PLANTS AS SOURCES OF ANTI-PROTOZOAL COMPOUNDS

Thesis presented by

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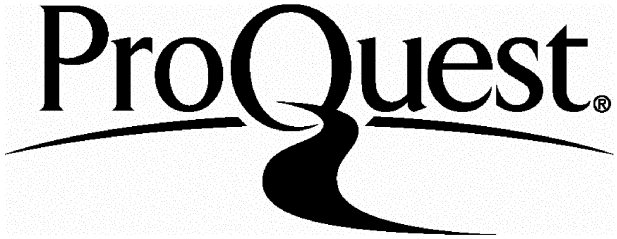

dedicated to

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, Lonchocarpus cyanensis, Tamarindus indica* and *Ximenia americana,* reputated 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 30µg/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 60-250µg/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 anti-malarial 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

indicate that cryptolepine interacts with DNA.

Contents

Aknowledgements

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.

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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*

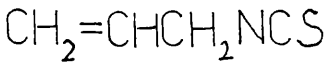
which is cultivated extensively in many parts of Asia (Samuelsson, 1992).

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

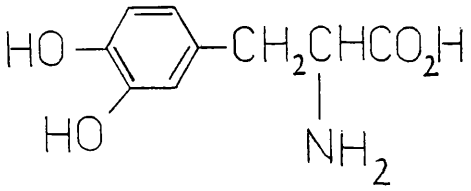
Alkaloids can be divided into two broad classes: non-heterocyclic alkaloids, (for example, ephedrine (**13**) and colchicine (**14**; Fig. 3; Page 6) and heterocyclic alkaloids, to which many important drugs belong, for example, hyoscine (**15**), atropine (**16**) and cocaine (**17**) (tropane alkaloids), 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).

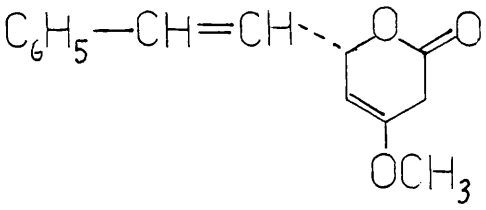
Fig. 1 Plant-derived compounds which may now be synthesized commercially.



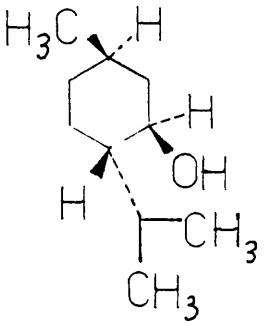
(**1**) allylisothiocyanate



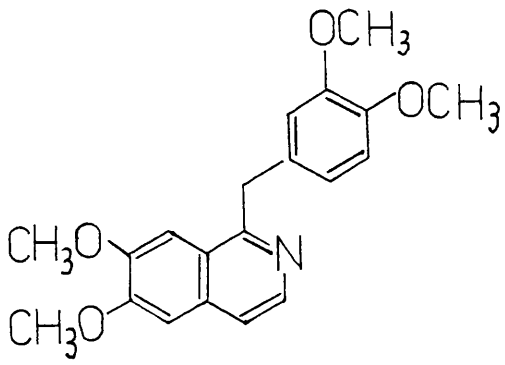
(**2**) L-dopa



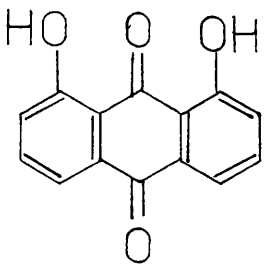
(**3**) kawain



(**4**) menthol

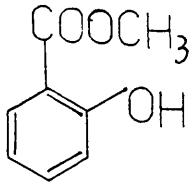


(**5**) papaverine



(**6**) danthron

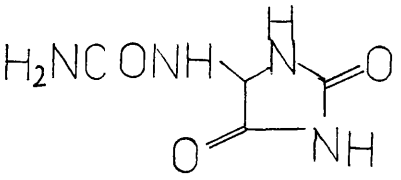
Fig. 1 continued



(7)

methyl

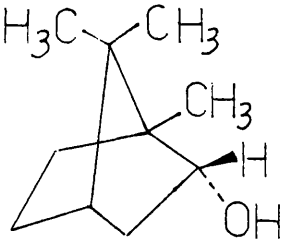
salicylate



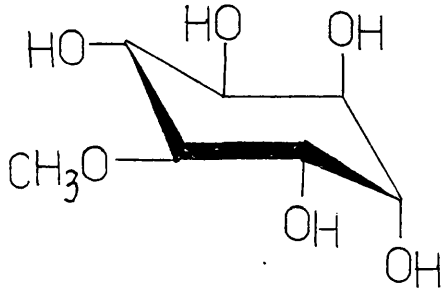
(8)

allantoin

(9) benzyl benzoate



(10) borneol



(11) pinitol

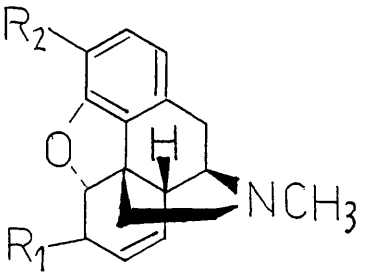
Fig 2. morphinane alkaloids

(**12**) morphine R_1 = OH

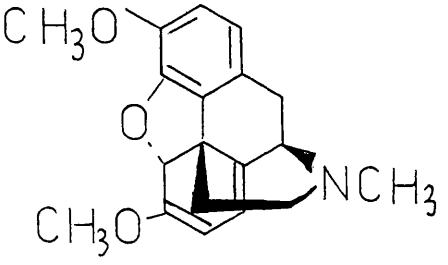
R_2 = OH

(**22**) codeine R_1 = OH

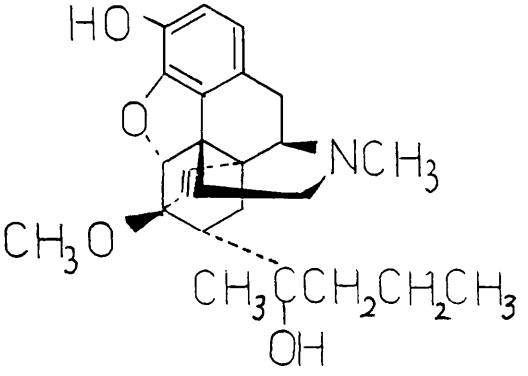
R_2 = OCH_3



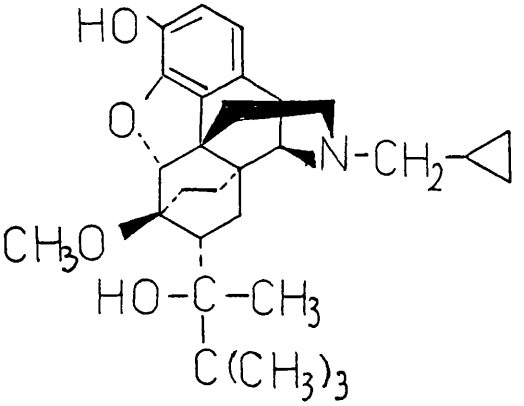
(**45**) thebaine



(**46**) etorphine



(**47**) buprenorphine



(**48**) nalbuphine

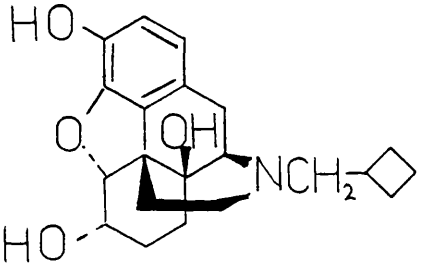
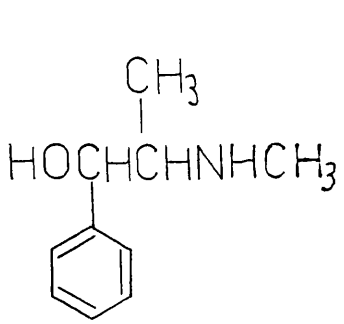
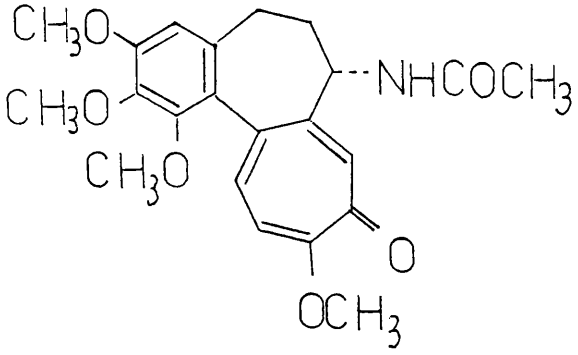


Fig. 3 Non-heterocyclic alkaloids

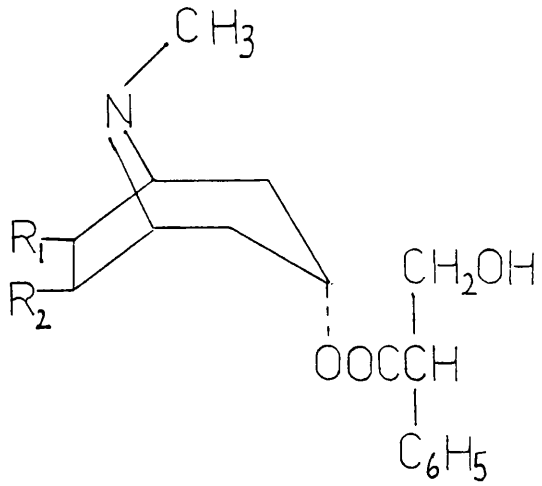


(**13**) ephedrine



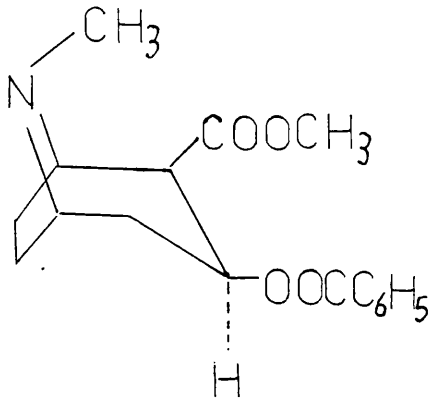
(**14**) colchicine

Fig. 4 Heterocyclic alkaloids

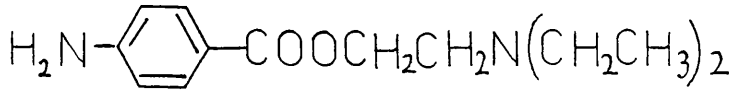


(**15**) hyoscine R_1-R_2 = -O-

(**16**) atropine R_1=R_2 = H

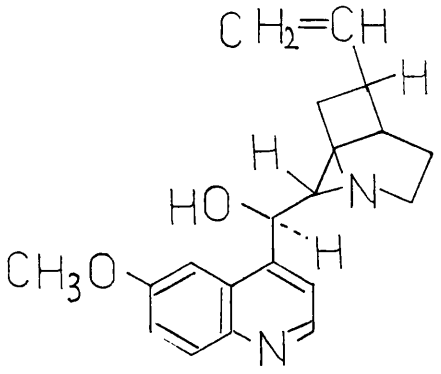


(**17**) cocaine

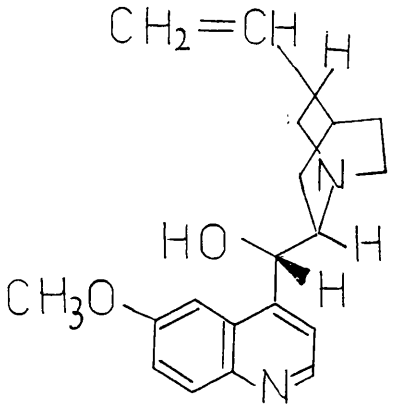


(**44**) procaine

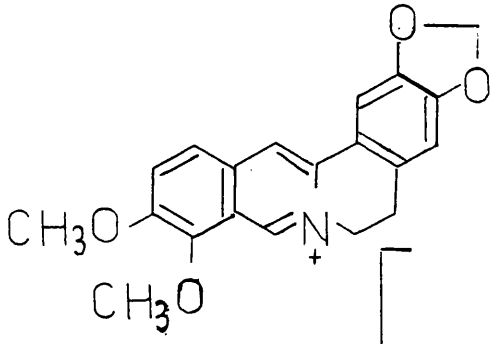
Fig. 4 continued



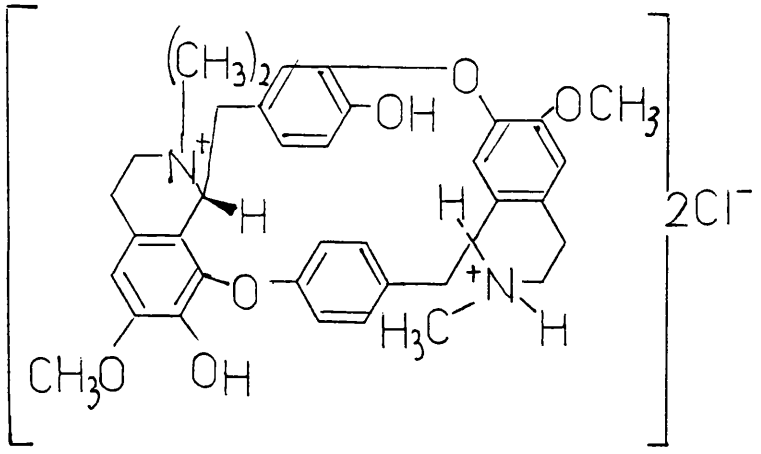
(**18**) quinine



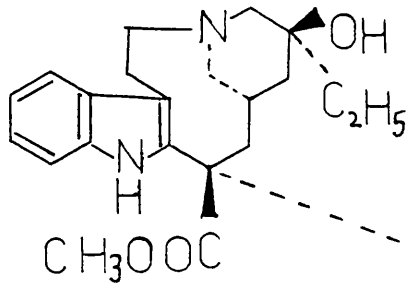
(**19**) quinidine



(**20**) berberine

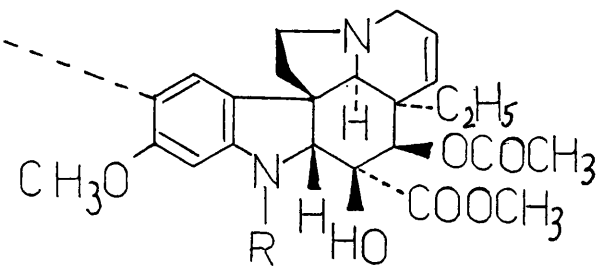


(**21**) tubocurarine chloride



(**23**) vinblastine

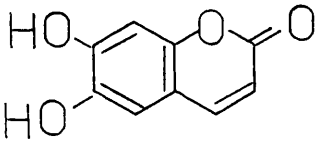
R = CH_3



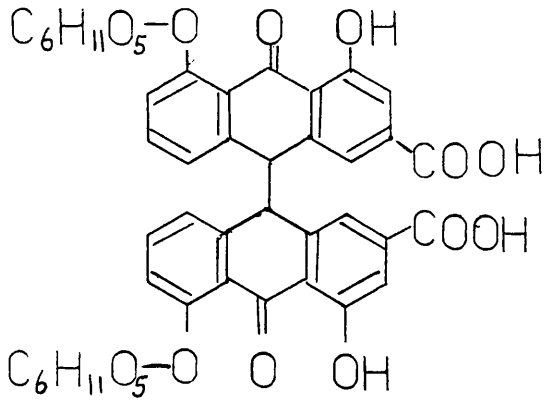
(**24**) vincristine

R = CHO

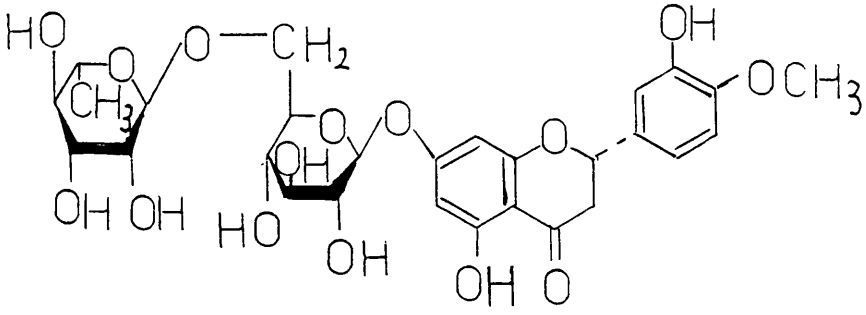
Fig. 5 Cardioactive glycosides (31 and 32), phenolic compounds (25 and 26), flavones (27 and 28), a terpene (29), a lignan (30) and a plant sterol (33).



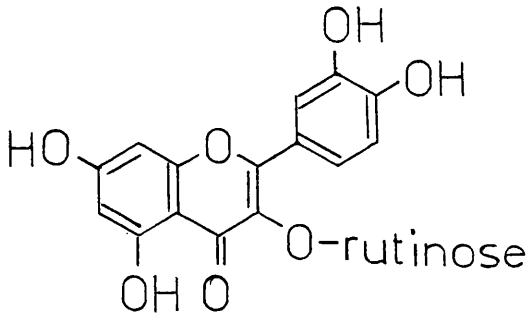
(**25**) esculetin



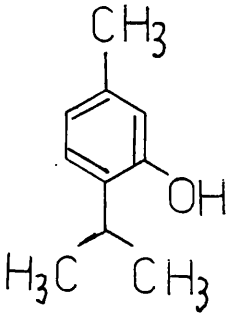
(**26**) sennoside A



(**27**) hesperidin

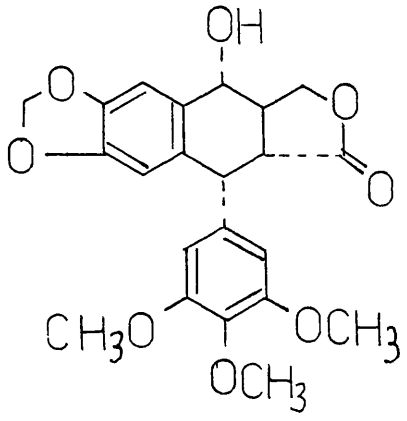


(**28**) rutin

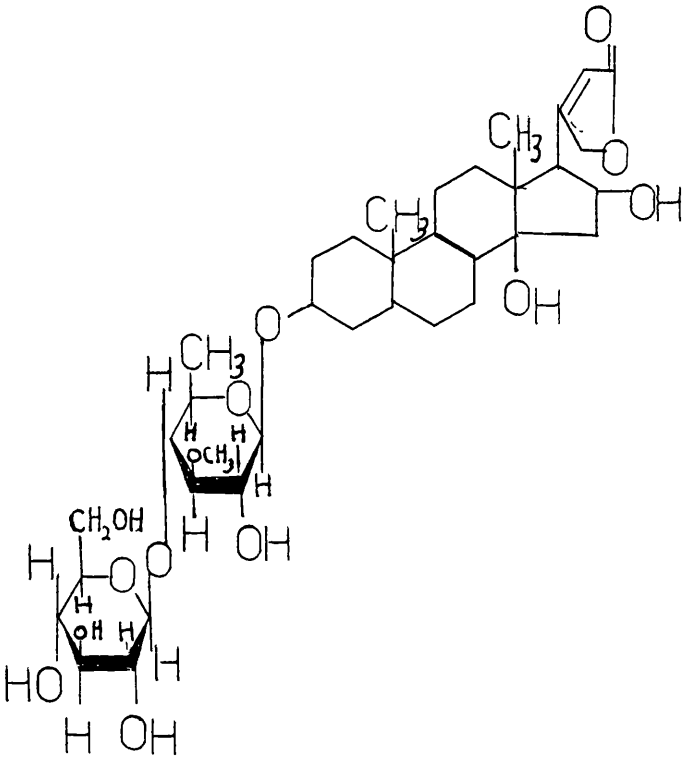


(**29**) thymol

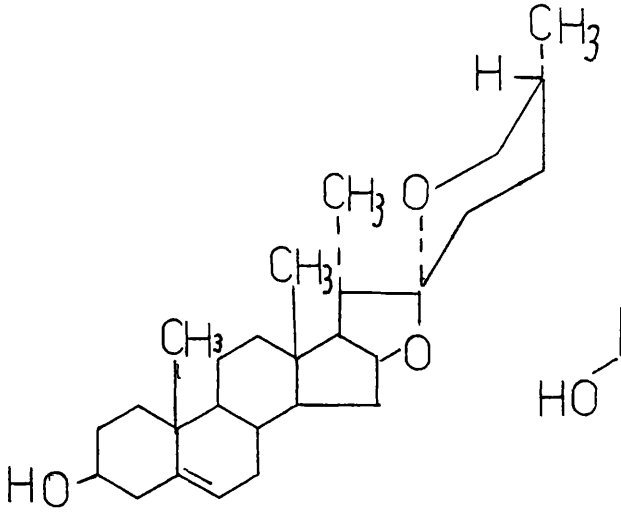
Fig. 5 continued



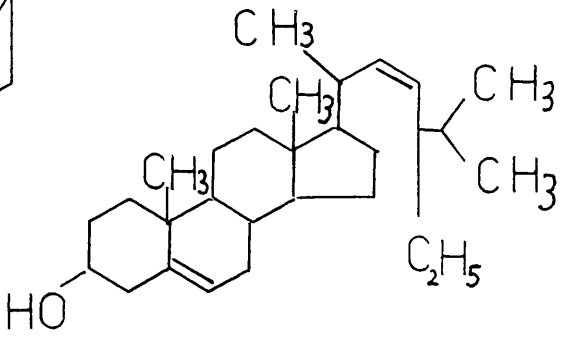
(**30**) podophyllotoxin



(**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).

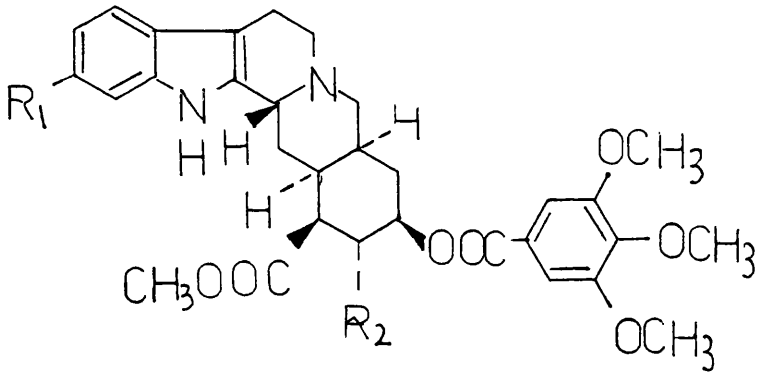
Modified active compounds include plant sterols which are used as starting materials for the partial synthesis of steroid drugs. Diosgenin (**32**) from *Dioscorea floribunda* has been used as a template for forty useful steroid drugs (Farnsworth and Soejarto, 1985) and stigmasterol (**33**),

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).

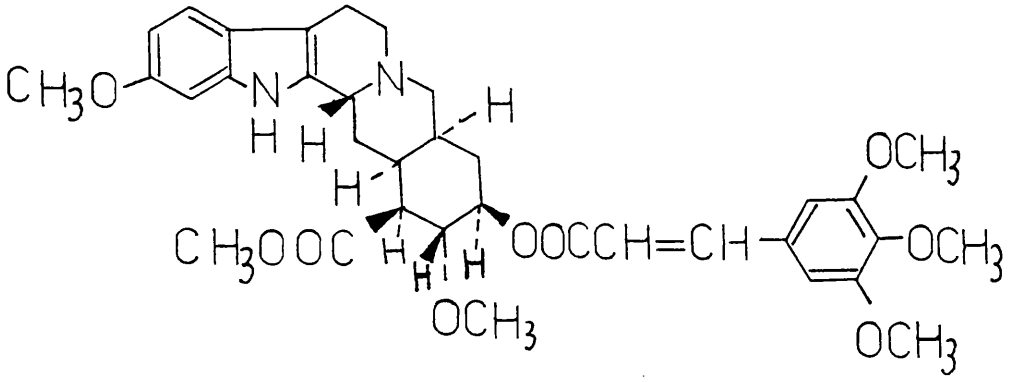
Fig. 6 **Rauwolfia alkaloids (34-38) and some plant compounds which have been used as templates for the synthesis of analogues (39-43).**



(**34**) reserpine $R_1 = CH_3O$ $R_2 = OCH_3$

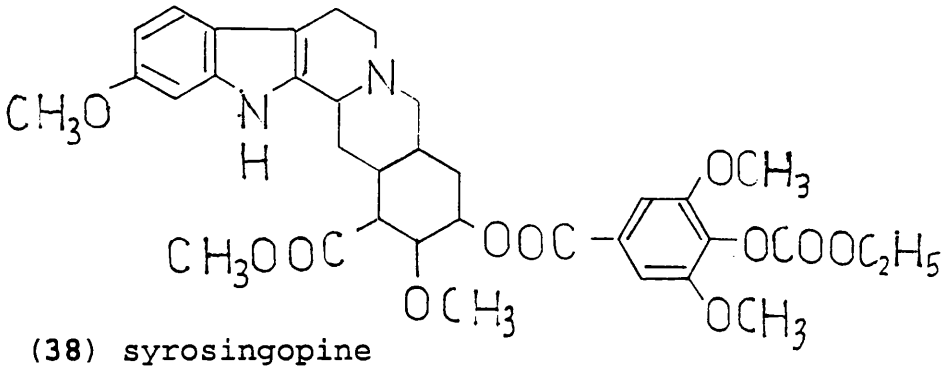
(**35**) deserpidine $R_1 = H$ $R_2 = OCH_3$

(**36**) raunescine $R_1 = H$ $R_2 = OH$

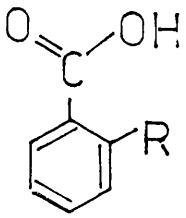


(**37**) rescinnamine

Fig. 6 continued

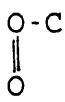


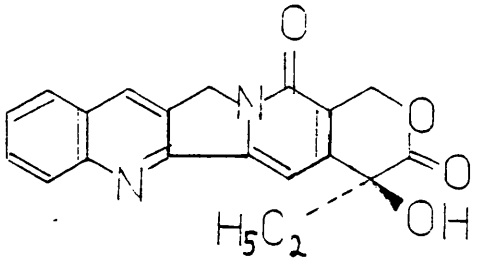
(38) syrosingopine



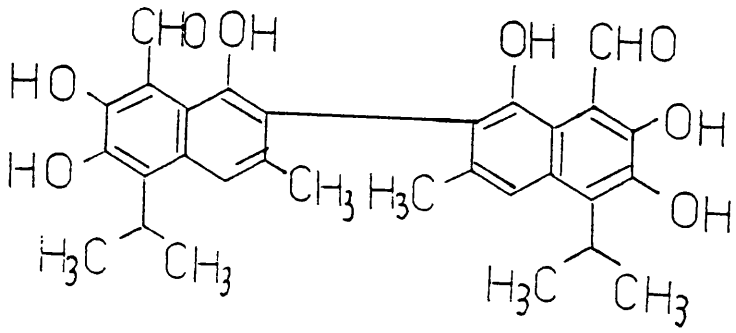
(39) salicin R = O-glucose

(40) salicylic acid R = OH

(41) acetyl salicylic acid R = 



(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 accessability 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 (Ginzbarg, 1977).

In the Caribbean a research project involving the participation of several Central American countries, in addition to Colombia, Venezeula 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,

1993).

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 (75,000km^2 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 world wide 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 gondi.*

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

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

* 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. brasiliensis* 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 lyze *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 anti-malarial (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).

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

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, Picrasma 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 traditonally 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 taditionally 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

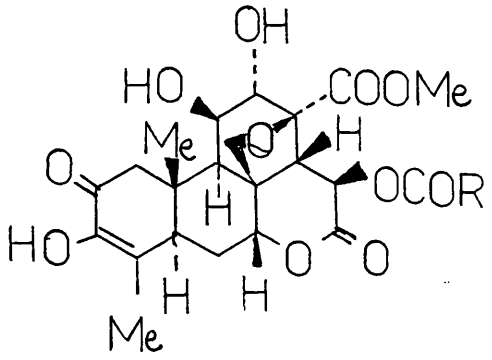
Studies have been carried out in many parts of the world on plants used traditionally to treat malaria, often screening crude extracts *in vitro* against *Plasmodium spp.* (Phillipson and Wright, 1991c; Anderson *et al.*, 1988; Ratsimamanga-Urverg *et al.*, 1991; Pavanand *et al.*, 1989) or *in vivo* (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 antimalarials 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).

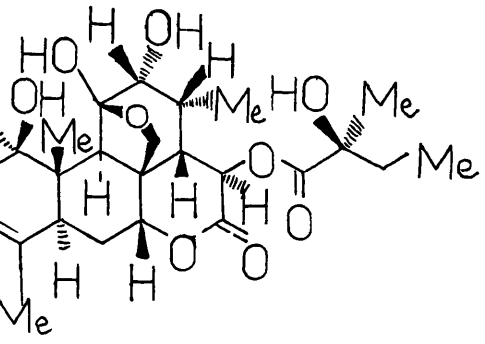
Fig. 7 Some anti-malarial terpenoids



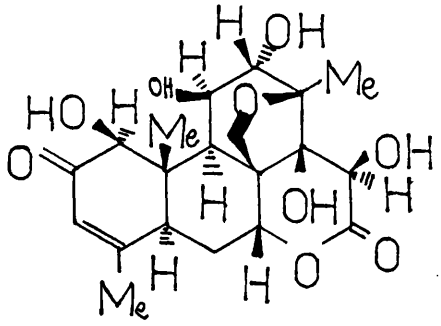
(50) bruceantin:

R = $CH{=}C(CH_3)CH(CH_3)_2$

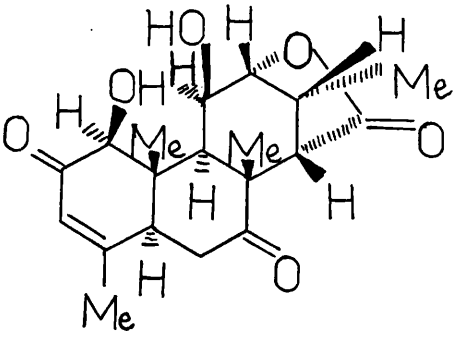
(69) bruceine A: R = $CH_2CH(CH_3)_2$



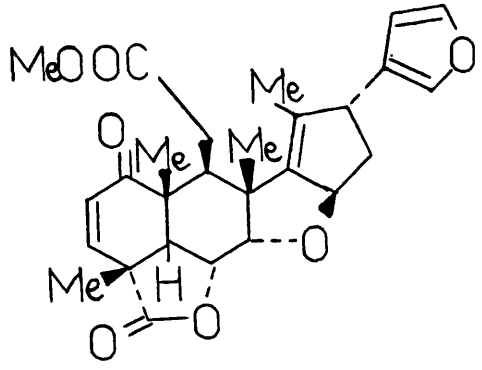
(51) glaucarubinone



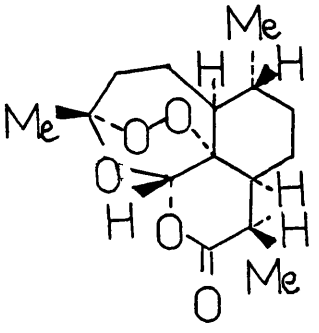
(53) bruceine D



(52) eurycomalactone

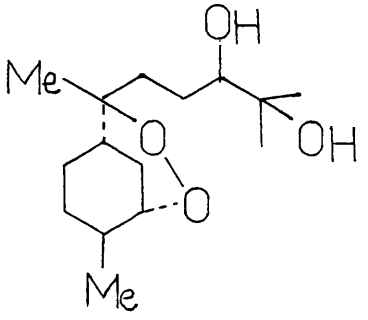


(54) nimbolide

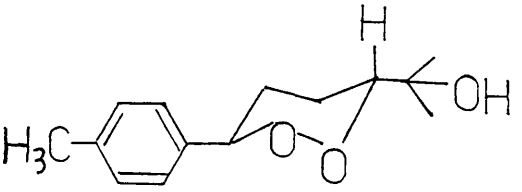


(55) artemisinin

Fig. 7 continued

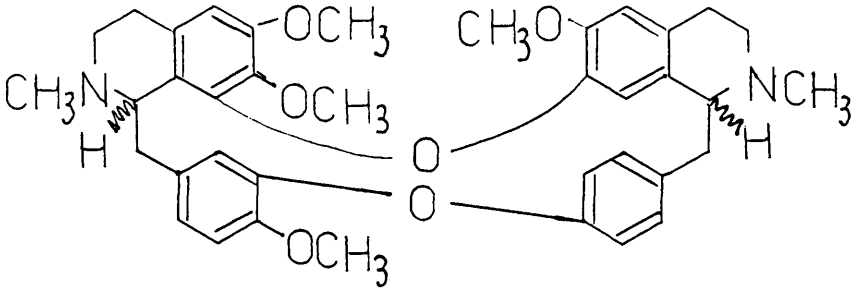


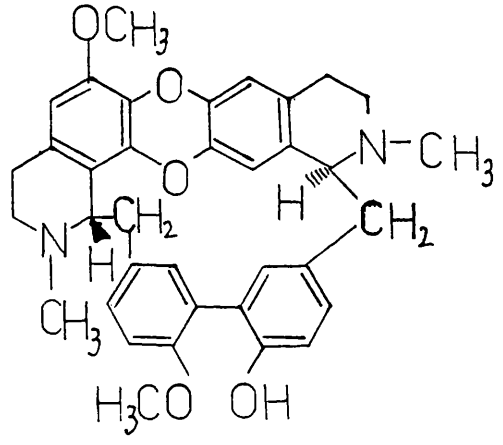
(56) yinghaosu A



(57) yinghaosu C

Fig. 8 Some anti-malarial alkaloids



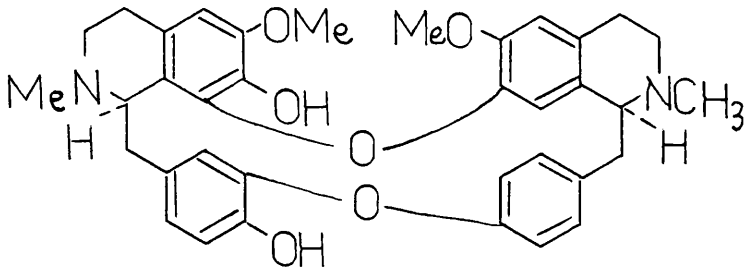


(58) tiliacorine

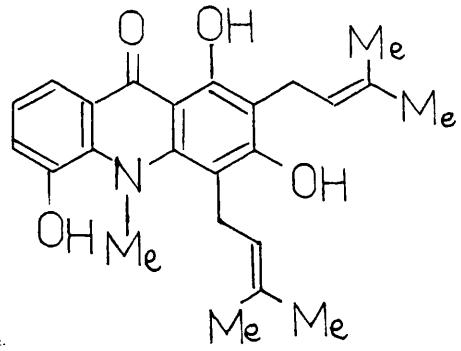
(59) phaeanthine: R, R.

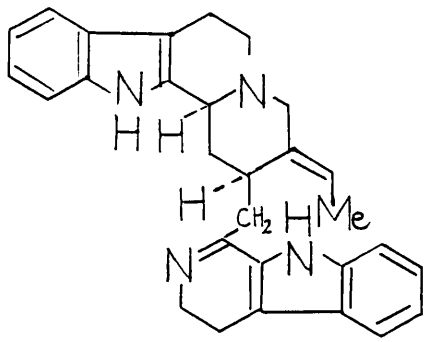
(60) isotetrandrine: R, S.

Fig. 8 continued



(**61**) obamegine





(**62**) atalaphilline (**63**) 3′,4′-dihydrousambarensine

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'dihydrousambarensine (**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

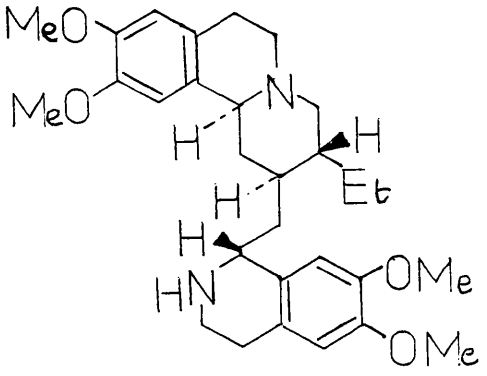
evaluated for activity against *Entamoeba histolytica* both *in vitro* and *in vivo* and the NAPRALERT data base lists 139 genera which have yielded extracts with antiamoebic effects (Phillipson and O'Neill, 1987). Anti-amoebic plant products have been reviewed by Phillipson and O'Neill (1987).

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 parallelled one another (Wright, 1989).

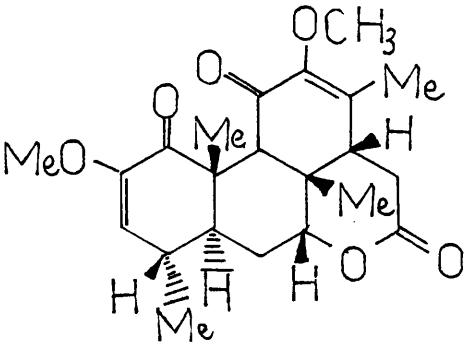
Similarly, the series of bisbenzylisoquinoline alkaloids derived from *Triclisia spp.* were also tested against both *P. falciparum* and *E. histolytica* but were less active against *E. histolytica* than against *P. falciparum*. However, isotrilobine (**65**), had antiamoebic activity similar to emetine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

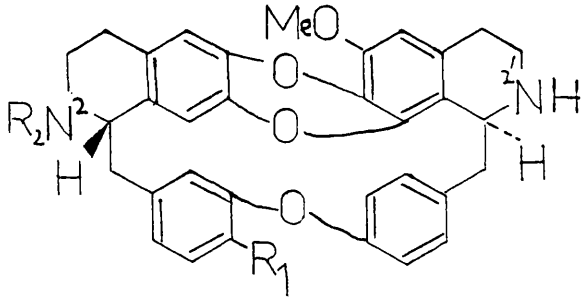
Fig. 9 **Some plant products with activity against *Entamoeba histolytica* and *Giardia lamblia*.**



(49) emetine

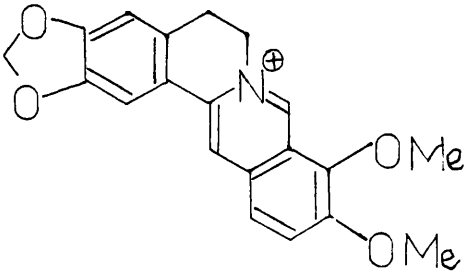


(64) quassin



(65) isotrilobine: S,S $R_1 = OCH_3$

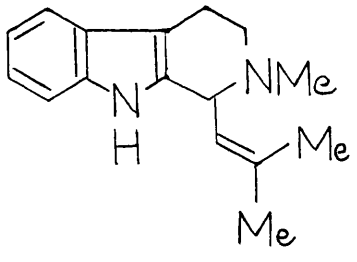
$R_2 = CH_3$



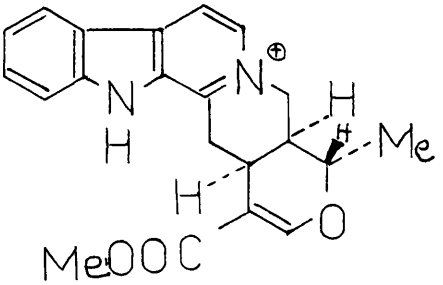
(66) berberine

(70) cocsoline: S,S $R_1 = OH$

$R_2 = H$



(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 *in vitro* and *in vivo* against several species of *Leishmania* (Ghosh *et al.*, 1985; Vennerström *et al.*, 1990) as well as against *E. histolytica* and *Giardia lamblia*, as previously mentioned.

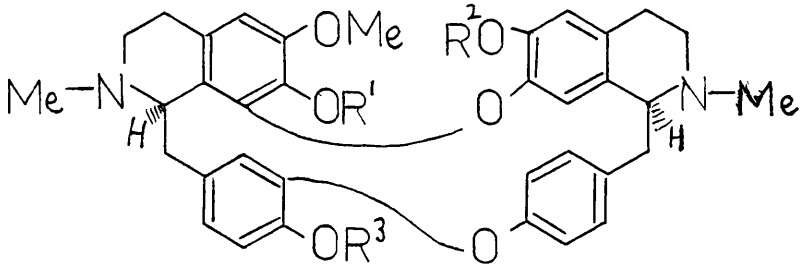
Ethnopharmacological studies carried out on plants used by native and migrant peoples in Bolivia demonstrated that a significantly higher number of plants used by native people (Chimane Indians) were active *in vitro* against *Leishmania spp.* than those used by migrants (Fournet *et al.*, 1994). Crude extracts of fifty three of these Bolivian medicinal plants were tested against *Leishmania spp.* and *T. cruzi* and active compounds were purified and identified from those extracts which were active *in vitro*. Thirty eight percent of the 26 species of Asteraceae screened were active and studies concentrating on genera from the families Annonaceae, Berberidaceae, Hernandiaceae and 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**) cocsoline (**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.

