PLANTS AS SOURCES OF
ANTI-PROTOZOAL
COMPOUNDS

Thesis presented by

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for the degree of

Doctor of Philosophy

in the Faculty of Medicine
of the University of London

Department of Pharmacognosy
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University of London

1995
dedicated to
my late father
Abstract

The majority of the world's population relies on traditional medicine, mainly plant-based, for the treatment of disease. This study focuses on plant remedies used to treat tropical diseases caused by protozoan parasites.

The following protozoal diseases: African trypanosomiasis, leishmaniasis, South American trypanosomiasis and malaria, and the traditional use of plant remedies in their treatment, are reviewed in a world wide context.

In the present work, vector and mammalian forms of Trypanosoma b. brucei, the vector forms of Leishmania donovani and Trypanosoma cruzi and the mammalian forms of Plasmodium falciparum were maintained in culture in vitro in order to evaluate the activity of a series of plant extracts, pure natural products and synthetic analogues against these protozoan parasites in vitro.

Six species of West African plants, Alstonia boonei, Alchornea cordifolia, Annona senegalensis, Lonchorcarpus cyanensis, Tamarindus indica and Ximenia americana, reputed to be used in the treatment of human African trypanosomiasis, were evaluated for activity against T. b. brucei, L. donovani and T. cruzi, in vitro. Crude extracts of three of these plants, A. boonei, A. cordifolia and A. senegalensis, were active in the range of 30 - 500 μg/ml
against T. b. brucei but inactive against T. cruzi and L. donovani. A. boonei was further investigated, resulting in the isolation of the indole alkaloids, echitamine, undulifoline, 12-methoxyechitmidine and a mixture of echitamidine and 12-methoxyechitamidine. These were identified on the basis of UV, MS, $^1$H NMR, $^{13}$C NMR and COSY spectroscopy. 12-methoxy-echitamidine and undulifoline have not previously been reported to have been isolated from A. boonei. Four mixtures of terpenoids were also isolated from A. boonei. The indole alkaloids caused a 100% inhibition of T. b. brucei mammalian forms at a concentration of 30|ig/ml in vitro and three of the mixtures of terpenoids inhibited the motility of T. b. brucei procyclic forms in a range of concentrations from 60-250|ig/ml in vitro.

Lignans representing the natural product surinamensin, from Virola surinamensis and a lignan from Virola pavonis from Brazil, together with twenty four synthetic analogues, were tested for activity against L. donovani promastigote and amastigote forms in vitro. Fourteen lignans, including surinamensin and the natural lignan from V. pavonis, supressed promastigote forms 100% at a range of concentrations from 30-100 μM, but ten of them were toxic to macrophages in the amastigote form test. Four synthetic lignan analogues with sulphur bridges between C-8 and C-4' were selectively active against L. donovani amastigotes at 30 and 100 μM and one of these (3,4-dimethoxyphenyl -(1-(4-methylphenyl)thio)ethyl ketone) had a 42% inhibition in
vivo at a dose of 100 mg/kg/day in the 5 day suppressive test in mice. Mode of action studies suggested that these lignans may inhibit microtubule formation or function.

The lignan podophyllotoxin, the alkaloids, camptothecin and ellipticine and the flavonoids, morin, phloridzin, phloretin and cosmetin and the phenolic compound, maclurin were tested against *L. donovani* amastigote forms *in vitro*. None of these compounds showed any selectivity in their action against *L. donovani* amastigotes in the mouse peritoneal macrophage test.

A traditional anti-leishmanial treatment from Iraq, known as Kubbal Azrak, was investigated for anti- *Leishmania* spp. activity and had ED$_{50}$ values of 0.05 and 0.01 µg/ml against *L. donovani* amastigote and promastigote forms *in vitro* but proved toxic to macrophages. When tested against *Leishmania major* in vivo it was inactive. The major component of Kubbal Azrak was identified as gentian violet, on the basis of thin layer chromatography, UV, $^1$H NMR and $^{13}$C NMR spectroscopy.

*Cryptolepis sanguinolenta* is traditionally used as an antimalarial remedy in Ghana and the major alkaloid, cryptolepine had an IC$_{50}$ value of 0.134µM against *P. falciparum* *in vitro*, comparable to chloroquine which has an IC$_{50}$ value of 0.023µM. Cryptolepine was not active against *P. berghei* in vivo in mice. UV spectroscopy was used to
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First of all I would like to thank my supervisors, Professor J. David Phillipson and Doctor Simon L. Croft, for their guidance and encouragement throughout these studies. I would also like to thank Dr G. C. Kirby and Dr D. C. Warhurst from the Department of Medical Parasitology at the London School of Hygiene and Tropical Medicine for their invaluable help.

My special thanks go to Doctor Peter Houghton at the Department of Pharmacognosy, Kings College, in whose laboratory some of the phytochemistry was carried out, and who supplied me with African plant material and to Doctor Lauro Barata of the Department of Chemistry, University of Campinas, Brazil, who supplied the semi-synthetic neolignans.

I am indebted to Kings College University NMR services, Department of Chemistry and Steve Vandehoven of Astra Research, for the NMR spectra and the Mass Spectroscopy Unit of the School of Pharmacy for the mass spectra. I am grateful to members of staff at the Royal Botanical Gardens of Kew, who authenticated the African plant material. I would like to thank Doctor B. K. Noamesi, of Kumasi University, Ghana, for the sample of cryptolepine and Professor Farnsworth for the data from the NAPRALERT data base.
I would like to thank the technical staff and PhD students who provided me with help from the School of Pharmacy, Department of Pharmacognosy and Computer Unit; The London School of Hygiene and Tropical Medicine, Department of Medical Parasitology; and Kings College, Department of Pharmacognosy. I would also like to thank the library staff of the latter three institutions as well as of the British Library.

Special thanks go to the Sir Halley Stewart Trust for the funding, without which this work would not have been possible.
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PLANTS AS SOURCES OF ANTI-PROTOZOAL COMPOUNDS

CHAPTER 1 INTRODUCTION

1.1 General Introduction. Plants as medicinal agents

Plant medicine has been used since the dawn of history and, even today, 80% of the world's population still depends on traditional medicine (based mainly on plant remedies) for the treatment of illnesses, both physical and psychological (Farnsworth et al., 1985). Forty percent of the manufactured drugs in use today are either natural products or are derived from natural products (Samuelsson, 1992). Farnsworth identified 119 plant-derived chemical compounds of known structure that are used as pharmaceutical agents (Farnsworth, 1990), only 11 of which are synthesized (Farnsworth et al., 1985; Fig. 1; Pages 3 and 4). These are: allyl isothiocyanate (1) from Brassica nigra; L-dopa (2) from Mucuna deeringiana; kawain (3) from Piper methysticum; menthol (4) from Mentha spp.; papaverine (5) from Papaver somniferum; danthron (1,8-dihydroxy anthraquinone) (6) from Cassia spp.; methyl salicylate (7) from Gaultheria procumbens, and allantoin (8), benzyl benzoate (9), borneol (10) and pinitol (11) from several genera. The remaining 108 have been isolated on a commercial basis directly from plants, e.g. the alkaloid morphine (12; Fig. 2; Page 5) is extracted and isolated from the capsules and poppy straw of Papaver somniferum.
which is cultivated extensively in many parts of Asia (Samuelsson, 1992).

Important classes of biologically active plant-derived compounds include alkaloids, phenolic compounds, terpenes, cardioactive glycosides, lignans, vitamins and plant steroids.

Alkaloids can be divided into two broad classes: non-heterocyclic alkaloids, (for example, ephedrine (13) and colchicine (14; Fig. 3; Page 6) and heterocyclic alkaloids, to which many important drugs belong, for example, hyoscine (15), atropine (16) and cocaine (17) (tropane alkaloids), quinine (18) and quinidine (19) (quinoline alkaloids), berberine, (20) tubocurarine (21; Fig 4; Pages 6 and 7), morphine (12) and codeine (22; Fig 2; Page 5) (isoquinoline alkaloids), vinblastine (23) and vincristine (24) (indole alkaloids) (Fig. 4; Page 7).

Phenolic compounds of pharmacological interest include coumarins, (for example, esculetin; 25) anthraquinones (for example, sennoside A; 26) and flavones (for example, hesperidin; 27) and rutin (28; Fig. 5; Page 8)). Terpenes are present in many volatile oils e.g. menthol (4) and thymol (29), (monoterpenes). Lignans (for example, podophyllotoxin; 30), are constituents of resins. Cardioactive glycosides include digitalin (31; Fig. 5; Pages 8 and 9).
Fig. 1  Plant-derived compounds which may now be synthesized commercially.

(1) allylisothiocyanate

(2) L-dopa

(3) kawain

(4) menthol

(5) papaverine

(6) danthron
Fig. 1 continued

(7) methyl salicylate

(8) allantoin

(9) benzyl benzoate

(10) borneol

(11) pinitol
Fig 2. morphinane alkaloids

(12) morphine \( R_1 = \text{OH} \)
\( R_2 = \text{OH} \)

(22) codeine \( R_1 = \text{OH} \)
\( R_2 = \text{OCH}_3 \)

(45) thebaine

(46) etorphine

(47) buprenorphine

(48) nalbuphine
Fig. 3 Non-heterocyclic alkaloids

(13) ephedrine

(14) colchicine

Fig. 4 Heterocyclic alkaloids

(15) hyoscine $R_1 R_2 = \text{O}$

(16) atropine $R_1 R_2 = \text{H}$

(17) cocaine

(44) procaine
Fig. 4 continued

(18) quinine

(19) quinidine

(20) berberine

(21) tubocurarine chloride

(23) vinblastine

\( R = CH_3 \)

(24) vincristine

\( R = CHO \)
Fig. 5 Cardioactive glycosides (31 and 32), phenolic compounds (25 and 26), flavones (27 and 28), a terpene (29), a lignan (30) and a plant sterol (33).

(25) esculetin

(26) sennoside A

(27) hesperidin

(28) rutin

(29) thymol
Fig. 5 continued

(30) podophyllotoxin

(31) digitalin

(32) diosgenin

(33) stigmasterol
Plant products may be used as crude plant extracts, semipurified mixtures of active compounds, or as purified and/or modified active compounds (Farnsworth and Soejarto, 1985).

Crude plant extracts still in use in the developed world include digitalis leaf from Digitalis spp., containing 0.3% cardenolic glycosides (British Pharmacopoeia, 1993), which has a positive inotropic effect on cardiac muscle and is used as a remedy for congestive heart failure (Bolognesi et al., 1992) and senna tablets, the standardized powdered pericarp of the fruit of Cassia senna and Cassia angustifolia, containing sennosides, (anthraquinone derivatives) which have a purgative action (British Pharmaceutical Codex, 1973).

Semipurified mixtures of active compounds include flavonoid preparations from citrus species, containing hesperidin (27) and rutin (28; Fig 5; Page 8) which decrease capillary fragility (Trease and Evans, 1989); and Veratrum rhizome from Veratrum viride, an alkaloid mixture used for the treatment of hypertension (Samuelsson, 1992).

Modified active compounds include plant sterols which are used as starting materials for the partial synthesis of steroid drugs. Diosgenin (32) from Dioscorea floribunda has been used as a template for forty useful steroid drugs (Farnsworth and Soejarto, 1985) and stigmasterol (33),
extracted from the unsaponifiable fraction of soya-bean oil, is now the principal starting material for the industrial semi-synthesis of steroids (Fig. 5; Page 9; Samuelsson 1992).

Examples of semisynthetic derivatives include the rauwolfia alkaloids deserpidine (35), raunescine (36), rescinnamine (37) and syrosingopine (38), all derived from reserpine (34), an antihypertensive, sedative drug isolated from *Rauwolfia spp.* (Bowman and Rand, 1980; Fig. 6; Pages 12 and 13).

Natural products have been used as templates for the synthesis of a broad array of analogues currently in use as pharmaceutical agents (Farnsworth, 1990): the glycoside salicin (39) derived from *Salix spp.* was the starting material for the synthesis of salicylic acid (40), from which a series of related drugs has been derived. These include acetyl salicylic acid (41; Fig. 6; Page 13) which was first synthesized in 1899 and marketed under the name aspirin and which became one of the cheapest and most widely used drugs in the world (Bowman and Rand, 1980). Camptothecin (42; Fig. 6; Page 13), derived from *Camptotheca acuminata*, is a planar aromatic alkaloid with anti-leukaemic activity *in vivo* which was used as a template for the synthesis of a series of analogues (Wall and Wani, 1984).
Rauwolfia alkaloids (34-38) and some plant compounds which have been used as templates for the synthesis of analogues (39-43).
Fig. 6 continued

(38) syrosingopine

(39) salicin $R = O$-glucose

(40) salicylic acid $R = OH$

(41) acetyl salicylic acid $R = O\cdot C\cdot CH_3$

(42) camptothecin

(43) gossypol
Analogues of gossypol (43; Fig. 6; Page 13), derived from Gossypium spp. (Malvaceae), have been synthesised and tested in the search for less toxic, more effective compounds for use as antifertility drugs (Fong, 1983). Cocaine (17), derived from Erythroxylon coca, was first introduced as a local anaesthetic in 1884 and gave rise to the development of hundreds of synthetic analogues including procaine (44; Fig. 4; Page 6; Liljestrand, 1971).

Thousands of derivatives and several synthetic analogues have been prepared from naturally derived opiates, still the first choice pain killers in terminal cancer (Scherpereel, 1991) in the search for stronger analgesic properties and fewer side effects (Rapoport, 1983). The 6,14-ethenomorphinan type compounds such as etorphine (46) (derived from thebaine; 45), are many times more powerful pain killers than morphine (12) which itself has many synthetic analogues such as buprenorphine (47) and nalbuphine (48; Fig 2; Page 5) currently in use (Bazin and Picard, 1992). Analogues of the psychoactive cannabinoids from Cannabis sativa are currently undergoing development for controlling nausea in cancer chemotherapy and in the treatment of glaucoma (Razdan, 1983).

1.1.1 Traditional medicines in the developing world

In the Developing World traditional medicines based on crude plant preparations are widely used to treat most
diseases and medical conditions (Waller, 1993). Ethnomedicine has several advantages in the Developing World over modern pharmaceutical products: for example, its relative cheapness, local availability, and its link with traditional society. The accessibility and acceptability of traditional plant-based remedies is especially important in areas where modern medicines are not available or where people cannot afford them (Waller, 1993).

For many countries in the Developing World the cost of importing pharmaceutical products on a large scale is prohibitive and the Developing World's capacity for the isolation and manufacture of large quantities of active compounds from plants or the synthesis of drugs is inadequate (Farnsworth et al., 1985).

In 1978 the World Health Organization (WHO) passed a number of resolutions in recognition of the importance of traditional plant-based medicine in the health systems of many developing countries and called for the therapeutic classification of medicinal plants used in a number of countries in order to assess their safety and efficacy. WHO advised the adoption of international standards for the establishment of identity, purity, and strength of plant preparations, the dissemination of information among member States of the WHO and the designation of research and training centres for the study and conservation of medicinal plants (Akerele, 1991).
The organisational framework of traditional medicine varies from country to country, with well documented and well researched systems in existence in India and China (Farnsworth, 1990), but frequently intermixing with magic procedures and religious ceremonies in many other countries (Schultes, 1979).

In China, where herbal medicine has a long history, over 300 books on Chinese herbal medicine have been published and a recent survey identified 4,877 plant species with useful medicinal properties (Hsiao, 1980).

The Chinese categorise traditional medicine into three types:

1) Chinese Traditional Medicine, a well-documented system utilizing several hundred well-known plants.
2) National minorities medicine, used by healers within the national minorities such as Mongolia, Uygur, and Tai.
3) Folk medicine, where remedies (many of which are kept secret) are passed on by word of mouth from generation to generation (Hsiao, 1980).

In China over 5,000 of the 35,000 indigenous species of plants are used as medicines (Farnsworth and Soejarto, 1991) and traditional medicine is an integral part of the formal health system. It is used in about forty percent of cases at primary care level (Akerele, 1991). In 1991 the
annual demand for medicinal plants was 700,000 tons.

The first volume of the Chinese Pharmacopoeia is devoted almost totally to traditional Chinese medicinal plants and their varied preparations (Xiao Pei-gen, 1991). Chinese Traditional Medicine is also popular in South East Asian countries such as Japan, Korea, Hong Kong, and Malaysia (Principe, 1991).

India's use of plants for medicinal treatment, one of which is the Ayurvedic system (containing over eight thousand remedies), dates back over five thousand years. Ayurvedic medicine is still used in over fourteen thousand dispensaries and recognises over two thousand five hundred medicinal plants (Principe, 1991).

In Africa ethnobotanical surveys have been carried out in twelve former French colonies and in Western Nigeria (Adjanohoun et al., 1988, 1991, Sofowara, 1993), in Uganda (Sofowara, 1993), Tanzania and in Somalia (Samuelsson et al., 1991). In addition, seventeen research centres have been financed by the Organization of African Unity/Scientific Technical Research Commission (OAU/STRC) to promote research into the therapeutic efficacy of traditional remedies (OAU/STRC, 1968) and the first pan-African pharmacopoeia is currently being compiled under the auspices of OAU/STRC (OAU/STRC, 1985, 1986).
Research programmes to evaluate medicinal plants are also being carried out in several South American countries, including Brazil, where phytotherapy forms an integral part of the mainstream health service in several states (Inoué, 1990; Elizabetsky and Wannmacher, 1993), and where the Brazilian Foundation of Medicinal Plants has developed a data base (Souza Brito and Souza Brito, 1993) and various government funded research programmes are in progress. In Panama ethnobotanical surveys have been carried out in collaboration with Native Indian Peoples with a view to promoting the cultivation of pharmacologically active medicinal plants in areas where economic and cultural factors severely limit the use of modern pharmaceutical products (Joly et al., 1987; Joly et al., 1990; Gupta et al., 1993). In Bolivia and Paraguay the Instituto Boliviano de Biologia de Altura (IBBA) and l’Institut Français de Recherche pour le Dévelopement en Coopération (ORSTOM) have undertaken ethnopharmacological studies in collaboration with local and indigenous groups (Fournet et al., 1994). Costa Rica has also initiated a research programme (Ginzburg, 1977).

In the Caribbean a research project involving the participation of several Central American countries, in addition to Colombia, Venezuela and all the Caribbean Islands, aims to evaluate scientifically traditional plant remedies with a view to publishing a plant pharmacopoea as part of the Caribbean Health Policy (Soejarto and Robineau,
There are also ethnopharmacological surveys currently under way in Samoa (Cox, 1993), Papua New Guinea (Holdsworth and Mahana, 1983), Belize, Honduras, Dominican Republic, Puerto Rico, Martinique, Guyana, Ecuador, Peru, and Guatemala (Balick, 1990).

Many pharmaceutical companies are funding ethnopharmacological surveys with a bias towards discovering plant products with anti-cancer or anti-AIDS activity. By 1990, 107 US pharmaceutical companies (223 worldwide) were involved in plant medicine research with several clinical trials in progress (Fellows, 1992).

1.1.2 Ethnopharmacology

The term ethnopharmacology was first used at an international symposium held in San Francisco, in 1967, to describe the historical, cultural, anthropological, botanical, chemical and pharmacological aspects of traditional psychoactive drugs (Del Pozo, 1979). Ethnopharmacology has since been defined as the collection of plants used by indigenous peoples in traditional medicine (Cox, 1990), as the collection of plants with the greatest amount of biological activity, as identified by their frequent use by traditional healers (Balick, 1990), and as an interdisciplinary science based upon the correct...
botanical identification, collection and preservation of plants used in traditional medicine, and for the isolation, pharmacological analysis and structural determination of their active compounds in order to discover plant products with potential as drugs (Cox, 1993).

Native people who have lived for many generations in tropical rain forests, whose rich diversity of flora contains a wealth of medicinal plants (Balick, 1990), possess extensive knowledge of the medicinally useful species (Elizabetsky and Wannamacher, 1993) which are more likely to contain pharmacologically active compounds than plants collected at random (Cox, 1990; Farnsworth, 1990; Balick, 1990).

Destruction of the rainforests by the mining and logging industries and by agriculture continues to destroy a biologically rich habitat and potential source of new products. It also destroys the primary health care network of native people since contact with non-local people who move into these areas results in the gradual loss of traditional healing systems together with the healers' store of knowledge (Balick, 1990). The depletion of this great natural resource (75,000km² of rainforest is lost annually - Fellows, 1992) makes the collection of ethnomedical data from forest-dwelling people a matter of urgency.
A data base called Napralert (Natural Products Alert) has been in existence since 1975 in the Department of Pharmacognosy and Pharmacology, University of Illinois, for the surveillance of world literature on the chemistry and pharmacology of natural products (Farnsworth, 1983). The NAPRALERT database documents the ethnomedical uses for approximately 9,200 species of monocotyledonous and dicotyledonous angiosperms, gymnosperms, pteridophytes, bryophytes and lichens, suggesting that 28% of the plants on earth have at some point been used in ethnomedicine (Farnsworth and Soejarto, 1991).

1.1.3 Biological screening of medicinal plants and plant products.

After plant material has been selected, carefully recorded and herbarium samples stored for future reference, crude extracts of plants may be evaluated for biological activity, prior to fractionation and elucidation of the structure of active compounds. Screening is usually carried out in at least four stages:

1 primary screening;
2 secondary screening;
3 toxicity testing;
4 clinical trials.

Primary screening is used for crude extracts of plant
material and may be divided into: (i) a prescreen, in which large numbers of initial samples are tested rapidly in order to discard inert material; (ii) a screen, in which material is selected for individual study; and (iii) a monitor screen used to guide fractionation of crude material towards isolation of pure active compounds. Secondary screening may consist of detailed testing of lead compounds in multiple models and test conditions in order to determine selectivity, but sometimes multiple models and test conditions are used in a primary screen, since crude extracts may vary considerably in their effect in different systems (Suffness and Pezzuto, 1991). Highly specific molecular assays may also be carried out during secondary testing, targeting enzymes, receptors, DNA etc. in order to determine the mode of action of the compound.

Both primary and secondary screening may be carried out in vivo (using whole animals), ex vivo (using isolated organs; for example guinea pig ileum) or in vitro on organisms in culture or on sub-cellular organelles (WHO, 1973). The choice of screen depends on the resources available and the type of organism being screened against.

Crude extracts may have low activity in a primary screen because the active compounds are present in such small quantities that their effect is masked. Alternatively, a low activity of crude extract may mirror low activity of large quantities of the active compounds. Crude extracts
with high activity, on the other hand may contain non-selective cytotoxic compounds, and/or selectively toxic compounds, so that after the isolation of active compounds from these crude extracts it is essential that they are tested for toxicity. Toxicity testing may be carried out in vitro or in an animal model (in vivo.)

Clinical trials are carried out in human volunteers and patients. An additional stage may be added between toxicity testing and the clinical trial stage to study the pharmacokinetics of a new drug (Hugo and Russel, 1980).

From the 1950’s primary screening of crude plant extracts for anti-cancer and pharmacological activity was based largely on in vivo and ex vivo techniques. For example, the Hippocratic screen, a comprehensive in vivo pharmacological screen, measured a wide variety of parameters in rats injected intraperitoneally with crude plant extracts or pure compounds (Malone and Robichaud, 1962); the egg- cultivated tumour screen (Dalal et al., 1958) used embryonated eggs inoculated with tumours, while the egg- cultivated virus screen (Taylor et al., 1954) used embryonated eggs inoculated with viruses to screen plant extracts. Ex vivo techniques were used for pharmacological screens of plant extracts using several isolated organ preparations from a variety of animals (Hooper and Leonard, 1965; Feng et al., 1965).
In 1960 the National Cancer Institute (NCI), which had been testing plant extracts for antineoplastic activity since 1956 (Hardinge et al., 1961), initiated a major plant extract screening programme, testing potential anticancer agents in mice bearing transplanted solid tumours closely resembling the major tumours in man (lung, breast, colorectal; Alley et al., 1988). 120,000 plant extracts were prepared from 35,000 different species from worldwide sources and screened over a period of several years. Those found to be active were sent to NCI-supported chemists for isolation and purification of active compounds (Johnson, 1982).

In the early 1960's, at Eli Lilly laboratories, an ethnopharmacological investigation (using mice bearing transplanted P-1534 leukemias) into the plant Catharanthus roseus (Vinca rosea) with reputed oral hypoglycaemic agent activity, led to the isolation of the anti-leukemic agents vincristine (24) and vinblastine (23) (Fig. 4; Page 7; Johnson et al., 1963).

For years in vivo systems were used as a primary screen for anticancer and pharmacological activity and sometimes the same screen was used to assess antineoplastic and pharmacological activity simultaneously (Farnsworth et al., 1966).

Gradually in vitro anti-cancer screens were developed and,
in 1986, the NCI phased out their in vivo primary screen, developing in its place panels of human tumour cell lines against which potential anti-tumour agents were screened, in vitro, for selective cytotoxicity (Alley, et al., 1988), retaining in vivo tests as a secondary screen. By 1991 the NCI was screening natural products against 60 human tumour cell lines (Monks et al., 1991).

In vitro antimicrobial assays were developed as a primary screen long before in vitro antineoplastic assays. The main methods used have been reviewed (Farnsworth et al., 1966; Rios et al., 1988; Janssen et al., 1987) and can be divided into diffusion, dilution and bioautographic techniques.

Diffusion methods utilise a semi-solid medium such as agar seeded with bacterial or fungal test organisms. Wells cut into the agar may be filled with plant extract (Villar et al., 1986) or stainless steel or porcelain cylinders (Barbagallo and Chisari, 1987), or paper disks (Bondi et al., 1947) containing plant extracts may be applied to the surface of the agar. After incubation the diameter of the clear zone (inhibitory zone) around the disk, hole or cylinder is measured to determine the degree of activity present in the test material, as compared with controls. Minimum inhibitory concentration (MIC) can be calculated from the width of the inhibitory zone (Hugo and Russell, 1980) but a more accurate MIC value can be obtained using
a dilution technique in liquid medium (Rios et al., 1988).

In dilution methods the microbe to be tested is seeded into a liquid medium (Fournier et al., 1978) or a semisolid medium (Mitscher et al., 1972) and, after incubation with the plant extract or compound, inhibition is assessed, for example, by turbidometry (Hugo and Russell, 1980).

Bioautography can be used to localise antimicrobial activity on a chromatogram by allowing diffusion of compounds from a chromatogram to the inoculated agar plate (contact bioautography; Betina, 1973), by applying a liquid suspension of the test microorganism directly onto the chromatogram (direct bioautography; Hamburger and Cordell, 1987) or by covering the chromatogram with agar seeded with the test organisms (immersion bioautography; Betina, 1973). Inhibition zones are then visualized by appropriate vital stains (Begit and Kline, 1972).

