IDENTIFICATION OF ANTIKINETOPLASTID COMPOUNDS FROM *PSOROTHAMNUS POLYDENIUS* AND *P. ARBORESCENS*

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

BY

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ABSTRACT

Parasitic diseases such as leishmaniasis and trypanosomiasis remain a major public health problem. They exact a heavy toll of illness and death especially in developing countries. Common chemotherapeutic agents currently used are often inadequate, requiring long courses of parenteral administration, having toxic side effects or are becoming less effective due to the emergence of resistant strains. Thus, there is an urgent need for new, effective, and inexpensive drugs.

Natural products are important sources of novel therapeutic agents in the fight against parasitic diseases. The goal of our research is to identify effective antileishmanial and trypanocidal agents from plant sources using a systematic approach. A natural products library of a representative diversity was screened for antiparasitic activity against *Leishmania donovani* and *Trypanosoma brucei brucei*. The active samples were further evaluated regarding their toxicity versus mammalian cell lines. From the results, the plant genus *Psorothamnus* was identified as a promising source of potential new antiparasitic compounds.

Bioactivity-guided fractionation of the methanolic extract of *Psorothamnus polydenius* yielded the new chalcone, 2,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone, together with six other known compounds, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone, dalrubone, demethoxymatteucinol, eriodictyol, oleanolic acid and photodalrubone. This was the first report of chalcones in *P. polydenius*. The extracts and isolated compounds were tested in vitro for their antiprotozoal activity against *Leishmania donovani* and *Trypanosoma brucei*. The isolated chalcones exhibited leishmanicidal (IC_{50} 5.0–7.5 μg/mL, respectively) and trypanocidal (IC_{50} 6.3–6.8 μg/ml, respectively) properties. Dalrubone displayed 6-fold selectivity for axenic *L. donovani* parasites over vero cells. Furthermore, treatment of *L. mexicana*-preinfected macrophages with the chalcones (12.5 μg/mL) or dalrubone (25 μg/mL) reduced the number of infected macrophages by at least 96% while posing no toxicity to the host cell. Flow cytometry analysis showed that dalrubone does not affect the cell cycle progression of the parasite. Electron microscopy revealed significant ultrastructural changes in the parasite suggesting perturbations in the secretory pathways of the parasite. However, dalrubone did not display a favorable in vivo activity against *L. donovani* infection in mice, possibly because of a low potency.

Another member of the same genus, *P. arborescens*, exhibited significant activity against *Leishmania donovani* axenic amastigotes and *Trypanosoma brucei brucei* bloodstream forms. Bioactivity-guided fractionation of the root extract of *P. arborescens* yielded the new isoflavone 5,7,3′,4′-tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (**5.1a**) and the new 2-arylbenzofuran 2-(2′-hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (**5.7**), together with seven other known compounds,

fremontin, glycyrrhisoflavone, calycosin, maackiain, 4-hydroxymaackiain, oleanolic acid, and isoliquiritigenin. In addition, the structure of the isoflavone fremontin was revised using spectroscopic and chemical methods and was assigned a new structure. The isoflavone **5.1a** and isoliquiritigenin displayed IC_{50} values of 4.6 and 5.3 μg/mL, respectively, against *L. donovani* axenic amastigotes. Calycosin exhibited selective toxicity against *T. b. brucei* (IC_{50} 3.6 μg/mL) compared to *L. donovani* amastigotes and Vero cells (IC_{50} values 28.5 and 45.1 μg/mL, respectively). These results prompted us to test a small group of structurally-related isoflavones for their antitrypanosomal activities. Genistein and 7,3′,4′-trihydroxyisoflavone displayed promising activity (IC_{50} values 1.1 and 1.9 μg/mL, equivalent to 4.2 and 7.1 μM, respectively) and selectivity (IC_{50} values 33 and 135 μM, respectively, versus Vero cells). The mechanism of the antikinetoplastid activity of isoflavonoids is not clear although electron microscopy studies indicated that treatment of *L. donovani* promastigotes with isoflavone **5.1a** resulted in significant changes in the mitochondrial ultrastructure. Our results suggest that the isoflavone skeleton deserves further investigation as a template for novel antileishmanial and trypanocidal compounds.

Dedicated to my parents

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FIELDS OF STUDY

Major Field: Pharmacy

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LIST OF ABBREVIATIONS

$[\alpha]_D$: Specific optical rotation

Ara: α-L-Arabinopyranose

Bz: Benzoyl

c: Concentration

Calcd: Calculated

COSY: Correlation Spectroscopy

δ (ppm): Chemical shift in parts per million

DEPT: Distortionless Enhancement by Polarization Transfer

DMSO: Dimethylsulfoxide

DPFGSE-NOE: Double Pulsed Field Gradient Spin Echo-NOE

EC_{50}: Effective concentration that inhibits a response by 50% relative to a control

ε: Molar absorptivity

Gal: β-D-Galactopyranose

Glc: β-D-Glucopyranose

h = Hour

HMBC: Heteronuclear Multiple Bond Correlation spectroscopy

HPLC: High-Performance Liquid Chromatography

HRESIMS: High-Resolution Electrospray Ionization Mass Spectrometry

HSQC: Heteronuclear Single Quantum Coherence spectroscopy

Hz: Hertz

IC_{50}: Sample contration that inhibits cell growth by 50% compared to untreated control

$[M]^+$: Molecular ion

i.p.: Intraperitoneal

IR: Infrared absorption

K_d: Dissociation constant

λ (nm): Wavelength in nanometers

mp: Melting point

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

m/z: Mass-to-charge ratio

NMR: Nuclear Magnetic Resonance

NOESY: Nuclear Overhauser Enhancement Spectroscopy

ν (cm^{-1}): Infrared absoption frequency in reciprocal centimeter

PBS: Phosphate-Buffered Saline

p.o.: By mouth (per os)

R_f: Migration distance relative to the solvent front in thin-layer chromatography

Rha: α-L-Rhamnopyranose

SAR: Structure-Activity Relationship

s.c.: Subcutaneous

SD: Standard Deviation

t_R: Retention time

TLC: Thin-Layer Chromatography

UV: Ultraviolet absorption

VLC: Vacuum Liquid Chromatography

WBA: World Botanical Associates (Bakersfield, CA)

CHAPTER 1

KINETOPLASTID PARASITES RESPONSIBLE FOR HUMAN DISEASES

Three distinct kinetoplastid parasites cause human diseases: *Trypanosoma brucei* complex (African sleeping sickness), *Trypanosoma cruzi* (Chagas disease), and *Leishmania* spp. (leishmaniasis). These flagellated protozoa are characterized by the presence of a sub-cellular structure known as the kinetoplast which is a distinct part of the mitochondrion and contains mitochondrial DNA.

1.1 LEISHMANIASIS

1.1.1 Parasite Transmission and Life Cycle

Leishmaniasis is a spectrum of disease caused by protozoan parasites belonging to the genus *Leishmania*. Twenty *Leishmania* species are pathogenic for humans and can be transmitted by the bite of an infected female sandfly, introducing parasites into the host. Thirty sandfly species are proven vectors and can become infected when taking a blood meal from a reservoir host. Hosts are infected humans and wild and domestic animals. Epidemiologically, the transmission cycle can be zoonotic, meaning animal reservoir hosts are involved, or anthroponotic, where man is the sole source of infection for the

insect vector. The life cycle of *Leishmania* (Figure 1.1) involves two parasite forms, the flagellated motile promastigote stage inhabiting the gut of the sandfly, and the non-flagellated amastigote stage growing intracellularly inside the phagolysosomes of mammalian macrophages.[1,2]

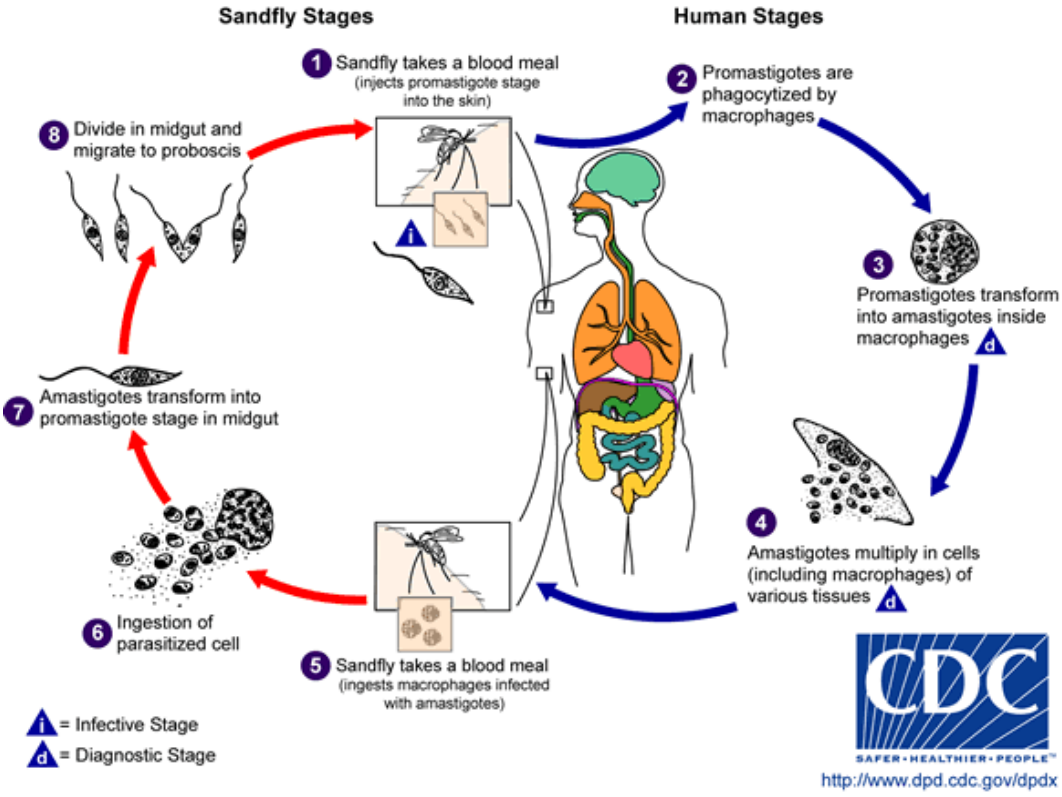


Figure 1.1: Life Cycle of *Leishmania*

(Reproduced by permission of the Centers for Disease Control and Prevention.)

1.1.2 Clinical Manifestations

The clinical manifestations of the disease depend on the infecting species. Leishmaniasis presents itself in three main clinical forms, all of which have devastating consequences. In visceral leishmaniasis (VL or "kala-azar"), the parasites reside in the liver, spleen and bone marrow causing a severe systemic disease that is fatal if untreated. Mucocutaneous leishmaniasis (MCL) is characterized by lesions in the mucous tissues of the nose and mouth and often progresses to massive tissue destruction and disfigurement. Cutaneous leishmaniasis (CL) involves the development of self-healing but chronic skin ulcers at the site of sandfly bites. There are also two rare types, post kala-azar dermal leishmaniasis (PKDL), which is a chronic CL form that develops in some patients after recovering from VL, and viscerotropic leishmaniasis, which is a mild visceral disease that does not progress to classic kala-azar. Viscerotropic leishmaniasis was observed in nine veterans (out of 500,000 U.S. soldiers) of the first Gulf War (Operation Desert Storm). Symptoms varied, including fevers, chronic fatigue, malaise, cough, intermittent diarrhea or abdominal pain.[3]

1.1.3 Epidemiology

The epidemic of VL in southern Sudan in the early 1990s resulted in an estimated overall death rate of 38–57% and up to 70% in the most affected areas, corresponding to about 100,000 deaths among approximately 280,000 people in the epidemic area.[4] According to World Health Organization (WHO) statistics (http://www.who.int/leishmaniasis/en), there are 12 million people currently affected by

leishmaniasis in 88 countries on five continents - Africa, Asia, Europe, North America and South America - with a total of 350 million people at risk. Two million new cases (1.5 million for CL and 500,000 for VL) are considered to occur annually. Of these, 90% of VL cases occur in five countries: Bangladesh, India, Nepal, Sudan and Brazil, while 90% of CL cases reside in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. There appears to be an increased incidence over the past decade (e.g. CL in Brazil: 1998, 21,800 cases; 2002, 40,000 cases and CL in Kabul, Afghanistan: 1994, 14,200; 2002, 65,000 cases). The reasons for this include rural to urban migration, projects bringing non-immune populations to endemic areas, malnutrition that decreases immunity, socioeconomic deterioration in crowded poor city suburbs, and the emergence of *Leishmania*/HIV coinfection. Even so, there is little doubt that the actual prevalence of leishmaniasis is considerably greater than the numbers officially reported because many cases go either undiagnosed or unreported, especially in places where patients have no access to medical facilities. Moreover, the economic impact of the disease is huge, with the serious costs involved (medical care/treatment, depletion of work force) negatively affecting the implementation of development projects.[5] In short, the situation is worsening and the progress being made against the disease is disappointing.[6]

1.1.4 Leishmaniasis in the Western Nations

The leishmaniases have been considered as diseases of tropical and subtropical countries. The WHO lists leishmaniasis as one of the six most important tropical diseases. Nevertheless, there is a growing interest in this disease in industrialized

countries due to the increase of *Leishmania*-HIV coinfection[7] and the increased importance of travel medicine. Moreover, leishmaniasis is an emerging zoonosis in the U.S.A.,[8-10] and 500–700 parasitologically confirmed cases were identified in U.S. soldiers in the Middle East between 2002–2004.[11,12] In addition, immunologists have studied *Leishmania* species as interesting models of intracellular parasitism where a microorganism survives and multiplies within macrophages.[2]

1.1.5 Control and Chemotherapy

Leishmaniasis control remains a serious problem. Transmission is difficult to interrupt, although some attempts to reduce vector and mammalian reservoir populations have been successful. Currently, no vaccines are available for either VL or CL inspite of various research efforts.[13] Available drugs are limited in number and suffer from various shortcomings.[3,14-19] Pentavalent antimonials, sodium stibogluconate (Pentostam™, Wellcome) and meglumine antimonate (Glucantime™, Farmitalia), have been the first line drugs for over 50 years. They require long courses with parenteral administration, and have toxic side effects and variable efficacy. Increasing resistance to antimonials is observed, particularly in northeast India.[18] The secondary treatment, amphotericin B, is given parenterally and has considerable renal toxicity. Newer agents with proven efficacy against Indian visceral disease include liposomal amphotericin B, which is easily tolerated and requires fewer injections, and the oral agent miltefosine. The former agent suffers from its high cost and the latter is extremely teratogenic.[16] Thus, there is an urgent need for new drugs against leishmaniasis.

1.1.6 History of the Disease

Leishmania parasites in a specimen from a "Delhi boil", a CL lesion, were first recognized by Cunningham in 1885 in India. Shortly after, the Russian army surgeon Borovsky gave an excellent description of the organisms in lesions from a "Sart sore" observed in Tashkent, Uzbekistan in 1889. In 1903, the American physician Wright discovered intracellular protozoal organisms inside a cutaneous lesion from a young Armenian immigrant. Wright gave the name "*Helcosoma tropicum*" to the organisms. Similar organisms, found in the spleens of "kala-azar" patients, were studied independently by Leishman and Donovan in 1903 and were later named *Leishmania donovani* by Ross in 1903. Wright's parasite was renamed *Leishmania tropica* by Lühe in 1906.[20]

1.2 AFRICAN SLEEPING SICKNESS

1.2.1 Parasite Transmission and Life Cycle

Human African trypanosomiasis (sleeping sickness) is caused by infection with the hemoflagellate parasites *Trypanosoma brucei rhodesiense* (in East and Southern Africa) and *T. b. gambiense* (in West and Central Africa). A third subspecies, *T. b. brucei*, causes animal trypanosomiasis in cattle but does not infect humans. The disease is distributed in 36 countries in sub-Saharan Africa. Infection is transmitted by the bloodsucking male and female tsetse flies (*Glossina* spp.). The life cycle (Figure 1.2) involves the infective metacyclic trypanosomes, which are injected in the saliva of the tsetse fly into the host. The metacyclic forms differentiate into bloodstream forms that

spread to the vasculature via the lymphatic system. The parasite maintains a chronic infection by evading the immune response of the host as it continuously changes its variant surface glycoprotein in the process of antigenic variation.[21]

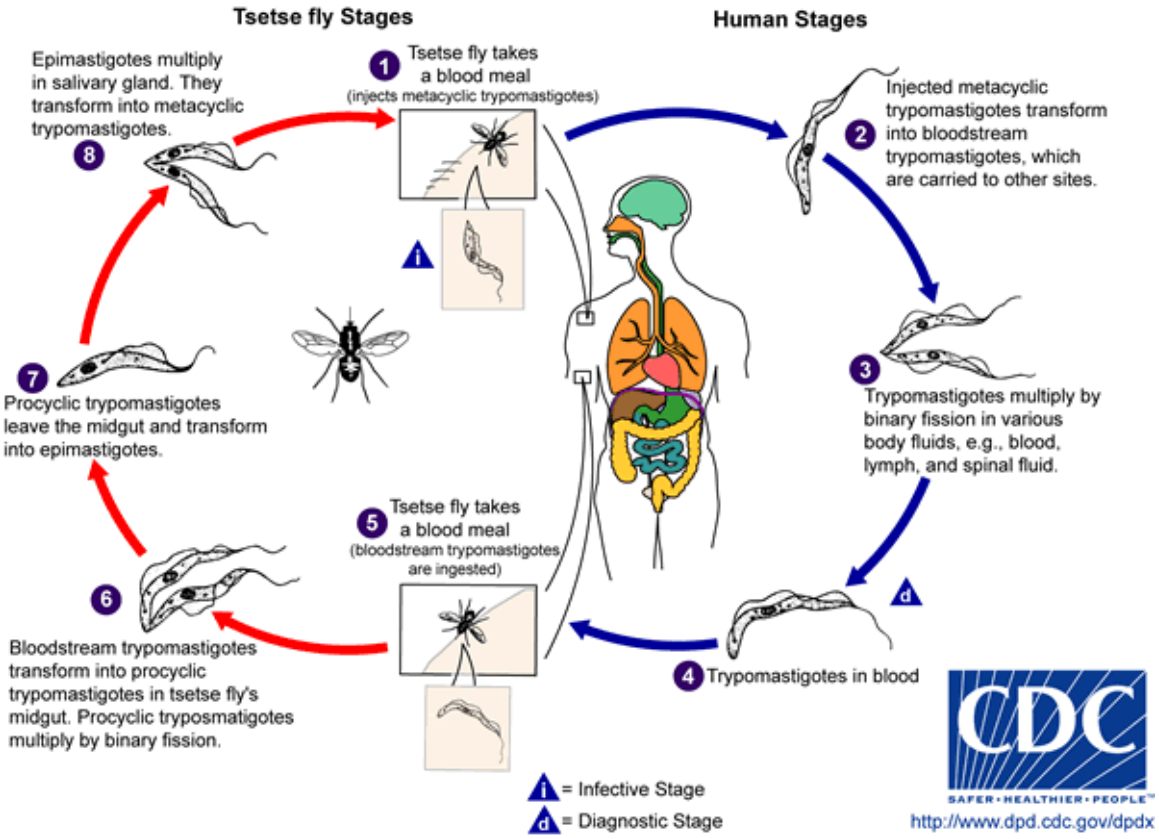


Figure 1.2: Life Cycle of *Trypanosoma brucei rhodesiense* and *T. b. gambiense* (Reproduced by permission of the Centers for Disease Control and Prevention.)

1.2.2 Clinical Manifestations

Early symptoms of the disease include malaise and irregular fevers as well as enlarged lymph glands and spleen. This is followed by headache, anemia, joint pain and

swollen tissues. Neurological and endocrine disorders develop in the neurological (meningoencephalitic) phase of the disease when the parasite invades the CNS, causing confusion, sensory and motor disturbances and sleep abnormalities. In the absence of treatment, the disease progresses to the final stage, leading to seizures, somnolence, coma and death. *T. b. rhodesiense* causes an acute form of the disease, leading to death within weeks. *T. b. gambiense* infections tend to progress more slowly over a period of years, causing a chronic disease that nonetheless ends in death.[21]

1.2.3 Epidemiology

The disease affects 36 countries in sub-Saharan Africa, threatening over 60 million people. About 45,000 cases were reported in 1999, but the WHO estimates that the incidence is 10 times higher with the real number of cases up to 500,000 per year. In some villages of Angola, Congo, and southern Sudan the prevalence is between 20 and 50%.[22]

1.2.4 Control and Chemotherapy

Control of the disease is difficult and is hampered by the large domestic and wild animal reservoirs of the zoonotic parasite and complicated by political instability, war and poverty. Pentamidine and suramin are used to treat the initial phase, but do not cross the blood-brain barrier and thus cannot be used to treat late stage disease. Melarsoprol, an arsenical drug, is used for treatment of the late stage disease but has drastic side effects including fatal reactive encephalopathy in 3–10% of cases. In addition, resistance to the

drug is rising to 30% in parts of central Africa. Eflornithine is an alternative to melarsoprol treatment but is effective only against *T. b. gambiense* and must be administered in high doses over a long course of treatment.[23]

1.3 CHAGAS DISEASE

1.3.1 Parasite Transmission and Life Cycle

American trypanosomiasis (Chagas disease) is caused by another trypanosomatid, *T. cruzi*. The parasite is zoonotic, mainly transmitted by blood-feeding "Assassin" or "Kissing" bugs of the sub-family Triatominae (belonging to the genera *Triatoma*, *Rhodinius*, and *Panstrongylus*). During a blood meal, the infective metacyclic trypomastigotes are released in the feces of the insect vector and enter the host through the bite wound or through an intact mucous membrane such as the conjunctiva. The parasite can also be transmitted through contaminated blood transfusion, organ transplantation, or transplacentally.

1.3.2 Clinical Manifestations

The disease begins with an initial acute stage that is usually asymptomatic and with a low mortality rate (< 10%). The disease then advances to a chronic stage, resulting in irreversible lesions in the gastrointestinal tract and the heart in 30–40% of cases.

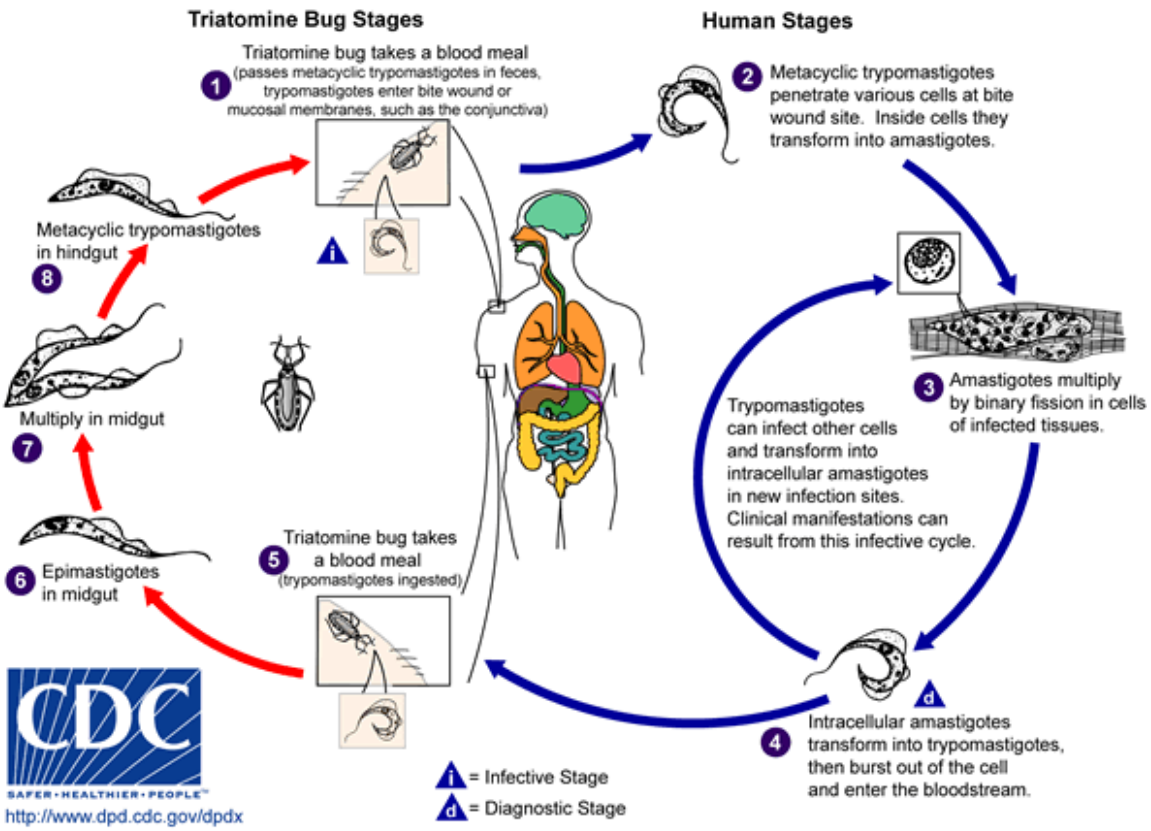


Figure 1.3: Life Cycle of *Trypanosoma cruzi*

(Reproduced by permission of the Centers for Disease Control and Prevention.)

1.3.3 Epidemiology

The disease is endemic in Latin America from northern Mexico to southern Argentina, affecting 18 countries. Currently, there are about 20 million infected individuals and 100 million at risk of acquiring the infection. The annual incidence of the disease is about 300,000 cases, resulting in 21,000 deaths per year (http://www.who.int/tdr/diseases/chagas).

1.1.4 Control and Chemotherapy

It is now accepted that the pathogenesis of the disease in its acute and chronic stages is related to the persistence of the parasite in the host in addition to an unbalanced immune response.[24] Thus, the disease should be treated as a parasitic rather than an autoimmune disease. The main chemotherapy currently available are the nitroheterocyclic compounds nifurtimox and benzinidazole. Both drugs suffer from serious side effects including anorexia, vomiting, peripheral polyneuropathy and allergic dermopathy. These adverse effects frequently lead to treatment discontinuation. However, the major drawback of these drugs is the low efficacy in the chronic form of the disease evidenced by failure to clear the parasites in > 80% of patients.[24] Gentian violet is the only effective compound that eliminates the parasites from blood before use for transfusion.

CHAPTER 2

ADVANCES IN NATURAL PRODUCTS RESEARCH FOR ANTIKINETOPLASTID AGENTS

2.1 DEVELOPMENT OF NATURAL PRODUCTS AS ANTIPARASITIC DRUGS

2.1.1 Why Natural Products?

Natural products continue to be an important source of chemotherapeutic agents, particularly those used to treat infectious diseases. Out of the 162 New Chemical Entities (NCE) approved as antiinfective drugs by the regulatory agencies over the period 1981–2002, 107 (66%) were from a natural origin (natural product, derived from natural products, or synthetic with a natural product pharmacophore).[25] However, over the past two decades, the pharmaceutical industry has turned away from natural product research. Instead, the focus shifted towards combinatorial chemistry, which seemed to fulfill the need for massive compound libraries to keep up with the high-throughput screens based on newly discovered molecular targets. However, this approach did not result in the expected enhanced productivity and increase in the number of novel drugs. The number of NCEs hit a 20-year record low of 37 in 2001 and is still decreasing.[25] In the same year, the number of New Drug Applications to the FDA dropped to only 16.[25] These facts emphasize the importance of natural product research to the drug discovery process. Drug

leads of novel molecular diversity can then be optimized by synthetic approaches. In addition, there is a growing interest among consumers in the use of alternative therapies and natural products, especially those from plant sources. The world market for over-the-counter phytomedicinal agents was estimated at $10 billion in 1997, with an annual growth of 6.5%. However, much remains to be explored concerning the use of higher plants as sources of drugs. Only a small percentage of the known plant species, estimated at 250,000–500,000, have been investigated phytochemically.[26]

2.1.2 Selection of Plant Material

Plant material used in screening for antiparasitic activity can be obtained by random collections or selected based on their ethnopharmacology/traditional medicinal use. Furthermore, plant collection for screening can focus on rare and uninvestigated species in hope of generating novel structures and activities or only collect samples on the basis of chemotaxonomy where botanically related species are expected to produce structurally-related metabolites.

2.1.3 Assaying Natural Products for Antiparasitic Activity

Screening of plant extracts for antiparasitic activity requires a simple and fast biological assay. Extracts displaying potency in the bioassay are then subjected to bioactivity-guided fractionation to isolate the active constituents. For antileishmanial activity, the promastigote stage is frequently used for the initial screening although promastigotes are not the clinically relevant form. Assays employing intracellular

amastigotes in macrophage models give more clinically relevant information about the activity and mammalian toxicity of the tested compounds.[27] Axenic cultures of *Leishmania* amastigotes that resemble intracellular amastigotes in many aspects have been established and used for screening antileishmanial drugs.[28] Axenic cultures of bloodstream trypomastigotes are commonly used for antitrypanosomal testing. Drug activities are determined by isotopic assays or by using colorimetric or fluorometric reagents. However, screening of drugs against parasitic cells in vitro has limitations, such as missing compounds that can act as prodrugs and those that have difficulty penetrating cells. Rodent models for leishmaniasis and African trypanosomiasis are available for in vivo testing which usually requires higher sample amounts than those necessary for in vitro testing.

2.2 NATURALLY OCCURRING ANTILEISHMANIAL AGENTS

2.2.1 Phenolic Compounds

2.2.1.1 Chalcones

Licochalcone A (**1.1**), isolated from the roots of the Chinese liquorice plant, *Glycyrrhiza glabra* (Fabaceae), strongly inhibited (68–82%) the multiplication of intracellular *L. major* amastigotes within human phagocytes at 0.5 μg/mL. Electron microscopy revealed that licochalcone A caused pronounced changes in the mitochondrial ultrastructure without apparent damage to the other organelles of the parasite. The compound did not affect the mitochondria of human phagocytic cells or cause any other major toxicity, suggesting safety for use as a drug.[29] The measurement of

O_2 consumption and CO_2 production confirmed that licochalcone A inhibits respiration of the promastigotes in a dose-dependent manner.[30] It was found to specifically inhibit mitochondrial fumarate reductase, which is a component of the parasite respiratory chain. The enzyme represents an excellent target for selective antileishmanial agents because it does not exist in mammalian cells.[31] In vivo testing against *L. major* cutaneous lesions in mice showed that treatment with 2.5 or 5 mg/kg/day i.p. starting at day 7 post-infection completely prevented lesion development. In addition, administration of licochalcone A at 20 mg/kg/day i.p. × 6 days to hamsters 1 day post-infection with *L. donovani* resulted in over 96% reduction in the hepatic and splenic parasite load. Oral administration at 5 to 150 mg/kg /day × 6 days resulted in 65–85% reduction in the parasite load compared to that in the untreated control.[32] A series of related oxygenated chalcones showed potency similar to that of licochalcone A, both in vitro and in vivo. For example, 2,4-dimethoxy-4′-allyloxychalcone and 3,5-dimethoxy-4′-allyloxychalcone inhibited the growth of intracellular *L. donovani* amastigotes with IC_{50} values 0.7 and 0.8 μg/mL, respectively (licochalcone A, IC_{50} 0.9 μg/mL). In the hamster model, these chalcones reduced *L. donovani* parasites in the liver by 97% and 88%, respectively, when administered i.p. at 20 mg/kg/day × 6 days.[33]

2′,6′-Dihydroxy-4′-methoxychalcone (**1.2**), obtained from *Piper aduncum* (Piperaceae), showed activity against *L. amazonensis* promastigotes (IC_{50} 0.5 μg/mL) and intracellular amastigotes (IC_{50} 24 μg/mL).[34] Furthermore, encapsulation of the chalcone in polymeric nanoparticles enhanced the antileishmanial activity. At 1 μg/mL, the encapsulated chalcone reduced intracellular *L. amazonensis* amstigotes in infected

macrophages by 53%. Moreover, the chalcone-nanoparticle formulation reduced the size of *L. amazonensis* cutaneous lesions in BALB/c mice by 60% and decreased the number of parasites in the lesions by 90% when administered for four doses (i.p. at 10 mg/kg/dose on days 42 and 48 post infection, as well as s.c. at 1 mg/kg/dose on days 27 and 54 post-infection). These results were comparable to those of equivalent doses of the standard antileishmanial drug, Glucantime.[35]

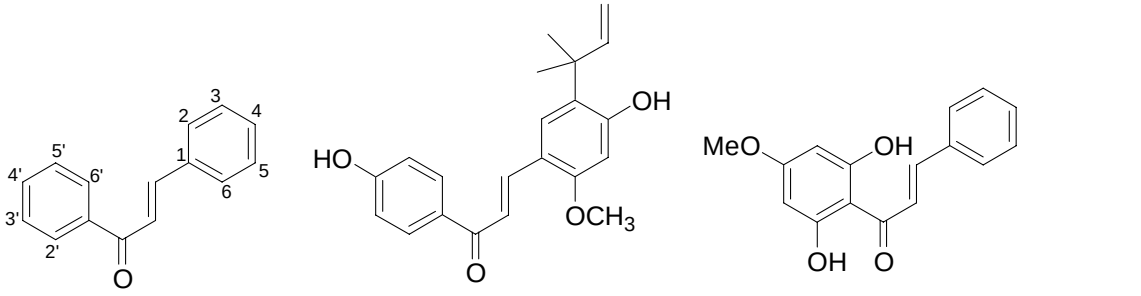


Figure 2.1: Structures of Some Antileishmanial Chalcones

In a study where 20 naturally occurring chalcones with different substitution patterns at C-3, -4, -2′, -3′, and -4′ were tested, most chalcones exhibited activity towards the promastigote form of *Leishmania* species at IC_{50} values between 0.07 and 2.0 μg/mL. Some displayed potent activity against the intracellular amastigote form, possessing IC_{50} values below 1 μg/mL. However, when tested against murine macrophages, all active compounds showed significant toxicity, with IC_{50} values 0.2 to 2.0 μg/mL. Chalcones with bulky substituents that hinder free rotation resulted in total loss of activity. Features of active chalcones include the presence of oxygenated substituents at positions C-2′ and

C-4′. The introduction of additional oxygenated substituents will increase activity only when the lipophilic nature of the skeleton is conserved, thus the introduction of methoxy groups is preferred. The introduction of hydroxyl groups or sugar units will render the compound too hydrophilic and reduce the leishmanicidal activity.[36] The acetylation of hydrophilic chalcones having many OH groups or sugar units enhances antileishmanial activity.[37] Substitution of the aromatic rings with various halogens reduces the antileishmanial and antitrypanosomal activity compared to the parent unsubstituted chalcone.[38] Chemical modification of the α,β-double bond of chalcones by reduction, substitution, or conversion into an acetylenic linkage, resulted in derivatives whose potencies differ only marginally from those of the parent compounds. This indicates that the propenone residue in the chalcone skeleton functions only as a spacer between the two aromatic rings that constitute the real pharmacophore.[39] The structure-activity relationships of a large number of synthetic antileishmanial chalcones was investigated by Nielsen et al.[40] and by Liu et al.[41]

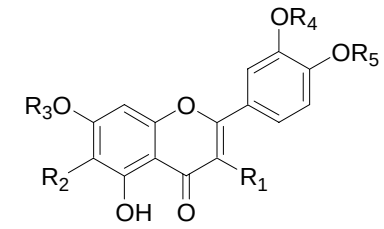
2.2.1.2 Flavonoids

2.2.1.2.1. Leishmanicidal Flavonoids

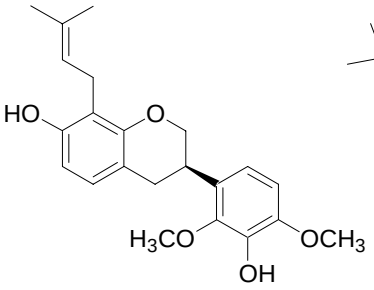
Luteolin (**2.3**), quercetin (**2.4**), isoorientin (**2.5**), and flavone A (**2.6**) displayed antileishmanial activity when tested in vitro against *L. donovani* promastigotes, while rutin was inactive. Luteolin and quercetin, with IC_{50} values of 12.5 and 45.5 μM, respectively, induced morphological changes and the loss of cellular integrity in the promastigotes. These flavonoids caused cell cycle arrest in the *G1* phase and a substantial

increase in apoptotic cells at their respective IC_{50} concentrations. Both agents reduced the intracellular *L. donovani* amastigote burden in mouse peritoneal macrophages by 70% at 12.5 and 45.5 μM, respectively. Luteolin and quercetin promoted kDNA minicircle linearization in a dose-dependent manner and were found to completely inhibit leishmanial topoisomerase II at 50 and 100 μM, respectively. Their cytotoxity was correlated to the formation of a cleavable complex. Notably, luteolin did not induce either cell cycle arrest or apoptosis in normal human T-cell blasts, revealing its antileishmanial selectivity. Moreover, when administered to *L. donovani*-infected hamsters at 3.5 mg/kg body weight orally twice weekly for 1 month, beginning 4 days post-infection, luteolin reduced the splenic parasite load by over 80%. The study suggested luteolin as a lead for the development of more effective antileishmanial chemotherapies.[42] Oral administration of quercetin (**2.4**) or flavone A (**2.6**) at 10 and 20 mg/kg, respectively, bi-weekly to *L. donovani*-infected hamsters, beginning 1 month post-infection, decreased the parasite load in the spleen by 77% and 35%, respectively, after 1 month of treatment. Moreover, the flavonoids prevented the development of anemia with hemoglobin (Hb) levels at 14 and 13 g/L, respectively, in infected animals versus 9 g/L in non-treated animals. The high antioxidant potency of quercetin protects the erythrocytes from oxidative damage, which is implicated in the premature hemolysis observed in visceral leishmaniasis. Quercetin in combination with sodium stibogluconate resulted in better reduction of parasitemia and higher enhancement of Hb levels than either of the agents alone, suggesting that quercetin is a promising candidate as an adjuvant therapy to combat VL infection and the anemia associated with it.[43] Luteolin 7-*O*-β-D-glucopyranoside (**2.7**) and

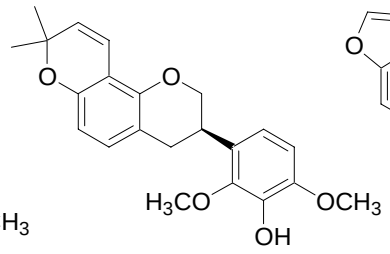
chrysoenol 7-*O*-β-D-glucopyranoside (**2.8**) showed potent inhibitory effects on *L. donovani* axenic amastigotes in vitro with IC_{50} values of 1.1 and 4.1 μg/mL, respectively. Interestingly, the first compound but not the second showed also a potent activity against plasmodial enoyl-acyl carrier protein reductase, which is a key regulator of type II fatty acid synthase (FAS-II). The compounds were non-toxic to L6 cells at up to 90 μg/mL and displayed weak to no activity against *T. b. rhodesiense* and *T. cruzi*.[44]



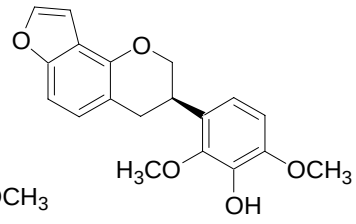
Luteolin (**2.3**) $R_1 = R_2 = R_3 = R_4 = R_5 = H$
Quercetin (**2.4**) $R_1 = OH, R_2 = R_3 = R_4 = R_5 = H$
Isoorientin (**2.5**) $R_2 = Glc, R_1 = R_3 = R_4 = R_5 = H$
Flavone A (**2.6**) $R_1 = R_2 = OCH_3, R_3 = R_4 = R_5 = CH_3$
2.7 $R_1 = R_2 = R_4 = R_5 = H, R_3 = Glc$
2.8 $R_1 = R_2 = R_5 = H, R_3 = Glc, R_4 = CH_3$



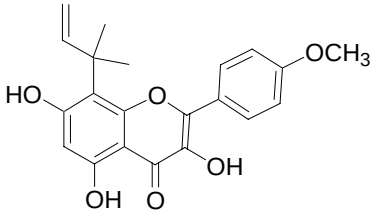
8-Prenylmucronulatol (**2.9**)



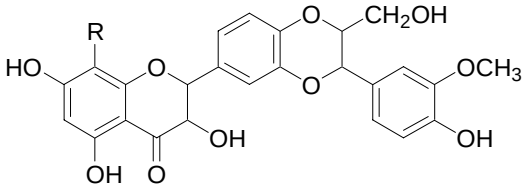
Glyasperin H (**2.10**)



Smiranicin (**2.11**)



8-(3,3-Dimethylallyl)-kaempferide (**2.12**)



Silybin (**2.13**) R = H
2.14 R = 3,3-dimethylallyl,Δ^2

Figure 2.2: Structures of Some Flavonoids with Antileishmanial Properties

Three isoflavans, 8-prenylmucronulatol (**2.9**), glyasperin H (**2.10**), and smiranicin (**2.11**), isolated from the Iranian plant *Smirnowia iranica* (Fabaceae), inhibited the growth of *L. donovani* promastigotes with IC_{50} values 6.9, 25, and 23 μM, respectively. The activity against the intracellular amastigote form was considerably lower, with the IC_{50} values being over 80 μM. The compounds were virtually nontoxic to mammalian RAW macrophages (IC_{50} around 200 μM).[45]

2.2.1.2.2 Flavonoids and modulation of multidrug resistance in *Leishmania*

Multidrug resistance (MDR) to cancer chemotherapy is often correlated to P-glycoprotein (Pgp) overexpression in tumor cells. Pgp is an ATP-dependent trans-membrane efflux pump of the ABCB (ATP-binding cassette B) family of transporter proteins that decreases the intracellular accumulation of cytotoxic drugs. The Pgp sequence contains 2 homologous halves, each composed of a transmembrane domain (TMD) involved in drug binding and efflux and a nucleotide-binding domain (NBD) where ATP binding and hydrolysis takes place.[46] Pgp modulators, including calcium channel blockers such as verapamil and immunosuppressants such as cyclosporin A, reverse MDR in tumor cells by competing with drug binding to the TMD of Pgp.[47] Recent investigations showed the involvement of ATP-dependent Pgp-like multidrug transporters in experimental multidrug resistance of *Leishmania* spp,[48-50] especially resistance to the promising alkyl-lysophospholipid miltefosine.[51,52] The *ldmdr1* gene from a *L. donovani* line selected for vinblastine resistance conferred resistance to unrelated drugs, like daunomycin and puromycin, when transfected into wild-type parasites.[49] A *L.*

tropica cell line selected for resistance to daunomycin was found to over-express LTRMDR1, a Pgp-like transporter encoded by the *ltrmdr1* gene. This cell line was found to be multdrug-resistent to other drugs, like doxorubicin HCl, vinblastine and puromycin, due to significant reduction of drug accumulation in the parasite.[48] Moreover, MDR *L. tropica* displayed cross-resistance to the antileishmanial drug, miltefosine.[51] However, multidrug resistance in *Leishmania* conferred by Pgp-like transporters is not reversed upon treatment with the classical Pgp inhibitors such as quinidine or verapamil.[48,50] The C-terminal NBD2 domain of *L. tropica* Pgp-like transporter was overexpressed in *Escherichia coli* and purified. The binding affinity of different flavonoids to the NBD2 was determined by monitoring the quenching of the intrinsic fluorescence of this domain. The recombinant protein bound flavonoids with the following efficiency: flavone > flavanone > isoflavone > glucorhamnosyl-flavone > chromone. The binding affinities of the flavones to the NBD2 was improved by the presence of OH groups in positions 3 or 5 and greatly enhanced by the introduction of a 1,1-dimethylallyl substituent at position 8 of the flavone skeleton. 8-(1,1-Dimethylallyl)-kaempferide (**2.12**) showed the best affinity of the flavones tested with a $K_d = 0.7$ μM. At 50 μM, it reversed parasite resistance to daunomycin, resulting in drug accumulation as assessed by flow cytometry and inhibition of parasite growth.[53] The flavanolignan silybin (**2.13**), which is the main constituent of the hepatoprotective herbal medication silymarin (from *Silybum marianum*), was recently shown to be synergistic with doxorubicin in a doxorubicin-resistant cell line, possibly by inhibiting Pgp. Silybin bound the recombinant C-terminal NBD of *L. tropica* Pgp-like transporter and sensitized the resistant promastigotes to

daunomycin. The binding affinity was increased by oxidation of silybin to the flavonol dehydrosilybin, and by the addition of the hydrophobic dimethylallyl moiety, especially at position 8 of ring A. In addition, the 3-OH group and the monolignol unit were important for binding. Thus, 8-(3,3-dimethylallyl)-dehydrosilybin (**2.14**) showed the highest affinity to the Pgp-like transporter, with a K_d = 0.11 μM. At 10 μM, it sensitized the MDR cells to daunomycin, resulting in more than 95% growth inhibition while displaying slight toxicity to the wild-type cells.[54]

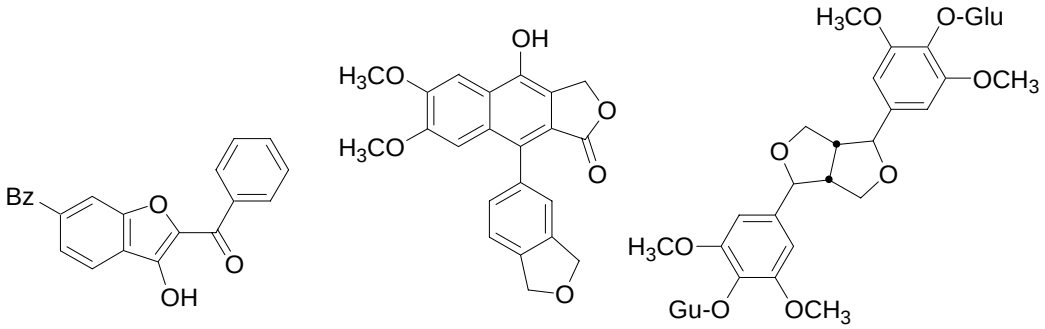
2.2.1.3 Aurones

A series of aurones showed antileishmanial activity, with aurone **2.15** being the most active. This compound is 58-fold more selective for the intracellular amastigotes (IC_{50} 0.04 μg/mL) than the murine host macrophages. The SAR drawn from this small set of compounds indicated that decreasing lipophilicity by the introduction of hydroxy and sugar groups and the introduction of bulky substituents (like benzyl or glucose moieties) decreases the antileishmanial activity, especially against intracellular amastigotes.[55] Aurones were shown to potently inhibit parasite mitochondrial fumarate reductase in a dose-dependent manner.[56]

2.2.1.4 Lignans

Diphyllin (**2.16**), isolated from *Haplophyllum bucharicum* (Rutaceae), an endemic plant of Uzbekistan, exhibited a moderate inhibitory effect on *L. infanatum* promastigotes (IC_{50} 14 μM), arresting the cell cycle in the *S* phase and causing a drop in intracellular

protein content. On the other hand, overnight pretreatment of macrophages with diphyllin resulted in potent inhibition of the internalization of amastigotes (IC_{50} 0.2 μM). The compound may act by modulating the surface molecules resposible for the attachment of *Leishmania* to the macrophages.[57] Liriodendrin (**2.17**), a lignan glycoside from *Phlomis brunneogaleata* (Lamiaceae), displayed activity against *L. donovani* amastigotes at an IC_{50} value 12 μg/mL while being much less active against trypanosomes and non-toxic to L6 mammalian cells at up to 90 μg/mL.[44]



2.15 Diphyllin (**2.16**) Liriodendrin (**2.17**)

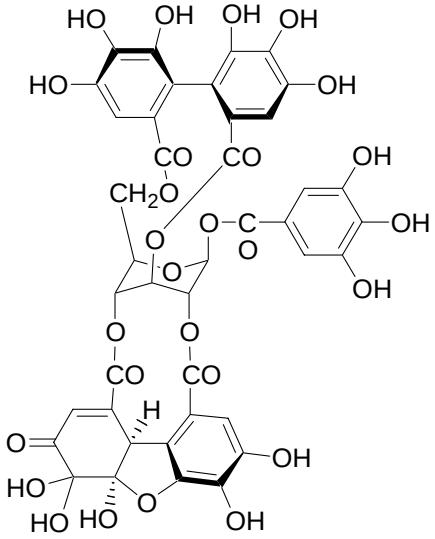
Figure 2.3: Structures of Some Antileishmanial Aurones and Lignans

2.2.1.5 Polyphenols/Vegetable Tannins

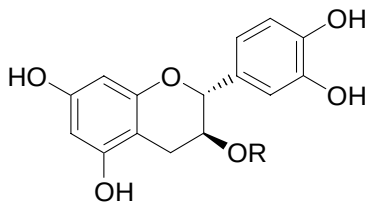
2.2.1.5.1 Hydrolyzable Tannins

A series of 27 hydrolyzable tannins was investigated for antileishmanial activity. All compounds exhibited potent activities against the intracellular amastigote form at IC_{50} values <0.4–12.5 μg/mL. In contrast, none of the compounds was significantly toxic to the parasite promastigote form. Most compounds were non-toxic to the mammalian

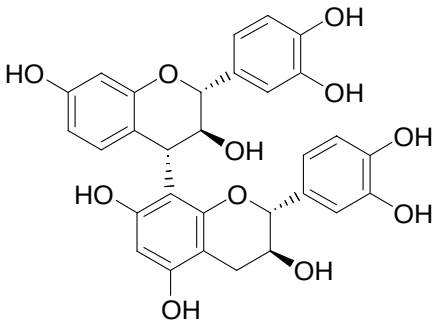
host cells (IC_{50} >25 μg/mL). All monomeric *C*-glucosidic ellagitannins and the majority of dehydroellagitannins induced potent tumor necrosis factor (TNF) and interferon-like (INF) activities in the host cells. Simple gallotannins and oligomeric ellagitannins did not show a similar effect. Structural features within monomeric ellagitannins that are associated with better TNF-inducing capabilities include the presence of a 3,6-bridging hexahydroxydiphenoyl (HHDP) group on a $^1C_4$ glucose core in addition to the presence of a second dehydro HHDP group in the molecule. For example, the dehydroellagitannin geraniin (**2.18**) displayed a potent activity against intracellular amastigotes (IC_{50} <0.4 μg/mL) and significantly induced TNF and INF with an EC_{50} values 0.7 and 0.3 μg/mL, respectively.[58]



Geraniin (**2.18**)



Gallocatechin (**2.19**) R = galloyl



Fisetinidol-4β,8-catechin (**2.20**)

