

Chapter 2

Parasitic Helminths of Humans and Animals: Health Impact and Control

Abstract Organic compounds from terrestrial and marine organisms have been used extensively in the treatment of many diseases and serve as compounds of interest both in their natural form and as templates for synthetic modifications. This chapter summarizes the present knowledge about anthelmintic effects of the extracts and some already purified natural compounds isolated from the lower marine organisms including bacteria, sponge, fungi, and algae as well as the higher plants. A brief summary on anthelmintics in use is also included to provide a background for the comparison of effective concentrations, mode of actions, and weaknesses in therapy. The main focus is placed on in vitro and in vivo activities of secondary plant metabolites (alkaloids, essential oils, flavonoids, saponins, amides, enzymes, condensed tannins, and lactones with endoperoxide bridge-artemisinins) against nematodes, trematodes, and cestodes of medical and veterinary importance, and experimental model infections. Several issues are highlighted; the synergistic effect of a number of bioactive components in plant extracts, multiple putative target sites in helminths for some of secondary plant metabolites, probably different from those of current anthelmintics, which is suggested by their modified mode of actions.

Keywords Helminths • Natural compounds • Drug discovery • Marine organisms • Terrestrial plants • Secondary plant metabolites • Anthelmintic activity

2.1 Anthelmintic Drugs: Mode of Action, Efficacy and Resistance

Anthelmintics are drugs that are used to treat infections with parasitic worms. This includes both flatworms, e.g., trematodes and cestodes and roundworms, i.e., nematodes. In the past, all the drugs used for humans were developed initially in

response to the considerable market for veterinary anthelmintics in high- and middle-income countries (Geary et al. 2010). Discovery of benzimidazoles (BZs), a very effective broad-spectrum group of anthelmintics dates back to 1961, when thiabendazole was synthesized. The subsequent cascade of patents during the next 25 years led to the experimental or commercial development of a further 15 BZs and central to their success is their selective toxicity for helminths (Lacey 1990). The most frequently used benzimidazoles in human medicine are albendazole and mebendazole. Discovery of ivermectins as natural compounds has enlarged the group of anthelmintic drugs indicated primarily for use in veterinary medicine (for details see Sect. 2.2). All of these drugs have been discovered by means of high throughput screening of a library of synthesised chemical compounds. Once the high anthelmintic activity and low toxicity of these molecules have been demonstrated, usually the next step is the evaluation of the mechanisms of action on parasites. Various issues relating to current anthelmintic drugs, such as in vitro drug effects, in vivo efficacy, pharmacokinetic characteristics, and mode of actions have been the subjects of numerous papers. The short overview of anthelmintic drugs included in this book should serve as background for the following chapter, where concentrations, activities, and putative target sites of natural compounds are often compared with the reference drugs. There are many excellent reviews dealing with these topics, for example: Keiser and Utzinger (2010), Holden-Dye and Walker (2007), Geary et al. (2010), McKellar and Jackson (2004), Frayha et al. (1997), Martin (1997), and others.

Drug treatment necessitates a thorough knowledge of the life cycle of the parasite as well as its physiology and biochemistry and surface of the worm (tegument or cuticle). Worm surface of flatworms as well as mouth in trematodes and gut in nematodes are the key factors in drug absorption. According to the target sites in helminths which are affected by anthelmintics, they form the following groups: *Nicotinic agonists* (e.g.: levamisole, pyrantel, morantel), *Acetylcholinesterase inhibitors* (haloxon, dichlorvos), *GABA agonist* (piperazine) and *GluCl potentiators* (avermectins, moxidectin, milbemycin D), *Calcium permeability increase* (praziquantel), *β -tubulin binding* (benzimidazole carbamates), *Proton ionophores* (for example: closantel, rafoxanide, niclosamide), *Inhibition of malate metabolism* (diamphenetide), *Inhibition of phosphoglycerate kinase* (clorsulon), and *Inhibitor of arachidonic acid metabolism* (diethylcarbamazine) (Martin 1997). However, no single drug available today has use for the treatment or prevention of both nematode and trematode infections in humans (Table 2.1) and there are also differences in susceptibility to individual chemical derivatives in the same class. The most important, in terms of the extent of their application in human and veterinary medicine and efficacy, are the following classes, for which we provide a concise description of their activities.

Nicotinic agonists act selectively as agonists at synaptic and extrasynaptic nicotinic acetylcholine receptors on nematode muscle cells and produce contraction and spastic paralysis. These anthelmintics have been shown to increase the membrane conductance and depolarize the membrane by opening non-selective cation ion-channels that are permeable to both Na^+ and K^+ . Levamisole, pyrantel,

Table 2.1 The key drugs registered for the treatment of parasitic worms in humans (adopted from Holden-Dye and Walker 2007)

Parasitic infection	Anthelmintic drugs
Schistosomiasis (blood fluke)	Antimonials, metrifonate, oxamniquine, praziquantel
Cestodiasis (tape worm)	Niclosamide, benzimidazoles, praziquantel,
Fascioliasis (liver fluke)	Praziquantel, closantel, (and halogenated salicylamides)
Intestinal round worms	Piperazine, benzimidazoles, morantel, pyrantel, levamisole, avermectins and milbemycins, closantel (and halogenated salicylamides) emodepside
Filariasis (tissue round worms)	Diethylcarbamazine, suramin, ivermectin

morantel, and oxantel are large organic cations, and could enter the nicotinic ion-channel from the outer cell membrane. Once they pass through these channels, they produce the block at the narrow region of the channels, which subsequently cause muscle contractions in nematodes. This leads to worm paralysis in a contractile state and, once rendered immobile, the worms are expelled (Martin and Robertson 2007).

Acetylcholinesterase inhibitors are selective organophosphorus anticholinesterases. The mode of action of these compounds is to block the action of the parasite enzyme, acetylcholinesterase, leading to the excessive build-up of the neurotransmitter, acetylcholine. Metrifonate, an organophosphorus compound, is rapidly absorbed after oral administration and transformed non-enzymatically to its active metabolite, dichlorvos. The drug displays its activity exclusively on *Schistosoma hematobium*, where it inhibits cholinesterase and acetylcholinesterase to produce reversible paralysis. The paralyzed worms quickly release their hold in the bladder veins of the host and are carried eventually to the lungs where they are trapped and encased and then die. The drug is more specific for helminth cholinesterase than mammalian cholinesterases.

The group of drugs acting as *GABA antagonists and GluCl channel potentiators*, which is the mode of action of avermectins has been extensively investigated. It was shown that drugs in this group act on the same receptor as the GABA neurotransmitter in nematodes that is a ligand-gated Cl^- channel found on the synaptic and extrasynaptic membrane of nematode muscle. Ivermectin is a macrocyclic lactone derivative of avermectin-B isolated from natural source and acts as γ -aminobutyric acid (GABA) antagonist in nematodes. Avermectins are involved in the opening of the GABA-dependent chloride channels, inducing release of this transmitter which leads to the complete paralysis and immobilization of the worms (see for review: Prichard et al. 2012). The avermectins also have a receptor-mediated effect on glutamate-gated chloride (GluCl) ion channels, which has been directly correlated to nematocidal activity and which is now considered as their major mode of action (Martin and Pennington 1988). P-glycoproteins, which have a significant role in transmembrane exclusion of avermectins from the CNS, gut, and hepatobiliary tract of hosts, could account for reduced oral bioavailability of some avermectins. This effect is selective only to nematodes and arthropods, and cestodes and trematodes are not susceptible to

ivermectin. Due to very low toxicity and high efficacy, ivermectins became the drugs of choice for the treatment of onchocerciasis in humans.

Benzimidazole carbamates have a broad spectrum of activities (vermicidal, ovicidal, and larvicidal activity) against many parasitic roundworms and several flatworm species, but they are not effective, for example on *Schistosoma* spp. Most human intestinal and systemic nematodes as well as systemic cestodes are susceptible to one or more of the benzimidazole compounds (Frayha et al. 1997). The most frequently used benzimidazoles in human medicine are albendazole and mebendazole (EMA 1997, 1999). Benzimidazoles bind to intracellular tubulin, preferentially affecting parasites, thus inhibiting the formation of microtubules. This subsequently leads to disruption of cell homeostasis due to the impaired transport of secretory granules and enzymes in the cytoplasm. The mechanism of action of albendazole is by blocking glucose uptake in larval and adult stages of susceptible parasites, and also depleting their glycogen reserves, thus decreasing ATP formation (Martin 1997). The drugs are relatively insoluble in water and partially soluble in most organic solvents what has an impact on their bioavailability in tissues. Whereas efficacy is high on gastrointestinal helminths, their limited absorption and rapid metabolism means that high and/or prolonged doses are effective in the treatment of human systemic infections (Dayan 2003).

Praziquantel (PZQ) acts primarily in the tegument, where it induces a Ca^{2+} influx and rise in intra-tegumental calcium leading to the increased concentration of Ca^{2+} in sarcoplasmic reticulum of muscle cells. Disturbance in calcium homeostasis causes immediate and paralytic muscular contractions, followed by death and expulsion of parasites. The possible target of this drug, at least in schistosomes, was proposed to be voltage-gated Ca^{2+} channels, which are important regulators of calcium homeostasis in excitable cells (Kohn et al. 2003). PZQ is practically insoluble in water, and partially soluble in ethanol and organic solvents. It has remarkable range of activity against trematodes, cestodes, and is the drug of choice for all *Schistosoma* species (Watson 2009). At the standard doses, no toxicity of PZQ has been recorded and extensive studies did not show mutagenic potential for humans (EMA 1999). It is well tolerated by patients and the major weakness of PZQ is its low efficacy against juvenile schistosomes and larval stages of cestodes (Cioli and Pica-Mattoccia 2003).

Understanding *drug resistance* is important for optimizing and monitoring, control, and reducing further selection for resistance. After decades of mass application of benzimidazole carbamates in the control of gastrointestinal nematodes in livestock, resistant strains have emerged all over the world. Recently, resistance has extended also to ivermectin and presents serious problem for the livestock industries and severely limits current parasite control strategies in humans (Prichard et al. 2012). The ATP-binding cassette (ABC) superfamily of proteins comprises several ATP-dependent efflux pumps involved in transport of toxins and xenobiotics from cells, which is essential for many cellular processes and are associated with development of multidrug resistance. P-glycoprotein (Pgp) and multidrug resistance-associated proteins (MDRP) represent two classes of these ABC transporters. They contribute to resistance to a number of

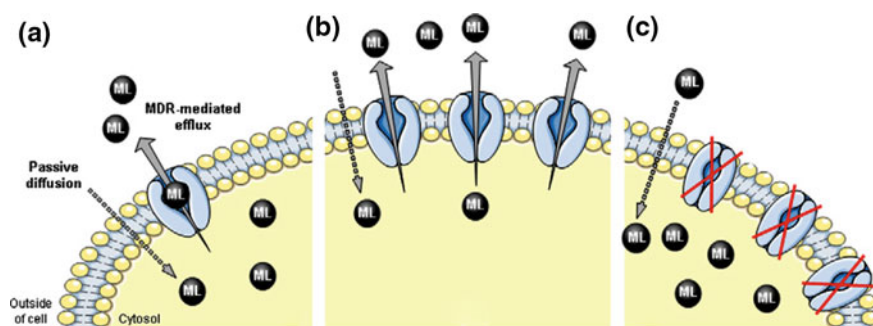


Fig. 2.1 Multidrug transporter-mediated efflux of drug, its contribution to the development of resistance and mechanism of reversion. *a* Constitutive expression of MDR transporters on cell membranes of target organism: basal efflux of drugs (example with macrocyclic lactones). *b* Overexpression of MDR transporters in response to drug pressure: increased efflux of drug and development of resistance. *c* Inhibition of MDR-mediated efflux with MDR reversal agents: enhancement of drug concentration and toxicity into the target cells after: Lespine et al. (2012)

anthelmintics, including macrocyclic lactones including ivermectin and moxidectin (see for review: Lespine et al. 2012) (Fig. 2.1). The high level of expression of MDRP to praziquantel was found in juvenile *Schistosoma mansoni* (Kasinathan et al. 2010). Several anthelmintics are inhibitors of these efflux pumps and appropriate combinations can result in higher treatment efficacy against parasites and reversal of resistance. There is the possibility that molecules with similar inhibitory action on multidrug resistance transporters will be present among a high number of naturally occurring compounds as indicated in a few studies reported in the following paragraphs.

2.2 Natural Compounds from Lower Terrestrial and Marine Organisms in Anthelmintic Drug Discovery

Marine-derived small molecules (MDSMs) from invertebrates comprise an extremely diverse and promising source of compounds from a wide variety of structural classes. They have been derived from marine plants, animals, algae, fungi, and bacteria and in total 106 marine chemicals discovered until 2002 were listed and characterized in the review of Mayer and Hamann (2005). Of these, 56 isolated marine chemicals showed one or more of anthelmintic, antibacterial, anticoagulant, antiprotozoal, antiplatelet, antituberculosis, or antiviral activities. The parasitic diseases caused by helminths and protozoan parasites that could be targets for the discovery of MDSMs were discussed in the review written by Crews and Hunter in 1993. They pointed out that very few antiparasitic drugs currently used for a spectrum of diseases were discovered after 1990 and stressed the great

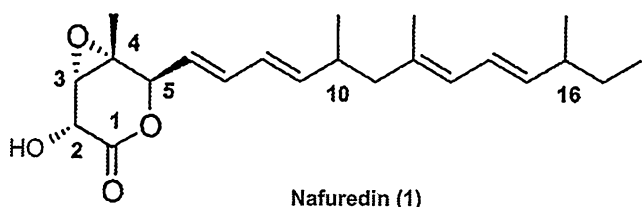


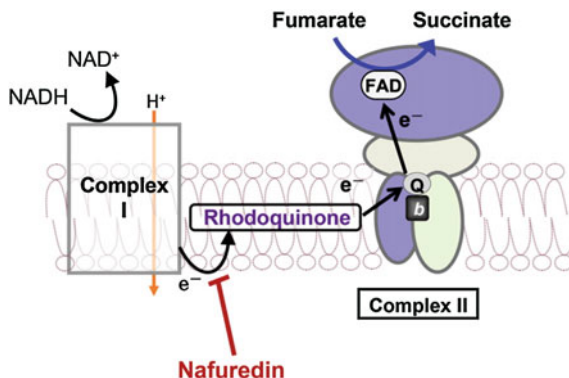
Fig. 2.2 Chemical structure of nafuredin

potential of MDSMs. In the recent review of Watts et al. (2010) six important parasitic diseases which affect the health and lives of over one billion people worldwide were selected and discussed in relation with natural products-based discovery. Included is a brief description of 133 marine-derived compounds displaying LC_{50}/IC_{50} below 30 μM , which were active against one or more selected parasites. The important issue highlighted in this and other reviews (e.g. Kita et al. 2007), that the majority of compounds exerted activity on protozoan species and much less invertebrate-derived molecules, were shown to be active against helminths.

Sponges (phylum: Porifera) are evolutionary ancient metazoans that have existed for 700–800 million years, populating mainly the tropical oceans in great abundance. So far about 15,000 species of sponges have been described, but their true diversity may be higher. Many species of marine sponges are associated with microbes and have been reported to produce pharmacologically active compounds (Thomas et al. 2010). An excellent example of a compound with very high anthelmintic activity which has developed from such association is *nafuredin* (Fig. 2.2).

It is chemically epoxy- δ -lactone with an olefine side chain, and was obtained from the fermentation (culture) broth of a fungal strain *Aspergillus niger* FT-0554 isolated from a marine sponge (Takano et al. 2001). Because helminths have exploited a variety of energy transducing systems in their adaptation to the peculiar habitats in their hosts, differences in energy metabolism between the host and helminths are attractive therapeutic targets for novel classes of anthelmintic compounds. NADH-fumarate reductase (NFRD) is part of a unique respiratory system in parasitic helminths (Fioravanti et al. 1998), representing a terminal electron transport system of anaerobic energy metabolism. Some kinds of parasites use this metabolism to generate ATP instead of classical glycolysis, TCA cycle, and electron transport systems. In the course of the screening of NFRD inhibitors, nafuredin was obtained and was originally tested on the nematode *Haemonchus contortus* and the tapeworm *Hymenolepis nana* in vivo (Omura et al. 2001). Sheep with *H. contortus* infection (5000 L3 larvae) were treated orally with 2 mg/kg of body weight of nafuredin. A greater than 90 % egg reduction was observed at day 11 post therapy and egg output was completely suppressed when the sheep were treated again 1 week after the first treatment. There were no signs of any side effects and no loss of body weight during the tests. The anthelmintic activity may be caused by the hampered energy metabolism of the parasite, because complex I

Fig. 2.3 NADH-fumarate reductase system of *Ascaris suum* as a target of chemotherapy. Nafuredin was found to be competitive inhibitor for rhodoquinone binding site of *A. suum* complex after Sakai et al. (2012)



from *H. contortus* was also sensitive to nafuredin, although the inhibitions were relatively weaker than those for *Ascaris suum*.

It was shown in this study and in the study of Sakai et al. (2012) that nafuredin inhibited NFRD of nematode *A. suum* adults with an IC_{50} value of 12 nM without showing cytotoxicity for mammalian cells. Recent research on the respiratory chain of the parasitic helminth, *A. suum* has shown that the mitochondrial NADH-fumarate reductase system (fumarate respiration), which is composed of complex I (NADH-rhodoquinone reductase), rhodoquinone and complex II (rhodoquinol-fumarate reductase), plays an important role in the anaerobic energy metabolism of adult parasites. They reside in the small intestine of hosts, where oxygen tension is low. Nafuredin competes for the quinone-binding site in complex I and shows high selective toxicity to the helminth enzyme. Furthermore, nafuredin inhibits complex I (NADH-ubiquinone oxidoreductase) in L2 larvae of *A. suum*, which possess aerobic energy metabolism similar as is present in mammals at the low concentration ($IC_{50} = 8.9$ nM) (Fig. 2.3). These data demonstrated that nafuredin is effective against both adult and larval stages. In contrast, the IC_{50} value for rat liver complex I was more than 1,000 times higher than for the *A. suum* complex (Omura et al. 2001). Authors also found that harzianopyridone and the chemically related atpenins inhibit complex II (succinateubiquinone reductase -SQR) in the mitochondria of *A. suum* nematode. Complex II is indispensable for the survival of anaerobic parasitic eukaryotes and, therefore, is also regarded as a good chemotherapeutic target for novel antihelmintics. These inhibitors were isolated from *Trichoderma* sp. FTD-0795, a terrestrial fungus (Miyadera et al. 2003).

