ANTITUBERCULAR AND ANTIMALARIAL ACTIVITY OF METABOLITES ISOLATED FROM CRUDE AND LEAD-LIKE ENHANCED (LLE) EXTRACTS FROM SELECTED NAMIBIAN MEDICINAL PLANTS

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN SCIENCE (CHEMISTRY AND BIOCHEMISTRY) OF THE UNIVERSITY OF NAMIBIA

BY

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Abstract

Medicinal plants remain an important source of new lead compounds and drugs, but the re-isolation of known compounds and the loss of bioactivity during purification impedes the discovery of novel bioactive compounds. Of interest to this study, is a protocol developed by Camp and co-workers, which involves enhancing the quality of plant extracts by frontloading them with metabolites with drug-like properties, that is, generating lead like enhanced (LLE) extracts. This approach was successfully applied and yielded novel antiplasmodial and antitrypanosomal compounds but has not been explored to search for antitubercular compounds or drug leads. The aim of this study was (i) to prepare crude organic and aqueous extracts of 25 plant parts obtained from eight indigenous Namibian medicinal plants, (ii) to evaluate their antiplasmodial and antimycobacterial activity, and (iii) to correlate their ethnomedicinal use with the biological results obtained in this study. It was further aimed at evaluating the antiplasmodial and antimycobacterial activity of the LLE extracts and MeOH fractions – obtained from the active crude extracts – as well as to isolate and characterize the bioactive compounds.

Eight plant species which are used traditionally for the treatment of tuberculosis, malaria and associated symptoms, were collected at Uis in the Erongo region and Tsumkwe in the Otjozondjupa region. Plants included were: *Terminalia sericea*, *Adansonia digitata*, *Ozoroa paniculosa*, *Diospyros lycioides*, *Albizia anthelmintica*, *Combretum imberbe*, *Aloe dichotoma* and *Sarcocaulon marlothii* Engl. The antiplasmodial activity was tested *in vitro* using the parasite lactate dehydrogenase assay against *Plasmodium falciparum* (CQS) NF54 and the *in vitro* antimycobacterial
activity testing was done using the standard broth microdilution method against *Mycobacterium tuberculosis* H37Rv-GFP strains.

The preliminary biological activity results showed that 10 crude extracts (8 organic and 2 aqueous) displayed antimycobacterial activity with an MIC$_{90} < 90.0 \mu g/mL$, whereas 4 crude extracts (1 organic and 3 aqueous) displayed antiplasmodial activity with IC$_{50} \leq 18.0 \mu g/mL$. The antimycobacterial and antiplasmodial activities for the fourteen crude extracts ranged from MIC$_{90}$ 9.94 – 86.8 $\mu g/mL$ and IC$_{50}$ 5.20 - 17.8 $\mu g/mL$, respectively. The African baobab tree, *A. digitata*, displayed the best antimycobacterial (bark, aq.: MIC$_{90} 9.94 \mu g/mL$) and antiplasmodial (stems, org.: IC$_{50}$ 5.20 $\mu g/mL$) activity. The stems of *S. marlothii*, an endemic and phytochemically unexplored medicinal plant, are traditionally used to treat tuberculosis. However, both the organic and aqueous extracts displayed poor antimycobacterial activity with MIC$_{90}$s of 103 $\mu g/mL$ and $>125 \mu g/mL$, respectively. Instead, both the organic and aqueous extracts displayed *in vitro* antiplasmodial activity with IC$_{50}$s of 8.80 $\mu g/mL$ and 17.8 $\mu g/mL$, respectively.

The antiplasmodial and antimycobacterial bioactive crudes were then subjected to solid phase extraction using Strata-X reversed phase cartridges prepacked with N-vinylpyrrolidone (NVP). The solvent systems used for elution was 70% MeOH:H$_2$O containing 1% trifluoroacetic acid followed by 100% MeOH, to yield the LLE and MeOH fractions, respectively. Compared to the bioactive antimycobacterial crudes, none of the LLE extracts displayed antimycobacterial activity. The MeOH fraction of the bark of *A. digitata* however, displayed a more than threefold increase in activity (MIC$_{90} 19.5 \mu g/mL$) compared to the organic crude (MIC$_{90} 70.7 \mu g/mL$). With regard to antiplasmodial activity, the MeOH fraction of the organic extract of the stems of *S.
*marlothii* displayed a twofold increase in antiplasmodial activity (IC$_{50}$ 4.30 μg/mL) compared to the crude (IC$_{50}$ 8.80 μg/mL). In accordance with the objective of the study, the crude organic extract of the stems of *S. marlothii* was subjected to flash chromatography, that is, to isolate and characterize potentially novel antiplasmodial compounds. Subfraction on E9 (IC$_{50}$ 6.464 ± 1.767 μg/mL), originating from the organic extract of the stems of *S. marlothii*, was obtained in small quantities (29.4 mg) and was impure as revealed by LC-MS data. The aromatic region of the $^1$H-NMR of subfraction E9 revealed that the major bioactive compound, contain a hydroxylated, trisubstituted aromatic ring with an unsaturated side chain, most likely a caffeic acid derivative.

The results obtained in this study supports the traditional use of *T. sericea* and *A. digitata* for the treatment of malaria and tuberculosis and their associated symptoms. It also supports the enrichment of extracts to expedite antimalarial drug research and recommends further purification of the crude or LLE extracts of *S. marlothii* for the unambiguously identification of the active compound/s which could serve as leads in antimalarial drug discovery.

**Keywords:** Medicinal plants; Namibia; malaria; antiplasmodial; tuberculosis; antimycobacterial; lead-like enhanced extracts; lead compounds
Conference Participation

1. Faculty of Science 6th Annual Science Research Conference, 14th-15th November 2019, University of Namibia, Main Campus, 2018.
   **Title:** Investigation of Biological Activity of Crude and Lead-Like Enhanced (LLE) Extracts from selected Namibian Medicinal Plants used in the Treatment of Malaria and Tuberculosis.

2. Faculty of Science 7th Annual Science Research Conference, 13th-14th November 2019, University of Namibia, Main Campus, 2019.
   **Title:** Exploration of Lead-Like Enhanced (LLE) front-loading to source lead compounds from medicinal plants in Namibia.
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<th>Definition</th>
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<tr>
<td>ADMET</td>
<td>Absorption, Distributions, Metabolism, Excretion, Toxicology</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>BPI</td>
<td>Base Peak Intensity</td>
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<tr>
<td>C</td>
<td>Carbon thirteen</td>
</tr>
<tr>
<td>13C</td>
<td>Carbon thirteen</td>
</tr>
<tr>
<td>CDD</td>
<td>Collaborative Drug Discovery</td>
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<tr>
<td>CM</td>
<td>Complementary medicine</td>
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<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<tr>
<td>CQS</td>
<td>Chloroquine sensitive</td>
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<td>DAD</td>
<td>Diode-Array Detection</td>
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<td>DCM</td>
<td>Dichloromethane</td>
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<td>Electrospray ionization</td>
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<td>Greater Mekong Subregion</td>
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<td>G Protein-Coupled Receptors</td>
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<td>Human Immune Virus</td>
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<td>HPLC</td>
<td>High-Performance Liquid chromatography</td>
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<td>H-bond</td>
<td>Hydrogen bond</td>
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<tr>
<td>1H-NMR</td>
<td>Proton nuclear magnetic resonance</td>
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<tr>
<td>IC50</td>
<td>Concentration inhibiting 50% of parasite growth</td>
</tr>
<tr>
<td>IR</td>
<td>Infra Red</td>
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<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
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<td>LLE</td>
<td>Lead-like enhanced extract</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Log $P$</td>
<td>Logarithm of the partition coefficient</td>
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<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant tuberculosis</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MHz</td>
<td>Megahertz</td>
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<tr>
<td>MIC$_{90}$</td>
<td>Minimum inhibitory concentration required to inhibit the growth of 90% of organisms</td>
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<td>MIC$_{99}$</td>
<td>Minimum inhibitory concentration required to inhibit the growth of 99% of organisms</td>
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<td>Medical Research Council</td>
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<td>National Botanical Research Institute</td>
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<td>Novel Chemical Entities</td>
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<td>Novartis Institute of Tropical Diseases</td>
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<td>NVP</td>
<td>N-Vinylpyrrolidone</td>
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<td>Trifluoroacetic acid</td>
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<td>TDR-TB</td>
<td>Totally drug resistant tuberculosis</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively drug resistant tuberculosis</td>
</tr>
</tbody>
</table>
Acknowledgements

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Declarations

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Name of Student                  Signature                  Date
Chapter 1: General Introduction

1.1 Background

Africa is considered to be the cradle of Mankind with a rich biological and cultural diversity revealed by marked regional difference in, for example, healing practices. The use of plants in traditional medicine (TM) is an integral part of the African culture despite the fact that this form of medicine is not as well documented as, for example Ayurvedic medicine in India and Chinese traditional medicine. Although the African continent has a high volume of endemic plant species, with the Republic of Madagascar topping the list at 82%, it is paradoxically also the continent to have one of the highest rates of deforestation in the world. Poor quality control and safety are also major obstacles in the use of African medicinal plants, and in addition, the African pharmacopoeia is incomplete, records are not comprehensive enough and remain so to date. The loss of indigenous knowledge is imminent and it is therefore becoming increasingly urgent to document the use of medicinal plants in Africa. The integration of TMs into the primary health care system will require the validation and standardization of TMs.

TM, sometimes referred to as complementary medicine (CM), is the main source, and in some cases the only source of health care for millions of people, because it is accessible and affordable, culturally acceptable and trusted. The fact that health care costs are on the rise, makes TM’s more attractive and it is used more frequently due to the increase in chronic, non-communicable diseases. Medicinal plants are widely used in traditional cultures all over the world, and are also becoming increasingly popular in modern society as natural alternatives to generic medicine. They continue to
represent an important resource for the discovery of new drugs, leads or richer sources of already known plant drugs\textsuperscript{5}. Direct ethnomedicinal selection is an important bioprospecting tool\textsuperscript{6} and this approach is described in literature as efficient when identifying plants of interest for phytochemical analyses\textsuperscript{7}.

A major challenge facing the use of traditional medicines is the proof that the active ingredient in medicinal plants are useful, not toxic and effective\textsuperscript{8}. Additionally, there are few reports on the adverse effects of TMs and herbal medicines and there exist clinical evidence on the life-threatening interactions of TMs and prescribed medicines\textsuperscript{9,10-12}.

Malaria is the one of the most worrisome parasitic diseases of human beings\textsuperscript{13}. Worldwide morbidity and mortality due to malaria continued to decrease and the global malaria community has grown increasingly supportive of the idea of malaria eradication\textsuperscript{14}. Disturbingly, the WHO World Malaria Report 2018, listed Namibia as the second highest country with respect to incidence of malaria in children under 5 years. Furthermore, this report revealed that Namibia, Botswana, South Africa and Eswatini are countries with an estimated increase of more than 20\% towards the burden of malaria\textsuperscript{15}. Low malaria transmission in Namibia suggests that elimination is possible, but the risk of imported malaria from Angola and neighbouring endemic countries, remains a challenge\textsuperscript{16}. Traditional and herbal remedies from medicinal plants, remain an alternative option of treatment in developing countries where malaria is endemic\textsuperscript{5,17-19}. 

2
Tuberculosis (TB) is the most important infectious disease and remains one of the major causes of death\textsuperscript{20}. It is a preventable disease, but the mortality rate among TB patients is unacceptably high according to the 2018 WHO Global report. Namibia is rated in the top 30 high TB burden countries in the world with estimates of 446 cases per 100 000 people since 2016\textsuperscript{21}. While TB occurs in every part of the world, Africa bears the greatest proportion of new cases. Reports on Sub-Saharan Africa reveal that 74\% of new TB cases are infected with HIV/AIDS, giving the region the highest co-infection rate in the world\textsuperscript{22}. Available drugs and vaccines have up to now made no significant impact on TB control. To add to this dilemma, the emergence of drug resistant TB is considered a public health crisis\textsuperscript{23-24}.

There is renewed interest in natural products and it is re-emerging as a major source of lead compounds as well as a major contributor to drug discovery and development. It is known that the classical process of isolating pure compounds from a bioactive extract is and has always been a long and labour-intensive process\textsuperscript{25}. Strategies to expedite drug discovery from plants such as dereplication studies\textsuperscript{25-28} and a protocol developed by an Australian research group, which is of interest to this study, are worth exploring. In brief the protocol entails the separation of compounds with drug-like properties in the crude extract called lead-like enhanced (LLE) extracts. Their approach is aimed at enhancing or prioritizing extracts and subsequent fractions with desired physicochemical properties, prior to isolation and characterization. According to reports, this method successfully yielded antiplasmodial and antitrypanosomal agents from extracts of plants and marine organisms\textsuperscript{29-30}.
1.2 Plants selected for the study

Namibia has a rich plant biodiversity and many rural communities make use of medicinal plants. In the Tsumkwe district of the Otjozondjupa region, there are more than 80 medicinal plant species identified that are used to treat various ailments. The plants shortlisted for this study were selected based on their ethnomedicinal uses, that is, for the treatment of malaria, TB and associated symptoms, as revealed by literature reports and personal communications with the local communities in the Tsumkwe and Uis [Mr. Thorou, Member of the Daureddaman Traditional Authority, Uis, 24th February 2015; 25th April, 2017; Naici, Field Guide in Tsumkwe, 21st January, 2015]. Symptoms of malaria, include high fever, sweating, chills, headache, vomiting and diarrhoea. TB symptoms (Fig. 1.1) include persistent coughing with or without blood, chest pain when breathing or coughing, unintentional weight loss, fatigue and fever. The plants selected for this study are indigenous to Namibia, not endangered, abundant and include: *Terminalia sericea* (antimalaria and antitubercular), *Adansonia digitata* (antitubercular and antimalaria), *Ozoroa paniculosa* (antitubercular), *Diospyros lycioides* (antitubercular), *Albizia anthelmintica* (antimalaria) from Tsumkwe, and *Combretum imberbe* (antitubercular), *Aloe dichotoma* (antitubercular) and *Sarcocaulon marlothii* Engl. (antitubercular) from Uis.

The scientific data obtained from the eight plants tested will be made available to the Khoisan and Damara communities and may be used to correlate their traditional use and the efficacy for which they are being used.
1.3 Statement of the problem

Malaria infections are decreasing, however parasite resistance to current antimalarial drugs for example artemisinin, and resistance to insecticides by vector mosquitoes, threaten to derail efforts to eliminate malaria\textsuperscript{15}. A survey on Namibian medicinal plants used in the treatment of malaria and associated symptoms revealed that many communities claim to use medicinal plants successfully\textsuperscript{36-38}. However, ethnomedicinal uses backed by scientific validation, need to be done. This is an important problem to be addressed by this study.

The TB burden is a concern in Namibia. There exist few reports that have claimed the emergence and increasing frequency of multidrug resistant (MDR-TB), extensively drug resistant (XDR-TB) and of totally drug-resistant TB (TDR-TB) strains, which have consequently led to a limited chance that the current drug therapies are successful\textsuperscript{23-24}. Although there are sufficient numbers of anti-TB drug candidates in the lead optimization stage, there is a worrying gap within the clinical trials stage that
needs to be filled\textsuperscript{24}. Considering that most currently used TB-drugs are derived from natural products, albeit that most were sourced from micro-organisms, new advances in isolation and method development could expedite the search for new drug-leads from plants. These developments highlight the urgent need to search and develop new antitubercular drugs so as to overcome the challenges of drug resistance and eventually to eradicate TB\textsuperscript{24}.

The classic process of isolating natural products, is slow, costly, separation is poor, loss of bioactivity in the purification process, and possibly only to discover that the compounds are known\textsuperscript{21}. This can be done by phytochemical screening, bioactivity-guided isolation and characterization and evaluation of pharmaceutical importance through the use of modern scientific methodology for example, chromatography and spectroscopic analyses, accompanied by biological and toxicity testing\textsuperscript{38}. The approach of preparing LLE extracts from crude extracts can expedite the discovery and, more specifically, isolation of natural product leads\textsuperscript{25}.

One of the plants selected for this study, \textit{Sarcocaulon marlothii}, is endemic to Namibia and has not been researched before. There is a lack of detailed phytochemical and biological data for this species which is used traditionally to treat the symptoms of tuberculosis. Although many studies have been done on Namibian medicinal plants, these studies are limited to biological activity screening. To date, a detailed in-depth phytochemical analysis is lacking, which could explain why there is little information correlating the ethnomedicinal use plants in Namibia, with sound scientific data.
1.4 Aim

The major aims of this project were as follows: firstly, to investigate the biological activities of extracts from plants commonly used to alleviate and/or treat the symptoms of tuberculosis and malaria. Secondly, to prepare the LLE extracts and methanol fractions from the biologically active extracts and to evaluate antimycobacterial and antimalarial activities of these. The final aim was to investigate constituents in the biologically active extracts and fractions by isolation, purification and structural elucidation to identify compounds which may have the potential to be developed into possible new drugs.

1.4.1 Objectives of study

The objectives were to,

- evaluate the antiplasmodial and antimycobacterial activity of organic and aqueous crude extracts of various parts of eight (8) plant species used ethnomedicinally to treat TB and malaria as well as their symptoms.
- correlate the ethnomedicinal use of the plants with the biological activities of the crude extracts.
- evaluate the antiplasmodial and antimycobacterial activity of LLE extracts and methanol fractions prepared from the biologically active crude extracts.
- identify the bioactive compounds from prioritized LLE and crude extracts.

1.5 Significance of study

This study intends to contribute towards the preservation of indigenous knowledge of Namibian medicinal plants used traditionally by the Khoisan and Daureddaman communities to treat malaria and tuberculosis. It is also envisaged that the study may contribute to and encourage sustainable harvesting and conservation of the flora as a
rich and biodiverse natural resource of Namibia. The phytochemical and bioactivity results obtained in this study will be available for documentation and could be included in local inventories.

A new methodology was applied in this study to expedite the search for lead- or drug-like compounds, by prioritizing extracts with favourable physicochemical properties. This lead-like enhanced (LLE) approach was applied as a first step to expedite the discovery of potential antiplasmodial and antitrypanosomal compounds, and for the first time in this study to source antimycobacterial agents. It is therefore expected that results from this study can either support previous reports on the utility and success of the LLE approach to source drugs for the two disease models under investigation.
1.6 References


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Chapter 2: Literature Review

2.1 Medicinal plants

Medicinal plants continue to play an important role in traditional medicine\(^1\) (TM)\(^1\) and represent an important resource for the discovery of new drugs, lead compounds or richer sources of already known plant drugs\(^2\). Well-known examples of plant-derived medicines include morphine, codeine, atropine, and digoxin as well as the important anticancer drug, taxol (Fig. 2.1)\(^2-3\). Medicinal plants of commercial importance in Africa include: *Agathosma betulina* (buchu), *Aloe ferox* (cape aloes), *Aloe vera* (true aloe, burn aloe or first aid plant), *Artemisia afra* (african wormwood), *Lessertia frutescens* (cancer bush), *Boswellia sacra* (frankincense), *Catha edulis* (khat), *Harpagophytum procumbens* (devil’s claw), *Hoodia gordonii* (bushman’s hat), *Hibiscus sabdariffa* (hibiscus) and *Hypoxis hemerocallidea* (african potato)\(^4-5\).

\[
\begin{align*}
\text{Morphine} & \quad \text{Codeine} & \quad \text{Atropine} \\
\text{Digoxin} & \quad \text{Taxol}
\end{align*}
\]

**Figure 2.1:** Structures of some important drug molecules isolated from medicinal plants.

---

\(^{1}\) A combination of knowledge and practice in the diagnoses, elimination and prevention of disease, is known as Traditional medicine (TM). Herbal medicine, also called botanical medicine, is part of TM where the herbalist (or traditional healer) use herbs and medicinal plants to treat various ailments\(^1\).
2.1.1 Herbal formulations from medicinal plants

Herbal medicines are defined by the WHO as “medicinal products that contain aerial or underground parts of plants, or other plant material, or combinations thereof, as active ingredients, either in the crude state or as plant preparations”\(^6\). Herbal medicines may contain excipients in addition to the active ingredients, which may be a combination of pharmacologically active plant substances that can work synergistically to produce an effect greater than the sum of the effects of the single constituents\(^6-7\). The defensive immune system is one of the most complex biological systems in the human body and survival is dependent on this mechanism. It assists the host to control microbes, allergens or toxic molecules and prevent the development of cancer. Medicinal plants with immunomodulatory properties are a recent concept in phytomedicine and herbal extracts can function as immunomodulatory suppressive or stimulants in the body. Immunomodulators can be defined as a chemical agent that modifies the immune response or functioning of the immune system. Examples of immunomodulatory herbal plants include, *Curcuma longa* (curcumin), *Rhododendron spisiferum* (proanthocyanidin A-1), *Allium sativum* (immunomodulatory proteins in raw garlic), *Echinacea purpurea*, *Calendula officinalis*, and *Panax ginseng*\(^8-11\).

