Search for natural sources with antiparasitic potentials using intracellularly persisting pathogens as test organisms

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ABSTRACT

Leishmaniasis is a prevalent disease in many parts of the tropical and subtropical world, causing significant morbidity or mortality. The rapid emergence of drug resistance by the treatment of parasites with common chemotherapeutics requires the development of new drugs for future therapy. Besides rational drug design, natural product research shows promise in finding new lead structures. Plants are valuable sources for the screening of bioactive secondary metabolites. Active compounds can be discovered successfully using modern biological screening assays and subsequent structural modifications can improve the therapeutic profile of lead substances.

In the present study we have investigated *in vitro* activity of some selected plants including *Drosera madagascariensis*, *Drosera peltata, Spathodea campanulata, Tabebuia avellanedae* and *Juglans regia* against both extracellular promastigotes and intracellular amastigotes of *Leishmania major* GFP. In parallel, the cytotoxic effects against bone marrow-derived macrophages as a mammalian host cell control were evaluated. In addition, a series of naphthoquinones was tested to gain insight into structure-activity relationships with a closer view on their mode of action.

The antileishmanial activity of *D. madagascariensis* was mainly attributed to 7-methyljuglone and quercetin, whereas plumbagin was the most active principal component of *D. peltata*. Ursolic acid and spathoside were apparently the most active compounds of *S. campanulata*. A bio-guided fractionation of the most active extract of *T. avellanedae* led to identification of lapachol and a mixture of isomeric furanonaphthoquinones [5-hydroxy- and 8-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-diones] as constituents predominantly responsible for the observed antileishmanial activities. Phenolic substances and terpenes which appeared enriched in *J. regia* leaves may be putative candidates as antileishmanial agents.

Regarding the tested series of naphthoquinones, most of the compounds exhibited noticeable leishmanlacidal activity against promastigotes (IC_{50} 0.5 to 6 µM) and intracellular amastigotes (IC_{50} 1 to 7µM) when compared with the antileishmanial drug amphotericin B (IC_{50} of 2.5 and 0.2µM, respectively). In general, host cell cytotoxicity and antiprotozoal activity seemed to increase in parallel. Conspicuously, the cytotoxic effect was less pronounced on infected host cells compared with that on noninfected cells. Concerning structure-activity relationships for the tested naphthoquinones, some interesting structural features emerged from this study. Introduction of a methyl or methoxyl group at C-2 of the parent 1,4-naphthoquinone slightly increased the leishmaniacidal activity, while the presence of a hydroxyl function in this

position dramatically reduced the effectiveness. In contrast, hydroxylation at C-5 and dihydroxy substitution at C-5 and C-8 appear to increase the antileishmanial activity, while the presence of hydroxyl groups in the quinone entity decrease dramatically the efficacy. Similarly, the presence of a side chain hydroxyl group *peri* to a carbonyl function as represented in the series of shikonin/alkannin derivatives increased the activity when compared with substituted analogs. Since furanonaphthoquinones tested were more active than corresponding naphthoquinones, it appears as if the presence of a furan ring increases the antileishmanial activity of naphthoquinones. Within the series of compounds tested, the dimeric mixture of vaforhizin and isovaforhizin showed the highest activity *in vitro* against intracellular amastigotes with an IC_{50} of 1.1 µM. The shikonin/alkannin derivatives proved to be similarly considerably leishmanicidal with IC_{50} values ranging from 1 to 3 µM. None of the naphthoquinones tested was capable to induce NO production known to play a crucial role in the host resistance against intracellular pathogens, excluding activation of microbicidal mechanisms in macrophages. The mode of action apparently depended on the nature and position of substituents, associated with the electrophilicity of the naphthoquinone or the efficiency of redox cycling. Conspicuously, members oxygenated in the quinone ring proved to be leishmanicidal when coincubated with glutathione, while the majority of the remaining compounds produced inactive glutathione-naphthoquinone conjugates.

ZUSAMMENFASSUNG

Leishmaniose ist eine verbreitete Krankheit in vielen Teilen der tropischen und subtropischen Welt mit einer hohen Erkrankungs- und Sterblichkeitsrate. Die rasche Selektion resistenter Parasiten gegenüber gängigen Chemotherapeutika macht die Entwicklung neuer Arzneimittel für zukünftige Therapien erforderlich. Neben dem strukturbasierten Drug Design ist die Naturstoff-Forschung eine vielversprechende Alternative bei der Suche nach neuen Leitstrukturen für innovative Arzneimittel. Pflanzen sind eine wertvolle Quelle für bioaktive sekundäre Metaboliten. Für das Auffinden von Wirkstoffen sind moderne biologische Screening Tests bestens geeignet. Mit strukturellen Modifikationen kann anschließend das therapeutische Profil von aufgefundenen Leitsubstanzen verbessert werden.

In der vorliegenden Studie haben wir einige ausgewählte Pflanzen (*Drosera madagascariensis*, *Drosera peltata*, *Spathodea campanulata*, *Tabebuia avellanedae* und *Juglans regia*) auf ihr antileishmanielles Potenzial gegenüber extrazellulären Promastigoten und intrazellulären Amastigoten von transgenen *Leishmania major* GFP untersucht. Parallel dazu wurden die Extrakte auf zytotoxische Effekte gegenüber Knochenmarksmakrophagen als Wirtszellen getestet. Darüber hinaus wurde eine Reihe von Naphthochinonen getestet, um Einblick sowohl in Struktur-Wirkungs-Beziehungen als auch Wirkmechanismen zu erhalten.

Die antileishmanielle Aktivität von *Drosera madagascariensis* beruhte hauptsächlich auf 7-Methyljuglone und Quercetin, während Plumbagin der aktivste Hauptbestandteil von *Drosera peltata* war. Ursolsäure und Spathosid waren offensichtlich die aktivsten Verbindungen von *Spathodea campanulata*. Eine wirkungsorientierte Fraktionierung (Bioassay guided fractionation) der aktivsten Fraktion aus *Tabebuia avellanedae* führte zur Identifizierung von Lapachol und einer Mischung von isomeren Furanonaphthoquinonen [5-Hydroxy- und 8-Hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]Furan-4,9-diones] als wesentliche Inhaltsstoffe für die beobachtete antileishmanielle Aktivität. Phenolische Verbindungen und Terpene, welche in *Juglans regia* Blättern angereichert vorliegen, wurden in dieser pflanzlichen Quelle als mögliche antileishmanielle Wirksubstanzen ausgemacht.

In der Reihe der getesteten Naphthochinone zeigten die meisten Verbindungen eine deutliche antileishmanielle Aktivität gegen Promastigoten (IC_{50} 0,5 bis 6 µM) und intrazellulären Amastigoten (IC_{50} 1 bis 7µM) im Vergleich zu Amphotericin B (IC_{50} 2,5 bzw. 0,2 µM) als Positivkontrolle. Im Allgemeinen schien die Zytotoxizität mit der antiparasitären Aktivität parallel anzusteigen. Auffälliger Weise war die zytotoxische Wirkung weniger stark ausgeprägt bei infizierten Wirtszellen im Vergleich zu nicht infizierten Zellen. Einige

interessante Struktur-Wirkungs-Beziehungen resultierten aus der Reihe der getesteten Naphthochinone in dieser Studie. Die Einführung einer Methyl- oder Methoxy-Gruppe an C-2 in das 1,4-Naphthochinongrundgerüst erhöhte leicht die antileishmanielle Aktivität, während die Anwesenheit einer Hydroxylgruppe in dieser Position die Wirksamkeit drastisch reduzierte. Im Gegensatz dazu verstärkte offensichtlich eine Hydroxylierung an C-5 oder auch die Anwesenheit einer Dihydroxygruppierung an C-5 und C-8 die antileishmanielle Aktivität, während die Anwesenheit von Hydroxylgruppen in der Chinonteilstruktur die antiparasitäre Wirksamkeit drastisch verringerte. Die Anwesenheit einer freien Hydroxylgruppe in der Seitenkette *peri* zur Carbonyl Funktion, wie in der Reihe der Shikonin / Alkannin Derivate vorzufinden, erhöhte die Aktivität im Vergleich zu substituierten Strukturanaloga.

Da die getesteten Furanonaphthoquinone sich als aktiver erwiesen als die entsprechenden Naphthochinone, erscheint es nahe liegend, dass die Anwesenheit eines Furanringes die antileishmanielle Aktivität von Naphthochinonen erhöht. Innerhalb der Reihe der untersuchten Verbindungen zeigte die Mischung der dimeren Vaforhizin und Isovaforhizin die höchste Aktivität *in vitro* gegen intrazelluläre Amastigoten mit einem IC_{50} von 1,1 µM. Die Shikonin / Alkannin Derivate erwiesen sich als ähnlich stark leishmanizid mit IC_{50}-Werten im Bereich von 1 bis 3 µM. Keine der getesteten Naphthochinone war fähig, eine NO-Produktion zu induzieren, welche eine entscheidende Rolle bei der Abwehr der Wirtszellen gegen intrazelluläre Pathogene spielt. Dies schließt eine Aktivierung von mikrobiziden Abwehrmechanismen in Makrophagen aus. Die Wirkungsweise hing offenbar von der Art und Position der Substituenten ab, verbunden mit den elektrophilen Eigenschaften des Naphthochinons oder das Ausmaß für einen Redoxzyklus. Auffällig war, dass die Vertreter mit einem oxygenierten Chinonring leishmanizide Aktivitäten zeigten, wenn sie mit Glutathion koinkubiert wurden, während der Großteil der übrigen Verbindungen inaktive Glutathion-Naphthochinon-Konjugate bildete.

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ABBREVIATIONS

BMMΦ	Bone marrow-derived macrophages
CC	Column chromatography
CFSPH	Center for Food Security and Public Health
ddH_2O	Double-distilled water
ELISA	Enzyme-linked immunosorbent assay
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
HIV	Human immunodeficiency virus infection
HPLC	High-performance liquid chromatography
LPS	lipopolysaccharide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PBS	Phosphate buffered saline
PI	Propidium iodide
PKDL	Post-kala azar dermal leishmaniasis
SI	Selectivity index
Th1	T-helper type-1
Th2	T-helper type-2
TLC	Thin layer chromatography
WHO	World Health Organization

1. INTRODUCTION

1.1 Leishmaniasis

Leishmaniasis is one of the most important parasitic diseases of humans, causing significant morbidity and mortality according to the World Health Organization (WHO) (WHO, 2002). Species of the genus *Leishmania* (Trypanosomatidae) were first described separately in 1903 by Leishman and Donovan in the splenic tissue of patients in India. Now a century later, millions are still afflicted by *Leishmania* and the WHO estimates that globally about 12 million people in 88 countries are infected with the pathogens, annually about 2 million new cases are added. Moreover, about 350 million people live at risk of infection with *Leishmania* parasites. The incidence of leishmaniasis is increasing, mainly because of man-made projects with environmental impact, like dams, irrigation systems and wells, as well as deforestation that increase human exposure to the sandfly vector. Immunosuppressive conditions such as acquired immunodeficiency syndrome (AIDS) increase the risk of *Leishmania*-infected people to develop potentially fatal visceral leishmaniasis by 100 to 2320 times (Alvar *et al*., 2008).

♦ Etiology

Leishmaniasis results from infection by various species of *Leishmania*, an obligate intracellular protozoan parasite that multiply in the white blood cells. Approximately thirty species have been described, and at least 20 thereof are pathogenic for mammals. Dogs and rodents seem to be the main reservoir of the pathogen.

Human visceral leishmaniasis is primarily caused by *L. donovani* and *L. infantum* in the Old World and by *L. chagasi* in the New World. *L. donovani* is anthroponotic; it is mainly transmitted between people, who act as the reservoir hosts while *L. infantum* is zoonotic. Most *Leishmania* species cause cutaneous leishmaniasis in humans. In the Old World, these organisms include the members of the *L. tropica* complex (*L. tropica, L. major* and *L. aethiopica*). New World species that cause cutaneous leishmaniasis include the members of the *L. braziliensis* complex (*L. braziliensis,* and *L. peruviana*) and the *L. mexicana* complex (*L. amazonensis, L. mexicana*, and *L. venezuelensis*), as well as *L. naiffi*, *L. lainsoni,* and *L. lindenbergi*. Additionally, some strains of *L. infantum* are a known cause of Old World cutaneous leishmaniasis without affecting the internal organs. All of these parasites, except of the anthroponotic species *L. tropica*, are zoonotic. In the New World, the organisms including *L. braziliensis* and *L. panamensis*/*L. guyanensis* may cause either cutaneous or mucocutaneous leishmaniasis. *L. infantum* is the most frequently species identified in animals (CFSPH, 2009).

♦ Geographic Distribution

Leishmania spp. have been reported to occur on all continents, except for Antarctica. These parasites are primarily endemic in many tropical and subtropical countries, predominantly in Africa, parts of Asia, the Middle East, Latin America, the Mediterranean region, and southern Europe (Figs. 1 and 2).

The spread of leishmaniasis depends strongly on the presence of vectors (insects, vertebrates), while the distribution of *Leishmania* species determines the type and severity of disease in geographic regions. *L. donovani* causes visceral leishmaniasis in Africa and South Asia, while *L. infantum* is the causative agent in the Middle East, Latin America, the Mediterranean, and parts of Asia. Cutaneous leishmaniasis is caused by *L. tropica* in the Mediterranean, the Middle East and parts of Asia, by *L. major* in the Middle East, Africa and parts of Asia, and by *L. aethiopica* in parts of Africa (CFSPH, 2009).

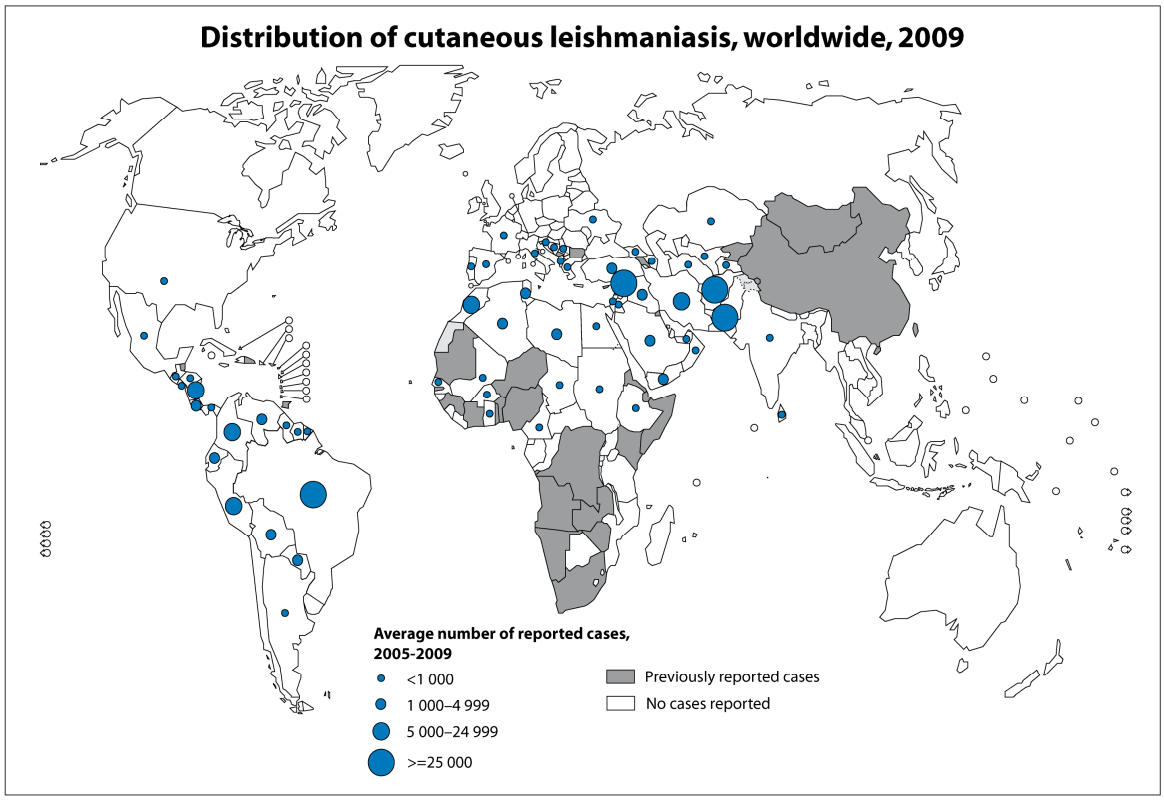


Fig. 1: The distribution areas of cutaneous leishmaniasis according to WHO, 2009 (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_leishmaniasis_cutaneous_2009.png)

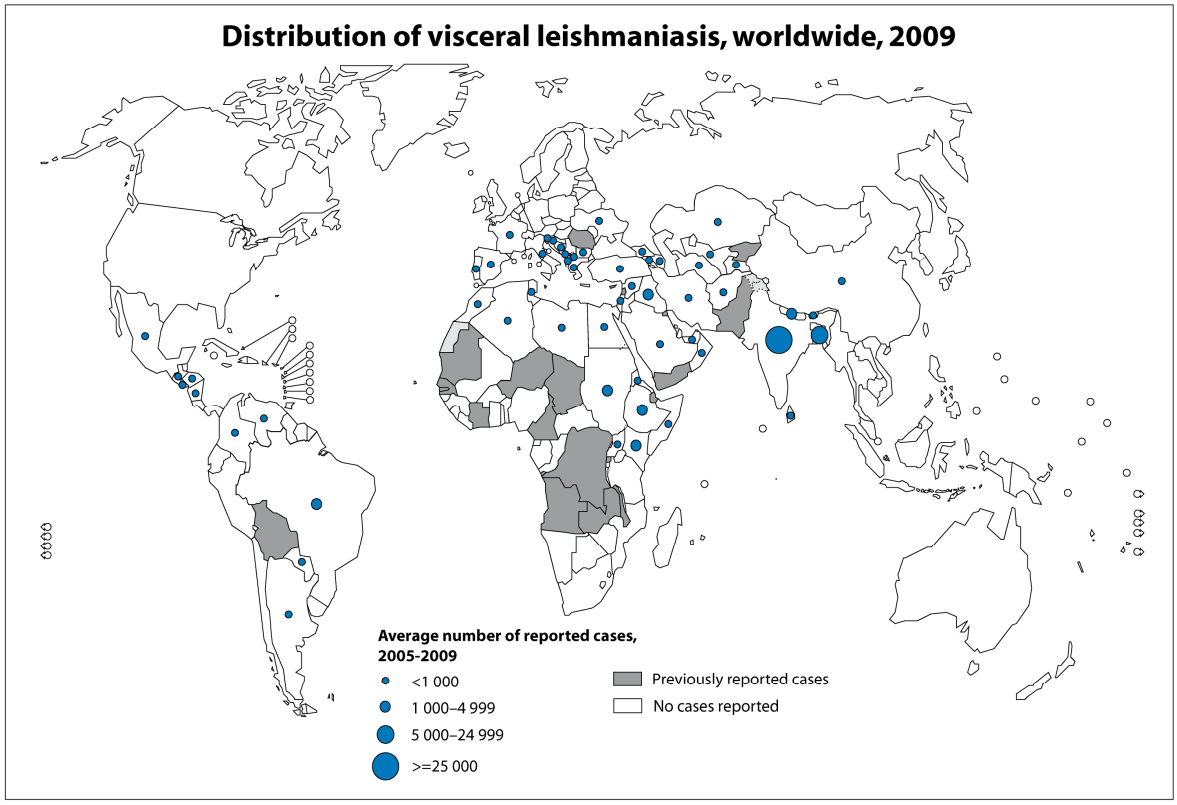


Fig. 2: The distribution areas of visceral leishmaniasis according to WHO, 2009 (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_leishmaniasis_visceral_2009.png)

♦ Transmission

Leishmania spp. are usually transmitted indirectly between hosts by sandflies of the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World. The distribution of sandflies largely affects the occurrence of disease. Each species of *Leishmania* is adapted to transmission in certain species of sandflies. Only the female sandfly transmits the parasite during blood feeding. Most species of sandflies bite when it is humid, and there is no rain or wind. These parasites have also been transmitted via blood transfusions and transplacental transmission in people. Venereal transmission of visceral leishmaniasis has been reported in dogs and humans, and other routes of transmission might be possible (CFSPH, 2009).

Humans and domestic animals are considered accidental hosts for many *Leishmania* spp., which are maintained in cycles between sandflies and mammalian hosts. Rodents and/or canines (wild or domestic) serve as the reservoir for most *Leishmania* species. *L. tropica* and *L. donovani* mainly are maintained in humans, but animals can also be infected occasionally. *L. peruviana* and *L. infantum* can be maintained in dogs, increasing the risk of transmission to people. Other domesticated animals might be involved as secondary maintenance hosts (CFSPH, 2009).

♦ Life cycle of *Leishmania*

The life cycle is relatively simple as illustrated in Fig. 3. When the infected sandfly bites a human, it injects small numbers of parasites which are in the promastigote stage and they will be rapidly phagocytized by macrophages. Once inside the cells, the parasites transform into amastigotes and enter the amastigote stage. The amastigotes begin to multiply and infect other cells and tissues. The life cycle is continued when the uninfected sandflies acquire the parasite by feeding on infected people or infected animals and ingest the amastigotes in the macrophages.

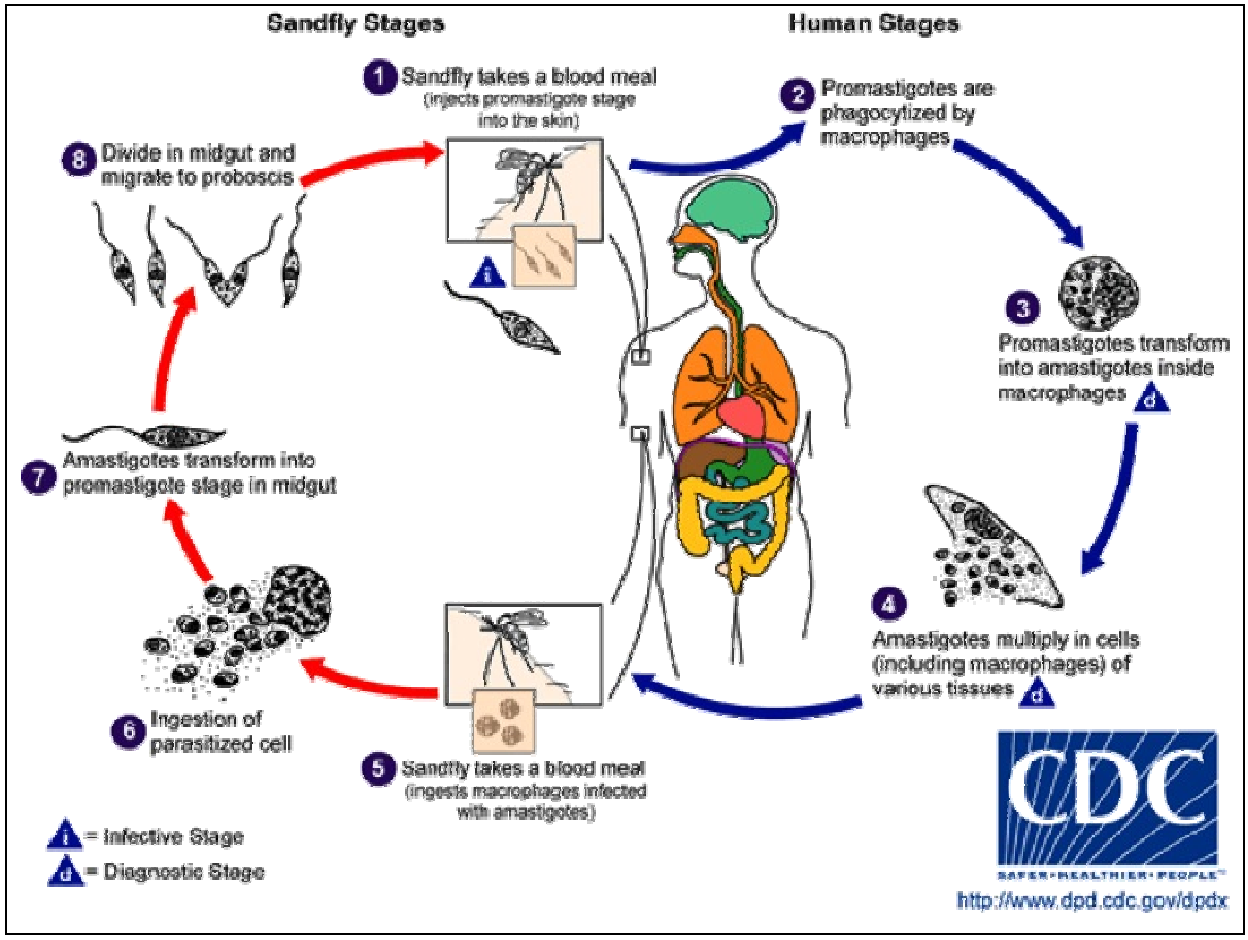


Fig. 3: The life cycle of *Leishmania* parasites.
Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies. The sandflies inject the infective stage (i.e., promastigotes) from their proboscis during blood meals(1). Promastigotes that reach the puncture wound are phagocytized by macrophages (2) and other types of mononuclear phagocytic cells. Promastigotes transform in these cells into the tissue stage of the parasite (i.e., amastigotes)(3), which multiply by simple division and proceed to infect other mononuclear phagocytic cells(4). Parasite, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Sandflies become infected by ingesting infected cells during blood meals ((5),(6)). In sandflies, amastigotes transform into promastigotes, develop in the gut (7) (in the hindgut for leishmanial organisms in the *Viannia* subgenus; in the midgut for organisms in the *Leishmania* subgenus), and migrate to the proboscis(8). SOURCE: Centers for Disease Control & Prevention (http://www.dpd.cdc.gov/dpdx/HTML/Leishmaniasis.htm)

♦ Incubation Period & Clinical Signs

People can be carriers for some species of *Leishmania* without any symptoms for long periods. Among symptomatic people, the reported incubation period can be as short as 1-2 weeks or as long as several months for cutaneous leishmaniasis when it is caused by New World species, and up to three years for Old World species. The incubation period for visceral leishmaniasis ranges typically from weeks to months; most cases appear clearly in two to six months. There are three clinically distinguishable types of leishmaniasis: cutaneous, mucocutaneous, and visceral. The species of *Leishmania* affect the form of the disease and the usual clinical signs. Some infections remain asymptomatic.

Cutaneous leishmaniasis

Cutaneous leishmaniasis involves the skin, and may be characterized by skin ulcers on the exposed parts of the body such as the face, arms and legs. It can produce a large number of lesions ranging from one to dozens. According to the infecting *Leishmania* species, the lesions may appear as smooth nodules, ulcers, flat plaques or hyperkeratotic wart-like lesions. The initial lesions are usually localized papules, but sometimes the parasites may spread via the lymphatic vessels and produce secondary lesions. The most lesions heal often spontaneously with permanent scars, but it may take several months to years according to the infecting *Leishmania* species. Immunosuppressive conditions like infection with the human immunodeficiency virus (HIV) or the steroid treatment can result in unusually severe cutaneous leishmaniasis.

Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis is a rare form of the disease usually occurs in Latin America. It tends to occur months or years after the healing of a cutaneous leishmaniasis ulcer, but it can also appear during the skin ulcers. Ulcerations and erythema at the nares are the first signs, which may develop into a destructive inflammation to involve the nasal septum, the pharynx or larynx. Mucocutaneous leishmaniasis does not heal spontaneously, and the destructive inflammation may lead to perforate the nasal septum causing severe disfigurement of the face, or block the pharynx or larynx. In some cases, the genitalia may also be involved (CFSPH, 2009).

Visceral leishmaniasis

The most common symptoms of visceral leishmaniasis, the most severe form, are an intermittent fever, decreased appetite, abnormal weight loss, anemia, and abdominal distension with enlargement of the liver and spleen. Thrombocytopenia, a low platelet count, may cause abnormal bleeding, including hemorrhages or petechiae on the mucous

membranes, and leukopenia can result in increased susceptibility to infections with other microorganisms. Other possible symptoms may include chronic diarrhea, darkening of the skin, coughing, lymphadenopathy, and in many cases, signs of chronic renal disease. Mild cases with only a few symptoms may resolve spontaneously. Without treatment, this form of the disease is nearly always fatal, often from secondary infections and other complications. Fulminant or fatal disease can also occur, especially in patients co-infected with HIV. Successfully treated people continue to carry the parasite, and the disease may recur if they become immunosuppressed. Similarly, asymptomatically infected individuals may later develop clinical signs (CFSPH, 2009).

Post-kala azar dermal leishmaniasis (PKDL) is a complication of visceral leishmaniasis caused by *L. donovani*. This syndrome is characterized by a macular, maculopapular, and nodular rash in a patient who has recovered from visceral leishmaniasis and who is otherwise well. The rash usually starts around the mouth and spreads to other parts of the body depending on severity. In Africa, PKLD is common, usually occurs within 6 months of visceral leishmaniasis, and typically disappears within a year without treatment. In South Asia, this syndrome is relatively rare, occurs several years after visceral leishmaniasis has been cured, and required prolonged treatment. In India, PKLD is seen in 1-3% of successfully treated cases of visceral leishmaniasis (CFSPH, 2009).

♦ Immune response

The immune response to *Leishmania* infection is cell mediated (Table. 1). *Leishmania* species are obligate intracellular protozoa that colonize macrophages in the mammalian host. The outcome of infection will depend on whether the host mounts primarily a T-helper type-1 (Th1) or Th2 response.

Table. 1: The immune response

Th1 immune response	• T-helper CD4 cells producing IL-2, IL-3, IFNγ • Will promote immune responses that are primarily cell mediated/inflammatory by activating cytotoxic T cells, natural killer cells and macrophages • In leishmaniasis, associated with disease resolution
Th2 immune response	• Th2 cells produce IL-4, IL-5, IL-6, IL-10 which favour induction of antibody responses by B cells • In leishmaniasis, associated with disease progression

In the Th1 response, promastigotes attach to reticuloendothelial cells and T helper CD4 cells produce different cytokines like IL-2, IL-3 and IFNγ which activate macrophages. IL-12 and tumor necrosis factor (TNF) are also important in this type of response. Activation of macrophage is a primary mechanism to eliminate the *Leishmania* parasite presumably mediated by toxic metabolites of oxygen, which may include super oxide anion (O^-_2), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Assreuy *et al*., 1994). The promastigotes are then phagocytosed by the activated macrophages into vacuoles which then fuse with lysosomes. In contrast, Th2 immune response limits the action of Th1 functions via IL-4 and IL-10, which deactivate macrophages helping intracellular parasite growth and disease progression.

Communicability

Although leishmaniasis is usually a vector-borne disease, person-to-person transmission including blood transfusion, venereal transmission and congenital transmission has been reported. Newborns can be infected whether or not the mother was symptomatic. Humans infected with some species of *Leishmania* can infect sandflies.

♦ Diagnostic Tests

Cutaneous leishmaniasis can already be diagnosed based on the skin symptoms. The diagnosis is confirmed by detection of pathogens in the impression smears of skin scrapings, or skin biopsies stained with Giemsa, Leishman's, Wright's or other stains. Intracellular amastigotes are directly detected in recent or active lesions. Moreover, promastigotes can be detected microscopically after 3-10 days in promastigote cultures. However, each species will grow only in certain media, but brain–heart infusion (BHI) medium, Novy-MacNeil-Nicole (NMN) medium, Evan's modified Tobie's medium (EMTM), Grace's medium and Schneider's *Drosophila* medium might be used initially. Inoculation of animals may be used especially with contaminated material, but the diagnosis may require weeks to months. Hamster (*Mesocricetus auratus*) is the most commonly used animal for *in-vivo* isolation.

The visceral leishmaniasis can be diagnosed through the clinical observations and by finding the amastigotes, which may be found in peripheral blood, or more often, in aspirates or biopsy smears from the spleen, bone marrow or lymph nodes. Culture or animal (hamster) inoculation may also be valuable, especially when parasite numbers are low.

The different *Leishmania* species, subspecies or strains can be identified by several techniques:

- Isoenzyme characterisation, also known as MLEE (multi-locus enzyme electrophoresis).

- The monoclonal antibody (MAb) technique.
- DNA hybridization and kinetoplast DNA restriction endonuclease analysis.
- Polymerase chain reaction (PCR)-based methods.

A delayed hypersensitivity test, the leishmanin skin test (Montenegro skin test), may be helpful in the diagnosis, while the serological tests including indirect immunofluorescence assay (IFA), direct agglutination test, enzyme-linked immunosorbent assay (ELISA), fast agglutination-screening test (FAST), and a rapid immunochromatographic assay (K39 dipstick or strip-test) are used for detecting antileishmanial antibodies.

Other assays including gel diffusion, complement fixation, indirect hemagglutination and countercurrent electrophoresis have also been used. Cross-reactions can occur in some serological tests with schistosomiasis, Chagas disease, leprosy, and malaria. Patients co-infected with HIV have difficulty in producing antibodies, especially at a late stage or during relapses; thereby the serological diagnosis of leishmaniasis is falsely negative in a high percentage of co-infected patients. Consequently, there is a need to use two or more serological tests and antigens freshly prepared in the laboratory to increase sensitivity.

♦ Treatment

DRUGS AVAILABLE AND IN CLINICAL TRIALS

The recommended drugs used for the treatment of leishmaniasis, the pentavalent antimonials, were first introduced 60 years ago. Even though antimonials are still the first-line drugs against all forms of leishmaniasis, their use in the clinical setting has several limitations, including severe side effects, the need for daily parenteral administration and drug resistance. Over the past decade alternative drugs or new formulations of other standard drugs have become available and registered for use in some countries, whilst other drugs are on clinical trial (Table. 2) (Croft *et al*., 2006).

Table. 2: Current drugs used for the treatment of leishmaniasis

Visceral leishmaniasis	
First line drugs	Sodium stibogluconate (Pentostam, SSG); meglumine antimoniate (Glucantime)
	Amphotericin B (Fungizone)
	Liposomal amphotericin B (AmBisome)
	Pentamidine
Clinical trials	Miltefosine (oral, Phase IV; registered in India)
	Paromomycin (Phase III)
	Sitamaqine (oral, Phase II)
	Other amphotericin B formulations
Cutaneous leishmaniasis	
First line drugs	Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)
	Amphotericin B (Fungizone)
	Pentamidine
	Paromomycin (topical formulations with methylbenzethonium chloride or urea)
Clinical trials	Miltefosine (oral, Phase III, registered in Colombia)
	Paromomycin (topical formulation with gentamicin and surfactants, Phase II)
	Imiquimod (topical immunomodulator, Phase II)
	Also anti-fungal azoles – ketoconazole, fluconazole, itraconazole

Parenteral administration unless otherwise stated

Pentavalent Antimonials

For almost seven decades, the pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) have been the standard antileishmanial therapeutic choice. Response rates are still over 95% in previously untreated visceral leishmaniasis patients in many parts of the world, but acquired resistance to pentavalent antimony (Sb^v) has developed in the high transmission epidemic region of Bihar, India (Croft *et al*., 2006). Failure rates of 65% have been reported and the use of antimony abandoned in this region (Sundar *et al*., 2000). However, outside Bihar, Sb^v remains the drug of choice to be used parenterally in a dose of 20 mg/kg daily for 30 days without any upper limit (Sundar and Chatterjee, 2006). Antimony therapy is often accompanied by local pain during intramuscular injections and by systemic side effects, requiring very careful medical supervision. Typical side-effects include nausea, vomiting, weakness and myalgia, abdominal colic, diarrhea, skin rashes, elevated hepatic transaminases blood levels, pancreatitis, and pneumonitis, together with the most important cardiotoxicity. Serious cardiotoxicity is seen in 9-10% of treated patients. Higher rates have

been reported due to improper formulation of drug and use of lots with high osmolarity (Sundar *et al*., 1998).

All pentavalent antimonials are now generally accepted as prodrugs which require biological reduction to the trivalent form (Sb^{III}) for antileishmanial activity. The site (macrophage, amastigote or both) and the mechanism of reduction (enzymatic or nonenzymatic) remain unclear. Trivalent antimony has recently been shown to directly interfere with thiol metabolism in drug-sensitive *L. donovani* and its mode of action involves several effects on glutathione and trypanothione metabolism (Wyllie *et al*., 2004).

Pentamidine

Pentamidine, an aromatic diamidine, still in use as first line drug for certain forms of cutaneous leishmaniasis, but in visceral leishmaniasis is used as second-line drug just because of efficacy and toxicity issues. It causes irreversible insulin dependent diabetes mellitus and death, therefore its use for visceral leishmaniasis in India has been abandoned (Olliaro *et al*., 2005). Pentamidine was found to be a competitive inhibitor of arginine transport and a noncompetitive inhibitor of putrescine and spermidine transport while its antileishmanial mechanisms of action, which possibly include inhibition of polyamine biosynthesis, DNA minor groove binding, and effect on mitochondrial inner membrane potential, are still not clearly defined (Bray *et al*., 2003). Recently mitochondria have been suggested as site of pentamidine accumulation in parasite and that the development of the resistance phenotype is accompanied with mitochondria alterations and by lack of mitochondrial accumulation and exclusion of the drug from the parasite (Mukherjee *et al*., 2006; Basselin *et al*., 2002).

Amphotericin B

Amphotericin B is an antifungal macrolide antibiotic, which was originally extracted from *Streptomyces nodosus*. Amphotericin B deoxycholate (Fungizone®), a micellar formulation, is highly effective. It has been used as a second line treatment for leishmaniasis since the 1960s. Need for hospitalization, constant monitoring of patients, prolonged duration of treatment and frequent adverse effects, including infusion-related fever and chills, nephrotoxicity, and hypokalemia are disadvantages of amphotericin B deoxycholate (Olliaro *et al*., 2005). Lipid based formulations of amphotericin B are available and liposomal amphotericin B (AmBisome®) was the first to be evaluated and is licensed in several European countries and USA for primary treatment of visceral leishmaniasis. Other commercially available lipid formulations, amphotericin B lipid complex (ABLC) and amphotericin B colloidal dispersion (ABCD) have also been tested but high cost and side effects were a limiting factor. Interestingly, the possibility of resistance cannot be ignored

with the increasing use of amphotericin B in lipid formulations that have longer half-lives. Moreover, there is a study on the emergence of amphotericin B resistance in *L. infantum*/HIV-infected cases (Di Giorgio *et al*., 1999).

Amphotericin B directly binds membrane-embedded ergosterol, the main sterol found in *Leishmania* membranes, to formation of transmembrane amphotericin B channels, aqueous pores, and leakage of cations (Pourshafie *et al*., 2004; Brajtburg and Bolard, 1996). The selectivity against *Leishmania* is due to its higher affinity for ergosterol over cholesterol, the predominant sterol in mammalian cells.

Miltefosine

Miltefosine is an alkylphosphocholine, initially developed as anticancer drug. It is first effective oral antileishmanial drug which became available and registered for treatment of visceral leishmaniasis in India and Germany and for cutaneous leishmaniasis in Colombia. Gastrointestinal toxicity and reversible, increased transaminase and creatinine levels were recognized as the most common adverse effect in clinical trials (Bhattacharya *et al*., 2007). Miltefosine is embryotoxic and fetotoxic in rats and rabbits, and teratogenic in rats. It is therefore contraindicated for use during pregnancy, and mandatory contraception is required for women in child-bearing age for the duration of therapy and 2-3 months beyond (Sindermann and Engel, 2006). A long terminal half-life of 150 – 200 hours has previously been reported and that raises concerns for emergence of resistance.

Uptake of miltefosine into *Leishmania* parasite is mediated by a plasma membrane P-type ATPase, which belongs to the partially characterized aminophospholipid translocase subfamily (Perez-Victoria *et al*., 2003). The mechanism of action of miltefosine is still not known. Several targets of miltefosine in *Leishmania* have been suggested, including perturbation of ether-lipid metabolism, glycosylphosphatidylinositol (GPI) anchor biosynthesis and signal transduction and induction of apoptosis (Lux *et al*., 1996; Paris *et al*., 2004; Verma and Dey, 2004) as well as inhibition of alkyl-specific acyl-CoA acyltransferase, an enzyme involved in lipid-remodeling (Lux *et al*., 2000). Recent studies suggest that mitochondria and specifically the cytochrome c oxidase is a potential target for miltefosine in *Leishmania* (Luque-Ortega and Rivas, 2007; Saugar *et al*., 2007). Effects on lipid metabolism, specifically phospholipid content, fatty acid and sterol content, have also been described in *L. donovani* promastigotes (Rakotomanga *et al*., 2007).

Paromomycin

Paromomycin, an aminoglycoside antibiotic, is the latest antileishmanial drug registered for visceral leishmaniasis in India. Paromomycin was shown to be noninferior to amphotericin B

for the treatment of visceral leishmaniasis in a randomized, controlled, phase 3 study in India (Sundar *et al*., 2007). Paromomycin has also been formulated for topical treatment of cutaneous leishmaniasis. The topical formulations offer the advantage of ease of administration, fewer side effects and cost-effectiveness in comparison to systemic treatment. Nephrotoxicity, ototoxicity and increased levels of transaminases are known drug class effects, but frequency of these reactions has been reported as low at therapeutic dosages for visceral leishmaniasis. Early studies on paromomycin in *Leishmania* spp. have implicated mitochondrial ribosomes and induction of respiratory dysfunction and mitochondrial membrane depolarization in the mechanism of action (Maarouf *et al*., 1995; Maarouf *et al*., 1997a; Maarouf *et al*., 1997b). The resistance was specific to paromomycin and stable and the resistance was related to decreased drug uptake in *L. donovani* (Maarouf *et al*., 1998).

Sitamaquine

Sitamaquine, a 4-methyl-6-methoxy-8-aminoquinoline (lepidine), was originally developed as WR6026 by the Walter Reed Army Institute in collaboration with GlaxoSmithKline for oral treatment of visceral leishmaniasis (Yeates, 2002). The most commonly reported side effects were abdominal pain, headache, vomiting, dyspepsia and cyanosis. It appears to have relative moderate toxicity and causes mild methemoglobinaemia and nephropathy (Dietze *et al*., 2001). Sitamaquine induced morphological changes in intracellular *L. tropica* amastigotes and host macrophages. Collapse of mitochondrial membrane potential in *L. donovani* promastigotes has also been shown as well as alkalisation of acidocalcisomes. Recently antileishmanial activity has been demonstrated as inhibition of the respiratory chain complex II, which in turn triggers oxidative stress and finally leads to an apoptosis-like death of parasites (Carvalho *et al*., 2011).

Imiquimod

Imiquimod (Aldara, 3M Pharmaceuticals) is a form of imidazoquinoline, which works as an antiviral, antitumor, and immunomodulator that stimulates a local immune response at the site of application. Imiquimod has been used for topical treatment of cutaneous leishmaniasis. It induces the production of cytokines and nitric oxide in macrophages. Monotherapy of imiquimod is less effective than the in conjunction with meglumine antimoniate (Shamsi Meymandi *et al*., 2011; Khalili *et al*., 2011). It was suggested that the topical treatment activates stimulate macrophages to kill the parasite, while meglumine antimoniate eliminates systemic amastigotes which are responsible for persistence of infection.

DRUGS IN LEAD OPTIMIZATION AND PRECLINICAL PHASES

Although many compounds have shown activity in *in vitro* models, only few have received thorough testing in relevant animal models of infection. Of these, a few have exhibited significant antileishmanial activity. For example, 8-aminoquinoline analogs, originally developed as antimalarials, have shown promise in the treatment of leishmaniasis as seen with NPC1161 (Nanayakkara *et al*., 2008), 8-aminoquinolines derivatives with a 5-(3-trifluoromethylphenoxy) substitution on the quinoline ring and methyl-substituted, ethyl-substituted or unsubstituted C4 positions (Jain *et al*., 2005), and 8-aminoquinoline tafenoquine (Yardley *et al*., 2010). Buparvaquone and two water soluble phosphate prodrugs have been tested in *in vivo* experimental models. In the visceral model, both prodrugs were significantly more effective in reducing the liver parasite burden compared with buparvaquone. Moreover, topical formulations of buparvaquone (or its prodrug) significantly reduced cutaneous parasite burden and lesion size (Garnier *et al*., 2007). Recently a novel liposomal formulation, containing phosphatidylserine, of buparvaquone showed high activity in *L. infantum chagasi*-infected hamsters (Reimão *et al*., 2012). β-Nitrovinylfuran derivatives were tested against a number of *Leishmania* species for the treatment of visceral leishmaniasis as well as for cutaneous and mucocutaneous leishmaniasis in *in vitro* assays with high activity (Castapedo *et al*., 2009). The same study investigated these agents in *in vivo* murine models and human's leishmaniasis and found that had a higher activity compared to reference drugs using different administration routes. On the other hand, plant products are an abundant source of leads evidenced by the use of medicinal plants for the treatment of parasitic diseases since ancient times. Licochalcone A, an oxygenated chalcone isolated from the roots of Chinese plant liquorice *Glycyrrhiza* has shown activity against *L. donovani* and *L. major* in experimental models (Chen *et al*., 1994). Furthermore, synthetic oxygenated derivatives of licochalcone A were also active. Flavonoids are widely distributed in the plant kingdom and a search for their antileishmanial activity have yielded compounds like quercetin derived from *Fagopyrum esculentum* and luteolin isolated from *Vitex negundo* (Mittra *et al*., 2000). *Kalanchoe pinnata* leaf extract, rich in flavonoids, exhibited activity in murine cutaneous leishmaniasis (da Silva *et al*., 1995) but its efficacy extends also to visceral leishmaniasis in *in vivo* murine models by increasing generation of reactive nitrogen intermediates (Gomes *et al*., 2010). Plant-derived saponins such as α-hederin, β-hederin and hederagenin isolated from *Hedera helix* show leishmanicidal activity against *L. infantum* and *L. tropica* whereas hederacolchiside A1 obtained from *Hedera colchica* exhibited strong activity against the promastigotes and amastigotes of *L. infantum*, but these saponins display a notable

antiproliferative activity on human monocytes (Ridoux *et al*., 2001). Alkaloids also have been abundantly used against leishmaniasis and include 2-substituted quinoline alkaloids isolated from a Bolivian medicinal plant *Galipea longiflora*, that shown activity for experimental treatment of New World cutaneous leishmaniasis (Fournet *et al*., 1993). Berberine, a quaternary isoquinolinic alkaloid found in a number of plant families (*e.g*. Annonaceae, Berberidaceae, Menispermaceae), is one of the alkaloids with the highest leishmanicidal activity. In both the 8-day and long-term models of *L. donovani* infection in hamsters, it markedly diminished the parasitic load and proved to be less toxic than pentamidine (Ghosh *et al*., 1985).

In view of the present scenario, development and introduction of new antileishmanial compounds would be an urgent need. The toxicity of the clinically used drugs and the persistence of side-effects even after modification of the dose level and duration of treatment as well as the development of drug-resistance by parasites are, however, severe drawbacks. The search for antileishmanial agents is on for new drugs that are less toxic, easily available and affordable for poor people most afflicted by the disease. In the ongoing search for new drugs, plant-derived products are gaining ground being easily available and relatively cheap. Isolation and purification of active constituents of medicinal plants was one of the most important powers that led to the birth of the pharmaceutical industry in the 19th century. After a long period of neglect, there has been reawakening of interest in the analysis of the natural products for the presence of therapeutic agents. Furthermore, the leads obtained from the medicinal plants with antileishmanial activity give new stimulus for obtaining valuable synthetic compounds. There are approximately 250,000 plant species worldwide of which only a fraction have so far been studied and they have shown potent chemotherapeutic properties (Kayser *et al*., 2003; Salem and Werbovetz, 2006). However, much remains to be explored to identify plants as sources of drugs especially as several plant metabolites have been shown to contain phytoconstituents with potent leishmanicidal activity. Interestingly, plants have exceptional ability to produce cytotoxic agents, which protect plants from invasive and pathogenic microbes in their environment and there is an ecological rationale that these agents are promising sources for finding new antiinfectious lead compounds. Thus, we have investigated in the present study antileishmanial activity of some selected plants including members of Droseraceae, Bignoniaceae and Juglandacea.

