ANTIPLASMODIAL ACTIVITY OF MEDICINAL PLANT PREPARATIONS T610 AND S076 USING PLASMODIUM FALCIPARUM IN VITRO CULTURE SYSTEM

A THESIS SUBMITTED

BY

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DEDICATION

To God Almighty, my husband, Kenneth and children

Dorcas and Lois Appiah-Opong
DECLARATION

This certifies that the experimental work described in this thesis was performed by me at the Noguchi Memorial Institute for Medical Research, Legon. The work has not been accepted for any degree and is not being submitted in candidature for any other degree. All references cited in the thesis have been fully acknowledged.

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To God be the glory
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LIST OF ABBREVIATIONS

CPD          Citrate-phosphate-dextrose
ACD          Acid-citrate-dextrose
DMSO         Dimethyl sulfoxide
HEPES        N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid RPMI
HPIA         Haem polymerization inhibitory activity
NHS          Normal human serum
RBC          Red blood cell
MTT          Methylthiazolyldiphenyl-tetrazolium bromide
DDT          Dichloro-diphenyl-trichloroethane
HPO          Hypoxanthine
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ABSTRACT

Some traditional medical practitioners use decoctions of the plants *Tridax procumbens* and *Phyllanthus amarus*, separately, to treat malaria in Ghana. These plants have however, not been investigated scientifically to establish their antimalarial activities. In this study, inhibition of chloroquine-resistant *Plasmodium falciparum* uptake of $^3$H-hypoxanthine was used as an *in vitro* assay to assess the antiplasmodial activities of aqueous, ethanolic, chloroform and ethyl acetate extracts of *Tridax procumbens* and *Phyllanthus amarus*. Chloroquine was used as a reference antimalarial drug. Cytotoxicities of the extracts to red blood cells were also investigated. Furthermore, the aqueous extracts of the plants were evaluated for haem polymerisation inhibitory activity.

The results show that high concentrations of chloroquine inhibited the uptake of $^3$H-hypoxanthine by *Plasmodium falciparum*, confirming the chloroquine-resistant nature of the parasites used. Both plant extracts also demonstrated antiplasmodial activity against the chloroquine resistant plasmodial parasites. Among the various extracts, the lowest 50% inhibitory concentrations (IC$_{50}$) of 24.8 and 11.7 μg/ml corresponded to the aqueous and ethanolic extracts, respectively, of *Phyllanthus amarus*. For *Tridax procumbens*, the lowest IC$_{50}$ values were 225.0 and 143.4 μg/ml for the ethanolic and aqueous extracts, respectively. Unlike chloroquine, none of the extracts inhibited haem polymerisation. Within the concentration range used, the least cytotoxicity to RBCs was observed in the aqueous extracts of both plants, the ethanolic extract of *Phyllanthus amarus* and the ethyl acetate extract of *Tridax procumbens*. These results suggest that the aqueous and ethanolic extracts of both plants were more effective as antiplasmodial preparations than the other extracts.
CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Malaria is a parasitic disease endemic in most tropical countries of the world, although individuals in other continents are potentially at risk. The annual global incidence of malaria is estimated to be approximately 200 million cases (Dyke and Ye, 1994). Tropical Africa accounts for about 80% of these clinical cases with over 250 million people infected and carrying the parasites (WHO, 1992). Malaria is still the most important parasitic disease in the world, causing 2-3 million deaths every year (WHO, 1997) with more than 1 million of this number being children under the age of 14 years.

The malaria situation in sub-Saharan Africa is ghastly. The disease now constitutes the leading cause of poverty, accounting for annual losses of up to US$ 12000 million (WHO, 2000). In Ghana, malaria is hyper endemic. It is a major cause of morbidity and mortality, especially among children below the age of 5 years, and accounts for 7.8% of all certified deaths in children and adults (Afari et al., 1995). In general malaria is considered to be the cause of the highest loss of the number of days of healthy life in Ghana. Transmission of the disease is intense, stable and perennial with slight seasonal variations. There is however, no reliable information on the impact of malaria on labour and productivity and the economy (Min. of Health. Rep. of Ghana, 1992).

The epidemiology of malaria is complex and may vary considerably even within relatively small geographic areas. Malaria transmission to man depends on several
interrelated factors (Gillies, 1988 and Molineaux et al., 1988). The most important pertains to the anopheline mosquito vector and in particular its longevity. As sporogony (development of sporozoite parasites in the vector) takes over a week (depending on ambient temperature), the mosquito must survive for longer than this after feeding on an infected human for transmission to occur. Malaria is usually a “rainy season disease” coinciding with increased mosquito abundance. However in some areas parasite rates (i.e. the proportion of people with positive blood smear) are relatively constant throughout the year (McGregor, 1965).

The principal vectors of malaria in Ghana are the Anopheles gambiae complex and An. funestus. An. melas found in salt-water bodies in the coastal areas, An. nili and An. hargreavesi are considered to be secondary vectors. Resistance of vector mosquitoes to some of the available insecticides has been reported, which has further exacerbated the threat of drug resistance (Werndorfer and Kousnetov, 1980).

Only three species of human plasmodia have been documented in Ghana. They are Plasmodium falciparum, P. malariae and P. ovale. Plasmodium falciparum is the most predominant parasite species, accounting for 80-90% of malarial infections. Therefore malaria control programs in Ghana often target P. falciparum. The Ministry of health of Ghana launched its action plan for malaria control in November 1992. The action plan had the principal aim of reducing malaria-related mortality through early diagnosis and effective treatment. In addition to this, is the effort to develop malaria vaccine although none has been ratified for use. The availability of an effective vaccine would undoubtedly facilitate the control of malaria. However for now chemotherapy is the only means available.
Antimalarial drugs used for effecting clinical cure include chloroquine, primaquine, mepacrine, quinine, proguanil, mefloquine and artemisinine. In Ghana the Ministry of Health has directed that chloroquine be used as the drug of choice for malaria treatment, while quinine and sulphadoxine-pyrimethamine (Fansidar) may serve as second and third line drugs for use against chloroquine-resistant plasmodial strains (Ministry of Health Poster, 1992). In spite of various strategies, malaria chemotherapy the world-over is confronted with the challenge of drug resistant malaria parasites. The emergence and spread of parasites resistant to both chloroquine and alternate drugs have been reported (Ofulla et al., 1996). This makes the search for new antimalarial drugs imperative.

Plant medicines handed down through folklore have been important sources of therapeutic agents. Several herbal remedies have also provided locally accessible alternatives to important drugs including antimalarials. It is estimated that more than 80% of the world’s population rely on indigenous systems of medicine that are mainly plant based (Principe, 1989). Many drugs that are currently on the market came into use through a systematic scientific study of herbal remedies. For example, the bark of the cinchona tree came to medical attention in Peru between 1628 and 1629 as a local remedy used against fevers (Lancet, 1992). In 1820 the French chemists Pierre Pelletier and Joseph Caventou isolated the alkaloid quinine from cinchona bark (Cook, 1996). Purification of the various cinchona alkaloids allowed standardization of dosage. In 1880, Laveran discovered that quinine cured the disease by killing the plasmodia parasites. As a result of these developments, diagnosis of malaria and prescription of cinchona alkaloids became more rational and logical. Furthermore, the most important development in recent years has been the
rediscovery and development of drugs related to artemisinine (qinghaosu), from the qinghao plant with China as its origin. Extracts of the qinghao plant have been used in traditional medical practice for over two millennia.

In Ghana a number of medicinal plants and preparations from them are used to treat malaria. Some of the plants are Tridax procumbens and Phyllanthus amarus, coded T610 and S076 respectively. However, these plants have not been investigated to establish their value as antimalarial agents. T610 has been reported to have antibacterial and antiprotozoal activities (Perumal et al., 1999 and Caceres et al., 1998). The antiprotozoal activities reported were against some forms of Trypanosoma cruzi but not P. falciparum. In Ghana T610 is also used as feed for guinea pigs and rabbits. Poultice of the plant are applied to whitlow and a decoction of it is used as antimalarial remedy. Plants of same genus as S076 are widely used for the treatment of jaundice and other diseases and are well tolerated. S076 inhibits the DNA polymerase of hepatitis B virus and reverse transcriptase (Mullen et al., 1996). There is no doubt that certain medicinal plants could be developed into acceptable antimalarials. Due to the high cost of alternative drugs to chloroquine in Ghana, it is important to undertake scientific investigations into the potential of these extracts and others as therapeutic antimalarial agents since they are cheaper.

**Rationale and Objective**

This study is based on reports by traditional medical practitioners of the efficacy of the decoction of plants T610 and S076 as antimalarials. These extracts are widely used in Ghana as antimalarial remedies without scientific proof of their efficacy. Since the cost of alternative drugs to chloroquine in Ghana is high, it is important to
undertake scientific investigations into these plant extracts in the search for potentially effective and affordable antimalarial drugs. The specific objectives were to:

1. investigate the activity of T610 and S076 against a chloroquine-sensitive strain of *P. falciparum* (3D7).
2. determine the activity of extracts of T610 and S076 against a chloroquine-resistant strain of *P. falciparum* (Dd2).
3. evaluate the cytotoxicity of the plant extracts on human RBCs.
4. assess whether the antiplasmodial effects of T610 or S076 were achieved through haem polymerization.
LITERATURE REVIEW

MALARIA

Malaria is an infectious parasitic disease, particularly prevalent wherever conditions are suitable for the maintenance of the anopheline mosquito vector. The causative organisms in man are four species of protozoan parasites of the genus Plasmodium, namely *P. vivax*, *P. malariae*, *P. ovale* and *P. falciparum* (Russell, 1993). Although all may cause severe illness, *P. falciparum* causes most of the serious complications and deaths. The classic malaria symptoms are chills, followed by fever and sweating. The clinical manifestations of malaria are dependent on previous immune status of the host.

LIFE CYCLE OF THE MALARIA PARASITE IN MOSQUITO

The female mosquitoes require blood for the maturation of their eggs. Following ingestion of the blood meal of a biting female anopheline mosquito (Appendix II), the mosquito proceeds to digest the blood in its alimentary tract. In the midgut, mature gametocytes freed of their red cell envelopes, are transformed into gametes. By the process of exflagellation, the male gametocyte forms eight threadlike structures. The motile male microgametes then separate and seek the female macrogametes. Fusion and meiosis then takes place resulting in a zygote formation. Within 24 hours the enlarging zygote becomes motile, and this form (the ookinete) penetrates the epithelial cell wall of the mosquito midgut where it encysts (oocyst). The nucleus of the young oocyst undergoes meiotic division about 48 hours after a blood meal. This is followed by successive mitotic divisions, resulting in enormous growth of the oocyst and its protrusion into the hemocoel (body cavity) of the mosquito. After it matures, the oocyst bursts and releases thousands of spindle-
shaped, motile sporozoites into the hemocoel. They migrate to all parts of the mosquito’s body and some enter the cells of the salivary glands. Just prior to the ingestion of blood, the infected mosquito inoculates salivary fluid containing some sporozoites. This initiates the cycle in the human host.

