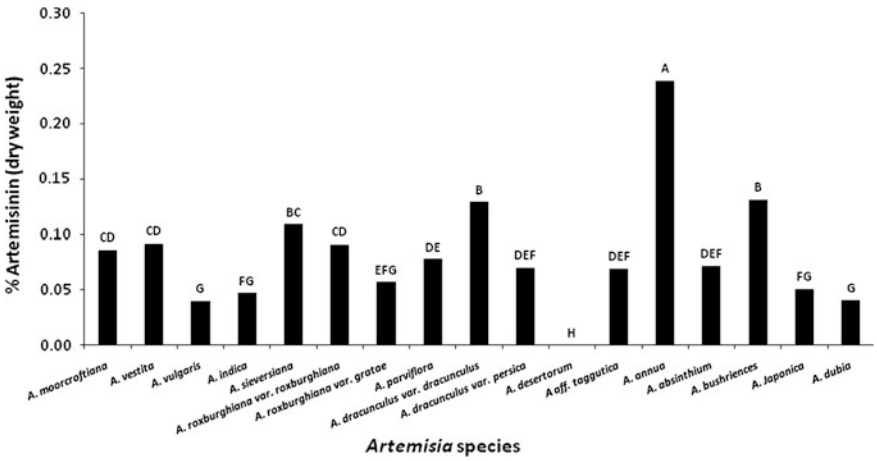

Fig. 15.2 Comparison of whole-plant parts (flowers, leaves, stem, and roots) artemisinin concentration among all seventeen *Artemisia* species (*Bar* represents the mean values of artemisinin in each treatment, and the alphabets above represent the LSD ranking of these values at $a = 0.05$) (Culled from Mannan et al. 2010)

the source plant is rich in artemisinin content, such as 1.5 % that has been reported recently (Graham et al. 2010). Such vegetative propagation methods will be equally useful for maintaining genetic fecundity. However, the cost benefits of crop establishment from seeds *versus* asexually propagated plantlets also need to be evaluated.

Meanwhile, there is great hope that global artemisinin requirements can be met by *in planta* production as potential new plant sources of artemisinin, apart from *A. annua*, have been identified (Mannam et al. 2010). This development is of great pharmacological importance since these *Artemisia* species are widely distributed and are abundant in many parts of Asia. Thus far, some of these diverse *Artemisia* species including *A. sieversiana, A. dracunculus var. dracunculus*, and others have shown that they can produce artemisinin, although at significantly lower concentrations than *A. annua* (Fig. 15.2). Consequently, these plants can be collected from the wild and screened further for genetic improvement and biological activity against the same diseases, which are being used to evaluate the efficacy of *A. annua* and artemisinin.

15.4.2 Biotechnology of A. annua and Enhanced Production of Artemisinin

Considering that *A. annua* is the only viable source of artemisinin at the moment, there is understandably a great degree of interest in enhancing its production. And although effective, the agronomic platform as the main production strategy seems

unlikely to solve the problem of global artemisinin availability due to the boom-and-bust cycles that its production has become associated with lately. As there are growing concerns that current artemisinin supply chain will be unable to meet future requirements, it is obvious that there is need for an additional source of artemisinin which supply will be consistent, reliable, and inexpensive. Consequently, a multifaceted approach using several strategies, including the utilization of the advanced techniques emerging from classical molecular biology, industrial fermentation, and genetic engineering research, would be of great interest. Some of these strategies include, but are not necessarily limited to the following.

15.4.2.1 Artificial Polyploidization of *Artemisia annua*

Artificial polyploidization is generally known to give rise to larger reproductive and vegetative organs (Adaniya and Shira 2001). It has also been shown to increase the production of important medicinal compounds and other secondary metabolites over those of their diploid counterparts (Griesbach and Kamo 1996). With this in mind, Wallaart et al. (1999) successfully induced tetraploid whole plants ($2n = 4x = 36$) from the diploid *A. annua* plants using colchicine. They reported a polyploidy production efficiency of 20 and a 30 % higher artemisinin yield in the tetraploid plants. And although the increased yields of these tetraploid clones did not reach commercially useful quantities (mg/g dry weight) of artemisinin, the work showed that there are certainly some advantages in selecting for high-yielding polyploids.