2.1.1.1 Herb-drug interaction

Herbal medicines are generally believed to be safe because they are “natural”. This assumption is hazardous and in recent reviews it has been reported that there are different and adverse side effects caused by herb-drug interactions\(^12-13\). The risk of herb-drug interactions is a real concern because the use of herbs may mimic, magnify or oppose the effect of the drugs. Some examples of well-known herb-drug interactions include the occurrence of bleeding when warfarin is combined with ginkgo (*Ginkgo*
biloba), and the noticing of mild serotonin syndrome in patients who mix St John’s wort (*Hypericum perforatum*) with serotonin-reuptake inhibitors. Another example of herb-drug interactions is purpura, also called blood spots or skin haemorrhages, resulting from the use of devil’s claw (*Harpagophytum procumbens*), which is indigenous to Southern Africa, with warfarin. In addition, the African potato (*Hypoxis hemerocallidea*) and cancer bush (*Lessertia frutescens*), formerly known as *Sutherlandia frutescens*, showed a negative interaction with antiretroviral medication.

The concomitant use of herbal formulations containing these plants with antiretrovirals, may put HIV patients at risk of treatment failure, viral resistance or drug toxicity\textsuperscript{14}. Plant medicines often contain a mixture of substances that have additive or even synergistic effects, so that health benefits are often difficult to test and verify\textsuperscript{15}. Despite these challenges, it has been shown that the scientific validation which includes the confirmation of the safety and efficacy of the traditional medicines, requires the isolation and characterization of secondary metabolites\textsuperscript{15}. It should therefore be used with care, proper consultation and information should be provided by health care practitioners when mixing herbs and pharmaceutical drugs as interaction with most drugs is not known\textsuperscript{14}.

2.1.1.2 Issues and challenges in natural product research

Selection of plants for phytochemical and/or pharmacological studies based on their traditional use, is considered an effective approach in the discovery of the active ingredient and novel compounds\textsuperscript{16-18}. One must however, be aware of the challenges posed by selecting plants on the basis of their ethnomedicinal uses. These challenges include the following: (i) plants as biological systems have the inherent potential to vary their chemistry and resulting biological activity, for example, plants that show
promising biological activity in initial assays may fail to display the activity on subsequent re-collections; (ii) the phytochemical profile usually varies between plant parts, within developmental periods and sometimes even diurnally; (iii) marked differences can usually be seen in the phytochemical profile between individual plants of a single population, and more so between members of different geographical populations\textsuperscript{15,17}. Additionally, countries with the most biological diversity, have either prohibited collection of plant material for export or have introduced laws on access benefit sharing, in accordance with the Kyoto Protocol\textsuperscript{19}. This explains why in the 1980’s until the early 2000’s, Big Pharma companies have terminated or scaled down their natural product operations\textsuperscript{20-21}.

2.2 Natural products: secondary metabolites

The chemical compounds in medicinal plants are known as secondary metabolites (natural products)\textsuperscript{15}. It is known that these secondary metabolites, in the form of nutraceuticals when taken as a decoction or infusion etc., contribute to the protection of human health, when their dietary intake is significant\textsuperscript{22}. Secondary metabolites or natural products (NP) are chemicals which are not directly involved in the normal growth and development, or reproduction of an organism, but offer a competitive advantage to the producer. Plants have evolved numerous defence strategies which include producing chemicals that deter herbivores, offer protection against microbial infections (bacteria, fungi) and even other plants competing for light, space and nutrients, to name a few. Also, secondary metabolites, can serve as signalling compounds to attract animals for pollination and seed dispersal. Secondary metabolites can be classified by structure, composition (nitrogen-containing or not), their solubility in various solvents or the pathway by which they are synthesized (Fig. 2.2). More than
one active chemical class of secondary metabolites are present in complex mixtures and it is therefore likely that several targets are affected simultaneously when taking herbal formulations\textsuperscript{15}.

\textbf{Figure 2.2:} Metabolic pathways for the production of secondary metabolites\textsuperscript{23}.

Secondary metabolites have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property\textsuperscript{22}. Classification based on biosynthetic origin lead to three classes: alkaloids (nitrogen-containing compounds), terpenoids and phenolic compounds as shown in Fig. 2.2\textsuperscript{16, 24}.
2.2.1 Classes of secondary metabolites

The classes of compounds discussed in this section was based on the chemotaxonomic information on the plant species collated during the literature review.

2.2.1.1 Terpenes

Terpenes form a large family with diverse structures of natural products, derived from \( C_5 \) isoprene units which are joined in a head-to-tail fashion. Typical structures contain carbon skeletons represented by \((C_5)^n\). Triterpenes \((C_{30})\) are compounds which consist of six five-carbon isoprene units and include steroids. There are at least 4000 known triterpenes and some occur freely, while others occur as glycosides (saponins). Different triterpenes were identified in members of the genus *Sarcocaulon* with tetracyclic and pentacyclic triterpenes being the two main classes. The pentacyclic triterpenoids are mainly classified as the \( \alpha \)-amyrin, \( \beta \)-amyrin, oleanane, friedelin, lupine and hopane type of triterpenoids. Examples of triterpenes isolated from medicinal plants in South Africa, *Mimisops caffra* (leaves) and *M. obtusifolia* (bark), include: ursolic acid, taraxerol, and sawamillitin. Ursolic acid showed appreciable antiplasmodial activity \((IC_{50} 6.8 \mu g/mL)\) against the chloroquine sensitive (CQS) strain of *P. falciparum*.

![Chemical structure of triterpene, ursolic acid.](image)

**Figure 2.3:** Chemical structure of triterpene, ursolic acid.
2.2.1.2 Phenolic acids

The term “phenolic acids” are assigned to phenols that possess one carboxylic acid functional group\textsuperscript{22} and they fall in the class of phenolic compounds which have at least one aromatic ring with one or more hydroxyl groups attached\textsuperscript{30}. Phenolic acids which occur naturally contain two carbon framework: the hydroxycinnamic (C\textsubscript{6}\textDash C\textsubscript{3}) and hydroxybenzoic (C\textsubscript{6}\textDash C\textsubscript{1}) structures\textsuperscript{22,31} and they are commonly found conjugated to sugars and organic acids (Fig. 2.4)\textsuperscript{32}.

![Structures of benzoic acid and cinnamic acid derivatives.](image)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Chemical Formula</th>
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<tr>
<td>p-hydroxybenzoic acid</td>
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</tr>
<tr>
<td>protocatectic acid</td>
<td>R=OH, R'=H;</td>
</tr>
<tr>
<td>vanillic acid</td>
<td>R=OCH\textsubscript{3}, R'=H;</td>
</tr>
<tr>
<td>gallic acid</td>
<td>R=R'=OH;</td>
</tr>
<tr>
<td>syringic acid</td>
<td>R=R'=OCH\textsubscript{3};</td>
</tr>
</tbody>
</table>

**Figure 2.4:** Structures of benzoic acid and cinnamic acid derivatives.

Hydroxycinnamic acids, have the role of basic precursors in the biosynthesis of various plant phenols. Phenolic acids function as reducing agents, free radical scavengers, and quenchers of singlet oxygen formation and are commonly researched for their antioxidant properties. Antioxidants are substances that significantly delay or prevent the oxidation of an oxidisable substrate when present in low concentrations. Oxidative stress and the damage it causes play a role in the development of heart, age-related neurodegenerative diseases, (e.g. Parkinson’s disease), and cancer\textsuperscript{32}. There is an increase in awareness of the importance of antioxidant activities of phenolic acids and their potential inclusion in processed food\textsuperscript{22}. Phenolics are responsible for antioxidant,
antimicrobial, anti-inflammatory, antihyperglycaemic and cytotoxic activity\textsuperscript{33}. Ellagitannins, are polyphenols that exhibit antioxidant, antimicrobial, anti-inflammatory, anticancer properties and more importantly, activity against methicillin-resistant \textit{Staphylococcus aureus}\textsuperscript{34-35}.

\textbf{2.2.1.3 Flavonoids}

Like phenolic acids, flavonoids also fall in the class of phenolic compounds which have at least one aromatic ring with one or more hydroxyl groups attached\textsuperscript{30}. All flavonoids are derived from the aromatic amino acids, phenylalanine and tyrosine and are C15 compounds arranged in three rings (\(\text{C}_6\text{C}_3\text{C}_6\)), consisting of two aromatic \(\text{C}_6\) rings (A and B) and a heterocyclic ring C that contains one oxygen atom (Fig.2.5)\textsuperscript{30-32}. More than 4000 flavonoids have been identified, and many occur in vegetables, fruits, and beverages like tea, coffee, and fruit drinks.

\begin{center}
\textbf{Figure 2.5:} Basic structure of flavonoids.
\end{center}

Flavonoids can be classified according to the substitution profile of the heterocyclic ring, the position of the secondary aromatic ring (B) as well as the oxidation state of the heterocyclic ring. The six major subclasses of flavonoids are:\textsuperscript{32}

1. Flavones (luteonin, apigenin, tangeritin).
2. Flavonols (quercetin, kaempferol, myricetin, isorhamnetin, pachypodol).
3. Flavanones (hesteretin, naringenin, eriodictyol).
4. Flavan-3-ols (catechins and epicatechins).
5. Isoflavones (genistein, daidzein, glycine).

6. Anthocyanidins (cyaniding, delphinidin, malvidin, pelagonidin, peonidin, petunidin).

Flavonoids have diverse biological and pharmacological activities, including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumour activities. The most well-known property, however, is their capacity to act as powerful antioxidants, which as described previously, can protect the human body from free radicals and reactive oxygen species\(^{22}\).

### 2.2.1.4 Alkaloids

Alkaloids are low molecular weight nitrogen-containing compounds found mainly in plants. They are present to a lesser extent in microorganisms and animals. More than 27,000 different alkaloid structures are known and 21,000 of these are from plants\(^ {25}\).

Alkaloids derive biosynthetically from amino acids and represent a large, diverse class of compounds which contain a basic amine group in their structure\(^ {24}\). This confers basicity on the alkaloids which facilitates their isolation and purification\(^ {25}\). They can be classified based on their biosynthetic precursor and heterocyclic ring system. Classification based on the latter yields the indole, piperidine, tropane, purine, pyrrolizidine, imidazole, quinolizidine, isoquinoline and pyrrolidine alkaloids\(^ {36}\).

Alkaloids derived from plants comprise about 15.6% percent of known natural products and they constitute almost 50% of the plant-derived natural products of pharmaceutical and biological value. In addition, more than one-third of the alkaloids that have been examined for biological activity in 20 or more assays, are known
potential pharmacological agents. They have given humanity a wide range of natural products that are useful in the cure of various ailments. Alkaloids are reported to have anti-cancerous, immune stimulant properties and antimicrobial activity.

2.2.2 Natural products from medicinal plants as a source of lead compounds in drug discovery

A lead compound, typically termed a developing drug candidate, is defined as a chemical compound that has some desirable biological activity as well as being therapeutically useful. This molecule can be characterized and modified to produce another molecule with better pharmacokinetic properties. A lead compound is therefore the first foothold on the drug discovery ladder. Lead compounds have structures that typically exhibit sub-optimal target binding affinity and should display the following properties, that is, to be considered for further development: 1) relatively simple features, amenable for combinatorial and medicinal chemistry optimization efforts; 2) membership to a well-established SAR (structure-activity relationship) series wherein compounds with similar (sub)-structures exhibit similar target binding affinity; 3) favourable patent situation; and 4) good ADMET (absorption, distribution, metabolism, excretion and toxicology) properties. Quinine and artemisinin are examples of lead compounds sourced from plants as antimalarial drugs. A lead compound with potent anti-TB activity, is the naphthoquinone, 7-methyljuglone (Fig. 2.12), from the genus Euclea.

According to a review by Newmann and Cragg, entitled: Natural Products as Sources of New Drugs from 1981 to 2014, natural products continue to play a significant role in the discovery of leads (Figure 2.6). As indicated in figure 2.6, natural products and
natural product-related compounds constitute 51% of all new drugs approved during this period\textsuperscript{47}.

![Figure 2.6: All new approved drugs covering the period 1981-2014; n = 1562\textsuperscript{47}. B = Biological macromolecule; N = Unaltered natural product; NB = Natural product botanical drug; ND = Natural product derivative; S = Synthetic drug, S* = Synthetic drug (Natural product pharmacophore); V = Vaccine; NM = Natural product mimic.]

\textbf{2.2.3 Isolation and characterization of bioactive and lead compounds}

It is known that the classical process leading from a bioactive extract to a pharmacologically pure compound is long, labour-intensive and starts with the preparation of an organic and/or aqueous extractions (Fig. 2.7). This is followed by condensing and drying the crude extracts in a short as possible time to avoid artefact formation. Measures need to be taken to prevent microbial growth in aqueous extracts. The crude extracts are then subjected to biological screening after which the active crude extracts are subjected to fractionation as part of a bioactivity guided isolation. The fractionation process involves several consecutive steps of preparative chromatographic separation, after which each fraction is submitted for biological testing until a pure bioactive compound is obtained. This has led to the successful isolation of many bioactive molecules in the past. The shortcomings of this process include the following: it is slow, costly, separation is poor, loss of bioactivity during
purification, and there is the likelihood of isolating compounds that are either known or uninteresting\textsuperscript{17,48}.

![Diagram of the isolation process of pure compounds from plants]

**Figure 2.7**: The isolation process of pure compounds from plants\textsuperscript{17}.

At the beginning of the 21\textsuperscript{st} century, modern drug discovery methods was revolutionized by the concerted use of so-called “hyphenated techniques” (Fig. 2.7). These techniques entail the combination of sensitive and rapid analytical techniques with on-line spectroscopic methods which simultaneously generate different and complementary chemical information\textsuperscript{16}. Examples of these are, High Performance Liquid chromatography with Diode-Array Detection (HPLC-DAD), -Mass Spectrometry (-MS) and -Nuclear Magnetic Resonance (-NMR) and these have increased the workflow and decreased the timelines significantly. The characterization of secondary metabolites in biological extracts using the above-mentioned methods provide a wealth of structural information with minute amounts of sample\textsuperscript{17,48}.
Camp *et al.* emphasized the importance of physicochemical properties of molecules in the development of drugs administered orally and the subsequent bioavailability of the drugs. In their approach, they enhanced both the extracts and subsequent fractions, which possess the desired physicochemical properties i.e. their log $P < 5$. Emphasis was placed on improving cell permeability, by prioritizing the physicochemical properties of biologically active small molecules early in the drug discovery process\textsuperscript{49}. Considering that some bioactive compounds are present in trace quantities and that the activity of some may be masked in plant extracts, as well as the fact that natural products do not always comply with Lipinski’s rule of five, it is plausible that, the aforementioned approach may lead to the premature elimination of plants.

Despite technological breakthroughs with the analysis, purification and structure elucidation of NPs over the last 15 years, tracking bioactivity in complex matrices such as plant extracts, remains a very challenging task.

### 2.3 Infectious diseases

#### 2.3.1 Malaria

Malaria is a mosquito-borne disease which in humans is caused by five protozoa: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and related sibling species *P. ovale*, and *P. knowlesi*. *Plasmodium vivax* is the most cosmopolitan of the human malarial species, reaching historical latitudinal extremes of 64° north and 32° south\textsuperscript{50}. The public health burden posed by *P. vivax* is no longer regarded as benign, because of the severe morbidity and death it causes\textsuperscript{51}. Nevertheless, *P. falciparum* remains the single most important threat to public health at a global scale, accounting for more than 90% of the world’s malaria mortality\textsuperscript{52}. In Namibia, transmission pockets remain primarily in the
Kunene -, Omusati -, Ohangwena –Kavango West -, Kavango East -, and the Zambezi regions, where malaria remains high and vulnerability is greater due to continuous population movement from neighbouring endemic countries53-55.

2.3.1.1 Medicinal plants with antimalarial activity

For decades, traditional plant-based herbal medicine made an immense contribution to malaria chemotherapy, either as direct antimalarial agents, or as important lead compounds for the discovery of more potent antimalarial drugs. Two of the most common and widely used antimalarials originated from plants namely artemisinin (Fig. 2.8) from Chinese _Artemisia annua_ and quinine (Fig.2.9) derived from the bark of the Peruvian _Cinchona_ L. tree56-58. The chemical structures of their respective semi-synthetic derivatives are shown in Figures 2.8 and 2.9.

The use of drugs in combating malaria has been faced with an important challenge: the emergence of drug resistance parasites. This has made many of the first line drugs such as chloroquine (CQ) ineffective57. _P. falciparum_ resistance to artemisinin has been detected in five countries of the Greater Mekong subregion (GMS): Cambodia, the Lao People’s Democratic Republic, Myanmar, Thailand and Vietnam. In many areas along the Cambodia–Thailand border, _P. falciparum_ has become resistant to most available antimalarial drugs59. Despite this, there has been a massive reduction in malaria cases and deaths in this subregion. In Africa, artemisinin resistance has not been reported to date60.
A review done by Chinsembu, reported on different plant species that are used to treat malaria in various countries of Africa\(^6\). In a study on medicinal plants which are traditionally used to combat malaria in the Ameru community in Kenya, it was found that the MeOH root bark extract of *Maytenus obtusifolia* (IC\(_{50}\) < 1.9 μg/mL) displayed promising antiplasmodial activity against *P. falciparum* D6 (CQS)\(^{57}\). Several species of *Maytenus* have their biological activities proven experimentally. Among them was the ethyl acetate extract of the stem bark of *M. senegalensis* which also revealed antiplasmodial activity (0.2 μg/mL)\(^{62-63}\).
Seven medicinal plants in Cameroon used in malaria treatment displayed antiplasmodial activity in vitro against the chloroquine resistant (W2) strain of *P. falciparum* with IC$_{50}$ < 5 μg/mL. They are *Uvariopsis congolana* (De Wild) Fries, *Polyalthia oliveri* Engl., *Artocarpus cummunis*. Forst, and the most active was *Enantia chlorantha* Oliv. with an IC$_{50}$ of 0.68 μg/mL$^{64-65}$.

In a study done on Namibian medicinal plants it was found that extracts of *Vahlia capensis*, *Nicolasia costata*, and *Dicerocaryum eriocarpum* displayed activities against the *P. falciparum* chloroquine sensitive strain (3D7)$^{66}$. The leaves of wild sesame, *Sesamum triphyllum* are used to treat malaria$^{67}$. Medicinal plants remain a very important source in which new antimalarial therapies may be discovered$^{57}$.
2.3.2 Tuberculosis

The spread of HIV is cited to be responsible for the increase in TB incidences in sub-Saharan Africa. Namibia is listed among the high TB and TB/HIV burden countries\textsuperscript{68}. Tuberculosis remains one of the major causes of death. It was estimated in 2014 that 9.6 million people developed TB and 1.5 million died from the disease, of whom 400 000 were HIV-positive\textsuperscript{69-70}. It generally infects the lungs and can be transmitted from person to person via droplets from the throat and lungs of people with the active disease\textsuperscript{71}. This contagious disease latently infects over 2 billion people worldwide\textsuperscript{72} and it has therefore been suggested that \textit{Mycobacterium tuberculosis}, the causative agent of TB, is responsible for more human deaths than any other single microbial pathogen\textsuperscript{73}. While TB occurs in every part of the world, Africa represents 28\% of the world TB burden\textsuperscript{70,74}. Neighbouring countries: South Africa, Zimbabwe and Angola, are listed as countries with the highest estimated numbers of multidrug-resistant TB (MDR-TB) cases\textsuperscript{68}. Namibia does not appear on this list which raises the question: if comprehensive surveys are conducted periodically to assess MDR-TB cases\textsuperscript{75}. The Ju/hoansi speakers, who live in the Tsumkwe area of the Otjozondjupa region in northeastern Namibia, are disproportionately affected by TB and many lives are claimed by it each year\textsuperscript{76}.

Tuberculosis is a preventable disease, however, the mortality rate among TB patients is unacceptably high according to the WHO Global report 2018\textsuperscript{68}. TB treatment comprised of a two-month intensive phase of isoniazid, rifampicin, ethambutol and pyrazinamide, followed by the continuation phase which lasts four months with isoniazid and rifampicin therapy (Fig. 2.10)\textsuperscript{77}. Interruptions in the use of these prescribed drugs and inefficient healthcare centres contributed to the development of
MDR-TB, which is defined as resistance to at least two front-line drugs, isoniazid and rifampicin. The duration of treatment of MDR-TB lasts at least twenty months with second-line drugs comprising capreomycin, kanamycin, amikacin and fluoroquinolones. These are more toxic and less efficient and MDR-TB cure rates are estimated at 60-75%\textsuperscript{77}. Riccardi \textit{et al.} estimated that of the 450 000 people who developed MDR-TB in the world, about 9.6% are extensively drug resistant (XDR-TB)\textsuperscript{78}. This means these patients developed additional resistance to at least one fluoroquinolone and one injectable second-line drug. For these patients, the chances of successful treatment are quite low. Major concerns are drug interactions during rifampicin treatment of HIV patients. The efficacy of HIV drugs is reduced or even completely absent when they are coadministered with rifampicin\textsuperscript{79}.

Progress has been made in research and development of new drugs for TB over the last decade. Bedaquiline (diarylquinoline) and delamanid (nitroimidazole) (Fig. 2.10) are two new drugs which received fast-tracked approval for use in the treatment of multidrug-resistant TB (MDR-TB) which have emerged over 2013–14, and the WHO has developed interim guidance on their use\textsuperscript{80-81}. There has been an increase in the number of new anti-TB drugs in the pipeline and there are currently a few drug candidates in the lead optimization stage, preclinical development, phase II and phase III clinical trials. However these new anti-TB compounds must overcome the problems with existing therapy, namely: lengthy treatment, high pill burden, high cost, side effects, interaction with other drugs, drug resistant \textit{M. tuberculosis} strains and lack of efficacy against latent TB\textsuperscript{81}. 
Further, novel drug regimens to shorten treatment of drug-susceptible and/or drug-resistant TB including new or re-purposed drugs, are under investigation\textsuperscript{80}. Efforts to combat TB deaths therefore need to be accelerated in order to meet the 2020 global targets\textsuperscript{68}. It has become urgent to prioritize the search and development of new antitubercular drugs so as to overcome the challenges of drug resistance and eventually to eradicate TB\textsuperscript{82}.