Antimicrobial plant product screening programmes are currently under way (Hernández-Pérez et al., 1994) in order to identify novel plant compounds for use as antimicrobial agents.

Early in vitro anti-viral tests identified few anti-viral compounds (Grunert, 1979). However, the development of a variety of cell culture systems suitable for infection with the relevant pathogenic viruses led to effective in vitro
anti-viral tests such as the virus yield reduction assay (Collins and Bauer, 1977), the plaque reduction assay (Field et al., 1986), and the inhibition of virus-induced cytopathic effect (CPE) assay (De Clercq et al., 1980). For viruses which do not produce plaques or CPE, haemagglutination or haemadsorption tests (Bauer, 1972), enzyme-linked immunosorbent assays (ELISA; Farber et al., 1987), or the inhibition of radiolabelled precursor uptake (Lin et al., 1983) may be used. The reverse transcriptase inhibition test has been used to screen 156 natural products (Tan et al., 1991).

Plant products are a major part of the NCI large scale in vitro multiparameter primary anti-HIV screen (Gulakowski et al., 1991) and are currently being screened in vitro for anti-viral activity by several other investigators (Hayashi et al., 1990) in addition to the NCI.

Plant products are also being screened in vitro for anti-inflammatory activity, (Benencia, et al., 1994; Pongprayoon, et al., 1991), anti-allergic activity (Tanno et al., 1989), anti-hormonal activity (Izzi et al., 1990), anti-free radical activity (Costantino et al., 1992) and immune stimulatory activity (Ushio and Abe, 1991).
1.1.3.1. Screening medicinal plants and plant products against disease-causing protozoan parasites.

Protozoan parasites (Table 1; Page 29) are responsible for a range of severe diseases in humans. Since the establishment of culture systems for most human disease-causing protozoan parasites, in vitro primary screens have been developed for a wide variety of these organisms including *Plasmodium falciparum*, *Entamoeba histolytica*, *Leishmania* spp., *Trypanosoma brucei* spp., *T. vivax*, *T. congolense*, *T. cruzi* spp., *Giardia lamblia* and *Toxoplasma gondii*.

1.1.3.2. *In vitro* screens against *Plasmodium falciparum*

Phillipson (1991) has reviewed *in vitro* and *in vivo* antimalarial plant product screening methods.

Early attempts to screen plant extracts for antimalarial activity used various avian malaria species *in vivo*, for example, *P. relictum* in canaries in the 1920's and *P. gallinaceum* in chicks in the 1930's. *P. cathemerium* was also used, in ducklings. However, there are major pharmacokinetic differences between the effects of drugs on *Plasmodium species* which infect birds and those which infect humans (Peters, 1970).
Table 1  Some major protozoal parasites which cause disease in humans

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>location</th>
<th>Disease caused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium falciparum, P. vivax, P. ovale and P. malariae</td>
<td>erythrocytes and liver</td>
<td>Malaria*</td>
</tr>
<tr>
<td>Trypanosoma brucei spp.</td>
<td>blood, lymph, and CNS</td>
<td>Sleeping sickness*</td>
</tr>
<tr>
<td>Trypanosoma cruzi</td>
<td>blood, lymph, muscle and nerves</td>
<td>Chagas' disease*</td>
</tr>
<tr>
<td>Leishmania spp.</td>
<td>macrophages of the reticulo-endothelial system</td>
<td>Visceral* and Cutaneous Leishmaniasis</td>
</tr>
<tr>
<td>Trichomonas spp.</td>
<td>intestinal and reproductive tracts</td>
<td>Trichomoniasis</td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>duodenum, jejunum and upper ileum</td>
<td>Giardiasis</td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td>large intestine, liver and other sites</td>
<td>Amoebic dysentery</td>
</tr>
<tr>
<td>Balantidium coli</td>
<td>caecum and colon</td>
<td>Balantidiosis</td>
</tr>
<tr>
<td>Cryptosporidium parvum.</td>
<td>epithelial cells of large and small intestines</td>
<td>Cryptosporidiosis</td>
</tr>
<tr>
<td>Dientamoeba fragilis</td>
<td>large intestine</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>muscle and intestinal epithelium and other tissue</td>
<td>Toxoplasmosis</td>
</tr>
<tr>
<td>Naegleria fowleri</td>
<td>brain</td>
<td>Meningo-encephalitis</td>
</tr>
<tr>
<td>Acanthamoeba spp.</td>
<td>eye and CNS</td>
<td>Keratitis and encephalitis</td>
</tr>
</tbody>
</table>

* severe tropical diseases
A rodent model was developed in mice by Lips, in 1948 (see Peters, 1970), using P. berghei, a parasite derived from the African tree rat Thamnomys surdaster, which has proved invaluable for in vivo screening ever since. Also in the 1940's simian models were developed using P. cynomolgi in Macacus mulatta, and P. knowlesi in Macacus irus, which closely parallel P. vivax and P. falciparum, respectively, in man (Peters 1970).

In vitro culturing techniques for Plasmodium falciparum (Trager and Jensen, 1976) provided the opportunity to develop an in vitro screen (Desjardins' et al., 1979). This technique has since been modified (Osisanya et al., 1981; Fairlamb et al., 1985) and measures the activity of potential antimalarial drugs against cultured intraerythrocytic asexual forms of the human malaria parasite, Plasmodium falciparum. Inhibition of the uptake of a radiolabelled nucleic acid precursor, hypoxanthine, by the parasites serves to indicate antimalarial activity.

Both the in vitro system (O'Neill et al., 1985; Kirby et al., 1989; Weenen et al., 1990) and in vivo systems (Peters, 1987), have been used to assess crude plant extracts, as well as pure compounds for anti-malarial activity.
1.1.3.3. In vitro screens against *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis*.

In vitro anti-amoebic screening techniques for plant products were developed using clonal growth of the parasites in semisolid agar media (Gillin and Diamond, 1978) then, subsequently, with *Entamoeba histolytica* axenic cultures in liquid medium (Bhutani et al., 1987; Keene et al., 1986; Wright et al., 1988). The latter test involves a simple microdilution technique and viability of amoebae, after inhibition by plant products, is assessed by staining with aqueous eosin (0.5%) and reading the optical density at 490 nm with a microplate reader. Medicinal plants and plant products have been screened in vitro, using this technique, by Ansari and Ahmad (1991) and Sharma and Bhutani (1988).

Plant products have also been screened against *Giardia lamblia* and *Trichomonas vaginalis* (Kaneda et al., 1991) axenic cultures in liquid medium.

1.1.3.4. In vitro screens against trypanosomatids

In vitro screens against the haemoflagellate trypanosomatid parasites of man, *Leishmania spp.* and *Trypanosoma spp.*, have been reviewed by Croft, (1986) and Gebre-Hiwot et al., (1992).
1.1.3.4.1. **In vitro** culture systems for the vector forms of trypanosomatids.

The vector stage of all three parasites, *Trypanosoma cruzi* epimastigote forms, *Leishmania* spp. promastigote forms and *Trypanosoma brucei* *brucei* procyclic trypomastigotes, were the first stages of trypanosomatids to be maintained axenically in culture. *T. b. brucei* procyclic trypomastigotes were first cultured in 1904 (Novy and MacNeal, 1904) but it was not until the 1970s that culture systems were developed for the bloodstream form trypomastigotes of *T. b. brucei* and *T. b. gambiense*. *L. tropica*, *L. major*, *L. aethiopica*, the *L. donovani* complex, promastigote forms of the *L. mexicana* complex and *L. braziliensis panamensis* (Taylor and Baker, 1968; Evans, 1978), and *T. cruzi* epimastigote forms (Bone and Parent, 1963) have also been cultured in various media.

1.1.3.4.2. **Screening plant products against the vector forms of trypanosomatids in vitro**

Early screens were developed against these stages. Plant products have been screened against *T. cruzi* epimastigote forms (Lopez et al., 1978; Cavin et al., 1987), against *L. donovani* promastigote forms (Robert-Géro et al., 1985; Hazra et al., 1987; Richomme et al., 1991), against *L. chagasi* promastigote forms (Iwu et al., 1992), against *L. brasilensis* promastigote forms, and against *L. mexicana*
amazonensis promastigote forms (Fournet et al., 1988a), using simple microdilution techniques and assessing viability of the parasites by microscopy or by counting motile forms (by haemocytometer or Coulter counter). The same plant products have also been screened against Leishmania spp. promastigote forms and T. cruzi epimastigote forms (Hocquemiller et al., 1991; Fournet et al., 1992a; Fournet et al., 1994).

Plant products have been screened against T. b. brucei procyclic trypomastigote forms (Eid et al., 1988; Owolabi et al., 1990) and Cenini et al., (1988) tested plant products for activity against both T. b. rhodesiense procyclic forms and Leishmania infantum promastigote forms.

However, drug susceptibility of the vector form does not always parallel that of the mammalian stage. For example, L. donovani promastigote forms are not affected by sodium stibogluconate or meglumine antimoniate (Jiminez and Ercoli, 1965), whilst ketoconazole, which inhibits intracellular T. cruzi amastigote forms (at a concentration of 0.1μg/ml), only inhibits replication of epimastigote forms at high concentrations (McCabe et al., 1984). With the development of intracellular culture systems for Leishmania spp. (Neal and Matthews, 1982) and T. cruzi (Williams and Remington, 1977), it became possible to assess potential therapeutic agents against the mammalian stages of these parasites.
1.1.3.4.3 In vitro culture systems for the mammalian forms of trypanosomatids.

T. cruzi amastigote forms have been cultured in a range of fibroblast cell lines (for example, subcutaneous mouse fibroblast; McCabe et al., 1984), and a range of muscle cell lines, (for example, a heart cell line; Gutteridge et al., 1969), African green monkey kidney (Vero) cells (Rovai et al., 1990) and human monocytes and macrophages (Williams and Remington, 1977). Amastigote forms of Leishmania spp. have been cultured in a dog sarcoma cell line (Mattock and Peters, 1975), a mouse macrophage cell line (Berens et al., 1980), human blood monocyte-derived macrophages (Berman and Wyler, 1980), and a human leukaemia monocyte cell line (Gebre-Hiwot et al., 1992). Both T. cruzi (Williams and Reminton, 1977) and Leishmania spp. amastigote forms (Bachrach et al., 1980) have been cultured in mouse peritoneal macrophages.

1.1.3.4.4 Screening plant products against amastigote forms in vitro.

Plant products have been screened in vitro against intracellular amastigote forms of Leishmania spp. in the mouse peritoneal macrophage model (Croft et al., 1985; Fournet et al., 1992a; Evans and Croft, 1987; Kaur et al., 1991) and against intracellular T. cruzi amastigote forms in Vero cells (Rovai et al., 1990). Leishmania spp.
amastigote forms can be maintained in mouse peritoneal macrophages for 8 days without destroying the macrophages and can be fixed and stained for counting by microscopy after treatment with drugs for seven days. Damage to the macrophages is assessed as an indication of toxicity to mammalian cells.

The advantage of using cultured cell lines is that they provide uniform samples of cells for infection with Leishmania spp. or T. cruzi, whereas mouse peritoneal macrophages may vary between samples. However, mouse peritoneal macrophages do not multiply so that the proportion of uninfected cells after drug treatment compared to the untreated group directly reflects the drug effect in killing intracellular amastigote forms. This permits an accurate comparison of the numbers of amastigote forms per host cell before and after drug treatment (Neal, 1987).

1.1.3.4.5. Screening plant products against Trypanosoma cruzi trypomastigote forms in vitro

Cover and Gutteridge (1982) developed a simple microslide technique to test drugs which might prevent the transmission of Chagas' disease through blood transfusion, since surveys of the incidence of T. cruzi infection in blood banks in South America range from 1.3 to 28% (Brener, 1979). The trypomastigote screen identifies compounds which
are active against the bloodstream forms. Plant products and extracts have also been screened in this system (González et al., 1990; Chiari et al., 1991; Cardoso et al., 1987; Sagua et al., 1987) in order to find a replacement for gentian violet which is used in many regions of South America to sterilise donor blood, despite severe drawbacks (Croft et al., 1988).

1.1.3.4.6. In vitro screens against Trypanosoma brucei spp. bloodstream forms.

Early in vitro test systems used T. b. brucei trypomastigote forms freshly isolated from infected rat blood. Williamson and Scott-Finnigan, (1978) assessed motility and infectivity of trypanosomes after the addition of drugs to freshly isolated infected blood and Desjardins et al., (1979) developed a test, similar to the antimalarial test, based on the inhibition of radiolabelled thymidine and L-leucine uptake by freshly isolated T. b. rhodesiense.

However, in order to develop screens with greater throughput capacity, T. b. brucei bloodstream forms needed to be cultured continuously. Early culture techniques for the vertebrate infective forms of T. b. brucei used a feeder layer of fibroblast-like cells (Hirumi et al., 1977). Subsequently, Chinese hamster lung cells, buffalo lung cells (Hill et al., 1978), rabbit fibroblast cells
(Brun and Schönenberger, 1979), *Microtus montanus* whole embryo fibroblasts (MEF; Brun et al., 1981), and embryonic cells from eland, impala, gazelle and goat (Brun et al., 1984) were used as feeder cell layers.

A model was developed for screening drugs, using the MEF system (Borowy et al., 1985), which enabled activity to be assessed against both the free bloodstream forms and the intercellular clusters of trypanosomes. The feeder cell layer in this system gives an indication of compound toxicity to mammalian cells in the same way that the mouse peritoneal macrophage cell layer indicates toxicity in amastigote tests. However, the MEF system is complex and time-consuming since *T. b. brucei* isolates, in contrast to *Leishmania* and *T. cruzi* amastigote forms, require a period of adaptation to growth on the feeder layer, and the MEF cell lines, unlike tumour cell lines, have a finite life. Not all *T. b. brucei* strains will grow on MEF cells and those which do tend to form intercellular clusters which cause problems if individual cell counts are used to assess trypanosome inhibition. If, on the other hand, a test is to be based on inhibition of the uptake of radiolabelled precursors, the presence of a metabolically active feeder cell layer makes the interpretation of the results difficult.

Breakthroughs in the development of *in vitro* screens against *T. b. brucei* were the first reports of successful
in vitro axenic culture systems for the mammalian forms (Baltz et al., 1985) and the Hirumis' continuous axenic culture system of T. b. brucei bloodstream forms in a modified Iscove's medium (HMI-18; Hirumi and Hirumi, 1989). This led to the development of a simple twenty four hour growth inhibition test against T. b. brucei bloodstream forms (Kaminsky and Zweygarth, 1989) and an incorporation test similar to the one described for Plasmodium falciparum by Desjardins et al., (1979) using the radiolabelled precursor $[^{3}H]$-hypoxanthine (Brun and Kunz, 1989). The plant product taxol had an EC$_{50}$ of 0.027µg/ml in the simple growth inhibition test (Kaminsky and Zweygarth, 1989), while gossypol, which was previously found to lyse T. b. brucei bloodstream and procyclic forms at a concentration of 20µM (Eid et al., 1988) was inactive in this system.

1.1.3.5. Relative advantages and disadvantages of in vitro and in vivo primary screens.

In vitro primary screens provide high throughput systems for screening small samples of plant material in order to obtain accurate, repeatable, data which can be compared with standard drugs used as a reference in the same system. The rapid results obtained make these systems ideal for the bioassay-guided fractionation of crude plant extracts in order to isolate and identify biologically active compounds in specific plant species. When looking for a compound with selective activity parallel assays can be carried out in
more than one system. For example, the NCI plant product screen uses a battery of different tumour cell lines (Monks et al., 1991). Fournet et al., (1992a; 1994) have carried out comparative screens of the same plant products against the vector forms of similar trypanosomatids (Leishmania spp. and T. cruzi).

In vitro tests can be used to compare the activity of analogues of active plant compounds in order to predict improved activity of new compounds. Drug-susceptible and drug-resistant strains of the test organism can also be compared in the same test.

However, the influences of metabolic, pharmacokinetic, and immune factors cannot normally be measured simultaneously in vitro (Gootz, 1990). A compound which is highly active in vitro may be toxic, or it may be ineffective in vivo because it is metabolised, or it may be so firmly bound to serum protein that it cannot exert its desired effect: furthermore, its distribution in the body may not allow it to encounter the challenge organisms for sufficient time to inhibit them (Hugo and Russell, 1980).

In vivo primary screens use large numbers of animals (Malone and Robichaud, 1962), are expensive (Holden, 1987), time consuming, complicated, sometimes not reproducible, require large amounts of plant extract (McLaughlin, 1991), and useful active compounds are sometimes missed due to the
presence of toxic constituents in the plant extract (Farnsworth et al., 1966). There are also emotional, moral and ethical reasons for seeking to limit the number of animals used in experiments.

1.1.4. Traditional medicine and protozoal diseases

Most of the populations of tropical countries still use plant extracts to treat diseases caused by protozoan parasites (Phillipson and O'Neill, 1987).

Certain plant families, e.g. Simaroubaceae, Annonaceae, and Rubiaceae, are used throughout the world to treat diseases caused by protozoal parasites. These have been reviewed by Phillipson et al., (1993). The largest number of antiprotozoal plants quoted in the literature are used traditionally to treat malaria; these have been reviewed by Phillipson and O'Neill (1987), Mukherjee (1991), Brandão et al., (1992) and Nkunya, (1992) and derive from the families Asteraceae, Meliaceae, Menispermaceae, and Rutaceae, in addition to the above mentioned families (Phillipson and Wright, 1991a).

Genera from the Simaroubaceae used in indigenous medicines include Picrasma javanica, a traditional Thai antimalarial (Pavanand et al., 1989); Brucea javanica, used in China and Thailand as an antimalarial; Simaba cedron and Ailanthus altissima (O'Neill et al., 1987) and Eurycoma
longifolia, a Malaysian febrifuge (Gimlette and Thomson, 1977). The Meliaceae, closely related to the Simaroubaceae, also contains a number of species which are used widely as anti-malarials e.g. Azadirachta indica (Bray et al., 1990) and Khaya grandifolia (Makinde et al., 1988).

Several well-known antimalarial plants are now cultivated in countries other than their country of origin. For example, Cinchona spp. (Rubiaceae), used since ancient times in South America (White, 1985), are now also cultivated extensively in Kashmir (Mukherjee, 1991) and many other tropical countries (Nkunya, 1992). Artemisia annua, which has been used in China for 2000 years, is now cultivated in India for the production of large quantities of artemisinin (Mukherjee, 1991).

Parallel use of plant species by widely separated groups to treat different diseases also occurs (Pavanand et al., 1988): for example, Brucea javanica is used as an antidysenteric in Thailand, as an antineoplastic in Ethiopia, while, in China, patients are treated with Brucea javanica for both malaria and dysentery (Pavanand et al., 1988).

The NAPRALERT natural product data base lists 565 records of plants used for malaria in traditional medicines (Farnsworth, 1993).
Table 2  Plants reputed to be used to treat African trypanosomiasis (Abbiw, 1990).

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Plant family</th>
<th>Part used</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haematostaphis barteri</em></td>
<td>Anacardiaceae</td>
<td>bark</td>
</tr>
<tr>
<td><em>Annona senegalensis</em></td>
<td>Annonaceae</td>
<td>root and bark</td>
</tr>
<tr>
<td><em>Cleistophophillus patens</em></td>
<td></td>
<td>leaf</td>
</tr>
<tr>
<td><em>Uvaria chamae</em></td>
<td></td>
<td>root</td>
</tr>
<tr>
<td><em>Cordia myxa</em></td>
<td>Boraginaceae</td>
<td>leaves</td>
</tr>
<tr>
<td><em>Ageratum conyzoides</em></td>
<td>Compositae</td>
<td>aerial parts</td>
</tr>
<tr>
<td><em>Afzelia africana</em></td>
<td>Leguminoseae</td>
<td>root</td>
</tr>
<tr>
<td><em>Lonchocarpus cyanensis</em></td>
<td></td>
<td>stem bark</td>
</tr>
<tr>
<td><em>Tamarindus indica</em></td>
<td></td>
<td>root</td>
</tr>
<tr>
<td><em>Costus afer</em></td>
<td>Zingiberaceae</td>
<td>root</td>
</tr>
<tr>
<td><em>Lophira lancelata</em></td>
<td>Ochnaceae</td>
<td>aerial parts</td>
</tr>
<tr>
<td><em>Ximenia americana</em></td>
<td>Olacaceae</td>
<td>stem bark,root</td>
</tr>
<tr>
<td><em>Opilia celtidifolia</em></td>
<td>Opiliaceae</td>
<td>root, leaf</td>
</tr>
<tr>
<td><em>Agraecum spp.</em></td>
<td>Orchidaceae</td>
<td>pseudo-bulbs</td>
</tr>
<tr>
<td><em>Ansella spp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bulbophyllum spp.</em></td>
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<tr>
<td><em>Eulophia spp.</em></td>
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<td><em>Habenaria spp.</em></td>
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<td><em>Listrostachys spp.</em></td>
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<tr>
<td><em>Polystachya spp.</em></td>
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<td></td>
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<tr>
<td><em>Lippia multiflora</em></td>
<td>Verbenaceae</td>
<td>aerial parts</td>
</tr>
<tr>
<td><em>Vitex fosteri</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The following plants are also allegedly used to treat sleeping sickness: *Argemone mexicana* (leaves, stems and root) and *Chasmanthera dependens* root, (Oliver-Bever, 1986)
Plants used traditionally in the treatment of amoebic dysentery have been reviewed by Phillipson and O'Neill (1987).

One of the first drugs reported for the treatment of amoebic dysentery was the isoquinoline alkaloid emetine (49; Fig. 9; Page 52), obtained from the roots and rhizomes of South American species of *Cephaëlis* (Rubiaceae), and which was used for centuries in Europe. Species of simaroubaceous plants used pantropically to treat dysentery include *Ailanthus altissima*, *Brucea javanica*, *Picrosma excelsa*, *Quassia amara* and *Simarouba glauca* (Keene et al., 1986). *Holarrhena floribunda* (Apocynaceae), which was introduced into Europe in the nineteenth century as a cure for dysentery is now cultivated in Burkina Faso; the bark is harvested for local use (Levy et al., 1990).

Traditional healers differ significantly from Western doctors in their descriptions of disease aetiology (Cox, 1990), sometimes treating symptoms rather than specific diseases. Thus diarrhoea may be treated with the same plant remedy, whether it is caused by *Entamoeba histolytica* or *Giardia lamblia*, and, indeed, there are few references in the literature specifically to plants used traditionally as antigiardial agents. A large number of plant species are also used to treat "fever" which can be caused by a number of diseases including malaria, sleeping sickness, viruses and numerous other aetiological agents and there are few
references in the literature to plants used traditionally to
treat sleeping sickness (Table 2; Page 41).

Medicinal plants used traditionally to treat leishmaniasis
have been reviewed by Iwu et al., (1994). Plant families
used to treat both malaria and leishmaniasis include
Annonaceae, Menispermaceae, Berberidaceae, Hernandiaceae
(Fournet et al., 1992a) and Bignoniaceae (Iwu et al.,
1994).

Plants from other families used traditionally to treat
leishmaniasis include *Pera benensis* (Euphorbiaceae), used
in Bolivia (Fournet et al., 1992a), *Picralima nitida*
(Apocynaceae), used in Nigeria (Iwu et al., 1992),
*Diospyros montana* (Ebenaceae), used in India (Hazra, et
al., 1987).

Plants used traditionally to treat leishmaniasis are being
investigated in several parts of the world, including
the Central Drug Research Institute, Lucknow, India (Kaur
et al., 1991), the Institute of African Studies, University
of Nigeria, Nsukka (as part of a Salvage Ethnography
Project; Iwu et al., 1992), the Instituto Boliviano de
Biologia de Altura (IBBA), and ORSTOM (French Institute of
Scientific Research for Development Cooperation) in Bolivia
(Fournet et al., 1994).

There are few references to plants used traditionally to
treat Chagas’ disease.
1.2 Anti-protozoal plant products

Antiprotozoal plant products have been reviewed several times in recent years (Phillipson and O’Neill, 1987; Wright and Phillipson, 1990; Phillipson and Wright, 1991a; 1991b; 1991c and Phillipson et al., 1993). Antiprotozoal compounds include quassinoids, quinoline alkaloids, indole alkaloids, bisbenzylisoquinoline alkaloids, protoberberine alkaloids and Strychnos alkaloids (Figs. 7 and 8; Pages 47-49).

1.2.1. Anti-malarial Plant Products

Studies have been carried out in many parts of the world on plants used traditionally to treat malaria, often screening crude extracts in vitro against Plasmodium spp. (Phillipson and Wright, 1991c; Anderson et al., 1988; Ratsimamanga-Urverg et al., 1991; Pavanand et al., 1989) or in vivo (Carvalho and Krettli, 1991; Makinde et al., 1990; Misra et al., 1991) or performing clinical trials with crude extracts of medicinal plants (Jurg et al., 1991). In addition, studies have been carried out on isolated antimalarial plant compounds, in vitro and in vivo, and these have been reviewed by Phillipson and O’Neill, (1987) Phillipson (1991); and Nkunya (1992).

The first anti-malarial plant compound to be isolated was the quinoline-quinuclidine alkaloid quinine (18), the principal alkaloid of South American Cinchona spp.
(Rubiaceae). For many years, quinine was used as a first line defence compound for the prevention and treatment of malaria and, although the development of synthetic 4-aminoquinoline antimalarial drugs led to a decline in its use, it is still used clinically due to resistance of P. falciparum in particular to synthetic antimalarials. The stereoisomer of quinine, the antiarrhythmic drug, quinidine (19), is also an effective antimalarial compound.

The family Simaroubaceae is rich in species used pantropically in traditional medicine to treat a wide range of diseases, including malaria. Studies focussing on this family (Trager and Polonsky, 1981; Guru et al., 1983; Chan et al., 1986; O’Neill et al., 1987; O’Neill et al., 1988; Anderson et al., 1991) have isolated several biologically active quassinoids, (polyoxygenated triterpenoids), β-carboline alkaloids, and indole alkaloids. The quassinoids are the more potent of these three groups of compounds; for example, bruceantin (50), glaucarubinone (51) and eurycomalactone (52), which are active in vitro against P. falciparum and cancer KB cells. Many quassinoids are cytotoxic but a few, such as bruceine D, (53) appear to be selectively toxic to P. falciparum in vivo (Fig 7; Page 47; Phillipson et al., 1993). Structural modifications to quassinoids have resulted in increased selectivity (Patel et al., 1991; Allen et al., 1993).
Fig. 7 Some anti-malarial terpenoids

(50) bruceantin: \[ R = \text{CH} = \text{C} \text{(CH}_3 \text{)} \text{CH} \text{(CH}_3 \text{)}_2 \]

(51) glaucarubinone

(69) bruceine A: \[ R = \text{CH}_2 \text{CH} \text{(CH}_3 \text{)}_2 \]

(52) eurycomalactone

(53) bruceine D

(54) nimbolide

(55) artemisinin
Fig. 7 continued

(56) yinghaosu A

(57) yinghaosu C

Fig. 8  Some anti-malarial alkaloids

(58) tiliacorine

(59) phaeanthine: R, R.