LIFE CYCLE OF THE MALARIA PARASITE IN THE HUMAN HOST

Infection with human malaria begins when the feeding female anopheline mosquito inoculates plasmodial sporozoites from its salivary glands into the host at the time of feeding (Appendix II). After injection they enter into circulation, either directly or via lymph channels, and rapidly target the hepatic parenchyma cells. The sporozoites bore into the hepatocytes and there begins a phase of asexual reproduction (exo-erythrocytic schizogony) during which many thousands of merozoites are produced. This phase is asymptomatic for the human host as only a few liver cells are usually infected. The merozoites are released from each ruptured infected hepatocyte into the blood stream where they initiate the erythrocytic phase of the cycle. The erythrocytic phase commences 5.5 days after the sporozoites are introduced in *P. falciparum*. This malaria parasite attacks red blood cells at all stages of maturity. The young intracellular trophozoite usually has a ring-shaped appearance. Parasites are freely motile within the erythrocyte and they consume its content. Proteolysis of haemoglobin within the digestive vacuole releases amino acids that are taken up and utilized by the growing parasite for protein synthesis. Haem, freed from its protein scaffold oxidizes to the toxic ferric form (ferriprotoporphyrin IX) but this toxicity is avoided by spontaneous polymerisation to an inert crystalline substance, haemozoin (Slater and Cerami, 1992).
At approximately 36 hours after merozoite invasion repeated nuclear division takes place to form a ‘segmenter’ or schizont. Eventually the growing parasite occupies the entire red cell, which has become circular, rigid, depleted in haemoglobin and full of merozoites. This then raptures destroying the remnants of the red cell and the merozoites released rapidly reinvoke other red cells and start a new asexual cycle (erythrocytic schizogony). Thus, the infection expands logarithmically and this phase in *P. falciparum* lasts 48 hours. After a series of asexual cycles a sub-population of parasites develop into sexual forms (gametocytes) that are long-lived and motile. Mature gametocytes do not develop further in the human host, but they are the only forms of the malaria parasite that are infective to susceptible anopheline mosquitoes.

**CLINICAL MANIFESTATIONS**

Chills followed by fever and sweating constitute the classic malaria paroxysm. Other commonly associated symptoms include headache, backache, myalgia, nausea, vomiting, anorexia, diarrhoea and cough. Common presenting features in severe falciparum malaria in childhood include generalized seizures, coma, lactic acidosis, hypoglycaemia and severe anaemia, with the latter being the most common presentation.

In cerebral malaria the onset of coma may be sudden, often following a generalized seizure with initial drowsiness, confusion, disorientation, delirium or agitation followed by unconsciousness (Bruce-Chwath, 1986). On examination the patient is febrile and there may be some passive resistance to hand flexion. A typical febrile paroxysm often starts in the afternoon or evening hours. It consists successively of a cold stage, associated with a shaking chill (usually less than 1 hour), a hot stage...
associated with a temperature of 40°-41°C, and a flushed, dry skin. The patient is usually warm and dry with a low or normal blood pressure.

**DIAGNOSIS**

Identification of the plasmodial species causing the infection is mandatory if the clinician is to select appropriate chemotherapy and prevent death from falciparum malaria. Examination of a thick blood film should be the first step in diagnosis, since this has the advantage of concentrating the parasites by 20 fold in comparison to a thin film, although the parasites may appear distorted making species identification difficult. If parasites are seen, then the species should be confirmed by the examination of a thin film. Ideally blood should be collected when the patient's temperature is rising.

In recent years a number of new techniques based on the "dipstick" format, have become available for the diagnosis of malaria (RPH Laboratory Medicine, 2001). These include the ICT-Malaria Pf, OptiMALr and the Determine kits. The methods are based on the principle of the detection of plasmodial histidine rich protein-2 (HRP-2) or parasite-specific lactate dehydrogenase (pLDH) that is present in *P. falciparum* infections. A number of reports claim sensitivities and specificities approaching 100% while other reports have claimed up to 6% cross reactivity with sera positive for rheumatoid factor. Some of these "dipstick" methods have been extended to include screening for other forms of malaria but to date results have not been quite so impressive. Dipstick tests have the potential of enhancing the speed and also the accuracy of diagnosing *P. falciparum*, particularly in non-specialised
laboratories where inexperienced or junior staff may be involved, since very little training is required for these techniques.

MALARIA CHEMOTHERAPY

Malarial chemotherapy is still one of the big areas of concern worldwide. A lot has been done to try and find the perfect drug that would bring about the eradication of malaria and alleviate suffering caused by this age-long disease. The antimalarial drugs have been classified by their selective actions on different phases of the parasite’s life cycle (Goldsmith, 1989). Drugs that eliminate developing tissue schizonts or latent hypnozoites in the liver, such as primaquine are called tissue schizonticides. Blood schizontocidal drugs which include chloroquine, amodiaquine, mefloquine and quinine, effective against asexual erythrocytic parasites, are used either as suppressive prophylactics to prevent the development of clinical attacks or as curative agents to achieve clinical cure. These eliminate parasites from the bloodstream. Furthermore gametocytocidal drugs such as primaquine act directly on the sexual forms (gametocytes), destroying them. Gametocytes are rendered non-infective in the mosquito by sporontocidal drugs, due to their interruption of sporogony in mosquitoes that feed on a treated person. Therefore gametocytocidal and sporontocidal drugs are important in the prevention of transmission of malaria.

Several new drugs have also been introduced due to the problem of \( P. falciparum \) resistance to chloroquine, which has been the drug of choice for a considerable period. However, it is worth noting that most of the drugs mentioned above are still in use. A perfect replacement for chloroquine must have all the qualities of chloroquine and if possible more. Artemisinine is the only compound that has come
top of the list in terms of efficacy, low toxicity, resistance, cost, rapid action and first order pharmacokinetics. Artemisinine is the most rapid-acting class of antimalarial drugs for both uncomplicated and severe malaria. It acts against the asexual stages, gametocytes and blocks sporogony, therefore its action is at all sites on the life cycle.

Major antimalarials are further classified by chemical groups and biological activity as follows:

(a) 4-aminoquinolines e.g. chloroquine, hydroxychloroquine and amodiaquine

(b) 8-aminoquinolines e.g. primaquine and trimethoprim

(c) Biguanides e.g. proguanil

(d) Diaminopyrimidines e.g. pyrimethamine

(e) Cinchona alkaloids e.g. quinine

(f) Sulphonamides e.g. sulfadoxine, sulfalene and sulfamethoxazole

(g) Sulfones e.g. dapsone

(h) 4-quinoline-carbinolamines e.g. mefloquine

(i) Sesquiterpene lactones e.g. artemisinine

(j) Antibiotics e.g. tetracycline and doxycycline

(a) 4-AMINOQUINOLINES: e.g. Chloroquine

7-chloro-4-(4’-diethylamine-1’-methylbutyl-amino) quinoline
As a 4-aminoquinoline, chloroquine has the quinoline nucleus in common with quinine and the 8-aminoquinolines and the alkyl side chain in common with mepacrine (Webster, 1990). The 4-aminoquinolines with the greatest antimalarial activity in both avian and human malarias have a chlorine atom in position 7 of the quinoline ring. The selective toxicity of 4-aminoquinolines and related blood schizontocides is based on their concentrative uptake by infected erythrocytes (Fitch, 1974). Chloroquine is the 4-aminoquinoline most widely used to prevent or terminate attacks of vivax, ovale, malariae, or sensitive falciparum malaria. It is a blood schizontocide although moderately effective against gametocytes of *P. ovale*, *P. vivax* and *P. malariae* but not against those of *P. falciparum*. Chloroquine does not effect radical cure of *P. vivax* or *P. ovale* infections, because it does not eliminate the persisting liver stages of those parasites.

The mechanism of action of chloroquine on *P. falciparum* has yet to be clearly defined, however there are prevailing views. Marchiafava and Bignami (1984) showed that chloroquine interferes with haem disposal mechanism of the parasitized erythrocyte. The also recognised that parasites were only susceptible to chloroquine when haemoglobin was actively digested to produce the malaria pigment haemozoin. There were also examples of chloroquine resistant, non-pigment producing parasites, which became susceptible when they began to produce pigment again (Ladda and Sprinz, 1969; Powers et al., 1969). The morphological response of chloroquine susceptible parasite to chloroquine is clumping of its pigment with the concomitant formation of lytic vesicles (Warhurst and Hockley, 1967; Macomber et al., 1966) and subsequent death of the parasite.
Free ferriprotoporphyrin (FP) is toxic to the parasite. It can also lyse normal erythrocytes at concentrations of 10 mmol, and has a similar effect on chloroquine resistant and sensitive *P. berghei* and *P. falciparum* parasite strains (Chou and Fitch, 1980, 1981; Orjih et al, 1986). It has been proposed that chloroquine binds to newly released FP and prevents its sequestration in malaria pigment, thereby causing lysis of the parasite (Chou and Fitch, 1980, 1981; Orjih et al, 1986; Fitch, 1986).

Another postulate of the mechanism of action is that, quinoline-containing antimalarial drugs accumulate in intracellular acid vesicles because of their weak base properties (Yayon et al, 1985; Homewood et al, 1972). It has been suggested that they may act by raising intravacuolar pH (Krogstad and Schlesinger, 1987). Ginsburg and Geary, (1987) estimated that during chemotherapy, chloroquine reaches millimolar concentrations in the food vacuole of susceptible malaria trophozoites. Other workers proposed that concentrations in this millimolar range will inhibit haem polymerase, the enzyme that polymerizes toxic FP to non-toxic haemozoin in the parasite, thereby disrupting the order of conversion of FP (haemoglobin-bound haem) into haemozoin.

(b) 8-AMINOQUINOLINES: e.g. primaquine

![Chemical structure of primaquine](image)

6-methoxy-8-(4’-amino-1-methylbutylamine) quinoline
The 8-aminoquinoline antimalarial drugs, of which primaquine is currently used, exert a marked gametocytocidal effect against all four species of plasmodia that infect man, especially *P. falciparum* (Webster, 1990). The methoxy group on the quinoline ring, as in quinine is believed to be important for antimalarial activity. The great clinical value of primaquine lies in its radical curative treatment of vivax and ovale (relapsing) malarias. Primaquine is used as a supplement to chloroquine in situations where treatment with the latter has proven unsatisfactory (Symposium, 1987). Primaquine is almost completely ineffective against the asexual erythrocytic forms of *P. falciparum* therefore it is normally combined with a schizontocidal drug.

Little is known about the mode of action of 8-aminoquinolines, especially why they are far more active against tissue forms and gametes than asexual blood forms of the malaria parasite. There is some evidence that primaquine itself accounts for the antimalarial activity, whereas metabolites of primaquine may be more active than the parent compound in causing hemolysis. Furthermore, based on findings from animal experiments, blocking action on the mitochondria of the pre-erythrocytic schizont has been suggested (Yusuf, 1992). The practical problems associated with 8-aminoquinolines are mainly related to their toxicity and the necessity for prolonged administration in radical treatment (Symposium, 1987). For example primaquine is known to induce haemolytic lesions in patients suffering from a deficiency in glucose-6-phosphate dehydrogenase, a genetic condition that is most common among inhabitants of regions in which malaria is endemic.
Biguanides are inactive against sporozoites but active against other asexual blood forms of all species of human malaria parasites. Proguanil is a pro-drug that is activated \textit{in vitro} by the mixed function oxidase system of the hepatic microsomes to form the active compound cycloguanil (Armstrong and Smith, 1974). It is an antifolate drug that has been shown to inhibit \textit{P. berghei} dihydrofolate reductase (DHFR) enzyme (Fernone \textit{et al.}, 1969), which exists in most protozoa as a bifunctional protein with thymidylate synthase (Garrett \textit{et al.}, 1984). Plasmodia, unlike humans retain the enzymes needed to synthesize folic acid from p-aminobenzoic acid (PABA), glutamic acid and pteridine, since the parasites cannot use pre-formed folic acid. Inhibition of DHFR in the parasite decreases the pool of tetrafolate co-factors, which are necessary for the synthesis of thymidylate, purines, methionine and the interconversion of glycine to serine. Therefore binding of antifolate drugs, such as cycloguanil to DHFR results in inhibition of DNA synthesis (Schellenberg and Coatney, 1961) and this as well as the depletion of folate co-factors (Gutteridge and Trigg, 1971) is responsible for the lethal effect of the drug on the malaria parasite. Outstanding features of proguanil include its extremely low toxicity, wide range of action and low cost.
Several diaminopyrimidines have high antimalarial activity, the most active being pyrimethamine which is effective against the plasmodia that infect man. The 2,4-diaminopyrimidines act by inhibiting DHFR of plasmodia at concentrations far lower than required to produce comparable inhibition of mammalian enzymes (Ferone et al., 1969). Consequently the effectiveness of pyrimethamine relies on its ability to inhibit the parasite enzyme, DHFR, resulting in the inhibition of DNA synthesis as found in proguanil. Pyrimethamine is slow in clearing parasitemia, but it prevents development of the fertilized gamete and has been used as an antimalarial prophylactic drug for three to four decades. It is frequently used in combination with sulphonamides or sulphones (Webster, 1990; Cowman and Foote, 1990).

