Chapter 9
Summary and Conclusion

9.1 INTRODUCTION

Malaria is the most important parasitic disease in the world, responsible for 500 million cases and 1 million deaths every year (WHO, 2003) and the number of clinical attacks due to *Plasmodium falciparum* seems to be 50% higher than WHO estimates (Snow et al., 2005). This situation has occurred due to progressive spread of resistance to almost every drug. This loss of effectiveness of the newer antimalarial drugs has also occurred at an alarming rate (Wongsrichanalai et al., 2002).

Resistance to artemisinin derivatives and artemisinin combination therapy (ACT) was also reported (Luxemburger et al., 1998; Gogtay et al., 2000; Sahr et al., 2001; Meshnick, 2002). To avoid resistance problem combination therapy associating long and short acting compounds with different modes of action was adopted. It offers efficient but expensive treatments (White, 1998). Hence, there is a clear need for a low cost, efficient, curative and preventive malaria treatment which does not induce resistance.

Plants have been an integral part of life in many indigenous communities including India. Leaman et al. (1995) showed that plants widely used as antimalarials by traditional healers are significantly more active *in vitro* against *P. falciparum* than plants that are not widely used or not used at all, for the treatment of malaria. This represents a potential source for the discovery of lead molecules for development into potential antimalarial drugs (Phillipson and Wright, 1991; Kayser et al., 2003). To support this, the molecular diversity and efficacy of antiparasitic plants, extracts, and herbal preparations have been intensively discussed in a few reviews (Schwikkard & Van-Heerden, 2002; Willcox & Bodeker, 2004; Wright, 2005). Also antimalarial properties of *Cinchona* bark, known for more than 300 years and several semisynthetic derivatives of artemisinin - the active ingredient of the Chinese herb ‘Qinghao’ (*Artemisia annua*, which was used traditionally for treating fevers) - being used increasingly over the past two decades reveals that the antimalarial drugs used historically have been derived from compounds from these medicinal plants or are
structures modeled on lead compounds from plants (PrayGod et al., 2008; Haynes, 2001).

However, WHO (2002) report states that although there is a widespread use of traditional herbal remedies in the management of malaria, scientific understanding of these plants is largely unexplored. By keeping this in view, the systematic work on collecting information on antimalarial plants based on traditional claims for Vishamajwara in Ayurveda (Satya Narayana Sastri, 2002) and further evaluation of the efficacy of the plant extracts, fractions and compound/s for antimalarial activity was undertaken.

9.2 PRELIMINARY ANTIPLASMODIAL SCREENING OF SELECTED MEDICINAL PLANTS

Fifteen medicinal plants were selected for preliminary antiplasmodial screening, based on their traditional claims for the treatment of fever and malarial fever (Vishamajwara in Ayurveda). Methanolic extract of the selected plants were subjected to antiplasmodial activity testing using schizont maturation inhibition assay and Plasmodium falciparum lactate dehydrogenase inhibition assay (PfLDH).

The results revealed that the methanolic extract of A. zeylanica, E. ribes, P. nigrum and P. zeylanica exhibited good activity in inhibiting schizont maturation and Plasmodium lactate dehydrogenase. As per WHO guidelines, among the plants screened A. zeylanica leaf (IC$_{50}$ 5.80 µg/ml) and E. ribes fruit (IC$_{50}$ 13.10 µg/ml) showed promising antiplasmodial activity in inhibiting PfLDH with IC$_{50}$ value below 15 µg/ml.

9.3 Isolation of vasicine and vasicinone from methanolic extract of Adhatoda zeylanica leaf and embelin from methanolic extract of Embelia ribes fruit

Since the methanolic extract of A. zeylanica leaf and E. ribes fruit were found to have good activity, they were further fractionated to isolate the antiplasmodial compound/s. Makler and others (1993) have shown that Plasmodium parasites can be accurately detected by the unique ability of the parasite lactate dehydrogenase (pLDH) to use 3-acetyl pyridine adenine dinucleotide (APAD) as a cofactor (Makler and Hinrichs, 1993; Makler et al., 1993). It was therefore thought worthwhile to espouse in vitro PfLDH assay to perform fractionation in order to isolate the bioactive compound/s. A.
zeylanica leaf and E. ribes fruit methanolic extracts were fractionated in solvents with increasing polarity viz, petroleum ether (60-80°C), chloroform, ethyl acetate and n-butanol. Chloroform fraction of A. zeylanica leaf methanolic extract showed highest activity, whereas petroleum ether (60-80°C) fraction of E. ribes fruit methanolic extract showed highest activity in inhibiting PfLDH. TLC of the fractions showed that in chloroform fraction of A. zeylanica leaf methanolic extract vasicine and vasicinone were major compounds and in petroleum ether fraction of E. ribes fruit methanolic extract embelin was major compound. Subsequently two compounds from A. zeylanica leaf and one compound from E. ribes fruit were isolated. The compounds, structure of which elucidated through interpretation of their spectral data, were identified as vasicine and vasicinone from A. zeylanica, and embelin from E. ribes.