1.2 Plants studied for antileishmanial activities

1.2.1 *Drosera species*

Drosera, commonly known as the sundews, is one of the largest genera of carnivorous plants, with at least 194 species. These members of the family Droseraceae characterized by leaves covered with glandular hairs which trap and digest insects. The hairs are stalked glands and produce digestive juices that decompose the trapped prey. Preying on insects is their primary means of obtaining nutrients. Various species, which vary greatly in size and form, can be found growing natively on every continent except Antarctica. The earliest documented use of *Drosera* species dates to the 12th century, when an Italian doctor named Matthaeus Platearius described the plants as a cough remedy. Traditionally, *drosera* species have been used for respiratory illnesses, such as coughs, wheezing, and asthma; however, limited evidence exists on how it may work. Additional study is needed in this area before conclusions can be made.

1.2.1.1 *D. madagascariensis*

D. madagascariensis is a robust stem-forming species with a clearly visible stem. The plant grows to a height of 25 cm, and the upper part is composed of carnivorous leaves while the lower part is covered with the dried remains of older leaves. The leaf arrangement on the stem is alternate. The pink petals are obovate, 6-12 mm long and 4-6 mm wide (Fig. 4). The seed capsules are dehiscent and bear numerous seeds. The root system is relatively undeveloped, serving mainly as an anchor and for water absorption, since nutrient uptake is achieved through carnivory. *D. madagascariensis* is native to the tropical Africa as far south as South Africa and east to the island of Madagascar.

Fig. 4: *Drosera madagascariensis*, (Droseraceae)
SOURCE: Botanical Conservatory (Yolo County, California, US)
(http://calphotos.berkeley.edu/cgi/img_query?seq_num=286399&one=T)

1.2.1.2 *D. peltata*

D. peltata, commonly called the shield sundew or pale sundew, is a climbing perennial tuberous plant. Its underground tuber is generally found 4 - 6 cm under the soil surface with a poorly developed root system. It usually begins growth in late autumn to early winter and grows to a height of 30 cm (Fig. 5). It forms aerial portion only for a few months during early summer. The stem emerges from the center of the rosette and rapidly grows upwards bearing shield-shaped cauline leaves every 1-2 cm each 2-3 mm long, 4-6 mm wide. The trapping and digestion mechanism usually depends on two types of glands: stalked glands that secrete sweet mucilage to attract insects and enzymes to digest them, and sessile glands which absorb the resulting nutrient soup. Upon touching the sticky dew, the prey becomes entrapped by sticky mucilage which prevents their progress or escape. The flowers have 5 white petals each 6-8 mm long, which are terminal on the stem. Stamens are 5, each 2-4 mm. *D. peltata* has a large range of distribution, which includes eastern and western Australia, New Zealand, India, and most of Southeast Asia.

Fig. 5: *Drosera peltata*, (Droseraceae), SOURCE: PlantSystematics.org (http://www.plantsystematics.org/cgi-bin/dol/dol_terminal.pl?taxon_name=Drosera_peltata)

1.2.2 *Spathodea campanulata*

Spathodea campanulata P.Beauv., commonly known as the fountain tree, African tulip tree, firebell, rarningobche, tulipàn Africano or tuliptree, has primarily described by the French naturalist Palisot de Beauvois (1752-1820). *S. campanulata* of the flowering plant family Bignoniaceae is a medium-sized tree that commonly reaches a height of 30 m (Fig. 6). This tree is planted extensively as an ornamental tree throughout the tropics and is much appreciated for its very showy reddish-orange campanulate flowers. Moreover, the wood is difficult to burn, so the tree is also valuable for fire resistant landscaping. Flowers are large, bell-shaped, up to 10 cm long and 5 cm wide with a peculiar smell. The pods are firm, thick, dark-brown, and 15 to 20 cm long, which contain a liquid that will squirt out if they are squeezed or pierced. Seeds, 5 months after flowering, are about 2.5 cm wide, with a broad, silvery white, transparent wing. Leaves are pinnately compound, 20 to 25 cm long, divided into 5 to 8 pairs of leaflets. Leaflets are elliptic-oblong, 7.5 to 11 cm long, and 3.5 to 7 cm wide, with a pointed tip, and several glands along the midrib near the base. African tulip tree comes from the rainforests of Equatorial Africa. Its native range extends along the Pacific Coast of Africa from Ghana to Angola and inland across the humid center of the continent to southern Sudan and Uganda. Traditionally, the stem bark has been used for wound healing, where it is applied as a paste to the wound (Mensah *et al*., 2003). Other folklore uses of the plant in Ghana include the treatment of dyspepsia and peptic ulcer (stem bark and leaf); arthritis and fracture (leaf, root bark and fruit); toothache and stomach ache (stem bark); and stomach ulcer (root bark and seed) (Agbovie *et al*., 2002).

Fig. 6: *Spathodea campanulata* P.Beauv, (Bignoniaceae), SOURCE: PlantSystematics.org http://www.plantsystematics.org/imgs/kcn2/r/Bignoniaceae_Spathodea_campanulata_8049.html

1.2.3 *Tabebuia avellanedae*

T. avellanedae LORENTZ *ex* GRISEB (syn. *T. impetiginosu* MART *ex* DC) is a native Bignoniaceae tree of the Amazon rainforest and other tropical parts of South and Latin America. Consequently it has a range of local names pau d'arco, ipê, ipê roxo, lapacho, tahuari, taheebo, trumpet tree, ipê-contra-sarna, tabebuia ipê and tajy. It is a large shrub or tree, growing to 50 m high and the base of the tree can be 2–3 m in diameter. Leaves are opposite and petiolate, 2 to 3 inches long, elliptic and lanceolate, with lightly serrated margins and pinnate venation. The leaves are palmately compound with usually 5 leaflets. The flower is large and 3-11 cm wide, sitting in dense clusters; its corolla is often pink or magenta. The calyx is campanulate to tubular, mostly five-lobed, and looking like a trumpet (Fig. 7). The fruit is a dehiscent pod, 10 to 50 cm long with several winged seeds. The pods often remain on the tree through the dry season until the beginning of the rainy time. Pau d'arco has a long and well-documented history of use by the indigenous tribes of the rainforest of South America. The inner bark of the trunk has been used for healing infections and against many ailments including malaria, anemia, colitis, respiratory problems, colds, cough, flu, fungal infections, fever, arthritis and rheumatism, snakebite, poor circulation, boils, and syphilis. Tea prepared from the inner bark is reputed to be useful in the treatment of cancer as well as in alleviating the ulcers, and the symptoms of disorders such as diabetes, and long term rheumatism in patients.

Fig. 7: *Tabebuia avellanedae* LORENTZ ex GRISEB (syn. *T. impetiginosu* MART ex DC), (Bignoniaceae). SOURCE: wikipedia.org

1.2.4 *Juglans regia*

The genus *Juglans* (family Juglandaceae) comprises several species and is widely distributed throughout the world. *Juglans regia* L., common walnut, is a deciduous tree growing to 20 m high and the trunk up to 2 m diameter, commonly with a short trunk and broad crown. The leaves are alternately arranged, 25–40 cm long, and imparipinnate with 5–9 leaflets, which are ovate to elliptic-ovate. The largest leaflets are the three at the apex, 10–18 cm long and 6–8 cm broad; the basal pair of leaflets are much smaller, 5–8 cm long, with the margins of the leaflets entire. The flowers are monoecious (individual flowers are either male or female, but both sexes can be found on the same plant). The male flowers are in pendant catkins 5–10 cm long, while the female are in spikes with pistillate flowers in clusters of three to nine. At the autumn the fruits green husks split open and the walnut will fall to the ground. The seed is large, 2 to 4-lobed at the base with a relatively thin shell and a rich flavor. Walnut is native to the region stretching from the Balkans eastward to the Himalayas and southwest China. The walnut tree has a long history of medicinal use. Its green fruit has been pickled in the vinegar and used as gargle for sore and slightly ulcerated throats, whereas the shell used to treat diarrhoea and anemia. Its leaves were described as anthelmintic, anti-inflammatory, astringent and depurative. The leaves are used internally for the treatment of chronic coughs, asthma, diarrhoea, dyspepsia. They are also used externally to treat skin disorders and purify the blood.

Fig. 8: *Juglans regia* L. (common walnut), (Juglandacea). SOURCE: wikipedia.org

1.3 Aims & objectives of the project

The increasing of international travel of people, global warming and climate change, the absence of a vaccine, and the development of drug-resistance by parasites, together with the increasing frequency of *Leishmania*/HIV co-infection, have enormous potential to increase the risk of leishmaniasis; therefore there is an urgent need for effective drugs to replace/supplement those in current use.

In recent years, there has been an increasing interest in natural products, especially those derived from higher plants, as a valuable source of drugs; in conjunction with the advances in chromatographic and analytical techniques. For these reasons, there was extensive search over the previous years by our working group for antiprotozoal and immunomodulatory agents (Latté and Kolodziej, 2000; Kayser *et al*., 2001; Kiderlen *et al*., 2001; Kolodziej *et al*., 2001a; Kolodziej *et al*., 2001b; Kolodziej *et al*., 2002; Tan *et al*., 2002a; Tan *et al*., 2002b; Sakar *et al*., 2002; Radtke *et al*., 2003; Radtke *et al*., 2004; Kolodziej and Kiderlen, 2005; Ercil *et al*., 2005; Kolodziej *et al*., 2005; Trun *et al*., 2006; Kolodziej , 2008; and Thäle *et al*., 2011). Because of the intracellular location of *Leishmania*, there were simultaneously attempts to develop the *in vitro* test systems, which enable us to assay the activity of natural products against intracellular amastigotes (Kram *et al*., 2008).

In our continuing search for antileishmanial compounds from plants, we have investigated in the present study the *in vitro* activity of some selected plants against both extra- and intracellular *L. major* parasites. Furthermore, it was important to identify the principal active components of the plant extracts. To achieve this aim it was useful to extract the plant materials with various solvents and to use different methods of extraction. The individual extracts were subjected to phytochemical analysis and tested *in vitro* for their antiprotozoal activities against both extra- and intracellular forms. As macrophages are host cells for *Leishmania* parasites, it was important to evaluate the cell cytotoxicity of extracts in parallel.

Recently, transgenic *Leishmania* GFP have opened the way for a FACS-based method to assess the killing of intracellular *Leishmania* parasites. This assay has several advantages. For example, the addition of PI, an intercalating fluorochrome that stains dead cells, permits simultaneous measuring of antiparasitic effects and cytotoxic effects on host cells. It appears worthwhile to compare the FACS-based method with that employing host cell lysis.

Fractionation of extracts exhibiting antileishmanial activities is a critical step to isolate the active compounds for establishing their structures by chemical and spectroscopic methods. Therefore, a combination of chromatographic techniques including TLC analysis, column

chromatography, and HPLC appears to be a useful strategy for successful isolation, while sophisticated NMR methods in conjunction with mass spectroscopy should meet the requirements for unambiguous structure elucidation.

Many medicinal plants are known to have immunomodulatory properties and have proved to maintain body resistance against infection by re-establishing the immune system reflected by, e.g., increased release of NO as antimicrobial effector molecule and enhanced expression of pro-inflammatory cytokines in infected cells. As leishmaniasis is associated with immunological dysfunction, experimental approaches have included the development of antileishmanial agents that activate macrophages, predominantly by enhancing the release of NO. In the present study we are trying to assess the ability of selected plant extracts for stimulating *Leishmania*-infected macrophages to activate cytotoxic defence mechanisms. Emphasis is placed on the NO-inducing potential, taking into account an alternative mode of action.

Many naphthoquinones have been identified as possible lead structures against *Leishmania* and other protozoa, but their mechanism of action is still unknown. To gain a better insight into the underlying principle, a series of naphthoquinones differing broadly in their chemical structures were included for testing.

2. MATERIALS & METHODS

2.1 Phytochemistry laboratory

2.1.1 Plant material

2.1.1.1 *Drosera* spp. *(D. madagascariensis; D. peltata)*, Droseraceae

The plant materials of *D. madagascariensis* DC and *D. peltata* SMITH were commercially obtained from Finzelberg, Andernach, Germany and authenticity of the plant material was confirmed by Dr. A. Langer, University of Vienna, Austria. Voucher specimens (Kol/DM and KOL/DP) are deposited at the Institute of Pharmacy, Pharmaceutical Biology, Freie Universität Berlin.

2.1.1.2 *Spathodea campanulata*, Bignoniaceae

The stem bark of *S. campanulata* P.Beauv was provided by the Botanical Garden of the Freie Universität Berlin, Germany. A voucher specimen (BKOL/RG 2) is deposited at the Institute of Pharmacy, Pharmaceutical Biology, Freie Universität Berlin.

2.1.1.3 *Tabebuia avellanedae*, Bignoniaceae

The stem bark of *T. avellanedae* LORENTZ *ex* GRISEB (syn. *T. impetiginosu* MART *ex* DC) was provided by the Botanical Garden of the Freie Universität Berlin, Germany. A voucher specimen (BKOL/RG 6) is deposited at the Institute of Pharmacy, Pharmaceutical Biology, Freie Universität Berlin.

2.1.1.4 *Juglans regia*, Juglandacea

The leaves and peels of *Juglans regia* L. were purchased from Alfred Galke GmbH, Gittelde, Germany.Voucher specimens (KOL/JR 1 and KOL/JR 2) are deposited at the Institute of Pharmacy, Pharmaceutical Biology, Freie Universität Berlin.

2.1.2 Chemicals

2.1.2.1 Solvents

All solvents used were purchased from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany).

2.1.2.2 Tested substances

- **Alkannin/shikonin derivatives**

The series of alkannin/shikonin derivatives, including monomeric alkannin, monomeric shikonin, *β,β*-dimethylacrylshikonin, isovalerylshikonin, acetylshikonin, deoxyshikonin, and

the dimeric compounds vaforhizin and iso-vaforhizin, was kindly provided by Prof. Papageorgiou and Prof. Assimopoulou, University of Athens, Greece. The identity and purity of these compounds were proved by HPLC analysis and NMR spectroscopy (Assimopoulou *et al*., 2008; Papageorgiou, 1979; Papageorgiou, 1980; Spyros *et al*., 2005).

♦ **Commercial samples**

All commercial samples used for testing and their providers are listed in Table. 3.

Table. 3: Tested substances listed in alphabetical order

Substances	Company
amphotericin B	Sigma-Aldrich (Germany)
2,3-dimethoxy-1,4- naphthoquinone	Sigma-Aldrich (Germany)
2-hydroxy-3-methyl-1,4-naphthoquinone	Sigma-Aldrich (Germany)
hyperoside	Sigma-Aldrich (Germany)
isoquercitrin	Sigma-Aldrich (Germany)
juglone	Sigma-Aldrich (Germany)
lapachol	MP Biomedicals, LLC (France)
lawsone	Sigma-Aldrich (Germany)
menadione	Sigma-Aldrich (Germany)
2-methoxy-1,4-naphthoquinone	Sigma-Aldrich (Germany)
1,4-naphthoquinone	Sigma-Aldrich (Germany)
oleanolic acid	Sigma-Aldrich (Germany)
plumbagin	Sigma-Aldrich (Germany)
quercetin	Sigma-Aldrich (Germany)
ursolic acid	Sigma-Aldrich (Germany)

2.1.2.3 Spray reagents for thin layer chromatography

➢ **Aluminium chloride reagent:** (modified from Wagner *et al.,* 1983)

1 g of aluminium chloride was dissolved in 100 ml of methanol. After spraying the TLC plate was heated at 90 °C for 5 minutes, and the response was visualized under UV at 365 nm. Flavonoids with a free OH group in position 3 or 5 show yellow to greenish fluorescence.

- **Anisaldehyde- sulphuric acid reagent:** (Wagner *et al.,* 1983)

0.5 ml of anisaldehyde was dissolved in 10 ml of glacial acetic acid, followed by addition of 85 ml of methanol and 5 ml concentrated sulphuric acid. TLC plates were sprayed with the reagent, heated at 100 °C for 5-10 minutes, and analysed in the daylight or under UV-365 nm. The reagent has only limited stability and is no longer useable when the colour has turned to red-violet. Phenols, terpenes, sugars, and steroids turn violet, blue, red, grey or green.

- **Antimony-III-chloride reagent (SbCl3):** (Wagner *et al.,* 1983)

20 g of antimony-III-chloride was dissolved in chloroform and the volume was adjusted to 100 ml.

TLC plates were sprayed with ca. 15-20 ml of the reagent and then heated at 110 °C for 5-6 minutes. The response was analysed in the daylight and under UV-365 nm. Terpenes, steroids, and saponins showed reddish, yellow or blue-violet spots.

- **Ethanolic potassium hydroxide reagent:** (Wagner and Bladt,1996)

10 g of potassium hydroxide was dissolved in 100 ml of ethanol. The plate was sprayed with 10 ml and evaluated in the daylight or in UV-365 nm. This reagent detects anthraquinones (red), anthrones (yellow, UV-365 nm), and coumarins (blue, UV-365 nm). Naphthoquinones show a red fluorescence in UV-365 nm and red to red-brown colour in the daylight.

- **Ethanolic vanillin in sulphuric acid:** (Wagner and Bladt,1996)

1 g of vanillin was dissolved in 100 ml of ethanol (solution I).

10% ethanolic sulphuric acid (solution II).

The plate was sprayed with 10 ml of solution I, followed immediately by 10 ml of solution II, and then heated at 110 °C for 5-10 minutes. It is a general reagent for terpenes, iridoides, and lignans.

- **Naturstoffreagenz A**

TLC plates were sprayed with ca. 10 ml of a 1% methanolic solution of Naturstoffreagenz A (diphenylboric acid 2-aminoethylester). An intensive fluorescence will appear immediately or within 15 minutes under UV-365 nm, typical for flavonoids. The response depends on the structure of flavonoids, showing yellow, orange or green fluorescent spots at 366 nm according to their substitution patterns of the B-ring. The reagent must be freshly prepared before use.

- **Ninhydrin reagent:** (Wagner and Bladt,1996)

30 mg ninhydrin was dissolved in 10 ml *n*-butanol, followed by the addition of 0.3 ml 98% acetic acid. After spraying the plate was heated at 100 °C for 5-10 minutes. Amino acids, amines and amino sugars show reddish spots.

2.1.3 Devices

All devices used in the phytochemical laboratory are listed in table. 4.

Table. 4: Devices used in the phytochemical laboratory

Devices	Company
Analytical balance	Sartorius (2254)
Milling plant materials	Retsch-Mühle, Typ. SK 25
	Janker & Kunkel Ultra Turrax IKA 10
Precision balance	Sartorius (LW 3008)
Rotary evaporator	Büchi (Rotavapor R114 and B-480)
Shaker	IKA-Werk
Vacuum drying cabinet	WTB Binder VD 23/53
Vacuum freeze dryer	Christ (alpha 2-4)
Vacuum pump of rotary evaporator	Vacuubrand CVC 2
Ultrasonic bath	Sonorex (Super RK 106)
UV-lamp	Camag (Universal UV-lamp)
Vortexer	Winn (Vortex Genie)
Water bath	Heraeus

2.1.4 Chromatographic methods

2.1.4.1 Thin layer chromatography

Analytical precoated TLC plates were used (silica gel 60 F_{254}, Merck, Darmstadt, Germany). Spray reagents for detection were freshly prepared and are described elsewhere (Section 2.1.2.3). The mobile phases (V/V) used are mentioned in Section 2.3.1.

2.1.4.2 Column chromatography

Column chromatography (CC) was carried out on silica gel 60 F_{254} (Merck, Darmstadt, Germany) using open glass columns (30 to 100 cm x 2 to 4.5 cm). All solvents used were purchased from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany).

2.1.4.3 High-performance liquid chromatography (HPLC)

HPLC separations were done with a Shimadzu instrument, equipped with a gradient former and a photodiode array detector, and Class-LC 10/ M10A software.

Semi-preparative HPLC

HPLC system	Shimadzu
Stationary phase	Vertex-Eurospher 100-C18 (5µm, 8 x 250 mm)
Sample loop	100 µl
Column oven	Shimadzu CTO-10 AS VP
Detector	Scimadzu SPD-M 10 A Diode-Array Shimadzu SPD-10A VP UV Detector
Pump	Shimadzu FCV-10 AL VP low-pressure gradient flow-control valve Shimadzu LC-10 AT VP pump system Shimadzu CBM-10 A
Degasser	Shimadzu DGU-14 A
Fraction collector	Shimadzu FRC-10 A
Software	Shimadzu Class-LC 10/ M10A software

2.1.5 Spectroscopic methods

2.1.5.1 Nuclear magnetic resonance spectroscopy

NMR spectra were recorded on a Bruker DPX-400 instrument (Rheinstetten, Germany), operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts are given in ppm and coupling constants (*J*) in Hz. The multiplicity is indicated with *s* for singlet, *d* for doublet, *t* for triplet and *m* for multiplet (*br* stands for broad signal).

2.1.6 Mass spectroscopy

EI-mass spectra were aquired with a Varian MAT CH_7A spectrometer.

2.2 Cell culture laboratory

2.2.1 Devices

All devices used in the cell culture laboratory are listed in table. 5.

Table. 5: Devices used in the cell culture laboratory

Devices	Company, City
Analytical balance (A200S)	Sartorius, Kreuzburg
Axio cam	Carl Zeiss, Göttingen
Biological safety cabinet (Class II) Type A/B3	NuAir, Illinois, USA
CO_2 Water-Jacketed incubator (Type 450 E)	NuAir, Illinois, USA
Digital camera (Power Shot A95)	Canon Germany, Krefeld
Electronic laboratory balance (MC1)	Sartorius, Kreuzburg
E.L.I.S.A. reader (SpectraFluor)	Tecan, Frankfurt am Main
Flow cytometer (FACS Calibur)	Becton Dickinson Biosciences, Heidelberg
Fluorescence microscope (Axioskop 2)	Carl Zeiss, Göttingen
Freezing container (Cryo 1°C)	Nalge Nunc, Rochester, New York, USA
Light microscope (Axiovert 25, Axiostar plus)	Carl Zeiss, Göttingen
Magnetic stirring hot plate	IKA Works Werke, Staufen
Microplate shaker MT4	IKA Works Werke, Staufen
pH-meter CG 825	Schott, Hofheim
Vortex minishaker KMS 1	IKA Works Werke, Staufen
Water bath	Dinkelberg-Labortechnik, Ulm

2.2.2 Materials and reagents

The source of materials and other reagents used are described where appropriate

2.2.2.1 Double-distilled water

Double-distilled water (ddH_2O) was met by reverse osmosis and ion exchange, followed by autoclaving.

2.2.2.2 RPMI 1640 medium

Powder of RPMI 1640 containing L-glutamine was purchased from Gibco-Invitrogen (Paisley, UK). It was dissolved in ddH_2O under sterile conditions and stored at 4 °C until use.

2.2.2.3 Phosphate buffered saline (PBS)

Phosphate buffered saline (PBS) was prepared under sterile conditions and adjusted to pH 7.2 (Tab. 6).

Table. 6: Composition of the PBS buffer

PBS	
NaCl	137.0 mM
KCl	27.0 mM
KH_2PO_4 x $2H_2O$	1.8 mM
Na_2HPO_4 x $2H_2O$	8.0 mM
dissolved in distilled H_2O and sterilized by autoclaving	

2.2.3 Cell lines

2.2.3.1 Bone marrow-derived macrophages (BMMΦ)

Mice were killed by cervical dislocation and bone marrow was obtained by flushing femora and tibiae with cold Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). The pooled bone marrow precursor cells were matured in polystyrene petri dishes (Sarstedt, Nümbrecht, Germany) for 8–10 days in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Bio Whittaker, Verviers, Belgium), 5% heat-inactivated horse serum (Gibco), 15% fibroblast (L929 cells)-conditioned medium, penicillin (100 U/ml), streptomycin (100 µg/ml), D-glucose (20 mM, Sigma), Na-pyruvate (10 mM), and HEPES (25 mM) at 37 °C in a humidified normal atmosphere with 5% CO_2.

For harvesting, the medium was replaced twice by cold PBS, thereby removing most non-adherent cells and debris. BMMΦ were chilled to 4 °C for 1 h, rinsed off the plastic with a 5-ml pipette, and washed by centrifugation (250 ×g, 10 minutes, 4 °C). The viable cells, as assessed by trypan-blue exclusion, were counted and kept at 4 °C until use.

2.2.3.2 L 929 (CSF)-cells

L929 (CSF)-cells belong to the murine fibroblast cell lines and are producers of macrophage colony-stimulating factor, involved in the differentiation of precursor BMMΦ cells. The L929 cells were kindly provided by the late Professor M.L. Lohmann-Matthes (Fraunhofer-Institute of Toxicology, Hannover). For production of L929-conditioned medium, in fluid nitrogen conserved L929 (CSF)-cells were thawed and cultured in 250 ml polystyrene culture bottles (Becton Dickinson Labware, Franklin Lake, USA) in RPMI 1640 medium supplemented with 5% FCS (R5) (Bio Whittaker), streptomycin (100 µg / ml) and penicillin G (100 U / ml). After two to three days of cultivation at 37 °C in a humidified normal atmosphere with 5% CO_2, medium was replaced by fresh medium containing 1% FCS (R1). To avoid the growth of a surplus of cells, the cultures were passaged every 3 to 4 days. Cells were harvested

following treatment with trypsin-EDTA solution (Gibco), washed by centrifugation (250 ×g, 10 minutes, 4 °C) with cold Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) and cultured in R5 medium for 3 to 4 days to allow proliferation. After this period R5 medium was replaced by R1 medium. This culture protocol was repeated for maximal 2 months. All R1 supernatants were collected, combined, sterile filtered (0.22 µM, Schleicher & Schuell, Dassel) and kept at -20 °C until use.

2.2.4 Mice

Six- to 12-week-old C57BL/6 mice were bred at and supplied by Zentrale Versuchstierzucht, Federal Institute for Risk Assessment (BfR), Berlin, Germany. Animal care and the experimental procedure were in accordance with the institutional guidelines and the German law.

2.2.5 Parasites

GFP-transfected *L. major* strain LT 52, clone CC-1pXG-GFP (*L. major* GFP;), stable under the influence of geneticin (Ha *et al*., 1996), were kindly provided by Prof. S.M. Beverley (Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, USA).

2.3 Phytochemical work-up procedures

2.3.1 Preliminary TLC analysis

2.3.1.1 Detection of naphthoquinones

Extracts were qualitatively analysed for the presence of naphthoquinones using silica gel 60 aluminium sheets (Merck) and the following mobile phases:

S-1: $CHCl_3$

S-2: toluene-formic acid (99:1)

S-3: petroleum ether-ethyl acetate (9:1)

All naphthoquinones present in the plant extracts showed quenching under UV-254 nm and yellow to orange colouration in the day light and under UV at 365 nm on TLC plates. After spraying with 10% ethanolic KOH reagent, naphthoquinones showed a red fluorescence under UV-365 nm and a red to red-brown colour in the day light.

2.3.1.2 Detection of flavonoids and flavonoid glycosides

Flavonoids and flavonoid glycosides were chromatographed on TLC plates using the following mobile phases:

S-1: ethyl acetate-water-formic acid (18:1:1)

S-2: 1-butanol-water-acetic acid (4:5:1, upper phase).

All flavonoids showed fluorescence quenching under the UV light at 254 nm and a dark yellow, green or blue fluorescence at 365 nm, which was enhanced by spraying with Naturstoffreagenz A.

2.3.1.3 Detection of sterols and triterpenes

Sterols and triterpenes were monitored on TLC plates using the following mobile phases:

S-1: $CHCl_3$

S-2: toluene-acetone (1:1)

S-3: toluene-ethyl acetate (5:2)

Most triterpenes are colourless substances and are not visible on TLC plates either in the day light or under UV exposure. After spraying with the anisaldehyde-sulphuric acid reagent, a characteristic blue colouration verified the presence of triterpenes. Besides, antimony (III) chloride reagent (Carr-Price reagent) was used to confirm the presence of triterpenes, appearing as red-violet spots.

2.3.1.4 Detection of glutathione

Glutathione was chromatographed on TLC plates using phenol-water (70:30) as mobile phase. The colourless substance was visualized by a reddish spot when using the ninhydrin reagent for detection.

2.3.2 Preparation of glutathione- naphthoquinone adducts

Solution I: 10 mM of naphthoquinone in DMSO (dimethyl sulfoxide, Merck, Darmstadt).

Solution II: 10 mM of reduced glutathione in distilled H_2O.

Solution I (1 ml) was mixed with solution II (1 ml) and stirred for 2 h at 37 °C. The reaction was monitored by TLC analysis with solution I and solution II as references. Phenol-water (70:30) was used as mobile phase and the ninhydrin reagent for detection, respectively (Section 2.3.1.4).

2.3.3 Isolation and purification of the plumbagin-glutathione conjugate formed

Plumbagin was reacted with glutathione as described above (Section 2.3.2).

For isolation, the mixture was subjected to HPLC purification to afford three distinct peaks (glutathione; Rt 3.5 min, plumbagin-glutathione conjugate; Rt 14.6 min, and plumbagin; Rt 27.3 min).

Experimental conditions: RP-18 column (5 µm; 8 x 250 mm); operating temperature (40 °C).

The mobile phase was composed of a water-methanol gradient as follows:

1:0 → 1:1 (20 min)

1:1 → 0:1 (5 min)

Flow rate: 3 ml/min, detection at 220 nm.

Overall yield of the plumbagin-glutathione conjugate: 2 mg.

2.3.4 *Drosera madagascariensis*

2.3.4.1 Preparation of *D. madagascariensis* extracts

Dried plant material of *D. madagascariensis* was extracted following two different protocols for the enrichment of compounds.

a) Method A

The plant material (25 g) was individually extracted with ethanol and CH_2Cl_2 (each 3 x1 l) at room temperature for 24 h to afford a residue (1.1 g and 0.6 g, respectively) on evaporation of either solvent (Fig. 9).

Fig. 9: Preparation of extracts of *D. madagascariensis* (method A)

b) Method B

Alternatively, the plant material (38 g) was successively stirred for 24 h at room temperatures with dichloromethane followed by ethanol (each 3 x1 l) to afford a residue (0.8 and 1.7 g, respectively) on evaporation of the solvents (Fig. 10).

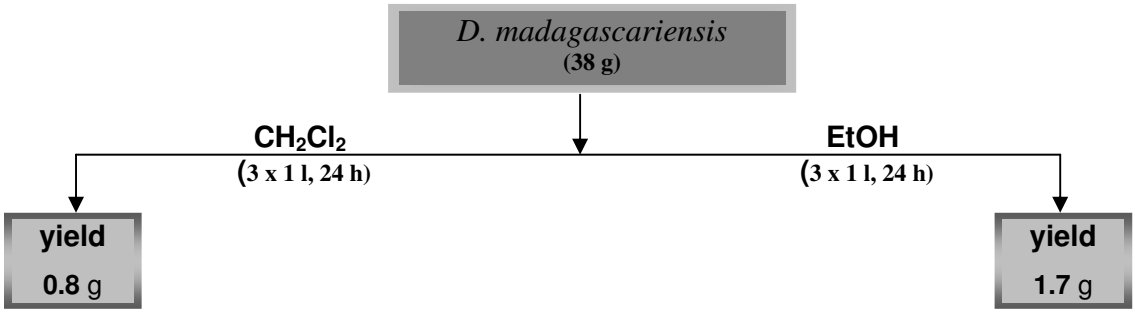


Fig. 10: Preparation of extracts of *D. madagascariensis* (method B)

2.3.5 *Drosera peltata*

2.3.5.1 Preparation of *D. peltata* extracts

Likewise, dried plant material of *D. peltata* was extracted following two different protocols for the enrichment of compounds as follows.

a) Method A

Plant material (25 g) was individually extracted with *n*-hexane, dichloromethane, ethanol, and ethyl acetate (each 5 x 500 ml) at room temperature for 24 h. The combined extracts were filtered to afford a brownish residue (0.2, 0.3, 0.3 and 1.6 g, respectively) on evaporation of the solvent under reduced pressure at 40 °C (Fig. 11).

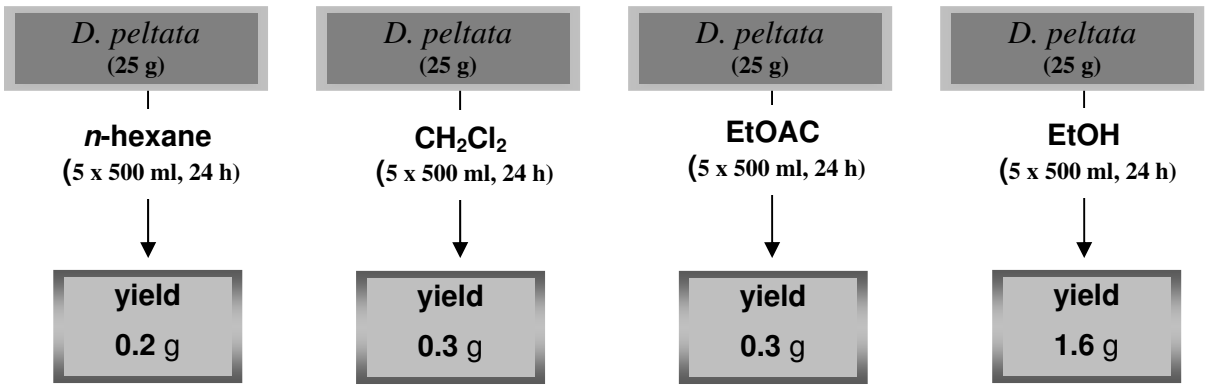


Fig. 11: Preparation of extracts of *D. peltata* (method A)

b) Method B

Alternatively, plant material (40 g) was successively extracted for 24 h at room temperatures with solvents of increasing polarity beginning with *n*-hexane followed by dichloromethane, ethyl acetate and ethanol (each 5 x1 l) to afford a residue (0.4, 0.8, 0.5 and 3.7 g, respectively) on evaporation of the solvents (Fig. 12).

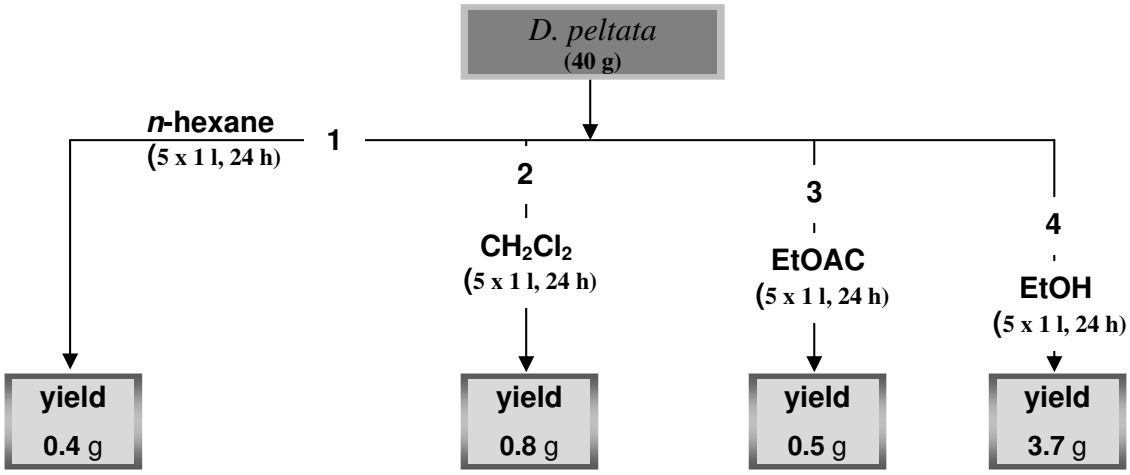


Fig. 12: Preparation of extracts of *D. peltata* (method B)

2.3.5.2 Quantification of plumbagin using (HPLC)

The amount of plumbagin in extracts of *D. peltata* was determined using HPLC analysis. Experimental conditions: RP-18 column (5 µm; 8 x 250 mm); operating temperature, room temperature. The mobile phase was composed of a water-methanol gradient 1:0 → 0:1 (40 min; flow rate 3 ml/min); detection at 254 nm. Quantification was performed by referring to a standard curve of plumbagin.

2.3.5.3 Column chromatography of the dichloromethane extract of *D. peltata*

The dichloromethane extract (0.8 g), obtained from applying method B, was fractionated by column chromatography over silica 60 (52 cm x 2 cm) eluted with an *n*-hexane-ethyl acetate gradient system (1:0 → 0:1). The content (25 ml fractions) of test fractions 20-23 (47 mg) (*n*-hexane - ethyl acetate, 97:3), 31-33 (12 mg) (*n*-hexane - ethyl acetate, 95:5) and 47-51 (4 mg) (*n*-hexane - ethyl acetate, 90:10) was subjected to HPLC purification to afford three yellow substances [(**5**), 38 mg, Rt 34.6 min; (**19**), 3.2 mg, Rt 39 min; and (**22**), oily, 2 mg, Rt 25.7 min, respectively). Experimental conditions: RP-18 column (5 µm; 8 x 250 mm); operating temperature, 40 °C. The mobile phase was composed of a water-methanol gradient 1:0 → 0:1 (40 min; flow rate 3 ml/min); detection at 254 nm.

2.3.5.4 Spectroscopic analysis of plumbagin

The spectroscopic data identified substance (**5**) as 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), confirmed by comparison with an authentic reference.

Plumbagin (5): 38 mg, orange-yellow needles.

EI-MS: *m/z* (rel. intensity %) 188 [M, $C_{11}H_8O_3]^+$ (100), 173 [M-$CH_3]^+$ (22), 160 [M-CO]$^+$ (21), 132 [M-CO-CO]$^+$ (15), 120 [$C_7H_4O_2]^+$ (23), 92 [$C_6H_4O]^+$ (20), 77 (10), 63 (15).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 11.98 (*s*, 5-OH), 7.62 (*t, J*= 7.8 and 2.4 Hz, H-6 and H-7), 7.24 (*d, J*=7.8 Hz, H-8), 6.81 (*s*, H-3), 2.20 (*s*, H_3C-2).

2.3.5.5 Spectroscopic analysis of 3,3'-biplumbagin

Substance (**19**) was shown to be 5-hydroxy-3-(8-hydroxy-3-methyl-1,4-dioxonaphthalen-2-yl)-2-methylnaphthalene-1,4-dione (3,3'-Biplumbagin).

3,3'-Biplumbagin (19): 3.2 mg, yellow needles.

EI-MS: *m/z* (rel. intensity %) 374 [M, $C_{22}H_{14}O_6]^+$ (100), 359 [M-$CH_3]^+$ (56), 357 [M-OH]$^+$ (34), 345 [M-CO]$^+$ (25), 331 [M-Me-CO]$^+$ (11), 317 [M-H-2CO]$^+$ (7), 121 (16), 120 [$C_7H_4O_2]^+$ (17), 92 [$C_6H_4O]^+$ (28).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 11.81 (*s*, 5-OH and 5´-OH), 7.73 (*br. d*, *J*=7.4 Hz, H-8 and H-8´), 7.67 (*dd, J*= 7.4 and 8.00, H-7 and H-7´), 7.30 (*br. d, J*=8.00, H-6 and H-6´), 2.08 (s, 2-CH_3 and 2´-CH_3).

2.3.5.6 Spectroscopic analysis of *cis*-isoshinanolone

Substance (**22**) was identified as the tetralone derivative *cis*-isoshinanolone.

***Cis*-isoshinanolone (22)**: 2 mg, oil.

EI-MS: *m/z* (rel. intensity %) 192 [M, $C_{11}H_{12}O_3]^+$ (99), 177 [M-$CH_3]^+$ (16), 174 [$C_{11}H_{10}O_2]^+$, (22), 163 [$C_9H_7O_3]^+$ (12), 150 (33), 121 [$C_7H_5O_2]^+$ (100).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.41 (*s*, 8-OH), 7.48 (*dd*, *J*= 8 and 7.3 Hz, H-6), 6.94 (*br, J*= 8.00, H-7), 6.92 (*br., J*=7.3, H-5), 4.75 (*d*, *J*= 4.44, H-4), 2.87 (*dd*, *J*= 17.7 and 11 Hz, H-2_{ax}), 2.57 (*dd*, *J*= 17.7 and 4.2 Hz, H-2_{eq}), 2.44 (*m*, H-3), 1.18 (*d*, *J*= 6.9, 3- CH_3).

2.3.6 *Spathodea campanulata*

2.3.6.1 Preparation of *S. campanulata* extracts

The plant material of *S. campanulata* (200 g) was individually extracted with *n*-hexane and dichloromethane (each 3 x1 l) at room temperature for 2 days to afford a residue (0.5 g and 1 g, respectively) on evaporation of the solvent (Fig. 13).

In parallel, the plant material was either extracted with methanol (200 g, 3 x1 l) or with ethanol (750 g, 10 x2 l) at room temperature for 24 h. The combined extracts were filtered and concentrated under reduced pressure at 40 °C to yield 28 g and 38.5 g, respectively as shown in Fig. 13.

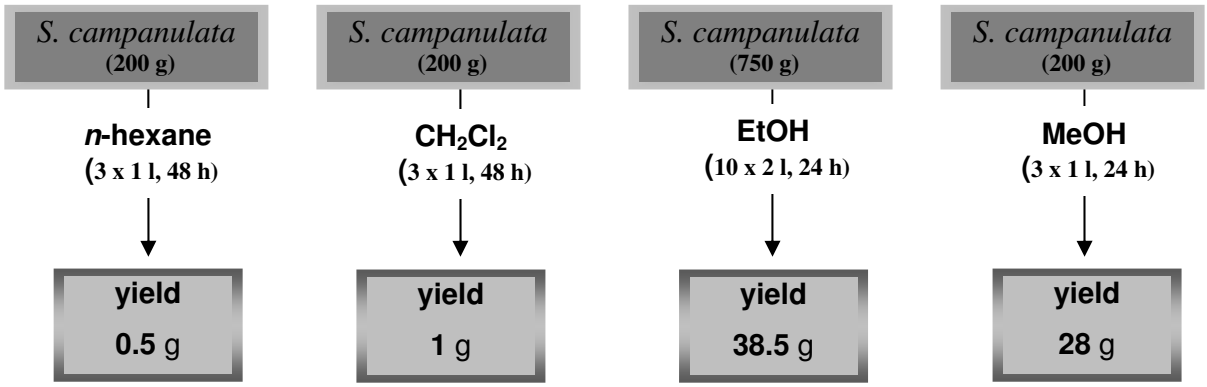


Fig. 13: Preparation of extracts of *S. campanulata*

3.3.6.2 Fractionation of *S. campanulata* ethanol extract

The ethanol extractives (17 g) were sequentially partitioned into *n*-hexane (2.6 g), dichloromethane (1.8 g), ethyl acetate (0.3 g), and methanol (12.2 g) fractions. Each fraction was concentrated to dryness by evaporation of the solvent under reduced pressure at 40 °C (Fig. 14).

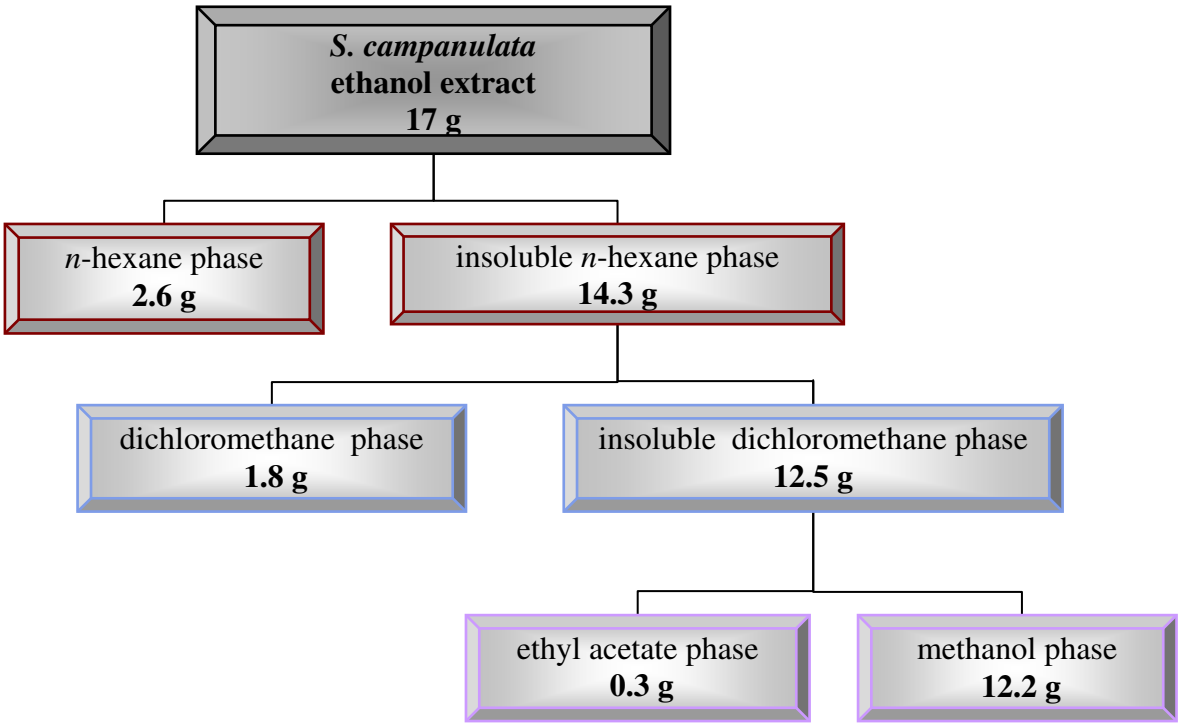


Fig. 14: Fractionation of *S. campanulata* ethanol extract

2.3.7 *Tabebuia avellanedae*

2.3.7.1 Preparation of *T. avellanedae* extracts

The dried inner and powdered bark of *T. avellanedae* (400 g) was extracted with methanol (2 x 2 l) for 24 h at room temperatures. The combined extracts were filtered and concentrated under reduced pressure at 40 °C to yield 51 g of a brown solid. A portion of the methanol extract (42 g) was sequentially partitioned into n-hexane (6.6 g), dichloromethane (6 g), ethyl acetate (9.7 g), *n*-butanol (13.2 g), and water-soluble (7 g) fractions for bioassay-guided activity studies. The fractions were concentrated to dryness by evaporation of the solvent under reduced pressure at 40 °C (Fig. 15).