Numerous species of marine sponges have been examined for the presence of compounds with antiparasitic activity and in several species nematocidal agents were found and chemically characterized. In a southern Australian sponge of the genus *Echinodictyum* a compound (–)-*echinobetaine A* with potent anthelmintic activity was isolated and synthesized (Capon et al. 2005). The marine natural product onnamide F, isolated from the Australian marine sponge *Trachycladus laevispirulifer*, showed potent inhibition of larval development of *H. contortus* with an in vitro value of 2.6 μ g/ml (Vuong et al. 2001). This compound contains a common structural motif

previously described in a number of natural products exhibiting interesting pharmacological activities. For both compounds, the mechanism of their anthelmintic activity has to be determined and low threshold concentrations indicate that they interfere with the essential physiological process in helminths.

Lymphatic filariasis, caused by nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, is a parasitic disease of high medical importance and prevalence in developing countries in Southeast Asia, and sub-Saharan Africa. Several marine organisms were found to contain structures which possess antifilarial activity against either adult worms or microfilaria, or both stages. The marine sponge *Haliclona oculata* is an important source of steroids, terpenoids, alkaloids, cyclic peptides, and unsaturated fatty acids. Some of these compounds have been reported to possess diverse biological activities. Methanol extract, chloroform fraction, and one of the chromatographic fractions of this sponge revealed IC₅₀ values of 5.00, 1.80, and 1.62 µg/ml, respectively, when adult *B. malayi* were exposed to these test samples for 72 h at 37 °C. Under similar exposure conditions, the IC₅₀ values for microfilariae were 1.88, 1.72, and 1.19 µg/ml, respectively. The samples were found to be safe revealing >10 selectivity indices (SI) on the basis of cytotoxicity to Vero cells (monkey kidney cells). In vivo on experimentally infected gerbils, the highest efficacy (70 %) was achieved with the chromatographic fraction, where the main constituents were determined as alkaloids *mimosamycin*, *xestospongins*-C, *xestospongins*-D, and *araguspongsin*-C (Gupta et al. 2012).

Marine alga, Botryocladia leptopoda (J. Ag.) Klyn. (order Rhodophyceae) is a red alga and this genus includes at least 24 different species which are found in the Mediterranean Sea, around South Africa, the Indian Ocean, Caribbean, around Indonesia, and in the Pacific Ocean. Antifilarial activity of the ethanol extract from this red alga was examined on a subperiodic *B. malayi* nematode, which was maintained in *Mastomys coucha*. The crude extract was active on adult worms in vitro and LC₁₀₀ = 125 µg/ml was determined by a complete loss of motility. After fractionation of crude extract the activity was localized only in the n-hexane fraction and at a dose of 200 mg/kg (p.o) administered to animals for 5 days, it showed about 45 % adulticidal efficacy. Moreover, substantial proportions (71.05 %) of adult female worms were found to be sterilized (Lakshmi et al. 2004a). In other studies, very similar antifilarial effects (LC₁₀₀ = 125 µg/ml) were observed on the same filarial nematode model with chloroform-methanol (1:1) extract from unidentified *green Zoanthus* (Phylum Cnidaria) and the active antifilarial principle was probably present in the most effective chloroform fraction obtained after fractionation (Lakshmi et al. 2004b).

With the recent emphasis of the World Health Organisation (WHO) on the development of novel antifilarial agents from natural products, screening programs involved extracts derived from both terrestrial plants and marine flora/fauna. Most of the observed microfilaricidal efficacy was slow and sustained in contrast to the dramatic microfilaricidal action of the standard drug diethylcarbamazine, indicating that the activity of crude extracts could be due to the combined or synergistic effects of more than one component. Besides, in vitro results may not always give a true picture of in vivo efficacy.

One of the most important milestones during intensive screening programs of compounds from natural sources was isolation of *avermectins* by Japanese scientists dating back to 1973 (Egerton et al. 1979). Eight active components of avermectins were isolated from the broth of lower terrestrial organism originated in a soil sample. The strain was classified as a new species of actinomycetes (bacteria) and named *Streptomyces avermectinius* (formerly *Streptomyces avermitilis*) (Takahashi et al. 2002). Avermectin activates parasite-specific glutamate-gated chloride channel (Omura Omura 2002), that leads to the neurological disruption of the parasite. Although avermectins also bind to γ -aminobutyric acid-gated (GABA) and glycine-gated chloride channels in mammals, their affinity for invertebrate receptors is more than 100 times higher.

Terrestrial *fungal species* from the genera *Trichoderma* and *Rosellinia* were identified as producers of highly specific compounds which can target an essential molecular mechanism in nematodes. For example, a fungus of the genus *Rosellinia* was identified as the source of a very effective compound (PF1022A), which is a cyclic octadepsipeptide with high nematocidal activity (Sasaki et al. 1992). *Emodepside*, a semisynthetic derivative of PF1022A was later developed by Bayer HealthCare (<http://www.bayerhealthcare.com>), Meiji Seika, and Astellas Pharma Inc. (<http://www.astellas.com>). Its target was identified as a novel 110 kDa heptahelical transmembrane receptor, named HC-110R, in nematodes, which is similar to mammalian latrophilins (Saeger et al. 2001). Latrophilins are latrotoxin specific G-protein-coupled receptors in helminths that are implicated in the regulation of exocytosis. Emodepside functions as an antagonist to latrotoxin signaling by impairing the influx of Ca^{2+} . It is highly effective against adult stages of the nematodes *Nippostrongylus brasiliensis* and *Strongyloides ratti* in rats and the nematode *Heligmosomoides polygyrus* in mice when used at an oral-dosage range of 1.0–10 mg/kg (Harder and von Samson-Himmelstjerna 2002).

2.3 Anthelmintic Potential of Higher Plants

Before commercial anthelmintics were introduced into the world market, worm infections were controlled using specific plants that, based more on belief rather than knowledge, were credited with having specific actions. Plants with antiparasitic properties could be found in temperate, tropical, as well as colder climates in the world. In traditional medicine, aqueous or powdered parts of plants were usually used at various dosages/concentrations, which could be the main reason for differences in treatment effects reported from different regions. The concentration of bioactive phytochemicals in the particular plant is also dependent on growing conditions such as climate, soil, and period of collection. Therefore the evaluation of the therapeutical potential of plant extracts must be performed in controlled in vitro and in vivo studies using established analyses and rationally designed experimental schedules.

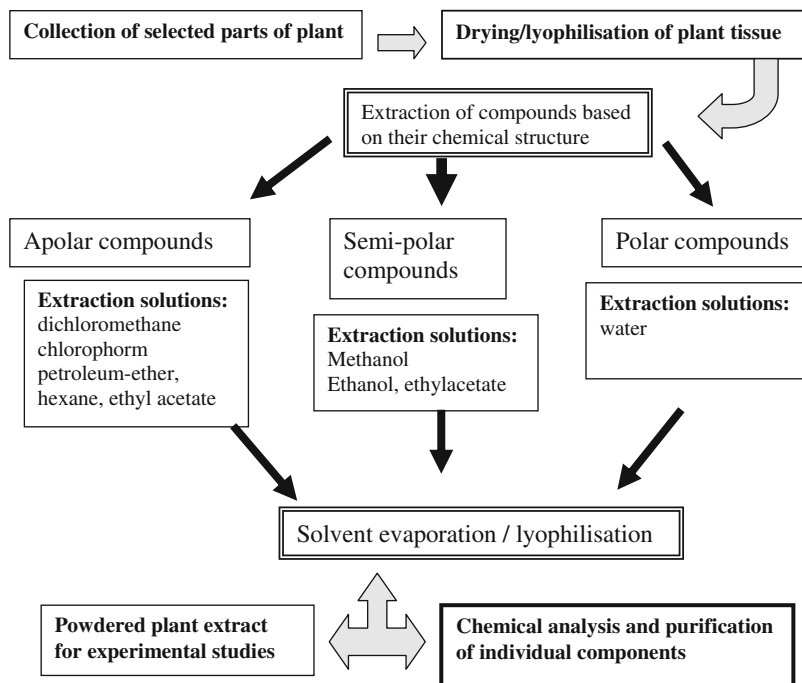


Fig. 2.4 Diagram showing the key steps of preparatory process for obtaining the single group of phytochemical/compound from the higher plants

The great advances in biochemical and analytical methods over the past 10 years allowed the separation of plant phytochemicals and consequently analysis of their chemical nature. The group of interest in this pharmacological screening program is a diverse group of secondary metabolites and usually several different classes are found together in each plant. Their basic chemical structure decides in which extraction medium (polar vs. non-polar) is dissolved in optimal quantity and quality for exerting their full bioactivity. However, many traditionally used plants also contain substances that are found to be extremely toxic. The most common extraction medium is water, however, in most studies higher therapeutical effects were observed for extracts prepared in ethanol/methanol or other organic solvents from the same plants, due to better solubility of secondary metabolites in semi-polar and apolar solutions. The schematic diagram showing the preparation of extracts for experimental testing *in vitro* and *in vivo* is shown in Fig. 2.4 and the powdered form of extract or isolated compounds is preferred. Then organic-solvent extracts are usually redissolved in DMSO, whereas water and alcoholic extracts in sterile water for further applications.

Plant-derived secondary metabolites can be divided, on the basis of their molecular formulas and structural motifs, into several classes and the most abundant are essential oils, flavonoids, alkaloids, saponins, glycosides, tannins,

sesquiterpene lactones, lactones with peroxidic structure, amides, and proteins with enzymatic activity. The first step in discovering a new lead compound with desired pharmacological potential against parasites is traditionally the screening of a number of plant extracts based on their long history of medicinal applications. Countries situated in tropical regions like India, China, and other parts of Asia, South America, and Africa are especially rich in plants with a variety of bioactive molecules.

2.3.1 Plant Extracts

After an intensive search of the literature it was revealed that the majority of studies focussed on helminths living in the gastrointestinal tract of definitive hosts with fewer reports focussing on tissue-dwelling larval stages of helminths infecting humans. More than one billion people are infected with gastrointestinal helminths, and these infections are more common in the tropics where poor hygienic conditions and poverty increase the risk of infection (Brooker et al. 2006; Hotez et al. 2008). Infections of animals with gastrointestinal nematodes (GIN) are highly prevalent in both temperate and tropical areas and represent a major threat for livestock production. Nevertheless, the largest number of plant extracts has been examined for their effect to significantly reduce or remove gastrointestinal nematodes of livestock (for example see reviews: Athanasiadou and Kyriazakis 2004; Hoste et al. 2006, 2012; Hoste and Torres-Acosta, 2011; Githiori et al. 2006; de Gives et al. 2012). Extracts from a wide range of higher plants or trees have been examined so far and we summarized the data about extracts with the significant anthelmintic activity and available composition of phytochemicals. In some of these reports information about the toxicity for the hosts were available.

2.3.1.1 Plants with Activity on Cestodes and Trematodes

In the screening of compounds, which would be effective against flatworms with medical and veterinary importance, the surface structures of the tegument of cestodes and trematodes represent the potential target sites as the small molecules can be absorbed in the tegument. In experimental studies, the model infections where the life cycle can be maintained in rodents and invertebrate intermediate hosts are preferred for evaluation of higher number of plant extracts. Cestode *Hymenolepis diminuta* is the parasite of rats where adults live in the intestine and release gravid segments containing eggs. In the invertebrate hosts (beetle) cysticercoids develop from eggs and after their ingestion by rats, the immature stages and adults develop. This cestode model is widely used for many research purposes, including evaluation of cestocidal effects of plant extracts.

India is a country rich in plants, which have a long history of traditional medicinal use, including expulsion of intestinal cestodes. The extracts from leaves

or plants of *Adhatoda vasica*, *Trifolium repens*, *Solanum myriacanthum*, and *Acacia* spp. were considered for a long time to contain substances with anticestodal activity. For in vivo effects, the production of eggs per gram of feces (EPG) by adult worms and a number of surviving immature and adult stages are common and suitable criteria. In the study of Yadav and Tangpu (2008) the efficacy of methanolic leaf extracts of *Adhatoda vasica* was evaluated using immature (larval) and mature stages of *H. diminuta*-albino rat experimental model. The extracts at two daily doses of 800 mg/kg reduced the EPG counts by 79.6 %, adult worm recovery was 16 %, and recovery of juvenile stages was 20 %. Effect was dose-dependent at the dose from 100 mg up to 3,200 mg/kg per os (p.o.) at which no mortality or any adverse signs with regard to body temperature or food uptake up to 72 h post therapy (p.t.) were observed. It was suggested that the anticestodal activity may be attributed to the two major constituents, the *alkaloids*: *vasicine* and *vasicinone*. In the work of Tangpu et al. (2004) the methanolic extract of *Trifolium repens* at a dose of 500 mg/kg reduced the mean number of excreted eggs per gram of feces (EPG) by 54.9 % and worm recovery by 40 %. In this study PZQ in the recommended dose of 5 mg/kg was only slightly more effective with an EPG reduction of 65.9 %. A reduction in EPG counts implies that phytochemicals present in extracts contributed to a higher elimination of adult worms from the intestine or to inhibition of egg production. *Solanum myriacanthum* Dunal is a perennial shrub that is used in Indian folk medicine. An oral dose of 800 mg/kg of extract, given for 3 days showed 60.49 % reduction in the EPG counts and 56.60 % reduction in the worm counts in the extract-treated group as compared to untreated controls (Yadav and Tangpu 2012). The effects of the extract were more apparent on the adult stages than on larval or immature stages of the parasite. It was assumed that the anthelmintic efficacy of plant extract may be due to the presence of secondary metabolites, particularly the *alkaloids*. Solanaceae is known for possessing a diverse range of alkaloids, like solasodine, solakhasanin, solamargine, and khasinin (Weissenberg 2001). Recently, Kamaraj and Rahuman (2011) studied the in vitro larvicidal and ovicidal activity of leaf and seed extracts of yet another *Solanum* species, i.e., *Solanum torvum* on nematode *H. contortus*. At the maximum concentration tested (50 mg/kg), a 100 % inhibition of egg hatching and larval development was recorded for an ethyl acetate extract of the plant. The extract also showed its antiparasitic effects on some hematophagous parasites of cattle and goat and also against a digenean fluke of sheep, namely *Paramphistomum cervi*. Many previous studies have assigned the antiparasitic effects of medicinal plants to these alkaloids (Athanasiadou and Kyriazakis 2004).

The anticestode activity on *H. diminuta* in the albino rat model had methanol leaf extract of *Strobilanthes discolor* (Acanthaceae). Tangpu and Yadav (2006) reported that a bioactive substance present in this plant at the dose of 800 mg/kg administered twice daily for 3 days resulted in the complete elimination of immature stages in treated rats. The doses up to 2000 mg/kg, given p.o. showed no mortality or any adverse signs in the animals, but so far the active plant component has not been determined. In the same experimental model and treatment design, the similar high activity against immature stages was obtained with methanol leaf

extracts from *Zanthoxylum rhetsa* DC (Yadav and Tangpu 2009). Treatments with extracts from *Z. rhetsa* resulted in 86.6 % reduction of immature stages and EPG counts dropped to zero. This anticestodal property could be attributed to plant components *terpenoids* (xantyletin, sesamin), *alkaloids*, *flavonoids*, and *essential oil* (sabinene), which have been described as the key constituents of this plant.

Anticestode effects were demonstrated also for ethanolic extract of stem barks from *Acacia oxyphylla* on the fowl gastrointestinal cestode *Raillietina echinobothrida* in vitro (Roy et al. 2007) and in vivo using extracts from *Acacia auriculiformis* against *H. diminuta* (Ghosh et al. 1996). Extracts decreased the motility of worms, induced distinct tissue damage in the subtegumental and somatic muscle layers, and mortality in a dose-dependent manner at concentrations between 0.5 and 20 mg/ml in vitro, indicating that condensed *tannins* as well as *saponins*, components found in herb extracts, can interact with the molecules in the tegument of cestodes. The cestode treated in vivo with 20 mg/kg showed irreversible destruction throughout the general topography of body, disorganization of the tegumental morphology, and deformation of microtriches (Table 2.4).

In recent studies, a very promising anthelmintic effect in vitro exerted extracts from plants found in tropical climates: *Lysimachia ramosa*, *Olea europaea*, and *Satureja khuzestanica* (Challam et al. 2010; Zibaei et al. 2012). The adult trematode, *Fasciolopsis buski*, nematode, *Ascaris suum* and a cestode, *Raillietina echinobothrida* were exposed to concentrations of 5–50 mg/ml of an alcoholic extract of *Lysimachia ramosa* Wall. Treated parasites revealed complete inactivation and loss of motility/flaccid paralysis that was followed by death at varying periods of time and deformity to the surface architecture of the worms was observed. The pharmacologically active components responsible for these effects were triterpenoids, *saponins*, *organic acids*, and *flavones*, which were recorded from different species of the genus *Lysimachia*. The protoscolicidal activity on *Echinococcus granulosus* in vitro was demonstrated for aqueous extracts of *Olea europaea* and ethanolic extracts from leaves of *Satureja khuzestanica*, which showed higher protoscolicidal activity than the aqueous extracts from *O. europaea* leaves. Loss of viability of protoscoleces was associated with a profound and characteristic morphological alteration to the surface of larvae.

Several studies showed that also extracts from plants grown to serve as human food for example: *coconut*, *onion*, *garlic*, *fig*, *date*, *annanas*, *chicory* have high anthelmintic potential against intestinal nematodes, cestodes, and trematodes. Active compounds were extracted into either chloroform, water, or polyethylene glycol/propylene carbonate (PEG/PC) and were examined on the cestode models *Hymenolepis diminuta*, *Hymenolepis microstoma*, *Taenia taeniiformis*, and the trematode models *Fasciola hepatica* and *Echinostoma caproni* (Abdel-Ghaffar et al. 2011). Of all extracts tested, it was found that single extract had a very low anthelmintic effect in vivo; however, treatment of infected animals with a combination of onion and coconut extracts in PEG/PC eliminated all cestodes. The same composition of extracts was effective against *E. caproni* but failed to kill the liver fluke *F. hepatica* in the final hosts. In contrast, total or partial failure of onion oil extracts and coconut extracts given alone to kill

Table 2.4 Anthelmintic effects of plant extracts on intestinal cestode *Hymenolepis diminuta* and/or *Hymenolepis microstoma*, resp. in albino rat model

Name of plant (extraction medium)	The most effective concentration/dose of extract in selected in vitro test or in vivo studies	Main plant secondary metabolites	References
<i>Acacia auriculiformis</i> (ethanol)	300 mg/kg, one dose = 100 % reduction of AWN	Triterpinoid saponins: Acaciaside-A Acaciaside-B	Ghosh et al. (1996),
Onion bulbs (ethanol) + coconut powder	500 mg/kg, 8 daily doses (4 g/kg) + 500 mg/kg, 8 daily doses (4 g/kg) = 100 % reduction AWC	Allicin, Essential oils	Abdel-Ghafar et al. (2011)
<i>Trifolium repens</i> (methanol)	200 mg/kg, 5 doses (total 1 g/kg): FECR = 47 % 500 mg/kg, 5 doses (2.5 g/kg): FECR = 65.9 % 100 mg/kg, 3 doses (total 0.3 g): FECR = 23.3 %, reduction AWN = 40 %	Not determined	Tangpu et al. (2004)
<i>Zanthoxylum rhetsa</i> (methanol)	800 mg/kg, 3 doses (2.4 g/kg): FECR = 100 %, reduction AWN = 86.6 %	Terpenoids, alkaloids, flavonoids	Yadav and Tangpu (2009)
<i>Solanum myriacanthum</i> (methanol)	800 mg/kg, 3 doses (total 2.4 g/kg): FECR = 60.4 %, reduction AWN = 56.6 % LD ₅₀ = 3.0 mg/kg in vitro	Alkaloids: solasodine, solakhasanin, solamargine, khasinin	Yadav and Tangpu (2012)
<i>Strobilanthes discolor</i> (methanol)	800 mg/kg, 3 doses (total 2.4 g/kg): FECR = 93.7 % reduction AWN = 90 %	Not determined	Tangpu and Yadav (2006)
<i>Adhoda vasica</i> (methanol)	800 mg/kg, 2 doses (total 1.6 g/kg): FECR = 79.5 % reduction AWN = 83.4 %	Alkaloids: vasicine, vasicinone, glycosides	Yadav and Tangpu (2008)

Legend

EPG eggs per gram

FECR fecal egg count reduction

AWN adult worm number

nematodes were reported by Abu-El-Ezz (2005) and Oliveira et al. (2009), indicating that anthelmintic effect of onion and coconut rely on the synergistic action of selected phytochemicals, which are able to extract with PEG/PC system and that several daily doses are necessary.