\textbf{Figure 2.10}: Chemical structure of antituberculosis drugs in current use.
2.3.2.1 Medicinal plants with antitubercular activity

Drugs currently used as first- or second-line drugs in the treatment of TB, e.g. rifampicin and streptomycin, are natural products from microorganism origin. Research which involved the screening of medicinal plant extracts showed that phytochemicals can serve as potential anti-tuberculosis drugs or lead compounds. Among the different classes of compounds identified are alkaloids, terpenoids, coumarins/chromones, peptides and phenolics.

It is noteworthy that most of the plants which displayed antimycobacterial activity, were used as ethnomedicine for the treatment of tuberculosis and/or related symptoms. A study by Adeleye et al., revealed that both the ethanolic and aqueous extracts of four Nigerian medicinal plants, *Allium cepa*, *Allium ascalonicum*, *Terminalia glaucescens* and *Securidaca longipedunculata* (ethanolic extract only), inhibited the growth of *M. tuberculosis* at a concentration of 0.05 mg/mL. A review by Oosthuizen et al., 2018, listed medicinal plants, with activity (MIC ≤ 100 μg/mL) against several *Mycobacterium* spp. Plants with MICs lower than 50 μg/mL were identified and included *Berchemia discolor*; (acetone extract of the bark; 12.5 μg/mL), *Warburgia salutaris* (acetone extract of the leaves; 25 μg/mL), *Terminalia sericea* (acetone extract of the bark; 25 μg/mL), and *Bridelia micrantha* (acetone extract of the bark; 25 μg/mL). Two plants, *Helichrysum melanacme* and *Euclea natalensis*, were the most effective extracts against *M. tuberculosis* displaying equipotent activity with MIC of 0.5 μg/mL. Diospyrin and its monomer, 7-methyljuglone or 7-methyl-5-hydroxynaphthalene-1,4-dione (Fig. 2.11), were isolated from *Euclea natalensis* (Ebenaceae), a plant used in South Africa to treat chest ailments. It displayed activity...
against drug-resistant strains of *M. tuberculosis* comparable to that of anti-TB drugs, streptomycin and ethambutol.

![Diospyrin](image1.png) ![7-methyljuglone](image2.png)

**Figure 2.11:** Structures of antimycobacterial napthoquinones isolated from *E. natalensis*

In Ghana, a research group identified and documented 15 medicinal plant species that are traditionally used to treat TB and in a follow-up study, investigated the antimycobacterial and cytotoxic activity of the various plant parts of these species. They found that the ethanolic extract of the leaves of *Solanum torvum* Sw. (Solanaceae) displayed an MIC value of 156.3 μg/mL against *M. tuberculosis* H37Ra (ATCC® 25 177TM) and it was recommended for further investigation to source potential anti-TB agents against sensitive and drug-resistant strains of *M. tuberculosis*. Several medicinal plants in Mozambique are used traditionally to treat tuberculosis and related symptoms. The n-hexane extracts of *Maerua edulis* and *Securidaca longipedunculata*, the EtOAc extract of *Tabernaemontana elegans* and DCM extract of *Zanthoxylum capense* were found to possess activity against *M. bovis* BCG and *M. tuberculosis* H37Ra with MIC values ranging between 15.6 – 62.5 μg/mL. In Senegal, the aqueous extracts of two Combretaceae sp. *Combretum aculeatum* and *Guiera senegalensis*, showed significant antimycobacterial against *M. marinum* with an IC50 of 0.5 mg/mL. Natural remedies, especially those derived from ethnomedicinal plants, are still being used worldwide in the management of TB.
Traditional healers among the Ju/'hoansi community, use various plant species to treat the symptoms of TB. Some of them are *Diplorhyncus condylocarpum*, *Combretum hereoensis*, *Peltophorum africanaum*, *Acacia erioloba*, and *Heteropyxis natalensis*. According to the traditional healers, the plant was perceived to be efficacious if the symptoms disappeared for some time. While the plants could be efficacious due to a placebo effect, preliminary phytochemical studies revealed that some of them contain antimicrobial and/or anti-inflammatory substances71,75,92. A USA-based company, Himalayan Herbal Healthcare, formulated a polyherbal remedy, Liv52, which is known as a potent hepatoprotectant, especially against chemically induced hepatotoxicity93. It is prescribed in Russia as an adjuvant for patients suffering from TB side-effects87.
### 2.4 Overview of plants selected for this study

Table 2.1: Ethnobotanical and phytochemical information on selected plants.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Classification and common names</th>
<th>Ethnobotanical uses</th>
<th>Phytochemical compounds &amp; pharmacology</th>
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<tbody>
<tr>
<td><em>Terminalia sericea</em></td>
<td><strong>Family</strong>: Combretaceae  &lt;br&gt; <strong>Common names</strong>: English: Silver cluster-leaf or Silver terminalia  &lt;br&gt; Afrikaans: Vaalboom  &lt;br&gt; Nama:</td>
<td>Strips of bark or leaves are chewed to cure bad colds and persistent coughing; chewed bark/leaf infusion taken as anti-malaria remedy and to reduce fever; root decoction is taken to treat severe cases of coughing*. Plant is used in the folk remedies for TB(^94).</td>
<td>1. Termilignan B, Arjunic acid(^95); are antibacterial.  &lt;br&gt; 2. Ellagittannins, ellagic acid and derivatives, gallo-tannins(^73). Ellagic acid has antiplasmodial activity; IC(_{50}) 0.5 μM(^96).  &lt;br&gt; 3. Tannin: Terminalin A(^73).  &lt;br&gt; 4. Terpenoids: Sericic acid &amp; sericoside(^97).  &lt;br&gt; 5. Stilbene glycoside; resveratrol-3-O-β-rutinoside, resveratrol-3-β-rutinoside glycoside, resveratrol, triterpenoid acid arjungenin and stigmasterol, β-sitosterol(^98).  &lt;br&gt; 6. Anolignan B(^99).</td>
</tr>
<tr>
<td>Plant species</td>
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<tr>
<td><em>Albizia anthelmintica</em></td>
<td>FAMILY: Fabaceae, Sub-family: Mimosoideae Common names§: English: Worm-cure albizia Oshiwambo: Omupopo Nama: Arub Damara: Arus</td>
<td>Roots are dried and pulverized and used to treat headaches, fever, stomach complaints, rheumatism, and syphilis**. An infusion is made from the crushed bark, and administered orally to malaria patients(^{100}). The bark is used as a purgative and anthelmintic. In Somalia it is used to treat gonorrhoea. Twigs are used as toothbrushes for oral hygiene(^{101}).</td>
<td>1. Gallic acid (an antioxidant), quercetin-3-(O-\beta-D)-glucopyranoside, kaempferol-3-(O-\beta-D)-glucopyranoside, 2. Kaempferol-3-(O-6\beta-O-galloyl-\beta-D)-glucopyranoside, quercetin-3-(O-6\beta-O-galloyl-\beta-D)-glucopyranoside(^{102}). Quercetin-3-(O-\beta-D)-glucopyranoside active against <em>M. tuberculosis</em> 0.15 mg/mL(^{103}).</td>
</tr>
<tr>
<td>Plant species</td>
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<td>Phytochemical compounds &amp; pharmacology</td>
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</table>
| *Ozoroa paniculosa* | FAMILY: Anacardiaceae  
Common names: English: common resin bush  
Jul’hoan: Baràtatà | Root decoction is used for persistent coughing and chest pains*.  
Used for kidney and lung complaints; also used for tuberculosis.  
The bark and roots are used to treat diarrhoea and abdominal pain$^{104-105}$. | 1. Flavonoids, phenols, tannins  
anacardic acid, ginkgoic acid  
and triterpenes$^{104-107}$.  
Anacardic acid - a phenolic  
lipid and a potent antibacterial.  
It is active against *M. smegmatis* (IC$_{50}$ 21.1 μg/mL)  
and *M. tuberculosis* (MIC 31.3 μg/mL)$^{107}$. |
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</table>
| *Diospyros lycioides* | FAMILY: Ebenaceae  
Common names*: English: bluebush, Otjiherero: Omuzeme  
Rukwamgali: Sihorowa | Chewing stick –root & twigs. Root decoction is taken as remedy against symptoms of TB (persistent coughing and expectoration of blood-tinged sputum)*. The twigs and roots from these plants are commonly used as chewing sticks for oral hygiene in Namibia108. | 1. Binaphthalenone glycosides: 1’,2-binaphthalen-4-one-2’,3-dimethyl-1,8’-epoxy-1,4’,5,5’,8,8’-hexahydroxy-8-O-β-xylopyranosyl(1→6)-β-glucopyranoside; 1,2’-binaphthalen-4-one-2’,3-dimethyl-1,8’-epoxy-1,4’,5,5’,8,8’-hexahydroxy-5’,8-di-O-β-xylopyranosyl(1→6)-β-glucopyranoside109.  
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<tr>
<td><em>Sarcocalon marlothii</em></td>
<td>FAMILY: Geraniaceae Common names³: Khoekhoe (Damara): //norab</td>
<td>The stems are used for the treatment of TB, high blood pressure and infertility**.</td>
<td>1. Triterpenes¹¹¹ From <em>Geranium robertinum</em> in Geraniaceae Family: Ellagic acid, quercetin, geraniin, caffeic acid Ellagic acid displays antiplasmodial activity IC₅₀ 0.5 μM⁹⁶.</td>
</tr>
<tr>
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</table>
| *Combretum imberbe* | FAMILY: Combretaceae  
Common names*:  
English: leadwood  
Afrikaans: hardekool  
Herero: morumboromboga  
Nama: !Hāb  
Silozi: Muzwili | Leave decoction is taken for coughing and bad cold and respiratory tract infections**. | 1. Pentacyclic triterpenes: Imberbic acid and derivatives of hydroxyimberbic acid, Rhamnosides\textsuperscript{112}.  
2. Oleanolic acid derivatives\textsuperscript{113-114}  
Imberbic acid – displays potent activity against *M. fortuitum* and *S. aureus*\textsuperscript{112}. |
<table>
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| *Adansonia digitata* | FAMILY: Malvaceae  
Common names:<br>English: baobab, cream-of-tartar tree  
Afrikaans: kremetart boom  
Setswana: Mowana  
German: Affenbrotbaum | Leaf decoction is taken as prophylactic for malaria symptoms and to regulate excessive diaphoresis. The leaves are also eaten fresh or decoction taken for chest complaints, severe coughs and asthma*. The bark is used to alleviate colds, fever and influenza. The fruit pulp is used to treat fever, diarrhoea, dysentery, smallpox, and measles, the coughing up of blood and as a painkiller.115. It is used in the treatment of TB and malaria71, 116. | 1. Tannins, phlobatannins, terpenoids, cardiac glycosides and saponins117.  
2. Isopropyl myristate118.  
3. (−)-epicatechin, and epicatechin derivatives118.  
3. Ascorbic, citric, tartaric, malic, succinic acid118.  
4. Campesterol, cholesterol, isofucosterol, β-sitosterol, stigmasterol and tocopherol (α,β,γ and δ)118.  
5. Linoleic, oleic, palmitic, linolenic, stearic and arachidic acids118-119.  
6. 3,7-dihydroxy-flavan-4-one-5-O-β-D-galactopyranosyl (1→4)-β-D-glucopyanoside; 3,3′,4′-trihydroxyflavan-4-one-7-O-α-L-rhamnopyranoside; quercetin derivatives118. |
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<tbody>
<tr>
<td>Aloe dichotoma</td>
<td>FAMILY: Asphodelaceae Common names: English: quiver tree Afrikaans: kokerboom Nama: //gàràs</td>
<td>The roots are used to treat TB symptoms, colds and chest pains. Roots are mashed, suspended in cold water and boiled. The infusion is taken 3 times a day**.</td>
<td>1. 8-C-glucosyl-7-O-methyl-aloediol; 2. Aloeresins B, C, D, E, F; isoaloeresin D; 3. 7-O-methylaloeresin A and 4-O-glucosyl-isoaloeresin D 5. (5/7)-hydroxyaloin, 6. 8-O-methyl-7-hydroxyaloin and 5-hydroxyaloin-6-O-acetate 7. Aloin, aloein, aloesin and aloenin B; aloeresin A; feroxin A; apigenin^1\textsuperscript{20-121}.</td>
</tr>
</tbody>
</table>

Ethnomedicinal use in Tsumkwe * and Uis ** regions
\^ All common and vernacular names were retrieved from the Namibia Biodiversity Database except S. marlothii
\* Common name used by Daureddaman community in Uis

All photographs displayed in this table, were taken during plant collection trips by C. V. Raidron, R. H. Hans and H. M. van Wyk, unless otherwise stated.
2.5 References


100. Nawinda TN. Antibacterial, antioxidant and phytochemical investigation of *Albizia anthelmintica* leaves, roots and stem bark. MSc Thesis. University of Namibia, Department of Chemistry and Biochemistry; 2016.


Chapter 3: Investigation of Biological Activities of Crude Extracts from Selected Namibian Medicinal Plants used in the Treatment of Malaria and Tuberculosis

3.1 Abstract

Ethnopharmacological information obtained literature sources and from the Daureddaman people of Uis in the Erongo region as well as the Khoisan people of Tsumkwe in the Otjozondjupa region, in Namibia, resulted in the identification of eight plant species which are used to treat malaria, tuberculosis and related symptoms.

This study evaluated the antiplasmodial and antimycobacterial potential of the 25 plant parts from eight selected indigenous Namibian medicinal plants. Plant parts were subjected to two separate extractions, one with DCM:MeOH(1:1) at room temperature and the other with distilled water at 60°C to yield the organic and aqueous extracts, respectively. Crude extracts of all plant parts were subjected to antiplasmodial and antimycobacterial activity testing. In vitro antiplasmodial activity was evaluated using the parasite lactate dehydrogenase assay against Plasmodium falciparum (CQS) NF54 and the in vitro antimycobacterial activity testing against Mycobacterium tuberculosis H37Rv-GFP strains was done using the standard broth microdilution method.

Reported here, are the preliminary biological activities of the crude organic and aqueous extracts. From the preliminary results obtained, 10 antimycobacterial active crude extracts (8 organic and 2 aqueous), with MIC$_{90} < 90 \, \mu g/mL$ and 4 antiplasmodial active crude extracts (1 organic and 3 aqueous), with IC$_{50} \leq 18 \, \mu g/mL$ were selected for further chemical analyses. The antimycobacterial and antiplasmodial activities ranged from MIC$_{90} 9.9 - 86.8 \, \mu g/mL$ and IC$_{50} 5.2 - 17.8 \, \mu g/mL$ respectively. The best
antimycobacterial and antiplasmodial activity was displayed by *A. digitata*. The stems of *S. marlothii*, are used by the community in Uis to treat tuberculosis, however poor antimycobacterial activity for the organic and aqueous extracts (MIC$_{90}$ 103 μg/mL and MIC$_{90}$ >125 μg/mL, respectively, were recorded. Instead, both organic and aqueous extracts displayed good to moderate antiplasmodial activity (IC$_{50}$ 8.8 μg/mL and IC$_{50}$ 17.8 μg/mL respectively). The preliminary antiplasmodial activity of *S. marlothii*, an endemic species to Namibia, is promising and it merits further phytochemical and pharmacological analyses.

**Keywords:** Medicinal plants; Tsumkwe; Uis; Namibia; malaria; antiplasmodial; tuberculosis; antimycobacterial; TB

### 3.2 Introduction

In 2018 it was reported that 22 people died of malaria in Namibia between January and March 2018, while 13 909 cases were recorded. The Kavango East and West regions contributed about 77.3% to the total cases, followed by the Zambezi (previously known as Caprivi) (11.1%) and the Ohangwena (7.6%) regions. Concerning the TB burden in Namibia, it was reported that 700 people died from tuberculosis-related infections, while over 8 800 new infections were recorded in 2017. As mentioned in section 2.3.2, the WHO currently rates Namibia in the top 30 high TB-burden countries in the world.

The ethnopharmacological approach for selecting plants to subject to phytochemical analysis, requires knowledge on the plant, the plant parts used and the method of preparation. The communities in Uis and Tsumkwe were visited to gather information
on and to collect medicinal plants used to treat symptoms of malaria and TB in accordance with the ethnopharmacological approach (Fig. 2.1). Available literature on the medicinal plants of the regions, was also consulted\(^4\). The species collected from Tsumkwe were *Terminalia sericea* (antimalaria and antitubercular), *Adansonia digitata* (antimalaria and antitubercular) *Ozoroa paniculosa* (antitubercular), *Diospyros lycioides* (antitubercular), *Albizia anthelmintica* (antimalaria), whereas *Combretum imberbe* (antitubercular), *Aloe dichotoma* (antitubercular) and *Sarcocaulon marlothii* Engl. (antitubercular) were collected from Uis. The communities interviewed during the plant collection trips, indicated that they prepare many of their remedies from medicinal plants with boiling water. Some secondary metabolites are soluble in non-polar organic solvents\(^5\) and it is for this reason that both aqueous and organic solvent extractions were prepared for this study.

*Figure 3.1:* Photographs taken during plant collection of medicinal plants in Uis (A & B) and Tsumkwe (C & D).
Of the plants collected for this study, *T. sericea* and *A. digitata* (baobab) are both traditionally used to treat TB\(^6\)\(^{-8}\) and malaria\(^9\)\(^{-11}\) and related symptoms. Compounds previously isolated from *T. sericea* include termilignan B and acid which display antibacterial activity\(^12\), as well as ellagic acid to which antiplasmodial activity (IC\(_{50}\) 0.5 μM) was ascribed\(^13\). The acetone extracts of the leaves of *A. digitata*, display activity against *M. smegmatis* (MIC 1.25 μg/mL)\(^8\). The baobab has been researched extensively and a wide variety of classes of compounds were isolated from it, for example, tannins, phlobatannins, cardiac glycosides and saponins, to name a few\(^11\). The crushed bark of *A. anthelmintica* is administered to malaria patients\(^14\). Compounds isolated from this plant include gallic acid\(^15\), an antioxidant, quercetin-3-Ο-β-D-glucopyranoside, which displayed activity against *M. tuberculosis* (0.15 mg/mL)\(^16\), and kaempferol derivatives\(^15\).

The plant species *O. paniculosa*\(^17\), *D. lycioides*, *S. marlothii* and *A. dichotoma* are all used ethnomedicinally to treat TB and related symptoms(Personal communication with Mr. Thoroub in Uis and !Naici in Tsumkwe). Compounds previously isolated from *O. paniculosa* include flavonoids, phenols, tannins, triterpenes, ginkgoic acid\(^18\) and anacardic acid\(^19\). It is reported that anacardic acid, isolated from the acetone extract of the bark, have moderate activity against *M. tuberculosis* with an MIC of 125 μg/mL\(^19\). Compounds isolated from *D. lycioides*, diospyrosides A, B, C and D are reported to have antibacterial activity whereas 7-methyljuglone is reported to be active against drug resistant *M. tuberculosis*\(^20\). Various triterpenes, one which is structurally similar to lupeol, have been isolated from the waxy layer of a member of the Sarcocaulon genus, *S. patersonii*\(^21\). Aloin, aloenin, aloesin and aloeresin A were previously isolated from *A. dichotoma*\(^22\)\(^{-23}\).
The purpose of this study is to analyse the antiplasmodial and antimycobacterial activities of the 27 plant parts, from eight selected indigenous Namibian medicinal plants.

3.3 Materials and methods

3.2.1 Collection of plant materials

Fresh plant material was collected from eight indigenous medicinal plants in the Tsumkwe and Uis in Namibia (Fig. 3.2). During collection the plants were indexed by GPS coordinates, geographic location, local name and date. Plant parts were vacuum sealed on site and were transported in a portable refrigerator to the University of Namibia (UNAM). Here the voucher specimens were prepared and delivered to the National Botanical Research Institute (NBRI) for taxonomic identification by Ms Frances Chase and Mr David Aiyambo.

![Figure 3.2: Map of Namibia, indicating the Uis and Tsumkwe areas where the medicinal plants for this study were collected.](image-url)
The identification reports are included in the Appendix, Fig. A2 & A3. All plant parts were separated, adulterants removed, cut into smaller pieces and allowed to dry for two weeks in the shade at room temperature. These were ground to a powder and stored in the refrigerator until extraction.

3.2.2 Preparation of the crude extracts

Each ground sample was subjected to both aqueous and organic solvent extraction and the ratio used was 1g of ground plant material mixed with 10 mL of solvent (water or organic solvent). The aqueous extractions were obtained by homogenizing the plant material in distilled water and shaking it in an incubator at 60°C for 48 h. The resulting mixture was filtered under vacuum with the aid of Celite and the filtrate volume reduced in vacuo followed by freeze-drying overnight. The dried aqueous crude extracts were weighed and stored in the freezer until further analyses. A total of 24 crude aqueous extracts were obtained in this manner. No crude aqueous extract of the stems of A. digitata was done, due to insufficient plant material available.

