CYTOTOXIC AND ANTIMALARIAL NATURAL PRODUCTS

Thesis presented by

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dedicated to

MY MOTHER

CONTENTS

ABSTRACT

Eighty percent of the world's population must rely on traditional medicine for the treatment of disease. In a report in 1978, the World Health Organisation, recognizing that modern medicine cannot meet its aim to make healthcare available to everyone by the year 2000, has recommended the improvement and use of herbal medicine.

This study focuses initially on a herbal remedy used traditionally in Chinese Medicine for the treatment of malaria. An aqueous infusion or "tea" was prepared from the fruits of the Simaroubaceae species *Brucea javanica*, and four quassinoids, bruceines A, B, C, and brusatol have been isolated and identified by the use of chromatographic and spectroscopic techniques.

The aqueous tea, together with crude extracts of two other related plant species, *Simarouba amara* and *Simarouba glauca* have been tested against *Plasmodium falciparum in vitro*, and *Plasmodium berghei in vivo*.

The development of a microdilution assay for the assessment of cytotoxicity against KB cells, a human epidermoid nasopharyngeal carcinoma cell line, is described. The test was used to evaluate the cytotoxic activity of crude plant extracts and isolated pure compounds, shown to demonstrate antiplasmodial activity.

Some 107 compounds and crude extracts, including 21 quassinoids, 12 semi- synthetic quassinoids, 46 isoquinoline alkaloids, 4 canthin-6-one alkaloids, several known cytotoxic and therapeutic agents, and a number of natural products have been tested in the *in vitro* KB cytotoxicity assay. Comparisons of their antiplasmodial and cytotoxic activities are made.

The effect of three potent antiplasmodial quassinoids, bruceantin, brusatol, and bruceine D, on protein and nucleic acid synthesis in KB cells has been investigated.

Transmission electron microscopy has been used to demonstrate visually the effect of the same three quassinoids on cellular morphology and on the membrane surface of KB cells grown in a monolayer.

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INTRODUCTION

1.1 Global Impact of Malaria

Malaria is currently the most significant parasitic disease of humanity. The numbers of cases of malaria, and resulting deaths, dwarf those of virtually all other infectious diseases, including human immunodeficiency virus infection (Schlesinger *et al*, 1988). Estimates of malaria incidence lie between 413 million (Loyola *et al*, 1988), and 489 million cases (Sturchler *et al*, 1989) per year, with more than 2 million deaths occurring annually worldwide (Wyler, 1983). Most deaths are among children below the age of five. Malaria has worldwide distribution in the tropics, subtropics, and also in areas in the temperate zone. With global warming, some regions which have previously been free of malaria will almost certainly become affected. Despite conscious research efforts orchestrated by the World Health Organisation (WHO) and other groups, there has been a dramatic resurgence of the disease over the last two decades. This can be attributed to the development of resistance by the malaria parasite to chemotherapeutic agents such as chloroquine and pyrimethamine, and also to the insecticide (eg. DDT) resistance demonstrated by the anopheline mosquito vector (Payne, 1987).

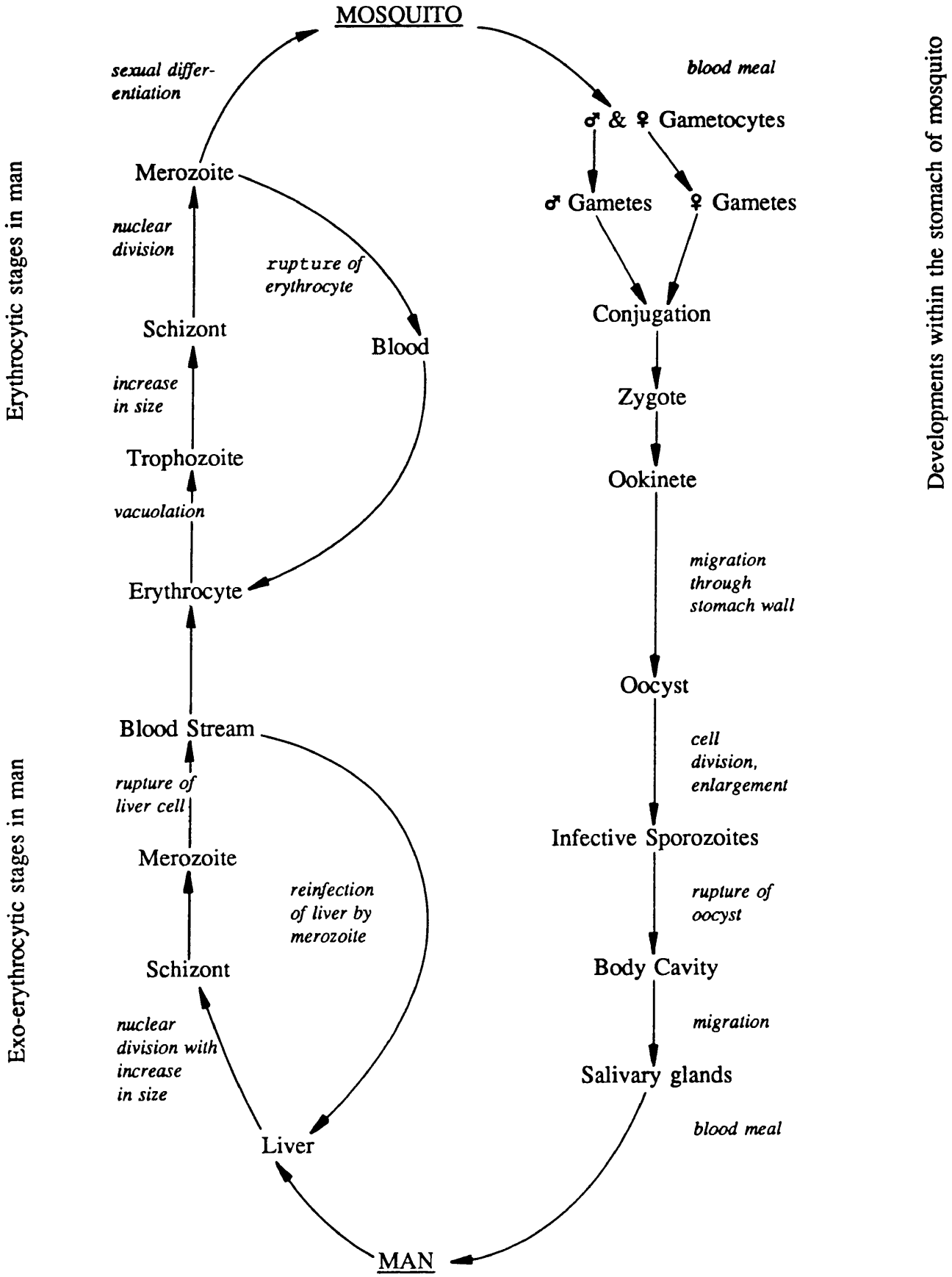
In Britain, cases of imported malaria recorded by the Malaria Reference Laboratory rose by 51% over the decade 1978-1989, with cases in 1989 totalling 1,987. During this period there was an increase in the potentially life- threatening *P. falciparum* infection, from 20 to 56 percent of all cases. More recent figures for 1990 show that cases continue to rise, with a sharp increase occurring in *falciparum* malaria from West Africa. Sub-Saharan Africa, notably Kenya, Tanzania, Uganda, Nigeria, Ghana, and Sierra Leone accounts for most *P. falciparum*, *P. ovale*, and *P. malariae* cases reported in Britain, whereas most *P. vivax* cases come from the Indian subcontinent (Conlon, 1990). The development of the tourist industry has increased the numbers of travellers exposed to malaria still further, and resistance to chemoprophylaxis by *P. falciparum* has become even more widespread.

1.2 Malaria Parasites of Man

The term *malaria* is derived from two Italian words, *mal* meaning bad, and *aria* meaning air. Malaria is due to infection with protozoan parasites of the genus *Plasmodium*, and is usually transmitted through the bite of an infected female anopheline mosquito. Four species infect humans; *P. vivax*, *P. falciparum*, *P. ovale*, and *P. malariae* , and all have similar morphology and life cycles. The asexual cycle (shizogony) takes place in the red blood cells, and the sexual cycle (sporogony) in mosquitoes (see Fig. 1.1).

Figure 1.1 Life Cycle of the Malaria Parasite

(Adapted from Brown and Neva, 1983)



When a human is bitten by an infected mosquito, the sporozoite form of the malaria parasite is injected into the blood stream. The sporozoites are taken up into the parenchyma cells of the host's liver, where they undergo asexual division to form schizonts. In the case of *P. ovale* and *P. vivax*, sporozoites can remain dormant in the liver (as hypnozoites) for up to 12 months. Eventually the schizonts rupture into the blood stream, releasing vast numbers of merozoites, which in turn invade red blood cells. Initially the merozoites take the form of a ring of bluish cytoplasm with a dot-like nucleus of red chromatin. These feed and grow into trophozoites, and take on a more typical appearance depending on the species. Blood smears stained by Giemsa or Wrights reveal the differential features in morphology, making it possible to identify the species responsible. Trophozoites grow until nuclear division which produces erythrocytic schizonts, and these in turn rupture to release merozoites that invade yet more red cells. Parasite density, described as the number of parasites in the peripheral blood, varies with each species. *P. falciparum* gives the highest parasitaemia ($\geq 10^6 mL^{-1}$ - up to 10-40% of red blood cells), and as such poses a significant risk of death in the non-immune patient. A predilection for red blood cells of certain ages, means that other species produce less morbidity and mortality, because they invade only young (*P. vivax* and *P. ovale*) or mature (*P. malariae*) erythrocytes. *P. falciparum*, on the otherhand, infects cells of all ages, and these adhere to the vascular endothelial cells in the deep tissues, especially in the brain. After several days some trophozoites differentiate into male and female gametocytes - the sexual forms which only develop further when taken into the mosquito in a blood meal. Multiplication of the parasite occurs as the sexual forms continue to develop in the mosquito, where mature sporozoites are eventually produced. Clinical symptoms of malaria only develop when there is sufficient asexual infection of the red blood cells. Liver forms and sexual forms of the parasite do not cause symptoms (Brown and Neva, 1983).

1.3 History

Antimalarial agents were in use during the time of Hippocrates, who divided miasmic fevers into continuous, quotidian, tertian, and quartan types, the latter three being attributed to malaria. In China, effective chemotherapy started some two thousand years ago with the use of extracts from the herb Qinghao (*Artemisia annua*, Compositae). One story, since disproved, suggested that in 1638 the Countess d'El Chinchon, wife of the Viceroy of Peru , was cured of malaria by the bark of a certain tree, later called *Cinchona*. The bark (known as Peruvian or Jesuits' bark), from which quinine and other cinchona alkaloids were found to be extractable, has been used successfully to treat malaria since the 17^{th} century.

It was not until the 1920's that the development of synthetic antimalarial drugs began. The most successful, chloroquine and amodiaquine were based on the structure of quinine and were widely used during World War II. However, in the late 1950's, the first suspected cases of *P. falciparum* resistance to chloroquine appeared in Thailand and Columbia, and this has now spread throughout most of the malaria endemic region. Resistance to synthetic drugs forced a return to the use of quinine, and stimulated the search for new antimalarial agents (Brown and Neva, 1983).

1.4 Chemotherapy

Thus far, no drug has been developed which is capable of destroying the infecting sporozoites before they enter the liver. It is therefore, not possible to provide a true antimalarial prophylaxis for those likely to be exposed to infected mosquitoes, although the parasite is susceptible to attack by drugs in the succeeding stages of its developmental cycle. It may seem on first inspection that there is an extensive chemotherapeutic armamentarium currently available to combat malarial infection. However, it is a disturbing fact that there is no present day drug in existence which is uniformly safe, effective, widely available, or can guarantee protection against infection.

Antimalarial drugs are categorised by the stage of *Plasmodia* against which they have therapeutic efficacy (Webster, 1985). Tissue schizonticides prevent the development of the erythrocytic stage of the disease by acting on the initial hepatic tissue form of the malarial parasite. Termed as causal prophylactics, they prevent the symptomatic stage of the disease, and destroy the parasite as it establishes infection in the liver. Tissue schizonticides used to prevent relapse of the infection, act on the liver on the latent hypnozoite stages found only in *P. vivax* and *P. ovale* infections. By far the largest group of drugs are blood schizonticides, and these act in the red blood cells on the asexual stage. The sexual forms of the parasite which are necessary to perpetuate the next stage of the cycle, are destroyed by gametocytocides. Sporontocides, on the other hand inhibit the formation of malarial oocysts and sporozoites in infected mosquitoes. It is necessary for the arthropod vector to ingest the sporonticide during a blood meal for it to be effective. Although an infected person would not derive any direct benefit from the latter two forms of antimalarial agent, further spread of infection would be limited in an endemic region.

The next section gives a brief review of antimalarial agents: drugs currently available, some no longer in favour, and those in development, on which the future control of malaria so heavily depends. The drugs can be conveniently divided into the following groups: quinoline derivatives (including *Cinchona* alkaloids, 4- and 8-aminoquinolines, and mefloquine), folate synthesis and dihydrofolate reductase inhibitors, biguanides, sesquiterpene lactones, 9-phenanthrene methanols, and antibiotics (W. Peters, 1987).

1.4.1 QUINOLINE DERIVATIVES (see Figure 1.2)

1.4.1.1 *Cinchona* Alkaloids

Quinine is the most important alkaloid of *Cinchona* bark, but other alkaloids (quinidine - the diastereomer of quinine, cinchonine, and cinchonidine) are found in such total alkaloid preparations as totaquine, tincture of cinchona and cinchona febrifuge. The *Cinchona* alkaloids act primarily as blood schizonticides, and have a rapid action in reducing parasitaemia, but are often used with a slower acting schizonticide to prevent recrudescence of infection (Panisko *et al*, 1990). The specific mechanism of antimalarial action of these drugs remains unclear. Previous theories which include drug-DNA binding (Hofheinz and Merkli, 1984), and alteration of parasite haemoglobin digestion which leads to the disruption of host- parasite membranes, have lost favour. A review by Schlesinger *et al*, 1988 has indicated that

quinine and quinidine are concentrated in mammalian and parasite acid vesicles as monoprotic weak bases. They then cause an elevation of the pH in these intracellular organelles. How pH elevation ultimately results in parasite death is as yet unclear. Serious adverse effects with *Cinchona* alkaloids are infrequent in the recommended doses. However, therapeutic doses above 5mg/mL produce an unpleasant group of symptoms known as cinchonism, a syndrome of tinnitus,vertigo, transient loss of hearing, nausea, abdominal pain, headachea, and blurred vision. Quinidine toxicity is similar to that of quinine, but is also potentially more cardiotoxic.

1.4.1.2 4-Aminoquinolines

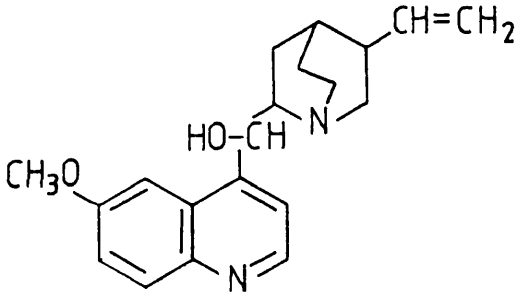
Chloroquine

Chloroquine (CQ) is a 4-aminoquinoline that underwent extensive testing during World War II. It remains the most widely used antimalarial drug, being the treatment choice for CQ-sensitive strains of *P. falciparum*, and for the three other species of human malaria. CQ is not a causal prophylactic as it has no effect against the exoerythrocytic tissue stages of malaria. The drug, however, acts rapidly and is highly effective against the asexual erythrocytic forms, and kills the gametocytes of *P. vivax, P. ovale*, and *P. malariae*.

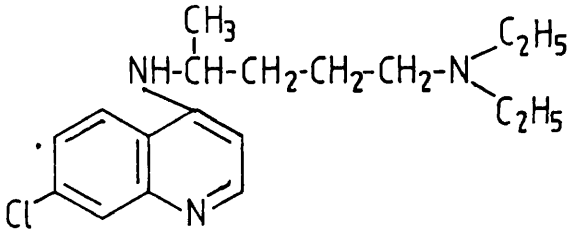
The antimalarial mechanism of action of CQ has been the subject of a number of recent reviews (Ginsburg and Geary, 1987; Krogstad and Schlesinger, 1987; Schlesinger *et al*, 1988). CQ is a weak base, and as such becomes concentrated within the parasite acid vesicles where the internal pH is raised. However, this alkalinising effect is very marked, and a mechanism termed the "non-weak base effect", appears to be responsible for the phenomenon. This occurs because the susceptible parasite has an active CQ-concentrating mechanism in its acid vesicles, further characterisation of which is not yet available. A raised intravesicular pH is the final common pathway present for CQ, the cinchona alkaloids, and also mefloquine. As mentioned earlier, how this chemical observation results in the destruction of the parasite is not yet clear. Although CQ is one of the safest antimalarial drugs, it it can nevertheless cause severe itching, and at concentrations above 250ng/mL causes dizziness, visual disturbances, nausea, vomiting, and fatigue.

The increasing incidence of drug resistance has meant that CQ is being used more frequently in combination with other antimalarial drugs. However, few studies have been carried out into the synergy or antagonism of such drug combinations. One report (Stahel *et al*, 1988), showed *in vitro* antagonism between CQ and the drugs quinine, mefloquine, amodiaquine, artemisinin, and pyrimethamine/sulphadoxine (Fansidar) against both CQ-sensitive, and CQ-resistant strains of *P. falciparum*. Whether this finding has clinical relevance, with a possible contribution to the reduction of drug efficacy, has yet to be determined.

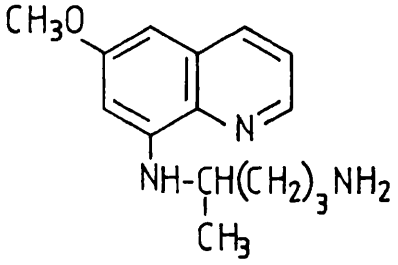
Figure 1.2. Quinoline Derived Antimalarial Drugs



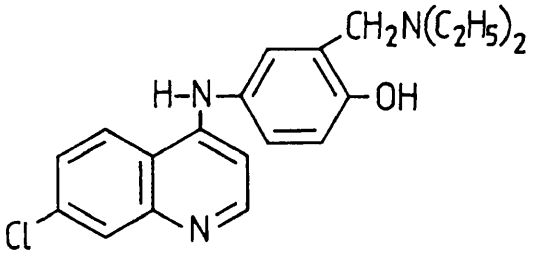
quinine



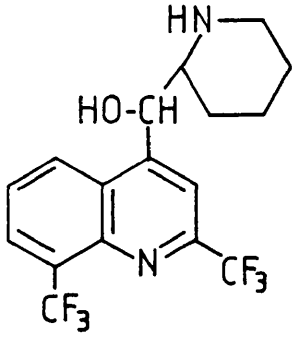
chloroquine



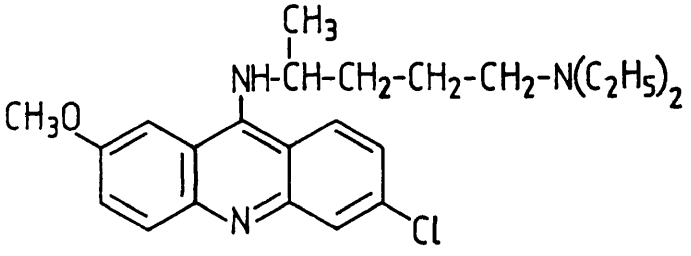
primaquine



amodiaquine



mefloquine



mepacrine

Amodiaquine

Amodiaquine is another 4-aminoquinoline which has been used as an antimalarial chemoprophylactic and therapeutic agent for many years. Reports of its improved efficacy over CQ in treating CQ-resistant *P. falciparum* (Hall *et al*, 1975; Watkins *et al*, 1984) led to a resurgence of its use in the 1980's. This in turn resulted in an increase in documented cases of severe toxicity to the liver and bone marrow (Centres for Disease Control, 1986), and its use as a prophylactic is no longer recommended. Minor toxicities are similar to those of CQ. However, because of increasing resistance, a high recrudescence rate (>50% with oral therapy in the Punjab - Khaliq *et al*, 1987), and the major toxicities described earlier, amodiaquine is now losing favour as an antimalarial agent.

Mepacrine

The chemical structure of mepacrine is similar to that of CQ, but with the addition of an extra ring. It was the first synthetic antimalarial to find widespread clinical application, being most active against the asexual erythrocytic form of all the plasmodia species and was a popular suppressive drug. However, mepacrine is much less widely used than formerly because of its tendency to produce serious side effects. Prolonged administration causes an unpleasant yellowing of the skin and the conjunctivae and effects on the nervous system can be severe or even fatal.

1.4.1.3 8-Aminoquinolines

Primaquine

Primaquine is the only agent in the group of 8-aminoquinolines currently employed as an antimalarial drug, and the only compound available for clinical use that acts in the liver on the hypnozoites of *P. vivax* and *P. ovale*. It is, therefore, a very useful antimalarial because it can prevent relapses of these infections from their dormant hepatic stages, and as a sporonticide and gametocytocide, has been used to prevent transmission of *P. falciparum*. Although the mechanism of drug action is unknown, the drug (or its metabolites) may affect plasmodial mitochondrial function and bind to DNA (Grewal, 1981). Adverse effects are minimal when administered to Caucasians, but primaquine can cause haemolysis of red cells deficient in glucose-6-phosphate dehydrogenase (G-6-PD), a more common condition in Negroes and people of Mediterranean or Asian ancestry.

1.4.1.4 4-Quinolinemethanol

Mefloquine

Mefloquine is a 4-quinolinemethanol which is effective against CQ-resistant and some quinine-resistant strains of *P. falciparum*. The drug acts to raise the pH in parasite acid vesicles in a similar manner to CQ, is well absorbed orally, but has proved too irritating for injection. It is an effective blood schizonticide, destroying the early asexual blood stages, but is not an effective tissue schizonticide. Clinical trials carried out in S. America, Africa, and S.E. Asia (WHO, 1984) showed mefloquine to be effective. However, almost immediately there were worrying reports of pre-existing "intrinsic", or rapidly developing

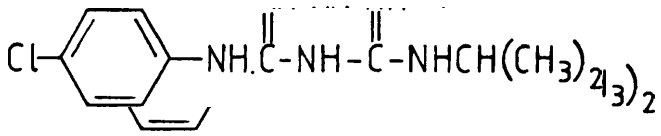
resistance of *P. falciparum* to the new drug. The WHO suggested that the drug be issued in combination with pyrimethamine/sulphadoxine (Fansidar) in an attempt to delay the development of resistance. It has since been suggested that antagonism exists between the mefloquine and pyrimethamine/sulphadoxine components in some *Plasmodium* isolates (Hoffman *et al*, 1985), and a high incidence of toxicity was reported when it was used for malaria prophylaxis (Miller *et al*, 1986).

1.4.2 FOLATE SYNTHESIS- AND DIHYDROFOLATE REDUCTASE INHIBITORS

Proguanil

Proguanil, and its analogue chloroguanil which act as dihydrofolate reductase inhibitors, were first synthesized in Britain in 1945. They destroy the asexual erythrocytic forms of all species of human malaria parasites, but their action is too slow for treatment of acute attack. Active against the exoerythrocytic forms of *P. falciparum*, the drugs provide a radical cure in cases of malaria due to this organism, and are effective suppressive agents for malaria due to the other species as well. Both proguanil and chloroguanil are pro-drugs, and exert their effect by being able to prevent the utilization of folic acid by the parasite. Their primary metabolites have similar structural features and antimalarial properties as pyrimethamine.

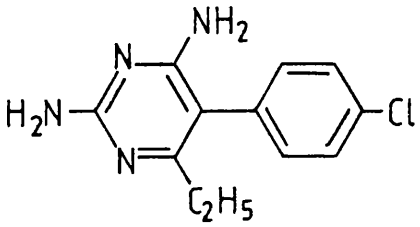
Proguanil has been considered to be one of the best tolerated antimalarial drugs, with mild gastric intolerance being the main complaint. Unfortunately, the malaria parasite develops resistance to proguanil with relative ease, and the drug can no longer be used in areas where resistant strains are appearing in large numbers. Recent data, however, on the efficacy of a proguanil/sulphonamide combination for malaria chemoprophylaxis is very encouraging (Pang *et al*, 1989).



proguanil

Pyrimethamine

Developed in the mid 1940's pyrimethamine is a synthetic 2,4-diamino pyrimidine, which has been widely used for prophylaxis both alone and in combination with sulphonamides. Unfortunately, widespread resistance to *P. falciparum* and *P. vivax* was noted within 2 years of its introduction, and although the drug continues to be effective in some areas (Bradley-Moore *et al*, 1985), most authorities no longer recommend it for chemoprophylaxis (Bruce-Chwatt, 1985; WHO, 1988), except in combination with sulphur drugs.



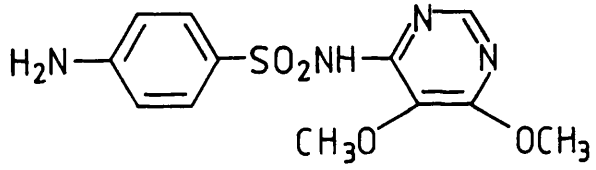
pyrimethamine

Pyrimethamine/sulfadoxine

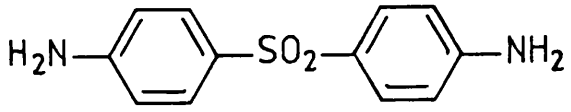
The combination of a diaminopyrimidine and a long acting sulphonamide was considered ideal because of a synergistic inhibitory effect exerted on the *Plasmodium* folic acid cycle (WHO, 1987). It was also hoped that by using the two drugs together, the development of resistance would be prevented or delayed (Pearson and Hewlett, 1987). Pyrimethamine, a dihydrofolate reductase inhibitor, is a slow-acting blood schizonticide for all 4 human plasmodia species, is active against exoerythrocytic stages of *P. falciparum*, and has weak sporonticidal activity (Goodwin, 1952). Sulfadoxine, in addition to being a dihydrofolate reductase inhibitor, is also a dihydropteroate synthetase inhibitor. It demonstrates slow activity against *P. falciparum* as a blood schizonticide, and shows weaker activity against the erythrocytic stages of the other species. Severe skin reactions were reported to have occurred in 1 per 5,000 to 8,000 Americans taking the pyrimethamine/sulfadoxine (Fansidar) combination for malaria prophylaxis, with fatal reactions in 1 per 11,000 to 25,000 (Miller *et al*, 1986). In view of this risk the drug is only recommended as a chemoprophylactic agent in persons at very high risk of CQ-resistant malaria in remote areas, or for presumptive self-treatment in travellers without access to medical care. A recent report, however, documented therapeutic failures when this regimen was used by US travellers to E. Africa (Centres for Disease Control, 1989).

Pyrimethamine/Dapsone

The combination of pyrimethamine with dapsone, a dihydropteroate inhibitor was introduced for chemoprophylaxis in 1968. Initially recommended weekly for prophylaxis, the regimen was changed to twice weekly, because of the discrepancy in the half-lives of the drugs. Between 1979 and 1982, 17 cases of agranulocytosis (some fatal) were reported with pyrimethamine/ dapsone use, most of these being associated with the twice weekly dose dapsone use, most of being associated with the twice weekly dose (Hutchison *et al*, 1986). It has since been suggested that haematological parameters should be monitored during long term use of the drug. The mechanism of action of pyrimethamine/dapsone (Maloprim) is similar to that of Fansidar, and therefore, the former agent is recommended for use in areas where CQ-resistant *P. falciparum* (CRPF) malaria occurs, but where Fansidar resistance is low . At present there is still a paucity of information on the efficacy of Maloprim in CRPF areas.



sulfadoxine



dapsone

1.4.3 SESQUITERPENE LACTONES

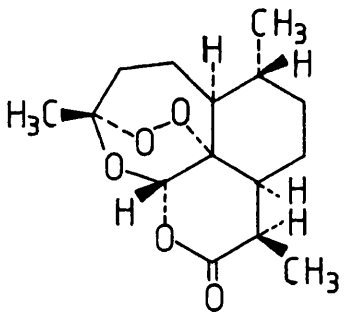
Artemisinin (Quinghaosu, QHS), is the parent compound of a unique group of antimalarials, which originated from the traditional Chinese medical pharmacopoeia. For centuries the Chinese have treated people suffering from malaria with a preparation made from the leaves and flowering tops of *Artemisia annua*,L., a traditional Chinese herb of the family Compositae. In 1972, Chinese scientists isolated the plant's antipyretic principle, which was shown to be an endoperoxide of a sesquiterpene lactone. It is poorly soluble in water or oil, a limiting factor in its absorption through the gut, but possesses good thermal stability. In an attempt to increase the solubility of artemisinin, as well as its antimalarial efficacy, several derivatives have been synthesized involving reduction of the C=O group at the C-12 position. The chemical structures of important derivatives of artemisinin with enhanced antimalarial activity are depicted in Fig. 1.3. Two resulting semi-synthetic forms, artemether (the methyl ether derivative of dihydroartemisinin), and sodium artesunate (the sodium salt of the dihydroartemisinin half-ester of succinic acid), are now employed as antimalarials in China. The former is indeed more oil soluble than the parent compound, but has more acute toxicity when used in laboratory animals. The latter, water-soluble derivative, whilst being 5.2 times more potent than artemisinin, and less toxic than artemether, is unfortunately unstable both within and outside the body. The lack of a stable water-soluble derivative is a major difficulty with the use of this group of drugs, which are not yet commercially available outside China (Woerdenbag *et al*, 1990).

The sesquiterpene lactones appear to be rapid-acting blood schizonticides of *P. falciparum* and *P. vivax*. Artemisinin acts early in the asexual parasite development cycle by destroying the very young, small ring forms (Li *et al*, 1984), and its ability to reduce levels of parasitaemia is even more rapid than quinine. Unfortunately, recrudescence rates with these drugs are very high, and for this reason the use of these drugs alone is not recommended. Their main contribution to the antimalarial armamentarium appears to be in rapid reduction of asexual parasitaemia in heavy *P. falciparum* infections. The mechanism of action of sesquiterpene lactones has yet to be fully elucidated. However, changes in the ultra structure of the parasite membranes after exposure to the drug have been reported. Morphological changes in the ribosomes, and the endoplasmic reticulum were described by Ellis *et al* in 1985, and nuclear swelling was noticed within the *P. innui* parasite in a study with monkeys (Jiang *et al*, 1985). In addition artemisinin and several derivatives have an *in vitro* inhibitory effect on the protein synthesis in human erythrocytes infected with *P. falciparum*, and a secondary effect on nucleic acid synthesis (Gu *et al*, 1983). The peroxide moiety of artemisinin is believed to be responsible for the parasiticidal effect of the molecule. It is thought that oxygen radicals are generated which bring about parasite destruction by attacking crucial cellular constituents (Ames *et al*, 1985). This is in agreement with observation that derivatives of artemisinin which lack the peroxide moiety, are devoid of antimalarial activity (China Cooperative Research Group, 1982). Several simple compounds containing a 1,2,4-trioxane ring have been prepared and evaluated for antimalarial activity in an attempt to gain information concerning the portion(s) of the QHS ring necessary for antimalarial activity (Kepler *et al*, 1988). The results indicated that the 1,2,4-trioxane ring system alone is insufficient for high activity, and that the 5-oxygen substituent of the 1,2,4-trioxane ring system of QHS is also important to its antimalarial action.

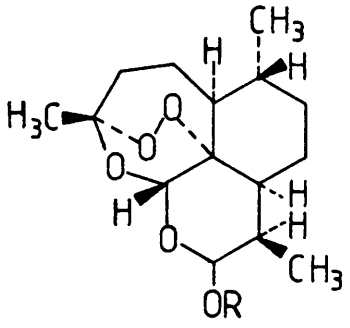
The effect of artemisinin has been tested in combination with other antimalarial drugs against CQ-resistant and CQ-sensitive strains of *P. falciparum* (Chawirat *et al*, 1987; Ye *et al*, 1989). In the resistant line, primaquine was potentiated, with mefloquine and tetracycline treatment showing synergism against both strains. Antagonism, however, was noted with sulphonamides, pyrimethamine, and CQ, and these findings were in agreement with those found *in vivo* in rodent malaria (Chawirat *et al*, 1986).

The Coordinating Clinical Study Group on QHS in China in 1979, (cited in Chang and But, 1986) successfully treated 2,099 patients in the 1970's with different artemisinin preparations. Among these were 1,511 cases of *P. vivax*, and 588 cases of *P. falciparum* malaria. In addition, 143 cases of CRPF malaria, and 144 cases of cerebral malaria were cured, with no clear adverse reactions or side-effects reported. Despite its effectivity against drug-resistant *Plasmodium* species, and a rather low toxicity, the high recrudescence rate is likely to limit the use of artemisinin. Arteether, an ethyl ether derivative which is more lipophilic than artemisinin should begin phase I trials in the near future, and the combination of sesquiterpene lactones with other antimalarial agents may prove very useful.

Fig. 1.3 Structure of Artemisinin and Important Derivatives



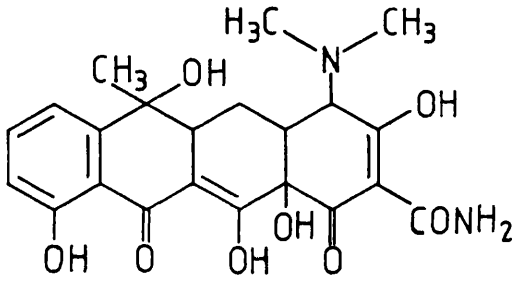
artemisinin



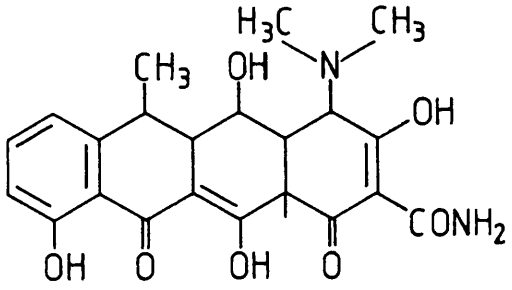
R	
CH_3	**artemether**
CH_2CH_3	**arteether**
$C(=O)CH_2CH_2COO^-Na^+$	**sodium artesunate**

1.4.4 ANTIBIOTICS

Over the years, several antibiotics have been screened for potential antiplasmodial activity. The efficacy of tetracyclines was already recognized in 1949 (Cooper *et al*, 1949), but were reconsidered as antimalarial drugs because of the progressive worldwide spread of CQ resistance. They now play a valuable role in the treatment of multiple drug-resistant *P. falciparum* infections, and can help to prevent recrudescence in other species. Tetracycline, and a derivative doxycycline are slow acting blood schizonticides, and are also effective against the primary exoerythrocytic phase of *P. falciparum* (Hoffman, 1986). Because of its slow action, tetracycline must be combined with rapidly acting quinine in treating acute *falciparum* infection in order to achieve a quick reduction in parasitaemia. On the other hand, doxycycline alone has proven effect in chemoprophylactic therapy (Pang *et al*, 1987), although gastrointestinal disturbances and vaginal candidiasis are common problems with this treatment regimen.

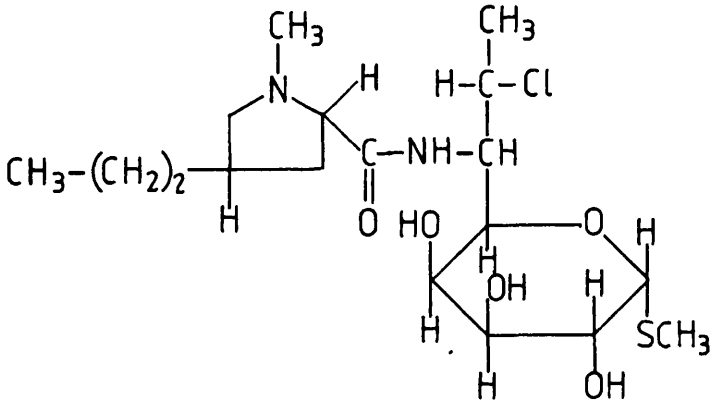


tetracycline

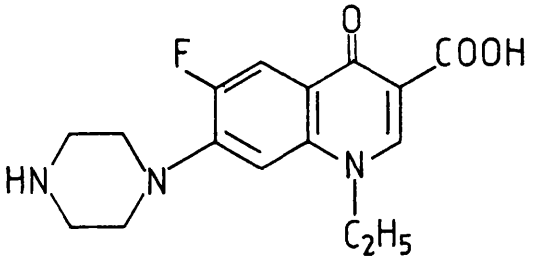


doxycycline

Clindamycin, a slow-acting blood schizonticide of *P. falciparum*, is employed in malaria treatment in a similar fashion to the tetracyclines. Another potent antibacterial agent which was accidently discovered to to have antiplasmodial activity, is norfloxacin. This DNA-gyrase inhibitor belongs to a highly active group of compounds known as fluoroquinolones, and its structure bears striking similarities to other quinoline derived antimalarials. A small preliminary study showed norfloxacin to be effective against asexual forms of *P. falciparum*, with no reported side-effects (Sarma, 1989).



clindamycin

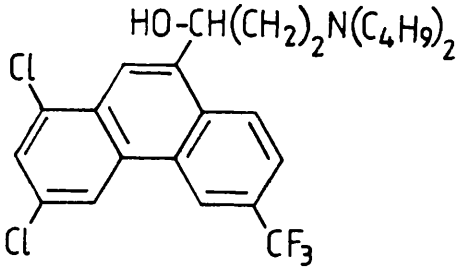


norfloxacin

1.4.5 9-PHENANTHRENE METHANOLS

Halofantrine

Halofantrine is a promising synthetic antimalarial drug recently developed by the Walter Reed Army Institute, which is effective against CRPF. Although early preparations of the drug showed variable bioavailability, it has proved effective in clinical trials and is now manufactured by Smith Kline and Beecham. It is slowly eliminated from the body, and hence could be used for a single-dose treatment of malaria (Sweeney, 1984).

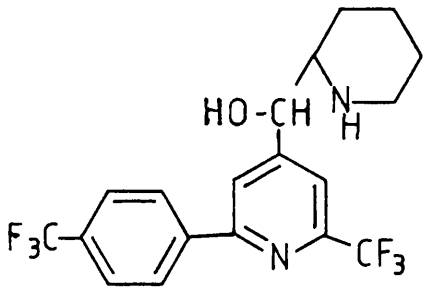


halofantrine

1.4.6 OTHER COMPOUNDS

Enpiroline

Enpiroline is another newly developed drug which has reputed efficacy comparable to that of mefloquine (Cosgriff *et al*, 1984).



enpiroline

1.5 THE URGENT NEED FOR NEW ANTIMALARIALS

The event of World War I saw the first modern attempts to identify compounds with antimalarial properties. During this time, quinine supplies (vitally needed as the sole therapeutic agent against malaria) became cut off from Austro-German and Turkish forces. Research efforts were similarly stimulated when the flow of quinine to both Allied and Axis forces became disrupted during World War II. As a result the first generation of modern synthetic antimalarials came into being, most notably CQ and primaquine (Wyler, 1984). However, after World War II, there was little incentive for industrial research into antimalarials, since little profit could be expected in return for what would necessitate a major investment. In 1956, the WHO started the Malaria Action Programme for the control and prevention of malaria world-wide, and in the United States, the Walter Reed Army Research Institute sustained the only major programme to identify new drugs. Approximately 30 compounds have reached late pre-clinical testing, a handful have qualified for clinical development, and only one - mefloquine, has enjoyed broad clinical application, and then only in certain regions. This is from more than 300,000 compounds tested at the institute (Harinasuta, 1983). Clearly, new antimalarials are not common place, and the hope that mefloquine would solve the widespread problem of CQ resistance, was quickly dashed with the rapid emergence of *P. falciparum* strains which were resistant to the new drug (Bugbjerg *et al*, 1983).

As already highlighted in the previous sections, it has become extremely difficult to propose simple, widely available, and uniformly acceptable recommendations for malaria prophylaxis. Important considerations in selecting a regimen include geographic regions to be visited, the prevalence of CRPF infections, the potential toxicity and lack of efficacy of alternative drugs, the time of year, and also the extent of exposure. It is essential to realise, however, that no drug can guarantee protection against the disease, and infection of a non-immune host by *P. falciparum* is a life-threatening illness. Management of the appropriate antimalarial therapy has become challenging in itself. A rapid diagnosis, accurate speciation, and an up-to-date knowledge of the distribution of drug resistance patterns are all required for a favourable outcome.

Man's failure to make any real impact on the malarial problem has been further exacerbated by the increasing resistance of the vector mosquitos to insecticides. Work is in progress to develop a vaccine against the disease, however, it will be some years before experimental studies can allow full scale clinical trials. Meanwhile, the number of individuals exposed to malaria is rising, and with no present-day antimalarial drug in existence which is uniformly safe, effective, and widely available, the urgent need for new antimalarials cannot be over-emphasized. It is a sobering thought that there is now a real prospect of encountering untreatable quinine-resistant malaria.

1.6 TRADITIONAL MEDICINE

The Alma-Ata Declaration of Member States of WHO to ensure "Health for all by the year 2000" has highlighted the vital role of plants and traditional medicine in reaching that goal. It is estimated that some 20 000 plant species are used medicinally (Penso, 1982), and that 75% of the world's population must rely upon these plants for the treatment of diseases (Marini-Bettolo, 1979). However, although medicinal plants have been used for millenia, relatively little is known about the efficacy and safety of traditional remedies, and only recently have they become the subject of scientific study.

It is known that malaria has been a scourge on mankind for thousands of years, and indeed malarial fevers are described in cuneiform tablets found in Mesopotamia, dating back to 2000 BC (Bruce-Chwatt, 1988). Therefore, it is not difficult to imagine that Man's earliest empiric efforts to find curative herbs for the disease must have been stimulated by the acute morbidity and mortality inflicted by falciparum malaria. Today, quinine and artemisinin are legacies from centuries of struggle to identify febrifuges and plants with antimalarial properties.

There is surely a strong probability that other plant antimalarial compounds (with novel chemical structures and novel modes of action) lie as yet undiscovered, and it seems only logical to investigate this largely untapped source. The NAPRALERT natural product data base maintained by the University of Illinois (a WHO Collaborating Centre for Traditional Medicine) lists species from 152 pantropically distributed genera which have folkloric reputations for antimalarial properties (Farnsworth, N.R, personal communication).

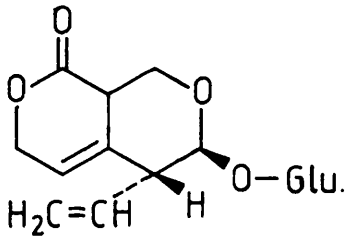
1.6.1 Plant Antimalarials in Chinese Traditional Medicine

Each developing country has its own indigenous system of traditional medicine. However, one of the most extensive and best documented source of materia medica is in Chinese traditional medicine. The written record dates back 3000 years, with its verbal tradition centuries earlier, and it is used by over one fifth of the world's poulation. Some uses have recently been scientifically substantiated, artemisinin from *A. annua* being a prime example, while many others await future research. In one review series, which includes monographs on some 250 kinds of Chinese materia medica stated to be in common use, 8 plants are documented as antimalarials, with comprehensive literature references to chemical, biological, and clinical findings (Chang and But, 1986, 1987).

Of those listed, probably the best known is **Qinghao**, the aerial part of *Artemisia annua* L. (Compositae). Highly esteemed for its antipyretic properties, it is mainly uesd in the treatment of malaria, low grade fever, scabies and ulcers. The antimalarial action of artemisinin and other sesquiterpene lactone constituents and derivatives of the herb are described in detail in section 1.4.3. Clinical studies using tablets prepared from the dilute alcohol extract achieved a 100% cure rate in 485 cases of tertian malaria, and 105 cases of subtertian malaria, when taken in divided doses spread over 3 days. A similar success rate was attained in another study in which 143 cases of CQ-resistant subtertian malaria were cured, and 131 out of 141 (i.e. 97%) cases of cerebral malaria were cured. Unfortunately, the relapse rate was high, although this

was reduced by increasing the dosage. A very important characteristic of the herb which makes it stand out from other antimalarials is its low toxicity. Only 3.4% of 590 malarial patients treated with the tablet of the herb extract developed gastrointestinal symptoms, such as nausea, vomiting, and abdominal pain. Another property of the plant which is highly relevant in this context, is its insecticidal activity. The acetone extract of the herb, or alternatively the dried plant material can also act as a mosquito repellant when applied to the skin.

Another herbal remedy **Longdan**, is derived from the root of *Gentiana manshurica* Kitag., (Gentianaceae). Amongst other conditions it is used for the treatment of jaundice, hepatitis, anorexia, eczema, and urinary tract infections. The root and rhizome of *Gentiana* spp. contains approx. 2% gentiopicrin, and this has been found to be lethal to *Plasmodium* sp. Side-effects were reported to be mild.



gentiopicrin

Qingfengteng is the stem of *Sinomenium acutum* Thunb., (Menispermaceae). The herb is recommended as an antirheumatic, but one of its constituents, sinomenine has been shown to be active against *Plasmodium* sp. (see Fig. 3.1.6).

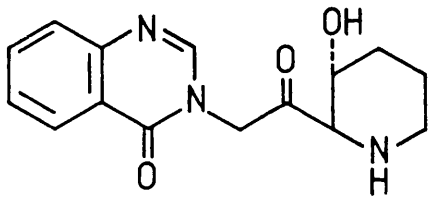
Chaihu refers to the root obtained from *Bupleurum chinense* DC., (Umbelliferae). It is used to treat fever and malaria, and like most of the traditional Chinese antimalarials, it has a bitter taste, and is described as having a slightly cold property. Saponins are the main constituents of the roots, and these appear to be the main active antipyretic components.

Another herb with a reputation for its antimalarial properties, **Huangjing** is derived from the fruit, root, and leaf of *Vitex negundo* L. (Verbenaceae). The bioactive components of the herb, which is mainly used to treat the common cold , coughs, and asthma, are thought to be flavonoid glycosides. The leaf decoction can kill *P. falciparum*, and when taken as a tea for 7 days was reported to be effective for malaria prophylaxis.

An antihelmintic from the Rosaceae family called **Hecaoya**, refers to the winter bud of *Agrimonia pilosa* Ledeb.var. Its parasiticidal effects are thought to be due to agrimophol, a phenolic constituent of the herb. Crude agrimophol given to rats intragastrically was weakly active against *P. berghei*, the ED_{50} being

34g (crude drug)/kg; it also markedly inhibited *P. innui.*

The root of *Dichroea febrifuga* (Saxifragaceae) is the Chinese medicinal herb **Changshan**, which is an antipyretic, effective against malaria, amoebiasis, and also giardiasis. Amongst other constituents, the root contains 3 alkaloid tautomers, α-, β-, and γ-dichroines. The former two compounds are more commonly called febrifugine and isofebrifugine. All show potency equivalent to, or greater than quinine when tested against *P. gallinaceum* (chicken malaria). Another report found isofebrifugine *P. gallinaceum* (chiken malaria). Another report found isofebrifugine to be 100 times more effective than quinine against *P. lophurae* (duck malaria).



febrifugine

Changshan was given in tablet form to 1926 patients. Those with tertian malaria were found to respond better to the herbal medicine than to chloroguanide and quinacrine. A further study was carried out involving 5984 malaria carriers below the age of ten, who were administered the herb in injection form. Parasitaemia was successfully reduced, although there was a high incidence rate of nausea and vomiting (40.1%). High liver toxicity caused by the febrifugine alkaloid has prevented its use else where, although the plant continues to be used in China (Steck *et al*, 1972).

Yadanzi is the name given to the ripe fruits of *B. javanica* L. Merr (Simaroubaceae), and is described as being toxic, extremely bitter, with a cold property. Apart from its antimalarial action, it is claimed to have antipyretic, detoxicant, anti-inflammatory, antidysenteric, and has a necrotizing effect on warts. Hence, the herb is a remedy for malaria, amoebic dysentery, and is used topically for corns and warts. The bark or root of the *Brucea* plant is a folk remedy in other countries for chronic diarrhoea, and cancer.

The active constituents of Yadanzi are bitter principles identified as quassinoids. Brusatol, bruceolide, and bruceines A, B, C, D, E, F, and G have been isolated, together with a number of glycosides. Bruceosides A and B were reported to have antineoplastic activity, as was oleic acid, one of the oils present in the herb. The structures of a number of bruceolides are given in Fig. 1.5.

Experiments using the orally administered kernel of *B. javanica* fruits, and the crude non-crystalline powder i.m., had a marked antimalarial action against *P. gallinaceum* in chickens, causing a rapid reduction (and eradication in some cases) of parasitaemia. The median effective dose (MED) of the kernel decoction was equivalent to that of quinine. However, a further study found it necessary to increase the dosage to half of the LD_{50}, and a number of toxic deaths were reported. It was shown that the therapeutic potency was greatly influenced by the variation in the sources of the herb, and also the duration of storage.

Conflicting reports have also arisen from clinical studies. The first report involved 27 cases (7 with malignant malaria) who were dosed orally 3 times daily for 5 days with capsules prepared from 5-15 *B. javanica* fruit kernels. After treatment, blood smears became negative in 25 cases, and 23 patients either suffered no further attacks, or suffered only one more. In a larger group of 89 cases treated with Yadanzi , 58 out of 67 cases with tertian malaria were cured, as were 6 of 13 subtertian, and 7 out of 9 quartan cases. No severe adverse reactions were reported. However, in a third study it was stated that the herb was ineffective against quartan malaria, susceptible to relapse, and with a high incidence of toxic reactions. Oral dosing causes abdominal pain, nausea, vomiting, and dizziness, and although the incidence of reactions was as high as 78.3% in one study, no deaths due to poisoning with the herb have been reported in over 200 years.

Interestingly, mosquito larvae and eggs were completely eradicated by contact with a 5-10% extract of the herb for 18-48 hours, and treatment of stagnant water by the stem and leaves was also effective.

1.7 PLANT ANTIMALARIAL AGENTS

An extensive investigation into the *in vivo* antimalarial activity of plants was carried out by scientists from Merck, as long ago as the 1940's (Spencer *et al*, 1947), but until recently there has been very little response to the findings. Some 600 species, representing 126 families of angiosperms were examined on the basis of literature references to their use as febrifuges or antimalarials. Methanolic or aqueous plant extracts were tested against *P. lophurae* or *P. cathemerium* (duck malaria), or *P. gallinaceum* (chicken malaria). Table 1.1 shows species from over 30 genera found to possess activity. Included in the list are three of the Chinese herbs discussed in the previous section. The Simaroubaceae family (from which *B. javanica* originates) was highlighted for its high number of active species.

The last 10 years have seen major break-throughs in the screening for antimalarial activity. The avian malarias of the Merck screen have been largely superceded by rodent models using *P. bergei* (Peters, 1970), and the cultivation of *P. falciparum in vitro* (Trager and Jenson, 1976) subsequently led to the development of a much needed *in vitro* microdilution assay (Desjardins *et al*, 1979). These techniques have proved to be invaluable tools for the rigorous screening of plants for antimalarial activity, and several research groups have now demonstrated activity for a large number of diverse plant products. The following section deals briefly with some of the different types of natural product molecules which exhibit antiplasmodial activity.

Table 1.1 Plant Species Demonstrating Activity Against Avian Malarias [a]

Amaryllidaceae spp.
Aristolochia sp..
Cissampelos pareira
Cooperia pedunculata
Cornus florida
Croton spp.
Datisca glomerulata
Dichroa febrifuga
Eryngium foetidum
Gentiana spp.
Hymenocallis caribea
Remijia peruviana
Schultesia lisianthiodes
Simaroubaceae spp.

a Spencer *et al*, 1947

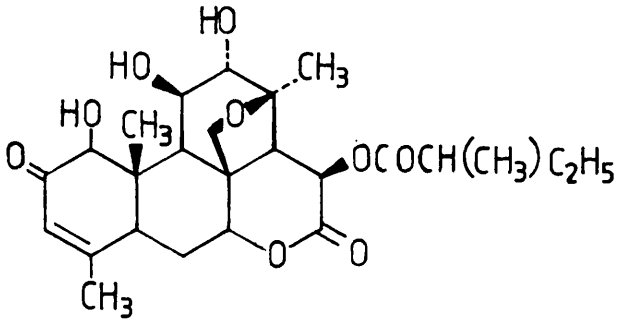
1.7.1 Quassinoids

The Simaroubaceae family is taxonomically related to the Meliaceae and the Rutaceae, and its species are used pantropically as antimalarials in Thailand, Malaysia, India, Africa, and South and Central America. Several species have been investigated, and the active principles identified as bitter pentacyclic terpenoids which are related to limonoids, and known collectively as quassinoids. The generic term arises from quassin, the name of the first structurally identified member of its class, of which over 120 quassinoids are now known. Most of the knowledge regarding the chemistry of quassinoids has been gained mainly from work carried out by J. Polonsky and colleagues over the last 30 years (Polonsky *et al*, 1973, 1985 and 1986).

