NATURAL PRODUCTS AGAINST PROTOZOAL DISEASES

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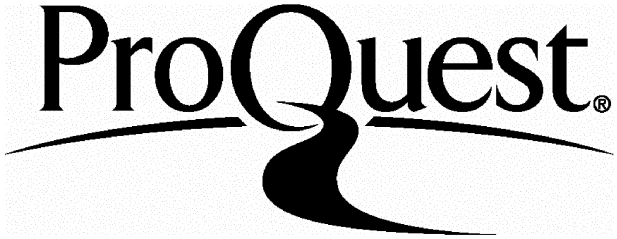

"Apply your heart to instruction
and your ears to words of knowledge"

Proverbs 23:12

I dedicate this thesis

to my late father

Mariano Camacho Garcia

ABSTRACT

Disease caused by parasite protozoa, including malaria, leishmaniasis and trypanosomiasis, result in considerable mortality and morbidity throughout the world. There is an urgent need for new chemotherapeutic drugs for the treatment of these diseases. Natural products represent one source of novel antiprotozoal drugs.

Forty-seven plant species obtained from Ghana, Panama, Mexico, Oman, Egypt and Kew Gardens, London were selected for antiprotozoal screening either because of their traditional use or because of chemotaxonomic considerations. Methanolic and aqueous extracts of these plants were prepared and screened against *L. donovani* promastigotes and *T. b. brucei* trypomastigotes *in vitro*. The cytotoxicity of the extracts was determined against KB cells and compared with their antiprotozoal activities *in vitro*. The toxicity of selected extracts was also assessed using P388D1 cells and brine shrimps. From the preliminary screening *Celaenodendron mexicanum, Galphimia glauca, Guarea rhopalocarpa, Stephania dinklagei, Triclisia patens, Cephaelis camponutans and Hintonia latiflora* were selected for further investigation in order to isolate their active principles. Bioactive-guided fractionation using *L. donovani* promastigotes, *P. falciparum*, KB cells or brine shrimps *in vitro*, and a combination of chromatographic techniques yielded some 38 pure compounds of which 11 were novel, and 9 semisynthetic derivates were prepared. The structure of isolated compounds was determined by UV, IR, HRMS, ^{1}H NMR, ^{13}C NMR, COSY-45, HMQC, HMBC, COLOC and NOESY experiments.

C. mexicanum yielded 3 active terpenes, epi-oleanolic acid, 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid and 3-oxo-tirucalla-7,24Z-dien-26 oic acid. In addition, 4 terpenes, friedelin, maytensifolin B, 3β-hydroxy-friedelan-16-one and celaenodendrolide as well as 3 biflavonoids, amentoflavone, podocarpusflavone A and podocarpusflavone B were also isolated from this plant. *G. glauca* afforded the active flavonol quercetin and the novel terpenes galphimine C, galphimine D, galphimine E and glaucamine, and the known steroids stigmasterol and sitosteryl-3-O-β-D-glucopyranoside. *G. rhopalocarpa* yielded 4 new active terpenes, 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, lanosta-7,9(11),24EZ-triene-3α,23-diol, ent-8(14),15-sandaracopimaradiene-

2β,18-diol, and ent-8(14),15-sandaracopimaradiene-2α,18-diol, together with the coumarin scopoletin. *S. dinklagei* afforded 6 aporphine alkaloids, the novel stepharandine, N-methyl-liriodendronine and 2-O,N-dimethyl-liriodendronine and the known corydine, liriodenine and dicentrinone, and the anthraquinone aloe-emodin. *T. patens* yielded 2 active principles the bisbenzylisoquinoline alkaloids phaeanthine and aromoline. Two active quinones, benzo[g]isoquinoline-5,10-dione and 1-hydroxybenzoisochromanquinone were isolated as the active principles of *C. camponuntants*. Two phenylcoumarins 5-O-β-D-glucopyranosyl-3',4'dihydroxy-7-methoxy-4-phenylcoumarin, 5-O-β-D-galactopyranosyl-3',4'dihydroxy-7-methoxy-4-phenylcoumarin; one cucurbitacin, 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F and one flavonoid, 7-methyl-luteolin were isolated from *H.latiflora*.

A total of 97 natural products including alkaloids, quinones, terpenes, flavonoids and coumarins, were screened for their antiprotozoal activity against *L.donovani, T.b.brucei* and cytotoxicity against KB and P388D1 cells *in vitro*, in order to establish structure activity relationships. This study revealed that the most active compounds were the bisbenzylisoquinoline alkaloids phaeanthine and fangchinoline with IC_{50} values at 1.5 μg/ml and 0.24 μg/ml respectively against promastigotes forms of *L.donovani*. The most active against *T.b.brucei* and *T.cruzi* was the quinone 1-acetyl-benzoisochromanquinone with IC_{50} values of 0.17 and 1.79 μg/ml, respectively. 7-Methyl-luteolin and quercetin were the most active against *P.falciparum* with IC_{50} values of 13.9 and 6.5 μg/ml respectively. Acetylstrictodine and acetylstrictosidine lactam gave a 30 and 33.2% of inhibition of liver parasites in experimental model of *L.donovani* compared to pentostam.

Selected natural products and crude extracts were assessed for their immunomodulatory activity *in vitro* using the mitogenesis of BALB/c mice spleen cells and superoxide release in murine macrophages cell line J774. In combination with Con A, the strongest synergistic effect on lymphocyte proliferation was observed with 6 compounds, having a maximum effects in the interval of 4-5 times over control at concentration range of 0.001-1 μg/ml. In the case of superoxide release, 31 compounds were able to promote spontaneous release of superoxide in the range of 2-6 times over control at concentrations equal or lower than 1 μg/ml.

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CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency syndrome
APC's	Antigen Presenting Cells
BBIQ	Bisbenzylisoquinoline
BCG	Bacille Calmette Guerin
br	Broad
^{13}C	Carbon thirteen
°C	Centrigrade
CC	Column Chromatography
$CDCl_3$	Deuterochloroform
C_5D_5N	Deuteropyridine
CD_3OD	Deuteromethanol
CF_3CO_2D	Deuterotrifluroacetic acid
CI	Chemical Ionization
CNS	Central Nervous System
CMC	Carboxymethylcellolose
COLOC	Correlation Spectroscopy via Long-range Coupling
Con A	Concanavaline A
COSY-45	Two dimensional ^1H -^1H correlation spectroscopy
CSF	Cerebro Spinal Fluid
d	Doublet
dd	Double doublet
ddd	Double double doublet
2D	Two Dimensional
DCL	Diffuse Cutaneous Leishmaniasis
DEPT	Distortionless Enhancement by Polarization Transfer
DHFR	Dihydrofolate reductase
DMAP	Dimethyl Amino Pyridine
DMFO	DL-α-Difluoromethylornithine

$DMSO\text{-}d_6$	Deuterodimethylsuphoxide
DNA	Deoxynucleic acid
ED_{50}	Effective Dose fifty
EIMS	Electron Impact Mass Spectrometry
FABMS	Fast Atom Bombardment Mass Spectrometry
FBS	Foetal Bovine Serum
FPIX	Ferriprotoporphyrin IX
FMLP	N-Formylmethionyl-leucyl-phenylalanine
GC-MS	Gas Chromatography-Mass Spectrometry
G6PD	Glucose-6-phosphate dehydrogenase
^1H	Proton
HAT	Human African Trypanosomiasis
HBSS	Hanks Balance Salt Solution
Hex	n-Hexane
HIFCS	Heated Inactivated Foetal Calf Serum
HMBC	Heteronuclear Multiple Bond Connectivity
HMQC	Heteronuclear multiple quantum coherence
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
Hz	Hertz
IC_{50}	Inhibition Concentration fifty
IC_{90}	Inhibition Concentration ninety
ID_{50}	Inhibitory Doses fifty
IL	Interleukin
IR	Infrared
IFN	Interferon
J	Coupling constant
kDNA	Kinetoplast Deoxyribonucleic acid
Kg	Kilogram
l	Litre
LC_{50}	Lethal Concentration fifty
LCL	Local Cutaneous Leishmaniasis

LPS	Lipopolissacharide
μM	MicroMolar
μCi	Microcurie
m	Multiplet
MDR	Multidrug resistant
mM	MilliMolar
M	Molar
MCL	Mucocutanous Leishmaniasis
mg	Milligram
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Normal
max	Maxima
MEC	Minimum Effective Concentration
Mel T	Melarsoprol Trypanothione
mg	Milligram
ml	Millilitre
m/z	Mass of the ion in Dalton divided by its charge
MHz	Mega Hertz
MIC	Minimum Inhibitory Concentration
MNOBA	Mononitro-Ortho-Benzoic Acid
MS	Mass Spectrometry
NK	Natural Killer
nm	Nanometre
nM	NanoMolar
NMR	Nuclear Magnetic Resonance
NOESY	Two dimensional Nuclear Overhauser Effect Spectroscopy
OB	Oxidative Burst
PABA	p-Amino Benzoic Acid
PBS	Phosphate Buffer Solution
PHA	Phytohemagglutinin
PMN	Polymorphonuclear leukocytes
ppm	Part per million

PTLC	Preparative Thin Layer Chromatography
RNA	Ribonucleic Acid
s	Singlet
Sb^v	Antimony pentavalent
SEM	Standard Error Media
SDS	Sodium Dodecyl Sulphate
sh	Shoulder
sp	Species
RDA	Retro Diels Alder
SOD	Superoxide Dismutase
SRBC	Sheep Red Blood Cell
t	Triplet
TCR	T Cell Receptor
td	Triplet doublet
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TNF	Tumour Necrosis Factor
TPA	12-O-Tetradecanoylphorbol-13-acetate
UV	Ultraviolet
VL	Visceral Leishmaniasis
WHO	World Health Organization

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CHAPTER 1

NATURAL COMPOUNDS AGAINST PROTOZOAL DISEASES

1.1 Introduction

Protozoan diseases are responsible for considerable mortality and morbidity throughout the world. Malaria caused by *Plasmodium* sp. is the most prevalent of these diseases and is one of the main causes of deaths in endemic areas. It is estimated that the disease afflicts 300-500 million people and kills about up to 2 million annually. In Africa alone the disease is responsible for the deaths of one million children each year. Kinetoplastida protozoa such as *Leishmania* spp., *Trypanosoma cruzi*, and *Trypanosoma brucei* sp. are the etiological agents of leishmaniasis, American trypanosomiasis (Chagas' disease), and Human African trypanosomiasis (sleeping sickness), respectively. Worldwide it has been estimated that some 12 million people have leishmaniasis and about 18 million people in Latin America have Chagas' disease with 45 000 deaths per year. There are 50 000 new cases of sleeping sickness reported each year. Other protozoal diseases such as amoebiasis and giardiasis, are responsible for 42 million and 200 million infections annually (WHO, 1995). In addition, opportunistic protozoal parasites including *Cryptosporidium parvum, Toxoplasma gondii*, *L. infantum* and *L. donovani* are responsible for a considerable number of deaths in immunocompromised patients (Zumla & Croft, 1992). Leishmaniasis, trypanosomiasis and malaria are briefly described below on account of their prevalence and mortality and because of the needs for effective control of these diseases (Table 1.1).

1.2 Leishmaniasis

Leishmaniasis is a protozoan disease whose diverse clinical manifestations are dependent both on the infecting species of *Leishmania* and on the immune response of the host. This complex disease is endemic in the tropical regions of America, Africa, India, South-

Table 1.1 Chemotheraphy and mode of action of some drugs used for the treatment of malaria, leishmaniasis and trypanosomiasis.

	Protozoa	Geographical distribution	Drugs	Chemical class	Mode of action
Malaria	*P. vivax*	Tropical, subtropical and temperate areas of Latin America and Asia.	Quinine, quinidine	Quinine alkaloids	FPIX-drug complex, inhibit haem polymerization
			Chloroquine	4-Aminoquinoline	"
			Mefloquine	4-Quinoline methanol	"
			Primaquine	8-Aminoquinoline	unknown
	P. falciparum	Tropical and subtropical regions of Africa, South-East Asia and Central America.	Pyrimethamine	Diaminopyrimidine	Inhibits dyhydrofolate reductase (DHFR)
			Proguanil	Biguanide	"
			Sulphadoxine	Sulphonamide	Inhibits dihydropteroate synthetase (DPS)
			Halofantrine	9-Phenantrene methanol	Unknown
		Central West Africa and	Artemisinin and derivates	Sesquiterpene lactone	Free radicals
	P. ovale	West Pacific regions.	Tetracycline	Tetracycline	Inhibits protein synthesis at the ribosomal level
			Clyndametacine,Clindamycin	Lincosamide	"
		Worldwide in malaria	Pyrimethamine/sulphadoxine		
	P. malariae	endemic areas	Quinine/tetracycline		
			Pyrimethamine/clindamycin		
Leishmaniasis [VL]	*L. donovani*	China, India	Sodium stibogluconate	Pentavalent antimonial	Unknown
			N-methylmeglumine antimonate	"	"
		Southwest Asia, North and East	Pentamidine	Diamidine	Inhibits DNA, RNA, phospholipid and protein
VL [LCL]	*L. infantum*	Africa, Southern Europe	Amphotericin B	Polyenentibiotic	Membrane function
		South and Central America			
VL [LCL]	*L. chagasi*	Asia, China, Africa			
LCL	*L. major*	North Africa			
LCL [DCL]	*L. aethiopica*	Asia, South Europe			
LCL [VL]	*L. tropica*	Central and South America			
LCL, MCL	*L. braziliensis* complex	Mexico, Central and South America			
LCL, DCL	*L. mexicana* complex	Mexico, Central and South America			
					Inhibits kinetoplast DNA, proteins and lipids
American trypanosomiasis (Chagas' disease)	*T. cruzi*	Latin America	Benznidazole	Nitroimidazole	Free radicals, inhibit trypanothione reductase
			Nifurtimox	Nitrofuran	
					Inhibits topoisomerase II and glycolisis
African trypanosomiasis (Slepping sickness)	*T. b. gambiense*	West Africa	Eflornithine	Diamidine	Inhibits RNA polymerase and other enzymes
			Pentamidine	Napthylamine	Inhibits trypanothione reductase and glycolysis
	T. b. rhodesiense	East Africa	Suramin	Arsenical	Inhibits ornithine decarboxylase
			Melarsoprol	Ornithine analogue	

VL: Visceral leishmaniasis; LCL: Local Cutaneous Leishmaniasis; DCL: Diffuse Cutaneous Leishmaniasis; MCL: Mucocutaneous Leishmaniasis. Brackets denote uncommon manifestation of disease. FPIX. Ferriprotoporphyrin IX. Adapted in part from Leiby *et al.*, 1994.

West Asia and the Mediterranean (Grevelink & Lerner, 1996). Transmission of the disease occurs through the bite of a sandfly (*Lutzomyia* or *Phlebotomus*). There are over 20 known species of *Leishmania* of which over a dozen are associated with various forms of leishmaniasis (Table 1.1). Two main clinical forms of leishmaniasis result from infection by different species of the parasites: cutaneous leishmaniasis and visceral leishmaniasis.

Cutaneous leishmaniasis (CL) has three variants: diffuse cutaneous leishmaniasis (DCL), recidiva leishmaniasis (RL) and mucocutaneous leishmaniasis (MCL). Cutaneous leishmaniasis is an infection restricted to the skin and is characterized by a number of small lesions at the site of the bite, which develops into a large sore. Without treatment the disease can last for several months or years leaving disfiguring scars. DCL is associated with cutaneous metastases from the initial skin lesion which occurs when the immune system fails to react effectively to infection. Lesions cover a large part of the body, never heal without treatment, and are extremely refractory to all forms of therapy (Evans, 1993). MCL is characterized by destruction of the nasopharyngeal mucosa and it is chronic and difficult to treat (Martinez *et al.*, 1992). RL is a chronic and progressive form of CL, which may last for years with little or no response to treatment.

Visceral leishmaniasis (VL) or kala-azar involves infection of the reticuloendothelial system. The disease is characterized by marked weight loss, fever, thrombocytopenia, massive enlargment of the liver and spleen (Grevelink & Lerner, 1996). VL has been characterized as an opportunistic infection in patients suffering from immunosupression due to HIV infection or immunosuppressant drugs taken for organ transplant or cancer (Alvar *et al.*, 1992; Da-Cruz *et al.*, 1992; Estambale & Knight, 1992; Golino *et al.*, 1992; Dereure *et al.*, 1995; Rosenthal *et al.*, 1995). VL is caused by *L. donovani, L. infantum* and *L. chagasi*, however, the relation between parasites and pathology is not clear. For example, some strains of *L. infantum* cause VL, others CL (Ho *et al.*, 1994), while other strains may be avirulent to immunocompetent individuals, and all three types can cause VL in immunocompromised individuals (Gradoni & Gramiccia, 1994). *L. tropica* associated with CL can also cause VL (Kreutzer *et al.*, 1993) and *L. amazonensis* is

associated with CL, VL and diffuse cutaneous leishmaniasis. Non-ulcerative cutaneous nodules known as post-kala-azar dermal leishmaniasis (PKDL) may develop in some patients as a complication during VL treatment.

1.2.1 Life cycle of *Leishmania* sp.

Leishmania as the other kinetoplastida protozoa have a specialized mitochondrial structure, and they exist as flagellates for part or all of their life-cycle and are transmitted by arthropod vectors. The life cycle of *Leishmania* parasites (Leiby *et al.*, 1994) involves two morphological stages, promastigotes and amastigotes (Figure 1.1).

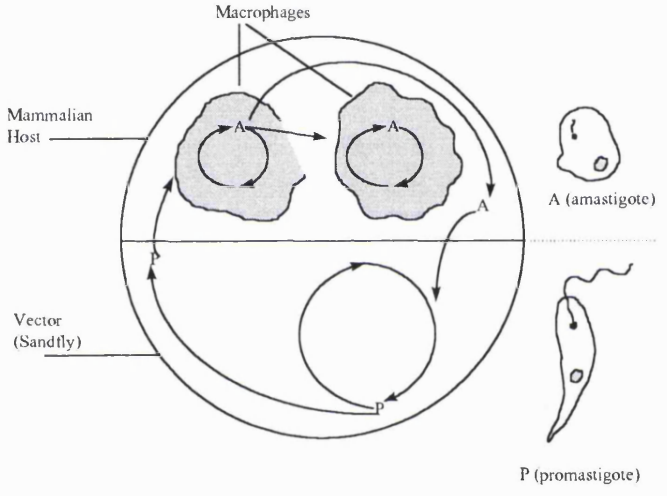


Figure 1.1 Life cycle of *Leishmania* sp.

Infective promastigotes (metacyclic forms) are deposited into the skin of the mammalian host along with salivary secretion, when an infective sandfly takes a blood meal; metacyclic promastigotes are rapidly phagocytosed by macrophages and/or Langerhans cells in the skin. The phagocytosed promastigotes are enclosed in a phagosome in the host cell, and transform into an amastigote, which adjusts to higher temperature, and lower pH enviroment within the phagolysosome. Replication by binary fission takes place until the initially infected cell ruptures and releases amastigotes that, in turn, are phagocytozed by other macrophages in the skin or visceral organs where they continue dividing. The cycle of infection and replication continues until the infection is

established. A female sandfly, during a blood meal, ingests amastigotes which are released into the midgut. The amastigotes replicate and transform into a non-dividing, highly infective metacyclic stage. The metacyclic forms move to the pharynx and proboscis and are transmitted during the next blood meal of the sandfly.

1.2.2 Drug treatment of Leishmaniasis

1.2.2.1 Antimonials

Two pentavalent antimonials, sodium stibogluconate (Pentostam^R) and N-meglumine antimonate (Glucantime^R), are considered the first line antileishmanial drugs. They have been used for the treatment of all forms of leishmaniasis, except DCL, for over the last 40 years. The chemical structure of sodium stibogluconate and N-meglumine antimonate has not been established. The efficacy of Sb^V in treating leishmaniasis depends upon species and strains of parasites, as well as the individual immune host response. Pentavalent antimonials are administrated by intramusclar or intravenous injections and require hospital admission for the whole period of treatment due to their toxicity. Pentavalent antimonials are rapidly excreted in the urine, this avoids acute toxicity, but cumulative toxicity may occur due to the production of a slowly excreted trivalent metabolite, which increases in proportion to dose and duration of treatment. Common side effects at normal therapeutic dose include: myalgia, anorexia, vomiting, lethargy, hepatic, as well as cardiac and renal damage (Olliario & Bryceson, 1993).

Treatment failures with pentavalent antimonials have long been observed in the chemotherapy of leishmaniasis. Reasons postulated for failure include, development of resistance, poor drug penetration into sites of infection, insensitivity of amastigotes, poor host immunity, lack of understanding of pharmacokinetics and perhaps most important, species and strain differences in drug sensitivity. In CL, many species of parasites are involved and there is increasing evidence to suggest innate differences in responsiveness. *L. aethiopica* and *L. mexicana*, for example are relatively insensitive to antimonials (Olliario & Bryceson, 1993). Experimental data suggests that parasite resistance to antimony emerges as a result of discontinous drug exposure or inadequate dosage, for

example significant resistance among isolates of *L. mexicana* and *L. brazilienses* has been documented (Grogl *et al.*, 1992). *In vitro* studies with promastigotes has indicated that the presence and expression of the gene PGPA in *L. major* and ltPGPA in *L. tarentolae* promoted the development of resistance to Sb^{III} and Sb^{V} respectively by decreasing influx of the drug (Olliario & Bryceson, 1993). On the other hand, it is generally accepted that chemotherapy of leishmaniasis may not completely eradicate the parasite; cell mediated immune responses control infection when low number of parasites persist. The appearance of VL in some AIDS and immunocompromised patients confirms the importance of the immune system in eradicating these parasites (Dereure *et al.*, 1995; Grammicia *et al.*, 1992; Gradoni & Gramiccia, 1994). Also, the ineffectiveness of antimonial therapy in DCL indicates that the drug alone is not capable of eradicating the parasites without the input of the immune system (De Rossell *et al.*, 1992), and in general, the treatment of leishmaniasis in immunocomprised patients is difficult to treat. The mode of action of pentavalent antimonial drugs against *Leishmania* is unknown, although studies *in vitro* with amastigotes have showed that they inhibit glycolitic enzymes and fatty acid oxidation (Berman, 1988).

In another approach, liposomal encapsulation of pentavalent antimonials has shown to increase the activity by 200 to 700 fold compared with the free drug in *Leishmania* infected rodents (Croft *et al.*, 1989). Combined immunochemotherapy (antimonials and interferon-γ-or Bacille Calmette-Guerin, BCG) has been studied in clinical trials for the treatment of VL with very good results but these studies are in early stage (Berger & Fairlamb, 1992).

1.2.2.2 Pentamidine

The diamidine pentamidine (Figure 1.2) is used for the treatment of DCL, unresponsive and relapsed cases of VL to antimonials, and for the treatment of early stage of African trypanosomiasis (see section 1.4.2.1). The long course of treatment, slow rate of excretion and undesirable side effects are the draw-backs of this drug. The mode of action of pentamidine in *Leishmania* is poorly understood, although it is known to damage the kinetoplast-DNA-mitocondrial complex (Olliario & Bryceson, 1993). In

another investigation pentamidine-bound nanoparticles have proved to be 25 times more active than the free drug against *L. donovani* promastigotes (Deniou *et al.*, 1993). Less toxic analogues of pentamidine have been synthesised and they are in process of evaluation for leishmaniasis (Chauhan *et al.*, 1993).

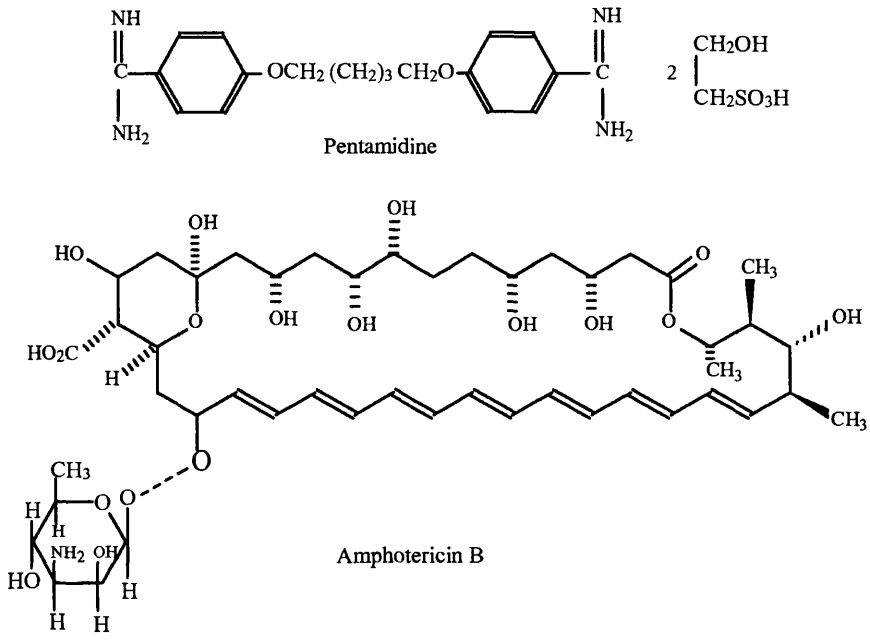


Figure 1.2 Second-line drugs used for the treatment of leishmaniasis

1.2.2.3 Amphotericin B

Amphotericin B (Figure 1.2) is a very toxic polyene antibiotic, used for the treatment of unresponsive and relapsed cases of VL and for advanced cases of MCL which fail to respond to antimonials (Sahay & Jha, 1996). The drug is excreted slowly after being administered by intravenous infusion; its use is limited because of its adverse reactions such as thrombocytopenia, convulsions, anorexia, and renal damage. Amphotericin B acts by binding to ergosterol, which is the major cell membrane sterol in these organisms, leading to pore formation and a consequent increased in cell permeability which causes cell death. In mammalian cells amphotericin B also binds to cell membrane cholesterol, causing cell death (Torre-Cisneros *et al.*, 1993). Drug-delivery systems based on lipid-associated amphotericin B have increased the therapeutic index of this drug. Liposomal

amphotericin B(AmBisome) has been used succesfully in a patient with VL unresponsive to standard treatment (Croft *et al.*, 1991; Davidson *et al.*, 1994; Davidson & Croft, 1993; Lazanas *et al.*, 1993); amphotericin B cholesterol sulphate (Amphocil) and amphotericin B lipid complex are now underway in human clinical trials (Bryceson, 1994; Dietze *et al.*, 1992). Finally, amphotericin B has also shown activity against *Trypanosoma cruzi* infected- mice (Castro *et al.*, 1993).

1.3 American trypanosomiasis (Chagas' disease)

Trypanosoma cruzi is the etiological agent of Chagas' disease which is endemic in Latin America. The disease is transmitted by many species of Triatomine bugs, although there are reports of infection as a result of blood transfusion, congenital transmission, via organ transplant and by accidental inoculation (WHO, 1995). Domestic animals, rodents and small feral species serve as reservoirs. Chagas' disease has three defined phases: acute, indeterminate and chronic. The acute phase is characterised by symptoms such as headaches, fever, anaemia and general malaise. In many cases the infection has an indeterminate phase during which no symptoms are detected and this may last for many years or for the rest of the life of the patient. About 30% of the infected people go on to develop a progressive chronic form associated with myocarditis and/or digestive system megasyndromes. In the chronic stage, Chagas' disease remains incurable (De Castro, 1993). Recently, the occurrence of leishmaniasis patients carryng a double infection with *T. cruzi* has been reported (Chiaramonte *et al.*, 1996). Immunosuppression and autoimmune reactions have been observed during acute or chronic Chagas' disease (Kierszenbaum & Sztein, 1994).

1.3.1 Life cycle of *Trypanosoma cruzi*

The life cycle of *T. cruzi* includes three main morphological stages: epimastigote, amastigote and trypomastigote (Figure 1.3). The metacyclic trypomastigote is deposited with the faeces of an infected triatomine vector, on the skin of a mammalian host during a blood meal. Once it has penetrated the skin through a wound, skin abrasion or mucosal surfaces, the trypomastigote passes into the blood-stream and rapidly invades different cell types. If they are to infect macrophages once inside these cells the parasites use a

protease to escape from their phagosome to live within the cytoplasm. The trypomastigotes transform into the amastigote, which is the reproductive form in the vertebrate host. Reproduction by binary fission continues until the host cell is destroyed and the parasites are liberated in the form of trypomastigotes. Trypomastigotes infect neighbouring cells and also remain in the blood-stream where they can be ingested by further triatomine bugs. In the midgut of the insect, trypomastigotes transform into epimastigotes and divide by binary fission. As the parasites migrates to the hind gut, it transforms to the metacyclic trypomastigote form. The cycle continues when the infected bug defecates on the skin of another vertebrate host (De Castro, 1993).

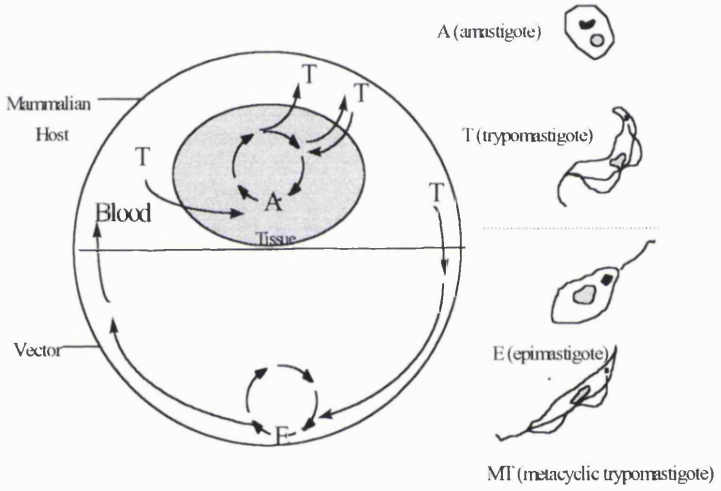


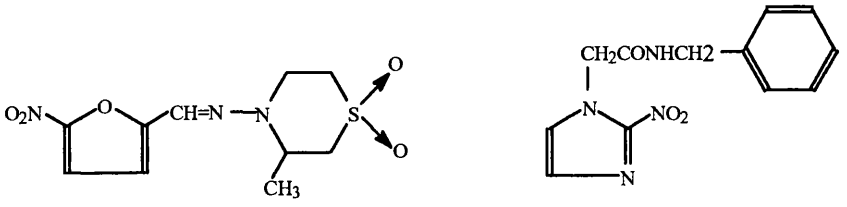
Figure 1.3 Life cycle of *Trypanosoma cruzi*

1.3.2 Drug treatment of Chagas' disease

1.3.2.1 Nifurtimox and Benznidazole

The nitrofuran derivative nifurtimox and the 2-nitroimidazole benznidazole (Figure 1.4) have been used for the for treatment of Chagas' disease since 1978, and nifurtimox is no longer manufactured. Both nitroheterocyclic drugs are only effective during the acute phase of the disease. Nifurtimox is readily absorbed after oral administration and rapidly metabolized, whereas benznidazole is rapidly absorbed and distributed through the body

tissues, metabolites being excreted in the urine. Both drugs produce anorexia, neurological toxic effects and bone marrow depression. *T. cruzi* infection has been reported in a few AIDS patients (Louis *et al.*, 1991), and in those receiving immunosuppressive therapy (Metze *et al.*, 1991); such cases are difficult to treat. Chagas' disease patients can be refractory to treatment with nifurtimox and benznidazole, a fact that may be due at last in part, to strain differences in drug sensitivity. Treatment failures have been ascribed to the presence of drug-resistant strains, although this remains to be proved (De Castro, 1993). Nifurtimox has been used in a few trials in patients infected with *T. b. gambiense* who relapsed with eflornithine or melarsoprol with good results but it has not yet used in *T. b. rhodesiense* cases (Pepin *et al.*, 1992a).



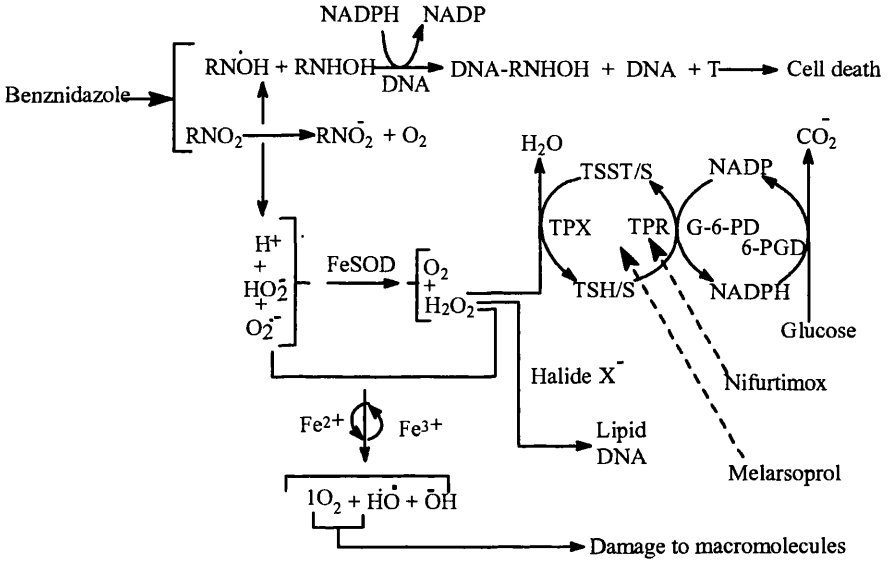
Nifurtimox Benznidazole

Figure 1.4 Drugs used for the treatment of Chagas' disease

1.3.2.1.2 Free radicals and trypanothione in the mode of action of some drugs.

Trypanothione reductase (Figure 1.5) is a flavoenzyme that has been found only in parasitic protozoa of the order Kinetoplastida. The enzyme catalyzes the NADPH-dependent reduction of glutathionylspermidine conjugates and is a key enzyme of the parasite's thiol metabolism (Krauth-Siegel & Schoneck, 1995; Kemp *et al.*, 1996). It maintains intracellular thiol-redox and is considered an important defense mechanism against oxygen free radicals (Fairlamb *et al.*, 1992). Nifurtimox, tricyclic compounds such as acridines and phenothiazines as well as crystal violet, quinones, and aminoquinolines competitively inhibit trypanothione reductase, resulting in the generation of oxygen free radicals (O_2.-, H_2O_2 ,OH^-) leading to cell death (De Campo 1993). It has

been reported that nitroaromatic compounds like nifurtimox form nitro-aryl anion radical (Ar NO_2.-) which may be oxidized to generate O_2.- , which in turn will produce H_2O_2 by superoxide dismutase (SOD). Then the free radicals bind covalently to cellular components (DNA, proteins) and/or triggering of lipid peroxidation leading to enzyme, genetic, protein, and lipid damage in the parasite causing its death. In the case of benznidazole, it does not produce free radicals but generates nitro-aryl anion radicals which bind covalently to DNA or proteins causing cell ceath.



FeSOD, Ironsuperoxide dismutase; GSH/S, glutathione/spermidine-trypanothione GSSG/S, reduced glutathione/spermidine-reduced trypanothione; GST, glutathioi S-transferase; TPX, trypanothione peroxidase; TPR, trypanothione reductase.

Figure 1.5 Free radicals and inhibition of trypanothione reductase in the mode of action of some drugs

1.4 Human African Trypanosomiasis (Sleeping sickness)

Human African Trypanosomiasis (HAT) is caused by infestation with the trypanosoma which is inoculated by the bite of the tsetse fly *Glossina*. The particular ecological conditions of parasites and vectors are such that the disease is only found in the intertropical regions of Africa. Although there are many species of trypanosomes, only two, belonging to the brucei group are likely to lead to HAT. These two species are quite similar

morphologically but have different pathogenicity. *Trypanosoma brucei gambiense* found in West and Central Africa leads to a chronic form of the disease or sleeping sickness, and *T. b. rhodesiense* leads to a more virulent and acute condition. Further, for each species of trypanosome there are strains of different virulence, which account, at least in part, for the interindividual variability in the clinical course (Dumas & Bouteille, 1996). Immediately after penetration into the human organism, the trypanosome multiplies at the point of inoculation, producing a local inflammatory reaction. It then invades the host blood stream, tissues and eventually the central nervous system (CNS). The involvement of the CNS leads to an irreversible damage process ending by death without treatment. As a response to the infection T cells produce interferon-gamma which activate macrophages to release tumor necrosis factor alpha and nitric oxide (NO) which are trypanostatic static and other cytokines and prostaglandins. However, macrophages release also substances which enhance immunosuppression and alter the blood brain barrier (BBB). Trypanosomes and inflammatory cells invade the CNS leading to a progressive meningoencephalitis leading to CNS immunopathological process (Sileghem *et al.*, 1994).

1.4.1 Life cycle of *Trypanosoma brucei* sp.

The pleomorphic trypomastigotes of *T. b. rhodesiense* and *T. b. gambiense* spend their entire life cycle extracellularly (Figure 1.6). A tsetse fly becomes infected when it ingests a blood meal containing bloodstream trypomastigotes. In the vector gut, they change morphologically into procyclic trypomastigotes in the midgut and after several cycles of cell division they migrate to the insect's salivary glands; here they transform to epimastigotes and divide before transforming into small infective metacyclic trypomastigotes, the infective stage for mammalian host. During a blood meal an infected tsetse fly introduces metacyclic trypomastigotes into the skin of the host along with the salivary secretion. The metacyclic trypomastigotes rapidly transform into bloodstream trypomastigotes within the extracellular spaces in the subcutaneous tissues, where the parasites replicate by binary fission. The trypomastigotes eventually find their way into the bloodstream and the lymphatics, where they continue the replication cycle. The life cycle continues when a tsetse fly ingests blood stream trypomastigotes during a blood meal from an infected individual (Despommier & Karapelou, 1987).

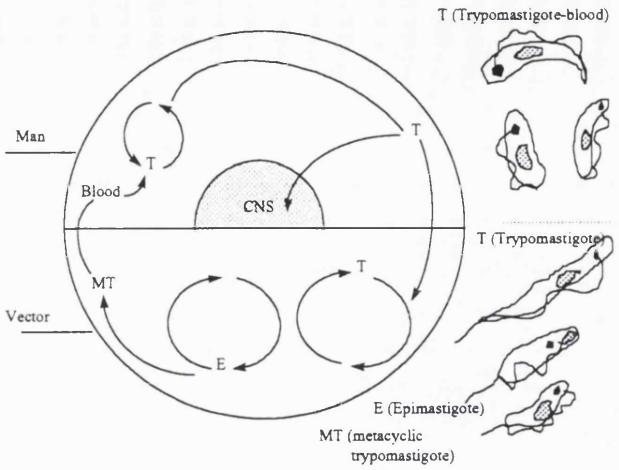


Figure 1.6 Life cycle of *Trypanosoma brucei* sp.

1.4.2 Drug treatment of Human African trypanosomiasis

1.4.2.1 Pentamidine

The diamidine pentamidine isethionate (Figure 1.7) has been been used for the treatment of trypanosomiasis for more than 50 years. Pentamidine isethionate is considered a second-line drug choice for early-stage of *T. b. gambiense.* It acts relatively slowly against trypanosomes and does not enter into the cerebro spinal fluid (CSF), although recently the presence of small amounts of pentamidine in CSF in patients has been reported (Bronner *et al.*, 1991). After intramuscular administration the drug has a prolonged action and a slow rate of excretion. This was the basis for its use in chemoprophylaxis. The side effects of this drug include pain and abscess formation at site of injection, myalgia, headache, nephrotoxicity, tachycardia, nausea, hypoglycemia, pancreatitis, hypocalcemia, and renal failure neutropenia (Conte, 1991). Pentamidine inhibits trypanosomal S-adenosyl-L-methionine decarboxylase, an enzyme involved in the synthesis of polyamines (Bitonti *et al.*, 1986a). Other possible modes of action of pentamidine include: inhibition of topoisomerase II, an enzyme involved in the replication of kinetoplast DNA (kDNA) (Shapiro, 1993); inhibition of glycolysis, or of DNA, RNA, or of protein and phospholipid synthesis,or of amino acid transport or of cation efflux (Sands *et al.*, 1985). It has been reported that melarsen-sensitive and -resistant *T. b. brucei* possess at least two nucleoside transporter systems designated P1

and P2, and the later being implicated in the selective uptake of melaminophenyl arsenical drugs (Carter *et al.*, 1995).

Combinations of pentamidine-suramin have been used as the first choice for the treatment of early-stage of *T. b. gambiense*. However, relapse and failure cases have been reported in *T. b. gambiense* infected patients (Pepin & Milord, 1994; Pepin & Khonde, 1996). In addition, *T. b. gambiense* pentamidine resistant strains have been documented (Bacchi *et al.*, 1990; Goa *et al.*, 1985). Another diamidine diminazene has been used against animal trypanosomiasis and for the treatment of human rhodesiense and gambiense early-stage trypanosomiasis, but neurological toxicity and relapse cases have been observed (Abaru *et al.*, 1984).

1.4.2.2 Suramin

The naphthylamine suramin (Figure 1.7) has been recommended for the treatment of early-stage rhodesiense trypanosomiasis, and has rarely been used for gambian early-stage disease. Suramin like pentamidine was also used by mobile teams in villages until they reached the hospital where melarsoprol is administrated. After intravenous administration the drug binds to all kinds of plasma proteins and can be found in the blood for several months after injection, being the basis for its use in chemoprophylaxis (Pepin & Milord, 1994). Suramin penetrates negligibly into CSF; patients with mildly abnormal CSF can be treated with suramin alone. However, failure rate is high as the drug acts slowly (Wellde *et al.*, 1989). When suramin is given in monotherapy, renal toxicity , polyneuropathy, stomatitis, liver dysfunction, adrenal insufficiency and thrombocytopenia are the most common side effects (La Rocca *et al.*, 1990). The mode of action of suramin is poorly understood. However, it has been reported, that it inhibits numerous enzymes including, L-α-glycerophosphate oxidase, RNA polymerase, topoisomerase II, an enzyme involved in the replication of kinetoplast DNA (kDNA) (Shapiro, 1993). Furthermore, it inhibits protein and phospholipid synthesis, or of amino acid transport of cation efflux (Sand *et al.*, 1985). Relapses have been reported in the treatment of early-stage T.b.gambiense sleeping sickness with a combination of pentamidine and suramin (Pepin & Khande, 1996).

1.4.2.3 Melarsoprol

Melarsoprol a highly toxic trivalent arsenical (Figure 1.7) was introduced in 1949 and, remains the most active trypanocidal drug available for the treatment of late-stage gambiense and rhodesiense trypanosomiasis. The intravenous administration of melarsoprol requires hospitalization because of immediate reactivity and hazard, involving up to 10% of patients with fatal outcome in 1-5% of cases. Toxic side effects of melarsoprol include reactive encephalopathy, polyneuropathy, renal failure and arrhythmia (Gherardi *et al.*, 1990). The remarkable efficacy of melarsoprol is more a consequence of its extraordinary activity against the parasite than of good CSF penetration (Pepin & Milord, 1994). Melarsoprol binds to trypanothione resulting in an adduct (Mel T) preventing trypanothione from its cellular function (Fairlamb *et al.*, 1989), and it is also a potent inhibitor of pyruvate kinase, a key glycolytic enzyme in the parasite. Resistance and failure to melarsoprol have been reported and the degree of sensitivity differs between the two subspecies (Yarlett *et al.*, 1991). Patients with rhodesiense trypanosomiasis who relapse after melarosprol will often respond favorably to a second course of the drug. However, those with gambiense trypanosomiasis who relapse after a first course of melarsoprol will rarely be cured by additional courses of melarsoprol. These cases are treated either with eflornithine or a combination of melarsoprol/eflornithine or nifurtimox alone or in combination with melarsoprol. Combination of eflornithine/ melarsoprol and nifurtimox/melarsoprol has also been used in murine models with late-stage of *T. b. rhodesiense* with promising results (Jennings, 1990; Jernigan & Pearson, 1993).

Resistance of *T. brucei* to arsenicals, diamidines and DFMO is related to reduced drug uptake. In arsenicals the resistance involves a loss or marked alteration in the adenosine transporter which decrease the uptake of melarsoprol into the parasite (Carter & Fairlamb, 1993; Carter *et al.*, 1995). Other factors of failure include innate differences in sensitivity between the two subspecies to eflornithine (Bacchi, 1993), melarsoprol (Yarlett *et al.*, 1991) and nifurtimox (Pepin *et al.*, 1992a) as well as behavioural changes of the parasite. The parasite can occupy areas such as the cerebral interstitium, where the drug penetrates poorly; thus the parasites escape the drug and generate relapses.

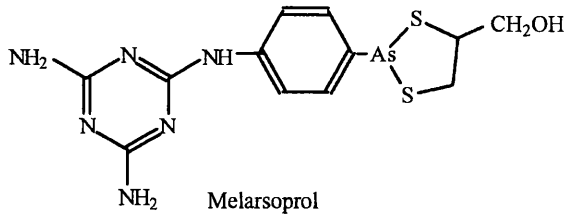
1.4.2.4 Eflornithine

DL-α-difluoromethylornithine (DFMO) known as eflornithine (Figure 1.7) was originally registered for the treatment of Human African trypanosomiasis in 1990, and has been used succesfully in arseno-resistant gambiense African trypanosomiasis (Van Nieuwenhove, 1992). Eflornithine has a good penetration into the CSF after intravenous administration, which probably contributes to a large extent to the efficacy of this drug (Milord *et al.*, 1993). Eflornithine has been used in AIDS patients infected with trypanosomiasis but with poor results maybe because eflornithine is trypanostatic rather than trypanocidal and a normal immune response is necessary to achieve a cure. The most adverse effects associated with eflornithine are bone marrow suppression, anaemia, thrombocytopenia, seizures and convulsions (Milord *et al.*, 1992; Pepin *et al.*, 1992b). Treatment failures have been reported for eflornithine and were more common among new cases than among patients pretreated with melarsoprol. The major drawbacks of this drug are unresponsiveness of *T. b . rhodesiense* strains, high cost and administration of the drug. There has been no reported cross-resistance between melarsoprol and eflornithine (Bacchi *et al.*, 1990). There is no alternative drug for melarsoprol resistant infection of *T. b. rhodesiense* and, the trials of the combination of eflornithine-suramin are in progress for the treatment of rhodesiense sleeping sickness (Kuzoe, 1993). Synergistic effects have been observed with combination eflornithine/nifurtimox in *T. b. brucei* murine models (Zwygarth *et al.*, 1990). Recently, DFMO has been evaluated in order to induce protective immunity against *P. berghei malaria* (Francois *et al.*, 1997).

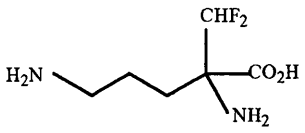
1.4.3 Mode of action of polyamine inhibitors

Polyamines are essential for the growth and multiplication of all eukaryotic cells, they affect nucleic acid synthesis and contribute to the regulation of protein synthesis. Ornithine decarboxylase is the enzyme that catalyses the conversion of ornithine to putrescine, the first and rate-limiting step in the synthesis of putrescine and of the polyamines spermidine and spermine (Marr, 1991). Eflornithine is a selective and irreversible inhibitor of ornithine decarboxylase thus decreasing putrescine and spermidine concentrations. Other enzymes also involved in the synthesis of polyamines are S-adenosylmethionine decarboxylase and methylthioadenosine phosphorylase; pentamidine

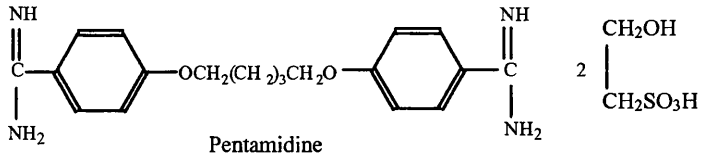
and diminazene have been shown to inhibit S-adenosylmethionine decarboxylase (Bitonti *et al.*, 1986a). Other polyamine inhibitors have been tested *in vitro* against trypanosomes (Majumber & Klerszenbaum, 1993; Byers *et al.*, 1991).



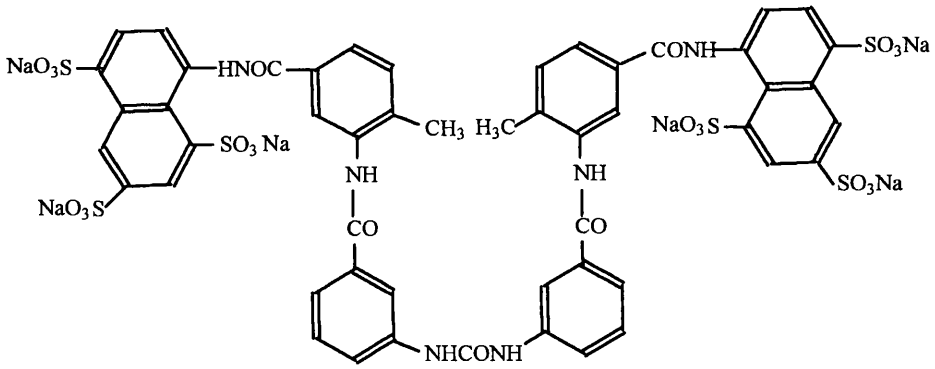
Melarsoprol



Eflornithine



Pentamidine



Suramin

Figure 1.7 Drugs used for the treatment of Human African trypanosomiasis

1.5 Drugs undergoing clinical trials or in experimental investigation in leishmaniasis and trypanosomiasis.

Some compounds undergoing on clinical trials or experimental investigation in leishmaniasis and trypanosomiasis are given in Table 1.2. Drugs in clinical trials will be described briefly.

Allopurinol (Figure 1.8) is an pyrazolopyrimidine compound and has been studied in the treatment of leishmaniasis. Oral administration of allopurinol has been shown to be

effective as and less toxic than pentavalent antimonials (Sb^v). Combination of Sb^v and allopurinol in the treatment of CL caused by *L. panamensis* (Martinez & Marr, 1992) and in the treatment of chronic Chagas' disease (Berens *et al.*, 1990; Gallerano *et al.*, 1990) gave good results. Allopurinol affects protein synthesis and RNA function by inhibiting purine metabolism (Marr, 1991). Allopurinol ribonucleoside, an analogue of allopurinol was linked to a drug delivery system and was found to be 50 times more active than the free drug *in vitro* (Negre *et al.*, 1992).

The azole ketoconazole (Figure 1.8) has been used for the treatment of CL caused by *L. mexicana* and *L. panamensis* (Brener *et al.*, 1993); and antimony-resistant VL patients pretreated with ketoconazole gave promising results (Rashid *et al.*, 1994). Experimental studies have been shown that ketoconazole had synergistic activity when was combined with miconazole or lovastatin for the treatment of Chagas disease or leishmaniasis (Armauer, 1993; Halim *et al.*, 1993; Urbina, 1993; Wali *et al.*, 1992). Another azole itraconazole was effective in the treatment of cutaneous leismanisis. Both ketoconazole and itraconazole inhibit cytochrome P-450 dependent lanosterol C_{14} demethylase, an enzyme involve in the formation of ergosterol, the main sterol component in cell membranes of kinetoplastida parasites (Haughan *et al.*, 1992). Ketoconazole and itraconazole were found to be effective against acute but not chronic Chagas' disease in mice. Furthermore, synergistic action has been observed with the combinations of lovastatin and miconazole against promastigotes and amastigotes of *L. donovani* and *L. amazonensis in vitro* (Haughan *et al.*, 1992); ketoconazole and terbinafine or lovastatin plus ketoconazole against *T. cruzi* in both *in vitro* and *in vivo* (Urbina *et al.*, 1993). Other inhibitors of sterol biosynthesis with antiprotozoal activity have been reported recently by Haughan *et al.* (1995).

Aminosidine/ paromomycin (Figure 1.8) has been used topically in combination with methylbenzethonium chloride or urea, in the treatment of patients with CL with promising results (Hepburn *et al.*, 1994; Olliario & Bryceson, 1993). Parenteral administration of aminosidine plus antimonials has been found effective against VL (Thakur *et al.*, 1992) and CL caused by *L. aethiopica* (Teklemariam *et al.*, 1994).

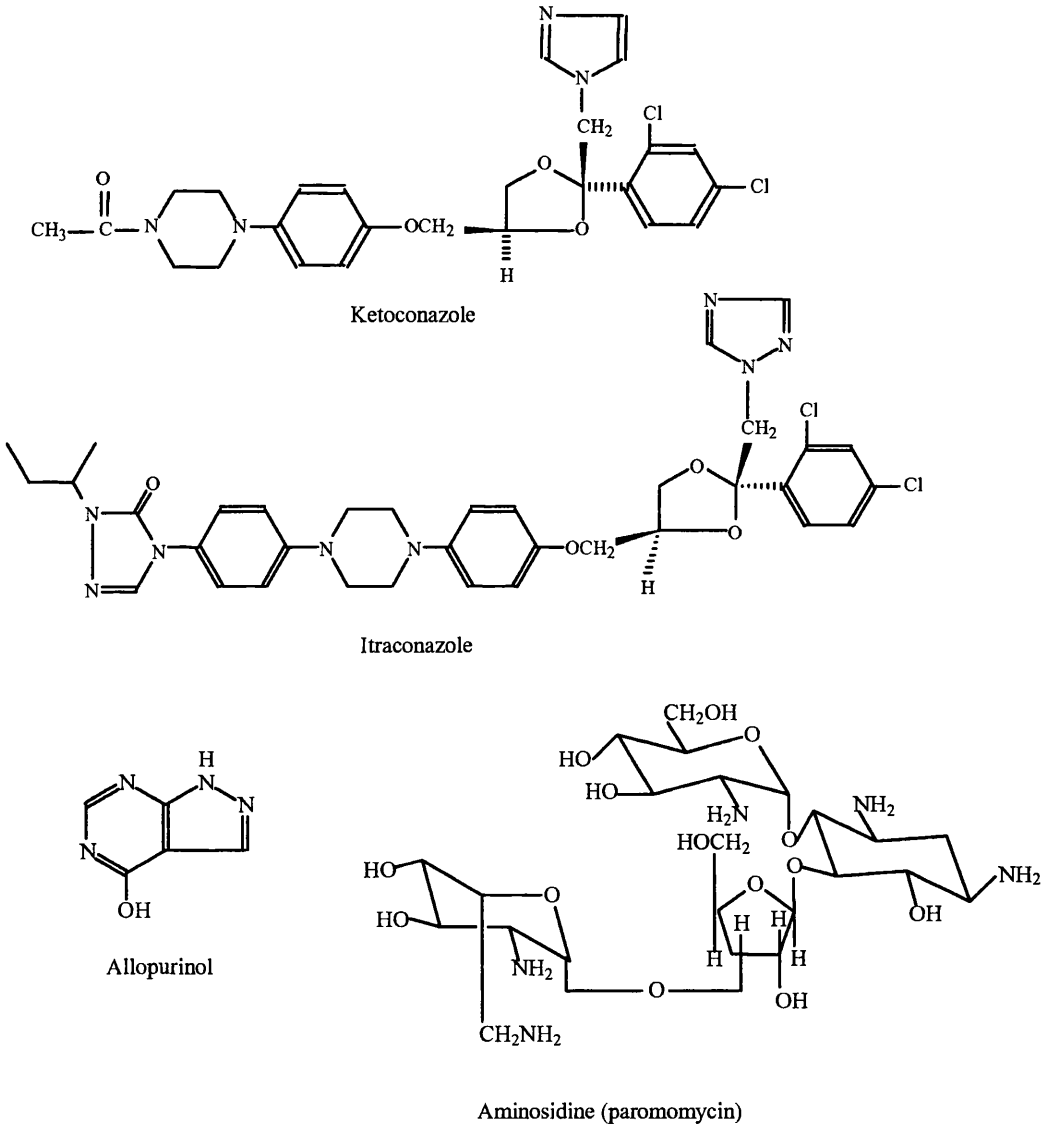


Figure 1.8 Some drugs used in clinical trials for the treatment of leishmanisis and trypanosomiasis

Table 1.2 Some antileishmanial and antitrypanosomal drugs undergoing clinical trials

Drug	Mode of action	Status	References
Lipid associated amphotericin B	Membrane function	Clinical trials leishmaniasis	Croft *et al.*, 1991
Paramomycin/ aminosidine	Inhibits protein synthesis and binds to ribosomes	Clinical trials leishmaniasis	Scott *et al.*, 1992
Allopurinol and allopurinol ribose	Inhibits purine synthesis	Clinical trials leishmaniasis and Chagas' disease	Martinez & Marr, 1992
Ketoconazole, intraconazole or ketoconazole/ miconazole, ketoconazole/ lovastatin	Ergosterol biosynthesis inhibitors	Clinical trials leishmaniasis and Chagas' disease *In vitro* and animal studies against trypanosomiasis and leishmaniasis	Wali *et al.*, 1992 Halim *et al.*, 1993
Platinum, iridium and rhodium	Unknown	*In vitro* and animal studies against *L.donovani*	Croft *et al.*, 1992
Terbinafine	Ergosterol biosynthesis inhibitors	*In vitro* and animal studies against *T. cruzi*	Haughan *et al.*, 1992 Urbina *et al.*, 1993
Dapsone	Folate inhibitors	*In vitro*, animal studies and one study in human leishmaniasis	Olliaro & bryceson, 1993
9-Aniloacridines	Topoisomerase II DNA binding	*In vitro* antitripanosomal and antimalarial studies	Figgit *et al.*, 1992 Mauel *et al.*, 1993
Difluoromethylarginine	Inhibits arginine decarboxilase	*In vitro* studies agaisnt *T. cruzi*	Yakubu *et al.*, 1992
Hydroquinones	Blocks mitocondrial electron transport	*In vitro* studies agaisnt *T. cruzi.* Experimental *Leishmania*	Aldunate & Morello, 1993 Croft *et al.*, 1992
Peptide-fluoromethyl ketones	Inhibits cysteine protease	*In vitro* studies agaisnt *T. cruzi*	Harth *et al.*, 1993
α-Monofluromethyl-dehydroornithine	Inhibitor of S-adenosylmethionine decarboxylase	*In vitro* studies agaisnt *T. brucei*	Byers *et al.*, 1991
Phospholipids ilmofosine	Lipid biosynthesis inhibitors	*In vitro* studies agaisnt *Leishmania* spp	Croft *et al.*, 1993
Primaquine analogues	Unknown	Animal studies against *T. cruzi*	Kinnamon *et al.*, 1996
5-nitroimidazoles, MK-436, or Fexindazole gels in combination with melarsoprol	Inhibits trypanothione reductase	Experimental murine CNS-*T. brucei* infection	Jennings *et al.*, 1996

1.6 Malaria

Malaria is caused by *Plasmodium* sp., and although more than 100 species of *Plasmodium* have been identified, only four are capable of infecting humans (*P. falciparum, P. vivax, P. malariae, P. ovale*), the rest parasitize a variety of animal hosts. *P. vivax* is the most widespread species, extending throughout the tropics, subtropics and into the temperate malarious areas of Latin America and Asia. *P. falciparum* predominates in the tropical and subtropical regions of Africa and South-East Asia. *P. ovale* occurs chiefly in Central West Africa and sporadically in the West Pacific regions. Mixed infections of two or more species are particularly frequent in endemic regions (Pasvol *et al.*, 1995). Fever and malaise with intermittent paroxysms are typical in all *Plasmodium* infections. Particularly, falciparum malaria is the most dangerous form of the disease, resulting in life-threatening complications such as anaemia and cerebral malaria (Bradley, 1995). Acute malaria caused by *P. falciparum* infection leads to a temporary but profound immunosuppression and autoantibody production (Riley *et al.*, 1994). Malaria in AIDS patients has been reported (Ho & Sexton, 1995).

1.6.1 Life cycle of malaria

The life cycle of all species of malaria consists of an exogenous sexual phase (sporogony) in the mosquito and endogenous asexual phase (schizogony) in man (Figure 1.9). The later phase consists of two developmental cycles, one in the liver parenchyma (exoerythrocytic) and the other in the erythrocytes (erythrocytic). Infection is initiated by the injection of sporozoites into the bloodstream during feeding by female infected *Anopheles* mosquitoes. The sporozoites migrate to the liver, where they invade hepatocytes and undergo a phase of maturation and asexual reproduction (schizogony) to release into the blood large numbers of free merozoites. The invasion of merozoites into circulating red blood cells initiates the erythrocytic phase of the life cycle. Schizogony is repeated within erythrocytes, and infected red cells rupture and release more merozoites into the circulation to begin another erythrocytic cycle. A proportion of invading merozoites differentiate into male or female gametocytes which, when ingested by a mosquito, emerge from the erythrocyte to form gametes. After fertilization, the zygote (ookinete) migrates across the mosquito midgut wall and matures within the

body cavity. The resulting oocysts produce sporozoites that migrate to the mosquito salivary glands ready for inoculation (Riley *et al.*, 1994).

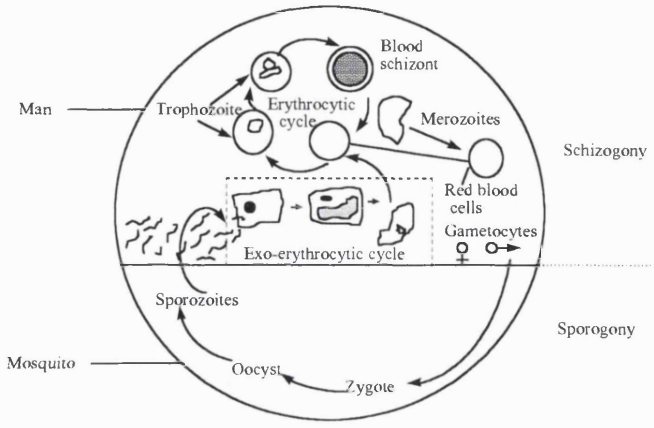


Figure 1.9 Life cycle of malaria

1.6.2 Drug treatment of malaria

1.6.2.1 Quinine

Quinine (Figure 1.10) the first antimalarial drug, was isolated from the bark of *Cinchona succiruba* (Rubiaceae) in 1820. Despite many years of use, quinine remains an important and effective antimalarial drug (White, 1996). *Cinchona* is cultivated in many tropical countries because of its uneconomic synthesis (Winstanley, 1995). Quinine acts on all human *Plamodium* species. Currently, quinine is used in the treatment of severe malaria and acute attacks of multiple drug-resistant strains of falciparum malaria and is the only drug accepted for treating chloroquine resistant malaria during pregnancy (Schapira *et al.*, 1993; Yen *et al.*, 1994). Quinine is completely absorbed after oral dosing and undergoes extensive hepatic biotransformation and is eliminated in the urine (Mansor *et al.*, 1991; Karbwang *et al.*, 1993). The most common side effect is the symtom complex known as cinchonism consisting of tinnitus, nausea, headache and visual disturbances. Dose related effects are limited to the cardiovascular, gastrointestinal and central nervous

system (Gerson, 1996; White, 1995). Resistance to quinine have been reported, although is still effective in most countries of the world. Rapid-efflux mechanisms are thought to be involved in mediating resistance. Mode of action of quinine and quinidine is by inhibition of parasite haem polymerase (Kain, 1993). Quinine is usually administrated in combination with pyremethamine/sulfadoxine or tetracyclines (White, 1995). The diasteromer of quinine, quinidine, widely used as an antirrhythmic is also effective for the treatment of severe malaria and may be used when quinine is not available (Karbwang *et al.*, 1993). Other cinchona alkaloids with antimalarial activity include cinchonine, cinchonidine and totaquine, a standarized alkaloid extract (Phillipson & Wright, 1991). Some antimalarial drugs have been found to act on the immune system, depressing PMN functions associated with antimicrobial activity and may also decreased antibody production. These drugs include: quinine, chloroquine, pyrimethamine, mefloquine and quinacrine (Targett, 1992). It has been reported that chloroquine or primaquine prophylaxis enhanced lymphocyte proliferation in patients (Fryauff *et al.*, 1996).

1.6.2.2 Chloroquine

Chloroquine (Figure 1.10) a 4-aminoquinoline, initially synthesised in 1934 as a substitute for quinine has been considered the drug of choice for the treatment of malaria caused by non-resistant strains of *P. vivax, P. ovale, P. malariae* and *P. falciparum*. The drug is almost completely absorbed after oral administration, with excretion mainly in the urine (White, 1995). The drug is well-tolerated in prophylactic doses, but side effects are common with therapeutic doses, those include headache, blurring of vision, gastrointestinal upset, and pruritis (Winstanley, 1995). Although chloroquine-resistant falciparum malaria has been documented for more than 30 years, only recently has the mechanism of resistance to chloroquine been elucidated. Resistance to chloroquine in *P. falciparum* is mediated by rapid-efflux of the drug. Drug-resistant strains of *P. falciparum* accumulate less chloroquine by effluxing this drug 40-50 times faster than do chloroquine-sensitive parasites (Wellems, 1992).

1.6.2.3 Mefloquine

Mefloquine (Figure 1.10) a 4-quinoline methanol, was first introduced in 1984 as a result of the increasing problems of drug resistance in *P. falciparum* (White, 1992). It is potent in all human malarias and multi-resistant *P. falciparum*. It is used in prophylaxis and for the treatment of acute attacks of malaria due to multidrug resistant strains of *P. falciparum*. After oral administration, mefloquine is rapidly absorbed and eliminated in the urine, and is well tolerated in prophylactic doses; treatment doses, however, may result in severe neuropsychiatric reactions (Kain, 1993). Resistance to mefloquine has been reported in the last decade from most malaria-endemic areas. The exact mechanism of drug resistance to mefloquine is unknown. However, recent evidence suggests the potential involvement of the *P. falciparum* multidrug resistant (MDR) gene family. Cross-resistance may develop between mefloquine and halofantrine when they are administered together (Winstanley, 1995). On the other hand, the use of chloroquine and proguanil combination as antimalarial prophylaxis has shown adverse reactions (Barret *et al.*, 1996).

1.6.2.4 Mode of action of aminoquinolines

Chloroquine, quinine and related quinoline drugs are effective against the erythrocytic stages of malaria parasites. The theory of mechanism of drug concentration suggests that because these compounds are weakly basic drugs they accumulate within the parasite's acidic digestive vacuoles (Schlesinger *et al.*, 1988; Warhurst, 1987), raising intravascular pH of the parasite and this may exert their toxicity. Digestion of haemoglobin into parasite's acidic vacuoles releases large amounts of the toxic haem moiety ferriprotoporphyrin IX (FPIX) which if soluble can damage biological membranes and inhibit different types of enzymes. It has been supposed that chloroquine and related quinoline antimalarial drugs complex with FPIX in the acidic digestive vacuole. Thus the toxic FPIX-drug complexes would not be incorporated into an insoluble material haemozoin, poisoning the acid digestive vacuole and starving the parasite (Warhust, 1987; Blauer *et al.*, 1993). In addition, it has been suggested that these drugs may act by inhibiting a putative plasmodial enzyme haem polymerase disrupting the conversion of haem into haemozin (Wellems, 1992; Slater & Cerami, 1992). Although recently it has

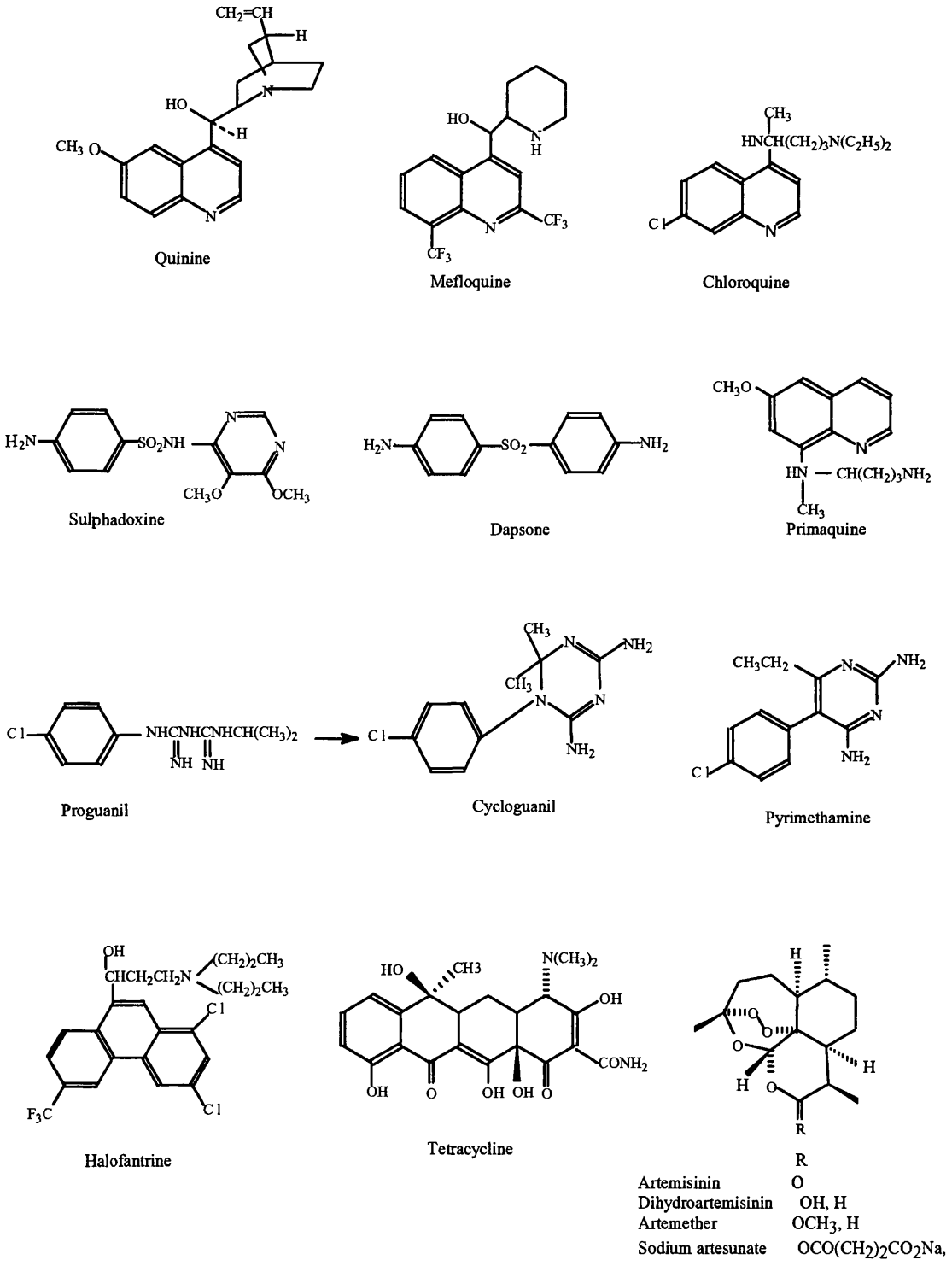


Figure 1.10 Some drugs used for the treatment of malaria

been suggested that such an enzyme does not exist. Since it has been shown that

polymerization can be induce by a physicochemical process in which haemozin served to seed or support its own polymerization, and therefore an enzyme is not required for polymerization (Ridley, 1996).

1. 6.2.5 Antifolates

1.6.2.5.1 Sulphadoxine

The sulphonamide, sulphadoxine (Figure 1.10) is used as a prophylactic and in uncomplicated cases of falciparum malaria. It is rapidly absorbed after oral administration, and is generally well-tolerated, although, skin reactions and bone marrow depression have been observed. The drug is effective against falciparum malaria when is given alone but it causes drug resistance. Thus, it is usually administrated in combination with pyrimethamine.

The combination of pyrimethamine and sulphadoxine known as Fansidar is used for chloroquine-resistance falciparum malaria and acute attacks of malaria by susceptible strains of *P. falciparum* in areas where chloroquine resistant malaria occurs. Strains of *P. falciparum* and *P. vivax* resistant to this combination are now wide widespread in many areas and fansidar is usually given with quinine which provides rapidly acting schizontocide effects. Currently, the use of Fansindar is limited due to severe skin reaction in some patients (WHO, 1993, White, 1996).

1.6.2.5.2 Dapsone

The sulphone dapsone (Figure 1.10) acts slowly and resistance develops easily when it is used alone. Dapsone is well absorbed after oral dosing; doses employed in prophylaxis are associated with low toxicity. At standard doses symptoms as nausea, vomiting, headache, blurred vision and insomnia may occur, and may induce hemolysis in glucose-6-phosphate dehydrogenase (G6PD)- deficient individuals. The combination of pyrimethamine/dapsone (Daraprim) has been used for prophylaxis (WHO, 1991).

1.6.2.5.3 Pyrimethamine

A 2,4-diaminopyrimidine, pyrimethamine (Figure 1.10) is used to prevent the transmission of malaria, since development of the asexual stage in the mosquito is prevented by administration of pyrimethamine to the human host. However, slow action and rapid development of resistance has limited the use of pyrimethamine, and is mostly used in combination with sulfadoxine or sulphone. Pyrimethamine has been found more potent to *P. falciparum* than mammalian cells. However, *P. falciparum* develops resistance to dihydrofolate reductase (DHFR)-inhibitors because it is capable of undergoing drug-specific point mutations of the gene for DHFR (Brooks *et al.*, 1994).

1.6.2.5.4 Proguanil

Proguanil (Figure 1.10) a biguanide is cyclised metabolically to the active metabolite cycloguanil (Figure 1.10). The drug is used in the prophylaxis and treatment of malaria infections in pregnant women and non-immune individuals in areas where resistance has not been stablished. Proguanil is slowly absorbed by the oral route with a following elimination mainly in the urine. Resistance to proguanil is now widespread due to mutations in specific aminoacids in *P. falciparum* DHFR (Winstanley, 1995). Proguanil plus atovaquone is effective effective for the treatment of *P.ovale* and *P.malariae* malaria (Radloff *et al.*, 1996). A series of antifolate drugs are being investigated for antimalarial activity (Canfield *et al.*, 1993; Rathod & Reshime, 1994).

1.6.2.5.5 Mode of action of antifolate drugs

Parasites cannot use preformed folic acid and must synthesise it from p-aminobenzoic acid (PABA), pteridine and glutamic acid from their host. Antifolate drugs inhibit the synthesis of folic acid which is reduced to tetrahydrofolic acid important in the synthesis of pyrimidines and purines which are essential in nucleic acid synthesis (Figure 1.11). Sulphonamides (e.g. sulphadoxine) and sulphones (e.g. dapsone) inhibit the incorporation of para-aminobenzoic acid (PABA) into dihydropteroate, a precursor of dihydrofolate, by competitive inhibition of dihydropteroate synthetase (DHPS). Both parasite and host use the enzyme dihydrofolate reductase (DHFR) to convert dihydrofolic acid to tetrahydrofolic acid. Drugs such as pyrimethamine and proguanil competitively inhibit DHFR by competing with dihydrofolic acid.

DHFR by competing with dihydrofolic acid.

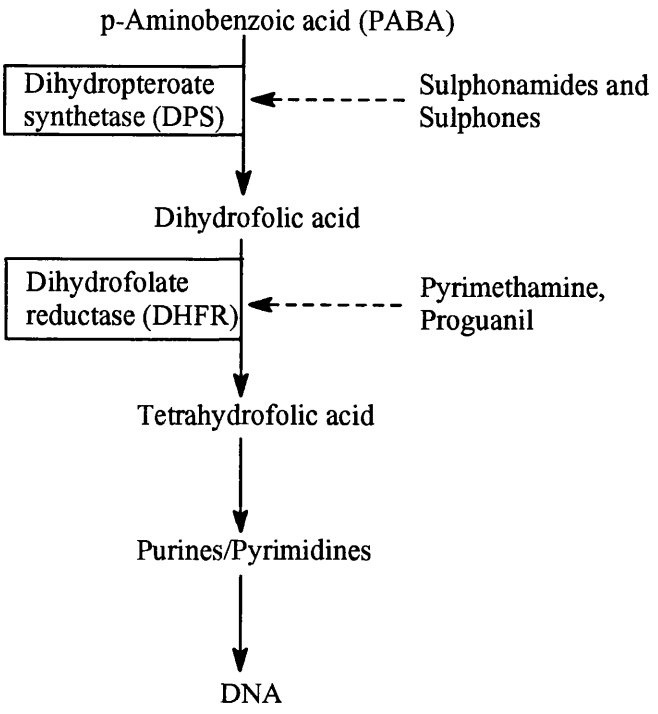


Figure 1.11 Mode of action of antifolate drugs

1.6.2.6 Primaquine

Primaquine an 8-aminoquinoline derivative (Figure 1.10) is the only drug effective against the liver exoerythrocytic forms of the malaria parasite. Primaquine is used against *P. vivax* and *P. ovale* after standard chloroquine therapy and towards *P. falciparum* after treatment with blood schizontocides. Primaquine is rapidly absorbed after oral administration. Dose related adverse effects include abdominal cramps, and anaemia as well as intravascular haemolysis occur in G6PD-deficient individuals (Kavin, 1993).

1.6.2.7 Halofantrine

A phenanthrene methanol, halofantrine (Figure 1.10) is effective against strains of *P. falciparum* resistant to chloroquine, Fansidar and mefloquine and for the treatment of acute malaria caused by single or mixed infections of *P. falciparum* or *P. vivax*. Although the mode of action of halofantrine is not fully understood, some studies indicate

that its mechanism is similar to quinine and chloroquine (Winstanley, 1995). Abdominal pain, diarrhoea, pruritis, and skin rash have been reported following its administration.

1.6.2.8 Artemisinin and its derivatives

Artemisin (Qinghaosu) is a naturally occurring sesquiterpene lactone endoperoxide (Figure 1.10), being the founder of a completely new class of antimalarials drugs. It was isolated in 1972 from *Artemisia annua,* a plant used in Chinese medicine for over 2000 years for the treatment of fever. Artemisinin and its semi-synthetic derivatives; a water-soluble hemisuccinate salt (artesunate); and two oil-soluble compounds, artemether and arteether, have lead to faster parasite and fever clearance times than any other antimalarials and are now being used clinically in much of the world (White *et al.*, 1992). Artemisinin and its derivates have an unusual mode of action involving the iron-catalized generation of a carbon-centered free radical followed by the alkylation of malaria-specific proteins within the parasite resulting in selective toxicity (Meshnick *et al.*, 1994; Kamchonwongpaison & Messhnick, 1996a,b). Artemisinin also affects membranes, mitochondria, food vacuoles and protein synthesis in the parasite (Meshnick, 1994).

Artemisinin and its derivates are potent blood schizonticides which are effective against strains of *P. falciparum* resistant to standard drugs and against *P. vivax*. The fat- and the water-soluble derivates are approximately twice and 4 to 5 times more active *in vitro* than artemisinin. Artemisinin is available only for oral and rectal administration. Absorption is incomplete and elimination is fast, with a elimination half-life of 2 to 5 hrs. Artesunate and artemether are administered by parenteral formulation and can be considered as prodrugs. Biotransformation into the active metabolite dihydroartemisin occurs rapidly. The reported elimination half-life of artesunate is less than 1 hr, and for artemeter is 3-11 hr (de Vries & Dien, 1996). Artemether is used for the treatment of severe malaria, while artesunate alone or in combination with mefloquine is effective against the highly multi-drug resistant strains of *P. falciparum* (White, 1994b). There is no evidence of high toxicity of artemisin and derivates in man although recent reports of the neurotoxicity of these compounds are of concern (Karbwang *et al.*, 1994). In China, artemisinin and derivatives have replaced chloroquine and quinine in the treatment of falciparum malaria

(Li *et al.*, 1994) and over one million people in the world have been treated with these compounds (White, 1994a; De Vries & Dien, 1996). The antimalarial activity of related trioxanes has been reported recently (Cumming *et al.*, 1997).

Artemisinin was found effective against promastigotes *in vitro*, with an ED_{50} at 7.5 x 10^{-7} M. Both artemisinin and artemether were leishmanicidal in the infected murine macrophages *in vitro* test with ED_{50} values at 3 x 10^{-5} M and 3 x 10^{-6} M respectively. These compounds were not toxic to macrophages even at 10^{-4} M. Intra-lesion injection intramuscular and oral routes for both artemisinin and artemether were effective in treating *L. major* infected-mice (Yang & Liew, 1993).

1.6.2.9 Antibiotics

Tetracyclines (Figure 1.10) are broad spectrum of antimicrobial antibiotics with antimalarial activities. Tetracycline is not administered alone because of its slow action, but as a supplement to quinine in the treatment of *P. falciparum* malaria (WHO,1991). The mode of action has not been established but it has been suggested that it might be similar to that in bacteria inhibiting protein synthesis at the ribosomal level. The most common side effects of tetracycline are abdominal discomfort, nausea, and vomiting.

1.6.2.10 Antimalarial drugs used in some countries or undergoing clinical trials

Despite the urgent need for new antimarial drugs, particularly those against multiresistant falciparum malaria, only a limited number of drugs are now at an advanced stage of preclinical or clinical development. Pyronaridine and benflumetol were developed and registered in China as antimalarial drugs since about 20 years ago. Pyronaridine is effective against some strains of multi-resistant *P. falciparum*. Combination benflumetol/artemeter is now used for treatment of falciparum malaria in China (Olliario & Trigg, 1995). A pyridinemethanol, enpiroline which is more active than chloroquine against chloroquine-sensitive *P. falciparum* , has also shown activity against the multidrug-resistance strain of *P. falciparum*. The 8-aminoquinoline, WR 238, 605, analogue of primaquine is more potent than primaquine against the liver exoerythrocytic forms of the malaria parasite (Cattani *et al.*, 1993). Atovaquone a

hydroxynaphthoquinone is effective in uncomplicated malaria and in combination with proguanil against multi-resistant falciparum malaria (WHO, 1995; Hudson, 1993). An analogue of proguanil WR 250 417 has been shown to be highly active against a pyrimethamine-resistant strain of *P. falciparum* in monkeys (Olliario & Trigg, 1995).

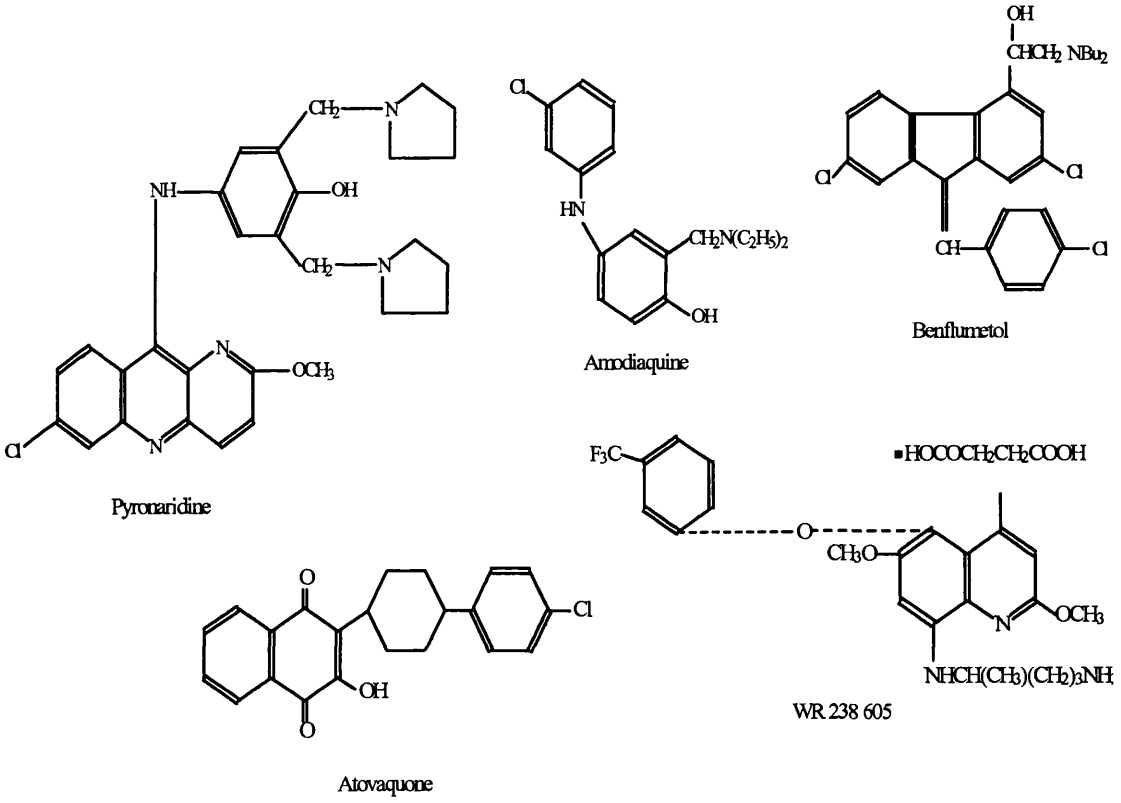


Figure 1.12 Some antimalarial drugs undergoing clinical trials

1.7 Higher plants as a source of new antiprotozoal drugs

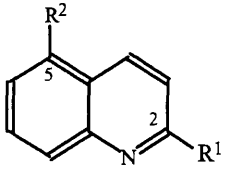
An estimated 80% of the world's population depends upon locally available herbal remedies for their medicines (Farnsworth, 1985; 1990) and the discovery of new drugs from traditional medicine is not a new phenomenon. Worldwide, more than 100 clinically useful prescription drugs are derived from higher plants and a number of the currently used antiprotozoal drugs are natural products (e.g., quinine, emetine, artemisinin) or semi-synthetic derivatives of natural products (e.g., artemether, arteether). The diversity of species and compounds which possess antiprotozoal activities has been well illustrated in several reviews (Phillipson & O'Neill, 1987; Wright & Phillipson, 1990; Phillipson & Wright 1991; Nkunya 1992; Phillipson *et al.*, 1993; 1995; Kirby, 1996; Iwu, 1994). Recent reports of new antiprotozoal drugs from higher plants and few examples including some of those mentioned above will be described below. The structures of the compounds referred in the text are included either in Tables 1.3-1.22 or in Figures 1.13-1.21.

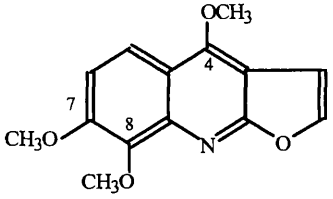
1.7.1 Alkaloids

1.7.1.1 Quinoline alkaloids

Galipea longiflora (Rutaceae) is used for the treatment of cutaneous leishmaniasis in South America. Bioactive guided fractionation afforded 12 active 2-substituted quinoline alkaloids (Table 1.3). All quinoline alkaloids were more potent than N-methylglucamine antimonate, but were 25-100 times less active than pentamidine against promastigotes forms of *Leishmania* species. The presence of a 4-methoxy group in these series of alkaloids produced decrease in antileishmanial activity (Fournet *et al.*, 1993c). Three quinoline alkaloids substituted in position 2 with a 3C chain showed *in vitro* activity against *Leishmania* sp., and *T. cruzi* at 50 μg/ml; 2-n-propylquinoline, chimanine B and chimanine D (Fournet *et al.*, 1994). Further studies *in vivo* (Table 1.3) indicated that 2-n-propylquinoline and chimanine D were more active than pentavalent antimony, in treating *L. amazonensis* PH8 infected mice. Furthermore, 2-(3,4-methylenedioxyphenylethyl) quinoline, cusparine; 2-(3,4-dimethoxyphenylethyl)quinoline, skimmianine and chimanine B were as effective as the reference drug. On the other hand, 2-phenylquinoline was as potent as pentavalent antimony in treating *L. amazonensis* H-

Table 1.3 *In vivo* antileishmanial effects of 2-substituted quinoline alkaloids from *Galipea longiflora*.





Skimmianine

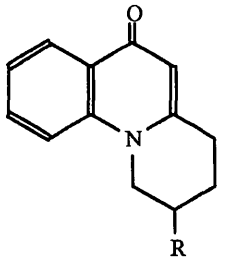
Compound	R^1	R^2	*Leishmania amazonensis* mm* PH8	H-142	*Leishmania venezuelensis* mm* H3	*L. donovani* % inhibition i.p	s.c	oral
2-Phenyl-quinoline	H	phenyl	5.0	2.54	NT	NT	NT	NT
2-n-Pentyl-quinoline	H	n-pentyl	5.6	NT	NT	NT	NT	NT
2-n-Propyl-quinoline	H	n-propyl	3.4	NT	4.12	62.4	76.3	99.9
4-Methoxy-2-phenyl-quinoline	OMe	phenyl	5.0	NT	NT	NT	NT	NT
2-(3,4-Methylenedioxyphenylethyl)-quinoline	H	3,4-methoxylenedioxyphenylethyl	4.6	NT	5.50	NT	NT	NT
4-Methoxy-2-n-pentyl-quinoline	OMe	n-pentyl	4.9	NT	NT	NT	NT	NT
4-Methoxy-2-n-propyl-quinoline, chimanine A	OMe	n-propyl	4.8	NT	NT	NT	NT	NT
4-Methoxy-2-(3,4-methylenedioxyphenylethyl) quinoline, cusparine	OMe	3,4-methylenedioxyphenylethyl	4.1	NT	NT	NT	NT	NT
2-(3,4-Dimethoxyphenylethy)-quinoline	H	3,4-dimethoxyphenylethyl	4.4	NT	NT	NT	NT	NT
2-(E)-Prop-1'-enyl-quinoline, chimanine B	H	(E)-prop-1'-enyl	4.3	NT	NT	NT	NT	NT
2-(1',2'-Trans-epoxypropyl)-quinoline, chimanine D	H	1',2'-trans-epoxypropyl	2.6	NT	NT	57	86.6	72.9
Skimmianine			4.2	NT	NT	NT	NT	NT
Control			6.6	3.49	6.60	NT	NT	NT
N-Methylglucamine antimonate			4.0	2.51	4.25	97.2	97.4	97.4

Subcutanous administration of alkaloids at 100 mg/kg/day and N-methylglucamine antimonate at 56 mg of Sb^V/kg/day for a period of 14 days. Treatment started 1 day after infection with *L. amazonensis strain* PH 8 and H-142 or *L. venezuelensis* strain H3. * Averange measurement in millimetres (mm) for 10 mice. Treatment of *L. donovani* was given for 10 days using i.p. intraperitoneal; s.c. subcutaneous or oral administration. NT, not tested

142 infected mice, but less active than the reference drug against the PH8 strain (Fournet *et al.*, 1994a). Treatment of *L. donovani*-infected mice with chimanine D, 2-n-propylquinoline and meglumine antimonate resulted in significant liver parasite suppression (Fournet *et al.*, 1994b). Finally, 2-n-pentylquinoline was activite against *P. vinckei petteri* infected mice with ED_{50} of 50 mg/kg (Gantier *et al.*, 1996).

Dictyoloma peruviana (Rutaceae) used for the treatment of leishmaniasis in South America yielded two piperidino [1,2a]4-quinolinones: dictyolomide A and dictyolomide B (Table 1.4). Both alkaloids induced complete lysis of promastigotes of *Leishmania* sp., at 100 µg/ml (Lavaud *et al.*, 1995).

Table 1.4 *In vitro* antileishmanial activity of 4-quinoline alkaloids isolated from *Dictyoloma peruviana.*



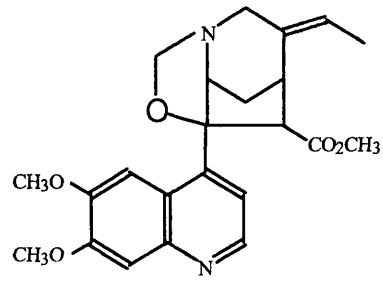
R

Dyctyolomide A $-CH_2-CH_2-CH=CH-CH_2-CH_3$
Dyctyolomide B $-CHOH-CH_2-CH_2-CH_2-CH_2-CH$

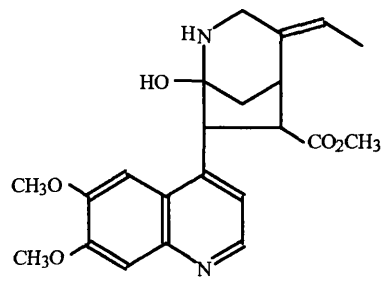
Compound	*Leishmania* sp. IC_{100} (µg/ml)
Dictyolomide A	100
Dictyolomide B	100

Alstonia coriacea (Apocynaceae) is used in South-East Asia for the treatment of malaria and dysentery. Investigation of this plant has resulted in the isolation of two structurally related quinine alkaloids, corialstonine and corialstonidine (Table 1.5). Both were active towards *P. falciparum in vitro* (Wright *et al.*, 1993a).

Table 1.5 *In vitro* antimalarial activity of some alkaloids from *Alstonia coriacea*



Corialstonine



Corialstonidine

Compound	*P. falciparum* (KI) IC_{50} (μM)
Corialstonine	5.7
Corialstonidine	5.4

1.7.1.2 Isoquinoline alkaloids

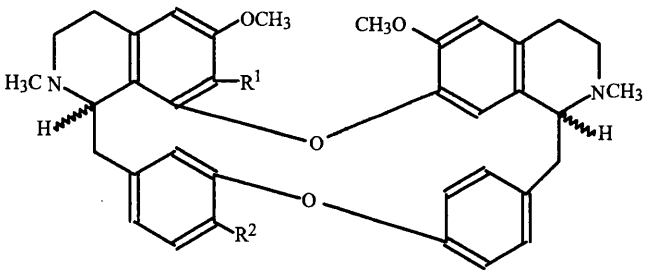
Berberine (Figure 1.13), a benzylisoquinoline alkaloid, occurs in a number of plant families including Annonaceae and Menispermaceae, and has been used to treat malaria, amoebiasis, and leishmaniasis. Independent investigations from two different laboratories have shown that berberine was active against three chloroquine-resistant strains of *P. falciparum in vitro* in the range (IC_{50}) of 0.14 to 0.36 μg/ml (Partridge *et al.*, 1990), but it was inactive against *P. berghei* in mice (Vennerstrom & Klayman, 1988). This finding contrasted with the activity against *P. falciparum in vitro* and the use of berberine in man. The same abnormality occurs for berberine against *E. histolytica*. In constrast, berberine, has demonstrated experimental and clinical efficaces against both visceral (Ghosh *et al.*, 1983) and cutaneous leishmaniasis (Vennerstrom *et al.*, 1990). The mode of action of berberine in *Leishmania* parasite involves the inhibition of nucleic acid, protein synthesis and endogenous respiration of the parasite (Ghosh *et al.*, 1985). *In vivo* antileishmanial studies of berberine and some of its derivatives have been shown that tetrahydroberberine was less toxic and more potent than berberine against *L. donovani* but was not as potent as the reference drug N-methylmeglumine antimonate. Further, berberine and 8-cyanodihydroberberine were as effective as the standard drug in treating *L. panamensis* infected-mice (Vennerstrom *et al.*, 1990).

Emetine (Figure 1.13), an isoquinoline alkaloid from *Cephaelis ipecacuanha* and related species, is still used as an amoebicidal drug. Emetine was effective in treating diffuse cutaneous leishmaniasis resistant to amphotericin B (Cohen & Wahaba, 1980). Studies of structure activity on *L. tropica* infected-mice have shown that antileishmanial activity of emetine is related to the configuration at the C-1' position of the molecule, similar to that for amoebicidal activity (Neal, 1970). Emetine and chemically related alkaloids inhibited protein synthesis in a wide range of cell types including mammalian tissues and *E. histolytica* (Neal, 1970). On the other hand, emetine possess immmunostimulant properties at low concentration of 0.0001% (Wagner & Proksch 1985).

1.7.1.2.1 Bisbenzylisoquinoline (BBIQ) alkaloids

Some 24 BBIQ alkaloids were assessed for antiprotozoal and cytotoxicity *in vitro* (Table 1.6), and 8 had IC_{50} values of <1μM against the multi-drug resistant strain of *P. falciparum* (K1) (Marshall *et al.*, 1994). A further eleven had IC_{50} values between 1 and 10 μM. Under the same test conditions, chloroquine had an IC_{50} value of 0.2 μM. The most potent antimalarial BBIQ alkaloid was thalisopodine with IC_{50} value of 0.09 μM. Phaeanthine was twice as potent against chloroquine-resistant *P. falciparum* (K1) *in vitro* as against a chloroquine-sensitive clone (T9-96) (Ekong *et al.*, 1991). The most active BBIQ alkaloid against *E. histolytica in vitro* was aromoline (IC_{50} = 5 μM). Emetine under the same conditions had an IC_{50} value of 2.2 μM. None of the 24 alkaloids tested showed significant cytotoxic activity against KB cells. Tetrandrine was the most potent against chloroquine-resistant strains of *P.falciparum* than to chloroquine-sensitive strains (Ye & Van Dyke, 1989). Combination of tetrandrine/artemisinin, provided long acting and synergistic activity against *P. falciparum in vitro* (Van Dyke, 1991). Gyrocarpine, daphnandrine and obaderine had activity against promastigotes of *Leishmania* sp., *in vitro* (Fournet *et al.*, 1988a), and against several strains of *T. cruzi* epimastigotes (Fournet *et al.*, 1988b) and trypomastigotes (Rojas de Arias *et al.*, 1994). Studies *in vivo* have shown that isotetrandrine was as effective as Sb^V in treating *L. amazonensis* infected-mice and less active against *L. venezuelensis* infected mice (Fournet *et al.*, 1993b). Limacine and phaeanthine alkaloids, were inactive against *T. b. brucei*, *P. falciparum* and *P. berghei* in mice.

Table 1.6 Biological activities of miscellaneous bisbenzylisoquinoline alkaloids



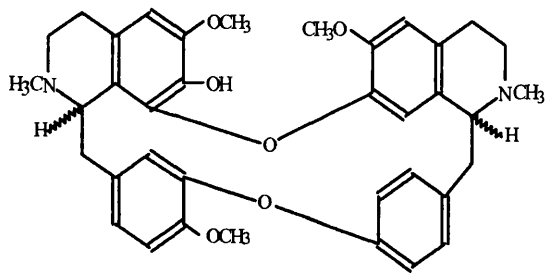
		R^1	R^2
Phaeanthine	(R,R)	OCH_3	OCH_3
Tetrandrine	(S,S)	OCH_3	OCH_3
Isotetrandrine	(R,S)	OCH_3	OCH_3
Berbamine	(R,S)	OCH_3	OH
Pycnamine	(R,R)	OCH_3	OH

BBIQ alkaloid	IC_{50} μM/ml		
	P. falciparum	*E. histolytica*	KB cells
Funiferine	0.63	45.5	108
Tiliagenine	6.32	31.6	>411
Daphnoline	0.96	>86.2	46.4
Aromoline	1.32	~5.0	105
Homoaromoline	3.46	17.3	>82.2
Oxyacanthine HCl	1.06	32.3	74.4
Thalisopidine	0.09	~80.1	41.2
Phaeanthine	1.46	17.4	43.6
Tetrandrine	0.57	16.9	NT
Isotetrandrine	0.16	22.2	NT
Tetrandrine methiodide	>65.4	39.5	>32.7
Pycnamine	0.83	NT	31.9
Fangchinoline	1.43	24.2	104
Berbamine	0.45	36.8	17.8
Obamegine	0.74	30.8	55.4
Dinklacorine	3.92	34.4	54.8
Isochondodendrine	22.0	17.7	>421
Trigilletime	42.1	41.7	>448
Cocsoline	1.16	18.0	NT
Cocsuline	~88.9	23.5	>222
Isotrilobine	2.09	10.8	18.8
Cocsuline methiodide	>17.8	36.2	>355
Gilletine	1.81	20.9	38.3
Insularine picrate	2.07	11.1	>294
Chloroquine diphosphate	0.09	NT	NT
Podophyllotoxin	NT	NT	0.008
Emetine dihydrochloride	NT	2.23	NT

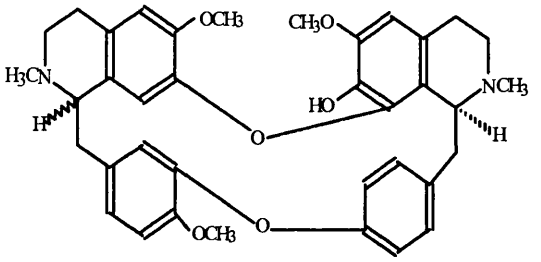
NT, not tested

Cyclea barbata (Menispermaceae) a plant used in East Asia and Thailand for the treatment of fevers yielded 5 bisbenzylisoquinoline (BBIQ) alkaloids (Table 1.7): (+)-tetrandrine, (-)-limacine, (+)-thalrugoside, (+)-homoaromoline and (-)-cycleapeltine]. These alkaloids were active in the range of (IC_{50}) 0.02-0.2 μg/ml against two strains of *P. falciparum*. These values were compared with cytotoxic effects obtained with a series of tumor cells, and the relation of activities yielded ratios in the range of 2-100, whereas agents such as quinine and artemisinin yielded ratios > 1000 (Lin *et al.*, 1993).

Table 1. 7 Biological activities of bisbenzylisoquinoline alkaloids isolated from *Cyclea barbata*



(-)-Limacine
(+)-Thalrugoside



(+)-Homoaromoline
(-)-Cycleapeltine

BBIQ alkaloid	*Plasmodium falciparum* IC_{50} (μM)		KB cells IC_{50} (μg/ml)		P338 cells IC_{50} (μg/ml)
	D-6	W-2	KB	KB-VI	
Tetrandrine[a]	0.179	0.160	2.1	3.7	0.40
Limacine	0.052	0.164	9.8	11	0.25
Thalrugosine	0.065	0.078	3.4	11	0.36
Homoaromoline	0.232	0.451	3.6	15	0.31
Cycleapeltine	0.029	0.451	2.2	4.4	0.57
Chloroquine	0.002	0.031	17.4	7.8	>5
Quinine	0.007	0.030	>20	5.6	>5
Mefloquine	0.009	0.001	1.0	1.1	0.2

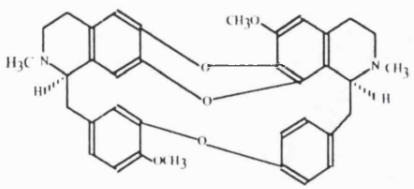
[a] For structure see Table 1.9. *P. falciparum* chloroquine-sensitive (D6) and -resistant (W-2) strains. KB and KB-V1 are vinblastine-sensitive and -resistant strains respectively.

Stephania erecta (Menispermaceae) has been used in Thai folk medicine as a skeletal muscle relaxant and an analgesic. The study of this plant yielded 13 BBIQ alkaloids (Table 1.8) which have shown antimalarial and cytotoxic properties (Likhitwitayawuid *et al.*, 1993). The alkaloids inhibited the growth of chloroquine sensitive (D-6) and chloroquine-resistant (W-2) *P. falciparum* strains having IC_{50} values in the range of 0.045 to 0.294 μg/ml (Table 1.8). These values were roughly equivalent or greater in the drug resistant strain W-2 as compared with strain D-6, with the exception of tetrandrine which showed greater activity with strain W-2. The cytotoxicity potential of these agents was measured with a battery of cultured mammalian cells. In general, all alkaloids displayed nonselective cytotoxicity with a series of 11 cultured mammalian cells. Ten of these showed greater cytotoxic activity with vinblastine-resistant KB cells (KB-VI) as compared with KB cells. (+)-2-N-Methyl-telobine against KB-IV cells was about 10-fold greater than against the KB cells. However, selectivity indices (cytotoxicity/antimalarial activity) of chloroquine (158 to > 8000), quinine (162 to 2631) or mefloquine (22 to 4417) were generally superior to the values calculated for the isolated compounds (1 to 357).

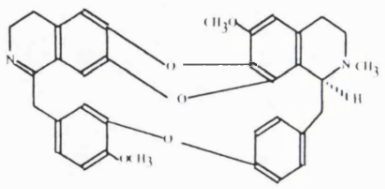
1.7.1.2.2 Other isoquinoline alkaloids

Pogonopus tubulosus (Rubiaceae) called "falsa quina" in South America used against malaria, afforded an isoquinoline-indole alkaloid tubulosine, and two emetine-like alkaloids psychotrine and cephaeline (Table 1.9). Tubulosine showed *in vitro* antiplasmodial activity against both chloroquine-sensitive (2089) and chloroquine-resistant (INDO) strains of *P. falciparum* (Sauvain *et al.*, 1996b), and the activitites were similar to those previously reported by Wright *et al.* (1991a). The *in vitro* activities of the cephaeline was similar to tubulosine whereas psychotrine was less active against INDO strain. Cephaeline and psychotrine were more cytotoxic than tubulosine, suggesting that the presence of an indole group in this series of compounds enhances its selectivity to protozoa. Tubulosine had significant activity *in vivo* (ED_{50} = 0.45 mg/kg/day) on the *P. berguei* strain, whereas psychotrine (ED_{50} >2 mg/kg/day) and cephaeline (ED_{50} = 6 mg/kg/day) were less active. Tubulosine is more active than usamberine group (Table 1.9) which are relatively active *in vitro* and inactive *in vivo*.

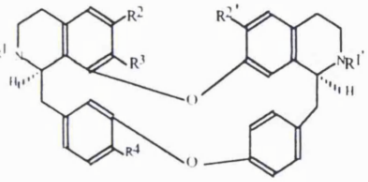
Table 1.8 *In vitro* biological activities of bisbenzylisoquinoline alkaloids from *Stephania erecta*



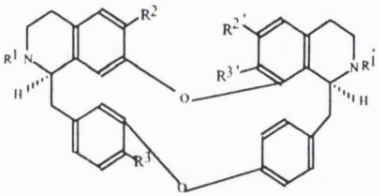
N-Methyltelobine



1,2-Dehydrotelobine



I

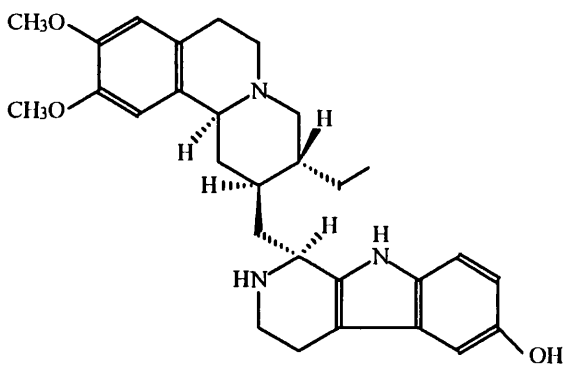


II

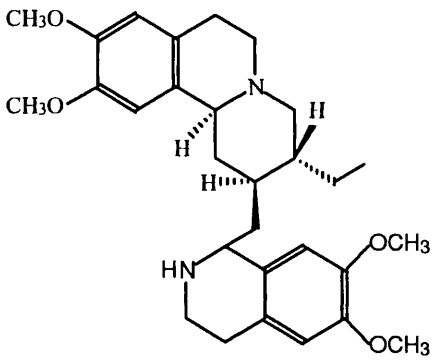
Bisbenzylisoquinoline alkaloid							*Plasmodium falciparum* IC_{50} µM		KB cells IC_{50} µg/ml		P338 IC_{50} µg/ml
							D-6	W-2	KB	KB-VI	
N-Methyltelobine							97.4	255.7	4.8	0.4	5.6
1,2-Dehydrotelobine							306.7	256.4	2.8	6.8	3.1
See structure I	R^1	R^2	R^3	R^4	$R^{2'}$	$R^{3'}$					
2-Norisotetrandrine	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	66.1	45.3	6.3	2.8	4.7
Isotetrandrine	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	165.1	54.6	6.6	1.5	5.6
2-Northalrugoside	OH	OCH_3	OH	OCH_3	OCH_3	OCH_3	68.6	125.1	6.3	8.1	0.1
Thalrugoside	OCH_3	OCH_3	OH	OCH_3	OCH_3	OCH_3	120.6	229.7	6.5	3.7	7.4
See structure II	R^1	R^2	R^3	$R^{2'}$	$R^{3'}$	$R^{3'}$					
Homoaromiline	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OH	104.6	288.3	6.3	3.1	3.7
Stephibaberine	OCH_3	OCH_3	OCH_3	OCH_3	OH	OCH_3	130.0	310.0	6.4	7.4	3.7
Daphnandrine	OH	OCH_3	OCH_3	OCH_3	OCH_3	OH	63.0	223.2	5.8	2.2	2.0
2-Norcepharanthine	OH	OCH_3	OCH_3	OCH_3	-CH-		46.6	129.4	3.9	1.4	0.1
Cepharanthine	OCH_3	OCH_3	OCH_3	OCH_3	-CH-		140.4	294.8	5.9	0.9	0.3
2-Norobaberine	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	45.9	93.7	6.0	2.0	3.5
Obaberine	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	231.0	216.0	8.6	2.6	4.1
Chloroquine							0.002	0.031	17.4	7.8	>5
Quinine							0.007	0.030	>20	5.6	>5
Mefloquine							0.009	0.001	1.0	1.1	0.2

D6 and W-2 are chloroquine-sensistive and -resistant strains of *P.falciparum*. KB and KB-VI are vinblastine-sensistive and -resistant strains of KB cells.

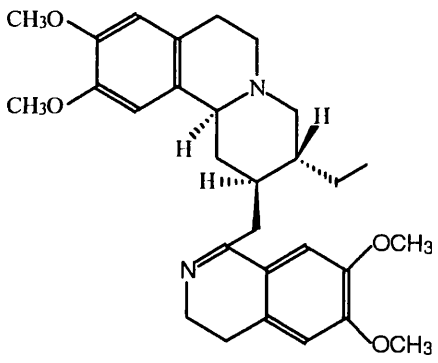
Table 1.9 *In vitro* antimalarial activity of alkaloids isolated from *Pogonopus tubulosus*



Tubulosine



Cephaeline



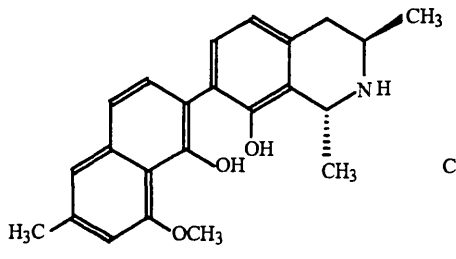
Psychotrine

Compound	*Plasmodium falciparum* IC_{50} (µg/ml)	
	2087	INDO
Tubulosine	0.006	0.011
Psychotrine	0.14	0.39
Cephaeline	0.027	0.011
Chloroquine	0.02	0.08

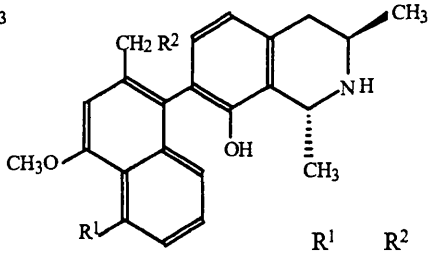
2087 and INDO are chloroquine-sensistive and -resistant strains of *P. falciparum*.

Triphyophyllum peltatum (Dioncophyllaceae), *Ancistrocladus abbreviatus* and *A.barteri* (Ancistrocladaceaea) are well-known in the traditional medicine of West Africa for the treatment of fevers and malaria. These species yielded some naphthylisoquinoline alkaloids (Table 1.10). Dioncophylline B and dioncopeltine A were found the most active compounds against chloroquine-resistant K1 and -sensitive NF 54/64 strains of *P. falciparum in vitro*. Dioncophylline A had IC_{50} values against both strains in the order of 1 μg/ml. Dioncophylline B, dioncopeltine A and dioncophylline A were also active against asexual blood forms of *P. berghei in vitro* (Francois *et al.*, 1994, 1995).

Table 1.10 *In vitro* antimalarial activity of some naphthylisoquinoline alkaloids



Dioncophylline B



	R^1	R^2
Dioncopeltine A	OH	OH
Dioncophylline A	OCH_3	H

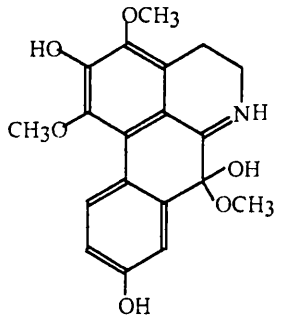
Compound	*P. falciparum* IC_{50} (μg/ml)	
	K1	NF 54/64
Dioncophylline B	0.06	0.224
Dioncopeltine A	0.33	0.021
Dioncophylline A	0.86	1.44
N-Methyldioncophylline	5.74	13.63
Chloroquine	0.068	NT

P. falciparum chloroquine-resistant (K1) and -sensitive (NF 54/65) strains.

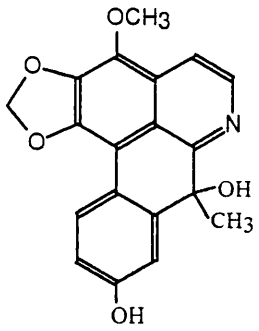
1.7.1.2.3 Aporphine alkaloids

Guatteria foliosa (Annonaceae) is used in Bolivia as an insect repellent and showed activity against *Leishmania* sp. and *T. cruzi in vitro*. Bioactive guided fractionation resulted in the isolation of two aporphine alkaloids: 3-hydroxynornuciferine and isoguatouregidine, and the aminoethylphenanthrene argentinine (Table 1.11) which had activity (≥60%) against trypomastigote forms of *T. cruzi* (Mahiou *et al.*, 1994).

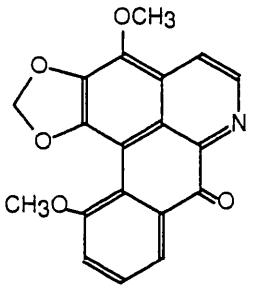
Table 1.11 *In vitro* activity of some aporphine alkaloids from *Guatteria foliosa* against *T. cruzi* Y



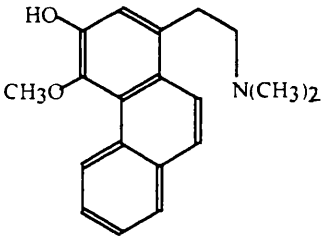
Isoguatouregidine



3-Methoxyguattescidine



3-Methoxyoxoputerine



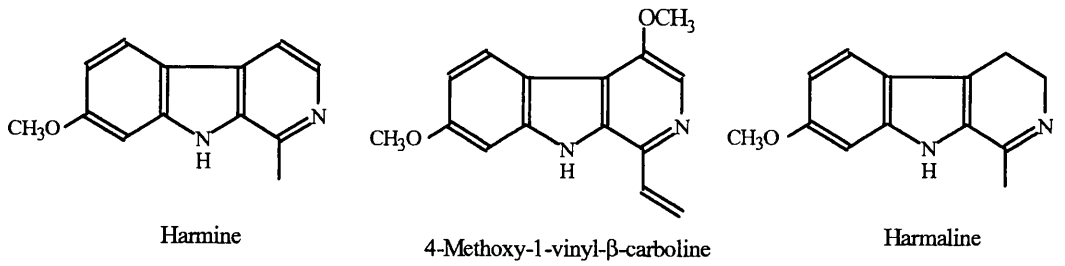
Argentinine

Compound	% lysis at 250 µg/ml
3-Hydroxynornuciferine	68.2
Isoguattouregidine	92
3-Methoxyguattescidine	0
3-Methoxyoxoputerine	46.6
Argentinine	81.3
Gentian violet	100

1.7.1.3 Indole alkaloids

The β-carboline harmine (Table 1.12) had significant activity against epimastigotes of *T. cruzi in vitro* (Cavin *et al.*, 1987); 4-methoxy-1-vinyl-β-carboline from *Picrasmia javanica* had *in vitro* activity against *P. falciparum*. The dihydrocarboline harmaline was active against *L. amazonensis* amastigotes *in vitro*, and its synthetic analogue α-ethyltryptamine was orally active (ED_{50} = 47 mg/kg). β-Carboline compounds may act by interfering with aromatic amino acid metabolism and this could be the mode of action in *Trypanosoma* sp. and *Leishmania* sp. (Evans & Croft, 1987).

Table 1.12 *In vitro* antiprotozoal activities of some β-carboline alkaloids



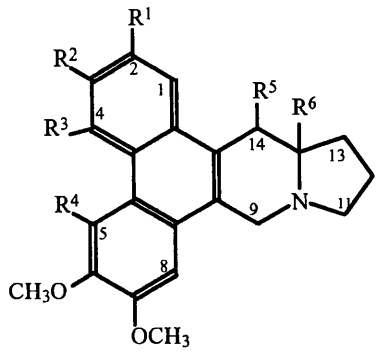
Compound	IC_{50} (μg/ml) *T. cruzi*	*P. falciparum*	*L. amazonensis*
Harmine	5.0	NT	NT
4-Methoxy-1-vinyl-β-carboline	NT	1.9	NT
Harmaline	NT	NT	24

NT, not tested

Cryptolepis sanguinolenta (Asclepiadaceae) clinically used in Ghana for the treatment of malaria yielded two indoloquinoline alkaloids (Figure 1.13) cryptolepine and isocryptolepine (Grellier *et al.*, 1996). Two independent studies have been shown that cryptolepine was active against two chloroquine-resistant strains of *P. falciparum* K1 (Kirby *et al.*, 1992) and FcB1/Colombia strains with an IC_{50} value in the range of 0.13 to 0.56 μM. Isocryptolepine had IC_{50} value of 0.3 μM against chloroquine-resistant strain FcB1/Colombia strain (Grellier *et al.*, 1996). The antiplasmodial activity of cryptolepine was confirmed *in vivo* on the rodent malaria parasites, *P. vinckei petteri* and *P. berghei* this latter being much less sensitive to cryptolepine (Kirby *et al.*, 1995; Grellier *et al.*, 1996). These differences of sensitivity could be explained in part by the biological peculiarities of these strains. Cryptolepine interacts with DNA in a similar way to 9-aminoacridine and this may explain its *in vitro* antimalarial activity (Noamesi *et al.*, 1991). In addition, the hypothermic activity of cryptolepine might also contribute to its activity by reducing fever associated with malaria. The indoloisoquinoline ellipticine from *Ochrosia elliptica* (Apocynaceae) and ollivacine (Figure 1.13) from *Aspidosperma nigricans* (Apocynaceae) have been shown to be active against *T. cruzi in vitro* (Phillipson *et al.*, 1995).

Tylophora indica a plant used as a substitute for ipecacuanha in the treatment of dysentery yielded three active phenanthroindolizidine alkaloids: Tylophorine, 4-methoxy-14-hydroxy-tylophorine and 14-deoxy-13a-methyl-tylohirsutinidine (Table 1.13). Tylophorine and 4-methoxy-14-hydroxy-tylophorine were 2 and 3 times more potent than the antiprotozoal drugs metronidazole and emetine dihidrochloride, whilst 14-deoxy-13a-methyl-tylohirsutinidine was as active as the standard drug. Tylophorine in a similar way to emetine inhibits protein synthesis. Tylophorine hydrochloride was found equally effective as emetine in the treatment of intestinal and hepatic infection in test animals, however its gross toxicity excluded the potential use in man (Bhutani *et al.*, 1987). In addition, tylophorine has been found to enhance phagocytosis at low concentration of 10^{-6} mg/ml (Wagner, 1990).

Table 1. 13 *In vitro* antiamoebic activity of some phenanthroindolizidine alkaloids from *Tylophora indica*



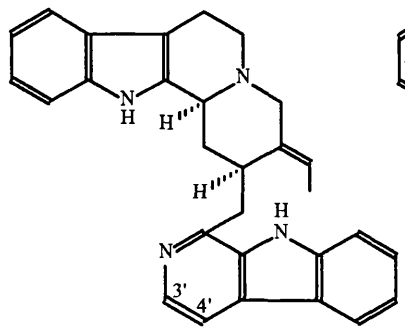
Compound	R¹	R²	R³	R⁴	R⁵	*E. histolytica* MIC (μg/ml)
Tylophorine	OCH_3	OCH_3	H	H	H	6.25
4-Methoxy-14-hydroxy-tylophorine	OCH_3	OCH_3	OCH_3	H	OH	3.12
14-Deoxy-13a-methyl-tylohirsutinidine	H	OCH_3	OH	H	OCH_3	12.5
Metronidazole						12.5
Emetine hydrochloride						12.5

Five alkaloids (Table 1.14) from *Strychnos usambarensis* (Loganiaceae) an antimalarial African plant were assessed *in vitro* for antiprotozoal and cytotoxic activities (Wright *et al.*, 1991a). Usambarensine and 3',4'-dihydro-usambaresine were highly active against *P. falciparum*, *E. histolytica* and *G. intestinalis*. Usambarensine possesses activities similar to those of emetine and metronidazole against *E. histolytica* and *G. intestinalis* but is about four times less potent as an antiplasmodial agent than chloroquine. In contrast, 3,4-dihydrousambarensine is 40 times more active against *P. falciparum* than usambarensine, and has weak antiamoebic and antigiardial properties. Usambarine had potent antiamoebic properties. Strychnofoline an oxindole alkaloid containing only one indole ring system was devoid of antiprotozoal activity suggesting that the presence of two indole moieties is essential for activity in these series of compounds. None of the alkaloids displayed high toxicity against KB cells (Wright *et al.*, 1993a).

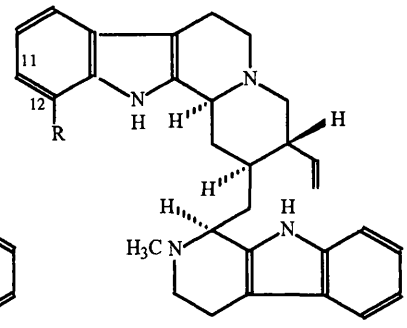
Alstonia angustifolia a Malaysian antimalarial plant has yielded the dimeric alkaloids of alstonerine and pleiocarpamine villastonine and macrocarpamine respectively, and macralstonine acetate a dimer of alstonine (Table 1.15) which had antiprotozoal activity *in vitro* (Wright *et al.*, 1992). However, they were 15-50 times less potent than chloroquine against *P. falciparum* and 4-8 times less potent than emetine against *E. histolica*. Macrocarpamine was active towards *G. intestinalis* (IC_{50} = 8.32 μM).