(60) isotetrandrine: R, S.
Studies of the closely related family Meliaceae, (Bray et al., 1990), which also contains modified terpenoids, focussed on a group of limonoids (oxidized triterpenes) with antimalarial activity, for example, nimbolide (54). Other antimalarial terpenoids include the sesquiterpene
lactone artemisinin (55), isolated from the Chinese antimalarial plant *Artemisia annua* (Compositeae) in 1972 (Klayman, 1985). Artemisinin was used as a template for the development of analogues with improved solubility and antimalarial properties which are currently in clinical use in China, Vietnam, Myanmar, Thailand and Brazil (WHO, 1994). Several antimalarial sesquiterpenoids have been isolated from another Chinese medicinal plant, *Arbotrys hexapetalus* (Annonaceae), including yinghaosu (56 and 57; Fig.7; Page 48).

*Triclisia* spp. (Menispermaceae) are used pantropically as antimalarials. Phytochemical investigations of these species yielded a series of antimalarial bisbenzylisoquinoline alkaloids including tiliacorine (58), phaeanthine (59), isotetrandrine (60) and obamegine (61) with potent, selective, anti-plasmodial properties *in vitro* (Marshal et al., 1994). Atalaphilline (62), an acridone alkaloid from *Atalantia monophylla*, Rutaceae, is selectively active against *P. berghei*, in mice and studies of some species of *Strychnos* yielded several (*Strychnos*) alkaloids with potent anti-plasmodial activity including 3',4'dihydrousambaresnine (63; Fig 8; Pages 48 and 49; Wright et al., 1991).

1.2.2 Anti-amoebic plant products

Crude plant extracts and isolated plant compounds have been
evaluated for activity against *Entamoeba histolytica* both in vitro and in vivo and the NAPRALERT data base lists 139 genera which have yielded extracts with antiamoebic effects (Phillipson and O’Neill, 1987). Anti-amoebic plant products have been reviewed by Phillipson and O’Neill (1987).

Since a number of simaroubaceous spp. are used traditionally to treat both amoebic dysentery and malaria, 25 quassinoids derived from these species, including quassin (64), were screened against both *P. falciparum* and *E. histolytica*, in vitro, and, in general, antimalarial activity and amoebicidal activity paralleled one another (Wright, 1989).

Similarly, the series of bisbenzylisoquinoline alkaloids derived from *Triclisia spp.* were also tested against both *P. falciparum* and *E. histolytica* but were less active against *E. histolytica* than against *P. falciparum*. However, isotrilobine (65), had antiamoebic activity similar to emetine and metronidazole (Marshall et al., 1994).

Other examples of antiamoebic compounds include the anti-leishmanial quaternary isoquinoline alkaloid berberine (66), from species of *Berberis* (Berberidaceae) which, interestingly, has low activity against *P. falciparum* but is highly active against *E. histolytica*. 
Fig. 9  Some plant products with activity against *Entamoeba histolytica* and *Giardia lamblia*.

(49) emetine  
(64) quassin

(65) isotrilobine: S,S  \( R_1 = \text{OCH}_3 \)  \( R_2 = \text{CH}_3 \)

(66) berberine

(70) coxsoline: S,S  \( R_1 = \text{OH} \)  \( R_2 = \text{H} \)

(67) borrerine  
(68) alstonine
The indole alkaloid, borrerine (67), from Borreria verticillata and the aromatic alkaloid, alstonine (68) (Fig. 9; Page 52) from Alstonia spp., Apocynaceae, have anti-amoebic activity in vitro (Phillipson and Wright, 1991b).

1.2.3. Plant products with activity against Giardia lamblia and Trichomonas vaginalis.

Plant products with activity against Giardia lamblia, in vitro, include the antimalarial quassinoids bruceantin (50), bruceine A (69); the antiamoebic compound emetine (49) (Wright et al., 1991); berberine (66; Kaneda et al., 1991); and cocsoline (70; Fig. 9; Page 52) derived from Anisocycla cymosa (Menispermaceae; Kanyinda et al., 1992). Gupte (1975) reported results of a clinical trial which showed that berberine from Berberis aristata had therapeutic activity comparable to mepacrine hydrochloride and metronidazole in children suffering from giardiasis. He also reported that berberine inhibited the growth of Trichomonas vaginalis in vitro.

1.2.4. Plant products with activity against trypanosomatids

Plant products with activity against Leishmania spp. have been reviewed by Iwu et al. (1994) who list 18 plant families which contain a wide variety of antileishmanial
compounds, including bisbenzylisoquinoline alkaloids, indole alkaloids, quinones and terpenes.

Possibly the most important antileishmanial plant product is berberine (66) which has been used clinically for the treatment of leishmaniasis (El-On et al., 1988) and is active \textit{in vitro} and \textit{in vivo} against several species of \textit{Leishmania} (Ghosh et al., 1985; Venneström et al., 1990) as well as against \textit{E. histolytica} and \textit{Giardia lamblia}, as previously mentioned.

Ethnopharmacological studies carried out on plants used by native and migrant peoples in Bolivia demonstrated that a significantly higher number of plants used by native people (Chimane Indians) were active \textit{in vitro} against \textit{Leishmania spp.} than those used by migrants (Fournet et al., 1994). Crude extracts of fifty three of these Bolivian medicinal plants were tested against \textit{Leishmania spp.} and \textit{T. cruzi} and active compounds were purified and identified from those extracts which were active \textit{in vitro}. Thirty eight percent of the 26 species of Asteraceae screened were active and studies concentrating on genera from the families \textit{Annonaceae, Berberidaceae, Hernandiaceae} and \textit{Menispermaceae}, which are also frequently used in traditional medicine, resulted in the isolation of several bisbenzylisoquinoline alkaloids (including berbamine (71) krukovine (72) limacine (73) phaeanthine (59) daphnoline (74) gyrocarpine (75; Fig. 10; Page 56) coxoline (70; Fig.
9; Page 52) and antioquine (76; Fig 10; Page 57) which were active in vitro against *L. donovani*, *L. braziliensis*, and *L. amazonensis* promastigote forms (Fournet et al., 1988a).

Three bisbenzylisoquinoline alkaloids, gyrocarpine (75), daphnandrine (77) and obaberine (78) were also active against epimastigote forms of three strains of *T. cruzi* in vitro (Fournet et al., 1988b) and another, isotetrandrine (79; Fig. 10, Pages 56, 57 and 58), isolated from *Limaciopsis loangensis* (Menispermaceae) was also significantly active in vivo against cutaneous leishmaniasis caused by three different strains of parasite (Fournet et al., 1993).

Indole alkaloids with antileishmanial activity in vitro include harmaline (80) (Evans and Croft, 1987), isolated from *Peganum harmala*, which is also active against *T. cruzi* epimastigote forms (Cavin et al., 1987), alstonine (68; Fig. 9; Page 52); akuammine (81), akuammicine (82) from *Alstonia species* and picraline (83) from *Picralima nitida* fruits (Iwu et al., 1992; Fig. 11; Page 58). Indole alkaloid precursors, iridoid glycosides, have also been reported to be active against *Leishmania donovani* amastigote forms in vitro, (Tandon et al., 1991) and in vivo due to immunostimulant activity (Puri et al., 1992). The Bignoniaceae contain several Bolivian medicinal plants from which antileishmanial naphthoquinones have been isolated.
Fig. 10 Bisbenzylisoquinoline alkaloids with activity against *Leishmania* spp. and *Trypanosoma cruzi*.

(71) berbamine $R^1=R^2=\text{Me}, R^3=\text{H}, 1'S$

(72) krukovine $R^1=R^3=\text{H}, R^2=\text{Me}, 1R, 1'R$

(73) limacine $R^1=\text{H}, R^2=R^3=\text{Me}, 1R, 1'R$

(74) daphnoline

(75) gyrocarpine
Fig. 10 cont.

(76) antioquine

(77) daphnandrine

(78) obaberine
Fig. 10 continued

(79) isotetrandrine

Fig. 11  Indole alkaloids with activity against *Leishmania* *spp.* and *Trypanosoma cruzi*.

(80) harmaline

(81) akuammine

(82) akuammicine

(83) picraline
These include plumbagin (84), 3,3′-biplumbagin (85) and 8,8′-biplumbagin (86), from *Pera benensis*, which were active *in vitro* against promastigote forms of three species of *Leishmania* and epimastigote forms of six strains of *T. cruzi* (Fournet et al., 1992a). Plumbagin and 8,8′-biplumbagin were also active *in vivo* against three species of *Leishmania* (Fournet et al., 1992b; Croft et al., 1985). Diospyrin (87), a bis-naphthoquinone derivative isolated from *Diospyros montana*, Bignoniaceae, is active *in vitro* against *L. donovani* promastigote forms (Hazra et al., 1987; Fig. 12; Page 60).

Robert-Géro *et al.* (1985) described the activity of a series of quassinoids such as chaparrinone (88), sergeolide (89) (Fig. 13; Page 61), glaucarubinone (51) and bruceantin (50) against *L. donovani* promastigote forms *in vitro* which, however, were not tested *in vivo* due to their toxicity to macrophages. Other antileishmanial terpenoids include the monoterpene espintanol (90) from *Oxandra espintana* (Annonaceae; Hocquemiller et al., 1991) which was active against several species of *Leishmania* promastigote forms and strains of *T. cruzi* epimastigote forms, *in vitro*, and against *L. amazonensis*, *in vivo*, and a labdane derivative (91) from *Polyalthia macropoda*, also Annonaceae, which was active against *L. donovani* promastigote forms *in vitro* (Richomme *et al.*, 1991; Fig. 14; Page 61).
Fig. 12 Naphthoquinones with activity against Leishmania spp. and Trypanosoma cruzi.

(84) plumbagin

(85) 3,3'-biplumbagin

(86) 8,8'-biplumbagin

(87) diospyrin
Fig. 13. Quassinoids with activity against *Leishmania* *spp.*
and *Trypanosoma cruzi*.

(88) chaparrinone

(89) sergeolide

Fig. 14 Some terpenoids with activity against *Leishmania* *spp.* and *T. cruzi*.

(90) espintanol

(91) *Polyalthia* labdane derivative
Fig. 14 continued

(92) dehydrozaluzanin C

Fig. 15  Plant compounds with activity against Trypanosoma cruzi.

(93) gossypol

(94) Z-ajoene

(95) E-ajoene
Fig. 15 cont.

(96) taxol

(97) tingenone

(98) lapachol

(99) β-lapachone

(100) (3R)-clussequinone
Emetine (49), better known for its antiamoebic activity, is also active in vivo against Leishmania tropica (Neal, 1970).

The sesquiterpene lactone dehydrozaluzanin C (92; Fig. 14; Page 62), isolated from Munnozia maronii, Asteraceae, was active against three species of Leishmania promastigote forms and three strains of T. cruzi epimastigote forms in vitro but not in vivo. It has recently been discovered that artemisinin (55), the Chinese antimalarial compound, and its derivatives, are highly active in vitro and significantly active in vivo against L. major. These preliminary findings, together with low toxicity, are encouraging (Yang and Liew, 1993).

Although many of the plants from which the above mentioned compounds were derived are used traditionally to treat leishmania but not Chagas’ disease, the same types of plant compounds tend to be active, in vitro, against Leishmania spp. promastigote and T. cruzi epimastigote forms. However, not all plant products which have been screened against Leishmania have also been screened against T. cruzi. Some plant products, for example quassinoids, have been tested only against Leishmania spp.: others have been tested only against T. cruzi.

Crude extracts of plant material chosen at random have been screened for anti-T. cruzi epimastigote and trypomastigote
activity (González et al., 1990; Sagua et al., 1987; Cardoso et al., 1987) as well as crude extracts of plants which are traditionally used as antipyretics and antimalarials (Castro et al., 1992).

Natural products with known specific activity are sometimes chosen for testing against T. cruzi. For example, the polyphenolic antifertility compound gossypol (93) inhibits motility of T. cruzi trypomastigote forms (Rovai et al., 1990) and ajoene, (94 and 95) a conversion product of the garlic compound allicin (which inhibits phospholipid biosynthesis) also selectively inhibits T. cruzi trypomastigote forms (Urbina, et al., 1993; Fig. 15; Page 62).

The microtubule-stabilizing agent taxol (96), isolated from Taxus brevifolia (Coniferae), inhibits replication of T. cruzi epimastigote and amastigote forms in vitro (Baum et al., 1981). Tingenone (97), a triterpene quinone-methide antineoplastic agent isolated from the Celastraceae, inhibits growth and macromolecule biosynthesis of T. cruzi epimastigote forms (Goijman et al., 1985).

Since naphthoquinones are potent inhibitors of electron transport (Howland, 1963), Lopez et al. (1978) tested a series of naphthoquinone derivatives of lapachol (98) against T. cruzi. One of these, β-lapachone (99) from Tabebuia spp., was highly active against T. cruzi
epimastigote forms in vitro but stimulated the conversion of oxyhaemoglobin to methaemoglobin and was inactivated in the presence of blood (Lopez et al., 1978).

Chiari et al. (1991) screened a random selection of compounds for activity against T. cruzi trypomastigote forms and found six flavonoids with low activity in vitro, including (3R)-claussequinone (100) which was chosen as a template for the synthesis of derivatives with increased activity and solubility (Fig. 15; Pages 62 and 63).

1.2.4.1 Plant products with activity against Trypanosoma b. brucei.

Studies have been carried out in Africa on plants used traditionally to treat a number of different conditions, screening crude extracts for activity against T. b. brucei in vivo. Extracts of Acalypha hispida (Euphorbiaceae; Okanla et al., 1990), Alchornea cordifolia (Euphorbiaceae; Agbe and Oguntimein, 1987), Morinda lucida (Rubiceae; Asuzu and Chineme, 1990), and Picralima nitida (Apocynaceae; Wosu and Ibe, 1989), showed little selectivity, suppressing parasitaemia temporarily but being relatively toxic to their rodent hosts.

However, crude extracts of Annona senegalensis (Annonaceae) used traditionally to treat sleeping sickness in Northern Nigeria, were active against T. b. brucei in vivo (Igweh
Khaya grandifolia crude extract inhibited the growth of T. b. brucei bloodstream forms grown over a Microtus montana embryonic fibroblast feeder cell layer (Owolabi et al., 1990). As yet, there have been no reports of compounds with selective activity against T. b. brucei isolated from these active African plant crude extracts.

A few plant compounds have been screened for activity against T. b. brucei in vitro and in vivo; Williamson and Scott-Finnigan (1978) reported activity for the flavonoid quercetin (101; Fig. 16; Page 68) in vitro but not in vivo, and for emetine both in vivo and in vitro.

Studies by Aboagye-Nyame (1993) on the bark of Kigelia pinnata, a West African tree used extensively in traditional medicine, demonstrated that several fractions of the crude extract were active in vitro against T. b. brucei. Kigelia pinnata bark contains naphthoquinones, including lapachol (98; Govindachari et al., 1971) and isopinnatal (102; Fig. 16; Page 68). Isopinnatal, isolated from one of the fractions, was significantly active against both bloodstream and procyclic forms of T. b. brucei in vitro (Aboagye-Nyame, 1993). Gossypol, which is active against T. cruzi, as already mentioned, lyses T. b. brucei by interfering with glycolysis in the parasite (Eid et al., 1988).
1.3 Aims of the present research

This research aims to evaluate the efficacy of a selection of plant-based traditional remedies, from Africa, South America and Iraq, for the treatment of diseases caused by the protozoan parasites, T. b. brucei, T. cruzi, L. donovani and P. falciparum.

Both vector and mammalian forms of the parasites will be cultured in order to test crude extracts of plant material and pure compounds derived from plant material against the different stages of the parasites.

The vector stages will be used for a rapid screen of crude
plant material in order to select those plants with *in vitro* activity for bioassay-guided fractionation, leading to the isolation of active compounds which will be tested against the mammalian stages of the parasites.

This work aims to elucidate the structure of active compounds by spectroscopy, to test eventual promising leads *in vivo* and to carry out mode of action studies on promising active compounds.

It aims, where possible, to test compounds with the same type of structures as those found to be active, whose mode of action is already known, in the same *in vitro* systems, for comparative purposes.

It also aims to test semisynthetic analogues of some active compounds, in the same *in vitro* systems, in order to assess structure-activity relationships.
2.1.1. The epidemiology of African sleeping sickness.

Human African trypanosomiasis occurs in 36 African countries between latitudes 14° North and 29° South (Kuzoe, 1993; Fig. 17; Page 71) and is caused by two species of African trypanosomes, Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense. T. brucei spp. are mainly transmitted from one mammalian host to another by haematophagous tsetse flies of the genus Glossina which infest 10.4 million square kilometres (WHO/VBC, 1989; Fig. 18; Page 71), the vectors of gambiense sleeping sickness tending to occur in West and Central Africa in areas of rivers and lakes and the vectors of rhodesiense sleeping sickness in East and Southern Africa in areas of savanna and woodlands (Connor et al, 1976).

Fifty million people are still exposed to the risk of contracting sleeping sickness (Kuzoe, 1993) despite the fact that this disease was almost brought under control in the early 1950s.
Fig. 17  Distribution of sleeping sickness foci in man in Africa (adapted from WHO/VBC/89)

T. b. gambiense areas to the left of dotted line, T. b. rhodesiense areas to the right of line.

Fig. 18  Distribution of Glossina spp. in Africa (adapted from WHO/VBC, 1989)

Key

tsetse-infested areas
Political and civil unrest and worsening economic conditions after the end of colonial rule led to a decline in trypanosomiasis control resulting in recrudescence of old foci in Cameroon, Chad, Congo and the Republic of Central Africa, where the disease had previously been brought under control (WHO, 1990a). Recent political unrest in Zaire and in the Sudan caused a complete abandonment of trypanosomiasis control and there is now a serious risk of epidemics occurring in both countries, especially in Zaire where in some villages 70% of the people are infected (TDR Progress, 1995). A number of sleeping sickness foci extend across borders between one country and another and difficult relations between countries often hinders control of the disease. The major epidemic in south-eastern Uganda (Mbulamberi, 1990) which began in 1975 has only recently been brought under control (Kuzoe, 1993) but the situation in northern Uganda, where infected refugees are arriving from the Sudan, is now critical (Mbulamberi, 1992).

Officially only twenty five thousand new cases are reported annually (Kuzoe, 1993) though this figure is an underestimate due to the inaccessibility of the affected areas, difficulty of diagnosis, and poor reporting. The true infection rate may be many times the official figure (TDR Progress, 1995). As long as economic conditions continue to worsen in Africa sleeping sickness epidemics remain a continuous threat (Kuzoe, 1993).
2.1.2 The life cycle of *Trypanosoma brucei* spp.

The life cycle of the *T. brucei* spp. is complex (Fig 19; Page 74). When the tsetse fly feeds on a host infected with trypanosomes of the *T. brucei* group, it ingests short stumpy trypomastigote forms which transform to procyclic trypomastigote forms in the midgut. After four days the procyclic trypomastigote forms invade the ectoperitrophic space and transform to rapidly dividing mesocyclic forms which move forward to the proventriculus, elongating and ceasing to divide. They then migrate via the oesophagus, mouthparts and salivary ducts to the salivary glands where they become dividing epimastigote forms which transform to infective non-dividing metacyclic trypomastigote forms (Vickerman, 1985). When the infected fly next feeds, metacyclic trypomastigote forms are injected into the skin of the host where a local inflammatory reaction (a chancre) sometimes develops (Barry and Emery, 1984). The trypomastigote forms enter the host's blood and lymphatic circulation, multiplying by binary fission as long, slender trypomastigote forms and eventually crossing the choroid plexus into the central nervous system.

Trypanosomes are covered by a replaceable surface coat of variant surface glycoprotein (VSG) which specifies the variable antigenic type (VAT) of the trypanosome. They switch from one VSG to another in order to evade the host's immune system.
Fig. 19 The developmental cycle of *Trypanosoma brucei* spp.
adapted from Vickerman, 1985.

**IN TSETSE SALIVARY GLANDS**

epimastigote

migration to tsetse salivary glands

mesocyclic

procyclic

metacyclic

injection into mammal

slender form

stumpy form

ingestion by tsetse

**IN TSETSE MIDGUT**

**IN MAMMAL**
A variable number of trypanosomes switch VSGs, thus eluding the host's immune system (Barry and Turner, 1991). Variant individuals give rise to a succession of new populations expressing new sets of VSGs to which the host continually produces VAT-specific IgM antibodies (Mansfield, 1994). This results in a continually relapsing growth pattern in the blood (Barry and Turner, 1991). As the parasitaemia displaying one VSG declines, non-dividing stumpy trypomastigote forms replace the slender dividing forms with that particular VSG. These stumpy forms are infective to feeding tsetse flies.

2.1.3 The course of the disease

*T. b. gambiense*, which infects humans and livestock, causes a chronic infection in humans with elusive and mild symptoms which can last for more than ten years (van Nieuwenhove, 1992) whereas sleeping sickness caused by *T. b. rhodesiense* is usually an acute disease, often fatal within weeks if not treated (Jernigan and Pearson, 1993).

Waves of parasitaemia are reflected by intermittent fever in the early stage of the disease. Other highly variable and often nonspecific clinical symptoms include headaches, giddiness, myalgia, pruritis, peripheral oedema, cardiovascular disorders, weight loss, and possible confusion with intercurrent infections (WHO, 1986). At this stage the lymph glands and spleen may also become swollen.
The frequency and severity of the early symptoms varies between *rhodesiense* and *gambiense* disease, symptoms of the former tending to be more severe and acute. The chancre, which appears a few days after the bite of the infected tsetse fly, is very rare in *gambiense* sleeping sickness but frequently seen in *rhodesiense* disease.

The early stage of the disease may last for several years in *gambiense* disease or as little as eight days in that caused by *T. b. rhodesiense* (Braendli *et al*, 1990). The parasites then invade the central nervous system (CNS), either via the choroid plexus, via regions where the blood brain barrier (BBB) is incomplete, or through transient leaks in the BBB (Pentreath, 1989), leading to the final stage of the disease.

Inflammation and oedema of the meninges and the brain give rise to characteristic symptoms such as abnormal movements, disturbed coordination, abnormalities of muscle tone, mood changes, nocturnal insomnia and severe daytime somnolence, followed by coma. Irreversible neurological lesions occur at this late stage of the disease which is fatal, unless treated (WHO, 1986).

2.2 Current clinical chemotherapy

A conventional vaccine has so far eluded researchers due to the antigenic diversity of bloodstream form trypanosomes
Few drugs are available for the treatment of sleeping sickness which can only be prevented by vector control and the treatment of established cases. Early stage *gambiense* sleeping sickness is treated with the naphthylamine suramin (103) or the diamidine pentamidine (104), both of which have been in use since the first half of this century. Late stage *gambiense* sleeping sickness is treated with the highly toxic organic arsenical melarsoprol (105) which crosses the blood brain barrier (Fig. 20; Page 79; Jernigan and Pearson, 1993). Late stage melarsoprol-resistant *gambiense* trypanosomiasis can be treated with eflornithine (DL-α-difluoromethylornithine - DFMO) (106), an irreversible inhibitor of ornithine decarboxylase, which was registered by the US Food and Drug Administration in 1990 under the trade name Ornidyl (Kuzoe, 1993), or with the nitrofuran nifurtimox (107) (van Nieuwenhove, 1992). Nitrofurazone (108) has also occasionally been used (Fig. 20; Pages 79 and 80).

Only two drugs are available for the treatment of *rhodesiense* sleeping sickness: suramin (103) in the early stages and melarsoprol (105) in the late stages (Wéry, 1991). There is no cure for melarsoprol-resistant *rhodesiense* late stage disease.

*Rhodesiense* trypanosomiasis is extremely virulent, trypanosomes sometimes being found in the brain as little as eight days after the infecting bite of the tsetse fly,
so that diagnosis and treatment in the early stages is difficult.

Side effects of suramin include vomiting, pruritis, urticaria, peripheral neuropathy, photophobia and renal impairment (Gutteridge, 1985). Side effects of melarsoprol are common and can include fever, chest pain, gripping abdominal pains and subjective sensations of heat and disturbances of smell (Gutteridge, 1985). More than 10% of patients treated with arsenicals develop reactive arsenical encephalopathy and 1-5% die as a result. Side effects of eflornithine include diarrhoea, dysphagia (difficulty with swallowing), debility, pruritus, palpitations, hair loss, abdominal pain, vomiting, seizures, anorexia and anaemia (Wéry, 1991).

In the Sudan and Zaire melarsoprol-refractory T. b. gambiense disease has been treated with nifurtimox (107) but results were not encouraging (Pépin and Milord, 1994). Side effects of nifurtimox are nausea, weight loss, memory loss, sleep disorders and depression (Gutteridge, 1985).

An increasing number of people are infected with drug-resistant disease (de Groof et al., 1992) and the use of pentamidine, suramin, melarsoprol and eflornithine is restricted to specialised treatment centres where parenteral administration can be carried out and the severe side effects monitored.
Fig 20. Structures of compounds currently in use for the chemotherapy of African Trypanosomiasis.

(103) suramin

(104) pentamidine

(105) melarsoprol

(106) DL-α-difluoromethylornithine (DFMO)
This renders these drugs useless in remote areas, especially in cases of fulminating *T. rhodesiense* disease.

New drugs which can be administered orally are thus urgently needed for the treatment of African trypanosomiasis.
2.3 Phytochemical studies

2.3.1 Materials

2.3.1.1 African plants chosen for screening against *Trypanosoma b. brucei*

The following sun-dried plant material, reputed to be efficacious in the treatment of Human African Trypanosomiasis, was obtained from Ghana and authenticated by Laura Hastings at the Royal Botanic Gardens, Kew.

- *Alstonia boonei* (Apocynaceae) bark
- *Alchornea cordifolia* (Euphorbiaceae) leaves, stem, bark
- *Annona senegalensis* (Annonaceae) leaves, stem, bark
- *Lonchocarpus cyanensis* (Papilionaceae) roots
- *Tamarindus indica* (Caesalpinaceae) roots
- *Ximenia americana* (Oleaceae) stem, bark

2.3.1.2 Solvents used for the crude extraction and chromatographic separation of plant material

<table>
<thead>
<tr>
<th>solvent</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.880 ammonia sol.</td>
<td>BDH</td>
</tr>
<tr>
<td>chloroform</td>
<td>BDH</td>
</tr>
<tr>
<td>hydrochloric acid</td>
<td>M and B</td>
</tr>
<tr>
<td>methanol</td>
<td>BDH</td>
</tr>
<tr>
<td>petroleum ether</td>
<td>BDH</td>
</tr>
</tbody>
</table>
propan-2-ol  BDH
ethyl acetate  BDH

All solvents used for crude extraction and chromatographic separation of plant material were general purpose grade.

