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Review

Ajuga remota Benth.: From ethnopharmacology to phytomedical perspective in the treatment of malaria

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ABSTRACT

Treatment and control of malaria have become more difficult with the spread of drug-resistant parasites and insecticide-resistant mosquito vectors. In the search for new antimalarial drugs, ethnopharmacological sources should merit more attention. Establishing the safety of traditional herbal medicines, along with identifying their active principles, are essential steps in the production of a properly standardized and accessible herbal medicine. Phytochemical characterization could also serve as a base for the development of new chemical compounds.

The genus of *Ajuga* belongs to the family Lamiaceae and contains at least 301 species. Many of these plants have been used in traditional medicine. *Ajuga remota* in particular is traditionally used as a herbal remedy for fever and infections, and is prescribed for malaria by 66% of the Kenyan herbalists.

A large number of compounds have already been isolated from *A. remota*, including ergosterol-5,8-endoperoxide (**6**), ajugarin-I (**1**), 8-*O*-acetylharpagide (**5**) and several phytoecdysteroids. *In vitro* pharmacological studies have been conducted on constituents of *A. remota* of which some of them displayed a concentration-dependent inhibition of chloroquine-sensitive and -resistant *Plasmodium falciparum* and *Mycobacterium tuberculosis*. Inhibition of parasitaemia was demonstrated in mouse models with *P. berghei*, supporting the traditional use of the plant against malaria.

In this state-of-the-art review, *A. remota* as a possible therapeutic tool for malaria is discussed.

Contents

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Introduction

Malaria is caused by *Plasmodium* protozoa and is transmitted by female Anopheles mosquitoes. Four species may cause malaria in man: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Malaria induced by *P. vivax* is the most common outside Africa, while the most lethal form is caused by *P. falciparum*. In recent years, some human cases of malaria have also occurred with *P. knowlesi*, a monkey malaria species that occurs in certain forested areas of South-East Asia. Although a rare disease in developed countries, malaria is highly prevalent in developing areas of the world. It is endemic in 105 countries and responsible for over 300–500 million clinical cases and more than a million deaths each year, particularly among children and pregnant women. Therefore, it is one of the most important tropical diseases. Travellers and immigrants are also at risk, with imported cases of malaria increasing in non-endemic areas (World Health Organisation 2007). Because malaria is not a public health priority in the industrialized countries, the market is seen as unprofitable and the disease has been classified as a 'neglected disease' (Eurordis 2005; Haffner et al. 2008). In view of the increasing drug resistance, new drugs or drug combinations are urgently needed for treatment. In Kenya, for example, 61–80% of parasites isolated from malaria cases are resistant to chloroquine and 30% of those treated with chloroquine are clinical treatment failures (Omar et al. 2001). Widespread resistance also occurs for most other antimalarial drugs. Artemisinin derivatives are currently the best available drugs, but resistance is developing and spreading from the Thai-Cambodian border (Dondorp et al. 2010).

Plants have always been considered as a possible alternative and rich source of new drugs. In Africa up to 80% of the population uses traditional medicine such as botanicals to help meet their health care needs. Antimalarial drugs such as quinine and artemisinin were either obtained directly from plants or developed using chemical structures of plant-derived compounds as templates. In tropical countries, modern medicines may not be available to most of the rural populations, and even if available, the cost of the drugs is usually prohibitive. This means that the majority of the populations in developing countries still largely depend on traditional medicinal remedies (World Health Organization 2002). Here we review the possible use of *Ajuga remota* in antimalarial therapy.

Botanical aspects

To what extent is Ajuga remota a unique species?

The genus of *Ajuga* L. or bugleweed belongs to the family Lamiaceae (the mint family), which also includes tall timber trees such as teak and aromatic or culinary species such as sage, thyme, mint, oregano, rosemary, lavender, basils and patchouli. Lamiaceae are also widely used in traditional medicine and to control insects (Kew Royal Botanic Gardens 2010). The *Ajuga* plant grows to 5–50 cm tall, with opposite leaves that are attractive. The flowers are mostly blue, purple or yellow. The petals are fused into an upper and a lower lip, hence the original family name 'Labiatae'. Many *Ajuga* species are used in horticulture as groundcover or border, and in rock gardens, and some are regarded as weeds. A large number of varieties (cultivars) are used in gardens because of their varied blooms of different colours. Ethnopharmacological surveys have revealed that about 20 species are used in traditional medicine mostly in Africa, Asia and China. *Ajuga remota* in particular is traditionally used as an herbal remedy for fever, infection, malaria and mycobacterial diseases (Israili and Lyoussi 2009). It is prescribed by 66% of Kenyan herbalists for the treatment of malaria (Kuria et al. 2001).

Different synonyms of this species can be found in literature: *Ajuga integrifolia* Buch.-Ham., *Ajuga bracteosa* Wall. ex Benth. (homotypic synonym) and *Ajuga remota* Benth. (heterotypic synonym) (Govaerts et al. 2010; Hsi-wen and Hedge 1994).

Phytochemical aspects

What is the phytochemical profile of Ajuga remota?

A large number of compounds has been isolated from *A. remota*/*A. bracteosa*, including neo-clerodane diterpenoids (e.g. ajugarin-I (**1**)), phytoecdysteroids (phytoecdysones, e.g. 20-hydroxyecdysone (**4**)), iridoid glycosides (e.g. 8-*O*-acetylharpagide (**5**)), flavonol glycosides and ergosterol-5,8-endoperoxide (**6**) (Israili and Lyoussi 2009; Kuria et al. 2002; Ramazanov 2005; Riaz et al. 2007).

A short overview of the constituents and their chemical structure can be found in Fig. 1. For the extensive chemical profile, we refer to the online supplement (Appendix A). Numbers in bold are also included in this table.