Figure 2.4: Structures of Some Antileishmanial Tannins

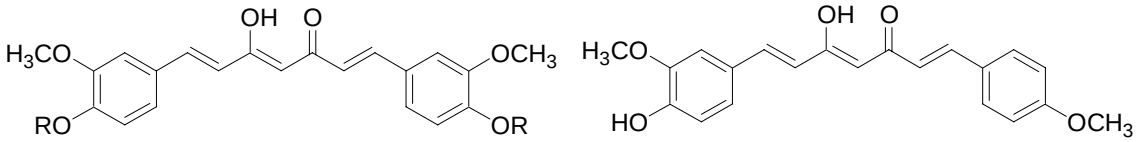
2.2.1.5.2 Proanthocyanidins (Condensed Tannins)

Another series of 19 proanthocyanidins and related compounds was evaluated for antileishmanial activity. Similar to the hydrolyzable tannins, most of the polyphenols tested inhibited the intracellular survival of *L. donovani* amastigotes with IC_{50} values in the range 0.5–15 μg/mL while not being active against *Leishmania* extracellular promastigote forms or being toxic towards mammalian macrophages. The proanthocyanidins induced tumor necrosis factor (TNF) release, though to a lesser extent than hydrolyzable tannins, in correlation with the intracellular antileishmanial effect. The induction of TNF was increased by the presence of galloyl moieties in the structure. The simple flavon-3-ol, gallocatechin (**2.19**) displayed an IC_{50} 4.7 μg/mL, while the proanthocyanidin fisetinidol-4β,8-catechin (**2.20**) inhibited the intracellular amastigotes at an IC_{50} 0.5 μg/mL. Both compounds showed no toxicity to the host cells at concentrations up to 25 μg/mL.[59]

2.2.1.5 Diarylheptanoids

The ground dried rhizomes of the plant *Curcuma longa* (Zingiberaceae), commonly called turmeric, is used as an herbal remedy for the treatment of many ailments and also as a food coloring agent and spice. Curcumin (**2.21**), the major active constituent, is a diphenylheptanoid that possesses potent antioxidant, anti-inflammatory and anti-carcinogenic properties as well as antibacterial and antifungal activities.[60] Moreover, in long-term feeding of curcumin to dogs, guinea pigs or monkeys, there was no evidence of toxicity.[61] Curcumin showed an average IC_{50} of 5.3 μM against the

promastigotes of various leishmanial strains including *L. major*, *L. tropica* and *L. infanatum* and an IC_{50} of 10 μM against *L. major* axenic amastigote-like cells.[60] Dimethylcurcumin (**2.22**) is more potent than curcumin, while bromination and partial or complete reduction of curcumin drastically decreased activity. In vivo testing of dimethylcurcumin (20 mg/kg s.c., one dose at the time of infection) in mice infected with *L. amazonensis* revealed a 66% reduction in the lesion size compared to the control group at the 45th day of infection. However, no difference was observed on further follow up.[62] Another study showed that diarylheptanoids are more efficient than diarylpentanoids against several *Leishmania* species. Substitution of the aromatic ring with 4-hydroxy and 3-methoxy groups, enhances the antileishmanial activity. The compound 1-(4-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione (**2.23**) was one of the most active compounds, with an IC_{50} of 8 μM against *L. amazonensis* promastigotes and 58 μM against axenic amastigotes (after 24 h incubation).[63]



Curcumin (**2.21**) R = H
Dimethylcurcumin (**2.22**) R = CH_3

2.23

Figure 2.5: Structures of Some Antileishmanial Diarylheptanoids

2.2.1.7 Other phenolics

Three compounds isolated from *Machaerium multiflorum* (Fabaceae) showed significant antileishmanial as well as antibacterial, antifungal and antimalarial activities. The hexahydrodibenzopyran (HHDBP) machaeriol C (**2.24**) and the 5,6-*seco*-HHDBPs machaeridiol B (**2.25**) and machaeridiol C (**2.26**) inhibited the growth of *L. donovani* promastigotes with an IC_{50} values of 6.0, 0.9, and 3.0 μg/mL. These compounds showed no cytotoxicity against Vero cells at 4.7 μg/mL.[64]

The phenylethanoid glycosides verbascoside (**2.27**), isoverbascoside, forsythoside B, echinacoside (**2.28**), glucopyranosyl-(1→G_i-6)-martynoside (**2.29**) and integrifolioside B from *Phlomis brunneogaleata* (Lamiaceae) showed activity against *L. donovani* axenic amastigotes at IC_{50} values of 8 to 13 μg/mL while displaying low or no toxicity against L6 cells. The glycoside, 4-hydroxyacetophenone 4-*O*-(6′-apiofuranosyl)-glucopyranoside from the same species was also active, showing an IC_{50} value of 11 μg/mL against the amastigotes, while displaying no toxicity to the mammalian cell line or *Trypanosoma* species.[44]

The common caffeic acid esters chlorogenic acid (**2.30**), 3-*O*-caffeoylquinic acid methyl ester (**2.31**) and 5-*O*-caffeoylshikimic acid (**2.32**) showed activity against *L. donovani* axenic amastigotes at IC_{50} values 7 to 9 μg/mL while exhibiting low or no toxicity against L6 cells.[44]

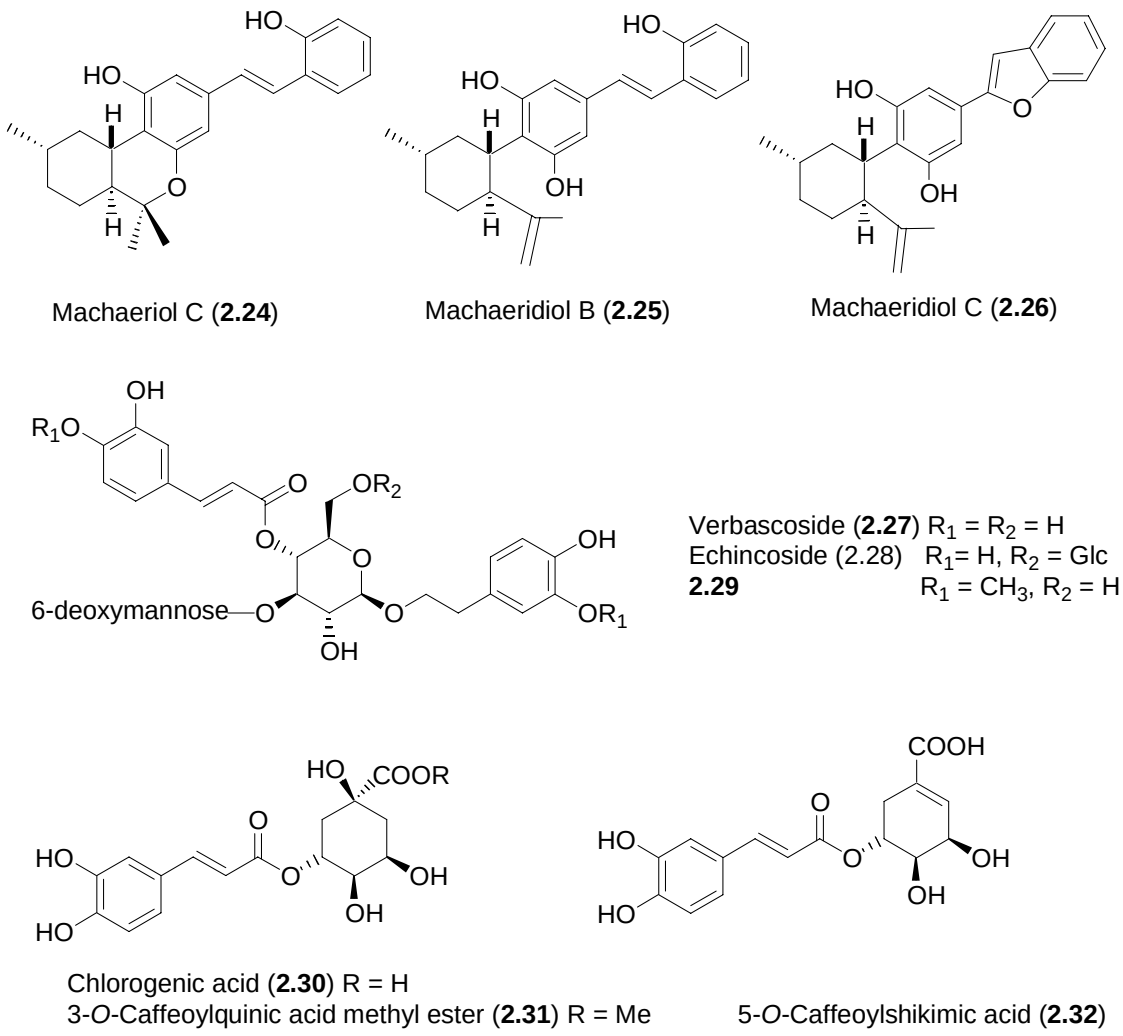


Figure 2.6: Structures of Some Phenolic Compounds with Activity against *Leishmania*

2.2.2 Terpenoids

2.2.2.1 Monoterpenes

The linalool-rich essential oil from the leaves of *Croton cajucara* was reported to exert potent leishmanicidal properties against the promastigotes of *L. amazonensis* (LD_{50} 8.3 ng/mL). Electron microscopy revealed morphological changes in the promastigotes

within 1 h of treatment with 15 ng/mL of the oil, including destruction of the nuclear and kinetoplast chromatin followed by cell lysis. At the same concentration, the oil was non-toxic to mouse peritoneal macrophages or Vero cells. Treatment of mouse peritoneal macrophages with 15 ng/mL of the essential oil for 20 min before or 90 min after infection with *L. amazonensis* promastigotes, resulted in an increase in NO level and a 50% reduction of intracellular amastigotes compared to control. The purified active constituent, linalool (**2.33**), is a terpene alcohol component of many essential oils. It was shown to exert an extremely potent antileishmanial activity against *L. amazonensis* promastigotes and amastigotes with the 50% lethal doses (LD_{50}) being 4.3 and 16 ng/mL, respectively. At these LD_{50} concentrations, linalool displayed no cytotoxic effect on mouse peritoneal macrophages or Vero mammalian cells.[65] On the other hand, the terpenic alcohol, terpinen-4-ol (4-carvomenthenol, **2.34**), and several other monoterpenes including α-pinene, β-pinene, and terpinolene were found to be only weakly active against *L. major* promastigotes, displaying LD_{50} values of >50 μg/mL.[66]

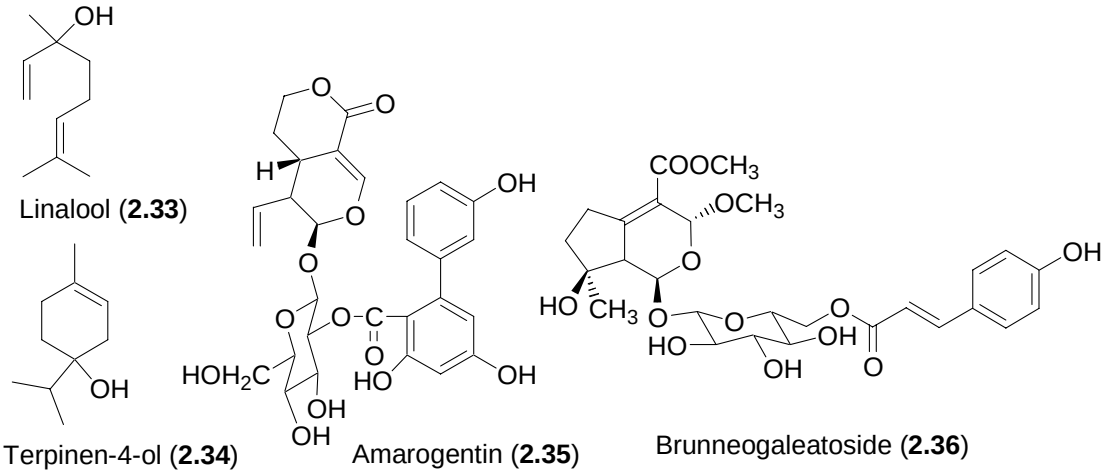


Figure 2.7: Structures of Some Antileishmanial Monoterpenes and Iridoids

2.2.2.2 Iridoids

Iridoids are cyclopentan[*c*]pyran monoterpenoids. Amarogentin (**2.35**) is a secoiridoid glycoside isolated from the Indian medicinal plant *Swertia chirata* (Gentianaceae). It potently inhibited the DNA relaxation activity of type I topoisomerase from *Leishmania donovani* in a dose-dependent manner. The inhibition was enhanced by preincubation of the enzyme with amarogentin before addition of the supercoiled DNA substrate and reversed by increasing the enzyme concentration. This evidence suggested that the compound works by binding with topoisomerase I, preventing binary complex formation between the enzyme and DNA. The inhibitory effect of amarogentin at 5 and 10 μM on DNA relaxation was comparable to that of camptothecin (at the same concentrations), an antitumor agent known to inhibit topoisomerase I by stabilizing the cleavable complex between the enzyme and DNA.[67] The antileishmanial effect of amarogentin was further investigated in a hamster model of visceral leishmaniasis. Thirty days post-infection with *L. donovani*, hamsters were given 2.5 mg/kg of amarogentin, free or intercalated in 0.5 mL liposomal or niosomal (non-ionic surfactant vesicles) suspension, s.c. every 3 days for a total of 6 doses. The niosomal amarogentin showed better efficacy compared to liposomal and free amarogentin; the reduction in the parasite load in the spleen upon treatment was 90%, 69%, and 34%, respectively, in relation to control. The drug did not exhibit any toxicity to the animals judging by histological examination of tissues, blood pathology and assessment of liver function.[68] Another iridoid glycoside, brunneogaleatoside (**2.36**), isolated from *Phlomis brunneogaleata*

(Lamiaceae), exhibited potent activity against *L. donovani* axenic amastigotes (IC_{50} 4.7 μg/mL) while being non-toxic to L6 cells at up to 90 μg/mL.[44]

Picrorhiza kurroa (Scrophulariaceae) is a well known medicinal plant in ayurvedic medicine for the treatment of liver ailments. Picroliv, a standardized fraction from the root and rhizome of the plant consisting of iridoid glycosides and shown to be responsible for its hepatoprotective activity, was investigated for the in vivo treatment of visceral leishmaniasis. The drug itself had no antileishmanial activity, but it significantly increased the potency of the oral antileishmanial drug miltefosine. Thus, a dosing regimen involving a low dose of miltefosine (25 mg/kg/day p.o. × 5 days) together with picroliv (10 mg/kg/day × 33 days) gave the same results as the standard regimen of miltefosine (50 mg/kg/day × 5 days, p.o.) in experimental visceral leishmaniasis in hamsters. Picroliv acts in two ways: it is an immunomodulator that enhances the immunocompetence of the host to control the parasite growth, and it is hepatoprotective. Thus, picroliv in combination with miltefosine appears to be a promising treatment strategy that might prevent the emergence of resistance to miltefosine and decrease its associated side effects due to the decreased dose.[69] Similarly, combination therapy of picroliv at 12.5 mg/kg × 7 days p.o. given 5 days after the last dose of sodium stibogluconate (10 mg/kg × 5 days i.p) displayed marked hepatoprotective effect and significant antileishmanial activity when used in the treatment of experimental *L. donovani* infections in hamsters.[70]

2.2.2.3 Sesquiterpenes

2.2.2.3.1 Sesquiterpenes and Modulation of Multidrug Resistance in *Leishmania*

Sesquiterpene esters based on the dihydro-β-agarofuran skeleton are characteristic of the Celastraceae family. They displayed immunosuppressive, anti-HIV, antitumor-promoting, insect-antifeedant, and insecticidal properties. They also showed activity in reversing MDR phenotypes in *Leishmania* and cancer cells.[71] A series of nine agarofuran sesquiterpenes isolated from the aerial parts of *Crossopetalum tonduzii* and the roots of *Maytenus macrocarpa* was investigated for reversal of daunomycin resistance in a laboratory-generated multidrug-resistant *Leishmania tropica* strain. At 15 μM of the sesquiterpene, 1,6-diacetoxy-9-benzoyloxy-15(2)-methylbutyroyloxy-8-nicotynoyloxy-4-hydroxy-dihydro-β-agarofuran (**2.37**), the growth of the resistant parasites in a culture medium containing 150 μM daunomycin was inhibited by 93%. Other agarofuran derivatives displayed the same effect with varying potencies, showing that these compounds reverse the daunomycin-resistant phenotype. The compounds showed low binding affinity for recombinant NBD2 of a *Leishmania* Pgp-like transporter, suggesting that they bind to the TMD of the transporter to block the daunomycin efflux.[72] An additional set of 14 sesquiterpenes from *Maytenus magellanica* and *M. chubutensis* was tested for the ability to reverse the resistance phenotype in a MDR *L. major* line. Some compounds showed potent activity, with 2-acetoxy-1-benzoyloxy-9-cinnamoyloxy-4-hydroxydihydro-β-agarofuran (**2.38**) being the most active compound. At 7.5 μM, it produced 88% growth inhibition of the MDR promastigotes cultured in a medium containing 150 μM daunomycin while showing no intrinsic cytotoxicity.[73] Interestingly, a

similar agarofuran sesquiterpene (**2.39**, isolated from *Maytenus canariensis*) was found to completely sensitize MDR *L. major* parasites to the promising new antileishmanial drug miltefosine at a concentration of 10 μM. A Pgp-like transporter was implicated in the experimental resistance of *Leishmania* to miltefosine and other alkyllysophospholipids.[51]

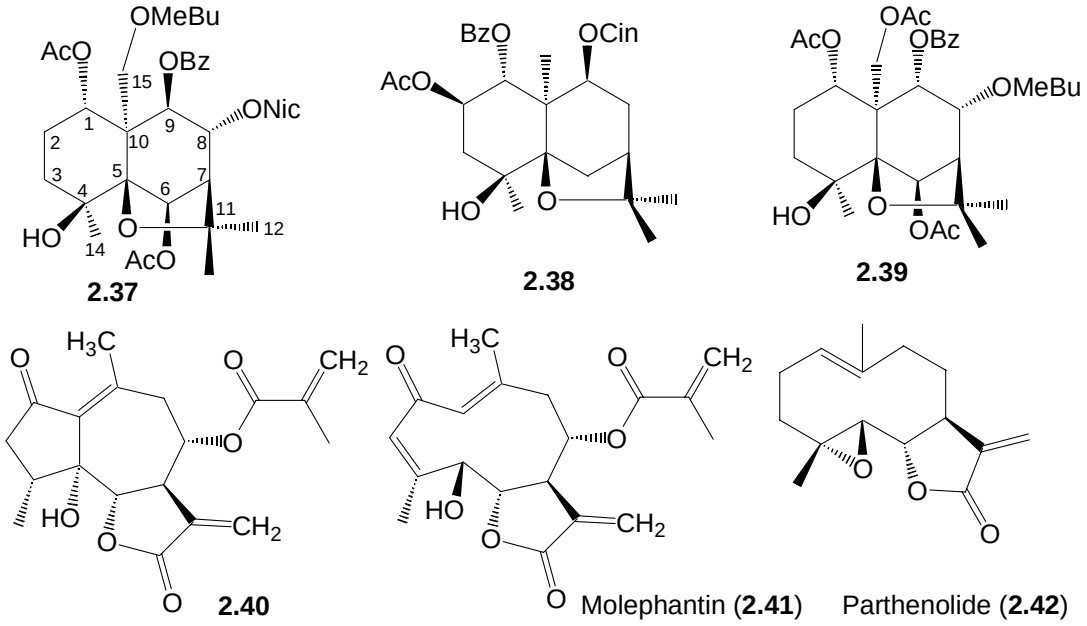


Figure 2.8: Structures of Some Antileishmanial Agarofurans and Sesquiterpene Lactones

2.2.2.3.2 Sesquiterpene Lactones

Several guaianolide-type sesquiterpenes including (**2.40**) and germacranolide-type sesquiterpenes including molephantin (**2.41**), elephantopin, and isoelephantopin showed potent in vitro leishmanicidal activities, with IC_{50} values ranging from < 0.1 to 1.0 μg/mL. The α-methylene-γ-butyrolactone moiety is essential for activity and can play the

role of a Michael acceptor that can easily react with biological nucleophiles (e.g., sulfhydryl-containing enzymes). Interestingly, the simple compound α-methylene-γ-butyrolactone showed moderate activity, with an IC_{50} of 3.5 μg/mL. Despite the high antileishmanial activity, some of the isolated sesquiterpene lactones are known to be cytotoxic. Thus, the therapeutic potential of these compounds is questionable.[74]

Parthenolide is a sesquiterpene lactone isolated from *Tanacetum parthenium*, commonly known as Feverfew, which has long been used in folk medicine for the treatment of fever, migraine, arthritis, stomachache, toothache, and insect bites. Pathenolide showed significant activity against *L. amazonensis* promastigotes (IC_{50} 0.4 μg/mL) and intracellular amastigote forms (IC_{50} 0.8 μg/mL). The compound did not show any cytotoxic effect against J774 macrophages nor did it cause lysis in sheep blood. Electron microscopy revealed marked morphological changes in the promastigotes treated with parthenolide, such as the appearance of large-lysosome-like structures in the cytoplasm and intense exocytic activity in the region of the flagellar pocket. The morphological changes were correlated to a process of exacerbated protein production by cells in attempt to survive. Cysteine protease activity was found to increase upon parthenolide treatment.[75]

2.2.2.4 Diterpenoids

Several tanshinones (20-norditerpenes with an abietane-type skeleton containing a quinone moiety in the C-ring) with antileishmanial activities were isolated from *Perovskia abrotanoides* (Lamiaceae), an Iranian medicinal herb used to treat cutaneous

leishmaniasis. The compounds cryptotanshinone (**2.43**) and 1-oximiltirone (**2.44**) were most active, both displaying IC_{50} values of 18 μM against *L. major* promastigotes. The compounds were also moderately toxic against proliferating phytohaemagglutinin A-stimulated human lymphocytes (IC_{50} values of 37 and 45 μM, respectively).[76]

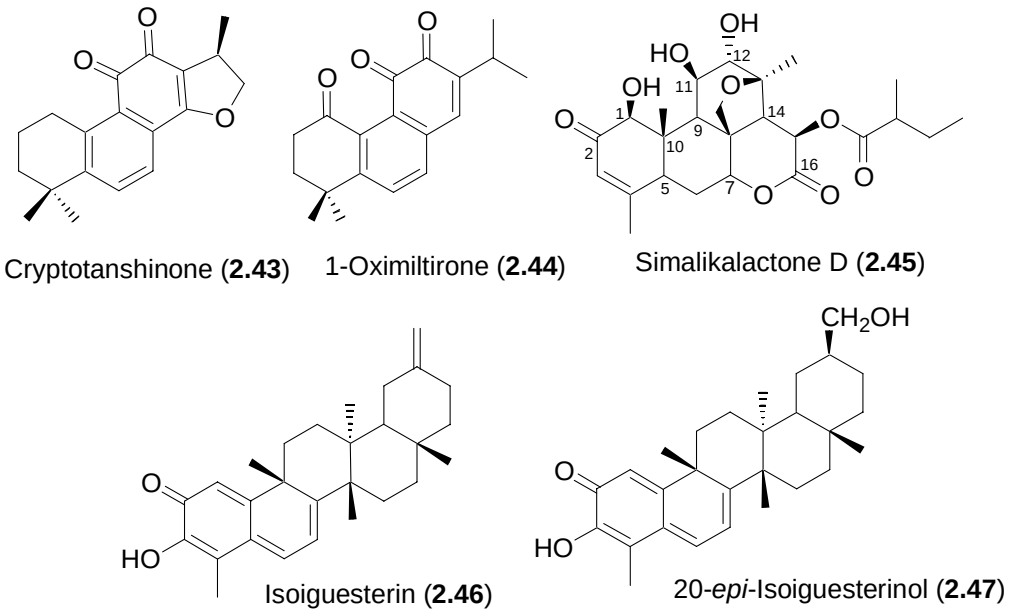


Figure 2.9: Structures of Some Antileishmanial Di- and Triterpenes

2.2.2.5 Triterpenes

Simalikalactone D (**2.45**) is a quassinoid (a decanortriterpenoid) isolated from the root bark of *Simaba orinocensis* (Simaroubaceae). It displayed potent activity against *L. donovani* promastigotes with an IC_{50} value 35 ng/mL. It was thus >46- and >31-fold more potent than the standard antileishmanial drugs pentamidine and amphotericin B (IC_{50} values 1.6 and 1.1 μg/mL, respectively). It showed good selectivity with an IC_{50} 2.3

μg/mL towards Vero cells. Acetylation of similakilactone D at positions 11 and 12 completely abolished activity. The carbonyl group at position 2 and/or the hydroxyl group at C-12α are critical for the antileishmanial activity. Orinocinolide, a related quassinoid with α-OH group at position 2 and β-OH group at C-12, has much weaker antileishmanial activity (IC_{50} values 25 μg/mL). Simalikalactone D was found to inhibit protein synthesis in vitro and in vivo, while the inactive acetylated derivative does not affect protein synthesis.[77]

Two bisnortriterpene quinone methides, isoiguesterin (**2.46**) and 20-*epi*-isoiguesterinol (**2.47**), were isolated from *Salacia madagascariensis* (Celastraceae), a plant used in the traditional medicine of East Africa to treat malaria, fever, and menorrhagia. They displayed potent antileishmanial activity against *L. donovani* (IC_{50} values 0.032 and 0.027 μg/mL, respectively; the parasite stage used in the assay was not specified) while showing much less toxicity towards Vero cells (IC_{50} values 1.6 and 2.1 μg/mL, respectively). Amphotericin B, used as a positive control in the assay, was less potent than either compounds with an IC_{50} of 0.11 μg/mL.[78]

2.2.2.7 Saponins:

In 1991, antileishmanial activity was reported for several saponins from English ivy, *Hedera helix* (Araliaceae), on promastigote and amastigote forms of *L. infantum* and *L. tropica*.[79] The in vitro antileishmanial activity of three saponins, α- (**2.48**) and β-hederin (**2.49**) and hederacolchiside A_1 (**2.50**), was investigated on *Leishmania infantum*. The results showed that the saponins exhibited strong antiproliferative activity on all life

stages of the parasite by altering membrane integrity and potential. α-Hederin and β-hederin exhibited potent activity, with IC_{50} values 0.35 and 0.25 μg/mL, respectively, against intracellular amastigotes. Hederacolchiside A_1 which differs from β-hederin only in having one additional glucose unit in the sugar side chain, displayed greater activity against both the promastigotes and amastigotes (IC_{50} 1.2 and 0.05 μg/mL, respectively). The saponins also exerted potent antiproliferative activity against human monocytes (hederacolchiside A_1 IC_{50} 0.45 μg/mL) by inhibiting DNA synthesis.[80] α-Hederin inhibited the proliferation of mammalian cancer and non-cancer cell lines at low micromolar concentrations, induced vacuolization of the cytoplasm and caused membrane alterations leading to cell death.[81] However, the favorable ratio between the antileishmanial activity versus amastigotes and toxicity to human cells suggested that the saponins could be considered as possible antileishmanial drugs.[80] In a recent study, α- and β-hederin and hederacolchiside A_1 were shown to be much more active on the intracellular amastigote stage of *L. mexicana* (IC_{50} values 0.41, 0.35 and 0.07 μM, respectively) than the promastigote stage (IC_{50} values 11, 18 and 1.5 μM, respectively). The compounds were only 8- to 13-fold selective for the parasite, as they displayed significant toxicity towards human THP1 monocytes (IC_{50} values 3.5, 4.6, and 0.8 μM, respectively). However, combinations of sub-toxic concentrations of saponins with antileishmanial drugs such as pentamidine and amphotericin B enhanced the efficiency of the conventional drugs in vitro. Moreover, the action of saponins on the promastigote membrane was cumulative with those of amphotericin B. The authors suggested the

possibility of combining the antileishmanial therapy with a saponin ointment for the treatment of cutaneous leishmaniasis.[82]

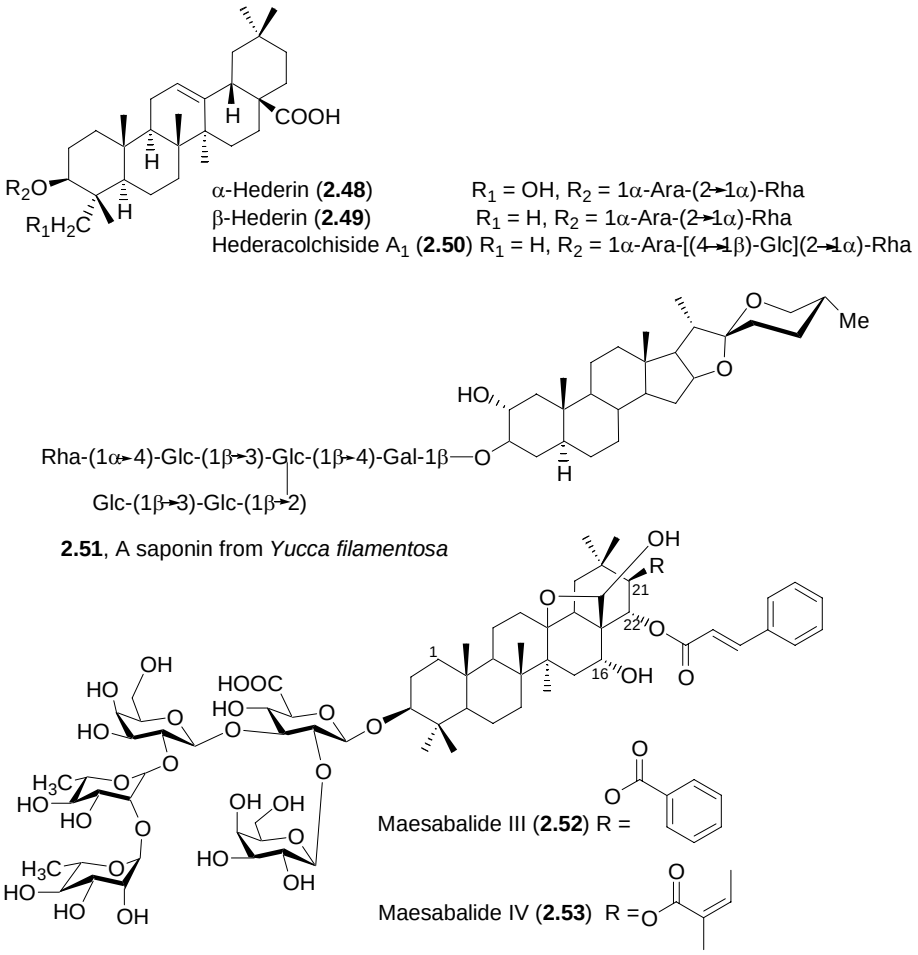


Figure 2.10: Structures of Some Antileishmanial Saponins

A steroidal saponin characterized as 3-*O*-((β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2))(α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3))-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)-25*R*,5α-spirostan-2α,3β-diol (**2.51**),

isolated from *Yucca filamentosa* (Agavaceae), showed a dose-related inhibitory effect on *L. mexicana amazonensis* promastigotes. Treatment with 10 μg/mL of this compound gave 100% leishmanicidal activity after only 1 hour, while 1 μg/mL resulted in about 35% inhibition during the same time interval. At 10 μg/mL, the saponin showed better efficacy than pentamidine on *L. major*-infected macrophages (infection rate was reduced by 72% versus 40% for pentamidine) after incubation for 72 h. The leishmanicidal activity of the saponin is at least partially due to an effect on the parasite membrane potentail.[83,84]

Oleanane-type triterpene saponins with very potent prophylactic as well as therapeutic antileishmanial activity have been isolated from *Maesa balansae* (Myrsinaceae). This Vietnamese shrub, which is used in traditional medicine for the treatment of anthelmintic infections, skin ulcers, allergies and headache, was found to possess strong antileishmanial properties during random screening. The saponins, named maesabalides I through VI, possess a hemiacetal moiety between C-13 and C-28. Maesabalides III (**2.52**) and IV (**2.53**), the two major saponin constituents (70% of total purified saponins), showed the highest activity against *L. infantum* intracellular amastigotes, with IC_{50} values of 20 ng/mL (13 nM) for both. Maesabalides V and VI, which are analogs of maesabalides III and IV, respectively, with acetylated 16-OH groups, are far weaker in activity with IC_{50} values of 3400 and 700 ng/mL, respectively. Maesabalides I and II, which are analogs of maesabalides III and IV, respectively, with a (*Z*)- instead of (*E*)-cinnamoyl moiety at C-22, retain antileishmanial potency, with IC_{50} values of 70 and 50 ng/mL, respectively.[85] Several derivatives were prepared to better

understand the structure-activity relationship. The results suggested that the saponin core structure can undergo minor structural changes without losing activity. The presence of a free OH group at C-16 seems to be critical for the antileishmanial activity, while removing the substituents at C-21 and C-22 to leave the free OH groups abolishes the activity. Also, the aglycone derivatives are completely inactive, suggesting that the carbohydrate portion of the molecules is necessary for activity. The dihydrocinnamoyl derivatives of maesabalides III and VI completely retain activity, indicating that the double bond in the cinnamoyl group is not needed for activity.[86] In comparison, the reference drug sodium stibogluconate has an IC_{50} 5.6 μg/mL in the same assay, that is 280-fold less potent than maesabalide III. PX-6518, a mixture of maesabalides I through VI, has an IC_{50} value of 50 ng/mL against the intracellular amastigotes of *L. donovani* and *L. infantum* (the causative agents of VL), 1 μg/mL against *L. mexicana* and 5 μg/mL against *L. major* (causative agents of CL).[87] It shows toxicity towards murine macrophages and human fibroblasts (MRC-5 cells) at IC_{50} values 1 and >32 μg/mL, respectivly. The higher cytotoxicity to the macrophage cells suggests a selective intracellular accumulation in phagocytic cells. At concentrations up to 32 μg/mL, PX-6518 did not have any inhibitory activity on several viruses (including HIV), bacteria, yeasts, fungi and parasites (including *Plasmodium* and *Trypanosoma*), suggesting a highly selective antileishmanial toxicity. Moreover, the saponins were totally inactive towards *Leishmania* promastigotes.[88] This selectivity indicates that the mode of action is not related to a non-specific cell or membrane toxicity, as is the case with most of the antimicrobial effects described for saponins. PX-6518 displayed very promising efficacy

and potency in vivo. In a prophylactic study design, where the first dose of drug is given either immediately before or at the time of the infection, the lowest active dose (LAD) against *L. donovani* was 1 × 0.4 mg/kg, against *L. mexicana* and *L. panamensis* was 6 × <0.5 mg/kg (administered in alternate days), against *L. major* was 6 × 2 mg/kg (in alternate days). In an early curative study design, LAD against *L. donovani* is 1 ×1.6 mg/kg, against *L. mexicana*, *L. panamensis* and *L. major* is 4 ×1 mg/kg (given over 4 weeks). In a late curative study design, where the first administration of the test drug is given when the dermal lesions are well established or become chronic, LAD against *L. mexicana* and *L. panamensis* was 1 mg/kg, 2 ×/week for 4 weeks, against *L. major* was 22 × 1 mg/kg (given over 11 weeks). The isolated saponins were patented for use against leishmaniasis.[87] Despite the effectiveness of the drug in eliminating the liver amastigote burden in animals infected with *L. donovani*, clearance from the spleen and bone marrow was more difficult. This is already documented for the reference drug, sodium stibogluconate, with which clearance from the spleen and bone marrow is much more difficult to achieve than from the liver.[88] Likewise, administration of a single dose of maesabalide III at a dose of 0.8 mg/kg s.c. to hamsters with an established *L. donovani* infection (28 days post-infection) reduced the liver amastigote burden by 94% at day 7 post treatment. However, clinical protection was not obtained, as 2 out of 5 hamsters died 78 days post-infection. The efficacy was comparable to that of a single dose of liposomal amphotericin B at 5 mg/kg, and thus the results are promising.[89] More pharmacological, toxicological and pharmacokinetic studies are needed to optimize a dosage schedule for this promising antileishmanial agent.

2.2.3 Quinones

2.2.3.1 Naphthoquinones

The antiprotozoal activity of hydroxynaphthoquinonoids is well established. These compounds share a structural similarity with reduced coenzyme Q (ubiquinone) which plays a role in the mitochondrial electron transport system. Thus, these agents would be expected to disrupt the mitochondrial electron transport chain, inhibiting parasite growth. Alternatively, it has been proposed that the interaction of naphthoquinones with the respiratory chain leads to the generation of free radicals capable of destroying parasites.[90] A problem with this group of compounds is the lack of adequate selectivity, as they might affect elements of the mamalian electron transport chain.[91]

Diospyrin (**2.54**), a bisnaphthoquinone isolated from the stem bark of *Diospyros montana*, showed a significant inhibitory effect on *L. donovani* promastigotes with an IC_{50} of 1.9 μM.[92,93] Diospyrin, or its methylated derivative (**2.55**), did not show selectivity against *L. donovani* intracellular amastigotes. The hydroquinonoid derivatives **2.56** and **2.57** showed appreciable activity, with ED_{50} values of 2.2 and 2.4 μM, respectively. However, those derivatives were toxic to macrophages at 10 μM.[90] Diospyrin was found to interact with type I DNA topoisomerase of *L. donovani*, stabilizing the enzyme-DNA cleavable complex. This is a reversible and selective inhibition of topoisomerase I, as it does not inhibit topoisomerase II of the same species and 10-fold higher concentrations of diospyrin are required to inhibit topoisomerase I from calf thymus. Diospyrin differs from camptothecin, a known topoisomerase I

inhibitor that stabilizes the topoisomerase I-DNA covalent complex without binding to either the enzyme or DNA alone, in that it binds to the enzyme alone. Thus, preincubation of the enzyme with diospyrin before DNA addition enhanced enzyme inhibition.[94]

Plumbagin (**2.58**), a major component from *Plumbago* species, has antileishmanial activity with an IC_{50} of 0.21 μM against *L. donovani* promastigotes. The hydroquinonoid derivative of plumbagin did not show a similar improvement of activity as with diospyrin derivatives.[93] Plumbagin delayed the development of *L. amazonensis* and *L. venezuelensis* infection in BALB/c mice when administered 24 h post-infection at a dose of 5 or 2.5 mg/kg/day.[95] The mechanism of action of plumbagin is probably different than that of diospyrin, as it was shown to induce topoisomerase II-mediated mammalian DNA cleavage in vitro.[96] Plumbagin and synthetic derivatives were also shown to act as subversive substrates of *T. cruzi* trypanothione reductase (EC value 28 μM).[97]

2.2.3.2 Anthraquinones and Anthranoids

The stem bark of the Tanzanian medicinal plant *Vismia orientalis* (Clusiaceae) afforded several anthranoid compounds possessing antiprotozoal activities against *T. b. rhodesiense*, *T. cruzi*, *L. donovani*, and *Plasmodium falciparum*. Emodin (**2.59**) and vismione D (**2.60**) were the most potent against *L. donovani* axenic amastigotes, with IC_{50} values 2.05 and 0.37 μg/mL, respectively. They were moderately toxic against L6 cells (rat skeletal myoblasts), with IC_{50} values 4.1 and 20 μg/mL, respectively.[98]

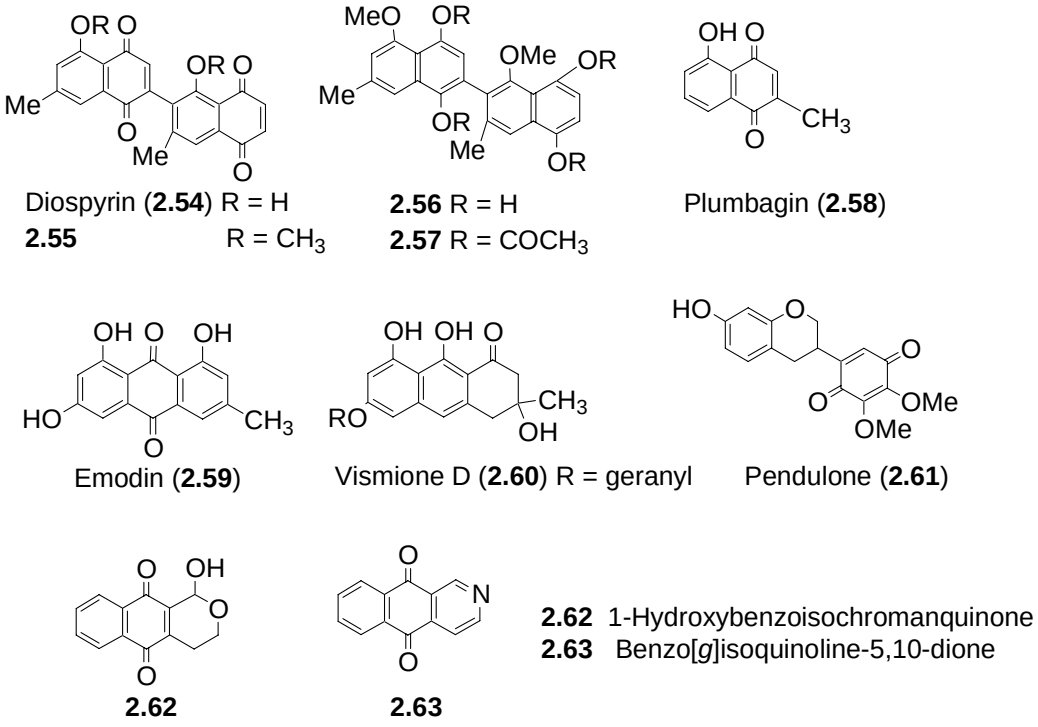


Figure 2.11: Structures of Some Quinones with Antileishmanial Activity

2.2.3.3 Other Quinones

Pendulone (**2.61**), an isoflavanquinone isolated from the Myanmar timber species *Millettia pendula*, possessed potent antileishmanial activity with an IC_{50} of 66 ng/mL against *L. major* promastigotes.[99]

1-Hydroxybenzoisochromanquinone (**2.62**) and benzo[*g*]isoquinoline-5,10-dione (**2.63**) from *Psychotria (Cephaelis) camponutans* showed activity against *L. donovani* promastigotes with IC_{50} values 37.7 and 6.6 μM and against the intracellular amastigotes of the same parasite at IC_{50} values of 14.1 and 16.5 μM, respectively. The acetyl

derivative of **2.62**,1-acetylbenzoisochromanquinone, showed more potent activity with an IC_{50} of 2.3 μM against the promastigote form and 2.0 μM against the intracellular amastigote form. It also displayed moderate toxicity against KB cells (IC_{50} 12 μM).[100]

2.2.4 Alkaloids

2.2.4.1 Quinoline alkaloids

2-Substituted quinolines isolated from the Bolivian medicinal plant, *Galipea longiflora* (Rutaceae), displayed efficacy in the experimental treatment of New World cutaneous leishmaniasis. 2-*n*-Propylquinoline (**2.64**), chimanine B (**2.65**), and chimanine D (**2.66**) displayed IC_{90} values of 50, 25 and 25 μg/mL, respectively, against *L. braziliensis* promastigotes.[101] Chimanine D and 2-*n*-propylquinoline given s.c. at a dose of 100 mg/kg for 14 days, beginning 1 day post-infection, were more potent than the classical antimonial Glucantime (*N*-methylglucamine antimonate) in clearing *L. amazonensis* infection in mice. 2-*n*-Propylquinone also showed activity similar to that of Glucantime against the virulent strain *L. venezuelensis*.[101] Twice daily oral treatment with chimanine B at 50 mg/kg for 15 days (starting 4–5 weeks after parasite inoculation) produced the same effects as treatment with Glucantime in treating *L. amazonensis* skin lesions in BALB/c mice.[102] Moreover, oral administration of 2-*n*-propylquinoline at 0.54 mM/kg (94 mg/kg) once daily for 5 or 10 days to *L. donovani*-infected mice (starting 1 weak after parasite inoculation) reduced the parasite burden in the liver by 88% and 100%, respectively. Subcutaneous treatment with chimanine D for 10 days at 0.54 mM/kg (100 mg/kg) causes 87% reduction of the parasite load in the liver.[103]

2.2.4.2 Isoquinoline alkaloids

Berberine (**2.67**) is a quaternary isoquinoline alkaloid found in a several plant species belonging to various families such as Annonaceae and Berberidaceae. It eliminates intracellular *L. major* amastigotes from mouse peritoneal macrophages at a concentration of 10 μg/mL. Tetrahydroberberine (**2.68**) was more potent and less toxic than berberine against *L. donovani* infection in hamsters, when administered at a dose of 54 mg/kg s.c. twice daily over a 4-day period starting 3 days post-infection. Neither alkaloid was as potent as meglumine antimonate (Glucantime), however. Against cutaneous *L. braziliensis* infection in hamsters, only berberine showed significant activity with greater than 50% suppression of lesion size when administered on day 19 post-infection at a dose of 26 mg/kg s.c. twice daily for 4 days.[104] In another report, topical administration of berberine as a 15% ointment appeared to be only partially effective. Twice daily application for 12 days beginning 35 days post-inoculation cured *L. major* cutaneous lesions in only 2 out of 3 mice. However, the lesions relapsed 10 to 30 days after the end of treatment.[105]

2.2.4.3 Benzoquinolizidine alkaloids

The benzoquinolizidine alkaloid, cephaeline (**2.69**), isolated from the stem bark of *Psychotria klugii* (Rubiaceae), exhibited a potent antileishmanial effect against *L. donovani* promastigotes (IC_{50} 30 ng/mL) while being 193-fold more selective against this parasite than mammalian Vero cells (IC_{50} 5.3 μg/mL). The related alkaloids, klugine (**2.72**) and isocephaeline (**2.70**), isolated from the same plant, were also active but less

potent (IC_{50} 400 and 450 ng/mL). Emetine (**2.71**), a known antileishmanial agent with a related structure, was found to be as potent as cephaeline but >12-fold more toxic to Vero cells (IC_{50} 0.42 μg/mL).[106] When tested for topical treatment of cutaneous leishmaniasis caused by *L. major* in experimental animals, emetine hydrochloride ointment was ineffective and was lethal to mice at concentrations higher than 0.5%.[105] In another recent investigation, the therapeutic effect of the alkaloid emetine hydrochloride administered intralesionally was compared with that of standard parenteral treatment with Glucantime in hamsters experimentally infected with amastigotes of *Leishmania braziliensis*. Both chemotherapeutic agents reduced significantly the average lesion sizes in experimental animals in comparison with those untreated. However, viable amastigotes in nodules and/or scars of all the evaluated hamsters were detected 75 to 230 days after the end of treatment.[107]

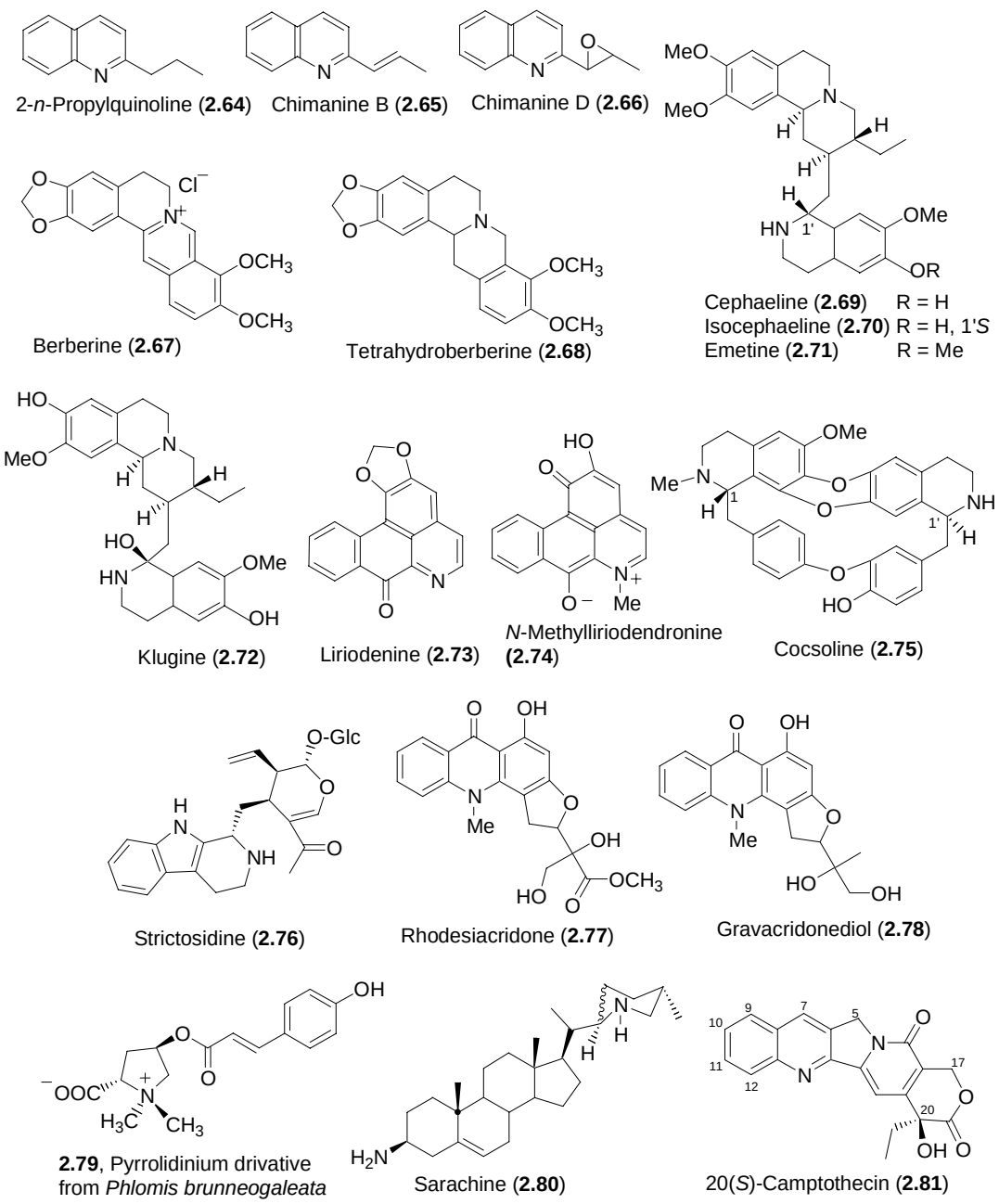


Figure 2.12: Structures of Some Alkaloids with Antileishmanial Activity

2.2.4.4 Other Alkaloids

The oxoaporphine alkaloid liriodenine (**2.73**) and the zwitterionic derivative *N*-methylliriodendronine (**2.74**) isolated from the African medicinal shrub *Stephania dinklagei* (Menispermaceae), exhibited activity against *L. donovani* promastigotes (IC_{50} values of 15 and 19 μM, respectively) and amastigotes (IC_{50} values of 72 and 36 μM, respectively). However, liriodenine showed cytotoxicity against KB cells at an IC_{50} value of 27 μM.[108] Liriodenine isolated from the stem bark of *Rollinia emarginata* (Annonaceae) inhibited the growth of the promastigotes of *L. braziliensis*, *L. amazonensis*, and *L. donovani* with an IC_{100} value of 5 μg/mL (18 μM).[109] Liriodenine was also isolated from the stem bark of the Bolivian *Unonopsis buchtienii* (Annonaceae), and displayed activity against *L. donovani* and *L. major* promastigotes at an IC_{100} of 3.1 μg/mL, and toxicity towards Vero cells at an IC_{50} value of 1 μg/mL.[110]

A series of bisbenzylisoquinolines (BBIQ) showed activity (IC_{50} values of 0.4–140 μM) against *L. donovani* promastigotes. However, most compounds were not as active towards intracellular *L. donovani* amastigotes; cocsoline (**2.75**) was the only alkaloid that showed selective toxicity with an IC_{50} value of 12 μM (8 μg/mL) against the amastigotes. Analysis of the SAR of this series of alkaloids showed that the oxidation state and nature of substitution of the nitrogen atoms are important for activity. Alkaloids with methylated nitrogen atoms were more active than those with non-substituted or aromatic nitrogens. Quaternization of one or more nitrogen atoms resulted in a loss of antileishmanial activity. Moreover, the stereochemistry of the alkaloids at the chiral centers (C-1 and C-1′) affected the antileishmanial activity.[111] Another series of BBIQ

alkaloids isolated from *Guatteria boliviana* (Annonaceae), showed only moderate inhibition on the promastigote forms of different strains of *Leishmania* at 100 μg/mL, while displaying IC_{50} values under 10 μg/mL towards the KB cell line. Interestingly, most of these alkaloids have non-substituted nitrogen atoms.[112]

The indole alkaloids, strictosidine (**2.76**) and acetylstrictosidine, obtained from *Cephaelis dichroea*, showed antiprotozoal activity toward *L. donovani* promastigotes (IC_{50} values of 14 and 6.3 μM, respectively) and toward *L. donovani* intracellular amastigotes (IC_{50} values of 39.3 and 2.1 μM, respectively). These compounds displayed low toxicity against KB cells, with IC_{50} values of 74 and 131 μM, respectively.[100]

The acridone derivatives rhodesiacridone (**2.77**) and gravacridonediol (**2.78**) were isolated from *Thamnosma rhodesica* (Rutaceae), a shrublet traditionally used in Zimbabwe as an ant and flea repellent and for the treatment of chest ailments. They showed some activity (69% and 46% inhibition, respectively) against *L. major* promastigotes at 10 μM. Interestingly, the compounds were more active on the amastigote form of the parasites, causing over 90% inhibition at 10 μM and around 50% at 1 μM for both. They did not show toxicity against murine macrophages at the same concentrations. Acridines with their planar tricyclic structure can intercalate DNA and interfere with various processes although they are likely to exert their antileishmanial effects by multiple cytotoxic mechanisms.