2.3.1.3 Chromatographic materials for the separation of *Alstonia boonei* and *Alchornea cordifolia*

**For analytical chromatography**
Pre-coated aluminium-backed silica gel (60) plates F$_{254}$ (0.25mm thick)  source Merck Ltd.

**For column chromatography**
Silica gel 60, 70-230 mesh ASTM  Merck Ltd.

**For preparatory thin layer chromatography**
Glass plates (20cm x 20cm)
Silica gel GF$_{254}$  Merck Ltd.

**Spray reagents**

**Dragendorff's reagent (Munier's modification)**
Preparation: Solution A: 0.8 g bismuth subnitrate + 10 ml glacial acetic acid, made to 50 ml volume with distilled water.
Solution B: 20 g potassium iodide, made to 50 ml volume with distilled water.
Immediately before use 5 ml each of solutions A and B were added to 20 ml of glacial acetic acid and made up to 100 ml volume with distilled water.

Procedure: the chromatogram was observed immediately after spraying.

**Ferric chloride in perchloric acid**

Preparation: 1 ml of 0.5 M iron (III) chloride solution was mixed with 50 ml of 35% perchloric acid.

Procedure: the chromatogram was observed immediately after spraying, after heating with hot air and after heating at 110°C for 30 minutes.

**1% Cerium (IV) sulphate in 10% sulphuric acid solution.**

Preparation: a saturated solution of cerium (IV) sulphate was prepared, using 10% sulphuric acid.

Procedure: the chromatogram was observed immediately after spraying, after heating with hot air and after heating at 120°C for 15 minutes.

**Nitric acid (conc.)**

Procedure: the chromatogram was observed immediately after spraying, after five minutes and after half an hour.

2.4 Methods
2.4.1 Extraction procedures
2.4.1.1 Aqueous extraction procedures

Since all of the above plant material is traditionally
administered orally in the form of aqueous teas in Ghana, (Abbiw, D., personal communication) 10 g each of the above plants was ground, macerated at 40-60°C in distilled water for one hour, then allowed to cool, filtered and freeze dried. Aqueous extracts were tested for biological activity against Trypanosoma b. brucei procyclic forms in vitro.

Since crude extracts of A. boonei and A. cordifolia were the most active against T. b. brucei, they were fractionated further.

2.4.1.2 Sequential extraction procedures
2.4.1.2.1 Small scale sequential extraction of A. cordifolia

15 g each of stem, stem bark, root and leaf were ground to powder. These were then sequentially extracted with petroleum ether, methanol and water (Fig 21; page 87). Petroleum ether and methanol extracts were filtered and evaporated to dryness using a rotary evaporator. Aqueous extracts were filtered and freeze dried. The extracts were tested against T. b. brucei procyclic forms in vitro.

2.4.1.2.2 Small scale sequential extraction of A. boonei

15 g of A. boonei root was ground to a powder and sequentially extracted with petroleum ether, methanol and water (Fig. 22; Page 88). Petroleum ether and methanol
extracts were filtered and evaporated to dryness. Aqueous extracts were filtered and freeze dried. These crude extracts were then tested against \textit{T. b. brucei} procyclic forms \textit{in vitro}.

2.4.1.2.3 Large scale alkaloidal extraction of \textit{A. boonei}

A crude alkaloidal extract was prepared from 100g of \textit{A. boonei} ground bark (Fig. 23; Page 89). Both the crude alkaloidal extract and the non-alkaloidal extract were tested against \textit{T. b. brucei} procyclic forms \textit{in vitro}.

2.4.1.3 Large scale petroleum ether extraction of \textit{A. boonei}

Since the petroleum ether fraction derived from the sequential extraction of \textit{A. boonei} was active against \textit{T. b. brucei}, 100g of ground \textit{A. boonei} bark was extracted with petroleum ether (Fig. 24; Page 90) with a view to isolating the active constituents from this fraction.

2.5 Isolation of compounds from \textit{A. boonei}

Analytical TLC with a solvent system of chloroform : methanol 85:15, revealed seven Dragendorff-positive spots in the crude alkaloidal fraction with \textit{R}_f values of 0.04, 0.13, 0.17, 0.25, 0.3, 0.38 and 0.45. The alkaloidal fraction was therefore separated on a silica column.
A rapid preliminary fractionation of the crude alkaloid extract of *A. boonei* was carried out using positive pressure chromatography (Wright et al., 1992) in a glass column, 40cm high and 2cm diameter. A slurry of silica gel and chloroform was allowed to stand for an hour and then packed into the glass column to a height of 25 cm and covered with 2 cm of fine sand (BDH 40-100 mesh). Chloroform was allowed to flow through the column for two hours before adding crude alkaloidal extract to the top of the column. The column was eluted with chloroform followed by a chloroform-methanol gradient, finishing with 50% chloroform and 50% methanol. Aluminium-backed silica gel plates were used routinely to monitor each fraction obtained from column chromatography; separation of alkaloids was checked by spraying with Dragendorff's reagent and appropriate fractions were pooled for further separation on TLC and tested against *T. b. brucei* procyclic forms *in vitro*. Inactive fractions were discarded.

50ml fractions were collected, the following active fractions eluting with the following solvent systems:

(i) 60-67 with CHCl₃:MeOH 95:5
(ii) 70-78 with CHCl₃:MeOH 94:6
(iii) 83-95 with CHCl₃:MeOH 90:10
(iv) 96-114 with CHCl₃:MeOH 50:50.
Fig. 22 Small scale sequential extraction of *Alstonia boonei* bark

15 g *A. boonei* bark, ground

petroleum ether

petroleum ether

residue

petroleum ether

residue

methanol

methanolic crude extract

residue

52 mg (0.35%)

water

aqueous extract

residue

847 mg (5.56%)
Fig. 23  Large scale alkaloidal extraction of *Alstonia boonei* bark.

100 g ground *A. boonei* bark

methanol

<table>
<thead>
<tr>
<th>crude extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% hydrochloric acid</td>
</tr>
<tr>
<td>adjust to pH 8.0 with ammonia</td>
</tr>
<tr>
<td>chloroform</td>
</tr>
</tbody>
</table>

non-alkaloidal extract  
2.7g (2.7%)

<table>
<thead>
<tr>
<th>crude alkaloidal extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>205mg (0.2%)</td>
</tr>
</tbody>
</table>
Fig. 24 Petroleum ether extraction of *Alstonia boonei* bark

100 g *A. boonei* bark, ground

petroleum ether

residue 547 mg extract (0.55%)
2.5.1.2 Preparative thin layer chromatography

45 g silica gel was shaken vigorously with 90 ml distilled water and spread to a thickness of 0.5 mm on glass plates (20 cm x 20 cm) using a Jobling Laboratory Division Moving Spreader. Plates were heated to 110°C for one hour, allowed to cool, then washed with methanol and allowed to dry before use. Samples were applied to plates using capillary tubes and allowed to air dry before development in chromatography tanks lined with chromatography paper saturated in the relevant solvent. In the case of \textit{A. boonei} alkaloidal separation, multiple development was used in order to augment separation efficiency.

The following pooled fractions from the column were separated by preparative thin layer chromatography, using the following solvent systems:

<table>
<thead>
<tr>
<th>fraction</th>
<th>solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>CHCl$_3$:MeOH 85:15</td>
</tr>
<tr>
<td>(ii)</td>
<td>ethyl acetate:isopropanol:ammonia 85:10:5</td>
</tr>
<tr>
<td>(iii)</td>
<td>ethyl acetate:isopropanol:ammonia 85:10:5</td>
</tr>
<tr>
<td>(iv)</td>
<td>ethyl acetate:isopropanol:ammonia 45:35:20</td>
</tr>
</tbody>
</table>

The petroleum ether fraction was separated by preparative thin layer chromatography using chloroform: hexane 95:5.

Developed plates were viewed under ultraviolet light at 254
and 366nm then their edges were sprayed with Dragendorff's reagent to detect the presence of alkaloids. Separated bands were scraped from the plates and compounds eluted from them through chloroform-washed cotton wool. Eluates were then evaporated to dryness under reduced pressure, redissolved in minimal quantities of solvent, then dried under nitrogen and stored in glass vials at 4°C.

Four alkaloidal fractions (a, b, c and d) were isolated by preparative TLC from the column fractions as follows:

correction

fraction d was isolated from (i)
fraction c was isolated from (ii)
fraction b was isolated from (iii)
fraction a was isolated from (iv)

Four petroleum ether fractions, 1, 2, 3 and 4 were isolated by preparative TLC. All the alkaloidal and petroleum ether fractions were tested against T. b. brucei in vitro.

2.5.2 Spectroscopic methods

2.5.2.1 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy was carried out at King's College, London, on a Bruker WM-250 MHz spectrophotometer with TMS as internal standard. CDCl₃ was used for alkaloidal fractions b, c and d and petroleum ether fractions. DMSO_d₆ was used for alkaloidal fraction a. The chemical shift values (δ) were recorded in ppm, and the coupling constant (J) as Hz.
2.5.2.2 Mass spectroscopy (MS)

MS was carried out at the School of Pharmacy on a VG MassLab 12/250 quadrupole mass spectrometer with a VG 11-73 data system. The approximate TSP ion source condition temperatures were: source- 200°C; nozzle- 190°C, and chamber- 230°C (though other temperatures were used in some cases).

2.5.2.3 Ultraviolet spectroscopy (UV)

UV spectroscopy was carried out using a Perkin-Elmer model 402 double beam spectrophotometer. All samples were dissolved in methanol except fraction d which was dissolved in ethanol.

Alkaloidal fractions a, b, and c were submitted to UV, EIMS, $^1$H NMR, and $^{13}$C NMR spectroscopy. $^1$H NMR, EIMS and UV spectroscopy were performed on fraction d and the petroleum ether fractions of Alstonia boonei were submitted to $^1$H NMR spectroscopy.

2.6 Spectral data for alkaloidal fractions from A. boonei

2.6.1 Echitamine (fraction a)

Crystals from MeOH, mp 283°C

UV $\lambda_{\text{max}}$ nm: 295, 236, 207 (Fig. 25; Page 100).

EIMS m/z (Table 3; Page 96; Fig. 26; Page 101).
2.6.2 Undulifoline (fraction b)

yellow/orange resin

UV $\lambda_{\text{max}}$ nm: 291, 281, 222, 206 (Fig. 30; Page 105).

EIMS m/z (rel. int.%) (Table 3; Page 96; Fig. 31; Page 106).

$^1$H NMR (CDCl$_3$) (Table 4; Page 97; Fig. 32; Page 107).

$^{13}$C NMR (Table 6; Page 99; Fig. 33; Page 108).

COSY spectrum (Fig. 34; Page 109).

2.6.3 Echitamidine (fraction c - major compound)

orange resin

UV $\lambda_{\text{max}}$ nm: 332, 294, 236, 204 (Fig. 35; Page 110).

EIMS m/z (Table 3; Page 96; Fig. 36; Page 111).

$^1$H NMR (CDCl$_3$) (Table 5; Page 98; Fig. 37; Page 112).

$^{13}$C NMR (Table 6; Page 99; Fig. 38; Page 113).

COSY spectrum (Fig. 39; Page 114).

12-Methoxyechitamidine (fraction c - minor compound)

EIMS m/z (rel. int. %): 370 (12), 271 (38) (Fig. 36; Page 111).

$^1$H NMR: 8.84 (1H, br s, NH) 6.75-7.2 (3H, m, H-9, H-11, H-10,), 3.45 (s, OMe) (Fig. 37; Page 112).

$^{13}$C NMR (Table 6; Page 99; Fig. 38; Page 113).
2.6.4 12-methoxyechitamidine (fraction d)

orange resin

UV $\lambda_{\text{max}}$ nm: 333, 284, 240, 210 nm (Fig. 40; Page 115).

EIMS: m/z (Table 3; Page 96; Fig. 41; Page 116).

$^1$H NMR (Table 5; Page 98; Fig. 42; Page 117).

TLC and chromogenic reactions

$R_f = 0.18$ in a chloroform:methanol 93:7 system.

Concentrated nitric acid produced a bright green colour which turned yellow after five minutes, then disappeared on standing.

Cerium sulphate in sulphuric acid produced a purple reaction immediately after spraying, which became dull yellow after heating.

Iron chloride in perchloric acid produced a yellow reaction immediately after spraying, which changed to greenish brown with a blue edge on heating.

2.6.5 Petroleum ether fractions from A. boonei

$^1$H NMR spectroscopy on the petroleum ether fractions 1, 2, 3, and 4 showed the presence of numerous methyl signals in the δ0.7-1.2 range of the spectrum, indicating that they consisted of mixtures of compounds, including triterpenes.
Table 3  EIMS assignments (rel. int. %) of echitamine, undulifoline, echitamidine (fraction c major peaks) and 12-methoxyechitamidine

<table>
<thead>
<tr>
<th></th>
<th>echitamine</th>
<th>undulifoline</th>
<th>echitamidine</th>
<th>12-methoxy-echitamidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>384 [M-H]^+ (96)</td>
<td>341 [M+H]^+ (100)</td>
<td>340 [M]^+ (33)</td>
<td>370 [M]^+ (30)</td>
</tr>
<tr>
<td></td>
<td>370 (100)</td>
<td>284 (34)</td>
<td>271 (38)</td>
<td>354 (3)</td>
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<td></td>
<td>353 (87)</td>
<td>281 (5)</td>
<td>241 (100)</td>
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<td>252 (22)</td>
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<td>232 (26)</td>
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<td></td>
<td>194 (37)</td>
<td>167 (32)</td>
<td>105 (2)</td>
<td>271 (100)</td>
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Table 5 \(^1\)H NMR assignments of echitamidine (major peaks of fraction c) and 12-methoxyechitamidine (fraction d)

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### Table 6  $^{13}$C NMR assignments [δ 125 MHz] of echitamine, echitamidine (fraction c major compound) 12-methoxyechitamidine (fraction c minor compound) and undulifoline.

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n.o. = not observed
Fig. 26 El Mass spectrum of echitamine
Current Data Parameters
NAME: marZ295205
EXPNO: 1
PAGENO: 1

F1 - Acquisition Parameters
Date: 950322
Time: 12.23
PULPROG: 123
SOLVENT: DMSO
AA: 1.958005 sec
F1RES: 0.254314 Hz
Dw: 50.0 usec
RE: 1024
NUCLEUS: 1H
ML1: 1 dB
D1: 1000000 usec
F1: 9.5 usec
DE: 75.0 usec
SF1D: 400.136796 MHz
SAH: 8333.37 Hz
T1: 20766
M: 16
D: 2

F2 - Processing parameters
SI: 262384
SF: 400.136795 MHz
MWM: 16
SB: 0
LB: 0.30 Hz
GB: 0
FC: 1.60

1D NMR plot parameters
CP: 40.00 cm
DF: 22.500 ppm
F1: 5141.72 Hz
F2P: 0.400 ppm
PM: 0.32500 ppm/cm
HCM: 130.04430 Hz/cm
Fig. 28 $^{13}$C NMR spectrum of echitamine
Fig. 29
COSY spectrum of choline
Fig. 30  UV spectrum of undulifoline
Fig. 31  EI Mass spectrum of undulifoline
Fig. 3.2 1H NMR spectrum of undulifoline
Fig. 33  $^{13}$C NMR spectrum of undulifoline
Fig. 34  COSY spectrum of undulifoline
Fig. 35  UV spectrum of alkaloidal fraction c of *Alstonia boonei* (mixture of echitamidine and 12-methoxyechitamidine)
Fig. 36  EI Mass spectrum of alkloidal fraction c of Alstonia boonei (mixture of echitamidine and 12-methoxyechitamidine)
Fig. 37 $^1$H NMR spectrum of alkaloidal fraction c of *Alstonia boonei* (mixture of echitamidine and 12-methoxyechitamidine)
Fig. 38  $^{13}$C NMR spectrum of alkaloidal fraction c of Alstonia boonei (mixture of echitamidine and 12-methoxyechitamidine)
Fig. 39 COSY spectrum of alkaloidal fraction c of Alstonia boonei (mixture of echitamidine and 12-methoxyechitamidine)
optical density

wavelength (nm)
Fig. 41 Mass spectrum of 12-methoxyechitamidine
Fig. 42 $^1$H NMR spectrum of 12-methoxyechitamidine
2.7 *T. b. brucei* culturing techniques

Both procyclic and bloodstream form trypomastigote cultures were initiated using *T. b. brucei* (strain S427)-infected blood, harvested aseptically from an infected Sprague Dawley rat by cardiac puncture.

2.7.1 *T. b. brucei* procyclic form culturing technique.

*T. b. brucei* procyclic forms were cultured according to the technique of Brun and Schonenberger (1979), with the following modifications: parasitaemia in the rat was monitored daily by examination of a sample of tail blood and counting the number of trypanosomes per microscope field of wet blood film at 40 X magnification. When the parasitaemia reached one trypanosome per field (approximately day 3) 1% eflornithine was added to the rat's drinking water for 24 hours, causing the trypanosomes to transform to short stumpy forms which are ideal for the initiation of procyclic cultures. A drop of infected tail blood was diluted in SDM-79 medium (Brun and Schonenberger, 1979) with 10% heat-inactivated foetal calf serum (Gibco Ltd.), haemin (10mg/l), and 5 mM sodium citrate (Brun, 1981) in 25 cm² culture flasks and incubated at 28°C. Antibiotics were not included in the culture medium.
2.7.2 *T. b. brucei* bloodstream form culturing technique.

*T. b. brucei*-infected blood was harvested aseptically from anaesthetised Sprague Dawley rats during the log phase of the parasitaemia (parasitaemia was monitored daily, as above). At this stage the majority of the trypanosomes are long and slender and ideal for the initiation of a bloodstream form culture (Hirumi *et al.*, 1977). Trypanosomes were separated from the infected blood by anion exchange DEAE cellulose chromatography (Lanham and Godfrey, 1970), after which the trypanosomes were cultured in HMI-9 medium (Gibco), a modification of Iscove’s medium with 20% heat-inactivated foetal calf serum (Gibco; Hirumi and Hirumi, 1989).

2.8 Tests against *T. b. brucei*.

2.8.1 Tests against *T. b. brucei* procyclic forms.

Crude plant extracts were screened against fast-growing *T. b. brucei* procyclic forms in SDM 79 medium in 96-well microtitre plates using a simple microdilution technique. Each extract was tested in triplicate in limiting dilutions from 500-15 μg/ml against trypanosomes at a starting concentration of $2 \times 10^5$/ml and incubated at 28°C for 48 hours. Drug-free controls were included in each test. Trypanosome motility was assessed through an inverted phase microscope at 40 X magnification after 24 hours and 48 hours and compared with controls. Motility of cultures
provided an indication of activity on a scale of +++ (zero motility) to 0 (full motility).

2.8.2 Tests against T. b. brucei bloodstream forms.

All samples were initially tested in triplicate in limiting dilutions from 500-15 μg/ml. Active compounds were further tested at lower concentrations. The starting concentration of T. b. brucei bloodstream forms was always 2 X 10^5/ml. Samples were tested in 96 well microtitre plates in HMI-9 medium against fast-growing bloodstream form trypanosomes (2 X 10^5/ml) and incubated at 37° in 5% CO₂ for 48 hours. Drug-free controls and the standard drug, pentamidine, were included in each test at 1, 0.3, 0.1 and 0.03μM. Trypanosome numbers in each well of the 96 well plate were counted by haemocytometer and inhibition was assessed by calculating survival as a percentage of the non-treated control.

In most cases, vector forms were used to screen crude plant extracts and the mammalian forms were used to test pure compounds.

The following crude extracts of African plant material were tested for activity against T. b. brucei procyclic forms in vitro:

aqueous extracts of:

Alstonia boonei bark
Alchonea cordifolia leaves, stem, bark
Annona senegalensis leaves, stem, bark
Lonchocarpus cyanensis root
Tamarindus indica root
Ximenia americana stem, bark
methanolic and petroleum ether extracts of:
Alstonia boonei bark
Alchornea cordifolia leaves, stem, bark;

The following semipurified fractions of Alstonia boonei bark were tested for activity against T. b. brucei procyclic forms:
petroleum ether fractions 1, 2, 3 and 4

The following pure compounds were tested for activity against T. b. brucei bloodstream forms:
echitamine, undulifoline and 12-methoxyechitamidine. The echitamidine/12-methoxyechitamidine mixture was also tested for activity against T. b. brucei bloodstream forms.
2.9 Results

This study has resulted in the isolation of four alkaloidal fractions (a, b, c and d) and three petroleum ether fractions (1, 2 and 4) from *Alstonia boonei* bark with activity against *T. b. brucei in vitro* (Tables 10 and 11, Pages 134 and 135).

Fraction a, the major alkaloid, was identified as echitamine (113, Page 127). This quaternary alkaloid was isolated as needle-like white crystals from the polar fractions obtained after silica gel chromatography. The UV spectrum (Fig. 25, Page 100) indicated the presence of an indoline chromophore. The EI mass spectrum (Table 3, Page 96; Fig. 26, Page 101) showed a prominent peak at m/z 384 which was assigned to [M-H]+. Intense ion peaks were observed at m/z 370 (loss of a methylene) and at m/z 353 (further loss of a hydroxyl). The 1H NMR spectrum (Fig. 27, Page 102, Table 4, Page 97) displayed three methyl singlets at δ3.75 (a methyl ester), at δ3.36 (an N-methyl) and at δ1.81 (an 18-methyl). The downfield part of the 1H NMR spectrum showed only the four unsubstituted aromatic protons and NH of the indole nucleus. The 13C NMR spectrum (Fig. 28, Page 103, Table 6, Page 99) displayed signals for 6 methines, three methyls, five methylenes, a COO (C-22) at δ173.57, a carboxymethyl (C-22) at δ52.32 an N-methyl signal at δ49.96 and four quaternary carbons. The COSY spectrum (Fig. 29, Page 104) showed coupling between proton
signals from adjacent carbons, for example H-14 and H-15 and H-5 and H-6. The melting point, UV, EIMS, $^1$H NMR and $^{13}$C NMR spectral data of this compound agree with those obtained by Keawpradub et al., (1994) for echitamine (113; Fig. 43, Page 127; see appendix Page 219). It is therefore concluded that fraction a is echitamine.

Fraction b was identified as undulifoline (123, Page 129). The UV spectrum (Fig. 30, Page 105) indicated the presence of an indole chromophore. The EI mass spectrum of undulifoline (Fig. 31; Page 106; Table 3, Page 96) showed an intense ion at m/z 341 [M+H]$^+$, and a small ion at m/z 281 (loss of a carboxymethyl). The FAB mass spectrum was dominated by an intense ion at m/z 341 (M+H)$^+$. The $^1$H NMR spectrum (Fig. 32, Page 107, Table 4, Page 97) showed the presence of an intact indole nucleus (four unsubstituted aromatic protons at $\delta$7.55, 7.36, 7.18, 7.10 and an NH at $\delta$8.52), a carboxymethyl at $\delta$3.78 and an N(4) methyl at $\delta$2.31. The $^{13}$C NMR spectrum (Fig. 33, Page 108, Table 6, Page 99) showed a signal for a carboxymethyl at $\delta$173.09 and an N-methyl at 44.35 and signals for seven methines, five methylenes, two methyls and five quaternary carbons. In the COSY spectrum the following coupling pattern was observed: H-21 ($\delta$3.97) with H-20 ($\delta$2.73), H-20 ($\delta$2.70) with H-19 ($\delta$1.25) and H-15 ($\delta$2.78), H-19 ($\delta$1.25) with H-18 ($\delta$3.50). The UV, EIMS, $^1$H NMR and $^{13}$C NMR spectral data agree with those obtained by Massiot et al., (1992) for undulifoline (123; Fig. 45, Page 129; see appendix, Page 219). Therefore
fraction b is identified as undulifoline. The absolute configuration of undulifoline was tentatively assigned by Massiot et al., (1992).

Fraction d and the minor component of fraction c were identified as 12-methoxyechitamidine (122, Page 129) and both exhibited UV spectra characteristic of anilinomethacrylate moieties (Figs. 35 and 40; Pages 110 and 115).

12-methoxyechitamidine (fraction d) showed a M⁺ at m/z 370 in the EI mass spectrum (Table 3, Page 96; Fig. 41, Page 116), a small peak at m/z 311 (loss of a carboxymethyl group), a small peak at m/z 293 (subsequent loss of an H₂O), and an intense ion at m/z 271. The indole with a methoxy group in the aromatic region plus a C₂H₄ group is represented by a peak at m/z 174 and the subsequent loss of a methyl by a peak at m/z 160. A prominent peak at m/z 94 is diagnostic of the akuammicine-type Strychnos alkaloids. The ¹H NMR spectrum (Table 5, Page 98; Fig. 42, Page 117) showed the presence of three methyl groups, at δ3.89 (a 17-carbomethoxy group), δ3.91 (an aromatic methyl) and δ1.14 (an 18-methyl group). Only three protons were discernable in the aromatic region (H-9, H-10 and H-11) and the ortho coupling of H-10 with H-11 and H-9 suggests that the methoxy group is at C-12. This is confirmed by the fact that the mass spectral and ¹H NMR data and the colour reactions of fraction d agree with that of Banerji and
Siddhanta, (1981) for scholarine ((±)-12-methoxyechitamidine) (122; Fig. 44, Page 129) and with that of Oguakwa et al., (1983) for (−)-12-methoxyechitamidine produced by hydrolysis of N-Formyl-12-methoxyechitamidine (110; Fig. 43, Page 127). On the basis of UV, EIMS, and 1H NMR fraction d is identified as 12-methoxyechitamidine.

The EIMS of fraction c (Fig 36, Page 111,) clearly indicated that it was a mixture of two compounds with the presence of ion peaks at m/z 370 and 340 and prominent peaks at 271 and 241. This was indicative of a one methoxy group difference between the two compounds. The similarities between the EIMS and 1H NMR spectra of the minor component of fraction c (Fig. 37, Page 112, Table 5, Page 98) and of 12-methoxyechitamidine clearly indicated that fraction c contained some 12-methoxyechitamidine. That it is the minor component is confirmed by the size of the NH peak at 8.48 which integrates for half the NH peak of the major component at 8.6.

The major component of fraction c was identified as echitamidine (111, page 127). The 1H NMR spectrum (Fig. 37, Page 112; Table 5, Page 98) differs from the minor component only in the aromatic region, where the four unsubstituted protons of the major component and the three aromatic protons of the minor component can be discerned. Coupling of the unsubstituted aromatic protons of the major component and coupling of H-10 with H-11 and H-9 of the
minor component can both be discerned in the COSY spectrum of fraction c, suggesting that the major component is echitamidine and the minor component is 12-methoxyechitamidine. This is further confirmed by the fact that the EI mass spectral data and the $^1$H NMR spectral data of the major component of fraction c correspond with that of echitamidine (Keawpradub et al., 1994; see appendix, Page 220).