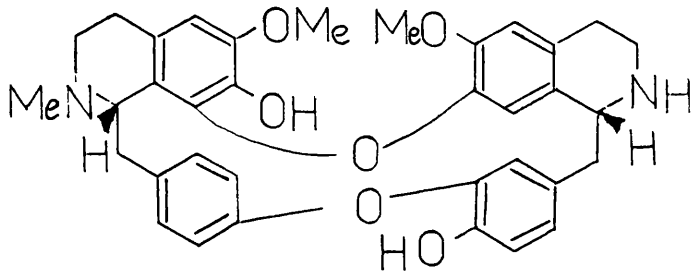
Fig. 10 Bisbenzylisoquinoline alkaloids with activity against *Leishmania spp.* and *Trypanosoma cruzi*.



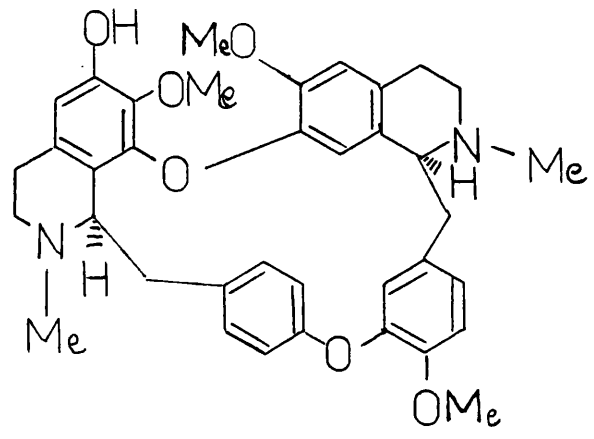
(71) berbamine $R^1=R^2=Me$, $R^3=H$, 1′S

(72) krukovine $R^1=R^3=H$, $R^2=Me$, 1R,1′R

(73) limacine $R^1=H$, $R^2=R^3=Me$, 1R, 1′R

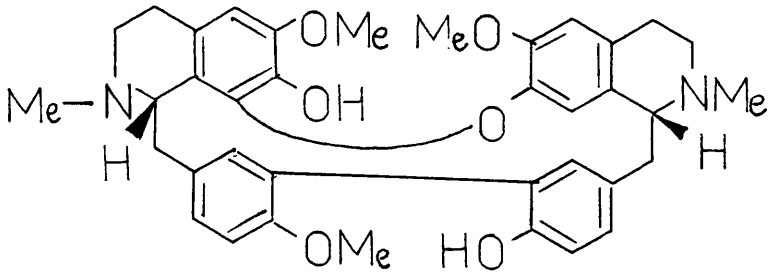


(74) daphnoline

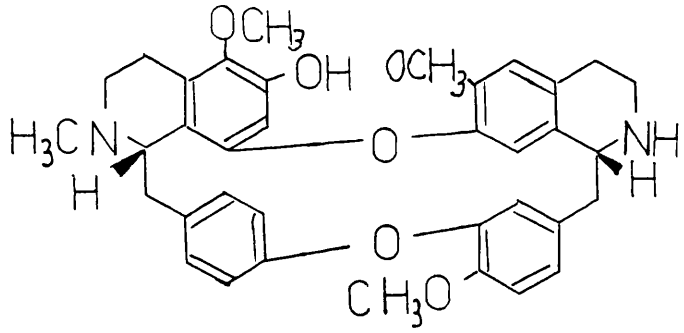


(75) gyrocarpine

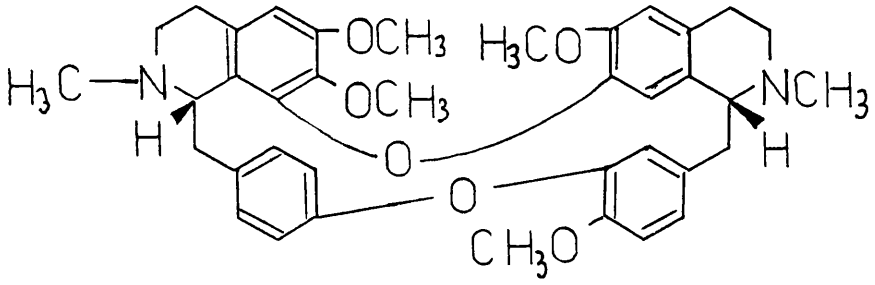
Fig. 10 cont.



(76) antioquine

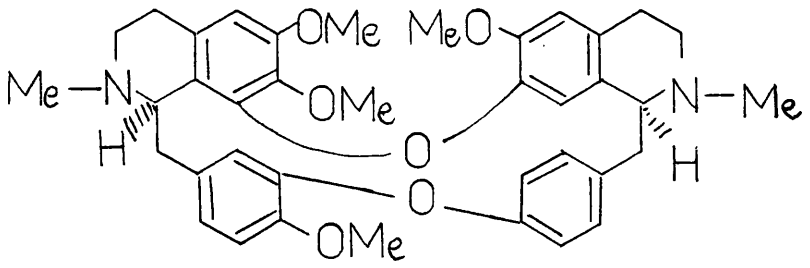


(77) daphnandrine



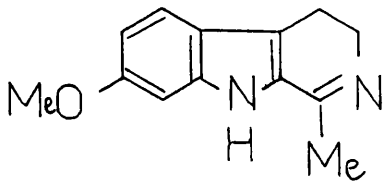
(78) obaberine

Fig. 10 continued

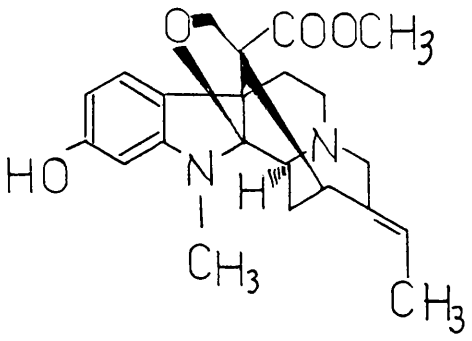


(**79**) isotetrandrine

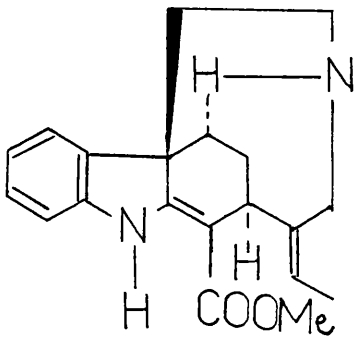
Fig.11 Indole alkaloids with activity against *Leishmania spp.* and *Trypanosoma cruzi* .



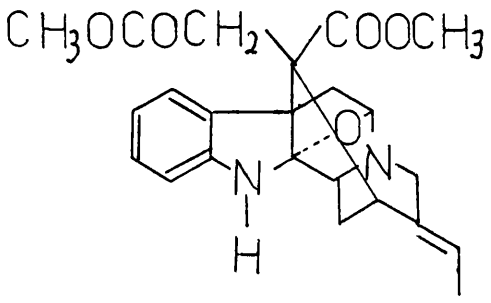
(**80**) harmaline



(**81**) akuammine



(**82**) akuammicine

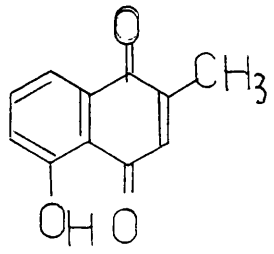


(**83**) picraline

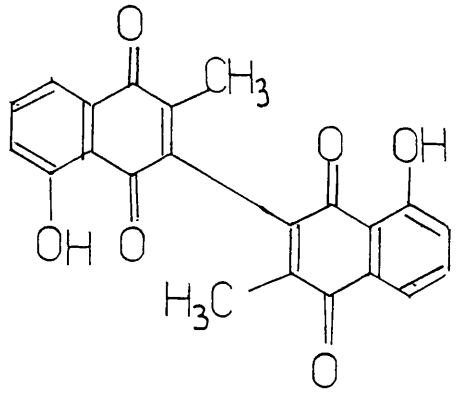
These include plumbagin (**84**), 3,3′-biplumbagin (**85**) and 8,8′-biplumbagin (**86**), from *Pera benensis*, which were active *in vitro* against promastigote forms of three species of *Leishmania* and epimastigote forms of six strains of *T. cruzi* (Fournet *et al.*, 1992a). Plumbagin and 8,8′-biplumbagin 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* (Annonceae; 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**).

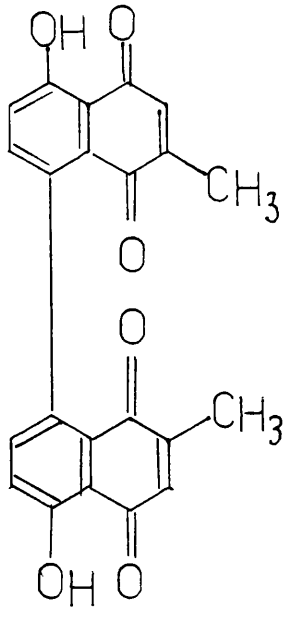
Fig. 12 **Naphthoquinones with activity against *Leishmania spp.* and *Trypanosoma cruzi*.**



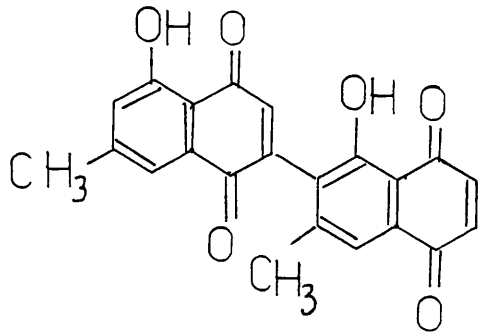
(**84**) plumbagin



(**85**) 3,3′-biplumbagin

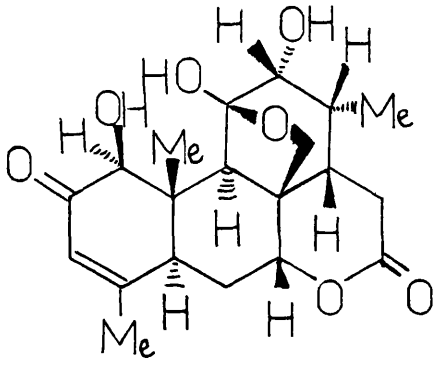


(**86**) 8,8′-biplumbagin

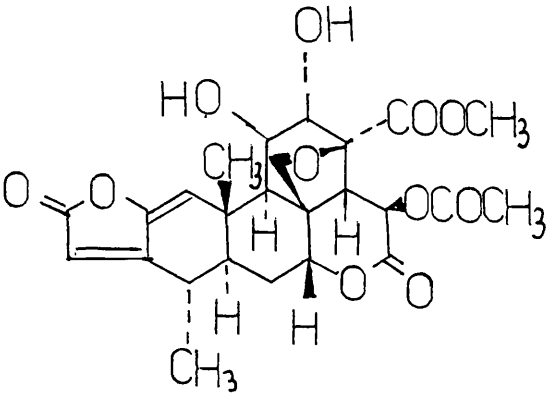


(**87**) diospyrin

Fig. 13. Quassinoids with activity against *Leishmania spp.* and *Trypanosoma cruzi*.

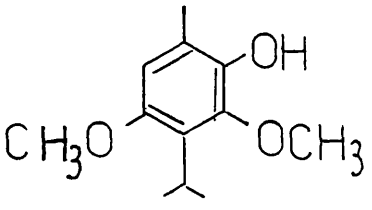


(**88**) chaparrinone

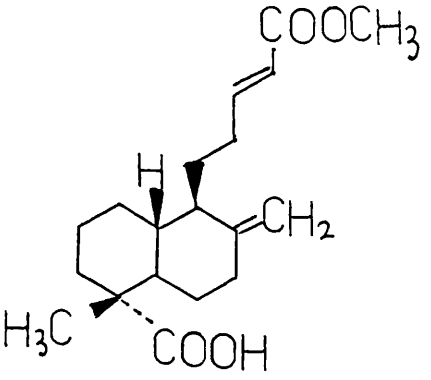


(**89**) sergeolide

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

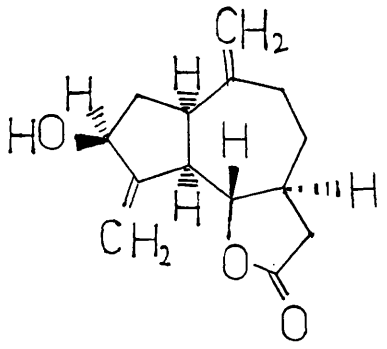


(**90**) espintanol



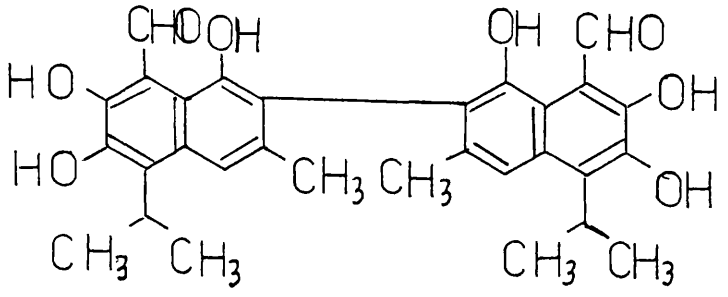
(**91**) *Polyalthia* labdane derivative

Fig. 14 continued

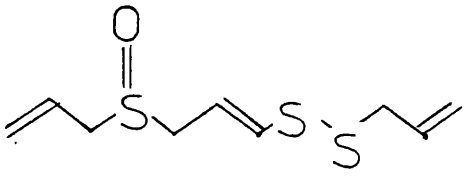


(**92**) dehydrozaluzanin C

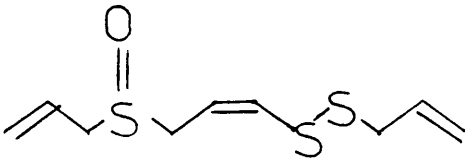
Fig. 15 Plant compounds with activity against *Trypanosoma cruzi*.



(**93**) gossypol

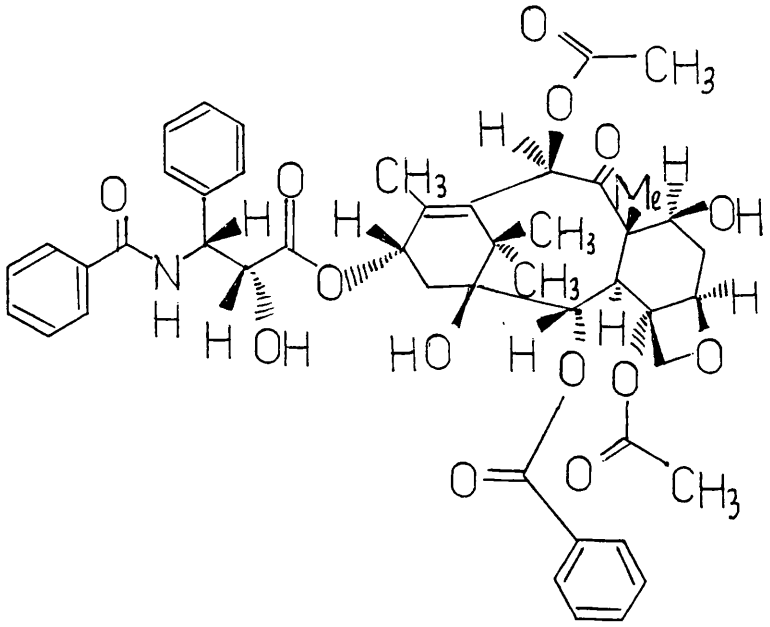


(**94**) Z-ajoene

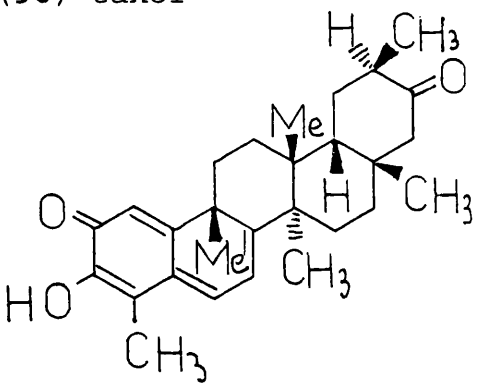


(**95**) E-ajoene

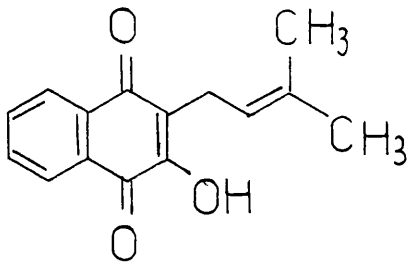
Fig. 15 cont.



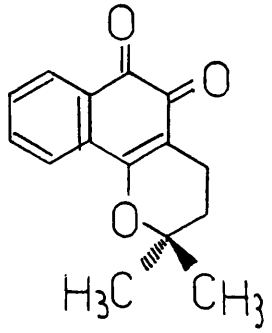
(**96**) taxol



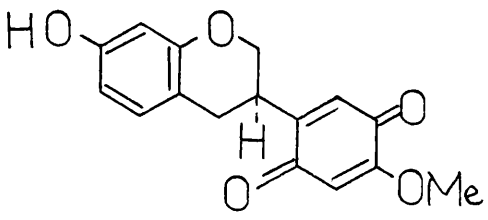
(**97**) tingenone



(**98**) lapachol



(**99**) β-lapachone



(**100**) (3R)-claussequinone

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), *Alchornia 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 senagalensis* (Annonaceae) used traditionally to treat sleeping sickness in Northern Nigeria, were active against *T. b. brucei in vivo* (Igweh

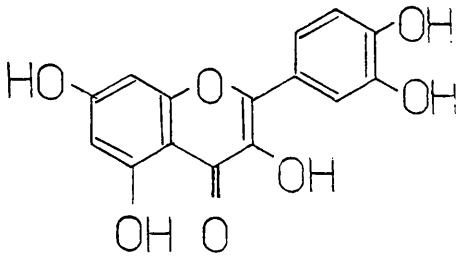
and Onabanjo 1989).

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.

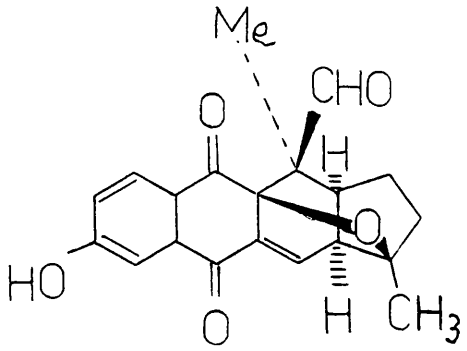
A few plant compounds have been screened for activity against *T. b. brucei in vitro* and *in vivo;* Williamson and Scott-Finnigan (1978) reported activity for the flavonoid quercetin (**101**; Fig. **16**; Page **68**) *in vitro* but not *in vivo*, and for emetine both *in vivo* and *in vitro.*

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).

Fig. 16 Compounds with activity against *Trypanosoma b. brucei*.



(**101**) quercetin



(**102**) isopinnatal

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 ellucidate 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.

CHAPTER 2 THE SEARCH FOR NOVEL PLANT PRODUCTS WITH ACTIVITY AGAINST *TRYPANOSOMA B. BRUCEI.*

2.1. Introduction

2.1.1. The epidemiology of African sleeping sickness.

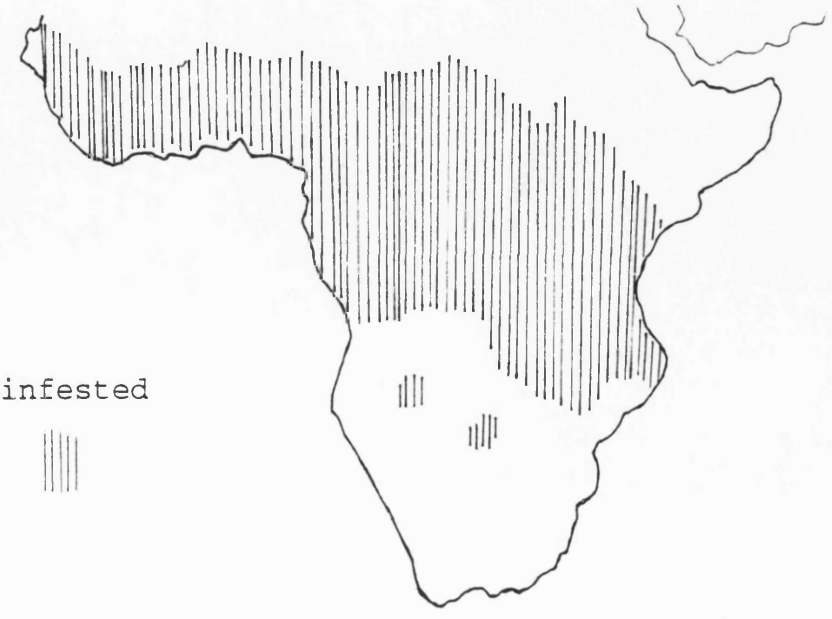
Human African trypanosomiasis occurs in 36 African countries between latitudes 14° North and 29° South (Kuzoe, 1993; Fig. 17; Page 71) and is caused by two species of African trypanosomes, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. *T. brucei spp.* are mainly transmitted from 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).

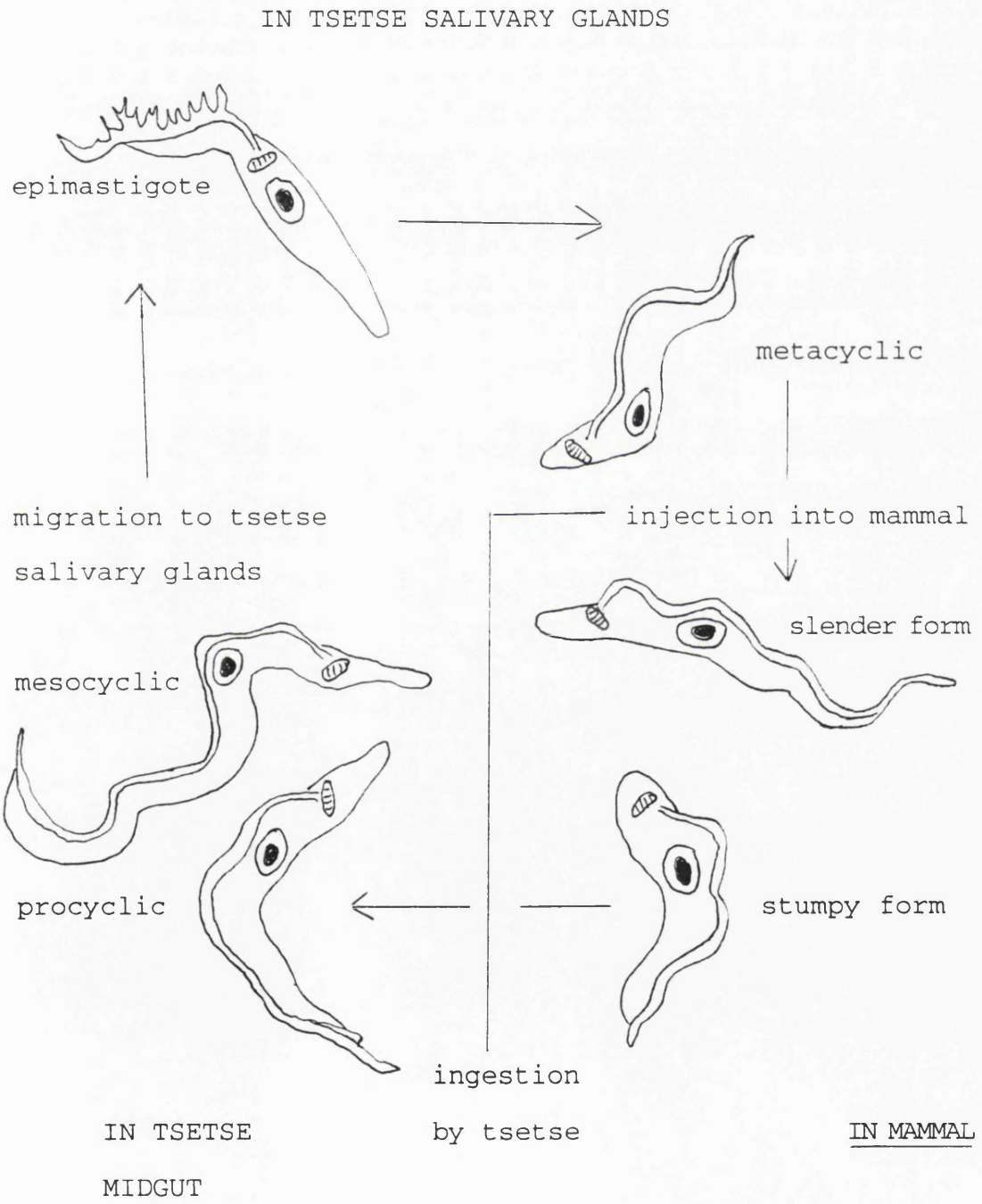
Officially only twenty five thousand new cases are reported annually (Kuzoe, 1993) though this figure is an underestimate due to the inaccessibility of the affected areas, difficulty of diagnosis, and poor reporting. The true infection rate may be many times the official figure (TDR Progress, 1995). 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.



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

(Borst and Rudenko, 1994). 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,

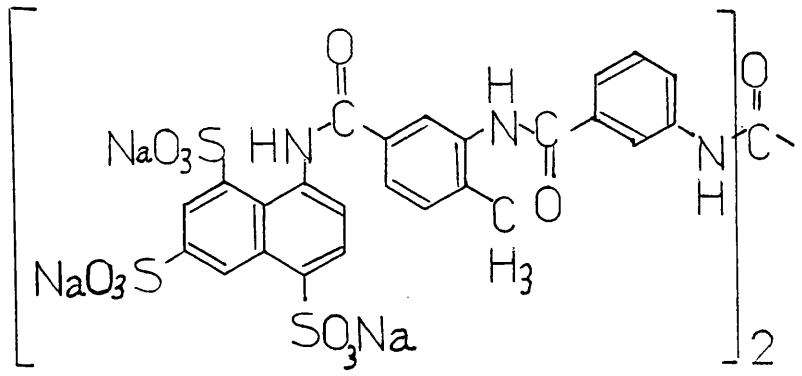
so that diagnosis and treatment in the early stages is difficult.

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).

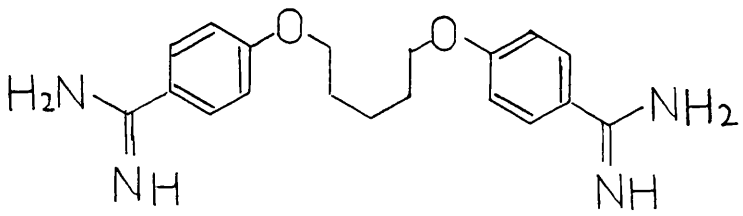
In the Sudan and Zaire melarsoprol-refractory *T. b. gambiense* disease has been treated with nifurtimox (**107**) but results were not encouraging (Pépin and Milord, 1994). Side effects of nifurtimox are nausea, weight loss, memory loss, sleep disorders and depression (Gutteridge, 1985).

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.

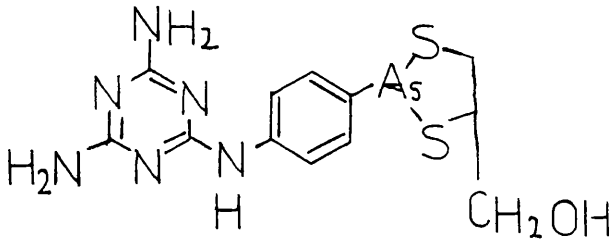
Fig 20. Structures of compounds currently in use for the chemotherapy of African Trypanosomiasis.



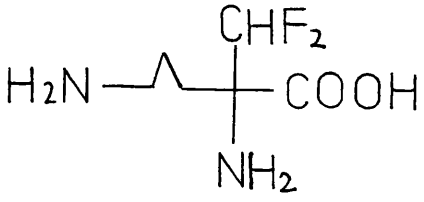
(**103**) suramin



(**104**) pentamidine



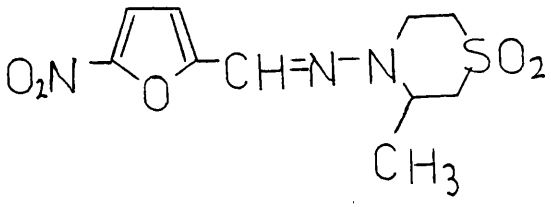
(**105**) melarsoprol



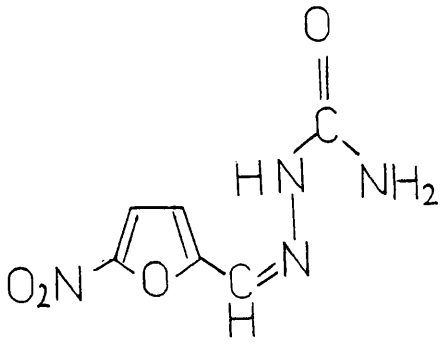
(**106**) DL-α-difluoromethylornithine

(DFMO)

Fig. 20 continued



(**107**) nifurtimox



(**108**) nitrofurazone

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	(Papillionaceae)	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

solvent	source
0.880 ammonia sol.	BDH
chloroform	BDH
hydrochloric acid	M and B
methanol	BDH
petroleum ether	BDH

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	**source**
Pre-coated aluminium-backed silica gel (60) plates F_{254} (0.25mm thick)	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 *T. b. brucei* procyclic forms *in vitro*.

2.4.1.2.3 Large scale alkaloidal extraction of *A. boonei*

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

2.4.1.3 Large scale petroleum ether extraction of *A. boonei*

Since the petroleum ether fraction derived from the sequential extraction of *A. boonei* was active against *T. b. brucei*, 100g of ground *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 *A. boonei*

Analytical TLC with a solvent system of chloroform : methanol 85:15, revealed seven Dragendorff-positive spots in the crude alkaloidal fraction with 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.

2.5.1 Chromatographic techniques

2.5.1.1 Column chromatography

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 $CHCl_3$:MeOH 95:5

(ii) 70-78 with $CHCl_3$:MeOH 94:6

(iii) 83-95 with $CHCl_3$:MeOH 90:10

(iv) 96-114 with $CHCl_3$:MeOH 50:50.

Fig. 21 **Small scale sequential extraction of *Alchornea cordifolia*.**

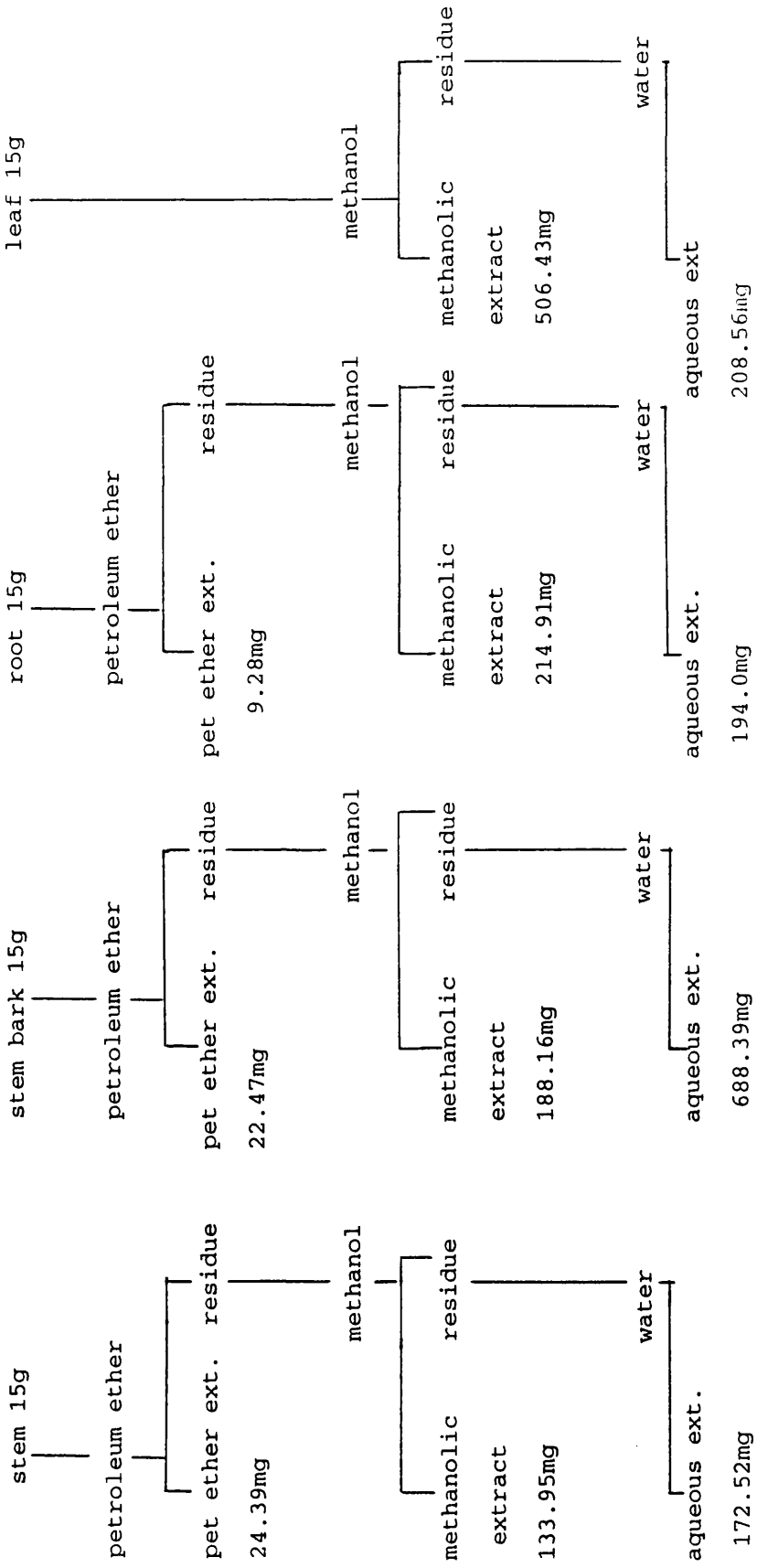


Fig. 22 Small scale sequential extraction of *Alstonia boonei* bark

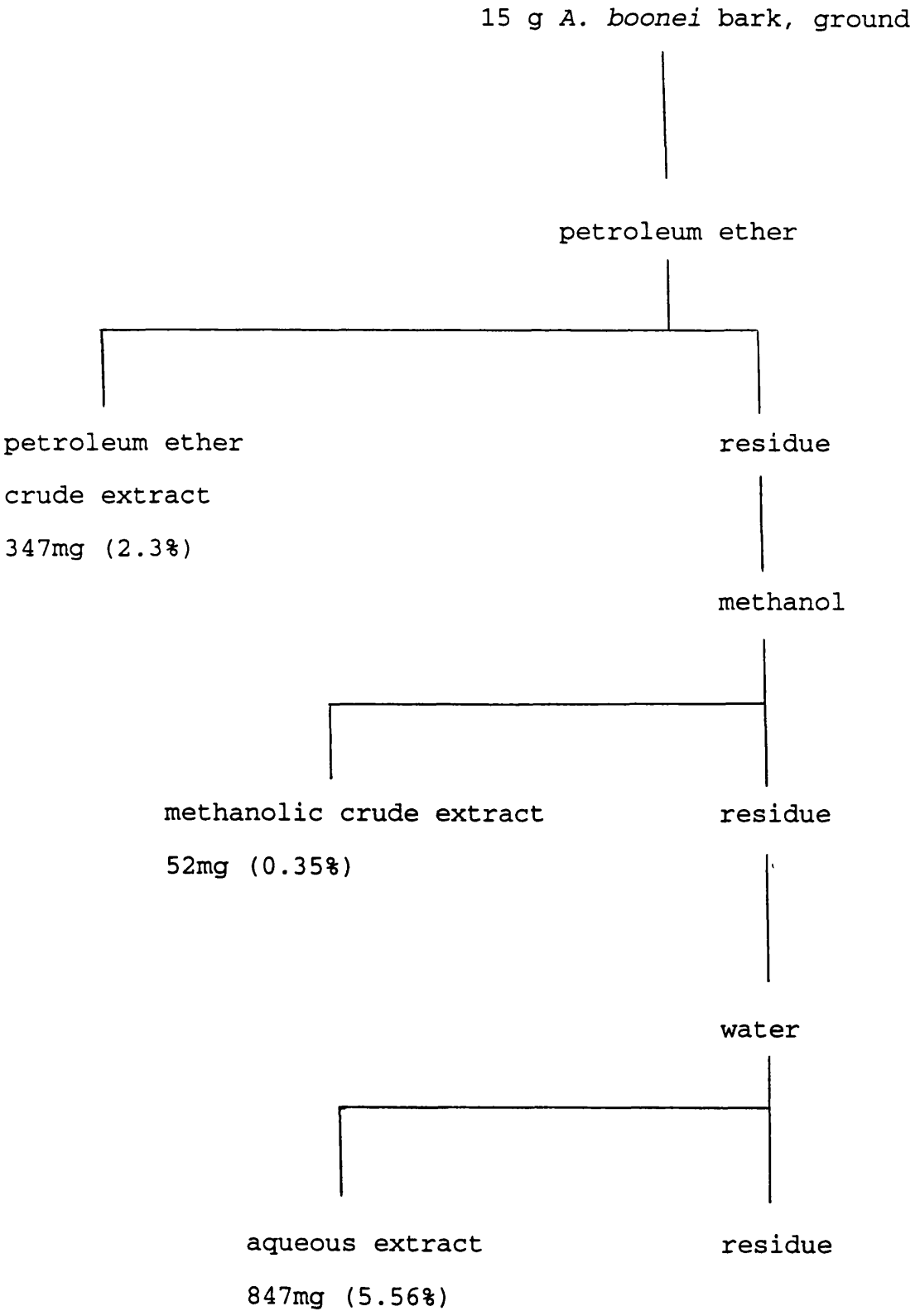


Fig. 23 Large scale alkaloidal extraction of *Alstonia boonei* bark.