The pyrrolidinium derivative, 2-carboxy-4-*p*-coumaroyloxy-1,1-dimethylpyrrolidinium inner salt (**2.79**), from *Phlomis brunneogaleata*, showed significant antileishmanial activity against *L. donovani* axenic amastigotes (IC_{50} value 4.7

μg/mL), while being inactive against *T. b. rhodesiense*, *T. cruzi* and non-toxic to L6 cells.[44]

Sarachine (**2.80**), an aminosteroid isolated from the Bolivian plant *Saracha punctata* (Solanaceae), inhibited the growth of *L. braziliensis* promastigotes (IC_{50} of 12.5 μM) and *T. cruzi* epimastigotes (IC_{50} of 25 μM). The compound was strongly cytotoxic towards macrophages (100% mortality at 12.5 μM). Interestingly, the compound displayed strong in vitro antiplasmodial activity with an IC_{50} of 25 nM.[113]

Camptothecin (**2.81**) inhibited leishmanial topoisomerase I and was found to be cytotoxic against *L. donovani* promastigotes with an IC_{50} value of 3.2 μM.[114]

2.2.4.5 Naphthylisoquinoline Alkaloids

The genus *Ancistrocladus* has afforded several monomeric and dimeric naphthylisoquinoline alkaloids with antiparasitic properties. Ancistroealaines A (**2.82**) and B (**2.83**), isolated from *Ancistrocladus ealaensis*, a liana indigenous to central Africa, exhibited activity against *Leishmania donovani* intracellular amastigotes (IC_{50} 4.1 and 10 μg/mL, respectively). The compounds did not show any cytotoxicity against L6 cells or murine macrophages.[115] Another species from central Africa, *A. likoko*, afforded the 5,8′-coupled naphthylisoquinoline alkaloid ancistrolikokine D (**2.84**). It showed significant antileishmanial activity versus the intracellular amastigotes at an IC_{50} of 5.5 μg/mL, while being weakly toxic towards L6 cells (IC_{50} 37 μg/mL).[116] The 7,8′-coupled naphthylisoquinoline alkaloids ancistrogriffines A (**2.85**) and C (**2.86**) were isolated from the Asian *A. griffithii*. They showed antileishmanial properties (IC_{50} 3.1 and 18 μg/mL,

respectively, against intracellular *L. donovani*) while displaying moderate toxicity towards L6 cells (IC_{50} 14 and 36 μg/mL, respectively).[117] The 5,8´-coupled naphthylisoquinoline alkaloids, ancistrocongolines B (**2.89**) and C (**2.91**) isolated from *Ancistrocladus congolensis*, showed moderate antileishmanial activity, both displaying IC_{50} values of 19 μg/mL versus *L. donovani* intracellular amastigotes. Korupensamine (**2.90**) showed a weaker activity, with an IC_{50} value of 25 μg/mL.[118] Ancistrotanzanines A (**2.92**) and B (**2.93**) and ancistrotectoriline A (**2.94**) were isolated from the East African *A. tanzaniensis*. Ancistrotanzanines A and B displayed potent activity against intracellular *L. donovani* (IC_{50} values of 1.8 and 1.6 μg/mL) while being moderately toxic towards L6 cells (IC_{50} 6.4 and 8.1 μg/mL). Ancistrotectoriline A was inactive up to a concentration of 10 μg/mL.[119] Ancistrocladinine (**2.95**) isolated from the same species also showed a potent antileishmanial effect, with an IC_{50} value of 2.9 μg/mL, while being only weakly toxic to L6 cells (IC_{50} 28 μg/mL).[120]

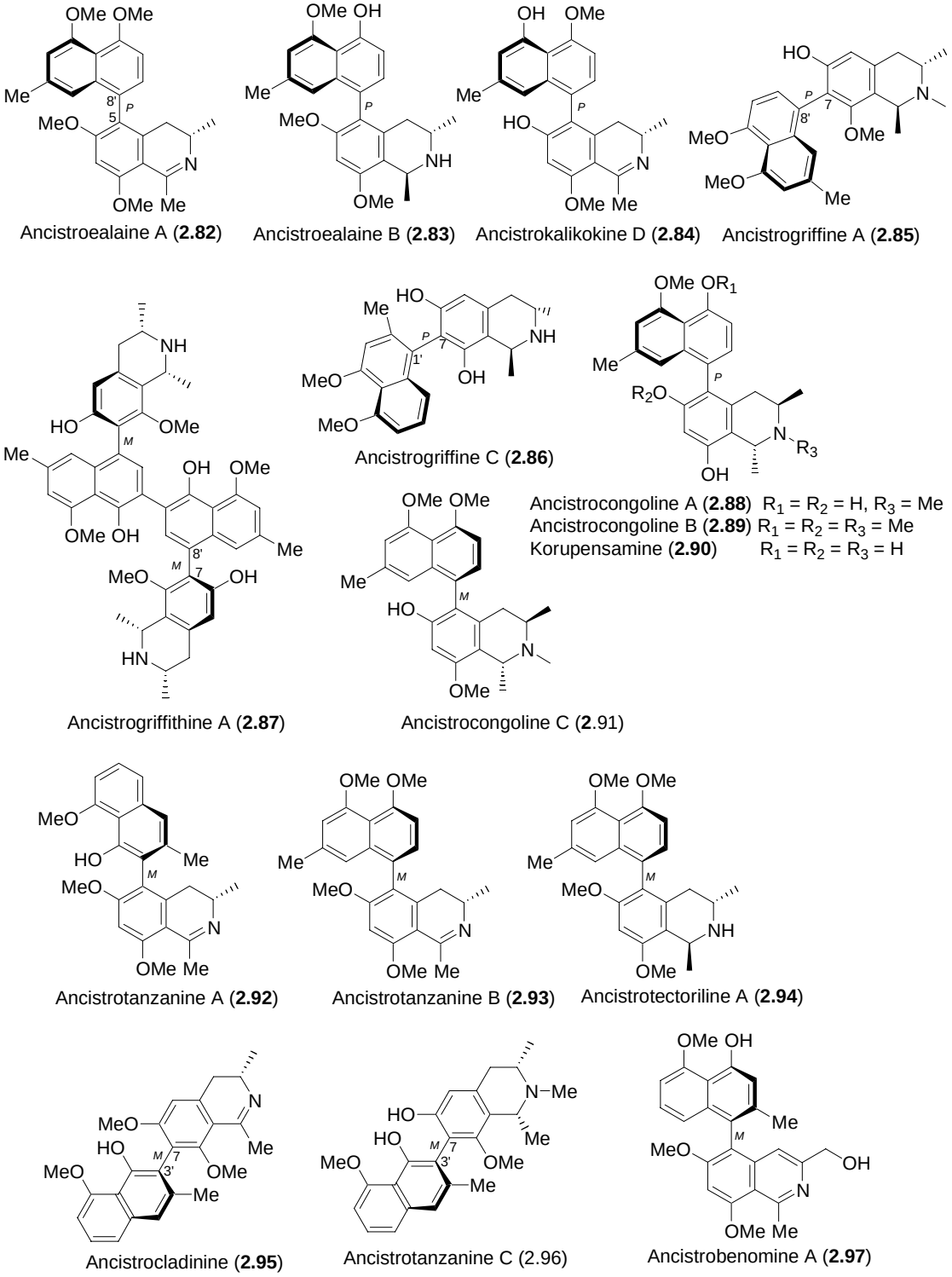


Figure 2.13: Structures of Some Antikinetoplastid Naphthylisoquinoline Alkaloids

2.2.5 Other Secondary Metabolites

2.2.5.1 Acetylenes

Bioassay-guided fractionation of the bark of the Peruvian medicinal tree *Minquartia guianensis* afforded minquartynoic acid (**2.98**) in high yield (2–3%). This showed significant antileishmanial activity, with an IC_{50} of 1.4 μg/mL versus *L. major* promastigotes, and inhibited the proliferation of human lymphocytes with an IC_{50} of 5.3 μg/mL.[121] This constituent was originally isolated in 1989 as a cytotoxic compound with activity against P-388 murine lymphocytic leukemia cells.[122]

2.2.5.2 Acetogenins

Several acetogenins isolated from the stem bark of *Rollinia emarginata* (Annonaceae) including rolliniastatin-1 (**2.99**), sylvaticin (**2.100**), and squamocin (**1.101**) inhibited the growth of *L. braziliensis*, *L. amazonensis*, and *L. donovani* promastigotes with IC_{100} values ranging from 5–10 μg/mL.[109]

2.2.5.3 Lactones

Argentilactone (**2.102**), isolated as a major constituent from *Annona haematantha* (Annonaceae), displayed activity against *L. donovani*, *L. major* and *L. amazonensis* at 10 μg/mL. The compound was assessed in vivo for the experimental treatment of cutaneous leishmaniasis. Mice were inoculated with *L. amazonensis* amastigotes, and four weeks later were given argentilacone at 25 mg/kg once daily for 14 days either orally or s.c. Treatment results were assessed seven weeks post-infection, showing that s.c.

argintilactone decreased the weight of cutaneous lesions by 95% and the parasite burden, both in the lesion and in the spleen, by 97% and 48%, respectively, compared to the control. The effects of this compound were similar to treatment with the reference drug meglumine antimonate, and the treatment was well tolerated by the mice.[123]

Klaivanolide (**2.103**), a bisunsaturated 7-membered lactone, was isolated from *Uvaria klaineana* (Annonaceae), a liana endemic to Gabon and Congo in Africa. The compound exhibited potent in vitro antileishmanial activity against the promastigote forms of *L. donovani*, with IC_{50} values of 1.8 and 3.1 μM, respectively.[124]

2.2.5.4 γ-Pyrone

Two γ-pyrones, **2.104** and **2.105**, isolated from *Podolepsis hieracioides* (Asteraceae), showed activity against the promastigotes of several *Leishmania* species (IC_{50} values of 3.8–5.4 μg/mL) and against the intracellular amastigotes of *L. donovani*. The compounds showed toxicity to KB and SK-mel cells at IC_{50} values of 11–15 μg/mL and to RAW macrophage-like cells at >25 μg/mL. The compounds were not active against *T. b. brucei* or *T. cruzi* at concentrations up to 30 μg/mL.[125]

2.2.5.5 Peroxides

Two cyclic peroxides isolated from the Palauan sponge *Plakortis* aff. a*ngulospiculatus* showed potent activity against *L. mexicana* promastigotes. The most potent peroxide (**2.106**, LD_{50} 0.29 μg/mL) caused lysis of the cell membrane after 24 h at 1 μg/mL and striking decrease in the parasite motility after 1 h.[126]

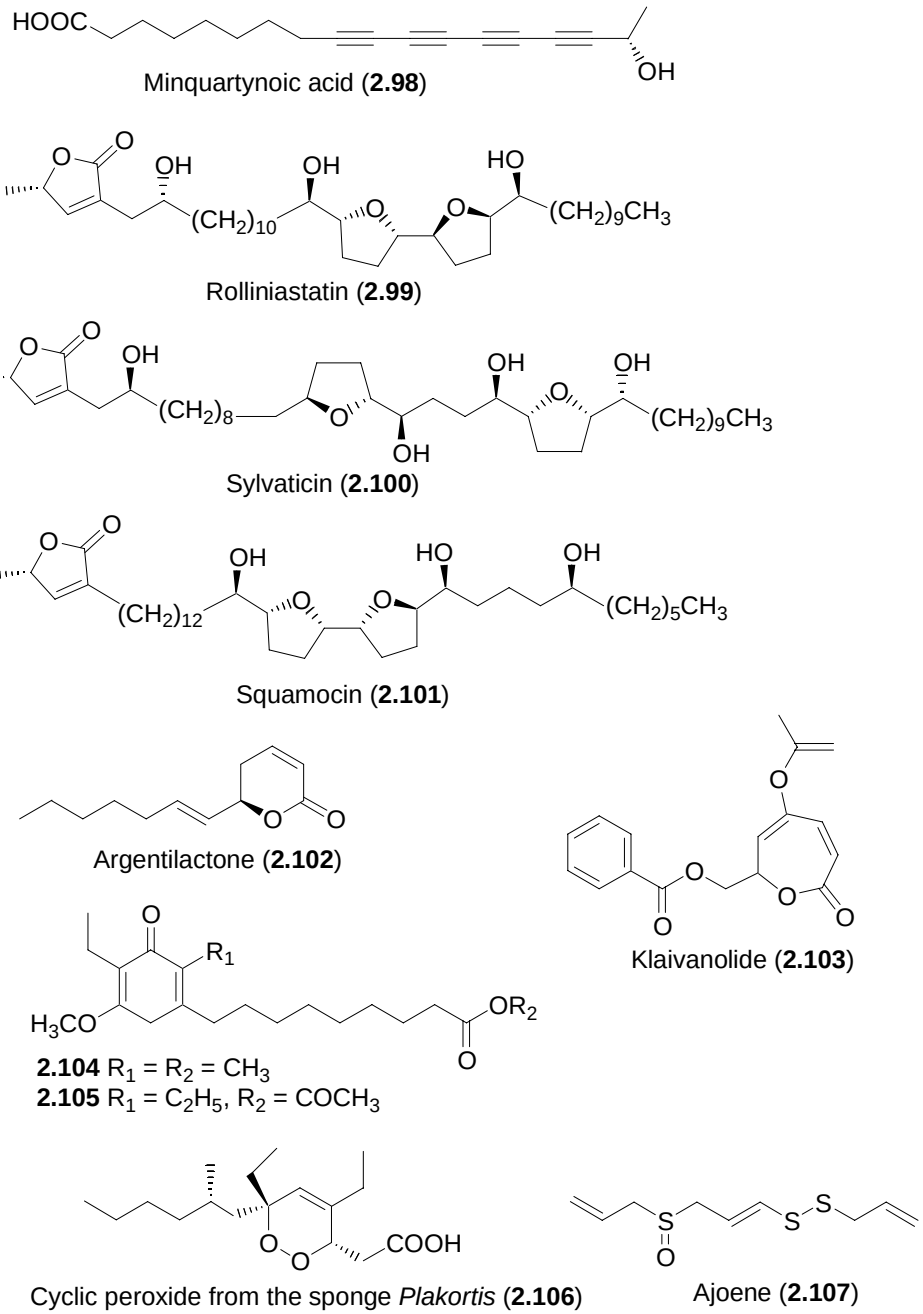


Figure 2.14: Structures of Some Antileishmanial Compounds from Various Chemical Classes

2.2.6 Miscellaneous Plant Extracts

Kalanchoe pinnata (Crassulaceae), a medicinal plant used in Brazil, India, China and Africa for the teatment of cutaneous wounds, arthritis and gastric ulcers, showed antileishmanial effects in vivo. The aqueous leaf extract given orally at daily doses of 8 mg per mouse significantly prevented lesion growth in BALB/c mice infected with *Leishmania amazonensis*, when given at an early stage of infection. The results were similar to those seen with the reference antileishmanial drug meglumine antimonate. The extract was also effective at a late stage of infection where it prevented lesion growth. In addition, the extract diminished the capacity of animals to develop delayed-type hypersensitivity and to produce parasite-specific antibodies. Administration of the extract i.v. or i.p. was not as effective as the oral route, possibly due to a slower persistent release from the mucosa into the circulation or a differential breakdown of the extract in the gastrointestinal tract.[127] In vitro, the extract exhibited a dose-dependent inhibition of the intracellular *L. amazonensis* parasite growth, with 60% inhibition at a concentration 500 μg/mL incubated with the macrophages 24 h before and after infection, or 42% when incubated with same concentration 4 h after infection. However, the extract did not inhibit the growth of the axenic promastigote form of the parasite, demonstrating that it has no direct antileishmanial effect. The extract inhibited parasite growth by activating the macrophages causing an increase in NO production. NO production is thought to inhibit the progress of leishmaniasis by two ways: inducing direct parasite killing by the macrophages and suppressing unwanted immune responses. The extract was suggested as an affordable substitute for INF-γ for treatment of leishmaniasis.[128] A study provided

evidence that the fatty acids palmitic, stearic and traces of arachidonic acid present in *Kalanchoe pinnata* may be responsible, at least in part, for its immunosuppressive effect in vivo.[129]

Garlic (*Allium sativum*) is known to enhance the delayed type hypersensitivity responses, T-lymphocyte proliferation and natural killer cell activity. Garlic extract, administered at 20 mg/kg/day × 2 weeks i.p., 30 days post-infection, suppressed lesion growth in *L. major* infections in BALB/c mice and was more effective than Glucantime alone (60 mg/kg/day × 2 weeks, s.c.). The combined treatment of garlic extract and Glucantime was more effective than either agent alone. Garlic extract was shown to induce the production of high levels of IFN-γ and IL-2, modulating the cytokine patterns towards a T helper 1-type response and the development of an effective cell mediated response in the infected mice. In addition, garlic extract contains organosulfur microbicidal compounds that might be responsible for the inhibition of the in vitro growth of *L. major* promastigotes.[130] Ajoene (**2.107**), a major active component isolated from garlic, was shown to have potent antileishmanial activity against *L. mexicana*, *L. m. venezuelensis*, *L. m. amazonensis* and *L. donovani chagasi*. Ajoene at 10 μM induced 100% lysis of *Leishmania* after 96 h of incubation with an IC_{50} value of about 2 μM or less for all species. Ajoene decreased intracellular *L. mexicana* infection of macrophages by 40% at 10 μM, by 95% at 50 μM and by 100% at 100 μM while not affecting the host cells. Ultrastructural studies showed a time- and dose-dependent morphological alteration of the mitochondrial membrane and nuclear envelope and the formation of large autophagic vacuoles and megasomes.[131]

Aqueous onion (*Allium cepa*) extract showed an average IC_{50} of 0.38 mg/mL against promastigotes of several *Leishmania* strains. Onion is again rich in sulfur-containing principles that are responsible for many of its biological effects and that make it a therapeutic agent. Sulfur-compounds are mainly in the form of cysteine derivatives which decompose by the enzyme allinase upon extraction into a variety of volatile thiosulfinates and polysulfides, and also in the form of non-volatile sulfur-containing peptides and proteins. Further investigation is needed, however, to determine the antiparasitic principles in the aqueous onion extract.[132]

2.3 NATURALLY OCURRING ANTITRYPANOSOMAL AGENTS

Contrary to the situation with leishmaniasis, the literature describes fewer studies that evaluate antitrypanosomal natural products especially those with activity against African trypanosomiasis.

2.3.1 Phenolic Compounds

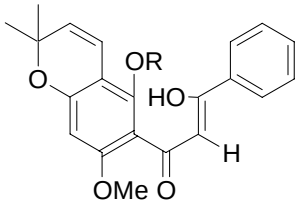
2.3.1.1 Chalcones

The β-oxygenated chalcones praecansone B (**2.108**) and demethylpraecansone A (**2.109**) displayed considerable activity against *T. b. rhodesiense* with IC_{50} values 5.9 and 5.1 μg/mL, respectively, while showing no cytotoxicity against L6 cells.[133]

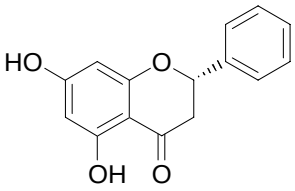
2.3.1.2 Flavonoids

The flavanone pinocembrin (**2.110**) and the flavones chrysin (**2.111**), and 7-methylluteolin (**2.112**) showed moderate activity against *T. b. brucei* bloodstream form

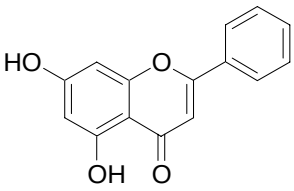
trypomastigotes with IC_{50} values of 11, 14, and 13 μM, respectively. The activity was affected by the status of B ring oxygenation, as is the case with the antileishmanial activity. Moreover, chrysin and pinocembrin displayed about 82- and 12-fold selectivity, respectively, for the parasites compared to the mammalian KB cells (IC_{50} values are 1110 and 124 μM, respectively) while 7-methylluteolin was considerably more toxic, with an IC_{50} value of 38 μM. Methylation or acetylation of pinocembrin greatly reduced its antitrypanosomal activity (IC_{50} >30 μM).[100] In another study, the flavonoids 3-methylquercetin (**2.113**), 3,6-dimethylquercetagetin (**2.114**), 7,3′-dimethylluteolin, and 3,6,7,3′-tetramethylquercetagetin showed weak activity against *T. cruzi* trypomastigotes with IC_{50} values of 128, 138, 145, and 102 μg/mL, respectively.[134] The methanolic extract of the leaves of *Lychnophora staavioides* (Asteraceae) exhibited trypanocidal activity against *T. cruzi*, with 99% lysis of trypomastigote forms at a concentration of 4 mg/mL. Quercetin 3-methyl ether (**2.113**) was the most active compound isolated from the extract showing 63% inhibition of the trypomastigotes at a concentration 500 μg/mL. This compound did not show any blood lysis activity and was considered a promising compound for use against *T. cruzi* in blood banks.[135]



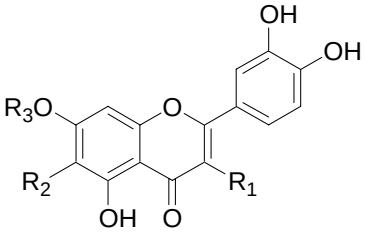
Praecansone B (**2.108**) R = Me
Demethylpraecansone A (**2.109**) R = H



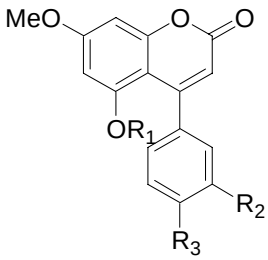
Pinocembrin (**2.110**)



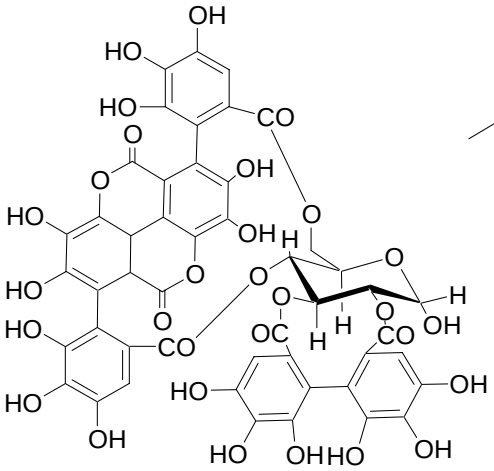
Chrysin (**2.111**)



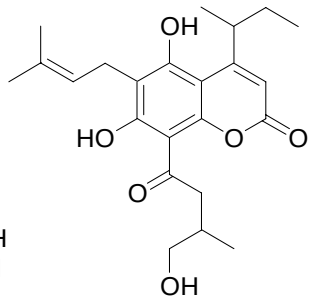
7-Methylluteolin (**2.112**) R_1 =R_2 = H, R_3 = CH_3
3-Methylquercetin (**2.113**) R_1 = OCH_3, R_2 = R_3 = H
3,6-Dimethylquercetagetin (**2.114**) R_1 = R_2 = OCH_3, R_3 = H



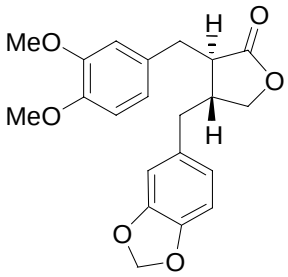
2.115 R_1 = tetraAcGlu, R_2 = R_3 = OAc
2.116 R_1 = tetraAcGal, R_2 = H, R_3 = OAc



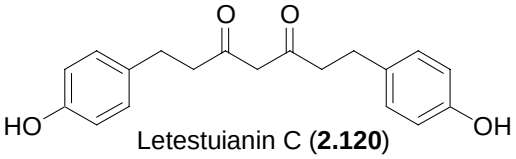
Punicalagin (**2.119**)



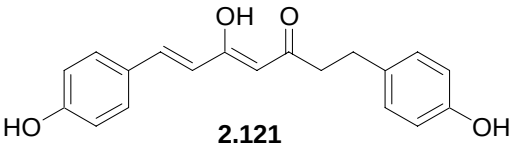
2.117



(-)-Methylpluviatolide (**2.118**)



Letestuianin C (**2.120**)



2.121

Figure 2.15: Structures of Some Trypanocidal Benzenoid Compounds

2.3.1.3 Coumarins

5-*O*-(β-D-Tetraacetoxyglucopyranosyl)-3′,4′-diacetoxy-7-methoxy-4-phenylcoumarin (**2.115**) as well as coumarin **2.116** showed moderate activity against *T.b. brucei* bloodstream trypomastigotes, both with an IC_{50} value of 11 μM, while being non-toxic to KB cells (IC_{50} values >170 μM).[100]

The coumarin 4-(1-methylpropyl)-5,7-dihydroxy-8-(4-hydroxy-3-methylbutyryl)-6-(3-methylbut-2-enyl)chromen-2-one (**2.117**) isolated from the stem bark of *Kielmeyera albopunctata* (Clusiaceae) was active in vitro against the trypomastigote form of *T. cruzi* in the infected-murine blood assay, causing 80% inhibition at 125 μg/mL after 24 h contact at 4 ºC.[136]

2.3.1.4 Lignans

The lignan (–)-methylpluviatolide (**2.118**) isolated from the leaves of *Zanthoxylum naranjillo* (Rutaceae) exhibited significant activity against two strains of *T. cruzi* in vitro, with 88–99% inhibition at 25 μg/mL. Animals inoculated with infected blood treated with 25 μg/mL of the lignan for 24 h at 4 °C did not become infected for the rest of their lives. Animals treated 2 days after parasite inoculation with the compound at 10 mg/kg/day i.p. for 10–20 days showed a significant reduction in parasitemia and an increase in the survival time in comparison with the negative control. The lactone ring appeared to be crucial for activity, as derivatives with the ketone group reduced into a hydroxyl are inactive. However, this compound was inactive against the tissue forms of the parasite.[137]

2.3.1.5 Tannins

The acetone extract of the stem bark of the Ethiopian medicinal plant *Combretum molle* (Combretaceae) showed significant activity against the *T. b. rhodesiense* bloodstream form trypomastigotes, with an IC_{50} value 2.2 μg/mL. However, the extract was not active against intracellular *L. donovani* amastigotes or *T. cruzi* in murine peritoneal macrophages. Punicalagin (**2.119**), an ellagitannin constituent of the extract, was shown to have significant antitrypanosomal activity (IC_{50} 1.75 μg/mL). The compound was relatively non-toxic, with an IC_{50} value of 132 μg/mL against KB cells (selectivity ratio = 70).[138]

2.3.1.6 Diarylheptanoids

The diarylheptanoids letestuianin C (**2.120**) and (4*Z*,6*E*)-5-hydroxy-1,7-bis(4-hydroxyphenyl)hepta-4,6-dien-3-one (**2.121**), isolated from the seeds of the African medicinal plant *Aframomum letestuianum* (Zingiberaceae), were active against bloodstream forms of African trypanosomes (*T. b. brucei* and *T. b. rhodesiense*), with IC_{50} values in the range 1–3 μg/mL after 48 h incubation. Interestingly, the presence of methoxy groups in the *meta* position of the phenyl rings greatly decreased activity.[139]

2.3.1.7 Other Phenolics

The phenylethanoid glycosides isoverbascoside, forsythoside B, and echinacoside (**2.28**) from *Phlomis brunneogaleata* (Lamiaceae) showed activity against *T. b. rhodesiense* at IC_{50} values of 6 to 9 μg/mL, while verbascoside (**2.27**) and

glucopyranosyl-(1→G_i-6)-martynoside (**2.29**) were less active (IC_{50} values of 14 and 28 μg/mL, respectively). Integrifolioside B showed no activity at concentrations up to 100 μg/mL. The compounds were not active against *T. cruzi* and exhibited low or no toxicity against L6 cells.[44]

The common caffeic acid esters chlorogenic acid (**2.30**), 3-*O*-caffeoylquinic acid methyl ester (**2.31**) and 5-*O*-caffeoylshikimic acid (**2.32**) showed weak activity against *T. b. rhodesiense* at IC_{50} values of 17 to 21 μg/mL, while showing low or no toxicity against L6 cells. The compounds were not active against *T. cruzi*.[44]

2.3.2 Terpenes

2.3.2.1 Monoterpenes

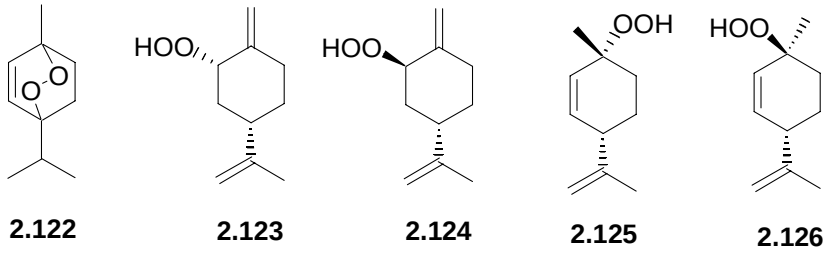
Terpinen-4-ol (**2.34**), the main component of the Australian *Melaleuca alternifolia* (tea tree) oil, was found to be 1025-fold more toxic to bloodstream forms of *T. brucei* than to human myeloid leukemic (HL-60) cells (IC_{50} values of 0.02 and 20.5 μg/mL, respectively.)[66]

Ascaridole (**2.122**) and four related monoterpene hydroperoxides were isolated from the aerial parts of *Chenopodium ambrosioides* (Chenopodiaceae) using an antitrypanosomal activity-guided fractionation. This plant is used for the production of chenopodium oil, which has long been used as an anthelmintic due to its content of ascaridole. Ascaridole (**2.122**), (–)-(2*S*-4*S*)- and (–)-(2*R*-4*S*)-*p*-mentha-1(7),8-dien-2-hydroperoxide (**2.123** and **2.124**), and (–)-(1*R*-4*S*)- and (–)-(1*S*-4*S*)-*p*-mentha-2,8-dien-1-hydroperoxide (**2.125** and **2.126**) showed in vitro activities against the epimastigotes of *T.*

cruzi with MLCs (minimum lethal concentrations) of 23, 1.2, 1.6, 3.1, and 0.8 μM, respectively. The alcohols prepared by treating each of the hydroperoxides **2.123–2.126** with triphenylphosphine (PPh_3) showed no antitrypanosomal effect even at 400 μM, indicating that the hydroperoxy group is essential for the activity of this group of compounds. The compounds were also evaluated against the bloodstream forms of the parasite in the HeLa cell infection assay. The compounds were toxic to HeLa cells at a concentration of 10 μg/mL (59 μM). At 1 μg/mL (5.9 μM), compounds **2.124** and **2.125** inhibited the infection of HeLa cells by trypanosomes by 63% and 88%, respectively, while compound **2.126** almost completely inhibited the infection. However, the compounds did not inhibit the proliferation of the amastigotes inside the infected cells (i.e. the infection rate was decreased but not the number of amastigotes per infected cell.)[140]

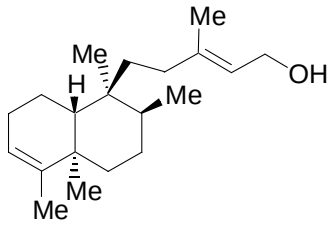
2.3.2.2 Diterpenes

Compound **2.127**, a diasteriosomer of kolavenol isolated from the root bark of *Entada abyssinica* (Fabaceae), a plant used by the traditional healers in Uganda for the treatment of sleeping sickness, showed trypanocidal activity with an IC_{50} value of 2.5 μg/mL (8.6 μM) against *T. brucei rhodesiense*.[141]

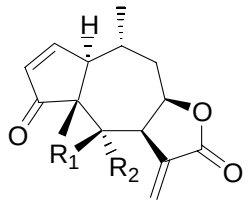


2.122 **2.123** **2.124** **2.125** **2.126**

Monoterpenes from chenopodium oil

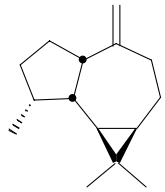


2.127

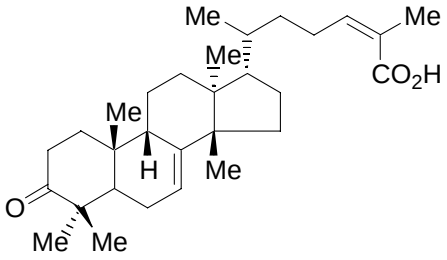


Helenalin (**2.128**) R_1 = H, R_2 = OH

Mexicanin (**2.129**) R_1 = OH, R_2 = H



Alloaromadendrene (**2.130**)



3-Oxotirucalla-7,24*Z*-dien-26-oic acid (**2.131**)

Figure 2.16: Structures of Some Trypanocidal Terpenoids

2.3.2.3 Sesquiterpenes

Helenalin (**2.128**), a sesquiterpene lactone with two α,β-unsaturated carbonyl groups is a bifunctional alkylating agent. It showed promising antitrypanosomal activity (IC_{50} values of 0.051 and 0.695 μM against *T. b. rhodesiense* and *T. cruzi*, respectively), while displaying only moderate toxicity against L6 cells with an IC_{50} value of 0.992 μM. Mexicanin (**2.129**), a diastereoisomer of helenalin, is much less active with IC_{50} values of

0.318 and 1.870 μM against *T. b. rhodesiense* bloodstream forms and *T. cruzi* trypomastigotes, respectively. Saturation of either of the cyclopentenone or the α-methylene-γ-lactone moieties resulted in a dramatic decrease of activity.[142] The mechanism of action is poorly understood, although it is known that the α-methylene-γ-lactone substituent is responsible for most of the biological properties of this class of compounds. In another study,[143] *T. cruzi* epimastigotes, the non-infective parasite form, were shown to be less sensitive to either helenalin or mexacanin with IC_{50} values 1.9 and 3.8 μM, respectively. The compounds induced cytoplasmic vacuolization and nuclear disorganization in the trypanosomes. The antitrypanosomal effect was not reversed by the reducing agents dithiothreitol or glutathione, arguing against the involvement of non-specific interaction with sulphhydryl-containing enzymes in the trypanocidal mechanism.

Alloaromadendrene (**2.130**), a sesquiterpene component of Australian *Melaleuca alternifolia* (tea tree) oil, exhibited moderate activity against *T. brucei* bloodstream forms (IC_{50} value of 1.9 μg/mL). It is 13.6-fold more selective against the parasites compared to the human myeloid leukemic (HL-60) cells (IC_{50} value = 25.8 μg/mL.)[66]

2.3.2.4 Triterpenes

3-Oxotirucalla-7,24*Z*-dien-26-oic acid (**2.131**), a triterpene carboxylic acid isolated from *Celaenodendron mexicanum*, showed activity against *L. donovani* promastigotes and amastigotes and *T.b. brucei* bloodstream trypomastigotes (IC_{50} values 13.7, 90, and 16.8 μM, repectively) while displaying low toxicity to KB and P-388 cells (IC_{50} values 137.6 and 107.4 μM, respectively.)[144]

2.3.3 Quinones

2.3.3.1 Naphthoquinones

Diospyrin (**2.54**), a bisnaphthoquinone isolated from the stem bark of *Diospyros montana*, showed a weak inhibitory effect against *T. b. brucei* bloodstream trypomastigotes (IC_{50} = 50 μM). The synthetic derivatives **2.55** and **2.56** demonstrated improved activity, with IC_{50} values of 2.1 and 0.7 μM, respectively. Derivative **2.56** inhibited intracellular *T. cruzi* by 100% at a concentration of 3 μM, while **2.55** was much less toxic to *T. cruzi* (IC_{50} 17 μM).[90]

2.3.3.2 Anthranoids

Vismione D (**2.60**), 3-geranyloxy-6-methyl-1,8-dihydroxyanthraquinone (**2.132**) and emodin (**2.59**), isolated from the stem bark of the Tanzanian medicinal plant *Vismia orientalis* (Clusiaceae), showed moderate toxicity against *T. b. rhodesiense* with IC_{50} values 9.0, 14 and 18 μg/mL The compounds were also toxic against L6 cells with IC_{50} values of 4.1, >90 and 20 μg/mL, respectively. Only vismione D and emodin showed activity against *T. cruzi*, with IC_{50} values 4.6 and 20 μg/mL, respectively.[98]

The anthraquinone aloe-emodin (**2.133**), isolated from the African medicinal shrub *Stephania dinklagei* (Menispermaceae), exhibited activity against *T. b. brucei* (IC_{50} = 14 μM) while showing no cytotoxicity towards KB cells (IC_{50} = 1059 μM).[108]

The anthraquinone knipholone (**2.134**), originally isolated from *Kniphofia foliosa* (Liliaceae), and its anthrone derivative (**2.135**) exhibited antitrypanosomal activity against intracellular *T. cruzi* trypomastigotes (IC_{50} values of 7.6 and 1.5 μg/mL,

respectively) and *T. b. rhodesiense* (IC_{50} values of 9.3 and 13 μg/mL, respectively) while showing toxicity to L6 cells at MIC 33 and 3.7 μg/mL, respectively. The compounds displayed no antileishmanial activity.[145]

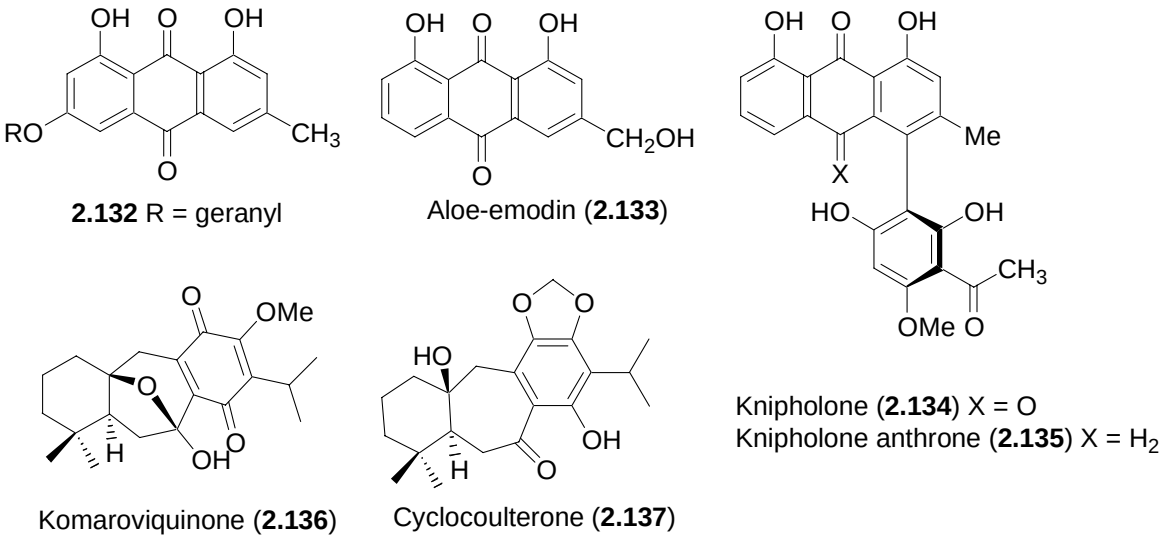


Figure 2.17: Structures of Some Trypanocidal Quinones and Related Compounds

2.3.3.3 Other Quinones

1-Hydroxybenzoisochromanquinone (**2.62**) and benzo[*g*]isoquinoline-5,10-dione (**2.63**) obtained from *Psychotria (Cephaelis) camponutans* showed activity against *T. b. brucei* bloodstream trypomastigotes (IC_{50} 3.3 and 7.5 μM, respectively) and *T. cruzi* amastigotes (IC_{50} of compound **2.62** is 8.1 μM). However, the compounds display toxicity against KB cells (IC_{50} 8.1 and 7.8 μM). The acetyl derivative of **2.62**, 1-acetylbenzoisochromanquinone, exhibited greater potency with an IC_{50} value of 0.65 μM

against *T. b. brucei* bloodstream trypomastigotes and 6.6 μM against *T. cruzi* amastigotes while still displaying moderate toxicity against KB cells (IC_{50} = 12 μM).[100]

The icetexane diterpene quinone, komaroviquinone (**2.136**), isolated from an Uzbekistan medicinal plant, *Dracocephalum komarovi* (Labiatae), showed strong trypanocidal activity against the epimastigotes of *T. cruzi*, with a MLC (minimum lethal concentration) of 0.4 μM. Cyclocoulterone (**2.137**), a structurally similar diterpene lacking the quinone moiety isolated from the same plant, showed only moderate trypnocidal activity (MLC of 20 μM). Under the same assay conditions, the MLC of gentian violet, which is used to disinfect trypanosomes from transfusion blood in Latin America, was 6.3 μM.[146]

2.3.4 Alkaloids

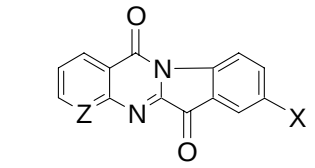
2.3.4.1 Indole Alkaloids

The indole alkaloids strictosidine (**2.76**) and acetylstrictosidine obtained from *Cephaelis dichroea* showed antiprotozoal activity toward *T.b. brucei* bloodstream form trypomastigotes (IC_{50} values of 6.1 and 17 μM, respectively). These compounds showed low toxicity against KB cells, with IC_{50} values of 74 and 131 uM, respectively. Only strictosidine showed activity against intracellular *T. cruzi* amastigotes, with an IC_{50} value of 28 μM.[100]

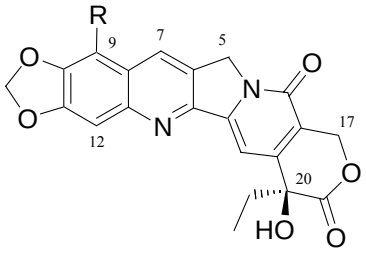
Tryptanthrin (**2.138**), the active constituent of *Strobilanthese cusia*, the traditional Japanese herbal remedy for fungal infections,[147] was found to exhibit antibacterial (including antimycobacterial), antiplasmodial and antitrypanosomal activities.

Tryptanthrin itself gave an IC_{50} value of 23.0 μM against bloodstream form *T. brucei*. Introduction of an electron-withdrawing group (halogen or nitro) at position 8 of the tryptanthrin core significantly increased the antitrypanosomal activity. The most active analogs, 8-nitrotrypanthrin (**2.139**) and 4-aza-8-bromotrypanthrin (**2.140**), showed submicromolar IC_{50}s (0.82 and 0.4 μM, respectively) against bloodstream form *T. b. brucei*.[148]

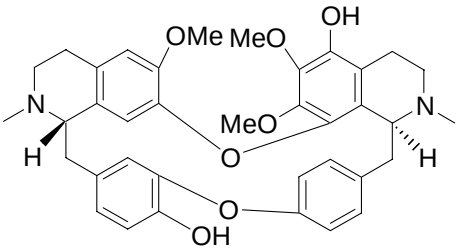
Camptothecin (**2.81**) is a pyrano-indolizinoquinoline alkaloid from the wood and bark extracts of *Camptotheca acuminata* (Nyssaceae) trees.[149] Camptothecin was found to be cytotoxic to *T. brucei*, *T. cruzi*, and *L. donovani* in vitro, with EC_{50} values of 1.5, 1.6, and 3.2 μM, respectively. It promotes the formation of nuclear and mitochondrial DNA-protein adducts in trypanosomes and *Leishmania* implicating topoisomerase I in the antiparasitic mechanism of action.[114] A series of camptothecin analogs were tested for trypanocidal acitivity to formulate a structure-activity relationship. Modification to the pentacyclic nucleus of camptothecin abolished activity, whereas the activity was increased by adding certain substituents at positions 7, 9, 10 and 11 of the parent ring system. For example, the 9-chloro-10,11-methylenedioxy (**2.141**) and the 9-amino-10,11-methylenedioxy (**2.142**) derivatives were 39- and 22-fold more active against *T. brucei*, while becoming only 10- and 4-fold more toxic to the mammalian L1210 leukemia cells than the parent camptothecin, respectively. The study identified the 9-substituted-10,11-methylenedioxy structural motif as a starting point to obtain camptothecin derivatives with trypanocidal selectivity.[150]



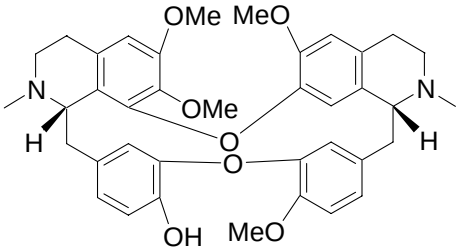
Trypanthrin (**2.138**) Z = C, X = H
8-Nitrotrypanthrin (**2.139**) Z = C, X = NO_2
4-Aza-8-bromotrypanthrin (**2.140**) Z = N, X = Br



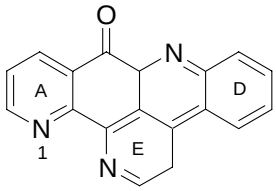
2.141 R = Cl
2.142 R = NH_2



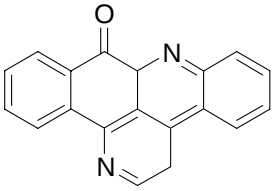
Thanlisopidine (**2.143**)



Funiferine (**2.144**)



Ascididemin (**145**)



146

Figure 2.18: Structures of Some Trypanocidal Alkaloids

2.2.4.2 Bisbenzylisoquinoline Alkaloids

A series of bisbenzylisoquinolines (BBIQ) showed activity (IC_{50} values of 1– 8 μM) against *T. b. brucei* bloodstream trypomastigotes. Thalisopidine (**2.143**) was the most active, with an IC_{50} value of 1.1 μM. The stereochemistry of the alkaloids at C-1 and C-1′ did not affect the antitrypanosomal activity that was observed for compounds

possessing the *R,R*, *S,S*, *R,S*, and *S,R* configurations at C-1 and C-1′. However, phenolic alkaloids showed less activity than their methyl ethers.[111] Another series of BBIQ alkaloids isolated from *Guatteria boliviana* (Annonaceae), showed only weak activity on the trypomastigote forms of *T. cruzi*. The most active alkaloid was funiferine (**2.144**) with an IC_{50} value of 30 μg/mL.[112]

2.2.4.3 Naphthylisoquinoline Alkaloids

The genus *Ancistrocladus* (Ancistrocladaceae) includes 30 species of lianas indigenous to the rain forests of Africa and Asia. Phytochemical and bioactivity studies revealed several monomeric and dimeric naphthylisoquinoline alkaloids with antiparasitic properties. Ancistroealaines A (**2.82**) and B (**2.83**), from the central African species *Ancistrocladus ealaensis*, exhibited activity against *T. b. rhodesiense* (IC_{50} values of 3.5 and 2.0 μg/mL) and *T. cruzi* trypomastigotes (IC_{50} values of 2.4 and 18 μg/mL). The compounds did not show any cytotoxicity against L6 cells (IC_{50} >90 μg/mL) or murine macrophages (IC_{50} >30 μg/mL for both compounds).[115] Ancistrolikokine D (**2.84**), from *A. likoko*, showed a significant activity versus *T. b. rhodesiense* (IC_{50} = 2.7 μg/mL) and *T. cruzi* (IC_{50} = 13 μg/mL) while being weakly toxic towards L6 cells (IC_{50} = 37 μg/mL).[116] Ancistrogriffines A (**2.85**) and C (**2.86**), and the naphthylisoquinoline dimer ancistrogriffithine A (**2.87**) were isolated from the Asian *A. griffithii*. They showed antitrypanosomal properties (IC_{50} values of 2.2, 3.0 and 0.9 μg/mL, respectively, against *T. b. rhodesiense* and 17, 41 and 14 μg/mL, respectively, against intracellular *T. cruzi*) while displaying moderate to weak toxicity towards L6 cells (IC_{50} 14, 36 and 5.8 μg/mL,

respectively).[117] Ancistrocongolines A (**2.88**) and B (**2.89**) and korupensamine A (**2.90**) isolated from *Ancistrocladus congolensis* showed moderate antitrypanosomal activity, with IC_{50} values of 3.0, 2.5 and 1.9 μg/mL, respectively, versus *T. b. rhodesiense*. Ancistrocongoline C (**2.91**) showed weaker activity, with an IC_{50} value of 16 μg/mL.[118] Ancistrotanzanines A (**2.92**) and B (**2.93**) and ancistrotectoriline A (**2.94**) were isolated from the East African *A. tanzaniensis*. They displayed activity against *T. b. rhodesiense* (IC_{50} 0.7, 0.7 and 2.1 μg/mL, respectively) and *T. cruzi* (IC_{50} 1.7, 1.5 and 18 μg/mL) while being moderately toxic towards L6 cells (IC_{50} 6.4, 8.1 and 6.5 μg/mL).[119] Ancistrocladinine (**2.95**) and ancistrotanzanine C (**2.96**) isolated from the same species also showed a significant effect against *T. b. rhodesiense* (IC_{50} 2.0 and 1.3 μg/mL, respectively) and a weaker activity towards *T. cruzi* (IC_{50} 23 and 14 μg/mL), while being only weakly toxic to L6 cells (IC_{50} values of 28 and 41 μg/mL, respectively).[120] Ancistrobeomine A (**2.97**) obtained from the Malysian *A. benomensis*, was active against *T. b. rhodesiense* (IC_{50} = 1.3 μg/mL) with weaker activity towards *T. cruzi* (IC_{50} = 4.8 μg/mL) and a weak toxicity against L6 cells (IC_{50} 12 μg/mL).[151]

2.2.4.4 Other Alkaloids

Liriodenine (**2.73**), isolated from the stem bark of *Rollinia emarginata* and *Unonopsis buchtienii* (Annonaceae), reduced the number of *T. cruzi* parasites in infected murine blood by 53% at a concentration of 250 μg/mL after incubation for 24 h at 4 ºC.[109] It also showed weak activity against *T. b. brucei*, with an IC_{100} value 50 μg/mL, while it displayed toxicity towards Vero cells at an IC_{50} value of 1 μg/mL.[110]

Ascididemin (**2.145**), a marine pyridoacridone alkaloid, displayed moderate activity against *T. b. rhodesiense* bloodsteam forms, with an IC_{50} 4.0 μg/mL. Replacement of the N at position 1 with a C atom resulted in a 2000-fold increase in potency, as compound **2.146** exhibited an IC_{50} of 2 ng/mL against the parasites. This derivative was less active against *T. cruzi* trypomastigotes with an IC_{50} 0.68 μg/mL. Moreover, compound **2.146** was highly selective. It was toxic to L6 cells at an IC_{50} 1.9 μg/mL. Other derivatives of the marine alkaloid inhibited the growth of trypanosomes at concentrations below 0.1 μg/mL. However, this class of compounds did not show potency against *Leishmania*.[152]

2.3.5 Other Secondary Metabolites

The acetogenins rolliniastatin-1 (**2.99**) and squamocin (**2.101**), isolated from *Rollinia emarginata* (Annonaceae), at a concentration of 250 μg/mL reduced the number of *T. cruzi* parasites in infected murine blood by 89% and 67%, respectively, after incubation for 24 h at 4ºC. Gentain violet, which is used to disinfect transfusion blood in Latin America, causes 100% inhibtion of the parasite at the same concentration.[109]

Klaivanolide (**2.103**), a bis-unsaturated seven-membered lactone was isolated from the stems of *Uvaria klaineana* (Annoonaceae), a liana endemic to Gabon and Congo in Africa. The compound exhibited weak in vitro activity against bloodstream form *T. b. brucei*, with MEC (minimum effective concentration) 33 μM.[124]

Ajoene (**2.107**), a potent antiplatelet compound derived from garlic, induced cell lysis of *T. cruzi* epimastigotes at 100 μM. Moreover, the intracellular amastigotes were

eradicated from Vero cells after 96 h incubation with 40 μM ajoene. In *T. cruzi*, ajoene was shown to interfere with the biosynthesis of phosphatidylcholine, leading to drastic perturbation of the plasma membrane and the death of the organism.[153] It also acts as a subversive substrate and inhibitor of the enzyme trypanothione reductase, increasing the oxidative stress on the parasite.[154]

2.4 CONCLUDING REMARKS

Parasitic diseases are still a threat to mankind in the 21st century. The lack of effective chemotherapy for Chagas disease, the emergence of *Leishmania*/HIV co-infection, and the alarming decrease in our arsenal of effective antiparasitic drugs due to the development of drug resistance, emphasize the need for new prophylactic and therapeutic agents.

