Antiplasmodial activity of Warburgia salutaris (Bertol. F.)Chiov.(Cannelaceae)

By

Zoxolo Nokulunga Nyaba (211529312)

Submitted in fulfilment of the requirements for the degree of Master of Science (MSc) in the Department of Biochemistry, Faculty of Agriculture, Engineering and Science, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg campus.

Supervisor: Doctor M.B.C Simelane

Co-Supervisor: Doctor P Murambiwa

DECLARATION

I, Zoxolo Nokulunga Nyaba, hereby declare that the dissertation titled "Antiplasmodial activity of Warburgia salutaris (Bertol. F).Chiov. (Cannelaceae)" is a result of my own research and investigation. This work has not been submitted in part or in full for any other degree or at another university.

Student: Ms Z.N Nyaba Signature:_____ Date: 20-12-2017

Supervisor: Dr M.B.C Simelane Signature:  Date: 20-12-2017

Co-Supervisor: Dr P Murambiwa Signature:_____ Date:_____

Acknowledgments

I would like to thank the following:

- The Lord Almighty for His unfailing love, mercy, and grace.
- My supervisor, Dr M.B.C Simelane, for the opportunity.
- Co-Supervisor Dr P Murambiwa for her tireless assistance.
- Prof S Mukaratirwa for the time I spent under his assistance and for allowing me to utilize his lab and equipment.
- The University of KwaZulu-Natal (Pietermaritzburg campus) Chemistry department.
- The department of Human Physiology staff and Biomedical research Unit staff for allowing me to use their facility and equipment in the study.
- The University of KwaZulu-Natal Pietermaritzburg campus Botanical gardens.
- The University of KwaZulu-Natal Research office and NRF for funding this research
- I would also like to thank the following people who variously contributed to my research, making it a success: Prof P Smith (UCT), Prof F.O Shode (CPU), Prof A.R Opoku (UZ) and Dr N N Mhlongo
- My research group colleagues and friends, Miss N.Z Msomi; Mr M Nsibi and Mr S Sibisi
- My amazing grandmother (Dr T.E Mgobhozi) for her prayers, love and support
- My entire family for the love and support
- Mr S.M Mbuli for the love, support and constant motivation.

May the Almighty God shower you all with blessings. This degree would not have been possible without each and every one of you. Thank you very much.

Dedication

I dedicate this work to my lovely mother, Ms N Mgobhozi. You are still my rock. Your love still keeps me going; I am forever blessed to have had you as my mother. May your soul rest in peace.

To my entire family for the encouragement and support you gave me. I am very grateful and deeply humbled. I love you all.

To Mr SM Mbuli, my unexpected blessing, thank you for pushing me, for being my friend and confidant and for the words of wisdom and encouragement when I felt like giving up. May the Lord continue to shower you with blessings.

My grandmother, there is a lot I could not have done without you. This is for all your prayers, love, motivation and support. I love you very much. I pray the Lord continues to bless you abundantly.

Abstract

Malaria is a deadly disease in sub-tropical and tropical regions. Despite extensive development in treatment options, it continues to be a major burden due to development of resistance to medications that are currently available. This has led to a demand for the production of more efficient, alternative antimalarial drugs. Dichloromethane was used to extract the stem-bark of *Warburgia salutaris*, a medical plant, used by Zulu traditional healers to treat malaria and a compound was isolated by column chromatography. Mukaadial Acetate (M. acetate) was identified and characterized by spectroscopic analysis (^{1}H NMR, ^{13}C NMR, IR and MS) and the structure was confirmed by X-ray crystallography. A chloroquine-sensitive *Plasmodium falciparum* strain was used to test *in vitro* antiplasmodial activity. The MTT assay was used to measure the cytotoxicity of *W. salutaris* and Mukaadial Acetate on HEK239 and HEPG2 cell lines. Molecular docking of the compound was conducted using AutoDock Vina (www.vina.scripps.edu). UCSF Chimera (www.cgl.ucsf.edu) was used for structural modifications and LigPlot was used to examine molecular interactions. Antiplasmodial studies (*in vivo*) were carried out using chloroquine-sensitive rodent malaria parasite, *Plasmodium berghei*, in Sprague-Dawley rats. Mukaadial Acetate and the crude extract were administered orally at 0.5; 1.5, 2.5 and 5mg/kg, chloroquine p.o. was used as the reference drug. Determination of percentage parasitemia, haematological parameters, and rat body weights was done throughout the experimental study period.

The crude extract inhibited parasite growth (IC 50 0.01±0.30 µg/ml). The compound, Mukaadial Acetate also exhibited activity (IC 50 0.44±0.10 µg/ml) against the parasite growth. Mukaadial acetate had a cytotoxicity activity of 36.7 ± 0.8 and 119.2 ± 8.8 (µg/ml) on HEK293 and HEPG2 cells respectively.

Docking showed that Mukaadial Acetate binds to the pyrophosphate, purine and ribose binding sites of the PfHGXPRT with an optimum binding conformation and forms steric and hydrophobic interactions as well as hydrogen bonds with the residues inhabiting the respective binding sites. The crude extract and the pure *W. salutaris* compound reduced percentage parasitemia in a dose-dependent manner in comparison to the control. There were no significant differences in the hematological parameters in all the experimental groups in comparison to control group.

It is evident that *W. salutaris* possesses constituents (including Mukaadial Acetate) that

display antiplasmodial properties. The current study scientifically validates the use of *W. salutaris* in traditional medicine.

Keywords: *Plasmodium falciparum*; antimalarial activity; *Warburgia salutaris*; Mukaadial Acetate; molecular docking; PfHGXPRT; *Plasmodium berghei.*

Contribution to knowledge

See appendix 5

A. Poster Presentation

Z.N. Nyaba, P. Smith and M.B.C. Simelane. Antiplasmodial activity of new sesquiterpene isolated from *Warburgia salutaris*. 25th South African Society of Biochemistry and Molecular Biology Congress: East London. Eastern Cape, South Africa. July 10-14, 2016.

B. Paper in preparation

Zoxolo N. Nyaba, Peter Smith, Ndumiso N. Mhlongo, Francis O. Shode, Andy R. Opoku and Mthokozisi B.C. Simelane. 2017. Antiplasmodial activity of drimane sesquiterpene isolated from Warburgia salutaris.

C. Oral Presentation

Antiplasmodial Activity of Drimane Sesquiterpene Isolated from *Warburgia salutaris*. 19th International Conference on Natural Products: London, United Kingdom. June 28-29, 2017. Digital Article Identifier (DAI): urn:dai:10.1999/1307-6892/70831

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List of abbreviations

ACT	Artemisinin combination therapy
ADA	Adenosine deaminase
AK	Adenosine kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ART	Artesunate
ATP	Adenosine triphosphate
CQ	Chloroquine
CQS	Chloroquine-sensitive
DCM	dichloromethane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
EMEM	Eagle's minimum essential medium
ETC	Electron transport chain
HEK293	Human embryonic kidney cells
HEPG2	Liver hepatocellular cells
HGXPT	Hypoxanthine-guanine phosphoribosyltransferase
HIV/AIDS	Human immunodeficiency virus infection and acquired immune deficiency syndrome
HSP	Heat shock protein
IC50	Inhibitory concentration
IMP	Inosine monophosphate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
NT1	Nucleoside transporter 1
PBS	Phosphate buffered saline
*pf*HGXPT	*Plasmodium falciparum* Hypoxanthine-

	guanine phosphoribosyltransferase
PNP	Purine nucleoside phosphorylase
PPM	Parasitic plasma membrane
PVM	Parasitic vacuole membrane
RBCM	Red blood cell membrane
RF	Retardation factor
RNA	Ribonucleic acid
TLC	Thin layer chromatography
UKZN	University of Kwa-Zulu Natal
UV	Ultra-violet
WHO	World health organisation

1. Chapter One

1.1. Introduction to malaria

Malaria is a deadly disease caused by parasitic protozoa of the *Plasmodium* genus. There are five, human malaria causing *Plasmodium* species, i.e. *Plasmodium vivax*; *Plasmodium ovale*; *Plasmodium malariaea*; *Plasmodium knowlesi* and *Plasmodium falciparum* [1]. The species differ in shape and size, pathogenic pattern, geographic distribution and resistance development [2]. *P. vivax* and *P. falciparum* are considered the most lethal [1]. The parasite is transmitted by a bite from a female *Anopheles* mosquito [1].

Malaria is prevalent in tropical and sub-tropical regions. In the year 2015, 3.2 billion people were at risk of malaria worldwide and from these, an estimated 438 000 deaths were reported [4]. Although the World Health Organisation (WHO) has reported a decrease in malaria cases and deaths from 2000-2015 [1], there is still an increasing panic due to the emerging resistance of some *P. falciparum* strains against the most prevalent malaria treatments [3].

A large majority (90%) of malaria caused fatalities in 2015 were found to be in Sub-Saharan Africa [4]. This is due to lack of effective medical treatment for some diseases such as malaria and/or HIV/AIDS, which, although globally distributed, affect Africa more than other areas in the world [5]. Literature indicates that due to this inaccessibility of western medicines in parts of the continent, a large majority of the African population mainly depend on traditional medicine as their primary health care provider [5].

Traditional healing has been practised for centuries. Traditional healers use plants, among other materials, with medicinal properties to treat and control various diseases. Traditional medicines are the source of the two main groups of current antimalarial drugs (artemisinin and quinine derivatives [5]. Scientists have been encouraged to look into indigenous medicinal plants for novel drugs of phytochemical origin as they provide potential pharmaceutical importance [68]. Research on traditional healers and malaria has led to the recognition of *Warburgia salutaris* plant parts as a potential malaria treatment. With

consideration of the extensive use of *W. salutaris* by Zulu traditional healers against malaria, this study was done to determine the antimalarial properties of this plant. [33]

1.2. Structure of dissertation

1. Chapter 1- Consists of a brief introduction and background of the research, centring on the target of the study.
2. Chapter 2- Lays out the background and current knowledge on malaria, including research, which has been done around the subject of this study. It also includes the aim and objectives of this study.
3. Chapter 3- Provides the methodology and some equipment used to collect data
4. Chapter 4- Consists of the results obtained in the study
5. Chapter 5- Consists of the discussion of the research findings, in contrast to literature.
6. Chapter 6- Provides the conclusion of the study based on the results obtained.

2. Chapter two

2.1. Malaria distribution

Malaria parasite survival and distribution mainly depends on factors of the climate. This disease is transmitted in subtropical and tropical areas, where *Anopheles* mosquitoes thrive and where the parasites can complete their mosquito growth cycle [61].

Temperature is very critical, as *Plasmodium falciparum*, which causes severe malaria, cannot complete its growth cycle at temperatures below 20^{o}C, and thus cannot be transmitted [61]. The map below shows an estimation of malaria transmission, worldwide [61].

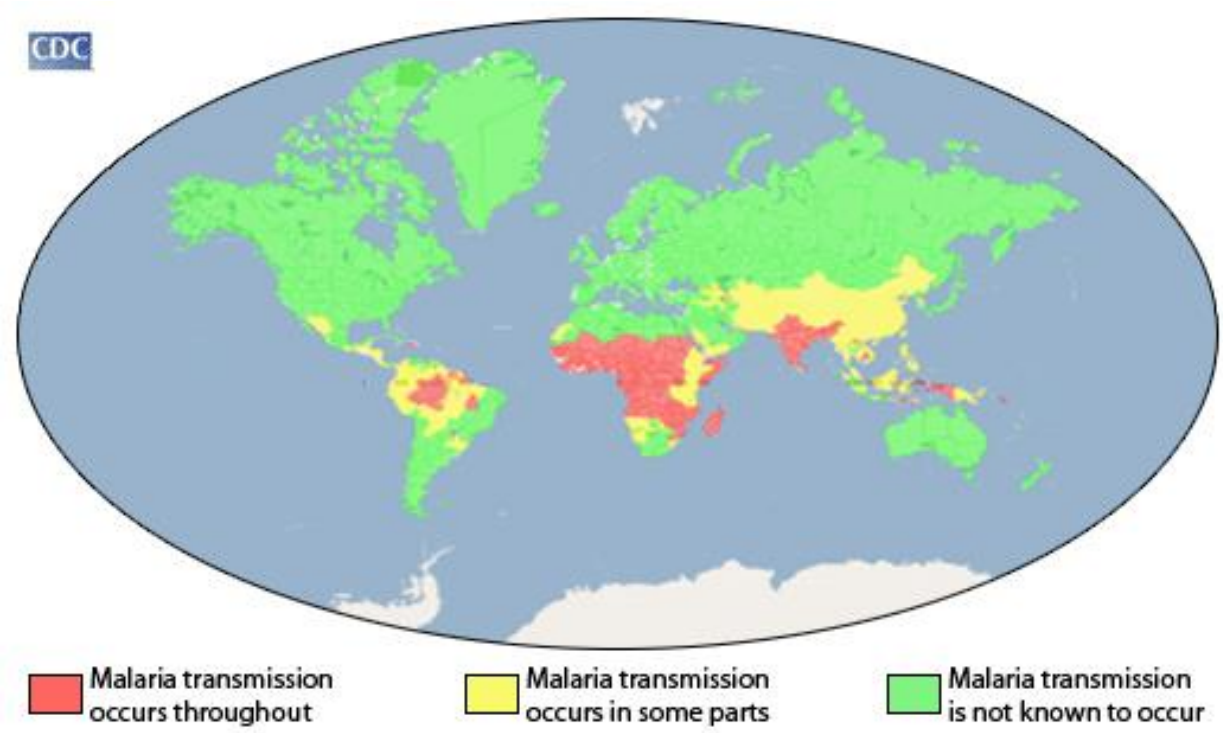


Figure 2.1: Map depicting the distribution of malaria in the world [61].

