Chapter 6
Safety Pharmacology of Herbal Products

Gert Laekeman

Abstract This chapter aims at a comprehensive review on safety pharmacology. Foxglove and the discovery of acetylsalicylic acid are given as historical examples emerging from benefit-risk balance. Before going to practical examples of *in vitro* research, a theoretical approach is given to competitive and non-competitive antagonism. Results of experimental work are presented in order to show how early toxic activity can be detected and can even be related to mechanisms of action (e.g., the indole alkaloids of *Pterotaberna inconspicua* and the sesquiterpene lactone vernolepin from *Vernonia amygdalina*). If substances can be detected as responsible agents for a biological activity of complex mixtures, the safety approach can be simplified (e.g., eugenol in powder of *Myristica fragrans*). Pharmacokinetic considerations are important when taking into consideration safety and therapeutic issues of red yeast rice. A pharmacokinetic schedule is given and illustrated with herbal examples. Finally, a framework of questions is proposed to cover quality as well as pre-clinical and clinical safety.

Keywords Pharmacology • Safety • History • Checklist • Pharmacokinetics • Pharmacodynamics

Safety Pharmacology: An Introduction

Although there seems to be no internationally accepted definition of safety pharmacology, Pugsley et al. (2008) define the concept as follows: "... Safety Pharmacology is the discipline that seeks to predict whether a drug (in the widest sense of the word), if administered to human (or animal) populations, is likely to be found unsafe, and its professional mandate is to prevent such an occurrence . . ."

In its *Guidance for Industry*, the Food and Drug Administration defines the objectives of safety pharmacology studies: (1) to identify undesirable pharmacodynamics properties of a substance that may have relevance to its human

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safety; (2) to evaluate adverse pharmacodynamic and/or pathophysiological effects of a substance observed in toxicology and/or clinical studies; and (3) to investigate the mechanism of the adverse pharmacodynamic effects observed and/or suspected (ICH 2001).

The experimental approach of safety pharmacology can be designed according to the hypothesized properties of the substance or preparation. The purpose is not to address an exhaustive list, but a few examples can be illustrative.

A therapeutic class to which the principle to be tested belongs, permits foreseeing possible toxic effects, e.g., an antispasmodic activity on smooth muscle cells can result in irreversible paralysis. However, adverse effects are sometimes surprising: for example, substances with an antipsychotic action can cause QT-prolongation. The last example points to the importance of taking vital organs and systems to the testing. Pugsley et al. (2008) present an overview of a multidisciplinary integration required to evaluate the safety profile of new chemical entities in safety pharmacology. According to their view, the central nervous activity, as well as respiratory and cardiovascular functions should be covered.

Ligand binding of molecules to receptors studies give factual evidence for specificity and affinity, but do not permit making predictions on the intrinsic activity that is related to the cascade of events triggered by the binding. The same can be said about enzyme-inhibiting assays. Moreover, experimental models should also take into account that substances can be metabolized, and that the safety of metabolites should be tested as well.

As a result of the above-mentioned considerations, pharmacological testing of safety remains a challenge. Reliable results can only be obtained by triangulation, i.e., combining various techniques and models, which often means combining in vitro and in vivo testing. For example, the European Medicines Agency (EMA) has established a Joint Expert Group for Reduction, Replacement and Refinement (JEG 3Rs) in order to reduce the number of animals in experimental pharmacological procedures (EMA 2012). The JEG3R examines possibilities and limitations of the 3R principle. In the near future, guiding documents will be available on the EMA website.

This chapter aims at a comprehensive view of historical and actual examples of how to get an estimate of the benefit-risk balance. It also presents a primary framework of binary questions that should be feasible for safety when considering herbal practice.

Historical Considerations

Throughout history, the beneficial or toxic effects of many plant materials must have been recognized by trial and error. Attempts were made to develop methods in order to turn pharmacological activity into therapeutic practice. Experimentation and observation suffered from the difficulty of characterizing real actions of natural substances and mixtures and making the difference between a real effect and a coincidence.

Around the end of the eighteenth century, developments in chemistry helped master the way of making preparations in a reproducible way. The time for standardization was still to come, but the notion of dose-response was something within reach.

Digitalis Purpurea as an Example

William Withering (1741–1799) was one of the first practitioners carrying out "phase one" experiments *avant la lettre* by administering increasing dosages of leaves of *Digitalis purpurea* to patients and studying their cardiac function. Firstly, he collected and prepared the leaves of the purple foxglove (*Digitalis purpurea*) to obtain a product of reasonable consistency. Secondly, he identified most of the adverse effects of digitalis and how toxicity could be minimized by dose reduction. Thirdly, he investigated the dose-response characteristics of digitalis, with respect to both slowing the heart rate and inducing diuresis. He also showed that some individuals were more sensitive and responsive to digitalis than others (Breckenridge 2006) (Fig. 6.1).

The lessons that Withering learned from his studies of digitalis are still relevant today. He dealt – probably for the first time in history – with the basic properties of medicines: quality, safety, and effectiveness.

Reasonable Consistency: The Quality Issue

A quote can be taken from William Withering's *Account of the Foxglove and Some of Its Medical Uses* (London: CGJ and Robinson 1785): cited by Breckenridge (2006)

> I was well aware of the uncertainty which must attend on the exhibition of the root of a biennial plant and therefore continued to use the leaves. These I found to vary much at different seasons of the year, but I found that if gathered at one time of year, namely when it

Fig. 6.1 *Digitalis pupurea* L. or foxglove, the plant from which William Withering evaluated therapeutic effects and safety, using the leaves in clinical experiments (Image taken from http://www.kuleuven-kulak.be/bioweb/?page=guide&lang=nl. Accessed on April 30, 2015)

> was in its flowering state and carefully dried, the dose could be determined as exactly as any other medicine. The more I saw of the great powers of this plant, the more it seemed necessary to bring the doses to the greatest degree of accuracy.

A clearer historical reference to the nature and quality of herbal raw materials can probably not be made. Until now, the composition of various preparations has been discussed. The definition of the preparations is an important issue in the making of herbal monographs within the Herbal Medicinal Product Committee of the European Medicines Agency (EMA). Very recently, the possible consequences of differences in the composition of *Serenoa repens* (saw palmetto) extracts (hexane, ethanol, and critical CO_2) were discussed. Advanced chemical analytical techniques go hand in hand with the evaluation of pharmacological and safety evaluation (Habib and Wyllie 2004; Scaglione et al. 2008, Scaglione et al. 2012; Booker et al. 2014; De Monte et al. 2014; De Combarieu et al. 2015.

Adverse Events: The Safety Issue

All cardioactive steroids share the property of being potent and highly specific inhibitors of the intrinsic Na1K1-ATPase membrane protein. This enzyme comprises the cellular "sodium pump" in which membrane ion translocation is coupled with the hydrolysis of a high-energy ATP phosphate. Despite their more than 200 years in use, the safety of cardiac glycosides still remains a matter of debate (Hauptman and Kelly 1999). Withering gained more experience with the use of digitalis, which helped to lower adverse effects. It is not clear whether or not this was related to various dosage regimens or to the use of various preparations of digitalis that probably had varying degrees of bioavailability (Breckenridge 2006).

Until now there has been an ongoing debate on the safety of digoxin. Results of a meta-analysis suggest that digoxin use is associated with a greater risk of mortality in patients with atrial fibrillation, regardless of concomitant heart failure. Indeed, shortening of the refractory period may enhance the risk of cardiac arrhythmia. Moreover, signs for under-treatment are similar to those of overtreatment (Ouyang et al. 2015).

Dose-Response Characteristics: The Efficacy Issue

Withering considered foxglove to be a useful therapeutic tool in case of edema and weakness of the heart. Clinical research shows that digoxin reduces hospitalization for worsening heart failure (HF) when patients are admitted with sinus heart rhythm and ejection fraction lower than 45 %. International guidelines recommend that digoxin be considered an add-on therapy in patients with symptomatic heart failure when insufficient therapeutic results are obtained with optimal doses of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, β-blockers, and mineralocorticoid receptor antagonists. However, there is a lack of definitive evidence from randomized studies for the efficacy of digoxin as add-on therapy in this context. Questions also remain regarding the optimal dosage of digoxin. Contemporary

data for digoxin in HF are derived from observational studies, and the findings are conflicting. Despite two centuries' experience with cardiac glycosides to treat HF, fundamental questions remain unanswered (Chaggar et al. 2015).

Observing and Understanding: The Key to Progress

From the current perspective, it seems self-evident that isolation and identification of active principles is the key to understanding the efficacy and safety of herbal medicines. In this respect, it is astonishing to know that at the beginning of the nineteenth century, scientists could isolate and identify quite complex structures, such as morphine from *Papaver somniferum* Friedrich Sertürner in 1804 (Schmidt 1985) and quinine from *Cinchona officinalis* Pierre Joseph Pelletier and Joseph Bienaimé Caventou in 1820 (Kyle & Shampe 1974). These isolations began from factual evidence based on observed efficient and safe use of the herbal preparations.

The definition of the symptoms of inflammation by *rubor*, *calor*, *dolor*, and *tumor* by Aurus Cornelius Celsus (ca 25 B.C. to ca 50 A.D.) helped make a therapeutic approach to willow leaves as a useful tool already in Roman times. It was the Rev. Edward Stone (1702–1768) who successfully tested willow as a possible alternative for the expensive *Cinchona* in case of fever. German scientists succeeded in isolating salicylic acid from meadowsweet (*Filipendula ulmaria*) Löwig and Weidman in 1839; unfortunately, the substance was revealed to have strong stomach-churning acidity. One hundred years after Edward Stone, Charles Gerhardt (1816–1856), a French professor of chemistry, synthesized acetylsalicylic acid for the first time, in an attempt to find a solution to the gastric irritation caused by salicylic acid. However, he found the synthesis procedure too complicated and left the findings in the drawer. The finding of a more suitable way to synthesize acetylsalicylic acid was claimed by Felix Hoffman (1868–1946) and Arthur Eichengrün (1867–1949), and the drug turned out to be the first commercial success of a herbal-derived chemical substance with feasible therapeutic properties and – for that time – an acceptable safety profile (Jeffreys 2005).

