ETHNOPHARMACOLOGICAL ASSESSMENT OF *GUIBOURTIA COLEOSPERMA* AND *DIOSPYROS CHAMAETHAMNUS* EXTRACTS AS ALTERNATIVE TREATMENT OPTIONS FOR MALARIA

A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF NAMIBIA

BY

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OCTOBER 2016

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ABSTRACT

Malaria, a parasitic infectious disease, remains one of the world’s foremost health concerns, even more so in developing countries. Much progress has been made in fighting the disease, particularly in Southern Africa where four countries (Namibia included) have targeted malaria elimination by 2020. Challenges such as the absence of a vaccine, resistance to insecticides, and particularly the emergence of resistance to current antimalarial treatment regimens threaten to undermine the current successes in malaria control efforts. Secondly, the lack of access and acceptance of conventional antimalarial treatment by populations in malaria endemic areas reduces the feasibility of eliminating and consequently eradicating malaria. Local communities in Namibia use plant-based medicines to treat malaria and malaria associated symptoms based on traditional observations or beliefs over decades. As to whether these plants are efficacious for the indication and cause toxicity is yet to be validated scientifically. The aims of this study were, therefore, to evaluate the biological activities of the extracts of two Namibian plant species to provide a scientific rationale for their traditional uses.

*Guibourtia coleosperma* and *Diospyros chamaethamnus*, which are used to alleviate symptoms of malaria in Namibia, were investigated using phytochemical analyses, and *in vitro* and *in vivo* bioassays. Extracts were prepared by using solvent extraction of varying polarities to obtain a wide range of metabolites. Ground plant material was macerated in distilled water (aqueous extracts) and dichloromethane-methanol (1:1v/v) (organic extracts) respectively. The extracts were dried *in vacuo*, and examined for six classes of compounds known to have antiplasmodial activity using TLC. GC-MS was used to identify compounds
in the plant extracts related to biological activity, using a BP5MS column. Radical scavenging abilities of the plant extracts were ascertained by the 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) method. \textit{In vitro} antimalarial activity was determined by reconstituting the plant extracts in water and DMSO at varying concentrations, and incubating extracts with \textit{Plasmodium falciparum} D10 infected RBCs for 48 hours. Subsequently, growth inhibition of the \textit{P. falciparum} parasites was determined using parasitaemia. \textit{In vitro} assays to determine cytotoxic effects were conducted with the plant extracts using a fibroblast cell line (W138). \textit{In vivo} inhibition of the growth of \textit{P. berghei} in Swiss albino mice (20±4 g) was evaluated using optical microscopy on blood smears. Survival curves post-infection were also used to determine suppressive and prophylactic activities of extracts. The plant extracts were also evaluated for their toxicity in healthy mice using a dose escalation method with a starting dose of 300 mg kg$^{-1}$.

The crude extracts contained alkaloids, anthraquinones, flavonoids, steroids and terpenoids. Secondary metabolites with antiplasmodial, antibacterial and antioxidant properties were tentatively identified as 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, phloroglucinol, stigmasterol, glycerol 2-hexadecanoate, $\alpha$-amyrin, 9,12-octadecadenoic acid (Z,Z)-, hexadecanoic acid, oleic acid, lanosterol, spiculesporic acid, squalene, campesterol and vitamin E. Antioxidant activity results showed that the extracts of \textit{D. chamaethamnus} yielded the highest antioxidant activities with IC$_{50}$ values of ranging from 7.63 to 10.74 $\mu$g/mL, whilst the extracts \textit{G. coleosperma} yielded antioxidant activities with IC$_{50}$ values of ranging from 22.03 to 36.05 $\mu$g/mL. Moderate \textit{in vitro} antiplasmodial activity (IC$_{50}$ < 50 $\mu$g/ml) against \textit{P. falciparum} D10 was observed for the two plants ranging from 18.30 to 31.61 $\mu$g/mL. All plant extracts showed no cytotoxicity with IC$_{50}$ values above 100
µg/mL, except for the organic extract of *D. chamaethamnus* (CC₅₀=29.73). The organic extracts for both *D. chamaethamnus* and *G. coleosperma* (800 mgkg⁻¹) exhibited significant 

\( (P< 0.05) \) blood schizonticidal activity in the 4-day early infection test with parasite growth inhibition of 44.66 and 29.59 %, respectively. This dose also prolonged the survival of the mice by 50 and 58 % (*i.e.* with 6 and 7 days), respectively. The plant extracts also exhibited prophylactic activity in the mice inhibiting parasitaemia with 56.13 (*D. chamaethamnus*) and 55.48 % (*G. coleosperma*) at the highest dose (800 mgkg⁻¹) and increasing survival by 155.6 and 22.2 % (*i.e.* with 14 and 3 days), respectively. Oral administration of crude extracts at the highest dose of 2000 mgkg⁻¹ resulted in no mortalities or evidence of adverse effects, indicating that *D. chamaethamnus* and *G. coleosperma* extracts were non-toxic.

The study showed promising antimalarial activities of *D. chamaethamnus* and *G. coleosperma*. The results show that the ethnomedicinal use of these plants to treat symptoms of malaria is rational and safe. This is a step in the right direction towards incorporating their use in mainstream health care policies as alternative treatment options for malaria. Identification of bioactive compounds to standardize extracts should be the next step. Further studies should also include the optimization of doses to improve efficacy, and studies to assess the antiplasmodial activities of the two plants in combination treatments as used in an ethnomedicinal setting. Lastly, this study has shown that the plant extracts can also be used as a prophylactic. This is new knowledge should be shared with indigenous communities to maximize the medicinal use of these plants.
Key words: medicinal plants, *Diospyros chamaethamnus*, *Guibourtia coleosperma*, malaria, phytochemical screening, antimalarial compounds, *in vitro* antiplasmodial activity, *in vivo* antiplasmodial activity, cytotoxicity, acute oral toxicity
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<table>
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ACT(s)</td>
<td>Artemisinin-based combination therapy/therapies</td>
</tr>
<tr>
<td>AEAC</td>
<td>Ascorbic acid equivalent antioxidant capacity</td>
</tr>
<tr>
<td>ART-LUM</td>
<td>Artemether-Lumefantrine</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BP5MS</td>
<td>5% diphenyl-95% dimethylpolysiloxane</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicine</td>
</tr>
<tr>
<td>CC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% cellular cytotoxic concentration</td>
</tr>
<tr>
<td>CHAI</td>
<td>Clinton Health Access Initiative</td>
</tr>
<tr>
<td>CTMDR</td>
<td>Centre for Traditional Medicine and Drug Research</td>
</tr>
<tr>
<td>DAPP</td>
<td>Development Aid for People to People</td>
</tr>
<tr>
<td>DCAQ</td>
<td><em>Diospyros chamaethamnus</em> aqueous extract</td>
</tr>
<tr>
<td>DCOR</td>
<td><em>Diospyros chamaethamnus</em> organic extract</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro-diphenyl-trichloroethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-Diphenyl-1-picryl-hydrazyl</td>
</tr>
<tr>
<td>DVS</td>
<td>Dominant vector species</td>
</tr>
<tr>
<td>E8</td>
<td>Elimination eight</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Authenticated of Cell Cultures</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's minimum essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Association</td>
</tr>
<tr>
<td>FPPIX</td>
<td>Ferriprotoporphyrin IX</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCAQ</td>
<td><em>Guibourtia coleosperma</em> aqueous extract</td>
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<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GCOR</td>
<td><em>Guibourtia coleosperma</em> organic extract</td>
</tr>
<tr>
<td>GFATM</td>
<td>Global Fund to Fight AIDS, TB and Malaria</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV/AIDS</td>
<td>Human immune virus/Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>IC50</td>
<td>50 % inhibitory concentration</td>
</tr>
<tr>
<td>i.d.</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IKS</td>
<td>Indigenous Knowledge System</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor residual spraying</td>
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<td>ITN(s)</td>
<td>Insecticide treated nets</td>
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<tr>
<td>IUPEC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography–mass spectrometry</td>
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<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>MDG</td>
<td>Millennium Development Goal</td>
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<tr>
<td>MoHSS</td>
<td>Ministry of Health and Social Services</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NVDCP</td>
<td>National Vector-borne Disease Control Programme</td>
</tr>
<tr>
<td>OP</td>
<td>Per Os (by mouth)</td>
</tr>
<tr>
<td>PSG</td>
<td>Phosphate saline glucose</td>
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<tr>
<td>RBC(s)</td>
<td>Red blood cell(s)</td>
</tr>
<tr>
<td>RBM</td>
<td>Roll back malaria</td>
</tr>
<tr>
<td>RDT(s)</td>
<td>Rapid diagnostic test(s)</td>
</tr>
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<td>RI(s)</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<td>SADC</td>
<td>Southern Africa Development Community</td>
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<td>SI</td>
<td>Selective index/indices</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SFH</td>
<td>Society for Family Health</td>
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<td>SRB</td>
<td>Sulforhodamine B</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<td>TCA</td>
<td>Trichloroacetic acid</td>
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<td>TIC(s)</td>
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<td>TLC</td>
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<td>TM</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ACKNOWLEDGMENTS

The German Academic Exchange Program (DAAD) together with the Multidisciplinary Research Centre (MRC) and the Staff Development Office at the University of Namibia funded this research. I would like to express my gratitude to the three entities for the financial support throughout the course of this study.

I would like to extend my heartfelt gratitude to my supervisor Dr. Davis Mumbengegwi for his guidance, mentorship and invaluable support over the last four years, for going above and beyond to assist and advise me. Thank you for giving me the opportunity to learn from you and to follow in your footsteps! Thanks also to my co-supervisor, Dr. Ronnie Böck. He was always ready and willing to extend a helping hand and give advice where needed.

I would like to express my sincere gratitude to Dr. Stefan Louw, for providing me with his technical expertise in gas chromatograph-mas spectroscopy and assisting me in the data analyses. Thank you for your time and valuable contribution!

I would also like to thank Dr. Myrtle Morkel who assisted in phlebotomy; as well as all the members of the Biomedical Research Laboratory, past and present, particularly Florence Dushimemaria, Munyaradzi Tambo, Sylvia Nafuka, Joyce Auala, Erastus Haindongo, Rahl-Jeanne Bussel, Waldimar Swartz and Tanga Kashupi for rendering
assistance during this study, specifically in donating blood. I would also like to thank Mildred Johnson and Julia Hoveka for always being readily available and willing to donate blood.

I would like to thank Mr. Enock Moindi, Dr. James Kuria and Mr. Chrispas Ngule from the Centre for Traditional Medicine and Drug Research (CTMDR), Kenya Medical Research Institute (KEMRI), Nairobi for training and hosting me with the animal work. I would also like to extend my gratitude to the Office of the Vice Chancellor for funding the training.

Lastly, I would like to thank the almighty God. Thank you Lord for this amazing opportunity and journey in which I was able to contribute to my field of study, and for your guidance in every step of the way!

I can do all things through Christ who strengthens me.

– Philippians 4:13
DECLARATION

I, Charwan Iwanette du Preez, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Charwan Iwanette du Preez
CHAPTER 1: INTRODUCTION

1.1 General introduction

The first drug recorded in history for malaria was quinine, an alkaloid isolated from the bark of the *Cinchona* tree in 1817 (WHO, 2007b). In the 1940s an analogue of quinine, chloroquine was synthesized due to shortages of the drug (Sá, Chong, & Wellems, 2011). The emergence of parasites resistant to this synthetic drug however, soon led to its demise in the late 1950s in some parts of the world, and by the 1980s in almost all regions with *P. falciparum* malaria (Achan *et al*., 2011) as the first line treatment for malaria. Artemisinin, another natural compound, was isolated from the Chinese herb *Artemisia annua* in 1971 (Hill & Staunton, 2010, p. 385). Artemisinin and derivatives such as artesunate, artemether and artether, are used in combination with partner drugs such lumefantrine, halofantrine and mefloquine. These are commonly known as artemisinin-based combination therapies (ACTs) and are currently the first line treatment for uncomplicated malaria (Skolnik, 2015, p. 336). However, due to monotherapy, indiscriminant use, as well as the use of counterfeit drugs, resistance to artemisinin has already been reported in five countries (Skolnik, 2015, p. 336) and reduced sensitivity of *P. falciparum* to ACTs in regions of South-East Asia including the Thailand–Cambodia border (Sá *et al*., 2011).

There are a limited number of effective alternatives to artemisinin-based drugs for the treatment of malaria (Skolnik, 2015, p. 336). The ever increasing emergence and spread
of drug resistance makes treatment of malaria increasingly difficult. Furthermore, lack of access and acceptance of conventional treatment for malaria is a reality in some communities (Worrall, Basu, & Hanson, 2005; Tipke et al., 2009). At present there is no functional, safe, and widely available malaria vaccine (Mata, Salvador, Igartua, Hernandez, & Pedraz, 2013), hence efforts to develop new antimalarial drugs are of even greater significance. Since existing antimalarial chemotherapeutic agents are based on natural products, plants continue to play an important role in the search for leads for antimalarial drugs (Wang, Hao, & Chen, 2007).

Plants have historically been used as traditional remedies and are still used up to this day, with about 80% of the population in rural parts of Africa and other developing countries relying on traditional herbal medicines to treat day to day infirmities (Bodeker & Kronenberg, 2002; Mahomoodally, 2013). This is a result of long-term use, allowing for a selection process to weed out plants causing adverse side-effects (Mahomoodally, 2013). Traditional medicines (TMs) are perceived not only as affordable, but also as effective and safe. However, this is not always true and because of a lack of scientific data, it is not possible to guarantee their safety for human consumption. When TMs are used casually or in inappropriate doses, they can elicit harmful effects on the body, which can be fatal (Street & Van Staden, 2009).

A vast knowledge of medicinal plants for treatment of malaria exists. According to Willcox et al. (2011) 1,277 plants are used traditionally to manage malaria worldwide.
In Namibia, some communities especially in rural areas use TMs to treat malaria and malaria-like symptoms, reinforcing the need for the integration of TM with modern medicine to achieve elimination of malaria. However, before plants can be established as alternative or complementary medicines for malaria they need to be scientifically valorized as safe and effective (Fokunang et al., 2011).

1.2 What is malaria?

Malaria is a treatable and preventable infectious disease. It is caused by protozoan parasites from the genus *Plasmodia*, which are transmitted from person to person through the bites of female *Anopheles* mosquitoes (Bope & Kellerman, 2015). Five species of *Plasmodia* are responsible for malaria in humans, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi*, and *P. malariae* (Antinori, Galimberti, Milazzo, & Corbellino, 2012); of which *P. falciparum* is the most common cause of malaria, especially in sub-Saharan Africa. It is responsible for severe malaria and the subsequent high mortality rate in this region (Snow & Omumbo, 2006), whilst *P. vivax*, has a high malaria incidence rate on the Asian and South American continents (Gething et al., 2012). Together they account for more than 90% of clinical cases worldwide. Drug resistance has been reported mostly in *P. falciparum* infections (Bope & Kellerman, 2015, p. 143). *P. knowlesi* was formerly found only in primates, but with changes in environmental conditions and the subsequent close proximity of humans with monkeys in Southeast Asian countries, infections have now been reported in the human population of these countries (Ramasamy, 2014; Bope & Kellerman, 2015).
The parasites have a complex life cycle; half of their cycle occurs in humans and the other half in mosquitoes. After a human is bitten by an infected mosquito, sporozoites travel to the liver and infect hepatocytes where they mature into schizonts. The schizonts are released into the blood as thousands of merozoites initiating the erythrocytic stage of their life cycle (Salton & Kim, 1996; Haussig, Matuschewski, & Kooij, 2011). However, in *P. vivax* and *P. ovale*, some schizonts become dormant and remain in the liver indefinitely as hypnozoites (Hulden & Hulden, 2011). In the red blood cell (RBC) the parasites replicate asexually, releasing more parasites into the blood stream. A portion of the merozoites differentiates into sexual forms known as gametocytes that are taken up by the mosquito when it feeds on blood of a malaria infected person (Baker, 2010).

In the gut of the mosquito, the male and female gametocytes develop into gametes, which in turn form ookinetes. The ookinetes transform into oocysts that eventually develop into sporozoites. The sporozoites travel to the salivary glands of the mosquito and are injected into the skin of a human during a blood meal to start the cycle again (Shamil, Ravi, & Chandra, 2014; Bope & Kellerman, 2015, p. 144).

The common symptoms of malaria are recurrent symptoms such as fever, shivering and sweating, often occurring concurrently with the rupture of schizonts and subsequent destruction of RBCs to release parasites. Flu-like symptoms including aches, coughing, malaise, diarrhoea and vomiting are also common in malaria infected patients, as well as infirmities such as jaundice, anemia (Bope & Kellerman, 2015, p. 144). However, in
cases of severe malaria, the nervous, respiratory and renal and or hematopoietic systems are often affected. Hypoglycemia, metabolic acidosis, kidney failure, and coma are common complications including severe anemia which are often fatal (Trampuz, Jereb, Muzlovic, & Prabhu, 2003).

1.3 Malaria, a health concern

Malaria is endemic in many parts of the world, where it continues to be a public health problem, with about 3.2 billion people affected worldwide. In 2013 alone, an estimated 198 million clinical cases were reported, most of which were in Africa. Africa accounted for 82% of all malaria cases, followed by South-East Asia with 12%. That same year, an estimated 584,000 malaria deaths were recorded worldwide. Africa accounted for 90% of the deaths, of which 78% fatalities were in children under the age of five. Malaria also puts a heavy burden on the economy of the developing world by exhausting health care resources and by associated loss of economic activity (WHO, 2014).

In Namibia the majority of malaria cases are found in the north central and north eastern parts of the country. The climate in these parts are conducive for breeding of the Anopheles arabiensis mosquitoes, the carriers of the P. falciparum parasites (MoHSS, 2014). More than half of the population lives in areas of high malaria transmission. Populations that are most at risk are in and around the Zambezi and Kavango regions and along the Namibia-Angolan border. The number of malaria incidences (out-patient and in-patient) reported in 2004 was 595,367, which reduced significantly to 3,213 in
2012. The number of deaths in 2004 (1,734) was relatively high compared to 2012 (4). However, between 2012 and 2014, a rise in malaria cases and mortality was observed. In 2013, reported cases had gone up from 3,213 to 4,844, as did the malaria deaths from 4 to 21. In 2014, the malaria incidence (17,166) and number of deaths (61) continued to increase (MoHSS, 2015).

Despite malaria posing a health threat, Namibia and several previously known endemic countries, such as South Africa, Swaziland and Botswana, are moving towards elimination of the disease. This change in trend is a result of implementation of interventions such as insecticide treated nets (ITNs), indoor residual spraying (IRS), larviciding, and intermittent preventive malaria treatment for pregnant women. Early diagnosis and appropriate treatment for all malaria cases using artemisinin-based combination therapy (ACT) regimens are also an important contributing factor in this decline (WHO, 2014). According to White (2008), treatment of malaria is of paramount importance in its elimination and eventual eradication. The demise of artemisinin derivatives would reverse the global efforts in malaria control, as well as negatively affecting the health of communities in malarious areas.

1.4 Statement of the research problem

Currently there are few available malaria medicines including WHO-recommended treatments, due to the emergence and spread of resistant malaria parasites and because of a reduced sensitivity of *P. falciparum* parasites to the drugs. This presents a challenge to
the country, as it aims to eliminate malaria by 2020. Hence, the removal of all *Plasmodium* reservoirs is critical. This means treating all malaria cases with medicines that are known to be effective. In Namibia, some communities prefer TM over allopathic medicine, and in some instances, they do not have readily available access to modern medicine. This presents another challenge in itself. This can be solved by the incorporation of medicinal plant use in healthcare policies. Many traditional methods, however, do not meet the criteria of standardized medicines, because there is no documentation on their safety and efficacy, nor is there scientific evidence on their medicinal uses. Plant-based treatments are nonetheless an important source of antimalarials. This is portrayed by the current WHO-recommended treatment, ACTs that consists of a plant-derived compound artemisinin and a synthetic partner drug, whilst the former first-line treatment chloroquine was a plant-based synthetic compound. Before medicinal plants can be considered for use of treatment for malaria, they need to be scientifically validated.

### 1.5 Aims of the study

The main aim of this study was to valorize the ethnomedicinal uses of two plant species in Namibia as alternative treatment options for malaria. The specific aims underlying the main aim were:

i. To determine the phytochemical profile of plant extracts;

ii. To evaluate the biological (potential antiplasmodial) activities of extracts from the plants in *in vitro* (cellular) and *in vivo* (small animal) models of malaria;
iii. To evaluate antioxidant properties of plant extracts using the DPPH radical scavenging method;

iv. To profile the toxicity and the therapeutic index for extracts from the plants.
CHAPTER 2: LITERATURE REVIEW

2.1 Malaria: an infectious disease

Malaria is one of the oldest parasitic diseases known to man. It was reported as early as 400 B.C. by Hippocrates, one of the earliest documented Greek physicians (Harrison, 1978). Malaria in his book "On Airs, Waters, and Places", was at the time known as fevers caused by bodies of standing water. The Romans too, associated malaria with wetlands. It was from this association that the name of the disease “mal aria” originated, which means “bad air” in Italian (Oaks, Mitchel, Pearson, & Carpenter, 1991). During the 1800s and 1890s the malaria parasite was identified as the causative agent and the mosquito as the vector (Meshnick & Dobson, 2001, p. 15).

The malaria parasites are protozoan in nature and are from the genus Plasmodia (Bope & Kellerman, 2015). Species from this genus require a host for survival, as well as for breeding purposes. Furthermore, these parasites are heteroxenous and have a complex life cycle which includes two hosts, a vertebrate and Anopheline mosquitoes (Gerald, Mahajan, & Kumar, 2011). The mosquito is defined as the definitive host; while mammals, birds and reptiles are considered as the intermediate host, in which over 250 species of Plasmodia have been reported to cause malaria (Ramasamy, 2014). Regardless of this high number of hosts, the parasites generally tend to be host specific. In humans, only four species of the Plasmodium parasites were thought to be responsible
for malaria infections, including *P. falciparum, P. vivax, P. ovale,* and *P. malariae* (Crutcher & Hoffman, 1996).

In 2004 an epidemic of *P. knowlesi,* normally found in pig- and long-tailed macaques (monkeys), was observed in humans in Malaysia. This cross-infection of malaria from animals to humans under natural conditions is considered to be extremely rare. Since then, ongoing transmission in all Southeast Asian countries excluding Laos has been reported (Singh & Daneshvar, 2013). *P. knowlesi* was subsequently recognized as the fifth parasite to cause malaria in humans (Zaki & Shanbag, 2011; Rajahram et al., 2012). Individuals at risk of contracting *Knowlesi* malaria are those coming in close proximity with these primates, particularly in and around forests (Ramasamy, 2014; Bope & Kellerman, 2015). These parasites are said to cause severe malaria in humans if untreated, because of the increasing parasite load every 24 hours in the circulatory system. Due to its similarities in morphological features with *P. falciparum* and *P. malariae,* some cases may go undetected. Confirmed cases are only made through molecular detection tools, being able to accurately identify the parasites (Singh & Daneshvar, 2013).

*P. falciparum* remains the most dominant and pathogenic causative agent of malaria, contributing to most severe malaria cases and deaths in sub-Saharan Africa (Snow & Omumbo, 2006). Multiple infections may also occur in persons infected with *Falciparum* malaria. This is because *P. falciparum* parasites distinctively invade and
infect erythrocytes of all ages, infecting a high percentage of RBCs (Zeibig, 2013, p. 150), unlike *Vivax* and *Ovale* that infect reticulocytes, which makes up 2 % of all RBCs. *Malariae* parasites infect an even smaller group of erythrocytes called senescent (mature) RBCs (Kerlin & Gatton, 2013). Moreover, *P. falciparum* parasites have developed resistance to almost all antimalarial treatment regimens (White, 2004), and is limited to the tropical and subtropical regions such as sub-Saharan Africa, Southeast Asia, Western Pacific and countries sharing the Amazon rainforest (Autino, Noris, Russo, & Castelli, 2012). Specifically, in Namibia, about 97 % of malaria cases are attributed to *P. falciparum* infections with *P. vivax* as the causative agent for the remaining 3 % (MoHSS, 2014).

*P. vivax* is also very common, and unlike *P. falciparum*, accounts for very few severe cases of malaria (Mendis, Sina, Marchesini, & Carter, 2001), and resistance to a lesser number of antimalarials (White, 2004). Resistance of *Vivax* was reported to chloroquine and sulfadoxine-pyrimethamine (White, 2004). In addition, *P. vivax* is more widespread than *P. falciparum* and is mainly found in the tropics and temperate zones (Autino et al., 2012) because of its ability to grow and mature in the *Anopheline* mosquito at lower temperatures and higher altitudes (WHO, 2014). *P. vivax* transmission occurs in Central and Southeast Asia, Western Pacific, Eastern and Southern Africa and South America (Autino et al., 2012). In Africa, there is a low distribution of *Vivax* malaria, because of a group of individuals in Central and West Africa that lacks the Duffy negative gene (Mendes et al., 2011) responsible for producing protein (*i.e.* Duffy antigen receptors),
that enables *P. vivax* merozoites to attach and enter RBCs (Autino *et al.*, 2012). As a result, complete immunity to *P. vivax* has been shown in these individuals (Packard, 2007). However, a recent study indicated the occurrence of *P. vivax* infections in Duffy negative individuals, which may be as a result of the co-existence of Duffy negative and Duffy positive individuals within a population (Mendes *et al.*, 2011).

*P. ovale* and *P. malariae* are relatively uncommon and represent only a small percentage of malaria infections, which are mild in clinical presentation, and easily treatable. *Ovale* malaria transmission occurs in Africa especially among the Duffy negative population (Barnes, 2007, p. 92), in China, Indonesia, Philippines and in parts of Asia, Far West and South America (Cheesbrough, 2005, p. 240). *P. vivax* and *P. ovale* often causes relapses months or years after the initial malarial infection, because the parasites’ dormant liver stages enable them to survive for long periods without causing disease (White, Dondorp, & Paris, 2002). Occurrences in infections with *P. malariae* occur in subtropical and subtropical areas and are very low. *Malariae* transmission also occurs in Guyana, India, Malaysia and Sri Lanka (Cheesbrough, 2005).

The female *Anopheles* (*Anopheline*) mosquitoes are mainly responsible for the transmission of the malaria parasites from one person to another (Bope & Kellerman, 2015). The *Anopheline* mosquitoes feed on blood every so often to provide the necessary protein for the development of their eggs, concomitantly injecting the parasites into the skin of a person, bringing about infection (Moss & Morrow, 2013, p. 888). Like the
male *Anopheles* mosquitoes, the female also uses nectar from plants as an energy source (Gu, Müller, Schlein, Novak, & Beier, 2011). These mosquitoes grow very well in wet and warm climates. Cold temperatures such as 16 °C hamper their development, as do high altitudes, lack of water and vegetation (WHO, 2007a). Hence, malaria occurrence coincides with the rainy season and increased farming activities. Sharing of hypodermic needles with an infected person, from mother to baby (congenital), receiving blood from an infected person during blood transfusion, and organ transplants can also result in transmission of the parasites (Sadanand, 2010).

There are 70 known *Anopheles* species that can transmit malaria to humans, of which 41 are dominant vector species (DVS). In most parts of the world, a high variability in vector species exists (Autino et al., 2012). In Africa, however, there are only four DVS namely *Anopheles gambiae* complex, *An. funestus*, *An. moucheti* and *An. nili* (Autino et al., 2012). The *An. gambiae* complex can be further divided into *An. gambiae*, *An. arabiensis*, *An. merus* and *An. melas*. The 3 *Anopheles* species primarily responsible for malarial infections in humans in sub-Saharan Africa include *An. arabiensis*, *An. funestus* and *An. gambiae* (Mwangangi et al., 2013); of which *An. gambiae* is the most efficient in spreading the disease. In Namibia, *An. arabiensis* is the only vector responsible for malaria transmission (MoHSS, 2014).
2.2 Developmental stages of the malaria parasites

The *Plasmodia* species are morphologically similar in many respects; therefore they infect and cause disease in the same way. A diagrammatic representation of the developmental stages of the *Plasmodium* species is presented in Figure 1.