15.4.2.2 Metabolic Engineering of the Artemisinin Biosynthesis Pathway

In recent years, the use of genetic engineering techniques to alter the metabolic pathway of artemisinin biosynthesis in transgenic *A. annua* has been attempted (Arsenault et al. 2008; Liu et al. 2011). This has been achieved mainly through the introduction of key genes encoding for enzymes regulating the biosynthetic pathway leading to the formation of artemisinin *in planta*. In this connection, the role of certain genes, especially those involving key enzymes in the biosynthesis of artemisinin such as farnesyl diphosphate synthase (FDS) and amorpha-4,11-diene synthase (AMS), readily comes to mind. It could be speculated that genes controlling these key enzymes can be manipulated such that the enzymes become overexpressed in *A. annua*. Alternatively, other enzymes that are involved in pathways competing for precursors of artemisinin, for example, squalene synthase (SQS) can be inhibited through genetic engineering such that the genetically modified plants produce more artemisinin.

Efforts are equally geared towards the development of transgenic plants by introducing the gene for artemisinin production (from *A. annua*) into a much faster-growing plant species, for example, chicory or tobacco (*Nicotiana tabacum*) with a

proportionately higher leaf biomass, possibly, to enhance higher artemisinin yield at a very low cost. Such efforts already appear to be largely rewarding as demonstrated recently where the introduction of a gene into *N. tabacum* resulted in the expression of an active enzyme and the accumulation of the first-dedicated precursor of artemisinin (amorpha-4,11-diene) ranging from 0.2 to 1.7 ng/g fresh weight of leaf tissue (Wallaart et al. 2001). Some studies have also transformed a cDNA encoding cotton FDS (farnesyl diphosphate synthase) under the control of CaMV 35S promoter into *A. annua* via *A. tumefaciens* or *A. rhizogenes*. By overexpressing FDS, a key enzyme in the biosynthesis of artemisinin, the content of the sesquiterpenoid antimalarial drug was increased by about 0.8–1 % dry weight in the transgenic plants (Chen et al. 2000). Lately, *N. benthamiana* has also been deployed at commercial scale for rapid production of several pharmaceutical precursors of artemisinin (van Harpen et al. 2010), further opening up the vista of opportunities that can be utilized for the production of this essential antimalarial drug.

15.4.2.3 Up-Scaling of *Ex Planta* Semi-synthesis of Artemisinin in Microbial Systems

Surprisingly, this feature does not seem to be unique to plants alone. Recent advances using recombinant microbes circumvented the poor performance of plant terpene cyclases by expressing a codon-optimized fold (Martin et al. 2003). In a remarkable series of metabolic engineering experiments, these authors equally used engineered mevalonate pathway gene from the yeast eukaryotic system, which was about 30 to 90 times more efficient than the normal pathway in *E. coli*. This combined approach highlights an increased production of amorpha-4,11-diene by approximately 1,000-fold (Martin et al. 2003), which taken further into the pathway would possibly lead to the production of artemisinic acid. In a more facile approach, a cytochrome P450 monooxygenase gene (*CYP71AV1*) isolated directly from glandular trichomes of *A. annua* (Teoh et al. 2006) and inserted in yeast cells performed a three-step oxidation of amorpha- 4,11-diene that allowed its conversion into artemisinic acid in yields that appear suitable for large-scale fermentation (Ro et al. 2006). These authors successfully added or tweaked a dozen genes in yeast in commercial fermentation tanks to produce artemisinic acid. Coming on the footsteps of this development, it is of special pharmacological interest that efforts are currently underway to optimize the *CYP71AV1* gene expression system in several prokaryotic strains in order to sustain high-level production of amorpha-4,11-diene that can be easily converted to artemisinic acid, which can be subsequently oxidized to yield artemisinin (Hale et al. 2007). The hallmark in all of these studies was the desire to modify the genomes of bacteria and yeast which can be fermented in huge bioreactors to yield a plentiful and inexpensive supply of artemisinic acid. This metabolically synthesized artemisinic acid can be obtained easily through a simple purification process, which can be converted to artemisinin through a few inexpensive chemical steps in the laboratory. The artemisinin thus produced can be further converted through simple