Pharmacological aspects

Is there scientific evidence to support the ethnopharmacological use?

A limited number of *in vitro* and *in vivo* studies have been conducted on different extracts and constituents of *A. remota* to determine its antiplasmodial potential.

In vitro assays

The antiplasmodial activity of the ethanolic leaf extract of *A. bracteosa* (ELEAB) was evaluated *in vitro*, assessing the inhibition of schizont maturation. The extract was found to inhibit *P. berghei* schizont maturation in a dose-dependent manner, with an IC_{50} of 10 μg/ml after 21 h. Maximum schizont maturation inhibition (72.4%) was observed with 100 μg/ml concentration of extract. Chloroquine was used as a positive control and exhibited 96.0% inhibition at a concentration of 10 μM (Chandel and Bagai 2011).

The *in vitro* antiplasmodial properties of aqueous decoctions, ethanolic macerates, and petroleum ether, methanol and water Soxhlet extracts of *A. remota* were investigated against a chloroquine-sensitive (FCA/20GHA) and -resistant (W2) strain of *P. falciparum*. The activity was assessed by the parasite lactate dehydrogenase (pLDH) assay method. A concentration-dependent growth inhibition was shown, the ethanol macerate being the most active with an IC_{50} of 55 and 57 μg/ml against respectively the chloroquine-sensitive and -resistant strain. Chloroquine showed an IC_{50} of 13 and >5000 ng/ml, respectively (Kuria et al. 2001).

Other researchers prepared several organic and aqueous extracts (hexane, chloroform, ethyl acetate, methanol and water) of the leaves of *A. remota*. *In vitro* tests showed a moderate activity against a chloroquine-sensitive (K39) strain of *P. falciparum* (IC_{50} = 20–100 μg/ml), in comparison to chloroquine

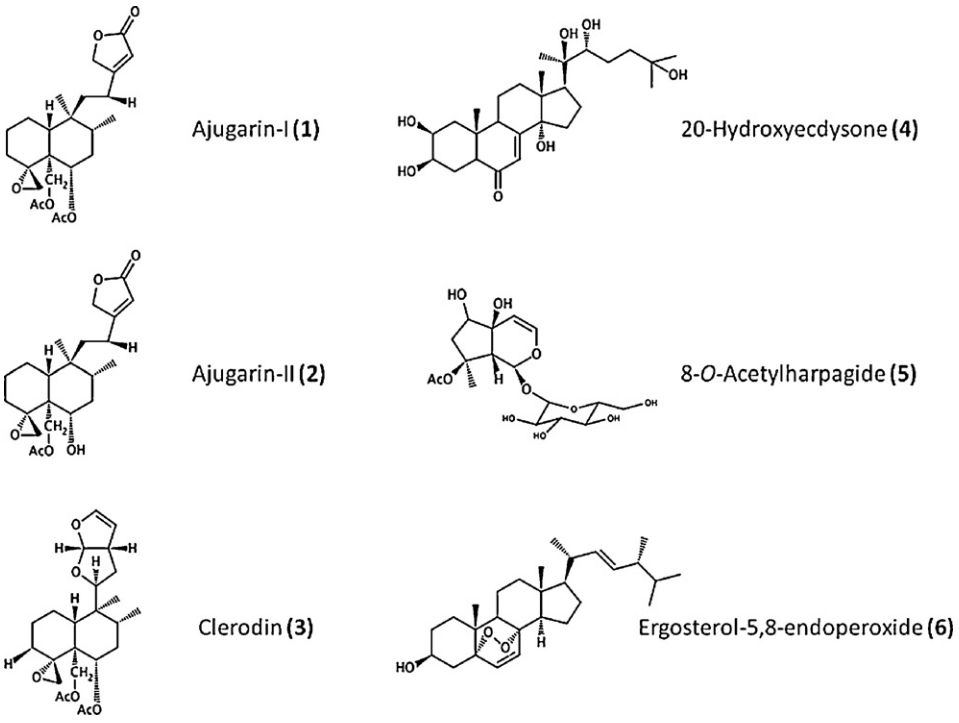

Fig. 1. Short overview of chemical profile of *Ajuga remota*/*Ajuga bracteosa*.

(IC_{50} = 0.019 μg/ml). The methanol extract presented the best activity (IC_{50} = 21.60 μg/ml) (Muregi et al. 2004).

Further phytochemical research focussed on three constituents: ajugarin-I (**1**), 8-*O*-acetylharpagide (**5**) and ergosterol-5,8-endoperoxide (**6**). These compounds were either isolated from the plant or synthesized. In the pLDH assay, ajugarin-I (**1**) was moderately active against the FCA20/GHA strain (IC_{50} = 23.0 μM), as compared to chloroquine (IC_{50} = 0.041 μM). The synthesized ergosterol-5,8-endoperoxide (**6**) was about three times more potent than ajugarin-I, with an IC_{50} of 8.2 μM. In contrast, 8-*O*-acetylharpagide (**5**) demonstrated no antiplasmodial activity even at the highest test concentration of 500 μM. Cytotoxicity was evaluated, using a skin carcinoma cell line (A431). Ajugarin-I (**1**) and ergosterol-5,8-endoperoxide (**6**) were not cytotoxic at the highest test concentrations used for antiplasmodial activity (ED_{50} > 88 μM and > 23 μM). In the cytotoxicity assay, 8-*O*-acetylharpagide (**5**) caused a concentration-dependent inhibition of cell proliferation (ED_{50} = 310 μM) which is approximately seven times less toxic than the standard antineoplastic reference fluorouracil used in the test (ED_{50} = 44 μM) (Kuria et al. 2002).