![Diagram of the developmental stages of the malaria parasites](image)

*Figure 1*: Diagram of the developmental stages of the malaria parasites in two hosts namely the mosquito and a person (Genome Research Limited, 2016).

Once an *Anopheline* mosquito bites a person, malaria spindle-shaped parasites called sporozoites (infective form), enter the blood circulation and travels to the liver, where they infect liver cells also known as hepatocytes. In the hepatocytes, the sporozoites
grow and mature into pre-erythrocytic schizonts. In the case of *P. vivax* and *P. ovale*, a portion of the parasites remain inactive (hypnozoites) for weeks to months, or even years. The reactivation of these dormant forms after the initial infection has cleared, causes relapses in individuals (Gill & Beeching, 2009, p. 55). The pre-erythrocytic oval-shaped schizonts contain 10,000 to 30,000 merozoites (Packard, 2007), which are released into the blood stream where each merozoite invades and infects erythrocytes and reticulocytes, starting the erythrocytic schizogony developmental stage. From the time of infection up to the time the parasites are released into the peripheral blood is known as the pre-patent period and takes 7-30 days (Gill & Beeching, 2009, p. 55).

In the RBC or erythrocyte, the merozoites develop into various stages namely the ring, trophozoite and the schizont stages, which take about 24-72 hours, depending on the species, to complete the cycle. *P. falciparum, P. vivax,* and *P. ovale* all have asexual cycles of 48 hours, whilst for *P. malariae* the asexual cycle lasts about 72 hours (White et al., 2002) and for *P. knowlesi* 24 hours (Daneshvar et al., 2009). The rings (also known as young trophozoites) are uninucleated and mature into trophozoites. The trophozoites continue to increase in size until the nucleus divides into 2 or more nuclei forming schizonts. One schizont, in turn divides asexually to form many merozoites. This marks the end of the schizogony developmental stage and the incubation period. The RBCs, as a result of the heavy parasite load, burst and release merozoites that invade new erythrocytes and reticulocytes. It’s only after a week, *i.e.* several generations of the erythrocytic schizogony, some of the merozoites differentiate into sexual forms
known as gametocytes (macrogametocytes: female and microgametocytes: male) (White et al., 2002).

The gametocytes remain inactive until taken up by the mosquito during a blood meal (Delves et al., 2013). Once in the midgut of the mosquito, the gametocytes transform into gametes and leave the RBCs for sexual reproduction to take place forming diploid zygotes. The latter matures into motile ookinete that penetrates the wall of the gut, and develops into oocysts. At this point, an oocyst matures and gives rise to hundreds of sporozoites. The sporozoites travel to the salivary glands of the mosquito and remain here until the mosquito takes a blood meal (Packard, 2007). As the latter feeds of a new person, the sporozoites, together with a small amount of saliva will be injected into the skin of this individual. The saliva serves as an anticoagulant disrupting the clotting process of blood, as well as providing a passage for the sporozoites into the host (Missant, 2013, p. 367). The sporozoites then find their way into the bloodstream. Hence, the cycle of the malarial parasites perpetuates.

2.3 Clinical manifestations of malaria

The major clinical manifestation of malaria is fever, accompanied with headache. Headache is described as an important presentation in malaria. According to Wiwanitkit (2009), 80 and 75.5% of patients with malaria in two separate studies had headaches, which persisted for the duration of the disease. Data from two other studies concurred and showed that 70% (Trampuz et al., 2003) and 74% (Robinson et al., 2001) of
malaria patients presented with headaches. It was also shown that headache is the direct cause of immunoregulatory proteins, cytokines especially tumor necrotic factor, which are produced as a result of RBC lysis. Furthermore, high levels of cytokines were found in people with acute malaria (Wiwanitkit et al., 2009).

The time of infection to the appearance of the first symptoms, known as the incubation period, usually varies for each Plasmodium species. For symptoms to appear in P. falciparum infections it takes approximately 9-14 days, for P. vivax infections it takes 12-17 days and for P. malariae 18-40 days (Brasil et al., 2011). For P. knowlesi, the incubation period is 9-12 days (Bronner, Divis, Färnert, & Singh, 2009), however more than 4 weeks was recently observed in Knowlesi infected individuals (Tanizaki et al., 2013). The first sporozoite load, and the resulting merozoite load released into the bloodstream may also play a role in the length of the incubation period. It is assumed that an increase in sporozoite and or merozoite load results in a shorter incubation period (Carosi & Castelli, 1997). In contrast, partial immunity to malaria may lengthen the incubation period (Bartoloni & Zammarchi, 2012).

2.3.1 Uncomplicated malaria

Flu-like prodromal symptoms such as headaches, myalgia, coughing, malaise, dizziness, backache, nausea, vomiting and a sense of chillness indicate the onset of clinical malaria (Bartoloni & Zammarchi, 2012). This is followed by paroxysm, also known as clinical episodes, and is characterized by shaking chills which lasts up to 10-15 minutes or more,
fever accompanied by body aches and disorientation lasting 2-6 hours or more and, profuse sweating. This happens when a large number of infected erythrocytes rupture, resulting in the release of merozoites (Bartoloni & Zammarchi, 2012) together with parasitic waste products and debris into the blood stream (Heelan & Ingersoll, 2002). Malarial paroxysm is usually irregular at first, and after several days it becomes periodic and coincides with the length of the erythrocytic cycle of infection, depending on the parasite strain. In *P. vivax* and *P. falciparum* malaria, clinical episodes occur every 2 days *(i.e. a 3 day interval)* and thus acquired the names benign tertian malaria and malignant tertian malaria, respectively. *P. ovale* too has a 3 day interval between paroxysms, whereas *P. malariae* has a 4 day interval and is the called quartan malaria (Heelan & Ingersoll, 2002). In *P. knowlesi* malaria, clinical episodes occurs every day (Fairhurst & Wellems, 2014, p. 3075), and is therefore referred to as quotidian malaria (Vadivelan & Dutta, 2014). As the disease advances, the pattern of the clinical episodes becomes inconsistent and may at times cease (Garcia, 2010).

Other symptoms also presented in patients with malaria are vomiting, abdominal pain, diarrhoea, poor appetite, mild jaundice and anemia (Bope & Kellerman, 2015). These signs are also non-specific and related to uncomplicated malaria. Anemia is caused by a noticeable reduction in RBCs. This is a result of lysis of cells during the erythrocytic stages of the parasites, together with splenic clearance of both infected and uninfected erythrocytes by macrophages (Safeukui *et al.*, 2015). In addition, the drop in the production of erythrocytes affected by bone marrow suppression also plays an important
role in the low levels of RBCs (Haldar & Mohandas, 2009). Jaundice, a condition in which the skin turns yellow, is a direct result of the hemolysis of both infected and uninfected erythrocytes (White & Breman, 2012). If not properly and promptly diagnosed and treated, complications can result, some of which may be fatal, especially in individuals with low immunity.

2.3.2 Complicated malaria

Complications of *P. falciparum* malaria are usually related to blocking of blood vessels resulting in the deprivation of blood to organs, in turn leading to a deficiency of oxygen supply to organs also known as hypoxia. The severity of these complications is dependent on the level of oxygen reaching the body tissues and the organ involved (Clark, Budd, Alleva, & Cowden, 2006). RBCs infected with mature *Falciparum* parasites (late trophozoites and schizonts) become sticky and adheres to the endothelial cells of blood vessels in internal organs, thereby blocking blood flow (Zaki & Shanbag, 2011). The sequestration of parasites, specifically in the cerebral micro-circulation, disrupts the blood supply to the brain resulting in severe cerebral malaria. This type of malaria is marked by hypoglycemia, metabolic acidosis, electrolyte imbalance, headaches, hyperpyrexia (extreme fevers) reduced consciousness, coma and/or seizures, especially in children (Idro, Marsh, John, & Newton, 2010). It is the most severe and often fatal complication (Heelan & Ingersoll, 2002).
More complications include pulmonary edema (buildup of fluid in the lungs), splenomegaly (enlarged spleen), hepatomegaly (enlarged liver), lowered immune system (susceptibility to other infections, acute respiratory distress syndrome, thrombocytopenia (decrease in blood platelets), nephrotic syndrome (chronic severe kidney disease), kidney failure, metabolic acidosis (excessive acidity in the blood and tissue fluids), hypoglycemia (low blood glucose), acute anemia, and shock (Bartoloni & Zammarchi, 2012). An uncommon complication known as Blackwater fever occurs with a sudden hemolysis of erythrocytes in blood vessels, releasing substantial amounts of hemoglobin that ends up in urine (Carosi & Castelli, 1997; Weatherall et al., 2002) resulting in a dark brown to black colour, hence the name. Individuals with glucose 6-phosphate dehydrogenase (G6PD) deficiency are more likely to develop Blackwater fever that may lead to acute renal failure (Bartoloni & Zammarchi, 2012). G6PD deficiency disorder is predominantly found in communities in parts of Africa, Asia and Mediterranean Europe (Peters & Van Noorden, 2009).

2.3.3 Immunity and malaria

The host-immune response to malaria involves the generation of ROS including nitric oxide (NO) and oxygen radicals. This increased production of free radicals or oxidants causes oxidative stress, which contributes to the development of systemic complications in malaria infected persons (Percário et al., 2012) such as cerebral pathology, sequestration, anemia, respiratory distress and placental malaria (Becker et al., 2004). Oxidative stress changes red blood and endothelial cells in blood vessels, enabling the
parasite to enter and cause pathology in organs such as the liver and brain (Percário et al., 2012). Alterations of the surface of the infected RBCs can also result in phagocytosis by macrophages (Becker et al., 2004). Free radicals are also involved in cellular signaling and as carriers for iron requirement needed by parasites for survival in a host.

Infection with malaria for the first time is often fatal for anyone in low or no malaria transmission areas if untreated. Such infections develop into severe malaria most of the time and subsequently death (Davies, Halablab, Young, Cox, & Clarke, 2002). Mostly tourists travelling to endemic areas, as well as migrant workers, refugees (Wilson, 1995), and airport staff are affected (Rodger, Cooke, Ord, Sutherland, & Pasvol, 2008). Individuals with low immunity are most vulnerable in high transmission areas including children under the age of five, pregnant women and immunocompromised individuals (Schantz-Dunn & Nour, 2009) such as those infected with HIV/AIDS, are at risk in developing severe malaria. Children are susceptible to infection because they do not have fully developed immune systems. Pregnant women are also more likely to contract malaria because their immunity to the disease is reduced during pregnancy (Misra, 2007, p. 244). They can develop miscarriages, intra-uterine growth retardation, severe illness and eventual death. In addition, premature births, low birth weight and stillbirths of newborns can result (Fairhurst & Wellems, 2014).

Individuals who have recovered from malaria often develop immunity, and in mature individuals, subsequent infections are asymptomatic or mild. Evidence has shown that
individuals with acquired immunity need continuous exposure to the parasites in order for the immunity to be maintained (Davies et al., 2002). Moreover, it has been found that persons lacking the Duffy trait have partial immunity to *Falciparum* malaria and complete immunity to *Vivax* malaria (Humphreys, 2003). Other haematological pathologies including sickle-cell anemia also known as hemoglobin S (heterozygous for sickle hemoglobin) (Packard, 2007), hemoglobin E trait (Hutagalung et al., 1999; Keohane, Smith, & Walenga, 2015, p. 4422), beta thalassemia (Garamszegi, 2014), and those with G6PD deficiency (Packard, 2007) also provides some protection against infection with *P. falciparum*.

### 2.4 Malaria burden

Malaria remains one of the most important causes of morbidity and mortality affecting about 3.2 billion people worldwide (WHO, 2014). According to World Health Organization (WHO), the number of clinical cases has reached 300 to 500 million per year, most of which are in sub-Saharan Africa. It thus, poses a health threat to more than half of the world’s population. Furthermore, it ranks in the top three most devastating infectious diseases known to mankind, particularly affecting those in resource poor settings along with TB and HIV/AIDS (Slater, 2009, p. 5). Malaria also puts a heavy economic burden on the developing world whether directly by medical costs, or indirectly by absence from work to seek treatment, thus hampering the growth and development of the economy (RBM, 2008; Tediosi, 2010, p. 10). In addition, health system resources including the maintenance of health facilities and health care
infrastructure; deployment of antimalarials and ITNs; vector control strategies, education and research also contributes immensely to the economic burden. Global funding for malaria control and elimination activities in 2013 was about US$ 2.7 billion, which was three times more than the amount in 2005. In Africa, the continent with highest malaria incidence, the disease accounted for as much as 72 % of the total funding (WHO, 2014).

2.4.1 The global malaria situation
Malaria affects 97 countries on a world-scale (Figure 2). The countries that are at risk are primarily situated in the tropical and subtropical regions of the world including: sub-Saharan Africa, the Americas (Central and South America), the Middle East, the Caribbean islands of Hispaniola, the Indian Subcontinent, South-East Asia and Oceania. It is projected that a total of 3.2 billion people live in these malaria endemic areas and that 1 out of a 1000 of the population will become infected and develop malaria (WHO, 2014).
Figure 2: Map showing countries with ongoing malaria transmission in 2013 adapted from the World Malaria Report, 2014 (Genome Research Limited, 2016).

In 2013 there were 198 million malaria incidences globally and approximately 584 000 deaths. The majority of malaria cases occurred in Africa with 163 million cases (82 %) followed by South-East Asia with 24 million (12 %), Eastern Mediterranean 9 million (5 %), Western Pacific 1 million (0.5 %), the Americas 700 000 (0.4 %) and Europe with 2 thousand cases. For all malaria deaths, Africa accounted for 90 % (528 000), followed by South-East Asia (7 %, 41 000), Eastern Mediterranean (2 %, 11 000), Western Pacific (0.6 %, 3 300) and the Americas (0.1 %, 800) (WHO, 2014). Seventy eight percent (453 000) of deaths globally were attributed to children under the age of 5 of whom an estimated 437 000 were from Africa.
Despite the high numbers in incidence and malaria deaths for 2013, there was a 30% reduction in malaria cases globally and 34% in Africa; and a decrease in mortality rates by approximately 47% worldwide and 54% in Africa. More importantly, in the under-five age group, mortality rates have declined by more than half both globally (53%) and in Africa (58%). This was a result of an up-scaling of malaria interventions between 2000 and 2013 to meet targets such as “Reversing the incidence of malaria” set by the Millennium Development Goal target (MDG 6 target C: to have halted by 2015), and “Reducing malaria incidence by 75% by 2015” set by the World Health Assembly and Roll Back Malaria Partnership (WHO, 2014).

Progress towards these targets have already been achieved as 64 countries are currently on the way to reverse the incidence of malaria within their borders by 2015, of which 55 are on track to meet the target of reducing malaria incidence by 75% by 2015 (RBM, 2015). Furthermore, the Malaria World report revealed that in 4 out of 5 Southern African countries there was a > 75% reduction in malaria case and mortality incidences between 2000 and 2013. Sadly, between 2012 and 2013 there was more than a 2-fold increase in malaria cases within these countries (WHO, 2014). The countries are as follow: Namibia, South Africa, Swaziland and Botswana (Figure 3). They make up the front-line countries who all aim for malaria elimination by 2020. Angola, Zambia, Zimbabwe, and Mozambique make up the second-line countries, who are aiming for malaria elimination from their southern districts by 2025 and country-wide elimination by 2030. The Elimination Eight (E8) regional initiative was formed in 2009 to help
coordinate the malaria elimination efforts of the eight countries shown here (WHO, 2014).

Figure 3: Map showing the SADC E8 countries with Namibia, South Africa, Swaziland and Botswana being the four front-line countries, targeting malaria elimination by 2020 (Brieger, 2015).
2.4.2 Malaria situation in Namibia

In Namibia, malaria is highly seasonal, and transmission usually occurs during October-April, after heavy summer rains. A great majority of infections and deaths attributed to malaria occur in the north central and north eastern parts of the country, including the Zambezi and Kavango regions and along the Namibia-Angolan border (MoHSS, 2015). These areas have a warm and wet climate that is conducive for breeding of Anopheles arabiensis mosquitoes, carriers of the P. falciparum parasites (MoHSS, 2014). Namibia has a population size of approximately 2.2 million, of which over 60% lives in areas of either moderate risk transmission or low transmission (Figure 4). Rates of infection decline progressively to the south-west of these areas, with malaria ‘risk free’ areas within the central and southern regions of Namibia including Khomas, Erongo, Karas and Hardap.

In Namibia, malaria case incidences and deaths have decreased considerably over the past decade with a reduction rate of 97% (Figure 5). The number of malaria incidences (both out- and in-patient) amounted to 595,367 in 2004, which had significantly reduced to 3,213 in 2012; and in 2004 1,734 deaths were reported compared to 4 in 2012 (MoHSS, 2014). Even though concerted efforts are being made towards the country’s goal to eliminate malaria by 2020, a rise in malaria cases and mortality rates was observed between 2012 and 2014. In 2013, reported cases had gone up from 3,213 to 4,844, and in 2014 the malaria incidence further increased to 17,166 as did the
malaria deaths from 4 to 21 (MoHSS, 2015); and in 2014 the number of deaths was 61 (MoHSS, 2015). This increase shows that more needs to be done in order to achieve elimination of the disease.

Figure 4: Map of Namibia showing malaria transmission risk areas for 2014 (MoHSS, 2015). Key: Zone 1 – moderate malaria transmission, Zone 2 – low malaria transmission, Zone 3 – malaria ‘risk-free’ areas.
Figure 5: The changes in confirmed malaria (A) cases and (B) deaths in Namibia between 2004 and 2014 – adapted from the Malaria Annual Report 2013/2014 (MoHSS, 2015).
2.5 Current efforts to control and eliminate malaria in Namibia

The WHO defines malaria elimination as “zero local transmission of the disease”. Namibia is already in the pre-elimination phase, i.e. < 1 case per 1000 population in each district. Namibia has targeted eliminating malaria by 2020 partnering with the WHO; Roll Back Malaria; Southern Africa Development Community (SADC); the Global Fund to Fight AIDS, TB, and Malaria (GFATM); the World Bank; the United Nations Children’s Fund (UNICEF); the Global Health Group; the Clinton Health Access Initiative (CHAI); the Malaria Atlas Project; Society for Family Health (SFH); Anglican Diocese/Nets for Life; Red Cross; and the Development Aid for People to People (DAPP). Together with government funding and initiatives such as the Elimination 8 (E8), i.e. SADC countries working together to fight malaria, the country makes available the necessary malaria interventions in the effort to eliminate the disease within its borders (MoHSS, 2010).

2.5.1 Control and prevention strategies

The scale-up and improved coverage of a combination of malaria control interventions, by the Ministry of Health and Social Services (MoHSS) through its National Vector-borne Disease Control Programme (NVDCP), has attributed to the dramatic decline in malaria mortality and morbidity (MoHSS, 2010). Such interventions included the distribution of vector control tools including long-lasting ITNs, IRS and targeted-larviciding; as well as active surveillance (MoHSS, 2010). Pyrethroid treated bed nets, IRS with Dichloro-diphenyl-trichloroethane (DDT) 75% WP and deltamethrine 250
WG, and larviciding all interfere with the transmission of the disease from vector to human host and *vice versa*.

Prophylactic drugs, another preventative measure, are taken orally to kill the malaria parasite as soon as it enters the circulatory system. These drugs are only made available to persons traveling to malaria endemic areas. Mefloquine, doxycycline or atovaquone/proguanil are the recommended prophylactic drugs in Namibia and in countries with chloroquine-resistant and multidrug resistant *P. falciparum*. Chloroquine/proguanil or atovaquone/proguanil are to be used in countries with low occurrences of chloroquine-resistant *P. falciparum* as the chemoprophylaxis of choice, while the use of chloroquine is only recommended in chloroquine sensitive and *P. falciparum* free areas (Amet, Zimner-Rapuch, Launay-Vacher, Janus, & Deray, 2013).

### 2.5.2 Diagnosis and treatment of malaria

The "golden standard" method for detection of malaria is microscopy (Mekonnen, Aseffa, Medhin, Berhe, & Velavan, 2014), even though clinical manifestation of the disease underlies the core of diagnosis in remote rural and/or traditional settings. Microscopy, involving staining a patient’s blood sample on a microscope slide and analyzing it under a light microscope, is effective, but yet labour intensive, time-consuming and subjective; this could mean that the results would bring about inaccurate diagnosis (Sadanand, 2010). Furthermore, microscopy was found to be unreliable in low transmission areas, where 88 % of cases went undetected (Mekonnen *et al.*, 2014). As a
result and due to a paucity of light microscopes in clinics and shortage of trained staff, the MoHSS introduced rapid diagnostic tests (RDTs) in 2007 (MoHSS, 2010). RDTs are available in all clinics and hospitals, and are used to confirm diagnosis in persons presenting with fever and malaria associated symptoms. The MoHSS has gone further and procured new RDTs with improved sensitivity and specificity for all four *Plasmodium* species (MoHSS, 2014). RDTs, however, are unable to reliably detect low-density parasitaemia (≤ 200 parasites/μL) (McMorrow, Aidoo, & Kachur, 2011); and with malaria transmission being low in Namibia, the use of RDTs has become limited.

Timely treatment with appropriate antimalarial chemotherapy greatly reduces the disease’s toll (Mekonnen et al., 2014). Artemether/lumefantrine (ART-LUM), an ACT is the first-line antimalarial for all uncomplicated malaria in Namibia, with exception to children under 5 kg and pregnant women in the first trimester. ART-LUM (Artefan or Coartem) is available in all health facilities and is given to individuals free of charge who test positive for malaria. A single low dose of primaquine is administered with ART-LUM on the first day of treatment to clear the gametocyte load and subsequently reduce malaria transmission. Other front-line antimalarials include sulphadoxine/pyrimethamine and quinine, which are administered orally to treat malaria infections in women 12 weeks pregnant and children weighing less than 5 kg. Oral quinine may also be used as a second-line treatment for uncomplicated malaria and in treatment failures of ART-LUM. Intravenous (IV) artesunate, on the other hand, is administered to children and adults with severe malaria, whereas IV quinine is given to
children under 5 kg and pregnant women in the first trimester with complicated malaria (MoHSS, 2014).

2.6 Antimalarials

Among the several interventions, antimalarials are the most important contributing factor in malaria control and the eventual elimination of the disease (White, 2008). Antimalarial drugs can be used in both the prevention and treatment of infection with the Plasmodium parasites. Antimalarials taken before infection are called prophylactics and should be consumed at fixed intervals before and after travelling to a malaria endemic region. Suppressive prophylactics kill erythrocytic parasites before they proliferate sufficiently in number to cause acute symptomatic malaria; whereas causal prophylactic drugs are capable of preventing erythrocytic infection by targeting parasites in the liver (White, 2014).

Treatment for malaria is characterized into 4-aminoquinolines, arylaminoalcohols, 8-aminoquinolines, artemisinins, antifolates, respiratory chain inhibitors and antibiotics (Melariri et al., 2015). Available agents for malaria treatment are also stage-specific and target various pathways. Antimalarials that act on erythrocytic stages of the parasites are called blood schizonticides, those that are active against the hepatic stages are referred to as tissue schizonticides, and those that eliminate the sexual forms of the parasites are called gametocides (John, Petri, Markell, & Voge, 2006, p. 98). There is currently no single readily available treatment that can kill all stages of the Plasmodia life cycle
(Delves et al., 2012). Such a treatment is the Holy Grail for malaria drugs, a single exposure radical cure and prophylaxis (SERCaP), which is essential in the eradication of malaria. It would have the following characteristics: single fixed dose combination tablet, radical cure of *Falciparum* and *Vivax* malaria, affordable, long duration/post-treatment prophylaxis, transmission blocking, target all life cycle stages, and high barrier to resistance (The malERA Consultative Group on Drugs, 2011).

### 2.6.1 4-Aminoquinolines and 8-aminoquinolines

Many of the current antimalarial agents are blood schizonticides, killing the erythrocytic stages of the parasite. The quinoline derivatives or quinolones such as 4- and 8-aminoquinolines (Figure 6) are good examples. They act by counteracting the crystallization of haemozoin. In the RBC, the parasite uses 60-80% of the haemoglobin (Egan, 2008) to synthesize amino acids which are vital for their being (Hempelmann, 2007). Haemoglobin is broken down in the food vacuole of the parasite; this degradation is carried out by enzymes called peptidases. The resultant waste product free-haem, also referred to as ferrriprotoporphyrin IX (FPPIX), is toxic to the parasites. The parasites, however, counteract the toxicity of the haem by the polymerization to haemozoin. This mechanism of conversion of haem to haemozoin is a good drug target, since humans do not produce haemozoin (Sadanand, 2010).

The 4-aminoquinoline antimalarials chloroquine and amodiaquine form complexes with haem. This prevents the formation of crystalline haemozoin, subsequently killing the
parasites (Schlitzer, 2008). Reports, however, have also indicated that chloroquine inhibit the synthesis of the haemozoin pigment when it gets trapped in the digestive vacuole of the parasite due to the addition of a proton (Jiang, Joy, Furuya, & Su, 2006), and or by capping haemozoin to prevent further polymerization of haem (Bray, Ward, & O’Neil, 2005).

Chloroquine was the first ever drug for treatment of malaria that was deployed on mass scale and is been in use for almost a hundred years (Meshnick & Dobson, 2001). However, widespread *P. falciparum* resistance has led to the demise of this safe and affordable drug. The first reports of resistance were in 1957 along the Thailand-Cambodia border (Dondorp *et al.*, 2010), and today about 80% of *P. falciparum* strains in malaria endemic areas except for Central America, the island of Hispaniola, and some areas of the Middle East and Central Asia are resistant to chloroquine (Bloland, 2001). Resistance to chloroquine has been well reported in South-East and South Asia, as well as in South America and East and West Africa (Dondorp *et al.*, 2010). In contrast, chloroquine remains effective against *P. vivax*, *P. ovale* and *P. malariae* parasites in most malaria endemic regions. However, *P. vivax* resistance to chloroquine has been observed in parts of Southeast Asia and South America (Wellems & Plowe, 2001). Amodiaquine, effective only against low level chloroquine-resistant *falciparum* infections, has been withdrawn from Western markets because of its adverse effects on the liver, and a marked decrease in granulocytes in the peripheral blood (Attia, 2010).
Primaquine, an 8-aminoquinoline is one of very few current antimalarials used as both blood and/or tissue schizonticides in the treatment of malaria. In *Vivax* malaria, it remains the only licensed drug to treat recrudescence and relapses, and is termed as the radical cure (John *et al*., 2012). It is also found to reduce the gametocyte load in malaria patients (Delves *et al*., 2013). This drug has been shown to cause life-threatening hemolysis and methemoglobinemia in persons with G6PD deficiency (Bope & Kellerman, 2012, p. 127). The WHO, however recently recommended low dose primaquine for treatment of malaria as partener drug for ACTs in pre-elimination or elimination settings (MoHSS, 2014).