9.4 EVALUATION OF THE ISOLATED COMPOUNDS FOR ANTIPLASMODIAL ACTIVITY

The three isolated compounds were evaluated for antiplasmodial activity in schizont maturation inhibition assay and PfLDH inhibition assay. In schizont maturation inhibition assay vasicine, vasicinone and embelin showed IC$_{50}$ of 8.75, 63.20 and 9.97 µg/ml respectively. In PfLDH inhibition assay vasicine, vasicinone and embelin showed IC$_{50}$ of 2.05, 57.20 and 3.33 µg/ml respectively. From the results it is clear that vasicine and embelin showed good activity in inhibiting schizont maturation and PfLDH. Further these compounds were assayed for mechanism of action in different assays.

9.5 EFFECT OF VASICINE, VASICINONE AND EMBELIN ON SOME IMPORTANT TARGETS OF PLASMODIUM FALCIPARUM

Most of the established drugs for malaria had developed resistance for malarial parasite due to mutation in the respective targets. So newer compounds acting on valid targets for P. falciparum are likely to be effective. Therefore we investigated the effect of vasicine, vasicinone and embelin on some important targets of P. falciparum.
9.5.1 Effect of vasicine, vasicinone and embelin on parasite invasion of red blood cells

There is a link between the erythrocyte membrane properties and the ability of erythrocytes to function as hosts for the *Plasmodium* parasites and this was exploited for antimalarial therapy (Ziegler et al. 2002; Ziegler et al., 2002). Vasicine showed 70.17 % inhibition of merozoite invasion of human RBC at 25 μg/ml while it showed 77.18 % inhibition of schizont maturation at 25 μg/ml. Embelin showed 73.65 % inhibition of merozoite invasion of human RBC at 25 μg/ml while it showed 71.44 % inhibition of schizont maturation at 25 μg/ml. From the above experiments it was observed that vasicine and embelin showed good activity in inhibiting schizont maturation and merozoite invasion of RBC. Both the compounds have the potential to block parasite development.

9.5.2 Effect of vasicine, vasicinone and embelin on hemozoin formation inhibition

There is a direct relationship between intra-erythrocytic anti-malarial activity and inhibition of β-haematin formation (Pandey et al., 1998). At 5 molar equivalents to hemin, embelin showed 85.21 %, vasicine showed 32.7 % and vasicinone showed only 9.68 % inhibition of hemozoin formation (compared with positive control clotrimazole 94.64 % inhibition). Further, embelin exhibited IC_{50} of 11.66 mM at 5 molar equivalents of heme.

9.5.3 Heme-embelin interaction assay

The inhibition of heme crystallization by antimalarial drugs is mediated by binding to heme (Fitch et al., 2004). Heme solution showed a broad Soret band from 370 to 390 nm resembling a dimer. The interaction of heme with embelin was monitored in the Soret region of the UV-visible spectrum. There is a decrease in the absorbance at 386 nm, with increase in embelin concentration. The interaction of heme and drugs in stoichiometries was further explored by Job’s plot (Huang et al., 1982). Formation of the complex is maximized when embelin and heme are present in a 2 : 3 ratio.

9.5.4 Effect of embelin on GSH (Glutathione reductase)-dependent heme degradation

The major degradation pathway of heme is not the biocrystallization, since around 70% of non-crystallized heme exists in the food vacuole and is subsequently catabolized by GSH, leading to the formation of oxidized glutathione (Ginsburg et al., 1998). The
absorption spectrum of heme (10 µM) in DMSO exhibited broad soret band at 386 nm resembling a dimer form. The maximal absorption of the soret of heme (10 µM) was shifted to broader soret band between 360 to 370 nm after adding GSH (10 mM), probably due to the formation of GSH-heme complex. After addition of embelin to GSH-heme complex, there is a shift of peak to 327 and 396 nm, may be due to altered effect of GSH on heme.

9.5.5 Effect of vasicine, vasicinone and embelin on *Plasmodium* protein kinase inhibition

In the catalytic domain of protein kinase, 40–60% of the residues are different between the mammalian and plasmodial enzymes, and the latter display unique structural features such as insertions or terminal extensions. These considerations suggest that the enzymes from *P. falciparum* have different susceptibilities to kinase inhibitors helped to develop kinase inhibitors against protein kinase enzymes from *P. falciparum* (Kappes B et al., 1999). Vasicine, vasicinone and embelin did not inhibit any of the protein kinase enzymes even at 100 µM concentration.

9.5.6 Effect of vasicine, vasicinone and embelin on plasmepsin inhibition

The plasmepsins produced by the *Plasmodium* parasite are aspartic proteases and have been recognized as attractive targets for the design of novel chemotherapeutic compounds for the control of malaria (Olliaro and Goldberg, 1995). Vasicinone exhibited IC₅₀ value of 13.3 µM in plasmepsin II inhibition while vasicine and embelin did not show any inhibition. None of the compounds showed Plasmepsin IV inhibition.