(e) CINCHONA ALKALOIDS: e.g. Quinine
Quinine acts primarily as a blood schizontocide. It has little effect on sporozoites or pre-erythrocytic forms of malaria parasites. This alkaloid is also gametocytocidal for *P. vivax* and *P. malariae* but not for *P. falciparum*. Quinine is especially valuable for the treatment of severe illness due to chloroquine-resistant and multidrug-resistant strains of *P. falciparum*. When quinine is repeatedly given in full therapeutic doses, a typical dose-related cluster of symptoms occurs, termed cinchonism as a result of some adverse effect on the central nervous system, cardiovascular system, skeletal muscle, gastrointestinal tract or the pancreas (Webster, 1990).

(f) **SULPHONAMIDES**: e.g. Sulphadoxine

![N'(5,6-dimethoxy-4-pyrimidinyl)-sulphanilide](image)

N'(5,6-dimethoxy-4-pyrimidinyl)-sulphanilide and

(g) **SULFONES**: e.g. Dapsone

![4,4'-diaminodiphenylsulfone](image)

4,4'-diaminodiphenylsulfone

Differences in folate metabolism in man, protozoa and bacteria led to the development of antiprotozoal antifolate drugs such as sulphonamides and sulfones with limited human toxicity. These drugs act mainly by substrate inhibition, having structural similarities either to para aminobenzoic acid (PABA) or folic acid. Thus they prevent the formation of folic acid from pteridine, glutamic acid and PABA.
This leads to adverse effects on nuclear division in erythrocytic schizonts. Sulfonamides and sulfones are therefore effective blood schizonticides against \textit{P. falciparum} (Goldsmith, 1989). However the combination of sulphadoxine or dapsone to a DHFR inhibitor such as pyrimethamine or proguanil offers two-step synergistic blockade of plasmodial division. The combination of sulphadoxine and pyrimethamine has been used extensively for the prophylaxis of chloroquine-resistant strains of \textit{P. falciparum} (Webster, 1990).

(h) 4-QUINOLINE-METHANOL: e.g. Mefloquine

\[
\text{d-} \text{(2-piperidyl)-2,8-bis (trifluoromethyl-)-4-quinoline-methanol}
\]

Mefloquine has strong schizonticidal activity against all malaria parasites. It is however not active against the hepatic stages of infection. The drug is effective against most resistant strains of \textit{P. falciparum}, producing fewer adverse effects than quinine (Goldsmith, 1989). Mefloquine has been associated with retinal and epididymal lesions in long-term animal studies; therefore it should not be used for long-term prophylaxis. Its mechanism of action is however not known.
Artemisinine (Qinghaosu) is the active principle of a Chinese herbal medicine (qing hao). The drug is an effective blood schizonticide against all types of malaria. It is especially useful in the treatment of falciparum cerebral malaria, including that of chloroquine-resistant strains, since it is a fast-acting drug. Due to the short half-life of artemisinine, it is not useful for prophylaxis (Goldsmith, 1989). Its mechanism of action is not clear, however, it has been suggested that the compound might have an oxidant mode of action due to the presence of the trioxane structure (Hamburger and Hostettmann, 1991). The mechanism of action of artemisinine appears to involve two steps. The first being the activation, intra-parasite iron catalyses of the cleavage of the endoperoxide bridge and the generation of free radicals. This is followed by a second step of alkylation and the formation of covalent bonds between artemisinine-derived free radicals and parasite proteins (Meshnick, 1994).
Antibiotics with antimalarial activity such as tetracycline and doxycycline have been proved to be useful addition to quinine especially in areas where resistance to quinine is emerging (Malaria information site, 1999). Tetracycline is a blood schizonticide often used in emergencies for the treatment of multi-resistant falciparum malaria. However due to the damaging effects the drug could have on bones and teeth its use by pregnant or lactating women or in young children is not recommended. The drug also shows marked activity against primary tissue schizonts of chloroquine-resistant strains of \textit{P. falciparum} but its long-term use as a prophylactic agent is not advised due to possible adverse effects. The mechanism of action of tetracycline is not clear.

**CONTROL OF MALARIA**

The most widely accepted strategy for malaria control is the Global Malaria Control Strategy, which is promoted by WHO (WHO, 1993a, 1993b). Provision of early diagnosis and prompt treatment is recognised as the cornerstone of all national malaria control programmes. It is a major component of national plans of action, and it is the basis for training courses in many countries, particularly in sub-Saharan Africa.
Implementation of vector control (mainly insecticide-treated bed-net and indoor residual spraying) is also important, but this varies widely in different regions. Indoor residual spraying plays a significant role in malaria prevention and control in many countries particularly outside Africa. This specific method of transmission control has been instrumental with DDT use, to achieve malaria eradication from most countries in the temperate zone (Bosman, et al., 1999). In other countries where it was possible to achieve a cost-effective reduction of malaria incidence, this approach is essential to maintain malaria at a low level with only sporadic cases. Several pilot projects in Africa during the eradication period, as well as the Garki Project (Molineaux and Gramiccia, 1980) have proven the effectiveness of indoor spraying on malaria transmission, morbidity and mortality. In Africa the use of insecticide-treated bed-nets is gradually shifting from the ‘project bases’ to operational implementation. The fact that only one class of insecticides (pyrethroids) can currently be used to treat nets and that multiple resistance to this class of insecticides is emerging in a number of countries is quite disturbing.

Alternative control methods such as environmental management and biological control have a limited role at present in malaria control. These methods could be applied whenever they are cost-effective in specific eco-epidemiological situations.

The development of a safe and effective method of immunizing against malaria (vaccine development) has been a goal of malariologists for more than 60 years (Ballou et al., 1999). A vaccine would be one of the most important new tools for reducing the global burden of the disease. Nearly all malaria vaccine research is currently focused on P. falciparum as this species is responsible for the vast majority
of deaths and severe morbidity caused by malaria. The sporozoite stage of the parasites has historically been a major target because they appear to be susceptible to attack by antibodies as they travel through the blood stream (Potocnjak, et al., 1980). However candidate antigens have been identified from different stages of the parasite and some have advanced to the point of preliminary clinical evaluation (NIAID Division of Microbiology and Infectious diseases, 2001). Unfortunately the results of these current formulations in achieving protective immunity in man have thus far been generally disappointing. One candidate vaccine Spf66, developed by Colombian investigators and based on antigens from two parasite stages, has been tested in widespread field trials. Although the results of early clinical trials in humans were encouraging, subsequent trials demonstrated marginal efficacy. More recently, a vaccine against pre-erythrocytic stages (a recombinant \textit{P. falciparum} circumsporozoite protein-hepatitis B surface antigen subunit vaccine) showed protective efficacy in a small clinical trial involving experimental challenge previously unexposed volunteers. This vaccine is currently being assessed in an endemic region of Africa.

The important bottleneck of malaria control in Africa is the allocation of resources by national governments to malaria control. In addition there is inadequate support from the international community to seriously reduce malaria morbidity and mortality in Africa.

\textbf{PLANTS AS A SOURCE OF ANTIMALARIALS}

The use of medicinal plants is found in almost all cultures. In the world today substances derived from higher plants constitute about 25\% of prescribed medicines
The study of the chemistry of plants (Phytochemistry) has been crucial in the chemical classification of medicinal plants, and the development of novel medicinal compounds of enhanced medicinal activity. The most important plant derived compounds found in plant medicines are terpenoids and alkaloids. Others such as anthraquinone glycosides and other types of glycosides are also widely used.

Quinine, the chief alkaloid in the bark of cinchona tree indigenous to certain regions of South America, was isolated in 1820 by Pelletier and Caventou for the treatment of fevers (Webster, 1990). Many natural alkaloids related to quinine have been identified, and semi-synthetic and synthetic chemicals have been derived from quinine. These differ mainly in the nature of substitution on the side chain. Each alteration in chemical structure of quinine causes corresponding quantitative but not qualitative changes in the pharmacological actions of the resulting compounds. The antimalarial activities for several plants used in treating malaria in Africa have been investigated (Gbeassor et al, 1989). These plants include *Jatropha gossypiifolia*, *Dichapetalum guineese*, *Paullinia pinnata*, *Cassia siamea* and *Nauclea latifolia*.

Investigation on the plant *Eugenia uniflora* indicated blood schizontocidal effect against early infection in mice (Agbedahunsi and Aladesanmi, 1993). The chemosuppressive effect of extracts of this plant is related to the polarity of solvent used for the extraction since increase in polarity yielded substances with corresponding increase in chemosuppression. Therefore it could be inferred that the antimalarial active principle in the plant could be a polar compound. Thus further purification of the aqueous fraction could increase chemosuppressive activity.
Another plant well established as having antimalarial activity is *Cryptolepis sanguinolenta*. A 5% aqueous decoction of its root powder is used to treat malaria at the Centre for Scientific Research into Plant Medicine (C. S. R.P. M.) at Mampong-Akwapim, and has been reported to clear parasites over a period of 7 days (Oku-Ampofo and Boye, 1983). Ghanaian traditional healers have used the decoction for many years. Traditional medical practitioners in Kenya use *Albizia gummifera* and *Aspilia mossambicensis* among others to treat malaria and febrile conditions (Gachathi, 1989; Kokwaro, 1993). Scientific investigations of these plants have shown antimalarial activities of fractions isolated (Ofulla et al., 1996). There are several plants used as antimalarials that have not been investigated scientifically for their therapeutic activity and these include *T. procumbens* and *P. amarus*.

**THE PLANTS USED, *Tridax procumbens* and *Phyllanthus amarus***

*Tridax procumbens* is a common plant in tropical countries. The plant could be found on wastelands, cultivated fields or by roadsides. It belongs to the family Compositae. *Tridax procumbens* is a very hairy plant with coarsely toothed leaves, composite flower with about six ray flowers that are yellow with three teeth and numerous dark yellow disc flowers. The plant is tap rooted, therefore an attempt to pull it out of the soil results in a break from its roots.

The other plant *P. amarus*, is a perennial herb found in Central and Southern India, Sri Lanka and Ghana. In Ghana the Twis call it “awommaguwakyi”. It can grow to 12-24 inches in height and blooms with many yellow flowers. This plant belongs to the family Euphorbiaceae. All parts of the plant are employed therapeutically. Phyllanthus species are also found in other countries, including China (e.g.,
Phyllanthus urinaria), the Philippines, Cuba, Nigeria, and Guam. Traditional medical practitioners use the plants, T. procumbens and P. amarus for the treatment of malaria.

**DRUG RESISTANCE IN FALCIPARUM MALARIA**

The chief obstacle to successful chemotherapy of malaria is the parasite’s development of resistance to available drugs, particularly chloroquine (Cowman and Foote, 1990). Of the plasmodial species that infect man drug resistance poses a serious clinical problem only with *P. falciparum*. Drug resistance is the ability of a parasite strain to survive and / or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within limits of tolerance of the subject (WHO, 1973). Although drug sensitivity of all stages of the plasmodium should be considered in this context, the term “resistance” is applied primarily to the asexual blood forms, presumably because this stage in the life cycle of the parasite produces the acute clinical episodes during malaria infection.