In a bioassay-guided search for antimycobacterial compounds from higher plants, the crude methanol extract of the aerial parts of *A. remota* gave 97% inhibition against *Mycobacterium tuberculosis* (H_{37}Rv) at 100 μg/ml (Cantrell et al. 1999). A detailed chemical investigation of the active fractions resulted in the isolation of ergosterol-5,8-endoperoxide (**6**), ajugarin-I (**1**), ajugarin-II (**2**) and clerodin (**3**). Ergosterol-5,8-endoperoxide, also synthesized from the commercially available ergosterol, was the only active compound (MIC = 1 μg/ml). Modification of the structure at the site of the free hydroxyl group or the endoperoxide bound reduced the activity, suggesting that both moieties are necessary for activity. Ajugarin-I, ajugarin-II and clerodin were inactive with MICs of >128 μg/ml. These bioassays were performed using the BACTEC 460TB Method (B460), a radiometric method for reliable and rapid testing of susceptibility of *M. tuberculosis* to different drugs (Cantrell et al. 1999). More recently, the BACTEC MGIT 960 System (M960) has been introduced as a less labour-intensive alternative. This fully automated, continuous-monitoring system exploits the fluorescence of an oxygen sensor to detect growth of *Mycobacteria* in culture. Research has shown that the M960 system performs as well as the B460 system for testing the susceptibility of *M. tuberculosis* to different drugs (Scarparo et al. 2004; Tortoli et al. 1999). However, it was shown that ergosterol-5,8-endoperoxide had no detectable antimycobacterial activity in the M960 system, but significant concentration-dependent inhibition in the B460 system. Concentrations used ranged from 0.625 to 50 μg/ml. These results suggest that the evaluation and interpretation of the antimycobacterial activity of a compound may be significantly affected by the method employed. The reason that the M960 system shows no activity may be due to the much faster growth rate of the organism in the used medium (Duarte et al. 2007). With the B460 method, ergosterol-5,8-endoperoxide has a high *in vitro* antimycobacterial activity compared to standard antituberculosis drugs. These include isoniazid, rifampin, streptomycin, ethambutol and pyrazinamide and have a MIC of 0.05, 0.25, 2.0, 3.8 and 100 μg/ml, respectively (Cantrell et al. 2001).

In vivo assays

The *in vitro* antiplasmodial activity has been confirmed *in vivo* (Chandel and Bagai 2010). The ethanolic leaf extract of *A. bracteosa* (ELEAB) was prepared using Soxhlet extraction. The LD_{50} of ELEAB was more than 5 g/kg body weight of naive mice, illustrating the safety of the extract. BALB/c mice were infected with the chloroquine-sensitive *P. berghei* (NK65) strain and three different protocols were conducted. First, the antiplasmodial activity was evaluated in early infection (suppressive test). During 4 days, infected mice were orally treated with different doses of ELEAB or chloroquine (positive control). Significant, dose-dependent parasitaemia suppression was witnessed for ELEAB. The activity of the highest concentration (1000 mg/kg/day) was comparable to the suppression by chloroquine (86.2%), however, 60% mortality was observed with this concentration on day 7. Therefore, this concentration was not tested further for repository and curative activity of ELEAB. Secondly, evaluation of the repository

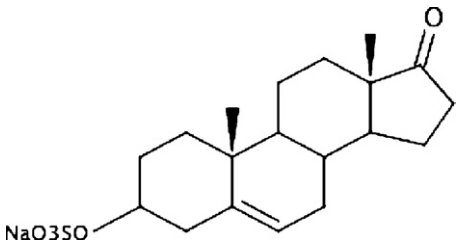

Fig. 2. Dihydroepiandrosterone sulphate (DHEAS).

activity was performed (preventive test). During 4 days, mice were administered orally the extract or pyrimethamine (positive control). The mice were infected on the fifth day and the parasitaemia was measured 72 h later. ELEAB produced a significant and dose-dependent repository activity. Pyrimethamine exerted a considerable higher chemosuppression compared to extract treated groups. Third, the antiplasmodial activity in established infection was evaluated (curative test). Seventy-two hours after infection, mice were administered orally ELEAB or chloroquine (positive control) for 5 days. Monitoring parasitaemia levels confirmed the dose-dependent suppression and showed an increase in mean survival time (MST). In conclusion, these results show that ELEAB can inhibit parasitaemia in a dose-dependent manner and also enhance the MST. These findings support the traditional use of the plant in the treatment of malaria (Chandel and Bagai 2010).

In contrast, an aqueous (hot water) extract of the whole plant was found ineffective in the 4-day suppressive model. On day 4 post-infection (p.i.), no suppression of the parasitaemia was observed and the 20% increase in mouse survival was not remarkably different from the untreated, infected group. In a drug combination study, synergistic effects of the extract with chloroquine were examined. Oral administration of the extract and chloroquine on day 4 p.i. showed good tolerance. No treated mice died within 48 h of extract administration, ruling out acute toxicity. Although no suppression of the parasitaemia was seen on day 11 p.i., there were 40% more surviving mice on day 14 p.i. compared to the control group (chloroquine). Nevertheless, the authors concluded that *A. remota* had no antiplasmodial activity *in vivo* (Muregi et al. 2006). The next year, they published a similar study, administering a methanol extract of the whole plant. Parasitaemia suppression and mouse survival did not improve significantly (Muregi et al. 2007).

Clinical aspects

How does sexual maturity affect the immune response?

Since *A. remota* contains molecules which resemble dehydroepiandrosterone sulphate (DHEAS, Fig. 2, an immunomodulatory steroid in humans), it is possible that the extracts have immunostimulatory properties. Therefore, we will first discuss some immunological aspects associated with malaria.