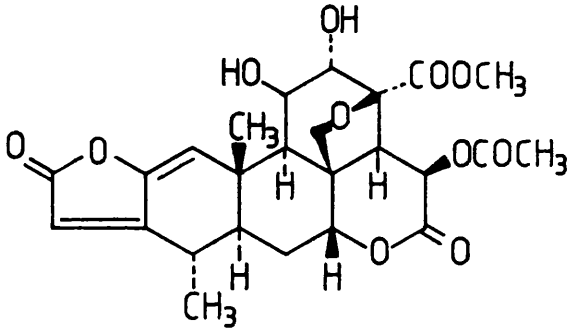
In 1981 Trager and Polonsky reported the antiplasmodial activities of 5 quassinoids, the most active of these was simalikalactone D (Fig. 1.4), which gave complete inhibition of *P. falciparum* at 0.002 μg/mL. Also in the early 80's, bruceantin, a quassinoid from the *Brucea* species was found to demonstrate high *in vitro* activity with an IC_{50} value of 0.001 μg/mL, whereas the parent compound quassin was inactive at 25 μg/mL (Guru *et al*, 1983).

Since then a number of Simaroubaceae species have been examined for their antiplasmodial activities, and bioassay-guided fractionation of the active extracts has lead to the isolation of a diverse array of quassinoids. A comparison of their relative IC_{50} values allows a number of conclusions about structure-activity relationships to be drawn. Quassinoids obtained from the genus *Brucea* are known as bruceolides and they comprise a series of C-15 esters of the parent alcohol bruceolide. For example, the majority of bruceolide quassinoids isolated from *B. javanica* differ only in the nature of the ester function at C-15 (see Fig. 1.5). If this moiety is changed, a ten-fold difference is observed between the most active compound bruceantin (IC_{50} 0.001μg/mL), and the least active, bruceine B (IC_{50} 0.01μg/mL). Removal of the ester group altogether, as in the case of bruceolide (IC_{50} 0.451μg/mL) reduces activity 500 fold. Similarly, a 3-fold difference in activity is noted for brusatol and bruceine A (IC_{50} 0.003μg/mL and 0.011μg/mL,

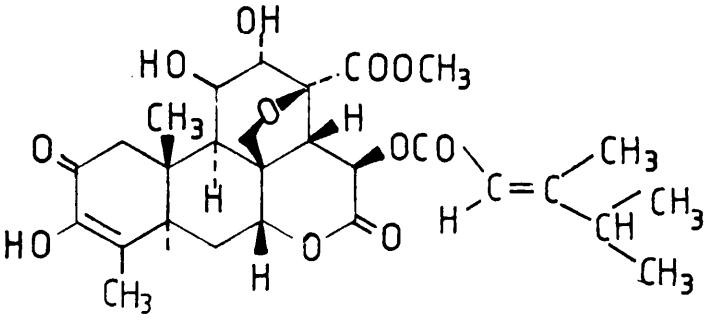
Figure 1.4 *In Vitro* Activities of Some Quassinoids Against *P. falciparum*



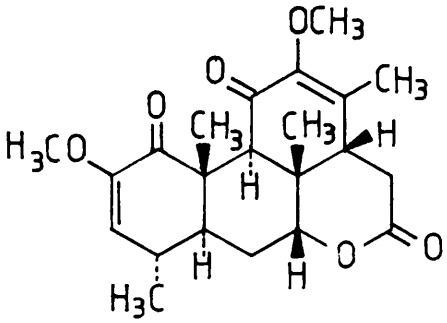
simalikalactone D



sergeolide



bruceantin



quassin

Quassinoid	IC_{50} µg/mL
Bruceantin	0.001[a]
Quassin	25.0[a]
Simalikalactone D	0.002[b]
Sergeolide	0.006[c]

a Guru *et al*, 1985

b Trager and Polonsky, 1981

c Fandeur *et al*, 1985

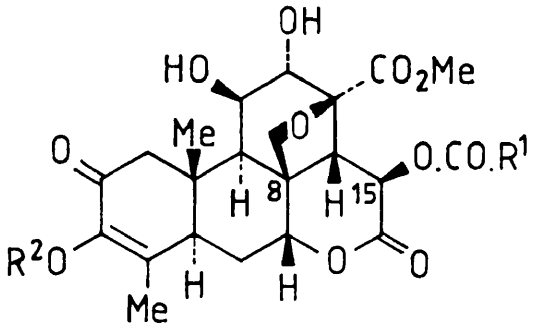
respectively), the latter being merely the dihydro derivative of the other. A 4-fold activity difference is observed between bruceine A and its dehydro derivative dehydrobruceine A, which has an IC_{50} value of 0.046µg/mL (O'Neill *et al*, 1987). The C-15 ester side-chain of bruceantin is slightly longer than brusatol, and the compound is more than 3 times as active against *P. falciparum in vitro* , illustrating further the importance of the C-15 ester moiety to lipophilicity, or other specific steric requirements in *in vitro* antimalarial activity.

The polar quassinoids bruceine D and yadanziolide A (IC_{50} 0.015 and 0.031μg/mL) were isolated from a butanolic extract of *B. javanica*, and exhibited slightly less activity than the more lipophilic, chloroform-soluble quassinoids. Two quassinoid glycosides, yadanziosides F and I demonstrated considerably less activity, having IC_{50} values of 5μg/mL and 22.04μg/mL, respectively. Indeed a 2000-fold decrease in activity is apparent from a comparison of the 3-O-glycoside (Yadanzioside I) with its aglycone bruceine B.

Several of the bruceolide quassinoids have been tested for *in vivo* antimalarial activity against *P. berghei*. Brusatol and bruceine B showed high activity, having ED_{90} values of 3.03 and 2.82 mg/kg/day respectively, and bruceine B appeared to cause fewer deaths. As might be expected, the *in vitro* anti-*P. falciparum* activities of the quassinoids do not parallel their *in vivo* activities against *P. berghei* after oral dosing. This indicates a difference in the pharmacokinetics of the compounds and warrants further investigation.

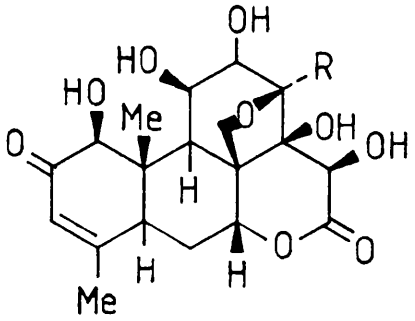
Sergeolide (Fig. 1.4), a natural quassinoid with a butanolide function isolated from *Picrolemma pseudocoffea* showed complete inhibition of growth of CQ-sensitive strains of *P. falciparum* at 0.006μg/mL, and had a marked effect against *P. berghei* in mice at 0.26 mg/kg/day. However, in its present form at least, the high degree of toxicity (LD_{50} 1.8 mg/kg/day) is thought to render the molecule unsuitable for malaria curative treatment (Fandeur *et al*, 1985).

Figure 1.5 Structures of Quassinoids Isolated from *Brucea javanica* Fruits

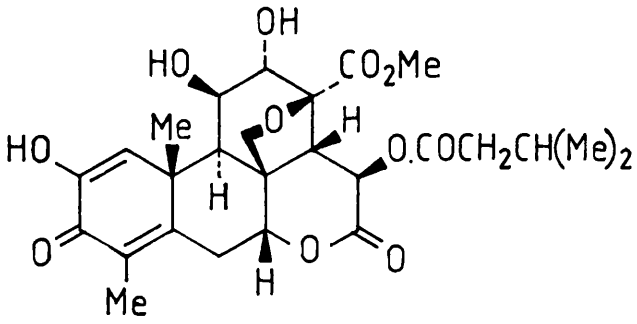


	R_1	R_2
Bruceine A	$CH_2CH(Me)_2$	**H**
Bruceine B	**Me**	**H**
Bruceine C	$CH=C(Me)C(OH)(Me)_2$	**H**
Brusatol	$CH=C(Me)_2$	**H**
Bruceolide	**C-15 β-OH**	**H**
Yadanzioside I	**Me**	**D-glucose**

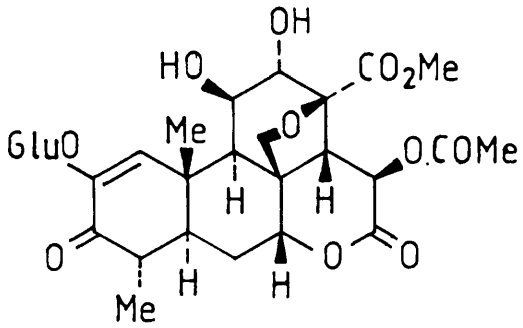
Figure 1.5 cont. Structures of Quassinoids Isolated from *Brucea javanica* Fruits



	R
Bruceine D	**Me**
Yadanziolide A	**CH_2OH**



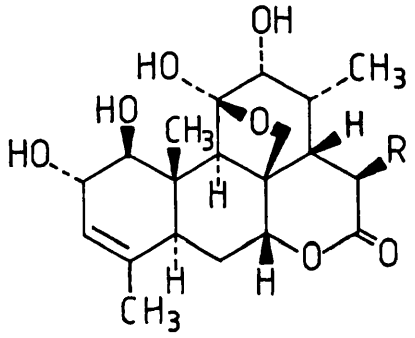
Dehydrobruceine A



Yadanzioside F

The importance of the ester function at C-15 is further illustrated by the compounds shown in Fig. 1.6 which possess a dihydroxy substituted A ring; glaucarubin is approximately 3 times more active than chaparrin, and has activity about 8 times greater than glaucarubol. However, all 3 compounds appeared to be inactive *in vivo* at 9 mg/kg/day. The *in vitro* activities of glaucarubinone suggest that an ester function at C-15 improves activity over one at C-6. In addition, if the C-15 position is already esterified, further esterification at C-6 produces little enhancement in activity as illustrated by the IC_{50} values of undulatone and holacanthone.

Figure 1.6 ***In Vitro*** **Activities of Chaparrin, Glaucarubol and Glaucarubin Against** ***P. falciparum*** **(K1)[a]**

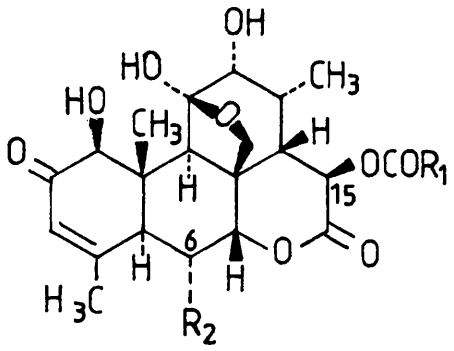


Quassinoid	R	IC_{50} µg/mL
Chaparrin	H	0.180
Glaucarubol	OH	0.410
Glaucarubin	$OCOC(CH_3)(OH)C_2H_5$	0.055

[a] O'Neill *et al*, 1986

The first 4 compounds shown in Fig. 1.7 were isolated from *Simarouba amara*, a species found in Central and South America (O'Neill *et al*, 1988). These compounds differ in their C-15 ester substituents, but unlike the bruceolides discussed above, their IC_{50} values are of the same order of magnitude. However, activity is markedly reduced if the ester is replaced by a straight alkyl chain as in 15-β-heptylchaparrinone, or by a sugar moiety as in 15-glucosylglaucarubolone.

Figure 1.7 *In Vitro P. falciparum* Activities of Quassinoids Differing in their C-6 and C-15 Substituents



Quassinoid	R_1	R_2	IC_{50} µg/mL
Ailanthinone	$OCOCH(CH_3)C_2H_5$	H	0.009
Glaucarubinone	$OCOC(CH_3)(OH)C_2H_5$	H	0.004
2^1-acetylglaucarubinone	$OCOC(CH_3)(OAc)C_2H_5$	H	0.008
Holacanthone	OAc	H	0.007
15β-heptylchaparrinone	$(CH_2)_6CH_3$	H	0.067
15-glucosylglaucarubolone	O.Glu	H	3.64
Undulatone	OAc	$OCOC(CH_3)=CHCH_3$	0.006
6α-senecioyloxychaparrinone	H	$OCOCH=C(CH_3)_2$	0.008
Chaparrinone	H	H	>0.01[b]

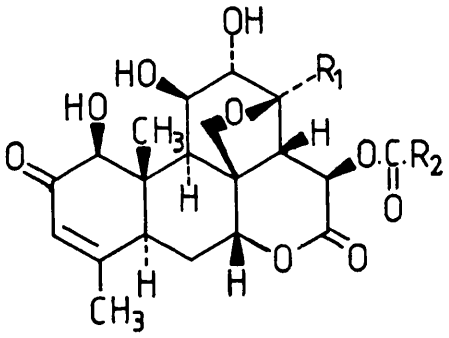
a O'Neill and Phillipson, 1989

b Trager and Polonsky, 1981.

The difference in the position of the methylene-oxygen bridge is also significant. This is the only structural difference, for example, between the two compounds ailanthone and simalikalactone D (see Figs. 1.4 and 1.9). In simalikalactone D, the bridge is positioned between C-8 and C-13, and in ailanthone it joins C-8 to C-11. A 10-fold difference in *in vitro* activity against *P. falciparum* is observed as a result if this difference.

If bruceines A and B are compared with iso-bruceines A and B, a 5-fold change in activities is found, the iso-derivatives showing the greatest activity (Fig. 1.8). Both series differ only in the oxidation pattern of the A ring - both have an α,β-unsaturated keto group and also a hydroxyl which is adjacent to the carbonyl ring. Glaucarubin, which lacks an α,β- unsaturated keto function in ring A, is 10 times less active than glaucarubinone.

Figure 1.8 ***In Vitro*** **Activities of Isobruceine A and B Against** ***P. falciparum*** **(K1)[a]**

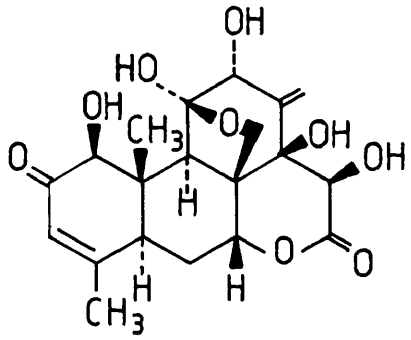


Quassinoid	R_1	R_2	IC_{50} µg/mL
Isobruceine A	CO_2CH_3	$CH_2CH(CH_3)_3$	0.002
Isobruceine B	CO_2CH_3	CH_3	0.002

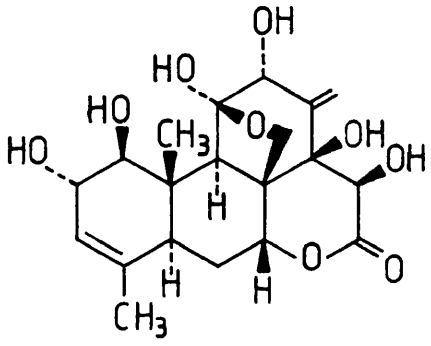
a O'Neill and Phillipson, 1989.

Two other compounds which differ in their A ring structure are eurycomanone and eurycomanol (see Fig. 1.9), and were isolated from *E. longifolia*. This is a species native of Burma, Thai, Indo-China and S.E. Asia, and is prescribed in Malay herbal medicine as a febrifuge (Chan *et al*, 1986). Eurycomanone has an α,β unsaturated ketone, and shows activity 2.5 times greater than eurycomanol which has a glycol function. Ailanthone, on the other hand, from *Ailanthus altissima* is 7 times more active than eurycomanone which has hydroxyl substituents at C-14 and C-15. When tested *in vivo* against *P. berghei*, ailanthone demonstrated similar activity to ailanthinone and glaucarubinone, whose structures also possess α-ketol groups in ring A.

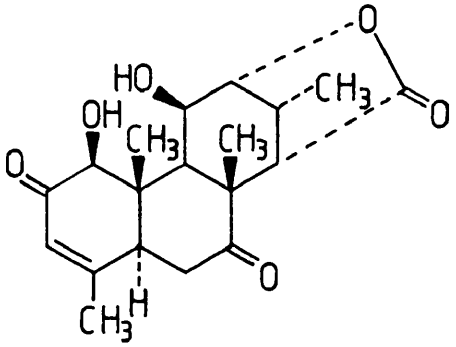
Figure 1.9 Structures and *In Vitro* Activities Against *P. falciparum* (K1) of Ailanthone, Eurycomanone, Eurycomanol, Eurycomalactone and Cedronine



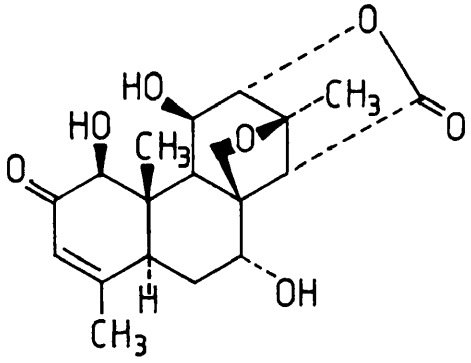
eurycomanone



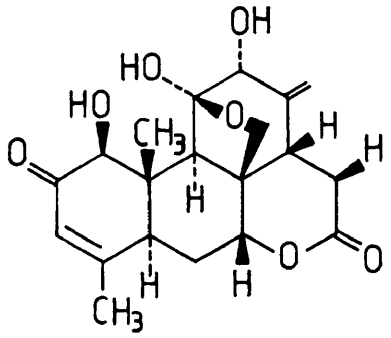
eurycomanol



eurycomalactone



cedronine



ailanthone

Figure 1.9 cont.

Quassinoid	IC_{50} µg/mL[a]
Ailanthone	0.015
Eurycomanone	0.110
Eurycomanol	0.280
Eurycomalactone	0.210
6-hydroxy-5,6-dehydroeurycomalactone	1.150
Cedronine	50.0[b]

a O'Neill *et al*, 1986.

b O'Neill unpublished result

Fig. 1.9 also shows a number of C-19 quassinoids. Eurycomalactone and 6-OH-5,6-dehydroeurycomolactone infer that hydroxylation at position C-6 results in a 5-fold decrease in antimalarial potency.

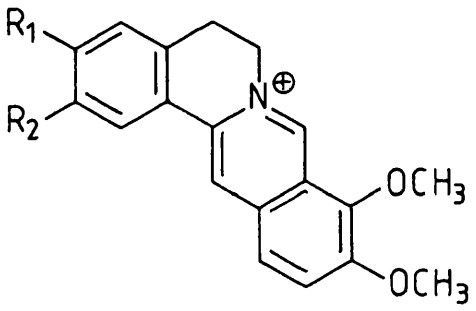
The loss of activity demonstrated by cedronine, a compound isolated from *Simaba cedron* (O'Neill unpublished result) is surprising since it possesses a methylene-oxygen bridge. However, the B ring carbonyl of this molecule is also reduced to secondary alcoholic function.

Some quassinoids (eg. bruceantin) have been reported to have antitumour activity (Cassady and Suffness, 1980). Undoubtedly, some quassinoids are highly toxic to mammalian cells, and the antiplasmodial activity is merely a reflection of general toxicity. However, *in vitro* activity against KB cells - a human epidermoid nasopharyngeal carcinoma (O'Neill, *et al*, 1986, Anderson *et al*,1989), and *in vivo* activity against *P. berghei* (O'Neill *et al*, 1987,1988) have provided strong evidence that quassinoid antimalarial activities do not necessarily parallel their cytotoxic activities. As yet, the mechanism of action of quassinoids against *Plasmodium* spp. is not fully understood, and further investigation is needed if the differences in specificity are to be exploited.

1.7.2 Alkaloids

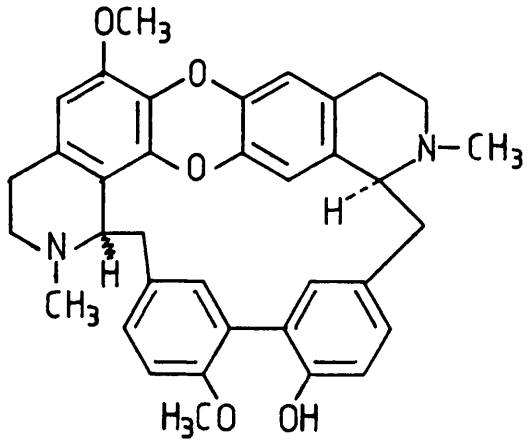
Quinine and its isomer quinidine from *Cinchona* species, and febrifugine, the active constituent of the Chinese malaria remedy *Dichroa febrifuga* have already been discussed in section 1.4. Berberine is an alkaloid constituent of 9 plant families, 2 examples being Berberidaceae and Menispermaceae. Berberine and three closely related alkaloids, palmatine, jatrorrhizine, and columbamine (Fig. 1.10) were shown to have *in vitro* activity against a number of *P. falciparum* strains, ranging from 0.14 - 1.61μg/mL (Vennerstrom and Klayman, 1988; Partridge *et al*, 1988). Although these alkaloids are obtained from plants widely used in the traditional treatment of malaria, leishmaniasis, and amoebiasis, and have been found to have *in vitro* activity, none have yet proved to be active *in vivo* (Vennerstrom and Klayman, 1988).

Figure 1.10 Structure of Berberine and Three Related Alkaloids



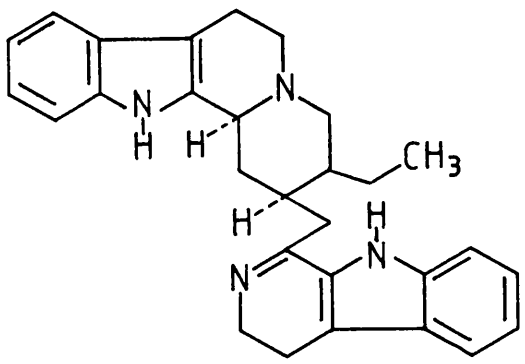
R_1	R_2	
$-OCH_2O-$		**berberine**
OCH_3	OCH_3	**palmatine**
OH	OCH_3	**jatrorrhizine**
OCH_3	OH	**columbamine**

An investigation of 18 plant species from Sierra Leone with reputations as antimalarials, led to the isolation of a series of bisbenzylisoquinoline alkaloids from *Triclisia patens* which were active against a multidrug resistant strain of *P. falciparum* (K1). The IC_{50} values of pycnamine and phaeanthine were 0.15 and 1.43μg/mL respectively (Partridge *et al*, 1988). Three other alkaloids were isolated during a separate study in Thailand of another Menispermaceae species *Triclisia triandra*. The most active tiliacorinine had a IC_{50} value of 0.68μg/mL.



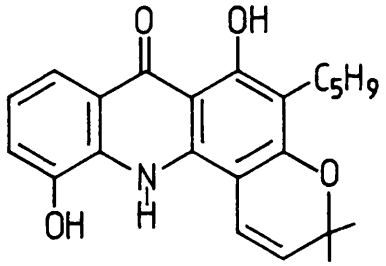
tiliacorine

Dihydrousambarensine is a bisindole alkaloid from the *Strychnos* species, *S. usamberensis* which had an IC_{50} value against *P. falciparum* of 0.01μg/mL, some 15 times more active than CQ (Wright *et al*, 1990).



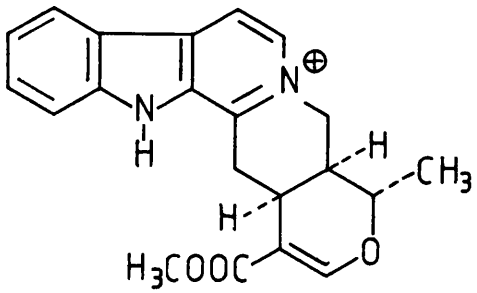
dihydrousambarensine

Citrus grandis (Rutaceae) is used as an antimalarial in Taiwan. Several acridone alkaloids from this and other Rutaceae species have demonstrated *in vitro* antiplasmodial activity (Fujioka *et al*, 1989). Atalaphillinine, from *Atalantia monophylla* was also active against *P. berghei in vivo*, with little toxicity.



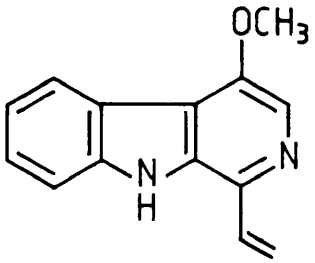
atalaphillinine

Investigation into the constituents of *Alstonia* species was stimulated by the knowledge that extracts from these plants were used as a cure for malaria in the Far East. Alstonine was one highly aromatic alkaloid isolated from *A. constricta*, and its hydrochloride was shown to have greater activity than quinine against *P. lophurae* in duck, but was also more toxic (Vasanth *et al*, 1990).



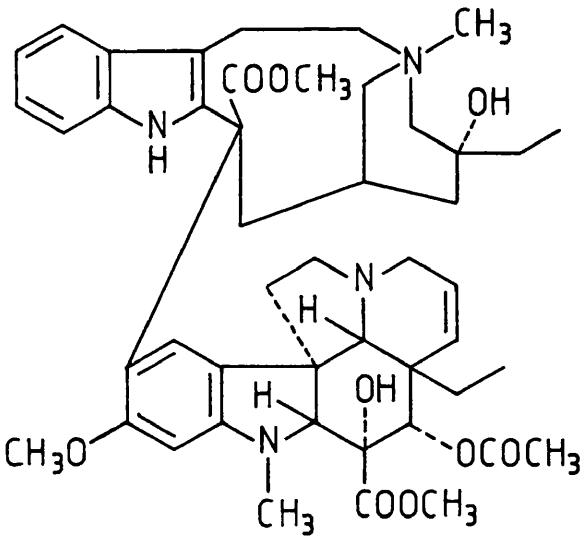
alstonine

In addition to the quassinoids discussed previously, simple β-carboline alkaloids may also contribute to the antimalarial activity of some species of Simaroubaceae. The 4-methoxy-1-vinyl-β-carboline was isolated from *Picrasma javanica*, and reported to have *in vitro* activity against *P. falciparum* with an IC_{50} value of 1.9 μg/mL (Pavanand *et al*, 1988).



4-methoxy-1-vinyl-β-carboline

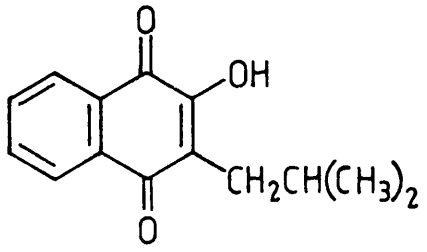
Another indole alkaloid, vinblastine which is an antineoplastic from *Catharanthus roseus* is also highly toxic to *P. falciparum*, arresting the growth of trophozoites (Usanga *et al*, 1986). Although the drug is too toxic for clinical use as an antimalarial, it has nevertheless provided valuable information on the mode of action.



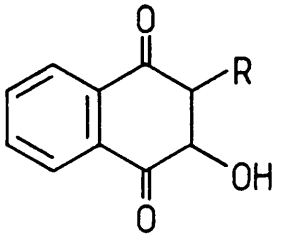
vinblastine

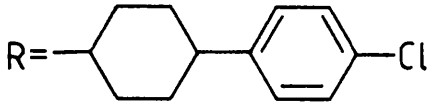
1.7.3 Quinones

Lapachol is a naphthoquinone present in some species of the Bignoniaceae (eg. *Tabebuia rosea*) which is shown to demonstrate weak activity against *P. falciparum*, and reduce the number of *P. lophurae* parasites in duck by 87% at doses >200 mg/kg (Carvalho *et al*, 1988; Fieser, 1948). Derivatives of lapachol and other hydroxynaphthoquinones have subsequently been synthesized which have activities against various species of protozoa (Hudson *et al*, 1985). One such compound, produced at the Wellcome Foundation and given the code BW566C was found to be more stable than previously developed naphthoquinone drugs, and highly active against *P. falciparum* infections in the Aotus monkey (Pudney, 1987).



lapachol

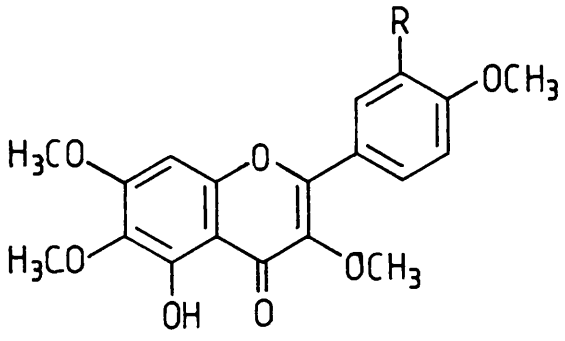




BW 566C

1.7.4 Phenols

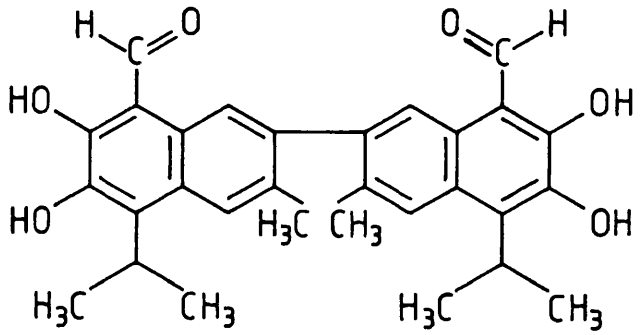
It has been established that flavonoids are common constituents of higher plants. Two methoxylated flavonoid constituents of *A. annua*, namely artemetin and casticin, potentiate the *in vitro* activity of artemisinin against *P. falciparum*, at concentrations in which they themselves exert no antiplasmodial activity (Elford *et al*, 1987). These flavonoids inhibit the influx of glutamine and myoinositol into parasitised erythrocytes.



R= OCH_3, **artemetin**

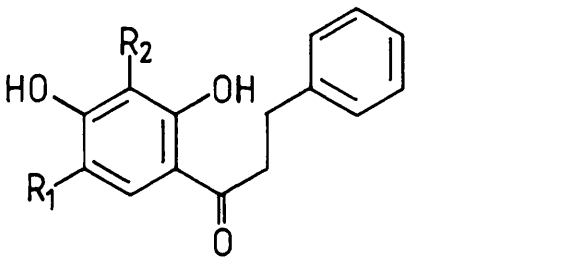
R= OH, **casticin**

A polyphenolic compound gossypol, from *Thespepsia populnea* and other species has been acredited with antispermatogenic and antiprotozoal activity (Gonzalez-Garza and Said-Fernandez, 1988). It inhibited the growth of *P. falciparum in vitro* at concentrations below 1μg/mL, and its antiparasitic effect was apparent at all stages of *P. falciparum* development in cultured human erythrocytes.

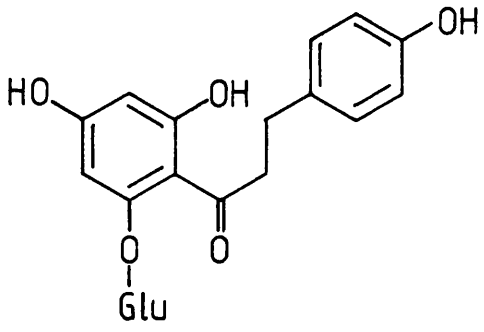


gossypol

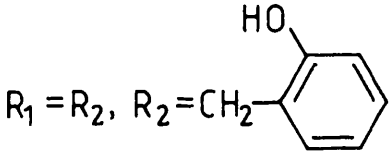
Phloridzin is a bitter tasting chalcone which has been used for its antimalarial properties, and subsequently found to block parasite induced permeability into erythrocytes (Silfen *et al*, 1988). Two other chalcones isolated from *Uvaria* species (Annonaceae); uvaretin and diuvaretin have *in vitro* IC_{50} values against *P. falciparum* of 3.49 and 4.2μg/mL, respectively (Phillipson and Wright, 1990).



uvaretin



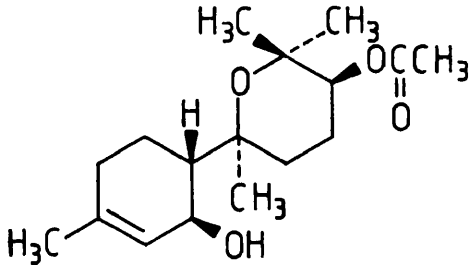
phloridzin



diuvaretin

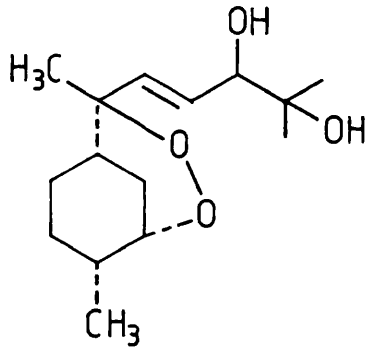
1.7.5 Other Terpenes

The sesquiterpene lactone, artemisinin from *A. annua*, and its derivatives have been mentioned in detail in section 1.4.3. Another *Artemisia* species , *A. abrotanum* is used in European herbal medicine for the treatment of fever. A novel sesquiterpene, 1(s)-hydroxy-α-bisaboloxide A was isolated from this plant, and shown to have an IC_{50} value against *P. falciparum* of 5.1μg/mL (Cubuku *et al*, 1990).

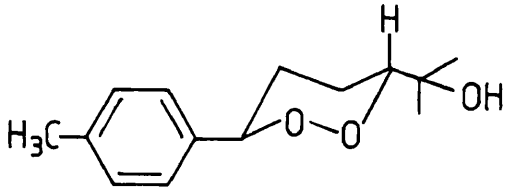


1(s)-hydroxy-α-bisaboloxide A

The highly active and rare endoperoxide moiety has also been found in species of the Annonaceae, a distinctly different family from the Compositae. Yingzhaosu A and C are 2 active terpenoids from *Arbatotrys unciatus* which incorporate this (Klayman *et al* , 1985).

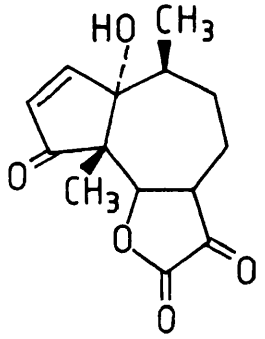


yingzhaosu A



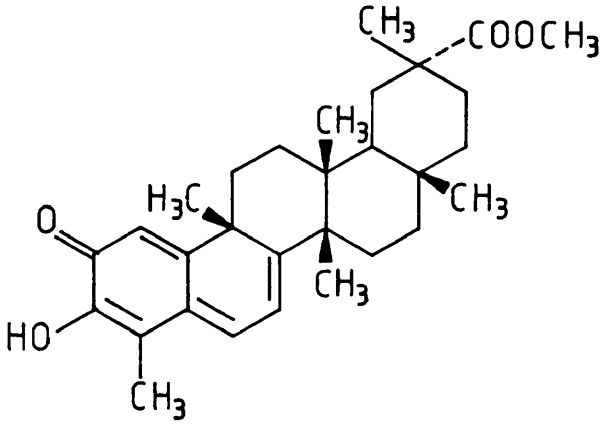
yingzhaosu C

Parthenin, from *Parthenium hysterophorus* is another sesquiterpene lactone which has an *in vitro* IC_{50} value of 1.29µg/mL against *P. falciparum*, somewhat higher than artemisinin (Hooper *et al*, 1990).



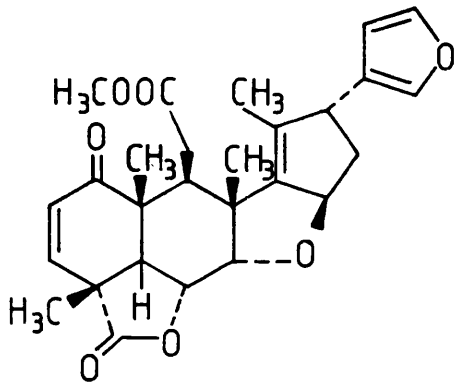
parthenin

The root, stems, and leaves of *Celastrus paniculatus* are prescribed in Thailand for the treatment of fever (Pavanand *et al*, 1989a),and the root bark is sold as an antimalarial. The active ingredient was discovered to be a triterpenoid, pristimerin, with an IC_{50} value against *P. falciparum* of 0.28µg/mL.



pristimerin

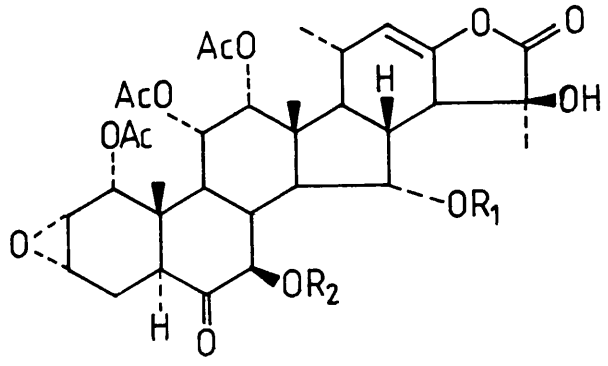
The Meliaceae is closely related to the Simaroubaceae, the plant family from which quassinoids originate. A group of compounds, known collectively as limonoids are bitter principle constituents found in species of the Meliaceae. The limonoids are biosynthetically related to the quassinoids. Nimbolide, is a limonoid isolated from the "Neem tree" *Azadirachta indica*, which has a reputation as an antimalarial throughout Asia and India. It was shown to have an IC_{50} value of 0.95µg/mL against *P. falciparum in vitro* (Rochanakij *et al*, 1985), and of 19 limonoids tested by Bray *et al*, 1985 and 1990, 3 others in addition to nimbolide were found to have activity with IC_{50} values between 0.5 - 3.0µg/mL.



nimbolide

1.7.6 Miscellaneous Natural Products

New steroidal bitter principles, taccalonolide A, B, C and D have been isolated from *Tacca plantaginea*, a Chinese medicinal plant which demonstrated antiplasmodial activity against *P. berghei* (Chen and Wang, 1987). Activities against *Plasmodium* and P-388 cell culture appear to indicate that the enol-γ-lactone portion of the molecule may be important for biological activity (Chen and Wang,1988).

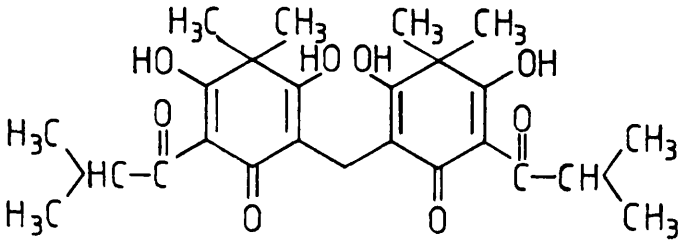


$R_1 = Ac$, $R_2 = H$ **taccalonolide A**

$R_1 = H$, $R_2 = H$ **II B**

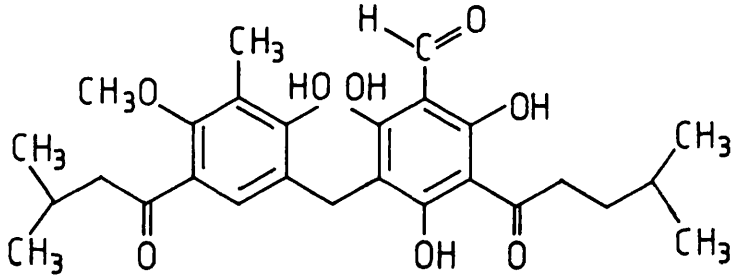
$R_1 = H$, $R_2 = Ac$ **II C**

Japonicine A, isolated from Diercao (*Hypericum japonicum*) is reported to show antimalarial activity in mice (Gu *et al*, 1984).

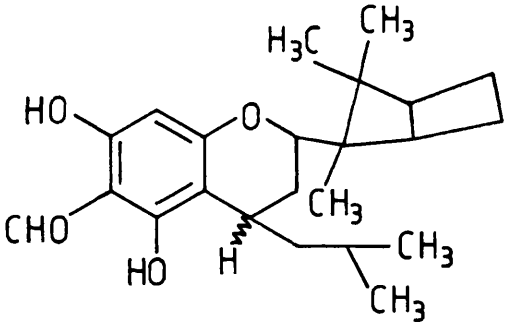


japonicine A

Yet another plant used in Chinese herbal medicine to treat malaria is *Eucalyptus robusta* (Myrtaceae). Several active compounds, robustaol A and 2 more active compounds structurally related to the euglobols, robustadial A and B were isolated. Investigations into their chemistry and biological activities are on-going (Xu *et al*, 1984).



robustaol A



αH = robustadial A

βH = II B

1.8 AIMS OF WORK

1. To investigate the antimalarial activity of an aqueous "tea" prepared from *Brucea javanica* fruits - a traditional Chinese medicinal.
2. To compare the cytotoxic and antiplasmodial activities of some plant derived antimalarials.
3. To develop a reliable microtitre test method for *in vitro* cytotoxicity testing of potential antimalarial agents.
4. To compare *in vitro* and *in vivo* test methods for chemotherapeutic activity and toxicity.
5. To investigate the modes of action of selected antimalarials against mammalian cells.

CHAPTER TWO — PHYTOCHEMICAL STUDIES

2.1 Materials and Methods

2.1.1 PLANT MATERIAL

The source of the plant material is given in the text and the name of the supplier (and also authenticator) is mentioned in the acknowledgements. All plant material was air dried in the country of origin before transportation, and powdered prior to extraction.

2.1.2 SOLVENTS AND REAGENTS

All solvents and reagents were supplied by BDH Ltd., May and Baker Ltd., or Sigma Chemical Co. Ltd., except where otherwise stated in the text. Methanol was redistilled before use.

2.1.3 EXTRACTION PROCEDURES

In extracting plant material, two principal schemes were followed. Fig. 2.1 illustrates the extraction procedure for the neutral compounds, including quassinoids. Ground plant material was extracted sequentially with petroleum ether, methanol and water. The concentrated methanolic extract was further partitioned between chloroform, n-butanol and water.

Fig. 2.2 shows the scheme employed for alkaloid extraction. Ground plant material was sonicated for 30 mins with methanol. The filtered extract was concentrated to dryness and the residue extracted with 2% sulphuric acid. The filtered acid extract was basified with dilute ammonium hydroxide solution and extracted into chloroform. The concentrated chloroform extract was further extracted with dilute acid, which was again rendered alkaline with ammonium solution, and shaken with chloroform. The chloroform solution, on evaporation to dryness yielded the crude alkaloid extract. Each chloroform and aqueous fraction was monitored throughout the extraction process for the presence of alkaloids, using Dragendorff reagent (see section 2.1.6.2).

2.1.4 AQUEOUS INFUSION PREPARATION

500 mg of *B. javanica* fruits from Hong Kong were boiled with 1L of distilled water for 60 mins. The viscous extract was allowed to cool, filtered and freeze dried (see Fig. 2.3). The product was then subjected to a series of chloroform extractions and acid hydrolyses (see Fig. 2.4 and section 2.1.5).

Figure 2.1 Extraction Procedure for Neutral Compounds

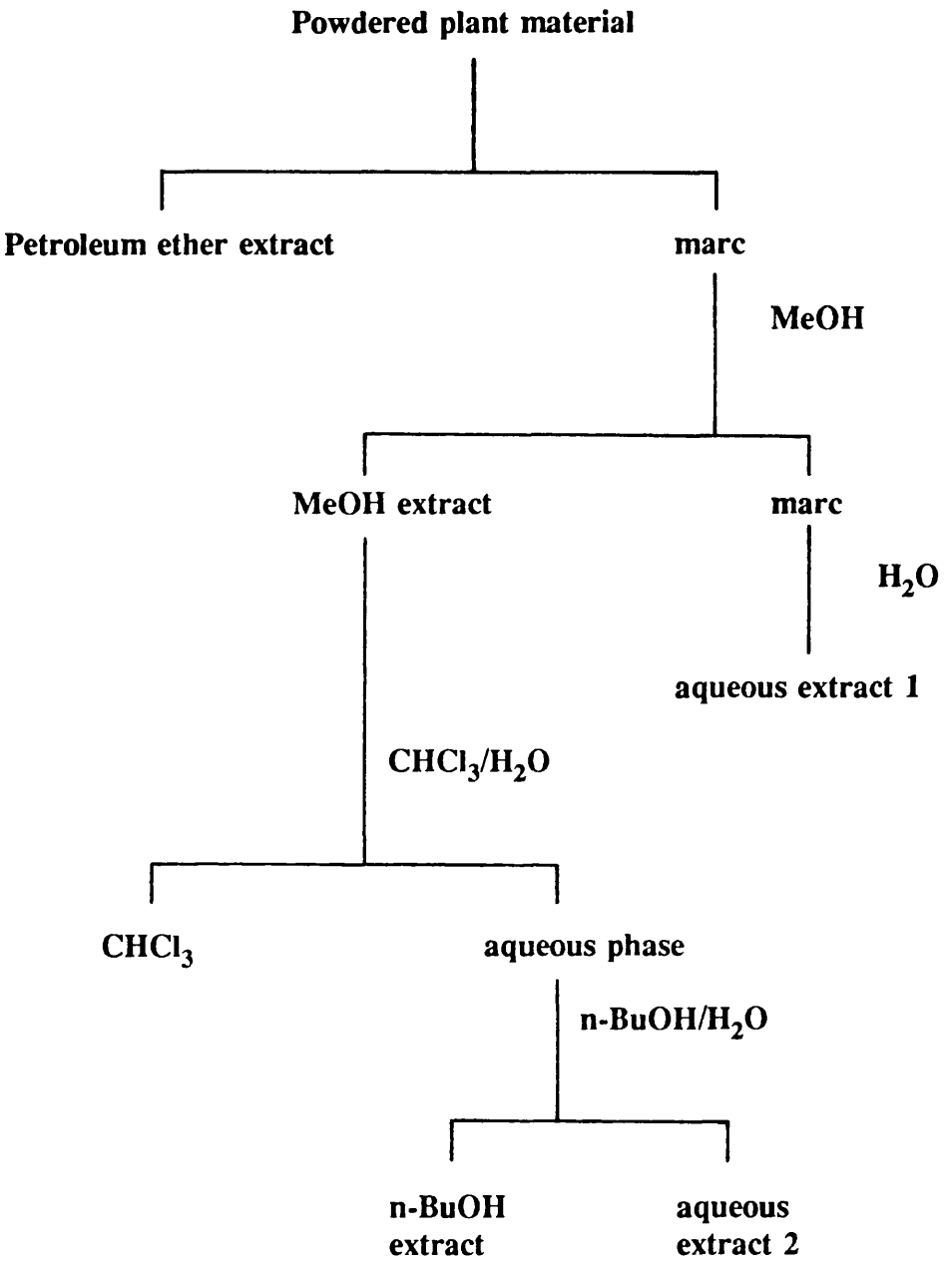


Figure 2.2 Extraction Procedure for Alkaloids

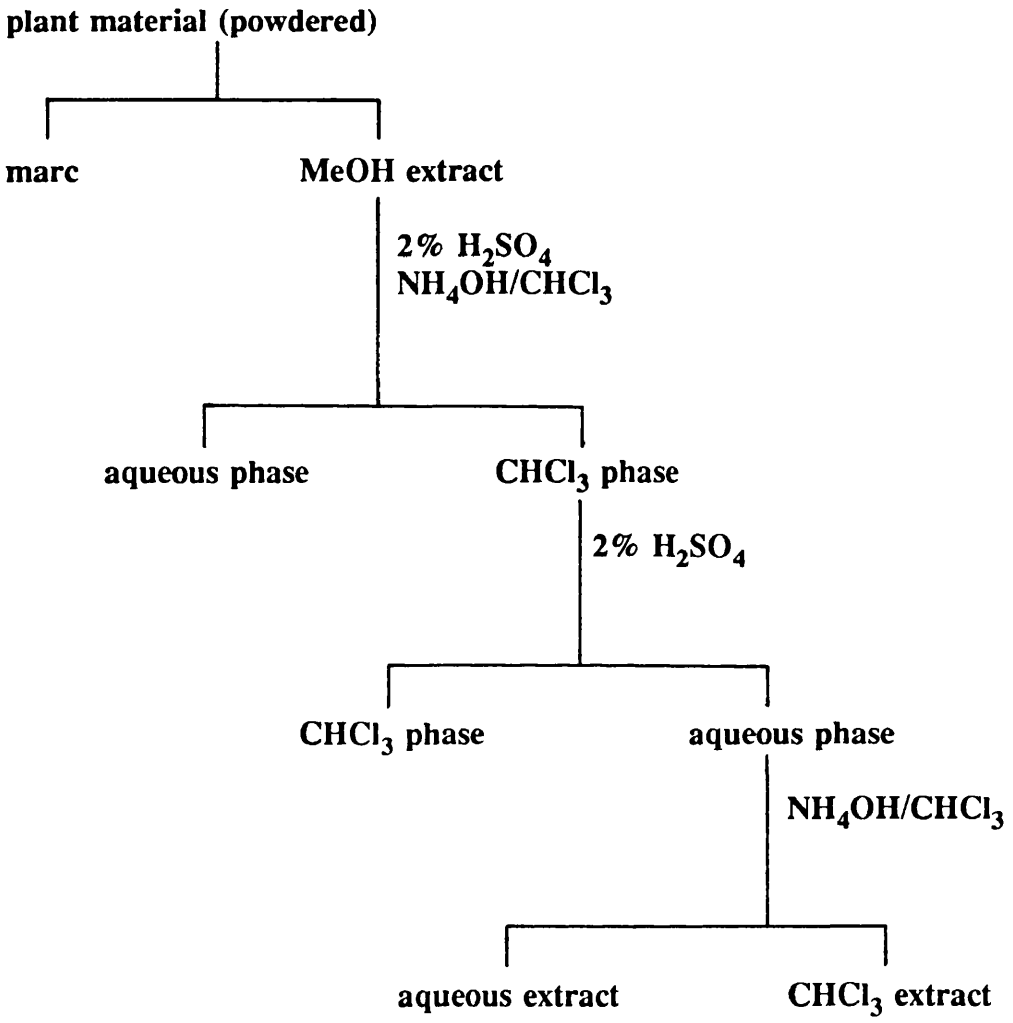


Figure 2.3 Preparation of Aqueous Tea (infusion)

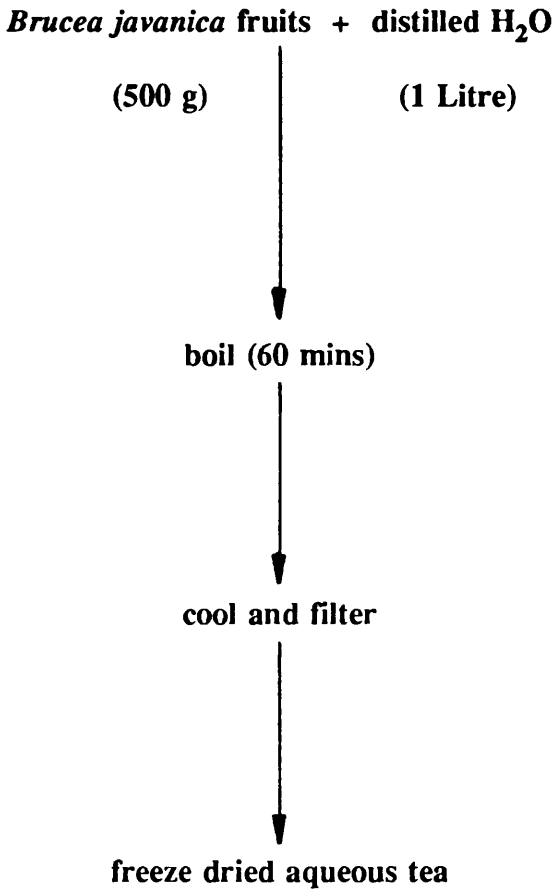


Figure 2.4 Separation Scheme for Aqueous Tea

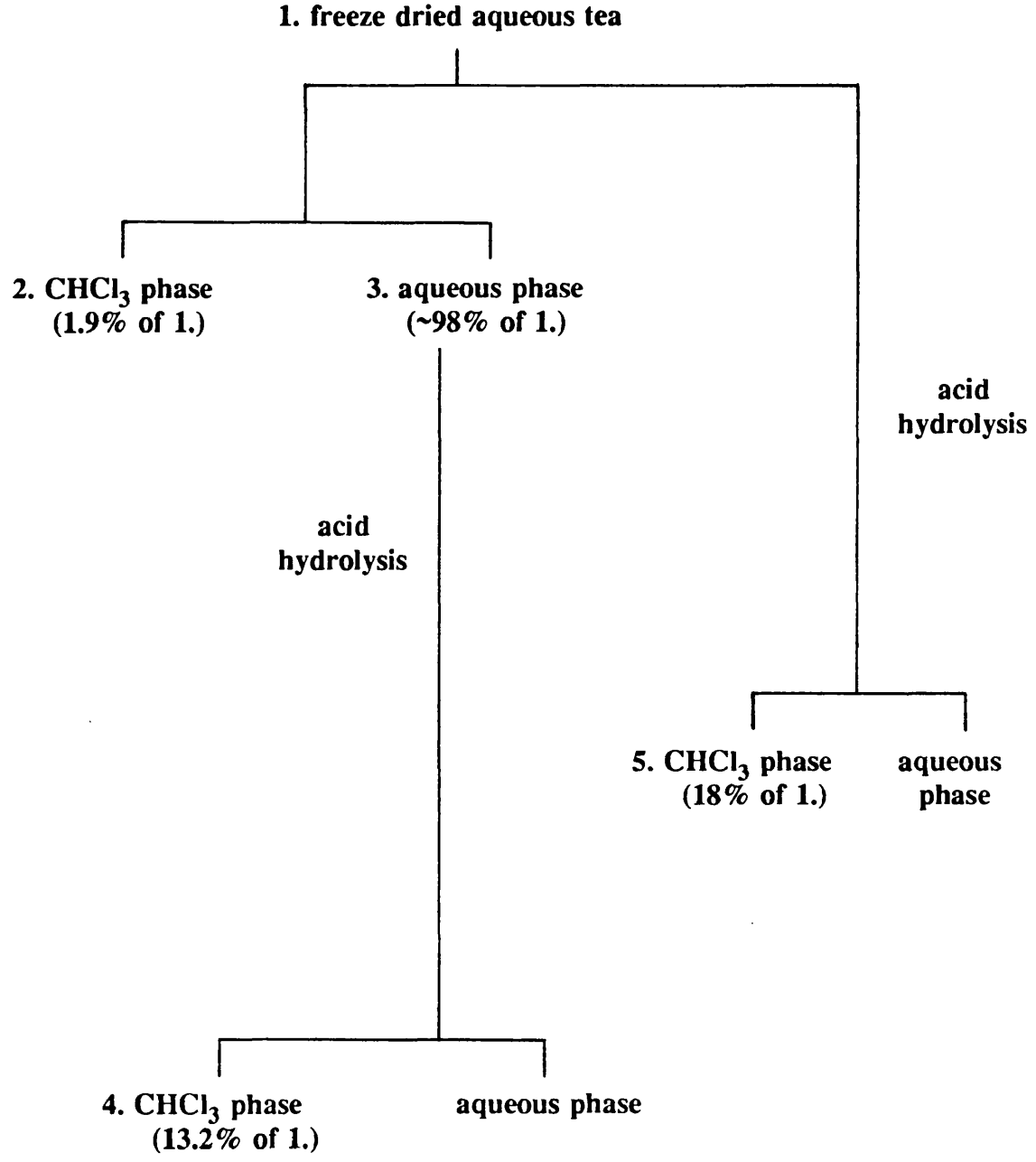
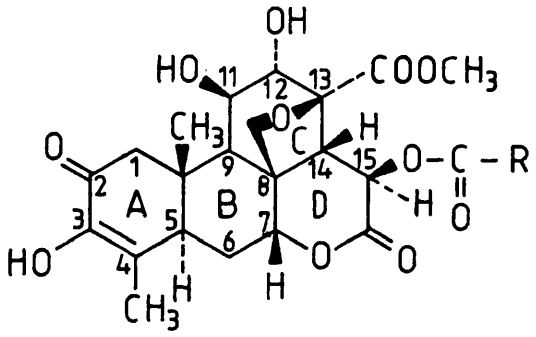


Figure 2.5 Structures of Bruceolides isolated from Aqueous Tea Prepared from *B. javanica* Fruits



R	
$CH_2CH(CH_3)_2$	**bruceine A**
CH_3	**bruceine B**
$CH{=}C(CH_3)C(OH)(CH_3)_2$	**bruceine C**
$CH{=}C(CH_3)_2$	**brusatol**

2.1.5 ACID HYDROLYSIS OF SAMPLES

In an attempt to remove the sugar moiety from any glycosides, and hence liberate any quassinoid aglycones, the aqueous tea (both before and after chloroform extraction) was subjected to acid hydrolysis (Lee *et al*, 1979). Approx. 18g of the freeze dried extract were dissolved in 500mL of 1.5M sulphuric acid : methanol (1:1) and heated under reflux for 5 hours. The hydrolysed products were poured into 500mL of iced distilled water, and extracted with three 50mL volumes of chloroform, before filtering through cotton wool. The chloroform filtrate was reduced under pressure and stored under nitrogen in small vials, below 5°C.