Peschiera van heurkii (Apocynaceae) a medicinal plant used in Bolivia for the treatment of leishmaniasis yielded some antileishmanial indole and bis- indole alkaloids. The strongest leishmanicidal activity was observed with the dimeric alkaloids: conodurine, N-demethylconodurine and conoduramine (Table 1.16), which were the most potent against promastigotes and amastigotes of *L. amazonensis*. Conoduramine was as active as the antileishmanial drug N-methylmeglumine antimonate whilst conodurine was 5 times less potent than the reference drug against amastigotes of *L. amazonensis*. Conodurine was found to be less active than glucamine antimonate, and gabunine was devoided of activity in treating *L. amazonensis* infected mice (Munoz *et al.*, 1994).

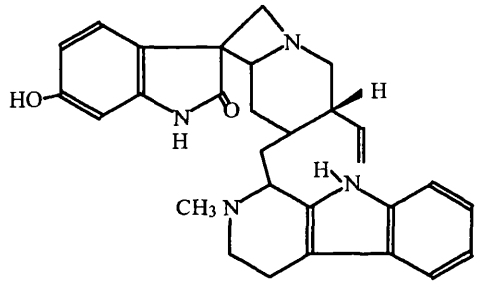
Table 1.14 *In vitro* biological activities of some alkaloids isolated from *Strychnos usambarensis*



Usambarensine



Usambarine R = H
Strychnopentamine R = 12-(N-methylpiperin

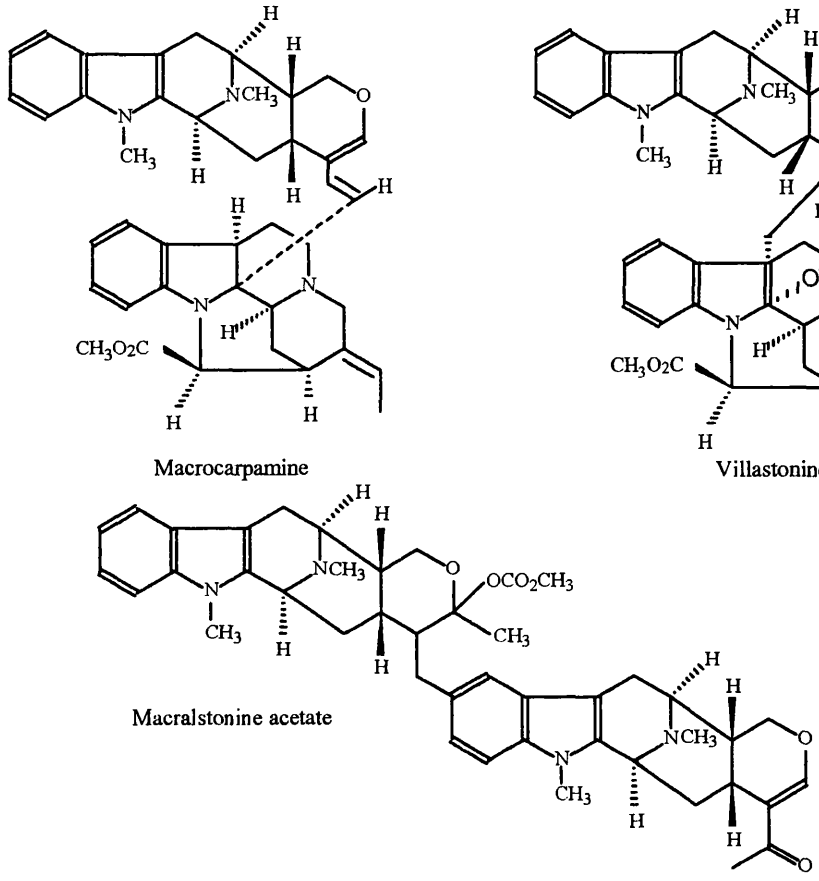


Strychnofoline

Compound	IC_{50} (μM)			
	P. falciparum	*E. histolytica*	*G. instestinalis*	KB cells
Usambarensine	0.88	1.13	3.89	21.2
3',4'-Dihydro-	0.023	5.02	5.14	33.9
usambarensine	~4.11	1.02	22.3	20.8
Usambarine	0.164	14	7.30	35
Strychnopentamine	13.80	>52	NT	NT
Strychnofoline	0.156	NT	5.58	153
Chloroquine diphosphate				
Emetine dihydrochloride	0.13	3.07	2.98	0.41
Metronidazole	NT	1.87	NT	NT

NT, not tested

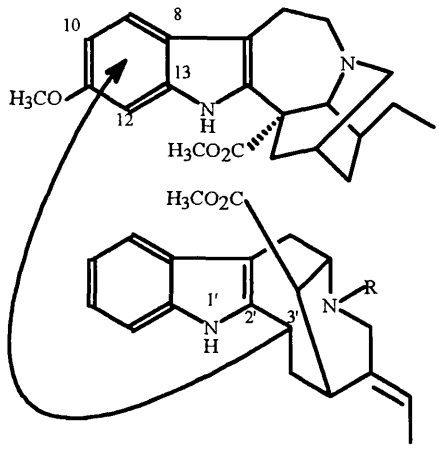
Table 1.15 *In vitro* antiprotozoal activities of some alkaloids from *Alstonia angustifolia*



Compound	IC_{50} (μM)	
	P. falciparum	*E. histolytica*
Macrocarpamine	3.43	15.51
Macralstonine acetate	9.36	8.12
Villastonine	2.92	11.8
Altonerine	46.3	75.3
Pleiocarpamine	20.5	47.4
Chloroquine diphosphate	0.168	NT
Emetine hydrochloride	NT	2.04

NT, not tested

Table 1.16 *In vitro* antileishmanial activity of some bis-indole alkaloids from *Peschiera van heurkii*



Alkaloid	R	bound	*L. amazonensis* amastigotes IC_{50} (μg/ml)
Conodurine	CH_3	C3' → C12	50
N-Demethylconodurine	H	C3' → C12	25-12.5
(gabunine)	H	C3' → C10	5-10
Conoduramine			

Other indole alkaloids with antiprotozoal activity include the antitumour drug, camptothecin from *Camptothera acuminata.* Camptothecin is an inhibitor of eukaryotic DNA topoisomerase I of trypanosomes and *Leishmania in vitro* with IC_{50} values of 1.5, 1.6 and 3.2 μM against *T. brucei T. cruzi* and *L.donovani* respectively (Bodley & Shapiro, 1995). The antileishmanial constituents of *P. nitida* (Iwu, 1994): alstonine (Figure 1.13), akuammine (Figure 1.13), akuammicine (Figure 1.13), picraline (Figure 1.13) and the dimeric compound picranitidine (Figure 1.13). Vinblastine from *Catharanthus roseus* active against *T. cruzi* epimastigotes (Cavin *et al.*, 1987) and *P. falciparum in vitro* (Usanga *et al.*, 1986).

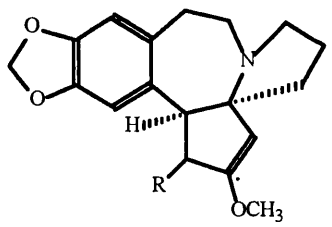
1.7.1.4 Other alkaloids

Other alkaloids with *in vitro* antiprotozoal activity include: febrifugine from *Dichroea febrifuga* (Steck, 1972); the steroidal alkaloid conessine from *Holarrhena pubescens*, the piperidine alkaloids piperine from *Piper nigrum* (Kapil *et al.*, 1993) and carpaine from

Carica papaya (Moraceae), the phenanthrene-quinolizidine cryptopleurine, the quinolizidines matrine and cystisine (Phillipson *et al.*, 1995), and the apomorphine alkaloids boldine, glaucine, predicentrine, and norarmepaine (Morello *et al.*, 1994).

The antitumor alkaloid homoharringtonine, from *Cephalotaxus* sp. (Cephalotaxaceae), was effective and highly potent against two strains *P. falciparum.* whereas, cephalotaxine was less active (Table 1.17). Cephalotaxine potentiated the activity of emetine and of anisomycin against *P. falciparum* (K1) *in vitro* (Al-Khayat *et al.*, 1991). Oral administration of homoharringtonine to *P. yoelii* infected mice resulted in 99.97% suppression of parasitemia (Whaum & Brown, 1990). Homoharringtonine was found also active against *E. histolytica in vitro* (Wright *et al.*, 1991b).

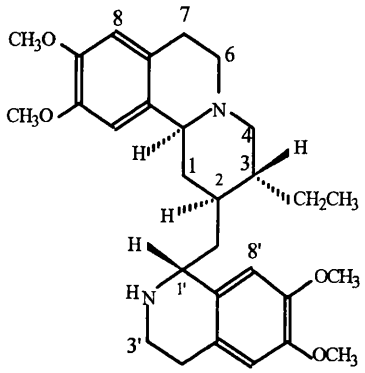
Table 1. 17 *In vitro* antiprotozoal activities of some alkaloids from *Cephalotaxus* sp.



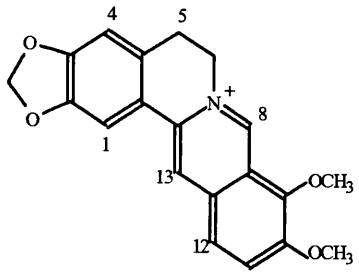
	R
Cephalotaxine	OH
Homoharringtonine	$CH_3C(OH)(CH_3)(CH_2)_3$-C(OH)(CO_2-)$CH_2CO_2CH_3$

Alkaloid	IC_{50} µM		
	P. falciparum		*E. histolytica*
	K1	T9-96	NIH 200
Homoharringtonine	0.004	0.004	0.35
Cephalotaxine	28.4	38.1	NT

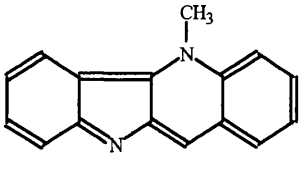
K1 and T9-96 chloroquine resistant and -sensitive strains of *P. falciparum.* NT, not tested



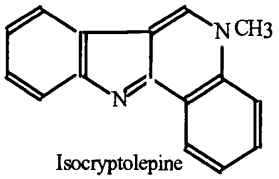
Emetine



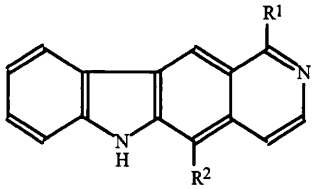
Berberine



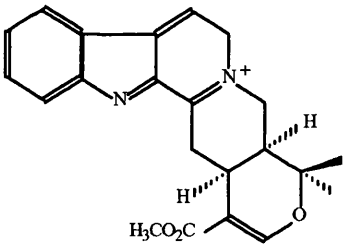
Cryptolepine



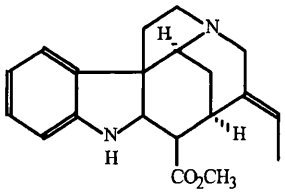
Isocryptolepine



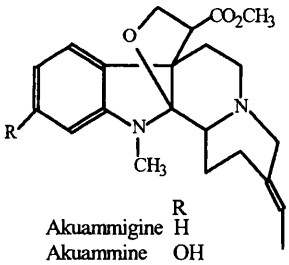
Ellipticine R = H
Olivacine R = CH_3



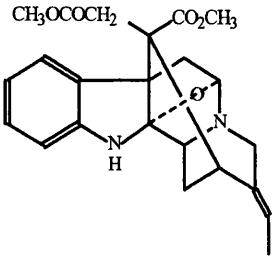
Alstonine



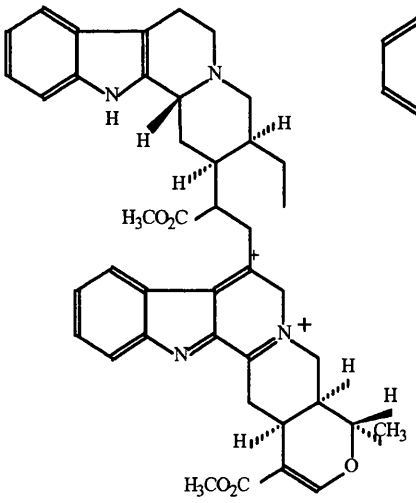
Akuammicine



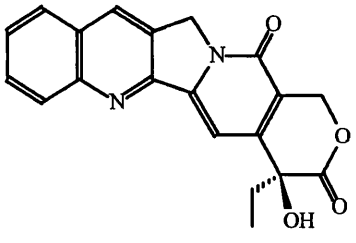
Akuammigine R = H
Akuammine R = OH



Picraline



Picranitidine



Camptothecin

Figure 1.13 Some alkaloids with antiprotozoal activity

1.7.2 Quinones

1.7.2.1 Benzoquinones

Jacaranda copaica (Bignoniaceae) afforded the benzoquinone jacaranone (Figure 1.14) effective against promastigotes of *Leishmania in vitro* (ED_{50} of 0.02 mM) but it was toxic for macrophages at the same doses. The subcutanous injection of this compound showed a slight activity, a strong inflammatory reaction, and cutaneous toxicity which masked the antileishmanial effects of the compound (Sauvin *et al.*, 1993). The bis-benzoquinone japonicine A from *Hypericum japonicum* (Guttiferae) possessed *in vivo* antimalarial activity (Gu *et al.*, 1984). The bioactive benzoquinones, 1-hydroxybenzoisochromanquinone and benz[g]isoquinoline-5,10-dione (Figure 1.14) were isolated from *Psychotria camponuntans* (Solis *et al.*, 1995a). Benz[g]isoquinoline-5,10-dione (IC_{50} = 2.45 μM) was twice as active against brine shrimp than 1-hydroxybenzoisochromanquinone (IC_{50} = 7.32). Benz[g]isoquinoline-5,10-dione (IC_{50} = 0.84 μM) was 3- and 10-times more active than1-hydroxybenzoisochromanquinone (IC_{50}=2.66μM) and 1-acetylbenzoisochromanquinone (IC_{50} = 6.02 μM) against *P. falciparum* (K1).

1.7.2.2 Naphthoquinones

Ampelocera edentula (Ulmaceae) is used in Bolivia for the treatment of cutaneous leishmaniasis. Bioassay-guided fractionation of this plant afforded a naphthoquinone 4-hydroxy-1-tetralone (Figure 1.14), which is active towards promastigotes of *Leishmania* and epimastigotes of *T. cruzi*. 4-Hydroxy-1-tetralone administrated subcutaneously (25 mg/kg/day) was slightly less effective than pentavalent antimony (56 mg Sb^{v}/kg/day) in treating both *L. amazonensis* or *L. venezuelensis* infected-mice However, single treatment near the site of infection, with 4-hydroxy-1-tetralone was more effective than the reference drug in treating *L. amazonensis* infected mice (Fournet *et al.*, 1994c).These results could explain the use of the above plant for the treatment of leishmanisis.

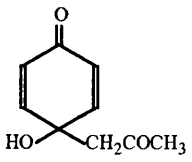
Diospyrin (Figure 1.14), a bis-naphthoquinone from *Diospyros montana* a plant, used for its antitumour properties exhibited antiprotozoal activity towards *L. donovani* promastigotes with a MIC value of 1μg/ml. In another study, diospyrin has shown to

be inactive to amastigote of *L.donovani* and active against *T.cruzi* and *T.b.brucei in vitro* with ED_{50} values of 27 and 50 μM, respectively (Yardley *et al.*, 1996). Plumbagin, diospyrin and β-lapachone inhibit the respiration of cells by interfering with electron transport (Hazra *et al.*, 1987).

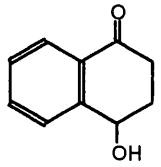
Lapachol (Figure 1.14), a naphthoquinone present in the South American Bignoniaceae *Tabebuia rosea* had antimicrobial and antineoplastic properties, and weak activity towards *T. cruzi* epimastigotes (Lopes *et al.*, 1978). Lapachol may be useful when added to blood to prevent transmission of *T. cruzi* (Goncalves *et al.*, 1980). Like other naphthoquinones, lapachol exerts its trypanocidal action by generation of free radicals, drug metabolites and/ or oxygen reduction derivatives (Ellis, 1994). Lapachol possesses immunostimulat properties (Wagner, 1990).

Lawsone (Figure 1.14) 3-(1,1)-dimethylallyl)-lawsone and the cyclized α-lapachone analogue (-)-2,3,3-trimethyl-2,3-dihydronaphto-[2,3-b]furan-4,9-quinoline, inhibited the proliferation of three strains of *T. cruzi* epimastigotes with IC_{50} values in the range of 20-50, 5.3-15.1 and 2.1-5.2 μM, respectively. The last compound and the most potent did not inhibit respiration of *T. cruzi* but produced a temporary increase in oxygen consumption suggestive of redoxcycling (Sepulveda-Boza & Cassels, 1996). Two semisynthetic derivates naphthooxazole and naphthoimidazole showed lytic activity towards bloodstream forms of *T. cruzi* (Pinto *et al.*, 1997).

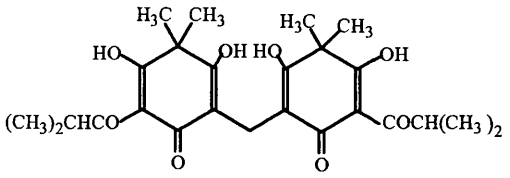
Pera benensis (Euphorbiaceae) an antileishmanial Bolivian plant yielded three active naphthoquinones (Fournet *et al.*, 1992): plumbagin, 3,3'-biplumbagin and 8,8'-biplumbagin (Figure 1.14). Plumbagin had *in vitro* activity on promastigotes and amastigotes of *Leishmania* sp., (Fournet *et al.*, 1990). In addition, treatment of *L. amazonensis* infected mice with plumbagin (2.5 mg/kg/day) developed an equivalent lesion size to those treated with N-methylglucamine antimoniate (200 mg/kg/d) (Croft *et al.*, 1985; Fournet *et al.*, 1992). Both dimers of plumbagin, 3,3'-biplumbagin and 8,8'-biplumbagin were less toxic but less active than plumbagin. On the other hand, 8,8'-biplumbagin (50 mg/kg/day) showed an equivalent activity as the pentavalent antimony



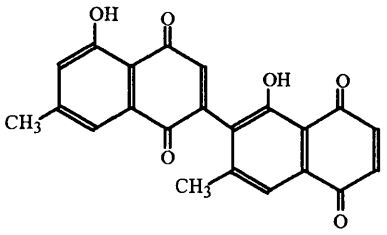
Jacaranone



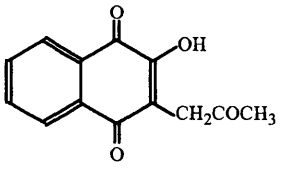
4-Hydroxy-1-tetralone



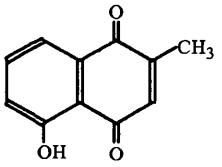
Japonicine A



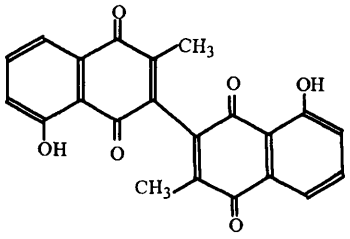
Diospyrin



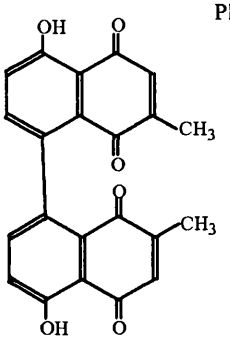
Lapachol



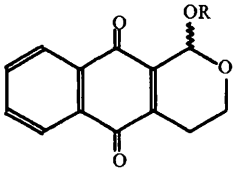
Plumbagin



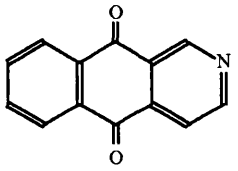
3', 3' -Biplumbagin



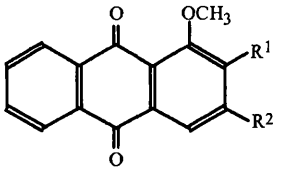
8',8'-Biplumgagin



R	
1-Hydroxybenzo chromanquinone	OH
1-Acetylbenzoiso chromanquinone	OAc



Benz[g]-isoquinoline-5, 10- dione



	R^1	R^2
Digitolutein	OH	CH_3
Rubiadin-1-methylether	CH_3	OH
Damnacanthol	CHO	OH

Figure 1.14 Some quinones with antiprotozoal activity

drug (400 mg/kg/day) when administered locally in the *L. amazonensis* infected rear footpad (Fournet *et al.*, 1992). Plumbagin as the standard pentavalent antimony drug delayed the development of *L. amazonensis* and *L. venezuelensis.* Plumbagin has been shown to increase the phagocytosis of human granulocytes *in vitro* in concentration range of 2.5 pg-25 pg/ml (Wagner, 1990), and also possess antibacterial properties. Naphthoquinones affect *Leishmania* sp., by generating free oxygen radicals withing parasites which are defective in protective mechanisms against oxygen radicals particularly catalasa (Docampo *et al.*, 1978).

1.7.2.3 Anthraquinones

Morinda lucida (Rubiaceae) is used in traditional medicine in West Africa to treat malaria. The study of this plant yielded 3 active anthraquinones: digitolutein, rubiadin-1-methylether, and damnacanthol (Figure 1.14)whichhad IC_{50} values in the range 8.1-12.9 µg/ml against *P. falciparum in vitro* (Koumaglo *et al.*, 1992).

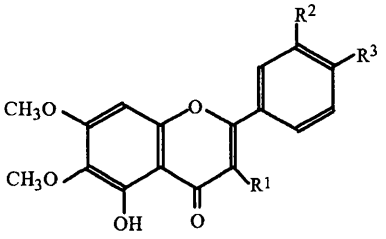
1.7.3 Flavonoids

Four flavonoids from *Artemisia annua*, artemetin, chrysoplenetin, chrysoplenol D, and circilineol (Figure 1.15) had IC_{50} values in the range of 2.3-6.5 x 10^{-5} M against *P. falciparum* (K1) *in vitro*. At lower concentrations of 5 x 10^{-6} M they potentiated the antimalarial effect of artemisinin *in vitro* (Elford *et al.*, 1987). Similar effects were observed with artemetin (Figure 1.15) and casticin (Figure 1.15).

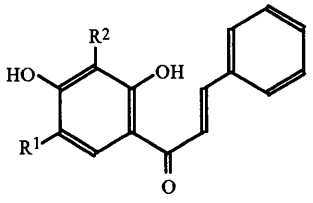
Uvaria spp (Annonaceae) has yielded two dibenzylated chalcones uvaretin and diuvaretin (Figure 1.15) with *in vitro* antimalarial activity at (IC_{50}) 3.49 and 4.20 µg/ml, respectively against *P. falciparum* K1 strain (Phillipson *et al.*, 1995).

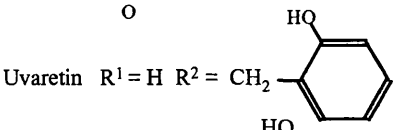
Licochalcone A (Figure 1.15), an oxygenated chalcone from the roots of Chinese liquorice plant, inhibited the growth of promastigotes and amastigotes of both *L. major* and *L. donovani.* The IC_{50} values of licochalcone A against logarithmic- and stationary-phase promastigotes of *L. major* were 4 and 2.5 µg/ml respectively. At a concentration peripheral of 0.5 µg/ml, licochalcone A markedly reduced the infection rate of *L. major*

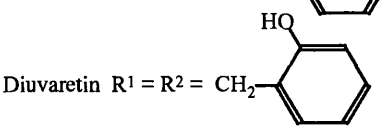
human blood monocyte-derived macrophages or U937 cells. Licochalcone A at this concentration was non toxic to host cells, and intracellular *Leishmania* amastigotes were more susceptible than promastigotes. Studies on the mode of action of licochalcone A indicated that the target organelle appears to be the parasite mitochondria (Chen *et al.*, 1993).

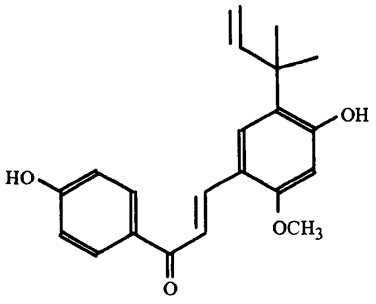


	R1	R2	R3
Artemetin	OMe	OMe	OMe
Casticin	OMe	OH	OMe
Chrysoplenetin	OMe	OMe	OMe
Chrysoplenol D	OMe	OH	OH
Circilineol	H	OMe	OH
Eupatorin	H	OH	OMe

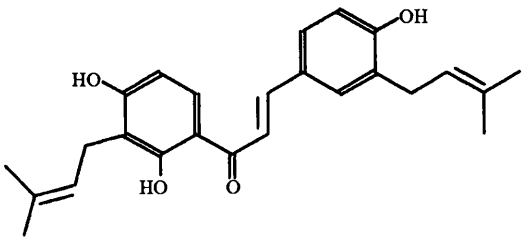




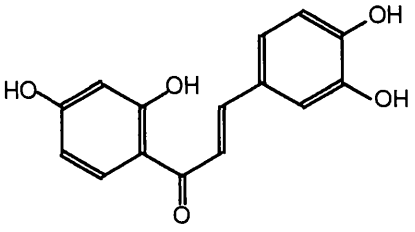




Licochalcone A



(E)-1-[2,4-Dihydroxy-3-(3-methyl-2-butenyl)phenyl]
-3-[4-hydroxy-3-(3-methyl-2-butenyl]phenyl-2-propen-1-



Butein

Figure 1.15 Some flavonoids with antiprotozoal activity

Intraperitoneal administration of licochalcone A (2.5-5 mg/kg/day) completely prevented lesion development in *L. major* infected-mice. Intraperitoneal (20 mg/kg/day), and oral administration of licochalcone A (150 mg/kg/day) resulted in a >96% and 85% reduction of parasite load in the liver and the spleen of *L. donovani* infected-hamsters compared with the values for untreated control animals (Chen *et al.*, 1994a). Furthermore, licochalcone A was active towards *P. falciparum* (Chen *et al.*, 1994b).

An analogue isolated also from liquorice roots: (E)-1-[2,4-dihydroxy-3-(3-methyl-2-butenyl)phenyl]-3-[4-hydroxy-3-(methyl-2-butenyl)phenyl]-phenyl-2-propen-1-one (Figure 1.15) showed potent *in vitro* activity (91% inhibition at 10 μg/ml) towards *L. donovani* after 20-h incubation exposure. In the same conditions Pentostam caused inhibition of 95% at 100 μg/ml and 35% at 10 μg/ml (Christensen *et al.*, 1994).

1.7.4 Terpenes

1.7.4.1 Sesquiterpenes

Several sesquiterpene lactones from a number of medicinal plants have been reported to possess antiprotozoal activity. Parthenin (Figure 1.16) from *Parthenium hysterophorus* is active at (IC_{50}) 1.29 μg/ml towards *P. falciparum* (Phillipson & Wright, 1991). Brevilin (Figure 1.16) from *Centipeda minima* (Asteraceae) is active at (IC_{50}) at 3.26 and 5.57 μg/ml against *P. falciparum* and *G. intestinalis* respectively (Yu *et al.*, 1994; 1995). Dehydrozaluzanin C (Figure 1.16) from *Munnozia maronii* inhibited the growth of 12 *Leishmania* and 15 *T. cruzi* strains with IC_{90} between 2.5 and 50 μg/ml respectively. Dehydrozaluzanin C reduced lesions of *L. amazonensis* in mice, but was less active than N-methylmeglumine antimonate (Fournet *et al.*, 1993). Goyazensolide, lychnopholide, eremantholide C and the caryophyllene derivate, lychnophoic acid (Figure 1.16) from *Lychnophora* sp. (Asteraceae) were active against blood stream forms of *T. cruzi* Y strain; the first two with IC_{100} values of 240 and 3600 μg/ml and the last two with IC_{50} values of 150 and 12 μg/ml respectively. Vernodalin, vernolide, hydroxyvernolide and vernodalol *Vernonia amygdalina* (Figure 1.16) inhibited *P. falciparum in vitro* with IC_{50} values between 4 and 11.4 μg/ml. In addition, vernodalin and vernolide had a MIC of 0.5 and <10 μg/ml respectively against *L. infantum* amastigotes (Ohigashi *et al.*, 1993).

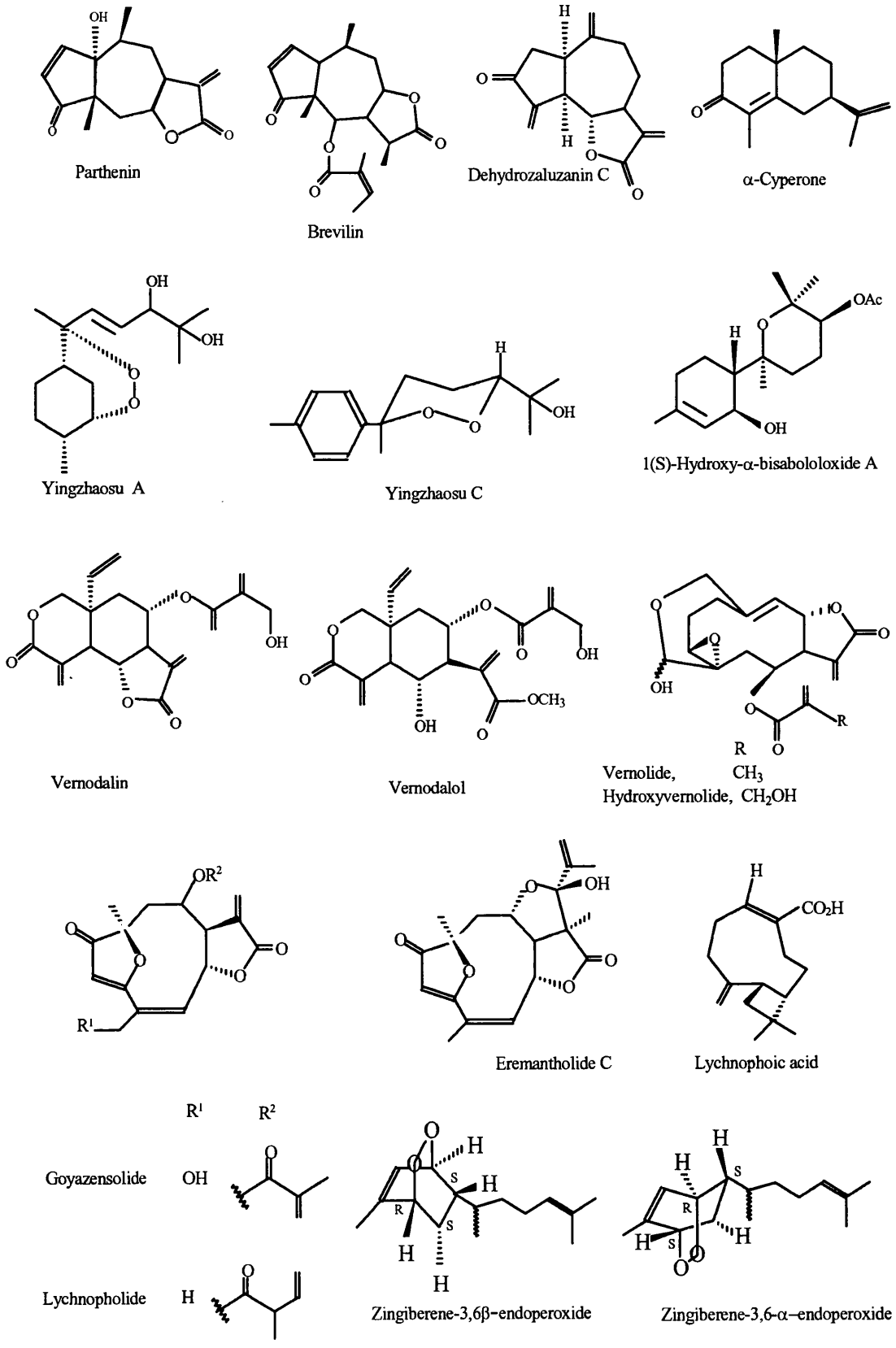


Figure 1.16 Some sesquiterpenes with antiprotozoal activity

Biological activity of parthenin and of other related sesquiterpene lactones containing an exocyclic methylene γ-lactone moiety is ascribed to their high reactivity forming Michael addition adducts with nucleophiles present in enzymes and proteins (Hooper *et al.*, 1990).

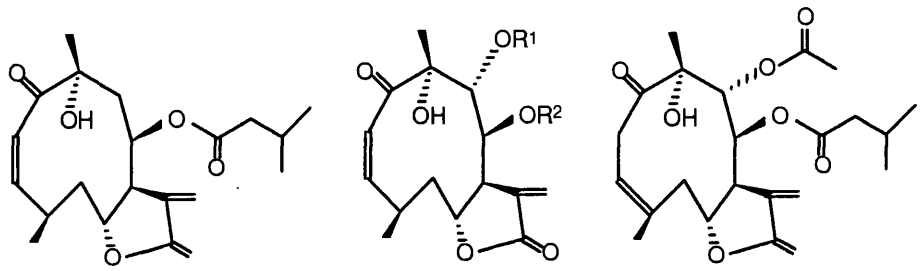
Artabotrys uniciatus (Annonaceae) yielded the sesquiterpene peroxides yingzhousu A and yingzhousu C (Figure 1.16). Both were effective in treating *P. berghei* infected mice their antimalarial activities are comparable to those of mefloquine and quinine, but they are 5-10 times less active than artemisinin (Zhang, 1988; Nkunya, 1992). A series of trioxane analogues have been synthesised and one of them (Ro 42-1611, arteflene) reached clinical trials, but was discontinued because of its high recrudescent rates (Olliario & Trigg, 1995). *Artemisia abrotanum* (Asteraceae) used for the treatment of fever, yielded the sesquiterpene (1S)-hydroxy-α-bisabolol oxide A (Figure 1.16) which is active (IC_{50}) at 5.1 μg/ml towards *P. falciparum* K1 strain (Cubukcu *et al.*, 1990). Two bisabobolane-type sesquiterpene, zingiberene 3,6β-endoperoxide and zingiberene-3,6-α-endoperoxide (Figure 1.16) from the *Senecio selloi* and *Eupatorium rufescens* (Asteraceae) were active against *P. falciparum* at (IC_{50}) 10 μg/ml (Rucker *et al.*, 1996).

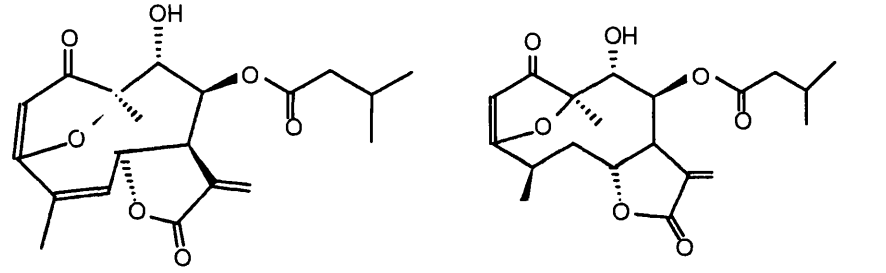
Phytochemical studies of *Neurolaena lobata* (Asteraceae) a plant used in Central America for the treatment of malaria yielded four germacranolide sesquiterpene lactones as well as two furanoheliangolides (Table 1.18). The germacranolide sesquiterpene lactones were highly active, while the furanoheliangolides show only moderate activities. Within the germacranolides, neurolenin C appears to possess the highest activity. The results suggested that one of the structural requirements for high antiplasmodial activity *in vitro* is an α,β-unsaturated keto function. Additionally, a free hydroxy function at C-8 increased the antiplasmodial activity, while a free hydroxyl group at C-9 decreased the activity (Francois *et al.*, 1996a).

α-Cyperone (Figure 1.16) from *Cyperus rotundus* had an IC_{50} value of 5.5 μg/ml against *P. falciparum* (Nkunya, 1992). *C. rotundus* afforded also patchoulenone, caryophyllene α-oxide, 10,12-peroxycalamene and 4,7-dimethyl-1-tetralone (Table 1.19). The antimalarial activities of these compounds are in the range of (EC_{50})10^{-4}-10^{-6} M, with the

endoperoxide sesquiterpene, 10,12-peroxycalamene, exhibiting the strongest effect at (EC_{50})2.33 x 10^{-6} M (Thebtaranonth, *et al.*, 1995).

Table 2.18 *In vitro* antimalarial activities of sesquiterpene lactones from *Neurolaena lobata*

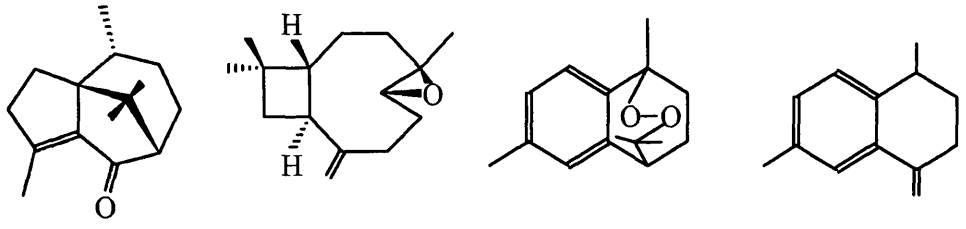




Compound	*P. falciparum* IC_{50} (µg/ml)	GLC_4	COLO 320
Neurolenin A	0.92	1.5	2.2
Neurolenin B	0.62	1.1	1.2
Neurolenin C/D (3:2)	0.62	1.8	2.4
Lobatin A	15.62	8.5	8.7
Lobatin B	16.51	0.6	1.1
9α-HYdroxy-8β-isovalerinyloxy-calyculatolide	19.27	10.1	9.0
Artemisinin	0.14	NT	NT
Quinine	0.19	NT	NT
Cisplatin	NT	1.0	2.7

NT: Not tested. GLC_4: a human small lung cell carcinoma cell line. COLO 320: a human colorectal cancer cell line.

Table 1.19 *In vitro* antimalarial activities of sesquiterpenes from *Cyperus rotundus*



Patchoulenone | Caryophyllene α-oxide | 10,12-Peroxycalamenene | 4,7-Dimethyl-1-tetralon

Compound	*Plasmodium falciparum* EC_{50} (M)
Patchoulenone	1.08×10^{-4}
Caryophyllene α-oxide	3.45×10^{-4}
10,12-Peroxycalamenene	2.33×10^{-6}
4,7-Dimethyl-1-tetralone	8.62×10^{-5}

1.7.4.2 Diterpenes

Two abietanes, 3-0-benzoylhosloppone and 3-O-cinnamoylhosloppone (Figure 1.17) from the antimalarial Tanzanian plant *Hoslundia opposita* (Laniaceae) had antimalarial activity at (IC_{50}) 0.41 and 3.8 μg/ml respectively against the multidrug resistant K1 strain of *P. falciparum* (Achenbach *et al.*, 1992).

Jatrogrossidione (Figure 1.17), the main diterpene of *Jatropha grossidentata* (Euphorbiaceae) has *in vitro* activity against promastigotes of *Leishmania* and epimastigotes of *T. cruzi* with IC_{100} values of 0.75 and 1.5-5.0 μg/ml respectively. The IC_{50} value of jatrogrossidione was < 0.25 μg/ml, against amastigotes of *Leishmania* infecting macrophages, with a cytotoxicity at concentrations higher than 0.5 μg/ml. Jatrophone (Figure 1.17) from *J. isabelli* was significantly active against the strain PH8 of *L. amazonensis* at a dose of 25 mg/kg/day, but at a dose of 112 mg/kg/day, showed toxicity in experimental models whilst jatrogrossidione at the same dose was inactive against *Leishmania* (Schmeda-Hirschmann *et al.*, 1996).

The labdane, (4S,9R,10R) methyl-18-carboxy-labda-8,13(E)-diene-15-oate (Figure 1.17) from *Polyalthia macropoda* (Annonaceae), has been shown to be effective (IC_{50}) at 0.75 mg/ml against *L. donovani* promastigotes (Richomme *et al.*, 1991). The tumour promoting and immunostimulant 12-O-tetradecanoyl phorbol-13-acetate (TPA) has been shown activity against *L. amazonensis* (Yamada, 1992) and *T. cruzi in vitro* (Vannier-Santos *et al.*, 1988).

The diterpenes (-)-en-kaur-16-en-19-oic acid, (-)-trachyloba-19-oic acid, and (-)-kauran-16α-ol (Figure 1.17) from *Viguiera aspillioides* (Asteraceae) and some synthetic derivatives of the acidic compounds were tested for their trypanocidal activity. The parent diterpenes showed IC_{50} values of 500 μg/ml. The lowest IC_{50} value was obtained for the synthetic derivative (-)-kaur-16-en-19-ol (200 μg/ml). By comparison, the more active compound, (-)-kaur-16-en-19-ol was approximately 10 to 20 times less active than gentian violet (Da Costa *et al.*, 1996).

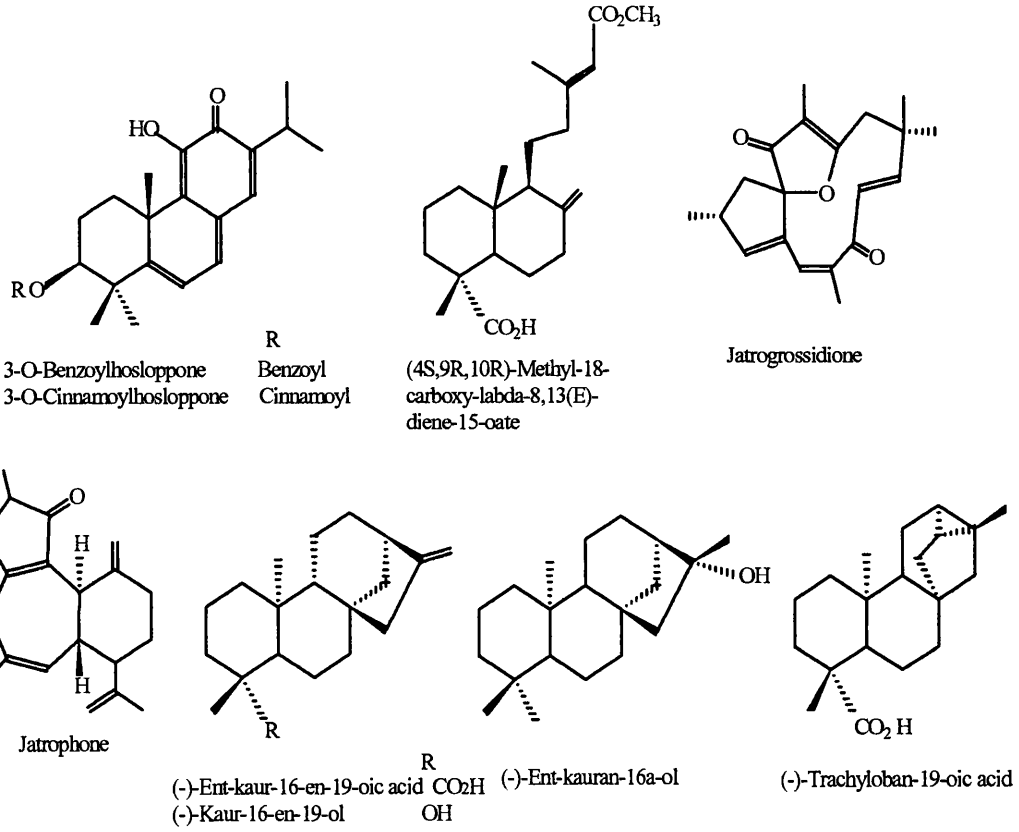
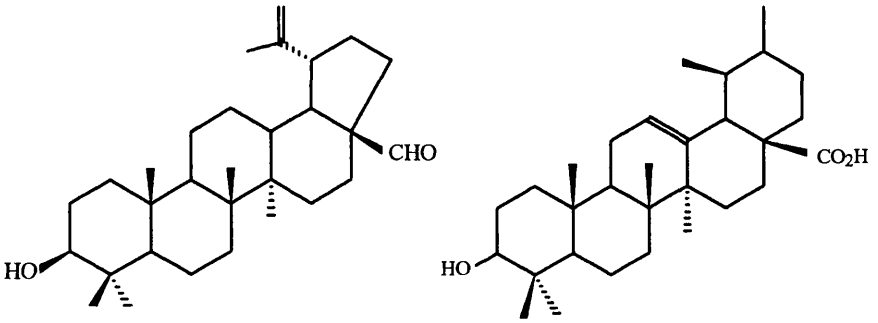


Figure 1.17 Some diterpenes with antiprotozoal activity

1.7.4.3 Triterpenes

Ursolic acid (Figure 1.18) a triterpene from the medicinal plant *Jacaranda copaica* (Bignoniaceae) possesses antileishmanial activity. Ursolic acid had an interesting activity *in vitro* with ED_{50} value of 0.02 mM against amastigotes and was not toxic to macrophages at twice this dose. Intralesional administration of this terpene (0.16 mM/kg/day) reducted development of leismaniasis nodules caused by *L. amazonensis* (Sauvain *et al.*, 1993). The triterpene betulin aldehyde (Figure 1.18) has been isolated as the active principle of *Doliocarpus dentatus* (Aublet) a plant used for the treatment of leishmanial ulcers in South America. Betulin aldehyde has an IC_{50} value of μg/ml against *Leishmania* sp promastigotes . However, toxicity was observed at this dose on macrophages (Sauvain *et al.*, 1996a).



Betulin aldehyde Ursolic acid

Figure 1.18 Some triterpenes with antiprotozoal activity

1.7.4.4 Quassinoids

The quassinoids are a group of heavily oxygenated terpenoid lactones (Figure 1.19) present in species of Simaroubaceae which are related to the Rutaceae and Meliaceae families. Bioassay-guided fractionation of some Simaroubaceae species led to the isolation of quassinoids as the major active principles of this family. A number of quassinoids were tested against *E. histolytica, G. intestinalis, P. falciparum* and *T. gondii* and their activities were compared with their *in vitro* cytotoxic effects on human epidermoid carcinoma KB cells (Anderson *et al.*, 1991; Wright *et al.*, 1993b).

Bruceantin (Figure 1.18) was found the most active of the quassinoids against each of the four species of protozoa with IC_{50} values between 0.0015 and 1.2 μM, however it was found to be highly toxic to KB cells. Studies of structure-activity relationships showed that the most potent quassinoids against *P. falciparum* multidrug-resistant strain K1, are pentacyclic and include a lactone ring and a methylene-oxygen bridge which links C-8 to either C-13 e.g., brusatol (Figure 1.19) or C-11 e.g., ailanthinone (Figure 1.19). Combinations of bruceantin and glaucarubinone potentiated the antimalarial effect (Allen, 1993b). A number of semi-synthetic and synthetic derivates were prepared and their antimalarial and cytotoxic properties were studied (Langa't, 1995). Some quassinoids have been shown to disrupt normal ribosomal function and cause irreversible inhibition of protein synthesis in eukaryotic cells. *In vivo* studies have shown that the ED_{50} values for nine quassinoids tested range from 0.76 to 3.36 mg/kg/day; the comparable value for chloroquine diphosphate is 2.27 mg/kg/day. However, 7 of the 9 quassinoids caused toxic death in dose ranges of 3-18 mg/kg/day; brucein D (Figure 1.19) was found to be the least toxic (Phillipson *et al.*, 1993).

Eurycomanol (Figure 1.19), eurycomanol 2-O-β-D-glucopyranoside, and 13β, 18-dihydroeurycomanol from *Eurycoma longifolia* inhibited the growth of nine chloroquine resistant *P. falciparum* isolates with IC_{50} values in the range of 1.23-4.90 μM, 0.38-3.49μM and 0.50-2.34 μM, compared with 0.32-0.77 μM for chloroquine (Ang *et al.*, 1995). *Simaba cedron* (Meliaceae) an Latin American antimalarial plant yielded cedronin (Figure 1.19) which is effective against KB cells (IC_{50} = 4 μg/ml; 10.4 μM) and *P. falciparum* FCC2 chloroquine-sensistive and FZR8 resistant strains with an equivalent IC_{50} value of 0.25 μg/ml. Cedronin is effective in treating *P. vinkei* infected rodents with an ED_{50} value of 1.8 mg/kg (Moretti *et al.*, 1994). However its toxic/therapeutic ratio (10/1.8) remains lower than chloroquine (10/0.5). A series of 12 quassinoids were assessed against *L. donovani* promastigotes *in vitro*. Seven had IC_{50} values between 0.5 and 1.85μM. 15-β-Heptyl-chaparrinone and simalikalactone D (Figure 1.19) were the most active with IC_{50}s of 0.5 and 0.7 μM. However, both were toxic to macrophages at the inhibitory concentration (Robert-Gero *et al.*, 1985).

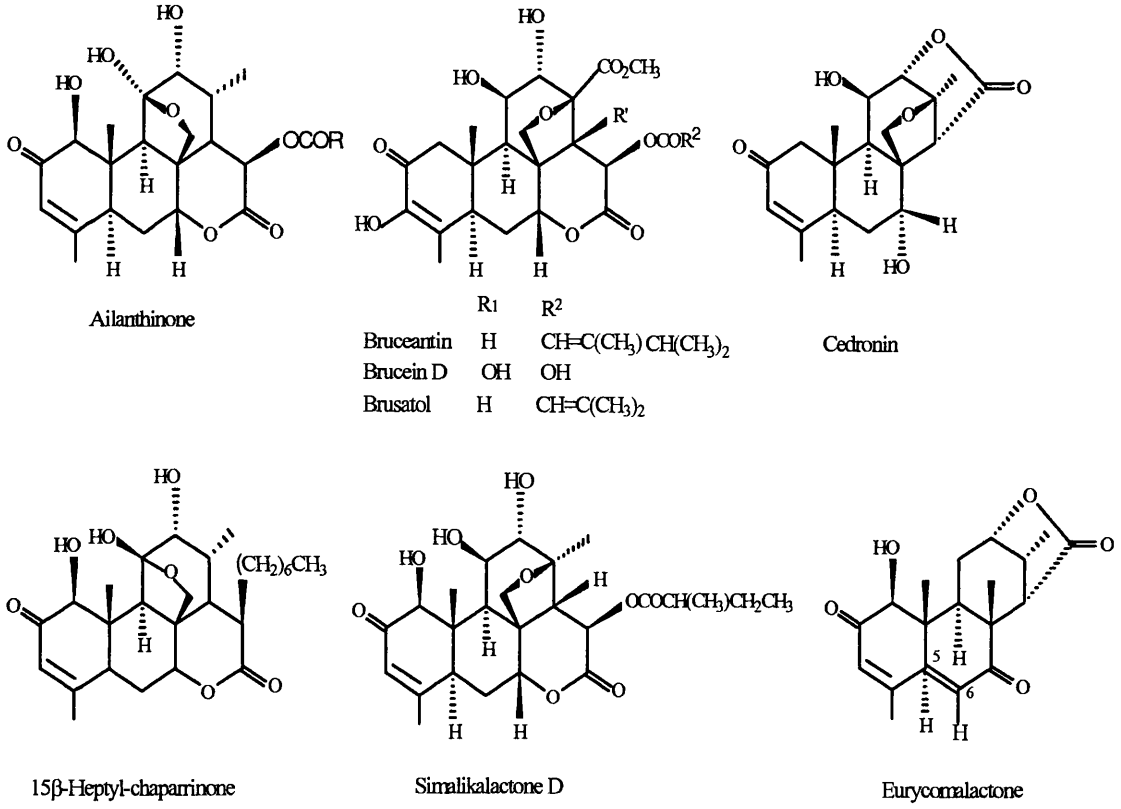
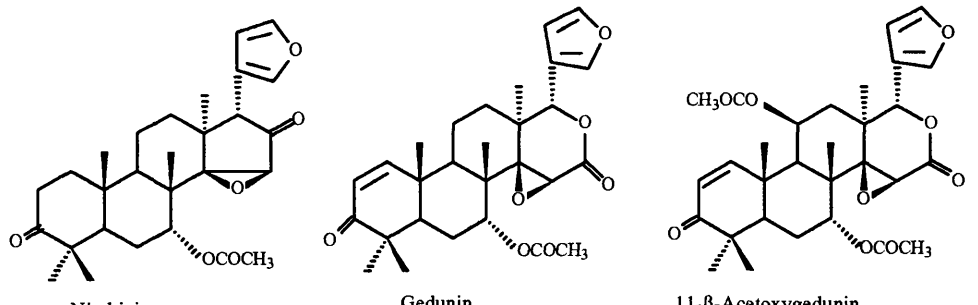


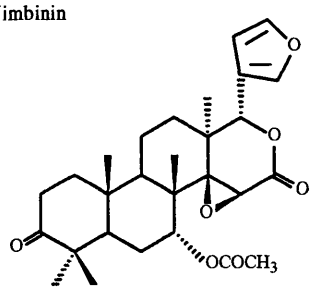
Figure 1.19 Some quassinoids with antiprotozoal activity

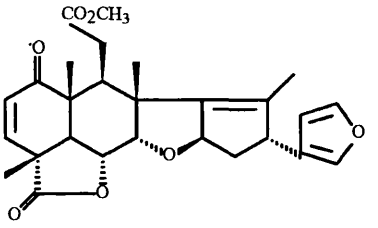
1.7.4.5 Limonoids

Limonoids are a group of oxidized triterpenes related to quassinoids and occur in species of the families Meliaceae, Rutaceae and Cneoraceae . Twenty-five limonoids obtained from several species of Meliaceae used traditionally as febrifuges were evaluated against *P. falciparum* (K1) *in vitro*. Only five compounds (Table 1.20) were found to be moderately active with IC_{50} values between 0.72 and 3.11 μg/ml (Bray *et al.*, 1990). However, the most potent, gedunin was not effective in treating *P. berghei* infected mice. Therefore, it has been suggested that the immunomodulatory activity of Meliaceae plants and some of their constituents might be responsible for some beneficial effects claimed by patients suffering from malaria infection (Phillipson *et al.*, 1993).

Table 1. 20 *In vitro* antimalarial activities of some limonoids




Dihydroxigedunin

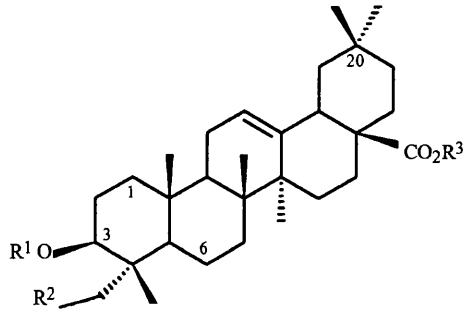

Nimbolide

Compound	*P. falciparum* K1 IC_{50} (μg/ml)
Nimbinin	0.77
Gedunin	0.72
11-β-Acetoxygedunin	3.11
Dihydrogedunin	2.63
Nimbolide	1.74
Quinine 2HCl	0.038
Chloroquine diphosphate	0.210

1.7.4.6 Other terpenes

Some triterpene saponins including the bidesmosides hederasaponin B, C and D, and their corresponding monodesmosides α-, β-, δ-hederin, and hederagenin (Table 1.21) were isolated from *Hedera helix*. *In vitro* studies showed that the bidesmosides are inactive whereas the monodesmosides are active against different strains of *Leishmania* sp. Monodesmosides against promastigote forms of *Leishmania* sp. had the same MIC as pentamidine (5 μg/ml), and against *L. infantum* and *L. tropica* amastigote forms only hederagenin exhibited a significant activity which is equivalent to pentavalent antimony. The use of salts of these compounds lead to improvement in their solubility and their antileishmanial activity (Majester-Savornin *et al.*, 1991).

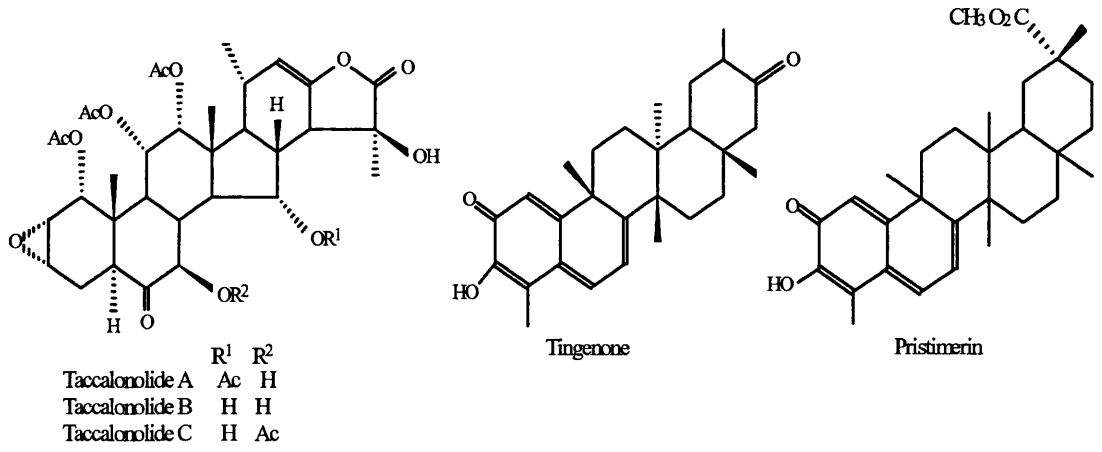
Table 1.21 *In vitro* antileishmanial activity of some saponins isolated from *Hedera helix*



Compound	R^1	R^2	R^3	*Leishmania* sp IC_{50} (μg/ml)
Hederagenin (Na)	H	OH	H	~25
α-Hederin (Na)	rham(1→2)ara(1→	OH	H	~10
β-Hederin (Na)	rham(1→2)ara(1→	H	H	>1[a]
δ-Hederin (Na)	ara(1→	OH	OH	>10[a]
Hederasaponin C	rham(1→2)ara(1→	OH	rham(1→4)glc(1→6)glc(1→	>100[a]
Glucantime[b]				~10

[a] The compound was toxic to infected macrophages and not active to amastigotes at this concentration.
[b] Reference compound glucantime=N-methylglucantime antimonate.

The terpenoids taccalonolides A,B, and C (Figure 1.20) isolated from the Chinese medicinal plant *Tacca plantaginea* (Taccaceae) was effective in treating *P. berguei* infected mice (Chen *et al.*, 1987). Tingenone (Figure 1.20), a quinone triterpene, present in various species of the Celastraceae and Hypocrataceae was highly active against *T. cruzi* epimastigotes *in vitro*, and its mode of action involves the inhibition of DNA synthesis (Goijman *et al.*, 1985). A quinoid triterpene, pristimerin (Figure 1.20) the active principle of the antimalarial plant *Celastrus paniculatus* Will (Celastraceae) has shown activity against *P. falciparum* K-3 with IC_{50} value of 256 ng/ml. However, it was found to be less potent than the standard antimalarial drugs quinine, chloroquine and mefloquine (Pavanand *et al.*, 1989a).



	R^1	R^2
Taccalonolide A	Ac	H
Taccalonolide B	H	H
Taccalonolide C	H	Ac

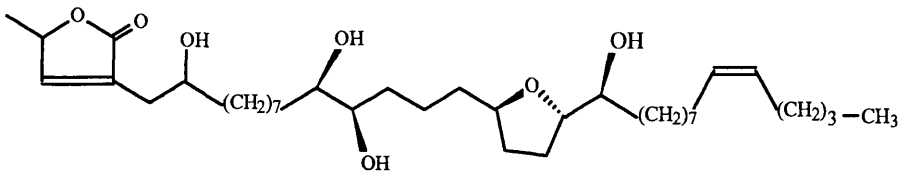
Tingenone

Pristimerin

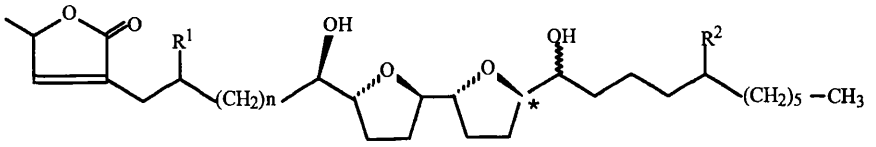
Figure 1.20 Other terpenes with antiprotozoal activity

Five acetogenins (Table 1.22) were isolated from *Annona senegalensis* (Annonaceae) by biologically guided fractionation using *Artemia salina* toxicity test. The acetogenins were evaluated against Kinetoplastidae parasites and against KB and VERO mammal cell lines. Senegalene was the most active (IC_{50}) at 50 µg/ml against *T. brucei* after 1 h incubation period of time. It was also active (IC_{50}) against *L. major* and *L. donovani* at the concentrations of 50µg/ml and 25 µg/ml respectively. Pentamidine, was twice as efficient as senegalene, squamocine and molvizarine. On the other hand the compounds were not as active as compared to melarsoprol. However, the evaluated acetogenins were more cytotoxic than the antitumor reference compound, vinblastine. The protozoa used for the testing were far less sensitive to the isolated acetogenins than the tested cell lines. However, senegalene, squamocine and molvizarine have the potential to be effective antileishmanial chemotherapeutic agents in developing countries (Sahpaz *et al.*, 1994).

Table 1.22 *In vitro* biological activities of acetogenins from *Annona senegalis*



Senegalene



Compound	R^1	R^2	n	*	MEC (µg/ml) *L.m*	*L.d*	*T.b.b*	KB	VERO
Senegalene					50	25	10	$5x10^{-4}$	$2x10^{-1}$
Squamocine	H	OH	10	erytro	50	25	10	10^{-5}	10^{-2}
Asimicine	OH	H	10	threo	100	100	10	$2x10^{-4}$	$7x10^{-2}$
Rolliniastatine-2	OH	H	10	erytro	200	>200	50	$3x10^{-5}$	$3x10^{-2}$
Molvizarine	OH	H	8	erytro	25	50	10	10^{-5}	10^{-2}
Pentamidine di-isetionate					25	25	0.2	NT	NT
Melarsoprol					NT	NT	0.05	NT	NT
Vinblastine					NT	NT	NT	10^{-3}	>3

[a] Determined after 24 h. NT, not tested. *L.m: L. major. L.d: L.donovani. T.b.b. :T.brucei brucei*

1.7.5 Other compounds

Two prenylated diphenol terpenoids grifolin (Figure 1.21) and piperogalin (Figure 1.21) from *Peperomia galioides* (Piperaceae) have antiprotozoal activity. Grifolin has been shown 60% inhibition against *T. cruzi* at 250µg/ml whereas piperogalin has been exhibited 90% of inhibition against three species of *Leishmania* at 10 µg/ml (Mahiou *et al.*, 1995).

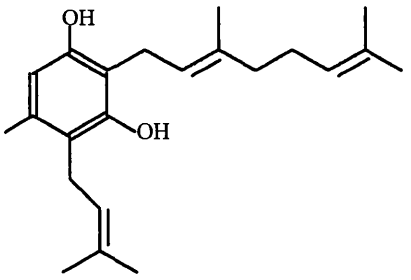
The aromatic monoterpene espintanol (Figure 1.21) from *Oxandra espintana* (Annonaceae) has been found active towards 12 strains of *Leishmania* promastigotes with IC_{90} values in the range of 10-15 µg/ml; in the same conditions the standard drug pentamidine had an IC_{50} value of 1.5 µg/ml. Espintanol has also been found effective against 20 strains of *T. cruzi* epimastigotes with IC_{90} values in the the range 25 to <100 µg/ml, and the standard drug benznidazole had an equivalent range of values

(Hocquemiller *et al.*, 1991). The polyphenolic triterpene gossypol found in cotton seed oil and known for its spermatogenic properties, has been shown to be effective towards *T. cruzi, P. falciparum* and *T. b. brucei* (Gonzalez-Garza & Said-Fernanadez, 1988). Gossypol has been found to possess immunomodulatory properties at very low concentrations. The aromatic compound, 2-benzoxazolinone from *Acanthus illicifolius* showed *in vitro* antileishmanial activity with an IC_{50} value of 37.3-42.7 μg/ml.

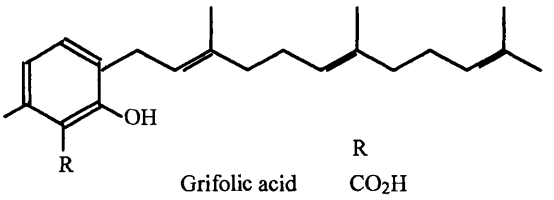
Two lignans, (+) medioresinol (Figure 1.21)and lirioresinol (Figure 1.21) from the antileishmanial South American plant *Doliocarpus dentatus* have shown antileishmanial activity with a survival index of amastigotes of around 40% at 60 μg/ml. At higher concentrations (125 μg/ml) the lignans were toxic to macrophages (Sauvain *et al.*, 1996a). It has been reported that lignans inhibit cyclic AMP phosphodiesterase and they may act in similar manner on *Leishmania* parasites.

Allicin is a diallyl trisulfide and is one of the major components of *Allium sativum*. Allicin has antimicrobial properties and possesses antiprotozoal activity against *T. brucei* sp., *E. histolytica* and *G. intestinalis in vitro* having IC_{50} values of 0.8-5.5, 59 and 14 μg/ml respectively (Lun *et al.*, 1994).

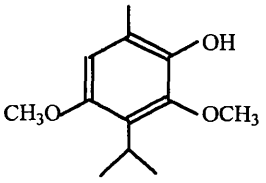
It has been shown that the flavonoid quinones (3R)-claussequinone, (R)-4-methoxydalbergione, and (S)-4,4'-dimethoxydalbergione, (Figure 1.21) lyse *T. cruzi* trypomastigotes in *vitro* at concentrations around 100 μM (~ 25 μg/ml). The related non-quinonic butein, (3S)-vestitol only exhibited effectivenes toward some strains tested, at only at 2 mM (see Sepulveda-Boza & Cassels, 1996).



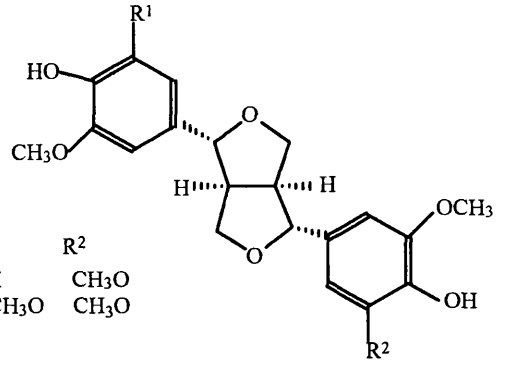
Piperogalin



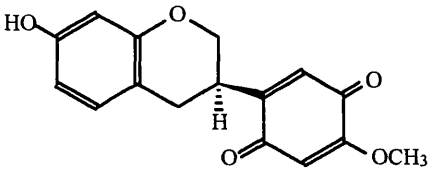
	R
Grifolic acid	CO_2H
Grifolin	H



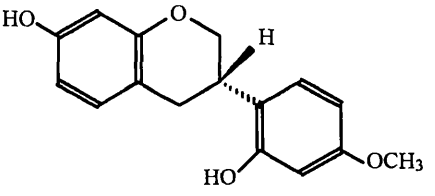
Espintanol



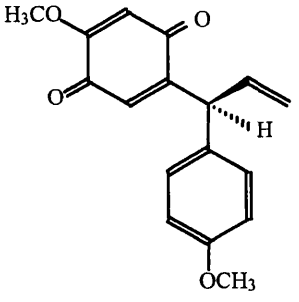
	R^1	R^2
(+) Mediioresinol	H	CH_3O
(-) Lirioresinol B	CH_3O	CH_3O



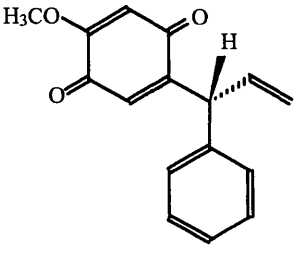
(3R)-Claussequinone



(3S)-vestitol



(S)-4,4'-Dimethoxydalbergione



(R)-4-Methoxydalbergione

Figure 1.21 Other compounds with antiprotozoal activity

1.8 Summary

Chemotherapy of parasitic diseases is complex and difficult to achieve. Therapies of malaria, leishmaniasis and trypanosomiasis have several problems of resistance, variable efficacy between strains or species of the causative organisms, toxicity, difficulty of administration, require long courses of administration or combination of these factors. In addition, opportunistic protozoan in immuno-compromised patients are difficult to treat. There is no ideal drug for the treatment of these serious protozoal diseases which mainly affect developing countries. There is a need of new antiprotozoal drugs.

Many taxonomic families have been shown to contain compounds with significant activity against various protozoa. The active constituents of some of these plants have been isolated, and they have a broad spectrum of chemical structures and may act by a variety of mechanisms, although in most cases this has not been clearly demonstrated for the majority of them. For some active compounds their activity has been confirmed by *in vivo* studies and/or clinical evaluation, and for others, only *in vitro* activity of extracts or compounds responsible for the biological activity has been reported and confirmatory *in vivo* evaluation are under way.

The activity of many of these compounds may be due to direct antiprotozoal action and this has been demonstrated for a number of isolated natural products. However, plant extracts contain complex mixtures of chemicals and other actions such as anti-inflammatory, antipyretic or immunostimulatory actions which may contribute to the overall curative effect. For example *Azadirachta indica* and *Melia azadirach* are used as febrifuges and contain limonoids, which were moderately active against malaria parasites. The plants also possess polysaccharides which have anti-inflammatory and anti-tumor activity (Fujiwara *et al.*, 1984) as well as peptidoglycans and phenolic compounds which activate the phagocytic function of polymorphonuclear leucocytes (Van der Nat *et al.*, 1991).

A crude extract prepared from five plants (*Boerhavia diffusa*, *Tinospora cordifolia*, *Berberis aristata*, *Terminalia chebula* and *Zingiber officinale*) reduced hepatic

amoebiasis in animals models and the same extract enhanced the humoral immunity in experimental models (Sohni & Bhatt, 1996). *Artemisia annua* displayed immunosuppressive effects in vitro on human complement, proliferation of T-cells and chemiluminescence by zymosan-stimulated PMN (Kroes *et al.*, 1995). On the other hand, glycyrrhizin a terpenoid saponin from liquorice roots increases lymphocyte transformation and possessed anti-inflammatory properties.

In addition, ursolic acid from *Jacaranda copaica* may not exert its activity solely via direct action against *Leishmania* parasites, and the beneficial therapeutic effects claimed by patients might be due to the antiinflammatory and immunomodulating activities described for this compound (Sauvain *et al.*, 1993). Glycyrrhizin inhibited the generation of free radicals by neutrophils (Akamatsu *et al.*, 1991). Other antiprotozoal compounds with immunomodulatory properties include: berbamine and tetrandrine (Schiff, 1991), cepharanthine and emetine (Wagner & Proksch 1985), artemisinin (Zhang *et al.*, 1995), plumbagin and lapachol (Wagner, 1990). Furthermore, it is likely that the immunomodulatory activity of some natural compounds present in the plant may influence the overall therapeutic effects of such medicinal plants.

There is a need to develop new antiprotozoal drugs with better activity and low toxicity. Higher plants represent an extraordinary source of antiprotozoal drugs with novel structures and mode of action.

1.9 Aims of this study

To isolate and identify active and other compounds present in the plants selected either because of their traditional use of because of chemotaxonomic considerations.

To determine the antiprotozoal activities *in vitro* against *Leishmania donovani*, *Trypanosoma cruzi*, *T. brucei brucei* and *Plasmodium falciparum* of crude extracts, isolated compounds, and some chemical derivatives.

To assess the toxicity against brine shrimp in a lethality test and cytotoxicity against KB and P388D1 cells *in vitro* of isolated compounds, derivatives and crude extracts.

To determine the specificity of action of the tested compounds and extracts by comparison between the cytotoxic and antiprotozoal activities

To compare the structure activity relationship for the tested compounds.

To investigate the antiprotozoal activity *in vivo* of selected compounds against *L. donovani* and *T. b. brucei*.

To determine the effects on lymphocyte proliferation and production of superoxide of selected pure compounds and crude extracts.

CHAPTER 2

PHYTOCHEMISTRY

2.1 Introduction

The aim of the phytochemical work described in this chapter, in conjunction with the biological screening outlined in Chapter 3 was to prepare extracts of higher plants, follow up those which were active and then isolate active constituents, as well as other compounds present in the same plants in order to stablish structure-activity relationships among them. Results from *in vitro* antiprotozoal and cytotoxic screening of crude extracts of plant material were used to direct subsequent phytochemical work and enable the eventual isolation of a number of pure compounds with antiprotozoal activity. Some compounds were extracted in sufficient amounts so that they could be assessed for *in-vivo* antileishmanial and trypanocidal activities. In addition, selected antiprotozoal medicinal plants and some of their constituents were evaluated for *in vitro* immunomodulatory activity (Chapter 4) in order to determine the interrelation between antiprotozoal and immunomodulatory effects of samples tested.

2.2 Material and General methods

2.2.1 Chromatographic techniques

Thin Layer Chromatography (TLC).- TLC analyses were performed using Merck aluminium backed precoated thin layer Kiesel gel 60 F-254 plates (0.25 mm thick).

Preparative Thin Layer Chromatography (PTLC).- PTLC separations were carried out on glass plates (20 cm x 20 cm) spread with 0.5 mm layer of silica gel GF254 (Merck) using a Camag silica gel spreader. Plates were activated at 120 ^{0}C 2 h. Once cool, the plates were washed with acetone and allowed to dry before use.

Column chromatography (CC).- CC was carried out on silica gel 60, 70-230 mesh (Merck), neutra aluminium or sephadex HL-20 (Pharmacia) in a gravity column using gradient of different solvents. In some instance column chromatograms were performed

by flash techniques using silica gel Sorbil C 60-H (40-60 μm) Rhone-Poulenc.

High Performance Liquid Chromatography (HPLC).- Semi-Preparative HPLCwas performed on Apex Prepsil column (10 mm x 25 cm, 8μm particle size, Jones Chromatography, UK). Isocratic elution was carried out with the mobile phase $CHCl_3$-MeOH (95:5) at a flow rate of 1 ml/min. All the solvents were HPLC grade andwere degassed by sonication prior to use. Absorbances between 230 and 400 nm were detected and data were processed using PDA software.

2.2.2 Spray reagents

Dragendorff reagent (Munier's modification)

Solution A: 0.8 g bismuth subnitrate +10 ml glacial acetic acid, made to 50 ml volume with H_2O. 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 H_2O.

H_2SO_4 40% in ethanol.- 40 ml of H_2SO_4 conc., made to 100 ml with EtOH.

0.1% $CeSO_4$-2N H_2SO_4.- 2 g $CeSO_4$ + 22 ml H_2SO_4 conc. + 350 g of H_2O.

1% Vanillin - 5% H_2SO_4 .- 1 g Vanillin + 5 ml H_2SO_4 conc., made to 100 ml with EtOH.

Anisaldehyde.- 0.5 ml anisaldehyde + 9 ml EtOH + 0.5 ml H_2SO_4 conc., + 1 ml AcOH.

5% KOH in EtOH.- 5 g KOH, made to 100 ml with EtOH.

1% Iodoplatinate - 5% H_2SO_4 .- 1g iodoplatinate + 5 ml H_2SO_4 conc., made to 100 ml with EtOH.

The TLC plates were initially observed under UV, then sprayed with chromogenic reagent and heated at 100 °C for several minutes, under observation. Plates sprayed with Dragendorff were visualised immediately.

2.2.3 Determination of Spectroscopic and Physical data

Ultraviolet Spectroscopy UV.- UV spectra were obtained on a Perkin-Elmer 402 Ultraviolet-Visible Spectrophotometer using spectroscopic grade methanol.

Infrared Spectroscopy IR.- IR spectra were recorded in KBr, preparing a thin film on NaCl cells or in solution with spectroscopic grade chloroform, using a Perkin Elmer model 841 infrared spectrophotometer.

Nuclear Magnetic Resonance NMR.- ^{1}H NMR spectra were recorded at 400 MHz and the ^{13}C NMR and DEPT at 100 MHz on a Bruker AMX-400 in the indicated solvent with TMS as internal standard. Two dimensional experiments: COSY-45, HMQC, HMBC, COLOC and NOESY were also recorded on an AMX-400 nuclear magnetic resonance spectrometer. NMR-Spectra for isolated compounds labelled S-1 - S-115 are given in the Appendix.

Mass spectrometry MS.- Electron impact (EI) mas spectra and accurate mass determination were recorded on a VG-Analytical instrument ZAB IF mass spectrometer at 70 eV with an inlet temperature of 180-200 ^{0}C unless otherwise stated. Chemical ionization (CI) mass spectra were recorded on a VG Mass lab 12-250 quadrupole instrument, using ammonia as ionising gas unless otherwise stated. Fast atom bombardment (FAB) mass spectra were recorded on a VG analytical ZAB SE spectrometer with xenon as the atom source at 8 eV. Samples were dissolved in a 2-nitrobenzyl alcohol plus sodium iodide matrix (MNOBA + NaI) unless otherwise stated. Laser mass spectra was recorded on tof laser VG TOF spectrometer. Gas chromatography-Mass spectra (GC-MS).- Samples were dissolved in $CHCl_3$, and injected into the column GC1 (Polly diethyl silane) in the following conditions: Length 25 m; Internal diameter 0.25 mm, Injector temp. 250 °C; Interface temp. 310 °C; Carrier gas Helium; Flow rate 1 cm^3 min^{-1}; Head pressure 10 lbs $sqin^{-1}$, Initial temp. 125 °C; 2nd temp. 175 °C; 3rd temp.220 °C; Final temperature 300 °C.

Optical activity ([α]).- The [α] of some compounds was recorded on a Bellingham & Stanley model P 506 polarimeter in $CHCl_3$, Temp. 26.6 °C, λ = 589 nm, and 10 cm cell.

Melting points.- The melting points were determined in open capillaries in a Stuart scientific melting point model SMP1, and are uncorrected.

2.2.6 Preparation of semisynthetic derivates

Acetyl derivates.- To each sample (10 mg) was added 1 ml of Ac_2O-pyridine (1:1) in a small flask. After standing at room temperature for 48 h, the reaction was stopped by adding MeOH at 0 °C; pyridine was removed by evaporation under reduced pressure as an azeotrope formed by adding benzene. The residue was purified by Sephadex HL-20 or silica gel column chromatographic eluted with the solvent indicated to yield the acetyl derivated compound.

Methyl derivates.- Ethereal CH_2N_2 (6 ml) was added, at 0 °C, to the sample in ether (50 mg in 6 ml). The mixture was allowed to stand at room temperature overnight and was then evaporated under a N_2 stream. Purification of the residue by Sephadex HL-20 or silica gel, eluted with the appropriate solvent produced the pure methyl ester derivated.

Oxidation.- The compound (20 mg) dissolved in pyridine (200 µl) was treated with a solution of CrO_3 (60 mg) in pyridine (600 µl) at 10 °C, and stirred at room temperature for 4 h. The reaction mixture was diluted with water and extracted with $CHCl_3$. The chloroformic layer was washed with HCl (0.1 N) and dried over Mg_2SO_4, the organic supernant was dried *in vacuo* and the product purified by CC over silica gel with the appropiate solvent.

Hydrolysis.- The compound was heated with 2 N HCl under reflux for 1-3 hr. After cooling at room temperature the reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The aglycone was detected in the $CHCl_3$ layer by TLC. The aqueous layer was neutralized with $NaHCO_3$, concentrated, then subjected to TLC analysis in direct comparison with sugar standards, using as developing solvent BuOH-HOAc-H_2O (4:1:5),

and detection reagent anisaldehyde. The $CHCl_3$ extracts were dried over Na_2SO_4 and dried *in vacuo* for further identification of the aglycone.

Molisch test.- Sample (1 mg) in H_2O or EtOH (3ml) was added 1 ml of α-naphthol (10% in EtOH), followed by the slow addition of two drops of H_2SO_4 concentrated, down the wall of the test tube. A violet color in the interface was indicative of the presence of carbohydrates.

2.2.5 Plant material

Forty-seven plant species belonging to 27 families (Table 2.1) were selected for screening either because of their traditional use or because of chemotaxonomic considerations. Plant material was obtained at several points, during the research work and were supplied and authenticated as indicated.

Eight plants species were supplied by Dr. Pablo N. Solis and authenticated by Prof. Mireya Correa. Voucher specimens have been deposited at the Herbarium of CIFLORPAN, Centro de Investigaciones Farmacognosticas, Facultad de Farmacia, Universidad de Panama, Panama. *Celaenodendron mexicanum*, leaves were collected on the route to Barra de Navidad Puerto Vallarta (Km 15) in Chamela, Jalisco, Mexico in January, 1990, by Ms. N. Martijena; eleven plant species were purchased in the Mercado de Sonora, Mexico City, in January, 1994. Voucher specimens have been deposited at the National Herbarium of Mexico (MEXU), Instituto de Biologia, Universidad Nacional Autonoma de Mexico, Mexico. Twelve plant extracts were provided by Dr. Sarah J. Marshall, and the the plant material was authenticated by Dr. Shanina A. Ghazanfar, Sultan Qaboos University, Sultanate of Oman. Voucher specimens have been deposited at the Department of Biology Herbarium, Sultan Qaboos University, Sultanate of Oman. *Triclisia patens* and *Stephania dinklagei* stems were supplied and authenticated by Mr. A. A. Enti in Ghana. A voucher specimen was deposited at the Herbarium, Department of Botany, University of Ghana, Legon. Triclisia *subcordata* Oliv. leaves were supplied and authenticated by Dr. Z.O. Gbile. A voucher specimen was deposited at the Forestry Research Institute of Nigeria, Ibadan,

Table 2.1 Plant species selected for biological screening

Plant	COUNTRY	USE	VOUCHER SPECIMEN
ALBERTICIAE			
Epinetrum ferruginum	Nigeria	fever	-
ANARCADIACEAE			
Rhus aucheri	Oman	malaria, fever	SQUH 3563
ANNONACEAE			
Annona purpurea	Mexico	fever	MC-94-1
APOCYNACEAE			
Alstonia macrophylla	Nigeria	malaria, fever	-
Rhazya stricta	Oman	malaria, fever	SQUH 3556
ASCLEPIADACEAE			
Calotropis procera	Oman	malaria, fever	SQUH 3552
ASTERACEAE			
Ratibida latipaliaris	Mexico	infections	MC-94-2
Ratibida mexicana	Mexico	dysentery, infections	MC-94-3
Sonchus oleraceus	Oman	malaria, fever	SQUH 3565
ASTERACEAE			
Vernonia cinerea	Oman	malaria, fever	SQUH 3562
BORAGINACEAE			
Arnebia hispidissima	Oman	malaria, fever	SQUH 3553
BRASSICACEAE			
Sysymbium irio	Oman	malaria, fever	SQUH 3559
CAPPARACEAE			
Capparis spinosa	Oman	malaria, fever	SQUH 3566
Maerua crassifolia	Oman	malaria, fever	SQUH 3555
CHENOPODIACEAE			
Teloxys graveolens	Mexico	helmints, infections	MC-94-4
COMPOSITAE			
Scoloymus hispanicus	Egypt	infections	UE993
EUPHORBIACEAE			
Celaenodendron mexicanum	Mexico	skin infections	Martijena 90-II
LEGUMINOSAE			
Parkia biglobosa	Ghana	fish poison	-
LECYTHIDACEAE			
Napoleona heudelotii	Nigeria	fever	-
LORANTHACEAE			
Struthanthus orbicularis	Nigeria	fever	-
MALPHIGEACEAE			
Galphimia glauca	Mexico	malaria, fever, dysentery, CNS	MC-94-5
MELIACEAE			
Guarea grandifolia	Panama	not used	CIFLORPAN 645 (PMA)
Guarea macropetala	Panama	not used	CIFLORPAN 572 (PMA)
Guarea rhopalocarpa	Panama	not used	CIFLORPAN 643 (PMA)
Malacea depresa	Mexico	malaria, fever	MC-94-6
Ruagea glabra	Panama	not used	CIFLORPAN 691 (PMA)
Swietenia hummillis	Mexico	dysentery, fever	MC-94-7
Swietenia macrophylla	Mexico	fever	MC-94-8
MENISPERMACEAE			
Stephania dinklagei	Ghana	vermiguge, aphrodisiac, fish poison	FE 2482
Triclisia patens	Ghana	malaria, fever	FH1 103433
Triclisia subcordata	Nigeria	malaria, fever	
ORCHIDACEAE			
Stenorrhynchos lanceolatus	Mexico	infections	MC-94-9
RUBIACEA			
Cephaelis camponutans	Panama	not used	CIFLORPAN 1220 (PMA)
Cephaelis dichroa	Panama	not used	CIFLORPAN 755 (PMA)
Cephaelis glomerulata	Panama	not used	CIFLORPAN 599 (PMA)
Cigarrilla mexicana	Mexico	dysentery, emetic	MC-94-10
Craterispermum laurinum	Nigeria	fever	MC-94-11
Hintonia latiflora	Mexico	malaria, infections, hypoglucemic	MC-94-12
RUTACEAE			
Stauranthus perforatum	Nigeria	fever	-
SIMAROUBACEAE			
Ailanthus altissima	London	malaria, fever	-
Brucea javanica	Thailand	dysentery, malaria	-
Picramnia antidesma	Panama	malaria	CIFLORPAN 331 (PMA)
SOLANACEAE			
Solanum nigrum	Oman	fever	SQUH 3564
Withania somnifera	Oman	malaria, fever	SQUH 3554
ZYGOPHYLLACEAE			
Fagonia indica	Oman	malaria, fever	SQUH 3557

Nigeria. *Brucea javanica* fruits were provided by Professor P. Tantivatana, Institue of Health Research, Chulalongkorn, University, Bangkok, Thailand and was authenticated by Mr. L. L. Forman of the Herbarium, Kew Garden London. *Ailanthus altissima* (Mill) Swingle was obtained as a gift from Kew Gardens U.K., after the trees blown by the storm in 1987. *Scoloymus hispanicus* was proportionated by Dr. Maha Aboul-Ela, Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria. A voucher specimen has been deposited at the Herbarium of the University of Alexandria, Alexandria, Egypt. Seven plant species were supplied by Professor J.D. Phillipson and voucher specimens has been deposited in the Department of Pharmacognosy, School of Pharmacy, University of London.

2.2.6 Preparation of plant extracts

In order to screen the plant material for biological activity preliminary methanolic and aqueous extracts were prepared according to the procedure described in Figure 2.1.

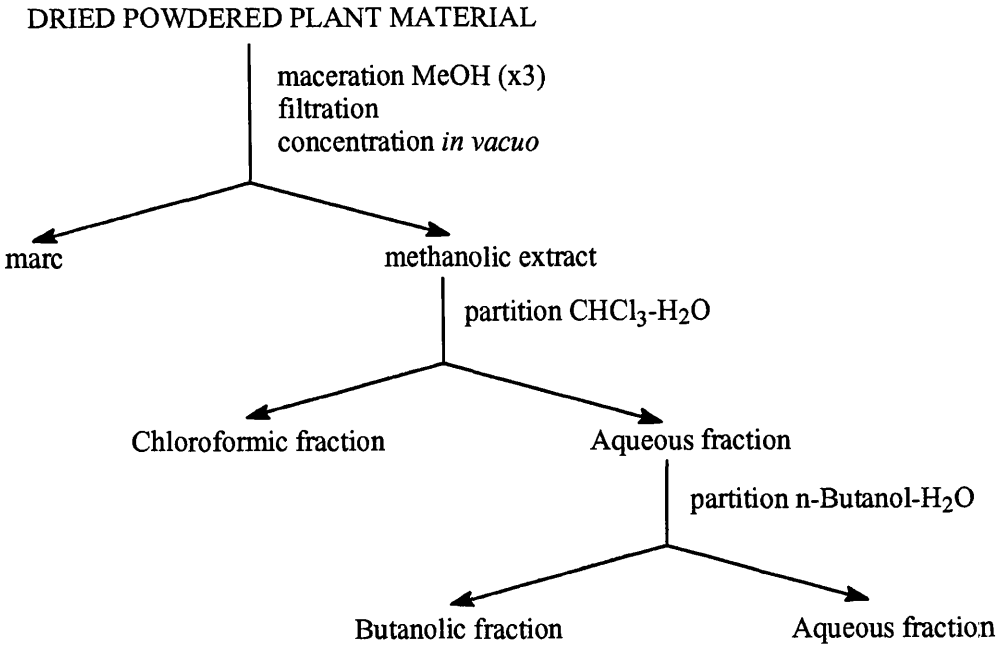


Figure 2.1 Extraction and fractionation of plant material

The air dried plant material (10 g) was powdered through a 2 mm Corning Mill. Ground, air-dried plant material was extracted with MeOH by maceration at room temperature. The supernatant was filtered and evaporated, *in vacuo* to obtain the methanolic extract, and the plant residue was air-dried and further extracted with distilled H_2O, using the same conditions described above. The supernatant was filtered, frozen and lyophilized in a freeze drier to obtain the aqueous extract. The crude extracts were tested for biological activity; the methodology used for the *in vitro* assays and the results of these screenings are discussed in full in Chapter 3. Once preliminary screening had indicated which plants were worth pursuing, larger scale extraction and further partition between $CHCl_3$, n-BuOH and H_2O (Figure 2.1) of the methanolic extract was carried out to isolate some active constituents. In addition, other compounds were also isolated during the chromatographic procedures. In general, the identification of the new natural products was carried out by spectroscopic methods, and the known compounds were identified by comparison of spectroscopic data with literature.

2.3 Plants selected for this study

From the results of *in vitro* screening, seven plants were selected for further investigation either because of their activity or because they were available in good quantity in our laboratory. The plants selected for this study were: *Celaenodendron mexicanum*, *Galphimia glauca*, *Guarea rhopalocarpa, Stephania dinklagei*, *Triclisia patens*, *Hintonia latiflora*, and *Cephaelis camponutans*. Each plant will be discussed separately and a summary will be given to the end of the chapter.

2.3.1 *Celaenodendron mexicanum* Standl (Euphorbiaceae)

Celaenodendron mexicanum is the only species of this monotypic genus which is endemic to Mexico, and it grows in the tropical Caducifolia forest on the pacific Coast. The tree is known commonly as "palo prieto" and its reported to be used as an antiseptic medicinal plant. Investigations on the leaves of *C. mexicanum* have reported the presence of friedelin, maytensifiolin B, ginkgetin, bilobetin, and amentoflavone and the herbicidal effects of the above compounds have been determined (Castaneda *et al.*, 1992). Further studies on the stem bark reported the isolation of celaenodendrolide, ellagic acid-4-O-β-

D-xylopyranoside-3,3'-dimethylether, friedelin-3β-ol, dihydroisohyenanchin, and (-)-(2S, 4R)-1-methyl-4-hydrooxypyrrolidine-2-carboxylic acid (Castaneda *et al.*, 1993). Because Euphorbiaceae species are source of biologically active compounds, and have potential for the development of novel antiprotozoal drugs, we decided to investigate this species further by bioactive-guided fractionation using *Artemia salina* lethality test. This test has proved to be useful tool for searching bioactive compounds (Mayer, 1982).

2.3.1.1 Extraction and Isolation

Air-dried ground leaves of *C. mexicanum* (70 g) were extracted and fractionated according to the procedure described in Figure 2.1. The extracts and fractions were monitored for *in vitro* toxicity against *A. salina* following the procedure described in Chapter 3. The active chloroformic fraction (IC_{50} = 71.35 μg/ml) was subjected to column chromatography on silica gel using a gradient of hexane/$CHCl_3$ and $CHCl_3$-MeOH. A total of 144 fractions of 100 ml each were collected and monitored on TLC. The eluates were combined on the basis of similar TLC profiles and tested for biological activity. Further rechromatography of the active fraction (F 15-18, IC_{50} = 30 μg/ml) on silica gel CC eluted with a gradient of hexane-acetone to yield two major bioactive triterpenes: 3-oxo-tirucalla-7,24Z-dien-26-oic acid (7.8 mg, Hex-acetone 96:4), and 3α-hydroxy-tirucalla-7,24Z-dien-26-oic acid (19 mg, Hex-acetone 95:5). Other more polar active fraction (F, 19) was purified on Sephadex LH-20 column chromatography in MeOH, followed by prep. HPLC (Silica 8u) eluted with $CHCl_3$-MeOH (95:5) at a flow rate of 1 ml/ml, to afford the active epi-oleanolic acid (20 mg), and additional amounts of 3α-hydroxy-tirucalla-7,24Z-dien-26-oic acid (5.6 mg).

In addition, from the original column chromatography of the chloroformic extract four terpenes crystallyzed spontaneously from different non-active fractions: friedelin (3.3 mg, Hex-$CHCl_3$ 3:7); maytensifolin B (67 mg, Hex-$CHCl_3$ 5:5), 3β-hydroxyfriedelan-16-one (4 mg, Hex-$CHCl_3$ 7:3), and celaenodendrolide (22 mg, $CHCl_3$-MeOH 1:1); which were purified by recrystallization in MeOH. On the other hand, the butanolic fraction was chromatographed on Si gel CC using $CHCl_3$-MeOH gradient as eluent to yield three biflavonoids: podocarpusflavone A (26.5 mg, $CHCl_3$-MeOH 8:2), podocarpusflavone

B (11 mg, $CHCl_3$-MeOH 3:7), and amentoflavone (40.8 mg, $CHCl_3$-MeOH 20:80), which were purified by Sepahadex HL-20 column chromatography in MeOH. The terpenes were monitored during their separation on TLC with 1% vanillin-5% H_2SO_4 in EtOH, and the biflavonoids with 0.1% $CeSO_4$ - 2N H_2SO_4.

2.3.1.2 Spectroscopic data

2.3.1.2.1 3α-Hydroxy-tirucalla-7, 24Z-dien-26-oic acid (Cm1)

Colourless crystals (MeOH), mp. 165-166 (MeOH). [α] -36.97 ($CHCl_3$, *c* 5.68) (λ = 584 nm; 26.6 °C). IRν max (KBr) cm^{-1}: 3450, 2950, 1690, 1640, 1460, 1380, 1250. HRMS: 456.3609 (calcd. 456.3604 $C_{30}H_{48}O_3$). FABMS (MNOBA + Na matriz) m/z (rel. int.): 456 (45), 439 (100), 423 (48), 307 (66), 289 (51), 273 (23), 255 (18), 245 (20), 235 (38), 213 (21), 201 (37), 187 (57). EIMS m/z (rel. int.): 456 $[M]^+$ (6), 441 $[M-15]^+$ (43), 438 $[M-H_2O]^+$ (2), 423 $[M-H_2O-15]^+$ (100), 397 $[M-CO_2-15]^+$ (9), 327 (7), 316 $[M-C_8H_{13}O_2]^+$ (6), 301 (8), 287 (7), 241 (9), 227 (9), 215 (7), 187 (18), 175 (20), 161 (16), 147 (17), 135 (21), 119 (25), 105 (29), 95 (46), 81 (31), 69 (27), 55 (46). ^1H and ^{13}C NMR data see Table 2.2 and Appendix S-1 - S-6.

2.3.1.2.2 3-Oxo-tirucalla-7,24Z-dien-26-oic acid (Cm2)

Colourless resin. [α] -18 ($CHCl_3$, *c* 5.68) (λ = 584 nm; 26.6 °C). IRν max (film) cm^{-1}: 3400, 2960, 1730, 1640, 1450, 1380, 1270, 1120. HRMS: 454.3447 (calcd. 454.3446 $C_{30}H_{46}O_3$). FABMS (MNOBA + Na matriz) m/z (rel. int.): 454 (57), 439 (100), 421 (18), 307 (27), 289 (28), 271 (17), 257 (24), 245 (24), 235 (35), 215 (17), 201 (26), 187 (34), 173 (33). EIMS m/z (rel. int.): 454 $[M]^+$ (12), 439 (100), 421 (45), 257 (16), 187 (15), 149 (30), 121 (34), 105 (37), 95 (83), 81 (37), 67 (55), 55 (95), 43 (100), 29 (40). ^1H and ^{13}C NMR data see Table 2.2 and Appendix S-7 and S-8.

2.3.1.2.3 Epi-oleanolic acid

White amorphous solid (MeOH), mp. 297-298 °C. FABMS (MNOBA): 456 ($C_{30}H_{48}O_3$) $[M]^+$ (7), 440 (13), 424 (4), 412 (8), 394 (6), 249 (39), 230 (15), 202 (22), 204 (72), 189 (88), 175 (44), 159 (38), 145 (62), 119 (90), 107 (100). IR ν max (KBr) cm^{-1}: 3400, 2970, 1700, 1460, 1390, 760. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.20, 1.38 (each, m, H-1), 1.58 (m, H-2), 1.92 (m, H-2'), 3.41 (brs, H-3), 1.60 (m, H-11), 1.90 (m, H-11'),

5.27 (t, J = 3.3 Hz, H-12), 2.81 (dd, J = 14, 4, H-18), 1.15, 1,60 (each, m, H-19), 0.95 (s, CH_3 -23), 0.83 (s, CH_3 -24), 0.90 (s, CH_3-25), 0.74 (s, CH_3 -27), 1.14 (s, CH_3-26), 0.92 (s, CH_3-29), 0.92 (s, CH_3 -30). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.50 (C-25), 17.58 (C-27), 18.62 (C-6), 23.27 (C-11), 23.99 (C-30), 22.64 (C-24), 23.70 (C-16), 25.54 (C-2), 26 (C-17), 28 (C-15), 28.69 (C-23), 31.07 (C-20), 32.84 (C-7), 32.88 (C-22), 33.22 (C-21), 33.48 (C-29), 34.18 (C-1), 37.55 (C-8), 37.72 (C-10), 39.81 (C-4), 42 (C-14), 46.91 (C-19), 47.79 (C-9), 49.35 (C-5), 76.62 (C-3), 123 (C-12), 144 (C-13), 184 (C-28). See Appendix S-9 - S-12.

2.3.1.2.4 Friedelin

White needles (Hex-$CHCl_3$), mp. 240-241 ^{0}C. EIMS m/z (rel. int.): 426 (C_{30} H_{50} O) $[M]^+$ (21), 411 (10), 341 (6), 302 (20), 273 (50), 246 (30), 231 (33), 219 (28), 205 (31), 179 (23), 163 (27), 123 (71), 109 (93), 96 (100), 81 (86), 69 (57). IR ν max cm^{-1} : 2930, 2875, 1720, 1460, 1390. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.71 (s, CH_3-24), 0.87 (d, J = 5.23 Hz, CH_3 -23), 0.94 (s, CH_3 -25), 0.99 (s, CH_3 -28), 0.99 (s, CH_3 -26), 1.04 (s, CH_3-27), 1.17 (s, CH_3 -29), 1.54 (s, CH_3 -30), 1.68 (dd, J = 14, 5.7 Hz, H-2 *ax*), 1.76 (dt, J = 12, 2 Hz, H-11), 1.97 (m, H-1 *ec*), 2.28 (m, H-10), 2.28 (td, J = 14, 7.6 Hz, H-1 *ax*), 2.39 (ddd, J = 14, 5.7, 1.9 H-2 *ec*). See Appendix S-13.

2.3.1.2.5 Maytensifolin B

White amorphous powder ($CHCl_3$), mp. >300 °C. EIMS m/z (rel. int.): 440 (C_{30} H_{48} O_2) $[M]^+$ (12), 425 (12), 409 (7), 355 (10), 273 (9), 247 (7), 232 (7), 220 (18), 189 (11), 175 (17), 163 (19), 151 (22), 135 (30), 123 (54), 109 (75), 95 (85), 81 (91), 67 (89). IRν max (KBr) cm^{-1}: 2925, 1700, 1684, 1440, 1380, 1210, 1970, 1100, 970. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.73 (s, CH_3-24), 0.88 (s, d, J = 5.3 Hz, CH_3-23), 0.89 (s, CH_3-25), 0.90 (s, CH_3 -28), 0.96 (s, CH_3 .26), 1.04 (s, CH_3 -27), 1.19 (s, CH_3 -29), 1.29 (s, CH_3-30), 1.68 (dd, J = 14, 5.7 Hz, H-2 *ax*), 2.08 (d, J = 19 Hz, H-15 *ax*), 2.41 (d, J = 19 Hz, H-15 *ec*), 2 (m, H-1). See Appendix S-14.

2.3.1.2.6 3β-Hydroxyfriedelan-16-one

White powder ($CHCl_3$), mp. 111-113 °C. EIMS m/z (rel. int.): 440 ($C_{30}H_{48}O_2$) $[M]^+$ (38), 425 (45), 355 (25), 275 (10), 219 (45), 124 (40). IRν max (KBr) cm^{-1}: 3460, 2920, 2860, 1680, 1440, 1380, 1230, 1190. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.84 (s, CH_3-26), 0.88 (s, CH_3-25), 0.92 (s, CH_3-28), 0.96 (d, J = 5.3 Hz, CH_3-23), 1.04 (s, CH_3 -27), 1.17 (s, CH_3 -29), 1.25 (s, CH_3 -24), 1.28 (s, CH_3 -30), 3.73 (d. J = 2 Hz, H-3). See Appendix S-15.

2.3.1.2.7 Celaenodendrolide

Colourless prisms (MeOH), mp. 254-255 °C. IR ν max (KBr) cm^{-1}: 3500, 1772, 1760, 1630, 980, 900, 820 cm^{-1}. FABMS (Thioglycerol + TFA) m/z (rel. int.): 411 ($C_{20}H_{26}O_9$) $[M + 1]^+$ (7), 391 (5), 369 (6), 293 (17), 277 (16), 217 (4), 185 (100). ^1H NMR (400 MHz, C_5D_5N) δ: 1.45 (d, J = 6.4 Hz, CH_3 -19), 1.86 (s, CH_3 -7), 2.39 (s, CH_3 -9), 2.99 (dd, J = 13, 9 Hz, H-14β), 3.27 (ddd, J = 13, 10, 3.9 Hz, H-16), 3.33 (s, 2-CH_3O), 3.63 (d, J = 1 Hz, H-5), 4.0 (d, J = 2.8 Hz, H-11), 4.22 (d, J = 2.7 Hz, H-12), 4.28 (s, H-2), 4.39 (dd, J = 13, 12 Hz, H-14α), 4.66 (m, H-18), 5.22 (brd, J = 7.4 Hz, H-10), 5.40 (s, H-3), 6.05 (d, J = 5.4 Hz, 18-OH). ^{13}C NMR (100 MHz, C_5D_5N) δ: 22.28 (C-19), 25.35 (C-7), 20.91 (C-9), 30.20 (C-14), 48.90 (C-16), 58.24 (C-5), 58.99 (2-OCH_3), 61.65 (C-12), 62.97 (C-11), 65.41 (C-18), 83.33 (C-3), 52.47 (C-1), 77.87 (C-6), 80.63 (C-4), 87.68 (C-2), 91.62 (C-13), 112.79 (C-10), 145.70 (C-8), 175.68 (C-15), 176.76 (C-17). See Appendix S-16 - S-19.

2.3.1.2.7.1 Acetyl-celaenodendrolide. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.36 (d, J = 6.4 Hz, CH_3-19), 1.37 (s, CH_3 -7), 1.93 (s, CH_3-9), 2.03 (s, CH_3CO_2), 2.06 (s, CH_3CO_2), 2.33 (dd, J = 13, 9 Hz, H-14β), 2.88 (ddd, J = 13, 10, 3.9 Hz, H-16), 3.27 (d, J = 1 Hz, H-5), 3.47 (d, J = 2.7 Hz, H-12), 3.5 (s, 2-OCH_3), 3.54 (dd, J = 13, 12 Hz, H-14α), 3.8 (d, J = 2.8 Hz, H-11), 3.95 (s, H-2), 5.24 (m, H-18), 5.4 (d, J = 7 Hz, H-10), 5.91 (s, H-3),. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.83 (C-9), 21.18 (C-19), 23.88 (C-7), 30.09 (C-14), 45.61 (C-16), 51.37 (C-1), 55.22 (C-5), 59.36 (C-12), 61.75 (C-11), 68.25 (C-8), 76.71 (C-3), 88.94 (C-4), 90.91 (C-13), 120 (C-10), 136.44 (C-8), 168.89 (CH_3CO_2), 170 (CH_3CO_2), 172.83 (C-15), 173.75 (C-17). See Appendix S-20 - S-21.

Table 2.2 ^{1}H NMR (400 MHZ) and ^{13}C NMR (100 MHZ) data of 3α-hydroxy-tirucalla-7, 24Z-dien-26-oic acid (Cm1) ,3α-acetyl-tirucalla-7, 24Z- dien-26-oic acid (Cm1a), and 3-oxo-tirucalla-7,24Z-dien-26-oic acid (Cm2).

Position	Cm1 δ_H C_5H_5N	Cm1 δ_H $CDCl_3$- CD_3OD	Cm1a δ_H $CDCl_3$	Cm1b δ_H $CDCl_3$	Cm2 δ_H $CDCl_3$	Cm1 δ_C C_5D_5N	Cm1 δ_C $CDCl_3$-CD_3OD	Cm1a δ_C $CDCl_3$	Cm1b δ_C $CDCl_3$	Cm2 δ_C $CDCl_3$
1	1.46 m			1.49 m	1.47	31.88	31.48	38.55	38.95	36.26
	2.02 m					-	-	-	-	-
2	1.84 d (9.4)			2.24 td (14,3.6)	2.25 td (3.6,14)	26.57	26.99	34.96	35.35	35.97
	2.01 m			2.76 td (14.5, 5.5)	2.76 dt (14,5)	-	-	-	-	-
3	3.69 brs	3.44 brs	4.68 brs		1.70 t (7)	75.28	76.29	217.03	217.43	218.67
4	-					37.94	37.55	47.88	47.64	48.28
5	2.22 dd (12,5)			2.09 m	2.08 t (3.8)	44.91	44.73	52.33	51.88	51.88
6	2.10 m					24.36	24.14	24.38	24.78	27.27
7	5.35 dd (2.68)	5.26 brd (3.12)	5.26 brd (3.3)	5.3 d (3)	5.30 dd (6,3)	118.55	118.20	117.80	118.21	118.21
8	-	-	-	-	-	146.38	146.35	145.97	145.96	145.96
9	2.50 d (12.76)	2.41 t (8)			2.28 t (7)	49.12	48.86	48.47	48.88	50.48
10	-	-	-	-	-	35.15	34.92	35.01	35.41	37.53
11	1.57 m				1.59 d (7.6)	18.34	18.22	18.28	18.71	20.66
12	1.60 d (12.3)			1.62 d (13)	1.84 m	34.04	34.07	34.04	34.04	34.46
	1.74 d (12.3)					-	-	-	-	-
13	-	-	-	-	-	43.75	43.73	43.51	43.92	44.53
14	-	-	-	-	-	51.52	51.47	51.18	51.58	51.58
15	1.49 m			1.73 t (8)	1.73 d (9)	34.37	34.22	33.62	30.19	31.097
16	2.03 m				1.97	28.47	28.42	28.20	28.45	28.61
17	1.42 m				1.49 m	53.17	53.15	52.88	52.74	53.28
18-CH_3	0.79 s	0.78 s		0.81 s	1.12 s	22.07	22.01	21.97	22.02	22.38
19-CH_3	0.87 s	0.82 s		1.12 s	1.005 s	13.45	13.15	12.80	13.21	15.95
20	1.47 d (12)	1.40	0.84			36.47	36.32	38.54	36.46	36.84
21-CH_3	0.97 d (5.2)	0.89 d (6.3)	0.92 d (6.2)	0.89 d (6.3)	0.89 d	18.54	18.34	18.20	18.64	18.97
22	1.30 m				2.52 m	36.23	36.00	34.94	34.98	34.46
	1.68 m					-	-	-	-	-
23	2.88 m	2.52 m		2.56 m	2.52 m	27.16	27.95	26.93	27.88	27.33
	2.79 m			2.46 m		-	-	-	-	-
24	6.06 brt	5.96 td (7, 0.88)	6.9 td (7.2, 0.8)	6.08 t (7)	6.08 t (7)	142.67	144.46	147.13	147.67	147.72
25	-	-	-	-	-	128.65	126.99	125.74	125.00	134.70
26-CH_3	2.15 s	1.89	1.84 s	1.92 s	1.92 s	21.53	20.86	20.59	20.97	20.19
27	-	-	-	-	-	170.72	171.05	172.46	173.64	173.64
28-CH_3	1.17 s	0.98 s	0.86 s	1.00 s	1.05 s	28.74	28.42	21.61	21.53	20.96
29-CH_3	0.98 s	0.91 s	0.98 s	1.04 s	0.89 s	22.19	21.97	24.54	24.59	24.96
30-CH_3	1.04 s	0.93 s	0.97 s	1.00 s	1.00 s	27.51	27.42	27.43	27.85	27.10
CH_3CO_2	-	-	2.07 s	-	-	-	-	-	-	-

Coupling constants (J in Hz). TMS was used as internal standard. Assignments were based on DEPT, COSY-45, HMQC, HMBC, and NOESY experiments.

2.3.1.2.8 Amentoflavone

Yellow amorphous powder (MeOH), mp. 222-224 °C. IR ν max (KBr) cm^{-1}: 3400, 1660, 1610, 1570, 1490, 1350, 1280, 1170. EIMS m/z (rel. int.): 538 ($C_{30}H_{18}O_{10}$) $[M]^+$ (38), 522 (100), 420 (5), 404 (13), 378 (80), 251 (23), 225 (29), 154 (30), 122 (59), 69 (42). ^1H and ^{13}C NMR data see Table 2.3. See Appendix S-22, S-23

2.3.1.2.9 Podocarpusflavone B

Yellow amorphous powder (MeOH), 219-221 °C. IR ν max (KBr) cm^{-1}: 3150, 1650, 1600, 1570, 1500, 1438, 1350, 1290, 1240, 1160, 1120. EIMS m/z (rel. int.): 552 ($C_{31}H_{20}O_{10}$) $[M]^+$ (93), 507 (12), 392 (17), 378 (44), 268 (8), 251 (18), 225 (17), 177 (19), 154 (17), 136 (100), 108 (13), 92 (18). ^1H and ^{13}C NMR data see Table 2.3. See Appendix S-24 - S-27.

2.3.1.2.10 Podocarpusflavone A

Yellow amorphous powder (MeOH), mp. 198-199 °C. IR ν max (KBr) cm^{-1}: 3200, 1660, 1600, 1575, 1500, 1440, 1340, 1250, 1160, 830. EIMS m/z (rel. int.): 566 ($C_{32}H_{22}O_{10}$) $[M]^+$ (65), 551 (100), 522 (7), 417 (4), 392 (38), 177 (13), 136 (67), 77 (27). ^1H and ^{13}C NMR data see Table 2.3. See Appendix S-28 - S-29.

2.3.1.3 Results and Discussion

Bioassay-directed fractionation of methanolic extract of *Celaenodendron mexicanum* using brine shrimps letality test led to the isolation of three bioactive carboxylic triterpenes, 3α-hydroxy-tirucalla-7,24Z-dien-26-oic acid (Cm1), 3-oxo-tirucalla-7,24Z-dien-26-oic (Cm2), and epi-oleanolic acid.

In addition, four terpenes (friedelin, maytensifolin B, 3β-hydroxyfriedelan-16-one, and celaenodendrolide) and three biflavonoids (amentoflavone, podocarpusflavone A, and podocarpusflavone B) were also isolated. No phorbols were detected in this species. The structures of isolated compounds from *C. mexicanum* are shown in the Figure 2.2.

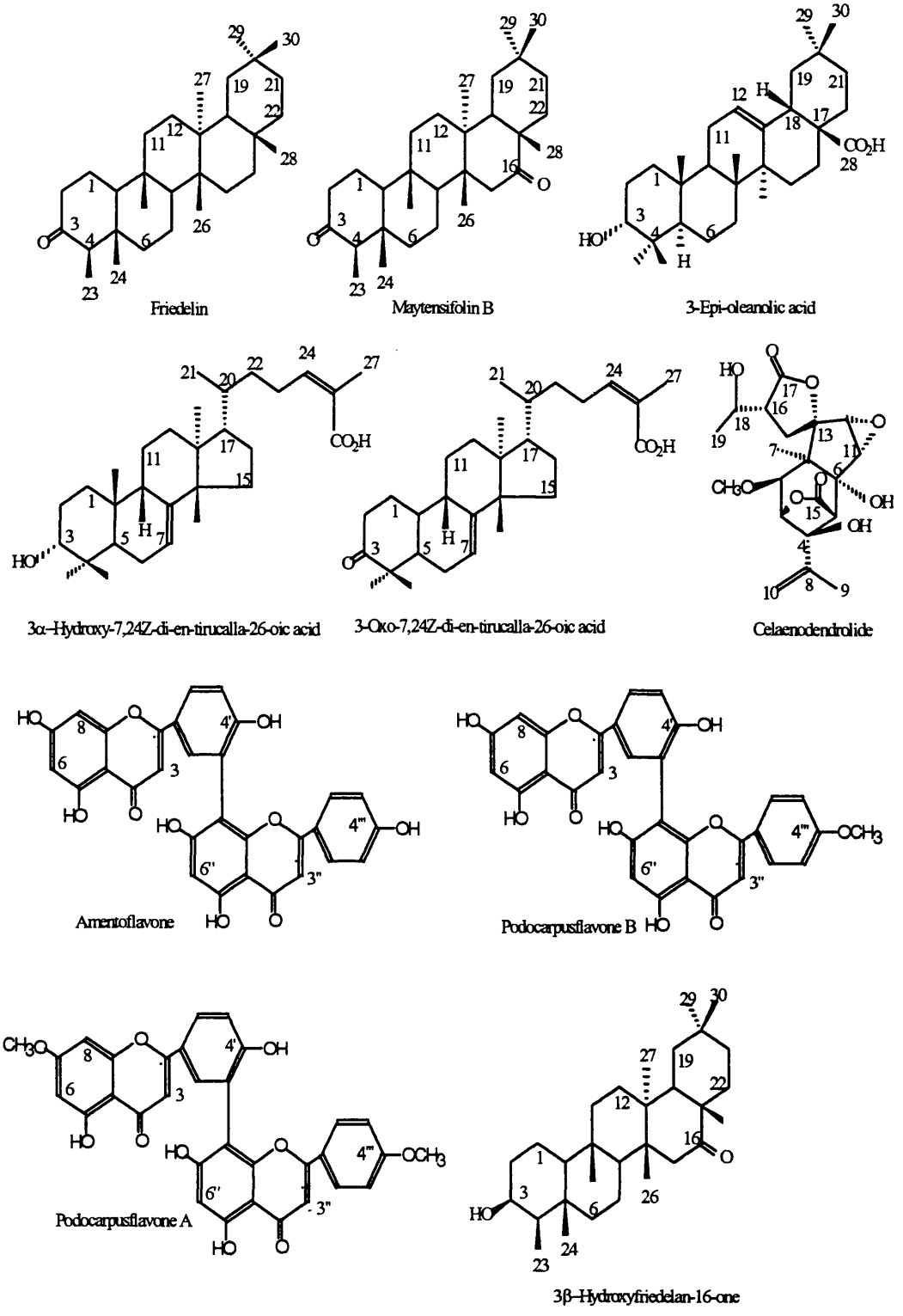


Figure 2.2 Structures of compounds isolated from *Celaenodendron mexicanum*

2.3.1.3.1 Structure determination and identification

2.3.1.3.1.1 3α-Hydroxy-tirucalla-7,24Z-dien-26-oic acid (Cm1)

High resolution EI mass spectrometry of Cm1 showed a $[M]^+$ peak at 456 m/z corresponding to the molecular formula $C_{30}H_{48}O_3$, indicating the presence of eight double bond equivalents, five of which were accounted for one pentacyclic ring nucleus, one carboxylic group and two double bonds, confirmed by DEPT spectrum (S-2). The IR spectrum of Cm1 showed absorptions for hydroxyl (3450 cm^{-1}), α,β-unsaturated carboxylic acid (1690 cm^{-1}) and double bond (1640 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra (S-1 and S-2) of Cm1 showed the presence of five ter-methyl (δ 0.79, 0.87, 0.98, 1.04, 1.17), one sec-methyl (δ 0.97, d, J= 6 Hz) and one vinylic methyl group (δ 2.15) signals, which are typical of a pentacyclic triterpene with a side chain. The ^1H and ^{13}C NMR spectra also showed two olefinic protons (δ_H 5.35, δc 118; δ_H 6.06, δc 142.67), one of which was deshielded, by the typical β position on an α,β-unsaturated carboxylic acid (δ_C 170.72) in the side chain of the tetracyclic triterpene. A significant fragment at m/z 316 $[M - C_8H_{13}O_2]^+$, resulting from the cleavage of the side chain of the tetracyclic nucleus required the presence of the α,β-unsaturated COOH at C-26 of the side chain (Figure 2.3). The geometry relatioship between the C-24/C-25 double bond and the carboxylic acid was identified from the chemical shift of H-24 (δ 6.06) and its comparison with methyl angelate (δ5.97, COOH and H-24 trans) and methyl tiglate (δ 6.72, COOH and H-24 cis) (Da Silva *et al.*, 1990), as well as NOESY spectrum (S-6). The structure of the side-chain was confirm from COSY-45 (S-3), HMQC (S-4) and HMBC (S-5) spectra. The COSY-45 spectrum (S-3) showed important coupling between the following resonances: H_3-21 *vs* H-20; H-23 *vs* H-22 and H-24; H-24 *vs* H_3-26. Futhermore, in the HMBC experiment (S-5) appreciable long-range shift correlations were observed between the following protons and carbons: H_3-26 *vs* C-24 and C-25. Furthermore, appreciable NOEs (S-6) were observed between H-24 and H_3-26, and between H-24 and H_2-23, thus confirming the geometry of the double bond located between C-24/C-25.

Placement of the second double bond at C7/C8 was indicated by fragments at m/z 316, 175, 140, 122 and 96, associated with Retro Diels Alder (RDA) clavage of ring B, see

Figure 2.3. This was supported by the ^{13}C NMR spectrum which was closely related with that reported for masticadienoic acid (Gewali *et al.*, 1990; Da Silva *et al.*, 1990). Thus, indicating a tirucall-7-en instead of a lanostan-7-en nucleus, this was further confirmed by COSY-45 spectrum, which showed cross-peaks between H-7 and H_2-6. Furthermore, the NOESY spectrum (S-6) showed strong interaction of H-7 with H_3-30 and H_2-6.

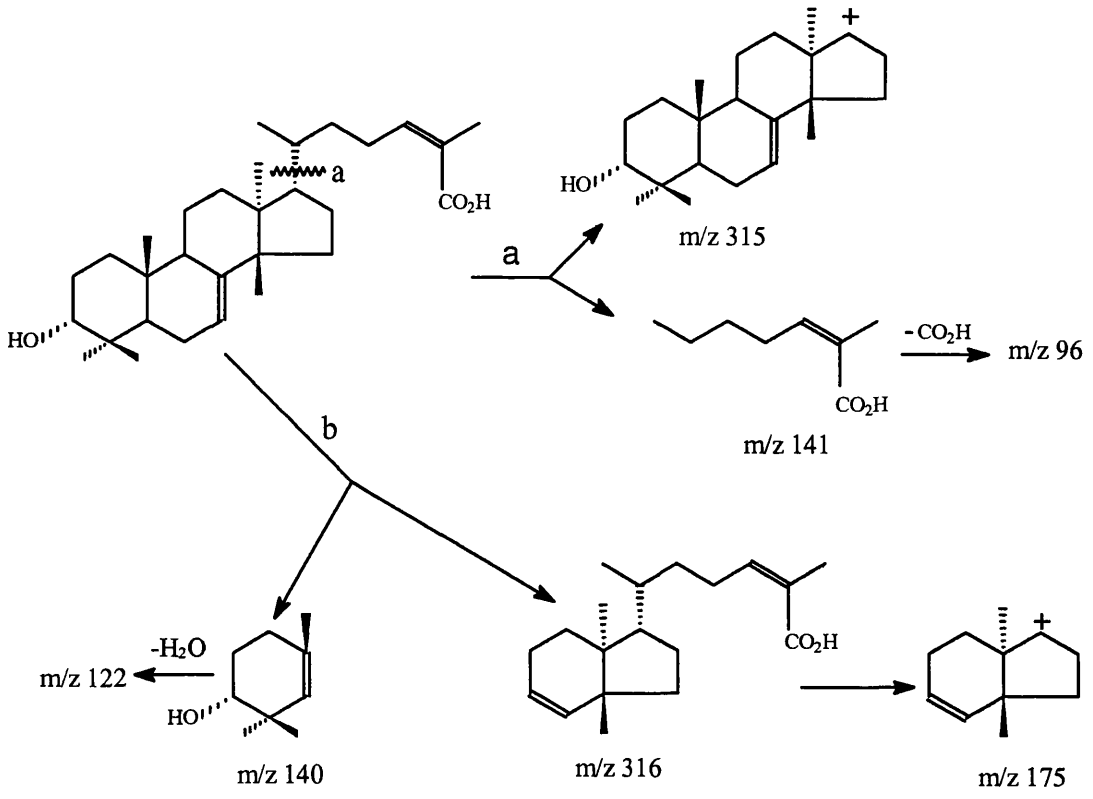


Figure 2.3 Mass spectral fragmentation of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

The ^1H NMR spectrum further showed a broad singlet (δ_H 3.69; δ_C 75.28) attributed to the proton geminal of a secondary hydroxyl group, which is placed at C-3 on biogenetic consideration. The coupling constant and chemical shift of H-3 favoured the α-orientation (axial) of the hydroxyl group at C-3. This was confirmed by the COSY-45 experiment of Cm1, which showed important cross-peaks between the oximethine CHOH (δ_H 3.69), and the two resonances of the neighbouring protons CH_2-2 (δ_H 1.84, d, J =

9.4 Hz and δ_H 2.01 m). Furthermore, appreciable NOEs were observed between H-3, H_2-2, and H_3-29. Finally, HMBC spectrum (S-5) of Cm1 showed long-range shift correlations between the following protons and neighbouring carbons: H_3-29 *vs* C-3, C-4, and C-28. Acetylation of Cm with pyridine-Ac_2O (Figure 2.4) afforded Cm1a, m/z 498 $[M]^+$ (C_{32} H_{50} O_4).