2.3.1.2 Nematocidal Activity of Plants

Parasitic nematodes represent a serious threat to humans, animals, and plants. Gastrointestinal nematode infections of livestock, which are bred for the production of meat, milk, or wool all around the world, lead to enormous economic losses. The control of these parasites has relied on the use of chemical anthelmintics, resulting in development of drug-resistant strains. Alternative control methods are biological control, vaccination, and traditional medicinal plants, which are the focus of examination over the world. The evidence of anthelmintic properties of plants is gained primarily from ethnoveterinary and ethnomedical knowledge. Novel approaches to use the plants for control of gastrointestinal nematodes in small ruminants are outlined in the excellent reviews of Githiori et al. (2006) and Hoste and Torres-Acosta (2011). In parallel with exploring non-chemical methods of control to reduce the infective larvae in the field, the search for novel pharmacologically active compounds in plant extracts is necessary for developing future anthelmintics. Obviously, *in vitro* assays are applied to pre-screen the activity of plant extracts and isolated components on free-living and consequently on parasitic stages of nematodes. Data collected by numerous authors to date indicate that concentrations of potentially active substances used *in vitro* do not always correspond to *in vivo* bioavailability. Therefore, *in vitro* assays should always be followed by *in vivo* controlled studies. Recently, the potential of several plant extracts with antiparasitic activities with emphasis on gastrointestinal nematodes of livestock was reviewed by de Gives et al. (2012) pointing to some other aspects. It should be taken into consideration that the different parts of higher plants (leaves, stems, roots, flowers, fruits) may contain different concentrations of the bioactive compounds that have to be determined early in the screening process. Moreover, the possible side effects on hosts should be established *in vitro* using cell lines or other sensitive tests to determine LC₅₀ (lethal concentration at which 50 % of cells is killed), before carrying out *in vivo* assays.

Validation of antiparasitic activities of compounds obtained from plant extracts requires standardization of methodologies and this issue is discussed in the review of Athanasiadou et al. (2007). The authors focussed on the strengths and weaknesses of the existing methodologies used in the controlled studies and also discussed issues like the seasonal variability of the plant composition and how this can affect their antiparasitic properties. In line with their report we want to highlight the importance of identification of mechanism of action of isolated phytochemicals, which would target the unique molecular and physiological pathways of parasites.

2.3.1.3 Plants with Activity Against Gastrointestinal Nematodes

Tannin-rich plants have attracted high attention for their effect on internal nematodes in ruminants and this topic was discussed in detail in the review of Athanasiadou et al. (2001); Hoste et al. (2006, 2012); Diaz et al. (2010); Min and Hart (2003) summarized the present knowledge on antiparasitic effects of tannin-rich plants from in vivo and in vitro studies. In the majority of reports the effect was usually examined in sheep and goats, which were fed with freshly harvested forage legumes, including sulla (*Hedysarum coronarium*), sainfoin (*Onobrychis vicifolia*), trefoils (*Lotus corniculatus* and *L. pedunculatus*) and other legumes *Sericea lespedeza*, *Lespedeza cuneata* (for example: Bernes et al. 2000). Some studies have also tested the properties of chicory (*Chicorium intybus*) (Fig. 2.5) and are listed in

Hedysarum coronarium (sulla)



Lotus corniculatus (trefoils)



Onobrychis vicifolia (sainfoin)



Chicorium intybus (chicory)



Fig. 2.5 The plants rich in condensed tannins used frequently as livestock forage

these reviews. Consumption of such bioactive plants had the beneficial effects on the host physiology and the ability to maintain homeostasis under parasitic challenge. However, the content of condensed tannins should be controlled and limited (see another part in this chapter). Such a diet has also been associated with nematodicidal effects and the most commonly reported effect was a substantial decrease in FEC (fecal egg counts), which was frequently related to significant effects on female worm fecundity. When using the whole plant extracts, these effects on nematodes can be associated with the presence of one or more plant secondary metabolites with a large range of biochemical characteristics, of which condensed tannins (CT) and sesquiterpene lactones (SL) are the most widespread in these forage legumes (Max et al. 2005). The direct role of condensed tannins on significant reduction of worm burden, hatching of eggs, and FEC in ruminants with GIN in vivo was shown after wattle tannin drenches to animals (Max 2010). Most in vitro studies reported the interference with hatching of eggs and inhibition of larval mobility for L1 and L3 stages, which can contribute to a gradual decrease in pasture contamination with infective stages. The other effect seen following incubation with tannin-rich extracts, for example, from tropical legume plant *Arachis pinto* and *Newbouldia laevis*, was a significant inhibition of the exsheathment process of *H. contortus* at concentration of 1.2 and 0.6 mg/ml, respectively (von Son-de-Fernex et al. 2012; Azando et al. 2011). Feeding with chicory, which has a specific chemical composition of CT and SL resulted in the most effective “green” therapy against GIN (Tzamaloukas et al. 2005 and the above-mentioned reviews).

Several in vitro tests have been developed and proposed for the screening of drug resistant strains of nematodes (Várady et al. 2009), which target either the larval stages (larval development assay-LDA, larval migration inhibition assay-LMIA, larval escheatment inhibition assay-LEIA), the eggs (egg hatch test-EHT), or the adult stage (the adult motility inhibition assay-AMIA). Some of these bioassays are employed in screening of nematodicidal effects of plants with a record in ethnoveterinary medicine.

Haemonchus contortus is a blood-feeding abomasal nematode parasite of small ruminants and is highly pathogenic capable of causing acute disease and high mortality. The disease is characterized by hemorrhagic anemia. Resistance of this nematode to commercial anthelmintics, mainly in the tropical regions, initiated intensive research on the search of novel anthelmintic lead compounds. The effects of several plant extracts on *H. contortus* and other important gastrointestinal nematodes using EHT and LDA tests have been examined by many authors, for example: Gabino et al. (2010), Aroche et al. (2008) and others. Relevant information is summarized in Table 2.5. The anti-nematode activity was confirmed in the plant extracts from *Adhatoda vasica* (L), *Annona squamosa*, *Cocos nucifera*, *Coriandrum sativum*, *Eucalyptus staigeriana*, *Hedera helix*, *Melia azedarach*, *Mentha piperita*, *Lippia sidoides*, *Piper tuberculatum*, *Phytolacca icosandra*, *Prosopis laevigata*, *Spigelia anthelmia*, *Spigella torvum*. Individual extracts, isolated mostly with alcoholic and other organic solvents (n-hexane, acetone, PEG) showed the dose-dependent inhibitory effects in both tests. The highest

Table 2.5 Nematocidal effects of plant extracts on several developmental stages of *Haemonchus contortus* examined in vitro and in vivo

Name of plant	Type of study	The most effective concentration/ dose of extract in selected in vitro test or in vivo studies	Plant secondary metabolites	References
<i>Piper tuberculatum</i>	In vitro	LD ₅₀ = 0.031 mg/ml in EHT LD ₅₀ = 0.02 mg/ml in LDA	Piplatrines, piperine, essential oils,	Carvalho et al. (2012)
<i>Lippia sidoides</i>	In vitro	LD ₅₀ = 0.04 mg/ml in EHT LD ₅₀ = 0.02 mg/ml in LDA	Essential oils (76 % thymol)	
<i>Mentha piperita</i>	In vitro	LD ₅₀ = 0.037 mg/ml in EHT LD ₅₀ = 0.018 mg/ml in LDA	Essential oils (menthol 27 %)	
<i>Coriandrum sativum</i> seeds	In vitro/in vivo (sheep)	LD ₅₀ = 0.12 mg/ml and 100 % inhibition in EHT at 0.5 mg/ml In vivo: (0.9 g/kg 1x): significant FECR reduction and 25.5 % reduction in worm counts.	Quercetin, 3-glucuronide, linalool, camphor, geraniol, coumarins	Eguale et al. (2007a)
<i>Eucalyptus staigeriana</i>	In vitro	1.35 mg/ml: 99 % inhibition in EHT 5.4 mg/ml: 99 % inhibition in LDA	Essential oils	Macedo et al. (2010)
<i>Melia azedarach</i>	In vitro	12.5 mg/ml: 99 % inhibition in EHT	Not known	Kamaraj et al. (2010a)
<i>Hedera helix</i>	In vitro/in vivo (sheep)	50 mg/ml: 91 % inhibition in LDA LD ₅₀ = 0.17 mg/ml in EHT In vivo: 2.2 g/kg-1x p.o. = 47.5 % reduction in FEC	Triterpenoid saponins, alkaloids, flavonoids	Eguale et al. (2007b)
<i>Cocos nucifera</i>	In vitro	5 mg/ml: 100 % inhibition in EHT 80 mg/ml: 99 % inhibition in LDA	Essential oils	Oliveira et al. (2009)

(continued)

Table 2.5 (continued)

Name of plant	Type of study	The most effective concentration/ dose of extract in selected in vitro test or in vivo studies	Plant secondary metabolites	References
<i>Prosopis laevigata</i>	In vitro/in vivo (gerbils)	20 mg/ml: 86 % larval mortality In vivo: 40 mg/kg 1x i.p. = 42.5 % reduction of larval numbers	Not determined	Gabino et al. (2010), Aroche et al. (2008)
<i>Annona squamosa</i> , <i>Solanum torvum</i> , <i>Terminalia chebula</i>	In vitro	50 mg/ml: 100 % inhibition in EHT 50 mg/ml: 100 % inhibition in LDA	Acetogenins Anthraquinones, glucopyranose, flavonoids (ellagic acid, tannic acid, gallic)	Kamaraj and Rahuman (2011)
<i>Phytolacca icosandra</i>	In vitro/ In vivo (goats)	0.15 mg/ml: 76 % inhibition in EHT 3 mg/ml: 67 % inhibition in LIM LD ₅₀ = 0.28 mg/ml in EHT 0.9 mg/ml: 90 % inhibition in EHT 250 mg/kg 2x p.o. = 72 % reduction in FEC on day 11 p.t.	Flavonoids, steroids, terpenoids, Saponins, coumarins,	Hernández-Villegas et al. (2011), (2012)
<i>Arachis pintoi</i>	In vitro	1.2 mg/ml: 100 % inhibition of larval exsheathment in LEIA	Tannins, polyphenols	Von Son-de Femex et al. (2012)
<i>Gratylia argentea</i>	In vitro	1.2 mg/ml: 66 % inhibition of larval migration in LMIA	Tannins, polyphenols	
<i>Artemisia annua</i>	In vitro/in vivo (sheep)	25 mg/ml: 99 % inhibition of LMIA 3 g/kg 1x p.o. = 67 % reduction in FEC		Tariq et al. (2009), Iqbal et al. (2004)

activity at lowest concentration of extract seems to be a good criterion for selection of these plants for further in vivo studies and characterization of bioactive compounds. The low LC_{50} resp. LD_{50} (lethal concentration/dose) values in both EHT and LDA tests between 0.02 and 0.040 mg/ml were found for *Lippia sidoides*, *Piper tuberculatum*, and *Mentha piperita* extracts, which were rich in essential oils. These components were probably responsible for nematodicidal activity, mainly the abundant components of oils, for example thymol or menthol (Carvalho et al. 2012). The complete inhibition of EHT was observed at concentrations less than 0.5 mg/ml of crude aqueous and hyquercentindro-alcoholic extracts of the seeds of *Coriandrum sativum* (ED_{50} of the aqueous extract was 0.12 mg/ml). The main components of *C. sativum* were determined as flavonoids and essential oils (camphor, geraniol, coumarins) (Egualde et al. 2007a). However, significant decrease in FEC in artificially infected sheep was observed only for a dose of 0.9 g/kg given at week 4 post infections with L3 stage of larvae, reaching the efficacy of only 25 %. Both aqueous and hydroethanolic (50 % ethanol solution in water) extracts from *Melia azedarach* inhibited nearly completely the egg hatching and larval development at the dose of 12.5 mg/ml, indicating the presence of bioactive compounds with the ovicidal and larvicidal effects (Kamaraj et al. 2010a, b). Hydroalcoholic extracts from ripe fruits of *Hedera helix* significantly inhibited egg hatching of this nematode and ED_{50} in this test was 0.12 mg/ml and fecal egg count reduction in infected sheep treated with dose of 2.25 g/kg was 47.5 % (Egualde et al. 2007b). Similar anthelmintic activity was reported in aqueous methanolic extract from *Caesalpinia crista* on *H. contortus* (Jabbar et al. 2007). LD_{50} was achieved at the concentration of 0.134 mg/ml in EHT and 3.0 g/kg of extract given in two doses to sheep caused 93.9 % reduction in EGP. These deleterious effects of plant components were probably due to the result of blocking some important physiological processes in helminths.

Anthelmintic activity (AH) had also ethanolic extract from leaves of *Phytolacca icosandra* against *H. contortus* in EHT and LDA in vitro and in vivo in infected goats. The dose of 250 mg/kg of extract given on two consecutive days caused the highest reduction of worms (72 %) on day 11 p.t. No adverse effects were observed in all animals for the entire trial and standard methods revealed presence of saponins, coumarins, flavonoids, steroids, and terpenes as well as the presence of three fatty acids having the highest abundance (Hernandez-Villegas et al. 2011, 2012).

Many other studies have focussed on plant extracts and their activity on other gastrointestinal nematodes (GIN) of ruminants including *Teladorsagia circumcincta*, *Trichostrongylus axei* (all living in the abomasum), *Trichostrongylus colubriformis*, and *Nematodirus battus*, which reside in small intestine, *Trichuris ovis*, with localization in the large intestine and other GIN (*Ostertagia circumcincta*, *Strongyloides papillosus*, *Chabertia ovina*). Our search of the literature revealed that the different plants native to the country, where studies were conducted, varied in anti-nematode activity in vitro and in controlled studies in vivo. Many plant extracts given in the range of grams per kilogram of body weight of hosts failed to reach the efficacy of commercially used anthelmintic drugs, which could be attributed to the specific digestive system of ruminants.

The anti-nematode activity against GIN of sheep in vitro and/or in vivo was found in extracts of *Anacardium humile* containing mainly tannins, flavonoids, and alkaloids with $LD_{50} = 10.14$ mg/ml for the aqueous extract (Nery et al. 2010), and in the aqueous and methanolic extract from aerial parts of *Adhatoda vasica* (L), which are rich in alkaloid vasicine, saponins, and glycosides. Different nematodes revealed different susceptibility to the same concentration of extracts in vitro and the highest effectiveness was seen at a concentration of 50 mg/ml, with inhibition between 80 and 88 % in EHT and LDA tests (Al-Shaibani et al. 2008). A significant anthelmintic effect on GIN was also demonstrated in the extracts of *Fumaria parviflora* (Papaveraceae) (Hördegen et al. 2003), in *Cichorium intybus* containing mostly tannins (Tzamaloukas et al. 2006), in *Melia azerdach* and *Trichilia clausenii*, both native to Asia, in which the main components were tannins, phenolic compounds, and steroids (Cala et al. 2012). *Agave sisalama*, sisal, is used as the source of fibers (4 % of plant) with many commercial applications. It has also a large amount of saponins composed of steroidal or triterpenoid glycosides. Silveira et al. (2012) confirmed very high effects (90–98 %) of low dose (0.12 mg/ml) of this extract in EHT and LDA tests against several GIN in vitro. A high dose of 50 mg/kg was required to reach similar anti-nematode activity in vitro with extracts from *Salvadora persica* and *Taminalia avicennoides* (Reuben et al. 2011) and chemical analysis of the aqueous extracts revealed the presence of tannins, flavonoids, saponins, sterols, terpens, and reducing sugars.

Acacia species are perennial climbing shrubs native to many Asian and African countries rich in condensed tannins. Lambs with natural GIN infection grazing *Acacia* for 4 weeks showed significantly lower mean fecal egg counts (FEC) (Akkari et al. 2008; Kahiya et al. 2003) and the anthelmintic effect of tannins against nematodes remains equivocal. The similar mode of action of tannins on nematodes as was reported for synthetic phenolic anthelmintics (oxyclozanide, niclosamide, nitroxynil) is possible, as these drugs interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation, consequently leading to depletion of parasite ATP (Martin 1997). Another report suggested tannin's ability to bind to glycoproteins on the cuticle of parasites (Hoste et al. 2006).

Artemisia species are the source of artemisinin, which was recognized as a new lead compound with high anti-protozoan effect. It was partially effective also against *Schistosoma* spp. and *Fasciola* spp. (Keiser and Utzinger 2007, see the paragraph on Sect. 2.3.8). Several studies examined the anthelmintic potential of the extracts from *Artemisia* spp. on nematodes with various levels of success. Significant inhibition (nearly 100 %) of *H. contortus* larval motility in vitro was found at the concentration of 25 mg/ml after several hours of exposure and worm paralysis and/or mortality was seen at 6 h post exposure. In sheep with this infection, and treated with a single dose of up to 3 g/kg of extract, the reduction of infection was only 67 % (Tariq et al. 2009; Iqbal et al. 2004).