2.1.6 CHROMATOGRAPHIC TECHNIQUES

2.1.6.1 THIN-LAYER CHROMATOGRAPHY (TLC)

Analytical TLC was carried out on pre-coated, aluminium backed silica gel GF_{254} plates (0.25 mm thick - Merck Ltd.). Analytical partition chromatography was carried out using aluminium backed pre-coated silica gel 60 F_{254} plates (0.25 mm thick - Merk Ltd.) which had been coated in 5% v/v liquid paraffin in diethyl ether and left to dry. For preparative TLC, glass plates (100 mm x 100mm) spread with a 0.5 mm layer of silica gel GF_{254} (Merck Ltd.) were used. It was necessary to activate the plates by heating at 110°C overnight. Once cool, the plates were washed with methanol, and allowed to dry before use. Samples were applied to analytical plates with capillary tubes, and finely drawn out glass tubes were used to streak samples onto preparative plates. The applied samples were left to air dry before the plates were developed in chromatography tanks which had been lined with chromatography paper and saturated in the relevant eluting solvent. Depending on the polarity of the plant constituents involved, several different solvent systems were employed, and the details of these are mentioned in the course of the text. A number of spray reagents (see section 2.1.6.1) were used for detection, as was visualisation under short (254 nm) and long (365 nm) wave ultra violet light. Compounds with a chromophore were seen under short wave as quenched spots contrasting with a bright green fluorescent background of F_{254}. The very edge of preparative plates were sprayed to guide the removal of separated bands, and this sprayed region then disgarded. The separated silica bands were scraped from the glass plates into sintered glass eluting tubes, and eluted with chloroform, or a chloroform/methanol mixture. The resulting eluates were evaporated to dryness under pressure, redissolved in a minimum of solvent (usually chloroform), and filtered through a plug of washed cotton wool to remove any traces of silica. Extracts were dried under nitrogen and stored in sample tubes below 5°C.

2.1.6.2 SPRAY REAGENTS

1. Iron (III) chloride reagent, 5% in ethanol.

2. Sulphuric acid (concentrated), 50% in ethanol.

3. Phosphomolybdic acid reagent (PMA), 5% in 95% ethanol.

4. Dragendorff reagent (Munièr's modification).

Solution A: 0.8g bismuth subnitrate + 10mL glacial acetic acid, made to 50mL volume with distilled water.

Solution B: 20g potassium iodide, made to 50mL volume with distilled water.

Immediately before use, 5mL each of solutions A and B were added to 20mL of glacial acetic acid and made up to 100mL volume with distilled water.

All chromatographed plates were initially observed under UV before treatment with a spray reagent. Plates visualised with ferric chloride were examined immediately, and plates sprayed with sulphuric acid or phosphomolybdic acid were heated at 100°C for several minutes, under observation.

2.1.6.3 COLUMN CHROMATOGRAPHY

Quantities of plant extract greater than 2g were separated by flash column chromatography. A 3 x 35cm eluting tube was packed with silicagel 60 (Fluka AG) and the extract applied over a layer of acid washed sand. The column was eluted with chloroform, proceeded by a chloroform-methanol gradient finishing with methanol. Fractions of 25mL were collected under reduced pressure and monitored on TLC. Fractions with similar TLC profiles were pooled.

2.1.6.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Semi-preparative HPLC was performed on an Altex model 334 gradient liquid chromatograph, using an Ultrasphere ODS 5µm (10 x 250mm) column, fitted with an Ultrasphere ODS 5µm (4.6 x 45mm) pre-column (Beckman). Gradient programme: solvent A = 20% methanol in water, solvent B = 80% methanol in water; flow rate 2.5mL/min; 0 → 2 min, % B = 50, 2 → 27 min, % B increases to 90, 27 → 32 min, % B decreases to 50. Detection was by an Altex model 153 UV spectrophotometer at 254nm. Solvents were de-gassed by sonication under reduced pressure.

The column was calibrated for 5 quassinoids: bruceantin, brusatol, and bruceines A, B, and C, using standard reference samples. Duplicate stock solutions of each quassinoid were subjected to serial dilution, and these applied in duplicate to the column in repetitive 10µL quantities with the aid of an autosampler.

Regression analysis was carried out on calibration curves of mean peak area versus weight of quassinoid. This data was used to estimate the amount of quassinoid in the aqueous tea samples.

2.1.7 SPECTROSCOPIC METHODS

2.1.7.1 ULTRAVIOLET SPECTROSCOPY (UV)

UV spectroscopy was carried out using a Perkin Elmer model 402 double beam spectrophotometer. Samples were taken up in methanol. Shifts in their absorbance maxima were determined by the addition of 50µL of 0.1M sodium hydroxide, and reversed by 50µL of 0.1M hydrochloric acid, both to the blank and sample cells.

2.1.7.2 PROTON MAGNETIC RESONANCE SPECTRA (PMR)

Spectra were determined in $CDCl_3$, with TMS as internal standard on a Bruker WM - 250 MHz spectrometer. The chemical shift values (δ) were recorded in ppm, and the coupling constant (J) as Hz.

2.1.7.3 THERMOSPRAY (TSP) MASS SPECTROSCOPY (MS)

Thermospray MS was carried out on a VG MassLab 12/250 quadrupole mass spectrometer fitted with a commercial TSP source and a VG 11-73 data system. The approximate TSP ion source condition temperatures were: source, 200°C, nozzle, 190°C, and chamber, 230°C.

2.2 Results and Discussion

2.2.1 ISOLATION AND IDENTIFICATION OF BRUCEOLIDES FROM AN AQUEOUS TEA PREPARED FROM *BRUCEA JAVANICA* FRUITS

Brucea javanica fruits (500g), from Hong Kong were boiled in 1L of distilled water for 60 mins. The resulting aqueous infusion or "tea" was left to cool for one hour before filtering, and the filtrate placed in a freezer overnight in preparation for freeze-drying. The freeze dried material obtained (23g) was very sticky in consistency, indicating a high sugar concentration. The tea was then subjected to a series of acid hydrolyses (as described in section 2.1.5), and partitions between chloroform and water (as illustrated in Fig. 2.4). The tea was found to consist of approximately 2% chloroform-soluble material. After removal of this $CHCl_3$-soluble material from the tea , acid hydrolysis of the remaining aqueous phase liberated a further 13.2% $CHCl_3$-soluble material. This was consistent with the finding that acid hydrolysis of the complete tea resulted in a total of approximately 18% $CHCl_3$-soluble material. Analytical TLC was performed on all extracts, using chloroform - isopropanol (9:1). Chloroform extracts 2 and 5 revealed at least 4 distinct ferric chloride positive blue/grey spots. No separation of the aqueous phase was achieved with

this system. However, as the base line material was negative with ferric chloride, and the aqueous phases were found to be inactive when tested against *P. falciparum in vitro*, no further attempt at their separation was made. Conversely, the bruceolide-containing chloroform extracts 2 and 5 were separately column-chromatographed as described in section 2.1.6.3. Eluted fractions were monitored by analytical TLC, using chloroform - methanol (9:1), chloroform - isopropanol (9:1) and hexane - ethylacetate - methanol (6:4:1) as developing solvents. The solvent system used for analytical partition chromatography was chloroform - methanol (1:1). A total of 8 semi-purified fractions were obtained from chloroform extracts 2 and 5. These were subjected to repetitive preparative TLC using hexane - ethylacetate - methanol (6:4:1) and chloroform - isopropanol (9:1). Two ferric chloride positive constituents with Rf values corresponding to bruceine A and brusatol were found to run very close together on TLC. This made their separation and isolation somewhat difficult, and preparative TLC resulted in a mixture of the two.

Samples of the aqueous tea extracts were analysed by HPLC. This indicated the presence of bruceines A,B,C and brusatol. Chloroform extract 2 yielded 4 quassinoids: 0.145% brusatol (equivalent to 0.0029% of the freeze dried aqueous tea - 1.), 0.072% bruceine A (0.0014% of 1.), 0.093% bruceine B (0.0019% of 1.), and 0.078% bruceine C (0.0016% of 1.). From this the total bruceolide content of the tea was calculated to be 0.0078%. It was found that chloroform extract 5 contained only 3 quassinoids: 0.342% brusatol (equivalent to 0.0616% of the freeze dried aqueous tea - 1.), 0.014% bruceine A (0.0025% of 1.), and 0.063% bruceine C (0.0113% of 1.). The total bruceolide content of the hydrolysed tea was found to be 0.075% - an approximate ten fold increase compared to the non-hydrolysed tea.

The major quassinoid in the $CHCl_3$ extracts of both the hydrolysed and non-hydrolysed tea was found to be brusatol. In the non-hydrolysed sample bruceine A, B and C occurred in similar amounts, although in the hydrolysed sample, bruceine C predominated over bruceine A. Also, the fact that bruceine B was not present in the chloroform extract following hydrolysis would appear to indicate the absence of a bruceine B -O- glycoside in the aqueous tea.

2.2.1.2 SPECTRAL DATA

Brusatol

PMR : $CDCl_3$, 250 MHz with TMS as internal standard

δ 1.4(3H, s, C-10 Me); 1.85(3H, d, J=13 Hz, C-4 Me); 1.9(3H, s, C-3^1 Me); 2.1(3H, s, C-3^1 Me); 3.79(3H, s, OMe); 3.82(1H, d, J=8Hz, C-8 OCH_2); 4.25 - 4.26(2H, m, C-11H, C-12H); 4.71(1H, overlapped d, J=5.3Hz, C-8 OCH_2); 4.78(1H, m, C-7H); 5.62(1H, s, C-2^1H); 6.08(1H, s, C-15H).

THERMOSPRAY MS : m/z (rel. int. %)

$[M]^+$ 520(23), $[M+H]^+$ 521(100), $[M+NH_4]^+$ 538(17), $[M+NH_4\text{-}2H_2O]^+$ 502(6), $[M+NH_4\text{-}3H_2O]^+$ 484(4), $[M+H\text{-}H_2O]^+$ 503(6), $[M+H\text{-}2H_2O]^+$ 485(5), $[2M+NH_4]^+$ 1058(1).

HPLC

The sample alone had a retention time, Rt of 17.5 mins, consistent with that of a reference sample using the solvent system given in section 2.1.6.4.

TLC

The sample gave a green/black colour with ferric chloride, a grey absorbance under UV_{254}, and had a hRf value of 53 with a chloroform - isopropanol (9:1) solvent system.

UV

λmax (MeOH), 218, 278nm; λmax (MeOH+50μL NaOH), 218,320nm - reversible on addition of 50μL HCl).

Bruceine A

PMR : $CDCl_3$, 250 MHz, TMS as internal standard.

δ 0.97(6H, d, J=8.7Hz, $CH(CH_3)_2$); 1.4(3H, s, C-10 Me); 1.85(3H, d, C-4 Me); 3.82(1H, d, J=8Hz, C-8 OCH_2); 3.85(3H, s, OMe); 4.25 - 4.26(2H, m, C-11H, c-12H); 4.71(1H, overlapped d, J=5.3Hz, C-8 OCH_2); 4.78(1H, m, J=2.7Hz, C-7H); 6.09(1H, s, OH); 6.35(1H, d, C-15H).

THERMOSPRAY MS : m/z (rel. int. %)

$[M]^+$ 522(40), $[M+H]^+$ 523(100), $[M+NH_4]^+$ 540(15), $[M+NH_4\text{-}2H_2O]^+$ 504(5), $[M+NH_4\text{-}3H_2O]^+$ 486(4), $[M+H\text{-}H_2O]^+$ 505(3), $[M+H\text{-}2H_2O]^+$ 487(5), $[M+H\text{-}3H_2O]^+$ 489(2).

HPLC

The Rt of both the isolated compound and standard sample, using the system described in section 2.1.6.4 was 23.6 mins.

TLC

The hRf value was 52, using chloroform - isopropanol (9:1), and a blue/ black colour was given with ferric chloride spray, and a grey absorbance was observed under UV_{254}. The characteristics were identical to those of a standard sample of bruceine A.

UV

λmax (MeOH), 218nm, 280nm; λmax (MeOH + 50μL NaOH) 218, ≈330nm - reversed on addition of 50μL HCl.

Bruceine B

PMR : $CDCl_3$, 250 MHz, TMS as internal standard.

δ 1.4(3H, s, C-10 Me); 1.85(3H, d, C-4 Me); 2.09(3H, s, COMe); 2.45(1H, d, J=3.6Hz, C-1H); 3.05(1H, d, J=2.3Hz, C-14H); 3.79(2H, d, J=3.1Hz, C-8 OCH_2); 3.85(3H, s, C-13, COOMe); 4.19(1H, s, C-12H); 4.26(1H, d, C-11H); 4.73(1H, overlapped d, J=5.7Hz, C-8 OCH_2) ; 4.46(1H, s, overlapped C-7H); 6.34(1H, d, C-15).

THERMOSPRAY MS : m/z (rel. int. %)

$[M]^+$ 480(35), $[M+H]^+$ 481(100), $[M+NH_4]^+$ 498(30), $[M+NH_4-H_2O]^+$ 482(35), $[M+NH_4-2H_2O]^+$ 462(1), $[M+H-3H_2O]^+$ 463(2), $[2M+NH_4-H_2O]^+$ 962(2), $[2M+H]^+$ 961(5).

HPLC

Using the system described in section 2.1.6.4, the Rt of both the sample and reference were found to be 9.6 mins.

TLC

The hRf value of 48 with chloroform - isopropanol was identical to that obtained for a standard reference sample, as was the corresponding grey/black colour observed with ferric chloride and a grey absorbance seen under UV_{254}.

UV

λmax (MeOH) 218, 279nm; λmax (MeOH+ 50μL NaOH), 218, ≈ 322nm - reversed on addition of 50μL of 0.1M HCl.

Bruceine C

PMR : $CDCl_3$, 250 MHz with TMS as internal standard.

δ 1.38(6H, s, $C(CH_2)_3OH$); 1.4(3H, s, C-10 Me); 1.85(3H, d, C-4 Me); 1.85(3H, d, C-4 Me); 2.19(3H, d, C-3^1 Me); 3.8(3H, s, OMe); 3.82(1H, overlapped d, J=4Hz, OCH_2); 4.20(1H, s, C-12H); 4.24(1H, d, C-11H); 4.72(1H, d, J=3Hz, OCH_2); 4.79(1H, br.s, C-7H); 6.09(1H, s,C-2^1H); 6.33(1H, s,C-15H).

THERMOSPRAY MS : m/z (rel. int. %)

$[M]^+$ 564(0), $[M+NH_4]^+$ 582(53), $[M+H]^+$ 565(90), $[M+H-H_2O]^+$ 547(45); $[2M+NH_4]^+$ 1146(25).

HPLC

The isolated sample had a Rt of 16.6 mins using the system described earlier in section 2.1.6.4. This corresponded to the Rt of the standard bruceine C sample.

TLC

The standard sample of bruceine C gave a corresponding grey/black colour with ferric chloride, an hRf value of 41 with chloroform - isopropanol (9:1), and a grey absorbance when seen under UV_{254}.

UV

λmax (MeOH) 219,280nm; λmax (MeOH+50μL 0.1M NaOH) 219, ≈330nm - reversible on addition of 50μL 0.1M HCl.

Fig. 2.6 PMR Spectrum of Brusatol (250MHz)

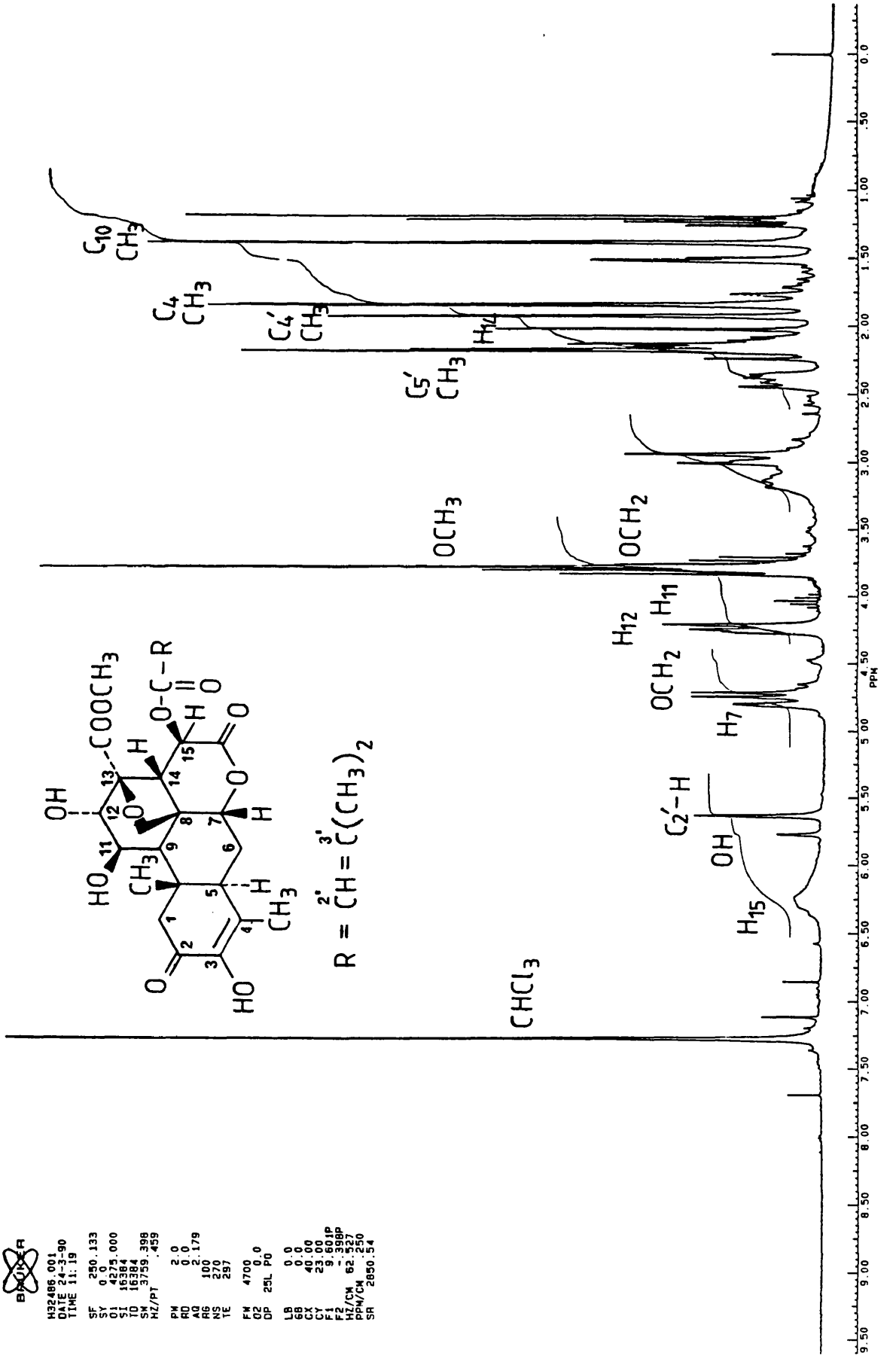


Fig. 2.7 Thermospray Mass Spectrum of Brusatol

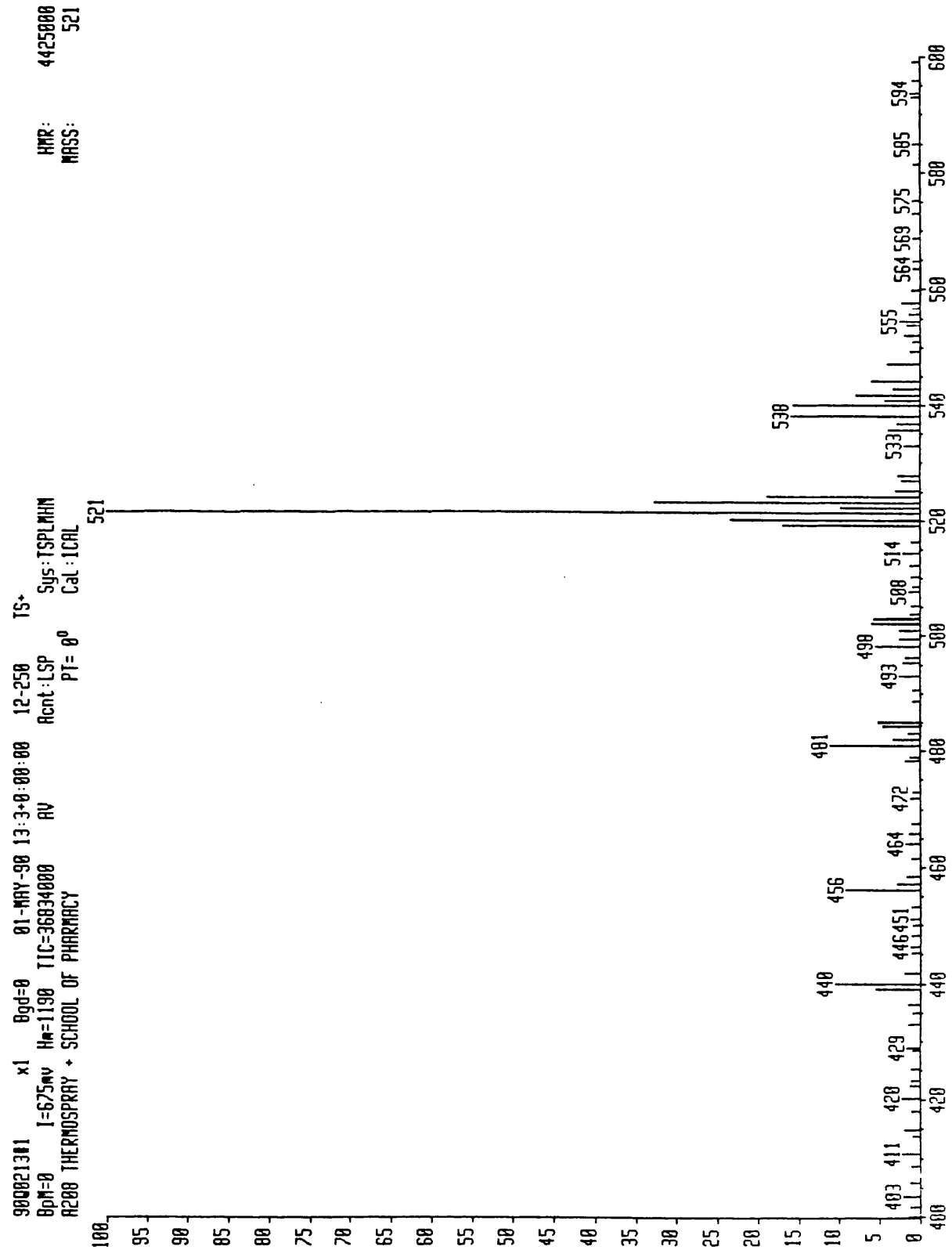


Fig. 2.8 PMR Spectrum of Bruceine A (250MHz)

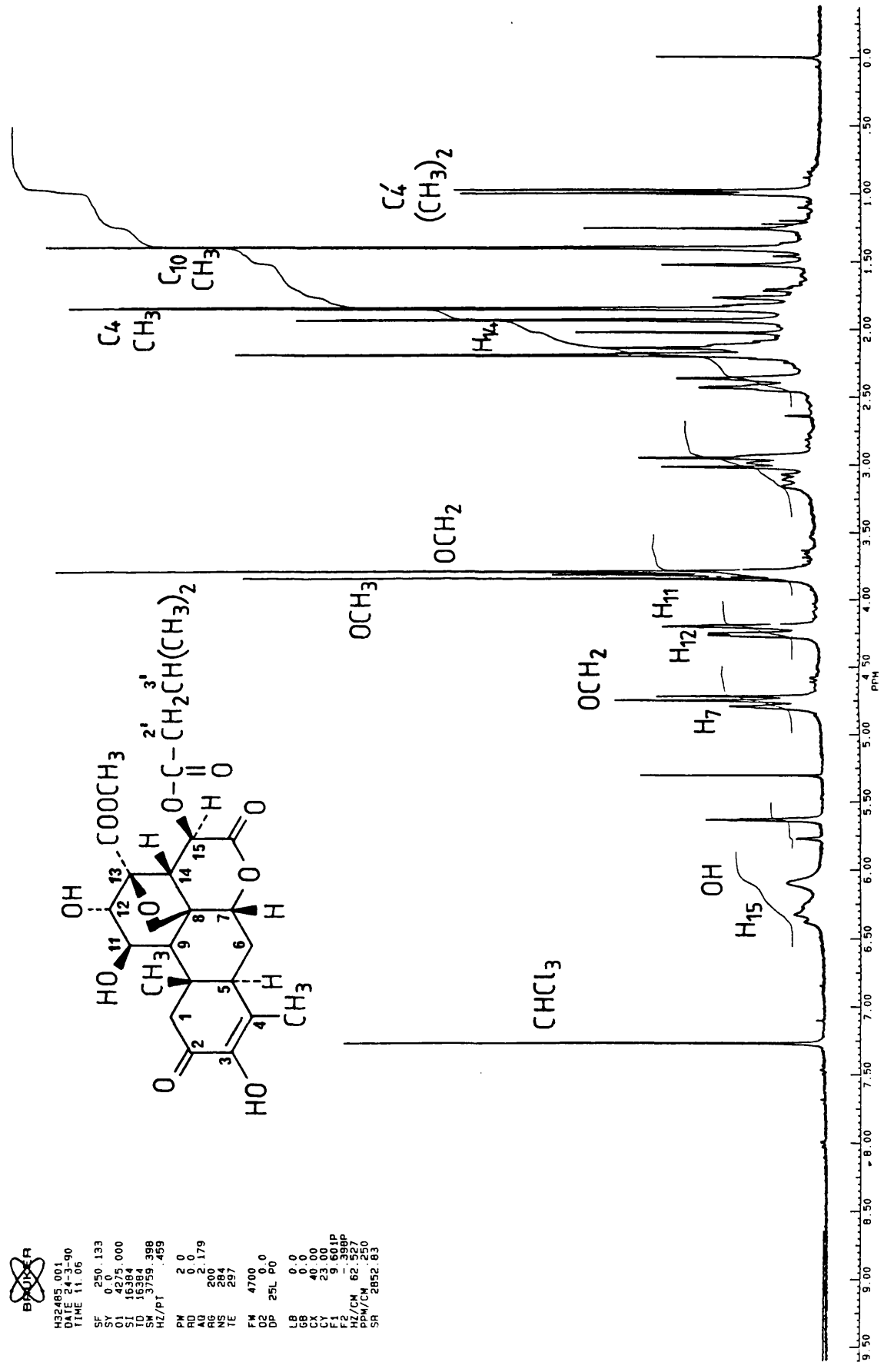


Fig. 2.9 Thermospray Mass Spectrum of Bruceine A

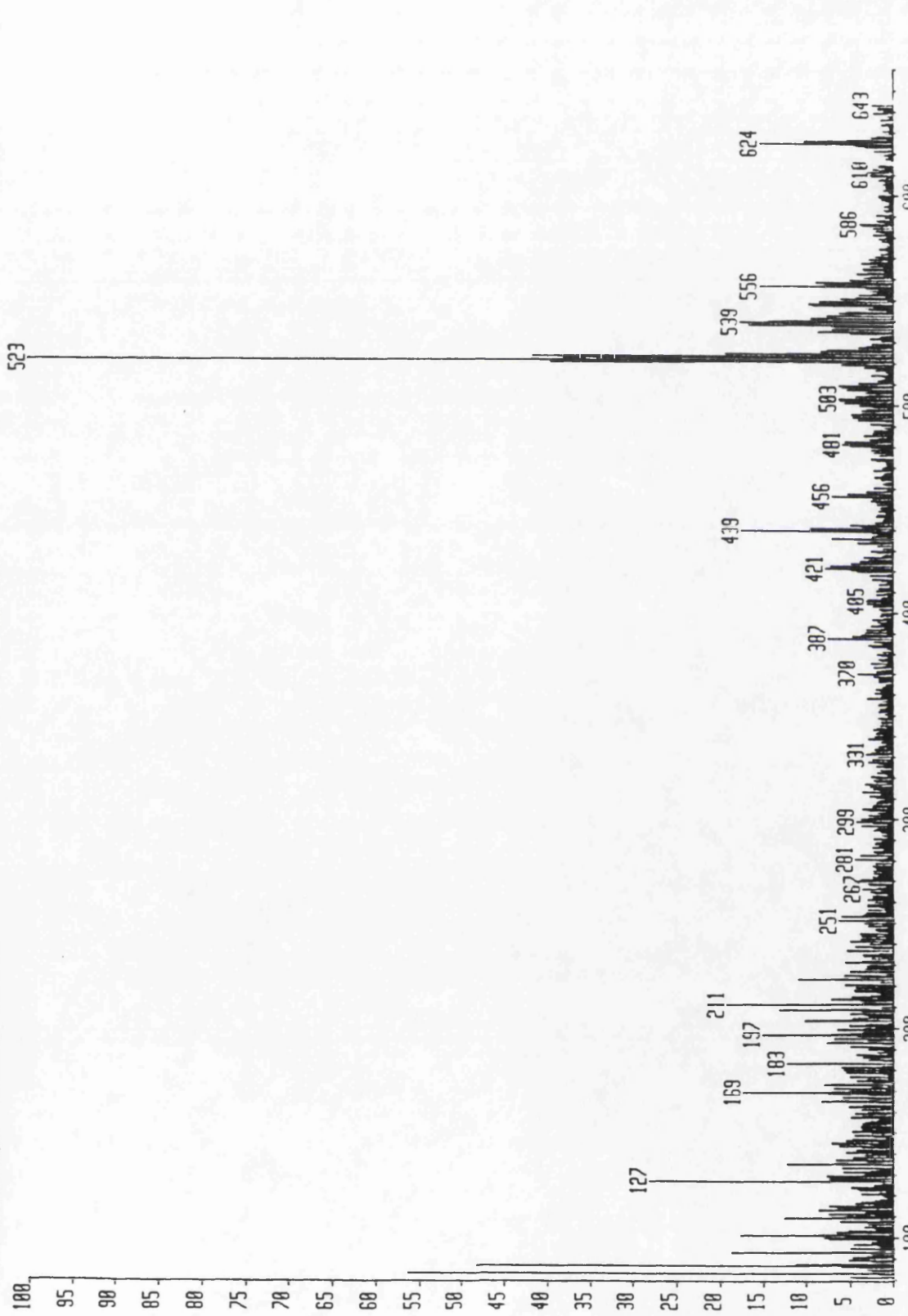


Fig. 2.10 PMR Spectrum of Bruceine B

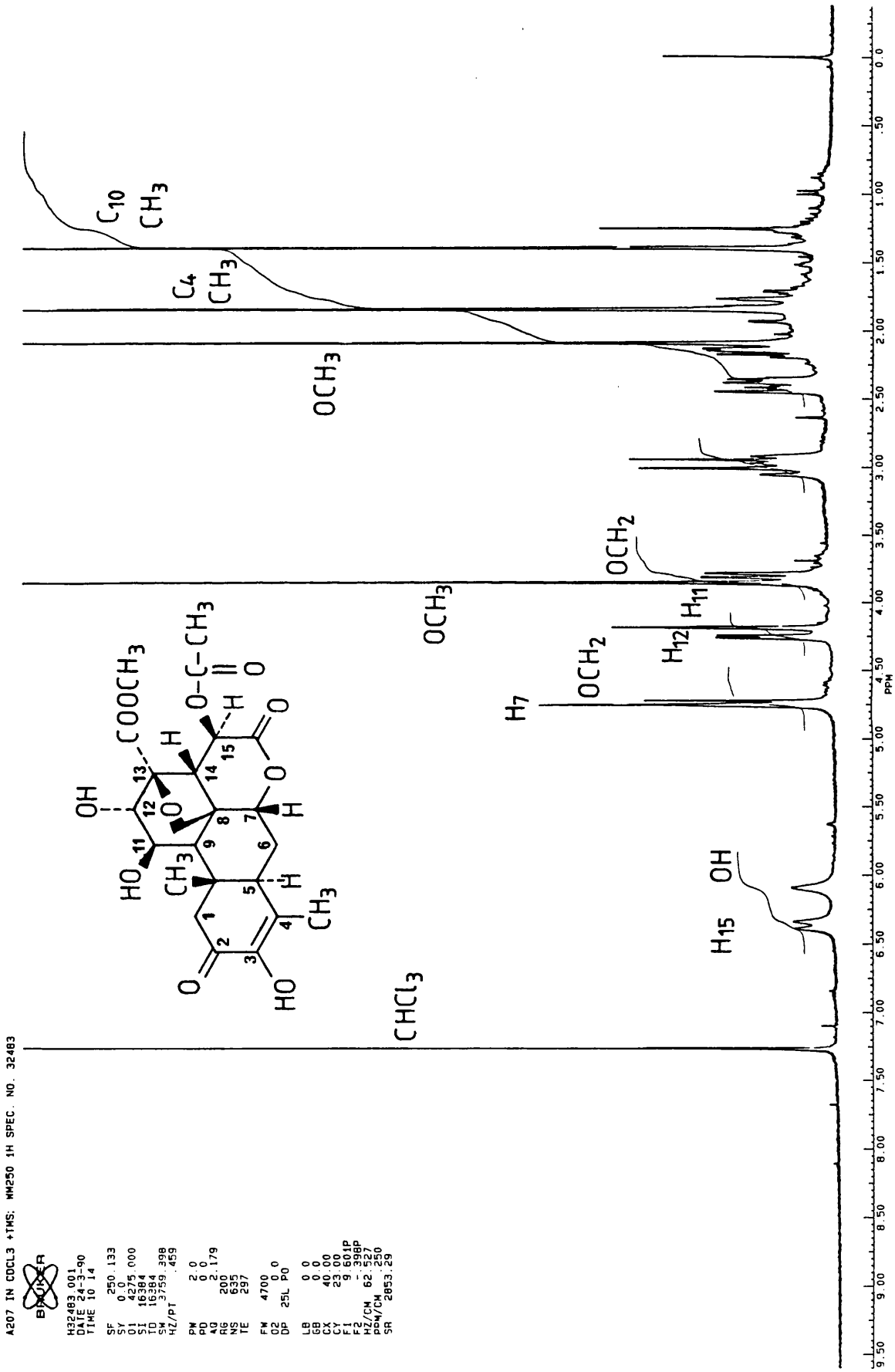


Fig. 2.11 Thermospray Mass Spectrum of Bruceine B

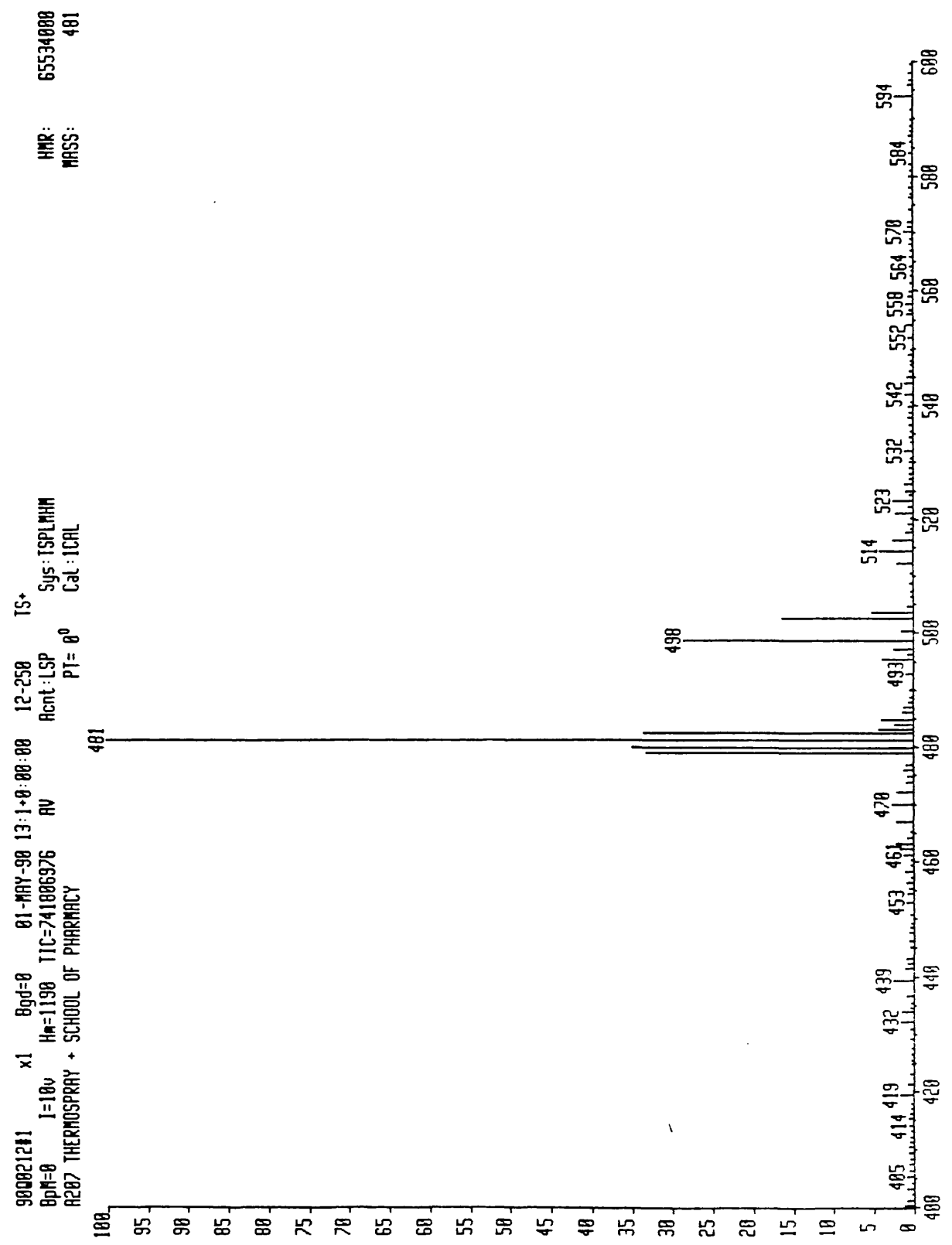


Fig. 2.12 PMR Spectrum of Bruceine C

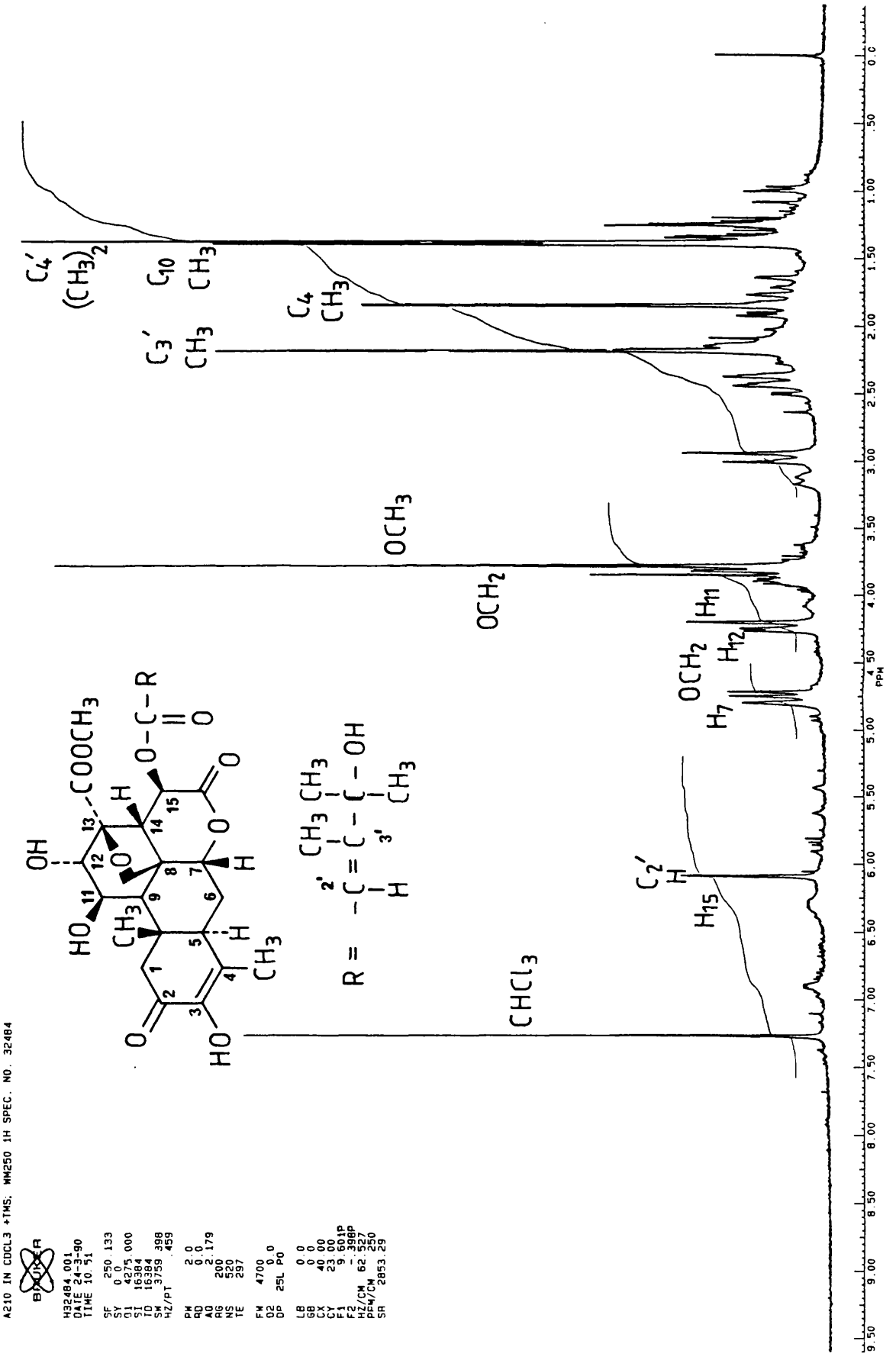
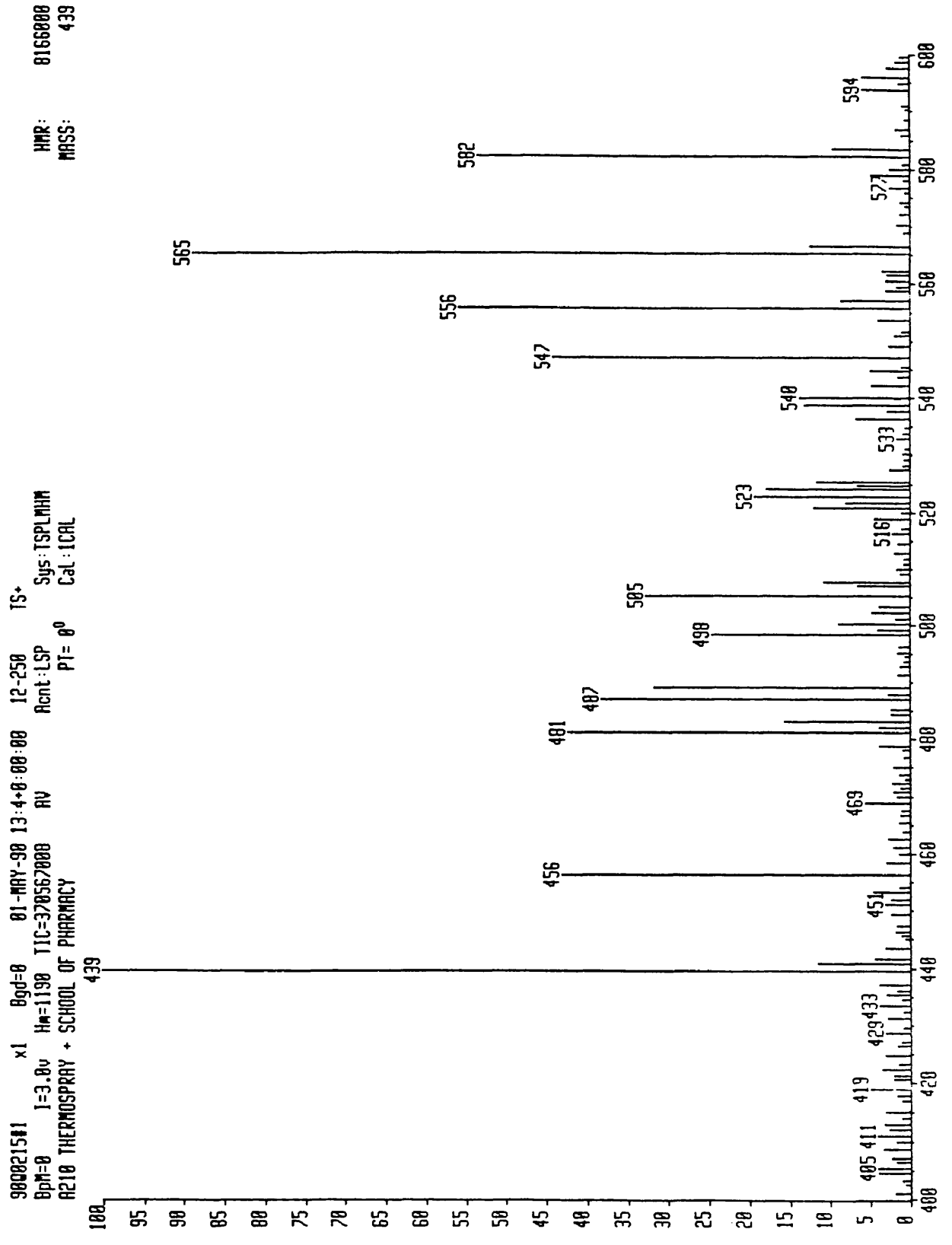


Fig. 2.13 Thermospray Mass Spectrum of Bruceine C



Discussion

The quassinoids isolated from the aqueous tea all have the bruceolide characteristics of a methylene-oxygen bridge between the C-8 and C-13 carbon atoms, and all possess a diosphenol moiety in ring A. This ring A substitution pattern reflects the similar ultra violet absorption spectra obtained for each of the compounds. The resulting spectra show a peak maxima at approximately 280 nm, which is seen to undergo a reversible bathochromic shift on addition of alkali. The diosphenol moiety also gives a colour reaction with ferric chloride which provides a very useful guide to identification in TLC.

The PMR spectra of bruceines A,B,C and brusatol have a number of characteristic signals in common. These include signals for the methyl groups at C-4 and C-10, the acetate group at C-13, the methylene-oxygen bridge, and the protons at C-7, 11, 12 and C-15. In particular three proton singlets at δ1.90 and δ2.11 disclose the presence of the senecioyl grouping of the side chain of brusatol. The C-2^1 proton appears further down field at δ5.62. In contrast the spectrum of bruceine A shows a 6H doublet at δ0.97 attributable to the $CH(CH_3)_2$ methyl groups of the ester side chain. Bruceine B has a 3H singlet at δ2.09 indicating the presence of an acetyl group, and bruceine C has a 3H doublet at δ2.19 due to a methyl group at C-3^1, and a single proton at δ6.09 which can be assigned to the proton at C-2^1.

Quassinoids (molecular mass approx. 500-600) are relatively non-volatile molecules which demonstrate a low abundance of molecular ions when examined by electron-impact MS, due to the thermal instability of the C-15 ester. Therefore, a soft ionisation method - Thermospray (TSP) mass spectroscopy was employed to give molecular weight information on the isolated bruceolides. Thermospray has been developed as a liquid chromatography/MS interfacing technique and can be defined as the production of a jet of fine liquid particles by heating (Vestal, 1985). Production of the visible thermospray jet is accomplished by partial vaporization of a liquid stream as it flows through a heated capillary tube. This produces a supersonic vapour jet which contains any unvaporized material as entrained liquid droplets or solid particles. Samples of interest in LC are usually less volatile than the solvent, so tend to remain preferentially in droplets. TSP gives protonated species with few fragment ions.