In high transmission areas, *P. falciparum* infection is more frequent and severe in children than in adults. Therefore it was thought that resistance to malaria results from cumulative exposure or from additional exposure to poorly immunogenic antigens. However, acquired immunity develops more quickly in adults than in children, as witnessed in malaria-naive migrants to areas of endemicity. This suggests that human pubertal development contributes greatly to resistance, separate from any effect of increasing cumulative exposure (Kurtis et al. 2001; Leenstra et al. 2003). The development of resistance before (12- to 14-years-old), during (15- to 20-years-old) and after (21- to 35-years-old) puberty was examined in Kenyan males. Resistance to *P. falciparum* increased with age during puberty but not before puberty and is demonstrated by a lower frequency and density of parasitaemia (Kurtis et al. 2001). In pubertal girls from an area of intense transmission in western Kenya, DHEAS level was significantly associated with decreased parasite density (even after adjustment for age) and was also positively correlated with haemoglobin level (Leenstra et al. 2003). These findings support the hypothesis that host pubertal development, indicated by DHEAS level, is a significant predictor of increasing resistance to malaria infection, independent of age and cumulative exposure (Kurtis et al. 2001; Leenstra et al. 2003).

DHEA (dehydroepiandrosterone) and DHEAS are synthesized from cholesterol in the adrenal glands and act as intermediates in sex steroid biosynthesis (Ayi et al. 2002). The maximum circulating levels of DHEA/DHEAS (10 nM/10 μM) are seen at the age of 20–30 years and thereafter levels gradually decrease. By 70 years of age, DHEA/DHEAS levels are 20–30% of those achieved in early adulthood. This fall in serum DHEA/DHEAS levels with age in men and women is termed the 'adrenopause' (Hazeldine et al. 2010). Although inactive as a hormone, DHEA has potent immune modulatory functions, exhibiting both immune stimulation and counteracting the immune-suppressive effects of glucocorticoids. In addition to controlling the inflammatory process, *in vitro* DHEAS toxicity has been demonstrated against a chloroquine-sensitive strain of *Plasmodium* parasites (IC_{50} = 19 μM). As a potent inhibitor of mammalian glucose 6-phosphate dehydrogenase (G6PDH), DHEA protects mammalians cells from an exacerbated immune reaction. G6PD deficiency is known to exert antimalarial protection via enhanced opsonization and phagocytosis of rings, the early forms of the parasite (Ayi et al. 2002; Cordeiro and Thiemann 2010; Hazeldine et al. 2010). Furthermore, DHEAS has a protective effect on haemoglobin, which could be explained by two mechanisms. First, because DHEAS is associated with decreased parasite density, higher DHEAS levels would be associated with decreased parasite-induced erythrocyte lysis. Secondly, malaria-associated anaemia is due, in part, to the anaemia of inflammation, which is characterized by dyserythropoiesis, shunting of iron to non-bioavailable forms, decreased erythropoietin production and responsiveness, and decreased erythrocyte survival. As a potent downmodulator of the proinflammatory cytokines (TNF-α, IL-6 and IL-1) that mediate this form of anaemia, DHEAS may attenuate the deleterious consequences on haemoglobin level (Leenstra et al. 2003). Lastly, early administration of DHEA (three subcutaneous doses of 50 μg per week) was protective in a model of pulmonary tuberculosis in mice (Hernández-Pando et al. 1998).

These findings are of particular relevance to the development and implementation of a malaria vaccine, because they imply that vaccination of prepubertal children may not result in adultlike immunity. The use of immunoadjuvants specifically designed to boost adultlike immunity, may be required (Leenstra et al. 2003). On the other hand, to increase vaccine efficacy in the elderly, it has been attempted to reverse their impaired immune response by administering DHEA as a vaccine adjuvant (Hazeldine et al. 2010). In animal studies, DHEA administration augmented the immune response of aged mice to a range of vaccines, such as the influenza vaccine (single subcutaneous injection of 10 mg DHEA, four hours prior to immunization) and the 23-valent pneumococcal polysaccharide (Pnu-Imune) vaccine (subcutaneous injection of 10 μg DHEA) (Danenberg et al. 1995; Garg and Bondada 1993). In one study among elderly volunteers, a single subcutaneous injection of 7.5 mg DHEAS administered simultaneously to influenza vaccination significantly increased antibody titers in individuals with low pre-vaccination antibody titers and circulating DHEAS levels. This suggests that low serum DHEAS may act as a biomarker for poor vaccine responses and indicates a beneficial response to DHEA or DHEAS supplementation (Hazeldine et al. 2010).

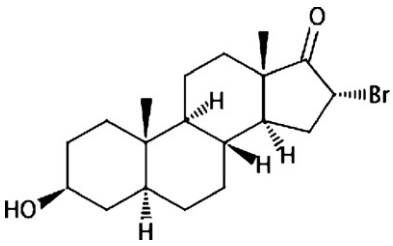

Fig. 3. 16α-Bromoepiandrosterone (HE2000).

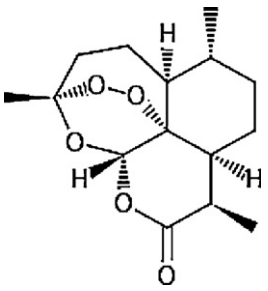

Fig. 5. Artemisinin.