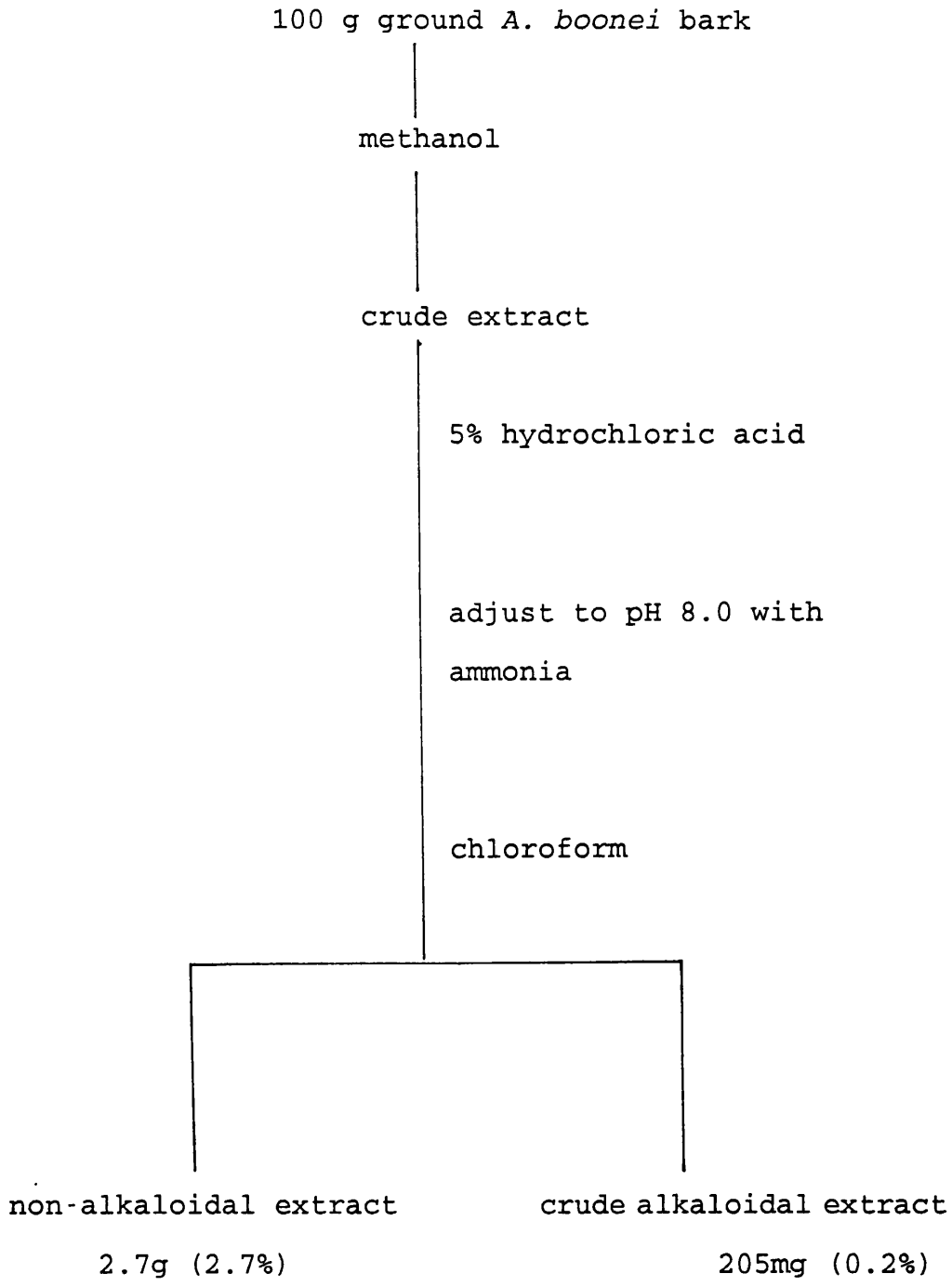
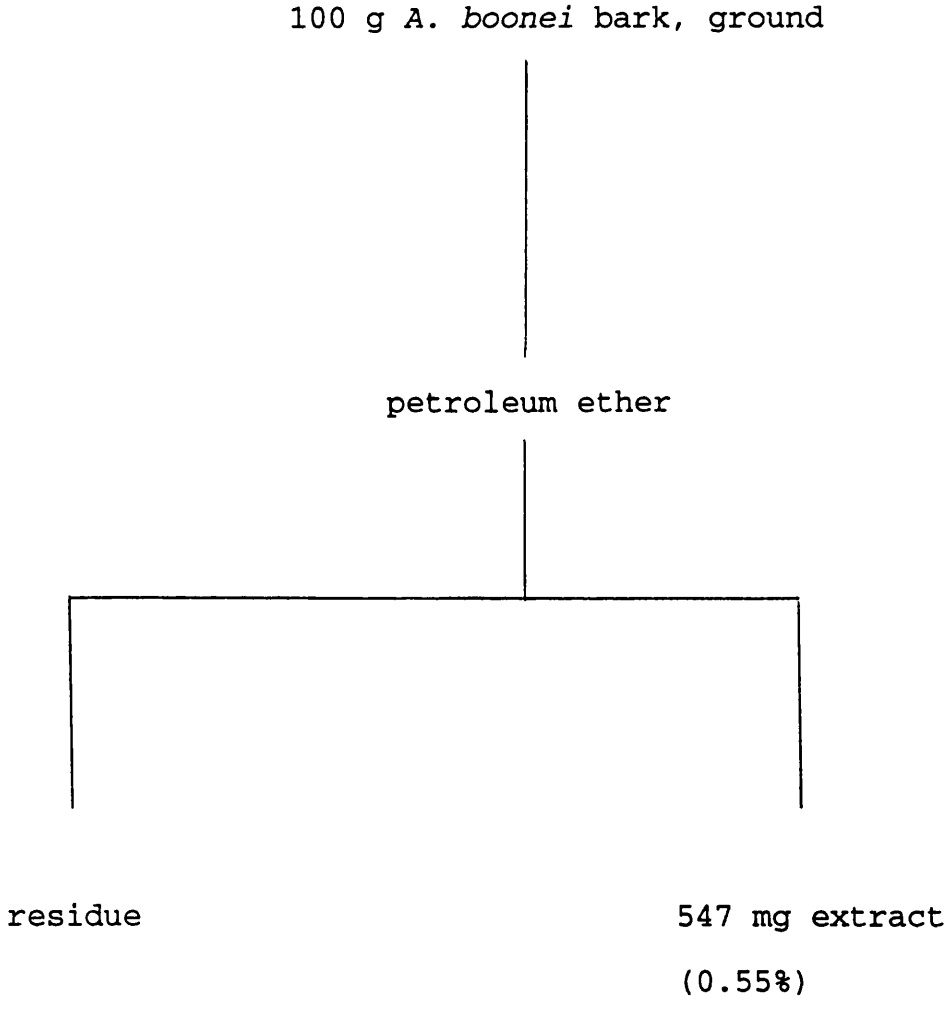


Fig. 24 Petroleum ether extraction of *Alstonia boonei* bark



2.5.1.2 Preparative thin layer chromatography

45 g silica gel was shaken vigorously with 90 mls distilled water and spread to a thickness of 0.5 mm on glass plates (20cm x 20cm) 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 *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:

fraction	solvent system
(i)	$CHCl_3$:MeOH 85:15
(ii)	ethyl acetate:isopropanol:ammonia 85:10:5
(iii)	ethyl acetate:isopropanol:ammonia 85:10:5
(iv)	ethyl acetate:isopropanol:ammonia 45:35:20

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:
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. $CDCl_3$ was used for alkaloidal fractions b, c and d and petroleum ether fractions. $DMSO_{d\text{-}6}$ 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 λ_{max} nm: 295, 236, 207 (Fig. **25**; Page **100**).

EIMS m/z (Table **3**; Page **96**; Fig. **26**; Page **101**).

^{1}H NMR (DMSO-d_6) (Table **4**; Page **97**; Fig. **27**; Page **102**).

^{13}C NMR (Table **6**; Page **99**; Fig. **28**; Page **103**).

COSY spectrum (Fig. **29**; Page **104**).

2.6.2 Undulifoline (fraction b)

yellow/orange resin

UV λ_{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 λ_{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: δ8.48 (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 λ_{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

echitamine	undulifoline	echitamidine	12-methoxy-echitamidine
384 $[M-H]^+$ (96)	341 $[M+H]^+$ (100)	340 $[M]^+$ (33)	370 $[M]^+$ (30)
370 (100)	284 (34)	271 (38)	354 (3)
353 (87)	281 (5)	241 (100)	352 (6)
267 (25)	252 (17)	225 (20)	338 (14)
252 (22)	239 (85)	180 (32)	311 (7)
232 (26)	181 (35)	139 (12)	293 (7)
194 (37)	167 (32)	105 (2)	271 (100)
153 (69)	136 (54)		255 (20)
	122 (37)		174 (14)
	109 (38)		160 (9)
			94 (42)

Table 4 **^{1}H NMR assignments of echitamine and undulifoline**

	echitamine			undulifoline		
H	δ	multi-plicity	J=Hz	δ	multi-plicity	J=Hz
H3	4.35	1H, m		2.45	1H, m	
H5	3.62 3.34	1H, dd 1H, m	13.5, 2.4			
H6	2.02 2.22	1H, dd 1H, dt	14.2, 8.4 13.2, 8.1			
H9	7.74	1H, d	7.5	7.36	1H, d	7.9
H10	6.75	1H, td	7.5, 0.6	7.10	1H, td	6.2,1.8
H11	7.09	1H, dt	7.4, 0.7	7.18	1H, td	6.8,1.3
H12	6.68	1H, dd	7.5	7.55	1H, d	7.7
H14 α H14 β	1.50 2.56	1H, dd 1H, m	14.5, 5.6	2.13 1.65	1H, m 1H, dd	 13.0, 2.4
H15	3.88	1H, d	5.0	2.78	1H, brd	3.1
H17 H17	3.16	1H, s		3.88 4.21	1H, d 1H, d	11.7 11.8
H18	1.81	3H, dd	6.9, 1.3	3.71 3.5	1H, dt 1H,brt	13.4, 2.6 12.4
H19	5.72	1H, q	6.7	2.13 1.25	1H, m 1H, m	
H20				2.70	1H, m	
H21 H21	4.46 4.26	1H, brd 1H, brd	14.1 14.9	3.97	1H, brd	2.4
NH	7.64	1H, brs		8.52	1H, brs	
OMe	3.75	3H, s		3.78	3H, s	
NMe	3.36	3H, s		2.31	3H, s	

Table 5 **^{1}H NMR assignments of echitamidine (major peaks of fraction c) and 12-methoxyechitamidine (fraction d)**

	echitamidine			12-methoxyechitamidine		
	δ	multi-plicity	J=Hz	δ	multi-plicity	J=Hz
H3	3.91	1H, br s		4.55	1H, brs	
H5	3.10 2.89	1H, m 1H, m		3.15	1H, dd	
H6	2.82 1.88	1H, m 1H, m				
H9	7.19	1H, d	7.3	6.88	1H, dd	8.96, 2.0
H10	6.93	1H, td	7.7,1	6.95	1H, dd	8.96, 8.96
H11	7.16	1H, td	6.9,0.7	6.85	1H, dd	8.96, 2.0
H12	6.84	1H, br d	7.7			
H14	1.39 2.04	1H, m 1H, m		2.79	1H, dt	13.4
H15	3.32	1H, br s		3.3	1H, td	
H18	1.17	3H, d	6.2	1.14	3H, d	6.5
H19	3.27	1H, m				
H20	1.79	1H, m				
H21						
NH	8.63	1H, br s				
COOMe	3.84	3H, s		3.89	3H, s	
OMe				3.91	3H, s	

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

	echit-amine	echi-tamidine	12-methoxy-echi-tamidine	unduli-foline
C2	100.41	172.24	172.97	135.12
C3	69.18	60.68	60.77	46.48
C5	62.17	53.85	n.o	
C6	41.48	43.36	43.12	
C7	60.96	57.06	57.71	107.51
C8	129.12	135.41	136.36	129.30
C9	127.09	121.50	112.29	120.07
C10	119.89	119.84	122.26	119.27
C11	129.18	127.74	110.13	122.35
C12	111.02	109.67	-	111.71
C13	147.92	143.70	144.27	137.26
C14	31.12	30.95	30.89	31.02
C15	34.81	28.74	28.76	38.32
C16	56.09	96.82	97.19	55.86
C17	64.89	168.94	168.99	79.48
C18	15.35	19.76	18.86	70.05
C19	130.20	68.38 68.42	68.22 68.01	33.49
C20	132.94	45.75	n. o.	40.67
C21	65.05	47.97	n. o	59.05
COO	173.57			52.78
Me	52.32	50.58	51.97	173.09
OMe			55.49	
N_b-Me	49.96			44.35

