Chapter - VII

Discussion
Common antiprotozoal activities of plant extracts prepared from the traditional remedies from *Brueca javanica* fruits and *Simarouba amara* stem and particularly their quassinoids have been studied extensively along with standard drugs for *E. histolytica* and *P. falciparum* (Wright et al. 1993). Purified compounds with activities against more than one protozoa may or may not parallel each other in most of the studies for screening *in vitro* and are even more diversified during *in vivo* studies. This has been further extended for multidrug resistant *P. falciparum* (Pavanand et al. 1986). No common mechanisms of action, course of pathogenesis and methods of cure can be found for protozoal diseases because of different host defense mechanisms and individual parasitic preferences.

Reviews of antimalarial agents from plants in the early 90’s do not show any significant presence of antimalarial active principles among the top 30 extensively investigated medicinal plants (Vohora 1992). However a compilation of 87 plants which are found effective against *P. falciparum* and *P. yoelli* have only one plant namely *Azadirachta indica* common with the earlier mentioned top 30 plants (Guru et al. 1996). In most reviews of plants used as antimalarials, ethnobotanical listings have no scientific support (Aminuddin et al. 1993). An extensive compilation of antimalarial plants brings out the better dose dependent effectiveness of crude extracts. The best three dose efficacies in terpenes and quassinoids categories are of *Upaca nitida* (IC$_{50}$ 0.0013 μg/ml), *Brueca javanica* (IC$_{50}$ 0.006 μg/ml) and *Bridelia catharctica* (ID$_{50}$ 0.005 μg/ml). Of these *Brueca javanica* has been studied most extensively and its quassinoids were found to be effective at IC$_{50}$ 0.0009 μg/ml against *P. falciparum* K1. Also the plants of the same family Simaroubaceae were found to be most effective at IC$_{50}$ of 0.0009 μg/ml. Alkaloids from Rutaceae, Ancistrocladaceae, Menispermaceae and Apocyanaceae shows the most effective IC$_{50}$. The family Dioncophyllaceae shows the most effective IC$_{50}$ at 0.014 and 0.015 μg/ml against *P. falciparum* NF 54 and *P. berghei* Anka respectively and *Alstonia angustifolia* shows ED$_{50}$ of 2.92 μg/ml. Under miscellaneous compounds the families Annonaceae and Meliaceae have the most effective IC$_{50}$. Ursolic acid from *Spathodea campanulata* produced the highest suppression *in vivo* of 97 % at 60 mg/kg/day. The other effective plants are *Melia azaderarch* at ID$_{50}$ of 0.08 μg/ml and *Glycrrhiza glabra* at IC$_{50}$ of 0.6 μg /ml. Compared to other plants *C. procera* was lowly placed in the table. (Sharma and Sharma 1998).
While these plants are comparable to *Artemisia annua* at IC<sub>50</sub> of 0.01 μg /ml from which artemisinin is commercially exploited the most extensively studied plants are of the family Simaroubaceae namely *Brucea javanica* which appears most frequently in research studies. *Eurycoma longifolia* also seems to be popular for research purposes with IC<sub>50</sub> of 0.11 μg /ml. Alstonia, Brucea, Simarouba, Eurycoma etc. are examples of some plants which have been studied most frequently against different *Plasmodium* strains by different methods and without conclusive evidences even after two decades of scientific evaluation.

Table 3a shows the best three dose efficacies of crude extracts and fractions with *Artemisia indica* (EC<sub>50</sub> 6.60 x 10<sup>-6</sup> g/ml), *Hannoa klaineana* (IC<sub>50</sub> 0.672 μg/ml) and *Azadirachta indica* (IC<sub>50</sub> 2.45 μg/ml). Table 3b shows the efficacies of isolated compounds in order of merit. 2-(2,4-dihydroxyphenyl)-5,6-methylenedioxybenzofuran (EC<sub>50</sub> 2.70 x 10<sup>-6</sup> g/ml) from *Artemisia indica* (Asteraceae) as also its other compounds, has put this plant second only to Cinchona for its antimalarial discoveries. The compounds from the plant *Castela texana* (Simaroubaceae) have active principles which range from the highly effective Holacanthone (IC<sub>50</sub> 0.010 & 0.012 μg/ml) along with isolated compounds with consistently significant activity at doses less than 10 μg/ml. *Hannoa chlorantha* of the same family also shows promising dose effective activity with Chaparrinone (IC<sub>50</sub> 0.037 μg/ml).