In the study of Mehlhorn et al. (2011a), especially prepared extracts from coconut dried endosperm and onion bulbs were examined in vivo in sheep infected with various gastrointestinal nematodes and/or *Moniezia expansa* (Cestoda). A combination of onion and coconut extracts each containing 60 g of dried mass was

given to sheep for 8 days combined with PEG/PC. In all cases, the worms disappeared from the feces indicating the 100 % de-worming effect. PEG alone probably improved the absorption of individual substances in garlic and coconut powders and when given alone, it had no effect on worm burden. Administration of onion powder alone showed low efficacy against worms (Klimpel et al. 2011; Abdel-Ghaffar et al. 2011). *Allium sativum* (garlic) extract exerted anthelmintic activity against *H. contortus* in vitro (Iqbal et al. 2001), but pure allicin (the leading substance in onion and garlic) remained ineffective against the worms. These studies demonstrated the synergistic effect of garlic and coconut given as powder to animals and was reviewed by Mehlhorn et al. (2011b).

High-throughput screening of chemical substances for their anthelmintic activity requires a well-established model. The use of parasitic nematodes as a screening system in vitro is hindered by the difficulty of maintaining them in vitro for prolonged periods of time and the need to have infected animals as sources of eggs or adults. In this respect, the free-living soil nematode *Caenorhabditis elegans* was proposed as a system to test products for potential anthelmintic effect against small ruminant gastrointestinal nematodes, including *H. contortus* (Katiki et al. 2011a, b). *Leucosidea sericea* is a plant native to southern Africa and was used to expel parasitic intestinal worms (vermifuge) and as an astringent in combination with other plants. The study of Aremu et al. (2010) aimed to examine whether extracts from this plant could exert both anthelmintic and anti-inflammatory activity. Cyclooxygenase enzymes (COX-1 and -2) were used to determine the anti-inflammatory potential of the plant extracts as their production is increased in acute and chronic inflammatory conditions associated with many diseases. An in vitro colorimetric assay for the determination of free living nematode larvae viability enabled the recording of the minimum lethal concentration (MLC) values of the extracts against *C. elegans* var. Bristol (N2). At the highest screening concentration (250 µg/ml), the PE (petroleum extract) of the leaves exhibited the highest COX-1 and COX-2 inhibition of 97 and 91 %, respectively. Alkaloids and saponins were only detected in the leaf and stem extracts, respectively, of *L. sericea*. MLC values for all the organic solvent extracts of the leaves between 0.26 and 0.56 mg/ml corresponded with high anthelmintic activity.

2.3.1.4 Plant extracts with activity on nematode species of hosts with monogastric digestive systems including *Heligmosomoides bakeri*, *Trichinella spiralis*, *Brugia malayi*, *Toxocara cati*, *A. suum*.

Trichinellosis is a worldwide zoonotic infection caused by nematodes of the genus *Trichinella* and in nature is widespread in wild carnivorous animals (Murrell and Pozio 2011). Pigs and also humans can be infected after the ingestion of meat containing live larvae and therapy for patients is not always successful (Pozio et al. 2001) with anthelmintics, due to low bioavailability for larvae localized in the muscles. Methanol extracts from *Artemisia absinthium* and *A. vulgaris* given to

rats at the dose of 300 mg/kg during enteral (adult) phase reached the rate of larval reduction in various muscles between 37 and 75 %. During the parenteral (encapsulated larvae) phase, the higher dose of 600 mg/kg decreased the larval rate between 43 and 66 % (Caner et al. 2008). These data suggest that concentration of artemisinin in extracts was not sufficient for complete worm elimination and longer administration could be the choice. The synergistic effect of other plant components like flavonoids is also possible.

Heligmosomoides bakeri infection in rodents is a suitable experimental model for human gastrointestinal nematodes. Extracts from leaves of *Ageratum conyzoides* (Asteraceae), used in ethnomedicine in Africa, showed 53.3 % inhibition of the embryonation process in eggs at concentration of 3.7 mg/ml and LC₅₀ was 1.5 mg/ml for L2 stage larvae (Poné et al. 2011). Lymphatic filariasis is a major health problem in the local population in tropical and subtropical countries and the disease is caused by the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus*. Treatment relies on two drugs, diethylcarbamazine and ivermectin, but they have poor activity on adult parasites. Several plant extracts have been examined for antifilarial activity in vitro and in laboratory animals. The crude extract of *Caesalpinia bonducella* showed very high effects against all stages of nematode *B. malayi* (Gaur et al. 2008) extract from stem portion of plant *Lantana camara* administered to rats at the dose of 1 g/kg for 5 days killed 43 % of adults and sterilized 76 % of the surviving female worms. Two isolated compounds, *oleanonic acid* and *oleanolic acids* might be responsible for this effect (Misra et al. 2007). All *B. malayi* worms were killed in vitro with extract at concentration of 0.031 mg/ml. Antifilarial activity on microfilaria of *B. malayi* in vitro was identified in methanolic extracts of roots of *Vitex negundo* L. and leaves of *Aegle marmelos* Corr by Sahare et al. (2008). Chromatographic analysis revealed the presence of *alkaloids*, *saponin*, and *flavonoids* in *Vitex negundo* and *coumarins* as the main component in plant *A. marmelos*, and both extracts at concentration of 0.1 µg/ml showed complete loss of motility of microfilaria after 48 h, indicating the inhibition of the essential physiological process in larvae. Transcuticular/tegumental diffusion is a common means of entry for non-nutrient and non-electrolyte substances into helminth parasites. It was shown that this route is predominant for the uptake of major groups of anthelmintics like benzimidazole, levamisole, and ivermectin, and also some of bioactive phytochemicals can enter tegument/cuticle in this way. Lipophilic anthelmintics have a greater capability to cross the external surface of helminths than hydrophilic compounds (Geary et al. 1999).

2.3.2 Alkaloids

Alkaloids represent a highly diverse group of compounds that are related only by the occurrence of a nitrogen atom in a heterocyclic ring. The nitrogen in the alkaloid molecule is derived from amino acid metabolism. Since the amino acid skeleton is often largely retained in the alkaloid structure, alkaloids originating

from the same amino acid show similar structural features and can be classified according to their biosynthetic origin. Plants are estimated to produce approximately 12,000 different alkaloids, which can be organized into groups according to their carbon skeletal structures (Ziegler and Facchini 2008).

Alkaloids often have pronounced bioactivities and are therefore thought to play an important role in the interaction of plants with their environment. Alkaloids and extracts of alkaloid-containing plants have been used throughout human history as remedies, poisons, and psychoactive drugs. Several alkaloids are used medicinally or provide lead structures for novel synthetic drugs (Fester 2010).

A great number of alkaloids originating from marine organisms showed a potent activity on parasitic protozoa and on several helminth species in vitro and some specifically inhibit essential enzymatic systems in parasites (see for review Watts et al. 2010 and Chap 1 in this book). However there are few studies, in which alkaloids isolated from higher plants, were examined as anthelmintic agents.

Many known anthelmintics are effective against nematodes living in gastrointestinal system, but limited efficacy has been reported against larvae which migrate to human tissues where they may cause a severe pathology. This is the case for *Toxocara canis* and *Toxocara cati* larvae, both zoonotic nematode infections. Migration of larvae is associated with many non-specific pathological syndromes, eosinophilia, and an allergic type of immune response. The seroprevalence of toxocariasis in the healthy human population was estimated to be 5–10 % and the disease ranks among the most frequent parasitic diseases in temperate climates. The search for plant phytochemicals effective against second stage larvae of *T.canis* were the focus of studies of several research groups. Satou et al. (2002a) developed a specific test for the evaluation of nematocidal activity in vitro and introduced the concept of Relative Mobility (RM) of larvae. This shows the concentration of a compound at which RM equals 50 % of the control, the RM₅₀, value. Using this test, a set of *isoquinoline alkaloids* isolated from the plants *Macleaya cordata*, *Chelidonium majus*, and *Corydalis turtschaninovani*, grown in Japan for various purposes, were examined for inhibition of mobility of larvae and their cytotoxicity. Some of the isolated alkaloids were highly cytotoxic in HL60 tissue-culture cells, so only those with a high RM₅₀/IC₅₀ ratio (Selectivity index: SI > 100) were proposed as potential anthelmintic alkaloid molecules, namely *allocryptopine*, *dehydrocorydaline*, and *papaverine*. However, the mechanism of inhibition of larval motility is so far unclear.

In an effort to identify a treatment for *Sstrongyloides stercoralis* larval migrans in humans, the effects of *isoquinoline alkaloids* were examined by Satou et al. (2002b) using infective third-stage larvae of *Strongyloides ratti* and *S. venezuelensis* as model nematodes for *S. stercoralis*. The nematocidal activity of a set of isoquinoline alkaloids isolated from the same plants as in the previous study was further evaluated in vitro on L3 stage larvae. Total inhibition of mobility corresponding to the complete paralysis of larvae was test of choice for

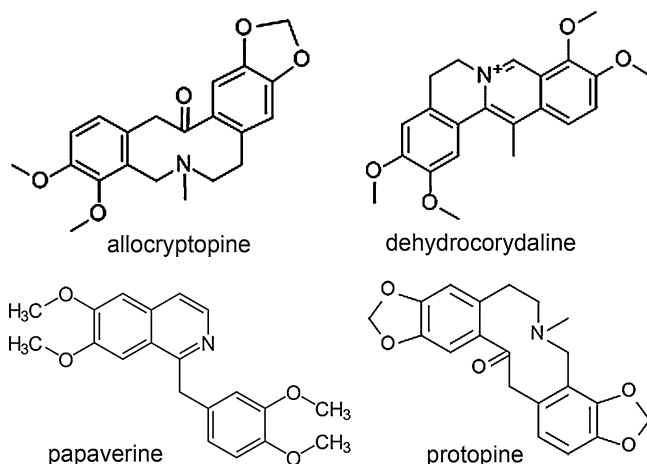


Fig. 2.6 Chemical structure of selected isoquinoline alkaloids with anthelmintic activity

comparison of nematocidal activity of all alkaloids. Three alkaloids: *protopine*, *d-corydaline*, and *l-stylopine* (Fig. 2.6) exhibited strong nematocidal activity at low concentrations (52/33 μM , 18/30 μM , 14/13 μM for *S. ratti*, and *S. venezuelensis*, respectively.), and showed little cytotoxicity (SI > 100) for HL60 tissue-culture cells. However, these concentrations were significantly higher than that found for ivermectin (2.2 and 2.3 μM , resp.), which was the most effective strongyloidosis treatment.

β -carboline alkaloids are a large group of natural and synthetic indole alkaloids with different degrees of aromaticity, some of which are widely distributed in nature, including various plants, foodstuffs, marine organisms, insects, mammals as well as human tissues, and body fluids. They possess diverse biological activities and can inhibit several enzymatic systems in mammals (Cao et al. 2007).

A set of 17 different *β -carboline alkaloids* from the plants *Picrasma quassoides* and *Ailanthus altissima* which grow in Japan was examined using *T. canis* larvae in in vitro screening (Satou et al. 2005). Inhibition of mobility (larval paralysis) and low cytotoxicity were found only for a few alkaloids and only one alkaloid with no cytotoxicity at 0.1 mg/ml was examined in vivo on infected mice. In attempt to slow-down metabolism of this alkaloid, it was entrapped in pegylated liposomal carriers and this drug formulation showed higher reduction of larval numbers in the brain of mice than reference drug albendazole, as well as decreased eosinophilia. No side effects in mice were observed. Alkaloids with reported high nematocidal potential on larvae migrating in the tissues of hosts which also showed very low cytotoxicity seem to be interesting molecules. Therefore, it is worth to elucidate their mode of action on larvae and inhibition of motility suggests that their target could be in neuromuscular regulation.

2.3.3 Essential Oils

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. In nature, essential oils play an important role in the protection of plants as antibacterials, antivirals, antifungals, insecticides, and also against herbivores by reducing their appetite for such plants. There are several methods for extracting essential oils. These may include use of liquid carbon dioxide or microwaves, and mainly low or high pressures distillation employing boiling water or hot steam.

Essential oils are very complex mixtures which can contain 20–60 components at quite different concentrations. They are characterized by two–three major components at fairly high concentrations (20–70 %) compared to others components present in trace amounts. The major component is composed of *terpenes* (for example geraniol, carvacrol, thymol, cymene, sabinene, alpha-pinene, betapinene, citronellol, sesquiterpene farnesol) or *terpenoids* (menthol, ascaridole), and the other components of *aromatic and aliphatic constituents*, all characterized by low molecular weight (for example cinnamyl alcohol, eugenol, safrole) (Fig. 2.7). In general, the abundant compounds determine the biological properties of the particular essential oil and it is likely that their mode of action involves several targets in the cells. (see for review: Bakkali et al. 2008). The hydrophobicity of essential oils enable them to pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids, rendering them permeable (Turina et al. 2006). This extensive change in fluidity/permeability of membranes, results in the leakage of radicals, cytochrome C, calcium ions, and proteins, as in the case of oxidative stress and bioenergetic failure. Cytotoxicity to eukaryotic cells appears to include such membranes damage. In general, the cytotoxic activity of essential oils is mostly due to the presence of phenols, aldehydes, and alcohols (Sacchetti et al. 2005). Clearly, it has been shown by Bakkali et al. (2005) that the tested essential oils presented a specificity in the amplitude, but not in the mode of action, and of the biological effects, i.e., cytotoxicity, cytoplasmic mutant induction, gene induction, and antigenotoxic effects.

Essential oils or some of their constituents are effective against a large variety of organisms including bacteria, fungi, viruses, protozoa as well as metazoan parasites (Bakkali et al. 2008). Plants known for a high content of essential oils are

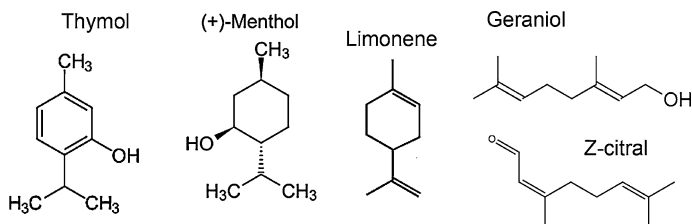


Fig. 2.7 Chemical structure of selected components of essential oils with anthelmintic activity

for example: *Origanum compactum*, *Coriandrum sativum*, *Eucalyptus staigeriana*, *Artemisia herba-alba*, *Cinnamomum camphora*, leaf and seeds of *Anethum graveolens*, *Mentha piperita*, *Salvia* spp., *Nigella sativa*, and others.

We showed in previous part of this chapter, that the extracts from several of these plants exerted significant anthelmintic activity and their essential oils probably contributed to the final effects in vitro and in vivo. To be able to study a direct effect of essential oils on helminths in vitro, their limited solubility in water requires using suitable solvents, usually non-ionic detergents (Tween 20, Tritox-X-100) or DMSO. However, the final effect on infections in animals is influenced by low absorption, the first pass effect in the liver and metabolism into individual components (Fandohan et al. 2008), each component having different mode of action. As the mode of absorption in monogastric and polygastric animals is different, this may influence also the fate of essential oils.

So far, only several essential oils have been examined for their activity on helminths in vitro and in controlled in vivo studies. Whereas some essential oils exerted nematocidal activity only in vitro (for example oil from *Cymbopogon* spp. and *Chenopodium ambrosioides*), others (for example oil from *Eucalyptus staigeriana*, *Ocimum gratissimum*) significantly reduced infection with GIN in ruminants and also in rodent models in vivo. Oil composition was different in these plants, which probably influenced the in vivo activity.

Herbs of *Cymbopogon* spp. belong to family Poaceae and the essential oil is used in Brasil for its characteristic aroma as an insecticide. In vitro activity of oils from *Cymbopogon schoenanthus*, *Cymbopogon martini*, and *Mentha piperita* was studied against developmental stages of trichostrongylidae from sheep naturally infected (95 % *Haemonchus contortus* and 5 % *Trichostrongylus* spp.) (Katiki et al. 2011a, b). The essential oil of *Cymbopogon schoenanthus* had LC₅₀ value of 0.045 mg/ml in EHT, 0.063 mg/ml in LDA, 0.009 mg/ml in LFIA, and 24.66 mg/ml in larval exsheathment assay (LEA), but oil from *M. piperita* was much less effective. The major constituent of the essential oil from *M. piperita* was menthol (42.5 %), while for *C. martinii* and *C. schoenanthus* the main component was geraniol (81.4 and 62.5 %, respectively). However, in young lambs experimentally infected with a multidrug-resistant *Haemonchus contortus* strain, administration of *C. schoenanthus* essential oil (180 mg/kg and 360 mg/kg BW) for 3 consecutive days failed as an anthelmintic treatment. No statistically significant reduction in fecal egg count, packed cell volume or total worm count was observed (Katiki et al. 2012) and toxicity symptoms were observed at 360 mg/kg, with signs of discomfort, apathy, lethargy, and drowsiness in treated animals. Similarly, oil-containing extracts from *Chenopodium ambrosioides* were effective on nematodes in vitro, but short-term treatment with up to 0.4 ml/kg BW of oil was not effective in reducing the number of nematode adults or eggs in infected goats (Ketzis et al. 2002). The major oil component was detected as the terpenoid *ascaridol*, which in the same way as the terpenoid *geraniol*, became probably ineffective following absorption in polygastric animals.

Eucalyptus staigeriana is most commonly used in Brazil for extraction of essential oil, in which several secondary metabolites were detected and the highest

concentration was found for (+)-*limonene* (28.8 %), α -*terpinolen* (9.4 %), *Z-citral* (10.77 %), and *E-citral* (14.16 %) (Macedo et al. 2010). Using the in vitro model of *H. contortus*, oil inhibited the in vitro egg hatching test (EHT) and larval development assay (LDA) by 99.2 % at low concentration of 1.35 mg/ml and 5.5 mg/ml, respectively. In a subacute toxicity study on mice, the LD₅₀ was 4 112.94 mg/kg after oral administration. Fecal egg count reduction (FECR) in goats after treatment with oils varied from 61.4 to 76.57 % at 8 and 15 days p.t. and biochemical tests indicated that kidney and hepatic functions were preserved, and this essential oil did not produce toxicity during the treatment period in experimental animals. Oil from *E.citriodora* at concentration of 5.3 mg/ml inhibited egg hatching of GIN by 98.8% (Macedo et al. 2011). The use of these essential oils would be justified even with effectiveness less than 95 %, especially in situations where the synthetic anthelmintic was not recommended, such as organic farms, in milk-producing animals.