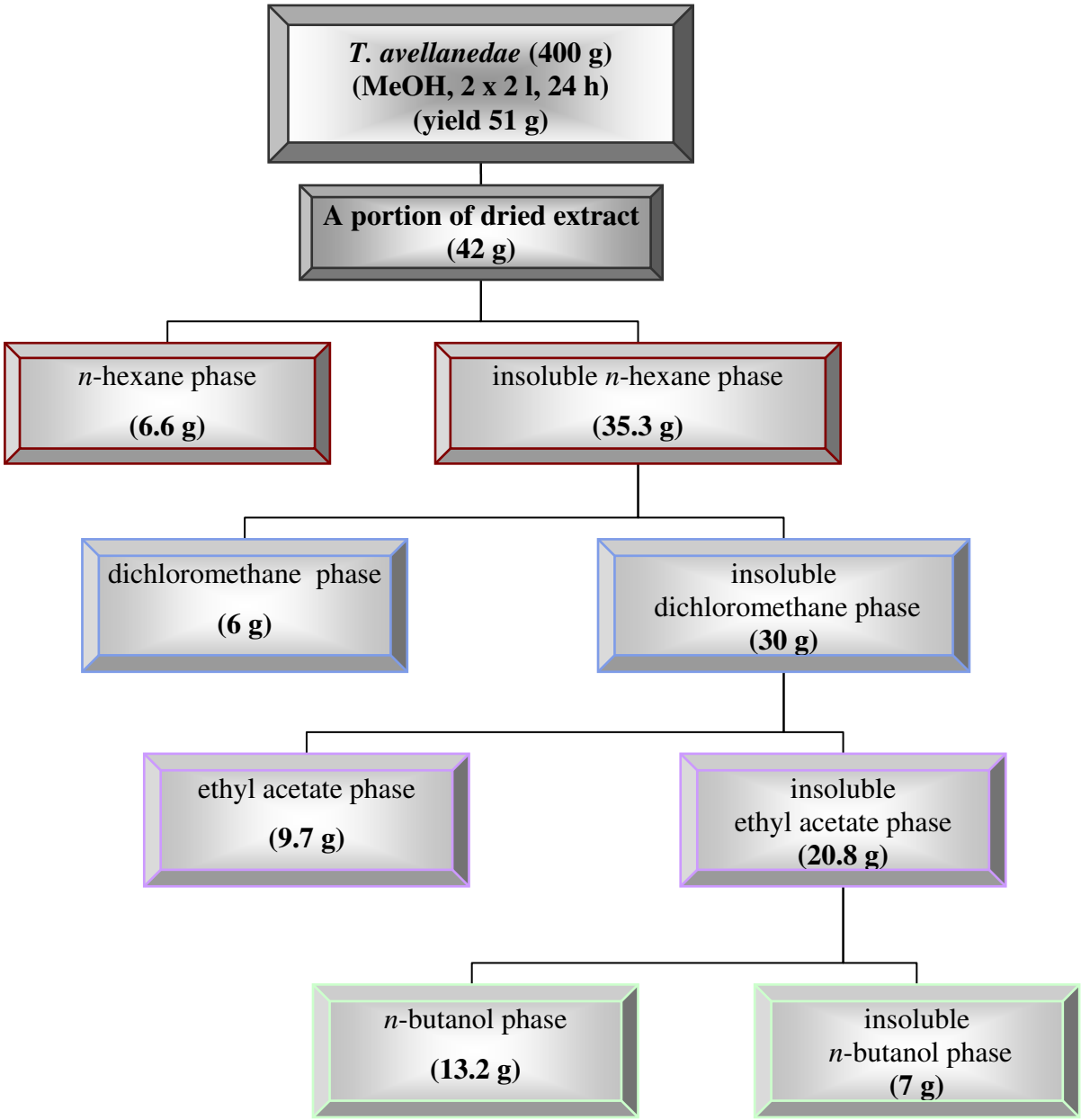


Fig. 15: Preparation and fractionation of *T. avellanedae* extract

2.3.7.2 Fractionation of *T. avellanedae* dichloromethane fraction using column chromatography:

The dichloromethane extractives (4 g) were fractionated by column chromatography over silica gel 60 (75 cm x 4 cm, 140 g silica gel 60) using a dichloromethane- ethyl acetate gradient system (1:0 → 0:1). Fractions (each 50 ml) were collected and grouped according to their TLC profiles to afford 20 subfractions. Fraction 5 (1650-1850 ml; CH_2Cl_2 - EtOAc, 95:5) was further separated by CC (30 cm x 2 cm; 50 g silica gel 60) using a petroleum ether-ethyl acetate gradient system (1:0 → 0:1). Test tubes (each 5 ml) were collected and pooled, according to the similarity of the TLC profile, affording 6 subfractions. A distinct yellow spot was detected in subfraction 4 (1600-1700 ml; petroleum ether- ethyl acetate 92:8) with orange colouration under UV at 365 nm and quenching under UV-254 nm on TLC plate. This substance (**20** & **21**) was isolated using HPLC separation (RP-18 column. 5 µm; 8 x 250 mm; operating temperature, 40 °C; water-methanol gradient 1:0 → 0:1; 40 min; flow rate 3 ml/min; detection at 254 nm; Rt: 32 min).

2.3.7.3 Spectroscopic analysis of isomeric naphtho[2,3-b]furan-4,9-diones

Spectroscopic analysis proved substance (**20** & **21**) as a mixture comprised of 5-and 8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione.

Yellow needles (4 mg).

EI-MS: m/z (rel. intensity) 258 $[M]^{+\bullet}$ (89), 243 $[M\text{-}Me]^+$ (100), 216 $[M\text{-}Me\text{-}CO]^+$ (27), 215 [M-Me-CO] (36), 187 $[M\text{-}Me\text{-}CO\text{-}CO]^+$ (16), 159 $[M\text{-}Me\text{-}CO\text{-}CO\text{-}CO]^+$ (5), 123 (8), 121 $[C_7H_5O_2]^+$ (11).

5- hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione (**20**)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.19 (*s*, 5-OH), 7.72 (*dd*, *J*= 7.2 and 1.3 Hz, H-8), 7.60 (*dd, J*= 8.1 and 7.7 Hz, H-7), 7.27 (*dd, J*= 8.3 and 1.2 Hz, H-6), 6.86 (*d, J*= 0.7 Hz, H-3), 5.05 (*m*, H-10), 2.1 (*br. s*, 10-OH), 1.66 (*d, J*= 6.5 Hz, 10-CH_3).

8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione (21)

^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.04 (*s*, 8-OH), 7.72 (*dd*, *J*= 7.2 and 1.3 Hz, H-5), 7.60 (*dd, J*= 8.1 and 7.7 Hz, H-6), 7.27 (*dd, J*= 8.3 and 1.2 Hz, H-7), 6.86 (*d, J*= 0.7 Hz, H-3), 5.05 (*m*, H-10), 2.1 (*br. s*, 10-OH), 1.66 (*d, J*= 6.5 Hz, 10-CH_3).

2.3.8 *Juglans regia*

2.3.8.1 Preparation of *J. regia* extracts

The dried and powdered leaves of *J. regia* L were individually treated with *n*-hexane (200 g, 3 x 2 l), dichloromethane (100 g, 3 x 2 l), ethyl acetate (100 g, 3 x 2 l), and ethanol (100 g, 3 x 2 l) at room temperatures for 2 days. The combined extracts were filtered and concentrated under reduced pressure at 40 °C to yield a solid residue in each instance (6.6, 4.8, 4.7, and 11 g, respectively) (Fig. 16).

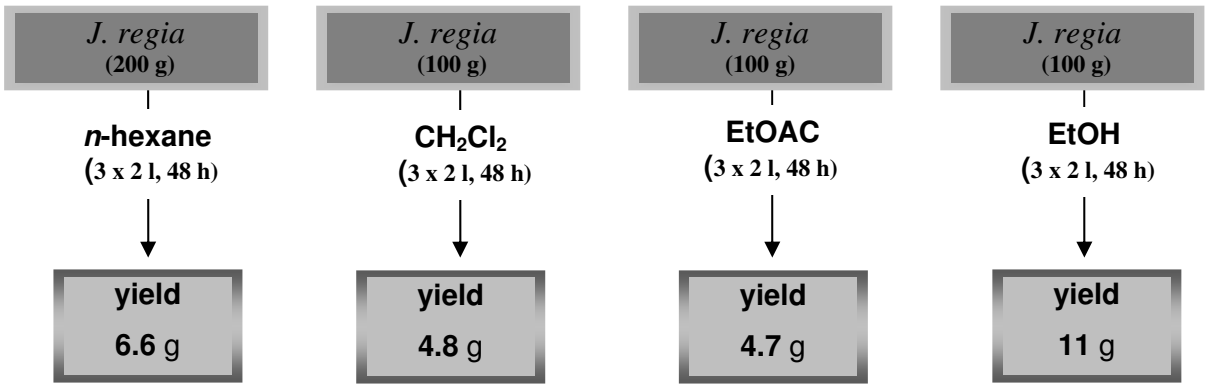


Fig. 16: Preparation of extracts of *J. regia* (leaves)

The extraction of dried and powdered peels of *J. regia* was performed as described above for leaves, including the same quantities of the plant material. The work-up procedure and the respective yields of extracts is shown in Fig. 17.

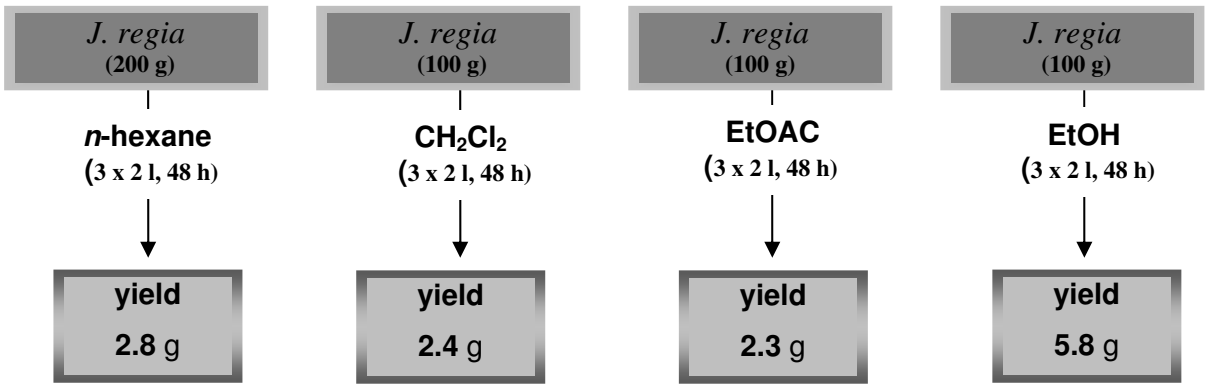


Fig. 17: Preparation of extracts of *J. regia* (peels)

2.3.8.2 Fractionation of the *n*-hexane extract of *J. regia* (leaves)

The *n*-hexane extract of leaves of *J. regia* (5 g) was chromatographed on a silica gel column (70 cm × 4 cm, silica gel 60) by eluting with a stepwise gradient of *n*-hexane/ethyl acetate (100/0, 90/10, 80/20, 25/75, 0/100). Fractions (each 50 ml) with a similar TLC pattern were pooled to afford 14 subfractions used for bioassay-guided activity studies.

2.4 Cell biology methods

2.4.1 Sample preparation

Extracts and compounds were dissolved in sterile DMSO. Appropriate stock solutions (20 mg/ml and 10 mM, respectively) were stored at -20 °C until use (antileishmanial activity studies). Test concentrations were prepared by serial dilutions with medium as described below.

2.4.2 In vitro infection of BMMΦ with GFP- transfected *L. major*

BMMΦ were infected with *L. major* GFP promastigotes in their infective "metacyclic" post-logarithmic growth phase. 1×10^7 BMMΦ/ml R10 medium were incubated with 8×10^7 *L. major* organisms/ml R10 medium in 5 ml polypropylene round-bottom tubes (Falcon, Becton Dickinson, Franklin Lake, NJ, USA) (ratio macrophage/*L. major* 1:8; final volume per tube of 1.5–2.0 ml containing ca. 1.5×10^7 macrophages). The cell suspension was centrifuged for 5 minutes at 250 ×g and then incubated for 120 minutes at 37 °C. Every 30 minutes the cells were gently resuspended as a precaution against local medium exhaustion using a 1 ml pipette tip. Subsequently the cells were centrifuged (200 ×g, 10 minutes, 4 °C), the supernatant discarded and replaced by cold PBS. After transfer of the cells into 50-ml tubes (Falcon), they were washed with 50 ml of cold PBS to remove the remaining extracellular parasites. Extracellular *L. major* promastigotes are temperature sensitive. After 24 h no or only very few, extracellular parasites were generally seen in control micrographs. Aliquots of the final suspension ($5x10^5$ infected BMMΦ/ml) were seeded in microtiter plates and incubated for 24 h at 37 °C to allow internalized *Leishmania* to transform into the amastigote form. Non–infected and infected but untreated BMMΦ were used as negative controls, while amphotericin B-treated infected BMMΦ served as positive control.

2.4.3 Cytotoxicity of extracts against BMMΦ using MTT assay

The viability of BMMΦ was determined by the trypan blue exclusion method. Viable cells were quantified in a Neubauer chamber and seeded into flat-bottom microtiter plates at $1x10^5$ per well in 100 µl R10 medium. Cells were allowed to settle and adhere to the plastic for 24 h. Then serial dilutions of extracts were added. After incubating for 48 h at 37 °C, 20µl/well MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5mg/1ml in PBS, Sigma-Aldrich] were added. MTT processing was stopped after 6 h and formazan crystals solubilised by adding 50 µl/well acidified SDS (20%). After incubating for 18 h at 37 °C, optical densities were determined spectrophotometrically at 570 nm using a semi-automated microplate reader. Cell free samples served as a blank.

2.4.4 Antileishmanial activity of extracts against promastigote *L. major* GFP

Transgenic *Leishmania major* promastigotes expressing green fluorescent protein (GFP) were cultured in *Leishmania* growth medium in flat-bottom microtiter plates. The *Leishmania* growth medium consisted of RPMI 1640 medium (Gibco- Invitrogen, Paisley, UK) supplemented with 5% heat inactivated fetal calf serum (FCS), penicillin G (100 U/ml; PAA Laboratories, Cölbel, BRD), streptomycin (100 µg/ml; PAA),15% MΦ-conditioned medium, sodium pyruvate (10 mM; Sigma-Aldrich Chemie, Steinheim, Germany), HEPES (25 mM; Gibco), Geneticin (25 µg/ml; G418, Gibco), and hemin (0.25% v/v solution B of Hosmem II medium; Berens and Marr, 1978).

Promastigotes in the stationary culture stage were counted using trypan blue and seeded at $1x10^5$ parasites per well in 100 µl *Leishmania* growth medium. Serial dilutions of extracts were added and the parasites then incubated at 25 °C, 5% CO_2 in a humidified atmosphere for 48 h. Thereafter, MTT (20 µl/well of a 5 mg/ml stock solution) was added. After 6 h MTT processing was stopped and formazan crystals solubilised by adding 50 µl/well acidified SDS (20%) and incubating for 18 h at 37 °C (Tada *et al*, 1968). The relative amount of formazan/well produced by viable parasites was determined spectrophotometrically at 570 nm by blanking against an appropriate vehicle control using a semi-automated microplate reader. Antileishmanial effects were expressed as IC_{50} values, indicating the concentration of an extract which provoked a 50% reduction in viability of the parasite. Non–treated parasites served as negative and amphotericin B-treated promastigotes as positive controls. All experiments were performed in triplicate.

2.4.5 Antileishmanial activity of extracts against intracellular *Leishmania*

2.4.5.1 Parasite retrieval assay

L. major-infected BMMΦ ($1x10^5$/100 µl/well) were incubated with 100 µl R10 medium/well containing different concentrations of test samples in 96-well microtiter plates at 37 °C for 48 h. Each concentration was tested in triplicate. Surviving parasites were retrieved by lysis of the host cells with 0.015% SDS solution and the lysates were cultured at 25 °C for 3-5 days to allow viable parasites to transform into promastigotes. Their viability was assessed by metabolic conversion of MTT as described (Kiderlen and Kaye, 1990).

2.4.5.2 FACS analysis

L. major-infected BMMΦ ($7x10^5$/750 µl/tube) were incubated with the test compounds in a final volume of 1.5 ml in 5-ml sterile polystyrene round-bottom tubes with caps (Falcon) for 48 h at 37 °C. For each sample concentration, non–infected sample-treated BMMΦ were used for facilitating the gating. Non-infected and infected BMMΦ were washed (200 ×g, 10 minutes, 4 °C) and re-suspended in 2 ml of cold PBS. The cells were centrifuged again, re-suspended in 300 µl of PBS, and stored on ice to facilitate detachment of remaining adherent cells. To discriminate dead from living cells, PI (Sigma) solution (150 µl/tube) was added ca. 30 s before the FACS measurement, giving a final concentration of 0.33 µg PI/ml. Information on the rate of infection was obtained by comparing the GFP signal with that of non-infected cells. A total of 20,000 events were counted using a FACS Calibur cytometer and CellQuest Pro software (both Becton Dickinson) with the following instrument settings: forward scatter (E-1, linear mode; threshold 52), side scatter (346 V, linear mode), fluorescence 1 (548 V, log mode) and fluorescence 3 (581 V, log mode).

2.4.6 Griess assay

L. major-infected BMMΦ were incubated with the test compounds in 96-well microtiter plates for 48 h as described above (see Section 2.4.5.1). The supernatants were collected as a source of secreted NO which was quantitated by determining the nitrite concentration using the Griess assay. Aliquots of 100 µl of the supernatant were mixed with 100 µl of Griess reagents [1:1 v/v mixture of 1% sulphanilamide, 0.1% N-(1-naphthyl) ethylendiamine dihydrochloride in 3% H_3PO_4]. After 5 minutes at room temperature, the absorbance was determined at 550 nm using an ELISA reader. The nitrite concentration was calculated from a standard curve generated with $NaNO_2$. Recombinant murine interferon-γ (100 U/ml) in

combination with LPS (10 ng/ml) were used as positive controls of activation of BMMΦ and NO production.

2.4.7 Inhibition of iNO synthase

For inhibition of the iNO synthase, infected untreated and infected treated BMMΦ were incubated with the nonspecific iNO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, Sigma-Aldrich).

All samples at their effective concentrations were tested alone or in combination with 300 µM of L-NMMA against *L. major* amastigotes using parasite retrieval assay and FACS analysis as described elsewhere (see Section 2.4.5). As controls, infected BMMΦ were treated with rIFN-γ (100 U / ml) + LPS (10 ng/ml) either in combination with the active form of iNO synthase inhibitor (L-NMMA, 300 µM) or its inactive enantiomer (D-NMMA, 300 µM, Sigma-Aldrich). In parallel, the NO-inducing potential was determined using the Griess assay (Section 2.4.6).

2.5 Statistical analysis

The statistical analyses were performed using Excel software 2010 for Windows®. Data in tables and figures were presented as mean ± standard deviation (SD). Differences between groups were assessed by Student's *t*-test. A probability of $P<0.05$ was considered significantly different.

3. RESULTS

3.1 *Drosera madagascariensis*

3.1.1 Phytochemical studies

3.1.1.1 Preliminary TLC analysis of extracts

Initially, extracts of *D. madagascariensis* were qualitatively analysed for the presence of naphthoquinones, sterols, triterpenes, and flavonoids on TLC plates (Table. 7).

3.1.1.1.1 Detection of naphthoquinones in *D. madagascariensis* extracts

All extracts except for the ethanol extract (method B) revealed a yellow to orange spot in the day light and under UV at 365 nm, and the absence of plumbagin and juglone as evident from comparing with authentic reference samples. After spraying with 10% ethanolic KOH, this striking spot responded in a red colour typical of naphthoquinones (Fig. 18). Colouration and chromatographic mobility suggested this compound to be 7-methyljuglone (Zenk *et al*., 1969). The absence of 7-methyljuglone in the ethanol extract (method B) may be explained by the successive extraction of the plant material starting with dichloromethane.

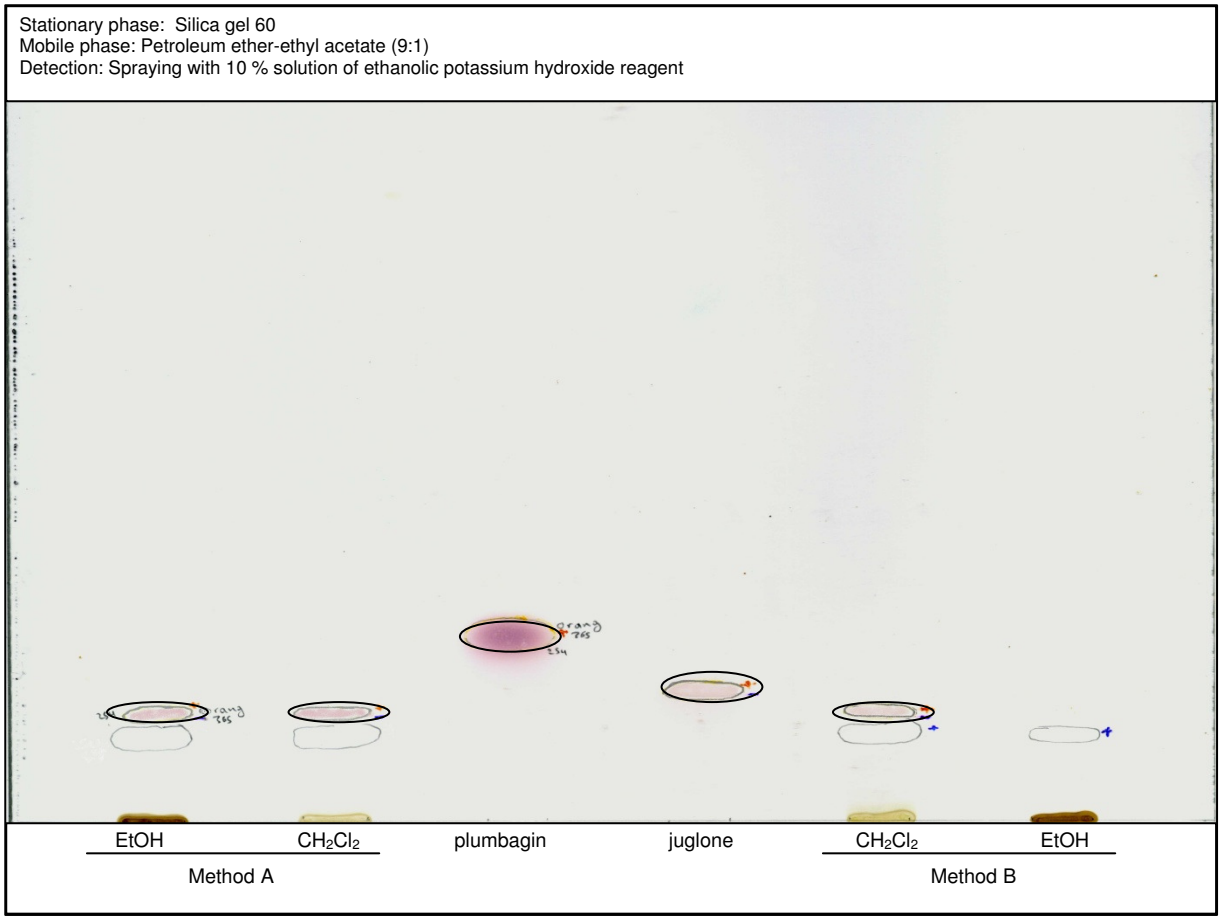


Fig. 18: Thin layer chromatography of *D. madagascariensis* extracts with plumbagin and juglone as references.

3.1.1.1.2 Detection of flavonoids and flavonoid glycosides in *D. madagascariensis* extracts

Quercetin and its glycosides such as hyperoside (quercetin-3-O-galactoside) and isoquercitrin (quercetin-3-O-glucoside) have been reported to be present in *D. madagascariensis*, associated with myricetin-3-O-galactoside (Kolodziej *et al*., 2002). Flavonoids and flavonoid glycosides were apparently absent from the dichloromethane extracts (Fig. 19), whereas quercetin and hyperoside were readily detected in the ethanol extract (method A) in comparison with reference samples. The ethanol extract (method B) revealed four yellow to orange spots in the day light and under UV at 365 nm corresponding to quercetin, isoquercitrin, and hyperoside, while the remaining compound was supposed to be myricetin-3-O-galactoside (Kolodziej *et al*., 2002).

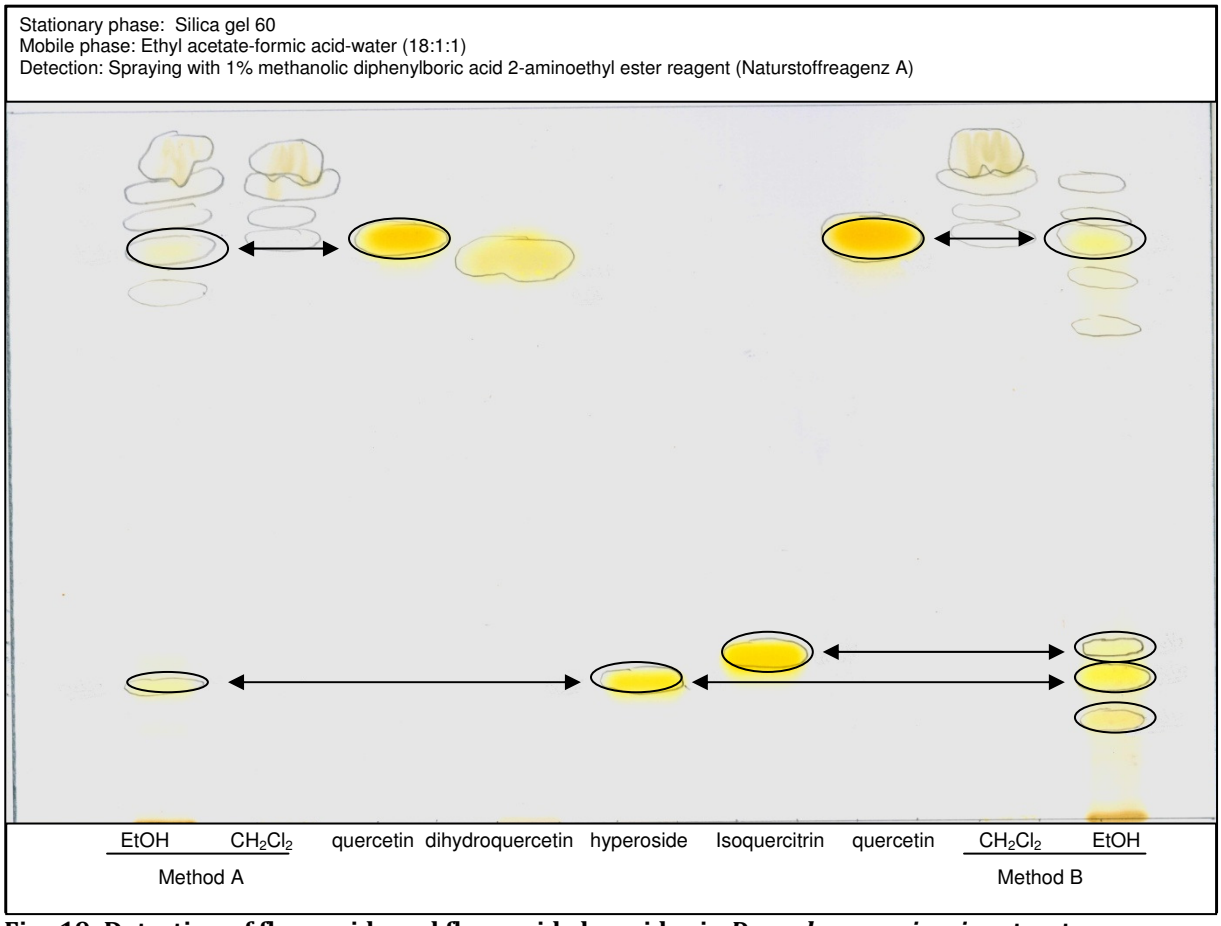


Fig. 19: Detection of flavonoids and flavonoid glycosides in *D. madagascariensis* extracts

3.1.1.1.3 Detection of terpenes in *D. madagascariensis* extracts

Steroids are derivatives of triterpenes with 26 or more carbon atoms that have undergone a characteristic type of rearrangement. Stigmasterol, β-sitosterol, and campesterol are the most ubiquitous plant sterols.

Most triterpenes are colourless substances and they are not visible on TLC plates either in day light or under UV exposure. However, after spraying with antimony (III) chloride reagent (Carr-Price reagent), followed by drying for 10 min under hot air and heating the TLC plate at 105 °C for 10 min the characteristic red-violet colouration verified the presence of five terpenes in the dichloromethane extracts and of four terpenes in the ethanol extract (method A) (Fig. 20). On the other hand, only one terpene was detected in the ethanol extract (method B) due to the extraction procedure with enrichment of lipophilic substances in the initial dichloromethane extract (method B).

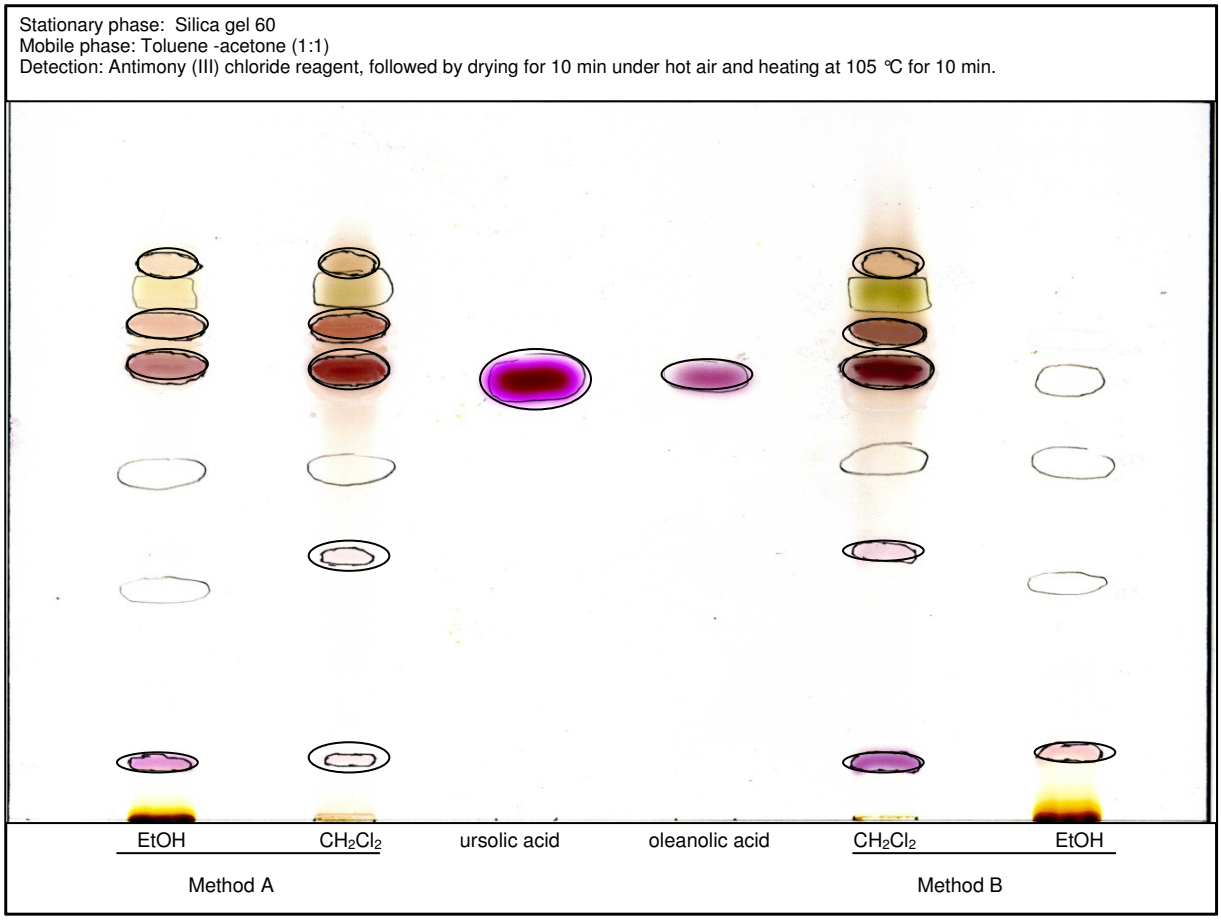


Fig. 20: Detection of terpenes in *D. madagascariensis* extracts

These results were confirmed using different mobile phases as referred to in experimental (Section 2.3.1.3) and additional reagents for detection such as ethanolic vanillin-sulphuric acid and anisaldehyde-sulphuric acid (Section 2.1.2.3).

All results of TLC analysis of *D. madagascariensis* extracts are summarized in Table. 7.

Table. 7: Results of the preliminary TLC analysis of *D. madagascariensis* extracts

Class of compounds	Compounds detected	Extraction procedure			
		Method A		Method B	
		CH_2Cl_2	EtOH	CH_2Cl_2	EtOH
Flavonoids	quercetin	-	+	-	+
Flavonoid glycosides	isoquercitrin	-	-	-	+
	hyperoside	-	+	-	+
	myricetin-3-O-galactoside	-	-	-	+
Naphthoquinones	7-methyljuglone	+	+	+	-
	plumbagin	-	-	-	-
Terpenes		+++++	++++	+++++	+

(+) present, (-) absent

3.1.2 Biological activity of *D. madagascariensis* extracts

3.1.2.1 Cytotoxicity of *D. madagascariensis* extracts against BMMΦ

In order to first assess possible toxic effects of *D. madagascariensis* extracts on macrophages as host cells of *Leishmania* parasites, bone marrow-derived macrophages (BMMΦ) were exposed to different extracts. As a parameter of cytotoxicity, the EC_{50} value, the sample concentration causing 50% toxicity, was used. The cytotoxic activities of *D. madagascariensis* extracts are shown in Table. 8.

As shown, the dichloromethane extracts obtained either from method A or method B exhibited similar cytotoxicities on macrophages but only at high concentrations (EC_{50} of 200 µg/ml and 191 µg/ml, respectively). Although there was a marked difference in the cytotoxicities between those of the ethanol extracts prepared according to method A (EC_{50} of 160 µg/ml) and method B (EC_{50} of 228 µg/ml), the data suggest again negligible cytotoxic effects on BMMΦ.

Juglone was found to be the most toxic compound of *D. madagascariensis* extracts against BMMΦ with an EC_{50} value of 2.4 µg/ml (14.1 µM), while quercetin and hyperoside showed weak cytotoxicities with EC_{50} values of 95 µg/ml (314 µM) and 105 µg/ml (226 µM), respectively. Isoquercitrin did not show any cytotoxicity on macrophages even at the highest test concentrations (EC_{50} > 20 µg/ml, >43 µM).

The observed, albeit weak cytotoxicities of the dichloromethane and ethanol extracts may be explained by the presence of the naphthoquinone 7-methyljuglone and flavonoids. Conspicuously, the absence of 7-methyljuglone from the ethanol extract (method B) resulted in a much lower cytotoxicity in spite of the presence of flavonoids and flavonoid glycosides.

3.1.2.2 Antileishmanial activity of *D. madagascariensis* extracts

All *D. madagascariensis* extracts exhibited moderate antileishmanial activity against both extra- and intracellular *L. major* parasites, with IC_{50} values ranging from 55 µg/ml to 100 µg/ml (Table. 8). In general, the dichloromethane extracts were more effective (IC_{50} 55-60 µg/ml) than the ethanolic preparations (IC_{50} 80-100 µg/ml). Also worthy of mention is that the dichloromethane extracts showed similar antileishmanial activity against both extra- and intracellular parasites. In contrast, method A produced a better antiparasitic ethanol extract than that obtained from method B, although the antileishmanial effects were very weak as evident from their IC_{50} values (*vide supra*).

In parallel, some major constituents of *D. madagascariensis* were tested against both extra- and intracellular *L. major* parasites (Table. 8). Interestingly, juglone was the most effective compound against both *Leihmania* forms within the range of samples tested (IC_{50} of 1 µg/ml, 6.5 µM). Members of flavonoids and flavonoid glycosides were generally ineffective against *L. major* parasites (isoquercetin: IC_{50} > 20 µg/ml, > 43 µM); hyperoside: IC_{50} > 80 µg/ml, > 172 µM). Only quercetin exhibited moderate antileishmanial activity with a difference in the sensitivity of promastigotes (IC_{50} of 15 µg/ml, 50 µM) and amastigotes (IC_{50} of 22 µg/ml, 72.8 µM).

The above results, when taken together with the findings of TLC analysis (Table. 7) indicate that the antileishmanial activity of *D. madagascariensis* may mainly be attributed to the naphthoquinone 7-methyljuglone and quercetin as concluded from the active dichloromethane extracts (7-methyljuglone; flavonoids absent) and the ethanol preparation (method B; quercetin; 7-methyljuglone absent)

Amphotericin B, a well-known antileishmanial drug, served as a positive control (promastigotes: IC_{50} of 2.3 µg/ml, 2.5 µM; amastigotes: IC_{50} of 0.18 µg/ml, 0.2 µM).

Table. 8: *In vitro* antileishmanial activities (*L. major*) and host cell (BMMΦ) cytotoxicity of *D .madagascariensis* extracts.

Extract	Antileishmanial activity		BMMΦ	*
	Amastigotes[a]	Promastigotes[a]	cytotoxicity[a]	SI
Method A				
CH_2Cl_2	60 ± 4.5	55 ± 3.8	200 ± 9	3.3
EtOH	80 ± 3	78 ± 6.4	160 ± 8.2	2
Method B				
CH_2Cl_2	56 ± 4.2	61 ± 3.6	191 ± 7.6	3.4
EtOH	95 ± 4.7	100 ± 5.7	228 ± 11	2.4
Juglone	1 ± 0.1 (6.5 µM)	1 ± 0.2 (6.5 µM)	2.4 ± 0.2 (14.1 µM)	2.3
Quercetin	22 ± 2.1 (72.8 µM)	15 ± 1.2 (50 µM)	95 ± 4.6 (314 µM)	4.3
Isoquercitrin	>20 (>43 µM)	>20 (>43 µM)	>20 (>43 µM)	-
Hyperoside	>80 (>172 µM)	>80 (>172 µM)	105 ± 4.1 (226 µM)	-
Amphotericin B	0.18 (0.2 µM)	2.3 (2.5 µM)	n.d.	-

[a] Values indicate the effective concentration in µg/ml necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * the ratio of EC_{50} to IC_{50}.

3.1.2.3 NO production in infected macrophages

Cell-mediated immunity is generally believed to play a causal role in the host resistance against the protozoa parasite *Leishmania* (Howard, 1985). NO is a simple gaseous, diatomic free radical molecule that has a wide array of physiological functions. It is synthesized by many cells types, including mast cells and macrophages, which play critical roles in immunity and inflammation. On the other, in vitro and in vivo infection models of leishmaniasis have contributed substantially to our understanding of cell-mediated immunity and intracellular killing mechanisms. The transformation of macrophages from host cells to cytotoxic effector cells is the major immunological pathway for parasite clearance (Alexander and Bryson, 2005). This process is mediated by interferon (IFN)-γ (Murray *et al*., 1983) plus a second signal (Pace *et al*., 1983) which, at least in *in vitro* experiments, is usually given by bacterial endotoxin (lipopolysaccharide, LPS). Activated macrophages release a variety of potentially cytotoxic molecules, of which nitric oxides, produced by inducible NO synthase (iNOS), are the most effective against *Leishmania* and other parasites (Lemesre *et al*., 1997; Green *et al*., 1990). Inducible NO synthase produces NO by oxidation of L-arginine to L-citrulline, and this pathway is inhibited by the L-arginine analog, L-N^G-monomethylarginine (L-NMMA). NO_2^- and NO_3^- are the terminal derivatives of NO in biological systems, because NO is a

very reactive gas and readily trapped by heme, reactive oxygen or other biological materials, such as glutathione, sulfhydryl (SH-) groups or unsaturated fatty acids. Nitrite formed by the spontaneous oxidation of NO was determined spectrophotometrically using the Griess assay (Fig. 21). The relative nitrite concentrations were calculated from a standard curve generated with sodium nitrite ($NaNO_2$).

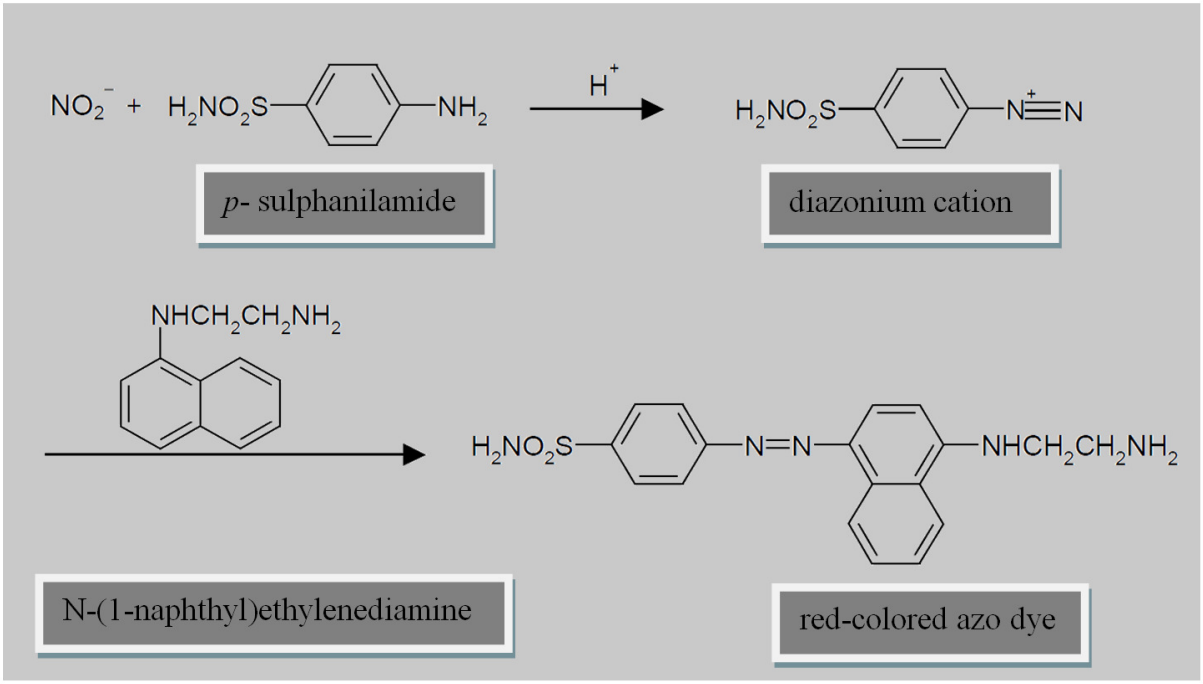


Fig. 21: The Griess reaction. Nitrite ($NO2^-$), stable decomposition product of NO, reacts with sulfanilamide to yield a diazonium derivative. This reactive intermediate will interact with N-(1-naphthyl)ethylenediamine to yield a coloured diazo product that absorbs strongly at 550 nm.

In order to assess the NO-inducing potential of *D. madagascariensis* extracts in parasitized macrophages, the supernatants of sample treated cell cultures collected as a source of secreted NO were subjected to the Griess assay. Furthermore, juglone and quercetin were included in the series of test samples. Incubations with L-N^G-monomethylarginine (L-NMMA), a well-known inhibitor of iNOS, or its ineffective enantiomer D-NMMA which does not have any significant effect on iNOS served as controls. In parallel, the rate of intracellular killing was determined by FACS analyses and the parasite retrieval assay (Figs. 22 and 23).

As evident from Fig. 22, just infected and non-infected macrophages exhibited very low induced nitrite concentrations (< 11 µM) compared to infected macrophages stimulated with IFN-γ plus LPS (58 µM). Interestingly, this high level of NO production was associated with a highly parasite killing rate of ca. 93%, while all *D. madagascariensis* extracts, juglone, and quercetin at their effective concentrations induced the release of NO at relatively low levels (< 11 µM) and in the same range of that of just infected macrophages.

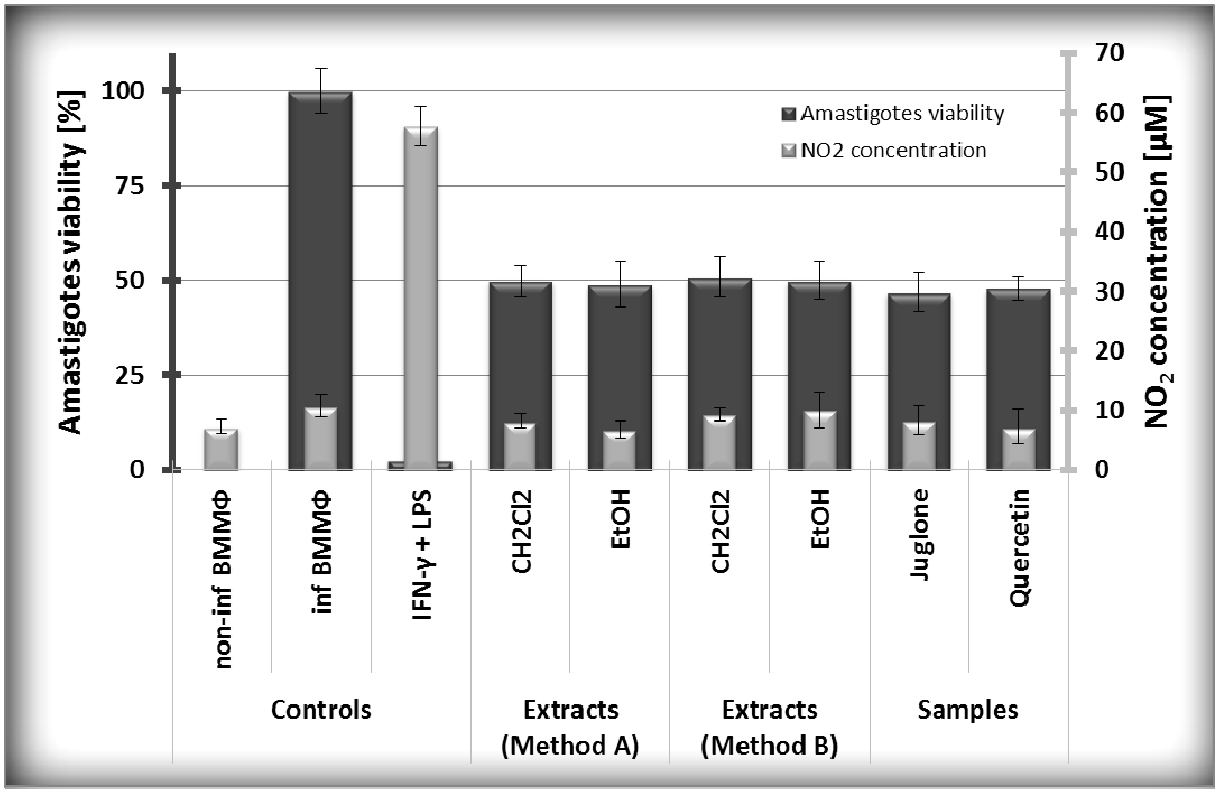


Fig. 22: In vitro antileishmanial activity (FACS analyses, ■), and induced nitrite concentrations (Griess assay, □) in the supernatants of non-infected macrophages, non-treated infected macrophages, infected treated with rIFN-γ 10 U/ml+ LPS 1 ng/ml, and infected treated with *D. madagascariensis* extracts, juglone, and quercetin at effective concentrations. (Mean values ± standard deviations were calculated from three independent experiments).

In the presence of L-NMMA, the stimulus IFN-γ plus LPS induced very low nitrite concentration (9 µM) and, in parallel, exhibited negligible antileishmanial activity as shown in Fig 23. In contrast, all *D. madagascariensis* extracts, juglone, and quercetin at their IC_{50} ranges showed similar antileishmanial activities when incubated with or without the inhibitor L-NMMA (Fig. 22 and Fig. 23). Conspicuously, these findings showed that the *D. madagascariensis* extracts, juglone, and quercetin did not stimulate the infected macrophages to produce NO and that the antileishmanial activity of the samples should be mediated by a different, possibly direct mode of action.