The organic solvent extractions were done by soaking the ground plant material in a 50% MeOH:DCM mixture and leaving it on an orbital shaker for 48 h at room temperature. The resulting mixture was filtered and the residue generated was further extracted with 100% MeOH. The combined filtrate was evaporated in vacuo, followed by freeze-drying overnight. The organic crude extracts were weighed and stored in the freezer until further analyses. A total of 25 crude organic extracts were obtained.

3.2.3 Antiplasmodial and antimycobacterial testing of crude extracts

All the aqueous and organic extracts fractions were tested for antiplasmodial activity against chloroquine sensitive strain (CQS) of P. falciparum NF54. The activity in vitro
was tested using the parasite lactate dehydrogenase assay as described by Makler (1993)\textsuperscript{25}. The antiplasmodial screening was coordinated by the H3D lab and done at the Division of Pharmacology, Department of Medicine, University of Cape Town, South Africa, coordinated by Prof. Peter Smith.

All the aqueous and organic extracts fractions were also tested for antimycobacterial activities against \textit{M. tuberculosis} H\textsubscript{37}Rv-GFP strain, using the standard broth micro-dilution method. The antimycobacterial screening was done at the MRC/NHLS Molecular Mycobacteriology Research Unit, DST/NRF Centre of Excellence for Biomedical TB Research, Department of Pathology, Faculty of Health Sciences, University of Cape Town, South Africa coordinated by Prof Digby Warner.

\textbf{3.2.3.1 \textit{In vitro} antiplasmodial assay}

The analyses were done against the NF54CQS strain of \textit{Plasmodium falciparum}. Continuous \textit{in vitro} cultures of asexual erythrocyte stages of \textit{P. falciparum} were maintained using a modified method of Trager and Jensen (1976)\textsuperscript{26}. Stock solutions of the test samples were prepared at a concentration of 20 mg/mL in 100\% DMSO and stored at –20 °C. Samples were tested as a suspension if it could not dissolve completely. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) and artesunate (Art) were used as the reference drug in all experiments. A full dose-response was performed for all compounds to determine the concentration inhibiting 50\% of parasite growth (IC\textsubscript{50} value). Test samples were tested at a starting concentration of 10 μg/mL, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 μg/mL. The same dilution technique was used for all samples. CQ and Art were also tested from a
starting concentration of 1 µg/mL. The IC\textsubscript{50} values were obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

### 3.2.3.2 In vitro antimycobacterial assay

The minimum inhibitory concentration (MIC) of the crude extracts was determined using the standard broth micro dilution method, where a 10 mL culture of *Mycobacterium tuberculosis* H\textsubscript{37}Rv-GFP\textsuperscript{27-29}, was grown to an optical density (OD\textsubscript{600}) of 0.6 – 0.7. Cultures were diluted 1:100, in Gaste-Fe (glycerol–alanine–salts) medium pH 6.6, and supplemented with 0.05% Tween-80 and 1% Glycerol\textsuperscript{30}, prior to inoculation of the MIC assay. The crude extracts were reconstituted to a concentration of 2 mg/mL in DMSO. Two-fold serial dilutions of the test compound were prepared across a 96-well micro titre plate, after which, 50 µL of the diluted *M. tuberculosis* cultures was added to each well in the serial dilution. Assay controls used were a minimum growth control (Rifampicin at 2 x MIC), and a maximum growth control (5% DMSO). The micro titre plates were sealed in a secondary container and incubated at 37 °C with 5% CO\textsubscript{2} and humidification. Relative fluorescence (excitation 485 nm; emission 520 nm) was measured using a plate reader (FLUOstar OPTIMA, BMG LABTECH), at day 7 and day 14. The raw fluorescence data were archived and analysed using the CDD Vault from Collaborative Drug Discovery, in which, data were normalized to the minimum and maximum inhibition controls to generate a dose response curve (% inhibition), using the Levenberg-Marquardt damped least-squares method, from which the MIC\textsubscript{90} and MIC\textsubscript{99} were calculated (Burlingame, CA www.collaborativemed.com). The lowest concentration of drug that inhibits growth of more than 90% of the bacterial population was considered to be the MIC\textsubscript{90} and the
lowest concentration of drug that inhibits growth of more than 99% of the bacterial population was considered to be the MIC$_{99}$.

3.3 Results and discussion

3.3.1 Yield of extracts

As mentioned in section 3.1, extraction is an important step in the separation of compounds from plant material and the yields obtained depend on the solvent, temperature, extraction time and composition of the sample$^{31}$. In this work, the extraction yields for the crude organic extracts ranged from 2.27 to 33.81% and the extraction yields for the crude aqueous extracts ranged from 2.20 to 33.05% (Table 3.2). From the results, the organic solvent extraction yields of a specific plant part are higher than that of the corresponding aqueous crude extract. A few exceptions were the following: leaves of *T. sericea*, roots of *A. anthelmintica*, leaves of *O. paniculosa*, twigs and roots of *D. lycioides*, bark of *A. digitata*, and roots of *A. dichotoma*. For *A. dichotoma* the yield obtained for the aqueous root extract (22.29%) was more than 2.5 times greater than that of the crude organic solvent (8.78%).
### Table 3.2: Percentage yield of crude organic and aqueous extracts.

<table>
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<tr>
<th>Plant Specie</th>
<th>Plant part</th>
<th>Extract</th>
<th>Dry plant material (g)</th>
<th>Crude Extract (g)</th>
<th>% Yield*</th>
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<td>Crude Extract (g)</td>
<td>% Yield*</td>
</tr>
<tr>
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<td>------------</td>
<td>----------</td>
<td>------------------------</td>
<td>-------------------</td>
<td>----------</td>
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<td>22.29</td>
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</table>

* % Extraction yield: expressed as 100 x (g dry extract / g dry plant material)

3.3.2 Biological activity

Although some plants selected are used to treat malaria and others for tuberculosis, all 53 crude extracts (27 organic and 26 aqueous) were submitted for both antiplasmodial and antimycobacterial analyses. The data of the biological activities of all the crude extracts are displayed in in table format, Table 3.3.
Table 3.3: Biological activities of the crude plant extracts.

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<tr>
<th>Plant specie</th>
<th>Plant part</th>
<th>Extract</th>
<th>% survival at 20 µg/mL</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/mL)</th>
<th>MIC&lt;sub&gt;99&lt;/sub&gt; (µg/mL)</th>
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<td>&gt;125</td>
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<td>95</td>
<td>&gt;125</td>
<td>&gt;125</td>
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</tr>
</tbody>
</table>
### 3.3.2.1 Antiplasmodial activity

The antiplasmodial results of the crude extracts obtained in this study were expressed in two ways: (i) % survival of the microorganism at 20 μg/mL concentration of the crude extract; and (ii) half-maximal inhibitory concentration (IC$_{50}$), which means that the value obtained is the lowest concentration (μg/mL) of the crude extract, which inhibits the microorganism by 50%. In this study, crude extracts were ranked according to IC$_{50}$ values, as being high activity (IC$_{50}$ ≤ 10 μg/mL); moderate activity (10 < IC$_{50}$ < 100 μg/mL); and low activity (IC$_{50}$ > 100 μg/mL)$^{32}$. The activity ranking for the crude extracts according to % survival, as being high activity (% survival ≤ 20); moderate activity (20 < % survival < 55); and low activity (55 < % survival < 95); inactive (% survival > 90)$^{32}$.

The plants *T. sericea*, *A. digitata*, and *A. anthelmintica* are traditionally used to treat or alleviate antimalarial symptoms. When comparing the antiplasmodial activity
results (% survival at 20 μg/mL) of these plants, it was observed that the crude organic extracts of the roots of *T. sericea*, displayed the best activity (40%), compared to the crude aqueous extract (57%). The crude organic extracts of the stems of *A. digitata* also displayed activity (44%), whilst both the crude organic and aqueous extracts of the stem bark of *A. anthelmintica*, displayed low activity (85% and 90%), respectively (Fig 3.3). The IC$_{50}$ values of some extracts were determined and are displayed in Table 3.3.

Four crude extracts (three organic and one aqueous) displayed antiplasmodial activity with IC$_{50}$ values ranging from 5.2 – 17.8 μg/mL. The crude organic extract of the twigs of *A. digitata* showed good antiplasmodial activity (IC$_{50}$: 5.2 μg/mL) and the crude aqueous extract of the stems of *S. marlothii*, intermediate antiplasmodial activity (IC$_{50}$: 17.8 μg/mL). Also noted was that the crude organic extract of the roots of *T. sericea* and the crude aqueous extract of the stems of *S. marlothii* showed intermediate antiplasmodial activity with IC$_{50}$ values 8.7 μg/mL and 8.8 μg/mL, respectively.
Figure 3.3: Antiplasmodial activity of the organic and aqueous crude extracts. Chloroquine and artesunate were used as controls.
3.3.2.2 Antimycobacterial activity

The antimycobacterial results of the crude extracts obtained in this study were expressed in MIC$_{90}$ and MIC$_{99}$, which means that the value obtained is the lowest concentration (μg/mL) of the crude extract which inhibits 90% or 99% growth of the mycobacterial population respectively. Some biological activity results are expressed in minimum inhibitory concentration (MIC), which means the value is the lowest concentration (mg/mL) of an antimicrobial substance that will inhibit the visible growth of a microorganism after overnight incubation. In this study we have ranked the values as follows: significant activity (MIC$_{90} < 10$ μg/mL); moderate (10 μg/mL < MIC$_{90} \leq 100$ μg/mL); and low or negligible (MIC$_{90} > 100$ μg/mL)$^{33}$.

Of the 49 crude extracts screened for antituberculosis activity, ten different crude extracts (eight organic and two aqueous) displayed antimycobacterial activity with MIC$_{90}$ values ranging from 9.9 – 86.6 μg/mL. The following plants are traditionally used to treat tuberculosis: A. digitata, O. paniculosa, D. lycioides, C. imberbe, A. dichotoma, and S. marlothii (Personal communication with Mr. Thoroub in Uis and !Naici in Tsumkwe). When comparing the antitubercular activity of their crude extracts, (MIC$_{90}$), the following were found: (i) the aqueous extract of the bark of A. digitata (baobab tree) displayed significant activity, (9.9 μg/mL) whilst the organic extract was seven times less active with 70.7 μg/mL; (ii) the aqueous extract of the roots of A. dichotoma also displayed moderate activity, 27.3 μg/mL, followed by the organic extract of the twigs of T. sericea, 40.1 μg/mL; (iii) the organic extracts of the stem bark of C. imberbe, and of the roots of D. lycioides, 47.2 μg/mL and 71.6 μg/mL moderate activity respectively; (iv) the organic extracts of the leaves of A. dichotoma, the twigs of C. imberbe and the twigs of A. digitata also displayed moderate activity,
86.8 μg/mL, 95.6 μg/mL and 97.9 μg/mL respectively; (v) the organic extracts of the stems of *S. marlothii* and the twigs of *O. paniculosa* displayed negligible activity, with values 103 μg/mL and 108 μg/mL, respectively (Fig.3.4). Other studies, confirm the antimycobacterial activity of *O. paniculosa*, albeit from the acetone extract of the bark (MIC 0.3 mg/mL against *M. tuberculosis*)$^{19}$ and the acetone extract of the leaves (MIC 0.52 mg/mL)$^{34}$. These results support the ethnobotanical use of this plant to treat tuberculosis and its related symptoms.
**Figure 3.4:** Antimycobacterial activity of the organic and aqueous crude extracts. Rifampicin was used as the control.
3.4 Summary and conclusions

In Africa, *Adansonia digitata* (baobab) is referred to as “The Tree of Life” and is revered for its nutritional and medicinal value\(^7,10\). From the biological results obtained, both the aqueous and organic solvent crude extracts of *A. digitata* displayed antimycobacterial activity, whereas the organic extract also displayed antiplasmodial activity. This confirms the traditional use for both antimalaria and antituberculosis\(^7,33-36\), and supports the medicinal value of this plant. A literature search revealed that the *T. sericea*, *A. anthelmintica*, *C. imberbe* and *A. digitata* were subjected to detailed phytochemical analyses, with different classes of phytochemicals isolated from them\(^6,11-12,14-14,37-43\). It was for this reason that these plants were not subject to further analyses, for example, fractionation and isolation studies.

The traditional use of *O. paniculosa*, as antitubercular medicinal plants was supported by the results obtained in this study. Of all the plant parts analysed, the organic extract of the twigs, was the only plant part which displayed moderate activity with an MIC\(_{90}\) 80.7 μg/mL recorded. A study done by T. Seaman, confirmed that the acetone extract of the bark of *O. paniculosa* displayed activity against *M. tuberculosis* H\(_{37}\)Ra (MIC 0.3 mg/mL with the BACTEC 460 method, as opposed to the standard broth microdilution methods used in this study\(^19\).

The traditional use of *S. marlothii*, for treating tuberculosis and associated symptoms, was not supported by the results obtained in this study. None of the plant parts extracted, both organic and aqueous, displayed antimycobacterial activity. Instead, the organic and aqueous crude extracts both displayed antiplasmodial activity, which is contrary to the traditional use of the plant. The organic crude extract displayed high
antiplasmodial activity (IC$_{50}$ 8.8 μg/mL) and the aqueous extract moderate activity (IC$_{50}$ 17.8 μg/mL). It is worth mentioning that ellagic acid which displayed antiplasmodial activity (IC$_{50}$ 0.5 μM)$^{13}$ was reported to have been isolated from members of the Geraniaceae family$^{44}$. $S. marlothii$, also from the Geraniaceae family, is endemic to Namibia and to date very little phytochemical studies have been done on it. Although many Aloe species have been subjected to phytochemical studies, e.g. Aloe vera, $A. dichotoma$ does not appear to have been studied extensively$^{22-23}$. 

The biological active crude extracts of $S. marlothii$ and $A. dichotoma$ will be subjected to SPE extraction to obtain antiplasmodial and antimycobacterial LLEs, respectively.
3.5 References


14. Nawinda TN. Antibacterial, antioxidant and phytochemical investigation of *Albizia anthelmintica* leaves, roots and stem bark. MSc Thesis. University of Namibia, Department of Chemistry and Biochemistry; 2106.


Chapter 4: Investigation of Biological Activities of Lead-Like Enhanced (LLE) Extracts from Selected Namibian Medicinal Plants used in the Treatment of Malaria and Tuberculosis

4.1 Abstract

Medicinal plants, remain an important source of new drugs and new drug leads and the importance of the physicochemical properties of these natural product-derived molecules has been acknowledged. There is a need to expedite the discovery of new lead compounds for malaria and tuberculosis through the application of innovative and easy to implement strategies. Of interest to this project, is a protocol developed by Camp et al. which involves the enrichment of crude extracts with desirable lead-like and drug-like properties. For this study, bioactive crude extracts which displayed antimycobacterial activity with \( \text{MIC}_{90} < 90 \mu g/mL \) (8 organic and 2 aqueous), and antiplasmodial activity with \( \text{IC}_{50} < 18 \mu g/mL \) (1 organic and 3 aqueous) were selected. Using the Camp method, lead-like enhanced (LLE) extracts were prepared using solid phase extraction (SPE) to separate drug-like compounds from the selected bioactive crude extracts. Reported here are the biological activities against \textit{Plasmodium falciparum} (CQS) NF54 and the \textit{Mycobacterium tuberculosis} H37Rv-GFP strains of the LLE-extracts and MeOH fractions prepared from previously selected crude extracts.

The bioactive crude extracts were subjected to SPE for the preparation of the LLE extracts and their resulting MeOH fractions. Solid phase extraction was performed using Strata-X 33 \( \mu \) reversed phase cartridges, prepacked with benzene-based functionalized polymer linked to \( N \)-vinylpyrrolidone (NVP), 3 mL or 6 mL tubes (Phenomenex). The elution solvent was 70:30 MeOH:H\(_2\)O with 1% TFA and yielded the LLE extract, followed by 100% MeOH rinse to yield the MeOH fraction. None of
the LLE extracts displayed antimycobacterial activity. The MeOH fraction of the bark of *A. digitata* displayed a threefold increase in activity (MIC$_{90}$ 19.5 μg/mL) compared to the organic crude (MIC$_{90}$ 70.7 μg/mL). The LLE extract of the twigs of *A. digitata* was devoid of antiplasmodial activity and the MeOH fraction was more active (IC$_{50}$ 2.4 μg/mL) than the crude (IC$_{50}$ 5.2 μg/mL). The MeOH fraction of the organic extract of the stems of *S. marlothii* also displayed increased antiplasmodial activity (IC$_{50}$ 4.3 μg/mL) than the crude (IC$_{50}$ 8.8 μg/mL).

The results of this study indicate that *S. marlothii* and *A. dichotoma* showed promising preliminary antiplasmodial and antimycobacterial activity, respectively. It can be concluded that these two plants are a potential source of compounds active against malaria and TB. *S. marlothii* is endemic to Namibia and has not been researched before, although other species in the Sarcocaulon family have been, and few phytochemical studies have been done on *A. dichotoma*. Future work will include the isolation and structure elucidation of the active compound(s).

**Keywords:** Medicinal plants; antiplasmodial; antimycobacterial; LLE, Solid phase extraction, SPE, lead compounds, log $P < 5$

### 4.2 Introduction

The search for new drug candidates to eradicate both TB and malaria successfully and safely, has become a worldwide focus. By virtue of their structural diversity and inherent biological activity, natural products sourced from medicinal plants, play an important role in the discovery of lead compounds which can expedite the discovery of new efficacious and safe drugs. This, moreover, requires the application of appropriate techniques to increase the pace of NP isolation, such as extract-enrichment...
protocols, as well as appropriate extraction, analytical and biological methodologies. One such protocol, developed by Camp et al.\textsuperscript{5}, and applied in this study, involves the upstream optimization of drug-like properties, using log $P$ as a filter to yield lead-like enhanced extracts for a quicker isolation of bioactive compounds.

### 4.2.1 Lead-like enhanced (LLE) approach to discover new drug leads

The term lead-like is conferred to molecules with physicochemical properties which satisfies the “rule of five” (RO5) and therefore, increases the odds that the optimization process will results in molecules with acceptable drug-like properties. The term drug-like implies that a molecule has acceptable ADMET properties to survive through the completions of human Phase 1 trials to become a successful drug product\textsuperscript{6}. These two terms will be used interchangeably throughout thesis. The discovery of new drug-like molecules involves the measurement and application of compound properties for candidate selection and optimization\textsuperscript{7}. Lipinski’s RO5 has become one of the most widely used tools to assess the relationship between structures and drug-like properties. It deals with orally active compounds and defines four simple physicochemical parameters associated with: molecular weight $\leq$ 500, Log $P \leq 5$, hydrogen-bond donors $\leq$ 5, and hydrogen-bond acceptors $\leq$ 10. The RO5 predicts that poor absorption is more likely when these values mentioned above are higher than those indicated\textsuperscript{6,8}. According to Camp et al., log $P$ lends itself most readily as filter descriptor in the development of a generic protocol that can be used in tandem with NP drug discovery. The coincidence is that log $P$ –a measure of lipophilicity or hydrophobicity- is also considered the “Lord of the Rules” for drug discovery and development. The partition coefficient, abbreviated $P$, is defined as a particular ratio of the concentration of a solute between two solvents; one solvent is water and the
other is a non-polar solvent (e.g. octanol) (Eq. 1). Camp et al., prioritized crude extracts by front-loading them with desirable log $P$ characteristics, and yielded antiplasmodial and antitrypanosomal compounds, which have the advantage of becoming successful drugs$^5$.

$$\log P_{\text{oct/wat}} = \log \left( \frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{water}}} \right)$$

…………….Equation 1

4.2.2 Development of protocol for LLE extraction according to Camp et al.$^{(5)}$

In brief, this chromatography-based method is aimed at enhancing the quality of plant extracts, which is generating the LLE extracts using solid-phase extraction (SPE) with an appropriate adsorbent and elution solvent. It was envisaged that with the right combination of the latter two, components in the extract with log $P > 5$ will be retained on the adsorbent whereas those with log $P < 5$ (drug-like compounds) eluted. In developing the protocol, four adsorbents were screened: octadecylsilica gel (Davisil C18-bonded silica); polystyrene (Diaion HP-20); cross-linked poly(styrene-divinylbenzene) (Amberlite XAD-16); and the cross-linked poly(divinylbenzene-$N$-vinylpyrrolidone) (DVB-NVP) co-polymer (Waters Oasis HLB). The eluting solvents tested were the combinations of MeOH/H$_2$O in the ratios: 70:30; 80:20; 70:30: TFA (1%) and 80:20:TFA (1%). Using a test sample comprising 14 NP drugs and 115 NPs with calculated log $P$ (clog $P$) values, the RP-HPLC retention times were calibrated. Eluents were collected and analyzed by RP-HPLC, that is in accordance with reports that log $P$ correlates with RP-HPLC retention times$^{5,9}$.

Development of the protocol continued by subjecting 221 plant and marine crude extracts, obtained through sequential extraction with DCM and MeOH, to LLE generation. Subsequent analyses of the eluents (LLE fractions) used RP HPLC fitted
with a series-connected photodiode array (PDA) detector and evaporative light scattering detector (ELSD). Results obtained revealed that (i) the DVB-NVP adsorbent was the best in retaining compounds with a log P > 5 and (ii) that the best recoveries of compounds with log P < 5 was obtained with a dual solvent system with the first being a mixture of 70:30 MeOH:H$_2$O with 1% TFA and the second 90:10 MeOH:H$_2$O with 1% TFA.