16α-Bromoepiandrosterone (α-epi-Br or HE2000, Fig. 3) is a synthetic DHEA analogue that showed significant *in vitro* growth inhibition of chloroquine-sensitive and -resistant strains of *P. falciparum* (IC_{50} between 4.8 and 7.5 μM). Activity in a rat model of *P. berghei* (ANKA) malaria was observed. Rats were intravenously injected with chloroquine (40 mg/kg) or α-epi-Br (30 mg/kg or 60 mg/kg) after infection, for a total of four injections. All the treated groups showed a significant decrease of parasitaemia on day four and on day 11, compared to the saline control. There was no significant difference between the different treatment groups and the effect of α-epi-Br appeared to be dose-independent. Immune modulation was cited as the probable mode of action (Freilich et al. 2000). In pilot studies on 42 patients with confirmed uncomplicated *P. falciparum* malaria, a seven-day course of 100 mg daily of α-epi-Br was given by either buccomucosal administration (25 mg tablets) or intramuscular injection (50 mg/1 ml vials). Within the treatment period, 41 patients achieved a 50% reduction in blood levels of parasites, of which 32 patients cleared malaria parasites below detectable levels. Proof of efficacy is not possible with this study because an experimental control group is lacking. Nevertheless, the possible role of α-epi-Br as an effective immune agent should be further investigated. α-epi-Br may be a useful addition to treatment strategies for drug resistant malaria strains (Frincke et al. 2007). α-epi-Br is a potent inhibitor of G6PDH, as is the case with DHEA and DHEAS (see above). Although plasmacompatible antimalarial concentrations of α-epi-Br did not inhibit G6PDH activity *in vitro*, a remarkable stimulation of ring phagocytosis took place (Ayi et al. 2002). α-epi-Br could also improve the course of *M. tuberculosis* infection, as observed in a preclinical model. Three times a week, doses of either 0.02 mg, 0.2 mg or 2.0 mg of α-epi-Br were subcutaneously administered to mice. This reduced the bacterial load associated with progressive tuberculosis, with or without antibiotic therapy (Hernández-Pando et al. 2005).

Secondary metabolites of Ajuga remota and sexual maturity

Among higher plants the *Ajuga* spp. produce considerable amounts of ecdysteroids, neo-clerodane diterpenes and other secondary plant metabolites having both toxic and insect developmental and reproduction disrupter (IDRD) activity. In terms of antiplasmodial activity, especially ergosterol-5,8-endoperoxide and the phytoecdysteroids seem of interest (Fekete et al. 2004).

Ergosterol-5,8-endoperoxide

As discussed above, ergosterol-5,8-endoperoxide (**6**) is not cytotoxic and is active against both *M. tuberculosis* and chloroquine-sensitive *P. falciparum* (FCA 20/GHA) (Cantrell et al. 1999; Kuria et al. 2002). Looking at the molecular structure of this compound (Fig. 4), the antiplasmodial action might be due to its endoperoxide structure and/or due to the steroid structure. Obviously, still other mechanisms may be involved.

Steroid structure. Because ergosterol-5,8-endoperoxide (**6**) has a similar steroid backbone as DHEAS (Fig. 4), we can hypothesize that it displays a similar mechanism of action as DHEAS. Possibly, ergosterol-5,8-endoperoxide contributes to the realization of immunity against *Plasmodium* as seen by DHEAS during pubertal development.

Endoperoxide structure. Endoperoxides such as artemisinin (Fig. 5) are fast acting drugs, which exert their antimalarial effect within an hour of administration (Pandey et al. 1999). They kill nearly all of the asexual stages of parasite development in the blood (including young ring-stages), and also affect the sexual stages of *P. falciparum* (gametocytes), but they do not affect pre-erythrocytic development or the latent stages of *P. vivax* and *P. ovale* in the liver (the hypnozoites). Artemisinin-based Combination Therapy (ACT) is currently recommended by the WHO as the first-line treatment for all falciparum malaria in malaria endemic countries. Artemisinins are also active against multidrug resistant (MDR) *P. falciparum*, but reduced susceptibility is emerging from the Thai-Cambodian border (White 2008).

Although the exact antimalarial mechanism is still an enigma, several hypotheses have been proposed. It was first believed that the haem-catalyzed cleavage of the endoperoxide bridge forms a free radical, followed by specific and selective alkylation of some malarial proteins (Fig. 6), but other mechanisms are involved (Meshnick et al. 1996).

Host haemoglobin is degraded inside the parasite by a series of proteolytic enzymes to release peptides and amino acids, required for development and to create space within its digestive vacuole. During this process a build up of haematin occurs which is potentially toxic to the parasite (Fig. 7). To circumvent this toxicity, the parasite has developed a mechanism whereby haematin undergoes biomineralization to form insoluble non-toxic

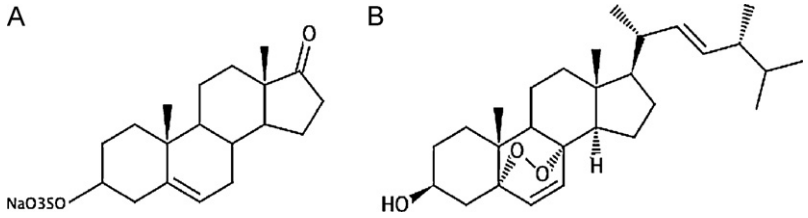

Fig. 4. Structure of (A) DHEAS and (B) ergosterol-5,8-endoperoxide.

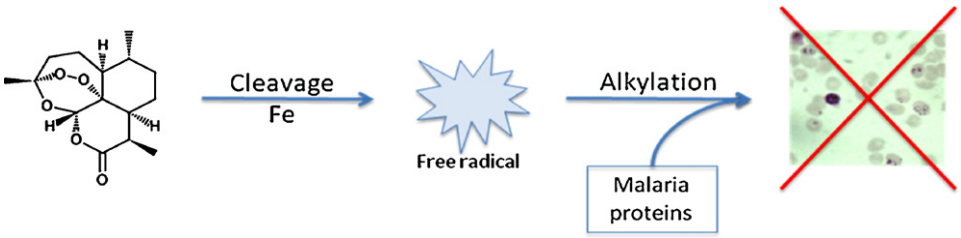

Fig. 6. Artemisinin: proposed mechanism of action by Meshnick et al. (1996).

haemozoin (malaria pigment) (O'Neill et al. 2010). Earlier findings point towards a three-step effect of endoperoxide drugs on the parasite (Fig. 7) (Pandey et al. 1999). (1) The formation of a covalent complex with haem as well as (2) direct inhibition of the haem polymerization pathway, would initiate accumulation of haem. Together with inhibition of malarial digestive vacuole proteases, these are important targets for the antiparasitic activity of the drugs but may not be sufficient to explain the selective and fast antimalarial action compared to quinoline antimalarials. (3) The endoperoxides additionally initiate the breakdown of the malaria pigment already present in the parasite food vacuole. This reaction could easily lead to a rapid increase in the endogenous haem level, which may not be detoxified by the usual haem polymerization pathway since this activity is already blocked by the endoperoxide.