n.o. = not observed

Fig. 25 UV spectrum of echitamine

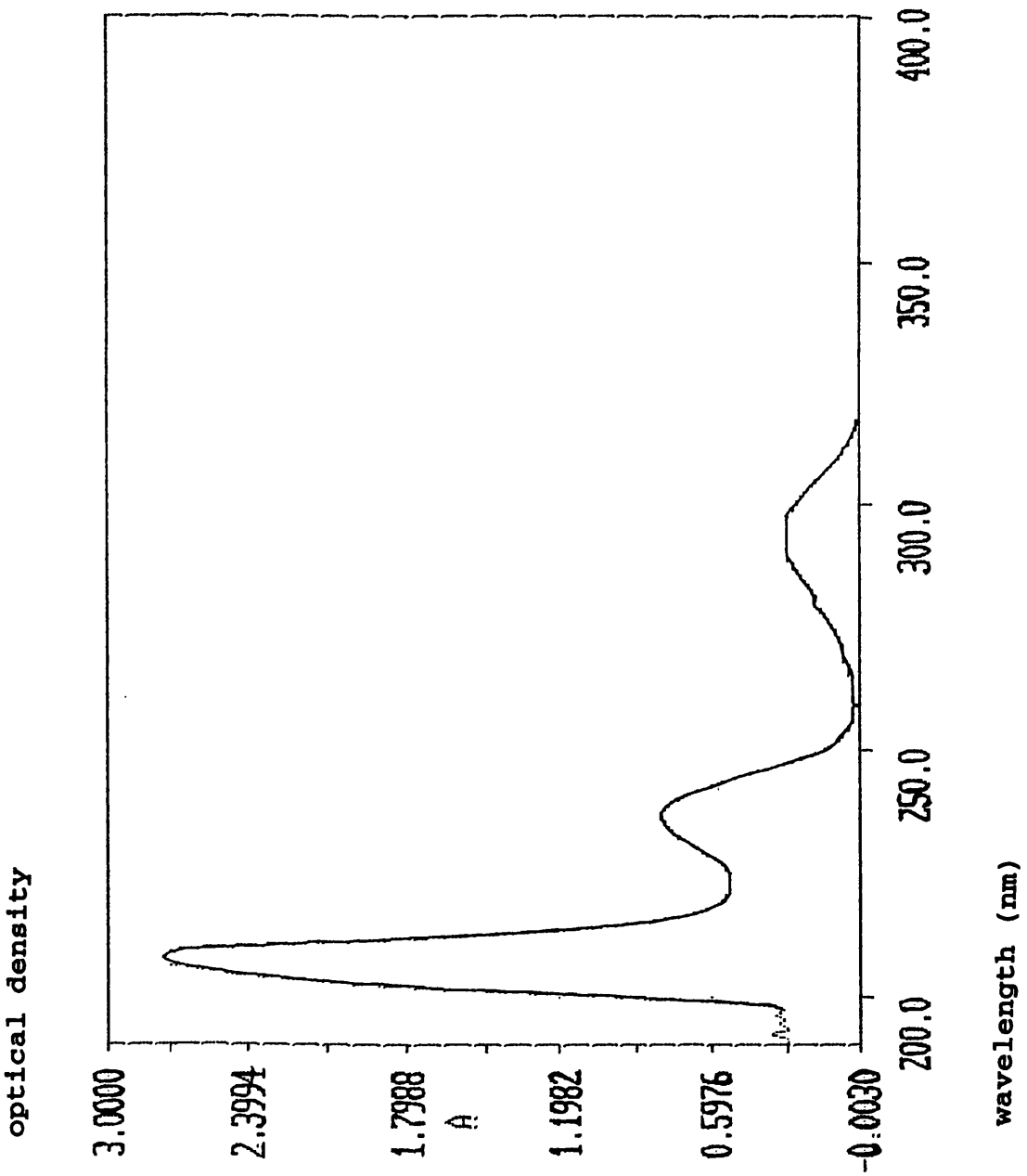


Fig. 26 EI Mass spectrum of echitamine

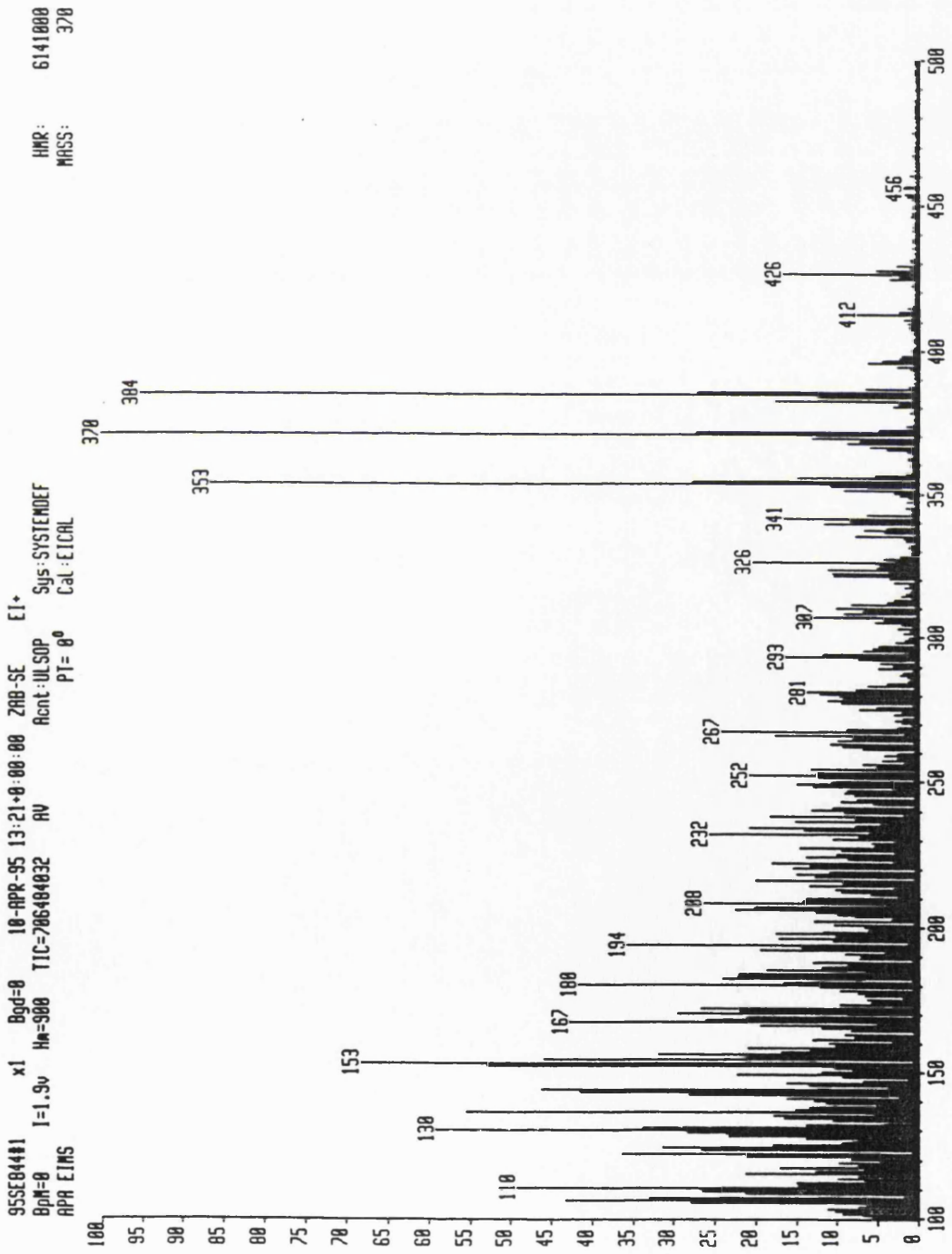


Fig. 27 ^{1}H NMR spectrum of echitamine

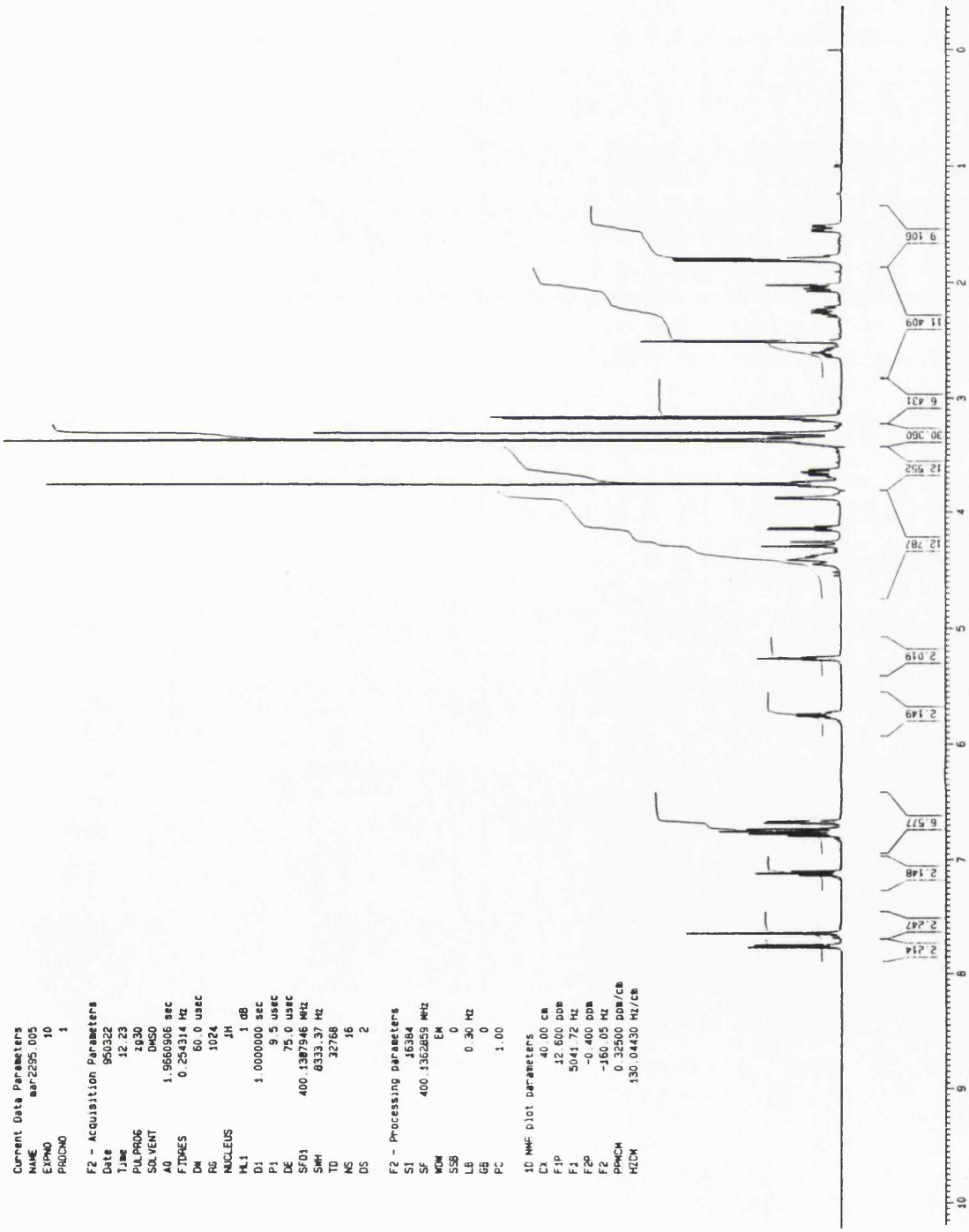


Fig. 28 ^{13}C NMR spectrum of echitamine

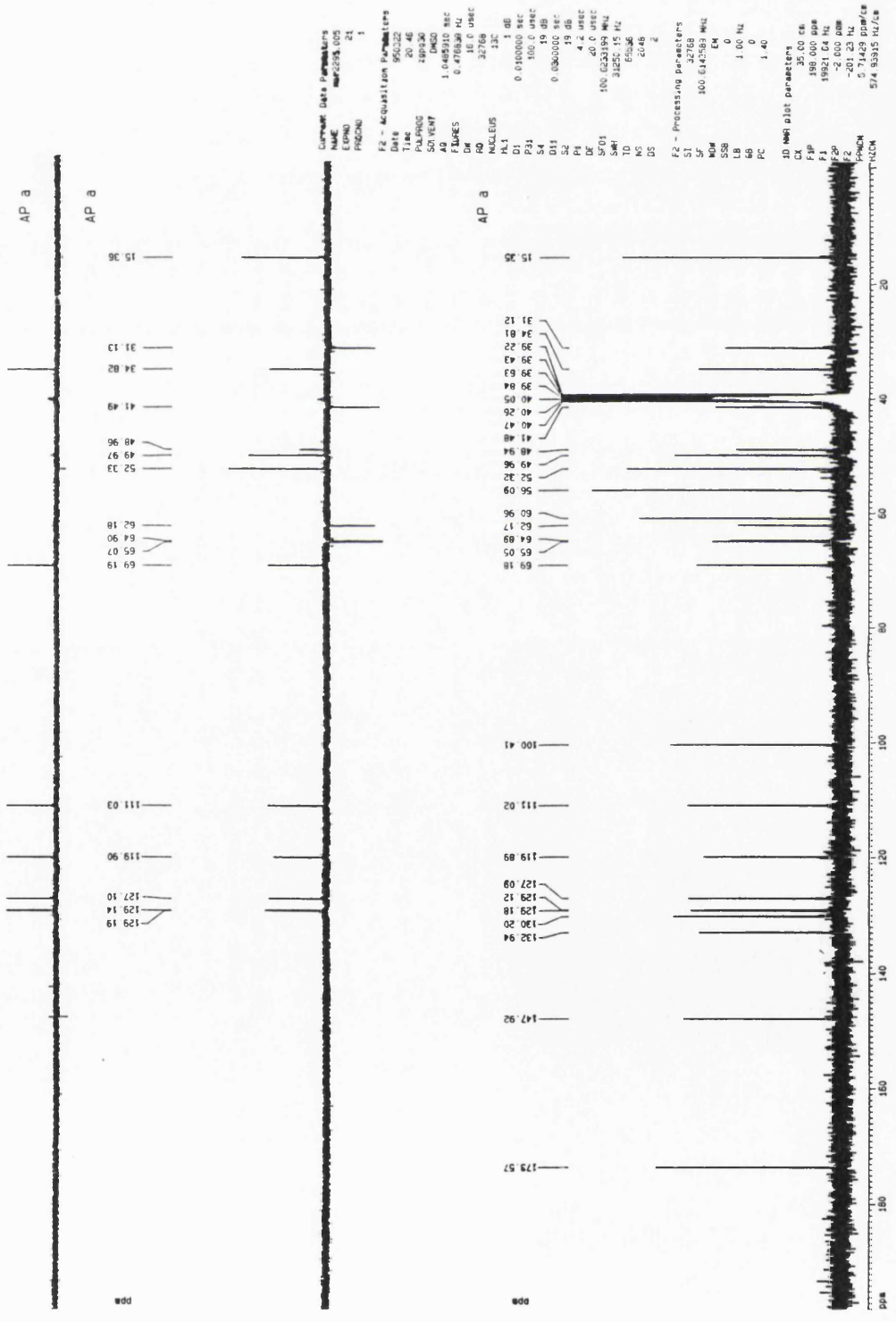


Fig. 29 COSY spectrum of echitamine

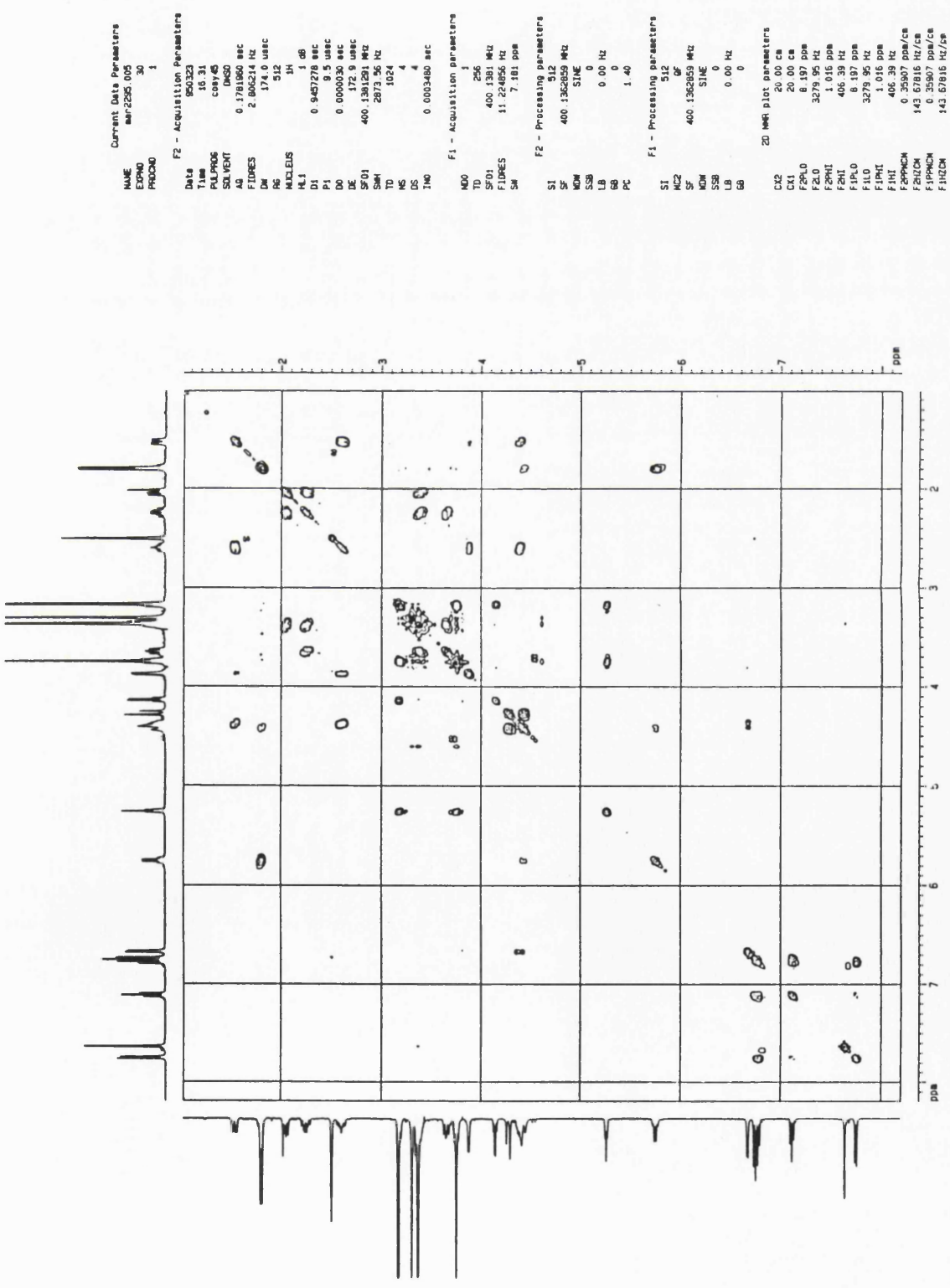


Fig. 30 UV spectrum of undulifoline

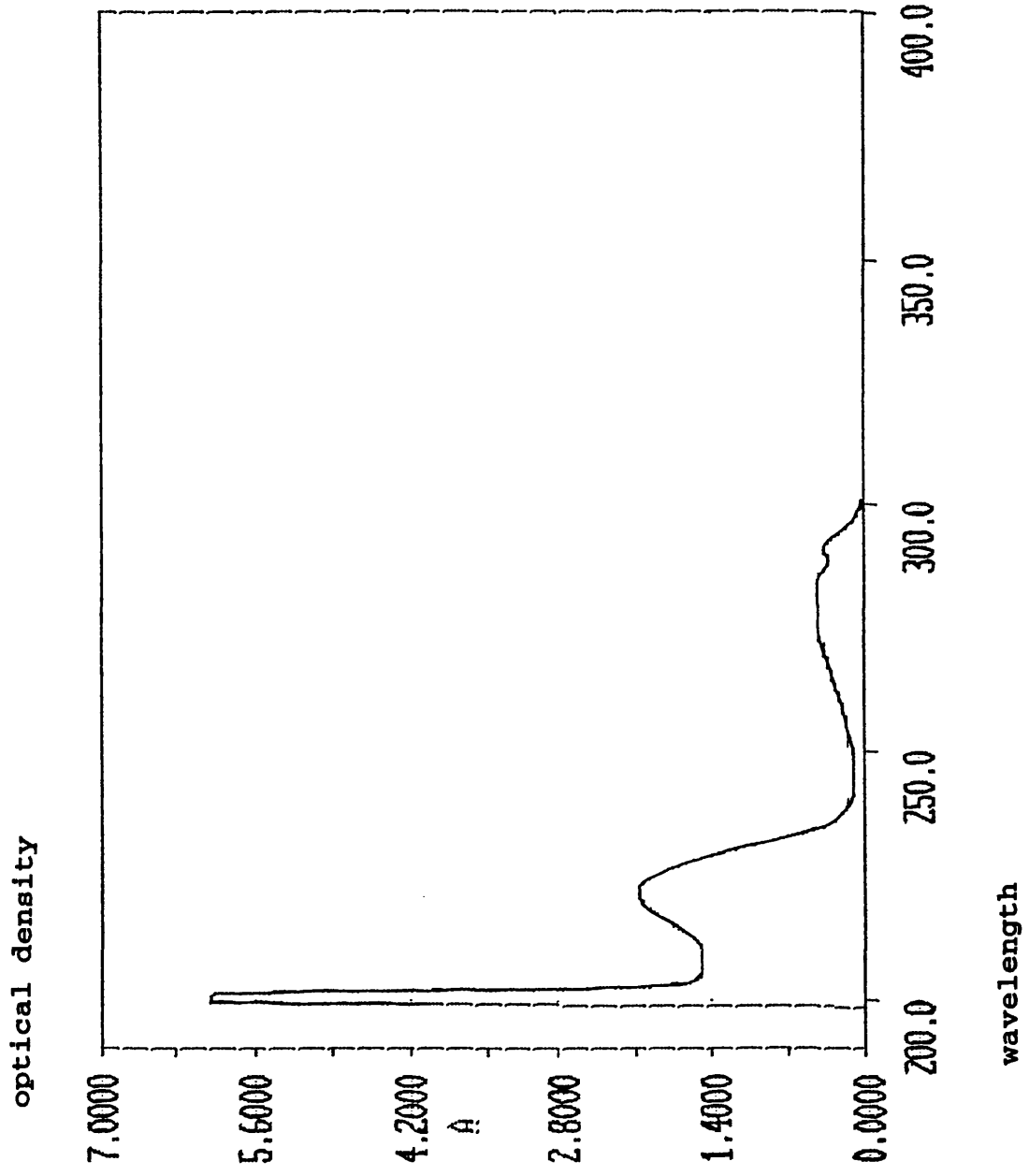


Fig. 31 EI Mass spectrum of undulifoline

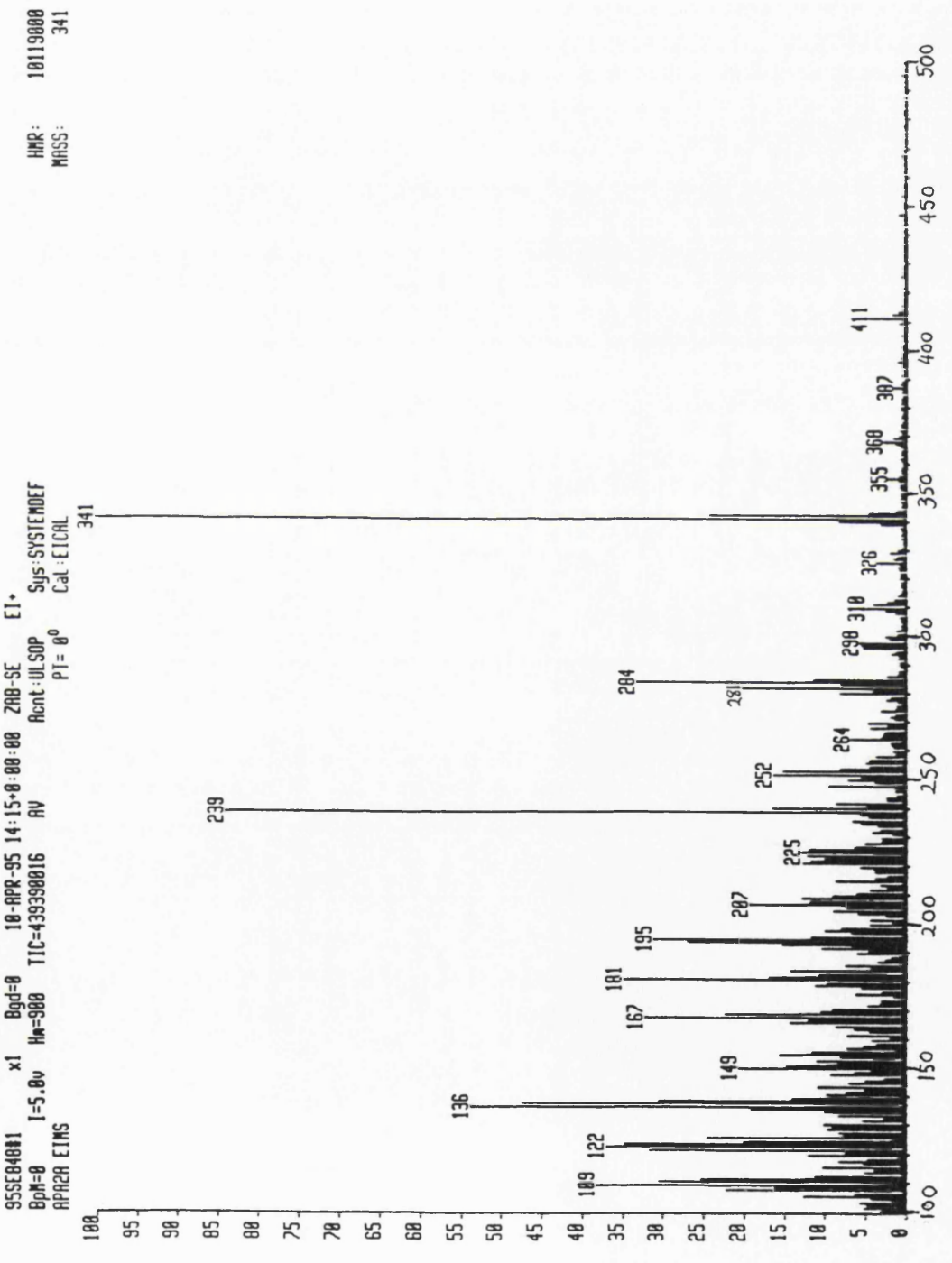


Fig. 32 ^{1}H NMR spectrum of undulifoline

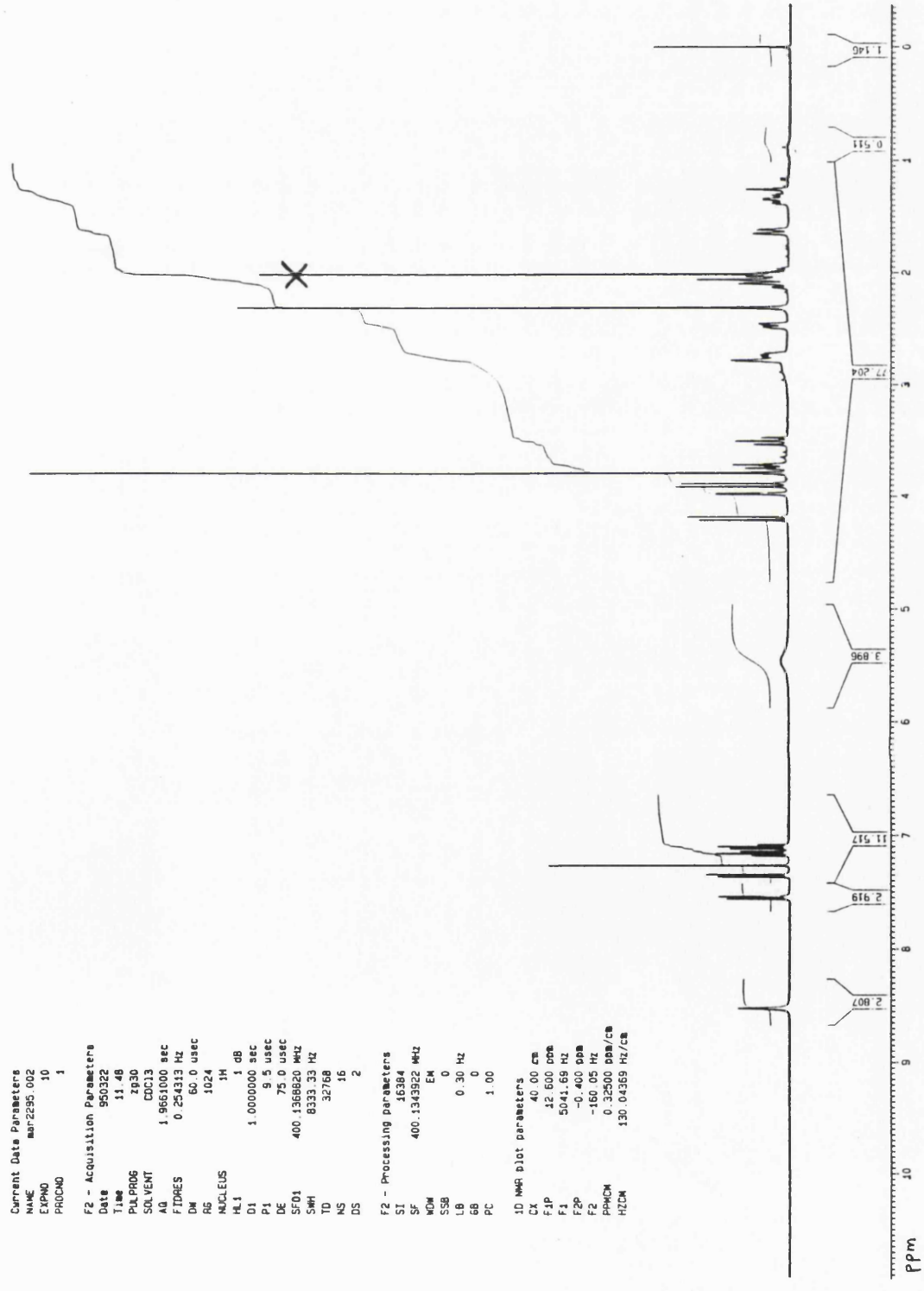


Fig. 33 ^{13}C NMR spectrum of undulifoline

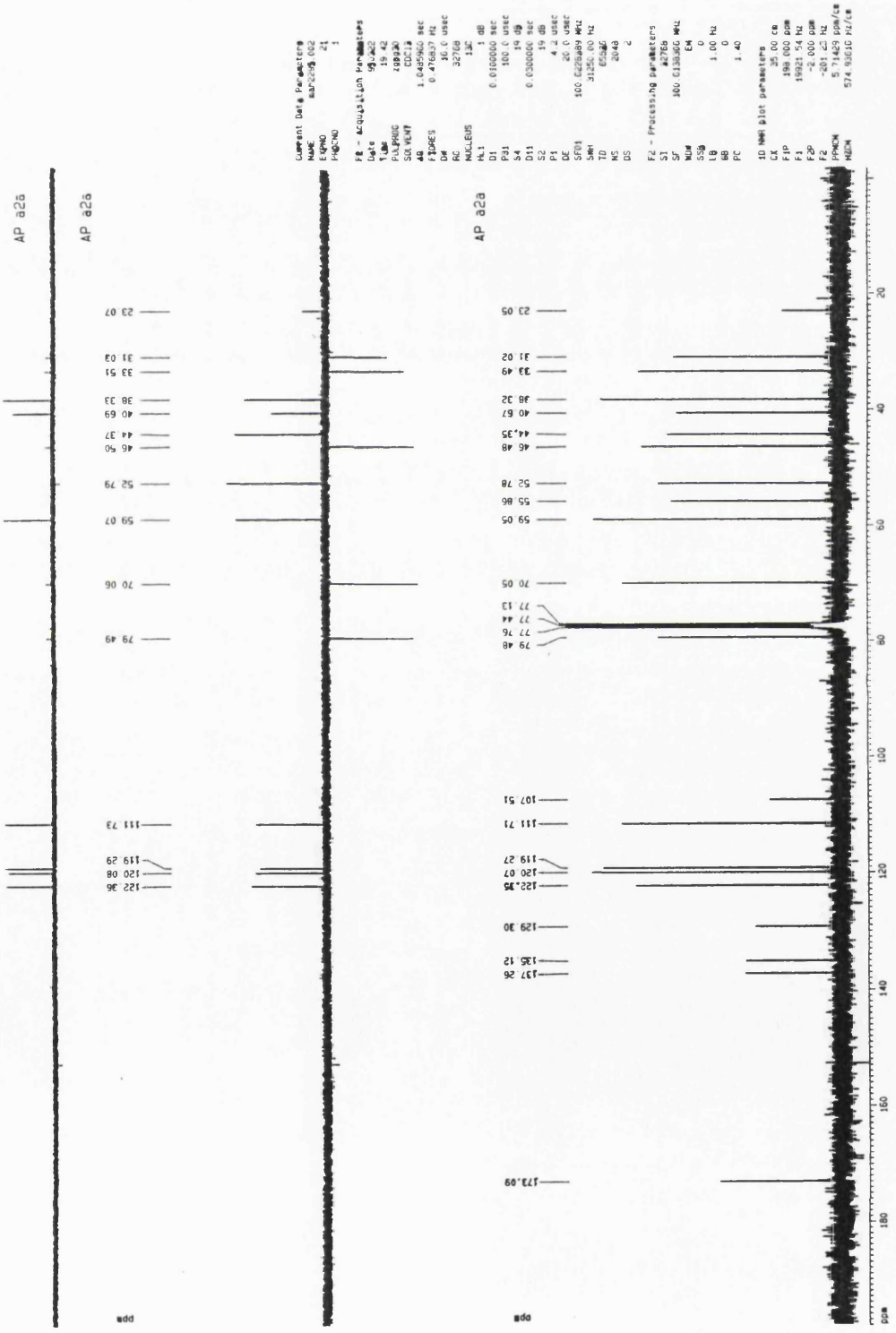


Fig. 34 COSY spectrum of undulifoline

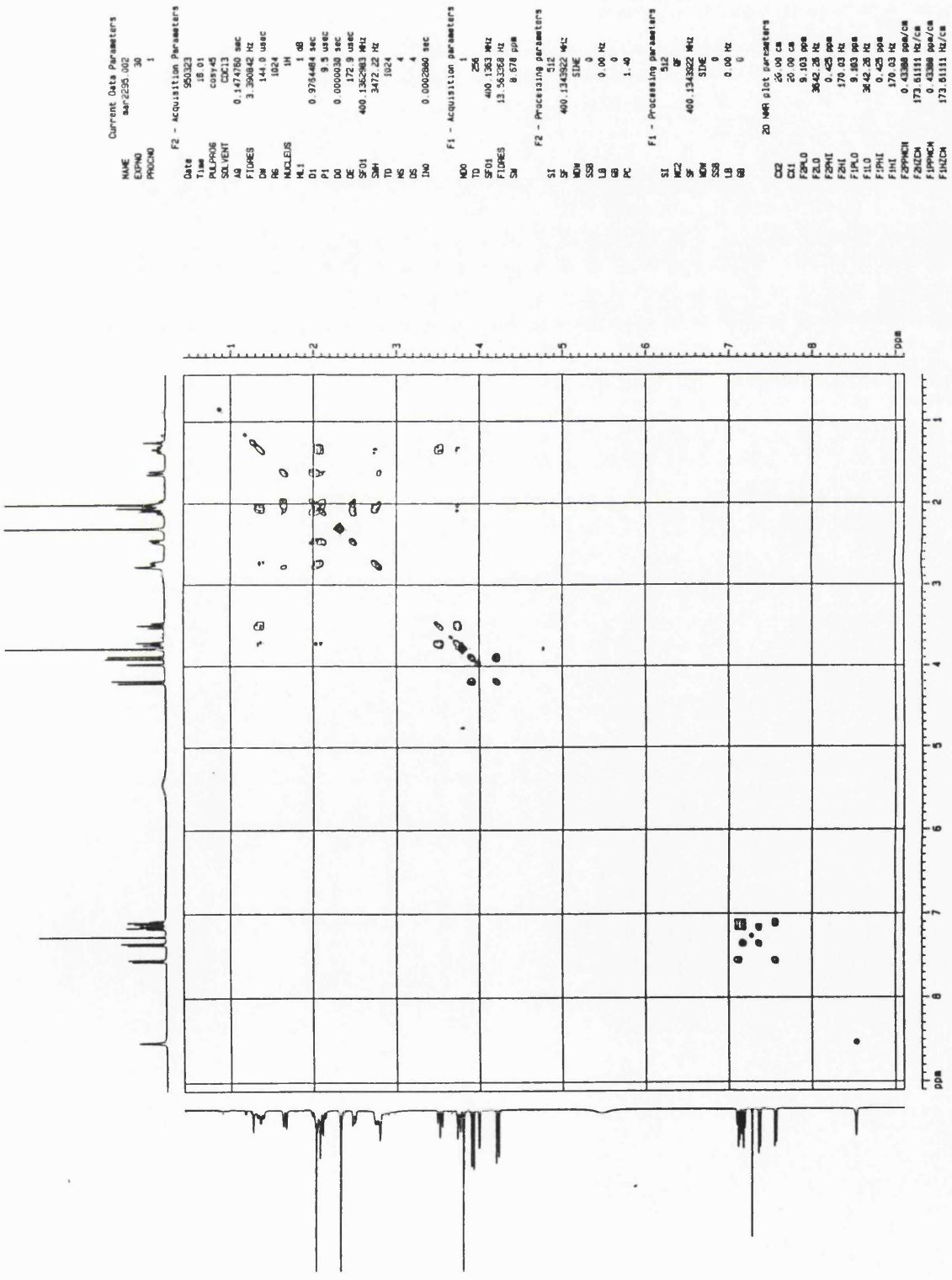


Fig. 35 UV spectrum of alkaloidal fraction c of *Alstonia boonei* (mixture of echitamidine and 12-methoxyechitamidine)

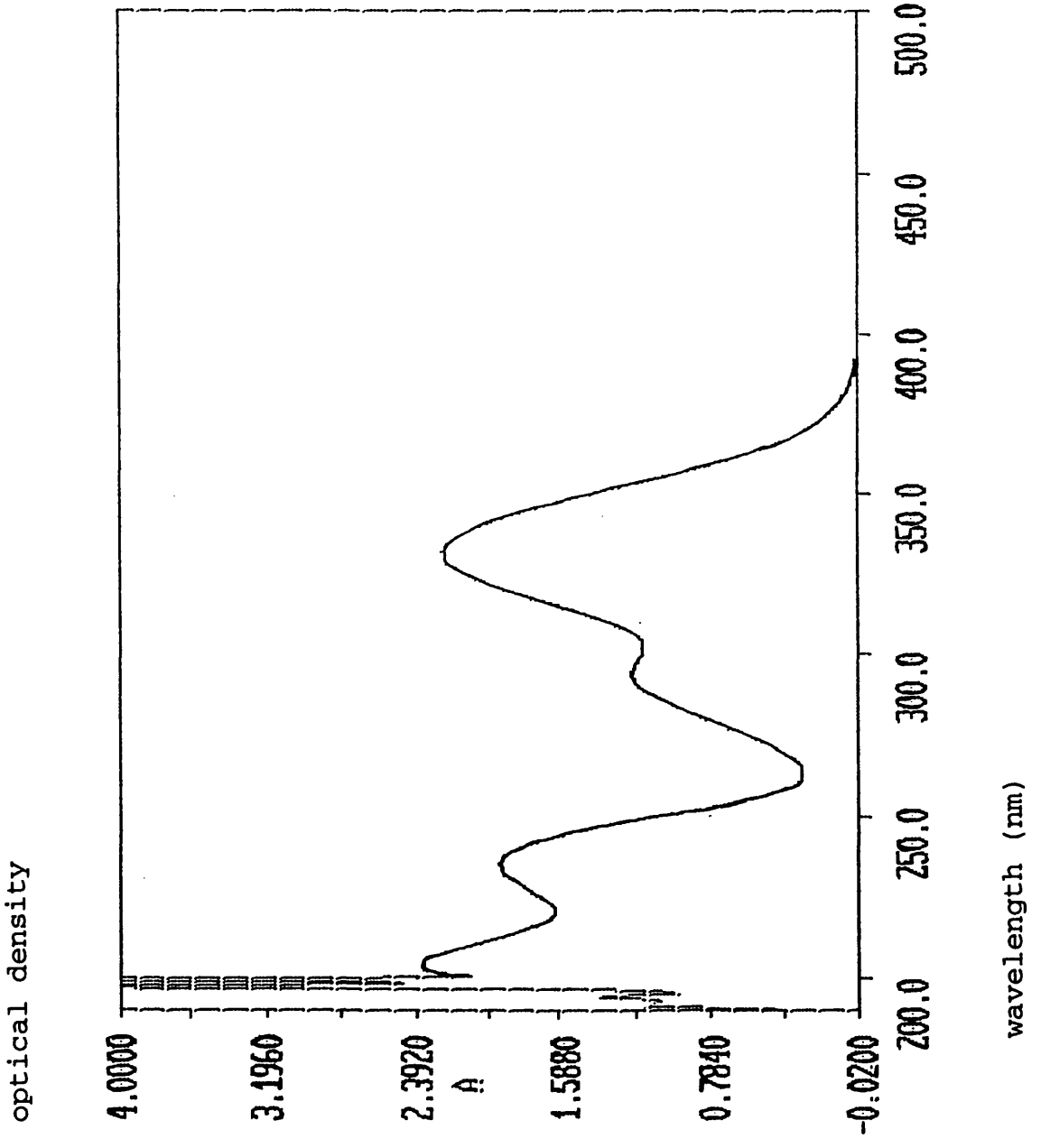


Fig. 36 EI Mass spectrum of alkloidal fraction c of *Alstonia boonei* (*mixture of echitamidine and 12-methoxyechitamidine*)

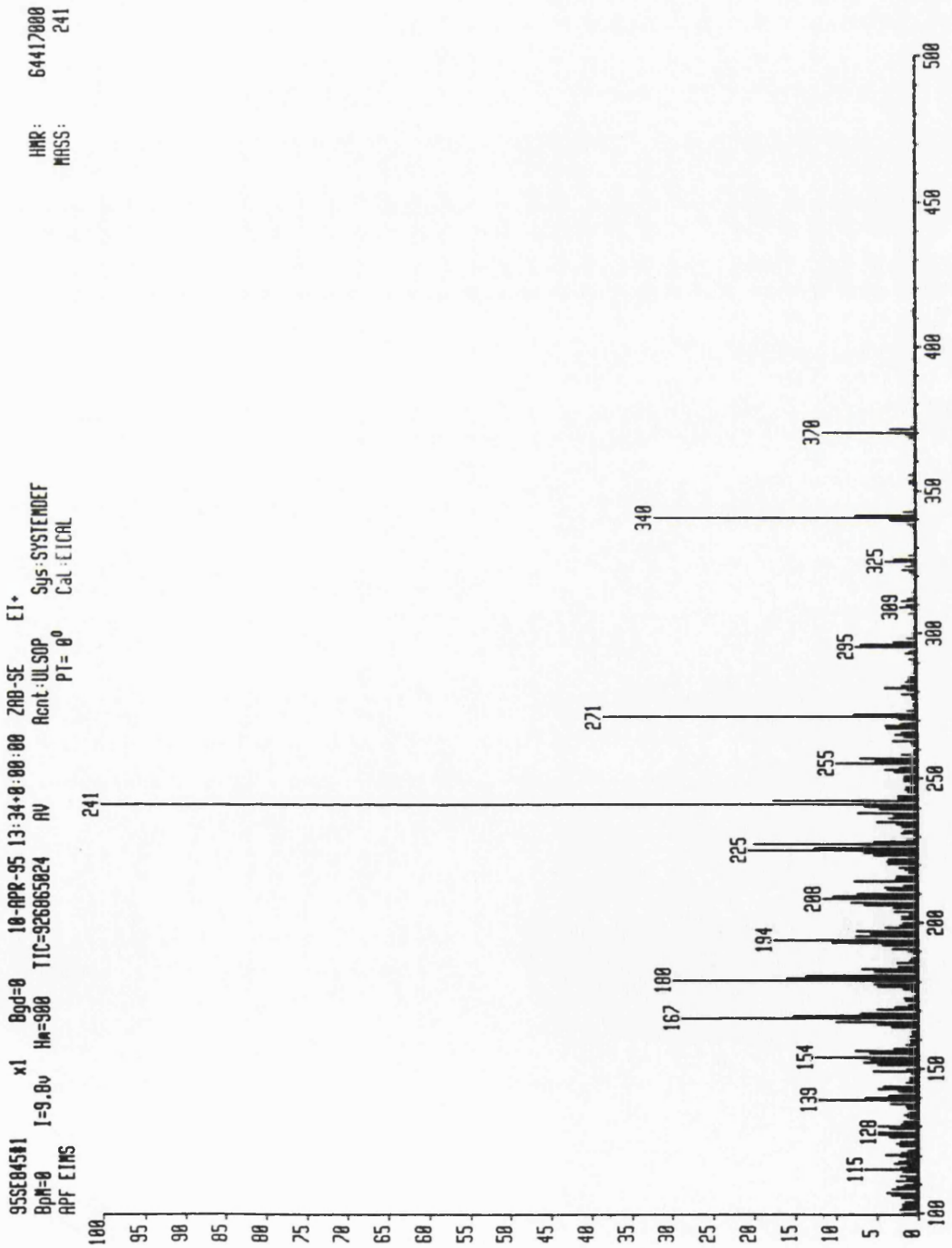


Fig. 37 ^{1}H NMR spectrum of alkaloidal fraction c of *Alstonia boonei* (mixture of echitamidine and 12-methoxyechitamidine)

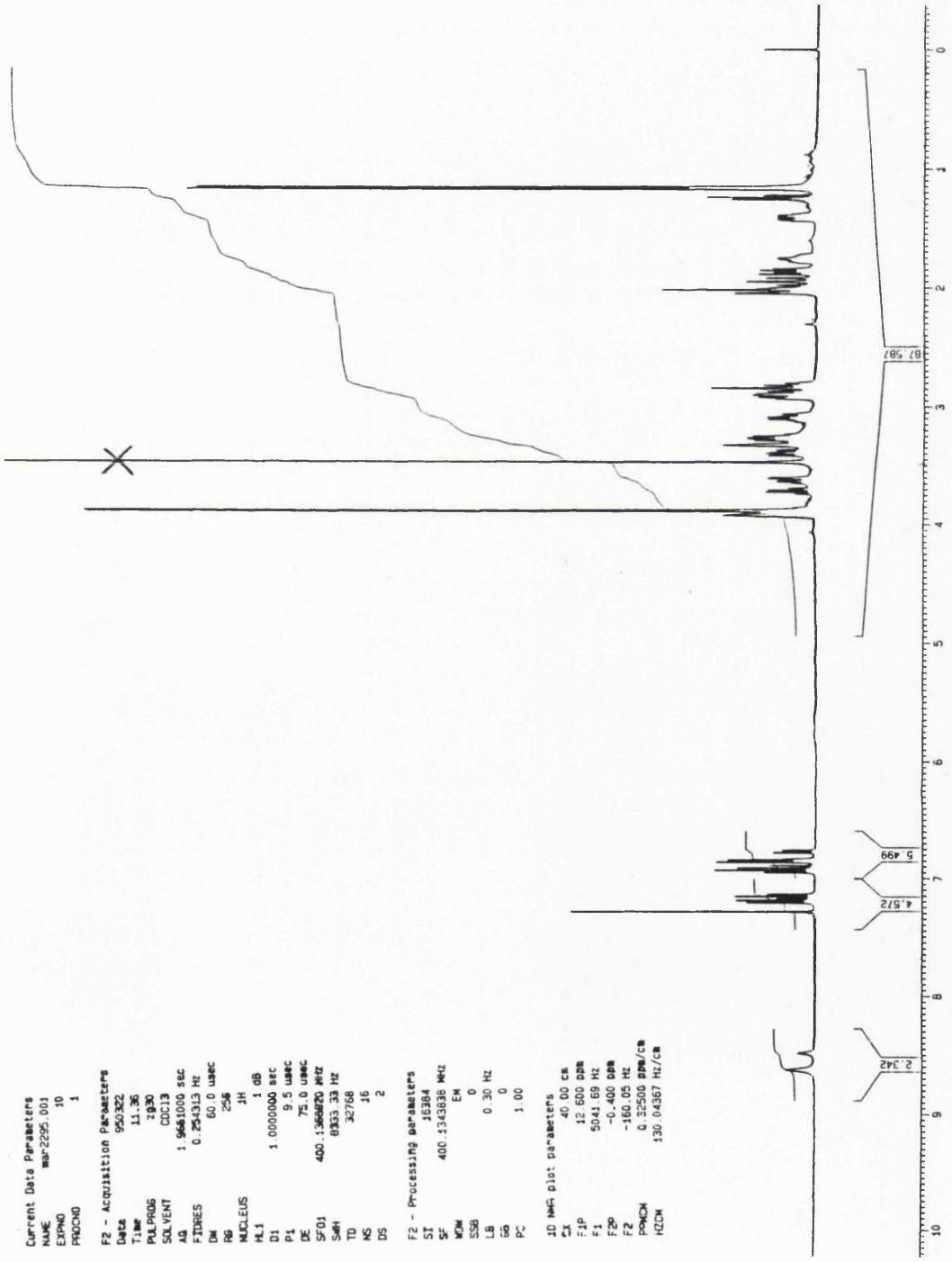


Fig. 38 ^{13}C NMR spectrum of alkaloidal fraction c of *Alstonia boonei* (mixture of echitamidine and 12-methoxyechitamidine)

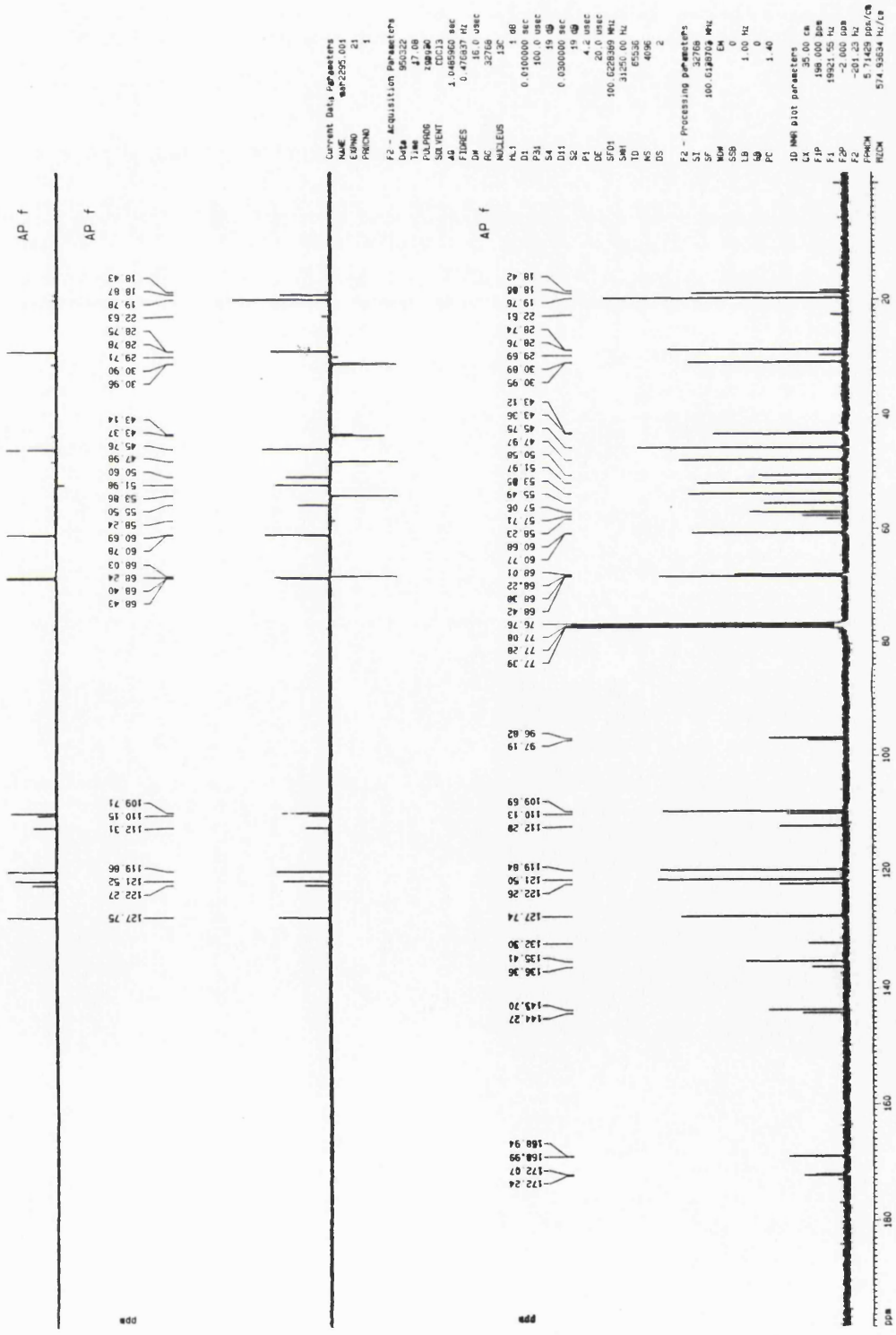


Fig. 39 COSY spectrum of alkaloidal fraction c of *Alstonia boonei* (mixture of echitamidine and 12-methoxyechitamidine)

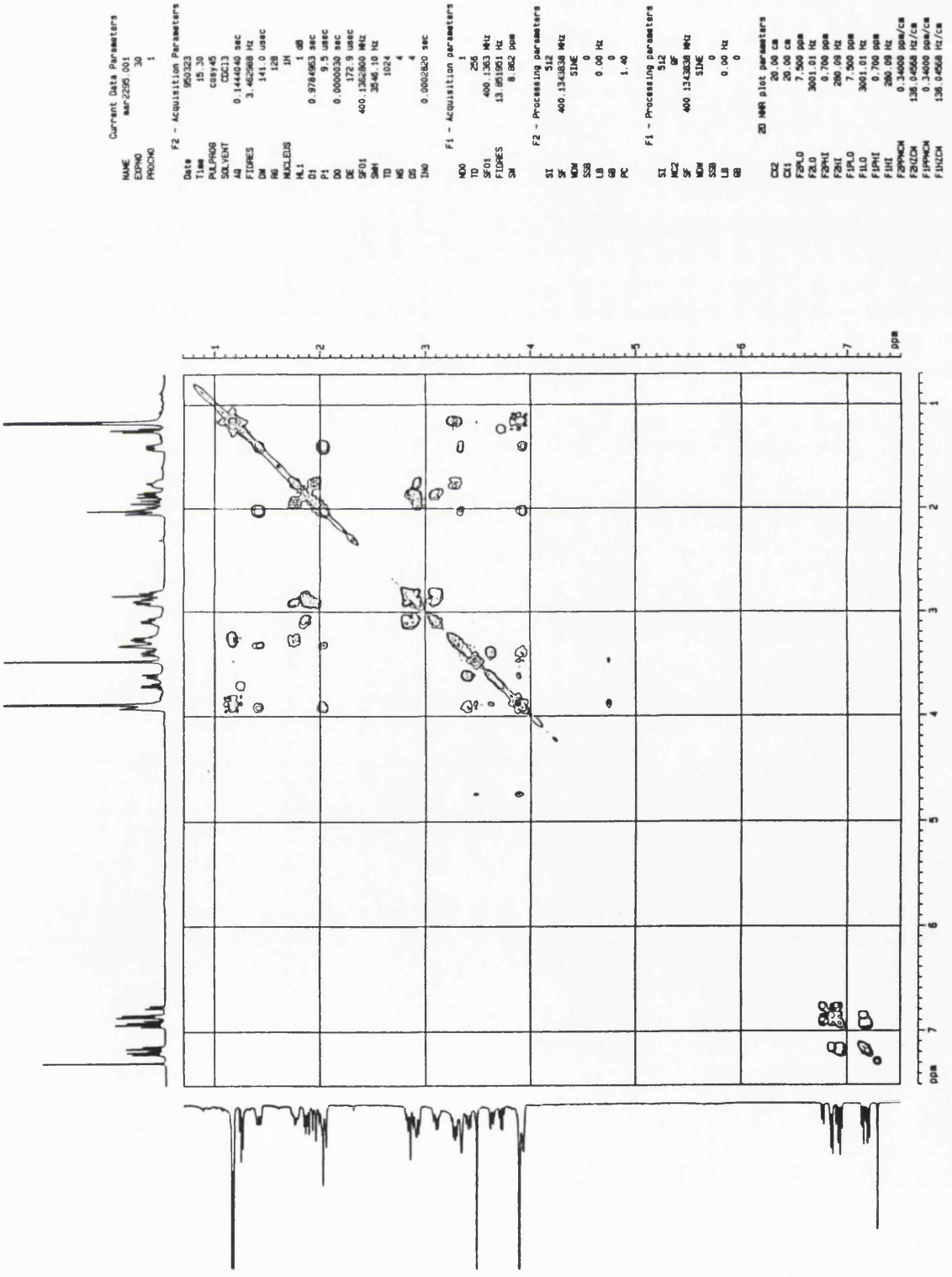


Fig. 40 UV spectrum of 12-methoxyechitamdine

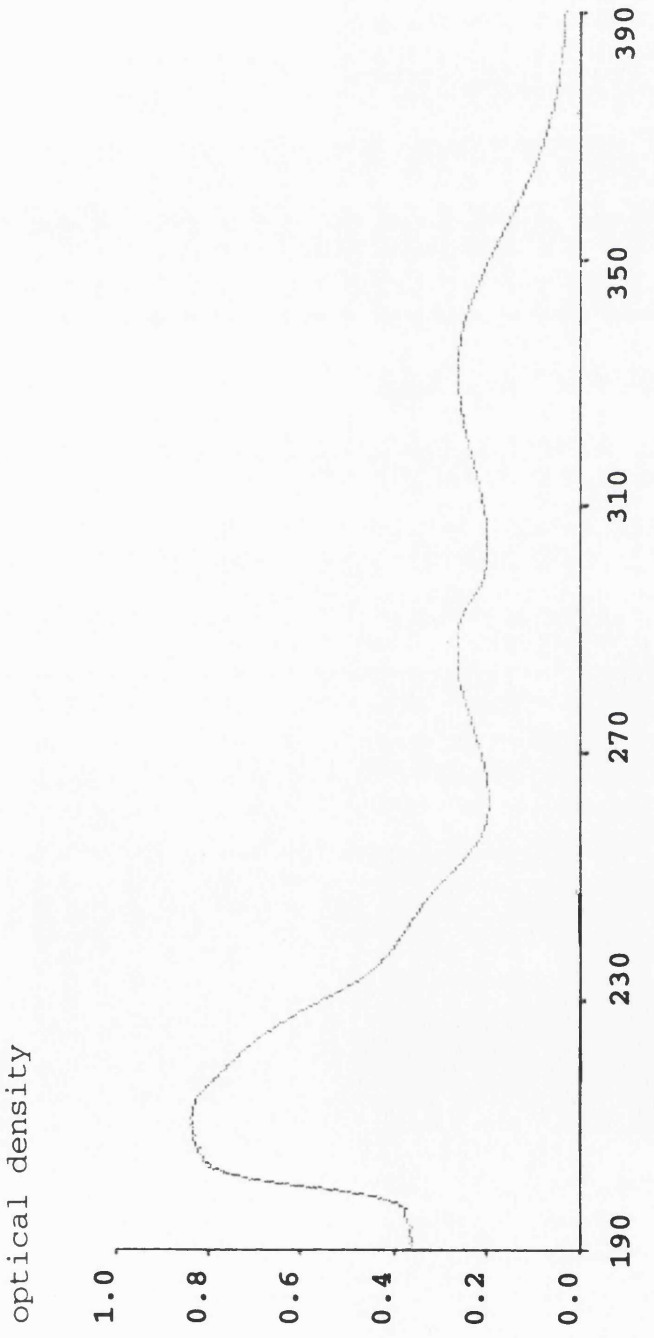


Fig. 41 EI Mass spectrum of 12-methoxyechitamidine

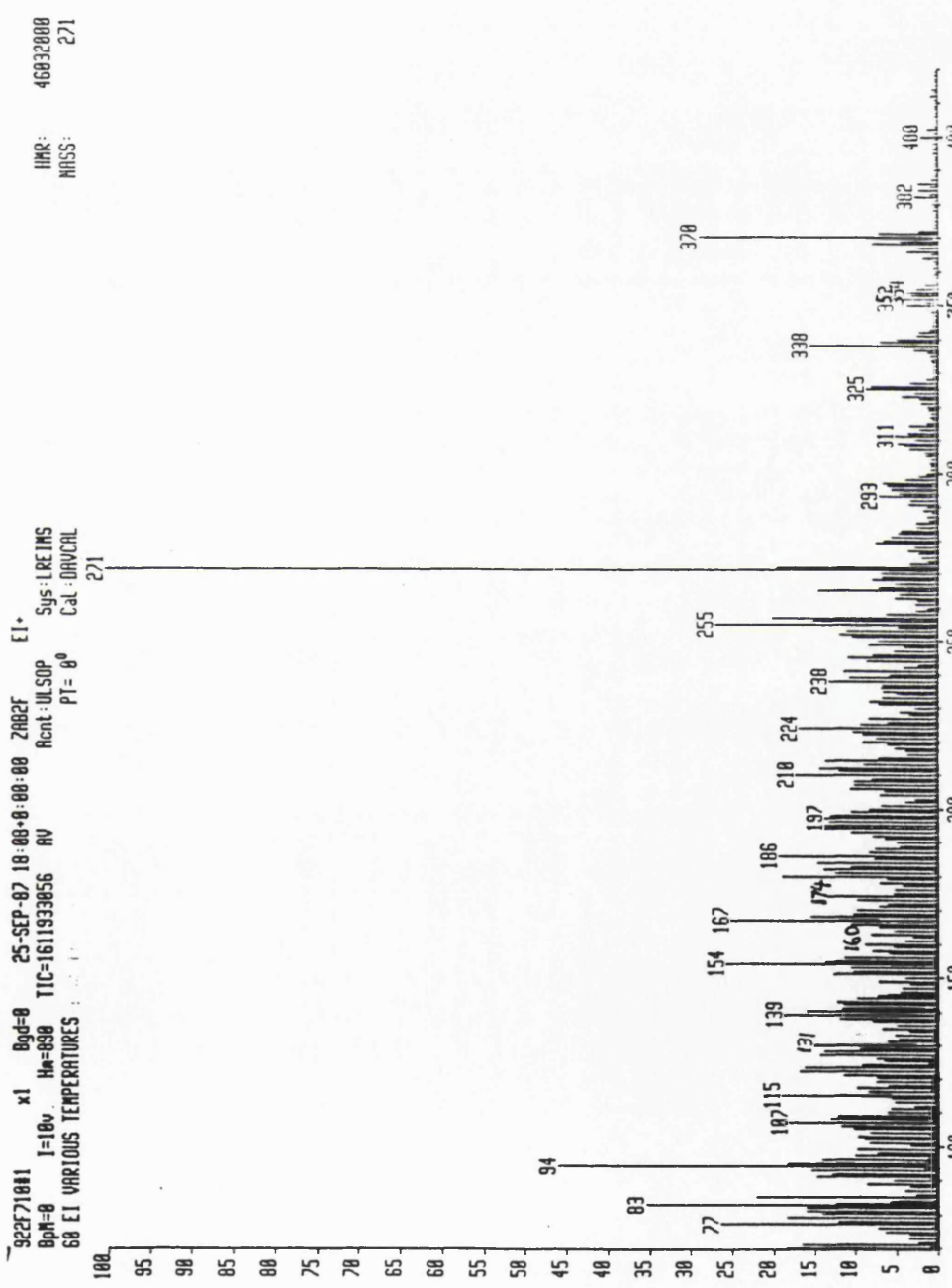
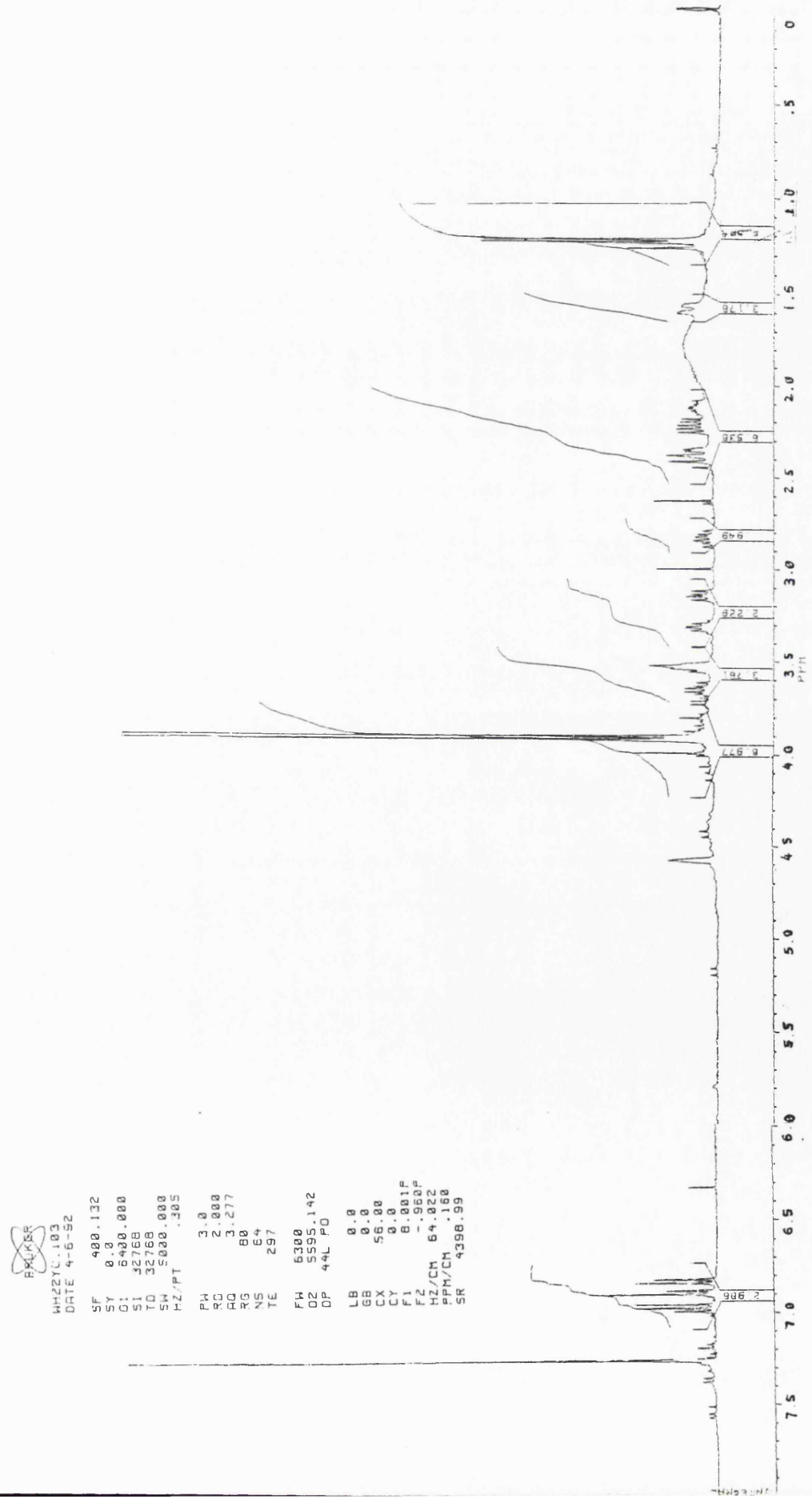


Fig. 42 ^{1}H NMR spectrum of 12-methoxyechitamidine



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 cm^2 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 $\mu g/ml^{-1}$ against trypanosomes at a starting concentration of 2 X $10^5/ml^{-1}$ 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% CO_2 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 ^{13}C 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_b-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 δ7.55, 7.36, 7.18, 7.10 and an NH at δ8.52), a carboxymethyl at δ3.78 and an N(4) methyl at δ2.31. The ^{13}C NMR spectrum (Fig. **33**, Page **108**, Table **6**, Page **99**) showed a signal for a carboxymethyl at δ173.09 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 (δ3.97) with H-20 (δ2.73), H-20 (δ2.70) with H-19 (δ1.25) and H-15 (δ2.78), H-19 (δ1.25) with H-18 (δ3.50). 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 H_2O), and an intense ion at m/z 271. The indole with a methoxy group in the aromatic region plus a C_2H_4 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_a-formyl-12-methoxyechitamidine (**110**; Fig. **43**, Page **127**). On the basis of UV, EIMS, and ^1H NMR fraction d is identified as 12-methoxyechitamidine.

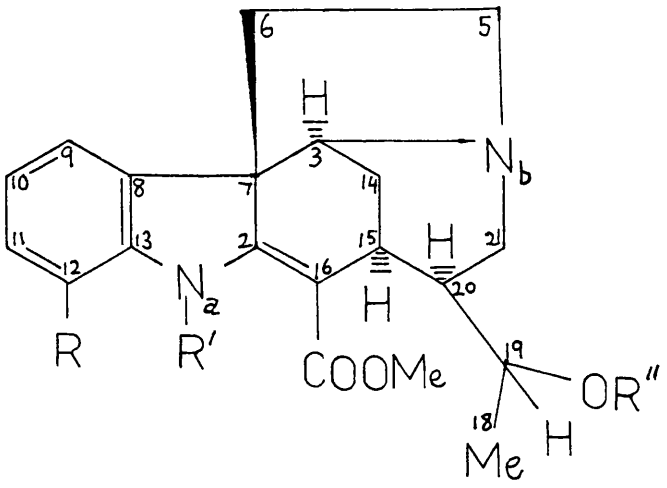
The EIMS of fraction c (Fig **36**, Page **111**,) clearly indicated that it was a mixture of two compounds with the presence of ion peaks at m/z 370 and 340 and prominent peaks at 271 and 241. This was indicative of a one methoxy group difference between the two compounds. The similarities between the 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 δ8.48 which integrates for half the NH peak of the major component at δ8.6.

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

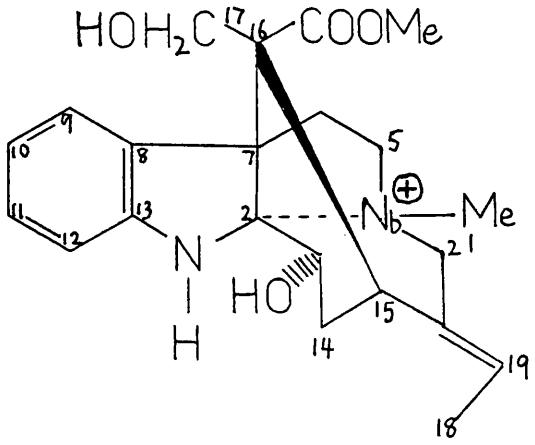
minor component can both be discerned in the COSY spectrum of fraction c, suggesting that the major component is echitamidine and the minor component is 12-methoxyechitamidine. This is further confirmed by the fact that the EI mass spectral data and the ^{1}H NMR spectral data of the major component 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.

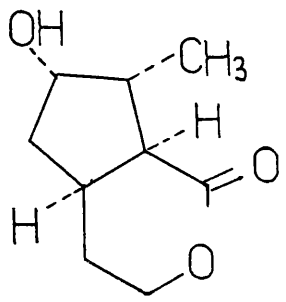
Fig. 43 Compounds previously isolated from *Alstonia boonei* bark (Oguakwa *et al*., 1983; Marini-Bettolo *et al*., 1983).