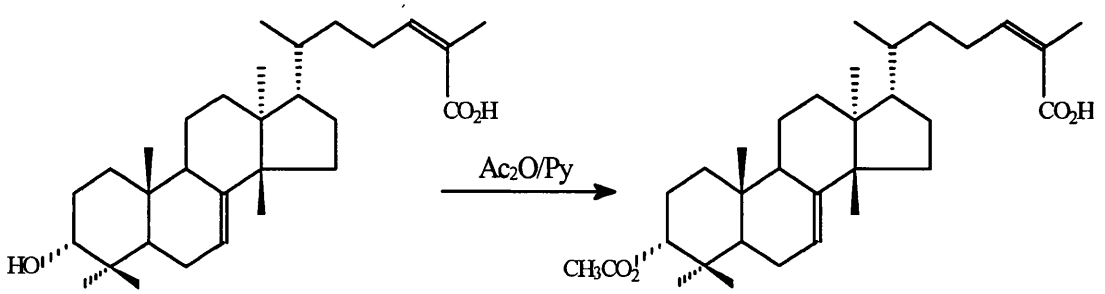


Figure 2.4 Acetylation of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

The IR spectrum of the acetylderivate showed absorptions for one acetate group at 1730 and 1210 cm^{-1}. The mass spectrum showed the highest ion peak at m/z 438 $[M\text{-}AcOH]^+$. The ^1H NMR data (Table 2.2) was similar to that of Cm1 except that the oxymethine group was deshielded paramagnetically to high field (δ_H 4.68 brs), confirming the presence of one secondary hydroxyl group in the molecule. Although, the methyl derivative of 3α-hydroxy-tirucalla-7, 24Z-dien-26-oic acid has been isolated and its physical properties has been reported (Monaco *et al.*, 1974), this is the first report of the isolation and determination of the physical and spectroscopical data of 3α-hydroxy-tirucalla-7, 24Z-dien-26-oic acid.

2.3.1.3.1.2 3-Oxo-tirucalla-7,24Z-dien-26-oic acid (Cm2)

The compound Cm2 showed a molecular ion at m/z 454 $[M]^+$ (C_{30} H_{46} O_3). Its IR spectrum showed a peak at 1710 cm^{-1} of ketone group. Comparison of ^1H and ^{13}C NMR spectra (S-7 and S-8) of Cm2 with 3α-hydroxy-tirucalla-7, 24Z-dien-26-oic, clearly,

indicated that the hydroxyl group at C-3 was replaced by a keto group in Cm2. Consistent with this proposal was the presence of two protons [δ_H 2.5 (td, J = 14,3 Hz) and δ_H 2.76 (dt, J = 14,5 Hz)] α to the carbonyl instead of a hydroxyl group, and the signal of C-2 was shifted about 7 ppm downfield to δ_C 34.96 owing to the presence of a carbonyl (δ_C 217) at C-3. Oxidation of 3α-hydroxy-tirucalla-7, 24Z-dien-26-oic acid with CrO_3-pyridine yielded 3-oxo-tirucalla-7,24(Z)-dien-26-oic acid (Figure 2.5), which had identical spectroscopic data to 3-oxo-tirucalla-7,24Z-dien-26 oic acid (Konno *et al*, 1981).

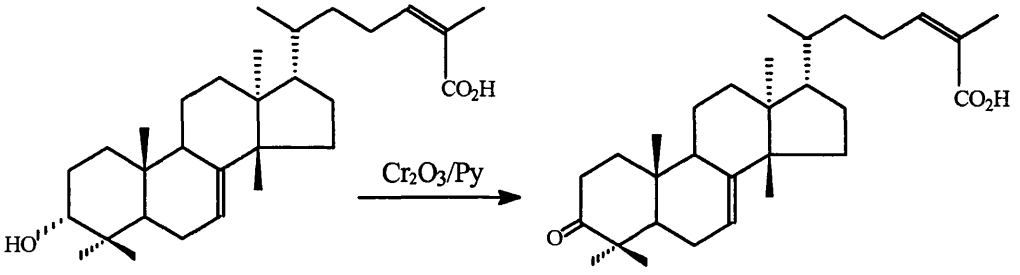


Figure 2.5 Chemical conversion of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid to 3-oxo-tirucalla-7,24Z-dien-26 oic acid.

2.3.1.3.1.3 Epi-oleanolic acid

The FABMS (MNOBA + Na matrix) and EIMS of epi-oleanolic acid revealed a $[M]^+$, m/z 456 (C_{30} H_{48} O_3), and fragments (m/z 249, 208, 204, and 190) typical of a RDA fragmentation of an olean-12-ene. The ^1H and ^{13}C NMR spectra (S-9 and S-10) exhibited signals due to seven tertiary methyl groups (δ 0.74-1.14), and three isolated hydrogens (δ 2.81, δ 3.41 and δ 5.27) of H-18, H-3 and H-12. The signal of H-18 (δ_H 2.81, dd, J = 14, 4 Hz), permitted the identification of an oleanen skeleton bearing a carboxyl (δc 184.29) group at C-28; the presence of H-3 as a broad singlet signal (δ_H 3.41; δc 76.62) indicative of the α-configuration of the hydroxyl group; and the chemical shifts of C-12/C-13 , and the olefinic proton on H-12 (δ_H 5.27, t, J = 3.3 Hz; δc 123, 144), indicated an Δ^{12} unsaturated oleaneno. 2D NMR experiments such as COSY-45 (S-11), HMQC (S-12), and NOESY spectra, confirmed the full structure of

epi-oleanolic acid. COSY-45 spectrum showed cross-peaks between the following protons: H-3 *vs* H-2; H-18 *vs* H-19; and H-12 *vs* H-11. NOESY interactions could be observed: H-3 *vs* 23-H_3, 24-H_3; H-12 *vs* H-11, H-18, H-26; and H-18 *vs* 30-H_3, 26-H_3. The spectroscopical data of epi-oleanolic (3α-hydroxy-12-oleanen-28-oic acid) acid was in agreement with literature (Chen *et al.*, 1983; Ikuta & Itokawa, 1988).

2.3.1.3.1.4 Friedelin, maytensifolin B and 3β-hydroxyfriedelan-16-one

The identification of friedelin (Gunatilaka *et al.*, 1983), maytensifolin B (Nosaki *et al.*, 1986) and 3β-hydroxyfriedelin-16-one (Castaneda *et al.*, 1992) was carried out by comparison of their spectral data with literature. Comparative analysis of ^{1}H NMR spectra (S-13, S-14 and S-15) of three terpenoids indicated that they were closely related friedelan-type triterpenes. The three triterpenes showed seven tertiary (δ 0.71 - 1.54), and one secondary (δ0.87 - 0.96, d, J= 5.3 Hz) methyl groups, however, some differences could be observed. In the case of friedelin one methylene group α to a keto group (δ 1.68, dd, J = 14, 5.7 Hz, H-2) was observed; maytensifolin B displayed two methylene groups and each has an α keto group (δ 1.68 and 2.35 each 1H, dd, J= 14, 5.7 Hz, H-2 and δ 2.08 and 2.41 each 1H, d, J = 19 Hz, H-15), and 3β-hydroxy-friedelan-16-one revealed one methylene group (2.41 each 1H, d, J=19 Hz, H-15) α to a keto group, and one methyne bearing an hydroxyl group (δ_H 3.74) at C-3.

2.3.1.3.1.5 Celaenodendrolide

The ^{1}H and ^{13}C NMR spectra (S-16 and S-17) of the picrotoxane celaenodendrolide [m/z 410 (C_{20} H_{26} O_9)] showed resonances typical of C-19 type picrotoxane terpenoid possessing two γ-lactone carboxylic groups (δ_C 175.68 and 176.76), three tertiary methyl groups (δ_H 1.45, 1.86 and 2.39); an isoprenyl moiety (δ_H 2.40 and 5.22; δ_C 145.7, 112.8, and 20.91); one quaternary carbon (δ_C 91.62) bearing a hydroxyl group, and signals of protons (H-14 and H-16) in the α-oriented side chain at C-16. Acetylation of celanodendrolide with Ac_2O and pyridine afforded the diacetyl derivated as the major product. The downfield shifts observed for H-18 (δ_H 5.24) and H-3 (δ_H 5.91) in the ^{1}H NMR (S-20) of acetylderivated were in agreement with the placement of the secondary and one tertiary carbinolic groups at C-18 and C-4 respectively. The chemical shift

observed for the carbinolic group at δ_C 77.87 in the ^{13}C NMR spectrum (S-21) was consistent with the disposition of a remaining tertiary group at C-6. ^{1}H and ^{13}C assignments were supported by HMQC spectrum (S-19). COSY-45 spectrum (S-18) showed the W-type long range coupling between H-3 and H-5 which is a common feature of the picrotoxin type compounds. Further correlations were observed with the following protons: H-2 *vs* H-10; H-3 *vs* H_3-9, H-10; H-10 *vs* H_3-9, H-5, H-2; H-18 *vs* H-19; H-11 *vs* H-12; H-16 *vs* H-14α, H-14β. The spectral data of celaenodendrolide was in agreement with literature (Castaneda *et al.* 1993).

2.3.1.3.1.6 Amentoflavone, podocarpusflavone A and podocarpusflavone B

Three biflavonoids with similar physical and spectroscopic properties were isolated. The absortions at 328-336 and 269-276 nm in the UV spectrums; and the peaks for hydroxyl (3100, 3400 cm^{-1}), carbonyl (1650 cm^{-1}) and aromaticity (1600, 1500 cm^{-1}) in the IR spectra; were typical of flavonoids. The molecular ions of the three flavonoids at m/z 536, 552 and 566 correspond to the molecular formulas $C_{30}H_{18}O_{10}$, $C_{31}H_{20}O_{10}$, and $C_{32}H_{22}O_{10}$, confirmed by DEPT spectra (S-23, S-25 and S-27) and correspond to the mono- and dimethoxy derivatives of amentoflavone. Comparison of ^{1}H and ^{13}C NMR spectra (S-22 and S-23) of amentoflavone with those of apigenine showed that the resonances of the A- and B-ring signals of amentoflavone were shielded by aryl substituents at C-8 and C-3', indicating that C-3' and C-8' were the positions of the two apigenin units linked together. Comparison of ^{1}H NMR spectra (S-22, S-24 and S-26) showed that the three biflavonoids have two singlets at δ 6.8 and 6.86 assigned to H-3 and H-3″ of the monomers of a biflavone; two meta-coupled protons at δ 6.20 and 6.50 (2H, d, each, J = 2 Hz) of H-6 and H-8; one A_2B_2 coupling system with signals at δ 6.80 (d, J = 8.8 Hz, H-3‴, H-5‴) and δ 7.60 (d, J = 8.8 Hz; H-2‴, H-6‴); a singlet appearing at δ 6.40 (s, H-6″) corresponded to an isolated aromatic hydrogen; one ABX coupling system at δ 7.1 (d, J = 9.28 Hz; H-5′), 7.9 (d, J = 2 Hz, H-2') and 8.0 (dd, J= 9, 2 Hz, H-6'); and two singlets at δ 12-13 corresponding to the 5 and 5″ hydroxyl groups chelated with 4 and 4″ carbonyl group of the γ-pirona (δc 181); differing for the presence of one and two methoxy groups in the spectra of the mono- and dimethoxy derivative of amentoflavone. Placement of methoxy groups were determined by NOESY spectrum (S-29). In the

case of the monomethoxy derivative of amentoflavone interactions were observed between between 4"-OMe *vs* H-3", H-5". In the case of dimethoxy derivatives, interactions were observed between 4"-OMe *vs* H-3", H-5", as well as 4'''-OMe *vs* H-3''' , H-5'''. The spectral and physical properties of the three biflavonoids were in good harmony with those described previously for amentoflavone (Markham & Geiger, 1994; Markham *et al.*, 1987), podocarpusflavone A (Geiger & Quinn, 1982), and podocarpusflavone B (Garg & Mitra, 1971).

Table 2.3 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data in DMSO-d_6 of podocarpusflavone A, podocarpusflavone B and amentoflavone.

Position	Amentoflavone δ_H	Podocarpusflavone B δ_H	Podocarpusflavone A δ_H	Amentoflavone δc	Podocarpusflavone B δc	Podocarpusflavone A δc
2	-	-	-	164.01	164.00	165.00
3	6.79 s	6.812 s	6.87 s	102.48	103.15	103.15
4	-	-	-	181.63	181.63	181.82
5	-	-	-	161.35	162.00	161.03
6	6.19 d (2)	6.18 d (2)	6.34 d (2)	98.60	98.72	98.67
7	-	-	-	163.72	163.10	163.99
8	6.46 d (2)	6.44 d (2)	6.73 d (2)	93.92	93.89	92.48
9	-	-	-	157.27	157.26	157.22
10	-	-	-	103.61	103.58	103.94
1'	-	-	-	120.76	120.85	120.72
2'	8.00 d (2)	7.98 d (2.)	8.01 d (2)	127.66	127.69	127.79
3'	-	-	-	121.31	122.90	122.90
4'	-	-	-	159.58	159.50	159.70
5'	7.15 d (9.28)	7.15 d (8.5)	7.16 d (8)	116.15	116.11	116.17
6'	8.01 dd (9, 2.4)	8.00 dd (8, 2.4)	8.03 dd (8, 2)	131.30	131.25	131.26
2"	-	-	-	163.56	163.68	163.08
3"	6.85 s	6.88 s	6.88 s	102.84	102.91	103.04
4"	-	-	-	182.01	182.05	182.04
5"	-	-	-	160.44	160.45	160.50
6"	6.39 s	6.41 s	6.41 s	98.75	98.64	97.94
7"	-	-	-	162.02	162.09	162.09
8"	-	-	-	103.93	103.93	104.64
9"	-	-	-	154.39	154.44	154.46
10"	-	-	-	103.49	103.62	103.58
1'''	-	-	-	119.96	119.90	120.00
2'''	7.58 (8.8)	7.68 d (8.96)	7.67 d (9)	128.09	127.88	127.86
3'''	6.72 (8.8)	6.92 d (9)	6.92 d (9)	115.66	114.36	114.35
4'''	-	-	-	160.91	161.36	161.08
5'''	6.72 (8.8)	6.92 d (9)	6.92 d (9)	115.66	114.36	114.35
6'''	7.58 (8.8)	7.62 d (8.96)	7.67 d (8.9)	128.09	127.88	127.86
7-OCH_3	-	3.76 s	3.76 s	-	55.39	55.80
4'''-	-	-	3.82 s	-	-	55.39
CH_3O	12.98 s, 13.11 s	12.96 s, 13.07 s	12.95 s, 13.05 s	-	-	-
5-OH	10.27 brs,	10.58 brs,	10.61 brs	-	-	-
OH	10.63 brs,	10.77 brs	-	-	-	-
OH	10.81 brs	-	-		-	-
OH						-

Coupling constants (J in Hz). TMS was used as internal standard. Assignments were based on DEPT, COSY-45, and NOESY experiments.

2.3.2 *Galphimia glauca* (Malpighaceae)

Galphimia glauca has been used in Mexican traditional medicine to treat mental disorders (Estrada, 1990), to alleviate heart pains, to calm the nerves (Linares *et al.*, 1988), to treat malaria, and to alleviate various gastrointestinal maladies such as diarrhea, dysentery, and gastroenteritis (Diaz, 1976). *G. glauca* has been pharmacologically evaluated for its activity on smooth muscle (Meckes *et al.*, 1993; Perrusquia *et al.*, 1995), and on central nervous system (Tortoriello & Lozoya, 1992), as well as its effect on inhibition of cona-induced histamine release from human adenoidal-mast-cells (Bent *et al.*, 1990). *G. glauca* has been introduced into homoeopathy as a remedy against pollinosis (Wiesenauer *et al.*, 1983; 1990a: 1990b; Wiesenauer & Gauss, 1985). The species has been found to contain tetragalloylquinic acid, gallic acid, methyl gallate, ellagic acid and flavonoid acylglycosides, which showed activity against allergic reactions *in vivo* models (Neszmelyl *et al.*, 1993). A norsecotriterpenoid named galphimine B was isolated recently and exhibited a strong depressant activity on the CNS (Tortoriello & Ortega, 1993). Considering that there are no previous reports validating the antimalarial properties of this herb remedy, we decided to perform a bioactive guided fractionation using *P. falciparum* and *L. donovani in vitro* tests in order to isolate potential antiprotozoal compounds.

2.3.2.1 Extraction and Isolation

The air-dried and powdered aerial parts of *G. glauca* (2.5 Kg) were extracted following to the procedure described in Figure 2.1. The extracts obtained were tested for antiprotozoal activity *in vitro*. The n-butanolic active fraction was subjected to silica gel CC using $CHCl_3$-MeOH gradient as eluent followed by Sephadex HL-20 column in MeOH to yield quercetin (81 mg) as the major active compound. Chromatograms were observed under UV and sprayed with $CeSO_4$-H_2SO_4 reagent. On the other hand, the chloroformic fraction (IC_{50} = 86.70 μg/ml) was chromatographed on silica gel column, with Hex-$CHCl_3$, and $CHCl_3$-MeOH gradient as eluent. The resulting fractions were further rechromatographed in silica gel CC using Hex-acetone gradient as eluent, to yield the following compounds: quercetin (27 mg), galphimine C (46 mg), galphimine D (30 mg), glaucamine (4018.58 mg), galphimine E (43.67 mg), stigmasterol (400 mg), and

sitosteryl-3-O-β-D-glucopyranoside (37 mg). Chromatograms were sprayed with 1% vanillin-5% H_2SO_4.

2.3.2.2 Spectroscopic data

2.3.2.2.1 Quercetin

Yellow needles (MeOH), mp. > 270 °C. FABMS (MNOBA + Na) m/z (rel.int.): 302 ($C_{15}H_{10}O_7$) [M^+] (6), 286 (7), 274 (3), 243 (4), 184 (16), 153 (38), 132 (100), 111 (25), 97 (43). Laser MS, m/z (rel.int) 302 (100). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 6.19 (d, J = 1.8 Hz, H-6), 6.41 (d, J = 1.76 Hz, H-8), 6.89 (d, J = 8 Hz, H-5'), 7.54 (dd, J = 8, 2 Hz, H-6'), 7.68 (d, J = 2 Hz, H-2'). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 93.34 (C-8), 98.17 (C-6), 103.03 (C-10), 115.03 (C-2'), 115.58 (C-5'), 119.99 (C-6'), 121.98 (C-1'), 135.72 (C-3), 144.99 (C-3'), 147.62 (C-4'), 156.17 (C-9), 160.49 (C-2), 160.76 (C-5), 163.85 (C-7), 175.83 (C-4). See Appendix S-30 and S-31.

2.3.2.2.2 Galphimine C

White needles ($CHCl_3$), mp. 246 °C. [α]$_D$ 0 ($CHCl_3$, *c* 5.86) (λ = 584 nm; 26.6 °C). IR ν max (KBr) cm^{-1}: 3540, 1760-1720, 1250, 1050. HRMS m/z (rel. int.): 616.2760 (calcd. 616.2760 $C_{34}H_{48}O_{10}$). FABMS (MNOBA + Na matrix) m/z (rel. int.): 617 [M + H]$^+$ (53), 599 (16), 557 [M-CH_3CO_2H + H]$^+$, 525 (14), 495 (10), 479 (9), 465 (23), 431 (12), 419 (13), 373 (22), 341 (10), 329 (18), 306 (64). ^{1}H and ^{13}C NMR data see Table 2.4. and Appendix S-32 - S-37.

2.3.2.2.3 Galphimine D

White needles ($CHCl_3$), mp. 285 °C. [α]$_D$ -18.03 ($CHCl_3$, *c* 6.10) (λ = 584 nm; 26.6 °C). IR ν max (KBr) cm^{-1}: 3400, 1720-1750, 1250, 1050. HRMS m/z (rel. int.): 674.3800 (calcd. 674.3800 $C_{36}H_{50}O_{12}$). FABMS (MNOBA + Na matrix) m/z (rel. int.): 675 [M + H]$^+$ (26), 615 (M - CH_3CO_2H + H] (100), 583 (6), 555 (7), 523 (3), 477 (2), 460 (10), 417 (5), 391 (29), 371 (59), 329 (19), 307 (93). ^{1}H and ^{13}C NMR data (Table 2.4) and Appendix S-38 - S-43.

2.3.2.2.4 Galphimine E

White needles ($CHCl_3$), mp. 271 °C. $[\alpha]_D$ -23.55 ($CHCl_3$, *c* 5.52) (λ = 584 nm; 26.6°C). IR ν max (KBr) cm^{-1}: 3400, 1750-1720, 1250, 1050. HRMS m/z (rel. int.): 732.2460 (calcd. 732.2418 $C_{38}H_{52}O_{14}$). FABMS (MNOBA + Na matrix) m/z (rel. int.): 733 $[M + H]^+$ (1), 673 (25), 613 (8), 553 (4), 431 (13), 371 (4), 329 (3), 307 (10), 289 (7), 273 (2), 243 (3), 214 (4), 176 (6), 154 (78), 136 (100), 121 (29). ^1H and ^{13}C NMR see Table 2.4. and Appendix S-44 - S-49.

2.3.2.2.5 Glaucamine

White needles ($CHCl_3$), mp. 234 °C $[\alpha]_D$ + 46.46 ($CHCl_3$, *c* 9.04) (λ = 584 nm; 26.6 °C). IR ν max (KBr)cm^{-1}: 3400, 1750-1720, 1250, 1050. HRMS m/z (rel.int.): 620.327049 (calcd 620.3270 $C_{34}H_{52}O_{10}$) (MNOBA + Na matrix) m/z (rel.int.): 621 $[M + H]^+$ (18), 603 (17), 585 (37), 567 (12), 543 (28), 525 (40), 507 (22), 483 (15), 465 (58), 447 (51), 433 (26), 421 (10), 410 (74), 387 (16), 375 (24), 307 (50), 289 (58), 243 (60), 433 (26), 421 (10), 410 (74), 387 (16), 375 (24), 307 (50), 289 (58), 243(60). ^1H and ^{13}C NMR see Table 2.4.

2.3.2.2.6 Stigmasterol

White amorphous powder ($CHCl_3$), mp. 171 °C IR ν max (KBr) cm^{-1}: 3200, 1640, 1450, 1380, 720. EIMS m/z (rel. int.): 412 ($C_{29}H_{48}O$) $[M]^+$ (37), 396 (14), 381 (7), 329 (13), 303 (14), 274 (11), 255 (16), 231 (10), 213 (16), 199 (8), 187 (7), 159 (21), 145 (26), 133 (20), 119(21), 105 (28), 95 (27), 81 (31), 69 (30), 55 (52), 43 (100). ^1H NMR (400 MHz, $CDCl_3$) δ: 0.68 (3H, s, 18-H_3), 0.81 (3H, s, 27-H_3), 0.83 (3H, s, 26-H_3), 0.84 (3H, t, J = 7 Hz, 29-H_3), 0.92 (3H, d, J = 6.5 Hz, 21-H_3), 1.00 (3H, s, 19-H_3), 1.83 (2H, m), 1.99 (2H, m), 2.27 (2H, m), 3.52 (1H, m, H-7), 5.08 (dd, J = 15, 7.5 Hz, H-22), 5.20 (dd, J = 15, 7.5 Hz, H-23), 5.35 (1H, d, J = 5.12 Hz, H-6). ^{13}C NMR DEPT (100 MHz, $CDCl_3$) δ: 12.28 (CH_3), 12.40 (CH_3), 19.19 (CH_3), 19.40 (CH_3), 19.44 (CH_3), 20.25 (CH_3), 21.53 (CH_2), 23.43 (CH_2), 24.72 (CH_2), 26.43 (CH_2), 28.67 (CH_2), 29.51 (CH), 32.29 (CH), 32.30 (CH_2), 32.33 (CH_2), 34.34 (CH_2), 36.55 (CH), 37.64 (CH_2), 42.69 (CH_2), 46.22 (CH), 50.52 (CH), 56.44 (CH), 57.17 (CH), 72.22 (CH), 122.15 (CH). See Appendix S-56 and S-57.

Table 2.4 ^{1}H NMR (400 MHz) ^{13}C NMR (100 MHz) spectral data in $CDCl_3$ for galphimine C, galphimine D, galphimine E and glaucamine

Position	Galphimine C	Galphimine D	Galphimine E	Glaucamine	Galphimine C	Galphimine D	Galphimine E	Glaucamine
	δ_H				δ_C			
1	3.55 dd (4)	3.55 dd (4)	3.56 dd (3.6)	1.55 m	52.73	52.70	53.86	16.21
2	3.56 dd (4, 1.2)	3.58 d (4)	3.57 d (4.8)	1.66 d (13)	56.78	56.43	57.61	31.51
				1.96 d (15.5)	-	-	-	-
3	-	-	-	4.93 brd (2.2)	168.57	168.55	169.70	74.75
4	5.32 q (6.16)	5.26 q (6.3)	5.2 q (6.48)	1.77 m	76.31	76.40	77.70	49.07
5	-	-	-	-	38.63	41.51	42.51	45.40
6	1.44 d (16.6)	1.43 d (16)	1.47 d (17)	3.21 d (8.4)	31.84	31.76	32.88	86.34
	1.73 dd (16.6, 5.6)	1.73 dd (16,6)	1.71 dd (16,5.8)		-	-	-	-
7	5.16 brt (7.96)	5.11 brt (7)	5.10 brt (5.8)	3.98 brt (8.8)	69.03	68.70	70.07	72.55
8	2.15 d (7.8)	1.96 brd (8.16)	2.01 d (5)	1.66 d (10.36)	50.40	51.27	52.40	49.96
9	-	-	-	-	37.86	37.78	38.95	37.51
10	1.66 brs	1.63 brs (9.7)	1.62 brs	1.06 dd (10.3, 2.6)	53.85	54.24	55.84	59.31
11	0.77 dq (12,2)	0.71 dt (10.4,3.8)	0.97 dd (12.6,4)	1.55 dd (12)	27.08	26.94	35.25	30.41
	2.82 td (13.7, 4)	2.69 td (13,3.9)	2.98 t (12.6)	3.22 dd (14.8, 2.6)	-	-	-	-
12	1.12 m	1.47 m	5.6 dd (12.7,4)	1.43 m	33.90	26.79	76.32	29.65
	1.98 m	1.67 m		2.18 m	-	-	-	-
13	-	-	-	-	57.14	57.93	59.54	79.87
14	-	-	-	-	41.13	41.94	49.19	43.80
15	1.04 dd (12,3)	5.56 dd (6.9, 6.7)	6.04 dd (12,6.2)	1.55 d (12)	36.10	70.94	70.06	39.84
	2.17 m				-	-	-	-
16	2.03 m	2.3 m	2.28 dd (6)	1.40 m	29.16	34.90	36.96	25.26
	2.25 m			1.95 m	-	-	-	-
17	-	-	-	-	42.15	43.14	43.93	41.53
18	-	-	-	-	76.39	76.72	80.66	79.51
19	2.08 d (12.2)	2.11 d (15)	2.16 d (15)	2.37 d (13.2)	42.61	41.95	42.78	45.05
	2.90 d (15)	2.89 d (15)	2.86 d (13)	2.66 d (13.2)	-	-	-	-
20	-	-	-	-	144.23	138.58	138.96	144.07
21	2.16 brt	2.15 brt (4.4)	2.14 m	2.20 d (13)	23.43	23.91	25.65	33.25
				2.54 d (13.24)	-	-	-	-
22	1.38 m	1.62 dd (5.4)	1.95 m	2.74 dd (17,3.5)	40.44	40.23	41.39	52.73
	1.98 m	1.96 (8.16)	1.71 m		-	-	-	-
23-CH_3	1.17 d (6.4)	1.17 d (6.4)	1.17 d (6.4)	1.35 d (7.16)	13.04	12.99	14.26	16.70
24	3.63 d (12.26)	3.61 d (12.88)	3.61 d (12)	4.5 0 d (12.5)	68.22	68.21	69.39	65.11
	5.07 d (12.84)	5.05 d (12.68)	5.06 d (12.7)	4.73 d (12.5)	-	-	-	-
25-CH_3	1.34 s	1.36 s	1.52 s	1.02 s	20.46	20.92	23.11	19.46
26-CH_3	1.31 s	1.30 s	1.35 s	1.18 s	22.72	23.13	25.08	23.04
28-CH_3	1.09 s	1.00 s	1.25 s	1.27 s	25.67	18.26	16.03	22.78
29	4.46 d (2)	4.6 d (1.5)	4.61 d (1.8)	4.79 brs	109.69	113.18	114.60	110.45
	4.89 d (2)	4.8 brs	4.81 d (1.8)	4.81 brs	-	-	-	-
30	-	-	-	-	170.21	170.20	170.71	170.04
CO_2CH_3	3.49 s	3.62 s	3.65 s	3.65 s	51.35	51.35	52.69	51.37
OCO_2CH_3	1.99 s	1.98 s	1.98 s	2.04 s	21.05	21.03	22.28	21.43
"	2.07 s	2.05 s	2.06 s	2.11 s	21.62	21.23	22.61	21.22
"	-	2.07 s	1.98 s	-	170.21	21.60	22.64	170.04
"	-	-	2.03 s	-	170.89	170.20	22.97	170.62
					174.62	170.88	171.39	174.84
					-	173.69	171.47	-
					-	-	171.85	-
					-	-	174.27	

Coupling constant (J in Hz). TMS as used as internal standard. Assignments were based on DEPT, COSY-45, HMQC, COLOQ, and NOESY experiments

2.3.2.2.7 Sitosteryl-3-O-β-D-glucopyranoside

White amorphous powder ($CHCl_3$), mp. 250 °C (MeOH). IR ν max (KBr) cm^{-1}: 3400, 1700, 1100, 950, 720. FABMS (MNOBA + Na matrix) m/z (rel. int.): 577 $[M + H]^+$ ($C_{35}H_{60}O_6$) (3), 532 (6), 501 (5), 484 (7), 460 (5), 451 (6), 411 (13), 415 (13), 395 (73), 381 (21), 363 (12), 343 (13), 335 (18), 321 (15), 313 (39), 303 (23), 287 (27), 273 (46), 255 (48), 243 (53), 226 (39), 214 (100). ^1H NMR (400 MHz, $CDCl_3$): δ 0.68 (3H, s, 18-H_3), 0.83 (d, J = 2 Hz, H_3), 0.85 (s, H_3), 0.93 (3H, d, J = 6.48 Hz, 21-H_3), 1.01 (s, 19-H_3), 2.37 (1H, t, J = 3 Hz), 2.02 (1H, td, J = 14.9, 4.5 Hz), 2.50 (1H, dq, J = 7, 3 Hz), 3.24 (td, J = 7.68, 2.00 Hz, H-2'), 3.37 (m, H- 5'), 3.42 (1H, dd, J = 6.8 Hz, H-4'), 3.43 (dd, J = 6.8 Hz, H-3'), 3.59 (m, H-3), 3.75 (dd, J = 12, 4.64 Hz, H-6'), 3.84 (dd, J = 12, 3 Hz, H-6'), 4.41 (d, J = 7.8 Hz, H-1'), 5.37 (d, J = 5.2 Hz, H-6). See Appendix S-58 and S-59.

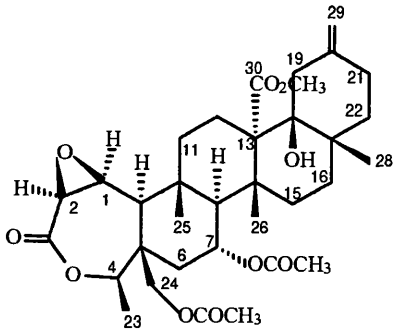
2.3.2.3 Results and Discussion

Bioactive guided fractionation of methanolic extract of aerial parts of *Galphimia glauca* using *Leishmania donovani promastigotes* and *Plasmodium falciparum in vitro* tests, resulted in the isolation of the flavonol quercetin as the major active compound. In addition, from the chloroform fraction, four novel nor-triterpenoids named: galphimine C, galphimine D, galphimine E, and glaucamine were obtained together with the known steroids: stigmasterol and sitosteryl-3-O-β-D-glucopyranoside. The structures of compounds obtained from *Galphimia glauca* are shown in Figure 2.6.

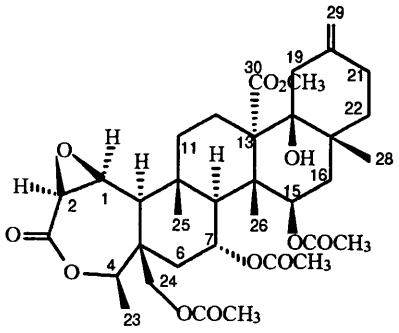
2.3.2.3.1 Structure determination and identification

2.3.2.3.1.1 Quercetin

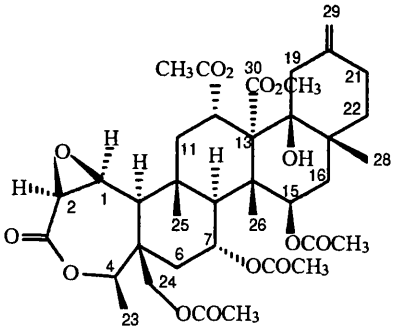
The EIMS and Laser MS of quercetin exhibited a molecular ion at m/z 302, which corresponds to the molecular formula $C_{15}H_{10}O_7$, confirmed by DEPT spectrum (S-31). The ^1H NMR spectrum (E-30) showed the presence of an AB system at δ 6.19 (H-8) and 6.41 (H-6) (each d, J = 1.8 Hz) of a 5,7-disubstituted ring A, an ABX system at δ 6.89 (d, J = 8.4 Hz, H-5'), 7.54 (dd, J = 8.4, 2.1 Hz, H-6') and 7.68 (d, J = 2 Hz, H-2'), of a 3',4'-disubstituted ring B, and a chelated hydroxyl group at C-5 (δ_H 12.49, δ_C 160.75). Furthermore, for diagnostic peaks at m/z 153 and 137, result of RDA cleavage



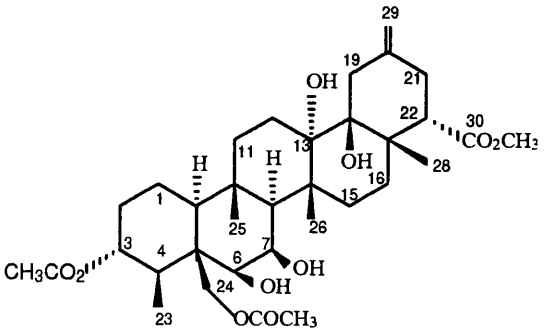
Galphimine C



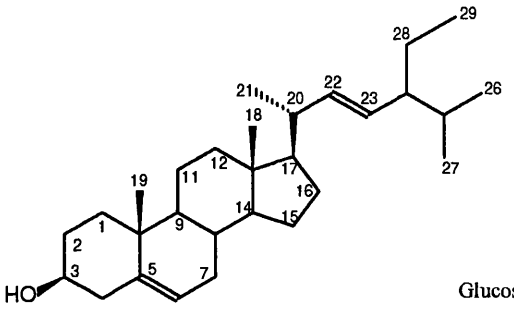
Galphimine D



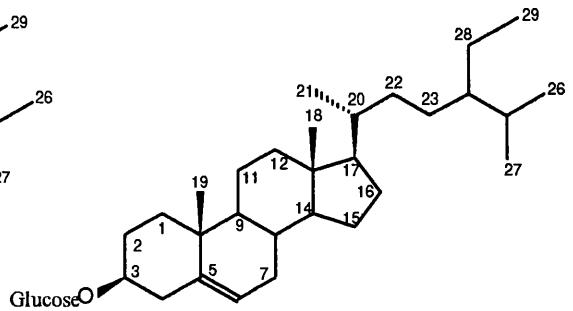
Galphimine E



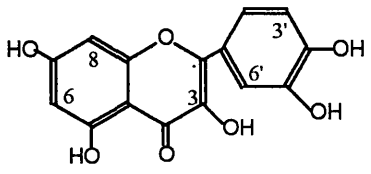
Glaucamine



Stigmasterol



Sitosteryl-3-O-β–D-glucopyranoside



Quercetin

Figure 2.6 Structures of compounds isolated from *Galphimia glauca*

of ring C, indicated the presence of two phenolic groups in ring A and two phenolic groups in ring B (Figure 2.7). Full assignation of protons and carbons was confirmed with HMQC spectrum. The physical and spectroscopical data of the flavonol quercetin was in agreement with those in literature (Pakulski *et al.*, 1996; Markham & Chari,1982).

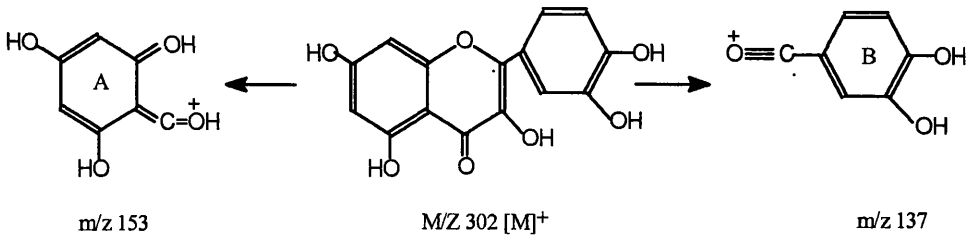


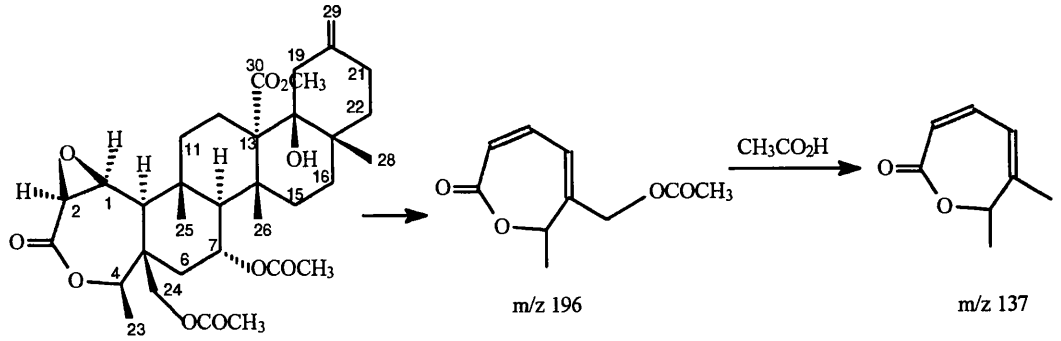
Figure 2.7 Mass spectral fragmentation of quercetin

2.3.2.3.1.2 Galphimine C

High-resolution FAB-mass spectrometry of galphimine C, showed a molecular ion at m/z 616 $[M]^+$ ($C_{34}H_{48}O_{10}$). The loss of a molecule of acetic acid led to the fragment, m/z 556 [$C_{32}H_{44}O_8$, $M^+ - CH_3CO_2 + H$], generated from the molecular ion, through a McLafferty rearrangement in the ring A of galphimine C (Figure 2.6). The IR spectrum showed the presence of hydroxyl (3500 cm^{-1}) and ester (1760-1720 cm^{-1}). The ^1H NMR spectrum (S-32) exhibited the presence of four methyl groups [δ 1.09 (s), 1.17 (d, J = 6.4 Hz), 1.31 (s), and 1.34 (s)], two acetoxyl groups [δ 1.99 (s) and 2.07 (s)], a methoxyl group [δ 3,49 (s)], two methine signals of an epoxide function [δ 3.55 (d, J = 4 Hz) and 3.56 (dd, J = 4, 1.26 Hz)], an exo-methylene [δ 4.46 and 4.49, (each 1H, AB, d, J = 2 Hz)], a methylene [δ 3.63 and 5.07, (each 1H, AB, d, J = 12.55 Hz)] and a methine [δ 5.16 (brt, J = 7.96 Hz)] bearing each one acetoxyl group.

^1H and ^{13}C NMR assignments of galphimine C (Tables 2.4) were greatly facilitated by HMQC spectrum (S-35). Long-range C-H correlations were observed in COLOC spectrum (S-36) with the following protons and carbons: H-1 (δ_H 3.55) and H-2 (δ_H 3.58) *vs* C-3 (δ_C 168.69); H-1 (δ_H 3.55) and H-10 (δ_H 1.63) *vs* C-2 (δ_C 56.80), which were utilized to confirm the epoxide function at C-1, C-2. The lactone system was

confirmed by ^{13}C NMR spectrum (S-33) through the signals at δ 52.73 (C-1), 56.38 (C-2) and 168.57 (C-3) and its localization in the heptacycle ring A, was done in accordance with the chemical shifts of carbon atoms: δ 76.31 (C-4), 38.63 (C-5), 53.85 (C-10), 13.04 (C-23), and the comparison with a model structure (Abreu *et al.*, 1990). The peak at m/z 212, 196, 152, and 137 in the mass spectrum (Figure 2.8), suggested the presence of methylene bearing an acetoxyl group at C-5. Long-range C-H correlations in COLOC spectrum (S-36) enabled the determination of the following carbon sequence: C-9 → C-8 → C-25; C-26 → C-14; C-5 → C-10; C-8 → C-14 → C-26 and C-9 → C-10 → C-11. On the other hand, the carbon sequence C-6 → C-7 → C-8; C-15 → C-16; C-11 → C-12; C-22 → C-21 → C-20 → C-29 and C-19 → C-20 → C-29, was disclosed by ^{1}H-^{1}H COSY correlations (S-34) arising from the geminal and vicinal sping coupling constants between involucrated protons. Further, the localization of the acetoxyl groups at C-7 and C-24, the hydroxyl group at C-18, the epoxide at C-1-C-2, and the methoxyl group at C-13 was made possible by ^{1}H and ^{13}C chemical shifts, multiplicity of the signals observed in the 2D NMR spectra (COSY-45, HMQC, HMBC, COLOC).



Galphimine C

Figure 2.8 Mass spectral fragmentation of galphimine C

The spectral analysis pointed to a nor-friedelane type skeleton for galphimine C. With the constitution establised, all that was left was to determine the configuration and conformation of the molecule, this was achieved by a NOESY spectrum (S-37). The analysis of results indicated that the position and orientation of 25, 26 and 28-CH_3 is consistent only with rings C and D in a chair-trans-chair arrangement with the three

methyl groups and the hydroxyl group at C-18 all axial and β-orientated. Furthermore, the hydrogens on C-10, C-8 are all axial and α-orientated, whereas H-1 and H-2 are pseudo equatorial and α-orientated. The α-axial carbomethoxy group at C-13 is consistent only with a cis D/E ring junction. The acetylated methine at C-7 is α-orientated, because cross-peaks were observed between 25- and 26-methyl-signals with the β-hydrogen at C-7. The B/C junction is trans, and ring B is in a twist-boat conformation. The coupling constants in ring B ($J_{6,7}$ = 16.6, 5.6 and $J_{7,8}$ = 7.8 Hz) confirm gauche dihedral angles for each pair of protons. Finally, the two 4-8-10 and 8-10-13 sequences must place these protons on the α-face of the molecule, which completes the assignment of the relative configuration as shown in spectrum (S-37). On the basis of the above information, galphimine C was identified as the novel compound 7α,24-diacetoxy-13α-carbomethoxy-1β,2β-epoxy-18β-hydroxy-27,30-bisnor-3,4-secofriedela-20(29)-en-3,4R-olide.

2.3.2.3.1.3 Galphimine D

The molecular formula of galphimine D, was determined to be C_{36} H_{50} O_{12} , $[M + H]^+$, m/z 674 by HRFABMS. ^1H and ^{13}C NMR spectra (S-38 and S-39) closely resembled those of the nor-triterpenoid galphimine C, except for the presence of additional signals of acetoxyl group [δ_H 2.05, s, CH_3CO_2 ; δc 21.23, 170.20] and the methine bearing this group [δ_H 5.56, dd, J = 12.3, 7 Hz; δc 70.94]. The location of the acetoxyl group at C-15 in the moiety was established by the analysis of the cross-peaks observed between H-15 and H-16 in the COSY-45 spectrum (S-40) of galphimine D. The assumption was confirmed by analysis of HMQC (S-41), HMBC and COLOC (S-42) experiments. ^1H - ^{13}C long range observed in COLOC and HMBC experiments showed as important features, a correlation of the signal of C-18 → C-28 → C-19; C-26 → C-10; C-5 → C-23 → C-10 → C-1. Finally, the stereochemistry of acetoxyl group at C-15 in galphimine D was determined by a NOESY spectrum (S-43). NOE's were observed between H-8 and H-11, thus H-15 is α and axial and 15-OAc must be β ecuatorial. On the basis of the above information galphimine D was identified as the novel compound 7α,15β,24-triacetoxy-13α-carbomethoxy-1β,2β-epoxy-18β-hydroxy-27,30-bisnor-3,4-secofriedela-20(29)-en-3,4R-olide.

2.3.2.3.1.4 Galphimine E

The molecular formula of galphimine E was determined to be $C_{38}H_{52}O_{14}$ $[M + H]^+$, m/z 733 by HRFABMS, and was consistent with an additional acetoxyl group in comparison with glaucamine D. This was corroborated by ^1H and ^{13}C NMR experiments (S-44 and S-45) comparison of galphimine E, with those of galphimine D. The NMR data of galphimine E was very similar to galphimine D, except for the presence of an additional acetoxyl group signal (δ_H 1.98, s, CH_3CO_2; δc 22.64, 171.49) and the methine bearing this group (δ_H 5.6, dd, J = 12.7, 4; δc 76.32). The position of the additional acetoxyl group at C-12 was determined by the analysis of COSY-45 (S-46), which showed cross-peaks between the protons H-11 (δ_H 0.97, dd, J = 12.6, 4 Hz and 2.98, t, J = 12.6 Hz; δc 35.26) and H-12 (δ_H 5.6, dd, J = 12.7, 4; δc 76.32). The above features were confirmed with HMQC (S-47), HMBC and COLOC (S-48) experiments. The ^1H - ^{13}C long range correlations , observed in COLOC experiment showed the following sequence of carbons: C-18 → C-28 → C-19; C-26 → C-10; C-5 → C-23 → C-10 → C-1. Furthermore, the NOESY spectrum (S-49) of galphimine E, showed the interactions between the following protons: H-12 / H-11 / 25-H_3 / 26-H_3, this allowed to conclude that the proton bearing the acetoxyl group at C-12 was β and axial and the acetoxyl group must be ecuatorial and α orientated. On the basis of the above information galphimine E was identified as the novel compound 7α,15β,12α,24-tetra-acetoxy-13α-carbomethoxy-1β,2β-epoxy-18β-hydroxy-27,30-bisnor-3,4-secofriedela-20(29)-en-3,4R-olide.

2.3.2.3.1.5 Glaucamine

The molecular formula of glaucamine was ascertained to be C_{34} H_{52} O_{10} $[M + H]^+$, m/z 621 by high resolution FAB-mass spectrometry. The IR spectrum of glaucamine showed as main features the presence of hydroxyl (3500 cm^{-1}) and ester (1720 cm^{-1}). The NMR data (Tables 2.4) of glaucamine resembles those of galphimine C-E, except for the absence of the lactone moiety in ring A. Analysis of the ^1H and ^{13}C NMR spectra (S-50 and S-51) of glaucamine revealed signals for four methyl groups [1.02 (s), 1.18 (s), 1.27 (s), 1.35 (d, J = 7.16 Hz)], two acetoxyl groups [δ 2.04 (s) and 2.11 (s)], one methoxyl [δ_H 3.65 (s); δc 51.37, 174.87] group, two secondary hydroxyl groups [δ 3.31, d, J = 8.36 Hz; 3.98, t, J = 8.8 Hz], one exomethylene group [δ_H 4.79, 4.81 (each 1H, s); δc 110.45,

144.07], one methylene bearing one acetoxy group [δ_H 4.5, 4.73 (each, d, J = 12.5 Hz); δc 65.11], and one methine bearing one acetoxy group [δ_H 4.93, brd, J = 2.2 Hz; δc 74.75]. Further, the presence of one tertiary hydroxyl group (δc 79.87) and the absence of the lactone moiety was revealed by DEPT and HMQC spectra (S-51 and S-53). The above spectral analysis pointed to a basic skeleton of a nor-friedelane type for glaucamine. Localization of acetoxyl groups at C-3 and C-24, the secondary hydroxyl groups at C-6 and C-7, and the methoxyl group at C-22 was made possible by ^1H and ^{13}C chemical shifts, multiplicity of the signals, ^1H - ^1H 2D homonuclear, and ^1H - ^{13}C 2D heteronuclear correlations. The long-range C-H correlation observed in COLOC spectrum (S-54) enabled determination of the following carbon sequence: C-3 → C-4 → C-5; C-19 → C-20→ C-21; → C-16 → C-14; C-17 → C-18 → C-22 → C-28; C-25 → C-9 → C-10 → C-8. Further the carbon sequence C-1 → C-2 → C-3; C-6 → C-7 → C-8; C-11 → C-12; C-15 → C-16; and C-20 → C-21 → C-22, was disclosed by ^1H - ^1H 2D homonuclear COSY-45 (S-52) correlations arising from the geminal and vicinal spin coupling constants between involucrated protons. The stereochemistry of glaucamine was determined by the coupling constants observed between the protons and confirmed by the NOESY spectrum (S-55). Cross-peaks were observed between: H-3 and H-2, H-4, H-23; H-29 and H-19, H-21; H-24 and H-25, H-7; H-7 and H-26, H-25, H-24; H-28 and H-22; and H-22 and H-21. On the basis of the above information glaucamine was identified as the novel compound 3β,24-diacetoxy-6β,7α,13α,18β-tetra-hydroxy-27,30-bisnor-friedela-20(29)-en-22α-carboxylate.

It is interesting to note that galphimine C, galphimine D and galphimine E are naturally occurring 3,4-seco(3 → 4 lactone)-27,30-bisnor-triterpenoid of the friedelane series with a carbomethoxy group at C-13. The biosynthesis of galphimine C, galphimine D and galphimine E has not yet been investigated, however might involved a ring A cleavage through an enzymatically controlled Bayer-Villiger reaction as proposed by Baas (1985).

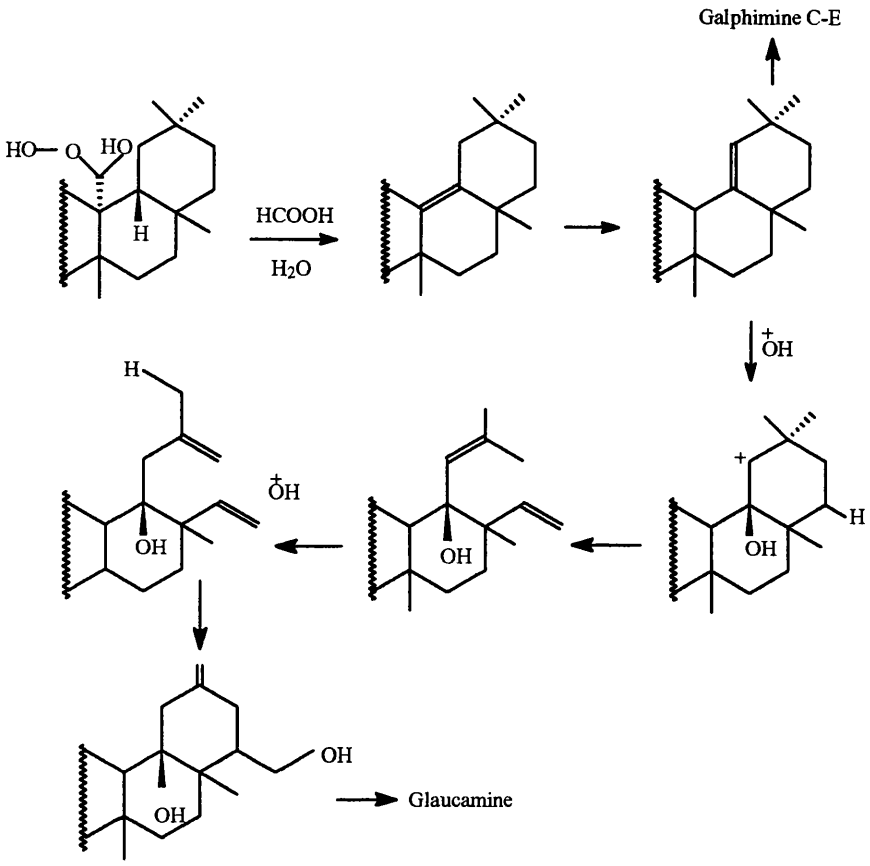


Figure 2.9 Putative Biosynthetic pathway of galphimine C, galphimine D, galphimine E and glaucamine

The biosynthesis of glaucamine has not yet been studied. However, it can be suggested that the secondary biomodification of the E ring might include elimination of the Me-20 by oxidative demethylation (as formic acid or decarboxylation) and methylation or formylation at C-22 (Torssell, 1983). Alternatively, this biotransformation might involve a ring E cleavage and induce biogenetic cyclization through the reactions suggessted in Figure 2.9. The other biosynthetic modifications observed in galphimine C → E and glaucamine (hydroxylation, olefinic groups, oxidation of the alcohol function to a carboxyl group, acetylation, methylation and epoxidation) are common processes of secondary metabolism (Torssell, 1983).

2.3.2.3.1.6 Stigmasterol

The molecular formula of stigmasterol (C_{29} H_{48} O) was deduced from EIMS, and DEPT spectrum (S-57). The ^{1}H and ^{13}C NMR spectra (S-56 and S-57) displayed signals typical of a tetracyclic terpenoid with six methyl groups [δ 0.68 (s), 0.81 (s), 0.83, 0.84 (s), 0.92 (d, J = 5 Hz), 1.00 (s)]; one methine bearing an hydroxyl group [δ_H 3.52, m; δc 72.22]; and one trisubstituted olefinic proton [δ_H 5.35 (d, J = 5.12 Hz); δc 122.15, 139]. The physical and spectroscopic data of stigmasterol (3β,22E-stigmasterol-5,22-dien-3-ol) was in agreement with literature (Rubinstein *et al.*, 1976).

2.3.2.3.7 Sitosteryl-3-O-β-D-glucopyranoside

Sitosteryl-3-O-β-D-glucopyranoside had a molecular formula of C_{35} H_{60} O_6, deduced from FABMS, and DEPT spectrum (S-59). The glycoside respond positively to Molisch test and showed IR absorptions bands at 3420 (hydroxyl), 1634 (insaturation) and 1077 (glycosidic linkage) cm^{-1}. The ^{1}H and ^{13}C NMR spectra (S-58 and S-59) of this glycoside showed six methyl groups [δ 0.68 (s), 0.81 (s), 0.83 (d, J = 2 Hz), 0.85 (s), 0.93 (d, J = 6.48 Hz), 1.014 (s)]; one trisubstituted olefinic proton [δ_H 5.35 (d, J = 5.12 Hz); δc 122.43, 140], displaying significant resemblance with those for βsitosterol (Khan *et al.*, 1997). Further, the existence of one anomeric proton [δ_H 4.41, d, J = 7.8 Hz, H-1'; δc 101.36], together with six oxymethines of the sugar portion [δ_H 3.24 - 4.41]; and one isolated oxymethine [δ_H 3.58, m; δc 79.41], reflected its glycosidic nature. A comparative study of the ^{1}H and ^{13}C NMR chemical shifts data for this compound with those observed for β-sitosterol were consistent with the position of the glucose at C-3. (Khan *et al.*, 1997). Acid hydrolysis of glycoside with 2 N HCl yielded β-sitosterol and glucose, which were detected by TLC in direct comparison with authentic samples. The spectroscopic data of sitosteryl-3-O- β-D-glucopyranoside was in agreement with literature (Khan *et al.*, 1997).

2.3.3 *Guarea rhopalocarpa* Radlkofer (Meliaceae)

The family Meliaceae is a tropical and subtropical family comprising 50 genera and more than 1000 species. It is known to contains limonoids (Connolly, 1983; Taylor, 1983) and tetraterpenoids (Banerji & Nigam, 1984). A number of limonoids have been shown to have antimalarial (Bray *et al.*, 1990) and cytotoxic activities *in vitro* (Pettit *et al.*, 1993). The genus *Guarea* has 20 species distributed in Africa, and 150 species in tropical America, and 8 species are found in Panama (Schultes & Raffouf, 1990). Chemically six species have been studied they include, *G. carinata*, *G. cedrata*, *G. glabra*, *G. kunthiana*, *G. thompsonii*, and *G. trichilioides*. They contain diterpenoids (Pereira *et al.*, 1990), triterpenoids (Akinniyi *et al.*, 1980; Ferguson *et al*, 1975; Moutoo *et al.*, 1992; Furlan *et al.*, 1993; Lukacova *et al.*, 1982), and steroids (Zelnik & Rosito, 1971). Biologically, some species have been studied for example *G. guidania* showed antiinflammatory activity in rats (Oga *et al.*, 1981); *G. sepium* displayed activity against various species of helminths including *Strongiloides stercoralis*, *Ancylostoma caninum*, and *A. duodenale* (Gilbert *et al.*, 1972), and *G.trichilioides* showed cytotoxic activity *in vitro* against P388 murine cell line (Lukacova *et al.*, 1982). *Guarea rhopalocarpa*, syn.*Guarea tuisiana* is a tree of lowland tropical rain forest and montane rain forest in Costa Rica and Western and Central Panama. There is no previous chemical studies on this plant. Recent studies hace been shown that the methanolic extract possessed cytotoxicity against KB cells and toxicity towards brine shrimp (Solis, 1994). Thus, it was decided to investigate this plant further in order to isolate bioactive compounds.

2.3.3.1 Extraction and Isolation

Dried, ground leaves (650 g) of *Guarea rhopalocarpa* were extracted following the procedure described in Figure 2.1. The extracts were evaluated for activity against *L. donovani* promastigotes. The $CHCl_3$ soluble fraction in which, the activity was concentrated was repeatedly chromatographed on silica gel CC, with Hex-acetone gradient as eluent to yield the novel bioactive terpenes named: 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one (63 mg), lanosta-7,9(11),24EZ-triene-3α,23-diol (1 g), ent-8(14),15-sandaracopimaradiene-2β,18-diol (45 mg), and ent-8(14),15-sandaracopimaradiene-2α,18-diol (35 mg). The coumarin scopoletin (25.12 mg), and

the steroid stigmasterol (22.6 mg) crystallized spontaneously from non-active fractions during the chromatographic procedure. Scopoletin was purified by Sephaex LH-20 in methanol and the other compounds were purified by re-crystalization in $CHCl_3$-MeOH. TLC plates were sprayed with 1% vanillin- 5% H_2SO_4.

2.3.3.2 Spectroscopic data

2.3.3.2.1 23-Hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, Gr7

Colourless needles (Methanol), mp. 95 °C. $[\alpha]_D$ -32.25 ($CHCl_3$, *c* 1) (λ = 584 nm; 26.6 °C). UV λ max nm: 250, 242, 235 (MeOH). IR ν max (KBr) cm^{-1}: 3400, 2980, 1710, 1450, 1380, 1050, 760. HRMS: 438.337000 (calcd 438.334131 $C_{30}H_{46}O_2$). EIMS m/z (rel. int.): 438 $[M]^+$ (5), 436 (55), 421 (100), 393 (34), 355 (7), 341 (32), 297 (40), 271 (18), 253 (16), 243 (11), 201 (12), 187 (17), 159 (16), 148 (42), 123 (29), 105 (28), 81 (41), 67 (15), 41 (18). ^1H and ^{13}C NMR data see Table 2.5 and Appendix S-60 - S-63.

2.3.3.2.2 Lanosta-7,9(11),24-triene-3α,23-diol, Gr11

Colourless needles (MeOH), mp. 105 °C. $[\alpha]_D$ - 20 ($CHCl_3$, *c* 1) (λ = 584 nm; 26.6 °C). IR ν max (KBr) cm^{-1}: 3400, 2980, 1440, 1380, 1220, 1060, 1000, 750. HRMS: 440.347000 (calcd 440.349781 $C_{30}H_{48}0_2$). EIMS m/z (rel.int.): 440 $[M]^+$ (25), 438 (76), 423 (28), 405 (30), 387 (6), 351 (3), 338 (4), 323 (6), 299 (10), 281 (9), 255 (14), 229 (7), 213 (7), 187 (10), 175 (10), 150 (100), 148 (38), 107 (41), 95 (48), 81 (18), 55 (14), 43 (12). ^1H and ^{13}C NMR data see Table 2.5 and Appendix S-64 - S-67.

2.3.3.2.3 Ent-8(14), 15-sandaracopimaradiene-2β,18-diol, Gr12

Colourless crystals (Methanol), mp. 156 °C. $[\alpha]_D$ = + 11.36 ($CDCl_3$, *c* 0.1) (λ = 584 nm; 26.6 °C). IR ν max (KBr) cm^{-1}: 3360-3340, 2989, 1450, 1390, 1100. HRMS: 304.243200 (calcd. 304.240231 $C_{20}H_{32}O_2$). EIMS m/z (rel.int.): 304 $[M]^+$ (43), 289 (14), 273 (100), 255 $[M-H_2O-CH_2OH]^+$ (86), 187 (34), 175 (26), 159 (17), 148 (26), 133 (46), 121 (87), 105 (72), 91 (96), 79 (56), 67 (39), 55 (98). ^1H and ^{13}C NMR data see Table 2.6 and Appendix S-68 - S-73.

2.3.3.2.4 Ent-8(14), 15-sandaracopimaradiene-2α,18-diol, Gr15

Colourless crystals (Methanol), mp. 182 °C. $[\alpha]_D$ = + 8.77 ($CDCl_3$, *c* 0.1) (λ = 584 nm; 26.6 °C). IR ν max (Kbr) cm^{-1}: 3360-3340, 2980, 1450, 1380, 1090. HRMS: 304.243200 (calcd. 304.240231 C_{20} H_{32} O_2). EIMS m/z (rel.int.): 304 $[M]^+$ (4), 286 (33), 271 (16), 255 (36), 241 (16), 227 (3), 213 (5), 199 (7), 187 (100), 173 (12), 159 (16), 145 (21), 133 (37), 121 (83), 105 (64), 91 (72), 79 (44), 67 (27), 55 (63). ^1H and ^{13}C NMR data see Table 2.6 and Appendix S-74 - S-79.

2.3.3.2.5 Stigmasterol

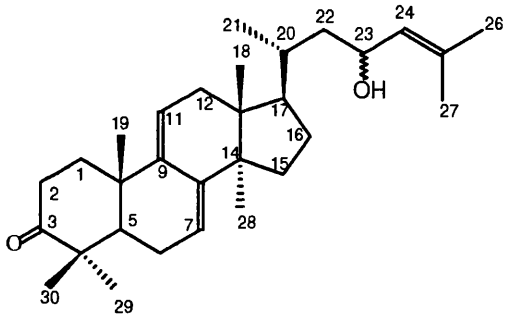
Physical and spectrosocopical data identical to those of secction 2.3.2.2.6.

2.3.3.2.5 Scopoletin

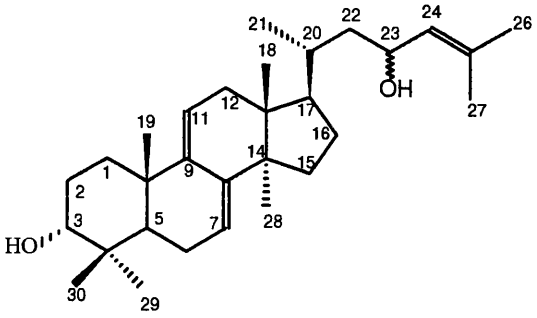
Yellow needles (Methanol), mp. °C . EIMS m/z (rel.int.): 192 (C_{10} H_8 O_4) $[M]^+$ (100), 181 (14), 177 (74), 169 (18), 164 (33), 157 (3), 149 (82), 141 (4), 135 (5), 131 (25), 121 (37), 115 (5), 105 (7), 100 (8), 93 (9), 79 (43). ^1H NMR (400 MHz, $CDCl_3$) δ: 3.95 (s, 7-OCH_3), 6.24 (d, J = 9.5 Hz, H-3), 6.89 (s, H-5), 6.91 (s, H-8), 7.56 (brs, 6-OH), 7.66 (d, J = 9.4 Hz, H-4). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 103.35 (C-8), 108.10 (C-5),111.43 (C-4a), 113 (C-3), 143.69 (C-4), 144.74 (C-8a), 150.28 (C-6), 150.41 (C-7), 161.63 (C-2). See Appendix S-80 - S-82.

2.3.3.3 Results and Discussion

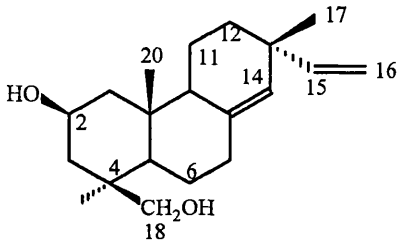
Bioactive guided fractionation of methanolic extract of *Guarea rhopalocarpa* using *L. donovani* promastigotes forms, led to the isolation of the new active terpenoids identified as 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one (Gr7), lanosta-7,9(11),24EZ-triene-3α,23-diol (Gr11), ent-8(14),15-sandaracopimaradiene-2β,18-diol (Gr12), and ent-8(14),15-sandaracopimaradiene-2α,18-diol (Gr15), together with the known coumarin scopoletin and the steroid stigmasterol, which precipitated from non-active fractions. The structures of the isolated compounds from *Guarea rhopalocarpa* are shown in Figure 2.10.



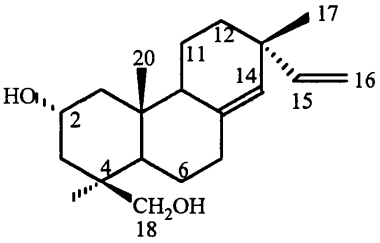
23-Hydroxylanosta-7,9(11),24EZ-triene-3-one



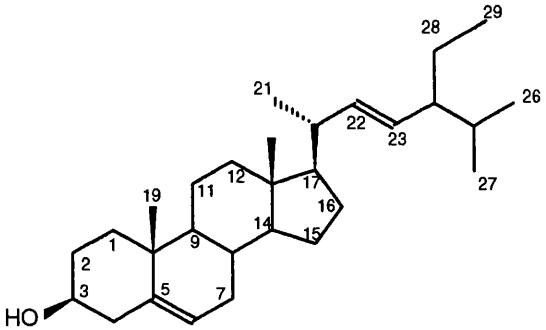
Lanosta-7,9(11),24EZ-triene-3α,24-diol



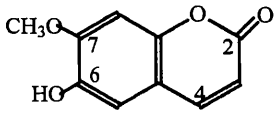
Ent-8(14),15-sandaracopimaradiene-2β,18–diol



Ent-8(14),15-sandaracopimaradiene-2α,18–diol



Stigmasterol



Scopoletin

Figure 2.10 Structure of compounds isolated from *Galphimia glauca*

2.3.3.3.1 Structure elucidation and identification

2.3.3.3.1.1 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, Gr7.

Compound Gr7 gave a molecular peak at m/z 438 (C_{30} H_{46} O_2), and showed absorptions in the UV spectra at 250, 242, 235 nm (MeOH) characterisitic of the presence of a transoid heteroannular diene moiety. The IR spectrum showed absorptions typical of

hydroxyl (3400 cm^{-1}) and keto (1700 cm^{-1}) groups. Analysis of NMR data (Table 2.5), indicated that Gr7 was a lanostane type triterpenoid (Shiao, *et al.*, 1988; Hirotani *et al.*, 1987; Gewali *et al.*, 1990; De Pascual *et al.*, 1987; Furlan *et al.*, 1993; Toth *et al.*, 1983), possessing a heteronuclear diene moiety, one allylic hydroxyl group and one keto group.

The ^{1}H and ^{13}C NMR spectra (S-60 and S-61) showed the presence of five tertiary methyl [δ_H 0.77, 0.85, 0.90 x 2 and 0.92 (each, s, 3H)], one secondary methyl (δ_H 0.98, d, J = 6.4 Hz), and two olefinic methyl (δ_H 1.70, 1.72) groups, as well as one triplet of doublets (δ_H 2.71; δc 38.87) characteristic of the axial H-2 proton, when a keto group is present at C-3, stablishing the position of the keto group at C-3 (δc 217). Furthermore, the signals due to one allyl oxymethine group (δ_H 4.78, 4.82; δc 74,76), three olefinic protons (δ_H 5.14-5.32; δc 101-146) signals were observed and these were duplicated, indicating that Gr7 was a mixture of $^{24}\Delta$ epimers. This was confirmed by GC-EIMS analysis which showed a mixture of two epimers at a ratio 8: 2. The EIMS of Gr7 exhibited two abundant mass fragments, one at m/z 313 $[M\text{-}C_8 H_{15} O]^+$ due to loss of the side chain, and another fragment at m/z 271, which represents further loss of 42 amu due to the loss of carbons at positions C-15, C-16 and C-17 (Figure 2.11).

The side chain loss of 127 amu ($C_8 H_5 O$) indicated that one trisubstituted alkene group and one allyl oxymethine were present in the side chain. Consequently, the heteroannular diene was in the ring system. The presence of the two olefinic methyl groups (δ_H 1.70, 1.72) in the ^{1}H NMR spectrum (S-60), indicated that the double bond in the side chain was at C-24 position. This, was further confirmed by the cross-peaks observed in the COSY-45 spectrum (S-62) for 26-H_3, 27-H_3 with the olefinic H-24, which in turn was coupled to the oxymethine at C-23 (δ_H 4.78, 4.82; δc 74,76), which in turn was coupled to the H-22. Complete ^{1}H and ^{13}C assignment was achieved by HMQC spectrum (S-63). On basis of the above information Gr7 was identified as the novel compound 23-hydroxy-lanosta-7,9(11),24EZ-triene-3-one.

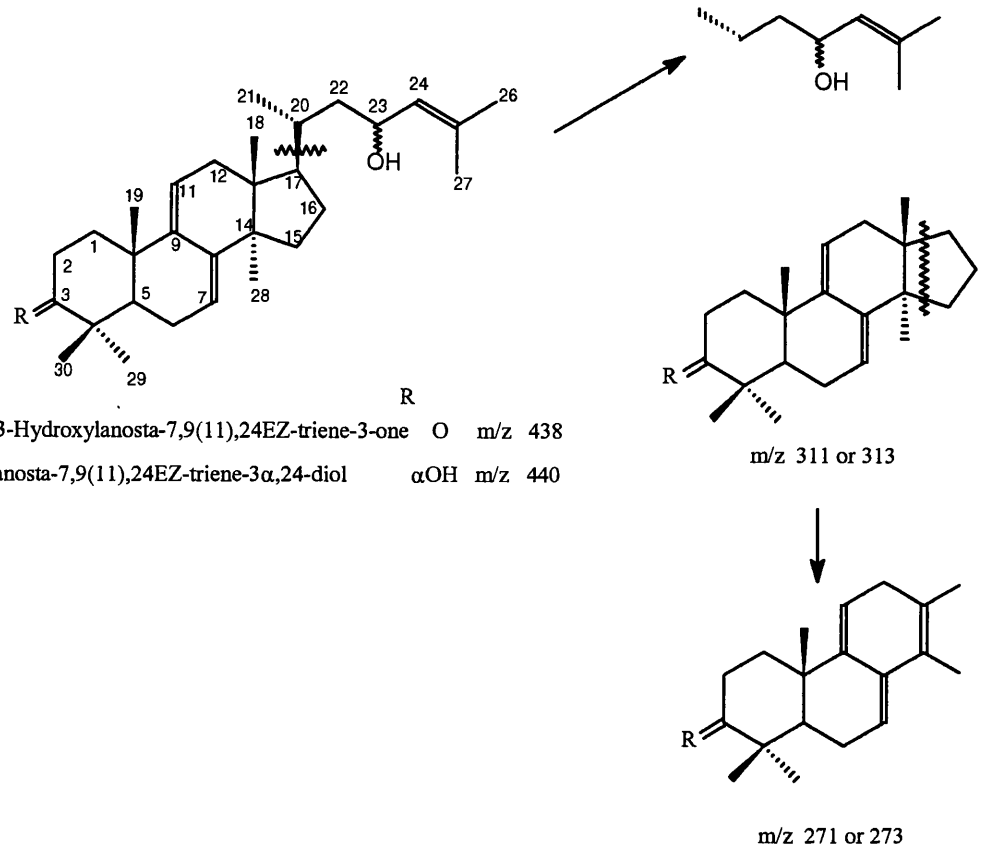


Figure 2.11 Mass spectral fragmentation of 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one and lanosta-7,9(11),24Ez-triene-3α,23-diol

2.3.3.3.1.2 Lanosta-7,9(11),24EZ-triene-3α,23-diol, Gr11

The compound Gr11, gave a molecular peak at m/z 440 (C_{30} H_{48} O_2), and absorptions in the UV spectrum at 235, 243, 251 nm (MeOH), indicating the presence of a heteroannular diene moiety in the molecule of Gr11 similar to that of 23-hydroxy-5α-lanosta-7,9(11),24-triene-3-one. Its IR spectrum showed hydroxyl (3400 cm^{-1}) and double bond groups (1620, 1640cm^{-1}). Comparison of ^{1}H and ^{13}C NMR spectra (S-64 and S-65) of Gr11 with those of 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one (S-60 and S-61) indicated that chemical shifts and coupling patterns for the olephinic protons, H-7, H-11, and H-24, were almost identical in both terpenes. However, the ^{1}H NMR spectrum of Gr11 showed a broad singlet at δ_H 3.46 brs (δc 76.30) of a α-hydroxyl group at C-3 instead of the carbonyl signal (δc 217) observed in 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one. Furthermore, cross-peaks in the COSY-45 spectrum (S-66)

Table 2.5 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHZ) data of 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene3-one and lanosta-7,9(11),24EZ-triene-3α,23-diol in $CDCl_3$

Position	23-hydroxy-5α-lanosta-7,9(11),24EZ-trien-3-one, Gr7 δ_H	lanosta-7,9(11),24EZ-triene-3α,23-diol, Gr11 δ_H	Gr7 δc	Gr11 δc
1	-	-	34.24	34.80
2	2.71 td (16,7)	1.9, 1.6 each, m	38.87	34.83
3	-	3.46 brs	217.33	76.30
				76.20*
4	-	-	51.39	50.36
5	2.36 m	2.36 m	50.96	48.43
6	1.80 m	1.55-2.0 m	24.77	23.91
7	5.28 m	5.25 m	118.48	118.13
8	-	-	146.21	145.98
9	-	-	146.08	145.85
10	-	-	35.31	34.80
11	5.32 d (3)	5.28 d (3)	101.82	101.44
12	2.0 m	2.20 m	35.50	34.83
13	-	-	44.10	43.67
14	-	-	48.27	48.43
15	-	-	31.95	33.78
16	-	-	27.78	27.34
17	-	-	52.76	50.56
18-CH_3	0.80 s	0.91 s	24.94	23.00
19-CH_3	1.04 s	0.85 s	25.02	25.00
20	-	-	27.68	27.32
21-CH_3	1.02 d (7)	0.98 d (7)	48.07	45.14
22	1.6, 2.15 each, m	1.6, 2.15 each, m	40.04	44.62
23	4.78, 4.82* each, m	4.78, 4.82* each m	74.37	73.90
			76.14*	75.66*
24	5.14 brd (7)	5.13 brd (7)	128.29	128.06
25	-	-	137.36	136.78
26 CH_3	1.70 d (0.6)	1.72 d (0.6)	26.92	25.84
27 CH_3	1.68 d (0.6)	1.70 d (0.6)	18.20	18.32
28-CH_3	1.002 s	0.77 s	13.15	12.92
29-CH_3	1.10 s	0.91 s	21.97	21.77
30-CH_3	0.90 s	0.92 s	22.99	27.77

Coupling constants (J in Hz). Assignments were based on DEPT, COSY-45, HMQC, and NOESY experiments. TMS was used as internal standard.

were observed for the following pair of signals: H-2/H-3; H-6/H-7; H-11/H-12; H-23/H-24. Complete assignation of ^{1}H and ^{13}C was carried out by HMQC spectrum (S-67). The duplicity of the signals for the olefinic protons indicated that Gr11 was a mixture of $^{24}\Delta$ epimers (1:1 ratio), this was confirmed with a GC-EIMS analysis of Gr11. On the basis of the above information Gr11 was identified as the novel compound lanosta-

7,9(11),24EZ-triene-3α,23-diol.

2.3.3.3.1.3 Ent-8(14),15-sandaracopimaradiene-2β,18-diol, Gr12

The HRMS of Gr12 showed a molecular ion peak at m/z 304 (C_{20} H_{32} O_2). The IR spectrum showed hydroxyl groups (3400-3000, 1080, 1045 cm^{-1}), and double bonds (1640, 915, 995 and 845 cm^{-1}). The ^{13}C NMR and DEPT spectra (S-69) revealed the presence of three methyl, six methylene, and two methine groups, three quaternary carbon atoms, one primary and one secondary hydroxyl group, and four olefinic carbons. Moreover, ^1H NMR spectrum (S-68) showed three tertiary methyl groups [δ 1.0, 1.04, and 1.07 (each, s H_3)], one AB system (δ 3.1, 3.3, 2H, dd, J = 11 Hz) of an α-equatorial C-4 hydroxymethyl, one proton geminal to a secondary hydroxyl group (δ 4.23, tt, J = 8, 4 Hz), and four olefinic protons. Three of the olefinic peaks were attributable to a monosubstituted olefin, constituting an ABX system (δ_A 4.87, δ_B 4.91, δ_C 5.77, J_{AB} = 1.4, J_{AX} = 10, J_{BX} = 17 Hz) , and one was isolated (δ 5.2 brs) and weakly coupled. ^1H and ^{13}C NMR signals assignment was achieved by COSY-45 (S-70), HMQC (S-71), HMBC (S-72) and COLOC spectra, which allowed the assumption of a 8(14),15-sandaracopimaradiene-18-ol, substituted with a secondary hydroxyl group (Van Puyvelde *et al.*, 1987; Candy *et al.*, 1970). The chemical shifts and vicinal coupling constant observed for the proton geminal to the secondary hydroxyl group in Gr12 (δ 4.23, tt, J = 8, 4 Hz), together with cross-peaks observed along the CH_2-1, CH_2-2 and CH_2-3, in the COSY-45 spectrum (S-70) led to the conclusion that the hydroxyl group was located at C-2, of a rigid chair-ring A, and had β-axial configuration. The position of the hydroxyl group was confirmed with the long-range proton-carbon correlation HMBC and COLOC experiments (S-72) which showed the following correlations: 19-H_3 *vs* C-4, 3-H_2, 18-H_2; 20-H_3 *vs* C-9, 1-H_2. The relative configuration of Gr12 was confirmed by NOESY spectrum (S-73). Cross-peaks were observed between the following protons: H-2 / 19-H_3 / 20-H_3; H-16 / H-15 / CH_3-17; 17-H_3 / H-14; and H-14 / H-7. Furthermore, the chair form of the B-ring with an exocyclic double bond was indicated by the vicinal axial-equatorial coupling J (ae) of 4 Hz and J (ee) of 2 Hz along the H-6-H-7 bond and the cross-peaks observed in the COSY-45 spectrum for the following resonances: H-14, H-9 and H-7α, but not to H-7β, which resides in the plane

of the double bond. The vicinal coupling (J = 7 Hz) along the H-9 to H-11 bond, and the cross-peaks in the COSY-45 spectrum are consistent with a non-chair C-ring containing the double bond between C-8 and C-14. Correlations were observed in the HMBC and COLOC experiments for the following protons and carbons: 17-H_3, CH-13, CH-15, C-14, CH_2-12. On the basis of the above information, Gr12 was identified as the novel compound ent-8(14),15-sandaracopimaradiene,2β,18-diol.

Table 2.6 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data for ent-8(14),15-sandaracopimaradiene-2β,18-diol (Gr12) and ent-8(14),15-sandaracopimaradiene-2α,18-diol (Gr15) in $CDCl_3$.

Position	Gr12	Gr15	Gr12	Gr15
	δ_H		δc	
1	1.55, 1.80 each, m	0.98 t (11.8) 2.06 td (11, 7.1)	45.10	47.84
2	4.23 tt (8,4)	3.86 tt (14, 3.6)	67.86	65.05
3	1.52, 1.74 each, m	1.39, 1.6 each, dd (11, 4)	40.59	44.2
4	-	-	37.83	39.40
5	1.38 m	1.37 m	46.69	46.89
6	1.38, 1.55 each, m	1.26, 1.48 each, m	22.92	22.10
7	2.11, 2.24 each, dd (4.2)	2.11, 2.24 each, dd (4, 2)	36.04	35.51
8	-	-	137.02	136.39
9	1.77 m	1.83 t (7.7 Hz)	51.29	50.51
10	-	-	38.27	39.62
11	1.50, 1.62 each, m	1.52, 1.63 each, m	19.19	18.78
12	1.36, 1.50 each, m	1.34, 1.49 each, m	34.84	34.46
13	-	-	37.59	37.45
14	5.24 brs	5.25 brs	129.49	129.35
15	5.77 dd (17,10)	5.77 dd (17.48, 10.6)	149.16	149.00
16	4.87 dd (10, 1.48) 4.91 dd (17, 1.48)	4.87 dd (10, 1.48) 4.91 dd (17, 1.48)	110.62	110.16
17-CH_3	1.04 s	1.04 s	26.56	25.92
18	3.10, 3.36 each, d (11)	3.10, 3.35 each, d (11)	71.61	71.28
19-CH_3	1.004 s	0.83 s	20.50	18.95
20-CH_3	1.07 s	0.87 s	18.88	16.50

Coupling constants (J in Hz). TMS was used as internal standard. Assignments were based on DEPT, COSY-45, HMQC, HMBC, and NOESY experiments.

2.3.3.3.1.4 Ent-8(14),15-sandaracopimaradiene-2α,18-diol, Gr15

The high resolution EIMS of Gr15, showed a molecular ion peak at m/z 304 (C_{20} H_{32} O_2), and the fragment ion at m/z 255 $[M\text{-}H_2O\text{-}CH_2O]^+$, as was observed for ent-8(14),15-sandaracopimaradiene,2β,18-diol, suggesting that both diterpenes were stereo- or positional isomers (Van Puyvelde *et al.*, 1987). Comparison of ^{1}H and ^{13}C NMR as

well as HMQC spectra (S-74, S-75 and S-77) of Gr15 with those of ent-8(14),15-sandaracopimaradiene,2β,18-diol, supported the above suggestion.

The chemical shifts and vicinal coupling constant observed for the proton geminal to the secondary hydroxyl group in Gr15 (δ 3.86, dd, J = 14, 3.6 Hz), and the tertiary methyl groups 19-H_3 (0.85, s), 20-H_3 (0.90, s), together with cross-peaks observed along the CH_2-1, CH_2-2 and CH_2-2, CH_2-3, in the COSY-45 spectrum (S-76) led to the conclusion of an α-equatorial configuration of the hydroxyl group at C-2 in the A-ring in Gr15. The position of the hydroxyl group was confirmed with the long-range proton-carbon correlations observed in HMBC (S-78) and COLOC experiments. Finally, the relative configuration of Gr15 was confirmed by NOESY spectrum (S-79). Cross-peaks were observed among the following protons: H-2 / H-3; H-16 / H-15 / 17-H_3; 17-H_3 / H-14; and H-14 / H-7. On the basis of the above information, Gr15 was identified as the novel compound ent-8(14),15-sandaracopimaradiene,2α,18-diol.

2.3.3.3.1.5 Scopoletin

The most polar compound showed a ion molecular at m/z 192 (C_{10} H_8 O_4) and exhibited IR signals characteristic of a coumarin with absorptions at 3420 (hydroxyl), 1719, 1709 (δ lactone) and 1613, 1557 and 1509 (aromaticity) cm^{-1}. The ^1H NMR spectrum (S-80) of the coumarin revealed an AB system (δ 6.24 and 7.66 each d, J = 9.4 Hz, for H-3 and H-4 respectively) characteristic of the protons of a δ-lactone (δc 161, S-81), and two singlets (δ 6.89 and 6.91) for the para-protons of a benzenoid nucleous, and a singlet at δ 6.91 assigned to H-8. The above information indicated that the coumarin could be scopoletin or isoscopoletin. Thus, a NOESY spectrum (S-82) was carried out to determine the position of methoxy and hydroxy groups on the coumarin. Interactions were observed between the MeO group at C-6 and H-5, which in turn interacted with H-4. This data confirmed that the coumarin was scopoletin (6-Hydroxy-7-methoxy-coumarin). Its physical and spectroscopical data were in agreement with those reported in the literature (Arisawa *et al.*, 1983).

2.3.4 *Stephania dinklagei* Diels (Menispermaceae)

Stephania dinklagei is a climbing shrub of the deciduous forest of both East and West Africa. The roots and stems have been used medicinally in Ghana as: vermifuge, analgesic, aphrodisiac, sedative drug, as well as in the treatment of infertility in the female and impotence in the male and as fish poison. Previous studies on *S. dinklagei* have resulted in the isolation of isocorydine, dicentrine (Quevauviller & Serrazin, 1967), corydine, roemerine (Debray *et al.*, 1967), norcorydine, steporphine, 1,2-methylenedioxy-3-methoxyaporphine, stepharine, stephalagine, N-methylglaucine chloride (Dwuma-Badu *et al.*, 1980), and N-methyl-corydine chloride (Tackie *et al.*, 1974). Although, the chemical constituents of this plant have been reported several times, the antileishmanial and antitrypanosomal activitites of this plant and its constituents has not been reported. Thus, it was decided, to investigate this plant further.

2.3.4.1. Extraction and Isolation

Ground, air-dried stem parts of *Stephania dinklagei* (100 g) were extracted successively with MeOH and H_2O at room temperature. The extracts were monitored for antileishmanial and cytotoxicity activities *in vitro*. Both MeOH and H_2O extracts showed *in vitro* activity (Chapter 3), and it was decided to investigate the methanolic extract. The methanolic extract was subjected to flash CC on several silica gel Sorbil C 60-H columns using $CHCl_3$-MeOH mixtures of increasing polarity as eluent. The chromatographic separation was monitored by TLC sprayed with Dragendorff's reagent. Further chromatography of the active fractions on Sephadex LH-20 column chromatography in $CHCl_3$-MeOH 30:70 allowed eventually the separation of the following compounds as the active principles of this plant stepharandine (5.1 mg), the zwitterionic aporphinic alkaloids, N-methyl-liriodendronine (9 mg) and 2-O,N-dimethyl-liriodendronine (2 mg), and the oxoaphorphine alkaloids liriodenine (8.2 mg) and dicentrinone (21 mg). In addition, the aporphine alkaloid corydine (3 mg) and the anthraquinone aloe-emodin (8 mg) crystallized from two different non-active fractions.

2.3.4.2 Spectroscopic data

2.3.4.2.1 Stepharandine

Orange crystalline solid (MeOH), mp. > 280 °C. HREIMS m/z (rel.int.): 297.2200 (calcd 297.2202 C_{16} H_{11} O_5 N). EIMS m/z (rel. int.): 297 ($[M]^+$ (4), 296 (17), 285 (23), 264 (32), 256 (100), 236 (42), 228 (54), 220 (26), 213 (92), 206 (32). ^{1}H NMR data see Table 2.7 and Appendix S-83 and S-84.

2.3.4.2.2 Corydine

Colorless needles (Methanol), mp. 148-150 °C. UV λ max nm: 215, 260, 270, 302 (MeOH). IR ν max (KBr) cm^{-1}: 3220, 3480. EIMS m/z (rel.int.): 341 (C_{20} H_{23} O_4 N) $[M]^+$ (100), 326 (39), 310 (61), 298 (35), 267 (11). ^{1}H NMR data see Table 2.7 and Appendix S-85.

2.3.4.2.3 2-O,N-Dimethyl-liriodendronine

Dark-violet needles (Methanol), mp. 274-276 °C. UV λ max nm: 243, 269, 308, 399, 426, 589 (MeOH); λ max nm: 251, 286, 387, 473 (MeOH+HCl). IR ν max (KBr) cm^{-1}: 2950, 1630, 1610, 1575, 1520, 1270, 830. HRMS: 291.056112 (calcd 291.012118 C_{18} H_{13} O_3 N). EIMS m/z (rel.int): 291$[M]^+$ (59), 276 (48), 262 (29), 248 (6), 233 (21), 207 (14), 191 (11), 149 (100), 104 (25), 91 (19), 71 (87). ^{1}H NMR data see Table 2.7 and Appendix S-86 and S-87.

2.3.4.2.4 N-Methyl-liriodendronine.

Brown-reddish solid(Methanol), mp. 269-270 °C (dec). UV λ max nm: 270, 308, 475, 590 (MeOH). λ max nm: 247, 275, 350. IR ν max cm^{-1}: 2900, 1630, 1600, 1570, 1440, 1280, 830. HREIMS: 279.123116 (calcd 279.344011 C_{17} H_{11} O_3 N). EIMS m/z (rel.int): 279 $[M + 2]^+$ (100), 270 (11), 256 (14), 239 (10), 223 (23), 213 (13), 205 (21). ^{1}H NMR data see Table 2.7 and Appendix S-88.

2.3.4.2.5 Liriodenine

Yellow needles (Methanol), mp. 280 - 283 °C (dec). UV λ max nm: 246, 264, 301, 409 (MeOH); λ max nm: 243, 249, 275, 290, 412, 503, 568, 608 (MeOH+HCl). IR ν

Table 2.7 ^{1}H NMR (400 MHz, δ) data of aporphine alkaloids isolated from *Stephania dinklagei*

Position	Stepharandine CF_3COOD	Corydine $CDCl_3$	Liriodenine $CDCl_3$	Dicentrinone $CDCl_3$	Dicentrinone C_5D_5N	Dicentrinone CF_3COOD	2-O,N-Dimethyl-liriodendronine C_5D_5N	N-Methyl-liriodendronine $CDCl_3$
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	7.76 s	6.69 s	7.18 s	7.13 s	7.13 s	7.54 s	7.053 s	6.58 s
4	4.23 s	2.51 dd (12.6, 3.4) 2.67 d (16)	7.77 d (5.16)	7.75 d (6.3)	7.72 d (5.3)	8.44 d (6.3)	7.74 d (6.12)	7.14 d (6.9)
5	4.34 s	3.04 dd (14, 3.3)	8.89 d (5.16)	8.89 d (6.3)	8.87 d (5.3)	8.75 d (6.3)	8.19 d (6.12)	7.39 d (6.9)
6	-	-	-	-	-	-	-	-
6a	-	3.17 m	-	-	-	-	-	-
7	4.28 s	2.42 t (13) 2.93 d (13)	-	-	-	-	-	-
8	8.73	7.09 d (8.2)	8.59 dd (7.86, 1.3)	7.98 s	7.99 s	7.99 s	8.87 dd (6.8, 1.48)	8.38 d (8.1)
9	-	6.88 d (8.2)	7.57 td (8, 1.3)	-	-	-	7.46 td (7.48, 1.04)	7.45 t (7.7)
10	-	-	7.75 td (8, 1.5)	-	-	-	7.82 td (7.7, 1.63)	7.74 t (8.3)
11	-	-	8.65 d (8)	7.99 s	8.05 s	8.27 s	10.90 d (8.56)	8.38 d (8.1)
1,2 -CH_2O_2	6.86 s	-	6.37 s	6.37 s	6.34 s	6.69 s	-	-
6-NCH_3	-	2.54 s	-	-	-	-	4.71 s	-
2- OCH_3	-	3.91 s	-	-	-	-	3.91 s	4.39 s
9-OCH_3	-	3.92 s	-	4.01 s	3.99 s	4.13 s	-	-
10-OCH_3	-	3.90 s	-	4.08 s	4.02 s	4.19 s	-	-
11-OCH_3	-	-	-	-	-	-	-	-

Coupling constants (J in Hz). TMS was used as internal standard. Assignments were based on DEPT, COSY-45 and NOESY experiments.

max (KBr) cm^{-1}: 1390, 1440, 1655, 1045, 955, 615. FABMS (Matriz MNOBA) m/z (rel.int.): 276 (C_{20} H_{23} O_4 N) $[M + 1]^+$ (100), 258 (44), 242 (48), 226 (41), 212 (32), 196 (35). ^1H NMR see data Table 2.7. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 102.47 (1,2-OCH_2O-), 103.27 (C-3), 108.14 (C-1a), 123 (C-16), 124.26 (C-4), 127.36 (C-11), 128.59 (C-8), 128.84 (C-9), 131.30 (C-7a), 132.88 (C-11a), 133.94 (C-10), 135.72 (C-3a), 144.95 (C-5), 145.30 (C-6a), 147.95 (C-1), 151.74 (C-2), 182.50 (C-7). See Appendix S-89 and S-90.

2.3.4.2.4 Dicentrinone

Yellow needles (Methanol), mp. > 300 °C. UV λ max nm: 213, 272, 310, 352, 388, 430 (MeOH). IR ν max (KBr) cm^{-1}: 1655, 1590, 1640, 1052, 962. EIMS m/z (rel.int.): 335 (C_{19} H_{13} O_5 N) $[M]^+$ (88), 328 (23), 304 (19), 293 (16), 275 (18), 249 (13), 213 (9), 185 (9), 165 (27), 149 (100), 136 (52). ^1H NMR see Table 2.7 ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.16 (9-OCH_3), 56.31 (10-OCH_3), 102.38 (1,2-OCH_2O), 102.81 (C-3), 108.82 (C-11), 109.62 (C-8), 124.02 (C-4), 135.58 (C-3a), 144.83 (C-5), 149.52 (C-10), 153.88 (C-9). ^{13}C NMR (100 MHz, CF_3CO_2D) δ: 58.48 (9-OCH_3), 58.78 (10-OCH_3), 106.23 (C-3), 108 (1,2-OCH_2O-), 110.45 (C-), 112.30 (C-11), 112.40 (C-8), 124.48 (C-1a), 125.15 (C-7a), 129.26 (C-4), 132.24 (C-11a), 135.90 (C-5), 137.48 (C-3a), 146.13 (C-1b). See Appendix S-91 -S-93.

2.3.4.2.7 Aloe-emodin

Yellow needles (MeOH), mp > 270 °C. EIMS m/z (rel. int.): 270 (C_{15} H_{10} O_5) $[M]^+$ (100), 241 (82), 224 (14), 213 (10), 207 (14), 167 (15), 121 (15), 109 (11), 97 (16), 91 (25), 85 (38), 83 (57), 71 (42). Laser MS (rel. int.): 272 $[M + 2]^+$ (100). FABS (MNOBA matriz) m/z (rel. int.): 270 (93), 241 (100), 257 (13), 224 (16), 194 (19), 183 (14), 149 (81), 137 (42), 121 (43), 109 (49). ^1H NMR (400 MHz, DMSO-d_6 -$CDCl_3$) δ: 4.65 (t, J = 5.76 Hz, 11-OH), 4.77 (d, J = 5.24 Hz, 11-H_2), 7.30 (dd, J = 8.26, 0.84 Hz, 7-H), 7.37 (brs, 2-H), 7.69 (t, J = 8.16 Hz, 6-H), 7.84 (d, J = 0.76 Hz, 4-H), 7.83 (dd, J = 8.28, 0.76 Hz, 5-H). 12.13 (s, 1-OH), 12.07 (s, 8-OH). ^{13}C NMR (100 MHz, DMSO-d_6 -$CDCl_3$) δ: 76.93 (C-11), 114.46 (C-1a), 115.86 (C-8a), 117.96 (C-2), 119.92 (C-4), 121.36 (C-5), 124.56 (C-7), 133.39 (C-4a), 133.64 (C-5a), 137.05 (C-6),

153.38 (C-3), 162.40 (C-1), 162.92 (C-8), 181.83 (C-10), 192.59 (C-9). See Appendix S-94 and S-95.

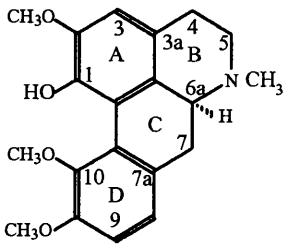
2.3.4.3 Results and Discussion

Bioactive guided fractionation of methanolic extract of stem parts of *Stephania dinklagei*, using *Leishmania donovani* promastigotes *in vitro* test led to the isolation of five alkaloids as the major active principles of this plant (see Figure 2.12). Three of them are new natural products: the aporphine alkaloid: stepharandine; the zwitterionic oxoaporphine alkaloids, N-methyl-liriodendronine and 2-O,N-dimethyl-liriodendronine, and the known oxoaporphine alkaloids, liriodenine and dicentrinone. From non-active fractions crystallized spontaneously the aporphine alkaloid corydine and the anthraquinone aloe-emodin. Apart from corydine, the isolated compounds have not been reported previously as constituents of *Stephania dinklagei*.

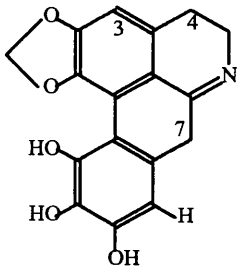
2.3.4.3.1 Structure determination and identification

2.3.4.3.1.1 Stepharandine

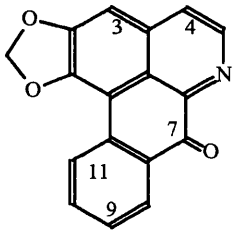
The molecular formula of stepharandine was established as $C_{16}H_{11}O_5N$ by HREIMS. Its UV spectrum displayed absorptions at 220, 250, 289, 420 nm (MeOH), which showed bathochromic shifts upon the addition of base and acid, indicating the phenolic nature of this alkaloid, and the presence of an imine function (Shamma, 1972; Mahiou *et al.*, 1994). The IR spectrum displayed absorptions for phenolic (3100, 1020 cm^{-1}), methylenedioxy (1070 and 950 cm^{-1}) and aromatic (1600, 1500 cm^{-1}) groups. The ^1H NMR spectrum (S-83) displayed six protons [δ 4.23, 4.29 and 4.34 (each 2H, s)] assignable to the three methylene units at C-4, C-5 and C-7, respectively. The aromatic region of the spectrum showed the presence of one 1,2-methylenedioxy group (δ 6.87, s, 2H) as a singlet, two isolated aromatic protons [δ 7.76 and 8.73 (each 1H, s)], assignable to the aromatic protons at C-3 and C-8 respectively, and three broad singlets (δ 5.72, 8.34 and 8.79) of phenolic groups.



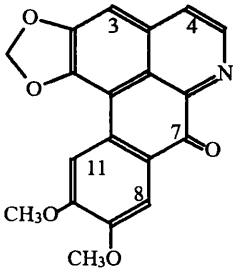
Corydine (S)



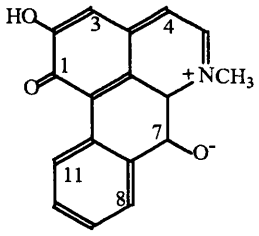
Stepharandine



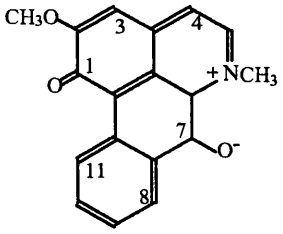
Liriodenine



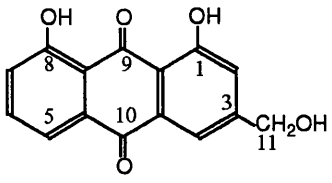
Dicentrinone



N-Methyl-liriodendronine



2-O,N-Dimethyl-liriodendronine



Aloe-emodin

Figure 2.12 Structures of compounds isolated from *Stephania dinklagei*

Analysis of the UV, IR and ^{1}H NMR data of stepharandine and a reviewing of ^{1}H NMR data of noraporphine alkaloids (Guinaudeau *et al.*, 1975; 1979; 1983; 1988; 1991) allowed the assumption of a noraporphine alkaloid with a methylenedioxy and three phenolic groups as substituents. Because of the small quantity of this alkaloid, ^{13}C NMR could not be done. However, a NOESY spectrum (S-84) allowed the location of methylenedioxy at C-1, C-2, on ring A, and the three phenolic at C-11, C-10, and C-9, on ring D. NOEs were observed for the following protons: H-3 / H-4 / H-7; and H-7 / H-9. Thus, in basis of the above information stepharandine was identified as the novel compound 9,10,11-trihydroxy-1,2-methylenedioxy-6,6a-dehydronoraphorphine alkaloid.

2.3.4.3.1.2 Corydine

The ^{1}H NMR spectrum (S-85) of corydine revealed seven aliphatic protons at δ 2.3 - 3.3, assigned to methylene units at C-4, C-5 and C-7, and to one methyne at C-6a, one singlet at δ 2.54 associated with NCH_3 group, and three aromatic methoxy groups at δ 3.74, 3.91, and 3.92. Further, the aromatic region of ^{1}H NMR spectrum showed one aromatic proton appearing as a singlet at δ 6.69 assigned to the isolated H-3, and a AB system of two ortho-coupled protons at δ 7.09 and 6.88 (each 1H, d, J = 8.2 Hz) assigned to H-8 and H-9. The spectroscopic data of corydine was in agreement with literature (Guinaudeau *et al.*, 1975, 1979).

2.3.4.3.1.3 2-Methyl-liriodendronine and 2-O,N-dimethyl-liriodendronine

Two oxo-aporphine zwitterion alkaloids present in small amounts were isolated and identified as 2-methyl-liriodendronine and 2-O,N-dimethyl-liriodendronine. These alkaloids had limited solubility in organic solvents, and high melting point. The $CHCl_3$ solution showed a blue and brown-reddish color respectively. Both, give a blue color in basic solution and red color when they are treated with mineral acids. The intense UV absorption band at λmax 308 nm observed for both alkaloids indicated that, they were zwitterionic oxo-aporphine alkaloids similar in structure to the corunnine-type alkaloid (Senter & Chen, 1977). On addition of acid, a hypsochromic shift of the absorption bands from those observed in ethanol was obtained. Furthermore, the absorption bands at 1630 and 1575 cm^{-1}, in the IR spectrum, correspond to a carbonyl with the corunnine-

type resonance forms: conjugated -C=O and -C=N+ (Ribas *et al.*, 1971). The ^{1}H NMR spectrum (S-86) of 2-O-N-dimethyl-liriodendronine revealed an +N-Me group at δ 4.71 and a OMe group at δ 3.91, and seven aromatic hydrogens assigned to: one isolated aromatic hydrogen (H-3) at δ 7.05, one AB quartet (each, d, J = 6.12 Hz) at δ 7.74 (H-4) and δ 8.19 (H-5), and one ABMX system [δ 10.89 (d, J = 8.56 Hz, H-11), δ 8.57 (dd, J = 6.8, 1.48 Hz, H-8), δ 7.82 (td, J = 7.7, 1.63 Hz, H-10), 7.46 (td, J = 7.48, 1.04 Hz, H-9)]. This assignment was corroborated with a COSY-45 spectrum (S-87). Cross-peaks were observed between H-11/H-10; H-8/H-9; H-9/H-10, and H-4/H-5. The ^{1}H NMR spectrum (S-88) of N-methyl-liriodendronine was almost identical to 2-O,N-dimethyl-liriodendronine, but with the absence of the O-methyl signal. Although, the zwitterionic oxo-aporphine alkaloids 2-O,N-dimethyl-liriodendronine and N-methyl-liriodendronine has been synthesised previously (Senter & Chen, 1977; Guinaudeau *et al.*, 1991; Saa *et al.*, 1976: Ribas & Castedo, 1971, Chen *et al.*, 1976), this is the first report of both alkaloids as plant-derived compounds.

2.3.4.3.1.4 Liriodenine and dicentrinone

The oxoaporphine alkaloids liriodenine and dicentrinone, in $CHCl_3$ solution showed a bright green-yellow fluorescence, and displayed a red coloration upon treatment with diluted mineral acids. Their UV spectra exhibited absorption bands above 350 nm which underwent a bathochromic hyperchromic shift in acid-basic medium, diagnostic of extensive conjugation of an oxoaphorphine chromophore (Chen *et al.*, 1976). Their IR spectrum showed absoptions for phenolic (3200, 3480, 1050 and 1300 cm^{-1}), aromatic (1600, 1500 cm-1) and carbonyl (1655 cm^{-1}) groups.

Comparison of ^{1}H NMR spectra (S-89 - S-92) of both liriodenine and dicentrinone, indicated that they possessed one singlet for 2-hydrogen atoms of the C-1 and C-2, methylenedioxy ring at δ_H 6.37 δc 102.38), one singlet at δ 7.1 assigned to the isolated aromatic hydrogen H-3 in ring A, and the signals of H- 4 and H-5, which resonated as a system of two doublets ($J_{4,5}$ = 5 Hz) at δ 7.7 and δ 8.8. The main differences observed in the ^{1}H NMR spectra were those for the ring-D. Liriodenine showed the presence of four protons of a ABMX (δ_A 8.65, d, J = 8 Hz; δ_B 8.59, dd, J = 7.8, 1.3 Hz; δ_M 7.75, td,

J = 8, 1.5; δ_X 7.57, td, J = 8, 1.3 Hz) spin-system of an unsubstituted ring D. Whereas, dicentrinone showed two aromatic protons at δ 7.98 and 7.99 (each, s, 1H) in para disposition of a tetrasustituted aromatic ring. The downfield signal at δ 7.99 observed for dicentrinone was indicative of H-11, which normally appears the farthest down-field (Chen *et al.*, 1976). Further, the ^{1}H NMR showed also two aromatic methoxy groups at δ_H 4.13; δc 56.16 and 4.19; δc 56.30, located at C-9 and C-10 in the ring D for dicentrinone. The exact position of methoxy groups in dicentrinone was confirmed by NOESY spectrum (S-93). Interactions were observed between the signal at δ 4.01 (9-OCH_3) and 7.98 (H-8), and the signal at δ 4.08 (10-OCH_3) and 7.99 (H-11). The spectroscopic data of liriodenine and dicentrinone was in agreement with literature (Cava et al., 1971; Chen *et al.*, 1976; Guinaudeau, 1994; Wu *et al.*, 1988, 1990).

2.3.4.3.1.5 Aloe-emodin

Aloe-emodin exhibited a molecular peak at m/z 270 corresponding with the molecular formula C_{15} H_{10} O_5 and its ^{1}H and ^{13}C NMR spectra (S-94 and S-95) were typical of anthraquinones. The COSY-45 spectrum (S-96) showed the correlation between the two signals assigned to CH_2OH at δ4.67 (t, J = 5.5 Hz, 11-OH), and 4.76 (d, J = 5.5 Hz, CH_2OH), and the second signal correlated also with the signals at 7.37 (brs, H-2), and 7.84 (brs, H-4). Furthermore, an ABX system of three methine protons was observed at δ 7.30 (dd, J = 8.26, 0.84 Hz, H-7), 7.69 (t, J =8.16, 7.72, H-6), and 7.83 (dd, J = 8.28, 0.76 Hz, H-5). Analysis of NOESY spectrum revealed cross-peaks between hydroxyl group at C-2 and the protons H-2 and H-4. ^{1}H and ^{13}C assignments were confirmed with HMQC spectrum (S-97). The HMBC spectrum showed long-range correlations with the following signals: H-2 (δ 7.17) *vs* C-1(δ 162.40), C-3(δ 153.38).

2.3.5 *Triclisia patens* Oliv. (Menispermaceae)

Triclisia patens is a woody climber of the forest and coastal areas of Ghana. *Triclisia* species have been used in the treatment of malaria, diarrhoea, pyorrhea, swelling in the extremities, anaemia, and joint pains as well as arrow poison. Previous studies on *T. patens* have reported the presence of a number of bisbenzylisoquinoline alkaloids including: phaeanthine (Dwuma-Badu *et al.*, 1975a), N,N-dimethyl-phaeanthine, cocsuline, pycnamine (Tackie *et al.*, 1974), trigilletimine (Dwuma-Badu *et al.*, 1975 b), and isotetrandrine (Marshall *et al.*, 1991), as well as the oxo-aphorphine alkaloid O-methyl-moschatoline (Dwuma-Badu *et al.*, 1975a). Some of the above BBIQ alkaloids possess a number of biological activities (Shiff, 1991; Marshall *et al.*, 1994). Since *T. patens* was found to be the most active against *Leishmania donovani* promastigotes, it was decided to study this species further.

2.3.5.1 Extraction and Isolation

Dried and powdered leaves (150 g) of *T. patens* were extracted subsequently with MeOH and H_2O at room temperature. The methanolic extract (6.5 g) was treated with 1% HCl, and the acidic solution was basified with NH_4OH to pH = 9, then extracted exhaustively with $CHCl_3$. Evaporation *in vacuo* of the $CHCl_3$ solution gave the crude alkaloid extract (1.10 g). The crude alkaloid extract was flash chromatographed on silica gel Sorbil C 60-H, and eluted with a gradient of $CHCl_3$ - MeOH. The compounds were monitored on TLC under UV and sprayed with Dragendorff reagent. Further rechromatography of the active fractions (F 1-3) using PTLC on silica gel and $CHCl_3$-MeOH (95:5) as eluent, followed by purification on Sephadex LH-20 column chromatography in MeOH, allowed eventually the separation and purification of two major active BBIQ alkaloids aromoline (2.7 mg) and phaeanthine (13 mg).

2.3.5.2 Spectroscopic data

2.3.5.2.1 Phaeanthine

Colorless needles ($CHCl_3$-MeOH), mp. 220-221 °C. UV λ max nm: 208, 237 (sh), 280 (MeOH). IR ν max (KBr) cm^{-1}: 3500, 1640, 1500, 1312. EIMS m/z (rel.int.): 622 (C_{38} H_{42} O_6 N_2) $[M]^+$ (67), 485 (79), 431 (34), 396 (12), 395 (66), 381 (58), 364 (80), 349

(56), 198 (15), 192 (9), 175 (11). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.33 (s, 2-NCH_3), 2.41 (dd, J = 15.3, 5.6 Hz, H-4'), 2.52 (d, J = 13.6 Hz, H-15' *ax*), 2.62 (s, 2'-NCH_3), 2.75 (d, J = 13.6 Hz, H-15' *ec*), 2.80 (d, J = 13.8 Hz, H-15 *ax*), 2.95 (d, J = 15 Hz, H-4), 3.19 (s, 7-OCH_3), 3.25 (dd, J = 12.5, 5.6 Hz, H-15), 3.43 (m, H-3'), 3.52 (m, H-3), 3.74 (brs, H-1'), 3.75 (s, 6-OCH_3), 3.87 (dd, J = 10.9, 5.6 Hz, H-1'), 3.93 (s, 12-OCH_3), 5.99 (s, H-8'), 6.30 (s, H-5), 6.31 (d, J = 2.1 Hz, H-10'), 6.51 (s, H-5'), 6.55 (d, J = 1.4 Hz, H-10), 6.81 (dd, J = 8, 2.5 Hz, H-11'), 6.86 (brs, 2 OH), 7.14 (dd, J = 8, 2.5 Hz, H-13'), 7.34 (dd, J = 8, 2 Hz, H-14'). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 22.39 (C-4), 25.61 (C-4'), 38.67 (C-15'), 42.34 (C-15), 43.02 (2'-NCH_3), 44.48 (C-3), 42.69 (2-NCH_3), 45.63 (C-3'), 56.20 (6-OCH_3), 56.24 (6'-OCH_3), 56.54 (12-OCH_3), 60.72 (7-OCH_3), 61.82 (C-1), 64.30 (C-1'), 106.07 (C-5), 111.87 (C-13), 113.07 (C-5'), 116.56 (C-10), 120.57 (C-8'), 122.35 (C-11'), 122.42 (C-13'), 123.11 (C-14), 123.31 (C-8a), 128.33 (C-4a), 128.40 (C-4a'), 128.52 (C-8a'), 130.57 (C-10', C-14'), 135.34 (C-9), 135.62 (C-9'), 138 (C-7), 144.14 (C-7'), 147.40 (C-12), 148.86 (C-8), 149.01 (C-6'), 149.76 (C-11), 151 (C-6), 154.10 (C-12'). See Appendix S-98 - S-101.

2.3.5.2.2 Aromoline

White crystalline solid (Methanol), mp. 198-200 °C. UV λ max nm: 208, 228 (sh), 285 (MeOH). IR ν max (KBr) cm^{-1}: 3515, 1660, 1580, 1480, 1467, 1391, 1312, 1205, 1094. EIMS m/z (rel.int.): 594 (C_{36} H_{38} O_6 N_2) $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.45 (brd, J = 12.5 Hz, H-15'), 2.60 (s, 2-NCH_3), 2.67 (dd, J = 15, 5 Hz, H-4'), 2.89 (m, H-15), 3.25-3.32 (m, H-3 *ax* and H-3 *ec*), 3.57 (brs, H-1'), 3.61 (s, 6'-OCH_3), 3.81 (s, 6-OCH_3), 4.18 (d, J = 5.16 Hz, H-1), 5.2 (brs, 12-OH), 5.6 (brs, 12'-OH), 6.35 (s, H-5), 6.36 (s, H-5'), 6.39 (d, J = 6.3 Hz, H-10), 6.67 (s, H-10'), 6.76 (d, J = 8 Hz, H-11), 6.81 (s, H-8'), 6.82 (d, J = 8 Hz, H-13), 6.91 (m, H-13'), 6.93 (m, H-14 and H-14'). See Appendix S-102 - 104.

2.3.5.3 Results and Discussion

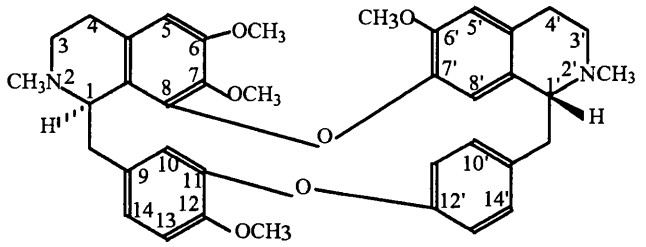
Bioassay-guided fractionation of MeOH extract of *T. patens* led to the isolation and identification of two major active compounds the known bisbenzylisoquinoline alkaloids phaeanthine and aromoline. In this study, ^{1}H NMR and ^{13}C NMR spectra (S-99,S-100) have now been completely assigned for phaeanthine by the use of 2D NMR experiments:

COSY-45, NOESY and HMQC spectra, and for aromoline this is the first report of its COSY-45 spectrum. The structures of compounds isolated from *Triclisia patens* are shown in Figure 2.13.

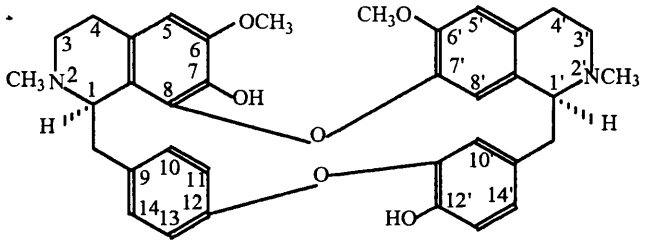
2.3.5.3.1 Structure determination and identification

2.3.5.3.1.1 Phaeanthine

Phaeanthine was identified by comparison of its spectroscopic data with those in literature (Guinaudeau *et al.*, 1986), and by comparison with an authentic sample available in our laboratory. The ^{1}H NMR spectrum (S-98) of phaeanthine showed resonances of two N-Me at δ 2.33, 2.62, and four aromatic MeO groups at δ 3.19. 3.37, 3.75, and 3.93. In the aromatic region 10 protons were observed as three singlets (δ 5.99, 6.3 and 6.51), one doublet (δ 6.55, J = 1.4 Hz), four doublet of doublets (δ 6.31, 6.81, 7.14, 7.34, J = 8.3, 2.5 Hz), and a cluster of 2 aromatic protons at δ 6.86 and 6.87. The above ^{1}H NMR spectral features together with the signals observed for in the ^{13}C NMR spectrum (S-99) were typical of a Head to Head and Tail to Tail BBIQ containing two diaryl ether bridges at C-8 - C-7' and C-11 - C-12' typical of the subgroup VIII (Guinaudeau *et al.*, 1986; Schiff, 1991). Furthermore, the peaks at m/z 396, 381, 198, 175 in the MS, together with the H-1 signal at δ 3.87 (dd, J= 10.92, 5.56 Hz) and H-1' signal at 3.74 (m) in the ^{1}H NMR spectrum are typical to the subgroup VIII (8-7', 11-12'), with three MeO groups on the upper and one on the lower part of the molecule, as occurs with phaeanthine. The COSY-45 spectrum (S-100) of phaeanthine showed cross peaks for the four protons of the F ring. Further, H-14 coupled to H-13' and H-10' ; H-11' coupled to H-13', H-10' ; H-14 coupled to H-13, H-10; and H-1 coupled to H-1'. The NOESY spectrum (S-101) showed the spatial correlation between H-5 and 6-CH_3O; H-13 and12-CH_3O; H-5' and 6'-OCH_3; and H-8' and H-14'.



Type VIII
Phaeanthine R,R



Type VI
Aromoline R,S

Figure 2.13 Structures of compounds isolated from *Triclisia patens*

2.3.5.3.1.2 Aromoline

Aromoline was identified by comparison of its spectroscopic data with literature data (Schiff, 1983; 1985; 1987). Its showed ^{1}H NMR (S-102) spectral features of a head to head, and tail to tail bisbenzylisoquinoline alkaloid containing two diaryl ether linkages between carbons C-8/C-7' and C-12/C-11' with two aromatic CH_3O (δ 3.61 and 3.81), and two NCH_3 (δ 2.60 and 2.62). Like phaeanthine, its H-8' signal was farther upfield than that of H-5'. COSY-45 spectrum (S-103) showed correlations for the following pair of protons: H-14/H-14'; H-14/H-13; H-1/H-15; and H-3/H-4. The stereochemistry of the molecule was determined by NOESY spectrum (S-104). Cross-peaks were observed for the following protons: 2-NCH_3/H-3/H-1/H-5; H-1/H-15; 6-OCH_3/H-5; H-10/H-14/H-13; 6-OCH_3/H-5; 2'-NCH_3/H-3'/H-1'; H-1'/H-15'/H-10'; H-14'/H-13'.

2.3.6 *Cephaelis camponutans* (Dwyer & Hayden) Hammel (Rubiaceae)

One of the largest plant families, the Rubiaceae has 7000 species distributed in 500 genera (Schultes & Raffauf, 1990). The family has been reported to possess iridoids, several type of alkaloids, triterpenes, sterols, quinones, naphthalene derivates, polyphenols and tannins. The most important medicinal compounds of the family include quinine from *Cinchona* species, emetine from *Cephaelis* species, and the widely distributed quinones. There are some 200 species of *Cephaelis* occuring through the tropics of which 21 occur in Panama. Some 24 monoterpenoid isoquinoline alkaloids have been isolated from *Cephaelis ipecacuanha* (Itoh *et al.*, 1991; Nagakura *et al.*, 1993); the simple indole alkaloid gramine from *Cephaelis stipulacea* (Yulianti & Djamal, 1991). Two benzoquinones: benzo[g]isoquinoline-5,10-dione and 1-hydroxybenzoisochromanquinone from *Cepahelis camponutants*; the activity of both quinones against brine shrimps, KB cells and *Plasmodium falcifarum* have been determined (Solis *et al.*, 1995). In this study we decided to investigate further this species to isolate other antiprotozoal compounds.

2.3.6.1 Extraction and Isolation

The dried and powdered woody parts (100 g) of *Cephaelis camponuntans* were extracted according to the procedure described in Figure 2.1. The antileishmanial activity *in vitro* was determined for all the extracts. The chloroformic fraction showed the highest activity against *Leishmania donovani* promastigotes, and was submitted to repeated silica gel column chromatography with $CHCl_3$-EtOAc gradient as eluent to yield two major active compounds, the known benzoquinones: benzo[g]isoquinoline-5,10-dione (8.6 mg) and 1-hydroxybenzoisochromanquinone (13.4 mg). TLC were observed under UV and sprayed either with 5% KOH in MeOH or iodoplatinate-5% H_2SO_4.

2.3.6.2 Spectroscopic data

2.3.6.2.1 Benzo[g]isoquinoline-5,10-dione

Yellow needles ($CHCl_3$-MeOH), mp. > 270 °C. IR ν max (KBr) cm^{-1}: 1680, 1580, 1300, 700. EIMS: 209 ($C_{12}H_7NO_2$) $[M]^+$ (100), 181 (50), 153 (65), 126 (40), 83 (10), 76 (20). ^1H NMR (400 MHz, $CDCl_3$) δ: 7.80 (m, H-7), 7.90 (m, H-8), 8.0 (1H, d, J =

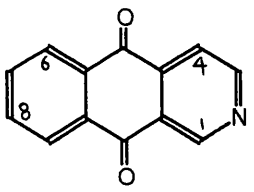
5 Hz, H-4), 8.30 (m, H-6), 8.34 (m, H-9), 9.12 (1H, d, J = 5 Hz, H-3), 9.57 (1H, s, H-1). See Appendix S-105.