Recently, insights from medicinal chemistry studies (Li and Zhou, 2010) led to three hypotheses for the mechanism of action of artemisinin (Fig. 8). The first model is that artemisinin binds specifically to a target, such as the protein PfATP6, in malaria parasites. PfATP6 is the only SERCA-type (sarcoplasmic/endoplasmic reticulum calcium ATPase) of *P. falciparum*. The second speculates that artemisinin is uniquely activated by haem in malaria vacuoles. The third hypothesizes that artemisinin is activated by malaria mitochondria and the resulting free radicals damage non-specifically the surrounding molecules.

Finally, the molecular mechanism of action of artemisinin has been reviewed by O'Neill et al. (2010). There is strong evidence to suggest that the primary activator is an iron source, in the form of Fe^{2+}, haem or both. Although protein alkylation in *Plasmodium* is well established, a single molecular target with a direct role in cell death is yet to be identified. Apparently, cellular response to artemisinin in *Plasmodium* is of a multi-faceted nature, which may explain the use against otherwise multi-drug resistant strains. In conclusion, the molecular target of artemisinin is still under debate.

Both artemisinin and ergosterol-5,8-endoperoxide have their endoperoxide in common. However, the steroid structure in ergosterol-5,8-endoperoxide may add to the targeting of the parasite.

Phytoecdysteroids

Ecdysteroids (also called ecdysones) are a group of natural polyhydroxysteroids present in plants (phytoecdysteroids) and animals (zooecdysteroids). Some ecdysteroids, such as ecdysone,

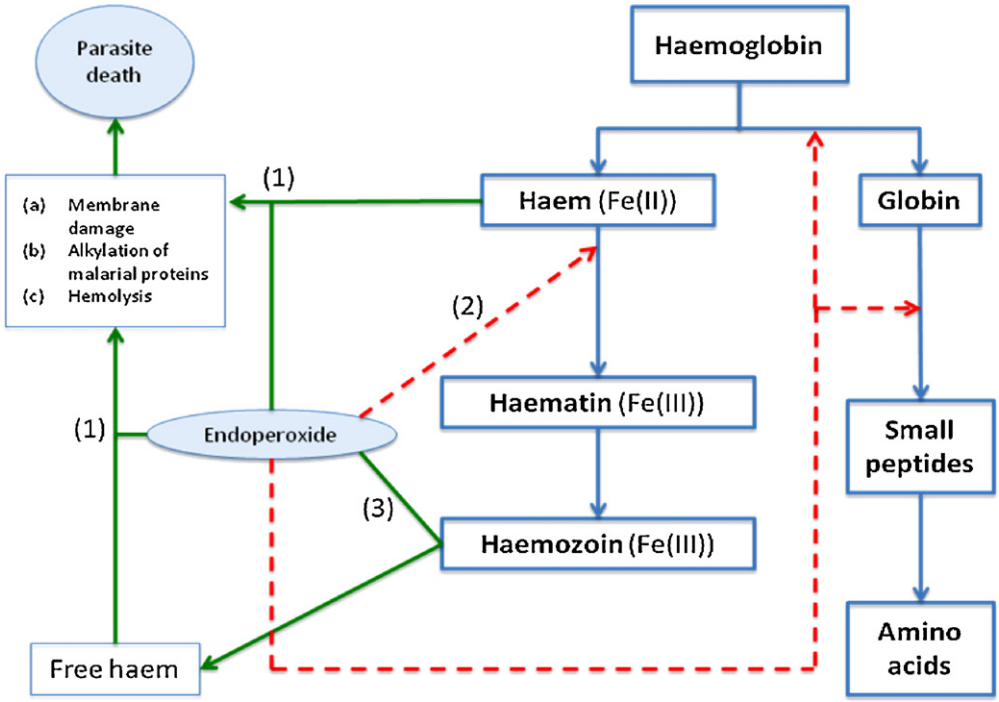

Fig. 7. Detoxification of haemoglobin (toxic haematin is converted by the parasite to an insoluble non-toxic compound called haemozoin) (O'Neill et al., 2010) and proposed mechanism of action of artemisinins (1, 2 and 3) (Pandey et al., 1999) Dashed red arrows: inhibition of the haem polymerization pathway (2) and of malarial digestive vacuole proteases. Solid green arrows: formation of a covalent complex with haem (1) and breakdown of the malaria pigment already present in the parasite food vacuole (3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