The predominant ionic species for all the isolated quassinoids was the $[M]^+$ ion, with weaker ammonium ion adducts also present. The TSP mass spectra of brusatol shows a high intensity $[M+H]^+$ 521 ion, a weaker molecular ion $[M]^+$ 520, and a $[M+NH_4]^+$ 538 ion. For bruceine A, the $[M]^+$ 522 and $[M+H]^+$ 523 ions occur at approximately the same intensities, and a weaker $[M+NH_4]^+$ 540 ion is observed. A high intensity peak attributable to the $[M+H]^+$ 481 ion is seen on the TSP mass spectrum of bruceine B. Also noteable are the $[M+NH_4]^+$ 498, $[M]^+$ 480, and $[M+NH_4\text{-}H_2O]^+$ 482 ions, together with the dimer $[2M+NH_4]^+$ 978 ion. Bruceine C does not show a molecular ion $[M]^+$ 564, but the major ions from the spectrum are the $[M+H]^+$ 439 ion (following ester cleavage) and the $[M+H]^+$ 565 ion. Three weaker ions: $[M+NH_4]^+$ 582, $[M+H\text{-}H_2O]^+$ 547, and $[2M+NH_4]^+$ 1146 are also seen. The technique provides very clear information for protonated species, but shows few fragment ions which are very useful in the identification of side chain substituents are not clear from the TSP spectra. For example, the ion formed by the ester group of bruceolides appears in the low mass region (see Table 2.1). Expansion of the TSP spectra in this region may have provided more information.

Table 2.1 m/z Values for Ester Ions of a Number of Bruceolides

Compound	R	m/z (R^+)
Brusatol	COC_4H_7	83
Bruceine A	COC_4H_9	85
Bruceine B	$COCH_3$	43
Bruceine C	$COC_6H_{11}O$	127

Consideration of all the spectroscopic data together confirmed that the bruceolides isolated from the aqueous tea are bruceines A,B and C, and brusatol (see Fig. 2.5).

2.2.2 ALKALOID EXTRACTION OF *B. JAVANICA* FRUITS

Powdered *B. javanica* fruits (100g) were extracted for alkaloids as illustrated in Fig. 2.2. The resulting product (0.39g) was found to be highly active against *P. falciparum in vitro* (see section 4.3.2). Analytical TLC using chloroform - ethylacetate - methanol (2:2:1) as developing solvent and Dragendorff reagent for visualization, revealed 3 orange spots. However, the same alkaloid sample also reacted with ferric chloride to give at least 2 blue/grey spots, suggesting the presence of quassinoids or phenols. Preparative TLC, using chloroform - isopropanol (9:1) was carried out to isolate the Dragendorff positive material. However, once isolated this no longer demonstrated *in vitro* antiplasmodial activity. A comparison with standard samples on HPLC, using 67% methanol - water showed they were not canthin-6-one, 1-methoxycanthin-6-one, or 5- methoxycanthin-6-one, which are 3 alkaloids isolated previously from Simaroubaceae species (Cordell *et al*, 1978).

2.2.3 EXTRACTION OF *SIMAROUBA AMARA* FRUITS

200g of powdered *S. amara* fruits were extracted and fractionated as outlined in Fig. 2.1. The dried extracts were tested for *in vitro* antimalarial activity, the results of which are given in section 4.3.1. Analytical TLC was carried out on the active chloroform and aqueous fractions, and also on the butanolic fraction, using chloroform - methanol - water (65:35:10), and chloroform - isopropanol (9:1) as developing solvents, with 60% ethanolic sulphuric acid and Dragendorff reagent for visualisation. At least 10 unknown components were revealed, giving a blue/grey or purple colouration with sulphuric acid, and one gave an orange colour with Dragendorff reagent. A *S. amara* alkaloid extract (0.0096g) prepared as shown in Fig. 2.2 from 10g of powdered fruits, was found to be inactive *in vitro* against *P. falciparum*. Therefore, identification of the unknown alkaloids was not pursued any further.

TLC investigation of all the fractions also failed to identify 4 highly active quassinoids - holacanthone, glaucarubinone, ailanthone and 2^1- acetylglaucarubinone, isolated previously from the chloroform fraction of *S. amara* fruits (O'Neill *et al*, 1988). Nevertheless, the moderate *in vitro* antiplasmodial activity shown by the chloroform and aqueous fractions warrants future investigation.

An aqueous infusion from *S. amara* fruits was also prepared, as described in section 2.1.4 for *B. javanica* fruits. TLC analysis, using the developing solvents and visualising agents described above, yielded at least 3 components. However, these were not identified after the aqueous tea was shown to demonstrate very little antimalarial activity (see section 4.3.1).

2.2.5 EXTRACTION OF *SIMAROUBA GLAUCA* FRUITS

Two samples of *S. glauca* fruits from San Jose and Heredia in Costa Rica were separated according to the scheme shown in Fig. 2.1. The 2 sets of extracts were compared by analytical TLC using chloroform - methanol - water (65:35:10), with 60% ethanolic sulphuric acid as visualising agent. The 2 groups of samples were found to have corresponding TLC profiles, so the fruits were combined. The extracts were tested for *in vitro* antimalarial activity, the results of which are shown in section 4.3.1.

CHAPTER THREE — *IN-VITRO* CYTOTOXICITY AND MODE OF ACTION AGAINST KB CELLS

3.1 Introduction

The *in-vitro* KB assay has been used by the National Cancer Institute (NCI) since 1960 as a preliminary screen for cytotoxicity and for fractionating plant samples prior to carrying out assays for *in-vivo* activity. Initially described by Eagle in 1955 and Oyama and Eagle in 1956, the assay has since been standardised by the NCI (Geran *et al*, 1972), and also modified by Wall *et al*, 1987. These pre-existing techniques are time consuming and/or require milligram to gram quantities of drug or crude extract. In a small laboratory where resources are limited and hence large scale drug synthesis or extraction procedures are neither affordable or practical, it is difficult to obtain pure compounds in the required amounts. A sensitive, simple and rapid procedure was needed for use in bioassay guided fractionation of plant extracts. Ideally, this should not necessitate the use of a radiolabelled compound, or a complicated protein determination.

An aim of the present study was to develop such an assay, and use it to determine the cytotoxicity of a range of compounds of varying chemical types , which have been shown to demonstrate antiplasmodial activity. A further objective was to investigate the effect of some of the most potent antiplasmodial quassinoids on protein and nucleic acid synthesis in KB cells, and to study their effects on cellular morphology.

3.2 Materials and methods

3.2.1 *IN-VITRO* CYTOTOXICITY AGAINST KB CELLS

3.2.1.1 KB Cells

The *in-vitro* cytotoxicity test described here utilizes cells derived from a human epidermoid carcinoma of the nasopharynx (Eagle 1955). The KB cell line was established by Eagle in 1954, when biopsy material from a tumor mass taken from the floor of the mouth and tongue of a 54 year caucasian (with the initials KB) was made available for tissue culture. The KB cell line maintains a generation time of approximately 30 hours. KB cultures were obtained from Flow Laboratories Ltd., grown as a monolayer in either 75 cm^2 or 120 cm^2 flasks. The cells had been routinely screened for mycoplasma contamination using a fluorochrome stain (Hoechst stain - Flow Labs. Inc.) by Flow Labs. Inc. and examined for the presence of extranuclear fluorescence.

3.2.1.2 Maintenance of Culture

All procedures were carried out under aseptic conditions in a laminar air flow cabinet and disposal of waste cultures, medium and equipment was achieved by autoclaving or decontaminating with sodium hypochlorite solution (0.5M).

Culture medium

The cells were cultured in Falcon flasks (80 cm^2 ; Nunc) and maintained in Eagle's Minimum Essential Medium modified with Earle's Salts (EMEM) with 0.85g/L sodium bicarbonate , 10% foetal bovine serum , 2mM glutamine, 1% antibiotic mixture and 1% Flow non-essential amino acids concentrate. Except where otherwise stated , all components of the medium were obtained from Flow Laboratories Ltd.

Eagle's Minimum Essential Medium (Modified with EARLE'S SALTS)

Ingredient	**mg/L**
L-Arginine HCl	126.4
L-Cystine disodium salt	28.42
L-Histidine HCl H_2O	41.9
L-Isoleucine	52.5
L-Leucine	52.5
L-Lysine HCl	73.06
L-Methionine	14.9
L-Phenylalanine	33.02
L-Threonine	47.64
L-Tryptophan	10.2
L-Tyrosine disodium salt	45.02
L-Valine	46.9
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic Acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.1
Thiamine HCl	1.00

Earle's Balanced Salts

Ingredient	mg/L
$CaCl_2.H_2O$	264.9
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6800
$NaHCO_3$	2000
$NaH_2PO_4.2H_2O$	158.3
D-Glucose	1000
Phenol red sodium salt	17.00

The medium was buffered by sodium bicarbonate at a concentration of 0.85 g/L (10mM).

Serum - Foetal bovine serum is processed from blood collected by cardiac puncture from bovine foetuses .

Glutamine - L-glutamine is omitted from most liquid media because of its instabililty at temperatures above -10°C . Therefore , 2mM of L-glutamine (BDH Ltd.) were added immediately prior to use.

Antibiotics - A 2mL volume (1%) of a mixture of penicillin (5000 IU/mL) and streptomycin (5000 µg/mL) was added to each batch of medium.

Flow Non-essential Amino Acids

Ingredient	mg/L
L-Alanine	8.90
L-Asparagine H_2O	15.00
L-Aspartic acid	13.30
Glycine	7.50
L-Glutamic acid	14.70
L-Proline	11.50
L-Serine	10.50

The medium was made up in 200 mL volumes and filtered through disposable, sterile membrane filter units (0.22 µm) obtained from Nalgene .

Trypsinizing Solution

A trypsin-EDTA mixture of 0.05% (w/v) trypsin (1:250) and 0.02% (w/v) EDTA in a special salt solution was used.

Ingredient	mg/L
EDTA disodium salt	200.0
D-Glucose	1000
KCl	400.0
NaCl	8000
Phenol red sodium salt	2.00
$NaHCO_3$	580.0
Trypsin (1:250)	500.0

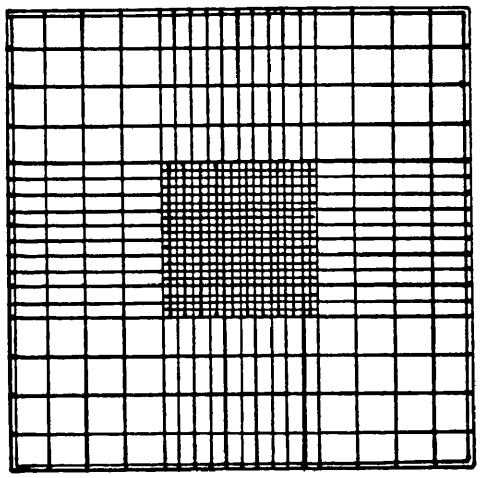
3.2.1.3 SUBCULTURING

The medium was decanted from the culture flask and the monolayer washed with sufficient trypsinizing solution to cover the cells . The flasks were left to incubate at room temperature for approximately 15 mins until the cells became detached . The resulting cell suspension was transferred to sterile centrifuge tubes (10mL ; Sterilin) and spun at 400 r.p.m. for approximately 15 seconds . The supernatant was discarded and the cells resuspended in complete medium . Any aggregates were broken down by pipetting the cell suspension up and down vigorously several times . The cells were counted using a haemocytometer and the appropriate number of cells added to fresh culture vessels containing complete medium to give a final concentration of 10^5 cells/mL . The flasks were gassed with a mixture of 5% $C0_2$ in air until the medium was pale orange in colour and incubated at 37°C .

3.2.1.4 USE OF THE HAEMOCYTOMETER

The chamber of a haemocytometer is divided into 9 large squares by triple white lines . The centre square is further divided into 25 squares which , in their turn are further sub-divided into 16 small squares . The four corner squares are divided into 16 squares . The large squares each have an area of $1mm^2$. When the coverslip is passed down over the grid so that interference patterns appear, the depth of the chamber is 0.1 mm. The total volume over each large square is therefore: $1x1x0.1 = 0.1\ mm^3$ (i.e. = $0.0001\ cm^3$, or $=10^{-4}$ mL).

The Haemocytometer



A dilution of the cell suspension was prepared in trypan blue such that , when the suspension was added to the counting chamber, approximately 50-100 cells were observed over each large square. A Pasteur pipette was used to suck up a small amount of suspension to fill a well of the haemocytometer. This was achieved by touching the edge of the coverslip with the pipette tip and allowing the well to be filled by capillary action . Care was taken to avoid flooding the chamber. Only the cells in the centre square and the four large corner squares were counted. Cells touching the upper and right hand perimeter lines were ignored; those touching the lower and left hand perimeter lines were counted. Dead cells stained blue. Thus, if the total number of cells counted was n and the solution was diluted y fold in Trypan blue, the number of cells in one large square = n/5. The no. of cells per mL diluted suspension = 10^4n/5 , so the no.of cells per mL undiluted suspension = 10^4ny/5.

3.2.1.5 CRYOPRESERVATION

On receiving a batch of cells, a proportion was established in storage in liquid nitrogen for later experimentation. For this, a sub-confluent flask of growing cells were trypsinized and resuspended in medium at a concentration of 2.5 - $4x10^6$ viable cells per mL. The cell suspension was then cooled on ice and dimethylsulphoxide (DMSO) added to give a final concentration of 10% (w/v). Using a sterile syringe (18G needle) a 1 mL aliquot was transferred to a sterile liquid nitrogen ampoule . The ampoule was sealed and frozen down in a biological freezer (Planer Biomed - Kryo 10 Series) to a temperature of -80 °C at a rate of 5°C per minute to 0°C and then 1°C per minute thereafter, before being transferred to the vapour phase of a liquid nitrogen cylinder for storage.

3.2.1.6 RECOVERY OF FROZEN CELLS

After a sample of cells had been subjected to between 10 and 15 passages, it was replaced by another batch taken from liquid nitrogen storage . Whilst wearing gloves and a face mask a liquid nitrogen ampoule was removed from the cylinder and thawed in a water bath at 37°C . The outside of the tube was sterilised with 70% ethanol and allowed to dry, before the contents were asceptically transferred to a culture flask . Sufficient complete medium was then added to dilute the DMSO at least tenfold.

The flask was then gassed and incubated as described previously. The cells were left to settle and after 24 hours the medium was replaced and incubation continued until the culture was almost confluent. Subculturing was then performed as usual.

3.2.1.7 CYTOTOXICITY TEST PROTOCOL

Development of the test is described in the discussion .

Test samples were dissolved with the aid of mild sonication (in a Sonicleaner bath - Ultrasonics Ltd.) in 50µL of ethanol , and diluted with complete medium to give concentrations of 1 mg/mL for pure compounds and 10 mg/mL for crude plant extracts uniform suspension by this process . However, on occasion it was possible to improve solubility by replacing ethanol with DMSO . When satisfactorily dissolved , the samples were further diluted with complete medium , the concentration of ethanol being diluted at least 1000:1 to a non-toxic level .

Twofold serial dilutions of the samples in duplicate were performed in 50µL of complete medium in 96 well microtitre plates (Linbro ; Flow Labs. Inc.) . Control wells were set up simultaneously. Each test included podophyllotoxin or 6-mercaptopurine as a standard cytotoxic drug , together with 2 rows of control wells (culture medium plus cells and a blank of culture medium only).

A cell suspension was prepared by trypsinizing a sub-confluent culture as previously described and estimating the number of cells per mL using a haemocytometer , with Trypan blue exclusion to confirm viability. The suspension was then diluted to a concentration of $5x10^5$ cells/mL with complete medium.

50µL of the $5x10^5$ cell/mL suspension was added to the test and control wells (except blank) giving a total

well volume of 100µL. The inoculum size was chosen so as to achieve a confluent monolayer in the control wells , without overgrowth by the end of the test. The plates were covered with lids, placed in a modular incubating chamber (Flow Labs Inc.) and gassed for approximately 5 mins with 5% CO_2 in air or until the medium became pale orange in colour. The plates were incubated at 37°C for 48 hours.

3.2.1.8 ASSESSMENT OF CYTOTOXICITY

After 48 hours incubation, the cells in the plates were fixed and stained shaking of the plates, and then the cells immediately washed with 0.9% sodium chloride solution at 37°C. After being allowed to dry at room temperature, the KB cells were fixed with methanol for 10 mins and again left to dry. A 0.5% aqueous eosin stain (George T. Gurr Ltd.) was then added to each well and left for a further 15 mins before the plates were rinsed three times with distilled water to remove the excess stain.

A 200µL volume of 0.1M sodium hydroxide solution was added to each well of the air dried plates and left for 20 mins to digest the cell protein and hence release the stain. After this time the optical density (O.D.) of the solution in each well was determined using a microplate reader (MR-700 ; Dynatech Labs. Inc.). The percentage inhibition of the KB cell proliferation was calculated by comparison of the O.D. readings of the control and test wells (see equation below), and plotted against concentration of sample being tested. Each sample was tested at least twice.

ANALYSIS OF RESULTS

Optical density readings were converted to % inhibition of cell growth by the following equation:

$$\%Inhibition = 100 - \frac{[\,OD\ of\ KB\ Cells + drug\,] - [\,OD\ of\ cell\text{-}free\ wells\,]}{[\,OD\ of\ KB\ Cells - OD\ of\ cell\text{-}freewells\,]} \times 100$$

% inhibition was plotted against drug concentration. ED_{50} values, standard deviations and 95% confidence limits were obtained from linear regression analysis of the resulting curves.

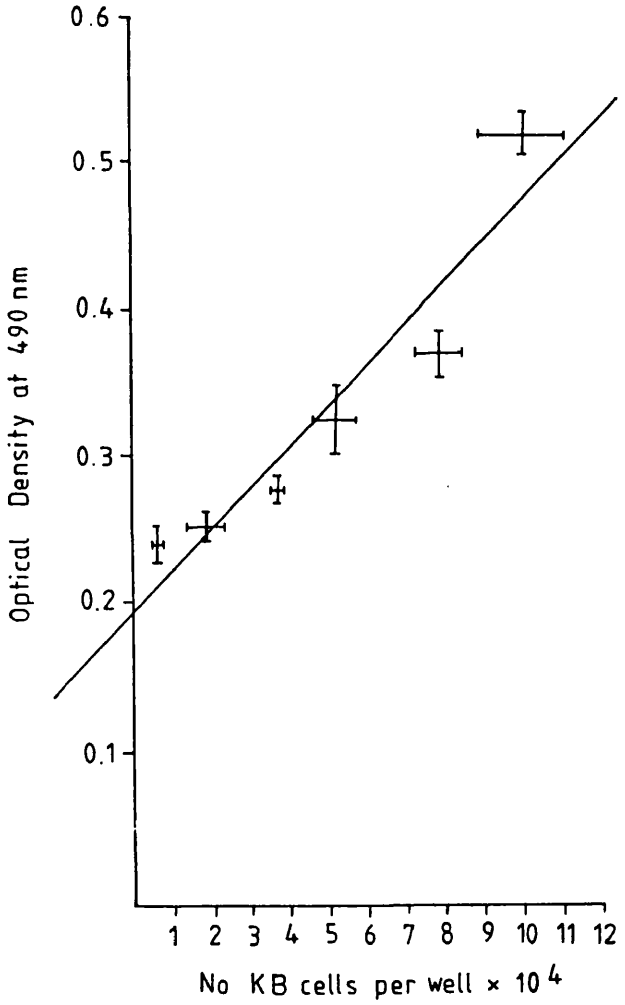
3.2.1.9 VALIDATION OF THE TEST

It has been shown that dye-binding to cells grown in monolayers occurs in a quantitative manner (Barer *et al*, 1986). In order to confirm this, and hence validate the new KB microplate cytotoxicity test, a correlation between numbers of KB cells and O.D. had previously been obtained. This was achieved by setting up 2 identical plates containing serial dilutions of a cell suspension. After 48 hours incubation the cells from plate 1 were trypsinized and counted with a haemocytometer, whilst the second plate was fixed and stained with eosin as described earlier and the O.D. measured. For each cell concentration cell counts and O.D. readings were obtained in triplicate.

Figure 3.1. shows the correlation between the numbers of KB cells and O.D. The correlation coefficient was shown to be 0.95 which is indicative of a good linear relationship and was found to be significant at the 0.1% level. At cell numbers above $2x10^5$ per well the correlation began to drift slightly. However, assuming a generation time of 30 hours, the test innoculum size of $2.5x10^4$ cells per well would give $8.0x10^4$ cells per well after 48 hours. This cell number fell well within the linear portion of the curve.

The cytotoxic drugs podophyllotoxin and 6-mercaptopurine, which were used as positive controls, were shown to give reproducible and consistent ED_{50} values during preliminary test experiments. The range of values obtained for podophyllotoxin was 0.002 -0.007μg/mL, and for 6-mercaptopurine the ED_{50} fell between 0.09 - 0.6μg/ml. The control limits for a valid test in the NCI KB screen on 6-mercaptopurine are 0.05 -0.5 μg/ml, which compares favourably to the values of the author.

Figure 3.1. Correlation of optical density and numbers of KB cells [1]



[1] Line of best fit determined by linear regression. Standard deviations are shown. Correlation coefficient was 0.95. For 16 degrees of freedom (n-2) and P = 0.001, statistic tables give r = 0.708. The correlation is significant at the 0.1% level.

3.2.1.10 THE 72 HOUR TEST

The pre-existing KB assays involve a 72 hour procedure in contrast to the 48 hour method employed here. As a comparison, therefore, a selection of compounds were subjected to both 48 and 72 hour microtitre tests. The method for the 72 hour test was identical to the 48 hour test described previously, except that the cell inoculum number per well was reduced from $5x10^5$ cell/mL to $2.75x10^5$. The test was stopped after 72 hours and the plates processed as for the 48 hour test method.

3.2.2 *IN-VITRO* STUDIES ON THE MODE OF ACTION OF QUASSINOIDS AGAINST KB CELLS

3.2.2.1 INTRODUCTION

The work was carried out in collaboration with Dr. G.C.Kirby at the London School of Hygiene and Tropical Medicine.

Previous *in vitro* and *in vivo* studies using yeast, rabbit reticulocyte, and P388 lymphocytic leukemia cell model systems on many quassinoids and their derivatives, have shown them to be primary inhibitors of protein synthesis (Fresno *et al*, 1978 ; Elgebaly *et al*, 1979 ; Hall *et al* , 1981/82 ; Liou *et al*, 1982). Quassinoids act at the ribosome level by inhibiting the elongation step of protein synthesis. However, there is additional evidence to show that some specific quassinoids also have an inhibitory effect on peptide bond formation, and on enzymes regulating nucleic acid synthesis (Hall *et al*, 1982). For example DNA polymerases, mRNA and tRNA-polymerases, nucleotide kinase, and purine synthesis; Quassinoids also have the ability to suppress oxidative phosphorylation (Hall *et al*, 1979). Work by Kirby *et al*, 1989, demonstrated that quassinoids can also inhibit protein synthesis in the malaria parasite *in vitro*.

The following experiments were designed to study the effect of three quassinoids, bruceantin, brusatol, and bruceine D (having different IC_{50} values against *P.falciparum*-infected erythrocytes) on protein and nucleic acid synthesis in KB cells. It was then hoped to compare and contrast these effects with those seen in the parasite.

In vitro incorporation of [^{3}H]-isoleucine and [^{3}H]-hypoxanthine into acid-insoluble products by KB cells was used as an index for protein- and nucleic acid synthetic activity, respectively.

3.2.2.2 LABELLED PRECURSORS

[G-^{3}H]-hypoxanthine ([^{3}H]hyp; sp. act. 43.3 mCi/mg) and L-[4,5-^{3}H]-isoleucine ([^{3}H]ile; sp. act. 754 mCi/mg) were obtained from Amersham. Lyophilised [^{3}H]hyp and [^{3}H]ile, supplied as an aqueous solution containing 2% ethanol (1.0 mCi/mL) were diluted in EMEM without serum to give 40µCi/mL prior to use.

3.2.2.3 DRUGS

The isolation and identification of the quassinoids bruceantin, brusatol and bruceine D, has been described in the previous chapter. Stock solutions of the drugs were prepared by the addition of a small volume of absolute ethanol (BDH) to give a final test concentration not greater than 0.1%. The stock solutions were further diluted with serum free EMEM, to give concentrations 300 times the required final concentration. A 10μL volume of drug solution was added to 3mL of KB cell suspension, giving a final drug concentration ten times the *in-vitro* ED_{50} value (see Table 3.9) determined previously. The change in volume brought about by the making of this final dilution was not deemed significant. Actinomycin D and cycloheximide which were used as controls for nucleic acid synthesis and protein synthesis respectively, were purchased from Sigma Chemical Co.

3.2.2.4 CELL PREPARATION AND SAMPLING

For the uptake experiments, rapidly growing, subconfluent cultures were used. The cells were subcultured as described previously, however, after centrifugation to remove the trypsin, the cells were resuspended in serum-free EMEM and diluted to a concentration of $5x10^5$ cells/mL. 3mL aliquots of cell suspension were pipetted into plastic screw-capped culture tubes (15mL; NUNC), and [^{3}H]hyp or [^{3}H]ile added to give a final radioactive concentration of 2μCi/mL. The tubes were pre-incubated at 37°C for 30 mins before drugs were added and incubation continued. Control flasks without drug were simultaneously set up. The tubes were aggitated at regular intervals throughout the experiment in order to prevent the cells from adhering to the flasks and hence ensure a uniform cell suspension. After mixing, duplicate 200μL samples of the suspension were taken and pipetted into microcentrifuge tubes containing 1mL of 0.9% saline on ice and stored until processed.

3.2.2.5 SAMPLE PROCESSING

The technique used was adapted from Gu *et al*, 1983 and based on the method described by Neame, 1977. Samples were taken from the ice, placed in a microcentrifuge (MSE Micro Centaur) and spun at 10 000g for approx. 5 seconds. Being careful not to disturb the cell pellet, a pipette was used to remove the supernatant and the pelletted cells lysed with 200μL of distilled water before the extracts were treated with 100μL of trichloroacetic acid (TCA; Sigma). The resulting precipitates were again washed with distilled water (800μL), and the acid-insoluble material was then recovered by centrifugation at 10 000g for 60 secs. The supernatant was now discarded by inverting the tubes and 300μL of formic acid (90% w/w; Hopkin and Williams) added. The TCA-insoluble material, which is a semi solid, was then transferred to plastic scintillation vials (7mL; Packard) to which was added 10mL of Emulsifier Scintillator 299 (Packard) and the tubes shaken. A Packard Tri-Carb Scintillation Spectrometer (Model 574) was used to determine the tritium activity. Counting efficiency was monitored using an external standard.

3.2.3 ELECTRON MICROSCOPICAL INVESTIGATIONS OF DRUG TREATED CULTURES

3.2.3.1 INTRODUCTION

This work was carried out in collaboration with Dr. S. Croft at the London School of Hygeine and Tropical Medicine.

The aim of this study was to use electron microscopy to investigate the qualitative effect of quassinoids on the morphology of KB cells, and to attempt to relate any apparent cellular disruptions to biochemical processes in the cell.

The work was carried out using the same three quassinoids (bruceantin, brusatol and bruceine D) as used in the mode of action studies described previously in section 3.3.2. Where possible the experiment was designed to mimic the conditions of the 48 hour *in vitro* KB cytotoxicity assay. Hence, culture flasks were incubated with drug for 48 hours before fixing and staining. However, in an attempt not to induce too much cellular damage which would mask any subtle drug effects, a lower drug concentration was used - equivalent to the known ED_{25} values already obtained.

3.2.3.2 SPECIMEN PREPARATION

Three cultures were incubated in $80cm^3$ culture flasks with the quassinoids at concentrations equivalent to their previously obtained ED_{25} values against KB cells. A control flask without drug was also set up. After 24 hours, the drug in medium was decanted from the flasks and the monolayer of cells fixed using the protocol described below. A 5mL volume of each solution was used per specimen.

(1) Fixation in 3% glutaraldehyde in 0.075M sodium cacodylate buffer (pH 7.4) at 4°C, overnight.

(2) Encapsulation in 2% aqueous agar.

(3) Washed in buffer at 4°C, overnight.

(4) Post-fixed in 1% osmium tetroxide in cacodylate buffer at 4°C for 1-2 hours.

(5) Washed in distilled water for 20 mins.

(6) Repeat of washing procedure (step 5) at least twice.

(7) Dehydration as follows: 10 mins in 30% methanol

Block stain :-R

25 mins in 30% methanol + 2% uranyl acetate

10 mins 1n 30% methanol

10 mins in 60% methanol

10 mins in 70% methanol

10 mins in 80% methanol

10 mins in 90% methanol

10 mins in 100% methanol (repeated twice)

(8) Solvent washed in 1,2-epoxy propane (2x10 mins)

(9) Infiltration with resin (TAAB Hard) at room temperature, overnight

(10) Repeated with fresh resin, overnight.

(11) Embedded in fresh resin and polymerised at 60°C for 24-48 hours.

Test specimen preparation for Transmission Electron Microsopy and the photography was carried out by Mr. D.G.Tovey and Dr. S.L.Croft in the E.M. and Histopathology Unit at the London School of Hygiene and Tropical Medicine.

Sections of 70nm thickness were cut from the resin blocks on a LKB III Ultramicrotome with a glass knife. These were then mounted on a 200 mesh copper grid and stained in Reynold lead citrate for 5 mins. The sections were viewed on a JEOL 1200 EX MKII transmission electron microscope.

3.3. Results and Discussion

The ED_{50} results of the compounds tested in the KB cytotoxicity assay are expressed in both µg/mL and µM, with the standard error of the mean (SEM) also given. The ED_{50} values are converted from µg/mL to µM by the following equation:

$$X\ \mu g/mL = \frac{X}{1000\ x\ RMM}\ \mu M$$

RMM = Relative molecular mass

Also the SEM is calculated from the standard deviation (SD) as shown by the equation below:

$$SEM = \frac{SD}{\sqrt{n}}$$

where n = no. of test repeats.

Any structures not shown in text are given in appendix.

3.3.1 CYTOTOXIC ACTIVITY OF BRUCEOLIDES

Table 3.1. *In vitro* Cytotoxicity against KB cells of a series of Bruceolide Quassinoids

Quassinoid	ED_{50} (μg/mL)[a]	ED_{50} (μM)	SEM (μM)
Bruceantin	0.008[b]	0.015	0.002
Brusatol	0.102[b]	0.196	0.02
Bruceolide	7.57[b]	17.3	1.69
Bruceine A	0.098[b]	0.188	0.008
Bruceine B	0.055[b]	0.115	0.008
Bruceine C	0.021[b]	0.037	0.01
Bruceine D	1.16[b]	2.82	0.88
Bruceine E	5.58	13.5	3.9
Dehydrobruceine C	3.27	5.82	0.53
Dehydrobruceantin	2.05	3.76	1.39
Dehydrobrusatol	0.299	0.577	0.08
Isobruceine A	0.071	0.136	0.06
Yadanzioside F	>50		

a Mean value of 4 determinations

b Based on 2-fold dilutions in duplicate

Some of the quassinoids tested differed only marginally in their structure, and apparently small variations nevertheless produced large differences in the *in-vitro* KB cytotoxicity of these compounds. For example, the first six compounds listed in Table 3.1 differ only in their C-15 substituents, but have ED_{50} values ranging from 0.015 μM for bruceantin, the most cytotoxic, to 17.3 μM for bruceolide (the parent alcohol), the least toxic. The importance of the C-15 ester function in contributing to both *in-vitro* antileukemic and antimalarial activity (O'Neill *et al*, 1987) has been noted.

Bruceines D and E, the more polar quassinoids were generally less toxic than the more lipophilic, chloroform-soluble compounds mentioned above. This difference was further highlighted by yadanzioside F - a polar quassinoid glycoside, which was shown to be in active against KB at 50 μg/mL. Also yadanzioside F, together with a number of other quassinoid glycosides have been found to possess little *in-vitro* activity against chloroquine- resistant *P.falciparum*, when compared to the aglycone.

Also worthy of mention is the alteration in cytotoxic activity produced as a result of modifications to the A-ring substitution pattern. Bruceantin is 250 times more toxic to KB cells than its ring A dehydro

derivative, dehydrobruceantin, and similarly, bruceine C is 157 times more toxic than dehydrobruceine C. However, the difference is less dramatic for brusatol which is only 3 times more toxic than dehydrobrusatol. Bruceine A and isobruceine A differ only in the oxidation pattern of the A-ring. However, despite a 5-fold change in activity seen against chloroquine-resistant *P.falciparum in-vitro* (the iso-derivative being more active, O'Neill *et al*, 1986), there is no significant change in activity against KB cells.

3.3.2 CYTOTOXIC ACTIVITY OF SOME NON-BRUCEOLIDE QUASSINOIDS

Table 3.2. *In vitro* Cytotoxicity against KB cells of a number of Non-Bruceolide Quassinoids

Quassinoid	ED_{50} (μg/mL)[a]	ED_{50} (μM)	SEM (μM)
Ailanthone	0.661	1.76	0.45
Ailanthinone	0.437	0.914	0.13
Holocanthone	0.139	0.319	0.14
Chaparrinone	0.624	1.65	0.32
6α-Senecioyloxy -chaparrinone	0.041	0.086	0.02
6α-Senecioyloxy -chaparrin	0.025	0.052	0.01
Glaucarubinone	0.112	0.228	0.022
Quassin	>50		

a Mean value of 4 determinations

Quassin, lacking the C-ring methyleneoxy bridge, was shown to be in-active against KB cells at 50μg/mL. It has also previously been found to have little *in vitro* activity against *P. falciparum* (Guru *et al*, 1983). The remaining compounds in Table 3.2 have a methyleneoxy bridge positioned between C-8 and C-11, as opposed to between C-8 and C-13 as seen in the bruceolide quassinoids in table 3.1. The only compounds having an ED_{50} above 1μM against KB cells were ailanthone and chaparrinone , which both lack an ester function at C-15. The most toxic of the group by almost a factor of 10 are 6α-senecioyloxy chaparrin and 6α-senecioyloxy chaparrinone, which are the only compounds to possess an ester function at C-6.

3.3.3 CYTOTOXIC ACTIVITY OF A SERIES OF SEMI-SYNTHETIC QUASSINOIDS

Table 3.3. ***In vitro*** **Cytotoxicity against KB cells of some Semi-synthetic Quassinoids**[a]

Quassinoid	ED_{50} (μg/mL)	ED_{50} (μ/M)	SEM (μ/M)
Bruceolide Esters			
3,15-diacetate	0.334	0.64	0.21
3,12,15-triacetate	3.08	5.46	0.51
3,15-dipropionate	0.298	0.546	0.11
3,15-dibutyrate	0.349	0.604	0.28
3,15-dipentanoate	0.124	0.205	0.02
3,15-dihexanoate	0.135	0.213	0.05
3,15-dioctanoate	0.136	0.197	0.03
3,15-didecanoate	0.231	0.310	0.07
3-monomyristate	4.03	6.22	0.97
3-monostearate	10.83	15.39	4.6
3-MEM Brusatol	15.7	25.9	4.5

a Mean value of 4 determinations

The importance of side-chain substituents, especially the ester function to quassinoid activity has already been mentioned. A series of bruceolide esters and an etherified quassinoid, 3-methoxyethoxymethyl (3-MEM) brusatol listed in Table 3.3 were synthesized by Dr. G. Patel (Patel *et al*, 1989). The 3-MEM brusatol, with an ED_{50} of 25.9μM was shown to be over 100 times less toxic against KB cells than the parent compound brusatol.

Several of the bruceolide esters were found to have ED_{50} values greater than 1μM. The longer chain mono-esters had ED_{50} values of 15.39μM and 6.22μM, and also worthy of a mention for its relatively low toxicity is the 3,12,15-tri-acetate (ED_{50} 5.46μM). Thus, subtle differences in the ester side-chain (and perhaps lipophilicity) may play an important role in determining the cytotoxicity of this group of compounds and hence their potential as therapeutic agents. In an attempt to establish a therapeutic index for the compounds tested, a comparison of cytotoxic and antimalarial activities is discussed in Chapter 5.

3.3.4 CYTOTOXIC ACTIVITY OF SOME KNOWN THERAPEUTIC AND CYTOTOXIC AGENTS

Table 3.4 *In vitro* Cytotoxicity against KB cells of some known therapeutic and cytotoxic agents[a]

Compound	ED_{50} (μg/mL)	ED_{50} (μM)	SEM (μM)
Quinine	108	333	43.8
Chloroquine diphosphate	72.8[b]	153	8.51
Artemisinin	54.6	194	17.4
Emetine hydrochloride	0.372	0.673	0.2
Podophyllotoxin	0.003[b]	0.007	0.0005
Teniposide	96.9	148	8.35
Etoposide	19.8	33.0	3.15
Actinomycin D	0.015	0.012	0.002
Cycloheximide	0.929	3.31	0.49
Nigericin	0.027	0.037	0.006
6-Mercaptopurine	0.581	3.42	1.0
Vincristine sulphate	0.003	0.004	0.0006
Vinblastine sulphate	0.008	0.01	0.002
Chloramphenicol	>50		

a Mean values of 4 determinations

b Based on 2-fold dilutions in duplicate

Three drugs used in the treatment of malaria - quinine, artemisinin and chloroquine diphosphate all have extremely high ED_{50} values above 150 μM. Teniposide and etoposide which are derivatives of the highly toxic antitumor compound podophyllotoxin (ED_{50} 0.007μM), also have relatively high ED_{50} results of 148μM and 33μM respectively, as did the antibiotic chloramphenicol (ED_{50} >50μg/mL). Another antibiotic tested, nigericin, which is known to affect ion transport and ATPase activity in mitochondria was almost a thousand fold more toxic than this.

Not surprisingly the lowest ED_{50} values were obtained for the anticancer agents, for example podophyllotoxin, actinomycin D, vinblastine sulphate and the N-formyl derivative vincristine sulphate. An exception to this was 6-mercaptopurine which, as an antineoplastic agent has a relatively high ED_{50} value of 3.42μM. This figure is comparable to that obtained for cycloheximide, a very potent inhibitor of protein synthesis.

Emetine hydrochloride is a valuable drug for the treatment of amoebiasis, despite having a low ED_{50} value of 0.673 μM (and hence high toxicity). Table 3.4 lists a small number of therapeutic and cytotoxic agents. However, many of the results obtained were in the same order of magnitude as those obtained for the quassinoids and semi-synthetic quassinoids in Tables 3.1 - 3.3. It is not possible, therefore, merely on the basis of their cytotoxicity to dismiss quassinoids as potential chemotherapeutic agents. Selectivity, as will be discussed in Chapter 5 is the critical factor.

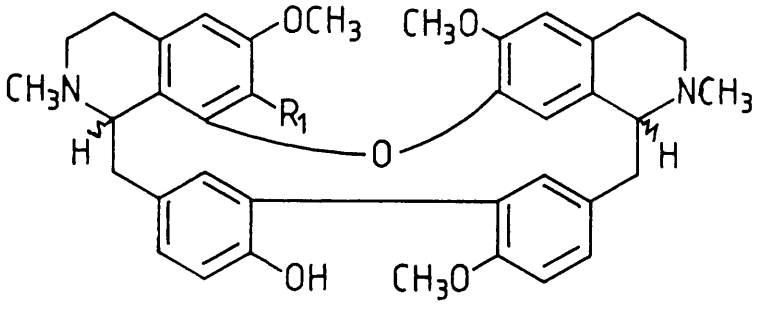
3.3.5 CYTOTOXIC ACTIVITY OF ISOQUINOLINE ALKALOIDS

Prof. P. Schiff provided the following alkaloids for biological investigations. Cytotoxic testing against KB cells was carried out in collaboration with Miss S. Partridge.

A. Bisbenzylisoquinoline alkaloids

1. Funiferine type

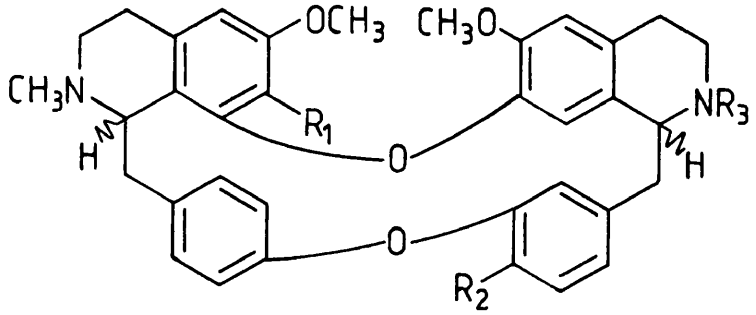
Fig. 3.2 Structures of Funiferine-type Alkaloids and *in vitro* Cytotoxic Activities Against KB Cells



Alkaloid	R_1	ED_{50} (μM)	SEM (μM)	N
Funiferine	OCH_3	108	25.69	4
Tigliageine	OH	>411	—	2

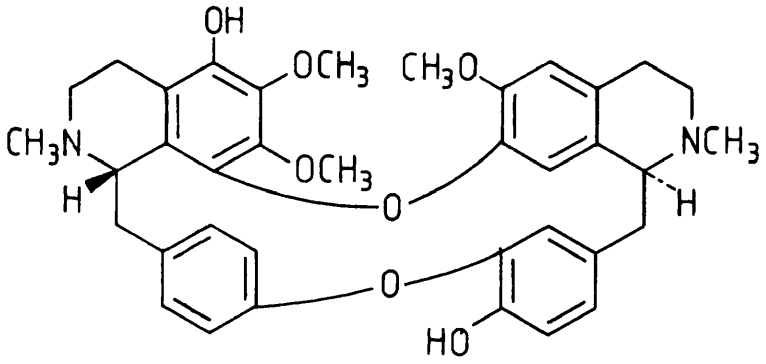
2. Thalisopidine type

Fig. 3.3 Structures of Thalisopidine-type Alkaloids and *in vitro* Cytotoxic Activities Against KB Cells



Alkaloid	R_1	R_2	R_3	ED_{50} (μM)	SEM (μM)	N
Daphnoline	OH	OH	H	46.4	6.21	4
Aromoline	OH	OH	CH_3	106	—	2
Homoaromoline	OH	OCH_3	OCH_3	>82.2	—	2
Oxyacanthine HCl	OCH_3	OH	CH_3	74.3	8.04	4

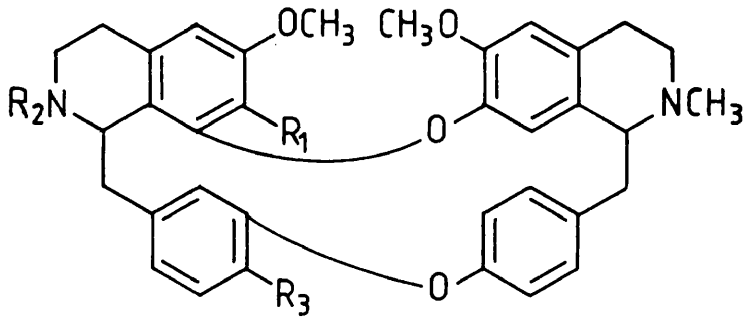
Fig. 3.4. Structure of Thalisopidine and *in vitro* Cytotoxic Activity Against KB Cells



Thalisopidine: ED_{50} >685μM (n=2).

3. Pycnamine type

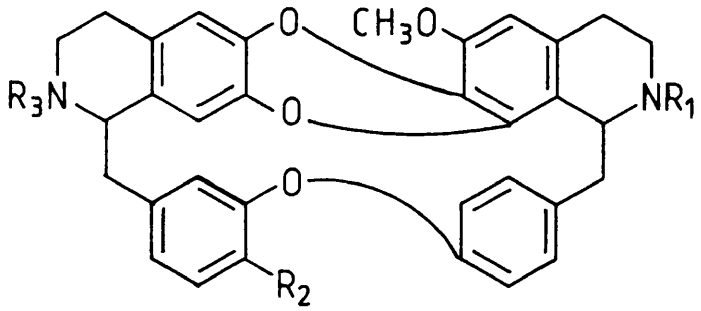
Fig. 3.5. Structures of Pycnamine-type Alkaloids and *in vitro* Cytotoxic Activities Against KB Cells



Alkaloid	R_1	R_2	R_3	ED_{50} (μM)	SEM (μM)	N
Phaeanthine	OCH_3	CH_3	OCH_3	43.6	5.95	4
Pycnamine	CH_3	CH_3	OH	31.9	6.83	4
Isotetrandrine	OCH_3	CH_3	OCH_3	105	16.4	4
Berbamine	OCH_3	CH_3	OH	0.454	0.818	4
Obamegine	OH	CH_3	OH	55.4	6.54	4
Fangchinoline	OH	CH_3	OH	104	4.42	4
Tetrandrine -methiodide	OCH_3	CH_3	OCH_3	>32.7	—	2

4. Cocsoline type

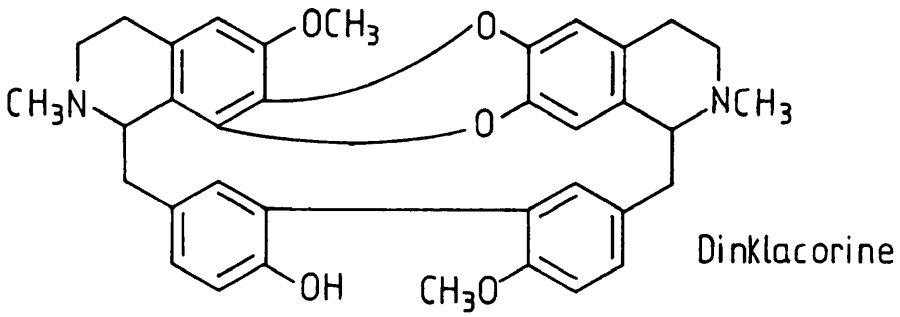
Fig. 3.6. Structures of Cocsoline-type Alkaloids and *in vitro* Cytotoxicity Against KB Cells



Alkaloid	R_1	R_2	R_3	ED_{50} (μM)	SEM (μM)	N
Cocsoline	CH_3	OH	H	>222	—	2
Cocsoline -methiodide	CH_3	OH	CH_3	>355	—	2
Isotrilobine	CH_3	OCH_3	CH_3	15.0	2.29	4
Trilobine	H	OCH_3	CH_3	20.8	3.32	4

5. Miscellaneous Bisbenzylisoquinoline Alkaloids

Fig. 3.7. Structures of Miscellaneous Bisbenzylisoquinoline Alkaloids and *in vitro* Cytotoxicity Against KB Cells



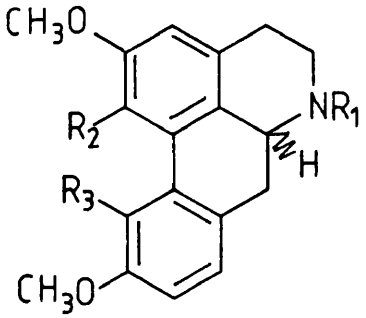
Alkaloid	ED_{50} (μM)	SEM (μM)	N
Dinklacorine	54.8	10.8	4
Gilletine	74.2	3.82	4
Insularine Picrate	>294	—	2
Isochondodendrine	>421	—	2
Trigilletimine	>448	—	2

Fig. 3.7 is continued over page

B. Aporphine Alkaloids

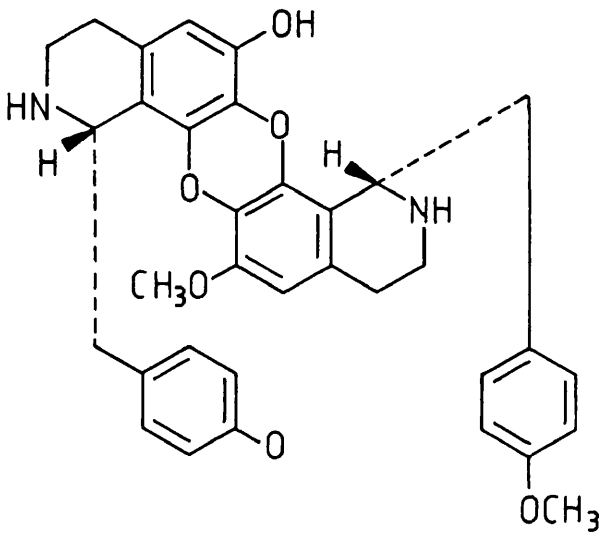
1. Corydine type

Fig. 3.8. Structures of Corydine-type Alkaloids and *in vitro* Cytotoxicity Against KB Cells

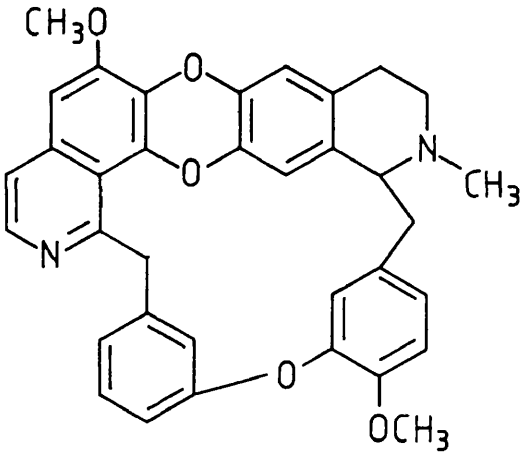


Alkaloid	R_1	R_2	R_3	ED_{50} (μM)	SEM (μM)	N
Corydine	CH_3	OH	OCH_3	>733	—	2
Norcorydine	H	OH	OCH_3	>764	—	2
Isocorydine	CH_3	OCH_3	OH	>733	—	2
Catalpifoline	H	OCH_3	OCH_3	>733	—	2

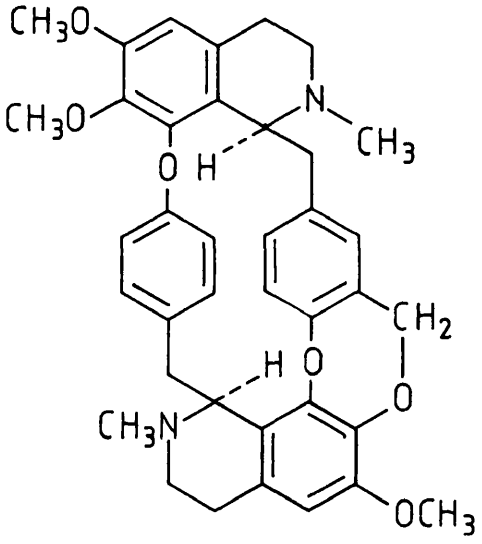
Figure 3.7. (continued)



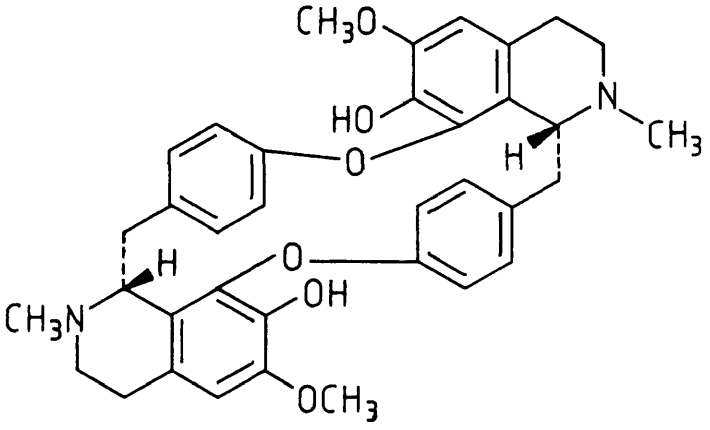
gilletine



trigilletimine



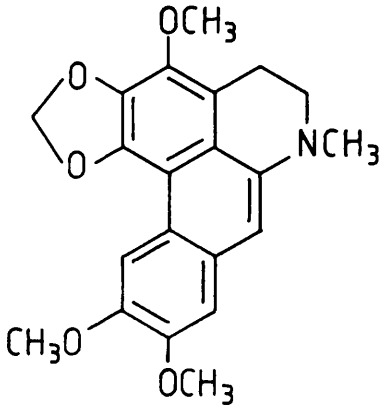
insularine



isochondodendrine

2. Dehydroaporphine type

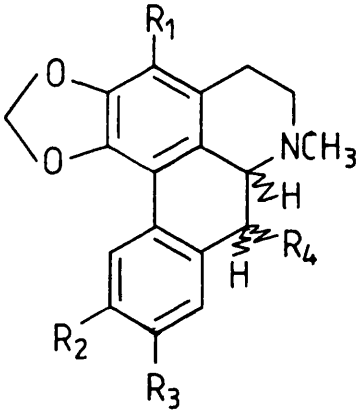
Fig. 3.9. Structures of dehydroocoteine and *in vitro* Activity Against KB Cells



Dehydroocoteine: ED_{50} > 341μM (n=2)

3. Ocoteine type

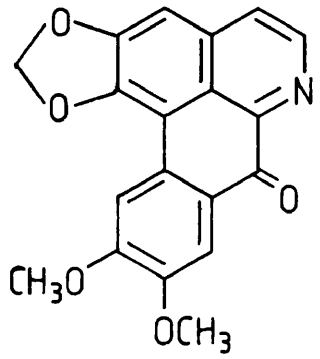
Fig. 3.10. Structure of Ocoteine-type Alkaloids and *in vitro* Activity Against KB Cells



Alkaloid	R_1	R_2	R_3	R_4	ED_{50} (μM)	SEM (μM)	N
Ocoteine	OCH_3	OCH_3	OCH_3	H	74.3	12.3	4
Ushinsunine	H	H	H	OH	42.5	4.85	4

4. Oxoaporphine type

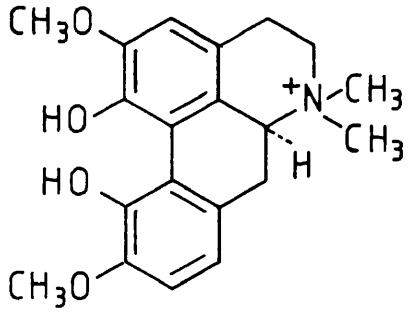
Fig. 3.11. Structure of Thalicminine and *in vitro* cytotoxicity Against KB Cells



Thalicminine: ED_{50} >685μM (n=2).