2.3.6.2.2 1-Hydroxy-benzoisochromanquinone

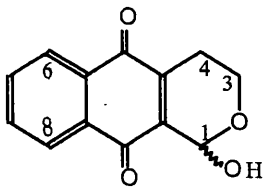
Yellow amourphous powder ($CHCl_3$), mp. 258-260 °C. IR ν max (KBr) cm^{-1}: 3450, 1660, 1595. EIMS m/z: 230 (C_{13} H_{10} O_4) $[M]^+$(3), 221 (43), 212 (63), 183 (21), 169 (4), 155 (15), 128 (74), 105 (93), 97 (24), 77 (100), 69 (38). ^1H NMR (400 MHz, $CDCl_3$) δ: 2.6 - 2.8 (ddd, J = 18, 3 Hz, H_2-4), 4.6 - 4.8 (2H, ddd, J = 18, 3 Hz, H-3), 5.3 (1H, t, J = 3.6 Hz, H-1), 7.72 - 7.76 (2H, m, H-6 and H-9), 8.0 - 8.10 (2H, m, H-7 and H-8). See Appendix S-106.

2.3.6.3 Results and Discussion

Bioactivity guided fractionantion of methanolic extract of *Cephaelis camponutans*, using *Leismania donovani* promastigotes test *in vitro*, led to the isolation of two known bioactive benzoisoquinones 1-hydroxyl-benzoisochromanquinone and benzo[g]isoquinoline-5,10-dione (Figure 2. 14).



Benzo[g]isoquinoline-5,10-dione



1-Hydroxy-benzoisochromanquinc

Figure 2.14 Structures of compounds isolated from *Cephaelis camponutans*

2.3.6.3.1 Structure determination and identification

2.3.6.3.1.1 Benzo[g]isoquinoline-5,10-dione

The ^1H NMR spectrum (S-105) of benzo[g]isoquinoline-5,10-dione [209 $[M]^+$ (C_{13} H_7 NO_2)] displayed a singlet at δ 9.57 corresponding to the proton on C-1, two doublets coupling to each other at δ 9.1 and 8 assigned to protons on C-3 and C-4 respectively; and two signals integrating for two protons (H-6 and H-8) characterisitic of a 1,2-

substituted benzene ring. This quinone was identified by analysis of its physical and spectroscopic data with literature (Solis *et al.*, 1995), and by comparison with an authentic sample available in our laboratory.

2.3.6.3.1.2 1-Hydroxybenzoisochromanquinone

The ^1H NMR spectrum (S-106) of 1-Hydroxybenzoisochromanquinone (m/z 230 $[M]^+$, C_{13} H_{10} O_4) showed 4 aromatic protons typical of a 1,2 substituted benzene ring, and a fine triplet at δ 5.3 assigned for the proton on C-1. A double doublet at δ 4.84 - 4.63 (J = 3 and 18 Hz) showing geminal coupling constant, assigned to proton on C-3 and the double doublet at δ 2.84 - 2.64, which showed similar coupling constant to the latter, was assigned to the protons on C-4. The identification of this benzoquinone was carried out by comparison of its physical and spectroscopic data with those reported in literature (Solis *et al.*, 1995), and by comparison with an authentic sample.

2.3.7 *Hintonia latiflora* (Sesse et Mocino ex DC.) Bullock (Rubiaceae)

The stem bark of *H. latiflora*, syn. *Coutarea latiflora* (Sesse et Mocino ex DC) popularly known as "copalchile, falsa quina, palo amargo or copalquin" (Martinez, 1987) is highly valued in Mexican traditional medicine , not only for the cure of malaria but also for treating wounds and diabetes mellitus (Bever & Zahnd, 1979). In 1984 Reher & Kraus reported the isolation and structure elucidation of the oxidocoumarin (Reher & Kraus, 1984). In 1987 Reguero *et al.* (1987) described the isolation of 23,24-dihydrocucurbitacin F and 23,24-dehydrocucurbitacin F-25 acetate. More recently from the defatted stem bark of *H. latiflora* the flavone 7-methyl-luteolin (Camacho, 1990), the polyalcohol manitol and six glycosides of phenylcoumarins have been obtained: 5-O-β-D-glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, 5-O-β-D-galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, 5-O-(6"-acetyl)-β-D-galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, 5-O-β-D-glucopyranosyl-7,3',4'-trihydroxy-4-phenylcoumarin, and 5-O-β-D-galactopyranosyl-4'-hydroxy-7-methoxy-4-phenylcoumarin (Mata *et al.*, 1990; Mata, 1993), the novel phenylstyrene 6-O-β-D-glucopyranosyl-2,3',4'-trihydroxy-4-methoxy-α-phenylstyrene (Mata *et al.*, 1992). The antimicrobial activity of the plant and its metabolites has been

determined (Rojas *et al.*, 1992). Although the galactoside possesses moderate activity against *C. albicans*, the crude methanol extract of *H. latiflora* was inactive against the yeast, gram-positive and gram-negative bacteria. None of the others glycosides exhibited antiseptic properties against the several organisms tested. The methanol extract were devoid of *in vitro anti-Plasmodium falciparum* activity. However, the butanolic fraction showed weak activity against *P. falciparum* and *Leishmania donovani*. Thus, it was decided to investigate this species further to isolate other compounds with potential antiprotozoal.

2.3.7.1 Extraction and Isolation

Hintonia latiflora stem bark (200 g) was powdered and extracted according to the procedure described in Figure 2.1. The n-butanolic active extract, was flash chromatographed over silica gel Sorbil C 60-H with $CHCl_3$-MeOH gradient as eluent. A total of 120 fractions of 100 ml each was collected and fractions combined on the basis of TLC composition. 40% H_2SO_4 in ethanol was used as spray reagent. Fractions 10-25 eluted with $CHCl_3$-MeOH (99:1) showed activy, and was rechromatographed over LH-20, eluted with MeOH to afforded the bioactive 7-methyl-luteolin. Fractions 67-75, eluted with $CHCl_3$-MeOH (80:20), also contained bioactive substances and was further subjected to flash CC over silica gel Sorbil C 60-H with mixtures of $CHCl_3$-MeOH, followed by Sephadex LH-20 column chromatography in MeOH, to yield bioactive 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F (7.9 mg), along with 5-O-β-D-glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin (22.8 mg), and 5-O-β-D-galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin (31 mg).

2.3.7.2 Spectroscopic data

2.3.7.2.1 7-Methyl-luteolin (5,3',4'-Trihydroxy-7-methoxy-flavone)

Yellow needles (MeOH), mp. 270-272 °C. IR ν max (KBr) cm^{-1}: 3420, 3220, 2940, 2700, 1660, 1600, 1500, 1440, 1340, 1300, 1270, 1190, 1100. EIMS m/z (rel.int.): 300 (C_{16} H_{12} O_6) $[M]^+$ (100), 299 (21), 271 (45), 257 (26), 243 (10), 228 (7), 167 (20), 153 (15), 151 (20), 134 (17), 123 (10), 95 (31), 69 (32). ^1H NMR (400 MHz, DMSO-d_6) δ: 3.87 (s, 7-OMe), 6.37 (s, H-3), 6.71 (d, J = 2 Hz, H-6), 6.72 (d, J = 2 Hz, H-8), 6.89 (d,

J = 8 Hz, H-5'), 7.43 (d, J = 2 Hz, H-2'), 7.44 (dd, J = 8, 2 Hz, H-6'), 12.98 (brs, 5-OH).See Appendix S-107.

2.3.7.2.2 5-O-β-D-Glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin

Yellow crystalline solid (MeOH), mp. 257-258 °C. IR ν max (KBr) cm^{-1}: 3500, 3400, 1698, 1615, 1520, 1160, 1110, 1080, 1050. FABMS m/z (rel. int.): 462 (C_{22} H_{22} O_{11}) $[M]^+$ (100), 300 (80), 272 (75), 257 (30). ^1H NMR (400 MHz, DMSO-d_6) δ: 5.87 (s, H-3), 6.56 (d, J = 3 Hz, H-6), 6.60 (d, J = 3 Hz, H-8), 6.80 (d, J = 3 Hz, H-2'), 6.82 (d, J = 8 Hz, H-5'), 6.55 (dd, J = 8,3 Hz, H-6'), 3.85 (s, 7-OCH_3), 4.70 (d, J = 8 Hz, H-1"), 3.10-4.60 (m, H-2"-H-6"). See Appendix S-108 and S-109.

2.3.7.2.2.1 5-O-β-D-Tetraacetoxyglucopyranosyl-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin

White crystalline solid (MeOH), mp. 89-91 °C. IR ν max (KBr) cm^{-1}: 3440, 2930, 1755, 1620, 1370, 1170, 1110, 1070. ^1H NMR (400 MHz, DMSO-d_6) δ: 1.90 (s, CH_3CO_2), 2.0 (s, CH_3CO_2), 2.04 (s, CH_3CO_2), 2.06 (s, CH_3CO_2), 2.33 9s, CH_3CO_2), 2.35 (s, CH_3CO_2), 3.89 (s, 7-OCH_3), 4-5.26 (m, H-1"-H-6"), 6.06 (s, H-3), 6.42 (d, J = 3 Hz, H-8), 6.62 (d, J = 3 Hz, H-6), 7.05-7.28 (m, H-2', H-3', H-5').

2.3.7.2.2.2 5-O-β-D-Glucopyranosyl-7,3',4'-trimethoxy-4-phenylcoumarin

White amourphous solid, mp. 192-193 °C. IR ν max (KBr) cm^{-1}: 3400, 1710, 1620, 1430, 1360, 270. FABMS m/z (rel. int.): 490 (C_{24} H_{26} O_{11}) $[M]^+$ (100). 1H NMR (400, DMSO-d_6) δ: 3.80 (s, OCH_3), 3.84 (s, OCH_3), 3.90 (s, OCH_3), 3-4 (m, H-2" - H-6"), 4.93 (d, J = 8 Hz, H-1'), 6.0 (s, H-3), 6.64 (d, J = 3 Hz, H-8), 6.76 (d, J = 3 Hz, H-6), 6.93-6.98 (m, H-2', H-3', H-5').

2.3.7.2.3 5-O-β-D-Galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin

Crystalline cream solid (MeOH-H_2O), mp. 229-230 °C. UV λ max nm: 212, 250, 327 (MeOH). IR ν max (KBr) cm^{-1}: 3400, 3300, 1719, 1613, 1160, 1070, 1049. FABMS m/z (rel.int.): 462 (C_{22} H_{22} O_{11}) $[M]^+$ (100), 300 (86), 272 (60), 257 (45). ^1H NMR (400 MHz, DMSO-d_6) δ: 3.70 s (7-OCH_3), 6.20 (s, H-3), 4 - 4.5 (m, H-2" - H-6"), 5.26 (d, J = 8 Hz, H-1"), 6.57 (d, J = 3 Hz, H-8), 6.9 (dd, J = 8, 3 Hz, H-6'), 7.0 (d, J = 3 Hz,

H-6), 7.20 (d, J = 8 Hz, H-5'), 7.38 (d, J = 3 Hz, H-2'). See Appendix S-110 and S-111.

2.3.7.2.3.1 5-O-β-D-Tetraacetoxygalactopyranosyl-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin

White amorphous solid (MeOH), mp. 80-83 °C. IR ν max (KBr) cm^{-1}: 1753, 1616, 1506, 1433. FABMS m/z (rel.int.): 714 (C_{34} H_{34} O_{17}) $[M]^+$ (100). ^1H NMR (400 MHz, $CDCl_3$) δ: 1.85, 1.92, 1.99 and 2.18 (s, 2"-CH_3CO_2), 2.18 (s, 6"-CH_3CO_2), 2.30 (s, 3' -CH_3CO_2), 2.30 (s, 4'-CH $_3CO_2$), 3.85 (s, 7-OCH_3), 5 (d, J = 8 Hz, H-1"), 3.8 - 4.8 (m, H-2"-H-6"), 6.15 (s, H-3), 6.65 (d, J = 3 Hz, H-6), 6.52 (d, J = 3 Hz, H-8), 7.10 - 7.20 (m, H-2', H-5' and H-6'). See Appendix S-112 and S-113.

2.3.7.2.3.2 5-O-β-D-Galactopyranosyl-7,3',4'-trimethoxy-4-phenylcoumarin

White amorphous powder (MeOH), 178-180 °C. IR ν max (KBr) cm^{-1}: 3400, 1710, 1620, 1430, 1360, 270. FABMS m/z (rel. int.): 490 (C_{24} H_{26} O_{11}) $[M]^+$ (100). ^1H NMR (400 MHz, DMSO-d_6) δ: 3.85 (s, 7-OCH_3 and 3'-OCH_3), 3.90 (s, 4'-OCH_3), 4.64 (d, J = 8 Hz, H-1"), 3 - 3.5 (m, H-2" - H-6"), 5.90 (s, H-3), 6.6 (s, H-6, H-8), 6.92 (brs, H-2', H-5', H-6').

2.3.7.2.3.3 5,3',4'-Trihydroxy-7-methoxy-4-phenylcoumarin

Green-yellowish powder (MeOH), mp. 138-140 °C. IR ν max (KBr) cm^{-1}: 3400, 1670, 1620, 1590, 1512, 1300. FABMS m/z (rel. int.): 300 (C_{16} H_{12} O_6) $[M]^+$ (100). ^1H NMR (400 MHz, DMSO-d_6) δ: 3.85 (s, 7-OMe), 5.88 (s, H-3), 6.25 (d, J = 3 Hz, H-6), 6.40 (d, J = 3 Hz, H-8), 6.86 (d, J = 3 Hz, H-2'), 6.87 (d, J = 8 Hz, H-5'), 6.72 (dd, J = 8, 3 Hz, H-6'). See Appendix S-114

2.3.7.2.3.4 5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin

Cream amorphous solid ($CHCl_3$-MeOH), mp. 160-162 °C. IR ν max (KBr) cm^{-1}: 1777, 1760, 1720, 1620, 1500, 1430, 1371, 1151. FABMS m/z (rel.int): 426 (C_{22} H_{18} O_9) $[M]^+$ (100). ^1H NMR (400 MHz, $CDCl_3$) δ: 1.60 (s, 5-$OCOCH_3$), 2.30 (s, 3'-$OCOCH_3$), 2.30 (s, 4'-$OCOCH_3$), 3.83 (s, 7-OCH_3), 6.07 (s, H-3), 6.82 (d, J = 3 Hz, H-6), 6.48 (d, J = 3 Hz, H-8), 7.10-7.30 (m, H-2', H-5' and H-6').

2.3.7.2.4 3-O-β-D-Glucopyranosyl-23,24-dihidrocucurbitacin F

Amorphous white solid (MeOH), mp. 200-203 °C. IR ν max (KBr) cm^{-1}: 3400, 2970, 1690, 1630, 1455, 1370, 1210, 1070, 1020. ^{13}C NMR (C_5D_5N) δ: 19 (C-19), 20.3 (C-18), 20.4 (C-30), 20.5 (C-28), 23.4 (C-29), 25.2 (C-7), 25.4 (C-21), 29.7 (C-27), 30 (C-26), 32.60 (C-23), 34 (C-1), 34.1 (C-10), 38.3 (C-24), 42.5 (C-4), 43.1 (C-8), 46.2 (C-15), 48.6 (C-13), 48.8 (C-9), 49.2 (C-12), 51 (C-14), 59 (C-17), 62.9 (C-6'), 69.2 (C-25), 70.4 (C-2), 71.20 (C-16), 71.7 (C-4'), 76.26 (C-2'), 78.40 (C-5'), 78.60 (C-3'), 80.10 (C-20), 93.70 (C-3), 107 (C-1'), 119.4 (C-6), 142 (C-5), 213 (C-11), 216 (C-22). See Appendix S-115.

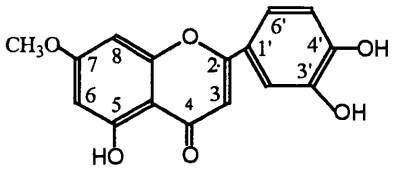
2.3.7.3 Results and Discussion

Bioactive guided fractionation of methanolic extract of *Hintonia latiflora* stem bark using *Leishmania donovani and Plasmodium falciparum in vitro* tests led to the isolation of 7-methyl-luteolin and 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F, 5-O-β-D-glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, and 5-O-β-D-galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin. Figure 2.15 showed the structures of compounds isolated from *Hintonia latiflora*.

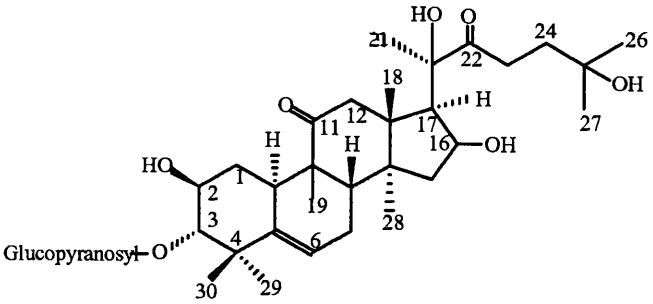
2.3.7.3.1 Structure determination and identification

2.3.7.3.1.1 7-Methyl-luteolin

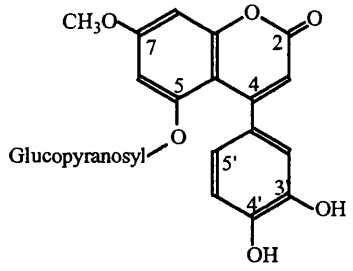
The flavone 7-methyl-luteolin exhibited a molecular ion at m/z 300 (C_{16} H_{12} O_6). Diagnostic peaks of RDA cleavage of ring C at m/z 167 and 134, in the EIMS indicated the presence of one methoxy and one hydroxyl group in ring A, as well as two hydroxyl groups in ring B. The ^1H NMR spectrum (S-107) of 7-methyl-luteolin showed one methoxy group at δ 3.87; one singlet at δ 6.37 typical for H-3 of flavones; one ABC coupling system of three aromatic proton signals at δ 6.89, 7.43, and 7.44; two doublets at δ 6.71 and 6.72 of a AB system of two meta related protons in the ring A, and one C-5 chelated hydroxyl (δ 12.98, brs). 7-Methyl-luteolin was obtained as yellow needles, and its identification was carried out by comparison of its spectroscopic data with those reported ion the literature (Aquino *et al.*, 1988).



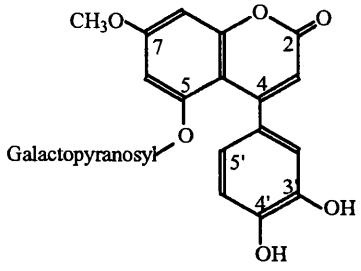
7-Methyl-luteolin



3-O-β–D-Glucopyranosyl-23,24-dihydrocucurbitacin F



5-O-β–D-Glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin



5-O-β–D-Galactopyranolyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin

Figure 2.15 Structures of compounds isolated from *Hintonia latiflora*

2.3.7.3.1.2 5-O-β-D-Glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin and 5-O-β-D-Galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin

Two glycosides of phenylcoumarin responded positively to Molisch and each had the same molecular formula of $C_{22}H_{22}O_{11}$. The presence of a 4-phenylcoumarin skeleton in the molecule was easily deduced by the UV and IR spectra along with the higly diagnostic resonance of the H-3 proton. The ^{1}H and ^{13}C NMR spectra (S-108 and S-111) exhibited, in addition to the sugar portion, and H-3 signals, an ABC system for a trisubstituted aromatic ring [δ 7.3 (d, J = 3 Hz), δ 7.10 (d, J = 8 Hz), δ 6.87 9dd, J = 8.3 Hz)], an AB system attributed to two mutually meta located protons [δ 6.98, H- and 6.61, H- (each, d, J =3 Hz)], and a singlet for aromatic methoxy (7-OCH_3). The spectral properties of both compounds were very similar, differing mainly in the ^{1}H NMR signals for the sugar portion (δ_H 3-4). The hydrolysis of both glycosides with 2 N HCl , yielded

glucose and galactose (tlc), respectively and a common aglycone that was characterized as 5,3',4'-trihydroxy-7-methoxy-4-phenylcoumarin. Treatment of both glycosides and the aglycone with Ac_2O/pyridine afforded the hexaacetyl derivatives, 5-O-β-D-Tetraacetylglucopyranosyl-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin, 5-O-β-D-Tetraacetylgalactopyranosyl-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin, and 3',4'-diacetoxy-7-methoxy-4-phenylcoumarin. The presence of a noticeable shifted upfield signal for an acetate at δ 1.59 in the ^1H NMR of the acetyl derivatives of the aglycone and the absence of such a signal in the ^1H NMR were consistent with the attachment of the sugar moiety at C-5 (Mata *et al.*, 1990). The β-O-glycoside linkage was inferred both from the coupling constant value (d, J = 8 Hz) observed for the anomeric proton (δ 4.67) in the ^1H NMR of both glycosides. Finally, methylation of both glycosides with ethereal CH_2N_2 yielded 5-O-β-D-glucopyranosyl-7,3',4'-trimethoxy-4-phenylcoumarin and 5-O-β-D-galactopyranosyl-7,3',4'-trimethoxy-4-phenylcoumarin. For structures of semi-synthetic derivatives see Chapter 3.

2.3.7.3.1.3 3-O-β-D-Glucopyranosyl-23,24-dihydrocucurbitacin F

The molecular formula was established as C_{36} H_{58} O_{12} by FABMS. The presence of hydroxyl (3400 cm-1) and ketone (1690) was inferred from the IR spectrum. The H NMR showed signals typical of a polyhydroxylated terpene (S-115). The ^{13}C NMR spectrum confirmed the presence of 36 carbons and supported the assignment of a glucocucurbitacin type of compound, which possesses two ketone groups (δ 216, 213), and five oxygenated functions (δ 93, 80, 71, 70, and 69), in addition to those of the β-D-glucopyranosyl moiety. Enzymatic hydrolysis with β-D-glucosidase afforded β-D-glucose and 23,24-dihydrocucurbitacin F, identical to an authentic sample (Reguero *et al.*, 1987). The remarkable low field of the C-3 signal (δ 93.7) in the ^{13}C NMR of glycoside compared with the corresponding resonance in the aglycone (δ 81.4) clearly indicated that the sugar residue was attached to the hydroxyl group at C-3 in the cucurbitacin . The physical and spectroscopic data of 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F was in agreement with those in literature (Mata *et al.*, 1990).

2.4 Summary

From a preliminary *in vitro* screening of 92 plant extracts *C. mexicanum*, *G. glauca*, *S. dinklagei*, *T. patens*, *H. latiflora*, *G. rhopalocarpa* and *C. camponutans* extracts were selected for further investigation to isolate some of their active principles, and other compounds present in these plants (Table 2.8). Bioactive guided fractionation of plant extracts using *in vitro* assays against *L. donovani*, *P. falciparum,* KB cells or *Artemia salina*, and a combination of chromatographic techniques yielded a series of active compounds which include alkaloids, quinones, terpenes, flavonoids and coumarins.

Three active triterpenes, were isolated from *C. mexicanum* 3α-hydroxy-7,24Z-di-en-tirucalla-26-oic acid and 3-oxo-7,24Z-di-en-tirucalla-26-oic acid, and epi-oleanolic acid. In addition, four terpenes (friedelin, maytensifolin B, 3β-hydroxyfriedelan-16-one, and celaenodendrolide) and three biflavonoids (amentoflavone, podocarpusflavone A and podocarpusflavone B) were also isolated. *G. glauca* yielded the flavonol quercetin as the active principle, together with the new terpenes, galphimine C, galphimine D, galphimine E and glaucamine, and the steroids stigmasterol and sitosteryl-3-O-β-D glucopyranoside. *G. rhopalocarpa* yielded four novel active terpenes, 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, lanosta-7,9(11),24EZ-triene-3α,23-diol, 8(14),15-sandaracopimaradien-2β,18-diol and 8(14),15-sandaracopimaradine-2α,18-diol; together with the steroid stigmasterol, and the coumarin scopoletin. *S. dinklagei* yielded five active aporphine alkaloids, the new natural zwitterionic oxoaporphine alkaloids N-methyl-lioriodendronine and 2-O, N-dimethyl-liriodendronine, the novel 6-6a-dehydronoraporphine, stepharandine, and the known oxoaporphine alkaloids: dicentrinone and liriodenine; together with the aporphine alkaloid corydine and the anthraquinone aloe-emodin. *T. patens* yielded two BBIQ alkaloids aromoline and phaeanthine as active principles. From *C. camponutans* two active quinones were isolated, the 1-hydroxy-benzoisochromanquinone and benzo[g]isoquinoline-5,10-dione. *H. latiflora* afforded the known flavone 7-methyl-luteolin, toghether with the cucurbitacin, 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F, and the phenylcoumarins, 5-O-β-D-glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin and 5-O-β-D-galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin.

Table 2. 8 Compounds isolated from plant species investigated in this study

Plant	Country	Use	Constituents
Euphorbiaceae *Celaenodendron mexicanum*	Mexico	Skin infections	**Terpenene** 3-Oxo-7,24Z-dien-tirucalla-26-oic acid 3α-Hydroxy-7,24Z-dien-tirucalla-26-oic acid Epi-oleanolic acid Friedelin Maytensifolin B 3β-Hydroxy-friedelan-16-one Celaenodenrolide **Flavonoid** Amentoflavone Podocarpusflavone B Podocarpusflavone A
Malphigeceae *Galphimia glauca*	Mexico	Malaria, dysentery, fever, CNS	**Flavonoid** Quercetin **Terpene** Galphimine C Galphimine D Galphimine E Glaucamine **Sterold** Stigmasterol Sitosteryl-3-O-β-D-glucopyranoside
Meliaceae *Guarea rhopalocarpa*	Panama	Not used	**Terpene** 23-Hydroxy-5α-lanosta-7,(9),24-triene-3-one Lanosta-7,9(11),24-triene-3α,24-diol Ent-8(14),15-Sandaracopimaradiene-2β,18-diol Ent-8(14),15-Sandaracopimaradiene-2α,18-diol **Sterold** Stigmasterol **Coumarin** Scopoletin

Table 2.8 Table 2. 8 Compounds isolated from plant species investigated in this study (Continuation).

Plant	Country	Use	Constituents
Menispermaceae *Stephania dinklagei*	Ghana	Vermifuge, analgesic, aphrodisiac, sedative, CNS, fish poison	**Alkaloid** Stepharandine Corydine N-Methyl-liriodendronine 2-O,N-Dimethyl-liriodendronine Liriodenine Dicentrinone **Quinone** Aloe-emodin
Triclisia patens	Ghana	Fever, malaria	**Alkaloid** Aromoline Phaeanthine
Rubiaceae *Cephaelis camponutans*	Panama	Not used	**Quinone** Benz[g]isoquinoline-5,10-dione 1-Hydroxy-benzoisochromanquinone
Hintonia latiflora	Mexico	Malaria, infections, hypoglucemic	**Flavonoid** 7-Methyl-luteolin **Coumarin** 5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin 5-O[-β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin **Terpene** 3-O-β-D-Glucopyranosyl-23,24-dihydrocucurbitacin F

CHAPTER 3

ANTIPROTOZOAL AND CYTOTOXIC ACTIVITIES OF SOME EXTRACTS AND PLANT-DERIVED COMPOUNDS

3.1 Antiprotozoal activity

3.1.1 Introduction

Diseases caused by parasitic protozoa including malaria, leishmaniasis and trypanosomiasis, are the cause of considerable mortality and morbidity throughout the world. There is an urgent need for new chemotherapeutic drugs for the treatment of these diseases which mainly affect developing countries. Plants have been used as an important source of clinical agents in the past and they still have considerable potential as sources of new drugs. The importance of natural product molecules to medicine lies not only in their pharmacological or chemotherapeutic effects for the production of new drugs substances, but also in their role as template molecules for the development of new drugs substances (Phillipson, 1995).

In vitro screening procedures are valuable as a means of initially assessing the activity of an extract or compound since they are rapid, sensitive, inexpensive and require only a small quantity of test material. Such features make these tests particularly useful for screening natural products. It is also possible to examine the effect of biologically active extracts upon species of parasites which infect humans cell when *in-vitro* screens are used.

When screening plant extracts, the bioassay can be used to determine fractionation of active plant extracts, the fraction(s) retaining activity being successively purified until the active pure constituents are isolated. Once *in vitro* activity has been noted, the active extract or pure plant products can then be further tested *in vivo* as a secondary stage to the screening procedure.

In this chapter *in vitro* screening techniques have been used to examine the antileishmanial, antitrypanosomal, antimalarial and cytotoxic properties of a group of plants with traditional reputation for the treatment of protozoal diseases. This represents the first step in validating their use in traditional medicine. In addition, other plants which were chemotaxonomically related were also screened.

Ninety-two crude extracts obtained from 47 plant species and 97 pure plant-derived compounds were screened for *in vitro* antiprotozoal activity against *Leishmania donovani* and *Trypanosoma b. brucei*. Further, selected drugs were tested against *Trypanosoma cruzi* and *Plasmodium falciparum*. The *in vitro* cytotoxicity towards KB and P388D1 cells was also determined for the samples. In addition, the toxicity against brine shrimps was assessed for some samples. Finally, in order to see the selectivity of the samples tested against protozoa parasite *vs* mammalian cells, the relation between cytotoxic activity/ antiprotozoal activity was determined for tested compounds.

The 92 crude extracts and 37 plant-derived compounds as well as 8 semi-synthetic derivates were obtained according to the procedures described in Chapter 2. Fifty-two compounds which were available in our laboratory were included in the screening. A number of alkaloids were supplied for testing by Professor P.L.Schiff Jr. of the University of Pittsburgh, Pennsylvania. The alkaloids comprised 18 bisbenzylisoquinoline (BBIQ) alkaloids, 7 aporphines, 6 protoberberines, 4 morphinanones, 2 protopines, 2 benzylisoquinolines, and 2 miscellaneous. Dr. P.N. Solis of the University of Panama provided several alkaloids including: vallesiacotamine, strictosidine lactam and strictosidine from *Cephaelis dichroa* (Solis *et al.*, 1995); glomerulatine A (8-8a, 8'-8'a tetradehydro (-) calycanthine) from *Cephaelis glomerulata* (Solis *et al.*, 1997), and four anthraquinones aloe-emodin, picramnioside A, picramnioside B and picramnioside C from *Picramnia antidesma* (Solis *et al.*, 1995). Pinocembrin , acetylpinocembrin , pinostrobin and chrysin were obtained from *Teloxys graveolens* (Camacho, 1990). Finally, 12 plant-extracts were supplied by Dr. S.J. Marshall from the University of Oman and one extract of *Scoloymus hispanicus* was supplied by Dr. M. Abdul from the University of Alexandria Egypt.

3.1.2 Materials and Methods

3.1.2.1 Preparation of samples for antiprotozoal testing *in vitro*

Samples were dissolved aseptically in absolute ethanol or dimethylsulphoxide and diluted with the appropriate culture medium prior to testing. The final concentration of EtOH or dimethylsulphoxide (DMSO) never exceeded 0.1% in test medium, a concentration which has no significant effect on the growth of the parasites.

3.1.2.2 Antileishmanial activity

3.1.2.2.1 *Leishmania donovani* promastigote cultures

The strain of *L. donovani* (MHOM/ET/67/L82; LV9), originally isolated in Ethiopia was used. Cultures of promastigotes of *L. donovani* were maintained in continuous logarithmic phase of growth in culture medium containing RPMI 1640 medium, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated foetal calf serum (HIFCS), at 26 °C in tissue culture flasks. The medium was changed twice a week. The parasites were checked under the microscope for flagellar motility as an indication of viability before each experiment.

3.1.2.2.1.1 *Leishmania donovani* promastigotes test protocol.

The effect of samples on promastigotes was evaluated by a method similar to the one described by Fournet *et al.* (1994d). The test was carried out in 96-well microtitre plates. Two-fold serial dilutions were performed to obtain a series of eight concentrations starting from 500, 200, 100, 50 or 5 µg/ml. Each test well contained 100 µl of diluted sample and all concentrations were performed in triplicate. To each well was added 200, 000 promastigotes in the exponential phase of growth suspended in 100 µl of culture medium. Two series of controls were performed, (a) parasites in cultured medium without sample and (b) parasites in cultured medium with EtOH or DMSO. Pentamidine was used in each test as standard reference antileishmanial drugs. The samples were tested at least twice on separate occasions. The plates were incubated at 26 °C; the activity of the compounds was evaluated after 48 or 72 h by counting the number of live parasites from each well using a normal phase microscope at 400 times magnification and a

haemocytometer. The counts were compared with those of controls grown without drug or with pentamidine.

3.1.2.2.2 *Leishmania donovani* amastigote cultures

Amastigotes were maintained in male golden hamsters (90-120 g) by serial passage every 6-8 weeks. Amastigotes were obtained from the spleen of a freshly killed golden hamster, infected for approximately 6-8 weeks with *L.donovani*. The spleen was placed in medium containing RPMI 1640 medium supplemented with 25 mM HEPES, 4 mM L-glutamine, 0.02 mg/ml gentamicin, and homogenized with a sterile Potter crusher. The suspension was spun and the pellets were washed three times with medium and resuspended in culture medium. The hamsters were infected by intracardiac injection of 10^8 of *L. donovani* amastigote in 100 µl of culture medium.

3.1.2.2.2.1 *Leishmania donovani* amastigote test protocol

The test was carried out in the mouse peritoneal macrophage system described by Neal & Croft (1984). Macrophages obtained from the peritoneum of a fresh killed male CD1 mice were suspended in culture medium and counted on a haemocytometer. 5×10^4 Macrophages in 500 µl of culturing medium were added to each glass-bottomed chamber of an eight chambered slide (Lab-Tek Products, Miles Lab.), and allowed to adhere at 37 °C in a mixture of 5%CO_2 and 95% air. After 24 hr the cultures were examined for absence of contamination and the supernatant on the plates was removed. The adherent macrophages were exposed to 5×10^5 amastigote (500 µl) per chamber (ratio of infection organism to host cell of 1:10). Infection took place overnight. Non-phagocytozed parasites were removed from adherent macrophages the following day by lavage. A control culture (day 1) was fixed and stained with 10% Giemsa' stain. The infected macrophages cultures were maintained in culture medium alone (control) or in medium containing a specific drug concentration in three fold dilutions starting from 90, 30 or 10 µM at 37°C in an atmosphere of 5% CO_2 - 95% air. Each concentration was tested in quadruplicate. The medium and the drug were changed on days 2 and 5, and after 7 days of culture (day 7) control and experimental samples were fixed with methanol and stained with 10% Giemsa. Drug activity was assessed by counting microscopically

(x1000 magnification) 100 macrophages in each well. Then for each well, the percentage of parasited cells was calculated in relation to the total number of macrophages on the plate. For each assay, the % of inhibition of amastigotes was calculated relative to the control. The antileishmanial activity of the different compounds was estimated by comparison of these results with those obtained with sodium stibogluconate as reference drug and the untreated group. Each test was performed twice in independent experiments. Cytotoxicity of the compounds was also evaluated at the same time by direct observation under the microscope of the non-parasitized macrophages exposed to the products.

3.1.2.2.3 *In vivo* antileishmanial test protocol

The methodology used was similar to that described by Croft *et al.*, (1992). Female BALB/c mice (weight 20 g) were inoculated via the tail vein by injection of 10^7 amastigote in 200 µl of medium. One week after infection the mice were randomly divided into groups of 5 and the treatments were initiated. Drugs were made up with 0.25 % carboxymethylcellulose (CMC)-Tween 80, and the reference drug sodium stibogluconate ($NaSb^v$) was dissolved in 0.25% CMC. The animals were treated subcutaneously once daily with the experimental drugs at a dose of 100 mg/kg/day during 5 days. Sodium stibogluconate ($NaSb^v$) was given subcutaneously at a dose of 45 mg Sb^v/kg/day. Untreated mice received subcutaneous phosphate buffer solution (PBS) and Tween 80. Three days after the last drug administration, the mice were weighed, killed and the livers removed and weighed. Liver impressions were methanol fixed and stained with Giemsa's stain and the numbers of amastigotes per host liver cell nucleus were counted (500 liver nuclei of each animal were examined under oil immersion). The number of amastigotes per liver cell nucleus x liver mass in mg x (2×10^5) is approximately equal to the total number of amastigotes per liver. Parasite suppression was calculated from the ratio of the mean liver amastigotes counts of drug-treated mice and the mean liver amastigote counts of untreated mice multiplied by 100 to obtain the percentage parasite suppression. The *in vivo* antileishmanial test was carried out by Miss V. Yardley.

3.1.2.3 *In vitro* activity against *Trypanosoma cruzi*

3.1.2.3.1 Trypomastigote cultures

Cultures of *T. cruzi* trypomastigotes were obtained by infecting confluent Vero cell monolayers in medium 199 supplemented with 100 U/ml penicillin, 100 µl/ml streptomycin and 3.5% HIFCS with trypomastigotes (MHOM/BR/OO/Y, a human Brazilian strain) at 37 °C in a mixture of 5%CO_2 95% air. Monolayers were incubated overnight at 37 °C, then washed with medium to remove residual metacyclic trypomastigotes. Flasks were reincubated at 37 °C and 5%CO_2, the medium being changed every 2 to 3 days. One to two weeks after infection trypomastigotes present in the culture supernatant could be harvested.

3.1.2.3.1.1 Culture of mammalian cells

Vero cells, a monkey kidney cell line, was grown in 199 medium supplemented with 3.5 % HIFCS at 37 °C in a humidified atmosphere containing 5% CO_2 - 95% air. Monolayers, in tissue culture flasks, were maintained by serial passage of cells after treatment with trypsin-EDTA solution for 5 min at 37 °C, to detach adherent cells from the plastic.

3.1.2.3.1.2 *Trypanosoma cruzi* amastigotes test protocol

The *in vitro* test was carried out in similar manner to the mouse peritoneal macrophage system described for *L. donovani* amastigotes (Neal & Croft, 1984) except that the ratio of infecting organisms to host cells used for this experiment was 5:1 rather than 10:1 and the time of incubation was 3 days rather than 5 days. Briefly, the macrophages were infected with *T. cruzi* for 24 h. The infected macrophages were treated with three different concentrations of the drug during 3 days. After culture incubation at 37 °C in 5% CO_2-95 % air mixture the proportion of infected macrophages in drug treated cells in Giemsa-stained preparations was determined microscopically and compared with those untreated control and with the standard trypanocidal drug nifurtimox.

3.1.2.4 *In vitro* activity against *Trypanosoma brucei brucei*

3.1.2.4.1 Trypomastigote cultures

The method of Hirumi & Hirumi (1989, 1994) was used. Blood stream form trypomastigotes (BSFs) of *T. b. brucei* (strain S427) were cultivated *in vitro* in complete medium containing modified Iscove's medium (HMI-18) supplemented with 0.05 mM bathocuproine sulphonate, 1.5 mM L-cysteine, 1 mM hypoxanthine, 0.2 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.16 mM thymidine, 2.23mM sodium bicarbonate and 20% (v/v) heat-inactivated foetal calf serum at 37 °C under atmosphere of 95% air + 5% CO_2. Subcultures were maintained by transferring an aliquot of BSFs into new tissue culture flasks and readjusting the BSFs density to 7-9 x 10^5/ml in fresh medium every 48 h.

3.1.2.4.1.1 *In vitro* trypomastigote test protocol

The methodology used was carried out according to the protocol described by Sahpaz *et al.* (1994). The test was performed in flat-bottomed 96 well microtitre plates. Each well received 100 µl of the drugs in two fold concentration series in triplicate. Then, the wells were inoculated with 20 000 trypanosomes in 100 µl of culture medium. Four control wells randomised in each plate received only medium. The viability of trypanosomes was assessed by direct observation and counting the live parasites after 24, 48 and 72 h in a Neubauer haemocytometer with a phase contrast microscope (x 400). The reference compound used for these experiments was pentamidine. Each experiment was performed twice on separate occasions. For each compound the Minimum Effective Concentration (MEC) after 24, 48 and 72 h of incubation was determined.

3.1.2.4.2 *In vivo* activity against *Trypanosoma b. brucei*

In vivo studies were performed in mice according to the method of Loiseau *et al.* (1992). Female mice CD1 (18-20g) were infected (0.2ml) intraperitoneally (i.p) with 1.75 x 10^5 trypanosomes/ml suspended in NaCl 0.15 M solution (day 1). One day after infection the mice were randomly divided into groups of five. Five infected mice were used as controls and received only vehicle (25% cellacol) subcutaneously. The mean survival time of control mice was standarized at 11 days. One group was treated intraperitoneally with

melarsoprol a standard trypanocide for purposes of comparison. The mice were injected intraperitoneally with 3mg/kg/day of melarsoprol for 4 days. The other groups of mice were treated subcutaneously once daily with the experimental drugs (100 mg/kg/day) suspended in 0.25 % cellacol for four days. The trypanocidal activity was evaluated by the mean survival time and parasitemia of treated mice for each dose compared with the untreated mice and those treated with melarsoprol. The *in vivo* antitrypanosomal test was carried out by Mr. P. Rock.

3.1.2.5 *In vitro* antimalarial activity

3.1.2.5.1 *Plasmodium falciparum* cultures

The chloroquine- and pyrimethamine-resistant strain (K1) of *P. falciparum*, obtained from Thailand was used. Cultures of *P. falciparum* were maintained *in vitro* in human A^+ erythrocytes suspended in RPMI 1640 supplemented with D-glucose and 10% human A^+ serum at 37 °C with 5% CO_2% - 95% air according to the method of Trager & Jenson (1976). *P. falciparum* cultures were maintained by Dr. G.C. Kirby.

3.1.2.5.1.1 Test protocol

The test measures the ability of compounds to inhibit the growth of *P. falciparum* by measuring the reduction in incorporation of [^{3}H]-hypoxanthine into the parasite. Determinations of 50% inhibitory concentrations (IC_{50}) were carried out as described by Desjardins *et al.* (1979) with modifications by Ekong *et al.* (1990). The test was carried out in 96-well microtitre plates. Two-fold serial dilutions were performed along the length of the microtitre plate to obtain a series of 12 concentrations; each test well contained 50µl of diluted sample. All tests were performed in duplicate and drugs were tested at least twice in separate experiments. To each well was added 50 µl of *P. falciparum*-infected human erythrocytes (A^+) suspended in RPMI 1640 supplemented with D-glucose and 20% human A^+ serum (5% haematocrit, 1% parasitaemia) to give a final serum, haematocrit, and parasitaemia of 10%, 2.5% and 0.5% respectively (dilutions to 1% parasitaemia were made with washed uninfected erythrocytes). Two series of controls were performed, (a) parasitised blood without sample and (b) uninfected red blood cells without sample. Chloroquine diphosphate, used in each test as a standard reference antimalarial drug, was prepared in filter-sterilised distilled water and four-fold

serial dilutions were made. After incubation of the parasites in an atmosphere of 3% O_2, 4% CO_2 and 93% N_2 for 24 hours at 37°C, in an incubator, 5 µl of ^{3}H-hypoxanthine (40µCi/ml) was added to each well and incubation continued for a further 18-24 hours.

3.1.2.5.1.2 Harvesting

Red blood cells were washed from the wells with 0.9% saline using a semi automated cell harvester (Skatron) onto glass fibre filter mats (Titertek) predampened with saline. The glass fibre mats were then flushed with distilled water to remove traces of haemoglobin and free radiolabelled hypoxanthine, while that incorporated into nucleic acids remained firmly attached to the filter discs. Dried discs were placed in 3 ml aliquots of Ecoscint scintillation fluid and counted on a Beckman LS 6000 TA scintillation spectrometer.

3.2 Cytotoxic activity

3.2.1 Introduction

The KB cells (human epidermoid carcinoma of the nasopharynx) have proved to be useful in the comparison for *in vitro* cytotoxicity of natural products with *in vitro* antiprotozoal activity (Anderson *et al.*, 1992). A prerequisite for potential antiprotozoal agents is that they should display a high degree of selectivity towards the parasite, *i.e.* have low toxicity to the host (Anderson *et al.*, 1992). An estimation of therapeutic index in which the desired biological activity is compared with general toxicity is one in which selectivity maybe assessed. For the plant extracts and plant-derived compounds tested, the cytotoxicity against human nasopharyngeal KB cells and antiprotozoal activity against *L. donovani, T. b. brucei* and *T. cruzi* and *P. falciparum* has been compared and a ratio (IC_{50} for KB cells/IC_{50} for protozoa) has been calculated. A value greater than 1 is considered to be more selective against the parasite, and a value less than one of more selectivity to KB cells. The selectivity of some compounds towards one or more species of protozoa has also been evaluated. In addition, the cytotoxicity of selected extracts and pure plant-derived compounds were also assessed against P388D1 murine lymphocytic leukemia cells in order to compare the cytotoxicity of some samples with other mammalian cells.

3.2.2 Materials and Methods

3.2.2.1 The KB cytotoxicity test

3.2.2.1.1 Maintenance of cultures

Human nasopharyngeal KB cells obtained from Flow laboratories (U.K) were cultures in tissue culture flasks in complete medium composed of Eagles Minimum Essential Medium with Earl's salts and 0.85% g/l of sodium bicarbonate, supplemented with non-essential amino acid (1%, v/v), HIFCS (10%, v/v), L-glutamine (2mM), penicillin (50 U/ml) and streptomycin (50 µg/ml); cells were incubated at 37 ^{0} C in 5% CO_2-air mixture. The cells were subcultured by pouring out the culture medium and detaching the cells by adding 5-10 ml of trypsin/EDTA solution (0.05%/0.02%). After 10-15 minutes the cell suspension was transfered into sterile centrifuge tubes, spun at 400 r.p.m. for approximately 20 seconds and the supernant discarded. The pellet was washed with complete medium, spun and supernant discarded twice. The cells were throughly mixed by pipetting the suspension to break up any aggregates. Cell number was determined using a haemocytometer; an appropriate number of cells transferred to new tissue culture flasks, and diluted with completed medium to give a final concentration of 10^5 cells/ml. The flasks with the cells were incubated at 37 ^{0}C in 5% CO_2 air-mixture. The medium was changed twice a week.

3.2.2.1.2 Test protocol

A modification of the method developed by Anderson *et al.* (1991) was used to assess *in vitro* cytotoxicity against KB cells. Test samples were dissolved in ethanol or DMSO and diluted with complete medium to give concentrations of 1µg /ml. The final concentration of ethanol or DMSO never exceeded 0.0.1 %, a non toxic level. The test was carried out in 96-well microtitre plates and each well contained 50 µl of diluted sample. Two fold serial dilutions were performed along the breadth of the microtitre plate to obtain 7 concentrations. The 8th well was left as a drug free control. Podophyllotoxin and emetine were used as control drugs. To each well was added 50 µl of a 1 x 10^6 cell/ml suspension giving a total volume of 100 µl/ml. The plates were covered with lids and placed in a 5% CO_2-air mixture incubator at 37 ^{0}C for 48 h.

The cells were fixed by adding 65 µl/ml of 25% trichloroacetic acid at 4 °C to each well and refrigerated at 4 °C for 1 hour. The plates were rinsed with distilled water 5 times and left to dry. Protein staining was performed by adding 100 µl of 1% aqueous eosin B stain to each well and left to stand for 1 h. The plates were washed 5 times with 1% acetic acid and allowed to dry. 200 µl of 5 mM sodium hydroxide solution was added to each well and kept for 20 minutes to digest protein and extract the dye. The optical density (O.D.) of the solution in each well was determined at 490 nm by a MR700 microplate reader (Dynatech Labs. Inc.). Using the absorbancy reading for each well the inhibition of KB cells growth was calculated as percentage of the control.

3.2.2.2 The P388D1 cytotoxicity test

3.2.2.2.1 Maintenance of culture

Murine lymphocytic leukemia P388D1 cells were cultured in tissue culture flasks with complete medium containing Dulbecco's Modified minimum essential medium (DMEM, GIBCO), with 2.5 µg/l $NaHCO_3$, supplemented with L-glutamine (20 mM), penicillin (100 U/ml), streptomycin sulphate (100 µg/ml), mercaptoethanol (0.05 mM) and 10% heated inactivated foetal bovine serum at 37 °C in 100% humidity with 5% CO_2 atmosphere in air. The cells were subcultured by pouring out the culture medium and detaching the cells by adding 5-10 ml of trypsin-EDTA solution (0.05%/0.02%). After 5 minutes the cell suspension was transfered into sterile centrifuge tubes, spun at 1050 r.p.m. for 10 minutes and the supernant discarded. The pellet was washed twice with complete medium, spun and the supernant discarded twice. Cell number was determined and the appropiate number of cells transferred to a new tissue culture flask and diluted with complete medium to give a final concentration of 10^4 cells/ml and incubated at 37 °C in 5% CO_2 air-mixture.

3.2.2.2.2 Test protocol

The method described by Likhitwitayawuid *et al.* (1993) was used to assess for *in vitro* cytotoxicity against P388D1 cells. Test samples were dissolved in EtOH or DMSO and diluted with complete medium to give concentrations of 1 µg/ml. The test was carried out in 96-well microtitre plates, each well contained 100 µl of diluted sample The final

concentration of EtOH or DMSO never exceeded 0.05%, a non toxic level. Two fold serial dilutions were performed along the breadth of the microtitre plate to obtain 7 concentrations. The 8th well was left as a drug free control. Podophyllotoxin was used as control drugs. To each well was added 100 µl of a 1 x 10^5 cell/ml suspension giving a total volume of 200 µl/ml. The plates were covered with lids and placed in a 5% CO_2-air mixture incubator at 37 ^{0}C for 48 h. After the incubation period, the cells were fixed by adding 100 µl/ml of 40% trichloroacetic acid (4 ^{0}C) to each well and refrigerated at 4 ^{0}C for 1 hour. The plates were rinsed 4 times with distilled water and air-dried. The trichloroacetic-acid-fixed cells were stained by the addition of 100 µl of 0.4% of sulforhodamine B (w/v) dissolved in 1% aqueous acetic acid for 30 minutes. Free sulforhodamine B solution was then removed by washing with 1% aqueous acetic acid (4x). The plates were then air-dried, and the bound dye was solubilized by the addition of 200 µl of 10 mM unbuffered Tris base, pH 10. Finally, the optical density was determined at 550 nm using a microplate reader.

3.3 Determination of IC_{50} values

The inhibitory concentration, IC_{50}, is the concentration of drug causing 50% inhibition of parasite or cell growth in the *in vitro* test. The IC_{50} values for each drug for antiprotozoal and cytotoxic activities as well as their standard deviations were calculated using the Minitab statistical programme package. Furthermore, the results obtained from the in vitro screening tests were analyzed using t' test statistical models to compared treated and untreated groups.

3.3. Brine Shrimp lethality bioassay

3.3.1 Introduction

The brine shrimp bioassay is a general bioassay which detects a broad range of biological activities and a diversity of chemical structures in higher plants. Bioactive compounds are always toxic in high doses. Thus *in vivo* lethality in a simple zoological organism maybe used as a rapid and simple monitor for the detection of bioactive compounds and plant extracts which may be manifested as toxic toward newly hatched nauplii (Mclaughlin *et al.*, 1991, 1993). The brine shrimp bioassay is not specific for any particular

physiological action but for a significant number of species with cytotoxic activity it may be possible to monitor fractionation using principally the brine shrimp rather than more expensive time consuming cytotoxic assays. Active compounds thus obtained could be subjected to more elaborated bioassays for specific pharmacological activities (Meyer *et al.*, 1982). The brine shrimp assay has the advantage of being rapid, inexpensive and simple. The assay provides a convenient means by which the presence of cytotoxic compounds may be detected during the fractionation of plant extracts.

3.3.2 Materials and Methods

3.3.2.1 Hatching of brine shrimp eggs

Brine shrimp eggs, *Artemia salina* obtained locally (Interpet Ltd. Dorking, England) were hatched in artificial sea water (40 g/l) prepared from sea salt (Sigma) and distilled water, and oxygenated with an aquarium pump. After 48 h incubation in a warm room (22-29 ^{0}C), nauplii were collected with a Pasteur pipette after attracting the organism to one side of the vessel with a light source. Nauplii were separated from the eggs by pipetting them 2-3 times in a small beakers containing sea water, and a suspension of 10-15 shrimps in 100 µl of sea water was prepared before each test.

3.3.2.2 Test protocol

The microdilution method developed by Solis *et al.* (1993) was used to assess *in vivo* lethal toxicity against brine shrimps. Samples were dissolved in DMSO and diluted with artificial sea water. The final concentration of DMSO never exceeded 0.05% which has no significant effect on the brine shrimps. The test was carried out in 96-well microtitre plates and each test well contained 100 µl of diluted sample. Twofold serial dilutions were performed along the breadth of the microtitre plate to obtain 7 concentrations in triplicate. The 8th well with DMSO was used as a drug free control. A suspension of nauplii containing 10-15 organisms (100 µl) was added to each well and the covered plate incubated at 22-29 ^{0}C for 24 h. Plates were examined under an inverted phase microscope (x12.5) and the number of dead nauplii in each well were counted. 100 µl methanol was added to each well and after 15 minutes the total number of shrimps in each well were counted. LC_{50} values were then calculated by Probit analysis (Finney, 1971).

3.4 Results and Discussion

The *in vitro* antiprotozoal and cytotoxic activitites (IC_{50} values) of standard drugs, extracts and pure plant-derived compounds are shown in Tables 3.1 to 3.14. The structures of plant-derived compounds are illustrated in Fig. 3.1 to 3.12. The IC_{50} values and MIC of samples tested are the means of two or three determinations. For the samples tested the toxicity to KB cells and activity against protozoa has been compared and a ratio of IC_{50} KB cells/ IC_{50} protozoa calculated. A value greater than 1 is considered as being of more selective against KB cells, and a value lower than 1 is considered as being more selective to the parasite.

3.4.1 Plant extracts

The results in Table 3.2 revealed that from 97 extracts screened for *in vitro* antiprotozoal activity against *L. donovani* promastigotes and *T. b. brucei* trypomastigotes, 12 crude extracts had IC_{50}s equal to or below 16.22 μg/ml; a further 19 had IC_{50} values in the range of 22.10 and 69.34 μg/ml with IC_{50} values of less than 10 μg/ml against *L. donovani* promastigotes. In the same conditions the standard drug pentamidine had an IC_{50} value of 0.24μg/ml against *L. donovani* promastigotes (Table 3.1). The most active extract was *T. patens* with an IC_{50} value of 1.5 μg/ml against *L. donovani* promastigotes. Fourteen plants had MEC values between 7.81 and 50 μg/ml against *T. b. brucei*. *Annona purpurea* and *Alstonia macrophylla* were the most active extracts against *T. b. brucei* with an IC_{50} value of 7.8 μg/ml. In the same conditions pentamidine had an IC_{50} value of 0.6 ng/ml.

Annona purpurea was found to be the most active extract against KB cells. It is 92 times as toxic as the standard podophyllotoxin (IC_{50} = 0.0073 μg/ml). Apart from *Annona purpurea*, none of the extracts screened for antiprotozoal activity were found as toxic to KB cells as the standard podophyllotoxin. Cytotoxicity did not always correlate with antitrypanosomal activity as can be seen with *G. grandifolia* and *P. antidesma*.

In assessing selectivity of action against protozoan versus mammalian cells, it can be seen that the antileishmanial and trypanocidal activities of *Swietenia humillis* and *Annona*

Table 3.1 *In vitro* activities of standard drugs

Drug	*L. donovani* Promastigotes IC_{50} µg/ml (B)	*L. donovani* Amastigotes IC_{50} µM ± SEM (A)	*L. donovani* Amastigotes IC_{50} µg/ml	*T. b. brucei* Trypomastigotes MEC µg/ml	*T. b. brucei* Trypomastigotes MEC µM ± SEM (C)	*T. cruzi* Amastigotes IC_{50} µg/ml	*T. cruzi* Amastigotes IC_{50} µM ± SEM (D)	*P. falciparum* Blood schizonts IC_{50} µg/ml	*P. falciparum* Blood schizonts IC_{50} µM ±SEM (E)	KB cells IC_{50} µg/ml	KB cells IC_{50} µM ± SEM (F)	F/A ratio	KB IC_{50}/ parasite IC_{50}	Brine shrimps IC_{50} µg/ml	Brine shrimps IC_{50} µM ± SEM
Pentamidine isothionate	0.24	0.40 ± 0.09	NT	$6x10^{-4}$	$3.4x10^{-4} \pm 5x10^{-5}$	NT	-	NT	-	0.10	0.17 ± 0.06	0.42	478.87 [a]	-	NT
Sodium stibogluconate	500	-	9.75	NT	NT	NT	-	NT	-	332.58	-	0.66	34.11 [b]	-	NT
Ketoconazole	232	-	NT	NT	NT	NT	-	NT	-	19.41	1.16 ± 0.51	0.08	-	-	NT
Nifurtimox	NT	-	NT	NT	NT	0.83	2.90 ± 0.1	NT	-	-	-				
Chloroquine diphosphate	NT	-	NT	NT	NT	NT	-	0.2	0.59 ± 0.10	51.36	160.56 ± 2.20	-	272.13 [c]	>1000	>3126.07
Emetine hydrochloride	0.30	0.62	NT	NT	NT	NT	-	NT	-	1.46	0.70± 0.09	1.13	-	13.94	29.0 ± 60 [d]
Podophyllotoxin	NT	-	NT	NT	NT	NT	-	NT	-	$6.21x10^{-3}$	0.015 ± 0.008	-	-	6.22	15.0 ± 2.1 [d]

[a]F/C; [b]F/B; [c]F/E · [d]Data obtained Solis (1994). NT: Not tested

Table 3.2 *In vitro* antiprotozoal and toxic activities of crude plant extracts.

Plant	Origin and Use	Part	Extract	*L. donovani* Promastigotes IC_{50} µg/ml ±SEM (A)	*T. b. brucei* Trypomastigote MEC µg/ml ±SEM (C)	KB cells IC_{50} µg/ml± SEM (F)	F/A	F/B	P388D1cells IC_{50} µg/ml ± SEM	Brine shrimps LC_{50} µg/ml±SEM
ALBERTICIAE										
Epinetrum ferruginum	8,f	L	MeOH	69.34 ± 3.76	125 ± 4.32	163.62 ± 1.58	2.36	1.31	NT	755.63 ± 4.90
			H_2O	250 ± 1.90	357 ± 2.90	432 ± 2.56	1.78	1.21	NT	>1000
ANARCADIACEAE										
Rhus aucheri	3,m,f	L	MeOH	55.85 ± 1.59	>500	142.32 ± 1.66	2.55	-	NT	NT
		B	MeOH	171 ± 2.60	NT	>500	-	-	NT	NT
		S	MeOH	141.87 ± 1.70	NT	136.32 ± 1.13	0.96	-	NT	NT
			H_2O	323.59 ± 4.67	NT	168.13 ± 3.80	0.52	-	NT	NT
ANNONACEAE										
Annona purpurea	1,f	B	MeOH	113.24 ± 1.20	125 ± 3.98	$0.0098 \pm 6x10^{-4}$	$8.7x10^{-5}$	$7.8x10^{-5}$	NT	0.065 ± 0.004
			H_2O	289 ± 3.70	>500	79.40 ± 4.20	0.27	-	NT	NT
		S	MeOH	28.57 ± 1.78	7.81 ± 1.45	$0.0001 \pm 4x10^{-5}$	$3.5x10^{-6}$	$1.28x10^{-5}$	NT	0.0013 ± 0.0006
			H_2O	179.90 ± 4.10	96.70 ± 3.90	59.40 ± 1.11	0.33	0.61	NT	NT
APOCYNACEAE										
Alstonia macrophylla	8,m,f	B	MeOH	4.09 ± 1.23	7.81 ± 2.70	49.86 ± 1.87	12.19	6.38	>100	NT
			H_2O	86 ± 1.38	170.07 ± 1.90	92 ± 3.33	1.39	0.54	NT	26.86 ± 2.70
Rhazya stricta	3,f,c	L	MeOH	3.50 ± 0.60	50.00 ± 4.80	101.50 ± 3.78	29.00	2.03	22.17 ± 3.44	NT
			H_2O	12.50 ± 1.98	360.70 ± 6.78	467.90 ± 6.12	37.36	1.29	NT	NT
ASCLEPIADACEAE										
Calotropis procera	3,f,m	S	MeOH	16.22 ± 0.68	NT	11.70 ± 2.59	0.72	-	NT	NT
			H_2O	76.19 ± 2.77	NT	380.19 ± 3.86	5.00	-	NT	NT
ASTERACEAE										
Ratibida latipaliaris	1,infec	AP	MeOH	28.84 ± 0.22	31.25 ± 4.14	75 ± 1.27	2.60	2.4	NT	141.04 ± 2.60
Ratibida mexicana	1,d, infec	AP	MeOH	31.60 ± 1.02	125 ± 3.50	66.71 ± 0.98	2.11	0.53	NT	84.11± 3.80
Sonchus oleraceus	3,f,m	WP	MeOH	440.55 ± 3.80	NT	>500	-	-	NT	NT
	3,f,m	WP	MeOH	69.18 ± 2.82	500.00 ± 2.20	>500	-	-	NT	NT

Table 3.2 *In vitro* antiprotozoal and toxic activities of crude plant extracts (Continued).

Plant	Origin and Use	Part	Extract	*L. donovani* Promastigotes IC_{50} µg/ml ±SEM (A)	*T. b. brucei* Trypomastigote MEC µg/ml ±SEM (C)	KB cells IC_{50} µg/ml± SEM (F)	F/A	F/B	P388D1cells IC_{50} µg/ml ± SEM	Brine shrimps LC_{50} µg/ml±SEM
ASTERACEAE										
Vernonia cinerea	3,f,m	WP	MeOH	142.90 ± 1.17	NT	181.92 ± 1.15	1.27	-	NT	NT
			H_2O	443.61 ± 2.35	NT	>500	-	-	NT	NT
BORAGINACEAE										
Arnebia hispidissima	3,f,m	WP	MeOH	66.68 ± 1.45	NT	227.00 ± 1.14	3.40	-	NT	NT
			H_2O	>500	NT	>500	-	-	NT	NT
		SP	MeOH	171.80 ± 1.12	NT	407.43 ± 3.70	2.37	-	NT	NT
			H_20	>500	NT	>500	-	-	NT	NT
BRASSICACEAE										
Sysymbium irio	3,f,m	AP	MeOH	>500	NT	214.83 ± 1.12	-	-	NT	NT
CAPPARACEAE										
Capparis spinosa	3,f,m	L	MeOH	199.53 ± 0.98	NT	114.01 ± 1.34	0.57	-	NT	NT
			H_2O	360.58 ± 3.02	NT	>500	-	-	NT	NT
Maerua crassifolia	3,f,m	S	MeOH	371.53 ± 1.98	NT	>500	-	-	NT	NT
			H_20	>500	NT	172.81 ± 2.56	-	-	80.50 ± 4.44	160 ± 3.80
CHENOPODIACEAE										
Teloxys graveolens	1,h, infect	AP	MeOH	42.30 ± 1.27	500 ± 3.66	63.92 ± 1.78	1.51	0.13	NT	NT
			H_20	255.34 ± 0.98	NT	356.89 ± 2.40	1.39	-	NT	>1000
COMPOSITAE										
Scoloymus hispanicus	7,v	AP	MeOH	>500	>500	380.90 ± 2.80	-	-	NT	>1000
			H_20	>500	NT	NT	-	-	20±0.60	154.56 ± 4.60
EUPHORBIACEAE										
Celaenodendron mexicanum	1,inf	L	MeOH	39.50 ± 1.10	500	122.59 ± 1.56	1.07	0.063	>100	530 ±1.4
			H_20	367.90 ± 1.56	>500	200 ± 1.90	0.70	-	NT	>1000
LEGUMINOSAE										
Parkia biglobosa	4,p	B	MeOH	47.32 ± 1.39	125.00 ± 2.22	297.26 ± 0.81	6.28	2.38	NT	>1000
			H_2O	150 ± 4.40	500	467 ± 1.12	3.11	-	NT	NT
Senna reticulata	8,f	L	MeOH	>500.00	>500.00	232.90 ± 0.29	-	-	>500	>1000
			H_20	>500.00	>500.00	>500.00	-	-	>500	>1000

Table 3.2 *In vitro* antiprotozoal and toxic activities of crude plant extracts (Continued).

Plant	Origin and Use	Part	Extract	*L. donovani* Promastigotes IC_{50} µg/ml ±SEM (A)	*T. b. brucei* Trypomastigote MEC µg/ml ±SEM (C)	KB cells IC_{50} µg/ml± SEM (F)	F/A	F/B	P388D1cells IC_{50} µg/ml ± SEM	Brine shrimps LC_{50} µg/ml±SEM
LECYTHIDACEAE										
Napoleona heudelotii	8,f	S	MeOH	41.20 ± 0.45	31.25 ± 1.30	63.71 ± 0.76	1.55	2.03	NT	>1000.00
			H_2O	>500	>500	124.80 ± 2.90	-	-	NT	>1000.00
LORANTHACEAE										
Struthanthus orbicularis	8,f	L	MeOH	54.83 ± 0.89	125.00 ± 6.70	201.12 ± 1.67	3.66	1.60	NT	>1000.00
			H_2O	198.98 ± 5.19	500 ± 3.70	NT	-	-	NT	>1000.00
MALPHIGEACEAE										
Galphimia glauca	1,m,f,d CNS	AP	MeOH	68.20 ± 0.24	125 ± 2.29	163.87 ± 0.33	2.40	1.31	94.91 ± 1.80	368.50 ± 6.80
			H_2O	103.67 ± 3.12	500 ± 6.90	>500	-	-	NT	NT
MELIACEAE										
Guarea grandifolia	2,nu	B	MeOH	162.18 ± 1.06	500 ± 3.77	1.96 ± 0.38	0.012	$3.9x10^{-3}$	NT	NT
			H_2O	>500	> 500	289.00 ± 1.12	-	-	NT	NT
Guarea macropetala	2,nu	BR	MeOH	38.70 ± 1.14	31.25 ± 6.80	128.82 ± 0.96	3.33	4.12	NT	93.30 ± 5.04
			H_2O	>500	500 ± 6.00	>500	-	-	NT	>1000
Guarea rhopalocarpa	2,nu	L	MeOH	14.00 ± 0.87	31.25 ± 2.59	32.91 ± 1.19	2.35	1.05	14.84 ± 2.22	5.80 ± 2.10
			H_2O	>500	500 ± 1.23	244.76 ± 1.38	-	0.49	NT	>1000
Malacea depresa	1,f,d	B	MeOH	107.15 ± 1.20	500 ± 4.80	204.17 ± 2.56	1.90	0.41	NT	NT
			H_2O	>500	>500	NT	-	-	NT	NT
Ruagea glabra	2,nu	SB	MeOH	53.70 ± 1.32	125.00 ± 1.11	123.03 ± 3.62	2.29	0.98	NT	228.80 ± 3.80
			H_2O	>500	>500	500 ± 3.00	-	-	NT	>1000
Swietenia hummillis	1,d,f	B	MeOH	13.67 ± 0.54	500 ± 3.45	11.48 ± 0.60	0.84	0.02	36.77 ± 5.66	NT
Swietenia macrophylla	1,f	B	MeOH	14.12 ± 0.92	500 ± 2.00	87.09 ± 2.19	6.17	0.17	>100	NT
		L	MeOH	9.18 ± 0.60	50 ± 1.34	24 ± 1.78	2.61	0.48	51.43 ± 1.29	NT

Table 3.2 *In vitro* antiprotozoal and toxic activities of crude plant extracts (Continued).

Plant	Origin and Use	Part	Extract	*L. donovani* Promastigotes IC_{50} µg/ml ±SEM (A)	*T. b. brucei* Trypomastigote MEC µg/ml ±SEM (C)	KB cells IC_{50} µg/ml± SEM (F)	F/A	F/B	P388D1cells IC_{50} µg/ml ± SEM	Brine shrimps LC_{50} µg/ml±SEM
MENISPERMACEAE										
Stephania dinklagei	4,p,v	AP	MeOH	8.00 ± 0.21	31.25 ± 3.30	63.90 ± 1.78	7.98	2.04	15.08 ± 1.67	69.38 ± 2.50
			H_2O	8.16 ± 0.80	125 ± 3.00	190.55 ± 1.58	23.35	1.52	NT	260.00 ± 3.0
Triclisia patens	4,f,m	AP	MeOH	1.50 ± 0.16	31.25 ± 2.00	45.90 ± 0.39	30.60	1.47	12.08 ± 2.45	821.023 ± 5.80
			H_2O	5.83 ± 0.22	50 ± 1.23	>500	-	-	NT	>1000
Triclisia subcordata	8,f,m	AP	MeOH	75.86 ± 0.95	500 ± 2.50	130.37 ± 2.67	1.72	0.26	NT	696.37 ± 2.40
			H_2O	240 ± 3.67	500 ± 2.20	>500	-	-	NT	>1000
ORCHIDACEAE										
Stenorrhynchos lanceolatus	1,inf	AP	MeOH	99.04 ± 1.47	NT	346.74 ± 0.70	3.50	-	NT	NT
			H_2O	>500	NT	>500	-	-	NT	NT
RUBIACEAE										
Cephaelis componutans	2,nu	R	MeOH	6.68 ± 1.07	50.00 ± 4.5	80.80 ± 0.73	0.41	0.06	18.56 ± 0.97	275 ± 4.0
			H_2O	>500	>500	NT	-	-	NT	NT
Cephaelis dichroa	2,nu	AP	MeOH	74.32 ± 1.25	125.00 ± 3.80	202.28 ± 1.12	2.72	1.62	NT	>1000
			H_2O	225 ± 3.90	>500	NT	-	-	NT	NT
Cephaelis glomerulata	2,nu	AP	MeOH	30.90 ± 1.13	31.25 ± 4.40	286.25 ± 2.93	9.26	9.16	NT	>1000
			H_2O	450 ± 3.96	>500	NT	-	-	NT	NT
Cigarrilla mexicana	1,d,e	AP	MeOH	>500	125 ± 1.78	13.04 ± 1.76	-	0.14	NT	440.80 ± 3.70
Craterispermum laurinum	8,f	B	MeOH	41.10 ± 5.5	31.25 ± 6.49	83.86 ± 0.98	2.04	0.67	NT	411.35 ± 2.20
Hintonia latiflora	1,m, infec	B	MeOH	85.10 ± 1.05	31.25 ± 2.20	56.91 ± 1.11	0.66	0.45	34.78 ± 1.45	NT
			H_2O	129.78 ± 1.56	400 ± 3.20	126.00 ± 4.50	-	-	NT	NT
Nauclea latifolia		R	MeOH	74.40 ± 1.16	>500.00	58.00 ± 2.95	0.77	-	28.56 ± 1.60	>1000

Table 3.2 *In vitro* antiprotozoal and toxic activities of crude plant extracts (Continued).

Plant	Origin and Use	Part	Extract	*L. donovani* Promastigotes IC_{50} µg/ml ±SEM (A)	*T. b. brucei* Trypomastigote MEC µg/ml ±SEM (C)	KB cells IC_{50} µg/ml± SEM (F)	F/A	F/B	P388D1cells IC_{50} µg/ml ± SEM	Brine shrimps LC_{50} µg/ml±SEM
RUTACEAE										
Stauranthus perforatum	8,f	AP	MeOH	131.22±1.30	31.25 ± 3.44	47.80±1.44	0.36	1.53	NT	NT
SIMAROUBACEAE										
Ailanthus altissima	6,f,m	B	MeOH	>500	500 ± 7.00	82.30 ± 0.45	-	0.16	NT	476.44 ± 3.0
			H_2O	>500	>500	NT	-	-	NT	399.05 ± 2.11
Brucea javanica	5,m,d	F	MeOH	246.68 ± 1.20	500 ± 2.60	9.20 ± 0.23	0.037	0.018	10.69 ± 3.21	NT
			H_2O	>500	>500	1.19 ± 0.33	-	-	NT	NT
Picramnia antidesma	2,m	L	MeOH	300 ± 2.30	500 ± 2.00	8.70 ± 1.10	0.029	0.017	3.98 ± 0.67	511.60 ± 5.30
			H_2O	>500	>500	370 ± 2.30	-	-	NT	NT
SOLANACEAE										
Solanum nigrum	3,f	WP	MeOH	197.67 ± 1.29	NT	88.70 ± 3.90	0.44	-	NT	NT
Withania somnifera	3,f,m	L	MeOH	22.10 ± 1.62	NT	19.70 ± 2.68	0.89	-	NT	NT
ZYGOPHYLLACEAE										
Fagonia indica	3,f,m	AP	MeOH	>500	NT	>500	-	-	NT	NT
			H_2O	>500	NT	NT	-	-	NT	NT

Country: Mexico (1), Panama (2), Oman (3), Ghana (4), Thailand (5), London (6), Egypt (7), Nigeria (8).
Usos: malaria (m), fever (f), infection (inf), dysentery (d), anti-inflammatory (inf)l, anthelmintic(h), fish poison (p). hypoglucemic (h), emetic (e).
Part: Leaves (L), Aerial Parts (AP), Bark (B), Fruit (F), Whole plant (WP), Seed (S), Root (R), Branch (BR), Stem Bark (SB). various uses (v), not used (nu), central nervous system (CNS). NT: not tested

purpurea was related to their toxicity to KB cells. This is demonstrated by their ratio F/A and F/C values of less than one (Table 3.2). This indicated that they are more toxic to mammalian cells than to protozoa. In contrast, the most active extracts were more selective against the parasites than the mammalian cells. This is demonstrated from their ratio (IC_{50} for KB cells/ IC_{50} for protozoan) values of more than one. *Triclisia patens* and *Rhazya stricta* were found to be the most selective against *L. donovani* promastigotes, with F/A ratios of 30.60 and 29 respectively whereas *Cephaelis glomerulata* and *Alstonia macrophylla* were the most selective against *T. b. brucei* with a favourable F/B ratios of 9.16 and 6.38 respectively.

Two indole alkaloids villastonine and macrocarpamine isolated from *Alstonia angustifolia* displayed anti-*Plasmodium falciparum* activity (Wright *et al.*, 1992). The indole alkaloids could explain the antileishmanial and trypanocidal activity of *A. macrophylla*, since related indole alkaloids have been found active against *L. donovani* (Iwu *et al.*, 1994). Comparison of the cytotoxic activity/antiprotozoal activity ratios for *Alstonia macrophylla* against both parasites indicate some degree of selectivity against *L. donovani* (12.19) rather than to *T. b. brucei* (6.38).

Rhazya stricta has been shown *in vitro* antimalarial properties (Marshall, personal communication). Phytochemical studies of this plant have revealed the presence of a number of alkaloids such as indole and 8-vinylquinoline (Atta-Ur-Rahman *et al.*, 1991); which could explain the antiprotozoal activity of the plant, since related indole (Iwu *et al.*, 1994) and quinoline (Fournet *et al.*, 1993c) alkaloids have been shown *in vitro* antiprotozoal activity.

Cephaelis glomerulata (Solis *et al.*, 1997) has shown the presence of three calycanthine-type alkaloids. It is known that quinoline derivatives alkaloids possess significant antiprotozoal activity *in vivo* studies (Fournet *et al.*, 1994b). Thus, it is possible that these quinoline analogues are responsible for the antitrypanosomal activity of this species.

Ratibida ratipaliaris was active against both *Leishmania* and *Trypanosoma*, probably the antiprotozoal activity found in this plant is related to sesquiterpenes lactones ratibinolide I and ratibinolide II , as well as the flavone hispiduline (Mata *et al.*, 1990b; Rojas *et al.*, 1991). Since some sesquiterpene lactones (Fournet *et al.*, 1993a), and flavonoids have been shown to possess antileishmanial activity (Chen *et al.*, 1993). This could explain the use of this plant for the treatment of skin wounds and inflammation. The antiprotozoal activity of some sesquiterpene lactones has been attributed to the exocyclic double bound which is able to form Michael adducts with free nucleophiles in the parasites or mammalian cells.

The antiparasitic activities of some plants (e.g. *Annona purpurea* bark and *Picramnia antidesma*) did not parallel the brine shrimp lethality test. However, the antiparasitic and cytototoxic activities of the seed of *Annona purpurea* , *Guarea rhopalocarla, Celaenodendron mexicanum, Cephaelis camponutans*, correlated with the toxicity against brine shrimps. These differences could be attributed to differences in the chemical compositions or differences in the concentration of metabolites present in the plants. A search in the literature indicates that there are, no previous reports about the antileishmanial and trypanocidal activities reported for the 47 plant-species screened in this study, thus they are worthy of further studies.

From this preliminary study, *Celaenodendron mexicanum*, *Galphimia glauca*, *Stephania dinklagei*, *Triclisia patens*, *Hintonia latiflora*, *Guarea rhopalocarpa* and *Cephaelis camponutans* extracts were selected for further investigation in order to isolate their active principles. Bioactive guided fractionation of plant extracts using *in vitro* assays against *L. donovani*, *P. falciparum,* KB cells or *Artemia salina*, and a combination of chromatographic techniques yielded a series of active compounds including alkaloids, quinones, terpenes, flavonoids and coumarins (see Chapter 2). In addition, to the bioactive compounds, others compounds present in the plants were also isolated, some semi-synthetic derivatives were prepared (see Chapter 2), and other related compounds which were available in our laboratory were also screened for *in vitro* antiprotozoal and cytotoxic activities, in order to establish a structure-activity relationship among them.

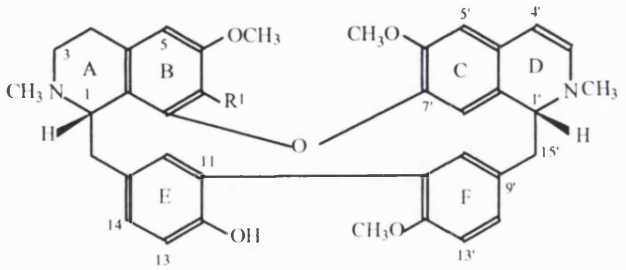
3.4.2 Plant-derived compounds

3.4.2.1 Alkaloids

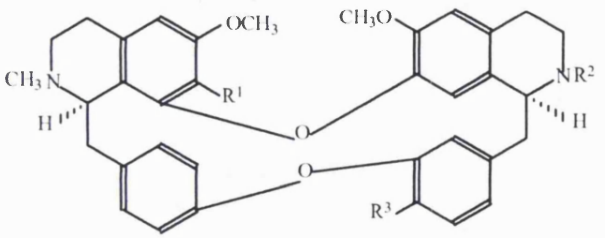
3.4.2.1.1 Bisbenzylisoquinoline (BBIQ) alkaloids

The bisbenzylisoquinoline (BBIQ) alkaloids constitute a series of almost 400 phenylalanine-derived metabolites with a rich and varied chemistry and pharmacology. They occur in 57 genera representing 14 families. BBIQ alkaloids comprise two isoquinoline moieties ("head" portions) linked to two benzyl moieties ("tail" portions). They have been classified into 26 structural types (denoted by Roman numerals) according to the number, position, and type of bridges linking the two monomers (Schiff, 1991). The alkaloids investigated in the present work are representative of nine of the known structural types: IV, VI, VII, VIII, XVIII, XX, XXIII, XXIV, and XXVI. Alkaloids within each group differ from one another by their stereochemistry at C-1' and/or their substituents and nitrogen functionalities (Figure 3.1). The results shown in Table 3.3 revealed that of the 20 BBIQ alkaloids tested 8 had IC_{50} values of less than 1 μg/ml against *L. donovani* promastigotes *in vitro*. A further 9 had IC_{50} values between 1 and 10 μg/ml. Under the same test conditions, pentamidine had an IC_{50} value of 0.24 μg/ml. The most potent BBIQ against promastigotes was found to be fangchinoline with an IC_{50} value of 0.24 μg/ml. In assessing structure activity relationships, it can be seen that the 17 BBIQ alkaloids with antileishmanial activity of less than 10 μg/ml (Table 3.3) have a range of different structures (Figure 3.1). It can be seen that dimers of the head-to-head and head-to-tail types, e.g. fangchinoline and insularine picrate are active. The status of the nitrogen atoms is fundamental for antileishmanial activity, as evidenced by trigilletimine, with an aromatic N-2', being 151-fold less active than isotrilobine, which is the corresponding D ring-saturated, N-2'-methylated analogue. Quaternization of N-2' results in the loss of antileishmanial activity, as indicated by a comparison of cosuline and cocsuline methiodide (Table 3.3), perhaps by limiting the drug's passage across cell membranes.

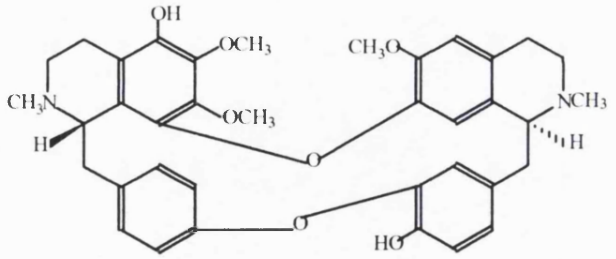
The substituents present in each monomeric half of the molecule may influence antileishmanial activity. A comparison of phenols and their corresponding methyl ethers indicates that a phenolic hydroxyl may result in less activity; cocsuline for example is 78 times less active than isotrilobine against *L. donovani* promastigotes



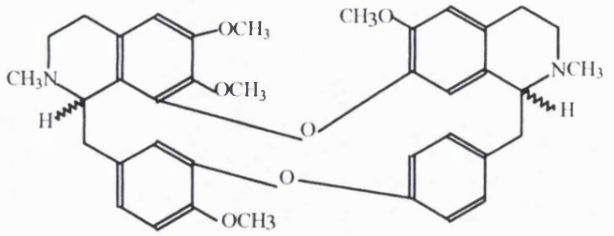
Type IV R1
Funiferine S,R OCH_3
Tiliageine S,R OH



Type VI R1 R2 R3
Daphnoline R,S OH H OH
Oxyacanthine R,S OCH_3 CH_3 OH
Aromoline R,S OH OH OCH_3

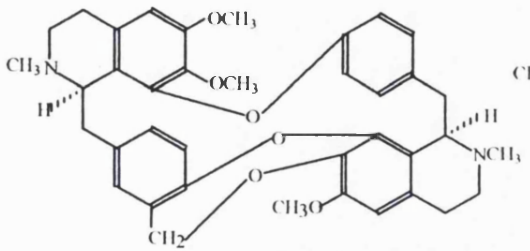


Type XXIII
Thalisopidine S,S

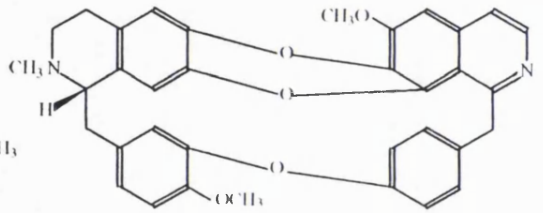


Type VIII
Phaenathine R,R
Isotetrandrine R,S

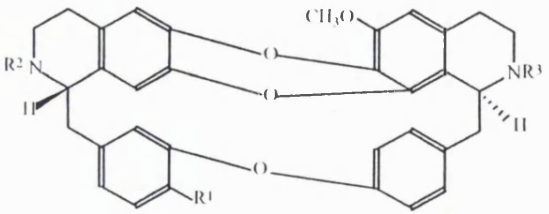
Figure 3.1 Bisbenzylisoquinoline alkaloids tested



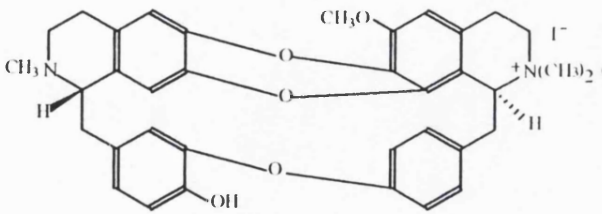
Type XXVI
Insularine R,R



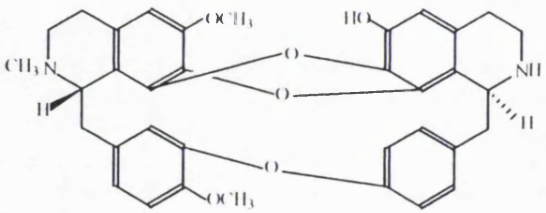
Type XXIII
Trigilletinine



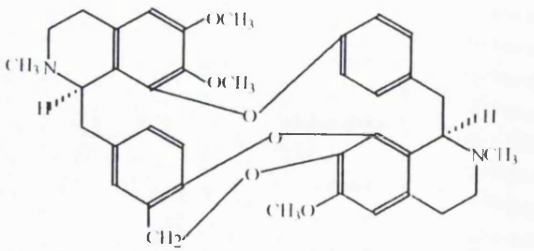
Type XXIII		R^1	R^2	R^3
Coesoline	S,S	OH	H	CH_3
Coesuline	S,S	OH	CH_3	CH_3
Isotrilobine	S,S	OCH_3	CH_3	CH_3
Trilobine	S,S	OCH_3	CH_3	H



Coesuline methiodide S,S



Type XXIV
Guilletine S,R



Type XXVI
Insularine R,R

Figure 3.1 Bisbenzylisoquinoline alkaloids tested (Continued)

Table 3.3 *In vitro* antiprotozoal and cytotoxic activities of Bisbenzylisoquinoline (BBIQ) alkaloids.

Bisbenzyl isoquinoline (BBIQ) alkaloids	*L. donovani* promastigotes IC_{50} µg/ml	µM ± SEM (A)	amastigotes µg/ml	µM ± SEM (B)	*T. b. brucei* trypomastigotes MEC µg/ml	µM ± SEM (C)	KB cells IC_{50} µg/ml	µM ± SEM (F)	F/A ratio	F/C ratio
Type IV										
Funiferine	0.33	0.53 ± 0.09	-	T[d]	2.41	3.88 ± 0.18	67.21	108.00 [a]	203.77	27.83
Tiliageine	0.48	0.79 ± 0.05	-	T[e]	5.0	8.22 ± 1.37	>250.00	>411.00 [a]	-	-
Type VI										
Daphnoline	>50.0	>86.21	-	T[e]	1.12	1.93 ± 0.21	26.91	46.40 [a]	-	24.04
Oxyacanthine HCl	0.44	0.68 ± 0.16	-	NT	-	NT	47.97	74.40 [a]	109.41	-
Aromoline	0.62	1.04 ± 0.16	-	NT	0.88	1.48 ± 0.02	64.15	108 ± 0.02	104	73
Type VII										
Thalisopidine	0.85	1.36 ± 0.04	T	T[d]	0.71	1.14 ± 0.01	25.72	41.20 [a]	30.26	36.14
Type VIII										
Phaeanthine[b,c]	9.79	15.74 ± 1.12	1.5	2.41 ± 0.23	1.07	1.73 ± 0.17	38.70	62.22 ± 0.53	3.95	35.96
Isotetrandrine	1.38	2.22 ± 0.22	T	T[e]	0.90	1.45 ± 0.04	65.31	105.00 [a]	47.30	72.41
Fangchinoline	0.24	0.39 ± 0.02	-	NT	-	NT	63.23	103.99 [a]	266.64	-
Berbamine	15.00	24.67 ± 1.22	T	T[d]	0.92	1.51 ± 0.18	10.82	17.80 [a]	0.72	11.78
Obamegine	0.75	1.26 ± 0.07	T	T[e]			32.91	55.40 [a]	43.97	-
Type XVIII										
Dinklacorine	2.39	4.15 ± 0.60	-	NT	-	NT	31.58	54.80 [a]	13.20	-
Type XX										
Isochondodendrine	7.00	11.78 ± 1.10	T	T[e]	>17.82	>30.00	>250.10	>421.00 [a]	-	-

Table 3.3 *In vitro* antiprotozoal and cytotoxic activities of Bisbenzylisoquinoline (BBIQ) alkaloids (Continued).

Bisbenzyl isoquinoline (BBIQ) alkaloids	*L. donovani* promastigotes IC$_{50}$ µg/ml	µM ± SEM (A)	amastigotes µg/ml	µM ± SEM (B)	*T. b. brucei* trypomastigotes MEC µg/ml	µM ± SEM (C)	KB cells IC$_{50}$ µg/ml	µM ± SEM (F)	F/A ratio	F/C ratio
Type XXIII Trigilletimine	4.79	8.58	>50.24	>90.00	-	NT	>249.98	>448.00 [a]	-	-
Type XXIII Cocsuline MeI	97.50	138.46 ± 2.74	>63.34	>90.00	-	NT	>222.00	>355.00 [a]	>2.56	-
Cocsoline [c]	9.10	16.59 ± 1.24	6.76	12.33 ±	-	NT	240.00	156.33 [a]	9.42	-
Isotrilobine	0.33	0.57 ± 0.014	-	1.29	0.86	1.49 ± 0.17	8.64	18.80 [a]	32.98	12.62
Trilobine	1.39	2.47 ± 0.26	-	NT NT	0.84	1.49 ± 0.16	11.69	20.80 [a]	8.42	13.96
Type XXIV Guilletine	0.74	1.28 ± 0.13	T	T [d]	1.50	2.60 ± 0.11	42.90	38.30 [a]	29.92	14.73
Type XXIV Insularine picrate	1.15	1.35 ± 0.18	T	T [d]	-	NT	249.73	>294.00 [a]	217.78	-

[a]Data obtained from Marshall *et al.*, (1994); T [d], T [e] = Toxic to macrophages at concentration range of 90-10 and 90-30 µM respectively at lower concentrations were not active. [b]Phaeanthine P388D1 IC$_{50}$ = 5.21 ± 0.07 µM (3.24 µg/ml). [c]Phaeanthine F/B = 25.82; Cocsoline F/B = 12.67.

(Table 3.3). In contrast, some BBIQ alkaloids possessing particular phenolic substituents increased activity in comparison with their corresponding methyl ethers, as exemplified by the BBIQ alkaloids of the type VIII, phaeanthine, isotetrandrine and berbamine, which are 40, 5 and 189 times less active than fangchinoline towards *L.donovani* promastigotes.

Antileishmanial activity was observed for alkaloids possessing R,R, S,S, R,S, and S,R configurations at C-1 and C-1'. A direct comparison of two alkaloids with identical ether linkages and substituents and differing only at C-1 and C-1' could be made for phaeanthine (R,R) and isotetrandrine (R,S). Phaeanthine and isotetrandrine differ significantly in their antileishmanial activity. Phaeanthine was found to be 7 times less potent than isotetrandrine.

Phaeanthine and cocsoline were found to be the most active compounds from this series of compounds against amastigote forms of *L. donovani.* Phaeanthine was 6-fold more potent than $NaSb^{v}$, but more toxic to macrophages, wheras cocsoline was as potent as $NaSb^{v}$ and less toxic than phaeanthine. Of the two, cocsoline has the best D/B ratio (35.5), indicating significantly higher activity against amastigotes than mammalian cells. In contrast, 8 BBIQ alkaloids including isotetrandrine were toxic to macrophages at the concentrations tested. It is possible that they may show activity at lower concentrations, since isotetrandrine has demonstrated *in vivo* antileishmanial activity against *L.venezuelensis* amastigotes (Fournet *et al.*, 1993b). Unfortunately, the quantity of these alkaloids available was insufficient to test them at different concentrations. Further work with these BBIQ might be worthwhile.

The IC_{50} values determined by other workers (Fournet et *al.*, 1988a) for daphnoline, berbamine, phaeanthine, cocsuline and isochondodendrine against the Indian strain of *L. donovani* promastigotes (MHOM/IN/83/HS 70) are in good agreement with those obtained in the present investigation against the Ethiopian strain (MHOM/ET/67/L82;LV9). The above five BBIQ alkaloids have also exhibited activity against different strains of *T. cruzi* trypomastigotes (Fournet *et al.*, 1988 b; 1994). The *in vitro* cytotoxic and antimalarial activities of other related BBIQ

alkaloids isolated from two Menispermaceous species: *Stephania erecta* (Likhitwitayawuid et *al.*, 1993) and *Cyclea barbata* (Lin et *al.*, 1993) have been reported.

None of the BBIQ alkaloids tested showed significant cytotoxic activity against KB cells, the most active being isotrilobine, which was 1880 times less toxic than the standard podophyllotoxin (Table 3.3). In assessing selectivity of action against *L.donovani* versus mammalian cells, the ratio (IC_{50} for KB cells/IC_{50} for *L.donovani* promastigotes) of IC_{50} values *in vitro* for the standard drug pentamidine is 0.41. This indicated that pentamidine is more toxic to mammalian cells than to the the parasite. The least cytotoxic alkaloids tiliageine, trigilletimine and insularine picrate showed high selective toxicity towards *L. donovani* promastigotes than to mammalian cells compared with the standard drug since they have ratio values of more than one (see Table 3.3).

The *in vitro* IC_{50} values of the 11 BBIQ alkaloids screened against the blood stream forms of *T. b. brucei* are given in the Table 3.3. The results indicated that 6 BBIQ alkaloids had IC_{50} values of less than 1 μg/ml against the trypomastigotes. In the same conditions pentamidine had an IC_{50} value of 6×10^{-3} μg/ml.

In assessing selectivity in antiprotozoal action it can be seen that the *in vitro* activity of funiferine, tiliageine and isochondodendrine was lower against *T. b. brucei* than against *L. donovani* promastigotes. However, the *in vitro* activity of daphnoline, phaeanthine and berbamine was lower against *L. donovani* promastigotes than *T. b. brucei.* The results indicated that these two groups of alkaloids had a high selective action against *L. donovani* and *T. b. brucei* respectively.

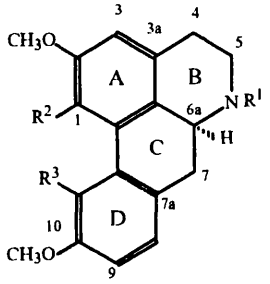
Other activities such as: antimicrobial, cardiovascular, central nervous, immunomodulatory, anti-inflammatory, and platelet aggregation activities (Schiff, 1991) have been reported for the BBIQ alkaloids berbamine, daphnoline, tetrandrine and trilobine.

3.4.2.1.2 Aporphine alkaloids

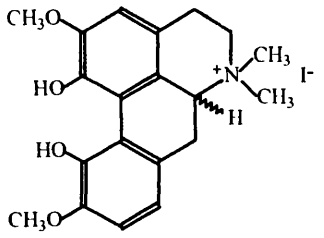
Ten aporphine alkaloids were tested for biological activity the results are given in the Table 3.4 and the structures in the Figure 3.2. Norcorydine and catalpifoline were the most active alkaloids of this group against *L. donovani* promastigotes and possess the most favourable F/A ratio (105.46, 114.7 respectively), *i.e* they have high selectivity to the promastigotes than to mammalian cells. Both alkaloids were inactive against amastigotes and *T. b. brucei* , thus they possess selective antiprotozoal action. It can be seen that norcorydine and catalpifoline bear a secondary amine (N-6), and are more active than corydine and isocorydine, which possess tertiary nitrogen (N-6), by a factor of 3 and 7 respectively. In addition,the quaternisation of N-6 also decreases the activity as it can be seen with magnoflorine, suggesting that a secondary amine is a structural requirement for *in vitro* antileishmanial activity in corydine-like alkaloids.

Ocoteine and dehydro-ocoteine alkaloids possess a methylene dioxy function on the A aromatic ring. Dehydro-ocoteine was not toxic to KB cells and amastigotes and has negligible activity against promastigotes of *L. donovani*, but did show antitrypanosomal activity in comparison to ocoteine which was inactive. This indicates the importance of the stereochemistry at C-6a-C7 in determining trypanocidal activity. Finally, the 6,6a-dehydronoraporphine stepharandine was twice as potent as pentostam against amastigotes.

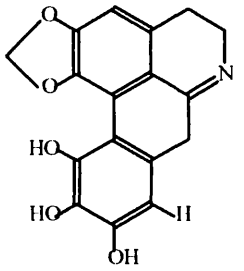
The oxo-aporphine alkaloids thalicminine, liriodenine and dicentrinone were also included in the screening. Thalicminine is the oxo-derivative of ocoteine with an aromatic nitrogen instead of the tertiary amine of ocoteine; it exhibited weak activity towards promastigotes and negligible activity in the other two screens. The zwitterionic oxoaporphine alkaloids 2-O,N-dimethyl-liriodendronine and N-Methyl-liriodendronine showed activity towards both promastigotes and amastigotes,N-methyl-liriodendronine being almost as active as pentostam towards amastigotes of *L. donovani*. Two analogues of thalicminine, dicentrinone and liriodenine displayed antileishmanial and cytotoxic activities but not trypanocidal activity. Liriodenine was found to be as potent as $NaSb^{v}$ against *L.donovani* amastigotes. *In vitro* cytotoxic activity of liriodenine against KB cells has been reported



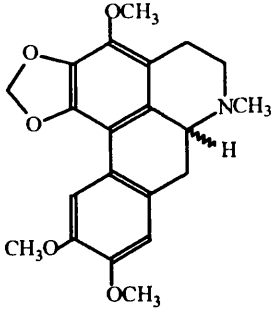
		R^1	R^2	R^3
Corydine	S	CH_3	OH	OCH_3
Norcorydine	S	H	OH	OCH_3
Isocorydine	S	CH_3	OCH_3	OH
Catalpifoline	S	H	OCH_3	OCH_3



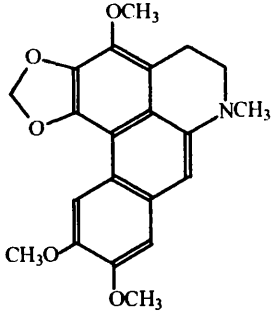
Magnoflorine iodide



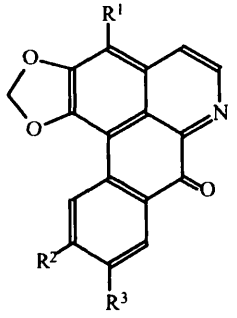
Stepharandine



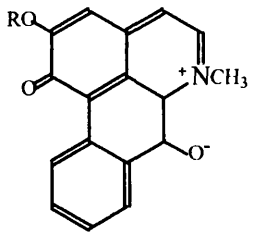
Ocoteine (+)



Dehydro-ocoteine



	R^1	R^2	R^3
Liriodenine	H	H	H
Dicentrinone	H	OCH_3	OCH_3
Thalicminine	OCH_3	OCH_3	OCH_3



	R
2-O,N-Dimethyl-liriodendronine	CH_3
N-Methyl-liriodendronine	H

Figure 3.2 Aporphine alkaloids tested

Table 3.4 *In vitro* antiprotozoal activities of Aporphine alkaloids

Aporphine alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml	µM ± SEM (A)	Amastigotes µg/ml	µM ± SEM (B)	*T. b. brucei* trypomastigotes MEC µg/ml	µM ± SEM (C)	*P. falciparum* blood schizonts IC_{50} µg/ml	µM ± SEM (E)
Corydine	9.10	26.7 ± 1.48	>30.69	>90.00	>10.23	>30.00	121.06	355.1 ± 1.74
Norcoridine	2.37	7.24 ± 1.66	>29.44	>90.00	>9.81	>30.00	-	NT
Isocorydine	18.20	53.37 ± 2.1	>30.70	>90.00	>10.23	>30.00	-	NT
Catalpifoline	2.18	6.39 ± 0.15	>30.69	>90.00	>10.23	>30.00	-	NT
Magnoflorine	15.80	33.68 ± 3.04	>42.21	>90.00	-	NT	-	NT
Ocoteine	29.00	78.56 ± 2.14	>33.22	>90.00	-	NT	-	NT
Dehydro-ocoteine	18.26	49.73 ± 1.87	>33.04	>90.00	8.37	22.80 ± 1.42	-	NT
Thalicminine	14.03	38.43 ± 1.39	>32.86	>90.00	-	NT	-	NT
Stepharandine	3.18	15.20 ± 0.44	2.60	8.75 ± 0.30	-	NT	-	NT
Dicentrinone	8.34	30.33 ± 1.23	15.28	55.56 ± 1.97	>8.25	>30.00	63.39	189.22 ± 1.57
Liriodenine	5.05	15.07 ± 0.15	6.89	20.57 ± 0.74	>10.05	>30.00	6.92	25.16 ± 2.92
2-O,N-Dimethyl-	6.00	20.62 ± 0.17	-	T^a	-	NT	-	NT
liriodendronine	4.10	18.56 ± 0.14	10.00	36.10 ± 0.11	-	NT	-	NT
N-Methyl-liriodendronine								

T^a = Toxic at concentration range of 30-10 µM. NT: Not tested

Table 3.4.1 *In vitro* cytotoxic activities of Aporphine alkaloids

Aporphine alkaloids	KB cells IC_{50} µg/ml	KB cells IC_{50} µM ± SEM (F)	P388D1 cells IC_{50} µg/ml	P388D1 cells IC_{50} µM ± SEM	F/A ratio	F/B ratio
Corydine	>250.00	>733.00	67.12	196.83 ± 1.32	-	-
Norcoridine	>250.00	>764.00 [a]	-	NT	-	-
Isocorydine	>250.07	>733.00 [a]	-	NT	-	-
Catalpifoline	>250.07	>733.00 [a]	-	NT	-	-
Magnoflorine	>250.00	>533.00 [a]	-	NT	-	-
Ocoteine	27.43	74.3 [a]	-	NT	-	-
Dehydro-ocoteine	>125.19	>341.00 [a]	-	NT	-	-
Thalicminine	>250.00	>685.00 [a]	-	NT	-	-
Dicentrinone	7.42	26.98 ± 2.40	9.47	136.73 ± 1.10	0.89	0.48
Liriodenine	3.51	10.48 ± 2.10	4.24	21.55 ± 1.04	0.69	0.17
2-O,N-Dimethyl-liriodendronine	14.39	49.45 ± 1.11	-	NT	-	-
N-Methyl-liriodendronine	-	NT	-	NT	-	-
Stepharandine	-	NT	-	NT	-	-

[a]Data obtained from Marhall *et al.*, (1994). NT: Not tested

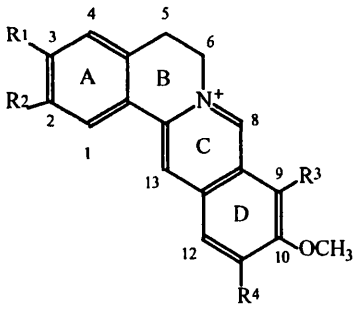
and the IC_{50} value found in this study is in agreement with that previously reported (Wu *et al.*, 1988). However liriodenine was not toxic *in vivo*. Further studies of this series type of alkaloids would be worthwhile in order to find the best structural requirements for antiprotozoal activity. Liriodenine is the most widely distributed oxoaporphine alkaloid and possesses cytotoxicity, analgesic, antimicrobial, antitumor, hypotensive, stimulant and sedative properties (Wu *et al.*, 1988, 1990, Rios *et al.*, 1989).

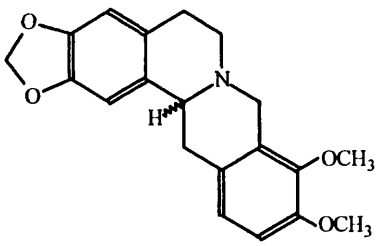
3.4.2.1.3 Protoberberine and Berberine-like alkaloids

Four protoberberine and two berberine alkaloids (Figure 3.3) were screened for biological activity (Table 3.5). Columbamine, dehydrodiscretine and canadine exhibited weak activity against *L.donovani* promastigotes and were devoid of activity towards amastigotes. In contrast, jatrorrhizine had activity against *L. donovani* promastigotes and *T. b. brucei*, and was not found toxic towards KB cells indicating its selective antiprotozoal action. Jatrorrhizine has been shown to possess *in vitro* antimalarial activity (Marshall, 1991) but was devoid of antimalarial activity *in vivo* (Schiff, 1991). Jatrorrhizine, columbamine and dehydro-discretine possess antimicrobial activities.

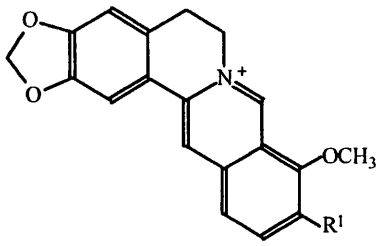
Comparison of berberine and thalifendine indicates that the presence of a phenolic function at C-10 considerably altered antileishmanial activity, with thalifendine being 6-fold less active than berberine against *L. donovani* promastigotes, and was devoid of activity towards amastigotes. However both alkaloids had similar activity towards *T. b. brucei*. These results indicate that the functional group at C-10 alters antileishmanial activity but not trypanocidal activity.

The reputation of berberine as an antiprotozoal agent has given rise to several studies on its activity *in vitro* and *in vivo*. Berberine, has been shown to be effective in treating visceral leishmaniasis (Ghosh et *al.*, 1983, 1985) but little activity on treating cutaneous leishmaniasis, and some of its derivates have been tested for their efficacy in *Leishmania* experimental models (Vennerstrom *et al.*, 1990). Studies on mode of action have revealed that berberine and pentamidine inhibit nucleic acids and protein synthesis (Venenerstrom *et al.*, 1990). They also decreased deoxyglucose uptake and were found





Canadine



		R^1
Berberine chloride	Achiral	OCH_3
Thalifendine chloride	Achiral	OH

Figure 3.3 Protoberberine and Berberine-like alkaloids tested

Table 3.5 *In vitro* antiprotozoal and cytotoxic activitites of Protoberberine and Berberine-like alkaloids

Protoberberine and Berberine-like alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml µM±SEM (B)	µM ± SEM	Amastigotes µg/ml (A)	µM ± SEM	*T. b. brucei* Trypomastigotes MEC µg/ml	µM ± SEM (C)	KB cells IC_{50} µg/ml	µM (F)	F/A ratio	F/B ratio	F/C ratio
Canadine	19.40	57.16 ± 2.35	>30.5	>90.00	-	NT	>250.14	>737.00[a]	-	-	-
Jatrorrhizine HCl	4.50	13.32 ± 1.36	5	NT	3.91	9.55 ± 1.45	>125.24	>334.99[a]	-	-	-
Columbamine HCl	21.76	58.20 ± 2.80	-	>90.00	-	NT	29.12	77.89[a]	1.34	-	-
Dehydrodiscretine HCl	11.38	30.44 ± 2.69	>33.6	NT	-	NT	>125.24	>334.99[a]	-	-	-
Berberine HCl	5.14	13.82 ± 0.87	5	29.38±1.1	0.93	2.50 ± 0.56	2.72	7.31[a]	0.53	0.24	2.93
Thalifendine	30.64	85.63 ± 2.40	- 10.90 >32.2 0	>90.00	0.61	1.71 ± 0.06	>250.11	>699.00[a]	-	-	-

[a] Data obtained from Marshall, (1991). NT: Not tested

to interact *in vitro* with nuclear DNA from *L. donovani* promastigotes. Furthermore, berberine has been reported to possess trypanocidal (Glasby, 1975), antimalarial, antiamoebic, cytotoxic (Dai *et al.*, 1993; Marshall *et al.*, 1994), antitumour (Suffness & Cordell, 1985), antibacterial (Hahn & Ciak, 1975), antifungal, anthelmintic and tuberculostatic properties (Schiff, 1987b). Berberine occurs in many species (e.g. *Berberis aristata, Fibraurea chloroleuca*) which have been used widely in traditional medicine for the treatment of leishmaniasis and other parasitic diseases for over 50 years (Iwu et *al.*, 1994).

3.4.2.1.4 Morphinanone alkaloids

Three simple morphinanone alkaloids together with metaphanine (hasubanan subclass of morphinanone alkaloids with a hemiketal linkage from C-8 to C-10) were also included in the screening (Figure 3.4). The four morphinanone alkaloids showed low grade of activity against promastigote and were devoid of activity to amastigote forms of *L. donovani, T. b. brucei* and KB cells (Table 3.6). O-Methyl-flavinantine has analgesic activity (Dwuma-Badu et *al.*, 1980).

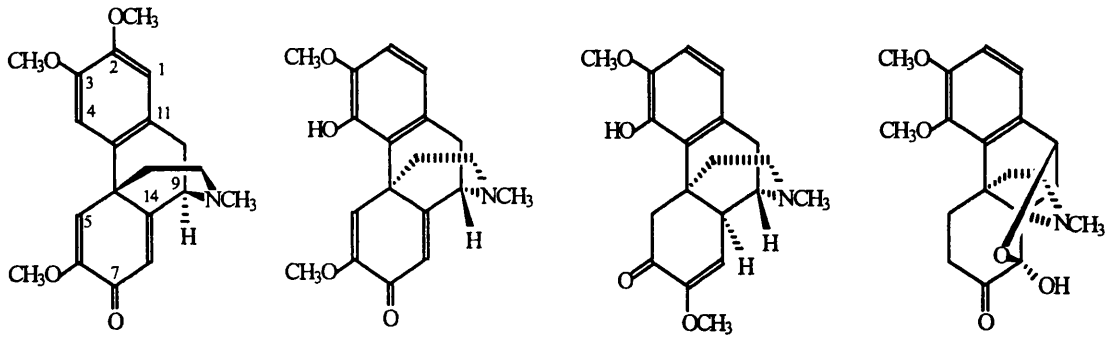


Figure 3.4 Morphinanone alkaloids tested

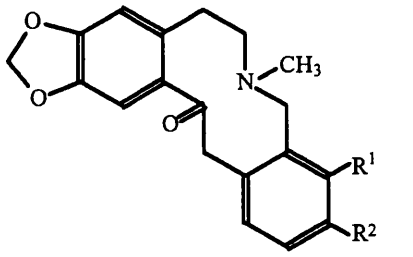
Table 3.6 *In vitro* antiprotozoal and cytotoxic activities of Morphinanone alkaloids

Morphinone alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml	µM (SEM) (A)	Amastigotes µg/ml	µM (B)	KB cells IC_{50} µg/ml	µM (F)
Sinoacutine	22.34	68.23 ± 1.13	>29.47	>90.00	>250.00	>767[a]
Sinomenine	18.20	55.25 ± 1.49	>29.65	>90.00	>250.00	>759[a]
Metaphanine HBr	17.01	39.90 ± 2.12	>38.37	>90.00	>250.00	>587[a]
O-Methylflavantine	23.03	67.65 ± 0.95	>30.64	>90.00	>250.00	>734[a]

[a]Data obtained from Marshall, (1991).

3.4.2.1.5 Protopine alkaloids

It can be seen that protopine and allocryptopine (Figure 3.5) showed weak activity against promastigotes, but were devoid of activity against amastigote forms of *L. donovani* and KB cells (Table 3.7). The results indicate that protopine has selective activity against *T. b. brucei*. Protopine has antibacterial properties (Schiff, 1991).



	R^1	R^2
Protopine	-OCH_2-	
Allocrytopine	OCH_3	OCH_3

Figure 3.5 Protopine alkaloids tested

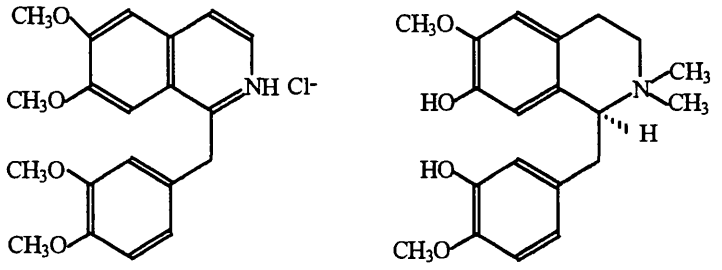
Table 3.7 *In vitro* antiprotozoal and cytotoxic activities of Protopine alkaloids

Protopine alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml	µM (SEM) (A)	Amastigotes µg/ml	µM (B)	*T. b. brucei* Trypomastigotes MEC µg/ml	µM (SEM) (C)	KB cells IC_{50} µg/ml	µM (F)
Protopine	18.50	52.35 ± 0.92	>31.81	>90.00	1.39	3.94 ± 1.48	>125.00	>354.10[a]
Allocryptopine	16.98	45.96 ± 1.45	>33.25	>90.00	NT	NT	>124.00	>338.87[a]

NT: Not tested. [a]Data obtained from Marshall (1991).

3.4.2.1.6 Benzylisoquinoline alkaloids

Two benzylisoquinoline alkaloids papaverine and tembetarine (Figure 3.6) were also tested for biological activity. Both alkaloids showed weak activity against promastigotes (Table 3.8) and they were devoid of activity against amastigotes.



Papaverine hydrochloride Tembetarine

Figure 3.6 Benzylisoquinoline alkaloids

Table 3.8 *In vitro* antiprotozoal and cytotoxic activitites of Benzylisoquinoline alkaloids

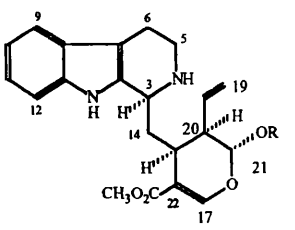
Benzylisoquinoline alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml (B)	µM (SEM) (A)	Amastigotes µg/ml	µM	KB cells IC_{50} µg/ml	µM (F)	F/A
Papaverine HCl	16.34	43.47 ± 1.60	NT	NT	2.43	6.47[a]	0.15
Tembetarine HCl	13.83	40.15 ± 1.18	>31.0 0	>90.0 0	>250	>726.00[a]	-

[a]Data obtained from Marshall, (1991). NT. not tested.

Tembetarine was not toxic towards KB cells whilst papaverine was highly toxic. If we compare papaverine hydrochloride and temberatine it can be seen that for this series of alkaloids structural requirements for antiprotozoal and cytotoxic activies differ.

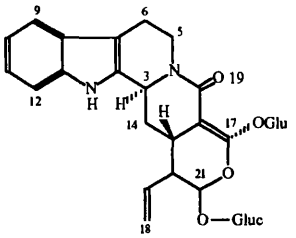
3.4.2.1.7 Strictosidine-like alkaloids

Five strictosidine-like alkaloids were tested for biological activity (Figure 3.7), acetylstrictosidine was found to be the most active (Table 3.9) of this series against both promastigotes and amastigotes forms of *L. donovani*. Comparison of the activities of

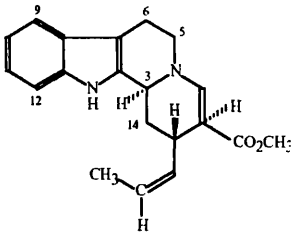


R

Strictosidine Glucose
Acetylstrictosidine Tetraacetylglucose



Strictosidine lactam



Vallesiacotamine

Figure 3.7 Strictosidine-like alkaloids tested

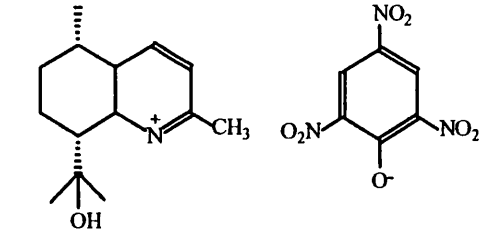
Table 3.9 *In vitro* antiprotozoal activities of Strictosidine-like alkaloids

Strictosidine-like alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml	µM ± SEM (A)	*L. donovani* Amastigotes IC_{50} µg/ml	µM ± SEM (B)	*T. b. brucei* Trypomastigotes MEC µg/ml	µM ± SEM (C)	*T.cruzi* Amastigotes IC_{50} µg/ml	µM ± SEM (D)	*P.falciparum* Blood schizonts IC_{50} µg/ml	µM ± SEM (E)
Strictosidine	7.60	14.34 ± 1.75	20.83	39.30 ± 1.04	3.25	6.14 ± 0.70	14.59	27.53 ± 1.00	79.87	150.70 ± 1.17
Acetylstrictosidine	4.36	6.25 ± 0.98	14.04	2.14 ± 0.60	11.67	16.72 ± 1.10	>63.18	>90.00	-	NT
Strictosidine lactam	>50.00	>100.40	>44.82	>90.00	14.94	30.00 ± 1.67	>44.82	>90.00	272.27	546.73 ± 1.95
Acetylstrictosidine lactam	56.23	84.43 ± 0.75	25.84	40.93 ± 1.55	19.50	29.80 ± 2.30	-	NT	94.19	141.43 ± 1.46
Vallesiacotamine	63.53	181.51 ± 2.75	>31.50	>90.00	-	NT	-	NT	-	NT

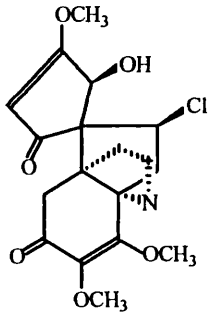
NT: Not tested

Table 3.9.1 *In vitro* cytotoxic activities of Strictosidine-like alkaloids

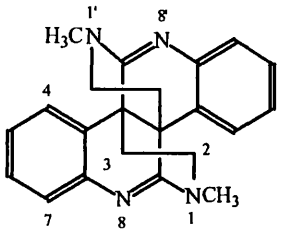
Strictosidine-like alkaloids	KB cells IC_{50} µg/ml	µM ± SEM (F)	P388D1 cells IC_{50} µg/ml	µM ± SEM	F/A	F/B	F/C
Strictosidine	39.14	73.86 ± 1.03	7.04	13.28 ± 1.47	5.15	1.88	12.04
Acetylstrictosidine	91.20	130.66 ± 1.15	43.56	62.41 ± 1.18	20.92	6.49	7.81
Strictosidine lactam	319.32	641.20 ± 1.27	481.94	967.75 ± 1.09	-	-	21.37
Acetylstrictosidine lactam	214.78	322.49 ± 2.79	9.36	14.05 ± 1.13	3.82	8.31	11.01
Vallesiacotamine	340.94	974.11 ± 3.70	196.00	784.00 ± 3.90	5.37	-	-



Fabianine picrate



Acutumine



8-8a,8'-8a-tetradehydro-(-)isocalycanthine

Figure 3.8 Miscellaneous alkaloids

Table 3.10 *In vitro* antiprotozoal and cytotoxic activitites of miscellanous alkaloids

Miscellaneous alkaloids	*L. donovani* Promastigotes IC_{50} µg/ml (A)	µM ± SEM (B)	Amastigotes µg/ml	µM	*T. b. brucei* Trypomastigotes MEC µg/ml	µM ± SEM (C)	KB cells IC_{50} µg/ml	µM (F)
Acutumine	30.90	77.66 ± 3.10	>35.81	>90.00	3.35	8.43 ± 1.45	>249.99 [a]	>628.30
Fabianine picrate	63.10	140.69 ± 1.11	>40.36	>90.00	>13.44	>30.00	>124.99 [a]	>278.70
8-8a,8'-8a-tetradehydro-(-)isocalycanthine	33.11	96.81 ± 1.20	>30.78	>90.00	>10.26	>30.00	>500.00	>1461.99

[a]Data obtained from Marshall, (1991)

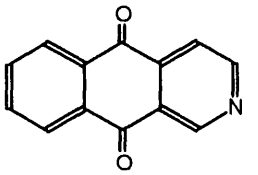
strictosidine and acetyl-strictosidine against *Leishmania* indicates that the presence of acetyl groups enhanced the antiprotozoal activity perhaps because of the increase of the lipophilicity of the original natural product. Vallesiacotamine and strictosidine lactam were devoid of interesting antiprotozoal and cytotoxic properties. None of these series of alkaloids were toxic to KB and P388 D1cells. This constituted the first report of antiprotozoal activity for the tested strictosidine alkaloids.

3.4.2.1.8 Miscellaneous alkaloids

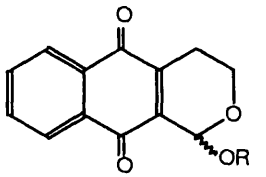
Fabianine picrate, and acutumine (Figure 3.8) showed little activity towards promastigote and were devoid of activity towards amastigote forms of *L.donovani* (Table 3.10). Acutumine had activity against *T. b. brucei*; this may indicate that acutumine has more structural requirements for antitrypanosomal activity than for leishmanial activity, and was not toxic to KB cells, making acutumine anew target for the development of antitrypanosomal drug. Glomerulatine A showed weak activity against promastigotes and was devoid of activity against amastigotes of *L. donovani* and KB cells.