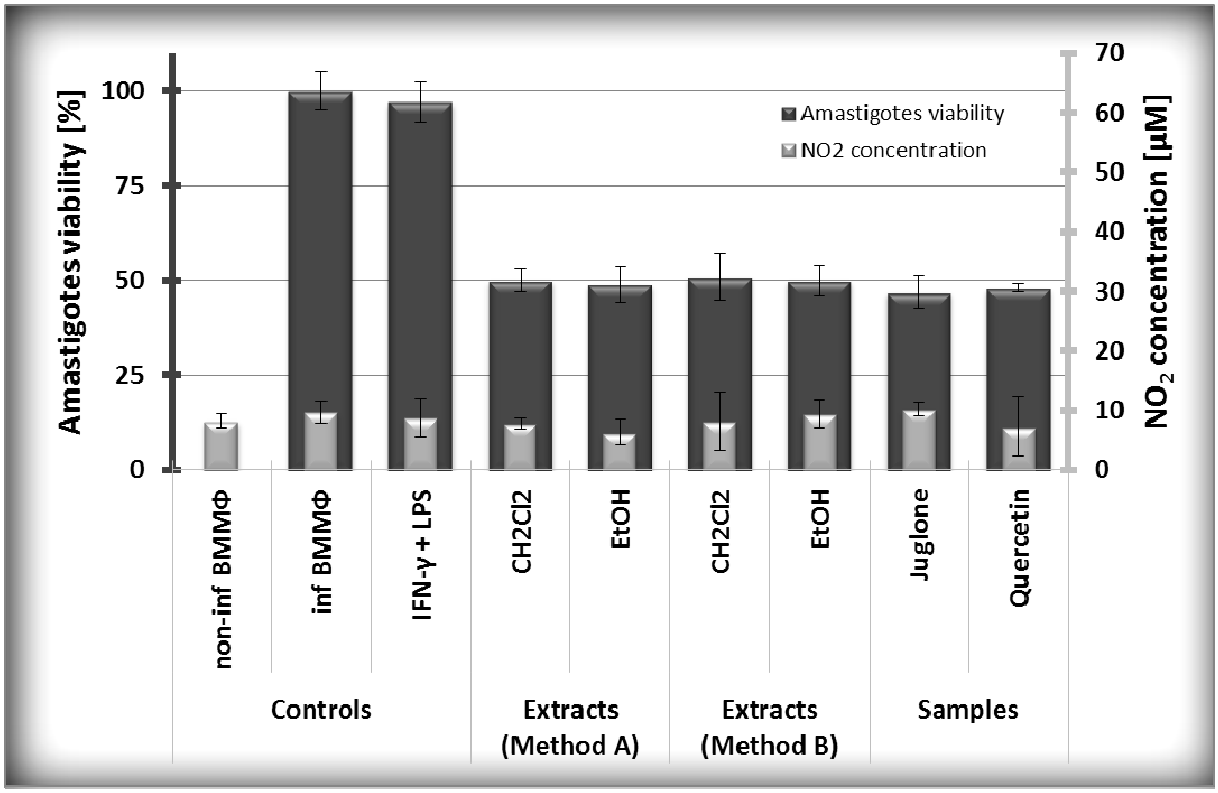


Fig. 23: ***In vitro*** **antileishmanial activity (FACS analyses, ■), and induced nitrite concentrations (Griess assay, □) in the presence of L-NMMA. (Non-infected macrophages, non-treated infected macrophages, infected treated with rIFN-γ 10 U/ml + LPS 1 ng/ml, and infected treated with** ***D. madagascariensis*** **extracts, juglone, and quercetin at effective concentrations). (Mean values ± standard deviations were calculated from three independent experiments).**

3.2 *Drosera peltata*

3.2.1 Phytochemical studies

3.2.1.1 Preliminary TLC analysis of extracts

3.2.1.1.1 Detection of naphthoquinones in *D. peltata* extracts

The characteristic yellow to orange colouration in the day light and under UV at 365 nm verified the presence of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), the major naphthoquinone reported to occur in aerial parts of the genus *Drosera*, in all extracts when compared with a reference sample of plumbagin, appearing at R_f value 0.35 under the experimental conditions (Fig. 24). Two additional naphthoquinones were detected in all extracts except for the ethanol extracts as yellow spot in the day light and as orange fluorescence spot under UV at 365 nm with R_f values 0.25 and 0.15, respectively. After spraying with ethanolic KOH, plumbagin and these two naphthoquinones responded in a typical red colour (Fig. 24).

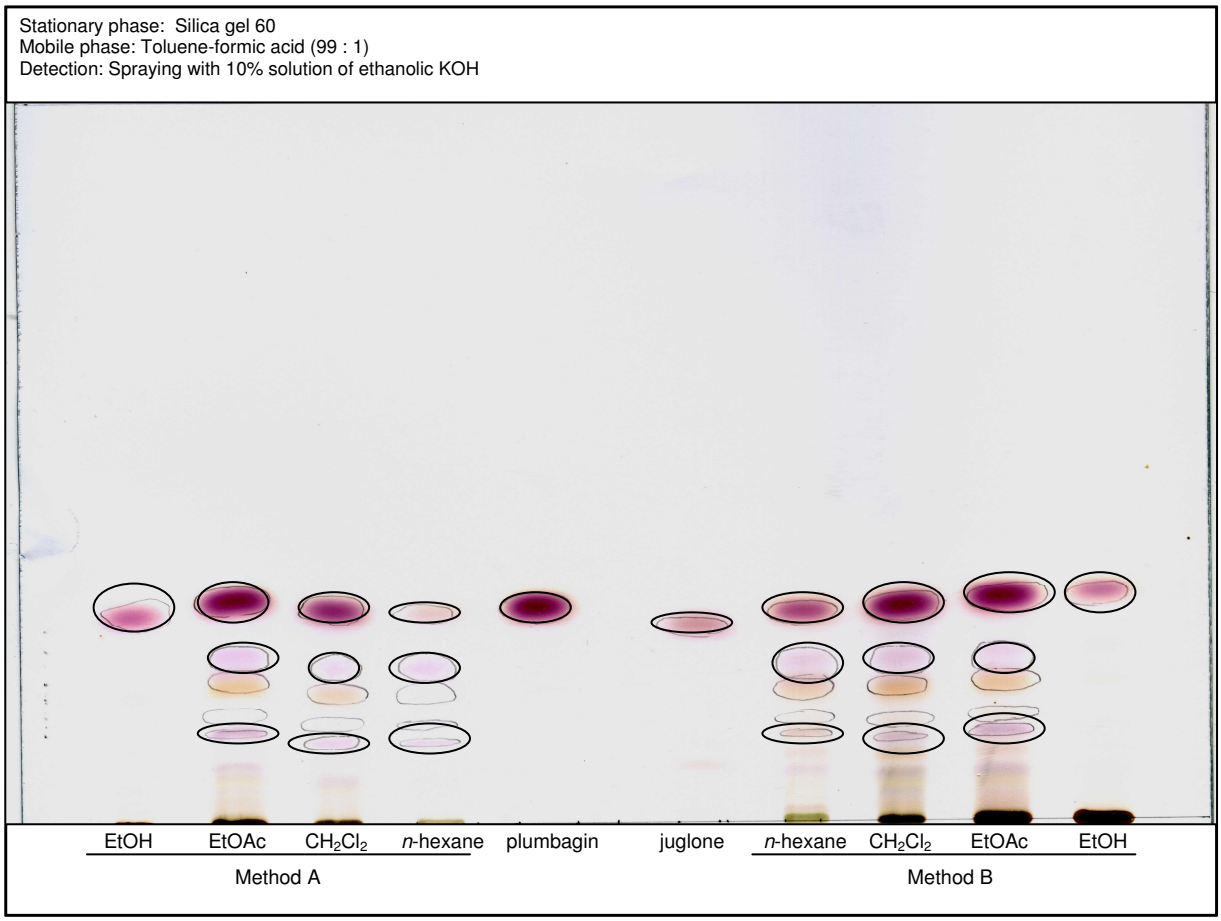


Fig. 24: Detection of naphthoquinones in *D. peltata* extracts.

The naphthoquinone with R_f 0.25 was tentatively identified as droserone (3,5-dihydroxy-2-methyl-1,4-naphthoquinone) on the basis of its chromatographic properties (Wang *et al*., 1998), while that with lower mobility was successfully isolated and characterized as 3,3'-biplumbagin [5-hydroxy-3-(8-hydroxy-3-methyl-1,4-dioxonaphthalen-2-yl)-2-methyl naphtha-lene-1,4dione] (Section 3.2.1.3.2). Notably, its occurrence in this plant source was demonstrated for the first time.

3.2.1.1.2 Detection of flavonoids and flavonoid glycosides in *D. peltata* extracts

All flavonoids cause fluorescence quenching under the UV light at 254 nm and show dark yellow, green or blue fluorescence at 366 nm. Fluorescence can be enhanced using 1% methanolic diphenylboric acid 2-aminoethyl ester reagent (Naturstoffreagenz A). After spraying the TLC plate, the flavonoids show yellow, orange or pink fluorescence at 366 nm according to substitution pattern of B-ring and structural type of flavonoids.

As for the *D. peltata* extracts, flavonoids and flavonoid glycosides were not detectable in any extract. For comparison quercetin, dihydroquercetin, isoquercitrin, and hyperoside were used as reference samples.

3.2.1.1.3 Detection of sterols and triterpenes in *D. peltata* extracts

Most triterpenes are not readily visible on TLC plates either in day light or under UV exposure. However, after spraying with antimony (III) chloride reagent (Carr-Price reagent), followed by drying for 10 min under hot air and heating at 105 °C for 10 min the appearance of a characteristic red-violet colouration of bands verified the presence of three triterpenes present in all extracts prepared according to method A. On the other hand, when using method B for extract preparation, most amounts of triterpenes remained in the *n*-hexane and dichloromethane phases, whereas members of this class of secondary products were apparently absent in the ethyl acetate and ethanol extracts. Anisaldehyde-sulphuric acid reagent was used to confirm the presence of triterpenes, with ursolic acid as control in terms of response in colour and chromatographic mobility. After spraying the TLC plate, it was heated at 105 °C for 10 min to give two violet spots (R_f value of 0.17 and 0.65) and grey spot (R_f value of 0.1) under the experimental conditions (Fig. 25). Based on their chromatographic behaviour the grey spot and the violet spot (R_f 0.17) were tentatively identified to be beta-sitosterol and stigmasterol, respectively (Wang *et al*., 1998), which was further substantiated by co-chromatography with authentic reference samples.

All results of preliminary TLC analysis of *D. peltata* extracts are summarized in Table 9.

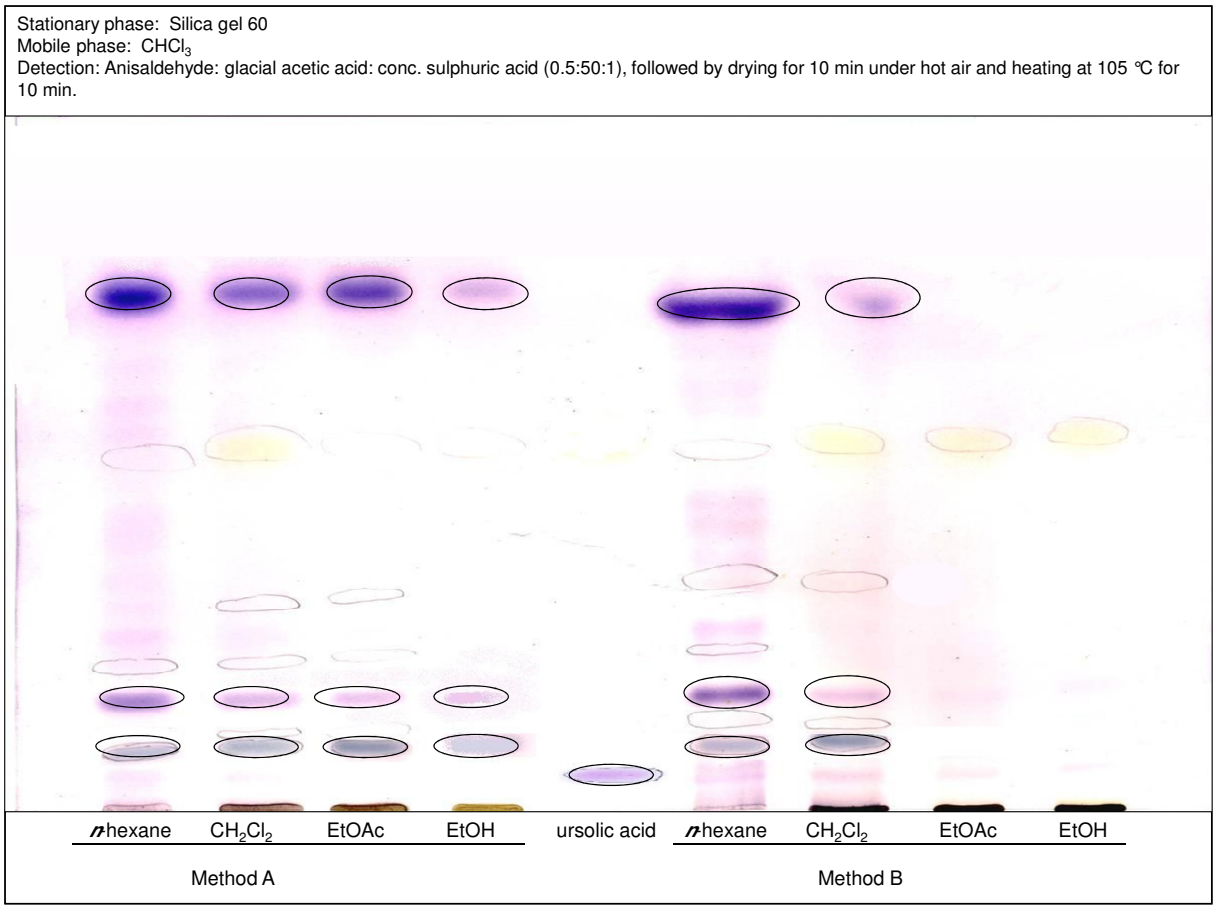


Fig. 25: Detection of triterpenes in *D. peltata* extracts.

Table. 9: Results of the preliminary TLC analysis of *D. peltata* extracts

Class of compounds	Compounds detected	Extraction procedure							
		Method A				Method B			
		n-hexane	CH_2Cl_2	EtOAc	EtOH	*n*-hexane	CH_2Cl_2	EtOAc	EtOH
Flavonoids		-	-	-	-	-	-	-	-
Tetralones	*cis*-isoshinanolone	-	+	+	-	-	+	+	-
Naphthoquinones	plumbagin	+	+	+	+	+	+	+	+
	droserone	+	+	+	-	+	+	+	-
	3,3'-biplumbagin	+	+	+	-	+	+	+	-
Terpenes	unidentified terpene	+	+	+	+	+	+	-	-
	beta-sitosterol	+	+	+	+	+	+	-	-
	stigmasterol	+	+	+	+	+	+	-	-

(+) present, (-) absent

3.2.1.2 Quantification of plumbagin in *D. peltata* extracts using HPLC

The amount of plumbagin in extracts of *D. peltata* was determined using high-performance liquid chromatography (HPLC) in reference with a solution of plumbagin of known concentration (Fig. 26). The plumbagin content of *D. peltata* extracts are shown in Table. 10. As can be seen, the highest amount of plumbagin resided in the dichloromethane and ethyl acetate extracts (5% and 3.5%, respectively) irrespective of applying method A or method B for extraction, while *n*-hexane and ethanol extracts contained only little quantities of plumbagin (0.6%, and 0.3%, respectively). These results indicate that there is no difference in the plumbagin content when using either method A or B for extraction, and that dichloromethane is the best suited solvent for extraction of plumbagin from the plant material.

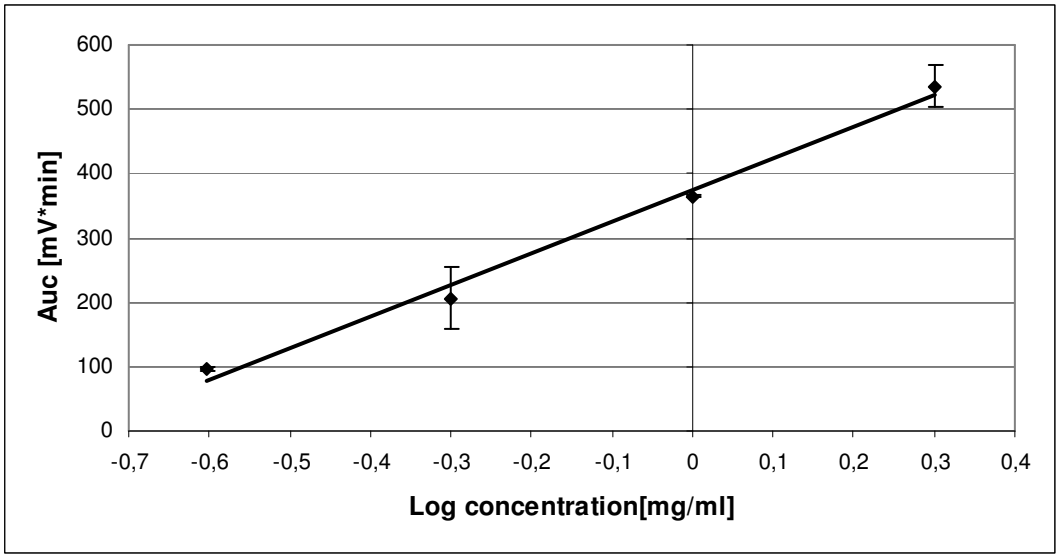


Fig. 26: Standard curve of plumbagin using high-performance liquid chromatography (Mean values ± standard deviations were calculated from three independent experiments).

Table. 10: Plumbagin content of *D. peltata* extracts using HPLC measurements

Extract	Plumbagin (%)*	
	Method A	Method B
***n*-hexane**	0.6 ± 0.02	0.6 ± 0.01
CH_2Cl_2	5.3 ± 0.35	5.0 ± 0.06
EtOAc	3.5 ± 0.09	3.2 ± 1.07
EtOH	1.9 ± 0.01	0.3 ± 0.01

(Mean values ± standard deviations were calculated from three independent experiments.
*** Based on the dry weight of the extracts)**

3.2.1.3 Isolation and purification of substances from dichloromethane extract of *D. peltata*

The dichloromethane extract which was obtained from method B of extraction was the most important one in terms of the content of naphthoquinones. A proportion (0.8 g) was further fractionated using column chromatography and an *n*-hexane-ethyl acetate gradient system (100:0 → 0:100) as eluant. Fractions (each 25 ml) were collected and grouped according to their TLC profiles to afford 8 fractions as shown in Table. 11.

Table. 11: Fractionation of dichloromethane extract (method B) using column chromatography

Fraction	Solvent system *n*-hexane – EtOAc/ v:v	Elution volume	Yield (mg)	TLC screening
1	100 : 0	1-14 (0 – 350 ml)	385	fatty acids- terpenes
2	98 : 2	15-19 (350- 475 ml)	163	terpenes
3	97 : 3	20-23 (475- 575 ml)	47	plumbagin- droserone- terpenes
4	95 : 5	24-30 (575- 750 ml)	35	plumbagin
5	95 : 5	31-33 (750-825 ml)	12	3,3'-biplumbagin
6	92 : 8	34-46 (825-1150 ml)	42	-
7	90 : 10	47-51 (1150-1275 ml)	4	tetralone (*cis*-isoshinanolone)
8	0 : 100	52-71 (1275-1775 ml)	64	-

3.2.1.3.1 Isolation and purification of plumbagin (5)

Plumbagin (**5**) was detected mainly in fraction 3 and fraction 4 of the dichloromethane extract (method B) when compared with a reference sample (R_f 0.54; mobile phase: 100% chlorophorm) (Leclercq and Angenot, 1984).

Fraction 3 was subjected to high-performance liquid chromatography using a water-methanol gradient (1:0 → 0:1) as mobile phase at a flow rate of 3 ml/min (40 min). Plumbagin was detected at 254 nm showing a retention time of 34.6 min. The HPLC separations afforded 38 mg of orange-yellow needles which were established as plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (**$C_{11}H_8O_3$**) using mass spectroscopy and nuclear magnetic resonance spectroscopy (Fig. 27).

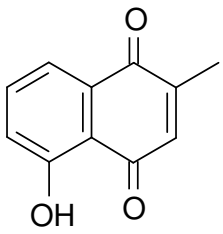


Fig. 27: Chemical structure of plumbagin (5-Hydroxy-2-methyl-1,4-naphthoquinone)

Its molecular formula, $C_{11}H_8O_3$, was concluded from the $[M]^+$ at *m/z* 188 in the EI-MS, supported by the fragmentation pattern (*m/z* 173 $[M\text{-}Me]^+$, 160 $[M\text{-}CO]^+$, 132 $[M\text{-}CO\text{-}CO]^+$, 120 $[C_7H_4O_2]^+$, 92 $[C_6H_4O]^+$, and 77) (Fig. 28).

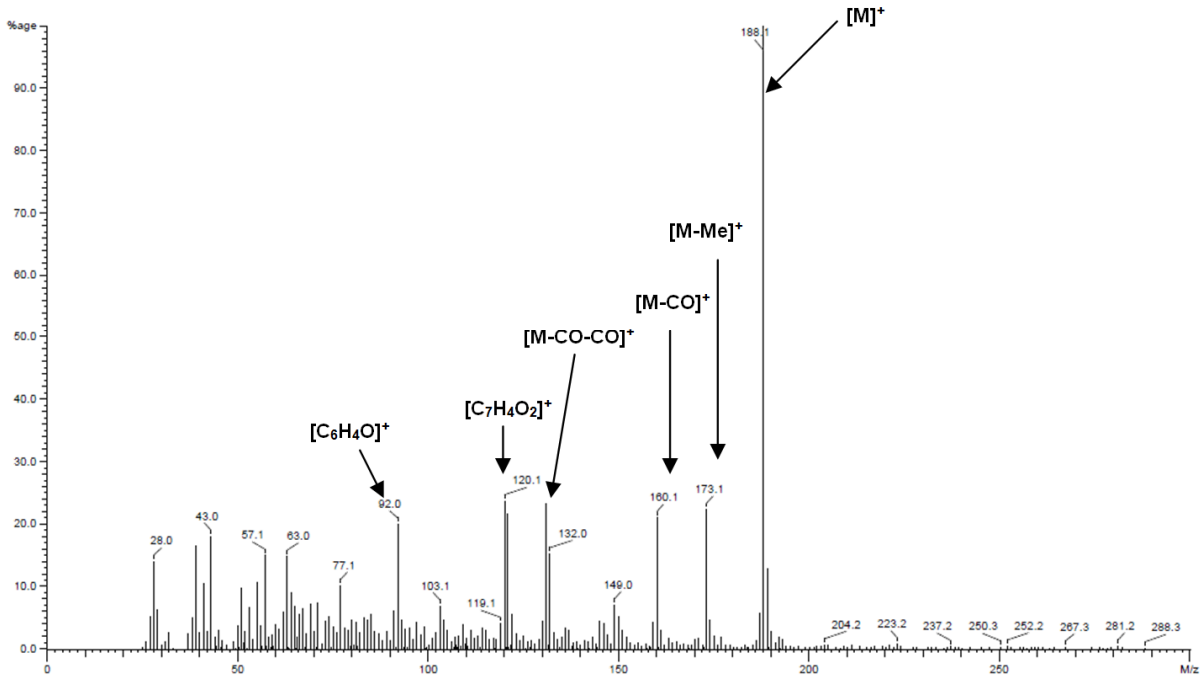


Fig. 28: EI-mass spectrum of plumbagin (5)

The ^{1}H-NMR spectrum of plumbagin revealed the presence of an AMX spin system {δ 7.24 (1H, *d,* H-8), 7.62 (2H, *t*, H-6 and H-7)}, an aromatic proton signal at δ 6.81 (1H, *s*, H-3), and a sharp low-field singlet at δ 11.98 (5–OH). The aliphatic region exhibited a methyl signal at δ 2.20 (3H, *s*, Me-2), (Fig. 29). The spectroscopic data were in agreement with those of an authentic sample.

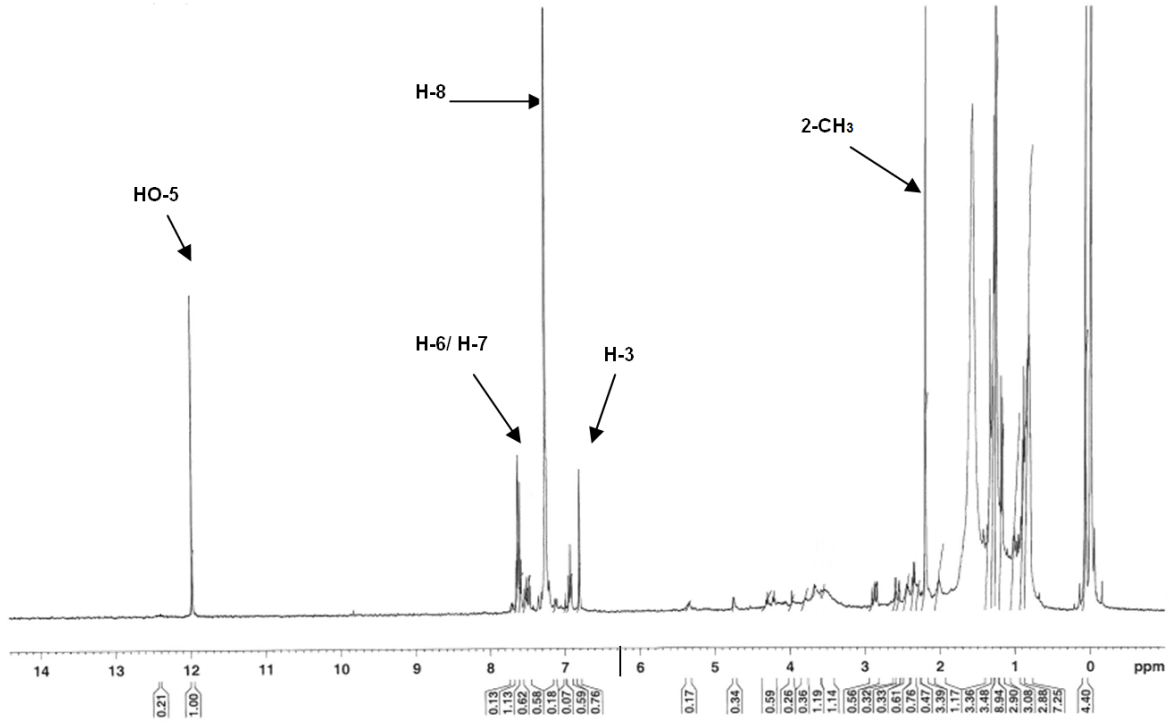


Fig. 29: ^{1}H-NMR spectrum of plumbagin (5)

3.2.1.3.2 Isolation and purification of 3,3'-biplumbagin (19)

3,3'-Biplumbagin (**19**) was obtained from *D. peltata* for the first time. It was detected in fraction 5 of the CC purification (Table. 11) by TLC monitoring (R_f 0.15) using toluene-formic acid (99: 1) as a mobile phase.

Fraction 5 was applied to high-performance liquid chromatography using a water-methanol gradient (1:0 → 0:1) as mobile phase at a flow rate of 3 ml/min. 3,3'-Biplumbagin (**19**) was detected at Rt 39 min. Due to the characteristic UV absorptions 254 nm was used as the acquisition wavelength in the HPLC analyses. The separations afforded 3.2 mg of yellow needles. Its identity was established by mass spectroscopy and nuclear magnetic resonance spectroscopy (Fig. 30).

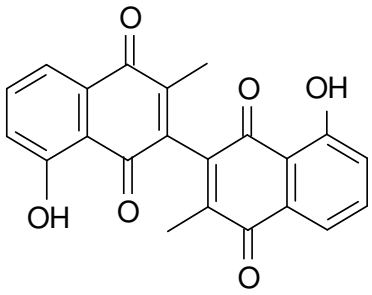


Fig. 30: Chemical structure of 3,3'-biplumbagin

The EI-MS showed an $[M]^{+\bullet}$ ion at *m/z* 374 indicating the molecular formula $C_{22}H_{14}O_6$, and fragments at *m/z* 359 $[M\text{-}Me]^+$, 357 $[M\text{-}OH]^+$, 345 $[M\text{-}CO]^+$, 331 $[M\text{-}Me\text{-}CO]^+$, 317 $[M\text{-}H\text{-}2CO]^+$, 120 $[C_7H_4O_2]^+$, and 92 $[C_6H_4O]^+$) (Fig. 31).

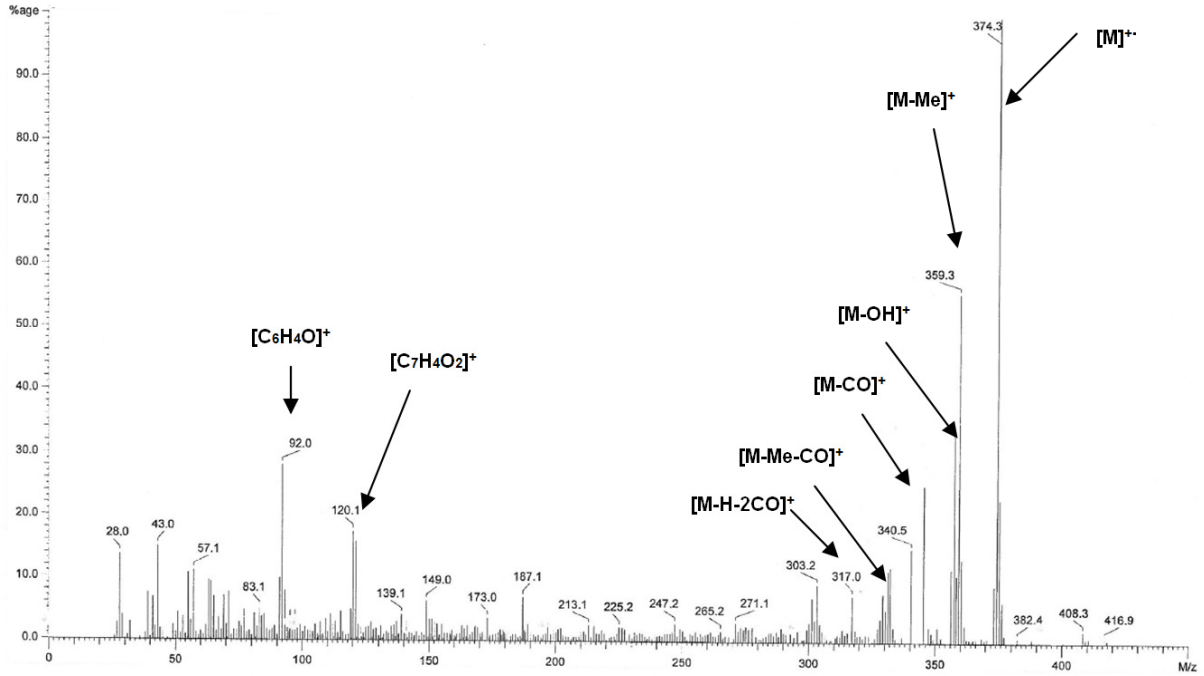


Fig. 31: EI-mass spectrum of 3,3'-biplumbagin (19)

The ^{1}H-NMR data of 3,3'-biplumbagin (**19**) are summarized in Fig. 32 and Table. 12.

Table. 12. ^{1}H-NMR data of 3,3'-biplumbagin[a] (400 MHz, $CDCl_3$)

Proton	Chemical shifts
2-CH_3 and 2´-CH_3	2.08 (*s*)
H-6 and H-6´	7.3 (*br. d*, *J*=8.00)
H-7 and H-7´	7.67 (*dd*, *J*= 7.4 and 8.00)
H-8 and H-8´	7.73 (*br. d*, *J*=7.4)
HO-5 and HO-5´	11.81 (*s*)

[a] **(δ in ppm from TMS, multiplicities and *J* values (Hz) are given in parentheses)**

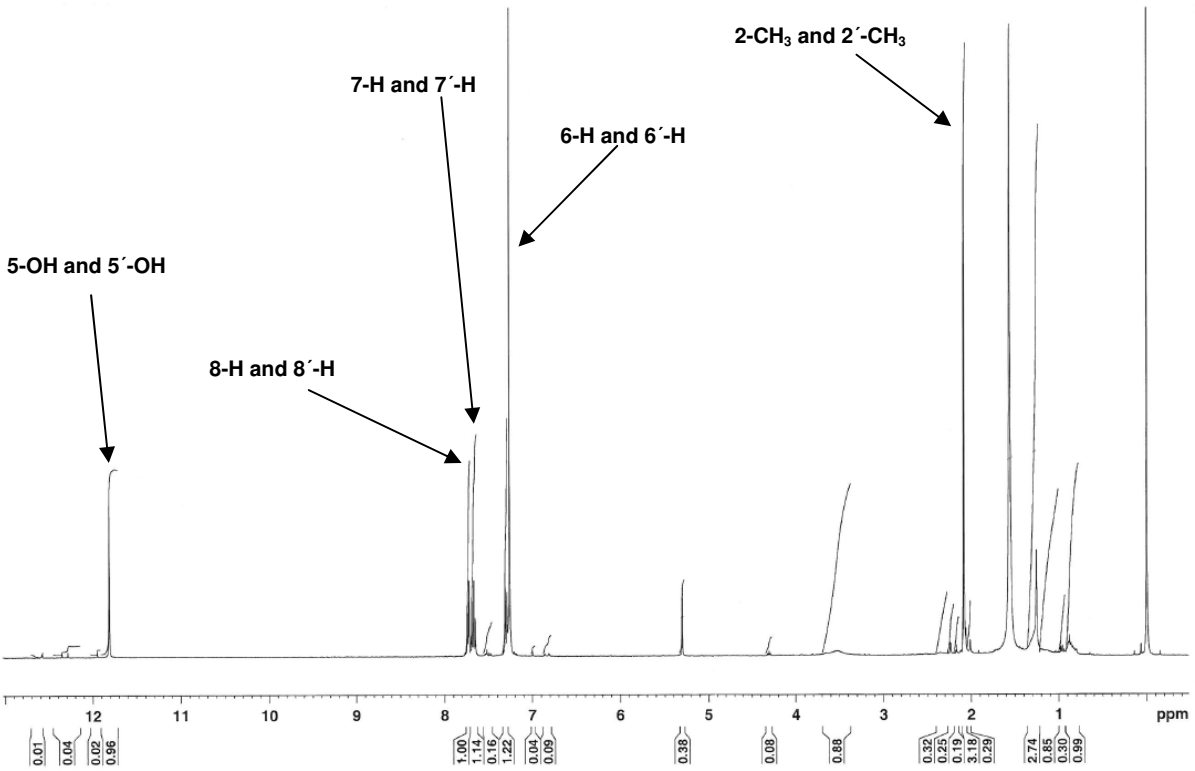


Fig. 32: ^{1}H-NMR spectrum of 3,3'-biplumbagin (19)

3.2.1.3.3 Isolation and purification of *cis*-isoshinanolone (22)

Cis-isoshinanolone was detected in fraction 7 of the dichloromethane extract (method B) of *D. peltata*, exhibiting chromatographic mobility similar to that of droserone with an R_f value of 0.25 (mobile phase: Toluene-formic acid 99 : 1), responding with a greyish colouration to the anisaldehyde-sulphuric acid reagent on TLC plates. It was isolated as a yellow oil following HPLC purification (water-methanol gradient 1:0 → 0:1; flow rate: 3 ml/min; Rt 25.7 min; detection 254 nm; yield 2 mg).

Cis-isoshinanolone (**22**) [(3R,4R)-4,8-dihydroxy-3-methyl-3,4-dihydro-2H-naphthalen-1-one], was readily identified by means of its spectroscopic data (MS and ^{1}H-NMR) which were identical to those of an authentic specimen (Fig. 33).

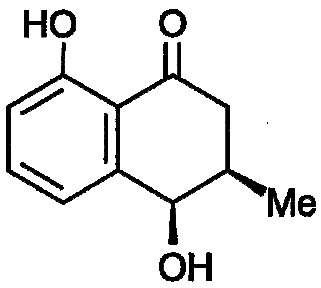


Fig. 33: Chemical structure of *cis*-isoshinanolone (22)

Its EI-MS showed an $[M]^{+\cdot}$ at *m/z* 192, consistent with a molecular formula $C_{11}H_{12}O_3$ for the metabolite. The detected fragments at *m/z* 177 $[M\text{-}Me]^+$, 174 $[C_{11}H_{10}O_2]^+$, 163 $[C_9H_7O_3]^+$, 150, and 121 $[C_7H_5O_2]^+$ lend support to the anticipated structure (Fig. 34).

The ^{1}H-NMR spectrum of *cis*-isoshinanolone (400 MHz, $CDCl_3$) showed a sharp low-field singlet at δ 12.41 (*s*, 8-OH), and signals in the aromatic and aliphatic regions (Fig. 35). In the aromatic region of the ^{1}H-NMR spectrum, the signals in the range of 6.8-7.5 ppm can be assigned to three aromatic protons {δ 7.48 (*dd*, *J*= 8.0 and 7.3 Hz, H-6), 6.94 (*br, J*= 8.0, H-7), 6.92 (*br., J*=7.3, H-5)}, while the aliphatic region exhibited signals at δ 4.75 (*d*, *J*= 2.94, H-4), 2.87 (*dd*, *J*= 17.7 and 11 Hz, H-2_{ax}), 2.57 (*dd*, *J*= 17.7 and 4.2 Hz, H-2_{eq}), 2.44 (*m*, H-3), and 1.18 (*d*, *J*= 6.9, 3-CH_3). The determination of the relative 3,4-configuration was readily deduced by the magnitude of the respective coupling constants [2.5 and 7.5 Hz for the 3,4-*cis*- and 3,4-*trans*-isomer, respectively (Tezuka *et al.,* 1973)]. The observed coupling constant of 2.9 Hz suggested that the methyl and hydroxyl groups are *cis*-configurated (the methyl group is in an equatorial orientation and the hydroxyl is placed in a pseudo-axial orientation).

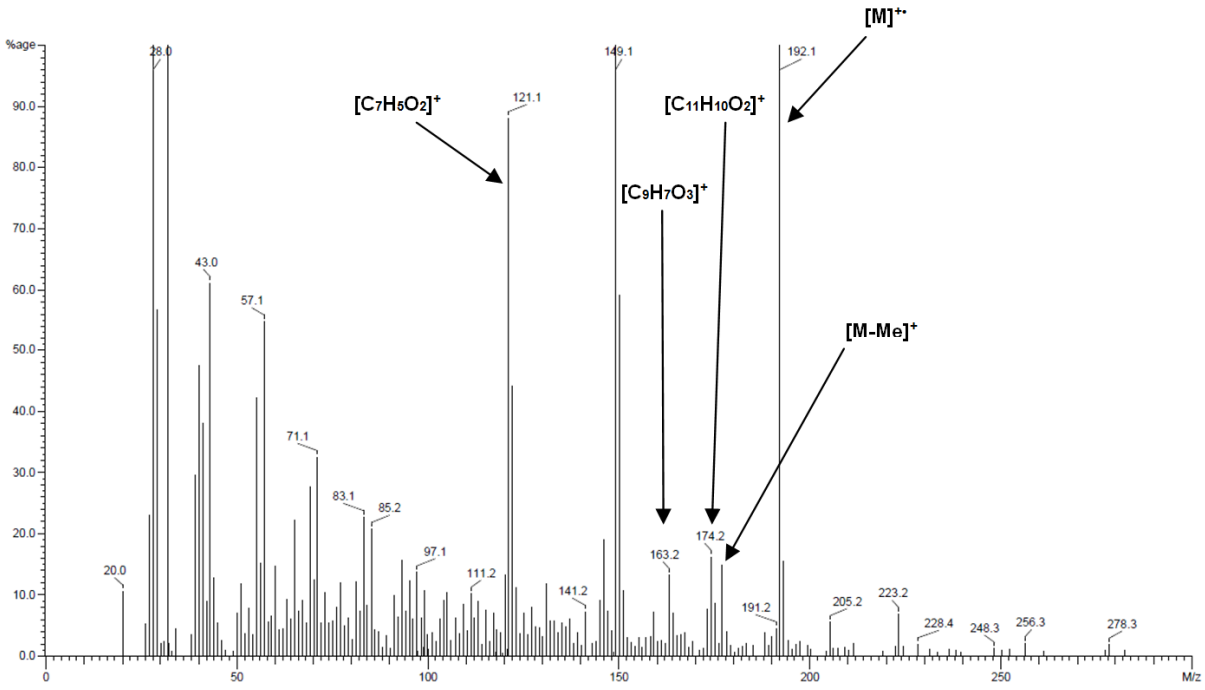


Fig. 34: EI-mass spectrum of *cis*-isoshinanolone (22)

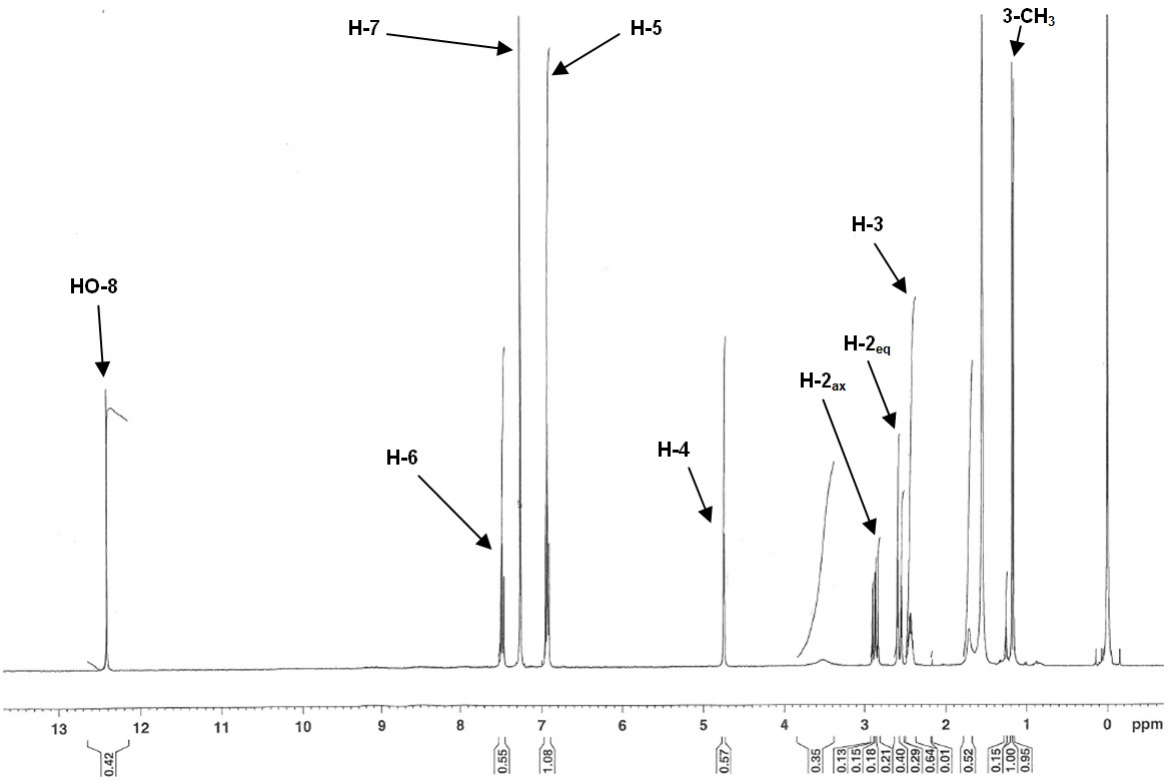


Fig. 35: ^{1}H-NMR spectrum of *cis*-isoshinanolone (22)

Unfortunately, the amount of the isolated *cis*-isoshinanolone (2 mg) was not enough to perform the additional examinations such as X-ray structure analysis and circular dichroism

for the determination of the absolute configuration. It should be noted that (+)-*cis*-isoshinanolone, an acetogenic metabolite of wide-spread occurrence in higher plants, was previously identified in a commercial *Drosera* fluid extract for the first time (Kolodziej *et al*., 2002).

3.2.2 Biological activity of *D. peltata* extracts

3.2.2.1 Cytotoxicity of *D. peltata* extracts against BMMΦ

Bone marrow-derived macrophages (BMMΦ) were exposed to different extracts of *D. peltata* using MTT assay to evaluate possible toxic effects on macrophages as host cells of *Leishmania* parasites. The EC_{50} values of *D. peltata* extracts are listed in Table. 13.

As can be seen, within the extracts obtained from method B the dichloromethane and ethyl acetate extracts showed high cytotoxicity on macrophages at low concentrations (EC_{50}< 9 µg/ml) compared with those of the *n*-hexane and ethanol extracts (EC_{50}> 80 µg/ml). A similar picture emerged from analysing the extracts prepared according to method A. Again, dichloromethane and ethyl acetate extracts exhibited more pronounced cytotoxic effects on macrophages (EC_{50} values of 17 mg/ml and 35 mg/ml, respectively) when compared with those of the *n*-hexane and ethanol extracts (EC_{50} of 90 µg/ml, and 50 µg/ml, respectively). In contrast, the ethanol extract (method B) did not show any cytotoxicity (EC_{50} > 80 µg/ml) compared with that of method A (EC_{50} 50 µg/ml).

According to the preliminary TLC analyses of *D. peltata* extracts, naphthoquinones were considered as the main constituents of the ethyl acetate extract (method B)(Table. 9). It was thus reasoned that the naphthoquinones significantly contributed to the cytotoxic activities of *D. peltata*. That plumbagin showed prominent cytotoxicity on macrophages at low concentrations (EC_{50} 1.5 µg/ml, 8µM) (Table. 13) strongly supports this conjecture. Moreover, plumbagin represents 3.2% of the dry weight of the ethyl acetate extract (method B).

Besides, the terpenes of *D. peltata* extracts did not show any cytotoxicity as concluded from effects of the ethyl acetate extract (method A) (EC_{50} of 35 µg/ml). Notably this phase contained significant amounts of plumbagin (3.5% on the basis of the dry weight) which proved relatively cytotoxic.

3,3'-Biplumbagin which was isolated for the first time from *D. peltata* showed a very weak cytotoxicity (EC_{50} 31µg/ml; 83 µM), while *cis*-isoshinanolone did not exhibit any cytotoxicity even at the highest test concentration (EC_{50} >17.3 µg/ml, >90 µM) (Table. 13).

3.2.2.2 Antileishmanial activity of *D. peltata* extracts

All *D. peltata* extracts except for the ethanol extract (method B) showed activity against both extra- and intracellular *L. major* parasites, with IC_{50} values ranging from 3 µg/ml to 37 µg/ml (Table. 13). The highest antileishmanial activity resided in the dichloromethane extract (method B), ethyl acetate extract (method B) and dichloromethane extract (method A) with IC_{50} values of 5 µg/ml, 5 µg/ml, and 3 µg/ml against extracellular promastigotes and 5 µg/ml, 4 µg/ml, and 10 µg/ml against intracellular amastigotes, respectively. The highly lipophilic and highly polar compounds apparently did not contribute to the antileishmanial activity as concluded from the antiparasitic activities of the *n*-hexane and ethanol extracts (method B) with IC_{50} values >29 µg/ml. Plumbagin itself showed activity against both extra- and intracellular *L. major* parasites at low concentration with IC_{50} values < 0.5 µg/ml. According to the prominent antileishmanial activity of plumbagin, it was reasoned that the relatively high content of this naphthoquinone in the dichloromethane extract (method B), ethyl acetate extract (method B) and dichloromethane extract (method A) (5%, 3.2%, and 5.3%, respectively) significantly contributed to the antileishmanial activity. Conspicuously, the extracellular promastigotes were more sensitive to 3,3'-biplumbagin (**19**) than intracellular amastigotes, as evident from the IC_{50} values (promastigotes: IC_{50} of 4.5 µg/ml; amastigotes: IC_{50} of 9.5 µg/ml). On the other hand *cis*-isoshinanolone (**22**) did not affect both extra- and intracellular parasites even at relatively high concentrations (IC_{50} >15 µg/ml) (Table. 13). Given the IC_{50} values resulting from the parasite retrieval assay and FACS analysis, similar values were noted for dichloromethane extract (method B), ethyl acetate extract (method B) and the tested naphthoquinones. Conspicuously, there was a significant difference between the IC_{50} values of the ethanol extract (method A) assessed by the retrieval assay and FACS analysis, respectively. It appears reasonable to consider the IC_{50} values obtained from FACS analysis to be more accurate because the GFP signal of live parasites was measured directly, while retrieval of viable parasites was determined after host cell lysis, transformation of amastigotes into promastigotes and 5 days of culture.

Table. 13: *In vitro* antileishmanial activities (*L. major*) and host cell (BMMΦ) cytotoxicity of *D. peltat* extracts.