All the signals for echitamidine and 12-methoxyechitamidine can be detected in the $^{13}$C NMR spectrum of fraction c (Fig. 38, Page 113; Table 6; Page 99), in which the major peaks correspond with the $^{13}$C NMR spectrum of echitamidine (111; Fig. 43, Page 127; Keawpradub et al., 1994) and the minor peaks with that of 12-methoxyechitamidine (122, Fig. 44, Page 129; Oguakwa et al., 1983; see appendix, Page 220). The carboxymethyl groups at C-17 and the methyl groups at C-18 of echitamidine and 12-methoxyechitamidine are represented by signals at δ168.94 (major peak), 168.99 (minor peak) and 19.76 (major peak) and 18.86 (minor peak) respectively. However, four distinct signals can be seen at 68.42, 68.38, 68.22 and 68.01, indicating that isomeric mixtures of S (H-19) and R (H-19) echitamidine and 12-methoxyechitamidine are present, since both echitamidine and 12-methoxyechitamidine have chiral centres at carbon-19. Therefore the main component of fraction c is (±)-echitamidine and the minor component is (±)-12-methoxyechitamidine.
Fig. 43 Compounds previously isolated from Alstonia boonei bark (Oguakwa et al., 1983; Marini-Bettolo et al., 1983).

127

(109) $N_a$-formylechitamidine

(110) $N_a$-formyl-12-methoxyechitamidine

(111) echitamidine

(112) O-acetyl-$N_a$-formyl-12-methoxy-echitamidine

(113) echitamine

(114) boonein
Fig. 44  Akuammicine-type alkaloids isolated from *Alstonia scholaris* (Atta-ur-Rahman et al, 1990).

(115) akuammicine-N$_b$-methiodide  R=CH$_3$

(116) akuammicine-N$_b$-oxide  R=O$^-$

(117) voacangine

(118) hydroxy-19,20-dihydroakuammicine

(119) tubotiawine

(120) lochneridine
Fig. 44 cont.

(121) 19-R-scholaricine (122) scholarine
(demethylscholarine) ((±)-12-methoxyechitamidine)

Fig. 45

(123) undulifoline
Three petroleum ether fractions, (1,2 and 4) with activity against *T. b. brucei* *in vitro* (Table 10, Page 134) were separated from the bark of *A. boonei*. $^1$H NMR spectroscopy and TLC indicated that these consisted of three distinct mixtures of compounds, including terpenoids. They were not Dragendorff-positive and therefore unlikely to have included alkaloids.

### 2.9.1 Results of the biological tests

Crude extracts of the following plants were active against *T. b. brucei* procyclic forms in the 48 hour test:
Alchornea cordifolia
leaf, bark and root aqueous extract
(which inhibited motility totally at 30, 250 and 250 µg/ml respectively) (Table 7, Page 133).
leaf, bark, and root methanolic extracts
(which inhibited motility totally at 250 µg/ml)
root petroleum ether extract
(which inhibited motility at 125 µg/ml) (Table 8, Page 133)

Alstonia boonei
bark petroleum ether, methanolic and aqueous alkaloidal extracts (which inhibited motility at 125, 250 and 250 µg/ml respectively (Table 9 Page 134).

Annona senegalensis
bark aqueous extract
(which inhibited motility totally at 500 µg/ml; Table 7, Page 133).

The following crude aqueous extracts were inactive at 500µg/ml:
Lonchocarpus cyanensis roots, Tamarindus indica roots, Ximenia americana bark and stem, Annona senegalensis leaves and root, A. cordifolia stem. The non-alkaloidal extract of A. boonei was also inactive at 500µg/ml.
The following semi-purified fractions of *A. boonei* were active against *T. b. brucei* procyclic forms in the 48 hour test:

Petroleum ether fractions 1, 2 and 4
(which inhibited motility totally of *T. b. brucei* procyclic forms at 250, 125 and 250μg/ml respectively after 48 hours (Table 10, Page 134).

The following pure compounds, isolated from *A. boonei* were active against *T. b. brucei* bloodstream forms in the 48 hour test:

echitamine (a) with an ED₅₀ of 3.78 μg/ml
undulifoline (b) with an ED₅₀ of 3.85 μg/ml
12-methoxyechitamidine (d) with an ED₅₀ 2.98 μg/ml
The mixture of 12-methoxyechitamidine and echitamidine (c) had an ED₅₀ of 6.01 (Table 11, Page 135).
Table 7  Activity of the aqueous extracts of some African plants against *T. b. brucei* procyclic forms in vitro after 48 hours.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>125</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. cordifolia</em> leaf</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>'' bark</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>'' root</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>A. boonei</em> bark</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>A. senegalensis</em> bark</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition at: (μg/ml)

Table 8 Activity of the petroleum ether, methanolic and aqueous extracts of *A. cordifolia* leaves, bark and root against *T. b. brucei* procyclic forms in vitro after 48 hours.

<table>
<thead>
<tr>
<th>type of extract</th>
<th>30</th>
<th>60</th>
<th>125</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>leaf methanolic</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>leaf aqueous</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>bark methanolic</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>bark aqueous</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>root pet. ether</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>root methanolic</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition at: (μg/ml)

Key to all tests against *T. b. brucei* procyclic forms

+++ total inhibition of motility
++ moderate inhibition of motility
+ slight inhibition of motility
0 no inhibition of motility
Table 9  Activity of the petroleum ether, methanolic and aqueous extracts of *A. boonei* bark against *T. b. brucei* procyclic forms in vitro after 48 hours.

<table>
<thead>
<tr>
<th>type of extract</th>
<th>30</th>
<th>60</th>
<th>125</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>pet. ether</td>
<td>0</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>methanolic</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>aqueous</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 10  Activity of the petroleum ether fractions of *A. boonei* bark against *T. b. brucei* procyclic forms in vitro after 48 hours.

<table>
<thead>
<tr>
<th>fraction</th>
<th>30</th>
<th>60</th>
<th>125</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

key: as for table 7
Table 11. Activity of echitamine (a), undulifoline (b), a mixture of echitamidine and 12-methoxiechitamidine (c) and 12-methoxyechitamidine (d) against T. b. brucei bloodstream forms in vitro after 48 hours.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>30</th>
<th>10</th>
<th>3</th>
<th>1</th>
<th>ED$_{50}$ ± SD</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>100</td>
<td>55.6</td>
<td>40.9</td>
<td>35.7</td>
<td>3.78±0.165</td>
<td>1.36, 19.36</td>
</tr>
<tr>
<td>b</td>
<td>100</td>
<td>45.0</td>
<td>55.0</td>
<td>33.3</td>
<td>3.85±0.228</td>
<td>2.48, 36.9</td>
</tr>
<tr>
<td>c</td>
<td>100</td>
<td>36.8</td>
<td>42.1</td>
<td>10.5</td>
<td>6.01±0.176</td>
<td>1.05, 34.4</td>
</tr>
<tr>
<td>d</td>
<td>100</td>
<td>64.3</td>
<td>42.7</td>
<td>41.5</td>
<td>2.98±0.16</td>
<td>1.64, 14.6</td>
</tr>
</tbody>
</table>

2.10 Discussion

The bark of A. boonei, the commonest species of Alstonia in several tropical West African countries, (Ojewole, 1984) is used to treat fever and "neural disease" in Ghana, fever, dizziness and a number of other symptoms in Nigeria (Asuzu and Anaga, 1991) and is taken internally in the form of teas or palm wine infusions.

The aqueous extract of A. boonei has been reported to produce a slight reduction in parasitaemia in T. b. brucei-infected mice (Asuzu and Anaga, 1991); extracts of

Footnote: ED$_{50}$ values were derived by linear regression, using a computer programme called nanostat, developed at the London School of Hygiene and Tropical Medicine. There were four replicates.
A. boonei twigs were active against gram-positive bacteria at 50 mg/ml (Verpoorte et al., 1983) and a methanolic extract of A. congensis (a species almost identical with A. boonei; Oguakwa et al., 1983) suppressed parasitaemia in P. berghei-infected mice by 74.9% at 200mg/kg/day in a four day test (Awe and Opeke, 1990). This low degree of activity compares well with the low in vitro activity of A. boonei crude extracts against T. b. brucei found in the present study.

Echitamine (113), a quaternary indole and the major alkaloid in Alstonia boonei, is a hypotensive agent, causing ganglion block and a fall in blood pressure when administered parenterally (Vasanth et al., 1990) and significantly reducing systemic arterial blood pressure in normotensive rats and cats at 0.05-10 mg/kg i.v. (Ojewole, 1984). Echitamine chloride also has antimalarial activity; it was effective at 1.6 mg/kg against P. berghei in mice (Vasanth et al., 1990) and had an IC_{50} value of 42.6 ± 3.41 μM against P. falciparum in vitro. Again, this low degree of activity agrees with the low in vitro anti-T. b. brucei activity of echitamine found in this study.

A. scholaris has been investigated extensively and twenty eight alkaloids have been isolated from this species, ten of which are akuammicine-type strychnos alkaloids: akuammicine (82; Fig. 11, Page 58), akuammicine-N\_b-
methiodide (115), akuammicine-\textsubscript{N\textsubscript{x}}-oxide (116), voacangine (117), hydroxy-19, 20-dihydroakuammicine (118), tubotiamine (119), lochneridine (120), 19-R-scholaricine (121), scholarine (12-methoxyechitamidine) (122) and echitamidine (111) (Atta-ur-Rahman et al., 1990; Fig. 44, Pages 128 and 129). Less attention has been paid to \textit{A. boonei} de Wild, which is considered to be another variety of the same species (Oguakwa et al., 1983), but akuammicine-type alkaloids have also been isolated from this species: N\textsubscript{x}-formylechitamidine (109), N\textsubscript{x}-formyl-12-methoxyechitamidine (110), echitamidine (111) and O-acetyl-N\textsubscript{x}-formyl-12-methoxyechitamidine (112) (Oguakwa et al., 1983; Fig. 43, Page 127). The akuammicine-type alkaloid, 12-methoxyechitamidine and the uleine-type alkaloid, undulifoline (123, Fig. 45, Page 129) have not previously been reported to have been isolated from \textit{A. boonei}.

The barks of \textit{A. boonei} and \textit{A. scholaris} both contain terpenoid compounds. Boonein (114; Fig. 43; Page 127), a C-9 terpenoid lactone, was isolated from \textit{A. boonei} (Marini-Bettolo et al., 1983) and the pentacyclic triterpenes \textalpha\ amyrin (124) and lupeol (125; Fig. 46, Page 130), steroid precursors (Cheung and Wong, 1972), have been isolated from \textit{A. scholaris} (Mukherjee and Ghosh, 1979).

The compounds isolated in this study had low activity \textit{in vitro} against \textit{T. b. brucei}. However, echitamine, the major compound present in \textit{A. boonei}, is relatively non-toxic,
having an LD₅₀ of 70.5 ± 2.3 mg/kg i.p. in mice (Ojewole, 1984) and, being very polar, it is soluble in water, always an important consideration when an aqueous infusion of a plant is administered. However, aqueous infusions of the bark would be unlikely to cure sleeping sickness, though they would have a febrifugal effect.

The aqueous extract of the fresh leaves of *A. cordifolia* inhibited *T. b. brucei* procyclic motility totally in vitro at 30 μg/ml. However, after fractionation, neither of the fractions were as active against *T. b. brucei* procyclic forms, the aqueous and methanolic fractions only inhibiting motility at 125 and 250 μg/ml respectively (Tables 7 and 8, Page 133). Further fractionation produced fractions which were even less active against *T. b. brucei* procyclic forms (results not shown), therefore fractionation was not continued.

*A. cordifolia* leaves are traditionally pulverised and applied to trypanosomal chancre, ulcers and yaws (Dalziel, 1937), or used in the form of a cold maceration which is applied externally to cuts, sores and burns (Ajao et al., 1984); preparations of the plant may be taken internally (Dalziel, 1937) to treat a number of conditions, including fever (Adjanohoun and Assi, 1972; see Oliver Bever, 1986). Activity of aqueous extracts of the fresh leaves of *A. cordifolia* against *T. b. brucei* in vitro may be a simple non-selective cytotoxic effect due to the presence of
polyphenols (Schneider and Kubelka, 1989) which are responsible for the marked antibacterial activity reported for this plant (Ajao et al., 1984). Agbe and Ogun-timein (1987) reported a cure for rats infected with T. b. brucei using an ethanolic fraction of an aqueous extract of A. cordifolia leaves administered intraperitoneally; they did not, however investigate the effect of oral administration of the same extract which would mimic traditional use of the aqueous tea. Further research should be carried out to isolate the compounds responsible for the anti-trypanosomal activity of fresh Alchornea cordifolia leaves.

The aqueous extract of the stem bark of Annona senegalensis had a low activity against T. b. brucei procyclic forms and the root was inactive. This was surprising since in West Africa the root of this plant is used traditionally to treat gambiense sleeping sickness (Igweh and Onabanjo, 1989) and, in 1989, Igweh and Onabanjo reported a complete cure for T. b. brucei-infected mice with an oral dose of aqueous extract of Annona senegalensis root given for four consecutive days after infection. They reported that the aqueous root extract contained alkaloids. However yields of active compounds from the root and stem barks of this plant may vary according to location and season.

Several genera, including Annona, from the family Annonaceae contain acetogenins, bioactive natural products with antitumour, pesticidal and antimalarial activities
(Fang et al., 1993). Annonaceae also contain bisbenzylisoquinoline, proaporphine and aporphine alkaloids (Hocquemiller et al., 1984).

The aqueous extracts of *Lonchocarpus cyanensis* root, *Tamarindus indica* root and *Ximenia americana* bark and stem were inactive against *T. b. brucei* procyclic forms. There could be many reasons for this. The active components of these plants may not be water soluble or they may not be present in the parts of the plants chosen for testing or the plants may have been harvested at a time of the year when they contained low yields of active compounds. Alternatively, these plants may not contain trypanocidal compounds.

Plants used traditionally as medicinal agents are chosen for a number of reasons, both physical and spiritual. Traditional healers administer mixtures of plants, out of which maybe one or two plants contain active principles and the others have been added for spiritual or other reasons (Oku-Ampofo, O. personal communication). The literature does not mention whether the plants chosen for the present study are administered in isolation or in mixtures.

The symptoms of early stage sleeping sickness are difficult to interpret, and, although a good traditional healer might recognize them, inexpert village people may treat only an individual symptom such as fever. The literature does not
state whether a plant has been recommended by a traditional healer or whether it is merely used by local people. Modern ethnopharmacologists often seek out the traditional healers, when looking for plants to treat specific diseases, since they have a considerably larger store of knowledge than the people whom they treat. This approach may not have been used in the search for antitrypanosomal plants.

There is still an urgent need for new chemotherapeutic agents for the treatment of African trypanosomiasis. It is encouraging to note that some of the plants used traditionally which were investigated in this study have some activity against T. b. brucei, although this activity is low. However it was not possible to acquire all the plants reputed to be effective in the treatment of sleeping sickness since the deforestation which has taken place in West Africa has made the collection of medicinal plants increasingly difficult and many of the species mentioned by Abbiw (1990) are now extremely rare. The screening of rare plants for biological activity, however, is important since, although their rarity may preclude their use as medicinal agents, the possibility of discovering novel active compounds which could be synthesized, or used as templates for the synthesis of analogues from which potential drugs could be derived, further highlights the importance of preserving the remaining tropical forest.
3.1 Introduction
3.1.1 The epidemiology and the course of the disease.

Leishmaniasis is a complex of diseases caused by haemoflagellate protozoan parasites, with an estimated global incidence of 400,000 new cases annually (Ashford et al., 1992). This figure is based on the number of reported incidences of the disease excluding cases who live in rural areas without access to treatment, misdiagnosed cases, and cases seen clinically but not reported (Ashford et al., 1992). An estimated 200 million people in Africa, the Americas, Asia and Europe are at risk of infection with Leishmania spp. (Ashford et al., 1992).

Leishmaniasis is transmitted by phlebotomine sandflies which inject the parasites into the skin of the mammalian host where they are phagocytosed by host macrophages, transform to amastigotes, and begin active division. Multiplication of amastigotes within the macrophages of the skin and viscera gives rise to cutaneous and visceral leishmaniasis, respectively, and expression of the disease is influenced by species of parasite and immune status of the host (Neva and Sacks, 1990).
Fig. 47 Old World cutaneous leishmaniasis (adapted from Minter, 1989).

Fig. 48 New World cutaneous leishmaniasis (adapted from Minter, 1989).
L. tropica, L. major and L. aethiopica cause cutaneous disease in the Old World (Fig. 47) whilst parasites of the Leishmania mexicana complex cause cutaneous disease in the New World and those of Leishmania braziliensis complex cause cutaneous and mucosal disease in the New World (Fig. 48; Page 143); species forming the Leishmania donovani complex (L. donovani, L. infantum and L. chagasi) cause visceral disease in both the New World and the Old World (Berman, 1988; Fig. 49; Page 145; Table 12; Page 146).

There are many different types of cutaneous leishmaniasis and location of the patient, host response, secondary infections, treatment and chronicity all affect the appearance of the lesion (Walton, 1987). Numerous species and subspecies cause single ulcers, multiple ulcers, dry lesions, non-healing lesions, indolent nodular lesions, diffuse cutaneous leishmaniasis, and mutilating secondary mucocutaneous lesions (WHO, 1990b). Characteristically, however, a cutaneous lesion begins as a red papule, enlarges to form a shallow ulcer with raised indurated red margins, then heals with scarring (Berman, 1988).

Special cases
Diffuse cutaneous leishmaniasis (DCL) (Table 12; Page 146) is a rare condition, occurring as a result of lack of immune response to Leishmania organisms. It is characterised by widely disseminated thickening of the skin in plaques, papules or multiple nodules (WHO, 1990b).
Fig. 49 Visceral leishmaniasis in humans (adapted from Minter, 1989).
Table 12  Geographical distribution of the clinical disease caused by different species of *Leishmania* (adapted from Neva and Sacks, 1990).

<table>
<thead>
<tr>
<th>Species</th>
<th>geographical distribution</th>
<th>clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. mexicana</em> complex</td>
<td>New World, from southern US to central S. America</td>
<td>cutaneous ulcers; small proportion of cases develop diffuse cutaneous (DCL) or mucocutaneous (MCL) leishmaniasis</td>
</tr>
<tr>
<td><em>L. mexicana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. amazonensis</em></td>
<td></td>
<td></td>
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<tr>
<td><em>L. venezuelensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. braziliensis</em> complex</td>
<td>New World, from central America to northern Argentina</td>
<td>cutaneous ulcers; some cases may later develop MCL (probably more likely if cutaneous lesion not treated adequately)</td>
</tr>
<tr>
<td><em>L. braziliensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. guyanensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. panamensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. peruviana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>Northern and central Africa, Middle East, and S. Asia</td>
<td>cutaneous ulcers</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>Middle East and S. Asia</td>
<td>cutaneous ulcers and chronic relapsing cutaneous disease</td>
</tr>
<tr>
<td><em>L. aethiopica</em></td>
<td>Ethiopia and contiguous countries</td>
<td>cutaneous ulcers, rarely diffuse cutaneous leishmaniasis (DCL)</td>
</tr>
<tr>
<td><em>L. donovani</em> complex</td>
<td>E. Africa and south of the Sahara, Indian subcontinent, S. Asia</td>
<td>visceral leishmaniasis; small proportion may develop post-kala-azar dermal leishmaniasis (PKDL)</td>
</tr>
<tr>
<td><em>L. infantum</em></td>
<td>N. Africa and S. Europe</td>
<td>visceral leishmaniasan</td>
</tr>
<tr>
<td><em>L. chagasi</em></td>
<td>Brazil, Venezuela, Colombia, and isolated cases in central and S. America</td>
<td>visceral leishmaniasan</td>
</tr>
</tbody>
</table>
Mucocutaneous leishmaniasis (MCL; espundia; Table 12; Page 146) occurs in 3-5% of patients suffering from L. braziliensis or L. panamensis leishmaniasis infections. Primary cutaneous lesions spread via the blood or lymphatic system to the oronasal/pharyngeal mucosa and symptoms may occur at any time up to 30 years after the primary lesion. Espundia is characterised by ulceration and erosion which progressively destroy soft tissue and cartilage resulting in a high degree of suffering and severe mutilation. Secondary bacterial infections are frequent and can be fatal (WHO, 1990b).

Visceral leishmaniasis (kala-azar) varies according to the age of the patient and geographical location. It may be endemic, sporadic or epidemic, with different clinical features in each situation (WHO, 1990b). Onset of the disease is usually gradual and common symptoms are fever, malaise, weight loss, splenomegaly, hepatomegaly and lymphadenopathy. Kala-azar, if left untreated, has a high mortality rate (WHO, 1990b); more than half of visceral leishmaniasis cases occur in India and Sudan (Ashford et al., 1992).

Post-kala-azar dermal leishmaniasis (Table 12; Page 146) is commonest in India. It is characterised by hypopigmented or erythematous macules and may become papular or nodular, especially on the face. It may occur at any time between one and several years after visceral leishmaniasis has
apparently been cured (WHO, 1990b).

3.2 The Life cycle of the parasite

*Leishmania* are digenetic protozoa which exist as flagellated extracellular promastigotes in the sandfly vector and as aflagellate obligate intracellular amastigotes in the macrophages of their mammalian hosts (Neva and Sacks, 1990; Fig. 50; Page 149).

When the sandfly takes a blood meal from an infected vertebrate host it ingests intracellular amastigote forms which divide actively before transforming to flagellated promastigote forms. These forms migrate to the midgut (or the hindgut in the case of *braziliensis* group) of the sandfly where they attach to the microvilli by insertion of the flagellum and further replication takes place (Killick-Kendrick, 1979). They then migrate to the foregut where they transform to paramastigote forms which attach to the cuticular lining by the tip of the flagellum. Migration ends in the mouthparts, where they change into highly motile, non-dividing, metacyclic promastigote forms, which are infective to the vertebrate host (Alexander and Russell, 1992).
Fig. 50  The life-cycle of *Leishmania* spp. in the sandfly and in mammalian hosts. (adapted from Chang and Bray, 1985)

In the mammalian host

- Intracellular amastigote
- Phagocytosis by macrophage
- Injection into mammalian host
- Metacyclic promastigotes
- Promastigotes attached to microvilli
- Replication of amastigotes and their differentiation into promastigotes
- Rupture of parasitized macrophage

In the sandfly
When the infected sandfly takes another blood meal, promastigote forms are deposited in the skin of the mammalian host where they are phagocytosed by macrophages, enter a parasitophorous vacuole and transform into actively dividing amastigote forms. Heavily-parasitised macrophages rupture, releasing amastigote forms which then invade other macrophages (Fig. 50; Page 149).

3.3 Current clinical chemotherapy

The first choice drugs, the pentavalent antimonials sodium stibogluconate (126; Pentostam, assumed structure) and meglumine antimoniate (127; Glucantime, assumed structure; Fig. 51; Page 151), require parenteral administration under medical supervision and side effects frequently occur (Olliaro and Bryceson, 1993). There is increasing resistance to antimonials in many countries (Davidson and Croft, 1993); for example, in 1990 more than 10,000 cases unresponsive to antimony were reported in Bihar State, India (TDR news, 1990).

A second-line drug, amphotericin B (128; Fig. 51; Page 151), has numerous side effects including anaemia, cardiotoxicity and nephrotoxicity (Hoeprich, 1992) and the anti-trypanosomal drug pentamidine (104), also used in cases of antimony-resistant visceral leishmaniasis, is associated with disturbances of glucose metabolism which result in severe side effects (Katiyar et al., 1992).
Fig. 51 Compounds currently in use or undergoing clinical trial for the treatment of leishmaniasis.

(126) sodium stibogluconate

(127) meglumine antimononiate

(128) amphotericin B
Fig. 51 cont.

(129) paromomycin

(130) ketoconazole

(131) allopurinol
Although a few new drugs such as paromomycin (129), ketoconazole (130), allopurinol (131) (Olliaro and Bryceson, 1993) and the 8-aminoquinolone WR6026 (132) (Fig. 51; Pages 152 and 153; Katiyar et al., 1992) are currently on clinical trial, results have been equivocal. There remains an urgent need for new therapeutic agents. The plant kingdom, which has provided templates for semi-synthetic and synthetic compounds, for use as pharmacological agents, as previously mentioned, is still a valuable source of novel compounds.

3.4 Reasons for the choice of plants for testing.

The delta of the Amazon river in Brazil, a tropical rainforest, one of the most biologically diverse ecosystems in the world, contains a vast potential of novel compounds.
For this reason, an ethnopharmacological project was carried out in this region by Dr L.E.S. Barata. This resulted in the selection of the leaves of Virola surinamensis (Rol) Warb. (Myristicaceae) for their antiparasitic activity (Barata et al., 1978).

3.4.1 Virola species

Forty species of Virola (Myristicaceae) have been identified, worldwide, in mixed hardwood tropical forests (Plotkin and Schultes, 1990). The Amazon basin is a particularly rich source of these species which are used traditionally by Amazonian Indians for medicinal purposes, as hallucinogenic snuffs and as arrow poisons (Schultes and Holmstedt, 1971). Various tribes use the leaves and bark exudate of different Virola spp. to treat rheumatism, asthma, intestinal worms, skin diseases and ulcerating sores (Plotkin and Schultes, 1990).

3.4.2 Neolignans

Neolignans, which occur in Myristicaceae and other primitive plant families, are usually dimers of the oxidative coupling of allyl and propenyl phenols (Gottlieb, 1978).
Fig. 52  Neolignans isolated from Virola surinamensis
(Barata et al., 1978).

(133) elemisin  (not a neolignan)  (134) galbacin

(135) veraguensin

(136) surinamensin R=OMe
(137) virolin  R=H
Fig. 53  Type I synthetic analogues of surinamensin from *Virola surinamensis*.