3.4.2.2 Quinones

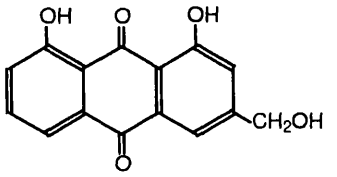
Three benzoquinones and four anthraquinones (Figure 3.9) were screened for biological activity (Table 3.11). Benzo[g]isoquinoline-5,10-dione and 1-hydroxy-benzo-isochromanquinone and the acetyl derivatived 1-acetyl-benzo-isochromanquinone were found active against *L. donovani*, *T. b. brucei* and *T. cruzi*. 1-Acetyl-benzo-isochromanquinone was 18 times as active as $NaSb^{v}$ whilst benzo[g]isoquinoline-5,10-dione and 1-hydroxy-benzoisochromanquinone were 2 and 3-fold more potent than $NaSb^{v}$ against amastigotes of *L. donovani*. The antimalarial and cytotoxicity of the six quinones tested have been reproted (Solis *et al.*, 1995a). Comparison of antileishmanial and antitrypanosomal and cytotoxic activities of 1-hydroxy-benzo-isochromanquinone and its acetyl derivative indicates that there is an increase in antiprotozoal activity rather than cytotoxic activity, when the hydroxyl group is replaced by an acetyl group. These results contrast with those obtained by Solis *et al.* (1995a), in which the acetyl derivatives was less active towards *P.falciparum* compared with the pattern compound. These results could be explained because *Leishmania* and *Trypanosoma* belongs to the



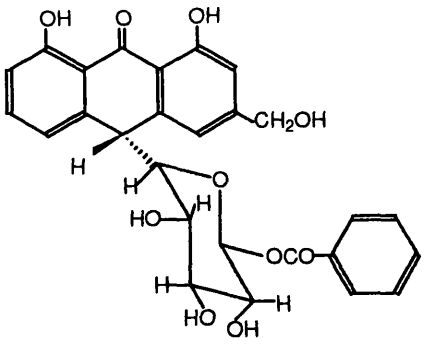
Benzo[g]isoquinoline-5,10-dione



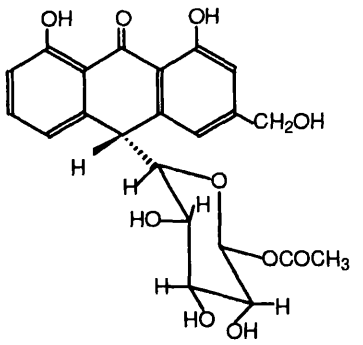
	R
1-Hydroxy-benzoisochromanquinone	H
1-Acetyl-benzoisochromanquinone	$OCOCH_3$



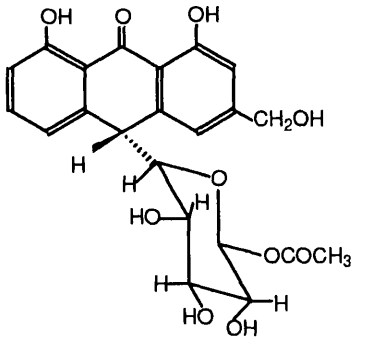
Aloe-emodin



Picramnioside A



Picramnioside B



Picramnioside C

Figure 3.9 Quinones tested

Table 3.11 *In vitro* antiprotozoal activities of Quinones

Quinones	*L. donovani* Promastigotes IC_{50} µg/ml	µM ± SEM (A)	Amastigotes µg/ml	µM ± SEM (B)	*T. b. brucei* Trypomastigotes MEC µg/ml	µM ± SEM (C)	*T. cruzi* Amastigotes IC_{50} µg/ml	µM ± SEM (D)	*P. falciparum* Blood schizonts IC_{50} µg/ml	µM ± SEM (E)
Benzo[g]isoquinoline-5,10-dione	1.38	6.60 ± 0.28	3.45	16.51 ± 0.07	1.57	7.51 ± 0.14	-	T [b]	0.84	4.02 [b]
1-Hydroxy-benzoisochromanquinone	8.66	37.65 ± 1.13	3.24	14.09 ± 0.12	0.76	3.30 ± 0.09	1.87	8.13 ± 1.23	2.66	11.56 [a]
1-Acetyl-benzoisochromanquinone	0.63	2.32 ± 0.08	0.54	1.98 ± 0.04	0.17	0.65 ± 0.007	1.79	6.60 ± 0.22	6.01	22.12 [a]
Aloe-emodin	>50.00	>185.18	>24.30	>90.00	7.81	28.93 ± 0.94	>24.30	>90.00	21.47	79.52 ± 0.88
Picramnioside A	25.70	50.59 ± 0.40	>45.72	>90.00	>15.24	>30.00	>45.72	>90.00	38.10	75.00 ± 2.14
Picramnioside B	20.75	46.52 ± 1.20	>40.14	>90.00	>13.38	>30.00	>40.14	>90.00	>500.00	>1121.08
Picramnioside C	65.00	145.74 ± 2.10	>40.14	>90.00	>13.38	>30.00	>40.14	>90.00	>500.00	>1121.08

[a]Data obtained from Solis *et al.*, 1995. T [b] = Toxic at concentration range of 90-3 µM, at lower concentrations they were not active.

Table 3.11.1 *In vitro* toxicity activities of Quinones

Quinones	KB cells IC_{50} µg/ml	µM (F)	P388D1 cells IC_{50} µg/ml	µM ± SEM	Brine shrimps LC_{50} µg/ml	µM	F/A	F/B	F/C	F/D	F/E
Benzo[g]isoquinoline-5,10-dione	1.62	7.75[a]	17.38	83.16 ± 1.22	2.45	11.72 [a]	1.17	11.07	1.03	-	1.93
1-Hydroxy-benzoisochromanquinone	1.86	8.09 [a]	2.85	12.39 ± 1.14	7.32	31.83 [a]	0.21	3.75	2.45	0.99	0.69
1-Acetyl-benzoisochromanquinone	3.21	11.80 [a]	2.03	7.46 ± 1.24	4.69	17.24 [a]	5.04	11.60	18.34	1.79	0.53
Aloe-emodin	286.00	1059.26 [a]	>250.00	925.93	>250.0	>926.0	-	-	36.62	-	13.32
Picramnioside A	35.70	70.27 [a]	22.91	45.10 ± 1.39	>250.0	>492.13	1.39	-	-	-	0.94
Picramnioside B	8.74	19.60 [a]	6.63	14.86 ± 1.20	>250.0	>560.54	0.42	-	-	-	-
Picramnioside C	8.32	18.65 [a]	4.70	10.54 ± 0.76	>250.0	>560.54	0.13	-	-	-	-

[a]Data obtained from Solis *et al.*, 1995.

Trypanosomatidae family possessing similar molecular and biochemical features which are unique to kinetoplastida order e.g. kinetoplast, trypanothione, respiratory pathway and $O_2^{\cdot}$ stress (Fairlamb et *al.*, 1992), and differ from those of *Plasmodium falciparum*. On the other hand, the toxicity of the three benzoquinones against KB cells (Solis *et al.*, 1995) was not parallel with that found for P388D1 cells. Since both cell lines have different susceptibility towards the quinones tested. Thus, further studies on the cytoxicity *in vitro* with a panel of cell lines as well as *in vivo* studies of the above 3 benzoquinones would be of great help in order to have a better understanding of the cytotoxicity of these compounds.

In contrast with the results obtained with benzoquinones, the C-xylosides of anthraquinone: picramnioside A, picramnioside B and picramnioside C (Figure 3.9) were inactive towards *L. donovani, T. b. brucei* and *T. cruzi*. The C-xylosides have been reported to be toxic against KB (Solis *et al.*, 1996). In this study the toxicity towards P388D1 cells has been determined. In contrast, with the above results the aglycone aloe-emodin, was found active towards *T. b. brucei,* and has a favourable F/C ratio of 36.62, suggesting that it is more selective to the parasite than to mammalian cells. This makes aloe-emodin an attractive target for the design of selective trypanocidal drugs.

Comparion of the anthraquinone aloe-emodin and its C-xylosides, picramniosides A, B and C indicates that the presence of the sugar on C-11 considerably decreases the trypanocidal activity and increases the cytotoxic activity. Thus structural requirements for antiprotozoal and cytotoxic activities differ for this type of quinone. The lack of antileishmanial activity of the C-xylosides could be explained by the fact that receptors interacting with terminal sugars such as mannose and glucose as ligands are involved in the binding of *L. donovani* promastigotes to the macrophage surface and their subsequent internalization. In similar manner the drugs could be bound to the receptor of the infected macrophages. Thus, the lack of recognition of xylose for the infected macrophages could explain the inactivity of these compounds against *L. donovani*, and this could be extended to *T. cruzi*.

Picramnia antidesma (Rubiaceae) has proved to be effective against *P. gallinaceum in vivo* (Spencer *et al.*, 1947) and subsequent work resulted in the isolation of the anthraquinones aloe-emodin, picramnioside A, picramnioside B and picramnioside C (Solis *et al.*, 1995b) which were evaluated for their cytotoxic against KB cells and anti-*Plasmodium falciparum* activities. Only aloe-emodin and picramnioside A showed weak activity against *P. falciparum in vitro*. It is possible that the plant does not exert its activity solely via direct action against the malaria parasite, and the beneficial therapeutic effects claimed for this plant might be due to the synergistic activity of the constituents of this plant, and antiinflammatory or immunomodulatory activities. Thus, immunommodulatory activity of some crude extracts and isolated compounds was determined in the present investigation (Chapter 4).

The antiprotozoal activity of the benzoquinones was paralleled with their toxicity to KB, P388D1 and brine shrimps. In contrast, the anthraquinone C-xylosides which were devoid of any antiparasitic activity, were toxic to KB cells but were not active towards brine shrimps. Thus, brine shrimp test could be used to guide the isolation of benzoquinones from plants, and avoiding the need for the antiprotozoal test at every stage in the isolation procedure.

3.4.2.3 Flavonoids

Nine flavonoids including 6 simple flavonoids and 3 biflavonoids (Figure 3.10) were screened for biological activity (Table 3.12). The most active flavonoid against *L . donovani* promastigotes was the flavone 7-methyl-luteolin, the active compound from *Hintonia latiflora* (Rubiaceae), and the flavonol quercetin the active compound from *Galphimia glauca* (Malphigeaceae). However, both flavonoids were toxic to the macrophages in the amastigote test system. The most active flavonoids against *T. b. brucei in vitro* were the flavanone pinocembrin, the flavones chrysin and 7-methyl-luteolin, and the flavonol quercetin, which had IC_{50} in the range of 3 - 4 µg/ml. If the antiprotozoal activities are compared for the last four flavonoids (Table 3.14). It can be seen that they were more selective to *T. b. brucei* than for *L. donovani* and *P. falciparum*,

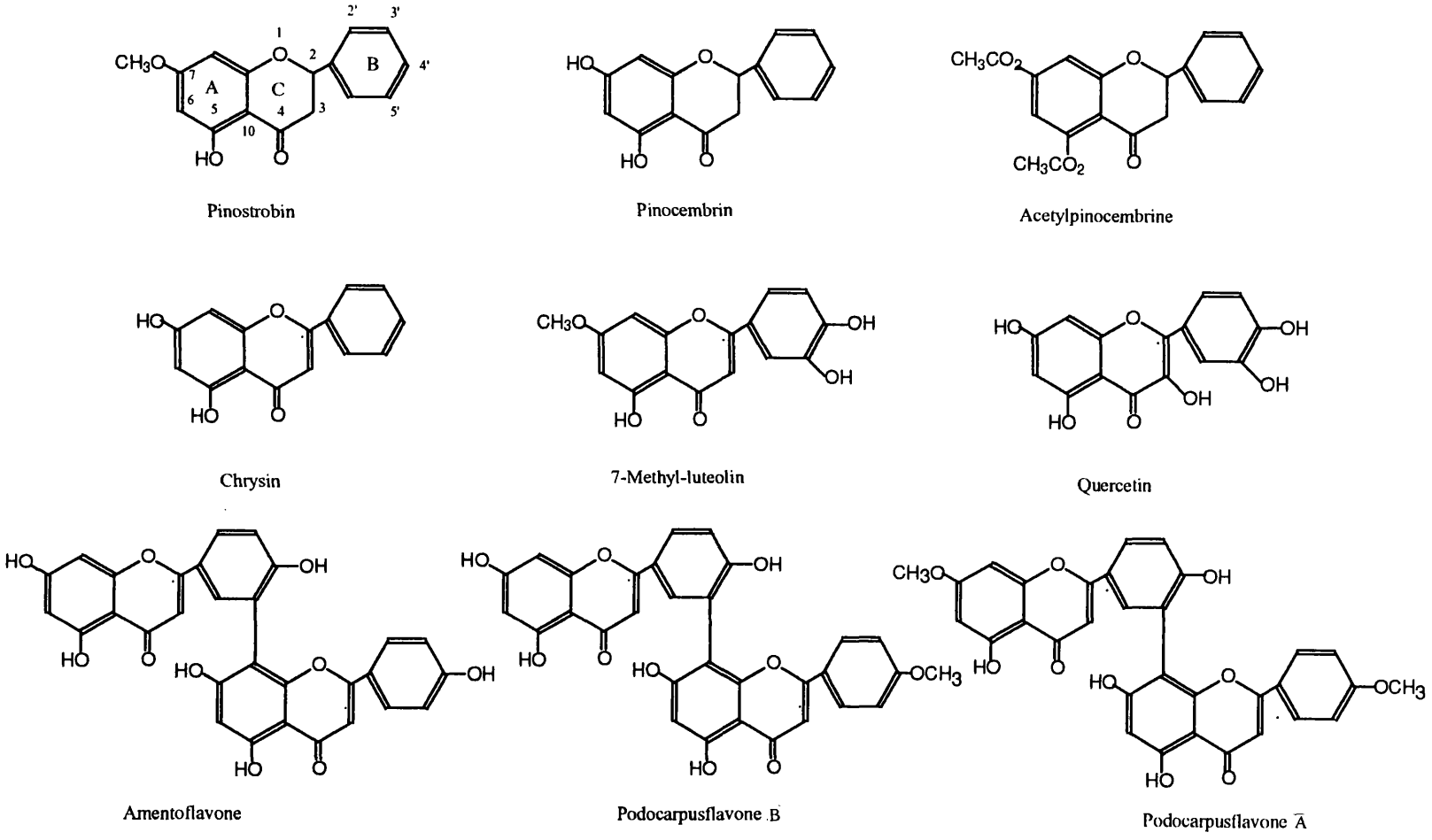


Figure 3.10 Flavonoids tested

Table 3.12 *In vitro* antiprotozoal activities of Flavonoids

Flavonoids	*L. donovani* Promastigotes IC_{50} µg/ml	µM ± SEM (A)	Amastigotes µg/ml	µM (B)	*T. b. brucei* Trypomastigotes MEC µg/ml	µM ± SEM (C)	*P. falciparum* Blood schizonts IC_{50} µg/ml	µM ± SEM (E)
Pinostrobin	148.52	550.00 ± 1.0	>24.30	>90.00	>8.10	>30.00	-	NT
Pinocembrin	48.75	190.40 ± 1.21	>23.84	>90.00	2.68	10.47 ± 1.30	39.65	154.90 ± 4.12
Acetylpinocembrin	132.75	390.10 ± 2.52	>30.60	>90.00	>10.20	>30.00	-	NT
Chrysin	52.63	207.00 ± 1.22	>22.86	>90.00	3.43	13.50 ± 1.06	49.47	194.76 ± 2.88
7-methyl-luteolin	10.71	35.70 ± 1.18	-	T[a]	4.00	13.33 ± 1.01	13.90	46.33 ± 2.68
Quercetin	29.51	63.87 ± 0.74	-	T[a]	4.58	13.24 ± 0.56	6.50	14.07 ± 1.11
Podocarpusflavone B	38.90	68.73 ± 3.82	>50.94	>90.00	>16.98	>30.00	47.13	83.27 ± 2.22
Podocarpusflavone A	41.53	75.23 ± 2.31	>49.68	>90.00	>15.56	>30.00	46.32	83.91 ± 2.10
Amentoflavone	54.45	101.21± 1.74	>48.42	>90.00	>16.14	>30.00	-	NT

[a] T = Toxic at 90 µM, not active at lower concentration. NT: Not tested

Table 3.12.1 *In vitro* cytotoxic activities of Flavonoids

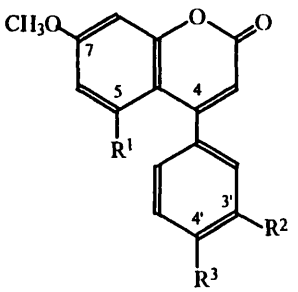
Flavonoids	KB cells IC_{50} µg/ml	µM ± SEM (F)	P388D1 cells IC_{50} µg/ml	µM ± SEM	Brine shrimps IC_{50} µg/ml	µM ± SEM	F/A	F/C	F/E
Pinostrobin	>500.00	>1851.85	226.67	839.52 ± 2.78	>500	>1851.86	-	-	-
Pinocembrin	31.62	123.51 ± 1.11	26.84	104.84 ± 2.30	8.20	32.03 ± 1.68	0.65	11.79	0.79
Acetylpinocembrin	37.78	111.12 ± 1.70	63.09	185.56 ± 0.78	>500	>1470.59	0.28	-	-
Chrysin	281.91	1109.88 ± 3.78	136.83	538.70 ± 1.90	390.90	1538.98 ± 5.70	5.36	82.19	5.70
7-methyl-luteolin	11.35	37.83 ± 2.00	2.66	8.86 ± 2.50	35.00	116.67 ± 3.70	1.06	2.84	0.82
Quercetin	136.68	295.84 ± 1.80	79.87	172.88 ± 0.07	260.70	564.28 ± 4.67	4.63	29.84	21.03
Podocarpusflavone B	77.23	136.45 ± 1.66	-	NT	37.77	66.73 ± 1.45	1.57	-	1.63
Podocarpusflavone A	61.08	110.65 ± 1.70	-	NT	52.00	94.20 ± 1.90	1.86	-	1.32
Amentoflavone	119.94	222.94 ± 0.98	-	NT	36.74	68.29 ± 2.60	2.20	-	-

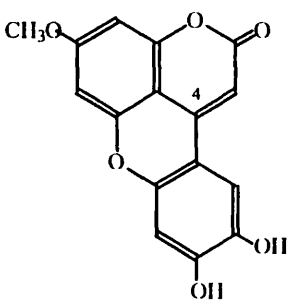
and were devoid of interesting activity against KB and P388D1 cells. These results indicated that these flavonoids were more selective towards *T. b. brucei* than to mammalian cells. This can be demonstrated by their favourable ratio F/C value compared with the of F/A and F/E ratios. The antihelmintic (Camacho *et al.*, 1991) and antimicrobial (Rojas *et al.*, 1992) properties of pinocembrin, pinostrobin and chrysin have already been described.

Comparison of the flavanones tested indicates that the presence of a phenolic function at C-7 is essential for the trypanocidal activity whilst for flavones this function is not relevant. Thus structural requirement for trypanocidal activity of flavanones and flavones differs for each group of flavonoids. Similarly, the structural requirement for antileishmanial, antiplasmodial activities could also be specific for each type of flavonoid, since the chalcone licochalcone A has been found highly active *in vitro* and *in vivo* against *L. donovani* promastigotes and amastigotes (Chen *et al.*, 1994a) as well as towards *Plasmodium falciparum* (Chen *et al.*, 1995b). None of the flavonoids assessed were found to be toxic to KB cells, the most toxic was 7-methyl-luteolin which is 2522 times less toxic than the standard podophyllotoxin.

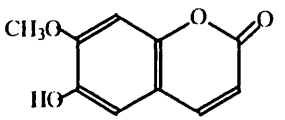
3.4.2.4 Coumarins

Twelve coumarins (Figure 3.11) were screened for biological activity. Results in Table 3.13 showed that the most active coumarins *in vitro* against *L. donovani* promastigotes were 5-O-[β-D-glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin, 5-O-[β-D-Tetra-acetoxyglucopyranosyl]-3',4'-diacetyl-7-methoxy-4-phenylcoumarin, and 5,3',4'-trihydroxy-7-methoxy-4-phenylcoumarin which had IC_{50} values of 5, 8.8 and 9 µg/ml respectively. If the above two derivatives are compared with the original natural product 5-O-[β-D-glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, it can be seen that by increasing the lipophilicity, either by acetylation or methylation, an increase in the activity towards promastigotes of *L. donovani* and *T. b. brucei* was observed. The coumarins tested were devoid of interesting activity against *P. falciparum* and KB cells. The antimicrobial properties of the eleven 4-phenylcoumarins tested in this study have already been reported (Rojas *et al.*, 1992).





4',5'-Dihidroxy-7-methoxy-
4-phenyl-5,2'-oxidocoumarin



Scopoletin

	R^1	R^2	R^3
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	5-O-β-D-galactopyranosyl	OH	OH
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	5-O-β-D-glucopyranosyl	OH	OH
5-O-[β-D-Tetra-acetoxygalactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	5-O-β-D-Tetra-acetylgalactopyranosyl	$OCOCH_3$	$OCOCH_3$
5-O-[β-D-Tetra-acetoxyglucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	5-O-β-D-Tetra-acetylglucopyranosyl	$OCOCH_3$	$OCOCH_3$
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	5-O-β-D-galactopyranosyl	OCH_3	OCH_3
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	5-O-β-D-glucopyranosyl	OCH_3	OCH_3
5,3',4'-Tri-hydroxy-7-methoxy-4-phenylcoumarin	OH	OH	OH
5,3',4'-Tri-acetoxy-7-methoxy-4-phenylcoumarin	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
5-O-[β-D-Tetra-acetoxygalactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin	5-O-β-D-Tetra-acetylglucopyranosyl	H	$OCOCH_3$

Figure 3.11 Coumarins tested

Table 3.13 *In Vitro* antiprotozoal activities of Coumarins

Coumarines	*L. donovani* Promastigotes IC_{50}		Amastigotes		*T. b. brucei* Trpomastigotes MEC		*P. falciparum* Blood schizonts IC_{50}	
	µg/ml	µM ± SEM (A)	µg/ml	µM (B)	µg/ml	µM ± SEM (C)	µg/ml	µM ± SEM (E)
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	19.86	42.98 ± 3.56	>41.58	>90.00	>13.86	>30.00	174.98	378.74 ± 1.67
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	13.33	28.85 ± 1.24	>41.58	>90.00	>13.86	>30.00	91.20	197.40 ± 2.78
5-O-[β-D-Tetraacetoxygalactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	36.31	50.85 ± 1.58	>64.26	>90.00	>21.42	>30.00	95.50	133.75 ± 1.67
5-O-[β-D-Tetraacetoxyglucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	8.85	12.39 ± 1.11	>64.26	>90.00	7.66	10.73 ± 1.31	86.60	121.29 ± 1.34
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy--4-phenylcoumarin	>500.00	>1020.40	>44.10	>90.00	>14.70	>30.00	-	NT
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	5.00	10.20 ± 0.85	>44.10	>90.00	>14.10	>30.00	-	NT
5,3',4'-Trihydroxy-7-methoxy-4-phenylcoumarin	9.00	30.00 ± 0.67	T[a]	T[a]	9.00	30.00	84.14	280.47 ± 2.59
5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin	478.63	1123.54 ± 2.44	>38.34	>90.00	>12.78	>30.00	25.70	60.33 ± 2.21
5-O-[β-D-Tetraacetoxygalactopyranosyl]-4'-acetyl-7-methoxy-4-phenylcoumarin	85.15	131.81 ± 3.56	>59.04	>90.00	6.88	10.65 ± 1.15	89.12	137.96 ± 2.30
4',5'-Dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin	42.78	143.56 ± 3.55	>25.56	>90.00	>8.52	>30.00	141.25	473.99 ± 1.49
Scopoletin	71.83	374.11 ± 2.22	>17.28	>90.00	>5.76	>30.00	-	NT

T[a] = Toxic at 90 µM, not active at lower concentrations.

Table 3.13.1 *In vitro* cytotoxic activities of Coumarins

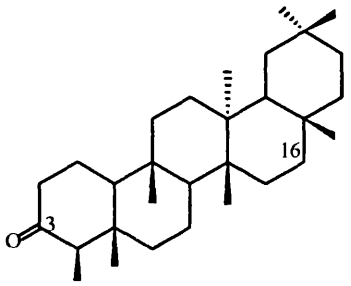
Coumarins	KB cells IC_{50} µg/ml	KB cells IC_{50} µM ± SEM	F/A	F/C
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	332.66	720.04 ± 1.89	16.75	-
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	>500.00	>1082.25 ± 4.56	-	-
5-O-[β-D-Tetraacetoxygalactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	77.12	108.02 ± 2.57	2.12	-
5-O-[β-D-Tetraacetoxyglucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	124.16	173.89 ± 3.20	14.37	16.20
5-O-[β-D-Galactopyranosyl]-3',4'-dimethoxy-7-methoxy-4-phenylcoumarin	193.19	394.26 ± 1.45	-	-
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy--4-phenylcoumarin	213.79	436.31 ± 3.30	42.80	-
5,3',4'-Trihydroxy-7-methoxy-4-phenylcoumarin	75.86	252.87 ± 2.20	8.43	-
5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin	89.91	211.06 ± 1.19	0.18	-
5-O-[β-D-Tetraacetoxygalactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin	121.62	188.27 ± 3.10	1.42	17.68
4',5'-Dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin	34.45	115.60 ± 1.33	0.80	-
Scopoletin	25.00	130.21 ± 1.60	0.35	-

3.4.2.5 Terpenes

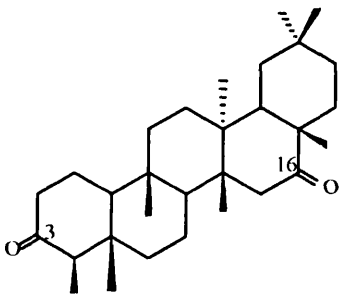
Twenty one terpenoids were screened for biological activity (Figure 3.12, Table 3.14), *Celaenodendron mexicanum* yielded three active triterpenes: 3-oxo-7,24Z-di-en-tirucalla-26-oic acid, 3α-hydroxy-7,24Z-di-en-tirucalla-26-oic acid and epi-oleanolic acid by means of bioactive guided fractionation using the *in vitro* toxicity test against brine shrimps. In this case, a correlation between the toxicity against brine shrimps and *L. donovani* promastigotes was observed. Thus, it was decided to guide the isolation of active compounds using brine shrimps and KB cells. The antileishmanial activity of 3-oxo-7,24Z-di-en-tirucalla-26-oic acid correlated with the activity against *T. b. brucei*, but it was not observed with the epi-oleanolic acid. These results suggested that the structural requirements for antiprotozoal activity are different for both tetracyclic and pentacyclic triterpenes. The activity found for the terpenes isolated from *C. mexicanum* could explain the use of this plant for the treatment of skin infections. However, since the biflavonoids isolated also from *C. mexicanum* had weak antileishmanial (Section 3.5.2.4) or antimicrobial activites (Mata, personal communication), it is possible that these compounds contribute with the global effects attributable to the plant and also they may exert other activities such as immunomodulatory effects.

It has been reported that the use of salts of triterpene saponins, monodesmosides and hederagenin leads to increase in solubility and the activity of this type of compounds (Majester-Savornin *et al.*, 1991). Thus, it is possible that the use of the salts of carboxylic acids: 3α-hydroxy-7,24Z-di-en-tirucalla-26-oic, 3-oxo-7,24Z-di-en-tirucalla-26-oic and epi-oleanolic lead to an increase in solubility in aqueous solution and their activities. In addition, bidesmosides which were inactive *in vitro* were active *in vivo*.

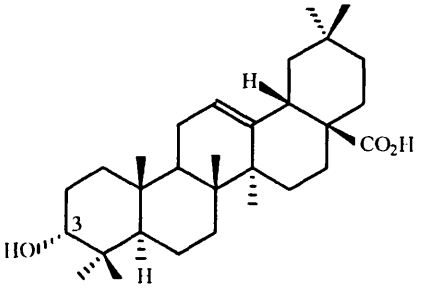
Bioactive guided-fractionation of *Guarea rhopalocarpa* using *L. donovani* promastigotes *in vitro* test led to the isolation of four novel terpenoids as the active principles of this plant: 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, lanosta-7,9(11),24EZ-triene-3α ,24-diol, ent-8(14),15-sandaracopimaradiene-2β ,18-diol and ent-8(14),15-sandaracopimaradiene-2α,18-diol as the active principles of this plant. Lanosta-



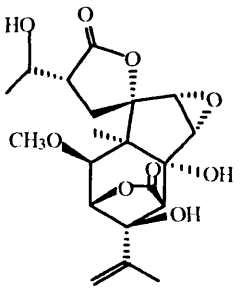
Friedelin



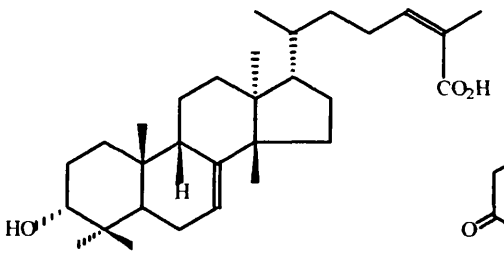
Maytensifolin B



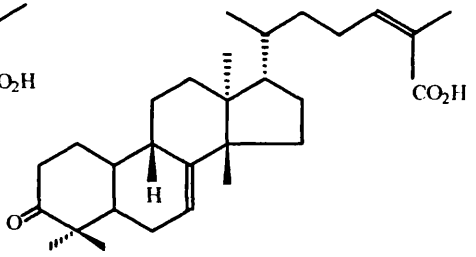
3-Epi-oleanolic acid



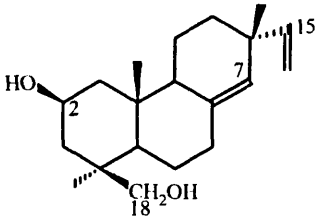
Celaenodendrolide



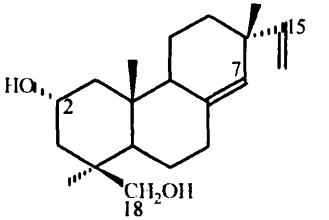
3α–Hydroxy-7,24Z-di-en-tirucalla-26-oic acid



3-Oxo-7,24Z-di-en-tirucalla-26-oic acid

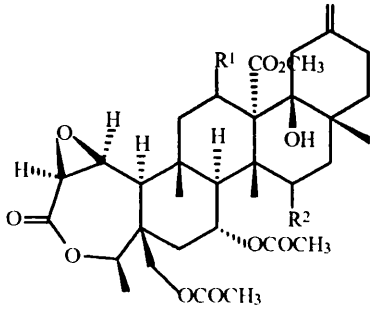


Ent-8(14),15-sandaracopimaradiene-2β,18–diol

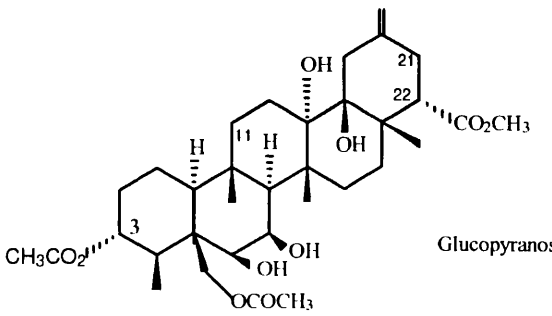


Ent-8(14),15-sandaracopimaradiene-2α,18–diol

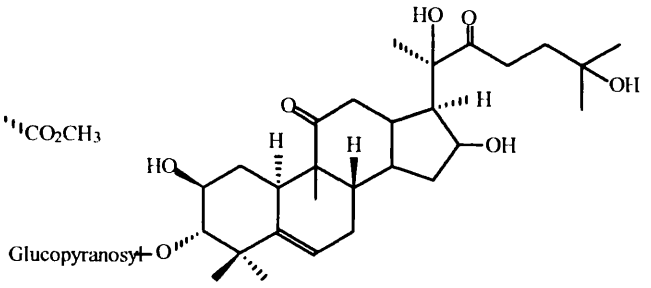
Figure 3.12 Terpenes tested



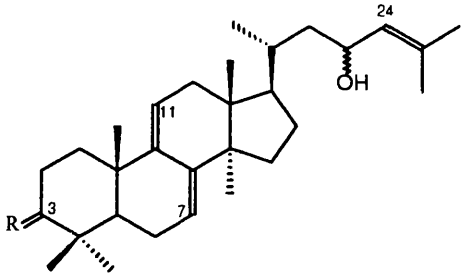
	R^1	R^2
Galphimine C	H	H
Galphimine D	H	β-$OCOCH_3$
Galphimine E	α-$OCOCH_3$	α-$OCOCH_3$



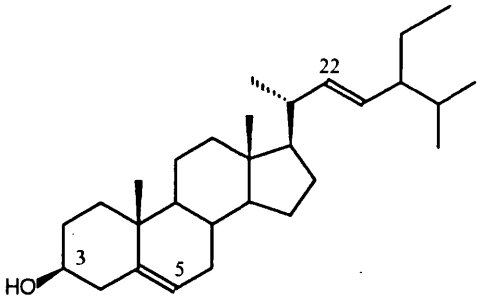
Glaucamine



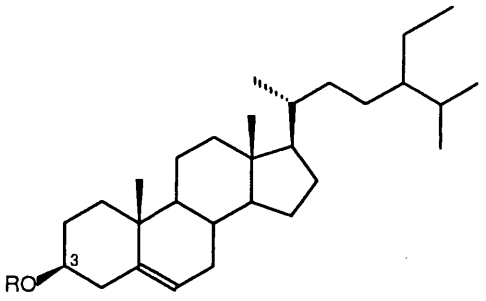
3-O-β-D-Glucopyranosyl-23,24-dihydrocucurbitacin F



	R
23-Hydroxylanosta-7,9(11),24EZ-triene-3-one	O
Lanosta-7,9(11),24EZ-triene-3α,24-diol	α-OH



Stigmasterol



	R
β-sitosterol	OH
sitosteryl-3-O-β-D-glucopyranoside	Glucopyranosyl

Figure 3.12 Terpenes tested (Continued)

Table 3.14 *In vitro* antiprotozoal activities of Terpenes

Terpenes	*L. donovani* Promastigotes IC_{50}		Amastigotes IC_{50}		*T. b. brucei* Trypomastigotes MEC		*T.cruzi* amastigotes IC_{50}		*P. falciparum* Blood schizonts IC_{50}	
	µg/ml	µM ± SEM (A)	µg/ml	µM ± SEM (B)	µg/ml	µM ± SEM (C)	µg/ml	µM ± SEM (E)	µg/ml	µM ± SEM (F)
Friedelin	>500.00	>1173.71	-	NT	-	>30.00	-	NT	>500.00	>767.54
Maytensifolin B	>500.00	>1136.36	>39.60	>90.00	>13.20	>30.00	-	NT	314.69	767.54 ± 4.50
Celaenodendrolide	>500.00	>1219.51	>36.90	>90.00	>12.30	>30.00	-	NT	63.30	138.81 ± 1.63
3α-Hydroxy-7,24Z-dien-tirucalla-26 oic acid	22.75	49.90 ± 2.57	>40.86	>90.00	>13.68	>30.00	>41.04	>90.00	34.67	139.43 ± 3.75
3-Oxo-7,24Z-dien-tirucalla-26 oic acid	6.23	13.72 ± 1.89	41.00	89.9 ± 2.70	7.65	16.85 ± 1.64	>40.86	>90.00	55.65	122.04 ± 2.05
Epi-oleanolic acid	8.57	18.79 ± 0.74	>41.04	>90.00	>13.20	>30.00	-	NT	19.32	28.33 ± 1.63
3-O-β-D-Glucopyranosyl-23,24-dihydrocucurbitacin F	257.00	376.80 ± 1.73	-	NT	>20.46	>30.00	>41.04	>90.00		
β-Sitosterol	>500.00	>1207.73	>37.26	>90.00	>12.42	>30.00	-	NT	>500.00	>1207.73
Stigmasterol	>500.00	>1209.73	>39.26	>90.00	>12.42	>30.00	-	NT	>500.00	>1209.73
23-Hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one	3.15	7.22 ± 0.03	32.43	74.38 ± 2.50	3.51	10.34 ± 1.53	11.39	26.14 ± 1.05	41.41	94.97 ± 1.59
Lanosta-7,9(11),24EZ-triene-3α,24-diol	9.20	20.17 ± 1.13	9.12	20.00 ± 0.80	1.45	3.25 ± 0.52	4.95	10.86 ± 1.28	31.73	69.58 ± 1.48
Epi-8(14),15-sandaracopimaradiene-2β-18-diol	15.12	49.74 ± 1.17	>27.36	>90.00	>9.12	>30.00	-	NT	31.67	104.18 ± 1.37
Epi-8(14),15-sandaracopimaradiene-2α,18-diol	5.11	16.81±1.43	>27.36	>90.00	>9.12	>30.00	-	NT	50.65	166.61 ± 1.64
Galphimine C	220.80	358.44± 1.84	-	-	>30.00	>48.70	-	NT	288.00	467.53
Galphimine D	412.10	611.42 ± 2.40	-	-	>30.00	>44.51	-	NT	>500.00	>742
Glaucamine	229.10	369.69 ± 1.17	-	-	>30.00	>48.39	-	NT	58.25	>94
Galphimine E	173.40	236.79 ± 1.91	-	-	>30.00	>41	-	NT	>500.00	>683
Sitosteryl-3-O-β-D-glucopyranoside	100.00	174.00 ± 1.39	-	-	>500.00	>868	-	NT	>500.00	>868

NT:Not tested

Table 3.14.1 *In vitro* cytotoxic activities of Terpenes

Terpenes	KB cells IC_{50} µg/ml	KB cells IC_{50} µM ± SEM (F)	P388D1cells IC_{50} µg/ml	P388D1cells IC_{50} µM ± SEM	Brine shrimps LC_{50} µg/ml	Brine shrimps LC_{50} µM ± SEM	F/A	F/B	F/C	F/D	F/E
Friedelin	>500.00	>1073.07	-	NT	>1000	>2347.42	-	-	-	-	-
Maytensifolin B	238.22	541.40 ± 2.51	-	NT	>1000	>2272.73	-	-	-	-	-
Celaenodendrolide	>500.00	>1219.51	-	NT	>1000	>2439.02	-	-	-	-	-
3α-Hydroxy-24Z-en-tirucalla-26 oic acid	46.81	102.65 ± 1.81	84.72	185.79±1.85	86.00	188.60 ± 3.50	0.49	-	-	-	0.73
3-Oxo-24Z-en-tirucalla-26 oic acid	62.49	137.64 ± 3.65	48.74	107.44±1.11	73.80	162.55 ± 2.80	10.00	1.14	8.17	-	0.98
Epi-oleanolic acid	16.27	35.68 ± 3.80	33.81	74.14±1.81	10.62	23.29 ± 1.11	1.90	-	-	-	0.29
3-O-β-D-Glucopyranosyl-23,24-dihydrocucurbitacin F	-	-	29.34	43.02±1.09	-	NT	-	-	-	-	6.45
β-Sitosterol	>500.0	>1270	103.27	249.44±1.01	-	NT	3.30	-	-	-	-
Stigmasterol	>500	>1272	105.27	251.44 ±3.2	-	NT	3.20	-	-	-	-
23-Hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one	13.19	30.25 ± 1.05	19.40	44.49±1.14	23.50	53.90 ± 3.70	4.19	0.41	3.75	1.16	0.32
Lanosta-7,9(11),24EZ-triene-3α,24-diol	9.69	21.25 ± 1.05	0.65	1.42±1.57	15.66	34.34 ± 2.90	1.05	1.06	6.68	1.96	0.30
Epi-8(14),15-sandaracopimaradiene-2β,18-diol	23.05	75.822 ± 1.17	11.54	37.96±1.08	192.70	633.88 ± 4.86	1.52	-	-	-	0.73
Epi-8(14),15-sandaracopimaradiene-2α,18-diol	14.58	47.96 ± 1.00	19.65	64.64±1.02	138.00	454.95 ± 2.70	2.85	-	-	-	0.29
Galphimine C	>500.00	>899.28	-	NT	-	NT	-	-	-	-	-
Galphimine D	>500.00	>975.90	-	NT	-	NT	-	-	-	-	-
Glaucamine	125.00	268.82 ± 1.98	-	NT	-	NT	-	-	-	-	-
Galphimine E	295.80	1006.12 ± 1.87	-	NT	-	NT	-	-	-	-	-
Sitosteryl-3-O-β-D-glucopyranoside	500.00	1240.70 ± 3.60	-	NT	-	NT	-	-	-	-	-

NT: Not tested

7,9(11),24EZ-triene-3α,24-diol was found to be the most active terpene against *L. donovani* promastigotes. 23-Hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, lanosta-7,9(11),24EZ-triene-3α,24-diol were active against *T. b. brucei*. The antiprotozoal activity of these two terpenoids was extended to *T. cruzi* and *P. falciparum*. If the (IC_{50} values for cytotoxic activity/ IC_{50} values for antiprotozoal activity) ratios of 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, and lanosta-7,9(11),24EZ-triene-3α,24-diol, are compared it can be seen that both terpenes had different degrees of selectivity towards the differents protozoa used in the *in vitro* screens. Furthermore, it can be seen that the oxidation stage at C-3 in the lanostane nucleus is important for antileishmanial activity. A comparison of 8(14),15-sandaracopimaradiene-2β ,18-diol and 8(14),15-sandaracopimaradiene-2α,18-diol indicates that the configuration of the hydroxyl group at C-2 is important for antileishmanial and cytotoxic activitites. The nor-seco-triterpenes galphimine C, galphimine D, and galphimine E, and glaucamine, and the steroids: stigmasterol and sitosteryl-3-O-β-D-glucopyranoside isolated from *Galphimia glauca* were devoid of any antiprotozoal and cytotoxic activity. However, they were found to modulate proliferation of lymphocytes (Chapter 4).

3.4.3 Activity against other protozoa: *T. cruzi and P. falciparum*

Sixteen compounds were screened for *in vitro* activity against *T. cruzi* amastigotes, of which the most active compounds were 1-hydroxy-benzoisochromanquinone and 1-acetyl-benzoisochromanquinone with IC_{50} values of 8.13 and 6.6 μM respectively (Table 3.11). In the same test conditions nifurtimox had an IC_{50} value of 2.89 μM.

Thirty-seven compounds were screened for *in vitro* anti-*Plasmodium falciparum* activity, and only 7-methyl-luteolin from *Hintonia latiflora,* quercetin from *Galphimia glauca* and liriodenine and stepharandine from *Stephania dinklagei* showed some activity against *P. falciparum*. These results in part support the use of *H.latiflora* and *G.glauca* for the treatment of malaria

The active principles of *Hintonia latiflora* and *Galphimia glauca*, 7-methyl-luteolin and quercetin possess weak *in vitro* anti-*Plasmodium falciparum* properties. However, the

results obtained in this study could explain in part the use of both plants as antimalarial drugs. It has been reported that some flavonoids from *Artemisia annua* have synergistic activity with the terpenoid artemisinin (Elford *et al.*, 1987), Thus it is possible that flavonoids and terpenoids isolated from *G. glauca* act synergistically to kill the parasite or by other mechanisms such as immunomodulation(see Chapter 4). The same principle could be applied to *C. mexicanum* which also posseses terpenoids and flavonoids.

Previously, a number of bioactivities have been described for quercetin including: antioxidant (Ioku *et al.*, 1995); inhibition of DNA synthesis of HL-60 cells (Uddin & Choudhry, 1995); potentiation of the effect of adriamycin in multidrug-resistant human breast-cancer cell line (Xu & Wei, 1995); inhibition of platelet aggregation (Chung-Kuo *et al.*, 1995), immunomodulating activity, inhibition of human topoisomerase I (Boege *et al.*, 1996), but there have not been reports of its antiprotozoal activity.

3.4.4 *In vivo* antiprotozoal studies

Strictosidine, acetylstrictosidine, acetylstrictosidiene lactam and 23-hydroxy-5 -lanosta-7,9(11),24EZ-triene-3-one were the only samples available in sufficient quantity to be tested for *in vivo* antiprotozoal activity towards *L. donovani* and *T. b. brucei.* The samples were tested at a single dose of 100 mg/kg, using the methodology described in sections 3.1.2.2.3 and 3.1.2.4.2. Results in Table 3.15, indicated that acetylstrictosidine and acetylstrictosidine lactam suppressed parasite burdens by 30 and 33.2% respectively. Whereas, the reference drug $NaSb^{v}$ at a dose of 45mg/kg/day suppressed parasite numbers by 72.79%. Comparation of activities of both strictosidine and its acetyl derivate indicated, that acetyl groups increases the antileishmanial activity of strictosidine. Strictosidine and 23-hydroxy-5 -lanosta-7,9(11),24EZ-triene-3-one were devoid of any activity *in vivo.* None of the compounds tested showed activity against *T. b. brucei in vivo.* From the results obtained *in vivo* it can be seen that a good *in vitro* antiprotozoal activity may no be paralleled by that *in vivo,* due to its bioavailability (*e.g.* solubility, instability, excretion, metabolism).

Table 3.15 *In vivo* antileishmanial activity of selected compounds against *L. donovoni.*

Sample	Daily dose mg/kg	Route	Mean parasites	% inhibition	Toxicity
Strictosidine	100	SC	1961896.80	5.56	nil
Acetylstrictosidine	100	SC	1387710.4	33.20	nil
Acetylstrictosidine lactam	100	SC	1452103.20	30.10	nil
23-hydroxy-5α-lanosta-7,9(11),24-triene-3-one	100	SC	2094328.40	0.0	nil
Sodium stibogluconate	45	SC	565147.60	72.79	nil
Control 0.25% celacol	-	SC	2077400.00	-	nil

n=5

3.4.5 Summary

Results shown in Tables 3.2 - 3.14 revealed that 10 compounds had IC_{50} values of less than 1 μg/ml against *L. donovani* promastigotes *in vitro*. A further 34 had IC_{50} values between 1 and 10 μg/ml. Among them, fangchinoline and isotrilobine were the most active with IC_{50} values of 0.24 and 0.33 μg/ml, respectively. In addition, fangchinoline and isotrilobine have a F/A ratio of 262.43 and 32.98, respectively. Under the same conditions, pentamidine had an IC_{50} of 0.24 μg/ml.

Fourteen compounds were active against amastigotes of *L. donovani* with IC_{50} values less than 50 μg/ml. In the same test conditions the standard $NaSb^{v}$ had an IC_{50} value of 9.75 μg/ml. Significant antileishmanial activity comparable or higher to that of $NaSb^{v}$ was demonstrated by the BBIQ alkaloid phaeanthine, the quinones benzo[g]isoquinoline-5,10-dione, 1-hydroxy-benzo-isochromanquinone and acetyl-benzo-isochromanquinone, which were 6,2,3, and 18 times as active as the standard $NaSb^{v}$.

From the results (Table 3.1 to 3.14) it can be seen that the activity against promastigotes does not necesarily confer activity against amastigotes since both stages of the parasite have different molecular and biochemical features, giving differences in drug sensitivity. For example, the well known antimonial drugs (Pentostam^{R} and Glucantime^{R}) are active against amastigotes but not towards promastigotes. Also, both compounds have different sensitivity to strains of visceral and cutaneous leishmaniasis. On the other hand, the same behaviour was observed with many of the tested compounds for example, 7-methyl-luteolin, epi-oleanolic acid, and norcorydine. Although a number of compounds (e.g., epi-oleanolic acid) inhibited promastigotes they were found inactive to amastigotes, therefore they have potential of preventing macrophage infection in a prophylactic manner.

Twenty-four compounds had IC_{50} values equal to or less than 10 μg/ml against *T. b. brucei in vitro*. Under the same conditions, pentamidine had an IC_{50} value of 6×10^{-3} μg/ml. The most potent trypanocidal compond was the benzoquinone 1-acetyl-benzo-isochromanquinone with an IC_{50} value of 0.17μg/ml.

In assessing structure-activity relationships of the screened compounds for antiprotozoal activity, it can be seen that the compounds with *in vitro* activity equal to or less than 10 μg/ml against *L. donovani* promastigotes and *T. b. brucei* trypomastigotes have a range of different structures including alkaloids, quinones, coumarins, flavonoids and terpenes. In general the activity against both parasites decreased in the order: alkaloids > quinones > terpenes > coumarins > flavonoids.

None of the compounds tested showed significant cytotoxic activity against KB and P388D1 cells. The most active being papaverine hydrochloride which is 647 times less active than the standard podophyllotoxin against KB and P388D1 cells respectively. However, papaverine was devoid of interesting activity against *L. donovani* and was inactive to *T. b. brucei;* in similar manner the cytotoxic naphthoquinones C-xylosides did not show antiprotozoal activity. Thus, it may concluded that cytotoxicity does not automatically confer antiprotozoal activity.

In assessing selectivity of antiprotozoal action, it can be seen from Tables 3.2 - 3.14 that most of the compounds with high antileishmanial activity (IC_{50} values of less than 10 μg/ml) had a parallel trypanocidal activity. Apart from thalisopodine, the IC_{50} values for antileishmanial and trypanocidal effects were found significantly different, according to t-student statistical treatment of the IC_{50} values.

The parallel antiprotozoal activity of the above samples could be explained by the fact that *Leishmania* and *Trypanosoma* are members of the family trypanosomatidae, and possess several molecular and biochemical features which are unique to the kinetoplatide order (e.g. kinetoplast, purine salvage pathway, trypanothione). However, the activity *in vitro* against *L. donovani* promastigotes does not necessarily imply that a sample will be active against other related protozoa. For example, the antileishmanial activity of norcorydine, catalpifoline, 5-O-[β -D-glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin and acetylstrictosidine did not extend to *T. b. brucei*, or other protozoa, whereas, the trypanocidal activity of daphnoline, thalifendine, protopine, acutumine,pinocembrine and 5-O-[β-D-tetraacetylgalactopyranosyl]-4'-acetyl-7-methoxy-4-phenylcoumarin did not

extended to *L. donovani.* These results suggests that the optimal structures for antileishmanial activity were not necessarily the same as those for trypanocidal activity.

The antiprotozoal activities did not parallel the brine shrimp test lethality test in the case of naphthoquinone C-xylosides. However, the cytotoxicity and brine shrimps test correlated with the antiprotozoal activitites of benzoquinones and cytotoxic terpenes. This approach indicated that the brine shimp lethality test could be useful for the screening of antiparasitic compounds with similar structures to those of benzoquinones and terpenes mentioned before. In addition, the brine shrimps lethality test could be useful also for other types of biological active compounds where the brine shrimps respond similarly to the corresponding mammalian cells. For example the DNA-dependent RNA polymerases of the *A. salina* have been shown to be similar to the mammalian type (Birndorf *et al.*, 1975).

In vitro and *in vivo* studies showed that marked improvement in the antiprotozoal activities of strictosidine lactam, acetyl-strictosidine lactam, 1-hydroxy-benzoisochromanquinone and 1-acetyl-benzoisochromanquinone could be brought by minor changes in the functional groups of the natural product.

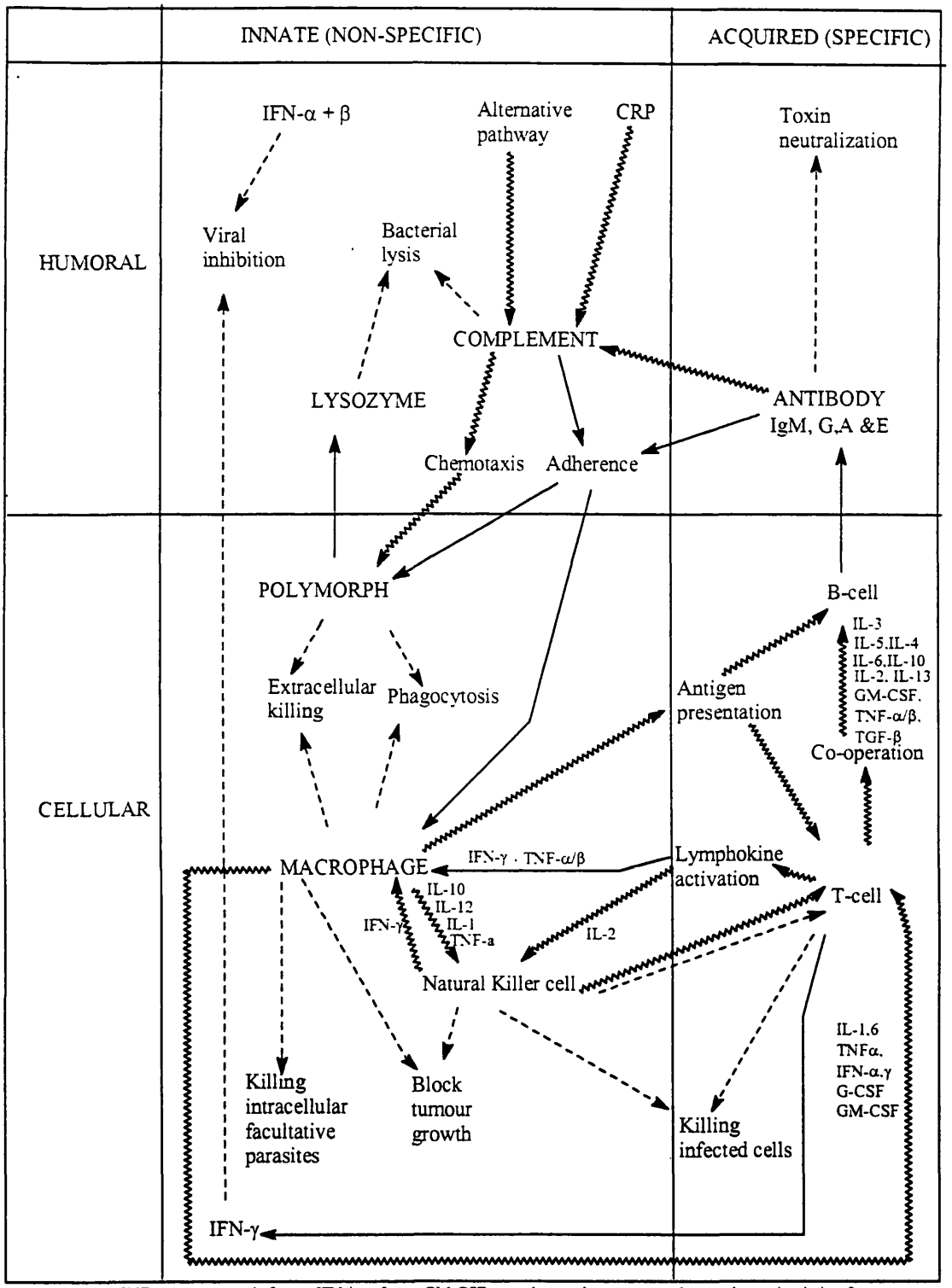
CHAPTER 4

IMMUNOMODULATORY EFFECTS OF SOME CRUDE EXTRACTS AND PLANT-DERIVED COMPOUNDS

4.1 Introduction

Immunomodulators are substances that enhance host defences or depress natural or acquired immunity (Nussler & Thomson, 1992). They may be divided into two categories, the specific and the non-specific immunomodulators. Specific immunomodulators are administered together with the antigen, for instance in vaccines where they are known as immunological adjuvants and enhance the immune response to vaccines. Non-specific immunostimulants are given to elicit a generalized state of resistance to pathogens or tumours. Non-specific immunosuppressants reduce the capacity of the immune system to respond to antigens by interfering with the function of the cells of the immune system. Immunological defense is a complicated interplay between non-specific and specific, cellular and humoral immune responses, stimulation and suppression of immunocompetent cells (Figure 4.1), and the influence of endocrine and other mechanisms upon the immune system (Wagner, 1990).

The processing and presentation of antigen to the specific effectors of immune responses (lymphocytes) is carried out by the antigen-presenting cells (APC's)-tissue macrophages or dendritic cells. Foreign antigen presented on the surface of APC's is recognized via the antigen-specific T-cell receptor (TCR). This results in lymphocyte activation and consequently, the secretion by these cells of a variety of cytokines which serve to amplify the immune sytem. Cytokines are produced by both mononuclear phagocytes (monokines) and by lymphocytes (lymphokines) and exert a diversity of effects on cells of the immune system and other cell types. Cytokines are responsible for T- and B-lymphocyte and natural killer (NK) cell activation and growth, macrophage activation, regulation of haematopoiesis and leukocyte migration. Not all the cytokines, however



IL-interleukins, TNF-tumour necrosis factor, IFN-interferon, GM-CSF-growth granulocyte-macrophage colony stimulating factor. GCSF-growth colony-stimulating factor, TGF-tumour granulocyte factor. ——➤ gives rises to - - -➤ inhibits ~~~➤ stimulates
Adaapted from Roitt (1991) and Gazzinelli, 1996.

Figure 4.1 Mechanisms involved in the immune system

are stimulatory, at least one cytokine (IL-10) is a cytokine synthesis inhibitory factor (Nussler & Thomson, 1992; Romagnani, 1996). Macrophage activation during infection occurs through a T cell-mediated process involving lymphokine synthesis. Activated macrophages become competent to kill several pathogens and lyse tumour cells. During this activation process, macrophages produce an array of biologically active products, e.g. proteases, reactive oxygen and nitrogen intermediates (ROI, NO), TNF and IL-1, known to have microbicidal activities. The oxidative burst is triggered by a variety of cytokines, mainly TNF-α, and IFN-γ (Langermans *et al.*, 1994). The precise mechanism by which oxygen radicals act on the parasite remains unclear. However, it has been proposed that ROI inhibits lipoprotein synthesis and causes DNA damage and lipid peroxidation in membranes. NO leads to inhibition of DNA synthesis, and decrease of total protein synthesis, and it is known that NO is involved in the control of parasite development such as the destruction of intracellular *Toxoplasma* and *Leishmania* and the hepatic stage of murine malaria (Langermans *et al.*, 1994).

4.1.1 Immunity to parasitic infections

Immunity to infection involves a constant battle between the host defenses and the parasites escaping destruction by the immune system. A wide variety of defensive mechanisms are deployed by the host but in general it may be stated that a humoral response develops when the organism invade the bloodstream (*P. falciparum*, *T. brucei*) whereas parasites which grow within the cells or tissues (e.g. *Leishmania spp, T. cruzi*) usually elicit cell mediated immunity. Antibodies present in adequate concentration and affinity are effective in providing protection against the blood forms of parasite such as *T. brucei* and the sporozoite and merozoite stages of malaria. Lymphokines produced by T-cells are of special importance for the activation of macrophages to kill intracellular organism such as *T. gondii*, *T. cruzi* (Kierzenbaum, 1995; Tarleton, 1995), and *Leishmania* sp. (Liew, 1986, Roach *et al.*, 1991, Ho *et al.*, 1994), which normally subvert the macrophages microbial mechanisms. Organisms such as *Plasmodium* sp., live part of their life-cycle in kupffer cells which are professional phagocytes and may be eliminated through activation of intracellular defense mechanisms by IFN-γ release from CD8 positive T-cells (Mauel *et al.*, 1996).

4.1.2 Resistant to host effector immunomechanisms

A number of parasitic protozoa gain access to resting macrophages in many ways and once inside the host cell, each has its own way of surviving the battery of attack mechanism including enzymic and pH-dependent processes and reactive oxygen species mounted by these professional killer cells. *T. gondii* which enters the cell via a nonphagocytic mechanism, inhibits phagosome-lysosome fusion by changing membrane; in addition host cell mitochondria live along the phagosome membrane. *T. cruzi* escapes from the confines of the phagosome into the cytoplasm, while *Leishmania* sp can survive within the fused phagolysosome. *Leishmania* have a membrane bond lipophosphoglycan (LPG) which helps to protect them from the oxidative burst by scavenging oxygen radicals. Some parasites such as *T. brucei* and *Plasmodium* sp escape from the cytocidal action of humoral antibody on their cycling blood forms by the ingenious trick of altering antigenic variation.

4.1.3 Deviation of the host immune response

Immunosuppression has been found in most of the parasite infections (e.g. *T. brucei*, *T. cruzi, Leishmania* sp). During infection by *T. brucei* for example, antibody cell-mediated immunity is only 5-10% of the normal value while T-suppressor activity is prominent, presumably related to an excessive load of antigen. *Streptococcus pneumonia* is always found as an opportunistic infection in Human African trypanosomiasis. *T. cruzi* and *Leishmania* sp depress both B and T cell activities. In addition, cross-reaction between parasite and host may give rise to auto-immunity and this has been proposed as the basis for the cardiomyopathy in Chagas' disease. On the other hand, there have been reports of AIDS patients suffering from leishmaniasis or Chagas's disease (Louis *et al.*, 1991; Pepin *et al.*, 1992a; Ferreira *et al.*, 1991; Zumla & Croft, 1992). Reactivation of *T. cruzi* in patients receiving immunosuppressive therapy or leukaemia (Metze *et al.*, 1991) have been also reported.

4.1.4 Combined immunochemotherapy

It is generally accepted that no chemotherapeutic agent is able to eradicate all infectious organisms, from the host without the help of the immune sytem. For example, in animal models, treatment of African trypanosomiasis with eflornithine or melarsoprol requires an

appropriate antibody-mediated immune response. An intact immune system is also necessary for rapid clearance of trypanosomes from the bloodstream following treatment with suramin. Similarly, an efficient cell-mediated immune response is required for maximal efficacy of pentavalent antimonials in the treatment of leishmaniasis. However, the potential relationship between parasite-induced or acquired immunosuppression and effective chemotherapy has been poorly studied. Macrophages which have been activated by bacterial cell wall components or gamma-interferon (IFN-γ) are known to display increased activity against *L. donovani* (Roach *et al.*, 1991) or *T. cruzi*. In experimental and clinical visceral leishmaniasis use of macrophage activators (e.g. *Corynebacterium parvum* or *Mycobacterium tuberculosis*, IFN-γ) together with pentavalent antimonials has lowered the dose of antimony required to cure the infection (Convit *et al.*, 1987; Badaro, 1990; Harms *et al.*, 1990).

The destruction of blood stage malaria by reactive oxygen intermediates has maintained interest in forms of therapy using BCG and *C. parvum* which non-specifically stimulate the immune system. In addition, the effectiveness of antimalarial drugs is also affected by the immune status of the host. Much of the evidence comes from practical experience, comparing drug dosages required for individuals with different degrees of exposure (Target, 1992). In addition, a number of anti-parasitic drugs perturb the immune system such as nifurtimox and amphotericin B by acting through metabolic activation leading to internal oxidant stress; the synergistic effect between such drugs and activation of the oxidant pathways in the immune system may be of importance (Berger & Fairlamb, 1992). Furthermore, some anti-malarial drugs such as: quinine, chloroquine, pyrimethamine, mefloquine and quinacrine suppress PMN functions associated with anti-microbial activity (Targett, 1992). They also have some effect on cell adherence, RNA or protein synthesis, and a marked effect on the respiratory burst and locomotion. Chloroquine at chemoprophylactic doses inhibited the release of interleukin (IL)-1 and tumor necrosis factor (TNF)-α from monocytes, as well as inhibited phagocytosis of zymosan particles or IgG-coated sheep erythrocytes (Targett, 1992). Amodiaquine stimulate the development of anti-IgG antibodies which produce agranulocytosis as a serious side-effect (Clarke *et al.*, 1991). Quinine and quinidine caused drug-induced immunologically mediated thrombocytopenia. The antibodies are directed against various glycoprotein

(GP) molecules on the surface of the platelets. Chloroquine or combination of chloroquine and pyrimethamine or sulphametoxine prophylaxis have been responsible for the reduction of normal human antibody response (Stevens, 1996). On the other hand, antibiotics may also alter, the normal immune response, particularly, the occurrence of neutropenia or agranulocytosis in patients receiving β-lactams, chloramphenicol, and sulphonamides (Labro, 1993).

4.1.5 Immunomodulators

In the past, living and attenuated microorganisms, autologous and heterologous proteins and injection of animal organ preparations have been used with the aim of restoring an impaired defense mechanisms in immunotherapy. At present some biological response modifiers such as interferon- (IFN) γ, tumour necrosis factors (TNF), and cytokines have potential for the treatment of cancer (Donmez & Groves, 1997; Anderson *et al.*, 1996; Lamont & Adorini, 1996; Lachman *et al.*, 1996); protozoal diseases (Ho *et al.*, 1994; Nacy *et al.*, 1985; Crutcher *et al.*, 1995; Gazzinelli, 1996); microbial infections (Romani *et al.*, 1995) heart diseases (Sasayama *et al.*, 1996; Maisch *et al.*, 1996), and autoimmune disorders (Grewal *et al.*, 1994).

A number of plant extracts (Ivanouska *et al.*, 1996; Sohni & Bhatt, 1996; Ottendorfer *et al.*, 1994; Labadie *et al.*, 1989) and plant-derived compounds have shown to possess immunomodulatory effects (Wang & Watson, 1994; Kensil, 1996; Wong *et al.*, 1994; Kosmiadi *et al.*, 1996; Lee *et al.*, 1997) and they have been excellently reviewed several times (Hikino, 1985; Wagner & Proksch, 1985; Bomford, 1988; Thatte & Dahanukar, 1989; Wagner, 1990;1994; Yamada, 1992; Hadden, 1993; Okuhara & Kino 1994; Zhang *et al.*, 1995; Zhang, 1996; Yang, 1996). These surveys revealed that potential immunostimulants can be found among both low- (e.g. alkaloids, terpenes, quinones, phenolic compounds) and high- molecular (e.g. polysaccharides, lectins, polypeptides) weight compounds. One compound in different concentrations can act as immunopotentiator or immunosuppresor and they influence mainly non-specific cellular and/or humoral immune defence systems. Some compounds with immunomodulatory properties are described below.

4.1.5.1 Alkaloids

Isopteropodine an oxindole alkaloid from *Uncaria* sp. stimulated *in vitro* phagocytosis by 55% at concentration range of 10^{-3} - 1^{-5} mg/ml. The immunostimulant activity of this alkaloid and other related alkaloids may explain the use of various *Uncaria* sp for wound-healing, intestinal infections and cancer treatment. Other alkaloids employed for similar purposes such as the isoquinoline alkaloid tylophorine from *Tylophora asthmatica*, the amoebicide, emetine from *C. ipecacuanha*, the bisbenzylisoquinoline alkaloid cepharanthine from *Stephania cepharantha* and aristolochic acid from *Aristolochia clematitis* (Wagner, 1990) enhanced phagocytosis.

The quinolizidine alkaloids matrine and oxymatrine (Figure 4.2) from *Sophora flavescens* are clinically effective in some allergic dermatopathies. Both have antiinflammatory activity in models mouse and suppress normal and mitogen induced proliferation of B lymphocytes (Wang *et al.*, 1994a). Sinomenine (Figure 4.2) an indole alkaloid from *Sinomenium acutum* used for the treatment of arthritis, was found to suppress spontaneous, lipopolysaccharide (LPS) and concanavalin A (Con A) induced proliferation of murine splenocytes (Wang *et al.*, 1992a). Morphine was found to inhibit chemotaxis, respiratory burst activity and phagocytosis of mononuclear phagocytes (Fecho *et al.*, 1995; Peterson *et al.*, 1993), and modulate some lymphocyte functions (Peterson *et al.*, 1995; Thomas *et al.*, 1995). Morphine also suppressed *P. berghei* infection in BALB/c mice, and this activity is due apparently to modulation of the macrophage-mediated protective mechanism (Singh *et al.*, 1994). Finally, semisynthetic derivates of ergot glycosides have been shown to have immunomodulatory properties (Kren *et al.*, 1996).

4.1.5.2 Quinones

The immunostimulatory activity of prenylated quinones ubiquinones Q_7 and Q_8 has been reported (Wagner & Prosk, 1985). Gossypol isolated from cotton seed oil known for its antispermogenic activity induces the production of IFN-γ. The naphthoquinone, plumbagin caused a marked stimulation of phagocytosis at high dilutions of 10^{-3} - 10^{-4} mg/ml, and also stimulated macrophages to secrete tumour necrosis factor. At concentrations of 10^{-1} - 10^{-2} mg/ml, this stimulatory effect was replaced by known cytotoxic activity. It is therefore possible that the anti-tumour and anti-infective actions

of the naphthoquinones-containing extracts of *Drosera* sp, *Dionaea muscipula* and the South American *Tabebuia avellanedae* arise partly from non-specific immunostimulation. The napthoquinones juglone and lawsone (Figure 4.2) conjugate effectively to protein thiol groups, but only juglone can undergo redox cycling. Juglone inhibited neutrophil superoxide production in a dose-dependent way (IC_{50} < 5 μM) whilst lawsone was less effective (IC_{50} > 100 μM). Juglone inhibited T cell proliferation (IC_{50} ~ 3μM), while hardly any effect of lawsone was observed (T'Hart, 1990).

4.1.5.3 Coumarins

Daphnetin (Figure 4.2) a coumarin-type compound from *Daphne giraldii*, was found to inhibit both humoral and cellular immunity, and enhance phagocytosis of macrophages of mice (Zhao, 1992). Osthol (Figure 4.2), an antiallergic compound from *Cnidium monnieri*, inhibits the passive cutaneous anaphylaxis (PCA) reaction in mice, and the degranulation of mast cells (Chen & Duan, 1988).

4.1.5.4 Flavonoids

Plantagoside (Figure 4.2) a flavone glucoside from *Plantago asiatica* decreased proliferation caused by Con A on spleen cells, but it did not suppress proliferation caused by phytohemagglutinin (PHA) or lipopolysaccharide (LPS) (Yamada *et al.*, 1989). Five isoflavanoquinones, abruquinone A, B, D, E and F (Figure 4.2) from *Abrus precatorios* (Leguminosae) showed anti-inflammatory, antiallergic effects, and inhibited superoxide production. These effects may explain the use of this plant as anti-inflammatory drug in traditional medicine (Kuo *et al.*, 1995). Quercetin 3-O-xylosyl (1→2)-rhamnoside and 3-O-rhamnoside from *Erythrospermum monticolum* (Flacourtiaceae) showed anti-inflammatory properties using as an experimental model for the inhibition the TPA-induced dermatitis of the mouse ear (Del Carmen Recio *et al.*, 1995).

4.1.5.5 Terpenes and saponins

Hydroxyachillin (Figure 4.2) a sesquiterpene lactone from *Tanacetum microphylum* (Compositae) has anti-inflammatory effects on murine carrageenin-induced oedema and this activity may be related to the use of the plant as an anti-inflammatory agent. The diterpene lactone epoxide: triptonide (Figure 4.2) from *Tripterygium wilfordii*

(Celastraceae) inhibited lipopolysaccharides (LPS), and concanavalin A (Con A) mitogen induced lymphocyte proliferation (Zhan *et al.*, 1995).

The triterpenes faradiol monoester, lupeol, ψ-taraxasterol, α- and β-amyrin and hydrophilic polysaccharides from *Calendula officinalis* have anti-inflammatory activity in the experimental model of the inhibition of the croton oil-induced dermatitis of the mouse ear (Yang, 1996).

Boswellic acid (Figure 4.2), a mixture of pentacyclic triterpenes from *Boswellia serrata* is used clinically for its antiinflammatory, antiarthritic and anticomplementary properties. Boswellic acid enhances humoral antibody synthesis, inhibits proliferative responsiveness of splenocytes to mitogens, and enhanced phagocytic function of macrophages in *vitro* (Sharma *et al.*, 1996).

Jaborosalactone-L and three 16α-oxygenated withanolides from *Dioscopodium penninervium* at non-toxic concentrations reduced the proliferative response of both inactive and *E. coli* lipopolysaccharide and concanavalin A activated rat spleen cells *in vitro* (Habtemariam, 1997).

Soybean an important legume that has been used for oil productions in food industry, has yielded soyasaponin Ab, a bisdesmosidic saponin which exhibited hydrogen peroxide production from polymorphonuclear lymphocytes (Yoshikoshi *et al.*, 1996). Other pharmacological effects reported for soybean saponins include: hypocholestemic, antitumor-promoting, and HIV infection inhibition.

The saikosaponins (Figure 4.2) from *Bupleurum kaoi* (Umbelliferae) have been found to increase thymus weight; IL-2 level; spleen weight; B-cell stimulation and the levels of IgA, IgG and IgM in serum animals treated (Yen *et al.*, 1995). The saponosides from *Mimosa tenuiflora* (Mimosaceae), mimonosides A, B and C, led to a synergic effect with Con A or LPS on activation of lymphocytes and had no effect on the growth of human and murine lymphoid tumour cells (Molt 4 and RDM 4) *in vitro* (Jian *et al.*, 1992).

Commercially available "saponin" is a mixture of saponins composed mainly of quillic acid has been isolated from *Quillagia saponaria*. Saponin is a powerful adjuvant for antibody response to antigens, eliciting both IgG1 and IgG2 antibody, cell-mediated immunity, tumour necrosisis factor, and IFN-γ production in mice (Kensil, 1996). Saponin has a wide application in veterinary vaccines, and has been successfully used in experimental immunisation against protozoal diseases such as malaria, babesiosis and trypanosomiasis (Bomford, 1988). Structure function analysis of the saponin QS21 has recently shown that adjuvant activity is largely dependent on the Schiff base-forming aldehyde (Dotsika *et al.*, 1997). In addition, its physico-chemical properties such as foaming, haemolytic and capacity to bind to cholesterol in cell membranes contributes to the activity of these saponins (Bomford, 1988). The antiviral effects of saponin has been also reported (Dotsika *et al.*, 1997).

Artemisinin a sesquiterpene from *Artemisia annua,* and its derivatives are well known as antimalarial drugs, and also have immunomodulatory properties. Artemisinin stimulates the phagocytosis of murine macrophages. Artemisinin and dehydroartemisinin suppress both humoral and cellular immunities in mice: the amount of anti-SRBC IgM were decreased as well as the proliferation response of murine splenocytes to Con A and LPS. Further studies indicated that IL-2 production of murine splenocytes was suppressed, suggesting that the inhibition of IL-2 production could be the most important of the mechanisms of immunosuppressive effects of artemisinin and dehydroartemisinin. Both arteether and artemether exhibited marked suppression of humoral responses. Artemether increased the weight of the spleen of normal and sheep red blood cell (SRBC)-treated mice and, reduced the percentage of phagocytosis in murine macrophages, and also markedly decreased the weight of thymus in mice sensitized with SRBC. Reduced artemisinin selectively promoted proliferation of T-lymphocytes and increased secretion of IL-2 (Zhang *et al.*, 1995: Yang, 1993).

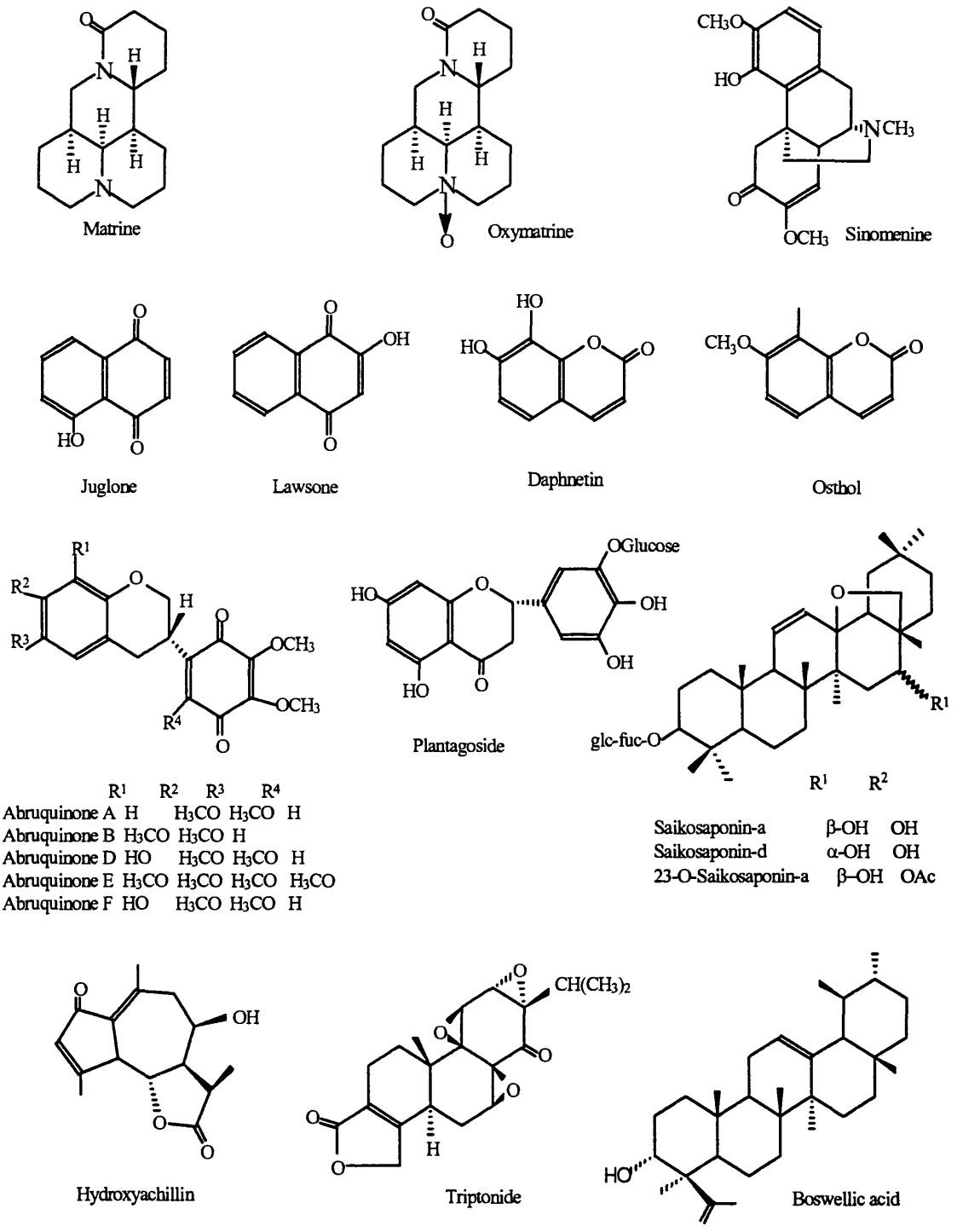


Figure 4.2 Some plant-derived compounds with immunomodulatory properties

4.2 Material and Methods

For this study murine lymphocyte proliferation and J774 murine macrophage superoxide-anion production were determined *in vitro* as two approaches to determine the immunomodulatory potential of 46 natural and semi-synthetic compounds and extracts of 16 plant species used in traditional medicine for the treatment of protozoal diseases in order to see the correlation between antiprotozoal and immunomodulatory activities. This is an approach which may be used to explain the curative effects attributed to some medicinal plants.

4.2.1 Preparation of samples for immunomodulating activity

The plant extracts and pure natural products were dissolved in EtOH, DMSO or in a mixture of 90 % EtOH/10 % DMSO. Further sterilization by filtration through 0.2 μm filters was carried out for each one of the samples tested. The stock solutions were stored in small sterile bottles at -20 °C. For mitogenic activity, the stock solutions were diluted with complete medium (4.2.2.2), and for production of superoxide, samples were diluted with phenol red-free Hanks balance salt solution (HBSS) to the desired concentration. Aqueous plants extracts were dissolved directly with cell culture medium. The final concentration of EtOH or DMSO in both test systems did not exceed 0.05%.

4.2.2 Mitogenic activity

4.2.2.1 Introduction

Lymphocytes comprise a population of cells with different immunological properties. They can be subdivided into T- and B-lymphocytes and the T-lymphocytes further divided into helper, suppressor, cytotoxic and memory cells. Assessment of lymphocyte function is performed by measuring the ability of lymphocytes to proliferate, produce mediators, induce cytotoxic responses and regulate immune response. The stimulation of lymphocyte proliferation has clinical relevance in patients with depressed cellular immunity, autoimmune disease and a variety of bacterial and viral infections as well as cancer. Lymphocyte proliferation can be measured by several methods, such as counting cells, measuring released ^{51}Cr-labelled protein after lysis, measuring incorporation of [^{3}H]thymidine, or by a colorimetric assay measuring the reduction of MTT to a formazan product by living metabolically active cells. In general, a basic amount of formazan is

generated by all living, metabolic active cells. Activated cells produce more formazan than resting cells which could allow the measurement of activation even in the absence of proliferation. The MTT colorimetric assay measures cytotoxicity, proliferation or activation of lymphocytes at the end of the assay, in response to a mitogen or any other appropriate stimulator.

4.2.2.2 Murine spleen cell cultures

Female BALB/c mice (6-8 weeks old), were used for the spleen cell cultures. Spleens were removed, minced and passed through a stainless 200-mesh in RPMI 1640 medium. Erythrocytes in the filtrate were lysed by treatment with 0.83% ammonium chloride-tris buffer (1 ml per spleen) and incubated in a water bath at 37 °C with continuous mixing for 3-5 minutes. The suspension was immediately diluted with RPMI-1640 medium and centrifuged at 1200 rpm for 10 min. Cells were washed twice with RPMI-1640 medium and suspended in culture medium containing RPMI-1640, L-glutamine (20 mM), 100 µg/ml streptomicin, 100 U/ml penicillin, $5x10^{-5}$ M 2-mercaptoethanol, 10 mM HEPES and 10% heat-inactivated fetal bovine serum. Then cells were cultured at a density of 2×10^5 cells/well with or without test sample in 0.1 ml cultured medium in 96 microculture plates at 37 °C for 3 days with 5% CO_2 - air mixture.

4.2.2.3 Test protocol

I*n vitro* lymphocyte proliferation was measured by the colorimetric MTT assay as described previously by Mosman (1983) and modified by Zhao *et al.* (1991). The mitogenic experiments were carried out in 96-well microtitre plates. Four-fold serial dilutions were performed along the breadth of the micro titre plate to obtain 6 concentrations, each well containing 50 µl of diluted sample. The last line of wells was left as a drug free control. To each well was added $2x10^5$ cells (50 µl) giving a total volume of 100 µl. The plates were covered and incubated at 37 ^{0}C for 3 days with 5% CO_2 - air mixture in a humidified atmosphere. Four hours prior to termination of the culture, 10 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] solution (5 mg/ml in phosphate buffered-saline) was added to each well of the culture plate. Cultures were stopped by adding 100 µl of 10% SDS solution into each well. The optical density at 540 nm was measured by a MR700 microplate reader

(Dynatech Labs. Inc). Samples were cultivated either in the presence or absence of Con A (0.5μg/ml) to investigate whether samples could directly affect the normal or mitogenic-induce lymphoproliferation rate. The known mitogenic agents Con A and LPS were used as control drugs. All concentrations were tested in triplicate and samples were tested at least twice on separate occasions.

4.2.3 Measurement of superoxide production

4.2.3.1 Introduction

Because one of the main functions of phagocytes in immune defense is to destroy viable bacteria intracellularly, it was interesting to evaluate a second parameter which correlated well with the killing of protozoa, bacteria, and tumour cells, namely the oxidative metabolism of neutrophils and macrophages (Ottendorfer *et al.*, 1994).

Phagocytic cells, such as polymorphonuclear leukocytes (PMNs) and macrophages, undergo an oxidative burst (OB) in response to phagocytic or membrane stimuli, with the generation and release of a variety of reactive oxygen metabolites, which destroy viable parasites, bacteria intracellularly (Rosen *et al.*, 1995). The oxidative burst can be monitored by assays of oxygen consumption, chemiluminescence, generation of reactive oxygen derivatives (H_2O_2, $O_2^{\cdot-}$, $HO^{\cdot}$), or formation of redox reaction products Since $O_2^{\cdot-}$ is the earliest product of the OB and the bulk of it is released into the medium surrounding the cell, the measurement of $O_2^{\cdot-}$ in the medium in which cells are suspended is an easy method for quantitation of the OB activity. The most common procedure for the assay of $O_2^{\cdot-}$ is the reduction of ferricytochrome c, a process in which $O_2^{\cdot-}$ serves as an electron donor. The amount of reduced cytochrome c is determined by measuring its absorbance at 550 nm. Proof that cytochrome c reduction was accomplished by $O_2^{\cdot-}$ is obtained by the fact that the reduction is eliminated when superoxide dismutase (SOD) is included in the reaction mixture.

4.2.3.2 Maintenance of J774 cell cultures

The murine macrophage cell line J774 has been used as a model for oxidative burst studies (Wang *et al.*, 1993) and is capable of phagocytosis. J774 cells were cultured in tissue cultured flasks (Falcon) in complete medium composed of DMEM , 10 % heat-

inactivated FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), 0.05 mM 2-mercaptoethanol, L-glutamine (20 mM), sodium pyruvate (10 mM) in a humidified 5% CO_2 - air mixture at 37 ^{0}C. Cells were released with 5 ml of trypsin-EDTA solution (0.05%/0.02%), after 5 minutes the trypsin was inactivated with completed medium. The cell suspension was then spun at 1050 rpm for 10 min and the supernant discarded. The pellet was washed with complete medium, spun and the supernant discarded twice. Then the pellet was washed with sterile HBSS and the supernant discarded. Cell density was determined using a Neubauer haemocytometer. Part of the subcultured cells were transferred to new flasks and diluted with complete medium and incubated in a humidified 5% CO_2 air mixture at 37 ^{0}C. A cell suspension of J774 cells in HBSS was prepared for each assay. The viability of cells used in experiments (>95%) was determined before each experiment by trypan blue exclusion.

4.2.3.3 Test protocol

The stimulation of macrophages was determined as superoxide-anion dependent reduction of ferricytochrome c according to the method of Pick and Mizel (1982). The test was carried out in flat-bottomed 96-well micro titre plate. 2 x 10^5 Cells/well (50 µl) were seeded and treated with samples (50 µl) in four different concentrations for a period of 2 h at 37 ^{0}C in 95% air-5% CO_2. Thereafter, cells were washed and received sterile cytochrome c solution in HBSS (150 µM, 100 µl). Blanks (cells with cytochrome c solution, SOD) and untreated controls (cells with cytochrome c solution) were included in each plate. The plates were covered and incubated for 90 minutes. Optical density was measured at 550 nm against blanks in a MR700 microplate reader (Dynatech Labs. Inc). The specificity of cytochrome reduction was demonstrated by its suppression in the presence of 300 U/ml of SOD (Type I, 3000 U/mg protein Sigma). LPS and TPA (12-O-Tetradecanoyl phorbol-13-acetate) were used as positive controls. The ability of the samples to promote macrophage $O_2^{\cdot-}$ production was determined either in the absence or presence of opsonized zymosan used at concentration of 5 mg/ml and included in cytochrome c solution at the beginning of the cultures. Each concentration was tested in triplicate. The samples were tested at least twice in independent experiments. The viability of the cells was determined at the end of the experiment using trypan blue exclusion. The amount of $O_2^{\cdot-}$ produced was calculated by using the extinction coefficient

E= $2.11x10^4$ M^{-1} cm^{-1}. The amount of $O_2^{\cdot}$ produced/well was calculated by the formula: nanomoles $O_2^{\cdot-}$ per well = (absorbance at 550 nm x 100)/ 6.3. Results were expressed as nM $O_2^{\cdot-}$ per well representing the amount of $O_2^{\cdot-}$ produced by stimulated cells of which nM $O_2^{\cdot-}$ per well in the absence of stimulation was deduced. Further, % of $O_2^{\cdot-}$ was also determined as compared with untreated control.

4.2.4 Statistical analysis

Statistical analysis was carried out using the Minitab statistical programme package. The data were analyzed statistically using one-way analysis of variance (ANOVA). The significance of difference between treated groups and the control group was assessed by unpaired student t' test. The differences were considered significant of $P < 0.05$. The % of lymphocyte proliferation or $O_2^{\cdot-}$ release for each sample are indicated as mean ± SEM of at least two independent deteminations, and each concentration was tested in triplicate .

4.3 Results and discussion

The *in vitro* effect of a number of samples on lymphocyte proliferation and macrophage superoxide production was determined as an approach to determine their immunomodulatory properties. The results of mitogenic activity and macrophage superoxide-anion production are given in percentages in comparison with untreated control in Tables 4.1 to 4.10. Figures 4.8 and 4.13 showed the comparative effects on the production of lymphocyte proliferation and induction of macrophage superoxide of the most active compounds. The discussion is divided into two sections, mitogenic and superoxide production, then a general discussion is given for both effects.

4.3.1 Mitogenic activity

The effect of 16 plant extracts and 52 compounds on murine lymphocyte proliferation was determined in the presence or absence of Con A. The mitogenic agents Con A (concanavaline A) and LPS (lipopolysaccharide) known to induce T- and B- lymphocyte proliferation, respectively were used as standard drugs. Con A was added at the beginning of the culture at concentration of 0.5 μg/ml in the presence of six different concentrations of samples in experiments of co-stimulation.

4.3.1.1 Plant extracts

The *in vitro* effects of 16 plant-extracts on lymphocyte proliferation in the absence or presence of Con A were studied. Results in Table 4.1 show that methanolic extracts of *Galphimia glauca, Hintonia latiflora* and aqueous extracts of *Guarea rhopalocarpa, Hintonia latiflora* and *Brucea javanica* enhanced proliferation of lymphocytes without the addition of a mitogen. They have a maximum response in the range of 1.43 - 2.17 times over the control at the concentration range of 10 to 0.01 μg/ml. The methanolic extract of *G. glauca* was the most active, having a maximum response of 2.17 times over control at 10 μg/ml. In the same conditions the standard Con A and LPS had their maximum mitogenic response of 3.64 and 4.10 over control at 1 and 10 μg/ml respectively.

The influence of samples tested on the response of lymphocytes to mitogens was also determined. Results in Table 4.1 indicate that methanolic extracts of *C. mexicanum, G. glauca, G. rhopalocarpa, S. dinklagei, T. patens, H. latiflora* and *B. javanica* in combination with Con A showed synergistic effects on the activation of T-lymphocytes, having a maximum co-stimulatory response in the range of 2.65 - 3.17-fold over untreated control at concentration range of 0.1 to 0.001 μg/ml. The strongest co-stimulatory activity was observed with methanolic extracts of *G. glauca, S. dinklagei* and *T. patens* which had a maximum co-stimulatory response of 3-times above control at concentration range of 0.1 to 0.001 μg/ml. Con A was used at a concentration of 0.5 μg/ml for the co-stimulatory experiments, having a mitogenic response of 2.23-fold over control.

Methanolic and aqueous extracts of *Picramnia antidesma* had suppressive effects on normal and mitogenic-induced proliferation in all the range of concentrations tested. The cytotoxicity of these extracts was ruled out by the observation that over 90% of the cells remained viable after 72 h incubation in the presence of extract at the range of concentrations tested, as assessed by trypan blue exclusion. Methanolic and aqueous extracts of *C. mexicanum, G. rhopalocarpa, S. dinklagei, T. patens, H. latiflora, B. javanica* and *P. antidesma* had suppressive effects related to their intrinsic cytotoxicity at 100 μg/ml.

Table 4.1 Mitogenic activitites of methanolic and aqueous extracts of some medicinal plants

Plant	Part	Extract	% Lymphocyte proliferation in the **absence** of Con A Concentration µg/ml **100**		**10**		**1.0**		**0.1**		**0.01**		**0.001**	
			O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
EUPHORBIACEAE														
Celaenodendrom mexicanum	L	MeOH	0	0	0.099±0.003	83	0.074±0.005	62	0.042±0.001	35	0.060±0.003	50	0.055±0.004	46
		H_2O	0.054±0.001	45	0.073±0.005	66	0.095±0.003	79	0.104±0.005	87	0.096±0.005	80	0.090±0.056	75
MALPHIGEACEAE														
Galphimia glauca	AP	MeOH	0.510±0.003	425	0.260±0.056	**217**	0.236±0.009	192	0.180±0.003	150	0.108±0.004	90	0.072±0.004	60
		H_2O	0.580±0.027	487	0.080±0.077	67	0.035±0.004	29	0.036±0.004	30	0.048±0.001	40	0.060±0.005	50
MELIACEAE														
Guarea rhopalocarpa	L	MeOH	0.026±0.002	22	0.110±0.056	92	0.069±0.004	58	0.048±0.005	40	0.043±0.002	36	0.024±0.004	20
		H_2O	0.061±0.012	51	0.217±0.045	**173**	0.108±0.067	90	0.072±0.002	60	0.078±0.001	65	0.048±0.003	40
MENISPERMACEAE														
Stephania dinklagei	AP	MeOH	0	0	0.025±0.033	21	0.039±0.006	33	0.109±0.004	88	0.144±0.034	**120**	0.130±0.056	108
		H_2O	0.099±0.005	83	0.033±0.056	25	0.145±0.004	**117**	0.024±0.001	20	0.021±0.044	18	0.012±0.004	10
Triclisia patens	AP	MeOH	0	0	0.112±0.077	92	0.157±0.088	**125**	0.125±0.003	105	0.108±0.066	90	0.085±0.006	70
		H_2O	0	0	0.010±0.004	8	0.025±0.005	21	0.082±0.011	68	0.066±0.033	55	0.050±0.003	42
RUBIACEAE														
Hintonia latiflora	B	MeOH	0.030±0.004	25	0.039±0.005	33	0.039±0.004	33	0.108±0.007	90	0.183±0.045	153	0.144±0.002	120
		H_2O	0.033±0.001	27	0.184±0.013	153	0.179±0.001	149	0.181±0.003	151	0.198±0.003	**165**	0.108±0.005	90
SIMAROUBACEAE														
Brucea javanica	F	MeOH	0.022±0.004	21	0.039±0.004	33	0.105±0.001	84	0.110±0.002	92	0.048±0.005	40	0.033±0.011	28
		H_2O	0.061±0.002	52	0.063±0.005	52	0.069±0.002	58	0.200±0.001	**167**	0.090±0.067	75	0.129±0.003	108
Picramnia antidesma	L	MeOH	0.083±0.001	68	0.105±0.012	86	0.129±0.002	108	0.066±0.003	55	0.108±0.044	90	0.096±0.004	80
		H_2O	0.079±0.005	66	0.102±0.034	85	0.082±0.007	68	0.110±0.004	96	0.124±0.056	103	0.108±0.002	90
Concanavaline (Con A)			0.088	73	0.126	107	0.435	**364**	0.296	247	0.323	269	0.195	162
Lipopolisaccharide (LPS)			0.261	218	0.570	**475**	0.360	289	0.320	267	0.150	125	0.079	66

L: Leaves. AP: Aereal Parts. B: Bark. F: Flowers. O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control: Mean O.D. ± SEM = 0.12 ± 0.017 . n=2

Table 4.1 Mitogenic activities of some medicinal plants (Continuation).

Plant	Part	Extract	% Lymphocyte proliferation in the **presence** of Con A Concentration µg/ml **100**		**10**		**1.0**		**0.1**		**0.01**		**0.001**	
			O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
EUPHORBIACEAE														
Celaenodendron mexicanum	L	MeOH	0.007±0.001	7	0.083±0.006	87	0.120±0.076	129	0.108±0.006	113	0.068±0.004	71	0.259±0.003	**265**
		H_2O	0.088±0.001	92	0.120±0.052	126	0.140±0.056	149	0.170±0.004	182	0.150±0.003	160	0.110±0.005	120
MALPHIGEACEAE														
Galphimia glauca	AP	MeOH	0.053±0.002	56	0.119±0.075	124	0.076±0.012	79	0.290±0.015	**306**	0.103±0.005	108	0.064±0.005	67
		H_2O	0.240±0.052	249	0.114±0.033	119	0.128±0.067	134	0.064±0.002	67	0.044±0.001	46	0.045±0.003	17
MELIACEAE														
Guarea rhopalocarpa	L	MeOH	0.040±0.013	42	0.164±0.070	171	0.276±0.045	288	0.158±0.012	259	0.248±0.047	165	0.124±0.007	129
		H_2O	0.076±0.005	80	0.134±0.050	138	0.140±0.034	146	0.144±0.016	150	0.192±0.023	**200**	0.108±0.004	113
MENISPERMACEAE														
Stephania dinklagei	AP	MeOH	0.049±0.006	52	0.068±0.003	71	0.089±0.030	93	0.304±0.004	**317**	0.058±0.004	61	0.144±0.080	150
		H_2O	0.048±0.003	51	0.114±0.043	119	0.119±0.005	124	0.148±0.002	155	0.144±0.066	150	0.130±0.067	137
Triclisia patens	AP	MeOH	0.009±0.002	9	0.016±0.002	17	0.018±0.004	19	0.019±0.003	20	0.038±0.003	40	0.280±0.030	**296**
		H_2O	0.013±0.004	14	0.040±0.015	42	0.092±0.006	96	0.144±0.002	150	0.114±0.002	119	0.064±0.056	67
RUBIACEAE														
Hintonia latiflora	B	MeOH	0.046±0.007	48	0.055±0.015	58	0.102±0.038	107	0.199±0.003	208	0.048±0.003	50	0.068±0.004	71
		H_2O	0.010±0.002	11	0.086±0.022	90	0.139±0.067	145	0.137±0.004	143	0.144±0.035	150	0.155±0.012	162
SIMAROUBACEAE														
Brucea javanica	F	MeOH	0.005±0.001	5	0.079±0.008	83	0.015±0.005	66	0.014±0.003	15	0.039±0.004	41	0.250±0.034	**265**
		H_2O	0.040±0.004	42	0.048±0.006	53	0.094±0.016	98	0.220±0.005	**230**	0.173±0.006	181	0.150±0.004	160
Picramnia antidesma	L	MeOH	0.036±0.001	38	0.083±0.002	87	0.103±0.045	108	0.089±0.001	93	0.168±0.004	176	0.128±0.005	134
		H_2O	0.054±0.005	56	0.110±0.043	115	0.139±0.036	145	0.103±0.004	108	0.033±0.006	35	0.053±0.007	56

L: Leaves. AP: Aereal Parts. B: Bark. F: Flowers. O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control: Mean O.D. ± SEM = 0.096 ± 0.004. In experiments of co-stimulation concanavaline A was used at the suboptimal concetration of 0.5 µg/ml, having a mitogenic response of 223 % as compared with untreated. control. n=2

4.3.1.2 Alkaloids

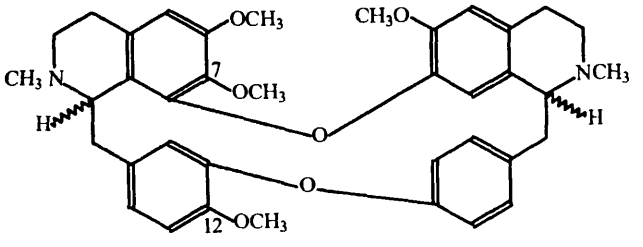
Results in Table 4.2 indicated that most of alkaloids (Figure 4.3) tested did not exert a mitogenic activity by themselves. Only strictosidine lactam showed a weak stimulation on spontaneous lymphocyte proliferation having a maximum response of 1.3-times above control at 0.1 μg/ml.

In combination with Con A, six alkaloids had a co-stimulatory effect including phaeanthine, stepharandine, dicentrinone, strictosidine, strictosidine lactam and acetylstrictosidine lactam, which had a maximum co-stimulatory effect corresponding to 3 - 4-times above control at concentrations of 0.1 or 0.01 μg/ml. Among them, the aporphine alkaloid stepharandine gave the strongest synergistic effect corresponding to 4-fold above control at 0.01 μg/ml. In contrast, the oxo-aporphine alkaloids liriodenine and 2-O,N-dimethyl-liriodendronine as well as acetyl-strictosidine inhibited normal and mitogenic-induced lymphocyte proliferation in all range of concentrations tested.

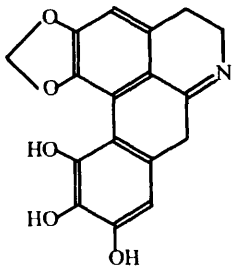
In assessing the structure-activity relationship it can be seen that the presence of a carbonyl function at C-7 in oxo-aporphine alkaloids confers immunosupppressive effects on the proliferation of lymphocytes, and the same structural requirement was observed for macrophage superoxide production such as liriodenine and dicentrinone. Comparison of strictosidine and acetylstrictosidine indicated that presence of acetyl-groups confer immunosuppressive effects on lymphocyte proliferation. In contrast, acetyl-strictosidine was found to possess immunostimulant effects on macrophage superoxide production. These results indicate that the same molecule can give different responses on different cells or mechanism involved in the immune system.

4.3.1.3 Quinones

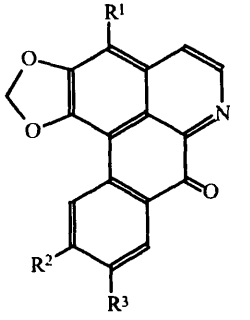
The effect of 6 quinones (Figure 4.4) on normal and mitogenic-induced lymphocyte proliferation was determined. Table 4.3 shows that most of quinones required a mitogenic agent in order to enhance the proliferation of lymphocytes. 1-Hydroxy-benzoisochromanquinone and aloe-emodin enhanced weakly the spontaneous proliferation of lymphocytes in 1.2 and 2.25 times above control at 1.0 μg/ml.



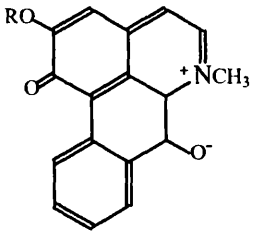
Phaeanthine R,R



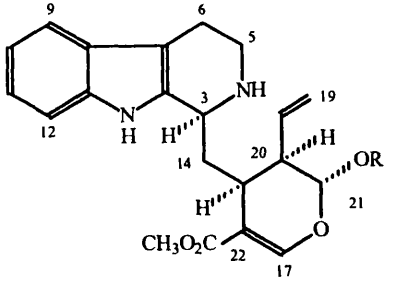
Stepharandine



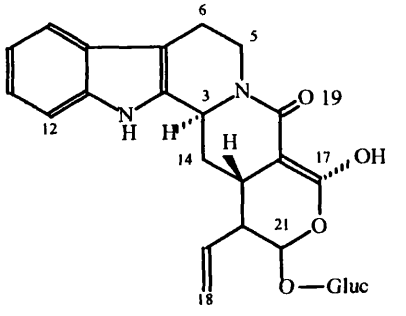
	R1	R2	R3
Liriodenine	H	H	H
Dicentrinone	H	OCH_3	OCH_3



	R
2-O,N-Dimethyl-liriodendronine	CH_3
N-Methyl-liriodendronine	H



	R
Strictosidine	Glucose
Acetylstrictosidine	Tetra-acetylglucose



	R
Strictosidine lactam	Glucose
Acetyl-strictosidine lactam	Tetra-acetyl-glucose

Figure 4.3 Alkaloids tested for immunomodulatory activity

Table 4.2 Mitogenic activities of some Alkaloids

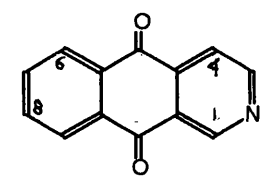
Alkaloids	% Lymphocyte proliferation in the **absence** of Con A Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Phaeanthine[1]	0	0	0.036±0.005	25	0.039±0.005	33	0.057±0.008	48	0.048±0.005	40	0.048±0.010	40
Stepharandine	0.020±0.005	17	0.047±0.006	39	0.037±0.001	31	0.033±0.003	28	0.048±0.008	40	0.057±0.007	48
Dicentrinone	0.009±0.001	8	0.064±0.570	54	0.072±0.004	60	0.094±0.001	75	0.083±0.009	69	0.072±0.002	60
Liriodenine	0.009±0.001	8	0.021±0.066	18	0.026±0.003	22	0.036±0.004	30	0.062±0.001	52	0.057±0.005	48
2-O,N-Dimethyl-liriodendronine	0.039±0.004	33	0.046±0.045	38	0.045±0.001	37	0.096±0.005	75	0.048±0.004	40	0.012±0.003	10
Strictosidine[1]	0.060±0.005	50	0.025±0.065	21	0.025±0.002	21	0.072±0.007	60	0.072±0.007	60	0.054±0.001	45
Acetylstrictosidine	0	0	0.031±0.003	25	0.050±0.008	42	0.052±0.004	44	0.038±0.006	32	0.033±0.005	28
Strictosidine lactam	0.039±0.006	33	0.061±0.004	50	0.062±0.009	51	0.156±0.002	**130**	0.158±0.077	125	0.138±0.067	115
Acetyl-strictosidinelactam[1]	0	0	0	0	0.020±0.005	17	0.045±0.009	38	0.025±0.004	21	0.010±0.056	9
	% Lymphocyte proliferation in the **presence** of Con A Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Phaeanthine[1]	0	0	0.014±0.005	15	0.133±0.056	139	0.246±0.067	254	0.290±0.044	**311**	0.078±0.064	82
Stepharandine	0.193±0.001	202	0.203±0.044	212	0.123±0.011	129	0.227±0.038	233	0.380±0.056	**400**	0.184±0.055	192
Dicentrinone	0.010±0.004	11	0.123±0.045	129	0.164±0.045	171	0.290±0.056	**306**	0.264±0.067	275	0.038±0.015	40
Liriodenine	0.044±0.002	46	0.128±0.032	134	0.150±0.047	160	0.144±0.069	150	0.093±0.011	97	0.144±0.067	150
2-O,N-Dimethyl-liriodendronine	0.011±0.005	12	0.013±0.023	14	0.019±0.001	20	0.010±0.001	11	0.010±0.005	11	0.23±0.006	243
Strictosidine[1]	0.035±0.001	37	0.098±0.067	103	0.123±0.001	129	0.319±0.066	**333**	0.170±0.066	181	0.164±0.056	171
Acetylstrictosidine	0.027±0.006	29	0.184±0.034	192	0.259±0.049	270	0.219±0.004	228	0.134±0.045	140	0.137±0.077	143
Strictosidinelactam	0.040±0.003	42	0.133±0.045	139	0.184±0.056	192	0.305±0.034	**318**	0.203±0.077	212	0.130±0.019	140
Acetyl-strictosidine lactam[1]	0.059±0.007	62	0.267±0.070	275	0.290±0.033	306	0.313±0.056	**327**	0.144±0.045	150	0.033±0.056	35

O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment.
Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. Untreated Control in the presence of Con A: Mean O.D. ± SEM = 0.096 ± 0.004. In experiments of co-stimulation concanavaline A was used at suboptimal concentration of 0.5 µg/ml, having a mitogenic response of 223 % as compared with untreated control. n=2. [1] n=3

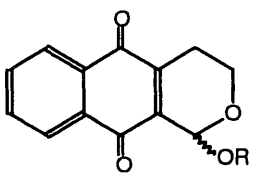
In combination with Con A, all quinones showed a co-stimulatory effect within the range of 3.53 - 5.1-fold over control at concentration range of 1.0 - 0.001 μg/ml. The strongest synergistic effect was observed with benzo[g]isoquinoline-5,10-dione and 1-acetyl-benzoisochromanquinone, which had a maximum effect of 5.51 and 4.62 times over control at 0.001 and 0.01 μg/ml respectively. Further, benzo[g]isoquinoline-5,10-dione was more active than 1-hydroxy-benzo-isochromanquinone. Thus it is possible that N on position 2 enhanced synergistic effects on T-cell activation.

If synergistic effects of 1-hydroxy-benzo-isochromanquinone and 1-acetyl-benzo-isochromanquinone are compared it can be seen that the presence of an acetyl group at C-1 enhanced the co-stimulatory response on the stimulation of T-cells. Similar effects were observed for both quinones on the stimulation of macrophages for superoxide release. Thus, it can be seen that, both quinones have similar structural requirements for both immunological effects and this is also extended to their antiprotozoal and cytotoxic properties. However, the concentrations necessary for both quinones to exert their antiprotozoal activity are higher than those required for the immunological response. If benzo[g]isoquinoline-5,10-dione, 1-hydroxy-benzo-isochromanquinone and 1-acetyl-benzo-isochromanquinone enhanced T-lymphocyte proliferation and macrophage superoxide at non-cytotoxic concentrations, it is possible that they may have applications as immunotherapeutic agents for the treatment of cancer. Further studies *in vitro* would be worthwhile in order to understand their mechanism of immunomodulation as well as to confirm their immunomodulatory effects *in vivo*.

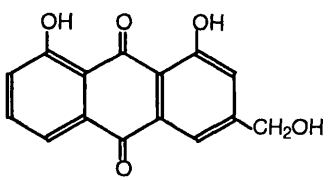
In the case of anthraquinones, comparison of aloe-emodin, picramnioside A and picramnioside C, indicated that the last two compounds were more active than their aglycone, aloe-emodin on lymphocyte proliferation and macrophage superoxide production, suggesting that xylose portion enhanced the immunostimulant response. Furthermore, picramnioside A with a benzoyl group at C-5' gave a stronger response on lymphocyte proliferation than picramnioside C which has instead an acetyl group. In contrast, picramnioside C was more active than picramnioside A on the superoxide release from macrophages. These results suggested that the structural requirements for both effects differ for these type of anthraquinones. Aloe-emodin enhanced mitogenic



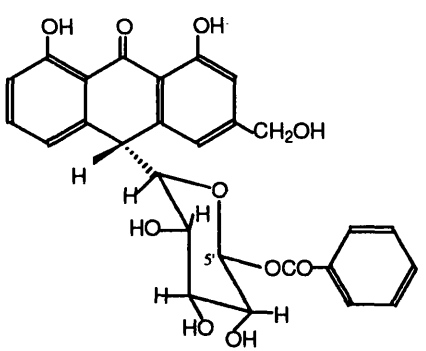
Benzo[g]isoquinoline-5,10-dione



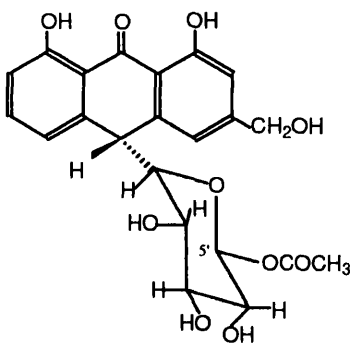
	R
1-Acetyl-benzoisochromanquinone	H
1-Hydroxy-benzoisochromanquinone	$OCOCH_3$



Aloe-emodin



Picramnioside A



Picramnioside C

Figure 4.4 Quinones tested for immunomodulatory activity

Table 4.3 Mitogenic activitites of some Quinones

Quinones	% Lymphocyte proliferation in the **absence** of Con A Concentration µg/ml									
	10		**1.0**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Benzo[g]isoquinoline-5,10-dione[1]	0	0	0	0	0.020±0.004	17	0.012±0.005	10	0.012±0.003	0
1-Hydroxy-benzoisochromanquinone[1]	0.043±0.002	36	0.144±0.056	**120**	0.026±0.006	22	0.069±0.002	58	0.066±0.005	55
1-Acetyl-benzoisochromanquinone[2]	0.056±0.010	47	0.048±0.011	40	0.039±0.001	33	0.033±0.001	28	0.024±0.001	20
Aloe-emodin	0.120±0.003	100	0.270±0.050	**225**	0.085±0.002	71	0.081±0.006	68	0.069±0.004	58
Picramnioside A	0.219±0.045	183	0.039±0.067	33	0.043±0.007	36	0.066±0.003	55	0.098±0.001	82
Picramnioside C	0.220±0.056	188	0.039±0.011	33	0.043±0.001	36	0.066±0.004	55	0.096±0.001	80
	% Lymphocyte proliferation in the **presence** of Con A Concentration µg/ml									
	10		**1.0**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Benzo[g]isoquinoline-5,10-dione[1]	0.010±0.001	11	0.210±0.002	223	0.340±0.056	358	0.318±0.045	332	0.528±0.056	**551**
1-Hydroxy-benzoisochromanquinone[1]	0.008±0.001	9	0.148±0.023	155	0.194±0.003	202	0.290±0.033	311	0.348±0.030	**363**
1-Acetyl-benzoisochromanquinone[2]	0.139±0.044	145	0.169±0.045	176	0.360±0.056	374	0.440±0.012	**462**	0.099±0.010	103
Aloe-emodin	0.030±0.003	32	0.190±0.056	202	0.289±0.045	302	0.338±0.045	**353**	0.323±0.055	337
Picramnioside A	0.033±0.007	35	0.385±0.023	396	0.320±0.010	337	0.360±0.043	**375**	0.343±0.011	358
Picramnioside C	0.148±0.067	155	0.309±0.012	322	0.304±0.033	317	0.35±0.033	**368**	0.279±0.023	291

O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment.
Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. Untreated Control in the presence of Con A: Mean O.D. ± SEM = 0.096 ± 0.004. In experiments of co-stimulation concanavaline A was used at suboptimal concentration of 0.5 µg/ml, having a mitogenic response of 223 % as compared with untreated control. n=2. [1]n=3. [2]n=4

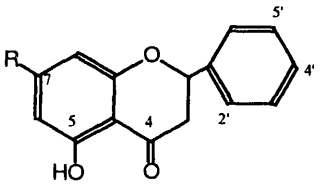
activity at concentrations equal or lower than 0.1 μg/ml, and inhibited macrophage superoxide production at the same concentration. From this results, it can be seen that the same compound at the same concentration can exert different immunological response on different cell or mechanisms involved in the immune system. Aloe-emodin has been reported to have mutagenic activity *in vitro* (Choi *et al.*, 1997) however, this activity was not observed *in vivo*.

4.3.1.4 Flavonoids

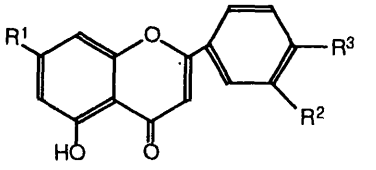
The effects of 8 flavonoids (Figure 4.5) on spontaneous and mitogenic-induced lymphocyte proliferation were determined. Results in Table 4.4 showed that 3 flavonoids enhanced normal proliferation of lymphocytes. They were the flavones, chrysin and 7-methyl-luteolin, and the flavonol quercetin. Chrysin and quercetin had a maximum effect of about 2-fold over control at 1 μg/ml; whereas 7-methyl-luteolin had mitogenic activity at the range of concentrations tested, having a maximum response of 2.2-fold over control at 0.01 μg/ml.

In combination with Con A no synergistic effect was observed for both flavones chrysin and 7-methyl-luteolin. Thus, it is possible that the enhancement observed for these flavones on normal proliferation is caused by the activation of B-lymphocytes. Therefore further studies of co-stimulation with other mitogenic agents will be necessary to confirm these preliminary findings.

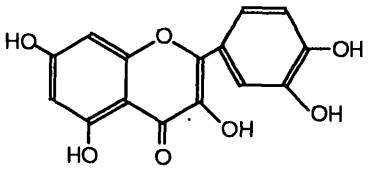
In the case of the flavanol quercetin, and bi-flavonoids podocarpusflavone A and podocarpusflavone B, synergistic effects were observed at concentration of 0.1 μg/ml, whereas at others concentrations they had suppressive effects. In a similar manner, quercetin showed immunosuppressive effects on normal and mitogenic-induce cultures at 0.1 μg/ml, whilst in other concentrations it was found to possess immunostimulant effects. Finally, it can be seen that flavanones pinocembrin and pinostrobin had suppressive effects on normal and mitogenic-induced lymphocyte cultures at all the concentrations tested.



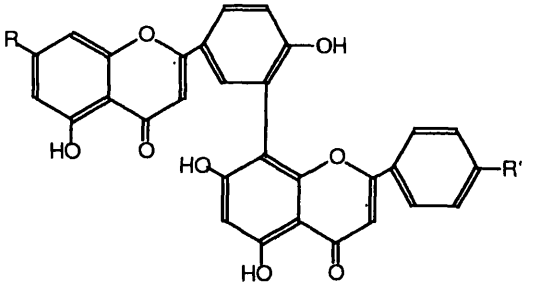
	R
Pinocembrin	OH
Pinostrobin	OCH_3



	R^1	R^2	R^3
Chrysin	OH	H	H
7-Methyl-luteolin	OCH_3	OH	OH



Quercetin



	R	R'
Amenthoflavone	OH	OH
Podocarpusflavone B	OH	OCH_3
Podocarpusflavone A	OCH_3	OCH_3

Figure 4.5 Flavonoids tested for immunomodulatory activity

Table 4.4 Mitogenic activities of some Flavonoids

Flavonoid	% Lymphocyte proliferation in the **absence** of Con A											
	Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Pinocembrin[1]	0.009±0.001	8	0.080±0.011	67	0.020±0.005	17	0.014±0.033	12	0.009±0.0008	8	0.009±0.0008	8
Pinostrobin	0.006±0.002	5	0.009±0.001	8	0.012±0.004	10	0.060±0.023	50	0.081±0.007	68	0.024±0.007	20
Chrysin	0.009±0.003	8	0.279±0.023	233	0.249±0.089	**208**	0.144±0.045	120	0.132±0.045	110	0.129±0.069	108
7-Methyl-luteolin[2]	0.200±0.005	167	0.309±0.045	258	0.260±0.067	217	0.210±0.057	175	0.270±0.004	**225**	0.252±0.056	210
Quercetin[2]	0.060±0.003	50	0.170±0.055	142	0.200±0.056	**167**	0.150±0.066	128	0.135±0.067	113	0.120±0.044	100
Podocarpusflavone B[2]	0.066±0.002	55	0.039±0.012	33	0.050±0.023	42	0.045±0.034	38	0.036±0.003	30	0.012±0.005	10
Podocarpusflavone A	0.240±0.034	200	0.099±0.008	83	0.030±0.014	25	0.037±0.011	25	0.012±0.008	10	0.006±0.0004	5
Amentoflavone	0.110±0.022	92	0.060±0.007	50	0.110±0.050	92	0.036±0.006	30	0.024±0.005	20	0.024±0.006	0
	% Lymphocyte proliferation in the **presence** of Con A											
	Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Pinocembrin	0.027±0.012	29	0.054±0.007	56	0.113±0.045	118	0.123±0.015	129	0.194±0.022	202	0.098±0.0014	103
Pinostrobin	0.031±0.011	33	0.019±0.008	20	0.069±0.012	72	0.033±0.002	35	0.024±0.006	25	0.083±0.012	87
Chrysin	0.075±0.006	79	0.049±0.004	51	0.160±0.010	167	0.144±0.006	150	0.074±0.004	77	0.108±0.067	113
7-Methyl-luteolin	0.144±0.004	150	0.198±0.006	207	0.220±0.045	232	0.153±0.067	160	0.154±0.012	160	0.150±0.010	160
Quercetin	0.024±0.007	25	0.203±0.023	212	0.230±0.010	212	0.298±0.045	**311**	0.161±0.022	168	0.130±0.040	140
Podocarpusflavone B	0.230±0.008	244	0.278±0.012	290	0.334±0.009	**348**	0.298±0.090	311	0.162±0.034	171	0.19 3±0.013	202
Podocarpusflavone A	0.026±0.0003	27	0.069±0.011	72	0.153±0.001	160	0.318±0.056	**332**	0.158±0.056	165	0.108±0.006	113
Amentoflavone	0.038±0.056	40	0.119±0.045	124	0.284±0.007	296	0.290±0.033	**311**	0.194±0.012	202	0.134±0.034	140

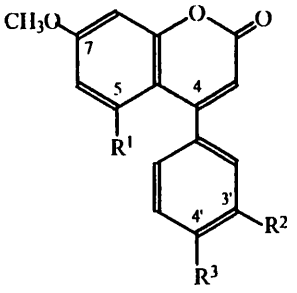
O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment.
Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. Untreated Control in the presence of Con A: Mean O.D. ± SEM = 0.096 ± 0.004. In experiments of co-stimulation concanavaline A was used at suboptimal concentration of 0.5 µg/ml, having a mitogenic response of 223 % as compared with untreated control. n=2. [1]n=3. [2]n=4

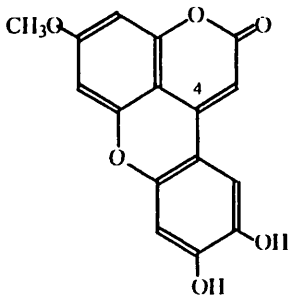
4.3.1.5 Coumarins

Eleven coumarins (Figure 4.6) were tested for mitogenic activity and the results given in Table 4.5 reveal that only two 4-phenylcoumarins promote lymphocyte proliferation. They were: the oxidocoumarin, 4',5'-dihydroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin and the acetyl aglycone: 5,3',4'-triacetoxy-7-methoxy-4-phenylcoumarin, which had a maximum response of 1.7 and 1.8 times over control at 0.1 μg/ml. However, the above 4-phenylcoumarins did not show co-stimulatory effect with Con A. It is known that murine splenocytes are composed of an average of 65% of B-lymphocytes and of 35% T-lymphocytes. Concanavalin A predominantly stimulates the T-lymphocytes. But it also stimulates the B-lymphocytes in the case of mixtures of B- and T-lymphocytes, while lipopolysaccharide (LPS) mainly stimulates B-lymphocytes (Jian *et al.*, 1992). Thus, it is possible that the enhancement observed on normal lymphocyte proliferation for the 4-phenylcoumarins is due to the activation of B-cells. Further studies of co-stimulation will be necessary to determine this preliminary findings.

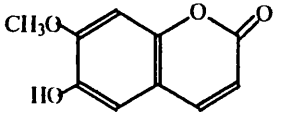
In combination with Con A, five coumarins showed synergistic effects including, the glucoside, 5-O-[β-D-glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, the galactoside, 5-O-[β-D-galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, the acetylgalactoside, 5-O-[β-D-tetraacetoxy-galactopyranosyl]-3',4'-diacetyl-7-methoxy-4-phenylcoumarin and the oxidocoumarin, 4',5'-dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin as well as scopoletin having a maximum co-stimulatory response in the range of 2.1 - 4.3-fold higher than control in a concentration range of 0.001 - 0.1 μg/ml respectively. The other coumarins suppressed normal and mitogenic-induced proliferation of lymphocytes in all the range of concentrations tested.

Comparison of mitogenic activities of glucoside and galactoside indicated that galactoside is twice times more active than the glucoside at concentrations equal or lower than 0.1 μg/ml. It is known that polygacturonan region of polysaccharides is involved in the expression not only of mitogenic activity but also of anti-complementary activity (Zhao *et al.*, 1992). This could explain why the galactoside gave a better co-stimulatory response than the glucoside. On the other hand, it is possible that the galactosides bind





4',5'-Dihidroxy-7-methoxy-
4-phenyl-5,2'-oxidocoumarin



Scopoletin

	R^1	R^2	R^3
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	5-O-β-D-galactopyranosyl	OH	OH
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	5-O-β-D-glucopyranosyl	OH	OH
5-O-[β-D-Tetra-acetoxygalactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	5-O-β-D-Tetra-acetylgalactopyranosyl	$OCOCH_3$	$OCOCH_3$
5-O-[β-D-Tetra-acetoxyglucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	5-O-β-D-Tetra-acetylglucopyranosyl	$OCOCH_3$	$OCOCH_3$
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	5-O-β-D-galactopyranosyl	OCH_3	OCH_3
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	5-O-β-D-glucopyranosyl	OCH_3	OCH_3
5,3',4'-Tri-hydroxy-7-methoxy-4-phenylcoumarin	OH	OH	OH
5,3',4'-Tri-acetoxy-7-methoxy-4-phenylcoumarin	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
5-O-[β-D-Tetra-acetoxygalactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin	5-O-β-D-Tetra-acetylglucopyranosyl	H	$OCOCH_3$

Figure 4.6 Coumarins tested for immunomodulatory activity

Table 4.5 Mitogenic activities of some Coumarins

Coumarins	% Lymphocyte proliferation in the **absence** of Con A Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	1.029±0.678	858	0.160±0.078	136	0.056±0.011	47	0.033±0.003	28	0.021±0.006	18	0.019±0.004	16
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	1.011±0.551	842	0.186±0.067	**155**	0.025±0.006	21	0.045±0.009	38	0.030±0.005	25	0.031±0.005	26
5-O-[β-D-Tetraacetoxy-glucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	0.819±0.090	683	0.046±0.007	39	0.063±0.005	53	0.046±0.003	38	0.036±0.004	30	0.033±0.003	28
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	0.870±0.067	725	0.140±0.007	**117**	0.110±0.004	92	0.086±0.004	70	0.078±0.003	65	0.072±0.002	60
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	0.080±0.012	67	0.230±0.068	**192**	0.129±0.004	108	0.050±0.001	42	0.045±0.002	38	0.045±0.005	38
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	0.074±0.011	62	0.069±0.005	58	0.064±0.002	54	0.054±0.003	45	0.046±0.003	39	0.048±0.001	40
5,3',4'-Trihydroxy-7methoxy-4-phenylcoumarin	0.770±0.023	642	0.039±0.005	33	0.086±0.008	72	0.036±0.005	30	0.033±0.003	28	0.030±0.002	25
5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin	0.440±0.013	367	0.092±0.006	77	0.075±0.003	63	0.204±0.045	**170**	0.192±0.004	160	0.180±0.005	150
4',5'-Dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin	0.520±0.056	438	0.024±0.007	205	0.220±0.068	**187**	0.220±0.067	**187**	0.180±0.009	150	0.060±0.003	50
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin	0.050±0.012	42	0.120±0.056	100	0.076±0.012	64	0.072±0.009	60	0.096±0.010	80	0.072±0.002	60
Scopoletin	0.056±0.008	47	0.133±0.044	111	0.039±0.004	33	0.084±-.006	70	0.078±0.002	65	0.078±0.001	65

O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment.
Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. n=2.