Nematodicidal activity of essential oils extracted from other plants reached values between 94.5 and 100 % and various concentrations of essential oils were used in the same tests and nematode model. Essential oils were isolated from *Eucalyptus globulus* (Macedo et al. 2009), *Ocimum gratissimum* Linn. (Labi-deae) with the main component *eugenol* (43.7 %) and 1,8-cineole (32.71 %) (Pessoa et al. 2002), *Croton zehntneri* and *Lippia sidoides* with the main components *anethole* and *thymol* (Camura-Vasconcelos et al. 2007). The similar inhibition of egg hatching as obtained with 1.0 % thiabendazole, were observed after exposure to oil of *O. gratissimum* (0.5 %) and *eugenol* (1.0 %) in vitro. At concentration of 800 mg/kg, the essential oils from *C. zehntneri* and *L. sidoides* were 46.3 % and 11.64 % effective against sheep gastrointestinal nematode *H. contortus*, indicating that *thymol* and *anethole* are the probable active substances having different mechanisms of action on nematode cells. Albuquerque et al. (1995) reported that *C. zehntneri* essential oil and *anethole* blocked muscle contractions and reduced the response in muscle to acetylcholine implying action sites in muscle fibers. In contrast, the chemical structure of *thymol* implies its possible amphipathic and/or hydrophobic behavior. This suggests an ability of *thymol* partition in the membrane from an aqueous part as well as a capacity to affect the membrane organization and the surface electrostatics. This assumption may explain the activity of *thymol* on the permeability of membranes and on the activity of membrane intrinsic proteins such as ATPases or membrane receptors (Sánchez et al. 2004).

Based on present data, the promising substances in terms of therapy of nematode infections in monogastric animals seem to be oil from *Cymbopogon* spp. and *Croton zehntneri*. Significant in vitro and in vivo nematocidal activity was found also in essential oils from *Eucalyptus* spp., in small ruminants and components of these oils deserve further investigations, namely citrals, limonene, *anethole*, and *eugenol*. Desired effects can result in reduced reinfection and reduced worm loads leading to decreased pasture contamination levels (Max 2010).

The protoscolicidal effect of *thymol* was tested in vitro against *E. granulosus* tapeworm at a concentrations of 10, 5 and 1 µg/ml in medium (Elissondo et al.

2008). At a concentration of 10 µg/ml thymol reduced viability to 53.5 % after 12 days of incubation and to 11.5 % after 42 days of culture. The primary site of damage was the tegument of protoscoleces and morphological changes included contractions of the soma regions, formation of blebs on the tegument, rostellar disorganization, loss of hooks, and destruction of microtriches, which are directly associated with nutrient absorption. Blebs and alteration of microtriches probably interfere with protoscoleces' nutrition explaining the later appearance and gradual elevation of the toxic effect of thymol. Stimulation of motility of protoscoleces was not observed. Also in another study (Moazeni et al. 2012) thymol as the main constituent in the essential oil from the fruits of plant *Trachyspermum ammi* (50 %) was probably the active molecule responsible for significant scolicidal effect in vitro. Addition of 5 mg/ml of essential oil killed 51.89, 72.20, 88.64, and 100 % of protoscolices after 10, 20, 30, and 60 min, respectively.

The tegument of cestodes and trematodes is morphologically and physiologically different than cuticle of nematodes and due to the absorption function for nutrients, essential oils will likely to cross individual layers of tegument. Based on our literature search it seems that *Schistosoma mansoni* has been the most intensively studied trematode in relation to the therapeutical potential of essential oils. Although praziquantel is effective against all medically important species of genus *Schistosoma*, it is ineffective against schistosomula, which motivates the search for new active compounds. Anti-schistosomal activity against various stages of this trematode was reported for several essential oils isolated from a variety of plants, mostly in recent years (Magalhaes et al. 2012; Ael-Banhawey et al. 2007; Parreira et al. 2010; de Melo et al. 2011; Caixeta et al. 2011; de Oliveira et al. 2012; Mahmoud et al. 2002). Available composition and the concentrations of those with the highest anthelmintic activity of essential oils so far examined are summarized in Table 2.6. The important observation reported by all these studies was that the concentration of about 100 mg/ml of oil was required to achieve effects similar to PZQ at a concentration of 12.5 µg/ml in vitro. At such high concentration of essential oils from *Piper cubeba*, *Baccharis dracunculifolia*, *Baccharis trimera*, *Ageratum conyzoides*, *Bidens sulphurea*, and *Plectranthus neochilus*, *Curcuma longa* oils caused the death of all adult worms and promoted separation of the couple pairs into individual male and female within 24–30 h. In vivo all of these oils given at higher doses were responsible also for remarkable reduction in the number of eggs and most of them reduced the viability of cercariae and schistosomula, an effect not seen with PZQ. Tegumental damage, destruction of tubercles and spines, and suckers of adult worms were observed in dead worms obtained from treated animals (de Oliveira et al. 2012).

Seeds of *Nigella sativa* have been employed for thousands of years as a spice. The immunomodulatory, therapeutic and anti-oxidant properties of oil and its constituents isolated from the seeds of this plant, in particular *thymoquinine* (TQ), have been reviewed by Salem (2005). Low doses (250 µl/kg) of the oil from *N. sativa* exerted trematocidal effect in vivo only when sidr honey was coadministered daily for 7 weeks post infection, whereas single therapy with oil was

Table 2.6 In vitro and in vivo anthelmintic effects of selected essential oils with determined components on nematodes (*H. contortus*), trematodes (*S. mansoni*, *F. gigantiga*) and cestodes (*E. multilocularis*)

Name of plant	Main constituents of essential oil	Parasitic infection	The most effective concentration/dose of oil in selected in vitro test or in vivo studies	References
<i>Croton zehntneri</i>	Anethole-64 % estragole < 15 %	<i>Haemonchus contortus</i>	1.25 mg/ml: 98 % inhibition in EHT 10 mg/ml: 99.2 % inhibition in LDA	Camura-Vasconcelos et al. (2007)
<i>Eucalyptus globulus</i>	(+) limonene	<i>Haemonchus contortus</i>	21.75 mg/ml: 99.3 % inhibition in EHT 43.5 mg/ml: 98.7 % inhibition in LDA	Macedo et al. (2009)
<i>Eucalyptus citriodora</i>	(+) limonene	<i>Haemonchus contortus</i>	5.3 mg/ml: 98.8 % inhibition in EHT 10.6 mg/ml: 99.7 % inhibition in LDA	Macedo et al. (2011)
<i>Eucalyptus staigeriana</i>	(+) limonene - 28.8 % Z-citral-10.77 % E-citral-14.16 %	<i>Haemonchus contortus</i>	1.35 mg/ml: 99.2 % inhibition in EHT 5.5 mg/ml: 99.0 % inhibition in LDA	Macedo et al. (2010)
<i>Cymbopogon schoenanthus</i>	Geraniol-62.5 %	<i>Haemonchus contortus</i>		Katiki et al. (2011a, b)
<i>Lippia sidoides</i>	Thymol < 50 %	<i>Haemonchus contortus</i>	LC ₅₀ = 0.045 mg/ml in EHT LC ₅₀ = 0.063 mg/ml in LDA 20 mg/ml: 94.5 % inhibition in LDA	Camura-v Vasconcelos et al. (2007)
<i>Piper cubeba</i> L.	Sabinene-19 %, eucalyptol-11 % 4-terpineol-6.3 % β -pinene-5.8 %	<i>Schistosoma mansoni</i>	0.012-0.05 mg/ml: reduction of cercarie viability; no effect on adult worms; separation of coupled adult worms	Magalhaes et al. (2012)
<i>Baccharis trimera</i> L.	Nerolidol-33 % Spathulenol-16 %	<i>Schistosoma mansoni</i>	0.130 mg/ml: 100 % mortality of adults, peeling of tegumental surface (tubercles, spines)	de Oliveira et al. (2012)
<i>Plectranthus neochilus</i>	β -caryophyllene - 28.2 % α -thujene-12.2 % α -pinene-12.6 %	<i>Schistosoma mansoni</i>	0.1 mg/ml for 24 h: 100 % mortality of adult worms, dose-dependent reduction in % of developed eggs	Caixeta et al. (2011)

(continued)

Table 2.6 (continued)

Name of plant	Main constituents of essential oil	Parasitic infection	The most effective concentration/dose of oil in selected in vitro test or in vivo studies	References
<i>Nigella sativa</i>	Diterpene alkaloids thymol, thymoquinone dithymquinone	<i>Schistosoma mansoni</i>	5.0 ml/kg daily (14 days) -32 % reduction of worm burden and reduction in eggs load in the intestine of treated mice	Mahmoud et al. (2002)
<i>Ageratum conyzoides</i> L.	Precocene I-74 % E-caryophyllene-14 %	<i>Schistosoma mansoni</i>	0.1 mg/ml for 120 h: 100 % mortality of adult worms, 75 % of separation of coupled adult worms	de Melo et al. (2011)
<i>Synthetic</i>	Thymol	<i>Echinococcus multilocularis</i>	0.010 mg/ml: 53.5 % reduction of protozoocytes viability after 12 days, massive tegumental damage	Elissondo et al. (2008)
<i>Allium sativum</i> (garlic)	allicin S-Allyl-L-cysteine g-glutamyl- Sallylcysteine	<i>Fasciola gigantica</i>	3 mg/ml: complete paralysis of worms after 15 min of incubation	Singh et al. (2009)
<i>Piper longum</i>	β -caryophyllene-33.44 % 3-carene-7.58 % Eugenol-7.39 %	<i>Fasciola gigantica</i>	3 mg/ml: initial excitation following paralysis of worms after 15 min of incubation	Singh et al. (2009)

ineffective (Mostafa and Soliman 2010). In another study on the same kind of infection, the higher daily doses (2.5 ml/kg or 5.0 ml/kg) of *N. sativa* oil administered orally to mice for 2 weeks reduced the number of *S. mansoni* worms in the liver only by 22 and 32 %, respectively, and decreased the total number of ova deposited in both the liver and the intestine (Mahmoud et al. 2002). Thymol and thymoquinone, as the main components of oil, could be responsible for observed trematocidal effect. Reductions in parasite burden and granuloma size coincided with partial amelioration of the *Schistosoma*-induced liver fibrosis and changes in ALT, GSH, AP activities in serum, suggesting that the schistosomicidal effect of *N. sativa* oil might be induced partly by its anti-oxidant effect documented in numerous studies (Ramadan et al. 2003). Similarly, treatment with *N. sativa* oil decreased the hepatocellular necrosis, degeneration, and advanced fibrosis in CCl₄-induced liver fibrosis in rabbits (Türkdoğan et al. 2001). The effect of the oil could, at least partly, be attributed also to drug-induced modulation of the immune response to *Schistosoma* eggs trapped in the liver as in vitro studies showed that oil enhanced the production of IL-3 by human lymphocytes and had a stimulatory effect on macrophages (Haq et al. 1995).

The relatively high effective concentration of essential oils and similar effects on flatworms indicates that individual components of oils probably did not selectively act on a parasite-specific molecular targets and that the final deleterious effect might occur due to synergism of all or some of the components.

More light onto the mechanism of action of essential oils on viability and motility of trematodes was brought by the study of Singh et al. (2009). In vitro exposure to essential oils from *Allium sativum* (garlic) and *Piper longum* (Indian long pepper) have markedly changed muscular activity of the whole worms and muscle strips of the liver fluke *Fasciola gigantica*. Essential oil from *A. sativum* caused complete paralysis of the fluke after 15 min of administration of 3 mg/ml and flaccid paralysis in the strip preparations. In contrast, essential oil from *P. longum* first induced marked excitatory effect and then the flaccid paralysis of the whole worm following 15 min exposure to the same concentration. These effects were irreversible and the rapid responses to oils suggest the involvement of neuromuscular system of worms. Many of the anthelmintics cause paralysis of helminth parasites by disrupting one or the other aspect of their neuromuscular system, but at much lower concentrations (Loukas and Hotez 2005). With regard to the effect of essential oil of *A. sativum* and *P. longum*, they produced grossly similar effect on both preparations, although the main components of both oils are different (Liu et al. 2007; Itakura et al. 2001). It was concluded that tegument did not interfere with the action of essential oils on smooth muscle actin of *F. gigantica*. Nevertheless, observations on strip preparations do not support the general assumption that the tegument provides a barrier in the translocation of drugs to neuromuscular targets in trematodes (Sobhona et al. 2000).

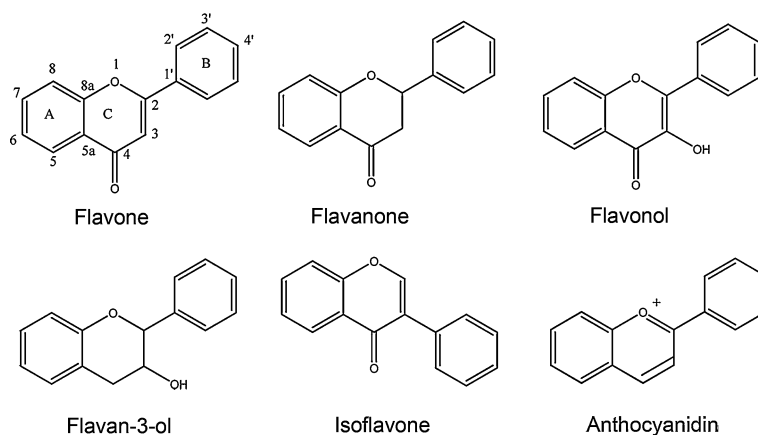


Fig. 2.8 Chemical structure of individual types of flavonoids

2.3.4 Flavonoids and Polyphenols

Flavonoids are plant pigments present in almost all terrestrial plants, where they provide UV protection and color. They are synthesized from phenylalanine and their basic chemical structure (C6-C3-C6 skeleton) has a fused ring system consisting of an aromatic ring and an oxygen-containing heterocyclic benzopyran ring with a phenyl substituent. Flavonoids can be divided into different classes depending on their oxidative status and substituents. In nature, flavonoids often occur as polymers, with dimers being the most common form. Most flavonoids, apart from catechins, are usually present in plants as β -glycosides. (Fig. 2.8).

The flavonoids appear to have an important role in the successful medical treatments of the ancient times, and since the last century they are subject of increasing interest of scientists working in various fields of medical research, including pharmacology of parasitic diseases. The excellent review on biochemistry and medical significance of the flavonoids was written by Havsteen (2002). His review deals also with a high spectrum of positive effects of various flavonoids on plant and mammalian cells in relation to therapeutical applications, their ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters, and to scavenge free radicals. It is well known that some flavonoids can inhibit or kill many bacterial strains, inhibit important viral enzymes, such as reverse transcriptase and protease, and destroy some pathogenic protozoans.

In the many plant extracts which showed a high anthelmintic activity, chemical analyses revealed the presence of flavonoids, along with other classes of phytochemicals. Although toxicity of most isolated flavonoids to animal cells is very low (Middleton et al. 2000), several ubiquitous flavonoids genistein, kaemferol,

rutin, quercetin, etc., showed deleterious effects on selected species of parasitic helminths. It is possible, that different developmental stages (larvae, juvenile or adult) of helminths might possess different susceptibility to selected flavonoids. For example the phenolic diketone curcumin and the flavonoid kaempferol exerted a strong adulticidal effect on *Schistosoma mansoni*, but no activity against nematodes was demonstrated. *Kaempferol* (flavonol) and its three derivatives, were isolated from two plant species of *Styrax camporum* and *Styrax pohlii* (Braguine et al. 2012), and were examined in vitro. Of these, kaempferol was the most effective in separating *S. mansoni* male and female couples and killing adult worms at a concentration of 100 µg/ml. The selective toxicity of the flavones *quercetin*, *chrysin*, and *3-hydroxyflavone* toward the several cancer cell lines but not to normal mammalian cells is worthy of mention (Pilatova et al. 2010). Selected flavones stimulated mechanisms leading to apoptosis of cancer cells and inhibited functions of important cell signaling molecules.

The polyphenol *curcumin* (phenolic diketone) is the major constituent in the rhizome of *Curcuma longa* (Zingiberaceae), which is responsible for the characteristic yellow pigment. Curcumin is well known to exhibit several biological activities, including anti-inflammatory, antioxidant, antiviral, anti-infectious, and anti-carcinogenic activities (for example Maheshwari et al. 2006). Angiogenesis is a key step in tumor growth and invasion and, in the recent review of Varinska et al. (2010), the anti-angiogenic potential of polyphenols including curcumin was discussed. Curcumin was shown to cause the death of all worms of *S. mansoni* at 50 and 100 µM concentrations due to the decreasing of their viability (Magalhães et al. 2009). All pairs of coupled adult worms were separated into individual male and female by the action of curcumin at the doses of 20–100 µM and it also reduced egg production by 50 %. An important issue in the drug development process is drug solubility in water and pharmacokinetic properties in vivo, which can be manipulated after their incorporation into drug delivery systems (DDS). The DDS should deliver a biologically active molecule at a desired rate for a desired duration and at a desired target, so as to maintain the drug level in the body at optimum therapeutic concentrations with minimum fluctuation. In the recent study of Luz et al. (2012) curcumin was incorporated into poly (lactico-glycolic) acid (PLGA) nanospheres by the nanoprecipitation technique. Incubation of adult *Schistosoma mansoni* with curcumin-loaded PLGA nanoparticles caused the death of all worms, a separation between 50 and 100 % of worm couples and the partial alterations in the tegument at concentrations from 30 µM. Nanoparticles contributed to the higher absorption rate of entrapped curcumin in comparison with free compound, thus decreasing the threshold concentration from 100 to 30 µM. Interestingly, in vivo studies with curcumin showed a lack of significant toxicity (Perkins et al. 2002). The mechanism by which curcumin exerts its in vitro schistosomicidal effect is not clear. However, it has been reported that it has a direct action involving parasite biochemical processes. One of the possible targets in *schistosomes* for the curcumin action is the ubiquitin–proteasome pathway. In the study of Allam (2009), a total dose of 400 mg/kg BW of curcumin was given to mice with *S. mansoni* infection (in 16 injections) and treatment was effective in

reducing worm burden by 44.4 % and tissue-egg burden by 30.9 %. Moreover, modulation of cellular and humoral immune responses was observed as well as a decrease of hepatic granuloma volume and overall collagenesis.

Genistein (4',5,7-hydroxyisoflavone), a major component of soya, is a well-known phytoestrogen, which was found also in the ethanol extracts of higher plants *Flemingia vestita*, *Accacia* spp., *Stephania glabra*, and possibly others. The extensive studies have indicated beneficial effect of genistein on a multitude of disorders, including cancer, cardiovascular diseases, osteoporosis, and postmenopausal symptoms (see for review Barnes 1998). It has strong anti-inflammatory and antibacterial properties in vitro and in vivo (for example, Verdreng et al. 2004), however, in mammals frequent administration of the higher doses either as therapeutical or dietary supply might cause undesirable side effects (Klein and King 2007).