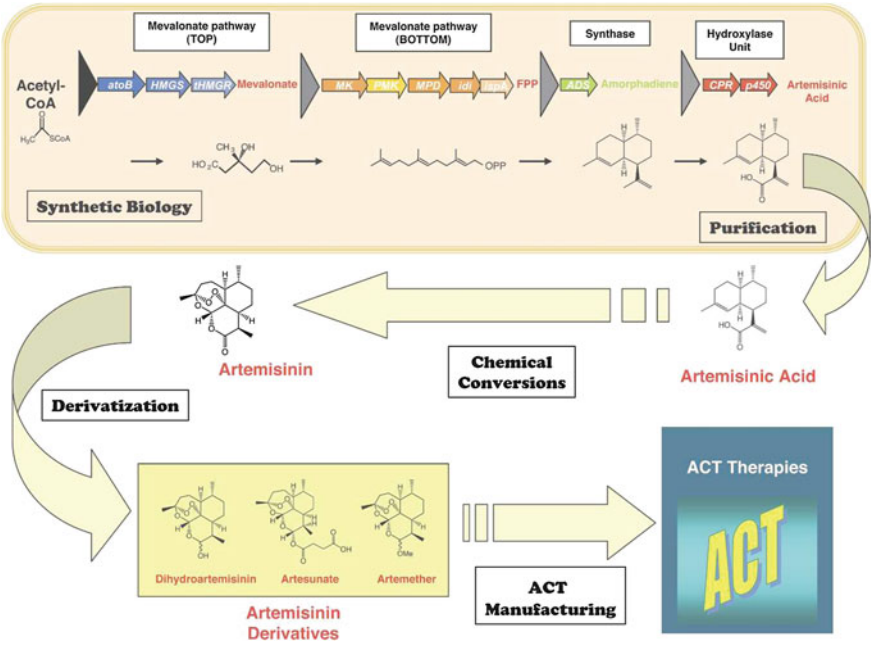

Fig. 15.3 The process for the microbial production of artemisinin (Adapted from Hale et al. 2007). Using synthetic biology, the metabolism of the microbe is engineered to produce artemisinic acid, a precursor to artemisinin. Starting from acetyl-CoA (an abundant product of the central metabolism of many microbes), the microbes produce, in turn, mevalonate, farnesyl pyrophosphate (FPP), amorphadiene, and, finally, artemisinic acid. The artemisinic acid is released from the microbes and purified from the culture media. The artemisinic acid is chemically converted to artemisinin. Once the artemisinin is produced, it must be further chemically converted into a derivative such as artesunate or artemether, which are integrated into ACTs for the treatment for malaria

downstream chemistry into derivatives such as dihydroartemisinin, artesunate, or artemether for possible integration with other antimalarial drugs for the production of low-cost, life-saving ACTs with a great impact on malaria mortality or the treatment for several neglected parasitic diseases in the tropics.