Table 4.5 Mitogenic activities of some Coumarins (Continuation)

Coumarins	% Lymphocyte proliferation in the **presence** of Con A Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O .D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	1.07±0.568	1119	0.318±0.005	343	0.218±0.045	228	0.239±0.045	249	0.284±0.030	**296**	0.203±0.067	212
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	1.00±0.670	1046	0.338±0.004	353	0.240±0.76	254	0.198±0.040	207	0.178±0.023	186	0.418±0.10	**436**
5-O-[β-D-Tetraacetoxy-glucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	0.640±0.005	666	0.103±0.006	108	0.128±0.050	134	0.103±0.067	108	0.103±0.002	108	0.054±0.079	56
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	0.698±0.002	728	0.230±0.006	244	0.214±0.068	223	0.139±0.066	145	0.078±0.004	82	0.203±0.056	212
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	0.078±0.003	82	0.033±0.001	35	0.133±0.045	139	0.178±0.034	186	0.084±0.003	87	0.078±0.030	82
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin	0.064±0.002	67	0.130±0.003	140	0.148±0.043	155	0.159±0.056	166	0.139±0.006	145	0.174±0.067	181
5,3',4'-Trihydroxy-7methoxy-4-phenylcoumarin	0.578±0.004	603	0.149±0.001	156	0.190±0.056	202	0.184±0.067	192	0.119±0.004	124	0.123±0.058	129
5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin	0.220±0.001	233	0.069±0.012	72	0.089±0.003	93	0.108±0.055	113	0.189±0.002	197	0.064±0.040	67
4',5'-Dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin	0.239±0.015	249	0.144±0.009	150	0.119±0.050	124	0.209±0.058	218	0.128±0.050	134	0.139±0.060	145
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin	0.015±0.006	16	0.159±0.087	166	0.179±0.060	187	0.171±0.011	179	0.200±0.070	209	0.133±0.080	139
Scopoletin	0.018±0.004	19	0.290±0.045	306	0.370±0.089	390	0.329±0.034	343	0.404±0.069	**421**	0.144±0.075	150

O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. Untreated Control in the presence of Con A: Mean O.D. ± SEM = 0.096 ± 0.004. In experiments of co-stimulation concanavaline A was used at suboptimal concentration of 0.5 µg/ml, having a mitogenic response of 223 % as compared with untreated control. n=2.

to Con A in such a way that galactose portions are exposed to the receptors on the membrane surface of lymphocytes leading to immunostimulation of lymphocytes. In addition, acetyl-galactoside was found less active than the original galactoside. Thus, it is possible that hydroxyl groups in the sugar portion of the 4-phenylcoumarin forms hydrogen bonds with the sugar portion of the mitogenic agent leading to the activation of T-cells.

Scopoletin did not induce normal proliferation of lymphocytes but showed synergistic activity with Con A having a maximum co-stimulatory response of 4.21 times above the control at 0.01 μg/ml. Comparison of daphnetin and osthol (Figure 4.2) which inhibited both humoral and cellular immunity, and enhanced phagocytosis of mice macrophages (Zhao *et al.*, 1992) with scopoletin which stimulate proliferation of T-cells, indicated that the phenolic group at C-8 is essential for immunosupressive effects of both humoral and cellular immunity for this type of coumarins.

4.3.1.6 Terpenes

Nineteen terpenes (Figure 4.7) were screened for mitogenic activity and results are given in Table 4.6. Results showed that 6 terpenes enhanced normal lymphoproliferation within the range of 1.3 - 2.4-times over the control in a concentration range of 1 - 0.01 μg/ml. Among them the cucurbitacin 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F gave the strongest effect, having a maximum response of about 2-times over control at concentration range of 0.1 - 0.01 μg/ml. In combination with Con A, 8 terpenes showed synergistic effects, having a maximum co-stimulatory response of 3-times over the control at concentration range of 0.1 - 0.01 μg/ml. Among them, epi-oleanolic acid, 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F, and galphimine C, showed the strongest synergistic effect, corresponding to 3 - 4-fold above control at concentration range of 0.1 to 0.01 μg/ml. Saponins Ma and Mb which are the heptasaccharide and hexasaccharide of oleanolic acid, stimulated normal Con A and LPS co-stimulated lymphocyte proliferation *in vitro* (Jian *et al.*, 1992). Thus, it is possible that epi-oleanolic acid also activates B-cells, and studies of co-stimulation with LPS and other mitogenic agents will be necessary to complete these preliminary findings.

If mitogenic activities of tirucall-type terpenoids, 3α-hydroxy-7,24Z-di-en-tirucalla-26-oic acid and 3-oxo-7,24Z-di-en-tirucalla-26-oic acid, are compared it can be seen that the second compound is more active. It is possible that the presence of the ketone group at C-3 enhances the activation of T-lymphocytes. Similar effects were observed with the lanostan-type triterpenes, 23-hydroxy-5α-lanosta-7, 9(11), 24EZ-triene-3-one and lanosta-7,9(11), 24EZ-triene-3α, 24-diol, being more active the second terpene.

Schiff base bonds formation, is essential in a number of dynamic physiological processes, including enzyme-substrate interaction, transamination, decarboxylation and other amino acid-modifying reactions mediated by pyridoxal phosphate. The Schiff base formation, occuring between specialised carbonyls and amines expressed reciprocally on the surfaces of antigen-presenting cells (APCs) and T cells plays an essential role in antigen-dependent T cell activation (Chen *et al.*, 1997). The identification of covalent chemical events, complementary to macromolecular interactions, occurring in the interaction of APCs and T cells is a novel target for the manipulation of immune responses and permits a rational approach for the design of immunopotentiatory drugs. Thus, the addition of small exogenous Schiff base forming molecules can substitute for the natural donor of carbonyl groups and provide a co-stimulatory signal to CD4+ T_H cells. Once triggered, $CD4^+$ T_H cells orchestrate the response largely through the production of cytokines which determine the direction an immune response may take. This could explain the higher activity observed in the tirucallan and lanostan terpenoids containing a ketone group at C-3 in their molecule on the activation of T-lymphocytes than those analogues having a hydroxyl group at C-3.

If co-stimulatory effects of pimaradienes type-diterpenes, ent-8(14),15-sandaracopimaradiene-2β,18-diol and ent-8(14),15-sandaracopimaradiene-2α,18-diol, are compared it can be seen that, the second compound showed strong synergistic activity, on the proliferation of T-lymphocytes and this activity parallel with the antiprotozoal activity. Thus, it is likely that position of hydroxyl group is important for mitogenic activity of these diterpenes.

Cucurbitacins are bitter constituents of Cucurbitaceae family and have antineoplastic

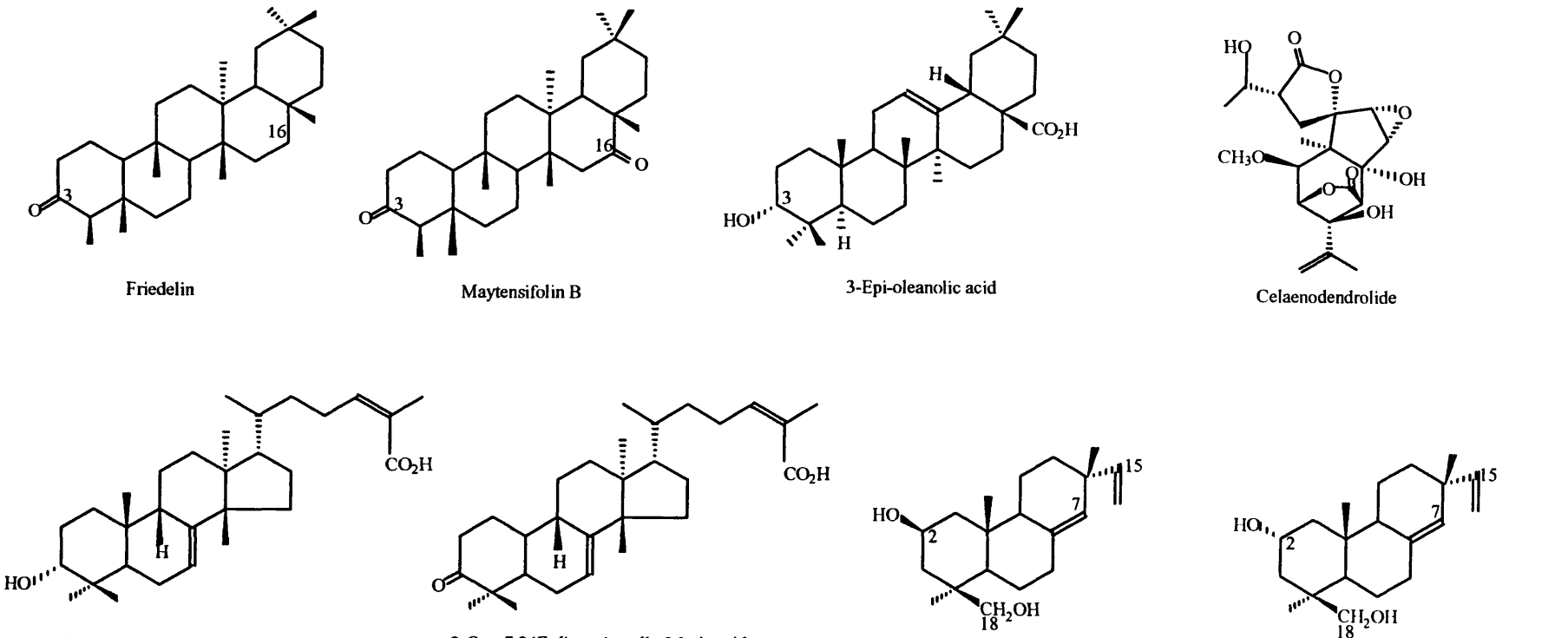
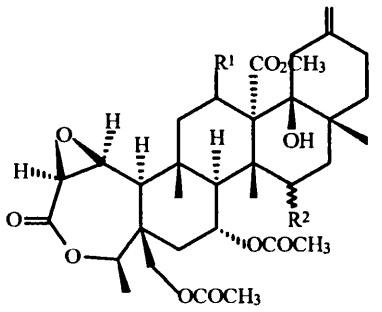
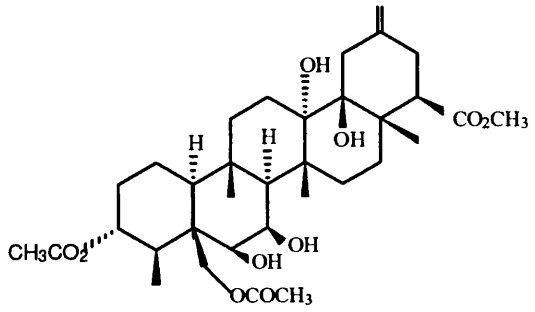


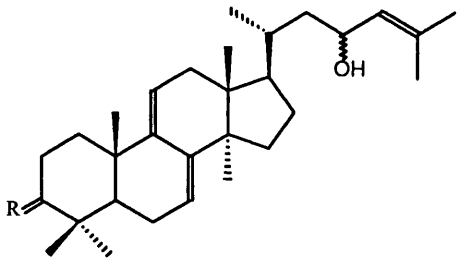
Figure 4.7 Terpenes tested for immunomodulatory activity



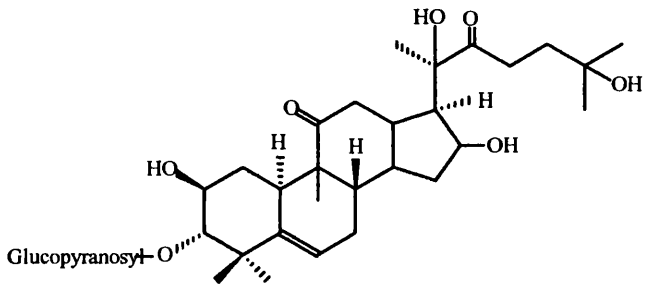
	R^1	R^2
Galphimine C	H	H
Galphimine D	H	β-$OCOCH_3$
Galphimine E	α-$OCOCH_3$	β-$OCOCH_3$



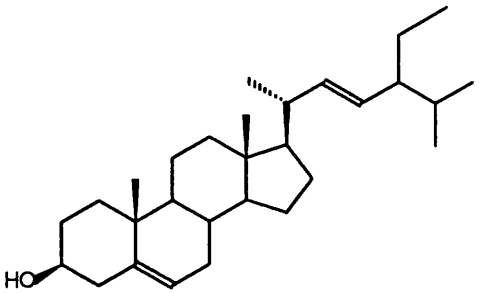
Glaucamine



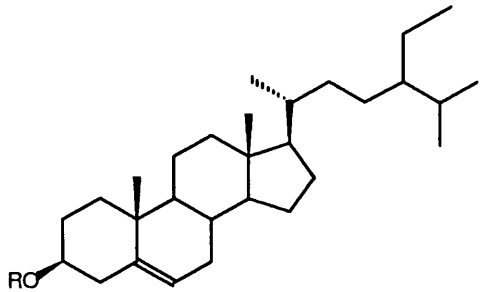
R	
23-Hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one	O
Lanosta-7,9(11),24EZ-triene-3α,24-diol	α-OH



3-O-β-D-Glucopyranosyl-23,24-dihydrocucurbitacin F



Stigmasterol



R	
β-Sitosterol	OH
sitosteryl-3-O-β-D-glucopyranoside	Glucopyranosyl

Figure 4.7 Terpenes tested for immunomodulatory activity (Continuation)

Table 4.6 Mitogenic activities of some Terpenes

Terpenes	% Lymphocyte proliferation in the **absence** of Con A Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Friedeline	0.069±0.002	58	0.180±0.004	150	0.025±0.005	21	0.081±0.007	68	0.067±0.005	56	0.048±0.006	40
Maytensifolin B	0.019±0.001	16	0.024±0.002	20	0.045±0.008	38	0.060±0.002	50	0.048±0.001	40	0.036±0.001	30
Celaenodendrolide[1]	0.090±0.005	75	0.020±0.006	17	0.030±0.009	25	0.012±0.005	10	0	0	0	0
3α-Hydroxy-7,24Z-dien-tirucalla-26 oic acid[1]	0.082±0.009	69	0.158±0.004	132	0.297±0.056	**248**	0.104±0.001	87	0.074±0.002	62	0.038±0.003	32
3-Oxo-7,24Z-dien-tirucalla-26 oic acid[1]	0.150±0.002	125	0.122±0.034	102	0.190±0.070	162	0.168±0.001	140	0.162±0.030	135	0.036±0.006	30
Epi-oleanolic acid[1]	0.008±0.001	7	0.061±0.010	51	0.210±0.040	175	0.276±0.078	**230**	0.260±0.056	217	0.180±0.04	150
3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F	0	0	0.120±0.050	100	0.129±0.067	108	0.189±0.45	**158**	0.159±0.067	133	0.132±0.080	110
23-Hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one[2]	0	0	0.060±0.009	50	0.030±0.006	25	0.129±0.032	108	0.120±0.059	100	0.114±0.003	95
Lanosta-7,9(11),24EZ-triene-3α,24-diol[1]	0.024±0.001	20	0.014±0.008	12	0.064±0.007	53	0.069±0.003	58	0.057±0.078	48	0.050±0.002	42
Ent-8(14),15-Sandaracopimaradiene-2β,18-diol[1]	0.159±0.006	133	0.115±0.004	96	0.025±0.002	21	0.074±0.004	62	0.061±0.005	51	0.054±0.007	45
Ent-8(14),15-Sandaracopimaradiene-2α,18-diol[1]	0.180±0.056	150	0.309±0.050	258	0.130±0.067	113	0.204±0.001	**170**	0.198±0.004	165	0.186±0.006	155
Galphimine C[1]	0.030±0.005	25	0.039±0.003	33	0.050±0.006	42	0.048±0.004	40	0.036±0.002	30	0.024±0.004	20
Galphimine D[1]	0.030±0.002	25	0.020±0.005	17	0.009±0.004	8	0.06±0.005	50	0.057±0.001	48	0.060±0.003	50
Galphimine E[1]	0.180±0.002	150	0.150±0.003	125	0.150±0.003	125	0.18±0.056	**150**	0.156±0.003	130	0.150±0.002	125
Glaucamine[1]	0.080±0.001	67	0.120±0.090	100	0.060±0.001	50	0.036±0.002	30	0.024±0.004	20	0.024±0.005	20
β-Sitosterol	0.033±0.002	28	0.106±0.013	87	0.140±0.005	**121**	0.016±0.005	14	0.033±0.002	28	0.024±0.007	20
Stigmasterol	0.060±0.007	50	0.108±0.056	89	0.030±0.004	25	0.024±0.004	20	0.036±0.005	30	0.042±0.003	35
Sitosteryl-3-O-β-D-glucopyranoside	0	0	0.009±0.006	8	0.039±0.009	33	0.036±0.006	30	0.024±0.001	20	0.012±0.004	10

O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment.
Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. n=2. [1]n=3. [2]n=4

Table 4.6 Mitogenic activities of some Terpenes (Continuation)

Terpenes	% Lymphocyte proliferation in the **presence** of Con A Concentration µg/ml											
	100		**10**		**1**		**0.1**		**0.01**		**0.001**	
	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%	O.D. ± SEM	%
Friedeline	0.015±0.004	16	0.086±0.005	90	0.270±0.006	285	0.139±0.056	145	0.003±0.0004	4	0.011±0.0002	12
Maytensifolin B	0.020±0.003	21	0.076±0.003	80	0.199±0.070	208	0.220±0.067	233	0.184±0.005	192	0.039±0.0004	41
Celaenodendrolide[1]	0.003±0.0005	4	0.040±0.001	42	0.108±0.050	113	0.049±0.054	51	0.161±0.059	167	0.160±0.001	167
3α-Hydroxy-7,24Z-dien-tirucalla-26-oic acid[1]	0.068±0.002	71	0.038±0.002	40	0.130±0.030	140	0.250±0.060	265	0.094±0.004	98	0.044±0.003	46
3-Oxo-7,24Z-dien-tirucalla-26-oic acid[1]	0	0	0.058±0.007	61	0.160±0.067	171	0.064±0.005	67	0.280±0.023	293	0.098±0.002	103
Epi-oleanolic acid[1]	0	0	0.190±0.002	202	0.218±0.056	228	0.320±0.070	**337**	0.304±0.004	317	0.040±0.00	42
3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F	0	0	0.110±0.001	119	0.160±0.089	171	0.169±0.052	171	0.329±0.045	**343**	0.203±0.067	212
23-Hydroxy-lanosta-7,9(11),24EZ-triene-3-one[2]	0	0	0.055±0.007	58	0.220±0.078	233	0.288±0.043	**301**	0.239±0.056	249	0.133±0.005	139
		0	0.048±0.003	50	0.320±0.090	332	0.329±0.023	**343**	0.235±0.060	245	0.123±0.056	129
5α-Lanosta-7,9(11),24EZ-triene-3α,24-diol[1]	0	29	0.068±0.005	71	0.150±0.060	160	0.318±0.012	**332**	0.133±0.050	139	0.108±0.004	113
Ent-8(14),15-Sandaracopimaradiene-2β,18-diol[1]	0.027±0.005	0	0.052±0.001	54	0.074±0.006	77	0.298±0.034	**311**	0.250±0.045	265	0.039±0.001	41
Ent-8(14),15-Sandaracopimaradiene-2α,18-diol[1]	0	8	0.130±0.04	137	0.190±0.004	202	0.390±0.050	**410**	0.053±0.050	56	0.114±0.002	119
		9	0.136±0.005	142	0.150±0.002	160	0.078±0.012	82	0.024±0.001	25	0.074±0.007	77
Galphimine C[1]	0.007±0.001	12.5	0.068±0.009	71	0.108±0.008	113	0.074±0.010	77	0.078±0.058	82	0.128±0.003	134
Galphimine D[1]	0.008±0.001	21	0.027±0.004	29	0.099±0.001	103	0.089±0.010	93	0.053±0.006	56	0.048±0.001	51
Galphimine E[1]	0.012±0.005	146	0.127±0.003	133	0.103±0.004	108	0.150±0.068	160	0.173±0.003	181	0.139±0.004	145
Glaucamine[1]	0.020±0.003	112	0.160±0.006	167	0.270±0.056	285	0.218±0.059	228	0.150±0.001	160	0.189±0.067	197
β-Sitosterol	0.140±0.056	29	0.088±0.002	92	0.099±0.034	104	0.148±0.098	155	0.083±0.004	87	0.104±0.078	109
Stigmasterol	0.107±0.077											
Sitosteryl-3-O-β-D-glucopyranoside	0.027±0.067											

O.D. Optical density was determinated at 550 nm. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment.
Untreated Control in the absence of Con A: Mean O.D. ± SEM = 0.12 ± 0.017. Untreated Control in the presence of Con A: Mean O.D. ± SEM = 0.096 ± 0.004. Concanavaline A was used at suboptimal concentration of 0.5 µg/ml, having a mitogenic response of 223 % as compared with untreated control. n=2. [1]n=3. [2]n=4.

activity. They have been found also in some species which are used in the treatment of malaria (Mata *et al.*, 1988; 1990). *Hintonia latiflora* (Rubiacea) a plant used for the treatment of malaria yielded 3-O-β-D-glucopyranosyl-23,24-dehydrocucurbitacin F which showed mitogenic activity in the absence or presence of Con A. Similar stimulant effects were observed for this cucurbitacin on macrophage superoxide-anion production.

Suppressive effects in the presence of mitogenic agent were observed with the following compounds: the picrotoxane: celaenodendrolide, the pentacyclic tritepenes: friedelin and maytensifolin B, the terpenes, galphimine D, galphimine E and glaucamine as well as the steroids: stigmasterol, and sitosteryl-3-O-β-D-glucopyranoside. If the effects of galphimine C are compared with galphimine D and galphimine E at 0.1 μg/ml, are compared it can be seen that presence of acetyl groups confers suppressive effects in this type of nor-secotriterpenes.

It has been reported that there are interactions between the immune system and central nervous system. It is likely that the brain is involved in the immune system regulation since activation of the immune system results in the elaboration of cytokines and antiinflammatory mediators; these mediators induce hypothalamic CRF, which stimulates the release of the same immunosuppressive molecules that mediate the response to stress. Stress induces proinflammatory cytokines which may contribute to both the pathogenesis of inflammatory diseases of unknown etiology and the progression of HIV infection to AIDS by activation of HIV replication (Blak, 1994). Thus, it is possible that suppressive effects observed in galphimine D, galphimine E and glaucamine can be extended to the CNS. This could be observed with galphimine B (Tortoriello & Ortega, 1993). Therefore, further studies *in vivo* will be necessary to confirm these preliminary findings. Then, if the activity is reproducible, they could useful for the treatment of stress or autoimmune diseases. The potential of these drugs is high since no toxicity to mammalian cells at high concentrations was observed.

4.3.2 Superoxide production

A total of 45 natural and semisynthetic compounds were evaluated *in vitro* for their ability to induce superoxide-anion production from J774 murine macrophage cell line

either in the presence or absence of zymosan as an approach to measure their immunomodulatory properties. Zymosan and PMA were used as control drugs during the assays *in vitro*. In experiments of co-stimulation, zymosan was used at concentration of 500 μg/ml, having a response corresponding to 20.36-times as compared with untreated control.

4.3.2.1 Alkaloids

Results in Table 4.7 showed that four of the nine alkaloids tested (Figure 4.3) enhanced macrophage superoxide release without addition of zymosan. They were, phaeanthine, stepharandine, acetyl-strictosidine and acetyl-strictosidine lactam, which have a maximum enhancement within the range of 1.5 - 5.5-fold higher than the control. Among them the most active alkaloids was acetyl-strictosidine lactam, having a maximum enhancement of 5.5-fold over control at 1 μg/ml.

In combination with zymosan, only phaeanthine and stepharandine showed synergistic effects of 24 and 24.9-fold above control at 0.1 and 1 μg/ml respectively. It has been reported that the BBIQ alkaloid tetrandrine scavenges superoxide anion in the hypoxanthine/xanthine oxidase (HX-XOD) system or by means of auto-oxidation of erythrocytes (Cao, 1996). If we compare phaeanthine with its analogue tetrandrine (Figure 4.2) it can be seen that stereochemistry at C-1' for these BBIQ alkaloids is a determinant for their capacity to enhance or suppress the release of macrophage superoxide.

It can be seen that strictosidine and strictosidine lactam had suppressive effects on macrophage superoxide release and that sustitution of their hydroxyl and phenolic groups for acetyl groups confers immunostimulant properties to both alkaloids. In addition, the acetyl derivates of strictosidine and strictosidine lactam were more active than the parent compounds in their antiprotozoal and cytotoxic properties.

The oxo-aporphine alkaloids liriodenine and 2-O,N-dimethyl-liriodendronine were found to suppress the release of superoxide from macrophages at all the concentrations tested. The analogue dicentrinone had a weak enhancement on macrophage superoxide of 1.2-

Table 4.7 Superoxide production of some Alkaloids

Alkaloid	% Macrophage superoxide production in **absence** of zymosan Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
Phaeanthine[2]	0.330 ± 0.09	108	0.57 ± 0.05	187	0.65 ± 0.06	**213**	0.55 ± 0.10	180
Stepharandine	1.280 ± 0.67	420	0.49 ± 0.09	161	0.65 ± 0.09	**213**	0.65 ± 0.13	**213**
Dicentrinone	2.320 ± 0.78	761	0.240 ± 0.12	79	0.39 ± 0.07	**128**	0.08 ± 0.01	26
Liriodenine	0.020 ± 0.004	5	0.004 ± 0.001	1.3	0.02 ± 0.006	5	0.25 ± 0.07	90
2-O,N-Dimethylliriodendronine	0.280 ± 0.56	20	1.050 ± 0.05	44	0.41 ± 0.07	**134**	0.05 ± 0.05	44
Strictosidine[1]	0.065 ± 0.11	2	0.330 ± 0.01	116	0.25 ± 0.06	82	0.41 ± 0.12	**134**
Acetyl-strictosidine[1]	0.010 ± 0.006	4	0.550 ± 0.002	108	0.40 ± 0.007	136	0.52 ± 0.006	**155**
Strictosidine lactam[1]	0.02 0± 0.005	5	0.007 ± 0.002	2	0.007 ± 0.001	2	0.02 ± 0.006	5
Acetyl-strictosidine lactam[2]	0.880 ± 0.040	255	0.410 ± 0.05	134	1.68 ± 0.07	**551**	1.13 ± 0.90	370
	Superoxide production in **presence** of zymosan Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
Phaeanthine[2]	6.12 ±1.2	2006	6.52 ± 1.18	2138	6.68 ± 1.14	2190	7.32 ± 1.11	**2400**
Stepharandine	6.82 ± 0.56	2236	5.65 ± 0.90	1852	7.62 ± 1.45	**2498**	5.87 ± 2.0	1924
Dicentrinone	3.81 ± 0.09	1249	1.27 ± 0.80	416	1.43 ± 0.09	467	0.55 ± 0.13	180
Liriodenine	1.27 ± 0.08	416	0.95 ± 0.10	311	1.35 ± 0.14	443	0.95 ± 0.10	311
2-O,N-Dimethylliriodendronine	3.41 ± 0.97	1118	1.82 ± 0.08	597	1.98 ± 0.19	649	1.43 ± 0.17	469
Strictosidine[1]	0.79 ± 0.087	659	0.08 ± 0.02	26	0.40 ± 0.07	131	0.08 ± 0.01	26
Acetyl-strictosidine[1]	0.79 ± 0.06	259	1.58 ± 0.08	518	0.79 ± 0.05	259	0.64 ± 0.08	210
Strictosidine lactam[1]	0.48 ± 0.07	157	0.63 ± 0.07	206	1.16 ± 0.030	352	1.27 ± 0.56	416
Acetyl-strictosidine lactam[2]	1.43 ± 0.09	469	0.63 ± 0.10	206	1.43 ± 0.06	469	1.5 ± 0.23	492

nM $O_2^{\cdot-}$: nanomoles of superoxide-anion release from macrophages. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control in the absence or presence of zymosan: nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. In experiments of co-stimulation, zymosan was used at suboptimal concentration of 500 µg/ml, having a response of 2036 % as compared with untreated control. n=2. [1]n=3. [2]n=4.

fold over the control at 1 μg/ml but at other concentrations displayed suppressive effects.

4.3.2.2 Quinones

Hydroxynaphthoquinone analogues known for their antitumour and antiprotozoal activity have the capacity to reduce coenzyme Q (ubiquinone) which has a wide range of functions in protozoan metabolism mainly in the electron transfer system (Ellis, 1994). This mechanism of action has been proposed to explain the broad-spectrum antiprotozal activity of the synthetic hydroxynaphthoquinone atovaquone (Hudson, 1993). An alternative mechanism involves the generation of free radicals during interaction of the drug with the respiratory chain, this theory has been proved by some naphthoquinones against *L. donovani* (Croft *et al.*, 1985) and *T. cruzi* (Aldunante & Moreno, 1993). By virtue of their structural analogy, the quinones tested (benzo [g]isoquinoline-5,10dione, 1-hydroxy-benzoisochromanquinoneand1-acetyl-benzoisochromanquinone)areexpected either to inhibit parasite growth by causing disruption in their mitochondrial electron transport chain or generating oxygen radicals producing stress and death of the parasite.

Six quinones (Figure 4.4) were evaluated *in vitro* for their ability to induce macrophage superoxide release. Results in Table 4.8 revealed that all quinones tested enhanced macrophage superoxide production without addition of zymosan. The benzoquinones benzo[g]isoquinoline-5,10-dione and 1-hydroxy-benzoisochromanquinone had a maximum enhancement of 1.34 and 2.88 times over control at 10 μg/ml, and at lower concentrations both quinones were found to have immunosuppressive effects. Furthermore, the acetyl derivative 1-acetyl-benzoisochromanquinone showed stimulatory effects at concentrations equal or lower than 10 μg/ml, having a maximum effect of 3.97-fold above control.

The anthraquinones picramnioside A and picramnioside C had immunostimulant effects at concentrations equal or lower than 1 μg/ml, having a maximum response of 1.6 and 3.1-times over control at 0.1 and 1 μg/ml respectively. Aloe-emodin showed stimulant response at 1 μg/ml and suppressive effects at 0.1 μg/ml.

In assessing the structure-activity relationships it can be seen that 1-acetyl-

Table 4.8 Superoxide production of some Quinones

	Superoxide production of in **absence** of zymosan Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	n M $O_2^{\cdot-}$ ± SEM	%
Benzo[g]isoquinoline-5,10-dione[2]	0.02 ± 0.006	5	0.88 ± 0.20	**288**	0.33 ± 0.06	108	0.007±0.001	2.3
1-Hydroxy-benzoisochromanquinone[2]	0.17 ± 0.07	56	0.41± 0.04	**134**	0.25 ± 0.04	82	0.09 ± 0.01	31
1-Acetyl-benzoisochromanquinone[2]	0.88 ± 0.05	288	1.21 ± 0.07	**397**	0.49 ± 0.04	161	0.81± 0.06	266
Aloe-emodin[1]	0.02 ± 0.004	5	0.25 ± 0.07	82	0.73 ± 0.18	**239**	0.08 ± 0.007	26
Picramnioside A[1]	0.81 ± 0.10	265	0.16 ± 0.01	119	0.41± 0.07	134	0.49 ± 0.01	**161**
Picramnioside C	0.49 ± 0.07	161	0.41± 0.08	134	0.97 ± 0.07	**318**	0.81± 0.07	265
	Superoxide production in **presence** of zymosan Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
Benzo[g]isoquinoline-5,10-dione[2]	0.40 ± 0.08	129	0.24 ± 0.06	79	0.48 ± 0.07	157	0.08 ± 0.006	26
1-Hydroxy-benzoisochromanquinone[2]	0.87 ± 0.02	285	1.19 ± 0.55	390	0.71± 0.01	233	0.03 ± 0.007	10
1-Acetyl-benzoisochromanquinone[2]	0.24 ± 0.08	79	1.11± 0.16	364	0.71± 0.09	233	0.24 ± 0.08	79
Aloe-emodin[1]	0.08 ± 0.01	26	0.40 ± 0.03	131	0.80 ± 0.03	26	0.55 ± 0.04	180
Picramnioside A[1]	1.43 ± 0.80	469	0.4 ± 0.04	131	1.19 ± 0.55	390	1.11± 0.045	364
Picramnioside C	0.64 ± 0.05	210	1.35 ± 0.12	443	1.03 ± 0.07	338	1.27 ± 0.12	416

nM $O_2^{\cdot-}$: nanomoles of superoxide. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control in the absence or presence of zymosan: nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. In experiments of co-stimulation, zymosan was used at suboptimal concentration of 500 µg/ml, having a response of 2036 % as compared with untreated control.
n=2. [1]n=3. [2]n=4

benzoisochromanquinone was more active than its parent compound. Similar effects were observed for both quinones in their mitogenic activities. Aloe-emodin was less active than picramnioside A and picramnioside C in the stimulation of macrophages. Similar effects were observed on lymphocyte proliferation for the three quinones. Further, picramnioside C was more active than picramnioside A. These results contrast with those obtained for their mitogenic activity. Thus, structural requirement for mitogenic and macrophage superoxide production differ for these quinones.

4.3.2.3 Flavonoids

Eight flavonoids (Figure 4.5) were evaluated for their effects on macrophage superoxide release. Table 4.9 revealed that apart from chrysin, all flavonoids tested were able to enhance macrophage superoxide production in the range of 1.61 to 4.20 times over the control at concentrations equal or lower than 10 μg/ml. Among these the strongest enhancement was observed with pinocembrine, 7-methyl-luteolin, podocarpusflavone A and podocarpusflavone B, having a maximum enhancement of 3-fold above control at concentration equal or lower than 1 μg/ml. In combination with zymosan only podocarpusflavone B showed synergistic effect corresponding to 33.8 times higher than control at 10 μg/ml.

Comparison of flavanones pinocembrin with pinostrobin indicated that the presence of a phenolic group at C-7 enhances the release of superoxide from macrophages in this series of flavanones. The same structural requirements were observed for the antimicrobial activities reported for these two flavanones (Rojas *et al.*, 1992). Thus it is possible that the antimicrobial activity observed in these flavanones is related to their ability to enhanced macrophage superoxide. In contrast, pinocembrin and pinostrobin were found to inhibit normal and mitogenic-induce lymphocyte proliferation. Thus, it can be seen that the same molecule can give different response on different mechanisms of the immune system.

In comparing the flavones 7-methyl-luteolin and chrysin are compared can be seen that the methoxy group at C-7 stimulates macrophage superoxide production. Luteolin from *Thymus vulgaris* was found to possess antioxidant properties in the

Table 4.9 Production of superoxide of some Flavonoids

Flavonoids	Superoxide production of in **absence** of zymosan Concentration µg/ml **100** nM $O_2^{\cdot-}$ ± SEM	%	**10** nM $O_2^{\cdot-}$ ± SEM	%	**1** nM $O_2^{\cdot-}$ ± SEM	%	**0.1** nM $O_2^{\cdot-}$ ± SEM	%
Pinocembrin[1]	0.23 ± 0.56	76	0.17 ± 0.05	56	1.21 ± 0.21	**397**	1.13 ± 0.55	370
Pinostrobin[1]	0.49 ± 0.11	161	1.13 ± 0.32	**370**	0.73 ± 0.23	239	0.57 ± 0.07	187
Chrysin[1]	0.88 ± 0.05	288	0.095 ± 0.01	31	0.02 ± 0.01	5	0.02 ± 0.007	5
7-Methyl-luteolin[1]	0.30 ± 0.50	98	0.41 ± 0.20	134	0.97 ± 0.15	**318**	0.65 ± 0.10	213
Quercetin	0.97 ± 0.23	318	0.57 ± 0.08	187	0.25 ± 0.08	82	0.65 ± 0.06	**213**
Podocarpusflavone B	2.55 ± 0.45	53	1.28 ± 0.25	**420**	0.41 ± 0.05	134	0.73 ± 0.12	239
Podocarpusflavone A	1.75 ± 0.98	574	0.33 ± 0.06	108	0.17 ± 0.06	56	0.73 ± 0.08	**239**
Amentoflavone	0.73 ± 0.11	239	0.25 ± 0.07	82	0.49 ± 0.17	**161**	0.08 ± 0.002	26
	Superoxide production in **presence** of zymosan Concentration µg/ml **100** nM $O_2^{\cdot-}$ ± SEM	%	**10** nM $O_2^{\cdot-}$ ± SEM	%	**1** nM $O_2^{\cdot-}$ ± SEM	%	**0.1** nM $O_2^{\cdot-}$ ± SEM	%
Pinocembrin[1]	2.62 ± 0.88	859	1.43 ± 0.33	469	0.95 ± 0.44	311	1.43 ± 0.26	469
Pinostrobin[1]	2.14 ± 0.85	702	2.06 ± 0.45	675	1.59 ± 0.44	521	1.75 ± 0.9	574
Chrysin[1]	1.11 ± 0.07	364	1.19 ± 0.18	390	0.55 ± 0.05	180	0.32 ± 0.06	105
7-Methyl-luteolin[1]	2.78 ± 0.66	911	0.55 ± 0.09	180	1.43 ± 0.22	469	1.43 ± 0.12	469
Quercetin	1.51 ± 0.67	495	2.22 ± 0.23	728	0.79 ± 0.06	259	0.08 ± 0.01	28
Podocarpusflavone B	4.13 ± 2.33	1354	1.03 ± 0.76	**3384**	2.38 ± 0.66	780	0.63 ± 0.15	206
Podocarpusflavone A	3.17 ± 1.14	1039	2.06 ± 0.89	675	1.67 ± 0.03	547	1.35 ± 0.35	443
Amentoflavone	1.43 ± 0.80	469	0.79 ± 0.13	259	0.87 ± 0.07	285	1.35 ± 0.32	443

nM $O_2^{\cdot-}$: nanomoles of superoxide anion. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control in the absence or presence of zymosan: nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. In experiments of co-stimulation, zymosan was used at suboptimal concentration of 500 µg/ml, having a response of 2036 % as compared with untreated control.
n=2. [1]n=3

xanthine/xanthine oxidase system (Haraguchi *et al.*, 1996). It is likely that the a hydroxy group in position 7 is an important structural requirement for radical scavenging of flavones. In comparing flavanones are compared with flavones it can be seen that for both type of flavonoids the double bound at C-2 / C-3 in conjugation with a keto-function in position 4 can exert stimulant or suppressive effects on superoxide production. The flavone chrysin showed scavenging properties in a wide range of concentrations, and it would be of interest to test this compound for anti-inflammatory properties *in vivo*, because of its low cytotoxicity properties,

It has been reported that unmetabolized flavonoids differ in their effects on free radical production of PMNs and their radical scavenging potencies as compared with their metabolites (Merfort *et al.*, 1996; Mathiesen *et al.*, 1995). Preliminary studies have been shown that flavonoids are absorbed only poorly after oral dosage, but they are extensively degraded by the intestinal microflora. These studies have shown that 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenyl-acetic acid, and 3-(4-hydroxyphenyl)-propionic acid, are metabolites which arise from quercetin glycosides, flavones and probably procyanidins by the human intestinal microflora. They have been tested for their effects on oxygen radical production by human PMNs stimulated with N- formyl-methionyl-leucyl-phenylalanine (FMLP) or opsonized zymosan. 3,4-Dihydroxyphenylacetic acid reduced chemiluminescence in PMNs stimulated by FMLP and opsonized zymosan, whereas, 3-(4-hydroxyphenyl)-propionic acid enhanced chemiluminescence in PMNs stimulated by FMLP and opsonized zymosan. Thus, it is possible that effects *in vivo* for flavonoids could differ from those *in vitro*. Thus, further studies *in vivo* with the flavonoids tested will be necessary to determine their immunomodulatory effects.

Quercetin had suppressive effects at 1µg/ml, but at other concentrations showed stimulant effects. The radical scavenging properties of quercetin on PMNs have been reported (Merfort *et al.* 1996) and are in agreement with the results found in this study. Quercetin possesses a number of biological activities (Ngomuo & Jones, 1996: Formica & Regelson, 1995).

If biflavonoids podocarpusflavone B, podocarpusflavone A and amentoflavone are compared it can be seen that substitution of phenolic groups for methoxy groups enhanced macrophage superoxide release, with podocarpusflavone B being the most active among the biflavonoids. Podocarpusflavone B showed a maximum response of 4.2 times above culture control at 10 μg/ml. Finally, amentoflavone has been reported to be a useful tool for exploring the mechanism of intracellular signalling pathways (Lee *et al.*, 1996).

4.3.2.4 Coumarins

The *in vitro* effects of 11 coumarins (Figure 4.6) on the release of superoxide from macrophages was determined. Results in Table 4.10 showed that eight coumarins were able to enhance spontaneous production of macrophage superoxide within the range of 1.6 - 4.20 times above untreated control at concentrations equal to or lower than 1 μg/ml. The strongest response was given by 5-O-[β-D-tetraacetoxy-glucopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin, having a maximum response of 4.20-fold over the control at 1 μg/ml. In combination with zymosan, none of the 4-phenylcoumarins tested showed a co-stimulatory response.

Suppressive effects at all the range of concentrations tested were observed for the glucoside, 5-O-[β-D-glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, the galactoside, 5-O-[β-D-galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin, and the acetyl-aglycone, 5,3',4'-triacetoxy-7-methoxy-4-phenylcoumarin. Suppressive and stimulant effects were observed with scopoletin which possess stimulant effects at 1 μg/ml, and suppressive response at other concentrations. Comparison of glucoside and galactoside with their acetyl and methyl-derivatives indicated that hydroxyl groups are determinants for suppressive effects on macrophage superoxide release. The substitution of hydroxyl groups in the sugar portion of galactoside and the glucoside for acetyl groups confer stimulant effects on macrophage superoxide release. In a similar manner, the aglycone 5,3',4'-trihydroxy-7-methoxy-4-phenylcoumarin showed suppressive effects and its acetyl derivative possess stimulant effects. The acetyl- and methyl-glucoside were more active than the galactoside and its acetyl- and methyl-derivatives on the activation of macrophages for superoxide release.

Table 4. 10 Superoxide production of some Coumarins

Coumarin	Superoxide production in **absence** of zymosan							
	Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	2 ± 0.5	656	0.57 ± 0.04	**187**	0.02 ± 0.007	5	0.33 ± 0.01	108
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	1.75 ± 0.67	574	0.57 ± 0.07	**187**	0.25 ± 0.07	82	0.17 ± 0.07	56
5-O-[β-D-Tetraacetoxy-glucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	2.48 ± 1.0	813	1.21 ± 0.35	**397**	0.49 ± 0.07	161	0.81 ± 0.11	265
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	1.84 ± 0.69	603	1.13 ± 0.17	**370**	0.88 ± 0.05	288	0.41 ± 0.13	134
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-7-methoxy-4-phenylcoumarin	0.73 ± 0.15	239	0.88 ± 0.12	288	0.97 ± 0.17	**318**	0.97 ± 0.11	**318**
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-7-methoxy-4-phenylcoumarin	0.09 ± 0.02	31	0.41 ± 0.12	134	0.77 ± 0.11	**252**	0.09 ± 0.01	31
5,3',4'-Tri-hydroxy-7-methoxy-4-phenylcoumarin	6.28 ± 2.33	2059	1.13 ± 0.13	**370**	0.49 ± 0.06	160	0.49 ± 0.05	160
5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin	2.24 ± 0.97	734	0.73 ± 0.13	239	0.25 ± 0.01	82	0.06 ± 0.001	20
4',5'-Dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin	1.44 ± 0.05	472	0.88 ± 0.11	288	0.81 ± 0.03	266	1.21 ± 0.03	**397**
5-O-[β-D-Tetraacetoxy-galactopiranosil]-4'-acetoxy-7-methoxy-4-phenylcoumarin	0.73 ± 0.10	239	1.05 ± 0.05	344	1.28 ± 0.45	**420**	0.97 ± 0.13	318
Scopoletin	0.49 ± 0.09	161	0.095 ± 0.04	31	0.57 ± 0.07	**187**	0.25 ± 0.06	82

nM $O_2^{\cdot-}$: nanomoles of superoxide anion. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated control: nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. n=2

Table 4.10 Superoxide production of some coumarins (Continuation)

Coumarins	Superoxide production in **presence** of zymosan Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
5-O-[β-D-Glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	1.98 ± 0.90	649	1.51 ± 0.08	31	1.58 ± 0.67	518	0.32 ± 0.06	105
5-O-[β-D-Galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin	2.14 ± 0.78	702	1.43 ± 0.33	469	1.35 ± 0.70	443	0.03 ± 0.01	10
5-O-[β-D-Tetraaacetoxy-glucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	2.38 ± 0.99	780	1.51 ± 0.56	495	1.19 ± 0.04	390	2.06 ± 0.07	675
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin	2.54 ± 1.11	833	1.98 ± 0.67	649	1.11± 0.02	364	1.03 ± 0.44	338
5-O-[β-D-Tetraacetoxy-galactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin	1.11 ± 0.19	364	1.43 ± 0.34	469	1.51 ± 0.44	489	1.27 ± 0.27	416
5-O-[β-D-Glucopyranosyl]-7,3',4'-trimethoxy-7-methoxy-4-phenylcoumarin	0.06 ± 0.01	20	1.66 ± 0.66	544	0.03 ± 0.005	10	0.24 ± 0.06	79
5-O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-7-methoxy-4-phenylcoumarin	0.16 ± 0.06	52	2.3 ± 0.45	754	0.55 ± 0.04	180	0.05 ± 0.007	18
5,3',4'-Trihydroxy-7-methoxy-4-phenylcoumarin	5.16 ± 1.12	1692	1.90 ± 0.56	623	1.90 ± 0.07	623	1.59 ± 0.78	33
5,3',4'-Triacetoxy-7-methoxy-4-phenylcoumarin	3.17 ± 0.96	1039	1.58 ± 0.78	518	1.27 ± 0.56	416	0.71 ± 0.11	233
4',5'-Dihidroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin	2.14 ± 0.06	702	1.67 ± 0.56	547	1.51 ± 0.05	495	1.75 ± 0.08	574
Scopoletin	1.27 ± 0.77	416	1.58 ± 0.67	518	0.95 ± 0.04	311	0.16 ± 0.02	52

nM $O_2^{\cdot-}$: nanomoles of superoxide anion. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated control : nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. In experiment of co-stimulation, zymosan was used at suboptimal concentration of 500 µg/ml, having a response of 2036 % as compared with untreated control. n=2.

These results contrast with those obtained for their mutagenic activity. The results could be explained by the fact that lymphocytes and macrophages have different membrane receptors, thus one compound could give a different response in different types of immune cells. Thus it is possible that the glucoside is better recognized by the macrophage membrane than the galactoside.

It has been reported that monocytes / macrophages possess cell surface receptors which are able to recognize specific carbohydrates such as mannose / fucose and β-glucan, and that stimulation of the receptor by a ligand induces various cellular responses, e.g. phagocytosis production of leukocytes, and the release of lysosomal enzymes (Matsumoto *et al.*, 1990; 1993). In addition, peritoneal macrophages of mice have receptors that specifically bind to C3b, this allows the phagocytes to recognize their targets (Wang et al., 1993).

4.3.2.5 Terpenes

Eleven terpenes (Figure 4.7) were evaluated for their ability to enhance the production of macrophage superoxide-anion. Results in Table 4.11 showed that 5 terpenes were able to enhance macrophage superoxide production without addition of zymosan. They were, 3α-hydroxy-7,24Z-dien-tirucalla-26-oic acid, 3-oxo-7,24Z-dien-tirucalla-26-oic acid, 3-O-β-D-23,24-dihydrocucurbitacin F, 5α-lanosta-7,9(11)-24EZ-triene-3α,24-diol and stigmasterol, having a maximum enhancement in the range of 1.34 - 2.39 times above control at concentrations equal or lower than 1 μg/ml. Among them, 3α-hydroxy-7,24Z-di-en-tirucalla-26-oic acid, 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F, and stigmasterol gave the strongest enhancement, having a maximum response of 2.13, 2.39 and 2.13-folds above control at 0.1 μg/ml. In combination with zymosan none of the terpenes tested showed a co-stimulatory response at the range of concentrations tested.

Six terpenes showed suppressive effects at concentrations equal or lower than 1μg/ml, they were, maytensifolin B, epi-oleanolic acid, 23-hydroxy-5α-lanosta-7, 9 (11)-24Z-triene-3-one, ent-8(14),15-sandaracopimaradine-2β-18-diol, ent-8(14),15-sandaracopimaradine-2α,18-diol and the sitosteryl-3-O-β-D-glucopyranoside.

Table 4.11 Production of superoxide of some Terpenes

Terpenes	Production of superoxide in **absence** of zymosan Concentration µg/ml							
	100		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
Maytensifolin B	0.013 ± 0.005	4	0.095 ± 0.01	31	0.013 ± 0.001	4	0.016 ± 0.005	5
3α-Hydroxy-7,24Z-dien-tirucalla-26 oic acid[1]	0.33 ± 0.05	108	0.49 ± 0.06	161	0.41± 0.08	134	0.65 ± 0.06	**213**
3-Oxo-7,24Z-dien-tirucalla-26 oic acid[1]	0.73 ± 0.12	239	0.41± 0.06	134	0.17 ± 0.05	56	0.49 ± 0.09	**161**
Epi-oleanolic acid[1]	0.02 ± 0.004	6	0.41± 0.05	**134**	0.01± 0.005	3	0.14 ± 0.06	46
3-[O-β-D-glucopyranosyl]-23,24-dihydrocucurbitacin F	1.52 ± 0.09	498	0.81 ± 0.08	265	0.65 ± 0.06	213	0.73 ± 0.05	**239**
23-Hydroxy-5α-lanosta-7,9(11),24-triene-3-one[1]	0.25 ± 0.09	82	0.095 ± 0.01	31	0.25 ± 0.03	82	0.33 ± 0.08	108
Lanosta-7,9(11),24-triene-3α,24-diol[1]	0.33 ± 0.06	108	0.25 ± 0.04	82	0.49 ± 0.07	161	0.57 ± 0.07	**187**
Ent-8(14),15-Sandaraco-pimaradiene-2β,18-diol[1]	0.25 ± 0.07	82	0.095 ± 0.01	31	0.016 ± 0.001	5	0.08 ± 0.02	26
Ent-8(14),15-Sandaraco-pimaradiene-2α,18-diol[1]	2.30 ± 0.90	754	0.006 ± 0.001	2.1	0.016 ± 0.001	5	0.016 ± 0.007	5
Stigmasterol	0.25 ± 0.06	82	0.33 ± 0.002	108	0.41± 0.12	134	0.65 ± 0.10	**213**
Sitosteryl-3-O-β-D-glucopyranoside	0.33 ± 0.08	108	0.33 ± 0.02	108	0.06 ± 0.008	19	0.25 ± 0.004	82

nM $O_2^{\cdot-}$: nanomoles of superoxide anion. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated Control : nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. n=2. [1]n=3.

Table 4. 11 Production of superoxide of some Terpenes (Continuation)

Terpenes	Production of superoxide in **presence** of zymosan Concentration µg/ml **100**		**10**		**1**		**0.1**	
	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%	nM $O_2^{\cdot-}$ ± SEM	%
Maytensifolin B	0.32 ± 0.07	105	1.90 ± 0.17	623	0.71± 0.06	233	0.63 ± 0.10	206
3α-Hydroxy-7,24Z-dien-tirucalla-26 oic acid[1]	1.19 ± 0.11	390	1.67 ± 0.08	547	1.51± 0.44	495	1.35 ± 0.13	443
3-Oxo-7,24Z-dien-tirucalla-26 oic acid[1]	1.35 ± 0.45	443	0.55 ± 0.07	180	0.71± 0.09	233	0.24 ± 0.04	78
Epi-oleanolic acid[1]	0.95 ± 0.12	311	0.71 ± 0.07	233	0.79 ± 0.09	259	0.24 ± 0.08	78
3-[O-β-D-glucopyranosyl]-23,24-dihydrocucurbitacin F	1.82 ± 0.07	597	1.43 ± 0.07	469	1.03 ± 0.07	338	0.40 ± 0.001	131
23-Hydroxy-5α-lanosta-7,9(11),24-triene-3-one[1]	0.71 ± 0.06	233	0.79 ± 0.06	259	1.11 ± 0.056	364	0.95 ± 0.13	311
Lanosta-7,9(11),24EZ-triene-3α,24-diol[1]	0.95 ± 0.06	311	0.014 ± 0.007	4.6	2.54 ± 0.44	834	1.35 ± 0.45	443
Ent-8(14),15-Sandaraco-pimaradiene-2β,18-diol[1]	1.27 ± 0.44	416	0.63 ± 0.16	206	0.48 ± 0.05	157	0.79 ± 0.07	259
Ent-8(14),15-Sandaraco-pimaradiene-2α,18-diol[1]	1.98 ± 0.08	649	0.87 ± 0.06	285	1.27 ± 0.12	416	1.19 ± 0.40	390
Stigmasterol	0.95 ± 0.07	311	1.75 ± 0.89	574	1.82 ± 0.60	597	0.0016 ± 0.0007	0.52
Sitosterol-3-O-β-D-glucopyranoside	0.03 ± 0.007	10	0.71 ± 0.05	232	0.11 ± 0.02	36	0.079 ± 0.002	26

nM $O_2^{\cdot-}$: nanomoles of superoxide anion. Values represent the mean of at least two independent determinations. Each concentration was tested by triplicate in each experiment. Untreated control : nM $O_2^{\cdot-}$ ± SEM = 0.305 ± 0.015. In experiments of co-stimulation zymosan was used at suboptimal concentration of 500 µg/ml, having a response of 2036 % as compared with untreated control.

n=2. [1]n=3.

and *in vivo* with this terpenes are required in order to complete their immunological effects.

Results in Tables 4.11 show that epi-oleanolic acid and 7,15-sandaracopimaradiene-2α,18-diol enhanced normal proliferation rate of splenocytes, but both compounds inhibited macrophage superoxide production, at the same concentration. This finding highlights again, that the same molecule at the same concentration can give different effects on different cells or mechanisms involved in the immune system.

4.3.3 General discussion

Results in Tables 4.1 to 4.11 revealed that effects on mitogenic activity and superoxide production occur in a narrow zone of concentrations for most of the tested extracts and pure compounds, and does not appear to be correlated with any particular type of extract or compound. Some compounds such as strictosidine lactam, ent-8(14),15-sandaracopimaradiene-2α,18-diol and the epi-oleanolic acid, possess two ways of immunomodulation in different immune cells. This could be explained by the fact that macrophages and lymphocytes have different membrane receptors and thus one compound could give a different response in different types of immune cells (Matsumoto *et al.*, 1993). In this respect, a number of tested compounds were found to stimulate normal and Con A induced lymphocyte proliferation, but they possess suppressive effects on macrophage superoxide production, such as the alkaloid strictosidine lactam, the triterpene epi-oleanolic acid and the diterpene ent-8(14),15-sandaracopimaradiene-2α,18-diol. The capacity of these compounds to enhance lymphocyte proliferation without undergoing redox cycling can be applied to control the cytotoxicity by producing free radicals without causing much damage to T lymphocytes and macrophages.

A number of compounds had suppressive effects on normal and mitogenic-induce lymphocyte proliferation, and enhanced macrophage superoxide release, such as the flavanones, pinocembrin and pinostrobin , the 4-phenylcoumarins: 5-O-[β-D-tetraacetoxy-glucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin, 5-O-[β-D-tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin, 5-O-[β-D-glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin, 5-O-[β-D-galactopyranosyl]-

7,3',4'-trimethoxy-4-phenylcoumarin, and 5-O-[β-D-tetraacetoxy-galactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin. On the other hand, some compounds such as quercetin acted as immunopotentiators or immunosuppressors at different concentrations on normal and mitogenic activity as well as macrophage superoxide production at different concentrations. The oxo-aporphine alkaloids, liriodenine and 2-O,N-dimethyl-liriodendronine, the triterpene, maytensifolin B; the picrotoxane, celaenodendrolide; and the steroids, β-sitosterol and sitosteryl-3-O-β-D-glucopyranoside were found to possess suppressive effects on normal and mitogenic-induce lymphocyte proliferation as well as macrophage superoxide release. If the results are reproducible in experimental models and, if these compounds are well tolerated by patients, then they may be developed into alternative coadjuvants for the treatment of disorders caused by an exaggerated or unwanted immune response, such as autoimmune diseases, inflammation and transplant rejection.

It should be emphasized, that lymphocyte populations studied were not homogeneous and that different lymphocyte subpopulations may react in different ways in the presence of the compounds. More detailed studies will be required with pure subpopulations of lymphocytes as well as studies of co-stimulation with other mitogenic agents to determine specificity of action and mechanism of immunomodulation of tested compounds. Furthermore, whether the tested compounds affect B-cell proliferation or other subpopulations or different pathways of signal transduction is not known yet. Thus, further studies of co-stimulation with LPS and other mitogenic agents will be required to determine the immunospecificity of tested compounds.

It has been reported that the function of T lymphocytes could be regulated by macrophages, inversely the function of macrophages could be activated by the lymphokines produced by T lymphocytes (Wang *et al.*, 1992). As, the interaction of macrophage and T lymphocytes is a complex phenomen, the question of the main activation pathway remains an interesting problem to be solved for tested compounds. Nevertheless, the present data at least indicate that there may be some connections between the activation of macrophages and enhancement of proliferation of lymphocytes both working together in addition to other factors such as humoral response, and

production of cytokines (e.g., IL-12) for the killing of pathogens in the host (Lamont & Adorini, 1996; Crutcher et al., 1995).

From the results obtained in this study, it can be observed that there are some correlations between mitogenic activity and macrophage superoxide production activities *in vitro*. This relationship could be extended to antiprotozoal, cytotoxic and antimicrobial activities for the most of the active principles isolated from plants studied. For example, phaenthine the active compound from *T. patens* which possesses antiprotozoal and cytotoxic properties was found to stimulate normal and mitogenic-induce lymphocyte proliferation and macrophage superoxide release. The same effects were observed for stepharandine from *S. dinklagei*; aloe-emodin, picramnioside A and picramnioside C from *P. antidesma;* podocarpusflavone B, 3α-hydroxy-7,24Z-dien-tirucalla-26-oic acid, 3-oxo-hydroxy-7,24Z-dien-tirucalla-26-oic acid and epi-oleanolic acid from *C. mexicanum;* 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacine F, 7-methyl-luteoline, 5-O-[β-D-glucopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin and 5-O-[β-D-galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin from *H. latiflora*; quercetin from *G. glauca*. 23-hydroxy-5α-lanosta-7,9(11), 24EZ-triene-3-one, 5α-lanosta-7,9(11), 24EZ-triene-3α,24-diol, ent-8(14),15-sandaracopimaradiene-2β, 18-diol, and ent-8(14),15-sandaracopimaradiene-2α, 18-diol from *G. rhopalocarpa*; and benzo[g]isoquinoline-5,10-dione and 1-hydroxy-benzoisochromanquinone from *C. camponutans*.

The key to many anti-protozoal defence mechanisms lies in the activation of macrophages, which requires an apparent synergism between two signals that operate sequentially, one a macrophage activation factor and the second usually supplied by a bacterial cell wall or cell wall component, such as LPS. A number of cytokines can provide the first signal but there is no doubt that a central role is played by IFN-γ, a molecule that has been implicated in virtually every parasitic infection. Thus a major mechanism involves the induction by IFN-γ in conjunction with other cytokines, of various low molecular weight and potentially toxic factors. It has been proposed that IFN-γ produced by T cells activates macrophages to secrete TNF which in turn sensitizes the same or other macrophages to secrete reactive oxygen species that rapidly destroy

parasites through a process of lipid peroxidation. However, several observations suggested that alternative mechanisms may be involved and attention has turned recently to the potential role of NO in the destruction of protozoal parasites.

4.4 Summary

In summary, from the 16 plant-extracts screened for mitogenic activity the most active was the methanolic extract of *G. glauca* (Figure 4.8), which had a maximum enhancement in the range of 1.92 - 2.17-fold higher than control at a concentration range of 1-10 μg/ml. In combination with Con A, methanolic extracts of *C. mexicanum*, *G. glauca*, *S. dinklagei*, and *T. patens* showed the strongest synergistic effect on activation of T-lymphocytes, having a maximum response in the range of 3.17 to 2.96 times above control at concentration range of 0.1 - 0.001 μg/ml.

From the 56 compounds screened for mitogenic activity, 4 compounds: aloe-emodin, chrysin, 7-methyl-luteolin, and 3α-hydroxy-7,24Z-dien-tirucalla-26-oic acid (Figure 4.9) were the most effective on the stimulation of normal proliferation of lymphocytes in comparison with control having a maximum enhancement corresponding to 2-fold over control at concentration range of 1 to 10 μg/ml. However, these compounds did not show co-stimulatory effect on the activation of T-cells. Thus, further co-stimulatory studies with other mitogenic agents will be required to understand their mechanism of immunomodulation. In combination with Con A, 26 compounds showed synergistic activity on the stimulation of T-lymphocytes having a maximum co-stimulatory response in the interval of 3 - 5.5-fold over control at concentration range of 0.1 - 0.001 μg/ml. The strongest synergistic effects were observed with the following compounds, the aporphine alkaloid, stepharandine, the quinones, benzo [g]isoquinoline-5,10-dione and 1-acetyl-benzoisochromanquinone, the coumarins, 5-O-[β-D-galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin and scopoletin, and the terpene galphimine C, which have a maximum co-stimulatory effect in the intervale of 4 - 5 times over control at concentration range of 0.001 - 1 μg/ml (Figure 4.9). Immunosuppressive effects on normal and mitogenic-induced proliferation of T-cells were observed with 20 compounds at concentrations equal or lower than 10 μg/ml, such as pinocembrin, pinostrobin, liriodenine, maytensifolin B, sitosteryl-3-O-β-D-glucopyranoside (Figure 4.11).

Toxicity of these compounds at concentrations lower than 10 μg/ml was ruled out by the observation of viability of the cells at the same concentrations by trypan blue exclusion. Whether these compounds suppress B-lymphocytes is not known yet. Thus further studies of co-stimulation with LPS will be necessary in order to determine their effects on humoral response.

In the case of macrophage superoxide production, from the 45 samples tested for superoxide-anion production, 31 compounds were able to promote spontaneous superoxide release from macrophages in the range of 2 to 5.97-times over untreated control at concentrations equal or lower than 1 μg/ml. Among them, 9 compounds gave the strongest enhancement corresponding to 2 - 5-fold above control. They were: acetyl-strictosidine lactam, picramnioside C, 5-O-[β-D-tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin, 5-O-β-D-glucopyranosyl-7,3',4'-trimetoxy-4-phenylcoumarin, 4',5'-dihidroxy-7-methoxy-4-phenyl-5,2'-oxydocoumarin, 3-O-β-D-glucopyranosyl-23,24-dihydroxycucurbitacin F, 3α-hydroxy-7,24Z-dien-tirucalla-26-oic acid, 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, ent-8(14),15-sandaracopimaradiene-2β-18-diol and ent-8(14),15-sandaracopimaradiene-2α-18-diol (Figures 4.10 to 4.12). In combination with zymosan, only phaenthine, stepharandine and podocarpusflavone B showed a co-stimulatory response corresponding to 24, 24.9 and 33.8-folds over control at 0.1, 1 and 10 μg/ml respectively (Figure 4.12). Inhibition of macrophage superoxide was observed in all the concentrations tested for liriodenine, 2-O-N-dimethyl-liriodenine, strictosidine lactam, chrysin, maytensyfolin B, epi-oleanolic acid, 23-hydroxy-5α-lanostan-7,9(11),24EZ-triene-3-one, ent-8(14),15-sandaracopimaradiene-2α-18-diol, ent-8(14),15-sandaracopimaradiene-2β-18-diol, sitosteryl-3-O-β-D-glucopyranoside (Figure 4.13).

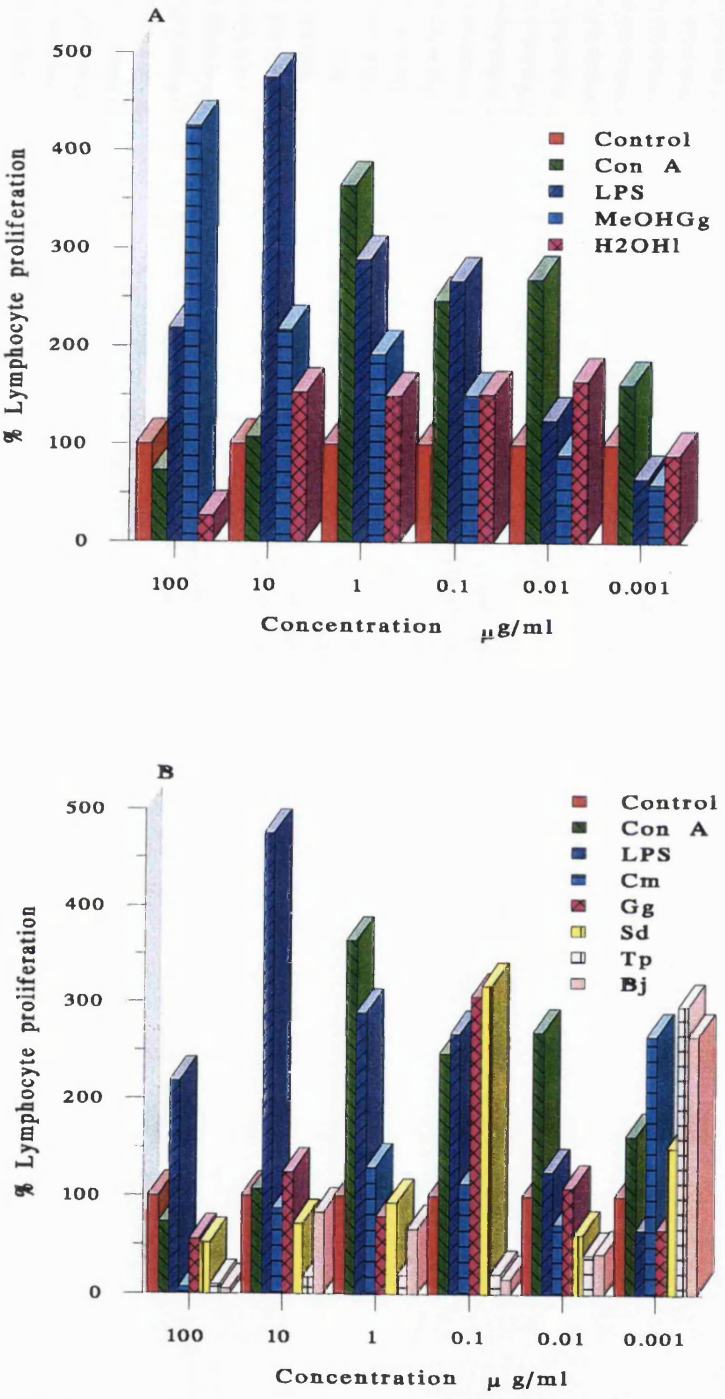


Figure 4.8 Effects of some crude extracts on normal (A) and Con A (B) induce lymphocyte proliferation. Results are expressed as the percentage of lymphocyte proliferation as compare with control culture. Con A: Concanavaline A. LPS: Lipopolysaccharide. MeOHGg: Methanolic extract of *Galphimia glauca*. H_2OHl: Aqueous extract of *Hintonia latiflora*. Methanolic extracts of Cm: *Celaenodendrom mexicanum*. Gg: *Galphimia glauca*. Sd: *Stephania dinklagei*. Tp: *Triclisia patens*. Bj: *Brucea javanica*.

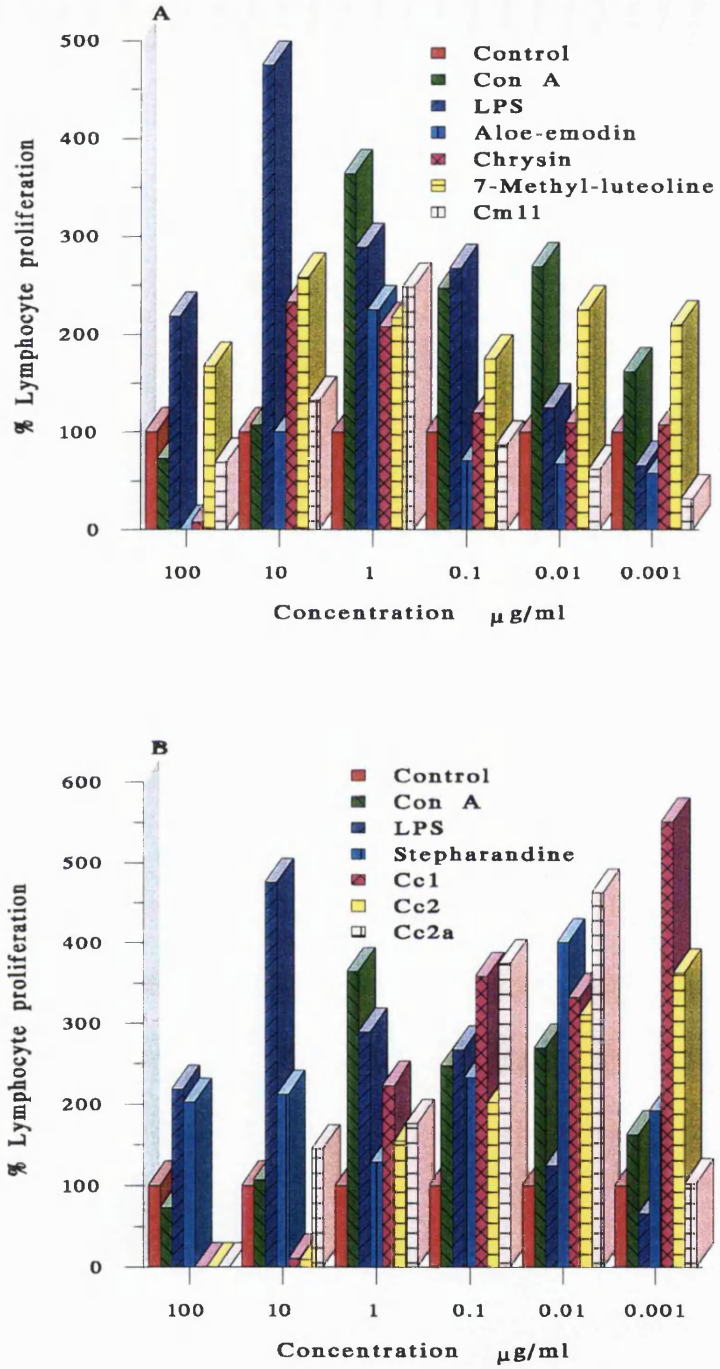
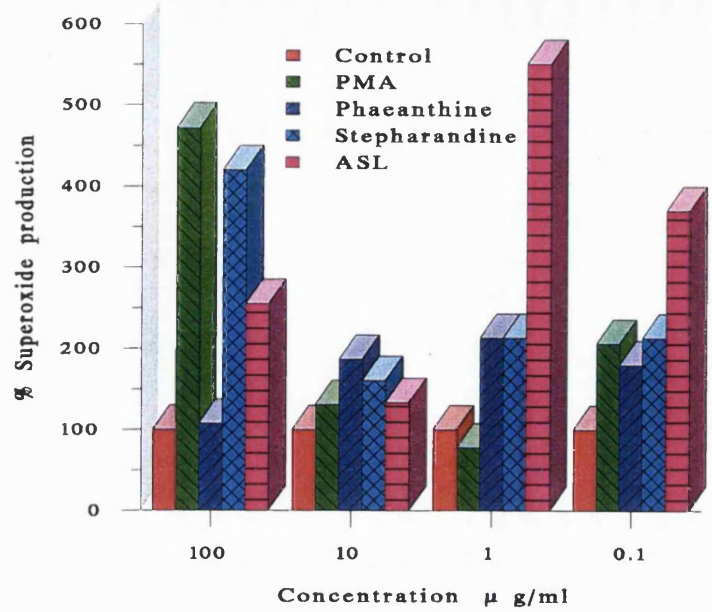


Figure 4.9 Effects of some compounds on normal (A) and Con A (B) induce lymphocyte proliferation .Results are expressed as the percentage of lymphocyte proliferation as compare with control culture. Con A: Concanavaline A. LPS: Lipopolysaccharide. Cm11: 3α-Hydroxy-5,24EZ-dien-tirucalla-26-oic acid. Cc1: Benzo[g]-isoquinoline-5,10-dione. Cc2: 1-Hydroxy-benzoisochromanquinone. Cc2a: 1-Acetyl-benzoisochromanquinone.



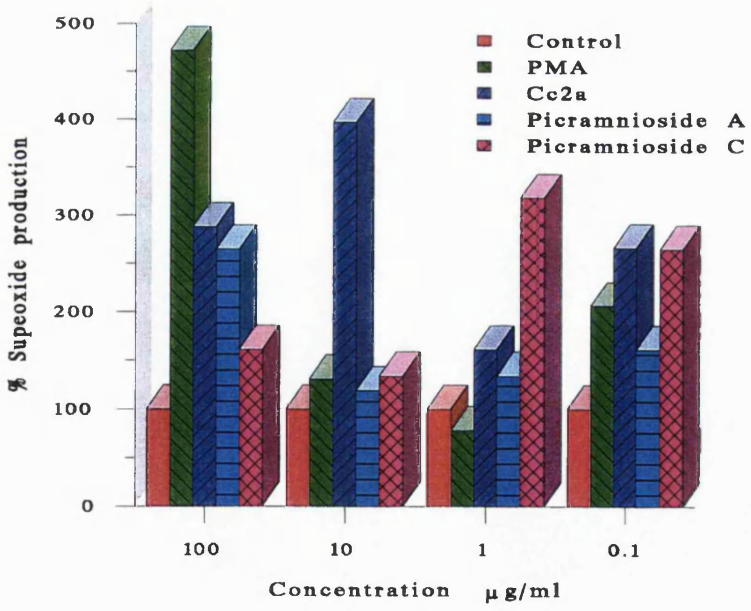
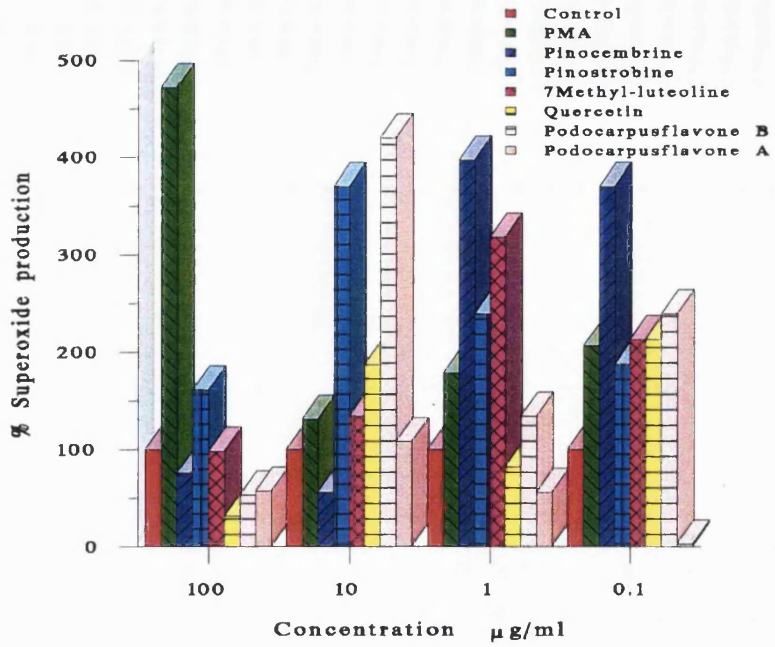


Figure 4.10 Effects of some compounds on macrophage superoxide production I. Results are expressed as the percentage of superoxide production as compared with control culture. PMA: Phorbol myristate acetate. ASL: Acetylstrictosidine lactam. Cc2a. 1-Acetyl-benzoisochromanquinone.



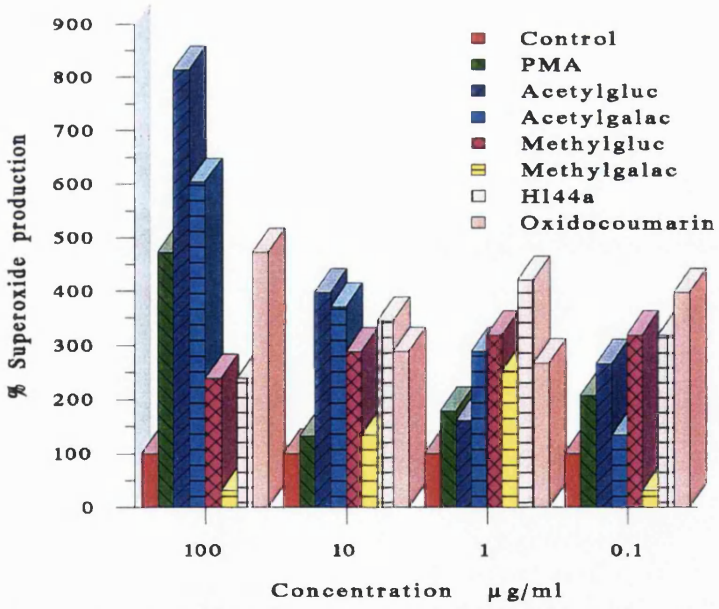


Figure 4.11 Effects of some compounds on macrophage superoxide production II. Results are expressed as the percentage of superoxide production as compared with control culture. PMA: Phorbol myristate acetate. Acetylgluc: 5-O-[β-D-Tetraacetoxyglucopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin. Acetylgal: 5-O-[β-D-Tetraacetoxygalactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin. Methylgluc: 5-O-[β-D-glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin. Methylgal: 5 -O-[β-D-Galactopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin. Hl44a: 5-O-[β-D-Tetraacetoxy-galactopyranosyl]-4'-acetoxy-7-methoxy-4-phenylcoumarin. Oxidocoumarin: 4',5'-Dihydroxy-7-methoxy-4-phenyl-5,2'-oxidocoumarin.

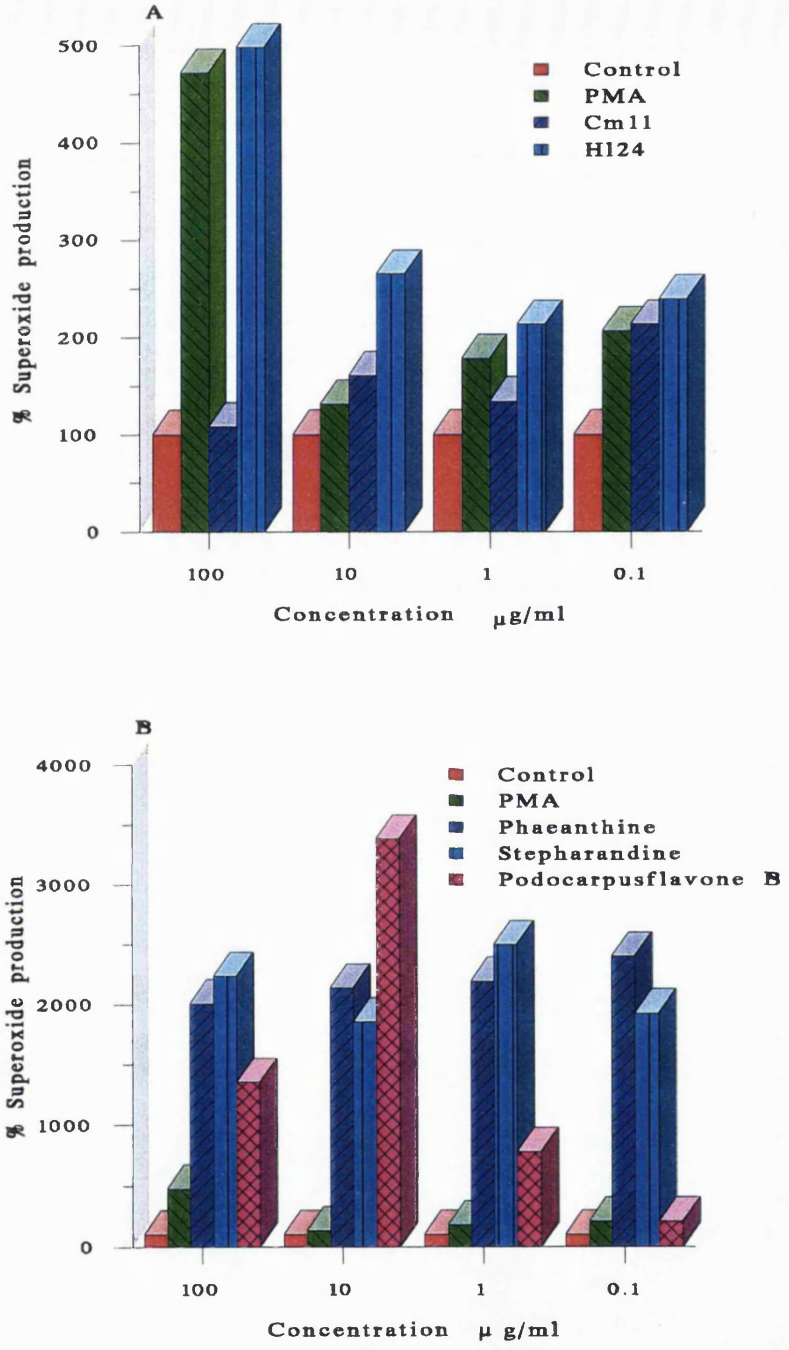


Figure 4.12 Effects of some compounds on macrophage superoxide production III. Results are expressed as the percentage of superoxide production as compared with control culture. PMA: Phorbol myristate acetate. Cm11: 3α-Hydroxy-7,24Z-dien-tirucalla-26-oic acid. Hl24: 3-O-[β-D-Glucopyranosyl]-23, 24-dihydrocucurbitacin F.

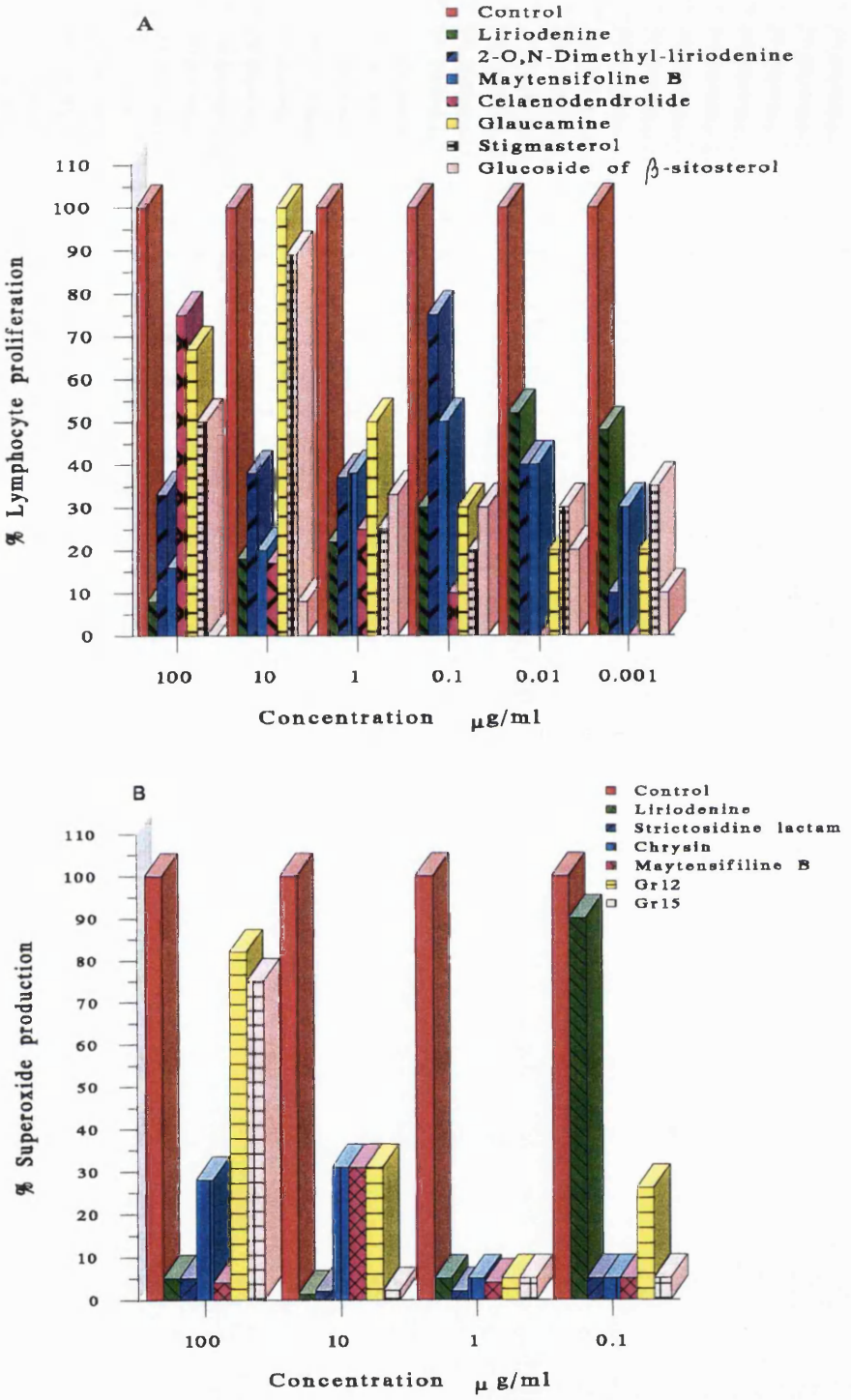


Figure 4.13 (A) Suppressice effects of some compounds on lymphocyte proliferation. Results are expressed as the percentage of lymphocyte proliferation. (B) Suppressive effects of some compounds on macrophage superoxide production. Results are expressed as the percentage of superoxide production as compared with control culture. Gr12: ent-7,15-sandaracopimaradiene-2β,18-diol. Gr15: ent-8(14),15-sandaracopimaradiene-2α,18-diol.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Parasitic protozoa remain a major health problem throught the world. Thre are few effective drugs for the treatment of many protozoal diseases. There is a need to develop new antiprotozoal drugs with novel structures and modes of action. Natural products from higher plants are a source of novel antiprotozoal drugs (see Chapter1).

Plant-derived compounds have long been, and continue to be, extremely important as sources of medicinal agents and models for the design, synthesis, and semisynthesis of novel substances for treating human diseases such as protozoan diseases (see Chapter 1). There are 119 drugs of known structure that are still extracted from higher plants and used globally in allopathic medicine (Farnsworth, 1990). About 74% of these were discovered by chemists who where attempting to identify the chemical substances in the plants that were responsible for their medical uses by humans. These 119 plant-derived drugs are produced commercially from less than 90 species of higher plants. There are at least 250 000 to 50 000 species of higher plants on earth, and only a small percentage has been investigated phytochemically and the fraction subjected to biological or pharmacological screening is even lower (Balandrin *et al.*, 1993). Therefore, it is logical to presume that many more useful drugs are being and will be found in the plant kingdom if the search for these entities is carried out in a logical and systematic manner.

The process to obtain a pharmacologically active pure plant constituent requires a multidisciplinary collaboration of botanist, pharmacognosists, chemists, pharmacologists, and toxicologists, and this approach has been described several times (Hamburger & Hostettman, 1991). The selection of the plant species to be studied is based on consideration of random collection, chemotaxonomic relationships, or traditional medicine (Hostettman, 1995). The use of ethnomedical information in the selection of plants to be investigated offers the advantage that the active compounds identified come to medicine with a long history information on efficacy and cytotoxicity in humnas.

For this study, 47 plant species, were selected either because of their traditional use or because of chemotaxonomic considerations. Plant species in investigation were screened for biological activity using *L. donovani*, *T. b.brucei, T.cruzi, P. falciparum,* KB cells or *A. salina, in vitro* assays. From this preliminary study *C. mexicanum*, *G. glauca*, *G. rhopalocarpa, S. dinklagei*, *T. patens*, *H. latiflora*, and *C. camponutans* were selected for further investigation to isolate some of their active principles, and other compounds present in these plants. Bioactive guided fractionation of selected plants and a combination of chromatographic techniques yielded 38 compounds of which 11 are novel. The compounds have a broad spectrum of chemical structures including, alkaloids, quinones, terpenes, flavonoids and coumarins.

From *C. mexicanum* three active triterpenes were isolated 3α-hydroxy-7,24Z-di-en-tirucalla-26-oic acid and 3-oxo-7,24Z-di-en-tirucalla-26-oic acid, and epi-oleanolic acid. In addition, the terpenes friedelin, maytensifolin B, 3β-hydroxyfriedelan-16-one, and celaenodendrolide, and three biflavonoids amentoflavone, podocarpusflavone A and podocarpusflavone B were also isolated.

G. glauca afforded the flavonol quercetin as the active principle, together with the new nor-secoterpenes: galphimine C, galphimine D, galphimine E and glaucamine, and the steroids stigmasterol and sitostèryl-3-O-β-D-glucopyranoside.

G. rhopalocarpa yielded four novel active terpenes: 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, lanosta-7,9(11),24EZ-triene-3,23-diol, ent-8(14),15-sandaracopimaradiene-2β,18-diol ent-8(14),15-sandaracopimaradine-2α,18-diol; together with the steroid stigmasterol and the coumarin scopoletin.

S. dinklagei yielded six active aporphine alkaloids, of which three are new, N-methyl-lioriodendronine, 2-O, N-dimethyl-liriodendronine, and stepharandine, and the known dicentrinone, liriodenine and corydine as well as the anthraquinone aloe-emodin.

T. patens yielded two bisbenzylisoquinoline alkaloids aromoline and phaeanthine as active principles. From *C. camponuntans* two active quinones were isolated, 1-hydroxy-benzoisochromanquinone and benzo[g]isoquinoline-5,10-dione.

H. latiflora afforded as active principle the flavone 7-methyl-luteolin, the cucurbitacin, 3-O-β-D-glucopyranosyl-23,24-dihydrocucurbitacin F, and the phenylcoumarins: 5-O-β-D-glucopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin and 5-O-β-D-galactopyranosyl-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin.

A total of 97 compounds including the above compounds, some semisynthetic derivates and other compounds available in our laboratory were screened for antiprotozoal activity against *L.donovani, T.b.brucei* and for cytotoxicity against KB cells *in vitro* in order to establish structure activity relationships among them. Results revealed that 10 compounds had IC_{50} values of less than 1 μg/ml against *L. donovani* promastigotes *in vitro*. A further 34 had IC_{50} values between 1 and 10 μg/ml. Among them, fangchinoline and isotrilobine were the most active with IC_{50} values of 0.24 and 0.33 μg/ml, respectively. In addition, fangchinoline and isotrilobine have a F/A (cytotoxic activity/antileishmanial activity) ratio of 262.43 and 32.98, respectively. The first having a high selectivity to promastigotes than to KB cells.

Fourteen compounds were active against amastigotes of *L. donovani* with IC_{50} values less than 50 μg/ml. In the same test conditions the standard $NaSb^{V}$ had an IC_{50} value of 9.75 Sb^{V} μg/ml. Significant antileishmanial activity comparable or higher to that of $NaSb^{V}$ was demonstrated by the BBIQ alkaloid phaeanthine, the quinones benzo[g]isoquinoline-5,10-dione, 1-hydroxy-benzo-isochromanquinone and acetyl-benzo-isochromanquinone, which were 6,2,3, and 18 times as active as the standard $NaSb^{V}$.

The activity against promastigotes does not necesarily confer activity against amastigotes since both stages of the parasite have different molecular and biochemical features, giving differences in drug sensitivity. This was observed with 7-methyl-luteolin, epi-oleanolic acid, and norcorydine. However, they may have potential as prophylactic agents.

Twenty-four compounds had IC_{50} values equal to or less than 10 μg/ml against *T. b. brucei in vitro*. Under the same conditions, pentamidine had an IC_{50} value of 6×10^{-3} μg/ml. The most potent trypanocidal compond was the benzoquinone 1-acetyl-benzo-isochromanquinone with an IC_{50} value of 0.17μg/ml.

In assessing structure-activity relationships of the screened compounds for antiprotozoal activity, it can be seen that the compounds with *in vitro* activity equal to or less than 10 μg/ml against *L. donovani* promastigotes and *T. b. brucei* trypomastigotes have a range of different structures including alkaloids, quinones, coumarins, flavonoids and terpenes.

None of the compounds tested showed significant cytotoxic activity against KB and P388D1 cells. The most active being papaverine hydrochloride which is 647 times less active than the standard podophyllotoxin against KB and P388D1 cells respectively. However, papaverine was devoid of interesting activity against *L. donovani* and was inactive to *T. b. brucei;* in similar manner the cytotoxic naphthoquinones C-xylosides did not show antiprotozoal activity. Thus, it may be concluded that cytotoxicity does not automatically confer antiprotozoal activity.

In assessing selectivity of antiprotozoal action, most of the compounds with IC_{50} values of less than 10 μg/ml had a parallel trypanocidal activity. Apart from thalisopodine, the IC_{50} values for antileishmanial and trypanocidal effects were found significantly different. The parallel antiprotozoal activity observed for these samples could be explained by the fact that *Leishmania* and *Trypanosoma* are members of the family trypanosomatidae, and possess similar molecular and biochemical features which are unique to the kinetoplast.

In contrast, the *in vitro* activity against *L. donovani* promastigotes does not necessarily imply that a sample will be active against other related protozoa. For example, the antileishmanial activity of norcorydine, catalpifoline, 5-O-[β-D-glucopyranosyl]-7,3',4'-trimethoxy-4-phenylcoumarin and acetylstrictosidine did not extend to *T. b. brucei*, or other protozoa, whereas, the trypanocidal activity of daphnoline, thalifendine, protopine, acutumine,pinocembrine and 5-O-[β-D-tetraacetylgalactopyranosyl]-4'-acetyl-7-methoxy-

4-phenylcoumarin did not extend to *L. donovani.* These results suggests that the optimal structures for antileishmanial activity are not necessarily the same as those for trypanocidal activity.

The antiprotozoal activities did not parallel the brine shrimp test lethality test in the case of naphthoquinones C-xylosides. However, the cytotoxicity and brine shrimps test correlated with the antiprotozoal activitites of benzoquinones and cytotoxic terpenes. This approach indicated that the brine shimp lethality test could be useful for the screening of antiparasitic compounds with similar structures to those of benzoquinones and terpenes isolated in this study.

Fifty six compounds were screened for mitogenic activity, and 4 compounds were more effective on the stimulation of normal proliferation of lymphocytes in comparison with control having a maximum enhancement corresponding to 2-fold over control at concentration range of 1 to 10 μg/ml. These were aloe-emodin, chrysin, 7-methyl-luteolin, and 3α-hydroxy-7,24Z-dien-tirucalla-26-oic acid. However, these compounds did not show co-stimulatory effect on activation of T-cells. Thus, further co-stimulatory studies with other mitogenic agents will be required to complete their mechanism of immunomodulation.

In combination with Con A, 26 compounds showed synergistic activity on the stimulation of T-lymphocytes having a maximum co-stimulatory response in the interval of 3 - 5.5-fold over control at concentration range of 0.1 - 0.001 μg/ml. The strongest synergistic effect was observed with the following compounds: the aporphine alkaloid, stepharandine, the quinones: benzo [g]isoquinoline-5,10-dione and 1-acetyl-benzoisochromanquinone, the coumarins: 5-O-[β-D-galactopyranosyl]-3',4'-dihydroxy-7-methoxy-4-phenylcoumarin and scopoletin, and the norsecotriterpene galphimine C, which have a maximum co-stimulatory effect in the range of 4 - 5 times over control at concentration range of 0.001 - 1 μg/ml (Figure 4.9).

Immunossuppressive effects on normal and mitogenic-induced proliferation of T-cells were observed with 20 compounds at concentrations equal or lower than 10 μg/ml. Whether these compounds suppress B-lymphocytes is not known yet. Thus further studies of co-stimulation with LPS will be necessary in other to determine their effects on humoral response.

In the case of macrophage superoxide production, from the 45 samples tested for superoxide-anion production, 31 compounds were able to promote spontaneous superoxide release from macrophages in the range of 2 to 5.97-times over untreated control at concentrations equal or lower than 1 μg/ml. Among them, 9 compounds gave the strongest enhancement corresponding to 2 - 5-fold above control. They were, acetyl-strictosidine lactam, picramnioside C, 5-O-[β-D-tetraacetoxy-galactopyranosyl]-3',4'-diacetoxy-7-methoxy-4-phenylcoumarin, 5-O-β-D-glucopyranosyl-7,3',4'-trimetoxy-4-phenylcoumarin, 4',5'-dihidroxy-7-methoxy-4-phenyl-5,2'-oxydocoumarin, 3-O-β-D-glucopyranosyl-23,24-dihydroxycucurbitacin F, 3α-hydroxy-7,24Z-dien-tirucalla-26-oic acid, 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one, ent-8(14),15-sandaracopimaradiene-2β-18-diol and ent-8(14),15-sandaracopimaradiene-2α-18-diol. In combination with zymosan, only phaenthine, stepharandine and podocarpusflavone B showed a co-stimulatory response corresponding to 24, 24.9 and 33.8-folds over control at 0.1, 1 and 10 μg/ml respectively.

Inhibition of macrophage superoxide was observed in all the concentrations tested for liriodenine, 2-O,N-dimethyl-liriodendronine, strictosidine lactam, chrysin, maytensyfoline B, epi-oleanolic acid, 23-hydroxy-5α-lanostan-7,9(11),24EZ-triene-3-one, ent-8(14),15-sandaracopimaradiene-2α-18-diol, ent-8(14),15-sandaracopimaradiene-2β-18-diol, and sitosteryl-3-O-β-D-glucopyranoside.

The results obtained in this study emphasize once again the potential of natural compounds from higher plants as modulators of non-specific immunity. Since few studies have been made on the immunological properties of the compounds tested in this study, the potential interest of these compounds is even greater. Thus, more detailed

studies will be required to understand their mechanism of immunomodulation. Further, more studies are need on the relationship between the immunomodulation effect and antiprotozoal, as well as antitumor activities. It must be kept in mind, however that results obtained *in vitro* in many cases may have no counterpart *in vivo*, thus studies *in vivo* will be necessary to determine their potential as immunomodulators agents. In addition to this, the efficacy of an immunostimulant is also strongly dependent on the immmune status of a patient at the onset of a treatment, on the dose and the mode of application (Wagner *et al.*, 1994). Moreover, it is also imperative to modify the chemical structures of some of the compounds which have seen shown to possess immunomodulatory effects and potential toxicity, in oder to develop immunomodulators of high efficacy and low toxicity.

From this study it can be seen that the activity of many plants may be due to direct antiprotozoal action of one of several of their components, and this has been demonstrated for a number of isolated natural products. However, plant extracts contain complex mixtures of chemicals and other actions such as immunostimulatory effects which may contribute to the overall curative effect.

It is known that a proportion of the non-polar compounds are extracted when aqueous teas are prepared, and also there are constituents present in the plant which aid solubilization or emulsification thus allowing lipophilic compounds to be extracted in very low concentrations necessary to give an immune response which could contribute to the anti-parasitic or anti-infections properties of the plants tested. Crude extracts contain not only low-moleculat-weight compounds but also high molecular-weight substances such as polysaccharides, proteins which contribute with the global properties of the plant activating on other cell components of the immune system such as complement (Yamada, 1992, Wagner, 1994). Thus it is likely that the activity of the plant is related not only to one compound but to several compounds which also might have synergistic activity contributing with the killing of patogens. It should also be remembered that many of these plant remedies are used in areas where people may be expected to have at least some degree of immune response to the pathogenic organisms causing the disease.

In vitro antiprotozoal activity of a natural product isolated from a plant used in traditional medicine provides some evidence to support the use of this plant but it does not verify clinical efficacy and it does not necessarily explain the mode of action. Thus, low molecular weight compounds with direct antiprotozoal activity may also modulate the immune sytem, acting synergistically or additively (Doenhoff & Chappell, 1992) with the antiprotozoan compound to overhelm the parasitic disease. From the results obtained in this study, it can be observed that there are some correlations between mitogenic activity and macrophage superoxide release activities *in vitro*. This relation could be extended to antiprotozoal, cytotoxic and antimicrobial activities for most of the active principles isoalted from the plants studied.

The increased awareness that drugs and host defense mechanisms can act synergistically or additively may result in the development of more effective treatment regimens or the testing of novel drugs and drug combinations. Furthermore, the incorporation of non-specific immunomodulators in the chemotherapeutic regimens might prove beneficial against intractable infections.

"So *in vivo* we have to imagine definitive sterilization in two ways:

1. The killing of a proportion of the parasites by the chemical;
2. Destruction of the remainder by antibody production induced by step 1."

Paul Ehrlich, 1909.

CHAPTER 6

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APENDIX

NMR-SPECTRA

S-71. HMQC spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

S-72. HMBC spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

S-73. NOESY spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

S-74. ^{1}H NMR (400 MHz, $CDCl_3$) of ent-8(14),15-sandaracopimaradiene-2α,18-diol

S-75. ^{13}C NMR (100 MHz, $CDCl_3$) of ent-8(14),15-sandaracopimaradiene-2α,18-diol

S-76. COSY-45 spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol

S-77. HMQC spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol

S-78. HMBC spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol

S-79. NOESY spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol

S-80. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of scopoletin

S-81. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of scopoletin

S-82. NOESY spectrum of scopoletin

S-83. ^{1}H NMR (400 MHz, CF_3CO_2D) spectrum of stepharandine

S-84. NOESY spectrum of stepharandine

S-85. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of corydine

S-86. ^{1}H NMR (400 MHz, C_5D_5N) spectrum of 2-O,N-dimethyl-liriodendronine

S-87. COSY-45 spectrum of 2-O,N-dimethyl-liriodendronine

S-88. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of N-methyl-liriodendronine

S-89. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of liriodenine

S-90. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of liriodenine

S-91. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of dicentrinone

S-92. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of dicentrinone

S-93. NOESY spectrum of dicentrinone

S-94. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of aloe-emodin

S-95. ^{13}C NMR (400 MHz, DMSO-d_6/$CDCl_3$) spectrum of aloe-emodin

S-96. COSY-45 spectrum of aloe-emodin

S-97. NOESY spectrum of aloe-emodin

S-98. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of phaeanthine

S-99. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of phaeanthine

S-100. COSY-45 spectrum of phaenthine

S-101. NOESY spectrum of phaenthine

S-102. ^{1}H NMR (400 HMz, $CDCl_3$) spectrum of aromoline

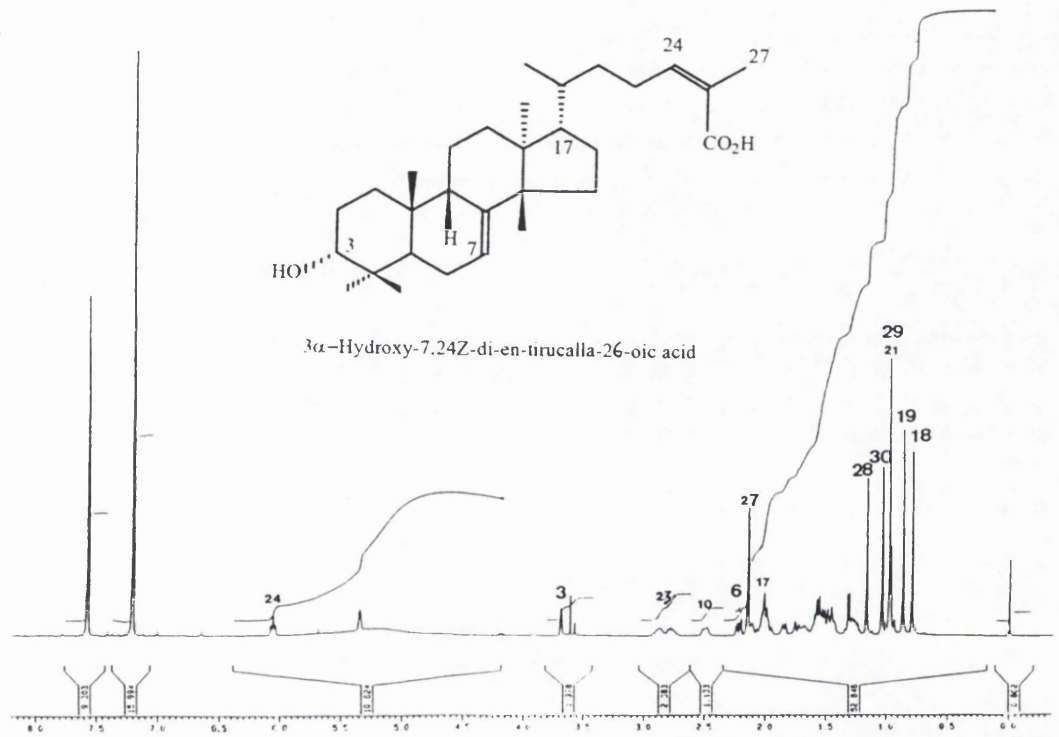
S-103. COSY-45 spectrum of aromoline

S-104. NOESY spectrum of aromoline

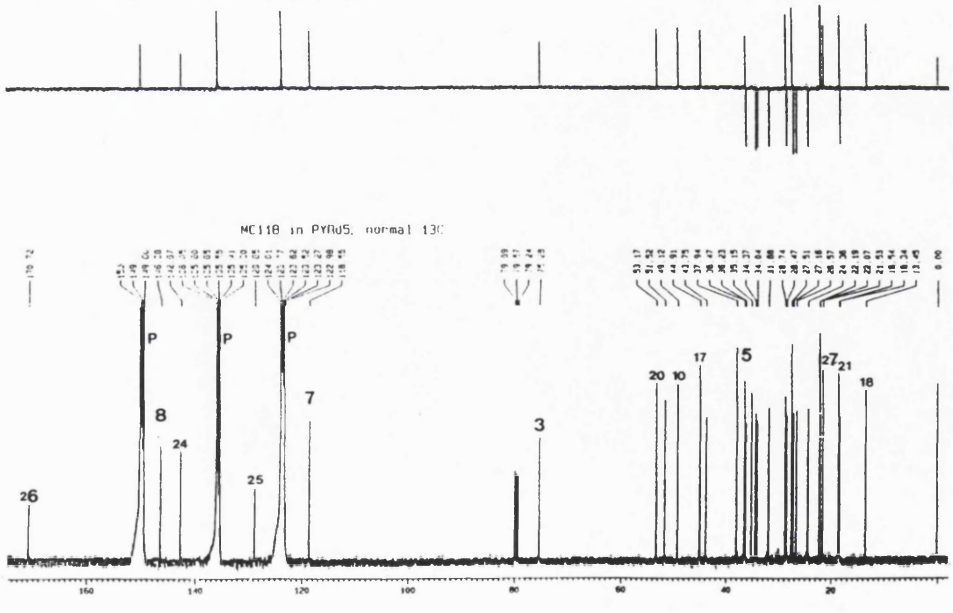
S-105. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of benzo[g]isoquinoline-5,10-dione

S-106. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 1-hydroxy-benzoisochromanquinone

S-107. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of 7-methyl-luteolin

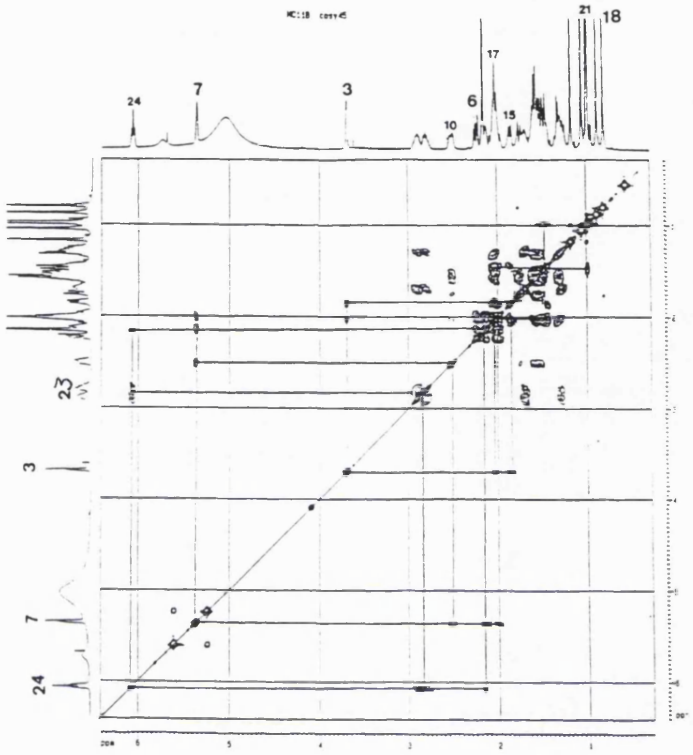


S-1. ^{1}H NMR (400 MHz, C_5D_5N) spectrum of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

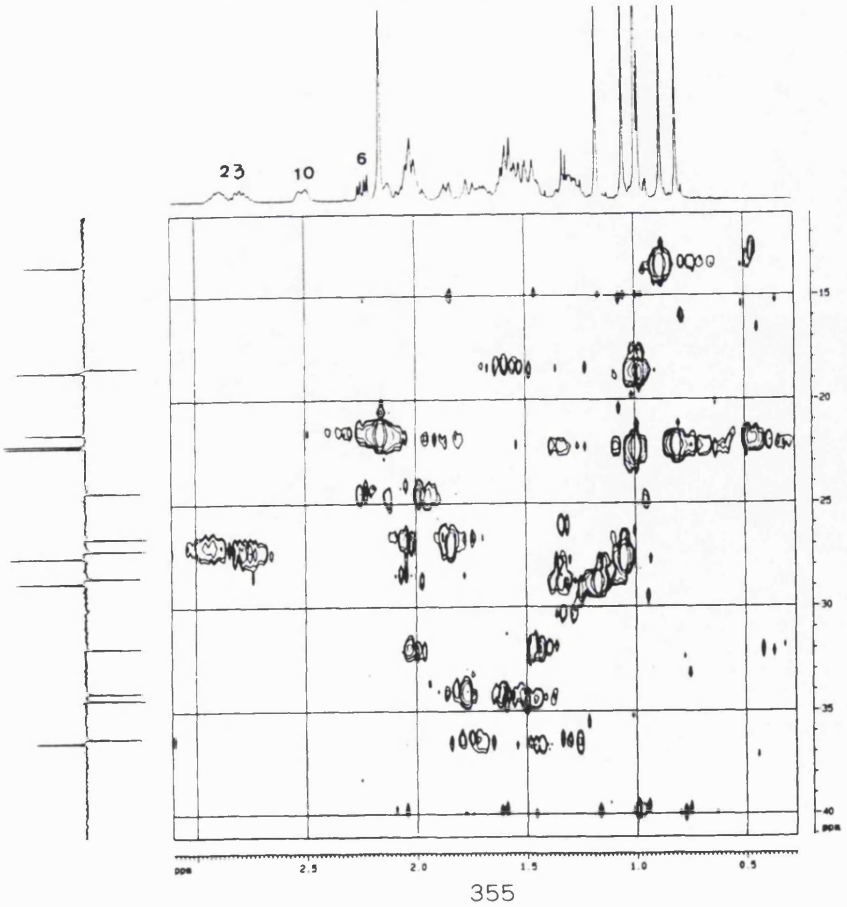


S-2. ^{13}C NMR (100 MHz, C_5D_5N) spectrum of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

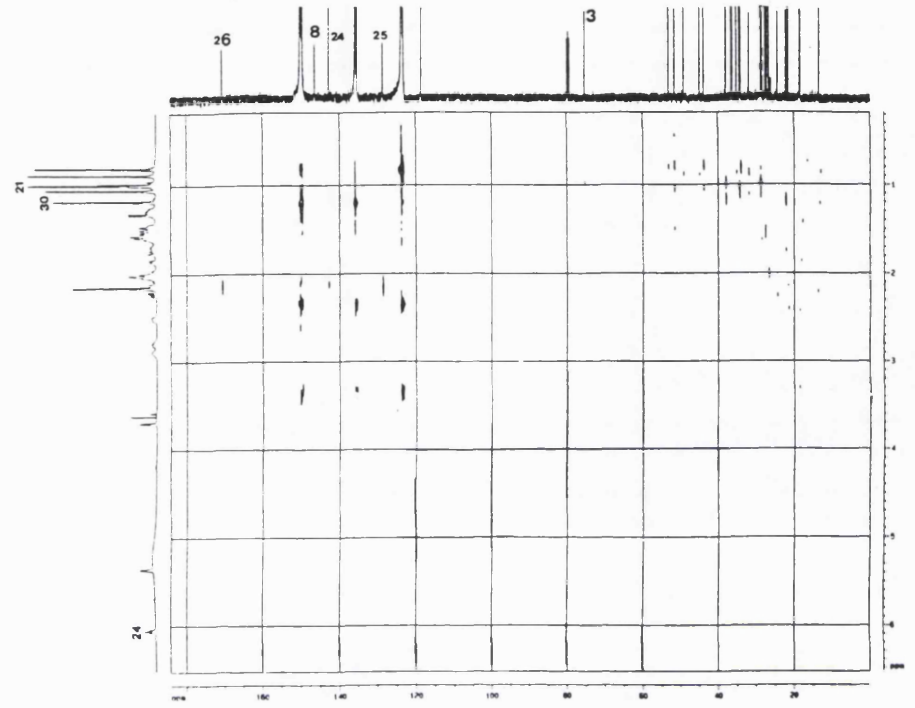
S-3. COSY-45 spectrum of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

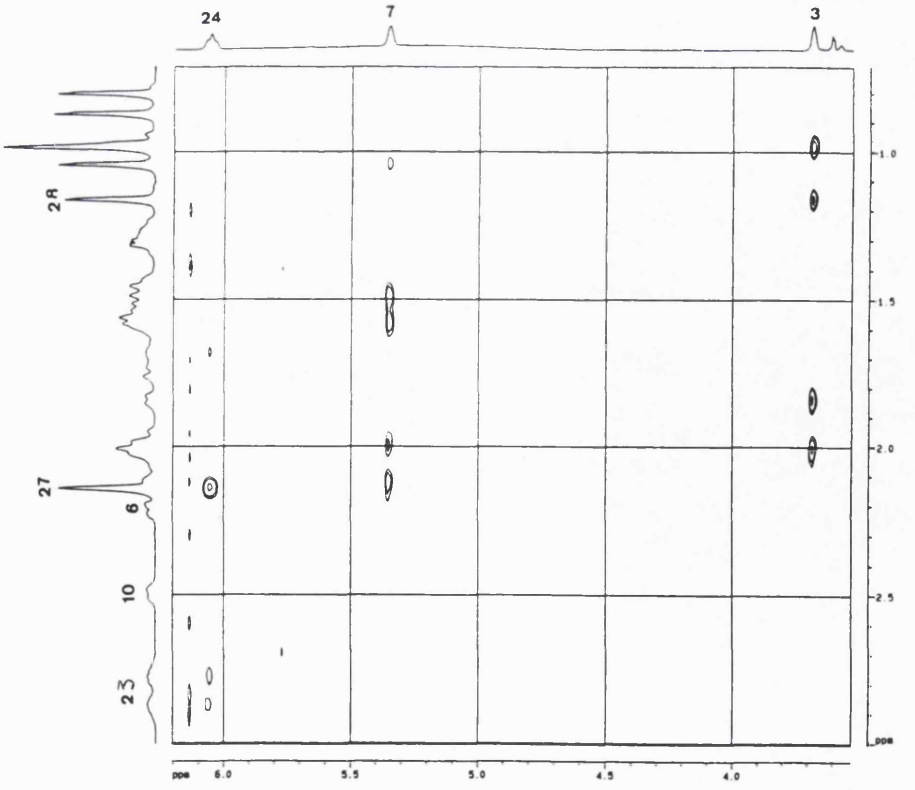


S-4. HMQC spectrum of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

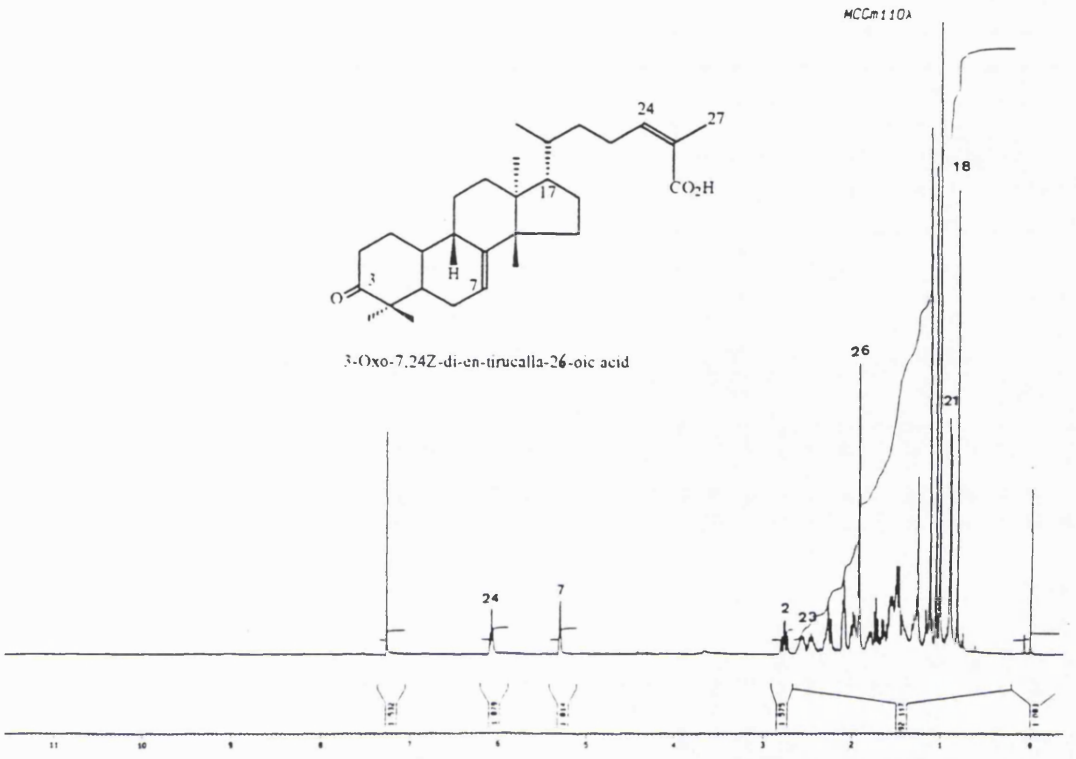


S-5. HMBC spectrum of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

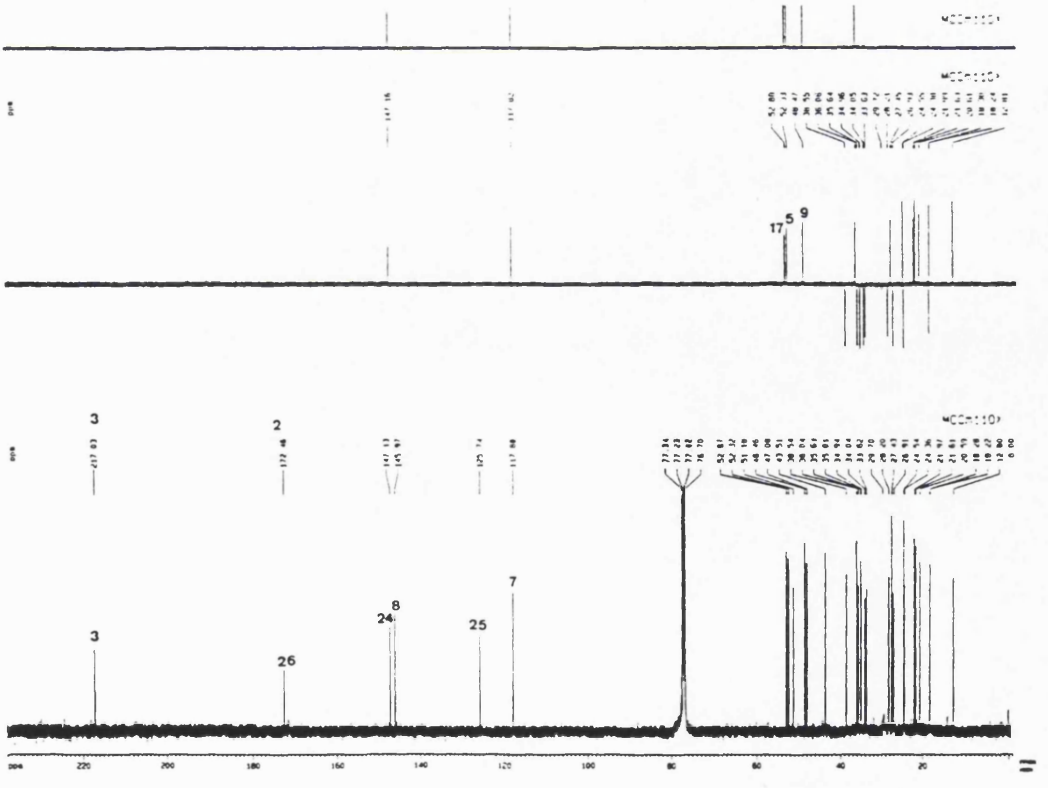




S-6. NOESY spectrum of 3α-hydroxy-tirucalla-7,24Z-dien-26 oic acid

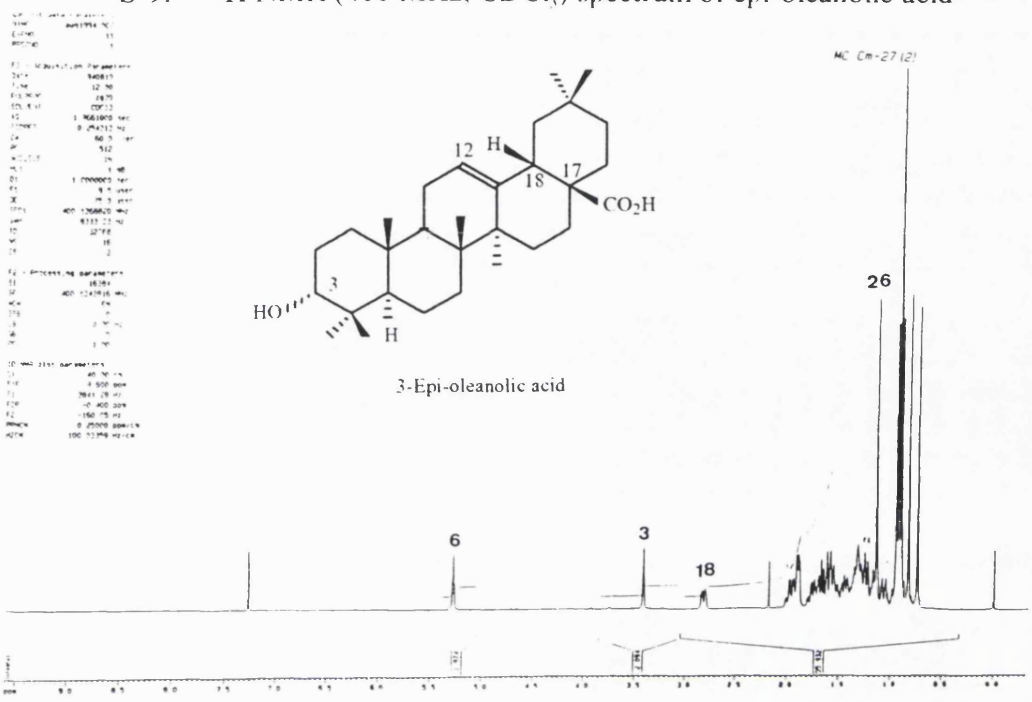


S-7. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 3-oxo-tirucalla-7,24Z-dien-26 oic acid