2.2. Lifecycle of the malaria parasite

Human malaria infection begins with a bite of an infected *Anopheles* mosquito. Where viable sporozoites are released into the bloodstream through saliva and invade liver cells [4], once, inside the hepatocytes, they undergo asexual multiplication to produce merozoites. The merozoites are released into the bloodstream and enter red cells, where they form characteristic rings (ring stage) which grow into mature trophozoites and go through asexual multiplication to form merozoites. The RBCs rupture releasing merozoites, which then invade other RBCs and begin a new cycle. The growth phase of the malaria parasite within the RBCs is 48-72 hours depending on the species, producing periodic fevers with continuous releases of the merozoites [6]. Some parasites develop in the RBCs to become male and female gametocytes (Figure 1). There is increased virulence and complications associated with *P. falciparum* species, which are due to the sequestration of infected erythrocytes in deep tissues [6].

Clinical symptoms of malaria include chills, delirium, fever, metabolic acidosis, multi-organ system failure, prostration and anaemia, cerebral anaemia, followed by coma and death, in pregnancy, symptoms may result in maternal and foetal deaths [7].

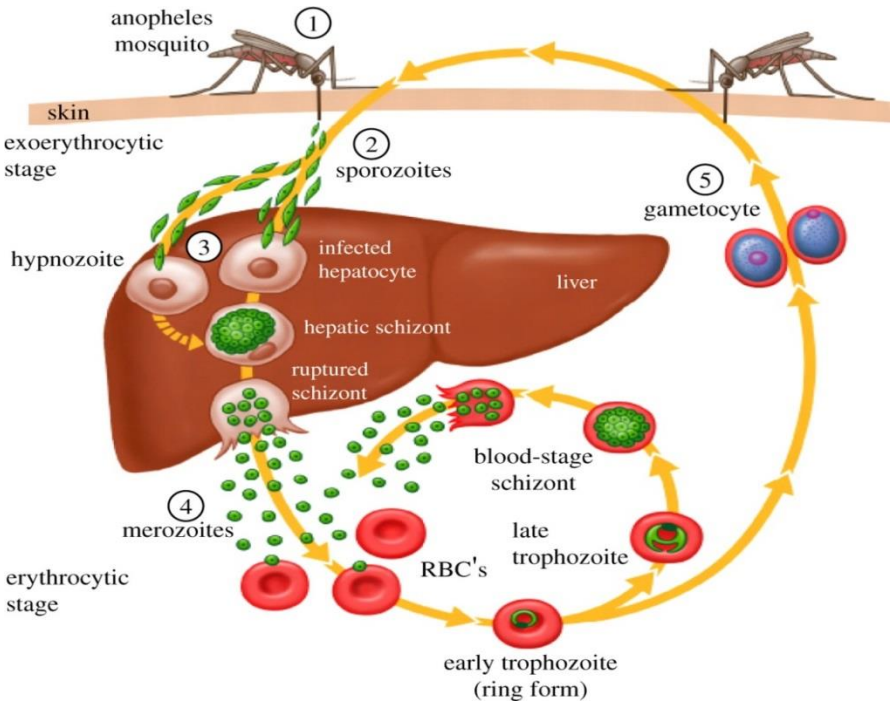


Figure 2.2: *Plasmodium* parasite life cycle [51]

2.3. Pathogenesis of malaria

When entering the human body from the body of a mosquito, there is a drastic change in environmental temperature for the parasite. Under normal circumstances the parasite would be destroyed, however, studies have shown that the *P. falciparum* parasite relies on heat shock proteins to survive this and other challenges the host presents [62]. Heat shock proteins (Hsps) are a class of molecular chaperones as they assist in the folding of other proteins in a cell. Some Hsps are expressed in response to cellular stress so as to facilitate protein folding in unfavourable conditions [69].

Heat shock protein 70 (Hsp70) and Hsp90 are the two prominently expressed chaperone proteins in *P. falciparum* [63]. These two proteins have been associated with development and pathogenesis in the blood stage of malaria. It has been suggested that they may also play a part in parasite drug resistance [63].

Hsp70 is one of the most common heat shock proteins in eukaryotic cells, with an ATP dependent function [70]. It switches between a low-affinity ATP binding state, where it binds to the peptide substrate allowing it to refold and then switches to a high-affinity ATP binding

state, releasing the substrate [71]. Studies have shown Hsp70 to have homologs of distinct forms found in various organelles. This is advantageous to the parasite as the functionally specialised Hsp 70s facilitate parasite survival [71]. This protein functions with chaperones such as Hsp90 and Hsp40. The relationship between Hsp70 and Hsp40 has not been studied in its entirety and is thus not fully understood [72].

Hsp90 is one of the chaperones known to be produced by the malaria parasite. It does not only work with its co-chaperones to assist newly synthesized proteins fold properly, it also assists with the regulation of transcription factors and protein kinases activity [73]. The latter function places Hsp90 at the centre of signal transduction within a cell, which is essential for its growth and survival [73]. Hsp70 and Hsp90 co-function in the folding of kinases and steroid hormone receptors, the association of these two is via an adaptor protein called the Hsp70-Hsp90 organising protein (Hop) [69]. Heat shock proteins have raised interest as drug targets since they are highly conserved proteins, they do not evolve as non-conserved proteins, which reduces the chance of drug resistance [74].

2.3.1. Digestion of haemoglobin

During infection, parasite multiplication is manifold, demanding a multitude of resources from the host to support its growth [8].

During the parasite growth phase in the erythrocytes, the parasite utilizes haemoglobin, from the early ring-from stage. The trophozoite is unable to metabolize haemoglobin completely, inside the food vacuole, as it does not have the enzyme heme-oxygenase, which is used by vertebrates for heme catabolism [9]. Proteolytic enzymes, called falcipains (serine proteases) and plasmepsins (aspartic proteases), mediate this metabolic process [10]. These enzymes are promising targets for drug discovery since haemoglobin digestion is vital for parasite survival. Ten aspartic proteases have been identified in the *P. falciparum* genome [11], four of which are found in the food vacuole. These four enzymes (plasmepsin I, II, IV and histoaspartic protease) are involved in heme digestion and differ in sequence specificity cleavage sites. Andrews *et al* (2007) discovered that individual knockout of each of these vacular aspartic proteases and the serine protease falcipain-2 demonstrate no effect on parasite growth. Furthermore, they revealed that accumulative knockout of the aspartic proteases and falcipan-2 enzymes, as well as the triple knockout of plasmepsin I/IV and falcipains-2, results in tempered growth of the *P. falciparum* parasite, which suggests that

combined inhibition of both aspartic proteases and serine proteases is toxic for the parasite. Therefore, novel inhibitors of the proteases from the parasite may serve as drugs for the treatment of malaria. [12]

The heme ion is very lethal to the parasite, but instead of being expelled from the RBC after separation from globin, heme is sequestered in the cell by being transformed into an insoluble form known as hemozoin. This appears as brown crystals in the digestive vacuole of the parasite [13]. The amount and the dimensions of the crystals in the RBC depend on the stage of the parasite development, with the least amount of the hemozoin detected in the ring stage and the highest amount in the schizont stage [14].

2.3.2. Purine salvage

P. falciparum is unable to produce its own purines. Instead it relies on purines of the host cell for biosynthesis of nucleic acids and cofactors [15]. Erythrocytes have mill molar concentrations of ADP and AMP in equilibrium with ATP, therefore the parasite has had no need to develop pathways for purine synthesis. Purine nucleosides and nucleobases are transported through the parasite membrane via the *P. falciparum* nucleoside transporter 1 (NT1) transporter Fig. (2.3). Mechanisms by which the parasite salvages purine during the blood stage differ in primary sources and routes of transformation. The main precursor for all purine synthesis in the metabolism of *P. falciparum* is Hypoxanthine. In vivo hypoxanthine is sourced from the erythrocyte purine pool, where ATP is in constant metabolic exchange with hypoxanthine through ADP, AMP, IMP, inosine and adenosine [16].

Human red blood cells are also unable to synthesize their own purine, the action of adenosine kinase (AK) helps to maintain adenine nucleotide pools by recovering adenosine from plasma. This action keeps erythrocytic adenosine concentrations low and since *Plasmodium* does not possess the enzyme adenosine kinase, *P. falciparum* salvages the adenosine from the erythrocytes by converting it to hypoxanthine using the sequential activities of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP). The transport of the majority of purine nucleosides and bases via the *P. falciparum* membrane is by the NT1 transporter Fig. (2.3). The hypoxanthine then gets transformed to IMP by hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT). Inosine 5'-Monophosphate serves as the

metabolic precursor for all purine deoxynucleotides and nucleotides required for DNA/RNA synthesis. HGXPRT, ADA and PNP are highly expressed proteins in *Plasmodium* [17]. The parasite is unable to directly convert adenosine to AMP since it does not have an adenosine kinase gene in its genome. However, metabolic analysis showed that *P. falciparum* is able to utilise AMP synthesized in the erythrocyte cytosol when high concentrations of exogenous adenosine are used to influence erythrocytic AMP production. [19]

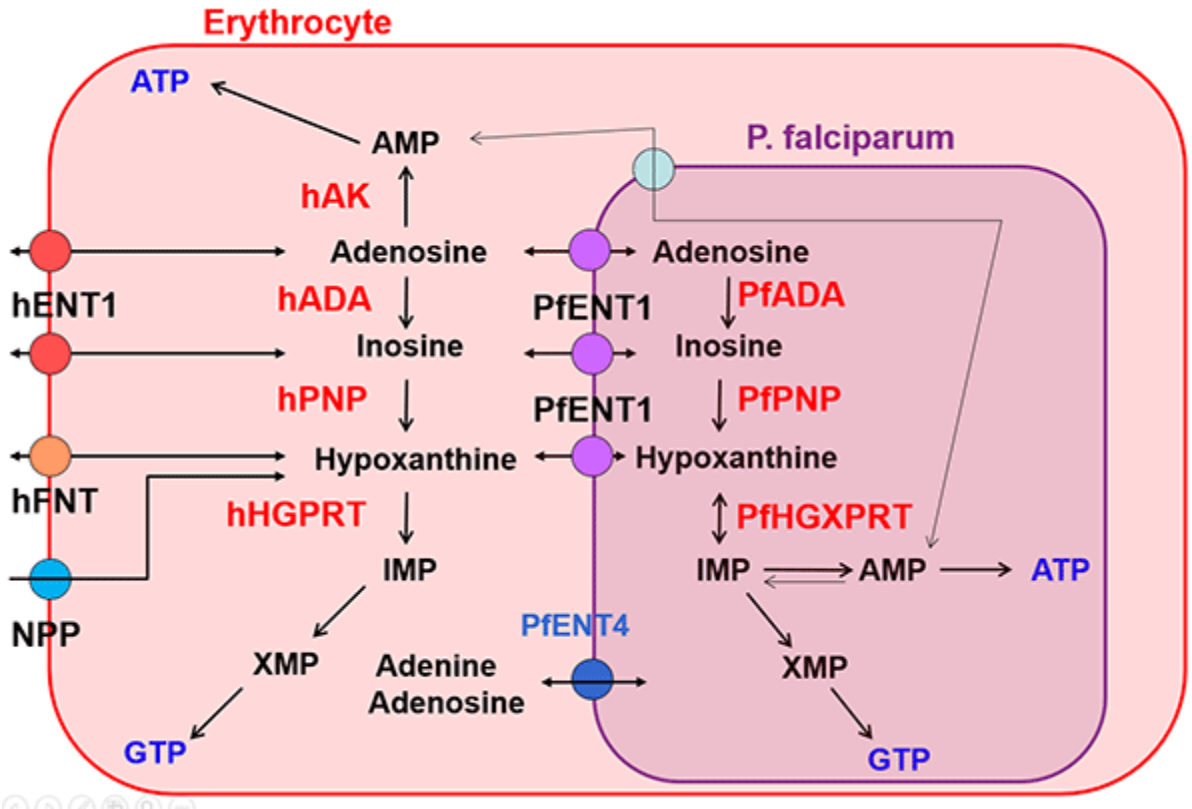


Figure 2.3: Purine salvage pathway in a *Plasmodium falciparum* infected-erythrocyte [85].