		R	R′	R″
(109)	N_a-formylechitamidine	H	CHO	H
(110)	N_a-formyl-12-methoxyechitamidine	OMe	CHO	H
(111)	echitamidine	H	H	H
(112)	O-acetyl-N_a-formyl-12-methoxy-echitamidine	OMe	CHO	Ac

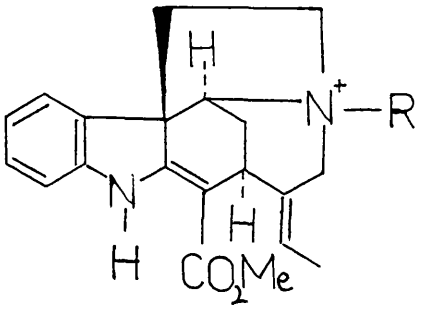


(113) echitamine



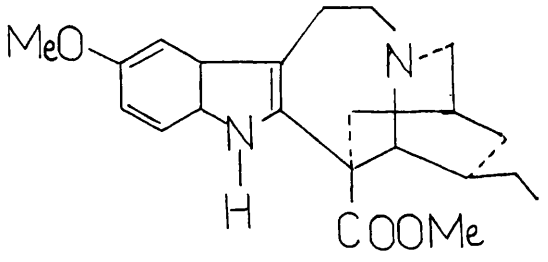
(114) boonein

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

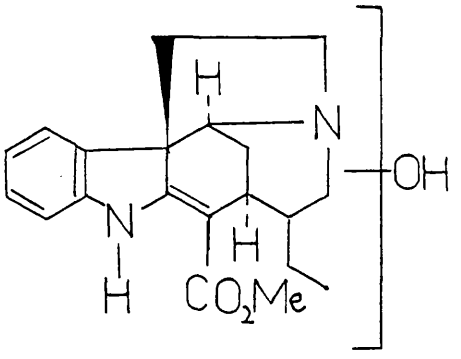


(**115**) akuammicine-N_b-methiodide R=CH_3

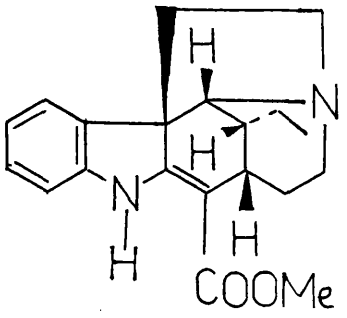
(**116**) akuammicine-N_b-oxide R=O^-



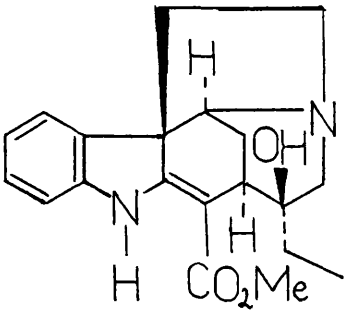
(**117**) voacangine



(**118**) hydroxy-19,20-dihydroakuammicine

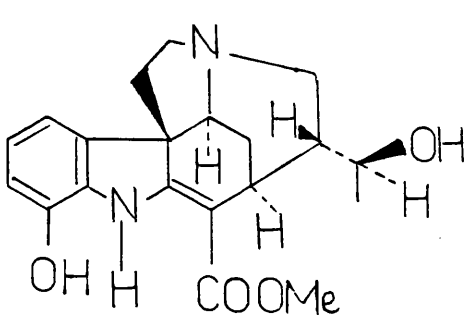


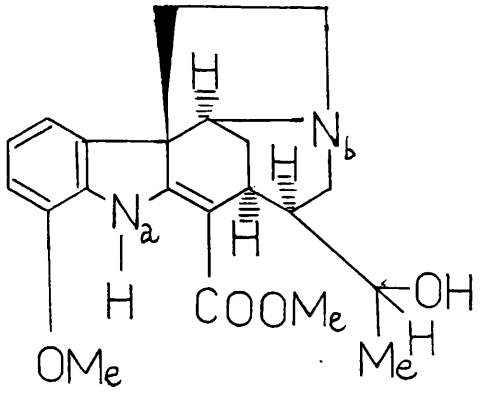
(**119**) tubotiawine



(**120**) lochneridine

Fig. 44 cont.

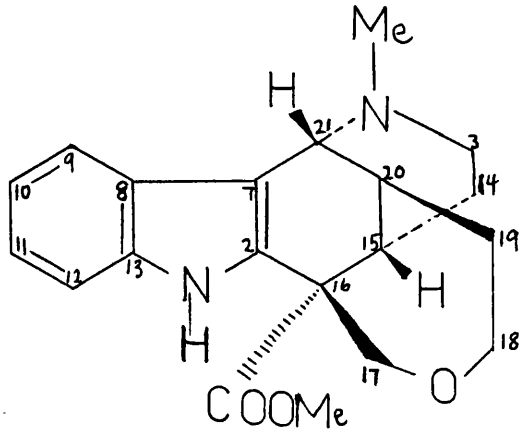




(121) 19-R-scholaricine (demethylscholarine)

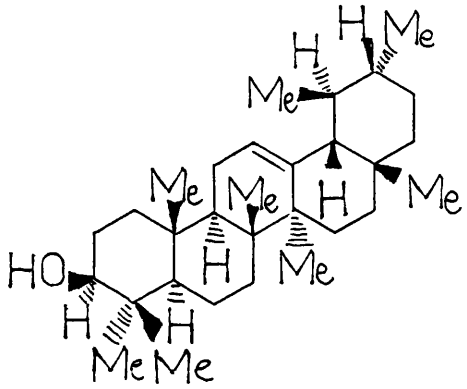
(122) scholarine ((±)-12-methoxyechitamidine)

Fig. 45

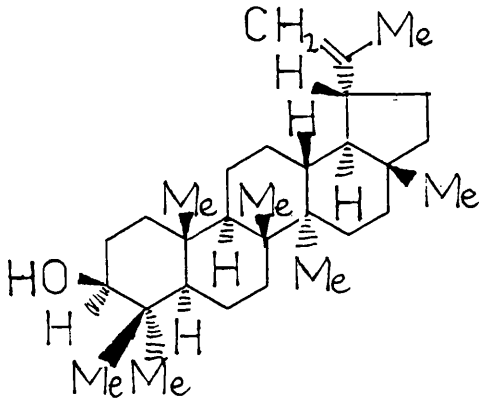


(123) undulifoline

Fig. 46 Terpenes from *Alstonia scholaris*



(124) α-amyrin



(125) lupeol

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 ED_{50} of 3.78 µg/ml
undulifoline (b) with an ED_{50} of 3.85 µg/ml
12-methoxyechitamidine (d) with an ED_{50} 2.98 µg/ml
The mixture of 12-methoxyechitamidine and echitamidine (c) had an ED_{50} 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.**

	inhibition at : (µg/ml)					
Plant species	15	30	60	125	250	500
A. cordifolia leaf	++	+++	+++	+++	+++	+++
'' bark	0	0	0	++	+++	+++
'' root	0	0	0	++	+++	+++
A. boonei bark	0	0	0	++	+++	+++
A. senegalensis bark	0	0	0	0	++	+++
control	0	0	0	0	0	0

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.**

	inhibition at: (µg/ml)				
type of extract	30	60	125	250	500
leaf methanolic	0	0	0	+++	+++
leaf aqueous	0	0	+++	+++	+++
bark methanolic	0	0	++	+++	+++
bark aqueous	0	0	0	0	0
root pet. ether	0	0	+++	+++	+++
root methanolic	0	0	+	+++	+++
control	0	0	0	0	0

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.

inhibition at: (µg/ml)

type of extract	30	60	125	250	500
pet. ether	0	+	+++	+++	+++
methanolic	0	0	+	+++	+++
aqueous	0	0	0	+++	+++
control	0	0	0	0	0

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

inhibition at : (µg/ml)

fraction	30	60	125	250	500
1	0	0	++	+++	+++
2	0	+	+++	+++	+++
4	0	0	+	+++	+++
control	0	0	0	0	0

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.

% inhibition at: (µg/ml)

fraction	30	10	3	1	ED_{50} ± SD	95% CI
a	100	55.6	40.9	35.7	3.78±0.165	1.36, 19.36
b	100	45.0	55.0	33.3	3.85±0.228	2.48, 36.9
c	100	36.8	42.1	10.5	6.01±0.176	1.05, 34.4
d	100	64.3	42.7	41.5	2.98±0.16	1.64, 14.6

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[1] 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-N_b-oxide (**116**), voacangine (**117**), hydroxy-19, 20-dihydroakuammicine (**118**), tubotiawine (**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 *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_a-formylechitamidine (**109**), N_a-formyl-12-methoxyechitamidine (**110**), echitamidine (**111**) and O-acetyl-N_a-formyl-12-methoxy-echitamidine (**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 *A. boonei*.

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

The compounds isolated in this study had low activity *in vitro* against *T. b. brucei*. However, echitamine, the major compound present in *A. boonei*, is relatively non-toxic,

having an LD_{50} 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 Oguntimein (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.

CHAPTER 3 PLANT PRODUCTS WITH ACTIVITY AGAINST *LEISHMANIA* SPECIES

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 exluding 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).

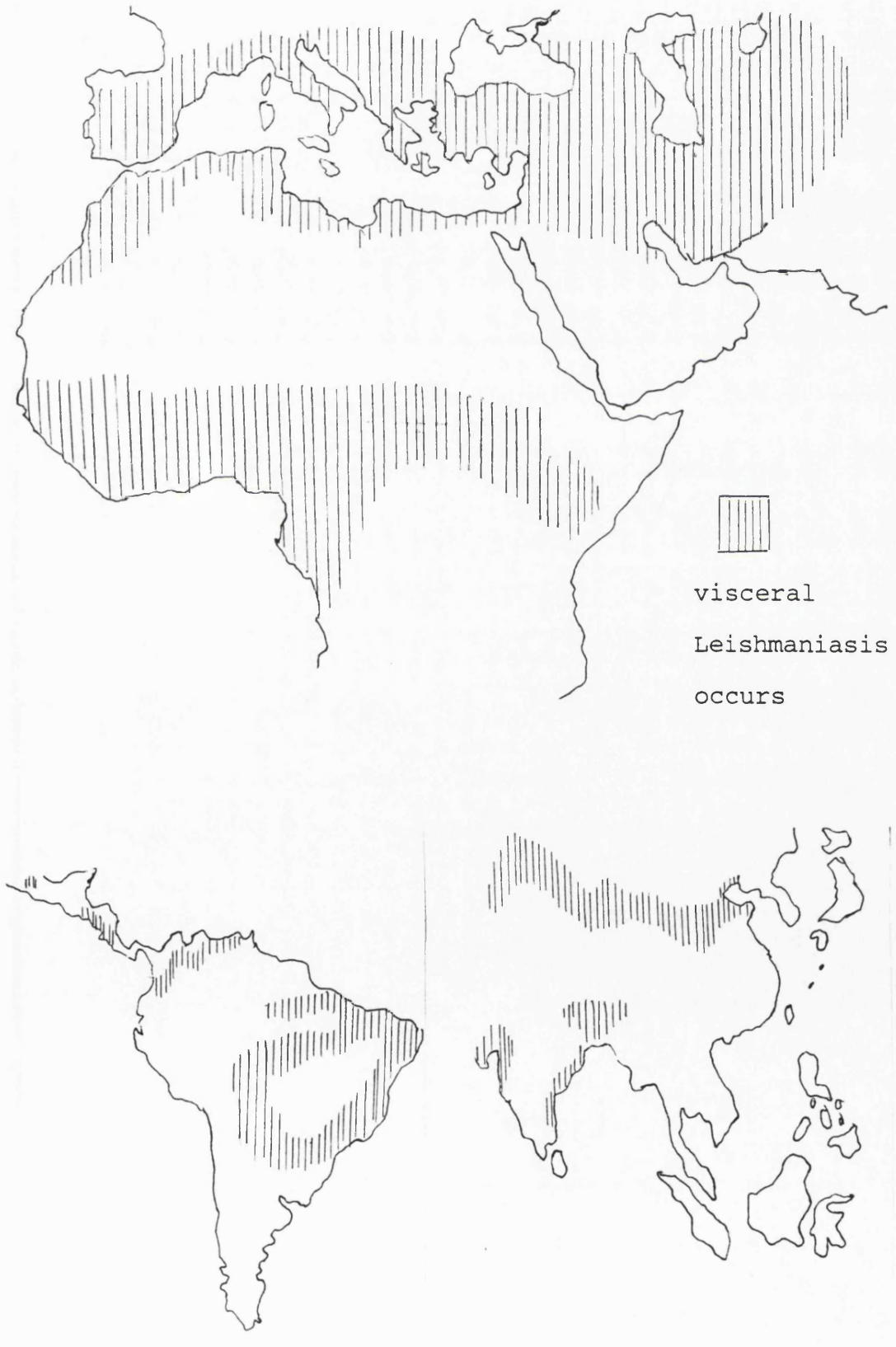


Table 12 Geographical distribution of the clinical disease caused by different species of *Leishmania* (adapted from Neva and Sacks, 1990).

Species	geographical distribution	clinical manifestations
L. mexicana complex *L. mexicana* *L. amazonensis* *L. venezuelensis*	New World, from southern US to central S. America	cutaneous ulcers; small proportion of cases develop diffuse cutaneous (DCL) or mucocutaneous (MCL) leishmaniasis
L. braziliensis complex *L. braziliensis* *L. guyanensis* *L. panamensis* *L. peruviana*	New World, from central America to northern Argentina	cutaneous ulcers; some cases may later develop MCL (probably more likely if cutaneous lesion not treated adequately)
L. major	Northern and central Africa, Middle East, and S. Asia	cutaneous ulcers
L. tropica	Middle East and S. Asia	cutaneous ulcers and chronic relapsing cutaneous disease
L. aethiopica	Ethiopia and contiguous countries	cutaneous ulcers, rarely diffuse cutaneous leishmaniasis (DCL)
L. donovani complex	E. Africa and south of the Sahara, Indian subcontinent, S. Asia	visceral leishmaniasis; small proportion may develop post-kala-azar dermal leishmaniasis (PKDL)
L. infantum	N. Africa and S. Europe	visceral leishmaniasis
L. chagasi	Brazil, Venezuela, Colombia, and isolated cases in central and S. America	visceral leishmaniasis

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

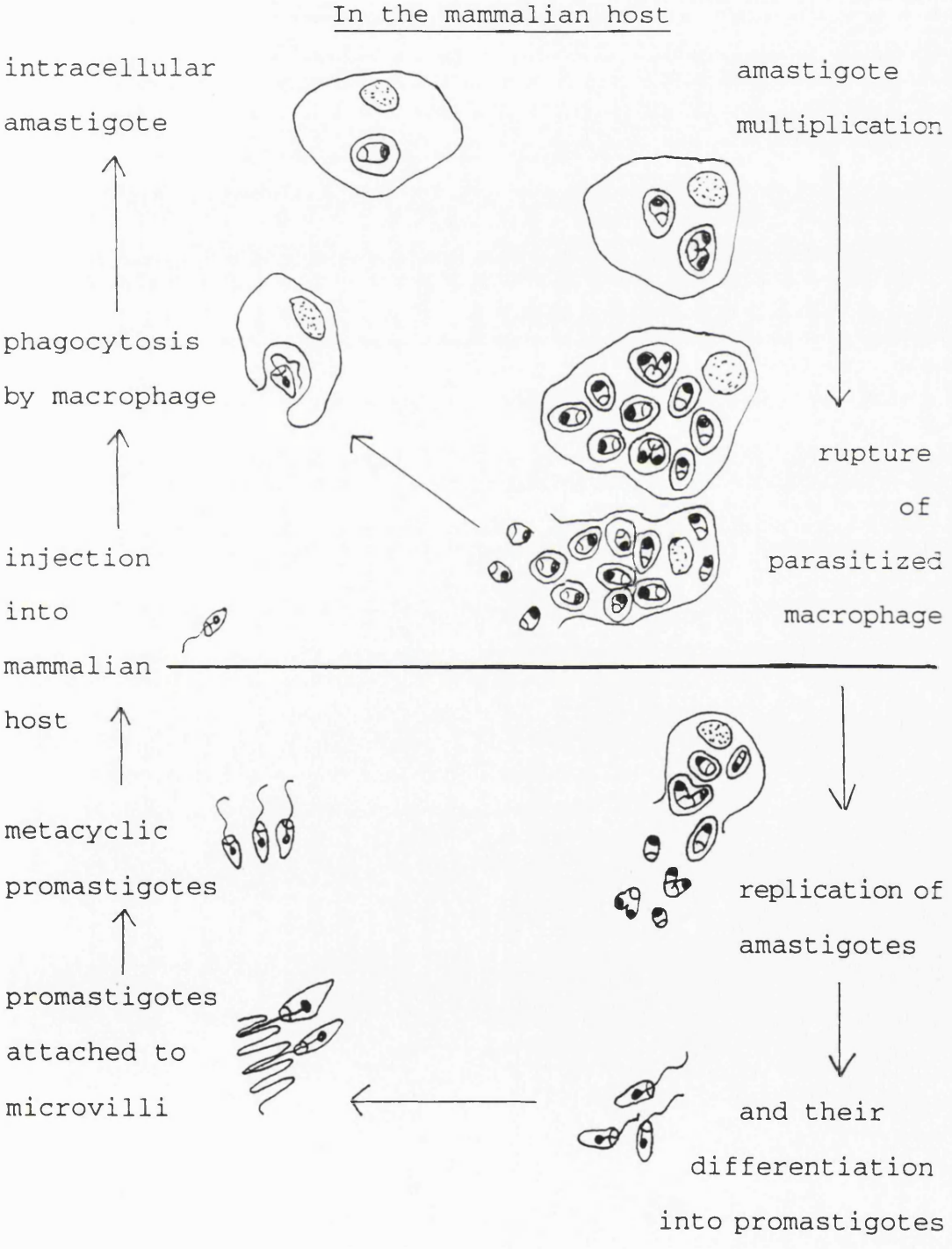
apparently been cured (WHO, 1990b).

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)



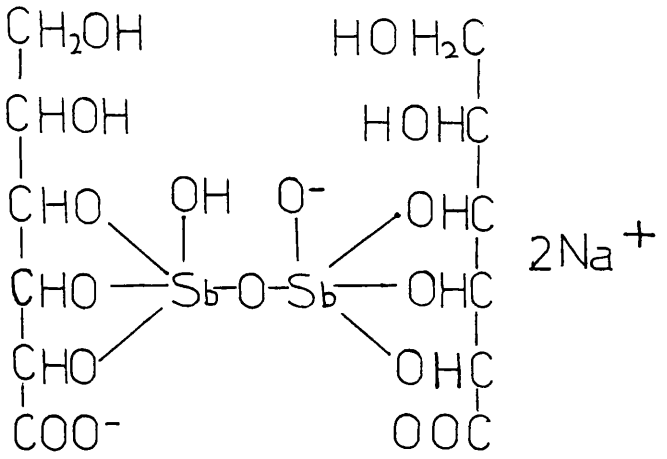
When the infected sandfly takes another blood meal promastigote forms are deposited in the skin of the mammalian host where they are phagocytosed by macrophages, enter a parasitophorous vacuole and transform into actively dividing amastigote forms. Heavily-parasitised macrophages rupture, releasing amastigote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

3.3 Current clinical chemotherapy

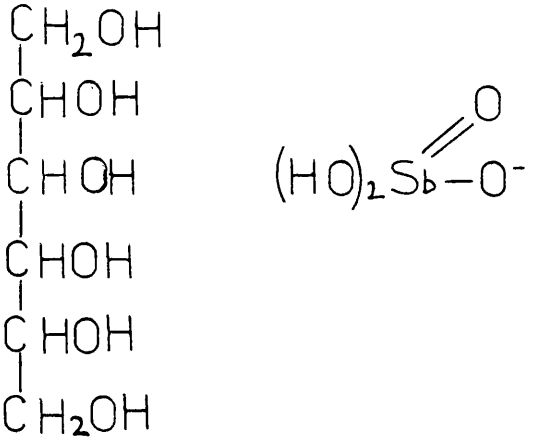
The first choice drugs, the pentavalent antimonials sodium stibogluconate (**126**; Pentostam, assumed structure) and meglumine antimoniate (**127**; Glucantime, assumed structure; Fig. **51**; Page **151**), require parenteral administration under medical supervision and side effects frequently occur (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).

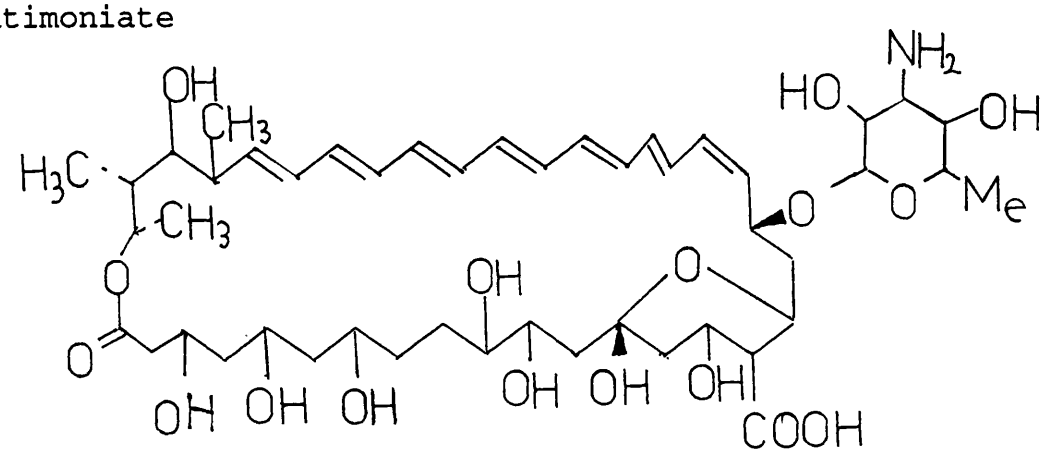
Fig. 51 Compounds currently in use or undergoing clinical trial for the treatment of leishmaniasis.



(**126**) sodium stibogluconate

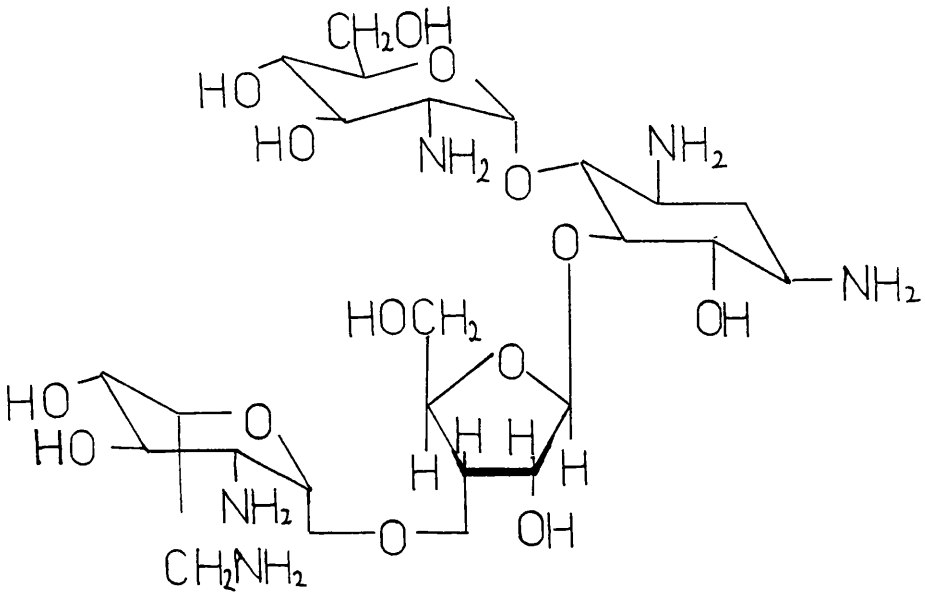


(**127**) meglumine antimoniate

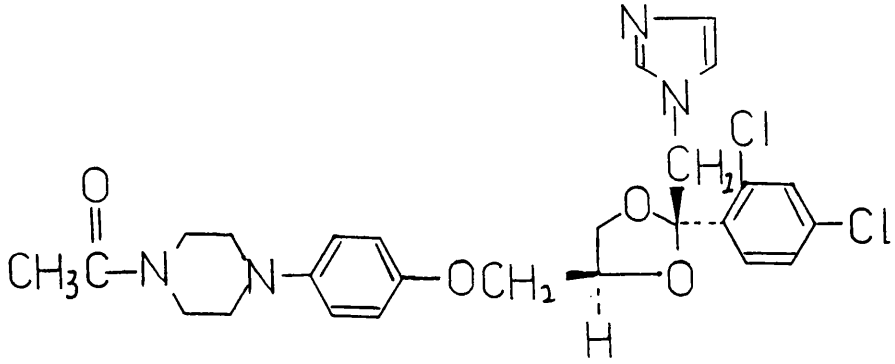


(**128**) amphotericin B

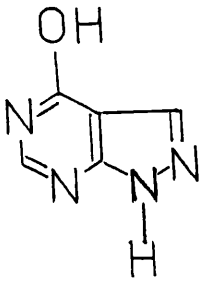
Fig. 51 cont.



(**129**) paromomycin

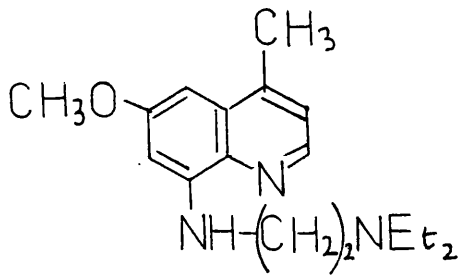


(**130**) ketoconazole



(**131**) allopurinol

Fig. 51 continued



(**132**) WR6026

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 ecosytems 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).

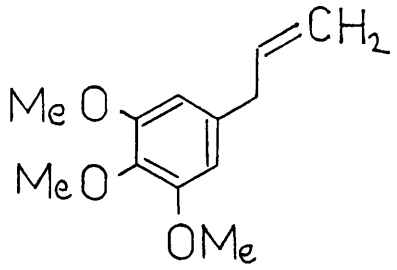
3.4.1 *Virola species*

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).

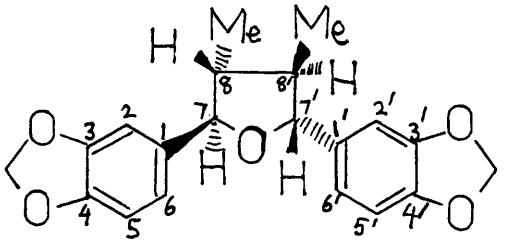
3.4.2 Neolignans

Neolignans, which occur in Myristicaceae and other primitive plant families, are usually dimers of the oxidative coupling of allyl and propenyl phenols (Gottlieb, 1978).

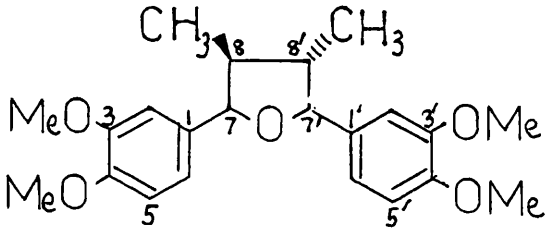
Fig. 52 Neolignans isolated from *Virola surinamensis* (Barata *et al.*, 1978).



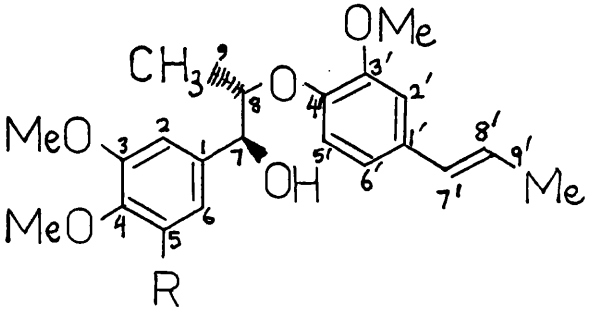
(**133**) elemisin
(not a neolignan)



(**134**) galbacin



(**135**) veraguensin



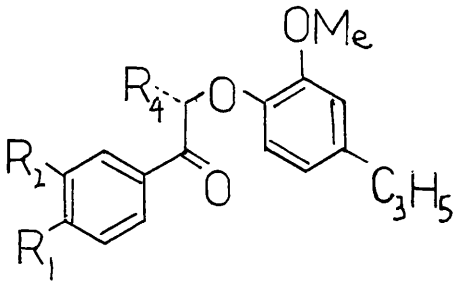
(**136**) surinamensin R=OMe

(**137**) virolin R=H

Fig. 53 **Type I synthetic analogues of surinamensin from *Virola surinamensis*.**

(138) R_1 = OMe R_2 = OMe
R_4 = CH_3

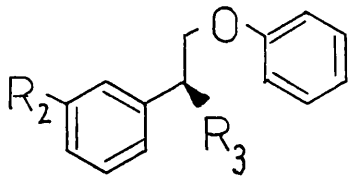
(139) R_1 = O R_2 = OCH_2O
R_4 = H

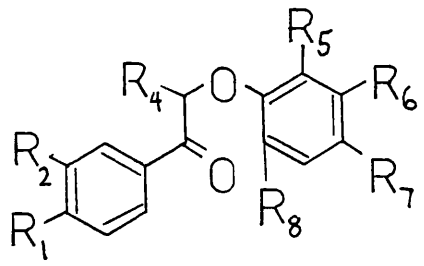


(140) R_3 = OH R_2 = H

(141) R_3 = NH_2 R_2 = Me

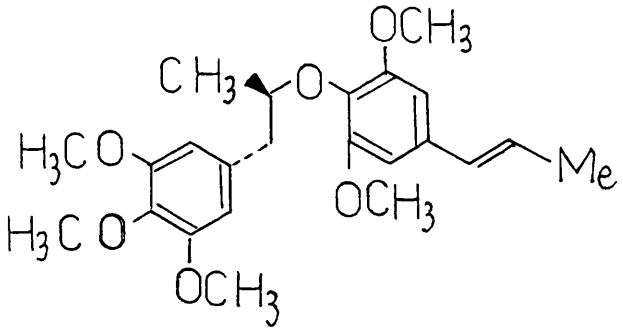
(142) R_3 = NOH R_2 = H



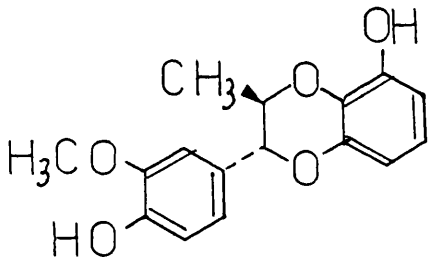


	R_1	R_2	R_4	R_5	R_6	R_7	R_8
(143)	H	H	H	H	H	H	H
(144)	H	H	H	OMe	H	H	H
(145)	H	H	H	H	H	OMe	H
(146)	H	H	H	H	H	Cl	H
(147)	H	H	H	NO_2	H	H	H
(148)	OMe	OMe	O	H	H	OMe	H
(149)	OMe	OMe	H	OMe	H	H	H
(150)	OMe	OMe	H	H	H	Cl	H
(151)	OMe	OMe	H	OMe	H	Propenyl	H
(152)	OMe	OMe	O	OMe	H	H	OMe
(153)	H	H	O	H	Me	H	H

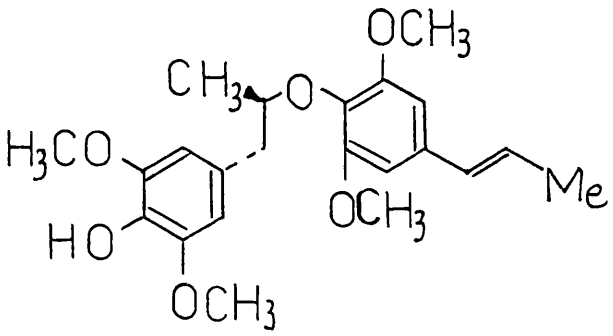
Fig. 54 **Natural neolignan (154) from *Virola pavonis* and synthetic derivatives (155 and 156)** (Ferri and Barata, 1992).



(**154**) natural neolignan from *V. pavonis*



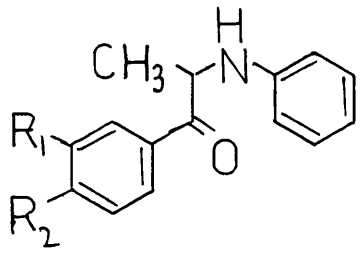
(**155**) synthetic analogue of a neolignan derived from *V. pavonis*



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

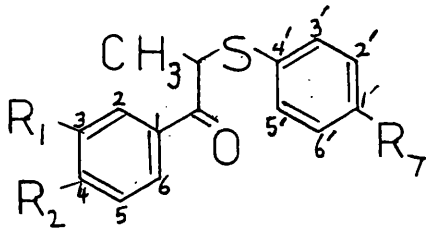
Fig. 55 Type II synthetic N- (157, 158) and type III S- (159-162) analogues of neolignans from *Virola surinamensis* (Ferri and Barata, 1991).

Type II



(**157**) R_1 = H R_2 = H

(**158**) R_1 = OMe R_2 = OMe



Type III

(**159**) R_1 = H R_2 = H R_7 = Cl

(**160**) R_1 = OMe R_2 = OMe R_7 = CH_3

(**161**) R_1 = OMe R_2 = OMe R_7 = Cl

(**162**) R_1 = OMe R_2 = OMe R_7 = H

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-O-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*

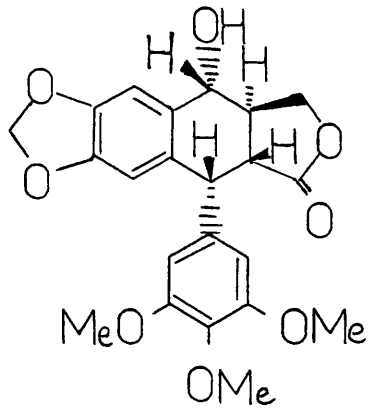
Several natural products are known to act on specific sites within the cell or to interfere with certain cell processes. In some cases these specific sites or cell processes may be the target of chemotherapeutic drugs. For example, inhibition of microtubule assembly:

3.5.1 Microtubule inhibitors

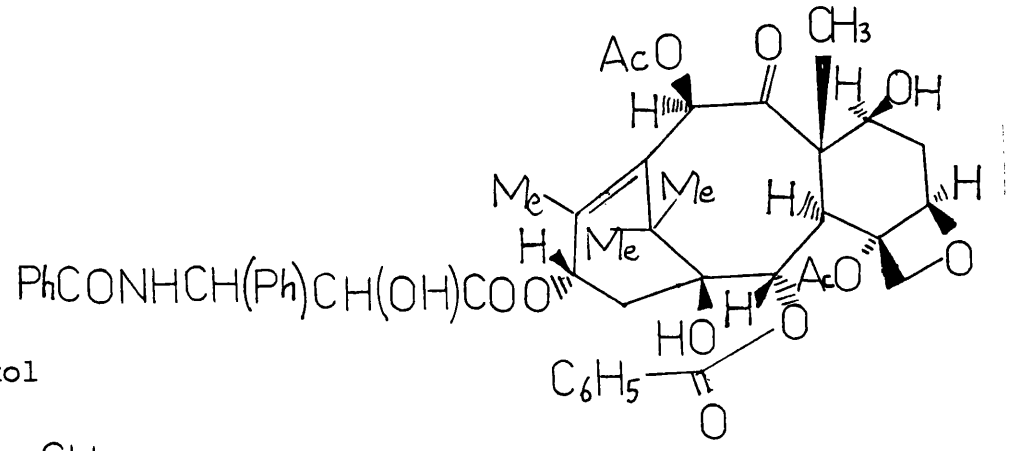
Microtubule assembly may be inhibited as a result of disruption of microtubule-assembly, for example by podophyllotoxin (**163**) from *Podophyllum spp.* rhizomes or *Vinca spp.* alkaloids, or as a result of microtubule-stabilization, for example, by taxol (**164**; Fig. **56**; Page **161**), isolated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

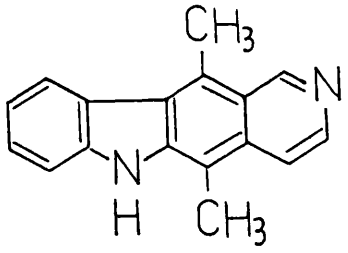
Fig. 56 Some microtubule and topoisomerase inhibitors



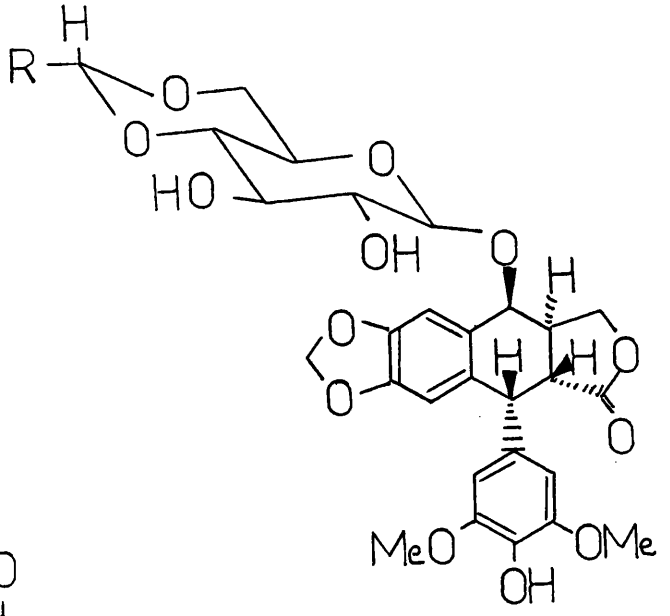
(**163**) podophyllotoxin



(**164**) taxol

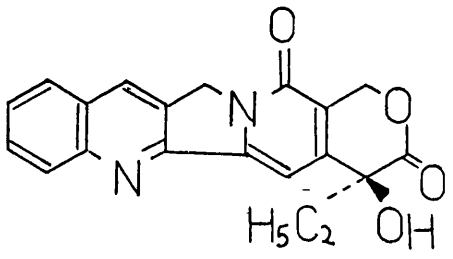


(**165**) ellipticine



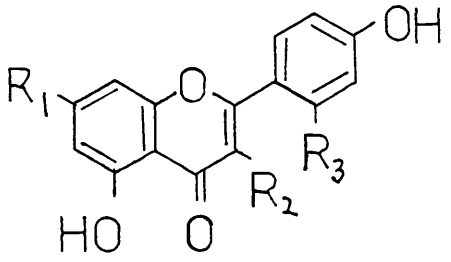
(**166**) etoposide R = Me

(**167**) teniposide R =



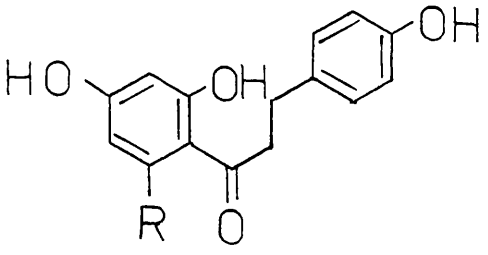
(**168**) camptothecin

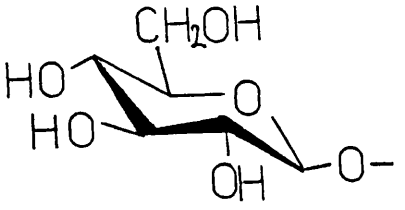
Fig. 57 Some glucose transport inhibitors (169- 171) and some chelators of peroxyl radicals (173, 172).



(**169**) cosmetin R_1 = O-glucose R_2 = H R_3 = H

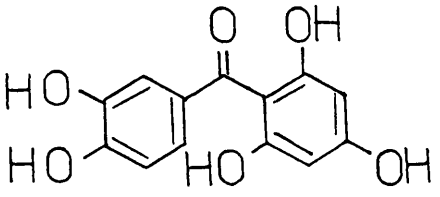
(**172**) morin R_1 = OH R_2 = OH R_3 = OH





(**170**) phloridzin R =

(**171**) phloretin R = 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 epipodophyllo-toxins 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^{0}C in tissue culture flasks. The medium was changed every four days.

3.6.2 *T. cruzi* epimastigote form culturing technique.

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 *L. donovani* test procedures

3.6.3.1 Tests against *L. donovani* promastigote forms *in vitro*.

In vitro promastigote assays were carried out in 96-well microplates using fast-growing *L. donovani* promastigote forms grown in Schneider's medium (Gibco) with 10% heat-inactivited 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 10^5 cells per ml and maintained in wells of sterile tissue-culture eight-chamber slides (Labtek Products, Miles Laboratories) at 37°C in a 5% CO_2/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

active at 30 μM and 100 μM (Table 13; Page 172). 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 100μM, 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 1μM. The antimitotic agent podophyllotoxin (**163**) was toxic to macrophages and amastigote forms at 30μM but inactive at 10μM, 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

forms and *T. cruzi* epimastigote forms:
aqueous extracts of *Alchornea cordifolia* leaf, bark, root and stem, *Alstonia boonei* bark, *Annona seneglensis* bark, leaves and root, *Lonchocarpus cyanensis* root, *Tamarindus indica* root, *Ximenia americana* bark and stem and the methanolic and petroleum ether extracts of *Alstonia boonei* bark and *Alchornea cordifolia* leaf, bark and root.