5. Quaternary Alkaloids

Fig. 3.12. Structure of Magnoflorine and *in vitro* cytotoxicity Against KB Cells

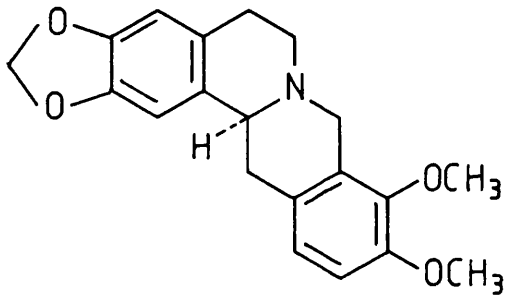


Magnoflorine (iodide): ED_{50} >533μM (n=2).

C. Protoberberine Alkaloids

1. Non-quaternary Alkaloids

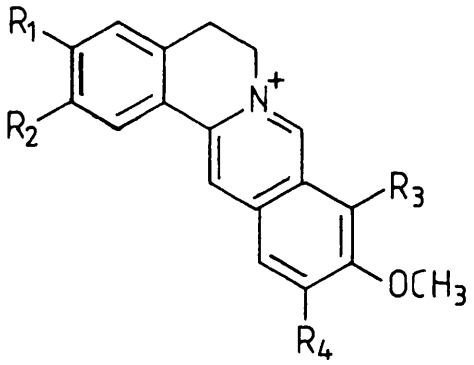
Fig. 3.13. Structure of Canadine and *in vitro* cytotoxicity Against KB Cells



+-Canadine: ED_{50} > 737μM (n=2).

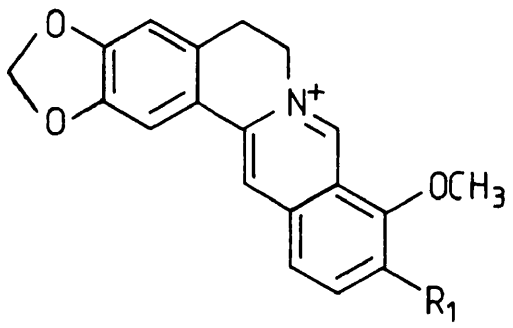
2. Jatrorrhizine type

fig. 3.14. Structure of Jatrorrhizine-type Alkaloids and *in vitro* Cytotoxic Activity Against KB Cells



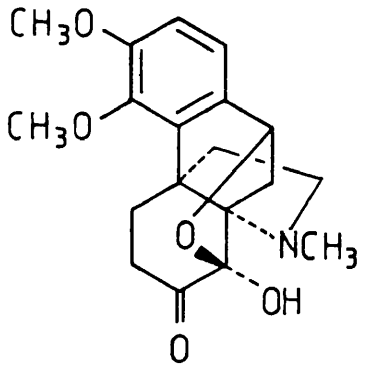
Alkaloid	R_1	R_2	R_3	R_4	ED_{50} (μM)	SEM (μM)	N
Jatrorrhizine	OH	OCH_3	OCH_3	H	>335	—	2
Columbamine	OCH_3	OH	OCH_3	H	77.9	18.4	4
Dehydrodiscretine	OH	OCH_3	H	OCH_3	>335	—	2

Fig. 3.15. Structures of Berberine-type Alkaloids and *in vitro* Cytotoxic Activity Against KB Cells



Alkaloid	R_1	ED_{50} (μM)	SEM (μM)	N
Berberine chloride	OCH_3	7.32	1.07	4
Thalifendine chloride	OH	>699	—	2

Fig. 3.16. Structures of Morphinandienone Alkaloids and *in vitro* Cytotoxic Activities Against KB Cells



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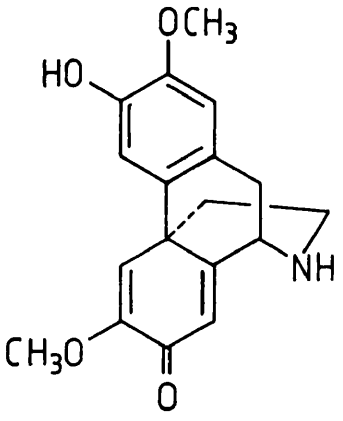
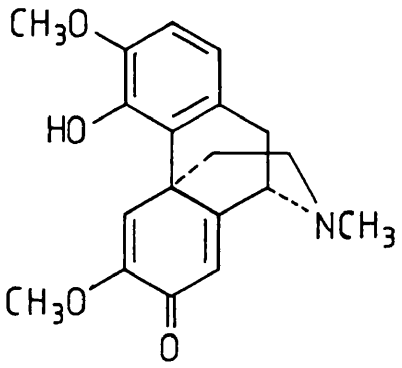


1

	Alkaloid	ED_{50} (μM)	SEM (μM)	N
1	O-Methylflavinantine	>734	—	2
2	Metaphanine hydrobromide	>586	—	2
3	Sinoacutine	765	—	2
4	Sinomenine	759	—	2



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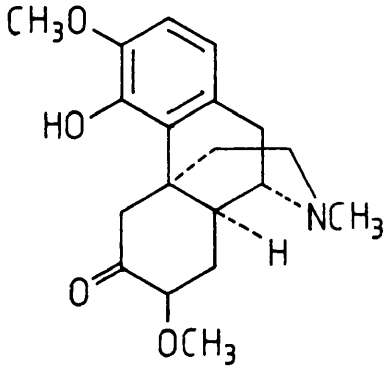
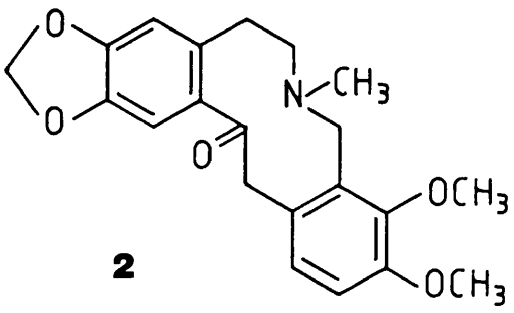


4

E. Protopine Alkaloids

Fig. 3.17. Structures of Protopine Alkaloids and *in vitro* Cytotoxic Activities Against KB Cells



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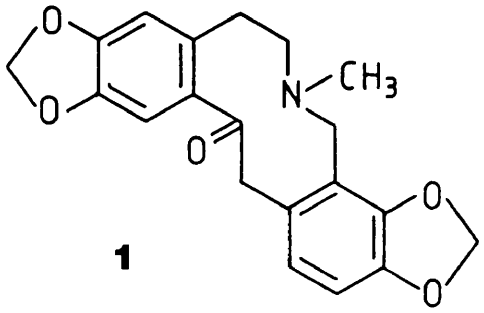
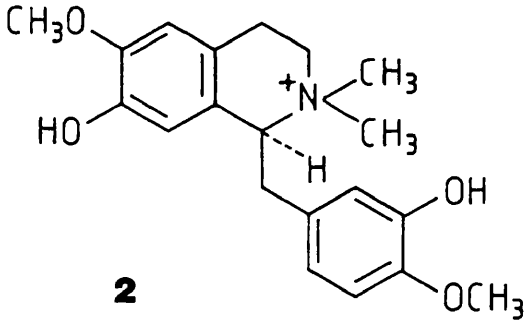


1

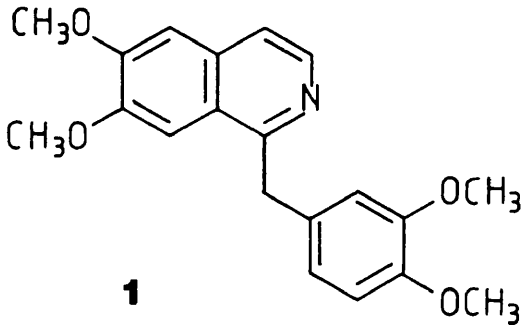
	Alkaloid	ED_{50} (μM)	SEM (μM)	N
1	Protopine	>354	—	2
2	Allocryptine	>338	—	2

F. Benzylisoquinoline Alkaloids

Fig. 3.18. Structures of Benzylisoquinoline Alkaloids and *in vitro* Cytotoxic Activities Against KB Cells



2

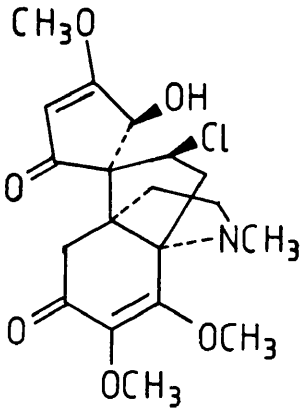


1

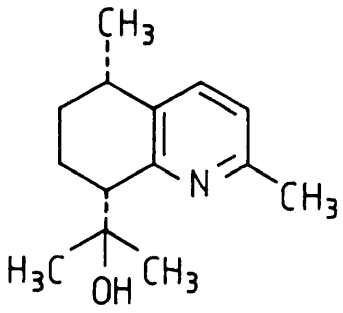
	Alkaloid	ED_{50} (μM)	SEM (μM)	N
1	Papaverine hydrochloride	6.47	1.99	4
2	Tembetarine	>726	—	2

Fig. 3.19 Structures of Acutumine and Fabianine picrate with *in vitro* Cytotoxic Activities Against KB Cells

1



2



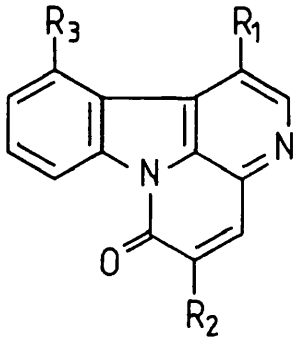
	Alkaloid	ED_{50} (μM)	SEM (μM)	N
1	Acutumine	>628	—	2
2	Fabianine picrate	>279	—	2

Very few of the isoquinoline alkaloids tested show activity in the KB assay. Only one, berbamine demonstrated activity below 1μM, with berberine chloride (ED_{50} 7.32μM) and papaverine hydrochloride (ED_{50} 6.47μM), the only compounds with activity between 10 and 1μM. A 100-fold difference in activity is observed between obamegine (ED_{50} 55.4 μM) and berbamine (ED_{50} 0. 454μM), for a very small structural change about a single carbon atom (see fig. 3.5).

There is, however, some disparity between several of the ED_{50} values, and previously published results using the NCI KB screen. More specifically, Hartwell and Abbott, 1969 reported tetrandrine, isotetrandrine, obamagine and fangchinoline to have ED_{50} values between 8 and 0.1μM, where in the present study the values were much higher - between 105 and 55 μM. There are several significant differences between the two assays which could explain the seemingly inconsistent data. These will be discussed in detail in Chapter 5. The NCI screen , for example adjust the pH of the drug-medium solution to neutral, and under these conditions the alkaloids would be in the salt form. In contrast, the present KB assay tests the alkaloids as free bases.

3.3.6 CYTOTOXIC ACTIVITY OF SOME CANTHIN-6-ONE ALKALOIDS

Table 3.5. ***In vitro*** **Cytotoxicity against KB cells of a number of Canthin-6-one Alkaloids[a]**



Alkaloid	R_1	R_2	R_3	ED_{50} (μg/mL)	ED_{50} (μM)	SEM (μM)
Canthin-6-one	H	H	H	5.55	25.3	2.91
1-Methoxy-canthin-6-one	OCH_3	H	H	7.60	30.4	7.56
5-Methoxy-canthin-6-one	H	OCH_3	H	>50		
11-Hydroxy-canthin-6-one	H	H	OH	1.98	8.46	1.44

a Mean value of 4 determinations

Four canthin-6-one alkaloids extracted from cell suspension cultures of *B. javanica* by Shi-lin Yang were tested for activity in the KB assay be sufficiently active to meet the NCI criteria of an active compound. The least toxic alkaloid, 5-methoxy canthin-6-one is inactive at 50μg/mL.

These findings are consistent with those of Cordell *et al*, 1978. It has been shown that either hydroxylation or methoxylation at either C-10 or C-11 are structural requirements for potent cytotoxic activity (Fukamiya *et al*, 1987). However, canthin-6-one, 1-methoxycanthin-6-one, and 5-methoxycanthin-6-one have been shown to be cytotoxic to guinea-pig ear keratinocytes (Anderson *et al*, 1983).

3.3.7 CYTOTOXIC ACTIVITY OF CRUDE PLANT EXTRACTS FROM *BRUCEA JAVANICA* AND *SIMAROUBA GLAUCA* FRUITS

Table 3.6. *In vitro* Cytotoxicity against KB cells of crude extracts from *B. javanica* and *S. glauca* fruits[a]

The method for the preparation of extracts shown in Table 3.6 is shown in Figs. 2.1, 2.3 and 2.4.

Extract	*B. javanica* fruits ED_{50} (μg/mL)	*S. glauca* fruits ED_{50} (μg/mL)
Aqueous tea	14.3	54.6
Chloroform extract of aq. tea	0.394	N.T.
Aq. tea (after chloroform ext.)	>100	>100
Hydrolysed Aqueous tea	1.07	N.T.
Hexane extract	>100	>100
Methanol extract	3.05	75.7
Chloroform extract	1.69	16.1
Aqueous 1	17.1	>100
Aqueous 2	46	>100
Butanol extract	10.9	>100

a Based on 2-fold dilutions in duplicate

N.T. Not Tested

The crude plant extracts from *S.glauca* exhibited very little cytotoxic activity against KB cells. However, four extracts from *B. javanica* were shown to have marked activity, and phytochemical investigation of the aqueous tea chloroform extract and hydrolysed aqueous tea yielded several lipophilic bruceolide quassinoids (see Chapter 2). Similarly, quassinoids were also isolated after fractionation of the chloroform and methanol extracts (O'Neill *et al*, 1987). The more polar quassinoids, bruceine D and several quassinoid glycosides were isolated from the butanol extract. These results illustrate the usefullness of the assay in guiding fractionation of plant extracts for the isolation of pure active constituents.

3.3.8 CYTOTOXIC ACTIVITY OF A RANGE OF COMPOUNDS TESTED OVER 72 HOURS

Table 3.7. *In vitro* Cytotoxicity of a range of compounds against KB cells in a 72 hour test[a]

Compound	ED_{50} (μg/mL)	ED_{50} (μM)	SEM (μM)
Bruceantin	0.009	0.016	0.005
Brusatol	0.047	0.09	0.021
Bruceine D	0.209	0.510	0.141
3,15 di-isobutyrate[b]	0.241	0.417	0.010
Chaparrinone	0.160	0.423	0.153
Chloroquine diphosphate	26.3	55.2	4.90
Artemisinin	36.6	130	19.8
Podophyllotoxin	0.004	0.009	0.001
Etoposide	3.00	5.00	0.325
Phaeanthine	5.65	9.09	1.35
Isotetrandrine	36.9	59.3	5.19

a Mean values of 4 determinations

b Di-ester of bruceolide

The 72 hour test results indicate that a 24 hour increase in the duration of the assay in conjunction with the NCI test, leads to a general increase in toxicity (i.e. a lowering of the ED_{50} values). Only the toxicity of podophyllotoxin and bruceantin did not significantly alter. If the compounds are listed in rank order of decreasing cytotoxicity (see Table 3.8), it can be seen that their relative cytotoxicities remain basically the same. However, the relative increase in the cytotoxicity from the 48 hour to the 72 hour test is not consistent for all the compounds, the range being a 2 to 7-fold increase. This may reflect the different modes of action and the differing membrane permeabilities of each of the drugs.

Table 3.8 Rank Order by Decreasing Cytotoxicity of Drugs Screened in a 72 Hour KB Test

Compound	Rank Order In 72 Hour Test	Rank Order In 48 Hour Test	Approx. Increase In Cytotoxicity (72hr/48hr)
1	Podophyllotoxin	(1)	x1
2	Bruceantin	(2)	x2
3	Brusatol	(3)	x6
4	Chaparrinone	(5)	x5
5	3,15 di-isobutyrate	(4)	x4
6	Bruceine D	(6)	x3
7	Etoposide	(7)	x1.5
8	Phaeanthine	(8)	x5
9	Isotetrandrine	(10)	x2
10	Chloroquine diphosphate	(9)	x3
11	Artemisinin	(11)	x2

Numbers in brackets represent rank order in 48 hour test and the numbers on right show approximate increase in cytotoxicity (i.e. decrease in ED_{50}).

3.3.9 EFFECTS OF QUASSINOIDS UPON PROTEIN SYNTHESIS AND NUCLEIC ACID SYNTHESIS IN KB CELLS

3.3.9.1 Effects of Drugs Upon Protein Synthesis

A preliminary experiment was carried out to determine a relationship between [^{3}H] ile incorporation by drug-free KB cells and incubation time (see Fig. 3.20). The linear relationship :

[^{3}H] ile incorp. (cpm) = 147.9 + 24.57 x incubation time,

where r = 0.98, continued for 260 mins. It was unnecessary to continue the experiments beyond this as the quassinoids exerted an inhititory affect very rapidly.

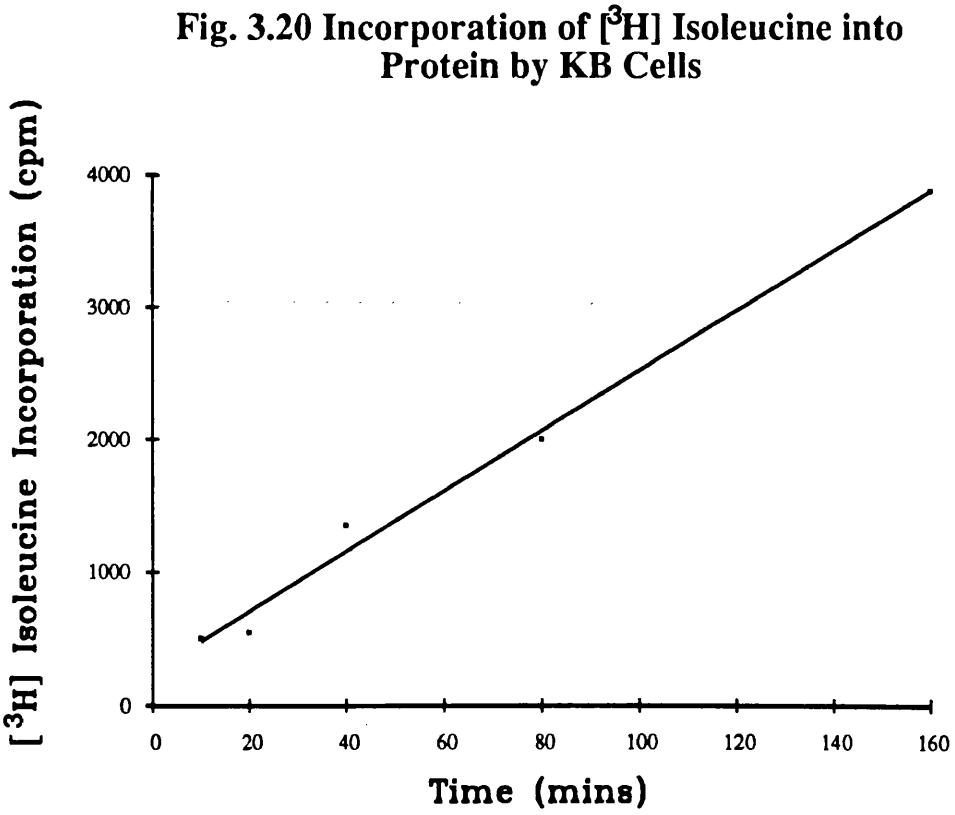


Fig. 3.20. Incorporation of [^{3}H]ile into protein by KB cells, in a preliminary experiment, over a period of 160 mins. The counts per minute illustrated are the means from cells harvested from duplicate 200 μL aliquots of culture suspension (5 x 10^5 cells/mL).

Table 3.9. Drug Concentrations Used and their Effects upon Protein and Nucleic Acid Synthesis in KB cells

Compound	Cytotoxicity Against KB Cells ED_{50} (μM)	Concentration at test xED_{50} (μM)	Protein Synthesis t_{50} ile (min)	N	Nucleic Acid Synthesis t_{50} Hyp (min)	N	t_{50} Ratio ile/Hyp
Bruceantin	0.015	10	45	2	324	1	0.14
Bruceine D	2.82	10	36	2	576	1	0.05
Brusatol	0.196	10	20	2	224	1	0.09
Actinomycin D	0.012	417	64	1	60	2	1.07
Cycloheximide	3.31	9.1	33	2	380	1	0.09
N = No. of duplicates							

3.3.9.1 (cont.)

The inhibitory effect of cycloheximide was apparent within 8 minutes of drug addition (see Fig. 3.21(a)). Actinomycin D had a less marked effect and was not noticeable until 32 mins, after which time [^{3}H]ile incorporation continued, but at a much reduced rate compared to the control. The effects of the three quassinoids are shown in Fig. 3.22(a). At ten times their *in vitro* ED_{50} concentrations against KB cells, all inhibit [^{3}H]ile incorporation within minutes of addition. Brusatol had the most potent effect, with bruceantin showing least inhibition. The concentrations of quassinoids used in this study which were shown to bring about inhibition of protein synthesis are of an order consistent with previously published values (Hall *et al.*, 1983).

3.3.9.2. Effects of Drugs Upon Nucleic Acid Synthesis

Figures 3.21(b) and 3.22(b) show the effects of drugs upon nucleic acid synthesis. In the non-drug treated controls, it can be seen that isotope incorporation increased with time. Of the drugs tested, actinomycin D had the greatest inhibitory effect and although cycloheximide also showed inhibition, it was less complete and occurred much later at 120 mins. The quassinoids showed inhibition comparable to cycloheximide, but rather less than actinomycin D. Again, brusatol had the biggest inhibitory effect of the quassinoids studied, and bruceantin had the least.

Fig. 3.21(a). Incorporation of [^{3}H]ile into protein and (b) incorporation of [^{3}H]hyp into nucleic acids by KB cells, in a single experiment: The cpm are the means from cells harvested from duplicate 200 µL aliquots of cell suspension (5 x 10^5 cells/mL). Drugs (cycloheximide at 3 x 10^5 M and actinomycin D at 10^{-5} M) were added at t=0.

Fig. 3.21 (a) Incorporation of [^{3}H] Isoleucine into Protein by KB Cells treated with Actinomycin D and Cycloheximide

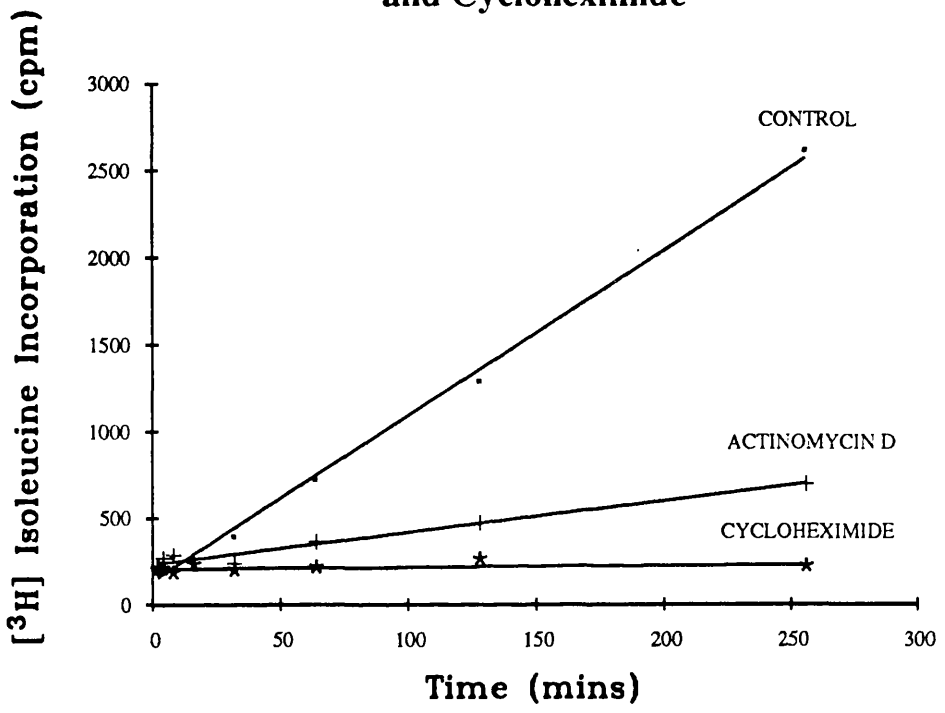


Fig. 3.21(b) Incorporation of [^{3}H] Hypoxanthine into Nucleic Acids by KB Cells treated with Actinomycin D and Cycloheximide

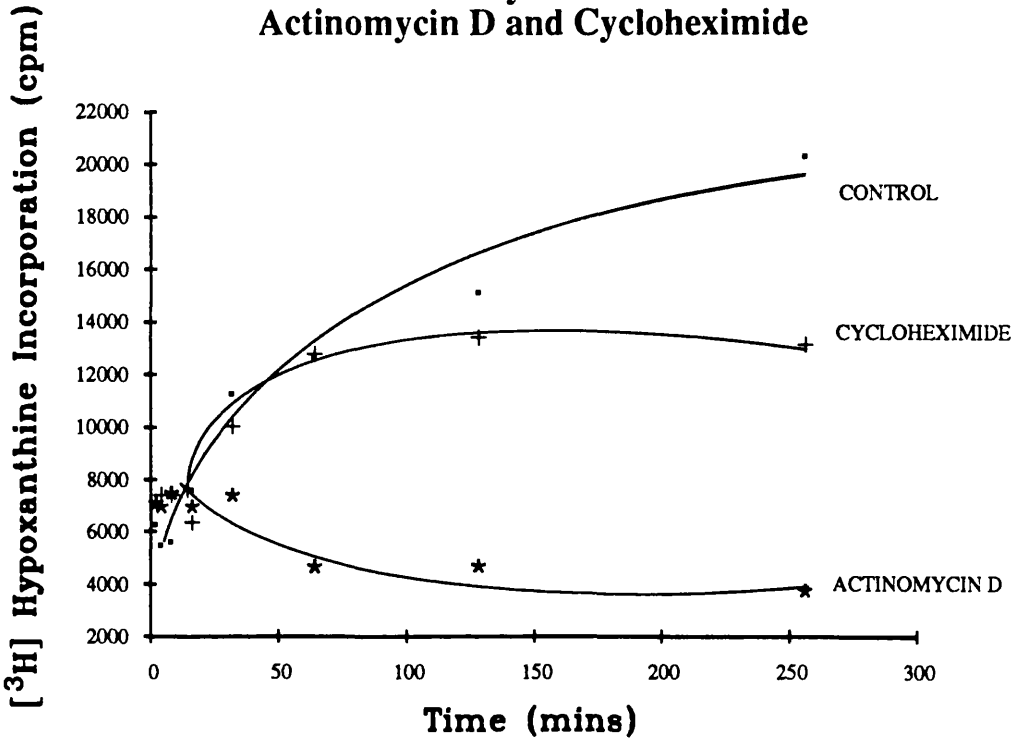


Fig. 3.22(a). Incorporation of [^{3}H]ile into protein and (b) incorporation of [^{3}H]hyp into nucleic acids by KB cells, in a single experiment, and showing the effects of a number of quassinoids. The cpm are the means from cells harvested from duplicate 200 μL aliquots of cell suspension (5 x 10^5 cells/mL). Drugs (bruceantin 0.15 μM, bruceine D 28.2 μM and brusatol 1.46 μM) are added at t=0.

Fig. 3.22(a) Incorporation of [^{3}H] Isoleucine into Protein by KB Cells treated with Bruceantin, Bruceine D and Brusatol

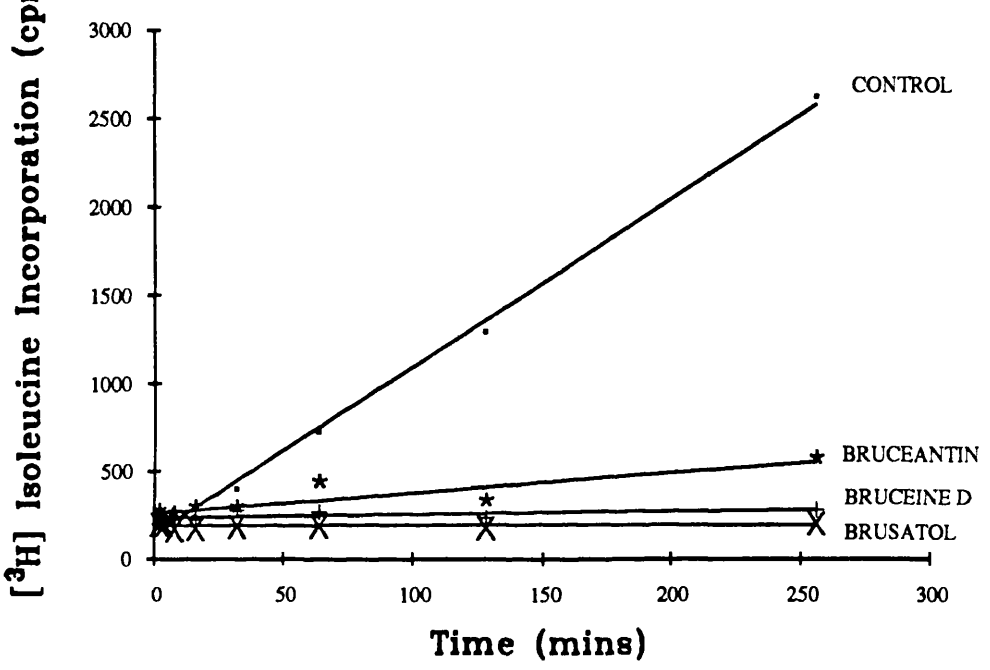
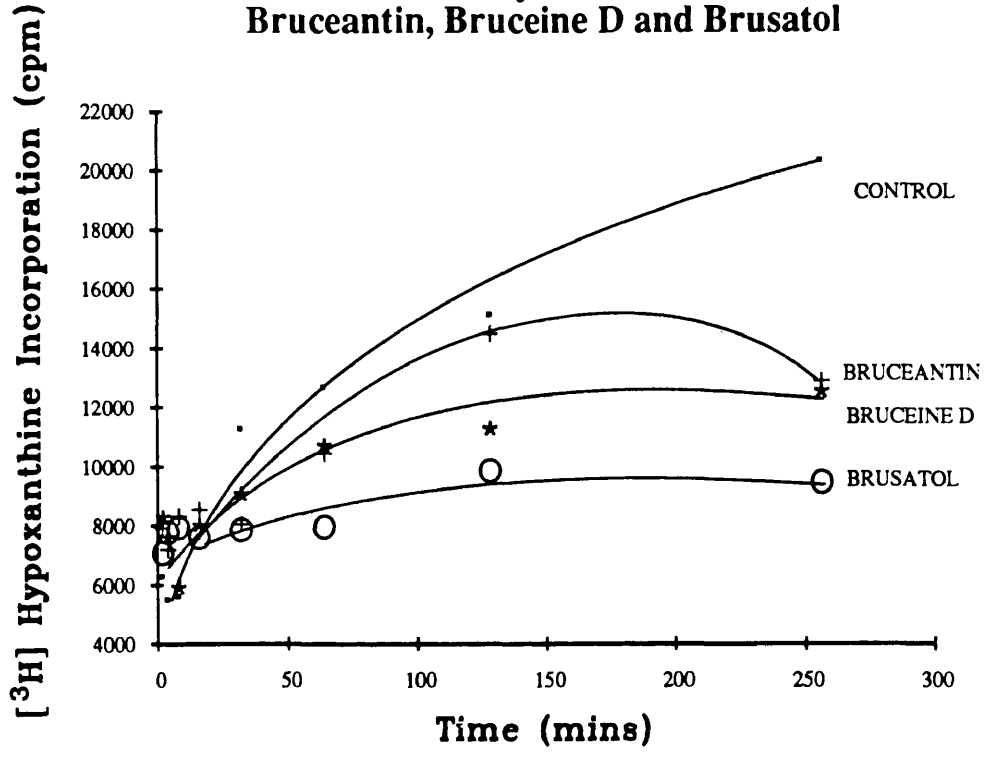


Fig. 3.22(b) Incorporation of [^{3}H] Hypoxanthine into Nucleic Acids by KB Cells treated with Bruceantin, Bruceine D and Brusatol



3.3.9.3 Correlation of Data

The percentage inhibition of control (non-drug treated) incorporation of radio-isotope was calculated for each drug and then plotted against time (log_{10})(see Fig. 3.23). Where the data points appeared to follow a linear relationship regression analysis was used to fit a straight line. From this it was possible to determine the t_{50} for each drug (i.e. the time in minutes after addition of drug at which incorporation of isotope represents 50% of control). The results are given in Table 3.8. The t_{50} (ile)/ t_{50} (hyp) ratio for each of the three quassinoids, and for cycloheximide was always below 1.0. However, the ratio for actinomycin D was 1.07.

Equivalent doses of the 3 quassinoids were used based on their *in vitro* cytotoxicity ED_{50} values against KB, in order to standardise conditions.

Fig. 3.23(a)

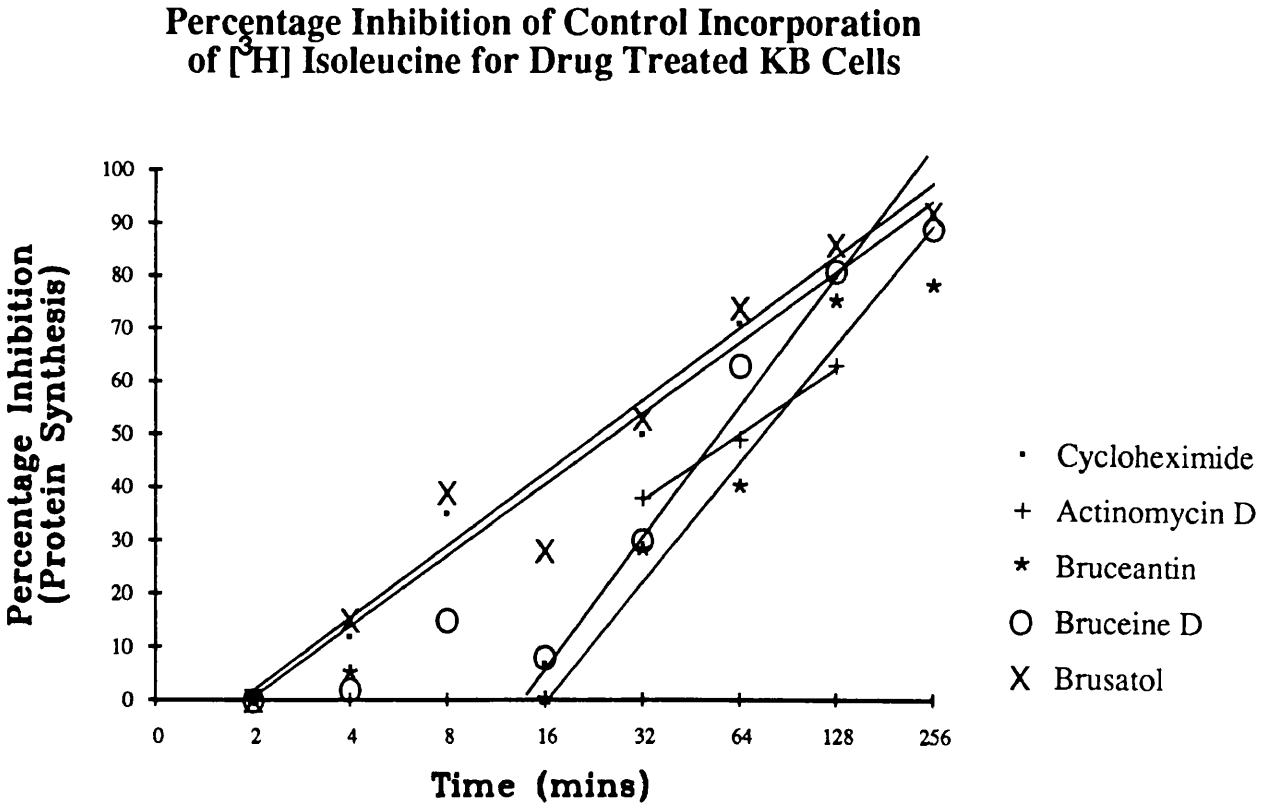
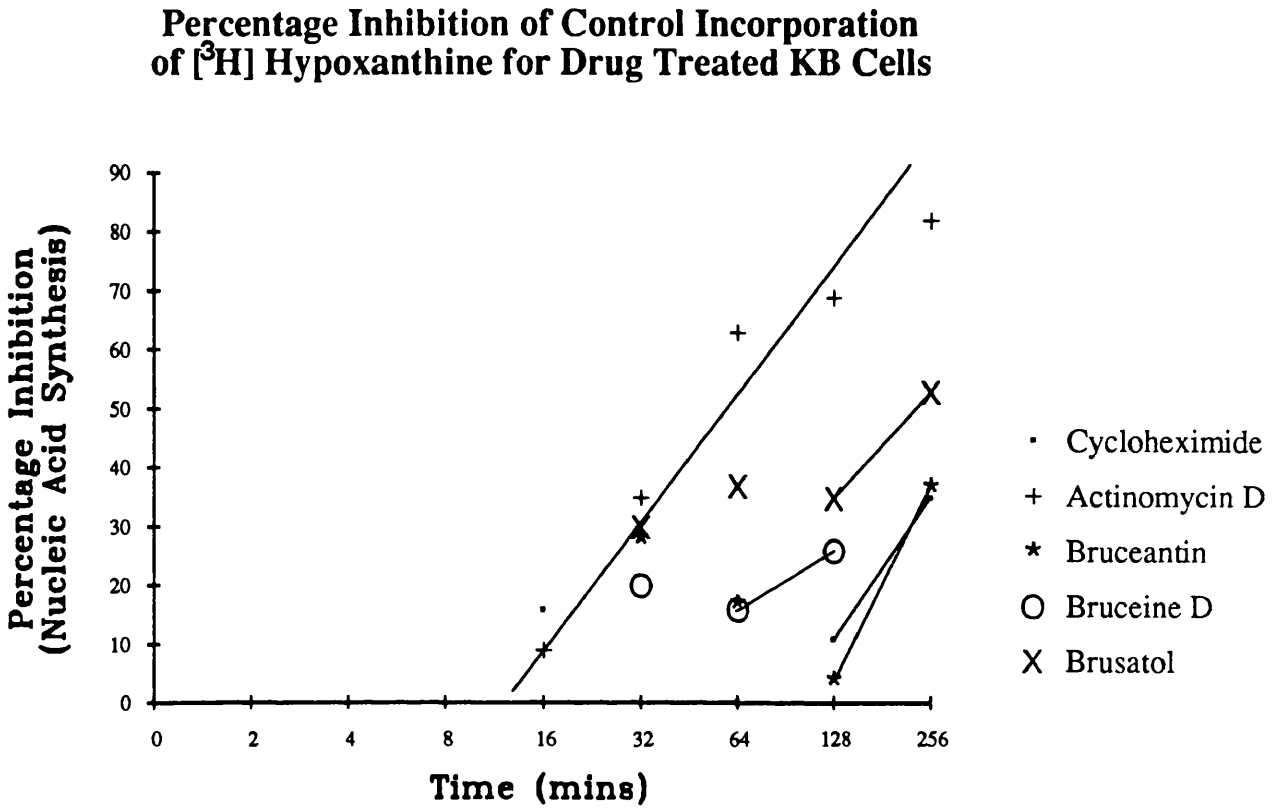


Fig. 3.23. The percentage inhibition of control incorporation of (a) [^{3}H]ile or (b) [^{3}H]hyp was calculated for each drug and plotted against time on a log scale. The time at which isotope incorporation represented 50% of control (t_{50}) was determined from linear regression analysis.

Fig. 3.23(b)



3.3.9.4 Discussion

In this investigation depression of nucleic acid synthesis in KB cells by the quassinoids was observed much later (at 120 mins.) than the inhibition of protein synthesis (found to occur at 32 mins.). The quassinoid results were comparable to those obtained for cycloheximide - a known protein synthesis inhibitor, yet differed greatly from the nucleic acid synthesis inhibitor actinomycin D. It was also interesting to note that although bruceantin is the most toxic of the 3 quassinoids against KB cells, it was the least potent inhibitor.

It appears from this study that the primary action of quassinoids in KB cells is against protein synthesis, followed later by the inhibition of nucleic acid synthesis. These results are in general agreement with previous investigations which show that quassinoids inhibit protein synthesis in rabbit reticulocytes, reticulocyte lysates (Beran *et al*, 1980), in Hela cells (Laio *et al*, 1976), P-388 lymphocytic leukaemia and Ehrlich and hepatoma carcinoma (Hall *et al*, 1983). In an early study brusatol, bruceine D and bruceantin (at 0.015 mM) were all found to inhibit protein and RNA synthesis in cultured P-388 leukemic cells. Of the three quassinoids brusatol was the most active inhibitor resulting in 86% suppression of protein synthesis and 62% suppression of RNA synthesis (Hall *et al.*, 1979). In HeLa cells bruceantin was found to inhibit protein synthesis by 90% at 2μM, whereas DNA and RNA synthesis were inhibited at 60% and 15% respectively. In rabbit reticulocytes 79% protein synthesis inhibition was observed with bruceantin at

0.1μM (Liao *et al.*, 1976). Also in rabbit reticulocytes brusatol was shown to be a potent inhibitor of peptidyl transferase (Willingham *et al.*, 1981.) Subsequent investigations have indicated that the mode of action of quassinoids as protein synthesis inhibitors is identical in all of the cells where inhibition is observed; i.e., the elongation step of protein synthesis is stopped. This inhibition of protein synthesis by quassinoids correlates positively with P-388 tumour cell growth inhibition (Hall *et al.*, 1983).

A recent study by Kirby *et al*, 1989, used a modification of the method described here to investigate the mode of action of quassinoids against CQ-resistant *P. falciparum.* It was concluded that the quassinoids also inhibit protein synthesis in the malaria parasite.

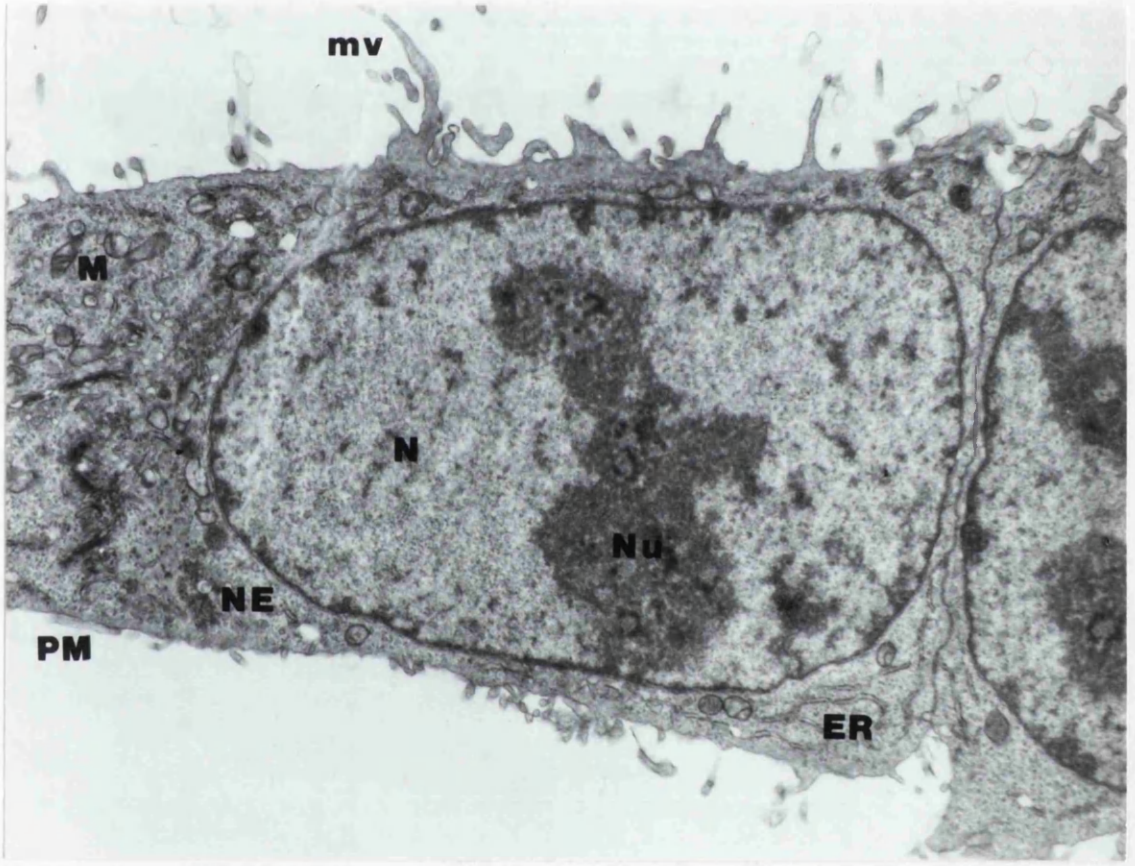
A comparison of the t_{50} values from the present study in KB cells with those of Kirby *et al* in *P. falciparum* reveals several differences. (The quassinoid concentrations in both investigations were equivalent to ten times the previously determined ED_{50} values against KB cells or ten times the IC_{50} value against *P. falciparum*). The t_{50}(ile) values in minutes for bruceantin, cycloheximide and actinomycin D in *P. falciparum* were 122 , 70 and 149 mins respectively, which compares with 45, 33 and 64 mins for the same compounds in KB cells. Hence it appears that protein synthesis inhibition occurs more quickly in the KB cell line, although a comparison of the inhibition of nucleic acid synthesis in both cell systems is more complex. The t_{50}(hyp) values for bruceantin, cycloheximide and actinomycin D in *P. falciparum* are 233, 184 and 64 respectively, whereas the corresponding values in KB cells are 324, 380 and 60 mins respectively. The t_{50}(hyp) values for actinomycin D are comparable in both systems, but the t_{50}(hyp) values for bruceantin and cycloheximide in KB cells are somewhat higher than in *P. falciparum.* Therefore, the results indicate that in KB cells protein synthesis inhibition by quassinoids is more rapid, and depression of nucleic acid synthesis is slower when compared with quassinoid-treated *P. falciparum.*

It has been shown that quassinoids are not in fact universal protein synthesis inhibitors. Brusatol and bruceantin have been found to be selective for Ehrlich hepatoma carcinoma, L-1210 lymphoid leukemia and P-388 lymphocytic leukemia as as for some normal tissue types such as lymphocytes, in which protein synthesis was observed. It has been suggested that observed differences in the magnitude of protein synthesis inhibition of two P-388 lymphocytic leukemia cell lines by the quassinoids occurs at the ribosomal level. Conversely, differences seen in normal livers from several mice strains may be the result of differences in cell membrane transport of the quassinoids into different tissues. This selectivity is very important if quassinoids are to be therapeutically useful as antimalarials as it is hoped that a quassinoid may be discovered which demonstrates selectivity for malaria parasites.

3.3.10 ELECTRONMICROSCOPICAL INVESTIGATION OF DRUG-TREATED KB CELLS

Representative examples of photographs from an EM investigation of quassinoid treated KB cells are given in figures 3.24 to 3.34. Figures 3.24 and 3.25 are of untreated KB cells used as control cells for comparison. Figures 3.26 to 3.29 show KB cells after incubation with bruceantin for 48 hours.

Figure 3.24 Electron micrograph of a KB cell (control) x 10 000



mv - microvilli

N - nucleus

Nu - nucleolus

NE - nuclear envelope

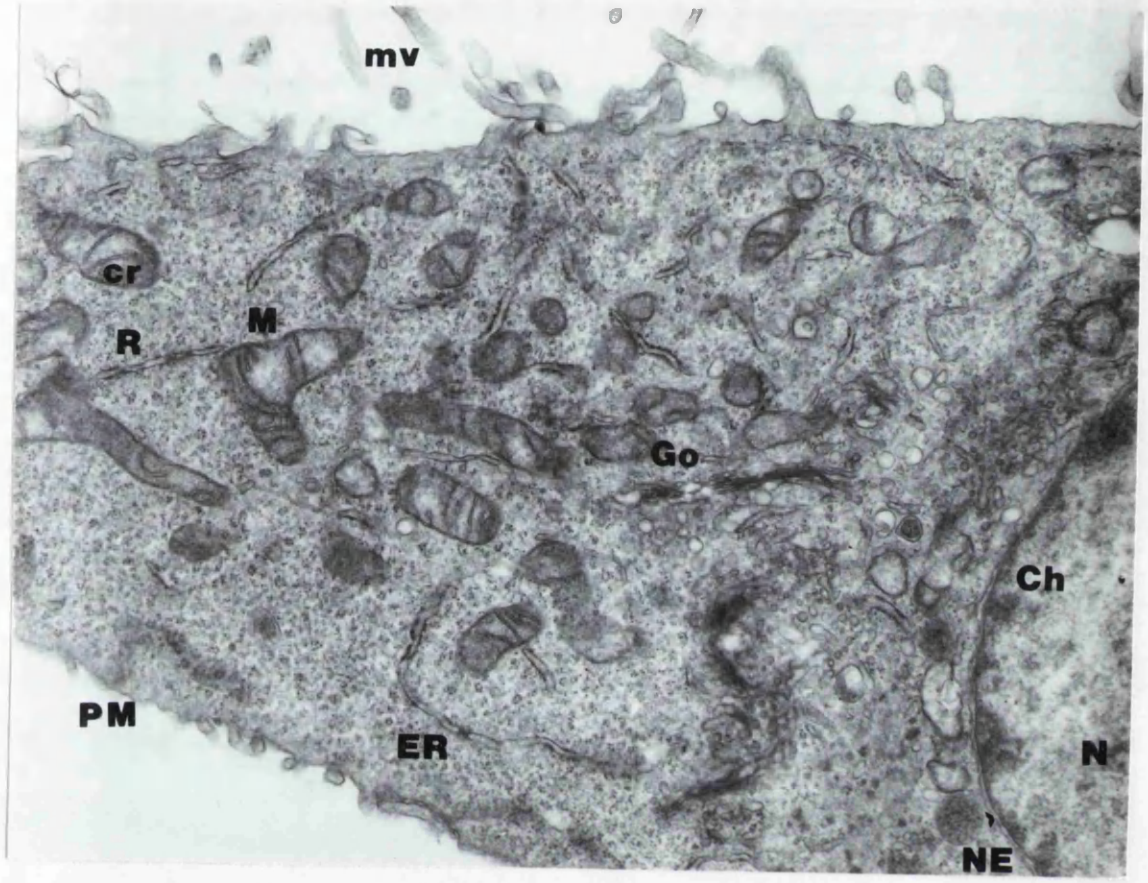
PM - plasma membrane

M - mitochondria

ER - endoplasmic reticulum

Fig. 3.24 shows a KB cell incubated for 48 hours without drug. Numerous microvilli extend from the plasma membrane; the nuclear envelope and plasma membrane appear regular in shape, with no obvious features of note.