For more than 2000 years, salicylic acid-containing plants have been used in traditional herbal medicinal practice. The acetylsalicylic acid that emerged from this use has been commercialized for more than 70 years, before the mechanism of action was discovered by Sir John Vane (1927–2004) (Vane 1971). For this discovery, Vane was awarded a Nobel Prize in Physiology or Medicine in 1982.

Pharmacodynamics and Safety Issues

Pharmacology is the scientific discipline that studies the interaction of a substance with the body, whereas pharmacokinetics deals with the action of the body on a substance. In a clinical environment, there is a tendency to replace pharmacology by pharmacodynamics. Pharmacokinetics and pharmacodynamics go in pair to the

so-called PK/PD approach, in which an attempt is made to translate the insight in the way the body deals with a medicine into a therapeutic perspective.

Having an insight into this process means knowing the pathway that the substances are following. The more specific and the more selective these substances are, the better they can be traced, and the better their safety can be controlled. An approach is made to competitive and non-competitive antagonism and to selectivity and specificity as important issues in the evaluation of safety and pharmacological perspectives.

Competitive and Non-competitive Antagonism: Theoretical Framework

A clear dose – or concentration relationship – of a natural substance or a plant extract is proof for pharmacological validation. Experimental data obtained with only one concentration or one dose should not be considered as proof of a pharmacological action. The description below is based on the models developed by Arunlakshana and Schild (1959) and Van Rossum (1963).

Agonists

A substance or an extract can behave as an agonist in an experimental model. In order to study real agonistic activity, the effects on an isolated organ are suitable for investigation. *In vitro* ligand binding to isolated receptors is a useful approach to the affinity of a substance to a receptor, but not to the intrinsic activity emerging from the binding of the substance with the receptor. An approach to affinity, as well as to intrinsic activity, can be made by considering the agonist "A" and the receptor "R" as two reacting principles. Once the agonist-receptor complex has been formed, an effect "E" can be the result.

A is the agonist, whereas R is the receptor. The effect E depends upon the affinity of the agonist A for the receptor and the intrinsic activity, i.e., the intensity with which the effect is generated. It can be written as the equation below. In this equation, α stands for the intrinsic activity.

$$AR = \alpha \times E$$

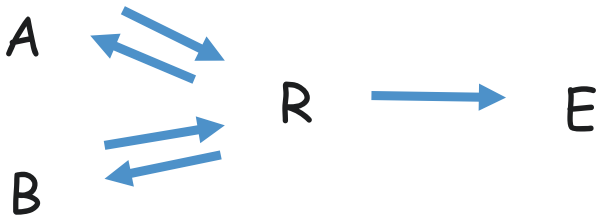
The model fits for a one-by-one relationship, hypothesizing the interaction of one molecule with one receptor. The stronger the intrinsic activity, the higher the effect. Affinity and intrinsic activity are specific for an agonist-receptor interaction.

Agonists can be compared by calculating the pD_2 defined as the negative logarithm of the molar concentration leading to 50 % of the maximal effect. The pD_2 can only be calculated when pure substances are tested. Plant extracts must be considered to be complex mixtures. Instead of molar concentrations of a pure compound, only EC_{50} values can be calculated, and these can be used to compare mutual activity. The EC_{50} value gives the concentration for which 50 % of the maximal effect is obtained.

Competitive Antagonist

Suppose an antagonist B is interfering with the activity of an agonist A. Again, the interaction of agonist, antagonist, and receptor R can be theorized by using a chemical approach. In the case of a competitive antagonist, this antagonist B competes for the same receptor as the agonist A; the affinity of both B and A for the receptor will have important consequences for the resulting effect E. The simplest approach is the one for which the antagonist has zero intrinsic activity – which is usually the case.

When a competitive antagonist binds reversibly to a receptor, increasing concentrations of agonist will chase it from the receptor. However, if the binding of B with the receptor is too strong and increasing concentrations of agonist cannot reverse the situation, this might be a sign of irreversibility and, as a consequence, of toxicity. In the case of competitive antagonism, concentration-effect curves will shift to the right and the same maximal effect will be obtained. Obtaining the maximal effect is a guarantee of non-toxicity. When the antagonist is washed out, the concentration-effect curves must be comparable to those obtained before the antagonist was brought in contact with the receptor.

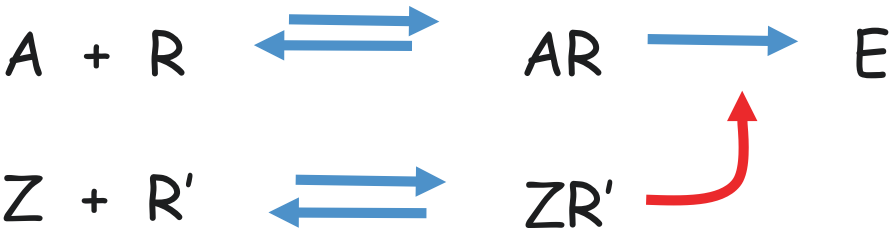


In order to compare competitive antagonists, the pA_2 has been introduced. It is the negative logarithm of the molar concentration of an antagonist for which the concentration of the agonist must be doubled in order to obtain the same effect as without an antagonist.

Non-competitive Antagonist

When the antagonist Z acts in a non-competitive way, it is hypothesized that it does not bind on the same place on the receptor as the agonist. The interaction between agonist and receptor takes place without hindrance, i.e., the affinity is not influenced.

Z will bind to another binding site, R, and the complex formed will negatively influence the effect E by interfering with the intrinsic activity. As a result, the maximum effect of the concentration-response curve will be lowered. The lowering is concentration-dependent and must be reversible once the antagonist has been washed out.



In order to compare non-competitive antagonists, the pD'_2 has been introduced. It is the negative logarithm of the molar concentration of the antagonist for which the maximal effect of the agonist is reduced to 50 %, as compared to the maximal effect without antagonist.

Natural Substances as Examples of Competitive and Non-competitive Antagonism

The theoretical framework of competitive and non-competitive antagonism is illustrated by the activity of indole alkaloids isolated from the leaves of *Pterotaberna inconspicua* Stapf. (*Apocynaceae*). This plant species grows as a shrub in Central Africa; its leaves are used in Congolese traditional medicines to treat hypertension, gastro-intestinal upsets, and several kinds of aches. The alkaloid content of the leaves was explored, having led to identification of 2-acylindole alkaloids of which the antihistaminic properties were studied on the isolated guinea-pig (Bakana et al. 1985).

The isolated ileum of the guinea pig offers opportunities to use many types of agonists that lead to contractions of the organ. One animal offers enough material to test properties of substances for at least one day. Several agonists can be used, giving typical patterns of contractions. Figure 6.2 gives examples of agonists that play a role in the human body; these can be administered to an ileum mounted in an isolated organ bath with *in vitro* conditions optimized by using a physiological mixture of electrolytes and glucose, at a temperature of 37 °C and oxygenated with a mixture of 95 % O_2 + 5 % CO_2. There is a nice concentration-response relationship that can be visualized by administering cumulative concentrations until a maximal response has been reached.

As can be seen in Fig. 6.2, contraction patterns differ from one agonist to another. Acetylcholine, bradykinine, and histamine give a sustained contraction, whereas contractions by the other agonists are rather short-lasting. Tachyphylaxis is observed for serotonin: even after washing, contractions with the same concentration fade.

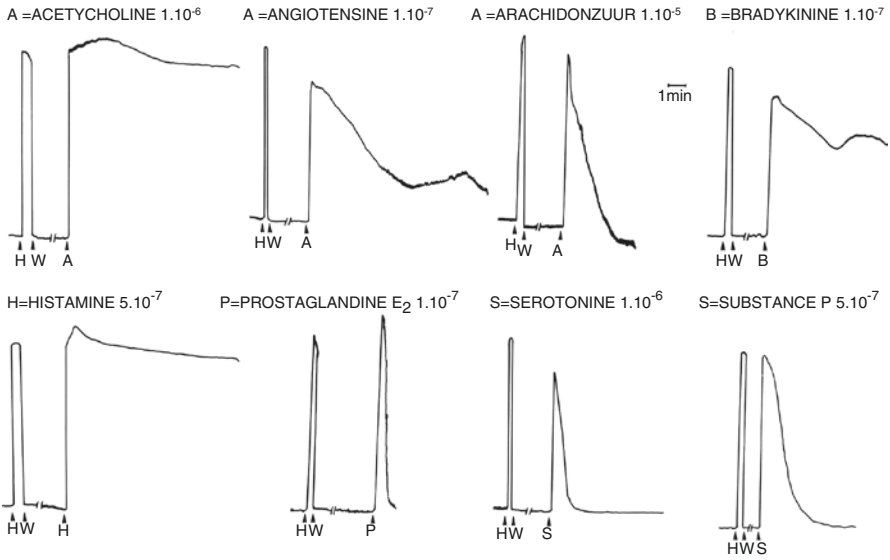


Fig. 6.2 Examples of contractions of different agonists on the isolated ileum of the guinea pig. All concentrations are expressed as g/ml (final concentrations in the isolated organ bath). Arachidonzuur = arachidonic acid (Laekeman 1980). *H* histamine 5.10^{-7} g/ml as initial positive control for contractility. *W* washing out histamine. Agonists were given within 10 min of the initial contraction by histamine

Epimethuenine and metheunine were the 2-acylindole alkaloids isolated from the leaves of *P. inconspicua*. Both substances are indole alkaloids with a different steric position of the substituents on C16 (Fig. 6.3).