However the overall non-specific activity of plant extracts is still apparently significant in traditional uses and cures. The cost of maintaining a minimum "freedom from disease" or its containment is met more effectively by such composite formulations than having to spend on both repeated diagnostic and prognostic investigations and the cost of drug along with its development. Crude extracts, which have composite activities, may find commonalties for antiprotozoal activity. Similar findings of *in vitro* activity of *C. wightii* in combination with other plants in Ayurvedic formulation have been reported (Shukla et. al. 1990). *In vitro* potencies may not reflect the situation, which occurs *in vivo*. It would be of great importance to elucidate the mechanism of action of these chemical compounds and to determine their structure in detail. Preliminary phytochemical screening in *C. procera* and *C. wightii* shows the presence of various compounds in specific parts of the plant and can be taken for pharmacotoxicological studies.
The extensive use of gum-oleo resin of *C. wightii* (Sharma and Sharma 1996) and plant parts of *C. procera* (Sharma and Sharma 1999) in traditional medicine is justifiable by the *in vitro* screening for both *E. histolytica* and *P. falciparum*. A similar study has been reported with 10% ethanolic extract of *C. wightii* (Ansari and Ahmad 1991). Based on Indian traditional medicine the usage of similar plants have been screened for *in vitro* antimalarial and antimicrobial activity from time to time with no conclusive activity many a times. e.g. Gentianine, which may be an artefact, produced during extraction (Natarajan et. al. 1974).

The popular consumption of flowers and buds of *C. procera* and gum-oleo resin of *C. wightii* are of special interest. The traditional uses of parts of *Calotropis procera* have been known and well documented in the subjective relief from fever and malaria as well as objective clinically acceptable cure by traditional practitioners of alternate medicine. Since records are sometimes available with Gunijis (Local Vaidyas), they should be encouraged to follow documentation along medically accepted norms. The preparation of ashes, concoctions, powders, pastes, decoctions are individual preferences of these practitioners and their patients. The root of *C. procera* is ashed and most commonly used.

In one of the earlier studies, ethanolic extract of whole plant *Calotropis procera* excluding root showed 35.57% inhibition at 100 μg/ml *in vitro* and nil under *in vivo* condition against *P. berghei* NK 65 (Mishra et.al. 1991). Further the inhibition of around 35% in Mishra’s study coincides well with the bud acetone, root ethylacetate and root acetone against MRC *P.f.* 20 and with flower methanol and root acetone against MRC *P.f.* 76 (Sharma and Sharma 1999). In the present study a comparable data set for the fractions of flower, bud and root lies within a dose range of 62-125 μg/ml. At the lower dose range the root extracts of *C. procera* seems to be the most effective for both the isolates, MRC *P.f.* 20 and MRC *P.f.* 76. (see Table 11c)

The ethanolic extracts yield from gum-olea resin, flower, bud and stem are more than the others. The bud ethanol shows a better ranked schizontocidal activity with both isolates MRC *P.f.* 20 and MRC *P.f.* 76 along with the hemolytic activity which is also better ranked showing less toxicity to the erythrocyte.

The two different isolates have a tendency to behave differently for various parameters studied for the derivatives from gum-oleo resin. The gum ethanol, gum hexane and Cembrene-A are steps in series for obtaining the active ingredients as
reflected in its effect in the CQ resistant isolate MRC P.f. 76. The use of plant parts of C. procera and gum resin of C. wightii needs much more justification as a chloroquine potentiating agents with minimal toxicity, inspite of its traditional medicinal value. With a significantly high AEI, reasonably synergistic effect with chloroquine and a lower hemolytic toxicity the gum-oleo resin shows some promise for these strains. Cembrene-A which is a purified compound from the gum-oleo-resin shows a consistent trend in schizonticidal and CQ potentiating activity with an A.E.I. of 6.17 for the CQ sensitive isolate MRC P.f.20 and an A.E.I. of 1.216 with CQ resistant isolate MRC P.f.76. Thus Cembrene-A shows a chloroquine potentiating effect with MRC P.f. 20 with no evidence of possible reversal in MRC P.f. 76.

The reversal of the response of the CQ resistant isolate to values as close to the CQ sensitive isolate is another indicator of the beneficial action of plant products, when in combination with chloroquine. This can be seen in the resistant strain reverting its response as seen in the IC₅₀ values (Table 21a) with gum ethanol, flower ethanol and flower ethylacetate.