Strains of *P. falciparum* that show resistance to some available drugs survive toxic doses by a number of mechanisms which include,

(i) Conversion of an active drug to an inactive derivative by enzyme(s) produced by the resistant cells.

(ii) Modification of drug sensitive sites.

(iii) Loss of cell permeability to drug.

(iv) Provision of an alternative enzyme or pathway that bypasses the inhibited reaction.
Increased levels of the enzyme inhibited by the drug.

Increased concentration of metabolite that antagonizes the inhibitor.

Decreased requirement for a product of the inhibited metabolic system.

Contributory factors to drug resistance include drug pressure, administration of sub-normal doses of the relevant drugs, self-medication, wrongful use of antimalarials for other diseases and the inability of policy makers to enforce regulations concerning the use of drugs.

The response of asexual forms of *P. falciparum* to normally recommended doses of chloroquine *in vivo* in man has been graded using a WHO field test (Bruce-Chwath, 1986). The graded responses are, sensitive (S), Level 1 resistance (RI), Level 2 resistance (RII) and Level 3 resistance (RIII). Description of the above responses are as follows:

(a) S: The parasites are considered sensitive to chloroquine if there is clearance of asexual parasitemia within 7 days of initiation of treatment, with no subsequent recrudescence.

(b) RI: Clearance of asexual parasitemia occurs within 7 days of initiation of treatment as in sensitivity, however, here it is followed by recrudescence.

(c) RII: There is marked reduction in asexual parasitemia but no clearance within 7 days of initiation of treatment.

(d) RIII: No marked reduction or a rise in asexual parasitemia is observed within 7 days of initiation of treatment.
Chloroquine-resistant *P. falciparum* was first reported simultaneously in South America and South East Asia in 1960-1961 (Moore and Lannier, 1961, Harinasuta *et al*., 1962). After nearly two decades, a third focus of chloroquine resistance emerged in East Africa (Campbell *et al*., 1979, Fog *et al*., 1979). Since then chloroquine-resistant *P. falciparum* has spread throughout almost all the malarious areas of the continent with heterogenic distribution both in frequency and degree (Bjorkman and Phillips-Howard, 1990). Most countries in West Africa have documented either *in vivo* or *in vitro* resistance of *P. falciparum* to chloroquine (Sansonetti *et al*., 1985; Neequaye, 1986; Le Bras *et al*., 1986; Salako and Fadeke, 1987; Charmot *et al*., 1988). In Ghana, clinical resistance of *P. falciparum* was first reported in 1986 by Neequaye. This was followed by the documentation of *P. falciparum* resistance at RI and RII level at the Korle-Bu Teaching Hospital (Ghana) (Ofori-Adjei *et al*., 1988, Neequaye *et al*., 1988).

Currently, *P. falciparum* resistance to several drugs that are alternatives to chloroquine has been reported. These include quinine, mefloquine and sulfadoxine / pyrimethamine (Fansidar). Quinine has been widely used for malaria treatment for a long time until the development of chloroquine in the early 1940s. Since then chloroquine replaced quinine because it is less costly, less toxic and requires smaller doses. However quinine reappeared after the development and spread of chloroquine-resistant *P. falciparum* and became an essential drug for treatment of both complicated *falciparum* infections and multidrug-resistant infections especially in S. E. Asia. Unfortunately resistance to quinine is now also appearing with about 50% treatment failure in infections observed in Thailand (Bunnag and Harinasuta, 1987, Giboda and Denis, 1988). From other areas including Africa there are reports of

Yet another drug that showed promising results upon clinical trials in various areas of the world in the early 1980s was mefloquine. This drug was reported to be active against chloroquine-resistant and fansidar-resistant strains of P. falciparum. However resistance to the drug in non-immune individuals was later reported from Thailand (Boudreau et al., 1982) and Tanzania (Bygbjerg et al., 1983). Full in vitro sensitivity to the drug has been reported from several areas of Africa (Bjorkman and Phillips-Howard, 1990). Furthermore decreased sensitivity of P. falciparum isolates to mefloquine has been reported in West Africa (Oduola et al., 1987).

The value of pyrimethamine- sulfadoxine combination lies in preventing or delaying strains of plasmodia from developing resistance to these drugs. This drug combination has been used successfully as an operational antimalarial drug in areas like South East Asia where chloroquine resistance is highly developed. However P. falciparum infections resistant to the drug combination at level III were observed in Thailand 10 years after its introduction (Doberstyn et al., 1976). Thereafter, treatment failure rates rapidly reached very high levels (Pinichpongse et al., 1982). Similarly, high rates (92%) of sulfadoxine-pyrimethamine resistance has been reported in vitro from Brazil (Kremsner et al., 1989).
REVIEW OF METHODS

In vitro cultivation of *P. falciparum*

The first attempt to culture malaria parasites was by Bass and Johns (1912). Since this first attempt as well as more recent work with the erythrocytic stages of both human malaria and malarial parasites of experimental animals, only one or at best a few cycles of development in vitro have been obtained (Developments in malaria immunology, 1975). It was in experiments of this type that the finding was made that Rosewell Parke Memorial Institute 1640 (RPMI 1640) medium supplemented with N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer and monkey serum, better supported development of monkey malaria parasite *P. coatneyi* than did a variety of other media. The RPMI 1640 medium was formulated by George Moore and his colleagues (Moore *et al.*, 1967) and no better replacement for this medium has yet been found. For example, any alterations to RPMI have been found to be generally deleterious to *P. falciparum* parasites (Jensen and Trager, 1977). In addition to the HEPES buffer in the medium is 5% sodium bicarbonate (NaHCO₃) that also acts as a buffer. It is worth noting that the best buffer system in the human body is that of bicarbonate.

L-glutamine and gentamicin are usually added to the medium as an amino acid supplement and bacteriostatic agent, respectively. The complete parasite culture medium is obtained by the addition of serum from non-immune donor. Media supplement of choice is human ABO blood group compatible sera (Jensen, 1983). Serum obtained from immune donors or persons living in malarious regions is not
suitable for the cultivation of malaria parasites due to the presence of antimalarial antibodies that would inhibit the growth of the parasites.

Erythrocytes used for culturing *P. falciparum*, are usually obtained from blood banks or asymptomatic volunteers who have not recently taken any antimalarial drug. The blood is aseptically collected into plastic bags or test tubes containing anticoagulants such as acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) adenine (Appendix III) and stored at 4°C for 4 days before washing and using it. This enhances its ability to support malaria cultures and also eliminate platelets and leucocytes. Erythrocytes in preservatives can be stored and used for culturing for up to 30 days after collection.

**Modified tetrazolium-based colorimetric assay**

The tetrazolium-based colorimetric assay was developed to assess the selectivity of drugs for pathogens such as the human immunodeficiency virus (HIV) and malaria parasites (Pauwels *et al.*, 1988). In this method, infected and uninfected red blood cells, treated or untreated with plant extracts or drugs are incubated for a period of 5 days to ensure that lysis of cells by extracts or parasites is complete. Tetrazolium salt, dissolved in phosphate buffer saline (PBS) is added to the contents of the microculture plate at the end of the fifth day of incubation. This results in the conversion of tetrazolium to formazan by mitochondrial enzymes. The concentration of formazan formed is directly proportional to the concentration of unlysed RBCs present in the culture after the incubation. Formazan formed is a solid precipitate, and this is dissolved with triton X-100 in acidified isopropanol (Appendix III) for spectroscopic determination (Appendix I). Four control experiments are set up in this
assay and these are, parasitized RBCs, uninfected RBCs, 500µg/ml of test extract and parasite culture medium. These control experiments are used to determine the independent effect of the four parameters mentioned above on the test experiment. Using Ayisi's modification (unpublished) of the formula of Pauwel et al (1988), the extract concentration needed to achieve 50% or 90% cell protection (effective concentration) can be computed. Furthermore the cytotoxic effect of extracts on RBCs and the selective indices of extracts can also be determined.

Inhibition of $^3$H-hypoxanthine uptake

This assay measures the uptake of radiolabeled nucleic acid precursor, $^3$H-hypoxanthine, by *P. falciparum* during short-term cultures in microtitration plates (Desjardins *et al.*, 1979). The parasites used are continually available from long-term maintenance cultures and the system is partially automated for rapid analysis of the data.

Hypoxanthine serves as the exogenous preformed purine, which the parasites require for growth. The inclusion of a preformed purine stems from the fact that although purines are required for growth of the parasites a functional *de novo* purine biosynthetic pathway has not been demonstrated for them. Thus the parasite depends on exogenous sources of purines. Hypoxanthine is derived intra-erythrocytically through the following pathway (Zubay, 1993):

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Adenosine triphosphate → adenosine diphosphate → adenosine monophosphate
hypoxanthine ← inosine ← Inosine monophosphate
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The preferred purine for *P. falciparum* appears to be hypoxanthine. For the purposes of quantitative assessment of the uptake by parasites *in vitro*, the hypoxanthine is radiolabeled. Scintillation counting which is used in this method gives reliable results and is less laborious as compared to the microscopic method of assessing antiplasmodial activity of plant extracts. Furthermore the use of 96-well microculture plates, makes the method simple and convenient as small volumes (microlitres) are used and several plant extracts could be tested on a single plate. Inhibition of uptake of hypoxanthine in the presence of a plant extract or drug is indicative of antimalarial activity.

**Haem polymerisation inhibition assay**

The main target of quinoline antimalarials is haem polymerisation, a process whereby intraerythrocytic-stage malaria parasites detoxify haem in the digestive vacuole. Haem, which is a by-product of haemoglobin digestion, is used by the parasites as a source of most of its essential amino acids (Goldberg *et al.*, 1990). It is potentially toxic to biological membranes and parasite enzymes (Fitch *et al.*, 1982; Gluzman *et al.*, 1994) and is thus sequestered in the form of an insoluble crystalline polymer, haemozoin (or malaria pigment).

Haem can be polymerised *in vitro*, in the presence of proteins, from a solution of haematin at 70°C or 37°C at acidic pH (Slater *et al.*, 1991; Egan *et al.*, 1994). The resulting polymer appears to retain the chemical, spectroscopic and biological
properties of the native pigment (Bohle et al., 1997; Taraselli et al., 1995). Quinoline antimalarials have been shown to inhibit both synthetic and native polymerisation (Raynes et al., 1996; Blauer and Akkawi, 1997). Chloroquine appears to act by forming quinoline-haem complexes, which terminate haemozoin chain extension (Sullivan et al., 1996; Dorn et al., 1995). The ability of drugs to inhibit haem polymerisation is directly related to their antimalarial potency.

A radiolabeled haem incorporation assay (Dorn et al., 1995) is currently used for testing antimalarial compounds. However, this method is expensive and uses trophozoite lysates and radiolabeled haemin. Furthermore, many laboratories are not equipped to conduct this assay routinely. Therefore, a method that does not require radiolabeled isotopes and is less expensive has been developed.