From the cumulative dose response curves, a pD_2 of the agonist histamine of 6.13 +/− 0.05 was calculated (mean ± SEM; n = 24). This value corresponds with the values found in the literature (Brownlee and Johnson et al. 1963; Van Rossum 1963), whereas methuenine showed a non-competitive type of antagonism (depression of the maximal response). This led to calculation of $pD'_2 = 5.13 \pm 0.14$ (+/- SEM) for methuenine. The type of antagonism found for epimethuenine was a competitive one (shifting the response by the agonist to the right). For epimethuenine, the pA2 equalled 6.55 ± 0.08 (± SEM) (Fig. 6.4).

Methuenine also depressed the maximum response by acetylcholine as an agonist, although the effect was inferior to that seen on histamine contractions, hypothesizing a certain selectivity towards muscarine and histamine receptors. Epimethuenine did not influence the concentration-response curve of acetylcholine (Fig. 6.5).

All inhibitions seen were reversible: maximal contractions by the agonists could be reached again, after washing the alkaloids from the isolated organ, by renewing the physiological fluid in the isolated organ bath. These findings clearly show the existence of specificity for natural products. Reversibility of the antagonism is a guarantee for the absence of toxicity on the organ used.

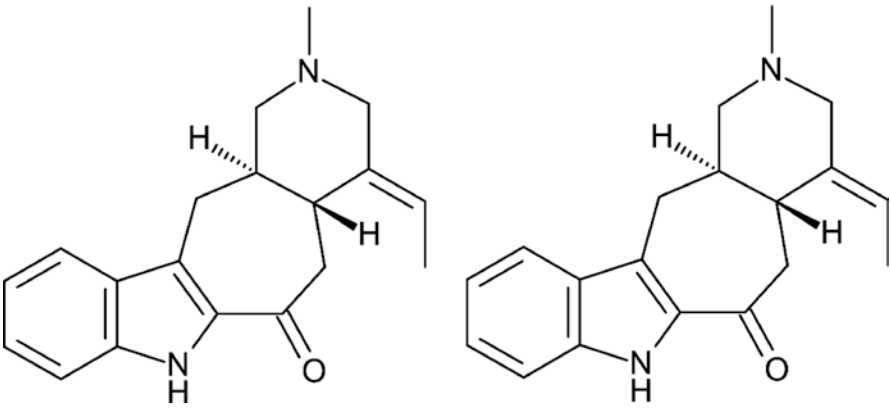


Fig. 6.3 2-acylindole alkaloids isolated from the leaves of *P. inconspicua*. Left: epimethuenine; right: methuenine

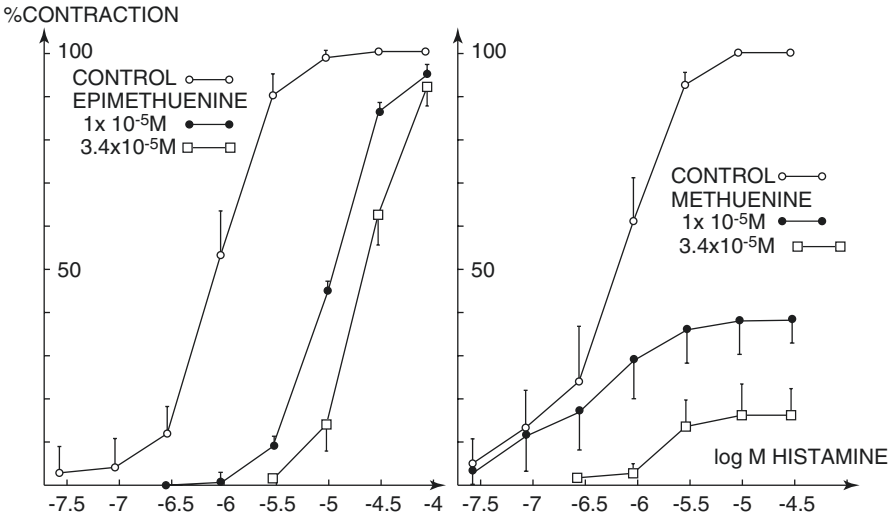


Fig. 6.4 Cumulative concentration-response curves with histamine on the isolated ileum of the guinea pig. Competitive antagonism obtained with epimethuenine and non-competitive antagonism by methuenine, 2-acylindole alkaloids isolated from *P. inconspicua*. Concentrations of alkaloids are given in Molar. Each point represents the mean ± SEM of at least five experiments, except for 3.4×10^{-5} M 16-epimethuenine where three experiments were carried out (Bakana et al. 1985)

Early Detection of Toxicity by an Experimental Pharmacological Approach

The isolated ileum of the guinea pig is a useful model for making predictions about the toxicity of natural compounds. In the case of methuenine and epimethuenine, the inhibiting activity was reversible, which is a guarantee of non-toxicity of the concentrations used.

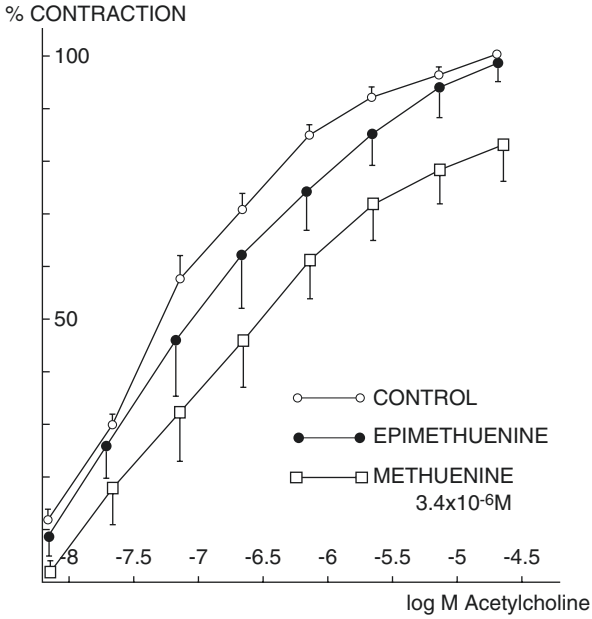


Fig. 6.5 Cumulative concentration-response curves with acetylcholine on the isolated ileum of the guinea pig, influenced by methuenine and 16-epimethuenine. Each point represents the mean ± SEM of six experiments (Bakana et al. 1985)

Another effect is seen with vernolepin, a sesquiterpene lactone isolated from *Vernonia amygdalina* Del. *(Asteraceae)* (Fig. 6.6). The plant is well known in African countries such as Cameroon, Guinea, Ghana, and Sao-Touré. It is also known in Angola, Congo, Ethiopia, Kenya, Rwanda, Tanzania, Uganda, Zambia, and Zimbabwe. The fruits of the plant are eaten; the leaves are used for various conditions, from infectious diseases to stimulation of circulation.

When vernolepin was tested on acetylcholine-induced contractions of the isolated guinea pig ileum, no inhibition was seen after an incubation time of less than 15 min. Enhancing the incubation time, however, resulted in an inhibition of the maximal response. After 60 min., contractions completely stopped. The total inhibition was irreversible, i.e., repeated washing was not able to restore contractions with any agonist, and the organ seemed completely paralyzed. This is proof of the fact that contact time can also be an important factor to take into account (Fig. 6.7) (Laekeman et al. 1983).

Interference with Neurotransmission

The coaxial stimulation of the guinea pig ileum gives insight into intestinal cholinergic neurotransmission. By the presence of neuronal plexi and their common embryonal origin, the isolated ileum of the guinea pig and the brain can be considered to be related. Dr. Paul Janssen, the founder of Janssen Pharmaceutica, once called the isolated guinea pig ileum a “mini central nervous system.” Indeed, most of the neurotransmitters that are expressed in the central nervous system are

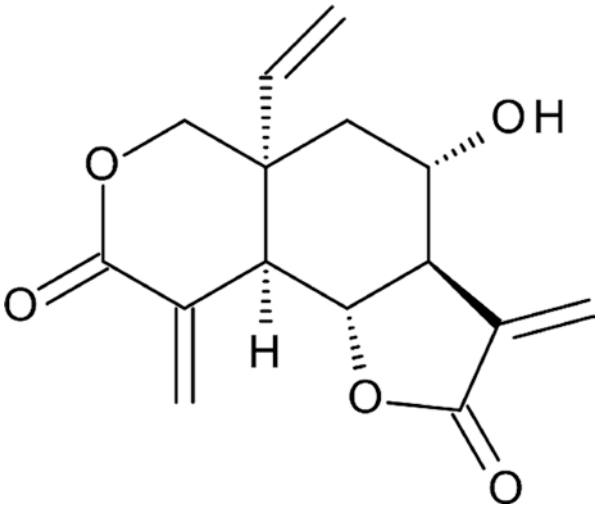


Fig. 6.6 Vernolepin isolated from *V. amygdalina*

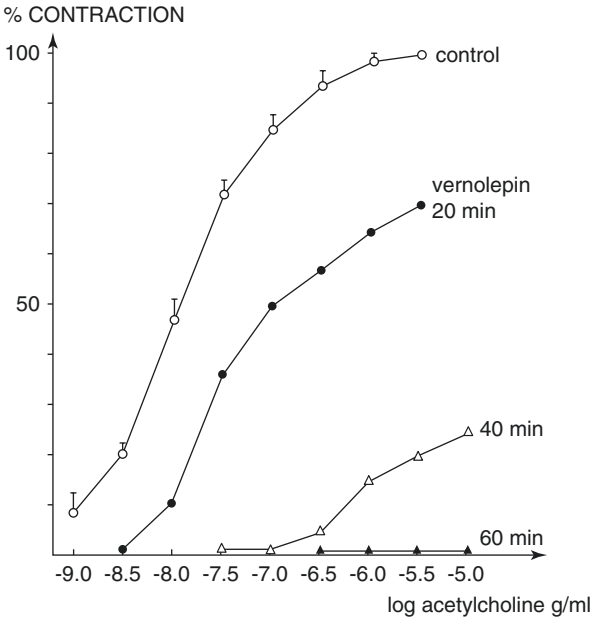


Fig. 6.7 Cumulative concentration-response curves with acetylcholine on the guinea pig ileum, influenced by vernolepin. A fixed concentration of 1.8×10^{-5}M was applied with the incubation time as a variable (Laekeman 1988)

represented within subclasses of the phenotypically diverse neurons of the enteric nervous system. The function of the intestine is influenced by nervous impulses, but the plexi of Auerbach and Meissner in the isolated ileum of the guinea pig can function independently (Nijenhuis et al. 2011). To trigger intestinal activity, acetylcholine is an important intestinal neurotransmitter, acting on local muscarine-2 receptors (Tayebati et al. 1999).