2.4. Treatment of Malaria

The choice of treatment is dependent on the level of infection and the type of strain. WHO recommends artemisinin-based combination therapies (ACTs) as treatment of non-severe *P. falciparum* malaria. The combination of two drugs with different mechanisms of action, makes ACTs the most effective currently available antimalarial treatment. There are five ATCs currently recommended by WHO for use against *P. falciparum* malaria. Results of therapeutic efficacy studies against local *P. falciparum* strains should dictate the choice of

ATC to be used [22]. People with severe malaria cases must first be treated intravenously or intramuscularly with Artesunate for a minimum of 24 hours until the patient is able to tolerate oral medication. Once the oral medication is tolerable, ACT treatment must be completed within 3 days, with single dose primaquine in areas of low transmission [22]. Although there are some effective treatment methods, the development of resistant strains is still a burden. Table 2.4 below shows targets and structures of some currently used antimalarial drugs.

2.4.1. Current anti-malarial drugs and their mechanisms of action

a) Chloroquine

This drug functions by inhibiting parasite DNA/RNA synthesis and causing rapid degradation of ribosomes. It accumulates in high concentrations in the parasite digestive vacuole. Once in the vacuole, chloroquine inhibits the detoxification of heme, by binding to it, thus leading to heme build-up. Toxic heme-chloroquine complex and heme promote cell lysis and eventually the auto-digestion of the parasitic cell. In other words, the parasitic cell drowns in its own metabolic products [23].

Resistance results in reduced accumulation of the chloroquine in the digestive vacuole [24]. Studies suggest that chloroquine resistance is due to reduced affinity of chloroquine for heme, thereby reducing chloroquine uptake by the parasite food vacuole [25].

b) Quinine

Quinine is used to treat uncomplicated as well as severe malaria in several therapeutic regimens and functions in a similar way to chloroquine but has a lower affinity for heme [23].

c) Artemisinin

Artemisinin falls under the antifolate antimalarial drugs. These drugs interfere with folate metabolism, a pathway essential to malaria parasite survival. Resistance is triggered by point mutations in the two key enzymes of the folate biosynthetic pathway, which are targeted by antifolates [23]. Artemisinin and its derivatives are sesquiterpene lactones, which treat malaria by interacting with iron or heme, disintegrating into free radicals inside the parasite.

Unfortunately, artemisinin resistance is constantly increasing [23].

d) Tetracycline

Cyclines are a form of antibiotic that act by inhibiting parasite protein synthesis. In *Plasmodium,* it is suggested that tetracycline may inhibit mitochondrial protein synthesis and decrease the activity of dihydroorotate dehydrogenase, a mitochondrial enzyme used for pyrimidine synthesis. Doxycycline blocks the production of nucleotides and deoxynucleotides in *P. falciparum;* it specifically targets the apicoplast of the parasite in two stages, one being with an immediate non-specific toxic effect on the apicoplast and the second being a characteristic cell death. Among the tetracycline family, doxycycline is the clinically recommended anti-malarial prophylaxis [64].

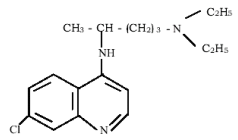
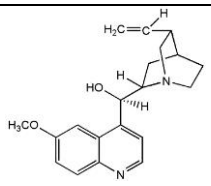
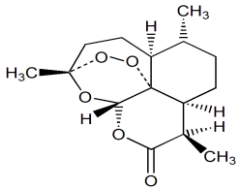
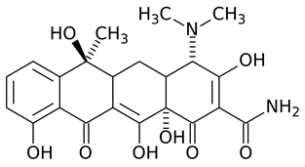
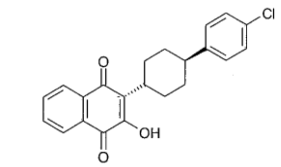
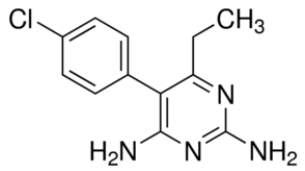
e) Atovaquone

This is an antimalarial drug used in both prevention and treatment of malaria. It acts by inhibiting the cytochrome c complex of the parasite, in the electron transport chain, and in that way collapsing the mitochondrial membrane potential. Since the malarial ETC does not contribute significantly to ATP synthesis, it is believed that parasitic cell death is caused by the indirect inhibition of dihydroorotate dehydrogenase, important for pyrimidine biosynthesis, which requires the ETC function [65].

f) Pyrimethamine

Pyrimethamine is a drug normally used in combination for the treatment of uncomplicated, chloroquine-resistant, *P. falciparum* malaria. It acts as a competitive inhibitor of dihydrofolate reductase (DHFR), a key enzyme in the production of tetrahydrofolate, which is a cofactor required for DNA and protein synthesis in the parasite [66].

Table 2.4: Targets and structures of some currently used antimalarial drugs.

Drug	Structure	Target
Chloroquine		Haemoglobin degradation, DNA/RNA synthesis, free radical generation [23]
Quinine		Membrane transport [23]
Artemisinin		Haemoglobin degradation, heme polymerization, free radical generation [23]
Tetracycline		Protein synthesis and DNA synthesis [64]
Atovaquone		Electron transport chain [65]
Pyrimethamine		Folate mechanism [66]

2.5. Potential targets for novel antimalarial drugs

The spread of multidrug-resistant *P. falciparum* has emphasised the necessity to develop new, affordable and effective antimalarial drugs, for developing countries, where malaria is prevalent [23]. Resistance to drugs may be due to defective nucleic acid repair or other genomic stability pathways. There have been several important and unrelated parasitic biochemical pathways studied in the search for drug target identification. Figure 2.5., below, summarizes the biochemical pathways which are potential targets for antimalarial drugs [67].

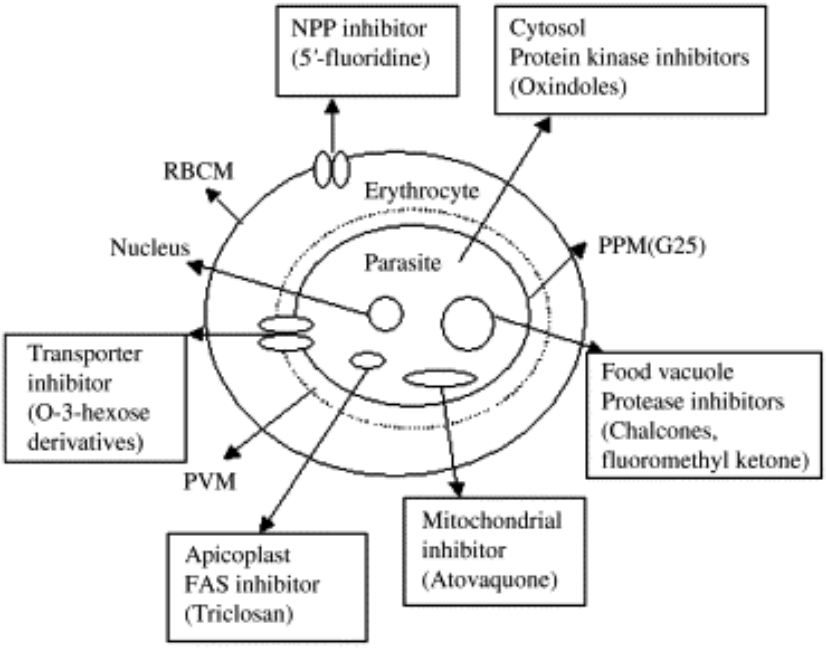


Figure 2.5: A diagrammatic representation of new molecular drug targets in plasmodium species. PVM: Parasitic vacuole membrane; RBCM: Red blood cell membrane; PPM: Parasite plasma membrane.

2.6. Traditional medicine

Traditional healers provide alternative health care and treatments that are indigenous to the culture and operate outside the government-funded healthcare systems as well as beyond the common medical profession practices. They are popular amongst many low to middle-class countries [75]. People regularly for maintaining good health [76] and sometimes for both chronic and acute diseases normally use traditional medicine, where conventional medicine is

inaccessible [77]. Plants have been used as treatment for centuries, and they still serve as a base for many pharmaceuticals to date [78].

Research on phytochemical compounds has contributed immensely to novel anti-malarial drugs. Drugs such as atovaquone; artemisinin and its derivatives; clindamycin; doxycycline; tetracycline, are all important examples of the contributions of natural products to the development of effective antimalarial drugs [78].

2.6.1. Medicinal plants used to treat malaria

Medicinal plants are the most popular form of traditional medicine in Africa. Plants are used for several ailments like skin disorders, ulcers, diarrhoea, colds, asthma, urinary tract infections, malaria, fever, sore throat, diabetes etc. [26]. For generations, numerous medicinal plants have been used to combat malaria, each with different mechanisms of action. Medicinal plants can be used for one of three characteristics i.e. synergic medicine; support of official medicine and Preventive medicine [27].

Researchers isolate compounds from medicinal plants, traditionally claimed to cure malaria or its symptoms, with the aim of finding a more effective, cheaper and less toxic antimalarial drug [28].

2.6.2. Antimalarial phytochemical compounds

One of the groups of compounds that have been explored is sesquiterpenes [29], which are a class of terpenes with three isoprene units and have the Molecular formula $C_{15}H_{24}$. They are poisonous to livestock and act as feeding insect deterrents. They also cause allergic contact dermatitis in humans [30].

Becker *et al*. used standard phytochemical analysis techniques to isolate a eudesmanolide-type sesquiterpene lactone, dehydrobrachylaenolide (**1**); this compound was found to be the key active component of *Dicoma anomala* subsp. *gerrardii* (Asteraceae) [31]. Compound **1** showed an *in vitro* IC_{50} value of 1.865 μM against a chloroquine-sensitive *P. falciparum*

strain (D10). Biological activities shown by synthetic derivatives of compound **1** revealed that a methylene lactone group should be present in the eudesmanolide for any noteworthy anti-malarial activity to occur. This is not the case with artemisinin and thus suggests that eudesmanolide-type sesquiterpene lactones use a different mechanism of action from artemisinin [31].

Wube *et al*. discovered that the antiprotozoal activity of *Warburgia ugandensis* (Cannelaceae) against *P. falciparum* and *Trypanosoma brucei rhodesiense* is due to drimane and coloratane sesquiterpenes found in the plant [32]. Compounds isolated from this plant include 4(13), 7-coloratadiene-12, 11-olide (**2**), 11α-hydroxymuzigadiolide (**3**), muzigadial (**4**), 6α, 9α-dihydroxy-4(13), 7-coloratadiene-11, 12-dial (**5**), cinnamolide (**6**), cinnamolide-3β-acetate (**7**), mukaadial (**8**) and ugandensidial (**9**) [32]. Amongst the discovered compounds, Compound **3** was found to be the most active against *P. falciparum* with an IC_{50} value of 6.40µM [32].