Figure 3.25 Electron micrograph of a KB cell (control) x 20 000



mv - microvilli

N - nucleus

Ch - chromatin

Ne - nuclear envelope

Go - Golgi complex

ER - endoplasmic reticulum

PM - plasma membrane

M - mitochondria

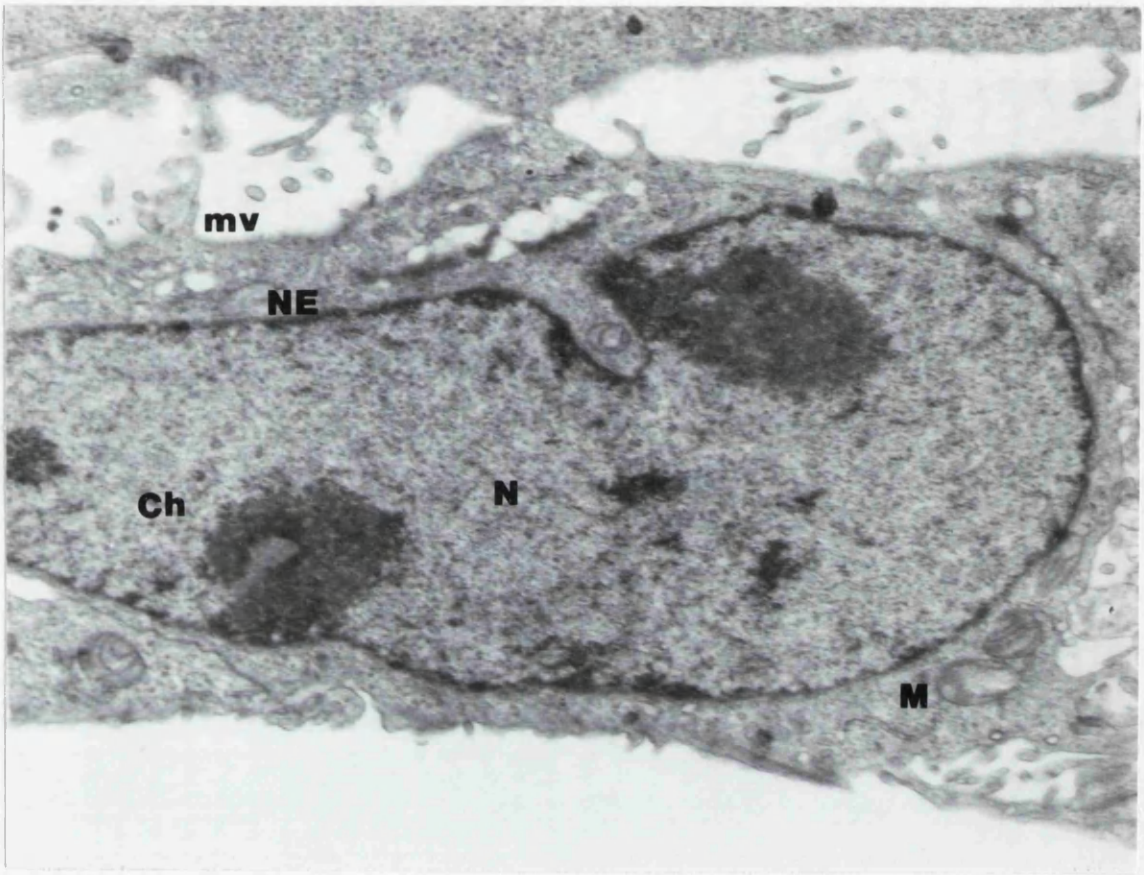
R - ribosomes

cr - cristae

> - pore complex

Fig. 3.25 shows an area to the left of the nucleus of the cell shown in Fig. 3.24 at a higher magnification. Inspection of the nuclear envelope region reveals nuclear pores and chromatin. The Golgi complex, mitochondria (with internal cristae), endoplasmic reticulum and ribosomes are also clearly visible, and their appearance is typical of the same organelles found in many other eukaryotic cells.

Figure 3.26 Electron micrograph of a KB cell following incubation with 0.0044 μM bruceantin for 48 hours (x 15 000)



mv - microvilli

N - nucleus

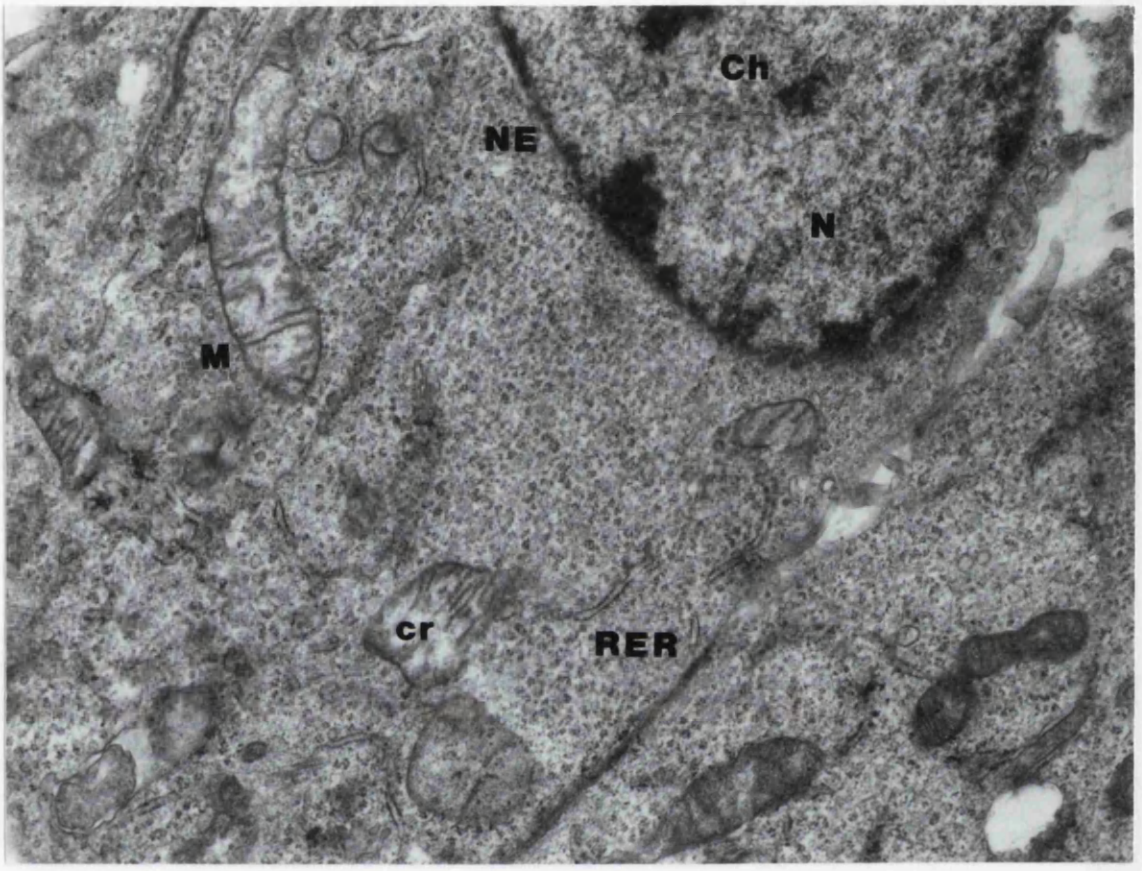
Ch - chromatin

M - mitochondria

NE - nuclear envelope

Fig.3.26 shows a KB cell after incubation with bruceantin at a concentration equivalent to the previously determined IC_{25} value against KB cells (0.0044 μM). The nuclear envelope shows a prominent invagination and the chromatin appears to have separated into discrete masses which have dispersed to the periphery of the nucleus.

Figure 3.27 Electron micrograph of a KB cell following a 48 hour incubation with 0.0044 μM bruceantin (x 20 000)



N - nucleus

NE - nuclear envelope

Ch - chromatin

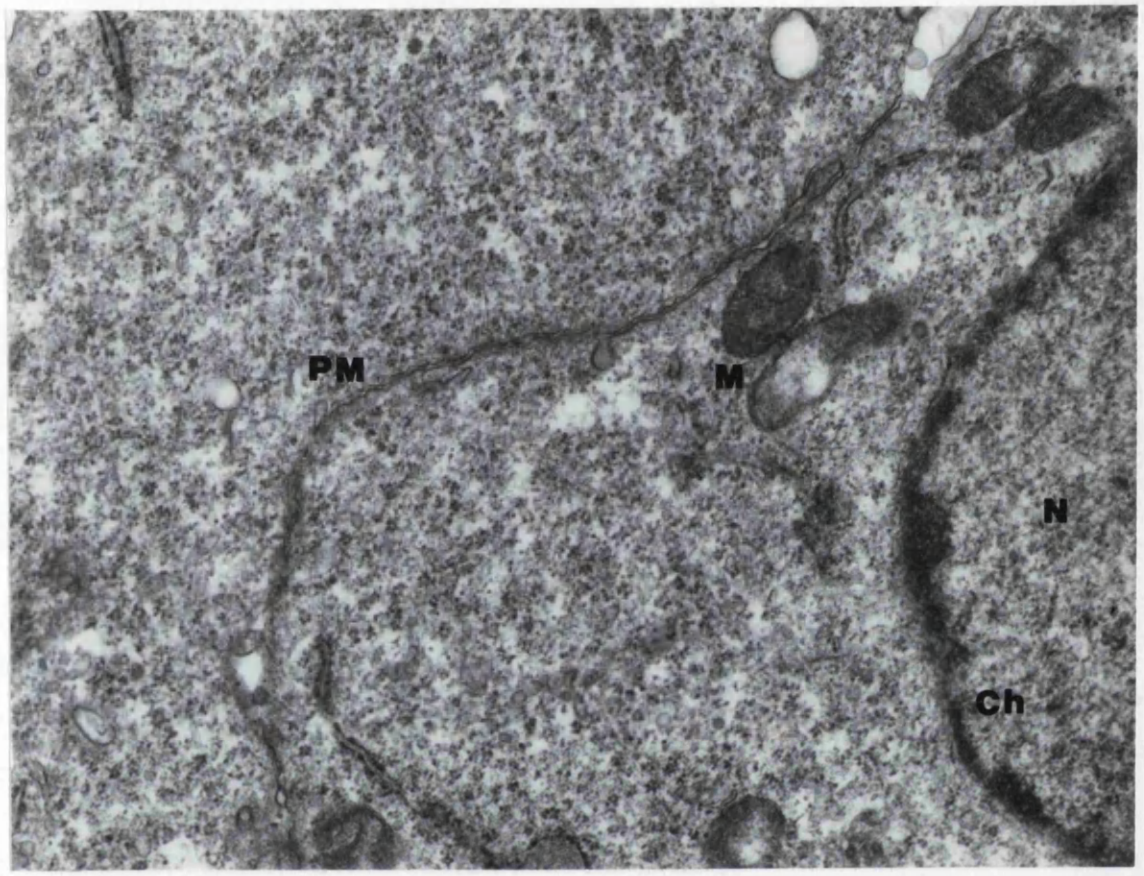
M - mitochondria

RER - rough endoplasmic reticulum

cr - cristae

In this KB cell treated with 0.0044 μM of bruceantin for 48 hours the mitochondria are swollen and the internal cristae are severely disrupted. Comparisons should be made with Fig. 3.25; both micrographs are taken at the same magnification, and the photographs are printed at the same size.

Figure 3.28 Electron micrograph of a KB cell following incubation with 0.0044 μM bruceantin for 48 hours (x 30 000)



N - nucleus

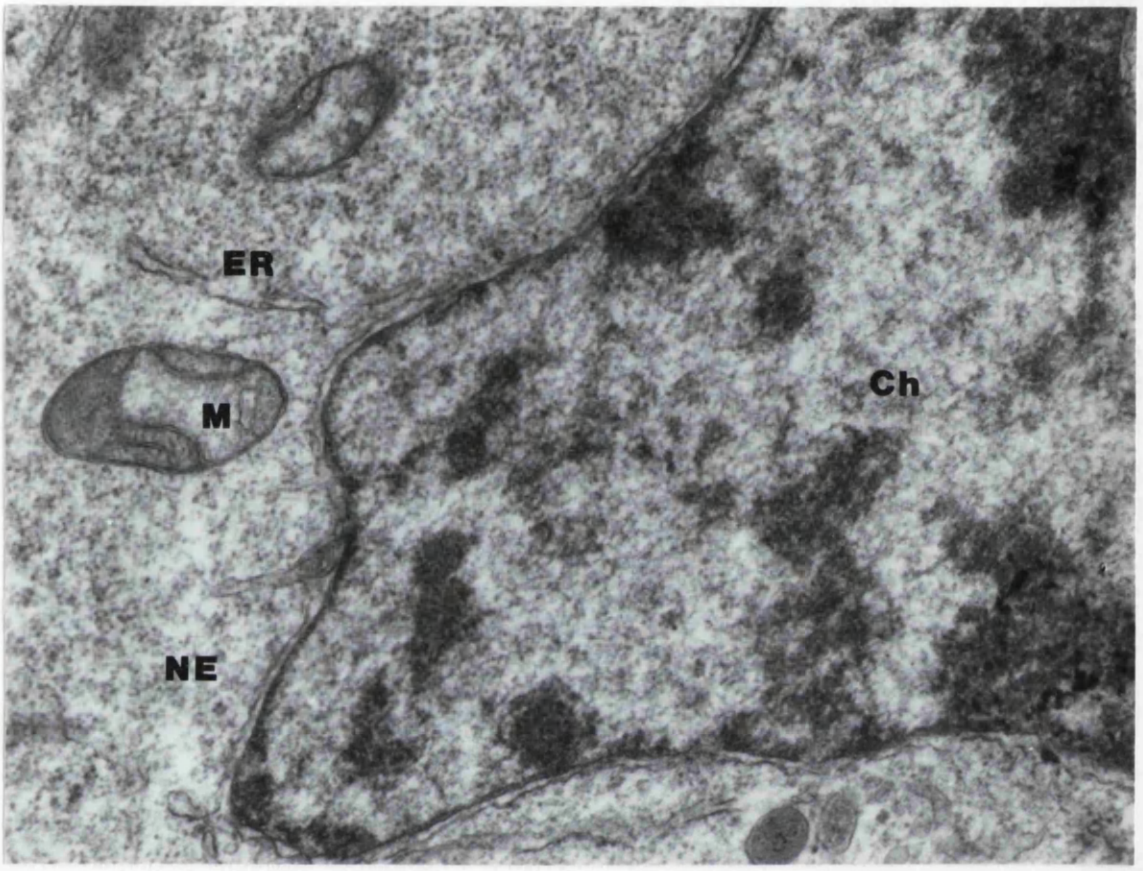
Ch - chromatin

M - mitochondria

PM - plasma membrane

After a 48 hour exposure to 0.0044 μM of bruceantin the structure of the mitochondria was clearly affected and the ribosomes became clumped in small masses. This photograph is included to demonstrate the second of these effects, although the abnormalities of the mitochondria are also seen.

Figure 3.29 Electron micrograph of a KB cell following incubation with 0.0044 μM bruceantin for 48 hours (x 40 000)



NE - nuclear envelope

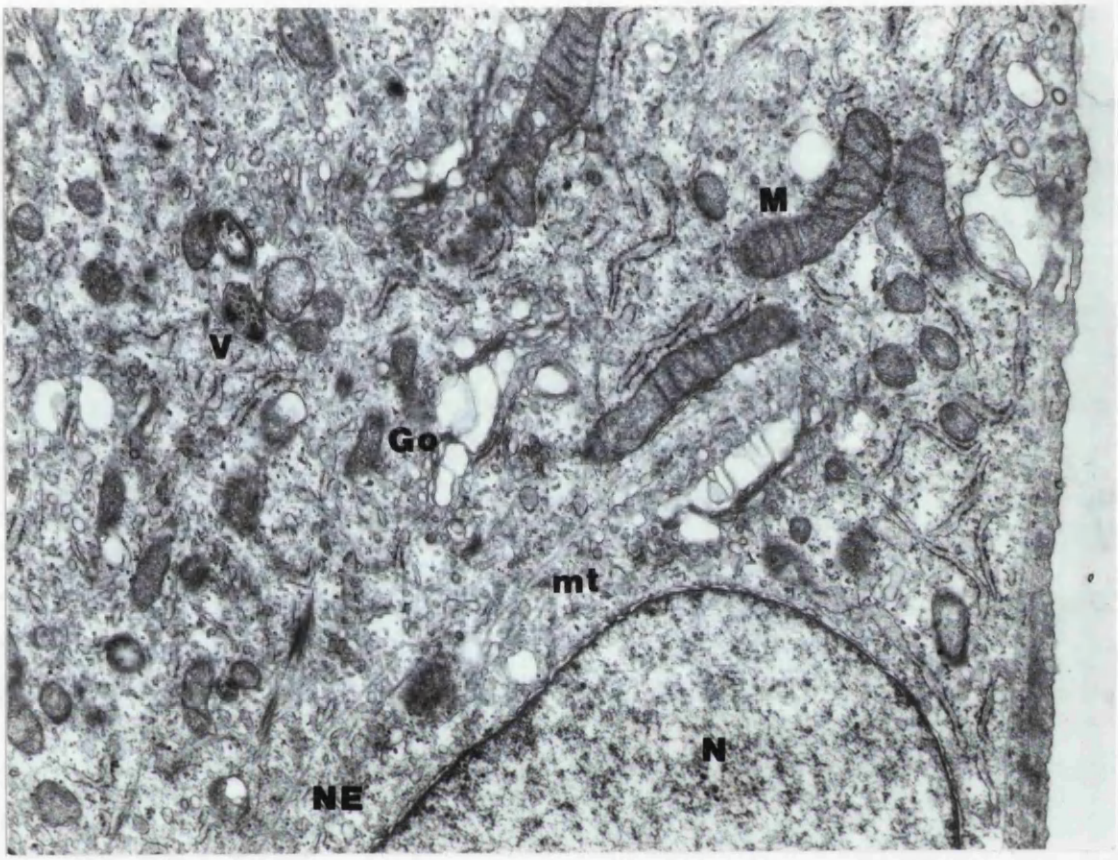
Ch - chromatin

M - mitochondria

ER - endoplasmic reticulum

The electron micrograph above shows the highly irregular nuclear envelope, the ribosomes are also depleted. It was commonly found that the nuclear envelope, in contrast with the smooth, regular pattern seen in Fig. 3.24. This type of irregularity is clearly featured in the above example.

Figure 3.30 Electron micrograph of a KB cell incubated with 0.075 μM brusatol for 48 hours (x 20 000)



N - nucleus

NE - nuclear envelope

M - mitochondria

Go - Golgi complex

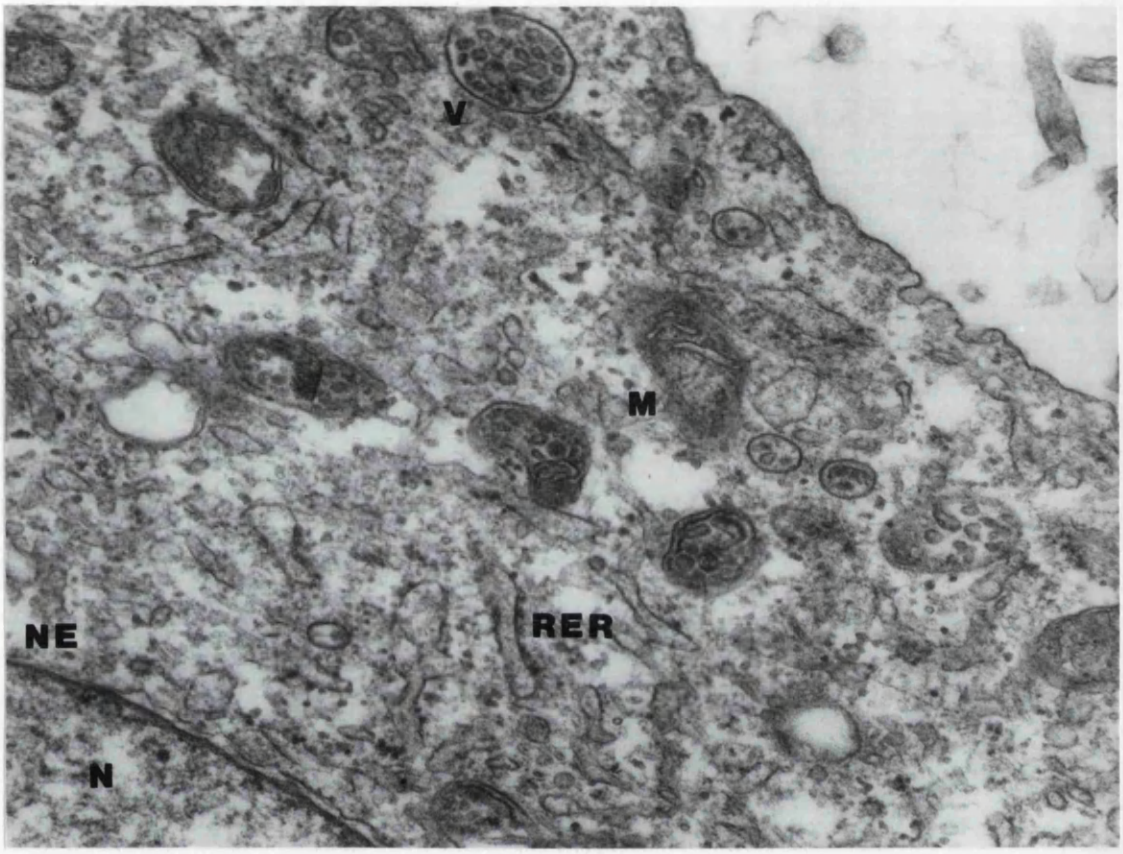
mt - microtubules

RER - rough endoplasmic reticulum

V - vesicles

In comparison with Fig. 3.25 the Golgi complex appears to be greatly swollen and numerous microtubules are also apparent, as well as aggregates of small vesicles.

Figure 3.31 Electron micrograph of a KB cell following incubation with 0.075 μM of brusatol for 48 hours (x 50 000)



N - nucleus

NE - nuclear envelope

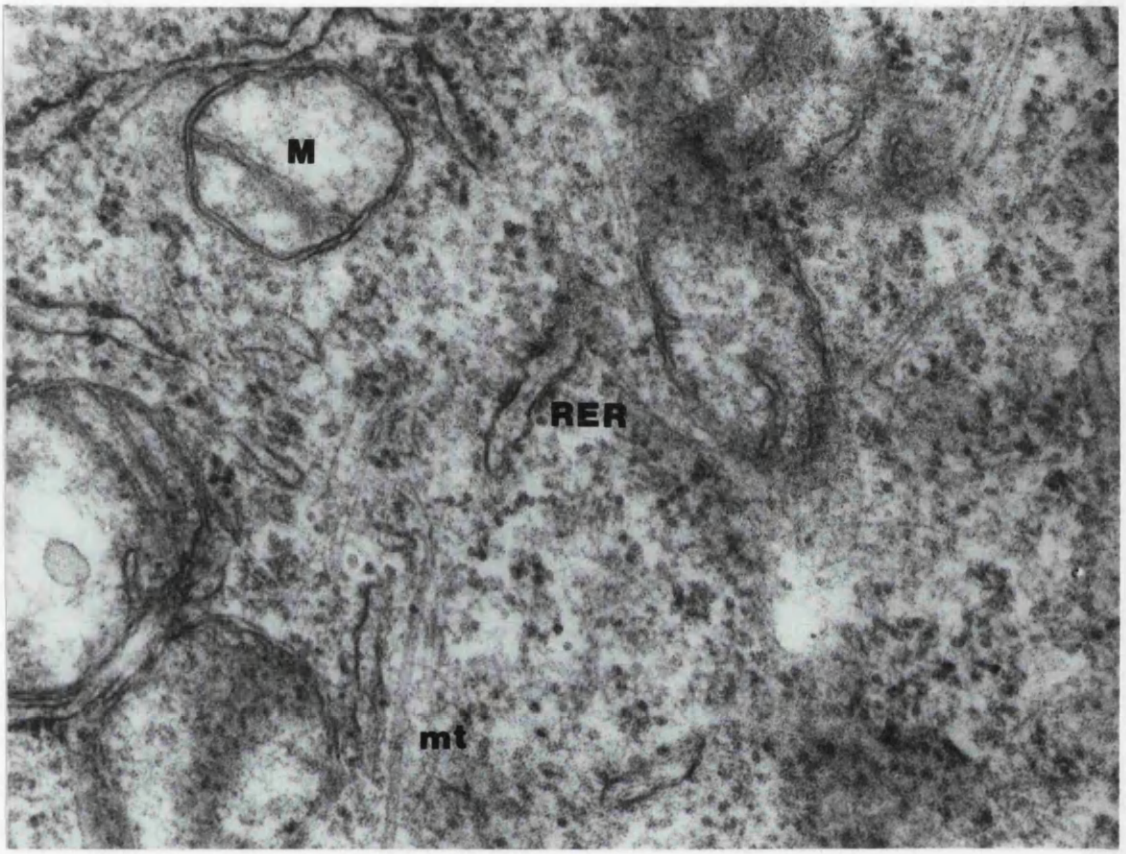
RER - rough endoplasmic reticulum

V - vesicles

M - mitochondria

This electron micrograph features a large number of vesicular bodies. A line of such structures appear horizontally towards the centre of the photograph.

Figure 3.32 Electron micrograph of a KB cell incubated for 48 hours with 0.075 μM brusatol (x 80 000)



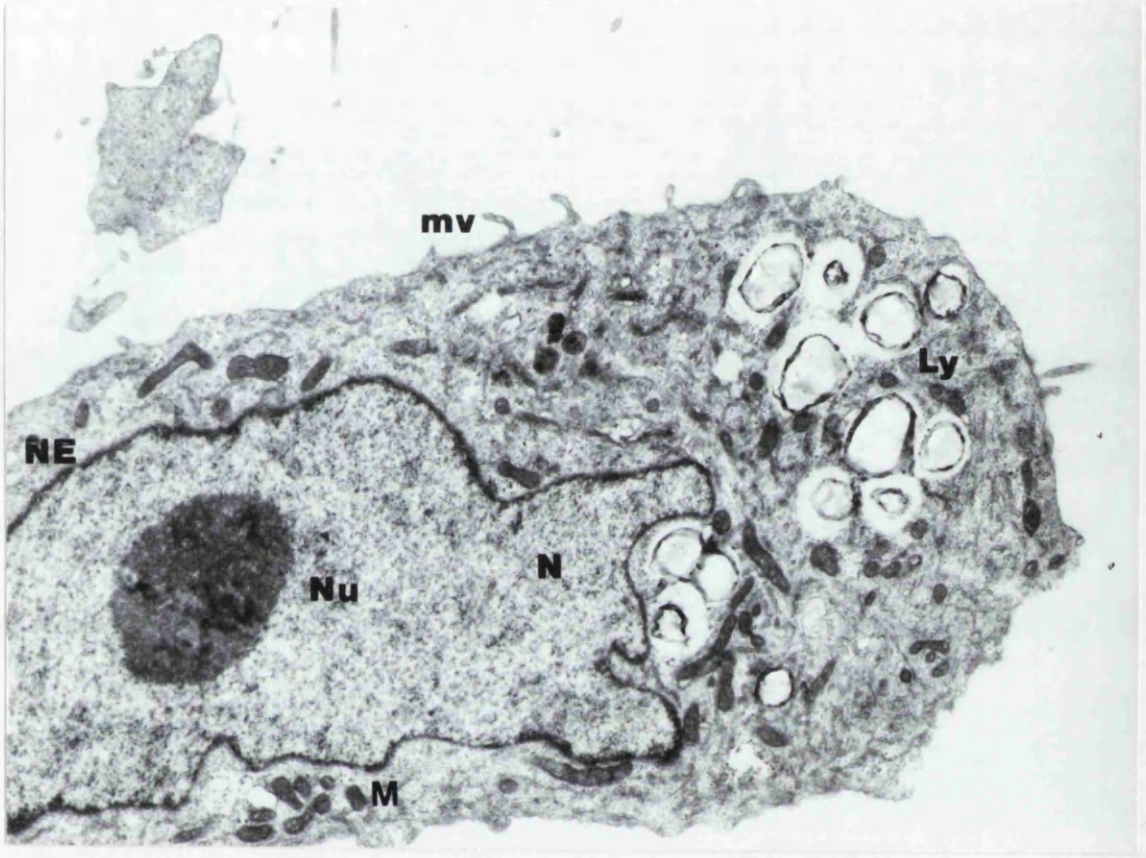
RER - rough endoplasmic reticulum

M - mitochondia

mt - microtubules

This photograph shows typical mitochondria with depleted internal cristae; ribosomal clumping is apparent and a microtubule is also demonstrated.

Figure 3.33 Electron micrograph of a KB cell incubated for 48 hours with 0.565 μM bruceine D (x 10 000)



N - nucleus

Nu - nucleolus

M - mitochondria

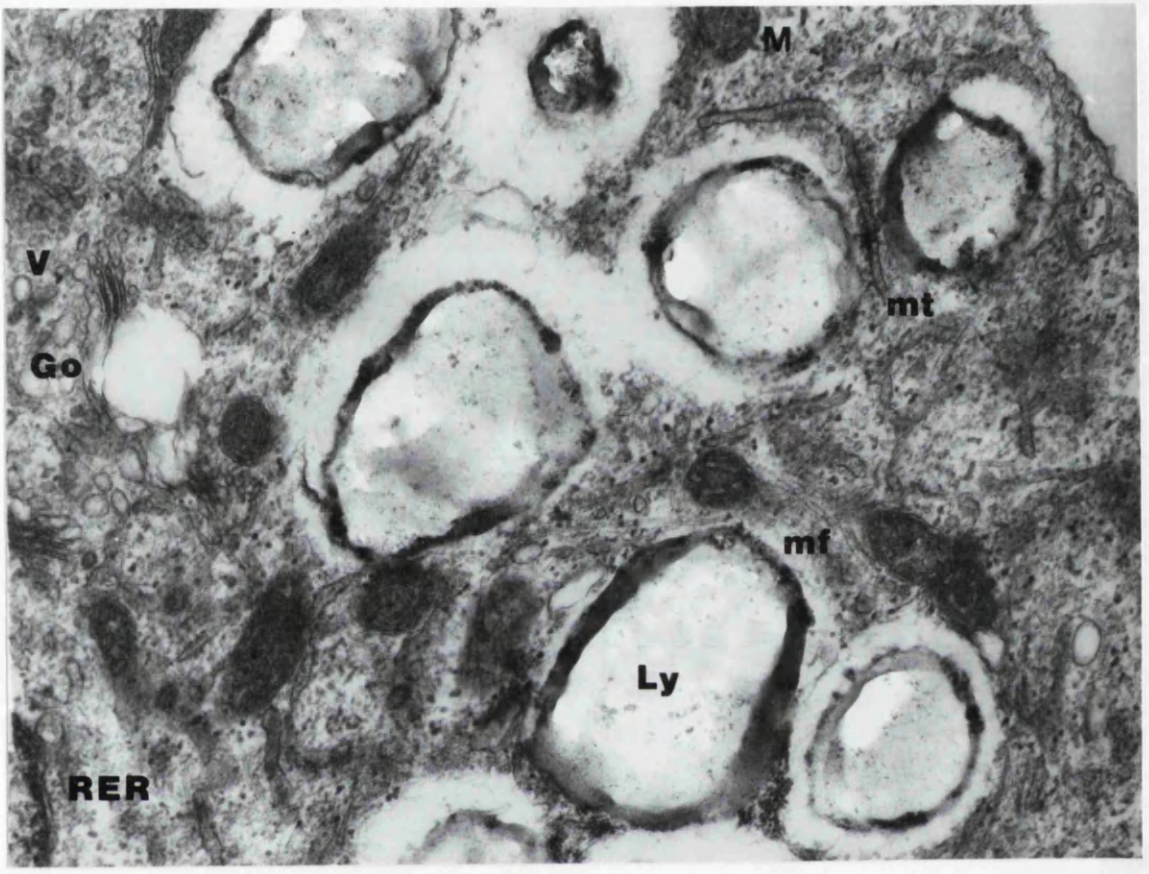
mv - microvilli

NE - nuclear envelope

Ly - lysosome

Bruceine D was the least toxic of the quassinoids included in the EM investigation. The nuclear envelope shows invagination (as in brusatol-treated cells), but there is also severe disruption of the cytoplasm surrounding several large lysosomes. This outstanding feature of lysosome aggregates was seen only in Bruceine D-treated preparations. Lysosomes were not prominent in control cells (Figs. 3.24 and 3.25)

Figure 3.34 Electron micrograph of a KB cell after 48 hour incubation with 0.565 μM bruceine D (x 40 000)



Go - Golgi complex

Ly - lysosome

M - mitochondria

mt - microtubules

V - vesicles

RER - rough endoplasmic reticulum

mf - microfilaments

This is an enlargement of the previous micrograph. Microtubules and smaller microfilaments surround the large lysosomes. The Golgi complex is also disrupted.

In the quassinoid-treated KB cells the appearance of many intracellular organelles is grossly disrupted when compared to control cells and to other normal eukaryotic cells. Although the plasma membrane remains intact, the nuclear membrane is seen to be very irregular with invaginations (Fig. 3.26), which is indicative of a cell under stress. When compared to the control, it can be seen from Fig. 3.27 that the number of organelles in this cell is reduced, the mitochondria present have lost internal cristae, the Golgi complex is not visible, the endoplasmic reticulum is less obvious and the ribosomes appear to be clumped.

Figures 3.30 to 3.32 inclusive show the EM results of brusatol-treated cultures. The effects appear to be less marked than those shown by bruceantin. The ribosomes are slightly depleted (this may result from clumping) and the mitochondria are condensed, although the cristae appear to be relatively normal. The most obvious effects are the 'blowing up' of the Golgi complex and the appearance of microtubules not obvious in control cells. Microtubules and microfilaments are protein-based structures (constructed from tubulin and actin respectively) which are closely involved in all eukaryotic cellular movements. They can act both separately and together. Microtubules are involved in the movement of flagella and cilia and microfilaments mediate intracellular motile processes, including cytoplasmic streaming and capping of particles embedded in or attached to the plasma membrane. Actin, in association with myosin, is part of the contractile protein complex of muscle cells. In addition, tubulin and actin coordinate closely in the division of animal cells, in which microtubules are associated with movement of chromosomes and microfilaments with the motile processes occurring during the division of the cytoplasm (Wolfe S. L. 1981).

It is also possible that vesicular bodies may be part of a pinocytotic process taking place in the stressed cell. Pinocytosis is a mechanism involving extensive rearrangements of the cell membrane by which molecules too large to be transported by active or passive transport can be taken into the cell. During pinocytosis, initially described by J. Edwards in 1925, the substance being taken up by the cell is bound to the plasma membrane in a mechanism involving receptor sites that "recognize" the substance and link specifically to it. The membrane region binding the substance then invaginates, producing a cup-shaped depression that deepens and pinches off forming an unattached vesicle that sinks into the underlying cytoplasm.

Smythies postulated in 1979 that some antitumour drugs including putative quassinoids and other antitumour agents may act as antagonists at certain prostoglandin receptors. For example, two bruceantin molecules may form a close interdigitating fit with the model receptor, with three hydrogen bonds linking each of the molecules to a phosphoserine portion of the receptor. Multiple van der Waals and lipophilic interactions would also occur. The entry of epidermal growth factor (EGF) into KB cells by such receptor-mediated endocytosis has been characterized by Willingham *et al*, 1984. It was shown that the EGF ligand is transported from the plasma membrane by endocytic vesicles termed receptosomes, delivered into the Golgi system, and ultimately transferred to lysosomes. Microtubules are also associated with this movement.

The results obtained for bruceine D are shown in Fig. 3.33 and 3.34. The microvilli around the cell are depleted and the nuclear envelope is very irregular. As seen with brusatol, microfilaments and

microtubules are also present in bruceine D-treated cells and are a further indication of a pinocytic process occurring in the cell. Osmium staining of lipid reveals a large number of lipid membrane bound lysosome-type structures. Lysosomes were first identified by Novikoff in 1961 and are a specialized class of secretion vesicles which contain a large number of hydrolytic enzymes. In general, hydrolysis reactions catalysed by lysosomal enzymes involve disassembly of macromolecules and polymers into their building block subunits (reviewed by Holtzman, 1976). Degradation within forms an important part of a cell's defense mechanism against toxic molecules. A number of quassinoids including bruceantin, bruceine D and brusatol (the most potent of the three) have been shown to demonstrate anti-inflammatory activity. One of the modes of action of quassinoids as anti-inflammatory agents is to stabilize lysosomal membranes, reducing the release of hydrolytic enzymes that cause damage to surrounding tissues (Hall *et al*, 1983).

It may appear from initial observation that the effects of the three quassinoids on the KB cells are rather different. However, some effects are consistent throughout: for example the increase in the number of mitochondria and the depletion of other organelles such as ribosomes. The differing observations may result from a time course effect, and reflect a difference in the speed of action due to permeability, or perhaps the cell's ability to isolate the drug within lysosomes or vesicles. The cells incubated with bruceine D and brusatol demonstrate an increase in the number of lysosomes and vesicles. The IC_{50} values for these two compounds against KB cells are also much higher than the value for bruceantin which does not show the same complement of lysosome and vesicular structures. A time course study is required to establish this theory further. Although this study is purely qualitative, it is possible to identify the Golgi complex, mitochondria and ribosomes as the main centres affected by the quassinoids, all of which are involved directly or indirectly with protein synthesis in the cell. Therefore the findings of this investigation are not inconsistent with work carried out previously (see section 3.3.9) which indicates that quassinoids exert their effects through the inhibition of protein synthesis. The presence of vesicles, lysosomes, microtubules and microfilaments may represent an increase in endocytosis occurring in response to, or as an effect of the treatment with the quassinoids.

CHAPTER FOUR — *IN-VITRO* AND *IN-VIVO* ANTIMALARIAL ACTIVITY OF CRUDE EXTRACTS FROM *BRUCEA* AND *SIMAROUBA* SPECIES

4.1 Introduction

Solvent extracts from the fruits of *S. amara S. glauca* and *B. javanica* fruits were tested for *in-vitro* antiplasmodial activity in an attampt to isolate and identify the constituents responsible for their reputed antimalarial activity. An aqueous infusion of *B. javanica* fruits prepared according to traditional methods and subjected to a series of chloroform extractions and hydrolyses (see section 2.1.4) was also tested. Several aqueous tea fractions were further screened for *in vivo* antimalarial activity.

This work was carried out in collaboration with Dr. G.C. Kirby and Dr. D.H. Bray in the Department of Medical Parasitology, at the London School of Hygiene and Tropical Medicine.

4.2 Materials and methods

4.2.1 *IN-VITRO* ANTIPLASMODIAL TEST SYSTEM

The test procedure is based on the method of Desjardins *et al.*, 1979 which has been published previously by O'Neill *et al.*, 1985, with modifications described by Ekong *et al.*, 1991. Cultures of *P. falciparum* (a chloroquine-resistant strain, K1, which originated from Thailand) were maintained *in-vitro* in human erythrocytes by a method described by Trager and Jensen, 1976 and later modified by Fairlamb *et al.*, 1985. The technique measures the incorporation of ^{3}H-hypoxanthine into drug-treated infected red blood cells compared with untreated infected red blood cells.

4.2.1.1 TEST PROTOCOL

Test compounds or crude extracts were dissolved in ethanol with the aid of mild sonication in a Sonicleaner bath (Ultrasonics Ltd.) and diluted with RPMI 1640 medium. The final concentration of ethanol was always less than 0.1% (i.e. a non-toxic level). Serial (2-,4-, and 10 fold) dilutions of test compounds or extracts were carried out in 96-well microtiter plates (Flow Labs. Inc. Ltd.). All tests were performed in duplicate and each well contained 50µL of drug solution in RPMI medium. To each well was added 50µL of human erythrocytes (A^{+}) , diluted to 5% haematocrit) with a 1% parasitaemia in complete medium (RPMI 1640 supplemented with D- glucose and 10% human A^{+} serum). Controls of uninfected red blood cells, infected red blood cells without drug and chloroquine as an internal standard were included in each experiment. After a 24 hour incubation at 37°C in a 3% oxygen, 4% carbon dioxide, 93% nitrogen

atmosphere, 5µL of ^{3}H-hypoxanthine (40µCi/mL ; Amersham) were added to each well and further incubation continued for a second 24 hours.

4.2.1.2 HARVESTING

This was achieved with the aid of a Cell Harvester (Skatron). Normal saline (0.9%) was used to wash the cells from the wells and onto a glass fibre membrane predampened with saline. The membrane was then flushed with distilled water for 12s to lyse the cells and to remove the remaining traces of haemoglobin and radiolabel not incorporated into nucleic acids. The glass fibre discs corresponding to each well were separated from the membrane and pushed into 4mL polypropylene scintillation vials. Finally, the discs in each vial were immersed in 4mL of scintillation fluid (Ecoscint; National Diagnostics) and the counts per minute determined for one-two minutes at about 30% efficiency.

4.2.1.3 ANALYSIS OF RESULTS

The percentage inhibition of uptake of ^{3}H-hypoxanthine at each dose level of drug or extract was calculated from the following equation :

$$\%Inhibition = 100 - \frac{(dpm\ infected\ blood + drug) - (dpm\ uninfected\ blood)}{(dpm\ infected\ blood) - (dpm\ uninfected\ blood)} \times 100$$

Linear regression analysis was used to interpret concentration versus % inhibition curves and from this the IC_{50} values with 95% confidence limits were determined.

4.2.2 *IN-VIVO* TESTING FOR ANTIMALARIAL ACTIVITY

The *in-vivo* test used was a four day suppressive test against *Plasmodium berghei*-infected mice (as described by Peters, 1984) and was carried out at the London School of Hygiene and Tropical Medicine. The procedure involves a comparison of the suppression of blood parasitaemia in drug-treated mice compared with untreated mice.

4.2.2.1 TEST PROTOCOL

DAY 0: The mice were inoculated i.v. in the tail with 0.2 mL of infected blood from a donor mouse, diluted in calf serum-Ringer solution, such that each 0.2 mL contained 10^{7} parasites. After 3 hours the mice were given an oral dose of drug micronised in distilled H_2O and 5% polyvinylpyrollidone (PVP).

DAYS 2-3: The mice were given the same dose of drug every 24 hours.

DAY 4: Blood smears were taken from the tip of the tail and the animals were killed with chloroform. Parasitaemia was determined microscopically from blood smears (fixed with methanol and stained with 10% Geimsa for 30 - 40 mins).

Controls: Mice dosed orally with H_2O.

Drug Doses: To obtain ED_{50} and/or ED_{90} values 5 concentrations of drug were tested, each using 5 mice. Each drug was diluted sequentially by a factor of three. Any deaths occurring during the course of the experiments were assumed to result from the toxicity of the drug or extract.

4.3 Results and Discussion

4.3.1 *IN-VITRO* ANTIPLASMODIAL ACTIVITIES OF *S. AMARA* AND *S. GLAUCA* FRUITS

Simarouba amara and *Simarouba glauca* fruits were extracted using the scheme described previously in section 2.1.3. Activities against *P. falciparum* (K1) are shown in Table 4.1.

Table 4.1 *In vitro* Activities of Crude Extracts from *S. amara* and *S. glauca* fruits against *P. falciparum* (K1)

Extract	*S. amara*	*S. glauca*
	P. falciparum[a] I.C.$_{50}$ µg/mL	***P. falciparum***[a] I.C.$_{50}$ µg/mL
Petroleum ether	>125	>500
Methanol	4.47	1.95
Aqueous fraction 1	1.65	17.13
Chloroform	0.151	0.49
Aqueous fraction 2	2.57	46.4
Butanol	9.95	4.88
Aqueous tea	29.1	N.R.

a Based on 2-fold dilutions in duplicate. N.R. No result.

In Panama *S. amara* fruits are used in traditional medicine for the treatment of malaria. Extracts from the fruits have previously been found to be active against avian malarias (Spencer *et al*, 1947), and also against *P. falciparum in vitro* and *P. berghei* in mice (O'Neill *et al*, 1988). During the latter study 4 active quassinoids were isolated from the chloroform extract and identified as ailanthone, 2'-

acetylglaucarubinone, holacanthone and glaucarubinone, having IC_{50} values between 4 and 9 ng/mL. Comparing the *in vitro* results of the present study with those reported by O'Neill *et al.*, 1988, the chloroform and methanol extracts are ten times less active than previously shown. Subsequent phytochemical investigation of the chloroform extract used in these experiments consistently failed to identify the 4 highly active quassinoids isolated earlier. This may help to explain why the ten fold increase *in vitro* antiplasmodial activity observed for the chloroform extract is 10 times less than previously reported. Conversely, the more polar aqueous extracts were approximately ten times more active than the previous findings of O'Neill *et al* and thus warrant further investigation. The aqueous tea extract of *S. amara* fruits showed relatively low activity against *P. falciparum* with an IC value of only 29 µg/mL.

S. glauca is a related species traditionally used to treat malaria in Central America. The seeds of the plant are also reputed to have amoebicidal properties (Cuckler *et al*, 1958). The greatest activity was to be found in the methanol and chloroform (IC_{50} 1.95 and 0.49µg/mL respectively) extracts of the fruits, indicating the presence of active lipophilic constituents. This suggests that there is some basis in the traditional use of the fruits for the treatment of malaria.

Two major compounds previously extracted from the seeds of *S. glauca* with hot water are glaucarubin and glaucarubinone (Hamm *et al*, 1954 and Polonsky *et al*, 1964). Two rare quassinoid glycosides (15-O-β-D-glucopyranosyl glaucarubolone and 15-O-β[B-D-glucopyranosyl glaucarubol) have also been isolated from the seeds, and were found to be active in the murine P-388 lymphocytic leukemia test system at 2 mg/kg/day (Bhatnagar *et al*, 1984). In addition to antileukemic activity, glaucarubin and glaucarubinone have been shown to possess *in vitro* antiplasmodial activity against *P. falciparum* (IC_{50} 0.055 µg/mL and 0.004 µg/mL respectively, Chan *et al*, 1985), *in vitro* cytotoxic activity against KB cells (ED_{50} 5.1 µg/mL and 0.04 µg/mL respectively) and *in vitro* antiamoebic activity against *E. histolytica* 1.57 µg/mL and 0.14 µg/mL respectively). In this study glaucarubinone was found to be three times less active against KB with an ED_{50} value of 0.113 µg/mL. Phytochemical investigation of the *S. glauca* fruits failed to isolate any of the compounds identified previously, or to identify the constituents responsible for the antiplasmodial activity shown in the methanol and chloroform extracts. Obviously it is not possible to attribute the antimalarial activity of the present extracts of *S. amara* and *S. glauca* to particular quassinoids previously identified. The possibility of the presence of other antimalarial compounds and their identity, or synergism between compounds present in the plant material remains to be investigated.

4.3.2 *IN VITRO* ANTIPLASMODIAL ACTIVITY OF *B. JAVANICA* FRUITS

An aqueous tea prepared from *B. javanica* fruits, as described in section 2.1.3, was subjected to solvent partition and to hydrolysis as outlined in Fig. 4.1. The extracts obtained were tested against *P. falciparum* (K1) *in vitro*, and the activities are shown in Fig. 4.1 and also in Table 4.2 (together with the cytotoxicity against KB cells (reported in section 3.36) for comparison).

The initial aqueous tea had an *in vitro* IC_{50} of 0.43 µg/mL. When the tea was subsequently partitioned between chloroform and water, the chloroform phase, having an IC_{50} of 5.5×10^{-3} µg/mL, was

Table 4.2 ***In vitro*** **Activities of Crude and Aqueous Tea Extracts from** ***B. javanica*** **Fruits Against** ***P. falciparum*****, with** ***In vitro*** **Cytotoxic Activity for Comparison**

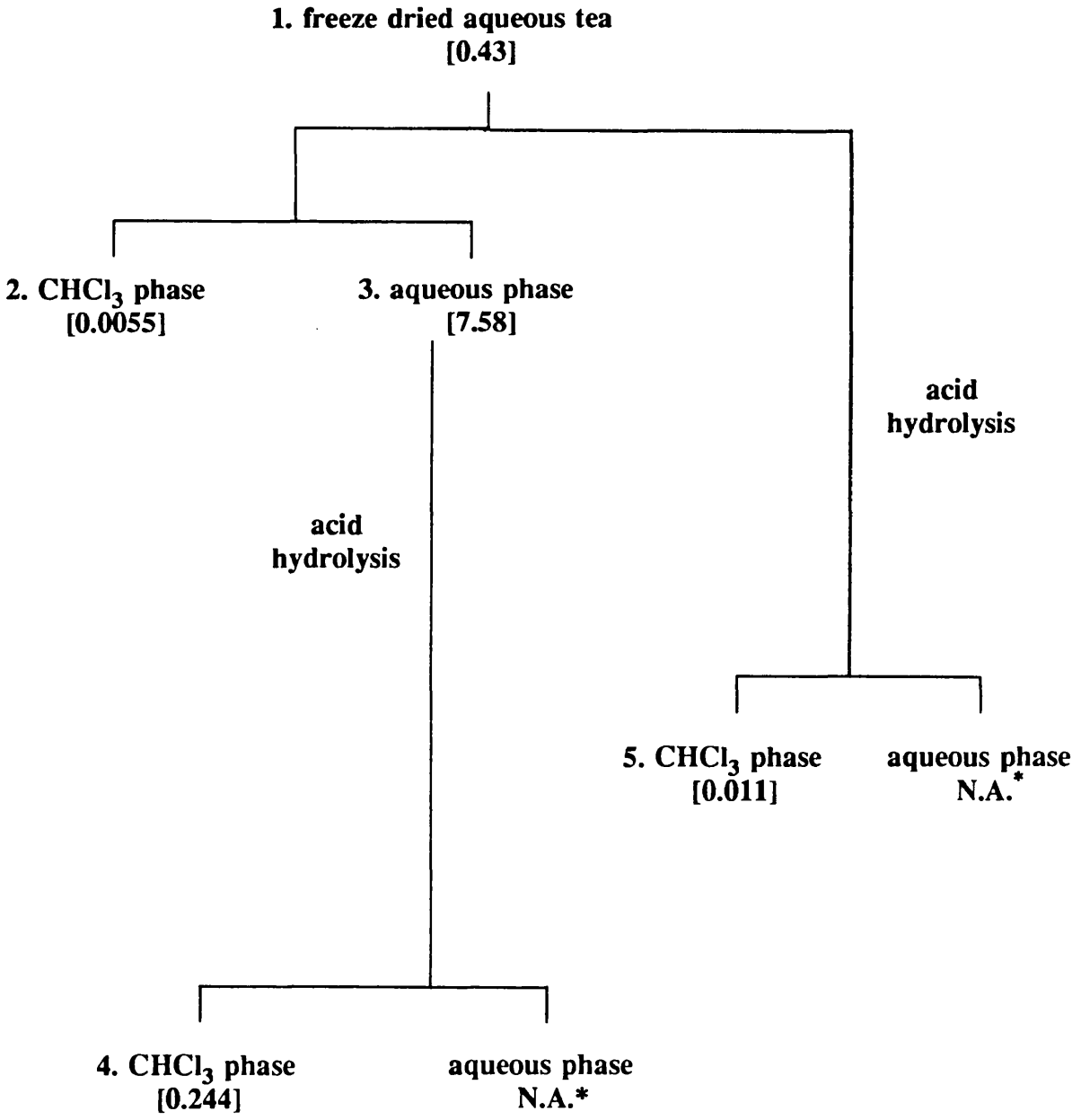
Extract	A	B	A/B
	KB Cells ED_{50} µg/mL	*P. falciparum* IC_{50} µg/mL	
Aqueous tea	14.3	0.43	33
Aqueous tea after $CHCl_3$ extraction	>250	7.58	-
Chloroform extract of Aq. tea	0.394	0.006	66
Hydrolysed aqueous tea	1.07	0.011	97
Hexane	>250	50[a]	-
Methanol	3.05	0.5[a]	6.1
Chloroform	1.69	0.5[a]	3.9
Aqueous 1	17.1	0.5[a]	34
Aqueous 2	46	50[a]	0.93
Butanol	10.9	0.5[a]	22

a O'Neill *et al*, 1987.

shown to be much more active than the aqueous phase which had an IC_{50} of 7.58 µg/mL. Thus it appears that the *in vitro* antiplasmodial activity of the aqueous tea is due principally to the presence of highly active chloroform-soluble substances, 'diluted' with relatively inactive polar quassinoids and other unknown constituents.

When the aqueous phase was treated with acid, a further supply of chloroform-soluble material was released. This was also shown to be active though less so at 0.244 µg/mL, than the initial chloroform extract. A number of lipophilic quassinoids were isolated from the active chloroform-soluble phases, both before and after hydrolysis. Bruceines A, B and C and brusatol were identified by comparison of their spectroscopic characteristics with those of reference compounds and literature values (see Chapter 2.2.1 and Fig. 4.2.5). These 4 quassinoids have previously been found to have very high *in vitro* antiplasmodial activities ranging from 0.003 µg/mL for brusatol, the most active to 0.011 µg/mL for bruceines A and B, the least active (O'Neill *et al*, 1987). The results of O'Neill *et al.*, 1987 are of the same order as those obtained in the present study for the chloroform extract of the aqueous tea and the hydrolysed aqueous tea shown in Table 4.2.