In order to study the influence of substances and complex mixtures, pieces of ileum are mounted over an electrode in an isolated organ bath. Every 10 s an electric pulse is delivered through the organ, leading to the release of small amounts of acetylcholine. This release causes a twitch contraction that lasts for less than one second, because the acetylcholine that is released is immediately hydrolyzed by cholinesterase. The quantity of acetylcholine per twitch is unmeasurably low. The coaxially stimulated ileum can be used to test antimuscarinic properties, but can be studied at the same possible interference with neuronal release of acetylcholine (Fig. 6.8) (Day and Vane 1963).

When testing the effect of methuenine and epimethuenine, it can be expected that the former will more likely inhibit contractions caused by acetylcholine released from the neuronal plexi in the ileum. But when twitch contractions are less prominent under the influence of natural substances, it must be determined whether the inhibition is caused by an inhibition of the release of acetylcholine, by a muscarinic antagonism, or by a non-specific activity.

Methuenine inhibited the coaxial stimulation more, and also inhibited contractions by exogenous acetylcholine. As it was shown that epimethuenine had a clear antihistaminic property, it inhibited twitch contractions only slightly. Prostaglandin E2 was able to partially restore the twitch contractions suppressed by methuenine. The action of methuenine could not be reversed by naloxone, which is additional proof that methuenine does not interfere with the neurotransmission (Van Nueten et al. 1976).

When applied to the coaxially stimulated, isolated ileum, vernolepin led to an initial stimulation of the twitch contractions and a time-dependent inhibition. It was proven that exogenous acetylcholine was also inhibited. As for the cumulative dose-response curves, the inhibition was irreversible, i.e., contractions could not be restored after washing (Fig. 6.9).

Morphine specifically inhibits cholinergic neurotransmission. Its action targets only the electrically evoked twitch contractions, whereas contraction by exogenous acetylcholine is not influenced. Moreover, the inhibiting activity of morphine is reversed by naloxone. Interpretation of the results obtained with coaxial stimulation can teach us more about specificity and reversibility of actions by natural substances (Roquebert et al. 1971).

Platelet Aggregation: Mechanisms *In Vitro* Predicting Safety In Vivo

Human or animal (e.g., rabbit) blood platelets are a useful experimental tool for studying the interaction of natural substances with an intracellular system. Platelet-rich plasma is obtained by centrifuging citrated blood samples at a lower speed. The aggregation of platelets can be triggered by several agents, a.o. arachidonic acid (AA), adenosine diphosphate (ADP), or collagen. Aggregation takes place in a small tube, filled with stirred platelet-rich plasma. The tube is placed between a light source and a photometer; as long as the platelet rich plasma is homogeneous,

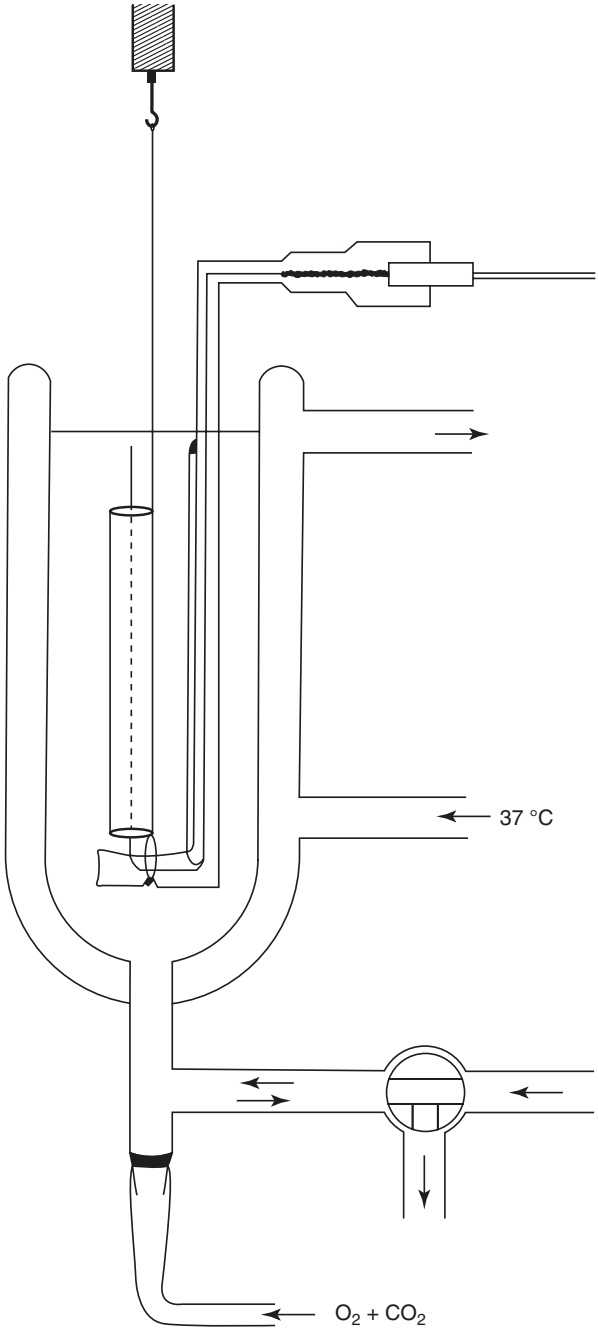


Fig. 6.8 Setup of the coaxial stimulation of the guinea pig ileum in an isolated organ bath. Twitch contractions are detected using an isometric transducer and signals are transmitted for recording

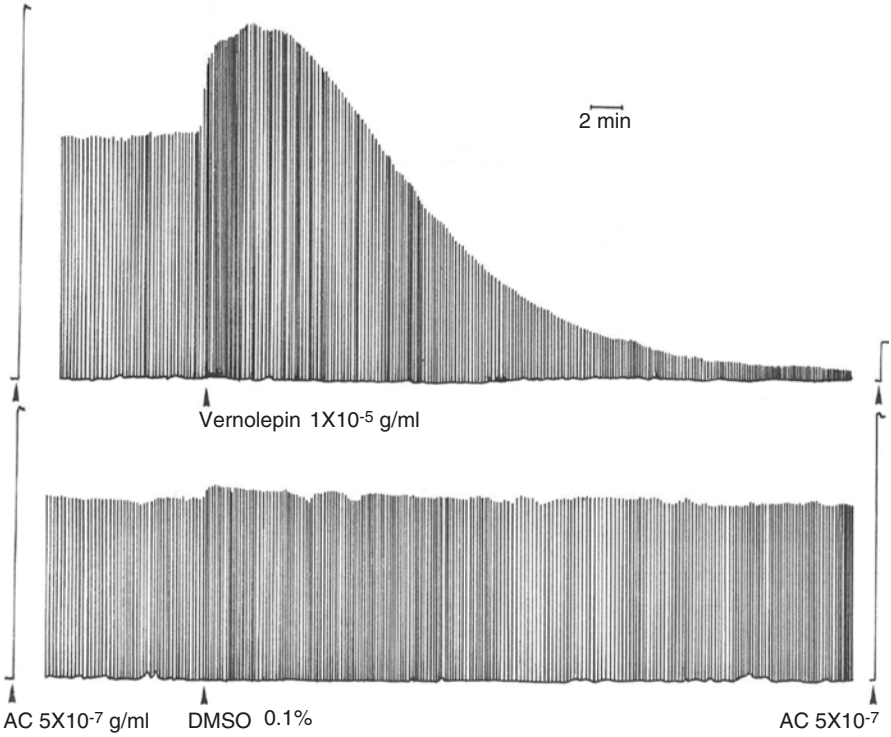


Fig. 6.9 Original tracing of a coaxially stimulated guinea pig ileum. Substances added to the isolated organ bath: *AC* acetylcholine, *DMSO* dimethylsulfoxide as solvent control, vernolepin (Laekeman 1988)

light transmission is hampered. When pro-aggregating agents are added, lumps of platelets are formed and sink to the bottom of the tube, as the stirring is not sufficient to keep the lumps afloat. Light transmission is enhanced when more aggregating lumps are formed, until all platelets have been coagulated (Born 1962).

Platelet aggregation triggered by AA, ADP and collagen was inhibited by vernolepin, and this inhibition was time-dependent (Fig. 6.10). The aggregation seemed to be reversible, at least after short incubation times, with the pattern passing by a maximum, close to the maximal aggregation, and then returning to baseline. When re-challenged, the platelets no longer responded to the aggregating agents, which seemed like they were protected against clotting.

In order to see what happened on a subcellular level, electron microscopy was performed at distinct phases before and during the aggregation process. Aliquots of the platelet suspension were stained with glutaraldehyde, the solution was decanted, and platelets were mixed with an ice-cold cacodylate-sucrose buffer, centrifuged and dehydrated with ethanol. Platelets were subsequently prepared for electron microscopy by embedding them in Epon-Epon in order to prepare ultrathin sections stained with uranyl acetate and lead citrate (Laekeman et al. 1985).