Extract	Antileishmanial activity Amastigotes[a]		Promastigotes[a]	BMMΦ cytotoxicity[a]	SI*
	Parasite retrieval assay	FACS analysis	MTT assay	MTT assay	
Method A					
n-hexane	26 ± 2	22 ± 1.4	26 ± 1.3	90 ± 5.3	3.4
CH_2Cl_2	10 ± 0.8	7 ± 0.3	3 ± 0.2	17 ± 2	1.7
EtOAc	16 ± 1.8	11 ± 1	18 ± 1.1	35 ± 2.6	2.2
EtOH	15 ± 2	8 ± 1	14 ± 0.8	50 ± 3.8	3.3
Method B					
n-hexane	35 ± 3.2	29 ± 2.4	37 ± 3	80 ± 6	2.3
CH_2Cl_2	5 ± 1	5 ± 0.5	5 ± 0.3	8 ± 0.2	1.6
EtOAc	4 ± 0.4	4 ± 0.5	5 ± 0.7	9 ± 1	2.2
EtOH	>40	40 ± 3	>80	>80	-
Plumbagin	0.5 ± 0.05	0.5 ± 0.04	0.47 ± 0.02	1.5 ± 0.1	3
3,3'-Biplumbagin	9.5 ± 0.7	9 ± 0.4	4.5 ± 0.4	31 ± 2.7	3.2
Cis-isoshinanolone	> 15	> 15	> 15	> 17.3	-
Amphotericin B	0.2	0.17	2.3	n.d.	-

[a] Values indicate the effective concentration in µg/ml necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * the ratio of EC_{50} to IC_{50}.

3.2.2.3 NO production in infected macrophages

L. major infected macrophages were incubated with *D. peltata* extracts and the tested naphthoquinones at their effective concentrations (Table. 13) alone or in the presence of L-NMMA. The nitrite ($NO2^-$) concentrations were determined in the supernatants of sample treated cell cultures to assess the NO-inducing potential of the tested samples. In parallel, the rate of intracellular killing activity of the tested samples was determined by FACS analyses.

Just infected and non-infected macrophages treated with or without L-NMMA exhibited very low induced nitrite concentrations (< 13 µM) when compared to infected macrophages stimulated with IFN-γ plus LPS (55 µM) (Fig. 36 and Fig. 37). Interestingly, the high NO-inducing potential of IFN-γ plus LPS was associated with a considerable parasite killing rate of ca 95%. Notably, the IFN-γ plus LPS stimulus "lost" its antileishmanial activity and showed a low induced nitrite concentration (11 µM) when concurrently incubated with L-NMMA as shown in Fig. 37.

In contrast all *D. peltata* extracts, plumbagin (**5**), and 3,3'-biplumbagin (**19**) induced the release of low levels of NO (< 14 μM) while exhibiting similarly antileishmanial effects, irrespective of incubating alone or in combination with L-NMMA as shown in Fig. 36 and Fig. 37. These conspicuous findings indicated that the above mentioned samples did not stimulate the infected macrophages to produce NO and that their antileishmanial activity should be mediated by a different, probably direct mode of action.

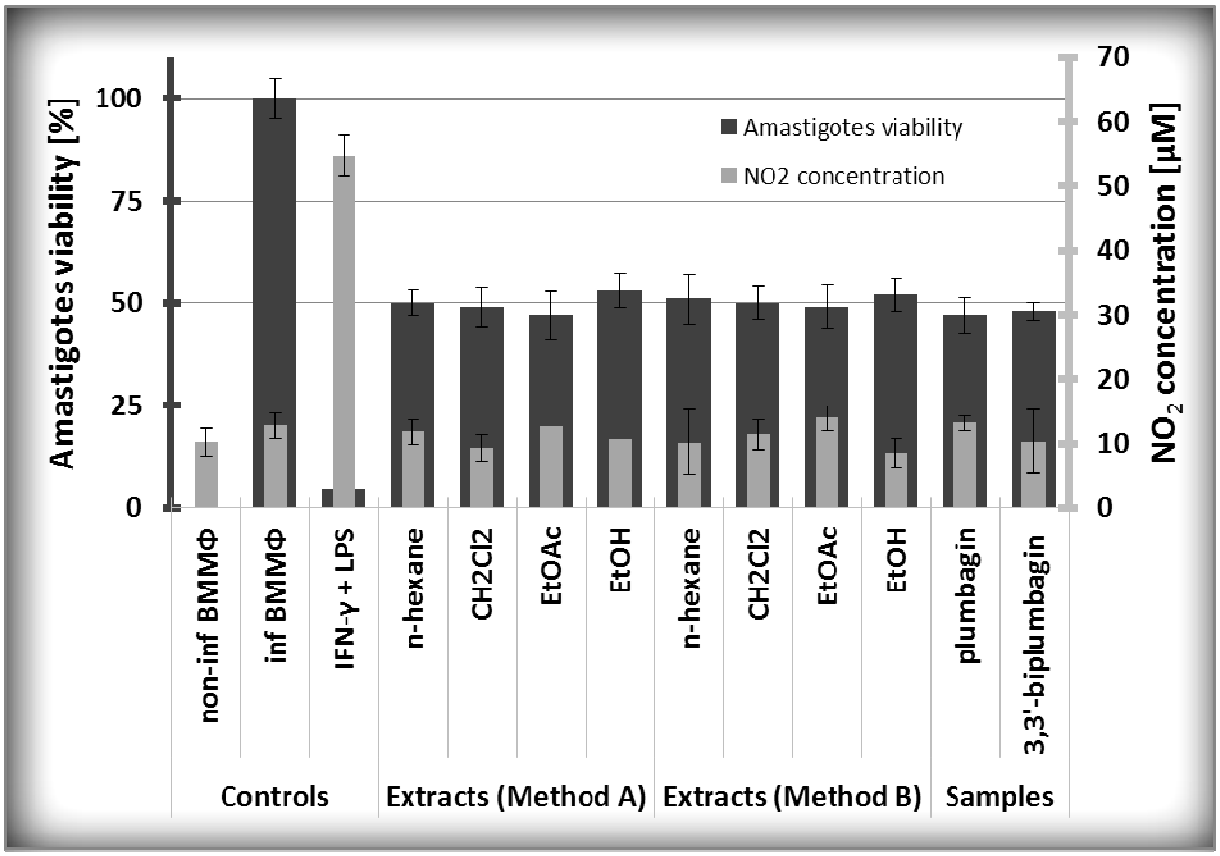


Fig. 36: In vitro antileishmanial activity (FACS analyses, ■), and induced nitrite concentrations (Griess assay, □) in the supernatants of non-infected macrophages, non-treated infected macrophages, infected treated with rIFN-γ 10 U/ml+ LPS 1 ng/ml, and infected treated with *D. peltata* extracts, plumbagin, and 3,3'-biplumbagin at their effective concentrations. (Mean values ± standard deviations were calculated from three independent experiments).

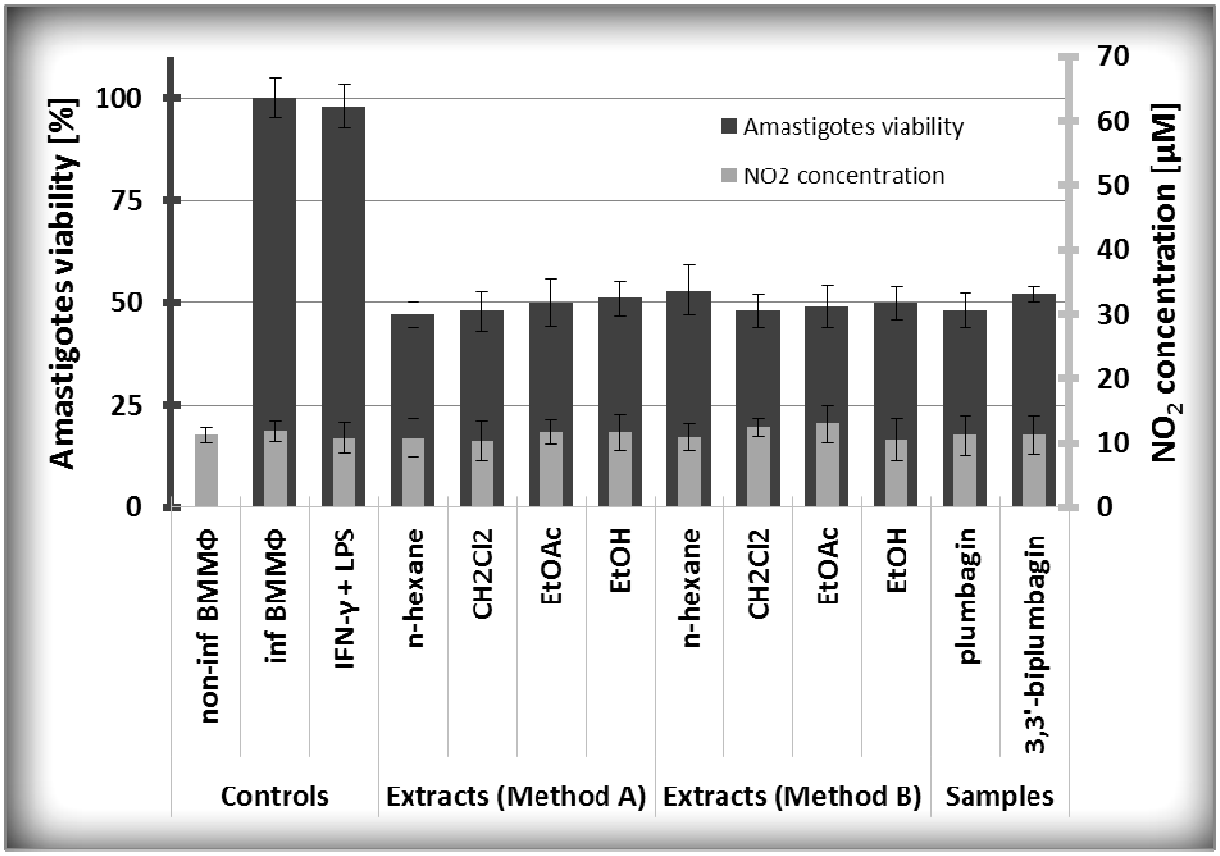


Fig. 37: *In vitro* antileishmanial activity (FACS analyses, ■), and induced nitrite concentrations (Griess assay, ■) in the presence of L-NMMA. (Non-infected macrophages, non-treated infected macrophages, infected treated with rIFN-γ 10 U/ml + LPS 1 ng/ml, and infected treated with *D. peltata* extracts, plumbagin, and 3,3'-biplumbagin at their effective concentrations). (Mean values ± standard deviations were calculated from three independent experiments).

3.3 *Spathodea campanulata*

3.3.1 Phytochemical studies

3.3.1.1 Preliminary TLC analysis of extracts

All extracts of *S. campanulata* were subjected to TLC analysis, using different solvent systems and spray reagents for the detection of constituents belonging to various groups of secondary metabolites.

None of the *S. campanulata* extracts studied showed the presence of naphthoquinones and flavonoids on TLC plates.

3.3.1.1.1 Detection of terpenes, glycosphingolipids, and saponins in *S. campanulata* extracts

Three terpenes were detected in the *n*-hexane and dichloromethane extracts of *S. campanulata* on a TLC plate with R_f values of 0.50, 0.35, and 0.32, respectively (mobile phase: toluene-ethyl acetate 5:2). They were detected as red-violet spots after spraying with the antimony (III) chloride reagent and heating the TLC plate at 105 °C for 10 min. Colouration and chromatographic mobility suggested the terpenes, in the order of decreasing R_f values, to be sitosterol, oleanolic acid and ursolic acid, respectively (Fig. 38). As expected, all these terpenes were only present in lipophilic extracts and absent in more polar phases including methanol and ethanol extracts. Conspicuously, the latter extract revealed a red spot after spraying with anisaldehyde-sulphuric acid reagent (R_f value of 0.17; mobile phase: toluene-ethyl acetate 5:2) corresponding to spathoside (Mbosso *et al*., 2008) (Fig. 39). In addition, two saponins were detected on TLC plates present in ethanol and methanol extracts using ethyl acetate-formic acid-water (18:1:1) as mobile phase (R_f values of 0.65 and 0.1).

The ethanol extract was fractionated into *n*-hexane, dichloromethane, ethyl acetate, and methanol fractions as described in Section 3.3.1.2. TLC analyses indicated that the spathoside spot resided merely in the dichloromethane fraction, while the presence of the two saponins bands was limited to the methanol fraction (Table. 14).

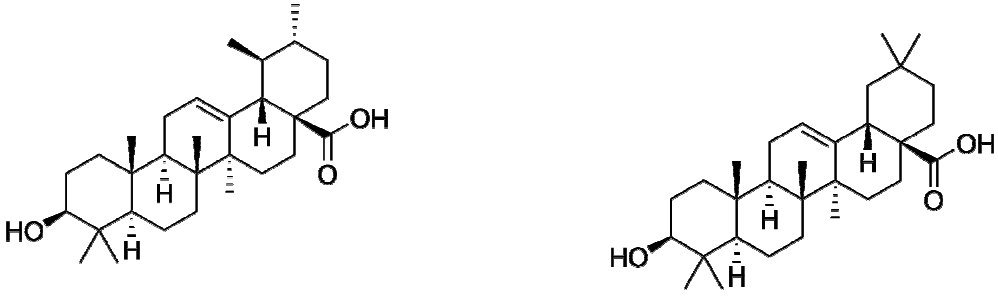


Fig. 38: Chemical structures of ursolic acid (left) and oleanolic acid (right)

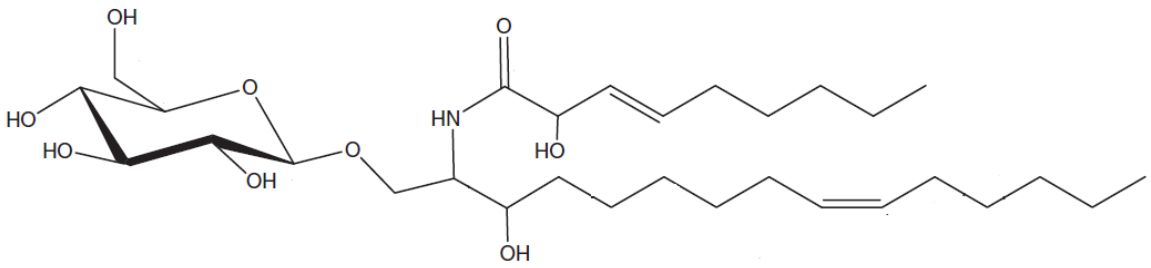


Fig. 39: Chemical structure of spathoside

Table. 14: Results of the preliminary TLC analysis of *S. campanulata* extracts

Class of compounds	Compounds detected	Extracts				EtOH extract			
		n-hexane	CH_2Cl_2	EtOH	MeOH	*n*-hexane phase	CH_2Cl_2 phase	EtOAc phase	MeOH phase
Flavonoids		-	-	-	-	-	-	-	-
Naphthoquinones		-	-	-	-	-	-	-	-
Saponins	unidentified	-	-	+	+	-	-	-	+
	unidentified	-	-	+	+	-	-	-	+
Terpenes	sitosterol	+	+	-	-	-	-	-	-
	oleanolic acid	+	+	-	-	-	-	-	-
	ursolic acid	+	+	-	-	-	-	-	-
Glycosphingolipids	spathoside	-	-	+	-	-	+	-	-

(+) present, (-) absent

3.3.1.2 Fractionation of *S. campanulata* ethanol extract

The ethanol extract of *S. campanulata* was sequentially partitioned into *n*-hexane (2.6 g), dichloromethane (1.8 g), ethyl acetate, and methanol fractions (0.3 g) (see Experimental Section 3.3.1.2).

3.3.2 Biological activity of *S. campanulata* extracts

3.3.2.1 Cytotoxicity of *S. campanulata* extracts against BMMΦ

None of the *S. campanulata* extracts showed selective toxicity when tested against bone marrow-derived macrophages (BMMΦ) (EC_{50} > 143µg/ml) (Table. 15). It is interesting to note that less polar compounds apparently contributed to a weak cytotoxicity of

S. campanulata extracts (EC_{50} of *n*-hexane, dichloromethane and ethanol extracts 140-180 µg/ml), while polar constituents seemed to be by far less cytotoxic as concluded from the EC_{50} of 750 µg/ml of the methanol extract. Following the detected profile of constituents, the toxic potential of the triterpenoids ursolic and oleanolic acids was assessed to gain insight into the cytotoxic principle of the above mentioned fractions against macrophages. Interestingly, ursolic acid showed a moderate cytotoxicity with an EC_{50} of 4.6 µg/ml (10.1 µM), while oleanolic acid did not exhibit any cytotoxic effects on macrophages even at the highest test concentration (EC_{50} > 80 µg/ml, >175 µM). With reference to the results of the TLC analysis (Section 3.3.1.1.1), it is likely that the very weak toxic effects of the *n*-hexane and dichloromethane extracts may be due to the presence of ursolic acid, while spathoside occurring in the ethanol extract may play a role in the harmful effects on macrophages.

3.3.2.2 Antileishmanial activity of *S. campanulata* extracts

All *S. campanulata* extracts showed activity against intracellular *L. major* parasites, with IC_{50} values ranging from 60 µg/ml to 340 µg/ml. Moderate antileishmanial activity resided in the *n*-hexane, dichloromethane and ethanol extracts with IC_{50} values in the range of 60 - 86 µg/ml. In contrast, the methanol extract proved to be rather inactive (IC_{50} of 340 µg/ml), suggesting that occurring polar constituents may not represent an antileishmanial active principle here. Regarding the activity against extracellular *L. major* parasites, only the ethanol extract exhibited a weak antileishmanial activity (IC_{50} of 140 µg/ml), while the remaining fractions were devoid of any activity (IC_{50} >160 µg/ml).

Ursolic acid showed activity against both extra- and intracellular *L. major* parasites, with IC_{50} values of 1.75 µg/ml (3.8 µM) and 2.5 µg/ml (5.5 µM), respectively. Conspicuously, oleanolic acid, structurally closely related to ursolic acid, did not show any antileishmanial activity even at high concentrations (IC_{50} >80 µg/ml, > 175 µM). This finding suggested that ursolic acid, abundantly present in most extracts studied, predominantly contributed to the observed antileishmanial activity of dichloromethane and *n*-hexane extracts. Interestingly, the ethanol extract showed a moderate antileishmanial activity despite the absence of ursolic acid (Table. 14). This observation suggested that spathoside or some unknown compounds may contribute to the antileishmanial activity of the ethanol extract. In order to get more information, the ethanol extract was sequentially partitioned into *n*-hexane, dichloromethane, ethyl acetate, and methanol fractions (Section 3.3.1.2).

Table. 15: *In vitro* antileishmanial activities (*L. major*) and host cell (BMMΦ) cytotoxicity of *S. campanulata* extracts.

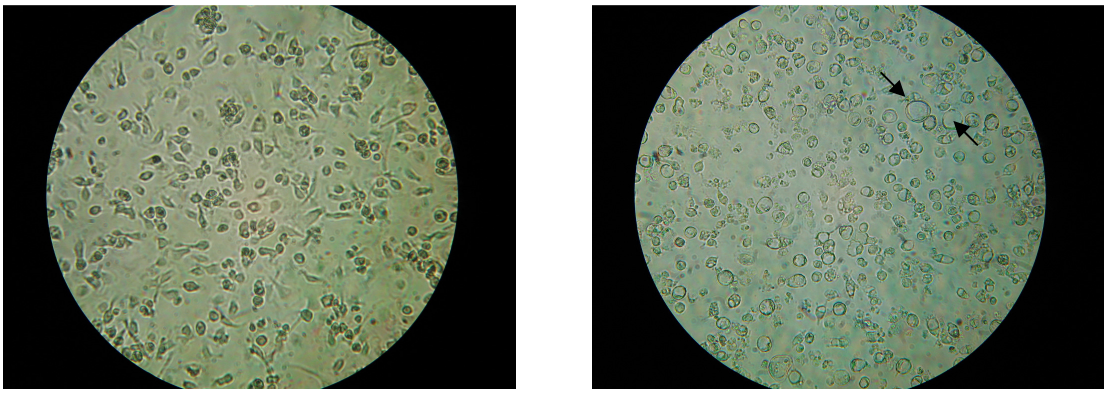
Extract	Antileishmanial activity		BMMΦ	*
	Amastigotes[a]	Promastigotes[a]	cytotoxicity[a]	SI
n-Hexane	60 ± 4.5	> 160	172 ± 9	-
CH_2Cl_2	63 ± 3	140	143 ± 8.2	2.2
EtOH	86 ± 4.2	> 160	182 ± 7.6	2.1
MeOH	340 ± 4.7	> 400	750 ± 11	2.2
Ursolic acid	2.5 ± 0.1 (5.5 µM)	1.75± 0.2 (3.8 µM)	4.6 ± 0.2 (10.1 µM)	1.8
Oleanolic acid	> 80 (> 175 µM)	> 80 (> 175 µM)	> 80 (> 175 µM)	-
Amphotericin B	0.18 (0.2 µM)	2.3 (2.5 µM)	n.d.	-

[a] Values indicate the effective concentration in µg/ml necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * the ratio of EC_{50} to IC_{50}.

3.3.2.3 Cytotoxicity of subfractions of the *S. campanulata* ethanol extract against BMMΦ

The *in vitro* cytotoxic activities of the subfractions of the parent *S. campanulata* ethanol extract are shown in Table. 15. As evident, none of the fractions tested showed selective toxicity against BMMΦ (EC_{50} > 180µg/ml), except for the dichloromethane fraction which exhibited a moderate cytotoxic activities (EC_{50} of 82 µg/ml). It is worth mentioning that spathoside resided in the dichloromethane fraction (Table. 14), indicating that the toxic effects of the dichloromethane fraction may possibly be associated with the presence of this compound.

However, microscopic examination of BMMΦ treated with nontoxic concentrations (80 µg/ml) of the methanol fraction showed changes in the form of cells when compared with non-treated BMMΦ (Fig. 40). It should be noted that evaluation of the cytotoxicity of this fraction using the MTT assay disclosed an EC_{50} value of 180 µg/ml. Most likely, the saponins of the methanol fraction caused the morphological changes without affecting the ability of mitochondrial function of MTT reduction. Independent support of this conjecture was obtained from saponin-free fractions which did not cause any morphological changes of macrophages.



Non-treated BMMΦ **Methanol fraction-treated BMMΦ**

Fig. 40. Microscopic examination of BMMΦ (left), and incubated with the methanol fraction for 48 h (right)

3.3.2.4 Antileishmanial activity of subfractions of *S. campanulata* ethanol extract

The dichloromethane subfraction was the most effective fraction of the parent *S. campanulata* ethanol extract against both forms of *L. major* parasites with IC_{50} value of <42 µg/ml (Table. 16). Since this fraction did not contain ursolic acid, which proved considerably active against *L. major* parasites (Table. 15), it appears reasonable that the antileishmanial activity may be due to spathoside. On the other hand, the remaining fractions (*n*-hexane, ethanol, and methanol fractions), which did not contain spathoside according to TLC analyses, exhibited only a weak antileishmanial activity against both extra- and intracellular *L. major* parasites, with IC_{50} values ranging from 104 µg/ml to 152 µg/ml (Table. 16). This finding supports the conclusion that spathoside has apparently remarkable antileishmanial activity.

Table. 16: *In vitro* antileishmanial activities (*L. major*) and host cell (BMMΦ) cytotoxicity of the subfractions of the *S. campanulata* ethanol extract

Extract	Antileishmanial activity		BMMΦ cytotoxicity[a]	SI*
	Amastigotes[a]	Promastigotes[a]		
n-Hexane phase	152 ± 8.5	134 ± 7	> 450	-
CH_2Cl_2 phase	42 ± 3	39 ± 4	82 ± 4.7	2
EtOAc phase	141 ± 6.2	127 ± 5.5	250 ± 16.6	1.77
MeOH phase	118 ± 5.5	104 ± 7	180 ± 11	1.5
Amphotericin B	0.18 (0.2 µM)	2.3 (2.5 µM)	n.d.	-

[a] **Values indicate the effective concentration in µg/ml necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * the ratio of EC_{50} to IC_{50}.**

3.3.2.5 NO production in infected macrophages

All *S. campanulata* extracts and investigated subfractions induced the release of NO at relatively low levels (< 12 µM). Conspicuously, similar antileishmanial activities were noted whether incubated alone or in combination with L-NMMA (data not shown). The same picture emerged from the data of ursolic acid, revealing a weak NO-inducing potential (11 µM) and similar parasite killing rates in the presence and absence of the iNOS inhibitor. These findings indicate that the *S. campanulata* extracts did not stimulate the infected macrophages to release NO and that this plant source is less suitable for finding new antileishmanial agents.

3.4 *Tabebuia avellanedae*

3.4.1 Phytochemical studies

3.4.1.1 Preliminary TLC analysis of extracts

3.4.1.1.1 Detection of naphthoquinones in *T. avellanedae* extracts

Two naphthoquinones were found in the parent methanol extract of *T. avellanedae***.** The extract was subjected to TLC analysis and chromatographed using petroleum ether-ethyl acetate (9:1). The naphthoquinones were located as yellow to orange spots in the day light and under UV at 365 nm, while spraying with ethanolic KOH produced typically red spots at R_f values of 0.2 and 0.12 (Fig. 41). A series of naphthoquinones including plumbagin, lapachol, and juglone was used for comparison. Lapachol, a major representative of this class of compounds has been reported to occur in the stem bark of *T. avellanedae* (Paterno *et al*., 1882), was detected with an R_f value of 0.2 by co-chromatography. Following fractionation of the methanol extract into different subfractions (Table. 17), lapachol was exclusively present in the *n*-hexane fraction and another naphthoquinone (isomeric mixture of **20** and **21**) was found in the dichloromethane fraction with R_f value of 0.12 (Fig. 41). In contrast, the polar fractions (ethyl acetate, *n*-butanol) did not contain any naphthoquinones.

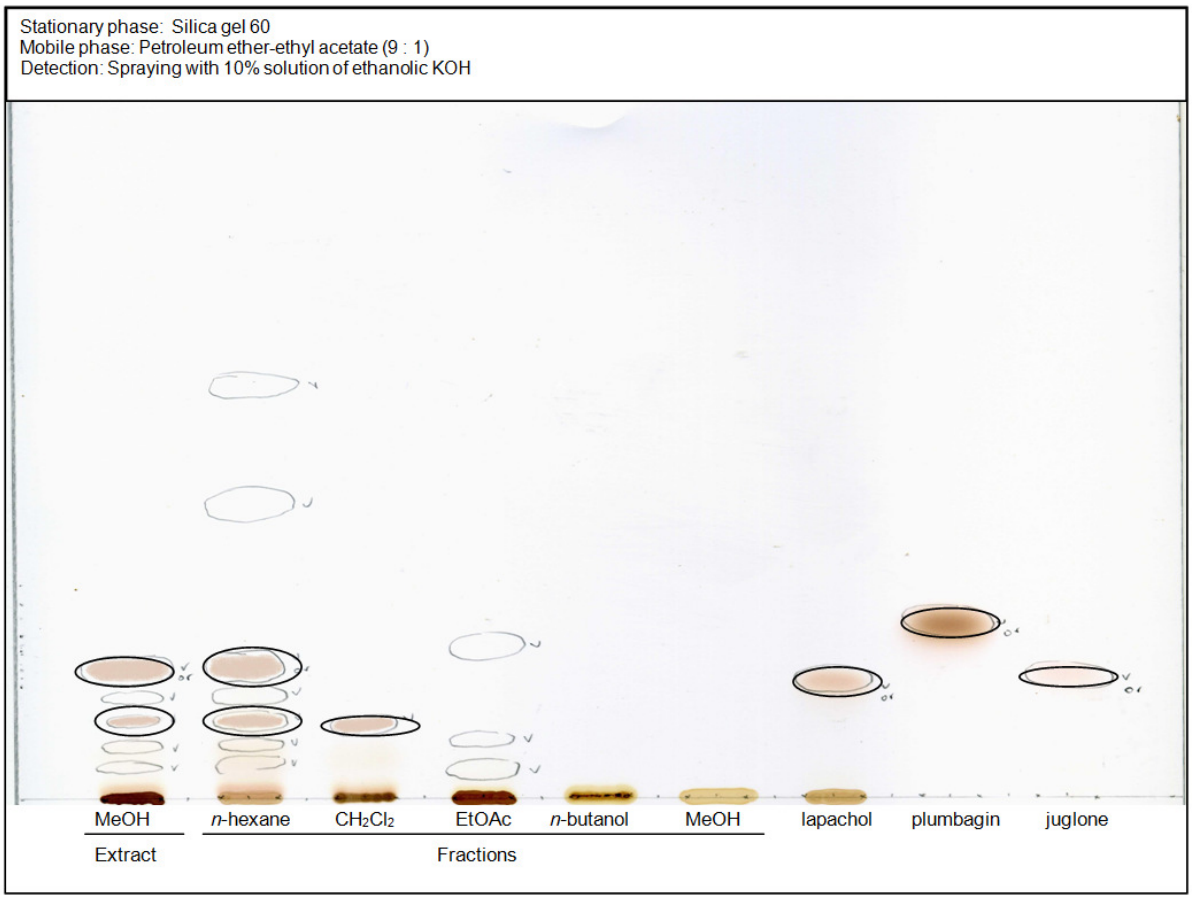


Fig. 41: Detection of naphthoquinones in the parent methanol extract of *T. avellanedae* and subfractions

3.4.1.1.2 Detection of terpenes and iridoides

TLC analysis of the methanol extract of *T. avellanedae* revealed the presence of four terpenes and four iridoides (chloroform-methanol, 4:1). The terpenes were detected with vanillin sulfuric acid reagent as violet spots with R_f values of 0.37, 0.6, 0.68, and 0.82, respectively. The iridoides were located on TLC plates at R_f values of 0.3, 0.57, 0.62, and 0.74, respectively, as blue fluorescence spots under UV at 365 nm and after spraying with vanillin sulfuric acid reagent turned into yellow-brown spots in the daylight. Fractionation of the methanol extract revealed differences in the distribution pattern of terpenes and iridoides among the fractions. While the *n*-hexane fraction retained all the terpenes, only the iridoid with R_f value of 0.74 remained. In contrast, all iridoides were found in the dichloromethane fraction but only one terpene with R_f value of 0.37 was detected. On the other hand, more polar fractions contained all iridoides and were devoid of the presence of terpenes as shown for the ethyl acetate fraction, while highly polar fractions including *n*-butanol and water phases were free of terpenes and iridoides (Table. 17).

3.4.1.1.3 Detection of flavonoids and flavonoid glycosides

Flavonoid aglycones were apparently absent from the parent methanol extract of *T. avellanedae*. However, the methanol extract and the ethyl acetate fraction revealed the presence of two yellow to orange spots in the day light and under UV at 365 nm with low mobility, indicative of flavonoid glycosides. These spots showed similar chromatographic behaviour as hyperoside and isoquercitrin on a TLC plate with R_f values of 0.25 and 0.32, respectively (mobile phase: ethyl acetate-formic acid-water, 18:1:1) (Fig. 42) (Table. 17).

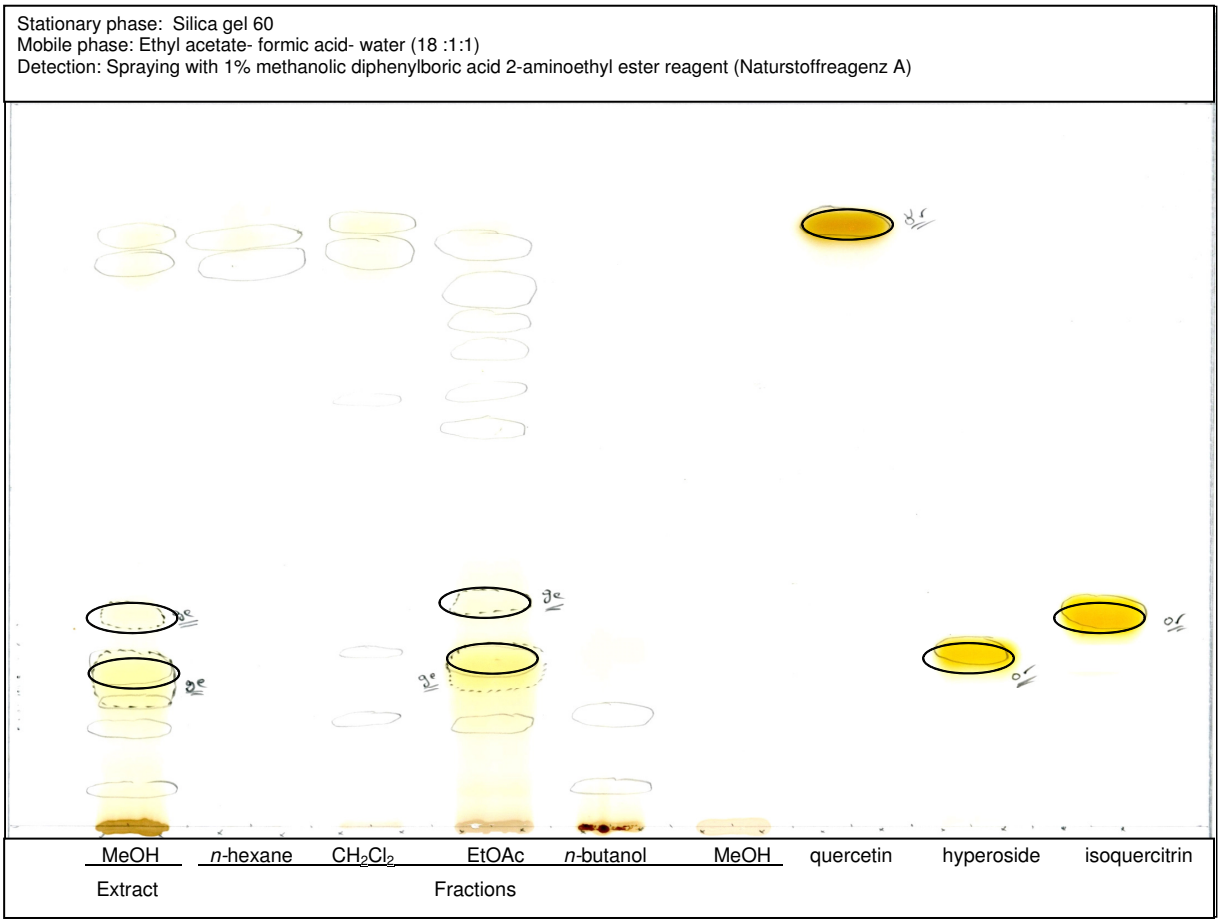


Fig. 42: Detection of flavonoids and flavonoid glycosides in *T. avellanedae* methanol extract and subfractions

Table. 17: Results of the preliminary TLC analysis of *T. avellanedae* methanol extract and subfractions

Class of compounds	Compounds detected	MeOH extract	Fractions of MeOH extract				
			n-hexane phase	CH_2Cl_2 phase	EtOAc phase	*n*-butanol phase	MeOH phase
Naphthoquinones	lapachol	+	+	-	-	-	-
	naphthoquinone	+	+	+	-	-	-
Flavonoids	hyperoside	+	-	-	+	-	-
	isoquercitrin	+	-	-	+	-	-
Terpenes	unidentified	+	+	-	-	-	-
	unidentified	+	+	-	-	-	-
	unidentified	+	+	-	-	-	-
	unidentified	+	+	+	-	-	-
Iridoides	unidentified	+	+	+	+	-	-
	unidentified	+	-	+	+	-	-
	unidentified	+	-	+	+	-	-
	unidentified	+	-	+	+	-	-

(+) present, (-) absent

3.4.1.2 Fractionation of the dichloromethane fraction

The dichloromethane phase of *T. avellanedae* extract was the most important extract in terms of naphthoquinones. Therefore a portion (4 g) was further fractionated using column chromatography in order to achieve isolation and purification of naphthoquinones. A dichloromethane-ethyl acetate gradient system (1:0 → 0:1) was used as a mobile phase. Fractions (each 50 ml) were collected and grouped according to their TLC profiles to yield 20 fractions (Table. 18).

Fraction 5 (1650-1850 ml; CH_2Cl_2 - EtOAc, 95:5) which exhibited the highest antileishmanial activity (Fig. 41) was further purified on silica gel using petroleum ether - ethyl acetate gradient system (1:0 → 0:1). Test tubes (each 5 ml) were collected, and 6 subfractions were obtained according to their behaviour on TLC plates (Table. 19).

Table. 18: Fractionation of the dichloromethane fraction using column chromatography

Fraction	Solvent system CH_2Cl_2 - EtOAc/ v:v	Elution volume	Yield (mg)	TLC screening
1	100 : 0	1-14 (0 – 700 ml)	126	Fatty acids- terpenes
2	100 : 0	15-23 (700- 1150 ml)	61	Terpenes
3	95 : 5	23-26 (1150- 1300 ml)	32	Fatty acids- terpenes
4	95 : 5	27-33 (1300- 1650 ml)	13	Quinoid - terpenes
5	95 : 5	34-37 (1650-1850 ml)	120	Quinoid - terpenes
6	90 : 10	38-41 (1850-2050 ml)	19	Quinoid - terpenes
7	90 : 10	42-55 (2050- 2750 ml)	144	Quinoid - terpenes
8	80 : 20	56-61 (2750- 3050 ml)	53	Iridoid
9	80 : 20	62-65 (3050- 3250 ml)	121	Quinoid
10	80 : 20	66-72 (3250- 3600 ml)	256	
11	70 : 30	73-79 (3600- 3950 ml)	169	Iridoid
12	70 : 30	80-89 (3950-4450 ml)	126	
13	60 : 40	90-100 (4450-5000 ml)	312	
14	50 :50	101-125 (5000-6250ml)	194	
15	30 : 70	126-137 (6250-6850ml)	243	Terpenes
16	30 : 70	138-143 (6850-7150ml)	124	
17	20 : 80	144-148 (7150-7400 ml)	316	
18	20 : 80	149-156 (7400- 7800 ml)	458	Iridoid
19	20 : 80	157-165 (7800- 8250 ml)	289	
20	0 : 100	166-180 (8250- 9000 ml)	460	

Table. 19: Fractionation of the fraction 5 of the dichloromethane phase using column chromatography

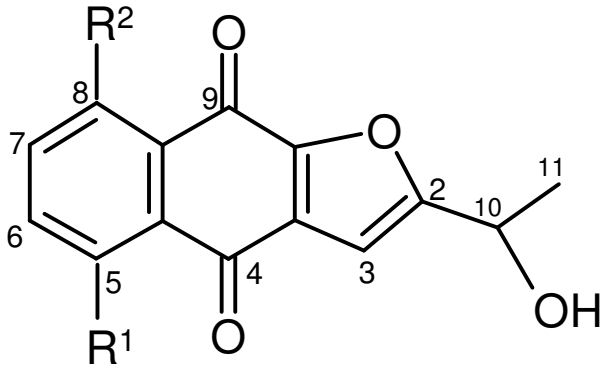
Fraction	Solvent system petroleum ether - EtOAc/ v:v	Elution volume	Yield (mg)	TLC screening
1	100 : 0	1-70 (0 – 350 ml)	22	Terpene
2	99 : 1	71-100 (350- 500 ml)	19	Terpene
3	98 : 2	100-320 (500- 1600 ml)	12	
4	92 : 8	321-340 (1600- 1700 ml)	32	Quinoid - terpene
5	50 : 50	341-400 (1700-2000 ml)	13	Quinoid - terpenes
6	0 : 100	401-500 (2000-2500 ml)	19	

3.4.1.2.1 Isolation and purification of isomeric naphtho[2,3-b]furan-4,9-diones

A distinct yellow spot in the subfraction 4 (1600-1700 ml; petroleum ether- ethyl acetate, 92:8) with orange colouration under UV at 365 nm and quenching under UV-254 nm was detected on TLC plates. This substance was isolated by HPLC separations (experimental conditions: RP-18 column, 5 µm; 8 x 250 mm; operating temperature, 40 °C; water-methanol gradient 1:0 → 0:1, 40 min; flow rate 3 ml/min; detection at 254 nm; retention time R_t: 32 min).

Structural assessment of this naphthoquinone was effected by analyses of MS and ^{1}H-NMR spectroscopic data. It was readily identified as a mixture of isomeric 5- (**20**) and 8-hydroxy naphtho[2,3-b]furan-4,9-dion (**21**) by comparison of the physical and spectroscopic properties with those reported in the literature (Wagner *et al*., 1989) (Fig. 43). The mixture of was obtained as an amorphous solid and its molecular formula, $C_{14}H_{10}O_5$, was concluded from EI-MS data: {$[M]^{+\bullet}$ at *m/z* 258; $[M\text{-}Me]^+$, *m/z* 243; $[M\text{-}Me\text{-}CO]^+$, *m/z* 216; [M-Me-CO], *m/z* 215;

[M-Me-CO-CO]$^+$ *m/z* 187; [M-Me-CO-CO-CO]$^+$ *m/z* 159; and $[C_7H_5O_2]^+$, *m/z* 121)} (Fig. 44).



(**20**) R^1= OH, R^2= H
(**21**) R^1= H, R^2= OH

Fig. 43: Chemical structure of 5-hydroxy- (20) and 8-hydroxy-2-(1'-hydroxyethyl)naphtho [2,3-b]furan-4,9-dione (21)

The ^{1}H-NMR spectrum of the isomeric mixture of (**20** & **21**) showed an aromatic ABX system (7.72, 7.6, and 7.27 ppm) and distinct signals at 12.19 and 12.04 ppm, respectively which disappeared on CD_3OD exchange. Accordingly, these low field resonances were readily assignable to chelated OH groups at positions 5 and 8 respectively, consistent with a mixture of isomeric 5-hydroxy (**20**) and 8-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione(**21**) (Fig. 45).

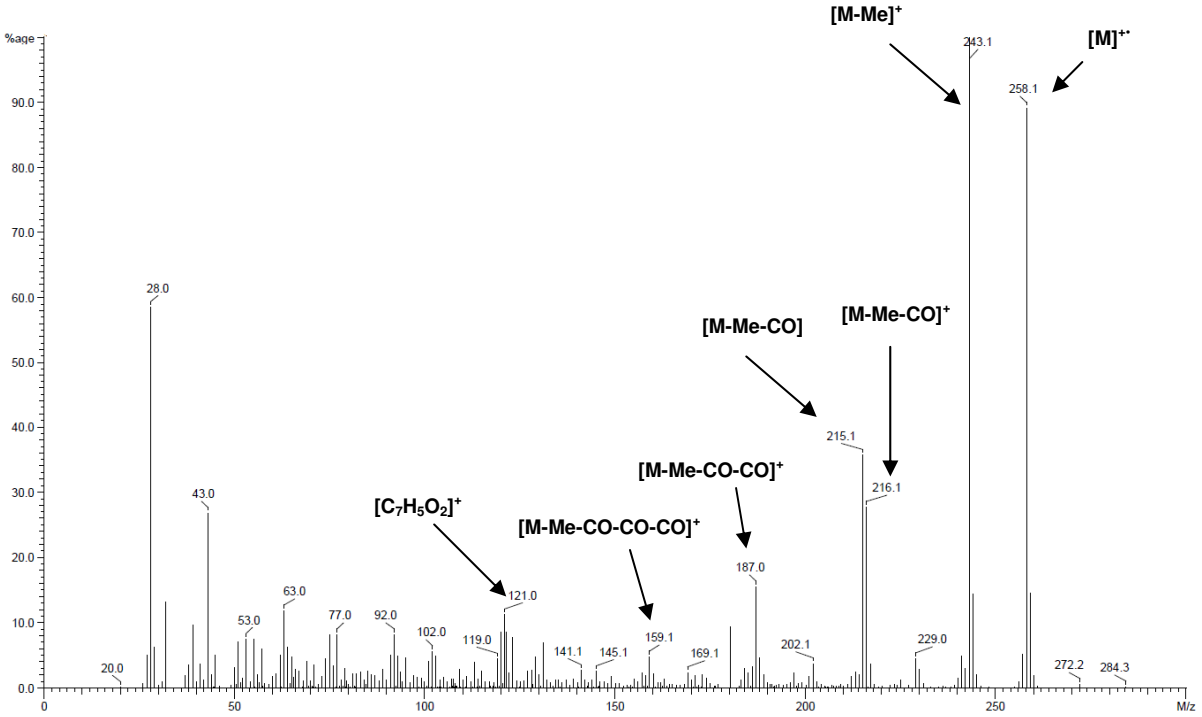


Fig. 44: EI-mass spectrum of isomeric mixture (20 & 21)

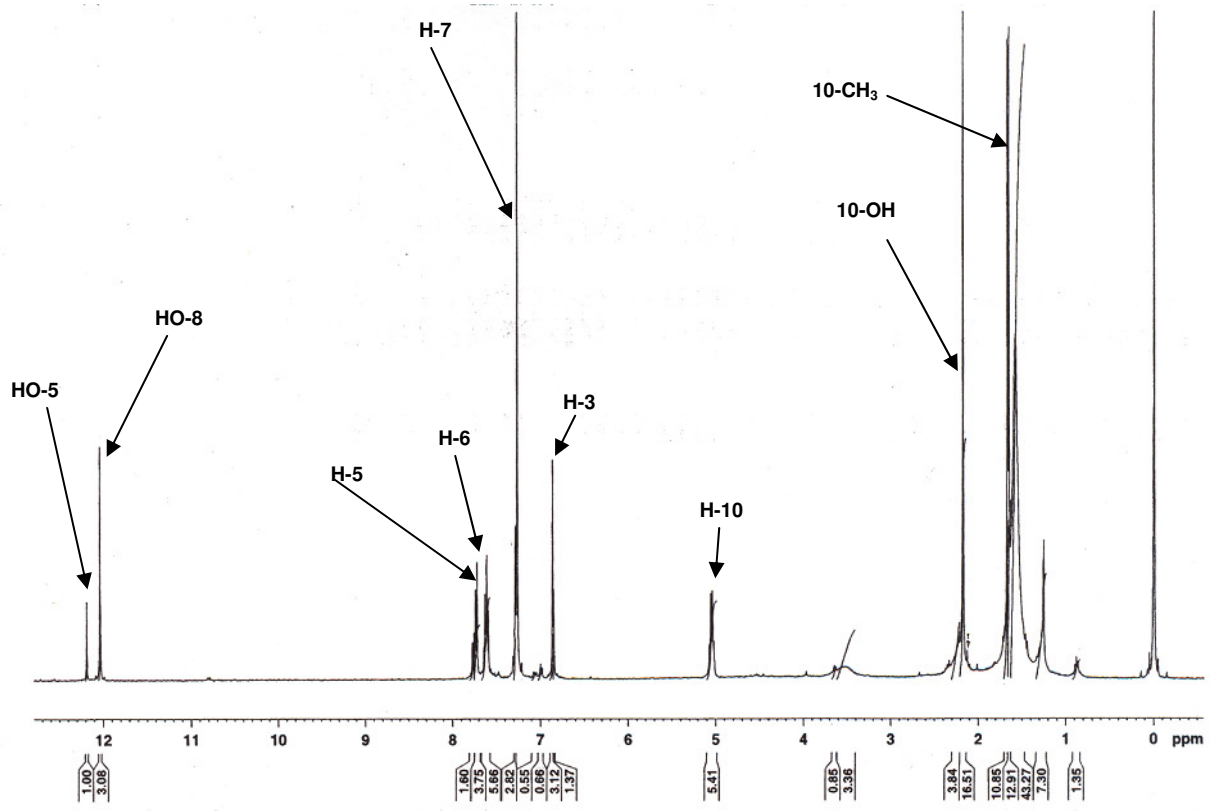


Fig. 45: ^{1}H-NMR spectrum of isomeric mixture (20 & 21)

3.4.2 Biological activity of *T. avellanedae* extract

3.4.2.1 Cytotoxicity of *T. avellanedae* methanol extract

The *in vitro* cytotoxic activities of the parent methanol extract of *T. avellanedae*, its subfractions, and lapachol are shown in Table. 20. As shown, the less polar compounds apparently contributed to the moderate cytotoxicity of *T. avellanedae* methanol extract, as concluded from the EC_{50} values of the *n*-hexane and dichloromethane fractions (117 µg/ml and 65 µg/ml, respectively). More polar constituents including naphthoquinone-free fractions appeared inactive as evidenced by EC_{50} > 400 µg/ml. These results suggested that the cytotoxic activities of *n*-hexane and dichloromethane fractions may be related to naphthoquinones present in these phases. However, lapachol did not show any cytotoxicity on macrophages even at high concentrations (EC_{50} > 72 µg/ml, 300 µM).