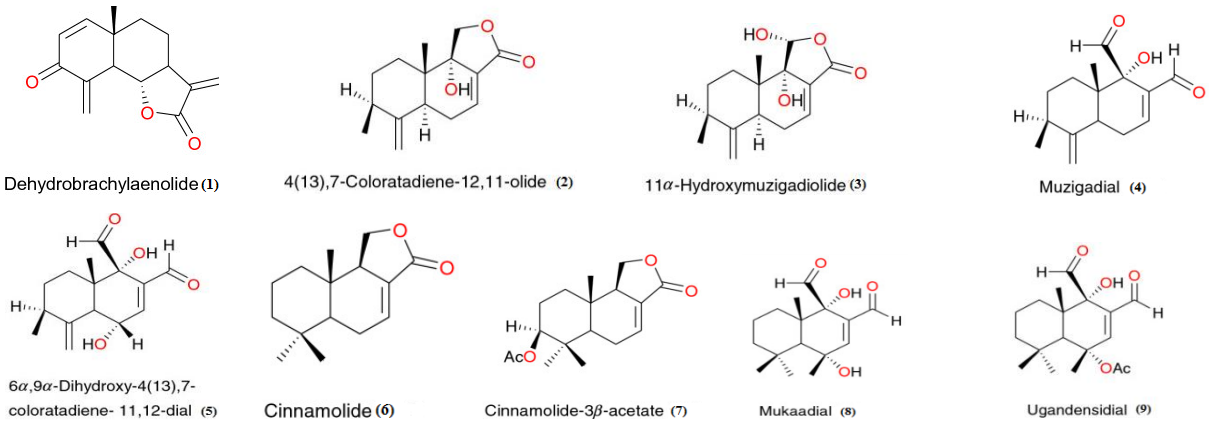
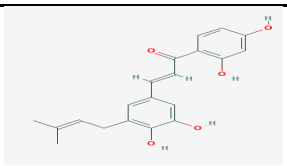
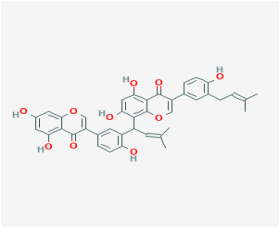
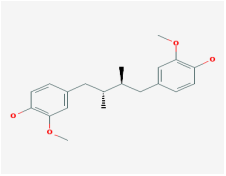
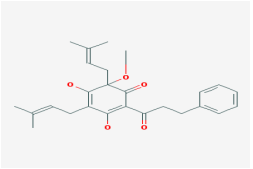
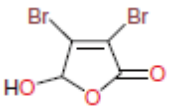
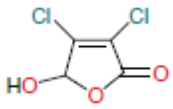
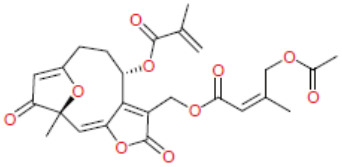


Figure 2.6.2: Compounds isolated from *Dicoma anomala* (1) and Warburgia *ugandensis* (2-9) [31, 32]

There are several other phytochemical compounds from different classes, which have been scientifically proven to possess antimalarial properties. Below is a tabulated summary of natural products isolated from medicinal plants, used traditionally to treat malaria [84].

Table 2.6.2: Natural compounds found to possess antiplasmodial activity.

Medicinal plant	Compound name and structure	Reference
Erythrinaabyssinica (stem bark)	 5-Prenylbutein	79
Ficus mucuso (figs)	 Mucusisoflavone C	80
***Lippia javanica* (leaves and stalks**	 Dihydroguaiaretic acid	81
***Helichrysum cymosum* (whole plant)**	 Helihumulone	82
Vernonia staehelinoides (leaves)	 Mucobromic acid	83
Vernonia staehelinoides (leaves)	 Mucochloric acid	83
Vernonia staehelinoides (leaves)	 Hirsutinolide	83

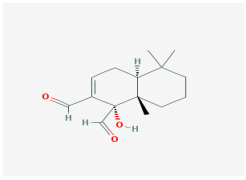
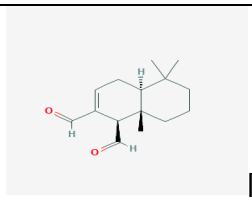
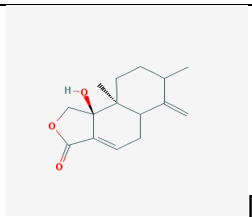
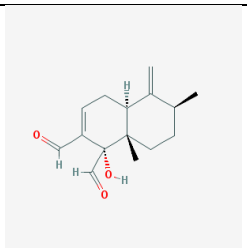
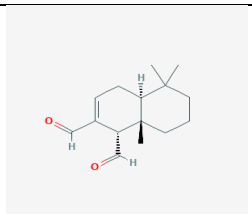
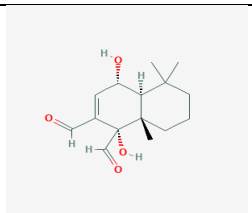
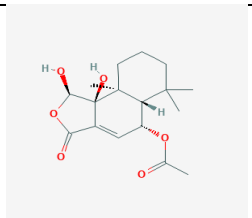
2.7. Warburgia salutaris

Warburgia salutaris (G. Bertol.) Chiov. (Canellaceae) is a commonly used medicinal plant found in eastern and southern Africa. It is an evergreen tree (Figure 2.6.2), with a fissured bark; glossy simple leaves; green flowers and plum-shaped fruits [52], that can grow up to 10 m in height [33]. The bark and leaves possess antimicrobial activity [37, 53 and 54] and are commercially available in a crude form (usually bark) from local muti markets and as over-the-counter products (containing leaves or leaf extracts) from pharmacies. *Warburgia salutaris* is widely used in traditional medicine to treat coughs, colds, bronchial infections, oral thrush, cystitis, malaria and many other ailments [55-59].

Figure 2.6.3: Bark samples on a traditional market (A) and examples of commercial bark and leaf samples (B–E) [60]. *Warburgia salutaris* tree (F) and flowers (G), taken at the UKZN botanical gardens.

The main compounds which have been isolated from this species include phenols which are responsible for its antioxidant effects [34], drimane sesquiterpenoids [35], antimicrobial sesquiterpenoid compounds [36, 37] furan and furanones [38]. Although none have been reported to possess anti-malarial activity, several drimane sesquiterpenes have been isolated from this plant (Table 2.4.1.1).

Table 2.4.1.1: Compound previously isolated from *W. salutaris*

Compound	**Structure**	**Activity**
Warburganal	 [39]	Molluscicidal [40, 41], antifungal and antibacterial [42, 43] activity
Polygodial	 [44]	Antifungal and antibacterial [42] activity
Salutarisolide	 [45-57]	None reported
Muzigadial	 [48]	Antimicrobial and antifungal [42, 37]
Isopolygodial	 [50]	None reported
Mukaadial	 [45]	Molluscicidal [45]
11-hydroxycinnamosmolide	 [28]	anti-mycobacterial activity [28].

2.8. Work scope:

Aim: To validate the use of *W. salutaris* in traditional medicine against malaria.

Objectives:

- To collect the plant material and prepare a voucher specimen.
- To isolate, purify and characterize active phytochemical compound(s) from the plant using chromatographic and spectroscopic techniques.
- To examine the *in vitro* antiplasmodial activity of the isolated compound(s) crude plant extract.
- To determine the *in vivo* antiplasmodial activity of the crude extracts and isolated compound(s).
- To investigate the cytotoxicity of the active compound(s)
- To carry out in silico studies on the active compound(s)

Chapter Three

Materials and methods

This chapter includes a brief description of all methods carried out in this study as well as a list of all important chemicals, reagents and equipment used in this study. Details of the preparation of reagents and the details of the methodology are in Appendix 1 and 2, respectively.

3.1. Materials

3.1.1. Chemicals

Unless otherwise stated, all chemicals and solvents used were of analytical grade from Merck chemicals (PTY) LTD, South Africa (SA). Chloroquine, artesunate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT salt), fetal bovine serum, penicillin, streptomycin, eagles minimum essential medium (EMEM) tween 20, dimethyl sulphoxide (DMSO), Giemsa DNA, isoform, silica gel (60: 0.063-0.200mm), sea sand (purified by acid and calcined for analysis), TLC Silica gel 60 F_{254} 20x20 cm Aluminium sheet, human embryonic kidney (HEK293) cells (Anti-viral Gene Therapy Unit, Medical School, University of the Witwatersrand, SA,), liver hepatocellular carcinoma (HepG2) cells (Highveld Biologicals (Pty) Ltd, Kelvin, SA).

Other materials and methods include:

Grinder- Hippo Mills (AC Trading)

Rotary evaporator- Heidolph Instruments GmbH & Co. Germany

UV Light- SPECTROLINE CL-150 Ultraviolet Fluorescence Analysis Cabinet

Nuclear Magnetic Resonance-Burker (Polychem suppliers)

Centrifuge- Eppendorf (Merck)

Sprague Dawley rats-Biomedical research unit, University of KwaZulu-Natal

3.2. Methodology (Appendix 2 for details)

3.2.1. Plant material

Fresh stem bark of *Warburgia salutaris* was collected from the University of KwaZulu-Natal, Pietermaritzburg campus, Botanical gardens (29° 37′ 30″ S, 30° 24′ 14″ E) in March 2016. The plant was identified by the UKZN botanical gardens horticulturist, Mrs Alison Young. A voucher specimen was prepared and stored at the University of KwaZulu-Natal Pietermaritzburg Herbarium, voucher number: NU0043932. The stem bark was air-dried, ground into powder and stored in sterile brown bottles until needed.

3.2.2. Extraction crude extract

The plant material (4.2 kg) was sequentially extracted (1:5 w/v) with dichloromethane (DCM), chloroform, ethyl acetate and methanol. Extracts were filtered through a Whatman (No. 1) filter paper; the residue was air dried and the extract concentrated using a rotary evaporator. The rotary evaporator ran at a reduced pressure with different temperatures per solvent i.e. DCM-45^{o}C; chloroform 61^{o}C; ethyl acetate-77^{o}C, and methanol-65^{o}C.

The concentrated extracts were dried overnight. The DCM extract weighed 85 grams; ethyl acetate extract=3.7 grams; chloroform extract=3.8 grams; and the methanol extract=83.3grams.

3.2.3. Isolation of pure compound

Column chromatography was done for the isolation of natural compounds from the DCM extract, Merck silica gel (60: 0.063-0.200mm) was used as the stationary phase, and Hexane: ethyl acetate ratio as the mobile phase. Hexane and ethyl acetate was distilled, at 67^{o}C and 77^{o}C respectively, prior to use in the column. The solvent ratios were from 9_{Hex}:$1_{Ethyl\ ac.}$ to 2_{Hex}:$8_{Ethyl\ ac.}$, 10 grams of the extract was used with 30x grams of silica gel, per column.

Thin layer chromatography was used to check the presence of a compound at each ratio, a 20% sulphuric acid in methanol solution was used to fix the TLC plates; they were then viewed under UV light. Fractions were collected per 80ml and combined according to their TLC profiles. At the ratio 8_{Hex}:2 $_{Ethyl\ ac.}$ A single bright spot appeared on the TLC plate when viewed under UV light, with a Retardation Factor (RF) value of 0.6.

The combined fractions were dried in the fume hood overnight, fractions from the 8_{Hex}:2 $_{Ethyl\ ac.}$, ratio dried into 6 grams of a white amorphous powder (NN-01), with a stinging effect to the nostrils.

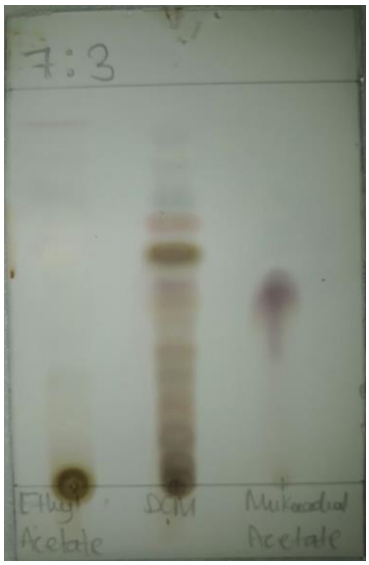


Figure 3.1: TLC plate showing compound present in the crude extract and the isolated compound.

3.2.4. Structural elucidation

The structure of compound NN-01 was determined using Nuclear magnetic resonance (NMR) techniques (^{1}H, ^{13}C, COSY, DEPT, NOESY, HMBC and, HSQC), infrared (IR) spectra and X-ray crystallography. The NMR data was collected using Filezilla 3.21.0 copyright © 2004-2016 Tim Kosse and processed, into spectra, using Spinworks 4.0 copyright ©2014, Kirk Marat, University of Manitoba to give the NMR spectrum.

The structure of compound NN-01 was determined through the study of the chemical shifts (table 3.1) and spectra, (Appendix 3). NN-01 was identified as, a new compound, Mukaadial

acetate (Figure 3.1). It was the first time Mukaadial Acetate was isolated from this plant or any higher plant species. Figure 3.2 below shows the single mass spectra of Mukaadial Acetate.