Natural products continue to be an attractive source of chemical diversity. Various compounds with antiprotozoal potential have been isolated from plants, primarily those used in tradional medicine, by activity-guided fractionation. However, many of them have not been further evaluated in vivo due to lack of selectivity, lack of sufficient potency or pharmacokinetic problems, especially those that interfere with absorption and attaining therapeutic drug concentrations at reasonable doses via the oral route. Funding and economic difficulties represent a large obstacle for research in this area, considering that the majority of cases with parasitic diseases occur in developing countries. It is crucial to bring together the efforts of academic research institutions, the pharmaceutical industry and international organizations to solve this problem.

CHAPTER 3

SCREENING A NATURAL PRODUCTS LIBRARY FOR LEISHMANIACIDAL AND TRYPANOCIDAL ACTIVITY

3.1 INTRODUCTION

Parasitic diseases remain a major public health problem. The dire need for novel and inexpensive antikinetoplastid agents was outlined in Chapters 1 and 2. The classic drug development process is extremely expensive and time-consuming. On average, screening 10,000 candidate compounds is needed to find a single agent that can make it through preclinical and clinical testing and the drug approval process to the market. This process, which is heavily regulated by the Food and Drug Administration in the United States, is estimated to take 8–15 years, consuming 150–500 million dollars.[155] Natural products represent an attractive source of a wide diversity of both chemical structures and biological activities for the drug discovery process. Moreover, they offer a great potential for accelerating and reducing the cost of the drug discovery process, especially when successful traditional phytomedicines are evaluated. An example is *Artemisia annua*, which had long been used in traditional Chinese medicine for the treatment of febrile illnesses. Upon phytochemical investigation, artemisinin was isolated as the active antimalarial principle.[156] Moreover, it was used as a lead compound to synthesize simpler

structures, retaining the antiplasmodial pharmacrophore and displaying remarkable potency.[157] The economic aspect of drug development is especially relevant in the area of parasitic diseases, which primarily affect people in the underdeveloped countries. The lack of a foreseeable profit or economic incentives prohibits pharmaceutical companies from investing in costly research in this area.

Our research approach was to screen a natural products library of a representative diversity, providing an opportunity to discover novel structures with high activity and selectivity. The ultimate goal was to generate new candidate antileishmanial and/or antitrypanosomal lead compounds from plant sources. Plants used in this screen are a collection of rare and uninvestigated species from the deserts in the western U.S.A., in addition to a collection of Chinese herbs. Many of these plants have traditional medicinal uses. These natural products library was originally collected for and extracted in the laboratory of Dr. John Cassady in the College of Pharmacy, The Ohio State University. The plant extracts were sampled and introduced them to medium-throughput screens for antiparasitic activity against the axenic amastigote form of *Leishmania donovani*. Extracts that showed an antileishmanial activity were also tested against *T. b. brucei* bloodstream form trypomastigotes. In addition, these samples were assayed for their cytotoxicity to the mammalian cell lines J774 (murine macrophages) and PC-3 (prostate cancer cells). Based on our results, we chose promising plant species for further investigation in order to isolate the antiparasitic constituents. Figure 3.1 provides a flow chart that outlines our research work.

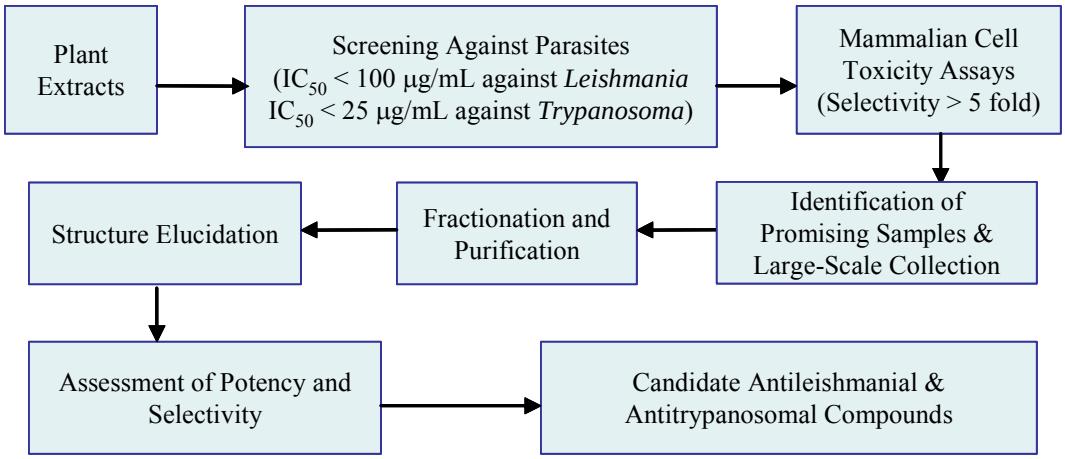


Figure 3.1: Flow Chart for the Identification of Novel Antiparasitic Compounds

3.2 EXPERIMENTAL SECTION

3.3.1 Plant Samples

The botanical specimens investigated in this study were obtained from the World Botanical Associates (WBA, Bakersfield, CA). Samples were collected from the western United States in California, Nevada and southern Oregon in quantities of 30–100 g from June 14th to July 10th, 1998, by Dr. Richard W. Spjut (SPJ), alone or with assistance from Mr. Richard Marin (S & M for Spjut & Marin). The emphasis was on samples not generally collected for the National Cancer Institute (NCI) based on the NCI requirement of 500 g (R. W. Spjut, personal communication; see also Spjut 1985[158]). The collections focused on root and stem bark from shrubs, small annual herbs, and flower and fruit parts of all plant species. Some species, such as *Ribes cereum* were sampled from up to three

different locations because the species exhibited different morphological features in different ecological environments. Vouchers were deposited in the United States National Herbarium (US), Smithsonian Institution, Department of Botany, Washington, DC. A few samples were collected from Florida (these were not part of the collection strategy in the western USA) by Patricia Gilliland (PG); vouchers were deposited at the University of Florida at Gainesville. Additional vouchers for all samples were deposited at WBA.

Note: In recent studies *Petasites palmatus* has been reduced to *Petasites frigidus* var. *palmatus*. The California plants grow in the understory of coastal and mixed evergreen forests. The plants are relatively large and easy to collect, in contrast to Alaskan plants that grow on open arctic slopes. For this reason, the older taxonomy seems more appropriate for this Californian species (R. W. Spjut, personnal communication).

The Chinese plant samples were obtained from Guang Zhou Yu Hai Phytochemical Products, Ltd., People's Republic of China (99 samples) and Guang Zhou Hong Xing Health Food Co., Ltd., People's Republic of China (50 samples).

3.3.2 Preparation of Extracts

For each plant sample, extracts were prepared (in Dr. John Cassady's laboratory) by macerating the powdered dry material with a sufficient volume of 95% EtOH. After filtration, the solvent was evaporated under reduced pressure at a temperature not exceeding 40 °C. Stock solutions of the extracts at 20 mg/mL in DMSO were prepared and stored at –20 C°.

3.3.3 Biological Assays

3.3.3.1 Antileishmanial Assay Using Axenic Amastigotes

The antileishmanial activity of the prepared extracts was tested in vitro against *Leishmania donovani* amastigote-like parasites (WHO designation: MHOM/SD/62/1S-$CL2_D$) in a three-day assay using the tetrazolium dye-based CellTiter reagent (Promega).[159,160] The amastigote medium used in this assay is based on the medium mentioned by Joshi *et al.*[161] Prior to addition of fetal bovine serum (FBS) to a final concentration of 20%, this amastigote medium contains 15 mM KCl, 115 mM KH_2PO_4, 10 mM K_2HPO_4, 0.5 mM $MgSO_4$, 24 mM $NaHCO_3$, a 1× concentration of RPMI-1640 vitamins and amino acids, 2.0 mM L-glutamine, 22 mM D-glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.1 mM adenosine, 1 μg/mL folate, and 25 mM MES (pH 5.5). *Leishmania* amastigotes, at 10^6 cells/mL, were seeded together with serial dilutions of the sample extracts in the individual wells of 96-well plates (final volume 60 μL/well). After 72 h incubation at 37 °C in a humidified 5% CO_2 incubator, cell viability was determined using the CellTiter reagent by adding 12 μL of assay solution to each well. After 3–6 h incubation at 37 °C to allow for color development, the optical densities were measured at 490 nm using SpectraMax Plus microplate reader. IC_{50} values, the concentration of the sample that inhibited cell growth by 50% compared to untreated control, were determined with the aid of the software program SoftMax Pro (Molecular Devices). For the IC_{50} determination, this program uses the dose-response equation $y = [(a-d)/(1+(x/c)^b)] + d$, where x = the drug concentration, y = absorbance at 490 nm, a = upper asymptote, b = slope, c = IC_{50} and d = lower asymptote.

3.3.3.2 Antitrypanosomal Assay

Extracts were tested for their activity against bloodstream-form *Trypanosoma brucei brucei* (MITat 1.2, variant 221) axenically cultured in HMI-9 medium,[162] which is composed of Iscove's Modified Dulbecco's Medium (IMDM) with 20% heat-inactivated FBS supplemented with 1 mM hypoxanthine, 0.05 mM bathocuproine sulfonate, 1.5 mM cysteine, 0.2 μM β-mercaptoethanol, 1 mM pyruvate, 0.16 mM thymidine, 100 units/mL penicillin, and 100 μg/ml streptomycin. Late log phase parasites were incubated in 96-well plates (Costar) at an initial concentration of 10^5 cells/mL in a volume of 100 μL/well, with or without test compounds at 37 °C in a humidified 5% CO_2 atmosphere for 72 h. Twenty-five μL of a 5 mg/mL solution of MTT (prepared in phosphate buffered saline and filter sterilized) was then added to each well, and plates were re-incubated at 37 ºC as before for 2 h. One hundred μL of 10% SDS lysis buffer (prepared in 50% aqueous DMF) were added to each well, and plates were incubated as before for an additional 3–4 h. Optical densities were then measured at 570 nm using a SpectraMax Plus microplate reader. IC_{50} values, the concentration of the compound that inhibited cell growth by 50% compared to untreated control, were determined with the aid of the software program SoftMax Pro (Molecular Devices) as mentioned previously.

3.3.3.3 Cytotoxicity Assays against J774 and PC-3 cells

The cytotoxicity of the extracts was evaluated against two cell lines, J774 macrophages and PC-3 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD). J774 macrophages, grown in Dulbecco's Modified Eagle's

Medium (DMEM, from ATCC) supplemented with 10% heat inactivated fetal calf serum, 2.0 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin, were added to individual wells of a 96-well plate at a concentration of 10^4 cells/mL and a total volume of 100 μL. Macrophages were allowed to adhere for 24 hours, and then the medium was removed and replaced with serial dilutions of the samples in the DMEM medium mentioned above without phenol red. After 72 hr incubation at 37 °C in a humidified 5% CO_2 incubator, cell viability was determined using the CellTiter reagent by adding 20 μL of assay solution to each well. After 6–7 h incubation at 37 °C to allow for color development, the absorbance of each well at 490 nm was measured in a SpectraMax Pro microplate reader (Molecular Devices). The toxicity of extracts to PC3 cells was assessed in the same manner as for the macrophages except that the medium used was RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2.0 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. IC_{50} values for the J774 and PC3 assays were determined with the software program SoftMax Pro as described previously.

3.3 RESULTS AND DISCUSSION

Out of 174 North American plant ethanolic extracts belonging to 130 species and 45 families, 19 samples showed activity against *L. donovani* axenic amastigotes at IC_{50} values under 100 μg/mL, with only 8 samples showing activity at IC_{50} values below 50 μg/mL (see Table 3.1). Samples that displayed some antileishmanial activity at or below 200 μg/mL were also assayed for antitrypanosomal activity. Twinty one extracts showed

activity against *Trypanosoma brucei brucei* with IC_{50} values less than 50 μg/mL, while 11 samples showed significant activity with IC_{50} values below 15 μg/mL. Some of the active samples displayed good selectivity with low toxicities to the mammalian cell lines. The most potent extracts are listed in Table 3.2. Promising plant species showing selective antileishmanial properties included *Psorothamnus polydenius* and *P. arborescens* (Fabaceae) and *Chamelaucium ucinatum* (Myrtaceae) while those exhibiting selective trypanocidal activities included *Chaetadelpha wheeleri* and *Petasites palmatus* (Asteraceae) and *Alnus rubra* (Betulaceae).

Many of the active samples or related species have been used in traditional medicine by the native tribes of California and Nevada, mostly for anti-infective applications. *Eriodictyon californicum* and *E. angustifolium* are both known as "Yerba Santa" (meaning holy weed). The tea made from the leaves was used for coughs, colds, sore throat, asthma, tuberculosis and rheumatism and as a "blood purifier". A poultice from the young leaves and stems was bound on the sores of man and animal.[163] A brew of the boiled leaves was taken to relieve stomachaches, vomiting and diarrhea, and for venereal diseases.[164] Some species of *Erigeron* were used to stop diarrhea and to prepare eyewashes. *Marrubium vulgare* (common name Horehound) was used as a counter irritant to stimulate blood circulation.[164] *Mimulus guttatus* (known as Common Monkeyflower) was used as a poultice for wounds and burns. *Psorothamnus polydenius*, or the smokebush, was known generally by all the native tribes of California as a treatment for coughs and colds. A tea made from boiled stems, leaves and flowers was also used for pneumonia, tuberculosis, venereal diseases, stomachaches, muscular pains,

kidney problems and diarrhea. The plant was used externally and internally for smallpox and measles. Additionally, the crushed fresh stems or powdered dried stems were rubbed on for treatment of sores. *Purshia tridentata* is known as Antelope Bush or Bitterbrush. The decoction made from the leaves, bark or roots was used as a tea for treatment of venereal diseases, especially gonorrhea. Remedies from this plant were also used internally and externally to treat smallpox, chicken pox, and measles. The leaf decoction was used for treatment of tuberculosis, pneumonia, colds or as a general tonic. An external wash was used for any itches, rash or insect bites. *Tanacetum vulgare* (Common Tansy) was used internally to treat bloody diarrhea and externally as an antiseptic wash. *Heracleum lanatum* (Cow Parsnip) was used to treat toothaches, coughs and colds.[164] The roots were also used to prepare a poultice for application to boils.[165]

Some of the samples that showed antiparasitic activity in our screen are reported in the literature to have other anti-infective properties. For example, extracts prepared from *Alnus rubra*, *Glehnia littoralis* and *Heracleum lanatum* are reported to possess significant activities against a panel of fungal species.[166]

Members of family Asteraceae exhibited significant activities in our assays, especially *Petasites palmatus* (Arctic Sweet Coltsfoot) and *Chaetadelpha wheeleri* (Dune Broom). The family is characterized by production of sesquiterpenes, which could be responsible for the antiparasitic activity. For example, the sesquiterpene lactone parthenolide from *Tanacetum parthenium* (commonly known as Feverfew) is reported to exhibit significant activity against *L. amazonensis* intracellular amastigote forms (IC_{50} 0.8 μg/mL).[75] However the contribution of constituents from other phytochemical classes

cannot be ruled out. *Brickellia grandiflora* (Large-Flowered Brickelbush), for example, is reported to contain pyrrolizidine alkaloids that might contribute to the antiparasitic activity.[167] The literature does not contain phytochemical reports concerning *Aralia californica* (California Spikenard). However, bioactive saponins and cerebrosides have been isolated from related *Aralia* species that are used as medicinal plants in Asia.[168,169] *Alnus rubra* (red alder) displayed a significant antitrypanosomal effect and is also reported to possess antibacterial and antifungal activities.[166,170] These activities might be attributed to the various diarylheptanoid constituents of the plant.[171] *Glehnia littoralis* (common name American Silvertop) is known to be rich in furanocoumarin phytoalexins[172] and contains antibacterial and antifungal acetylenic compounds.[173] The related species *Heracleum lanatum* (Cow Parsnip) is also rich in bioactive furanocoumarins.[174] *Lonicera involucrata*, commonly known as the Twinberry, displayed significant trypanocidal activity in our assays. It is not investigated in the literature but iridoid glycosides[175] and saponins[176] have been isolated from related *Lonicera* spp. *Escallonia rubra* was shown to exert an antifungal effect due to its content of furanocoumarins.[177] *Calystegia soldanella*, commonly known as Shore Morning Glory, is known to contain tropane alkaloids in addition to caffeic and coumaric acid esters.[178] Members of the genus *Psorothmnus* showed significant antileishmanial and trypanocidal activity in our assays. *P. arborescens* had not been investigated previously, while *P. polydenius* is reported to contain the red dye dalrubone and the flavanone demethoxymatteucinol.[179] *Eriodictyon californicum* (California Yerba Santa) and *E. angustifolium* (Narrowleaf Yerba Santa) are both rich in flavonoids.[180] In addition,

benzyl-*trans*-coumarate had been identified as a major antibacterial and antifungal compound in *E. angustifolium*.[181] *Marrubium vulgare* contains the labdane diterpenoids, marrubenol and marrubin, in addition to flavonoids and phenylpropanoids including the glycoside, forsythoside B.[182] The latter compound was previously shown to exert potent antileishmanial and antitrypanosomal activity.[44] *Chamelaucium ucinatum* (Wax-flower) is a native Australian plant cultivated for the floriculture industry; there are no reports in the literature about any biological activity or medicinal use of this species. The *Chamelaucium* samples used in this study were possibly escapees from a nursery in the Fallbrook, California area that no longer exists (R. W. Spjut, personnal communication). Interestingly, it showed selective potency against *Leishmania* amastigotes but not *T. b. brucei*, and the activity was confined to the aerial tops (flowers with leaves and twigs) but not the root or stem bark. Phytochemical investigations are limited to reports about the anthocyanidin pigments of the flowers[183] and the essential oil composition.[184] The literature is lacking in phytochemical investigations of *Ludwigia sphaerocarpa* (Globefruit Primrose-willow), although the closely related medicinal herb *L. octovalvis* contains cytotoxic oleanane-type triterpenes.[185] *Mimulus bigelovii* (Desert Monkey Flower) showed antileishmanial activity in our screen and was not investigated previously. Other *Mimulus* species were reported to contain flavonoids, carotenoids and geranyl pyrones.[186]

For the Chinese plants, ten out of 149 samples showed antileishmanial activity at IC_{50} values below 100 μg/mL and only two samples possessed IC_{50} values below 50 μg/mL. *Acroptilon repens*, commomly known as Russian Knapweed, displayed potent

activity but it was almost equally cytotoxic to the mammalian cell lines. Indeed, repin, one of the sesquiterpene lactone constituents of the herb, was shown to be neurotoxic to chick embryos.[187] *Saussurea involucrate* (a popular Chinese herb known as Snow Lotus) showed a significant activity against the parasite with an IC_{50} 42 μg/mL. *Heracleum dissectum* and *Hypericum hirsutum* (Hairy St. John's Wort), also showed good activities. It is interesting that *Marrubium vulgare* from the Chinese source showed the same activity as the North American species.

#	Species and Taxonomic Family	Plant Part	Voucher Number	IC_{50} (µg/mL) *L. donovani*
	Anacardiaceae			
1	*Malosma laurina* (Nutt.) Nutt. ex Engl.	sb	WBA-3461, S & M-14211	>200
2	*Rhus ovata* S. Watson	rb	WBA-3458, S & M-14210	>200
3	*R. ovata* S. Watson	sb	WBA-3459, S & M-14210	>200
	Apiaceae			
4	*Glehnia littoralis* F. Schmidt ex Miq.	rt-lf	WBA-3596, SPJ-14318	148 ± 24
5	*G. littoralis* F. Schmidt ex Miq.	fr	WBA-3597, SPJ-14318	112 ± 3
6	*Heracleum lanatum* Michx.	fr-ped	WBA-3557, SPJ-14283	62.5 ± 7.6
7	*Sanicula crassicaulis* Poepp. ex DC.	rt-st-lf-fr	WBA-3572, SPJ-14296	>200
	Araliaceae			
8	*Aralia californica* S. Watson	rt	WBA-3574, SPJ-14298	24.5 ± 2.0
	Asteraceae			
9	*Ambrosia chamissonis* (Less.) Greene var. *bipinnatisecta* (Less.) J. T. Howell	infl (fl)	WBA-3530, SPJ-14257	>200
10	*Baccharis pilularis* DC.	sb	WBA-3542, SPJ-14269	>200
11	*Brickellia grandiflora* (Hook.) Nutt.	lf-fl-st	WBA-3580, SPJ-14303	38.3 ± 2.2

Continued

Table 3.1: Activity of North American Botanical Samples against *L. donovani* Axenic Amastigotes
Plant parts in bold indicate that the sample was primarly from this part of the plant, which may include other parts as indicated. Abbreviations: rt = root, rb = root bark, rh = rhizome, st = stem of a herb, sb = stem bark, tw = twig, infl = inflorescence/infructescence, fl = flower, fr = fruit, ped = peduncle, immat = immature. IC_{50} values are given as the mean ± SD of three determinations. Pentamidine was used as the positive control, IC_{50} = 1.4 ± 0.2 µM.

Table 3.1 continued

12	*Chaetadelpha wheeleri* A. Gray ex S. Watson	rt	WBA-3625, SPJ-14344	159 ± 23
13	*Crepis occidentalis* Nutt.	rt	WBA-3623, SPJ-14342	>200
14	*Erigeron glaucus* Ker Gawl.	rt-rh-lf	WBA-3598, SPJ-14319	134 ± 12
15	*E. linearis* (Hook.) Piper	rt-st-lf-fl-fr	WBA-3622, SPJ-14341	196 ± 70
16	*Grindelia stricta* DC.	rt-st	WBA-3599, SPJ-14320	209 ± 52
17	*Hymenopappus filifolius* Hook. var. *megacephalus* B. Turner	st-lf-fl	WBA-3656, SPJ-14369	109 ± 26
18	*Petasites palmatus* (Aiton.) A. Gray	rt-rh	WBA-3560, SPJ-14286	27.7 ± 2.3
19	*P. palmatus* (Aiton.) A. Gray	lf-blade	WBA-3561, SPJ-14286	77.6 ± 12.7
20	*Tanacetum camphoratum* Less.	infl (fl)	WBA-3531, SPJ-14258	53.0 ± 1.5
	Betulaceae			
21	*Alnus rubra* Bong.	sb	WBA-3541, SPJ-14268	157 ± 34
22	*A. rhombifolia* Nutt.	sb	WBA-3579, SPJ-14302	>200
	Boraginaceae			
23	*Cryptantha confertiflora* (Greene) Payson	rt-lf	WBA-3665, SPJ-14378	>200
24	*C. confertiflora* (Greene) Payson	st-lf-fr	WBA-3666, SPJ-14378	>200
25	*C. confertiflora* (Greene) Payson	rt-lf	WBA-3669, SPJ-14381	>200
26	*C. confertiflora* (Greene) Payson	st-lf-fl	WBA-3670, SPJ-14381	>200
27	*C. micrantha* (Torr.) I. M. Johnst.	rt-st-lf-fl-fr	WBA-3634, SPJ-14351	>200
	Brassicaceae			
28	*Cakile maritima* Scop.	fr-fl	WBA-3600, SPJ-14321	119 ± 22
29	*Caulanthus crassicaulis* (Torr.) S. Watson	st- lf-fl	WBA-3649, SPJ-14363	>200
30	*C. crassicaulis* (Torr.) S. Watson	fl	WBA-3650, SPJ-14363	>200
31	*Rorippa nasturtium-aquaticum* L.	fl-lf-st	WBA-3558, SPJ-14284	>200

Continued

Table 3.1 continued

32	*Stanleya elata* M. E. Jones	rt	WBA-3504, SPJ-14239	>200
33	*S. elata* M. E. Jones	fl	WBA-3505, SPJ-14239	>200
34	*S. elata* M. E. Jones	fl-fr	WBA-3662, SPJ-14375	>200
35	*S. pinnata* (Pursh) Britton	fl	WBA-3503, SPJ-14238	>200
36	*S. pinnata* (Pursh) Britton	rt	WBA-3502, SPJ-14238	>200
37	*Thelypodium laciniatum* (Hook.) Endl.	st-lf-fl-fr	WBA-3645, SPJ-14359	>200
	Capparaceae			
38	*Cleome lutea* Hook.	rt-st-lf-fl-fr	WBA-3627, SPJ-14345	>200
	Caprifoliaceae			
39	*Lonicera involucrata* (Richardson in Franklin) Banks ex Spreng.	sb	WBA-3543, SPJ-14270	124 ± 32
40	*Sambucus mexicana* C. Presl. ex DC.	sb	WBA-3582, SPJ-14311	167 ± 3
41	*S. racemosa* L.	sb	WBA-3547, SPJ-14274	>200
42	*Symphoricarpos longiflorus* A. Gray	sb	WBA-3675, SPJ-14384	>200
43	*S. longiflorus* A. Gray	tw-lf	WBA-3676, SPJ-14384	>200
	Caryophyllaceae			
44	*Arenaria congesta* Nutt.	rt-st-lf-fl	WBA-3651, SPJ-14364	>200
	Chenopodiaceae			
45	*Atriplex confertifolia* (Torr. & Frém.) S. Watson	fr-tw-lf	WBA-3648, SPJ-14362	>200
46	*Grayia spinosa* (Hook.) Moq.	sb	WBA-3512, SPJ-14244	>200
47	*G. spinosa* (Hook.) Moq.	fr	WBA-3513, SPJ-14244	>200
48	*Krascheninnikovia lanata* (Pursh) A. Meeuse & A. Smit	fl-fr	WBA-3501, SPJ-14237	>200
49	*Sarcobatus baileyi* Coville	tw-lf-fl	WBA-3628, SPJ-14346	>200

Continued

Table 3.1 continued

	Convolvulaceae			
50	*Calystegia soldanella* (L.) Roem. & Schult.	rh-rt	WBA-3532A, SPJ-14259	43.9 ± 7.2
51	*C. soldanella* (L.) Roem. & Schult.	lf	WBA-3532B, SPJ-14259	171 ± 35
	Ephedraceae			
52	*Ephedra viridis* Coville	Pollen cones	WBA-3637, SPJ-14354	>200
	Equisetaceae			
53	*Equisetum telmateia* Ehrh. ssp. braunii (Milde) R. L. Hauke	upper st-lf	WBA-3554, SPJ-14280	>200
	Ericaceae			
54	*Arbutus menziesii* Pursh	sb	WBA-3562, SPJ-14287	>200
55	*Arctostaphylos manzanita* Parry	fl	WBA-3521, SPJ-14251	>200
56	*A. glandulosa* Eastw.	sb	WBA-3567, SPJ-14291	>200
57	*Gaultheria shallon* Pursh	infl-fr (immat)	WBA-3540, SPJ-14267	>200
58	*Rhododendron macrophyllum* D. Don ex G. Don	sb	WBA-3545, SPJ-14272	>200
59	*R. occidentale* (Torr. & A. Gray) A. Gray	sb	WBA-3573, SPJ-14297	>200
60	*Vaccinium ovatum* Pursh	sb	WBA-3535, SPJ-14262	>200
61	*V. parvifolium* Sm.	sb	WBA-3536, SPJ-14263	>200
	Escalloniaceae			
62	*Escallonia rubra* (Ruiz & Pav.) Pers. var. *macrantha* (Hook. & Arn.) Reiche	sb	WBA-3549, SPJ-14276	86.6 ± 16.7

Continued

Table 3.1 continued

	Euphorbiaceae			
63	*Tetracoccus dioicus* Parry	rt-rb	WBA-3465, S & M-14213	>200
64	*T. dioicus* Parry	tw-lf-fl	WBA-3467, S & M-14213	>200
	Fabaceae			
65	*Cercis occidentalis* Torr.	sb	WBA-3601- SPJ-14322	>200
66	*Lathyrus littoralis* (Nutt. ex Torr. & A. Gray) Endl. ex Walp.	rh-rt	WBA-3533A,SPJ-14260	>200
67	*L. polyphyllus* Nutt. ex Torr. & A. Gray	st-lf-fl	WBA-3583, SPJ-14305	>200
68	*Lotus aboriginus* Jepson	rt	WBA-3590, SPJ-14313	>200
69	*L. aboriginus* Jepson	lf-fl	WBA-3591, SPJ-14313	>200
70	*L. salsuginosus* Greene var. *brevivexillus* Ottley	rt-st-lf- fl-fr	WBA-3509, SPJ-14242	>200
71	*Mucuna pruriens* (L.) DC.	–	–	>200
72	*Psorothamnus arborescens* (Torr. in A. Gray) Barneby var. *minutifolius* (Parish) Barneby	fr-tw-lf	WBA-3647, SPJ-14361	11.7 ± 1.1
73	*P. polydenius* (Torr. ex S. Wats.) Rydb.	tw-lf-fl	WBA-3624, SPJ-14343	45.2 ± 15.4
	Fagaceae			
74	*Castanopsis chrysophylla* (Douglas ex Hook.) A. DC.	sb	WBA-3592, SPJ-14314	>200
75	*Lithocarpus densiflorus* (Hook. & Arn.) Rehder	lf	WBA-3594, SPJ-14316	>200
76	*L. densiflorus* (Hook. & Arn.) Rehder	sb	WBA-3564, SPJ-14289	>200
77	*Quercus agrifolia* Née	sb	WBA-3569, SPJ-14293	>200
78	*Q. chrysolepis* Liebm.	sb	WBA-3568, SPJ-14292	>200
79	*Q. kelloggii* Newb.	sb	WBA-3563, SPJ-14288	>200
80	*Q. vaccinifolia* Kellogg ex Curran	lf	WBA-3577, SPJ-14300	>200

Continued

Table 3.1 continued

	Garryaceae			
81	*Garrya elliptica* Douglas ex Lindl.	sb	WBA-3548, SPJ-14275	>200
82	*G. fremontii* Torr.	sb	WBA-3678, SPJ-14386	>200
83	*G. fremontii* Torr.	tw-lf	WBA-3679, SPJ-14386	>200
	Grossulariaceae			
84	*Ribes cereum* Douglas	sb	WBA-3642, SPJ-14357	>200
85	*R. cereum* Douglas	sb	WBA-3673, SPJ-14383	130 ± 18
86	*R. cereum* Douglas	tw-lf	WBA-3674, SPJ-14383	89.7 ± 23.1
87	*R. cereum* Douglas	sb	WBA-3522, SPJ-14252	>200
88	*R. cereum* Douglas	tw-lf-fl	WBA-3643, SPJ-14357	>200
89	*R. indecorum* Eastw.	rb	WBA-3449, S & M-14204	>200
90	*R. indecorum* Eastw.	sb	WBA-3450, S & M-14204	>200
	Hippocastanaceae			
91	*Aesculus californica* (Spach.) Nutt.	sb	WBA-3602, SPJ-14323	>200
92	*A. californica* (Spach.) Nutt.	fl	WBA-3605, SPJ-14323	>200
	Hydrophyllaceae			
93	*Draperia systyla* Torr. ex A. Gray	rt-st-lf-fl	WBA-3585, SPJ-14307	69.8 ± 21.5
94	*Eriodictyon angustifolium* Nutt.	rt	WBA-3525, SPJ-14233	52.5 ± 2.2
95	*E. angustifolium* Nutt.	lf-fl	WBA-3526, SPJ-14233	60.8 ± 23.5
96	*E. californicum* (Hook. & Arn.) Torr.	lf-fl	WBA-3593, SPJ-14315	79.5 ± 16.3
97	*E. crassifolium* Benth.	rb	WBA-3452, S & M-14206	>200
98	*E. crassifolium* Benth.	sb	WBA-3453, S & M-14206	>200
99	*Nama aretioides* (Hook. & Arn.) Brand	rt-st-lf-fl-fr	WBA-3508, SPJ-14241	>200

Continued

Table 3.1 continued

	Iridaceae			
100	*Iris douglasiana* Herbert	rt-st-lf	WBA-3537, SPJ-14264	>200
	Lamiaceae			
101	*Marrubium vulgare* L.	lf-fl-fr	WBA-3633, SPJ-14350	64.9 ± 21.2
102	*Salvia mellifera* Greene	sb	WBA-3456, S & M-14208	>200
103	*Stachys chamissonis* Benth.	infl (fl-fr)	WBA-3555, SPJ-14281	>200
	Lauraceae			
104	*Umbellularia californica* (Hook. & Arn.) Nutt.	sb	WBA-3565, SPJ-14290	>200
105	*U. californica* (Hook. & Arn.) Nutt.	lf	WBA-3566, SPJ-14290	>200
	Liliaceae			
106	*Chlorogalum pomeridianum* (DC.) Kunth	lf	WBA-3520, SPJ-14250	>200
107	*Disporum smithii* (Hook.) Piper	lf-st	WBA-3571, SPJ-14295	>200
108	*Leucocrinum montanum* Nutt. ex A. Gray	rt-st-lf-fl	WBA-3517, SPJ-14248	>200
109	*Maianthemum dilatatum* (Alph. Wood) A. Nelson & J. F. Macbr.	fr (immat)	WBA-3546, SPJ-14273	>200
110	*Triteleia hyacinthina* (Lindl.) Greene	fl-st	WBA-3620, SPJ-14339	>200
	Loasaceae			
111	*Petalonyx thurberi* A. Gray	infl (st-lf-fl)	WBA-3667, SPJ-14379	>200
	Malvaceae			
112	*Sphaeralcea ambigua* A. Gray	fr	WBA-3663, SPJ-14376	>200

Continued

Table 3.1 continued

	Myricaceae			
113	*Myrica californica* Cham. & Schltdl.	sb	WBA-3544A, SPJ-14271	>200
114	*M. californica* Cham. & Schltdl.	lf-fl	WBA-3544B, SPJ-14271	>200
	Myrtaceae			
115	*Chamelaucium uncinatum* Schauer	rb	WBA-3462, S & M-14212	>200
116	*C. uncinatum* Schauer	sb	WBA-3463, S & M-14212	>200
117	*C. uncinatum* Schauer	tw-lf-fl	WBA-3464, S & M-14212	19.4 ± 6.6
	Oleaceae			
118	*Menodora spinescens* A. Gray	rt	WBA-3506, SPJ-14240	>200
119	*M. spinescens* A. Gray	st	WBA-3507, SPJ-14240	>200
	Onagraceae			
120	*Fuchsia magellanica* Lam.	fl	WBA-3570, SPJ-14294	>200
121	*Jussiaea leptocarpa* Nutt.	rt-st-lf-fl	WBA-2999, PG-96-12	>200
122	*Ludwigia sphaerocarpa* Elliot		JB-2-139	43.8 ± 6.9
	Orchidaceae			
123	*Platanthera leucostachys* Lindl.	rt-st-lf-fl	WBA-3604, SPJ-14324	>200
	Polemoniaceae			
124	*Ipomopsis congesta* (Hook.) V. E. Grant	rt-st-lf-fl	WBA-3661, SPJ-14374	>200
125	*Leptodactylon pungens* (Torr.) Nutt.	rt	WBA-3640, SPJ-14356A	>200
126	*Phlox diffusa* Benth.	rt-st-lf-fl	WBA-3515, SPJ-14246	>200
127	*P. stansburyi* (Torr.) A. Heller	rt-st-lf-fl-fr	WBA-3660, SPJ-14373	118 ± 3

Continued

Table 3.1 continued

	Polygonaceae			
128	*Eriogonum heermannii* Durand & Hilg.	st-lf-fr	WBA-3668, SPJ-14380	>200
129	*E. inflatum* Torr. & Frém.	rt-st-lf-fl-fr	WBA-3500, SPJ-14236	>200
130	*E. nidularium* Coville.	rt-st-lf-fl-fr	WBA-3630, SPJ-14348	>200
131	*E. umbellatum* Torr. var. *nevadense* Gand.	st-lf-fl	WBA-3618, SPJ-14337	>200
	Pyrolaceae			
132	*Sarcodes sanguinea* Torr.	st-fl	WBA-3613, SPJ-14334	>200
	Ranunculaceae			
133	*Delphinium nudicaule* Torr. & A. Gray	st-lf-fl	WBA-3587, SPJ-14309	>200
	Rhamnaceae			
134	*Ceanothus cordulatus* Kellogg	tw-lf-fl	WBA-3677, SPJ-14385	>200
135	*C. crassifolius* Torr.	rb	WBA-3455, S & M-14207	>200
136	*C. crassifolius* Torr.	sb	WBA-3454, S & M-14207	>200
137	*C. integerrimus* Hook. & Arn.	sb	WBA-3527, SPJ-14254	>200
138	*C. thyrsiflorus* Eschsch.	sb	WBA-3534, SPJ-14261	>200
139	*C. tomentosus* Parry	sb	WBA-3446, S & M-14202	>200
140	*C. tomentosus* Parry	rb	WBA-3445, S & M-14202	>200
141	*C. velutinus* Douglas ex Hook.	sb	WBA-3629, SPJ-14256	>200
142	*C. velutinus* Douglas ex Hook.	fl-lf	WBA-3595, SPJ-14317	>200
143	*Rhamnus californica* Eschsch.	sb	WBA-3457, S & M-14209	114 ± 8
144	*R. tomentella* Benth.	sb	WBA-3576, SPJ-14299	>200

Continued

Table 3.1 continued

	Rosaceae			
145	*Adenostoma fasciculatum* Hook. & Arn.	rt	WBA-3447, S & M-14203	>200
146	*A. fasciculatum* Hook. & Arn.	sb	WBA-3448, S & M-14203	>200
147	*Amelanchier utahensis* (A. Chev.) Koehne	sb	WBA-3615, SPJ-14335	>200
148	*Chamaebatia foliolosa* Benth.	sb	WBA-3671, SPJ-14382	>200
149	*C. foliolosa* Benth.	tw-lf	WBA-3672, SPJ-14382	>200
150	*Cercocarpus betuloides* Torr. & A. Gray	rb	WBA-3443, S & M-14201	>200
151	*C. betuloides* Torr. & A. Gray	sb	WBA-3444, S & M-14201	>200
152	*C. ledifolius* Nutt.	sb	WBA-3616, SPJ-14336	>200
153	*Heteromeles arbutifolia* (Lindl.) M. Roem.	sb	WBA-3451, S & M-14205	>200
154	*H. arbutifolia* (Lindl.) M. Roem.	lf-fl (bud)	WBA-3578, SPJ-14301	>200
155	*Holodiscus discolor* (Pursh) Maxim.	sb	WBA-3581, SPJ-14304	>200
156	*Horkelia fusca* Lindl. ssp. *parviflora* (Nutt.) D. D. Keck	rt-st-lf-fl	WBA-3516, SPJ-14247	>200
157	*Purshia tridentata* (Pursh) DC.	sb	WBA-3510, SPJ-14243	117 ± 19
158	*P. tridentata* (Pursh) DC.	lf-fr	WBA-3511, SPJ-14243	>200
159	*Rubus spectabilis* Pursh	sb	WBA-3539, SPJ-14266	>200
	Rutaceae			
160	*Ptelea crenulata* Greene	sb	WBA-3518, SPJ-14294	155 ± 50
161	*P. crenulata* Greene	lf-fr	WBA-3519, SPJ-14294	218 ± 32
	Salicaceae			
162	*Salix hookeriana* Barratt ex Hook.	sb	WBA-3552, SPJ-14278	>200
163	*S. sitchensis* Sanson ex Bong.	sb	WBA-3528, SPJ-14255	>200

Continued

Table 3.1 continued

	Scrophulariaceae			
164	*Collinsia rattani* A. Gray	rt-st-lf-fl	WBA-3584, SPJ-14306	169 ± 25
165	*Mimulus bigelovii* A. Gray	rt-lf	WBA-3664, SPJ-14377	67.3 ± 13.7
166	*M. guttatus* Fisch. ex DC.	fl-st-fr	WBA-3556, SPJ-14282	>200
167	*Orthocarpus hispidus* Benth.	rt-st-lf-fl	WBA-3619, SPJ-14338	210 ± 33
168	*Penstemon deustus* Douglas ex Lindl.var. *suffrutescens* L.F.Henderson	rt	WBA-3523, SPJ-14253	>200
169	*P. deustus* Douglas ex Lindl.var. *suffrutescens* L.F.Henderson	lf-fl	WBA-3524, SPJ-14253	>200
170	*P. newberryi* A. Gray	rt-st-lf-fl	WBA-3680, SPJ-14387	>200
171	*P. scapoides* D. D. Keck	rt-st-lf-fl	WBA-3655, SPJ-14368	>200
172	*Scrophularia californica* Cham. & Schltdl.	infl (fl-fr)	WBA-3553, SPJ-14279	>200
	Solanaceae			
173	*Solanum aviculare* G. Forst.	sb	WBA-3538, SPJ-14265	>200
	Vaerianaceae			
174	*Valeriana sitchensis* Bong.	st-lf-fl	WBA-3586, SPJ-14308	218 ± 30

Plant and Taxonomic Family	Plant Part[a]	IC_{50} (µg/mL)			
		L. donovani[b]	*T. b. brucei*[c]	J774[d]	PC-3[d]
Apiaceae					
Glehnia littoralis	rt-lf	148 ± 24	>100	82.9	>200
G. littoralis	fr	112 ± 3	12.1 ± 1.1	25.8	>200
Heracleum lanatum	fr-ped	62.5 ± 7.6	33.3 ± 3.7	50–100	50–100
Araliaceae					
Aralia californica	rt	24.5 ± 2.0	21.2 ± 3.9	10.6	71.8
Asteraceae					
Brickellia grandiflora	**lf-fl-**st	38.3 ± 2.2	29.7 ± 4.1	54.1	29.4
Chaetadelpha wheeleri	rt	159 ± 23	5.67 ± 0.26	71.9	83.4
Erigeron glaucus	**rt-rh-**lf	134 ± 12	28.4 ± 0.7	>100	52.9
E. linearis	rt-st-lf-fl-fr	196 ± 70	23.3 ± 1.5	>200	>200
Grindelia stricta	rt-st	209 ± 52	>100	>100	>200
Hymenopappus filifolius var. *megacephalus*	st-lf-fl	109 ± 26	50–100	>100	50–100
Petasites palmatus	rt-rh	27.7 ± 2.3	3.04 ± 0.70	~ 50	41.0
P. palmatus	lf-blade	77.6 ± 12.7	12.7 ± 6.0	6.25	>100
Tanacetum camphoratum	infl (fl)	53.0 ± 1.5	23.7 ± 10.0	>100	~50
Betulaceae					
Alnus rubra	sb	157 ± 34	3.6 ± 1.4	>100	37.2
Brassicaceae					
Cakile maritima	fr-fl	119 ± 22	38.9 ± 15.3	>100	>100
Caprifoliaceae					
Lonicera involucrata	sb	124 ± 32	19.0 ± 0.9	74.7	>100
Sambucus mexicana	sb	167 ± 3	50–100	40.2	>200
Convolvulaceae					
Calystegia soldanella	rh-rt	43.9 ± 7.2	7.4 ± 0.5	22.7	26.8
C. soldanella	lf	171 ± 35	13.8 ± 0.7	50–100	55.1
Escalloniaceae					
Escallonia rubra var. *macrantha*	sb	86.6 ± 16.7	58.9 ± 3.4	>100	>200

Continued

Table 3.2: Growth Inhibitory Activities of Selected Active North American Plant Samples against *L. donovani*, *T. b. brucei*, and Mammalian Cell Lines
[a] Abbreviations of plants parts are the same as in Table 3.1. [b] IC_{50} values against *L. donovani* amastigotes are given as the mean ± SD of at least three determinations (pentamidine was used as the positive control, IC_{50} = 1.4 ± 0.2 µM). [c] IC_{50} values against *T. b. brucei* are given as the mean ± SD or as a range of at least three determinations (suramin was used as the positive control, IC_{50} = 0.21 ± 0.07 µM). [d] IC_{50} values against J774 macrophages and PC-3 prostate cells are given as the result of one determination. [e] ND, not determined.

Table 3.2 continued

Fabaceae					
Psorothamnus arborescens var. *minutifolius*	**fr**-tw-lf	11.7 ± 1.1	8.3 ± 2.3	>100	50–100
P. polydenius	tw-lf-fl	45.2 ± 15.4	12.3 ± 11.6	>100	>100
Grossulariaceae					
Ribes cereum (WBA- 3673)	sb	130 ± 18	50–100	39.4	25–50
R. cereum (WBA- 3674)	tw-lf	89.7 ± 23.1	50–100	>100	50–100
Hydrophyllaceae					
Draperia systyla	rt-st-lf-fl	69.8 ± 21.5	>200	>100	>100
Eriodictyon angustifolium	rt	52.5 ± 2.2	20.4 ± 3.1	>100	38.3
E. angustifolium	lf-fl	60.8 ± 23.5	12.6 ± 0.2	>100	87.1
E. californicum	lf-fl	79.5 ± 16.3	113 ± 12	>100	>100
Lamiaceae					
Marrubium vulgare	lf-fl-fr	64.9 ± 21.2	42.3 ± 9.9	~ 100	108.2
Myrtaceae					
Chamelaucium uncinatum	tw-lf-fl	19.4 ± 6.6	>200	>200	>100
Onagraceae					
Ludwigia sphaerocarpa	–	43.8 ± 6.9	11.5 ± 3.5	69.5	22.8
Polemoniaceae					
Phlox stansburyi	rt-st-lf-fl-fr	118 ± 3	90.5 ± 15.0	>100	>100
Rhamnaceae					
Rhamnus californica	sb	114 ± 8	>200	>100	>100
Rosaceae					
Purshia tridentata	sb	117 ± 19	50–100	37.9	ND[e]
Rutaceae					
Ptelea crenulata	sb	155 ± 50	55.6 ± 28.7	52.9	>100
P. crenulata	lf-fr	218 ± 32	>100	43.2	>200
Scrophulariaceae					
Collinsia rattani	rt-st-lf-fl	169 ± 25	>100	>200	>200
Mimulus bigelovii	rt-lf	67.3 ± 13.7	>100	>100	>100
Orthocarpus hispidus	rt-st-lf-fl	210 ± 33	>100	>200	>200
Vaerianaceae					
Valeriana sitchensis	st-lf-fl	218 ± 30	58.3 ± 14.4	>100	ND

#	Sample	Code	Family	IC_{50} (µg/mL) *L. donovani*)
1	*Allium mongolicum* Regel	498	Alliaceae	>100
2	*Heracleum dissectum* Ledeb.	289	Apiaceae	51.7 ± 1.8
3	*Arctium leiospermum* Juz. et Serg.	458	Asteraceae	>100
4	*Echinops gmelini* Turcz.	466	Asteraceae	>100
5	*Erigeron altaicus* Popov	469	Asteraceae	>100
6	*Karelinia caspia* (Pall.) Less.	476	Asteraceae	>100
7	*Leontopodium leontopodioides* (Willd.) Beauv.	477	Asteraceae	>100
8	*Saussurea alpina* DC.	481	Asteraceae	>200
9	*S. involucrata* (Karel. & Kir.) Sch.Bip.	483	Asteraceae	41.7 ± 0.7
10	*Scorzonera divaricata* Turcz	485	Asteraceae	100–200
11	*Berberis heteropoda* Schrenk.	136	Berberidaceae	>100
12	*Lappula consanguinea* (Fisch. et Mey) Guerke.	330	Boraginaceae	>100
13	*Lycopsis orientalis* L.	336	Boraginaceae	>100
14	*Trigonotis peduncularis* Benth. ex S.Moore & Baker	338	Boraginaceae	>200
15	*Descurainia sophia* (L.) Webb. ex Prantl.	154	Brassicaceae	>100
16	*Capparis spinosa* L.	153	Capparaceae	>100
17	*Lonicera tatarica* L.	451	Caprifoliaceae	>100
18	*Acanthophyllum pungens* (Bunge.) Boiss.	076	Caryophyllaceae	>100
19	*Arenaria serpyllifolia* L.	077	Caryophyllaceae	100–200
20	*Dianthus chinensis* L.	078	Caryophyllaceae	>100
21	*D. soongoricus* Schischk.	081	Caryophyllaceae	>100
22	*Silene gavrilovii* (Krasnov) Popov	083	Caryophyllaceae	100–200
23	*Stellaria brachypetala* Bunge	087	Caryophyllaceae	>100
24	*Agriophyllum arenarium* Bieb.	037	Chenopodiaceae	>100
25	*Chenopodium glaucum* L.	049	Chenopodiaceae	>100
26	*Sympegma regelii* Bunge	075	Chenopodiaceae	>100
27	*Convolvulus arvensis* L.	322	Convolvulaceae	>100
28	*Rhodiola algida* (Ledeb.) Fisch. et C. A. Mey.	162	Crassulaceae	>200
29	*Hippophae rhamnoides* L.	258	Elaeagnaceae	>100
30	*Ephedra intermedia* Schrenk. ex Mey.	001	Euphorbiaceae	>100
31	*Euphorbia soongarica* Boiss.	238	Euphorbiaceae	>100

Continued

Table 3.3: Antileishmanial Activity of Plant Samples from Yuhai Phytochemical Products, Ltd., Guangzhou, People's Republic of China
IC_{50} values against *L. donovani* amastigotes are given as the mean ± SD of three determinations. Pentamidine was used as the positive control, IC_{50} = 1.4 ± 0.2 µM.