Figure 4.1 ***In vitro*** **Activities of Tea Extracts -** ***B. javanica*** **fruits**



[ ] IC_{50} μg/mL *P. falciparum* (K1)

* N.A. Not Active

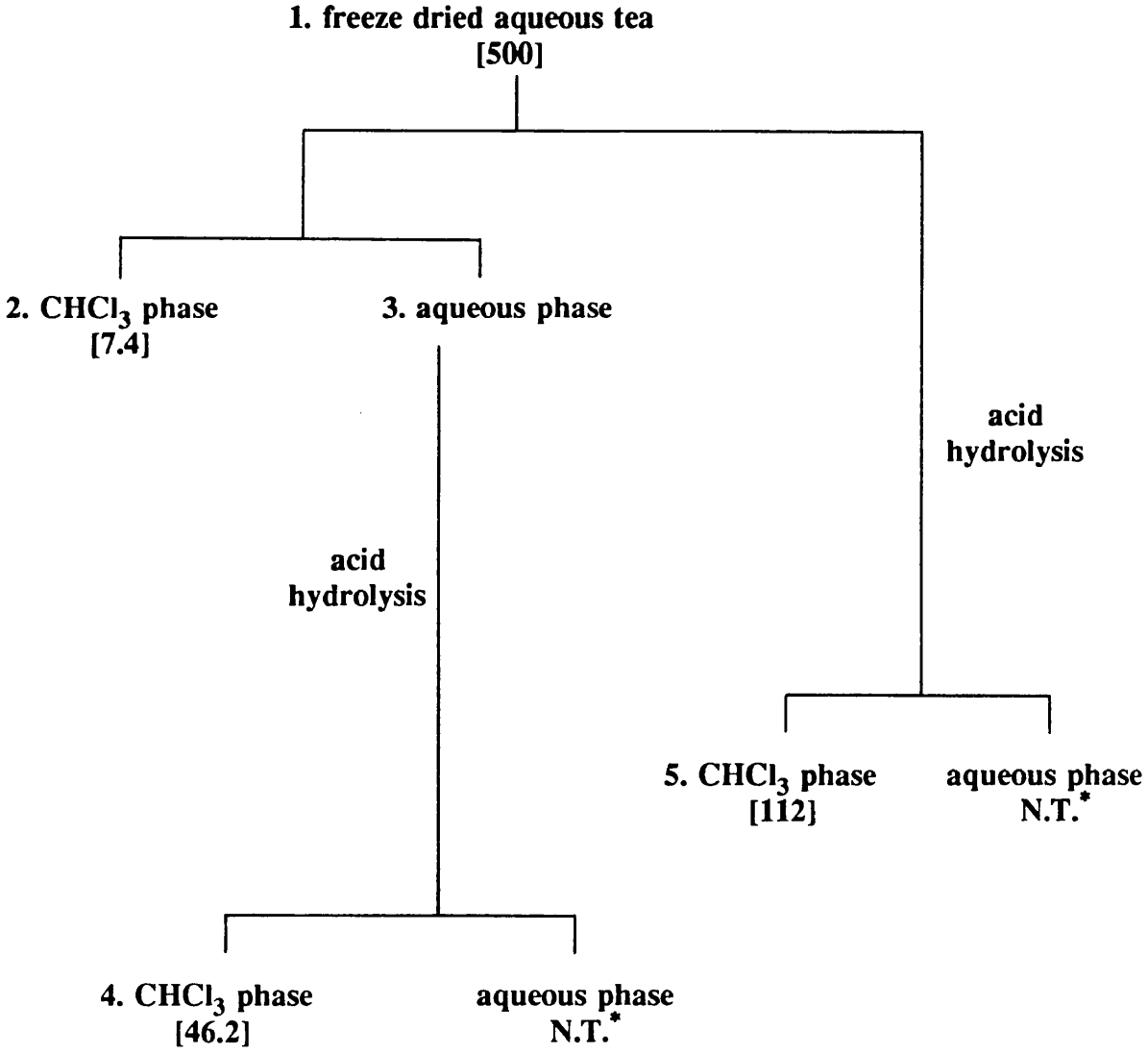
The inactive polar forms of the quassinoids which release active lipophilic quassinoids after acid hydrolysis have been found to be glycosidic forms (O'Neill *et al.*, 1987). Other workers have described the isolation and structural characterization of 13 glycosidic quassinoids, yadanziosides A - J, N and O from *B. javanica* fruits (Sakaki *et al*, 1984, Yoshimura *et al*, 1985, and Sakaki *et al*, 1986). Yadanziosides C, F and I are three glycosidic forms of bruceines B and C which were isolated during purification of active fractions from the polar n-butanol extract of *B. javanica* fruits (O'Neill *et al*, 1987). In that same study, *in vitro* testing against *P. falciparum* found the quassinoid glycosides, yadanzioside F and I to be much less active than the lipophilic chloroform-soluble quassinoids, having IC_{50} values of 5 µg /mL and 22.04 µg/mL respectively. For example the 3-O-glycoside yadanzioside I, was over 2000 times less active than its aglycone, bruceine B. Therefore, it seems likely that the *in vitro* antiplasmodial activity of the aqueous tea extract from *B. javanica* fruits could be is attributable to the highly active non-glycosidic quassinoids

In Table 4.2 the cytotoxicity against KB cells of crude and aqueous tea extracts from *B. javanica* fruits has been compared to the antiplasmodial activity. The ratio of these 2 values is taken as a measure of selective antiplasmodial activity. A value greater than one is indicative of greater activity against plasmodia. The 3 aqueous tea extracts show very favourable ratios between 33 and 97. It was, therefore, decided to monitor these extracts for *in vivo* antiplasmodial activity.

4.3.3 *IN VIVO* ANTIPLASMODIAL ACTIVITY OF AQUEOUS TEA EXTRACTS FROM *B. JAVANICA*

A large scale preparation and extraction was carried out as described in section 2.1.3 in order to obtain sufficient quantities of tea extracts for *in vivo* testing.

Figure 4.2 *In vivo* Activities of Aqueous Tea Extracts from *B. javanica* Fruits



[ ] ED_{50} mg/kg/day against *P. berghei* (N strain)

* N.T. Not tested - considerable degradation; not active *in vitro*

The original aqueous tea (top dose 4000 mg/kg/day), had an ED_{50} value of 500 mg/kg/day. After partitioning between chloroform and water the chloroform phase (2) (top dose 847.3 mg/kg/day) showed the greatest activity of the extracts tested with an ED_{50} of 7.4 mg/kg/day, comparable to the results obtained for pure quassinoids. Brusatol, for example, had an ED_{50} value of 1.27 mg/kg/day (O'Neill *et al.*, 1987). The aqueous phase was not tested for *in vivo* activity having previously been shown to be inactive *in vitro*. The chloroform extract (5) (top dose 600 mg/kg/day) produced after hydrolysis had an ED_{50} value of 112 mg/kg/day. However, acid hdrolysis of the of aqueous phase (3) yielded a further quantity of chloroform soluble material (4) (top dose 283.5 mg/kg/day) which was found to have greater activity than extract (5) with an ED value of 46.2 mg/kg/day.

The toxicity parameter used for studies in mice is LD_{50} (dose per unit body weight of test mice which results statistically in an average lethality of 50%). The procedure to determine the median lethal dose was derived by Trevan in 1927, who simply plotted the doses used in groups of animals against the percentage of mortality. Today LD_{50} numbers are used to classify chemicals by extent and kind of danger to human health. Lethality, however, is only one of the many possible signs of acute toxicity, and a difference in LD_{50} by a factor of two, for instance, may be of no relevance for hazard or risk assessment (Goldberg, 1984). Table 4.3 shows the Home Office classification of toxicity according to LD_{50}.

Table 4.3. Home Office Classification of Toxicity (Pascoe D., 1983)

LD_{50} (mg/kg)	Classification
< 5	Super-toxic
5 - 50	Extremely toxic
50 - 500	Very toxic
500 - 5000	Moderately toxic
5000 - 15000	Slightly toxic
>15000	Practically non-toxic

In terms of lethality it is the pure quassinoid brusatol which demonstrates the greatest toxicity; 4 out of 5 deaths occurring at 3 mg/kg/day. Indeed the LD_{50} value of 3.4 mg/kg/day for brusatol (i.e. 'super-toxic') is less than three times its ED_{50} value (1.27 mg/kg/day). This comparison is depicted in the therapeutic ratio (A/B) shown in Table 5.4. Chloroquine diphosphate (LD_{50} 79 mg/kg/day), the chloroform extract of the aqueous tea (2) (LD_{50} 54.4 mg/kg/day) and the hydrolysed extract of the aqueous tea (4) (LD_{50} 283 mg/kg/day) fall into the 'very toxic' category. However,the correspondingly low ED_{50} value for chloroquine diphosphate (1.52 mg/kg/day) results in the highest (and therefore most favourable) therapeutic ratio of 52 (see Table 5.4). The least toxic of the agents tested which falls into the 'moderately' toxic

category (with a LD_{50} value of 2400 mg/kg/day) is the initial freeze dried aqueous tea. It is also worth noting that no deaths were found to occur at the dose concentration corresponding to the tea's ED_{50} value (500 mg/kg/day). The tea has been used for several centuries in China for the treatment of malaria and there have been no reported deaths (Chang and But., 1989) and the findings of the present study provide further evidence of the efficacy of this traditional remedy. The *in vitro* cytotoxic and antiplasmodial activities of the aqueous tea, its chloroform extract, brusatol and chloroquine diphosphate are compared together with their *in vivo* activity against *P. berghei* in mice in Chapter 5 - Table 5.4.

CHAPTER FIVE — DISCUSSION

5.1 DEVELOPMENT OF A NEW MICRODILUTION ASSAY FOR THE ASSESSMENT OF *IN VITRO* CYTOTOXICITY

Previous KB assays (Geran *et al*, 1972 and Wall *et al*, 1987) have necessitated the use of milligram to gram quantities of test substances, together with the accompanying large volumes of culture medium, and amounts of reagents. Hence the cost in terms of time, resources and test compounds can be extremely high. The aim of the author was to establish KB cell growth in 96-well microtitre plates in an attempt to replicate, as far as possible, the experimental conditions of the *in vitro* antiplasmodial test system using *P. falciparum* (K1). This would enable a direct comparison of antiplasmodial and cytotoxic activity to be made and would simultaneously allow the use of microgram quantities of test substances, as well as being far less demanding on expensive tissue culture reagents and equipment. Previously published KB assays are based on the Lowry method of protein determination for the measurement of cell growth, employing a phenol reagent (Folin-Ciocalteau) for colour development. Such colorimetric methods can be preferable to the laborious procedure of actual cell enumeration, or to the use of radiolabelled compounds. However, it was not clear if a colorimetric procedure could be adapted for the growth of a monolayer culture in microtitre trays as desired here.

A 48 hour assay was considered appropriate (rather than the 72 hour protocols of Geran or Wall) to enable a direct comparison with the 48 hour *in vitro* antimalarial screen, which would also allow several tests to be performed per week. The KB cell line maintains a generation time in the logarithmic growth phase of approximately 30 hours, and thus at least one full cell cycle would occur in the 48 hour time span. During a 72 hour regime, the cells would be exposed for at least 2 cycles. Not surprisingly, therefore, an increase in the duration of drug exposure to the KB cells as in the 72 hour test described in section 3.3.8 was seen to have a profound effect on the ED_{50} value of each drug. This is consistent with previous studies. Freshney, for example, carried out work in 1976 with HeLa cells and found several compounds, including 6-mercaptopurine and methotrexate, to show increasing toxicity, and also a change in their relative toxicities with time. In the present investigation a comparison of the 48 and 72 hour tests showed that all drugs tested in both assays showed an increasing effect with time. In contrast to Freshney's work, there was little effect in the rank order of the ED_{50} values of the different drugs. It may, however, be of no value to know what happens when a cell line is exposed to a drug in culture for 3 days instead of 2 if, owing to pharmacokinetic factors, the drug remains available in the *in vivo* situation for only a few hours! Indeed, the duration of exposure to effective concentrations of drug is probably one of the major disparities between an *in vitro* technique and *in vivo* chemotherapy. Taking all these factors into consideration it was decided to persist with the 48 hour protocol.

Initial experiments were carried out to determine the inoculum size of KB cell suspension per well sufficient to provide confluency, but not overgrowth after 48 hours. Serial dilutions of a KB cell suspension in 100 µL volumes of Eagle's Minimum Essential Medium were carried out in 96-well microtitre plates. Placed in a modular incubator, the plates were gassed with 5% CO_2 in air and left for 48 hours at 37°C. The effect of the CO_2 on the medium bicarbonate buffer was critical for cell proliferation. The cells showed very slow growth in a red alkaline medium, whereas a yellow acid medium caused the cells rapidly to detach and to die. A 'tangerine' coloured medium (pH 7.3-7.4) was found to sustain maximum growth. Problems were initially encountered during the trypsinization of the KB cultures when exposure to the protease caused the cells to aggregate into clumps. However, if carried out at room temperature rather than at 37 °C, the process could be speeded up by several minutes, and gentle agitation of the cell suspension with a pipette was then sufficient to break up any cell aggregates.

The innoculum size chosen to achieve a confluent monolayer in the control wells after 48 hours was 2.5×10^4 cells, and this was reduced to 1.38×10^4 cells per well for the 72 hour test. Provided overgrowth had not occurred the cells were found to adhere quite strongly to the well bottoms and it was possible to remove the drug-containing medium merely by inverting the tray and tapping gently. The residual medium was removed by washing with 0.9% sodium chloride solution, which was used at 37°C to prevent detachment of the cells. The plates were left to dry in air prior to fixation for 10 mins with methanol and were again left to dry.

Lowry's reagent was used for the first attempt at staining but proved unsuitable because it was found also to stain the microtitre plates. Numerous washings with distilled water failed to remove the excess stain which in turn resulted in in high absorbance readings for the blank control wells containing no cells. The same problem was encountered using Coomassie blue. It was thought that water-soluble eosin might possibly be a suitable alternative as Wright and colleagues (1987) had successfully utilised this stain in an antiamœbic microdilution assay. An aqueous 0.5% eosin solution was tried, and it was found that any excess dye could be removed easily with just three washes of distilled water. A 200µL volume of a 0.1M sodium hydroxide solution was added to the individual wells in order to release the protein-bound dye. At least 20 minutes were allowed for this to occur, after which time the absorbance readings of the wells became stable. The optical densities at 490 nm of the resulting well solutions were recorded on a microplate reader.

A correlation obtained between cell number and optical density, together with reproducible ED_{50} values for cytotoxic control compounds (e.g. 6-mercaptopurine, cycloheximide and podophyllotoxin), served to validate the assay as a cytotoxic screen. In addition, a known antineoplastic agent, actinomycin D, was found to be very highly active against KB cells, whereas the antimalarials quinine and chloroquine, and the antibacterial agent chloramphenicol showed extremely low activity which demonstrates that the test is selective for compounds possessing different modes of cytotoxic activity. Hence the KB test can be said to meet the necessary requirements for a valid assay, including correlation and reproducibility.

5.2 THE MERITS AND LIMITATIONS OF THE KB ASSAY

The KB cell culture has been used since 1960 by the National Cancer Institute (NCI) as an antitumour assay for screening plant extracts, playing a powerful role in the discovery of antitumour agents from higher plants. Had KB alone been used as a preliminary screen, with *in vivo* screening limited to KB-active extracts, fractions or compounds, KB activity of crude products might have lead to the discovery of vinblastine, vincristine, podophyllotoxin (from which semi-synthetics have been derived) and all but one of the antitumour agents which underwent development for clinical evaluation up until the mid 1980's. These include bruceantin, bouvardin, ellipticine, camptothecin, homoharringtonine, taxol and thalicarpine. Indicine-N-oxide, however, would have been discovered only by *in-vivo* screening of crude plant products. In addition, KB cells are known not to be sensitive to tannins which can create false-positives against other cell lines, despite having no promise as useful anticancer drugs. The NCI KB assay was shown to be more sensitive to most antitumour agents than *in vivo* assays. This can be an advantage when screening plant products, since biologically active compounds are usually present in very small quantities and may go undetected in an *in vivo* screen. However, this sensitivity has led to the identification of too many false-positives, and the KB assay has emerged from NCI investigations as acceptible only for screening pure compounds available in amounts too small for *in vivo* assay, and for monitoring fractionation of natural products which are active *in vivo* as well as *in vitro*. Thus, the NCI now screen plant extracts for antitumour activity against KB in conjunction with slow growing human tumour cell lines *in vitro*, and more than 20 human tumour lines in athymic mice.

The use of KB cells during this study to monitor the cytotoxicity of potential antimalarials failed to detect any experimental agents which demonstrated selective activity against KB cells. However, the anti-cancer aspect of the KB test and the possibility of discovering a novel antitumour compound is always kept in mind, although it would be necessary to compare activity against KB cells with a non-cancerous cell line.

The KB microdilution assay described in this study incorporates the advantages and disadvantages inherent in any *in vitro* system, and these are equally applicable to the antiplasmodial test against *P. falciparum*. *In vitro* tests are simple and rapid methods, economising on animals and space, whilst at the same time allowing good control of experimental conditions. Certainly they are the only practical means of screening drugs which are available only in milligram quantities, a common occurrence when isolating active constituents from natural products, or resulting from the recently developed microtechniques for drug synthesis and analysis.

Nevertheless, *in vitro* tests also create problems of their own. Consider the use of human cancer cells in culture. KB cells, for instance, probably constitute only a small proportion of the cells present in the original biopsy sample, namely those which are particularly well-suited to the culture conditions used. Such cells may not be representative in their biochemistry of the malignant cells that composed the tumour *in situ* and, may therefore, differ in their chemosensitivity.

It has also become apparent in recent years that cultures can be subject to contamination by other cells. Indeed, there is evidence from chromosomal and isoenzyme studies that the KB cell culture has been contaminated by HeLa cells. The necessity for tests to ascertain that cell lines remain uncontaminated would possibly detract from the advantages of *in vitro* tests, whose simplicity is one reason why they are considered useful.

The cell kinetics of cells in culture or of transplanted animal tumours are often quite different from many human cancers, with a large proportion of cells in cycle with short generation times. However, in most human cancers *in situ*, intermitotic times are much longer and many cells are out of cycle. Differences in response to chemotherapy, even with similar cell lines, can be attributed to differences in cell kinetics and it has been demonstrated that some cells are sensitive to drugs only at particular stages of the cell cycle (Connors and Phillips, 1975). Also, the manner in which intact animals respond to any given pharmacological agent is highly variable. This may not necessarily be due to basic differences between cells of different species but may result from variations in absorption, distribution, metabolism and excretion of the drug. Rate of absorption, metabolism and excretion, protein binding and the occurrence of tissue depôts of the active drug will vary between different animal species. Clearly, these variables cannot be predicted when testing novel structures in the *in vitro* system.

Another very important factor is the host immune response. Sometimes the direct cytotoxicity of a drug can be relatively small, whilst stimulation of the immune response is large so that, *in vitro*, the effectiveness of the drug could be overlooked. In an *in vivo* model a drug may prove to be "effective", whilst in vitro it appears to be inactive.

A well-recognised advantage of *in vivo* tests is that, unlike many *in vitro* assays, they can detect agents which are themselves biologicaly inert and only act after some form of metabolism. In the case of the KB test, cytotoxicity will only be seen *in vitro* if this metabolism can be performed by the cells themselves; for example, conversion of purine and pyrimidine analogues to their nucleotides. However, there are many examples where the enzymatic activation takes place predominantly in the liver. Cyclophosphamide is considered to be one of the best alkylating agents in clinical use so far, with an *in vitro* ID_{90} value of 8000µg/mL; it is hardly cytotoxic compared with agents of the same type. On the otherhand, if incubated *in vitro* with liver microsomes and an NADPH generating system, the *in vitro* ED_{90} decreases to 1.82µg/mL. Hence, in order not to miss so called pro-drugs, a cell system is required which incorporates drug metabolising liver fractions in the presence of the drug under test. Although far more complex, such a system would prevent at least some of the false-negatives that would otherwise slip through *in vitro* systems (Connors and Phillips, 1975). An *in vitro* microassay system for the identification of antimalarials that act through their metabolites has been devised (Ramanaiah and Gajanana, 1985) using hepatic microsomes and standardised using proguanil hydrochloride: proguanil *per se* is inactive and its antimalarial activity is due to its main metabolite, cycloguanil.

For the reasons discussed above *in vitro* tests can scarcely approximate to the complexity of interactions that take place in a living animal. Nevertheless, the *in vitro* protocols already in existence are a legitimate complement to the current panoply of whole-animal procedures, reducing considerably the number of

animals that are subject to testing. The relative advantages and disadvantages of *in vitro* and *in vivo* bioassays are compared in Table 5.1.

Cytotoxicity testing represents only a very small proportion of the *in vitro* techniques in existence today. Literally, *in vitro* means "in glass", but scientists interpret the term more broadly to mean research that does not involve intact higher animals. *In vitro* testing includes a battery of living systems (e.g. bacteria, protozoa, fungi, molluscs), isolated subcellular systems (enzymes, receptors), and cultured cells (both animal and human, including fertilized eggs and embryos), all of which can be employed to evaluate the toxicity or biological activity of chemicals in humans. The issue of whole-animal testing has become more urgent and contentious in the past decade, and *in vitro* methodologies offer alternative strategies in the precision-monitoring of biological processes at the cellular, rather than at the organismal level.

Table 5.1 Comparison of *In Vitro* and *In Vivo* Bioassays for Assessing Biological Activities of Plant Extracts

-	*IN VIVO*	*IN VITRO*
ADVANTAGES	Influences of: uptake, kinetics, and metabolism	Speed Low cost Small sample size Whole-animal tests reduced
DISADVANTAGES	Long turn around Expensive Often less sensitive Large sample required Morally unacceptable	No uptake, kinetics and metabolism effects, therefore, activity may not correspond to *in vivo* activity

5.3 Therapeutic Ratio: *In Vitro* Cytotoxicity Against KB Cells versus *In Vitro* antimalarial Activity Against *P. falciparum* (K1)

It has been recognised for centuries that all chemicals are toxic. It was Paracelsus (1492-1541), who said: "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy." It follows, therefore, that it is the *degree* of toxicity and the *nature* of the toxicity of a compound which should be identified. In drug development it is the ratios between the *therapeutic* and the *toxic* doses, and between the *desired* and *unwanted* effects which are important.

In the present study, in order to obtain some estimation for the therapeutic index of the potential antimalarial agents investigated (i.e. selective antiprotozoal activity), the cytotoxicity to KB cells (A) has

been compared to antiplasmodial activity against *P. falciparum* (B), and a ratio (A/B) for the two calculated. A value greater than one is considered indicative of more selective activity against *Plasmodium*. Alternatively of course, it is possible to define the ratio in the opposite manner, (eg. B/A), in which case a ratio value less than one would then be indicative of antimalarial selectivity. To avoid confusion, it is necessary when using such a (derived) parameter to ensure that it is always correctly defined.

A number of known therapeutic and cytotoxic agents have been compared for their activities (see Table 5.2). The antimalarial drugs quinine, chloroquine and artemisinin all have very favourable ratios of 600, 347 and 1.39 x 10^4, respectively, which demonstrates the expected high selectivity for the malaria parasite. The ratio for chloroquine against a chloroquine-sensitive strain of malaria *in vitro* would be at least one order of magnitude (x10) greater. Conversely, the antineoplastic agents vincristine, vinblastine, 6-mercaptopurine and podophyllotoxin are selective for KB cells, all with ratios less than one (between $4x10^{-1}$ and $5x10^{-4}$). However, etoposide and teniposide, the two derivatives of podophyllotoxin, show much lower activity than the parent compound in both assays. Actinomycin D (an antibiotic and antineoplastic compound) and nigericin are two known cytotoxic agents which show high activity against KB cells, although *P. falciparum* demonstrates much greater sensitivity to the two drugs, and this is reflected especially in a ratio greater than 29 for actinomycin D. The cytotoxic agent cycloheximide is moderately toxic against KB cells, but is much more toxic against *P. falciparum*, hence producing a high A/B ratio of 61. The antiamœbic drug emetine hydrochloride has a ratio of 2.1 and as such is only twice as active against *P. falciparum* as against KB cells , illustrating the narrow margin between the therapeutic and toxic doses of this compound. Another therapeutic agent chloramphenicol, is an antibiotic produced by *Streptomyces venezuelae* which is effective against gram-positive bacteria but shows no significant cytotoxic or antimalarial activity in the two *in vitro* assays used in this study.

Table 5.2 *In Vitro* Therapeutic Ratios of Some Known Therapeutic and Cytotoxic Agents

Compound	A	B	A/B
	KB Cells ED_{50} μM	*P. falciparum* IC_{50} μM	
Actinomycin D	0.012	<0.0004[g]	>29
Artemisinin	194	0.014[b]	13900
Quinine	333	0.556[c]	600
Chloroquine diphosphate	153	0.441[d]	347
Chloroquine diphosphate	153	0.005*[f]	30600
Cycloheximide	3.31	0.054[f]	61
Cycloheximide	3.31	0.031*[f]	107
Emetine hydrochloride	0.673	0.325[e]	2.1
Etoposide	33.0	22.0[a]	1.5
6-Mercaptopurine	3.42	8.12[a]	0.42
Vinblastine sulphate	0.01	1.61[a]	0.006
Podophyllotoxin	0.007	13.2[a]	0.0005
Vincristine sulphate	0.004	1.61[a]	0.0005
Teniposide	148	>50.0[a]	<2.9
Nigericin	0.037	<0.1	>0.37
Chloramphenicol	>50	>50[a]	-

a Kirby G.C. unpublished result; b Kirby *et al.*, 1988; c O'Neill *et al.*, 1985; d O'Neill *et al.*, 1986; e Partridge S. unpublished result; f Ekong *et al.*, 1990; g Kirby *et al.*, 1989; * Tested against T9-96, a chloroquine-sensitive strain

Table 5.3 *In Vitro* Therapeutic Ratios of Quassinoids Tested

Quassinoid	A	B	A/B
	KB Cells ED_{50} µM	*P. falciparum* IC_{50} µM	
3-MEM Brusatol	25.9	0.0008[a]	32400
Glaucarubinone	2.28	0.008[b]	284
Bruceine E	13.5	0.151[c]	90
Bruceine D	2.82	0.037[d]	76
Ailanthinone	0.914	0.019[b]	48
(Ailanthinone	0.914	0.09[$i]	10)
Ailanthone	1.76	0.040[h]	44
Isobruceine A	0.136	0.004[e]	36
Brusatol	0.196	0.006[d]	34
3,15-dipropionate*	0.542	0.018[a]	30
Holacanthone	0.319	0.016[b]	20
Dehydrobrusatol	0.577	0.033[f]	18
Bruceolide	17.3	1.03[c]	17
3,15-diacetate*	0.64	0.04[a]	16
Dehydrobruceantin	3.76	0.256[h]	14
Dehydrobruceine C	5.82	0.424[c]	14
3,15-dibutyrate*	0.604	0.052[a]	12
Bruceantin	0.015	0.0015[d]	10
(Bruceantin	0.015	0.008[$i]	1.9)
Bruceine A	0.188	0.021[d]	8.9
Chaparrinone	1.65	0.194[f]	8.5
6α-Chaparrinone	0.086	0.017[e]	5.1
3-monostearate*	15.4	3.01[a]	5.1
Bruceine B	0.115	0.023[d]	5.0
3-monomyristate*	6.22	1.39[a]	4.5
3,12,15-triacetate*	5.46	1.24[a]	4.4
Bruceine C	0.037	0.009[d]	4.1
3,15-dipentanoate*	0.205	0.116[a]	1.8
3,15-dihexanoate*	0.213	0.189[a]	1.1
3,15-dioctanoate*	0.197	0.29[a]	0.7
3,15-didecanoate*	0.31	2.86[a]	0.11
Yadanzioside F	>50.0	7.59[h]	>6.6
Quassin	>50.0	95.9[g]	>0.52

a Patel *et al.*, 1989; b O'Neill *et al.*, 1988;
c O'Neill M.J. unpublished value; d O'Neill *et al.*, 1987;
e Cassady and Suffness, 1980; f Kirby G.C. unpublished value;
g Allen D. unpublished value; h Chan *et al.*, 1985; i Ekong *et al.*, 1990;
* bruceolide ester; $Tested against T9-96, a CQ-sensitve strain.

Table 5.3 lists the quassinoids in descending order of therapeutic ratio. The outstanding compound is clearly the semi-synthetic 3-MEM derivative of brusatol, with a value of 32400. This compound is highly active against *P. falciparum* but with a noticeably reduced degree of cytotoxic activity when compared to other naturally occurring quassinoids. Further work on this compound is currently in progress. Glaucarubinone has a ratio of 284 which is of the same order of magnitude as quinine and chloroquine diphosphate. The more polar bruceolide quassinoids, bruceines D and E have relatively favourable ratios of 76 and 90 respectively. Of the other bruceolides, brusatol and the parent alcohol bruceolide demonstrate greater antimalarial selectivity than bruceines A, B, C and bruceantin. However, from the table there are no obvious trends or patterns in structure-activity relationships. For example, the position of the methylene-oxygen bridge does not seem to confer any advantage, although it does appear that the shorter chain 3,15-diesters of brusatol feature higher in the table than the longer chain derivatives. At the concentrations tested, quassin and the glycoside yadanzioside F are not considered to be active against *P. falciparum in vitro*, (based on the criterion of activity selected earlier, $IC_{50} < 5\mu M$), and hence only a ratio of >6.6 can be given for yadanzioside F.

The bisbenzylisoquinoline alkaloids listed in section 3 have also been tested for *in vitro* antiplasmodial activity against *P. falciparum* (Marshall S.J., Antimalarial Activity of Some West African Plants, 1991; the reader is refered to this thesis for a detailed discussion of these results). In total 10 of the alkaloids were found to have IC_{50} values against *P. falciparum* (K1) between 0.1 and 1μM, and 11 more were active between 1 and 5μM. Thalisopidine demonstrated the greatest activity with an IC_{50} value of 0.089μM.

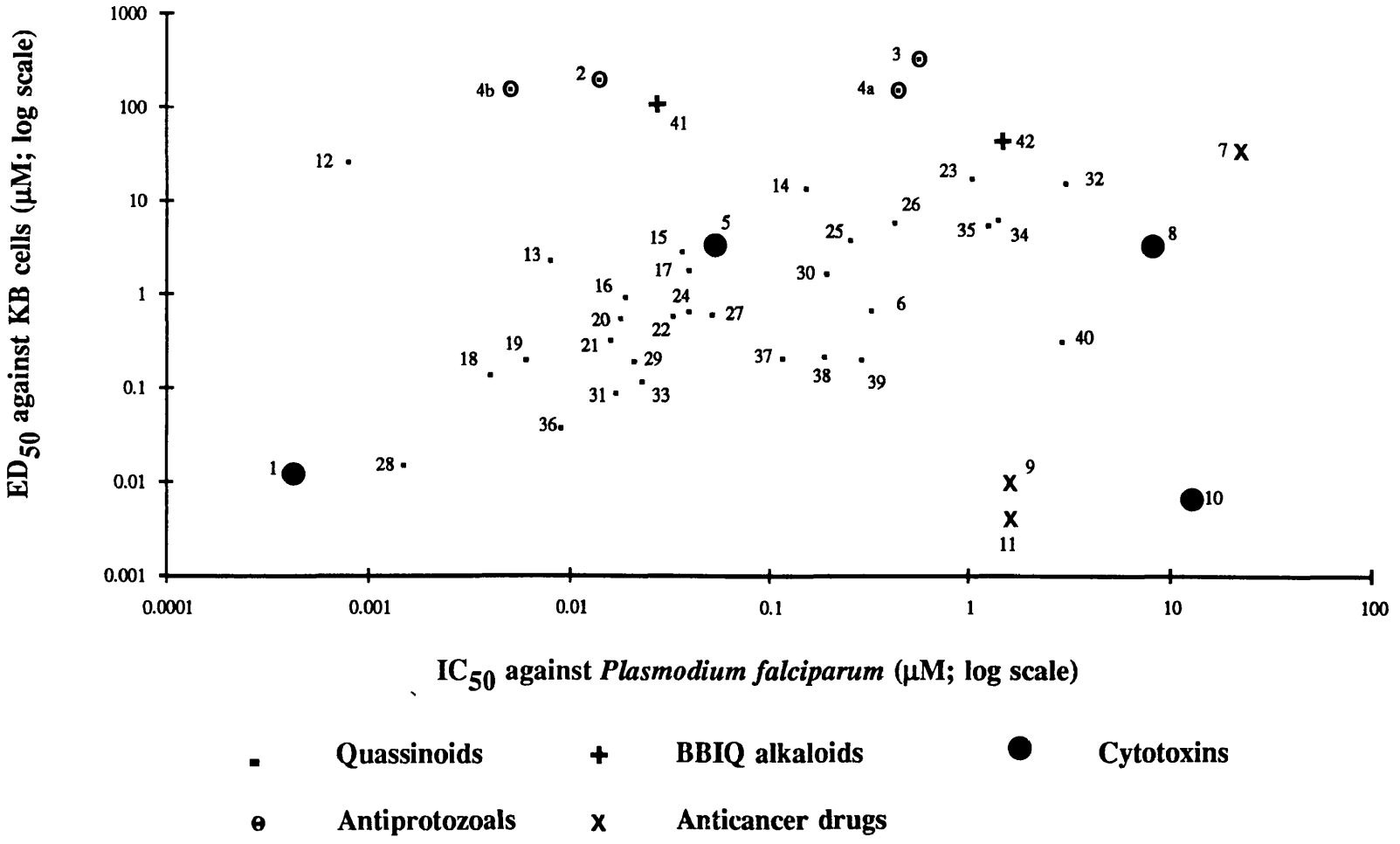
In contrast to the quassinoids, very few of the alkaloids tested in the KB screen demonstrated a high degree of cytotoxicity. Hence, the resulting therapeutic ratios for many of the alkaloids are very favourable. The lowest ratios of 8 and 9 are obtained for berberine and trilobine; eight compounds, including phaeanthine, have ratios in the range of 11 to 50, and the ratios of four other compounds lie between 50 and 100. Six of the active alkaloids have ratios greater than 100, whilst the outstanding value of 3889 was obtained for isotetrandrine - the diastereomer of phaeanthine. The canthin-6-one alkaloids did not demonstrate any antiplasmodial activity below 5μM and, therefore, no therapeutic ratio is given.

It is also possible to represent the cytotoxic and antimalarial data in graphical form. In Fig. 5.1 the *in vitro* KB cytotoxicity ED_{50} values are plotted against the *in vitro* IC_{50} values for *P. falciparum* (K1). The result for chloroquine tested against the chloroquine-sensitive strain of *P. falciparum* (T9-96) is also shown. The resulting graph reveals several regions. The top left hand sector includes the experimental compounds 3-MEM brusatol (12) and isotetrandrine (41), together with the therapeutic agents chloroquine (tested against T9-96; 4b) and artemisinin (2), and represents high antimalarial activity with low cytotoxicity. Since this region incorporates the data for chloroquine when tested against a CQ-sensitive strain of *P. falciparum* it might be considered to contain the drugs or compounds which possess the optimum criteria for antimalarial - toxic activity. Positioned slightly to the right of this region and demonstrating less *in vitro* antimalarial activity are quinine (3) and chloroquine (4a; tested against a CQ-resistant strain of *P. falciparum* (K1)). The quassinoid distribution is not random, but falls along a broad band which also

incorporates the protein synthesis inhibitor cycloheximide (5), and the antiamœbic agent emetine hydrochloride (6). The quassinoids with high therapeutic ratios (eg. glaucarubinone (13) and bruceines D (15) and E(14)) are positioned to the top right of the scatter, with the more active, yet concomitantly more cytotoxic compounds such as bruceantin (28) forming the lower left region of the distribution. Actinomycin D (1) is found in the very far lower left corner of the graph, illustrating its very high antimalarial activity and high cytotoxicity.

Fig. 5.1

Comparison of the *in vitro* cytotoxic and antimalarial activities of compounds studied.



KEY — Compounds Shown in Fig. 5.1

1. Actinomycin D
2. Artemisinin
3. Quinine
4a. Chloroquine diphosphate
4b. Chloroquine diphosphate*
5. Cycloheximide
6. Emetine hydrochloride
7. Etoposide
8. 6-Mercaptopurine
9. Vinblastine sulphate
10. Podophyllotoxin
11. Vincristine sulphate
12. 3-MEM brusatol[1]
13. Glaucarubinone
14. Bruceine E
15. Bruceine D
16. Ailanthinone
17. Ailanthone
18. Isobruceine A
19. Brusatol
20. 3,15-dipropionate[1]
21. Holacanthone
22. Dehydrobrusatol
23. Bruceolide
24. 3,15-diacetate[1]
25. Dehydrobruceantin
26. Dehydrobruceine C
27. 3,15-dibutyrate
28. Bruceantin
29. Bruceine A
30. Chaparrinone
31. 6-α senecioyl chaparrinone
32. 3-monostearate[1]
33. Bruceine B
34. 3-monomyristate[1]
35. 3,12,15-triacetate
36. Bruceine C
37. 3,15-dipentanoate[1]
38. 3,15-dihexanoate[1]
39. 3,15-dioctanoate[1]
40. 3,15-didecanoate[1]
41. Isotetrandrine
42. Phaeanthine

* Tested against a chloroquine-sensitive strain (T9-96)

[1] bruceolide ester

TABLE 5.4

A COMPARISON OF *IN VITRO* AND *IN VIVO* THERAPEUTIC RATIOS

	IN VITRO			*IN VIVO*		
	A KB ED_{50}(μg/ml)	B *P. falciparum* IC_{50}(μg/ml)	A/B	A *P. berghei* (mg/kg/day)[c] LD_{50}	B ED_{50}	A/B
Aqueous Tea	16.9	0.43	39	2400	500	5
$CHCl_3$ extract	0.55	0.006	92	54.4	7.4	7
Hyd. extract	1.07	0.011	97	283	46.2	6
Brusatol	0.102	0.003[a]	34	~3.4[d]	1.27[d]	3
Chloroquine Diphosphate	72.8	0.0097[f]	7505	79[e]	1.52[e]	52

[a] - From O'Neill et al, 1987
[b] - From O'Neill et al, 1986
[c] - Based on 4 oral doses given daily for 4 days
[d] - Unpublished result from Kirby, 1990
[e] - Given I.P; from Peters, 1965
[f] - Tested against T9-96; from Ekong *et al.*, 1990
~ - Approximate result

5.4 CORRELATION BETWEEN *IN VITRO* AND *IN VIVO* RESULTS

It was not presupposed that there should be any well-defined correlation between cell toxicity — antimalarial activity *in vitro* , and whole-animal toxicity — antimalarial activity *in vivo*. However, an attempt to correlate the *in vivo* and *in vitro* results has been made by comparing the relative therapeutic ratios. Table 5.4 shows the *in vitro* results against KB and *P. falciparum* for the aqueous tea (and chloroform and hydrolysed extracts of the tea) prepared from *B. javanica* fruits. The values for brusatol (the main quassinoid constituent of the tea) and for chloroquine diphosphate are also shown. These are compared with the corresponding *in vivo* results against *P. berghei*. The ratio of *in vitro* toxicity and antiplasmodial activity for each agent is always greater than the relative *in vivo* results. However, with the exception of chloroquine, a comparison of the A/B (therapeutic) ratios for the *in vitro* and *in vivo* pairs of results reveals a simple relationship whereby the *in vitro* ratios are approximately ten times greater than those derived for the *in vivo* results; this stems from the consistently low *in vitro* IC_{50} values . This ten-fold difference could possibly reflect the effects of drug concentration and duration of exoposure in the *in vitro* systems are greater than in the *in vivo* systems, where the agents are possibly rapidly deactivated in the liver and excreted.

It can be seen from Table 5.4 that chloroquine has very high antimalarial activity and correspondingly low toxicity *in vitro*. The aqueous tea also shows low cytotoxicity and is relatively non toxic *in vivo*, but its antimalarial activity is much lower than the other agents shown. When compared to the original aqueous tea the chloroform and hydrolysed extracts show increased *in vitro* cytotoxicity (30 and 16 times, respectively) and increased *in vitro* antimalarial activity (72 and 39 times, respectively). It is clear that in both the cytotoxic and antimalarial screens the activity of the chloroform extract is approximately 2 times that of the hydrolysed extract.

Considering the *in vivo* portion of the table, the *in vivo* potency of chloroquine is over 100 times less than *in vitro*. Brusatol and the aqueous tea preparations all show *in vivo* toxicity at a dose not much higher than their *in vivo* antimalarial activity. Interestingly, chloroquine and brusatol have approximately 200-400 times more antimalarial activity *in vitro* than *in vivo*, whereas this value for the crude extracts is approximately 1000-4000.

It is helpful to make a separate comparison of *in vitro* and *in vivo* antiplasmodial activity (see Table 5.5) and of *in vitro* and *in vivo* toxicity (see Table 5.6).

The IC_{50} value quoted in Table 5.5 is against *P. falciparum* T9-96 (a chloroquine-sensitive strain), to enable a comparison with the *in vivo P. berghei* strain which is also chloroquine-sensitive. The other *in vitro* values given are against a multidrug-resistant strain, K1 (note that the antimalarial activity of a number of typical quassinoids and some other protein synthesis inhibitors is found to be approximately equal against both strains, Ekong *et al*, 1990).

Table 5.5 shows that chloroquine and brusatol are approximately equipotent *in vitro* and *in vivo*, although brusatol shows slightly more *in vitro* activity. Both pure compounds also give much lower ratio values than the complex mixtures of crude extracts. When compared to brusatol, the freeze dried aqueous

Table 5.5 A Comparison of *in vitro* and *in vivo* antimalarial activity

Test Agent	*IN VITRO* IC_{50} µg/mL	*IN VIVO* ED_{50} mg/kg/day	*IN VIVO/IN VITRO*
Aqueous Tea (1)	0.43	500	1163
Chloroform extract (2)	0.006	7.4	1233
Hydrolysed extract (5)	0.011	46.2	4223
Brusatol	0.003	1.27	423
Chloroquine diphosphate	0.0097	1.52	157
Numbers in brackets refer to the extraction scheme in Fig. 2.4			

tea (1) is the least potent of the extracts of *Brucea javanica* fruits. Further purification of the tea results in enhanced antimalarial activity, *in vitro* and *in vivo*, with both the chloroform (2) and the hydrolysed (5) extracts demonstrating lower IC_{50} and ED_{50} values than the original aqueous tea. The *in vivo/in vitro* ratios for the aqueous tea (1) and chloroform (2) extracts are comparable, and are approximately 3 times greater than the ratio for brusatol. This reflects their paralleled *in vitro* and *in vivo* activities when compared to brusatol. Conversely, the *in vivo* activity of the hydrolysed extract is considerably lower than the *in vitro* activity and this is shown by the ratio of 4223 which is approximately 10 times greater than that of brusatol.

It has been reported in Chinese literature that experiments on chickens dosed with both orally administered *B. javanica* fruits and intramuscularly injected crude noncrystalline powder had a marked antimalarial action. In contrast, the "pure crystal" showed a weaker antimalarial activity. The antimalarial principle is also descibed as being highly soluble in water, heat-stable (unlike pure quassinoids) and has antiplasmodial effect (Chang and Butt, 1989 and references therein). Phytochemical investigation of the crude extracts from the freeze dried aqueous tea, as described in Section 2 revealed several differences in the constituents of the chloroform (2) and hydrolysed (5) extracts. The more potent chloroform extract contains 4 quassinoids: brusatol (0.003%), bruceine A (0.0014%), bruceine B (0.0019%) and bruceine C (0.0016%), whereas the hydrolysed extract contains just 3 quassinoids; brusatol (0.062%), bruceine A (0.0025%) and bruceine C (0.0113 %). In addition the total quassinoid content of the less active hydrolysed extract is 10 times greater than that of the more active chloroform extract. These results, therefore, appear rather anomalous. How these differences are affecting the antimalarial activity of the tea and its extracts is not known; perhaps there is synergism between the different quassinoids identified or perhaps some other (non-quassinoid) component not yet determined is required for antimalarial efficacy.

Table 5.6 A Comparison of *In Vitro* and *In Vivo* Cytotoxicity

Test Agent	*IN VITRO* ED_{50} µg/mL	*IN VIVO* LD_{50} mg/kg/day	*IN VIVO/IN VITRO*
Aqueous tea	16.85	2400	142
Chloroform extract	0.55	54.4	99
Hydrolysed extract	1.07	283	265
Brusatol	0.102	3.4	33
Chloroquine diphosphate	72.8	79	1

If it is considered that an *in vitro* ED_{50} value expressed in µg/mL or mg/L may be directly compared to an LD_{50} value in mg/kg (assuming 1L of $H_2O \approx$ 1Kg) it can be said that chloroquine is equipotent in both systems. Brusatol, however, is considerably more toxic, being 23 times more active *in vivo* and approximately 700 times more cytotoxic *in vitro* than chloroquine. This does not parallel the *in vivo/in vitro* antimalarial comparison between brusatol and chloroquine, and suggests a large increase in *in vitro* toxicity for brusatol. *In vivo* the uptake or distribution in a whole animal of brusatol is far from comparable to the *in vitro* situation; where parasites, albeit contained within red blood cells, are virtually directly exposed to the drug in the culture medium. It is also more likely that in the *in vivo* model brusatol is undergoes metabolism to inactive forms. Nevertheless, the LD_{50} for brusatol is still very low indicating its relatively high *in vivo* toxicity. All crude extracts and brusatol are more toxic than chloroquine *in vitro*, yet the aqueous tea and the hydrolysed preparation are less toxic than chloroquine or brusatol *in vivo*. Also, both brusatol and the chloroform extract show greater toxicity *in vivo* when compared to chloroquine. The *in vivo/in vitro* toxicity ratios of the aqueous tea and the chloroform extract are very similar, as were their equivalent antimalarial ratios. However, as was seen in Table 5.5, the hydrolysed sample behaves somewhat differently in the two systems. Its *in vitro* and *in vivo* toxicities are greater than those of the original aqueous tea, some 16 times and 8 times respectively. It is possible that in the *in vivo* situation there is reduced uptake of components of the hydrolysed aqueous tea extract, or perhaps metabolism to inactive forms is occurring. Alternatively, hydrolysis may remove some unknown quassinoid-related compound which competes with other "active" quassinoids for uptake in the *in vitro* system. This presupposes that an active (saturable) uptake process exists for quassinoids.

Clearly the comparison of *in vitro* and *in vivo* data, comparing effects upon two independent parameters (antimalarial and cytotoxic activity) is complex. It is essential that derived parameters such as cytotoxic/antimalarial ratios are clearly defined when attempting to compare or even standardise the effects of different types of drug or compound, or different drugs or compounds within the same family or series. An appreciation of the balance between cytotoxicity (*in vitro*), gross toxicity (*in vivo*) and antimalarial

activity (both *in vitro* and *in vivo*) is crucial when considering the possible development of a novel chemotherapeutic agent. The wider the safety margin the better the drug.

CHAPTER SIX — CONCLUSIONS

The urgent need for new antimalarials has been outlined in the introduction. Herbal remedies used in traditional medicine remain a relatively unexplored source of potential therapeutic agents and the investigation of plants used for the treatment of malaria is essential. The efficacy of such a herbal remedy used traditionally in Chinese Medicine for the treatment of malaria and fever has been investigated. This is in contrast with previous work which has focussed on the chemistry and biological activity of pure isolated compounds. An aqueous infusion or 'tea' prepared from the fruits of the Simaroubaceae plant *B. javanica* was shown to have both *in vitro* and (to a lesser extent) *in vivo* antimalarial activity, lending support to its use in traditional medicine. Phytochemical analysis of the tea revealed the presence of small quantities of highly active lipophilic quassinoids (≈2%), diluted with a much larger quantity of less active polar derivatives (18%). Four lipophilic quassinoids were subsequently isolated from the tea. Synergism between the various constituents of the tea may account (in some part at least) for the observed activity and experiments are being carried out to explore the possible potentiating effects of various quassinoid combinations. Some of the polar compounds, which are glycosidic forms can be converted into lipophilic quassinoids by acid hydrolysis. Partial hydrolysis of the polar forms into lipophilic quassinoids may occur *in vivo* in the stomach following oral administration.

It is possible that reduced uptake and metabolism of the quassinoids to less active forms may explain the reduction of *in vivo* activity of the tea when compared to its *in vitro* activity. *Purification of the both *in vitro* and *in vivo* activity, and also to an increase in toxicity. When the highly active (and toxic) lipophilic principles are diluted in the form of an aqueous infusion, as required by the traditional remedy the toxicity is reduced. Crude plant extracts from two other related plant species *S. amara* and *S. glauca* were also found to demonstrate *in vitro* antimalarial activity and warrant further investigation.

In order to distinguish between the specificity of antimalarial action and the non-selective cytotoxicity of potential antimalarial agents under study it is necessary to establish their toxicity to mammalian cells. An economical and convenient 48 hour microdilution assay using KB cells has been developed for the determination of *in vitro* cytotoxicity. The technique - a simplified modification of the standard NCI KB cytotoxicity assay, is shown to be reproducible and has been validated with known cytotoxic and non-toxic antimicrobial agents. The microplate test has been used successfully to monitor the cytotoxic activities of some 107 compounds and crude extracts. Taken as a whole the quassinoids were found to be a very toxic series of compounds, whereas the bisbenzylisoquinoline alkaloids (with the exception of berberine) demonstrated very low cytotoxicity.

A comparison of *in vitro* cytotoxicity and *in vitro* antimalarial activity can be represented in the form of a therapeutic ratio and *in vitro* selectivity for *P. falciparum* may be an indication of a therapeutically

useful antimalarial agent. The *in vivo* LD_{50}/*P. berghei* IC_{50} ratios have been compared with the *in vitro* KB/*P. falciparum* therapeutic ratios and for quassinoids the *in vivo* ratios are in the order of one tenth of the values obtained for*in vitro* ratios. These findings lend support to the value of *in vitro* testing and to the use of the KB assay as a preliminary step to *in vivo* testing.

The effects of three quassinoids upon KB cells were studied to try to determine whether their mode of action is similar to that observed in malaria parasites. All three compounds were shown to rapidly inhibit protein synthesis. Nucleic acid synthesis was suppressed to a lesser extent and (of the quassinoids) brusatol was the most potent inhibitor of both protein and nucleic acid synthesis. The results of this study may indicate that as inhibitors of protein synthesis quassinoids are more active against KB cells than against *P. falciparum*. Further work with other cell types including non-cancerous cell lines are required for comparisons.

Transmission electron microscopy of quassinoid-treated KB cells demonstrated visually their drastic effects on intracellular organelles and revealed several differences between the effects of the three quassinoids. These differences relating to their uptake and deposition in the cells could explain the disparity in the ED_{50} values obtained for brusastol, bruceantin and bruceine D against KB cells. A time course study is necessary to elucidate the intracellular events occurring in the cells following exposure to the quassinoids.

It is hoped that future investigations of natural products using similar techniques to those described here will lead to the discovery of a compound (naturally occurring or semi-synthetic) that is selctive for the malaria parasite.

* should read: Purification of the "active" tea constituents by solvent extraction leads to an increase in both *in vitro* and *in vivo* activity, and also to an increase in activity.