Table 3.1: Proton (^{1}H) and carbon 13 (^{13}C) NMR chemical shifts (δ, ppm) of M. Acetate

Position	**δc (ppm)**	**CHn**	**δH (ppm)**
1	66.13	CH	5.91(t, H, J = 4.8 Hz)
2	148.60	CH	7.01(d, H, J = 4.8 Hz)
3	141.00	C	
4	32.56	CH	4.07
5	41.66	C	
6	31.83	CH2	
7	17.70	CH2	
8	44.02	CH2	
9	34.00	C	
10	44.97	CH	2.06(d, H, J = 4.8 Hz)
11	193.01	CHO	9.76
12	201.44	CHO	9.48
13	19.95	CH3	1.34
14	24.75	CH3	1.17
15	21.44	CH3	1.03
16	170.01	COOR	
17	19.95	CH3	2.14

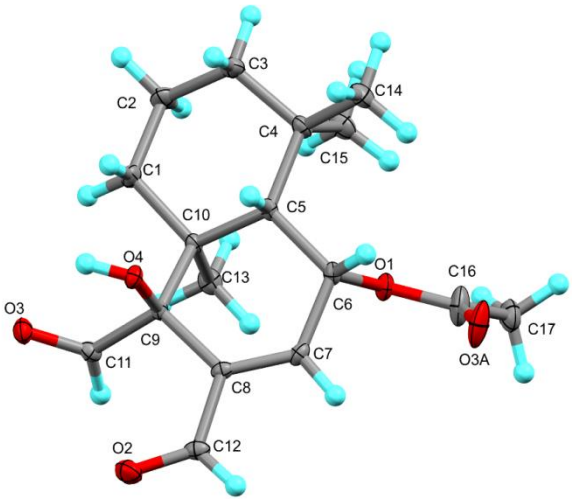


Figure 3.2: X-ray structure of Mukaadial Acetate ($C_{17}H_{24}O_4$; 292 g/mol)

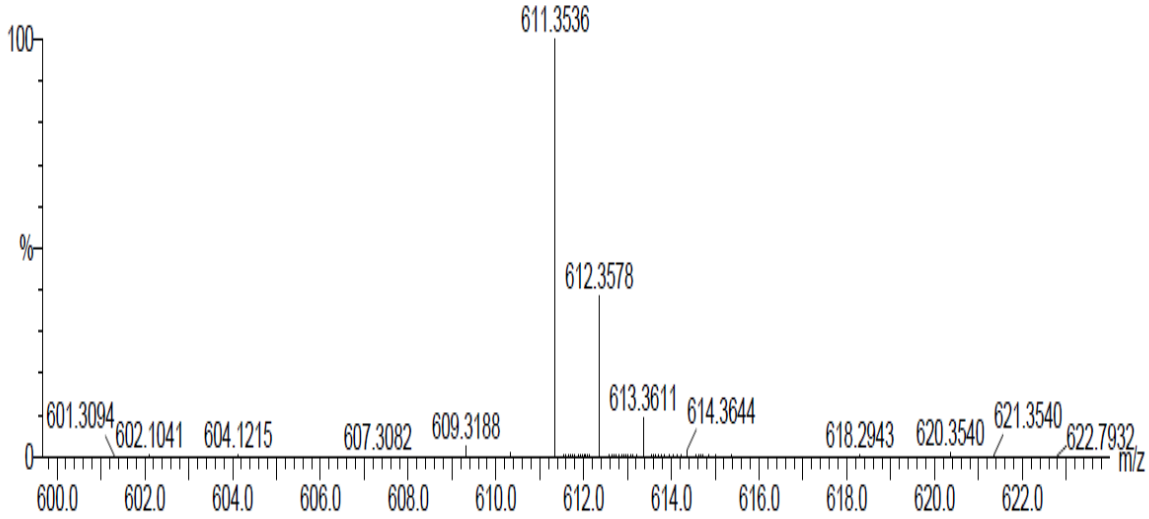


Figure 3.3: Single mass analysis of Mukaadial Acetate

3.3. Section A

3.3.1. Cytotoxicity MTT assay

The University of the Witwatersrand, Medical school, Antiviral Gene Therapy Unit, SA provided the HEK293 (Human embryonic kidney) cells, while the HepG2 (liver hepatocellular carcinoma) cells were obtained from the Highveld Biologicals (Pty) Ltd, Kelvin, SA. The assay method was a modification of that described by Mosman (1983)[40], it is used to calculate the cells' metabolic activity, via reducing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan salt by the enzyme succinate-tetrazolium reductase within the cell. The cells were developed to semiconfluent in the flasks for tissue culture (25 cm^2) in Eagle's minimal essential medium containing 100 U/ml penicillin, 100 µg/ml streptomycin as well as fetal bovine serum (10%). Cells were then prepared to a density of 21 x 10^3 cells in each well, with medium (100 µl) in a 96 well plate. A 24 hour incubation (37°C; 5% CO_2) followed, the old medium was then removed, while fresh medium (100 µl) was added. Varying (50µg/ml, 100µg/ml and 150µg/ml) of the compound in triplicates were added onto the cells and were then further incubated (48 hours; 37°C). Cells without the experimental compound were used as the positive controls which will show viability at 100%.

The original medium was removed, after 48 hours of incubation and 100 µl new medium as well as 5 mg/ml MTT in PBS added. This went into incubation (37^oC) for another 4 hours. MTT and medium were then discarded, and 200 µl of DMSO was added per well, for dissolving the formazan. The absorbance of resulting mixture was then read with a Mindray 96A micro plate reader (Vacutec, Hamburg, Germany). A wavelength of 570 nm (detection λ) was used and that of 630 nm as the reference wavelength of nonspecific signals. The percentage viability of the cell was linked to absorbance and worked out in reference to the positive control using the equation: [(OD_{570} Treated)/ (Control)] x 100. All experimentations were done in triplicates.

3.3.2. In vitro antiplasmodial activity

The *in vitro* antiplasmodial activity was carried out at the University of Cape Town, Division of Pharmacology, South Africa. The test samples were tested in triplicate on one occasion

against chloroquine-sensitive (CQS) strain of *Plasmodium falciparum* (NF54). Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976) [87]. Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase assay using a modified method described by Makler (1993) [88].

The test samples were prepared to a 20 mg/ml stock solution in 100% DMSO. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at -20ºC. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) and artesunate were used as the reference drugs in all experiments. A full dose-response was performed for all compounds to determine the concentration inhibiting 50% of parasite growth (IC_{50}–value). Test samples were tested at a starting concentration of 100 µg/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 µg/ml. The same dilution technique was used for all samples. Reference drugs were tested at a starting concentration of 1000 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC_{50}-values were obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

3.3.3. Molecular Docking

The crystal structure of *Pf*HGXPRT in complex with hypoxanthine (PDB: 3OZF) [89] was retrieved from the Protein Data Bank. This protein structure exists as a tetramer, therefore, chain A was selected to reduce computational cost. Protein structure and ligand modifications were conducted in UCSF Chimera [90] in preparation for docking. M. acetate docking into the *Pf*HGXPRT binding site was conducted using AutoDock Vina [91]. Non-polar hydrogen's, Geister charges, possible torsional degrees of motion and rotatable bonds of M. acetate, as well as Kollman charges [91] for all atom types, were assigned in AutoDock Tools interface. The Lamarckian Genetic Algorithm [92] was applied to generate a docked conformation. M. acetate was docked using a grid box size of 66×64×54 Å which was generated to encapsulate the active site amino acid residues of the *Pf*HGXPRT structure. Coordinates of a docked M. acetate-*Pf*HGXPRT complex were saved for further molecular analyses conducted in UCSF Chimera and LigPlot [94].

3.4. Animal Experiment

3.4.1. Ethical consideration

Ethical clearance was obtained from the University of KwaZulu-Natal Ethics committee (reference: AREC/053/016, appendix 4)

3.4.2. Animals

Male Sprague-Dawley rats, 16-18 weeks old (80-120 g) bred and housed at the University of KwaZulu-Natal, Westville campus, Biomedical Resource Unit were used in this study. The animals were maintained under standard laboratory conditions. The rats had free access to food and water.

3.4.3. Preparation of drugs and extract

Concentrations 0.5 mg/kg; 2.5 mg/kg and 5 mg/kg were prepared by dissolving the Mukaadial Acetate or crude extract in tween 20 and distilled H_2O was added to the required volume. For the main study, 1.5mg/kg of the crude extract and Mukaadial Acetate was prepared and 60 mg/kg of chloroquine was prepared, in the same way, for the control group. The drugs were administered via oral gavage.

3.4.4. Induction of malaria

Malaria was induced by a single intra-peritoneal injection of 10^5 *Plasmodium berghei* parasitized RBC [9]. The rodent CQ sensitive *P. berghei* parasite was obtained from Prof. Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). Successful malaria infection was confirmed by microscopic examination of Giemsa stained thin blood smears, from the rat tail. Percentage parasitemia greater than or equal to 20% was considered as stable malaria state before commencing with experimental procedures.

3.4.5. Experimental design

Acute studies were carried out to determine whether proposed concentrations of the Crude extract and Mukaadial Acetate would have any adverse effects on the animals. Four male Sprague Dawley rats were treated with two different Mukaadial Acetate and extract concentrations (0.5 mg/kg and 5 mg/kg) for five consecutive (n=1/group). The rodents were

closely monitored during the course of the treatment period and for five consecutive days post-treatment. The experimental animals were then sacrificed on day ten and blood was collected for a haematology profile.

Following the acute study, a 21-day experimental pilot study was conducted to investigate the antiplasmodial efficacy of selected extract and Mukaadial Acetate concentrations (0.5; 2.5; and 5 mg/kg). Malaria induction was done on day 0 of the experimental study using 10^5 *P. berghei* infected RBCs on Sprague-Dawley rats. The experimental study was divided into pre-treatment (day 0-7), treatment (day 8-12) and post-treatment period (day 13-21) Percentage parasitemia determination using Giemsa stained thin film blood smears was done daily post-*P. berghei* infection. On day 21, all the animals were sacrificed by isoform inhalation. Blood was collected by cardiac puncture into EDTA tubes for haematology parameters measurement, as shown in the schematic diagram below (Figure 3.4.1).

Pilot study animal design:

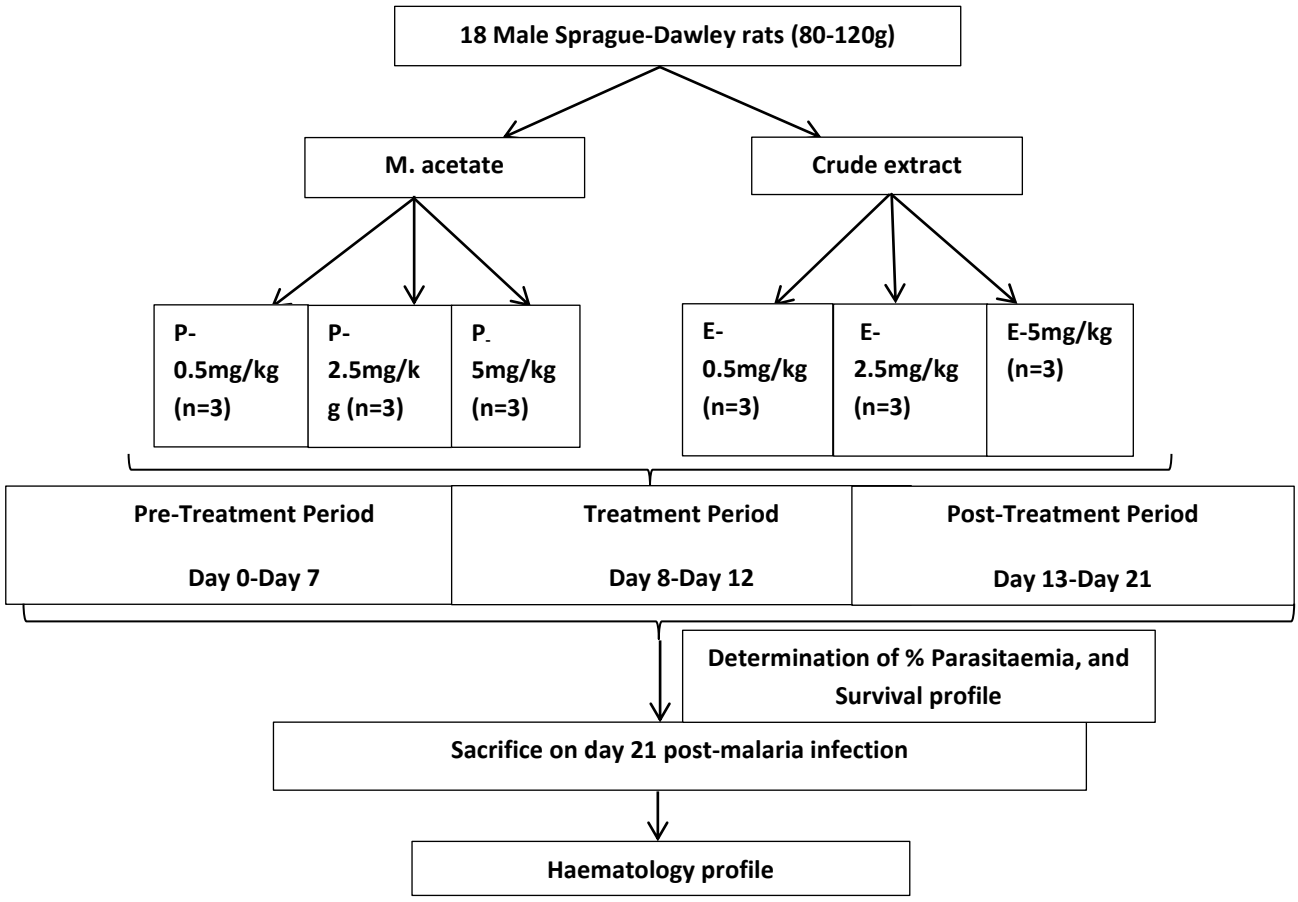


Figure 3.4.1: Schematic diagram of pilot study animal design.