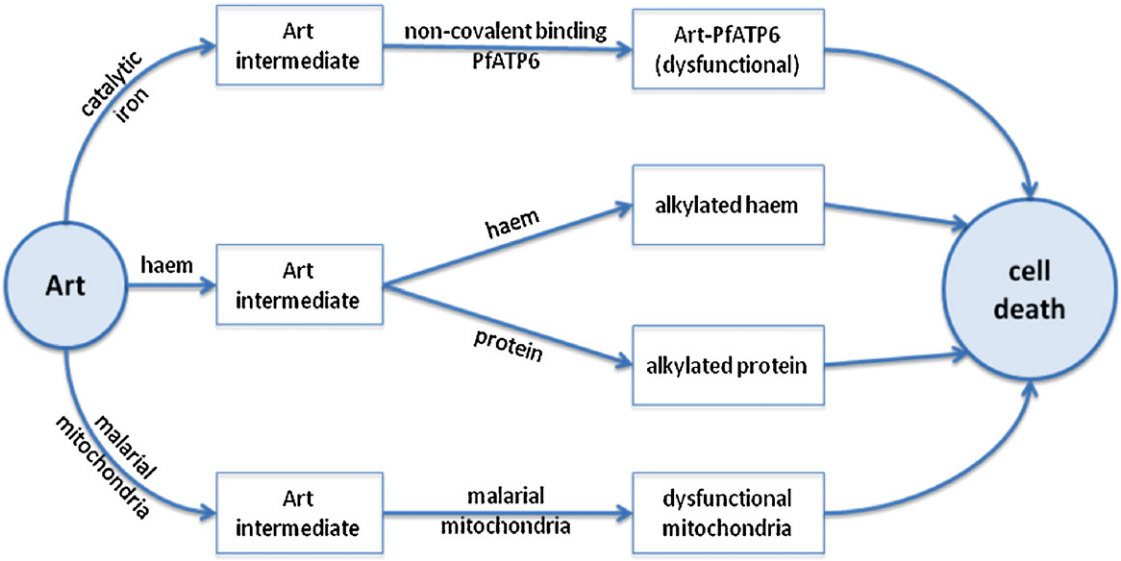

Fig. 8. Proposed biological models for the action of artemisinin (Art). (1) The PfATP6 model: artemisinin undergoes activation by reacting with catalytic iron, and after non-covalent specific interaction with PfATP6, the formed free radicals then exert irreversible damage to this target protein. (2) The haem model: artemisinin is activated by haem, followed by alkylation of haem and/or other malarial proteins. (3) The mitochondria model: malarial mitochondria specifically activate artemisinin. The activated artemisinin then induces free radicals production and mitochondrial membrane depolarization. No specific protein targets are implicated (Li and Zhou, 2010).

20-hydroxyecdysone (**4**) and ajugasterone C, can be found in plants and animals (Dinan 2001). The phytoecdysteroid profile varies in amount and composition between plant species, but also depends on plant organs, season, origin and habitat (Fekete et al. 2004).

Production of phytoecdysteroids by Ajuga species. The *Ajuga* genus is unique for the great variety of phytoecdysteroids, with a broad spectrum of biological and pharmacological actions. They accumulate in various plant organs such as flowers, stems, leaves, roots and fruits, usually present in small quantities of the order of 0.01–0.1% of the plant dry weight. The major phytoecdysteroid is 20-hydroxyecdysone (**4**), which is also the active moulting hormone of insects (Israili and Lyoussi 2009; Ramazanov 2005; Rharrabe et al. 2007).

As secondary plant metabolites, phytoecdysteroids stimulate protein synthesis in plants, activate cell mitosis and possibly act as plant growth regulators. They also protect plants against phytophagous insects either by feeding deterrence or endocrine disruption upon ingestion, which leads to death. However, several insect species remain unaffected by ecdysteroids present in their food even at high concentrations. These resistant insects have developed effective detoxification/inactivation mechanisms. On the other hand, a beneficial effect of ecdysteroids is improvement of silk yield by feeding 20-hydroxyecdysone (**4**) to silkworm (Israili and Lyoussi 2009; Rharrabe et al. 2007).

Possible antiplasmodial activity of phytoecdysteroids. Although detailed toxicological data are lacking for phytoecdysteroids, their acute toxicity to mammals seems to be extremely low (Israili and Lyoussi 2009). In mice, LD_{50} values of 20-hydroxyecdysone (**4**) after i.p. injection and oral application, were 6.4 g/kg body weight and 9 g/kg body weight respectively. Furthermore, 20-hydroxyecdysone was not embryotoxic when injected into developing chicken eggs (Dinan 2009).

The literature reporting the effects of ecdysteroids on mammals has been reviewed by several researchers and summarized by Dinan (2009). A wide range of beneficial pharmacological activities is claimed for phytoecdysteroids. In *A. remota* they can contribute to a possible antiplasmodial activity by their anabolic, adaptogenic and immunoprotective activities.

Anabolic activity: The backbone of phytoecdysteroids is identical to that of steroid hormones, which led to the comparison of their anabolic effects. As summarized by Sláma and Lafont (1995), 20-hydroxyecdysone (**4**) increased physical performance without training in a forced swimming test in mice and also caused anabolic increase of body mass. Stimulation of protein synthesis by up to 20% in mouse and human skeletal muscle cells by ecdysteroids (20-hydroxyecdysone, polypodine B and ponasterone) has been demonstrated (Gorelick-Feldman et al. 2008). According to other researchers, 20-hydroxyecdysone increased body mass and muscle fibre size in rats and may provide an alternative to anabolic–androgenic steroids in the treatment of muscle atrophy (Tóth et al. 2008). In contrast to anabolic vertebrate steroid hormones, the anabolic actions of 20-hydroxyecdysone is supposedly not associated with the adverse androgenic, antigonadotropic or thymolytic side effects. Phytoecdysteroids also appear not to have androgenic or (anti)oestrogenic effects (Sláma and Lafont 1995). However, in a double-blind, placebo-controlled trial 30 mg/day of 20-hydroxyecdysone did not significantly affect anabolic or catabolic response to resistance training, body composition, or training adaptations (Wilborn et al. 2006).

Adaptogenic activity: Ecdysteroids are believed to possess adaptogenic, antidepressive, tonic, and roborant properties, i.e., they enhance the ability to cope with stress and enhance resistance to tiredness. Certain plants (*Achyranthes* spp., *Cyathula* spp., *Leuzea carthamoides*), have been used as tonics, diuretics, and adaptogens in traditional Chinese and Asiatic medicines long before they were known to contain large amounts of ecdysteroids (Sláma and Lafont 1995). *L. carthamoides* is the basis of a green "tea", maralan, consumed extensively in Central Europe and said to improve general well-being, increase appetite, and improve digestion. Although these effects have been repeatedly ascribed to the ecdysteroid content of the plant, there is little evidence to directly associate them with this particular class of compounds (Dinan 2009).