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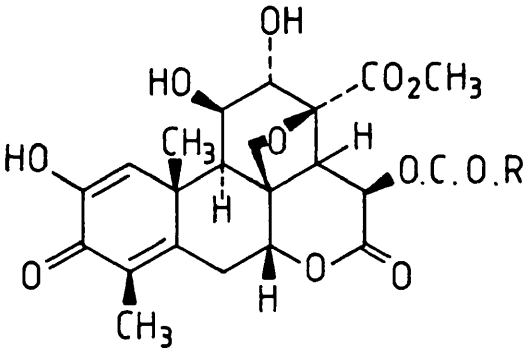
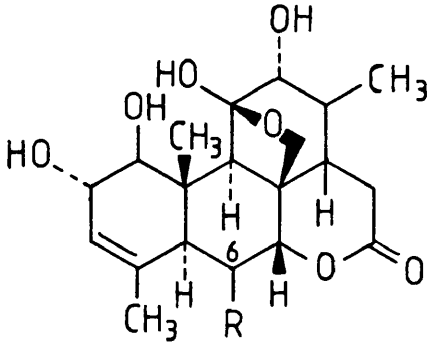
APPENDIX

Figure A1. Structures of Quassinoids in Tables 3.1 and 3.2 not given previously in text

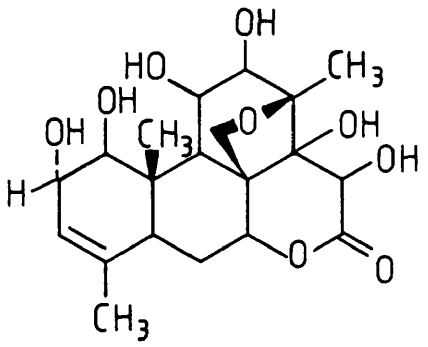


	R
Dehydrobruceantin	$CH=C(CH_3)CH(CH_3)_2$
Dehydrobruceine C	$CH=C(CH_3)C(OH)(CH_3)_2$
Dehydrobrusatol	$CH=C(CH_3)_2$



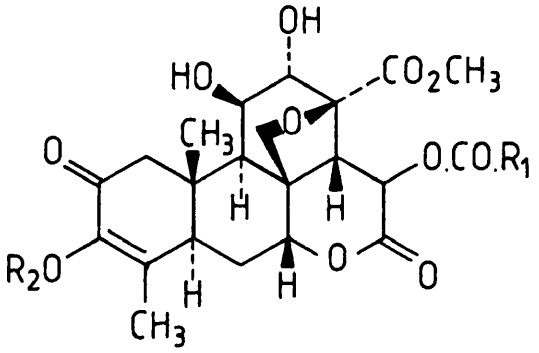
6α-senecioyloxychaparrin

R = $OCOCH=C(CH_3)_2$

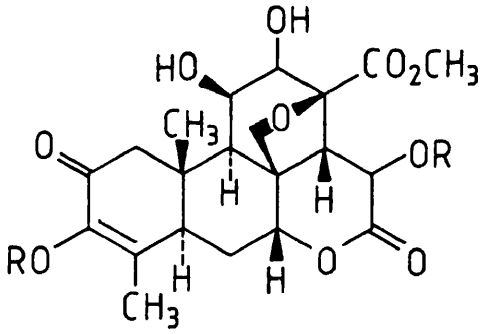


Bruceine E

Figure A2. Structures of Semi-synthetic Quassinoids in Table 3.3



	R1	R2
3-methoxyethoxymethyl brusatol	$CH=C(CH_3)_2$	$CH_2O(CH_2)_2OCH_3$

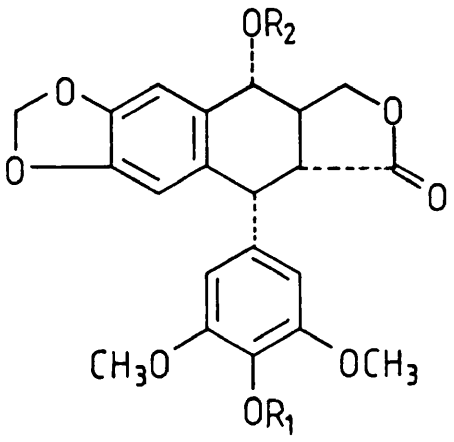


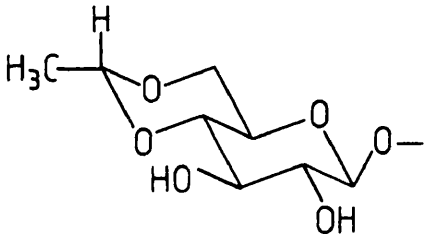
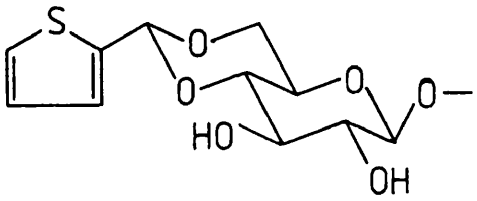
Bruceolide Esters

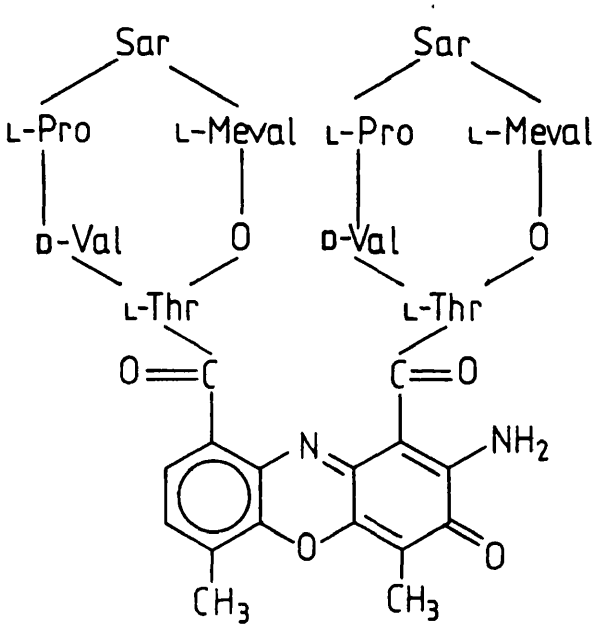
Ester Moieties (R)

Acetate	$CH_3.C=O$
Propionate	$CH_3.CH_2.C=O$
Butyrate	$CH_3.(CH_2)_2.C=O$
Pentanoate	$CH_3.(CH_2)_3.C=O$
Hexanoate	$CH_3.(CH_2)_4.C=O$
Octanoate	$CH_3.(CH_2)_6.C=O$
Decanoate	$CH_3.(CH_2)_8.C=O$
Myristate	$CH_3.(CH_2)_{12}.C=O$
Stearate	$CH_3.(CH_2)_{16}.C=O$

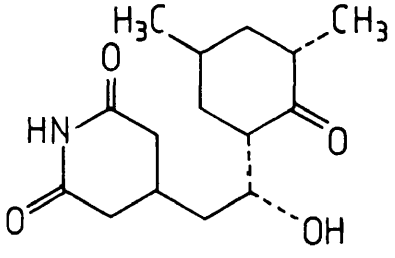
Figure A3. Structures of Therapeutic and Cytotoxic agents in Table 3.4



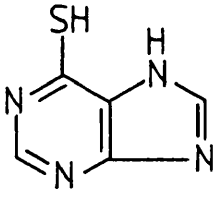
	R1	R2
Etoposide	H	
Teniposide	H	
Podophyllotoxin	CH_3	OH



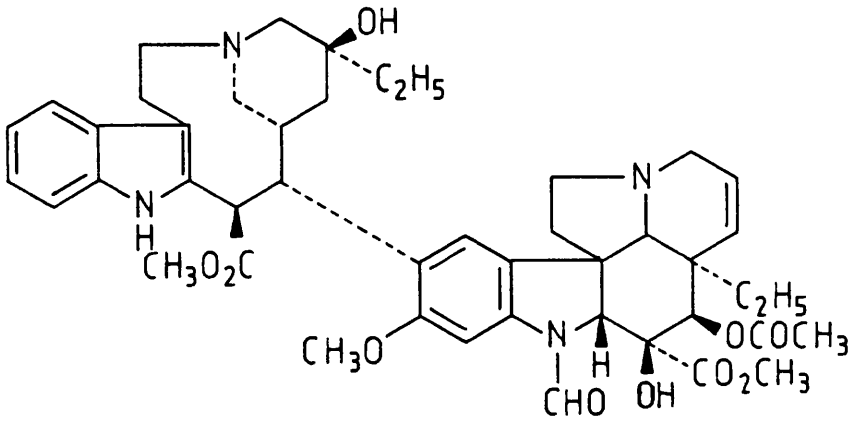
Actinomycin D



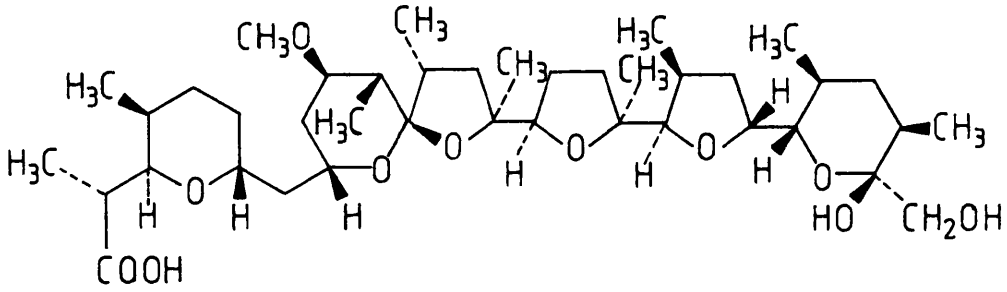
Cycloheximide



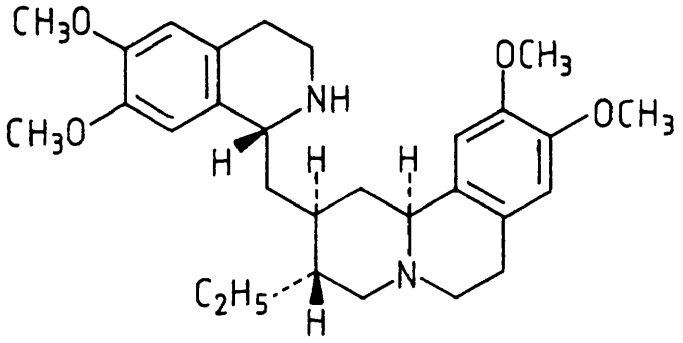
6-Mercaptopurine



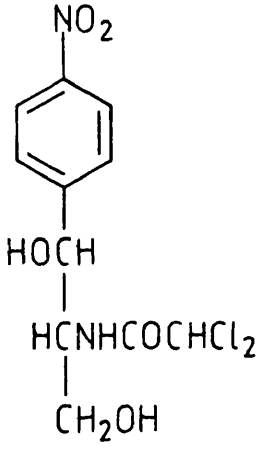
Vincristine



Nigericin



Emetine



Chloramphenicol

Publications

In Vitro Cytotoxicity of a Series of Quassinoids from *Brucea javanica* Fruits Against KB Cells

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Abstract

A microdilution technique was developed for the assessment of *in vitro* cytotoxicity against KB cells derived from a human epidermoid carcinoma of the nasopharynx. The test was used to determine the cytotoxicity of a series of quassinoids, isolated from *Brucea javanica*, and which have previously been shown to demonstrate activity against *Plasmodium falciparum*. The 50% effective dose (ED_{50}) for 7 quassinoids tested against KB ranged from 0.008 µg/ml for bruceantin, the most cytotoxic of the compounds tested to > 5 µg/ml for bruceolide, the least toxic tested. The activities of the quassinoids against KB did not parallel the known activities of the quassinoids against *Plasmodium falciparum* suggesting that the quassinoid mode of antimalarial action is not a simple cytotoxic effect and lends support to further investigation of the structure activity relationships within this group of compounds.

Key words

Brucea javanica, Plasmodium falciparum, in vitro cytotoxicity, quassinoids, antimalarial action.

Introduction

The development of a novel antimalarial drug is of paramount importance as the spread of chloroquine-resistant *falciparum* malaria becomes worldwide and the search for mechanistically novel compounds has led to the investigation of natural products, some of which have been used in traditional medicine for many hundreds of years. Some selected compounds from particular classes of natural products, such as lignans, cardenolides, and sesquiterpene lactones (1, 2, 3) show non-selective cytotoxicity and it is important, therefore, when searching for potential chemotherapeutic agents to distinguish between specificity of action and non-selective toxicity. An estimation of therapeutic index in which the desired biological activity is compared with general toxicity (3, 4) is one way in which selectivity of activity may be assessed.

In vitro testing is an important preliminary step to *in vivo* screening and gives potential for the reduction of whole animal experiments. In addition *in vitro* tests are relatively inexpensive, have a short time course, yet demonstrate high sensitivity. The standard KB cytotoxicity assay, initially described by Eagle (5) and later standardised by the National Cancer Institute (6) utilises cells derived from a human epidermoid carcinoma of the nasopharynx. The assay has since been further modified by Wall et al. (7), but all these techniques are time consuming and/or require relatively large quantities of test compounds and materials. We have developed a sensitive yet rapid microdilution method that does not necessiate the use of radiolabelled compounds nor require an involved protein determination procedure. (8).

Some plant species of the family Simaroubaceae are widely used in traditional medicine. *Brucea javanica* (L.) Merr. is distributed through S.E. Asia to S. China and its fruits have been used in traditional Chinese Medicine for thousands of years to treat both malaria and amoebiasis. Extracts of the fruits are highly active against *Plasmodium falciparum in vitro* and against *Plasmodium berghei in vivo* (11), and some of the bitter terpenoid quassinoids have also been the focus of extensive investigations as antitumour agents (9). In this study we have assessed the cytotoxicity of a series of quassinoids isolated from *Brucea javanica* fruits and compared these findings with the known *in vitro* antiplasmodial activity of the same compounds.

Materials and Methods

Test compounds

Chloroquine diphosphate was obtained from Sterling-Winthrop. Bruceines A, B, C, D, bruceantin and brusatol were available from previous extractions of *B. javanica* fruits (11). Bruceolide was obtained from brusatol by hydrolysis (11).

Maintenance of culture

KB cells (10) supplied by Flow Laboratories were cultured in Falcon flasks (80 cm^2; Nunc) using Eagles Minimum Essential Medium with Earle's salts and 0.85 g/l sodium bicarbonate. 1% Non-essential amino acids, 10% foetal bovine serum and L-glutamine (2 mM) were added immediately prior to use and the complete medium filtered using a 0.22 µm filter.

When the monolayer became fully confluent the cells were subcultured using 0.25 % trypsin solution. The detached cells were transferred to sterile centrifuge tubes (10 ml) and spun at 400 r.p.m. for approximately 15 seconds. After twice washing with complete medium the cells were resuspended in 1 ml of complete medium and mixed thoroughly to break down any cell aggregates. The cells were counted using a haemacytometer and the appropriate number of cells added to fresh culture vessels containing complete medium to give a final concentration of 10^5 cells/ml. The cells had been routinely screened for mycoplasma contamination using a DNA fluorochrome stain (Hoechst stain – Flow Labs. Inc.) by Flow Labs. Inc. and examined microscopically for the presence of extranuclear fluorescence.

Cytotoxicity test procedure

The test samples were dissolved in 50 µl of ethanol and diluted with complete medium to give concentrations of 1 mg/l for pure compounds and 10 mg/ml for crude extracts. The concentration of ethanol was diluted 1000 : 1 to a non-toxic level.

Two fold serial dilutions were performed in 50 µl of complete medium in 96 well microtiter plates (Linbro; Flow Labs. Inc.). Control wells without test samples were simultaneously used. 50 µl of a 1×10^6 cell/ml suspension was added to the test and control wells giving a total well volume of 100 µl. The plates were covered with lids, placed in a modular incubating chamber (Flow Labs. Inc.) and gassed for 5 minutes with 5 % carbon dioxide in air until the medium was pale orange in colour. The plates were incubated at 37 °C for 48 hours.

Assessment of cytotoxicity

After 48 hours incubation the cells in the plates were fixed and stained. To do this the drug in medium was removed by inversion and gentle shaking of the plates and the cells immediately washed with 0.9 % sodium chloride solution at 37 °C. After being allowed to dry at room temperature the KB cells were fixed with methanol for 10 minutes and again left to dry. A 0.5 % aqueous eosin stain (George T. Gurr Ltd.-21050) was then added to each well and left for a further 15 minutes before the plates were rinsed 3 times with distilled water to remove the excess stain.

A 200 µl volume of 0.1 M sodium hydroxide solution was added to each well and left for 20 minutes to digest the cell protein and hence release the stain. After this time the optical density (O.D.) at 490 nm of the solution in each well was determined using a microplate reader (MR-700: Dynatech Labs. Inc.).

A correlation between numbers of KB cells and O.D. had previously been obtained by setting up 2 plates containing serial dilutions of a cell suspension. After 48 hours incubation the cell from 1 plate were trypsinised and counted with a haemacytometer, and the other plate was fixed and stained with eosin as described above and the O.D. measured. For each cell concentration, cell counts and O.D. readings were obtained in triplicate. Fig. 2 shows the correlation between number of KB cells and O.D. The percentage inhibition of KB cell growth was calculated by comparison of the O.D. readings of the control and test samples, and plotted against concentration of sample being tested. Each sample was tested at least twice.

Results and Discussion

In order to obtain an estimate for the therapeutic index of a group of potential antimalarial agents, the cytotoxicity of a series of quassinoids was investigated: bruceine A (1), bruceine B (2), bruceine C (3), bruceantin (4), brusatol (5), bruceolide (6), bruceine D (7). The cytotoxicity to KB cells has been compared to antiplasmodial activity and a ratio for the two calculated (Table 1). Bruceantin had a 50 % effective dose (ED_{50}) of 0.008 µg/ml, which is comparable to the previously reported ED_{50} value of 0.007 µg/ml (9) obtained against KB using the original method described by Eagle (5). Podophyllotoxin (8), with an ED_{50} value of 0.003 µg/ml was found to be only slightly more cytotoxic than bruceantin. Phase I clinical trials of bruceantin as an antileukemic agent established hypotension, nausea and vomiting as dose-limiting toxicities (12). However, all further clinical trials have since been closed after disappointing responses during Phase II studies.

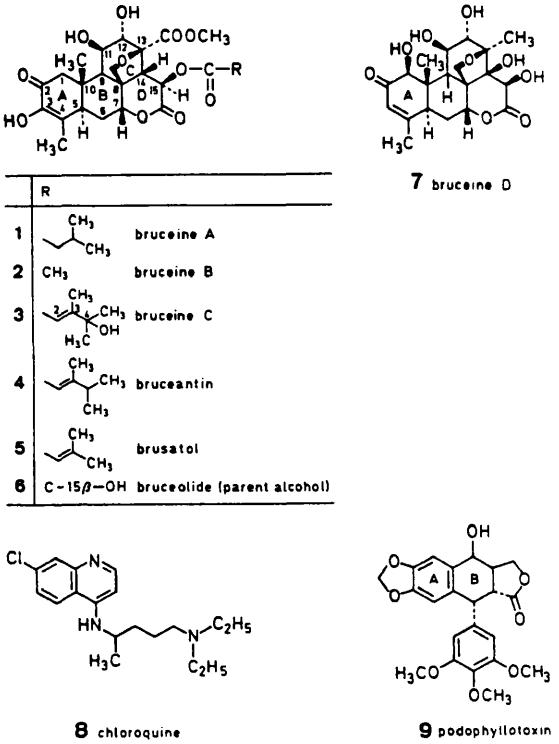

Fig. 1 Structures of the quassinoids (1–7), chloroquine (8), and podophyllotoxin (9).

Our results demonstrate that, although the quassinoids tested differ only marginally in their molecular structure, these small variations produce large differences in the *in vitro* cytotoxicity of these compounds. For example, compounds 1–6 differ only in their C-15 substituents, yet have ED_{50} values ranging from 0.008 µg/ml for bruceantin, the most toxic, to 7.56 µg/ml for bruceolide, the least toxic. The C-15 ester function (1–5) has been noted earlier as being important in contributing to *in vitro* antimalarial and antileukemic activity (13).

Bruceine D, a 14,15-diol is more polar than compounds 1–6, has a methyl function at C-13 and lacks an ester function at C-15, but was found to be 145 times less toxic than bruceantin, yet demonstrated relatively high antimalarial activity. This difference was reflected in the high

Table 1 *In vitro* cytotoxicity against KB cells of a series of isolated quassinoids from *B. javanica* fruits compared to *in vitro* antiplasmodial activity[a] against chloroquine resistant *P. falciparum* (K1).

	Quassinoid	A KB cells ED_{50} (μg/ml)[b]	CC[d]	B *P. falciparum* ED_{50} (μg/ml)[]	A/B
1	Bruceine A	0.098 (0.072–0.131)[c]	0.935	0.011[e]	9.9
2	Bruceine B	0.55 (0.047–0.063)[c]	0.980	0.011[e]	5.0
3	Bruceine C	0.021 (0.015–0.031)[c]	0.904	0.005[e]	4.2
4	Bruceantin	0.008 (0.006–0.01)[c]	0.948	0.0008[e]	10
5	Brusatol	0.102 (0.086–0.119)[c]	0.972	0.003[e]	34
6	Bruceolide	7.57 (6.86–8.44)[c]	0.978	0.451[f]	17
7	Bruceine D	1.158 (0.966–1.398)[c]	0.977	0.015[e]	77
8	Chloroquine Diphosphate	72.8 (53.9–98.3)[c]	0.925	0.210[g]	347
9	Podophyllotoxin	0.003 (0.002–0.004)[c]	0.887	NT	–

[a] Inhibition of incorpration of ^{3}(*H*)-hypoxanthine.
[b] Based upon 2-fold dilutions in duplicate.
[c] 95% confidence limits.
[d] Correlation coefficient.
[e] From O'Neill et al. (11).
[f] Unpublished result (value of O'Neill).
[g] From O'Neill et al. (13).
NT Not tested.

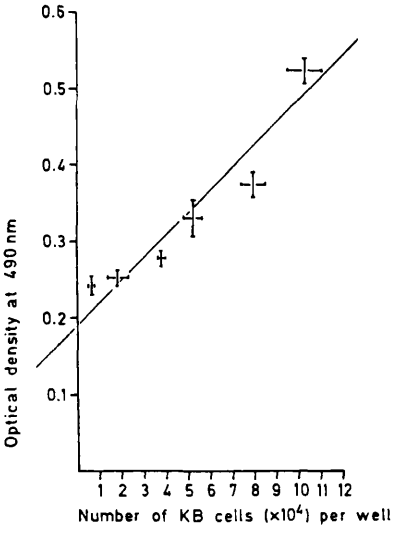

Fig. 2 Correlation of optical density and number of KB cells. [Line of best fit determined by linear regression. Standard deviations are shown. Correlation coefficient was 0.95. For 16 degrees of freedom (n-2) and P = 0.001, statistic tables give r = 0.708. The correlation is significant at the 0.1% level.]

value of 77 obtained for the cytotoxicity to antiplasmodial activity ratio (A/B) for bruceine D. Also noteworthy for their relatively high A/B values are brusatol and bruceolide with values of 34 and 17 respectively. However, the most favourable ratio by far was obtained for chloroquine (8) with an A/B value of 347. Brusatol and bruceine A are almost identical molecules except for the presence in brusatol of an unsaturated C-2′/C-3′ bond; they have comparable cytotoxic activities with ED_{50} values of 0.102 μg/ml and 0.098 μg/ml respectively. However, brusatol is more than 3 times more active *in vitro* than bruceine A against *P. falciparum* (K1). Hence, it can be seen that there is no direct relationship between cytotoxicity and antiplasmodial activity, which lends support to further investigation of these compounds and their analogues as potential antimalarials.

Some quassinoids are known to inhibit protein synthesis in mammalian cells and *Plasmodium* (9, 14, 15) and investigations into the uptake of a number of quassinoids by KB cells are presently underway. As a first approximation of potential therapeutic selectivity it is possible to use KB cells *in vitro* to monitor cytotoxicity and allow for comparison with *in vitro* tests for specific biological activity. However, it is important to realize that in order to expose different modes of cytotoxic action other cell lines and test systems must be used. The cytotoxic results obtained in this study enable the selection of compounds which show some specificity of action in the *in vitro* situation, prior to more detailed *in vivo* investigations.

Acknowledgements

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Plasmodium falciparum: effects of phaeanthine, a naturally-occurring bisbenzylisoquinoline alkaloid, on chloroquine-resistant and -sensitive parasites *in vitro*, and its influence on chloroquine activity

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Phaeanthine, a bisbenzylisoquinoline alkaloid which occurs naturally in *Triclisia* species, was extracted from *Triclisia patens* (Menispermaceae) obtained from Sierra Leone (West Africa). *In vitro*, phaeanthine was found to be twice as potent against a chloroquine-resistant *Plasmodium falciparum* strain (K1), as against a chloroquine-sensitive clone (T9-96), with 50% inhibitory concentrations of 365·85 (± 11·41) nM and 704·87 (± 81·48) nM respectively. At a sub-inhibitory concentration of 80·35 nM, chloroquine resistance was not reversed by phaeanthine. Isobolograms constructed from experiments with chloroquine/phaeanthine combinations showed antagonism in T9-96 and an additive effect in K1. In a 48-hour microtest, phaeanthine at antimalarial concentrations showed no cytotoxicity to mammalian (KB) cells *in vitro*.

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Abbreviations used in text: Chloroquine-resistant (CQ-R); chloroquine-sensitive (CQ-S); Phaeanthine (PH); chloroquine (CQ); culture medium (CM); 50% inhibitory concentration (IC_{50}); Bisbenzylisoquinoline (BBIQ); Tetrandrine (TT); Haematocrit (HC); Adenosine triphosphate (ATP).

Resistance of *Plasmodium falciparum* to CQ has become increasingly widespread (Peters, 1987) to the extent that it is now found in most areas where malaria is endemic. Indeed, the problem is not limited to one drug. For example, there have been reports of resistance, both *in vivo* (Karwacki *et al.*, 1989) and *in vitro* (Kilimali *et al.*, 1989), to mefloquine, which has only recently been introduced for treatment and prophylaxis. Such problems have led to a continued search for new antimalarial agents, and one possible source is plants with a traditional reputation as antimalarials or antipyretics. The *Cinchona* plant already provides useful drugs such as quinine and other cinchona alkaloids used in the treatment of malaria (White *et al.*, 1981; Bunnag *et al.*, 1987; Warhurst, 1987). Another compound of plant origin is qinghaosu (artemisinin) isolated from *Artemisia annua*, a herb used traditionally in China for more than 2000 years to treat fevers, including malaria (Klayman, 1985). Reports that this compound is effective against both CQ-R and CQ-S falciparum malaria (Klayman, 1985; Li *et al.*, 1984) point to the fact that plants can act as alternative sources of substances of therapeutic value.

BBIQ alkaloids are a group of compounds isolated primarily from the plant families Berberidaceae, Menispermaceae, Monimiaceae and Ranunculaceae (Guha *et al.*, 1979). Continued research has identified more BBIQ alkaloids from other plant families, and some of the compounds isolated have been found to exhibit a variety of pharmacological activities (Schiff, 1983, 1987). TT is one of the BBIQ alkaloids which has been studied more extensively. This alkaloid has been shown to be effective in the treatment of silicosis (Yu *et al.*, 1983), and to have anti-inflammatory (Seow *et al.*, 1988*a*, 1989), antimalarial (Ye and Van Dyke, 1989), antiallergic (Teh *et al.*, 1988) and immunosuppressive (Seow *et al.*, 1988*b*) properties. TT is also reported to be a calcium antagonist (Zeng *et al.*, 1982), and to be weakly mutagenic (Whong *et al.*, 1989).

PH [Fig. 1(A)], the enantiomer of TT, occurs in several plants of the families Gyrocarpaceae, Annonaceae and Menispermaceae (Guha *et al.*,

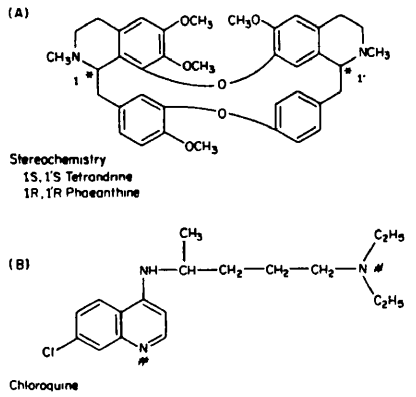

Fig. 1. Structures of compounds used: (A) phaeanthine and tetrandrine; (B) chloroquine (# = protonatable nitrogen atom).

1979; Schiff, 1983, 1987). Information on its pharmacological activity is scarce; a study carried out by Kronlund *et al.* (1970) showed that PH was devoid of muscle relaxant activity. *Triclisia patens* (Menispermaceae) is one of the plant species from which PH has been extracted (Boissier *et al.*, 1963; Kronlund *et al.*, 1970; Tackie *et al.*, 1974), and is sold in herb markets in Sierra Leone (West Africa) as a traditional remedy for malaria.

The antimalarial activity of PH *in vitro* has been reported in an abstract (Partridge *et al.*, 1988). In this paper we describe its effects on CQ-R and CQ-S falciparum malaria parasites *in vitro*. In addition, we examined the interaction of PH with CQ in antimalarial tests *in vitro*, and its cytotoxicity on KB cells.

MATERIALS AND METHODS

Parasite Strains and Cultivation

Two lines of *P. falciparum* were used in this study: T9-96, a CQ-S clone (Thaithong *et al.*, 1984), and K1, a highly CQ-R strain that also exhibits resistance towards pyrimethamine (Thaithong and Beale, 1981). Both were obtained from the WHO Reference Centre in Edinburgh. *In vitro* cultures of T9-96 and K1 were maintained (Trager and Jensen, 1976) as described by Fairlamb *et al.* (1985) in human A^+ erythrocytes using A^+ human serum-supplemented RPMI 1640 culture medium.

Plant Material

Our plant material (*Triclisia patens*) was purchased from a herb market in Sierra Leone (West Africa) in 1985 and 1988. Initial identification and authentication of the material was carried out by Dr. C. A. MacFoy of the Department of Botany, University of Sierra Leone, Freetown.

Extraction and Isolation of Phaeanthine

Phaeanthine was isolated using a combination of previously described methods (Boissier *et al.*, 1963; Tackie *et al.*, 1974). Dried wood from *Triclisia patens* was ground into a coarse powder (60 g), exhaustively extracted with 2% H_2SO_4, and then filtered. The filtrate was brought to pH 10 with concentrated ammonia, then extracted with $CHCl_3$ (4 × 500 ml). After evaporation of the $CHCl_3$ fraction at 40°C (Buchi Rotavapor-R) to a volume of 50 ml, the organic phase was re-extracted with 2% H_2SO_4 (7 × 50 ml) and brought to pH 10 with concentrated NH_4OH. The aqueous phase obtained was again extracted with $CHCl_3$ (5 × 150 ml). The $CHCl_3$ fraction was then evaporated to dryness, yielding 495 mg of extract.

A concentrated solution of 400 mg of the extract was adsorbed onto basic alumina (800 mg, Merck) and chromatographed over an alumina column (10 g) with ether (300 ml) as eluant. Preparative TLC of the eluate was performed on silica (Fluka) gel plates made up in 0·1 M NaOH and developed three times using $CHCl_3$:ethyl acetate:methanol (2:2:1). Three distinct bands were obtained. Band 2 was re-extracted as above (2% H_2SO_4 [10 ml] → concentrated ammonia → $CHCl_3$ [2 × 10 ml]). The final $CHCl_3$ phase was dried using anhydrous Na_2SO_4, filtered, and evaporated (Rotaevaporator, 40°C) to dryness. The material was then redissolved in methanol (HPLC grade, May & Baker) and dried under nitrogen to yield 37·3 mg of PH.

The material obtained was identical to pure PH reference standard with respect to its mass spectrum, proton magnetic resonance, melting point and optical density properties (Tackie *et al.*, 1974).

Drug Preparation

Chloroquine diphosphate (Sigma) was prepared as a 4 mM stock solution in distilled water, filter-sterilized (0·45 μm Millipore filter), and kept at −20°C. PH was freshly prepared for each experiment. PH (1 mg) was weighed into a clean glass vial (Trident) and dissolved in 200 μl of 100% ethanol. CM was then added to obtain a stock concentration of 1·607 mM (1 mg ml^{-1}) in 20% ethanol.

Before use, both drugs were serially diluted to twice the required top concentration in CM. For PH, the final concentration of ethanol in the highest drug concentration when tested alone was 1%. This concentration of ethanol was previously shown to have no significant effect on

the malaria parasites. In combination experiments, the concentration of ethanol was not higher than 0·004%.

Determination of Inhibitory Concentrations of Each Drug Alone

Drug sensitivity tests were carried out in 96-well micro-titre plates according to the method of Desjardins *et al.* (1979) with modifications by O'Neill *et al.* (1985). CQ was tested in two-fold serial dilutions, while PH was tested in four-fold serial dilutions. Each well contained a final volume of 100 µl: 50 µl of medium (with or without drug) and 50 µl of red blood cells (parasitized at 0·5% or non-parasitized) at 5% HC. The final HC per well was 2·5%. Asynchronous cultures with mainly ring stages were used. Tritiated hypoxanthine (0·2 µCi) (Amersham) was added to each well after 24 hours incubation, then cells were harvested after a further incubation period of 18–24 hours onto glass fibre filter mats (Titertek) using a semi-automatic cell harvester (Skatron, 7020). The dried discs were placed in 3 ml aliquots of ecoscint (National Diagnostics) and counted in a Tri-Carb scintillation spectrometer (Packard, model 574).

IC_{50} values were obtained for each drug using a computer programme which corrected for incorporation of ^{3}H-hypoxanthine into uninfected cells and non-drug treated infected cells, and converted the counts per minute at each drug concentration into percentage inhibition.

Determination of the Effect of Phaeanthine on the IC_{50} of Chloroquine

The ability of PH to reverse CQ resistance in the two lines of *P. falciparum* was determined using the method of Martin *et al.* (1987). PH concentrations tested in combination with CQ were 80·34 nM, 160·7 nM and 321·4 nM.

Potentiation

The effect of PH on the activity of CQ was examined *in vitro*. The two drugs were first tested individually, then as a combination in various proportions of their IC_{50} values (De Jongh, 1961; Berenbaum, 1978).

From the IC_{50} values derived for the single drugs, different concentrations corresponding to different fractions of the IC_{50} values of each drug were prepared in CM at twice the required final concentration. Both compounds were then tested in various combinations, either with CQ at a fixed concentration throughout while the concentration of PH was varied, or with PH at a fixed concentration while the concentration of CQ was varied. At the same time, CQ and PH were tested alone so that IC_{50} values could be obtained for use in analysis of the potentiation results. Controls, in this case, were in medium without drug.

Analysis of Data: Potentiation

IC_{50} values for the single drugs alone were obtained from dose–response curves, and concentrations used in setting up the experiments were based on these values. The concentrations of both CQ and PH used in the potentiation experiments were expressed as fractions of the IC_{50} values for the single drugs measured in the drug combination experiments (i.e. as isobolar units where IC_{50} of the single drug = 1·0 isobolar unit).

Likewise, IC_{50} values were determined for one drug in the presence of different concentrations of the other. These values were derived from the linear portions of the sigmoidal semi-log dose response curves. The IC_{50} values were then converted into fractional isobolar units using the equation:

$$\text{Fractional isobolar unit for drug A} = \frac{IC_{50} \text{ of drug A in combination of drugs B (fixed) and A (varied)}}{IC_{50} \text{ of drug A alone}}$$

When, in combinations, IC_{50} values higher than the measured IC_{50} values of the individual drugs were observed, they were plotted as 1·0 isobolar unit.

Construction of Isobolograms

The isobolar units obtained when the CQ concentration was fixed and PH varied, or vice versa, were plotted on isobolograms [Fig. 2(A) and (B)]. A straight line was drawn joining the IC_{50} values (i.e. 1·0 isobolar unit) for the individual drugs. Points above the line represent antagonism, and points falling below the line indicate potentiation (synergism). Points close to or on the line indicate an additive effect.

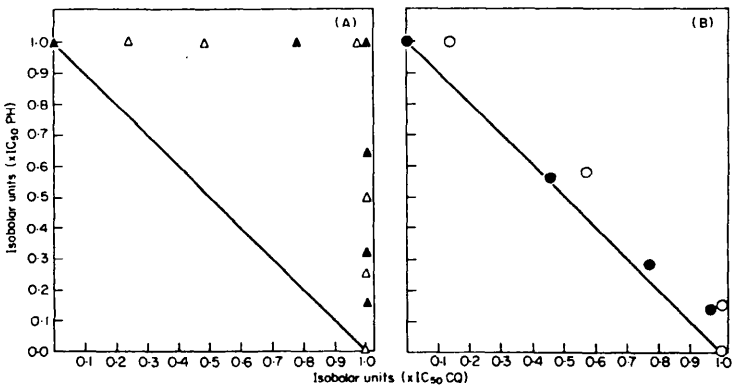

Fig. 2. Isobolograms showing the effects of using combinations of chloroquine and phaeanthine in T9-96 (CQ-S) and K1 (CQ-R). (A) Antagonism observed in T9-96 when CQ concentrations were fixed and PH concentrations varied (△), or when PH concentrations were fixed and CQ concentrations varied (▲); (B) additive effect in K1 at fixed CQ/varied PH concentrations (○), or at fixed PH/varied CQ concentrations (●).

TABLE I

IC_{50} values of single drugs alone when tested against Plasmodium falciparum *strains*

	T9-96		*K1*		*Ratio*
Drugs	*$\bar{x}$ (nM)*	*(S.E.; N)*	*$\bar{x}$ (nM)*	*(S.E.; N)*	*K1/T9-96*
CQ	13·325	(1·2; 4)	221·5	(19·00; 3)	16·62
PH	704·87	(81·48; 4)	365·85	(11·41; 4)	0·52
Ratio PH/CQ	52·90		1·65		

$\bar{x}$ = Mean IC_{50} value.
S.E. = Standard error.
N = No. of experiments.
nM = nanomolar.

RESULTS

Sensitivities to Single Drugs

Table I shows how the two lines of *P. falciparum* (T9-96 and K1) differ in their sensitivities to CQ and PH alone. The degree of resistance is

reflected by the ratio of the IC_{50} of resistant parasites to the IC_{50} of sensitive ones. T9-96 is more sensitive to the action of CQ than is K1 by a factor of almost 17, whereas K1 is twice as susceptible as T9-96 to PH ($P < 0.02$; Student's *t*-test). When the activities of the two compounds are compared within each line of *P. falciparum*, CQ exhibits greater activity (almost 53-fold) than PH in T9-96 while there is not much difference in activity between CQ and PH in K1.

Isobolograms

The effects of using a combination of PH and CQ on CQ-S and CQ-R *P. falciparum in vitro* are represented in Fig. 2(A) and (B). Against the CQ-S clone (T9-96) the combined drugs produced clear antagonism, but an additive effect was observed against the CQ-R K1 strain of *P. falciparum*. These results were reproduced in six separate experiments.

Cytotoxicity of PH to KB cells

KB cells were used to assess cytotoxicity of the BBIQ alkaloid, PH, in a microtechnique developed by Anderson *et al.* (1991). CQ was also evaluated in this system. The ratio of cytotoxicity to antimalarial activity for both PH and CQ suggests that both compounds are more active against malaria parasites than against mammalian cells (Table 2). At the highest concentration of PH tested (321·4 nM), which had a marked effect on malaria parasites (data not shown), cytotoxicity was negligible *in vitro*. There is a 1·72-fold difference between the calculated IC_{50} values of CQ and PH against the tumour cell lines (Table 2).

DISCUSSION

Triclisia species are used in West Africa for the treatment of malaria (Irvine, 1961), and our results show that PH, a BBIQ alkaloid isolated from the woody part of *T. patens*, had *in vitro* antimalarial activity against *Plasmodium falciparum*. The *in vitro* IC_{50} of PH against T9-96, the CQ-S clone, was approximately twice the value obtained for K1, the CQ-R strain. This pattern of sensitivity of CQ-S and CQ-R lines to BBIQ alkaloids has also been shown by Ye and Van Dyke (1989) using TT, the enantiomer of PH, where the difference in sensitivity to the BBIQ alkaloid was three-fold. Similarly, Shiraishi *et al.* (1987) have shown that a multidrug-resistant cancer cell line was more sensitive to two BBIQ alkaloids tested than was the sensitive parent line.

CQ and PH, in combination, showed an additive effect upon the CQ-R strain, but a definite antagonistic effect upon the CQ-S clone. It is possible that these effects are related to structural similarities between CQ and PH. Molecular modelling reveals that, apart from both agents having conjugated aromatic rings, the variable distance between the two protonatable nitrogen atoms of CQ [‡ in Fig. 1(B)] can extend to 9·75 Å, which is the measured distance between the two similar nitrogen atoms in PH (Partridge and Blagborough, unpubl. obs.). This suggests that competition of PH for the chloroquine uptake system (Homewood *et al.*, 1972) could account for mutual antagonism. In a chloroquine-resistant strain, competition might take place between the agents at both the uptake system and the export system, the latter apparently the basis of resistance (Krogstad *et al.*, 1987). This would have opposite effects of antagonism and potentiation, which might account for the additive effect observed in the resistant strain.

KB cells (mammalian epidermal cells) from carcinoma of the nasopharynx (Eagle, 1955) have been used to evaluate the cytotoxic potential of various compounds, both natural and synthetic (Sun *et al.*, 1987; Pirker *et al.*, 1988). In addition, it has been possible to separate cytotoxicity from antiviral activity using this cell line (Nasr *et al.*, 1988). According to the Cancer Chemotherapy National Service Centre, a pure compound is considered active in the 72-hour KB assay system if the average ED_{50} from two tests is 4 μg ml^{-1} or less (Kupchan *et al.*, 1965). Results we have obtained indicate a clear selective toxicity of PH against *P. falciparum* in comparison with KB cells. The IC_{50} of PH against KB cells in our test was 42·285 μg ml^{-1} (Table 2).

Varying degrees of cytotoxicity against different tumour cell lines have been recorded

TABLE 2
Comparison of antimalarial activity and cytotoxicity of PH and CQ

	IC_{50} values (nM)			*Ratio: cytotoxic IC_{50}/antimalarial IC_{50} (B/A)*	
	Malaria (A)				
Drugs	*K1*	*T9-96*	*KB cells (B)*	*KB/K1*	*KB/T9-96*
PH	365·85	704·87	67 949 (42·285 μg ml^{-1})	185·72	96·40
CQ	221·5	13·32	117 000 (72·8 μg ml^{-1})	528·2	8783·8
PH CQ	1·65	52·92	0·58		

for TT (Suffness and Cordell, 1985), with an ED_{50} value of 146·23–273·18 nM (0·091–0·17 μg ml^{-1}) against KB cells (Kupchan *et al.*, 1965). Certain studies have accorded the stereochemistry at C-1 and C-1′ some importance (review by Suffness and Cordell, 1985), as only BBIQ alkaloids with S,S configuration (except cepharanthine) exhibited good antitumour activity in both *in vitro* and *in vivo* test systems.

In this study, PH, with its own intrinsic antimalarial activity, shows antagonism in CQ-S T9-96 and additive effects in CQ-R K1 when combined with CQ. It is not certain how PH acts, but the parasite lysosome and membranes of the host/parasite complex are possible sites of action.

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Antimalarial activity of an aqueous tea prepared from Brucea javanica fruits

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We reported previously (O'Neill et al 1987) that Brucea javanica fruits contain quassinoids, some of which possess antiplasmodial activity both in-vitro and in-vivo. The most active of these quassinoids are generally highly lipophilic compounds. In traditional medicine, however, an aqueous tea is prepared from the fruit and is drunk as a treatment for malaria (Reid 1986). We were therefore interested to examine the antiplasmodial activity and quassinoid constituents of such a tea.

Fruits obtained from Hong Kong were boiled in water for 1 hour and after cooling, the tea was filtered and freeze dried. This material was tested for activity against Plasmodium falciparum in-vitro, using an assay which measures inhibition of incorporation of [^{3}H]-hypoxanthine (Desjardins et al 1979) and for its ability to suppress P. berghei infections in mice (Peters 1984). Results are given in Table 1. Phytochemical investigation of the freeze dried tea showed the presence of very small quantities (1.9%) of lipophilic quassinoids together with much larger quantities of polar quassinoids. A portion of the aqueous tea was subjected to acid hydrolysis by refluxing with 3N H_2SO_4 aq/methanol (1/1) for 5 hours. The mixture was cooled and extracted with chloroform. This chloroformic extract (18% of unhydrolysed tea) was found to be rich in lipophilic quassinoids. Bruceines A and C, and brusatol were subsequently isolated and identified by their NMR, MS and chromatographic characteristics (O'Neill et al 1987). The total chloroformic extract after hydrolysis was also examined for antiplasmodial activity in-vitro and in-vivo and results are given in Table 1. The chloroformic extract which was obtained after hydrolysis of the tea, and which was rich in lipophilic quassinoids was some 39 times more active in-vitro than the unhydrolysed tea. In-vivo, the chloroformic extract was also the more active but there was a mere 4.5 fold difference between the activities of the two preparations. The results indicate that the aqueous tea prepared from B. javanica fruits contains very small quantities of highly active lipophilic quassinoids together with much larger quantities of less active polar derivatives. Some of these polar compounds, which are glycosidic forms can be converted into lipophilic quassinoids by acid hydrolysis. Partial hydrolysis of the polar quassinoids into lipophilic quassinoids by stomach acid may explain the relatively small difference in the activities of the two preparations following oral administration.

Table 1. In-vitro and in-vivo antiplasmodial activities of an aqueous tea from B. javanica fruits and a hydrolysed extract

	A Aqueous tea	B Chloroformic extract after hydrolysis of tea	A/B
In-vitro IC_{50}* P. falciparum $\mu g.ml^{-1}$	0.43	0.011	39.1
In-vivo IC_{50}** P. berghei $mg.kg^{-1}.day^{-1}$	500	112	4.5

*Based upon duplicate 4 fold dilutions from 50 μgmL^{-1}

**Tested at 4 concentrations using 5 mice at each dose level. Each mouse received 4 doses of drug.

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A microdilution assay for the determination of *in vitro* cytotoxicity of potential antimalarial agents against KB cells

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Quassinoids are responsible for the *in vitro* antiplasmodial activity of *Brucea javanica* fruits (O'Neill et al 1987) which are used in Chinese traditional medicine (Reid, 1986). With any potential chemotherapeutic agent, it is important to distinguish between specificity of action and non-selective cytotoxicity and to this end we have developed a microdilution assay in order to assess the *in vitro* cytotoxicity of potential antimalarial compounds.

The technique is a modification of the standard 9-KB cytotoxicity assay initially described by Eagle (1958) and utilises cells derived from a human epidermoid carcinoma of the nasopharynx. For the assay, 50 µl of a 1 x 10^6 cell ml^{-1} suspension in Eagles Minimum Essential Medium with Earle's salts, sodium bicarbonate, foetal bovine serum and glutamine, are added to a 96-well microtitre plate containing two-fold serial dilutions of test compounds. The plates are incubated at 37°C in a 5% CO_2 atmosphere for 48 hours. After removal of medium, the remaining layer of cells is fixed with methanol before staining with 0.5% aqueous solution of eosin. When dry, 0.1 M NaOH is added to each well and the optical density of the resulting solution determined at 490 nm. The percentage inhibition of cell growth is calculated by comparison of control and test wells and IC_{50} values determined. Four quassinoids isolated from *B. javanica* fruits (O'Neill et al 1987) have been assessed for their cytotoxicity against KB cells and comparisons made with their activities against multi-drug resistant *Plasmodium falciparum* (K1) (Table 1).

Table 1. *In vitro* cytotoxicity compared to *in vitro* antiplasmodial activity[a] of quassinoids isolated from *Brucea javanica* fruits

Quassinoids	A KB cells (IC_{50} µg ml^{-1})[b]	B *P. falciparum* (IC_{50} µg ml^{-1})[c]	A/B
Bruceantin	0.008	0.0008	10.0
Brusatol	0.102	0.003	34.0
Bruceine A	0.098	0.011	8.9
Bruceine D	1.158	0.015	77.2

[a]Inhibition of incorporation of [^{3}H]-hypoxanthine
[b]Based on two-fold dilutions in duplicate
[c]From O'Neill et al 1987

Bruceantin was the most potent of the four quassinoids tested against KB cells, brusatol and bruceine A had similar activity whilst brusatol was the least cytotoxic (Table 1). Bruceantin and bruceine A had similar cytotoxic to antiplasmodial ratios (10.0, 8.9, respectively) whereas brusatol and bruceine D had corresponding values of 34.0 and 77.2, respectively. Of the four quassinoids tested, bruceine D was the least cytotoxic and possessed the most favourable cytotoxic to antiplasmodial ratio. The results indicate that cytotoxicity to KB cells does not necessarily parallel *P. falciparum* activity. The microtitre test developed for KB cells is rapid and sensitive, does not necessitate the use of radiolabelled compounds or require an involved protein determination procedure. It provides a useful comparison for *in vitro* antiplasmodial activity.

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ANTIPLASMODIAL ACTIVITY OF AN AQUEOUS TEA PREPARED FROM *BRUCEA JAVANICA* FRUITS AGAINST *P. FALCIPARUM* AND *P. BERGHEI*

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The fruits of the Simaroubaceae plant *Brucea javanica* are used in Chinese herbal medicine for the treatment of malaria. Extracts of the fruits contain quassinoids (O'Neill M.J. *et al*, 1987: J. Nat. Prod. 5(1), 41-48), some of which demonstrate *in vitro* and *in vivo* antiplasmodial activity. The most lipophilic quassinoids possess the greatest activity, yet the traditional treament for malaria involves drinking an aqueous extract or 'tea'. An aqueous tea was prepared from the fruits and was found to contain approximately 2% chloroform-soluble material, which included lipophilic quassinoids. A further 18% chloroform-soluble material was generated after acid hydrolysis of the aqueous tea. A series of lipophilic quassinoids was isolated from the chloroform extract and these had *in vitro* IC_{50} values between 0.011 - 0.0008 $\mu g\ ml^{-1}$ against *P. falciparum* (K1). The chloroform extract after hydrolysis was some 39 times more active than the unhydrolysed tea *in vitro*. *In vivo*, against *P. berghei* (N) the chloroform extract (ED_{50} 112 $mg.kg^{-1}.day^{-1}$) was, however, only 4.5 times less than that for the complete tea (ED_{50} 500 $mg.kg^{-1}.day^{-1}$). This difference between the *in vivo* and *in vitro* activities of the two preparations may be explained by conversion of polar forms into more active lipophilic quassinoids by stomach acid following oral administration.

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In vitro cytotoxic, antimalarial and antiamoebic activities of protoberberine alkaloids

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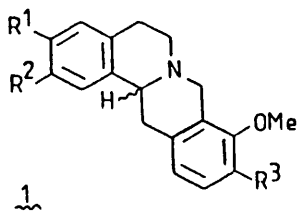
A wide range of plants is used in traditional medicine for the treatment of protozoal diseases (Phillipson and O'Neill, 1987). Some of these plants contain protoberberine alkaloids which may be responsible for their claimed therapeutic effects (Vennerstrom and Klayman, 1988). We have tested five alkaloids for their activities against KB cells as a measure of cytotoxicity to mammalian cells and against two protozoa, Plasmodium falciparum (K1, multi-drug resistant strain) and Entamoeba histolytica (NIH 200). The results are given in Table 1.

Table 1. In vitro cytotoxic, antimalarial and antiamoebic activities of protoberberine alkaloids



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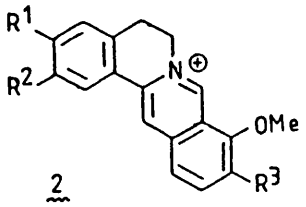

2

Alkaloid[a]	R^1	R^2	R^3	KB(A)	IC_{50} values (μM) P. falciparum (B)	E. histolytica	A/B
(±)-canadine 1	$-OCH_2O-$		OMe	>730	>147	126	-
berberine 2	$-OCH_2O-$		OMe	7.3	0.97	111	8
thalifendine 2	$-OCH_2O-$		OH	>698	7.9	115	>88
jatrorrhizine 2	OH	OMe	OMe	>334	3.1	83	>106
columbamine 2	OMe	OH	OMe	78	1.9	156	41

[a]Alkaloids (2) tested as chloride salts

Berberine was the only alkaloid which was toxic to KB cells and the other four alkaloids were non-toxic. Berberine also proved to be the most active alkaloid against P. falciparum and showed marked contrast with the non-active (±)-canadine, the corresponding tetrahydroprotoberberine alkaloid with identical substituents in rings A and D. Thalifendine which differs from berberine in having R^3=OH instead of OMe was approximately ten times less active against P. falciparum. Jatrorrhizine and columbamine were similar to berberine in their activity against P. falciparum and they differ only in the nature of their R^1 and R^2 substituents in ring A (Table 1). None of the alkaloids showed significant activity against E. histolytica and hence showed some selective action. It is surprising that berberine was inactive because it is used in some countries as an amoebicide. In assessing relative in vitro cytotoxicity to antiplasmodial activity, berberine proved to have the least favourable ratio while the closely related jatrorrhizine had a more favourable ratio. These results lend some support to the use of plants containing protoberberine alkaloids for the treatment of malaria but not for the treatment of amoebiasis.

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Title*

Antimalarial activity of 3-methoxyethoxymethyl brusatol, an etherified quassinoid.

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Laboratory Meeting

Text*

Quassinoids are probably responsible for the anti-malarial properties of traditional remedies prepared from simaroubaceous plants. Treatment of the quassinoid brusatol with methoxyethoxmethyl (MEM) chloride produced a 3-MEM ether with 6-times more activity against Plasmodium falciparum, in vitro, than the parent quassinoid. In vitro cytotoxicity screening against human KB cells showed the compound to be markedly less toxic than brusatol. Subsequent testing in P. berghei-infected mice showed that, at doses of 3-MEM brusatol of up to 275 mg/kg/day subcutaneously for four days, no mice died. Whilst these animals displayed none of the overt symptoms of malaria (lethargy, poor temperature control, reduced grooming reflexes), parasitaemias at the end of the test did not, however, differ significantly from those of non drug-treated controls. Only two out of four mice treated with brusatol (3.4 mg/kg/day s.c.) survived to day four, and tail-blood smears showed only occasional parasites. Further studies are required to confirm the apparent enhanced in vitro antimalarial toxicity of the etherified quassinoid and to establish whether it has any useful antimalarial activity *in vivo* at higher doses.

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