Table 3.3 continued

32	*Alhagi pseudalhagi* Desv.	197	Fabaceae	>100
33	*Ammodendron argenteum* (Pall.) Lipskiy.	198	Fabaceae	>100
34	*Astragalus aksuensis* Bunge.	199	Fabaceae	100–200
35	*Caragana camilli-schneideri* Kom.	205	Fabaceae	>100
36	*Hedysarum semenowi*Regel. et Herd.	210	Fabaceae	>100
37	*Oxytropis falcata* Bunge.	212	Fabaceae	>100
38	*Gentiana tianschanica* Rupr.	315	Gentianaceae	100–200
39	*Gentianopsis barbata* (Froel.) Ma.	317	Gentianaceae	>100
40	*Achnatherum inebrians* (Hance.) Keng.	490	Gramineae	>100
41	*Stipa breviflora* Griseb.	494	Gramineae	>200
42	*Hypericum hirsutum* L.	244	Guttiferae	73.5 ± 19.2
43	*Iris loczyi* Kanitz	509	Iridaceae	>100
44	*Iris sogdiana* Bunge	510	Iridaceae	>100
45	*Agastache rugosa* (Fisch. et Mey) Kuntze	330	Labiatae	>100
46	*Leonurus glaucescens* Bunge.	366	Labiatae	>100
47	*Marrubium vulgare* L.	370	Labiatae	62.3 ± 15.7
48	*Origanum vulgare* L.	382	Labiatae	>100
49	*Thymus asiaticus* Kitag.	396	Labiatae	81.1 ± 22.3
50	*Fritillaria pallidiflora* Schrenk.	505	Liliaceae	>100
51	*Malva verticillata* L.	243	Malvaceae	>100
52	*Paeonia anomala* L.	120	Paeoniaceae	>100
53	*P. sinjiangensis* K. Y. Pan	121	Paeoniaceae	>200
54	*Papaver tianschanicum* Popov	152	Papaveraceae	>200
55	*Polemonium caeruleum* L.	325	Polemoniaceae	>200
56	*Calligonum caput-medusae* Schrenk.	012	Polygonaceae	>100
57	*Oxyria digyna* (L.) Hill.	017	Polygonaceae	>100
58	*Polygonum songaricum* Schrenk.	024	Polygonaceae	>200
59	*Rheum wittrockii* Lundstrom	031	Polygonaceae	>200
60	*Glaux maritima* L.	298	Primulaceae	>100
61	*Aconitum sibiricum* Poir.	097	Ranunculaceae	100–200
62	*Anemone silvestris* L.	103	Ranunculaceae	>100
63	*Delphinium grandiflorum* L.	114	Ranunculaceae	>100
64	*D. tianshanicum* W. T. Wang	117	Ranunculaceae	>100
65	*Halerpestes ruthenica* (Jacq.) Ovczinn.	118	Ranunculaceae	>100
66	*Pulsatilla turczaninovii* Krylov & Sergievsk.	123	Ranunculaceae	>200
67	*Thalictrum alpinum* L.	127	Ranunculaceae	>100
68	*Trollius altaicus* C. A. Mey.	133	Ranunculaceae	72.1 ± 25.7

Continued

Table 3.3 continued

69	*Cotoneaster megalocarpa* Popov	178	Rosaceae	>100
70	*Cotoneaster* songorica (Regel. et Herd.) Popov	180	Rosaceae	>100
71	*Cotoneaster uniflora* Bunge.	181	Rosaceae	>100
72	*Crataegus songorica* K. Koch.	182	Rosaceae	>100
73	*Dasiphora parvifolia* Fisch.	183	Rosaceae	>100
74	*Fragaria vesca* L.	184	Rosaceae	>100
75	*Rubus* idaeus L.	190	Rosaceae	100–200
76	*Sorbus tianschanica* Rupr.	192	Rosaceae	>100
77	*Spiraea tianschanica* Pojark.	195	Rosaceae	>100
78	*Galium songaricum* Schrenk.	445	Rubiaceae	>100
79	*Rubia deserticola* Pojark.	448	Rubiaceae	>200
80	*Peganum harmala* L.	228	Rutaceae	>200
81	*Cistanche chinensis* (= *C. deserticola* Y. C. Ma)	437	Scrophulariaceae	>100
82	*Linaria longicalcarata* D.Y.Hong	411	Scrophulariaceae	>100
83	*L. vulgaris* Mill.	413	Scrophulariaceae	100–200
84	*Pedicularis soongarica* Schrenk	419	Scrophulariaceae	>200
85	*Scrophularia heucheriaeflora* Schrenk	423	Scrophulariaceae	>200
86	*Reaumuria kaschgarica* Rupr.	246	Tamaricaceae	>200
87	*Stelleropsis tianschanica* Pobed.	256	Thymelaeaceae	>100
88	*Aegopodium alpestre* Ledeb.	262	Umbelliferae	>100
89	*Angelica sinensis* (Oliv.) Diels	264	Umbelliferae	>100
90	*Archangelica brevicaulis* (Rupr.) Rchb.	266	Umbelliferae	>100
91	*A. decurrens* Ledeb.	267	Umbelliferae	>100
92	*Bupleurum aureum* Fisch.	269	Umbelliferae	>200
93	*B. exaltatum* Marsch. Bieb.	272	Umbelliferae	>100
94	*Peucedanum morisoni* Bess. ex Schult	291	Umbelliferae	52.7 ± 15.2
95	*Sium medium* Fisch. & Mey.	293	Umbelliferae	>100
96	*Urtica angustifolia* Fisch. ex Hornem.	004	Urticaceae	>200
97	*Valeriana officinalis* L.	455	Valerianaceae	>200
98	*Nitraria sibirica* Pall.	227	Zygophyllaceae	>100
99	*Zygophyllum xanthoxylum* (Bunge.) Maxim.	234	Zygophyllaceae	>200

#	Sample	Code	Family	IC_{50} µg/mL (*L. donovani*)
1	*Asparagus gobicus*	501	Asparagaceae	>200
2	*Acroptilon repens*	457	Asteraceae	7.91
3	*Artemisia annua*	459	Asteraceae	50–100
4	*Carduus crispus*	465	Asteraceae	50–100
5	*Heteropappus altaicus*	470	Asteraceae	100–200
6	*Senecio dubius*	486	Asteraceae	100–200
7	*Lepidium apetalum*	158	Brassicaceae	>200
8	*Codonopsis clematidea*	456	Campanulaceae	>200
9	*Atriplex cana*	042	Chenopodiaceae	>200
10	*Halogeton arachnoides*	053	Chenopodiaceae	>200
11	*Kalidium caspicum*	058	Chenopodiaceae	>200
12	*Kochia sieversiana*	064	Chenopodiaceae	>200
13	*Salsola abrotanoides*	066	Chenopodiaceae	>200
14	*Suaeda glauca*	072	Chenopodiaceae	>200
15	*Sedum aizoon*	172	Crassulaceae	>200
16	*Eremosparton songoricum*	207	Fabaceae	>200
17	*Hedysarum multijugum*	209	Fabaceae	>200
18	*Vicia cracca*	217	Fabaceae	>200
19	*Gentiana algida*	308	Gentianaceae	>200
20	*Swertia marginata*	319	Gentianaceae	100–200
21	*Erodium hoefftianum*	221	Geraniaceae	>200
22	*Geranium saxatile*	224	Geraniaceae	>200
23	*Iris songarica*	511	Iridaceae	>200
24	*Dracocephalum heterophyllum*	344	Labiatae	>200
25	*Elsholtzia densa*	351	Labiatae	>200
26	*Hyssopus cuspidatus*	355	Labiatae	>200
27	*Ocimum basilicum*	380	Labiatae	>200
28	*Scutellaria alberti*	391	Labiatae	107
29	*Althaea officinalis*	240	Malvaceae	>200
30	*Glaucium fimbrilligerum*	147	Papaveraceae	>200
31	*Papaver croceum*	149	Papaveraceae	>200
32	*Plantago depressa*	440	Plantaginaceae	>200
33	*Acantholimon tianschanicum*	301	Plumbaginaceae	>200
34	*Limonium aureum*	302	Plumbaginaceae	>200
35	*Polygonum amphibium*	018	Polygonaceae	>200

Continued

Table 3.4: Antileishmanial Activity of Plant Samples from Hongxing Health Food Co, Ltd., Guangzhou, People's Republic of China
IC_{50} values against *L. donovani* amastigotes are given as the result of one or two determinations. Pentamidine was used as the positive control, $IC_{50} = 1.4 \pm 0.2$ µM.

Table 3.4 continued

36	*Adonis tianschanicus*	102	Ranunculaceae	>200
37	*Clematis glauca*	107	Ranunculaceae	>200
38	*C. sibirica*	110	Ranunculaceae	>200
39	*C. tianschanica*	113	-	>200
40	*Thalictrum collinum*	128	Ranunculaceae	>200
41	*Alchemilla vulgaris*	177	Rosaceae	>200
42	*Potentilla biflora*	185	Rosaceae	>200
43	*Rosa spinosissima*	189	Rosaceae	>200
44	*Sanguisorba officinalis*	191	Rosaceae	>200
45	*Pedicularis resupinata*	417	Scrophulariaceae	>200
46	*Verbascum blattaria*	427	Scrophulariaceae	>200
47	*Myricaria elegans*	245	Tamaricaceae	>200
48	*Berula erecta*	268	Umbelliferae	100–200
49	*Patrinia sibirica*	453	Valerianaceae	>200
50	*Patrinia intermedia*	452	Valerianaceae	>200

Sample	**Family**	***L. donovani*[a]**	**J774[b]**	**PC-3[b]**
Heracleum dissectum	Apiaceae	50.7 ± 1.8	94.0	96.2
Acroptilon repens	Asteraceae	7.91	13.7	6–12
Artemisia annua	Asteraceae	50–100	50–100	25–50
Carduus crispus	Asteraceae	50–100	50–100	43.1
Saussurea involucrata	Asteraceae	41.7 ± 0.7	61.7	31.2
Hypericum hirsutum	Guttiferae	73.5 ± 19.2	64.4	51.8
Marrubium vulgare	Labiatae	62.3 ± 15.7	50–100	60.9
Thymus asiaticus Kitag.	Labiatae	81.1 ± 22.3	50–100	71.4
Trollius altaicus	Ranunculaceae	72.1 ± 25.7	60.9	52.2
Peucedanum morisoni	Umbelliferae	52.7 ± 15.2	50–100	100–200

Table 3.5: IC_{50} values of Selected Active Chinese Plant Samples against *L. donovani* and Mammalian Cell Lines

[a] IC_{50} values against *L. donovani* amastigotes are given as the mean ± SD of three determinations except for *A. repens* (1 determination) and *A. annua* and *C. crispus* (2 determinations). Pentamidine was used as the positive control, IC_{50} = 1.4 ± 0.2 μM. [b] IC_{50} values against J774 macrophages and PC-3 prostate cells are given as the result of one determination.

CHAPTER 4

ANTIPROTOZOAL COMPOUNDS FROM *PSOROTHAMNUS POLYDENIUS*

4.1 INTRODUCTION

The genus *Psorothamnus,* family Fabaceae, consists of nine North American species confined to the Sonoran, Chihuahuan, and Mojave deserts and deserts of the Colorado Plateau and the Great Basin. One member, *Psorothamnus polydenius* (S. Watson) Rydb.,[188] also known as *Dalea polyadenia* or the smoke bush, is a fragrant desert shrub characterized by numerous tiny orange glands scattered over the stems that give the plant its distinguishing citrus-like odor.[189] Other common names for this species include Dotted Dalea, Nevada Dalea, and Nevada Indigobush. *P. polydenius* was used by the desert Native Americans for dyeing deer skins and baskets. More importantly, they used it to treat numerous ailments ranging from colds and coughs to influenza, pneumonia, tuberculosis, smallpox and kidney problems. *P. polydenius* has thus been described as "the medicinal cure-all of the desert tribes".[163,164] One previous phytochemical investigation of *P. polydenius* resulted in the isolation of the red dye dalrubone and demethoxymatteucinol.[179] However, there is no previous report of any antiprotozoal activity associated with this plant.

In our preliminary screening of 323 American and Chinese plants for antikinetoplastid properties (as described in Chapter 3), the ethanolic extract of *P. polydenius* exhibited significant activity and selectivity for the parasites compared to mammalian cells. Therefore, we decided to investigate the active constituents in hope of finding new antiprotozoal compounds. Herein, we report on the isolation and characterization of compounds **4.1–4.7** and their activity against *Leishmania* and *Trypanosoma brucei*.

4.2 EXPERIMENTAL SECTION

4.2.1 General Experimental Procedures

Melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer 241 polarimeter using a 100 mm glass microcell. UV-vis spectra were taken in methanol using a SPECTRAmax PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA). IR spectra were obtained in KBr on a Nicolet Protégé 460 FT-IR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker 250, 300, and 600 MHz spectrometers using the solvents $CDCl_3$, methanol-d_4, or acetone-d_6 (Sigma) with TMS as the internal standard. ^1H-^1H COSY, HSQC, and HMBC NMR spectra were obtained using standard Bruker pulse sequences. All accurate mass experiments were performed on a Micromass ESI-Tof™ II (Micromass, Wythenshawe, UK) mass spectrometer. Column chromatography was conducted using silica gel 60 (63-200 μm particle size) from EM Science or Sephadex LH-20 from Amersham Biosciences. Silica

gel 60 H (15-45 μm particle size) from EM Science was used for vacuum-liquid chromatography (VLC). Precoated TLC silica gel 60 F_{254} plates from EM Science were used for thin-layer chromatography (0.25 mm and 2 mm layer thickness for analytical and preparative TLC, respectively). Spots were visualized using either anisaldehyde/sulfuric acid reagent or Natural Products reagent.[190] HPLC runs were carried out using a System Gold model 127 pump equipped with a model 166 UV detector (Beckman) and 4.6 × 250 mm or 10 × 250 mm C18-A Polaris columns (Varian) for analytical or semi-preparative runs, respectively.

4.2.2 Plant Material

Psorothamnus polydenius (Fabaceae) was initially collected from Washoe Co., Nevada, Highway 50 south of Highway 395 between Fenley and Benton Springs at an elevation of approximately 5,000 ft. in July 1998 by Dr. Richard W. Spjut (Spjut 14343, WBA 3643), World Botanical Associates (WBA, Bakersfield, CA). A second collection was made near the same location in September 2002 (Spjut 14964, WBA-4401-11). A third collection of the plant was made from Inyo Co., California, Owens Valley near Lone Pine just off Highway 193 at an elevation of ~3600 ft. in June 2003 (Spjut 15358, WBA-4842-22); see Figure 4.1. Voucher specimens of all collections were deposited at the U.S. National Herbarium (US), Smithsonian Institution, Washington DC. Additional vouchers for WBA-4401-11 and WBA-4842-22 were deposited at the herbaria of the Botanical Research Institute of Texas (BRIT) and the WBA (Bakersfield, CA).

Figure 4.1: Photos of *Psorothamnus polydenius*

A. A shrub growing in Inyo County, California. The photo was taken by Larry Blakely in 2003 and permission for use was granted.[191]
B. Voucher for *P. polydenius* collected June 12th, 2003 (Spjut 15358). The photo was obtained from the WBA (www.worldbotanical.com) and permission for use was granted.

4.2.3 Extraction and Isolation

The dried and powdered twigs with leaves and flowers (6.4 kg) were extracted with 95% EtOH (see Figure 4.2 for the general solvent extraction and fractionation scheme). A portion (446 g) of the dried extract was suspended in water and extracted with CH_2Cl_2. The dried CH_2Cl_2 fraction (223.6 g) was suspended in 90% MeOH and extracted with hexane to give a 90 % MeOH fraction (F001, 169.2 g) and a hexane fraction (F002, 59.0 g). The water layer was further extracted with EtOAc to give, after drying, an EtOAc fraction (F003, 6.8 g), a water fraction (F004, 194.3 g), and an insoluble fraction (F005, 10.7 g). The antileishmanial activities of F001, F002, F003, F004, and F005 in the axenic amastigote assay were 24, 45, > 200, > 200, and > 200 μg/mL, respectively. F001 was further investigated on the basis of the high bioactivity. A portion of F001 (46.0 g) was subjected to silica gel (440 g) column chromatography eluted with a gradient mixture of $CHCl_3$−MeOH (1:0 → 0:1, 1 L per fraction) to give nine fractions, A−I. Demethoxymatteucinol (**4.4**; 970 mg) was crystallized directly from fraction A in EtOAc−hexane. Crude dalrubone (**4.3**) in fraction B (2.5 g, eluted with $CHCl_3$) was purified over a silica gel (71 g) column using EtOAc−hexane as the solvent system. Purified dalrubone resisted crystallization except after additional purification by preparative HPLC using the solvent system 60% MeOH in water to give dalrubone (**4.3**; t_R 26 min, 517 mg equivalent to 1.1% of the MeOH fraction) that was crystallized from EtOH (130 mg). The actual amount of dalrubone (**4.3**) in the MeOH-soluble extract is estimated at 4−5%. F002, the hexane-soluble extract, was found to contain an additional quantity of dalrubone that was responsible for the bioactivity of that fraction.

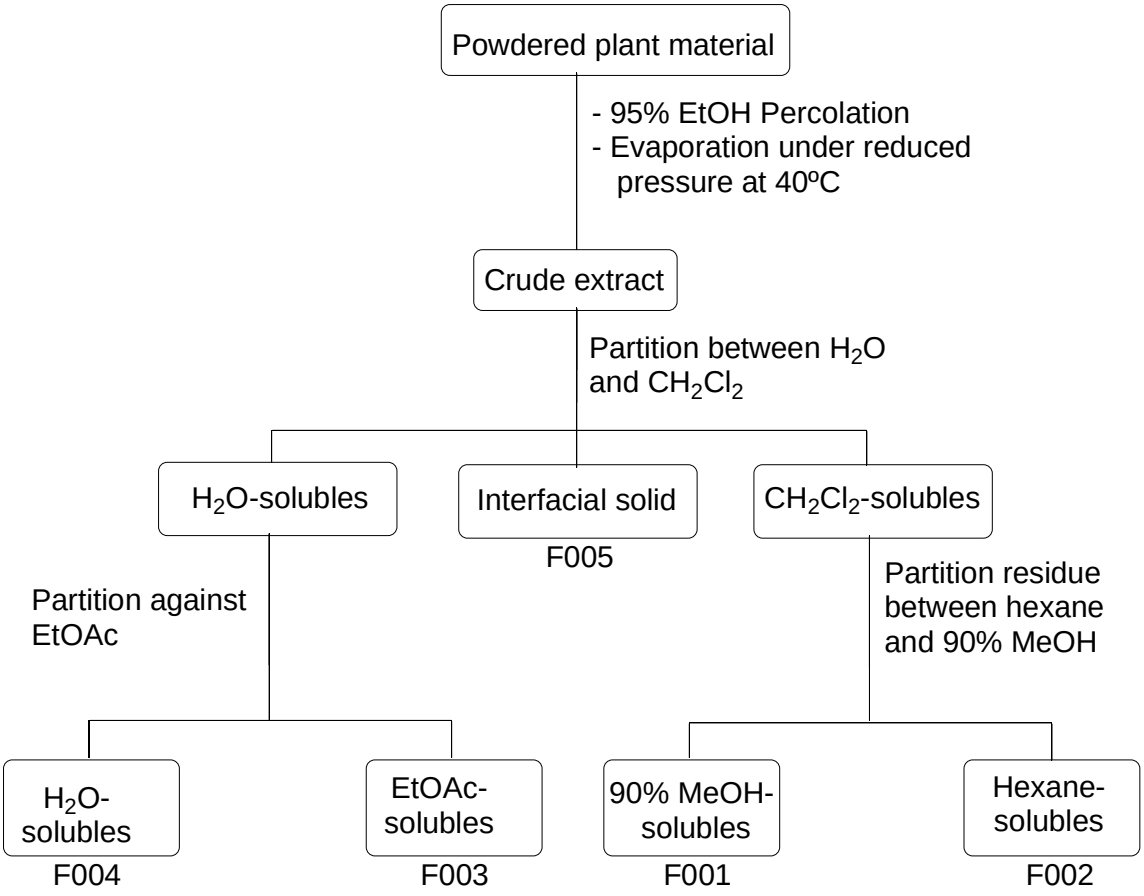


Figure 4.2: General Solvent Extraction and Fractionation Scheme Used for the Investigated Plant Samples

In an alternative procedure, another portion of F001 (21.0 g) was chromatographed over Sephadex LH-20 eluting with MeOH at a flow rate of 1 mL/min to give five fractions, F006−F010. F007−F010 showed activity in the bioassay (IC_{50}'s of 20.2, 11.7, 11.8, and 23.0 μg/mL, respectively, against axenic amastigotes). VLC of F007 yielded oleanolic acid (**4.7**) that was inactive in our assay. F008 (2.6 g) and F009 (3.1 g) contained crude dalrubone, while F010 (1.6 g) contained a mixture of flavonoids dominated by the flavanone, demethoxymatteucinol (**4.4**). F010 (1.58 g) was subjected to VLC using a solvent gradient of EtOAc in $CHCl_3$ and then a gradient of MeOH in EtOAc (100 mL per fraction). Fractions were pooled on the basis of the TLC behavior to give 11 fractions, F011−F021. Demethoxymatteucinol (**4.4**, 329 mg) was separated by crystallization from F013 (454 mg) as pale yellow crystals from EtOAc−MeOH. The mother liquor left after separation of demethoxymatteucinol crystals was subjected to preparative HPLC using a solvent system composed of 70% MeOH in water with 0.05% AcOH to give chalcone **4.1** at t_R 33 min (22 mg) and an additional quantity of demethoxymatteucinol at t_R 17 min (**4.4**, 43 mg). F016 (134 mg) was applied to a preparative TLC plate and eluted with the solvent system toluene−EtOAc−AcOH (20:10:1). The band with $R_f \sim 0.63$ was separated and subjected to preparative HPLC with a gradient of 50% solvent B in A (MeOH and water, respectively, each with 0.05% AcOH) increased to 80% in 60 min to elute chalcone **4.2** at t_R 41 min (4.0 mg). The band with $R_f \sim 0.2$ was separated and subjected to preparative HPLC with a gradient of 40% solvent B in A increased to 45% in 20 min to elute a mixture of compounds **4.6a** and **4.6b** as one peak (t_R 14 min, 4.2 mg). F019 (206 mg, eluted with 50% EtOAc in $CHCl_3$ from

VLC) was subjected to preparative HPLC using a gradient of 40% solvent B in A increased to 60% B in 40 min to give eriodictyol (**4.5**, 19 mg) at t_R 18 min. The purity of the isolated compounds was ascertained by carrying out HPLC and TLC analysis in at least two different solvens.

4.2.4 Characterization of the Isolated Compounds

2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (4.1): Orange needles; mp 120–122 ºC (lit.[192] 126-128 ºC); UV (MeOH) λ_{max} (log ε) 223 sh (4.87), 337 (4.42) nm; IR υ_{max} (KBr) 3331 (br OH), 2924, 2853, 1628, 1606, 1546, 1357, 1228, 1168, 1114 cm^{-1}; UV and IR data consistent with literature values;[192-194] ^1H, ^{13}C, and HMBC NMR ($CDCl_3$, 300 MHz), see Table 4.1; HRESIMS *m/z* 321.1106 $[M + Na]^+$ (calcd for $C_{18}H_{18}O_4Na$, 321.1103).

2,2′,4′-Trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (4.2): Orange powder; mp 155–160 ºC (dec.); UV (MeOH) λ_{max} (log ε) 300 sh (4.16), 366 (4.35) nm; IR υ_{max} (KBr) 3390 (br OH), 2927, 1621, 1556, 1539, 1456, 1350, 1165, 1110 cm^{-1}; ^1H, ^{13}C, and HMBC NMR ($CDCl_3$, 300 MHz), see Table 4.1; HRESIMS *m/z* 337.1050 $[M + Na]^+$ (calcd for $C_{18}H_{18}O_5Na$, 337.1052).

	Chalcone **4.1**			Chalcone **4.2**		
position	δ_C	δ_H (mult.; J_{HH})	HMBC	δ_C	δ_H (mult.; J_{HH})	HMBC
1	134.9			122.7		
2	127.9	7.65 (d; 7.2)	C-4, β	155.0		
3	128.4	7.41 (m)	C-1	116.4	6.84 (d; 8.0)	C-1, 5
4	129.7	7.41 (m)	C-2, 6	131.4	7.26 (ddd, 1.5, 7.3, 8.0)	C-2, 6
5	128.4	7.41 (m)	C-1	121.3	6.98 (dd; 7.3, 7.6)	C-1, 3
6	127.9	7.65 (d; 7.2)	C-4, β	129.2	7.62 (dd; 1.5, 7.6)	C-2, 4, β
α	126.2	7.99 (d; 15.7)	C-1, β, CO	127.6	8.05 (d; 15.8)	C-1, β, CO
β	142.4	7.84 (d; 15.7)	C-1, 2, 6, α, CO	137.8	8.15 (d; 15.8)	C-1, 2, 6, α, CO
1'	108.6			109.2		
2'	161.6			162.1		
OH-2'		13.60 (s)	C-1', 2', 3'		13.67 (s)	C- 1', 2', 3'
3'	106.1			106.5		
4'	158.7			159.1		
5'	108.4			108.8		
6'	158.4			158.9		
CH_3-3′	7.1	2.14 (s)	C-2', 3', 4'	7.6	2.14 (s)	C-2', 3', 4'
CH_3-5′	7.7	2.16 (s)	C-4', 5', 6'	8.2	2.15 (s)	C-4', 5', 6'
OCH_3-6′	61.9	3.66 (s)	C-6'	62.5	3.67 (s)	C-6'
CO	192.9			193.7		

Table 4.1: ^{1}H and ^{13}C NMR Assignments and HMBC Correlations for 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.1**) and for 2,2′,4′-Trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.2**) in $CDCl_3$

Dalrubone (4.3): Dark red plates; mp 99–100 ºC (lit.[195] 98–100 ºC); UV, IR, and ^{1}H and ^{13}C NMR data consistent with literature values;[195] UV (MeOH) λ_{max} (log ε) 225 sh (4.28), 306 (3.85), 426 (4.09) nm; IR υ_{max} (KBr) 3050, 2964, 2924, 2850, 1679, 1626, 1578, 1559, 1529, 1485, 1445, 1398, 1382, 1254, 1234, 1148, 1139, 1121 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.70 (1H, d, *J* = 10.0, H-3), 7.44 (1H, ddd, *J* = 1.6, 7.5, 8.5, H-7), 7.32 (1H, dd, *J* = 1.6, 7.6, H-5), 7.27 (1H, d, *J* = 7.5, H-8) (the second *J* value corresponding to meta coupling with H-6 could not be obtained as the $CDCl_3$ peak overlapped), 7.21 (1H, ddd, *J* = 1.0, 7.6, 8.5, H-6), 7.17 (1H, d, *J* = 10.0, H-4), 3.82 (3H, s, OCH_3), 1.95 (3H, s, CH_3-7′), 1.34 (6H, s, CH_3-8′, 9′); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 202.1 (C, C-2′), 199.4 (C, C-4′), 166.3 (C, C-6′), 156.5 (C, C-2), 152.7 (C, C-9), 133.5 (CH, C-4), 131.5 (CH, C-7), 127.3 (CH, C-5), 124.8 (CH, C-6), 120.8 (C, C-10), 119.4 (CH, C-3), 118.2 (C, C-5′), 116.2 (CH, C-8), 105.6 (C, C-1′), 59.5 (CH_3, OCH_3), 57.6 (C, C-3′), 23.0 (CH_3, C-8′, 9′), 9.1 (CH_3, C-7′); HRESIMS *m/z* 333.1103 $[M+ Na]^+$ (calcd for $C_{19}H_{18}O_4Na$, 333.1103).

Demethoxymatteucinol (6,8-dimethylpinocembrin) (4.4): Pale yellow needles; mp 201–202 ºC (lit.[196] 211 ºC); $[\alpha]_D$ –57.4º (*c* 0.5, MeOH at 24 ºC) (lit.[196] –46.0º); UV, IR, and ^{1}H and ^{13}C NMR data consistent with literature values;[196] UV (MeOH) λ_{max} (log ε) 205 (4.55), 230 (sh 4.21), 297 (4.24), 345 (3.59) nm; IR υ_{max} (KBr) 3235 (br OH), 1633, 1608, 1590, 1471, 1370, 1323, 1291, 1227, 1196, 1174, 1130, 1110 cm^{-1}; ^{1}H NMR (acetone-$d_{6,}$, 600 MHz) δ 12.41 disappeared on D_2O addition (1H, s, OH-5), 8.44 disappeared on D_2O addition (1H, br s, OH-7), 7.58 (2H, d, *J* = 7.4 Hz, H-2′ and H-6′), 7.45 (2H, dd, *J* =7.4, 7.4 Hz, H-3′ and H-5′), 7.39 (1H, dd, *J* = 7.4, 7.4 Hz, H-4’), 5.54

(1H, dd, J = 3.8, 12.7 Hz, H-2), 3.11 (1H, dd, J = 12.7, 17.0 Hz, H-$3_{ax(\alpha)}$), 2.85 (1H, dd, J = 3.8, 17.0 Hz, H-$3_{eq\ (\beta)}$), 2.06 (3H, s, CH_3-8), 2.05 (3H, s, CH_3-6); ^{13}C NMR (acetone-d_6, 150 MHz) δ 197.3 (C, C-4), 163.2 (C, C-7), 159.7 (C, C-5), 158.5 (C, C-9), 140.3 (C, C-1′), 129.4 (CH, C-3′, 5′), 129.1 (CH, C-4′), 126.9 (CH, C-2′, 6′), 104.3 (C, C-6), 103.5 (C, C-8), 102.9 (C, C-10), 79.4 (CH, C-2), 43.5 (CH_2, C-3), 8.2 (CH_3, C-8), 7.5 (CH_3, C-6); HMBC correlations H-2 to C-1′, -2′, -6′, H-3_{ax} to C-2, -4, -1′, H-3_{eq} to C-4, 6-CH_3 to C-5, -6, -7, 8-CH_3 to C-7, -8, -9, H-2′/H-6′ to C-2, -2′, -4′, -6′, H-3′/H-5′ to C-1′, -3′, -5′, H-4′ to C-2′, -6′; EIMS *m/z* (rel. int. %) 284 $[M]^+$ (74), 207 $[M\text{-}C_6H_5]^+$ (37), 180 $[M\text{-}C_6H_5\text{-}CH{=}CH_2]^+$ (67), 152 $[M\text{-}C_6H_5\text{-}CH{=}CH\text{-}CHO]^+$ (100); HRESIMS *m/z* 307.0960 [M + Na]$^+$ (calcd for $C_{17}H_{16}O_4Na$, 307.0946).

Eriodictyol (4.5): White powder; mp 263−265 ºC (dec., lit.[197] 268 ºC); $[\alpha]_D$ − 21.5º (*c* 1.0, MeOH at 25 ºC) [lit.[197] 0º (*c* 0.47, MeOH)]; UV, IR, and ^{1}H and ^{13}C NMR data consistent with literature values;[113,197] UV (MeOH) λ_{max} (log ε) 205 (4.85), 230 (sh 4.56), 287 (4.45), 330 (sh 3.82) nm; IR υ_{max} (KBr) 3355 (br OH), 1636, 1604, 1474,1450, 1311, 1274, 1259, 1159 cm^{-1}; ^{1}H NMR (methanol-d_4, 300 MHz) δ 6.91 (1H, s, H-2'), 6.78 (2H, m, H-5' and H-6'), 5.89 (1H, d, J = 2.2 Hz, H-8), 5.88 (1H, d, J = 2.2 Hz, H-6), 5.27 (1H, dd, J = 3.1, 12.7 Hz, H-2), 3.06 (1H, dd, J = 12.7, 17.2 Hz, H-3_{ax}), 2.68 (1H, dd, J = 3.1, 17.2 Hz, H-3_{eq}); ^{13}C NMR (methanol-d_4, 75 MHz) δ 197.9 (C, C-4), 168.5 (C, C-7), 165.6 (C, C-5), 162.0 (C, C-9), 147.0 (C, C-4′)[a], 146.6 (C, C-3′)[a], 131.9 (C, C-1′), 119.4 (CH, C-6′), 116.4 (CH, C-5′), 114.9 (CH, C-2′), 103.5 (C, C-10), 97.3 (CH, C-6)[b], 96.3 (CH, C-8)[b], 80.6 (CH, C-2), 44.2 (CH_2, C-3), (assignments with the same superscript might be interchanged); HMBC correlations H-2 to C-2′, -6′, H-3_{ax} to C-2, -4, -1′, H-3_{eq}

to C-4, -10, H-2′ to C-2, -4′, -6′, H-5′/H-6′ to C-2, -1′, -3′, -4′, -5′; HRESIMS *m/z* 311.0522 $[M+ Na]^+$ (calcd for $C_{15}H_{12}O_6Na$, 311.0532).

Photodalrubone (4.6a and 4.6b): Dark yellow residue; ^1H and ^{13}C NMR data consistent with literature values;[179] ^1H NMR ($CDCl_3$, 600 MHz) δ 8.53 and 8.35 (1H each, d, *J* = 9.5, H-3), 7.95 and 7.95 (1H each, d, *J* = 9.5, H-4),7.74 – 7.67 and 7.74 – 7.67 (2H each, m, H-7, 8), 7.614 and 7.607 (1H each, m, H-5), 7.475 and 7.462 (1H each, ddd, *J* = 2.0, 6.3, 8.3 or 1.5, 7.0, 8.1, respectively, H-6), 4.17 and 4.11 disappear on D_2O addition (1H each, s, OH), 2.28 and 2.26 (3H each, s, acetyl CH_3), 1.20, 1.18, 1.172, 1.166 (3H each, s, CH_3-3′ or 4′); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 208.7, 207.8 (C, *C*OCH_3), 203.1, 201.2 (C, C-5′ or 2′), 198.2, 196.0 (C, C-2′ or 5′), 165.9, 165.4 (C, C-2), 152.6 (C, C-9), 142.7, 142.5 (CH, C-4), 133.8, 133.7 (CH, C-7), 128.15, 128.10 (CH, C-5), 126.6, 126.5 (CH, C-6), 121.3, 121.2 (C, C-10), 118.3, 118.2 (CH, C-3), 118.0 (CH, C-8), 104.9, 104.2 (C, C-1′), 92.8, 92.7 (C, C-3′ or 4′), 53.6, 53.0 (C, C-4′ or 3′), 27.6, 27.4 (CH_3, CO*C*H_3), 24.6, 24.2 (CH_3, C-3′ or 4′ methyl), 16.8 (CH_3, C-3′ or 4′ methyl); NOE: irradiation of the H-4 resulted in enhancement of the H-3 and the H-5. Irradiation of the δ 2.28 Me resulted in enhancement of the δ 4.11 OH and δ 1.18 Me while irradiation of the δ 2.26 Me resulted in enhancement of the δ 4.17 OH and δ 1.172 Me. HSQC showed that the ^{13}C NMR resonance of δ 1.166 methyl is δ 24.2 and that of δ 1.20 methyl is 24.6. HMBC showed a correlation between δ 4.17 OH and both δ 207.8 and 198.2 keto groups, also a correlation between δ 4.11 OH and both δ 208.7 and 196.0 keto groups. The four Me groups at δ 1.20–1.166 showed correlations to the adjacent δ 203.1 and 201.2 keto groups although there was not enough resolution to determine which

methyls belong to what isomer; HRESIMS *m/z* 335.0909 $[M + Na]^+$ (calcd for $C_{18}H_{16}O_5Na$, 335.0895).

Oleanolic acid (4.7): amorphous white solid; mp 288–291 ˚C (lit.[198] 275–276 ºC); $[\alpha]_D$ +67º (*c* 0.046, MeOH at 25 ºC) [lit.[198] +82º (*c* 0.10, MeOH)]; IR υ_{max} (KBr) 3421, 2944, 2874, 1697, 1458, 1387, 1210, 1167, 1029, 996; ^1H and ^{13}C NMR data consistent with literature values;[199] ^1H NMR (pyridine-d_5, 400 MHz) δ 5.49 (1H, br t, *J* = 3.5 Hz, H-12), 3.43 (1H, dd, *J* = 5.6, 10.1 Hz, H-3α), 3.30 (1H, dd, *J* = 4.1, 13.5 Hz, H-18β), 2.21–1.75(10H, m, H-15β, H-16α, H-22β, H-11α, H-11β, H-16β, H-2α, H-2β, H-19α, H-22α), 1.68 (1H, tr, *J* = 8.8 Hz, H-9α), 1.60–1.29 (7H, m, H-1β, H-6α, H-7α, H-21α, H-6β, H-7β, H-19β), 1.27, 1.23 (3H each, s, CH_3-27, 23), 1.24–1.02 (3H, m, H-15α, H-21β, H-1α), 1.01, 1.01, 0.99, 0.94, 0.88 (3H each, s, CH_3-24, 26, 30, 29, 25), 0.86 (1H, m, H-5α); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 180.2 (C, C-28), 144.8 (C, C-13), 122.6 (CH, C-12), 78.1 (CH, C-3), 55.8 (CH, C-5), 48.1 (CH,C-9), 46.7 (C, C-17), 46.5 (CH_2, C-19), 42.2 (C, C-14), 42.0 (CH, C-18), 39.7 (C, C-8), 39.4 (C, C-4), 38.9 (CH_2, C-1), 37.4 (C, C-10), 34.2 (CH_2, C-21), 33.29 (CH_2, C-22) [a], 33.26 (CH_3, C-29), 33.2 (CH_2, C-7) [a], 31.0 (C, C-20), 28.8 (CH_3, C-23), 28.3 (CH_2, C-15), 28.1 (CH_2, C-2), 26.2 (CH_3, C-27), 23.82 (CH_2, C-11) [b], 23.76 (CH_3, C-30), 23.72 (CH_2, C-16) [b], 18.8 (CH_2, C-6), 17.4 (CH_3, C-26), 16.5 (CH_3, C-24), 15.5 (CH_3, C-25), [a,b] values with the same superscript maybe interchanged.

4.2.5 Biological Assays

Antileishmanial Assay Using Axenic Amastigotes. The antileishmanial activity of the isolated compounds was tested in vitro against *Leishmania donovani* amastigote-

like parasites (WHO designation: MHOM/SD/62/1S-CL2$_D$) in a three-day assay using the tetrazolium dye-based CellTiter reagent (Promega) as described previously in Chapter 3.

Antitrypanosomal Assay. Compounds were tested for their activity against bloodstream-form *Trypanosoma brucei brucei* (MITat 1.2, variant 221) axenically cultured in HMI-9 medium using the tetrazolium dye MTT as described earlier Chapter 3.

Cytotoxicity Assay. Cytotoxicity was evaluated against two cell lines, Vero cells and J774 A.1 macrophages, obtained from the American Type Culture Collection (ATCC, Rockville, MD). Vero cells were grown in Eagle's Minimum Essential Medium with Earle's Balanced Salt Solution (ATCC) supplemented with 50 units/mL penicillin, 50 µg/mL streptomycin, and 10% fetal bovine serum. The growth medium used for J774 A.1 cells was Dulbecco's Modified Eagle's Medium (DMEM, from ATCC) with the same supplements as for Vero cells. Cells (1000 Vero cells/well or 5000 J774 A.1 cells/well) were seeded together with serial dilutions of the test compounds in the individual wells of 96-well plates (final volume 100 µL/well). After 72 h incubation at 37 °C in a humidified 5% CO_2 incubator, cell viability was determined using the CellTiter reagent by adding 20 µL of assay solution to each well. After 12–14 h incubation at 37 °C to allow for color development, the absorbance of each well at 490 nm was measured in a SpectraMax Pro microplate reader as described previously in Chapter 3.

***Leishmania*-Infected Macrophage Assay.** Mouse peritoneal macrophages, obtained from 5–6 month old female mice (C57BL/6 strain),[200] were mixed with late log phase *L. mexicana* (WHO designation: MNYC/BCZ/62/M379) promastigotes to give a suspension containing 5×10^5 macrophages/mL and 50×10^5 promastigotes/mL in

DMEM supplemented with 4 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum. A 200 μL aliquot of this suspension was then pipetted into the individual wells of a 16-well glass chamber slides (Lab-Tek). Infection and attachment of the macrophages was allowed to occur over a period of 24 h at 33 °C in a humidified 5% CO_2 incubator. Wells were washed with Hank's Balanced Salt Solution (HBSS) to remove extracellular parasites, and then serial dilutions of drugs in supplemented DMEM were added to each well. After incubation for 72 h, the medium was discarded, the growth chamber was removed and the slides were thoroughly washed with phosphate-buffered saline (PBS). The cell films were immediately fixed for 5 s in methanol and stained with 0.04% Giemsa stain (Fisher) for 35 min. After thorough washing in flowing tap water, the slides were allowed to air-dry. The percentage of infected cells was determined after examination of at least 200 cells in each sample by oil immersion microscopy.

Flow Cytometry and Fluorescence Cell Cycle Analysis. *L. donovani* promastigotes (maintained at 26 °C in RPMI medium supplemented with 10% fetal bovine serum) or *T. b. brucei* bloodstream form trypomastigotes (maintained at 37 °C in HMI-9 medium) were incubated in T25 flasks (3 mL final volume) under the appropriate conditions described above for 24 or 48 h, respectively. Cultures contained 1% DMSO (v/v) in the presence or absence of test compounds. Cells were counted using a hemacytometer, then centrifuged (1200 × g) at 4 °C for 10 min. Cells were resuspended in 150 μL of PBS (0.01 M phosphate buffer, 0.137 M NaCl, and 2.7 mM KCl), then 350 μL of ice-cold methanol was added and the samples were fixed at –20 °C for 2–3 h. Cells

were centrifuged as before and resuspended in PBS containing 0.1% TX-100, 5 μg/mL RNase A, and 10 μg/mL propidium iodide for 20–30 min. Centrifugation was repeated, then cells were resuspended in PBS to a final concentration of 1×10^7 cells/mL and placed at 4 °C until analysis (typically 1–2 h). Fluorescence flow cytometry was conducted on a Becton Dickinson FACSCalibur flow cytometer. Results were analyzed using Cell Quest Pro software from BD Biosciences (San Jose, CA). In each case, gating was performed to exclude doublets and aggregates.

Transmission Electron Microscopy. Parasites were treated with test compounds for 12–48 h, centrifuged as described earlier, and fixed in 4% formaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 3 h at room temperature. The samples were then post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h, stained with 2% uranyl acetate and 2% lead citrate for 1 h, and dehydrated in increasing concentrations of ethanol followed by propylene oxide. Finally, the samples were embedded in Spurr resin which was then allowed to polymerize overnight at 60 °C. Thin sections were cut and examined with a Philips CM 12 transmission electron microscope.

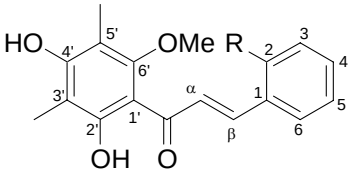
In Vivo *Leishmania* Assay. BALB/c mice 8–12 weeks old were infected via the lateral tail vein with 1×10^7 *L. donovani* amastigotes harvested from the spleen of infected hamster and suspended in 100 μL of RPMI medium. The infection was allowed to establish for 7 days. On day 7 post-infection, the mice were randomly separated into groups of five. Compounds were diluted to the required concentration in a vehicle composed of 10% DMSO, 0.5% methylcellulose and 0.1% Tween-80 in sterile water. For five days, mice received daily injections of 200 μL of the vehicle alone (control group),

dalrubone (50 mg/kg/day s.c.), or the standard antileishmanial agent miltefosine (10 mg/kg/day i.p.). Four days post-treatment the mice were sacrificed. The livers and spleens were collected, weighed and used to prepare impression smears that were fixed and stained (Giemsa) as before. The number of amastigotes per 200 macrophage nuclei were determined microscopically for each sample, adjusted to the number per 1000 macrophage nuclei and multiplied by the organ weight (in grams) to obtain the LDU (Leishman Donovan Unit) values.[201]

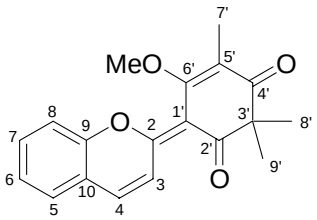
4.3 RESULTS AND DISCUSSION

Two chalcones, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.1**) and the new 2,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.2**), two flavanones, demethoxymatteucinol (**4.4**)[196] and eriodictyol (**4.5**),[113] two benzopyran pigments, dalrubone (**4.3**)[195] and photodalrubone (**4.6a** and **4.6b**),[179] and the triterpene oleanolic acid (**4.7**),[199] were isolated from the methanolic extract of *P. polydenius* (see Figure 4.3 for the structures of the isolated compounds). Separations were guided by bioactivity assays using *L. donovani* axenic amastigotes. The structures of the known compounds were determined by 1D and 2D NMR techniques and confirmed by comparing the physical and spectral data with those from the literature (mp, NMR, and MS). Demethoxymatteucinol (**4.4**) and dalrubone (**4.3**) represented the major metabolites in the extract. However, it appears that the amount of the red pigment dalrubone varies with the environmental conditions and time of collection. The plant collected in September 2002, after a relatively hot, dry summer, contained only traces of dalrubone, while the same

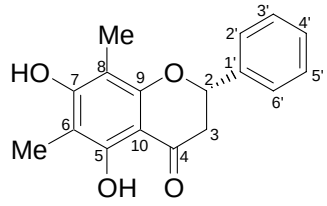
plant collected in June 2003 in a rainy season contained copious amount of the dye that imparted the methanolic extract with its characteristic dark reddish color. The demethoxymatteucinol content, however, was comparable in both collections.