3.4.5.3. Main study

Forty-eight male Sprague Dawley rats (80-120g) were randomly divided into two major groups: non-*P. berghei* infected and *P. berghei* infected (infected with 10^5 *Plasmodium berghei* parasitized erythrocytes, intra-peritoneally (i.p.) on day zero) (n=6/group).

Treatment commenced on day 8 of the study. The non-infected group had two sub-groups (n=6/group), receiving oral dosing of 2.5mg/kg crude extract and 2.5mg/kg Mukaadial Acetate each. The infected group had six sub groups (n=6/group): absolute control-vehicle; positive control-CQ (day 8-11); crude extract x5$^{\#}$-2.5 mg/kg (day8-12); M. acetatex5$^{\#}$-2.5mg/kg (day8-12);; Crude extract x1*-2.5 mg/kg (day8); M. acetatex1*-2.5 mg/kg (day8). The animals were then monitored for 9-13 days (varies per group) after the last day of treatment, until day 21. *x1-treated for one day; $^{\#}$x5-treated for five days

Main study design

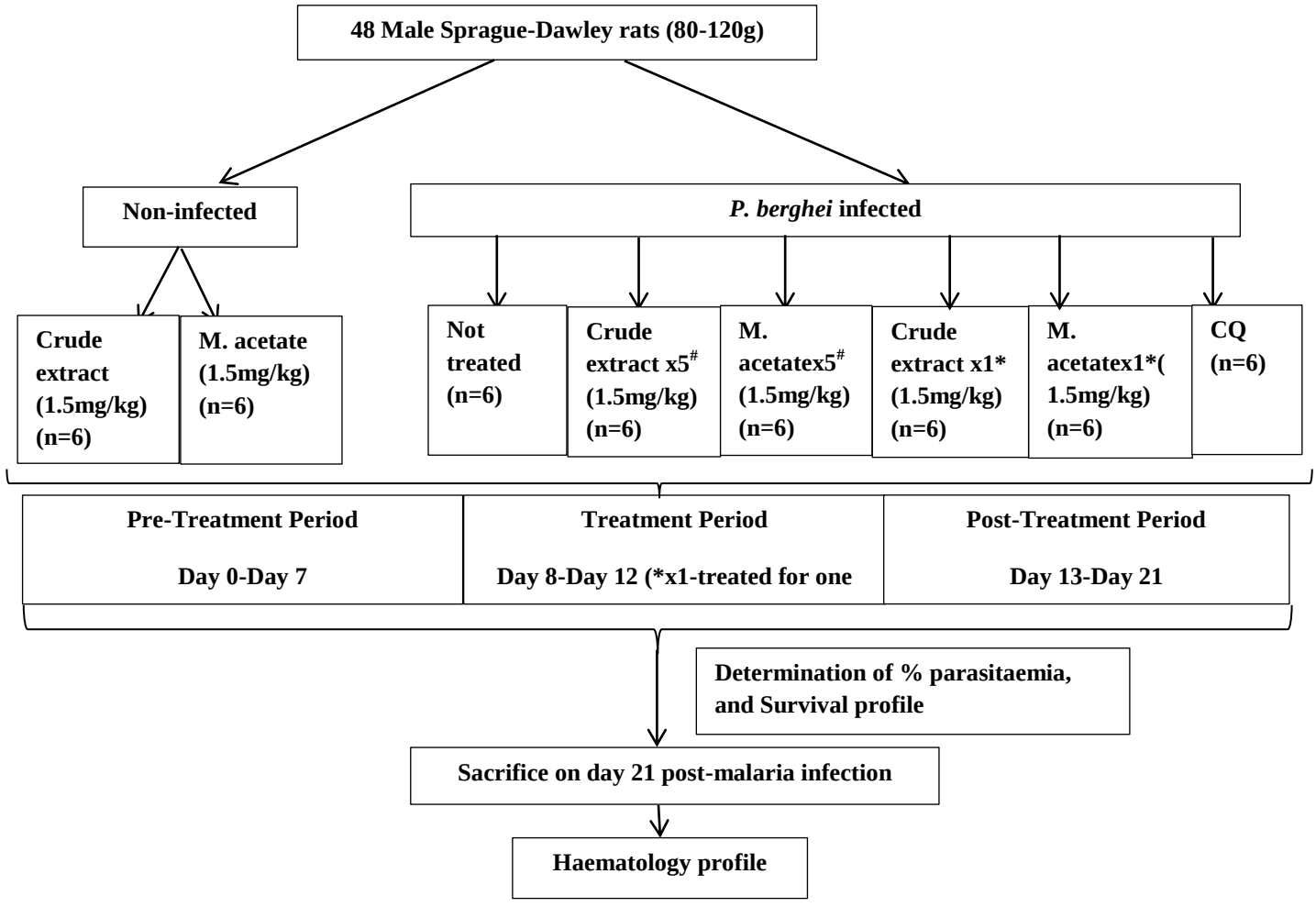


Figure 3.4.2: Schematic diagram of main study animal design

Percentage parasitemia was determined by thin blood smears prepared from the tail blood of each rat, fixed with methanol and stained with 20 % Giemsa every day from day 3 to day 21. The animals were sacrificed on day 21 and determination of, haematology profile and survival profile was done.

The body weights of the rats were monitored to observe whether the isolated compound prevents weight loss that is commonly observed with increasing parasitemia in infected rats. Weights were taken at 3-day intervals until day 21.

The rat grimace scale [10], from the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs), was used to ensure animal welfare was observed during the experiment. Orbital tightening; nose and cheek flattening; ear change; and whisker change was used as parameters for the grimace scale.

3.5. Calculating parasitemia

The parasitemia level was determined by counting the number of parasitized erythrocytes out of a hundred RBC in five random fields of the microscope. Average percent parasitemia was calculated by using the formula below [11, 12].

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC count}} X100$$

3.6. Statistical analysis

Data was analysed using two-way analysis of variance and group statistics, followed by Benferroni post hoc test. The mean ± SEM was calculated. The 2010 Microsoft Excel Program and version.4.0 Graph Pad Prism software (Graph Pad Prism, Inc.: San Diego, CA, USA, 1994–2003) were used for the statistical analysis of differences amongst the mean values calculated for the different trial groups, for IC_{50}. *P* values ≤ 0.05 were taken as significant.

Chapter Four

Results

4.1. Section A

4.1.2. Anti-plasmodial and cytotoxic activity

The bioassay separation of the DCM crude extract of *W. salutaris* led to the isolation and characterization of Mukaadial acetate. This was the first time that this compound was isolated from this plant, and it is the first time its antimalarial activity is being reported. The observed antimalarial and cytotoxic activity are depicted in table 4.1. The DCM extract showed higher activity than the methanol and hexane extracts.

Table 4.1. *In vitro* anti-plasmodial and cytotoxic activity of M. acetate

Sample code	***IC_{50} (µg/ml)**	**Cytotoxicity (µg/ml)**	
		HEK293	HEPG2
***W. salutaris* (DCM)**	0.01±0.30		
***W. salutaris* (Methanol)**	11.32±61	36.7 ± 0.8	119.2 ± 8.8
***W. salutaris* (Hexane)**	167.91±08[#]		
M. acetate	0.44±0.10		
CQ	4.9±0.07[#]		
Art	<2[#]		

*IC_{50}: Inhibitory concentration; [#]=ng/ml

4.1.2. Active Site Analysis

Molecular docking is an indispensable tool in molecular biology and computer-aided drug design as it predicts the binding modes of a ligand to a 3D structure of a target. It was appropriate therefore that the molecular docking of M. acetate be studied with its target protein. The active site of the *Pf*HGXPRT is subdivided into four functional regions namely;

the purine, the phosphate, the ribose and the magnesium ion-pyrophosphate binding site [93]. Amino acid residues predominantly forming a purine binding site include; Ile146, Asp145, Phe197, Leu 203, Lys176 and Val198 [93]. The phosphate binding site comprises Tyr116, Asp148, Thr149, Gly150 and Thr152 [93]. The magnesium-pyrophosphate binding site is primarily composed by residues Lys77, Gly78, Arg112, Ser115, Tyr116, Arg210 [93].

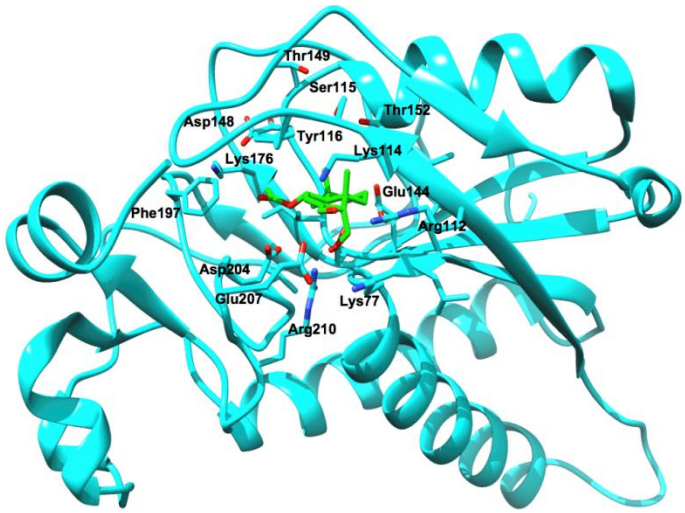


Figure 4.1: Representation of the 3D structure of *Pf*HGXPRT protein with mukaadial acetate (green) bound to the active site.

4.1.3. Mukaadial acetate Binding Mode

It was determined that M. acetate binds to the pyrophosphate and ribose binding sites of *Pf*HGXPRT protein with a docking score of -5.9 kcal/mol. The predicted binding mode of M. acetate o *Pf*HGXPRT binding site and interactions with residues inhabiting the binding site are depicted in Figure 4.2. The generated intermolecular interactions between M. acetate and binding site residues were predicted to include hydrogen bonding and hydrophobic interactions. Four hydrogen bond interactions were formed in the binding site: two between the third oxygen (O_3) of M. acetate and Arg210 amide group atoms (NH_1 and NH_2); another two between the fourth oxygen atom (O_4) of M. acetate and Arg112 amide group (NH_2) and Lys114 amide group (NZ).