![Chemical structures of 4- and 8-aminquinolines: A - amodiaquine, B - chloroquine and C – primaquine (Delves *et al*., 2013).](image)

**Figure 6:** Chemical structures of 4- and 8-aminquinolines: A - amodiaquine, B - chloroquine and C – primaquine (Delves *et al*., 2013).
2.6.2 Arylminoalcohols

Antimalarials from the arylaminoalcohol group (Figure 7), including quinine, lumefantrine, halofantrine and mefloquine also function as inhibitors of the haemozoin polymerization process (Hempelmann, 2007); though the exact mechanism is not known. Quinine, the first drug recorded in history for malaria was isolated directly from the bark of the Cinchona tree and is used in the treatment for both uncomplicated and severe malaria in limited malarious areas (Achan et al., 2011); and is effective against the blood stages for all four human malaria species (Bope & Kellerman, 2012, p. 126). It remains one of the crucial drug regimens for uncomplicated malaria in some parts of the world especially in malarious areas where ACT stocks have ran out (Achan et al., 2011), or where it is a recommended treatment option (Bope & Kellerman, 2012, p. 126), in spite of its multiple side-effects including slight impairment of hearing, headaches, nausea, vertigo, vomiting, abdominal pain, diarrhoea, marked auditory loss, loss of vision, hypotension, venous thrombosis, sterile abscesses, hypoglycemia skin eruptions, asthma, thrombocytopenia, hepatic injury and psychosis (Achan et al., 2011).
Both mefloquine and halofantrine efficiently kill chloroquine-resistant parasites, but have accompanying side-effects (Saifi, Beg, Harrath, Altayalan, & Quraishy, 2013). The prophylactic use of mefloquine results in insomnia, dizziness, hallucinations depression, psychosis and panic attacks (Tran, Browning, & Dell, 2006); whereas prolonged use of halofantrine may cause cardiac arrhythmias and as a result has been withdrawn from the market in some countries (Traebert & Dumotier, 2005). Resistance

Figure 7: Chemical structures of arylaminoalcohols: A - lumefantrine, B – halofantrine, C – mefloquine and D - quinine (Delves et al., 2013).
to quinine has been reported in South East Asia, Western Oceania and Sudan (Lin, Juliano, & Wongsrichanalai, 2010) and reduced sensitivity of mefloquine and halofantrine has also been reported (Saifi et al., 2013). Lumefantrine, on the other hand has not been used in monotherapy before (Lin et al., 2010), is highly lipophilic, in other words it is only available in the blood at therapeutic doses if taken with oily food or drink (Arrow, Panosian, & Gelband, 2004), and displays high activity with artemether, an artemisinin derivative, especially against multidrug-resistant *Falciparum* malaria (Lin et al., 2010).

### 2.6.3 Artemisinins

Malaria parasites (blood stage) have also been found to be sensitive to free radicals produced by artemisinin, a sesquiterpene lactone isolated from *Artemisia annua*. In the presence of haem, the endoperoxide-bridge in artemisinins are cleaved, producing free carbon centered radicals (Mercer et al., 2007). How these unstable and highly reactive molecules act against the asexual stages of the parasites are unclear, and may involve the alkylation of proteins and haem altering multiple targets, or inhibition of the ER-located calcium pump of the parasite (Ivers & Ryan, 2012, p. 634). These endoperoxides are exceedingly effective as blood schizonticides (Ivers & Ryan, 2012, p. 634) and can reduce the parasite load up to 10,000 fold within 48 hours (Sukumaran, 2013, p. 408). Additionally, they block parasite transmission by acting as gametocides (Petersen, Eastman, & Lanzer, 2011).
Artemisinin and its derivatives (Figure 8) are highly active as antimalarials. They have short half-lives, remaining in the blood for up to 30-84 minutes, which reduces the mode of resistance (Petersen et al., 2011). Because of their inability to remain in the blood for long, retaining therapeutic doses, artemisinins are used in combination with partner drugs that have long half-lives to clear the residual parasites. ACTs are recommended by the WHO as first line treatments for chloroquine-resistant *Falciparum* malaria in many parts of the world, and have been promoted to being a crucial tool in the efforts to eradicate the disease (Ivers & Ryan, 2012, p. 634).

Two well-known semi-synthesized artemisinins, artemether and artesunate are used extensively in the treatment of malaria. Artemether used in combination with Lumefantrine has been adopted in more than 30 countries as the first-line treatment for malaria since 2009 (Lin et al., 2010). In spite of their current role in reducing malaria morbidity and mortality, clinical resistance against these rapid acting drugs have been established in western Cambodia, where resistance to artemisinins was first reported. Reduced sensitivity of ART-LUM in western Cambodia and Uganda, with failure rates of approximately 15 % were reported (Lin et al., 2010), thus making the threat to global malaria eradication real.
2.6.4 Antifolates

Antifolates (Figure 9) are a group of antimalarials that prevents DNA synthesis in *Plasmodia* parasites (Spina, 2008, p. 25). They were discovered in the 1940s (Nzila, 2006). These drugs are effective causal prophylactics and treatment regimens (Gregson & Plowe, 2005), and act synergistically when used in combinations with dihydrofolate-reductase inhibitors (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and dihydropteroate synthetase inhibitors also known as sulfa drugs (dapsone, sulfalene, sulfamethoxazole, sulphadoxine) (Nzila, 2006). Since the parasites are unable to use pyrimidines from the host, they are forced to produce their own via the folic acid

*Figure 8:* Chemical structures of artemisinin and derivatives: A - artemisinin, B – artesunate, and C – artemether (Delves et al., 2013).
synthesis pathway. Antifolates block this pathway, and thus hinder parasite growth (Gregson & Plowe, 2005). A typical combination includes sulfadoxine/pyrimethamine which had replaced chloroquine as the first line treatment in the 1960s, however reports of resistance were reported soon afterwards (Pohlit et al., 2013). It is also being used as a prophylactic agent against malaria and intermittent preventative treatment for pregnant women (Deloron, Bertin, Briand, Massougbojdi, & Cot, 2010). Its persistent use, however, resulted in the withdrawal from the markets in many regions because of adverse side effects such as agranulocytosis and toxic epidermal necrolysis (Steven-Johnson syndrome) (Gutman, Kachur, Slutsker, Nzila, & Mutabingwa, 2012), the latter being a life-threatening skin condition, whereby a split in the epidermis and dermis occurs due to cell death (Rajkumar, Gertz, & Witzig, 2000). Sulfadoxine/pyrimethamine is still used today in African countries, mainly because it’s affordable. Recent data, however, shows that resistance to this drug combination is spreading in Africa (Deloron et al., 2010).

Proguanil, on the other hand is commonly used in conjunction with atovaquone, a respiratory chain inhibitor. This combination is commonly used as a chemoprophylactic (Nzila, 2006). Proguanil inhibits the dihydrofolate reductase inhibitors via its metabolite chlorcycloguanil (Nzila, 2006), and when used alongside atovaquone, activity is enhanced in the latter (Srivastava & Vaidya, 1999). Side-effects including gastrointestinal complications, headaches, maculopapular rash, nausea and diarrhea may
occur as a result, whereas adverse effects are uncommon in treatment of the disease; (Baggish & Hill, 2002).

![Chemical structures of antifolates](image)

**Figure 9**: Chemical structures of antifolates: A - proguanil, B – chlorproguanil, and C – pyrimethamine (Delves et al., 2013).

### 2.6.5 Respiratory chain inhibitors

The malaria parasites are highly dependent on *Plasmodia* mitochondria in all stages of their life cycle. Furthermore, molecular and functional differences exist between mitochondria of *Plasmodia sp.* and those of human cells. As a result, the mitochondrial electron transport chain was explored as a possible drug target as early as the 1940s. Atovaquone (Figure 10), a broad-spectrum antiprotozoal used in the prevention and treatment of malaria, directly affects the mitochondrial electron transport chain (MacRae
et al., 2013). It inhibits the co-enzyme Q (also called ubiquinone) whose function is to receive electrons from dehydrogenase enzymes and carry them to electron transport cytochromes (cytochrome complex III in this case). In turn several *Plasmodium* enzymes in the mitochondrial electron transport system are inhibited, particularly dihydroorotate dehydrogenase which is essential in the production of pyrimidines. The parasites are unable to synthesize pyrimidines, which then leads to parasite death (Baggish & Hill, 2002). Occurrences of drug resistant parasites and resulting treatment failures, however, were reported against this hydroxynaphthoquinone particularly when used as a single agent (Srivastava & Vaidya, 1999).

![Chemical structure of respiratory chain inhibitors: atovaquone](image)

**Figure 10:** Chemical structure of respiratory chain inhibitors: atovaquone (Delves *et al.*, 2013).

### 2.6.6 Antibiotics

Antibiotics (Figure 11) are presently used in combination with classical antimalarials including quinine or chloroquine to treat both uncomplicated and severe malaria (Pradel & Schlitzer, 2010). This is due to their slow removal of the malaria parasites within the circulatory system, which may have detrimental outcomes in non-immune persons, and
especially in patients with acute malaria. This class of drugs binds to ribosomal units, and thus acts by halting the process of protein synthesis. Fortunately, no resistant parasites against antibiotics have been described (Tan, Magill, Parise, & Arguin, 2011). Tetracycline and its derivative doxycycline is the most widely used (White et al., 2002), are both prophylactic and treatment options, especially in areas where chloroquine resistance exists and mefloquine is contraindicated in malaria prophylaxis (Tan et al., 2011). In 2010 the WHO recommend the use of quinine in conjunction with doxycycline, tetracycline or clindamycin as second-line treatment for uncomplicated malaria, and quinine with clindamycin for treatment of malaria in pregnant women in their first trimester (Achan et al., 2011). Long-term use of antibiotics, however, may disturb normal flora harboring the human body. Following a recent study, it was shown that tetracyclines suppress vaginal bacterial flora, causing increased growth of candida (Tan et al., 2011).

Clindamycin is also a slow acting schizonticide with low activity in comparison to tetracyclines (Bloland & Williams, 2002, p. 149), and because of its short half-life it has to be taken 3-4 times a day (Derouin, 2000, p. 102). It is only used in settings where tetracyclines are contraindicated because of its costly and toxic nature. Prolonged use especially, in high doses, may be toxic to some human tissues. Adverse effects of doxycycline have been described, such as esophagitis and esophageal ulcerations, gastrointestinal disturbances such as nausea, vomiting and abdominal pain, vaginitis, and some increased levels of photosensitivity (Tan et al., 2011).
2.7 Challenges in the efforts to eliminate malaria

Obstacles such as the lack of access and acceptance of conventional antimalarial treatment by populations in malaria endemic areas threaten to undermine the current successes in malaria control efforts. As do the absence of a vaccine, resistance to insecticides, and particularly the emergence of resistance to current antimalarial treatment regimens. The feasibility of eliminating and consequently eradicating malaria is thereby decreasing.

2.7.1 Lack of access and acceptance of conventional treatment for malaria

Treatment with conventional drugs plays a crucial role in malaria elimination. Despite the use of modern advances in malaria control, concerns about accessibility and acceptability of orthodox medicines persist in some communities. With malaria being a disease of poverty, access to treatment may not be readily available (Chuma, Okungu, & Molyneux, 2010). Malaria infected persons sometimes have to travel vast distances to

Figure 11: Chemical structures of antibiotics: A – tetracycline and B – doxycycline (Delves et al., 2013).
the nearest clinic or health facility before receiving medical attention. In addition, public health care facilities are faced with regular stock outs of appropriate medicines, delaying treatment. This, consequentially, may have fatal outcomes.

Furthermore, a heavy reliance on ethnomedicinal plants as primary health care exist, especially in rural African and developing countries (Graz, Kitua, & Malebo, 2011), which may provide treatment options mitigating the delay in treatment. There is a preference of TM over conventional medicines. Such communities opt for TM because of their perception that they are more effective with minimal side-effects (Galabuzi, Agea, Fungo, & Kamoga, 2009). Also TMs are believed to be affordable and accessible (Debas, Laxminarayan, & Straus, 2006; Chuma et al., 2010).

2.7.2 Drug resistance

Drug resistance is one of the major challenges facing malaria control efforts (Tanner et al., 2015). It is defined as the “ability of the parasites to persist and multiply despite the administration of antimalarials in optimal or higher doses for the duration in which these doses are effective in killing the parasites” (WHO, 2015). There are several factors that fuel the development of drug resistant Plasmodium parasites, including incorrect usage of treatment regimens, non-compliance with the course of treatment, usage of counterfeit drugs mainly of low quality, drug interactions, poor erratic absorption, and rapid elimination as a result of diarrhoea or vomiting (Vestergaard & Ringwald, 2007). In addition, drugs with long half-lives remain in the blood long after infection has cleared.
with drug levels high enough to cause drug pressure and low enough to increase the likelihood of recrudescence of the parasites in subsequent infection (Phillips, 2001; Fong, 2013).

Resistance to antimalarial drugs is mainly through spontaneous mutations, being point or multiple mutations. These mutations undergo meiosis in the mosquito and might as a result be lost. However, if they have survived sporogony, resistant parasites will be transferred to a new person (Olliaro, 2005). In addition, numerous mechanisms underlying the development and spread of resistance exist, including drug pressure. Drug pressure or selective pressure involves the elimination of sensitive parasites while those that are resistant survive (Bloland, 2001).

In parasite populations, parasites with varied susceptibilities to drugs exist, and because of drug pressure, over time resistance to a given drug becomes established in that area. Cross-resistance may also develop in parasites. This is when the parasites become resistant to a drug that is chemically related or have similar modes of action, especially to one in which resistance has already been shown. Also multidrug resistant parasites may develop because of their ability to adapt quickly to drugs from a different chemical class with a different mode of action (Sinha, Medhi, & Sehgal, 2014).

Resistance to antimalarials has become a problem in formerly drug resistant free regions and areas where malaria was previously wiped out. This is mainly because of population
movements and environmental changes (Bloland, 2001). The malaria parasites, specifically *P. falciparum* have developed resistance to most currently available antimalarial drugs which are safe and cheap (Vestergaard & Ringwald, 2007); including chloroquine a former first-line treatment, sulphadoxine-pyrimethamine a replacement for chloroquine, and ACTs the current first line antimalarials. Apart from *P. falciparum*, *P. vivax* and recently *P. malariae* have also shown resistance to malaria treatment (Vestergaard & Ringwald, 2007).

### 2.7.3 Insecticide resistance

Insecticide resistance is defined as “the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations” (Corbel & N’Guessan, 2013). The widespread use of insecticides at the beginning of 1950 has contributed to the development of resistance. Though, resistance to currently used insecticides has been shown in many *An.* species, vector control remains the backbone of malaria control programmes (S. Tiwari, Ghosh, Ojha, Dash, & Raghavendra, 2010). The *Anopheles* mosquitoes have developed resistance to the main chemical classes such as DDT, pyrethroids, carbamates and organophosphates (Corbel & N’Guessan, 2013). Pyrethroids are used in ITNs as approved by the WHO (Nkya et al., 2014), mainly because they are efficacious, safe, inexpensive, and long-lasting (Jamali, 2011). Resistance to this class of insecticides, however, has been reported, particularly in Africa (Ranson et al., 2011). DDT is used for IRS in some countries
(Fong, 2013), including Namibia, and fortunately, malaria species found in the country are susceptible to both DDT and pyrethroids (MoHSS, 2010).

The malaria vectors have developed mechanisms responsible for insecticide resistance. Two such mechanisms include modifications in the target site in mosquito reducing the chance for active insecticide substance to bind to and kill mosquito as a result, and the rapid degradation of insecticide in mosquito preventing much of the insecticide reaching the target site. Mosquitoes also changes their feeding and resting behavior to reduce contact with insecticide-treated surfaces of walls and bed nets (Corbel & N’Guessan, 2013). There are however, discussions on whether this behavioral avoidance is an adaptive or genetic response (Ranson et al., 2011). In addition, malaria vectors may also develop thick cuticles on their appendages reducing the uptake of insecticides; and is therefore called cuticular resistance (Corbel & N’Guessan, 2013). Cross-resistance in malaria vectors may also result due to the use of insecticides in agriculture. Most insecticides used in agriculture have the same target sites as those used in vector control, therefore when mosquitoes are exposed to the former; resistance against the former as well as to the latter develops. What’s more, urban pollutants are said to enhance insecticide tolerance levels in mosquitoes (Nkya et al., 2014).

### 2.7.4 The development of a malaria vaccine

No vaccine to date has been developed for malaria despite 30 years of research efforts (Arama & Troye-Blomberg, 2014). In fact, a number of vaccines have undergone
clinical trials and only one has made it to Phase 3 (Targett, Moorthy, & Brown, 2013), which is an indication of the struggle in this quest for a licensed vaccine. Vaccines in clinical trials at present are shown in Table 1. Vaccines have the ability to induce permanent protective immune responses, thus providing protection to subsequent infections. Vaccines have proved in the past to be efficient and cost-effective in controlling infectious diseases (Greenwood, 2014). Past studies show that the parasites express different antigens at each stage allowing the parasite to undermine the host immune responses and thus making it difficult for a vaccine to be fully functional as a malaria elimination tool. Given the complexity of the life cycle of the parasites, malaria vaccines target less than 0.5% of thousands of \textit{P. falciparum} antigens (Crompton, Pierce, & Miller, 2010).

The current vaccines in clinical development either blocks transmission, or are effective against pre-erythrocytic or blood stage parasites. Transmission-blocking vaccines are active against gametes, zygotes, and ookinetes inducing an antibody response in host. Consequently, when a mosquito feeds on an individual, blood consisting of antibodies is ingested; this in turn prevents parasite development in mosquito gut. This type of vaccine, however, does not confer protection against individuals, but prevents the transmission of the disease from person to person (Arama & Troye-Blomberg, 2014), which is rather an important aspect in the control of the disease (Targett \textit{et al.}, 2013). The pre-erythrocytic stage vaccine targets the sporozoites of the malaria parasites. Once these forms enter the blood stream, an immune response is initiated inhibiting parasite
development in the liver and thus blocking infection. Irradiated whole sporozoites and circumsporozoite protein-based vaccines are said to offer complete protection against infection, however this protection is found to be brief as in the case of acquired immunity where individuals require continued exposure to parasites, otherwise immunity wanes off (Okie, 2005). In addition, administration of circumsporozoite vaccines may result in insufficient levels of antibodies, making it impossible to clear infection resulting in delayed onset of acute symptoms (White et al., 2013).

Vaccines targeting the blood stage, on the other hand, prevent merozoites from invading and infecting RBCs, as well as inhibiting the rapid multiplication of Plasmodia in the blood, and thus block the development of clinical disease. According to Crompton et al., (2010) important obstacles in the development of such a vaccine is the multiplicity of genomic material in the malaria parasites as a result of selective pressures by human immune responses, making it impossible to create an effective erythrocytic-stage vaccine. In addition, Plasmodia proteins are highly redundant being able to side-step essential pathways involved in causing disease. Apart from vaccines as a tool in malaria control, adjuvants were also introduced as immunostimulants. These are substances or devices considered to enhance the body’s immune response to antigens, with no or little adverse effects on recipient, and are administered with vaccines as formulations within vectors of viral or bacterial origin (Arama & Troye-Blomberg, 2014).
Table 1: Malaria vaccines currently under clinical trial adapted from Arama and Troye-Blomberg (2014).

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Phases</th>
<th>Trial registration no.</th>
<th>Trial sponsor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRE-ERYTHROCYTIC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RTS,S/AS01E</td>
<td>1a 1b 2a 2b 3</td>
<td>NCT00866619</td>
<td>GlaxoSmithKline, Belgium</td>
</tr>
<tr>
<td>RTS,S/AS01 delayed fractional 3rd dose</td>
<td></td>
<td>NCT01857869</td>
<td>GlaxoSmithKline, Belgium</td>
</tr>
<tr>
<td>PfCelTOS FMP012</td>
<td></td>
<td>NCT01540479</td>
<td>US Army Medical Research and Materiel Command</td>
</tr>
<tr>
<td>CSVAC</td>
<td></td>
<td>NCT01450280</td>
<td>University of Oxford, UK</td>
</tr>
<tr>
<td>Adenovirus (Ad35) vectored CS and RTS,S-AS01 in heterologous prime-boost regimen</td>
<td></td>
<td>NCT01366534</td>
<td>GlaxoSmithKline, Belgium</td>
</tr>
<tr>
<td>ChAd63/MVA (CS; ME-TRAP)</td>
<td></td>
<td>NCT01364883</td>
<td>University of Oxford, UK</td>
</tr>
<tr>
<td>PISPZ</td>
<td></td>
<td>NCT01001650</td>
<td>Sanaria Inc., USA</td>
</tr>
<tr>
<td><strong>BLOOD-STAGE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAd63.AMA1/MVA.AMA1+Al/CPG7909</td>
<td>1a 1b 2a 2b 3</td>
<td>NCT01142765</td>
<td>University of Oxford, UK</td>
</tr>
<tr>
<td>AMA1-C1-Alhydrogel + CPG 7909</td>
<td></td>
<td>NCT00414336</td>
<td>National Institute of Allergy and Infectious Diseases (NIAID)</td>
</tr>
<tr>
<td>BSAM-2-Alhydrogel + CPG 7909</td>
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<td>NCT00889616</td>
<td>National Institute of Allergy and Infectious Diseases (NIAID)</td>
</tr>
<tr>
<td>EBA 175.R2</td>
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<td>NCT01026246</td>
<td>National Institute of Allergy and Infectious Diseases (NIAID)</td>
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<td>SE36</td>
<td></td>
<td>ISRCTN7161971</td>
<td>Research Foundation for Microbial Diseases of Osaka University, Japan</td>
</tr>
<tr>
<td>ChAd63/MVA AMA1 FMP2.1-AS01 B (AMA1 3D7)</td>
<td></td>
<td>NCT01142765</td>
<td>University of Oxford, UK</td>
</tr>
<tr>
<td>NMRC.M3V.Ad.PfCA</td>
<td></td>
<td>NCT00385047</td>
<td>US Army Medical Research and Materiel Command</td>
</tr>
<tr>
<td>MSP3 [181-276]</td>
<td></td>
<td>NCT00392015</td>
<td>US Army Medical Research and Materiel Command</td>
</tr>
<tr>
<td>GMZ2</td>
<td></td>
<td>NCT00424944</td>
<td>African Malaria Network Trust (AMANET)</td>
</tr>
<tr>
<td><strong>TRANSMISSION-BLOCKING</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pf625-EPA</td>
<td></td>
<td>NCT01434381</td>
<td>National Institute of Allergy and Infectious Diseases (NIAID)</td>
</tr>
</tbody>
</table>
2.8 A need for new antimalarials

There is an urgent need for new antimalarials with relatively low costs, high efficacy and minimal toxicity. With the development and spread of resistant parasites to available treatment for malaria and with no effective vaccine in place, a search for new treatment regimens is on the frontiers of ongoing research efforts. Several approaches are being adopted in this regard, including the development of analogs from existing antimalarials, combining two or more treatment regimens, the use of compounds which was intended for a different indication, testing and utilization of compounds to reverse drug resistance, and or looking into new drug targets (Rosenthal, 2003; Guantai & Chibale, 2011).

The use of natural products has also received considerable attention over the past decade as therapeutic agents for malaria mainly because of classical antimalarials such as quinine and artesiminin, which were isolated from plants (Mojab, 2012). Approximately 62% of new drug entities are either from plants or are plant derivatives or plant-inspired analogs. The development of drugs from plants, however, comes with its own limitations. For example, therapeutically significant compounds cannot be developed further due to factors such as toxicity, low solubility and yield, as well as low bioavailability (Ginsburg & Deharo, 2011). Another underlying issue with drug development from natural products is that a single bioactive compound may become inactive when isolated, however, when in the presence of other bioactive compounds such as in a herbal decoction, these constituents may interact to produce therapeutic effects or enhanced efficacy (Ginsburg & Deharo, 2011). Furthermore, to isolate a pure
bioactive compound from plants is far more challenging than is made or believed. Over a period of 4 years (2005-2008) not more than 500 new antimalarial compounds were isolated from medicinal plants used in folkloric remedies for malaria (Bero, Frédérich, & Quetin-Leclercq, 2009) compared to 31,000 synthetic compounds being tested for antimalarial activity (Pink, Hudson, Mouriès, & Bendig, 2005).

The classical drug discovery and development process is laborious taking up to 15 years or more depending on factors such as funding and attrition; and is costly spending up to US$800 million in search for new antimalarials (Willcox et al., 2011). These new drugs, most of the time does not reach the intended markets because of their high prices. In addition, often compounds of interest are found in low concentrations when isolated from their natural source, and their stability as well as their solubility properties has barricaded their development into clinical candidates (Guantai & Chibale, 2011).

This being said, a “reverse pharmacology” approach was established in Malawi in the hopes of developing an affordable standardized plant-based treatment for malaria, especially in remote areas where treatment is delayed. This approach encompasses 4 stages (Figure 12) with stage one involving the identification of lead plants with detailed information on preparation, doses, and duration of treatment through interviews with former malaria patients of traditional healers. This approach gave rise to a standardized phytomedicine for treatment of malaria after 6 years of research with minimum costs (Willcox et al., 2011).
Figure 12: The stages involved in developing a standardized phytomedicine using the reverse pharmacology approach adopted from Willcox et al. (2011).

The Food and Drug Association (FDA) has adopted the “Guidance for Industry: Botanical drug Products” in 2006 that does not require botanical drugs to undergo rigorous experimental phases, but because of their past use in TM they can proceed to phase II clinical trials with less preclinical and toxicological studies than chemical entities (Ginsburg & Deharo, 2011). What’s more, the WHO and its members support
TM being integrated into the health care system and used alongside conventional medicines, with safety and quality assurance policies in place (Graz et al., 2011).

2.9 Traditional herbal medicine as treatment options

Traditional medicine (TM) is described by the WHO as “the sum total of the knowledge, skills, and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in prevention, diagnosis, improvement or treatment of physical and mental illnesses” (WHO, 2000). In short, it is any form of medical treatment that is not Western or conventional (Fabricant & Farnsworth, 2001). Whereas, the term complementary and alternative medicines (CAM) is referred to as “the broad set of health-care practices that are not part of a country’s own tradition and are not integrated into the dominant health-care system” (WHO, 2000).

TM has contributed to the well-being and general health of people in many parts of the world for thousands of years; in fact their first use dates back to 2600 BC. Today, according to the WHO, approximately 80 % of the world uses TM as some form of health care, particularly herbal remedies (Mohammed, 2009). In Europe, North America and Australia TM has taken on the role as complementary or alternative medicines due to adverse effects of synthetic drugs; whilst in rural impoverished areas in Africa and developing countries the use TM serves as the first or only option for health care (Graz et al., 2011). In certain parts of Africa and Asia, an estimate of 80 % of the people rely
on TM (Ginsburg & Deharo, 2011). Reasons for the latter, being the ease and accessibility of TMs in remote areas, affordability TMs, unavailability of conventional medicines at health facilities, less side effects, influence from community members, negative perceptions of modern health care facilities, and the awareness of counterfeit drugs in the market (Bodeker & Graz, 2013; Rutebemberwa et al., 2013).

The use of plants in medicine, also referred to as ethnomedicine or herbal medicine, was first described about 60,000 years ago (Solecki & Shanidar, 1975 in Fabricant & Farnsworth, 2001), and was discovered through trial and error (Wang et al., 2007). In addition, the medicinal use of plants such as poppy, mint and senna was reported as early as 2500 BC. Due to their long-term use, medicinal plants are perceived as safe to use (Mohammed, 2009). However, because of a lack of scientific data, it is not possible to guarantee their safety for human consumption. When ethnomedicines are used casually or in inappropriate doses, these can elicit harmful effects on the body (Wang et al., 2007; Street & Van Staden, 2009), and can at times be fatal (Table 2). Signs of acute toxicity include that of dehydration, vomiting, jaundice, diarrhea, altered mental state and oligoanuria (Street & Van Staden, 2009).

Every so often the harmful effects go undetected or unnoticed. In spite of producing acute toxicity, these plant remedies cause chronic toxic effects contributing to severe damage in vital organs such as the liver and kidneys. The incidences of liver lesions in children from parts of East Africa were reported with prior use of Crotalaria sp. to treat
measles. Acute renal failure, a more serious complication brought about by the widespread use of herbal remedies has also been reported (Meyer, Chen, & Bennett, 2000; Saxena & Panhotra, 2003; Jha, 2010). This calls for revision of regulatory policies of TM where they are in place and to set up regulation of TM in countries which are lacking. Moreover, ethnomedicines need to be scientifically validated to show efficacy and safety for them to be used alongside allopathic medicine (Ginsburg & Deharo, 2011).