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*.**

Compound	inhibition at 100μM	inhibition at 30 μM
138	++++	++++
139	++++	++++
141	++++	++++
146	++++	++++
147	++++	++++
148	++++	++++
150	++++	++++
151	++++	++++
152	++++	++++
153	++++	++++
154	++++	++
155	++++	++
156	++++	+++
157	+++	++
158	++++	++++
159	++	-
160	++++	++
161	++++	++++
162	++++	++++

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*.

com-pound	%I at 100 µM ± S.E.	%I at30 µM ± S.E.	%I at 10µM	IC_{50}	MTC (µM)
159	100 ± 0	46.3 ± 5	0	31.3	300
160	100 ± 0	70.6 ± 3.3	0	29.6	300
161	100 ± 0	90.2 ± 0.9	0	27.5	300
162	83.3 ± 1.5	0	0		300
con-trol	%I at 27* ± S.E. 81.3 ±1.5	%I at 9* ± S.E. 77.4 ± 5.2	%I at 3* ± S.E 23.0 ± 3.8	5.32	27*

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. $IC_{50}$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.

compound	Dose level (mg/kg/day for 5 days)	%I
159	100	-1
160	100	42 P >0.5
161	100	-19
162	100	17
Na-stibo-gluconate	45	7 P >0.5
	15	74 P >0.5

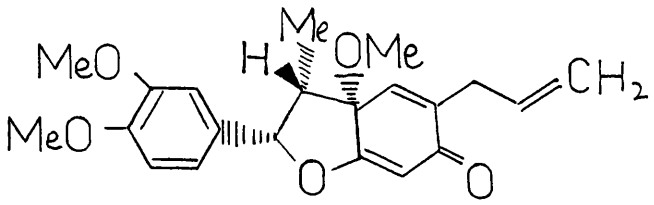
Footnote I = inhibition.
The test used five mice per group.
P values were derived by a T test.

3.10 Discussion

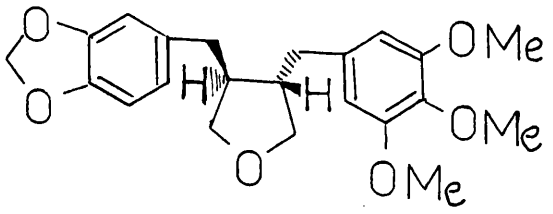
Lignans have shown promise in areas of cancer chemotherapy (for example etoposide (**166**) and teniposide (**167**), two semi-synthetic derivatives of podophyllotoxin (**163**; Fig. **56**; Page **161**; O'Dwyer *et al.*, 1985), which is used in the treatment of venereal warts. The neolignans kadsurenone (**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.

Fig. 58 Some biologically active neolignans



(**174**) kadsurenone



(**175**) burseran

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% CO_2 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). $5x10^6$ *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 MmMHz spectrophotometer in $CDCl_3$, with TMS as internal standard. The chemical shift values (δ) were recorded in ppm, and the coupling constant (J) as Hz. ^1H NMR, ^{13}C 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

δ3.2 (18H, s, $6CH_3$), 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).

^{13}C NMR 67.80MHz (CD_3OD)

δ40.70 ($6CH_3$: 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

λ_{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

δ3.25 (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

δ40.70 ($6CH_3$: 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. 59 ^{1}H NMR spectrum of Kubbal azrak, fraction F15

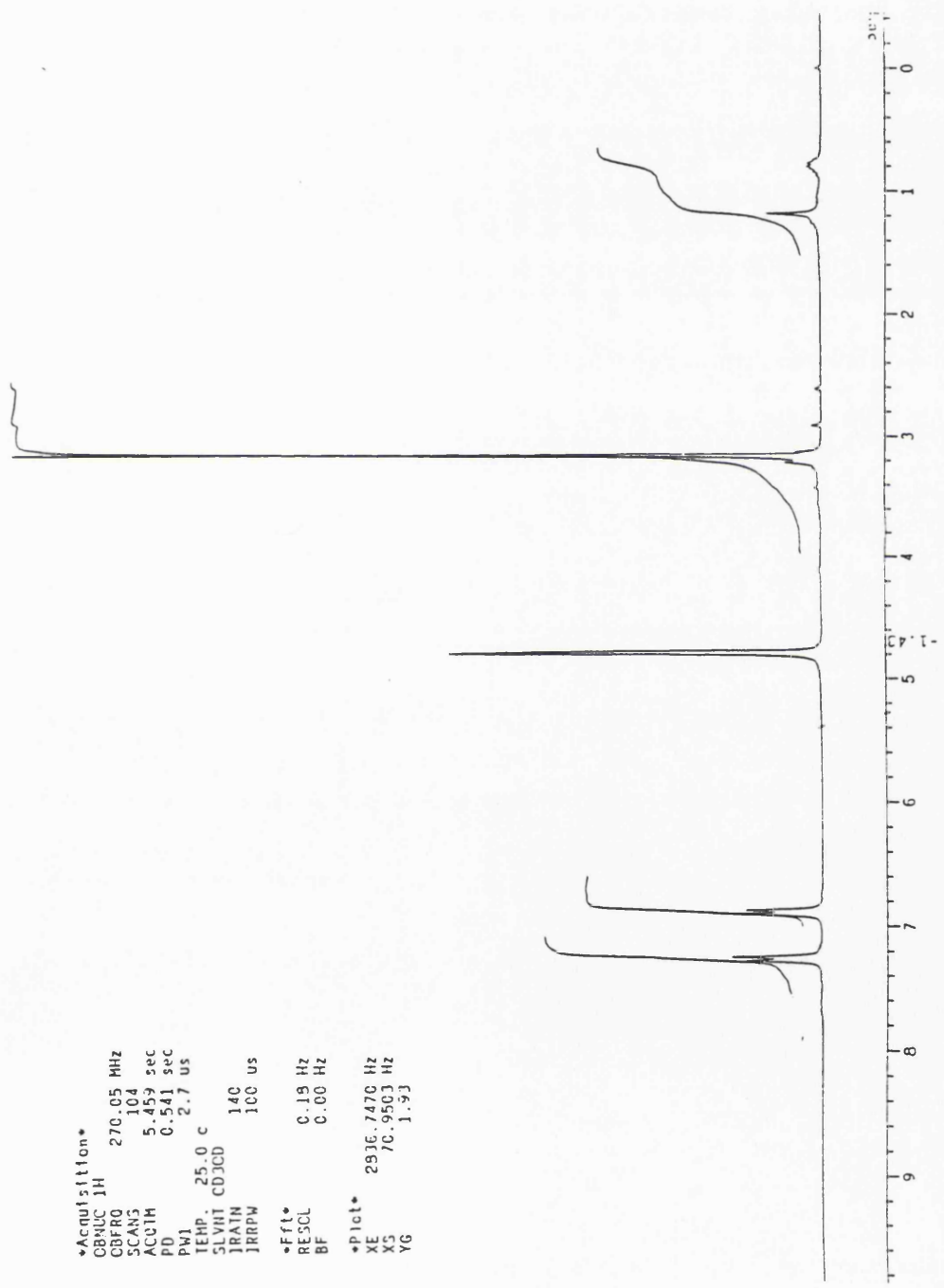


Fig. 60 ^{13}C NMR spectrum of Kubbal azrak, fraction F15

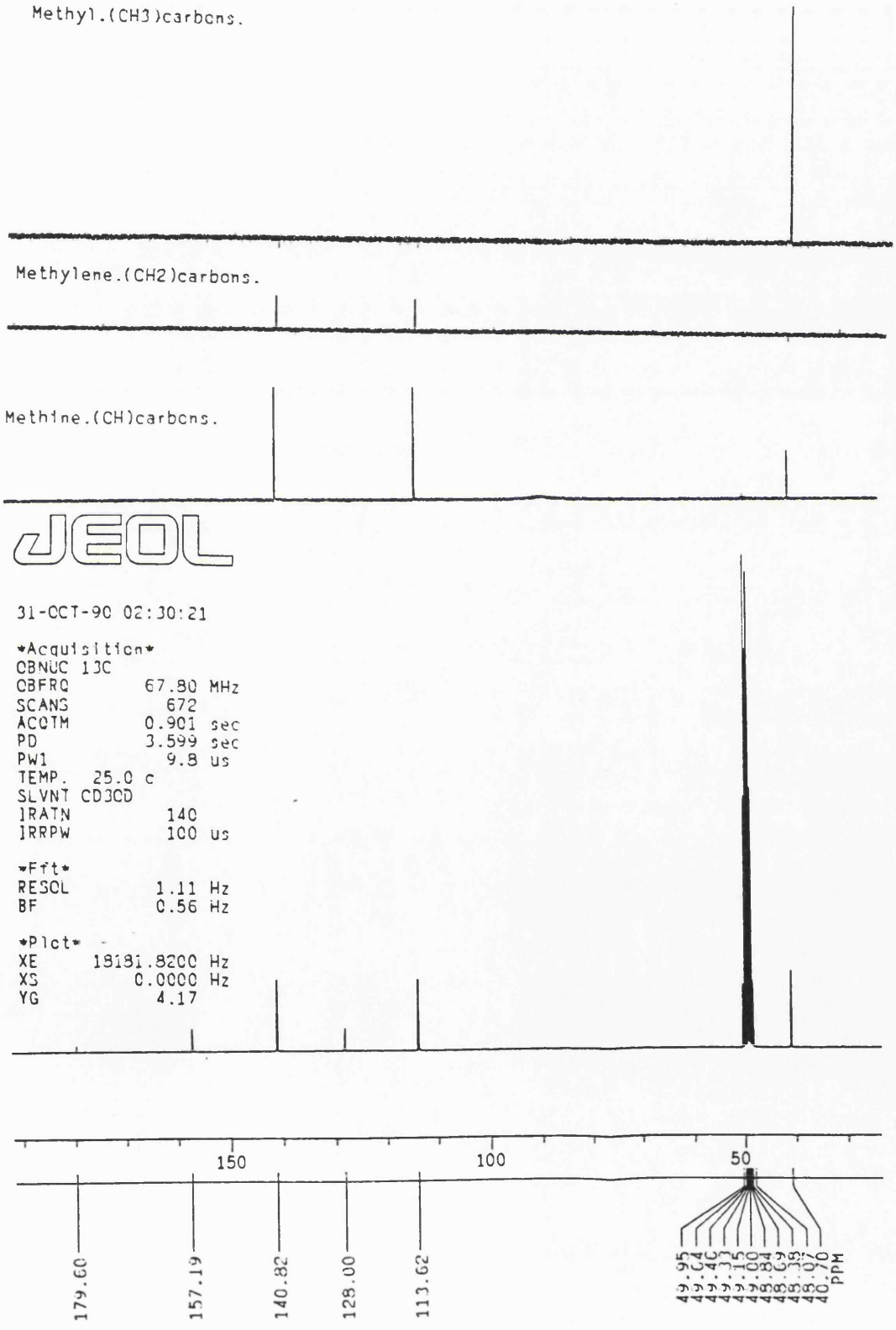
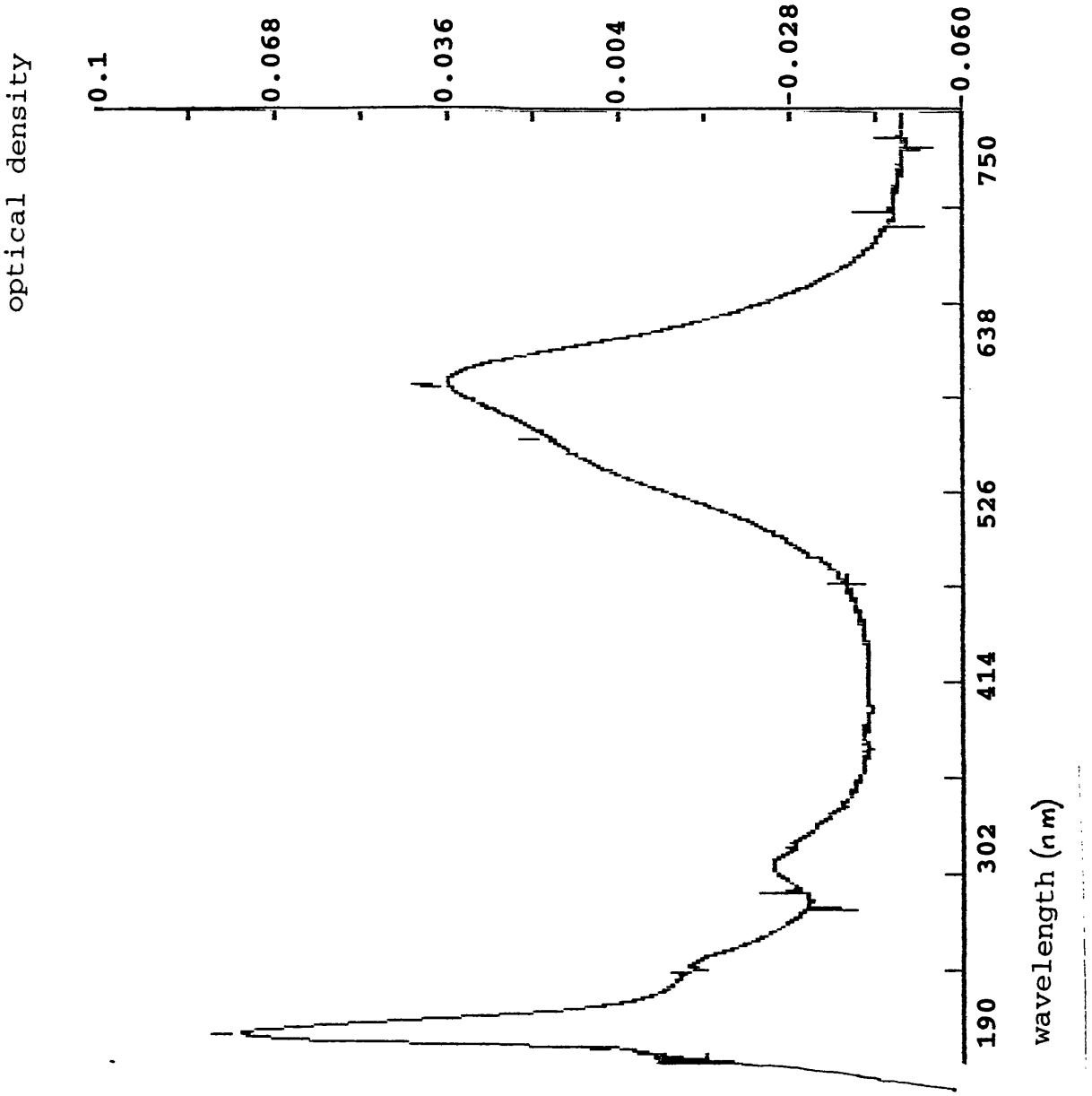


Fig. 61 UV spectrum of Kubbal azrak, fraction F15



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 δ3.2, 6.8 and 7.3 and the ^{13}C NMR spectrum showed five signals indicating $6CH_3$ at δ40.70, 6CH at 113.62, 6CH at 140.82, There were three quaternary carbon signals, 1, 1', 1'' at δ157.19, and three quaternary carbon signals, 4, 4' 4'' at δ128.00 and one quaternary carbon signal -C at δ179.60.

4.6.1 Results of *in vitro* tests

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

Fractions F15 and F18 caused total inhibition of amastigotes at 1 µg/ml, but were also toxic to macrophages. Fraction F19 caused 52.8% inhibition at 10 µg/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

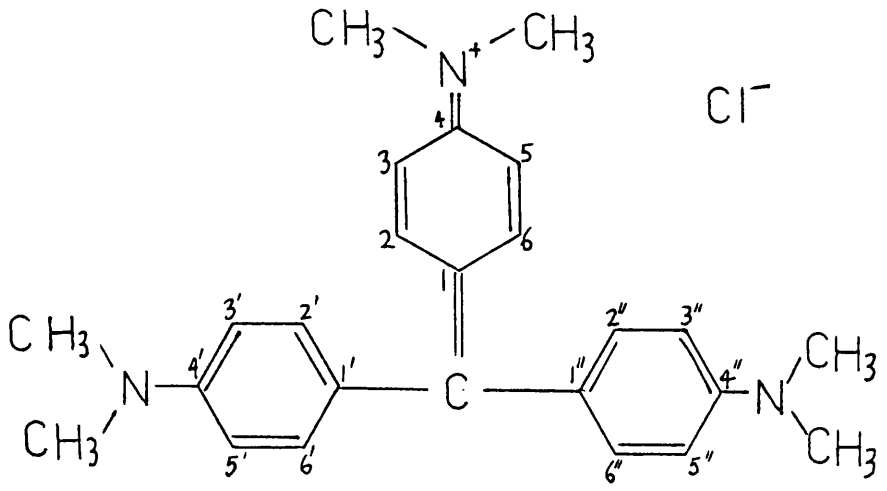
The sample of Kubbal azrak was obtained from Iraq in the beleif that it contained plant material but when examined microscopically the powder completely dissolved in chloral hydrate solution and there was no microscopic evidence for the presenc of plant material. It was composed mainly of gentian violet.

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 x 10^{-5} M (Pal and Ghosh, 1994).

Fig. 62 Gentian violet (176)



CHAPTER 5 ANTIMALARIAL ACTIVITY OF CRYPTOLEPINE, A PLANT-DERIVED INDOLOQUINOLINE *IN VITRO* AND *IN VIVO*

5.1 Introduction

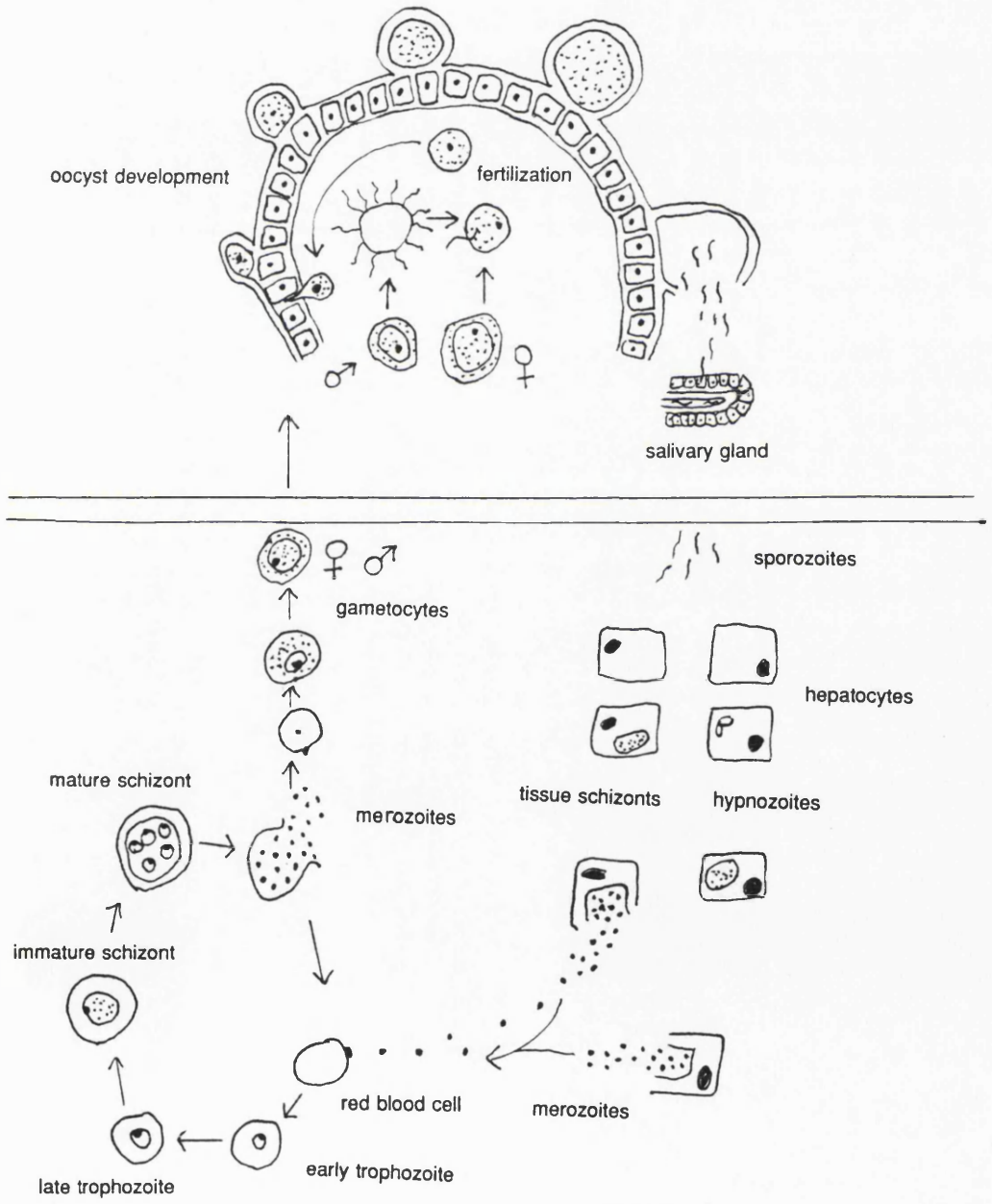
Worldwide, an estimated 300 million people are infected with malaria (of which 275 million in Africa; TDR News, 1992). The number of clinical cases per year is estimated to be over 120 million (of which 110 million in Africa) and the annual number of deaths caused by malaria is estimated to be between 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)



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

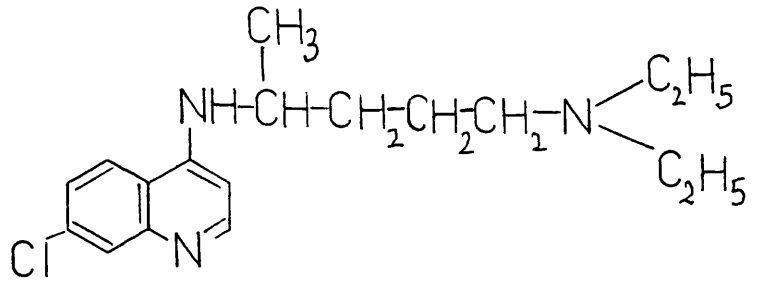
Malaria caused by chloroquine-sensitive species of *Plasmodium vivax, P. malariae* and *P. ovale* can be treated with chloroquine (**177**), amodiaquine (**178**) or quinidine (**19**). However, these drugs only eradicate intra-erythrocytic *Plasmodium* organisms so that a second drug is required for 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).

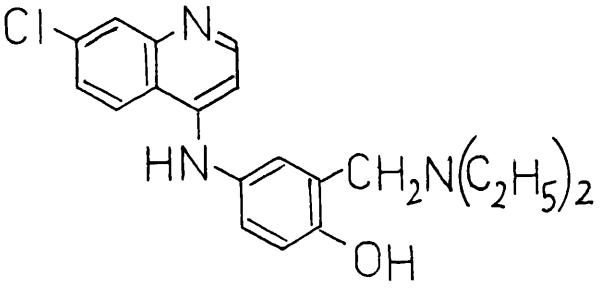
Resistance of *P. falciparum* to chloroquine can now be described as occurring worldwide and there is increasing resistance to other standard antimalarials (Wernsdorfer, 1991). This makes the treatment of this type of malaria difficult. Quinine (**18**) or mefloquine (**180**) may be used to eradicate 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 sulfa-pyrimethamine 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.

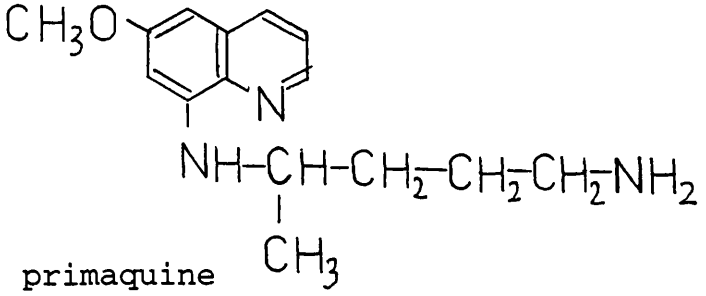
Fig.64 Some antimalarial Drugs



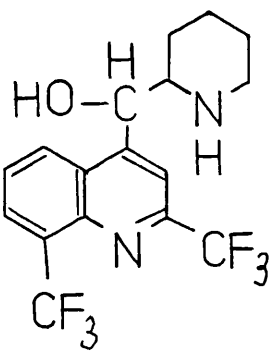
(177) chloroquine



(178) amodiaquine

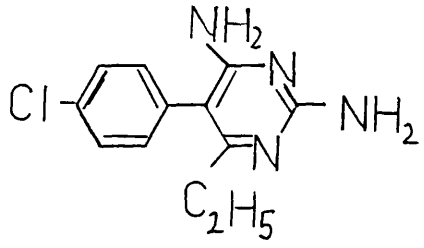


(179) primaquine

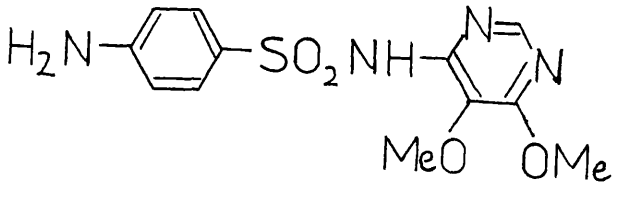


(180) mefloquine

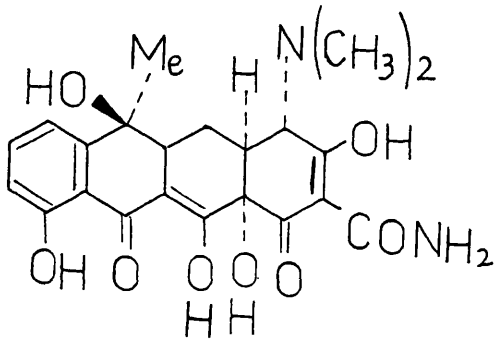
Fig. 64 cont.



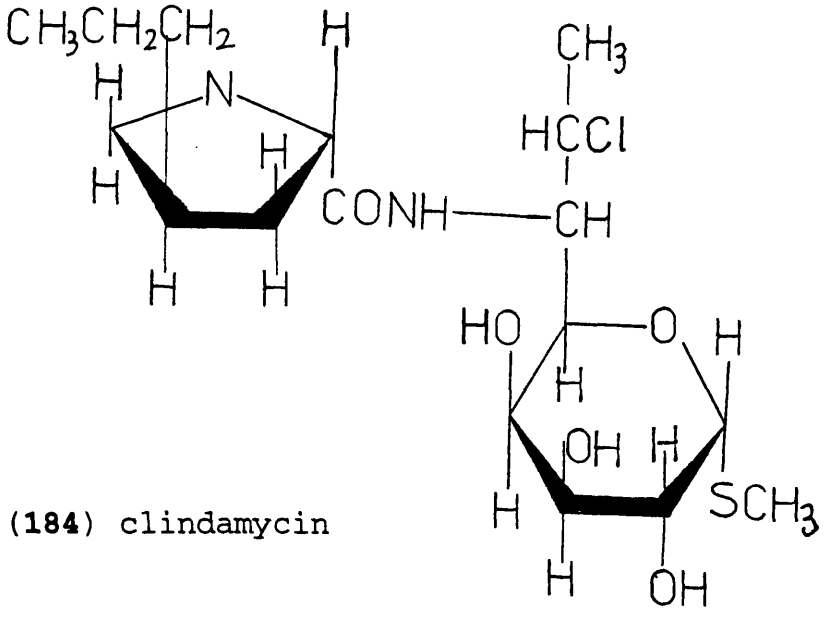
(**181**) pyrimethamine



(**182**) sulfadoxine

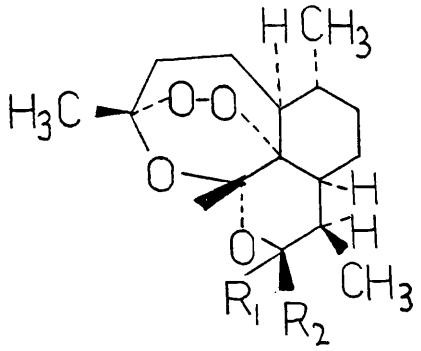


(**183**) tetracycline



(**184**) clindamycin

Fig. 64 cont.



(**185**) artesunate R_1 = H R_2 = ----$COOCH_2CH_2COOH$

(**186**) artemether R_1 = ----H R_2 = OCH_3

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:

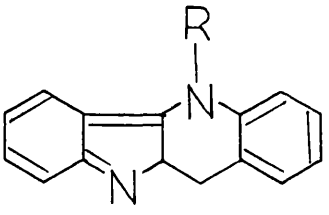
i) Hypotensive vasodilatation effects were demonstrated in the dog (Raymond-Hamet, 1938);
ii) Carrageenin-induced oedema-reducing effects were demonstrated in the rat hind paw subplantar tissues (Bamgbose and Noamesi, 1981);
iii) Prostaglandin E_2 antagonism effects were demonstrated in the superfused, isolated, rat stomach strip preparation (Bamgbose and Noamesi, 1981) and in

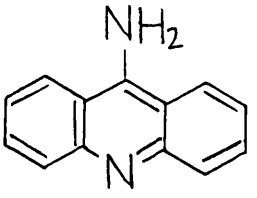
the isolated rabbit duodenum (Noamesi and Bamgbose, 1984).

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 (**189**) 9-aminoacridine

(**188**) quindoline R= H

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}H]-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 [^{3}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 *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 (10^7 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*.

mean IC_{50} ± standard error

	µg/ml	µM	n
cryptolepine	0.031 ± 0.0085	0.134 ± 0.037	3
chloroquine	0.073 ± 0.016	0.230 ± 0.05	3
9-aminoacridine	8.69	44.74	1

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

Dose ($mg/kg^{-1}/d^{-1}$ X4)	Mean % parasitaemia	±	standard deviation
control	34.22	±	4.16
7.04	42.54	±	12.03
14.08	38.48	±	12.79
28.16	40.80	±	0.38
56.32	46.44	±	8.01
112.63	33.04	±	10.44

Fig. 66 UV spectrum of cryptolepine

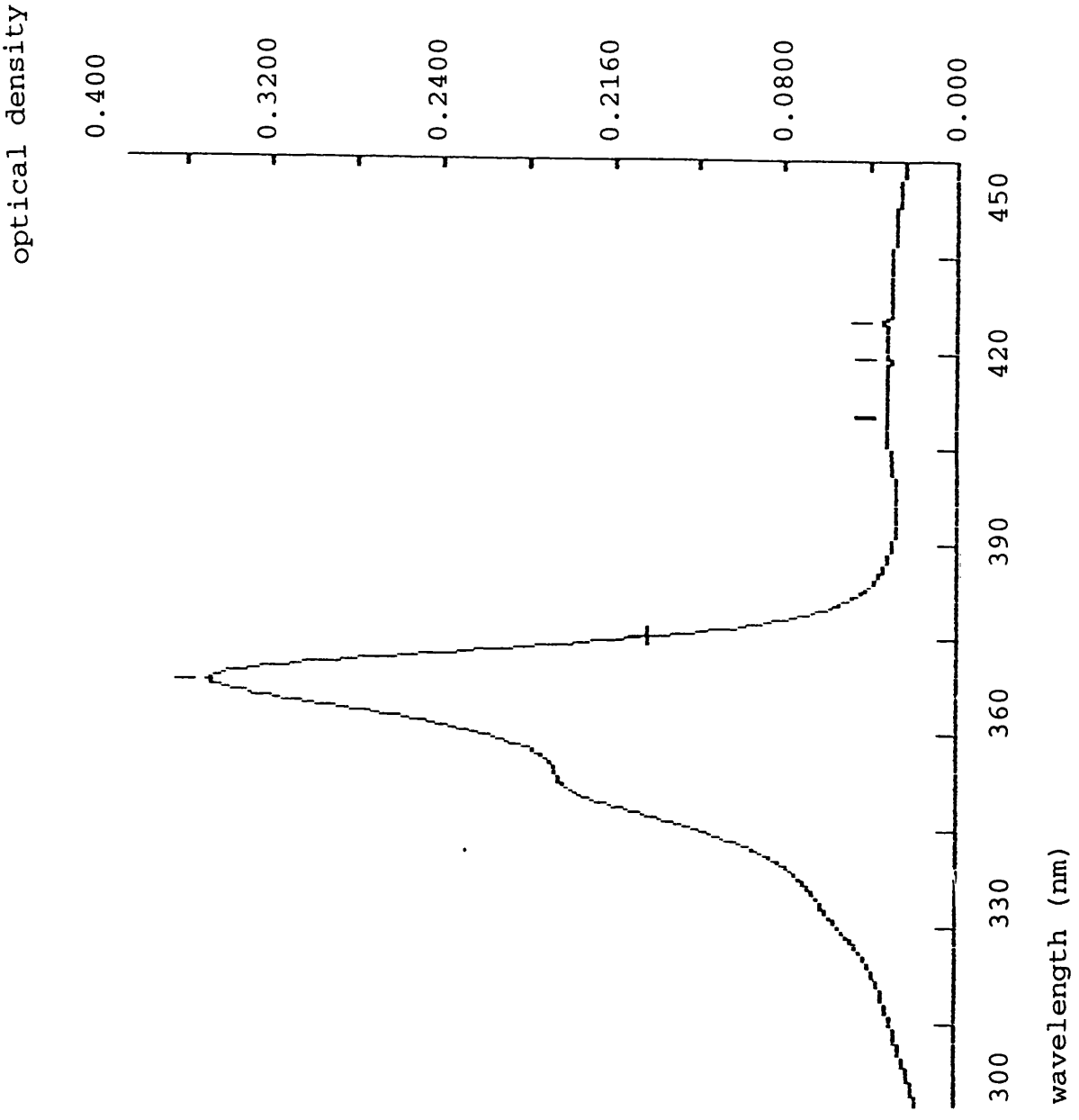


Fig. 67 Changes in the UV spectrum of cryptolepine on addition of DNA solution at pH 7.4

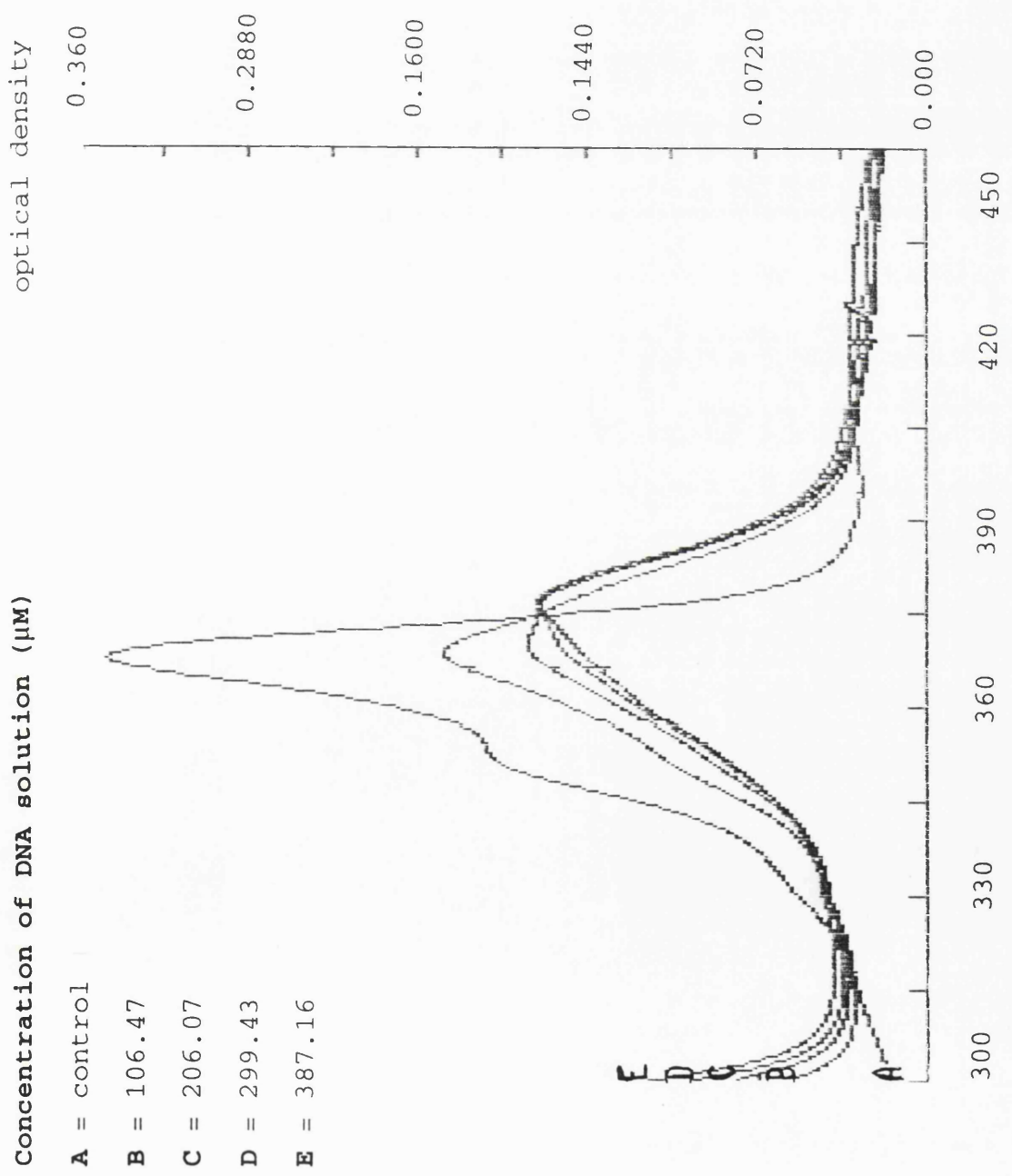
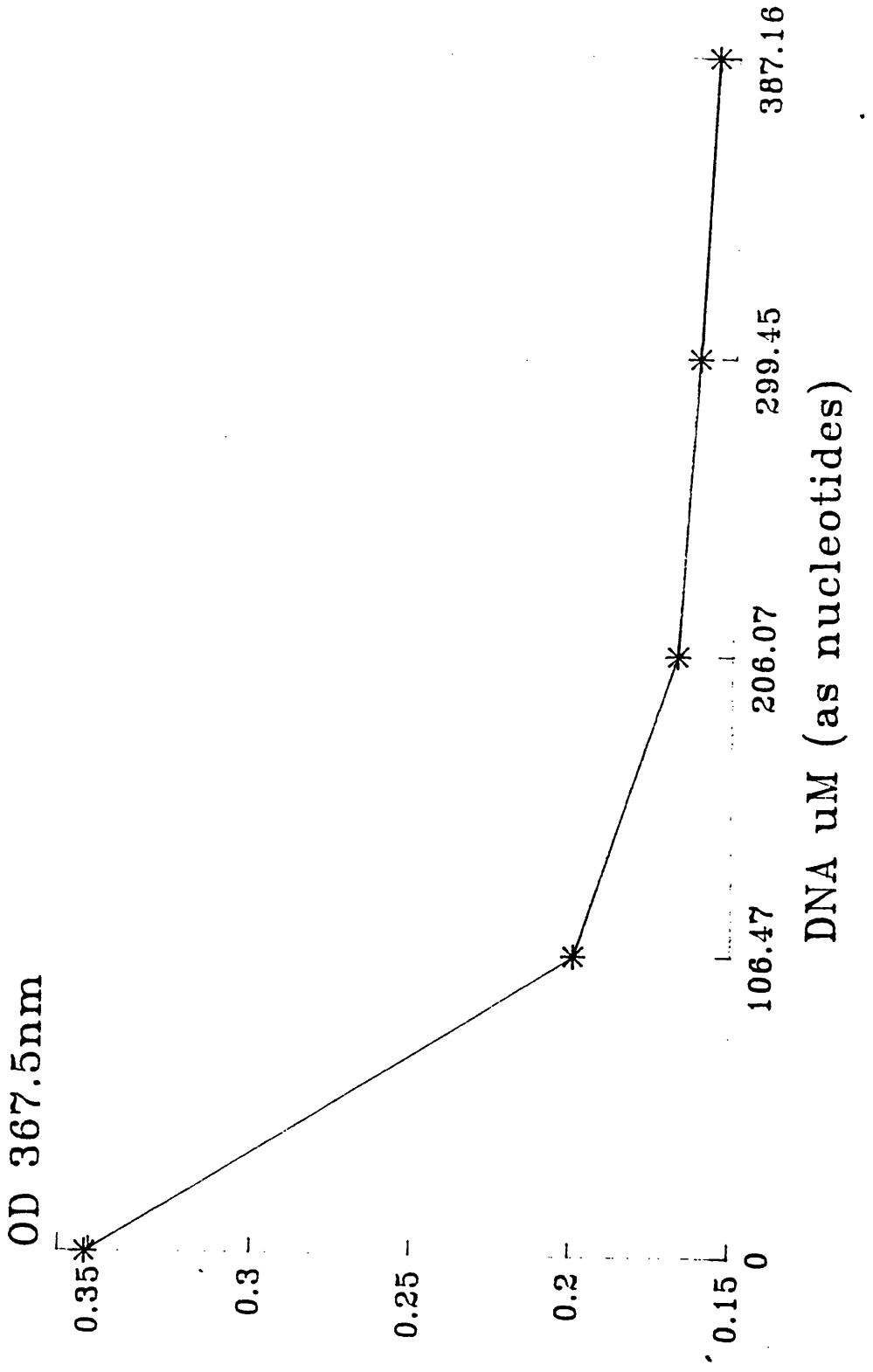


Fig. 68 Hypochromic effect of DNA on optical density of cryptolepine



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 IC_{50} 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 falciprum, 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.