To further assess the application of the method, a scaled up protocol yielded 70 000 (Fig. 4.1) extracts, of which a subset (18 453 LLE extracts) were subjected to fractionation with RP-HPLC (Onyx Monolithic). The 202 983 LLE fractions so obtained, were screened for activity against the CQS 3D7 Plasmodium falciparum strain, CQR Dd2 Plasmodium falciparum strains and Trypanosoma brucei, with the latter being the causative agent for human African Trypanosomiasis (HAT)$^5$.$^{10}$.

![Figure 4.1: Flow chart for the preparation of the LLE extracts and subsequent screening](image)

Figure 4.1: Flow chart for the preparation of the LLE extracts and subsequent screening.$^{10}$.
Biological results obtained for the LLE fractionated library revealed that 60 compounds with antimalarial activity and 58 with trypanosomal activity, were isolated. The activity of these compounds ranged from good (IC$_{50}$ 10 μM for 48 antimalarials and 30 antitrypanosomal) to poor (IC$_{50}$ > 50 μM for 8 antimalarials and 18 antitrypanosomal$^5$).

### 4.2.3 Limitations of the RO5

Natural products tend to deviate from Lipinski’s RO5 in that they have evolved to be bioactive and they often make use of transmembrane transporters rather than passive diffusion to enter cells$^8,11$. The RO5 was originally conceived for orally administered drugs$^6$. A review on advanced drug delivery by Pär Matsson et al. investigated the idea of exploring cell permeability beyond the “rule of 5” (bRO5). They concluded that there is too strict reliance on the RO5 and this has led to reduced investigation of beyond RO5 (bRO5) space$^{12}$. Also on reviewing the compliance of isolated compounds with RO5, Camp et al. ascribed the violations detected, to the TFA used in the LLE extract generation and its ability to ionize basic NP and subsequently influencing their log $P_s$$^5$.

Thomas Keller and research team concluded that Lipinski’s “Rule of five” (RO5), which are physicochemical properties that drug-like compounds have in common, have short comings, as there are exceptions to the RO5$^{13}$.

### 4.2.4 Background to the present study

In Chapter 3, it was described in detail how eight plants species, comprising 49 plant parts, prepared as both organic and aqueous extracts, were subjected to antiplasmodial
and antimycobacterial activity testing. The stems of *A. digitata*, however, was only extracted with organic solvent, because not enough sample was collected to perform an aqueous extraction. Four crude extracts displayed antiplasmodial activity (IC$_{50}$ $\leq$ 18 $\mu$g/mL) (Table 4.1) whereas ten crude extracts displayed antimycobacterial activity (MIC$_{90}$ $\leq$ 90 $\mu$g/mL) (Table 4.2). This part of the study explored the application of the method developed by Camp *et al*.$^{5}$, with modifications, by front-loading the active crude extracts, listed in Tables 4.1 and 4.2, with compounds with desirable log $P$ characteristics, and by testing the resulting LLEs for antimycobacterial and antiplasmodial activity.

**Table 4.1:** Antiplasmodial activity of crude extracts (IC$_{50}$ $\leq$ 18 $\mu$g/mL and their corresponding % survival at 20 $\mu$g/mL).

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant part</th>
<th>Extract</th>
<th>IC$_{50}$ (µg/mL)</th>
<th>NF54 % survival at 20 $\mu$g/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A. digitata</em></td>
<td>Twigs</td>
<td>Organic</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td><em>T. sericea</em></td>
<td>Roots</td>
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<td>11.4</td>
</tr>
<tr>
<td>3</td>
<td><em>S. marlothii</em></td>
<td>Stems</td>
<td>Organic</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td><em>S. marlothii</em></td>
<td>Stems</td>
<td>Aqueous</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>Chloroquine</td>
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<td></td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Artesunate</td>
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</tr>
</tbody>
</table>

**Table 4.2:** Antimycobacterial active crude extracts (MIC$_{90}$ values $< 90$ $\mu$g/mL and their corresponding MIC$_{99}$ values in $\mu$g/mL).

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant part</th>
<th>Extract</th>
<th>MIC$_{90}$ (µg/mL)</th>
<th>MIC$_{99}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A. digitata</em></td>
<td>Bark</td>
<td>Aqueous</td>
<td>9.9</td>
</tr>
<tr>
<td>2</td>
<td><em>A. digitata</em></td>
<td>Bark</td>
<td>Organic</td>
<td>70.7</td>
</tr>
<tr>
<td>3</td>
<td><em>A. dichotoma</em></td>
<td>Roots</td>
<td>Aqueous</td>
<td>27.3</td>
</tr>
<tr>
<td>4</td>
<td><em>A. dichotoma</em></td>
<td>Leaves</td>
<td>Organic</td>
<td>86.8</td>
</tr>
<tr>
<td>5</td>
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<td>Twigs</td>
<td>Organic</td>
<td>40.1</td>
</tr>
<tr>
<td>6</td>
<td><em>T. sericea</em></td>
<td>Leaves</td>
<td>Organic</td>
<td>71.8</td>
</tr>
<tr>
<td>7</td>
<td><em>C. imberbe</em></td>
<td>Stem bark</td>
<td>Organic</td>
<td>47.2</td>
</tr>
<tr>
<td>8</td>
<td><em>A. anthelmintica</em></td>
<td>Bark</td>
<td>Organic</td>
<td>70.8</td>
</tr>
<tr>
<td>9</td>
<td><em>A. anthelmintica</em></td>
<td>Roots</td>
<td>Organic</td>
<td>71.8</td>
</tr>
<tr>
<td>10</td>
<td><em>D. lycioides</em></td>
<td>Roots</td>
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<td>71.6</td>
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<tr>
<td></td>
<td>Rifampicin</td>
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<td></td>
<td>0.0066 $\mu$M</td>
</tr>
</tbody>
</table>

MIC$_{90}$ = Minimum Inhibitory Concentration required to inhibit the growth of 90% of organisms

MIC$_{99}$ = Minimum Inhibitory Concentration required to inhibit the growth of 99% of organisms
4.3 Materials and Methods

4.3.1 Preparation of lead-like enhanced (LLE) extracts

All four crude extracts which displayed antiplasmodial activity (Table 4.1) and the ten crude extracts (Table 4.2) which displayed antimycobacterial activity, were subjected to SPE for the preparation of the LLE extracts and their resulting MeOH fractions. Solid phase extraction was performed using a 20-place VacMaster™ pressure manifold, using Strata-X 33 μ reversed phase cartridges, prepacked with benzene-based functionalized polymer linked to N-vinylpyrrolidone (NVP), 3 mL or 6 mL tubes (Phenomenex). Due to limited funds, the closest cost-effective equivalent in terms of solid phase chemistry, was the choice of adsorbent used in this study, Strata-X, which is, as mentioned above, a benzene-based functionalized polymer linked to N-vinylpyrrolidone (NVP). The Camp method used a polydivinylbenzene (DVP)-NVP from Waters Oasis HLB®. Furthermore, pre-fractionation with n-hexane, to remove non-polar interferences and passing the crude extract through a polyamide column to remove tannins, were not done.

A pre-weighed mass of the crude extract (~50 mg for 3 mL cartridge and ~100-125 mg for 6 mL cartridge) was dissolved in minimum eluting solvent (600 - 800 μL of 70% MeOH:H2O containing 1% trifluoroacetic acid (TFA)), loaded onto the stationary phase of the SPE cartridges and allowed to adsorb onto the stationary phase. The crude extract was eluted with 3 x 3 mL of the eluting solvent for a 3 mL cartridge or 3 x 6 mL of the eluting solvent for a 6 mL cartridge. Eluents collected were dried in vacuo and then freeze-dried to give fraction A which represented the LLE extract. The SPE tubes were further eluted with 100% MeOH (~ 20 mL) until clear, and the eluents collected were dried in vacuo and freeze dried to give fraction B, which represented
the MeOH fraction. Figure 4.2 displays the flow charts of the preparation of the LLE extracts and MeOH fractions of *S. marlothii* and *T. sericea* whilst Figure 4.3 displays the same preparation method for *A. digitata* and *A. dichotoma*.

### 4.3.2 Biological activity testing

Both the LLE extract and the MeOH fractions were screened for biological activities against *P. falciparum* (CQS) NF54 and the *M. tuberculosis* H37Rv-GFP strains. The assays were done as described in section 3.2.3.

### 4.3.3 Nuclear magnetic resonance (NMR) analysis

$^1$H-NMR analysis were done on the crude, LLE extracts (fraction A) as well as the MeOH fractions (fraction B) of *S. marlothii* (stems, organic) and also on the crude and LLE extract of *A. dichotoma* (roots, aqueous). The NMR analyses were performed on a Bruker 600-MHz spectrophotometer equipped with a 5-mm Prodigy cryoprobe. $^1$H-NMR chemical shifts ($\delta$, ppm) were relative to residual solvent signals. NMR samples were dissolved in either deuterated methanol (CD$_3$OD), deuterated dimethyl sulfoxide (DMSO-$d_6$), or deuterated water (D$_2$O).
S. marlothii, stems, (org)
Antiplasmodial: IC\textsubscript{50}: 8.8 µg/mL

50.2 mg of dried organic crude:
SPE, Strata-X

70% MeOH:H\textsubscript{2}O with 1% TFA

A
23.0 mg
IC\textsubscript{50}: 10.0 µg/mL

B
8.6 mg
IC\textsubscript{50}: 4.3 µg/mL

LLE Extract
MeOH Fraction

62.9\% recovery
(31.6 mg)

T. sericea, roots, (org)
Antiplasmodial activity: IC\textsubscript{50}: 11.4 µg/mL

123.8 mg of dried organic crude:
SPE Strata-X

70% MeOH:H\textsubscript{2}O with 1% TFA

A
86.7 mg
IC\textsubscript{50}: 3.3 µg/mL

B
10.3 mg
IC\textsubscript{50}: 0.6 µg/mL

LLE Extract
MeOH Fraction

78.3\% recovery
(97.0 mg)

Figure 4.2: Flow charts depicting the preparation of the LLE extracts and MeOH fractions of S. marlothii and T. sericea, both with antiplasmodial activity.
A. digitata, bark, (aq.)
Antimycobacterial: MIC<sub>90</sub>: 9.9 µg/mL

50.8 mg of dried aqueous crude:
- SPE, Strata-X
- 70% MeOH:H<sub>2</sub>O with 1% TFA

LLE Extract

84.2% recovery
(42.8 mg)

A. dichotoma, roots, (org.)
Antimycobacterial activity: MIC<sub>90</sub>: 27.3 µg/mL

50 mg of dried aqueous crude:
- SPE Strata-X
- 70% MeOH:H<sub>2</sub>O with 1% TFA

LLE Extract

87% recovery
(43.6 mg)

**Figure 4.3:** Flow charts depicting the preparation of the LLE extracts and MeOH fractions of *A. digitata* and *A. dichotoma*, both with antimycobacterial activity.
4.4 Results and discussion

4.4.1 Percent yield of the LLE extracts and the MeOH fractions

Table 4.3: Percentage yields obtained after SPE of the biologically active crude extracts.

<table>
<thead>
<tr>
<th>Plant species &amp; extract</th>
<th>Crude loaded (mg)</th>
<th>Mass recovered (mg)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 A. digitata, twigs, organic</td>
<td>49.6</td>
<td>LLE 35.3</td>
<td>71</td>
</tr>
<tr>
<td>2 A. digitata, bark, aqueous</td>
<td>50.8</td>
<td>LLE 40.2</td>
<td>79</td>
</tr>
<tr>
<td>3 A. digitata, bark, organic</td>
<td>51.8</td>
<td>LLE 27.6</td>
<td>53</td>
</tr>
<tr>
<td>4 A. anthelmintica bark, organic</td>
<td>49.1</td>
<td>LLE 22.9</td>
<td>46</td>
</tr>
<tr>
<td>5 A. anthelmintica roots, organic</td>
<td>48.9</td>
<td>LLE 38.7</td>
<td>79</td>
</tr>
<tr>
<td>6 A. dichotoma roots, aqueous</td>
<td>50</td>
<td>LLE 40</td>
<td>80</td>
</tr>
<tr>
<td>7 A. dichotoma leaves, organic</td>
<td>54.9</td>
<td>LLE 38.3</td>
<td>69</td>
</tr>
<tr>
<td>8 C. imberbe stem bark, organic</td>
<td>49.3</td>
<td>LLE 21.5</td>
<td>43</td>
</tr>
<tr>
<td>9 D. lycioides roots, organic</td>
<td>50.8</td>
<td>LLE 43.2</td>
<td>85</td>
</tr>
<tr>
<td>10 S. marlothii stems, organic</td>
<td>50.2</td>
<td>LLE 23.0</td>
<td>45</td>
</tr>
<tr>
<td>11 S. marlothii stems, aqueous</td>
<td>51.1</td>
<td>LLE 47.8</td>
<td>93</td>
</tr>
<tr>
<td>12 T. sericea roots, organic</td>
<td>123.8</td>
<td>LLE 86.7</td>
<td>70</td>
</tr>
<tr>
<td>13 T. sericea twigs, organic</td>
<td>49.8</td>
<td>LLE 35.3</td>
<td>70</td>
</tr>
<tr>
<td>14 T. sericea leaves, organic</td>
<td>48.7</td>
<td>LLE 36.5</td>
<td>74</td>
</tr>
</tbody>
</table>

Overall, the yield recorded for the LLE extract was higher than the yields for the MeOH fractions (Table 4.3). The highest percentage yield obtained for the LLE extract, was from the crude aqueous extract of the stems of *S. marlothii* (93%).
4.4.2 Biological activity of the LLE extracts and the MeOH fractions obtained from SPE

Comparing the antiplasmodial activity of the crude, organic extract of the twigs of *A. digitata* (5.2 µg/mL), with the LLE extract and the MeOH fraction (Fig. 4.4), showed that the LLE extract was inactive (100 µg/mL), and the MeOH fraction was more than twofold more active than the crude (2.4 µg/mL). Significant differences were observed in the antiplasmodial activity of the crude organic extract (11.4 µg/mL), LLE extracts and MeOH fraction of the roots of *T. sericea*. The most active was the MeOH fraction (0.6 µg/mL), followed by the LLE extract (3.3 µg/mL) and then the organic crude extract. For *S. marlothii*, the MeOH fraction of the organic crude stems was the most active followed by the LLE extract of the aqueous extract of the stems (Fig. 4.4).

Reference has been made in sections 3.3.2.1 and 3.4, to the discrepancy in the traditional use of some of the plant species and the antimycobacterial activity recorded for this study; for example, the results obtained showed that *S. marlothii* displayed antiplasmodial activity for both the organic and crude extracts of the stems, whereas it is ethnomedicinally used for the treatment of tuberculosis.
Figure 4.4: Comparison of the antiplasmodial activity of crude extracts with their respective LLE extracts and MeOH Fractions.

The LLE extract of the roots of *T. sericea* and the stems of *S. marlothii* displayed increased antiplasmodial activity compared to their respective crude extracts. Whereas the methanol fractions obtained from the organic extracts of *A. digitata* twigs, *T. sericea* roots and *S. marlothii* stems, showed superior antiplasmodial activity compared to their respective crude extracts (Figure 4.4). The roots of *T. sericea* is a possible source antiplasmodial lead compounds. As mentioned in section 2.4 in Table 2.1, ellagic acid which was previously detected in the butanol extract of the roots of *T. sericea* was found to display antiplasmodial activity with IC$_{50}$ 0.5 μM$^{14-15}$. Surprisingly, the LLE extract of the twigs of *A. digitata* was devoid of antiplasmodial activity despite the crude displaying good antiplasmodial activity (IC$_{50}$ 5.2 μg/mL). This may indicate a synergistic effect in action and that activity is lost when separating the compounds during the fractionation process. In addition, the MeOH fraction displayed a more than twofold increase in antiplasmodial activity (IC$_{50}$ 2.4 μg/mL).
The antiplasmodial activity results of the crude extracts, the LLE extracts and the MeOH fractions are displayed in Table A1 in the Appendix.

With reference to the antimycobacterial results, both MIC$_{90}$ and MIC$_{99}$ were recorded for the LLE extracts and the MeOH fractions. Surprisingly, the LLE extracts of all the plants did not display any antimycobacterial activity (MIC$_{90} > 100$ µg/mL) (Fig. 4.5). Antimycobacterial activity was lost for *T. sericea* (twigs, org.), *A. anthelmintica* (bark, org. and roots, org.), *A. dichotoma* (leaves, org.), and *D. lycioides* (roots, org.), as both their respective LLE extracts and MeOH fractions were inactive. The MeOH fractions of *A. dichotoma* (roots, aq.), *C. imberbe* (stem bark, org.) and *T. sericea* (leaves, org) displayed some antimycobacterial activity, albeit weaker than their crude extracts. The MeOH fraction of *A. digitata* (bark, organic) displayed an increased antimycobacterial activity compared to its crude extracts. The antimycobacterial activity results of the crude extracts, the LLE extracts and the MeOH fractions are displayed in Table A2 in the Appendix.

An interesting observation, when comparing the crude organic extracts of the leaves of *T. sericea* and that of the stem bark of *C. imberbe* with their respective MeOH fractions, was that they both displayed equipotent antimycobacterial activity.
Figure 4.5: Comparison of the antimycobacterial activity of crude extracts with their respective LLE extracts and MeOH fractions.
4.4.3 NMR analyses

A few researchers have documented the use of NMR fingerprinting to guide the isolation of novel bioactive compounds\textsuperscript{16-18}. In the hope of identifying the compounds which may account for the antiplasmodial and antimycobacterial activity, a phytochemical investigation was carried out guided by comparing the \textsuperscript{1}H-NMR spectra of the crude, LLE and MeOH fractions of antiplasmodial active \textit{S. marlothii} (Fig. 4.6), \textit{T. sericea} (Fig. 4.7) and antimycobacterial active \textit{A. digitata} (Fig. 4.8) and \textit{A. dichotoma} (Fig. 4.9).

\textbf{Figure: 4.6:} \textsuperscript{1}H-NMR (600 MHz) spectra of crude organic extract (c) of the stems of \textit{S. marlothii} compared to its LLE extract (b) and MeOH fraction (a).
The $^1$H-NMR spectrum of the crude extract was complex and the extensive overlap of signals in the aliphatic region of the spectrum, did not allow for the identification of metabolites (Fig. 4.6 (c)). Differences in the profiles of the crude and the slightly less active LLE was noted in the aliphatic region ($\delta 0.5 – \delta 2.5$) of the spectra (Fig. 4.6 (b)). Differences were also detected in the aromatic region ($\delta 6.0 – \delta 7.5$) of the LLE extract but signal overlap with MeOD and D$_2$O in the carbinolic region ($\delta 3.0 – \delta 5.5$), hindered interpretation. The $^1$H-NMR spectrum of the MeOH fraction was less complex in that minimal signal overlap was detected in the aromatic region ($\delta 6.0 – \delta 7.5$) and the carbinolic region ($\delta 3.0 – \delta 5.5$) (Fig. 4.6 (a)).

![NMR Spectra](image)

**Figure: 4.7:** $^1$H-NMR (600 MHz) spectra of crude organic extract (c) of the roots of *T. sericea* compared to its LLE extract (b) and MeOH fraction (a).

Of all the fractions, the MeOH of *T. sericea* showed the best antiplasmodial activity.

From the NMR data, it was clear that this fraction was not pure. The aromatic regions...
(δ 6.0 – δ 7.5) and the aliphatic regions (δ 0.5 – δ 2.5) of the $^1$H-NMR spectra of the crude extract (Fig. 4.7 (c)) and the MeOH fraction (Fig. 4.7 (a)) showed similarities.

The $^1$H-NMR spectra of all three extracts of *A. digitata* (Fig. 4.8), showed that the aromatic region (δ 6.0 – δ 7.5) of the inactive LLE extract (Fig. 4.8 (b)) displayed no signals, whereas in the active crude extract (Fig. 4.8 (c)) and the slightly active MeOH fraction (Fig. 4.8 (a)), these regions displayed signals.

![Figure 4.8: $^1$H-NMR (600 MHz) spectra of crude organic extract (c) of the roots of *A. digitata* displaying antimycobacterial activity compared to its LLE extract (b) and MeOH fraction (a).](image)

By virtue of the nature and the extensive overlap of signals of the $^1$H-NMR spectra of *A. dichotoma* (Fig. 4.9), a meaningful interpretation of the $^1$H-NMR spectra of the LLE and crude extracts could not be done. The $^1$H-NMR of the MeOH fraction (MIC$_{90}$: 97.1 μg/mL) was not preformed.
Figure 4.9: $^1$H-NMR (600 MHz) spectra of the crude organic extract (b) of the roots of *A. dichotoma* which displayed antimycobacterial activity compared to its LLE extract (a).

4.5 Conclusions

According to the literature, the Camp method was developed and its application to source antimalarial and antitrypanosomal compounds, demonstrated. The application of this method to source antimycobacterial lead compounds is reported here for the first time. Deviations mentioned (section 4.2.1) for example the tannins and non-polar interferences that were not removed prior to the analyses, could have caused false positive and false negative biological results. What’s more, the relationship between antimycobacterial potency and the reduced permeability of the mycobacterial cell wall has been documented$^{19}$. The antimycobacterial results obtained revealed that the LLE extracts (with log $P < 5$) were devoid of any activity. This observation is in line with a study that found that an increase in antimycobacterial activity is linked to an increase
in log $P$ for a series of hit compounds. It is also interesting to note that bedaquiline, a recently approved MDR-TB drug, has a c log $P$ of 6.41$^{19}$.