Table 2: Statistics of acute poisonings from TM at Ga-Rankuwa hospital in Gauteng, South Africa adopted from Street & Van Staden (2009).

<table>
<thead>
<tr>
<th>Year</th>
<th>Total no. of poisonings</th>
<th>Poisonings from TMs</th>
<th>Deaths due to TM poisonings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981-1985</td>
<td>1164</td>
<td>204 (17.5 %)</td>
<td>31</td>
</tr>
<tr>
<td>1987-1992</td>
<td>3394</td>
<td>313 (9.2 %)</td>
<td>42</td>
</tr>
<tr>
<td>1996-2000</td>
<td>2067</td>
<td>98 (4.7 %)</td>
<td>5</td>
</tr>
</tbody>
</table>

Another common concern with TM is that the phytochemical content varies from plant to plant, hence the doses may vary. These variations are due to genetic, cultural, environmental (Kunle, Egharevba, & Ahmadu, 2012), physiological, and geographical factors among others (Figueiredo, Barroso, Pedro, & Scheffer, 2008). A high genetic variability among wild plants was reported. This affects the secondary metabolite
production in plants. Conditions in which plants grow also affect their chemical composition, for e.g. warm weather increases secondary metabolite production, while rain inhibits secondary metabolite production (Saxena, 2001). Factors such as collection methods, cultivation, harvest, post-harvest processing, and transport and storage conditions may also compromise the medicinal content of herbal medicines (WHO, 2003). The variation in chemical composition, fortunately, can be solved by having a wide-range dose that is not toxic but still effective. It is, however, also crucial to establish the phytochemical composition of herbal remedies for quality assurance purposes (Graz et al., 2011).

### 2.10 Secondary metabolites

Plants produce a number of secondary metabolites due to adverse conditions such as extreme temperatures, drought, flooding, injury, infections, soil and air pollutants, as well as to avoid being eaten (Iriti & Faoro, 2009). They are, therefore, described as chemical stores of a wide range of phytochemicals (Hussain et al., 2012) of which only a few are responsible for biological activity. The composition of these bioactive constituents varies from plant to plant (Doughari, 2012, p. 2). Medicinal and poisonous plants usually contain higher concentrations of these compounds than edible plants such as fruit, vegetables and feed for animals (Bernhoft, 2010).

Secondary metabolites contribute to the healing properties of plants and have played a prominent role in the development of chemotherapies. They can provide drugs directly
such as artemisinin from the Chinese herb *Artemisia annua* (Hill & Staunton, 2010, p. 385), or provide template molecules on which drug molecules can be synthesized organically such as quinine from Cinchona bark (WHO, 2007b). Furthermore, a large number of natural occurring compounds in plants with antiplasmodial properties have been described in the literature. However, for the purpose of this study only six classes namely, alkaloids, anthraquinones, coumarins, terpenoids, steroids and flavonoids will be discussed in terms of their chemical and their therapeutic properties.

### 2.10.1 Alkaloids

Alkaloids are usually bitter in taste, and as a result act as deterrents against herbivores and insects. Many of these compounds consist of nitrogen molecules with an amino acid as precursors including omithine, leucine lysine, tyrosine, tryptophan, anthranilic acid, phenylalanine, histidine, purine, geranyl, acetate, nicotinic acid, cholesterol and geranylgeranyldiphosphate (Iriti & Faoro, 2009; Roberts, Ryan, Moore, & Gulder, 2010). Some alkaloids are of pharmaceutical importance, and are used as narcotics such as cocaine and morphine, and as stimulants such as adrenaline (Brielmann, Setzer, Kaufman, Kirakosyan, & Cseke, 2006). Many are pharmacologically active at low concentrations, but in large quantities, these compounds can become toxic (Batista, De Jesus Silva Júnior, & De Oliveira, 2009).

Alkaloids are considered the largest class of compounds with antimalarial activity (Birhanu, Abula, & Wuhab, 2015), and quite a number of these compounds have been
reported in the literature. These include echitamine (a major alkaloid), corialstonine and corialstonine (two minor quinoline alkaloids), reticuline and laudanosine (benzylisoquinoline alkaloids) and laudanosine; exhibiting either moderate or high antimalarial activities in vitro and in vivo. Thirty one types of indole alkaloids active against the malaria parasites were also reported (Tringali, 2001; Batista et al., 2009). Furthermore, over one hundred alkaloids in vascular plants exhibiting significant antimalarial activities were documented between 1990 to 2000, of which some were more effective than chloroquine (Saxena, Pant, & Bhaluni, 2003).

2.10.2 Anthraquinones

The characteristic feature of anthraquinones is their orange-red colour. Many are found to have a bitter taste, and consist of an anthracene, the main nucleus for anthraquinone compounds. Anthraquinones were first described in 1909 by Robinson and Simonsen and are used as laxatives and purgatives (Kar, 2008, p. 761). In addition, anthraquinones isolated from medicinal plants have been shown to exert a number of biological activities such as antibacterial, antifungal, antimalarial (Wiart, 2012, p. 202) and anti-inflammatory activities (Kar, 2008, p. 761).

Anthraquinones isolated from Morinda lucida, a plant that is used to treat febrile illnesses, displayed good antiplasmodial activity against both chloroquine sensitive and resistant strains of P. falciparum (King & Lawton, 2005, p. 202). Sterekunthal A, an anthraquinone isolated from Stereospermum kunthianum is highly active against P.
*falciparum* (Wiart, 2012, p. 309). These anthraquinones may inhibit or suppress parasite growth by DNA intercalating activity, as a result of its cyclic planar structure (Sittie *et al.*, 1999).

### 2.10.3 Coumarins

Coumarins form part of benzo-pyrones, a chemically related group of compounds all of which consist of a benzene ring combined to a pyrone ring (Jain & Joshi, 2012). These compounds can be classified into 4 smaller groups including simple coumarins, pyranocoumarins, pyrone-substituted coumarins and furanocoumarins (Tiwari, Brunton, & Brennan, 2013). These compounds are common in the Rutaceae and Umbelliferae families, and are found in the leaves, fruit, roots and stems of most vascular plants (O’Kennedy, 2004). The fruit and then the roots have the highest coumarin content, and are especially found in high quantities in essential oils.

Ojala, (2001) reported coumarins with numerous pharmacological activities including anticoagulant, estrogenic, antimicrobial antithelminitic, sedative, analgesic, hypnotic, as well as antimalarial activities. Coumarins such as 5, 6, 7-trimethoxycoumarin and isofraxidin show moderate activity against the malaria parasites; whereas scopoletin and pectachol show less activity (Tringali, 2001). Some coumarins have also been reported to have immunostimulatory properties, and as a result are used in the treatment of diseases such as chronic brucellosis, mononucleosis, mycoplasmosis, toxoplasmosis and Q fever (Jain & Joshi, 2012).
2.10.4 Flavonoids

Flavonoids belong to the chemical sub-group, benzo-gama-pyrones (Jain & Joshi, 2012); and are the most common phenolic compounds in the plant kingdom (Harborne, 1993). Flavonoids are divided into flavones, flavonols, flavanones, chalcones, anthocyanins and isoflavones (Brielmann et al., 2006); of which many exert pharmacological activities including anti-inflammatory, antimicrobial, antioxidant, anticancer activities, antidiarrhoeal and antimalarial activities (Gurib-Fakim, 2006; López-Lázaro, 2009). Flavonoids such as deguelin and obouvatin exhibited notable antiplasmodial activities ranging between 12.4 and 27.6 µM (Batista et al., 2009). Flavones displayed moderate activity, whereas the isoflavonoids (3-phenylbenzoprans) have higher antimalarial activity. These compounds may act by inhibiting L-glutamine and myoinositol from entry of infected RBCs (Elford, 1986). They also have the ability to chelate/sequester iron needed by the Plasmodium parasite, thus adversely affecting the survival of the parasite in a host (Munro & Silva, 2012; Boampong, Karikari, & Ameyaw, 2015).

2.10.5 Terpenoids

Terpenes are made up of isoprenes only, which are simple hydrocarbon molecules; whilst terpenoids consist of isoprenes and oxygen molecules (Kar, 2008, p. 230). Examples of terpenoids or isoprenoids in plants include sterols in the membrane constituents, essential oils, and vitamins (Iriti & Faoro, 2009). Some of these compounds attract pollinators thereby acting as seed dispersers and preventing plants from growing close to one another, limiting competition of resources. Terpenoids including mono- and
sesquiterpenoids are volatile in nature, producing a scent that wards off insects, and prevents fungal and bacterial infections (Loughrin, Manukian, Heath, Turlings, & Tumlinson, 1994).

Terpenoids/terpenes are functionally and chemically diverse lipids (Iriti & Faoro, 2009) and forms the largest class of secondary metabolites from plants of which many possess antimicrobial (Chandramu, Manohar, Krupadanam, & Dashavantha, 2003), antimalarial (Fotie et al., 2006; Kar, 2008, p. 231), antipyretic and anti-inflammatory activities (Antonisamy, Duraipandiyan, & Ignacimuthu, 2011) among others. Triterpenes such as ursolic acid and oleanolic acid have been shown to exhibit very potent antibacterial activity against both *Escherichia coli* and *Bacillus subtilis* (Liu, 1995). Peroxides such as artemisinin (sesquiterpenoid), quinone ethides such as pristimerin (triterpenoid) and tingenone (limonoid), isonitrile (diterpenoid), quassinoids and triterpenes on the other hand, elicit harmful effects on *P. falciparum*. Quassinoids acts by inhibiting protein synthesis of the malaria parasites while the mechanism of action for limonoids are not clear (Kirby, O’Neill, Phillipson, & Warhurst, 1989). According to Sisodia, Negi, Darokar, Dwivedi, & Khanuja (2012), terpenoids may also prevent the uptake of nutrients by parasites.

### 2.10.6 Steroids

Steroids or sterols are abundant in plants (Adesina, 2005), and belongs to the group of secondary metabolites, terpenoids. Steroids consist of an isoprene (C5 unit) (Havlicek &
Spizek, 2014, p. 54). These compounds are responsible for a number of biological activities, including antiplasmodial activities (Haines, 2001); such as lupeol (Ziegler et al., 2002), triterpenoid saponins (Traore et al., 2000), and betulinic acid and its derivatives (Ziegler et al., 2004)). These bioactive compounds act against the *Plasmodia* parasites by preventing of the maturation of the parasites from rings to trophozoites (Londoño et al., 2006).

### 2.11 Traditional medicine used to manage malaria and symptoms

Over a thousand plants are used traditionally to manage malaria and fevers worldwide (Willcox et al., 2011); moreover, such plants have been described in African countries including Burkina Faso, Cameroon, Ethiopia, East and Central Africa, Ghana, Madagascar, Kenya, Mali, Uganda and French Guiana (Mohammed, 2009). However, it is believed that there are an ever increasing number of anti-malarial plants that are being reported due to current research interests (Rasoanaivo, Wright, Willcox, & Gilbert, 2011). This being said, a vast knowledge of medicinal plants for treatment of malaria exists that needs to be tapped into, especially in Namibia where little has been done.

Of the 1,277 plants used for treatment of malaria, only a small proportion has been tested for antimalarial activity *in vitro* and *in vivo*, and even less plants has been studied to show effectiveness and safety in humans. Only ten plant-based medicines have been reported in the literature that has undergone clinical trials (Willcox *et al*., 2011). Sufficient data on ethnomedicines are necessary for them to be incorporated into malaria
control programmes, although, for some no record exists, and their uses have not been valorized. Furthermore, the wealth of knowledge of traditional healers is passed on orally from generation to generation, usually to an apprentice and is not shared with the public; and because of much influence from the Western culture, this body of knowledge is starting to disappear (Fabricant & Farnsworth, 2001; Alves & Rosa, 2007). It is therefore, imperative that the uses of these ethnomedicines in the treatment for malaria are documented and verified scientifically.

In Namibia there are about 3,159 plants species from which 615 are medicinal and (Cheikhyoussef, Mapaure, & Shapi, 2011). Some are used to treat malaria and symptoms of said indication. In Namibia, there is a paucity of information on these plants in terms of ethnomedicinal uses, pharmacological efficacy, phytochemical composition, as well as their toxicity levels. Thus, there is a need for plants to be investigated as potential antimalarials to assure the development of efficient and safe malaria phytomedicines.
CHAPTER 3: MATERIALS AND METHODS

The schematic diagram below outlines the steps used in this study to assess plants used to manage malaria and symptoms in rural areas of Namibia as potential treatment options for malaria. Each step will be explained in more detail in Sections 3.1-3.11.

**Figure 13**: Schematic overview of the activities carried out in this study.
3.1 Selection of plants

Following an ethnobotanical survey to document medicinal Indigenous Knowledge (IK) in the Zambezi region, two plants were selected for the purpose of assessing them as alternative treatment options for malaria. During the survey, traditional practitioners and/or knowledge holders who use TM on a non-commercial basis were interviewed with standardized questionnaires (Appendix A). Information was gathered for each medicinal plant collected: its vernacular name, information on the part used and its preparation, and the route of administration.

Candidate plants, *Muzauli* and *Mundjongolo*, were selected based on their local use to either treat what is perceived as malaria, or to treat symptoms of malaria from the survey (Table 3). This selection method is called the ethnopharmacological approach (Silva *et al.*, 2013), and has been shown to be more efficient in the discovery of lead compounds or lead plants for the development of phytomedicines to treat malaria (Willcox *et al.*, 2011). Another criterion used in the selection of plants was that no previous studies should have been done on the plants describing antiplasmodial activities.
### Table 3: Ethnobotanical information on candidate plants

<table>
<thead>
<tr>
<th>Local name</th>
<th>Plant part</th>
<th>Use in combination with*</th>
<th>Ethnomedicinal use</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muzauli</strong></td>
<td>Root</td>
<td><em>Mukwenkwebo, Mungongolo</em></td>
<td>Shivering and sweating (fever and chills) in children</td>
<td>Oral, topical</td>
</tr>
<tr>
<td><strong>Mundjongolo</strong></td>
<td>Root</td>
<td><em>Situnduwanga, Kabubo, Mutjokela, Mukungu, Mushakashela, Mufula</em></td>
<td>Headaches</td>
<td>Oral, steam bath, topical</td>
</tr>
</tbody>
</table>

*Vernacular names of plants in SiLozi*
3.2 Collection of plants

Roots of the candidate plants were harvested in April 2012 in the Zambezi region of Namibia. Voucher specimens of the plants were also prepared using a plant press. These were deposited in National Herbarium at the National Botanical Research Institute (NBRI) of Namibia for the confirmation of the scientific nomenclature of the plants. They were identified as *Guibourtia coleosperma* (*Muzauli*, CID11) and *Diospyros chamaethamnus* (*Mundjongolo*, CID12) as shown in Table 3. Figure 14 shows the collection sites of where *D. chamaethamnus* and *G. coleosperma* samples and voucher specimens were collected.

**Figure 14:** Map of Namibia showing the geographical location of where the plants under investigation were collected. The GPS coordinates for *G. coleosperma* and *D. chamaethamnus* are 17°48′633″S; 023°08′986″E and 17°55′296″S; 023°09′03″E, respectively.
*D. chamaethamnus* (Figure 15) also known as the Gingerbread plum, Sand apple or dwarf Jackal-berry (English), *Mukushuwa, Mundjongolo* (SiLozi) belongs to the Ebenaceae family. This large woody shrub is found in the northeastern parts of Namibia largely on sandy plains, dunes and along dry river beds. It is less than one meter tall and grows in a suffrutex form. It is an evergreen annual shrub with round fruiting bodies that are only found in certain months of the year ranging from March-July, September-November; and small cream-coloured flowers that bloom only in March-May. The fruit are edible and covered in red-brown hairs. The leaves on the other hand, are evergreen, elongated, dark olive-green in colour and are arranged spirally (Curtis & Mannheimer, 2005, p. 518).

In TM, the roots are applied to incisions made into the skin to alleviate headaches, and may also be administered as a vapour bath or orally in the Zambezi region of Namibia. Antimalarial activity, as well as biological activities ranging from antibacterial to anticancer activities has been described for several species of *Diospyros* other than *D. chamaethamnus*, especially those producing edible fruit (Pinho, Sousa, Valentao, & Andrade, 2011). Such species include *D. rubra* (Prachayasittikul, Saraban, Cherdtrakulkiat, Ruchirawat, & Prachayasittikul, 2010), *D. quaesita* (Ma *et al.*, 2008), *D. glandulosa*, and *D. rhodocalyx* (Theerachayanan, Sirithunyalug, & Piyamongkol, 2007).
Figure 15: Photograph showing the characteristic features of *D. chamaethamnus* – it has an estimated height of less than one meter (left) and elongated, evergreen, spirally-arranged leaves (right).

*G. coleosperma* (Figure 16), also known as *Copaiba coleosperma* (family: Fabaceae) is widely distributed and is naturally found in open forests and woodlands in Namibia, Angola, southern Congo, Zambia, Zimbabwe, Swaziland, Cameroon and Botswana. In Namibia it grows abundantly in the north-eastern parts of the country known as the Kavango and Zambezi regions. It can grow up to 30 meters tall; and has a smooth-sometimes-flaking red or grey bark, with butterfly-shaped leaves, red-brown seeds with red arils, and small cream-coloured to white star-shaped flowers. Common names of *G. coleosperma* include *Baster mopanie* (Afrikaans), *Muzauli* (SiLozi), *Mussii* (Oshiwambo), *Ghushi* (Tswana), *False mopane* and or *African rosewood* (English) (Curtis & Mannheimer, 2005, p. 204). The young leaves are eaten to treat coughs whilst leaf infusions are drunk to treat severe coughs (Umberto Quattrocchi, 2012). The roots
and bark of *G. coleosperma* can also be taken for headaches, while the bark can be used in a vapour bath to treat headaches, whereas roots and leaves are ingredients in mixtures for the treatment of fever and psychological problems (Lemmens, Louppe, & Oteng-Amoako, 2012). In the Ohangwena region of Namibia it is used to treat headaches, fever, coughs and malaria (Nafuka, 2014), while in the Zambezi region of Namibia the roots are used to treat febrile illnesses in children.

**Figure 16**: Photographs showing the characteristic features of *G. coleosperma* – it has an estimated height of 20 meters (left) and butterfly-shaped leaves (right).

### 3.3 Preparation of plant material

After collection, the fresh plant material (*i.e.* roots) was allowed to dry at ambient temperatures for 4-6 weeks away from direct sunlight to avoid changes in chemical composition and properties of the plants. The dried plant material was ground to obtain a fine powder (particle size less than 1 mm) using a heavy duty blender, followed by storage in airtight 50 mL Falcon tubes at -20°C.
3.4 Method of extraction

3.4.1 Organic extraction

Organic extracts were prepared by maceration methods as outlined by Njateng, Gatsing, Mouokeu, Lunga, and Kuiate (2013) with some modifications. Ground plant material was macerated in methanol-dichloromethane (1:1 v/v) at sample-to-solvent ratio of 1:20 (w/v) for 48 hours at room temperature with intermittent shaking. This was followed by gravity filtration using Whatman qualitative filter paper, Grade 1 (110 mm) to make sure no plant matter remained in the supernatant. The filtrates were concentrated under reduced pressure using a rotary evaporator (Heidolph GI, Germany) at 60 °C followed by freeze-drying using a freeze-dryer (Christ Alpha 1-2 LD Plus, Germany) to obtain a solid extract. The dry organic extracts were labeled as OR, and designated as DC (for D. chamaethamnus) and GC (for G. coleosperma), which were weighed and stored at -20°C for further analysis.

3.4.2 Aqueous extraction

Aqueous extracts were prepared using a method that is in line with those used in a traditional setup by soaking ground plant material in distilled deionized water (1:20 w/v), which was placed in a water bath set at 60 °C for 2 hours with occasional shaking. The plant suspension was spun down at 800 x G for 30 minutes in an Avanti™ J-25 centrifuge (Beckman Coulter, USA). The extracts were filtered using gravity filtration, and concentrated using a rotary evaporator (water bath set at 60 °C). They were further dried using a freeze-dryer. The dry aqueous extracts were labeled as AQ and designated
as DC (for *D. chamaethamnus*) and GC (for *G. coleosperma*), which were weighed and stored at -20°C for further analysis.

The percentage yield of the extracts was determined using the dry weight of the extract (x) and the initial plant material (y) as follow:

\[
\text{Percentage yield} = \left( \frac{x}{y} \right) \times 100
\]

### 3.5 Characterization of plants by TLC

The phytochemical profiles of the plant extracts were determined using thin layer chromatography (TLC) by loading 7.5 μL of extract at a concentration of 40 mg/mL onto MERCK precoated Silica gel 60 F254. The plates were allowed to run in suitable solvent systems (Table 4) up until the solvent front reached a height of 15 cm; which were then dried and visualized using special spray reagents for detection of major antimalarial constituents namely alkaloids, coumarins, anthraquinones, flavonoids, steroids and terpenoids. The developed TLC plates were documented as pictures (Appendix B).
Table 4: The solvent system(s) and visualization reagents of selected classes of compounds that were used for determining the phytochemical profiles of the plant extracts in thin-layer chromatography.

<table>
<thead>
<tr>
<th>Class of compounds</th>
<th>Reference compound</th>
<th>Solvent system</th>
<th>Spray reagent</th>
<th>Colour change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Quercetin in ethanol (4:1000 w/v)</td>
<td>Chloroform-methanol (19:1 v/v)</td>
<td>1% Ethanolic solution of aluminum chloride</td>
<td>Yellow fluorescence in UV light at 366 nm</td>
<td>1 Mallikharjuna, Rajanna, Seetharam, &amp; Sharanabasappa (2007) 2 Gage, Douglas, &amp; Wender (1951)</td>
</tr>
<tr>
<td>Coumarins</td>
<td>MERCK Coumarin in di-ethyl ether (4:1000 w/v)</td>
<td>10% Acetic acid</td>
<td>Benedict's reagent</td>
<td>Blue-green fluorescence under long-wave UV light at 366 nm</td>
<td>1 Harborne (1998) 2 Reznik &amp; Egger (1961)</td>
</tr>
<tr>
<td>Steroids</td>
<td>SIGMA β-Sitosterol in chloroform (4:1000 w/v)</td>
<td>Chloroform-acetic acid-methanol-water (64:34:12:8 v/v/v/v)</td>
<td>Freshly prepared 0.5 mL p-anisaldehyde in 50 mL glacial acetic acid and 1 mL 97% sulfuric acid</td>
<td>Heat at 100-105°C until the maximal visualization of spots; green</td>
<td>1 Mallikharjuna et al. (2007) 2 Stahl &amp; Kaltenbach (1961)</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>SIGMA β-Sitosterol in chloroform-acetate (1:1)</td>
<td>Hexane-ethyl acetate (1:1)</td>
<td>Freshly prepared 0.5 mL p-anisaldehyde in 50 mL glacial acetic acid and 1 mL 97% sulfuric acid</td>
<td>Heat at 100-105°C until the maximal visualization of spots; purple-violet</td>
<td>1 Harborne (1998) 2 Stahl &amp; Kaltenbach (1961)</td>
</tr>
</tbody>
</table>
3.6 GC-MS analyses

3.6.1 Sample preparation

Plant extracts prepared as described in Section 3.3, were resuspended in methanol to obtain final concentrations of 10 mg/100 µL for the organic extracts, and 2 mg/100 µL for the aqueous extracts.

3.6.2 Instrument control and parameters

Gas chromatography-mass spectrometry (GC-MS) analyses of the samples were performed as described in Lytovchenko et al. (2009) with minor modifications. The GC-MS system consisted of a Focus GC coupled to an ITQ 700 MS (ThermoScientific, Italy), using Xcalibur Software version 2.1 for data acquisition. One microliter of sample was injected with a split ratio of 1:10 using the hot-needle technique. The injector temperature was maintained at 220 °C, the MS transfer line at 250 °C, and the ion source at 200 °C. Helium was used as the carrier gas set at a constant flow of 1 mL/min. A 5 % diphenyl-95 % dimethylpolysiloxane (BP5MS) (SGE Analytical Science, Australia) capillary column with dimensions of 30 m x 0.25 mm i.d., and a coating thickness of 0.25 µm was used for the stationary phase. The oven temperature was programmed at 2 °C/min⁻¹ from 40 °C to 300 °C and held isothermal for 60 min. The electron ionization mass spectra were recorded with an ionization voltage of 70 eV and an m/z 25-515 scan range.
Compounds were tentatively identified by comparing their mass spectra to those in the National Institute of Standards and Technology (NIST 11) mass spectra database. Furthermore, the identities of the compounds were also supported by their Kovats retention indices relative to C₁₀-C₄₀ n-alkanes (even number of carbons only) (Sigma Aldrich, Germany) to those of available values in the NIST RI search database, and others reported in the literature. The identities of a number of compounds were confirmed by retention time comparison using authentic reference standards.

3.7 Antioxidant activity

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) assay was used for the assessment of antioxidant activity of the plant extracts according to Mensor et al., (2001). DPPH is an organic chemical composed of stable free radicals that are reduced when they accept electrons or free radical species, resulting in a colour change from purple to yellow (Esmaeili, Taha, Mohajer, & Banisalam, 2015). According to Da et al. (2014) this reducing capacity of extracts is a good indicator of potential antioxidant activities. The scavenging activity of plant extracts was measured following mixing different concentrations (20, 40, 60, 80 µg/mL) of the extracts (100 µL) with 100 µL of 0.3 mM methanolic Aldrich DPPH solution to obtain final concentrations of 10, 20, 30 and 40 µg/mL. SIGMA Ascorbic acid was used as the positive control. The absorbance was measured at 518 nm after 30 minutes of incubation using a multimode plate reader. Three replicates of each sample were used for statistical analysis and the mean ± S.E.M. reported. The inhibitory effect of plant extracts of each parameter was calculated as:
\[
\% \text{ Inhibition} = \frac{(AC - AE)}{AC} \times 100;
\]

where, \( AC \) is the absorbance of the fully oxidized control and \( AE \) is the absorbance of the extract. The concentration required to scavenge 50 \% of the DPPH radical (IC50) was calculated using linear regression analysis by plotting the percentage inhibition against the concentrations of the plant extracts. The antioxidant activity of the plant extracts was also expressed as the ascorbic acid equivalent antioxidant capacity (AEAC) using the equation below:

\[
\text{AEAC (mg AA / 100 g)} = \frac{(\text{IC50 ascorbic acid})}{\text{IC50 extract}} \times 100.
\]

3.8 \textit{In vitro} antimalarial analyses

3.8.1 Culturing of erythrocytic asexual stages of \textit{Plasmodium falciparum}

Laboratory-adapted \textit{P. falciparum} D10 (chloroquine-sensitive strain) parasites were obtained from BEI resources, American Type Culture Collection (ATCC) (Manassas, VA, USA). The \textit{Plasmodium} parasites were grown and maintained in a continuous culture in sealed NEST flasks (25 and 75 cm², canted) using the method described by Trager and Jensen (1976) with modifications. The culture consisted of O⁺ human erythrocytes in culture medium and pooled human serum, which was incubated at 37 °C in a Scientific Series 2000 incubator. The growth medium of the \textit{Plasmodia} cultures was replaced on a daily basis, together with a special gas mixture of 5 \% carbon dioxide (CO₂), 5 \% oxygen (O₂) and 90 \% nitrogen (N₂). The estimation of the parasite load \textit{i.e.}
parasitaemia of the culture, as well as parasite visualization was done using a light (Olympus CX21, Japan) and fluorescence (Olympus BX51TRF, Japan) microscope. Detailed parasite culturing techniques are mentioned in Sections 3.7.1.1 to 3.7.1.6.