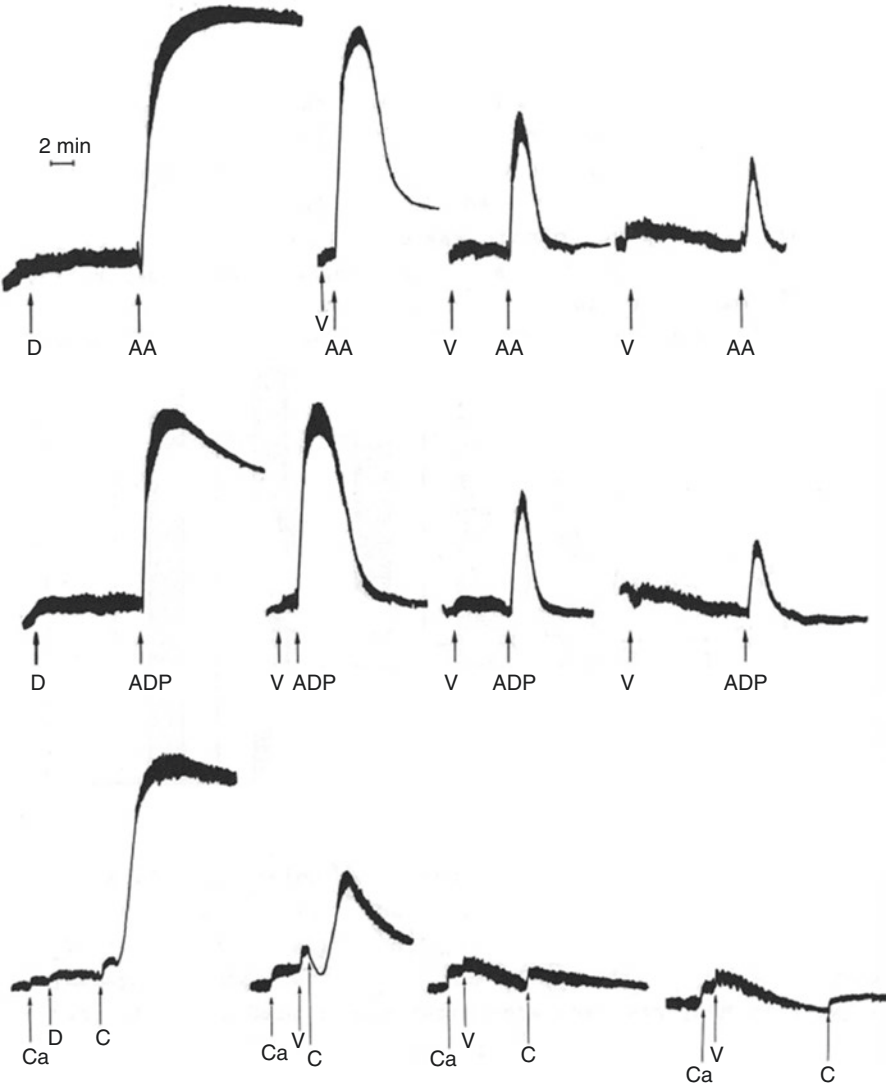


Fig. 6.10 The effect of vernolepin on rabbit platelets aggregated with various aggregating agents. Recording based on light transmission (Laekeman 1988). *AA* arachidonic acid (1×10^{-4} g/ml). *ADP* adenosinediphosphate (4×10^{-6} g/ml). *C* collagen (1×10^{-5} g/ml). *Ca* $CaCl_2$ (2 mM). *D* dimethylsulfoxide (0.1 %). *V* vernolepin (1×10^{-5} g/ml). All concentrations are final concentrations in the cuvette

Subcellular structures were seen by enlarging them more than 10,000 times. It was seen that although platelets tended to stick together by forming typical pseudopods, the aggregation did not take place. Platelets remained in suspension individually, but they did not return to their flattened discoid form, and some pseudopods

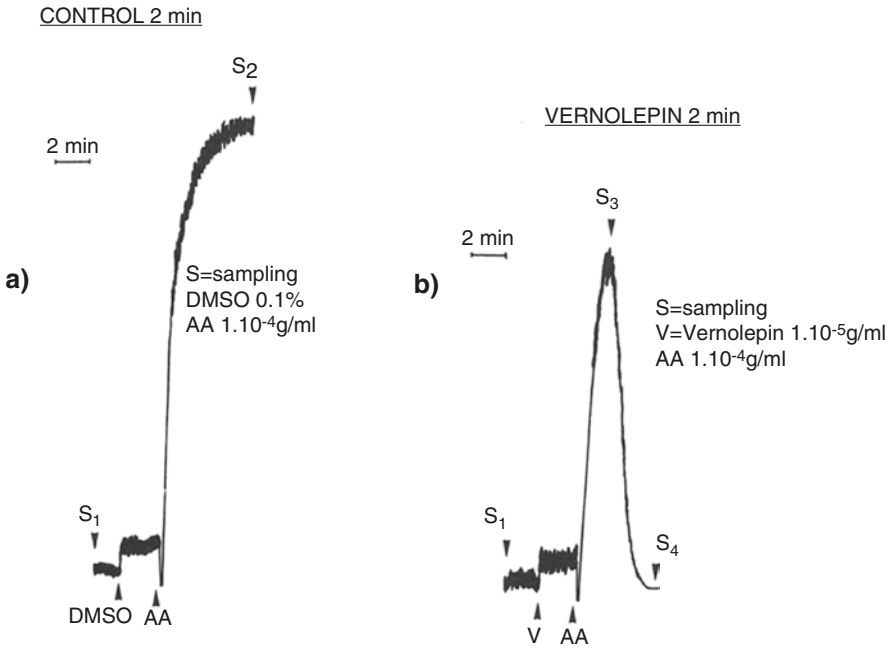


Fig. 6.11 Sampling of platelets for electron microscopy (Laekeman et al. 1985) published with permission of Springer. *AA* arachidonic acid (1×10^{-4} g/ml)

were kept. The intracellular granules were still visible, but not redistributed over the cellular surface (Figs. 6.11 and 6.12).

When platelets were incubated with vernolepin alone, a certain shape change occurred. The majority of platelets were swollen, and some of them had pseudopods. The main activity of vernolepin seemed to be concentrated on the platelet membrane. The microtubular system plays an important role in the aggregation process (Crawford 1976). An interaction of vernolepin at this level could be hypothesized. From subsequent *in vivo* experiments on rats, it could be seen that vernolepin had too small a therapeutic index to create perspectives. Intraperitoneal doses, from 10 mg/kg on up, resulted in lethal outcomes (Fig. 6.13).

The Relationship Between Secondary Metabolites and Pharmacological Activity: Simplification for Safety

The ripe seeds of *Myristica fragrans* Houtt. (family *Myristicaceae*) or nutmeg are used for digestive purposes. In China, the powdered nut is used as a warming and astringent remedy against dysentery, especially in children and the elderly. In Indochina, the powdered seeds are mixed with boiled rice against dysentery, anorexia, and colic (Perry 1980).

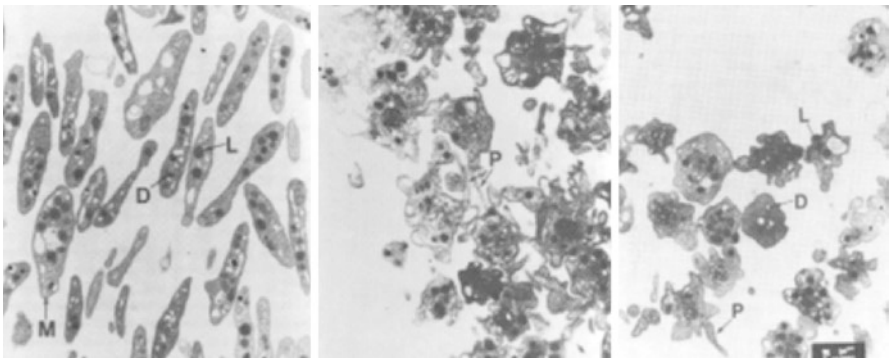


Fig. 6.12 Electron microscopic pictures of rabbit platelets activated by arachidonic acid. *Left* (=S_1 on Fig. 6.11): normal platelets in resting situation (*D* dense granule or body, *L* large granule, *M* microtubular system; enlargement 5,050 × 2.2). *Middle* (=S_3 on Fig. 6.11): platelets activated with arachidonic acid with formation of pseudopods (=P; enlargement 6,900 × 1.6). *Right* (=S_4 on Fig. 6.11): platelets back to baseline after nearly aggregation (*D* dense granule or body; *L* large granule, *P* pseudopods enlargement 6,900 × 1.6) (Laekeman et al. 1985). Published with permission of Springer

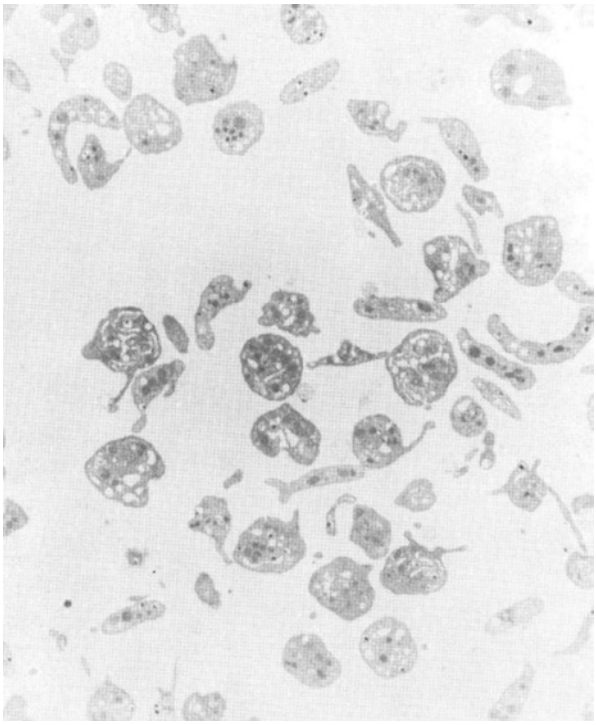


Fig. 6.13 Shape change of non-activated rabbit blood platelets after exposure to vernolepin 1×10^{-5} g/ml (enlargement 2,290 × 2.2) (Laekeman et al. 1985). Published with permission of Springer

Crude extracts of nutmeg inhibit the formation of prostaglandin-like compounds in vitro. Rasheed et al. (1984) reported strong evidence that the essential oil is the most important part of nutmeg with regard to pharmacological activity. They found eugenol and isoeugenol to be the most important compounds with regard to pharmacological activity in vitro. Bennett et al. (1988) have widened knowledge on the biological activity of eugenol.