(138) \( R_1 = \text{OMe} \quad R_2 = \text{OMe} \\
      \quad R_4 = \text{CH}_3 \\
(139) \ R_1 = \text{O} \quad R_2 = \text{OCH}_2\text{O} \\
      \quad R_4 = \text{H} \\
(140) \ R_3 = \text{OH} \quad R_2 = \text{H} \\
(141) \ R_3 = \text{NH}_2 \quad R_2 = \text{Me} \\
(142) \ R_3 = \text{NOH} \quad R_2 = \text{H}

\[
\begin{array}{cccccccc}
 R_1 & R_2 & R_4 & R_5 & R_6 & R_7 & R_8 \\
(143) & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} \\
(144) & \text{H} & \text{H} & \text{H} & \text{OMe} & \text{H} & \text{H} & \text{H} \\
(145) & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{OMe} & \text{H} \\
(146) & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{Cl} & \text{H} \\
(147) & \text{H} & \text{H} & \text{H} & \text{NO}_2 & \text{H} & \text{H} & \text{H} \\
(148) & \text{OMe} & \text{OMe} & \text{O} & \text{H} & \text{H} & \text{OMe} & \text{H} \\
(149) & \text{OMe} & \text{OMe} & \text{H} & \text{OMe} & \text{H} & \text{H} & \text{H} \\
(150) & \text{OMe} & \text{OMe} & \text{H} & \text{H} & \text{H} & \text{Cl} & \text{H} \\
(151) & \text{OMe} & \text{OMe} & \text{H} & \text{OMe} & \text{H} & \text{Propenyl} & \text{H} \\
(152) & \text{OMe} & \text{OMe} & \text{O} & \text{OMe} & \text{H} & \text{H} & \text{OMe} \\
(153) & \text{H} & \text{H} & \text{O} & \text{H} & \text{Me} & \text{H} & \text{H} \\
\end{array}
\]
Fig. 54  Natural neolignan (154) from Virola pavonis and synthetic derivatives (155 and 156) (Ferri and Barata, 1992).

(154) natural neolignan from *V. pavonis*

(155) synthetic analogue of a neolignan derived from *V. pavonis*

(156) synthetic analogue of a neolignan derived from *V. pavonis*
Fig. 55 Type II synthetic N-(157, 158) and type III S-(159-162) analogues of neolignans from Virola surinamensis (Ferri and Barata, 1991).

Type II

\[
\begin{align*}
\text{(157) } R_1 &= H \quad R_2 = H \\
\text{(158) } R_1 &= \text{OMe} \quad R_2 = \text{OMe}
\end{align*}
\]

Type III

\[
\begin{align*}
\text{(159) } R_1 &= H \quad R_2 = H \quad R_7 = \text{Cl} \\
\text{(160) } R_1 &= \text{OMe} \quad R_2 = \text{OMe} \quad R_7 = \text{CH}_3 \\
\text{(161) } R_1 &= \text{OMe} \quad R_2 = \text{OMe} \quad R_7 = \text{Cl} \\
\text{(162) } R_1 &= \text{OMe} \quad R_2 = \text{OMe} \quad R_7 = H
\end{align*}
\]
They have a wide range of biological effects including antifungal, anti-inflammatory, neuroleptic, antihepatotoxic, anticancer and anti-PAF activities (MacRae and Towers, 1984).

Barata et al., (1978) isolated five neolignans from *V. surinamensis*: elemisin (133), the 8,8'-neolignans galbacin (134) and veraguensin (135) and the 8-0-4' neolignans surinamensin (136) and virolin (137; Fig. 52; Page 155). Surinamensin and virolin were active against *Schistosoma mansoni* (Barata et al., 1978). Further studies on the leaves of *Virola pavonis* (A.DC.) A.C. Smith resulted in the isolation of a natural neolignan (154; Fig. 54; Page 157 Ferri and Barata 1991, 1992)) which was also used in this study.

3.4.3 Semi-synthetic neolignan analogues of Virola natural products.

Three types of analogues of surinamensin, were synthesized through the condensation of α-bromo ketones with phenols, anilines and thiophenols in basic solution (Ferri and Barata, 1991). The three types of analogues were as follows: type I, with ether linkages (138-153, 155 and 156; Figs. 53 and 54; Pages 156 and 157), type II with nitrogen linkages (157, 158), and type III with sulphur linkages (159-162; Fig. 55; Page 158).
Type I analogues (155 and 156) were synthesized based on structures of neolignans isolated from *Virola pavonis*.

### 3.5 Natural products with known activities chosen for screening against *Leishmania donovani* amastigote forms in vitro

Several natural products are known to act on specific sites within the cell or to interfere with certain cell processes. In some cases these specific sites or cell processes may be the target of chemotherapeutic drugs. For example, inhibition of microtubule assembly:

#### 3.5.1 Microtubule inhibitors

Microtubule assembly may be inhibited as a result of disruption of microtubule-assembly, for example by podophyllotoxin (163) from *Podophyllum spp.* rhizomes or *Vinca spp.* alkaloids, or as a result of microtubule-stabilization, for example, by taxol (164; Fig. 56; Page 161), isolated from *Taxus brevifolia* (Northover and Northover, 1993).

Microtubules are an important target in parasites such as trypanosomatids which undergo modulations of cell shape and capacity for motility as well as periods of rapid division at various stages during their life cycle.
Fig. 56  Some microtubule and topoisomerase inhibitors

(163) podophyllotoxin

(164) taxol

(165) ellipticine

(166) etoposide  $R = \text{Me}$

(167) teniposide  $R = \text{S}$

(168) camptothecin
Fig. 57  Some glucose transport inhibitors (169-171) and some chelators of peroxyl radicals (173, 172).

(169) cosmetin  $R_1 = \text{O-glucose}$  
$R_2 = H$  $R_3 = H$

(172) morin  $R_1 = \text{OH}$  
$R_2 = \text{OH}$  $R_3 = \text{OH}$

(170) phloridzin  $R = \text{CH}_2\text{OH}$

(171) phloretin  $R = \text{OH}$

(173) maclurin
3.5.2 Topoisomerase inhibitors

Enzymes such as topoisomerases I and II which regulate the topological conformation of DNA and participate in DNA replication, transcription, and sister chromatid exchange (Wang, 1987) may offer targets for chemotherapy, especially in rapidly proliferating cells such as cancer cells and parasites. There is convincing evidence that DNA topoisomerases are important chemotherapeutic cellular targets (Schneider et al., 1990).

Topoisomerase II enzymes catalyze double stranded DNA cleavage and resealing (Douc-Rasy et al., 1988) and are a major component of the mitotic chromosome scaffold (Earnshaw et al., 1985). Their levels are significantly increased in proliferating cells (Riou et al., 1985). Ellipticine (165) from Ochrosia spp., Apocynaceae (Douc-Rasy et al., 1988), and the semi-synthetic epipodophyllotoxins etoposide (166) and teniposide (167; Fig. 56; Page 161) are topoisomerase II inhibitors. Etoposide and teniposide trap nuclear enzyme DNA topoisomerase II on DNA in a sequence-specific manner (Smith and Soues, 1994) while ellipticine intercalates between the purines and pyrimidines of the DNA, thus inhibiting the unwinding of the DNA helix (Fosse et al., 1994).

Topoisomerase I catalyses transient single-strand breaks in DNA (Prost and Riou, 1994) and is specifically inhibited by
camptothecin (168; Fig. 56; Page 161) from *Camptotheca acuminata* (Prost and Riou, 1994).

### 3.5.3 Glucose transport inhibitors

Inhibitors of energy metabolism such as cosmetin (169), from *Anthemis nobilis* flowers (Compositeae), phloridzin (170) and its aglycone, phloretin (171; Fig. 57; Page 162) from the skins and leaves of apples (*Malus* spp., Rosaceae) inhibit transmembrane glucose transport and have been used to inhibit selectively cells such as cancer cells which are dividing rapidly (Nelson and Falk, 1993).

### 3.5.4 Chelators of peroxoyl radicals

Maclurin (173) and the flavonoid morin (172; Fig. 57; Page 162) from *Morus* spp., Moraceae, chelate peroxoyl radicals (Wu et al., 1994).

### 3.6 Experimental

#### 3.6.1 *L. donovani* promastigote form culturing technique

*L. donovani* (MHOM/ET/67/L82) promastigote forms were grown in Schneider’s medium (Gibco) with 10% foetal calf serum (Gibco) and gentamycin (50 μg/ml) at 24°C in tissue culture flasks. The medium was changed every four days.
165

3.6.2  \textit{T. cruzi} epimastigote form culturing technique.

\textit{T. cruzi} (MHOM/BR/78/SYLVIO/XIO-1) epimastigotes were grown in medium consisting of RPMI 1640 (Gibco) with 0.5% HEPES (Sigma), 0.5% (w/v) trypticase (BBL), 0.3 M haemin (Sigma) and 10% heat-inactivated foetal calf serum (Gibco) (Gibson and Miles, 1986). Epimastigotes were grown in tissue culture flasks at 24°C and medium was changed every four days.

3.6.3  \textit{L. donovani} test procedures

3.6.3.1 Tests against \textit{L. donovani} promastigote forms \textit{in vitro}.

\textit{In vitro} promastigote assays were carried out in 96-well microplates using fast-growing \textit{L. donovani} promastigote forms grown in Schneider's medium (Gibco) with 10% heat-inactivated foetal calf serum (Gibco). Each compound was tested in quadruplicate at 100 and 30 μM with 100 μl of promastigote suspension at $10^6$/ml per well, and incubated at 24°C for forty eight hours. Two sets of controls were included in each test: drug-free controls and standard drug controls.

Crude extracts were tested in triplicate in limiting dilutions from 500-15 μg/ml. Drug-free controls were included in each test. Promastigote motility was assessed.
through a phase contrast microscope at 40 X magnification after 24 hours and 48 hours. Motility of cultures provided an indication of activity on a scale of +++ (maximum inhibition) to 0 (no inhibition).

3.6.3.2 Test against *L. donovani* amastigote forms in vitro.

The amastigote-mouse peritoneal macrophage model (Neal and Croft, 1984) was used to assess the *in vitro* sensitivity of *L. donovani* parasites (MHOM/ET/67/L82) to test compounds. Mouse peritoneal macrophages from CD1 mice were suspended in RPMI 1640 medium (Gibco) with 10% heat-inactivated foetal calf serum (Gibco) and gentamycin (50 μg/ml) at a density of 10⁵ cells per ml and maintained in wells of sterile tissue-culture eight-chamber slides (Labtek Products, Miles Laboratories) at 37°C in a 5% CO₂/air mixture. After twenty four hours the macrophages were infected with *L. donovani* amastigote forms isolated from the spleen of an infected hamster at a ratio of 10:1.

Test compounds were added to the infected macrophages in fresh medium twenty four hours later, on day 2, and subsequently on days four and seven. Each compound was tested in quadruplicate at 100, 30 and 10 μM. On day nine the macrophage monolayers were fixed with methanol and stained with 10% Giemsa's stain for 10 minutes. Drug activity was assessed by counting the number of amastigote
forms per 100 macrophages. ED_{50} values were calculated by linear regression analysis. Damage to macrophages was assessed in order to determine toxicity. Where selective anti-leishmanial activity was found the compound was re-tested over an appropriate range of concentrations.

3.6.3.3 Test against *L. donovani* in vivo

Female BALB/c mice (B and K Ltd.), 18-20g, were infected by the tail vein with 5 x 10^6 amastigotes of *L. donovani* derived from a hamster spleen. One week after infection mice were dosed with compounds 159-162 suspended in 0.25% cellacol, by the subcutaneous route, daily for five days. Three days after completion of drug treatment mice were sacrificed, their livers removed, weighed and smears prepared. The liver amastigote load of untreated and treated mice was calculated from liver weight (mg) x number of amastigotes/500 liver cells.

3.6.3.4 Test against *T. cruzi* epimastigote forms in *vitro*.

Crude plant extracts were screened in triplicate in limiting dilutions from 500-15 µg/ml, in RPMI 1640 medium with the additions described above, against fast-growing *T. cruzi* epimastigotes in 96-well microplates and incubated at 24°C. Drug-free controls were included in each test. Motility was assessed through a phase contrast microscope
at 40 X magnification after 24 hours and 48 hours. Motility of cultures provided an indication of activity on a scale of +++ (maximum inhibition) to 0 (no inhibition).

In most cases, trypanosomatid vector forms were used to screen crude plant extracts and the mammalian forms were used to test pure compounds.

3.7 Compounds tested

The natural neolignan, surinamensin (136) together with type I (138-153; Fig. 53; Page 156), type II (157 and 158) and type III (159-162; Fig. 55; Page 158) synthetic analogues were assessed against L. donovani promastigote forms and amastigote forms in vitro. Two synthetic analogues (155 and 156) and a natural neolignan (154) from Virola pavonis leaves (Ferri and Barata, 1992) were also tested against L. donovani promastigote forms in vitro. The Type III compounds (159-162) were assessed for activity in vivo.

The following natural products were also screened for activity against L. donovani amastigote and promastigote forms in vitro:

the glucose-transport inhibitors phloridzin (170), cosmetin (169) and phloretin (171), the peroxoyl radical chelators morin (172) and maclurin (173), the topoisomerase I
inhibitor camptothecin (168), the topoisomerase II inhibitor ellipticine (165) and the microtubule-assembly disrupter, podophyllotoxin (163).

Crude extracts of the following African plants were screened against *L. donovani* promastigote forms and *T. cruzi* epimastigote forms:

*Alchornea cordifolia*
leaf, bark and root aqueous extracts
leaf, bark, and root methanolic extracts
root petroleum ether extract

*Alstonia boonei*
bark petroleum ether, methanolic and aqueous extracts

3.8 Results of the biological tests

3.8.1 Results of in vitro tests

Surinamensin (136), the neolignan isolated from *Virola surinamensis* and selected for its anti-schistosomal activity (Barata *et al*., 1978), was active *in vitro* against *L. donovani* promastigote forms at 50 μM but showed no selective toxicity when tested against *L. donovani* amastigote forms in the mouse peritoneal macrophage system. Tests using extracellular promastigote forms showed that compounds 138, 139, 141, 146-148, 150-153, and 155-162 were
The natural neolignan from *V. pavonis*, 154 was active at 100 μM. The remaining compounds were inactive. In the tests against amastigote forms in macrophages compounds 138, 139, 141, 148, 151-153 and 157 were active at 100 μM but toxic to macrophages, while compounds 146 and 150 were selectively toxic to macrophages. Compounds 159-162 (all type III compounds with a sulphur bridge) were active against both amastigote and promastigote forms (Tables 13 and 14; Pages 172 and 173) and showed the greatest selectivity. Compounds 140, 142-145 and 149 were inactive against promastigote and amastigote forms at 100 μM.

The chelators morin (172) and maclurin (173) were inactive at 100 μM, although maclurin damaged macrophages. The glucose transport inhibitors phloridzin (170) and cosmetin (169) were inactive against amastigote forms at 100 μM, though they were toxic to macrophages at the same concentration. The topoisomerase I inhibitor camptothecin (168) was toxic to macrophages at 10 μM. The topoisomerase II inhibitor ellipticine (165) was toxic to macrophages at 1 μM. The antimitotic agent podophyllotoxin (163) was toxic to macrophages and amastigote forms at 30 μM but inactive at 10 μM, although it was toxic to macrophages at this concentration.

The crude extracts of the following African plants were inactive at 500 μg/ml against *L. donovani* promastigote

3.8.2 Results of in vivo tests

Four of the compounds which showed activity in vitro were further tested against *L. donovani* in the BALB/c mouse model. Only compound 160 showed any significant anti-leishmanial activity in vivo, reducing the liver amastigote load by 42% (*P* > 0.5) at a dose of 100 mg/kg/day for five days (Table 15; Page 174).

3.9 Preliminary mode of action studies

*L. donovani* promastigote forms were cultured in 24 well plates with limiting dilutions, from 25-100 μM, of Type III compound 159 for 48 hours, after which they were fixed and stained with Giemsa stain. A sublethal concentration of compound 159, (50 μM), induced aberrant non-motile forms, with displaced nuclei and absent or reduced flagella, maybe compatible with inhibition of microtubule formation or function.
Table 13 Activity of synthetic neolignan analogues and the natural neolignan (154) from *V. pavonis* against *L. donovani* promastigote forms *in vitro*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>inhibition at 100μM</th>
<th>inhibition at 30 μM</th>
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<td>138</td>
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<td>161</td>
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</tr>
<tr>
<td>162</td>
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</tr>
</tbody>
</table>

Compounds 140, 142-145 and 149 were inactive at 100 μM.

**Key**

++++ All promastigotes rounded up and non-motile after 48 hrs.
+++ All promastigotes rounded up and only slight movement visible after 48 hrs.
++ Moderate inhibition of promastigote motility after 48 hrs.
+ Slight inhibition of promastigote motility after 48 hrs.
- No inhibition of promastigote motility.
Table 14 Activity of synthetic neolignan analogues against *L. donovani* amastigote forms *in vitro*.

<table>
<thead>
<tr>
<th>compound</th>
<th>%I at 100 µM ± S.E.</th>
<th>%I at 30 µM ± S.E.</th>
<th>%I at 10µM</th>
<th>IC₅₀ (%)</th>
<th>MTC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>100 ± 0</td>
<td>46.3 ± 5</td>
<td>0</td>
<td>31.3</td>
<td>300</td>
</tr>
<tr>
<td>160</td>
<td>100 ± 0</td>
<td>70.6 ± 3.3</td>
<td>0</td>
<td>29.6</td>
<td>300</td>
</tr>
<tr>
<td>161</td>
<td>100 ± 0</td>
<td>90.2 ± 0.9</td>
<td>0</td>
<td>27.5</td>
<td>300</td>
</tr>
<tr>
<td>162</td>
<td>83.3 ± 1.5</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>27*</td>
</tr>
<tr>
<td>control</td>
<td>%I at 27* ± S.E.</td>
<td>%I at 9* ± S.E.</td>
<td>%I at 3* ± S.E.</td>
<td>5.32</td>
<td>27*</td>
</tr>
<tr>
<td></td>
<td>81.3 ± 1.5</td>
<td>77.4 ± 5.2</td>
<td>23.0 ± 3.8</td>
<td></td>
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</tbody>
</table>

control = Na-stibogluconate

* = µg Sb/ml⁻¹

MTC = maximum tolerated concentration

P95 values

159 = 54.26-46.39
160 = 29.66-25.52
161 = 26.52-21.73

Footnote: I = inhibition. IC₅₀ and P95 values were derived by linear regression, using the nanostat programme (see footnote, page 135).
Table 15  Activity of sodium stibogluconate and synthetic neolignan analogues against *L. donovani* in BALB/c mice.

<table>
<thead>
<tr>
<th>compound</th>
<th>Dose level (mg/kg/day for 5 days)</th>
<th>%I</th>
</tr>
</thead>
<tbody>
<tr>
<td>159</td>
<td>100</td>
<td>-1</td>
</tr>
<tr>
<td>160</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P &gt;0.5</em></td>
</tr>
<tr>
<td>161</td>
<td>100</td>
<td>-19</td>
</tr>
<tr>
<td>162</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Na-stibogluconate</td>
<td>45</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P &gt;0.5</em></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P &gt;0.5</em></td>
</tr>
</tbody>
</table>

Footnote: I = inhibition.
The test used five mice per group.
P values were derived by a T test.
Lignans have shown promise in areas of cancer chemotherapy (for example etoposide (166) and teniposide (167), two semi-synthetic derivatives of podophyllotoxin (163; Fig. 56; Page 161; O'Dwyer et al., 1985), which is used in the treatment of venereal warts. The neolignans kadsurenone (174; Ayres and Loike, 1990), burseran (175; Fig. 58, Page 176; Braquet and Godfroid, 1986), and veraguensin (135; Barata et al., 1978) inhibit platelet activating factor (PAF), an important mediator of inflammatory responses. Previous studies showed that surinamensin (136) and virolin (137), inhibit penetration of Schistosoma mansoni into mice (Barata et al., 1978).

In the present study, podophyllotoxin, like surinamensin, was active against L. donovani amastigote forms at 30 μM, but showed no selectivity, being toxic to macrophages at this concentration. However the semisynthetic type III neolignans 159-162 were selectively active against amastigote forms, suggesting that the C8 sulphur bond may play a part in the anti-L. donovani activity of these compounds. One of these compounds (160) was significantly active in vivo, producing a 42% reduction in amastigote liver load which suggests that the OMe groups at C-3, C-4 and C-1' of this compound may be responsible for the increase in antileishmanial activity.
None of the topoisomerase I and II inhibitors, chelators, glucose transport inhibitors or antimitotic agents showed selectivity in their action towards *L. donovani* amastigote forms. Maclurin, phloridzin and cosmetin were selectively...
toxic to macrophages while the topoisomerase I inhibitor, camptothecin, and the topoisomerase II inhibitor, ellipticine were unselectively toxic to both L. donovani amastigote forms and mammalian macrophages.

The preliminary mode of action studies mentioned previously suggested that type III semisynthetic neolignans (159-162) may be inhibiting microtubule formation or function. These compounds are more selective in their action against L. donovani amastigote forms than their neolignan natural precursor, surinamensin in much the same way as etopocide and teniposide are more selective in their action against tumour cells than their natural precursor, the 2,7’-cyclolignan-9’-olide, podophyllotoxin, which disrupts microtubules. Since microtubules are important targets in trypanosomatids, as previously mentioned, these type III neolignans could provide useful leads for studying differences between trypanosomatids and mammalian cells.
CHAPTER 4 ANTI-LEISHMANIAL ACTIVITY OF KUBBAL AZRAK

4.1 Introduction

Kubbal azrak is a black powder used traditionally in Iraq to treat cutaneous leishmaniasis. It was decided to test a sample for anti-leishmanial activity in vitro and in vivo and to isolate its active compounds by bioactivity-guided fractionation.

4.2. In vitro L. donovani promastigote form test

L. donovani promastigote forms were cultured and in vitro promastigote assays were carried out in ninety six well microplates as described in section 3.6.3.1 (Page 165). Kubbal azrak was tested in triplicate in limiting dilutions from 0.002-5 μg/ml, with 100 μl of promastigote suspension at 2 X 10^5/ml per well, and incubated at 24°C in 5% CO₂ for forty eight hours. Drug-free controls were included in the test.

4.2.1 In vitro L. donovani amastigote form test

The L. donovani amastigote form test was carried out as described in section 3.6.3.2. (Page 166). Kubbal azrak was tested in limiting dilutions from 0.125-1 μg/ml. Controls were included as described in Part 2.
4.2.2 In vivo L. major test.

A suspension was made with 200 mg of gum tragacanth and 102.9 mg kubbal azrak, mixed with 20 ml distilled water and filtered through grade 1 filter paper (Whatman). $5 \times 10^6$ *L. major* promastigotes in 0.1 ml Schneider's drosophila medium plus 15% foetal calf serum was inoculated into the shaved rump of a Balb/c mouse and 2 days later 0.14 g ointment was applied, once daily for 10 days. After 5 days, the lesion diameter was measured. The lesion diameter was measured subsequently once a week for 3 months and compared with controls.

4.3 Kubbal azrak fractionation procedure.

290 mg of sample was dissolved in methanol and the resulting solution was decanted. The remaining solid was washed repeatedly with methanol to leave 40 mg of a pale blue solid. The rest of the sample was absorbed onto 2 g of silica and fractionated by column chromatography, as described in 2.5.1.1, (Page 86) except that no pressure was applied.

The column was eluted with a chloroform:ethanol gradient starting with 9:1 chloroform:ethanol and finishing with 100% ethanol. 100 ml fractions were collected and the main purple band eluted with fractions 12-15. Fraction 17 showed an intense green colouration and the final fraction, 19,
eluted with methanol/water mixtures. Fractions were monitored by TLC and identical fractions were pooled to give 39 mg of fraction F15, 11 mg of F18, 140 mg of F19 with 40 mg of solid residue remaining.

These fractions were tested against *L. donovani* promastigote forms and against *L. donovani* amastigote forms in mouse peritoneal macrophages in vitro.

4.4 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy was carried out by S. Vandehoven, Astra Research, on a 270.05 MHz spectrophotometer in CDCl₃, with TMS as internal standard. The chemical shift values (δ) were recorded in ppm, and the coupling constant (J) as Hz. ¹H NMR, ¹³C NMR and UV spectra were carried out on fraction F15. Fraction F15 and a sample of gentian violet were run concurrently on TLC in a chloroform:methanol 80:20 system, and a chloroform:methanol 60:40 system.

4.5 Spectral Data

4.5.1 Fraction F15

¹H NMR

83.2 (18H, s, 6CH₃), 6.8 (6H, d, J=8.5 Hz, H-6, H-2, H-6', H-2', H-6'', H-2''), 7.3 (6H, d, J=8.5 Hz, H-5, H-3, H-5', H-3', H-5'', H3'') (Fig 59; Page 182).
181

$^{13}$C NMR 67.80MHz (CD$_3$OD)

$\delta$ 40.70 (6CH$_3$: 3-[N(CH$_3$)$_2$]), 113.62 (6CH: C-6, C-2, C-6', C-2', C-6'', C-2''), 128.00 (3C: C-4, C-4', C-4''), 140.82 (6CH: C-5, C-3, C-5', C-3', C-5'', C-3'''), 157.19 (3C: C-1, C-1', C-1''), 179.60 (-C) (Fig. 60; Page 183).

UV

$\lambda_{\text{max}}$ (methanol) 589.25, 587.75, 555.75, 206.50, 197.75 nm (Fig 61; Page 184).

TLC

$R_f$: 0.45 in chloroform: methanol 80:20 system and
$R_f$: 0.72 in chloroform: methanol 60:40 system.

4.5.2 Gentian Violet

$^1$H NMR

$\delta$ 3.25 (18H, s, 6CH$_3$), 6.95 (6H, d, J=8.5 Hz, H-6, H-2, H-6', H-2', H-6'', H-2''), 7.3 (6H, d, J=8.5 Hz, H-5, H-3, H-5', H-3', H5'', H3'').

$^{13}$C NMR

$\delta$ 40.70 (6CH$_3$: 3-[N(CH$_3$)$_2$]), 113.2 (6CH: C-6, C-2, C-6', C-2', C-6'', C-2''), 127.90 (3C: C-4, C-4', C-4''), 140.70 (6CH: C-5, C-3, C-5', C-3', C-5'', C-3'''), 157.20 (3C: C-1, C-1', C-1''), 180 (-C).

TLC

$R_f$: 0.45 in chloroform: methanol 80:20 system and
$R_f$: 0.72 in chloroform: methanol 60:40 system.
Fig. 60 $^{13}$C NMR spectrum of Kubbal azrak, fraction F15

Methyl (CH$_3$) carbons.

Methylene (CH$_2$) carbons.

Methine (CH) carbons.

**JEOL**

31-CCT-90 02:30:21

*Acquisition*

CBFRO 67.80 MHz
SCANS 672
ACQTH 0.901 sec
PD 3.599 sec
PW1 9.8 us
TEMP 25.0 c
SLVNT CD3OD
IRATN 140
IRRPW 100 us

*Fft*

RESOL 1.11 Hz
BF 0.56 Hz

*Plot*

XE 1918.0000 Hz
XZ 0.0000 Hz
YG 4.17
4.6 Results

Identification of Kubbal Azrak

The black powder did not dissolve completely in methanol and the methanol-soluble portion was shown to be a mixture which gave dark purple spots on TLC when viewed in daylight. One pure compound was separated by column chromatography and shown to be identical to gentian violet by UV, TLC, \(^1\)H NMR, \(^{13}\)C NMR analysis (176; Fig. 62, Page 187). The \(^1\)H NMR spectrum showed only three clear signals at 83.2, 6.8 and 7.3 and the \(^{13}\)C NMR spectrum showed five signals indicating 6CH, at 840.70, 6CH at 113.62, 6CH at 140.82. There were three quaternary carbon signals, 1, 1', 1'' at 8157.19, and three quaternary carbon signals, 4, 4' 4'' at 8128.00 and one quaternary carbon signal -C at 8179.60.