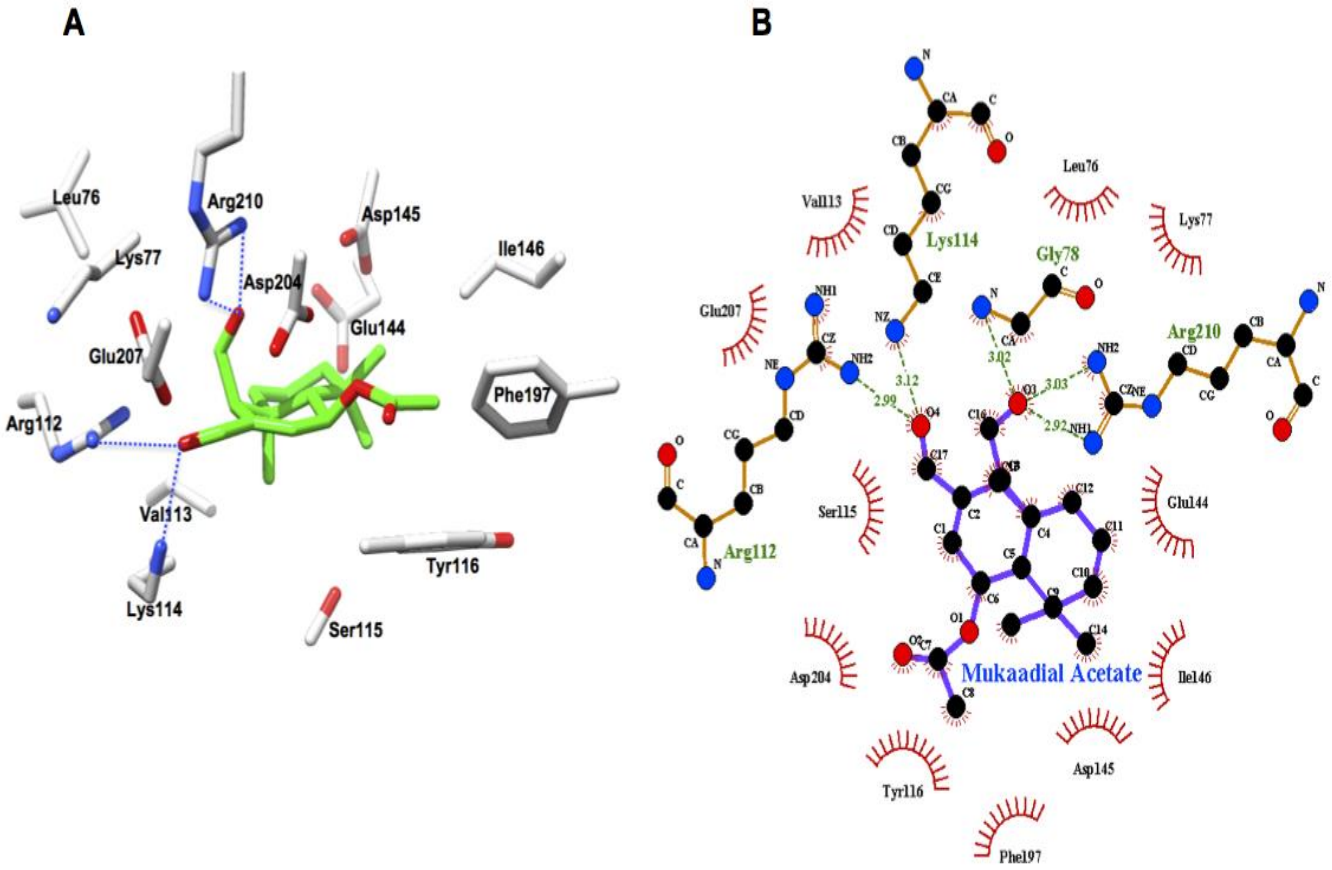


Figure 4.2: Predicted binding modes of M. acetate in 3D (A) [94] and generated intermolecular interactions (B) [95] within the *Pf*HGXPRT binding pocket.

4.2. Section B

4.2.1. Acute study results and observations

An acute toxicity study is done to determine the adverse effects of a substance from multiple exposure in a short period of time [110]. The study was carried out in accordance to with the OECD guidline 423 [110], using concentrations of 0.5, 1.5 and 2.5 mg/kg.

The animals showed no negative behavioural changes in all experimental groups, as they remained active and no adverse effects were observed.

4.2.2. Pilot study results and observations

The pilot study results showed increased antiplasmodial effect of both the *W. salutaris* crude extract and M. acetate, in comparison to the control. All the selected doses had no negative haematology effects on the experimental animals. These results lead to further studies (main study) using the lowest active concentration with a difference in dosing.

4.2.2.1. Effects of *W. salutaris* and mukaadial acetate on percentage parasitemia

W. salutaris and M. acetate, reduced % parasitemia in a dose-dependent manner, in comparison to control, though the differences between the *W. Salutaris* and M. acetate were not statistically significant as Figure 4.3. Peak % parasitemia was on days 7-8 post *P berghei* infection. Following the first day of treatment (day8), there was a decrease in % parasitemia, which gradually continued to decrease until day 21 of the experimental study for all the experimental groups.

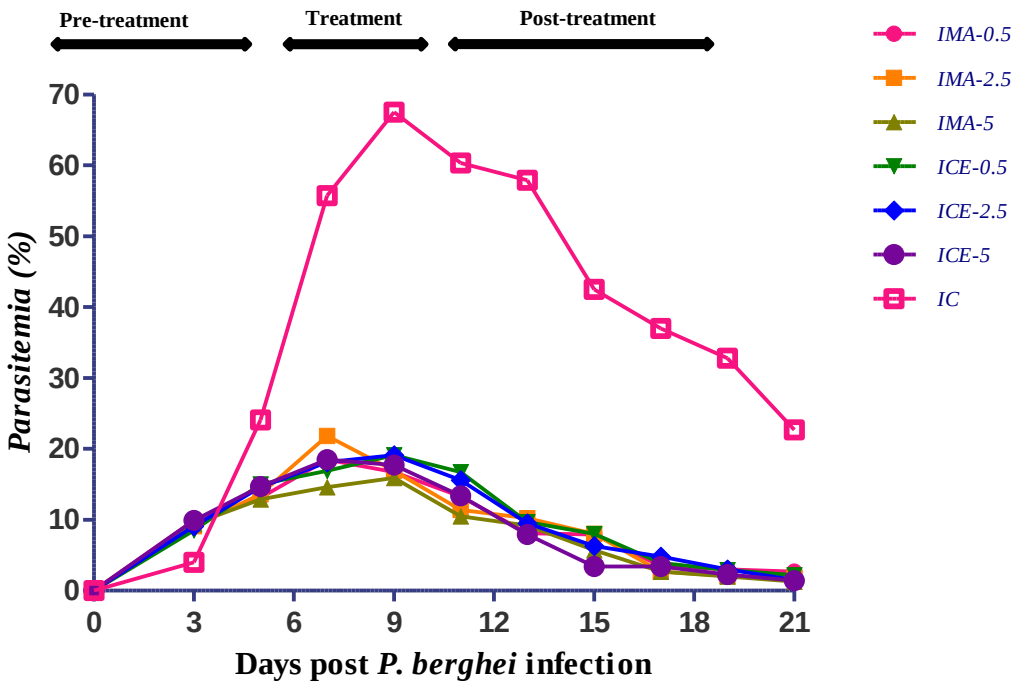


Figure 4.3: Percentage parasitemia of *Plasmodium berghei* infected SD rats treated with *Warburgia salutaris* and M. acetate. Where IMA-O.5: Infected mukaadial acetate treated 0.5 mg/kg; IMA-2.5: Infected mukaadial acetate treated 2.5 mg/kg; IMA-5: Infected mukaadial treated 5 mg/kg; ICE-0.5: Infected crude extract treated 0.5 mg/kg; ICE-2.5: Infected crude extract 2.5 mg/kg; ICE-5: Infected crude extract 5 mg/kg and IC: Infected control.

4.2.2.2.Haematology results

All the haematology parameters were within the respective reference ranges, for all the experimental groups as shown in Table 4.2 below. Indicating that the chosen compound and crude extracts have no adverse effects on the animals blood parameters. Values are presented as means (n=3 for each group). IMA-0.5:0.5mg/kg M. acetate treated; IMA-2.5:2,5mg/kg M. acetate treated; IMA-5:5mg/kg M. acetate treated; ICE-05:05mg/kg *W. salutaris* crude extract treated; ICE-2.5:2.5mg/kg *W. salutaris* treated; ICE-5:5mg/kg *W. salutaris* treated. Levels of significance, *P<0.05, ** P<0.01, *** P<0.001 in comparison to control group.

Table 4.2: Haematology parameters post *Plasmodium berghei* infection, treated with *Warburgia salutaris* and mukaadial acetate.

Groups	Haematology parameters								
	WBC (10^3/µl)	RBC (10^6/µl)	HGB (g/dL)	HCT (%)	NE (%)	LY (%)	MO (%)	EO (%)	BA (%)
IMA-0.5	4.93	8.10	14.97	42.67	17.73	67.07	13.40	0.93	0.87
IMA-2.5	4.60	8.26	15.73	44.67	14.20	72.40	14.17	1.40	0.77
IMA-5	6.03	8.14	14.93	42.97	19.60	56.70	16.90	1.60	0.40
ICE-0.5	6.73	7.74	13.53	39.26	13.23	72.87	14.73	1.27	0.30
ICE-2.5	5.70	7.82	14.07	40.73	10.00	78.13	11.33	0.20	0.33
ICE-5	7.00	8.01	14.37	42.00	17.73	68.73	17.03	0.20	0.50

4.2.3. Main animal study results

The study results showed varied antiplasmodial activity with the five-day treatment groups showing increased antiplasmodial effects than those of the one-day treatment. The selected dose had no negative haematology effects on the experimental animals.

4.2.3.1. Effects of W. salutaris and Mukaadial acetate on percentage parasitemia

Percentage parasitemia data is shown in table 4.3 below. Values are presented as means. Where UMA-Uninfected M. acetate treated; UCE-Uninfected crude extract treated; IC-infected without treatment; ICQ-infected chloroquine treated; ICE1-infected crude extract 1-day treatment; ICE2-infected crude extract 5-day treatment; IMA1-infected M. acetate 1-day treatment; IMA2-infected M. acetate 5-day treatment.

Levels of significance, *P<0.05, ** P<0.01, *** P<0.001 in comparison to control group.

Table 4.3: Percentage parasitemia *Plasmodium berghei* infected SD rats infected treated with *Warburgia salutaris* and mukaadial acetate.

Groups	Percentage parasitemia									
	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17	Day 19	Day 21
IC	9.20	12.97	18.20	39.70	52.37	52.20	43.13	35.77	28.30	25.27
ICQ	7.70	12.00	17.77	33.60	22.77***	13.23***	9.30***	6.67***	4.67***	0.53***
ICE1	6.87	10.20	27.23**	39.47	26.53***	20.00***	20.96***	25.97***	27.87	25.27
ICE2	8.40	17.50	28.07***	39.90	25.13***	16.13***	10.90***	7.03***	4.30***	1.40***
IMA1	6.27	15.63	29.20***	43.37	25.97***	23.57***	21.27***	24.5***	26.13***	28.87***
IMA2	4.97	12.17	22.43	33.03*	21.70***	14.57***	8.90***	5.90***	3.87***	0.70***

4.2.3.2. Effects of W. salutaris and Mukaadial acetate on haematology profile.

The table below represents haematology parameters. The study groups are represented as, UMA-Uninfected M. acetate treated; UCE-Uninfected crude extract treated; IC-infected without treatment; ICQ-infected chloroquine treated; ICE1-infected crude extract 1-day treatment; ICE2-infected crude extract 5-day treatment; IMA1-infected M. acetate 1-day treatment; IMA2-infected M. acetate 5-day treatment.). Levels of significance, *P<0.05, ** P<0.01, *** P<0.001 in comparison to control group.

Table 4.4: Haematology profile of study groups post *Plasmodium berghei* infection treated with *Warburgia salutaris* and mukaadial acetate.

Groups	Haematology parameters								
	WBC (10^3/µl)	RBC (10^6/µl)	HGB (g/dL)	HCT (%)	NE (%)	LY (%)	MO (%)	EO (%)	BA (%)
UMA	6.52	2.69	14.47	17.05	33.23	53.18	2.32	9.20	2.07
UCE	8.67	3.43	14.88	20.45	26.83	59.27	2.78	9.18	1.93
IC	8.35	*3.25	13.35	18.35	27.32	52.07	3.73	13.70	3.18
ICQ	15.02	3.99	12.48	21.88	21.67	57.82	2.18	7.98	***11.15
ICE1	2.45	1.26	15.25	8.95	23.48	51.57	3.82	10.98	10.15
ICE2	4.05	2.77	14.02	16.43	18.50	61.43	2.38	8.12	9.57
IMA1	2.23	2.93	13.05	16.50	18.72	62.42	2.85	7.67	8.35
IMA2	2.38	2.23	13.63	12.90	27.25	50.93	2.73	9.32	9.72

4.2.3.3. Effects of W. salutaris and Mukaadial acetate on animal weight.

The difference in weight was measured at two-day intervals to determine whether the *W. salutaris* crude extract and M. acetate prevent weight loss, commonly observed in malaria.