Production of artemisinin in large fermentation vessels through microbial engineering and simple chemistry, as illustrated in Fig. 15.3, may pave the way for an industrial process capable of supplementing the global supply of the drug from a second source, independent of the boom-and-bust uncertainties associated with *in planta* production (Paddon et al. 2013), which have had a highly negative impact both on the producers and on health outcomes. This *ex planta* approach came as a promise to increase supplies of high-quality artemisinin and, overall, lower the cost of ACTs in the near future (Ro et al. 2006). However, seven years down the road, this has not yet materialized because the science-related logistics are still beset with a lot of problems as the process has only recently moved into

commercial production and distribution. Consequently, it is expected that production from the crop will remain a crucial source of artemisinin for the foreseeable future, though promise of the arrival of semi-synthetic artemisinin to international commerce has put considerable pressure on prices of the plant-derived compound, which fell from US$ 800—950/kg to US$420—550/kg in October 2012. Meanwhile, factory produced *ex planta*-derived artemisinin, when the process becomes commercially successful, could serve as a supplemental source of the drug and not necessarily as the single magic bullet for its production. This is especially so as artemisinin semi-synthesized in microbial systems in fermentation tanks might not be any cheaper than the *in planta*-derived version. In this regard, artemisinin derived from *ex planta* sources could be used to simply smooth shortfalls that are presently experienced in agricultural production. This will be inevitable, as the loss of a child every 40 s to malaria (Bowles et al. 2008; Sachs and Malaney 2002) in parts of the world should prompt everyone to focus on enhancing the present supply of plant-derived artemisinin by cultivating genetically improved varieties and increasing the land area dedicated to the crop. The above scenario has actually led the Royal Tropical Institute of The Netherlands to assert that sufficient supplies of artemisinin could be met by increasing cultivation of *A. annua* in suitable regions of the developing world, especially Africa, where farmers have become quite used to its field management (Heemskerk et al. 2006). Now, since approximately 90 % of deaths from malaria occur in Africa, malaria is an African 'problem'. Thus, it is not surprising that African producers of the crop also have a strong incentive to remain in the forefront of the development of artemisinin-based drugs, which can be considered a locally based remedy for a locally based disease (Ellman 2010). Such developments will certainly bring immediate benefits to the existing artemisinin supply chain by reducing production costs, stabilizing supplies, and improving grower confidence in the crop.

15.4.3 Enhanced Semi-synthesis of Artemisinin Through Conversion of Sesquiterpenic Precursors of Artemisinin

Apart from *in planta* approach in *A. annua* and biotechnological means for enhancing the production of artemisinin in microbial systems, a new and efficient method being touted involves the establishment of commercial-scale extraction of artemisinic acid and dihydroartemisinic acid, two major sesquiterpenoid precursors of artemisinin, which have been identified from most commercial cultivars of the plant. A Chinese cultivar that was cultivated in 2006 in a West Virginia, USA, field and analysed for artemisinin, dihydroartemisinic acid, and artemisinic acid by HPLC throughout the growing season showed a peak in artemisinin production between August 28th and September 1st (Ferreira 2008). These plants had 0.93 % artemisinin, 1.6 % dihydroartemisinic acid, and 0.28 % artemisinic acid, respectively. The author suggested

that artemisinin production could, at least, be doubled by using both dihydroartemisinic acid and artemisinic acid eliminated in the by-product of artemisinin production (Jorge Ferreira, pers. comm.). Also, approximate quantifications for these sesquiterpenoid precursors indicate that there were about 24 % of dihydroartemisinic acid and 5 % of artemisinic acid, respectively, from the high artemisinin-containing cultivar Artemis (Ferreira, unpublished). Unfortunately, both dihydroartemisinic acid and artemisinic acid, which are usually extracted with refluxing in the extraction solvent, are presently discarded in the artemisinin purification steps, where artemisinin is pooled into non-polar fractions. It is, therefore, of immense economic importance that methods for extraction and conversion of dihydroartemisinic acid and artemisinic acid into artemisinin are optimized. This can potentially increase the final artemisinin profile derivable from a given quantity of dry leafy biomass by approximately 30 % (Brisibe et al. 2008b), especially against the backdrop of a recent finding where a photochemistry-based method, developed by researchers at the Max Plank Institute, Germany transformed dihydroartemisinin into artemisinin without enzymes but just with the use of light and oxygen (Lévesque and Seeberger 2012). It is obvious, therefore, that this approach is feasible and can be used to increase artemisinin production from the crop.