4.6.1 Results of in vitro tests

Kubbal azrak had an \(ED_{50}\) of 0.05 ± 0.003 \(\mu g\) against \(L.\) donovani promastigote forms and an \(ED_{50}\) of 0.15 ± 1.55 \(\mu g\) against \(L.\) donovani amastigotes. It was toxic to macrophages at 0.25 \(\mu g/ml\).

Fractions F15 and F18 caused total inhibition of amastigotes at 1 \(\mu g/ml\), but were also toxic to macrophages. Fraction F19 caused 52.8% inhibition at 10 \(\mu g/ml\), while the residue was inactive.
4.6.2 Results of in vivo test.

Kubbal azrak was inactive in vivo. Initially the lesion appeared to be healing, which may have been the result of the suppression of secondary bacterial infection, but subsequently there was no difference in the rate of growth of the lesion from that of the controls.

4.7 Discussion

The sample of Kubbal azrak was obtained from Iraq in the belief that it contained plant material but when examined microscopically the powder completely dissolved in chloral hydrate solution and there was no microscopic evidence for the presence of plant material. It was composed mainly of gentian violet.

Gentian violet or aniline violet \((C_{25}H_{30}ClN_3)\), a dye originally derived from coal tar, belonging to the triphenyl-methane class of dyes (Pal and Ghosh, 1994), is an effective antifungal and antiparasitic agent (Hodge et al., 1972) and has been used as an anti-bacterial agent (Rushing and Bowman, 1980). It has also been used to inhibit fungal growth in poultry feeds and to combat microbial infections in domestic animals (Allen and Meinertz, 1991).

It has been widely used in blood banks in Latin America in
an attempt to prevent transmission of T. cruzi through blood transfusions (Docampo et al. 1983). Gadelha et al. (1989) showed that the action of gentian violet against T. cruzi is the result of the uncoupling of oxidative phosphorylation in mitochondria which thus inhibits respiration. Docampo et al. (1983) showed that, in the presence of light, T. cruzi can enzymatically reduce gentian violet to a carbon-centered free radical which reacts with oxygen to form a reactive peroxyl free radical. If this light-enhanced free radical formation is the basis of the leishmanicidal action of gentian violet when applied topically to cutaneous leishmaniasis, there is a basis for its traditional use in Iraq although it is carcinogenic (Rushing and Bowman, 1980) and a non-intercalating dye which binds irreversibly to DNA in solution at \(1 \times 10^{-5} \text{ M}\) (Pal and Ghosh, 1994).

Fig. 62  Gentian violet  (176)
5.1 Introduction

Worldwide, an estimated 300 million people are infected with malaria (of which 275 million in Africa; TDR News, 1992). The number of clinical cases per year is estimated to be over 120 million (of which 110 million in Africa) and the annual number of deaths caused by malaria is estimated to be between 1.4 and 2.8 million (TDR News, 1994). In 1986 it was estimated that, worldwide, 1,600 million people were at risk of infection (Targett, 1986).

There are four species of malaria which infect humans: Plasmodium falciparum, P. vivax, P. malariae and P. ovale. P. falciparum is the most dangerous, causing the greatest mortality and morbidity (Schlesinger et al., 1988). Untreated, falciparum malaria can lead to cerebral malaria which is often fatal.

5.1.1 Life cycle of Plasmodium spp.

The life cycle of Plasmodium sp. occurs in two hosts, an insect vector (Anopheles spp. in the case of humans) and a vertebrate host.
Fig. 63  Lifecycle of a relapsing mammalian species of *Plasmodium*, (adapted from Peters, 1987)

In the mosquito

---

In the human host
The sexual phase of the cycle, sporogony, takes place in the mosquito, giving rise to sporozoites which are infective to their mammalian, avian or reptilian hosts.

When an infected mosquito takes a blood meal it introduces sporozoites into the host's blood stream. These enter the liver parenchymal cells and undergo a cycle of asexual division (pre-erythrocytic schizogony), later giving rise to merozoites which re-enter the blood stream, penetrate erythrocytes, and undergo further cycles of asexual division (erythrocytic schizogony). Some intraerythrocytic forms develop into male or female sexual stages (gametocytes) which are infective to the mosquito. When a mosquito takes a blood meal from an infected host, it may ingest gametocytes, which fuse to form zygotes in the stomach of the mosquito (Fig. 63; Page 189) thus initiating a new cycle of sporogeny.

5.1.2 Current chemotherapy

Malaria caused by chloroquine-sensitive species of Plasmodium vivax, P. malariae and P. ovale can be treated with chloroquine (177), amodiaquine (178) or quinidine (19). However, these drugs only eradicate intra-erythrocytic Plasmodium organisms so that a second drug is required for a complete cure, in order to destroy the exo-erythrocytic phase of the parasite which is sequestered in the liver. Primaquine (179) can be used for this purpose.
Unfortunately resistance of these species to chloroquine is on the increase (Rosenblatt, 1992).

Resistance of *P. falciparum* to chloroquine can now be described as occurring worldwide and there is increasing resistance to other standard antimalarials (Wernsdorfer, 1991). This makes the treatment of this type of malaria difficult. Quinine (18) or mefloquine (180) may be used to eradicate the intra-erythrocytic stage of the parasite, in conjunction with the sporontocide, pyrimethamine (181) and a sulfonamide such as sulfadoxine (Fansidar; 182), or an antibiotic such as tetracycline (183) or clindamycin (184). Artemisin (55) and its derivatives artesunate (185) and artemether (186; Fig. 64, Pages 192, 193 and 194) are now produced and marketed by a growing number of pharmaceutical companies. Side effects of quinine include cinchonism, urticaria and gastro-intestinal (GI) upset while dizziness and vomiting are common side effects of mefloquine. Allergic reactions to sulfonamides are common while tetracycline can cause GI upset and rashes from photosensitivity (Rosenblatt, 1992). In many parts of the world *P. falciparum* has developed resistance to sulfapyrimethamine combinations. Resistance to quinine and mefloquine are also on the increase (WHO 1994).

The need to develop new chemotherapeutic agents, in particular those which display novel modes of action, is pressing.
Fig. 64 Some antimalarial Drugs

(177) chloroquine

(178) amodiaquine

(179) primaquine

(180) mefloquine
Fig. 64 cont.

(181) pyrimethamine

(182) sulfadoxine

(183) tetracycline

(184) clindamycin
5.1.3 Healthcare in Ghana

Before the arrival of western medicine in Ghana traditional healers played an important role, administering plant medicines to the sick and, to a large extent, they continue to do so. A WHO-collaborating Government-funded centre, the Centre for Scientific Research into Plant Medicine, was established in 1973 at Mampong-Akuapim near Accra in order to promote scientific research related to the improvement of plant medicine so that the Government could cut down on the expensive importation of drugs. This centre liaises with the Ghana Psychic and Traditional Healers' Association and with scientific research institutions (Centre for Scientific Research into Plant Medicine, Official Leaflet, 1990).
The centre operates a regular outpatient system for the treatment of the following diseases: anaemia, arthritis, asthma, diabetes mellitus, epilepsy, hypertension, infective hepatitis, malaria, peptic ulcer, sickle cell disease, rheumatic diseases and skin diseases. All patients are diagnosed by a medical doctor and treated with plant extracts prescribed by a traditional healer working in collaboration with the medical doctor (Boye, personal communication).

The centre aims to develop appropriate technology for packaging its preparations for use throughout Ghana in order to promote the use of effective herbal remedies and make the 'Health for All by the Year 2000' programme a reality.

5.2 Cryptolepine

The stem and roots of Cryptolepis sanguinolenta (Lindl.) Schlechter (Asclepiadaceae or Periplocaceae), a scrambling shrubby plant known locally as 'nibima' or 'Ghana quinine', are used in traditional medicine in Ghana, West Africa, to treat malaria (particularly in children), fever, rheumatism and venereal disease (Boakye-Yiadom, 1979; Boye and Oku-Ampofo, 1983); in Zaire and Senegal infusions of the root are used in the treatment of stomach and intestinal disorders (Sofowora, 1982).
Cryptolepine (187) (Fig. 65; Page 197), a violet alkaloid (the salts of which are yellow), is a derived anhydro indoloquinoline base, 5-methyl quinolo [2’:3’:3:2] indole, first isolated earlier this century from the roots of C. triangularis N.E.Br. (synonymous with C. sanguinolenta) from the Congo (Clinquart, 1929).

Though concentrated in the root bark of the plant the alkaloid is also found in the leaves and stem.

In addition to cryptolepine (0.15%), quindoline (188) (quinolo [2’:3’: 3: 2] indole), has also been isolated from the plant (Raymond-Hamet, 1937; Dwuma-Badu et al., 1978; Cordell, 1981).

5.2.1 Pharmacological Properties of Cryptolepine

The pharmacological activity of cryptolepine has been demonstrated in a number of different systems:

i) Hypotensive vasodilatation effects were demonstrated in the dog (Raymond-Hamet, 1938);

ii) Carrageenin-induced oedema-reducing effects were demonstrated in the rat hind paw subplantar tissues (Bamgbose and Noamesi, 1981);

iii) Prostaglandin E₂ antagonism effects were demonstrated in the superfused, isolated, rat stomach strip preparation (Bamgbose and Noamesi, 1981) and in
the isolated rabbit duodenum (Noamesi and Bamgbose, 1984).

iv) Noradrenaline antagonism effects were demonstrated in the rat isolated vas deferens (Noamesi and Bamgbose, 1980).

v) Vasoconstrictor antagonism effects were demonstrated on isolated perfused rat mesenteric artery (Noamesi and Bamgbose, 1983a and 1983b).

Fig. 65 Structures of cryptolepine, 9-aminoacridine and quindoline (Cordell, 1981).

(187) cryptolepine \( R = CH_3 \)  
(188) quindoline \( R = H \)  
(189) 9-aminoacridine

5.2.2 Antimicrobial effects of cryptolepine

The results of extensive antimicrobial testing (Boakye-Yiadom and Heman-Ackeh, 1979; Gunatilaka et al., 1980; Boakye-Yiadom, 1983) show that the compound possesses
significant activity against several pathogenic organisms (60µl of cryptolepine hydrochloride solution, 1mg/ml v/v, produced inhibition zones between 15 mm and 30 mm on Neisseria gonorrhoeae, Escherichia coli, Staphylococcus aureus, Proteus vulgaris and the yeast Candida albicans in the agar diffusion method).

Because of the long-standing reputation of infusions of Cryptolepis root in the treatment of malaria in Ghana, it seemed appropriate to assess the activity of the purified alkaloid as a possible novel antimalarial agent. The clear structural similarities between cryptolepine (187) and 9-aminoacridine (189), which intercalates with DNA (Wright et al., 1980), suggested that the interaction of cryptolepine with DNA should also be studied.

5.3 Materials and Methods

Cryptolepine, isolated and purified from an ethanolic extract of the root bark of Cryptolepis sanguinolenta (see Dwuma-Badu, 1983) was supplied from Ghana.

5.3.1 Plasmodium falciparum culturing technique.

The multidrug-resistant K1 strain of Plasmodium falciparum was cultured in human A+ erythrocytes suspended in RPMI 1640 culture medium supplemented with D-glucose and 10% human A+ serum by a method described by Trager and Jensen, (1976)
and later modified by Fairlamb et al., (1985).

5.3.2 Test against *P. falciparum*

Vigorously growing cultures with a predominance of young ring forms were used in the determination of drug sensitivity (Ekong et al., 1990) which was based upon the microtitre technique first developed by Desjardins' group (Desjardins et al., 1979) and measures the inhibition of \(^3\)H-hypoxanthine uptake by drug-treated infected red blood cells. Compounds, including cryptolepine and 9-aminoacridine, (dissolved initially in ethanol and then diluted as necessary in serum-free culture medium) were tested in duplicate at twelve concentrations in four-fold dilutions; chloroquine diphosphate (dissolved initially in water) was tested concomitantly on each occasion as a control. The final haematocrit, parasitaemia and serum concentration were 2.5%, 1% and 10% respectively. Parasites were incubated for 24 hours in the presence of drugs before adding \([G-^3\text{H}]-\text{hypoxanthine}\) (0.02 µCi per well). After a further 18-24 hours incubation parasites were harvested onto glass fibre filters using a semi-automatic cell harvester (Skatron). Incorporation of radiolabel was determined by liquid scintillation using a Beckman LS 6000 LL scintillation counter with a counting efficiency for tritium of about 45-50%.
5.3.3 Data processing

Two sets of controls were included in each test: infected red blood cells with no drug (dpm - disintegrations per minute - equivalent to 0% inhibition or maximum growth) and uninfected red blood cells, again with no drug (dpm equivalent to 100% inhibition or total suppression of parasite growth). Computer programmes were used to convert dpm (proportional to the amount of \(^{1}\text{H}\)hypoxanthine incorporated by the parasites) for each well to percentage inhibition (relative to controls) and then to plot this as a function of the logarithm of drug concentration. Linear regression analysis (using the method of least squares) was applied to those parts of the sigmoidal curves obtained where response (percent inhibition) was dependent upon concentration; IC\(_{50}\) values (concentrations of drugs at which inhibition of parasite growth represents 50%) were derived for each drug. Data from different experiments were accumulated allowing calculation of the mean IC\(_{50}\) for each drug.

5.3.4 In vivo drug testing against \textbf{P. berghei}.

Random-bred, male, Swiss albino mice (TO strain, Eperythrozoon-free; A. Tuck and Son, Rayleigh, Essex, UK) with a mean weight of 25 g were used for in vivo drug testing.
P. berghei, strain NK65 (chloroquine-sensitive) was recovered from storage in liquid nitrogen and passaged once in uninfected 'donor' mice.

A four-day suppressive test was carried out as previously described (Peters, 1987). Infected erythrocytes, harvested from the donor mice, were injected intravenously (10⁷ per mouse) on day 0; cryptolepine was given subcutaneously in 0.2 ml sterile distilled water on days 0, 1, 2 and 3. Water alone was administered by the same route to a group of control mice. On the fourth day tail blood smears were prepared from each mouse; animals were then killed painlessly by gradual asphyxiation in carbon dioxide.

Parasitaemia was determined microscopically from the tail blood smears after fixation with methanol and staining with Giemsa stain (10% for 40 minutes). Doses of cryptolepine studied were 7.0, 14.1, 28.2, 56.3 and 112.6 mg/kg⁻¹; each dose-group and the control group contained three mice.

5.4 Studies on the interaction of cryptolepine with DNA

Cryptolepine was initially dissolved in phosphate-buffered saline (PBS; pH 7.4) to give a solution containing 3.25 μg/ml (14.0 μM). The absorption spectrum of the alkaloid was scanned between the wavelengths 300-450 nm using a Beckman DU-70 spectrometer. The quartz cuvette contained
8120 nmoles of cryptolepine in 580 μl PBS and scanning of the absorption spectrum was repeated after the addition of four successive 20μl aliquots of herring testis DNA (Sigma) in PBS, readings being made against a blank of 580 μl of the same solution of cryptolepine, with the addition of four successive 20μl aliquots of PBS. The cumulative concentrations of DNA added (as nucleotides) were 106.5, 206.1, 299.5 and 387.2 μM.

5.5 Results

5.5.1 Results of in vitro tests

In vitro tests were carried out on three separate occasions and the mean IC_{50} values for cryptolepine and chloroquine are shown in Table 16 (Page 204), together with those obtained for 9-aminoacridine (tested once only, in duplicate). The activity of cryptolepine was found to be approximately twice that of chloroquine, whilst 9-aminoacridine was markedly less active in vitro.

5.5.2 Results of in vivo tests

In control (untreated) mice mean parasitaemia on the fourth day of the test was 34.22%, with a standard deviation (SD) of 4.16%. Mean parasitaemia in the drug-treated groups is shown in Table 17 (Page 204). It is clear that, at the doses tested, cryptolepine had no significant effect upon
P. berghei infection in these mice.
Table 16 Mean IC$_{50}$ values derived for cryptolepine, chloroquine and 9-aminoacridine tested in vitro against *P. falciparum*.

<table>
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<th>µg/ml</th>
<th>µM</th>
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<tr>
<td>cryptolepine</td>
<td>0.031 ± 0.0085</td>
<td>0.134 ± 0.037</td>
<td>3</td>
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<tr>
<td>chloroquine</td>
<td>0.073 ± 0.016</td>
<td>0.230 ± 0.05</td>
<td>3</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>8.69</td>
<td>44.74</td>
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</table>

Table 17 Mean parasitaemias recorded from Giemsa-stained tail blood smears taken from control and cryptolepine-treated mice. Each group contained three animals.

<table>
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<th>Dose (mg/kg$^{-1}$/d$^{-1} \times 4$)</th>
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<tr>
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<tr>
<td>7.04</td>
<td>42.54 ± 12.03</td>
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<td>14.08</td>
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<td>40.80 ± 0.38</td>
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<td>56.32</td>
<td>46.44 ± 8.01</td>
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<tr>
<td>112.63</td>
<td>33.04 ± 10.44</td>
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</table>
Fig. 66
UV spectrum of cryptolepine
Concentration of DNA solution (μM)

A = control
B = 106.47
C = 206.07
D = 299.43
E = 387.16

optical density

Fig. 67: Changes in the UV spectrum of cryptolepine on addition of DNA solution at pH 7.4
Hypochromic effect of DNA on optical density of cryptolepine.
5.5.3 Results of the studies on the interaction of cryptolepine with DNA

The addition of DNA to cryptolepine caused a hypochromic shift in the peak absorbance (367.5 nm) of the pure alkaloid (Fig. 66; Page 205). As increasing amounts of herring testis DNA were added, a progressive bathochromic shift occurred in the peak of the absorbance spectrum to 376.25 nm (Fig. 67; Page 206). An isosbestic point (the wavelength at which the absorptivity of two or more compounds is identical, or the wavelength at which the absorption spectra of two or more compounds intersect when the absorbances are measured from solutions of equimolar concentration) was found to occur at approximately 375 nm (Fig. 68; Page 207).

5.6 Discussion

The mean IC50 value of cryptolepine, an indoloquinoline alkaloid isolated from the Ghanaian medicinal plant Cryptolepis sanguinolenta, against a multidrug-resistant strain of Plasmodium falciparum (K1) was approximately half that of chloroquine, in vitro. In view of this high degree of activity in vitro and the long-standing reputation of the plant in the treatment of malaria in West African countries such as Ghana, it was surprising that the isolated alkaloid proved to be inactive in the mouse-P. berghei model. This does correlate, however, with earlier
reports of the inactivity of the crude extract of root bark of the plant when tested against *P. berghei* in the mouse model (Boye and Oku-Ampofo, 1983).

Other drugs which have demonstrated a similar marked in vitro activity without significant activity in vivo include phaeanthine, a bisbenzylisoquinoline alkaloid isolated from *Triclisia patens*, a traditional antimalarial plant from Sierra Leone (Ekong et al., 1991) and the synthetic antihistaminic drug terfenadine (Kirby and Warhurst, 1992).

In a clinical trial at the Centre for Scientific Research into Plant Medicine, Mampong-Akwapim, Ghana an aqueous decoction of *C. sanguinolenta* root was used to treat malaria, apparently with great success (Boye and Oku-Ampofo, 1983). The results of this trial must, however, be treated with considerable caution since the report does not describe what, if any, controls were carried out. The study was performed in an area of endemicity where many individuals might be expected to display some degree of immunity to malaria.

Stemming from the apparent structural similarities shared between cryptoplepine and 9-aminoacridine, the simple experiments using herring testis DNA confirm the hypothesis that the alkaloid might interact with DNA. These experiments do not, of course, indicate whether the higher
degree of antiplasmodial activity of cryptolepine, compared with that of 9-aminoacridine, is due to differences in the effectiveness of binding of the two agents to DNA or whether it results from factors such as permeation of the two different compounds to their site of action.

Synthesis of many plant derived compounds is often difficult or impractical; cryptolepine, however, may be readily synthesised by a number of methods from simple starting materials such as O-nitrobenzyl malonic ester, isatinic acid and indoxyl and O, N-diacetyl indoxyl with isatin (Ablordeppey, 1983), which makes it attractive as a potential antimalarial compound. Because cryptolepine does show significant activity against *Plasmodium falciparum*, *in vitro*, it is suggested that additional *in vivo* models must be tested for the proper evaluation of this and other traditional medicines which show activity *in vitro* but not in the customary mouse model. Studies on appropriate methods of formulation are also clearly desirable.

These results do, however, suggest that there is some rational basis to the use in the traditional medicine of Ghana of the plant from which cryptolepine is derived. It may be that plant-derived medicines such as those prepared from *Cryptolepis* are indeed effective in the treatment of clinical malaria locally, where the benefits to be obtained from their use may stem from properties other than or additional to plasmodicidal activity.
The urgent need for new anti-protozoal drugs has been outlined in the introductions to Chapters 2, 3 and 5. The discovery of new drugs for the treatment of diseases caused by tropical protozoan parasites has been slow, especially in the case of African trypanosomiasis, Chagas' disease and leishmaniasis, where most of the drugs in use today were developed in the first half of this century. Traditional plant medicine remains a relatively unexplored source of potential therapeutic agents, despite recommendations by the WHO in 1978 that medicinal plants used in the treatment of diseases in the developing world should be assessed for efficacy and safety (Akerele, 1991).

The paucity of research into traditional plant remedies for diseases caused by trypanosomatids is due to lack of funding. The worsening economies of the countries where these diseases are endemic and competing health priorities leave meagre resources available for research into plant medicine, and in the developed world research into the chemotherapy of diseases which primarily affect the developing world is given a low priority.

This work investigated the antiprotozoal effects of selected plants from West Africa, three of which, *Alstonia boonei*, *Alchornea cordifolia* and *Annona senegalensis* (crude extracts) had low activity against *T. b. brucei* procyclic
forms but were inactive against *L. donovani* promastigote and *T. cruzi* epimastigote forms. These three African indigenous plants thus show a selectivity in their action against *T. b. brucei*, a uniquely African parasite, possibly due to metabolic or biochemical differences between the trypanosomatids. The traditional plant remedies *Lonchocarpus cyanensis* roots and *Tamarindus indica* roots, are ineffectual in eradicating the causative organisms of sleeping sickness in vitro.

The major compound present in *A. boonei* is the indole alkaloid, echitamine which, since it is water soluble, is therefore likely to contribute to activity of the crude aqueous extract. However, several non-alkaloidal semipurified mixtures, possibly containing terpenoids, in addition to three other alkaloids, were also isolated from this plant, all of which had low anti-*T. b. brucei* activity. It would therefore appear that the activity of *A. boonei* bark is due to the combined effects of a number of different compounds, including alkaloids.

The low degree of activity of both the crude extract and the pure compounds isolated preclude the potential development of *A. boonei* alkaloids as a novel drugs for the treatment of African sleeping sickness. However, there is a rational basis for its use as a febrifuge, in view of the known pharmacological activity of echitamine, the main alkaloid. There is a danger, in the case of African
trypanosomiasis, that symptoms, such as fever, may be treated, using ineffectual plant remedies, such as those tested in the present work, when the effective treatment of the disease depends on its early diagnosis and treatment with an effective drug. This further highlights the need to test plant remedies which may be used to treat life-threatening diseases, and since 80% of the world's population relies on plant medicine to treat protozoal diseases, it is essential that the remedies used be assessed for their efficacy and safety.

There remains an urgent need for new, safe drugs for the treatment of African sleeping sickness. The usefulness of a simple in vitro test for screening crude plant extracts against *T. b. brucei* procyclic forms was demonstrated in the present work and this test was also used to guide the fractionation of *A. boonei*. Four alkaloids with anti-*T. b. brucei* activity were isolated, two of which had not previously been isolated from *A. boonei*. The pure compounds were tested against *T. b. brucei* bloodstream forms in vitro, and were again found to have a low activity. Although there are biochemical differences between the two stages of the parasites, results of the tests were comparable.

The in vitro anti-*T. b. brucei* procyclic form and bloodstream form tests provide rapid screens for potential plant extracts and isolated compounds for anti-*T. b. brucei*
activity. Promising lead compounds can then be tested in vivo in the rat model. Further studies need to be carried out on a wider range of medicinal plants in order to discover new potential trypanocidal agents.

Research into plant products as anti-leishmanial agents in South America has been outlined in Chapter 1, section 1.2.4. Ethnopharmacological studies of plants used by native peoples in the treatment of leishmaniasis continue in South America, where more funding is available for plant product research than in Africa. The present study did not undertake a similar survey but rather concentrated on a comparative study of a series of semi-synthetic neolignans based on the structure of a bioactive plant compound, originally derived from the Amazonian tree, *Virola surinamensis*, in order to study the structure-activity relationship of the series.

The *L. donovani* amastigote form test was used to assess the activity and toxicity of these analogues and the *L. donovani* promastigote form test was used for comparative purposes. It was found that type III neolignans with a C-8 sulphur bond were the most selective anti-leishmanial agents *in vitro*, and of these, the compound with the greatest activity *in vivo* (160) had OMe substitutions at C-3 and C-4. Interestingly, compounds with the greatest selective activity in vitro, were inactive in vivo, only differing in structure from compound 160 at C-1' (compound
Preliminary mode of action studies indicated that these compounds may be interfering with microtubule formation or function. Microtubules are important targets for chemotherapeutic agents in parasites such as trypanosomatids.

Further studies to elucidate the mode of action of the type III neolignans would provide useful information regarding the parasite target of these compounds and types of structures which can be used selectively against this target, in order to design new anti-leishmanial drugs.

The present study also investigated an anti-leishmanial Iraqi traditional remedy kubbal azrak. This did not contain plant material. Traditional medicine, though primarily based on plant remedies, also makes use of non-plant components. For example, up until the end of the last century several remedies derived from non-plant material were listed in the American Materia Medica, such as bromine, gold and sodium chlorides, phosphorous, copper sulphate, mercury, potassium permanganate, sulphur and arsenic (Ellingwood, 1985). When traditional remedies have been ground into a powder, it is difficult to tell what the constituent components are but it is important to investigate such remedies in order assess their safety and efficacy.
Anti-malarial plant research has been outlined in Chapter 1, section 1.2.1. Throughout the world, studies continue to be carried out on plants used traditionally to treat malaria. The present work concentrated on one plant, Cryptolepis sanguinolenta, which is of significance in Ghana, where a standardized aqueous extract has been used routinely in the treatment of malaria since 1983, when the decision was taken at the International Seminar on Cryptolepine to promote the use of this plant remedy on a large scale in all health care institutions in the sub-region (Ocran, 1983).