When comparing the antiplasmodial activity of the crude extracts with the LLE extracts and the MeOH fractions, it is evident that for most plant species, the activity was retained or enhanced through the LLE generation/fractionation process. However, in the case of antimycobacterial activity, a loss in activity of the LLE extracts and MeOH fractions, were noticed for *A. digitata*, *A. anthelmintica*, *A. dichotoma*, *D. lycioides* and *T. sericea*. Speculatively, this could be due to the constituents in the crude extracts displaying a synergistic or additive effect, which is lost upon fractionation$^{20}$.

In the case of the antiplasmodial activity, the LLE extracts of *T. sericea* (roots, org.) and *S. marlothii* (stems, aq.) displayed superior activity compared to their crude counterparts, whereas for *A. digitata* (stems, org.) and *S. marlothii* (stems, org.) the MeOH fractions, which supposedly contain non-drug-like compounds, were more active than the crude. It is likely, that the compounds associated with antiplasmodial activity may be non-drug-like with log $P$ values $> 5$. It is also evident that the antiplasmodial activity was not lost during fractionation and that both the LLE extract and MeOH fraction merit further study. It is plausible that the compounds with log $P < 5$ are not responsible for observed antiplasmodial activity and that the MeOH fraction merits further purification to source antiplasmodial agents. Speculatively, partial purification of the crude extracts or LLE generation, unmasked the active compounds of some of these extracts, resulting in an increase in antiplasmodial activity.
The literature review in section 3.1.2, discussed the overview of the plants selected for this study, and revealed that *A. digitata* and *T. sericea* were extensively researched. It is for this reason that these plants, albeit among the most active, were not considered for further phytochemical analyses. For the antimycobacterial disease investigation, further analyses were conducted on the crude aqueous extract of the roots of *A. dichotoma*. The plant *S. marlothii* was selected for the antiplasmodial disease investigation in this study (chapter 5), because both its LLE extract and MeOH fraction displayed superior activity to its crude. In fact, the LLE extracts displayed a fourfold increase compared to its parent crude extract. Another important factor is because this plant is endemic to Namibia and literature search yielded limited data on it. *Sarcocaulon marlothii* displayed good antiplasmodial activity and it was envisaged that the $^1$H-NMR profile, of the most active MeOH fractions, will guide the isolation and subsequent identification of the compound/s responsible for the observed antiplasmodial activity. Even though the active methanol fraction do not display the desired lead-like and drug-like properties, it is plausible that novel antiplasmodial compounds may be isolated from it.
4.6 References


Chapter 5: Phytochemical Analyses and Antiplasmodial activity of *Sarcocaulon marlothii*

5.1 Abstract

*Sarcocaulon marlothii* is endemic to Namibia, and no phytochemical studies has been done on it before. The Daureddaman community in Uis use its stems ethnomedicinally for the treatment of tuberculosis and its symptoms. Results obtained earlier in this project, revealed poor correlation between the ethnomedicinal use and the antimycobacterial activity data. The organic and aqueous crude extracts displayed MIC\textsubscript{90} s of 103 μg/mL and > 125 μg/mL, respectively against *Mycobacterium tuberculosis*. The purpose of this study was to conduct the isolation and characterization of the antiplasmodial compounds. Earlier antiplasmodial activity data of the organic and aqueous extracts of its stems displayed reasonable activity against *Plasmodium falciparum*, with IC\textsubscript{50} s 8.8 μg/mL and 17.8 μg/mL, respectively. From follow-up analyses, it was found that the LLE extract and MeOH fraction, originating from the crude organic extract (IC\textsubscript{50} 8.8 μg/mL), displayed good antiplasmodial activity (IC\textsubscript{50} 10.0 μg/mL and 4.3 μg/mL respectively).

In this study, the crude organic extract of the stems of *S. marlothii* was subjected to a combination of normal phase flash chromatography, normal phase preparative TLC. Eight fractions were recovered from flash chromatography, and guided by \textsuperscript{1}H-NMR, fraction E was subjected to normal phase prep TLC. Nine subfractions were obtained and the \textsuperscript{1}H-NMR of subfraction E9 displayed it to contain a hydroxylated trisubstituted aromatic ring system, and most likely a caffeic acid derivative with no glycoside moiety attached. LC-MS/MS analyses confirmed that subfraction E9 was a mixture of three compounds, and minor impurities. The purified fractions were subjected to \textsuperscript{1}H-
NMR and LC-MS/MS analyses and they revealed that the purified fractions were still mixtures. However, based on the antiplasmodial results of subfraction E9, the antiplasmodial activity was still retained (6.464 ± 1.768 μg/mL) due to its superior activity compared to the crude extract (8.8 μg/mL), from which it was obtained. Further purification of the subfraction is needed to unambiguously identify the active component/s. Speculatively, *S. marlothii* may be a source for new antimalarial compounds or drugs.

**Keywords:** *Sarcocaulon marlothii*, antiplasmodial, malaria, lead compounds, LLE

### 5.2 Introduction

#### 5.2.1 Taxonomy, geographical distribution and habitat of *Sarcocaulon*

The genus *Sarcocaulon* belongs to the order Geraniales and the family Geraniaceae. Other genera in this family include *Geranium, Monsonia, Erodium* and *Pelargonium*.

The Geraniaceae family is widely distributed and consists mainly of annual or perennial herbs and shrubs. Members of the genus *Sarcocaulon* are spiny, fleshy shrubs with delicate white, yellow, salmon-pink or pink flower petals (Fig. 5.1 A).

The fleshy branches are prostrate, semi-erect or erect, covered with a waxy, translucent bark. These wax-covered fleshy branches are flammable and even when wet can be used as a kindling to light fires (Fig. 5.1 B). The local name in Uis is //norab (Damara).

The common names bushmen’s candle (English) or boesmankers (Afrikaans) is used for another species, *Sarcocaulon patersonii*. 
Figure 5.1: Photographs taken during plant collection trip to Uis, of *S. marlothii*. **A**: small shrub with green leaves and flower, and **B**: the stems covered with a waxy layer. (CV Raidron: Photos taken in its natural habitat).

The Geraniaceae family comprises about 700 species, whereas Sarcocaulon consists of 14 species and are mainly found in deserts or semi-desert regions in southern Africa\(^2,3\). Figure 5.2 shows the distribution of the genus Sarcocaulon in South Africa and Namibia. The most widespread species are *S. salmoniflorum* and *S. vanderietiae*, and one species, *S. mossamedense*, also occurs in Angola\(^2\). Three species are endemic to Namibia viz. *S. inerme, S. peniculinum* and *S. marlothii*, of interest to this study\(^2-4\).

Figure 5.2: Distribution of the genus *Sarcocaulon* in Namibia and South Africa\(^2\).
5.2.2 Ethnobotany of the Geraniaceae family

The tea made from the dried roots of *Pelargonium antidysentericum*, a member of the Geraniaceae family, is taken to control diabetes and also for a variety of stomach ailments. The root extract of *P. sidoides* is used traditionally in South Africa as a treatment for respiratory infections. It is marketed as Linctagon®, an over-the-counter medicine, and has been shown to successfully combat respiratory infections associated with colds and flu. All *Sarcocaulon* species have a resinous substance and is used as a popular kindling. An ethnobotanical study done in Namaqualand, South Africa, revealed that *Sarcocaulon* is used as an abortion causing agent and for the treatment of veterinary ailments, for example, parturient in sheep. Some communities in Namaqualand, use the stems for stomach ailments. *Sarcocaulon marlothii* is used by the Damara communities in Namibia to prevent miscarriage. The communities in Uis, Namibia, indicated that they use the stems of *S. marlothii* to treat symptoms of tuberculosis [Personal communication, Mr Thoroub, Uis 2015].

5.2.3 Previous phytochemical and pharmacological studies on members in the Geraniaceae family

Cinnamic acid derivatives such as ferulic acid, caffeic acid, components of ellagitannins such as hexahydroxydiphenic acid, ellagic acid, and geraniin, were identified from *Geranium robertianum*. It is reported that ellagic acid (Fig. 5.4) displayed *in vitro* antiplasmodial activity (IC$_{50}$ 0.5 μM) against chloroquine-sensitive and-resistant strains of *P. falciparum*. Another study concluded that geraniin (Fig. 5.4) displayed antiplasmodial activity (IC$_{50}$ 11.19 μg/mL) against cultured *P. falciparum* 3D7, and could be a potential option for malaria treatment. Known flavonoids, identified in *Geranium robertianum*, are quercetin, kaempferol,
myricetin, and luteolin. Quercetin (Fig. 5.4) displays antiplasmodial activity (IC$_{50}$ 4.11 μM) against *P. falciparum* 3D7 strain. Some quercetin analogues also displayed antiplasmodial activity with IC$_{50}$s ranging between 3.53 – 58.31 μM). Other classes of compounds isolated in this genus are small amounts of lectins, saponins and alkaloids. *Monsonia burkeana* contains a high content of polyphenols and tannins.

Phenolic acids, which include vanillic acid, *p*-hydroxybenzoic acid, and *α*-resorcylic acid were identified in members of the genus *Sarcocaulon*. It was reported that *p*-hydroxybenzoic acid displays some antimalarial activity. A GC-MS analysis of the waxy layer on the stems of *S. patersonii* showed that most of the 30 components identified were pentacyclic triterpenes. One in particular was structurally similar to lupeol, which displays anti-inflammatory action (Fig. 5.3), whilst other triterpenes were structurally similar to the triterpene amyrine. It is known that triterpenes have a wide spectrum of pharmacological effects and important biological activity, including anti-inflammatory, analgesic, liver protection, anti-ulceronic, anti-hyperglycemic, anti-tumor and immune regulation.

**Figure 5.3:** Structure of triterpene lupeol.

Despite being traditionally used for the treatment of TB and associated symptoms [personal communication, Mr Taurob, Uis], some phytochemicals isolated from the genus *Sarcocaulon* has been associated with antiplasmodial activity.
Figure 5.4: Chemical structures of some antiplasmodial polyphenols from Geraniaceae.

5.3 Application of LC-MS and identification of metabolites in complex plant mixtures

Liquid chromatography (LC) used in combination with mass spectrometry (MS) has become the method of choice in various laboratories, because it is selective, sensitive and versatile. A single wavelength detector (UV/Vis) or photodiode array (PDA) detectors are traditionally used for LC and the latter allows the parallel acquisition of a wide wavelength area. Natural products very often contain chromophores, for
example aromatic rings or double bonds, which make them fairly easy to detect using a UV detector. The use of mass spectrometer (MS) as detector for LC, has become popular, especially after the introduction of ionization techniques such as, atmospheric pressure ionization (API), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI is the most popular ionization method for LC-MS, because of the polar nature of most molecules. A positive voltage is used in positive ion mode, ESI+, and the negative ions move towards the capillary walls where they are neutralized, whereas the positive ions move towards the end of the capillary. When a negative voltage is used in negative ion mode, ESI– and the opposite happens.

ESI is classified as a “soft” ionization technique because the fragmentation of the analytes is low during ionization. Usually only protonated ([M+H]⁺) or deprotonated ([M–H]⁻) molecular ions together with some adduct ions e.g. sodium, potassium, lithium, and ammonium are formed during the ionization process. After ionization, the ions move towards a mass analyzer, which is situated in the mass spectrometer, and here the ions that were formed are separated based on their mass-to-charge (m/z) ratio. In secondary metabolite analyses, the time-of-flight (TOF) mass analyzer is commonly used. Modern TOF instruments are often hybrid instrument, Q-TOF, which are equipped with a quadrupole and a collision cell before the TOF mass analyzer. This makes it possible to acquire MS/MS measurements. In MS, a single precursor mass that is characteristic of a given analyte is selected. In MS/MS spectrum, the fragment ions are separated according to mass and the resulting MS/MS spectrum consists only of product ions from the selected precursor.
5.4 Background, scope and limitations of present study

*S. marlothii* is endemic to Namibia and no phytochemical studies have been done on it previously. This study embarked upon the further purification of the organic crude extract. Results obtained from this study (Chapter 3, section 3.3.2.1) revealed that the aqueous and organic crude extracts of the stems, were devoid of antimycobacterial activity (MIC$_{90}$ values of $>125$ μg/mL and 103 μg/mL respectively), but that it instead displayed antiplasmodial activity with an IC$_{50}$ of 17.8 μg/mL for aqueous and 8.8 μg/mL for the organic extract. Also observed, was that the LLE and MeOH fractions (Chapter 4, section 4.3.2) obtained from the antiplasmodial-active crude organic extract, displayed antiplasmodial activity with IC$_{50}$s 10.0 μg/mL and 4.3 μg/mL, respectively.

On the basis of the above, it was decided to continue with the purification of the potential antiplasmodial agents from the active MeOH fraction of the organic crude stem extract, albeit that they will be nondrug-like or Lipinsky-noncompliant. However, the yields obtained for the LLE extract (23.0 mg) and MeOH fractions (8.6 mg), were too low to continue with the fractionation thereof. Additionally, a bioassay-guided fractionation, requires that fractions and subfractions obtained be subjected to biological testing, but for this study, this could not be done because of the large number of fractions obtained and the costly nature of the antiplasmodial testing. In view of these limitations, the study continued with the fractionation and purification of the organic crude extract of the stems (IC$_{50}$ 8.8 μg/mL), guided by the $^1$H-NMR spectral data.
5.5 Materials and methods

5.5.1 Sample information

The crude organic extract of the stems of *S. marlothii* was subjected to a combination of normal phase flash chromatography and preparative TLC.

5.5.2 Reagents, solvents and instruments

All reagents used were of analytical reagent (AR) grade. The solvents used for LC-MS/MS were bought from Merck and Sigma-Aldrich, and were of HPLC grade. They were used without further purification. Thin layer chromatography (TLC) was performed on aluminium backed pre-coated silica gel 60 F<sub>254</sub>, 20 x 20 cm sheets, (Merck, 1.05554.0001). TLCs were visualized under ultraviolet light (UV) at 245 or 365 nm. Reagents used to visualize spots included: Iodine; p-anisaldehyde prepared by mixing solution A (5% p-anisaldehyde in EtOH) with solution B (5% H<sub>2</sub>SO<sub>4</sub> and 20% acetic acid in MeOH) in a 3:1 ratio; ceric ammonium sulphate; acid spray stain (1:1 mixture of H<sub>2</sub>SO<sub>4</sub> and MeOH) and vanillin spray reagent (vanillin dissolved in 95% EtOH (6.3% (w/v)). Preparative thin layer chromatography was performed on silica gel GF UV 254, 2000 micron glass plates (Uniplate, Analtech, P02015), or PLC Silica Gel 60 F<sub>254</sub>, 1 mm glass plates, 20 x 20 cm, Merck, (1.13895.0001). Normal phase flash chromatography was performed with silica gel 60, high purity, pore size 60 Å, 70-230 mesh, 63-200 μm (Fluka). The column used for flash chromatography (diameter 25 mm; length 24 cm and fitted with a glass frit) was packed with 40 mL of silica gel.

The Nuclear Magnetic Resonance (NMR) experiments were performed on a Bruker 600 MHz spectrometer equipped with a 5 mm Prodigy cryoprobe. <sup>1</sup>H and <sup>13</sup>C chemical shifts (δ, ppm) are relative to residual solvent signals. NMR samples were dissolved
in either deuterated methanol (CD$_3$OD), deuterated dimethyl sulfoxide (DMSO-$d_6$),
deuterated water (D$_2$O), or deuterated chloroform (CDC$_3$). In some cases mixtures of
the deuterated solvents listed above were used. $^1$H–NMR splitting patterns were
abbreviated as follows: s (singlet), br s (broad singlet), d (doublet), dd (doublet of
doublets), t (triplet), q (quartet) and m (multiplet). Coupling constants ($J$) were
reported in Hertz (Hz). MestreNova version 7 was used to process the NMR spectra
off-line. All NMR analyses were done in the Chemistry department at the University
of Cape Town, South Africa.

The LC-MS/MS analyses of selected subfractions were performed at the Central
Analytical Facilities (CAF), at Stellenbosch University, South Africa. The instrument
used was a Waters equipped with a photodiode array detector and UPLC coupled to a
Synapt G2 quadrupole. The analysis was performed on a Waters BEH C18 column,
with dimension of 2.1 x 100 mm. The mobile phase A was 0.1% formic acid and
mobile phase B was 0.1% formic acid in acetonitrile. The run time was 15 minutes.
MassLynx version 4.1 was used for the instrument control and data acquisition. The
samples were analyzed in both ESI positive and negative modes using a cone voltage
of 15 V. MS$^E$ (an acquisition mode performed concurrently with full scan acquisition
in a single analysis where fragmentation is performed using a collision energy ramp of
50-100 V). Data were acquired across a mass range of 150-1500 amu for MS and 100-
1500 for MS/MS. The PDA was set to acquire UV data in the 230-500 nm range. The
off-line software MassLynx 4.1 was used to process the chromatograms.
5.5.3 Flash chromatography of the crude organic extract of the stems of *S. marlothii*

The crude extract (1.928 g) was adsorbed separately onto silica gel (10 mL), allowed to dry and poured onto the silica gel in the column (dry packing). Cotton wool was placed on top to avoid disturbing the silica layer. Sequential elution was done with the following mobile phases: Fraction C: 100% Hexane; Fraction D: 80% Hexane:EtOAc; Fraction E: 60% Hexane:EtOAc; Fraction F: 100% EtOAc; Fraction G: 50% EtOAc:MeOH; Fraction H: 100% MeOH. During the elution of fractions D and F, different colour bands were observed, collected separately and labelled D1, D2, F1 and F2, respectively. Overall eight fractions were obtained and labelled: C, D1, D2, E, F1, F2, G, and H, (Fig.5.5), dried *in vacuo*, weighed and stored in the freezer until further analyses and purification. All eight fractions obtained from flash chromatography were submitted for $^1$H-NMR analysis (Fig. 5.8). Fractions E and F1 were combined based on their $^1$H-NMR profiles to yield combined fraction E (Fig. 5.8). As mentioned in section 5.4, the fractions were not submitted for antiplasmodial testing, instead, the selection of fractions for further purification (E and G) was done based on NMR profiles and yields obtained.

5.5.4 Further purification of combined fraction E and G obtained from flash chromatography

5.5.4.1 Preparative TLC of combined fraction E

Thin layer chromatography (TLC) of combined fraction E was performed and revealed that 7:3 Hexane:EtOAc mobile phase gave the best separation. Combined fraction E was subsequently subjected to preparative TLC. A pre-weighed sample (305.1 mg) was dissolved in minimum DCM and loaded onto 2 x 2 mm thick normal phase preparative TLC plates. After development in 7:3 Hexane:EtOAc mobile phase, the
plates were visualized under UV and the bands that separated were marked, each band was scraped and eluted with 50% DCM:MeOH. After filtration, the mother liquor obtained was dried *in vacuo* and then freeze dried. Ten subfractions were obtained (labelled E1 – E10, Fig. 5.6) and submitted for $^1$H-NMR analysis (Fig. 5.9). The $^1$H-NMR data of the subfractions were examined and subfraction E9 was selected for LC-MS/MS analysis (Fig. 5.12) and antiplasmodial testing.

### 5.5.4.2 Preparative TLC of fraction G

Thin layer chromatography (TLC) of fraction G revealed that 6:3 CHCl$_3$:H$_2$O sat. H$_2$O gave the best separation. Fraction G was subjected to preparative TLC. A pre-weighed sample (265 mg) was dissolved in minimum DCM and loaded onto 2 x 1 mm thick normal phase preparative TLC plates. After complete development in 6:3 CHCl$_3$:H$_2$O sat. H$_2$O mobile phase and air dried, the plates were visualized under UV and the separated bands marked, each band was scraped off and eluted with 50% DCM:MeOH. After filtration the mixture was dried *in vacuo* and then freeze dried. Six subfractions were obtained and labelled G1 – G6 (Fig. 5.7). Only subfractions G1 and G2 were submitted for antiplasmodial testing due to a lack of resources.
S. marlothii, stems, (org)
Antiplasmodial: IC₅₀: 8.8 µg/mL

1.928 g of dried organic crude: silica gel flash column chromatography

- C: 3.0 mg
- D1: 101.1 mg
- D2: 71.3 mg
- E: 401.0 mg
- F1: 163.3 mg
- F2: 7.7 mg
- G: 755.0 mg
- H: 97.1 mg

Fractions:
A: 70% MeOH:H₂O with 1% TFA
B: 100% MeOH
C: 100% Hexane
D: 80% Hexane: EtOAc
E: 60% Hexane: EtOAc
F: 100% EtOAc
G: 50% EtOAc: MeOH
H: 100% MeOH

50.2 mg of dried crude:
SPE, Strata-X

- A: 23.0 mg IC₅₀: 10.0 µg/mL
- B: 8.6 mg IC₅₀: 4.3 µg/mL

62.9% recovery (31.6 mg)

LLE Extract

MeOH Fraction

62.9% recovery (31.6 mg)

Figure 5.5: Flow-chart depicting the fractionation of the crude organic extract of the stems of S. marlothii.
**Figure 5.6:** Flow-chart depicting the subfractions obtained from combined fraction E after preparative thin layer chromatography.