15.5 Conclusion and Future Perspective

Thomas Jefferson in 1813 wrote that 'The greatest service which can be rendered any country is to add a useful plant to its culture'. There is no doubt that the spotlight on international malaria therapy is presently focused on the availability of artemisinin and the supply of ACTs from a seemingly simple, yet versatile plant of Asian origin that is suddenly found at the forefront of global efforts aimed at the eradication of malaria. In the current setting, it is essential that the production of artemisinin and its use as the key active ingredient in the manufacture of ACTs should be seen as the central focus (Brisibe et al. 2008b). Not surprisingly, the heavy demand placed on artemisinin due to its huge pharmacological benefits, especially in the treatment for malaria, would necessitate that possible alternatives for its larger-scale production, apart from *in planta* extraction, be considered. Under this circumstance, *ex planta* synthesis in microbial systems to produce some of its isoprenoid precursors such as dihydroartemisinic acid and artemisinic acid, which can subsequently be converted to artemisinin through inexpensive chemical procedures (Roth and Acton 1989), holds some promise. Still, this approach clearly has its own limitations of producing only artemisinin. The corollary implications of the *ex planta* method of artemisinin production include eliminating the natural germplasm of *A. annua* and the impact this would have on biodiversity and on the livelihoods of small- and medium-scale farmers already invested in the agricultural production of *A. annua* for its artemisinin-rich dried leaves. This is aside the unnecessary limitations that would be placed on other potential medicinal and industrial benefits of the plant that are conferred on it by the rich portfolio of biologically active compounds such as antioxidants, which

in turn, synergize with artemisinin to enhance its potency. In addition to artemisinin, other bioactive components including other antimalarial compounds present in the leaves may become available in the traditional *Artemisia* tea therapy, indicating that the plant is already a combination drug that may offer a cost-effective and affordable solution for malaria for low-income patients in developing countries. Currently, there are already pointers in this direction. All that would be required is for thoroughly controlled clinical studies to be conducted on the effectiveness and potential risks of treatment with the Artemisia tea, encapsulated dried biomass or fresh plant material.

A 2006 report of The Netherland's Tropical Institute actually warned that the prospect of synthetic production of artemisinin could further destabilize a very young market for natural artemisinin derived from *A. annua*, undermining the security of farmers just beginning to plant it for the first time. Surely a stable and adequate source of artemisinin supply would be fundamentally important in the global fight against malaria (Assured Artemisinin Supply System 2012—http://www.a2s2.org/index.php?id=50) and many other neglected tropical diseases. However, both plant-derived and *ex planta* production of artemisinin should be encouraged and adequately supported as they will collectively play a pivotal role in the supply of artemisinin for ACT production as well as bring immediate benefits to the existing artemisinin supply chain by reducing production costs, stabilizing supplies, and improving grower confidence in the crop in countries such as Kenya, Tanzania, Madagascar, Uganda, and Nigeria, where *A. annua* has been cultivated by smallholders for the past 10 years. Presently, an estimated land area of 2,000 ha in Kenya (all processed by East African Botanicals), 800 ha in Madagascar, 200 ha in Uganda, 20 ha in Tanzania (Malcolm Cutler, pers. comm.) and 156 ha in Nigeria are devoted to the cultivation of *A. annua* by small-scale, rural-based farmers, who take a net revenue of about US$ 600 from the dried leaf biomass per ha of land. The figures may be similar in other African countries where *Artemisia* cultivation has started. The income derived by rural families from the cultivation of the crop is very beneficial and help to solve several socio-economic problems in rural communities. Consequently, encouragement of *ex planta* production of artemisinin to the detriment of agricultural production can disrupt the livelihoods of thousands of farmers in Africa, who cultivate *A. annua* and earn reasonable incomes by supplying dried leaves to companies that specialize in the extraction of artemisinin, which is currently exported to pharmaceutical factories in India and Europe for the manufacture of live-saving ACTs.

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