3.8.1.1 Preparation of culture medium

Liquid RPMI 1640 (500 mL) supplemented with 25mM HEPES and L-glutamine was used to prepare the culture medium, both incomplete and complete. Using sterile techniques 1.25 mL of 10 mg/mL gentamicin, 5 mL of 20 % glucose and 3 mL 1 M sodium hydroxide (NaOH) were added to the 500 mL bottle to obtain the following final concentrations: 2 mM NaOH, 4 % D-glucose, and 0.02 mg/mL gentamicin. The culture medium was mixed thoroughly by inverting 10 times, and passed through a Corning filter system with a Millipore filter of 0.22 µm porosity. This medium was referred to as the incomplete medium and was stored at 4 °C and used within one month. To make up the final medium for culturing the *Plasmodium* parasites, 10 % sterile non-immune human serum was added to the incomplete medium. The serum used, was pooled (all blood groups) from local donors at the University of Namibia. This medium was referred to as the complete medium and was used within 7 days of preparation.

3.8.1.2 Preparation of red blood cells

Human blood for *Plasmodium* culture was collected from malaria-free volunteers by a medical doctor. The use of materials of human origin was ethically cleared by the MoHSS Biomedical Research Ethics Committee. O+ blood was collected in 4 mL SG
Plain/No Additive Vacutainer 4 mL tubes using BD Vacutainer Precision Glide 21Gx1.5 mm needles. Four milliliters of the blood was transferred to sterile 15 mL Falcon tubes under aseptic conditions \textit{i.e.} in a Biosafety Level II Cabinet. The tubes were topped up to 10 mL by adding incomplete media. The media-RBC suspension was then centrifuged at 690 x G (HERMLE 2326K, Germany) for 5 minutes. The supernatant was aspirated and more media was added to make a final volume of 14 mL. Centrifugation was done at 440 x G for 5 minutes with breaks. The buffy coat above RBC pellet was carefully removed by pipetting out a few milliliters of supernatant and then removing the coat with a sterile pipette. The washing of the RBCs was repeated four times, before the appropriate volume of incomplete media was added to give a 50 \% RBC suspension.

3.8.1.3 Preparation of serum

Fresh human whole blood (Groups A, AB, B, and O) was collected in BD Vacutainer SSTM\textsuperscript{TM} II Advance Plus blood collection tubes (5 mL) with coagulant using BD Vacutainer Precision Glide 21Gx1.5 mm needles. The manufacturer’s protocol was used, which involved the inversion of blood 6 times, following incubation at room temperature for 30 minutes. Centrifugation was done at 2000 x G for 30 minutes. The serum and erythrocytes was fractionated during the process of centrifugation. The serum was then collected and transferred under sterile conditions to new sterile 15 mL tubes and was heat inactivated by incubation in a water bath for 20 minutes at 56 °C. The serum was then stored at -20° C in the 15 mL Falcon tubes until further use.
3.8.1.4 Reviving of parasites

Cryovials containing cryopreserved *Plasmodium* strains were removed from a Nuaire Glacier -86 °C ultra-low freezer (NU-9334E, Japan) and were thawed rapidly by rolling the cryovials between two gloved hands; which were opened slowly inside an ESCO Class II Biosafety hood (AC2-4E1, Singapore). The parasitized erythrocytes were transferred from the cryovials gently (1mL) to 50 mL Falcon tubes. Two hundred microliters (0.2 mL) of 12 % sodium chloride (NaCl) solution was added dropwise using a sterile Serological pipette, with continuous agitation (tapping/shaking of tube) for approximately one minute. After 5 minutes, 10 mL of 1.8 % NaCl solution was added dropwise, again with continuous tapping of the tube. The first 1 mL was spread over one minute as it is the point where lysis of RBCs occurs.

The culture-NaCl solution was centrifuged at 250 x G for 5 minutes (with slow acceleration). The supernatant was aspirated and 10 mL of 0.9 % NaCl/0.2 % d-glucose solution was added dropwise with continuous agitation. The sample was then centrifuged at 440 x G for 5 minutes. Again the supernatant was aspirated. The cells were washed twice with 10 mL of incomplete media. Centrifugation and aspiration of supernatant was done between washes. After the last aspiration, 5 mL of the culture complete medium was added. The contents of the tube were then transferred to a small culture flask. An aliquot was removed and placed in a 1.5 mL Eppendorf tube to determine the parasitaemia before setting up the culture. The culture was fed with the gas mixture for 30 seconds (25 cm² flask) before being sealed and placed in a 37 °C incubator.
3.8.1.5 Maintenance of a continuous culture

The *Plasmodia* cultures were maintained by changing the culture medium daily. Thin smears were also made to determine the percentage parasitaemia, which was calculated by counting the infected RBCs in a total of 10,000 RBCs. The cultures were maintained at 2% haematocrit and parasitaemia. Briefly, the cultures were transferred from culture flasks in sterile conditions to 15 mL Falcon tubes, which were centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded and an appropriate volume (5 or 10 mL) of fresh, warm (37 °C) complete media was added according to the pellet size. If the haematocrit was less than 2%, fresh O+ erythrocytes (not more than 2 weeks old) was added; and if the parasitaemia was > 2%, the culture was diluted with the fresh O+ RBCs or split into 2 or more culture flasks. The parasitized RBCs and medium suspension were transferred into sterile culture flasks (25 and 75 cm²), and were fed with gas for 30 seconds or 1 minute relative to the size of the flask and incubated at 37 °C.

3.8.1.6 Preparation of thin smears

Thin blood smears were made to determine the parasitaemia and stages of parasites microscopically. One hundred microliters of the cultures were transferred to 1.5 mL Eppendorf tubes and were spun down by placing it in the microcentrifuge (HERMLE Z326K, Germany) for 30 seconds at a maximum speed. An aliquot of the supernatant was aspirated leaving an equal amount of the supernatant and pellet volume for mixing. Less than 20 µl of resuspended pellet solution was picked and placed on a frosted microscope slide. A second slide was placed on the first slide at 45 to 60 ° angle and
moved back into the drop, and then moved forward, smearing a film of blood across the
first slide. The smear was dried, and thereafter it was fixed with methanol. Thereafter,
the slide was covered with 10 % Giemsa solution for 20 minutes to stain the parasites
and RBCs. The Giemsa stain was then rinsed off with water and allowed to dry, after
which the smears were examined microscopically at X 1,000. Parasitaemia was
determined by counting both uninfected and infected erythrocytes in 10 or more fields
with approximately 100 RBCs in each field, expressing the parasitaemia as a percentage
of infected erythrocytes per total erythrocyte count.

3.8.2 Preparation of stock and working solutions
Aqueous and organic extracts of G. coleosperma and D. chamaethamnus were
resuspended in distilled deionized water and dimethyl sulfoxide (DMSO) respectively to
produce 10 mg/100µL solutions. Aliquots of the suspensions, i.e. stock solutions were
stored at -20°C until needed. Subsequently, the stock solutions were diluted to 1mg/ml,
in RPMI 1640 incomplete media, filter sterilized using 0.22 µm Millipore nylon syringe
filters, and then diluted further in RPMI 1640 complete media to prepare working
solutions at concentrations of 200 µg/mL; thus reducing the concentration of the DMSO
to 0.2 %, which was shown to have no measurable effect on parasite viability
(Ramazani, Zakeri, Sardari, Khodakarim, & Djadidt, 2010). The working solutions were
kept at 4 ºC for several days only. SIGMA Chloroquine diphosphate was dissolved in
distilled deionized water and DMSO respectively, 1 mg/mL each.
### 3.8.3 Bioassay for antiplasmodial activity

One hundred microliters (100 μL) of unsynchronized cultures with ~4% parasitaemia and ~4% haematocrit was placed in flat bottomed 96-well plates. Working solutions of plant extracts diluted in complete media (100 mL) were added to each well to obtain final concentrations of 5, 25, 50 and 100 μg/mL (Figure 17), cultures with a ~2% parasitaemia and ~2% haematocrit, and a final total volume of 200 μL. This was followed by incubation at 37°C for 48 hours. Chloroquine diphosphate (25 μg/ml) was used as the positive control for the D10 *Plasmodia* strain, whilst the negative control was void of any treatment. The experiment was done with all treatments in triplicate.

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**Figure 17:** A representative 96-well microtitre plate indicating final concentrations of plant extracts. NC = negative control, PC = positive control (chloroquine diphosphate). The outside wells were filled with water (W) to prevent the cultures from drying out.
3.8.4 Parasitaemia assessment

Percentage parasitaemia was used to assess the sensitivity of the *Plasmodium* parasites to the plant extracts, which is the ‘golden-standard’ for detection of malaria parasites. The number of parasites observed for treatments (plant extracts), as well as for negative and positive controls were quantified. Briefly, the parasites were harvested 48 hours post treatment, fixed on microscope slides and stained for 20 minutes in a 10 % Giemsa stain. Each smear was observed at 10 different fields and at 3 different parts of the slide under a light microscope. Infected red blood cells (iRBCs) were counted against a 1000 total red blood cells (tRBCs) *i.e.* both iRBCs and RBCs for each concentration and triplicate. The mean percentage parasitaemia and percentage growth inhibition were calculated. The latter was expressed as a percentage of the mean percentage parasitaemia for each concentration compared with the mean percentage parasitaemia for the untreated controls:

\[
\text{% Growth inhibition} = 100 - \frac{\text{Parasitaemia of treatment}}{\text{Parasitaemia of control}} \times 100.
\]

The growth inhibition values were plotted against corresponding concentrations of the plant extracts to generate log dose-response curves from which IC\textsubscript{50} values were obtained at a 95% confidence interval.

3.9 Cytotoxicity evaluation

The toxic effects of *G. coleosperma* and *D. chamaethamnus* extracts were investigated on mammalian cells using a protein dye, sulforhodamine B (SRB) (Fouche *et al.*, 2008).
SRB has the ability to bind to protein basic amino acid residues of trichloroacetic acid (TCA)-fixed cells. The incorporated dye can be solubilized for measurement, and the results are linear with cell number. For this assay, human fetal lung fibroblast cells W138 obtained from the European Collection of Authenticated of Cell Cultures (ECACC) was used for the assay. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 % FBS, 2 mM L-glutamine and 50 µg/mL gentamicin. Seeding of W138 cells was at 10,000 cells/well. Plant extracts were incubated with fibroblast cells in a 96 well plate in 5 % CO₂, 100 % humidified incubator at 37 °C. After 48 hours, cells were fixed with TCA, stained with 0.4 % SRB and solubilized with tris base. Etoposide was used as positive control. Absorbance was measured at 540 nm using plate reader as an indicative of the total cell protein. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Percentage cell viability was calculated as follows, in relation to the viability of cells without treatment (Control wells):

\[
\text{% Cell viability} = \frac{\text{OD}_{540} \text{ treatment} - \text{OD}_{540} \text{blank}}{\text{OD}_{540} \text{control} - \text{OD}_{540} \text{blank}} \times 100\%.
\]

The concentrations (µg/mL) were expressed in log form and used to plot the non-linear graphs against percentage cell viability. Also, the \( \text{CC}_{50} \) value, which is the concentration which reduces cell viability by 50 % was determined using a non-linear sigmoidal curve of cell viability and log extract concentrations. The selectivity index defined as \( \text{SI} = \frac{\text{CC}_{50}}{\text{IC}_{50}} \) (Singh, Kaushik, Mohanakrishnan, Tiwari, & Sahal, 2015) was also calculated.
3.10 *In vivo* antimalarial analyses

Swiss albino mice [*Mus musculus* L. (Muridae)] weighing 20±4 g were obtained from the animal house situated at the Kenya Medical Research Institute (KEMRI), Nairobi. This work was conducted with the approval of KEMRI’s Animal Care and Use Committee. The mice were fed with growers pellet Feed with water given *ad libitum*. The floor of the cages was covered with wood shavings serving as bedding for the mice. The shavings were replaced twice a week or as necessary to avoid a buildup of ammonia. The rodent parasite, chloroquine-sensitive *P. berghei* ANKA was used and sourced from the American Type Culture Collection (ATCC), USA (BEI resources).

3.10.1 Revival and maintenance of parasites

Cryopreserved *P. berghei* parasites were thawed at room temperature and were injected into healthy mice. The parasites were maintained by continuous reinfection of the parasites in healthy mice (*i.e.* serial passaging) every four days. Blood samples were collected from infected mice with a parasitaemia of 20-30 % by cardiac puncture. The blood was diluted to 1 % parasitaemia with phosphate saline glucose (PSG) buffer. Each mouse was inoculated intraperitoneally (IP) with 0.2 mL of 1 x 10^7 (*i.e.* 1 % parasitaemia) *P. berghei* infected RBCs using a 21G needle. The mice were monitored daily to determine the parasitaemia for 7 days using thin smears (Section 3.8.1.6) by bleeding the tail vein. Mice with a parasitaemia of 18-25 % were used to infect different groups of mice for experimental purposes.
3.10.2 Four-day-suppressive test

For the in vivo antimalarial evaluation of the plant extracts, the Peter’s 4 day suppressive test was employed (Akuodor, Anyalewechi, et al., 2010). A total of eighty mice (40 for D. chamaethamnus and 40 for G. coleosperma) were used. Each one was inoculated IP on day zero (D₀) with 0.2 mL of 1 x 10⁷ P. berghei infected RBCs. The mice were randomly divided into eight groups of five mice each (Figure 18). The aqueous and organic extracts were resuspended in distilled water and 10% Tween 80 respectively to obtain different doses of 200, 400 and 800 mg/kg/day relative to the average weight of the mice in each group. Groups 1-6 were treated with the aqueous and organic extracts at the different doses orally (PO). Group 7 served as the positive control consisting of 25 mg/kg of chloroquine diphosphate and Group 8 received a 10% Tween 80 solution OP (vehicle), which served as the control.

Each treatment was given as a single dose daily for four consecutive days (D₀ to D₃) and was started 3 hours post infection. Doses were administered PO due to traditional applications even though IP route is more efficacious due to bioavailability. On day 4 (D₄), thin blood smears were made to determine the percentage parasitaemia and suppression of growth for each dose by comparing the parasitaemia in infected controls with those of treated mice, using the following formula: \( \frac{A-B}{B} \times 100 \), where A is the average parasitaemia in the negative control group, and B the average percentage parasitaemia in the test group. The median survival time (MST) of each group was also
determined over 29 days using a Kaplan-Meier plot (GraphPad Prism 6) and the weight changes in the mice were also recorded.

**Figure 18:** Diagram showing the layout for the four-day-suppressive antimalarial test for each plant.
3.10.3 Repository (prophylactic) test

The prophylactic activity of selected plant extracts were determined using the method described by Tarkang, Okalebo, Ayong, Agbor, & Guantai (2014). Forty mice were randomly divided into eight groups of five mice each (Figure 19). Different treatments were administered to each group for three consecutive days (D₀ to D₂) before infection. Groups 1, 2 and 3 were treated with different doses of the aqueous extract of *D. chamaethamnus* (200, 400 and 800 mg/kg) OP; whereas groups 4, 5 and 6 were treated with the aqueous extract of *G. coleosperma* in the same doses. Group 8 served as the positive control consisting of 10mg/kg/day of pyrimethamine, while Group 7 received 10 ml/kg of distilled water OP (control). On day 4 (D₃), each mouse was challenged with an IP injection of 0.2 ml of 1 x 10⁷ *P. berghei* infected RBCs. Thin blood smears were prepared 72 hours post infection to assess the percentage parasitaemia and average chemosuppression (See Section 3.10.2). The median survival time (MST) of each group was also determined over 29 days using GraphPad Prism 6 and the weight changes in the mice were also recorded.
Figure 19: Diagram showing the layout for the repository antimalarial test.
3.11 Acute oral toxicity

The plant extracts were evaluated for their toxicity in non-infected female Swiss albino mice weighing 18-20 g, using the Acute Toxic Class Method with a starting dose of 300 mg kg\(^{-1}\) (OECD, 2001). This method was adopted from the Organization for Economic and Cultural Development (OECD) guidelines for testing of chemicals, and uses pre-defined doses that allow toxicity to be ranked according to the Globally Harmonized System for classification of chemicals which cause acute toxicity. Female mice were used, because this group shows a slightly higher sensitivity to chemicals than males (Narayan & Mittal, 2015).

The mice were deprived of food, but not water, for 3 hours prior to and 1 hour post administration of the aqueous and organic plant extracts (0.2 mL) PO in a single dose. The extracts were administered in stepwise manner starting with a dose of 300 mg kg\(^{-1}\) (Figure 20) since the toxicity of the plant extracts was not known. The mice were observed for signs of toxicity such as rough hair coat (hair erection), diarrhoea, vomiting, foaming in the mouth, incoordination of gait, difficulty in breathing (dyspnoea), unprovoked behavior (\textit{i.e.} fighting or biting), alertness, restlessness and clonic convulsions (jerking), lethargy or recumbency and coma after treatment for the first four hours, which are the most critical, then over a period of 24 hours, thereafter daily for 14 days. Mortality occurring at a particular dose will indicate either to continue administration of a subsequent higher dose or to estimate the LD\(_{50}\) by comparing the mortality to a fixed LD\(_{50}\) cut-off value provided in the said guideline.
The weight changes of the mice were noted shortly before administration of treatments and at the end of the observation period. The gravely ill mice were sacrificed or euthanized; firstly by injecting the mice intraperitoneally with pentobarbital, thereafter they were killed by cervical dislocation. These mice were recorded as having died. The mice that survived until the end of this period (day 14) were also killed in a humane manner. Post-mortem exams were performed in all of the animals to macroscopically observe the appearance of the vital organs for any signs of toxicity that may cause impending death in mouse. The heart, kidneys, lungs, spleen and liver were observed for changes in shape, colour, size and texture. The accumulation of body fluids surrounding organs were also observed and signs of inflammation (injected veins).

### 3.12 Data analysis

A statistical software package GraphPad Prism 6 was used to analyze the data. The results are reported as mean ± standard error (SEM) and were analyzed statistically using one-way and two-way ANOVA followed by post hoc tests, *i.e.* the Tukey Krammer (Tukey's) and Dunnett’s multiple comparisons tests, to compare mean differences for all groups and within treatment groups. IC$_{50}$ and CC$_{50}$ values were determined by nonlinear interpolation from inhibition curves generated in GraphPad Prism. Kaplan-Meier Plots were generated to show the survival times (ST) for mice and the results between test and control groups were analyzed using a Log-rank (Mantel-Cox) test. Values of $P < 0.05$ were considered significant.
**Figure 20:** Test procedure with a starting dose of 300 mg/kg body weight adopted from the OECD guidelines (2001) for testing of chemicals using the Acute Toxic Class Method. For each step three mice (females) were used. The 0, 1, 2, 3 indicates the number of moribund or dead mice at each step. At each step a new group of healthy mice were used.

Key: GHS = Globally Harmonized Classification System; ∞ = unclassified; LD$_{50}$ = lethal dosage in mg/kg of body weight that is required to kill 50 % of population; b.w. = body weight
CHAPTER 4: RESULTS

4.1 Effect of solvent on yield of plant extracts

The percentage yield of aqueous and organic plant extracts obtained after maceration with water and dichloromethane-methanol (DC-MeOH) (1-1 v/v) respectively, and drying are given in Figure 21. The highest yield was obtained from the organic extract of *Guibourtia coleosperma* (14.25±1.58 %), followed by the aqueous extract of *G. coleosperma* (11.07±1.98 %) and the organic extracts of *Diospyros chamaethamnus* (10.33±0.56 %). The aqueous extract of *D. chamaethamnus* (8.20±1.80 %) yielded the least. The percentage extraction yield for both plants with methanol-dichloromethane (1:1 v/v) was not better than water, even though a difference in the mean percentage yield was observed (P > 0.05). A difference was also observed between extracts of the two plants under investigation; however this difference was not statistically significant (P > 0.05).
Figure 21: The mean percentage yield of the root extracts of plants post extraction with water (aqueous extract) and DCM-MeOH (organic extract). The mean differences were not significant (P > 0.05) as analyzed by Tukey’s multiple comparisons test.

KEY: DCAQ = *D. chamaethamnus* aqueous extract, DCOR = *D. chamaethamnus* organic extract, GCAQ = *G. coleosperma* aqueous extract, GCOR = *G. coleosperma* organic extract.

4.2 Characterization of plant extracts by TLC

The TLC chemical characterization for the plant extracts investigated is presented in Table 5. *G. coleosperma* and *D. chamaethamnus* were assayed for classes of compounds with known antimalarial activity. Colorimetric changes were visualized as described in Section 3.3 giving an indication of the presence of compounds tested for. The chromatograms (Appendix B) revealed the presence of alkaloids (orange-brown zones), anthraquinones (pink-violet zones), flavonoids (yellow fluorescent zones), steroids (greenish-black zones), and terpenoids (purple or violet zones) in the organic
extracts for both *D. chamaethamnus* and *G. coleosperma*. In the aqueous extracts of the two plants, alkaloids, anthraquinones, steroids and flavonoids were present; terpenoids however, were absent. Coumarins (blue-green fluorescent zones) were only detected in the extracts of *G. coleosperma*, aqueous and organic. All classes tested for were present in the organic extracts of *G. coleosperma*; however in the aqueous extract 5 of the 6 classes were present. In the aqueous and organic root extracts of *D. chamaethamnus* only 4 and 5 of the six compounds, respectively were present.

**Table 5**: Presence of known antimalarial classes of compounds in *D. chamaethamnus* and *G. coleosperma* extracts.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Extract</th>
<th>Chief constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Al An C F S T</td>
</tr>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>+ + - + + -</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>+ + - + + +</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>+ + + + + -</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>+ + + + + +</td>
</tr>
</tbody>
</table>

+ = detected, - = not detected, Al = alkaloids, An = anthraquinones, C = coumarins, F = flavonoids, S = steroids and T = terpenoids
4.3 Characterization of plant extracts by GC-MS

Chemical characterization of the volatile constituents in the aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma* was performed using GC-MS. The resulting total ion chromatograms (TICs) are shown in Figures 23-26 and the identified compounds are listed in Tables 6-9 in order of elution from the apolar BP5MS column. The chemical structures of selected compounds (excluding those with IUPEC names) are shown in Figure 22. Possible contaminants, such as phthalate esters, in the extracts were also identified. These compounds were considered contaminants as they are industrial chemicals, and are usually found in plasticizers. The source of these compounds could be from the plastic Eppendorf tubes in which the extracts were stored (McDonald, Cummins, Barkley, Thompson, & Lincoln, 2008).

The chromatograms of the aqueous and organic extracts of *D. chamaethamnus* revealed the presence of twenty compounds each. The phytoconstituents present in the aqueous extract were alkanes (tetradecane, hexadecane, octadecane, octacosane), fatty acids and their esters (hexadecanoic acid, hexyl hexadecanoate and two octadecanoic acid esters), phenolic compounds (3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 1,3,5-benzentriol, an unidentified phenol), terpenoids (an unidentified sesquiterpenoid, α-amyrin, vitamin E, an unidentified triterpenoid) and steroids (stigmasterol, two unidentified sterols). Glycerol 2-hexadecanoate was also present. The organic extract had a similar chemical profile. The compounds present included alkanes (tetradecane hexadecane, heptadecane, octadecane), fatty acids (hexadecanoic acid, 9,12-
octadecanoic acid (Z,Z)-, oleic acid, octadecanoic acid), a fatty acid ester (methyl hexadecanoate), steroids (stigmasterol, lanosterol, 2 unidentified sterols), terpenoids (α-amyrin, 4 unidentified triterpenoids) and spiculesporic acid.

**Figure 22:** Chemical structures of selected compounds from Tables 5-8. (Compounds with IUPEC names were not included.)
Figure 23: Total ion chromatogram of the aqueous extract of *D. chamaethamnus* obtained by GC-MS analysis. The identified compounds are numbered in order of elution from the GC column. The numbers correspond to those in Table 6. *Possible contaminants.*
Table 6: Volatile and semi-volatile compounds identified in the aqueous extract of *Diospyros chamaethamnus*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time</th>
<th>Retention index</th>
<th>Name of compound</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.15</td>
<td>1147</td>
<td>3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one</td>
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<td>2</td>
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<td>1382</td>
<td>1,3,5-Benzentriol</td>
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</tr>
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<td>Hexadecane</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>1963</td>
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<td>B</td>
</tr>
<tr>
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<td>2323</td>
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</tr>
<tr>
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<td>2386</td>
<td>Hexyl hexadecanoate</td>
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</tr>
<tr>
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<tr>
<td>19</td>
<td>123.47</td>
<td>3362</td>
<td>α-Amyrin</td>
<td>B</td>
</tr>
</tbody>
</table>

*In order of elution from apolar BP5MS column. Retention index relative to C_{10}-C_{40} n-alkanes, on BP5MS column. Identification: A – comparison of mass spectrum and retention time with those of an authentic reference compound; B – comparison of mass spectrum and RI with NIST databases and published data (tentative identification); C – comparison of mass spectrum with NIST databases (tentative identification); D – retention index estimation.*
**Figure 24:** Total ion chromatogram of the organic extract of *D. chamaethamnus* obtained by GC-MS analysis. The identified compounds are numbered in order of elution from the GC column. The numbers correspond to those in Table 7. *Possible contaminants.*
Table 7: Volatile and semi-volatile compounds identified in the organic extract of *Diospyros chamaethamnus*.