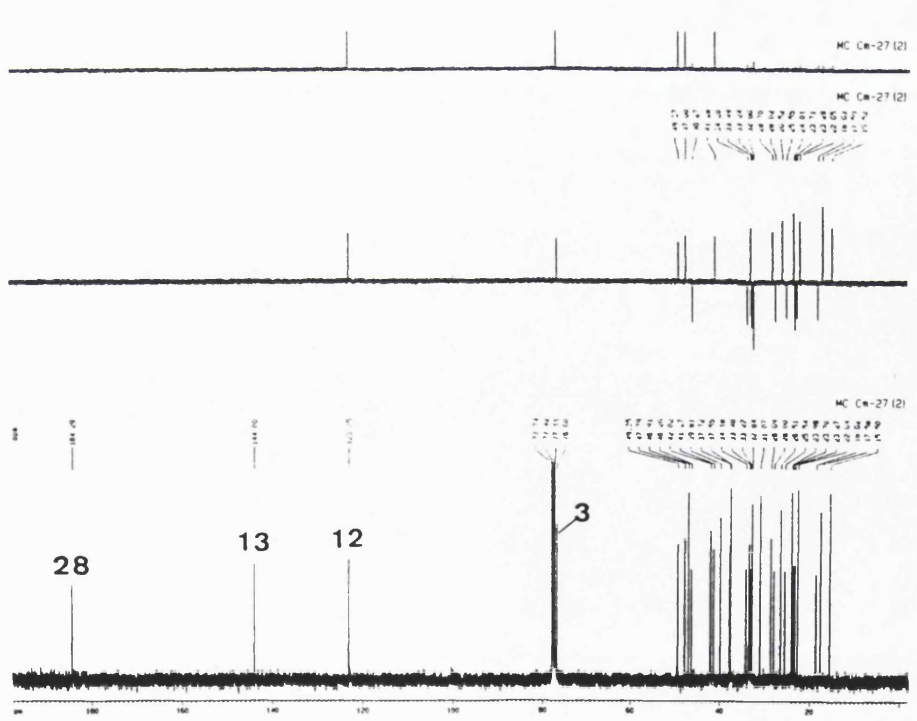


S-8. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of 3-oxo-tirucalla-7,24Z-dien-26 oic acid

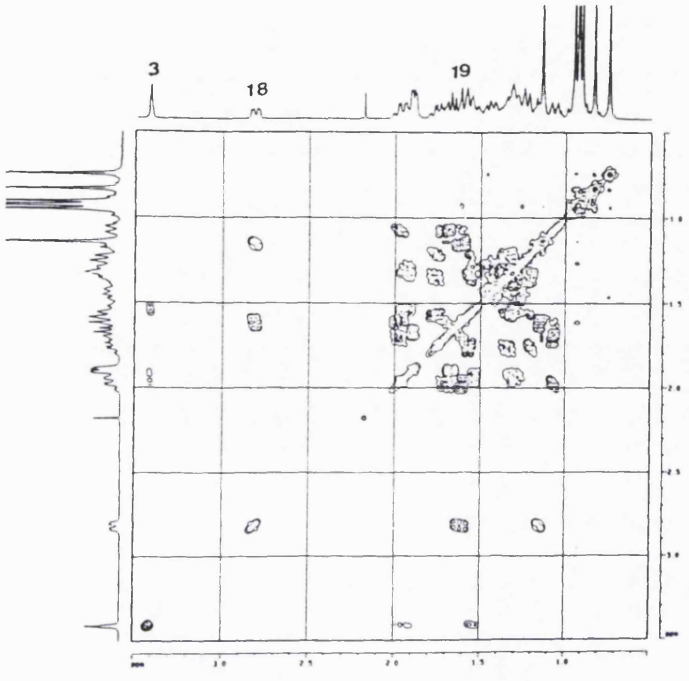
S-9. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of epi-oleanolic acid

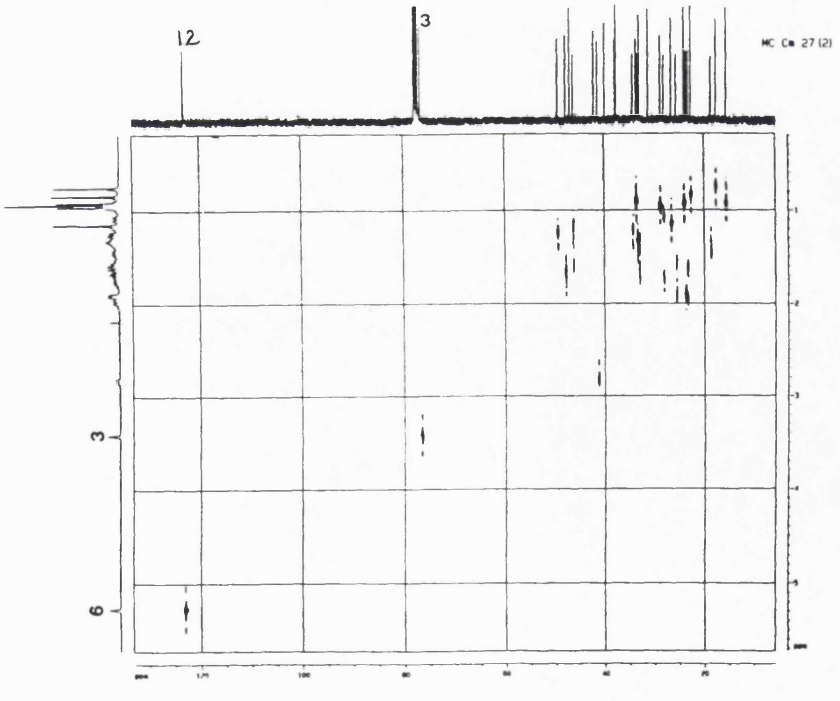


S-10. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of epi-oleanolic acid

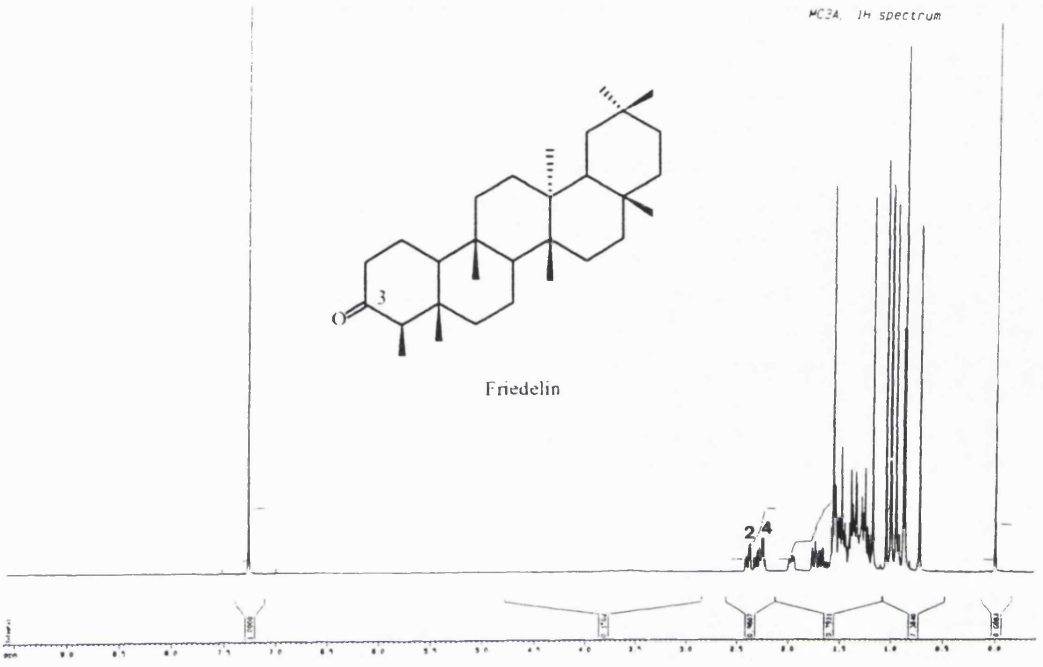


S-11. COSY-45 spectrum of epi-oleanolic acid

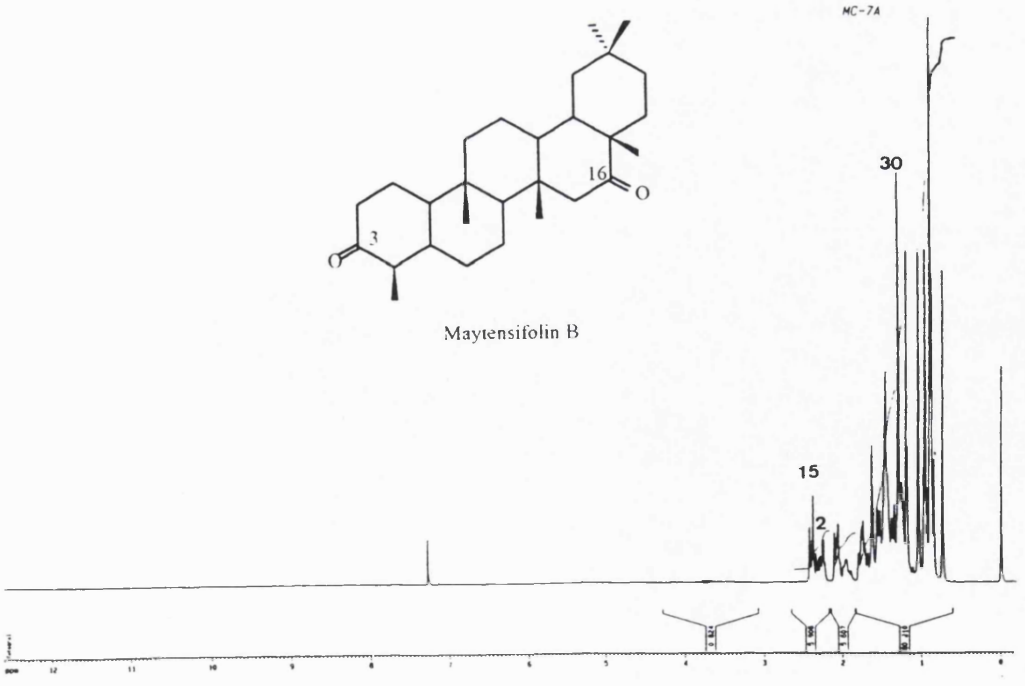




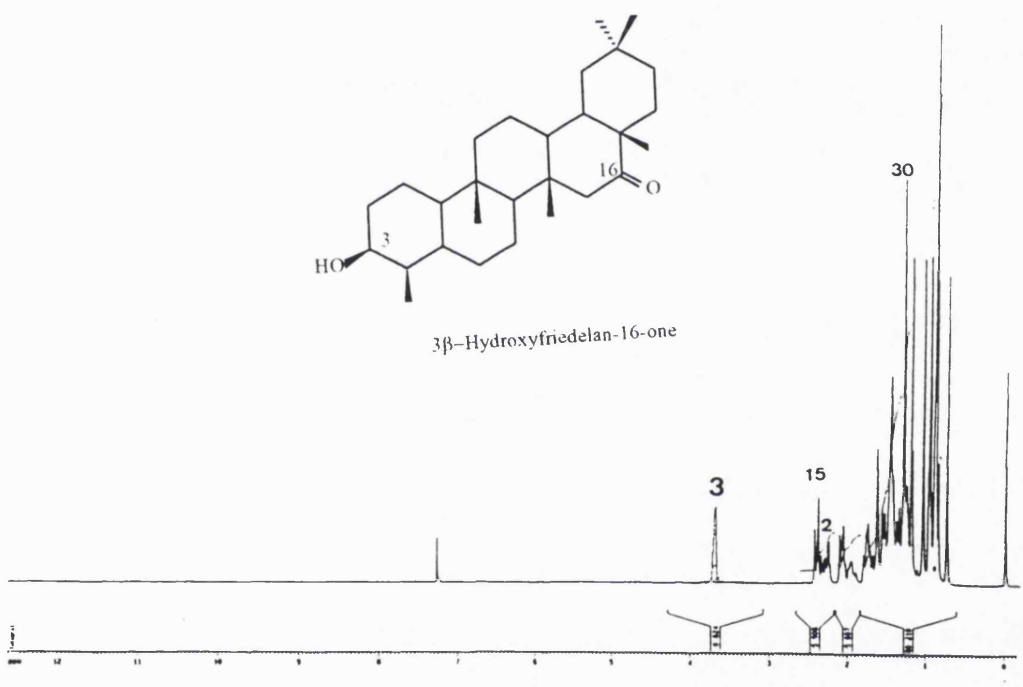
S-12. HMQC spectrum of epi-oleanolic acid



S-13. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of friedelin

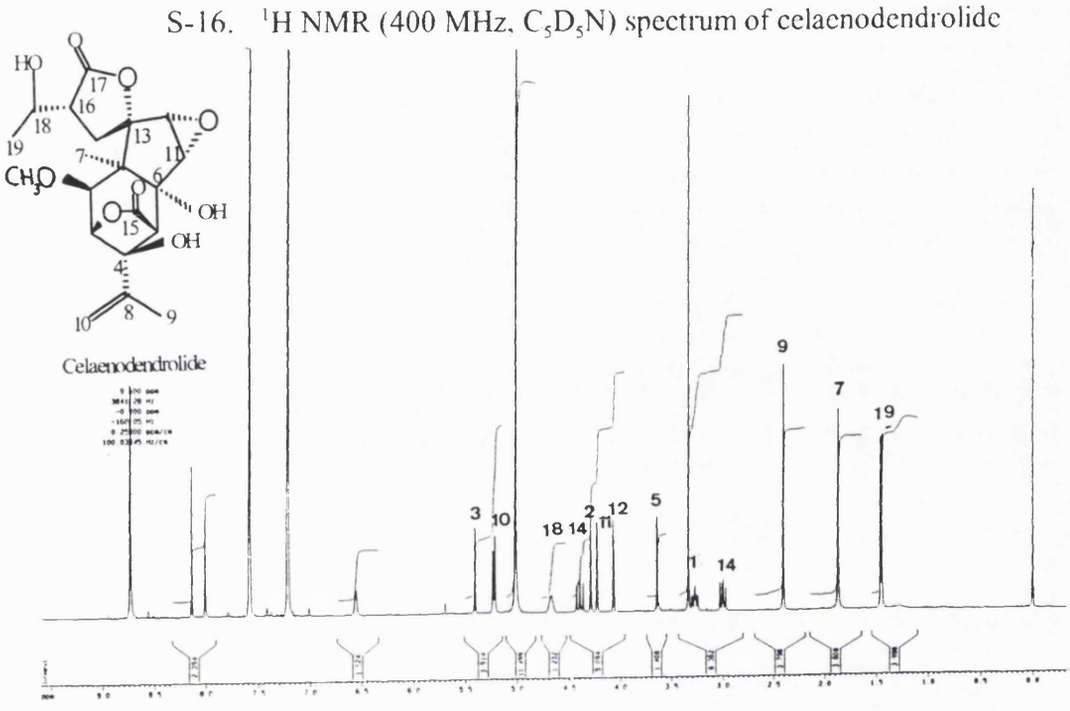


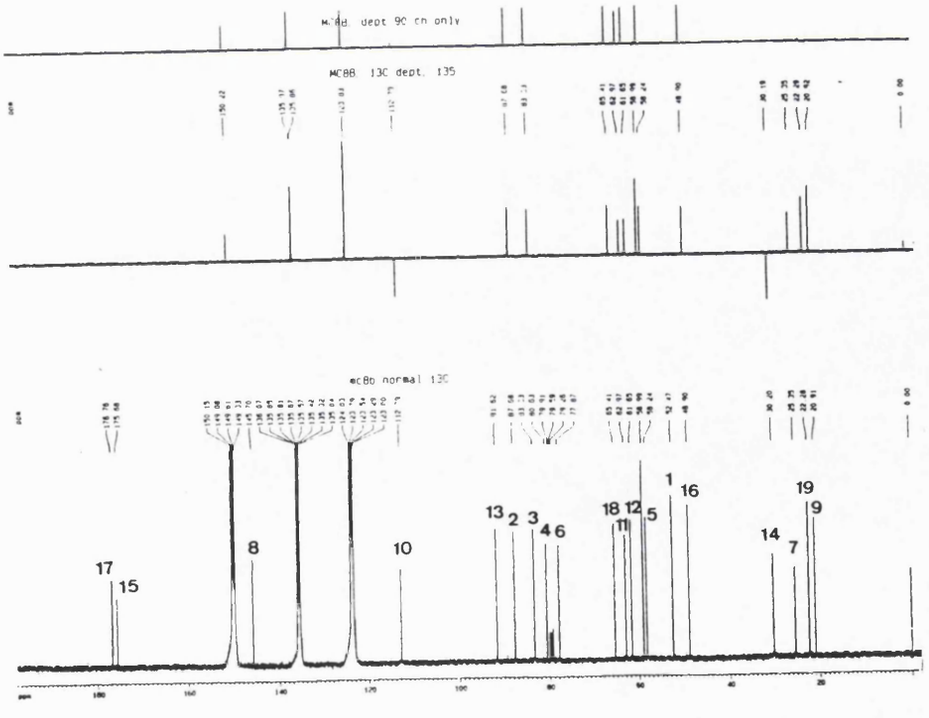
S-14. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of maytensifolin B



S-15. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 3β-hydroxy-friedelan-16-one

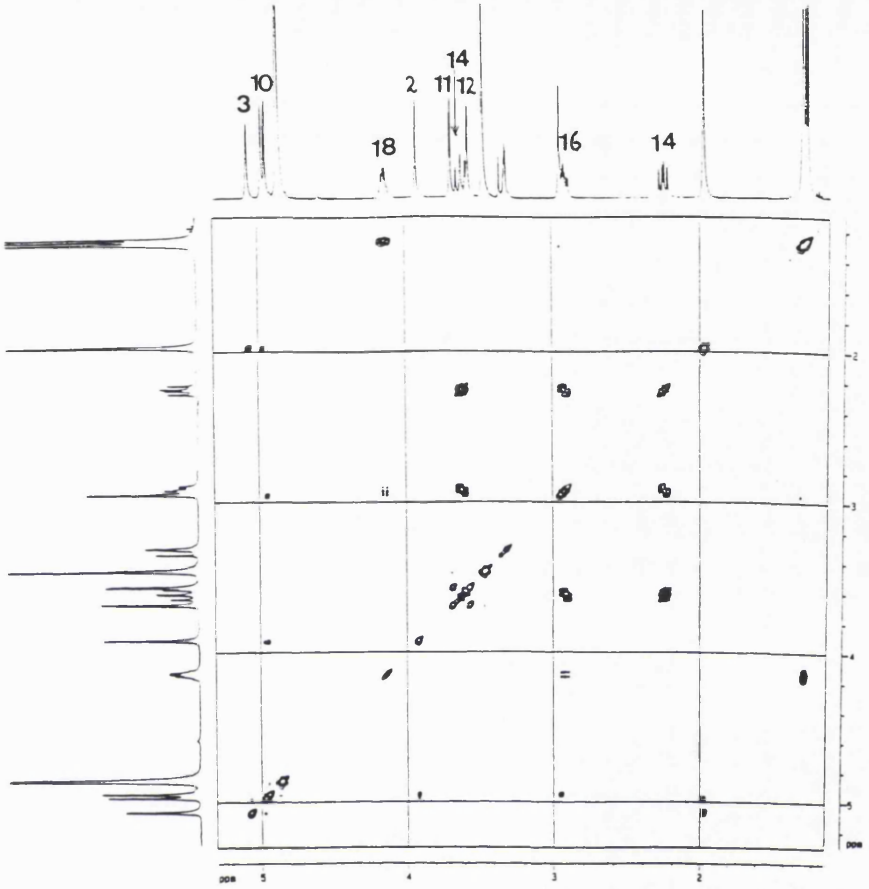
S-16. ^{1}H NMR (400 MHz, C_5D_5N) spectrum of celaenodendrolide



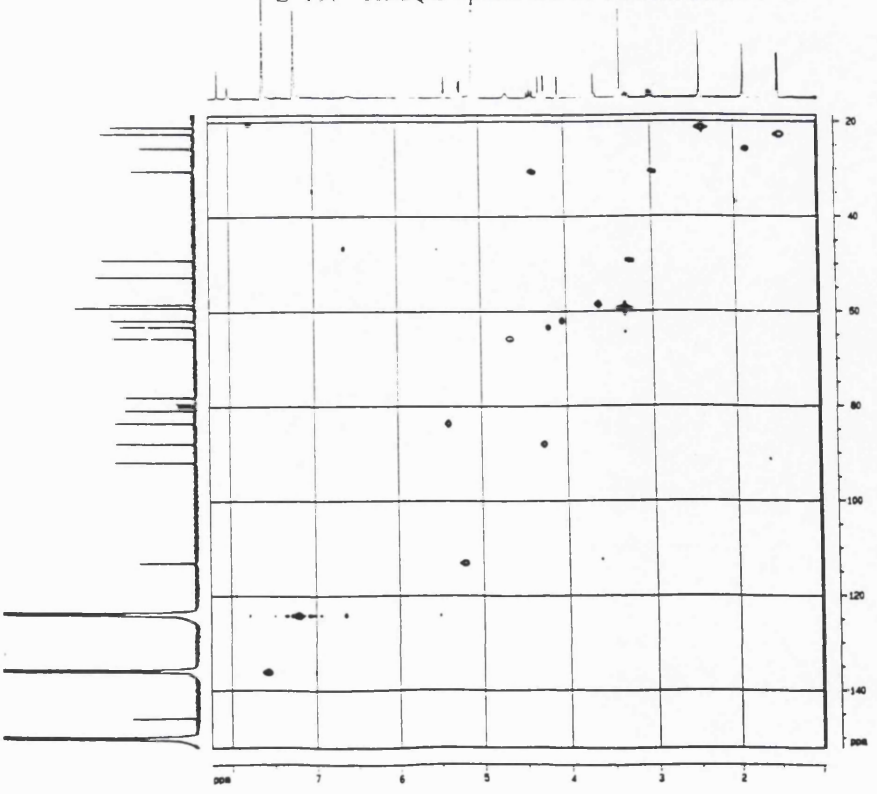


S-17. ^{13}C NMR (100 MHz, C_5D_5N) spectrum of celaenodendrolide

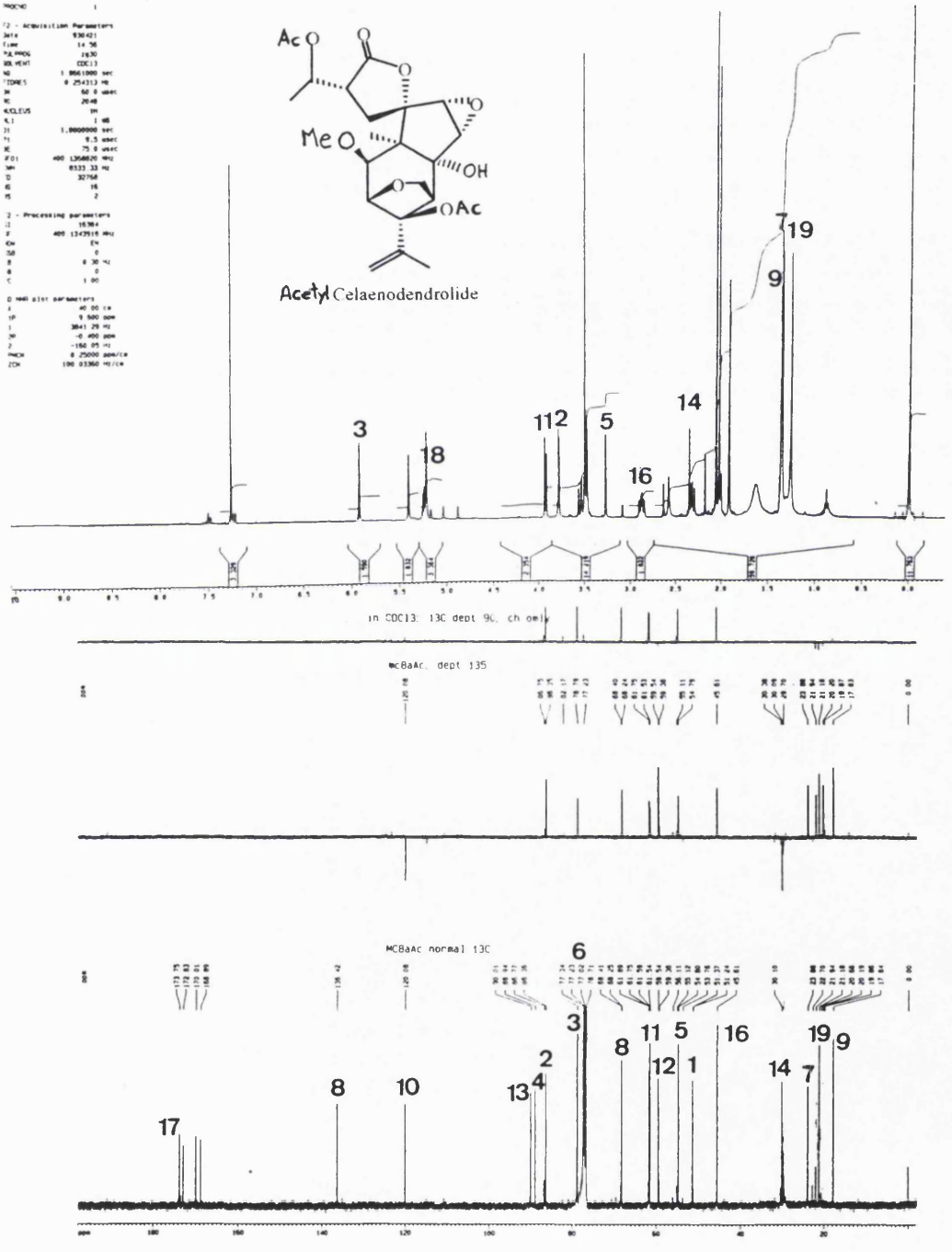
S-18. COSY-45 spectrum of celaenodendrolide



S-19. HMQC spectrum of celaenodendrolide

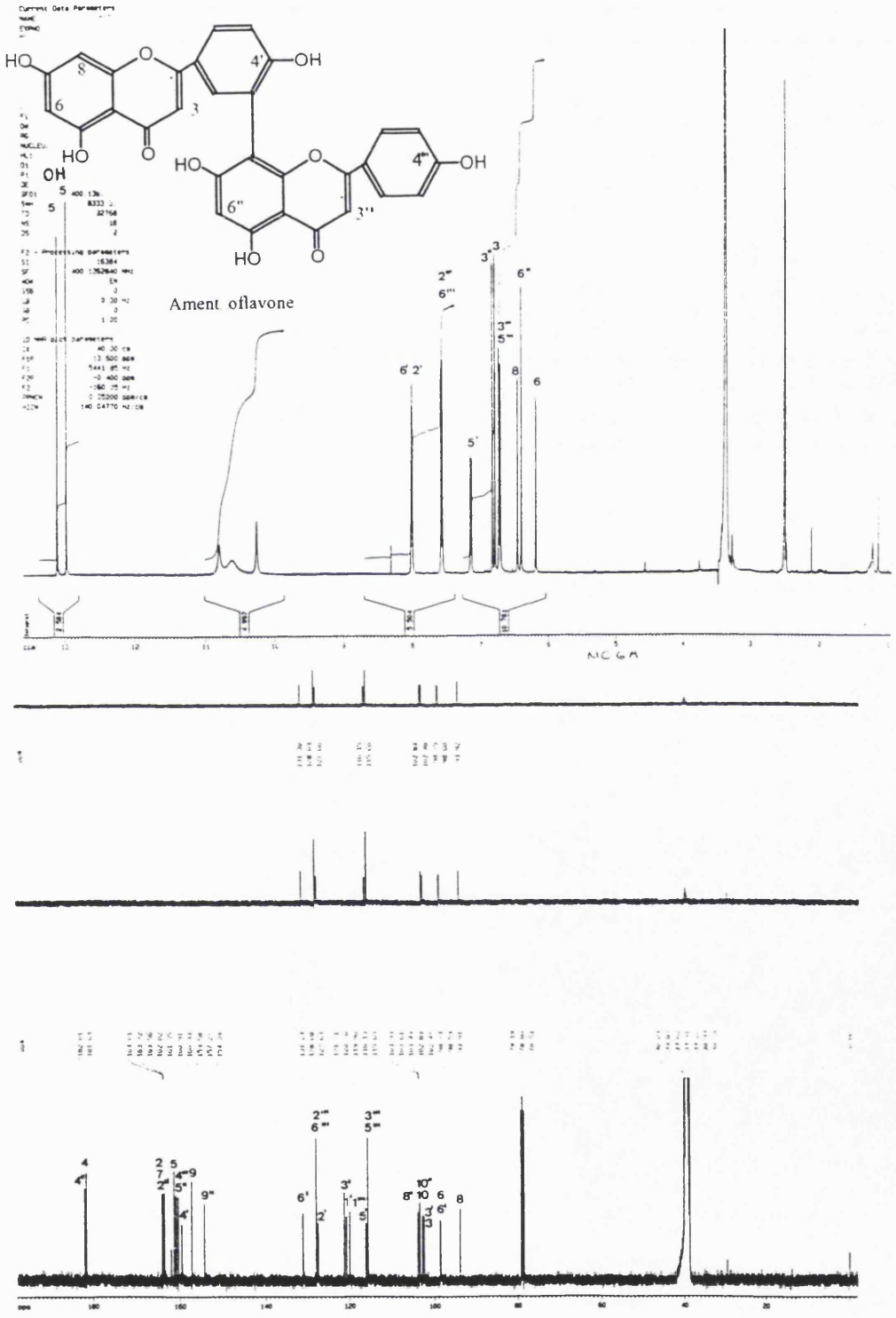


S-20. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of acetylcelaenodendrolide



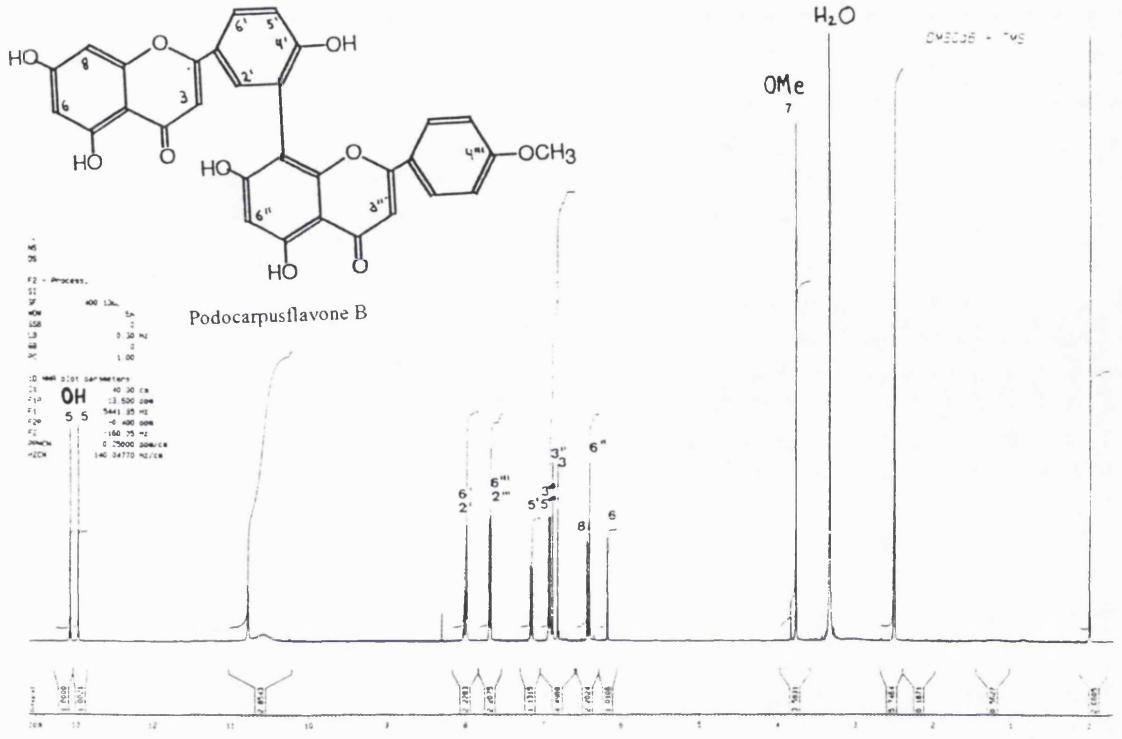
S-21. ^{13}C NMR (100, CDCl3) spectrum of acetylcelaenodendrolide

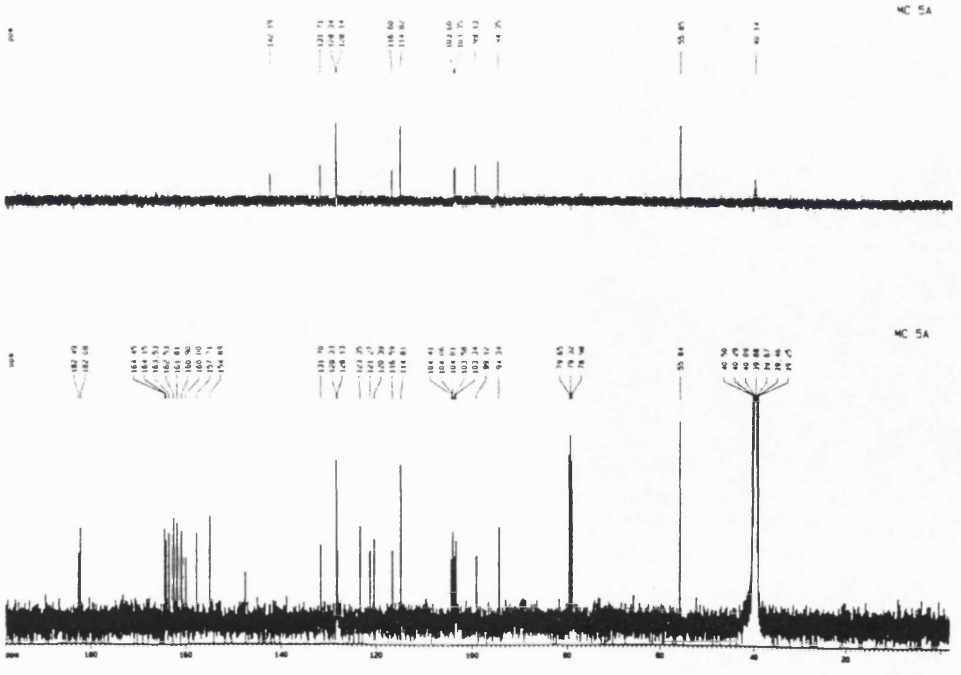
S-22. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of amentoflavone



S-23. ^{13}C NMR (100, DMSO-d_6) spectrum of amentoflavone

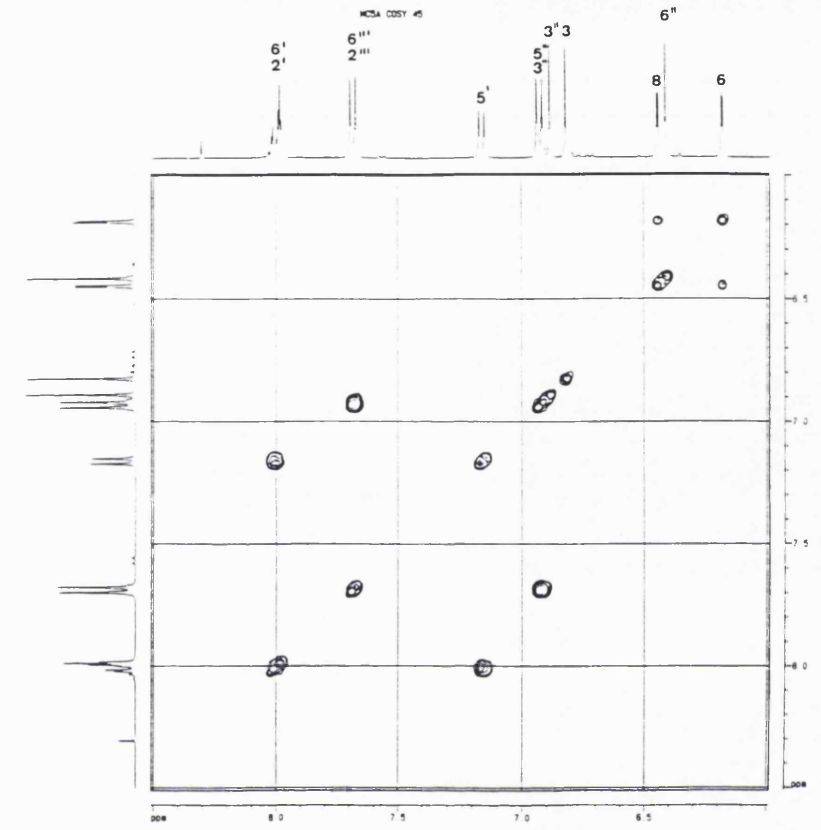
S-24 ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of podocarpusflavone B

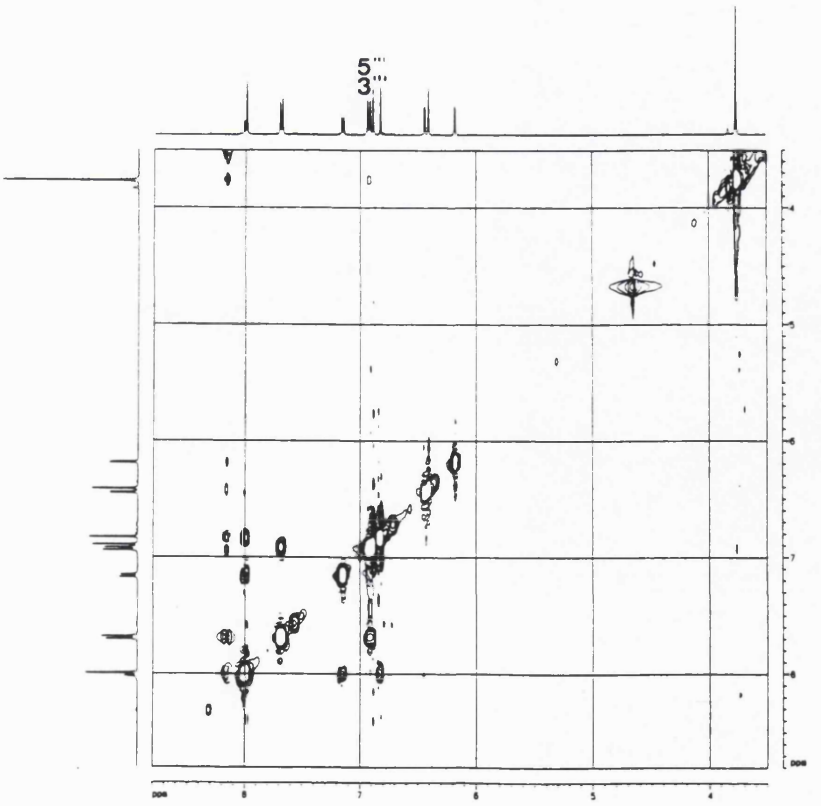




S-25 ^{13}C NMR (100, DMSO-d_6) spectrum of podocarpusflavone B

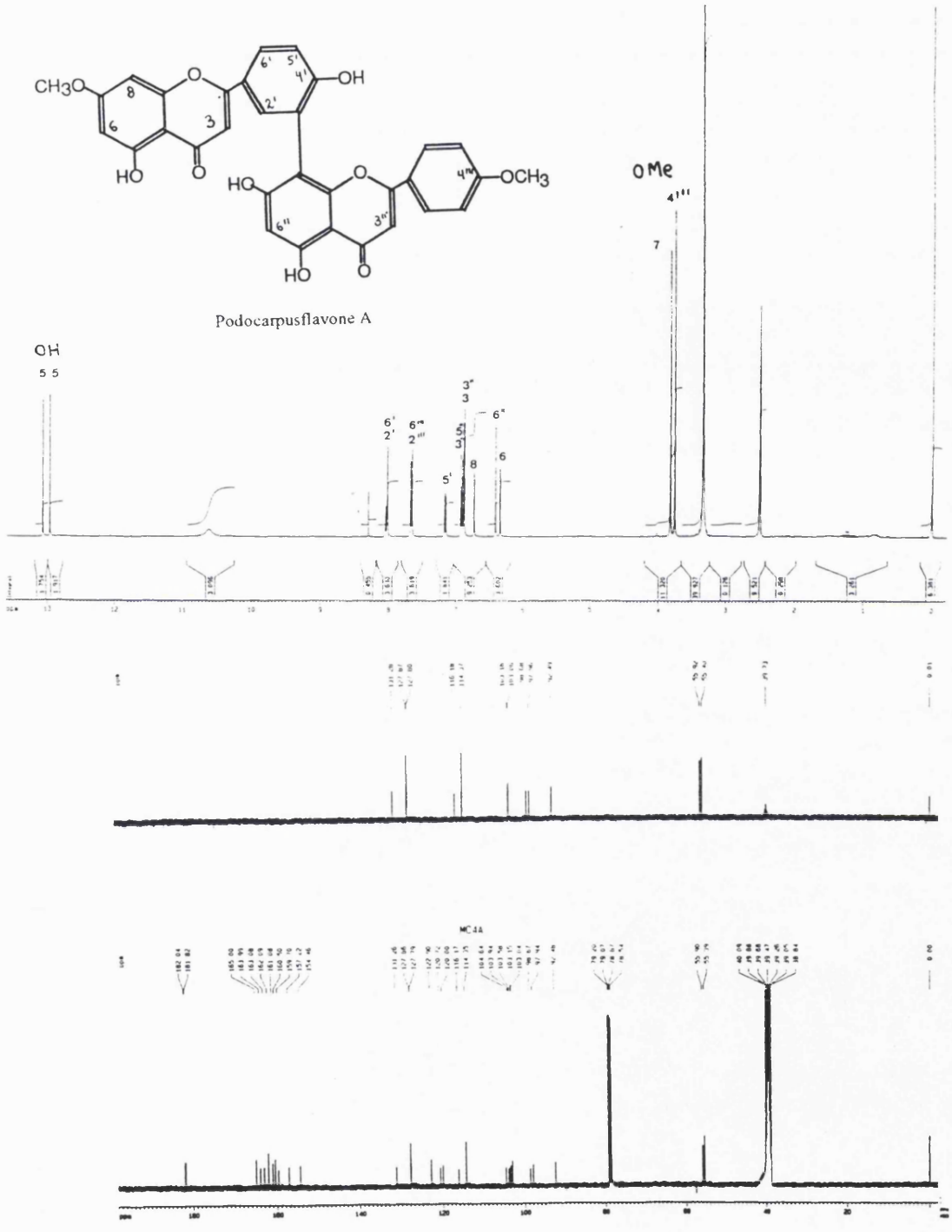
S-26 COSY-45 spectrum of podocarpusflavone B





S-27 NOESY spectrum of podocaarpusflavone B

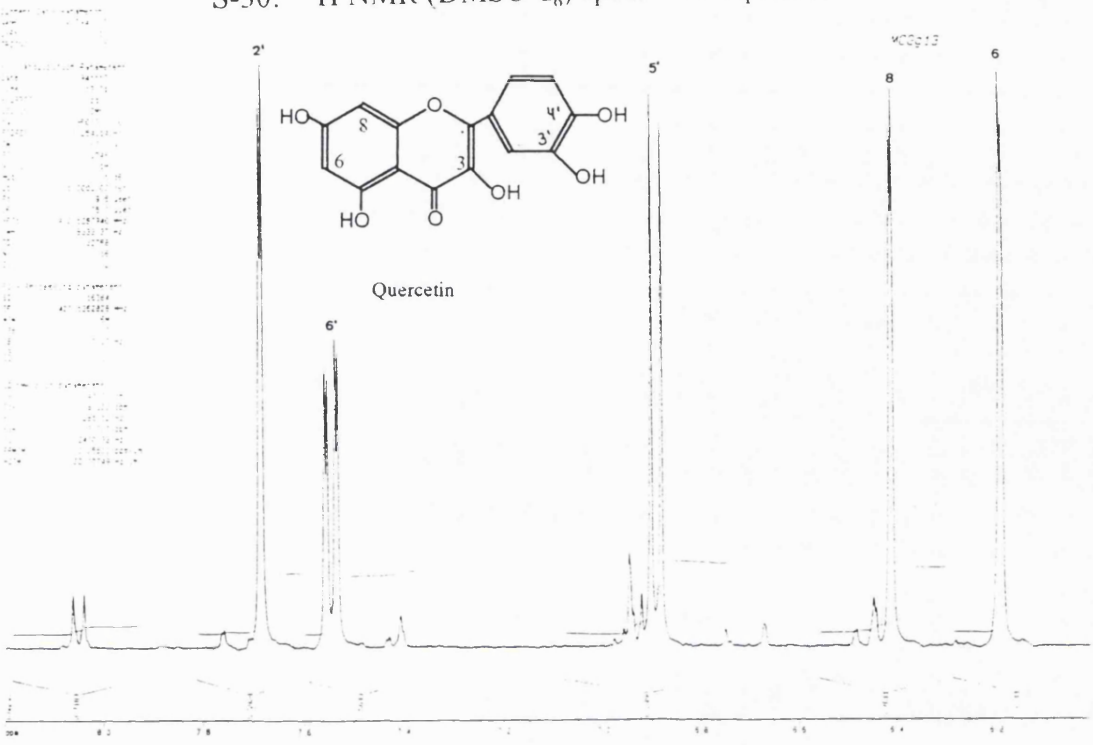
S-28 . ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of podocarpusflavone A

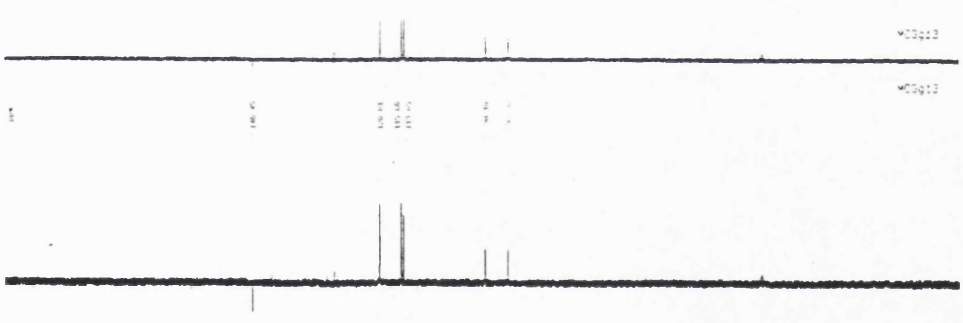


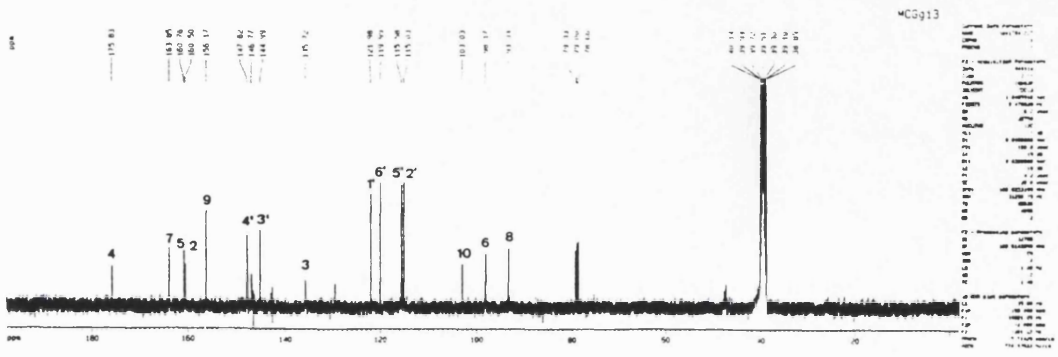
Podocarpusflavone A

S-29 . ^{13}C NMR (100, DMSO-d_6) spectrum of podocarpusflavone A

S-30. ^{1}H NMR (DMSO-d_6) spectrum of quercetin

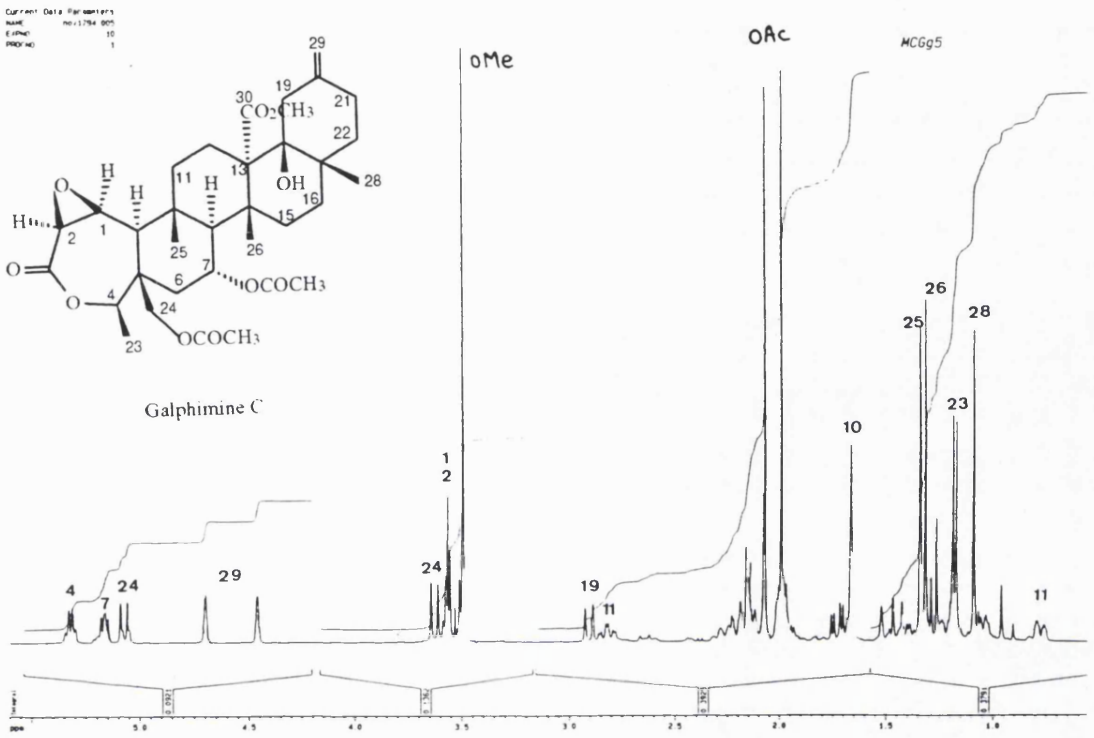


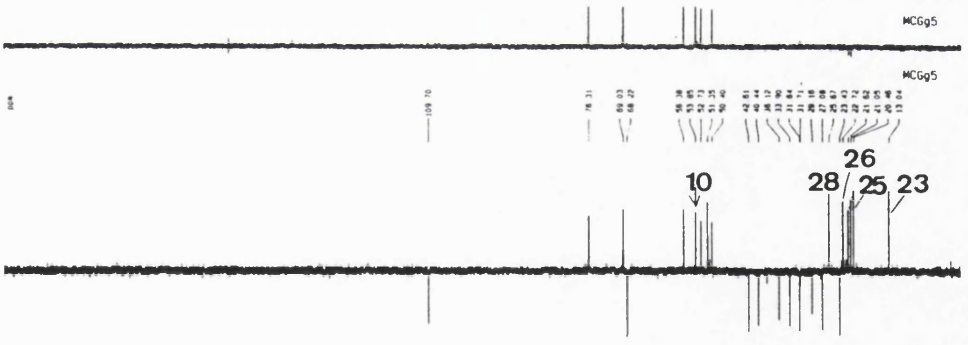


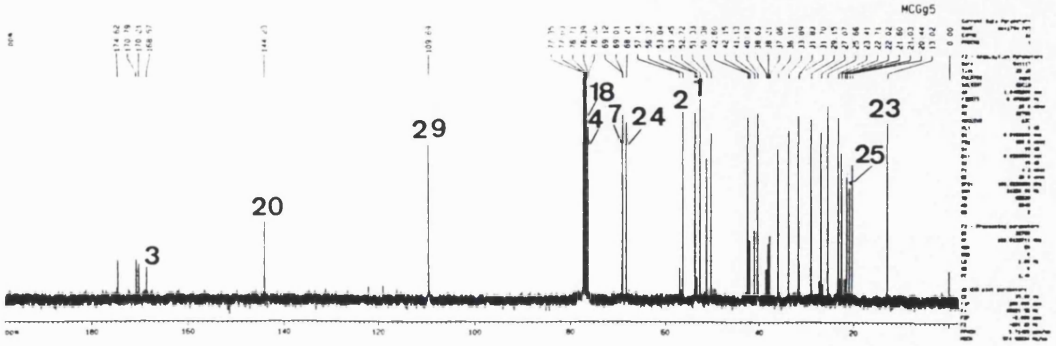


S-31. ^{13}C NMR (DMSO-d_6) spectrum of quercetin

S-32. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of galphimine C

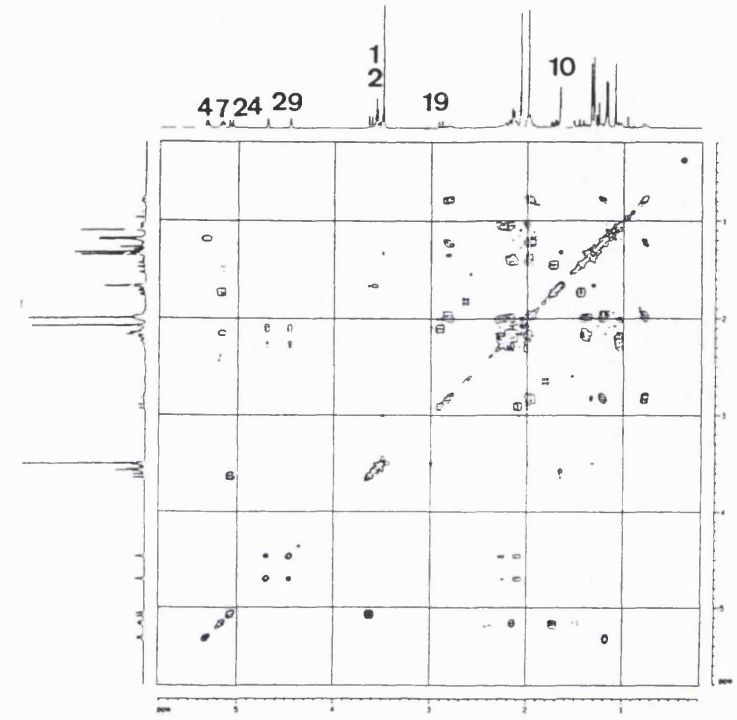


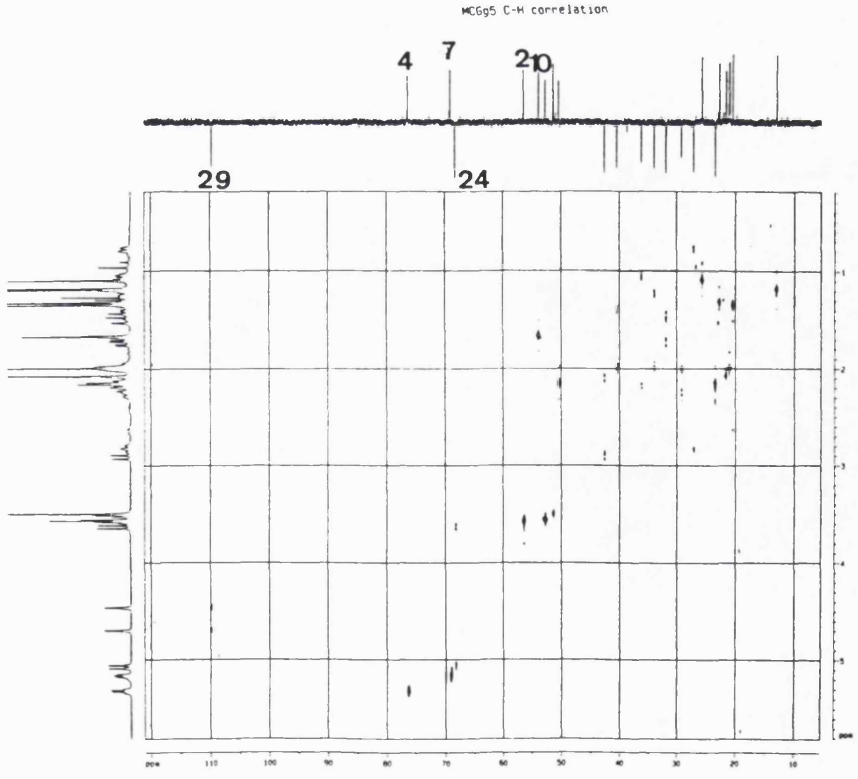




S-33. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of galphimine C

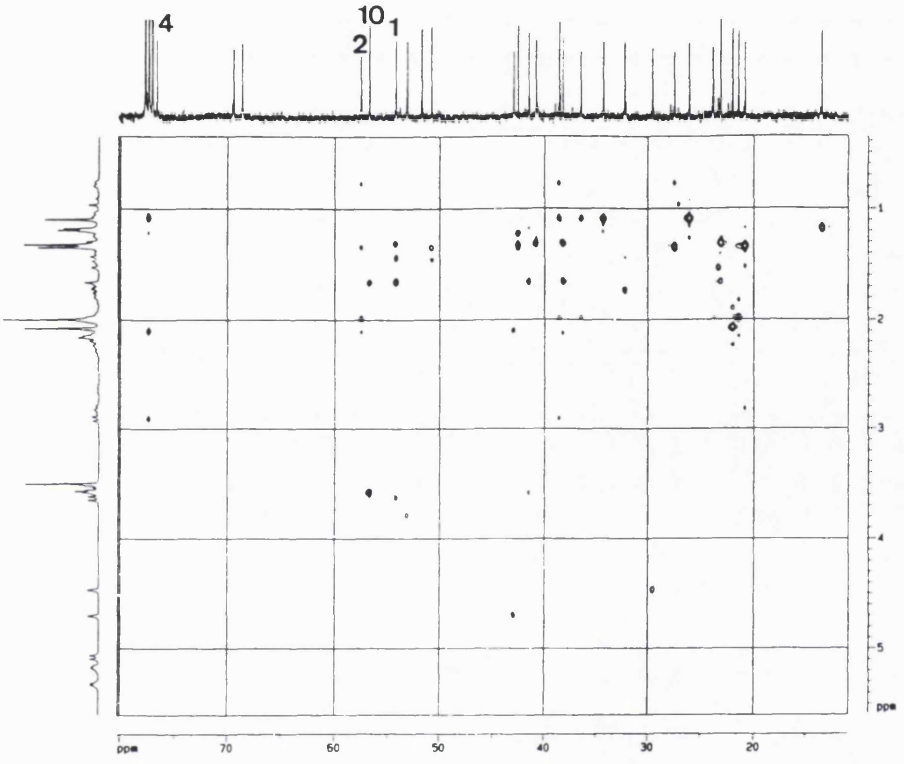
S-34. COSY-45 spectrum of galphimine C

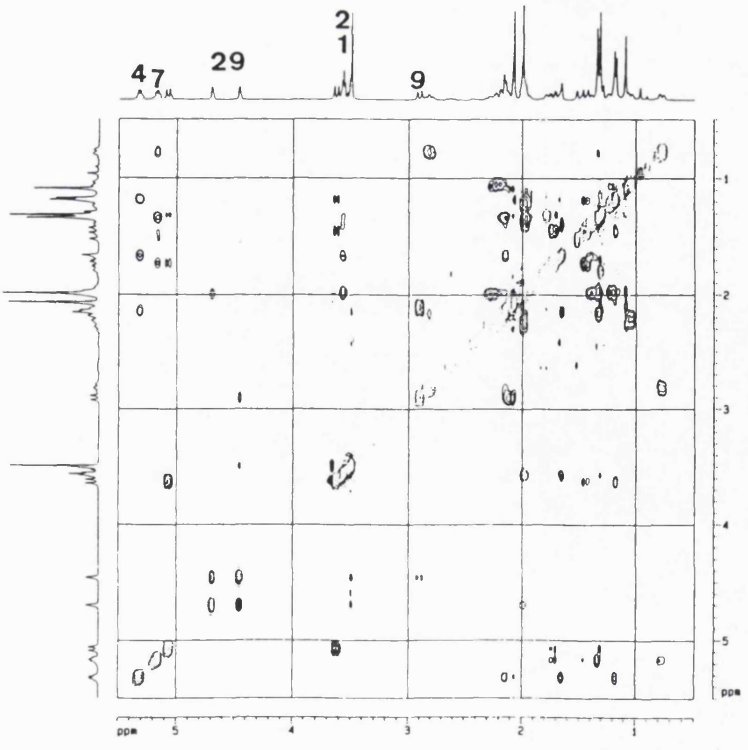




S-35. HMQC spectrum of galphimine C

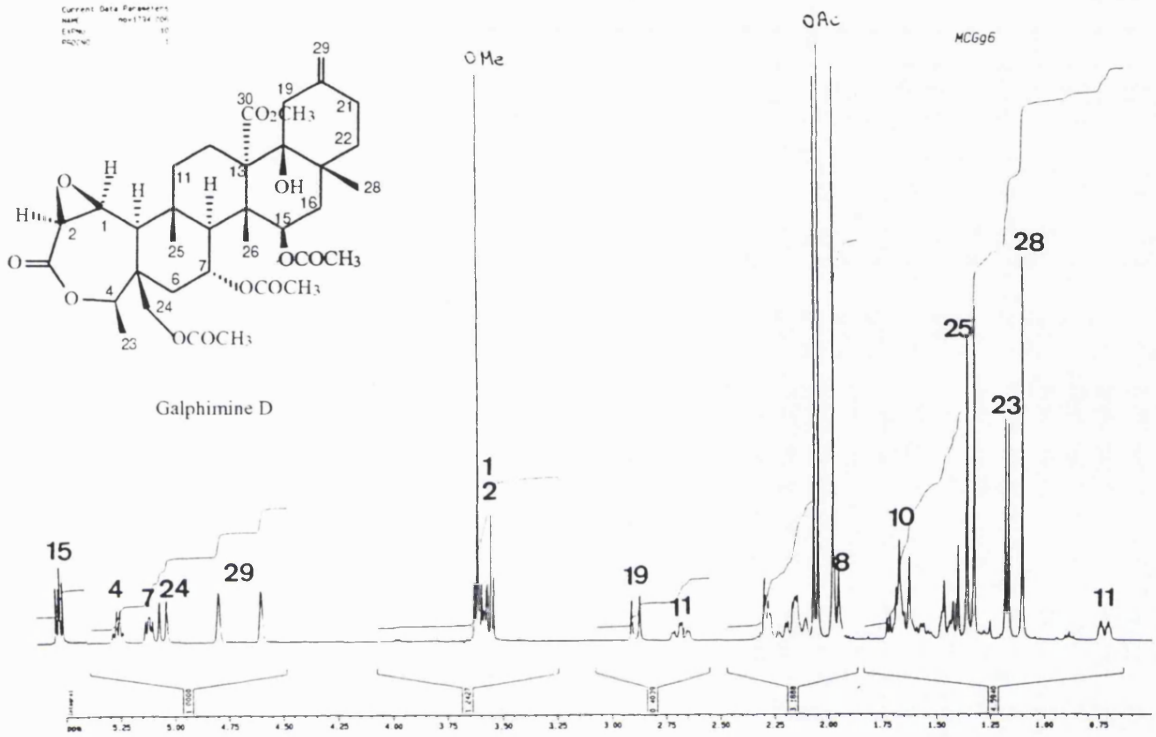
S-36. COLOC spectrum of galphimine C

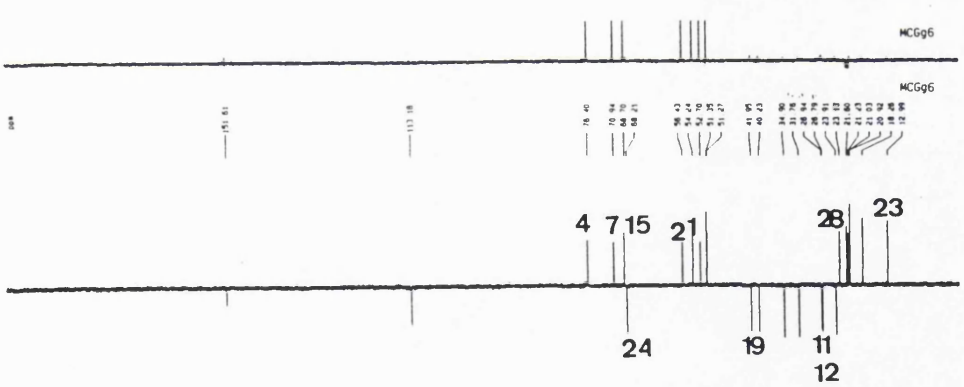


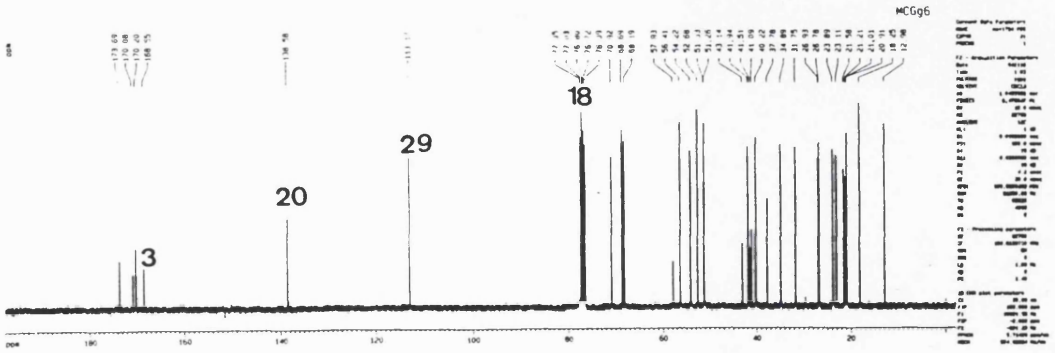


S-37. NOESY spectrum of galphimine C

S-38. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of galphimine D

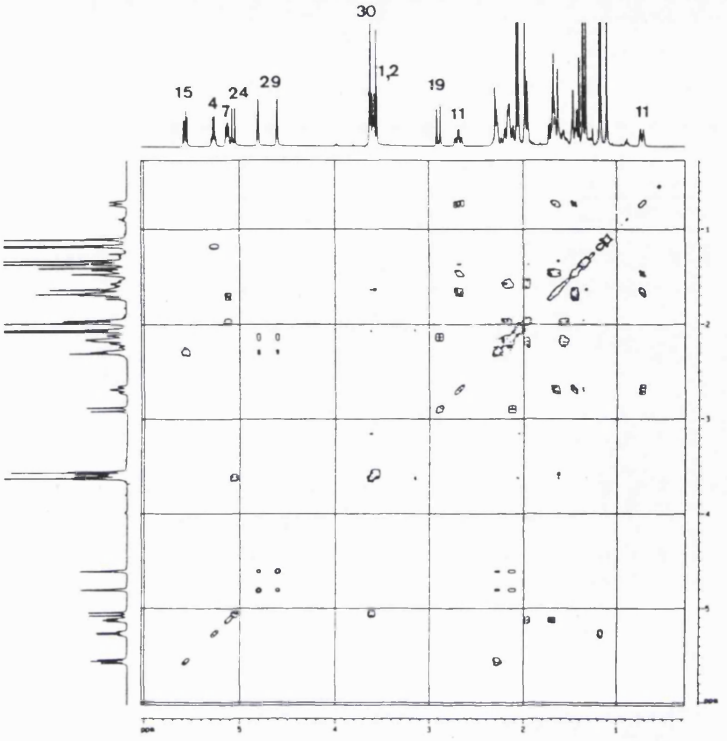


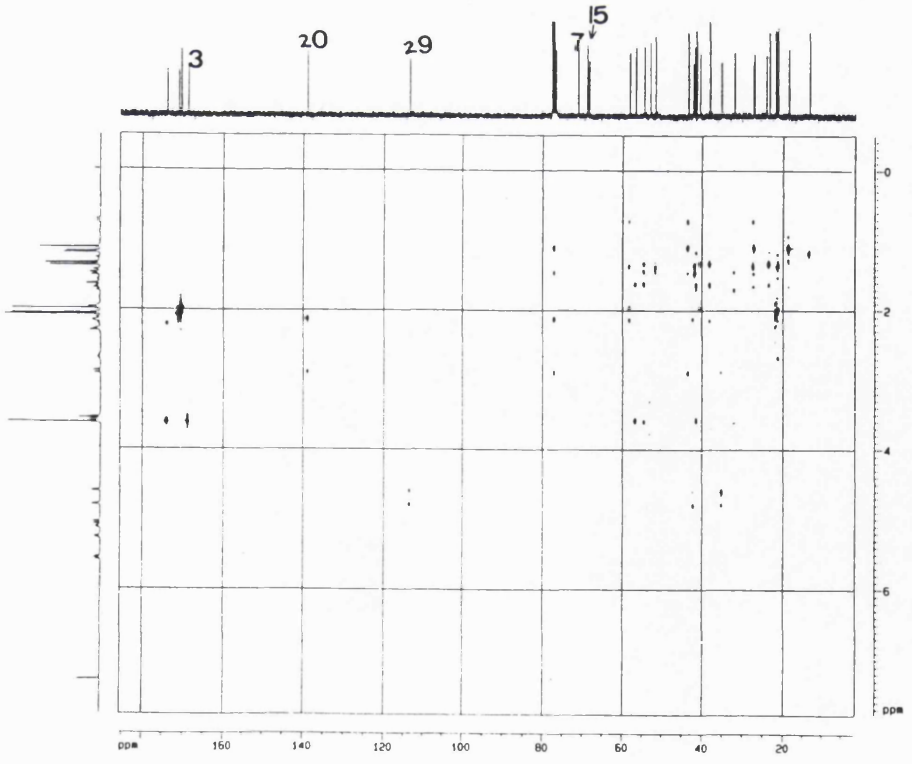




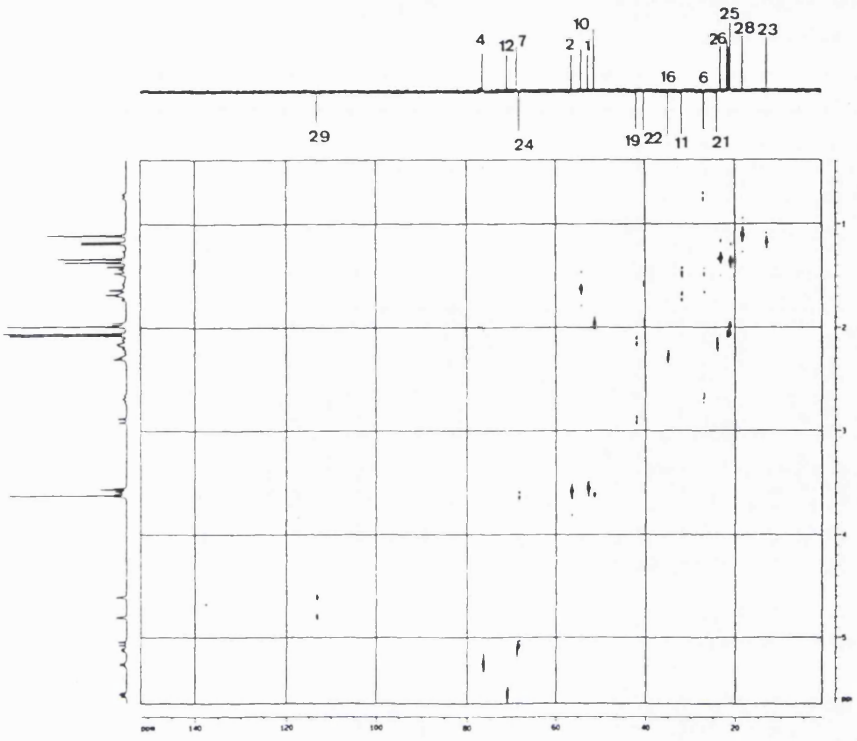
S-39. ^{13}C NMR (100MHZ, $CDCl_3$) spectrum of galphimine D

S-40. COSY-45 spectrum of galphimine D

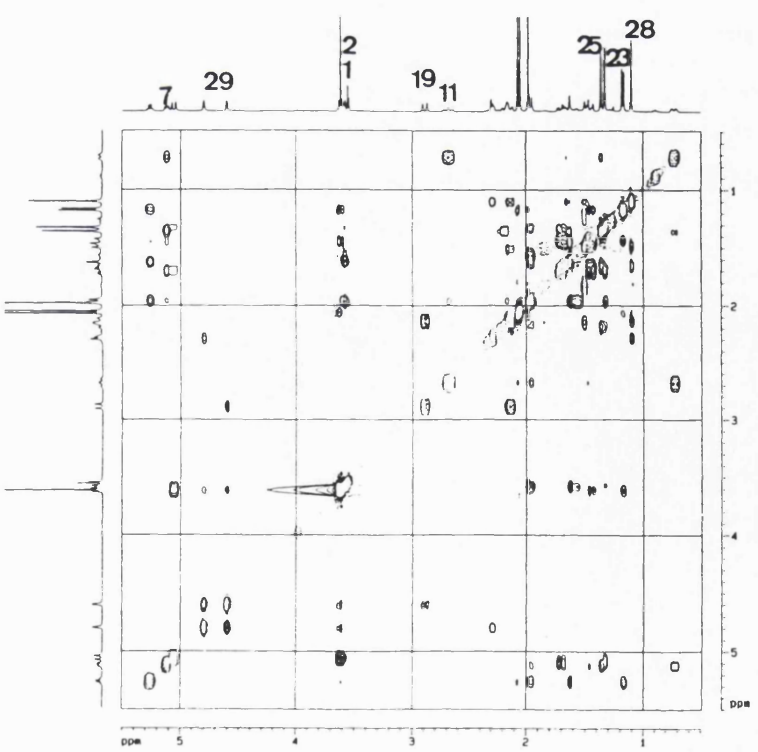




S-41. HMQC spectrum of galphimine D

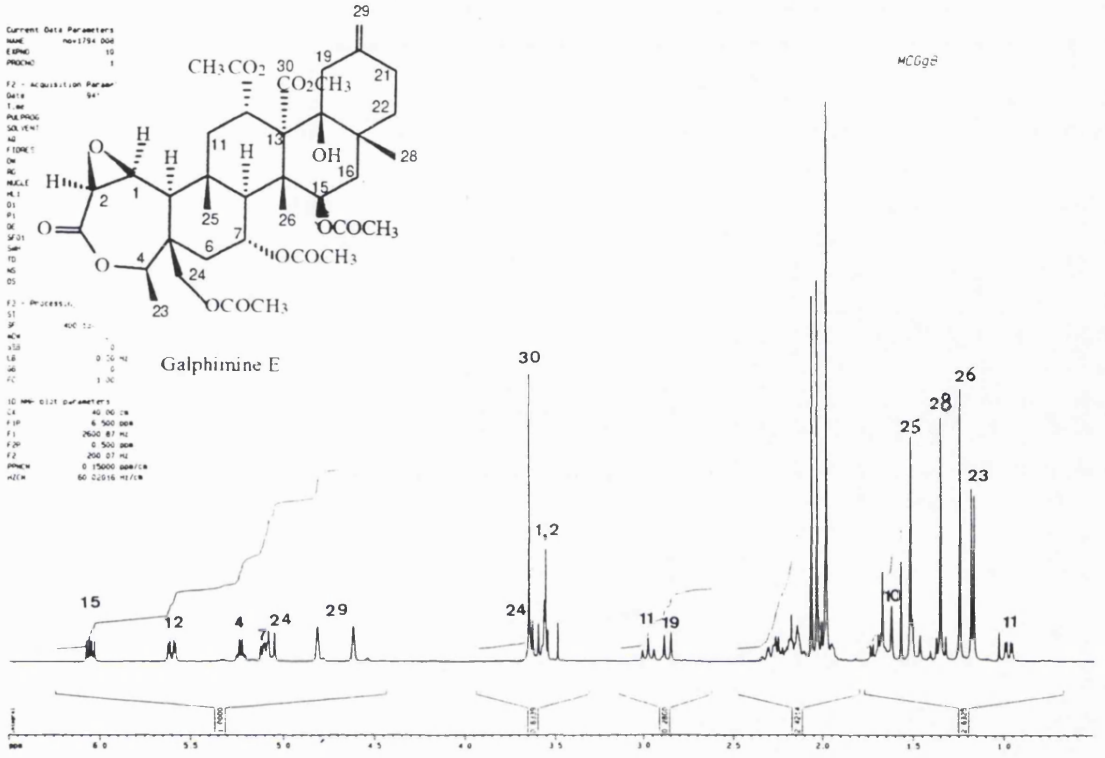


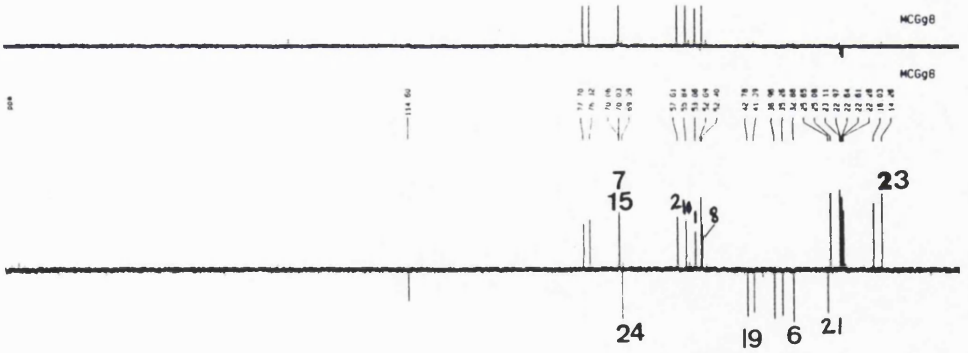
S-42. COLOC spectrum of galphimine D

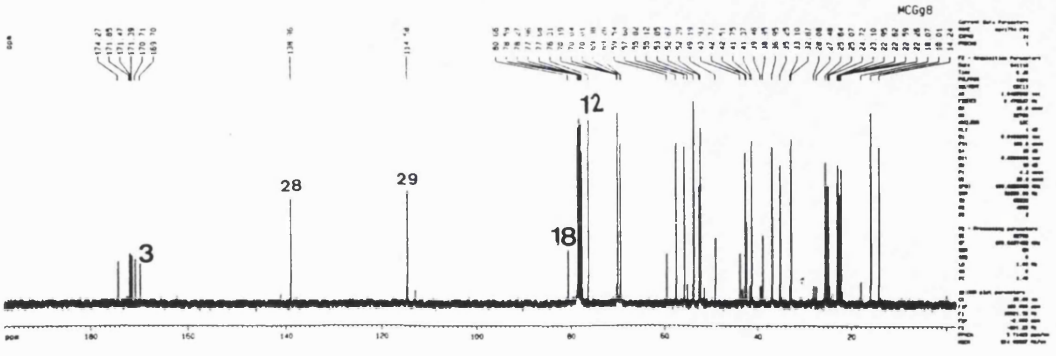


S-43. NOESY spectrum of galphimine D

S-44. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of galphimine E

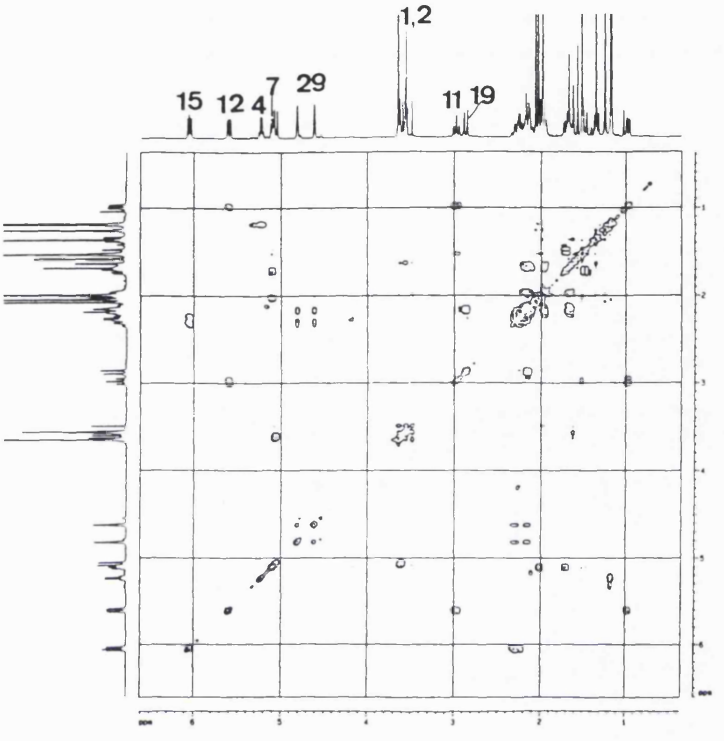


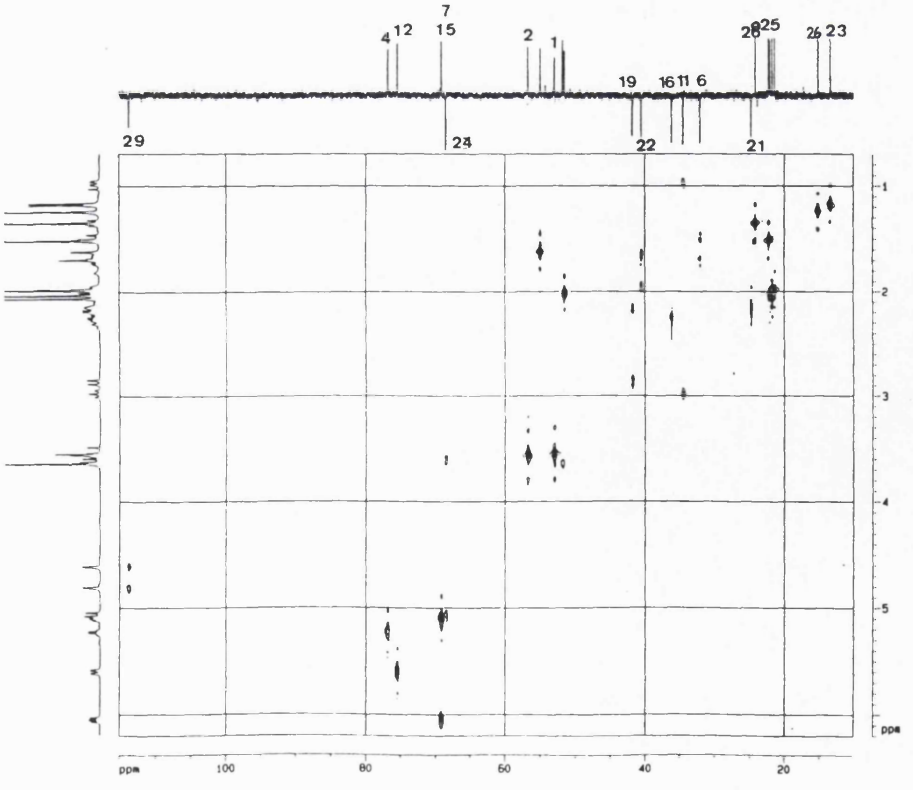




S-45. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of galphimine E

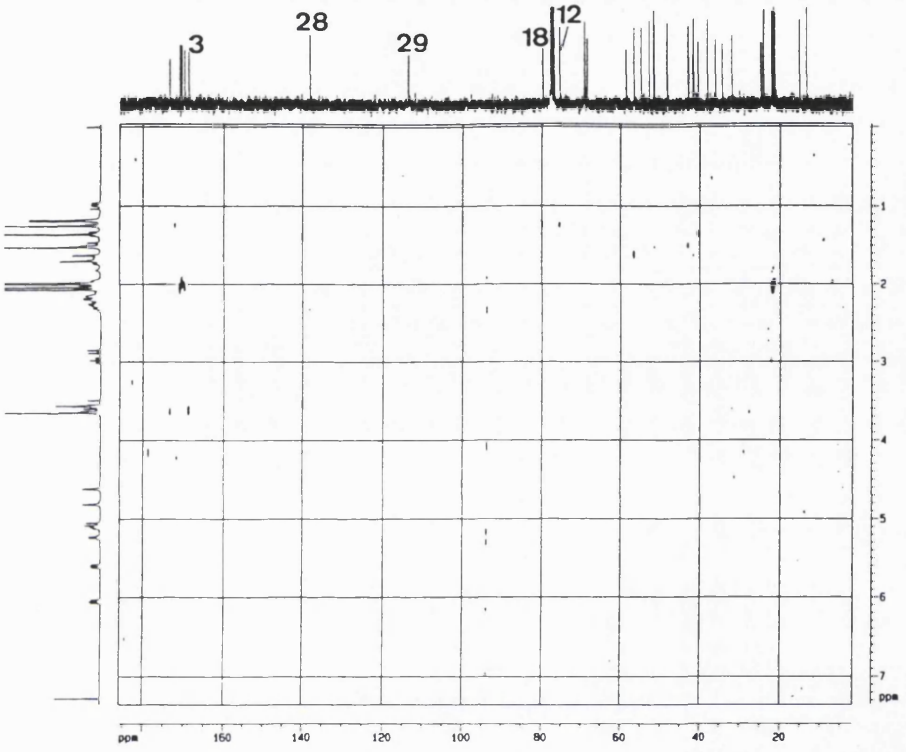
S-46. COSY-45 spectrum of galphimine E

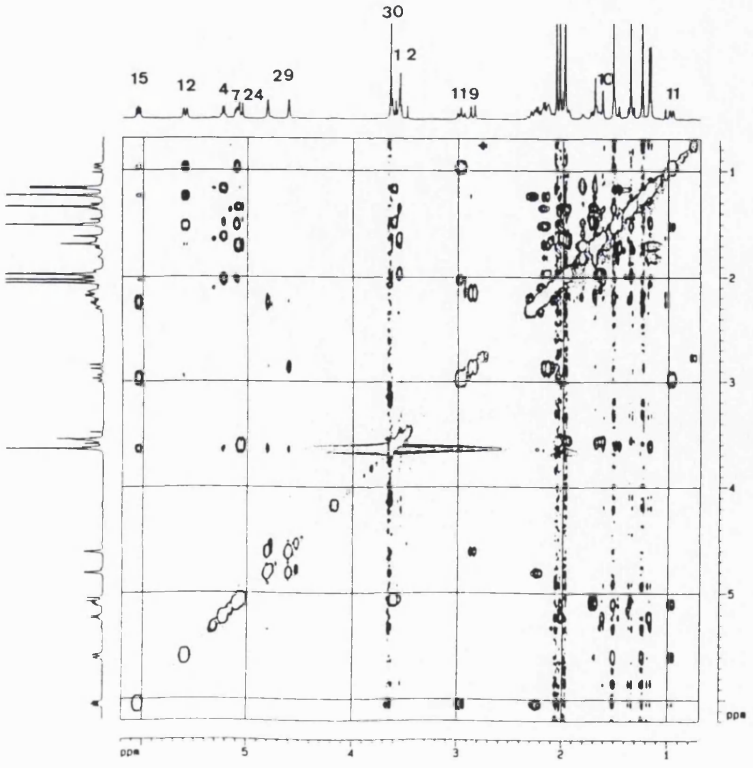




S-47. HMQC spectrum of galphimine E

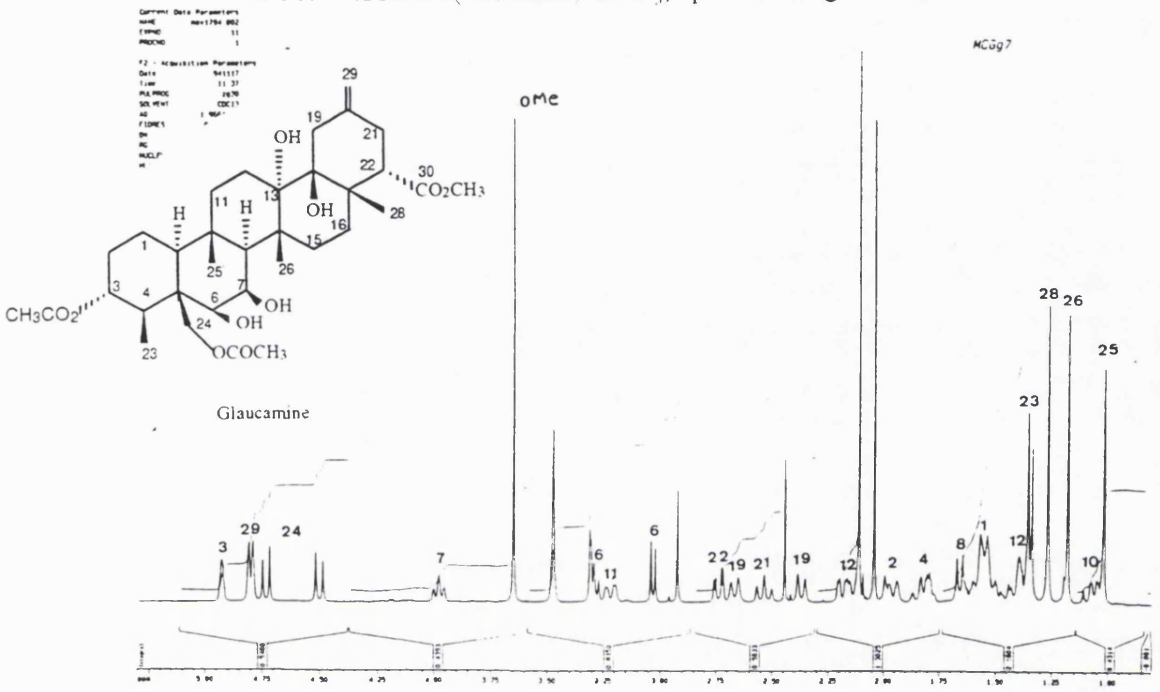
S-48. COLOC spectrum of galphimine E

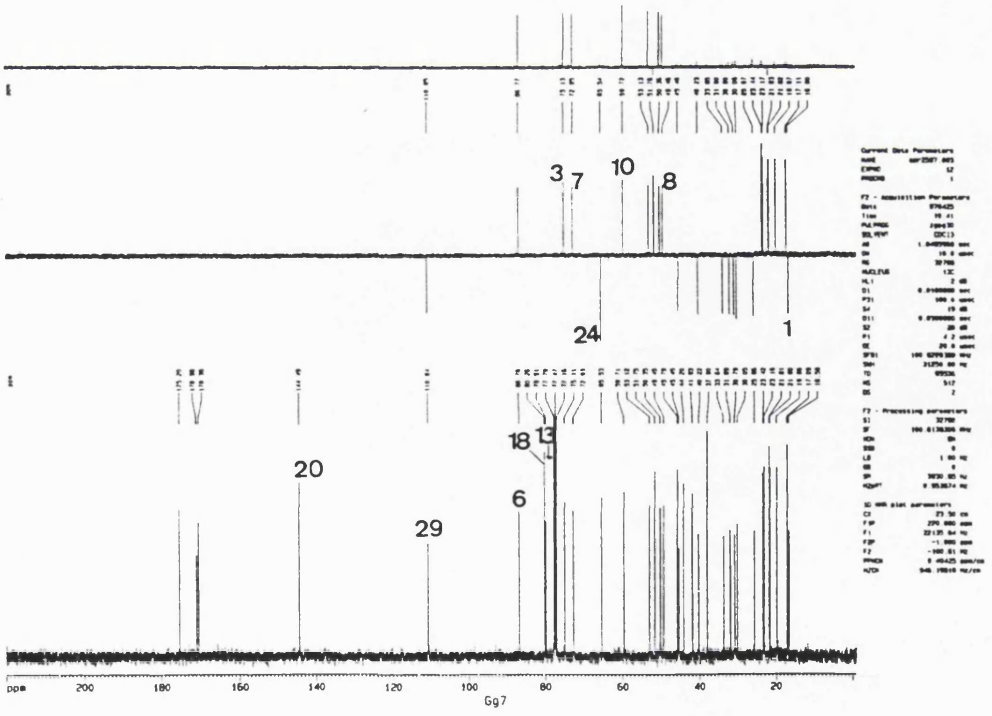




S-49. NOESY spectrum of galphimine E

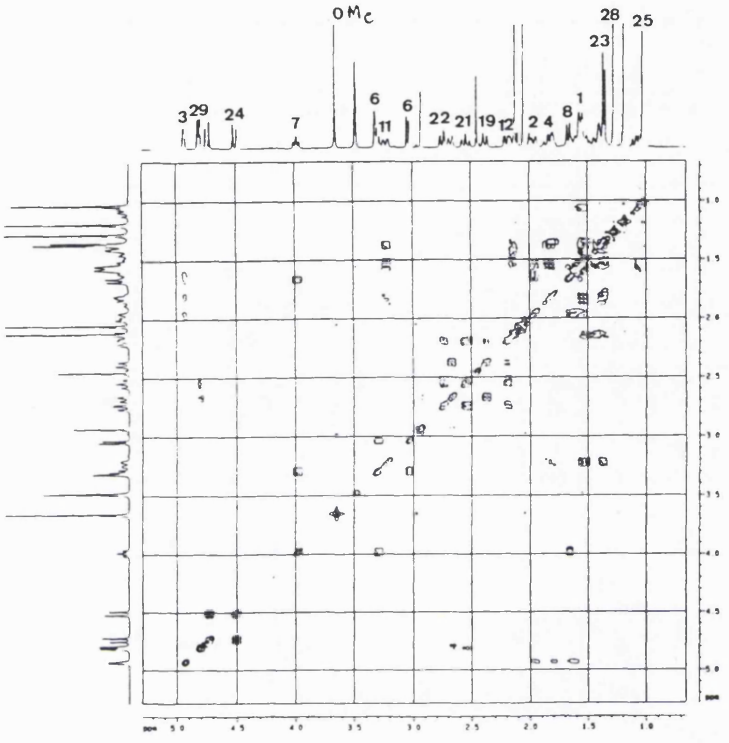
S-50. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of glaucamine

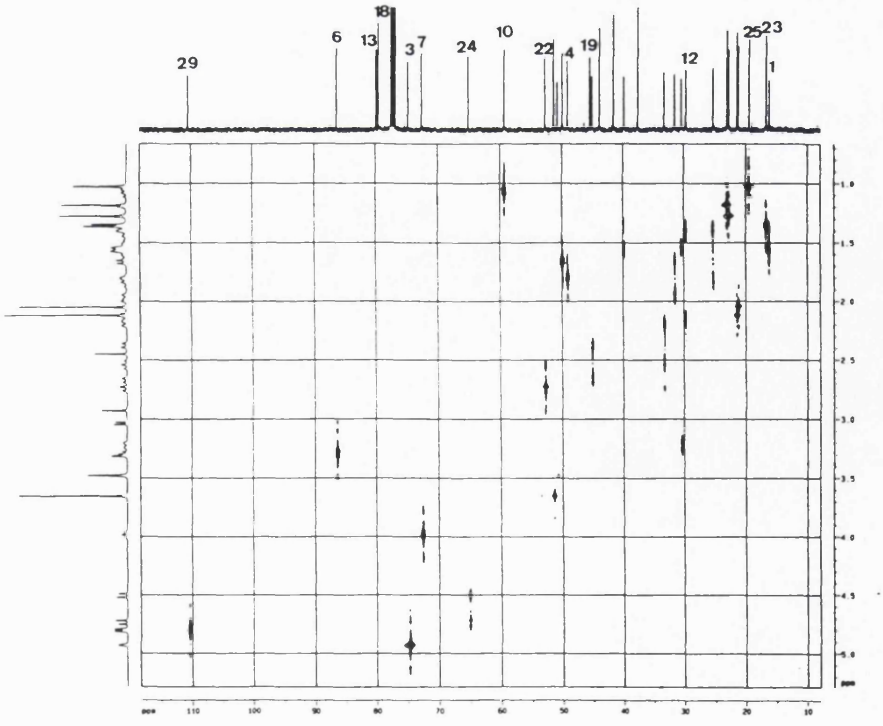




S-51. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of glaucamine

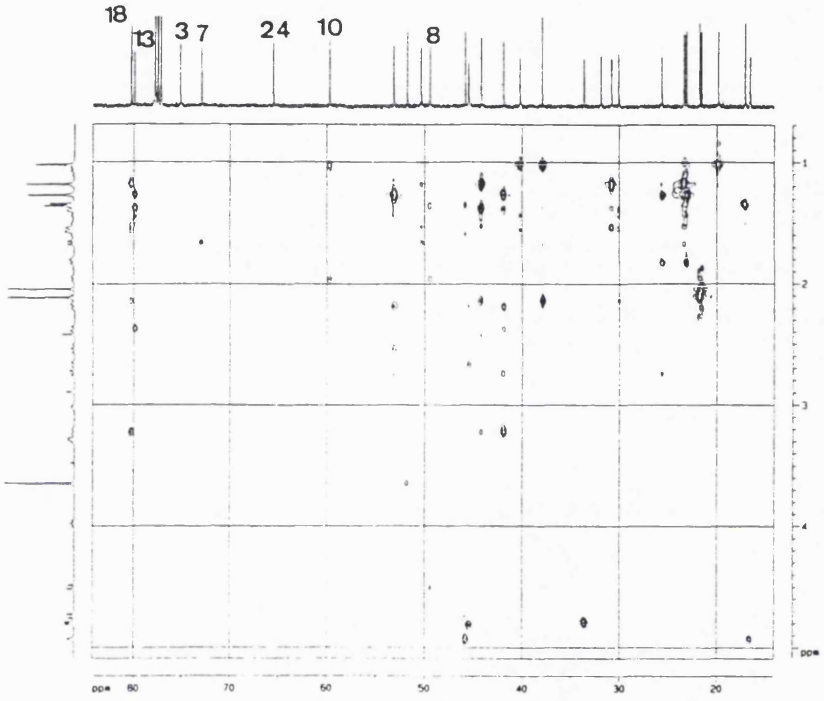
S-52. COSY-45 spectrum of glaucamine



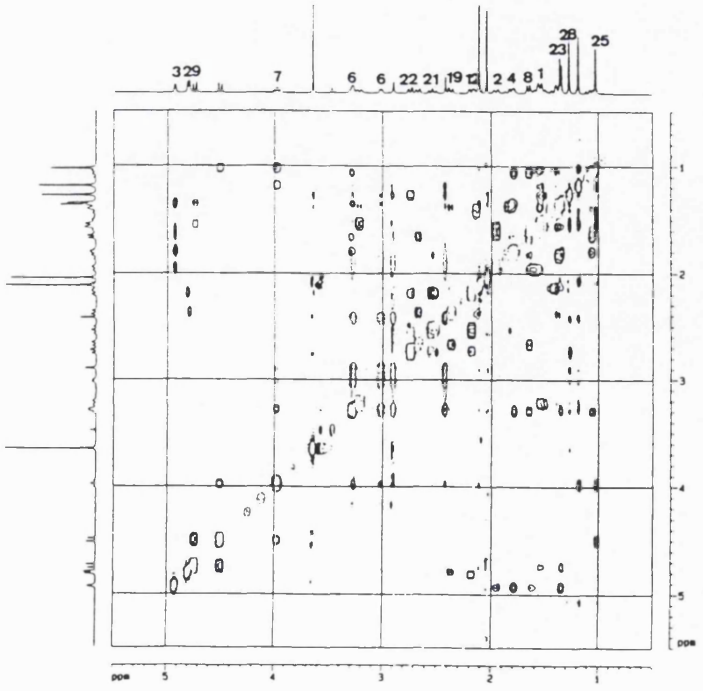


S-53. HMQC spectrum of glaucamine

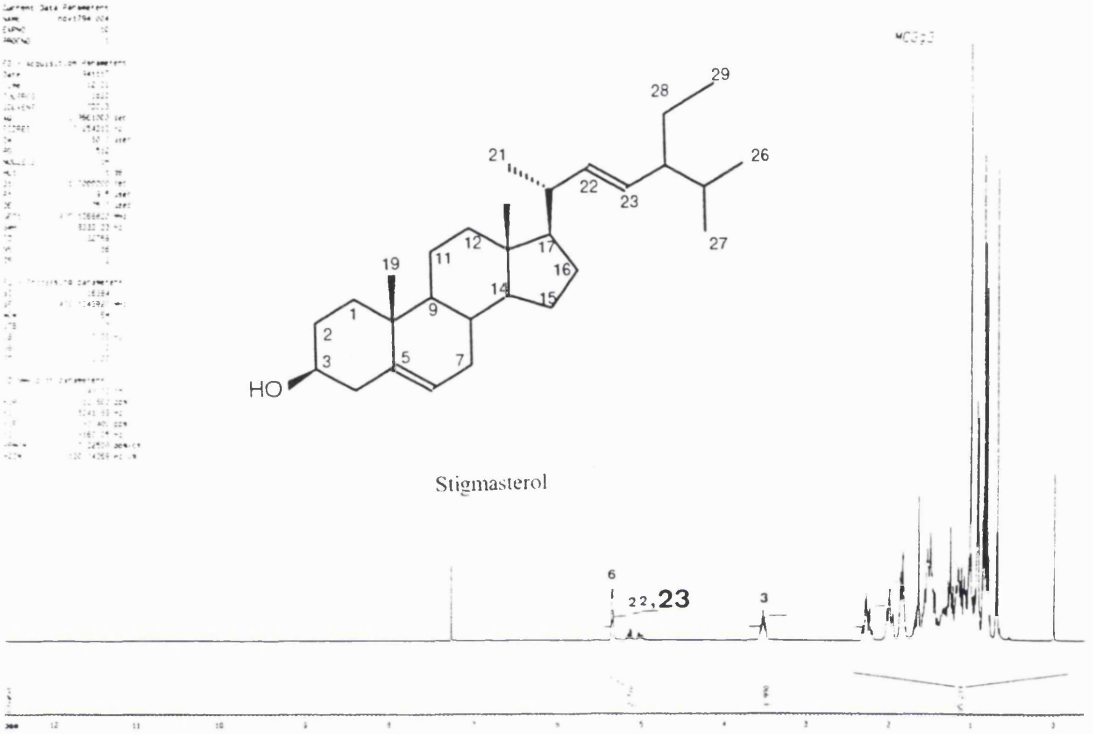
S-54. COLOC spectrum of glaucamine

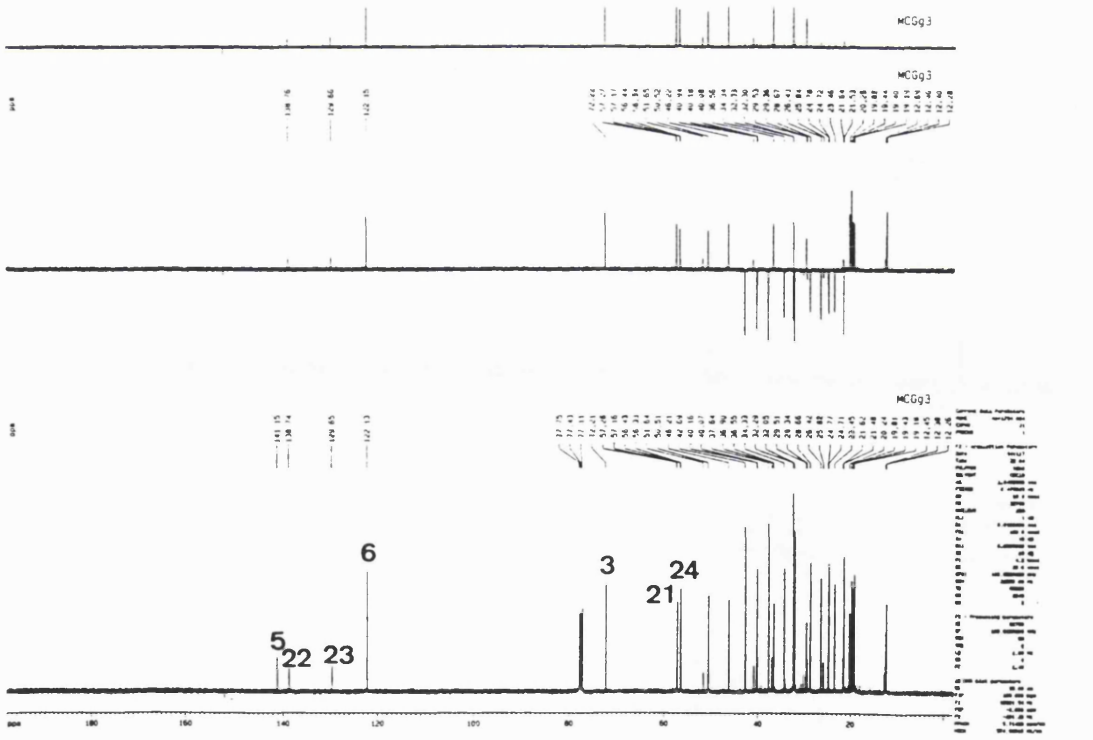


S-55. NOESY spectrum of glaucamine

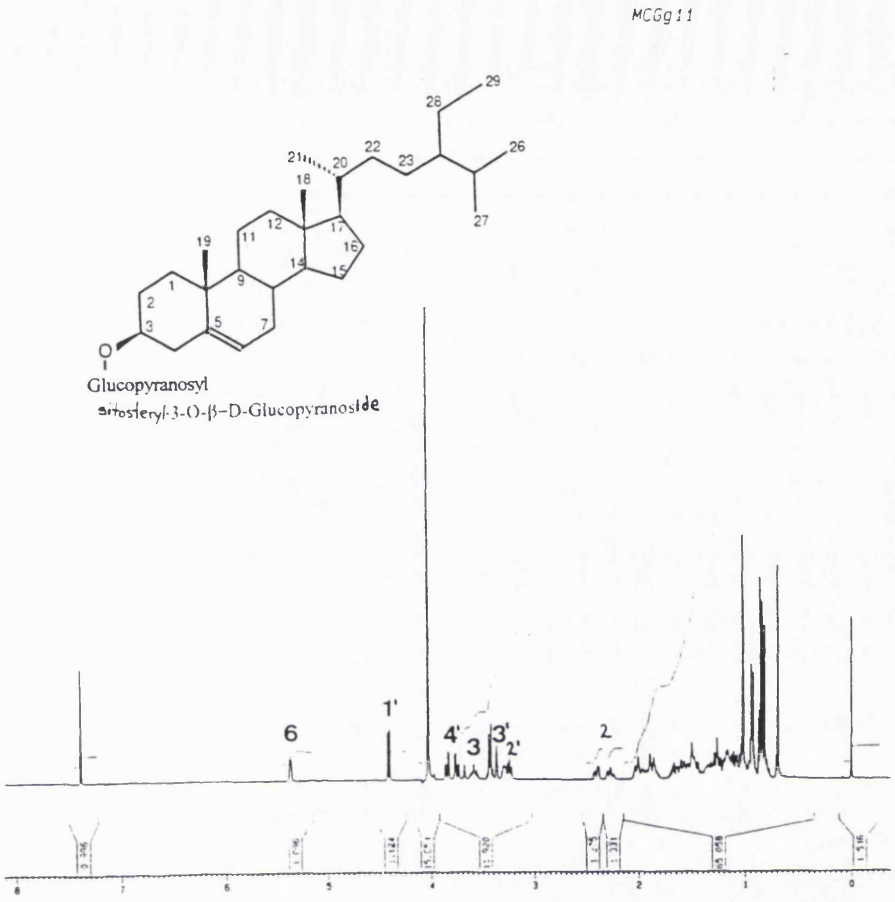


S-56. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of stigmasterol

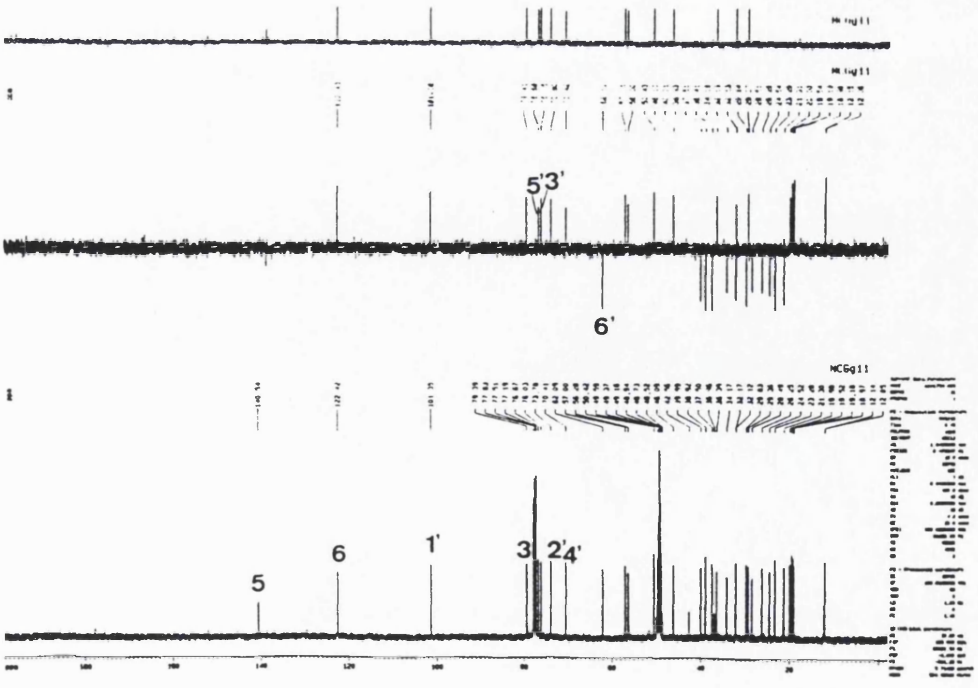




S-57. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of stigmasterol

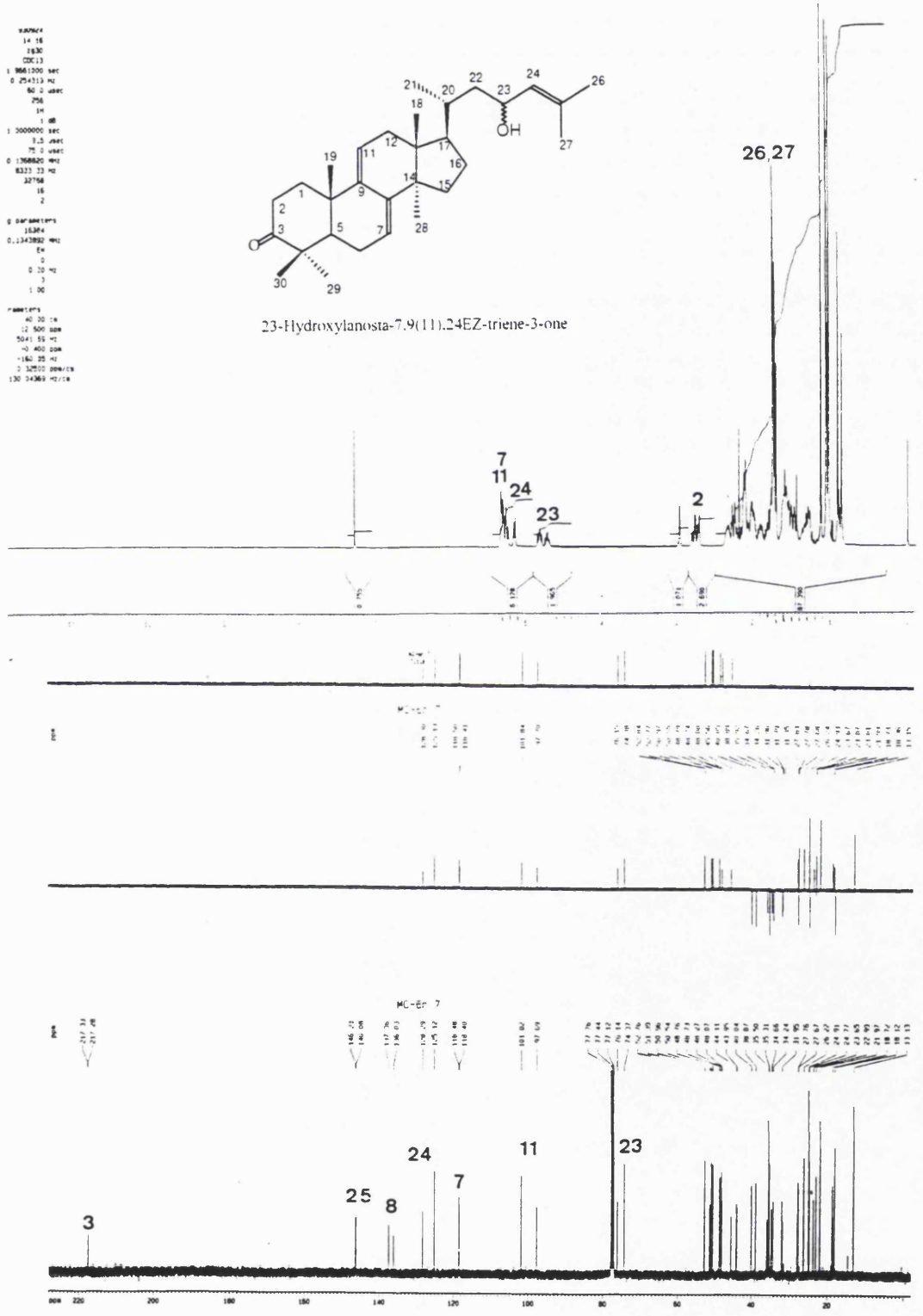


S-58. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of sitosteryl-3-O-β-D-glucopyranoside



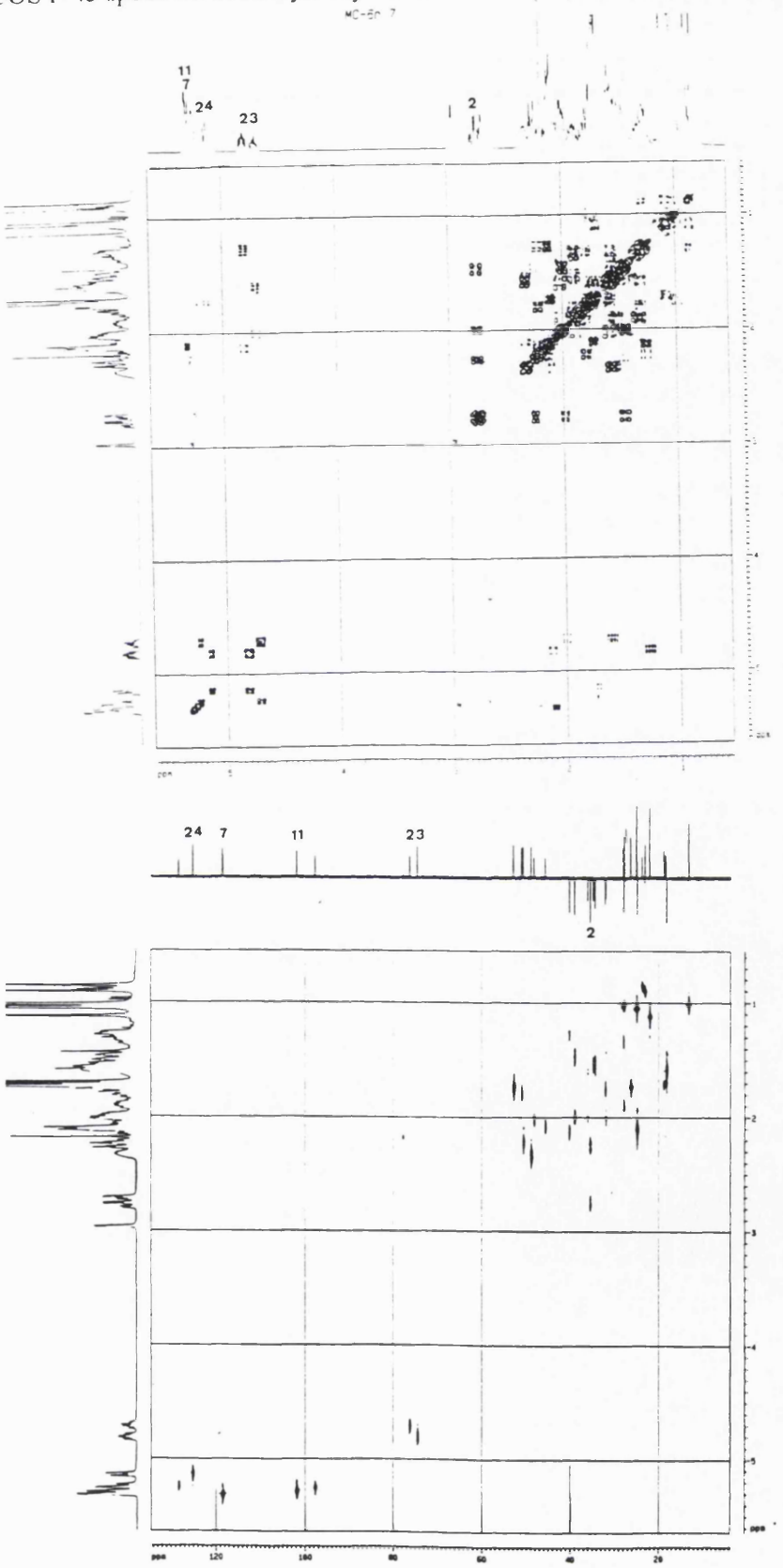
S-59. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of sitosteryl-3-O-β-D-glucopyranoside

S-60. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 23-hydroxy-5α-lanosta-7,9(11),24EZ- triene-3-one