The anthelmintic activity of genistein or genistein-rich extracts from root of plant *Flemingia vestita* has been proven on the cestodes *Raillietina echinobothrida*, *Echinococcus multilocularis* and *Echinococcus granulosus* and the trematode, *Fasciolopsis buski* in vitro. *Flemingia vestita* is an indigenous medical plant of north-east India and, according to the ethnomedical experiences, the crude extract of the root-tuber peel was effective against trematodes and cestodes, but not nematodes. The high concentration of *isoflavones* was found in the extract from this plant (Rao and Reddy 1991). The anthelmintic activity of genistein is mediated by its action of several cellular/molecular targets, and such a complex mode of action of this flavonoid was also found for mammalian cells. In parasitic flatworms, the primary or vital targets of *genistein* were suggested to be the tegumental enzymes, and their inhibition probably resulted in the paralysis and worm death (Pal and Tandon 1998). In vitro exposure to this compound resulted in tetanic contractions, flaccid paralysis, and disruption of tegument in *R. echinobothrida* (Tandon et al. 1997). After incubation of worms with genistein (0.5 mg/ml) activity of enzymes AcPase, AlkPase, ATPase, and 5'-Nu was found to be suppressed by 97, 95, 88, and 57 %, respectively. The effect of genistein on a neuromuscular signaling pathway in worms is implicated by results of another study (Kar et al. 2002). Nitric oxide (NO) is the important neuronal messenger, and the enzyme nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to citrulline and nitric oxide (NO). The enzyme NOS exists in three isoforms, which are either constitutively expressed in endothelial cells (cNOS), neurons (nNOS) or are induced by endotoxin and the inflammatory cytokines (iNOS). The activity of the first two NOS depends on the intracellular concentration of Ca^{2+} . In the trematode *Fasciolopsis buski*, which is the large intestinal fluke of swine and humans, genistein treatment in vitro increased nNOS activity in the neuronal tissues and consequently, elevated production of NO, which might also account for, among other factors, onset of paralysis—a manifestation of neurotoxicity. In helminths, NO has been suggested to have many physiological roles as a neurotransmitter at neuromuscular junctions, and in control of embryogenesis (Pfarr et al. 2001).

At the molecular level, NO exerts its most relevant physiological action by activating the soluble form of guanylyl cyclase, leading to the accumulation of

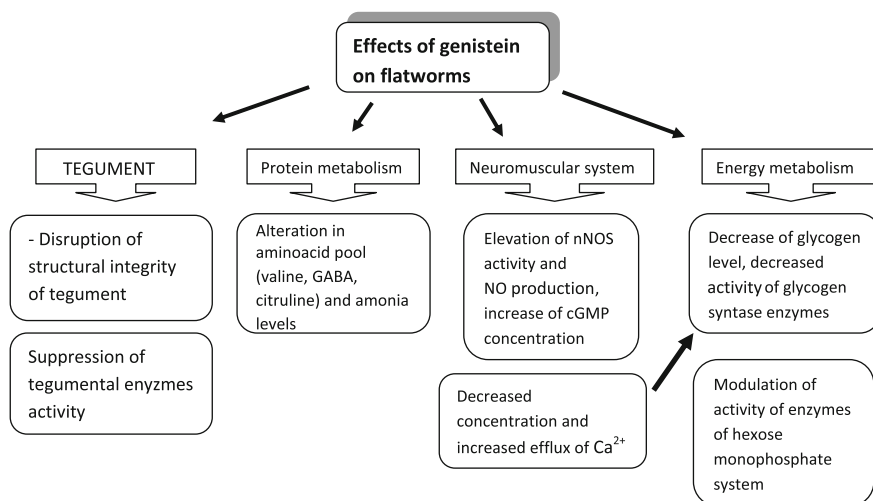


Fig. 2.9 Multitargeted effect of genistein in flatworms

cGMP, an important messenger mediating the functions of NO inside the cells. In the cestode *R. echinobothrida*, incubation of worms with genistein (0.5 mg/ml) modulated this physiological pathway in parasitic tissue (Das et al. 2009). At the time of onset of paralysis in the parasites, a significant increase (32–87 %) in the NOS activity, a two to three fold increase of NO efflux into the incubation media as well as the elevation of cGMP concentration in the treated parasite tissues by 44–103 % were observed. Data indicate that genistein can disturb the downstream signaling pathway of NO, as indicated by the change in cGMP concentrations in parasites. Calcium, which is stored in the calcareous corpuscles of many cestodes, especially the larval (metacestode) stages, is intimately involved in both muscle contractions and signal transduction of many receptors. In *R. echinobothrida* following in vitro treatment with genistein, the Ca^{2+} concentration was decreased significantly by 39–49 % in parasite tissue and also an increase of Ca^{2+} efflux by 91–160 % into the culture medium. The changes in Ca^{2+} homeostasis may be related to the anthelmintic stress caused by the phytochemicals (Das et al. 2006). A similar effect on breakdown of Ca^{2+} homeostasis in the tegument of flatworms is known for praziquantel (Cioli and Pica-Mattoccia 2003). It seems that neuromuscular activity of genistein is one of several effects, indicating multiple targets in parasitic cells which are summarized in Fig. 2.9. It was shown that genistein can interfere with the energy metabolism of flatworms. After exposure to genistein at a concentration of 0.2 mg/ml, the glycogen concentration in *R. echinobothrida* decreased by 15–44 %, which was accompanied by increase of activity of the active form of glycogen phosphorylase by 29–39 % and decrease of activity of the active form of glycogen synthase by 36–59 %. (Tandon et al. 2003). PZQ (1 $\mu\text{g}/\text{ml}$) the reference drug, also caused quantitative reduction in glycogen level and

alteration in enzyme activities. With limited ability to metabolize lipids and amino acids, cestodes and trematodes mainly utilize glucose and other simple carbohydrate molecules to meet their energy requirements (Bryant and Behm 1989) and the alterations in the activity of enzymes regulating glycogen metabolism may also be related to Ca^{2+} efflux. Several other enzymes are involved in energy metabolism of flatworms and genistein significantly influenced the key enzyme of hexose monophosphate pathway: glucose 6-phosphatase dehydrogenase and also enzymes of gluconeogenesis: pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and fructose 1,6-bisphosphatase in *R. echinobothridia* in vitro (Das et al. 2004), which is perhaps a function of high energy demand of the parasite under anthelmintic stress.

Proteosynthesis and proteolysis in the worms are highly regulated and interconnected processes and genistein was able to induce the alterations in the free amino acid pool and ammonia levels in the fluke *Fasciolipis buski* (Kar et al. 2004). Valine was found to be the most elevated amino acid as well as the levels of GABA and citrulline, which could be associated with the elevated activity of nNOS. Ammonia in the tissue homogenates as well as in incubation medium increased (66.4 %) compared to the controls. These data might indicate that genistein could be an effective compound in the therapy of gastrointestinal cestode and trematode infections, but it is necessary to determine the effective concentration and treatment schedule on animal models. There is a risk that the higher doses might lead to the uncontrolled stimulation of selected immune mechanisms, which are known to be the targets of genistein in mammals. It was shown that genistein, kaempferol, and quercetin can inhibit activation of the signal transducer and activator of transcription 1 (STAT-1), important transcription factor for iNOS and NO production in activated macrophages (Hämäläinen et al. 2007). The estrogen-like activity of genistein is a major concern during long-term chemotherapy as it binds to estrogen receptors and can induce estrogenic effects. Prolonged treatment with current anthelmintics is required to inhibit growth of parasitic cysts of *E. multilocularis*. It was shown that genistein exhibited significant metacestodicidal activity against *E. multilocularis* in vitro as well as against *E. granulosus* metacestodes and protoscoleces (Nagulesvaran et al. 2006). The native compound and synthetic derivatives of genistein, Rm 6423, and Rm 6426 induced truncation of microtriches, nuclear pyknosis, and vesiculations of protoscoleces. In addition, Rm 6423 specifically induced dramatic breakdown of the structural integrity of the germinal layer in metacestode cysts and a decrease of activity of metalloproteases, which allows the growth of cysts in the host tissues. Inter-individual, species and sex differences in gastrointestinal metabolism of this phytoestrogen may be critical factors in determining the efficacy of these various compounds in vivo.

There are a few studies, in which nematocidal activity of other plant-derived flavonoids was demonstrated in vitro and in vivo. The antifilarial activity of 6 flavonoids against the human lymphatic filarial parasite *B. malayi* was evaluated using an in vitro motility assay with adult worms and microfilariae, a biochemical test for viability (MTT-reduction assay), and two animal models, *Meriones unguiculatus* (implanted adult worms) and *Mastomys coucha* (natural infections)

(Lakshmi et al. 2010). All six flavonoids showed antifilarial activity in vitro, which can be classed in a decreasing order: *naringenin* > flavone = hesperetin > rutin > naringin > chrysin. IC₅₀ of naringenin was 2.5 µg/ml and adulticidal effect was seen at 125 µg/ml. In jirds, naringenin and flavone killed or sterilized adult worms at dose of 50 mg/kg, but in *Mastomys*, where the parasite produces a patent infection, only naringenin was filaricidal. All the flavonoids tested were well tolerated in both the animal models and there were no signs of behavioral or other changes that can be related to flavonoid treatment in the animals. Killing the adult worms or sterilizing the female worms is considered to be one of the best strategies, however, the mechanism by which these flavonoids affect the viability of filarial parasites is unknown.

The methanol extract of *Struthiola argentea* whole plant exhibited in vitro activity on nematodes and was therefore selected for bioassay-guided fractionation using the in vitro *Haemonchus contortus* assay. The newly isolated methoxylated flavone, *flavone 3* exhibited the most potent activity with an EC₉₀ of 3.1 µg/ml, which is significantly (>17-fold) less active than the ivermectin control (EC₉₀ = 0.18 µg/ml). (EC = effective concentration). However, in vivo evaluation using the *Heligmosomoides polygyrus* mouse model revealed that flavone 3 did not have any in vivo activity at 25 mg/kg (Ayers et al. 2008). In the case when high in vitro and much lower in vivo activities are observed, the possibility should be taken into consideration that host metabolism might transform one class of flavonoid into another, resulting in generation of new pharmacological activity or the loss of previous activity.

2.3.5 Glycosides and Saponins

Saponins and glycosides are naturally occurring chemical compounds found in a wide variety of higher plants, for example in lucerne (*Medicago sativa*), sisal (*Agave sisalama*), and seeds like soyabeans (*Glycine max*) (Francis et al. 2002). In chemistry, glycoside is a molecule in which a sugar is bound to a non-carbohydrate moiety, usually small organic molecules. They play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides, which are activated by enzyme hydrolysis. Saponins are chemically *glycosides*, which are composed of a lipid-soluble aglycon consisting of either a sterol or more commonly a triterpenoid with different, water-soluble, sugar residues. Saponins also have surfactant properties and sterol-group-containing saponins can particularly affect eukaryotic organisms that contain steroids in their membrane. They also demonstrate hemolytic action toward red blood cells, and can be toxic if given intravenously (Osborn et al. 2011). Moreover, consumption of food with a high saponin concentration by humans and animals can be potentially harmful (Milgate and Roberts 1995). Glycosides and saponins were detected in many higher plants used in the traditional ethnomedicine at various concentrations. So far, only several of these molecules isolated from the plants have been examined for their

anthelmintic activity under the experimental conditions or in vivo controlled studies with promising results (Makkar et al. 2007).

In vivo study conducted on rats with *H. diminuta* infection, saponins extracted from *Acacia auriculiformis* were administered orally at the dose of 200 mg/kg for several days. Adult worms were expelled within several days from intestinum indicating that saponins induced tegumental damage followed by the worm paralysis, which usually precedes expulsion. At such relatively higher dose of crude extracts no side effects on the host were observed (Ghosh et al. 1996). Chemical analysis of extract from the funicles of *A. auriculiformis* revealed the presence of triterpenoid saponins *acaciaside A and B*. They are the unique molecules because they contain a conjugated unsaturated system, which is highly susceptible to peroxidation (Ghosh et al. 1993). Except of cestocidal effect, these saponins showed also strong antifilarial activity, when concentration of 4 mg/ml killed 97 % of microfilaria of *Setaria cervi* and 100 % of adults within 100 min (Ghosh et al. 1993). Significant reduction of infection was found in vivo after repeated doses of 100 mg/kg without seeing any toxic effects in rats. Drugs probably caused a very high physiological stress on adult worms, resulting in their death and expulsion which is opposite to the low efficacy of antifilarial drugs toward adults. Sinha Babu et al. (1997) showed experimentally that these saponins enhance the cell membrane lipid peroxidation. They and others (Nandi et al. 2004) suggested that the conjugated unsaturated system of selected saponins is involved in the formations of free radicals, which induce membrane damage through peroxidation of membranes in helminths.

Glycosides with strong antifilarial activity were isolated from the crude extracts of the stem bark of *Streblus asper*, traditional medical plant of India. Two cardiac glycosides *asperoside* and *strebloside* were highly effective at the dose of 50 mg/kg against *Brugia malayi* in vivo and also in vitro, however several cardiac glycosides of other origins did not show any comparable antifilarial efficacy (Chatterjee et al. 1992). Different types of saponins were found in a high concentration in sisal (*Agave sisalana*), which is an important crop in Brazil. About 60 % of plant material is composed of liquid, containing mostly *sapogenins*, the non-glycosidic portion of saponins, which demonstrated significant anthelmintic activity on L1 stage of gastrointestinal nematodes (Silveira et al. 2012). Three types of sapogenins were detected in sisal, of which *hecogenin* was found of the greatest quantity. Saponins also have surfactant properties and can particularly affect eukaryotic organisms that contain steroids in their membrane (Osbourne 1996). Steroidal saponins are considered to be the active ingredients of plant extracts of sisal, which have detergent properties similar to those of polyene antibiotics. According to Francis et al. (2002), saponins present in *A. sisalana* caused their effect by intercalation in the cell membranes by their hydrophobic fraction, causing the formation of pores in tegument of helminths. Other steroidal saponins were isolated from the methanol extract of *Dracaena fragrans* (Agavaceae) and were tested on adult worms of trematode *Schistosoma mansoni* in vitro.

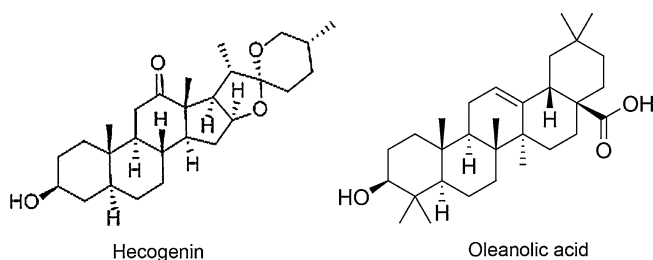


Fig. 2.10 Chemical structure of selected components of saponins with anthelmintic activity

Lethal effect on adults was achieved with LC_{50} of 18.4 $\mu\text{g/ml}$ 4 days after exposure (Tadros et al. 2008).

Calendula officinalis and *Beta vulgaris* are plants native in countries with temperate climate and their saponin components were detected as *triterpenic pentacyclic oleanolic acid* (OA, oleanane-type triterpene) *glucosides with glucuronic acid* attached to the hydroxyl group at the C-3 position of aglycone (oleanolic acid) (Fig. 2.10).

The glycoside compounds connected to glucuronic acid differ in saponins of both plant species (Doligalska et al. 2011). The anthelmintic activity of oleanane-type glucuronides (GlcUAOA) was examined on the development of free-living stages of *Heligmosomoides bakeri*, a parasitic nematode of the mouse intestine. Both *C. officinalis* and *B. vulgaris* GlcUAOA affected the development of the free living stages and interfered with function of the major membrane transporter for xenobiotics, P-glycoprotein (Pgp) in *H. bakeri*. The GlcUAOA inhibited egg hatching and molting of larvae and also changed their morphology. In nematodes, the availability of drugs is modulated by physical and biochemical barriers such as cuticle and intestinal epithelium (Kennedy et al. 1987; Kerboeuf et al. 2010). The function of these barriers depends on specific membrane transport systems such as P-glycoprotein, and nematode resistance to anti-parasitic treatment may be mediated by Pgp-related pathways (Kerboeuf et al. 2003, Kerboeuf and Guegnard 2011). Pgp plays a crucial role in the distribution, metabolism, excretion and absorption of toxic molecules (Riou et al. 2010). The mechanism of action of these saponins is not yet understood, but the anthelmintic activity could be attributed to the molecular structure of GlcUAOA, which is based on a 30-carbon skeleton comprising 5 six-membered rings (*ursanes and oleananes*). Oleanolic acid, when glycosylated at both C-3 and C-28, induces a permeability change in the cell membranes (Hu et al. 1996) probably also in cuticles and cell membranes of *H. bakeri* larvae (Doligalska et al. 2011). The integrity of cell membrane is critical for the barrier function and its loss results in cell death. Interestingly, the increasing level of Pgp has been observed in nematode strains resistant to anthelmintic treatment (Kerboeuf et al. 2003).

2.3.6 Enzymes, Amides and Other Specific Compounds

A broad group of plants, which has been used traditionally for the treatment of helminth infections, includes papaya (*Carica papaya*), fig (*Ficus* spp.), and pineapple (*Ananas comosus*). Papaya and fig release latex upon injury, which is rich in proteolytic enzymes, whereas other plants, such as the pineapple, contain large amounts of cysteine proteinases in the juices extracted from the stems or fruits (Rowan et al. 1990). These enzymes are already in use in medicine for their beneficial effects for example, during inflammatory diseases, and, interestingly, they were shown as the active anthelmintic principle toward nematodes (see reviews: Behnke et al. 2008; Stepek et al. 2004). Cysteine proteinases (CPs) present in the fiber lattices and extracts of fruits, all have a neutral pH optimum of around 7 and the enzymatic activity is associated with the soluble fraction after centrifugation. All of these cysteine proteinases from plants have similar, but not identical activities, and they vary in other important characteristics such as resistance to acidic conditions and susceptibility to digestion by the enzymes of the alimentary tract. The primary site of their proteolytic activity in nematodes is the cuticle but, surprisingly, free-living and soil-dwelling stages of parasitic nematodes, as well as totally free-living species, are resistant to their action (Behnke et al. 2008). The different composition of the free-living nematode outermost cuticular layers and the presence of biochemical defenses—proteinase inhibitors, is the possible explanation. In the case of parasitic stages of nematodes living in the gut, in the absence of host secreted intestinal CPs, it was unreasonable for parasitic stages to develop the defences against any CPs (Zang and Maizels 2001).

Nematocidal activity of enzymes present in papaya latex (*papain*, *chymopapain*, *caricain*, and *glycyl endopeptidase*) was demonstrated on *Ascaris suum* in naturally infected pigs (Satrija et al. 1994), on *Heligmosoides polygyrus* (syn. *Heligmosomoides bakeri*), *Trichuris muris* and *Protospirura muricola* infections in mice (Satrija et al. 1995; Stepek et al. 2006; 2007a, b, c; Behnke et al. 2008), all in monogastric animals. Experimental *H. bakeri* infection in mice is used as model nematode infection for monogastric animals and humans. A daily administration of papaya latex to infected mice for 7 days (133 nmol active cysteine proteinase/mouse/day) resulted in a nearly complete elimination of worms by day 25 post-therapy, indicating by 97 % reduction of *H. bakeri* fecal egg counts (Behnke et al. 2008). Recently, Buttle et al. (2011) showed that enzymes present in papaya latex posses potent anthelmintic activity capable of clearing the adult parasitic nematode *Haemonchus contortus* from the sheep abomasum. The lack of efficacy of a single dose compared with the use of 4 daily doses suggests that, following dilution in the rumen, the enzymes require prolonged contact time with the worms in order to prove effectivity.