In our study the IC₅₀ values of chloroquine related drugs ranges from 10 times in CQ sensitive isolate MRC P.f 20 to 440 times in CQ resistant isolate MRC P.f 76. The artemisinin related drugs shows an overall much better schizontocidal activity as compared to chloroquine related drugs. Primaquine, which has an exo-erythrocytic specific activity, shows a truly different response as compared with other drugs regarding the schizontocidal effect in both these isolates. The CQ resistant isolate MRC P.f 76 shows a very high resistance in vitro to Metronidazole, which is a specific antiamoebic drug and also equally strong contender as a broad-spectrum antiprotozoal drug as chloroquine in malaria. (Raju 1979). Arteether, Artemisinin and Halofantrine show the least difference in resistance in these two isolates.

In our study the results of the antiamoebic screening have been divided into 4 parts based upon the use of i) ethanolic extracts ii) the fractions as obtained from the ethanolic extracts iii) some more purified compounds iv) the standard antiprotozoal drugs. The most effective have been ranked under these categories and the similarities are reasonably obvious in each category. The similarities between the activity of the gum-oleo resin ethanol extract of C. wightii, its further fractionation with hexane and derived purified compounds, Guggulsterone-E and Cembrene-A show a similar trend. The most effective IC₅₀'s of C. procera show the flower ethanol extract and its ethylacetate fraction to be promising alongwith Calactin.
Among the various standard antiprotozoal drugs Metronidazole and Chloroquine are still the most effective. Purified drugs which are individually more effective as antimalarials have little in common with the antiamoebic metronidazole.

Phytochemical screening of these extracts show the presence of alkaloids, carbohydrates, glycosides, phenolic compounds/tannins, proteins and amino acids, flavonoids, saponins, sterols, acidic compounds and resins (Sharma and Sharma 1999). This mixture of organic compounds may result in the latency, which may be required for stages of colloid osmotic nature and destabilises the erythrocytic membrane, for binding with it before entry within the cell. Further metabolism and other intracellular effects can then followed in more sophisticated and expensive methodologies. The hemolytic activity with various ethanol extracts show a latency of almost 20 minutes before they seem to precipitate hemolysis, which increases to 30 minutes with further fractionation of these crude extracts. Suchethanolic extracts from Fenugeek (Trigonella foenum graceum) have an isolated saponin fraction with hemolytic activity (Stark and Madar 1993). In another study a steryl glycoside fraction from tubers of Momordica cochinichinensis has been shown to possess hemolytic activity (Ng et. al. 1986). Hemolysis is best evaluated using an in vitro method, which can show the effect of increasing concentration and to be sigmoidally related to the logarithm of contact time, as studied for various surfactants (Mohanet .al. 1992). The hemolysis process for ethanol-treated RBCs was preceded by the leakage of the small cation K⁺ and by considering the data with molecular sieving a pore size of approximately 13Å was found presumably at the deranged membrane cytoskeletal protein (Chi and Wu 1991 ). NaCl or KCl can increases the efficiency of the hemolysis at the pH 7.2-7.3 (Zavodnik et. al. 1994).

Our extracts show a certain amount of membrane instability with ethanolic extracts as reflected in the k1 range of value, which results in greater hemolysis with increase in dose. The saturation of the substrate at which the initial velocity which is one-half of the maximum velocity is reflected by k2 is found to be better at 5 mg /ml. Our extracts do not show a decrease in the velocity of the reaction with the increase in doses which results in near cent- percent hemolysis. Since these extracts are composite mixtures and their surface active properties are not known, the cooperativity parameter between its individual components cannot be reflected by any greater affinity or other similarities in their action.
The relative complete hemolytic response of the RBC suspension to various extracts from different parts of *C. procera* and gum-oleo resin of *C. wightii* show better hemolysis isotherms with extracts from Flower, gum and root ethanol extracts and their corresponding fractions.

An extract of *Ginko biloba* and chlorpromazine showed comparative effects on *in vitro* osmotic fragility of rat erythrocytes (Etienne et. al. 1982). The antimalarial action of quinoline related compounds particularly Chloroquine is related to RBC membrane changes and characterisation of immune autoantibodies on erythrocytes. The k1 values of the antimalarial plant ethanolic extracts studied can be related with a common effect of RBC membrane instability similar to that induced by Chloroquine. The advantage of using the rate of hemolysis for studying the toxicity and effectiveness of plant extracts at various stages of their fractionations till the final purification and identification of the structure of the ultimate effective compounds, is its being a simple and elegant experiment which can be set up without sophisticated equipments. It links the study of crude extracts to the designing of modern plant medicinal products with scientific validation of the ethnomedical history and prevalence of their usage.