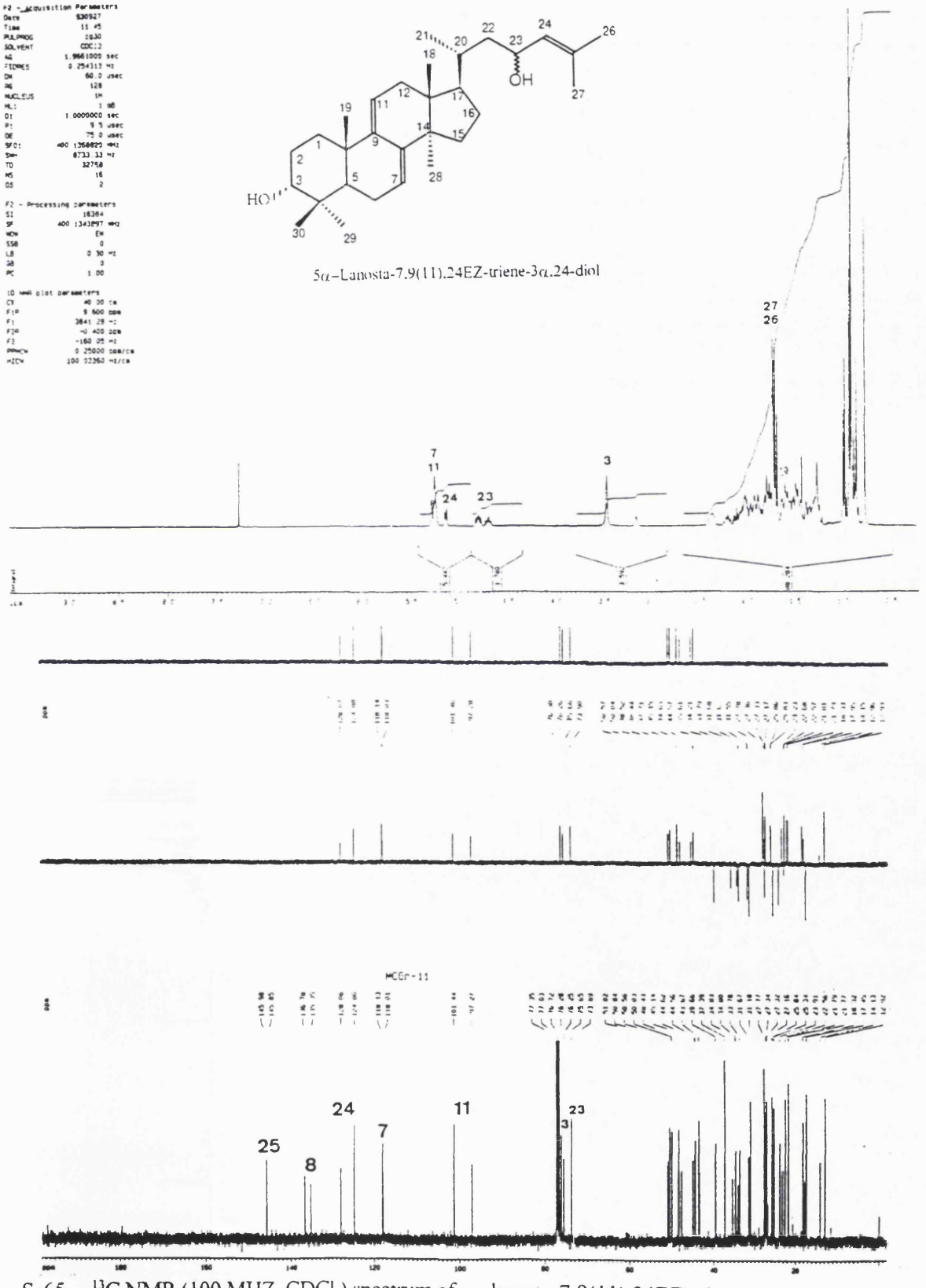
S-61. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of 23-hydroxy-5α-lanosta-7,9(11),24EZ- triene-3-one

S-62. COSY-45 spectrum of 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one

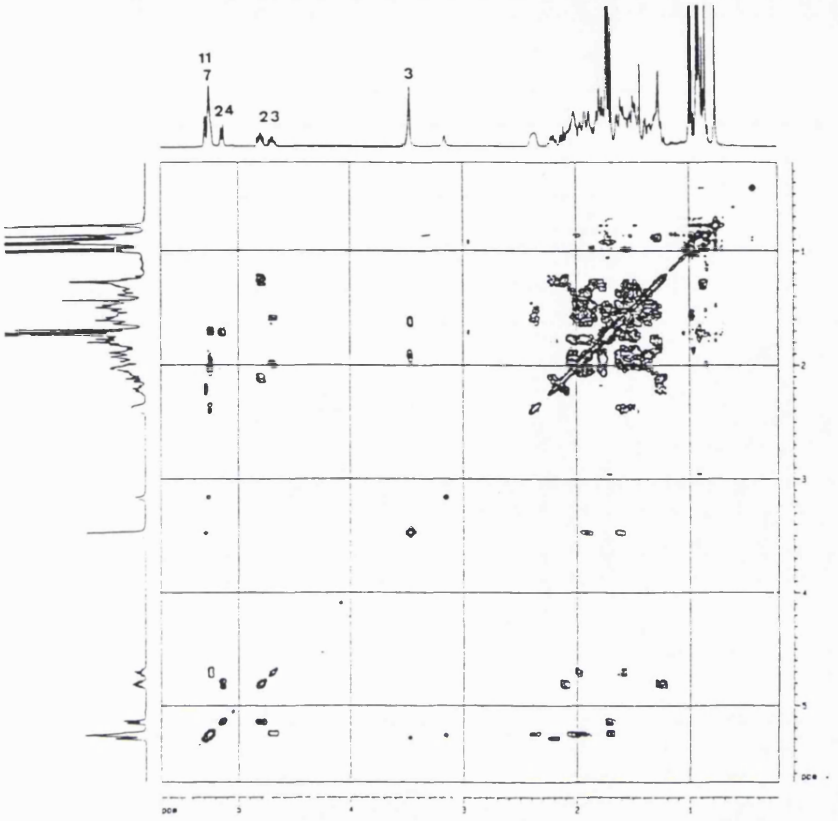


S-63. HMQC spectrum of 23-hydroxy-5α-lanosta-7,9(11),24EZ-triene-3-one

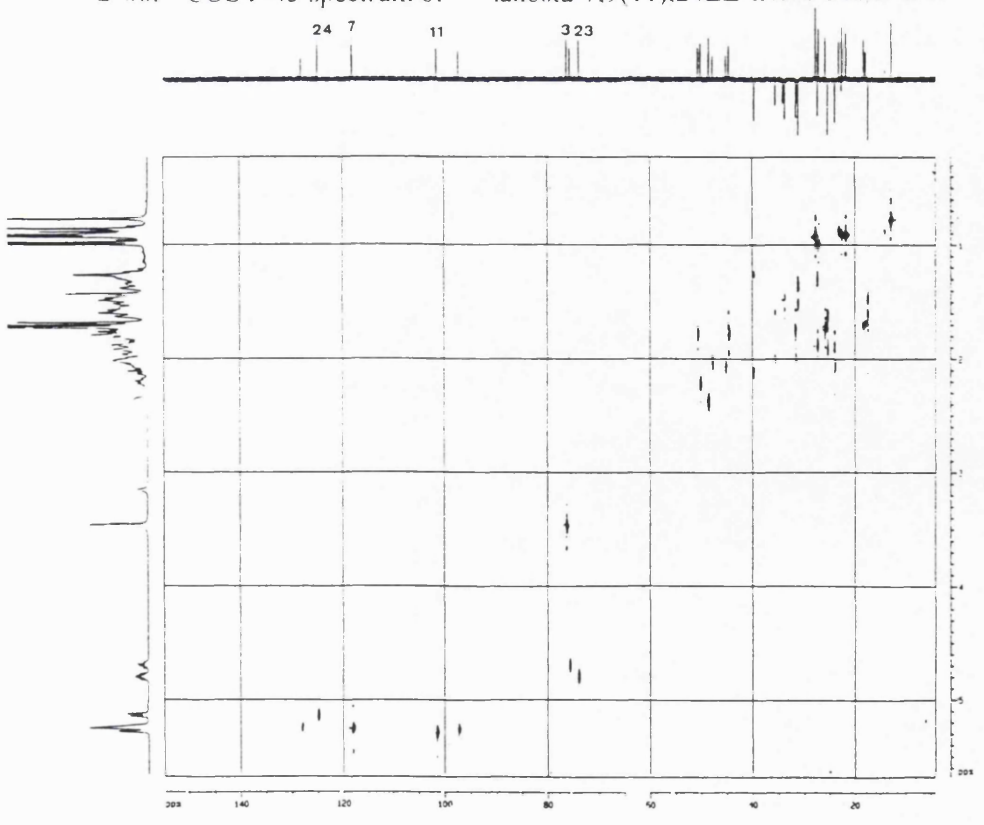
S-64. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of lanosta-7,9(11),24EZ-triene-3α,23- diol



S-65. ^{13}C NMR (100 MHZ, $CDCl_3$) spectrum of lanosta-7,9(11),24EZ-triene-3α,23- diol

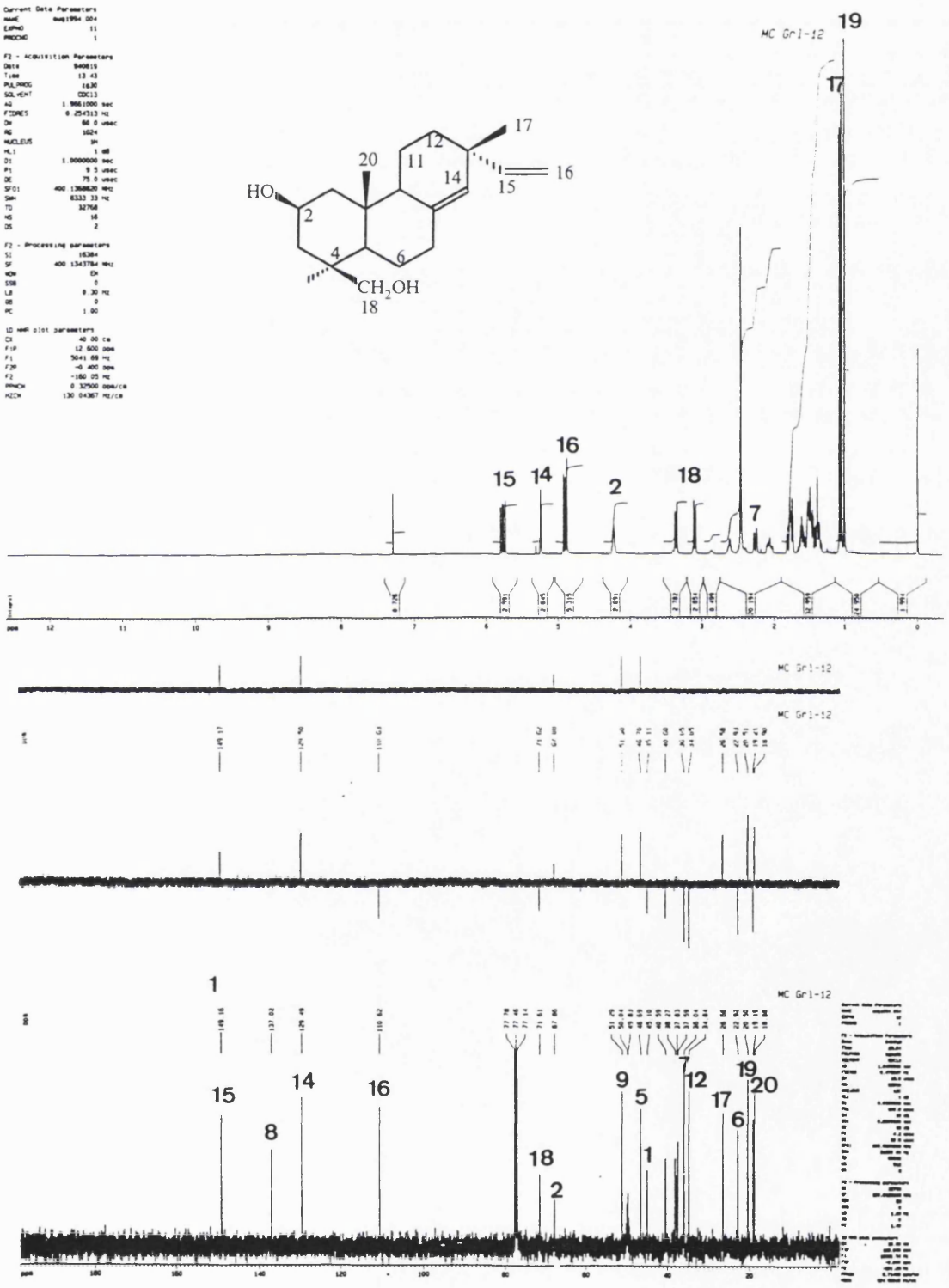


S-66. COSY-45 spectrum of lanosta-7,9(11),24EZ-triene-3α,23-diol



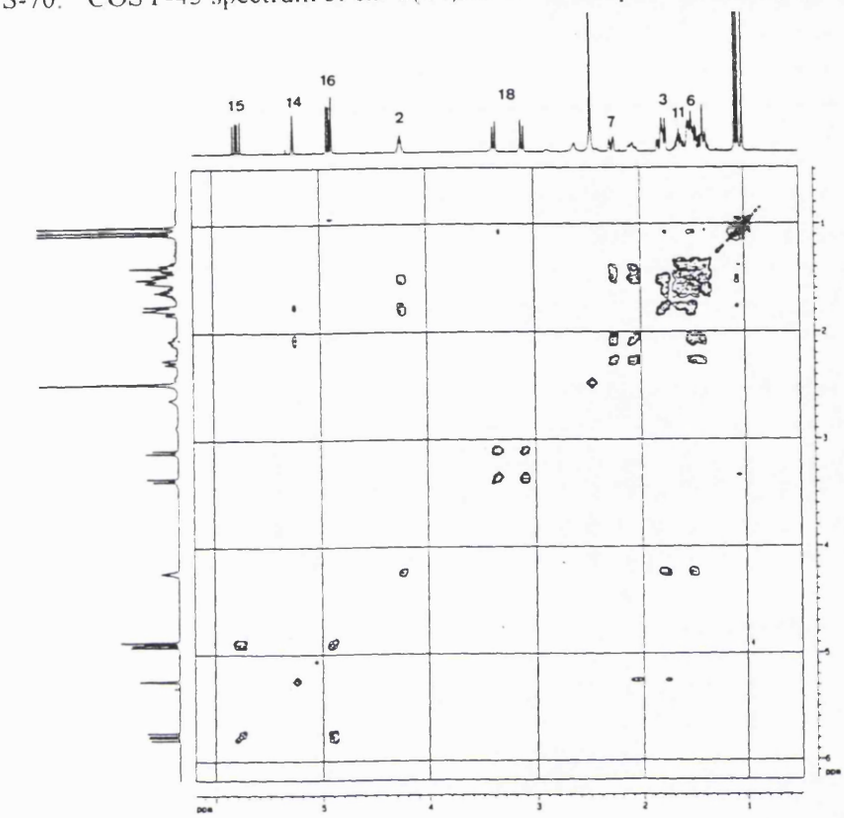
S-67. HMQC spectrum of lanosta-7,9(11),24EZ-triene-3α,23-diol

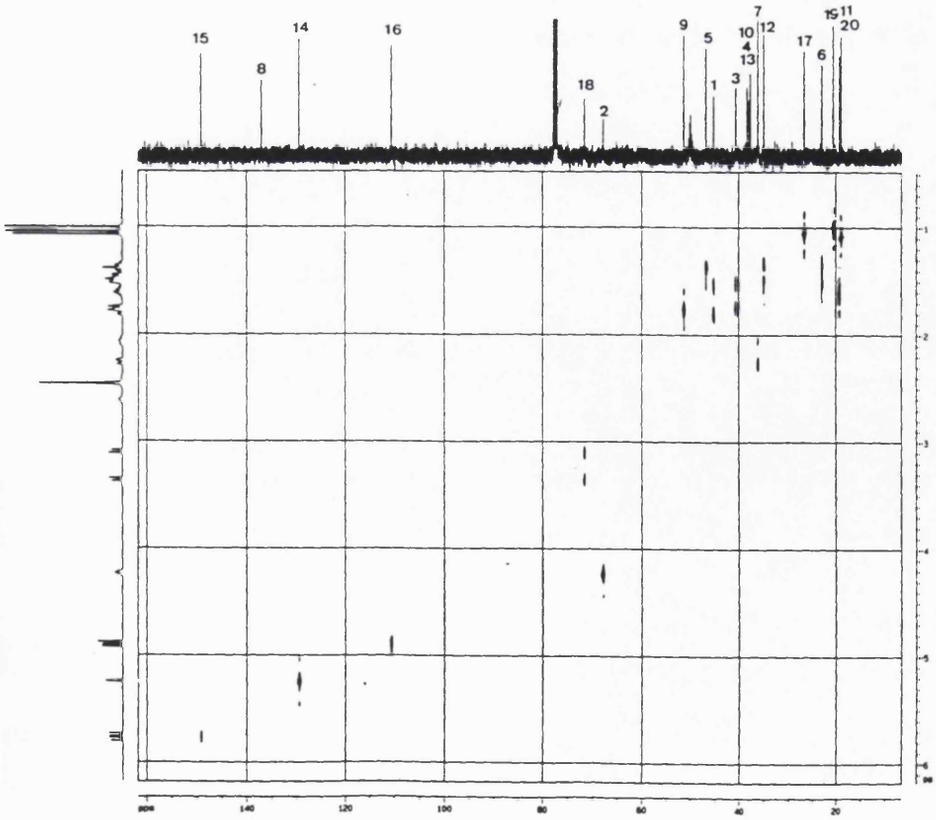
S-68. ^{1}H NMR (400 HMZ, $CDCl_3$) spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol



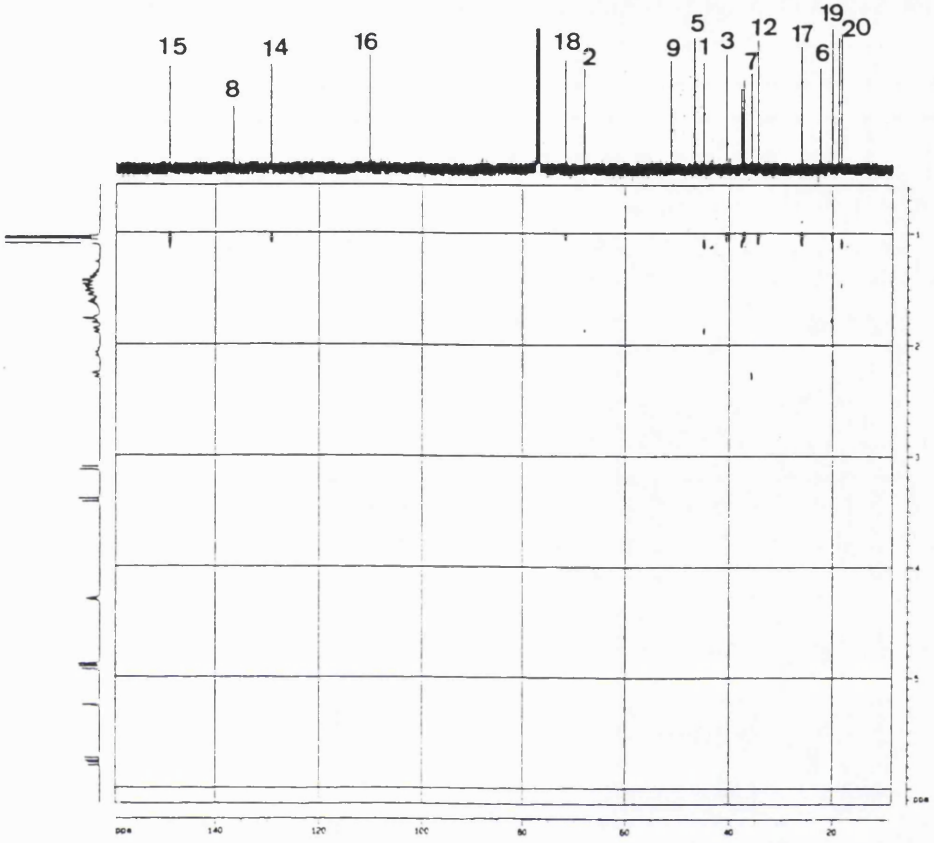
S-69. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

S-70. COSY-45 spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

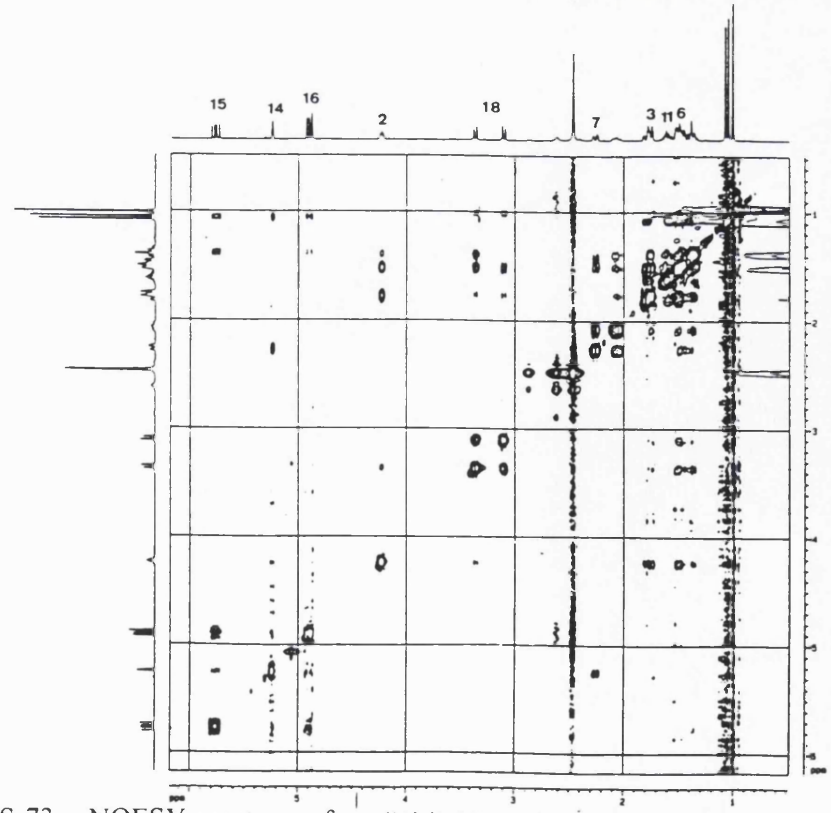




S-71. HMQC spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

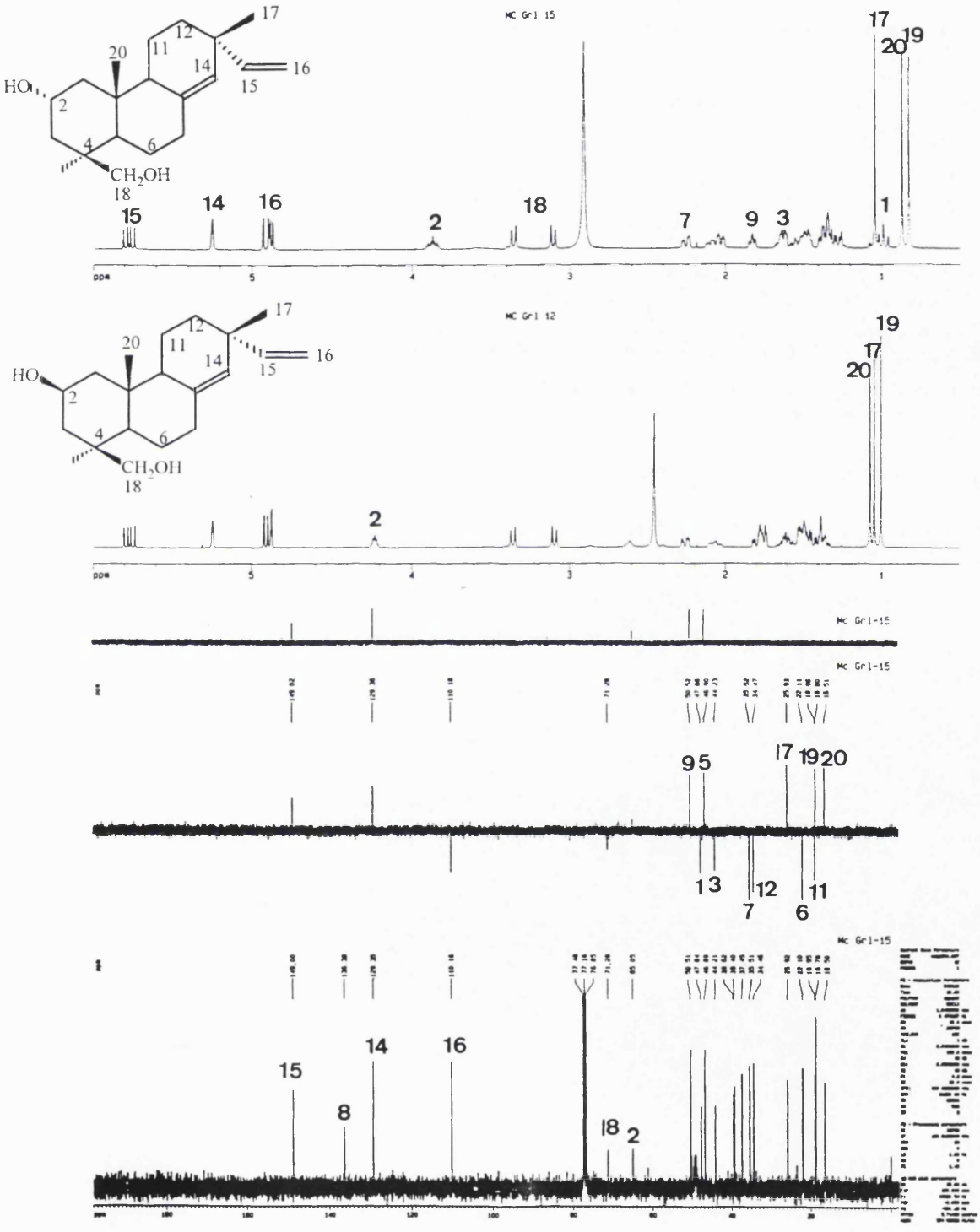


S-72. HMBC spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol



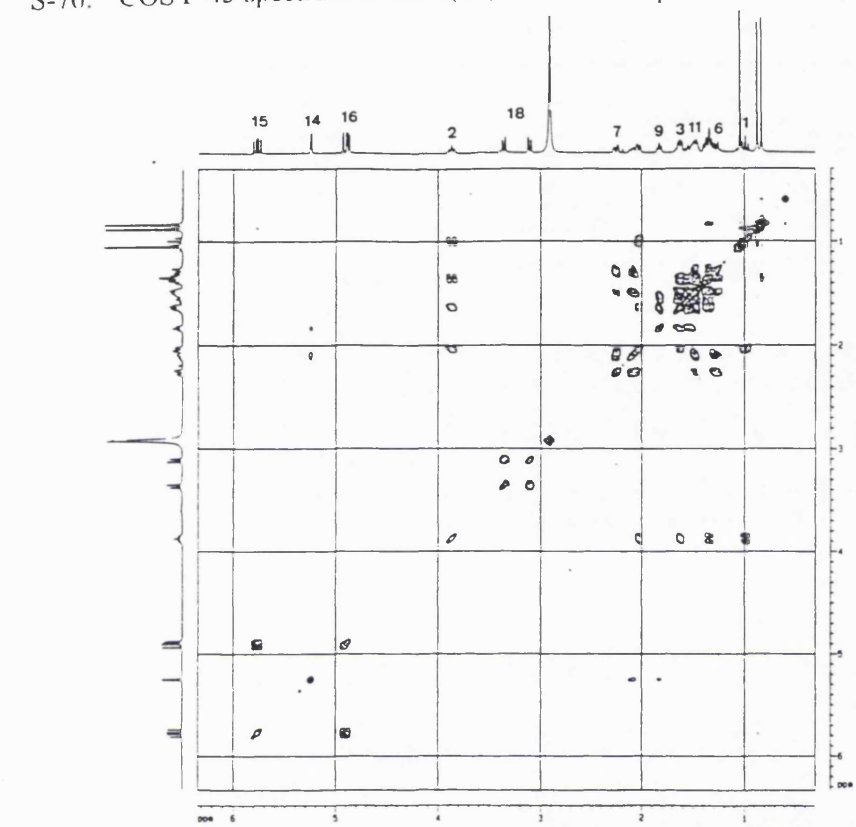
S-73. NOESY spectrum of ent-8(14),15-sandaracopimaradiene-2β,18-diol

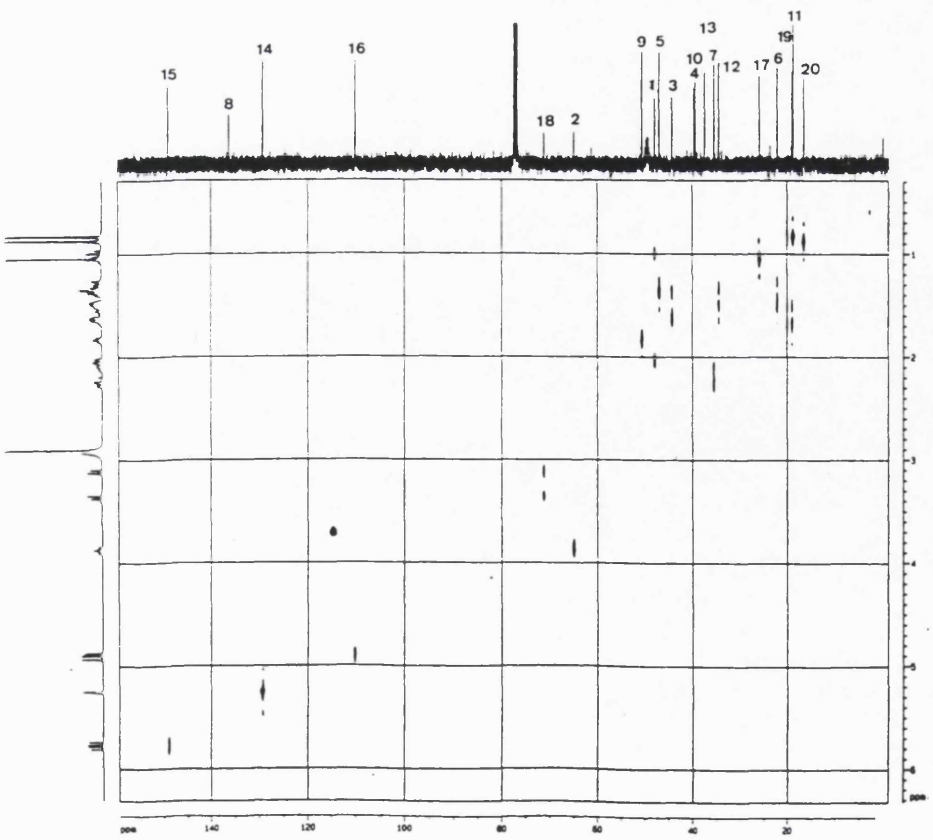
S-74. ^{1}H NMR (400 MHz, $CDCl_3$) of ent-8(14),15-sandaracopimaradiene-2α,18-diol



S-75. ^{13}C NMR (100 MHz, $CDCl_3$) of ent-8(14),15-sandaracopimaradiene-2α,18-diol

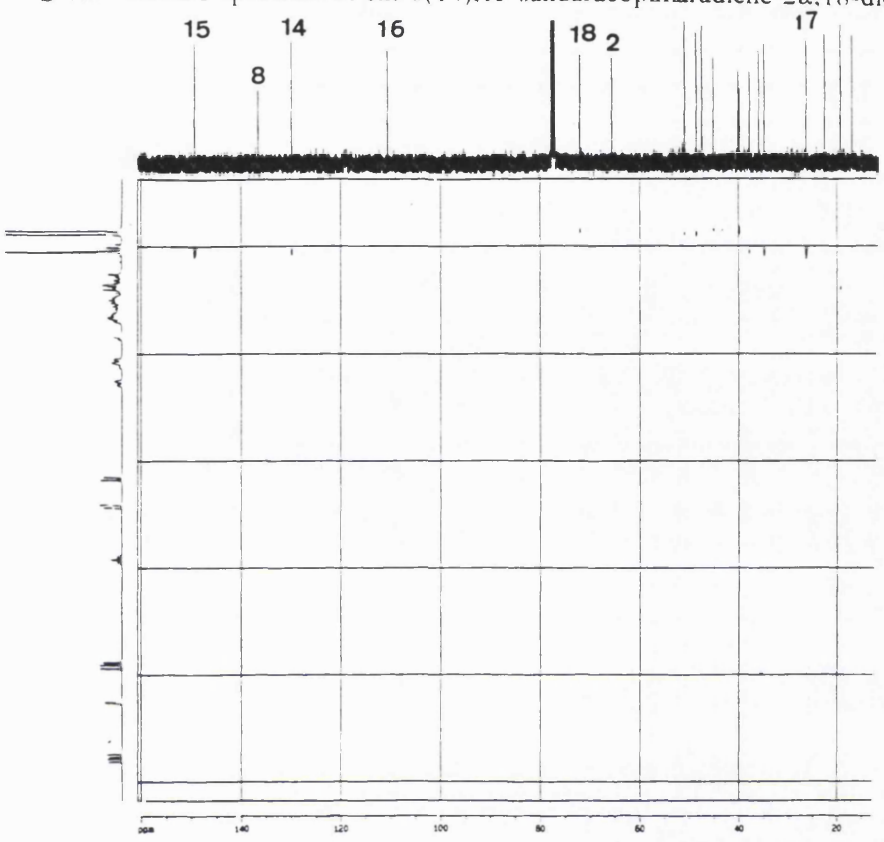
S-76. COSY-45 spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol



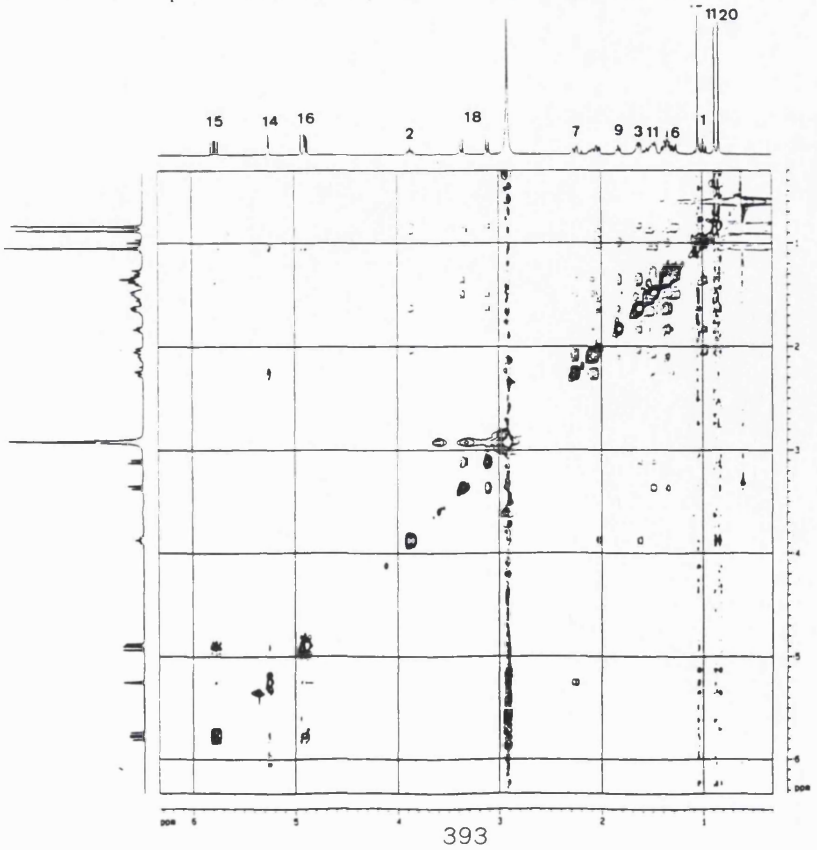


S-77. HMQC spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol

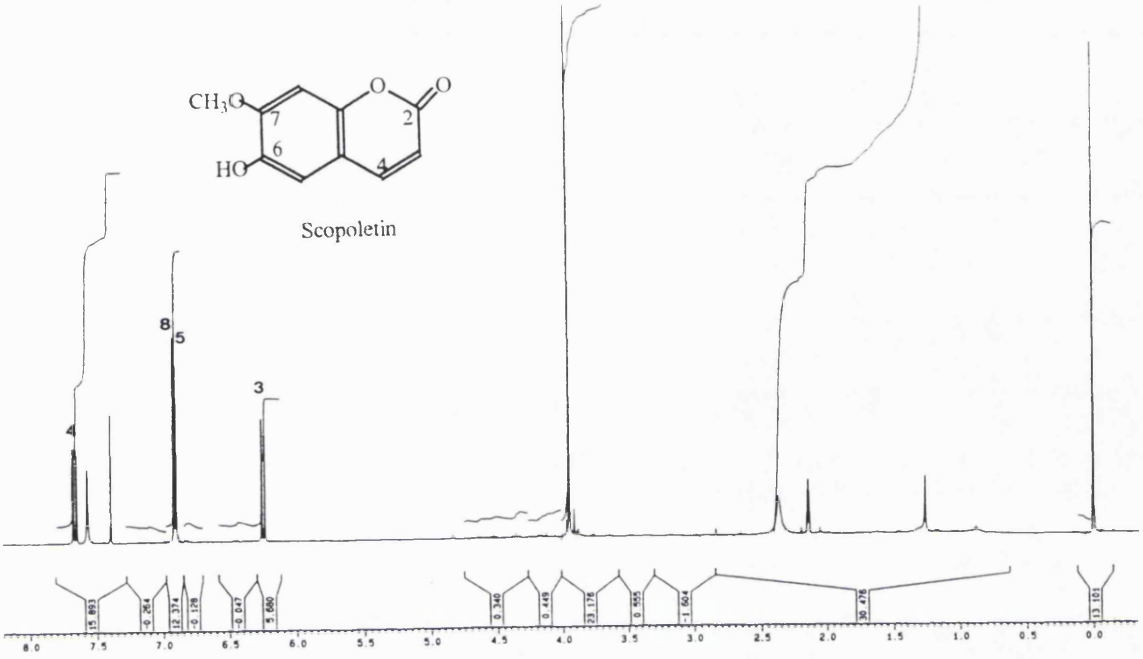
S-78. HMBC spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol



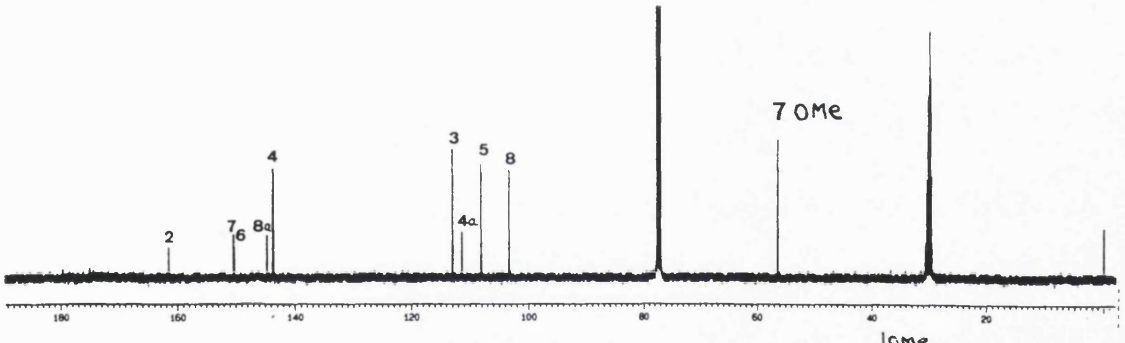
S-79. NOESY spectrum of ent-8(14),15-sandaracopimaradiene-2α,18-diol



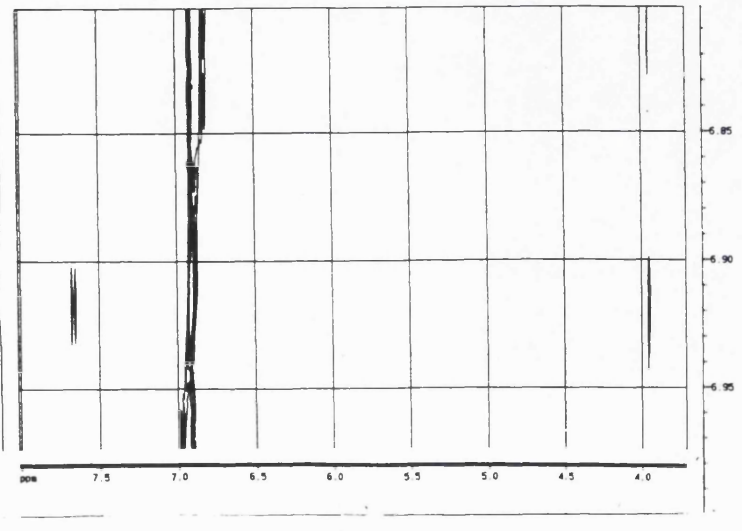
S-80. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of scopoletin



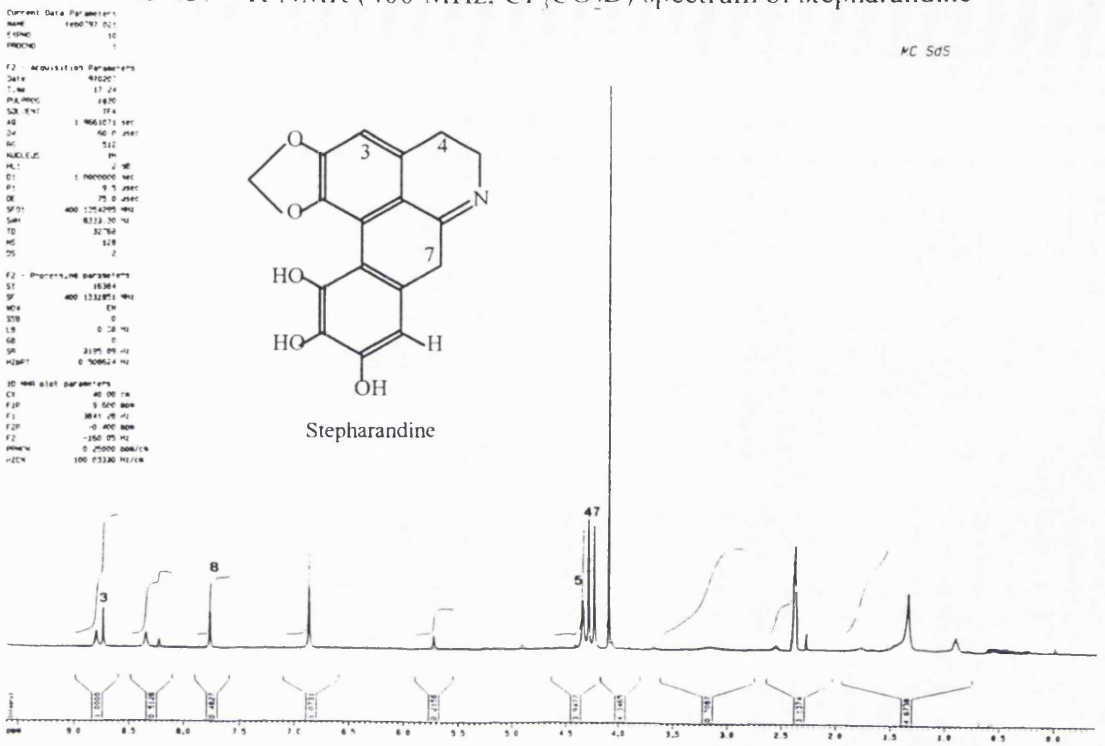
S-81. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of scopoletin

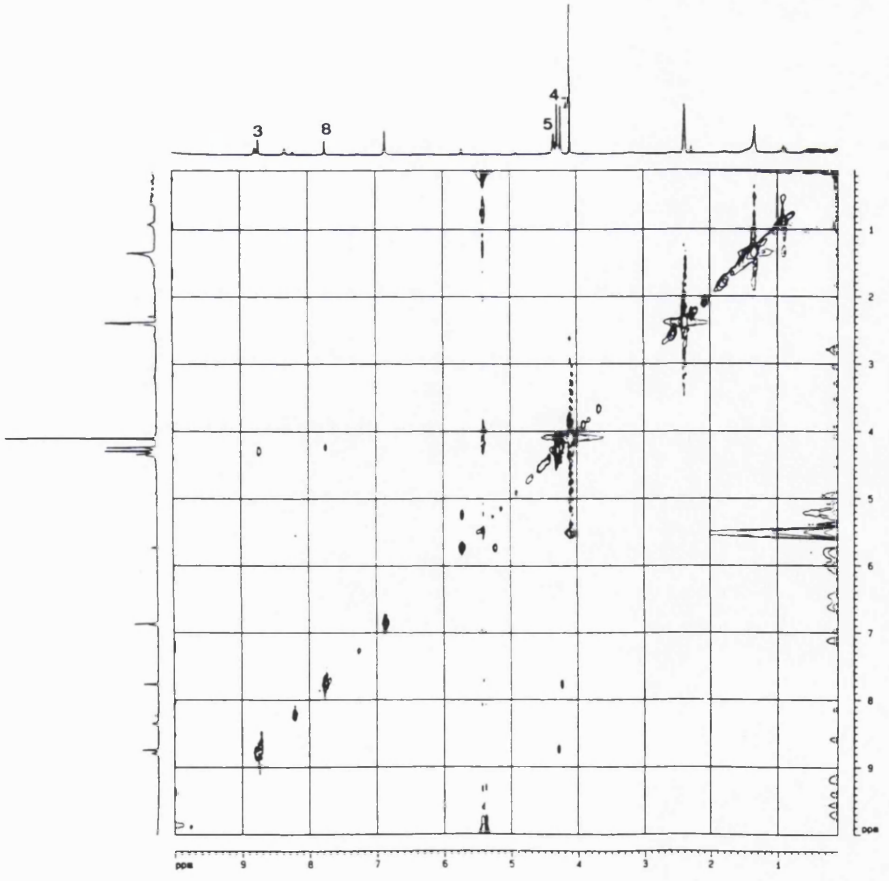


S-82. NOESY spectrum of scopoletin



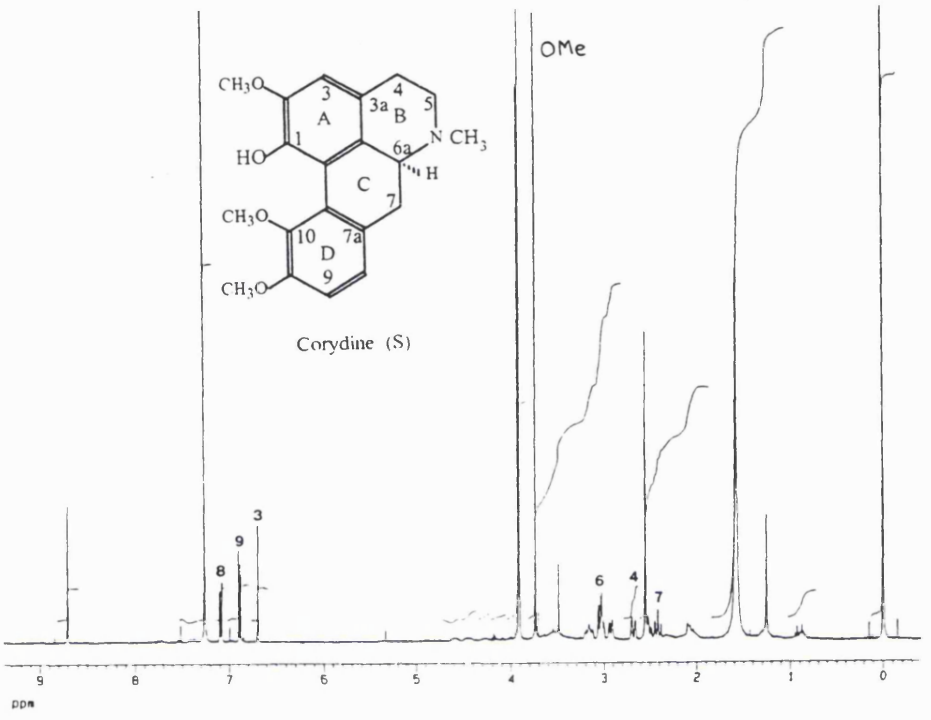
S-83. ^{1}H NMR (400 MHz, CF_3CO_2D) spectrum of stepharandine



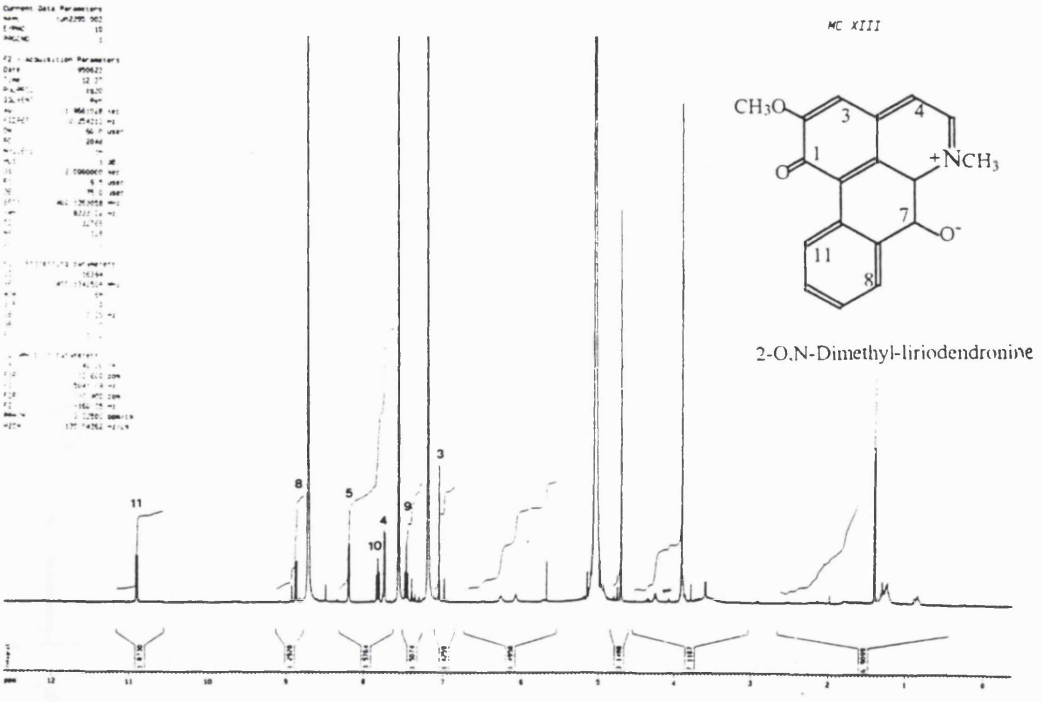


S-84. NOESY spectrum of stepharandine

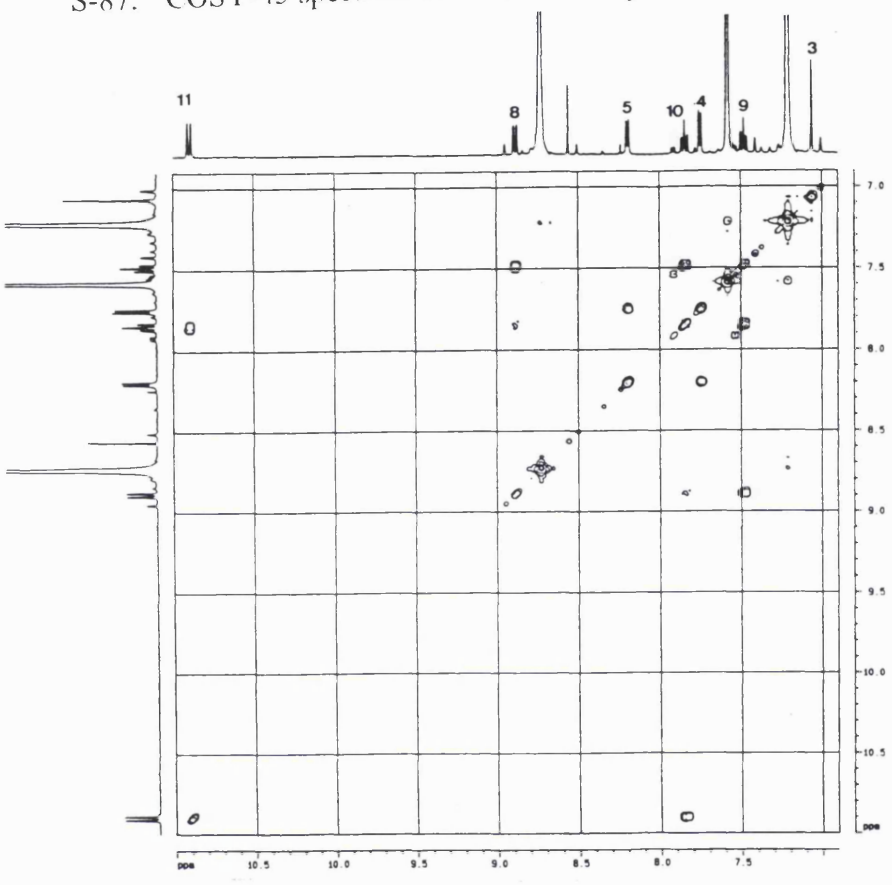
S-85. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of corydine

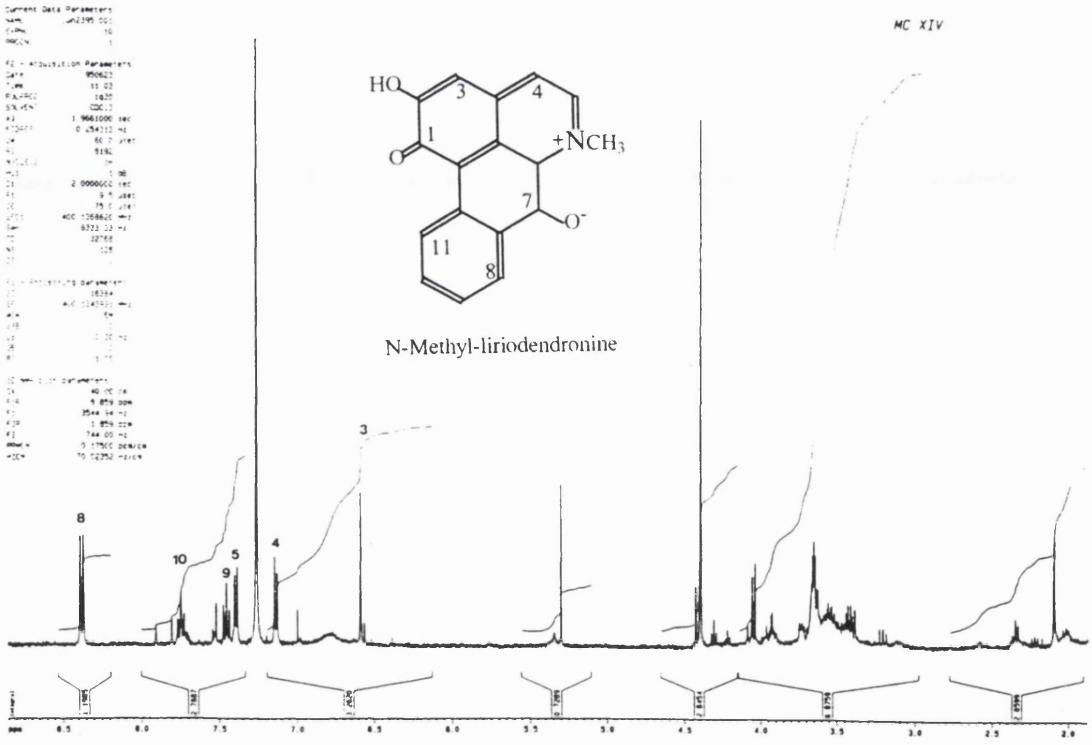


S-86. ^{1}H NMR (400 MHz, C_5D_5N) spectrum of 2-O,N-dimethyl-liriodendronine



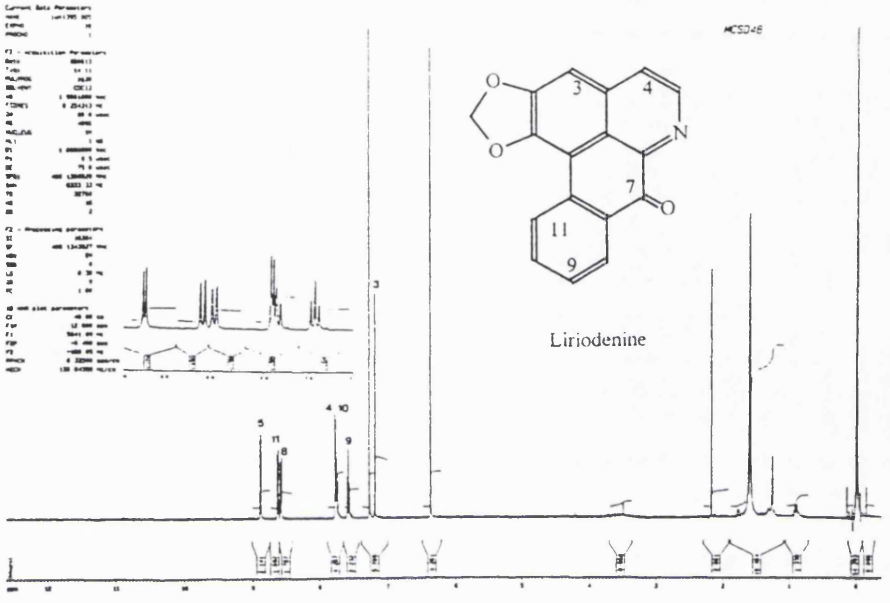
S-87. COSY-45 spectrum of 2-O,N-dimethyl-liriodendronine

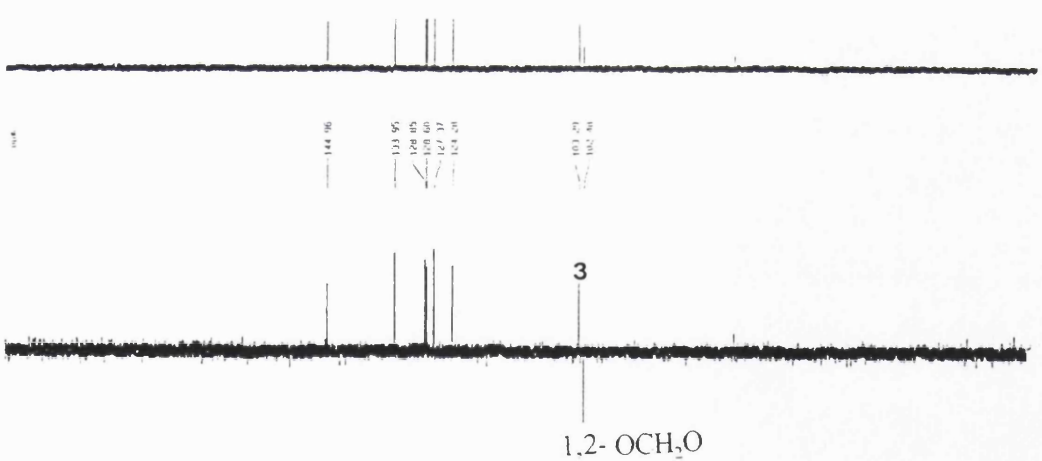


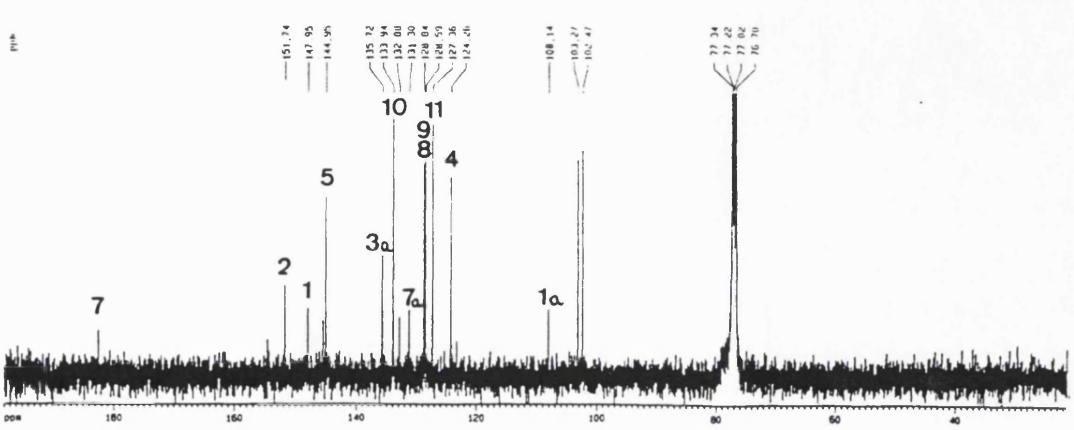


S-88. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of N-methyl-liriodendronine

S-89. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of liriodenine

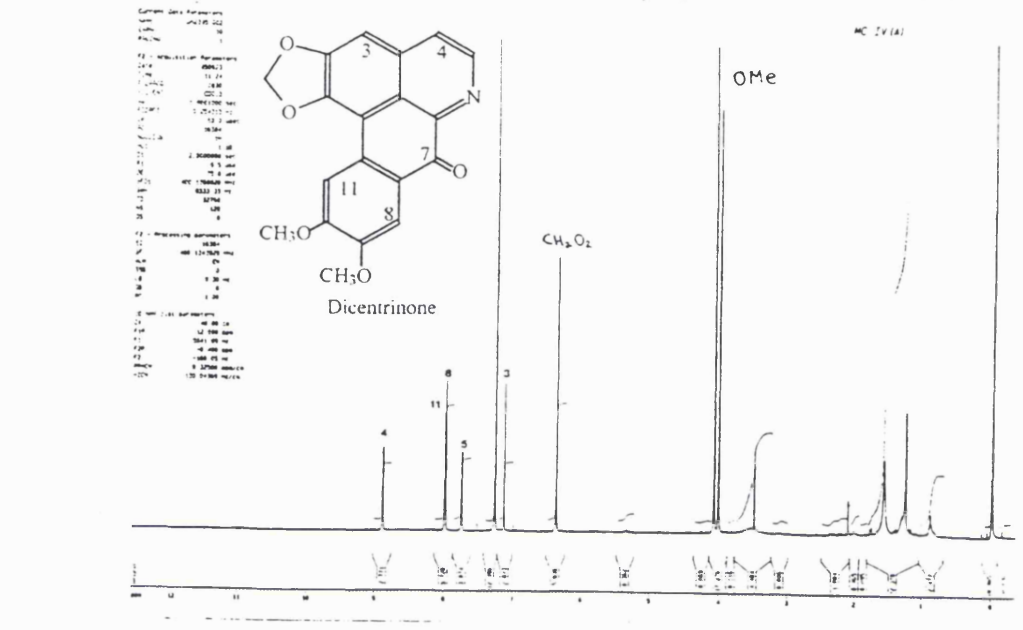


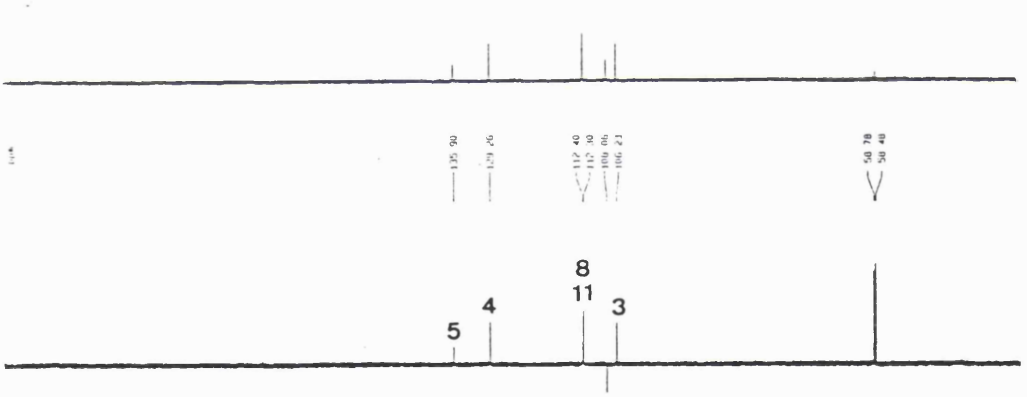


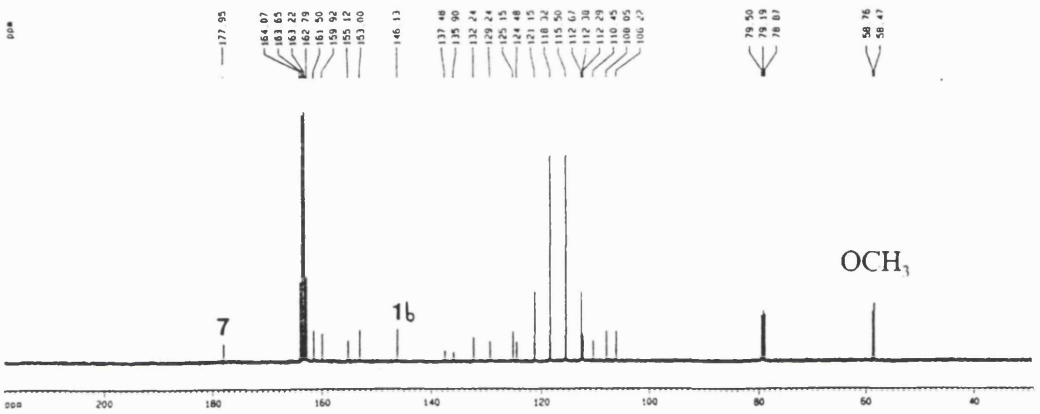


S-90. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of liriodenine

S-91. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of dicentrinone

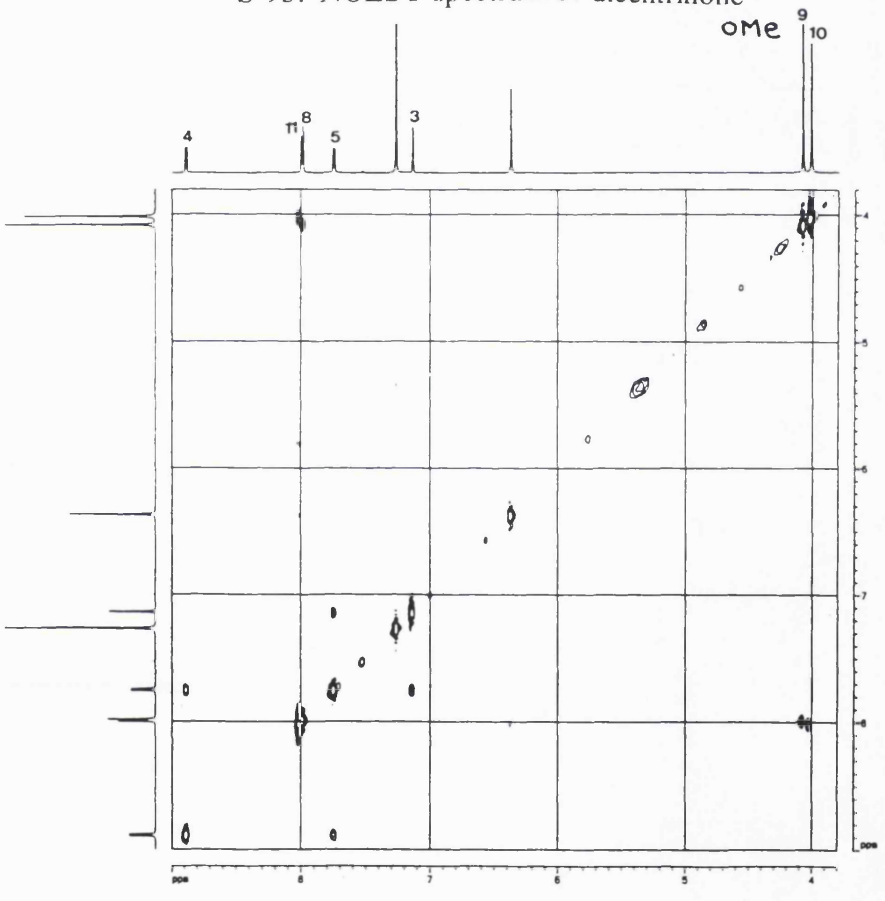




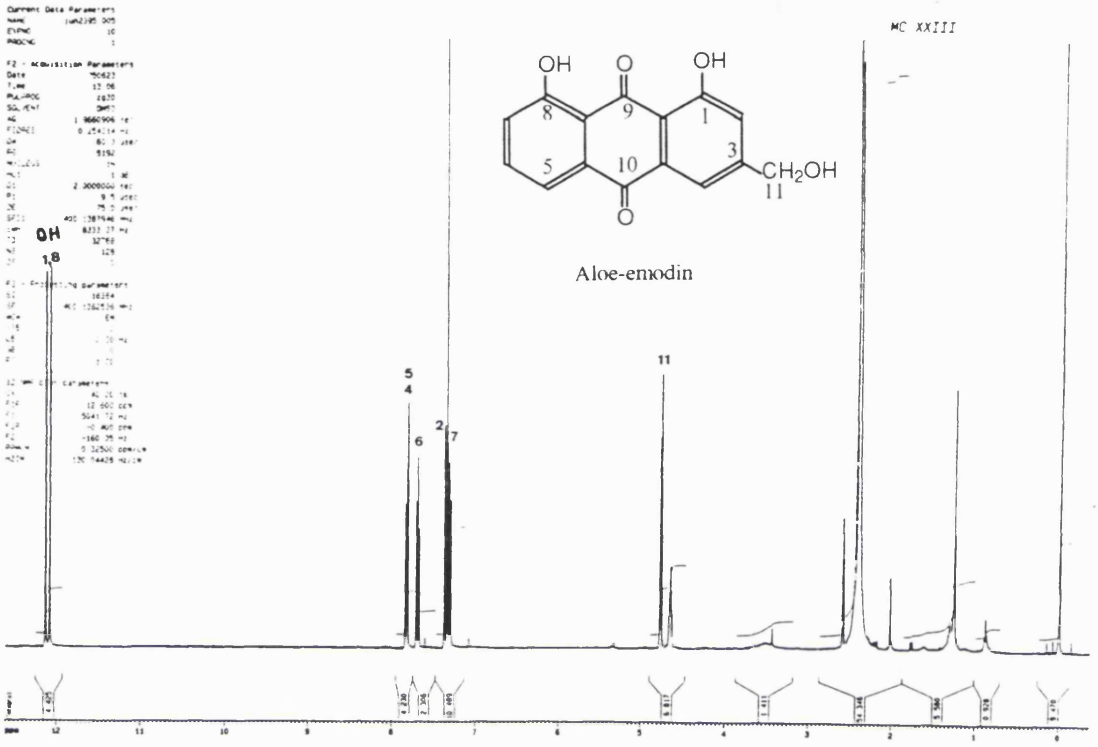


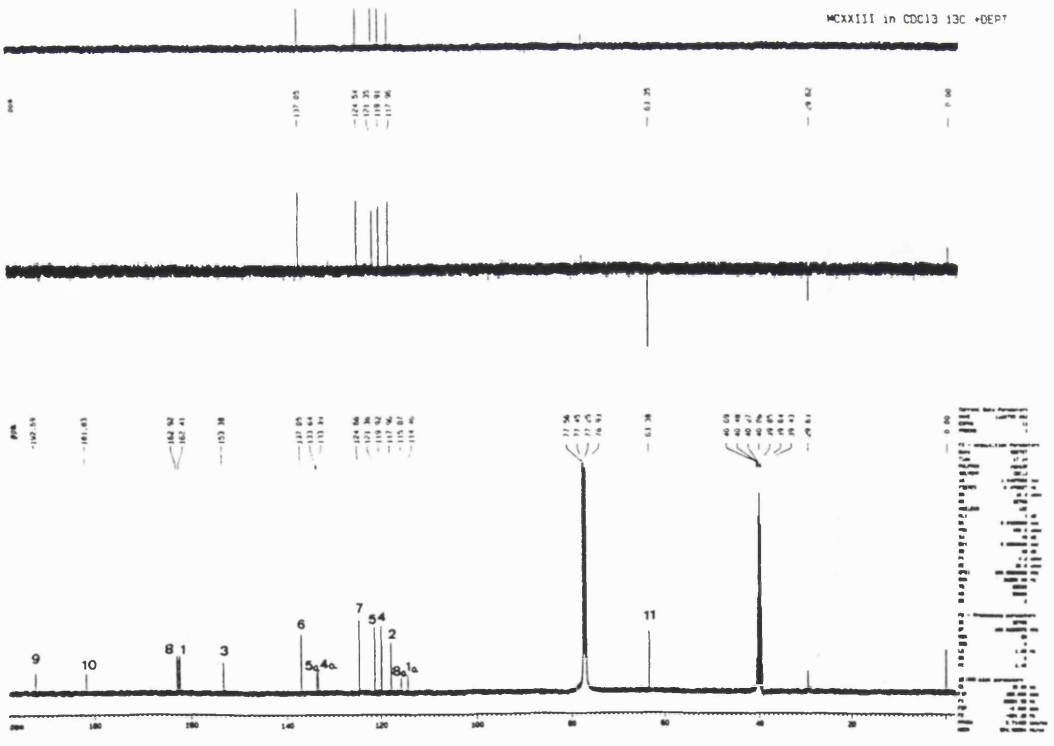
S-92. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of dicentrinone

S-93. NOESY spectrum of dicentrinone



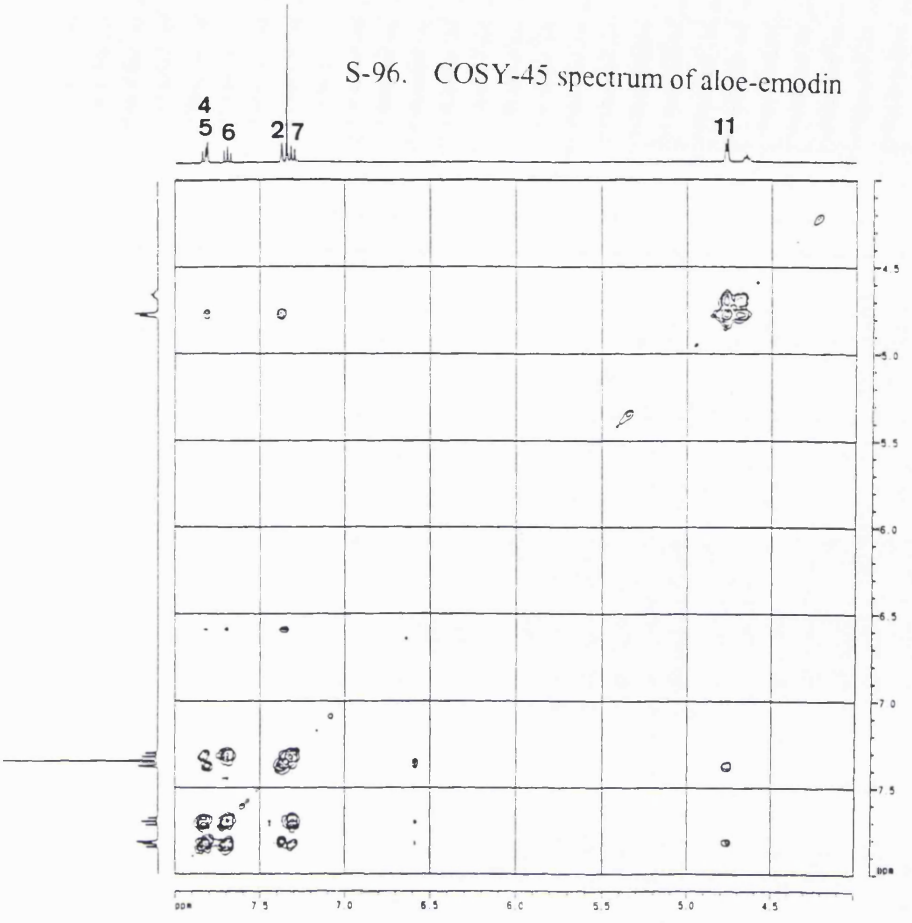
S-94. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of aloe-emodin



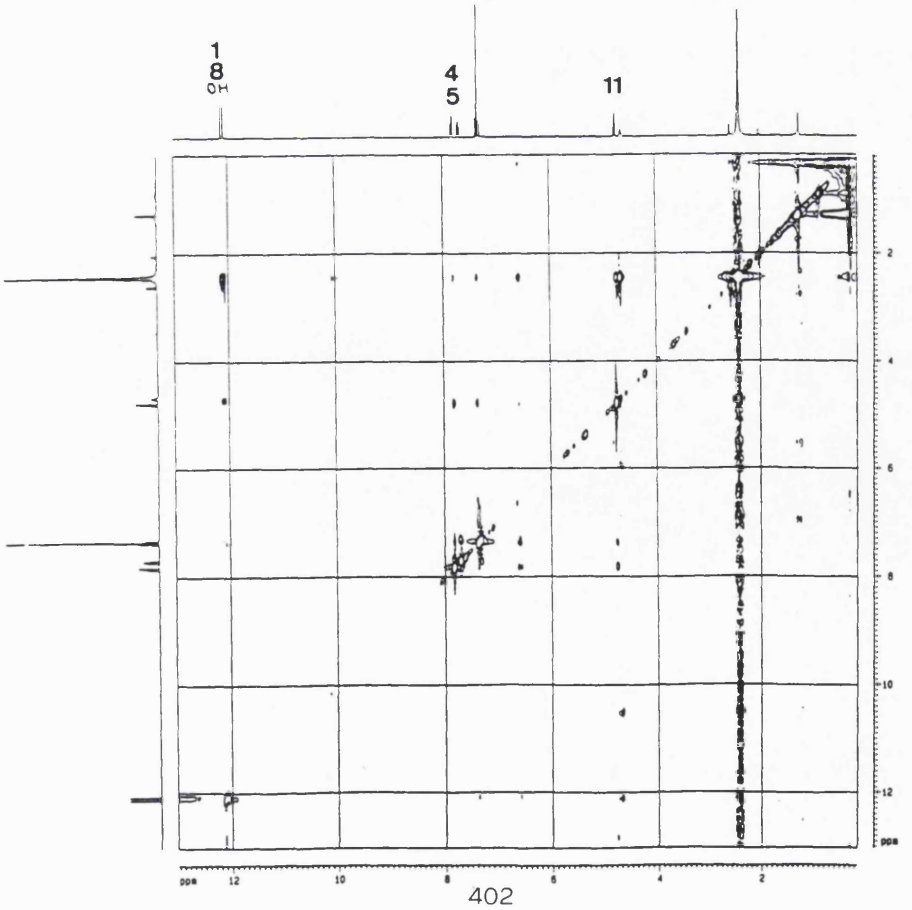


S-95. ^{13}C NMR (400 MHz, DMSO-d_6/$CDCl_3$) spectrum of aloe-emodin

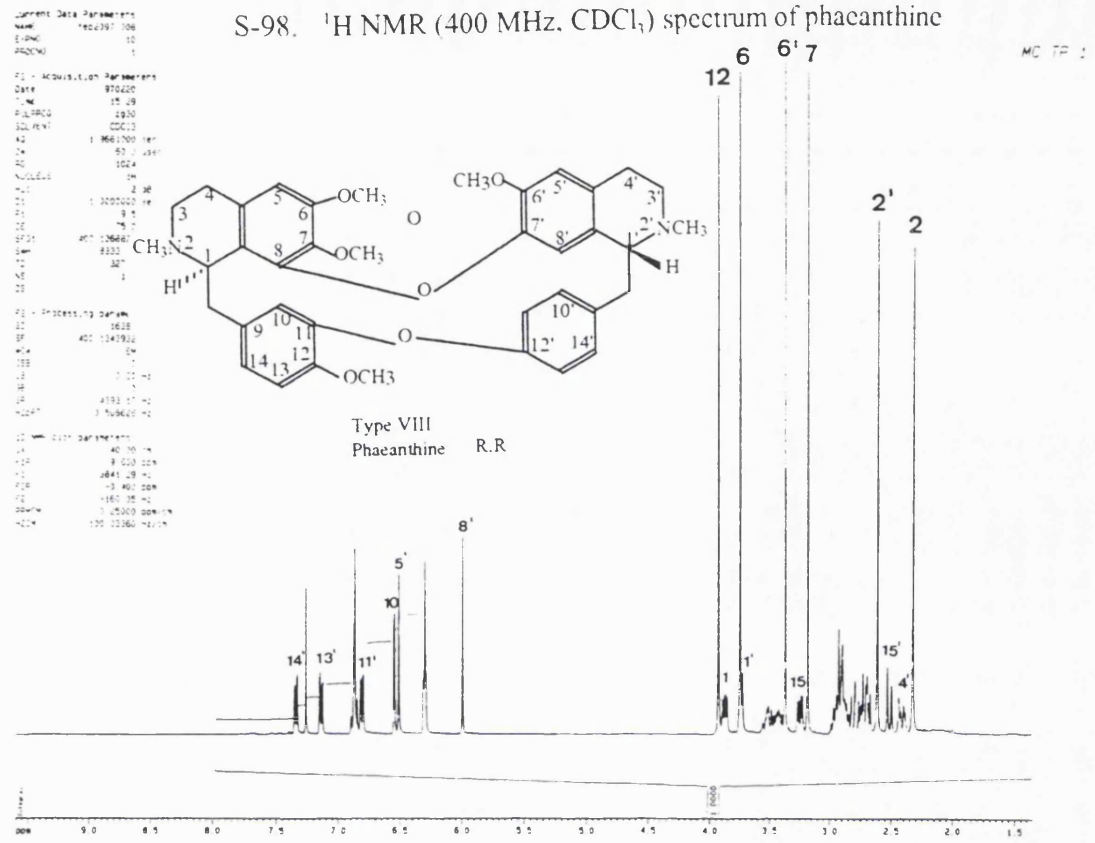
S-96. COSY-45 spectrum of aloe-emodin

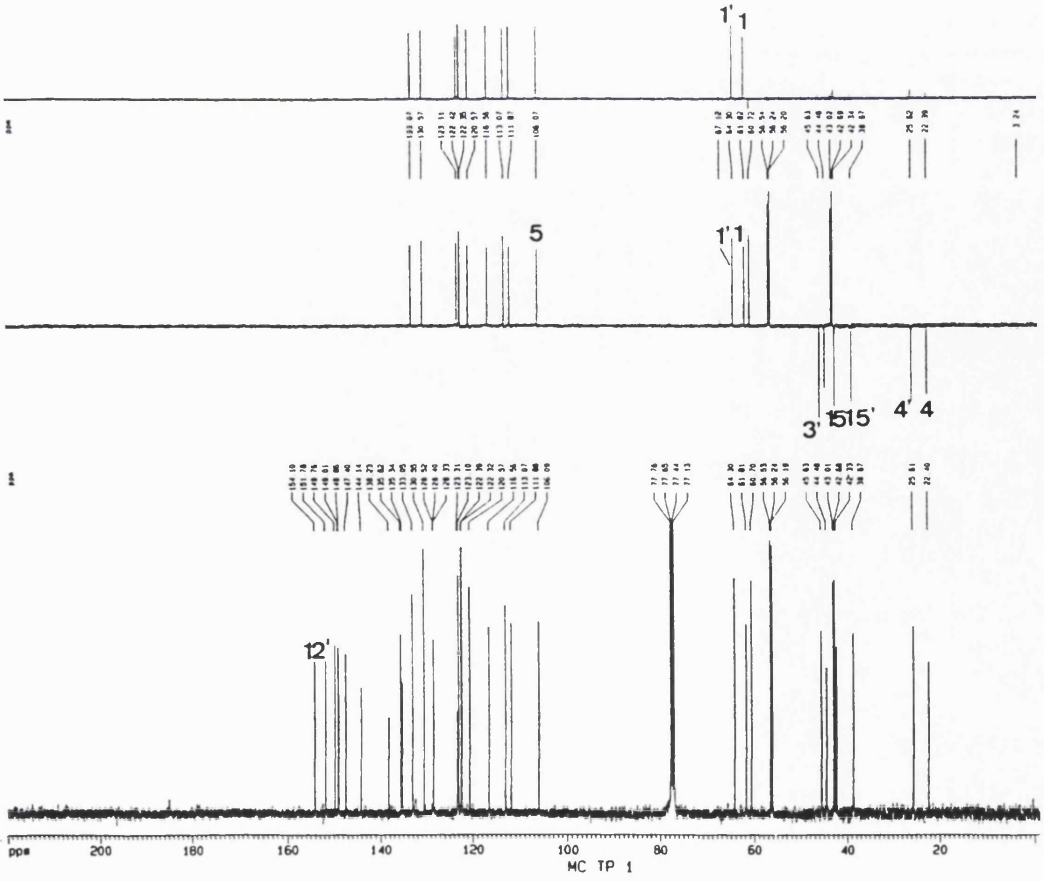


S-97. NOESY spectrum of aloe-emodin



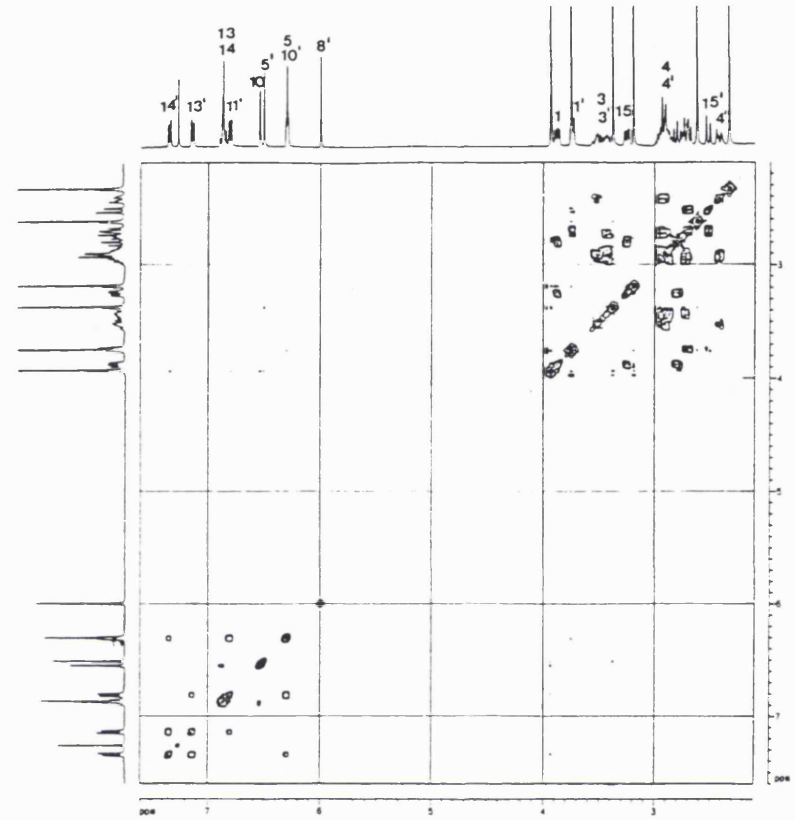
S-98. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of phaeanthine

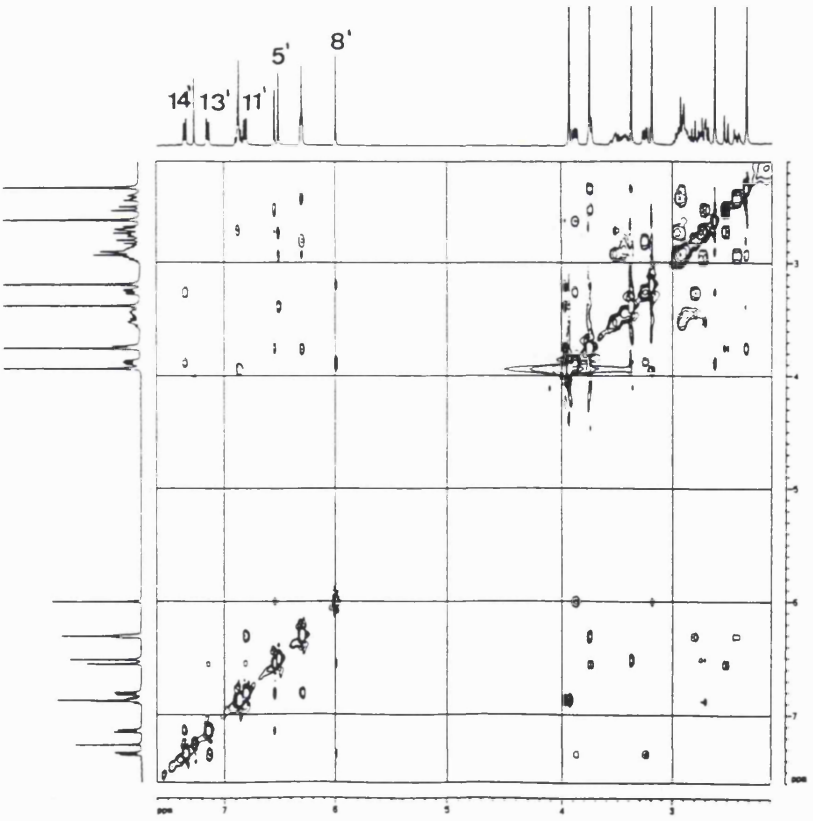




S-99. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of phaeanthine

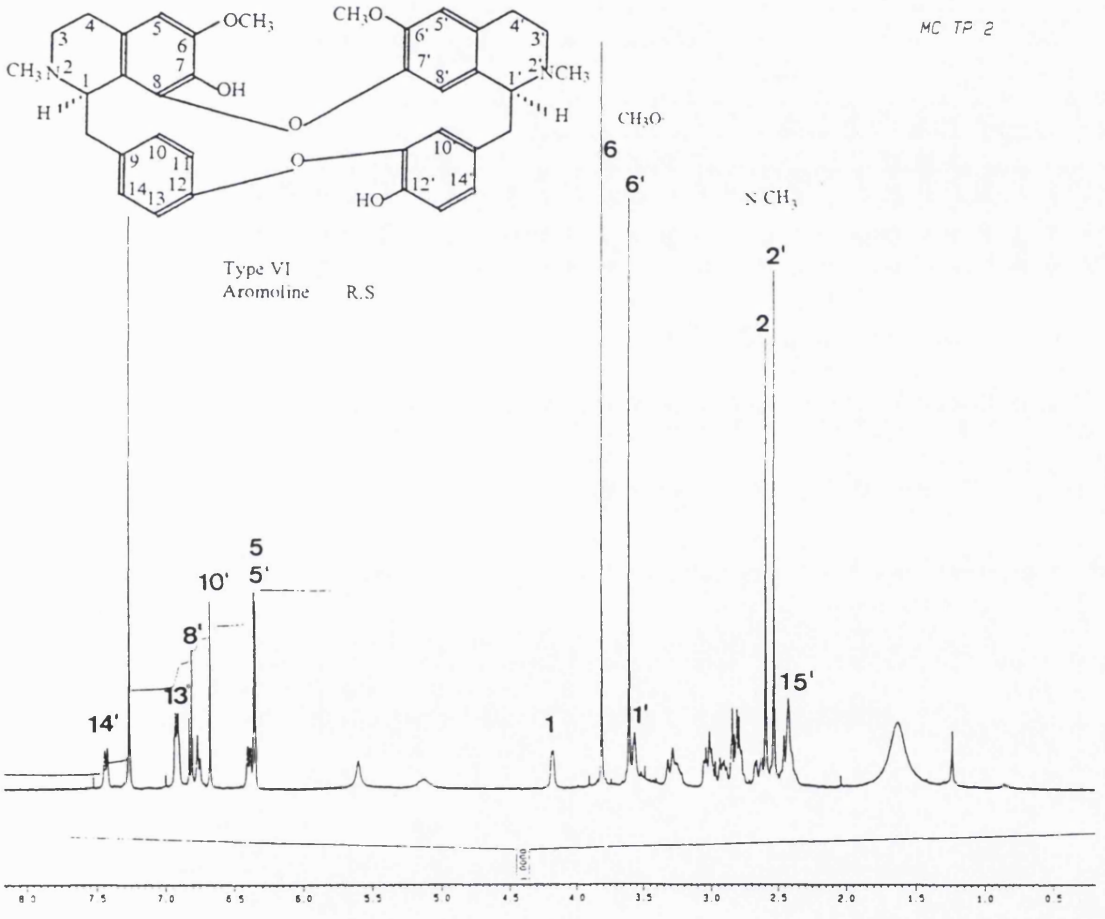
S-100. COSY-45 spectrum of phaenthine



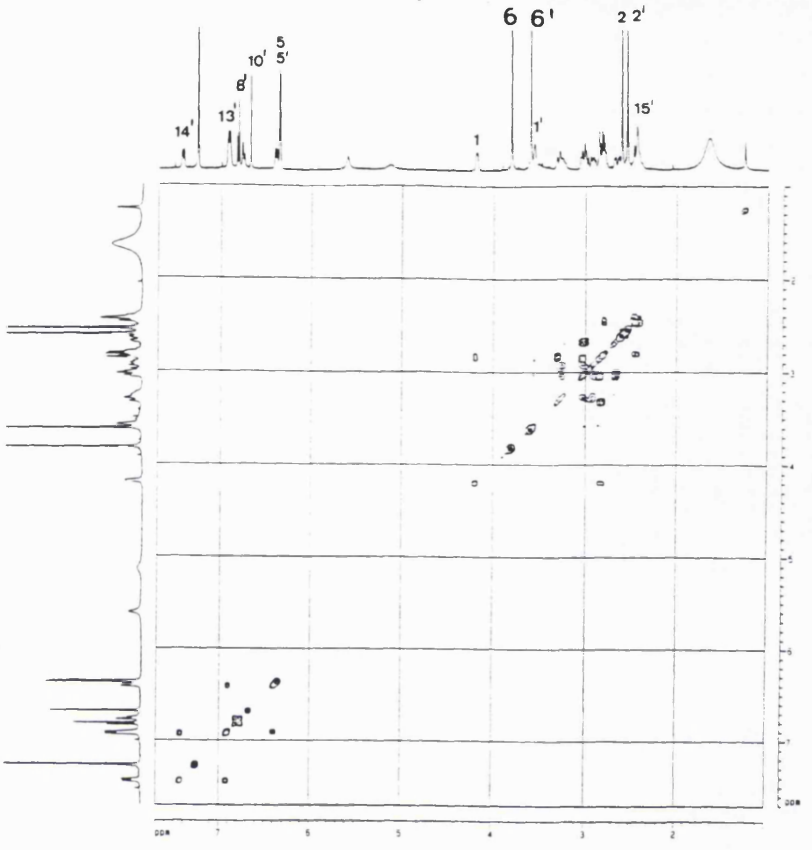


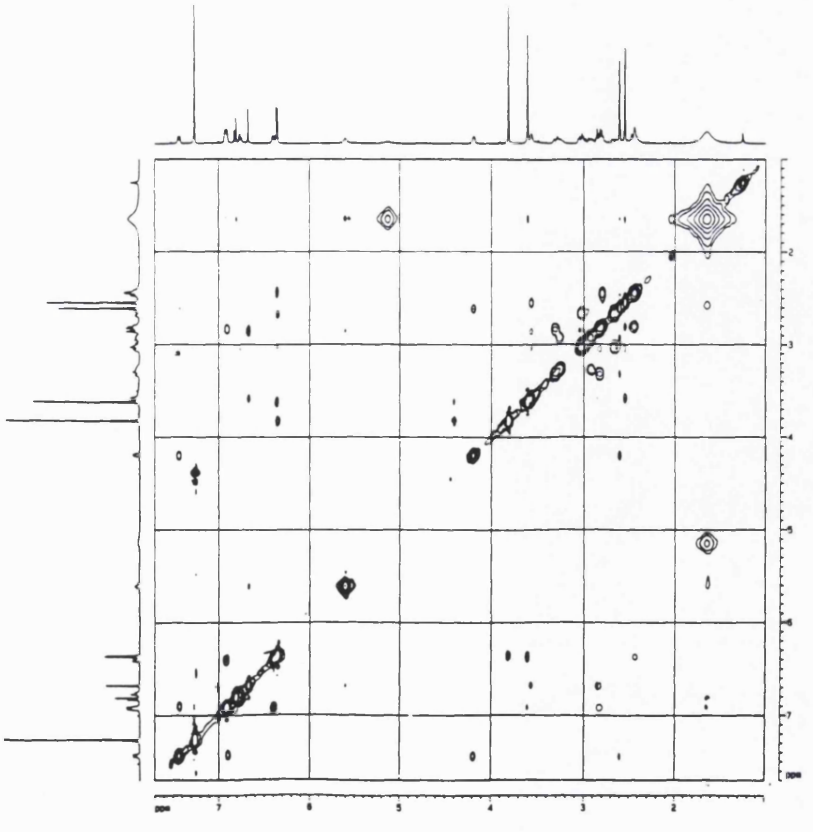
S-101. NOESY spectrum of phaenthine

S-102. ^{1}H NMR (400 HMz, $CDCl_3$) spectrum of aromoline



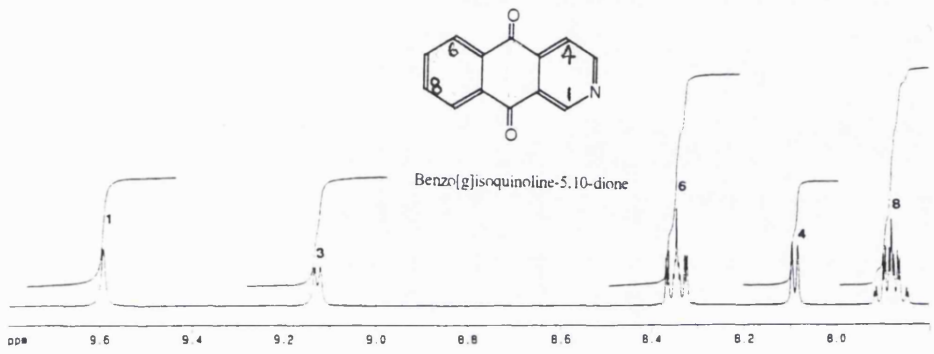
S-103. COSY-45 spectrum of aromoline



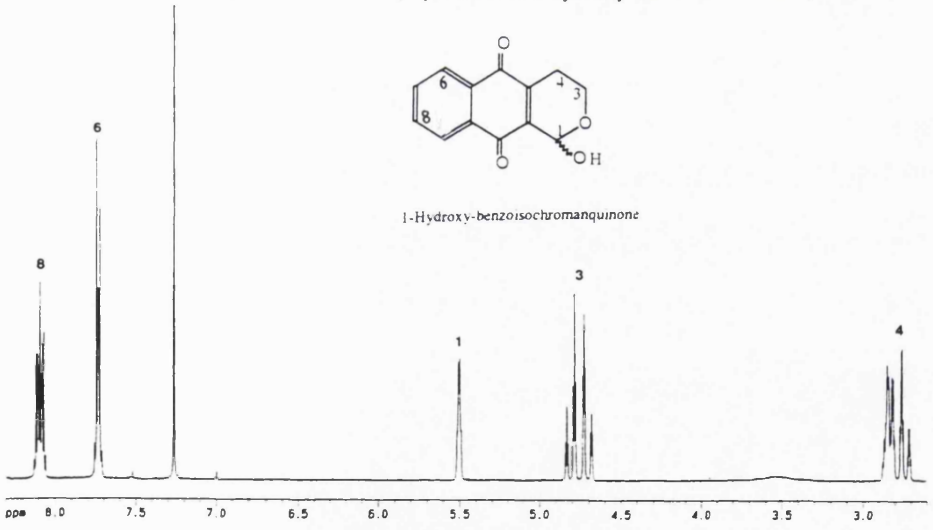


S-104. NOESY spectrum of aromoline

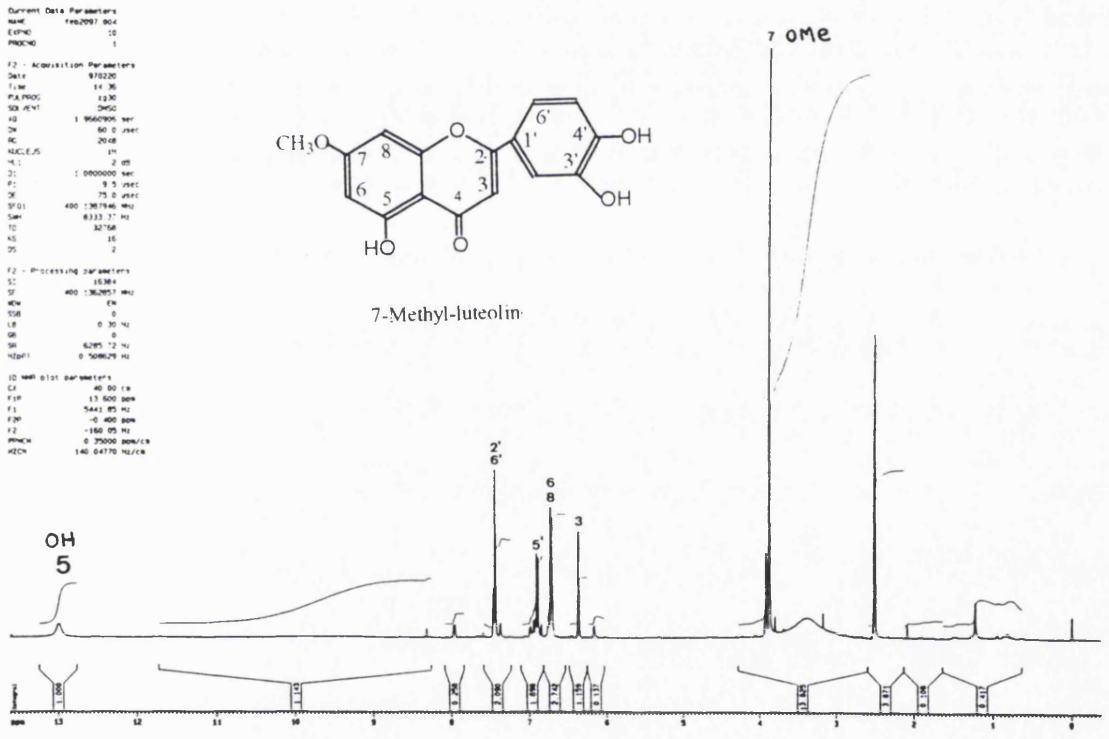
S-105. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of benzo[g]isoquinoline-5,10-dione



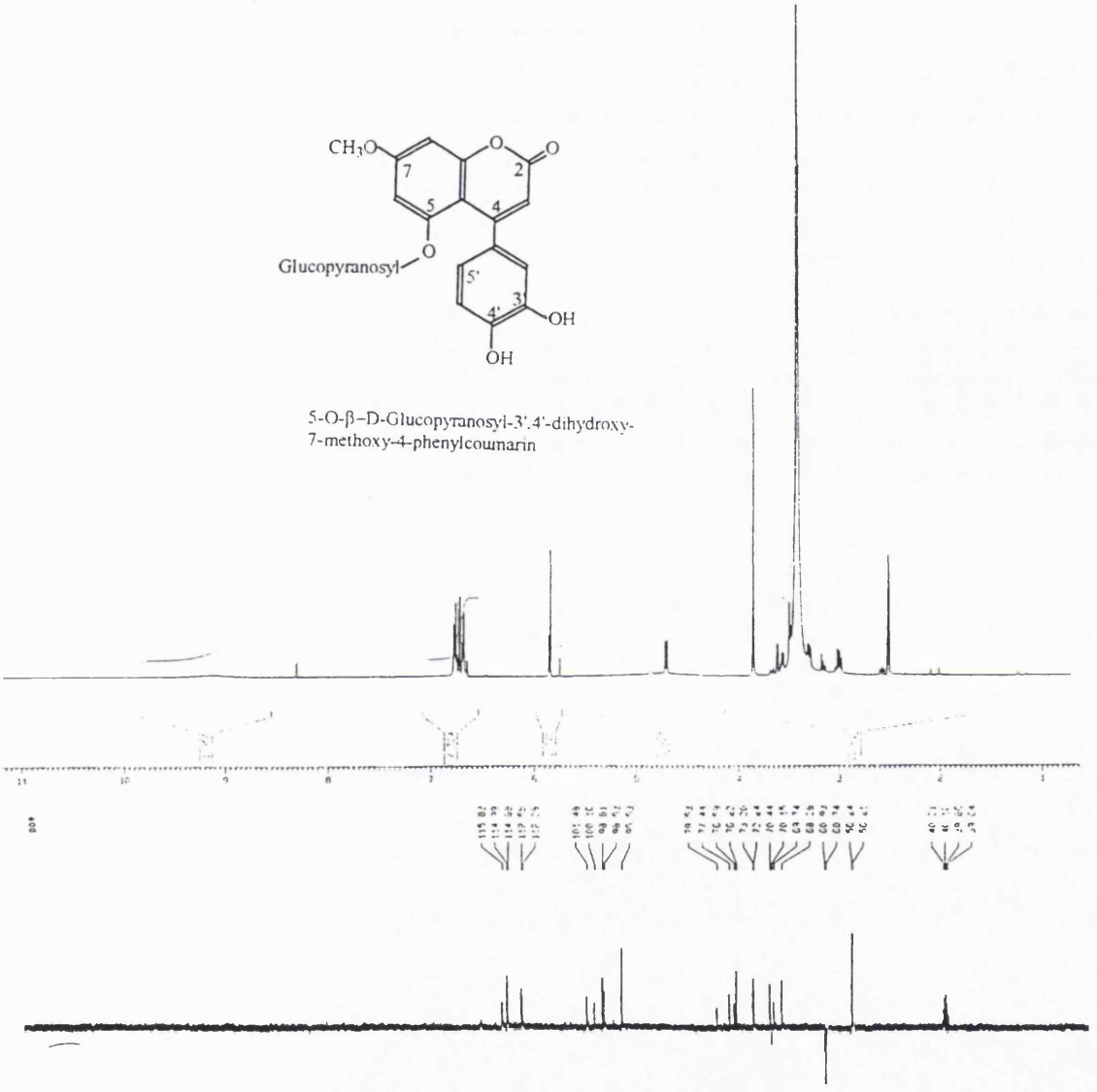
S-106. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 1-hydroxy-benzoisochromanquinone



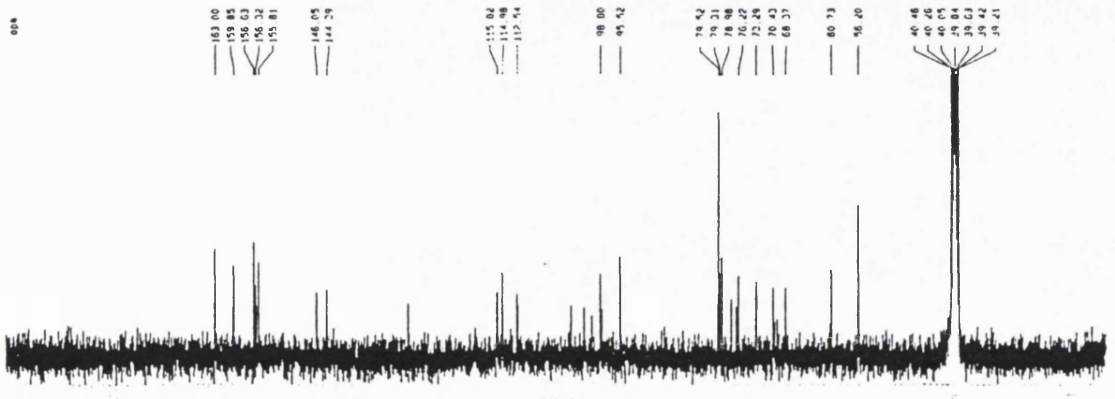
S-107. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of 7-methyl-luteolin



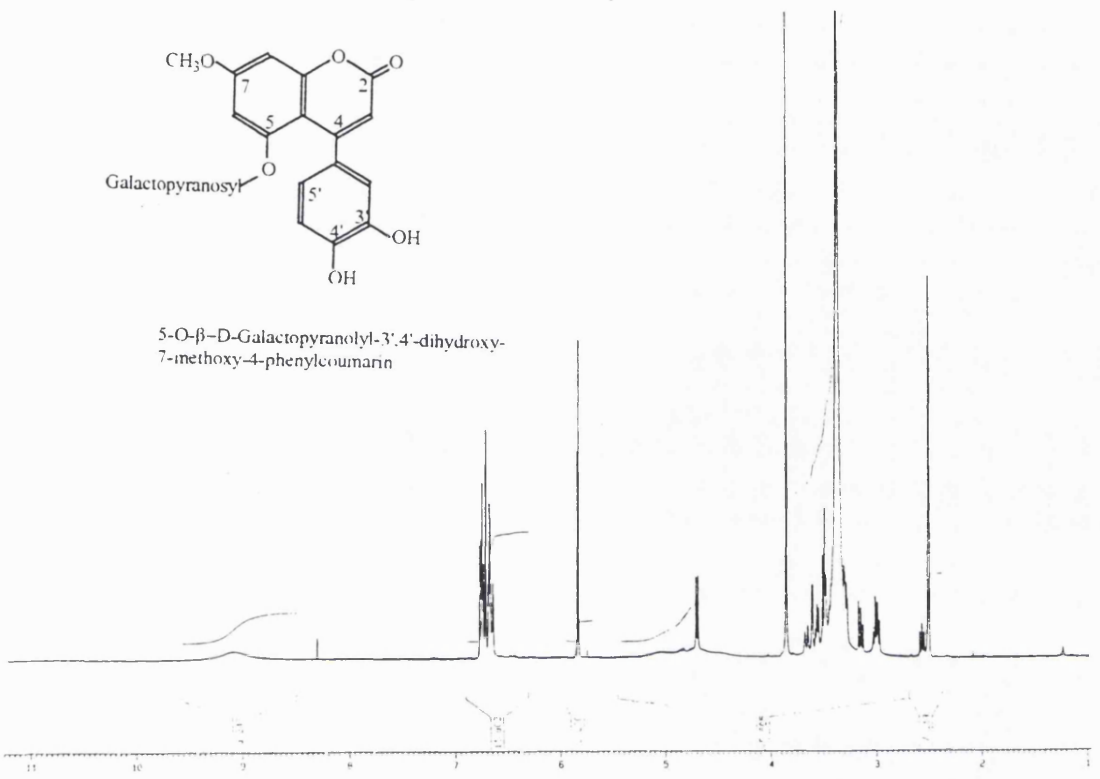
S-108. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of 5-O-β-D-glucopyranosyl]-3',4'- dihydroxy-7-methoxy-4-phenylcoumarin

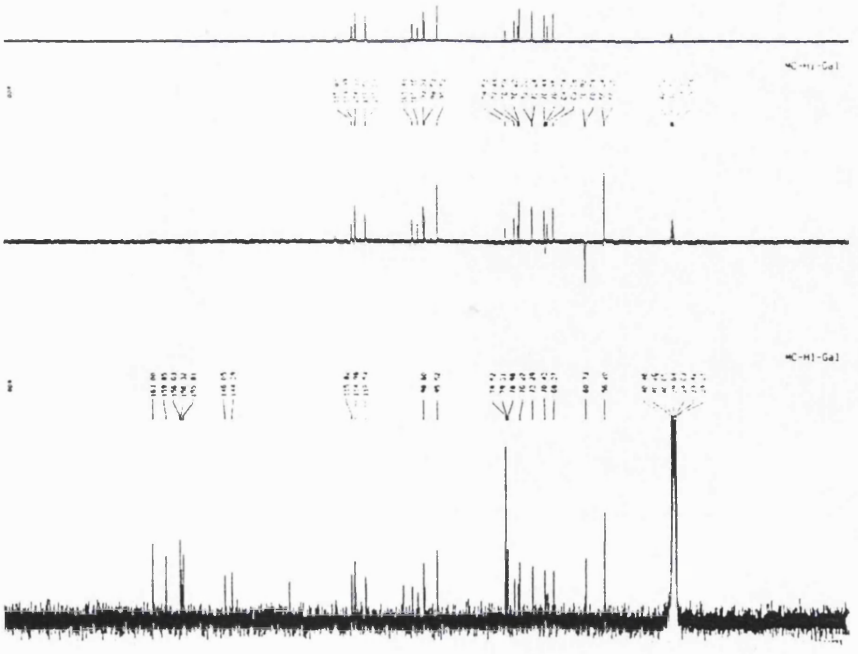


S-109. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of 5-O-β-D-glucopyranosyl]-3',4'- dihydroxy-7-methoxy-4-phenylcoumarin



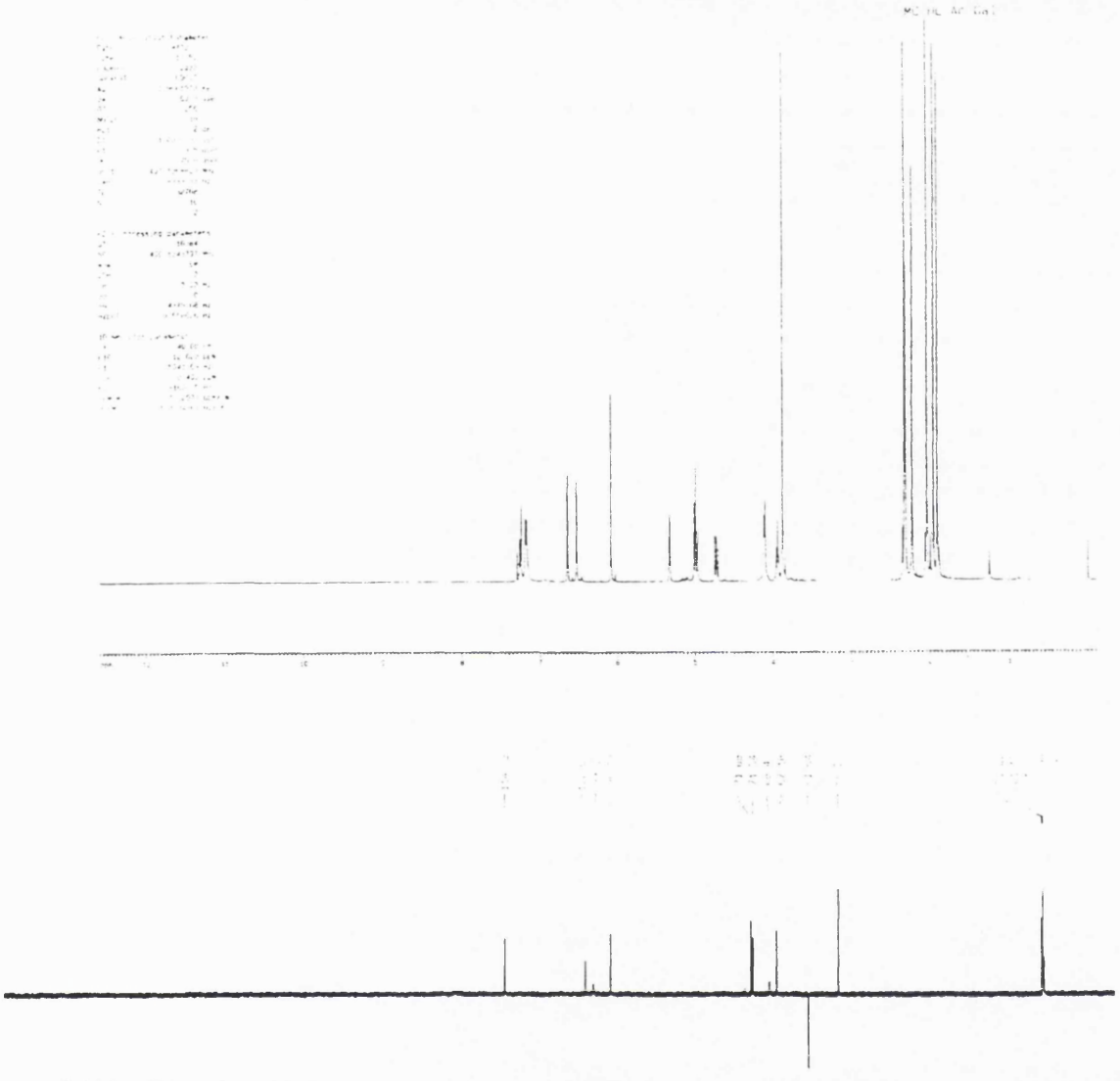
S-110. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of 5-O-β-D-galactopyranosyl]-3',4'- dihydroxy-7-methoxy-4-phenylcoumarin



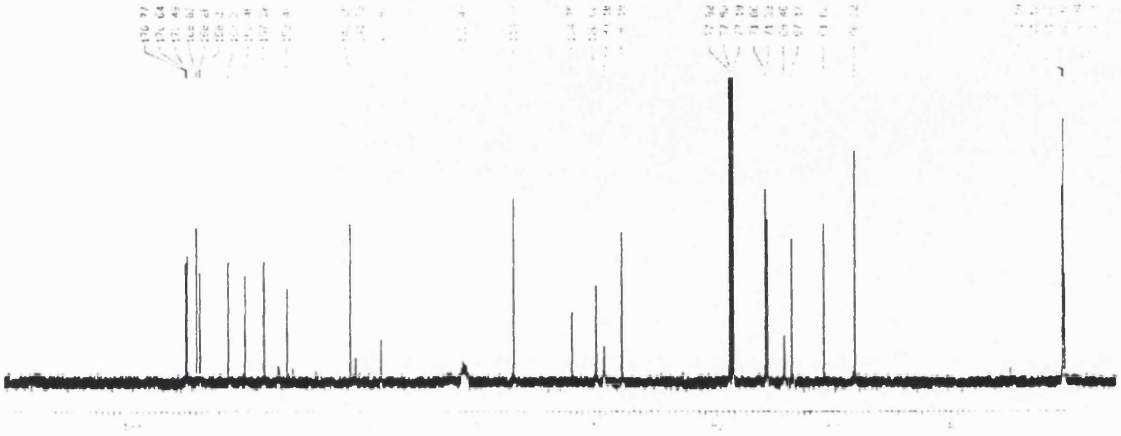


S-111. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of 5-O-β-D-galactopyranosyl]-3',4'- dihydroxy-7-methoxy-4-phenylcoumarin

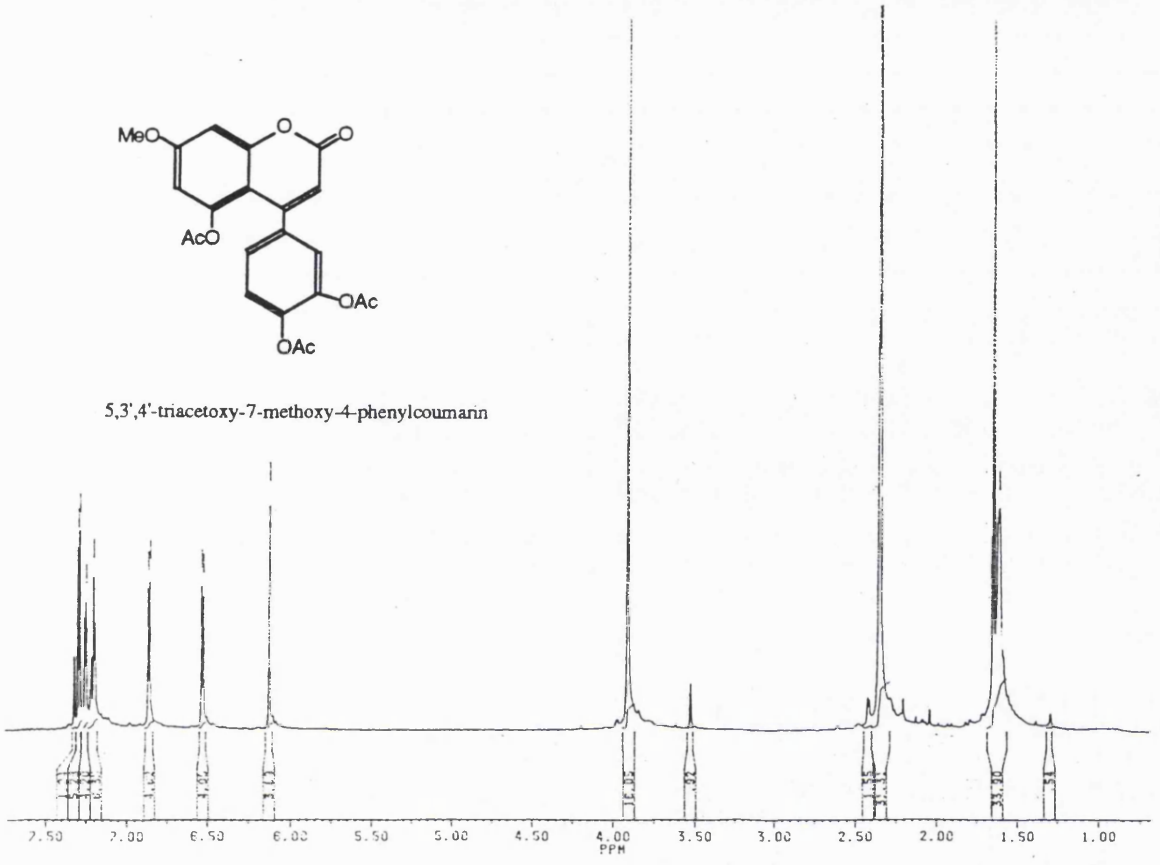
S-112. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 5-O-β-D-tetraacetylgalactopyranosyl]-3',4'-diacetyl-7-methoxy-4-phenylcoumarin



S-113. ^{13}C NMR (100 MHz, $CDCl_3$) spectrum 5-O-β-D-tetraacetoxygalactopyranosyl]-3',4'-diacetyl-7-methoxy-4-phenylcoumarin



S-114. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 5,3',4-triacetoxy-7-methoxy-4-phenylcoumarin



S-115. ^{1}H NMR (400 MHz, C_5D_5N) spectrum of 3-O-β-D-glucopyranosyl-23, 24-dihydrocucurbitacin F