<table>
<thead>
<tr>
<th>No.</th>
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<th>ID</th>
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<td>3820</td>
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<td>C</td>
</tr>
</tbody>
</table>

*a* In order of elution from apolar BP5MS column. *b* Retention index relative to C_{10}-C_{40} n-alkanes, on BP5MS column. *c* Identification: A – comparison of mass spectrum and retention time with those of an authentic reference compound; B – comparison of mass spectrum and RI with NIST databases and published data (tentative identification); C – comparison of mass spectrum with NIST databases (tentative identification).
In the aqueous extract of *G. coleosperma* roots, a total of only 7 compounds were found: an alkane (tetradecane), a fatty acid (hexadecanoic acid), terpenoids (squalene, a triterpenoid), phenolic compounds (2 unidentified phenols), and a steroid (an unidentified sterol). The TIC of the organic extract of *G. coleosperma* indicated the presence of nineteen compounds including alkanes such as tetradecane, hexadecane and octadecane; a phenolic compound (an unidentified phenol); fatty acids (hexadecanoic acid and oleic acid); terpenoids (α-amyrin and a triterpenoid); and steroids such as campesterol, lanosterol, and 2 unidentified steroids. Six of the compounds were not identified.
Figure 25: Total ion chromatogram of the aqueous extract of *G. coleosperma* obtained by GC-MS analysis. The identified compounds are numbered in order of elution from the GC column. The numbers correspond to those in Table 8. *Possible contaminants.*
Table 8: Volatile and semi-volatile compounds identified in the aqueous extract of *Guibourtia coleosperma*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time</th>
<th>Retention index(^b)</th>
<th>Name of compound</th>
<th>ID(^c)</th>
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</tr>
<tr>
<td>6</td>
<td>121.29</td>
<td>3288</td>
<td>Unidentified sterol</td>
<td>C</td>
</tr>
<tr>
<td>7</td>
<td>121.57</td>
<td>3297</td>
<td>Unidentified triterpenoid</td>
<td>C</td>
</tr>
</tbody>
</table>

\(^a\)In order of elution from apolar BP5MS column. \(^b\)Retention index relative to C\(_{10}\)-C\(_{40}\) \(n\)-alkanes, on BP5MS column. \(^c\)Identification: A – comparison of mass spectrum and retention time with those of an authentic reference compound; B – comparison of mass spectrum and RI with NIST databases and published data (tentative identification); C – comparison of mass spectrum with NIST databases (tentative identification).
Figure 26: Total ion chromatogram of the organic extract of *G. coleosperma* obtained by GC-MS analysis. The identified compounds are numbered in order of elution from the GC column. The numbers correspond to those in Table 8. *Possible contaminants.
Table 9: Volatile and semi-volatile compounds identified in the organic extract of *Guibourtia coleosperma*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time</th>
<th>Retention index</th>
<th>Name of compound</th>
<th>ID&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.77</td>
<td>1400</td>
<td>Tetradecane</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>53.51</td>
<td>1598</td>
<td>Unidentified phenol</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>53.66</td>
<td>1600</td>
<td>Hexadecane</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>64.41</td>
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</tr>
<tr>
<td>5</td>
<td>72.34</td>
<td>1964</td>
<td>Hexadecanoic acid</td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>80.19</td>
<td>2137</td>
<td>Oleic acid</td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>81.38</td>
<td>2163</td>
<td>Octadecanoic acid</td>
<td>B</td>
</tr>
<tr>
<td>8</td>
<td>86.03</td>
<td>2273</td>
<td>Unknown</td>
<td>C</td>
</tr>
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<td>9</td>
<td>86.88</td>
<td>2294</td>
<td>Unidentified steroid</td>
<td>C</td>
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<td>10</td>
<td>89.78</td>
<td>2364</td>
<td>Unknown</td>
<td>C</td>
</tr>
<tr>
<td>11</td>
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<td>C</td>
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<td>14</td>
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<td>3195</td>
<td>Campesterol</td>
<td>B</td>
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<tr>
<td>15</td>
<td>119.82</td>
<td>3237</td>
<td>Unknown</td>
<td>C</td>
</tr>
<tr>
<td>16</td>
<td>121.29</td>
<td>3288</td>
<td>Unidentified sterol</td>
<td>C</td>
</tr>
<tr>
<td>17</td>
<td>121.58</td>
<td>3298</td>
<td>Unidentified triterpenoid</td>
<td>C</td>
</tr>
<tr>
<td>18</td>
<td>122.50</td>
<td>3329</td>
<td>Lanosterol</td>
<td>B</td>
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<tr>
<td>19</td>
<td>123.51</td>
<td>3363</td>
<td>α-Amyrin</td>
<td>B</td>
</tr>
</tbody>
</table>

<sup>a</sup>In order of elution from apolar BP5MS column.  
<sup>b</sup>Retention index relative to C<sub>10</sub>-C<sub>40</sub> n-alkanes, on BP5MS column.  
<sup>c</sup>Identification: A – comparison of mass spectrum and retention time with those of an authentic reference compound; B – comparison of mass spectrum and RI with NIST databases and published data (tentative identification); C – comparison of mass spectrum with NIST databases (tentative identification).
4.4 Antioxidant activities of the plant extracts

The antioxidant activity of the plant extracts that is, their ability to scavenge DPPH free radicals is presented in Table 10. Antioxidant activity was observed for the aqueous and organic extracts of both *D. chamaethamnus* and *G. coleosperma*. The activities were concentration dependent, although at higher concentrations the activities were not statistically different (P > 0.05). The extracts of *D. chamaethamnus* exhibited higher antioxidant activity compared to the extracts of *G. coleosperma*. The aqueous (DCAQ) and organic (DCOR) extracts of *D. chamaethamnus* at the lowest concentration (10µg/mL) had observed antioxidant activities of 90.40±1.33 and 39.92±2.12 %, respectively; whereas at the highest concentration (40 µg/mL), the observed antioxidant activities were 94.19±0.31 and 94.27±0.37 %, respectively. The antioxidant activities for the aqueous (GCAQ) and organic (GCOR) extracts of *G. coleosperma*, conversely, were much lower. At 10 µg/mL antioxidant activities of 4.24±4.19 and 26.17±2.29 %, respectively were observed, and at 40 µg/mL the observed activities were 23.90±2.48 and 91.05±0.55 %, respectively.
Table 10: DPPH radical scavenging activity observed for the aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma*.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCAQ</td>
</tr>
<tr>
<td>10</td>
<td>90.40±1.33</td>
</tr>
<tr>
<td>20</td>
<td>92.75±0.30</td>
</tr>
<tr>
<td>30</td>
<td>93.27±0.62</td>
</tr>
<tr>
<td>40</td>
<td>94.19±0.31</td>
</tr>
</tbody>
</table>

Values in the table are expressed as means ± SEM of three replicates relative to control (vehicle), *n*=3.
DCAQ: *D. chamaethamnus* aqueous extract, DCOR: *D. chamaethamnus* organic extract, GCAQ: *G. coleosperma* aqueous extract, GCOR: *G. coleosperma* organic extract

From the estimated IC$_{50}$ values (Table 11) which are the concentrations of the extracts that was able to scavenge 50 % of the DPPH radicals, the order of potency was DCAQ (7.63 µg/mL) ˃ DCOR (10.74 µg/mL) ˃ GCOR (22.03 µg/mL) ˃ GCAQ (36.05 µg/mL). The calculated IC$_{50}$ for ascorbic acid was 5.97 µg/mL. The ascorbic acid equivalent antioxidant capacity (AEAC) of the test plant extracts are also shown in Table 11 and were expressed as mg of ascorbic acid (AA) equivalent antioxidant content per 100 g of plant extract. For the aqueous and organic extracts of *D. chamaethamnus*, AEAC was 78244 mg AA/100 g extract and 55587 mg AA/100 g extract, respectively. For *G. coleosperma* aqueous and organic extracts, values were 16560 mg AA/100 g extract and 27099 mg AA/ 100 g extract, respectively. The greater the scavenging activity (decolourization of DPPH solution), the greater the antioxidant activity of the plants, and this was reflected in the high AEAC values. The plant extract with an AEAC of over 600 mg AAeq/100 g is regarded as displaying very high antioxidant activities (Leong &
Shui, 2002). The AEAC values for the root extracts, aqueous and organic, of both plants were between 27000-79000 mg AAeq/100 g hence, they were considered sources of potent antioxidants.

Table 11: The antioxidant activity of aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma*.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extract</th>
<th>IC$_{50}$ (µg/mL)</th>
<th>[R$^2$]</th>
<th>AEAC$^*$ (mg AA/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>7.63 [0.9748]</td>
<td></td>
<td>78244</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>10.74 [0.9971]</td>
<td></td>
<td>55587</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>36.05 [0.9808]</td>
<td></td>
<td>16560</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>22.03 [0.9989]</td>
<td></td>
<td>27099</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
<td>5.97 [0.9968]</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Nonlinear regression analysis was used to calculate IC$_{50}$ values in GraphPad Prism 6. *Ascorbic acid equivalent antioxidant capacity.*
4.5 Determination of *in vitro* antimalarial activity

The antimalarial activities of the plant extracts were determined by quantifying parasitaemia and percentage growth inhibition of the chloroquine sensitive *P. falciparum* D10 strain, which were then compared with untreated parasite cultures (Figure 27). At each of the four concentrations (*i.e.* 5, 25, 50 and 100 µg/mL) for all the extracts (Figures 28 and 29), there was a significant reduction in the number of parasite infected RBCs relative to the control (*P* < 0.05) indicating antimalarial activity. There was, however, no statistical difference between organic and aqueous extracts of the plants under investigation (*P* > 0.05). In addition, the percentage parasitaemia were not significantly different with increasing concentrations of the plant extracts.

![Figure 27: Microphotographs showing a non-parasitized culture (uninfected RBCs) (left) and a parasitized culture of *P. fall iparaum* D10 (right) consisting of 1) rings, 2) trophozoites and 3) schizonts (magnification at x 1000).](image-url)
Figure 28: Percentage parasite survival of *P. falciparum* D10 48 hours post treatment with *D. chamaethamnus* (DC) aqueous (AQ) and organic (OR) extracts at various concentrations. Data are presented as means ± SEM. *: P < 0.05, **: P < 0.01, ***: P < 0.001, compared to negative control (one-way ANOVA followed by Tukey’s post hoc test). NC: negative control or vehicle and CQ: chloroquine or positive control.
Figure 29: Percentage parasite survival of *P. falciparum* D10 48 hours post treatment with *G. coleosperma* (GC) aqueous (AQ) and organic (OR) extracts at various concentrations. Data are presented as means ± SEM. ∗: P < 0.05, ∗∗: P < 0.01, ∗∗∗: P < 0.001, compared to negative control (one-way ANOVA followed by Tukey's post hoc test). NC: negative control or vehicle and CQ: chloroquine or positive control.
Chloroquine showed a mean percentage suppression of 92.55 %, compared to the aqueous (46.18 %) and organic (28.31 %) extracts of *D. chamaethamnus*, and *G. coleosperma* aqueous (49.30 %) and organic (49.13 %) extracts at the highest concentration (100 µg/mL) (Table 12). The aqueous and organic extracts of *D. chamaethamnus* showed the highest antiplasmodial activities with IC$_{50}$ values of 18.30 and 19.51 µg/mL, respectively. The *G. coleosperma* extracts on the other hand, exhibited lower antiplasmodial activities compared to *D. chamaethamnus* extracts with IC$_{50}$ values of 31.61 and 28.17 µg/mL for aqueous and organic, respectively. *In vitro* antimalarial activity of the plant extracts were classified either as highly active (IC$_{50}$ < 10 µg/mL), moderately active (10 ≤ IC$_{50}$ ≤ 50 µg/mL), minimally active (50 < IC$_{50}$ ≤ 100 µg/mL), and or inactive (IC$_{50}$ >100 µg/ml) (Dolabela et al. 2008). All tested extracts, that is the organic (28.17 µg/mL) and aqueous (31.61 µg/mL) extracts of *G. coleosperma* and the aqueous (18.30 µg/mL) and organic extracts (19.5 µg/mL) of *D. chamaethamnus* showed moderate activity with IC$_{50}$ values < 50 µg/mL.
**Table 12:** *In vitro* antiplasmodial activity of aqueous and organic extracts from *D. chamaethamnus* and *G. coleosperma* against erythrocytic stages of the *P. falciparum* D10 strain at 48 hours.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extract</th>
<th>% Parasite growth inhibition</th>
<th>IC$_{50}$ (µg/mL) [R$^2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 µg/mL</td>
<td>25 µg/mL</td>
</tr>
<tr>
<td><em>Diospyros chamaethamnus</em></td>
<td>Aqueous</td>
<td>47.36</td>
<td>33.01</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>50.04</td>
<td>33.51</td>
</tr>
<tr>
<td><em>Guibourtia coleosperma</em></td>
<td>Aqueous</td>
<td>28.46</td>
<td>36.72</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>29.75</td>
<td>37.34</td>
</tr>
<tr>
<td>Chloroquine (25 µg/mL)</td>
<td>-</td>
<td>-</td>
<td>92.54</td>
</tr>
</tbody>
</table>

IC$_{50}$ values were determined by nonlinear interpolation from each of the inhibition curves in GraphPad Prism 6.
4.6 Determination of cytotoxicity

To determine the potential toxic effects of the plant extracts on normal human fetal lung fibroblast cells, the SRB staining method was used. This assay determines the cell viability after exposure to the extracts. Dose response curves of the cytotoxic effects of the extracts on W138 cell lines are shown in Figures 30 and 31. The plant extracts generally exhibited weak toxicity except for one, the organic extract of *D. chamaethamnus* (DCOR) (Table 13). This extract had the highest toxicity on the fibroblast cells with a markedly reduced cell viability of 33.92±0.32 % followed by GCAQ (64.28±0.26 %), then DCAQ (81.76±1.84 %) and GCOR (101.88±1.54 %) at the highest concentration of 100 µg/mL. Differences in cytotoxicity were observed between various concentrations of the plant extracts, demonstrating a concentration dependent effect (P < 0.05). As the concentration increased, cell viability decreased.

The 50 % cellular cytotoxic concentration (CC\(_{50}\)) showed a similar trend in toxicity with GCOR (>100 µg/mL), DCAQ (150.8 µg/mL) and GCAQ (136.7 µg/mL), However, for DCOR, its CC\(_{50}\) (29.73 µg/mL) was closer to the CC\(_{50}\) of the control, etoposide (5.104 µg/mL). In addition, selective toxicity of the extracts towards the malaria parasites was also determined. The organic extract of *D. chamaethamnus* showed the lowest selective index (1.52), whilst its aqueous extract showed a high SI of 8.24. The aqueous extract of *G. coleosperma* showed an intermediary therapeutic index value of 4.32 and its organic extract showed the highest SI (> 8).
Figure 30: Dose response curves showing the cytotoxic effects of *Diospyros chamaethamnus* extracts, both (a) aqueous and (b) organic, on human fetal lung fibroblast W138 cells. Concentrations (µg/mL) were expressed in log form and used to plot the non-linear graphs against percentage cell viability.

Figure 31: Dose response curves showing the cytotoxic effects of *Guibourtia coleosperma* extracts, both (a) aqueous and (b) organic, on human fetal lung fibroblast W138 cells. Concentrations (µg/mL) were expressed in log form and used to plot the non-linear graphs against percentage cell viability.
Table 13: Cytotoxic activities of *Diospyros chamaethamnus* and *Guibourtia coleosperma* extracts, both aqueous and organic compared to etoposide (control) against human fetal lung fibroblast W138 cells.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Extract</th>
<th>% Cell viability</th>
<th>Concentrations (µg/mL)</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µg/mL) [R&lt;sup&gt;2&lt;/sup&gt;]</th>
<th>SI&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.25</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>102.78±1.04</td>
<td>102.04±2.52</td>
<td>103.37±0.95</td>
<td>97.62±1.30</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>97.78±0.40</td>
<td>70.82±0.28</td>
<td>41.66±0.90</td>
<td>33.98±0.27</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>100.8±1.17</td>
<td>97.58±0.47</td>
<td>92.84±0.55</td>
<td>90.48±0.02</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>106.2±1.55</td>
<td>103.36±1.06</td>
<td>105.38±1.34</td>
<td>100.65±1.39</td>
</tr>
<tr>
<td>Etoposide</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values in the table are expressed as means ± SEM of three replicates relative to control (vehicle), n = 3. CC<sub>50</sub> values were determined using non-linear regression analysis in GraphPad Prism 6. <sup>a</sup> Selectivity index = IC<sub>50</sub> fibroblast cells /IC<sub>50</sub> *Plasmodium.*
4.7 Determination of in vivo antiplasmodial activity

4.7.1 Suppressive activity of selected plant extracts

The efficacy of *D. chamaethamnus* and *G. coleosperma* extracts as antimalarials, both aqueous and organic extracts were evaluated using the Peter’s 4-day suppressive test. This test evaluates the schizonticidal activity of the plant extracts. The parasite load (Figure 32), survival time and weight changes for all groups of mice with *P. berghei* infection were determined.

![Microphotographs showing healthy (uninfected) RBCs in Swiss albino mice (left) and a *P. berghei* infected RBCs (established infection) in Swiss albino mice (right) consisting of 1) trophozoites and 2) rings (magnification at x 1000).](image)

**Figure 32:** Microphotographs showing healthy (uninfected) RBCs in Swiss albino mice (left) and a *P. berghei* infected RBCs (established infection) in Swiss albino mice) (right) consisting of 1) trophozoites and 2) rings (magnification at x 1000).

4.7.1.1 Parasite load

The data reveals there was a reduction in mean parasite count in some of the treatment groups for *D. chamaethamnus* as compared to the vehicle group (control) (P < 0.05) (Figure 33). The mean parasite count for DCAQ was 5.94, 28.10 and 25.04 % at doses of 200, 400, 800 mg kg\(^{-1}\), respectively, with a higher % parasite reduction at 400 than 800 mg kg\(^{-1}\). At 200 mg kg\(^{-1}\), the parasite count was at its lowest. For DCOR the trend in
parasitaemia was reversed with parasitaemia of 27.28, 22.43, 15.23 % at doses of 200, 400, 800 mgkg\(^{-1}\). The organic extracts exhibited higher antiplasmodial effects than the aqueous extracts, showing a lower parasite load at 400 and 800 mgkg\(^{-1}\) (P < 0.05). The extracts did not exhibit a dose dependent reduction in parasite counts in the two treatment groups, even though for DCOR, as the dose increased, % parasitaemia decreased. For GCOR, there was no dose dependency, since at different doses; parasite count was similar (P > 0.005).

*G. coleosperma* extracts reduced the *Plasmodium* load in infected mice as compared to the vehicle group (14.35 %), although this reduction was not significant (P > 0.05). The mean parasite count for *G. coleosperma* aqueous extract was 20.16, 12.57 and 11.68 at doses of 200, 400, 800 mgkg\(^{-1}\), respectively, with 11.05, 9.54, 10.10 mgkg\(^{-1}\) for the organic extract (Figure 34). Although not statistically significant, the organic extracts of *G. coleosperma* indicated a higher parasite reduction than the aqueous extracts (P > 0.05). In addition, the reduction of the parasite levels in mice did not decrease with escalating doses of the plant extracts.
Figure 33: Schizonticidal effects of aqueous (DCAQ) and organic (DCOR) extracts of *D. chamaethamnus* on the parasite load (i.e. parasitaemia) in male Swiss albino mice 4 days post infection compared to the vehicle (control group) and chloroquine (CQ). Data are presented as means ± SEM. *: $P < 0.05$; **: $P < 0.01$, compared to vehicle treated group (one-way ANOVA followed by Dunnett’s *post hoc* test).
Figure 34: Schizonticidal effects of aqueous (GCAQ) and organic (GCOR) extracts of *G. coleosperma* on the parasite load (i.e. parasitaemia) in male Swiss albino mice 4 days post infection compared to the vehicle (control group) and chloroquine (CQ). Data are presented as means ± SEM. *: P < 0.05; **: P < 0.01, ***: P < 0.001 compared to vehicle treated group (one-way ANOVA followed by Dunnett's post hoc test).

Percentage inhibition analysis showed that the extracts displayed chemosuppressive activities against *P. berghei*, although the extent of reduction was less than that of CQ which produced close to 100 % suppression (Table 14). The rank in decreasing order of chemosuppression for *D. chamaethamnus* extracts was: DCOR800 (44.66 %) > DCOR400 (18.52 %) > DCOR200 (0.89 %); and DCAQ200 (78.44 %) > DCAQ800 (9.01 %) > DCAQ400 (<1.00 %). For *G. coleosperma* extracts the order of activity was GCOR400 (33.48 %) > GCOR800 (29.59 %) > GCOR200 (23.00 %); and GCAQ800
(18.61 %) > GCAQ400 (12.37 %) > GCAQ200 ((<1.00 %). The organic extracts overall had the highest chemosuppressive effects for both plants. The aqueous extracts of *D. chamaethamnus* and *G. coleosperma* at doses of 400 and 200 mgkg$^{-1}$, respectively, no chemosuppressive activities were observed. Of the two plants, *G. coleosperma* indicated a better effect on % suppression of parasite growth.

**Table 14**: Percentage growth inhibition of *Plasmodia* parasites activities of aqueous and organic root extracts of *D. chamaethamnus* and *G. coleosperma* in *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extract</th>
<th>Dose (mgkg$^{-1}$, PO)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>200</td>
<td>78.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>&lt; 1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>9.01</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>200</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>18.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>44.66</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>200</td>
<td>&lt; 1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>12.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>18.61</td>
</tr>
<tr>
<td></td>
<td>Organic</td>
<td>200</td>
<td>23.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>33.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>29.59</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>-</td>
<td>25</td>
<td>99.37</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
4.7.1.2 Body weight

The changes in body weight were observed in mice treated with *D. chamaethamnus* extracts over a period of 4 and 6 days post infection with *P. berghei* (Figure 35). There was a reduction in weight associated with infection on day 4 for all treatment groups. An increase in weight was observed for chloroquine on Day 4. The weights of the mice in the treated groups were comparable to that of the control group (vehicle) and to those that received chloroquine (P > 0.05).

On day 6, a greater reduction in weight in the mice was observed. This was indicated in treatment groups of DCAQ at 200 mgkg⁻¹ (-14.29 %), 400 mgkg⁻¹ (-19.64 %) and 800 mgkg⁻¹ (-17.09 %); and for DCOR at 200 mgkg⁻¹ (-22.22 %), 400 mgkg⁻¹ (-20.20 %) and 800 mgkg⁻¹ (-12.87 %). For the chloroquine treated mice, the weight that was gained was lost over days 5 and 6. Compared to the vehicle (-20.67 %) and chloroquine (2.04 %) groups, the changes in weight for the treated mice were not statistically significant (P > 0.05) (Table 15). This indicates that both the *D. chamaethamnus* extracts (treatment) and chloroquine did not prevent weight loss in mice with *P. berghei* infection. The change in body weight for the groups of mice was not dose dependent. Furthermore, there were no detectable differences in weight changes between groups that received the aqueous and organic extracts for both day 4 and day 6 (P > 0.05).
Figure 35: Effects of aqueous and organic extracts of *D. chamaethamnus* on weight in mice infected with *Plasmodium berghei* on Day 4 and 6 post infection compared to vehicle (control) and chloroquine (CQ: standard).

Table 15: Weight changes in malaria infected mice, treated with *D. chamaethamnus* extracts, over 4 and 6 days compared to vehicle (control) and chloroquine (standard).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weigh (g)</th>
<th>Difference in weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>20.80</td>
<td>19.00</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>19.60</td>
<td>21.40</td>
</tr>
<tr>
<td>DCAQ200</td>
<td>19.60</td>
<td>18.80</td>
</tr>
<tr>
<td>DCAQ400</td>
<td>19.60</td>
<td>17.50</td>
</tr>
<tr>
<td>DCAQ800</td>
<td>19.60</td>
<td>17.40</td>
</tr>
<tr>
<td>DCOR200</td>
<td>19.80</td>
<td>17.80</td>
</tr>
<tr>
<td>DCOR400</td>
<td>19.80</td>
<td>18.40</td>
</tr>
<tr>
<td>DCOR800</td>
<td>20.20</td>
<td>19.20</td>
</tr>
</tbody>
</table>
On day 4, an increase in weight was observed in all groups treated with aqueous and organic extracts of *G. coleosperma* extracts (Figure 36), with the exception of GCOR400 and GCAQ800. On day 6, a decrease in weight was observed, although these changes were not statistically significant (P > 0.05). The weight percentage reduction of the treatment groups on day 6 were as follow: GCAQ at 200 mgkg\(^{-1}\) (-1.15 %), 400 mgkg\(^{-1}\) (-1.88 %) and 800 mgkg\(^{-1}\) (-9.30%); and for GCOR at 200 mgkg\(^{-1}\) (-8.82 %), 400 mgkg\(^{-1}\) (-9.57 %) and 800 mgkg\(^{-1}\) (-10.00 %) (Table 16). Weight gain observed in mice treated with chloroquine on day 4 was kept constant by day 6. These changes in body weight were not dose dependent and the differences between the extracts and between the extracts and chloroquine were not statically significant. The weight changes in these mice, as well as those treated with chloroquine were comparable to those in the control group (vehicle) (-13.83 %) on day 4 and 6 (P > 0.05). Both chloroquine and *G. coleosperma* extracts were therefore unable to prevent weight loss.
Figure 36: Effects of aqueous and organic extracts of *G. coleosperma* on weight in mice infected with *Plasmodium berghei* on Day 4 and 6 post infection compared to vehicle (control) and chloroquine (CQ: standard).

Table 16: Weight changes in malaria infected mice, treated with *G. coleosperma* extracts, over 4 and 6 days compared to vehicle (control) and chloroquine (standard).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>D0</th>
<th>D4</th>
<th>D6</th>
<th>D0-D4</th>
<th>D0-D6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>17.60</td>
<td>18.75</td>
<td>18.75</td>
<td>6.53</td>
<td>6.53</td>
</tr>
<tr>
<td>GCAQ200</td>
<td>17.40</td>
<td>19.20</td>
<td>17.20</td>
<td>10.34</td>
<td>-1.15</td>
</tr>
<tr>
<td>GCAQ400</td>
<td>18.60</td>
<td>19.60</td>
<td>18.25</td>
<td>5.38</td>
<td>-1.88</td>
</tr>
<tr>
<td>GCAQ800</td>
<td>15.80</td>
<td>15.67</td>
<td>14.33</td>
<td>-0.82</td>
<td>-9.30</td>
</tr>
<tr>
<td>GCOR200</td>
<td>17.00</td>
<td>18.40</td>
<td>15.50</td>
<td>8.24</td>
<td>-8.82</td>
</tr>
<tr>
<td>GCOR400</td>
<td>18.80</td>
<td>18.80</td>
<td>17.00</td>
<td>0</td>
<td>-9.57</td>
</tr>
<tr>
<td>GCOR800</td>
<td>18.00</td>
<td>18.80</td>
<td>16.20</td>
<td>4.44</td>
<td>-10.00</td>
</tr>
</tbody>
</table>
4.7.1.3 Survival time

The effect of the plant extract on the survival of the mice in each group was observed. *D. chamaethamnus* extracts were able to increase survival of the malaria infected mice in all groups (Figure 37) which were statistically significant compared to the negative control (P < 0.05). The median survival time (MST) for the infected mice is shown in Table 17. The least MST of 12 days was recorded for the control group (vehicle) that was left untreated; whereas that of the chloroquine group was undefined as 3 of the 5 mice were still alive on day 29, the end of the observation period. The MST of 13, 16 and 18 days was recorded for the groups that received 200, 400 and 800 mgkg\(^{-1}\) of the organic extract, respectively while 17, 12.5 and 13 days were recorded for the groups that received 200, 400 and 800 mgkg\(^{-1}\) of the aqueous extract, respectively.

*G. coleosperma* extracts increased the survival of mice with established infection of malaria (Figure 38). The survival time for the test groups were statistically significant compared to the negative control (P < 0.05). The MST for the control group (vehicle) was 12 days; while for the groups that received 200 and 400 mgkg\(^{-1}\) of the organic extract, 16 days each was recorded and 19 days for 800 mgkg\(^{-1}\); while 17, 14 and 15 days were recorded for the groups that received 200, 400 and 800 mgkg\(^{-1}\) of the aqueous extract, respectively.

Overall, the survival time increased in the mice treated with the maximum dose of the organic extract of both *G. coleosperma* and *D. chamaethamnus*; while the survival time
decreased in the mice treated with the maximum dose of the aqueous extract. However, the differences in median survival time of the mice in these groups were comparable (P > 0.05). There was also no significant difference between the MST for *D. chamaethamnus* and *G. coleosperma* as indicated by the MST for their aqueous and organic extracts (P > 0.05).

![Figure 37: A Kaplan-Meier Plot showing the survival time (ST) for mice treated with different doses (200, 400, 800 mg/kg) of aqueous (AQ) and organic (OR) extracts of *D. chamaethamnus* (DC) administered once daily O.P. for four consecutive days. Results between test and control groups were significant (P = 0.0344) as analyzed by Log-rank (Mantel-Cox) test.](image-url)
Figure 38: A Kaplan-Meier Plot showing the survival time (ST) for mice treated with different doses (200, 400, 800 mg/kg) of aqueous (AQ) and organic (OR) extracts of *G. coleosperma* (GC) administered once daily P.O. for four consecutive days. Results between test and control groups were significant (P = 0.0026) as analyzed by Log-rank (Mantel-Cox) test.
**Table 17:** Median survival time (MST) for mice subjected to different doses of aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma*.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Extract</th>
<th>Dose (mg kg⁻¹)</th>
<th>MST (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>200</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>13</td>
</tr>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Organic</td>
<td>200</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>18</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>200</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>15</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Organic</td>
<td>200</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>19</td>
</tr>
<tr>
<td>Chloroquine</td>
<td></td>
<td>25</td>
<td>Undefined</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>-</td>
<td>12</td>
</tr>
</tbody>
</table>

*Tabulated using GraphPad Prism 6 (Survival analyses).*
4.7.2 Prophylactic activity of selected medicinal plants

The prophylactic activities of the crude extracts of *D. chamaethamnus* and *G. coleosperma* were tested using the repository test or residual infection procedure. Only the aqueous extracts were investigated. This is because no significant difference was observed for the suppressive antiplasmodial activities between aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma*; also because water is the solvent most frequently used to prepare herbal remedies in ethnomedicinal settings. Moreover, the plants under investigation are prepared as decoctions in the rural areas of the Zambezi region in Namibia. The parasite load, survival time and weight changes for all groups of mice with *P. berghei* infection were also estimated.