3.4.2.2 Antileishmanial activity of *T. avellanedae* methanol extract

T. avellanedae methanol extract showed modest activity against both extra- and intracellular *L. major* parasites, with IC_{50} values of 150 µg/ml and 217 µg/ml, respectively (Table. 20). The highest antileishmanial activity resided in the dichloromethane and *n*-hexane fractions

showing IC_{50} values of 41 µg/ml, and 64 µg/ml, while high polar constituents appeared inactive as concluded from the activity of the remaining fractions with IC_{50} > 350 µg/ml. Since the naphthoquinones resided in the dichloromethane and *n*-hexane fractions (Fig. 41), it was suggested that they are responsible for the observed antileishmanial activity. Lapachol exhibited prominent antileishmanial activity against promastigotes (IC_{50} value of 8 µg/ml, 33 µM) and a moderate activity against amastigotes (IC_{50} value of 28 µg/ml, 115 µM). Because the dichloromethane fraction was more effective than the others, despite the absence of lapachol, it appeared useful to refine the fractionation in order to facilitate the isolation of the active antileishmanial agents.

Table. 20: *In vitro* antileishmanial activities (*L. major*) and host cell (BMMΦ) cytotoxicity of *T. avellanedae* methanol extract and its fractions

Extract	Antileishmanial activity		BMMΦ cytotoxicity[a]	SI*
	Amastigotes[a]	Promastigotes[a]		
MeOH extract	217 ± 11	150 ± 7	> 300	-
n-Hexane phase	64 ± 5	13 ± 2	117 ± 5	1.8
CH_2Cl_2 phase	41 ± 4	20 ± 3	65 ± 4	1.6
EtOAc phase	350 ± 17	>450	>400	-
n-butanol phase	>400	>900	>400	-
MeOH phase	>400	>900	>900	-
Lapachol	28 ± 3.7 (115 µM)	8 ± 1.3 (33 µM)	>72 (>300 µM)	-
Amphotericin B	0.18 (0.2 µM)	2.3 (2.5 µM)	n.d.	-

[a] Values indicate the effective concentration in µg/ml necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * the ratio of EC_{50} to IC_{50}.

3.4.2.3 NO production in infected macrophages

As shown for the above-mentioned plant extracts studied the methanol extract of *T. avellanedae* did not stimulate the *Leishmania*-infected macrophages to produce nitric oxide. Furthermore, the extracts exhibited similar antileishmanial activities whether incubated alone or in combination with L-NMMA (data not shown).

3.4.2.4 Antileishmanial activity of the sub-fractions of the dichloromethane phase

The dichloromethane fraction was further fractionated using column chromatography to yield 20 fractions, grouped according to their behaviour on TLC plates (Table. 18). These 20 fractions were tested against intracellular *L. major* parasites at concentrations of 20 µg/ml and 30 µg/ml (Fig. 46). The figure shows that the most active agents resided in fractions 4, 5, 6 and 7. The characteristic yellow to orange colouration in the day light and under UV at 365

nm with quenching under UV-254 nm verified the presence of naphthoquinones in these active fractions. Fraction 5 caused killing of ca. 70% and 90% of intracellular *L. major* parasites at concentrations of 20 µg/ml and 30 µg/ml, respectively. Therefore, this fraction was further fractionated as described in Table. 19.

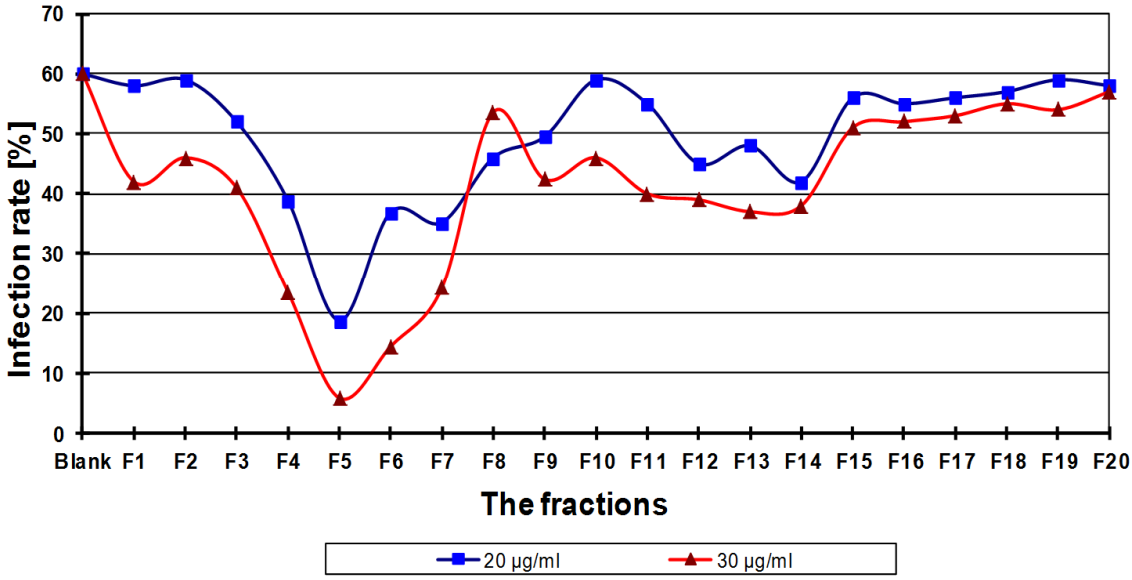


Fig. 46: Antileishmanial activity (FACS analysis) of the 20 fractions of the dichloromethane phase

3.4.2.5 Antileishmanial activity and cytotoxicity of isomeric naphtho[2,3-b]furan-4,9-diones

The isomeric mixture, comprised of 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione(**21**), showed a potent activity against intracellular amastigotes of *L. major* in a dose-dependent manner (Fig. 47). Its IC_{50} value was 1 µg/ml (4 µM), while cytotoxic effects on macrophages were not evident at 2 µg/ml (8 µM). These results indicate that the isomeric naphtho[2,3-b]furan-4,9-diones (**20** & **21**) isolated from *T. avellanedae* are potential antileishmanial compounds.

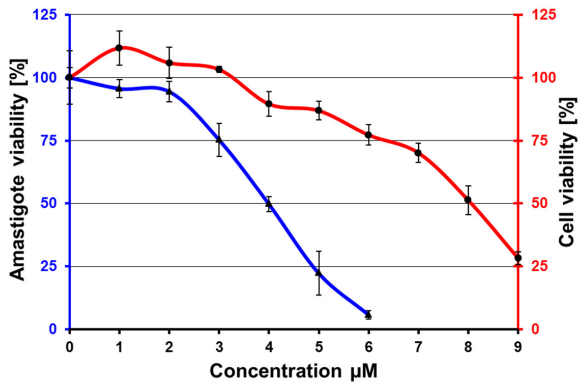


Fig. 47: Antileishmanial activity (FACS analysis) and cell cytotoxicity (FACS analysis) of isomeric mixture (20 & 21)

3.5 *Juglans regia*

3.5.1 Phytochemical studies

3.5.1.1 TLC analysis of extracts

TLC analysis was performed to detect the presence of chemical constituents such as naphthoquinones, flavonoids, terpenes and phenols. Notably, all extracts of *J. regia* under study did not show the presence of any naphthoquinones, even in glycosidic forms

3.5.1.1.1 Detection of flavonoids in *J. regia* extracts

Flavonoids were apparently absent from *J. regia* extracts prepared from peels, whereas quercetin and its 3-O-galactoside, 3-O-arabinoside, 3-O-xyloside, and 3-O-rhamnoside were readily detected in the ethyl acetate and ethanol extracts of leaves. These flavonoids were identified by comparison with reference samples. Furthermore, flavonoids were absent in highly lipophilic extracts of leaves including *n*-hexane and dichloromethane extracts (Table. 21).

3.5.1.1.2 Detection of terpenes and phenols in *J. regia* extracts

Five terpenes were detected in the *n*-hexane and dichloromethane extracts of leaves on TLC plate with R_f values of 0.75, 0.4, 0.33, 0.27, and 0.13 using toluene-ethyl acetate 5:2 as mobile phase. In contrast, the ethyl acetate and ethanol extracts of leaves exhibited only the three spots with low mobility (Table. 21). Regarding extracts of peels, three terpenoids were found to be present in the *n*-hexane and dichloromethane phases with R_f values of 0.65, 0.45, and 0.3. Only the latter was detected in the ethyl acetate and ethanol extracts of peels.

Additionally, four phenols were found in all extracts of leaves with R_f values of 0.63, 0.56, 0.55, and 0.35, while detection of phenolic compounds failed in extracts of peels (Table. 21).

TLC analysis of subfractions of the *n*-hexane extract of leaves revealed that the two terpenes appearing at R_f values of 0.75 and 0.4 resided in subfractions 3 and 4, while subfractions 5-9 showed terpenoid spots at R_f values of 0.33 and 0.27. The remaining terpene was found in fractions 13 and 14. The phenolic spots accumulated in subfractions 7 and 8 (R_f values of 0.63 and 0.56, respectively) in subfractions 9 and 10 (R_f values of 0.55, and 0.35).

Table. 21: Results of the preliminary TLC analysis of *J. regia* extracts

Class of compounds	Compounds detected	Leaves				Peels			
		n-hexane	CH_2Cl_2	EtOAC	EtOH	*n*-hexane	CH_2Cl_2	EtOAC	EtOH
Flavonoids	quercetin	-	-	+	+	-	-	-	-
	quercetin 3-O-galactoside	-	-	+	+	-	-	-	-
	quercetin 3-O-arabinoside	-	-	+	+	-	-	-	-
	quercetin 3-O-xyloside	-	-	+	+	-	-	-	-
	quercetin 3-O-rhamnoside	-	-	+	+	-	-	-	-
Naphthoquinones	juglone	-	-	-	-	-	-	-	-
Phenols	phenol (R_f = 0.63)	+	+	+	+	-	-	-	-
	phenol (R_f = 56)	+	+	+	+	-	-	-	-
	phenol (R_f = 0.5)	+	+	+	+	-	-	-	-
	phenol (R_f = 0.35)	+	+	+	+	-	-	-	-
Terpenes	terpene (R_f = 0.75)	+	+	-	-	-	-	-	-
	terpene (R_f = 0.65)	-	-	-	-	+	+	-	-
	terpene (R_f = 0.45)	-	-	-	-	+	+	-	-
	terpene (R_f = 0.4)	+	+	-	-	-	-	-	-
	terpene (R_f = 0.33)	+	+	+	+	-	-	-	-
	terpene (R_f = 0.3)	-	-	-	-	+	+	+	+
	terpene (R_f = 0.27)	+	+	+	+	-	-	-	-
	terpene (R_f = 0.13)	+	+	+	+	-	-	-	-

(+) present, (-) absent; R_f values were obtained using toluene-ethyl acetate (5:2) as mobile phase

3.5.1.2 Fractionation of the *n*-hexane extract of *J. regia* (leaves)

The *n*-hexane extract of *J. regia* (leaves) was the most important extract in terms of antileishmanial activity and non-cytotoxicity as shown in Table. 23. Consequently, a portion of the *n*-hexane extract (5 g) was further fractionated using column chromatography to gain insight into the compounds possessing antileishmanial activities.

The extract was chromatographed on a silica gel column by eluting with a stepwise gradient of *n*-hexane/ethyl acetate as described in Table. 22. Fractions (each 50 ml) with similar TLC patterns were pooled to afford 14 subfractions which were subjected to phytochemical analysis and biological tests.

Table. 22: Fractionation of *n*-hexane extract of *J. regia* (leaves) using column chromatography

Fraction	Solvent system *n*-Hexane - EtOAc	Elution volume	Yield (mg)	TLC screening
1	100 : 0	1-50 (0 – 2500 ml)		
	90 : 10	51-63 (2500 – 3150 ml)	94	Fatty acids
2	90 : 10	64-70 (3150- 3500 ml)	1870	Fatty acids- terpenes
3	90 : 10	71-78 (3500- 3900 ml)	413	Terpenes
4	90 : 10	79-95 (3900- 4750 ml)	612	Terpenes
5	90 : 10	96-100 (4750-5000 ml)		
	80 : 20	101-104 (5000-5200 ml)	106	Terpenes
6	80 : 20	105-108 (5200-5400 ml)	151	Terpenes
7	80 : 20	109-118 (5400-5900 ml)	308	Terpenes- phenols
8	80 : 20	119-145 (5900-7250 ml)	270	Terpenes- phenols
9	80 : 20	146-160 (7250-8000 ml)		
	50 : 50	161-170 (8000-8500 ml)	820	Terpenes- phenols
10	50 : 50	171-177 (8500-8850 ml)	142	Terpenes- phenols
11	50 : 50	178-183 (8850-9150 ml)	71	-
12	50 : 50	184-207 (9150-10350 ml)	82	-
13	25 : 75	208-217 (10350-10850 ml)	60	Terpenes
14	0 : 100	218-243 (10850-12150 ml)	55	Terpenes

3.5.2 Biological activity of *J. regia* extracts

3.5.2.1 Antileishmanial activity and cytotoxicity of *J. regia* extracts against BMMΦ

Antileishmanial activities of *J. regia* extracts on both extra- and intracellular *Leishmania* parasites were evaluated using MTT assay and parasite retrieval assay, respectively. Their cytotoxic side effects on BMMΦ as host cells were assessed using the MTT assay. Extracts of leaves exhibited prominent activities against both forms of *Leishmania* parasites with IC_{50} values ranging from 19 µg/ml to 44 µg/ml, except for the anti-promastigote activity of the ethanol extract (IC_{50} values of 123 µg/ml). In contrast, all extracts of peels were inactive against both forms of the parasite (IC_{50} > 160 µg/ml) (Table. 23). In terms of cytotoxicity, only the dichloromethane extract of leaves was slightly toxic with an EC_{50} value of 82 µg/ml (SI= 3.7), while all other extracts did not show any cytotoxic effects on macrophages (EC_{50} > 180 µg).

Interestingly, the leaf extracts showed a correlation between the polarity of solvents and antileishmanial activity as evident from IC_{50} values indicating decreased efficiency with increasing polarity. This finding suggests that lipophilic compounds represent the major active principle. Therefore, flavonoids residing in the ethyl acetate and ethanol extracts are considered to play a minor contributing role to the observed antileishmanial effects. Following TLC analysis, terpenes appeared to be putative candidates, while phenolic compounds as polar constituents may be less important (Tables. 21 and 23). Support of this conjecture should be obtained from the fractionation of the *n*-hexane extract.

Tale. 23: *In vitro* antileishmanial activities (*L. major*) and host cell (BMMΦ) cytotoxicity of *J. regia* extracts.

Extract	Antileishmanial activity Amastigotes[a]	Promastigotes[a]	BMMΦ cytotoxicity[a]	SI*
	Parasite retrieval assay	MTT assay	MTT assay	
Leaves				
n-hexane	19 ± 2	20 ± 1.3	> 180	-
CH_2Cl_2	22 ± 2	23 ± 1.7	82 ± 6	3.7
EtOAc	31 ± 1.8	44 ± 2.8	> 180	-
EtOH	42 ± 3	123 ± 6	> 180	-
Peels				
n-hexane	> 180	> 160	> 180	-
CH_2Cl_2	> 180	> 160	> 180	-
EtOAc	> 180	> 160	> 180	-
EtOH	> 180	> 160	> 180	-
Amphotericin B	0.2	2.3	n.d.	-

[a] **Values indicate the effective concentration in µg/ml necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * the ratio of EC_{50} to IC_{50}.**

3.5.2.2 Antileishmanial activity of fractions of *n*-hexane extract (leaves)

The *n*-hexane extract of leaves was fractionated using column chromatography to yield 14 fractions, grouped according to their behaviour on TLC plates (Table. 22). These were tested against intracellular *Leishmania* parasites at concentrations of 5 µg/ml and 10 µg/ml using the parasite retrieval assay. As evident from Fig. 48, the fractions 3–5 and 7–10 were the most effective ones. Fraction 4 caused killing of ca. 60% and 85% of intracellular *L. major* parasites at concentrations of 5 µg/ml and 10 µg/ml, respectively. The highest degree of antileishmanial activity was displayed by fraction 9 with parasite killing rates of ca. 88 % and 97 % at concentrations of 5 µg/ml and 10 µg/ml, respectively. As concluded from TLC analysis (Section 3.5.1.1.2), characteristic compounds were represented by terpenes and phenolic substances which appeared enriched therein.

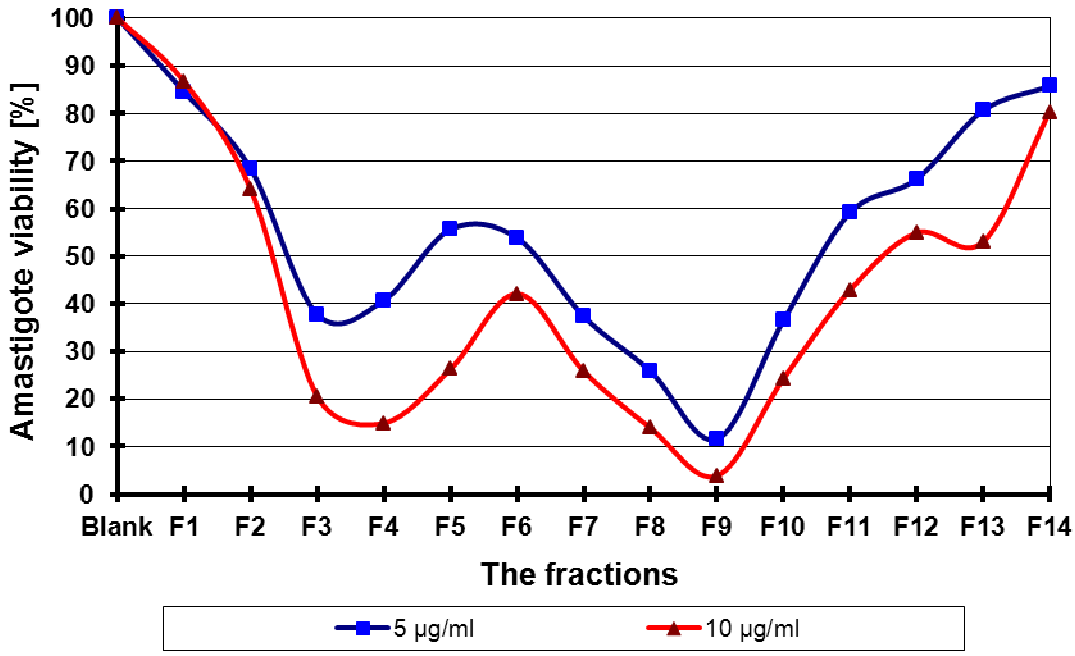


Fig. 48: Antileishmanial activity (parasite retrieval assay) of the 14 subfractions of *n*-hexane extract of *J. regia* leaves

3.5.2.3 NO production in infected macrophages

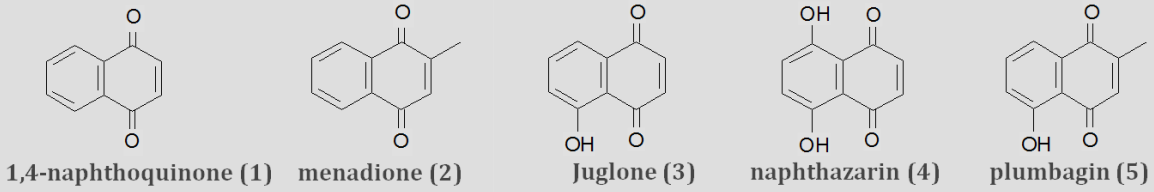
The extracts of *J. regia* leaves were tested alone or in combination with L-NMMA against intracellular amastigotes in the range of their IC_{50} concentrations. All extracts induced the release of NO at relatively low levels (< 14 µM), while exhibiting similar antileishmanial activities in the absence or presence of L-NMMA (data not shown). These findings indicate that the extracts of *J. regia* leaves induced the killing of parasites but did not stimulate the infected macrophages to produce microbicidal nitric oxide.

3.6 Naphthoquinones

Naphthoquinones are one of the groups of secondary metabolites that are widespread in nature. They are commonly present as monomers but also occur as dimers or trimers. The basic chemical structure of monomeric naphthoquinones is based on a bicyclic system – an naphthalene skeleton substituted in position C1 and C4 (1,4-naphthoquinones) or C1 and C2 (1,2-naphthoquinones). Plants containing naphthoquinones have been used in folk medicine for hundreds of years, and many studies have been performed to assess their biological activities including antibiotic, antiviral, anti-inflammatory, antipyretic, antiproliferative and cytotoxic effects (Babula *et al*., 2009). It is interesting to note that naphthoquinones were the most effective components of the plants under study. Therefore, it was useful to assess the cytotoxicity and antileishmanial activity of a series of naphthoquinones including the following groups (Fig 49):

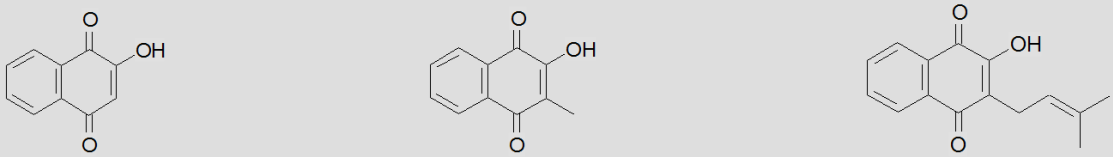
I. Simple naphthoquinones: 1,4-Naphthoquinone (**1**) and menadione (**2**).
II. Naphthoquinones oxygenated on the aromatic ring: Juglone (**3**), naphthazarin (**4**), and plumbagin (**5**).
III. Naphthoquinones oxygenated on the quinoid ring: Lawsone (**6**), 2-hydroxy-3-methyl-1,4-naphthoquinone (**7**), lapachol (**8**), 2-methoxy-1,4-naphthoquinone (**9**), and 2,3-dimethoxy-1,4-naphthoquinone (**10**).
IV. Alkannin/shikonin derivatives: Alkannin (**11**), shikonin (**12**), β,β`dimethylacrylshikonin (**13**), isovalerylshikonin (**14**), acetylshikonin (**15**), and deoxyshikonin (**16**).
V. Dimeric naphthoquinones: Mixture of vaforhizin (**17**) & iso-vaforhizin (**18**), and 3,3`-biplumbagin (**19**).
VI. Furanonaphthoquinones: 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione(**21**).

This diversity in the chemical structure of the range of naphthoquinones to be studied should facilitate to gain insight into structure-activity relationships, and possibly their mode of action.

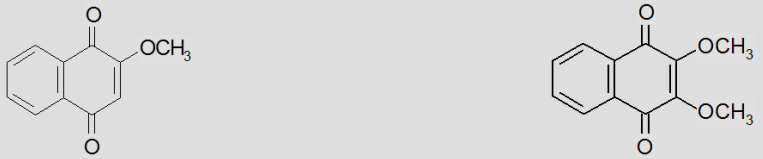


1,4-naphthoquinone (1) menadione (2) Juglone (3) naphthazarin (4) plumbagin (5)

I. Simple naphthoquinones **II. Naphthoquinones oxygenated on the aromatic ring**

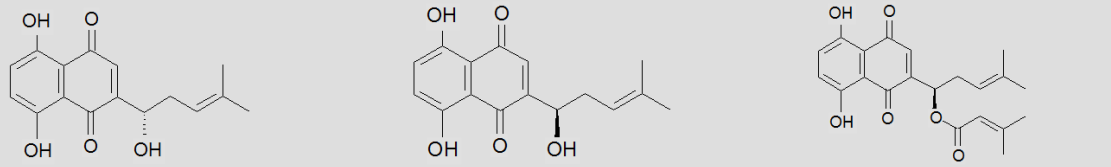


lawsone (6) 2-hydroxy-3-methyl-1,4-naphthoquinone (7) lapachol (8)

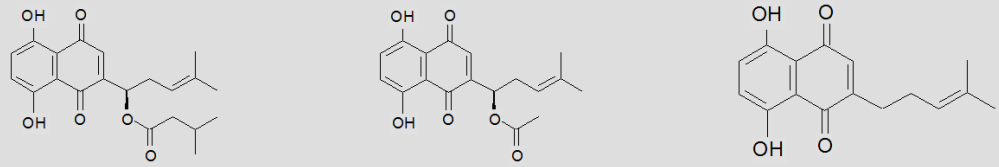


2-methoxy-1,4-naphthoquinone (9) 2,3-dimethoxy-1,4-naphthoquinone (10)

III. Naphthoquinones oxygenated on the quinoid ring

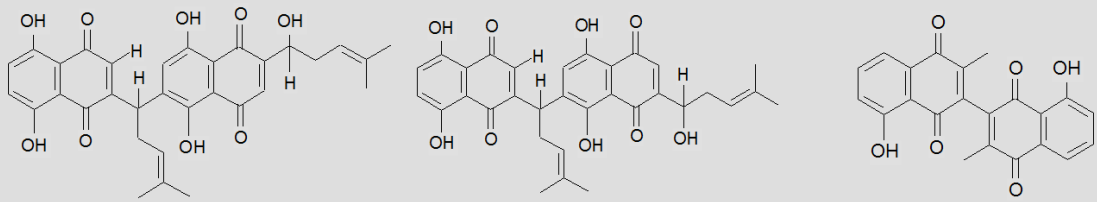


alkannin (11) shikonin (12) β,β`dimethylacrylshikonin (13)



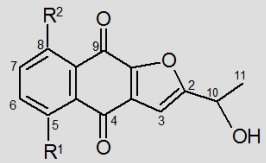
isovalerylshikonin (14) acetylshikonin (15) deoxyshikonin (16)

IV. Alkannin/shikonin derivatives



vaforhizin (17) iso-vaforhizin (18) 3,3`-biplumbagin (19)

V. Dimeric naphthoquinones



(20) R_1= OH, R_2= H: [5-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione]
(21) R_1= H, R_2= OH: [8-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione]

VI. Furanonaphthoquinones

Fig. 49 Chemical structures of naphthoquinones 1-21

3.6.1 Antileishmanial activity and cytotoxicity against BMMΦ

The *in vitro* leishmanicidal activity of the naphthoquinones tested against both extra- and intracellular *L. major* parasites and cell cytotoxicity are shown in Table. 24. With IC_{50} values ranging from 0.5 to 4 µM, 13 out of 21 test compounds were considerably active against promastigotes, when compared with the IC_{50} value 2.5 µM of amphotericin B serving as a positive control. A similar picture emerged from examining their anti-amastigote activity, though the antileishmanial activity was less effective (IC_{50} values of 1.1 to 6.5 µM) when compared with the IC_{50} value 0.2 µM of amphotericin B. On the other hand, the test naphthoquinones revealed a moderate cytotoxicity against BMMΦ as a mammalian host cell control, as evidenced by selectivity indices ranging from 1.1 to >7. In general, antileishmanial activity and cytotoxicity seemed to increase in parallel.

Starting with the simple naphthoquinones, 1,4-naphthoquinone (**1**) showed pronounced effects against promastigotes (IC_{50} of 2 µM), but only relatively low potencies against amastigotes (IC_{50} = 8.5 µM). The antileishmanial activity was associated with toxic effects for BMMΦ, as concluded from the SI of 1.2. Introduction of a methyl group at C-2, as represented by menadione (**2**), slightly increased the antileishmanial activity against amastigotes (IC_{50} of 6.2 µM) with a decrease in cytotoxicity against BMMΦ (SI of 2). The reduced activity of menadione (**2**) against promastigotes (IC_{50} of 10.8 µM) may be indicative of differences in amastigote/promastigote-specific activity.

As for the oxygenated members tested, juglone (**3**) and plumbagin (**5**), having an additional hydroxyl group at C-5 in common, exhibited pronounced effects against both forms of the parasite (IC_{50} values of 6 µM and 2.6 µM against promastigotes and amastigotes, respectively, in each instance). Notably, juglone (**3**) and plumbagin (**5**) were more active and less toxic against BMMΦ (SI values of 2.3, and 3.1, respectively) compared with 1,4-naphthoquinone (**1**) and menadione (**2**), respectively. Naphthazarin (**4**), 8-hydroxylated derivative of juglone (**3**), was the most effective compound in this group against both amastigotes and promastigotes with IC_{50} values of 2.3 µM and 1.2 µM, respectively, and showed similarly weak toxic effects against host cell controls (SI of 3.2).

In contrast, the third group which includes naphthoquinones substituted at the quinone ring exhibited negligible (**6**)-(**8**) or only moderate antileishmanial effects (**9**)-(**10**). Lawsone (**6**) showed only activity against amastigotes with an IC_{50} value of 32 µM and was devoid of any cytotoxicity against macrophages even at high concentrations (EC_{50} > 225 µM). Interestingly, its prenylated analogue lapachol (**8**) was less active against amastigotes (IC_{50} of 115 µM) and exhibited a weak anti-promastigote activity (IC_{50} of 33 µM). On the other hand, replacement

of the prenyl residue by a methyl group at C-3 led to the loss of the antileishmanial activity as evident by 2-hydroxy-3-methyl-1,4-naphthoquinone (**7**) (IC_{50} > 225 µM). Conspicuously, compounds which have a methoxy group at position 2 (**9**) or at C-2 and C-3 (**10**) were effective against amastigotes (IC_{50} of 6.5 µM and 7 µM, respectively) but also relatively toxic against BMMΦ (SI values of 2, and 1.3, respectively).

In the series of alkannin/shikonin derivatives, the principal compounds alkannin (**11**) and shikonin (**12**) exhibited the highest activity against amastigotes with IC_{50} values of 1.3 µM and 1.9 µM, respectively. Comparison of these compounds with the parent analogue naphthazarin (**4**) suggested that the presence of the 1´-hydroxy-3´-isohexenyl substituent at C-3 enhanced the activity. Since esterification of this side chain hydroxyl as reflected in β, β dimethylacrylshikonin (**13**), isovalerylshikonin (**14**), and acetylshikonin (**15**) reduced the antileishmanial activity (IC_{50} values of 2.7 µM, 3 µM, and 5 µM, respectively), the presence of this free hydroxyl group apparently represents a major contributing factor towards antileishmanial activities. Additional support of this surmise was evident from similar decreased antileishmanial activity of deoxyshikonin (**16**) (IC_{50} of 4.1 µM).

For dimers, the mixture of vaforhizin (**17**) and iso-vaforhizin (**18**) showed prominent antileishmanial activities against both extra- and intracellular *L. major* parasites with IC_{50} values of 0.7 µM and 1.1 µM, respectively, while 3,3`-biplumbagin (**19**) which shows a quinoid-quinoid type coupling of two plumbagin entities (**5**) proved to be a weak agent against amastigotes and promastigotes with IC_{50} value of 25.5 µM and 12 µM, respectively.

Finally, a mixture of isomeric 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**) was tested as an example of furanonaphthoquinones. It exhibited pronounced effects against promastigotes and amastigotes (IC_{50} of 4 µM), while toxic effects on BMMΦ were obvious at an increase of at least two-fold in sample quantity (SI of 2).

Table. 24: *In vitro* antileishmanial activity (parasite retrieval assay and MTT assay for amastigotes and promastigotes, respectively) and host cell (BMMΦ) cytotoxicity (MTT assay) of naphthoquinones.

Compound	Antileishmanial activity		BMMΦ	
	Amastigotes[a] IC_{50} (µM)	Promastigotes[a] IC_{50} (µM)	cytotoxicity[a] EC_{50} (µM)	SI*
Simple naphthoquinones				
1,4-naphthoquinone (**1**)	**8.5 ± 0.6**	**2 ± 0.2**	**9.8 ± 0.8**	**1.2**
menadione (**2**)	**6.2 ± 0.8**	**10.8 ± 0.6**	**12.2 ± 1.1**	**2**
Naphthoquinones oxygenated on the aromatic ring				
juglone (**3**)	**6 ± 0.5**	**6 ± 0.9**	**14 ± 1**	**2.3**
naphthazarin (**4**)	**2.3 ± 0.2**	**1.2 ± 0.4**	**7.3 ± 0.7**	**3.2**
plumbagin (**5**)	**2.6 ± 0.2**	**2.6 ± 0.3**	**8 ± 0.9**	**3.1**
Naphthoquinones oxygenated on the quinoid ring				
lawsone (**6**)	**32 ± 4.2**	**>80**	**>225**	**>7**
2-hydroxy-3-methyl-1,4-naphthoquinone (**7**)	**>225**	**>225**	**>225**	**-**
lapachol (**8**)	**115 ± 9**	**33 ± 2.6**	**>300**	**>2.6**
2-methoxy-1,4 naphthoquinone (**9**)	**6.5 ± 0.4**	**12.6 ± 0.9**	**13.1 ± 0.7**	**2**
2,3-dimethoxy-1,4-naphthoquinone (**10**)	**7 ± 0.2**	**>30**	**9.4 ± 0.6**	**1.3**
Alkannin/shikonin derivatives				
alkannin (**11**)	**1.3 ±0.2**	**1.5 ± 0.1**	**4 ± 0.4**	**3.1**
shikonin (**12**)	**1.9 ± 0.1**	**1.3 ± 0.6**	**3.7 ± 0.6**	**1.9**
β,β`dimethylacrylshikonin (**13**)	**2.7 ± 0.4**	**0.5 ± 0.2**	**7.2 ± 0.4**	**2.6**
isovalerylshikonin (**14**)	**3 ± 0.5**	**0.8 ± 0.4**	**9.5 ± 1**	**3.2**
acetylshikonin (**15**)	**5 ± 0.6**	**0.5 ± 0.1**	**11 ± 1.2**	**2.2**
deoxyshikonin (**16**)	**4.1 ± 0.8**	**2.2 ± 0.5**	**7.8 ± 0.8**	**1.9**
Dimeric naphthoquinones				
vaforhizin & iso-vaforhizin (**17** & **18**)	**1.1 ± 0.6**	**0.7 ± 0.2**	**2.4 ± 0.2**	**2.2**
3,3`-biplumbagin (**19**)	**25.5 ± 2.4**	**12 ± 1.2**	**83 ± 4.6**	**3.2**
Furanonaphthoquinones				
isomeric naphtho[2,3-b]furan-4,9-diones (**20** & **21**)	**4 ± 0.5**	**4 ± 0.5**	**8 ± 0.9**	**2**
Control				
amphotericin B	**0.2**	**2.5**	**n.d.**	**-**

[a] **Values indicate the effective concentration in µM necessary to achieve 50% growth inhibition of parasite (IC_{50}) and BMMΦ (EC_{50}); mean values ± standard deviations were calculated from three independent experiments; n.d. = not determined; * Selectivity index (ratio of EC_{50} to IC_{50}).**

3.6.2 Cytotoxicity against parasitized BMMΦ

The selectivity index is the ratio of host cell cytotoxicity (EC_{50}) versus antileishmanial activity (IC_{50}). It is generally derived from independent experiments, e.g., the MTT assay for cell cytotoxicity of sample-treated non-infected cells and the parasite retrieval assay in combination with the MTT assay for the viability of *Leishmania* using infected cells. Having in mind that non-infected and infected BMMΦ may respond to naphthoquinones differently, it seemed useful to also evaluate their cytotoxic effects on infected macrophages using FACS analysis. The discrimination between naphthoquinone-mediated cytotoxic effects on host cells and simultaneous killing of intracellular *L. major* GFP was assessed using PI-positive events (dead host cells) and the parasites' GFP signal as sensitive parameters, respectively. The ratios of cytotoxicity (EC_{50}; dead infected BMMΦ) to antileishmanial activity (IC_{50}) of naphthoquinones are shown in Table. 25. Interestingly, there was a significant difference between selectivity indices resulting from infected and non-infected cells ($p < 0.05$, Student's *t* test), where host cell cytotoxicity was clearly more pronounced by a factor of at least two (Tables. 24 & 25).

3.6.3 Mode of action

3.6.3.1 NO production in infected macrophages

Activated macrophages release many potentially cytotoxic molecules, e.g., nitric oxide and its congeners (NO), which are the most microbicidally effective against *Leishmania* and other parasites. Compared with the stimulus IFN-γ + LPS, all tested naphthoquinones showed similar antileishmanial activities at concentrations of their IC_{50} ranges when incubated with or without L-NMMA (as exemplarily shown for menadione in Fig. 50). Conspicuously, the NO-inducing potential of the tested naphthoquinones was relatively weak (ca. 7 µM) with menadione showing the highest NO-inducing capability (12 µM). This finding suggested that the naphthoquinones did not stimulate the infected macrophages for NO release and that their antileishmanial activity was apparently mediated by a different mode of action.

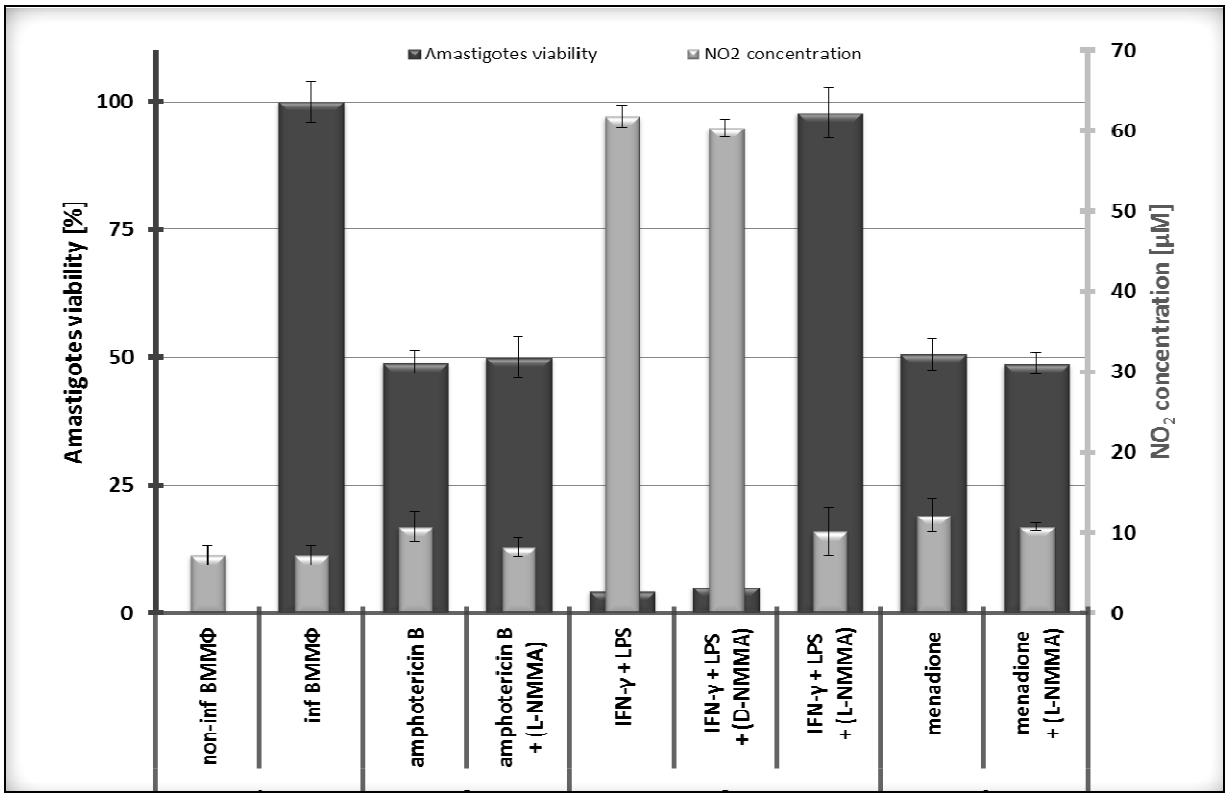


Fig. 50: *In vitro* antileishmanial activity (FACS analyses, ■), and induced nitrite concentrations (Griess assay, ■). (Non-infected macrophages, non-treated infected macrophages, infected treated with amphotericin B, infected treated with amphotericin B plus L-NMMA, infected treated with rIFN-γ 10 U/ml + LPS 1 ng/ml, infected treated with rIFN-γ + LPS plus D-NMMA, infected treated with rIFN-γ + LPS plus L-NMMA, infected treated with menadione, and infected treated with menadione plus L-NMMA). (Mean values ± standard deviations were calculated from three independent experiments).

3.6.3.2 Role of glutathione in the antileishmanial activity of naphthoquinones

Naphthoquinones are potent electrophiles capable of reacting with thiol groups in proteins as well as glutathione through arylation. Glutathione is the most abundant non-protein thiol and a multifunctional intracellular antioxidant in cells. In addition, glutathione is considered to be the major thiol-disulphide redox buffer of the cell, highly abundant in the cytosol, nuclei and mitochondria (Valko *et al.*, 2006). Based on previous findings, arylation of parasitic glutathione by a quinone moiety could well be the underlying mechanism of antileishmanial activity. Therefore, it appeared useful to investigate the role of glutathione in the antileishmanial activity of naphthoquinones for a better understanding of their mode of action.

❖ **Amastigotes**

FACS-analysis exhibited that ca. 80% of macrophages were successfully infected with GFP-transfected *L. major* (GFP signal = 79%) and that only 13% of the host cells were dead (PI signal = 13%) (Fig.51). Exposure of infected cells to the antileishmanial drug amphotericin B decreased the GFP signal by ca. 60% but without any effect on the viability of macrophages (PI signal was at 12% and in the same range of that of just infected

macrophages). Glutathione itself did not show any effects on intracellular parasites (GFP signal = 82%) nor toxic effects on the macrophages (PI signal = 11%).

All tested naphthoquinones at concentrations indicated above exhibited activity against intracellular amastigotes, as concluded from reduced GFP signal intensity. Notably, there was no significant difference between IC_{50} values resulting from the parasite retrieval assay (Table. 24) and FACS analysis (Table. 25) ($p > 0.05$, Student's *t* test). Moreover, about 90% of the parasites were killed at higher test concentrations but that was often associated with high toxicity for macrophages (data not shown).

Interestingly, 3,3`-biplumbagin (**19**), 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**), and oxygenated quinones (**6–10)** were found active when coincubated with or without glutathione, while the remaining naphthoquinones exhibited antileishmanial activity only in the absence of glutathione. The different effects of plumbagin (**5**) and lapachol (**8)** on the viability of *L. major* GFP amastigotes in BMMΦ are exemplarily shown in the absence (Fig. 52A) and presence of glutathione (Fig. 52B).

❖ Promastigotes

Regarding promastigotes, the picture was similar to that of amastigotes. Again, 3,3`-biplumbagin (**19**), 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**), and oxygenated quinones (**6–10)** exhibited antileishmanial activity when coincubated with or without glutathione, while the remaining naphthoquinones tested were only active in the absence of glutathione and did not show any antileishmanial activity or cell cytotoxicity even at the highest concentrations tested when coincubated with glutathione (Table. 25).

These results suggest at least two hypotheses regarding the role of glutathione in the antileishmanial activity of naphthoquinones: the possibility to modify the impact of naphthoquinones and, alternatively, the ability of glutathione to react with naphthoquinones to form inactive glutathione-conjugates via sulfhydryl arylation. Following reports on the formation of naphthoquinone-glutathione conjugates from a chemical viewpoint (Takahashi *et al*., 1987; Inbaraj and Chignell, 2004), similar studies were employed with a focus on their antileishmanial activity. Results are summarized in Table. 25 and Figures 51, 52A, and 52B.

Table. 25: *In vitro* antileishmanial activity (*L. major* GFP) of naphthoquinones (1)–(21) alone and in combination with glutathione as assessed by FACS analysis

Compound	Amastigotes[a] IC_{50} (µM)		Promastigotes[a] IC_{50} (µM)		* SI
	- Glutathione	+ Glutathione	- Glutathione	+ Glutathione	
Simple naphthoquinones					
1,4-naphthoquinone (**1**)	**8.3**	**> 80**	**1.9**	**> 80**	**2.4**
menadione (**2**)	**5.4**	**> 80**	**10.2**	**> 80**	**7.1**
Naphthoquinones oxygenated on the aromatic ring					
juglone (**3**)	**5.3**	**> 80**	**5.6**	**> 80**	**4.5**
naphthazarin (**4**)	**1.9**	**> 80**	**1.6**	**> 80**	**8.8**
plumbagin (**5**)	**2.5**	**> 80**	**2.4**	**> 80**	**>15**
Naphthoquinones oxygenated on the quinoid ring					
lawsone (**6**)	**42.7**	**41.8**	**186**	**176**	**>15**
2-hydroxy-3-methyl-1,4-naphthoquinone (**7**)	**344**	**336**	**714**	**740**	**>15**
lapachol (**8**)	**106**	**107**	**31.2**	**30.5**	**>15**
2-methoxy-1,4 naphthoquinone (**9**)	**6.7**	**6.8**	**12.3**	**11.8**	**>15**
2,3-dimethoxy-1,4-naphthoquinone (**10**)	**5.5**	**5.8**	**67.5**	**> 50**	**4.3**
Alkannin/shikonin derivatives					
alkannin (**11**)	**1.3**	**> 80**	**1.4**	**> 80**	**>15**
shikonin (**12**)	**2.3**	**> 80**	**1.3**	**> 80**	**4**
β,β`dimethylacrylshikonin (**13**)	**2.6**	**> 80**	**0.7**	**> 80**	**12**
isovalerylshikonin (**14**)	**2.5**	**> 80**	**1**	**> 80**	**>15**
acetylshikonin (**15**)	**4.4**	**> 80**	**0.7**	**> 80**	**>15**
deoxyshikonin (**16**)	**4.5**	**37**	**2.5**	**41**	**6**
Dimeric naphthoquinones					
vaforhizin & iso-vaforhizin (**17** & **18**)	**1**	**20**	**0.9**	**22**	**4.6**
3,3`-biplumbagin (**19**)	**25.4**	**25.7**	**14.1**	**13.3**	**>15**
Furanonaphthoquinones					
isomeric naphtho[2,3-b]furan-4,9-diones (**20** & **21**)	**3.5**	**3.7**	**3.5**	**3.7**	**8.9**
Control					
amphotericin B	**0.2**	**0.2**	**2.5**	**2.5**	**-**

[a] **Values indicate the concentration of a compound in µM effective in reducing the GFP signal by 50% and are derived from two independent experiments with similar results; n. d. = not determined. * Ratio of cytotoxicity (EC_{50}; dead infected BMMΦ based on PI staining) to antileishmanial activity (IC_{50}; GFP signal reduction) in the absence of glutathione.**

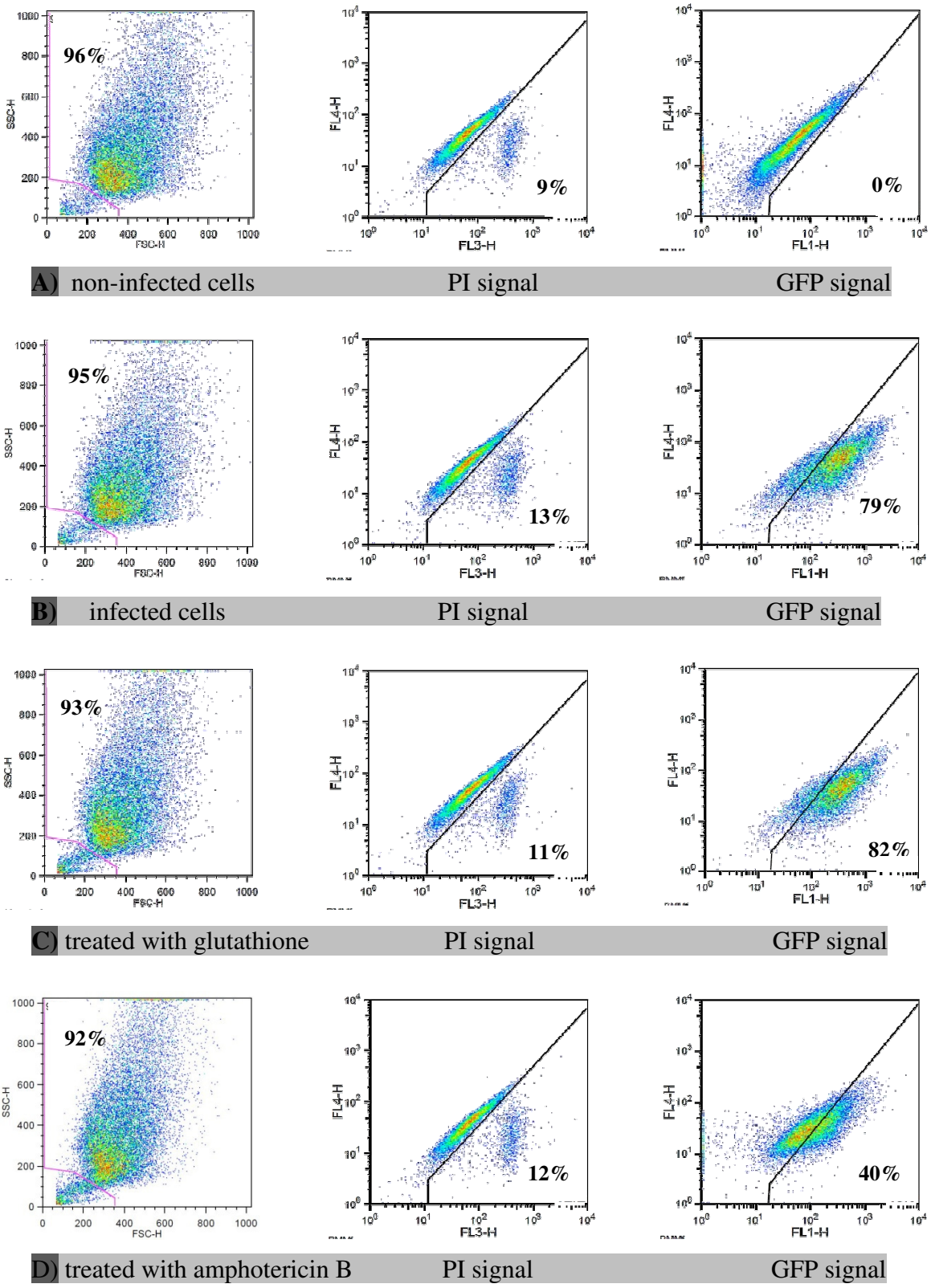


Fig. 51: Forward (FSC) and sideward (SSC) light scatter characteristics analyzed by FACS of BMMΦ infected with *L. major* GFP. Dot plots show all events with PI-positive events (dead host cells) and all living cells with the GFP-positive (viable intracellular *Leishmania* parasites) events gated. A non-infected; B infected cells; C infected cells + glutathione; D infected cells + amphotericin B (0.2 µM). One of three experiments with similar results.