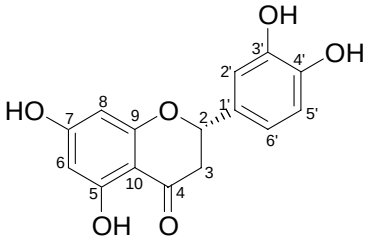
4.1 R = H
4.2 R = OH



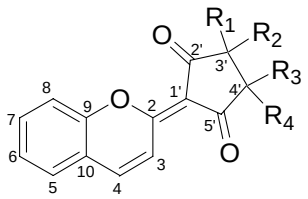
Dalrubone (**4.3**)



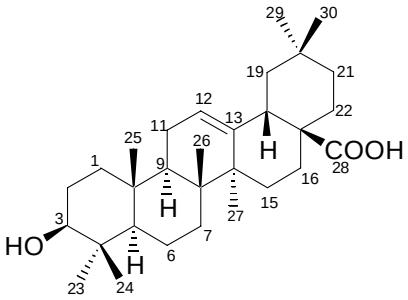
Demethoxymatteucinol (**4.4**)



Eriodictyol (**4.5**)



4.6a R_1 and R_2=OH and Ac, R_3=R_4=Me
4.6b R_1=R_2=Me, R_3 and R_4=OH and Ac



Oleanolic acid (**4.7**)

Figure 4.3: Structures of Compounds Isolated from *Psorothamnus polydenius*

4.3.1 Identification of 2′,4′-Dihydroxy-6′-methoxy-3′,5′–dimethylchalcone (1)

The NMR data for compound **4.1** (Table 4.1 and Figure 4.4) confirmed a chalcone structure with a non-substituted A ring and a highly substituted B ring with two methyls, two hydroxyls and a methoxy group. The methoxy group was assigned to the 6′ position based on the HMBC correlation between the methoxy singlet at δ 3.66 and the C-6′ peak at δ 158.4 which was in turn correlated to the 5′-methyl protons at δ 2.16 (Figure 4.5). Furthermore, the position of the methoxy group was confirmed by an NOE experiment where the OMe resonance at δ 3.66 was irradiated. The resulting NOE spectrum showed an enhancement of only one methyl signal (at δ 2.16) and the α-olefinic proton (Figure 4.6 B). Conversely, irradiation of the α-olefenic proton signal at δ 7.99 resulted in enhancement of the OMe signal at δ 3.66 (Figure 4.6 C). Irradiating the β-olefinic proton resulted in a weaker NOE effect at the methoxy group signal (Figure 4.6 D). Thus, the assignment of each atom within the molecule was established. The spectroscopic data for chalcone **4.1** are given in Table 4.1 for comparison with the new chalcone **4.2** (see Section 4.3.2) and because of some disagreement among literature data;[192-194] this is the first report of chalcone **4.1** in *P. polydenius.*

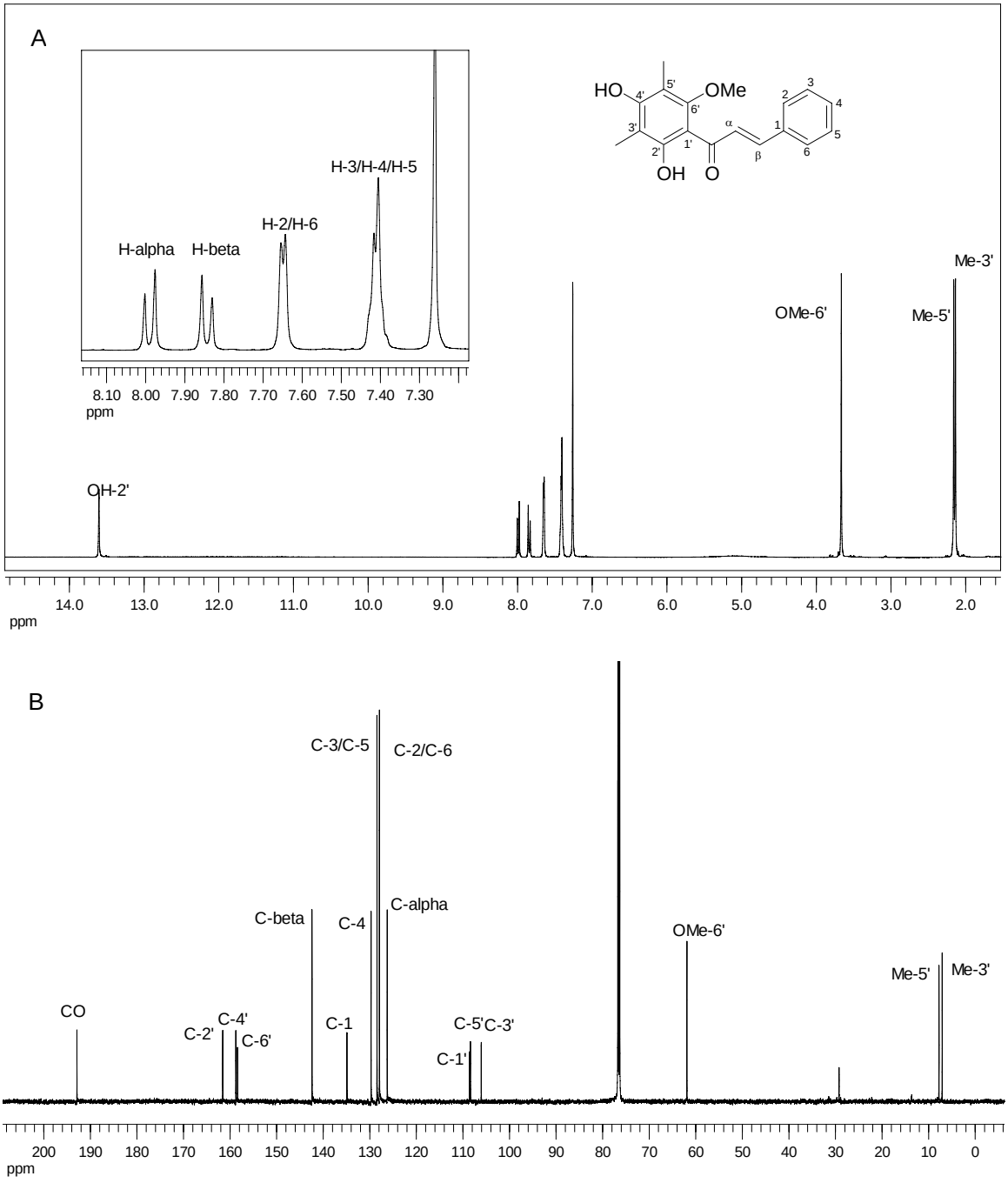


Figure 4.4: ^{1}H and ^{13}C NMR Spectra of 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.1**) in $CDCl_3$

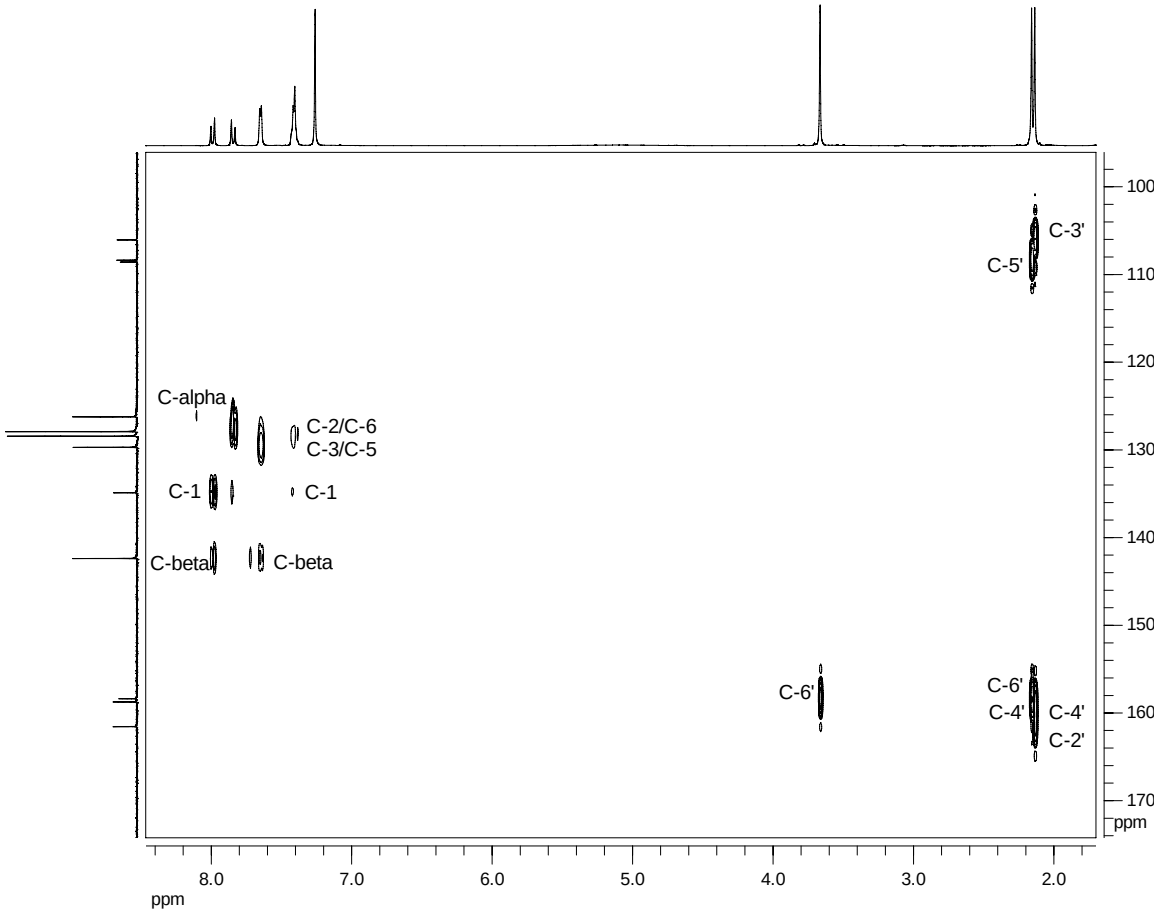


Figure 4.5: ^{1}H-^{13}C HMBC Spectrum of 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.1**) in $CDCl_3$

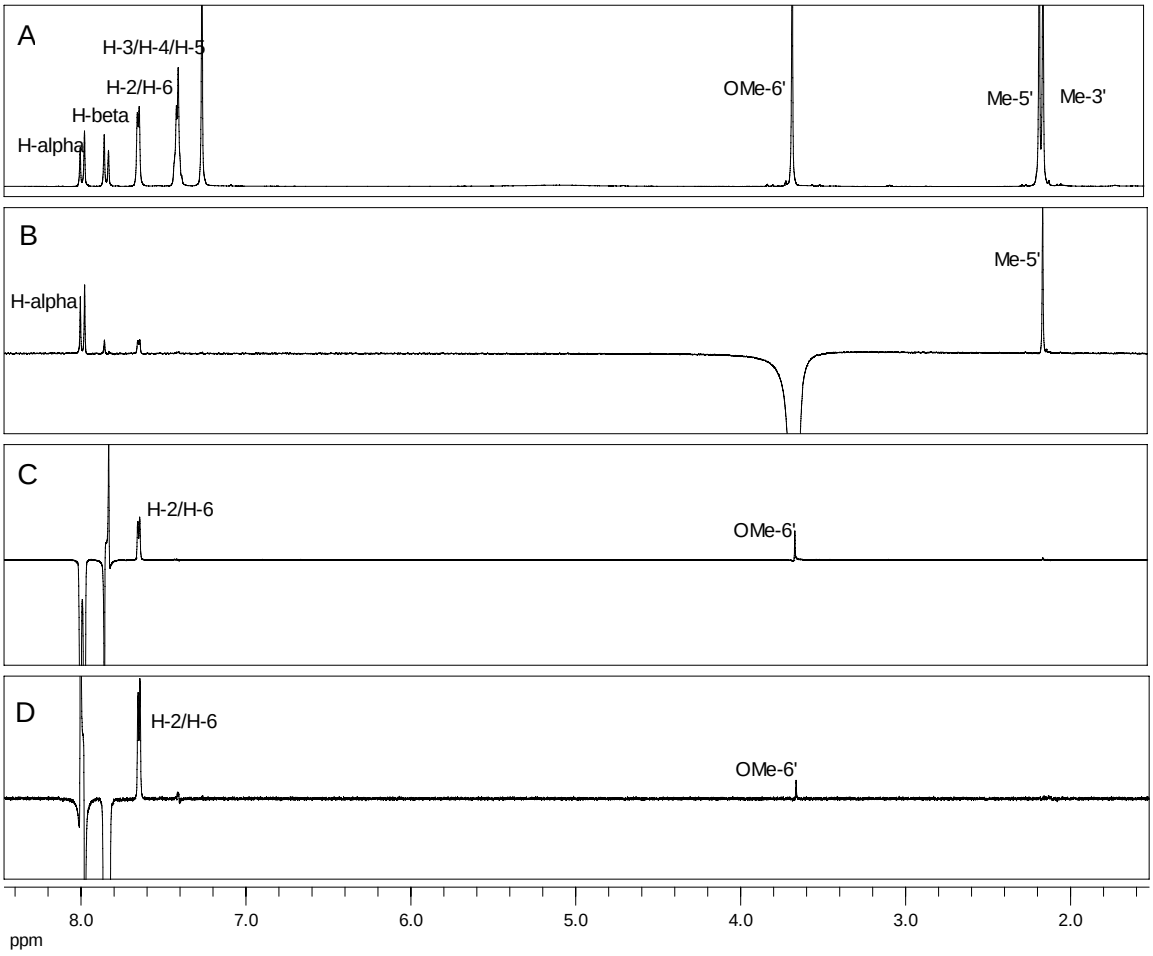


Figure 4.6: DPFGSE-NOE Spectra of 2′,4′-Dihydroxy-6′-methoxy-3′,5′-

dimethylchalcone (**4.1**)

Panel A shows the standard ^{1}H NMR spectrum. In B, a selective excitation pulse was adjusted on the the methoxy group singlet. A strong NOE effect was observed for the Me-5′ and H-α. A weaker NOE effect was observed for H-β and H-2/H-6. In C, selective irradiation of the H-α signal resulted in an NOE effect at 6′-OMe and H-2/H-6 signals. In D, the H-β signal was selectively irradiated resulting in a strong NOE effect at H-2/H-6 and a weaker effect at the OMe-6′.

4.3.2 Structure Elucidaiton of 2,2′,4′-Trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (4.2)

Chalcone **4.2** was also isolated from the flavonoid fraction eluted from Sephadex LH-20 by preparative HPLC. It showed a ^{1}H NMR spectrum similar to that of chalcone **4.1** except for a broad hydroxyl peak at δ 5.35 integrating to two protons and the A ring possessing only four aromatic protons, indicating di-substitution (Figure 4.7). The ^{1}H-^{1}H COSY spectrum showed that the four aromatic protons are adjacent, indicating an *o*-substituted aryl ring. HRESIMS gave $[M + Na]^{+}$ at *m/z* 337.1050 consistent with the proposed molecular formula $C_{18}H_{18}O_5$. The HSQC and HMBC spectral data confirmed the proposed structure (see Table 4.2 and Figure 4.8). The methyl group at δ 2.14 was assigned to the C-3′ position based on its HMBC correlation to the C-2′ and C-4′ carbons while the methyl group at δ 2.15 was assigned to the C-5′ position based on its correlation to the C-4′ and C-6′ carbons. The methoxy group was assigned to the C-6′ position based on the HMBC correlation between the methoxy proton singlet at δ 3.67 and the C-6′ peak at δ 158.9 ppm which was in turn correlated to the 5′ methyl protons at δ 2.15 (Figure 4.8). This is the first report of chalcone **4.2**.

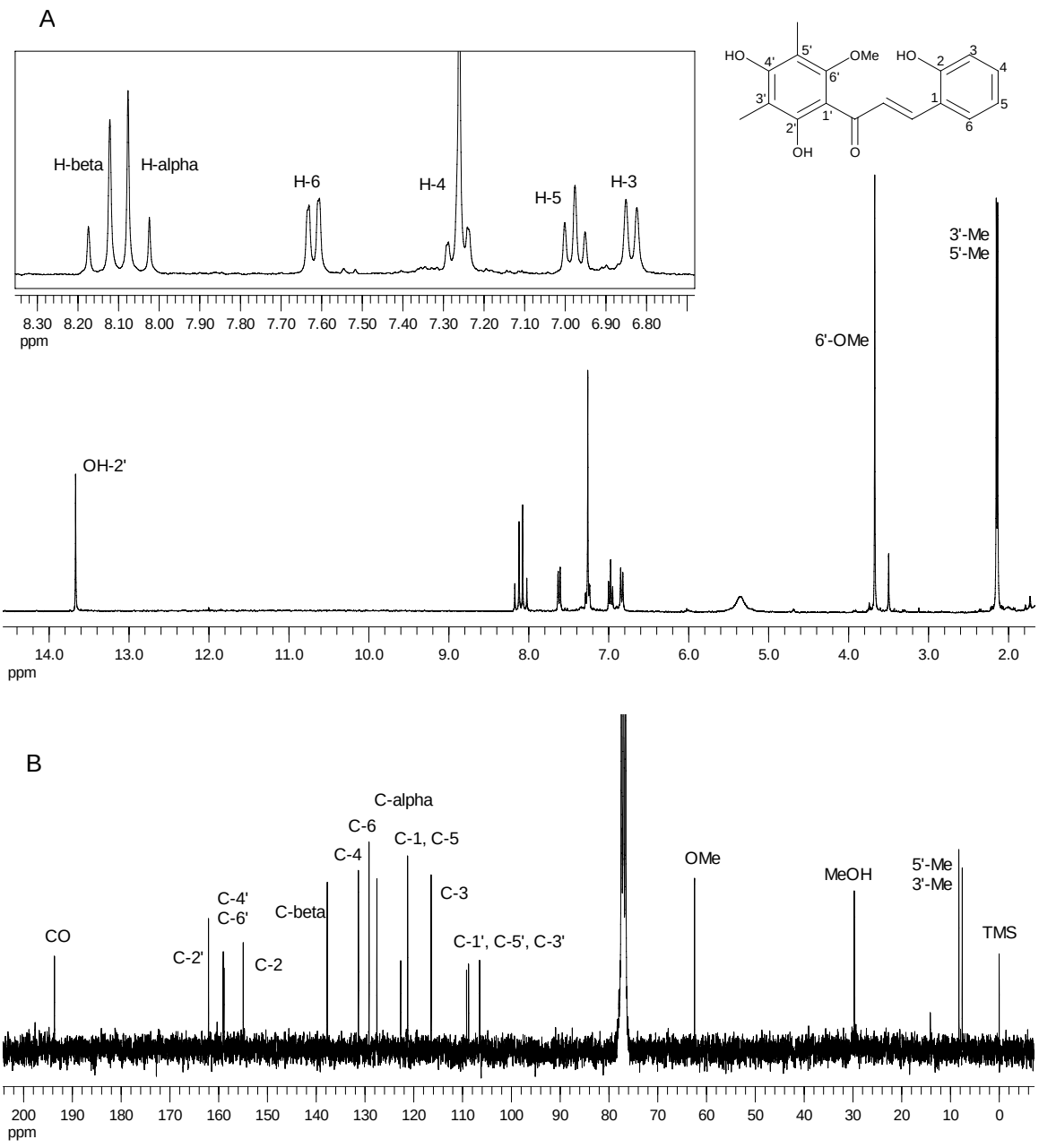


Figure 4.7: ^{1}H and ^{13}C NMR Spectra of 2,2′,4′-Trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.2**) in $CDCl_3$

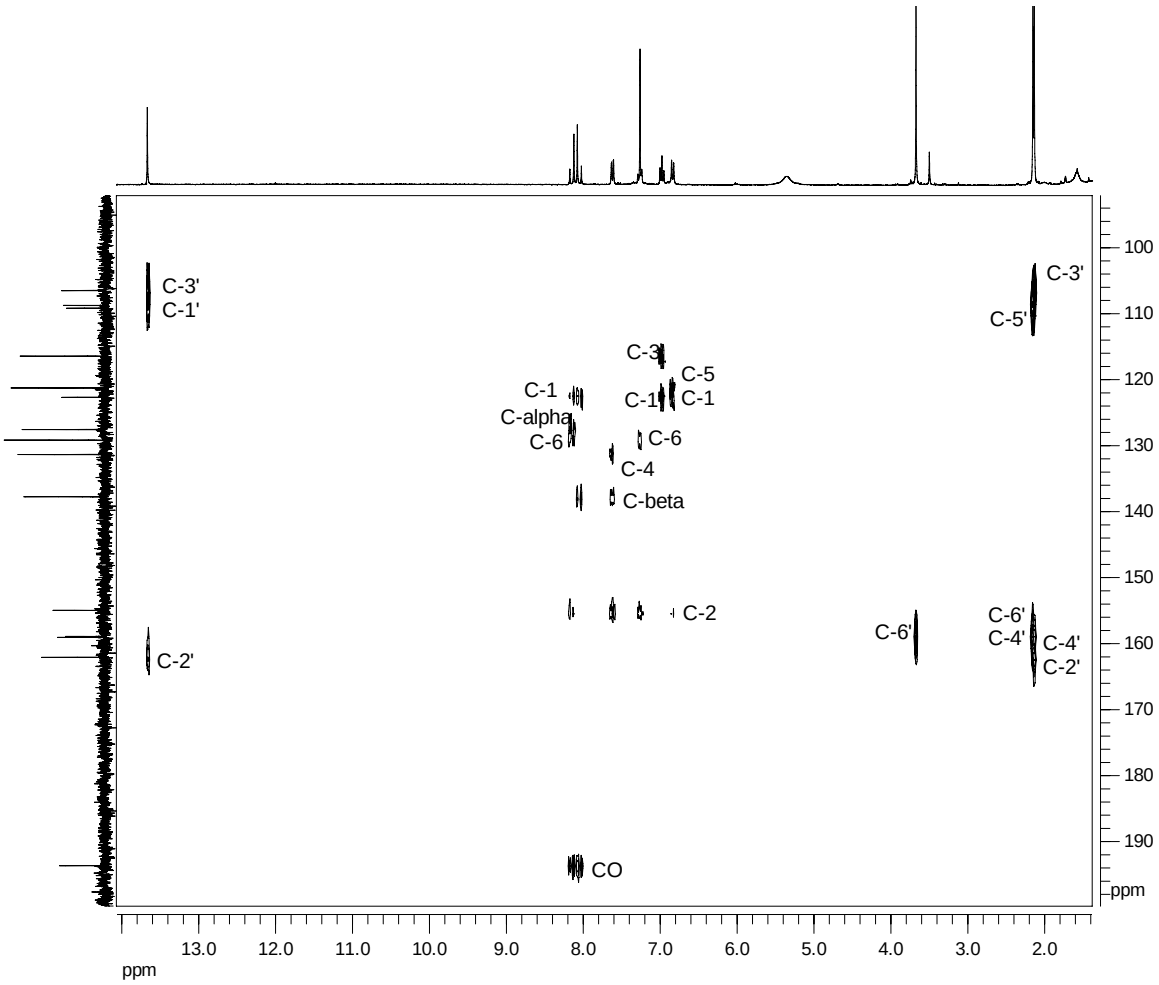


Figure 4.8: ^{1}H-^{13}C HMBC Spectrum of 2,2′,4′-Trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.2**) in $CDCl_3$

4.3.3 Photodalrubone (4.6a and 4.6b)

Compounds **4.6a** and **4.6b** eluted as one broad peak from preparative HPLC and showed a ^{13}C NMR spectrum similar to dalrubone (**4.3**) in the aromatic region with doubling of the peaks. The doubled peaks in the ^{1}H NMR spectrum consistently displayed slight differences in intensity and integration, suggesting the presence of two isomeric forms rather than a dimeric structure. HRESIMS gave $[M + Na]^+$ at *m/z* 335.0909, consistent with a molecular formula $C_{18}H_{16}O_5$. The HSQC and HMBC data confirmed the photodalrubone structure (**4.6a** and **4.6b**) which was proposed earlier by Dreyer et al.[179] as a photodegradation product of dalrubone (**4.3**). Oxygen undergoes a 1,2-addition to the 5′-6′ double bond of dalrubone followed by ring contration and liberation of formaldehyde (see Figure 4.9). Compounds **4.6a** and **4.6b** are isomeric structures that may result from rotation around the C-2–C-1′ single bond in the enol tautomer, and thus were not separated.

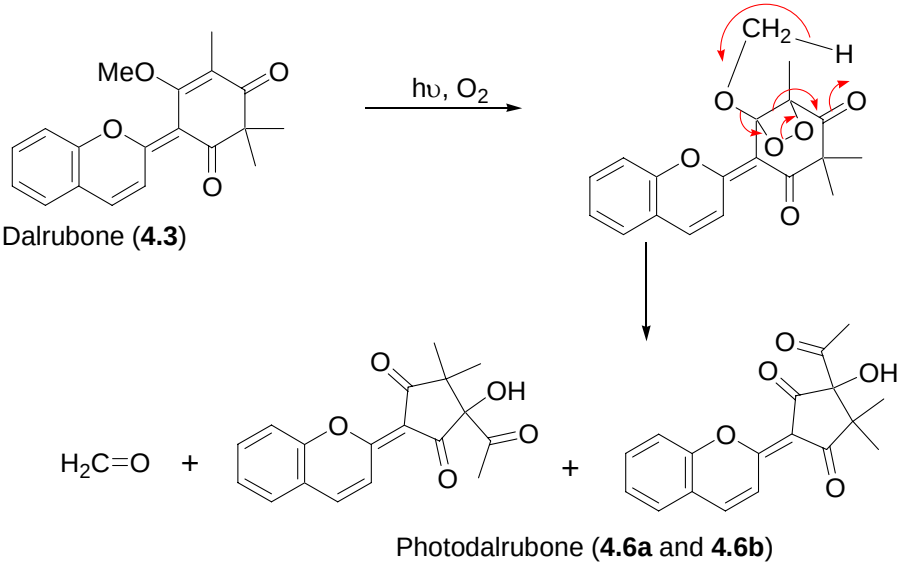


Figure 4.9: Photooxidation of Dalrubone to Form Photodalrubone[179]

4.3.4 Biosynthesis

Dalrubone (**4.3**) has a C_6–C_3–C_6 skeleton with a cyclohexadione moiety attached at position 2 of a 2H-chromene ring while demethoxymatteucinol (**4.4**) is a flavanone. Dryer et al.[179] suggested that both compounds are biosynthesized from a common chalcone intermediate, 2′,4′,6′-trihydroxychalcone. They also suggested that *C* and *O*-methylations occur late in the biosynthetic pathways to form both compounds. In the biogenetic scheme of Dryer et al., the cyclohexadione moiety of dalrubone originates from the A ring (the polyketide portion) of the chalcone precursor. On the other hand, Zhang et al.[195] suggested that the chalcone precursor of dalrubone is 4,2′-dihydroxychalcone and postulated an alternative biogenetic pathway in which the cyclohexadione portion of dalrubone originates from the phenyl ring of phenylalanine (i.e., from ring B of the chalcone precursor). The isolation of the chalcones **4.1** and **4.2** together with dalrubone (**4.3**) and demethoxymatteucinol (**4.4**) from *P. polydenius* strongly supports the proposed biogenesis of Dryer et al. (note the close similarity between ring A of the chalcones and the quinone ring of dalrubone). In addition, it implies that most of the methylations to the chalcone precursors occur early in the biosysnthetic pathway. These methylated chalcone precursors then undergo further modifications to produce dalrubone and demethoxymatteucinol. A modification of the biogenetic scheme of Dryer et al., incorporating chalcones **4.1** and **4.2,** is presented in Figure 4.10. The proposed pathway involves the formation of a flavylium ion intermediate from chalcone **4.2**. The flavylium intermediate then converts to a quinone that undergoes one additional *C*-methylation to form dalrubone.

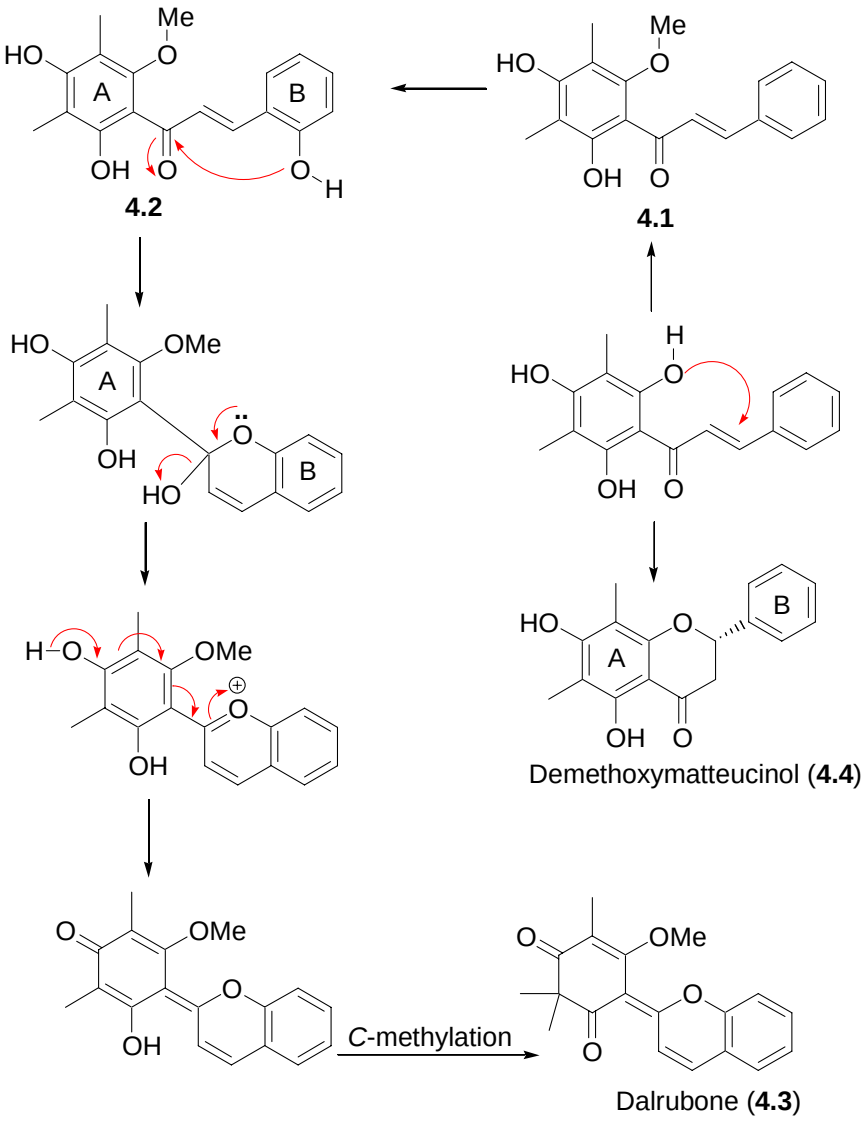


Figure 4.10: Biogenetic Relationships between Chalcones **4.1** and **4.2**, and Demethoxymatteucinol (**4.4**) and Dalrubone (**4.3**)

4.3.5 Antikinetoplastid Activities of the Isolated Compounds

The antiparasitic activities of compounds **4.1**–**4.6** were evaluated against *L. donovani*, the causative agent of visceral leishmaniasis, and *T. brucei brucei*, a *Trypanosoma* subspecies related to the causative agent of African sleeping sickness (see Tables 4.2 and 4.3). Compounds **4.1** and **4.2** exhibited significant activity against both parasites, with IC_{50} values in the range 5.0–7.5 μg/mL, while dalrubone (**4.3**) was about three times more active against *L. donovani* than *T. brucei brucei* (IC_{50} 7.5 and 21.6 μg/mL, respectively). Moreover, compounds **4.1**–**4.3** displayed 2.6-, 1.8- and 5.9-fold selectivity, respectively, against the *Leishmania* parasites compared to African Green Monkey kidney (Vero) cells. Dalrubone (**4.3**) showed low toxicity to the malignant J774A.1 macrophages compared to chalcones **4.1** and **4.2** (IC_{50} 30.1, 7.7, and 9.3 μg/mL, respectively). In addition, intracellular *Leishmania mexicana* amastigotes were essentially cleared at concentrations of 12.5, 12.5 and 25 μg/mL of compounds **4.1**–**4.3**, respectively, in murine peritoneal macrophages without harming the host mammalian cells.

compound	*L. donovani* axenic amastigotes[a]	*T. b. brucei* variant 221[a]	Vero cells[a]	J774 A.1 macrophages[a]
4.1	5.0 ± 1.3	6.3 ± 1.1	12.9 ± 0.1	7.7 ± 3.3
4.2	7.5 ± 0.9	6.8 ± 0.5	13.3 ± 0.5	9.3 ± 3.3
4.3	7.5 ± 1.0	21.6 ± 5.0	44.5 ± 2.3	30.1 ± 4.7
4.4	>100	28.9 ± 3.0	>50	>50
4.5	25.0 ± 4.4	25-50	36.5 ± 4.5	32.8 ± 12.3
4.6a and **4.6b**	45.2 ± 5.2	ND[b]	ND	ND
pentamidine	1.4 ± 0.2	0.007 ± 0.002	>50	12.9 ± 2.1
suramin	ND	0.193 ± 0.039	ND	ND

Table 4.2: IC_{50} Values (μg/mL) of Compounds **4.1**–**4.6** against Axenic *L. donovani*, *T. brucei*, and Mammalian Cell Lines

[a] IC_{50} values are given as the mean ± SD of three determinations. [b] ND: not determined.

compound	concentration (μg/mL)	% reduction in infection[a]
4.1	12.5	96 ± 2
	6.3	61 ± 13
	3.1	28 ± 3
4.2	12.5	96 ± 2
	6.3	34 ± 11
	3.1	6 ± 6
4.3	25	97 ± 3
	12.5	41 ± 10
	6.3	1 ± 8
4.5	50	76 ± 13
	25	55 ± 16
	12.5	30 ± 8
amphotericin B	0.116	95 ± 5
	0.029	85 ± 13
	0.014	59 ± 22

Table 4.3: Antileishmanial Activity of Compounds **4.1**–**4.3** and **4.5** against Intracellular *L. mexicana*

[a] % Decrease of infected macrophages in treated vs. non-treated wells.

4.3.6 Flow Cytometry

In an attempt to investigate the mechanism of action of dalrubone, cell cycle analysis of *L. donovani* promastigotes treated with the compound was performed. The parasites were incubated for 48 h at 26 °C in the presence of 50 μM (15.5 μg/mL) dalrubone, corresponding to the IC_{50} of dalrubone against the promastigotes, or 1% DMSO (control). The promastigotes were fixed, stained with propidium iodide, and analysed by flow cytometry as described in the experimental section. There was no significant difference between percentages of cells in each stage of the cell cycle compared to those of the control (see Figure 4.11 and Table 4.4). It appears that dalrubone does not affect the cell cycle progression in the parasites.

drug	*G1* phase %[a]	*G2/M* phase %[a]	*S* phase %[a]
1% DMSO (control)	79.1 ± 6.8	16.8 ± 5.7	4.1 ± 1.2
dalrubone	80.1 ± 1.4	16.3 ± 2.7	3.6 ± 2.6

Table 4.4: Summary of Fluorescence Cell Cycle Analyses of *L. donovani* Promastigotes Treated with Dalrubone

[a] The results represent the percentages of cells in each phase of the cell cycle ± the standard deviation from experiments done on 3 separate occasions.

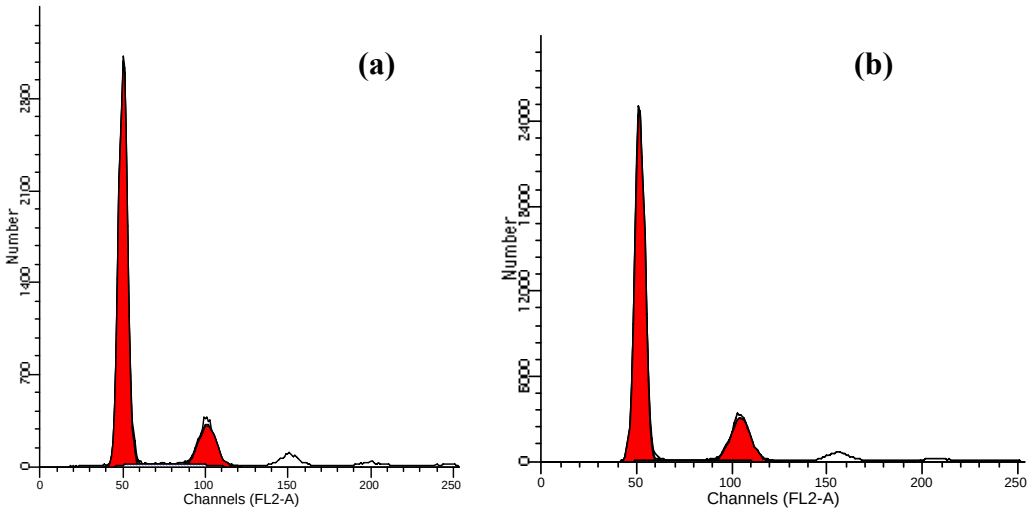


Figure 4.11 Flow Cytometry Analysis of *L. donovani* Promastigotes Treated with Dalrubone

Parasites were incubated at 26 °C for 48 h in in the presence of 1% DMSO (a) or 50 μM dalrubone (b), then fixed, stained with propidium iodide, and analysed by flow cytometry as described in the experimental section. The results shown are from a representative experiment performed in 3 separate occasions. The *y*-axis represents the number of particles (cells) and the *x*-axis (FL2-A) refers to the fluorescence intensity of each particle as measured by the flow cytometer. The shaded area represents the cell cycle analysis using Cell Quest Pro software.

4.3.7 Transmission Electron Microscopy

Electron microscopy of *L. donovani* promastigotes treated with dalrubone at 40 μM, a concentration slightly lower than the IC_{50} of dalrubone against this parasite form (50 μM), was carried out to gain insight regarding the antileishmanial mechanism of action. Promastigotes rather than amastigotes were chosen because they are well-characterized morphologically and thus it is easier to interprete their electron micrographs. Parasites incubated with dalrubone for 48 h showed significant ultrastructural changes (Figure 4.12). Most evident is the appearance of large concentric membranous structures in the cytoplasm. In addition, an increase in number of cytoplasmic vacuoles was also seen. It is known that in trypanosomatids (including *Leishmania*), proteins are synthesized in the rough endoplasmic reticulum (ER), exported to the Golgi apparatus via a specialized transfer ER, and then transported to the flagellar pocket via pleiomorphic vesicles and vacuoles. After delivery to the flagellar pocket, proteins can be directed to the cell body and/or the flagellum while proteins destined for degradation are transported to the lysosomes.[202] It seems that dalrubone interferes with these secretory pathways of the promastigotes. Parthenolide, a sesquiterpene lactone with antileishmanial properties, was found to produce similar morphological changes, such as the appearance of lysosome-like structures in addition to inducing an intense exocytic activity in the region of the flagellar pocket. These structural changes were suggested to indicate "a process of exacerbated protein production by cells as they attempt to survive."[75]

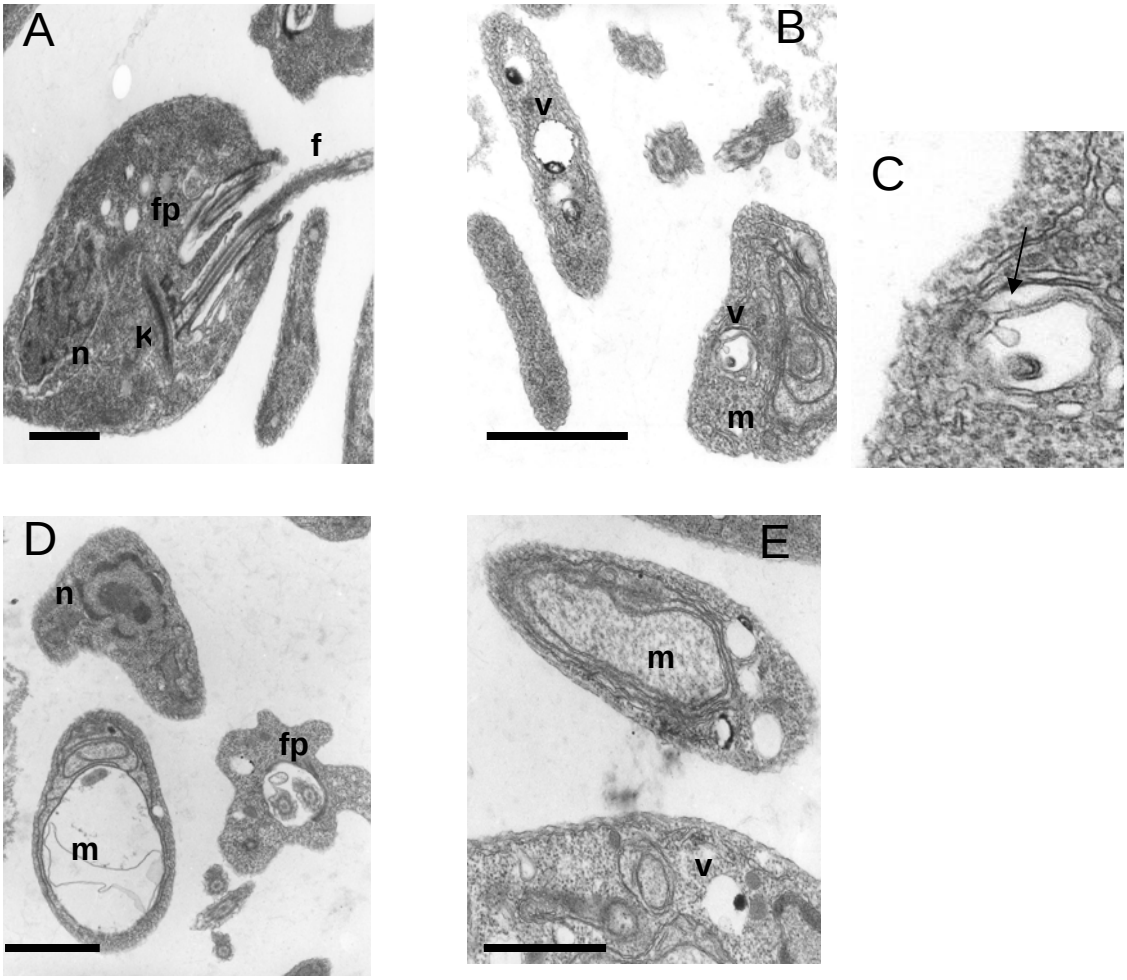


Figure 4.12: Transmission Electron Micrographs of *L. donovani* Promastigotes

In panel A, a control parasite treated with 1% DMSO showing a kinetoplast (k), two flagella (f) inside a flagellar pocket (fp), and a nucleus (n) is pictured. In panels B, D and E, Parasites treated with 40 μM dalrubone for 48 h are shown. Note the appearance of concentric membranous structures (m) and vacuoles (v) containing dark pigment. An enlargement of one of the vacuoles in panel B is presented in panel C showing a secretory activity towards the lumen of the vacuole. Bar, 1 μm.

4.3.8 In Vivo Antileishmanial Activity

To examine the in vivo antileishmanial potential of dalrubone, it was tested for activity against *L. donovani* infection in BALB/c mice. On day 7 post-infection, mice were given dalrubone at a subcutaneous dose 50 mg/kg/day, the standard antileishmanial drug miltefosine at an intraperitoneal dose 10 mg/kg/day, or vehicle only for 5 days. Mice were sacrificed 4 days after the end of treatment. Impression smears were prepared from the livers, Giemsa-stained and assessed microscopically, with LDU (Leishman Donovan Unit) values calculated as the number of amastigotes per 1000 macrophage nuclei multiplied by the liver weight in grams. While dalrubone-treated mice decreased liver parasitemia on average by 21%, this effect was judged as statistically non-significant (p value = 0.13 using the Student's t-test). In contrast, the standard antileishmanial drug miltefosine decreased the parasitemia in the liver tissues of infected mice by 78% (p = 0.0011); the results are shown in Figure 4.13. The drug-treated mice did not show any signs of toxicity during the treatment period. However, when the drug was given at the same dosage by the intraperitoneal route, it was immediately lethal to the mice. This appears to be related to the fast drug delivery to the systemic circulation in case of i.p. injection, whereas the same dose of the drug given subcutaneously and thus slowly absorbed into the systemic circulation was well-tolerated by the animals. A similar situation is encountered with other drugs when given by intravenous bolus injection, including calcium chloride which can lead to arrhythmia and verapamil which can cause bradycardia and heart block. If administered parenterally, these drugs are given by intravenous drip or slow intravenous injection. It worth mentioning that dalrubone is the

major component of *P. polydenius* which is a herbal remedy extensively used by the desert tribes of Nevada over generations via the oral and topical routes without reports of acute toxicity.[163,164]

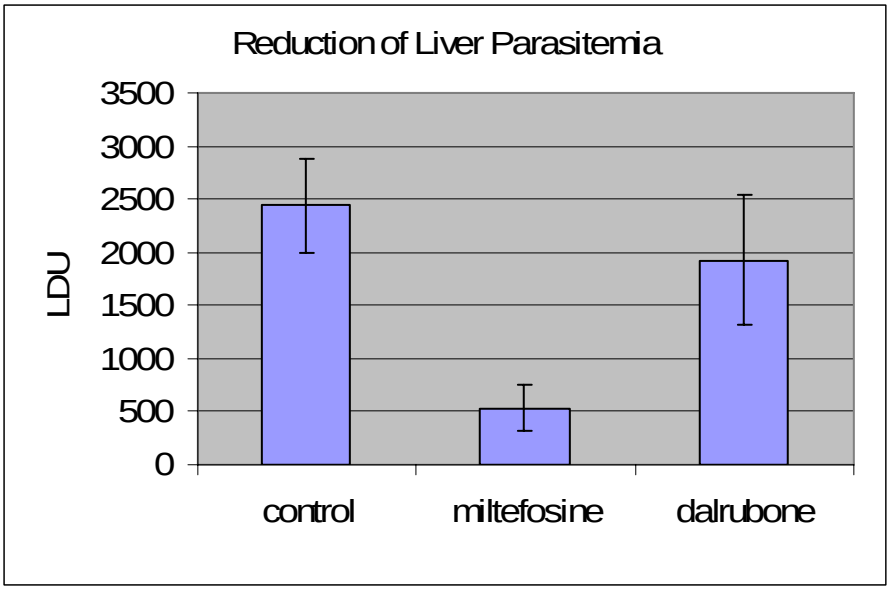


Figure 4.13: In Vivo Activity of Dalrubone against *L. donovani* Infection in BALB/c Mice.

Mice were infected with *L. donovani* amastigotes and 7 days later administered dalrubone (50 mg/kg/day s.c.), miltefosine (10 mg/kg/day i.p.) or vehicle alone for 5 days. Four days post-treatment, mice were sacrificed and liver parasitemia assessed microscopically. LDU values are indicative of the level of the parasitemia in the liver and were calculated from the number of amastigotes per 1000 macrophage nuclei multiplied by the liver weight in grams. The error bars represent the 95% confidence range.

4.4 SUMMARY AND CONCLUSIONS

Bioactivity-guided fractionation of the methanolic extract of *Psorothamnus polydenius* yielded the new chalcone, 2,2′,4′-trihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.2**), together with six other known compounds, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (**4.1**), dalrubone (**4.3**), demethoxymatteucinol (**4.4**), eriodictyol (**4.5**), photodalrubone (**4.6a** and **4.6b**), and oleanolic acid (**4.7**). This is the first report of chalcones in *P. polydenius*. The extracts and isolated compounds were tested in vitro for their antiprotozoal activity against *Leishmania donovani* and *Trypanosoma brucei*. Chalcones **4.1**, **4.2**, and dalrubone (**4.3**) exhibited leishmanicidal (IC_{50} 5.0, 7.5, and 7.5 μg/mL, respectively) and trypanocidal (IC_{50} 6.3, 6.8, and 21.6 μg/mL, respectively) properties. Dalrubone (**4.3**) displayed 6-fold selectivity for axenic *L. donovani* parasites over Vero cells. Furthermore, treatment of *L. mexicana*-preinfected macrophages with chalcones **4.1**, **4.2**, and dalrubone (**4.3**) (12.5, 12.5, and 25 μg/mL, respectively) reduced the number of infected macrophages by at least 96% while posing no toxicity to the host cell.

Our results revealed that *P. polydenius* is worthy of further consideration as an inexpensive herbal remedy for leishmaniasis as evidenced by the *in vitro* results. The major active metabolite, dalrubone (**4.3**), can be easily isolated in reasonable amounts from the aerial parts of the plant and was shown for the first time to possess significant activity and selectivity towards the *Leishmania* parasite.

We attempted to investigate the antileishmanial mechanism of action of dalrubone. Flow cytometric analysis of the cell cycle of *L. donovani* promastigotes treated with 50 μM dalrubone for 48 h showed no significant difference compared to that of control parasites. However, transmission electron micrographs of dalrubone-treated promastigotes revealed major ultrastructural changes in the parasite, including the emergence of large concentric membranous structures in the cytoplasm together with dark pigment-containing vacuoles that were not present in the non-treated cells. It appears that the antileishmanial mechanism of action of dalrubone involves an interference with the secretory pathways of the parasite. Further investigations are needed to clarify the specific intracellular target and the mechanism of action.

Dalrubone did not show activity in the in vivo murine *Leishmania* model. This could be due to a lack of sufficient potency and/or pharmacokinetic problems (possibly extensive drug metabolism to inactive metabolites). However, these problems may be overcome by modifying the structure using medicinal chemistry approaches to obtain more potent analogs.

CHAPTER 5

ISOFLAVONOIDS AND OTHER COMPOUNDS FROM *PSOROTHAMNUS ARBORESCENS* WITH ANTIPROTOZOAL ACTIVITIES

5.1 INTRODUCTION

The genus *Psorothamnus* (name from the Greek *psoros* and *thamnus*, meaning scurfy or scab shrub) belongs to the legume family (Fabaceae). It has been shown in our laboratory to be a rich source of antiparasitic compounds. The genus includes nine species found in the deserts of southwestern North America.[203] In the previous chapter, we isolated antileishmanial and trypanocidal compounds from *P. polydenius* (S. Watson) Rydberg that displayed selectivity for these organisms.[204] The plant *Psorothamnus arborescens* (Torrey ex A. Gray) Barneby var. *minutifolius* (Parish) Barneby, Fabaceae-Papilionoideae[188,203] [synonym *Dalea fremontii* Torrey ex A. Gray var. *minutifolia* (Parish) L. Benson][205] is also known as Mojave dalea[206] or indigo bush.[207] This plant is a small, thorny desert shrub usually up to 1 meter in height with deep indigo blue or violet flowers and is locally common through the west-central and north Mojave Desert, California, growing chiefly on granitic and volcanic bedrock.[203,206] *P. arborescens* var. *minutifolius* and also var. *simplicifolius* can have a distinct basal stem that extends above the ground to about 30 cm and the shrub can reach up to 2 meters in height, hence the

epithet *arborescens* (personal communication with Dr. R. W. Spjut, WBA). This species has not been investigated before and there are no previous reports of any antiparasitic activity associated with this plant. The closely related *P. fremontii* (*D. fremontii*), which occurs in California only on the sedimentary ranges of the far eastern Mojave Desert,[203] is reported to have been used by the indigenous tribes to stop internal hemorrhage and for the treatment of stomach problems.[164] Another related species, *P. polydenius* (*D. polyadenia*), is a medicinal plant generally well-known by all the native tribes of Nevada for the treatment of numerous ailments including respiratory infections, venereal diseases, smallpox and measles, in addition to kidney problems, muscular pains and sores.[164]

Psorothamnus arborescens extracts exhibited significant activity against *Leishmania donovani* axenic amastigotes and bloodstream form *Trypanosoma brucei brucei.* Bioassay-guided fractionation of the methanolic root extract from this plant resulted in the isolation of several bioactive compounds. In this chapter, we report on the structure elucidation and antiprotozoal activities of compounds **5.1a–5.8**. The structure of fremontin, an isoflavone previously isolated from *P. fremontii*,[208] has also been revised.