Janssens et al. (1990) investigated the pharmacological activity of aromatic compounds of commercially available nutmeg oil. The composition of the oil was characterized by gas chromatography, with an OV-17 glass capillary column and equipped with a flame ionization detector. The temperature of the column gradually rose from 100 to 200 °C. 3-t-butyl-4-hydroxanisole was added as an internal standard. An identical response was assumed for eugenol and isoeugenol; terpinen-4-ol and terpineol; safrole, myristicin, and elemicin. Peak areas were integrated. Fractions to be tested were prepared, using silicagel column chromatography with benzene, chloroform, and ethyl acetate as eluents. Fractions were pharmacologically tested.

In vitro rabbit blood platelets' aggregation was used to quantify the pharmacological activity of nutmeg oil samples (Born 1962). Aggregation curves were recorded as described earlier in this chapter. Arachidonic acid was used as a pro-aggregating agent, and polysorbate 80 (tween 80) was used as an emulsifying agent for the essential oil samples and standard compounds.

The qualitative composition of essential oil samples was roughly checked via thin layer chromatography. The following components could be compared with standard spots: safrole, myristicin, eugenol and/or isoeugenol, terpinen-4-ol, elemicin, and alpha-terpineol. A more detailed quantification of the oils was carried out by GC/MS.

Only five batches were found to be active: (1) fraction A contained large amounts of myristicin and a little safrole; (2) fraction B contained equal amounts of isoeugenol and myristicin; (3) fraction C contained methyleugenol and a trace of eugenol and isoeugenol; (4) fraction D contained myristicin, a small amount of eugenol but no isoeugenol; and (5) fraction E contained elemicin. Other volatile monoterpenes, such as alpha- and betapinene, were also found, but these showed no pharmacological activity. The activity of methyleugenol was largely inferior to that of eugenol and isoeugenol.

Eugenol and isoeugenol are the most potent constituents of nutmeg oil for the inhibition of platelet aggregation induced by arachidonic acid; all other constituents were 100-1,000 times less potent. No IC_{50} could be determined for linalool, alpha- and betapinene, alpha terpineol, terpinene4-ol and camphene, due to lack of activity. There was no potentiating action of one compound on another, when equal amounts of eugenol, safrole, myristicin and a-terpineol were mixed. In Table 6.1 the IC_{50} values are given in molar concentrations in order to compare the activity of the different compounds on a molecular basis.

Table 6.1 Platelet aggregation IC_{50} values of nutmeg oil constituents and indomethacin (positive control) (Janssens et al. 1990)

Product	IC_{50} (M)
Eugenol	3.0×10^{-7}
Isoeugenol	7.2×10^{-7}
Elemicin	3.6×10^{-4}
Myristicin	2.5×10^{-4}
Safrole	1.1×10^{-4}
Indomethacin	2.2×10^{-7}

Table 6.2 Comparison between experimentally obtained IC_{50} values (g/ml) of nutmeg oil and calculated activity based on eugenol and isoeugenol contents in the oils (Janssens et al. 1990)

Oil batch	Experimental $IC_{50} \pm SEM$ ($\times 10^{-5}$ g/ml)	Calculated IC_{50} (95 % CI) ($\times 10^{-5}$ g/ml
Donck	1.17 ± 0.63	0.86 (0.67–1.17)
Liebig	1.35 ± 0.89	1.21 (1.17–1.39)
Universal flavor	1.59 ± 1.16	0.90 (0.79–1.05)

When rough calculations are made as to the pharmacological activity of different oils using the concentrations of eugenol and isoeugenol in the oils as a starting point, comparison can be made between the activity of the oil and its most active compounds. Theoretical IC_{50} values were calculated, as if the oils were only composed of eugenol and isoeugenol diluted with pharmacologically inert material. These calculations resulted in values that could be compared to the actual IC_{50} values obtained for the real oils using *in vitro* blood platelet aggregation; the comparisons are shown in Table 6.2.

The results indicate the usefulness of the identification of highly active substances and of comparing their share in pharmacological activity of the complex mixture. It can be concluded that the matrix effect is not valid for nutmeg essential oil, when investigating possible anti-inflammatory action, eugenol and iso-eugenol as the most prominent active substances. At the same time, it showed that substances for which there is a safety concern, like safrole, can be eliminated without losing pharmacological activity (EMA 2014).

Pharmacokinetics and Safety Issues

The Case of Red Yeast Rice Extracts

The importance of pharmacokinetics can be illustrated by the actual development of red yeast rice preparations. Extracts of red yeast rice are used to lower cholesterol in mild hypercholesterolemia. Preparations of red yeast rice have been used for more than 2000 years in China; long-term cardiovascular beneficial effects have been claimed, even in patients at risk (Lu et al. 2008). Monacoline K, which is the most important active substance in red yeast rice extracts, has been identified as the inspiring natural substance that led to the discovery of simvastatin (Fig. 6.14).

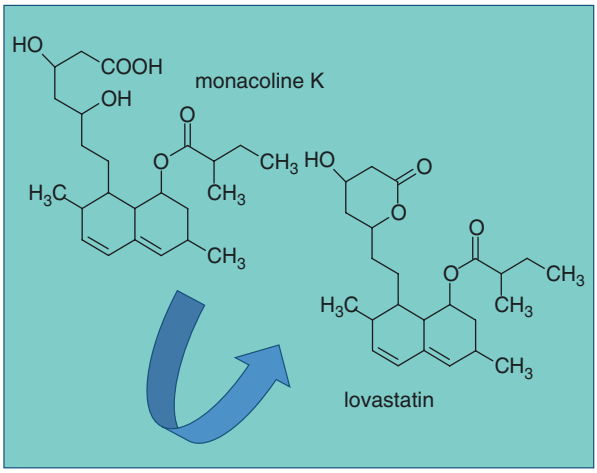


Fig. 6.14 Structures of monacoline K and lovastatin present in red yeast rice

There has been a debate with regard to the relationship between the composition of extracts and the benefit-risk ratio, with extrapolation to all statins. Indeed, monacoline K can be in its lactone form (lovastatin), or its open ring structure (with OH- and COOH-groups free = active configuration). The latter has a more hydrophilic character than the former. There is much (commercial) speculation about the prevailing configuration in extracts available on the market. Most of the preparations are food supplements, and there are no valid comparative data on possible undesirable effects of one extract versus another; consequently, it is difficult to address a risk-benefit balance of various extracts. The fact that mono-preparations as well as combinations are on the market hampers straightforward evaluation, so there is little underpinning evidence for making predictions. Due to the lack of data on monacoline K, research on commercialized statins may be inspiring. It seems that desolvation and resolvation of the statins before and after binding to the OH-Methyl-Glutaryl Coenzyme A Reductase (HMGCR) are the major determinants of the energetics of the binding process. An analysis of the amphiphilic nature of lovastatin anion, acid, and lactone, and their abilities to cross the blood brain barrier, has indicated that this process may be dominated by desolvation and resolvation effects as well. Crossing the blood brain barrier may influence central nervous system cholesterol physiology with unknown consequences. (Fong 2014).

Applying these hypotheses to a pharmacokinetic model can inspire further investigation of benefit-risk aspects related to the prevalence of one form or another in commercialized extracts. The findings should be linked to clinical observations. Figure 6.15 can help identify important issues. Herbal examples of absorption-distribution-metabolism-excretion processes can be seen.

Absorption: The Case of Anthraquinones-containing Plants

Anthraquinones can be present as C-O or C-C glycosides and have to be considered as prodrugs. A bacterial conversion is necessary to produce the bioactive forms. Anthracenic glycosides are not absorbed in the small intestine; they can resist

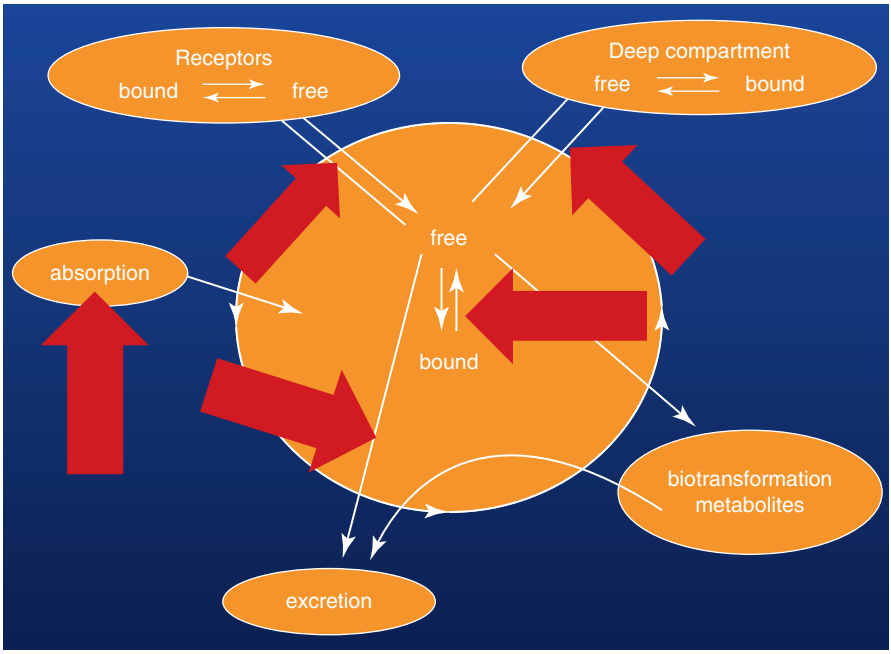


Fig. 6.15 Red arrows indicate possible critical points in the transfer of hydrophilic and lipophilic forms of statin-like compounds in red yeast rice extracts. Several processes ask for a transfer from hydrophilic to lipophilic configuration and vice versa: (1) absorption through the enterocytes; (2) binding to the plasma proteins; (3) migration to the deep compartment (more particularly, skeletal muscle cells); (4) binding to the HMGCR; (5) biotransformation and excretion

stomach acid and intestinal glycosidases and arrive at the colon intact, where they are converted to aglycones that can irritate the mucosal layers and invert the absorption of water and electrolytes. It is argued that plant species mainly containing anthrone C-glycosides (present for example in *Rhamnus purshianus* D.C.) or dianthrone O-glycosides (for example in *Cassia senna* L. and *Cassia angustifolia* Vahl) are preferable over species that contain more aglycones, which can be already absorbed in the small intestine (for example preparations from *Aloe* species that can contain *Aloe* emodins originating from the pericycle cells and adjacent leaf parenchyma) (De Witte et al. 2008; De Witte and Lemli 1990).