4.7.2.1 Parasite load

The aqueous extracts of *D. chamaethamnus* and *G. coleosperma* showed a reduction in the parasite load compared to the control group (vehicle) (P < 0.05) (Figure 39). There, however, was no significant difference in parasitaemia between DCAQ and GCAQ (treatment groups). The parasite load of the group of mice to which the aqueous extracts of *D. chamaethamnus* were administered to was 1.96 % at 200 mgkg⁻¹, 2.84 % at 400 mgkg⁻¹ and 2.14 % at 800 mgkg⁻¹. For *G. coleosperma* it was 2.33 % at 200 mgkg⁻¹, 2.33 % at 400 mgkg⁻¹, and 2.17 at 800 mgkg⁻¹. The antiplasmodial effects of the varying doses of extracts were comparable (P > 0.05), meaning the reduction effect of extracts on parasite load was not dose-dependent for either plant.
The percentage inhibition for the aqueous extract of *G. coleosperma* was 52.11 %, 52.16 % and 44.48 % for 200, 400, 800 mg/kg/day, respectively (Table 18). The least antiplasmodial effect was observed for 800 mgkg⁻¹ (highest dose), and the highest effect was observed for 400 mgkg⁻¹, whereas the 200 mgkg⁻¹ had an intermediary effect on the growth of the parasites. The aqueous extract of *D. chamaethamnus* exhibited antiplasmodial activities of 59.86 %, 41.68 %, 56.13 % for 200, 400, 800 mg/kg/day respectively; in addition, the highest activity was observed at the lowest dose (200 mgkg⁻¹) followed by the highest dose (800 mgkg⁻¹), and the least activity was observed at 400 mg/kg⁻¹. Overall, the antiplasmodial activities for the aqueous extracts for both plants were similar across doses (P > 0.05). Pyrimethamine, the positive control had a percentage growth inhibition of 88.79 % at a dose of 10 mgkg⁻¹.
Figure 39: Prophylactic effects of aqueous extracts of *D. chamaethamnus* and *G. coleosperma* extracts on the parasite load (*i.e.* parasitaemia) in male Swiss albino mice 72 hours post infection. Data are presented as means ± SEM. *: P < 0.05; **: P < 0.01, compared to vehicle treated groups (one-way ANOVA followed by Dunnett’s *post hoc* test).
**Table 18:** Prophylactic effects of aqueous extracts of *D. chamaethamnus* and *G. coleosperma* on *P. berghei* parasites.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extract</th>
<th>Dose (mg kg⁻¹, p.o.)</th>
<th>Growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>200</td>
<td>59.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>41.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>56.13</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>200</td>
<td>52.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>52.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>55.48</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>-</td>
<td>10</td>
<td>88.79</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

4.7.2.2 Body weight

Body weight reduction caused by inoculation of the parasites was observed in all mice in the aqueous extracts of both *D. chamaethamnus* and *G. coleosperma* on day 4 (Figure 40). However, in GCAQ200 (1.9 %) no decrease in weight was observed. On day 7 an increase in weight was observed for DCAQ200 (1.0 %), GCAQ200 (5.6 %), GCAQ400 (1.0 %) and GCAQ800 (2.6 %) (Table 19). However, these changes were not significant compared to the controls (vehicle and pyrimethamine) (P > 0.05). Also changes in weights showed no significant differences between doses of extracts administered and between the *D. chamaethamnus* and *G. coleosperma* extracts, for both Day 4 and Day 7 (P > 0.05).
Figure 40: Prophylactic effects of aqueous extracts of *D. chamaethamnus* and *G. coleosperma* on weight in mice infected with *Plasmodium berghei* on Day 4 and 7 compared to vehicle (control) and pyrimethamine (standard). Results between treatment groups were not significant by P > 0.005 as analyzed by Tukey’s multiple comparisons test.

Table 19: Weight changes in *D. chamaethamnus* and *G. coleosperma* treated malaria infected mice over 7 days compared to vehicle (control) and pyrimethamine (standard).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (g)</th>
<th>Difference in weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>18.8</td>
<td>17.6</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>20.2</td>
<td>20.2</td>
</tr>
<tr>
<td>DCAQ200</td>
<td>20</td>
<td>19.8</td>
</tr>
<tr>
<td>DCAQ400</td>
<td>19.8</td>
<td>17.8</td>
</tr>
<tr>
<td>DCAQ800</td>
<td>20.6</td>
<td>17</td>
</tr>
<tr>
<td>GCAQ200</td>
<td>20.4</td>
<td>20.8</td>
</tr>
<tr>
<td>GCAQ400</td>
<td>19</td>
<td>18.2</td>
</tr>
<tr>
<td>GCAQ800</td>
<td>22.4</td>
<td>21.6</td>
</tr>
</tbody>
</table>
4.7.2.3 Survival time

Comparison of the survival time of the mice in the experimental groups with the control group (vehicle) was performed (Figure 41) and was statistically significant (P < 0.05). The results indicated that the mice treated with all doses of the aqueous extracts for both *D. chamaethamnus* and *G. coleosperma*, lived longer than those in the control group (vehicle), although no dose-dependent effect was observed. The MST for the infected mice is shown in Table 20. The survival time increased in the mice treated with the maximum dose of the aqueous extract of both *G. coleosperma* and *D. chamaethamnus*. However, the differences in median survival time of the mice in these groups were comparable (P > 0.05). The longest survival times of 23 and 11 days were achieved at the highest dose (800 mgkg⁻¹) for DCAQ and GCAQ, respectively. The MST of 17 days was recorded for the groups that received 200 and 400 mgkg⁻¹ of DCAQ while 10 days were recorded for both groups that received 200 and 400 mgkg⁻¹ of GCAQ, respectively; whereas the MST for the control group (vehicle) was 9 days and for the group that received pyrimethamine the MST of the mice was 13 days. Furthermore, survival time of the mice treated with *D. chamaethamnus* aqueous extracts were longer than that observed in the groups treated with *G. coleosperma* aqueous extracts, and were also longer than those for the pyrimethamine treated mice (P > 0.05).
Figure 41: A Kaplan-Meier Plot showing the survival time (ST) for mice treated with different doses (200, 400, 800 mg/kg) of aqueous extracts (AQ) of *D. chamaethamnus* (DC) and *G. coleosperma* (GC) administered once daily P.O. for four consecutive days. Results between test and control groups were significant by $P < 0.005$ as analyzed by Log-rank (Mantel-Cox) test.
Table 20: Median survival time (MST) for infected mice subjected to different doses of aqueous extracts of *D. chamaethamnus* and *G. coleosperma*.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Extract</th>
<th>Dose (mg kg⁻¹)</th>
<th>MST (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chamaethamnus</em></td>
<td>Aqueous</td>
<td>200</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>23</td>
</tr>
<tr>
<td><em>G. coleosperma</em></td>
<td>Aqueous</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>11</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td></td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>

*Tabulated using GraphPad Prism 6 (Survival analyses).*
4.8 Acute oral toxicity evaluation

4.8.1 Clinical observations

The safety of the plant extracts was further ascertained in mice. Clinical signs of toxicity such as rough hair coat (hair erection), diarrhoea, vomiting, foaming in the mouth, incoordination of gait, difficulty in breathing (dyspnoea), unprovoked behavior (i.e. fighting or biting), alertness, restlessness and clonic convulsions (jerking), lethargy or recumbency and coma were used to evaluate the safety of the plant extracts in healthy female mice. Observations 24 hours post PO administration and throughout the 14-day study period revealed that the doses of aqueous and organic extracts of both *D. chamaethamnus* and *G. coleosperma* in the mice (up to 2000 mg/kg) did not produce significant changes in behavior (Table 21). The number of mice alive at the end of the study period is also shown in Table 21. No deaths were observed in all doses indicating that the medium lethal dose (LD$_{50}$) to be greater than 5000 mgkg$^{-1}$. In addition to clinical observations, gross post mortem examination was performed macroscopically on each mouse at the end of the study. No gross abnormality of the lungs, liver, kidneys, heart and spleen was observed. Abnormalities included hemorrhagic, lesions, enteritis and congestion, and size and colour change in these organs.
Table 21: Acute oral toxicity of the aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma* extracts in healthy female mice.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dose (mg kg⁻¹)</th>
<th>No. of mice</th>
<th>No. of dead mice</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCAQ</td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>DCOR</td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>3</td>
<td>0</td>
<td>None</td>
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<tr>
<td></td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>GCAQ</td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>GCOR</td>
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<td>None</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>3</td>
<td>0</td>
<td>None</td>
</tr>
</tbody>
</table>

* A repeat of dose to a second group of mice when 0 or 1 died in previous group.
4.8.2 Body weight changes

Each mouse was weighed at the start and end of experiment and the change in body weight for each group is shown in Figures 42 and 43; and the mean percent weight change tabulated in Table 22. All the animals from treated groups (300, 300* and 2000, 2000* mgkg\(^{-1}\) of aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma*) did not show any decrease in body weight for the 14 day period; in fact, a significant increase in body weight was observed (P < 0.05). There was however, no consistent difference in weight gain between varying doses within a treatment group. Percentage weight gain was less in the group that had received *G. coleosperma* extracts compared to those of *D. chamaethamnus*. Also, percentage weight gain was less in the group that had received 2000 mgkg\(^{-1}\) dose of extracts compared to those that received 300 mg/kg\(^{-1}\). In GCAQ, weight gain in mice was least for 2000* mg/kg\(^{-1}\) with a 6.1 % increase and greatest for 300 mg/kg\(^{-1}\) with a 19.3 % increase, whilst in GCOR weight gain was least for 2000 mg/kg\(^{-1}\) with a 26.7 % increase and greatest for 300 mg/kg\(^{-1}\) with a 39.1 % increase; in GCOR the least weight increase was observed for 2000 mg/kg\(^{-1}\) (21.7 %) and the greatest was for 300 mg/kg\(^{-1}\) (33.7 %), whereas for DCOR there was inconsistency in weight increase across concentrations. Overall, more than a 20 % weight gain was observed in all mice excluding those for GCAQ.
Figure 42: Weight changes in mice over 14 days for acute oral toxicity analysis following administration of aqueous (AQ) and organic (OR) extracts of *D. chamaethamnus* (DC) at fixed doses of 300 and 2000 mg/kg. Values for dosed groups between D0 and D14 are significantly different (P < 0.05). *A repeat of dose to a second group of mice when 0 or 1 died in previous group.
Figure 43: Weight changes in mice over 14 days for acute oral toxicity analysis following administration of aqueous (AQ) and organic (OR) extracts of *G. coleosperma* (GC) at fixed doses of 300 and 2000 mg/kg\(^{-1}\). Values for dosed groups between D0 and D14 are significantly different (P < 0.05). *A repeat of dose to a second group of mice when 0 or 1 died in previous group.*
Table 22: Summary of weight changes in mice treated with aqueous and organic extracts of *D. chamaethammus* and *G. coleosperma* at varying doses (300 and 2000 mgkg$^{-1}$) over 14 days (D$_{0}$-D$_{14}$) for the acute oral toxicity test.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dose (mgkg$^{-1}$)</th>
<th>Weight (g)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D$_{0}$</td>
<td>D$_{14}$</td>
</tr>
<tr>
<td>DCAQ</td>
<td>300</td>
<td>19.3±0.67</td>
<td>25±0.00</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>18.3±0.88</td>
<td>25.5±0.41</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>19.7±0.33</td>
<td>24.7±0.67</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>20±1.15</td>
<td>25.3±0.88</td>
</tr>
<tr>
<td>DCOR</td>
<td>300</td>
<td>17±0.58</td>
<td>22.7±1.45</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>20.7±0.67</td>
<td>26.3±0.33</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>19.3±0.67</td>
<td>24.7±0.67</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>18.7±0.33</td>
<td>25±1.15</td>
</tr>
<tr>
<td>GCAQ</td>
<td>300</td>
<td>19±0.58</td>
<td>22.7±0.67</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>19.7±0.88</td>
<td>21.7±0.88</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>22±1.15</td>
<td>24.7±0.88</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>21.7±0.88</td>
<td>23±1.15</td>
</tr>
<tr>
<td>GCOR</td>
<td>300</td>
<td>18.3±0.33</td>
<td>24.5±2.04</td>
</tr>
<tr>
<td></td>
<td>300*</td>
<td>18.3±0.67</td>
<td>24±1.15</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>20±0.58</td>
<td>24.3±1.45</td>
</tr>
<tr>
<td></td>
<td>2000*</td>
<td>19.7±0.67</td>
<td>24.7±2.40</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM, n=3
CHAPTER 5: DISCUSSION

5.1 Effect of solvent on yield of plant extracts

The extraction process is key in the analysis of medicinal plants (Gu et al., 2015), thus a solvent or solvent system which is able to obtain extracts with a high yield and that produces insignificant changes to the biological activities of the extract, is necessary (Quispe-Condori, Foglio, Rosa, & Meireles, 2008). The mean yield of the dry extracts obtained with the different solvents in this study shows that both dichloromethane-methanol and water were quantitatively good solvents for extraction in the two plants. This also demonstrates the importance of hot water extraction, as it closely mimics the traditional method of preparation. The efficiency of methanol and hot water in the extraction of phytochemicals has been reported in previous studies (Bushra, 2009; Njume 2011). However, it is important to note that the high yield of plant extracts do not necessarily mean higher biological activity (Anwar et al., 2013).

The yield was comparable between the two plants, however, this does indicate that the same compound quantities, or similar compounds were isolated. Water being a very polar solvent with a polarity index of 9.0, the aqueous extracts is thought to consist of polar compounds and a small number of less polar compounds. The solvent system consisting of methanol, an organic polar solvent with intermediate polarity (5.1) and dichloromethane with the least polarity (3.1) suggests that compounds of intermediate polarity and non-polar compounds were isolated (Seidel, 2006, p. 36).
5.2 Characterization of plant extracts by TLC

The presence of the classes of compounds in both the aqueous and organic plant extracts could be explained by the solubility of the compounds in the respective solvents. Previous studies reported that some terpenes/terpenoids to be soluble in either nonpolar or polar solvents (Colombini & Modugno, 2009, p. 330). The terpenoids produced in the roots of *D. chamaethamnus* and *G. coleosperma* may be non-polar, hence these compounds were solubilized in the organic solvent system (dichloromethane-methanol 1/1 v/v), and were therefore present only in the organic extracts of both *G. coleosperma* and *D. chamaethamnus*.

Alkaloids, anthraquinones, flavonoids and steroids were detected in both aqueous and organic extracts of the two plants; whereas, coumarins were identified only in the extracts of *G. coleosperma*. The presence of the classes of compounds in both the aqueous and organic plant extracts could be explained by the solubility of the compounds in the respective solvents. Compounds such as alkaloids and flavonoids are soluble in solvents of intermediate polarity, while more polar solvents isolate more polar compounds such as flavonoid glycosides and some alkaloids, and water-soluble plant constituents including quaternary alkaloids (Seidel, 2006, p. 36). Some coumarins are not very soluble in water (Lacy & O’Kennedy, 2004), but are solubilized to some extent, and are completely solubilized by non-polar solvents. Sterols and alkaloids are also soluble in less polar solvents. Anthraquinones in its free form are soluble in apolar
solvents (Ibrahim, Mdau, Ahmed, & Ilyas, 2010), while anthracene glycosides are soluble in polar solvents (Houghton & Raman, 2012).

The reported classes of compounds in this study are supported by similar findings from studies described in the literature. Flavonoid glycosides and flavans were reported in the bark extracts of *G. coleosperma* (Bekker, Bekker, & Brandt, 2006), whereas flavonoids, alkaloids, anthraquinones and coumarins of methanolic leaf extracts and anthraquinones from methanolic root extracts were reported (Nafuka, 2014). In *D. chamaethamnus* not much has been reported except for the isolation of naphthoquinones (Costa, Alves, Seabra, & Andrade, 1998) which have been shown to exhibit significant activity against *P. falciparum* (Kapadica, Azuine, Balasubramanian, & Sridhar, 2001; Ehrhardt *et al.*, 2013). There are also reports on the phytochemical composition of other *Diospyros* species. Earlier studies have reported the presence of anthraquinones, terpenoids, alkaloids and flavonoids in the root extracts of *D. lycioides* (Nyambe, 2014); anthraquinones, steroids, alkaloids (Ebbo, Mammam, Suleiman, Ahmed, & Bello, 2014), flavonoids and terpenoids (Nafuka, 2014) in the root extracts of *D. mespiliformis*; and anthraquinones, terpenoids and steroids in the extracts of *D. lotus* (Uddin, Rauf, Siddiqui, & Shah, 2011). Chemotaxonomy, the classification of plants exhibiting similar phytochemical properties, shows a strong correlation with plant genus’s and families. The same genus or species have been shown to produce similar phytoconstituents (Bernhoft, 2010).
Furthermore, a correlation between the biological activity of a plant and its phytoconstituents was shown (Crozier, Jaganath, & Clifford, 2006). The reported antiplasmodial activities of plants in the literature has been linked to the presence of alkaloids (Chea et al., 2007), terpenoids (Amoa Onguéné et al., 2013), steroids (Pabón, Carmona, Maestre, Camargo, & Blair, 2002), coumarins, anthraquinones and flavonoids (Ntie-Kang et al., 2014). The data therefore supports the claim that D. chamaethamnus and G. coleosperma treat symptoms of malaria. These compounds may work singly or in combination causing parasite growth inhibition or suppression.

5.3 Characterization of plant extracts by GC-MS

5.3.1 Identification of volatile constituents

The dry extracts of the plants were reconstituted in methanol for GC-MS analyses using an apolar column. The phytoconstituents in the aqueous extracts of the roots D. chamaethamnus and G. coleosperma were expected to be highly polar and mostly non-volatile showing fewer peaks in the chromatograms. However, the aqueous extracts were prepared at elevated temperatures (60 °C), and therefore will also contain some non-polar (hence volatile) compounds due to improved solubility of apolar compounds in water at such temperatures. Furthermore, it was not possible to completely resuspend the dried aqueous extract of G. coleosperma in a suitable solvent for GC-MS analysis, presumably due to the high polarity of the extracted compounds, and as a result only
seven compounds were identified, whilst in its organic extract which was highly soluble in the solvent, more compounds were identified.

The compounds were identified using a number of diagnostic tools. Most compounds were tentatively identified by comparison of their experimentally determined mass spectra and RIs with those in the NIST databases. Alkanes in the extracts were confirmed with the retention times of authentic reference compounds. The interpretation of the mass spectra of some of the compounds using diagnostic ions was also performed (Appendix C). Compounds 17-20 in the organic extract of *D. chamaethamnus* were identified as triterpenoids, as well as compound 18 in the aqueous extract. The most important feature in the mass spectra of the unidentified triterpenoids was the base peak at m/z 189 followed by peaks at m/z at 203/204, which is characteristic of the mass spectra of 18-oleanenes (triterpenoids) (Assimopoulou & Papageorgiou, 2005). The unidentified triterpenoid for the aqueous extract (compound 18) also show intense fragment ion peaks at m/z 204, 175 and 137, which are typical for simiarenol (a triterpenoid) (Hemmers, Gulz, & Marner, 1988). The mass spectra of the unidentified triterpenoids of *G. coleosperma*, compounds 7 and 17 in the aqueous and organic extracts, respectively displayed prominent peaks at m/z 218, 204 and 189. Such ions are consistent with those commonly found in the mass spectra of triterpenoids including those for lupenone, α-amyrinone, β-amyrinone, α-amyrin and lupeol (Hemmers *et al.*, 1988). The RI data of the triterpenoids suggested by the MS database however, did not correspond with the experimentally determined RIs of these compounds.
Compounds 17 and 13 from aqueous and organic extracts of *D. chamaethamnus*, respectively, and compounds 6 and 16 from the aqueous and organic extracts of *G. coleosperma*, respectively were identified based only on the fragmentation patterns observed in their mass spectra. Their MS data showed fragmentation patterns typical of steroids, with a prominent ion at m/z 329 (base peak) and accompanying ions at m/z 213, 255, 303, 381 and 414 similar to that of β-sitosterol (Bataglion et al., 2015). In addition, two compounds (13 in DCAQ and 16 in DCOR) displayed MS fragmentation patterns very similar to those of cholest-4-en-3-one and lanosterol with peaks at m/z 124 and 43, and 255 and 215, respectively (Louw, Burger, Le Roux, & Van Wyk, 2011) suggesting that these two compounds can be steroids, however, the identity of these phytoconstituents need to be confirmed. The fragmentation patterns of compounds 10 and 12 in the aqueous extract of *D. chamaethamnus* corresponded with that of an octadecanoic acid ester (Duraisamy Gomathi, Kalaiselvi, Ganesan, Devaki, & Uma, 2013) with prominent ions at m/z 267/269, 197/199, 129 and 55/57.

In the mass spectra of compounds 11 (DCAQ), 3 (GCAQ), and 2 (GCOR) prominent fragment ion peaks were observed at m/z 180/181, 76/77, and 51 corresponding to the mass spectra of 4-propyl-phenol (Wheeler, Heim, LoTorre, & Janes, 1997) and 4-((1E)-Hydroxy-1-propenyl)-2-methoxyphenol (Gopalakrishna & Vadivel, 2011), both phenolic compounds. Compound 5 (GCAQ), on the other hand had a mass spectrum that resembles that of 2-biphenylol, also a phenolic compound with prominent ions at m/z 242 (base peak), 225/227, 141/145 and 196 (Zhong et al., 2011). Compounds 11
(DCAQ), 3 (GCAQ), 2 (GCOR) and 5 (GCAQ) were therefore tentatively identified as phenolic compounds. Constituents 8, 10, 11, 12, 13, 15 in the organic extract of G. coleosperma, however, could not be identified by comparison of their mass spectra with those of known compounds in the MS library, nor could they be identified by interpretation of their mass spectra.

5.3.2 Reported biological activities
The extracts, both aqueous and organic, of D. chamaethamnus and G. coleosperma displayed similar chemical profiles. A reason for this could be because the two plants are found in the same geographical location (Figueiredo et al., 2008). The most common volatile and or semi-volatile phytoconstituents found in the plant extracts were alkanes, fatty acids, terpenoids, phenolic compounds and steroids with a trace of flavonoids. The presence of these phytochemicals in D. chamaethamnus and G. coleosperma have not been reported before, and correlate well with the TLC findings which also indicated the presence of terpenoids, flavonoids (phenols), anthraquinones (phenols) and steroids in the roots of these plants.

Alkanes are known to occur in cuticular waxes and functions in the rigidity of plants to prevent water loss (Luckner, 2013, p. 156). According to Leray (2012, pp. 3–4) long-chained hydrocarbons (C18-C36) are components of paraffin found in fossil fuels. Paraffin oil and waxes are often times used externally, while the light fraction of gasoline has been reported in the degreasing of wounds. Alkanes with shorter chains
(C8-16) such as dodecane and pentadecane, on the other hand are at times found in perfumes and flavors of plants. As a result, not much has been reported on the biological activities of these compounds, with a few exceptions. Antifungal and antibacterial activities have been reported for tetradecane, a cyclic alkane (Ozdemir, Karabay, Dalay, & Pazarbasi, 2004); hexadecane with the inclusion of antioxidant activities (Yogeswari, Ramalakshmi, Neelavathy, & Muthumary, 2012); as well as for eicosane (Hsouna et al., 2011). Dodecane, on the other hand has been shown to enhance antifungal activity (Stopiglia et al., 2012).

Terpenoids including α-amyrin and squalene were found in the extracts of the two plants. α-Amyrin possess antioxidant and analgesic properties (Bhalla, Gupta, & Bhargava, 1971; Chen et al., 2002, p. 2), as well as antiplasmodial activities (Hrckova & Velebny, 2012, p. 15). Previous studies have also shown α-amyrin as a growth inhibitor of some microbes. Squalene has also been implicated in antiplasmodial (Banzouzi et al., 2015) and antioxidant activities (Huang, Lin, & Fang, 2009). Vitamin E has been shown to be active against potentially pathogenic microorganisms (Kumar, Kumaravel, & Lalitha, 2010). In addition, triterpenoids were identified in the extracts. This may indicate peroxidation as a possible mode of action. Sesquiterpenoids such as artemisinin and its derivatives act by generating carbon centered free radicals and other reactive oxygen species (ROS) during alkylation of the haem protein by the endoperoxide bridge of compounds resulting in damage to parasite DNA and eventual death of the parasites (Gopalakrishnan & Kumar, 2015).
Stigmasterol, one of the most common phytosterols, have been shown to exert antimicrobial (antibacterial and –fungal) (Ritthiwigrom, Laphookhieo, & Pyne, 2013), anti-inflammatory (Yinusa, George, Shuaibu, & Ayo, 2014), and antiplasmodial activities (Banzouzi et al., 2015), as well as analgesic properties (Githinji, Mbugua, Kanui, & Kariuki, 2012). Campesterol, another phytosterol, also reported to have analgesic activity (Hemalatha, Sumalata, Sunandan, & Muvvla, 2013). Campesterol and stigmasterol, both scavenge oxidants, lower cholesterol, and possess anti-inflammatory, antibacterial, antifungal, anticarcinogenic properties (Choi et al., 2007; Sumit, Surendra, Mruthunjaya, Sachin, & Manjunath, 2013).

Fatty acids are present in all plant cells and together with lipids they act as membrane compounds, storage products, metabolites and a source of energy (Wada, Gombos, & Murata, 1994). A number of fatty acids, however, are well-known for their antibacterial and antifungal properties (Li et al., 2004), including long-chain unsaturated fatty acids such as oleic acids (McGaw, Jäger, & van Staden, 2002). Oleic acids are found to be active against *Mycobacterium aurum* and *M. phlei* (Agoramoorthy, Chandrasekaran, Venkatesalu, & Hsu, 2007). These fatty acids most likely are involved in the inhibition of FabI, an important drug target for antibiotics (Zheng et al., 2005). What’s more, these compounds, have recently been shown to possess activity against the *Plasmodium* parasites (Carballeira, 2008) and anti-inflammatory activity (Li et al., 2004). 9,12-Octadecanoic acid also possesses anti-inflammatory activity (Maruthupandian & Mohan,
Octadecanoic acid, on the other hand displayed inhibitory activity against bacterial and fungal infections (Abou-Elela, Abd-Elnaby, Ibrahim, & Okbah, 2009) and antimalarial activity (Zito et al., 2010).

Hexadecanoic acid, the most common fatty acid, and which is present in both aqueous extracts of *D. chamaethamnus* and *G. coleosperma* is said to have antifungal, antioxidant, antimalarial, antimicrobial activities (Hema, Kumaravel, & Alagusundaram, 2011; Hsouna et al., 2011). What’s more, fatty acid esters have been shown to also possess a number of biological activities as well. Octadecanoic acid esters have been shown to exhibit antifungal, antimicrobial, antibacterial, hypocholesterolemic (Abou-Elela et al., 2009), and antioxidant activities (Syeda, Habib-ur-Rahman, Choudahry, & Atta-ur-Rahman, 2011); and hexadecanoic acid esters exhibited some antiplasmodial activities with IC$_{50}$ greater than 100 (Banzouzi et al., 2015), anti-inflammatory, antimicrobial, anti-cholesterolemic, hepatoprotective, antihistimic properties (Kumar et al., 2010). Previous studies have shown specifically that hexadecanoic acid methyl ester or methyl hexadecanoate exert antifungal, antioxidant, hypocholesterolemic, nematicidal, insecticidal, haemolytic and potent antimicrobial activities (Hema et al., 2011). The ethyl ester hexadecanoic acid also exhibit hypocholesterolemic, antioxidant, nematicidal activities among others (Jargalsaikhan et al., 2013), as well as antimicrobial activities (Kumar et al., 2010).
Phenolic compounds or phenols are also known to demonstrate a variety of pharmacological activities. According to Bruins et al. (2006) these phytoconstituents display antimicrobial, anti-inflammatory, antiviral, antidiarrhoeal, immune modulatory activities; and are of interest to scientists because of their antioxidants activities. Phenols have recently also been shown to exhibit toxicity towards *Plasmodia* parasites (Ramanandraibe et al., 2008). 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, also known as pyranone, is a flavonoid and has been found to actively inhibit the growth of microorganisms and to mitigate against inflammatory responses (Gopalakrishnan & Kalaivarasi, 2013). 1,3,5-Benzentriol or phloroglucinol, a polyphenol, has been shown to exert antimicrobial, antiviral and antioxidant activities (Mammino, Gurib-Fakim, & Eloff, 2013, p. 97). *In vitro* activity of phloroglucinol derivatives against protozoal infections including schistosomiasis (Magalhães et al., 2010) and malaria (Moon, 2010) has been reported.