The promising results of the in vitro tests of the active constituent of C. sanguinolenta, cryptolepine, in the present study indicate that there is a rational basis to the use of decoctions of this plant in Ghana, although the results of the in vivo test were discouraging. Mode of action studies indicated that cryptolepine might intercalate with DNA.

In the developing world the majority of people cannot afford to buy pharmaceutical products and rely on plant medicine for the treatment of illness. It is urgent therefore that traditional remedies based on plants growing in these countries be thoroughly investigated.

Sharp reductions in subsidies for health care have occurred throughout the developing world as a result of the
imposition of the World Bank's Structural Adjustment Programmes (George and Sabelli, 1994). Health care provision in rural areas has declined as a result so that reliance on traditional remedies is likely to increase rather than to decrease in the near future, thus putting pressure on a dwindling resource. Deforestation makes the identification of plants which are effective medicinal agents even more urgent, in order that these plants may be cultivated.

On a more optimistic note, promising plant products for the future treatment of protozoal diseases include licochalcone A, a flavonoid isolated from the roots of Chinese licorice, which protects mice and hamsters from infections with *L. major* and *L. donovani*, and might provide a lead for the development of a new class of antileishmanial drugs (Chen et al., 1994); while potential new agents for the treatment of malaria include analogues of artemisinin such as arteflene, which is already in phase II trials at Hoffman-La Roche, Basel, Switzerland (Posner et al., 1994).
Table 18  $^{13}$C NMR assignments of echitamine (1) (Keawpradub et al., 1994), echitamidine (2) (Keawpradub et al., 1994) 12-methoxyechitamidine (3) (Oguakwa et al., 1983) and undulifoline (4) (Massiot et al., 1992).

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2 Spectral data for echitamine (Keawpradub et al., 1994)

UV $\lambda_{\text{max}}$ (methanol) nm: 294, 236, 207 EIMS m/z (re. int.): 384 [M]+ of Hofmann base (40), 252 (13), 232 (34, 194 (15), 152 (39), 58 (100). $^1$H NMR (500 MHz, DMSO-d$_6$): 8.774 (1H, dd, J=7.6, 1.0 Hz, H-9), 7.61 (1H, brs, MH), 7.10 (1H, td, J=7.6, 1.0 Hz, H-11), 6.75 (1H, td, J=7.6, 1.0 Hz, H-10), 6.73 (1H, dd, J=7.6, 1.0 Hz, H-12), 5.73 (1H, q, J=6.4 Hz, H-19), 4.42 (1H, brd, J=14.9 Hz, H-21a), 4.36 (1H, dd, J=10.6, 5.5 Hz, H-3), 4.25 (1H, d, J=14.9 Hz, H-21b), 3.86 (1H, d, J= 4.7 Hz, H-15), 3.74 (1H, d, J=10.2 Hz, H-17), 3.73 (3H, s, OMe), 3.63 (1H, dd, J=12.7, 8.5 Hz, H-5), 3.37 (1H, m H-5), 3.29 (3H, s, N-Me), 3.16 (1H, d, J=10.2 Hz, H-17), 2.59 (1H, ddd, J=15.0, 10.6, 5.5 Hz, H-14β), 2.24 (1H, dt, J= 14.2, 8.5, Hz, H-6), 2.02 (1H, dd, J=14.2, 8.4 Hz, H-6), 1.79 (3H, dd, J=6.4, 1.5 Hz, H-18), 1.52 (1H, ddd, J=15.0, 5.9, 1.0 Hz, H-14α).

3 Spectral data for undulifoline (Massiot et al., 1992)

UV $\lambda_{\text{max}}$ (methanol) nm: 218, 282, 290, EIMS m/z (rel. int.): 340 [M]+, 80, calc. for $\text{C}_{26}\text{H}_{34}\text{N}_{10}\text{O}_3$: 340.1785, 309 (2), 283 (30), 281 (15), 251 (20), 238 (75), 180 (30), 167 (25), 96 (20), 70 (20), 58 (100); $^1$H NMR (CDCl$_3$, 300 MHz) δ: 8.3 (1H, brs, NH), 7.55 (1H, d, J=8 Hz, H-12), 7.36 (1H, d, J=8Hz, H-9), 7.2-7.07 (2H, m, H-10, H-11), 4.18 (1H, d, J=11.8 Hz, H-17), 3.95 (1H, brd, J=2.6 Hz, H-21), 3.89 (1H, d, J=11.8 Hz, H-17), 3.79 (3H, s, OMe), 3.72 (1H, dt, J=12.6, 3.6 Hz,
H-18), 3.5 (1H, brt, J=12.6 Hz, H-18), 2.78 (1H, brq, J=3.3 Hz, H-15), 2.73 (1H, m, H-20), 2.45 (1H, m, H-3), 2.3 (3H, s, NMe), 2.12-1.91 (3H, m, H-3, H-14, H-19), 1.65 (1H, m, H-14), 1.35 (1H, dddd, J=3.5, 5.1, 11.8, 15 Hz, H-19); 13C NMR see table 18.

4 Spectral data for echitamidine (Keawpradub et al., 1994)

λmax (methanol) nm: 330, 296, 235) and the major peaks in the EIMS (Fig. 36), 1H NMR (Fig. 37) and 13C NMR (Fig. 38) spectra correspond to those of echitamidine (111, Fig. 30; Keawpradub et al., 1994): EIMS m/z (rel. int.): 340 [M]+ (19), 296 (5), 241 (100), 225 (11), 180 (33), 139 (10), 105 (3). 1H NMR (500 MHz, CDCl3): δ 8.64 (1H, brs, NH), 7.19 (1H, brd, J=7.6 Hz, H-9), 7.15 (1H, td, J=7.6, 1.0 Hz, H-11), 6.93 (1H, td, J=7.6, 1.0 Hz, H-10), 6.85 (1H, brd, J=7.6 Hz, H-12) 3.91 (1H, brs, H-3), 3.88 (3H, s, OMe), 3.33 (1H, brd, J=1.7 Hz, H-15), 3.27 (1H, dq, J=11.8, 6.1 Hz, H-19), 3.10 (1H, m, H-5), 2.91 (1H, dd, J=11.4, 4.3 Hz, H-21), 2.87 (1H, dd, J=13.0, 1.6 Hz, H-5), 2.82 (1H, m, H-6), 2.04 (1H, ddd, J=13.0, 3.0, 1.8 Hz, H-14), 1.94 (1H, brt, J=11.4 Hz, H-21), 1.86 (1H, m, H-6), 1.77 (1H, m, H-20), 1.42 (1H, ddd, J=13.0, 3.0, 2.0 Hz, H-14), 1.16 (3H, d, J=6.1 Hz, H-18). 13C NMR see table 18.

5 Spectral data for 12-methoxyechitamidine (Oguakwa et al., 1983)
EIMS, m/z (%): 370 [M]+, 40, 271 (100), 258 (17), 256 (20).

$^1$H NMR, δ: 1.14 (3H, d, J=7 Hz, Me-18), 3.90 (6H, s, COOMe and OMe arom), 6.7-7.0 (3H, m, H-9, H-10 and H-11), 8.56 (1H, s, exchangeable with D$_2$O, NH). It is therefore suggested that the main component of fraction f is echitamidine and the minor component is 12-methoxyechitamidine.

Spectral data for N$_b$-demethyl echitamine (Boonchuay and Court, 1976)

N$_b$-demethyl echitamine has a UV spectrum of 302, 243 and 215 nm, MS m/z (%) 370 (M$^+$ 100), 353 (84), 326 (18), 267 (9), 249 (8), 214 (6), 207 (6), 180 (9), 171 (11), 157 (24), $^1$H NMR: δ, 1.5 (3H, d, J=6.5 Hz), 3.2 (1-2H, br. s), 3.85 (3H, s), 5.5 (1H, q, J=6.5 Hz), 6.4-7.7 (5H, m)
REFERENCES


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pharmacology of natural products series, Cambridge University Press; 103-104.


Leishmania donovani. Biochemical Pharmacol. 29; 2397-2398.


Trypanosoma brucei, T. rhodesiense and T. gambiense.
J. Protozool., 28; 470-479.

Brun, R., Grootenhuis, J.G., Kunz, C. and Schönenberger, M.


Plant-derived alkaloids active against *Trypanosoma cruzi*. J. Ethnopharmacol. 19; 89-94.


De Clercq, E., Descamps, J., Verhelst, G., Walker, R.T.,


(-)Di-de-O-methylgrandisin, a lignan from Virola pavonis leaves. Phytochem. 30; 4204-4205.


Fournet, A., Angelo, A., Muñoz, V., Roblot, F.,


George, S. and Sabelli, F. (1994). Faith and Credit. The
*Leishmania donovani*: amastigote inhibition and mode of 
action of berberine. Exper. Parasitol. 60; 404-413.

Gibson, W.C. and Miles, M.A. (1986). The karyotype and 
ploidy of *Trypanosoma cruzi*. The EMBO Journal; 1299-
1305.

Gillin, F.D. and Diamond, L.S. (1978). Clonal growth of 
*Entamoeba histolytica* and other *Entamoeba* species in 
agar. J. Protozoal. 25; 539-543.

of Malayan Medicine, Oxford University Press, Kuala 
Lumpur; 183.

Ginzburg, C.N. (1977). Medicinal Plants of Bribris and 
Cabecar Indians of Costa Rica. America Indigena, 37; 
367-398.

Goijman, S.G., Turrens, J.F., Marini-Bettolo, G.B. and 
Stoppani, A.O.M. (1985). Effect of tingenone, a 
quinonoid triterpene, on growth and macromolecule 
biosynthesis in *Trypanosoma cruzi*. Experientia, 41; 
646-648.

González, J., Sagua, H., Araya, J., Loyola, A., Morales, 
G., Pereira, J., and Estrada, DM (1990). In vitro 
activity of natural products against the 
trypomastigote form of *Trypanosoma cruzi*. Phytother. 
Res. 4; 1-4.

Gooz, T., (1990). Discovery and development of new 
antimicrobial agents. Clinical Microbiol. Reviews; 13-


the Guami Indians in Western Panama. Part I, J. Ethnopharmacol., 20; 145-171.


contraction and cholinergic nerve transmission of isolated guinea-pig ileum. Planta Med. 48; 48-51.


Patel, G., Kirby, G. C., Phillipson, J.D. and Warhurst,


Ratsimamanga-Urverg, S., Rasoanaivo, P., Rakoto-


Samuelsson, G., Farah, M.H., Claeson, P., Hagos, M.,


Schultes, R.E., (1979). The place of ethnobotany in the ethnomedicinal search for psychotomimetic drugs.


isoquinoline alkaloids II: Relation between the chemical composition and antimicrobial activity. Planta Med. 52; 556-557.


85; D.M. Minter and C.J. Schofield (eds.); 78-106.
Wright, R.G. McR., Wakelin, L.P., Fieldes, A., Acheson,


INTRODUCTION

The ever-increasing emergence, worldwide, of strains of Plasmodium falciparum resistant to chloroquine and to other standard antimalarials underlies the need to develop new chemotherapeutic agents, in particular to other standard antimalarials. The introduction of medicines for the treatment of many illnesses, including malaria, represents the sole source of those with different or novel modes of action (Peters, example, Phillipson, 1993)—the successes with the plant-derived drugs quinine and artemisinin (or, more especially its derivatives) being examples of particular importance.

The stem and roots of Cryptolepis sanguinolenta (Lindl.) Schlechter (family: Aselepiadaceae or Periploaceae), a scrambling shrub plant known locally as 'nibima', or 'Ghana quinine' are used in traditional medicine in Ghana, West Africa, to treat malaria (particularly in children), fever, rheumatism and venereal disease (Boakye-Yiadom, 1979; Boye and Oku-Ampofo, 1983; Boye and Oku-Ampofo, 1990). In the former Belgian Congo (Zaire) and in Casamance (Senegal) the stem and roots of the plant are used in the treatment of stomach and intestinal disorders (Kerharo and Adams, 1974; Sofowora, 1982). The root is also sold and used as a yellow dye-stuff amongst the Hausa of northern Nigeria and in other parts of West Africa and as far south as Angola (Dalziel, 1956).

The sap of the plant is characteristically intensely bitter and rapidly turns to dark red upon exposure to air. Cryptolepine (Fig. 1), a violet alkaloid (the salts of which are yellow), is a derived anhydro indoloquinoline base, 5-methyl quinol[2':3':3;2]indole, first isolated earlier this century from the roots of C. triangularis N.E.Br. (synonymous with C. sanguinolentia) from the Congo (Clinguart, 1929). Though concentrated in the root bark of the plant the alkaloid is also found in the leaves and stem and is also reported (Dwuma-Badu, 1983) to have been isolated from the Sri Lankan plant Sida acuta Bum. F. (family: Malvaceae), although there appears to be no locally reported use of this latter plant in the treatment of malaria (Perry and Metzger, 1981). In addition to cryptolepine (0.15%), quindoline (quinol[2':3':3;2]indole; 0.0075%) and CSA-3 (0.052%), a phenolic derivative of cryptolepine, have also been isolated from the plant (Raymond-Hamet, 1937; Dwuma-Badu et al., 1978; Cordell, 1981).

Because of the long-standing reputation of infusions of Cryptolepis root in the treatment of malaria in Ghana, as evident from the convening in 1983 of an international conference devoted to the discussion of the potential use of cryptolepine in medicine, it seemed appropriate to assess the activity of the purified alkaloid as a possible novel antimalarial agent. Apparent structural similarities between cryptolepine and 9-aminocaridine (Fig. 1), which intercalates with DNA (Wright et al., 1980), suggested that the interaction of cryptolepine with DNA should also be studied.

Cryptolepine is an indoloquinoline, high yields of which may be extracted from the roots of the West African shrub Cryptolepis sanguinolenta. The use of this plant as a traditional treatment for malaria is widespread in Ghana and is reported to be clinically effective. We have tested cryptolepine for in vitro antiplasmodial activity against the multidrug resistant (K1) strain of Plasmodium falciparum and found it to be highly active with an IC50 value of 0.031 ± 0.0085 (SE) µg/mL, equivalent to 0.134 ± 0.037 µM (n = 3). In a 4-day suppression test there was, however, no significant reduction in parasitaemia in P. berghei-infected mice treated subcutaneously with cryptolepine (7-113 mg·kg·d·X4), when compared with untreated controls. Like 9-aminocaridine, this compound appears to intercalate with DNA and this may explain the high degree of antimalarial activity demonstrated in vitro.

Keywords: cryptolepine; indoloquinolines; Cryptolepis sanguinolenta; antiplasmodial activity; Plasmodium falciparum; Plasmodium berghei; 9-aminocaridine.
Cryptolepine was cultured in human erythrocytes and purified from an ethanol extract of the root bark of *Cryptolepis sanguinolenta* (see Dwuma-Badu, 1983).

Parasite cultivation and drug testing. In these experiments the multidrug-resistant K1 strain of *Plasmodium falciparum* was cultured in human A" erythrocytes with a predominance of young ring forms were determined by microscopic examination of thin blood smears stained with Giemsa's stain (10% for 40 min). Parasites were incubated for 24 h in the presence of drugs before adding [G-^3H]-hypoxanthine (0.02 pCi per well; Amersham UK). After a further 18–24 h incubation parasites were harvested onto glass fibre filters using a semi-automatic cell harvester (Skatron). Incorporation of radiolabel was determined by liquid scintillation using a Beckman LS 6000 LL scintillation counter with a counting efficiency for tritium of about 45%–50%.

Data processing. Two sets of controls were included in each test: infected red blood cells with no drug (dpm—disintegrations per minute—equivalent to 0% inhibition or maximum growth) and uninfected red blood cells, again with no drug (dpm equivalent to 100% inhibition or total suppression of parasite growth). Computer programs were used to convert dpm (proportional to the amount of [H]-hypoxanthine incorporated by the parasites) for each well to percentage inhibition (relative to controls) and then to plot this as a function of the logarithm of drug concentration. Linear regression analysis (using the method of least squares) was applied to those parts of the sigmoidal curves obtained where response (percent inhibition) was dependent upon concentration; IC₅₀ values (concentrations at which inhibition of parasite growth represents 50%) were derived for each drug. Data from independent experiments were accumulated allowing calculation of the mean IC₅₀ for each drug.

In *vivo* drug testing. Random-bred, male, Swiss albino mice (TO strain, *Eperithrozoon*-free; A. Tuck & Son, Rayleigh, Essex, UK) with a mean weight of 25 g were maintained on breeding diet No 3 (801180 W; Special Diets Services Ltd, Whitham, Essex, UK) and water ad libitum. *P. berghei*, strain NK65 (chloroquine-sensitive), was recovered from storage in liquid nitrogen and passaged once in uninfected 'donor' mice.

A 4-day suppressive test was carried out as previously described (Petters, 1987). Infected erythrocytes, harvested from the donor mice, were injected intravenously (10⁷ per mouse) on day 0; cryptolepine was given subcutaneously in 0.2 mL sterile distilled water on days 0, 1, 2 and 3. Water alone was administered to a group of control mice. On day 4 tail blood smears were prepared from each mouse; animals were then killed painlessly by gradual asphyxiation in carbon dioxide.

Parasitaemia was determined microscopically from the tail blood smears after fixation with methanol and staining with Giemsa's stain (10% for 40 min). Doses of cryptolepine studied were 7.0, 14.1, 28.2, 56.3 and 112.6 mg/kg/d; each dose-group and the control group contained three mice.

Studies on the interaction of cryptolepine with DNA. Cryptolepine was initially dissolved in phosphate-buffered saline (PBS; pH 7.4) to give a solution containing 3.25 µg/mL (14.0 µM). The absorption spectrum of the alkaloid was scanned between the wavelengths 300–450 nm using a Beckman DU-70 spectrometer. The quartz cuvette contained 8120 nmol cryptolepine in 580 µL PBS and scanning of the absorption spectrum was repeated after the addition of four successive 20 µL aliquots of herring testis DNA (Sigma) in PBS, readings being made against a blank of 580 µL of the same solution of cryptolepine, with the addition of four successive 20 µL aliquots of PBS. The cumulative concentrations of DNA added (as nucleotides) were 106.5, 206.1, 299.5 and 387.2 µM.

RESULTS

In *vivo* screening. Tests were carried out on three separate occasions and the mean IC₅₀ values for cryptolepine and chloroquine are shown in Table 1, together with

<table>
<thead>
<tr>
<th>Drug</th>
<th>µg/mL</th>
<th>mean IC₅₀ (SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptolepine</td>
<td>0.031</td>
<td>0.134 (0.037)</td>
<td>3</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.073</td>
<td>0.230 (0.05)</td>
<td>3</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>8.69</td>
<td>44.74 (—)</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1. Mean IC₅₀ values (with standard error of the mean) derived for cryptolepine, chloroquine (tested three times each, in duplicate) and 9-aminoacridine (tested once only, in duplicate)
Table 2. Mean parasitaemias recorded from Giemsa-stained tail blood smears taken from control (non drug-treated) and cryptolepine-treated mice. Each group contained three animals.

<table>
<thead>
<tr>
<th>Dose (mg/kg × 4)</th>
<th>Mean parasitaemia %</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>34.22</td>
<td>4.16</td>
</tr>
<tr>
<td>7.04</td>
<td>42.54</td>
<td>12.03</td>
</tr>
<tr>
<td>14.08</td>
<td>38.48</td>
<td>12.79</td>
</tr>
<tr>
<td>28.16</td>
<td>40.80</td>
<td>0.38</td>
</tr>
<tr>
<td>56.32</td>
<td>46.44</td>
<td>8.01</td>
</tr>
<tr>
<td>112.63</td>
<td>33.04</td>
<td>10.44</td>
</tr>
</tbody>
</table>

those obtained for 9-aminoacridine (tested once only, in duplicate). The activity of cryptolepine was found to be approximately twice that of chloroquine, whilst 9-aminoacridine was markedly less active in vitro.

In vivo screening. In control (untreated) mice mean parasitaemia on day 4 of the test was 34.22%, with a standard deviation (SD) of 4.16%. Mean parasitaemia in the drug-treated groups is shown in Table 2. At the doses tested, cryptolepine had no significant effect upon P. berghei infection in these mice.

Studies on the interaction of cryptolepine with DNA. The addition of DNA to cryptolepine caused a hypochromic shift in the peak absorbance (367.5 nm) of the pure alkaloid (Fig. 2A); as increasing amounts of herring testis DNA were added a progressive bathochromic shift occurred in the peak of the absorbance spectrum to 376.25 nm (Fig. 2B). An isosbestic point was found to occur at approximately 375 nm (Fig. 2A).

DISCUSSION

We have determined the in vitro activity of cryptolepine, an indoloquinoline alkaloid isolated from the Ghanaian medicinal plant Cryptolepis sanguinolenta, against a multidrug-resistant strain of Plasmodium falciparum (K1) and found the mean IC₅₀ value to be approximately half that of chloroquine in the same experiments. In view of this high degree of activity in vitro and the long-standing reputation of the plant in the treatment of malaria in West African countries such as Ghana, it was surprising that the isolated alkaloid proved to be inactive in the mouse-P. berghei model. This does correlate, however, with earlier reports of the inactivity of a crude extract of root bark of the plant when tested against P. berghei in the mouse model (Boye and Oku-Ampofo, 1983; Boye and Oku-Ampofo, 1990) and we have observed that some other drugs may also show marked in vitro activity with no significant activity in vivo: for example, phaeanthine (a bisbenzylisoquinoline alkaloid isolated from Tridisa patens, a traditional antimalarial plant from Sierra Leone—Ekong et al., 1991; Marshall, 1991), the synthetic antihistaminic drug terfenadine (Kirby and Warhurst, 1992) and the synthetic fluoroquinolone ciprofloxacin. In P. berghei-infected mice we found that ciprofloxacin does not reduce parasitaemia unless large doses are used. Salmon et al. (1990) have reported that ciprofloxacin reduces parasitaemia in the mouse model if multiple large doses are used. When we tested ciprofloxacin in P. berghei-infected hamsters, however, the drug proved to be much more effective than in mice, with lower ED₅₀ and ED₉₀ values being obtained even with single daily doses (Kirby et al., 1994).

In a clinical trial in Ghana an aqueous decoction of C. sanguinolenta root was used to treat malaria, apparently with great success (Boye and Oku-Ampofo, 1983). The results of this trial must, however, be treated with caution since the report does not describe what controls were carried out and it should be borne in mind that the study was performed in an area of endemicity where many individuals might be expected to display some degree of immunity to malaria.

Stemming from the apparent structural similarities shared between cryptolepine and 9-aminoacridine, our simple experiments using herring testis DNA confirm

**Figure 2.** (A) Spectral changes occurring on addition of herring testis DNA solution to cryptolepine at pH 7.4. Four increments of 20 µL of DNA at 1 mg/mL in PBS were added to 580 µL cryptolepine solution at 3.25 µg mL. The uppermost peak is that obtained before the addition of any DNA. Readings were made against a blank of 580 µL of the same solution of cryptolepine, with the addition of four successive 20 µL aliquots of PBS. (B) Hypochromic effect of DNA upon the optical density of cryptolepine at 367.5 nm (corrected for dilution).
Figure 3. (A) Putative complex formed between 9-aminoacridine and a thymine–adenine base pair. (B) Putative complex formed by cryptolepine interacting with a thymine–adenine base pair, showing the possibility of better overlay of the alkaloidal ring systems with respect to the purine–pyrimidine pair. In both parts of the figure the double bonds of the interacting drugs have been omitted for clarity (cf Fig. 1). Nitrogen atoms are denoted by solid circles, oxygen atoms of the base pairs by open circles (oxygen atoms of the ribose sugars are not highlighted).

the hypothesis that the alkaloid might interact with DNA. We went on to construct molecular models of 9-aminoacridine and cryptolepine using HyperChem software on an IBM computer. Geometric optimization was performed using an MM^+ force field and energies of the lowest unoccupied molecular orbitals; charge distribution was computed using the AM1 semiempirical molecular orbital program. Both molecules are planar conjugated systems containing two amino groups. In 9-aminoacridine the distance between ring and side chain nitrogen atoms is 4.0408 Å whereas the distance between the two nitrogen atoms (both occurring in ring systems) in cryptolepine is only 3.4002 Å. However, the distance between the negative charges associated with the two nitrogen atoms is in both cases in the region of 4 Å since the charge of the second nitrogen atom in cryptolepine is delocalized towards the methyl group (cf Fig. 1). The probable interaction with an adenine–thymine base pair is shown in Fig. 3. The positioning of 9-aminoacridine is taken from Stenlake (1979) and the interaction of cryptolepine is based upon this. It appears that, with similar interaction possible for the two nitrogen atoms of 9-aminoacridine and the nitrogen-nitrogen-methyl of cryptolepine, there is greater possibility for formation of \( \pi \rightarrow \tau \) charge transfer complexes between the purine–pyrimidine bases and cryptolepine than with 9-aminoacridine. As an explanation of the higher antiplasmodial activity of cryptolepine, compared with 9-aminoacridine, better intercalation with DNA may not be as satisfactory as an explanation founded upon the less basic nature of the former agent, allowing easier passage into the infected erythrocyte.

The results of extensive antimicrobial testing (Boakye-Yiadom and Heman-Ackah, 1979; Gunatilaka et al., 1980; Boakye-Yiadom, 1983) show that the compound possesses significant activity against several pathogenic organisms including Neisseria gonorrhoeae, Escherichia coli, Staphylococcus aureus, Proteus vulgaris and the yeast Candida albicans.

Synthesis of many plant-derived compounds is often difficult or impractical; cryptolepine, however, may be readily synthesized by a number of methods from simple starting materials (for summary see Ablordepepy, 1983) which makes it attractive as a potential antimalarial compound. Because cryptolepine does show significant activity against *Plasmodium falciparum in vitro* it is suggested that additional *in vivo* models must be tested for the proper evaluation of this and other traditional medicines which show activity in *vitro* but not in the customary mouse model; studies on appropriate methods of formulation are also clearly desirable. Our results do, however, suggest that there is some rational basis to the use in the traditional medicine of Ghana of the plant from which cryptolepine is derived. It may be that plant-derived medicines such as those prepared from *Cryptolepis* are indeed effective in the treatment of clinical malaria locally, where the benefits to be obtained from their use may stem from properties other than or additional to plasmoidal activity.

Acknowledgements

The generous financial support of the Wellcome Trust, the Medical, the Science and Engineering Research Councils, and the Sir Halley Stewart Trust, is gratefully acknowledged; DCW is funded by the PHLS.

**References**


Dwuma-Badu, D. (1983). Review of the isolation and characteri-