Immunoprotective activity: According to Dinan (2009), ecdysteroids may show some immunomodulatory effects in mice and rats and have anti-inflammatory activity in rodents. The immunomodulatory effect of 20-hydroxyecdysone (**4**) has been studied in humans. It acts as a lymphocyte and neutrophil modulator *in vivo*. *In vitro*, it activates T-cell CD2 presentation that is

suppressed both in secondary immunodeficient persons and pharmacologically by increased cAMP levels (Trenin and Volodin 1999).

Discussion

Which questions remain?

There is evidence for the traditional use of *Ajuga remota* in the treatment of malaria. *In vitro* research identified ergosterol-5,8-endoperoxide as the most potent antiplasmodial constituent, followed by ajugarin-I. These compounds were not cytotoxic at concentrations needed for antiplasmodial activity. In contrast, 8-*O*-acetylharpagide was inactive against *Plasmodium*, but showed *in vitro* cytotoxicity (Kuria et al. 2001, 2002; Muregi et al. 2004). The crude methanol extract of *A. remota* also presented antimycobacterial activity; ergosterol-5,8-endoperoxide was identified as the active compound with a MIC of 1 μg/ml (Cantrell et al. 1999).

In one *in vivo* study, the ethanolic leaf extract of *A. bracteosa* not only inhibited parasitaemia in a dose-dependent manner, but also enhanced the mean survival time of the treated mice. The extract appeared to be safe, with an LD_{50} of more than 5 g/kg body weight (Chandel and Bagai 2010). Other *in vivo* studies did not confirm the antiplasmodial action (Muregi et al. 2006, 2007). However, these researchers point out that bioactive compounds may not necessarily possess direct parasiticidal effect but may have other pharmacological properties such as antipyretic, analgesic or immunostimulatory. It is also known that the presence or quantities of bioactive compounds in plants may be influenced by several factors including season, weather conditions, environment, plant part used, intra-species variations and plant age, among other factors. Also, the nature of the solvent used and traditional extraction methods such as direct boiling of plant material are significant factors worthy of re-evaluation (Muregi et al. 2006, 2007). We can add to the remarks of the authors that only one dose of the extract was administered, while an adequately broad dose range enables dose–response curves (Cos et al. 2006). Also, a positive control group is lacking in the 4-day suppressive test of (Muregi et al. 2006).

In conclusion, we propose two hypotheses for the possible mechanism of action of *A. remota*. First, *A. remota* could have a direct antiplasmodial activity, as demonstrated by *in vitro* and some *in vivo* studies (Chandel and Bagai, 2010; Kuria et al. 2001, 2002; Muregi et al. 2004). Ergosterol-5,8-endoperoxide (the most potent antiplasmodial constituent *in vitro*) contains an endoperoxide group similar to artemisinin, which might be partly responsible for the activity. Secondly, *A. remota* could have immunostimulatory properties. Dehydroepiandrosterone sulphate (DHEAS) is an independent significant predictor of increasing resistance to malaria (Kurtis et al. 2001; Leenstra et al. 2003). Its immunomodulatory action could be mimicked by ergosterol-5,8-endoperoxide and by the phytoecdysteroids present in *A. remota*, which both have a similar steroid backbone as DHEAS. For the phytoecdysteroids, immunoprotective activities have been reported; anabolic and adaptogenic activity could add up to the effect (Dinan 2009).

Additional preclinical research might confirm the *in vivo* activity. Further phytochemical analysis is needed to identify the relation between secondary metabolites and the biological activity, especially regarding the antiplasmodial and immunostimulating activity. These should be carefully balanced against possible toxicity (acute, chronic, embryotoxicity and genotoxicity). Clinical evidence for antiparasitic and antimicrobial action is not yet available, while this is needed to validate the ethnopharmacological data.

It is advisable to use standardized extracts to conduct these assays, in order to have interpretable results. Therefore, secondary metabolites must be identified that are fit for standardizing extracts. Starting from preclinical and clinical evidence, the development of lead antimalarial compounds can be considered.

Apart from monotherapy, combination therapy should also be taken into account. There are several reasons why possible synergistic effects should be investigated thoroughly. As malaria has a complex pathophysiology, the disease can be treated more effectively with well-chosen pharmaceutical combinations, than with a single drug that only acts on one single target. Secondly, true synergism means that the overall effect of two drugs that are applied together as a mixture is larger than the summation of the separate effects. Therefore, lower amounts of both agents are necessary to achieve the desired effect, which means that dose reduction may be possible and, consequently, potential side effects may be decreased (Wagner and Ulrich-Merzenich 2009). Third, drug combinations can increase the 'life span' of individual antimalarials, as it delays the emergence and spread of resistant parasites. This is one of the reasons why ACTs are currently strongly advocated as first-line treatment of falciparum malaria, replacing artemisinin monotherapy (Dondorp et al. 2010). Furthermore, drug combinations can sometimes partly reverse resistance of the malaria parasites against common antimalarial drugs. Combination of chloroquine with DHEAS, for example, significantly increased sensitivity of resistant parasites to chloroquine and increased the survival period of infected mice (Safeukui et al. 2004). Pharmacological *in vitro* and *in vivo* investigations can detect and determine synergy effects of herbal drug combinations. However, these results must also be verified in humans by means of controlled clinical trials. Possible side effects of herbal drug extracts when combined with any synthetic drug must also be taken into consideration. The main criterion is a significant therapeutic equivalence and side effects equal or lesser to those of the reference drug (Wagner and Ulrich-Merzenich 2009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2011.08.063.

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