5.2 EXPERIMENTAL SECTION

5.2.1 General Experimental Procedures

Melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer polarimeter using a 100 mm glass microcell. UV-vis spectra were taken in methanol

using a SPECTRAmax PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA). IR spectra were obtained in KBr or as a film on a NaCl disc using a Nicolet Protégé 460 FT-IR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker 300 and 400 MHz spectrometers using the solvents $CDCl_3$, CD_3CN, methanol-d_4, or acetone-d_6 (Aldrich) with TMS as the internal standard. ^{13}C DEPT, ^{1}H-^{1}H COSY, DPFGSE-NOE (selective NOE),[209] NOESY, HSQC and HMBC NMR spectra were obtained using standard Bruker pulse sequences. All accurate mass experiments were performed on a Micromass ESI-TOF™ II (Micromass, Wythenshawe, UK) mass spectrometer. Column chromatography was conducted using silica gel 60 (63-200 μm particle size) from EM Science or Sephadex LH-20 from Amersham Biosciences. Silica gel 60 H (15–45 μm particle size) from EM Science was used for vacuum-liquid chromatography (VLC). Precoated TLC silica gel 60 F_{254} plates from EM Science were used for thin layer chromatography (0.25 mm and 2 mm layer thickness for analytical and preparative TLC, respectively). Spots were visualized using UV light or vanillin/sulfuric acid reagent (1% vanillin in 10% ethanolic H_2SO_4 containing 15% H_2O). HPLC runs were carried out using a System Gold model 127 pump equipped with a model 166 UV detector (Beckman) and 4.6 × 250 mm or 10 × 250 mm C18-A Polaris columns (Varian) for analytical (flow rate = 1 mL/min) or semi-preparative runs (flow rate = 5 mL/min), respectively. The solvents used were water, MeOH and CH_3CN, each with 0.1% AcOH, and are designated as A, B and C, respectively. Daidzein, 7,4′-dimethoxyisoflavone, 7,3′,4′-trihydroxyisoflavone and apigenin were purchased from Alfa Aesar, and genistein

was from Indofine Chemical Company (Somerville, NJ). Other compounds and reagents were obtained from Sigma-Aldrich.

5.2.2 Plant Material

Psorothamnus arborescens var. *minutifolius* root was collected on June 11th, 2003 from E. Tulare Co., California, on the eastern slopes of the southern Sierra Nevada in a desert-chaparral transitional vegetation by Dr. Richard W. Spjut (WBA 4841-13, SPJ-15357), World Botanical Associates (Bakersfield, CA). Voucher specimens are deposited at the Botanical Research Institute of Texas (BRIT), the United States National Herbarium, the Smithsonian Institution (US), and the World Botanical Associates (WBA).

Figure 5.1: Photos of *Psorothamnus arborescens* var. *minutifolius*

A. A shrub growing in Inyo Co., California
B. Voucher for the sample collected on June 11th, 2003 (Spjut 15357)
Photos were obtained from the WBA (www.worldbotanical.com) with permission.

5.2.3 Extraction and Isolation

The dried and powdered root material (0.6 kg) was percolated with 2.9 L of 95% EtOH. The resulting gummy extract (38 g) was partitioned between H_2O and CH_2Cl_2. The organic layer was then partitioned between 90% MeOH (F001, 12.0 g) and hexane (F002, 2.0 g). The aqueous fraction was further partitioned between EtOAc (F003, 0.5 g) and H_2O (F004, 10.4 g). In addition, an insoluble fraction (F005, 8.1 g) was also separated (see Figure 4.2 for the general solvent extraction and fractionation scheme). The IC_{50} values of F001 to F005 were 16.5, 143, 66.6, >400, and 36.1 μg/mL, respectively, in the axenic amastigote assay. F001 also showed a good antitrypanosomal activity (IC_{50} = 6–12 μg/mL). F001 was chromatographed over Sephadex LH-20 (100 g resin pre-swollen in MeOH) eluting with MeOH. The fractions were combined according to their TLC profile into seven pools (F006—F012). The bioactivity was found to be highest in fractions F008, F009 and F010. F008 (0.86 g) was chromatographed on a silica gel column eluting with a gradient of 20% EtOAc–hexane to 100% EtOAc. The fraction eluted with 25% EtOAc–hexane was purified using preparative HPLC with an isocratic solvent system of 90% B in A, λ_{max} 210 nm, to collect 23 mg of compound **4.7** (oleanolic acid, t_R 11 min). F009 (9.4 g) was subjected to silica gel chromatography using a solvent gradient from 30% EtOAc–hexane to 100% EtOAc to 25% MeOH–EtOAc to obtain nine fractions, F009-1 to F009-9. F009-4 (750 mg) was subjected to Sephadex LH-20 chromatography followed by HPLC using a gradient from 50% to 60% B in A over 30 min then isocratic 60% B in A to yield compound **5.3a** (22.5 mg, t_R 53 min), compound **5.7** (3.5 mg, t_R 37 min), and crude compound **5.8** (20 mg, t_R 8–12 min). The latter was

purified by additional HPLC using a gradient solvent of 40% to 60% B in A in 60 min to isolate 9.1 mg of **5.8** (t_R = 48–54 min). F009-5 (500 mg) was chromatographed on a silica gel column using a gradient solvent of 4% EtOAc–$CHCl_3$ to 100% $CHCl_3$ to 5% MeOH–$CHCl_3$. Fractions were combined on the basis of their TLC profile to give 150 mg of crude compound **5.1a** that was purified by HPLC using a solvent gradient of 50% B in A increased to 60% over 30 min then kept isocratic at 60% B, collecting fractions eluting at t_R 28–34 min to give 94 mg of pure compound **5.1a**. F009-8 (917 mg) was subjected to silica gel chromatography eluting with a gradient of 100% CH_2Cl_2 to 100% EtOAc. Additional silica gel column chromatography of the fraction eluting with 35% EtOAc in CH_2Cl_2 (F009-8-3, 260 mg) using the isocratic solvent $CHCl_3$–MeOH–H_2O (90:6:1, lower phase) followed by HPLC of sub-fraction 3 to obtain compound **5.4** (6 mg, isocratic 20% C in A, t_R 32 min) and subfraction 4 (50% B in A increased to 58% over 24 min then kept isocratic, collecting fractions eluting at t_R 24–28 min) to give 42.6 mg of compound **5.2a**. A portion (95 mg) of F010 was purified using preparative HPLC with an isocratic solvent system of 50% solvent B in A to obtain 24 mg of compound **5.6** (t_R 13.5 min) and 57 mg of compound **5.5** (t_R 29 min). The purity of the isolated compounds was ascertained by carrying out HPLC and TLC analysis in at least two different solvens.

5.2.4 Isolated Compounds and Semisynthetic Derivatives

5,7,3′,4′-Tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (5.1a): Greenish white residue; mp 203–205 °C; UV (MeOH) λ_{max} (log ε) 210 (4.53), 260 (4.42), 285 sh (4.00) nm; IR υ_{max} (KBr) 3446, 3223 (br OH), 3081, 2976, 2927, 1704, 1654, 1619, 1570, 1492, 1444, 1360, 1304, 1200, 1051 cm^{-1}; ^{1}H, ^{13}C (acetone-d_6, 400 and 100 MHz, respectively), and HMBC NMR data, see Table 5.1; HRESIMS *m/z* 377.1000 $[M+Na]^+$ (calcd for $C_{20}H_{18}NaO_6$, 377.1001).

C	**5.1a**			**5.1b**		
	δ_C	δ_H (mult.; J_{HH}, Hz)	HMBC	δ_C	δ_H (mult.; J_{HH}, Hz)	HMBC
2	155.2	7.89 (s)	C-3, 4, 9, 10[a],1′	155.5	8.02 (s)	C-3, 4, 9, 1′
3	125.3			124.9		
4	182.0			181.5		
5	163.8			163.9		
6	99.8	6.29 (d; 1.3)	C-4[a], 5, 7, 8, 10	99.9	6.30 (d; 1.3)	C-4[a], 5, 7, 8, 10
7	165.0			165.1		
8	94.5	6.43 (d; 1.3)	C-4[a], 6, 7, 9, 10	94.6	6.44 (d; 1.3)	C-4[a], 6, 7, 9, 10
9	159.2			159.3		
10	106.1			106.0		
1'	123.9			122.9[c]		
2'	129.2			122.7[c]		
3'	144.2			142.4		
4'	145.6			147.5		
5'	113.1	6.76 (d; 8.0)	C-1′, 2′[a], 3′	112.7	6.70 (d; 8.0)	C-1′, 3′
6'	123.0	6.56 (d; 8.0)	C-3, 2′, 4′, 1′′[a]	122.8	6.64 (d; 8.0)	C-3, 2′, 4′
1"	27.3	3.30 (br s)	C-1′, 2′, 3′, 2′′, 3′′	21.3	2.66 (br t; 6.8)	C-1′, 2′, 3′, 2′′, 3′′
2"	124.4	5.08 (m)	C-2′, 1′′, 4′′, 5′′	33.3	1.78 (t; 6.8)	C-2′, 1′′, 3′′, 4′′, 5′′
3"	130.8			75.2		
4"[b]	17.6	1.43 (s)	C-2′′, 3′′, 5′′	26.8	1.35 (s)	C-2′′, 3′′, 5′′
5"[b]	25.6	1.52 (s)	C-2′′, 3′′, 4′′	26.8	1.35 (s)	C-2′′, 3′′, 4′′
5-OH		13.00 (s)	C-5, 6, 10		12.98 (s)	C-5, 6, 10

[a] Weak 4-bond HMBC correlations. [b,c] Entries with the same superscript might be interchangeable.

Table 5.1: ^{1}H and ^{13}C NMR Assignments and HMBC Correlations for Compounds **5.1a** and **5.1b** in acetone-d_6

Acid-catalyzed cyclization of compound 5.1a: Compound **5.1a** (9.5 mg, 0.03 mmol) was stirred with 88% HCOOH (1 mL) at 80 °C overnight. The product **5.1b** (8.0 mg, 84% yield) was purified by HPLC (50 to 100% solvent B in A in 50 min; t_R = 22 min; mp 247–249 °C; ^{1}H, ^{13}C (acetone-d_6, 400 and 100 MHz, respectively), and HMBC NMR data, see Table 5.1).

Fremontin (5.2a): Greenish white powder, mp 140–142 °C and 236–240 °C (lit.[208] 244–247 °C); UV (MeOH) λ_{max} (log ε) 259 (4.37), 292 (3.99) nm; IR υ_{max} (film) 3366 (br OH), 1652, 1617, 1576, 1508, 1437, 1361, 1277, 1169 cm^{-1}; ^{1}H, ^{13}C (acetone-d_6, 400 and 100 MHz, respectively), and HMBC NMR data, see Table 5.4; HRESIMS *m/z* 377.1027 $[M+Na]^+$ (calcd for $C_{20}H_{18}NaO_6$, 377.1001).

Acetylation of fremontin: Fremontin (4 mg, 0.01 mmol) was acetylated with acetic anhydride (0.3 mL) in pyridine (0.3 mL) by stirring at room temperature overnight. Fremontin tetraacetate (**5.2b,** 3.5 mg, 59% yield) was purified by HPLC (gradient B in A, 70 to 100% in 30 min; t_R = 7.5 min; mp 195–197 °C (lit.[208] mp 195 °C); ^{1}H NMR (acetone-d_6, 400 MHz) δ 8.00 (1H, s, H-2), 7.39 (1H, s, H-5′), 7.35 (1H, d, *J* = 2.2 Hz, H-8), 6.97 (1H, d, *J* = 2.2 Hz, H-6), 6.95 (1H, s, H-2′), 5.99 (1H, dd, *J* = 10.6, 17.5 Hz, H-2″), 4.83 (1H, dd, *J* = 1.1, 17.5 Hz, H-3″a), 4.72 (1H, dd, *J* = 1.1, 10.6 Hz, H-3″b), 2.34, 2.29, 2.26, and 2.25 (3H each, s, CO*CH*$_3$), 1.38 and 1.33 (3H each, s, CH_3-4″ and -5″); ^{13}C NMR (acetone-d_6, 100 MHz) δ 175.9 (C, C-4), 169.3, 168.8, 168.7, and 168.5 (C each, *CO*CH_3), 158.6 (C, C-9), 155.2 (C, C-7), 153.6 (CH, C-2), 151.6 (C, C-5), 148.7 (CH, C-2″), 147.3 (C, C-4′), 143.1 (C, C-3′), 141.0 (C, C-6′), 130.2 (C, C-1′), 128.9 (CH, C-2′), 128.3 (C, C-3), 123.3 (CH, C-5′), 116.1 (C, C-10), 114.8 (CH, C-6), 110.8 (CH_2,

C-3″), 109.9 (CH, C-8), 42.8 (C, C-1″), 29.9 and 29.5 (CH_3 each, CH_3-4″ and -5″), 21.0, 21.0, 20.53, and 20.45 (CH_3 each, CO*CH*$_3$); HMBC correlations: H-2 to C-3, -4, -9, -1′, H-6 to C-5, -7, -8, -10, H-8 to C-6, -7, -9, -10, H-2′ to C-3, -3′, -4′, -6′, H-5′ to C-1′, -3′, -6′, -1″, H-2″ to C-1″, -4″, -5″, H-3″ to C-1″, -2″, CH_3-4″ to C-6′, -1″, -2″, -5″, CH_3-5″ to C-6′, -1″, -2″, -4″, CO*CH*$_3$ to *CO*CH_3.

Methylation of fremontin: Fremontin (20 mg, 0.06 mmol) in anhydrous acetone (9 mL) was refluxed for 2 h with excess K_2CO_3 (125 mg) and Me_2SO_4 (0.1 mL, 1.06 mmol) with molecular sieves (4 Å). PTLC (2% MeOH in $CHCl_3$) of the products yielded trimethylfremontin (**5.2c**, 10.5 mg, 47% yield) that was recrystallized from EtOAc/hexane as grayish needles (R_f 0.47; mp 163–164 °C; ^{1}H, ^{13}C ($CDCl_3$, 400 and 100 MHz, respectively), and HMBC NMR data, see Table 5.4) and tetramethylfremontin (**5.2d**, 11.0 mg, 47% yield, R_f 0.23, yellow residue, mp 98–100 °C; ^{1}H NMR (acetone-d_6, 400 MHz) δ 7.62 (1H, s, H-2), 7.05 (1H, s, H-5′), 6.59 (1H, s, H-2′), 6.56 (1H, d, *J* = 2.3 Hz, H-8), 6.48 (1H, d, *J* = 2.3 Hz, H-6), 6.05 (1H, dd, *J* = 10.6, 17.6 Hz, H-2″), 4.82 (1H, dd, *J* = 1.2, 17.6 Hz, H-3″a), 4.68 (1H, dd, *J* = 1.2, 10.6 Hz, H-3″b), 3.93 (3H, s, 7-OCH_3), 3.86 (3H, s, 5-OCH_3), 3.84 (3H, s, 4′-OCH_3), 3.75 (3H, s, 3′-OCH_3), 1.38 (3H, s, CH_3-4″), 1.33 (3H, s, CH_3-5″); ^{13}C NMR (acetone-d_6, 100 MHz) δ 176.0 (C, C-4), 164.8 (C, C-7), 162.3 (C, C-5), 160.8 (C, C-9), 151.6 (CH, C-2), 150.2 (CH, C-2″), 149.5 (C, C-4′), 147.9 (C, C-3′), 140.8 (C, C-6′), 129.7 (C, C-3), 125.1 (C, C-1′), 118.1 (CH, C-2′), 112.7 (C, C-5′), 110.5 (C, C-10), 109.5 (CH_2, C-3″), 96.7 (CH, C-6), 93.6 (CH, C-8), 56.47, 56.25, 56.21, and 56.09 (CH_3 each, OCH_3-5, 7, 3′, and 4′), 42.8 (C, C-1″), 29.7 (CH_3, CH_3-4″), 28.9 (CH_3, CH_3-5″); HMBC correlations: H-2 to C-3, -4, -9, -1′, H-6 to

C-5, -7, -8, -10, H-8 to C-6, -7, -9, -10, H-2′ to C-3, -4′, -6′, H-5′ to C-1′, -3′, -1″, H-2″ to C-1″, -4″, -5″, H-3″ to C-1″, -2″, CH_3-4″ to C-6′, -1″, -2″, -5″, CH_3-5″ to C-6′, -1″, -2″, -4″, OCH_3-3′ to C-3′, OCH_3-4′ to C-4′, OCH_3-5 to C-5, OCH_3-7 to C-7.

Glycyrrhisoflavone (5.3a): Brownish white residue, mp 98–100 °C; UV (MeOH) λ_{max} (log ε) 261 (4.48), 292 sh (4.13) nm; IR υ_{max} (film) 3373 (br OH), 1651, 1622, 1575, 1504, 1442, 1367, 1307, 1284, 1178, 1051, 838 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 13.07 (1H, br s, 5-OH), 8.09 (1H, s, H-2), 7.02 (1H, d, J = 1.2 Hz, H-2′), 6.84 (1H, d, J = 1.2 Hz, H-6′), 6.40 (1H, br s, H-8), 6.28 (1H, br s, H-6), 5.37 (1H, m, H-2″), 3.37 (2H, d, J = 7.3 Hz, H-1″), 1.73 (3H, s, CH_3-4″), 1.70 (3H, s, CH_3-5″); ^{13}C NMR (acetone-d_6,100 MHz) δ 181.6 (C, C-4), 165.1 (C, C-7), 163.9 (C, C-5), 159.0 (C, C-9), 154.2 (CH, C-2), 144.9 (C, C-4′), 144.2 (C, C-3′), 132.3 (C, C-3″), 128.8 (C, C-5′), 124.3 (C, C-3), 123.7 (CH, C-2″), 122.7 (C, C-1′), 122.1 (CH, C-6′), 114.7 (CH, C-2′), 106.0 (C, C-10), 99.8 (CH, C-6), 94.4 (CH, C-8), 29.0 (CH_2, C-1″), 25.9 (CH_3, CH_3-4″), 17.8 (CH_3, CH_3-5″); HMBC correlations: H-2 to C-3, -4, -9, -1′, H-6 to C-5, -7, -8, -10, H-8 to C-6, -7, -9, -10, H-2′ to C-3, -3′, -4′, -6′, H-6′ to C-3, -2′, -4′, -1″, H-1″ to C-4′, -5′, -6′, -2″, -3″, H-2″ to C-5′, 1″, -4″, -5″, CH_3-4″ to C-2″, -3″, -5″, CH_3-5″ to C-2″, -3″, -4″; HRESIMS *m/z* 377.1012 $[M+Na]^+$ (calcd for $C_{20}H_{18}NaO_6$, 377.1001).

Acid-catalyzed cyclization of glycyrrhisoflavone (5.3a): Glycyrrhisoflavone (**5.3a**, 4.0 mg, 0.01 mmol) in 1 mL HCOOH (88%) was stirred at 90 °C for 5 h. The reaction mixture was partitioned between H_2O (2 mL) and EtOAc (3 × 2 mL). After drying the organic layer in vacuo, the product **5.3b** (2.5 mg, 63% yield) was purified by HPLC (50 to 100% B in A in 50 min.; t_R = 25 min); ^1H NMR (acetone-d_6, 400 MHz) δ

13.07 (1H, br s, 5-OH), 8.13 (1H, s, H-2), 6.93 (1H, d, J = 2.1 Hz, H-2′), 6.84 (1H, d, J = 2.1 Hz, H-6′), 6.41 (1H, d, J = 1.9 Hz, H-8), 6.28 (1H, d, J = 1.9 Hz, H-6), 2.81 (2H, t, J = 6.7 Hz, H-1″), 1.86 (2H, t, J = 6.7 Hz, H-2″), 1.35 and 1.35 (3H each, s, CH_3-4″ and -5″); ^{13}C NMR (acetone-d_6, 100 MHz) δ 181.5 (C, C-4), 165.8 (C, C-7), 163.6 (C, C-5), 159.1 (C, C-9), 154.2 (CH, C-2), 146.6 (C, C-3′), 142.7 (C, C-4′), 124.1[a] (C, C-3), 123.2[a] (C, C-1′), 122.4 (CH, C-6′), 121.9 (C, C-5′), 114.3 (CH, C-2′), 105.8 (C, C-10), 100.0 (CH, C-6), 94.6 (CH, C-8), 75.7 (C, C-3″), 33.5 (CH_2, C-2″), 27.0 and 27.0 (CH_3 each, CH_3-4″ and -5″), 22.9 (CH_2, C-1″) (assignments with the same superscript might be interchangeable); HMBC correlations: H-2 to C-3, -4, -9, -1′, H-6 to C-5, -7, -8, -10, H-8 to C-6, -7, -9, -10, H-2′ to C-3, -3′, -4′, -6′, H-6′ to C-3, -2′, -4′, -1″, H-1″ to C-4′, -5′, -6′, -2″, -3″, H-2″ to C-5′, 1″, -3″, -4″, -5″, CH_3-4″ and -5″ to C-2″, -3″.

Methylation of glycyrrhisoflavone: Glycyrrhisoflavone (**5.3a**, 9.0 mg, 0.03 mmol) was heated to reflux with excess K_2CO_3 (280 mg) and excess Me_2SO_4 (0.5 mL, 5.3 mmol) in 9.0 mL of anhydrous acetone under 4 Å molecular sieves for 5 h. The product **5.3c** (6.2 mg, 59% yield) was purified by HPLC (50 to 100% B in A in 50 min.; t_R 34 min.; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.77 (1H, s, H-2), 7.11 (1H, d, J = 1.9 Hz, H-2′), 6.81 (1H, d, J = 1.9 Hz, H-6′), 6.45 (1H, d, J = 2.2 Hz, H-8), 6.38 (1H, d, J = 2.2 Hz, H-6), 5.28 (1H, m, H-2″), 3.94 (3H, s, 5-OCH_3), 3.89 (3H, s, 7-OCH_3), 3.88 (3H, s, 3′-OCH_3), 3.82 (3H, s, 4′-OCH_3), 3.36 (2H, d, J = 7.2 Hz, H-1″), 1.72 (3H, s, CH_3-4″), 1.71 (3H, s, CH_3-5″); ^{13}C NMR ($CDCl_3$,100 MHz) δ 175.3 (C, C-4), 163.8 (C, C-7), 161.4 (C, C-5), 159.8 (C, C-9), 152.2 (C, C-3′), 150.4 (CH, C-2), 146.8 (C, C-4′), 135.2 (C, C-5′), 132.1 (C, C-3″), 127.7 (C, C-3), 126.2 (C, C-1′), 123.0 (CH, C-2″), 122.1 (CH, C-6′),

111.8 (CH, C-2′), 109.9 (C, C-10), 96.2 (CH, C-6), 92.5 (CH, C-8), 60.5, 56.4, 55.8, and 55.7 (CH_3 each, 5-, 7-, 3′-, 4′-OCH_3), 28.6 (CH_2, C-1″), 25.8 (CH_3, CH_3-4″), 17.8 (CH_3, CH_3-5″); HMBC correlations: H-2 to C-3, -4, -9, -1′, H-6 to C-5, -7, -8, -10, H-8 to C-6, -7, -9, -10, H-2′ to C-3, -3′, -4′, -6′, H-6′ to C-3, -2′, -4′, -1″, H-1″ to C-4′, -5′, -6′, -2″, -3″, H-2″ to 1″, -4″, -5″, CH_3-4″ to C-2″, -3″, -5″, CH_3-5″ to C-2″, -3″, -4″).

Calycosin (5.4): White residue; UV (MeOH) λ_{max} (log ε) 248 (4.44), 260 (sh 4.41), 290 (4.21) nm; IR υ_{max} (film) 3205 (br OH), 1623, 1585, 1511, 1454, 1280, 1196, 1133, 1024, 852 cm^{-1}; ^1H, ^{13}C, and HMBC NMR data consistent with literature values;[210-212] ^1H NMR (methanol-d_4, 400 MHz) δ 8.12 (1H, s, H-2), 8.04 (1H, d, J = 8.8 Hz, H-5), 7.04 (1H, br s, H-2′), 6.96 (2H, m, H-5′ and H-6′), 6.93 (1H, dd, J = 2.0, 8.8 Hz, H-6), 6.83 (1H, d, J = 2.0 Hz, H-8), 3.88 (3H, s, 4′′-OCH_3); ^{13}C NMR (methanol-d_4, 100 MHz) δ 178.0 (C, C-4), 164.9 (C, C-7), 159.8 (C, C-9), 154.8 (CH, C-2), 149.2 (C, C-4′), 147.4 (C, C-3′), 128.5 (CH, C-5), 126.2 (C, C-1′), 125.8 (C, C-3), 121.6 (CH, C-6′), 118.1 (C, C-10), 117.4 (CH, C-2′), 116.6 (CH, C-6), 112.6 (C, C-5′), 103.3 (CH, C-8), 56.4 (CH_3, 4′′-OCH_3); HRESIMS *m/z* 307.0578 $[M+Na]^+$ (calcd for $C_{16}H_{12}NaO_5$, 307.0582).

Maackiain (5.5): white powder; mp 186–188 °C, $[\alpha]_D$ = –33.5°; UV (MeOH) λ_{max} (log ε) 213 (4.38), 286 (3.66), 310 (3.85) nm; IR υ_{max} (KBr) 3393 (br OH), 2927, 1624, 1474, 1456, 1347, 1320, 1186, 1144, 1122 cm^{-1}; ^1H, ^{13}C, and HMBC NMR data consistent with literature values[213-216] ^1H, ^{13}C (acetone-d_6, 300 and 75 MHz, respectively), see Table 5.2; HRESIMS *m/z* 307.0603 $[M+Na]^+$ (calcd for $C_{16}H_{12}NaO_5$, 307.0582).

3,4-Dihydroxy-8,9-methylenedioxypterocarpan (4-hydroxymaackiain, 5.6): Amorphous white powder, mp 186–189 °C, $[\alpha]_D = +62.2°$; UV (MeOH) λ_{max} (log ε) 213 (4.66), 310 (3.85) nm; IR υ_{max} (film) 3423 (br OH), 1625, 1476, 1459, 1338, 1206, 1144, 1052, 936 cm^{-1}; ^1H, ^{13}C, and HMBC NMR data consistent with literature values,[213,217] ^1H, ^{13}C (acetone-d_6, 300 and 75 MHz, respectively), see Table 5.2; HRESIMS *m/z* 323.0521 $[M+Na]^+$ (calcd for $C_{16}H_{12}NaO_6$, 323.0532).

	Maackiain (**5.5**)		4-Hydroxymaackiain (**5.6**)	
position	δ_C	δ_H (mult.; J_{HH})	δ_C	δ_H (mult.; J_{HH})
1	133.0	7.30 (d; 8.4)	121.9	6.85 (d; 8.4)
2	110.4	6.55 (dd; 2.2, 8.4)	110.1	6.57 (d; 8.4)
3	159.7		146.8	
4	103.9	6.36 (d; 2.2)	133.8	
4a	157.7		145.3	
6	67.0	4.27 (dd; 4.0, 10.0)	67.3	4.32 (dd; 4.5, 10.5)
		3.62 (dd; 10.0, 10.0)		3.65 (dd; 10.5, 10.5)
6a	41.0	3.56 (ddd; 4.0, 6.7, 10.0)	41.2	3.59 (ddd; 4.5, 7.1, 10.5)
6b	119.5		119.4	
7	105.9	6.89 (s)	105.9	6.88 (s)
8	142.4		142.4	
9	148.9		148.9	
10	93.9	6.40 (s)	93.9	6.39 (s)
10a	155.3		155.3	
11a	79.3	5.48 (d; 6.7)	79.6	5.52 (d; 7.1)
11b	112.8		113.6	
OCH_2O	102.1	5.922 (d; 11.0)	102.1	5.923 (d; 8.0)
		5.920 (d; 11.0)		5.919 (d; 8.0)

Table 5.2: ^1H and ^{13}C NMR Assignments and HMBC Correlations for Compounds **5.5** and **5.6** in acetone-d_6

2-(2′-Hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (5.7): White residue, mp 230–235 °C; UV (MeOH) λ_{max} (log ε) 247 (4.21), 295 (3.93), 346 (4.05) nm; IR υ_{max} (KBr) 3281 (br OH), 2867, 1656, 1618, 1589,1506, 1461, 1314, 1202, 1141, 1034, 950, 893, 802 cm^{-1}; ^{1}H, ^{13}C (acetone-d_6, 400 and 100 MHz, respectively), and HMBC NMR data, see Table 5.3; HRESIMS *m/z* 335.0550 $[M+Na]^+$ (calcd for $C_{17}H_{12}NaO_6$, 335.0532).

position	δ_C	δ_H (mult.; J_{HH})	HMBC
2	163.5		
3	118.3		
4	133.5	7.50 (d; 8.4)	C-6,8
5	108.9	6.66 (dd; 2.2,8.4)	C-6,7,9
6	159.9		
7	100.5	6.71 (d; 2.2)	C-5,6,8,9
8	162.5		
9	109.8		
1'	119.5		
2'	150.4		
3'	94.1	7.16 (s)	C-1′,2′,5′
4'	147.8		
5'	146.7		
6'	100.9	7.52 (s)	C-2,2′,4′
3-CHO	187.7	10.00 (s)	C-3
6-OCH_3	56.1	3.86 (s)	C-6
–OCH_2O–	102.7	6.08 (s)	C-4′,5′

Table 5.3: ^{1}H and ^{13}C NMR Assignments and HMBC Correlations for Compound **5.7** in acetone-d_6

Isoliquiritigenin (5.8): Yellow powder, mp 190–191 °C (lit.[218] mp 193.5 – 195 °C); UV (MeOH) λ_{max} (log ε) 242 sh (3.86), 298 sh (3.78), 371 (4.30) nm; IR υ_{max} (KBr) 3357 (br OH), 1630, 1605, 1586, 1547, 1514, 1503, 1220, 1166, 1142; ^{1}H, ^{13}C, and HMBC NMR data consistent with literature values;[218,219] ^{1}H NMR (CD_3CN, 400 MHz) δ 13.50 (1H, br s, OH-2′), 7.98 (1H, d, *J* = 8.9 Hz, H-6′), 7.80 (1H, d, *J* = 15.4 Hz, H-α), 7.65 (2H, d, *J* = 8.6 Hz, H-2 and H-6), 7.58 (1H, d, *J* = 15.4 Hz, H-β), 6.88 (2H, d, *J* = 8.6 Hz, H-3 and H-5), 6.42 (1H, dd, *J* = 2.4, 8.9 Hz, H-5′), 6.32 (1H, d, , *J* = 2.4 Hz, H-3′); ^{13}C NMR (CD_3CN, 100 MHz) δ 193.0 (C, CO), 167.4 (C, C-2′), 165.6 (C, C-4′), 160.6 (C, C-4), 144.9 (CH, C-β), 133.5 (CH, C-6′), 131.8 (CH, C-2 and C-6), 127.8 (C, C-1), 118.7 (CH, C-α), 116.8 (CH, C-3 and C-5), 114.5 (C, C-1′), 108.8 (CH, C-5′), 103.8 (CH, C-3′); HRESIMS *m/z* 279.0647 $[M+Na]^+$ (calcd for $C_{15}H_{12}NaO_4$, 279.0633).

5.2.5 Biological Assays

Antileishmanial Assay Using Axenic Amastigotes. The antileishmanial activity of the isolated compounds was tested in vitro against *Leishmania donovani* amastigote-like parasites (WHO designation: MHOM/SD/62/1S-$CL2_D$) in a three-day assay using the tetrazolium dye-based CellTiter reagent (Promega) as described previously in Chapter 3.[159,160,220]

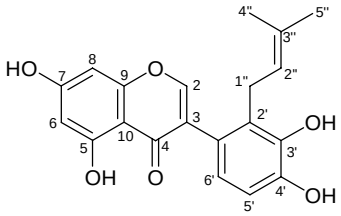
Antitrypanosomal Assay. Compounds were tested for their activity against bloodstream-form *Trypanosoma brucei brucei* (MITat 1.2, variant 221) axenically cultured in HMI-9 medium as described by Bodley et al.,[221] with minor modifications.

Briefly, 100 μL of late log phase parasites were incubated in 96-well plates (Costar) at an initial concentration of 10^5 cells/mL with or without test compounds at 37 °C in a humidified 5% CO_2 atmosphere for 72 h. Ten μL of a 20 mg/mL solution of *p*-nitrophenylphosphate (prepared in 1 M sodium acetate, pH 5.5, 1% TritonX-100) was then added to each well and plates were re-incubated at 37 ºC as before for 6–8 h. Optical densities were then measured at 405 nm using a SpectraMax Plus microplate reader (Molecular Devices). IC_{50} values, the concentration of the compound that inhibited cell growth by 50% compared to untreated control, were determined with the aid of the software program SoftMax Pro (Molecular Devices). This program uses the dose-response equation $y = ((a-d)/(1+(x/c)^b)) + d$, where x = the drug concentration, y = absorbance at 405 nm, a = upper asymptote, b = slope, c = IC_{50} and d = lower asymptote. This assay has the advantage of not being subject to interference by samples with a reducing potential (such as the *o*-catechols isolated and assayed in this study) as it depends on measuring acid phosphatase activity in surviving parasites.

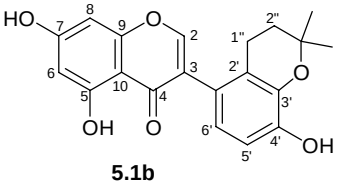
Cytotoxicity Assay. Cytotoxicity was evaluated against two cell lines, Vero cells and PC-3 prostate cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD) as described previously in Chapters 3 and 4.

5.3 RESULTS AND DISCUSSION

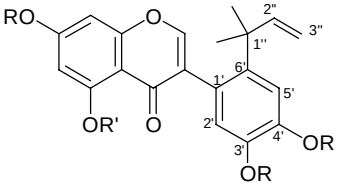
Bioactivity-guided fractionation of the methanolic extract of *P. arborescens* yielded four isoflavones, the new 5,7,3′,4′-tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (**5.1a**), fremontin (**5.2a**),[208] glycyrrhisoflavone (**5.3a**),[222] and calycosin (**5.4**),[210-212] the pterocarpans maackiain (**5.5**)[213-216] and 4-hydroxymaackiain (**5.6**),[213,217] the new 2-arylbenzofuran, 2-(2′-hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (**5.7**), the triterpene oleanolic acid (**4.7**),[199,212] and the chalcone isoliquiritigenin (**5.8**),[218,219] (Figure 5.2). The structures of the known compounds were determined by 1D and 2D NMR techniques and confirmed by comparing the physical and spectral data with those from the literature (mp, NMR, and MS).



5.1a



5.1b



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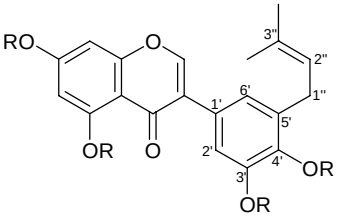
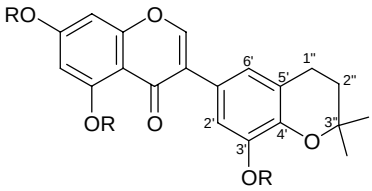
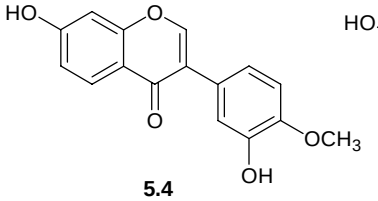
5.2b R = R' = Ac
5.2c R = Me, R' = H
5.2d R = R' = Me



5.3a R = H
5.3c R = Me



5.3b R = H



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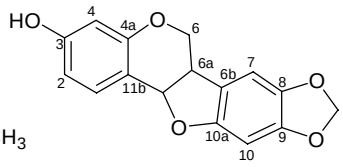

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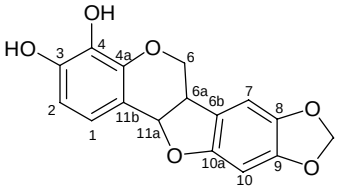

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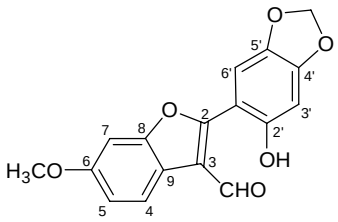

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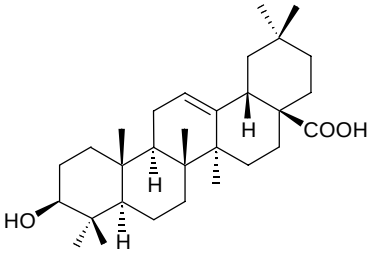

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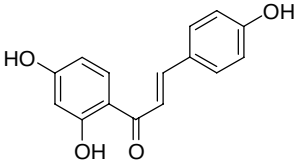


5.8

Figure 5.2: Structures of Compounds Isolated from *Psorothamnus arborescens* and Their Semisynthetic Derivatives

5.3.1 Structure Elucidation of 5,7,3′,4′-Tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (5.1a)

Isoflavone **5.1a** isolated from *P. arborescens* gave a ^{1}H NMR spectrum (see Figure 5.3) showing a chelated OH group at δ 13.00, a downfield singlet at δ 7.89, *o*-coupled aromatic protons at δ 6.56 and 6.76 (J = 8.0 Hz), *m*-coupled aromatic protons at δ 6.29 and 6.43 (J = 1.3 Hz), a methylene group at δ 3.30 displaying ^{1}H–^{1}H COSY correlation to a downfield methine at δ 5.08, in addition to two methyls at δ 1.43 and 1.52. The ^{13}C and DEPT NMR spectra (Figure 5.3) showed a carbonyl at δ 182.0, six oxygenated carbons in the aromatic region at δ 165.0, 163.8, 159.2, 155.2, 145.6, and 144.2, three methines at δ 124.4, 123.0 and 113.1, in addition to two relatively upfield methines at δ 99.8 and 94.5, one methylene at δ 27.3, two methyls at δ 25.6 and 17.6, and five quaternary carbons at δ 130.8, 129.2, 125.3, 123.9 and 106.1. HRESIMS confirmed the deduced molecular formula (measured $[M+Na]^{+}$ *m/z* 377.1000, calcd 377.1001 for $C_{20}H_{18}O_6$). The HMBC data (Figure 5.4) suggested an isoflavone structure hydroxylated at positions 5 and 7 of ring A and at 3′ and 4′ of ring B. H-2 at δ 7.89 showed a 3-bond HMBC correlation to C-1′ which is correlated to H-5′ and H-1′′. H-6′ shows 3-bond HMBC correlations to C-3 and C-2′. H-1′′ and H-2′′ of the side chain prenyl group are correlated to C-2′, thus establishing the position of the prenyl group. Further confirmation was provided via a selective NOE experiment in which H-2 showed NOE correlations to H-6′, H-1′′, and H-2′′. Assignment of ring B oxygenated carbons is based on the HMBC correlation of H-1′′ to C-3′, and that of H-6′ to C-4′. Furthermore, acid-catalyzed cyclization of the 2′-isoprenyl group gave the dihydrobenzopyran derivative

5.1b, verifying the presence of an OH group at the 3′ position. Another piece of evidence that would rule out the presence of an OH group in the 2′ position is the relatively downfield position of the OH-5 signal (δ 13.0); a 2′-OH group in an isoflavone structure would be expected to exert a significant shielding effect on the 5-OH, resulting in a more upfield chemical shift (δ 12.51–12.79).[22,23] The isoflavanone arizonicanol D, isolated from the root of *Sophora arizonica* (Fabaceae), possesses a B ring structure similar to that found in compound **5.1a** and their ^{1}H and ^{13}C NMR data are in agreement.[223] The structure of compound **5.1a** is also closely related to that of the isoflavone piscidone isolated from the root bark of the Jamaican dogwood *Piscidia erythrina* (Fabaceae).[224]

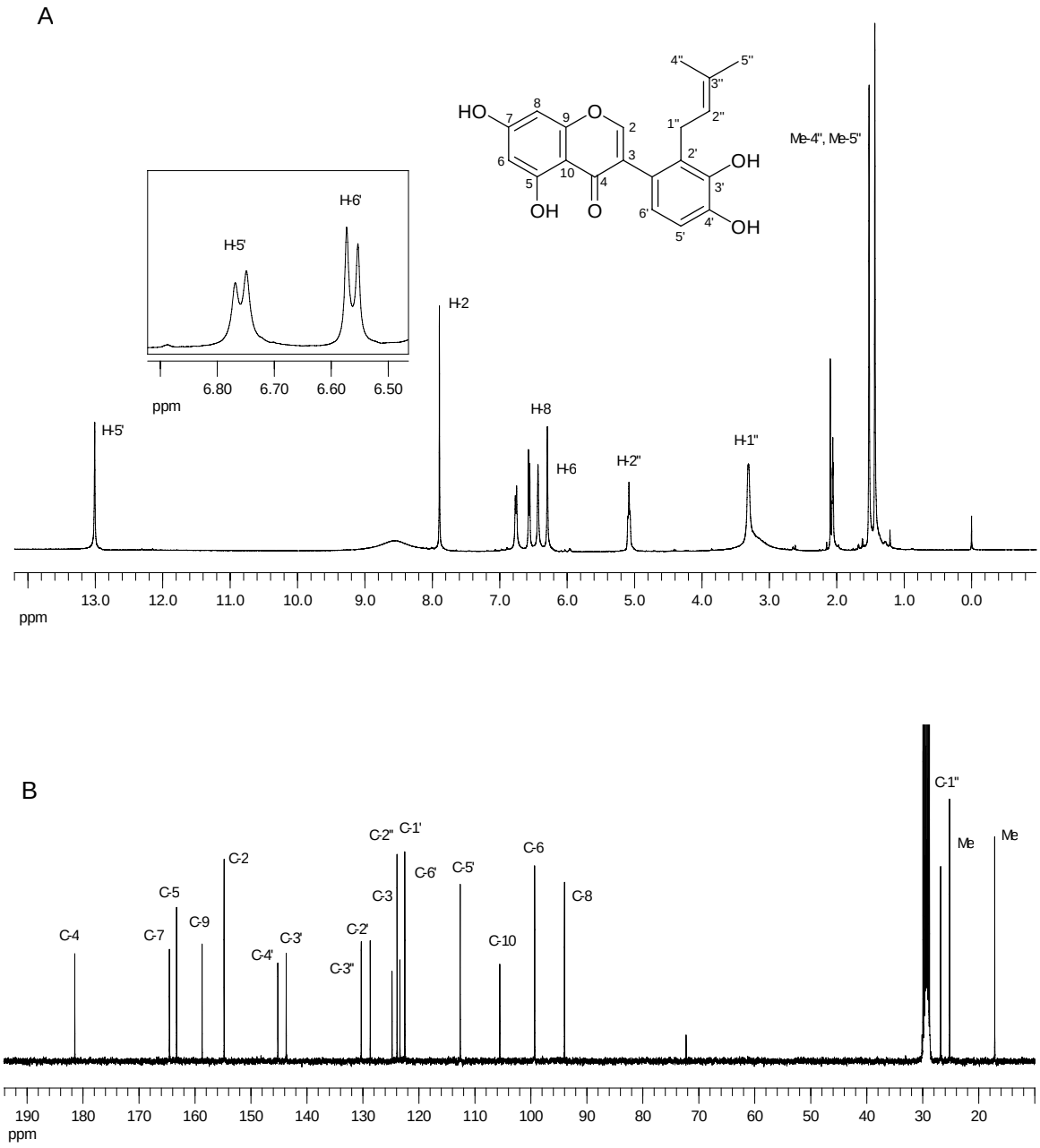


Figure 5.3: ^{1}H and ^{13}C NMR Spectra of 5,7,3′,4′-Tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (**5.1a**) in acetone-d_6

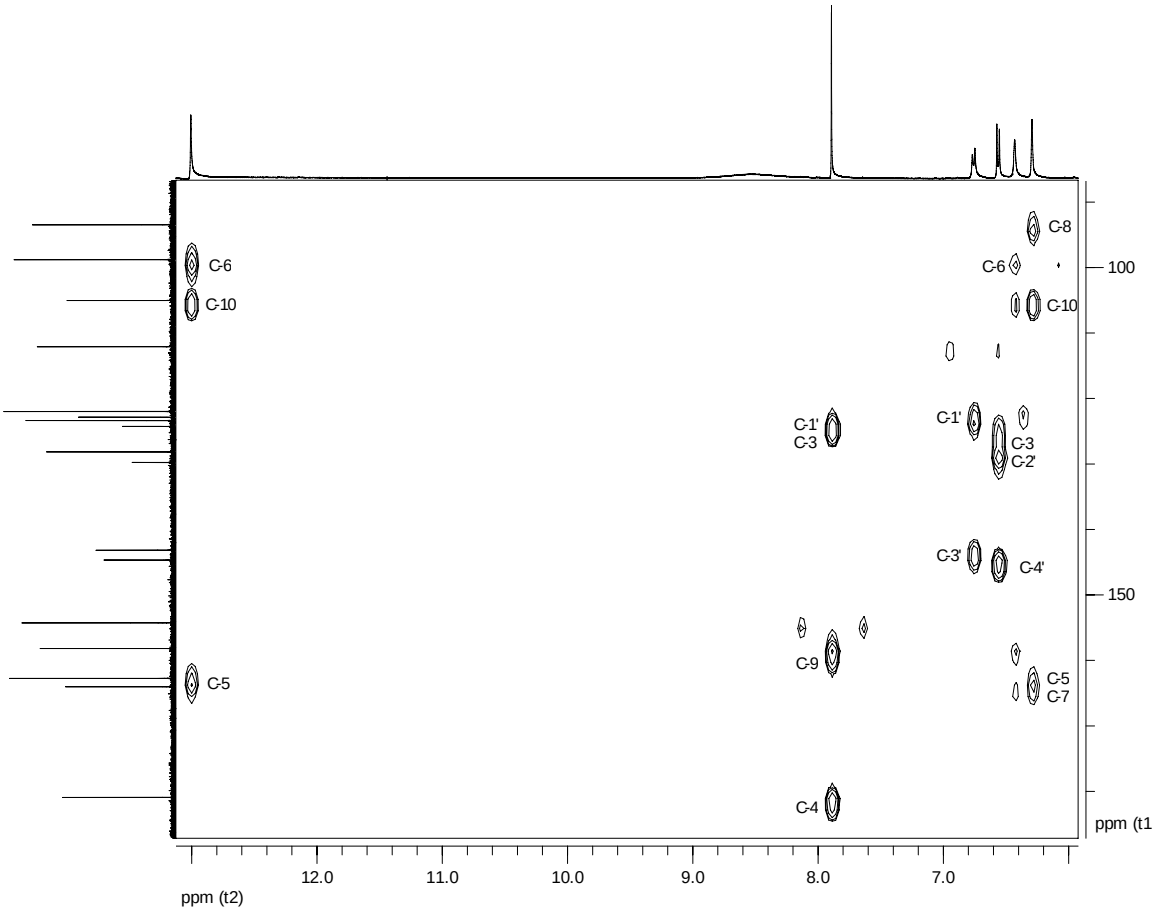


Figure 5.4: ^{1}H-^{13}C HMBC Spectrum of 5,7,3′,4′-Tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone **(5.1a)**

5.3.2 Structure Elucidation of 2-(2′-Hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (5.7)

The new 2-arylbenzofuran **5.7** exhibited a ^{1}H NMR spectrum (Figure 5.5) showing a downfield singlet at δ 10.00, a 1,2,4-trisubstituted benzene ring with protons at δ 7.50 (d, *J* = 8.4 Hz), 6.66 (dd, *J* = 2.2 and 8.4 Hz) and 6.71 (*J* = 2.2 Hz), two isolated aromatic singlets at δ 7.52 and 7.16, a downfield methylene singlet at δ 6.08 indicating a methylenedioxy group, and a methoxy group at δ 3.86. The ^{13}C and DEPT NMR spectra (Figure 5.5) showed a carbonyl signal at δ 187.7, six oxygenated aromatic carbons at δ 163.5–146.7, five aromatic methines and three quaternary carbons at δ 133.5 – 94.1, one methylenedioxy group at δ 102.7, and one methoxy group at δ 56.1. The deduced molecular formula $C_{17}H_{12}O_6$ was confirmed by HRESIMS (measured $[M+Na]^+$ *m/z* 335.0550, calcd 335.0532). The carbonyl signal at δ 187.7 showed an HSQC correlation to the proton singlet at δ 10.00, thus indicating a carbaldehyde group that was assigned to position 3 on the basis of an HMBC correlation between the aldehydic proton and C-3 (Figure 5.6). The position of the methoxy group was established by an HMBC correlation to C-6 and a NOESY correlation to H-5 and H-7. The methylenedioxy protons showed HMBC correlations to the *o*-oxygenated carbons of ring C (δ 146.7 and 147.8). The aromatic singlet at δ 7.52 was assigned to H-6′ on the basis of its 3-bond HMBC correlations to C-2 while H-3′ at δ 7.16 was correlated to C-1′ and 5′. Thus, compound **5.7** was shown to be 2-(2′-hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde. The compound closest in structure to **5.7** from the literature is cicerfuran isolated from the wild chickpea *Cicer bijugum*.[225]

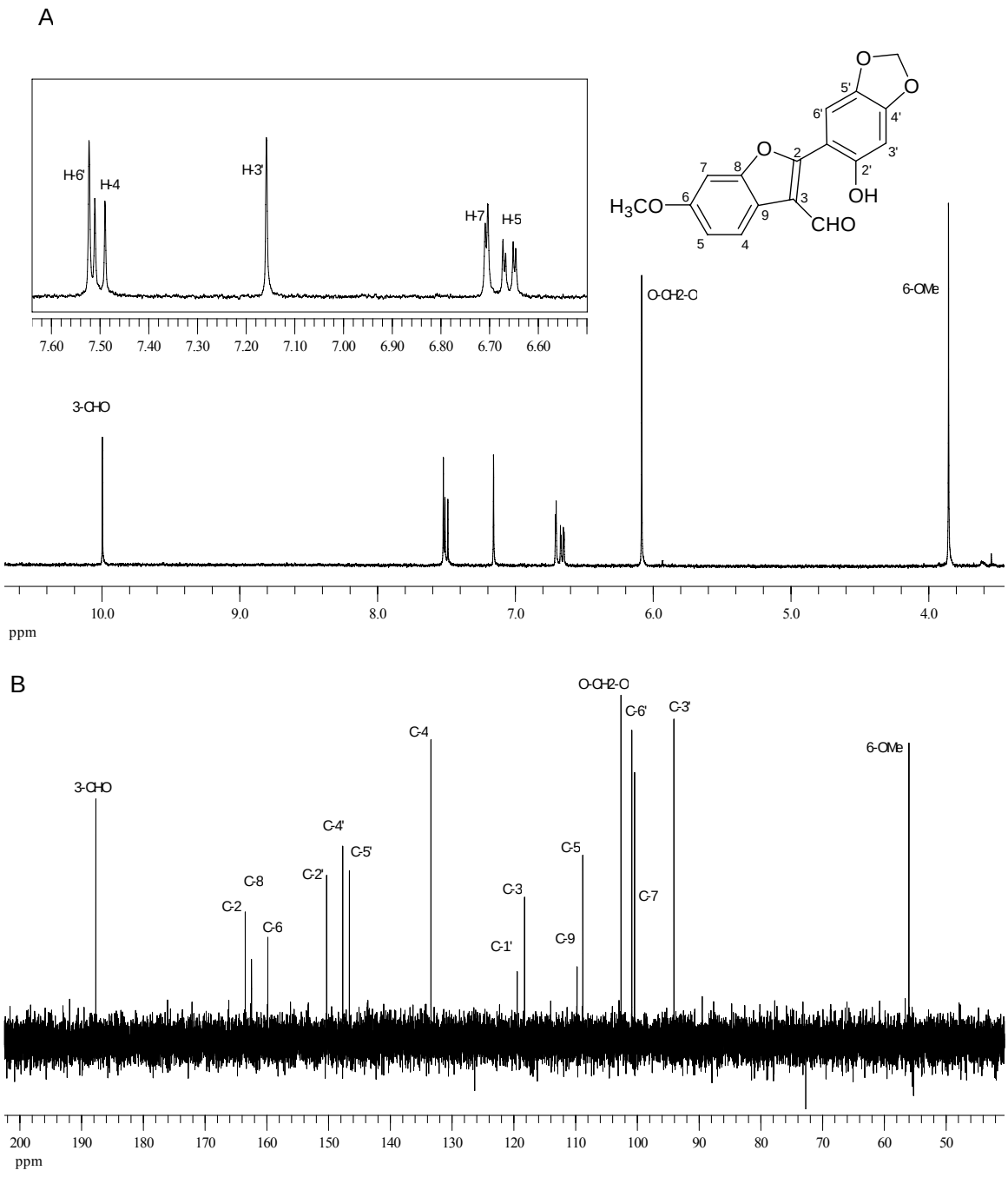


Figure 5.5: ^{1}H and ^{13}C NMR Spectra of 2-(2′-Hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (**5.7**) in acetone-d_6