Decoctions of plants T610 and S076 are widely used in Ghana as antimalarial remedies without scientific proof of their efficacy. In view of the high cost of alternative drugs to chloroquine in Ghana, these plant extracts were investigated to determine their potential as effective and affordable antimalarial phytomedicines. The methods used were the isotopic microtest (IMT), based on the incorporation of $^3$H-hypoxanthine (Desjardins et al., 1979) and the modified tetrazolium (MTT)-based colorimetric method (Ayisi et al., unpublished). The aqueous extracts of both plants used were tested for haem polymerisation inhibition using the haem polymerization inhibition activity (HPIA) method (Basilico et al., 1998).
CHAPTER 2

MATERIALS AND METHODS

Chemicals and Reagents

The following chemicals were purchased from Sigma (USA): RPMI-1640 with HEPES modification and NaHCO$_3$ without glutamine, L-Glutamine, Sodium Chloride, Chloroquine diphosphate salt, Tetrazolium salt (MTT), Isopropanol, Ethanol, Methanol, Triton-X 100, Dimethylsulfoxide (DMSO) and Haematin. Glycerol, Sorbitol, Giemsa stain and Immersion oil were obtained from BDH (England). Other chemicals are Gentamicin from GIBCO (Scotland), $^3$H-hypoxanthine from NEN BOSTON (USA), HCl from PHARMACOS LTD (England), and Acetic acid from KANTO CHEMICAL CO.INC. (Japan) and Sodium hydroxide from CHAMELEON SPECIAL REAGENT (Japan).

Red Blood Cells and Serum

RBCs from group O Rh$^+$ (Volunteers)

Normal Human Serum (NHS) from blood group AB Rh$^+$ (SIGMA, USA)

Malaria Parasite Strains

Chloroquine-resistant *P. falciparum* Dd2 strain and chloroquine sensitive *P. falciparum* 3D7 strain (both strains were obtained from the Centre for Medical Parasitology, Copenhagen, Denmark)

Plant materials

Whole plant of *P. amarus* and *T. procumbens* (without roots) coded as S076 and T610 respectively, were supplied by a herbalist called Papa Issah Yemoh in Accra.
METHODS

Preparation of extracts of T610, S076 and chloroquine

The T610 and S076 plants were air-dried and a portion (4g) of each was boiled separately in 100 ml of water. A portion of each of the aqueous extracts was freeze-dried and refrigerated for later use. Ethanol was added to the remainder of the aqueous extract in a 1:1 ratio (50%) and this was sequentially extracted with chloroform and ethyl acetate in a 2:3 ratio of sample to solvent in each case. The resulting chloroform and ethyl acetate extracts were concentrated using a rotary evaporator.

Portions of the air-dried T610 and S076 plants were extracted separately for 24 h at room temperature with 150 ml of 50% ethanol using cold maceration. The process was repeated two more times for each sample. The three successive extracts of each plant were filtered, pooled together and concentrated by rotary evaporation followed by freeze-drying.

One milligram of each of the freeze-dried aqueous samples was weighed out and dissolved in 1 ml of complete RPMI medium, while 5 mg of the chloroform or ethyl acetate extracts were dissolved with agitation in 50 μl of DMSO. Ten microlitres of each of the extracts in DMSO were dissolved in 990 μl complete RPMI. These stock solutions were filtered through 0.45μm millipore filters. Fifty microlitres (50 μl) of each of these solutions were then added to 50 μl of parasitized or non-parasitized red blood cells in a microtitre well to give a final concentration of 500 μg/ml. Nine concentrations from 2-fold serial dilutions were made for each extract using complete RPMI medium as the diluent (Range of dilution 1.9 to 500μg/ml).
Chloroquine was used as the standard drug. A concentration of 100 \(\mu\)g/ml of chloroquine was prepared and serially diluted two-fold to obtain a range of 0.39 \(\mu\)g/ml to 100 \(\mu\)g/ml.

**Preparation of RPMI culture medium**

The RPMI culture medium was prepared by adding to 500 ml of neutral RPMI (without Glutamine but with NaHCO\(_3\)), 2.5 ml L-Glutamine (stock 200 mg/ml) and 4 ml of gentamicin (stock 10 mg/ml) with mixing under sterile conditions. This mixture, hereafter referred to as the incomplete RPMI medium, was dispensed in 45 ml aliquots into sterile 50 ml tubes and stored at 4°C. For use, 50 mg D-Glucose was added to a 45 ml aliquot of RPMI. The mixture was filter sterilized through a 0.22\(\mu\)m Millipore filter. A parasite culture medium (complete RPMI medium) containing 10% normal human serum (NHS) was prepared by adding 5 ml of sterile NHS to the above mixture. The complete medium was discarded after 4 – 5 days when the solution turned pink.

**Preparation of uninfected RBCs**

Blood from group O Rh\(^+\) volunteers were aseptically drawn into tubes containing Citrate-Phosphate-Dextrose (CPD)-adenine as anticoagulant, mixed and stored at 4°C for 3 – 4 days before washing. The RBCs were washed 4 times with incomplete RPMI medium (without NHS). Each wash was centrifuged at 2000 rpm for 5 minutes. The plasma-CPD and buffy coat were removed, and the packed cells resuspended in equal volume of incomplete RPMI medium and re-centrifugated at 2000 rpm for 5 min. The washed RBCs were suspended in incomplete RPMI and stored at 4°C.
Thawing cryopreserved malaria parasites

Cryopreserved parasites were thawed as follows: screw caps of vials containing the parasites were slightly loosened and set upright in a water bath set at 37°C to thaw. Thawed suspensions were centrifuged at 1500 rpm for 5 min, and their supernatants removed. The infected RBCs were washed by resuspending them in an equal volume of hypertonic saline (3.5% NaCl, sterile). The mixture was then centrifuged and the supernatant again discarded. The cells were further washed in equal volume of complete RPMI before transferring them into a culture flask.

Culture Procedure

Chloroquine resistant strain of *P. falciparum*, Dd2, was maintained in continuous culture by the method of Jensen and Trager (1980). In a sterile hood, 5 ml of complete RPMI medium was placed into two separate 25 cm² culture flasks and 200 μl of packed washed uninfected RBCs were added, to obtain a hematocrit of 4%. The appropriate dilution of the infected RBCs was done with the washed uninfected RBCs already placed in the flask resulting in an initial parasitemia of 0.1 – 0.5%. Culture flasks were placed in a candle jar (desiccator) containing about 30 ml sterile water and two 1 lit candles. The vent was completely closely when the candlelights were at the point of extinction. These manipulations were all performed in a sterile hood (Hitachi Clean Bench, Japan). The cultures were maintained at 37°C in an incubator (Hirasawa Ter-her cubic incubator type HT, Japan).

After every 24 h-incubation, the candle jar was removed from the incubator, placed in the sterile hood and the spent culture medium removed and replaced with fresh medium. During this period parasitemia was determined.
Assessment of parasitemia

Parasitemia was assessed daily, during the period of changing of culture medium. A thin blood film was made by placing a small drop of blood from the culture (5 – 8μl) at one end of a microscope slide and the blood spread out to cover almost the whole length of the slide using a smooth edge spreader. The blood film was then thoroughly air-dried.

Subculturing of malaria parasites

Cultures with parasitemia of 3% and above were subcultured. Parasitized RBCs from the old cultures were transferred to culture flasks containing culture medium and uninfected RBCs (as in the culture procedure above) to obtain parasitemia of 0.1 – 0.5%. The culture flasks were placed in the candle jar and incubated at low levels of oxygen and carbon dioxide as described above.

Dried slides were fixed in methanol and air-dried before placing them on a staining rack. The slides were flooded with 10% Giemsa solution and left to stain for 20min. Stained slides were rinsed carefully and thoroughly under running tap water and left standing to air-dry.

The slides were examined microscopically under oil immersion using the 100X objective lens. Parasitemia was computed as a percentage of the ratio of the number of parasitized RBCs to the total number of RBCs in 5 – 10 microscope fields for each slide.
Cryopreservation of malaria parasites

Ring stages of malaria parasite cultures with parasitemia 3 % were centrifuged at 2000 rpm for 5 min. The supernatants were removed, and the cells were resuspended in an equal volume of freezing solution (appendix III). The parasite suspension was distributed in 0.2 ml aliquots into sterile small screw cap vials and quickly frozen by immersion in liquid nitrogen.

Preparation of infected RBCs for $^3$H-hypoxanthine uptake and MTT assays

Infected RBCs were diluted to the required parasitemia with washed uninfected RBCs. Two microlitres each of uninfected RBCs and diluted infected cells were suspended in 500 times their volume of complete culture medium. The viability of the cells were assessed by making a 1:1 (v/v) suspension of the cells in 0.5 % trypan-blue solution, and aliquots were placed on a hemocytometer. Viable cells that actively extruded the trypan-blue were observed under a microscope using the 20X objective lens and counted. The cells were then diluted with complete RPMI to $5 \times 10^8$ cells / ml for the $^3$H-hypoxanthine uptake assay or to $2.3 \times 10^8$ cells / ml for the tetrazolium-based colorimetric assay.

Inhibition of $^3$H-hypoxanthine uptake

This test procedure was based on the method of O’Neil et al., (1985). Wells in rows B – H on a 96-well microculture plate contained test extracts, whilst wells in row A were used for controls. Two-fold serial dilution was made for each plant extract with concentrations ranging from 1.9 to 500 µg/ml. Fifty microlitres of each concentration was placed into the flat-bottom microculture well in triplicate. Fifty microlitres of diluted infected RBCs ($5 \times 10^8$ cells / ml) were added to the wells with test extracts
using a multichannel pipette. For controls, 50 µl of diluted infected RBCs were placed in the first eight wells while 50 µl of diluted uninfected RBCs were placed into the remaining four wells as parasite and RBCs controls, respectively. Fifty microlitres of parasite culture medium were then added to each of the control wells. The plates were covered, placed in a candle jar and incubated under low oxygen and carbon dioxide levels at 37°C for 24 h as described above (Page 37). Nine concentrations of 2-fold serial dilutions of chloroquine, (0.39 to 100 µg/ml) was used as a reference drug. After 24 h of incubation, 20 µl of $^3$H-hypoxanthine (40 µCi/ml) were added to each well. The plates were then incubated again for 24 h, after which they were left in a refrigerator at 4°C to stop the reaction. Cells were harvested unto glass fiber filters using a Packard Filter Mate 96 Cell Harvester, and the filters were dried in an incubator for 24 h at 37°C. Incorporation of radioactive hypoxanthine was counted by a Direct Beta Counter Matrix 96.