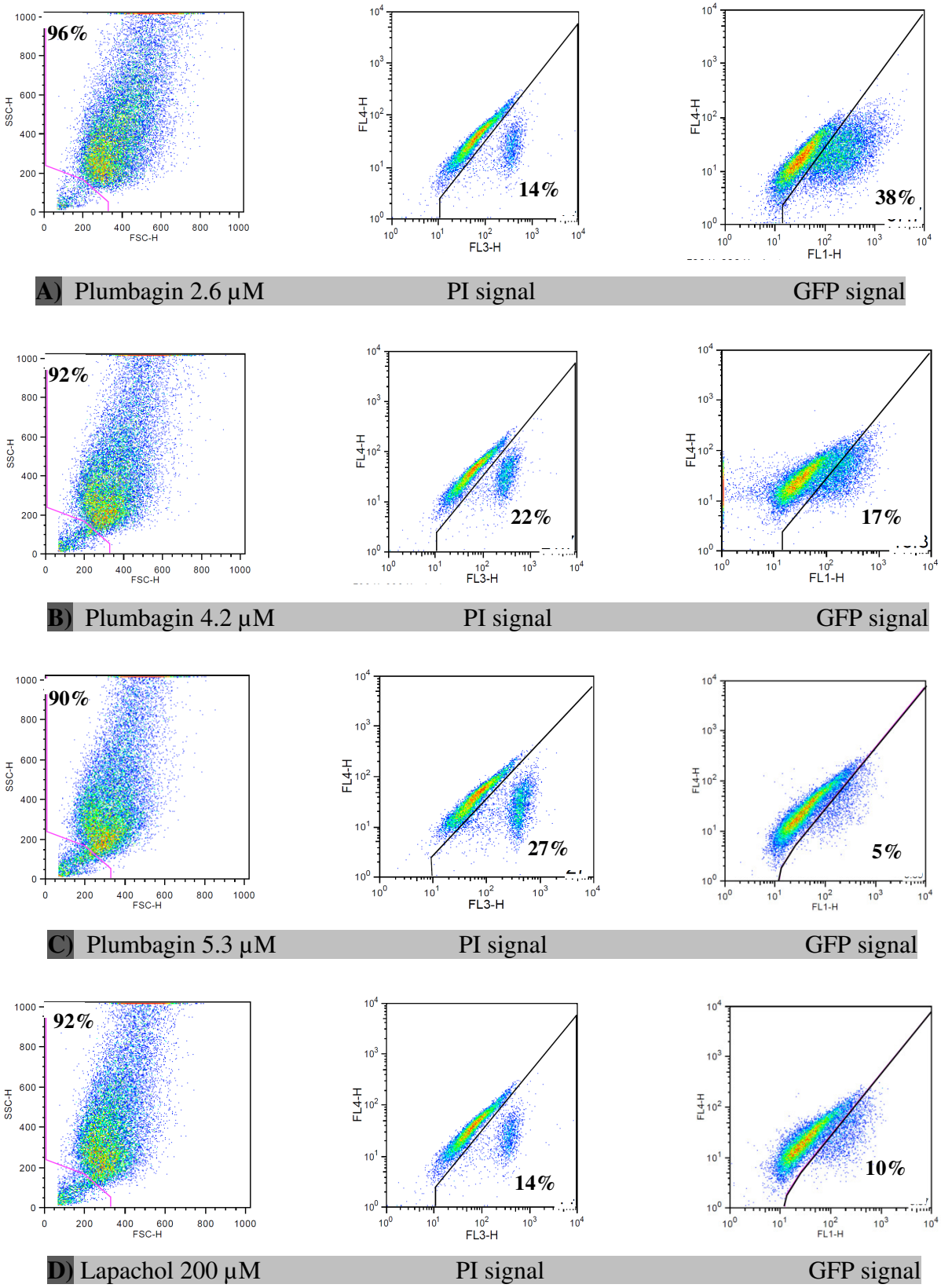


Fig. 52A: Effect of naphthoquinones as exemplarily shown for plumbagin (5) and lapachol (8) on the viability of *L. major* GFP amastigotes in infected BMMΦ as assessed by FACS analysis. Dot plots show all events with PI-positive events (dead host cells) and all living cells with the GFP-positive (viable intracellular *Leishmania* parasites) events gated. A, B, and C infected BMMΦ treated with plumbagin (5) at 2.6 µM, 4.2 µM, and 5.3 µM respectively in the absence of glutathione; D infected BMMΦ treat with lapachol (8) at 200 µM in the absence of glutathione. One of three experiments with similar results.

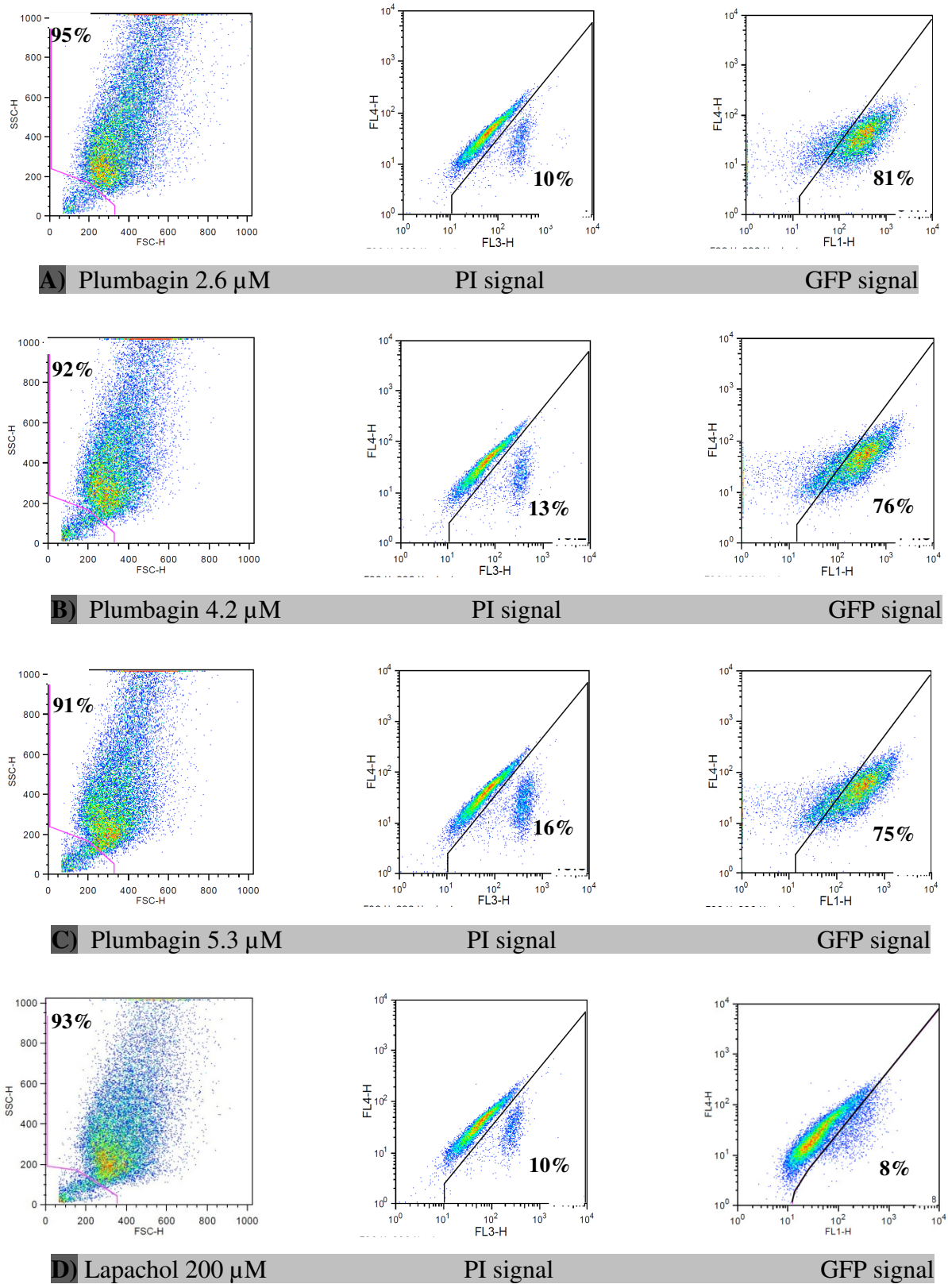
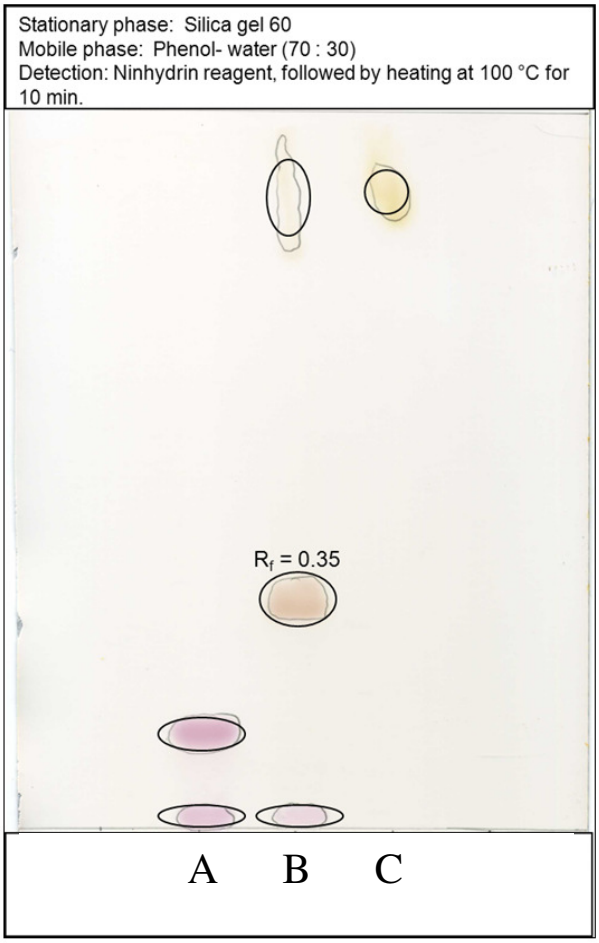


Fig. 52B: Effect of naphthoquinones as exemplarily shown for plumbagin (5) and lapachol (8) on the viability of *L. major* GFP amastigotes in infected BMMΦ as assessed by FACS analysis. Dot plots show all events with PI-positive events (dead host cells) and all living cells with the GFP-positive (viable intracellular *Leishmania* parasites) events gated. A, B, and C infected BMMΦ treated with plumbagin (5) at 2.6 µM, 4.2 µM, and 5.3 µM respectively in the presence of glutathione of glutathione (300 µM); D infected BMMΦ treat with lapachol (8) at 200 µM in the presence of glutathione (300 µM). One of three experiments with similar results.

3.6.3.2.1 TLC monitoring of the reaction of glutathione with naphthoquinones

Naphthoquinones were reacted with reduced glutathione as described in Section 2.3.2. The resultant mixtures were chromatographed on TLC plates using the mobile phase phenol-water (70:30). Glutathione and parent naphthoquinones were co-chromatographed on TLC plates for comparison. After spraying with the ninhydrin reagent and heating at 100 °C for 5-10 minutes, the reduced and oxidized form of glutathione and glutathione-naphthoquinone conjugates showed reddish spots. All naphthoquinones except for those oxygenated on the quinoid ring (**6**–**10**), 3,3`-biplumbagin (**19**), 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**) unable to arylate nucleophiles, produced glutathione-naphthoquinone conjugates as exemplarily shown for plumbagin (Fig. 53).

Taking into account the observed antileishmanial activities of naphthoquinones studied in the presence of glutathione (*vide supra*), it was evident that all naphthoquinones which were found to be inactive formed glutathione-naphthoquinone conjugates.



(A) Glutathione
(B) A mixture of plumbagin and glutathione
(C) Plumbagin

Fig. 53: Detection of glutathione-naphthoquinone conjugates on TLC plate as exemplarily shown for plumbagin (5)

3.6.3.2.2 Isolation of the plumbagin-glutathione conjugate

Plumbagin was one of the naphthoquinones which arylated nucleophiles and yielded plumbagin-glutathione conjugate as indicated above (*vide supra*).

It appeared useful to isolate this product and to evaluate its antileishmanial activity to verify the conjecture of the production of inactive naphthoquinone-glutathione conjugates. For this, the reaction mixture was subjected to HPLC purification using a water-methanol gradient (see Experimental Section 2.3.3) to afford three distinct peaks (glutathione; Rt 3.5 min, plumbagin-glutathione conjugate; Rt 14.6 min, and plumbagin; Rt 27.3 min) (Fig. 54). Appropriate fractions were combined and concentrated under reduced pressure at 40 °C to yield 2 mg of plumbagin-glutathione conjugate.

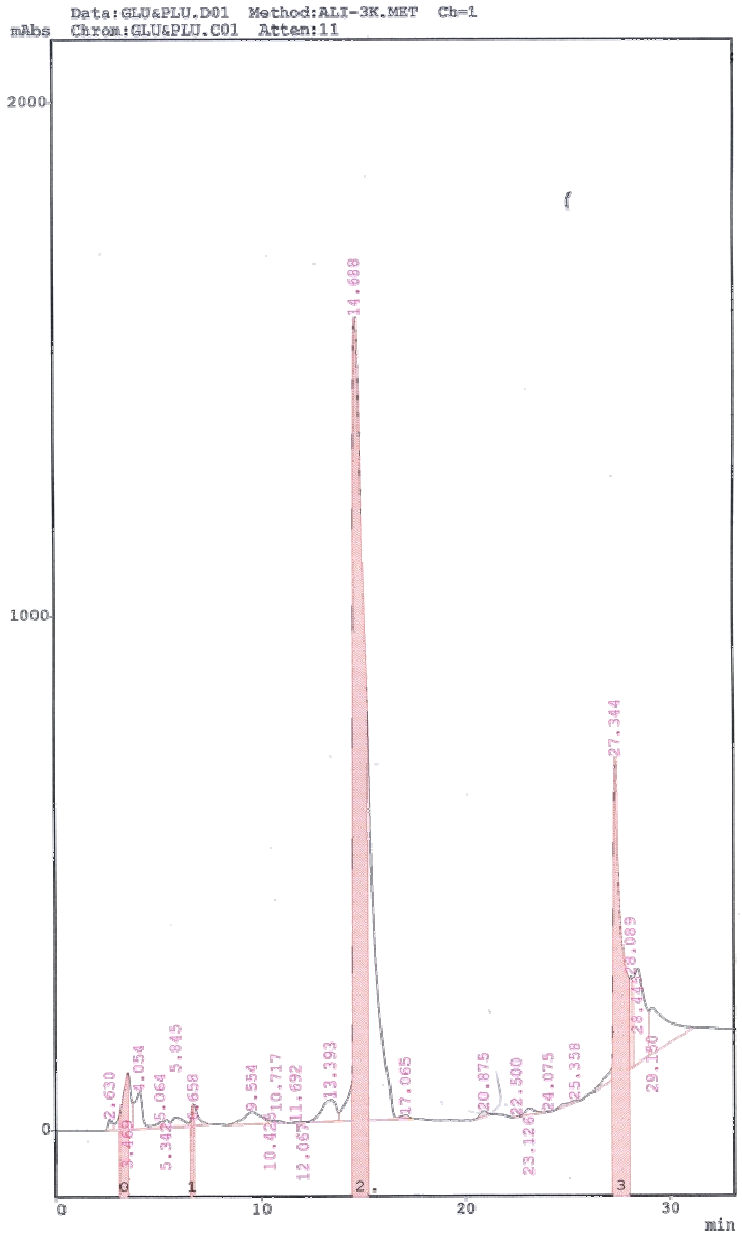


Fig. 54: HPLC purification of plumbagin-glutathione conjugate

3.6.3.2.3 Antileishmanial activity and cytotoxicity of plumbagin-glutathione conjugate

The plumbagin-glutathione conjugate did not exhibit any cytotoxic effects on macrophages even at the highest test concentration (EC_{50} > 80 µg/ml). Also, it was ineffective against both extra- and intracellular *L. major* parasites (IC_{50} > 80 µg/ml) (Fig. 55).

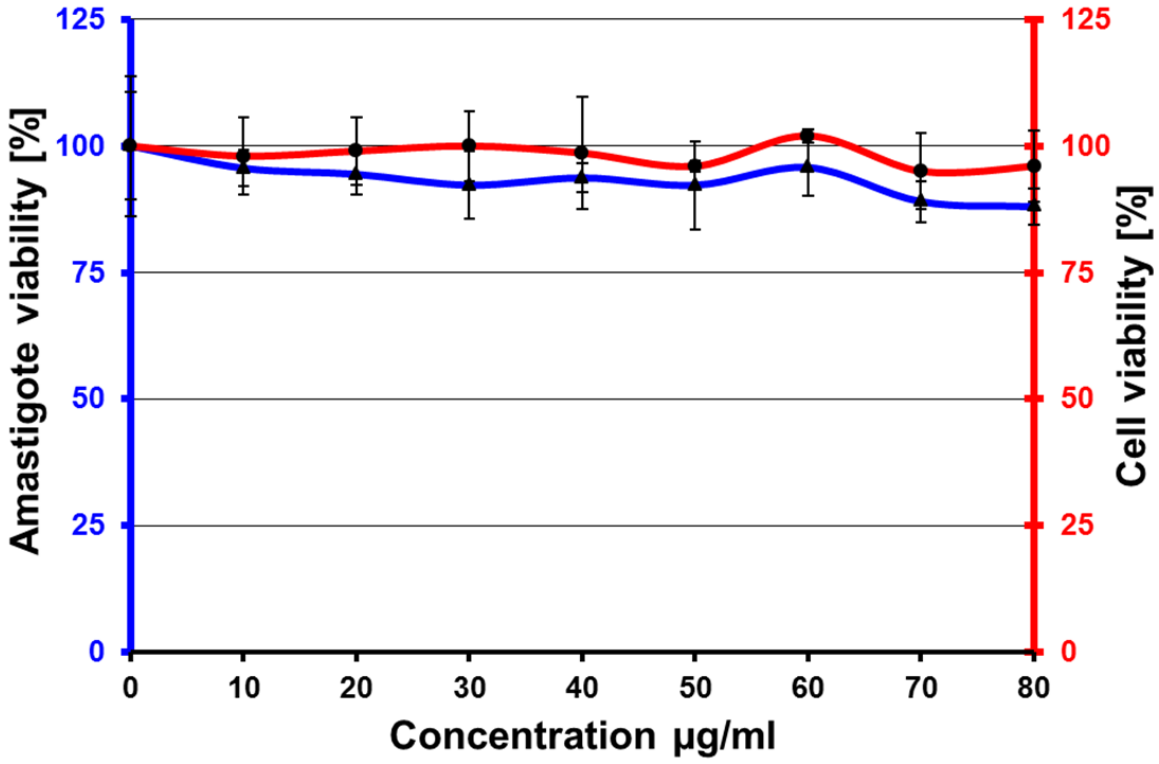


Fig. 55: Antileishmanial activity (parasite retrieval assay) and cell cytotoxicity (MTT assay) of plumbagin-glutathione conjugate

The current results show that distinct naphthoquinones preferably undergo electrophilic addition to produce naphthoquinone-thiol conjugates, which are devoid of any antileishmanial potential. For this reason, it is clear that glutathione plays an important role in their antileishmanial activity and effective concentrations may therefore only be reached by levels exceeding the cellular thiol level. In contrast, the naphthoquinones which were less prone to produce naphthoquinone-thiol conjugates retain antileishmanial activity in the presence of glutathione and they show a different mode of action.

4. DISCUSSION

4.1 *D. madagascariensis*

Droserae Herba has been used in therapy of respiratory tract infections like convulsive cough or whooping cough since the seventeenth century (Paper *et al*., 2005). Mainly *Drosera rotundifolia* L. was the source for this herbal medicine. The imminent extinction of *D. rotundifolia* and other European species due to the restriction of their habitat led to the use of species from Africa or Asia, e.g. *D. madagascariensis* or *D. peltata,* for pharmaceutical purposes (Krenn *et al.*, 1995).

For many years the beneficial effects of *Drosera* spp. have been attributed to naphthoquinones including plumbagin and 7-methyljuglone, but recent studies have shown that the anti-inflammatory and spasmolytic effects may be correlated to the flavonoid content (Melzig *et al.*, 2001; Paper *et al*., 2005). In this work we have investigated the antileishmanial activity and host cell cytotoxicity of *D. madagascariensis.* The extracts studied were prepared in two methods as described in Section 2.3.4 in an attempt to identify the active antileishmanial constituents of this plant source. TLC analysis demonstrated the presence of 7-methyljuglone, while plumbagin was absent (Culham and Gornall, 1994; Zenk *et al*., 1969). Furthermore, the flavonoids hyperoside, isoquercitrin, quercetin and myricetin-3-O-galactoside were detected, consistent with previous reports (Kolodziej *et al*. 2002; Melzig *et al*., 2001).

The results of biological activity studies indicated that the antileishmanial activity of *D. madagascariensis* extracts may mainly be attributed to 7-methyljuglone and quercetin as concluded from the metabolic pool of dichloromethane (7-methyljuglone; flavonoids absent) and ethanol extracts (quercetin; 7-methyljuglone absent) (Tables. 7 and 8). In general, their antileishmanial activity and cytotoxicity against host cells seemed to increase in parallel. However, 7-methyljuglone was reported to possesses a wide range of biological activities including antibacterial (Bapela *et al*., 2006; Mahapatra *et al*., 2007) and antifungal (Marston *et al*., 1984; Wang *et al*., 2011) effects. Moreover, this compound also has been found to be cytotoxic against various cancer cell lines (Mebe *et al*., 1998; Gu JQ *et al*., 2004). It is therefore likely that its activity extends to other protozoan. Interestingly, its chemical analogue juglone was effective against both extra- and intracellular *L. major* parasites at low concentrations with a similar IC_{50} value (1 µg/ml, 6.5 µM). Juglone is a naturally occurring naphthoquinone found in members of the Juglandaceae family. It has been shown to be inhibitory to oral pathogens, notably *Streptococcus mutans*, *Streptococcus sanguis*, *Porphyromonas gingivalis* and *Prevotella intermedia* (Didry *et al*., 1994; Cai *et al*., 2000), as well as other Gram-positive bacterial pathogens such as *Listeria monocytogenes* and

methicillin resistant *Staphylococcus aureus* (MRSA) (Neamatallah *et al*., 2005). Moreover, Clark *et al*. (1990) reported juglone to be as effective as certain commercially available antifungal agents. On the other hand, juglone received broad interest due to carcinogenic/anti-carcinogenic effects (Sugie *et al*., 1998; Segura-Aguilar *et al*., 1992).

However, the mode of antiparasitic action is unknown. Juglone was reported as subversive substrate of trypanothione reductase and lipoamide dehydrogenase from *Trypanosoma cruzi* (Salmon-Chemin *et al*., 2001). The similarity of biological activity of juglone and 7-methyljuglone may suggest similar mechanisms. It is interesting to note that juglone was relatively non-toxic for BMMΦ in this study, as concluded from the SI of 2.3, although there has been some indication of cytotoxicity (Inbaraj and Chignell, 2004).

Flavonoids of *D. madagascariensis* were moderately active, if at all, against *Leishmania* parasites (Table. 8). Quercetin reported to possess a wide range of biological activities including antioxidant, anti-hypertension, anti-inflammatory, antimicrobial and antiprotozoal activities (Bischoff, 2008). It exhibited a moderate activity against *L. major* parasites with a difference in the sensitivity between amastigotes (IC_{50} of 22 µg/ml, 72.8 µM) and promastigotes (IC_{50} of 15 µg/ml, 50 µM). These findings are consistent with those of del Rayo Camacho *et al*. (2002), indicating an anti-promastigote activity against *L. donovani* with IC_{50} of 63.8 µM. Moreover, it has been reported that the oral administration of quercetin reduced the splenic parasite load in animal models by 90% at a concentration of 14 mg/kg body weight (Mittra *et al*., 2000), and that it possessed potent oral efficacy in a murine model of cutaneous leishmaniasis caused by *L. amazonensis* (Muzitano *et al*., 2009). Interestingly, the current study revealed that quercetin showed a relatively good selectivity towards *Leishmania* parasites (SI of 4.3). Quercetin can induce the production of superoxide anion, hydrogen peroxide, and other reactive oxygen species (ROS), although, the precise molecular mechanism of action has not yet been demonstrated. Recently Fonseca-Silva *et al*. (2011) demonstrated that quercetin eventually exerts its antileishmanial effect on *L. amazonensis* promastigotes by the generation of ROS and disrupted parasite mitochondrial function. Furthermore, quercetin has been described as a mixed inhibitor of *L. amazonensis* arginase, which plays a role in the biosynthesis of polyamines and the conversion of arginine into ornithine and urea (da Silva *et al*., 2012). Ornithine is required for the polyamine pathway that is essential for cell proliferation and ROS detoxification by trypanothione. In the same study, isoquercitrin was a non-competitive inhibitor of *L. amazonensis* arginase and can be a possible antileishmanial agent. Contrary to expectations, our study showed that isoquercitrin was generally ineffective against both extra- and intracellular *L. major* parasites and did not

show any activity or cell cytotoxicity even at the highest test concentrations (IC_{50} , EC_{50} > 20 µg/ml, > 43 µM). A possible explanation for this might be the difference in *Leishmania* test organism. Furthermore, differences in cell permeability may also be considered. The higher antileishmanial activity of quercetin compared to glycosides such as isoquercitrin could be due to a better cell permeability.

Similarly, hyperoside co-occurring in ethanolic extracts of *D. madagascariensis* was inactive against both forms of *Leishmania* parasites (IC_{50} > 80 µg/ml, > 172 µM). To date, only a single study has reported an antileishmanial activity of hyperoside that was against *L. amazoniensis* promastigotes with IC_{50} of 3.03 µg/ml (Clavin *et al*., 2010). The contrasting results may be explained by different test organisms and differing methods. The susceptibility of parasites was assayed based on (3H) thymidine uptake, while the MTT assay was used in the current experiments. Further research is needed to place the claimed antileishmanial activity of hyperoside beyond doubt. Notably, hyperoside showed a weak cytotoxicity against host cells with EC_{50} value of 105 µg/ml (226 µM). On the other hand, *D. madagascariensis* and its active constituents juglone and quercetin did not show any significant NO-inducing potential in parasitized macrophages. Moreover, they retained their activity in the presence of the iNOS inhibitor L-NMMA (Fig. 22 and Fig. 23), suggesting an NO independent mode of action.

Taken together, our study showed that the antileishmanial activity of *D. madagascariensis* may be attributed to the naphthoquinone 7-methyljuglone and the flavonoid quercetin, whereas flavonoid glycosides and co-occurring terpenes were inactive.

Given the results of antileishmanial activity of *D. madagascariensis*, it appears reasonable to extend the activity studies to other members of the genus Drosera.

4.2 *Drosera peltata*

D. peltata, a tuberous sundew, has been used for centuries in traditional medicine to treat, i.e. dry coughs, bronchitis, whooping cough and asthma (Juniper *et al*., 1989). Moreover, the medicinal use of *Drosera* spp. extracts has been mentioned in the literature since the seventeenth century as an important antitussive for different respiratory diseases, including tuberculosis (Schnell, 1984). Previous research has noted the importance of the antimicrobial activity of extracts of aerial parts of *D. peltata* against oral bacteria (Didry *et al*., 1998). The current study set out with the aims of assessing antileishmanial activities and host cell cytotoxicity of *D. peltata* extracts, which were prepared as described in Section 2.3.5.1.

Plumbagin (**5**), the major naphthoquinone reported to occur in relatively large amounts in *D. peltata* (Leclercq and Angenot, 1984; Krenn *et al*., 1995), was identified in all extracts using TLC analysis, while two additional naphthoquinones, droserone and 3,3`-biplumbagin, were found in all extracts except for the ethanol extracts (Fig. 24). The different chromatographic behaviour prompted the isolation of the dimeric naphthoquinone, the structure of which being unambiguously elucidated by spectroscopic means. Droserone, an oxygenated derivative of plumbagin, has been reported to occur in *D. peltata* Smith var. lunata collected in Tibet (Wang *et al*., 1998). 3,3`-Biplumbagin has previously been shown to occur in the roots of *Plumbago zeylanica (*Zhong *et al*., 1984), the leaves and rhizomes of *Aristea ecklonii* (Kumar *et al*., 1985) and *Pera benensis* (Fournet *et al*., 1992a). Notably, its occurrence in *D. peltata* was demonstrated here for the first time. Moreover, *cis*-isoshinanolone (**22**), possibly a bio-synthetic precursor of plumbagin, was obtained from *D. peltata* extracts and spectroscopically characterized, while flavonoids were completely absent. These findings are consistent with those of Kolodziej *et al*. (2002) who found that the commercial plant material of *D. peltata*, non-accepted by the commission E, was devoid of flavonoids. The use of two different methods of extraction led to terpene-free and terpene-containing extracts which allowed evaluating the impact of the terpenes on the growth and viability of *Leishmania* parasites. Beta-sitosterol and stigmasterol were previously isolated from *D. peltata* Smith var. lunata (Wang *et al*., 1998) and their presence confirmed by chromatography using authentic samples. Besides, TLC analysis revealed the presence of additional but unidentified terpenes in the studied plant

Trials on finding the best solvent for extraction of plumbagin revealed that ethyl acetate gave the highest recovery rate from *Plumbago zeylanica*, while acetone proved best from *Plumbago rosea* (Kurian and Shankar, 2007). In the current study the highest amount of

plumbagin resided in the dichloromethane and ethyl acetate extracts (5% and 3.5%, respectively), while *n*-hexane and ethanol extracts contained only little quantities of plumbagin (0.6%, and 0.3%, respectively) (Table. 10). This finding indicated that dichloromethane is the best suited solvent for extraction of plumbagin here.

Several studies have reported that plumbagin shows a variety of therapeutic functions such as antimicrobial, antifungal, anticancer (Krishnaswamy and Purushothaman, 1980), antimycobacterial (Kuete *et al*., 2009), antimalarial (Likhitwitayawuid *et al*., 1998), antifertility (Premakumari *et al*., 1977) and antigonatropic (Santhakumari *et al*., 1980). Furthermore, biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis, demonstrated that plumbagin was the most active compound against five strains of *Leishmania* (promastigote), six strains of *Trypanosoma cruzi* (epimastigote) and the intracellular form (amastigote) of *L. amazonensis* (Fournet *et al*., 1992a). In prior study, plumbagin had *in vitro* an IC_{50} values of 0.42 and 1.1 µg/ml against *L. donovani* and *L. amazonensis* respectively, but subcutaneous plumbagin treatment of infected mice for 5 days at a dose of 16 mg/kg body weight produced no suppression of *L. donovani* amastigotes in the liver and only a 24% suppression of *L. amazonensis* lesions at the base of the back (Croft *et al.,* 1985). In contrast, in the same study, plumbagin gave 81% suppression of the *L. amazonensis* lesions when applied topically (0.02 ml of 1% plumbagin ointment) to the lesions for 15 days. Consistent with these findings, plumbagin showed *in vitro* pronounced activity against both extra- and intracellular *L. major* parasites at low concentration with IC_{50} values < 0.5 µg/ml (2.6 µM) (Table. 13). It is suggested that plumbagin may contribute to the bioactivity of the *D. peltata* extracts. The high content of plumbagin in the dichloromethane and ethyl acetate extracts (Table. 10) may explain the highly antileishmanial activity (Table. 13). Moreover, there was a strong relationship between the host cell cytotoxicity of extracts and their content of plumbagin (Tables. 10 and 13). In this context it should be noted that plumbagin showed prominent cytotoxicity on macrophages at low concentrations (EC_{50} 1.5 µg/ml, 8µM) (Table. 13). However, plumbagin showed fairly good selectivity (SI= 3). Plumbagin-induced cell death has been determined in many cell types including cancer cell lines such as HaCaT keratinocytes (Inbaraj and Chignell, 2004) and HeLa (Montoya *et al*., 2004). Its cytotoxic effect was attributed to generation of ROS and induction of apoptosis. Consequently, the parasites may be similarly affected by ROS but other mechanisms may be involved to rationalize differences in the selectivity. The mammalian host cells possess a glutathione/glutathione reductase (GR)-based system which is, at least in part, responsible for

maintaining the redox balance. Glutathione reductase maintains the glutathione level, thereby inactivating ROS. In *Leishmania* and other trypanosomatids, this redox system is replaced by a unique trypanothione/trypanothione reductase-based metabolism with trypanothione reductase (TryR) acting as the key enzyme (Krauth-Siegel and Comini, 2008). Thus, the difference in the specificity of glutathione reductase and trypanothione reductase toward their substrates is due to the difference in their respective active sites. The sensitivity of *Leishmania* against oxidative stress makes the components of the redox thiol metabolism in the parasite an attractive drug target for exploitation of promising antileishmanial chemotherapeutics. Plumbagin has been reported as potential modulators of redox thiol metabolism of *Leishmania* parasites (Sharma *et al*., 2012) and subversive substrate of trypanothione reductase (TryR) from *Trypanosoma cruzi* (Salmon-Chemin *et al*., 2001). Therefore, plumbagin has a significant effect on redox homeostasis and maintains increased ROS levels. Taking into account the sensitivity of *Leishmania* parasites against oxidative stress, this effect results in morphological changes and finally parasite kill (Sharma *et al*., 2012). The metabolism of plumbagin involves enzymatic reduction by one or two electrons, yielding the corresponding semiquinone radical or hydroquinone, respectively. The consequence of these enzymatic reductions is that the semiquinone transfers its extra electron to oxygen with the formation of superoxide radical anion and the original quinone. This reduction by a reductase followed by oxidation involving molecular oxygen (dioxygen) is known as redox cycling and continues until the system becomes anaerobic. In the case of a two electron reduction, the hydroquinone could become stable, and as such, excreted by the organism in a detoxification pathway (Gutierrez, 2000). Interestingly, the acetogenic tetralone (*cis*-isoshinanolone) did not show any significant activity either against *Leishmania* parasites or BMMΦ at the highest test concentration (15 µg/ml). Since *cis*-isoshinanolone was less active than the corresponding naphthoquinone (plumbagin), the quinone structure seems to be a better structural determinant for antileishmanial activity. However, there is no previous study on the antileishmanial activity of *cis*-isoshinanolone. Although the amount of the isolated *cis*-isoshinanolone (2 mg) was not enough to determine its absolute configuration, the presence of (+)-*cis*-isoshinanolone in a fluid extract of the aerial parts of *Drosera* species has been demonstrated for the first time by Kolodziej *et al*. (2002).

On the other hand, naphthoquinones commonly occur in the reduced and glycosidic forms in plants. In some species (e.g. *Diospyros*, *Ebenaceae*) are naphthoquinones present as monomers as well as dimers or trimers. Interestingly, previous studies have documented the presence of only monomeric naphthoquinones in *Drosera* species. In the current study we

have isolated 3,3`-biplumbagin from *D. peltata* for the first time. Fournet *et al*. (1992b) have found that 3,3`-biplumbagin was significantly less potent than plumbagin in BALB/c mice infected with *L. amazonensis* and *L. venezuelensis*. Furthermore, they have observed that 3,3`-biplumbagin was 10- to 20-fold less active than plumbagin *in vitro* against 5 strains of *Leishmania* (promastigote), 6 strains of *T. cruzi* (epimastigote) and the intracellular form of *L. amazonensis* (Fournet *et al*., 1992a). Conspicuously, 3,3`-biplumbagin showed very weak cytotoxicity on BMMΦ (EC_{50} 31µg/ml; 83 µM) compared with that of plumbagin (EC_{50} 1.5 µg/ml, 8µM) (Table. 13). The extracellular promastigotes were more sensitive to 3,3'-biplumbagin than intracellular amastigotes, as evident from the IC_{50} values (promastigotes: IC_{50} of 4.5 µg/ml; amastigotes: IC_{50} of 9.5 µg/ml). The difference in the effectiveness of plumbagin and 3,3`-biplumbagin may be due to their different capability to generate ROS. Other parameters that have to be taken into account include steric constraints of the dimeric form and hindered rotation but require further experiments. Another natural naphthoquinone, droserone, related to *D. peltata* has been recorded that it lacks immunostimulating activity (Kreher *et al*., 1988), but exhibits antimalarial (Likhitwitayawuid *et al*., 1998) and possibly pesticidal activity (Bringmann *et al*., 1993; Bringmann and Feineis, 2001). Moreover, droserone exerted an antifungal effect on a broad range of human and plant fungal pathogens (Eilenberg *et al*., 2010). However, there was an association between the presence of droserone in the most effective extracts of *D. peltata* and leishmanicidal activity, and it seems likely that droserone contributes to the antiparasitic activity of the *D. peltata* extracts. As for the terpenes present in *D. peltata* extracts, there was no evidence that they provide any cytotoxicity or activity as concluded from effects of the ethyl acetate extract (method A) (Table. 13). Furthermore, Waechter *et al*. (1999) have reported that beta-sitosterol and stigmasterol did not exhibit any activity against *L. major* and *L. donovani* at a concentration of 100 µg/ml.

On the basis of the present results, it is concluded that *D. peltata* has potent antileishmanial activity. The most active principles of this plant are represented by the naphthoquinones, while the tetralone, *cis*-isoshinanolone, and terpenes did not have any significant efficacy. Additionally, *D. peltata* and its active constituents did not show any significant NO-inducing potential in parasitized macrophages. Also, their activity was not affected by the presence of the inhibitor L-NMMA (Fig. 36 and Fig. 37). This finding proved that *D. peltat*a extracts did not activate the infected macrophages to release nitric oxide and that their antileishmanial activity may be mediated by ROS generated by naphthoquinones. Finally, it should be mentioned that using transgenic *L. major* GFP in the current study has allowed us to

evaluate the antileishmanial activity by FACS and host cell lysis in parallel. Interestingly, there was no significant difference between the IC_{50} values of all extracts except for the ethanol extract (method A) assessed by either assays (Table. 13). It appears reasonable to consider the IC_{50} values obtained from FACS analysis to be more accurate because the GFP signal of live parasites was measured directly, while retrieval of viable parasites was determined after host cell lysis, transformation of amastigotes into promastigotes and 5 days of culture. Furthermore, the addition of PI permits simultaneous assessment of antileishmanial and cell cytotoxicity effects by the FACS-based method.

4.3 *Spathodea campanulata*

S. campanulata P. Beauv (Bignoniaceae) also known as the "African tulip tree" is native to the West African tropical forests. It has been widely introduced as an ornamental plant in several regions of tropical America. This species has many uses in folk medicine for the treatment of many diseases: edema, dysentery, ulcers, filaria, gonorrhoea, diarrhoea, and as a poison antidote (Irvine, 1961). Previous reports have described a broad spectrum of biological activities of various parts of the plant. Extracts of flowers have shown molluscicidal activity (Mendes *et al*., 1986), potential antioxidant and free radical scavenging activities (Hareesh *et al*., 2010), and a broad-spectrum antimicrobial potency (Antonisamy *et al*., 2012), while stem bark preparations are reported to have wound healing (Houghton *et al*., 2005; Sy *et al*., 2005; Mensah *et al*., 2006), antimalarial (Makinde *et al*., 1988; Amusan *et al*., 1996), antimicrobial (Ofori-Kwakye *et al*., 2009), molluscicidal (Amusan *et al*., 1995), hypoglycemic, anticomplement and anti-HIV activities (Niyonzima *et al*., 1993, 1999). Furthermore, Pianaro *et al.* (2007) have reported antifungal activity of *S. campanulata* roots against *Cladosporium herbarum*. Similarly, *in vitro* antimalarial (Dhanabalan *et al.,* 2008), antibacterial and antioxidant (Akharaiyi *et al*., 2012) activities of leaf extracts have been documented. In parallel, several phytochemical studies of *S. campanulata* were performed including stem barks, leaves, flowers and fruits. The leaves furnished spathodol, caffeic acid, flavonoids (Ngouela *et al.,* 1991; Subramanian *et al*., 1973; El-Hela, 2001), alkaloids, saponins, steroids, phenols and terpenoids (Akharaiyi *et al*., 2012), while fruits were shown to contain polyphenols, tannins, saponins and glycosides. Phenols, alkaloids, coumarins and carbohydrates (Antonisamy *et al*., 2012) as well as anthocyanins (Banerjee and De, 2001) occurred in flower extracts, while the root material yielded the iridoid glucoside ajugol and two phenolic derivatives, *p*-hydroxybenzoic acid and *p*-hydroxybenzoic acid methyl ester (Pianaro *et al*., 2007). The broad spectrum of reported biological activities and the great diversity of the plant constituents stimulated the present study on the antileishmanial activity of *S. campanulata* stem bark extracts and their active constituents. It should be noted that this particular biological activity has not yet been reported for this plant.

Preliminary phytochemical analysis of *S. campanulata* stem bark extracts under study did not show the presence of any naphthoquinones nor flavonoids, consistent with phytochemical studies to date. Earlier work has revealed the presence of ursane- and oleanane-type triterpenes (Niyonzima *et al*., 1999; Amusan *et al*., 1996; Ngouela *et al*., 1990). In the present work, sitosterol, oleanolic acid and ursolic acid were present in lipophilic extracts as may be expected, while more polar phases including methanol and ethanol extracts revealed the

presence of two saponins. Furthermore, the ethanol extract afforded spathoside, which was first isolated by Mbosso *et al*. (2008).

It is interesting to note the significant difference in activity between the lipophilic extracts and highly polar extracts represented by methanol extraction. The polar constituents seemed to be nontoxic against host cells and leishmanicidal inactive as concluded from activities of the methanol extract (EC_{50} of 750 µg/ml; IC_{50} of 340 µg/ml). In contrast, less polar compounds apparently contributed to a weak cytotoxicity and moderate antileishmanial activity (EC_{50}: 140–180 µg/ml; IC_{50}: 60–86 µg/ml (Table. 15).

The presence of ursolic acid in the *n*-hexane and dichloromethane extracts may explain their efficacy, exhibiting pronounced activity against both extra- and intracellular *L. major* parasites, with IC_{50} values of 1.75 µg/ml (3.8 µM) and 2.5 µg/ml (5.5 µM), respectively. Also several studies have demonstrated *in vitro* prominent activity of ursolic acid against a panel of parasites including *L. amazonensis* (Sauvain *et al*., 1993; Torres-Santos *et al*., 2004; Odonne *et al*., 2011), *Trypanosoma brucei* and *L. mexicana* (Bero *et al*., 2011), *L. donovani* (da Silva Filho *et al*., 2009; Moulisha *et al*., 2010), *Plasmodium falciparum* (Van Baren *et al*., 2006) and *L. major* parasites (Takahashi *et al*., 2004). Conspicuously, it exhibited limited activity *in vivo* (Sauvain *et al*., 1993 and 1996). On the other hand, ursolic acid has been extensively studied for its hepatoprotective (Liu, 1995), anti-ulcer (Ovesná *et al*., 2004), cardioprotective (Senthil *et al*., 2007; Chen RJY *et al*., 2010), antimicrobial (Horiuchi *et al*., 2007), anti-inflammatory, and analgesic (Vasconcelos *et al*., 2006) activities. Ursolic and oleanolic acids have similar chemical structures but differ in their antiprotozoal activities, which has been attributed to the different position of the methyl group (oleanolic acid, at C-20 instead of C-19) (Abe *et al*., 2002; Tan *et al*., 2002b; Cunha *et al*., 2003; Cimanga *et al*., 2006; Van Baren *et al*., 2006; Hoet *et al*., 2007; Ferreira *et al*., 2010; Bero *et al*., 2011; Peixoto *et al*., 2011). Once again, ursolic acid has repeatedly been shown to exhibit potent antiprotozoal activities and, more significantly, proved to be a better candidate than oleanolic acid. It seems possible that the hydrogen at C-20 of ursolic acid is an important structural feature for its antiparasitic activity. In the current study, a significant difference in activity between these two compounds has also been shown. Oleanolic acid did not exhibit any effects on either macrophages or parasites even at the highest test concentration (EC_{50}, IC_{50} > 80 µg/ml, >175 µM). As already discussed (Section 4.1), sitosterol occuring in lipophilic extracts did not show any antileishmanial activity. Thus, the antileishmanial activity of *n*-hexane and dichloromethane extracts may be attributed to triterpenes, mainly ursolic acid.

Interestingly, the ursolic acid-free ethanol extract showed a moderate antileishmanial activity (IC_{50} of 140 µg/ml), suggesting that additional but unknown compounds may also contribute to the overall activity of the ethanol extract. Therefore, the ethanol extract was sequentially partitioned into *n*-hexane, dichloromethane, ethyl acetate, and methanol fractions, as described in Section 3.3.1.2, to get more information about the active principles of the plant. The dichloromethane fraction was the most effective fraction of the parent *S. campanulata* ethanol extract against both forms of *L. major* parasites with IC_{50} value of <42 µg/ml (Table. 16). Since this fraction did not contain ursolic acid, which proved considerably active against *L. major* parasites (Table. 15), it appears reasonable that the antileishmanial activity may be due to spathoside. On the other hand, the remaining fractions (*n*-hexane, ethanol, and methanol fractions), which did not contain spathoside according to TLC analyses, exhibited only a weak antileishmanial activity against both extra- and intracellular *L. major* parasites, with IC_{50} values ranging from 104 µg/ml to 152 µg/ml (Table. 16). This finding supports the conclusion that cerebroside (spathoside) has apparently remarkable antileishmanial activity. Generally, cerebrosides are a unique class of metabolites, some of which are reported to have hepatoprotective, antiulcerogenic, neuritogenic, immunostimulatory, antitumor, anti-HIV-1, antifungal, and antimicrobial activities (Tan and Jen, 2003).