Field work exploring the medicinal uses of plants by indigenous people in remote parts of the world require the use of assays which can determine whether plants exert a biological effect and have facilitated the discovery of bioactive molecules made by medicinal plants. The Brine shrimp toxicity microplate assay, Wheat rootlet growth inhibition assay are simple to be run in field tests as shown with *Sapium marmieri*, *Ficus insipida* and *Croton erythrochilus* without showing a positive interaction with the DNA-Methyl green assay (Mongelli et. al. 1995). None of the 39 extracts of 13 plants of the southwest Amazon rain forest showed no decrease of the initial absorbance of DNA-MG complex (Desmarchelier et. al. 1996). Further antioxidants from plant extracts of *Baccharis cordifolia* are known to show a 56% decrease in the initial absorbance of DNA-Methyl green complex at 1 mg/ml (Mongelli et. al. 1997). In our study the overall plant extracts showed little DNA binding except in cases where scattered 50% reduction in the final absorbance was observed. The decrease in absorbance upon hydrolysis of DNA-Methyl green substrate is the result of two sequential reactions. In the first reaction, enzymatic hydrolysis of phosphodiester bonds in DNA produces free methyl green with a decrease in absorbance which represents the initial rapid displacement of methyl green from DNA. The free methyl green decolorizes and yields a colourless carbinol in the second slower nonenzymatic,
reaction (Sinicropi et al. 1994). The reliability and reproducibility of this microassay differentiates between DNA intercalating agents that do not interact with the dye methyl green (Bonjean et al. 1996).

A simplified HPLC system for the detection of natural products capable of binding DNA has been used for various plants e.g. *Albizzia amara* and its extracts (Pezzuto et al. 1991), also the DNA affinity of active alkaloids from *Arisarum vulgare* (Melhaoui and Belouali 1998). The interaction of plant alkaloid Berberinium chloride with DNA has been investigated using spectrophotometry, viscometric titrations (Davidson et al. 1977). The binding of the alkaloid sanguinarine to natural DNAs of differing GC content has been studied by spectrophotometry and viscometry techniques. Binding parameters determined from spectrophotometric measurements by Scatchard analysis, according to an excluded-site model, indicate a very high specificity of sanguinarine binding to GC rich DNA (Nandi and Maiti 1985).

Antimalarials are among the most commonly used drugs today, yet there are only a few efforts in the world directed at new antimalarial drug development. The time frame from discovery through development to clinical trials and drug registration is such that the drugs currently in the pipeline cannot be expected to be available for general use in near future (Oliaro et al. 1996).

The results obtained from all these studies indicate that a variety of secondary plant metabolites display antimalarial activity against Plasmodium. The different profiles of activity seen with individual class of compounds suggest that there may be subtle differences in the site of action of these compounds. The isolation and elucidation of the chemical structure of different active components in the medicinal plants is of major value in that it identifies a new lead compound for chemical synthesis of candidate antimalarial drugs. The results of Cembrene-A, guggulsterone-E, Calactin and mixture of acetates in this study is an attempt to show the antiprotozoal activity and chloroquine potentiating action. In the pursuit of drug discoveries of the long drawn battle between parasite and man, the dichotomy of chance and choice, has been governed by market forces that govern the pharmaceutical industry. A whole new family of compounds derived from the Chinese herbal remedy artemisinin is currently under investigation. Some of its derivatives, such as artemether, artesunate, and dihydroartemisin, are already in use in various formulations. Such new compounds are needed for the continued struggle against multidrug resistant falciparum malaria.
After making crude extracts, sophisticated chemical works are required for identification of individual compounds using techniques such as chromatography, Nuclear Magnetic Resonance and Mass spectroscopy. Better biological work is required for screening and elucidation of the mechanism of action, metabolism of plants products for example the unique artemisinin endoperoxide, stable ozonoides, trioxanes etc. to synthesize molecules modeled on these moieties. Such a modern approach which prefers the use of individual compounds has emerged from the wealth of data of medicinal plants which belong to the oriental systems of medicine which are considered to be more holistic (Kirby 1997).