**Modified tetrazolium (MTT)-based colorimetric assay**

The procedure used was based on the modification of Pauwel et al., (1988). In this modified method, 100 µl of each of the extract concentrations were placed into wells of microculture plates (row B to row H) in duplicate (Ayisi et al., 1991). To the extract treated wells were added 100 µl of infected RBCs (2.3 X 10$^8$ cell/ml) at parasitemia of 1.5%. The same design was adopted for control experiments using uninfected RBCs. Wells in row A, contained the blank, parasite control, drug control, RBC control and parasite culture medium (PCM). Sterile phosphate buffer saline (PBS) was used as the blank, the parasite and RBC control well contained no extracts but 100 µl PCM and 100 µl of infected and uninfected RBCs respectively. The drug control wells had 100 µl of each of the extracts (500 µg/ml) and 100 µl of PCM in
duplicates. The plates were transferred into a candle jar and incubated at 37°C for 5 days under low levels of oxygen and carbon dioxide. After 5 days of incubation 20 μl of MTT (7.5 mg/ml) were added to each well and the plates incubated for another 2 h. One hundred and fifty microlitres of culture medium was then taken from each well and discarded. Two hundred microlitres of Triton X-100 in acidified isopropanol (See appendix) was added to each well to dissolve the formazan formed. With the aid of a multichannel pipette, the contents of each well were mixed several times, and the plates kept at room temperature in the dark for 24 h. The optical density (OD) of each well was read by an Emax Precision Microplate reader (Molecular Devices Corporation, Menlo Park, California) at 565 nm and 690 nm using phosphate buffered saline (PBS) for a blank. The microplate reader automatically subtracted readings at 690 nm from readings at 565 nm. The percent red blood cell protections by the different extracts were determined by the following formula of Ayisi et al (1991), equation (1)

\[
\text{Percent Cell Protection} = 1 + \frac{(\text{ODT})_{pf} - (\text{ODT})_{mock}}{(\text{ODC})_{mock} - (\text{ODC})_{pf}} \times 100
\]

where \((\text{ODT})_{pf}\) is the mean optical density for a given concentration of the plant extract in \(P. falciparum\) infected red blood cells \((\text{ODT})_{mock}\) is the mean optical density for a given concentration of the plant extract in uninfected red blood cells, \((\text{ODC})_{mock}\) is the mean optical density for untreated uninfected red blood cells (RBC control), and \((\text{ODC})_{pf}\) is the mean optical density for the untreated \(P. falciparum\) infected red blood cells. Values for the percent cell protection were plotted against the concentrations of the extracts to determine the concentrations required to achieve 50% and 90% cell protection (EC\(_{50}\) and EC\(_{90}\) respectively). The toxicity of the
extracts to the red blood cells was computed as percent cell survival using the following formula, equation (2)

\[
\text{Percent Cell Survival} = \frac{(\text{ODT})_{\text{mock}} - (\text{ODT})_{\text{cf}}}{(\text{ODC})_{\text{mock}} - (\text{ODC})_{\text{cf}}} \times 100
\]  

where \((\text{ODT})_{\text{cf}}\) is the mean optical density for cell-free treated well (Drug control), \((\text{ODC})_{\text{cf}}\) is the mean optical density for cell-free untreated well (Parasite culture medium) and the other parameters remain as defined in equation (1). The percent cell survival values were plotted against the concentrations of the drugs to determine the concentrations needed to cause 10\% and 50\% cytotoxicity (CC_{10} and CC_{50}, respectively). Selective indices (SI) of the extracts were then computed as CC_{50}/EC_{50}.

**Effect of dimethyl sulfoxide (DMSO) on \(^3\)H-hypoxanthine uptake by *P. falciparum***

Experiments were conducted to determine the possible effect of DMSO, which was used to dissolve some of the extracts, on 3D7 and Dd2 strains of *P. falciparum*. The assay for inhibition of \(^3\)H-hypoxanthine uptake was employed to monitor the effects of DMSO in these trials. The highest concentration of DMSO used in the preparation of extracts for the assays was 0.5\%, and this concentration was placed in culture wells in triplicate for each parasite strain. Fifty microlitres of parasitized RBCs were added to each of the wells with 0.5\% DMSO and to control wells without DMSO, and the assay for inhibition of \(^3\)H-hypoxanthine uptake was performed as described above.
Haem polymerization inhibition assay

One hundred microlitres of 4 mM solution of haematin dissolved in 0.1M NaOH, were aliquoted in eppendorff tubes. Fifty microlitres of different concentrations of chloroquine at a drug:haem ratio of between 1:1 to 8:1 were added to duplicate test tubes and 50μl of the aqueous extracts of T610 and S076 (500 μg/ml each) were also added to tubes with 100 μl haematin solution. Fifty microlitres of water was added to control wells. Haematin polymerization was initiated by adding 50μl of 0.8 mmol of acetic acid at a final pH of 3. The suspension was incubated at 37°C for 24 h to allow for complete polymerization. The tubes were centrifuged at 3300g for 15 min and the soluble fraction of unprecipitated material collected as fraction I. The remaining pellet was resuspended in 200μl of DMSO to remove unreacted haematin. Tubes were then centrifuged again at 3300g for 15 min. The DMSO-soluble fraction (fraction II) was collected and the pellet, consisting of a pure precipitate of β-haematin, was dissolved in 200 μl of 0.1 M NaOH (fraction III) for spectroscopic quantification. A 100μl aliquot of each fraction was transferred on to a new plate and 4-fold serial dilutions in 0.1 M NaOH were performed. The amount of haematin was determined by measuring the absorbance at 450 nm using a microplate reader (Molecular Devices Corporation, Menlo Park, California). A standard curve for haematin dissolved in 0.1 M NaOH was used to calculate the amount of porphyrin in each fraction.
CHAPTER 3

RESULTS

In this study the tetrazolium-based colorimetric method was essentially used to screen the effects of chloroquine and crude extracts of T610 and S076 on RBCs and the antimalarial activities of these extracts were tested using the radiolabeled hypoxanthine uptake method. Chloroquine was used as the reference antimalarial drug.

Effect of chloroquine on RBC protection and survival

The concentration-dependent effects of chloroquine on red blood cell protection from chloroquine-resistant strain of *P. falciparum*, Dd2, and survival are shown in figures 1 and 2 respectively. The figures show that chloroquine offered 50% cell protection at an effective concentration (EC50) of 80.0 ug/ml and a cytotoxic concentration of 85.7 ug/ml at 50% cell survival.

Effects of aqueous extracts of T610 and S076 on cell protection and survival

The effect of crude aqueous extracts of T610 and S076 on RBC protection and survival are shown in figures 3 and 4. Figure 3 shows that there was increased cell protection with increasing concentration of each of the two aqueous extracts. However, extracts S076 showed higher percent protection of RBCs than T610. Figure 4 shows that the percent survival of RBCs incubated with the T610 and S076 extracts were greater than 74.9% and 68.8% respectively.
Fig. 1 Concentration-dependent effect of chloroquine on RBC protection. Infected and uninfected RBCs were treated with chloroquine diphosphate salt dissolved in complete RPMI and serially diluted to obtain 9 concentrations, from 0.39 to 100 μg/ml. Points on the curve represent percent RBC protection from parasite infection calculated from the formula given earlier (page 41) using mean values from a single experiment calculated from duplicate wells.
Fig. 2 Concentration-dependent effect of chloroquine on RBC survival. From the same experiment described earlier (Fig. 1) percent RBC survival after drug treatment and parasite infection was calculated for each concentration using the formula given earlier (Page 42).
Fig. 3 Concentration-dependent effect of aqueous extracts of T610 and S076 on RBC protection. Experimental conditions were the same as mentioned earlier. However, for each of the two extracts, the 9 concentrations used were obtained from 2 fold serial dilution of the extracts from the concentration range 1.9 to 500 μg/ml. Percent RBC protection at each concentration was calculated as indicated earlier.
Fig. 4 Concentration-dependent effect of aqueous extracts of T610 and S076 on RBC survival. Results from the same experiment in Fig. 3 were used to compute the percent RBC survival at each given concentration from the formula shown earlier (Page 42).
Effects of ethanolic extracts of T610 and S076 on cell protection and survival

Figures 5 and 6 summarize the effects of the crude ethanolic extracts of T610 and S076 on RBC protection and survival. The S076 extracts showed increased cell protection with increasing concentration. The results for the T610 extract followed a similar trend except that a rapid decrease in cell protection was observed above the concentration of 62.5 μg/ml. Figure 6 shows that RBC survival decreased with increasing concentrations for both extracts. However, between 31.2 – 125.0 μg/ml of T610 extract, cell survival increased from 81.3 to 99.6 %. At the highest concentration of 500.0 μg/ml used for both extracts, T610 and S076, RBC survival was 62.9 and 70.8%, respectively.
Fig. 5 Concentration-dependent effect of ethanolic extracts of T610 and S076 on RBC protection. Experimental conditions and formula used for calculating percent RBC protection were the same as that used for Fig. 1.
Fig. 6 Concentration-dependent effect of ethanolic extracts of T610 and S076 on RBC survival. Results from the experiment in Fig. 5 were used to compute the percent RBC survival at each given concentration from the formula shown earlier (Page 42).
Effects of chloroform extracts of T610 and S076 on cell protection and survival

The effect of total chloroform extracts of T610 and S076 on RBC protection and survival are shown in figures 7 and 8. Figure 7 shows that for extract T610, minimum RBC protection was observed at the concentrations used. This trend was similar for the S076 extract, which showed less than 12% RBC protection at 125.0 μg/ml and below. However, above this concentration, there was a rather sharp concentration-dependent increase in cell protection. RBCs survival was greater than 75.1% and 16.7% for T610 and S076 respectively (Fig. 8).
Fig. 7 Concentration-dependent effect of chloroform extracts of T610 and S076 on RBC protection. Experimental conditions and formula (page 41) used for calculating percent RBC protection were the same as that used for Fig. 3.
Fig. 8 Concentration-dependent effect of chloroform extracts of T610 and S076 on RBC survival. Results from the experiment in Fig. 7 were used to compute the percent RBC survival at each given concentration from the formula shown earlier (Page 42).
Effects of ethyl acetate extracts of T610 and S076 on cell protection and survival

Figures 9 and 10 show the effects of ethyl acetate extracts of T610 and S076 on RBC protection and survival. Percent protection of RBCs by these extracts was below 13%, but for S076, a sharp increase in cell protection at the extract concentration of 250.0 μg/ml was observed. For both extracts, RBC survival was seen to decrease gradually with increasing concentration between the concentration of 1.9-15.6 μg/ml. Above this concentration range, percent cell survival appeared to be constant in both extracts until the concentration of 250.0 μg/ml where the S076 extract showed a sharp decline.
Fig. 9  Concentration-dependent effect of ethyl acetate extracts of T610 and S076 on RBC protection. Experimental conditions and formula (page 41) used for calculating percent RBC protection are on page 40.
Fig. 10 Concentration-dependent effect of ethyl acetate extracts of T610 and S076 on RBC survival. Results from the experiment in Fig. 9 were used to calculate the percent RBC survival at each given concentration from the formula shown earlier (Page 42).
Table 1 shows the extract concentrations at which 50% cell protection (Effective Concentration EC$_{50}$) and 50% cell survival (50% Cytotoxic Concentration CC$_{50}$) were obtained from the extracts incubated. Selective indices (SI) of the extracts computed from the formula,

$$SI = \frac{CC_{50}}{EC_{50}}$$

are also shown in table 1. Selective indices were not computed for extracts in which 50% cytotoxic concentration could not be estimated. The highest SI value computed was 32.1, and this corresponded to the crude ethanolic extract of *P. amarus* (S076), and the next was the ethanolic extract of *T. procumbens* (T610) that had a value of 6.2.
Table 1

Fifty percent effective \((EC_{50})\) and cytotoxic \((CC_{50})\) concentrations and selective indices \((SI)\) of T610 and S076 extracts in chloroquine-resistant \(P. falciparum\) (Dd2 strain) infected RBCs.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>(EC_{50}) ((\mu g/ml))</th>
<th>(CC_{50}) ((\mu g/ml))</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T610</td>
<td>S076</td>
<td>T610</td>
</tr>
<tr>
<td>Aqueous</td>
<td>&gt;500</td>
<td>34.9</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Ethanol</td>
<td>121.3</td>
<td>31.2</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Chloroform</td>
<td>&gt;500</td>
<td>263.9</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>&gt;500</td>
<td>368.4</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

*nd: not determined as a result of one of the determinants being out of range.*
Inhibition of radiolabeled hypoxanthine uptake in *P. falciparum*

The inhibition of uptake of $^3$H- hypoxanthine by *P. falciparum* parasitized RBC was used to assess the antimalarial activity of the various extracts of T610 and S076. Chloroquine inhibition of $^3$H- hypoxanthine uptake by chloroquine-resistant strain of *P. falciparum* (Dd2) is shown in figure 11. Increase in inhibition of uptake from 0.5-99.5% was observed with increasing concentration of 0.4-100 μg/ml. There was concentration-dependent increase in inhibition of $^3$H- hypoxanthine incorporation into the parasites.