Treatment with latex from the South American fig (*Ficus glabrata*) containing the enzymes *ficin* and *ficain*, was evaluated in preclinical study on groups of residents infected with one or more of gastro-intestinal nematodes *Ascaris*, *Ancylostoma*, and/or *Necator*, *Trichuris* or *Strongyloides* (Hansson et al. 1986). A dose of 1 ml/kg significantly reduced eggs per gram (EPG) for all of these

nematodes but complete elimination of worms was not achieved. Latex from other *Ficus* species, given at the dose of 4 ml/kg for 3 days, was examined as a vermifuge in mice naturally infected with several gastro-intestinal nematodes (de Amorin et al. 1999). The weak anthelmintic efficacy between 2.6 % up to 41 % as well as a high acute toxicity in gut, exclude ficin, the main principle of latex from fig, from therapeutical purposes. The enzymatic activity in *Ananas comosus*—pineapple is attributed to *ananain*, *fruit bromelain*, *stem bromelain*, and *comosain*, but their anthelmintic activity has been little examined in controlled in vivo experiments. Regarding the side effects of CPs, the immunogenic properties of orally administered fruit-derived CPs have only been examined by Hale (2004), who detected relatively low levels of circulating bromelain specific IgG after 18 weeks of daily oral treatment with bromelain, however, papain is known to be allergenic when inhaled.

Amides are small organic substances containing nitrogen in their molecule, similar to alkaloids. Amides are found in plants in much lower concentration than other secondary metabolites, where they usually play role in the defense against insects or fungi. The anthelmintic activity on *Schistosoma mansoni* was reported for amide *piplartine*, isolated from roots of plant *Piper tuberculatum* (de Moraes et al. 2011; de Moraes et al. 2012). The genus *Piper* includes species that are widely distributed throughout the tropical and subtropical regions of the world. Piplartine, 5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H)-pyridinone, is found in several *Piper* species and has shown several biological activities on humans as well as antifungal, insecticide (Navickiene et al. 2000, 2003) and anti-protozoan activities against visceral leishmaniasis (Bodiwala et al. 2007). Piplartine concentration of 15.8 μM reduced the motor activity of adults *Schistosoma* worms and caused their death within 24 h in vitro, probably also due to extensive tegumental destruction and damage to the tubercles. A concentration of 6.3 μM caused a 75 % reduction in egg production, however, separation of worm couples was not observed. It is known that the larval stage of this trematode, the schistosomulum, which infects humans via skin, is not sensitive to praziquantel therapy. In the recent study of de Moraes et al. (2012) piplartine reveals interesting anti-schistosomal properties also on schistosomula of different ages (3 h old and 1, 3, 5, and 7 days old). An extensive tegumental destruction, including blebbing, granularity and a shorter body length was observed, followed by the death of parasites, after 120 h of incubations with 7.5 μM of this amide. The mechanism by which piplartine exerts its in vitro schistosomicidal effects is not clear and, according to de Moraes et al. (2011), in vitro antischistosomal effects of piplartine may be related to the inhibition of neurotransmission system pathway in *S. mansoni*. They observed that in vitro effects of piplartine on *S. mansoni* adults better correlated with the muscular function (motor activity) than with tegumental destruction.

The rhizomes of fern *Dryopteris* spp. have popularly been used as vermifuge in flatworm infections, where the active anthelmintic principles were considered to be *phloroglucinol derivatives aspidin*, *flavaspidic acid*, *methylene-bisaspidinol*, and *desaspidin* (Socolsky et al. 2009). The main effects were tegumental alterations, decrease of motor activity of adult worms, egg production and decreased

development of eggs produced by the adult worms, which were inhibited by the incubation with these compounds in the concentrations range from 10 to 100 μM (Magalhães et al. 2010). These authors suggest that these effects may be related to the inhibition of oxidative phosphorylation pathway in *S. mansoni*.

Very promising compounds isolated from *Xylocarpus granatum* (local name in India is fruit from Andaman) with high antifilarial activity are *gedunin* and *photogedunin*. Of several isolated compounds, only two: gedunin (IC = inhibition concentration, CC = cytotoxicity concentration determined on VERO cell lines, SI = selectivity index) ($\text{IC}_{50} = 0.239 \mu\text{g/ml}$, $\text{CC}_{50} = 212.5 \mu\text{g/ml}$, $\text{SI} = 889.1$) and photogedunin ($\text{IC}_{50} = 0.213 \mu\text{g/ml}$, $\text{CC}_{50} = 262.3 \mu\text{g/ml}$, $\text{SI} = 1231.4$) at five daily doses of 100 mg/kg given by subcutaneous route revealed excellent adulticidal efficacy resulting in the death of 80 and 70 % transplanted adult *B. malayi* in the peritoneal cavity of jirds (Misra et al. 2011). The high IC_{50} may indicate that these compounds blocked an essential metabolic pathway in the parasites and are worth of further examination (Fig. 2.11).

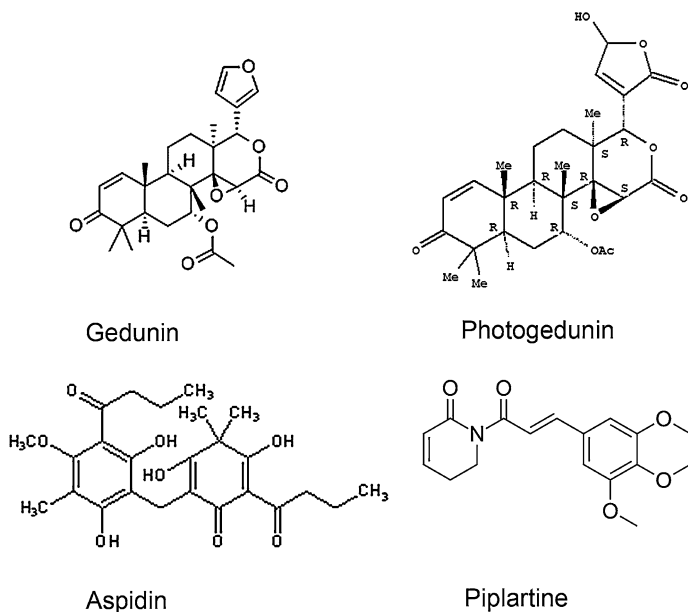


Fig. 2.11 Chemical structure of molecules with marked anthelmintic activity: piplartine, aspidin, gedunin and photogedunin

2.3.7 Condensed Tannins and Sesquiterpene Lactones

Tannins are group of secondary plant metabolites formed by water-soluble phenolic compounds with a great diversity, which can be divided into two major

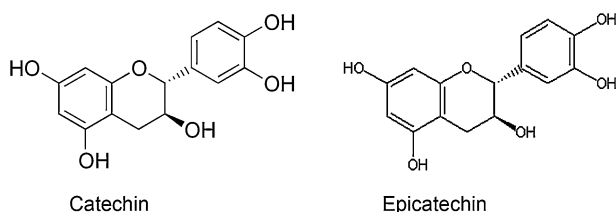


Fig. 2.12 Chemical structure of components of condensed tannins (flavan-3-ols) catechin and epicatechin

groups: the hydrolyzable and the condensed tannins (Waterman 1999). Condensed tannins are *polyphenols* (=proanthocyanidins) with high molecular weight from 500 to 20,000 kDa, which consist mainly of oligomers or polymers of monomeric units of flavan-3-ols (catechin, epicatechin, and so on) (Fig. 2.12). Depending on the chemical structure of the monomeric unit, in particular the number of hydroxyl radicals, they are classified into four sub-classes: the prodelphinidins (PDs), procyanidins (PCs), prorobetinidins (PRs), and profisetinidins (PSs). In legume forages, PCs and PDs are mainly found, in various proportions. The ratio between PDs/PCs differs widely between plant species and/or varieties (Mueller-Harvey 2006). Condensed tannins have a high affinity for proteins and polysaccharides, which can precipitate from the aqueous solutions. The affinity of condensed tannins for proteins is determined by the molecular mass and the molecular configuration of both the tannin and the proteins. Tannin–protein binding is usually reversible in acid or alkaline pH or after treatment with detergents (surfactants).

Condensed tannins (CTs) have high relevance for livestock production as tannin-rich plants have a long tradition of use not only as forages but also as “green” control of gastrointestinal nematode infections. Several excellent reviews deal with the various aspects of feeding of small ruminants with forages containing tannin-rich plants or even fodder trees (Diaz et al. 2010; Hoste et al. 2005, 2006, 2012; Sandoval-Castro et al. 2012 and others). They pointed that bioactive tanniferous plants represent a valuable option as an alternative to commercial drugs for the control of gastro-intestinal nematodes (GINs) as consumption of these plants has been associated with antiparasitic effects: reductions in nematode numbers, worm fecundity, and nematode eggs excretion. The main threat to the use of solely chemical drugs is the rapid development of resistance to any anthelmintic drug in worm populations after commercialization (Waller 2006) and the spread of anthelmintic resistance within worm populations (Kaplan 2004). These problems have stimulated research on plant-based phytochemicals with anthelmintic activity against gastrointestinal nematodes. Although consumption of high concentrations of condensed tannins (>7 % of DM) had a number of detrimental effects on ruminants, such as reduction in food intake, growth inhibition and interference with the morphology and the proteolytic activity of microbes in the rumen (Min et al. 2003; Waghorn and McNabb 2003), low or moderate concentrations of condensed tannins (<6 % of DM) have resulted in the positive

effects on animals. The pertinent use of *tannin-rich fodders* as *nutraceuticals* supposes a clear understanding of the mode of action against the worms. The term “nutraceutical” results from a contraction of nutrition and pharmaceutical. It is defined as “any substance that may be considered a food or part of a food which provides health benefits, including the prevention and treatment of disease” (Andlauer and Furst 2002).

Several aspects of direct and indirect effects of bioactive tannin-rich tropical and temperate legumes against nematode infections have been analyzed in detail in the recent review of Hoste et al. (2012). They discussed high variability of the effects of plant extracts on GIN and pointed that a way to overcome the origins of such variability is to better understand the mode of action of the bioactive compounds against the various nematode stages. This means: (i) to discuss the nature of the secondary metabolites involved in the activity; (ii) to better understand how these compounds affect the different parasitic stages and their biological or functional traits; and (iii) to relate these changes to possible consequences on the parasites’ life cycles.

The other group of the biochemical compounds with anthelmintic activity found in plants used as forage, for example in chicory, are *sesquiterpene lactones* (Foster et al. 2006). Within the last decade a number of studies focused on isolation of condensed tannins and sesquiterpene lactones from various legume forages and plants with the aim to reveal their effects in vitro and in vivo on various species and developmental stages of nematodes. Differentiated action of *condensed tannins* on parasite stages was observed by Athanasiadou et al. (2001), which were more effective against larvae than adults. This can also be explained by the difference between the cuticular components of the pre-parasitic stages (eggs to L3) and the parasitic stages (L4 and adults), as demonstrated by the study of Stepek et al. (Stepek et al. 2007a, b). CTs do have significant negative effects upon egg hatching and larval hatching in vitro as was demonstrated for example by Molan et al. (2002) and Novobilský et al. (2011).

Forage CTs are polymers of flavan-3-ol units, with a considerable range of structural variations. The constituent flavan-3-ol units in procyanidin (PC) polymers are either catechin (C) or epicatechin (EC), while prodelphinidin (PD) polymers contain either gallocatechin (GC) or epigallocatechin (EGC). *Trichostrongylus colubriformis* infective larvae were exposed to flavan-3-ols and their galloyl derivatives under in vitro conditions to compare their effects on the viability of eggs, development of first stage (L1) larvae, and the viability of the infective larvae determined by their mobility (Molan et al. 2003a). The flavan-3-ol gallates were effective in all three tests, with epigallocatechin gallate being the most effective in the egg hatch test (100 % inhibition at 1 mg/ml), also in inhibition of viability of larvae at 500 µg/ml. There was complete inhibition of development by all compounds at 100–200 µg/ml and 50 % inhibition between 42 and 59 µg/ml for flavan-3-ols, while values between 32 and 48 µg/ml were observed for flavan-3-ol gallates. Flavan-3-ols caused some inhibition of viability, but were not effective on other developmental stages (Molan et al. 2003a). The active CT extracts from forage legumes have epigallocatechin as the dominant

flavan-3-ol extender unit, and epigallocatechin was the most active flavan-3-ol in both the EHT and LDA assays. Results may indicate that the anti-parasite properties of CTs are not significantly dependent on their structure in terms of 2,3-stereochemistry of the heterocyclic C-rings (2,3-cis or 2,3-trans), but rather on the number of hydroxy groups in the B-ring (PD:PC ratio). The degree of polymerization and the inter-flavanoid linkages may play a significant role in the effects of CTs. *Onobrychis vicifolia* (sainfoin) is other leguminous forage rich in condensed tannins, which significantly inhibited migration ability of *H. contortus* L3 stage of larvae in vitro (Barrau et al. 2005). Inhibition was seen also for other bioactive compounds flavonol glycosides (rutin, nicotiflorin, and narcissin) present in low molecular weight fraction (up to 2 kDa) implying that both classes of phytochemicals contributed to the anthelmintic activity of this plant in vivo.

Chicory (*Cichorium intybus*) is a widespread herb with high nutritional value, that contains several secondary compounds, with sesquiterpene lactones and the major phenolics (condensed tannins) being the most abundant. The levels of the sesquiterpene lactones (*lactucin*, *lactupicrin*, and *8-deoxylactucin*) and the *hydroxyl coumarin chicorin* were found to be highest in the most actively growing regions of the plant (Rees and Harborne 1985). CT and crude sesquiterpene lactones (CSL) extracted from chicory have been shown to have direct effects on the motility of first-stage (L1) and third-stage (L3) larvae of deer lungworm (*Dictyocaulus viviparus*) and L3 larvae of gastrointestinal nematodes in vitro using the larval migration inhibition (LMI) assay (Molan et al. 2003b). Condensed tannins appeared to be more effective than CSLs at inactivating L1 and L3 lungworm and L3 gastrointestinal larvae in rumen fluid, but CSLs were particularly effective against L3 lungworm larvae in abomasal fluid. Condensed tannins were also effective at reducing the motility of infective larvae of gastrointestinal nematodes. Moreover, the L3 larvae of gastrointestinal nematodes were more sensitive to CT than L1 and L3 larvae of lungworms. This may be attributed to the fact that gastrointestinal L3 larvae were exsheathed, while the lungworm larvae were not. The protein surface of the sheath may interact with the CT and protect the larvae, whilst the absence of a sheath brings the larvae into direct contact with the CT and thus exposes them to a greater paralyzing effect. This was confirmed in the study of Molan et al. (2000b) who found that exsheathed larvae of deer gastrointestinal nematodes were more susceptible to the actions of CT than larvae with protecting layers. Indeed, Brunet and Hoste (2006) showed that monomers of condensed tannins affect directly the larval exsheathment of parasitic nematodes of ruminants. CSLs were effective at reducing the motility of lungworm and gastrointestinal nematode larvae, but their inhibitory activity in rumen fluid was lower than found for CT. This finding may be of practical value in controlling infection of the abomasum and intestine. In contrast to CT, pH probably does not affect the reactivity of CSL. The addition of PEG did not affect the biological activity of CSL, which may indicate that CSLs inhibit these larvae by a different mechanism than do CT.

The proportions of the *sesquiterpene lactones* (*lactucin* = LAC, *lactupicrin* = LPIC and *8-deoxylactucin* = DOL) vary among forage chicory cultivars, that could modify the final anthelmintic activity of CSL (Foster et al. 2011). After

using the combined LAC, DOL, and LPIC concentrations in the range from 0 to 5.0 mg/ml, egg hatching of *Haemonchus contortus* decreased sharply in a linear fashion. Concentrations of sesquiterpene lactones required for 50 % lethality were 2.6 mg/ml for cultivar containing higher amount of DOL and 6.4 mg/ml for cultivar with higher concentration of LAC, suggesting that LAC has minimal effect on egg hatching and that DOL or other constituent(s) in the CSLs are inhibitory (Foster et al. 2011). Metabolism or breakdown of free and bound sesquiterpene lactones in the rumen of animals is likely, and degradation rates of the individual chemical structures may vary, influencing what compounds and amounts reach the relevant section of the gastrointestinal track. Further studies are needed to determine the extent to which sesquiterpene lactone glycosides might influence the anthelmintic potential of chicory herbage.

Variations in the concentration and biochemical composition of condensed tannins extracted from several legume forages (*Lotus* spp., *Hedysarum coronarium*, *Onobrychis viciifolia*, *Dorycnium* spp.) were probably directly responsible for altered behavior of *Trichostrongylus columbriformis* exsheathed L3 stage larvae in a migration inhibition assay (Molan et al. 2000a, c). At 100 µg/ml, purified CTs from *Dorycnium* spp. were more effective in larval inhibition than CT from other forages. Similarly, at concentration of 1,000 µg/ml CT from *Dorycnium* spp. had the highest inhibitory activity (63 %) also on L1 stage of larvae, followed by CT from *Onobrychis viciifolia* (59 %). Data from selected studies briefly indicate the complexity of actions of condensed tannins from various sources on different developmental stages of GIN. The specific composition of CT and the stage-specific protein antigenic array of nematodes, which interact together may explain the observed variability. It is possible that CT can interfere with proteins involved in glucose metabolism as CT from *Chicorium intybus* inhibited glucose uptake in mammalian cells (Muthusamy et al. 2008).

2.3.8 Endoperoxide Sesquiterpene Lactone Artemisinin and Derivates

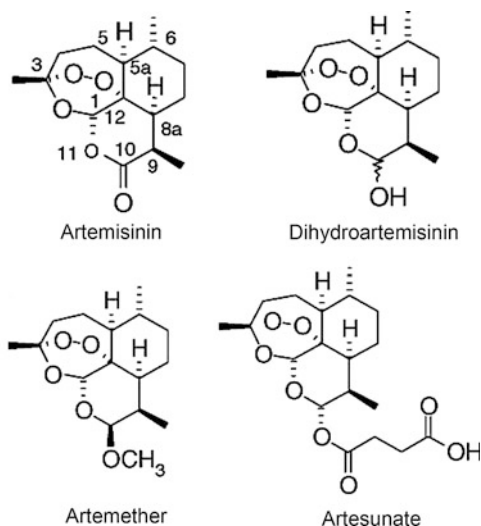
Plant extracts from the genus *Artemisia* (Asteraceae) have been used for long time in traditional Chinese medicine for treatment of parasitic infections in humans and animals. *Artemisia* is now growing in many European countries and has also become naturalized in North America (Baraldi et al. 2008). Antiparasitic activity is attributed to the unique bioactive sesquiterpene lactone with an endoperoxide bridge, artemisinin, which was found in the high concentration in *Artemisia annua* (Fig. 2.13), *A. vulgaris*, and *A. absinthium*. A recent review reported that *A. annua*, the only commercial source of artemisinin, also contains over 40 flavonoids, some of which might potentiate artemisinin effect in extracts by inhibiting cytochrome P-450 enzymes, which degrade artememisinin (Ferreira et al. 2010).