Biotransformation: Hepatotoxicity of Concentrated Camellia Extracts

Preparations of *Camellia sinensis* (L.) Kuntze are considered to be safe. Their traditional use has been approved by the EMA, and a European Union monograph was made after the assessment by the Herbal Medicinal Product Committee (Grigoras and Purdel 2014). However, the safety of orally taken preparations depends on the concentration of polyphenols circulating through the liver. A case of acute hepatitis

in a 46-year-old woman was related to the use of a highly concentrated *Camellia* extract made with 80 % ethanol (Vial et al. 2003). Mazzanti et al. (2015) report on 19 cases of hepatotoxicity due to green tea preparations. The high content of epigallocathechines may be the most probable cause, although factors related to the patient are also thought to be involved.

Binding to Proteins: Digoxin Binding Ab

Digoxin isolated from *Digitalis purpurea* L. leaves is a molecule with a particular pharmacokinetic profile. The distribution volume is estimated to be 510 L. This is rather high and means that there is an important intracellular penetration with concentrations in the heart muscle more than 30 times the plasma concentrations. The circulating digoxin is only for 30 % bound to plasma proteins. In the case of intoxications or overdose, hemodialysis makes no sense (Lanoxin® SPC 2015). Digoxin-specific antibody fragments (digoxin-Fab) are widely regarded as a safe and effective treatment for the management of digoxin poisoning. Although there are no randomized clinical trials, reports on more than 2,000 cases of acute or chronic poisoning show a therapeutic response from 50 % to 80–90 %. The time for reversal of digoxin toxicity varies between 30 and 45 min. Studies with pharmacokinetic data show that free digoxin concentration fell to almost zero within a few minutes following the administration of digoxin-Fab, which is a good example of applied safety pharmacokinetics (Chan and Buckley 2014).

Receptor Binding: Berberin As an Emerging Phytochemical Substance

PCSK9, or Protoprotein Convertase Subtilisine/Kexine type 9, downregulates the LDL-receptor. The protein can be seen as a negative factor hampering the elimination of LDL-cholesterol. Berberin, an isoquinoline alkaloid isolated from *Berberis* and *Hydrastis* species, lowered circulating PCSK9 concentrations and hepatic PCSK9 mRNA levels. The mechanism seems to be related to overexpressing ubiquitin, which in turn inhibits activation of the Hepatocyte Nuclear Factor 1-alpha (HNF1-alpha), a factor essential to PCSK9 expression. Berberin is a good example of a substance that binds to receptors at a subcellular level, with a mechanism of action closely related to recent developments in lipid lowering. These discoveries are contributing to clinical studies with plant species containing berberin, but a thorough risk-benefit analysis must go hand in hand with therapeutic development (Dong et al. 2015).

Excretion: The Example of Arbutin

The leaves of *Arctostaphylos uva ursi* (L.) Spreng or bearberry are granted a traditional use status by EMA, and an assessment of this plant has been carried out (Heroutová 2012). The toxicity of hydroquinone is an apparent risk related to its

use. However, the amount of hydroquinone corresponding to the recommended dose of bearberry leaf extract is considered to be safe for short-term use. In order to remain in a safe area, it is important to obtain a maximal yield within the dosage range applied. In a pilot study, coated tablets with bearberry extract were administered together with 10 g of sodium hydrogen carbonate. The pH of the urine changed from 6.5 to 7.4, and in one case to pH 8 for 1 h. Free hydroquinone was found in a therapeutic concentration in urine only if the pH was alkaline (pH 8) (Frohne 1970).

Safety Frame

Until now, no algorithm has been available for safety evaluation in general, and safety evaluation on pharmacological grounds in particular. The question of whether or not an herbal medicinal product is safe may be answered by using a multifocal approach.

As already shown by William Withering, the triad of "quality-safety-efficacy" plays a crucial role (Breckenridge 2006). Aspects related to quality and efficacy are dealt with in other chapters. Safety can be subdivided into several topics. The research question to be answered when considering the framework below can be formulated as "To what extent can a framework be considered to evaluate the safety of herbal products in a reproducible and comparative way and contribute to risk-benefit analysis?"

Ideally, the quality issue can be covered by Pharmacopoeia monographs. There is close co-operation between the Herbal Medicinal Product Committee within the EMA and Group 13B and 13C within the European Pharmacopoeia. The quality of the herbal material should be taken into consideration before beginning pharmacological investigations (Table 6.3).

As proven by a few examples, an experimental pharmacological approach focused on safety should take into account a number of the issues listed in Table 6.4, which deals with content and methodology. When extrapolating pharmacological,

Table 6.3 A series of questions related to the quality of herbal materials. The materials should yield as many green fields as possible

Question	Answer	
	NO	YES
1. Is the herbal medicinal product subject of a European Pharmacopoeia monograph?		
2. Is the herbal medicinal product subject of a monograph in an accessible Pharmacopoeia other than European Pharmacopoeia?		
3. Is an unambiguous macroscopic identification of the herbal medicinal product possible?		
4. Is an unambiguous chemical characterization of the herbal medicinal product possible?		
5. Is adulteration/contamination of the herbal medicinal product possible/probable?		

pharmacodynamic, and pharmacokinetic findings to clinical practice, a series of questions could be examined as presented in Table 6.5.

Conclusions

Safety pharmacology needs a practical approach, beginning from fundamental pharmacodynamics and pharmacokinetic properties of substances alone, or as a component in a complex matrix. An *in vitro* approach seems to be the most suitable way to begin. Irreversibility of the observed effects must be interpreted as an early warning for toxicity. *In vitro* techniques allow the study of types of antagonisms and mechanisms of action. Safety is linked to well-documented pharmacodynamics; in complex mixtures, it is useful to try to attribute pharmacological effects to individual substances. Pharmacokinetics of complex mixtures remains tricky, especially when families of related substances are present.

The proposed safety framework may help evaluate a safety profile of a substance or a complex mixture.

Table 6.4 A series of questions related to a safety pharmacology approach. The materials should yield as many green fields as possible

Question	Answer	
	NO	YES
1. Is the preparation tested related to the traditionally used preparation according to an ethnopharmacological approach?		
2. Does a concentration-response relationship exist in an *in vitro* experimental pharmacological approach?		
3. Does a dose-response relationship exist in an *in vivo* experimental pharmacological approach?		
4. Does an effect seen in an *in vitro* pharmacological approach correspond to an identifiable mechanism of action?		
5. Does an effect seen in an *in vivo* pharmacological approach correspond to an identifiable mechanism of action?		
6. Is there a comparison between the concentrations needed for a pharmacological effect *in vitro* and the doses needed for a pharmacological effect *in vivo*?		
7. Does an effect seen in an *in vitro* experimental model correspond to a possibly toxic action?[1]		
8. Does a relationship exist between an *in vitro* or *in vivo* pharmacological effect and identified natural substances?		
9. Does an affinity exist for certain types of CYP-isoenzymes or PgP-proteins?		
10. Can undesirable effects be hypothesized, starting from experimental pharmacological findings?		
11. Does the experimental pharmacological approach contribute to the underpinning of existing therapeutic indications?		
12. Does the experimental pharmacological approach open new perspectives for new therapeutic indications?		

Table 6.5 A series of questions related to clinical safety aspects of herbal medicines. The materials should yield as many green fields as possible

Question	Answer	
	NO	YES
1. Are non-serious undesirable effects[a] with a therapeutic posology of the herbal medicinal product reported in literature or reference sources?		
2. Were serious undersirable effects[b] with a therapeutic posology of the herbal medicinal product reported in literature or reference sources with a well-documented history[c]?		
3. Were serious undesirable effects with or without specifications of the therapeutic posology of the herbal medicinal product reported in literature or reference sources in general?		
4. Were one or more intoxications due to (in)voluntary overdose with the herbal medicinal product reported in literature or reference sources as documented case studies?		
5. Were one or more intoxications due to (in)voluntary overdose with the herbal medicinal product reported in literature or reference sources in general?		
6. Is (are) there (a) well defined therapeutic indication(s) for the herbal medicinal product according to international standards[d]?		
7. Can the symptoms of the conditions covered by the claimed indications be related to serious health deficiencies creating the need for medical supervision?		
8. Are there constitutional groups[e] at risk by using the herbal substance?		
9. Are drug-drug interactions of the herbal medicinal product with other medicines reported in literature or reference sources as case studies?		
10. Does there exist a plausibility for drug-drug interactions of the herbal medicinal product?		
11. Are there patient groups[f] at risk when considering the use of the herbal medicinal product?		
12. Can the herbal medicinal product be replaced by other medicines with stronger therapeutic activity and less undesirable effects?		
13. Can the herbal medicinal product be replaced by other medicines with stronger therapeutic activity but more undesirable effects?		
14. Is the use of the herbal medicinal product known in western traditional medicine?		
15. Is the herbal medicinal product studied in one or more placebo controlled clinical trials?		
16. Is the herbal medicinal product subject of one or more meta-analysis or reviews published in international scientific journals?		
17. Is the herbal medicinal product on the market in EU countries since 30 years or more?		

[a] Non serious undesirable effects = not leading to hospitalisation and reversible.