**S. marlothii, stems, (org)**

IC$_{50}$: 8.8 µg/ml

Silica gel flash column chromatography

**Combined E**

305.1 mg

Preparative TLC

2 mm plate; 7:3 Hexane: EtOAc

E1
1.2 mg

E2
1.3 mg

E3
5.9 mg

E4
23.4 mg

E5
26.0 mg

E6
22.3 mg

E7
28.3 mg

E8
22.4 mg

E9
29.4 mg

E10
4.8 mg

54% recovery

(165 mg)
S. marlothii, stems, (org)
IC₅₀: 8.8 µg/ml

Silica gel flash column chromatography

G
Loaded 265 mg on 3 x 1 mm preparative TLC plates
Mobile phase: 6:3 CHCl₃: MeOH sat. H₂O

G1, 6.4 mg
G2, 4.7 mg
G3, 32.7 mg
G4, 30.2 mg
G5, 85.0 mg
G6, 37.2 mg

74 % Recovery
(196.2 mg)

Preparative TLC fractions

Figure 5 7: Flow-chart depicting the subfractions obtained from fraction G after preparative thin layer chromatography.
5.5.5 Antiplasmodial testing of subfractions

Samples were tested in triplicate over 72 hours against the wild-type drug sensitive strains of the human malaria parasite, *Plasmodium falciparum* NF54. Continuous cultures of asexual erythrocyte stages of *P. falciparum* were maintained using the method described by Trager and Jensen\textsuperscript{27} with minor modifications. Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase assay using the method described by Makler *et al.* (1993)\textsuperscript{28}. Parasite viability was determined colourimetrically using the breakdown of a dye by metabolic enzymes of the glycolytic pathway taking place in living parasites as a marker for survival.

The test samples were prepared as 20 mg/mL stock solutions in 100% DMSO. Samples were tested as a suspension if not completely dissolved. Further dilutions were prepared in growth media on the day of the experiment. The standard antimalarial drugs chloroquine (CQ) and artemisin (Arts) were used as reference drugs in all experiments. A full dose-response was performed for all compounds in a 96-well plate to determine the concentration inhibiting 50% of parasite growth (IC\textsubscript{50} value). Test samples were tested at a starting concentration of 10 μg/mL, which was then serially diluted 2-fold in growth medium to generate the tested concentration range. The same dilution technique was used for all samples. (CQ) and (Arts) were tested from a starting concentration of 1 μg/mL. The highest concentration of solvent to which the parasites were exposed was < 0.1% and had no measurable effect on the parasite viability (data not shown). The assay plate was incubated at 37 °C for 72 h in a sealed gas chamber under 3% O\textsubscript{2} and 4% CO\textsubscript{2} with the balance being N\textsubscript{2}. After 72 h, the wells in the assay plate were gently suspended, and 15 μL from each well was transferred to a duplicate
plate containing 100 μL of Malstat reagent and 25 μL of nitroblue tetrazolium solution in each well. Plates were left to develop for 20 minutes in the dark and then absorbance of each well was quantified using a spectrophotometer at 620 nM wavelength. The remaining population of parasites at each concentration of the test compound was determined by comparing the absorbance of each well to the absorbance of a well containing the drug-free control. Survival was plotted against concentration and the IC₅₀-values were obtained using a non-linear dose-response curve fitting analysis via the Dotmatics software platform.

5.6 Results and discussion

The purification of the organic crude extract of the stems, commenced with flash chromatography and the eight fractions obtained had a combined mass of 1.615 g (83% recovery, Fig. 5.5). Both fractions E and G were subjected to PTLC (section 5.5.5) and fraction E afforded ten subfractions labelled E1 – E10 with a combined mass of 165 mg (54% recovery, Fig.5.6). PTLC of fraction G afforded 6 subfractions with a combined mass of 196.2 mg (74% recovery, Fig.5.7).

¹H-NMR spectroscopy provides a rapid and non-destructive identification of a wide range of compounds. It is used with success to indicate whether a biologically active extract contains novel or known metabolites²⁹. In order to identify the components which may account for the antiplasmodial activity of the stems, a comparison of ¹H-NMR spectra (Fig. 5.6) of the crude extract, LLE extract, MeOH fraction with those of the eight fractions (C, D1, D2, E, F1, F2, G, and H1) was done. The comparison of their ¹H-NMR spectral data revealed the separation by flash chromatography was not effective. Although it is not expected of flash chromatography to provide the resolution or reproducibility of HPLC, it is helpful to improve the purity of samples to an
acceptable level\textsuperscript{30}. Examination of the $^1$H-NMR spectra of fractions E and F revealed similar signal patterns in the aromatic region of the spectra. The two fractions E and F\textsubscript{1} were therefore combined (564.3 mg), and labelled combined E.

After preparative TLC of combined fraction E, ten subfractions with a total mass of 165 mg (54\% recovery) were obtained and 46\% of the sample loaded, remained stuck on the silica gel, which is a non-negligible loss. Examination of the $^1$H-NMR spectral data of the subfractions E\textsubscript{1}-E\textsubscript{10} (Fig. 5.9), revealed that the separation was still poor and although E\textsubscript{10} appeared to be pure, it could not be submitted for $^1$- and 2D-NMR analysis because of the low yield (4.7 mg). E-3 could also not be pursued further due to the low yield (5.9 mg).
Figure. 5.8: *S. marlothii*: $^1$H-NMR (600 MHz) spectra. Comparison of crude organic, LLE extracts (Fraction A) and MeOH fractions (Fraction B) with fractions C – H obtained from flash chromatography.
**Figure 5.9:** *S. marlothii:* $^1$H-NMR (600 MHz) spectra. Comparison of combined fraction E with subfractions E1-E10.
Figure 5.10: Comparison of the $^1$H-NMR (600 MHz) spectra of subfraction E9 with the crude organic extract and MeOH fraction from the stems of *S. marlothii*.

Figure 5.10 compares the $^1$H-NMR of crude, MeOH fraction and subfraction E9 and the following were inferred:

1. Extensive overlap in the aliphatic region of spectra was indicative that subfraction E9 was still impure. This was confirmed with the LC-MS data (section 5.5.3).

2. Notably identical signals, labelled 1-5 in Fig. 5.10 were observed in the aromatic region ($\delta$ 6.0 – $\delta$ 7.5 ppm) of the crude extract, MeOH fraction and subfraction E9 (Fig. 5.10). These signals can most likely be assigned to the bioactive compound, that is the compound responsible for the observed antiplasmodial activity of subfraction E by virtue of their presence in the active MeOH, and absence in the less active LLE (10.0 µg/mL).
Figure 5.11 (a) showed the $^1$H-NMR spectra of subfraction E9 as well as the expansion of the aromatic region (Fig. 5.11 (b). The spectrum indicates that subfraction E9 still contains impurities. From the expansion two spin systems (AB and ABX) could be identified. The AB spin system include vinylic protons resonating at $\delta$ 6.24 and $\delta$ 7.54, each appearing as doublets (d) with a typical trans coupling ($J = 15.6$ Hz). The multiplicity of the three signals for the ABX spin system, resonating at $\delta$ 6.85, $\delta$ 6.98 and $\delta$ 7.08, indicate the presence of a hydroxylated, trisubstituted aromatic ring system. Speculatively, the above indicate the presence of a trisubstituted hydroxycinnamic acid derivative and most likely a caffeic acid derivative, due to the absence of the methoxy signals in the carbinolic region (Fig 5.11 (c).
Figure 5.11: (a) The $^1$H-NMR (600 MHz) spectra of subfraction E9; (b) Expanded aromatic region: $6.2 - 7.6$ ppm; (c) Partial structure of a caffeic acid derivative.
5.6.1 LC-MS/MS analysis of subfraction E9

The sample was analyzed in both ESI positive and negative modes. Since the ESI negative analyses did not reveal additional information compared to the ESI positive results, only the ESI positive results are reported here.

An analysis of the diluent used to prepare the sample solutions, was analyzed as a blank sample; the total wavelength chromatogram (TWC), total ion chromatograms (TIC) of base peak intensities (BPIs) of the ESI+ and ESI– modes can be found in the Appendix, Fig. A1. Figure 5.12 displays the TWC and TIC of base peak intensities (BPIs) of electron spray ionization positive (ESI+) and negative (ESI–) modes of subfraction E9. Three major peaks were observed in the TIC ESI+. The ESI+ BPI chromatogram revealed a peak at retention time 6.92 minutes (labeled peak 1) that coincides with a peak in the TWC. The base and pseudomolecular ion, [M+H]+ peak was observed at m/z 419.0978 with another prominent peak at m/z 837.1889. Upon further inspection of the MS spectrum of peak 1 (Fig. 5.13(A)) it was deduced that m/z 837.1889 is the pseudo-molecular ion ([2M+H]⁺) of a dimer, which is confirmed by the peaks at m/z 854.2158 and 859.1726 identified as the ammonium adduct ([2M+NH₄]+) and sodium adduct ([2M+Na]+) ions, respectively. The MS/MS spectrum shows the pseudomolecular ion at m/z 419.0978, (Fig. 5.13 (B)) which is confirmed by the peaks at m/z 441.0794 and 457.0541 identified as the sodium adduct ([M+Na]+) and potassium adduct ([M+K]+) ions, respectively. Thus, the compound has a molecular mass of 418.0978 amu and the molecular formula of the neutral molecule fragment at m/z 419.0978 was calculated by the MassLynx 4.1 software to be C₂₀H₁₈O₁₀ and entered in the Dictionary of Natural Products (DNP)³¹. Details of the individual peaks are shown in Table 5.1.
A number of matches were obtained including coruleoellagic acid, fukiic acid, piscidic acid, salvianolic acid D, kaempferol 3-glucosides and other flavonoid glycoside derivatives. The available LC-MS/MS- and $^1$H-NMR spectral data of the matches were consulted and none coincided with the spectral data obtained for this peak 1.

The ESI$^+$ BPI chromatogram revealed a second peak at retention time 9.46 minutes for a UV-inactive compound as revealed by the TWC chromatogram. In the MS spectrum, the base peak was observed at $m/z$ 899.7508 and another prominent peak at $m/z$ 881.7410 (Fig. 5.14(A)). Upon further inspection of the MS spectrum it was deduced that $m/z$ 899.7508 was the ammonium adduct ion [M+NH$_4$]$^+$ and also the base peak. The peak at 881.7410 was the pseudomolecular ion [M+H]$^+$. Hence, the compound has a molecular mass of 880.7410. The molecular formula of the neutral molecule
fragment at m/z 881.7410 was calculated by the MassLynx 4.1 software to be C_{60}H_{97}O_{4} or C_{53}H_{101}O_{9}. Details of the individual peaks are displayed in Table 5.1.

**Figure 5.13:** The complete ESI+ MS spectrum (A) of the peak at retention time 6.92 mins. and the MS/MS spectrum (B) of subfraction E9.

Subsequently the molecular formula of the neutral molecule fragment at m/z 423.362 was calculated by the software to be C_{30}H_{46}O_{1} and entered in the Dictionary of Natural Products (DNP)\textsuperscript{31}. A number of matches were obtained including furospinulosin 2, many derivatives of lanosta, taraxastratrien-3-ol, and ursadien-3-ol. The available MS/MS- and \textsuperscript{1}H-NMR spectral data were consulted and none coincided with the spectral data obtained for this study.
Figure 5.14: The complete ESI+ MS spectrum (A) of the peak at retention time 9.46 mins. and the MS/MS spectrum (B) of subfraction E9.

The ESI+ BPI chromatogram revealed a third peak at retention time of 10.76 for a UV-active compound as presented in the TWC chromatogram. In the MS spectrum, the base peak was observed at \( m/z \) 457.3685 and is also the pseudomolecular ion, \([M+H]^+\), and \([2M+H]^+\) at \( m/z \) 913.7306 (Fig. 5.15(A)). Confirmation of this was revealed upon further inspection of the MS/MS spectrum (Fig.5.15 (B)), and it was deduced that the following peaks at \( m/z \) 479.3508, and 935.7141 were identified as the \([M+Na]^+\), and \([2M+Na]^+\) adducts. Hence, the compound at this peak has a molecular mass of
456.3685 amu. Details of the individual peaks are displayed in Table 5.1. Subsequently, the molecular formula of the neutral molecule fragment at \( m/z \) 457.3685 was calculated by the software to be \( C_{30}H_{48}O_3 \) and entered in the Dictionary of Natural Products (DNP). A total number of 686 hits were obtained, however analysis of literature data yielded no matches.

**Figure 5.15:** The complete ESI+ MS spectrum (A) of the peak at retention time 10.76 mins., and the MS/MS spectrum (B) of subfraction E9.

Using the LC-MS data for subfraction E9 none of the three peaks could be tentatively identified.
**Table 5.1:** Individual peaks of subfraction E9 by ESI-MS.

<table>
<thead>
<tr>
<th>Peak #.</th>
<th>t&lt;sub&gt;r&lt;/sub&gt;(min)</th>
<th>[M+H]&lt;sup&gt;+&lt;/sup&gt; m/z</th>
<th>MW / (Da)</th>
<th>Molecular Formula</th>
<th>Adducts m/z</th>
<th>Fragmentation (m/z)</th>
<th>Tentative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.92</td>
<td>419.0978 (100)</td>
<td>418.0978</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;O&lt;sub&gt;10&lt;/sub&gt;</td>
<td>[M+Na]&lt;sup&gt;+&lt;/sup&gt; 441.0794 [M+K]&lt;sup&gt;+&lt;/sup&gt; 457.0541 [2M+H]&lt;sup&gt;+&lt;/sup&gt; 837.1889 [2M+NH&lt;sub&gt;4&lt;/sub&gt;]&lt;sup&gt;+&lt;/sup&gt; 854.2158 [2M+Na]&lt;sup&gt;+&lt;/sup&gt; 859.1437</td>
<td>877.1468; 875.1437; 824.2062; 457.0541; 404.0746; 389.0864; 375.0710; 285.0762</td>
<td>Unknown</td>
</tr>
<tr>
<td>2</td>
<td>9.46</td>
<td>881.7410</td>
<td>880.7410</td>
<td>C&lt;sub&gt;60&lt;/sub&gt;H&lt;sub&gt;97&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt; or C&lt;sub&gt;53&lt;/sub&gt;H&lt;sub&gt;101&lt;/sub&gt;O&lt;sub&gt;9&lt;/sub&gt;</td>
<td>[M+NH&lt;sub&gt;4&lt;/sub&gt;]&lt;sup&gt;+&lt;/sup&gt; 899.7508 (100)</td>
<td>939.7446; 901.7598; 900.7538; 482.3679; 481.3667; 459.3843; 442.3768; 441.3730; 423.3626 (100); 411.3626; 405.3524; 393.3510; 273.2204; 245.1894; 217.1948; 177.1637</td>
<td>Unknown</td>
</tr>
<tr>
<td>3</td>
<td>10.76</td>
<td>457.3685 (100)</td>
<td>456.3685</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;46&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>[M+Na]&lt;sup&gt;+&lt;/sup&gt; 479.3508 [2M+H]&lt;sup&gt;+&lt;/sup&gt; 913.7306 [2M+Na]&lt;sup&gt;+&lt;/sup&gt; 935.7141</td>
<td>673.3380; 480.3531; 439.3578; 341.2844; 231.1747; 177.0546</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
5.6.2 Antiplasmodial activity testing of subfractions E9, G1 and G2

The test compounds showed no significant activity against the *P. falciparum*. Subfraction E9 displayed the best activity with G1 showing activity only at the upper end of the concentration range. Subfraction G2 was inactive even at the maximum tested concentration.

Table 5.1: Activity of subfractions tested in vitro against *P. falciparum* NF54. Data shown are represented in micrograms/mL with standard error mean where it could be calculated.

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>NF54 IC$_{50}$ (μg/mL)</th>
<th>Standard Error Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9</td>
<td>6.464</td>
<td>1.768</td>
</tr>
<tr>
<td>G1</td>
<td>8.595</td>
<td>1.601</td>
</tr>
<tr>
<td>G2</td>
<td>&gt;10</td>
<td>NA</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.009 μM</td>
<td>0.002</td>
</tr>
<tr>
<td>Artesunate</td>
<td>&lt;0.005 μM</td>
<td>NA</td>
</tr>
</tbody>
</table>

5.7 Conclusion

The aim of this part of the study was to isolate and identify the antiplasmodial compound(s) from the crude organic extract of the stems of *S. marlothii*, using the antiplasmodial data recorded, NMR profiles and LC-MS/MS data of fractions and subfractions obtained. The chromatographic methods employed were not effective as was confirmed by $^1$H-NMR profiles and the LC-MS data. However, based on the antiplasmodial results of subfraction E9, the antiplasmodial activity was still retained (6.46 μg/mL) due to its superior activity compared to the crude extract (8.8 μg/mL) from which it was obtained. The similarity of the $^1$H-NMR profiles of E9 and the crude extract (Fig. 5.10), albeit it impure, indicated the presence of compounds to which the antiplasmodial activity can be ascribed. The attempts to identify, even tentatively, using LC-MS data were unsuccessful. $^1$H-NMR data, speculatively led to the
identification of a partial structure, being a caffeic acid derivative with no glycoside moiety attached. *S. marlothii* therefore merits a more detailed phytochemical analysis.
5.8 References


8. Nortje JM. *Medicinal ethnobotany of the Kamiesberg, Namaqualand, Northern Cape Province, South Africa*. MSc Thesis. Johannesburg: University of Johannesburg, Department of Botany, Faculty of Science; 2011.


Chapter 6: Partial Phytochemical Analyses and Antimycobacterial Activity of Aloe dichotoma (Aloidendron dichotomum)

6.1 Abstract

*Aloe dichotoma*, is indigenous to Namibia and the Northern Cape. For this study, it is important to note that *A. digitata* displayed the best antimycobacterial activity (9.9 μg/mL), but has been subjected to extensive phytochemical studies. For this reason, *A. dichotoma* was selected because of the paucity of literature date on the phytochemical and pharmacological properties. Other members of the Aloe species has been extensively researched. The Daureddaman community in Uis use its roots ethnomedicinally for the treatment of tuberculosis. Results from this study confirmed the correlation between the ethnomedicinal use and the biological activity of the roots displayed activity against *Mycobacterium tuberculosis* (MIC<sub>90</sub> 27.3 μg/mL). The purpose of this study was to conduct the isolation and characterization of the active antimycobacterial compounds.

The crude aqueous extract of the roots was subjected to a combination of C-18 flash chromatography and normal phase preparative TLC. Five fractions were recovered from flash chromatography, and ¹H-NMR spectra revealed that none of the fractions were pure. Guided by TLC analyses, fractions D and E were subjected to preparative TLC after which three and four subfractions were recovered, respectively. Subfraction D1-A was analysed using LC/MS/MS and it revealed that it was still a mixture. A two-fold decrease in antimycobacterial activity of the subfraction was observed (MIC<sub>90</sub> 62.5 μg/mL) compared to the crude extract (MIC<sub>90</sub> 27.3 μg/mL).
Further purification of the subfraction is needed to unambiguously identify the active component/s.

**Keywords:** Aloe dichotoma,

### 6.2 Introduction

#### 6.2.1 Taxonomy, geographical distribution and habitat of Aloe

Prior to 2014, the Aloe genus was placed in the family Xanthorrhoeaceae subfamily Asphodeloideae. The family name Asphodelaceae is more widely used\(^1\) and presently includes over 500 species with nearly 420 species confined to Africa\(^1^3\).

In 2013, name changes were recommended, which affected Aloe and related genera. One of the suggestions were that the tree aloes be placed in a genus of its own, viz. *Aloidendron*. Aloes are one of the most popular components of the diverse flora in southern Africa and in particular South Africa. They are adapted to grow in areas of low water availability. Members of the genus are well represented on the African continent, Arabian Peninsula, Madagascar and eastern Indian Ocean islands\(^4^5\). The genus range from very small shrubs, to large trees\(^2\) and the species of interest to the present work, *Aloe dichotoma* (Fig. 6.1), now known as *Aloidendron dichotomum*\(^6^7\), is a tall branching tree, indigenous to Southern Africa, specifically in the Northern Cape and Namibia (Fig. 6.2). The bark on the trunk have razor sharp golden brown scales\(^6\).
Figure 6.1: Photographs taken during plant collection trip to Uis of A. dichotoma. A: Aloe tree with dichotomous branches and leaves, and B: tree bark. (C. Raidron: Photos taken in its natural habitat).

Figure 6.2: Distribution of A. dichotoma in Namibia and South Africa. This species is listed in Appendix II of the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), which means that it is not threatened, but trade must be controlled in order to avoid utilization incompatible with their survival.
6.2.2 Ethnobotanical and other uses of Aloe

Aloe species are used traditionally for over 2000 years in various cultures for their curative and therapeutic properties such as anti-inflammatory, antimicrobial, immune boosting, hypoglycemic, hypolipidemic, antidiabetic, wound-healing, antitumour and antioxidant properties. Usage also includes the treatment of constipation, burns and dermatitis\(^{5,9-10}\). *Aloe ferox*, also known as Cape aloes, is most widely used as a potent laxative but it is also reported to relieve arthritis, and conjunctivitis and other eye ailments. In Namibia, the sap of *Aloe hereoensis* and *A. littoralis* diluted in water, are reported to be used to treat sexually transmitted infections. The species endemic to Namibia, *A. asperifolia*, is used traditionally for epilepsy and arteriosclerosis. It is reported that *Aloe dichotoma* is used in Namibia to treat TB\(^{11}\). The genus is of great importance from taxonomic, ethnomedicinal, chemotaxonomic, ecotouristic and horticultural perspectives\(^{12}\). Consequently, there has been extensive research done on the some Aloe species, however, limited phytochemical research has been done on *A. dichotoma* and the other tree species,\(^{11-14}\). A comprehensive review detailing the ethnomedicinal use, pharmacological and toxicological studies carried out on indigenous and exotic Namibian aloes is lacking.