CHAPTER 6 CONCLUSIONS

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

161) and at C-3, C-4 and C-1′ (compound **159**).

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 ellucidate 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 artemisisin such as arteflene, which is already in phase II trials at Hoffman-La Roche, Basel, Switzerland (Posner *et al.*, 1994).

APPENDIX

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).

	1	2	3	4
C2	100.0	168.8	172.2	134.6
C3	68.8	60.9	61.0	46.1
C5	61.8	54.0	54.1	
C6	41.1	43.4	43.4	
C7	60.6	57.1	57.9	107.4
C8	128.7	135.5	136.5	129.0
C9	126.7	121.4	112.2	119.7
C10	119.5	119.8	122.0	118.9
C11	128.7	127.6	110.0	121.9
C12	110.6	109.6		111.2
C13	147.5	147.7	138.3	136.9
C14	30.7	31.0	31.1	30.7
C15	34.4	28.8	29.0	37.9
C16	55.7	96.9	97.2	55.4
C17	64.5			79.2
C18	14.9	19.8	19.9	69.6
C19	129.8	68.4	68.3	33.1
C20	132.6	45.8	46.0	44.0
C21	64.7	48.1	48.3	58.7
COO	173.1	172.3		52.3
COOMe	51.9	51.9	51.8	172.7
OMe			55.5	
N^b-Me	49.6			40.4

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

UV λ_{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). ^1H NMR (500 MHz, DMSO-d_6): δ7.74 (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-21α), 4.36 (1H, dd, J=10.6, 5.5 Hz, H-3), 4.25 (1H, d, J=14.9 Hz, H-21β), 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)

UVλ_{max} (methanol) nm: 218, 282, 290, EIMS m/z (rel. int.): 340 $[M]^+$, 80, calc. for\$C_{20}H_{24}N_2O_3$: 340.1785, 309 (2), 283 (30), 281 (15), 251 (20), 238 (75), 180 (30), 167 (25), 96 (20), 70 (20), 58 (100); ^1H NMR ($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); ^{13}C NMR see table **18**.

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

λ_{max} (methanol) nm: 330, 296, 235) and the major peaks in the EIMS (Fig. **36**), ^{1}H NMR (Fig. **37**) and ^{13}C 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). ^{1}H NMR (500 MHz, $CDCl_3$): δ8.64 (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). ^{13}C 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). ^1H 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_2O, 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-demethylechitamine (Boonchuay and Court, 1976)

N_b-demethylechitamine has a UV spectrum of 302, 243 and 215nm, MS m/z (%) 370 (M^+ 100), 353 (84), 326 (18), 267 (9), 249 (8), 214 (6), 207 (6), 180 (9), 171 (11), 157 (24), ^1H 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

Abbiw, D. (1990). Useful plants of Ghana. Intermediate Technology Publications and the Royal Botanic Gardens, Kew; 185.

Ablordeppy, S.Y.S. (1983). Some synthetic aspects of the chemistry of cryptolepine and its derivatives. Proceedings of the First International Seminar on Cryptolepine, 27-30 July 1983, University of Science and Technology, Kumasi, Ghana; 30-36.

Aboagye-Nyame, F. (1993). Antiprotozoal activity of compounds from *Kigelia pinnata*. MSc project. Dept. Pharmacy, Kings College, London; 79p.

Adjanohoun, *et al.*, (1988). Contribution aux études ethnobotanique et floristique en République Populaire du Congo. Agence de coopération culturelle et technique, Paris; see Sofowara, 1993.

Adjanohoun, *et al* (1991). Contribution to Ethnobotanical and Floristic studies in Western Nigeria. Organisation of African Unity, Scientific Technical Research Commission (OAU/STRC), Lagos; see Sofowara, 1993.

Agbe, S.A.O. and Oguntimein, B., (1987). Anti-Trypanosomal activity of *Alchornea cordifolia*. Phytother. Res. 4; 151-153.

Ajao, A.O., Shonukan, O. and Femi-Onadeko, B. (1984). Antibacterial effect of aqueous and alcoholic extracts of *Spondias monbin* and *Alchornea cordifolia*. Fitoterapia LV (6); 337-339.

Akerele, O., Heywood, V., Synge, H., (1991). Conservation of Medicinal Plants, Proceedings of an International Consultation, 21-27 March 1988, Chiang Mai, Thailand, 3-7.

Akunyili, D.N. and Houghton, P.J. (1993). Meroterpenoids and naphthaquinones from *Kigelia pinnata*. Phytochem. 32; 1015-1018.

Alexander, J. and Russell, D.G. (1992). The Interaction of *Leishmania species* with macrophages. Advances in Parasitol. 31; 175-254.

Allen, D., Toth, I., Wright, C.W., Kirby, G.C., Warhurst, D.C. and Phillipson, J.D. (1993). *In vitro* antimalarial and cytotoxic activities of semi-synthetic derivatives of brusatol. Eur. J. Med Chem. 28; 265-269.

Allen, J.L. and Meinertz, J.R. (1991). Post-column reaction for simultaneous analysis of chromatic and leuco forms of malachite green and crystal violet by high-performance liquid chromatography with photometric detection. J. Chromatogr. 536; 217-222.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H. and Boyd, M.R., (1988). Feasability of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. Cancer Res. 48; 589-601.

Anderson, M.M., O'Neill, M.J., Phillipson, J.D., Bray, D.H., Kirby, G.C. and Warhurst, D.C., (1988).

Antimalarial activity of an aqueous tea prepared from *Brucea javanica* fruits. J. Pharm. Pharmacol. Suppl. 40; 52p.

Anderson, M.M., O'Neill, M.J., Phillipson, J.D. and Warhurst, D.C. (1991). *In vitro* cytoxicity of a series of quassinoids from *Brucea javanica* fruits against KB cells. Planta Med 57; 62-64.

Ansari, M.H. and Ahmad, S.(1991). Screening some medicinal plants for antiamoebic action. Fitoterapia LXII, 2; 171-175.

Ashford, R.W., Desjeux, P. and de Raadt, P. (1992). Estimation of population at risk of infection and number of cases of leishmaniasis, Parasitol. Today, 8; 104-105.

Asuzu, I.U. and Anaga, A.O. (1991). Pharmacological screening of the aqueous extract of *Alstonia boonei* bark. Fitoterapia LXII; 411-417.

Asuzu, I.U. and Chineme, C.N., (1990). Effects of *Morinda lucida* leaf extract on *Trypanosoma brucei brucei* infection in mice. J. Ethnopharmacol., 30; 307-313.

Atta-ur-Rahman, M.S. Qureshi, M.M. and Ali, S.S. (1990). Chemical constituents of *Alstonia species*. J. Chem. Soc. Pak. 12; 355-406.

Awe and Opeke (1990). Effect of *Alstonia congensis* on *Plasmodium berghei berghei* in mice. Fitoterapia LXI; 225-229.

Ayres, D.C. and Loike, J.D. (1990). Lignans, chemical, biological and clinical properties. Chemistry and

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

Bachrach, U., Schnur, L.F., El-On, J., Greenblatt, C.L., Pearlman, E., Robert-Géro, M. and Lederer, E. (1980). Inhibitory activity of sinefungin and SIBA (5'deoxy-5'-S-isobutylthioadenosine) on the growth of promastigotes and amastigotes of different species of Leishmania. FEBS Letts. 121; 287-291.

Balick, M.J. (1990). Ethnobotany and the identification of therapeutic agents from the rainforest. In: Bioactive Compounds from Plants. CIBA Foundation Symposium 154, John Wiley and sons; 22-39.

Baltz, T., Baltz, D., Giroud, Ch. and Crockett, J., (1985). Cultivation in a semi defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. The EMBO Journal, 4; 1273-1277.

Bamgbose, S.O.A. and Noamesi, B.K. (1981). Studies on cryptolepine. II: inhibition of carrageenin-induced oedema by cryptolepine. Planta Med. 41; 392-396.

Banerji, A. and Siddhanta, A.K. (1981). Scholarine: an indole alkaloid of *Alstoni scholaris*. Phytochem. 20; 540-542.

Barata, L.E.S., Baker, P.M., Gottlieb, O.R. and Ruveda, E.A. (1978). Neolignans of *Virola surinamensis*. Phytochem. 17; 783-786.

Barbagallo, C. and Chisari, G. (1987). Antimicrobial activity of three *Hypericum species*. Fitoterapia 58;

175-177.

Barry, J.D. and Emery, D.L. (1984). Parasite development and host responses during the establishment of *Trypanosoma brucei* infection transmitted by tsetse fly. Parasitol. 88; 67-84.

Barry, J.S. and Turner, C.M.R. (1991). The dynamics of antigenic variation and growth of African trypanosomes. Parisotol. Today, 7; 207-211.

Bauer, D.J.(1972). Introduction to antiviral chemotherapy. In: Chemotherapy of Virus Diseases, Vol 1, D.J. Bauer (ed.), Pergamon Press, Oxford; 1-33.

Baum, S.G., Wittner, M., Nadler, J.P., Horwitz, S.B., Dennis. J.E., Schiff, P.B. and Tanowizt, H.B. (1981). Taxol, a microtubule stabilizing agent, blocks the replication of *Trypanosoma cruzi*. Proc. Natl. Acad. Sci. USA. 75; 4571-4575.

Bazin, J.E. and Picard, P. (1992). Drug treatment of perioperative pain. Thérapie 47; 481-484.

Begit, W.J. and Kline, R.M. (1972). The use of tetrazolium salt in bioautographic procedures. J. Chromatog. 64; 182-183.

Benencia, F., Courrèges, M.C., Massouh, E.J. and Coulombié, F.C.(1994). Effect of *Melia azedarach* L leaf extracts on human complement and polymorphonuclear leukocytes. J. Ethnopharmacol. 41; 53-57.

Berens, R.L., Marr, J.J., Nelson, D.J., and La Fou, S.W. (1980). Antileishmanial effect of allopurinol and allopurinol riboside on the intracellular forms of

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

Berman, J.D. (1988). Chemotherapy for Leishmaniasis: Biochemical Mechanisms, Clinical Efficacy and Future Strategies. Review of Infect. Dis. 10; 560-586.

Berman, J.D. and Wyler, D.J. (1980). An *in vitro* model for investigation of chemotherapeutic agents in Leishmaniasis. J. Infect. Dis. 142; 83-86.

Betina, V. (1973). Bioautography in paper and thin-layer chromatography and its scope in the antibiotic field. J. Chromatography 78; 41-51.

Bhutani, K.K., Sharma, G.L. and Mohd Ali (1987). Plant-based antiamoebic drugs; part 1. Antiamoebic activity of phenanthro-indolizidine alkaloids; common structural determinants of activity with emetine. Planta Med. Vol 53; 532-536.

Boakye-Yiadom, K. (1979). The antimicrobial activity of some West African medicinal plants. Quarterly Journal of Crude Drug Research, 2; 78-80.

Boakye-Yiadom, K. (1983). Antimicrobial properties of cryptolepine - a review. Proceedings of the First International Seminar on Cryptolepine, 27-30 July 1983, University of Science and Technology, Kumasi Ghana; 57-64.

Boakye-Yiadom, K. and Heman-Ackeh, S.M. (1979). Cryptolepine hydrochloride: effect on *Staphylococcus aureus*. Journal of Pharmaceutical Sciences, 68; 1510-1514.

Bolognesi, R., Tsialtas, D. and Manca, C. (1992). Digitalis and heart failure: does digitalis really produce beneficial effects through a positive inotropic action? Cardiovascular Drugs and Therapy. 6; 459-464.

Bone, G. and Parent, G. (1963). Stearic acid, an essential growth factor for *Trypanosoma cruzi*. J. Gen. Microbiol. 31; 261-266.

Boonchuay, W. and Court, W.E. (1976). Alkaloids of *Alstonia scholaris* from Thailand. Planta Med. 29; 380-390.

Borowy, N.K., Fink, E. and Hirumi, H. (1985). *Trypanosoma brucei*: five commonly used trypanocides assayed *in vitro* with a mammalian feeder layer system for cultivation of bloodstream forms. Exp. Parasitol. 60; 323-330.

Borst, P. and Rudenko, G. (1994). Antigenic variation in African Trypanosomes. Science, 264; 1872-1873.

Bowman, W.C. and Rand, M. J.(1980). Textbook of Pharmacology 2nd ed., Blackwell, 11.13, 11.38, 16.18.

Boye, G.L. and Oku-Ampofo (1983). Clinical uses of *Cryptolepis sanguinolenta*. Proceedings of the First International Seminar on Cryptolepine, 27-30 July 1983, University of Science and Technology, Kumasi, Ghana; 37-40.

Braendli, B. Dankwa, E. and Junghanss, T. (1990). East African sleeping sickness (*Trypanosoma rhodesiense* infection) in 2 Swiss travelers to the tropics. Schweiz Med. Wochenschr, 120; 1348-1352; see Wery, 1991.

Brandao, M.G.L., Grandi, T.S.M., Rocha, E.M.M., Sawyer, D.R. and Krettli, A.U. (1992). Survey of medicinal plants used as antimalarials in the Amazon. J. Ethnopharmacol. 36; 175-182.

Braquet, P. and Godfroid, J.J. (1986). PAF-acether specific binding sites. Part 2. Design of specific antagonists. Trends in Pharm. Sci.; 397-399.

Bray, D.H., Warhurst, D.C., Connolly, J.D. and Phillipson, J.D., (1990) Plants as sources of antimalarial drugs. Part 7. Activity of some species of Meliaceae. Plants and their constituent limonoids. Phytother. Res. 4; 29-35.

Brener, Z. (1979). Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Western hemisphere. Pharmacol. and Therapeut., 7; 71-90.

British Pharmacopoeia, (1993). Vol. 1; HMSO; 193.

British Pharmaceutical Codex, (1973). The Pharmaceutical Press; 437.

Brun, R, (1981). The stimulatory effect of citrate and cis aconitate on the transformation of *T. brucei* bloodstream forms to procyclic forms *in vitro*. Z. Parasitenkd, 66; 17-24.

Brun, R. and Schönenberger, M. (1979). Cultivation and *in vitro* cloning of procyclic culture forms of *T. brucei* in a semi-defined medium. Acta Tropica 36; 289-292.

Brun, R., Jenni, M., Schönenberger, M. and Schell, K.F., (1981). *In vitro* cultivation of bloodstream forms of

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

Brun, R., Grootenhuis, J.G., Kunz, C. and Schönenberger, M. (1984). *In vitro* cultivation of *Trypanosoma* (*T.*) *brucei* bloodstream forms, using cell lines and sera from African wild Bovidae. J. Parasitol. 70; 836-837.

Brun, R. and Kunz, C. (1989). *In vitro* incorporation test for determining the drug sensitivity of *Trypanosoma* (*T.*) *brucei*. Acta Tropica, 46; 369-376.

McCabe, R.E., Remington, J.S. and Araujo, F.G., (1984). Ketoconazole inhibition of intracellular multiplication of *Trypanosoma cruzi* and protection of mice against lethal infection with the organism. J. Infectious Dis. 150; 594-601.

Cardoso, J.E., Ghezzi, Z. and Lopes, J.L.C. (1987). Atividade *in vitro* dos estratos das plantas *Vernonia sp.* e *Eupatorium sp.* contra formas sanguineas da cepa y do *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Suppl. 82; 178.

Carvalho, L.H. and Krettli, A.U. (1991). Antimalarial chemotherapy with natural products and chemically defined molecules. Mem. Inst. Oswaldo Cruz. Rio de Janeiro, 86, Suppl 11; 181-184.

Castro, C., Jimenez, M. and Gonzales de la Parra, M.,(1992). Inhibitory effect of piquerol A on the growth of epimastigotes of *T. cruzi*. Planta Med. 58; 281-282.

Cavin, J.C., Krassner, S.M. and Rodriguez, E. (1987).

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

Cenini, P., Bolognesi, A. and Stirpe, F. (1988). Ribosome-inactiviating proteins from plants inhibit ribosome activity of *Trypanosoma* and *Leishmania*. J. Protozool., 35; 384-387.

Centre for Scientific Research into Plant Medicine, Mampong, Akuapim, Ghana, (1990). Official Leaflet; 1p.

Chan, K.L., O'Neill, M.J., Phillipson, J.D., Warhurst, D.C. (1986). Plants as sources of antimalarial drugs. Part 3. *Eurycoma longifolia*. Planta Med. 2; 105-107.

Chang, K. P. and Bray, R. S. eds. (1985). Leishmaniasis. Vol 1. Human Parasitic Diseases, E.J. Ruitenberg and A.J. MacInnis (series eds.) Elsevier. Amsterdam, New York and Oxford; 16.

Chen, M., Christensen, S. B., Theander, T. G. and Kharazmi A. (1994). Antileishmanial activity of licochalcone A in mice infected with *Leishmania major* and in hamsters infected with *Leishmania donovani*. Antimicrob. Agents Chemother. 38; 1339-1344.

Cheung, H.T. and Wong, W.C. (1972). Structures of triterpenes from *Dryobalanops aromatica*. Phytochemistry 11; 1771-1780.

Chiari, E., de Oliviera, A.B., Raslan, S. D., Mesquita, A.A.L. and Tavares, K.G., (1991). Screening *in vitro* of natural products against blood forms of *Trypanosoma cruzi*. Trans. R. Soc. Trop. Med. Hyg. 85; 372-374.

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

Jones, A.S., Torrence, P.F. and Shugar, D. (1980). Comparative efficacy of antiherpes drugs against different strains of *Herpes simplex* virus. J. Infect. Dis. 141; 563-574.

Clinquart, E.D. (1929). La composition chimique du *Cryptolepis triangularis* - une plante du Congo de Belgique. Bull. Soc. R. Med. Pub. du Royaume de Belgique, 9; 627-635.

Collins, P. and Bauer, D.J. (1977). Relative potencies of anti-herpes compounds. Ann. N.Y. Acad. Sci. 284; 49-59.

Connor, D.H., Neafie, R.C. and Dooley, J.R. (1976). African trypanosomiasis. In: Pathology of Tropical and Extraordinary Diseases. C. H. Binford, D. H. Connor (eds.), Washington, DC, Armed Forces Institute of Pathology; 308-316.

Cordell, G.A. (1981). Introduction to alkaloids: a biogenetic approach. John Wiley and Sons, New York; 273-274.

Costantino, L., Albasini, A., Rastelli, G. and Benvenuti, S. (1992). Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine oxidase. Planta Med. 58; 342-344.

Cover, B. and Gutteridge, W.E. (1982). A primary screen for drugs to prevent transmission of Chagas' disease during blood transfusion. Trans. R. Soc. Trop. Med. Hyg. 76; 633-635.

Cox, P.A., (1990). Ethnopharmacology and the search for

new drugs. In: Bioactive Compounds. CIBA Foundation Symposium 154; 40-47.

Cox, P.A., (1993). Saving the ethnopharmacological heritage of Samoa. J. Ethnopharmacol. 38; 181-188.

Croft, S.L. (1986). *In vitro* screens in the experimental chemotherapy of leishmaniasis and trypanosomiasis. Parasitol. Today, 2; 64-69.

Croft, S.L., Evans, A.T. and Neal, R.A. (1985). The activity of plumbagin and other electron carriers against *Leishmania donovani* and *Leishmania amazonensis*. Ann. Trop. Med. Parasitol. 79; 651-653.

Croft, S.L., Walker, J.J. and Gutteridge, W.E. (1988). Screening of drugs for rapid activity against *Trypanosoma cruzi* trypomastigotes *in vitro*. Trop. Med. Parasitol. 39; 145-148.

Dalal, V.C., Taylor, A. and McKenna, G.F. (1958). The effect of plant extracts on egg-cultivated tumour tissue. Tex. Rept. Biol. Med. 16; 439-442.

Dalziel, J. M. (1937). Useful plants of West Tropical Africa. Appendix to the flora of West Tropical Africa; J. Hutchinson and J.M. Dalziel (eds), Pub. Crown Agents for the Colonies, London; 135-136.

Davidson, R.N. and Croft, S.L. (1993). Recent advances in the treatment of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 87; 130-131 and 141.

Desjardins, R.E., Caufield, C.J., Haynes, J.D., and Chulay, J.D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution

technique. Antimicrob. Agents Chemother. 16; 710-718.

Docampo, R., Moreno, S.N.J., Muniz, R.P.A., Cruz, F.S. and Mason, R.P. (1983). Light-enhanced free radical formation and trypanocidal action of gentian violet (crystal violet). Science, 220; 1292-1295.

Donelson, J.E. and Turner, M.J. (1985). How the trypanosome changes its coat. Scientific American 252; 32-39.

Douc-Rasy, S., Riou, J. F., Ahomadegbe, J. C., and Fiou, G. (1988). ATP-independent DNA topoisomerase II as a potential drug target in trypanosomes. Biology of the Cell, 64; 145-156.

Dwuma-Badu D., Ayim, J.S., Fiagbe, N.I., Knapp, J.E., Schiff Jnr., P.L. and Slatkin, D.J. (1978). Constituents of West African medicinal Plants. XX: quindoline from *Cryptolepis sanguinolenta*. J. Pharm. Sci. 67; 433-434.

Dwuma-Badu, D. (1983). Review of the isolation and characterisation of the alkaloids of *Cryptolepis sanguinolenta*. Proceedings of the first international seminar on cryptolepine, 27-30 July 1983, University of Science and Technology, Kumasi, Ghana, 23-29.

Earnshaw, W.C., Halligan, B., Cooke, C.A. Heck, M.M.S. and Liu, L.F. (1985). Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell Biol. 100; 1706-1715.

Eid, J.E., Ueno, H., Wang, C.C. and Donelson, J.E. (1988). Gossypol-induced death of African trypanosomes. Exper. Parasitol. 66; 140-142.

Ekong, R.M., Kirby, G.C., Patel, G., Warhurst, D.C. and Phillipson, J.D. (1990). Comparison of the *in vitro* activities of quassinoids with activity against *Plasmodium falciparum*, anisomycin and some other inhibitors of eukaryotic protein synthesis. Biochem. Pharmacol. 40; 297-301.

Ekong, R., Partridge, S.J., Anderson, M.M., Kirby, G.C., Warhurst, D.C., Russel, P.F. and Phillipson, J.D. (1991). *Plasmodium falciparum*: effects of phaeanthine, a naturally-occurring bisbenzylisoquinoline alkaloid, on chloroquine-resistant and -sensitive parasites *in vitro*, and its influence on chloroquine activity. Ann. Trop. Med. Parasitol. 85; 205-213.

Elisabetsky, E., and Wannmacher, L.(1993). The status of ethnopharmacology in Brazil. J. of Ethnopharmacol. 38; 137-143.

Ellingwood, F. (1985). American Materia Medica, Therapeutics and Pharmacognosy. Botanical, Vol. II. Eclectic Medical Publications; 115-122, 199-202, 320, 494-498.

El-On, J., Jacobs, G. P., and Weinrauch, L. (1988). Topical chemotherapy of leishmaniasis. Parasitol. Today, 4; 76-81.

Evans, D.A. (1978). Kinetoplastida. In: Methods of cultivating parasites *in vitro*. A.E.R. Taylor and J.R. Baker, (Eds.), Academic Press, London; 55-88.

Evans, A.T. and Croft, S.L. (1987). Antileishmanial activity of harmaline and other tryptamine

derivatives. Phytother. Res. 1; 25-27.

Fairlamb, A.H., Warhurst, D.C., and Peters, W. (1985). An improved technique for the cultivation of *Plasmodium falciparum in vitro* without daily medium change. Ann. Trop. Med. Parasit. 79; 379-384.

Fang, X., Rieser, M.J., Gu, Z., Zhao, G., and McLaughlin, L. (1993). Annonaceous acetogenins: an updated review. Phytochem. Anal. 4; 27-48.

Farber, I., Klinger, C., Wutzler, P., Thiel, K.D., Reefschlager, J. and Herrmann, G. (1987). Effect of (E)-5-(2-bromovinyl-and 5-vinyl-1-β-D-arabinofuranosyluracil on Epstein-Barr virus antigen expression in P3HR-1 cells : comparison with acyclovir. Acta Virol. (Praha); 31, 13-18.

Farnsworth, N.R., Henry, L.K., Svoboda, G.H., Blomster, R.N., Yates, M.J. and Euler, K.L. (1966). Biological and phytochemical evaluation of plants. I. Biological test procedures and results from 200 accessions, Lloydia, 29; 101-122.

Farnsworth, N.R. (1983). The NAPRALERT database as an information source for application to traditional medicine. In: Traditional Medicine and Health Care Coverage, WHO, Bannerman, R.H., Burton, T., Ch'en, W.C. (eds); 184-193.

Farnsworth, N. R., Akerele, O., Bingel, A.S., Soejarto, D.D. and Guo, Z., (1985). Medicinal plants in therapy. Bulletin of the World Health Organization, 63; 965-981.

Farnsworth, N.R., and Soejarto, D.D., (1985). Potential consequence of plant extinction in the United States on the current and future availability of prescription drugs. Economic Botany, 39; 231-240.

Farnsworth, N.R.,(1990). The role of ethnopharmacology in drug development. in: Bioactive Compounds from Plants; CIBA Foundation Symposium 154, John Wiley and Sons, 2-21.

Farnsworth, N. R. and Soejarto, D. D. (1991). Global Importance of Medicinal Plants. In: Conservation of Medicinal Plants, O. Akerele, V. Heywood and H. Synge (eds); Proceedings of an international consultation 21-27 March, 1988, Chiang Mai, Thailand; 25.

Farnsworth, N.R. (1993). Ethnopharmacology and future drug development. The North American experience. J. Ethnopharmacol., 38; 145-152.

Fellows, L. (1992). What are the forests worth? The Lancet, 339; 1330-1333.

Feng, P.C., Haynes, L.J., Magnus, K.E. and Plinner, J.R. (1965). Further pharmacological screening of some West Indian medicinal plants. J. Pharm Pharmacol. 16; 115-117.

Ferri, P.H. and Barata, L.E.S. (1991). (-)Di-de-O-methylgrandisin, a lignan from *Virola pavonis* leaves. Phytochem. 30; 4204-4205.

Ferri, P.H. and Barata, L.E.S. (1992). Neolignans and a new phenylpropanoid from *Virola pavonis* leaves. Phytochem. 31; 1375-1377.

Field, A.K., Davis, M.E., de Witt, C.M., Perry, H.C., Schofield, T.L., Karkas, J.D., Germershausen, J., Wagner, A.F., Cantone, C.L., Maccoss, M. and Tolman, R.L. (1986). Efficacy of 2'-norcyclic GMP in treatment of experimental herpes virus infections. Antiviral Res. 6; 329-341.

Fong, H.H.S. (1983). Current status of gossypol, soapatanol and other plant-derived fertility regulating agents. In: Natural Products and Drug Development. P. Krogsgaard-Larsen, S. Brogger Christensen and Kofod, H. (eds), Munksgaard; 355-368.

Fosse, P., Charra, M., Paoletti, C., Saucier, J.M. and Rene, B. (1994). *In vitro* stimulation by ellipticine derivatives of DNA cleavage by topoisomerase II. Bulletin du Cancer, 81; 194-202.

Fournet, A., Muños, V., Munjon, A.M., Angelo, A., Hocquemiller, R., Cortés, D., Cavé, A., and Bruneton, J. (1988a). Activité antiparasitaire d'alcaloides bisbenzylisoquinoléiques. I: Activité *in vitro* sur des promastigotes de trois souches de *Leishmania*. J. Ethnopharmacol. 24; 327-335.

Fournet, A., Munjon, A.M., Muños, V., Angelo, A., Bruneton, J. Hocquemiller, R., Cortés, D. and Cavé, A., (1988b). Activité antiparasitaire d'alcaloïdes bisbenzylisoquinoléiques. II: Activité *in vitro* sur des epimastigotes de trois souches typifiées de *Trypanosoma cruzi*. J. Ethnopharmacol. 24; 337-343.

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

Hocquemiller, R. and Cavé, A., (1992a). Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis. J. Ethnopharmacol. 37; 159-164.

Fournet, A., Barrios, A.A., Muñoz, V., Hocquemiller, R. and Cavé, A. (1992b). Effect of natural naphthoquinones in BALB/c mice infected with *Leishmania amazonensis* and *L. venezuelensis*. Trop. Med. Parasitol. 43; 219-222.

Fournet, A. Barrios, A.A., Muñoz, V., Hocquemiller, R. and Cavé, A. (1993). Effect of some bisbenzyl isoquinoline alkaloids on American Leishmania sp. in BALB/c mice. Phytother. Res. 7; 281-284.

Fournet, A., Barrios, A.A. and Muñoz, V. (1994). Leishmanicidal and trypanocidal activities of Bolivian medicinal plants. J. Ethnopharmacol. 41; 19-37.

Fournier, G., Paris, M.R., Fournial, M.C. and Quero, A.M. (1978). Activité bacteristatique d'huiles essentielles de *Cannabis sativa*. L. Annales Pharmaceutiques Français 36; 603-606.

Gadelha, F.R., Moreno, S.J.J., De Souza, W., Cruz, F.S. and Docampo, R. (1989). The mitochondrion of *T. cruzi* is a target of crystal violet toxicity. Mol. Biochem. Parasitol. 34; 117-126.

Gebre-Hiwot, A., Tadesse, G., Croft, S.L. and Frommel, D. (1992). An *in vitro* model for screening anti-leishmanial drugs: the human leukaemia monocyte cell line. Acta Tropica, 51; 237-245.

George, S. and Sabelli, F. (1994). Faith and Credit. The

World Bank's Secular Empire. Penguin; 18.

Ghosh, A. K., Bhattacharyya, F.K. and Ghosh, D.K. (1985). *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.

Gimlette, J.D. and Thomson, H.W. (eds. 1977). A Dictionary of Malayan Medicine, Oxford University Press, Kuala Lumpur; 183.

Ginzbarg, 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.

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

31.

Gottlieb, O.R. (1978). Neolignans. Fort. Chem. Org. Nat. 35; 1-72.

de Groof, D., Bruneel, H., Musumari, T.S. and Ruppol, J.F. (1992). Traitement de la maladie du summeil a *Trypanosoma brucei gambiense* avec le DL-α-difluoromethylornithine (DFMO) dans un hôpital rural au Zaïre. Médecine Tropicàle, 52; 369-375.

Grunert, R.R. (1979). Search for antiviral agents. Ann. Rev. Microbiol. 33; 335-353.

Gulakowski, R.J., Mc Mahon, J.B., Staley, P.G., Moran, R.A., and Boyd, R., (1991). A semi-automated multiparameter approach for anti-HIV drug screening. J. Virological Methods, 33; 87-100.

Gunatilaka, A.A.L., Sotheeswaran, S., Balasubramaniam, S., Chandrasekara, A.I. and Sriyani, H.T. (1980). Studies on medicinal plants of Sri Lanka III. Pharmacologically important alkaloids from some *Sida* species. Planta Med. 39; 66-72.

Gupta, M.P., Correa, M.D., Solis, P.N., Jones, A., Galdomes, C. and Guionneau-Sinclair, F. (1993). Medicinal plant inventory of Kuna Indians: Part 1. J. Ethnopharmacol. 40, 77-109.

Gupte, S. (1975). Use of berberine in treatment of giardiasis. Am. J. Dis. Children, 129; 866; see Kaneda *et al*, 1991.

Guru, P.Y., Warhurst, D.C., Harris, A. and Phillipson, J.D. (1983). Antimalarial activity of bruceantin *in vitro*.

Ann. Trop. Med. Parasitol. 77; 433-435.

Gutteridge, W.E., (1985). Existing chemotherapy and its limitations (including drug resistance). Brit. Med. Bulletin 41; 1-36.

Gutteridge, W.E., Knowles, J., and Coombes, J.D. (1969). Growth of *Trypanosoma cruzi* in human heart tissue culture cells and effects of aminonucleoside of puromycin, trypacidin and aminopterin. J. Protozool. 16; 521-525.

Hamburger, M.O. and Cordell, G.A. (1987). A direct bioautographic TLC assay for compounds possessing antibacterial activity. J. Nat. Prod. 50; 19-22.

Hardinge, M.G., Courville, D.A., Hardinge, M., Fujikawa, B. and Harvey, R. (1961). Action of plant extracts on transplanted mouse tumours. Cancer Res. Suppl. 21; 573-608.

Hayashi, K., Hayashi, T., Morita, N. and Niwayama, S. (1990). Antiviral activity of an extract of *Cordia salicifolia* on *Herpes simplex* virus type 1. Planta Med. 56; 439-443.

Hazra, B., Saha, K., Ray, R., Roy, D.K. Sur, P. and Banerjee, A. (1987). Antiprotozoal activity of diospyrin towards *Leishmania donovani* promastigotes *in vitro*. Trans. R. Soc. Trop. Med. Hyg. 81; 738-741.

Hernández-Pérez, López-García, R.E., Rabanal, R.M., Darias, V. and Arias, A., (1994). Antimicrobial activity of *Visnea mocanera* leaf extracts. J. Ethnopharmacol. 41; 115-119.

Hill, G.C., Shimer, S., Caughey, B. and Sauer, S. (1978). Growth of infective forms of *Trypanosoma* (*T.*) *brucei* on buffalo lung and Chinese hamster lung tissue culture cells. Acta Tropica 35; 201-207.

Hirumi, H., Doyle, J.J. and Hirumi, K., (1977). Cultivation of bloodstream *Trypanosoma brucei*, Bulletin of the World Health Organization, 55; 405-409.

Hirumi, H. and Hirumi, K., (1989). Continuous Cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. J. Parasitol. 75; 985-989.

Hocquemiller, R., Cortes, D., Arango, G.J., Myint, S.H. and Cavé, A. (1991). Isôlement et synthèse de l'éspintanol, nouveau monoterpène antiparasitaire. J. Nat. Prod. 54; 445-452.

Hocquemiller, R., Cabalion, P., Fournet, A. and Cavé, A. (1984). Alcaloides des Annonacées XLIX: alcaloïdes d'*Isolona hexaloba*, *I. zenkeri* et *I. pilosa*. Planta Med. 1; 23-25.

Hodge, H.C., Indra, J., Drobeck, H.P., Duprey, L.P. and Tainter, M.L. (1972). Acute oral toxicity of methylrosaniline chloride. Toxicol. and Appl. Pharmacol. 22; 1-5.

Hoeprich, P.D. (1992). Clinical use of amphotericin B and derivatives: lore, mystique and fact. Clin. Infec. Dis. 14, Suppl 1; S114-119.

Holden, C. (1987). Animal Regulations: so far, so good. Science 238; 880-882.

Holdsworth, D. and Mahana, P. (1983). Traditional Plants of the Huon Peninsula, Morobe Province, Papua New Guinea. Int. J. Crude Drug Res., 21; 121-133.

Hooper, P.A. and Leonard, B.E. (1965). Pharmacological properties of some West Indian plants. J. Pharm. Pharmacol., 17; 98-107.

Howland, J.L. (1963). The reversibility of inhibition by 2-heptyl-4-hydroxyquinoline-N-oxide and 2-hydroxy-3 (-3-methylbutyl)-1,4-naphthoquinone of succinate oxidation. Biochemica et Biophysica Acta. 73; 665-667.

Hsiao, P.K. (1980). Traditional experience of Chinese herb medicine. Its application in drug research and new drug searching. In: Natural Products as Medicinal Agents. J.L. Beal and E. Reinhard (eds); Hippokrates; 351-394.

Hugo, W.B., and Russell, A.D. (1980). Pharmaceutical Microbiology, Blackwell Scientific Publications; 100-122.

Igweh, A.C. and Onabanjo, A.O. (1989). Chemotherapeutic effects of *Annona senegalensis* in *Trypanosoma brucei brucei*. Annals Trop. Med. Parasitol., 83; 527-534.

Inoué, A.S. (1990). Projeto de Fitoterapia do Estado de São Paulo; Annals do XI Simposio de Plantas Medicinais do Brasil. Comm. 4.69.

Iwu, M.M., Jackson, J.E., Tally, J.D. and Klayman, D.L. (1992). Evaluation of plant extracts for antileishmanial activity using a mechanism-based radiorespirometric microtechnique (RAM). Planta Med.

58; 436-441.

Iwu, M.M., Jackson, J.E. and Schuster, B.G. (1994). Medicinal plants in the fight against leishmaniasis, Parasitol. Today, 10; 65-68.

Izzi, A.E., Benie, T., Thienlant, M. L. and Dural, J. (1990). Inhibitory Effects of saponins from *Tetrapleura tetraptera* on the LH released by cultured rat pituitary cells. Planta Med.; 357-359.

Janssen, A.M., Scheffer, J.J.C. and Baerheim Svendsen, A., (1987). Antimicrobial activity of essential oils: A 1976-1986 literature review. Aspects of the test methods. Planta Med., 53; 395-398.

Jernigan, J.A. and Pearson, R.D. (1993). Chemotherapy of leishmaniasis, Chagas' disease and African trypanosomiasis. Current Opinion in Infectious Diseases, 6; 794-802.

Jiminez, G. and de Ercoli, N. (1965). Effect of drugs on various leishmania isolates and succinic dehydrogenase inhibition. Experimental Parasitology, 17; 302-308.

Johnson, S. (1982). Plant alkaloids. In: Cancer Medicine, J.F. Holland and E. Frei III (eds), Lea and Febiger, New York; 910-920.

Johnson, I.S., Armstrong, J.G., Gorman, M. and Burnett, J.P. (1963). The *Vinca* alkaloids: a new class of oncolytic agents. Cancer Res. 23: 1390-1427.

Joly, L.G., Guerra, S., Septimo, R., Solis, P.N., Correa, M., Gupta, M., Levy, S. and Sandberg, F. (1987). Ethnobotanical Inventory of Medicinal Plants Used by

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

Joly, L.G. Guerra, S., Septimo, R., Solis, P.N., Correa, M., Gupta, M., Levy, S., Sandberg, F., Perera, P. (1990). Ethnobotanical inventory of medicinal plants used by the Guami Indians in Western Panama. Part II, J. Ethnopharmacol., 28; 191-206.

Jurg, A., Tomas, T. and Pividal, J. (1991). Antimalarial activity of some plant remedies in use in Marracuene, Southern Mozambique. J. Ethnopharmacol. 33; 79-83.

Keawpradub, N., Takayama, H., Aimi, N. and Sakai, S. (1994). Indole alkaloids from *Alstonia claucescens*. Phytochem. 37; 1745-1749.

Kaminsky, R. and Zweygarth, E. (1989). Feeder layer-free *in vitro* assay for screening anti-trypanosomal compounds against *Trypanosoma brucei brucei* and *T. b. evansi*. Antimicrob. Agents Chemother.; 881-885.

Kaneda, Y., Torii, M., Tanaka, T. and Aikawa, M. (1991). *In vitro* effects of berberine sulphate on the growth and structure of *Entamoeba histolytica, Giardia lambla* and *Trichomonas vaginalis*. Annals of Trop. Med. and Parasitol. 85; 417-425.

Kanyinda, B., Francois, G., Vanhaelen, M. and Wery, M. (1992). Activity of three bisbenzylisoquinoline alkaloids from *Anisocyda cymosa* against *Giardia lamblia*. Planta Med. Suppl. 1; A635.

Katiyar, J.C., Anuradha, and Sharma, S. (1992). Kala-azar: current status of experimental chemotherapy. Med. Res.

Rev. 12; 473-504.

Kaur, S., Ganguly, N.K., Sodhi, S., Malia, N., and Mahajan, R.C. (1991). Effect of indigenous plant extracts on *Leishmania donovani* infection *in vitro* and *in vivo*, a preliminary report. Immunol. and Infect. Dis. 1; 219-222.

Keene, A.T., Harris, A., Phillipson, J.D. and Warhurst, D.C., (1986). *In vitro* amoebicidal testing of natural products. Part 1. Methodology. Planta Med.; 278-284.

Killick-Kendrick, R. (1979). Biology of *Leishmania* in phlebotomine sandflies. In: Biology of the Kinetoplaastida; W.H.R. Lumsden and D.A. Evans, (eds), Academic Press, New York; 395-460.

Kirby, G.C., O'Neill, M.J., Phillipson, J.D. and Warhurst, D.C.(1989). *In vitro* studies on the mode of action of quassinoids with activity against chloroquine-resistant *Plasmodium falciparum*. Biochem. Pharmacol. 38; 4367-4374.

Kirby, G.C. and Warhurst, D.C. (1992). Antimalarial activity of the antihistamine drug terfenadine. Trans. R. Soc. Trop. Med. Hyg. 86; 343.

Klayman, D.L. (1985) Quinghaosu (artemisinin): an antimalarial drug from China. Science, 228; 1049-1055.

Kuzoe, F.A.S. (1993). Current situation of African trypanosomiasis. Acta Tropica, 54; 153-162.