[b] Serious side effects = leading to hospitalization or dead

[c] Reference sources are accessible written sources, pharmacovigilance reports included. In clinical studies there exists a need for immediate reporting serious side effects in the pharmacovigilance cascade reported to the ethical committee when submitting the protocol of the study. Four conditions have simultaneously to be present in order to report: there must be *drug* involved (= one or more well defined medicinal products, among them the herbal medicinal product dealt with), there must be an *event* (= well documented phenomenon happening to the patient), there must be a *source* (= a well-defined source: first order = patient her/himself or people immediately involved in the event) and there must be a *patient* (= a well-defined person taking among others the herbal medicinal product)

[d] These therapeutic indication(s) are referring to international standards, e.g. ICD-10 criteria (http://www.who.int/classifications/apps/icd/icd10online/) and/or internationally recognised diagnostic references (e.g. DSM-IV criteria for mood disorders; NYHA for cardiac insufficiency; Vahlensieck for BPH).

[e] Constitutional groups = children, pregnant and lactating women and elderly

[f] Patient groups are considered as being at risk by the pathology they are suffering from

References

Arunlakshana O, Schild M (1959) Some quantitative uses of drugs antagonists. Br J Pharmacol 14:48–58

Bakana P, Laekeman GM, Totte J, Herman AG, Vlietinck AJ (1985) Stereochemical considerations in relation to the pharmacological activity of Pterotaberna alkaloids. J Nat Prod 48:766–771

Bennett A, Stamford IF, Tavares IA et al (1988) The biological activity of eugenol, a major constituent of nutmeg (*Myristica fragrans*): studies on prostaglandins, the intestines and other tissues. Phytother Res 2:124–130

Booker A, Suter A, Krnjic A et al (2014) A phytochemical comparison of saw palmetto products using gas chromatography and 1H nuclear magnetic resonance spectroscopy metabolomic profiling. J Pharm Pharmacogn 66:811–822

Born GVR (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927–929

Breckenridge A (2006) William Withering's legacy – for the good of the patient. Clin Med 6: 393–397

Brownlee G, Johnson ES (1965) The release of acetylcholine from the isolated ileum of the guinea-pig induced by 5-hydroxytryptamine and dimethylphenylpiperazine. Br J Pharmacol 24: 689–700.

Chaggar PS, Shaw SM, Williams SG (2015) Is foxglove effective in heart failure? Cardiovasc Ther. doi:10.1111/1755-5922 [Epub ahead of print]

Chan BS, Buckley NA (2014) Digoxin-specific antibody fragments in the treatment of digoxin toxicity. Clin Toxicol 52:824–836

Crawford N (1976) Platelete microfilaments and microtubules. In: Gordon JL (Ed) Platelets in biology and pathology. North-Holland Publishing Company, Amsterdam pp. 121–157.

Day M, Vane JR (1963) An analysis of direct and indirect actions of drugs on the isolated guinea-pig ileum. Br J Pharmacol Chemother 20:150–170

de Combarieu E, Martinelli EM, Pace R, Sardone N (2015) Metabolomics study of Saw palmetto extracts based on ^{1}H NMR spectroscopy. Fitoterapia 102:56–60

De Monte C, Carradori S, Granese A, Di Pierro GB, Leonardo C, De Nunzio C (2014) Modern extraction techniques and their impact on the pharmacological profile of *Serenoa repens* extracts for the treatment of lower urinary tract symptoms. BMC Urol 14:63–74

De Witte P, Lemli L (1990) The metabolism of anthranoid laxatives. Hepatogastroenterology 37:601–605

De Witte P, Demeyer K, Delmulle L et al (2008) Pharmacokinetics. In: Delmulle L, Demeyer K (eds) Anthraquinones containing plants reconsidered. Standaard Uitgeverij, Antwerpen, pp 99–107

Dong B, Li H, Singh AB, Cao A, Liu J (2015) Inhibition of PCSK9 transcription by berberine involves down regulation of hepatic HNF1-alpha protein expression through the ubiquitin-proteasome pathway. J Biol Chem 290:4047–4058

EMA (European Medicines Agency) (2012) Recommendation to marketing authorisation holders, highlighting the need to ensure compliance with 3Rs methods described in the European Pharmacopoeia. Committee for Medicinal Products for Human Use (CHMP) and Committee for Medicinal Products for Veterinary Use (CVMP), EMA

EMA (European Medicines Agency) (2014) Public statement on the use of herbal medicinal products containing estragole. Committee on Herbal Medicinal Products, EMA

Fong CW (2014) Statins in therapy: understanding their hydrophilicity, lipophilicity, binding to 3-hydroxy-3-methylglutaryl-CoA reductase, ability to cross the blood brain barrier and metabolic stability based on electrostatic molecular orbital studies. Eur J Med Chem 85:661–674

Frohne D (1970) Untersuchungen zur Frage der harndesinfizierenden Wirkungen von Bärentraubenblatt-Extrakten. Planta Med 18:1–25

Grigoras N, Purdel C (2014) Assessment report on *Camellia sinensis* (L.) Kuntze, non fermentatum folium. European Medicines Agency. http://www.ema.europa.eu/ema/

Habib FK, Wyllie MG (2004) Not all brands are created equal: a comparison of selected components of different brands of *Serenoa repens.*. Prostate Cancer Prostatic Dis 7:195–200
Hauptmann PJ, Kelly RA (1999) Digitalis. Circulation 99: 1265–1270.
Heroutová M (2012) Assessment report on *Arctostaphylos uva-ursi* (L.) Spreng., folium. EMA. http://www.ema.europa.eu/ema/
ICH (2001) Guidance for Industry: S7A safety pharmacology studies for human pharmaceuticals. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER)
Janssens J, Laekeman GM, Pieters LAC, Totte J, Herman AG, Vlietinck AJ (1990) Nutmeg oil: identification and quantification of its most active constituents as inhibitors of platelet aggregation. J Ethnopharmacol 29:179–188
Jeffreys D (2005) The remarkable story of a wonder drug Aspirin. Bloomsbury, London, pp 5–74
Kyle R, Shampe M (1974). Discoverers of quinine. JAMA. 229 (4): 462.
Laekeman G (1980) Pharmacological study of the Schulz-Dale reaction. University of Antwerp, PhD thesis
Laekeman G (1988) Phytopharmacological study of vernolepin and eugenol. University of Antwerp, PhD aggregation thesis
Laekeman GM, Mertens J, Totte J, Bult H, Vlietinck AJ, Herman AG (1983) Isolation and pharmacological characterization of vernolepin. J Nat Prod 46:161–169
Laekeman GM, De Clerck F, Vlietinck AJ, Herman AG (1985) Vernolepin: an antiplatelet compound of natural origin. Naunyn Schmiedeberg's Arch Pharmacol 331:108–113
Lanoxin (2015) Summary of Product Characteristics last update June 2015
Löwig C, Weidmann S (n.d.) Beiträge zur organischen Chemie (Contributions to organic chemistry); "III. Untersuchungen mit dem destillierten Wasser der Blüthen von *Spiraea Ulmaria*" (III. Investigations of the water distilled from the blossoms of *Spiraea ulmaria*). Annalen der Physik und Chemie 46:45–91; 57–83
Lu Z, Kou W, Du B et al (2008) Effect of Xuezhikang, an extract from red yeast Chinese rice, on coronary events in a Chinese population with previous myocardial infarction. Am J Cardiol 101:1689–1693
Mazzanti G, Di Sotto A, Vitalone A (2015) Hepatotoxicity of green tea: an update. Arch Toxicol 89:1175–1191
Nijenhuis CM, ter Horst PGJ, de Jong-van den Berg LTW, Wilffert B (2011) Disturbed development of the enteric nervous system after *in utero* exposure of selective serotonin re-uptake inhibitors and tricyclic antidepressants. Part 1: literature review. Br J Clin Pharmacol 73:16–26
Ouyang AJ, Lv YN, Zhong HL, Wen JH, Wei XH, Peng HW, Zhou J, Liu LL (2015) Meta-analysis of digoxin use and risk of mortality in patients with atrial fibrillation. Am J Cardiol 115(7):901–916
Perry LM (1980) Medicinal plants of East and Southeast Asia: attributed properties and uses. MIT Press, Cambridge, MA, pp 141–142, 153 and 227–228
Pugsley MK, Authier S, Curtis MJ (2008) Review: frontiers in pharmacology. Principles of safety pharmacology. Br J Pharmacol 154:1382–1399
Rasheed A, Laekeman G, Totte J, Vlietinck AJ, Herman AG (1984) Eugenol and prostaglandin biosynthesis. NEJM 310:50–51
Roquebert J, Dufour P, Canellas J (1971) Action of some morphine analgesics on the effects of coaxial electric stimulation of the isolated guinea-pig ileum. C R Seances Soc Biol Fil 165:577–580
Scaglione F, Lucini V, Pannacci M, Caronno A, Leone C (2008) Comparison of the potency of different brands of *Serenoa Repens* extract on 5alpha-reductase Types I and II in prostatic co-cultured epithelial and fibroblast cells. Pharmacology 82:270–275
Scaglione F, Lucini V, Pannacci M, Dugnani S, Leone C (2012) Comparison of the potency of 10 different brands of *Serenoa repens.*. Eur Rev Med Pharmacol Sci 16:569–574
Schmitz, R (1985). Friedrich Wilhelm Sertürner and the discovery of morphine". Pharmacy in history. 27 (2): 61–74.

Tayebati SK, Piergentili A, Natale D, Amenta F (1999) Evaluation of an agonist index: affinity ratio for compounds active on muscarinic cholinergic M2 receptors. J Auton Pharmacol 19:77–84

Van Nueten JM, Janssens AJ, Fontaine J (1976) Unexpected reversal effects of naloxone on the guinea pig ileum. Life Sci 18:803–809

Van Rossum JM (1963) Cumulative dose-response curves II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. Arch Int Pharmacodyn 143:299–330

Vane J (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 231:232–235

Vial T, Bernard G, Lewden B, Dumortier J, Descotes J (2003) Hépatite aiguë imputable à l'Exolise® (*Camellia sinensis*). Gastroenterol Clin Biol 27(12):1166