6.2.3 Previous phytochemical studies and pharmacological data on the Genus Aloe

Previous studies on the Aloe spp., focused primarily on the isolation of biologically active compounds from the leaves. For species *A. pulcherrima* and *A. hijazensis*, and *A. ferox*, the roots were investigated\(^{13,15-17}\). The acetone extract of the roots of *A. pulcherrima* displayed superior antibacterial activity and three anthraquinones were isolated from it, namely chrysophanol, aloesaponarin I and aloesaponarin II (Fig. 6.3). These anthraquinones displayed moderate *in vitro* antiplasmodial activity against both chloroquine-resistant (W2) and chloroquine-sensitive (D6) strains of *Plasmodium*
The highest activity was exhibited by aloesaponarin II (IC$_{50}$ 5.00 ± 0.36 μg/mL).

In another study, the methanol extract of the roots of *A. hijazensis* showed pronounced antimicrobial activity against different pathogenic species of bacteria and fungi, on the basis of the agar disc diffusion method. The following compounds were isolated from the methanol extract of its roots: aloesaponarin II, 3-methyl ether, ziganein, 4, 7-dichloroquinoline, chrysophanol, lupeol, aloe-emodin, aloesaponarin I, ferulic acid acid, caffeic acid, isovitexin, chrysophanein and aloenin B.

A chemotaxonomic survey of the roots of 172 Aloe species was done. From the acetone extract of *Aloe ferox*, the following compounds were identified: chrysophanol, asphodelin, aloesaponarin I, aloesaponarin II, aloesaponol I and aloesaponol II.

A review on the medicinal potential of South African *Aloe* species, revealed that the methanolic extract of the leaves of *A. dichotoma* displayed weak antiplasmodial activity (% parasite growth at 50 μg/mL: 72.35 ± 18.08$^{4,19}$). The genus *Aloe* has over the years proven to be an important source of biologically active compounds. More than 130 compounds belonging to different classes: anthrones, chromones, pyrones, coumarins, alkaloids, glycoproteins, naphthalenes and flavonoids have so far been isolated from this genus$^{3,10,13,20}$. Many of these compounds are responsible for some pharmacological activities previously described. For example, chrysophanol, an anthraquinone, has been reported to display antioxidant, anti-ulcer, anti-inflammatory, anticancer, neuroprotective, anti-aging, lung protective and hepatoprotective properties$^{15,21}$.

![Chemical Structures](image)

**Figure 6.3:** Chemical structures of some anthraquinones isolated from the roots of *Aloe ferox*, *A. pulcherrima*, and *A. hijazensis*.
6.3 Background and scope of current work

In Chapter 3, the biological activities of the crude extracts were discussed and the results obtained showed that the aqueous crude extract of the roots of *A. dichotoma* displayed the second best antimycobacterial activity ($\text{MIC}_{90}$ $27.3 \mu g/mL$) compared to the organic crude extract of the leaves ($\text{MIC}_{90}$ $86.8 \mu g/mL$). As discussed in Chapter 4, the LLE extract was inactive ($\text{MIC}_{90} >125 \mu g/mL$) and MeOH fraction displayed poor activity ($\text{MIC}_{90} 97.1 \mu g/mL$). The aqueous crude extract of the roots was therefore selected to be subjected to fractionation and isolation. This study focused on isolation and identification of antimycobacterial active compounds from the roots of *Aloe dichotoma*.

6.4 Materials and Methods

6.4.1 Sample information

The crude aqueous extract of the roots of *A. dichotoma* was subjected to a combination of reversed phase flash chromatography and normal phase preparative TLC. $^1$H-NMR spectroscopy was used to gain insight into the nature of the fractions. Please refer to The reagents, solvents and instruments used (NMR and LC-MS/MS) for the analyses of the fractions was covered in Chapter 5, section 5.5.2.

6.4.2 Reversed phase flash chromatography of the crude aqueous root extract of *Aloe dichotoma*

The crude aqueous extract of the roots was subjected to reversed phase flash chromatography with C-18 silica gel, KP-C18-HS Bulk sorbent (Biotage), 90 Å pore size. The column used for flash chromatography (diameter 25 mm; length 24 cm and fitted with a glass frit) was packed with 40 g of the C-18 silica. The pre-weighed crude extract (504.5 mg) was adsorbed onto C-18 silica (10 mL), allowed to dry and poured
onto the stationary phase. Cotton wool was placed on the top of the silica and eluted with the following mobile phases: Fraction 1 (C): 100% H\textsubscript{2}O; Fraction 2 (D): 75% H\textsubscript{2}O:MeOH; Fraction 3 (E): 50% H\textsubscript{2}O:MeOH; Fraction 4 (F): 25% H\textsubscript{2}O:MeOH; Fraction 5 (G): 100% MeOH (Fig. 6.4). Fractions D and E were selected, based on NMR profiles and yields obtained, for further purification. Fraction C was not selected, on the basis that it was obtained by eluting with 100% H\textsubscript{2}O and would most likely contain sugars.

6.4.3 Purification of fractions D and E obtained from flash chromatography

6.4.3.1 Preparative TLC (PTLC) of fractions D and E

Thin layer chromatography (TLC) of fractions D, E, the crude extract and MeOH fraction, revealed that 5:7:3 EtOAc:Acetone:H\textsubscript{2}O gave the best separation. Both fractions (D and E) were subsequently subjected to preparative TLC. Pre-weighed samples (176 mg of D and 98.3 mg of E) were separately dissolved in minimum EtOAc and loaded onto separate 1 mm normal phase preparative TLC plates (Merck). After development, the plates were visualized under UV\textsubscript{254} nm and the bands scraped and eluted with 50% DCM:MeOH. After filtration, the mother liquor obtained was dried in vacuo and then freeze dried (Fig. 6.5). Three subfractions (bands) were obtained from D, which were labelled D1-A, D1-B and D2 and five subfractions from E, labelled E1 – E5 (Fig. 6.5). Only subfraction D1-A was submitted for antimycobacterial testing and LC-MS/MS analysis, based on TLC profiles, \textsuperscript{1}H-NMR and yields.
6.5 Results and discussion

6.5.1 Flash chromatography: crude aqueous extract of the roots of A. dichotoma

Five fractions, labelled C - G were obtained (Fig 6.4) with a total mass of 565.3 mg (94% recovery). The fractions were stored in the freezer until further analyses and purification. All the fractions were submitted for $^1$H-NMR analysis (Fig. 6.6). The spectra revealed that none of the fractions were pure and further purification was needed. Ideally, a bioassay guided fractionation should have been followed, however this was not feasible, due to limited resources.
A. dichotoma, roots, (aq)
Antimycobacterial activity:
MIC\textsubscript{90}: 27.3 µg/mL

504.56 mg: C-18 silica gel flash column chromatography

50 mg: SPE Strata-X

A: 40 mg
MIC\textsubscript{90}: >125 µg/mL

B: 3.6 mg
MIC\textsubscript{90}: 97.1 µg/mL

87% recovery (43.6 mg)

LLE Extract

MeOH Fraction

Fractions:
A: 70% MeOH: H\textsubscript{2}O with 1% TFA
B: 100% MeOH
C: 100% H\textsubscript{2}O
D: 75% H\textsubscript{2}O: MeOH
E: 50% H\textsubscript{2}O: MeOH
F: 25% H\textsubscript{2}O: MeOH
G: 100% MeOH

94% recovery (565.3 mg)

504.56 mg: C-18 silica gel flash column chromatography

C 363.0 mg

D 106.9 mg

E 70.1 mg

F 19.8 mg

G 5.5 mg

TLC & preparative TLC analyses

Figure 6.4: Flow-chart depicting the fractionation of the crude aqueous extract of the roots of A. dichotoma.
A. dichotoma, roots, (aq) antimycobacterial activity: 
\[ \text{MIC}_{90}: 27.3 \, \mu g/ml \]

C-18 silica gel flash column chromatography

**D**

Loaded 176 mg on 3 x 1 mm preparative TLC plates; mobile phase 5:7:3 EtOAc:Acetone:H\textsubscript{2}O

- D-1A, 15.2 mg
- D-1B, 19.7 mg
- D-2, 21.0 mg

**E**

Loaded 98.3 mg on a 1 mm preparative TLC plates; mobile phase 5:7:3 EtOAc:Acetone:H\textsubscript{2}O

- E-1, 4.4 mg
- E-2, 11.7 mg
- E-3, 8.5 mg
- E-4, 10.4 mg
- E-5, 11.0 mg

32% Recovery (55.9 mg)

47% recovery (46.0 mg)

**Figure 6.5:** Flow-chart displaying the subfractions obtained from fractions D and E respectively after preparative TLC.
Figure 6.6: Comparison of the $^1$H-NMR (600 MHz) spectra of the crude - and the LLE extracts with the fractions (C, D, E, F, and G) obtained from flash chromatography of the aqueous crude extract of the roots of *A. dichotoma*.

The fractions obtained from reversed phase flash chromatography, were eluted with different solvent systems starting, with 100% H$_2$O (fraction C; 363.0 mg), followed by increasing concentrations of MeOH in a combination of H$_2$O: MeOH mixture.
(fractions D, E, and F; 106.9 mg, 70.1 mg and 19.8 mg respectively), and concluding with 100% MeOH (fraction G; 5.5 mg).

\(^1\)H-NMR of the fractions obtained from reversed flash chromatography, revealed that C contains mostly sugars, as is observed by a series of overlapping signals between δ 3.0 and 4.0 (Fig. 6.6). The spectra of fractions D, E and F were not well resolved and little structural information was deduced from this. The spectrum of fraction G, although well resolved, indicated that it was still a mixture, but the quantities obtained did not allow for the further purification or analysis.

6.5.2 Purification of fractions D and E by preparative TLC

As mentioned earlier, three subfractions were obtained from fraction D and labelled D1-A, D1-B and D2 with total mass 55.9 mg (32% recovery) (Fig. 6.5). Five subfractions were obtained from fraction E and labelled E1 – E5 (Fig. 6.5) with a total mass of 46.0 mg (47% recovery).

6.5.3 LC-MS/MS analysis of subfraction D1-A

The sample was analyzed in both ESI positive and negative modes. Since the ESI positive analyses did not reveal additional information compared to the ESI negative results, only the ESI negative results are reported here.

As mentioned in Chapter 5, an analysis of the diluent used to prepare the sample solutions, was analyzed as a blank sample, prior to the analysis of the subfraction. The total wavelength chromatogram (TWC), total ion chromatograms (TIC) of base peak
intensities (BPIs) of electron spray ionization positive mode and negative mode (ESI⁺) and (ESI⁻) of the blank appears in Fig. A1 in the Appendix.

Figure 6.7: TWC (A), TIC ESI⁻ (B) and ESI⁺ (C) BPI chromatograms of subfraction D1-A.

Figure 6.7 displays the TWC and TIC of base peak intensities (BPIs) of electron spray ionization positive (ESI⁺) and negative (ESI⁻) modes of subfraction D1-A. Two peaks of interest were observed in the TIC ESI⁻. The ESI⁻ BPI chromatogram revealed a peak at retention time 3.39 minutes (labeled peak 1) and another at retention time 3.59 minutes (labeled 2). The MS revealed identical fragmentation patterns and only the peak at retention time 3.39 minutes was considered to be analysed further. The base peak was observed at m/z 474.0803 and another peak at m/z 949.1693 (Fig. 6.8). The MS/MS spectrum of the peak at retention time 3.39 (Fig. 6.9), displayed a prominent peak at m/z 949.1693, and a reduction in the size of the base peak at m/z 474.0803. This may indicate that this could be the pseudomolecular ion ([M+H]⁺), and hence, the compound has a mass of 473.0803 amu. Upon further inspection of the MS/MS
spectrum of peak 1 (Fig. 6.10) it was deduced that $m/z$ 949.1693 is the pseudo-molecular ion ([2M+H]$^+$) of a dimer.

Figure 6.8: Complete MS spectrum of peak at retention time 3.39 minutes of subfraction D1-A.

Figure 6.9: Complete MS/MS spectrum of peak at retention time 3.39 minutes of subfraction D1-A.

After consultation of the National Institute of Standards and Technology (NIST) mass spectrometry data centre, analysis of the data as well as the MS$^E$ and UV data, yielded no matches.
6.5.4 Antimycobacterial testing

The antimycobacterial activity tests was performed for subfraction D1-A, using the microdilution method as described in Chapter 3. The antimycobacterial activity of subfraction D1-A was calculated as MIC$^{90}_{\text{MIC}}$ 62.5 μg/mL, which is less active than the crude (27.3 μg/mL).

6.6 Conclusion

These results confirm the ethnobotanical use of the roots of *A. dichotoma* by the Uis community. A loss in antimycobacterial activity was observed after fractionation and purification and might be indicative of a synergistic effect of compounds in the crude extract. Antimycobacterial activity in other fractions could have been missed, and this highlights the importance of a bioactivity guided isolation and characterization with NMR, which is highly recommended for future studies.
6.7 References


Chapter 7: Summary, conclusions and recommendations

The eradication of tuberculosis and malaria, still remain a challenge worldwide. In Namibia, these two diseases have a great impact especially on low-income communities, who rely on traditional medicine to treat these diseases and their symptoms. The safe and efficacious use of these traditional plants needed to be verified scientifically. This study intended to contribute to the chemical knowledge, reveal the antiplasmodial and antimycobacterial activity and explore the potential of medicinal plants used by local communities in Uis and Tsumkwe in Namibia, as sources of drug-like compounds. Eight plants were selected based on their ethnomedicinal uses and these included: *T. sericea* (antimalarial), *A. anthelmintica* (antimalarial), *O. paniculosa* (antitubercular), *D. lycioides* (antitubercular), *S. marlothii* (antitubercular), *C. imberbe* (antitubercular), *A. digitata* (antitubercular and antimalarial) and *A. dichotoma* (antitubercular). Due to an increase in drug resistance, as discussed in Chapters 1 and 2, strategies to accelerate the discovery of lead compounds from medicinal plants has become urgent. Also mentioned in Chapter 2, section 2.2.2, are the challenges that exist in natural product research with one being the labor-intensive nature of classical isolation methods. Costly equipment is needed to rapidly analyze large numbers of extracts, fractions and subfractions for bioactivity, as well as to aid the early identification of classes of compounds to be eliminated or otherwise, prioritized. In recognition of the importance of medicinal plant extracts as a source of lead-like or drug-like compounds, this study applied the method of Camp *et al.*\(^1\) to front-load (prioritize) plant extracts with drug-like properties.
In total, 25 plant parts were extracted using organic solvents and, in accordance with the traditional use and preparation methods, aqueous extracts were also prepared. Not enough sample was available to perform the aqueous extract of the stems of *A. digitata*. The total number of extracts (25 organic and 24 aqueous), were screened for both antimalarial and antimycobacterial activity. Of all the plants collected, *A. digitata* displayed the best antimycobacterial (MIC\(_{90}\) 9.9 μg/mL) and antiplasmodial activity (IC\(_{50}\) 5.2 μg/mL). The second best antimycobacterial and antiplasmodial activity was displayed by *A. dichotoma* (MIC\(_{90}\) 27.3 μg/mL) and *S. marlothii* (IC\(_{50}\) 8.8 μg/mL), respectively. The results obtained in this study supports the traditional use of *T. sericea* (org, roots; 40% survival and IC\(_{50}\) 8.7 ± 0.28 μg/mL), *A. digitata* (org. twigs; 44% survival and IC\(_{50}\) 5.2 μg/mL) and *A. anthelmintica* (85% survival) for the treatment of malaria. *Albizia anthelmintica*, however, displayed very weak antiplasmodial activity (85% survival at 20 μg/mL). The bark of *A. digitata* is used ethnomedicinal to treat TB symptoms and the biological results revealed that the aqueous extract of the bark, indeed displayed very good antimycobacterial activity (MIC\(_{90}\) 9.9 μg/mL). It thus merits further studies as a source of potential antitubercular agents. Ethnomedicinally, *O. paniculosa*, *D. lycioides*, *S. marlothii*, *C. imberbe*, and *A. dichotoma* are used to treat TB symptoms. The results obtained in this study, showed that *O. paniculosa* and *S. marlothii* display very weak antimycobacterial activity (MIC\(_{90}\) 108 μg/mL and 103 μg/mL, respectively); it is plausible that the ethnomedicinal use of the roots of *O. paniculosa* and the stems of *S. marlothii* do not correlate with the biological results obtained for this study. This study, however, supported the antitubercular use of the remaining plants species, *C. imberbe* and *A. dichotoma* for which MIC\(_{90}\)s of 27 μg/mL and 47 μg/mL, respectively, were recorded.
The active crude extracts (4 antiplasmodial and 10 antimycobacterial), were subjected to SPE fractionation to obtain the LLE extracts, which had drug-like properties. In half of the cases, the LLE extract displayed increased antiplasmodial activity compared to the crude extract (Fig. 4.4). The LLE extracts of the stems of *S. marlothii*, and the roots of *T. sericea*, displayed increased antiplasmodial activity (IC\textsubscript{50}s 3.3 and 12.1 μg/mL, respectively), compared to their respective crude extracts (IC\textsubscript{50}s 11.4 and 17.8 μg/mL, respectively). The MeOH fractions (regarded as the rinse/washing of the SPE cartridge) which were collected and subjected to biological testing, was not part of the original method of Camp *et al.* The biological results obtained were surprising in that three out of four MeOH samples, displayed increased activity, compared to the antiplasmodial activity of the crude extracts. With regard to antimycobacterial activity, neither the MeOH fractions nor the LLE extracts displayed activity.

It is worth mentioning that the method applied in this study deviated from the one by Camp *et al.*, in that the dried powdered plant materials in this study were not pre-extracted with hexane, that is, to remove fats and other nonpolar interfering compounds. Also, tannins, which can interfere in biochemical assays and lead to false positive results\(^1\), were not removed from the crude extracts. This would have required the passing of the crude extracts through a polyamide CC-6 column, prior to the SPE procedures. Nonetheless, in light of the above-mentioned deviation we cannot conclude on the application and effectiveness of the Camp method in the search for antiplasmodial and antimycobacterial drug leads.
Also, due to the low yields obtained for the LLE extracts none were submitted for LC-MS analysis. Instead, selected fractions, obtained by subjecting the active crudes to flash chromatography and subfractions obtained by preparative TLC, were subjected to LC-MS/MS. The LC-MS/MS analysis revealed that none of the fractions or subfractions were pure enough to allow for the tentative identification of metabolites.

None of the extracts of *O. paniculosa* were subjected to SPE for LLE extracts and may have been prematurely excluded. Little scientific data currently exists on phytochemical studies done on this plant species and it is must be considered for further analyses.

The structure elucidation of the antiplasmodial compound(s) present in the stems of *S. marlothii* was not possible with the data obtained. The promising antiplasmodial activity of *S. marlothii* (IC$_{50}$ 4.3 μg/mL), it being an endemic species to Namibia and the fact that it has not been researched before, serve as strong motivation for an in-depth, phytochemical study to be conducted. It is recommended that this follow-up research should include a pre-extraction of the plant material with hexane followed by the removal of tannins. Up-scaling of the LLE extracts and MeOH fractions, originating from the organic crude extract of the stems, must be done, to ensure that enough biomass will be available for bioactivity-guided isolation of the MeOH fraction alongside NMR and LC-MS/MS analyses.

In support of previous reports on the Baobab tree, also known as the *Tree of Life,* this study confirmed the therapeutic value to use its part to treat TB and malaria and their
symptoms. Results of this study will be shared with the Uis and Tsumkwe communities, who shared their indigenous knowledge. Note however that the result obtained for study do not serve as complete scientific validation, but instead made a contribution towards the validation of TM by correlation of the data obtained in this study and the ethnomedicinal uses of different Namibian medicinal plants. A full validation will include cytotoxicity and info on phytochemicals present and will be pursued in a follow-up study.

References

Appendix

Figure A1: TWC Chromatogram (A), TIC ESI (B) and ESI+ (C), BPI chromatograms of the blank sample.

<table>
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<th>Plant species</th>
<th>Plant part</th>
<th>Extract/Fraction</th>
<th>IC₅₀ (µg/mL)</th>
<th>NF54 % survival at 20 µg/mL</th>
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<td>MeOH</td>
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<td>&gt;125</td>
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### Figure A2: NBRI Identification Report 1

#### National Herbarium of Namibia (WIND)

**Identification Report**

Report No.: 2015/372  
17 August 2015

<table>
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<td>Diospyros lyciodes Decf. subsp. lyciodes</td>
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<td>Combretum mukwe Wurmb</td>
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<td>Synkielospermum brasiliense (Benth.) D. F. Oliver</td>
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<td>Fergusia abakana (Forsk.) C. M. van Zanten</td>
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**Identification categories:**  
1. Certain identification  
2. Closest to  
3. Certain to genus only  
4. Unable to identify
Figure A3: NBRI Identification Report 2

Ministry of Agriculture, Water and Forestry
National Herbarium of Namibia (WIND)
Identification Report
Report No.: 5 June 2017

Collector/s: Hans, R.
Address: UNAM
          Department of Chemistry
          Private Bag 13301
          Windhoek

<table>
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<td>Salvadora persica L.</td>
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Comment:

Curator
National Herbarium of Namibia (WIND)