Inhibition of *P. falciparum* uptake of radiolabeled hypoxanthine by aqueous extracts of T610 and S076

Figure 12 shows the percentage inhibition of *P. falciparum* uptake of $^3$H-hypoxanthine by aqueous extracts of T610 and S076. For S076 the lowest extract concentration (1.9 μg/ml) inhibited $^3$H- hypoxanthine uptake by 27.6%. Inhibition of $^3$H- hypoxanthine uptake by aqueous extract of S076 increased marginally up to an extract concentration of 15.6 μg/ml. Following that, a sharp increase in inhibition of $^3$H- hypoxanthine uptake occurred between the extract concentration range of 15.6-62.5 μg/ml. This rate reduced after the concentration of 62.5 μg/ml, reaching a maximum inhibition of 88.6% at a concentration of 500 μg/ml. For the T610 extract, the lowest concentration of 1.9 μg/ml inhibited *P. falciparum* uptake of $^3$H-hypoxanthine by 29.0%, rising marginally to 34.1% at an extract concentration 125.0 μg/ml. This was followed by a 19.7% increased inhibition of $^3$H- hypoxanthine uptake between extract concentrations of 125.0 to 500.0 μg/ml.
Fig. 11 Inhibition of $^3$H-hypoxanthine ($^3$H-HPO) uptake in *P. falciparum* (strain Dd2) by chloroquine. The parasite infected RBCs were treated with 9 different concentrations of chloroquine (see Fig. 1). After 24h incubation 20μl of $^3$H-hypoxanthine solution were added to each well. Uptake of $^3$H-hypoxanthine by viable parasites was determined using a scintillation counter and the percent inhibition of $^3$H-hypoxanthine uptake calculated from the formula mentioned earlier (page 43).
Fig. 12 Inhibition of $^3$H-hypoxanthine uptake in *P. falciparum* (strain Dd2) by aqueous extracts of T610 and S076. Infected RBCs were treated with aqueous extracts of T610 and S076 (at concentrations indicated above (Fig. 3)). Using experimental conditions as described above (Fig 11) inhibition of uptake of $^3$H-hypoxanthine was determined.
Inhibition of *P. falciparum*-infected RBC uptake of radiolabeled hypoxanthine by ethanolic extracts of T610 and S076

Inhibition of *P. falciparum* uptake $^3$H- hypoxanthine of by ethanolic extracts of T610 and S076 are shown in figure 13. For the ethanolic extract, 1.9 μg/ml of S076 inhibited uptake of $^3$H- hypoxanthine by 33.1%, increasing sharply between 7.8 – 62.5 μg/ml, and levelled off at 125.0 μg/ml. In the case of T610, 1.9 – 31.2 μg/ml of ethanolic extract inhibited $^3$H- hypoxanthine uptake by *P. falciparum* up to 25%. Inhibition of $^3$H-hypoxanthine uptake increased by 67.1% between 31.2 - 500.0 μg/ml of the ethanolic extract of T610.
Fig. 13 Inhibition of $^3$H-hypoxanthine uptake in *P. falciparum* (strain Dd2) by ethanolic extracts of T610 and S076. Percent inhibition of $^3$H-hypoxanthine uptake by the parasites after treatment with the different concentrations of ethanolic extracts of T610 and S076 (concentrations used were the same as for Fig. 5) was obtained using the same experimental conditions above (Fig. 12).
Inhibition of *P. falciparum*-infected RBC uptake of radiolabeled hypoxanthine by chloroform extracts of T610 and S076

Figure 14 shows the inhibition of *P. falciparum* uptake of $^3$H- hypoxanthine by chloroform extracts of T610 and S076. Inhibition of *P. falciparum* uptake of $^3$H- hypoxanthine by S076 was about 17.0%, between extract concentrations of 1.9-62.5 μg/ml. However, increase in inhibition of $^3$H- hypoxanthine uptake of 75.1% was realised with increasing concentration from 62.5-500.0 μg/ml. Similarly, the T610 extract showed constantly low inhibition of $^3$H- hypoxanthine uptake of about 25% within the concentration range of 1.9-250.0 μg/ml. A sharp increase in inhibition of $^3$H- hypoxanthine uptake of 38.3% occurred between the concentrations of 250.0 to 500.0 μg/ml.
Fig. 14 Inhibition of $^3$H-hypoxanthine uptake in *P. falciparum* (strain Dd2) by chloroform extracts of T610 and S076. Experimental conditions and formula used for computing percent inhibition of $^3$H-hypoxanthine uptake were the same as that used above (Fig. 13).
Inhibition of *P. falciparum*-infected RBC uptake of radiolabeled hypoxanthine by ethyl acetate extracts of T610 and S076

The inhibition of *P. falciparum* uptake of $^{3}\text{H}$- hypoxanthine by ethyl acetate extracts of T610 and S076 are shown in figure 15. There was increased inhibition of growth with increasing concentration of each extract. Within the concentration range 1.9-500.0 µg/ml percent inhibition of growth for S076 and T610 were of the ranges 24.3 - 48.6% and 28.9 - 53.7%, respectively.
Fig. 15 Inhibition of $^3$H-hypoxanthine uptake in *P. falciparum* (strain Dd2) by ethyl acetate extracts of T610 and S076. Infected RBCs were treated with ethyl acetate extracts of T610 and S076 under the same experimental conditions mentioned above and percent inhibition of $^3$H-hypoxanthine calculated.
Table 2 shows the extract concentrations at which 50% inhibitions of growth were obtained.

**Table 2**

Fifty percent inhibition concentrations (IC$_{50}$) of T610 and S076 extracts

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T610</td>
</tr>
<tr>
<td>Aqueous</td>
<td>225.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>143.4</td>
</tr>
<tr>
<td>Chloroform</td>
<td>430.6</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>250.0</td>
</tr>
</tbody>
</table>
Haem polymerisation inhibitory activity (HPIA)

Results of the haem polymerisation inhibitory activity (HPIA) of the crude aqueous extracts (T610 and S076) determined according to the method of Basilico et al., (1998) are shown in figures 16 and 17, respectively. Neither of these extracts caused an obvious increase in the amounts of porphyrin in the DMSO-soluble fractions (fraction II) containing unreacted haematin that would result in a corresponding decrease in the amounts of porphyrin in the DMSO-insoluble fractions (fraction III), consisting of a pure precipitate of β-haematin. However the results of HPIA of chloroquine as shown in figure 18 indicate that increasing the chloroquine concentration caused increased porphyrin levels in fraction II with corresponding decrease in fraction III. The amount of unprecipitated haematin (fraction I) did not vary significantly with increasing concentration in either of the tested extracts or chloroquine.
Fig. 16 H aematin polymerization in the presence of increasing concentrations of aqueous extract of T610. Data represents the means (with SD) of duplicate experiments at extract concentrations shown on the abscissa. Fraction of unpolymerized haematin (T610 I); DMSO-soluble fraction (T610 II); Final pellet, DMSO-insoluble fraction (T610 III).
Fig. 17 Haematin polymerization in the presence of increasing concentrations of aqueous extract of S076. The data shown represents: Fraction of unpolymerized haematin (S076 I); DMSO-soluble fraction (S076 II); Final pellet, DMSO-insoluble fraction (S076 III).
Fig. 18 Haematin polymerization in the presence of increasing concentrations of chloroquine. Experimental conditions are the same as above (Fig. 16). Fraction of unpolymerized haematin (CHQ I); DMSO-soluble fraction (CHQ II); Final pellet, DMSO-insoluble fraction (CHQ III). One equivalent of chloroquine as shown on the graph corresponds is 0.4μmol of chloroquine.
DISCUSSION AND CONCLUSION

DISCUSSION

The aim of this research project was to investigate the antiplasmodial activities of the plants *T. procumbens* (T610) and *P. amarus* (S076). The screening of crude extracts of medicinal plants for their medicinal properties has the potential of being more meaningful than the screening of pure compounds isolated from such plants (Kusumoto *et al.*, 1995; Cordell, 1995). Therefore crude extracts of each of the plants were prepared for the investigations described.

Antiplasmodial activities of these plant extracts serially diluted to cover a concentration range of 1.9 – 500.0 μg/ml were assessed *in vitro* using the chloroquine-resistant strain Dd2 of *P. falciparum*. A tetrazolium-based colorimetric assay (Ayisi *et al.*, 1991) was employed to screen the plant extracts for toxicity to RBCs. This assay had the added advantage of evaluating the selective activity of the extracts against malaria parasites. The formula for calculating percent cell protection and survival eliminates the background optical density readings due to plant extract, chloroquine and parasite culture medium colours. Tetrazolium salt reduction as an indicator of cell growth has been used in models for screening cytocidal chemical agents (Alley *et al.*, 1988; Scudiero *et al.*, 1988). The isotopic microtest that involves the inhibition of uptake of $^3$H-hypoxanthine by *P. falciparum*-infected RBC was the method used to determine the antiplasmodial activities of the extracts.

In this study, the protective effect of chloroquine on chloroquine-resistant *P. falciparum* infected RBCs was concentration-dependent, with a 50% effective
concentration (EC\textsubscript{50}) of 80.0 \mu g/ml. This figure is much higher than the WHO recommended concentration range of chloroquine for \textit{in vitro} microtest, which is in the nanogram range (WHO, 1990). The EC\textsubscript{50} reported here is consistent with the fact that the resistant strains of \textit{P. falciparum} were used in the present study, as inhibition of growth of these parasites would require high chloroquine concentrations (WHO, 1973). Survival of RBCs decreased with increasing concentration of chloroquine (Fig. 2), yielding a 50% cytotoxic concentration (CC\textsubscript{50}) of 85.7 \mu g/ml. At high concentrations of chloroquine, the RBCs undergo lysis (John \textit{et al.}, 1994), consequently causing a reduction in the number of viable RBCs. According to Finlay \textit{et al.}, (1984), only viable RBCs are capable of taking up and reducing tetrazolium via a mitochondrial dehydrogenase enzyme to yield the purple product, formazan that was used to monitor the assays.

The EC\textsubscript{50} crude extracts of \textit{P. amarus}, increased in the order of: ethanolic < aqueous < chloroform < ethyl acetate extract (Table 1). Therefore the ethanolic extract contained most of the active component(s) for inhibiting the growth of chloroquine-resistant \textit{P. falciparum} (Dd2) than the other extracts. This may explain why the ethanolic extract appeared to be more effective in protecting the RBCs from the destruction by the parasites. The protective effect found in the ethanolic extract, is consistent with reports of alcoholic extracts of other plants exhibiting protection against cytotoxic agents (Martinez, \textit{et al.}, 1998).

Furthermore in decreasing order the computed CC\textsubscript{50} of the extracts of \textit{P. amarus} were ethanolic > ethyl acetate > chloroform extract. Although the CC\textsubscript{50} of the aqueous extract could not be determined since over 50% cell survival values were
obtained, the high percent cell survival values obtained even at the highest concentration used indicates a much higher \( CC_{50} \) than that found in the other extracts. The aqueous extract followed by the ethanolic extracts showed the least toxicity to RBCs, considering that a high \( CC_{50} \) value means a high concentration of the extract is required to cause 50% cytotoxicity (Kusumoto et al., 1995). Comparison of the computed selective indices (SI) derived from the \( EC_{50} \) and \( CC_{50} \) values of the \textit{P. amarus} extracts revealed that the ethanolic extract had higher SI value. This value was 29 times that of the chloroform and ethyl acetate extracts which had equivalent SI values. Therefore the ethanolic extract of \textit{P. amarus} showed the highest selectivity against the chloroquine-resistant \textit{P. falciparum} (Dd2). The chloroform extract did not have a better selectivity against the parasites compared to the ethyl acetate extract despite the fact that the former had lower \( EC_{50} \), an indication of greater activity. Since the ethyl acetate extract was comparatively less cytotoxic to RBCs than the chloroform extract neither of the two extracts was found to have the advantage of having a greater selectivity. This illustrates the importance of determining not only the \( EC_{50} \) but also the \( CC_{50} \) in such investigations (Cordell, 1995). Percent cell protection or survival values greater than 100% indicate that an extract has the potential of enhancing longevity of RBCs. This is supported by reports by Ayisi et al., (1991) that showed cell protection and survival values greater than 100%.