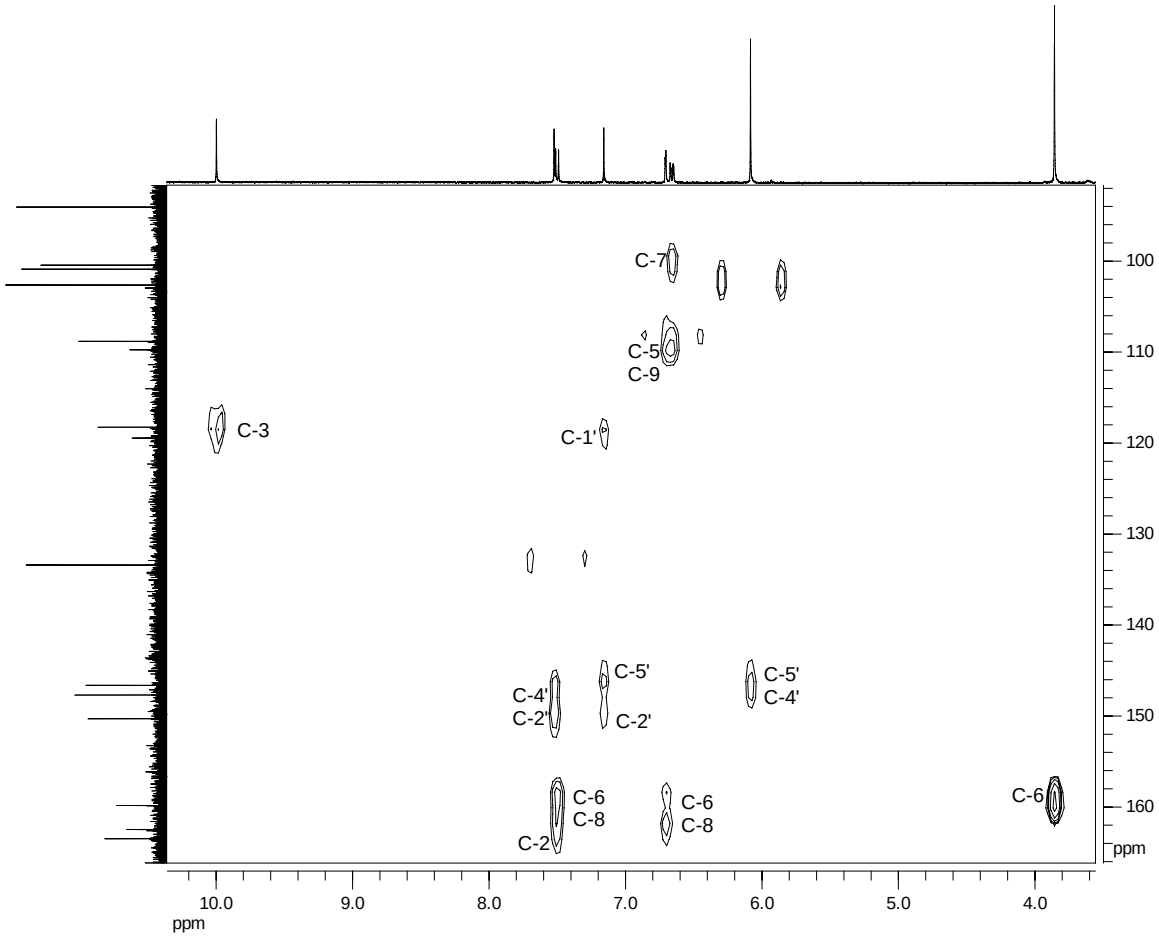
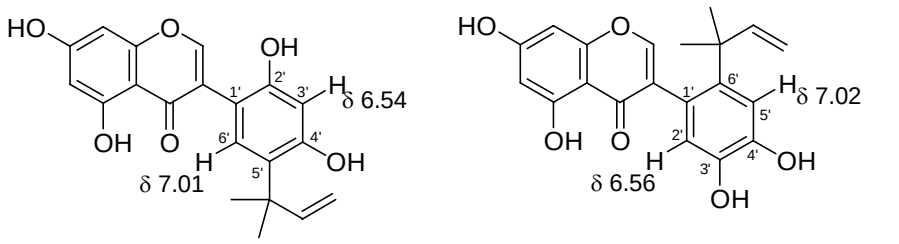


Figure 5.6: ^{1}H-^{13}C HMBC Spectrum of 2-(2′-Hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (**5.7**)

5.3.3 Revision of Fremontin Structure

The ^{1}H and ^{13}C NMR data (Table 5.4) as well as the physical data for compound **5.2a** closely matched those reported by Manikumar et al.[208] for the isoflavone fremontin (see Figure 5.7) isolated from the related species *P. fremontii*. However, the ^{13}C NMR data showing ring B oxygenated carbons at relatively upfield positions (δ 143.1 and 147.3) seemed more consistent with the OH groups in *ortho* rather than *meta* positions to each other; oxygenated carbons with no *ortho*/*para* oxygenation are expected to be more downfield (δ 155–165).[226] Furthermore, the OH-5 signal in acetone-d_6 occurred at δ 13.00, a more downfield value than that expected for a 2′-hydroxylated isoflavone (δ 12.51–12.79),[227,228] implying that postion 2′ is not hydroxylated. Tahara et al. suggested that the structure of fremontin should be reexamined on the basis of the OH-5 chemical shift,[227] and later proposed a possible B ring structure with two *o*-OH groups at positions 3′ and 4′ and the isoprenyl group assigned to position 6′.[229]



Fremontin (literature structure) Fremontin (proposed structure)

Figure 5.7: Fremontin; Literature and Proposed Structures

Our HMBC data (see Table 5.4) supported Tahara's proposed structure (Figure 5.7), showing correlations between H-2′ at δ 6.56 and C-3 and C-6′ while H-5′ at δ 7.02 was correlated to C-1′ and -3′. To shed further light on the structure of this compound, we performed selective NOE experiments to irradiate the methyl group at δ 1.33, resulting in enhancement of the H-2 singlet and the H-5′. Alternatively, irradiating the H-2 singlet enhanced the signals of the nearby isoprenyl methyl groups in the 6′ positions of ring B while irradiating the H-5′ resulted in strongly enhancing the prenyl methyls. In addition, acetylation of fremontin to give the tetraacetyl derivative led to a significant downfield shift of 0.39 and 0.37 ppm for H-2′ and H-5′, respectively, indicating that both protons are *ortho* to the acetylated OH group(s).[210] Since those protons are in the *para* position to each other, it follows that the only arrangement possible is that of the structure **5.2b**. Additional evidence comes from the trimethylated derivative **5.2c** prepared by reacting fremontin with dimethylsulfate in the presence of K_2CO_3, followed by separating the produced tri- (**5.2c**) and tetramethylfremontin (**5.2d**). The OMe groups in **5.2c** at δ 3.83, 3.88, and 3.92 are assigned at the 3′, 7 and 4′ positions, respectively, due to HMBC correlations (Table 5.4) with C-3′, -7, and -4′, respectively. Irradiation of H-5′ at δ 7.06 resulted in enhancement of the two isoprenyl methyls (by 3.4 and 4.1 %, respectively) and only the 4′-OMe group at δ 3.92 (by 6.5%), while irradiating the H-2′ at δ 6.54 resulted in enhancing the 3′-OMe peak at δ 3.83 by 5.6% and the H-2 signal at δ 7.59 by 0.5% (Figure 5.9). On the other hand, irradiating the H-2 proton at δ 7.59 resulted in enhancing the H-2′ at δ 6.54 (0.5%) and the isoprenyl group methyls (0.7 and 0.5%).

Thus, the structure of fremontin (**5.2a**) was shown to be 5,7,3′,4′-tetrahydroxy-6′-(1,1-dimethylallyl)isoflavone.

	5.2a			**5.2c**		
C	δ_C	δ_H (mult.; J_{HH}, Hz)	HMBC	δ_C	δ_H (mult.; J_{HH}, Hz)	HMBC
2	155.0	7.79 (s)	C-3, 4, 9, 1′	153.6	7.59 (s)	C-3, 4, 9, 1′
3	126.8			126.1		
4	183.0			182.1		
5	163.7			162.6		
6	99.7	6.27 (d; 1.9)	C-5, 7, 8, 10	98.1	6.38 (d; 2.1)	C-5, 7, 8, 10
7	165.1			165.5		
8	94.4	6.39 (d; 1.9)	C- 6, 7, 9, 10	92.4	6.40 (d; 2.1)	C- 6, 7, 9, 10
9	159.1			158.0		
10	105.9			106.0		
1'	122.2			121.6		
2'	121.1	6.56 (s)	C-3, 3′,4′, 6′	115.7	6.54 (s)	C-3, 3' , 4', 6'
3'	143.5			146.9		
4'	145.7			148.7		
5'	115.4	7.02 (s)	C-1′, 3′, 4′, 1′′	111.2	7.06 (s)	C-1′, 3′, 4′, 6′ , 1′′
6'	140.2			140.3		
1"	42.2			42.0		
2"	150.0	6.01 (dd; 10.6, 17.6)	C-6′, 1′′, 4′′, 5′′	148.5	6.02 (dd; 10.6, 17.5)	C-1′′, 4′′, 5′′
3"	109.4	4.77 (dd; 1.2, 17.6)	C-1′′, 2′′	109.8	4.81 (d; 17.5)	C-1′′, 2′′
		4.64 (dd; 1.2, 10.6)	C-1′′		4.73 (d; 10.6)	C-1′′, 2′′
4"	29.4	1.29 (s)	C-6′, 1′′, 2′′, 5′′	28.9	1.37 (s)	C-6′, 1′′, 2′′, 5′′
5"	30.0	1.33 (s)	C-6′, 1′′, 2′′, 4′′	29.7	1.40 (s)	C-6′, 1′′, 2′′, 4′′
5-OH		13.01 (s)	C-5, 6, 10		12.81 (s)	C-5, 6, 10
3'-OMe				55.78[a]	3.83 (s)	C-3′
7-OMe				55.84[a]	3.88 (s)	C-7
4′-OMe				55.94[a]	3.92 (s)	C-4′

[a] Assignments might be interchangeable.

Table 5.4: ^{1}H and ^{13}C NMR Assignments and HMBC Correlations for Compounds **5.2a** (acetone-d_6) and **5.2c** in $CDCl_3$

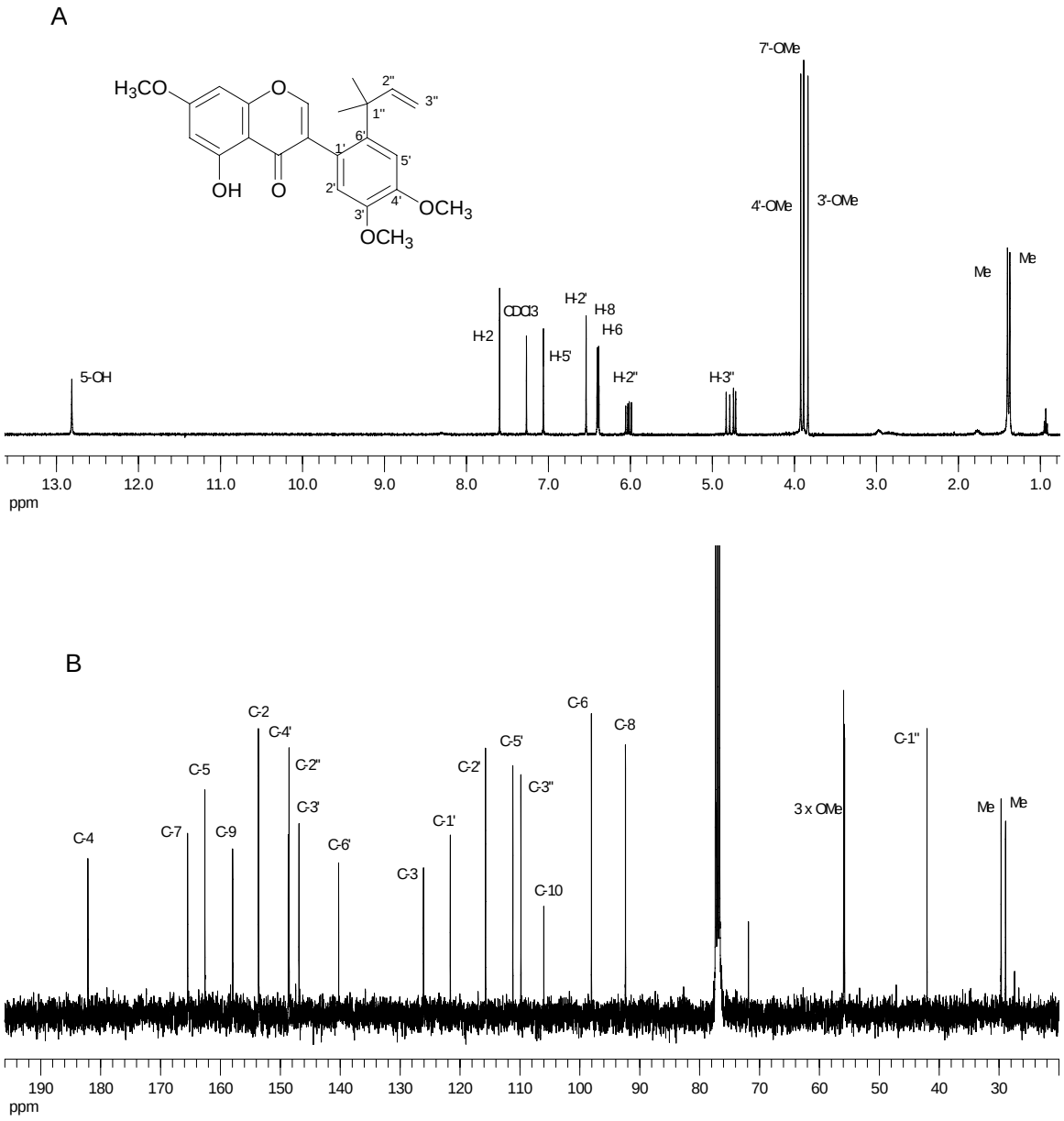


Figure 5.8: ^{1}H and ^{13}C NMR Spectra of Trimethylfremontin (**5.2c**) in $CDCl_3$

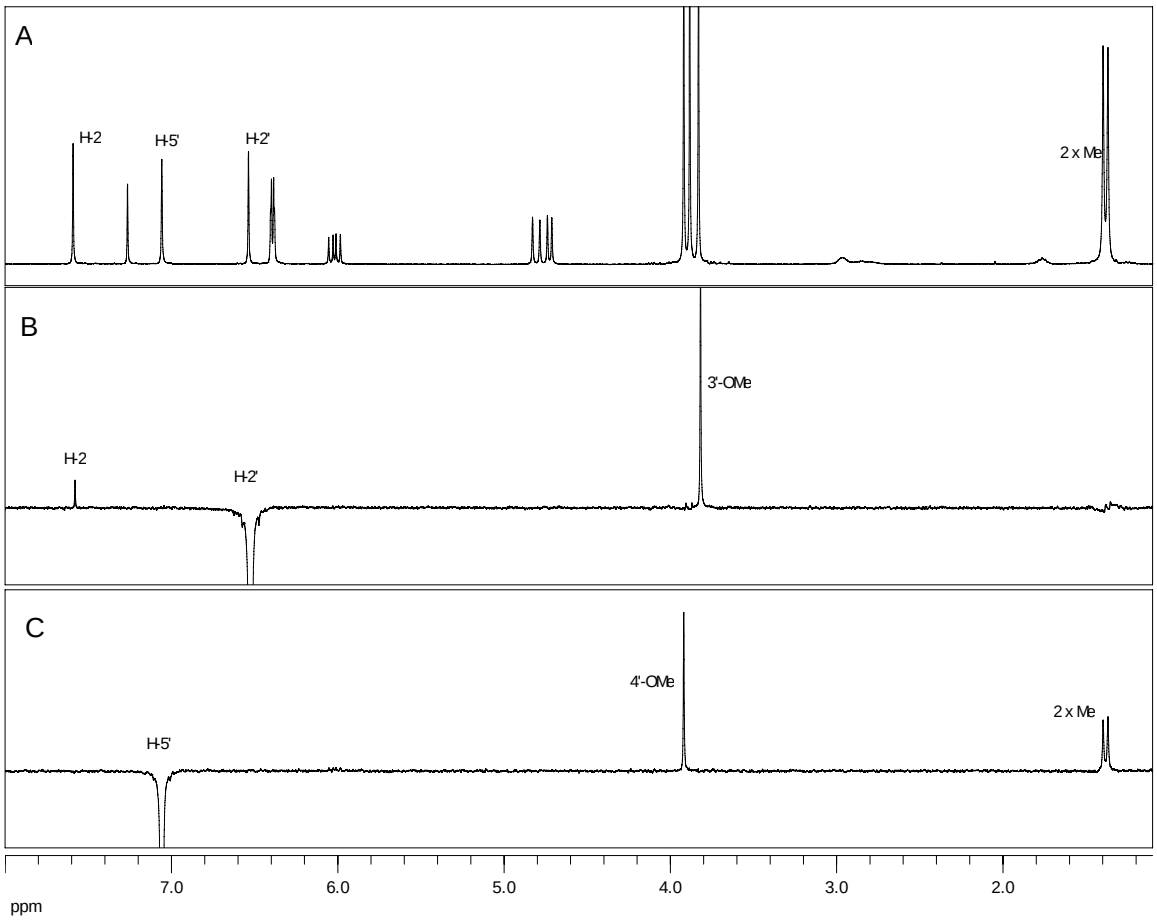


Figure 5.9: DPFGSE-NOE Spectra of Trimethylfremontin (**5.2c**)

Panel A shows the standard ^{1}H NMR spectrum of **5.2c**. In B, a selective excitation pulse was adjusted on the the signal of H-2´. A strong NOE effect is observed for the 3´-OMe and a weaker effect is observed for H-2. In C, selective irradiation of the signal of H-5´ resulted in an NOE effect at the 4´-OMe and the two prenyl methyls.

5.3.4 Glycyrrhisoflavone (5.3a)

Glycyrrhisoflavone (**5.3a**), which is closely related in structure to compound **5.1a**, showed ^{1}H and ^{13}C NMR spectra consistent with those reported in the literature.[222] However, we assigned some of the signals differently due to the current availability of HMBC data. The peak at δ 6.84 is assigned to H-6′ on the basis of its 3-bond correlation to C-3 and C-1″, while the peak at δ 7.02 is assigned to H-2′ as it shows correlation to C-3, -4′ and -6′. C-5′ is assigned to δ 128.8 on the basis of its correlation to H-2″. Further support for the structure was obtained in an NOE experiment where the H-2 was irradiated, resulting in enhancement of the H-6′ and to a lesser extent H-2′. The cyclized (**5.3b**) and methylated (**5.3c**) derivatives were prepared for biological testing.

5.3.5 Pterocarpans (5.5 and 5.6)

The NMR, UV and IR data of maackiain (**5.5**) were in agreement with the literature values.[213,215,216] In spite of the presence of two chiral centers in the structure, only the 6a*R*,11a*R* and 6a*S*,11a*S* *cis*-configurations are sterically possible.[230] However, the melting point and optical rotation data did not completely match those reported for either the pure isomers or the racemate. The melting point of **5.5** (186–188 °C) is higher than that reported for the (–)- or (+)-isomers (180 and 180–181 °C, respectively)[215] but lower than that of the racemate (195–196 °C).[215] It was reported that mixing the racemic maackiain with the (–)- or (+)-isomers results in depressing the racemate melting point.[215] The optical rotation is negative ($[\alpha]_D = –33.5°$), but the absolute value is much less than that reported in the literature for the optically-pure (–)-isomer ($[\alpha]_D = –267°$).[216] This

suggests that the compound isolated is a mixture of (–)-6a*R*,11a*R*-maackiain and the (+)-6a*S*,11a*S*-isomer with an excess of the (–)-form. The same situation is true for the isolated 3,4-dihydroxy-8,9-methylenedioxypterocarpan (**5.6**), which is a 4-hydroxy analog of maackiain (**5.5**). The melting point, 186–189 °C, is consistent with that reported for the (–)-6a*R*,11a*R*-isomer (186–187 °C);[217] no melting point was reported for the (+)-isomer.[213] The optical rotation of compound **5.6**, $[\alpha]_D = +62.2°$, is less in magnitude than that reported for the (+)-isomer ($[\alpha]_D = +154°$). This, again, suggests the the presence of a mixture of (–)-6a*R*,11a*R*-4-hydroxymaackiain and the (+)-6a*S*,11a*S*-isomer with an excess of the (+)-form.

5.3.6 Antiparasitic Activities of the Isolated Compounds, Their Semisynthetic Derivatives and Related Isoflavones

The activity of the isolated compounds against *L. donovani* axenic amastigotes and *T. b. brucei* bloodstream forms, as well as against two mammalian cell lines (Vero cells and PC-3 prostate cancer cells) is summarized in Table 5.5. Compounds **5.1a** and **5.8** showed significant antileishmanial and trypanocidal activities, displaying IC_{50} values below 10 μg/mL. However, they exhibited low selectivity (around three-fold) for the parasites over the mammalian Vero cells. The selectivity is worse for pterocarpan **5.6**, which shows higher toxicity towards Vero cells than to the parasites. On the other hand, calycosin (**5.4)** displayed selective toxicity (about 13-fold) towards *T. b. brucei* bloodstream forms (IC_{50} 3.2 μg/mL) over Vero cells. Calycosin is reported in the literature to have antiplasmodial,[231,232] antileishmanial,[233] and antigiardial effects.[234] In

addition, calycosin was found to protect human umbilical vein endothelial cells from hypoxia-induced impairment.[235] Methylation of the isoflavones **5.2a** and **5.3a** moderately improved the trypanocidal but not the antileishmanial activity. Tetramethylglycyrrhisoflavone (**5.3c**) is 3.7-fold more active towards trypanosomes than the parent glycyrrhisoflavone (**5.3a**). Interestingly, compound **5.7** did not show any antikinetoplastid activity in our assays. Related Fabaceae-derived 2-arylbenzofurans were reported to possess antifungal[225] and antimalarial[231] activities. The trypanocidal selectivity of calycosin prompted us to assay a group of related natural isoflavones that are commercially available (see Figure 5.10); the results are summarized in Table 5.6. Genistein, a common dietary isoflavone (especially in soy-based diets), was the most potent against trypanosomes. This compound displayed an IC_{50} of 4.2 μM, making it three-fold more active than calycosin (IC_{50} of 12.7 μM) on a molar basis. Past reports have shown that genistein inhibits trypanosomal protein tyrosine kinase (PTK) activity.[236] Later, it was shown that genistein is an efficient inhibitor of trypanosomal protein synthesis and phosphorylation (which is primarily serine and threonine phosphorylation in these organisms) indicating that the antitrypanosomal effect of this compound is not due to a specific effect on the tyrosine kinase activity.[237] Daidzein, a genistein analog that is known to be inactive against PTK,[238] still displayed an inhibitory effect (IC_{50} of 23.7 μM) against the parasites. Genistein was also reported to show activity against both chloroquine-sensitive and -resistent *Plasmodium falciparum*.[232] 7,3',4'-Trihydroxyisoflavone also showed a significant antitrypanosomal effect (IC_{50} of 7.1 μM) while being virtually non-toxic to Vero cells. Apigenin, a flavone with the same

substitution pattern as that of genistein, was about three-fold less active than genistein against the *T. b. brucei*.

compound	IC_{50} (μg/mL)[a]			
	L. donovani axenic amastigotes	*T. b. brucei* variant 221	Vero cells	PC-3 cells
5.1a	4.6 ± 0.3	4.3 ± 0.7	13.2 ± 3.6	4.5 ± 1.1
5.1b	13.6 ± 2.2	20.5 ± 0.9	NT[b]	38.0 ± 5.7
5.2a	43.8 ± 1.5	26.7 ± 2.1	47.5 ± 9.4	27.8 ± 9.7
5.2b	22.3 ± 0.8	35.4 ± 2.9	NT	34.6 ± 14.9
5.2c	>100	12.8 ± 0.8	NT	57.8 ± 1.4
5.2d	35.3 ± 4.5	17.1 ± 5.0	NT	55.1 ± 12.3
5.3a	16.4 ± 0.9	11.7 ± 0.9	18.7 ± 4.0	22.0 ± 2.6
5.3b	24.2 ± 2.3	27.3 ± 1.2	NT	28.1 ± 3.4
5.3c	17.0 ± 1.1	3.2 ± 0.9	18.2 ± 3.0	12.7 ± 1.9
5.4	28.5 ± 2.7	3.6 ± 0.2	45.1 ± 4.9	54.7 ± 8.3
5.5	>100	37.0 ± 3.8	>100	>100
5.6	11.2 ± 0.3	1.1 ± 0.2	1.0 ± 0.5	8.4 ± 2.7
5.7	>100	>100	>100	>100
4.7	>100	19.7 ± 3.8	>100	96.5 ± 4.5
5.8	5.3 ± 0.6	8.4 ± 0.5	16.8 ± 5.0	11.9 ± 2.0
ansamitocin P3	NT	NT	1.2 ± 0.3[c]	NT
pentamidine	2.3 ± 0.4[d]	NT	NT	NT
podophyllotoxin	NT	NT	NT	12.5 ± 1.1[c]
suramin	NT	0.190 ± 0.028[d]	NT	NT

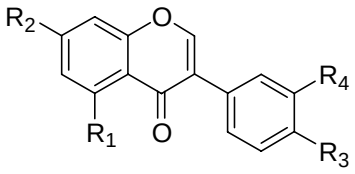
[a] Values represent the mean ± standard deviation of at least 3 independent experiments. [b] NT: Not tested. [c] IC_{50} value in nM. [d] IC_{50} values in μM.

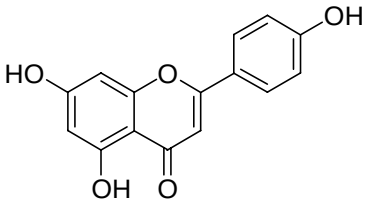
Table 5.5: Bioactivity of Compounds **5.1a–5.8** and **4.7** against Axenic *L. donovani*, *T. b. brucei* and Two Mammalian Cell Lines

compound	IC_{50} (μM)		
	L. donovani axenic amastigotes	*T. b. brucei* variant 221	Vero cells
genistein	73.0 ± 5.6	4.2 ± 0.5	32.9 ± 8.4
biochanin A	89.9 ± 1.8	12.3 ± 1.0	NT[b]
7-hydroxyisoflavone	113.0 ± 6.4	94.2 ± 4.0	NT
formononetin	NT	90.2 ± 14.1	NT
prunetin	NT	20.2 ± 4.3	NT
daidzein	NT	23.7 ± 5.0	NT
4',7-dimethoxyisoflavone	NT	>100	NT
3',4',7-trihydroxyisoflavone	NT	7.1 ± 1.6	134.5 ± 12.7
apigenin	NT	11.9 ± 0.9	NT
pentamidine	1.5 ± 0.2	NT	NT
suramin	NT	0.183 ± 0.025	NT

[a] Values represent the mean ± SD of at least 3 independent experiments. [b] NT: Not tested.

Table 5.6: Bioactivity of Selected Isoflavones against Axenic *L. donovani*, *T. b. brucei* and Vero Cells





Apigenin

Genistein	$R_1 = R_2 = R_3 = OH, R_4 = H$
Biochanin A	$R_1 = R_2 = OH, R_3 = OMe, R_4 = H$
7-Hydroxyisoflavone	$R_1 = R_3 = R_4 = H, R_2 = OH$
Formononetin	$R_1 = R_4 = H, R_2 = OH, R_3 = OMe$
Prunetin	$R_1 = R_3 = OH, R_2 = OMe, R_4 = H$
Daidzein	$R_1 = R_4 = H, R_2 = R_3 = OH$
4',7-Dimethoxyflavone	$R_1 = R_4 = H, R_2 = R_3 = OMe$
3',4',7-Trihydroxyisoflavone	$R_1 = H, R_2 = R_3 = R_4 = OH$

Figure 5.10: Structures of Commercially Available Flavonoids Tested for Antiprotozoal Activity

5.3.7 Cell Cycle Analysis

To assess the effects of these compounds on the cell cycle of *L. donovani*, drug-treated promastigotes were fixed, stained with propidium iodide and examined by flow cytometry. The results were analysed by the software Cell Quest Pro to obtain the percentage of cells in each phase of the cell cycle. The concentrations of compounds used in this assay were chosen to be close to the respective IC_{50} values against the promastigotes. Isoflavone **5.1a**, glycyrrhisoflavone (**5.3**), and 4-hydroxymaackiain (**5.6**) were less toxic to the promastigote forms (IC_{50} values are 13.1, 27.8, and 12.2 μg/mL, respectively) than to the amastigotes. Results revealed a dose-dependent increase of the percentage of cells in the *S* phase of the cell cycle as shown in Figure 5.11 and Table 5.7. Compound **5.1a** at 10 and 15 μg/mL increased the percentage of cells in the *S* phase of the cell cycle by 3- and 5-folds, respectively. Glycyrrhisoflavone displayed the same effect although with a lower potency than that of compound **5.1a**, in correlation with its lower antileishmanial activity. Similarly, 4-hydroxymaackiain at 20 μg/mL induced a 7-fold increase of parasites in the *S* phase. On the other hand, compound **5.1a** and calycosin did not significantly affect the phases of the cell cycle of *T. b. brucei* bloodstream forms (see Table 5.7 and Figure 5.11).

parasite	drug	conc. (μg/mL)	*G1* phase %	*G2*/*M* phase %	*S* phase %
L. donovani promastigotes					
	1% DMSO		79.1 ± 6.8[a]	16.8 ± 5.7[a]	4.1 ± 1.2[a]
	isoflavone **5.1a**	5	76.7 (9.6)	19.4 (7.9)	3.9 (1.6)
		10	65.6 (20.5)	22.7 (11.6)	11.7 (7.9)
		15	55.4 (6.4)	22.0 (9.2)	22.5 (2.8)
		20	66.1 (0.7)	13.9 (0.1)	20.0 (0.6)
	glycyrrhisoflavone (**5.3a**)				
		15	72.7 (21.3)	18.6 (12.5)	8.7 (8.7)
		30	68.9 (1.6)	18.6 (2.0)	12.5 (0.4)
	4-hydroxymaackiain (**5.6**)	10	56.2 (38.8)	21.7 (8.3)	22.1(31.5)
		20	38.2 (15.1)	32.9 (9.7)	28.9 (5.5)
T. b. brucei					
	1% DMSO		51.5 (1.7)	27.7 (8.7)	20.8 (7.0)
	isoflavone **5.1a**	2.5	50.8 (3.7)	32.5 (5.7)	16.7 (9.4)
		5	51.7 (2.8)	32.8 (9.8)	15.5 (7.0)
	calycosin (**5.4**)	2.5	45.9 (3.3)	34.0 (4.8)	20.2 (8.0)
		5	42.5 (1.5)	36.6 (5.8)	20.9 (4.3)

Table 5.7: Summary of Flow Cytometry Analysis of Some Isoflavonoids

The results represent the percentages of cells in each phase of the cell cycle from experiments done on two separate occasions; the range of each result is shown between brackets. [a] Entries for control (1% DMSO) parasites represent percentages of cells in each of the phases of the cell cycle ± the standard deviation from experiments done on four separate occasions.

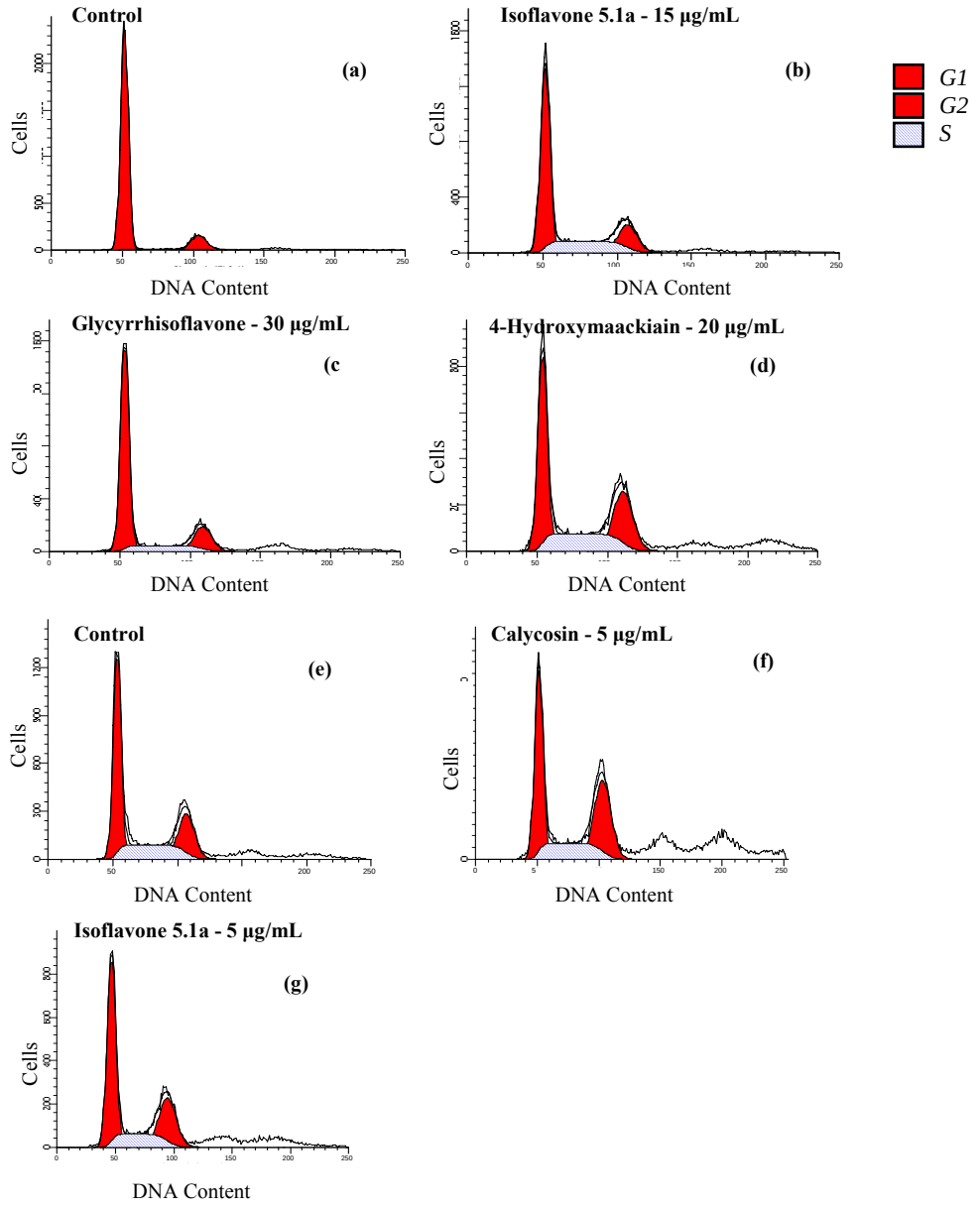


Figure 5.11: Effect of Isoflavonoids on the Cell Cycle of *L. donovani* and *T. brucei*

After 48 h treatments with 1% DMSO (a), 15 µg/mL isoflavone **5.1a** (b), 30 µg/mL glycyrrhisoflavone (c), or 20 µg/mL 4-hydroxymaackiain (d), *L. donovani* promastigotes were fixed, stained with propidium iodide and analyzed by flow cytometry. After 24 h treatment with 1% DMSO (e), 5 µg/mL calycosin (f), or 5 µg/mL isoflavone **5.1a** (g), *T. b. brucei* trypomastigotes were similarly processed and analyzed.

5.3.8 Transmission Electron Microscopy

Transmission electron microscopy was also employed to compare the ultrastructural morphology of control *L. donovani* promastigotes to those treated with compound **5.1a** at a concentration 13 μg/mL. After 24 h incubation, drug-treated parasites showed swollen mitochondria and a decrease in the cytoplasmic density, while other intracellular organelles including the nuclei and the kinetoplasts appeared normal (Figure 5.12, panels C and D). These ultrastructural changes look similar to those induced by licochalcone A[30] and other antileishmanial chalcones[33] in *L. major* promastigotes. Licochalcone A was found to inhibit the parasite respiration through inhibition of the parasite mitochondrial enzymes, especially fumarate reductase. After 48 h of incubation with compound **5.1a**, a large percentage of disintegrating cells can be seen.

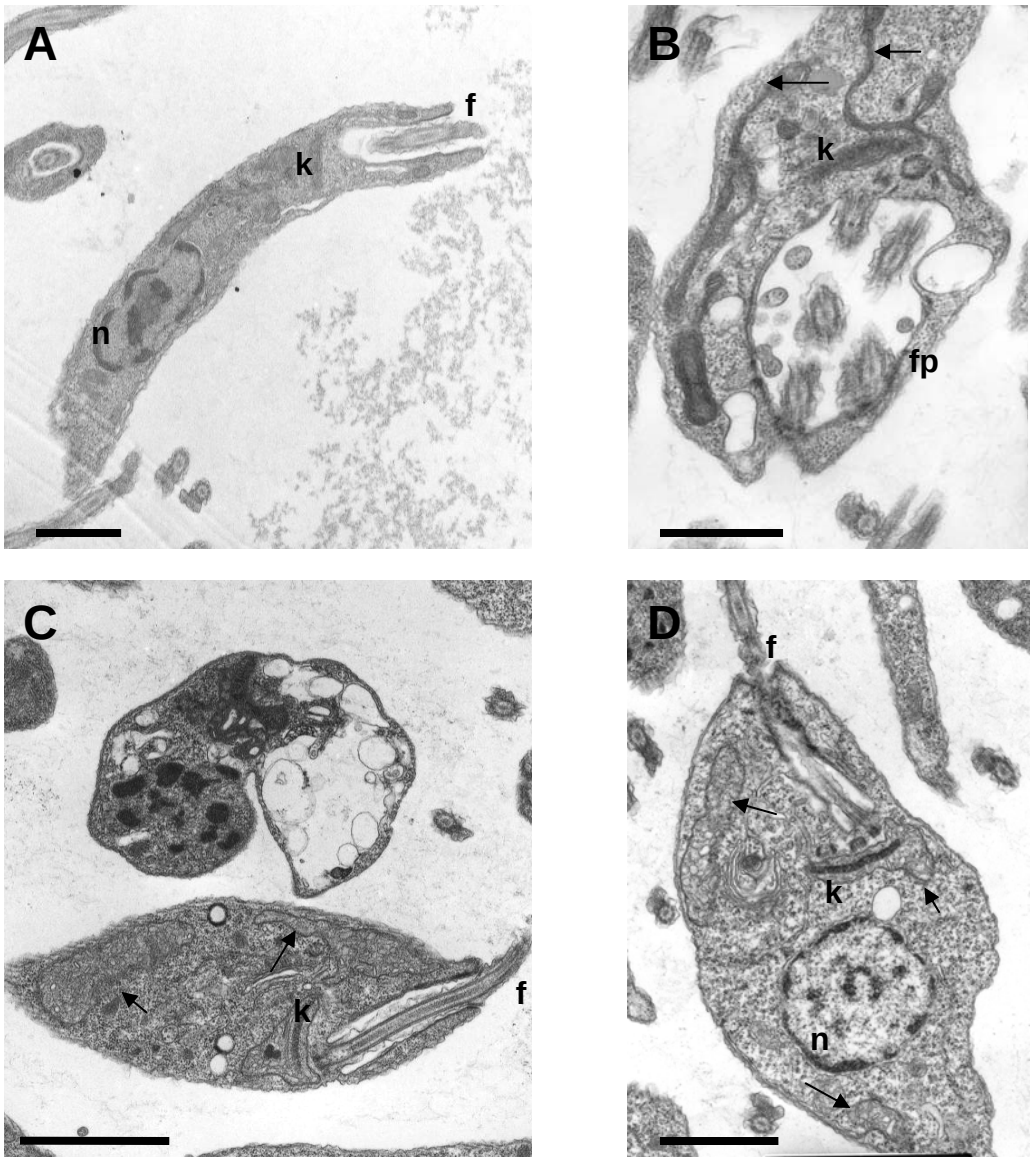


Figure 5.12: Transmission Electron Micrographs of *L. donovani* Promastigotes

A control parasite treated with 1% DMSO showing a nucleus (n), kinetoplast (k), and a flagellum (f) is pictured in panel A. Panel B shows a transverse section in a parasite treated with compound **5.1a** (13 μg/mL) for 48 h. An exocytic activity can be seen towards the lumen of the flagellar pocket which contains multiple cross sections of flagella. The cytoplasm around the flagellar pocket shows a kinetoplast and a mitochondrial network. Panels C and D show parasites treated with compound **5.1a** (13 μg/mL) for 24 h. The arrows show enlarged mitochondria while the kinetoplasts appear unaffected. A disintegrating promastigote appears in the top of panel C. Bar, 1 μm.

5.4 SUMMARY AND CONCLUSIONS

The plant genus *Psorothamnus* was identified in our laboratory as a rich source of potential new antiparasitic compounds. In the initial work carried out, we isolated antileishmanial and antitrypanosomal compounds from *P. polydenius* that displayed selectivity for these organisms.[204] Another member of the same genus, *P. arborescens*, exhibited significant activity against *Leishmania donovani* axenic amastigotes and *Trypanosoma brucei brucei* bloodstream forms. Bioactivity-guided fractionation of the root extract of *P. arborescens* yielded the new isoflavone 5,7,3′,4′-tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (**5.1a**) and the new 2-arylbenzofuran 2-(2′-hydroxy-4′,5′-methylenedioxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (**5.7**), together with seven other known compounds, fremontin (**5.2a**), glycyrrhisoflavone (**5.3a**), calycosin (**5.4**), maackiain (**5.5**), 4-hydroxymaackiain (**5.6**), oleanolic acid (**4.7**), and isoliquiritigenin (**5.8**). In addition, the structure of the isoflavone fremontin was revised using spectroscopic and chemical methods and was assigned the new structure **5.2a**. The prenylated isoflavone **5.1a** and the chalcone **5.9** displayed IC_{50} values of 4.6 and 5.3 μg/mL, respectively, against *L. donovani* axenic amastigotes. Non-prenylated isoflavones, such as calycosin or genistein, exhibit much lower antileishmanial activity. Cell cycle analysis of *L. donovani* promastigotes treated with 15 μg/mL isoflavone **5.1a** revealed a 5-fold increase in the cells in the *S* phase, in relation to control, after 48 h incubation. However, transmission electron micrographs of *L. donovani* promastigotes treated with **5.1a** for 24 h show significant swelling of the parasite mitochondria suggesting a possible effect on the parasite respiration. Several antileishmanial chalcones

that inhibit parasite mitochondrial enzymes were shown to induce similar ultrastructural changes in the promastigotes. Calycosin (**5.4**) exhibited selective toxicity against *T. b. brucei* blood stream forms (IC_{50} 3.6 μg/mL) compared to *L. donovani* amastigotes and Vero cells (IC_{50} 28.5 and 45.1 μg/mL, respectively). These results prompted us to test a small group of structurally-related isoflavones for their antitrypanosomal activities. Genistein and 3′,4′,7-trihydroxyisoflavone displayed promising activity (IC_{50} values 1.1 and 1.9 μg/mL, equivalent to 4.2 and 7.1 μM, respectively) and selectivity (IC_{50} versus Vero cells: 33 and 135 μM, respectively). It is hard to draw a structure activity relationship for isoflavones from the small set of compounds assayed for antitrypanosomal activity. It appears that isoflavones with more hydroxy substituents like genistein and 3′,4′,7- trihydroxyisoflavone have more potent antitrypanosomal activity compared to the less hydroxylated isoflavones like formonentin and 7-monohydroxyisoflavone (IC_{50} values > 90 μg/mL). However, methylation of glycyrrhisoflavone increased the antitrypanosomal activity of the compound. Flow cytometric analysis of *T. b. brucei* treated with calycosin revealed no significant effects on the cell cycle compared to control. The mode of antitrypanosomal activity of isoflavonoids is not known although the isoflavone genistein was reported to inhibit protein synthesis and phosphorylation in trypanosomes. This investigation suggests that the isoflavone skeleton deserves further investigation as a template for novel antileishmanial and trypanocidal compounds.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 NATURAL PRODUCTS IDENTIFIED IN THIS STUDY AS PROMISING SOURCES OF ANTIKINETOPLASTID COMPOUNDS

Screening of a library of natural products containing 323 plant extracts from North America and China revealed several species with promising antikinetoplastid activities. Out of 174 North American samples, 19 extracts showed antileishmanial activities with IC_{50} values below 100 μg/mL against *L. donovani* axenic amastigotes and 21 extracts displayed activity against *T. b. brucei* bloodstream forms at IC_{50} values below 50 μg/mL. Some of the active samples displayed good selectivity with low toxicities to mammalian cell lines. Promising plant species showing selective antileishmanial properties included *Psorothamnus polydenius* and *P. arborescens* (Fabaceae), *Mimulus bigelovii* (Scrophulariaceae) and *Chamelaucium ucinatum* (Myrtaceae) while those exhibiting selective trypanocidal activities included *Chaetadelpha wheeleri* and *Petasites palmatus* (Asteraceae), and *Alnus rubra* (Betulaceae). An investigation of the antikinetoplastid constituents of *P. polydenius* and *P. arborescens* was presented in this dissertation. Other promising plant species identified in our screen remain to be further studied.

6.2 IDENTIFICATION OF DALRUBONE AS A PROMISING ANTILEISHMANIAL LEAD COMPOUND

Dalrubone (Figure 6.1) is the major pigment in *P. polydenius*, a shrub from the Mojave Desert that was medicinally used by the native tribes in this area. The compound exhibited promising in vitro antileishmanial activity against both axenic and intracellular *L. donovani* amastigotes. Flow cytometry analysis showed that dalrubone does not affect the cell cycle progression of the parasite. An electron microscopy study revealed significant ultrastructural changes in dalrubone-treated *L. donovani* promastigotes including the appearance of concentric membranous structures and vacuoles. These ultramorphological perturbations suggest some kind of interference with the secretory pathways of the parasite. However, the exact drug target and the identity of the perturbed intracellular secretory structures (e.g., Golgi apparatus, endoplasmic reticulum, endosomes, etc.) remain to be identified. Dalrubone did not show a favorable in vivo antileishmanial activity when administered at a dose of 50 mg/kg/day for 5 days to *L. donovani* infected BALB/c mice, possibly because of its low potency or because of a pharmacokinetic problem. Improvement of the antileishmanial activity can be achieved by modifying the structure of dalrubone, including both the chromene and the cyclohexadione moieties, using medicinal chemistry approaches. This would also serve to explore the SAR and identify the pharmacophore, in addition to generating analogs that are more stable than the photosensitive dalrubone. The prepared derivatives will be assessed for their antikinetoplastid activity and stability. From the results, promising

candidates will be further assessed for their in vivo effect and the mode of action as outlined in Figure 6.2.

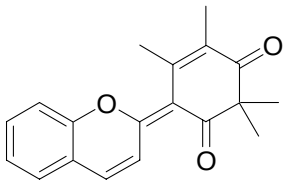


Figure 6.1: Structure of Dalrubone

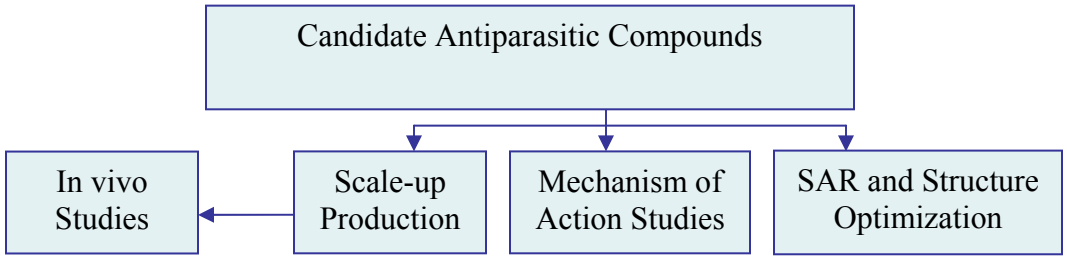
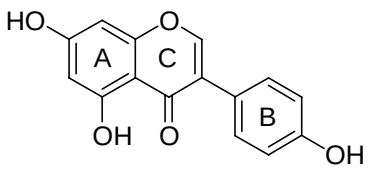


Figure 6.2: Scheme of Proposed Future Work

6.3 ISOLAVONES AS ANTIKINETOPLASTID COMPOUNDS

The isoflavone skeleton has not been previously systematically investigated as a template for antikinetoplastid compounds up to date. Our studies with compounds from *P. arborescens* and related commercially available molecules revealed several

isoflavonoids with remarkable antiprotozoal activities. In isoflavones, it appears that the structural requirements and the mode of action of antileishmanial compounds differ from those of antitrypanosomal structures. The simple dietary isoflavone genistein (Figure 6.3) was shown to be significantly active against *T. b. brucei* at low micromolar concentrations. This compound could be a new lead for the development of new antitrypanosomal compounds. Modification of rings A, B, and C of the core structure by the introduction of various substituents would generate an array of compounds that would help to draw the SAR requirements of this interesting class of compounds. Assessment and optimization of promising candidates according to the scheme in Figure 6.2 is needed to identify potentially more potent antikinetoplastid agents.



Genistein

Figure 6.3: Structure of Genistein

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