9.5.7 Effect of vasicine, vasicinone and embelin on Histidine rich protein–2 (HRP-2) inhibition

HRP-2 is largely made up of repeats involving three amino acids: histidine (34%), alanine (37%) and aspartic acid (10%) (Wellems and Howard, 1986). HRP-2 has been established as a catalyst for haemzoin formation, and haem metabolism has emerged as a potential drug target in the malaria parasite (Padmanban and Rangarajan et al., 2000). In HRP-2 inhibition assay vasicine, vasicinone and embelin exhibited IC₅₀ value of 20.03 µM, 18.48 µM, and 38.06 µM respectively as compared to chloroquine with IC₅₀ of 0.042 µM.
9.6 SYNERGISTIC EVALUATION OF ANTIPLASMODIAL COMPOUNDS IN VITRO

A combination of vasicine and embelin with the ratio of 2: 4 µg/ml and 3: 3 µg/ml showed 50 % inhibition with interaction index 0.64 (Figure 7.4 A). A combination of vasicinone and embelin with the ratio of 30:3 µg/ml showed 50 % inhibition with interaction index 0.77 (Figure 7.4 C). A combination of vasicine and vasicinone with the ratio of 8:60 µg/ml showed 50 % inhibition with interaction index 1.85 (Figure 7.4 B). A combination of quinine dihydrochloride and vasicine with the ratio of 4 ng/ml: 2 µg/ml showed 50 % inhibition with interaction index 0.54 (Figure 7.4 D). A combination of quinine dihydrochloride and vasicinone with the ratio of 2 ng/ml : 20 µg/ml showed 50 % inhibition with interaction index 0.63 (Figure 7.4 E). A combination of quinine dihydrochloride and embelin with the ratio of 1 ng/ml : 5 µg/ml showed 50 % inhibition with interaction index 0.52 (Figure 7.4 F). A combination of artemisinin and vasicine with the ratio of 0.4 ng/ml: 2 µg/ml showed 50 % inhibition with interaction index 0.55 (Figure 7.4 G). A combination of artemisinin and vasicinone with the ratio of 0.4 ng/ml : 20 µg/ml showed 50 % inhibition with interaction index 0.63 (Figure 7.4 H). A combination of artemisinin and embelin with the ratio of 1 ng/ml : 5 µg/ml showed 50 % inhibition with interaction index 0.55 (Figure 7.4 I).

9.7 QUANTIFICATION OF VASICINE AND VASICINONE IN THE LEAF OF ADHATODA ZEYLANICA LEAF AND EMBELIN IN THE FRUIT OF EMBELIA RIBES

The developed HPTLC method for the quantification of vasicine, vasicinone and embelin was found to be simple, precise, specific and sensitive. The amount of vasicine and vasicinone in Adhatoda zeylanica leaf was found to be 1.25 and 0.039 % (w/w) respectively. The amount of embelin in Embelia ribes fruit was found to be 3.21 % (w/w).

9.8 CONCLUSION

The objective of isolating antiplasmodial compound (s) was fulfilled. Preliminary antiplasmodial screening for search of better schizont maturation and PfLDH inhibiting plant confers Adhatoda zeylanica and Embelia ribes. Subsequently vasicine and vasicinone from A. zeylanica and embelin from E. ribes fruit were isolated. These
isolated compounds were tested for schizont maturation inhibition and PfLDH inhibition. Vasicine and embelin were found to have good antiplasmodial activity in inhibiting schizont maturation and PfLDH. Vasicinone was also screened in all mechanism of action based assays because of the same structural scaffold of vasicine and vasicinone. Out of the three compounds screened embelin demonstrated significant effect in hemozoin formation inhibition, heme binding, GSH dependent heme degradation and inhibited three enzymes of fatty acid biosynthesis. Eventhough vasicine showed better antimalarial activity in vitro in inhibiting schizont maturation and PfLDH than embelin, it did not show any effect on heme inhibition, plasmodium fatty acid biosynthesis (FAB) and plasmodium protein kinase. Vasicine, vasicinone and embelin showed moderate effect on Histidine rich protein-2 (HRP-2) and vasicinone showed moderate effect on Plasmepsin II (Figure 9.1).

Embelin was found to target lysosomal food vacuole and apicoplast of malarial parasite by demonstrating significant effect on respective heme and fatty acid biosynthesis enzymes Fab I, Fab G and Fab Z. Since embelin was found to act on two different targets of the parasite development, chances of development of resistance are less. Embelin is present in E. ribes fruit in good amount. Structurally it is a simple molecule, which facilitates modifications and synthesis of embelin derivatives.

Further embelin showed synergistic effect with vasicine and vasicinone also all the three isolated compounds showed synergistic effect with quinine dihydrochloride and artemisinin.

These findings reflect on its potential for the treatment of resistant malaria and its clinical efficacy. However the complete mechanism of action of vasicine and embelin remains to be determined.

References


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