Fig. 2.13 Image of plant *Artemisia annua*, the primary source of natural artemisinin



Artemisinin is considered as the most important plant secondary metabolite with respect to toxicity to a number of parasitic protozoan species (for review see: Muraleedharan and Avery 2009). The authors of these review summarized the progress in the development of anti-parasitic agents based on artemisinin and data from in vitro and preclinical studies on medically important protozoan and metazoan infections. Although effective, artemisinin suffers from drawbacks such as short plasma half-life, limited bioavailability and poor solubility, either in oil or water. On the basis of observations that the peroxide group in the molecule is responsible for its anti-parasitic action, initial efforts to improve these properties led to the development of first-generation artemisinin derivatives in the early 1980s. Of these, *dihydro-artemisinin*, was almost six times more potent in vitro than the parent compound on *Plasmodium* parasites in vivo (Janse et al. 1994). Within the next decade other semi-synthetic derivatives, *artemether*, *arteether*, and *artesunate* were prepared from the parent compound (Fig. 2.14). It is believed that their activity is released when the endoperoxide bridge is open, giving rise to peroxide molecules, which are the source of reactive oxygen species (Olliaro et al. 2001). Being recognized as an effective drug against malaria and other protozoan parasites of humans, the pharmacological potential of artemisinin and derivatives have been evaluated in a numerous studies employing several helminth species in

Fig. 2.14 Chemical structure of artemisinin and derivatives



vitro and in vivo (see reviews: Utzinger et al. 2001, 2007; Keiser and Utzinger 2007; Xiao 2005a, b; Xiao et al. 2010). Thus these compounds, which were primarily explored as novel anthelmintics in the therapy of human parasitic infections, were recognized recently as the drug candidates for veterinary medicine. (Keiser et al. 2008; Squires et al. 2011).

The zoonotic disease fascioliasis, caused by the liver flukes *Fasciola hepatica* and *Fasciola gigantica* is of considerable public health relevance in Bolivia, Cuba, Egypt, the Islamic Republic of Iran, Peru and Vietnam. An estimated 91 million people are at risk and 2.4–17 million people are infected (Keiser and Utzinger 2005). Fascioliasis is also a problem for livestock production and a threat for global food safety (Schweizer et al. 2005). The only approved human anthelmintics for trematodiasis are praziquantel and triclabendazole (TBZ), however the rapid spread of TBZ resistance is an important motivation for drug discovery of novel trematocidal drugs.

The effects of artemether and artesunate, derivatives of artemisinin, were evaluated on adults and juvenile stages of both *Fasciola* species in the series of in vitro and in vivo studies. In vitro incubation in 10 µg/ml of artemether or artesunate caused severe tegumental damage on adult *F. hepatica* (Keiser and Morson 2008) comprising swelling of tegumental ridges, followed by blebbing and later rupturing of the blebs, leading to erosion and lesion, and disruption of the tegument. Similar alterations of the surface were also observed in adult *F. gigantica* when treated in vitro with 10–30 µg/ml of artemether (Shalaby et al. 2009) and in 3-weeks old juvenile stages after incubation with 20–80 µg/ml of artesunate for minimum 6 h. No fluke was found to be dead after incubation for 24 h (Tansatit et al. 2012).

An oral administration of artesunate at a single dose of 200 mg/kg resulted in 95 and 56.4 % reduction of worm burden in rats harboring adult and juvenile

Fasciola hepatica, respectively (Duthaler et al. 2010). A high oral dosage of 400 mg/kg artesunate completely eradicated adult *F. hepatica* in infected rats (Keiser et al. 2006a). Ultrastructural changes of the tegument and gut of the triclabendazole-resistant adult *F. hepatica* in the rats were observed following 24–72 h in vivo treatment with 200 mg/kg artemether (O'Neill et al. 2009). In all of these studies, it was concluded that the tegument of *F. hepatica* and *F. gigantica* is a primary drug target for artemisinin derivatives and that they could be considered as drug candidates in the therapy of fascioliasis in monogastric animals.

Considering the veterinary importance of *Fasciola* spp. and the promising in vitro and in vivo activities of the artemisinins, trials were expanded to ruminants and their efficacy was assessed in sheep naturally infected with *F. hepatica*. In the study of Keiser et al. (2008) it was shown that single oral dose of 40 and 80 mg/kg body weight (BW) of artemether had no effect of FEC and worms burden, however a single dose of 160 mg/kg given intramuscularly significantly reduced the egg burden (64.9 %) and worm burden (91.3 %). A similar efficacy was achieved in naturally infected sheep treated with artesunate (Keiser et al. 2010). This indicates that the bioavailability after oral administration of artemether in sheep is much lower compared to drug bioavailability in monogastric animals that is related to the digestive physiology of the rumen. There were no adverse reactions observed in sheep after various single doses. The evaluation of the pharmacokinetic profile of both derivatives under the same experimental design in the sheep model explained, at least partially, the ineffectiveness of the oral treatment (80 mg/kg) compared to intramuscular (i.m.) treatment (160 mg/kg). The observed plasma concentrations for derivatives and their metabolites were significantly higher following i.m. compared to oral treatment with an especially large difference observed for the metabolite dihydroartemisinin (DHA). This metabolite rapidly reduced the viability of worms in vitro, while other metabolites did not (Duthaler et al. 2012). In rats, artemisinins are primarily converted to DHA via ester hydrolysis and further to inactive metabolites by hepatic cytochrome P450 and other enzyme systems. DHA exhibits a low bioavailability after oral administration, with a short elimination half-life (Li et al. 1998).

The problem of high multiple doses of artemisinins could be the embryotoxicity which was observed in laboratory animals (Clark et al. 2004). In addition, intramuscular administration of high doses of artemether has been shown to produce selective damage to the brain stem center of rodents, but only when multiple high doses of drug were administered. For example, intramuscular artemether (50–100 mg/kg day for 28 days) caused dose-dependent neuropathologic damage to the brain stem of mice (Nontprasert et al. 2002).

Schistosomiasis is a parasitic disease with a chronic debilitating character in humans and is ranked at second position of the world's parasitic diseases in terms of the extent of endemic areas and the number of infected people. Approximately 600 millions of people in 74 countries live at risk of infection (Lotfy 2009; Utzinger et al. 2009). Praziquantel is current drug of choice for the treatment of schistosomiasis. It is highly effective against adult stages and young developmental stages of all human schistosome species but is inactive on schistosomula

(Sabah et al. 1986; Xiao et al. 1987). There is a serious concern that a large scale application of praziquantel with having no similarly effective drug as an alternative, could lead to the development of tolerance/resistance to this drug. The first initiative in examination of artemisinins as anti-schistosomal compounds was taken by a group of Chinese scientists. They found that artemisinin administered to experimentally infected animals with *Schistosoma japonicum* resulted in marked reduction of adult worm burden (Chen et al. 1980). Since then numerous studies and reviews have been published dealing with in vitro and in vivo effects of artemisinin and its derivatives on *Schistosoma species* (*S. mansoni*, *S. japonicum*, *S. mekongi* and *S. haematobium*). In the review of Utzinger et al. (2001) the potential of artemether for control of schistosomiasis was described in detail. Based on results obtained through extensive laboratory and clinical investigations it was concluded that oral artemether at a dose of 6 mg/kg administered in 2- or 3-weeks intervals is safe and is effective in the prevention of patent *S. japonicum* and *S. mansoni* infections, thus preventing the onset and evolution of pathology. Secondly, a combination therapy of praziquantel with artemether was proposed as effective alternative and this strategy has been recommended for effective transmission control. Tegumental changes induced by the semi-synthetic artemisinin derivatives artemether and artesunate have been assessed in different schistosome species, and were reviewed by Utzinger et al. (2007) showing a great similarity with *Fasciola* spp. In addition, they summarised data from preclinical studies and clinical trials showing the safety and efficacy in human. Only about 1 % of the participants with schistosomiasis and fascioliasis involved in the clinical trials reported mild abdominal pain, dizziness, headache, and diarrhea, or slight fever (Wu et al. 1995; Li et al. 2005; Keiser et al. 2011).

The extent of worm burden reduction following artemether therapy is dependent on the period of drug administration, since the drug selectively kills the larval migratory stage of parasite. Very high worm reductions (more than 90 %) were observed for 5–14 day-old schistosomula of *S. japonicum* (Xiao et al. 1995) and 14–21- day-old worms of *S. mansoni* (Xiao and Catto 1989; Xiao et al. 2000b). In the case of *S. haematobium*, which has the longest developmental period of 61–65 days until worms reach sexual maturity, the highest susceptibility to artemether was detected in 28-day-old schistosomula (Yang et al. 2001). In line with this, the highest efficacy (95–99 %) was achieved when the initial dose of artemether was administered to mice 3 weeks post infection (Xiao et al. 2000c).

It has been found in many studies that antiprotozoan activity is mediated by the endoperoxide bridge in the molecule of artemisinins. The mechanism of their action was subjected to the intensive research not only by parasitologists, but also immunologists as it was shown that these compounds have anti-cancer and immunomodulatory effects (Meshnick 2002; Shakir et al. 2011). Regarding their effect on trematodes, it was revealed that, in *S. mansoni*, sites of action of artemether include enzymes of glucose utilization. Worms recovered from artemether-treated hosts had an increased activity of glycogen catabolism and decreased glucose uptake (Xiao et al. 2000a). In mice infected with adult *Schistosoma japonicum*, the effect of artemether was evaluated on glutathione S-transferase

(GST) and superoxide dismutase (SOD), which are major antioxidant enzymes of schistosomes involved in detoxification processes (Callahan et al. 1988; Scott and McManus 2000a, b). In adult worms recovered from mice treated with either a subcurative (100 mg/kg) or a curative dose (300 mg/kg) of drug, significantly decreased activity of both enzymes was detected (Xiao et al. 2002). The higher inhibition was found for GST than for SOD, with female worms being more affected than males, resulting in about 55 % decrease of enzyme activity. It was suggested that this enzyme might increase the schistosome susceptibility to oxidative attack, and consequential to this, might be linked with the antischistosomal action of artemether.

The trematocidal effect of artemether and artesunate was confirmed also for *Clonorchis sinensis* and *Opisthorchis viverrini*. In rats infected with 40–50 metacercariae of *C. sinensis* efficacy of treatment with a single oral dose of 150 mg/kg of artesunate, artemether was compared with that of praziquantel and tribendimidine. Reduction of adult worm burden was 100 % for artemether and 89 and 80 %, respectively, for both anthelmintics. However, efficacy of the same dose of artemisinin against the juvenile stage of this trematode was considerably lower (57–59 %) in comparison with a high worm reduction with these drugs (Keiser et al. 2006b). Combination of PZQ or tribendimidine with either artesunate or artemether showed promising clonorchicidal properties (Xiao et al. 2008). The most effective combination was PZQ with artemether, at which many worms died due to extensive damage to tegument indicating the synergistic effect (Keiser and Vargas 2010). Worms collected from treated rats showed extensive damage to tegument already 8 h after artemether administration, including severe swelling, fusion, and vacuolization. Interestingly, the severity of tegumental changes did not progress further with time (Xiao et al. 2009). In rabbits infected with 300 metacercariae, the significant reduction of *C. sinensis* adult worm burden was achieved after oral administration of 120 mg/kg of artesunate (88.8 %) and artemether (67.2 %) (Kim et al. 2009). Rats are less suitable final hosts of *C. sinensis*, whereas rabbits are much more susceptible hosts to this fluke. Differences in the efficacy of two artemisinins in two different experimental models seem to be influenced by host factors, probably the physiology of rabbits and absorption/metabolisation rates. It is believed that a mechanism of action of artemisinins on *C. sinensis* and other liver flukes involves degradation of hemoglobin and generation of free heme, a possible target for peroxidic drugs (Uttinger et al. 2007).

Opisthorchiasis is a neglected tropical disease caused by the liver fluke *Opisthorchis viverrini* that affects the poorest people in Cambodia, Laos, north-eastern parts of Thailand, and Vietnam and praziquantel is the only available drug for this infection (Keiser and Uttinger 2005). Artesunate and artemether at a dose of 400 mg/kg given to hamsters infected with *O. viverrini* resulted in worm-burden reductions of 77.6 % and 65.5 %, respectively (Keiser et al. 2006b), but when given to patients during randomized trial who had *O. viverrini* infections, artesunate did not show any trematocidal effect (Soukhathammavong et al. 2011).

In contrast with a considerable interest in extracts from *Artemisia* spp. or pure artemisinins as trematocidal agents, to date a few studies examined their effects on

other classes of parasitic helminths. In the recent study of Squires et al. (2011) artemisinin was tested for efficacy against *Haemonchus contortus* in a gerbil model of infection. Gerbils (*Meriones unguiculatus*) were recognized as a suitable model of *H. contortus* infection for anthelmintic testing, which offers the advantages of requiring smaller product quantities, greater standardization in testing conditions and evaluation of adverse effects on host pathology (Conder et al. 1990, Königová et al. 2008). Single oral doses of 400 mg/kg given on day 6 after infection or 200 mg/kg BW artemisinin administered daily for 5 days (between days 4 and 8 post infection) had no effect on reduction of pre-adult stages (L4) of this nematode. The lack of activity for L3 and L4 stages in gerbils, can not exclude that artemisinin and derivatives, when given intramuscularly, will be effective on the adult stage.

The susceptibility of cestodes to treatment with artemisinin derivatives was evaluated on the larval stage (protoscoleces) of the medically important cestodes *Echinococcus multilocularis* and *Echinococcus granulosus* (Spicher et al. 2008). As in the case of experimental infection with *H. contortus* in gerbils, no in vivo effect of a total dose of 200 mg/kg of artemisinin, artesunate, artemether or dihydroartemisinin was observed in infected and treated mice evaluated as the reduction in parasite cyst weight. Treatment began on week 8 post infection and a daily dose of 100 µl/mouse was applied by intragastric inoculation. This observation was in contrast with in vitro study, in which dihydroartemisinin and artesunate (10–40 µM) caused a 90 % reduction in viability of protoscoleces occurring on day 6 or 4 of incubation, respectively. Two other artemisinins were less effective. According to the authors, artemisinins and their metabolites were perhaps not delivered and accumulated in the parasite tissues in adequate quantities, because parasites are surrounded by an acellular laminated layer that represents a barrier for drugs. A short-elimination half-life of the active metabolite DHA probably contributed to its low bioavailability for echinococcus cysts (Li et al. 1998).

2.4 Concluding Remarks

Many people in developing countries still suffer or die from malaria, schistosomiasis, filariasis, and other infectious diseases, whereas, in developed countries, parasitic infectious diseases, especially those that are caused by opportunistic infection resulting from immunosuppressants and HIV/AIDS, are increasing. Moreover, the emergence of strains that are resistant to the current front-line drugs is reported nearly in all countries with intensive livestock production, emphasizing the need to search for compounds with antiparasitic activity for further drug development. Richness of bioactive molecules present in marine and terrestrial organisms and numerous records from traditional medicine and ethnoveterinary practice provide a starting point for pharmacological research. Essential parasite-specific systems, that differ from those of the hosts, represent attractive targets for

specialized chemotherapy, as illustrated by glutamate-gated chloride channels and the specific activator avermectin. Isolation and chemical synthesis of sesquiterpene lactones with a unique endoperoxide bridge in their molecule—artemisinins, is another example of high potential of natural compounds as the source of novel anti-infective drugs. Very promising materials, so far very little explored in anthelmintic drug discovery, are marine organisms. In this respect the organic compound, nafuredin is a very potent agent against nematodes due to specific inhibition of their essential metabolic pathways in nanomolar concentrations and a very low toxicity to mammalian cells.

Plant extracts are good starting point and the active principles that induced anthelmintic activities might be found in one or more classes of phytochemicals. The variations in activities of certain plant are due to the differences in the proportion of the active components responsible for the tested activity. Their proportions might be different in extracts obtained with different types of extraction solutions. The synergistic effect of several bioactive components is considered an important factor responsible for the variations in anthelmintic activity of extracts from different plants. Although a clear dose-dependent effect has been found for some plant extracts, the biochemical nature of the secondary compounds is also suspected to partly explain the variability of results found under *in vitro* and *in vivo* conditions. Only limited available data support the “indirect” hypothesis which relates the effects on the worms to an improved host immune response.

It is possible that extracts showing high efficacy in the range of 10–100 µg/ml *in vitro* contain a few compounds, which target different sites in helminths. Thus, plants with a high proportion of essential oils and flavonoids were the most effective against gastro-intestinal nematodes, however similar nematocidal effect of both components when tested individually, was observed in the range of milligrams. Two separate models of action could be attributed to the efficacy of essential oils in the treatment of protozoan and probably also helminth infections: combination of immunomodulatory and direct antiparasitic effects. Plants containing high amounts of polyphenols might also have applications for protecting proteins from degradation in the rumen, increasing the efficiency of microbial protein synthesis in the rumen and decreasing methane emission, for using as antioxidants, antibacterial as well as anthelmintic agents. Many studies concluded that the higher efficacy of a plant extract is usually achieved by increasing the dose or by repeated dosing for few days. Some of the active components (for example: condensed tannins) may have direct nematocidal and also anti-nutritional effects in livestock, such as reduced food intake and performance, therefore it is essential to validate both anthelmintic and side effects of isolated plant products. Flavonoids, in particular some of their components like thymol, have also exerted activity *in vitro* and *in vivo* against flatworms, whereas in nematodes their cuticle seems to prevent absorption necessary for further effects. Nevertheless, they can be absorbed via the oral route. Preliminary data obtained in flatworm models suggested their interaction with the worm-specific physiological processes. Other mode of anthelmintic activity was suggested for glycosides against filarial nematodes, whereas saponins probably affected permeability of the nematode cuticle as result

of their surfactant properties. Higher plants-derived alkaloids so far examined, were shown to interfere with vital functions of tissue-dwelling nematode larvae at low concentrations. The special group of molecules isolated from several fruits tissues are cysteine proteases, which are able to selectively affect parasitic nematodes in vitro and showing high efficacy in vivo in monogastric animals. Therefore, through the knowledge and understanding gained from basic pharmacological research in in vitro and in vivo controlled studies, an array of bioactive molecules could be discovered for further clinical applications in human and veterinary parasitology.

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