A similar trend as pertained in the \textit{P. amarus} extracts was exhibited in the \textit{T. procumbens} extracts. The \( EC_{50} \) of crude extracts of \textit{T. procumbens} were of increasing order, ethanolic < aqueous < chloroform extract. The ethyl acetate extract gave \( EC_{50} \) values less than 50%, an indication of rather minimal activity against the
chloroquine-resistant \textit{P. falciparum} strain. Hence the greatest activity of the \textit{T. procumbens} extracts against the parasites was found in the ethanolic extract.

The CC$_{50}$ values of the aqueous and ethyl acetate extracts \textit{T. procumbens} could not be computed since high percent cell survival recorded for these extracts were well above 50\% even at the highest concentration used. Therefore, the above extracts showed less toxicity to RBCs than the aqueous and chloroform extracts. The CC$_{50}$ of the chloroform extract was about 2 times greater than that of the ethanolic extract, thus the former was less toxic to RBCs than the latter.

The SI values of the aqueous and ethyl acetate extracts of \textit{T. procumbens} could not be computed since either CC$_{50}$ or EC$_{50}$ or both (Table 1) could not be determined from the concentration range used in the assays. The SI values determined show that the ethanolic extract had 6 times selectivity for malaria parasites than the chloroform extract. Although the chloroform extract showed less toxicity to RBCs than the ethanolic extract, the latter had greater activity against the parasites and hence greater selective activity.

The feasibility of using the uptake of $^3$H-hypoxanthine to measure antimalarial activity of plant extracts stems from the fact that culture medium composition was devoid of hypoxanthine, the preferred purine of malaria parasites. This is coupled with the capability of hypoxanthine to cross malaria parasite membrane, unlike thymidine (Manandhar and Van Dyke, 1975). Hence radiolabeled hypoxanthine is ultimately incorporated into ribonucleic acid and deoxyribonucleic acid of viable malaria parasites and therefore provides a reasonably broad index of parasite
metabolism. Suppression of the uptake and incorporation of \(^3\)H-hypoxanthine into nucleic acids by \textit{P. falciparum} has been used as an indicator of antimalarial drug activity (Desjardins \textit{et al.}, 1979).

The fifty percent inhibition concentration (IC\(_{50}\)) is an estimate of the concentration of drug inhibiting the maximum rate of metabolism of a fixed concentration of substrate by 50%, and as such a measure of inhibitory potency (Therapeutic products guidance document, 2000). Indeed the determination of IC\(_{50}\) is considered to be the most relevant single toxicity measurement for the evaluation of drugs.

Chloroquine caused a concentration-dependent inhibition of incorporation of \(^3\)H-hypoxanthine into the chloroquine resistant strain of \textit{P. falciparum} (Dd2) with an IC\(_{50}\) of 4.6 \(\mu\)g/ml. This confirms that the strain of \textit{P. falciparum} used was resistant to chloroquine as discussed previously.

The various crude extracts of \textit{P. amarus} inhibited \(^3\)H-hypoxanthine uptake by \textit{P. falciparum}. The IC\(_{50}\) were in the following order: ethanolic < aqueous < chloroform < ethyl acetate extract. Thus using the radiolabeled hypoxanthine uptake method, the ethanolic extract gave the highest activity of 11.7\(\mu\)g/ml, against chloroquine-resistant strain of \textit{P. falciparum} (Dd2). These results are consistent with those obtained using the MTT method.

The ethanolic extract of \textit{T. procumbens} with IC\(_{50}\) of 143.4 \(\mu\)g/ml demonstrated the highest activity with respect to inhibition of \(^3\)H-hypoxanthine uptake by the malaria parasites. The IC\(_{50}\) in increasing order were ethanolic < aqueous < ethyl acetate <
chloroform extract. The trend of activity in the extracts of *T. procumbens* was similar to that obtained using the MTT method except that in the inhibition of $^3$H-hypoxanthine uptake method the ethyl acetate extract showed greater activity against the parasites than the chloroform extract. The reason for the reversal in trend in these two extracts is not clear and would require further investigation.

Although the ethanolic extracts of both *T. procumbens* and *P. amarus* appeared to be the most active extracts against the chloroquine-resistant strain of *P. falciparum* (Dd2), selectivity of the extracts appears to depend on the toxicities they exhibit. For example a comparison of the toxicities of the various extracts with highest inhibitory activities to the malaria parasites revealed that the ethanolic extracts were also more toxic to RBCs than the aqueous extracts. Comprehensive toxicity studies of these extracts are required to determine the less toxic extract of each of the two plants and hence the most appropriate extract to develop and use in malaria chemotherapy. The concentrations of active chemical constituents in plant extracts are usually very low implying that a lot of plant material is required to obtain a substantial amount of active constituents, and hence biological or pharmacological activity. This would explain why in this work higher concentrations of the crude plant extracts were used in this work as compared to chloroquine, the standard drug that is a pure chemical compound. Thus the activity of total extracts of the plants cannot be compared with concentrations of isolated active ingredients (Phillipson, 1994).

Low inhibitory activity exhibited by some of the extracts on *P. falciparum in vitro* is not enough evidence for their being ineffective. These extracts may contain chemical substances that may require activation *in vivo*, by drug metabolising system. Certain
allopathic drugs are also known to be pro-drugs that need to be metabolised to their active forms. A typical example is proguanil an antimalarial drug that has to be converted to the active triazine derivative, cycloguanil, *in vivo* in order for it to exhibit appreciable effect on *P. falciparum* (Rieckmann *et al.*, 1968).

Although the $^3$H-hypoxanthine uptake assay for evaluating antiplasmodial activity is different from the MTT assay it is obvious that the trend of activities observed using both methods was similar, considering that EC$_{50}$ is equivalent to IC$_{50}$. It is worth noting that the duration of incubations of *P. falciparum* infected cells with the plant extracts in both assays were different. Although the length of incubation for the $^3$H-hypoxanthine uptake assay lasted for only 1 day and therefore more convenient to perform, it only determines the IC$_{50}$. However the hypoxanthine assay that lasted for 5 days had an added advantage of the possibility to determine both EC$_{50}$ and CC$_{50}$ in the same set-up.

Malaria parasites metabolise haemoglobin and detoxify the resulting haem by polymerizing it to form haemozoin (malaria pigment) (Goldberg *et al.*, 1990). Quinoline antimalarials have been shown to inhibit both synthetic and native polymerization of haem. Haem polymerization is therefore an essential and unique pharmacological target.

Chloroquine, a quinoline was effective in inhibiting polymerization of haematin to form β-haematin. The mechanism of action of chloroquine including its interaction with haem and its polymerization is only partly understood. The polymerization is commonly ascribed to the following proposed mechanism: $\pi-\pi$ interaction between the chloroquine and the haem electronic system (Adams *et al.*, 1996). Basilico *et al.*,
(1997), have substantiated the \( \pi-\pi \) adduct hypothesis and the stabilizing role of oxo bridges by showing that non-iron porphyrins can effectively prevent haem polymerization.

The aqueous extracts of *T. procumbens* and *P. amarus* unlike chloroquine did not effect any such inhibition of haem polymerization. This may be an indication that the antiplasmodial activities of these extracts are not related to the inhibition of haem polymerisation but may have different mechanisms of action. Alternatively it is possible that the concentrations of crude aqueous extracts used in the assay were too low to exhibit any appreciable inhibition of haem polymerization.

**CONCLUSION AND RECOMMENDATION**

The results obtained lend support to the claims of the herbalists that the decoction of either *T. procumbens* or *P. amarus* is active against chloroquine-resistant *P. falciparum*. Furthermore the aqueous and ethanolic extracts of both T610 and S076 showed remarkable antiplasmodial activity and considerably low toxicities to RBCs. However more comprehensive toxicity studies on these plant extracts are required especially since these extracts are currently being used by humans to manage malaria.

Moreover evaluation of the antiplasmodial activities of these extracts *in vivo* would be worthwhile in determining whether any of the extracts would be bioactivated to produce enhanced antiplasmodial activity. Determination of the antiplasmodial activity of these extracts against chloroquine-sensitive strains of *P. falciparum* will be useful since these plants appear to be novel. As there have been no isolation of
compounds with antimalarial activity from these plants, it is recommended that bioactivity-guided studies be carried out in order to isolate the active principles that may be novel first line antimalarial agents. These plants may be useful in containing chloroquine-resistant malaria infection, which is now prevalent in all malaria endemic parts of the world.

In view of the cost of radiolabeled hypoxanthine coupled with the hazards associated with the use of such materials, it is recommended that the MTT assay be further optimised for use in evaluating new antimalarial agents, since reagents required for the MTT assay are safer and less expensive.

No haem polymerization inhibitory activity was found in the aqueous extracts of both *T. procumbens* and *P. amarus* at the concentrations used. Higher concentrations of the extracts should be used to exclude the possibility of low extract concentration being responsible for the lack of extract-mediated inhibition of haem polymerization.
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LIST OF APPENDICES

APPENDIX I
Examples of microtitre plates with products of MTT-based colorimetric assay

Plant extract A

Plant extract B

Extract A, demonstrated activity against *P. falciparum*, decreasing lysis of RBCs at concentrations above 4.56 µg/ml, whilst extract B showed no activity against the parasites, resulting in destruction of RBCs in wells labelled ‘infected’.
APPENDIX II

Schematic life cycle of malaria parasites in man and mosquito
APPENDIX III

Preparation of solutions

10% Triton-X-100 in acidified isopropanol solution

Two hundred microlitres of HCl was added 50 ml of isopropanol and mixed. Five millilitres of the mixture was discarded and 5 ml of Triton-X-100 added and mixed.

CPD-adenine solution

The following reagents were weighed out, mixed and made up to 1 litre with distilled water;

Citric acid 5.0 g (17 mM)
Sodium citrate 26.67 g (90 mM)
Glucose 31.54 g (175 mM)
NaH$_2$PO$_4$ 2.21 g (16 mM)
Adenine 270 mg (2 mM)

The resulting solution was filter sterilized through a 0.22 µm Millipore filter and stored at 4°C.

Malaria parasite thawing solution

3.5 g of NaCl was dissolved in 100 ml of distilled water to obtain 3.5% hypertonic saline. The solution was sterilized by autoclaving.

Malaria parasite freezing solution

4.2 g of sorbitol was dissolved in 0.9% NaCl solution to obtain 4.2% sorbitol solution. To 180 ml of the sorbitol solution was added 70 ml glycerol. The mixture was sterilized by Millipore filtration.
10% Giemsa stain

1 ml of giemsa stain was dissolved 9 ml of in water and filtered through a Millipore filter.
APPENDIX IV

Haematin concentrations in fractions I, II and III obtained from the polymerisation of 0.4 µmol/well of haematin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Porphyrin µmol/well</th>
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<tbody>
<tr>
<td>I Unpolymerized haematin</td>
<td>0.002 ± 0.003</td>
</tr>
<tr>
<td>II DMSO-soluble</td>
<td>0.193 ± 0.052</td>
</tr>
<tr>
<td>III DMSO-insoluble, NaOH-soluble</td>
<td>0.212 ± 0.032</td>
</tr>
<tr>
<td>Total</td>
<td>0.407 ± 0.060</td>
</tr>
</tbody>
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