Specifically, Mbosso *et al*. (2008) have reported antibacterial activity of spathoside. Based on the current results, spathoside as possible lead structure against *Leishmania*, may give new impetus for obtaining valuable synthetic compounds. Currently, there are extensive investigations into the synthesis of (±)-spathoside and a number of derivatives (Rollyson and Fultz, 2012). The successful synthesis and the proposed completion have shown how all 8 stereoisomers can be derived from cheap, chiral starting materials for continued biological studies (Rollyson and Fultz, 2012). Consequently, the availability of spathoside derivatives in the near future could provide new antileishmanial agents.

On the other hand, some plant constituents may have toxic effects on the host cells; therefore, fractionation of extracts may provide advantages such as the exclusion of cytotoxic compounds. Here, the cytotoxicity of extracts on BMMΦ was evaluated using MTT assay. Notably, microscopic examination of BMMΦ treated with nontoxic concentrations of the methanol fraction showed changes in the form of cells when compared with non-treated BMMΦ (Fig. 40). Although the cytotoxicity of this fraction as assessed by the MTT assay disclosed an EC_{50} value of 180 µg/ml, an increase in cell size with the presence of large vacuoles was noted at the concentration of 80 µg/ml. Most likely, the occurring saponins caused the morphological changes without affecting the metabolic conversion of

MTT by mitochondria. Independent support of this conjecture was obtained from saponin-free fractions, including the most effective fraction containing spathoside, which did not cause any morphological changes of macrophages.

Although the anti-amastigote activities of ursolic acid and the dichloromethane fraction (IC_{50} of 2.5 and 42 µg/ml, respectively) were relatively similar to their anti-promastigote activities (IC_{50} of 1.75 and 39 µg/ml, respectively), an indirect activation of microbicidal mechanisms of macrophages via production of parasite-toxic NO intermediates was evaluated. Indeed, all *S. campanulata* extracts, subfractions and ursolic acid induced the release of NO at relatively low levels (< 12 µM), similarly to that observed for just infected and non-infected macrophages (10 µM). In contrast, IFN-γ plus LPS stimulated infected macrophages to produce relatively high NO level (58 µM). Interestingly, all samples exhibited similar antileishmanial activities whether incubated alone or in combination with the inhibitor L-NMMA, indicating that the antileishmanial effect of *S. campanulata* extracts might be direct against the parasite.

Taken together, our study revealed that the stem bark of *S. campanulata* has remarkable antileishmanial effects. Ursolic acid and spathoside are apparently the most important active compounds of the stem bark, whereas saponins should be removed from extracts due to their toxic effects on the host cells. However, in future investigations it might be possible to choose spathoside and ursolic acid as lead compounds for the development of new drugs against leishmaniasis.

4.4 *Tabebuia avellanedae*

Tabebuia avellanedae LORENZ [syn.: *T. impetiginosa* MART. ex DC, Bignoniaceae] is a popular tree distributed throughout the tropical rain forests of Central and South America. It has been used in folk medicine by native Americans for thousands of years, with indications that may pre-date the Incas (Duke, 1985; Schwontkowski, 1993). The herbal product obtained from the bark of tabebuia trees is called "taheebo", "lapacho", "pau d'arco", or "ipe roxo". The material is traditionally used for treating ulcers, syphilis, gastrointestinal problems, candidiasis, cancer, diabetes, prostatitis, constipation, and allergies. Some studies have reported that this plant exerts a number of pharmacological activities, including antiedematogenic, antinociceptive (De Miranda *et al*., 2001), antiulcerogenic (Twardowschy *et al*., 2008), anti-angiogenic (Kung *et al*., 2007), antifungal (Portillo *et al*., 2001), antimicrobial (Pereira *et al*., 2006), anti-inflammatory (Byeon *et al*., 2008), anticancer (Pardee *et al*., 2002; Kim *et al*., 2007), antipsoriatic (Son *et al*., 2006) and anticoagulation (Müller *et al*., 1999). Red Lapacho tea refers to decoctions prepared from the inner bark and heartwood of *T. avellanedae*. It has been used for many centuries as an analgesic, anti-inflammatory, antineoplasic, antimicrobial, and diuretic in the northeast of Brazil (de Almeida *et al*., 1990; Ueda *et al*., 1994).

Quinones in general and particularly hydroxyquinones as well as their reduced analogues, have been tested against several parasite species (Croft *et al*., 1992; Sauvain *et al*., 1993; Iwu *et al*., 1994; Ray *et al*., 1998; Kayser *et al*., 2000; Pinto *et al*., 2000; Ferreira *et al*., 2002; Hazra *et al*., 2002). Therefore, *T. avellanedae*, which has a great diversity and high content of naphthoquinones, appeared to be a promising material to search for new lead-compounds having antileishmanial properties. As the inner bark is the most important part of the plant, we investigated the antileishmanial activity and host cell toxicity of a methanolic extract of the stem bark and aimed to isolate active constituents.

Lapachol, the most abundant quinone in the family Bignoniaceae, was the first member of this class of compounds isolated from *T. avellanedae* (Paterno *et al*., 1882). In this study, it was identified in the methanol extract by TLC analysis, while fractionation of this extract led to enrichment of lapachol in a *n*-hexane fraction. Besides, an isomeric mixture of furanonaphthoquinones was found in *n*-hexane and dichloromethane fractions, while the polar fractions (ethyl acetate, *n*-butanol) did not contain any quinones. However, several studies have reported the presence of furanonaphthoquinones in *T. avellanedae* (Kreher *et al*., 1988; Wagner *et al*., 1989; Fujimoto *et al*., 1991; Ueda *et al*., 1994), whilst Steinert *et al*. (1996) have restricted their occurrence to the inner bark. In addition, four terpenes, four iridoides and

flavonoids including hyperoside and isoquercitrin were detected in the extract. Phytochemically, this plant has been previously extensively studied, and several biologically active constituents have been isolated, such as furanonaphtoquinones, quinones, benzoic acid, cyclopentene dialdehydes, flavonoids, iridoides and phenolic glycosides (Alonso, 2004).

Interestingly, the methanol extract showed modest activity against both extra- and intracellular *L. major* parasites, with IC_{50} values of 150 µg/ml and 217 µg/ml, respectively, while it did not exhibit any cytotoxicity on macrophages even at high concentrations (EC_{50} > 300 µg/ml). Following fractionation of the methanol extract, the highest antileishmanial activity resided in the dichloromethane and *n*-hexane fractions, while highly polar subfractions appeared inactive (Table. 20). Quercetin glycosides (hyperoside and isoquercitrin) residing only in the ethyl acetate fraction have been previously discussed as inactive agents (Section 4.1). The negligible activity of the latter fraction (IC_{50} > 350 µg/ml) may support this finding. Similarly, co-occurring iridoides should also be excluded from the list of active antileishmanial constituents for the same reason. Furthermore, although iridoids isolated from *Swertia chirata* (Loganiaceae), *Nyctanthes arbortristis* (Oleaceae) and *Picrorhiza kurroa* (Scrophulariaceae) have been reported as promising compounds with leishmanicidal activity (Ray *et al*., 1996; Tandon *et al*., 1991; Puri *et al*., 1992), no studies on the antiprotozoal activity of iridoides from *T. avellanedae* have been reported to date. That iridoids obtained from plant sources noted above exhibited antileishmanial activity may be attributed to structural differences. Therefore, the active principle appears limited to lapachol and furanonaphthoquinones present in the *n*-hexane and dichloromethane fractions, respectively (Fig. 41). Interestingly, lapachol showed prominent antileishmanial activity against promastigotes (IC_{50} value of 8 µg/ml, 33 µM) and a moderate activity against amastigotes (IC_{50} value of 28 µg/ml, 115 µM) which may explain the prominent antileishmanial activity of the *n*-hexane fraction (IC_{50} value of 64 µg/ml). However, several pharmacological activities have been attributed to lapachol and its semi-synthetic derivatives, such as antifungal and antimicrobial action (Portillo *et al*., 2001; Pereira *et al*., 2006), anticancer (Silva *et al*., 2003), antiviral (Li *et al*., 1993), antimalarial (Carvalho *et al*., 1988), cercaricidal and schistosomicidal (Pinto *et al*., 1977), and also a potential activity against *Trypanosoma cruzi*, the causal agent of Chagas' disease (Lopes *et al.,* 1978). Teixeira *et al*. (2001) have reported that lapachol exhibits marked *in vitro* activity (76–89 %, inhibition) against intracellular amastigotes of *L. (Viannia) braziliensis* at concentrations of 12.5 to 50 µg/ml and no toxicity to macrophages at concentrations <100 µg/ml. More interestingly, lapachol exhibited a more powerful antiamastigote effect and a lower host cytotoxicity than

pentostam. In the same study, lapachol did not prevent the development of *L. (Viannia) braziliensis*-induced lesions in an animal model (hamster) at an oral dose of 300 mg/kg/day for 42 days. It was suggested that lapachol could be transformed *in vivo* into an inactive metabolite (Teixeira *et al*., 2001). Lima *et al*. (2004) reported the antileishmanial activity of lapachol and its derivatives against metacyclic promastigotes of two different species of *Leishmania*: *L. amazonensis* and *L. braziliensis*. Lapachol has been shown to exert significant activity with IC_{50} values of 5.2 µg/ml and 11.9 µg/ml, respectively, while isolapachol acetate was the most active derivative (IC_{50} of 1.6 µg/ml and 3.4 µg/ml, respectively) (Lima *et al*., 2004). Furthermore, the leishmanicidal activity of lapachol against amastigotes of *L. donovani* in mouse peritoneal macrophages has been reported (Borris and Schaeffer, 1992). Although the antileishmanial effect of the *n*-hexane fraction may well be attributed to lapachol, the question remains regarding the active principle of the lapachol-free dichloromethane fraction with IC_{50} values of 41 µg/ml for amastigotes and 20 µg/ml for promastigotes, respectively (Table. 20). Therefore, the dichloromethane fraction was subjected to bioguided fractionation and separation by chromatographic methods. A total of twenty fractions with different TLC profiles obtained by column chromatography were tested for their antileishmanial activity. Fraction 5 exhibiting the highest antileishmanial activity (killing of ca. 70% and 90% of amastigotes at concentrations of 20 µg/ml and 30 µg/ml, respectively) was further purified by column chromatography to yield 6 subfractions (Table. 19). Following HPLC purification, subfraction 4 afforded an isomeric mixture of furanonaphthoquinones, identified as 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione (**21**) by means of spectroscopic data (MS and ^{1}H-NMR), which were consistent with those previously reported (Wagner *et al*., 1989). Kigelinone, 8-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione, was first obtained from the wood of *Kigelia pinnata* DC (Inoue *et al*., 1981), but the structure of kigelinone was also established to be that of the 5- hydroxy isomer (Wagner *et al*., 1989; Fujimoto *et al*., 1991). Wagner and his colleagues have previously reported the isolation of compounds **(20)** and (**21)** from *T. avellanedae* and have successfully separated these isomers by preparative HPLC on an ODS-phase column using THF/H_2O (Wagner *et al*., 1989). In the current study, the isomeric mixture of 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione(**21**) showed a potent activity in a concentration-dependent manner with an IC_{50} value of 1 µg/ml (4 µM) against both extra- and intracellular *L. major* parasites, when compared to the reference drug amphotericin B (IC_{50} values of 2.5 µM, 0.2 µM, respectively). Compared with lapachol, the mixture of furanonaphthoquinones (**20**) and (**21**) showed antileishmanial activity which was ca. 28 fold

more potent than lapachol. Notably, its toxic effects on BMMΦ were obvious at an increase of at least two-fold in sample quantity (SI of 2). It appears as if the presence of a furan ring increases the antileishmanial activity and cell cytotoxicity of naphthoquinones. However, extensive studies demonstrated that naphtho[2,3-b]- furan-4,9-dione congeners including compounds (**20**) and (**21**) showed potent cytotoxicity against numerous tumour cell lines (Saizarbitoria *et al*., 1992; Ueda *et al*., 1997). Besides, the same mixture of furanonaphthoquinones (**20**) and (**21**) has been isolated from *T. ochracea* ssp. *neochrysantha* and shown to possess important activity against rodent and human malaria parasites *Plasmodium berghei* and *P. falciparum,* respectively (Pérez *et al.*, 1997). Yamashita *et al.* (2009) have stereoselectively synthesized the compounds (**20**) and (**21**) and evaluated their biological activities. They found that compound (**20**) has a potent antiproliferative effect and *in vitro* and *in vivo* cancer chemopreventive activity. In addition, it displayed relatively strong antimicrobial activity against several Gram-positive bacteria and fungi, while its positional isomer (**21**) was less active in terms of antiproliferative and *in vitro* cancer chemopreventive activity, but similarly antimicrobial active (Yamashita *et al*., 2009). Therefore, it is likely that the two isomers differ in their antileishmanial activities, which should be evaluated in future studies. On the other hand, the mechanism of action of lapachol, furanonaphthoquinones (**20**) and (**21**) is still unknown, despite some insights. For example, these hydroxynaphthoquinones were suggested to perturb the electron transport chain in the mitochondria of the parasite or to generate free radicals (Yardley *et al*., 1996). The involvement of reactive oxygen species in the mechanism of action of lapachol was proposed by electrochemical studies in the presence of oxygen (Goulart *et al*., 2003). In the current study, the methanol extract of *T. avellanedae* and the isolated naphthoquinones did not show any effect on nitric oxide production in *Leishmania*-infected macrophages. The measured NO levels were similar to that observed for just infected and non-infected macrophages. In contrast, potent inhibition of NO production in LPS-activated macrophage-like J774.1 cells was observed by iridoids isolated from an aqueous extract of Brazilian *T. avellanedae* (Awale *et al*., 2005). Regardless, our result show that the extracts exhibited similar antileishmanial activities whether incubated alone or in combination with L-NMMA, suggesting an NO independent mode of action.

In conclusion, this study shows the antileishmanial activity of a methanolic extract of *T. avellanedae.* Its leishmanicidal activity could be related to lapachol and a mixture of furanonaphthoquinones, identified as isomeric 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**). However, due to the observed cell toxicity

of the furanonaphthoquinones, structural modifications can be an important strategy to obtain less toxic molecules, to be used as antileishmanial agents.

4.5 *Juglans regia*

Walnut (*Juglans regia* L., Juglandaceae), the most widespread nut tree in the world, has been used globally in human nutrition since ancient times. Furthermore, it has been widely used as herbal medicine in the treatment of various health disorders. All parts of the plant (green walnuts, shells, kernels, seeds, bark and leaves) have been used in both cosmetic and pharmaceutical industries (Stampar *et al*., 2006). Walnut fruit extracts exhibited antioxidant activities in a concentration-dependent manner in different assays. Their antimicrobial capacity was also checked against a panel of gram-positive and gram-negative bacteria as well as fungi (Pereira *et al*., 2008). Similarly, Noumi *et al*. (2011) reported antibacterial, anticandidal and antioxidant activities of extracts of *J. regia* bark. They suggested that these activities may be correlated with the high phenolic content and tannins in the bark of *J. regia.* However, the contribution of walnut oil to the total antioxidant capacity of walnut is less than 5% (Arranz *et al*., 2008), while phenolic compounds (Fukuda *et al*., 2003), especially hydrolysable tannins (Anderson *et al*., 2001; Arranz *et al*., 2008), tocopherols (Kornsteiner *et al*., 2006) and melatonin (Reiter *et al*., 2005) were the principle antioxidant constituents. Furthermore, walnut leaves have been widely used in traditional medicine for the treatment of ulcers and skin inflammations and for its antiseptic antihelmintic, antidiarrheic, and astringent properties (Evans, 2002). Anti-inflammatory, antinociceptive and antidiabetic activities of walnut leaves (Erdemoglu *et al*., 2003; Asgary *et al*., 2008), antioxidant effects of the seeds and radical scavenging of leaves have been reported (Almeida *et al*., 2008, Fukuda *et al*., 2003). The leaves of *J. regia* are listed in the German Drug Codex (DAC, 2001), are approved by the Kommission E, and the decoction form for external use is official in the standard license monographs (Deutscher Arzneimittel-Codex, 1997; German Komission E monograph, 1990; Wicht, 2002). It has been used as a topical remedy for the treatment of certain skin diseases, including dermal inflammation, excessive eczema and lymphatic disorders such as scrofula (Gruenwald *et al*., 2001). In traditional medicine *J. regia* stem bark was also used as an anthelmintic remedy. Recently, Kale *et al*. (2011) have tested the anthelmintic activity of different stem bark extracts of *J. regia* against *Eicinia foetida* as a test worm. Parameters of the bioassay included the determination of the time of paralysis and the time of death control. Crude acetone and methanolic extracts significantly exhibited paralysis and also caused death of worms in a dose dependent manner as compared to the standard reference albendazole. Ethanolic extracts showed quite weak anthelmintic effects (Kale *et al*., 2011).

Also, extracts of *J. regia* stem bark have been investigated for its anthelmintic activity on adult Indian earthworm, *Pheretima posthuma*, to substantiate the fokloric claim. Benzene,

methanol and ethanol extracts exhibited prominent anthelmintic activity, comparable to that of the standard drug piperazine citrate (Upadhyay *et al*., 2010). In another study the traditional use of *J. regia* stem bark against nematode infections has been demonstrated. An ethanol extract showed strong anthelmintic effects on the embryonating eggs of *Ascaris suum* and against the infective third-stage larvae of *Trichostrongylus colubriformis* in comparison with synthetic anthelmintic zentel (albendazole) (Urban *et al*., 2008). However, plant-derived products have been used as antimicrobial agents, particularly those rich in polyphenols (Cowan, 1999). In this regard, many polyphenols (hydrolysable tannins, proanthocyanidins and caffeic acid derivatives) have been reported to reduce the survival of the intracellular amastigote parasite form of *L. donovani* or *L. major* strains *in vitro* (Kolodziej and Kiderlen, 2005). However, there is no report on the leishmanicidal activity of *J. regia* extracts, although they are rich in phenolic compounds. In the present study, the antileishmanial and cell cytotoxicity activities of different extracts of *J. regia* leaves or peels were evaluated.

Preliminary phytochemical analysis of all extracts of *J. regia* under study did not show the presence of any naphthoquinones. Although the characteristic naphthoquinone juglone has been reported to be present in significant amounts (0.5 g /100 g dry weight) in fresh leaves of *J. regia* (Harborne, 1988; Gîrzu *et al*., 1998), only low amounts occur in dried leaves due to polymerization phenomena, (Wichtl and Anton, 1999). In the current study, dried material has been used for the extraction, thereby explaining the apparent absence of juglone. On the other hand, flavonoids appeared absent from *J. regia* extracts prepared from peels, whereas quercetin and its 3-O-galactoside, 3-O-arabinoside, 3-O-xyloside, and 3-O-rhamnoside were readily detected in the ethyl acetate and ethanol extracts of leaves, which is in accordance with findings by Jalili and Sadeghzade (2012). Furthermore, flavonoids were absent in highly lipophilic extracts of leaves including *n*-hexane and dichloromethane extracts. Besides, four unidentified phenols were found in all extracts of leaves, while detection of phenolic compounds failed in extracts of peels. However, several hydroxycinnamic acids (3-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids) were reported in methanolic extracts of *J. regia* leaves (Amaral *et al*., 2004; Jalili and Sadeghzade, 2012) as well as the occurrence of 5-caffeoylquinic acid (Wichtl and Anton, 1999). Additionally, five terpenes were detected in the *n*-hexane and dichloromethane extracts of leaves by TLC analysis. Of these, three with low mobility were found in the ethyl acetate and ethanol extracts of leaves. Regarding extracts of peels, three terpenoids were found in the *n*-hexane and dichloromethane phases and one terpene spot with low mobility in the ethyl acetate and ethanol extracts (Table. 21).

Biological activity studies revealed that the extracts of leaves exhibited prominent activities against both forms of *Leishmania* parasites with IC_{50} values ranging from 19 µg/ml to 44 µg/ml, except for the anti-promastigote activity of the ethanol extract (IC_{50} value of 123 µg/ml) (Table. 23). The highest antileishmanial activity resided in the *n*-hexane and dichloromethane phases (IC_{50} of 19 and 22 µg/ml, respectively). In contrast, all extracts of peels were inactive against both forms of the parasite (IC_{50} > 160 µg/ml). In terms of cytotoxicity, only the dichloromethane extract of leaves was slightly toxic with an EC_{50} value of 82 µg/ml (SI= 3.7), while the remaining extracts did not show any cytotoxic effects on macrophages (EC_{50} > 180 µg).

Given these findings, a correlation between the polarity of compounds and antileishmanial activity can be noted (Table. 23). Lipophilic compounds represented the major active principle in *J. regia* leaves. Therefore, flavonoids residing in the ethyl acetate and ethanol extracts are considered to play a minor contributing role to the observed antileishmanial effects. However, this conjecture was further supported by moderate to weak leishmanicidal activities of quercetin and its glycosides (Table. 8). Following TLC analysis, terpenes and phenolic constituents of *J. regia* leaves appeared to be putative antileishmanial candidates. The *n*-hexane extract of *J. regia* leaves was the most important one in terms of terpenes and phenolic compounds, antileishmanial activity, and non-cytotoxicity (EC_{50} > 180 µg). Therefore, a portion of the *n*-hexane extract was further fractionated using column chromatography to identify distinct antileishmanial compounds. Following column chromatography, fourteen subfractions were obtained (Table. 22), which were subjected to TLC analysis and biological tests. These were tested against intracellular *Leishmania* parasites at concentrations of 5 µg/ml and 10 µg/ml using the parasite retrieval assay. The fractions 3–5 and 7–10 were the most effective ones as shown in Fig. 48. Fraction 4 caused killing of ca. 60% and 85% of intracellular *L. major* parasites at concentrations of 5 µg/ml and 10 µg/ml, respectively, while fraction 9 exhibited marked *in vitro* activity (88 % and 97 %, inhibition) at concentrations of 5 µg/ml and 10 µg/ml, respectively. These results, when taken together with the findings of TLC analysis (Table. 22), indicate that the antileishmanial activity of *J. regia* leaves may mainly be correlated with terpenes and the phenolic content. However, it should be noted that no studies on the antileishmanial activity of reported phenolic compounds in *J. regia* leaves including 3-caffeoylquinic, 5-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids were reported to date. Caffeoylquinic acid derivatives are known to occur, for example, in Brazilian propolis (Tatefuji *et al*., 1996). Interestingly, there are several reports about the effect of propolis on protozoa that cause

diseases in humans and animals such as trichomoniasis, toxoplasmosis, giardiasis, Chagas disease, leishmaniasis and malaria (De Castro, 2001). These biological activities of propolis were associated mainly with phenolic compounds such as flavonoids and derivatives of hydroxycinnamic acids (De Castro, 2001). Independent support is provided by studies on propolis extracts collected in Bulgaria and Brazil which proved antileishmanial activities against different *Leishmania* strains (*L. major*, *L. amazonensis*, *L. chagasi*, *L. brazilensis*) at concentrations of 2.8–229.3 µg/ml and suggested phenolic compounds and flavonoids as the active constituents (Machado *et al*., 2007). These findings suggest that hydroxycinnamic acids derivatives occurring in *J. regia* leaves may represent, at least in parts, the antileishmanial principle. The leaves are easily available in abundant amounts and may therefore provide a convenient source of these active compounds. In addition, this plant material is much cheaper than propolis.

Bearing in mind that the effects of polyphenols on intracellular *Leishmania* parasites are due to macrophage activation rather than direct antiparasitic activity (Kolodziej and Kiderlen, 2005), the extracts of *J. regia* leaves were evaluated in terms of intracellular leishmanicidal effects and release of nitric oxide as indicator of macrophage activation. All extracts induced the release of NO at relatively low levels (< 14 µM) and in the same range of that of just infected macrophages. All *J. regia* leaves at their IC_{50} ranges showed similar antileishmanial activities when incubated with or without the inhibitor L-NMMA. These findings indicate that the extracts of *J. regia* leaves induced the killing of parasites but did not stimulate the infected macrophages to produce microbicidal nitric oxide.

In summary, the presented results provide data that *J. regia* leaves have promising *in vitro* antileishmanial properties with minimal or no cytotoxicity. In contrast, all extracts prepared from peels did not have any effects on either *Leishmania* or host cell macrophages. Phenolic substances and terpenes which appeared enriched in *J. regia* leaves may be putative candidates as antileishmanial agents, while quercetin and its glycosides may be less important. Consequently, the reported series of quinic acid derivatives of *J. regia* leaves including 3-caffeoylquinic, 5-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids may be interesting candidates to be tested for intracellular pathogens such as *Leishmania* parasites.

4.6 Naphthoquinones

Naphthoquinones are considered privileged structures in medicinal chemistry due to their structural properties and biological activities (Constantino and Barlocco, 2006). They are present in various families of plants and serve as vital links in the electron transport chains in the metabolic pathway, participating in multiple biological oxidative processes (Powis, 1987; O'Brien, 1991). The essential feature of naphthoquinone chemistry is its ease of reduction and, therefore, its ability to act as an oxidizing or dehydrogenating agent. This redox property is driven by the formation of a fully aromatic system (Abreu *et al*., 2002; Hillard *et al*., 2008). In folk medicine, plants containing naphthoquinones are often employed for the treatment of various diseases (Arenas, 1987; Bastien, 1983), and several quinonoids isolated from traditional medicinal plants are being investigated for antimicrobial and anticancer properties (Hazra *et al*., 2004).

Quinones are oxidants and electrophiles, and the relative contribution of these properties to both their toxic and therapeutic activities is influenced by their chemical structure, particularly substituent effects and the characteristics of the quinone nucleus (Monks and Jones, 2002). Previously, several naphthoquinones have been identified as antiprotozoal principles of plant extracts (Croft *et al*., 1992; Sauvain *et al*., 1993; Iwu *et al*., 1994; Ray *et al*., 1998; Kayser *et al*., 2000; Pinto *et al*., 2000; Ferreira *et al*., 2002; Hazra *et al*., 2002), but a comprehensive study of their structure-activity relationships (SARs) has not been conducted so far. Therefore, it was our interest to assess the *in vitro* leishmanicidal activities of a series of naphthoquinones and to determine their antileishmanial potential. The present study underlines the *in vitro* ability of naphthoquinones to inhibit leishmanial infections without significant toxicity to mammalian host cells.

The mechanism of action of naphthoquinones has not yet been fully investigated. Earlier reports suggested inhibition of the catalytic activity of DNA topoisomerase I of the parasite, as exemplified for diospyrin isolated from the stem bark of *Diospyros montana* Roxb. (Ray *et al*., 1998). However, most papers proposed the generation of oxygen radical species, which damage cells of various organisms. From a toxicological perspective, two major mechanisms have been proposed for the cytotoxic action of quinones in a variety of biological systems (Monks and Jones, 2002).

First is redox cycling of quinones that may be initiated by either a one- or two-electron reduction. The one electron reduction of quinones is catalysed by microsomal NADPH-cytochrome P-450 reductase or mitochondrial NADH ubiquinone oxidoreductase, yielding the corresponding semiquinone radicals. Under aerobic conditions, the semiquinone radical

participates in redox cycling to generate ROS like superoxide anion ($^{\cdot}O_2$) and hydrogen peroxide (H_2O_2). Superoxide anion can be converted to hydrogen peroxide (H_2O_2) via a superoxide dismutase (SOD)-catalysed reaction, followed by the formation of a hydroxyl radical ($^{\cdot}HO$) by the iron-catalysed reduction of peroxide via the Fenton reaction (Pinto and de Castro, 2009). All of these highly reactive species may react directly with DNA or other cellular macromolecules, such as proteins and lipids, leading to cell damage (Vilamil-Fernandez *et al*., 2004). The capacity to produce free oxygen radicals is dramatically influenced by the nature and position of substituents and contributes to both therapeutic and toxic actions of these substances.

The two-electron reduction of quinones is catalysed by NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase) (Ross and Siegel, 2004), and generates hydroquinones (QH2). This enzyme saves the cell from ROS formation, bypasses the production of toxic intermediates (e.g. semiquinone radicals), and reduces reactive and unstable quinones. Nevertheless, the cytotoxicity induced by hydroquinones can be attributed to activation of oxidative stress resulting from the autoxidation of the formed hydroquinone (O'Brien, 1991). However, as hydroquinones are readily autoxidised back to the quinone producing hydrogen peroxide, it is also conceivable that this metabolic route could in fact lead to toxicity (Gant *et al.,* 1988).

Second, quinones are potent electrophiles, capable of reacting with the thiol groups of proteins and glutathione. Indeed, generation of glutathione-conjugates catalyzed by glutathione tranferase isoforms (GST) with depletion of glutathione has been associated with quinone-induced cytotoxicity and oxidative stress (Monks and Lau, 1998; Chung *et al.,* 1999; Monks and Jones, 2002; Rodriguez *et al*., 2004).

In the current study, we evaluated *in vitro* susceptibility of extracellular promastigotes and intracellular amastigotes of transgenic *L. major* expressing GFP to a series of naphthoquinones including alkannin/shikonin derivatives, which are found mainly in members of the Boraginaceae, and some parent analogs obtained from *Tabebuia avellanedae* (Bignoniaceae) and *Drosera peltata* (Droseraceae). The antileishmanial activities were compared with their direct cytotoxic effects on macrophages in order to evaluate their antiprotozoal selectivities. In addition, structure-activity relationships are discussed with a closer view on their mode of action.

4.6.1 Structure-activity relationship

Our results showed that 13 out of 21 tested naphthoquinones were considerably leishmanicidal against promastigotes with IC_{50} values ranging from 0.5 to 4 µM, when

compared with the IC_{50} value 2.5 μM of amphotericin B as positive control (Table. 24). Among intracellular amastigotes, the picture was similar to that of promastigotes but their antiprotozoal activity was less effective (IC_{50} values of 1.1 to 6.5μM) when compared with the IC_{50} value 0.2 μM of amphotericin B. All tested naphthoquinones exhibited moderate toxicity with SI values ranging from 1.1 to >7. In general, antiprotozoal activity and cytotoxicity of naphthoquinones seemed to increase in parallel, suggesting similar mechanisms regarding the antileishmanial action and cytotoxicity.

The simple naphthoquinone 1,4-naphthoquinone (**1**) showed pronounced effects against promastigotes (IC_{50} of 2 μM) but only relatively moderate leishmanicidal potency against amastigotes (IC_{50} of 8.5 μM). The antileishmanial activity was associated with *in vitro* toxic effects for BMMΦ, as evident from the SI of ca. 1.1. Methylation of 1,4-naphthoquinone (**1**) at position 2 slightly increased the antileishmanial activity against amastigotes as concluded from the IC_{50} value of menadione (**2**) (IC_{50} of 6.2 μM). A possible explanation of this increase in efficiency may be the tendency of menadione (**2**) to be a more efficient superoxide generator when compared with 1,4-naphthoquinone (**1**) (Klaus *et al.,* 2010). However, the reduced potency of menadione (**2**) against promastigotes (IC_{50} of 10.8 μM) may be indicative of differences in amastigote/promastigote-specific activity. In contrast, the presence of a hydroxyl group at C-2 of 1,4-naphthoquinone (**1**), as represented by lawsone (**6**), reduced the antileishmanial activity and showed only activity against amastigotes with an IC_{50} value of 32 μM. On the other hand lawsone (**6**) did not show any cytotoxicity against macrophages even at high concentrations (EC_{50} > 225 μM). A plausible interpretation is that the acidic *ortho*-hydroxy groups render the molecule incapable of continuous redox cycling due to the stabilizing influence of keto-enol tautomerization. In fact, 2-hydroxy-1,4-naphthoquinone analogs are very poor electrophiles with high electron density at C-3, thereby precluding electrophilic addition. Furthermore, deprotonation of the 2-OH and tautomerization of the quinoid form stabilizes the quinoid structure, assisted by the donation of electrons from the deprotonized hydroxyl group to the quinone ring. As a consequence, such derivatives possess a very low one-electron reduction potential (Öllinger and Brunmark, 1991; Inbaraj and Chignell, 2004). Thus, introduction of a hydroxyl group at C-2 reduced effectiveness, while the additional presence of an aliphatic substituent such as a prenyl group at position 3 led to a significant loss of activity as evident from the antiprotozoal activity of lapachol (**8**). It was less active than lawsone (**6**) against amastigotes (IC_{50} of 115 μM) and exhibited a weak anti-promastigote activity (IC_{50} of 33 μM). Furthermore, lapachol (**8**) did not show any cytotoxicity on macrophages even at high concentrations (EC_{50} > 300 μM). Nevertheless, the

replacement of the prenyl group by a methyl group led to an inactive naphthoquinone, 2-hydroxy-3-methyl-1,4-naphthoquinone (**7**) (IC_{50} > 225 µM, EC_{50} > 225 µM). On the other hand, 2-methoxy-1,4-naphthoquinone (**9**) and 2,3-dimethoxy-1,4-naphthoquinone (**10**), unable to undergo tautomerization, were more active against amastigotes (IC_{50} of 6.5 µM and 7 µM, respectively) when compared with lawsone (**6**) or lapachol (**8**). Furthermore, methoxyquinones (**9**) and (**10**) were more active than the unsubstituted 1,4-naphthoquinone (**1**) or 2-methyl-1,4-naphthoquinone (**2**). Although 2,3-dimethoxy-1,4-naphthoquinone (10) does not react with free thiol groups of proteins and glutathione, it is able to redox cycle to the same degree as menadione (**2**)(Gant *et al.,* 1988).

Among the oxygenated members tested, juglone (**3**), naphthazarin (**4**), and plumbagin (**5**) exhibited pronounced antileishmanial activities against both amastigotes and promastigotes with IC_{50} values ranging from 1.2 to 6 µM (Table. 24). Interestingly, the common presence of a hydroxyl group at C-5 increased the antileishmanial activity when compared with 1,4-naphthoquinone (**1**). Notably, introduction of an additional hydroxyl group at C-8 further enhanced the antileishmanial efficacy as evident from naphthazarin (**4**) (IC_{50} of 2.3 µM). Indeed, it appears that the presence of a 5,8-dihydroxy pattern was crucial for marked antileishmanial potency of naphthoquinones as is also evident from the IC_{50} values of alkannin/shikonin derivatives (Table. 24). Previous studies have shown that naphthoquinones with electron –repelling hydroxyl group in the benzene moiety such as juglone (**3**) and shikonin (**12**) stimulated the microsomal lipid peroxidation. In contrast, lawsone (**6**) and lapachol (**8**) with hydroxyl group in the quinone moiety did not enhance the formation of lipid peroxides (Murakami *et al.,* 2010). Furthermore, 5-hydroxy- and 5,8-dihydroxy-1,4-naphthoquinones were more susceptible to one electron reduction and therefore more likely to redox cycle (O'Brien, 1991). This finding reflects an important role of these phenolic groups in stabilizing the semiquinone anion by intramolecular H-bonding (Mukerjee, 1987). Interestingly, the significant antileishmanial activities of compounds **3–5** were associated with relatively weak toxic effects against host cell controls (SI 2.3–3.2). On the other hand, in the series of alkannin/shikonin derivatives, the principal enantiomeric compounds alkannin (**11**) and shikonin (**12**) exhibited the highest toxicity for intracellularly residing amastigotes with IC_{50} values of 1.3 µM and 1.9 µM, respectively. Comparison of these naphthoquinones with the parent analogue naphthazarin (**4**) suggested that the presence of the 1′-hydroxy-3′-isohexenyl substituent at C-3 enhanced the antileishmanial activity. Since esterification of this side chain hydroxyl as reflected in compounds β,β`dimethylacrylshikonin (**13**), isovalerylshikonin (**14**), and acetylshikonin (**15**) reduced the antileishmanial activity (IC_{50}

values 2.7 µM, 3 µM, and 5 µM, respectively), the presence of this free hydroxyl group apparently represents a major contributing factor towards antileishmanial activities. Independent support of this conjecture was evident from similar decreased antiprotozoal activity of deoxyshikonin (**16**) with an IC_{50} value of 4.1 µM. A plausible explanation may be the formation of a well-stabilized semiquinone through the hydrogen bonded hydroxyl group *peri* to a carbonyl function similar to 5,8-dihydroxy analogs. That a free hydroxyl group at C-1′ may well enhance the activity was most recently concluded from calculated C–H bond dissociation enthalpy values showing higher stability of the derived carbon centered radical (Ordoudi *et al.,* 2011). Conspicuously, the effect of esterification differs with respect to promastigotes, demonstrating once more dissimilar biochemical and metabolic characters of the two stages of the parasite and, hence, rendering studies limited to extracellular forms little useful.

Although limited in the number of dimers tested, this study revealed marked differences in their antileishmanial activities. While the mixture of vaforhizin (**17**) and iso-vaforhizin (**18**) exhibited the highest antileishmanial activity within the series of compounds tested, 3,3`-biplumbagin (**19**) proved to be a weak antileishmanial agent (promastigotes: $IC_{50}$ of 12 µM; amastigotes: $IC_{50}$ of 25.5 µM). It would therefore appear that a quinoid-quinoid coupling was less favorable than the indicated dimerisation of alkannin/shikonin entities. Interestingly, 3,3`-biplumbagin (**19**) was less active than the corresponding monomer plumbagin (**5**), consistent with findings of Fournet *et al*. (1992b). Furthermore, Fournet *et al*. (1992a) have observed that 3,3`-biplumbagin was 10-to 20-fold less active than plumbagin *in vitro* against 5 strains of *Leishmania* (promastigote), 6 strains of *T. cruzi* (epimastigote) and the intracellular form of *L. amazonensis*. Conspicuously, the results demonstrated that 3,3`-biplumbagin has very weak cytotoxicity on BMMΦ (EC_{50} of 83 µM) compared with that of plumbagin (EC_{50} of 8 µM) (Table. 24). Most likely, the antileishmanial activity of dimeric naphthoquinones depended largely on the mode of coupling, associated with steric constraints and blocking the active sites. Other parameters that have to be taken into account include hindered rotation and steric constraints but require further experiments. Lastly, furanonaphthoquinones represented by a mixture of isomeric 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**) exhibited higher effects against both promastigotes and amastigotes (IC_{50} of 4 µM) when compared with juglone (**3**) (IC_{50} of 6 µM). It therfore appears as if the presence of a furan ring increases the antileishmanial activity of naphthoquinones. On the basis of these results, it is concluded that the leishmanicidal capability of naphthoquinones depend strongly on the nature and position of substituents.

Distinct naphthoquinones, most notably members of the A/S group, exhibited pronounced antileishmanial activities with less toxic effects against host cell controls at the tested concentrations. Hydroxylation at C-5 and dihydroxy substitutions at C-5 and C-8 appear to increase the efficiency of redox cycling and thus antileishmanial activity, while the presence of hydroxyl groups in the quinone entity decrease dramatically the efficacy. Dimerization of naphthoquinones may lead to increase or decrease the antileishmanial activity depending on the type of coupling.

4.6.2 Mode of action

As leishmaniasis is associated with immunological dysfunction of T cells, natural killer cells and in particular incapacitation of macrophages which ultimately leads to establishment of the parasite, experimental approaches have included developing antileishmanial compounds capable of recovering the Th1 immune response (Murray, 2001) via activation of macrophages (Kaye *et al.,* 2004). Activated macrophages release a variety of potentially cytotoxic molecules, of which nitric oxide and its congeners (NO), produced by inducible NO synthase (iNOS), are the most microbicidally effective against *Leishmania* and other parasites. In order to assess the NO-inducing potential of naphthoquinones in parasitized macrophages, the supernatants of sample treated cell cultures collected as a source of secreted NO were subjected to the Griess assay for determining the total amount of NO. In parallel, the rate of intracellular killing was determined by FACS analyses and the parasite retrieval assay. In a first set of experiments, the effects of IFN-γ+LPS on the parasites' viability was tested for validating the test model. These stimuli mediated significant antileishmanial activity, as evidenced by a killing rate of ca. 90% in parallel with a high NO production (56 µM) (Fig. 50). In contrast, just infected macrophages induced the release of NO at relatively low levels (7 µM). Notably, inhibition of iNOS by incubating with L-NMMA showed significantly reduced parasite killing rates and the induction of relatively low NO levels (8 µM).

Compared with the stimulus IFN-γ + LPS, all tested naphthoquinones exhibited similar antileishmanial activities when incubated with or without the inhibitor L-NMMA. Conspicuously, the NO-inducing potential of the tested naphthoquinones was relatively weak (ca. 7 µM) with menadione showing the highest NO-inducing capability (12 µM). This finding suggested that the naphthoquinones did not stimulate the infected macrophages for NO release and that their antileishmanial activity was apparently mediated by a direct mode of action. Independent support of this conjecture was provided by the antileishmanial activity of amphotericin B that was similarly independent of NO production (Fig. 50). Its mechanism

has been attributed to preferred interaction with ergosterol resulting in formation of transmembrane amphotericin B channels, aqueous pores, and leakage of cations (Pourshafie *et al*., 2004; Brajtburg and Bolard, 1996).

Having in mind that naphthoquinones are potent electrophiles capable of reducing cellular thiol levels, this finding prompted us to also investigate the antileishmanial activity in the presence of glutathione in order to get a better understanding of the mode of antiprotozoal action. FACS analysis showed that about 80% of the macrophages were successfully infected when coincubated with *L. major* GFP and that only 13% of the host cells were dead (Fig. 51). When *L. major* GFP-infected macrophages were exposed to amphotericin B, the GFP signal was reduced by ca. 60% but without any effect on the viability of the macrophages (PI signal=12%). On other hand, glutathione did not show any killing of the intracellular parasites (GFP signal=82%) nor toxic effects on the host cells (PI signal=11%). Treatment of infected macrophages with the naphthoquinones at effective concentrations reduced the GFP signal at similar rates as the viability of the parasites decreased in the parasite retrieval assay (Tables. 24 and 25). At higher sample concentrations, about 90% of the parasites were killed, generally associated with high toxicity for macrophages as evident from plumbagin (**5**) and lapachol (**8**) (Fig. 52A).

Interestingly, 3,3`-biplumbagin (**19**), 5-hydroxy- (**20**) and 8-hydroxy-2-(1'-hydroxyethyl) naphtho[2,3-b]furan-4,9-dione (**21**), and oxygenated quinones (**6**–**10)** were found active when coincubated with or without glutathione. In contrast, the remaining test compounds were only active in the absence of glutathione and did not show any antileishmanial activity or cell cytotoxicity even at the highest concentrations tested when coincubated with glutathione (Table. 25). It may be assumed that glutathione modifies the toxic effect of naphthoquinones, or formed inactive glutathione-naphthoquinone conjugates. From a chemical viewpoint, glutathione can react with the quinoid ring at C-2 or/and C-3 as the preferential sites via sulfhydryl arylation (Takahashi *et al*., 1987; Inbaraj and Chignell, 2004). Our TLC analysis confirmed this assumption and showed that all naphthoquinones except (**6**–**10**), (**19**), (**20**), and (**21**), produced glutathione-naphthoquinone conjugates (Section 3.6.3.2.1). Furthermore, this assumption has been confirmed for 2,3-dimethoxy-1,4-naphthoquinone (**10**) and has been attributed to the fact that the two electrophilic positions of the quinoid moiety are blocked by methoxy groups, and its activity was due to redox cycling (Gant *et al.,* 1988). The same probably holds true for 2-hydroxy-3-methyl-1,4-naphthoquinone (**7**), lapachol (**8**), 3,3`-biplumbagin (**19**), and the furanonaphthoquinones (**20**) and (**21**) with substituents at both C-2 and C-3. As a consequence, they are devoid of electrophilic addition. Furthermore, ESR

studies have shown that the reaction of naphthoquinones with glutathione occurs mainly at C-2 of the quinoid nucleus (Öllinger and Brunmark, 1991; Takahashi *et al*., 1987), which emphasizes that glutathione poorly reacts with lawsone (**6**) or 2-methoxy-1,4-naphthoquinone (**9**) substituted only at C-2 (Kot *et al.,* 2010). Within the group of the remaining naphthoquinones, which were only active in the absence of glutathione, 1,4-naphthoquinone (**1**), menadione (**2**), juglone (**3**), and plumbagin (**5**) have been reported to react with glutathione (Takahashi *et al*., 1987; Ross *et al.,* 1985; Doherty *et al.,* 1987; Castro *et al.,* 2008). Also, naphthazarin (**4**) and A/S have been shown to react with glutathione (Öllinger and Brunmark, 1991; Gao *et al.,* 2002). It may be assumed that naphthoquinones of the latter group formed inactive glutathione-conjugates via sulfhydryl arylation at the quinone entity (Gant *et al.,* 1988), thereby preventing redox cycling. Plumbagin (**5**) was one of the naphthoquinones which arylated nucleophiles and yielded plumbagin-glutathione conjugate as shown in Fig. 53. Independent support of this conjecture was obtained from HPLC analysis affording three distinct peaks (glutathione; Rt 3.5 min, plumbagin-glutathione conjugate; Rt 14.6 min, and plumbagin; Rt 27.3 min) (Fig. 54). Interestingly, the plumbagin-glutathione conjugate was ineffective against both extra- and intracellular *Leishmania* (IC_{50} > 80 µg/ml) (Fig. 55) thus supporting the above-mentioned findings. Also, it did not exhibit any cytotoxic effects on macrophages even at the highest test concentration (EC_{50} > 80 µg/ml). The production of reactive oxygen species may well contribute to the antileishmanial activity of plumbagin (**5**). Therefore, these candidates preferably undergo electrophilic addition to produce naphthoquinone-thiol conjugates, which are devoid of any antileishmanial potential. Effective concentrations may therefore only be reached by concentrations exceeding the cellular thiol level. Indeed, depletion of thiol levels in cells by, e.g., naphthoquinones, has been shown to induce apoptosis (Gao *et al.,* 2002). In contrast, 2-hydroxy-1,4-naphthoquinone (**6**) and related analogues are less prone to produce naphthoquinone-thiol conjugates, and therefore retain antileishmanial activity in the presence of glutathione. In summary, the naphthoquinones did not stimulate the infected macrophages for NO release and their mode of antileishmanial actions is based on NO-independent mechanisms, with activities strongly depending on the nature and position of substituents.

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6. Publications

Ali A, Assimopoulou A.N, Papageorgiou V.P, Kolodziej, H. (2011).
Structure-antileishmanial activity relationship study of naphthoquinones and dependency of the mode of action on the substitution patterns. *Planta Medica*, 77: 2003–12.

Poster

Ali A, Kiderlen AF, Kolodziej H.
Antileishmanial activity and cytotoxicity of *Drosera peltata* extracts. Deutsche Pharmazeutische Gesellschaft, Landesgruppe Berlin, Berlin, Germany, 4th July 2008.

Ali A, Kiderlen AF, Kolodziej H.
Lapachol and a mixture of 5- and 8-hydroxy naphtha [2,3-b]furan-4,9-diones are prominent antileishmanial constituents of *Tabebuia avellanedae*. Deutsche Pharmazeutische Gesellschaft, Landesgruppe Berlin, Berlin, Germany, 2nd July 2010.

Ali A, Kiderlen AF, Kolodziej H.
Lapachol and isomeric 5- and 8-hydroxy-2-(1'-hydroxyethyl)naphtho [2,3-b]furan-4,9-diones are effective antileishmanial constituents of *Tabebuia avellanedae*. 58th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Berlin, Germany, 29th August – 2nd September 2010. *Planta Medica,* 76: 1312 (P471), Abstract volume.

Ali A, Kiderlen AF, Kolodziej H.
Antileishmanial mode of action of lapachol and plumbagin. 58th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Berlin, Germany, 29th August – 2nd September 2010. *Planta Medica*, 76: 1312 (P472), Abstract volume.

Lectures

Ali A, Kiderlen AF, Kolodziej H.
The naphthoquinones lapachol and plumbagin exhibit different antileishmanial mode of actions. Pharmazeutische Gesellschaft, Landesgruppe Berlin, Berlin, Germany, 2nd July 2010.

7. Statutory Declaration

Hereby, I testify that this thesis is the result of my own work and research, using only the cited references and resources. I certify that this thesis has not been accepted as a doctorate to date or has been judged to be inadequate. The examination process underlying the PhD regulations is known to me.

Berlin, 15.09.2012
Ahmad Ali