Immunosuppression is common in patients with severe malaria. The patients, as a result are often at risk of secondary bacterial infections such as septicemia urinary tract infections, meningitis or bacterial pneumonia, which often times leads to shock and multi-organ failure. Fever blisters caused by the Herpes simplex virus, are also an accompaniment of the disease (Berkley, Mwarumba, Bramham, Lowe, & Marsh, 1999). It is probable that the reported antibacterial activity of some of the compounds present in the aqueous and organic extracts of the plants under investigation may act against some of the organisms causing these infections, whilst compounds implicated in
antiplasmodial activities inhibit the growth of the *Plasmodia* parasites. This is a classic example of synergism in plant extracts. Thus, antimicrobial and anti-inflammatory compounds can be of therapeutic value for prevention and treatment of secondary infections in malaria infected persons.

5.4 Antioxidant activity of the plant extracts

Radical scavenging activities observed for both *D. chamaethamnus* and *G. coleosperma* root extracts may be attributed to flavonoids and anthraquinones (both phenolic compounds) present in the aqueous and organic extracts of the two plants (Scalbert, Johnson, & Saltmarsh, 2005). The aqueous extract of *D. chamaethamnus* exhibited higher antioxidant activity than its organic extract, while the organic extract of *G. coleosperma* exhibited higher antioxidant activity than its aqueous extract. The latter may not be a true reflection of the antioxidant activity, because the dry aqueous extract of *G. coleosperma* when reconstituted in methanol did not dissolve completely due to its highly polar compounds.

The differential DPPH scavenging activities between the organic and aqueous extracts can be attributed to the presence of different phenolic compounds in their extracts, as well as the presence of these compounds in varying quantities. According to Brahmi *et al.* (2012), methanol is a suitable solvent for extraction of phenolic compounds. However, in a different study, hot water extraction was more successful at extracting
phenols; maximum radical scavenging activity was reported for extracts prepared with hot water (Goldsmith, Vuong, Stathopoulos, Roach, & Scarlett, 2014).

Concentration dependent antioxidant activity was observed for the aqueous and organic extracts. At higher concentrations the effects were quantitatively similar. This can be explained using the saturation factor model proposed by Salisbury & Ross (1992). This model suggests that as the concentration of the extract (independent factor) increases, the activity also increases (dependent factor), until saturation has been achieved. Thereafter, as the concentration (stimulus) continues to increase, the activity (response) remains constant. The activity of the plant extracts may also decrease when a certain concentration has been obtained at which it becomes inhibitory.

The free radical scavenging activities of *D. chamaethamnus* and *G. coleosperma* have not been reported before. However, reports on the antioxidant properties of related species for *D. chamaethamnus* (Mondal, Chakraborty, Gupta, & Mazumder, 2006; Baravalia, Kaneria, Vaghasiya, Parekh, & Chanda, 2009; Loizzo et al., 2009) and *G. coleosperma* (Fernande, Marthe, Laure, & Enyong, 2013) has been documented, and is consistent with the findings of this study further confirming the plants’ antioxidant activities. The ability of the tested plant extracts to scavenge or reduce DPPH radicals suggests that they may exert antiplasmodial activities through scavenging of ROS that is involved in the pathogenesis of malaria (Gopalakrishnan & Kumar, 2015), and as a result prevent progression of the disease (Da et al., 2014). The antioxidant effects of the
extracts of these plants may therefore, represent a possible mechanism contributing to their antiplasmodial activity.

5.5 Determination of *in vitro* and *in vivo* antimalarial activity

Aqueous and organic extracts of *D. chamaethamnus* and *G. coleosperma* were screened for antimalarial activities using *in vitro* and *in vivo* malaria models. Crude plant extracts were used because of the heterogeneous complex mixture of compounds that exists within a plant and because herbal remedies are commonly used in the traditional setting. Plant metabolites interact either in an additive or synergistic manner, contributing to the efficacy of these remedies. Increased activity (Cseke, Setzer, Vogler, Kirakosyan, & Kaufman, 2006, p. 264), as well as delaying the onset of resistance in pathogenic organisms (Kaufman, Cseke, Warber, Duke, & Brielmann, 1999; Wynn & Fougere, 2007) has been associated with these types of interactions.

Antiplasmodial activity of *D. chamaethamnus* and *G. coleosperma* against the *Plasmodia* parasites (*P. falciparum* and *P. berghei*) was evaluated by determining mean parasitaemia and percentage growth inhibition of parasites, as it is the most reliable measure for determining antimalarial activity (Nafiu, Abdulsalam, & Akanji, 2013). A small animal model of malaria was used for *in vivo* antimalarial activity, even though primate models have been shown to provide a better prediction of efficacy in humans. However, the former have been validated through the use of several conventional antimalarials, such as chloroquine, halofantrine, mefloquine, and more recently
artemisinin derivatives (Nafiu et al., 2013). Since *P. berghei* is sensitive to chloroquine, it was employed as the standard drug; its suppression activity of 99.37% in this study is in agreement with a previous study by Birhanu et al. (2015) which exhibited 100% suppression against the malaria parasites. Pyrimethamine was also used in this study because of its clinical use as a prophylactic (Root, 1999).

A reduction in the number of parasitized erythrocytes was observed for the aqueous and organic extracts of both plants *in vitro* and *in vivo* compared to the negative control (*i.e.* no treatment), thus indicating a suppression of the growth of the *Plasmodium* parasites. The antiplasmodial activity of the plant extracts may be attributed to presence of phytochemicals with antimalarial properties such as those tested for in this study including alkaloids, terpenoids, flavonoids, coumarins, anthraquinones, fatty acids, alkanes and steroids.

Antiplasmodial activities of plant extracts, both *in vitro* and *in vivo* were independent of concentration. In the mice, this may be explained by possible immunosuppressive activities of phytochemicals like saponins, tannins, and phenols that possesses the ability to suppress cellular immunity (Sankari, Chitra, Jubilee, Silambu, & Raju, 2010). Thereby with increased doses, the extracts may have exacerbated the infection resulting in increased parasite load or similar parasite loads within treatments for different concentrations. Whereas in the cell based model of malaria, the concentration independent effect exerted by the plant extracts may be as a result of saturation of
receptors or drug targets. Many pharmaceuticals produce therapeutic effects by binding to the receptors either causing agonist or antagonist effects, and at high concentrations the response reaches a maximum due to saturation of available receptors by a drug (Harro, 2004, p. 662). In addition, the observed antimalarial activities of the extracts at the highest doses or concentrations were not significantly high, in other words the maximal response was not produced. This may be as a result of compounds known as partial agonists. Normally a drug produces a maximal response when all of the receptors are occupied, and the half-maximal response is produced when 50% of the receptors are occupied. However, in this scenario it may be that the compounds present in the extracts are partial agonists. Regardless of maximal occupancy of receptors by these compounds, only a partial response will be elicited (Harro, 2004, p. 666).

Low suppressive/schizonticidal activities with *P. berghei* in mice were observed for both *G. coleosperma* and *D. chamaethamnus*. However, the aqueous extract of *D. chamaethamnus* at 200 mg kg\(^{-1}\) (*i.e.* the lowest dose), exhibited good schizonticidal activity (78.44%). This may be because the dosing of the plant extracts may have not been optimized. Perhaps more frequent exposure to plant extracts or administration of lower doses would have made them more active against the malaria parasites. However, the increased survival time of mice treated with the aqueous and organic extract of the test plants provided evidence that the plants as possible therapeutic options for malaria cannot be totally discounted.
The plants lacked the ability to fully suppress the growth of the erythrocytic stages of the parasites, but their valuable role could be in their antipyretic and analgesic effects (Wanyoike, Chhabra, Lang’at-Thoruwa, & Omar, 2004) as demonstrated by their traditional use and by the presence of antioxidants and compounds with immunoregulatory properties in their extracts. Furthermore, in the traditional setting, the plants are used in concoctions made up of different plant species as reported in this study. *D. chamaethamnus* also known as *Mundjongolo* in SiLozi is used in combination with *Situnduwanga, Kabubo, Mutjokela, Mukungu, Mushakashela* and *Mufula*. *G. coleosperma* (*Muzauli*) is also used in combination with other plants namely, *Mukwenkwebo* and *Mungongolo*. According to the literature different plants in a polyherbal remedy are responsible for efficacy in such remedies (Tarkang, Franzoi, et al., 2014).

The antiparasitic activities of the *D. chamaethamnus* showed a higher inhibition of growth of *P. falciparum* than *G. coleosperma* extracts. This is consistent with the antioxidant data; and may indicate that the extracts of *D. chamaethamnus* contain greater quantities of active compounds compared to those of *G. coleosperma*, since the TLC and GC-MS data revealed a similar chemical profile for the two plants. Furthermore, the antiplasmodial activities for the organic and aqueous extracts of each plant were comparable, suggesting that the active phytoconstituents responsible for the observed antimalarial effects are present in both the water and methanol-dichloromethane (1:1
v/v) extracts, further substantiating the use of water as an extractant in the traditional setting.

The aqueous root extracts of both *D. chamaethamnus* (59.86 %) and *G. coleosperma* (52.16 %) exhibited good antiplasmodial activity according to Deharo *et al.* (2001) with a 50 % reduction in parasite load at a dose of 200 mgkg$^{-1}$. Similar results were reported in previous studies done by Akuodor, Idris-Usman, *et al.*, (2010) and Alli, Adesokan, Salawu, Akanji, and Tijani (2011), which showed prophylactic activities of 60% (*Verbena hastata*) and 48.5 % (*Acacia nilotica*), against *P. berghei* at a dose of 200 mgkg$^{-1}$. The results further indicated that the mice treated with all doses of the aqueous extracts for both *D. chamaethamnus* and *G. coleosperma*, lived longer than those in the control group (vehicle). Terpenoids in the extracts of these plants may be responsible for this activity. This group of phytochemicals have been reported to be active against a broad range of blood stage parasites, including rings, trophozoites and schizonts (Boampong *et al.*, 2015). The ability of the extracts to kill all the erythrocytic blood forms of the parasites, makes the test plants good candidates as suppressive prophylactics (Castelli, Odolini, Autino, Foca, & Russo, 2010).

Weight loss is associated with malaria pathology (Langhorne, Quin, & Sanni, 2002). Therefore, analysis of the changes in body weight was done as a parameter to determine or confirm antimalarial activity of plant extracts. For the suppressive activities of *G.*
coleosperma and four days post infection, no weight loss in the mice in all groups (vehicle, chloroquine and treated) were observed, indicating a lack of malaria pathology. The parasite infestation of the RBCs in the mice may not have progressed to such an extent to cause pathology. On day 6 weight loss was observed for treatment groups and control groups (vehicle and chloroquine), and on both day 4 and 6 D. chamaethamnus treated groups experienced weight loss suggesting that chloroquine and test plant extracts were not efficient at preventing weight loss in malaria infected mice. The plant extracts and controls in the prophylactic test also did not prevent weight loss. As a result, it can be concluded that weight was not a good indicator of the antimalarial activity of plant extracts in this study. There were also no detectable differences in weight changes between the aqueous and organic extracts. This was in correlation with parasitaemia levels in the mice and the in vitro data.

In vitro and in vivo antiplasmodial activities of D. chamaethamnus and G. coleosperma have not been reported before. On the other hand, in vitro activity for related plant species has been reported. According to Prachayasittikul et al. (2010), antiplasmodial activities (IC\textsubscript{50}) of 176.20 µg/mL, 23.95 µg/mL, 33.58 µg/mL and 135.05 µg/mL were exhibited by hexane, dichloromethane, ethyl acetate and methanol extracts, respectively of D. rubra. D. mespiliformis displayed an IC\textsubscript{50} of 2.91 µg/mL for its aqueous extract (Nafuka, 2014). For D. quaesita, betulinic acid was isolated and tested against P. falciparum D6 and W2 strains, which exhibited IC\textsubscript{50} values of 0.9 and 0.6 µg/mL,
respectively. For *Guibourtia* species, a minimum inhibitory concentration of 2.4 µg/mL (Tantchou & Jensen, 1986) was reported for *G. tessmannii*.

### 5.6 Cytotoxicity evaluation

The concern with traditional herbal remedies is the lack of evidence to show they are safe (Jayasinghe, Udagama-Randeniya, & Ratnasooriya, 2008). This was addressed in the present study by determining the cytotoxicity and acute oral toxicity effects of the plants under investigation. *In vitro* toxicity of the plant extracts was determined against normal cells using the human fetal lung fibroblast W138 cell line. The aqueous root extract of *D. chamaethamnus* and both the aqueous and organic root extracts of *G. coleosperma* had no effect on the cell viability (CC\(_{50}\) > 100), whereas the organic extract of *D. chamaethamnus*, displayed a visible reduction in the viability of the fibroblast cells (CC\(_{50}\) = 29.73), as did the standard cytotoxic drug etoposide (CC\(_{50}\) = 5.10 µg/mL). Likhitwitayawuid *et al.* (1993) and Abdel-Hameed *et al.* (2012), however, regard an extract as non-toxic if CC\(_{50}\) values are greater than 20 µg/mL, therefore asserting all plant extracts under investigation as non-toxic.

The aqueous extract of *G. coleosperma* showed a greater reduction in cell viability compared to the organic extract, as did the organic extract of *D. chamaethamnus* compared to its aqueous extract. This corresponds with the *in vitro* antimalarial activities of the plant extracts. These differences in toxicity of the extracts may be as a result of the varying concentrations of certain bioactive compounds in them (Bernhoft, 2010). For
example, compounds such as alkaloids and terpenoids are therapeutic in low doses, however, when consumed in large quantities, they become toxic. Increased concentrations of bioactive compounds means increased levels of toxicity either to the parasite or healthy human cells, or both. In this case, it was both. However, use of a selectivity index can help to explain the therapeutic window in which a plant extract can have curative properties without being toxic.

A good antimalarial drug or herbal antimalarial remedy should show toxicity to the parasite, but not to the host cells (Chu, 2002, p. 387). This selective toxicity of the plant extracts was determined using the therapeutic or SI, which is defined as the ratio of the CC$_{50}$ value on the normal fibroblast cell lines to the IC$_{50}$ value against $P. falciparum$ D10 strain. Low SIs indicate that the antiplasmodial activity is due to general toxicity rather than specifically affecting the growth of the parasites. High SI on the other hand, with 4 as the minimum indicates a strong selective toxicity towards the malaria parasite, thereby offering potential and or safer therapies (Valdés et al., 2010).

The 50% cellular cytotoxic concentration of $G. coleosperma$ organic extract was not a fixed value (CC$_{50} > 100 \, \mu g/mL$), therefore, its SI could not be determined. The plant extracts of $G. coleosperma$ (aqueous and organic) and $D. chamaethamnus$ (aqueous), displayed high selectivity for $P. falciparum$ D10 with SI > 4 (Valdés et al., 2010); in contrast the organic extract of $D. chamaethamnus$ did not display selective toxicity towards the parasites with SI of 1.52. The cytotoxic activities of the plants under investigation have not been previously reported.
5.7 In vivo toxicity evaluation

The loss of body weight is frequently the first indicator of the onset of an adverse effect. A dose which causes 10 % or more reduction in body weights considered to be a toxic one (Jothy et al., 2011). The safety of the plant extracts were ascertained when a 20 % increase in weight was observed. Furthermore, no obvious clinical signs of toxicity were observed in the mice after PO administration of the aqueous and organic root extracts of *D. chamaethamnus* and *G. coleosperma*. These are important symptoms of toxic effects of compounds in animals (CDER, 1996). In addition, no gross abnormality of the lungs, liver, kidneys, heart and spleen was observed, confirming the toxicity status of the plant extracts. Hence, at doses less than or equal to 2000 mgkg$^{-1}$, the extracts were not toxic. This was in correspondence with the data for cytotoxicity, for which the plant extracts showed no toxicity. In vivo toxicity of the *D. chamaethamnus* and *G. coleosperma* have also not been reported before.

5.8 Summary

*D. chamaethamnus* and *G. coleosperma* have been shown to have moderate *in vitro*, low *in vivo* suppressive and good *in vivo* prophylactic antiplasmodial activities. The extracts were also found to be non-toxic in cell-based and small animal models. These findings corroborate the folkloric use of the roots for the treatment of symptoms of malaria in the traditional setting. It also validates the use of water as an extractant in such settings.
The antioxidant activities and presence of phenolic compounds in the plant extracts indicates that the extracts may act by forming some kind of protection against the oxidative damage induced by the parasites in malaria patients. As a result the use of the roots can be suggested for use in prevention of complications in malaria, especially in remote areas until diagnosis is confirmed and treatment is available or accessed at health care facilities. The presence of compounds with reported antibacterial and antifungal properties including stigmasterol, \( \alpha \)-amyrin, 1,3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, tetradecane, dodecane and octadecanoic acid in the root extracts of both plants, are essential in the treatment of opportunistic infections or secondary infections in malaria patients. This further supports the use of these plants as alternative treatment options.

The antiplasmodial activity has been linked to the classes of phytochemicals shown to be present in plant extracts such as alkaloids, terpenoids, steroids, coumarins, anthraquinones and flavonoids. Compounds such as \( \alpha \)-amyrin and hexadecanoic acid found in both *D. chamaethamnus* and *G. coleosperma*; stigmasterol and phloroglucinol in *D. chamaethamnus*; and squalene and octadecanoic acid in *G. coleosperma* have been implicated in this activity and have been linked to either anti/protozoal or antiplasmodial activity. Furthermore, the presence of phenolic compounds in these extracts together with the high antioxidant activities of the plant extracts, suggests that the observed antimalarial activities may be as a result of multiple modes of action including blocking of iron uptake by the parasite, a process necessary for its multiplication and survival. In
addition, these compounds may have prevented the progression or development of the disease in the mice.

5.9 Limitations of the study

Some of the limitations of the study include the use of GC-MS, which only identified the volatile and semi-volatile compounds in the plant extracts. Compounds of a non-volatile nature found in the roots of these plants may also be linked to the antiplasmodial activities of the plants. Another limitation is that of the Acute Toxic Class Method that was used to assess the toxicity of the extracts. This method does not depict the “true” LD50 values, but gives a more or less stringent classification of the toxic effects of the plant extract. In addition, lab strains of *P. falciparum* and *P. berghei* were used, both being chloroquine sensitive strains. Lab strains of the *P. falciparum* parasites have been adapted to grow outside the human body and are different from clinical strains that are found in malaria patients; *P. berghei* strains, on the other hand, do not cause malaria in humans, hence these parasites are used to infect only mice in the study to allow an animal model of malaria to test the activity of the plant extracts. Moreover, animal disease models do not always reflect the *in vivo* situation in humans. Some of the most common side-effects caused by medicines including heartburn and headache are difficult to observe in animal models.
CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

There is a growing recognition and acceptability of the use herbal medicines, although many have not been scientifically validated. This study aimed at validating the medicinal use of two plant species found in Namibia, namely Diospyros chamaethamnus and Guibourtia coleosperma. The results from the study showed that the extracts from the two plants exhibits antiplasmodial activities and are safe, and it reported the presence of known antiplasmodial compounds. Furthermore, this study is the first of its kind to report on the chemical properties, biological activities (antiplasmodial and antioxidant) and toxicity profile of D. chamaethamnus and G. coleosperma.

However, before these plants can be used or incorporated into mainstream health care policies for treatment of malaria, studies on safety in humans are still required. Another requirement before the two can be used as complementary or alternative medicines is the standardization and quality control of these herbal medicines. Lastly, this study has shown that the plant extracts can also be used as a prophylactic. This is new knowledge should be shared with indigenous communities to maximize the medicinal use of these plants.
Further studies should include the:

i. Identification of all known compounds (volatile and non-volatile) using LC-MS;

ii. Bioassay-guided fractionation of the crude plant extracts of *D. chamaethamnus* and *G. coleosperma* to isolate bioactive compounds responsible for antimalarial activity for quality control purposes, and for developing standardized ingredients;

iii. Isolation and structure elucidation of antiplasmodial and novel compounds using NMR;

iv. Optimization of doses to improve treatment efficacy since the plant extracts are non-toxic;

v. Testing of plants against *P. falciparum* resistant strains and multidrug resistant strains;

vi. Testing of individual plants and combination treatments with other plants as they are used in the traditionally;

vii. Testing of individual plants in combination with antimalarial drugs, since these combinations have been shown to achieve enhanced efficacy.
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EASE%20-%20Malaria%20MDG%20Achieved,%20Nov%202015_FINAL.pdf


APPENDICES

APPENDIX A

Survey on Indigenous Medicinal Knowledge in the Zambezi region

Date of Interview: ....................

Village GPS Coordinates: E..................  S..................

Thank you very much for taking time to respond to the questions contained in this research questionnaire. The data collected during this investigation is for academic and scientific purposes only. Your participation in this investigation is of vital importance and due care will be taken to ensure confidentiality is maintained with regard to your personal details. Please answer the questions fully. If you, as a respondent, wish your name to be used in the actual report writing please let me know. (NB: write it here)

Biographical Data of Informant

Type of Informant:

<table>
<thead>
<tr>
<th>Type of Informant</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Herbalist</td>
<td>1</td>
</tr>
<tr>
<td>Traditional doctor/healer</td>
<td>2</td>
</tr>
<tr>
<td>Spiritual healer</td>
<td>3</td>
</tr>
<tr>
<td>Non-practicing Knowledge holder</td>
<td>4</td>
</tr>
</tbody>
</table>

Gender:
Age:
Professional Occupation:
Level of Education:
Main Source of Income:
Number of dependents in your household:
Number of own children:
Constituency:
Name of Village:
Background Information

1. Ask the respondent to give a brief history about him/herself
2. When he/she become a "practitioner"?
3. Was the knowledge learnt and if so from whom?
4. Ask where the mentor is located. How was the learning or skills transfer conducted?
5. If not learnt, was it ancestral call?
6. How was the call experienced?
7. Are you transferring these skills you have to anyone?
8. If, yes, whom and why?

Observations

1. Describe the characteristics of the area in terms of:
   a. Soil type:
   b. Water (type of water source):
   c. Vegetation

2. Describe the loss of vegetation/degradation level, if any

Major diseases and treatment

Disease/ailment
Perceived cause
Symptoms
Human/animals affected
Gender of the affected
Name of plant (local or English)
Plant part used (roots, leaves, etc.)
Voucher specimen number
State of use (dry or fresh)
Mode of preparation (infusion, decoction, etc.)
Route of administration (oral, topical, nasal, etc.)
Time of recovery
Locality of plant (GPS location) and seasons when available
Abundance of plants

* And ask whether the quantity of the medicinal plants has decreased or increased, in the last decade?
* If so, what are the reasons?
* Are there ways through which you conserve these plants?
* How do you harvest the plants?
* Are there any you cultivate at home?
* If you use roots, have you tried using leaves of the same plant? And do they have the same healing effect?

* Scattered, Threatened, Presently safe, Doubtful presence
1. Probe issues of supply: where the plant is obtained and by what methods?
2. Also probe: combinations of plants for treatment. Ask why such a combination?
3. What leads to the identification of certain plants for treatment and not the others?
4. Which plants are the most important for your treatments?
5. Also probe methods of preparation and treatment, why such method(s) are used?

Thank you very much for your co-operation!
APPENDIX B

Figure 1: TLC fingerprints observed of plant extracts under visible light (A) and visible light after spraying with Dragendorff’s reagent (B) for the detection of alkaloids. The TLC solvent system used was methanol-ammonium hydroxide (200:3 v/v) and quinine hydrochloride was used as the standard. A colour change to orange gave an indication of the presence of alkaloids.

**Figure 2**: TLC fingerprints observed of plant extracts under visible light (A) and visible light after spraying with 10 % KOH ethanolic solution (B) for the detection of anthraquinones. The TLC solvent system used was ethyl acetate-methanol-water (100:17:13 v/v/v), and alizarin (B) was used as the standard. A colour change to pink-violet gave an indication of the presence of anthraquinones.

Figure 3: TLC fingerprints observed of plant extracts under visible light (A), and UV 366 nm after spraying with 1% ethanolic solution of aluminum chloride (B) for the detection of flavonoids. The TLC solvent system used was chloroform-methanol (19:1 v/v) and quercetin was used as the standard. Yellow fluorescence is an indication of the presence of flavonoids

Figure 4: TLC fingerprints observed of plant extracts under visible light (A) and visible light after spraying with freshly prepared p-Anisaldehyde solution (B) for the detection of steroids. The TLC solvent system used was chloroform-acetic acid-methanol-water (64:34:12:8 v/v/v/v) and β-Sitosterol was used as the standard. A colour change to green-black is an indication of the presence of steroids.

Figure 5: TLC fingerprints observed of plant extracts under visible light (A) and visible light after spraying with freshly prepared p-Anisaldehyde solution (B) for the detection of terpenoids. The TLC solvent system used was hexane-ethyl acetate (1:1) and β-Sitosterol was used as the standard. A colour change to purple-violet is an indication of the presence of terpenoids.

Figure 6: TLC fingerprints observed of plant extracts under visible light (A), and UV 366 nm after spraying with Benedict's reagent (B) for the detection of coumarins. The TLC solvent system used was 10 % acetic acid and Coumarin was used as the standard. Blue-green fluorescence is an indication of the presence of coumarins

APPENDIX C

Figure 7: Mass spectrum of compound 10, an octadecanoic acid ester in the aqueous extract of *D. chamaethamnus*.

Figure 8: Mass spectrum of compound 11, a steroid in the aqueous extract of *D. chamaethamnus*. 
Figure 9: Mass spectrum of compound 12, an octadecanoic acid ester in the aqueous extract of *D. chamaethamnus*.

Figure 10: Mass spectrum of compound 13, a steroid in the aqueous extract of *D. chamaethamnus*. 
Figure 11: Mass spectrum of compound 17, a steroid in the aqueous extract of *D. chamaethamnus*.

Figure 12: Mass spectrum of compound 13, a steroid in the organic extract of *D. chamaethamnus*.
Figure 13: Mass spectrum of compound 16, a steroid in the organic extract of *D. chamaethamnus*.

Figure 14: Mass spectrum of compound 17, a triterpenoid in the organic extract of *D. chamaethamnus*. 
**Figure 15:** Mass spectrum of compound 18, a triterpenoid in the organic extract of *D. chamaethamnus*.

**Figure 16:** Mass spectrum of compound 19, a triterpenoid in the organic extract of *D. chamaethamnus*. 
Figure 17: Mass spectrum of compound 20, a triterpenoid in the organic extract of *D. chamaethamnus*.

Figure 18: Mass spectrum of compound 6, a steroid in the aqueous extract of *G. coleosperma*.
Figure 19: Mass spectrum of compound 3, a phenolic compound in the aqueous extract of *G. coleosperma*.

Figure 19: Mass spectrum of compound 5, a phenolic compound in the aqueous extract of *G. coleosperma*. 
Figure 20: Mass spectrum of compound 7, a triterpenoid in the aqueous extract of *G. coleosperma.*

Figure 21: Mass spectrum of compound 2, a phenolic compound in the organic extract of *G. coleosperma.*
**Figure 22:** Mass spectrum of compound 16, a steroid in the organic extract of *G. coleosperma*.

**Figure 23:** Mass spectrum of compound 17, a triterpenoid in the organic extract of *G. coleosperma*. 