Lanham, S.M. and Godfrey, D.G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-Cellulose. Experimental Parasitol. 28; 521-

534.

Levy, J., Le Men, J. and Janot, M. M. (1960). Sur l'akuammicine, alcaloïde du *Picralima nitida* Stapf (Apocynacées) (1er memoire). Societé Chimique de France, Bulletin; 979-981.

Levy, O., Duez, P., Lejoly, J., Chamart, S., Sawadago, M. and Hanocq, M. (1990). Cultivation of *Holarrhena floribunda* for conessine production in Burkina Faso. Int. J. Crude Drug Res., 28; 285-295.

Liljestrand, G., (1971). The historical development of local anaesthesia. In: The Encyclopedia of Pharmacology and Therapeutics. Section 8, Vol 1. Local Anaesthetics; P. Lechat (ed); 28-30.

Lin, J.C., Smith, M.C., Cheng, Y.C. and Pagano, J.S. (1983). Epstein-Barr virus: inhibition of replication by three new drugs. Science 221; 578-579.

Lopez, J. N., Cruz, F.S., Docampo, R., Vasconcellos, M.E., Sampaio, M.C.R., Pinto, A.V. and Gilbert, B. (1978). *In vitro* and *in vivo* evaluation of the toxicity of 1,4-naphthoquinone and 1,2-naphthoquinone derivatives against *Trypanosoma cruzi*. Annals of Trop. Med. and Parasitol., 72; 523-553.

Luduena, R.F. (1979). Microtubules. In: Biochemistry of tubulin, K. Roberts and J.S. Hyams (eds.). Academic Press; 66-116.

MacRae, W.D. and Towers, G.H.N. (1984). Biological activities of lignans, Phytochem. 23; 1207-1220.

Makinde, J.M., Amusan, O.O.G., Adesogan, E.K. (1990). The

antimalarial activity of chromatographic fractions of *Spathodea campanulata* stem bark extracts against *Plasmodium berghei berghei* in mice. Phytother. Res. 4; 53-56.

Makinde, J.M., Awe, S.O. and Agebedahunsi, J.M. (1988). Effect of *Khaya grandifolia* extract on *Plasmodium berghei berghei* in mice. Phytother. Res. 2; 30-32.

Malone, M.H. and Robichaud, R.C. (1962). A Hippocratic screen for pure and crude drug materials. Lloydia, 25; 320-332.

Mansfield, J.M. (1994). T-cell responses to the Trypanosome variant surface glycoprotein: a new paradigm ? Parasitol. Today, 10; 267-270.

Marini-Bettolo, G.B., Nicoletti, M. Messana, I. and Patamia, M. (1983). Research on African medicinal plants - IV Boonein, a new C-9 terpenoid lactone from *Alstonia boonei*: a possible precursor in the indole alkaloid biogenesis. Tetrahedron, 39; 323-329.

Marshall, S.J., Russell, P.F., Wright, C.W., Anderson, M.M., Phillipson, J.D., Kirby, G.C., Warhurst, D.C. and Schiff, P.L. (1994). *In vitro* antiplasmodial, antiamoebic and cytotoxic activities of a series of bisbenzyl isoquinoline alkaloids. Antimicrobial Agents and Chemother., 38; 96-103.

Massiot, G., Boumendjel, A., Nuzillard, J.M., Richard, B., Le Men-Olivier, L., David, B. and Hadi, H.A. (1992). Alkaloids from *Alstonia undulifolia*. Phytochem. 31; 1078-1079.

Mattock, N.M. and Peters, W. (1975). The experimental chemotherapy of leishmaniasis. 1. Techniques for the study of drug action in tissue culture. Annals of Trop. Med. Parasitol. 69; 349-357.

Mbulamberi, D.B. (1990). Recent epidemic outbreaks of human trypanosomiasis in Uganda. Insect Sci. Applic. 11; 289-292.

Mbulamberi, D.B. (1992) National sleeping sickness control Programme, Jinja, Uganda. Second Quarterly Report, Summary; 1. see Kuzoe, 1993.

Mitscher, L.A., Leu, R.P., Bathala, M.S., Wu, W. N., Beal, J.L. and White, R. (1972). Antimicrobial agents from higher plants. I: Introduction , rationale and methodology. Lloydia 35; 157-166.

Misra, P., Pal, N.L., Guru, P.Y., Katiyar, J.C. and Tandon, J.S. (1991). Antimalarial activity of traditional plants against erythrocytic stages of *Plasmodium berghei*. Int. J. Pharmacog. 29; 19-23.

Monks, A., Scudiero, D. L., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J. and Boyd, M. (1991). Feasability of a high-flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. JNCI, 83; 757-766.

Mukherjee, K.S. and Ghosh, P.K. (1979). Chemistry of *Alstonia scholaris*, Visva-Bharati Journal of Research, 4; 36-68.

Mukherjee, T. (1991). Antimalarial herbal drugs. A Review. Fitoterapia 62; 197-204.

Nantulya, V.M. and Moloo, S.K. (1989). Recent developments in trypanosomiasis. International Journal of Animal Science 4; 71-84.

Neal, R.A. (1970). Effect of emetine and related compounds on experimental cutaneous leishmaniasis. Ann. Trop. Med. Parasitol. 64; 159-165.

Neal, R.A. and Matthews, P.J. (1982). *In vitro* antileishmanial properties of pentavalent antimonial compounds. Trans. R. Soc. Trop. Med. Hyg. 76; 21-27.

Neal, R.A. and Croft, S.L. (1984). An *in vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. J. antimicrob. Chemother. 14; 463-475.

Neal, R.A. (1987). Experimental Chemotherapy. In: The Leishmaniases, Vol 2; 793-845.

O'Neill, M.J., Bray, D.H., Boardman, P., Phillipson, J.D., and Warhurst, D.C. (1985). Plants as sources of antimalarial drugs. Part 1. *In vitro* test method for the evaluation of crude extracts from plants. Planta Med.; 394-398.

O'Neill, M.J., Bray, D.H., Boardman, P., Phillipson, J.D., Warhurst, D.C. and Peters, W. (1987). Plants as sources of antimalarial drugs, part 4. Activity of *Brucea javanica* fruits against chloroquine-resistant *Plasmodium falciparum, in vitro,* and against *P. berghei, in vivo*. J. Nat. Prod. 50; 41-48.

O'Neill, M.J., Bray, D.H., Boardman, P., Wright, C.W., Phillipson, J.D., Warhurst, D.C., Gupta, M.P., Correya, M. and Solis, P. (1988). Plants as sources of antimalarial drugs, Part 6. Activities of *Simarouba amara* fruits. J. Ethnopharmacol 22; 183-190.

Nelson, J.A.S. and Falk, R.E. (1993). The efficacy of phloridzin and phloretin on tumour cell growth. Anticancer Res. 13; 2287-2292.

Neva, F. and Sacks, D. (1990). Leishmaniasis. In: Tropical and Geographical Medicine. 2nd edition. K. S. Warren and A. A. F. Mahmoud (eds); 296-308.

van Nieuwenhove, S., (1992). Advances in sleeping sickness therapy. Ann. Soc. Belg. Med. Trop, 72, Suppl. 1; 39-51.

Nkunya, M.H.H. (1992). Progress in the search for antimalarials. NAPRECA monograph series, 4, published by NAPRECA Addis Ababa; 1-36.

Noamesi, B.K. and Bamgbose, S.O.A. (1980). The alpha-adrenoceptor blocking properties of cryptolepine on the rat isolated vas deferens. Studies on cryptolepine. Planta Med. 39; 51-56.

Noamesi, B.K. and Bamgbose, S.O.A. (1983a). studies on cryptolepine. Cryptolepine antagonism of noradrenaline and modification of this effect by calcium ions and prostaglandin E_2 on rat isolated mesenteric artery. Planta Med. 47; 100-102.

Noamesi, B.K. and Bamgbose, S.O.A. (1983b). Studies on cryptolepine. Effects of cryptolepine on smooth muscle

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

Noamesi, B.K. and Bamgbose, S.O.A. (1984). Studies on cryptolepine III: effects of cryptolepine on the tone and prostaglandin production in isolated rabbit duodenum. Planta Med. 51; 98-101.

Northover, A.M. and Northover, B.J. (1993). Possible involvement of microtubules in platelet-activating factor-induced increases in microvascular permeability *in vitro*. Inflammation, 17; 633-639.

Novy, F.G. and Mc Neal, W.J. (1904). On the cultivation of *Trypanosoma brucei*. J. Infect. Dis., 1; 1-30.

OAU/STRC, (1968), First inter-African symposium on traditional Pharmacopoeia and African medicinal plants. Publication No. 104 OAU/STRC, Lagos, see Sofowara, 1993.

OAU/STRC, (1985), African Pharmacopoeia, Vol 1. OAU/STRC Lagos, see Sofowara, 1993.

OAU/STRC, (1986). African Pharmacopoeia, Vol 2. OAU/STRC, Lagos, see Sofowara, 1993.

Ocran, J. (1983). Preface to the International seminar on Cryptolepine, Kumasi, Ghana, 27-30 July; 6-7.

O'Dwyer, P.J., Leyland Jones, B., Alonso, M.T., Marsonis, S. and Wittes, R.E. (1985). Etoposide (VP-16-213): Current status of an active anticancer drug. N. Eng. J. Med. 312; 697-700.

Oguakwa, J. U., Galeffi, C., Messana, I., Patamia, M., Nicoletti, M. and Marini-Bettolo, G.B. (1983).

Research on African medicinal plants III. New alkaloids from *Alstonia boonei* De Wild. Gazzetta Chim. Ital. 113; 533-535.

Ojewole, J.A.O. (1984). Studies on the pharmacology of echitamine, an alkaloid from the stem bark of *Alstonia boonei* L. (Apocynaceae). Int. J. Crude Drug Res. 22; 21-143.

Okanla, E.O., Owoyale, J.A. and Akinyanju, J.A., (1990). Trypanocidal effect of an aqueous extract of *Acalypha hispida* leaves. J. Ethnopharmacol., 29; 233-237.

Oliver-Bever, B. (1986). Medicinal Plants in Tropical West Africa. Cambridge University Press; 160-168.

Olliaro, P.L. and Bryceson, A.D.M. (1993). Practical progress and new drugs for changing patterns of leishmaniasis. Parasitol. Today, 9; 323-328.

Osisanya, J.O.S., Gould, S., and Warhurst, D.C. (1981). A simplified culture technique for *Plasmodium falciparum*. Ann. Trop. Med. Parasitol. 75; 107-109.

Owolabi, O.A., Makanga, B., Thomas, E.W., Molyneux, D.H. and Oliver, R.W.A. (1990). Trypanocidal Potentials of African Woody Plants: *in vitro* trial of *Khaya grandifolia* seed extracts against *Trypanosoma brucei brucei*. J. Ethnopharmacol. 30; 227-231.

Pal, M. K. and Ghosh, J. K. (1994). Circular dichroic and spectrophotometric probes of the competitive binding of crystal violet between DNA and other anionic polymers. Spectrochimica Acta. 50A; 119-126.

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

D.C. (1991). Antimalarial activity of 3-methoxyethoxymethyl brusatol, an etherified quassinoid. Trans. R. Soc. Trop. Med. Hyg. 85; 316.

Pavanand, K., Yongvanitchit, K., Webster, H.K., Dechatiwongse, T., Nutakul, W., Jewvachdamrongkul, Y. and Bansiddhi, J. (1988). *In vitro* antimalarial activity of a Thai medicinal plant *Picrasma javanica* BI. Phytother. Res. 2; 33-36.

Pavanand, K., Webster, H.k., Yonvaritchit, K., Kun-anake, A., Dechatiwongse, T., Nutakul, W. and Bansiddhi, J. (1989). Schizontocidal activity of *Celastrus paniculatus* Willd. against *Plasmodium falciparum, in vitro.* Phytother. Res. 3; 136-139.

Pentreath, V.W. (1989). Neurobiology of sleeping sickness. Parasitology Today, 5; 215-218.

Pépin, J. and Milord, F. (1994). The treatment of human African trypanosomiasis. AAdv. Parasitol, 33; 2-47.

Peters, W. (1970). Chemotherapy and Drug Resistance in Malaria. Academic Press, London; 77-106.

Peters, W. (1987). Chemotherapy and drug resistance in malaria, Vol 1, 2nd ed. Academic press, London; 16, 111-115.

Phillipson, J.D. (1991). Assays for antimalarial and amoebicidal activities. In: Methods in Plant Biochemistry, Vol. 6. P.M. Dey and J.B. Harborne (eds.) Assays for Bioactivity. ed. K. Hostettman. Academic Press; 135-152.

Phillipson, J.D. and O'Neill, M.J., (1987). Antimalarial

and amoebicidal natural products. In: Biologically Active Natural Products. Proceedings Phytochem. Soc. Europe, 27, K. Hostettmann and P.J. Lea (eds.), Clarendon Press, Oxford; 46-64.

Phillipson, J.D. and Wright, C.W., (1991a). Medicinal plants against protozoal diseases. Trans. R. Soc. Trop. Med. Hyg. 85; 18-21.

Phillipson, J.D. and Wright, C.W., (1991b) Antiprotozoal agents from plant sources. Planta Med., 57, Suppl; 53-59.

Phillipson, J.D., and Wright, C.W. (1991c). Can ethnopharmacology contribute to the development of antimalarial agents ? J. Ethnopharmacology, 32; 155-165.

Phillipson, J.D., Wright, C.W., Kirby, G.C. and Warhurst, D.C. (1993). Tropical plants as sources of antiprotozoal agents. In: Recent Advances in Phytochemistry, Vol. 27, Phytochemical potential of tropical plants, K.R. Downum, J.T. Romeo and H.A. Stafford (eds.), Plenum Press, New York and London; 1-40.

Plotkin, M.J. and Schultes, R.E. (1990). *Virola*: a promising genus for ethnopharmacological investigation. J. Psychoactive Drugs, 22; 357-361.

Pongprayoon, U., Baeckström, P., Jacobsson, U., Lindström, M. and Bohlin, L. (1991). Compounds Inhibiting Prostaglandin Synthesis Isolated from *Ipomoea pes-caprae*. Planta Med. 57; 515-518.

Posner, G. H., Oh. C. H., Webster, H. K. Ager, A. C., Rosson, R. N. (1994). New antimalarial, tricyclic 1,2,3-trioxanes: evaluation in mice and monkeys. Am. J. Trop Med. Hyg. 50; 522-526.

del Pozo, E.C. (1979). Empiricism and magic in Aztec pharmacology. In: Ethno-pharmacologic Search for Psychoactive Drugs; D.H.E. Efron, B. Holmstedt and N. S. Kline (eds.), Raven Press; 59.

Principe, P.P. (1991). Valuing the Biodiversity of Medicinal Plants. In: Conservation of Medicinal Plants, O. Akerele, V. Heywood and H. Synge (eds.); 84 and 229.

Prost, S and Riou, G. (1994). A human small-cell lung carcinoma cell line, resistant to 4'-(9-acridinylamino)-methanesulfon-M-anisidide and cross-resistant to camptothecin with a high-level of topoisomerase I. Biochem. Pharm. 48; 975-984.

Puri, A., Saxena, R.P., Guru, P.Y., Kulshreshtha, D.K., Saxena, K.C. and Dhawan, B.N. (1992). Immunostimulant activity of picroliv, the iridoid glycoside fraction of *Picrorhiza kurroa* and its protective action against *Leishmania donovani* infection in hamsters. Planta Med. 58; 528-532.

Rapoport, H. (1983). Morphine and related opioid analgesics. In: Natural Products and Drug Development P. Krogsgaard-Larsen, S. Brogger Christensen and H. Kofod (eds.), Munksgaard, 439-447.

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

Ratsimamanga, le Bras, J., Ramiliarisoa, O and Savel, J. (1991). Antimalarial activity and cytotoxicity of *Ficus pyrifolia* and *Rhus* (=*Baronia*) *taratana* leaf extracts; Phytother. Res. 5; 32-34.

Raymond-Hamet (1937). Sur quelques proprietés physiologiques des alcaloïdes du *Cryptolepis sanguinolenta* Schlechter. Comptes Rendus de la Soc. Biol. 126; 768-770.

Raymond-Hamet (1938). Sur les éffets hypotenseurs et vaso-dilateurs de la cryptolepine. Comptes Rendus de l'Academie des Sciences, 207; 1016-1018.

Razdan, R.K. (1983). Structure-activity studies and development of drugs from cannabinoids. In: Natural Products and Drug Development. P. Krogsgaard-Larsen, S. Brogger Christensen and H. Kofod (eds), Munksgaard; 486-499.

Richomme, P., Godet, M.C., Foussard, F., Toupet, L., Sévenet, T. and Bruneton, J. (1991). A novel anti-leishmanial labdane from *Polyalthia macropoda*. Planta Med. 57; 552-554

Rios, J.L., Recio, M.C., and Villar, A. (1988). Screening methods for natural products with antimicrobial activity: a review of the literature. J. Ethnopharmacol., 23; 127-149.

Riou, G., Gabillot, M., Barrois, M., Breitburd, F. and Orth, G. (1985). A type-II DNA topoisomerase and a catenating protein from the transplantable VX2 carcinoma. Eur. J. Biochem. 146; 483-488.

Robert-Géro, M., Bachrach, U., Bhatnagar, S. and Polonsky, D.J. (1985). Inhibition *in vitro* de la croissance des promastigotes de *Leishmania donovani* par des quassinoïds. C. R. Acad. Sc. Paris, t. 300, Série II; 803-806.

Rosenblatt, J.E. (1992). Antiparasitic Agents. Symposium on antimicrobial agents - Part VII. Mayo Clin. Proc. 67; 276-287.

Rovai, L.E., Aoki, A., Gerez, N.M., de Burgos, G. and Blanco, A. (1990). Effect of gossypol on trypomastigotes and amastigotes of *Trypanosoma cruzi*. J. Protozool, 37; 280-286.

Roy, S.K. and Chatterjee, A. (1968). Triterpene constituents of *Alstonia neriifolia* D.Don. J. Ind. Chem. Soc., 45; 21-22.

Rushing, L.G. and Bowman, M.C. (1980). Determination of gentian violet in animal feed, human urine and wastewater by high pressure liquid chromatography. J. Chromatogr. Sci. 18; 224-232.

Sagua, H., González, J., Araya, J., Loyola, L., Morales, G., Roco, R. and Isla, S. (1987). *In vitro* activity of natural products against *Trypanosoma cruzi:* trypanocidal effect and bioassays with *Artemia salina*. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Suppl. 82; 180.

Samuelsson, G., (1992). Drugs of natural origin, Swedish Pharmaceutical Press, 13, 266, 292, 179.

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

Thulin, M., Hedberg, O., Warfa, A.M., Hassan, A.O., Elmi, A.H., Abdurahman, A.O., Elmi, A.S., Abdi, Y.A., and Alin, M.H., (1991). Inventory of plants used in Traditional Medicine in Somalia. 1; Plants of the Families Acanthaceae - Chenopodiaceae. J. Ethnopharmacol. 35; 25-63.

Santos, L.S. and Barata, L.E.S. (1990). Determination of absolute configuration of (+) surinamensin via diastereomeric O-methyl mandelic esters. J. Brazil. Chem. Soc. 1; 47-49.

Scherpereel, P. (1991). Patient-controlled analgesia. Annales Françaises d'Anesthésie et de Réanimation 10; 269-83.

Schlesinger, P.H., Krogstad, D.J. and Herwaldt, B.L. (1988). Antimalarial agents: Mechanisms of action. Antimicrobial Agents, Chemother. 32; 793-798.

Schneider, K. and Kubelka, W. (1989) The antibacterial activity of *Alchornea cordifolia*. Planta Med. 55; 651.

Schneider, E, Hsiang, Y.H. and Liu, L.F. (1990). DNA topoisomerases as anticancer drug targets. In: Advances in Pharmacology, 21, T. August, M. W. Anders, F. Murad and A. Nies (eds.) Academic Press, Inc.; 149-183.

Schultes, R.E. and Holmstedt, B. (1971). Miscellaneous notes on Myristicaceous plants of South America. Lloydia (J.Nat. Prod.) 34; 61-78.

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

In: Ethnopharmacologic Search for Psychoactive Drugs, D.H. Efron, B. Holmstedt and N.S. Kline (eds)., Raven Press, 33-55.

Sharma, G.L. and Bhutani, K.K. (1988). Plant-based antiamoebic drugs; part 11. Amoebicidal activity of parthenin isolated from *Parthenium hysterophorus*. Planta Med. 54; 120-122.

Smith, P.J. and Soues, S. (1994). Multilevel therapeutic targetting by topoisomerase inhibitors. Brit. J. Cancer, 70, S23; S47-51.

Soejarto, D.D. and Robineau, L. (1993). TRAMIL: a research project on the medicinal plant resources of the Caribbean. Proceedings of a Symposium on Tropical Forest Medicinal Resources and the Conservation of Biodiversity. New York. January 24-25, 1992, see Farnsworth, 1993.

Sofowora, A. (1982). Medicinal Plants and traditional medicine in Africa. John Wiley and Sons Ltd.; 221-223.

Sofowora, A., (1993). Recent trends in research into African medicinal plants. J. Ethnopharmacol, 38, 209-214.

Souza Brito, A.R.M. and Souza Brito, A.A. (1993). Forty years of Brazilian medicinal plant research. J. Ethnopharmacol 39; 53-67.

Stanghellini, A. (1989). Situation de la trypannosomiase dans les états membres de l'OCEAC. Bull. Liais. Doc. OCEAC, 87; 7-11.

Suffness, M. and Pezzuto, J.M. (1991). Anticancer assays

in: Methods in Plant Biochemistry, P.M. Dey and J.B. Harborne, (eds.), Vol. 6; Assays for Bioactivity, K. Hostettmann, (ed.), Academic Press; 77.

Tan, G.T., Pezzuto, J.M., Kinghorn, A.D. and Hughes, S.H. (1991). Evaluation of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. J. Nat. Prod. 54; 143-154.

Tandon, J.S., Srivastava, V. and Guru, P.Y. (1991). Iridoids: a new class of leishmanicidal agents from *Nyctanthes arbor-tristis*. J. Nat. Prod. 54; 1102-1104.

Tanno, Y., Shindoh, Y. and Takishima, T. (1989). Modulation of Human Basophil Growth *in vitro* by Xiao-Quing-Long-Tang, Chai-Pu-Tang, Quing-Fei-Tang, Baialein and Ketotifen. Am. J. Chinese Med. XVII, 1-2; 45-50.

Targett, G. (1986). Malaria; current trends in treatment. MIMS, 15th January; 34.

Taylor, A.E.R. and Baker, J.R. (1968). The cultivation of parasites *in vitro*. Blackwell Scientific Publication: Oxford; 8-52.

TDR News, 34 (1990); 1-7.

TDR News, 41 (1992); 3-5.

TDR News, 42 (1993); 1-2

TDR News, 44 (1994); 1-2.

TDR Progress (1995); 115-123.

Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science 193; 673-679.

Trager, W. and Polonsky, J. (1981). Antimalarial activity of quassinoids against chloroquine-resistant

Plasmodium falciparum, in vitro. Am. J. Trop. Med. Hyg. 30; 531-537.

Trease, G.E. and Evans, W.C. (1989). Pharmacognosy, 13th ed. Bailliere Tindal; 10.

Urbina, J. A., Marchan E., Lazardi, K., Visbal, G., Apitz-Castro, R., Gil, F., Aguirre, T., Piras, M. M. and Piras, R., (1993). Inhibition of phosphatidylcholine biosynthesis and cell proliferation in *Trypanosoma cruzi* by ajoene, an antiplatelet compound isolated from garlic. Biochem. Pharmacol. 45; 2381-2387.

Ushio, Y., and Abe, H., (1991). The effects of Saikosaponin on macrophage functions and Lymphocyte Proliferation. Planta Med., 57; 515-518.

Vasanth, S., Gopal, R.H. and Rao, R.B. (1990). Plant antimalarial agents. J. Ind. Sci. Res. 49; 68-77.

Vennerström, J.L., Lovelace, J.K., Waits, V.B., Hanson, W.L. and Klayman, D.L., (1990). Berberine derivatives as antileishmanial drugs. Antimicrob. Ag. Chemother. 34; 910-921.

Verpoorte, R., Van Beek, T.A., Thomassen, P.H.A.M., Aandewiel, J. and Baerheim Svendsen, A (1983). Screening for antimicrobial activity of some plants belonging to the Apocynaceae and Loganiaceae. J. Ethnopharmacol. 8; 287-302.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. Brit. Med. Bull. 41; 105-114.

Villar, A., Rios, J.L., Recio, M.C., Cortes, D. and Cave, A. (1986). Antimicrobial activity of benzyl

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

Wall, M.E. and Wani, M.C., (1984). Antineoplastic structure activity relationships of camptothecin and related analogues. In: Natural Products and Drug Development, P. Krogsgaard-Larsen, S. Brogger Christensen and H. Kofod (Eds.), Munksgaard, 253-265.

Waller, D.P., (1993). Methods in Ethnopharmacology. J. Ethnopharmacol. 38; 189-195.

Walton, B.C., (1987). American cutaneous and mucocutaneous leishmaniasis. In: The Leishmaniases. Vol II, W. Peters and R. Killick-Kendrick (eds.); 637-664.

Wang, J.C. (1987). Recent studies of DNA topoisomerases. Biochimica et Biophysica Acta 909; 1-9.

Weenen, H., Nkunya, M.H.H., Bray, D.H., Mwasumbi, L.B., Kinabo, L.S. and Kilimali, V.A.E.B. (1990). Antimalarial activity of Tanzanian plants. Planta Med. 56; 368-370.

Wernsdorfer, W. H., (1991). The development and spread of drug-resistant malaria. Parasitol. Today, 7; 297-303.

Wéry, M. (1991). Therapy for African trypanosomiasis. Current Opinion in Infectious Diseases, 4; 838-843.

White, N.J. (1985). Clinical Pharmacokinetics of antimalarial Drugs. Clinical Pharmacokinetics 10; 187-215.

WHO/VBC/89. 967 (1989). Geographical distribution of arthropod-born diseases and their principal vectors;

85; D.M. Minter and C.J. Schofield (eds.); 78-106.

Williams, D.M. and Remington, J.S. (1977). Effect of human monocytes and macrophages on *Trypanosoma cruzi*. Immunology, 32; 19-23.

Williamson, J. and Scott-Finnigan, T.J. (1978). Trypanocidal activity of antitumor antibiotics and other metabolic inhibitors. Antimicrobial Agents and Chemother. 13; 735-744.

World Health Organization Regional Office for Europe (1973). Clinical pharmacological evaluation in drug control: report on a symposium, Heidelberg, 1972. Copenhagen, 1-89.

World Health Organization (1986). Epidemiology and control of African trypanosomiasis. Report of a WHO Expert Committee, Technical Report Series, 739; 1-125.

World Health Organization (1990a). Weekly Epidemiological Record No. 50; 14.

World Health Organization (1990b). Technical Report Series 793. Control of the Leishmaniases; 158 p.

World Health Organization (1994). The Role of Artemisin and its derivatives in the current treatment of Malaria. Report of an informal consultation convened by WHO in Geneva 27-29 Sept. 1993. WHO/MAL/94; 1067.

Wosu, L.K.O. and Ibe, C.C. (1989). Use of extracts of *Picralima nitida* bark in the treatment of experimental Trypanosomiasis: a preliminary study. J. Ethnopharmacol., 25; 263-268.

Wright, R.G. McR., Wakelin, L.P., Fieldes, A., Acheson,

R.M. and Waring, K.J. (1980). Effects of ring substituents and linker chains on the bifunctional interaction of diacridines into deoxyribonucleic acid. Biochemistry, 19; 5825-5836.

Wright, C.W., O'Neill, M.J., Phillipson, J.D. and Warhurst, D.C. (1988). Use of microdilution to assess *in vitro* antiamoebic activities of *Brucea javanica* fruits, *Simarouba amara* stem, and a number of quassinoids. Antimicrob. Agents and Chemother.; 1725-1729.

Wright, C.W. (1989) Antiamoebic and antimalarial natural products. Ph.D. thesis, University of London.

Wright, C.W. and Phillipson, J.D. (1990). Natural products and the development of selective antiprotozoal drugs. Phytother. Res. 4; 127-139.

Wright, C.W., Bray, D.H., O'Neill, M.J., Warhurst, D.C., Phillipson, J.D., Quentin-Leclercq, J. and Angenôt, L. (1991). Antiamoebic and antiplasmodial activities of alkaloids isolated from *Strychnos usambarensis*. Planta Med. 57; 337-340.

Wright, C.W., Allen, D., Cai, Y., Phillipson, J.D., Said, I.M., Kirby, G.C. and Warhurst, D.C. (1992). *In vitro* antiamoebic and antiplasmodial activity of alkaloids isolated from *Alstonia angustifolia* roots. Phytother. Res. 5; 121-124.

Wright, C.W., Allen, D., Phillipson, J.D., Kirby, G.C., Warhurst, D.C., Massiot, G. and Le Men-Olivier, L. (1993). Alstonia species: are they effective in malaria treatment ? J. Ethnopharmacol. 40; 41-45.

Wu, T.W., Zeng, L.H., Wu, J. and Fung, K.P. (1994). Morin, a wood pigment that protects three types of human cells in the cardiovascular system against oxyradical damage. Biochem. Pharmacol 47; 1099-1103.

Xiao Pei-gen (1991). The Chinese approach to medicinal plants - their utilization and conservation. In: Conservation of Medicinal Plants, O. Akerele, V. Heywood, and H. Synge (eds.); 305-313.

Yang, D.M. and Liew, F.Y. (1993) Effects of quinghaosu (artemisinin) and its derivatives on experimental cutaneous leishmaniasis. Parasitol. 106; 7-11.

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In vitro and *in vivo* Antimalarial Activity of Cryptolepine, a Plant-derived Indoloquinoline

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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 IC_{50} 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 × 4), when compared with untreated controls. Like 9-aminoacridine, 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-aminoacridine.

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 those with different or novel modes of action (Peters, 1982; White, 1985). For many living in areas of endemicity, traditionally used plants represent the sole source of medicines for the treatment of many illnesses, including malaria; such plants represent an important potential source of novel drug compounds (see, for example, Phillipson *et al.*, 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: 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; Boye and Oku-Ampofo, 1990). In the former Belgian Congo (Zaïre) and in Casamance (Senegal) infusions of the root are used in the treatment of stomach and intenstinal 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 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 and is also reported (Dwuma-Badu, 1983) to have been isolated from the Sri Lankan plant *Sida acuta* Burn. 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 (quinolo[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-aminoacridine (Fig. 1), which intercalates with DNA (Wright *et al.*, 1980), suggested that the interaction of cryptolepine with DNA should also be studied.

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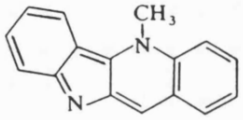

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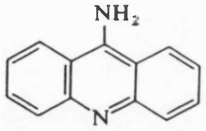

B

Figure 1. Structures of (A) cryptolepine, (B) 9-aminoacridine.

MATERIALS AND METHODS

Cryptolepine. This was supplied from Ghana, isolated 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 suspended in RPMI 1640 culture medium supplemented with D-glucose and 10% human A^+ serum. In the determination of drug sensitivity vigorously growing cultures with a predominance of young ring forms were used in a microtitre technique (Desjardins *et al.*, 1979; Ekong *et al.*, 1990). Cryptolepine and 9-aminoacridine (dissolved initially in ethanol and then diluted as necessary in serum-free culture medium) were tested in duplicate at 12 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 h in the presence of drugs before adding [G $-^3$H]-hypoxanthine (0.02 μCi 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 [^{3}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 at which inhibition of parasite growth represents 50%) were derived for each drug. Data from independent experiments were accumulated allowing calculation of the mean IC_{50} for each drug.

***In vivo* drug testing.** Random-bred, male, Swiss albino mice (TO strain, *Eperythrozoön*-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 (Peters, 1987). Infected erythrocytes, harvested from the donor mice, were injected intravenously (10^7 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 vitro* screening.** Tests were carried out on three separate occasions and the mean IC_{50} values for cryptolepine and chloroquine are shown in Table 1, together with

Table 1. Mean IC_{50} 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)

	mean IC_{50} (SE)		
	μg/mL	μM	*n*
Cryptolepine	0.031 (0.0085)	0.134 (0.037)	3
Chloroquine	0.073 (0.016)	0.230 (0.05)	3
9-aminoacridien	8.69 (–)	44.74 (—)	1

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

Dose (mg/kg/d × 4)	Mean parasitaemia %	Standard deviation %
0 (Control)	34.22	4.16
7.04	42.54	12.03
14.08	38.48	12.79
28.16	40.80	0.38
56.32	46.44	8.01
112.63	33.04	10.44

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 IC_{50} 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 *Triclisia 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 ED_{50} and ED_{90} 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

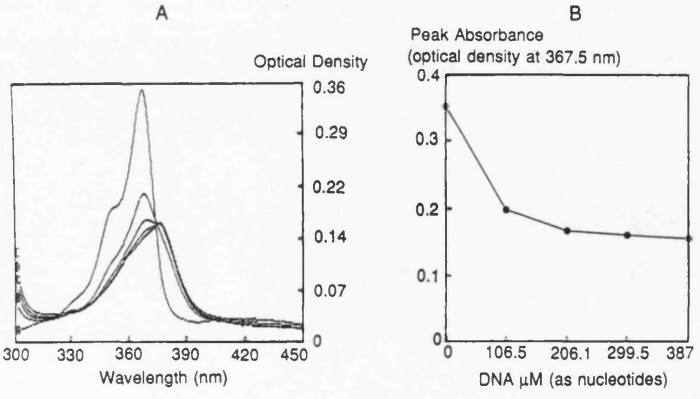

Figure 2. (A) Spectral changes occurring on addition of herring testis DNA solution to cryptolepine at pH 7.4. Four increments of 20 μL of DNA at 1 mg/mL in PBS were added to 580 μL cryptolepine solution at 3.25 μg mL. The uppermost peak is that obtained before the addition of any DNA. Readings were 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).

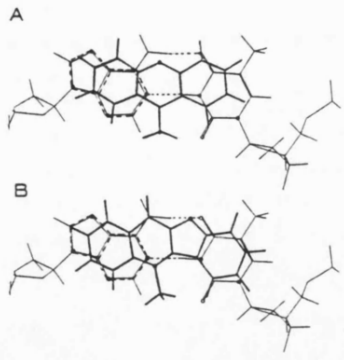

Figure 3. (A) Putative complex formed between 9-aminoacridine and a thymine–adenine base pair. (B) Putative complex formed by cryptolepine interacting with a thymine–adenine base pair, showing the possibility of better overlay of the alkaloidal ring systems with respect to the purine–pyrimidine pair. 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 AMI 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 $\pi - \pi$ 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-Ackeh, 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 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. 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 plasmodicidal activity.

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REFERENCES

Ablordeppey, S. Y. S. (1983). Some synthetic aspects of the chemistry of cryptolepine and its derivatives. *Proceedings of the First International Seminar on Cryptolepine, 27–30 July 1983, University of Science and Technology, Kumasi, Ghana*, pp. 30–36.

Boakye-Yiadom, K. (1979). The antimicrobial activity of some West African medicinal plants. *Q. J. Crude Drug Res.* **2**, 78–80.

Boakye-Yiadom, K. (1983). Antimicrobial properties of cryptolepine—a review. *Proceedings of the First International Seminar on Cryptolepine, 27–30 July 1983, University of Science and Technology, Kumasi, Ghana*, pp. 57–64.

Boakye-Yiadom, K., and Heman-Ackeh, S. M. (1979). Cryptolepine hydrochloride: effect on *Staphylococcus aureus*. *J. Pharmac. Sci.* **68**, 1510–1514.

Boye, G. L., and Oku-Ampofo (1983). Clinical uses of *Cryptolepis sanguinolenta*. *Proceedings of the First International Seminar on Cryptolepine, 27–30 July 1983, University of Science and Technology, Kumasi, Ghana*, pp. 37–40.

Boye, G. L., and Oku-Ampofo (1990). The rôle of plants and traditional medicine in primary health care in Ghana. In *Economic and Medicinal Plant Research*, Vol. 4 ed. by H. Wagner and N. R. Farnsworth, Academic Press, London.

Clinquart, E. D. (1929). La composition chimique du *Cryptolepis triangularis*—une plante du Congo de Belgique. *Bull. Soc. Roy. Méd. Publ. Royaume de Belgique*, **9**, 627–635.

Cordell, G. A. (1981). *Introduction to Alkaloids: A Biogenetic Approach*. John Wiley & Sons, New York.

Dalziel, J. M. (1956). *The Useful Plants of West Tropical Africa*. The Crown Agents for the Colonies, London.

Desjardins, R. E., Canfield, C. J., Haynes, J. D., and Chulay, J. D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.* **16**, 710–718.

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

sation of the alkaloids of *Cryptolepis sanguinolenta*. *Proceedings of the First International Seminar on Cryptolepine, 27–30 July 1983, University of Science and Technology, Kumasi, Ghana,* pp. 23–29.

Dwuma-Badu D., Ayim, J. S., Fiagbe, N. I., Knapp, J. E., Schiff Jnr, P. L., and Slatkin, D. J. (1978). Constituents of West African medicinal plants. XX: quindoline from *Cryptolepis sanguinolenta*. *J. Pharmac. Sci.* **67**, 433–434.

Ekong, R. M., Kirby, G. C., Patel, G., Warhurst, D. C., and Phillipson, J. D. (1990). Comparison of the *in vitro* activities of quassinoids with activity against *Plasmodium falciparum*, anisomycin and some other inhibitors of eukaryotic protein sysnthesis. *Biochem. Pharmacol.* **40**, 297–301.

Ekong, R. M., Partridge, S. J., Anderson, M. M. *et al.* (1991). *Plasmodium falciparum*: effects of phaeanthine, a naturally occurring bisbenzylisoquinoline alkaloid, on chloroquine-resistant and sensitive parasites *in vitro*, and its influence on chloroquine activity. *Ann. Trop. Med. Parasitol.* **85**, 205–213.

Gunatilaka, A. A. L., Sotheeswaran, S., Balasubramaniam, S., Chandrasekara, A. I., and Sriyani, H. T. (1980). Studies on medicinal plants of Sri Lanka III. Pharmacologically important alkaloids from some *Sida* species. *Planta Med.* **39**, 66–72.

Kerharo, J., and Adams, J. G. (1974). *La Pharmacopée Senegalaise Traditionelle*. Vigot Frères, Paris.

Kirby, G. C., and Warhurst, D. C. (1992). Antimalarial activity of the antihistamine drug terfenadine. *Trans. Royal Soc. Trop. Med. Hyg.* **86**, 343.

Kirby, G. C., Ekong, R. M., Keter, D. W., Midgeley, J. M., Phillipson, J. D., and Warhurst, D. C. (1994). Antiplasmodial activity *in vivo* of ciprofloxacin: *Mus musculus* versus *Mesocricetus auratus*. *Trans. Royal Soc. Trop. Med. Hyg.* **88**, 375.

Marshall (née Partridge), S. J. (1991). *Antimalarial Activities of Some West African Plants*. PhD Thesis, Brighton Polytechnic.

Perry, L. M., and Metzger, J. (1981). *Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses*. MIT Press, Massachusetts and London.

Peters, W. (1982). Antimalarial drug resistance: an increasing world problem. *Br. Med. Bull.* **38**, 187–192.

Peters, W. (1987). *Chemotherapy and Drug Resistance in Malaria* (revised 2nd edn), Academic Press, London.

Phillipson, J. D., Wright, C. W., Kirby, G. C., and Warhurst, D. C. (1993). Tropical plants as sources of antiprotozoal agents. In *Recent Advances in Phytochemistry*, Vol. 27, *Phytochemical Potential of Tropical Plants*, ed. by K. R. Downum, J. T. Romeo and H. A. Stafford, pp. 1–40. Plenum Press, New York.

Raymond-Hamet (1937). Sur quelques propriétés physiologiques des alcaloïdes du *Cryptolepis sanguinolenta* Schlechter. *Comptes Rendus de la Société de Biologie*, **126**, 768–770.

Salmon, D., Deloron, P., Malhotra, K., Gaudin, C., and Salmon, D. (1990). Effective treatment of malaria with ciprofloxacin and pefloxacin. *Antimicrob. Agents Chemother.* **34**, 2327–2330.

Sofowora, A. (1982). *Medicinal Plants and Traditional Medicine in Africa*. John Wiley & Sons, Chichester.

Stenlake, J. B. (1979). *Foundations of Molecular Pharmacology. Volume 2: The Chemical Basis of Drug Action*, p. 57. The Athlone Press of the University of London.

White, N. J. (1985). Clinical pharmacokinetics of antimalarial drugs. *Clin. Pharmacokinetics* **10**, 187–215.

Wright, R. G. McR., Wakelin, L. P., Fieldes, A., Acheson, R. M., and Waring, M. J. (1980). Effects of ring substituents and linker chains on the bifunctional interaction of diacridines into deoxyribonucleic acid. *Biochemistry* **19**, 5825–5836.