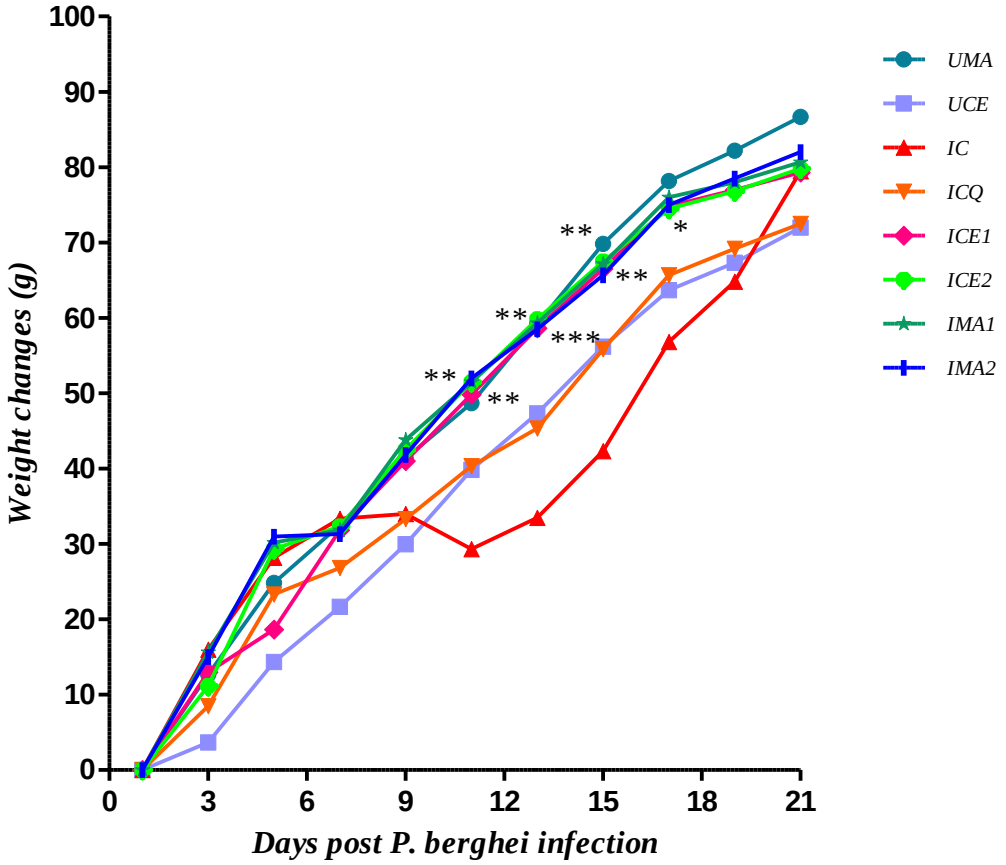


Figure 4.4: Difference in weight of *Plasmodium berghei* infected SD rats, treated with *Warburgia salutaris* and M. acetate over a 21-day period. Values are presented as mean ±SEM. UMA-Uninfected M. acetate treated; UCE-Uninfected crude extract treated; IC-infected without treatment; ICQ-infected chloroquine treated; ICE1-infected crude extract 1-day treatment; ICE2-infected crude extract 5-day treatment; IMA1-infected M. acetate 1-day treatment; IMA2-infected M. acetate 5-day treatment. Two-way analysis of variance was carried out for all percentage parasitemia data.

Levels of significance, *P<0.05, ** P<0.01, *** P<0.001 in comparison to control group.

Chapter Five

Discussion

5.1. Anti-plasmodial and Cytotoxic activity

Most people, living in malaria endemic areas, do not to afford antimalarial drugs, and thus rely on medicinal plants as primary treatment for malaria [97]. Discovering novel compounds of plant origin, which may be used in drug development, is still the main goal of ethno pharmacology [98]. In order for a drug to be considered effective, it should have an IC_{50} ≤1μg/ml and a selectivity index that is at least ten times more active on the parasite than it is on human cell-lines [99]. Mukaadial Acetate conforms to these standards. The *W. salutaris* DCM (0.1±0.03 μg/ml) extract showed greater activity than the methanol (11.32±61 μg/ml) and hexane (167±0.8 μg/ml) extracts. Therefore, the DCM extract was further purified to isolate the active compound, M. acetate (0.44±0.10 μg/ml), which also showed *in vitro* antimalarial activity and was later used with the DCM extract on *in vivo* experiments. Previous studies have shown extracts and isolated compounds with great antiplasmodial activity and toxicity values, such as; the methanol fraction of *Clerodendrum myricoides* leaves, an Ethiopian medicinal plant which was proven to have good antimalarial properties with an IC_{50} of 16.8 ± 2.65 μg/ml [107]. Myristic acid and β-caryophyllene, purified compounds, from *Murraya Koenigii*, were also found to be very active with IC_{50} values of 10.5 ± 0.8 and 8.25 ± 0.2 μg/mL, respectively [108]. However, the current study shows greater activity and lower cytotoxic effects of the DCM extract and its isolated compound M. acetate.

Literature shows that there has been no report of any other isolated antimalarial compound from *W. salutaris*. According to Katiyar, *et al.* (2012), natural sources hold a promise of delivering natural compounds as candidates or as a base for synthetic analogues to overcome any limitations of the natural compound in drug discovery [100]. The appreciable antiplasmodial activity of M. acetate makes it eligible for structural modification studies to reduce cytotoxicity while retaining or improving antiplasmodial activity. Based on the literature there have been no reports on M. acetate.

5.2. In vivo Anti-plasmodial efficacy

The rodent parasite *P. berghei* has been proven as the best in the early investigation of antimalarial activity of new test compounds [101]. Compounds that increase the survival time of rodents [102] and have more than 30% parasite inhibition [103] in comparison to the absolute control group are considered effective in standard screening tests, which is in agreement with the findings of this study as shown in Figures 4.13-4.16. M. acetate and *W. salutaris* showed dose-dependent parasite reduction in the pilot study, and optimum parasite inhibition is observed in the main study, five-day treatment groups, for both M. acetate and *W. salutaris*.

Gebretsadik and Mekonnen reported the *C. myricodies* ethyl acetate and methanol extracts to have 77.24% and 65.21% suppressive effects on *P. berghei* infection in mice at 300mg/kg doses [107].The root extract of *Echinops kebericho*, an Ethiopian medicinal plant was reported to have 57.29±1.76% parasite suppression at 500mg/kg on a chloroquine (CQ) sensitive *P. berghei* strain [109]. *In vivo* studies of the *M. koenigii* leaf crude ethyl acetate extract (600mg/kg) showed a 86.6% *P. berghei* malaria parasite reduction in mice. The two purified compounds of this extract, myristic acid and β-caryophyllene, were found to significantly reduce parasitemia by 82.6 and 88.2 % at 100mg/kg/body weight [108]. Although these extracts and compounds function by reducing infection in the blood stage as observed in the current study, the *W. salutaris* DCM extract and M. acetate possess higher affinity for the parasite with suppression values of 94.46 and 97.2%, respectively, at a significantly lower dosage of 1.5mg/kg. Therefore, the current study results supersede most of those reported in literature.

Effective anti-plasmodial products are expected to have protective effects against the loss of weight commonly observed in malaria infection [104]. In this study, these protective effects were observed in the M. acetate and *W. salutaris* groups as shown in Figure 4.26. Furthermore, the significant suppression of parasitemia at 1.5mg/kg for both the *W. salutaris* crude extract and M. acetate confirms that this plant has antimalarial effects in the late stages of *P. berghei* infection.

Although not significant, the difference in activity between M. acetate and *W. salutaris* crude extract may be justified by the presence of other secondary metabolites in the impure crude extract that do not possess antimalarial activity and may, therefore, have complex

biochemical effects contributing to slightly reduced therapeutic effects when compared to the pure M. acetate.

Low red blood cell count and reduced haematocrit in malaria infection are consistent with previous severe malarial anaemia studies [104]. Evans *et al* (2006) showed that the decrease in RBC production and RBC destruction in response to parasite invasion may indirectly lead to haematocrit and haemoglobin levels in the blood of infected animals [104]. CQ treatment also reduced haematocrit levels, which is supported by previous reports, that suggest that the binding of CQ to infected RBC leading to the eventual destruction of the parasite along with the RBC, has anaemic effects [105]. However, the mechanisms by which haematology parameters were affected are not described in this study. The measurement of plasma chemokine and cytokines may provide mechanisms by which observed malaria-induced haematology changes occurred.

Substances with antioxidant activity have antimicrobial activity, associated with elevated oxidative stress upon infection [106]. Previous studies have confirmed antioxidant activity of this plant [34], which may explain reported anti-microbial and antifungal [43,42] and anti-malarial activity.

5.3. Mukaadial acetate Binding activity

An interaction between M. acetate O_3 and Gly78 nitrogen atom was observed and is attributed to steric forces generated between the two atoms. M. acetate also formed hydrophobic interactions with residue Leu76, Lys77, Val113, Ser115, Tyr116, Glu144, Asp145, Ile146, Phe197 and Asp204. The observed interactions involved hydrophobic residues known to participate in purine binding (Tyr116, Ile146 and Phe197) [96], pyrophosphate and ribose binding (Tyr116, Ser115, Glu144, Tyr116, Asp204, Arg210, Arg112, Lys77, Gly78 and Asp145) [93] thus implying that M. acetate fitted in the active site with an optimum conformation.

Chapter Six

Conclusion

The target for this study was to scientifically validate the use of *W. salutaris* in Zulu traditional medicine. Results obtained show that *W. salutaris* possesses antimalarial activity, the active compound found to be M. acetate, which shows appreciable inhibition of parasite growth.

Molecular docking revealed that the isolated compound effectively binds to the active site of the parasite, forming respective hydrogen bonds, steric and hydrophobic interactions which largely contribute to its binding affinity.

The *in vivo* antiplasmodial studies with chloroquine-sensitive *P. berghei* show that *W. salutaris* has very good anti-malarial activity and protective effects on the survival of malaria-infected rodents.

Mukaadial acetate could serve as a lead compound for the development of a relatively potent drug in the treatment of malaria. However, more studies are required to determine the compound's mechanism of action on other *in vitro* and *in vivo* models. The results obtained support the use of *W. salutaris* in traditional medicine, to treat malaria.

6.1. Suggestion for future studies

The future direction for the current study would be the measurement of plasma chemokine's and cytokines to clarify the mechanism on blood parameters. Determination of the bioavailability of M. acetate using different modes of drug administration. Histological analysis of the harvested organs may give information regarding possible adverse effects of M. acetate on animal organs. The production and analysis of the bioactivity M. acetate chemical derivatives may lead to compounds with greater activity.

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Appendix 1
Preparation of reagents

1.1 20% sulphuric acid

20ml of sulphuric was prepared in 80ml methanol

1.2 Phosphate buffered saline (for infection)

8g of sodium chloride, 0.2g of potassium chloride, 0.24g of potassium dihydrogen phosphate and 1.44g of disodium hydrogen phosphate were prepared into 800ml of distilled water, the pH was adjusted to 7.4 with HCl and distilled water was added to a volume of 1litre.

1.3 Phosphate buffered saline (for Giemsa staining)

0.7g of anhydrous potassium dihydrogen phosphate and 1.0g disodium hydrogen phosphate was prepared in 800ml of distilled water, the pH was adjusted to 6.8 and distilled water was added to a volume of 1 litre.

1.4 Giemsa stain

Concentrated Giemsa was diluted to 20% in the PBS Giemsa stain buffer.

Appendix 2
Details of methodology

2.1 Packing the column

The column was packed with cotton at the base; hexane was added to allow a thin line of Merck; which was followed by silica gel (300grams), mixed with solvent to ensure no bubbles were formed; another thin sea sand layer was added and finally 10 grams of the DCM crude extract.

2.2. Structural studies

2.2.1. Nuclear magnetic resonance

NMR studies are done in order to determine the structure of organic molecules, in this study, the white amorphous powder obtained from the column, was sent to an NMR specialist Mr Craig Grimmer, at the UKZN-Pmb campus Chemistry department. The techniques carried out were ^{1}H NMR; ^{13}C NMR; DEPT; COSY; NOESY; HSQC; and HMBC. The NMR data was collected using Filezilla 3.21.0 copyright © 2004-2016 Tim Kosse and processed, into spectra, using Spinworks 4.0 copyright ©2014, Kirk Marat, Univeristy of Manitoba to give the NMR spectrum.

2.2.2. Mass spectroscopy

Low resolution and high resolution mass spectroscopy was done at the UKZN-Pmb campus Chemistry department. MS measures the masses of atoms within a sample.

2.2.3. Infrared spectroscopy

IR is used to determine functional groups in molecules by measuring the vibrations of atoms. Stronger bonds and light atoms generally vibrate at a high stretching frequency.

2.3. Molecular Docking

It is a tool used, in structural molecular biology and computer-assisted drug design, to predict the predominant binding mode/s of a ligand with a protein of known three-dimensional structure.

3.4. X-ray crystallography

This a tool used to identify the atomic and molecular structure of a crystal. The measure of angels and intensities of beams is used to produce a three-dimensional picture of the density of electrons within the crystal. The compound was recrystallized using hexane i.e. it was boiled in hexane for 5-10 minutes, then left to evaporate at room temperature until just the crystals were left behind.

Appendix 3
Spectra

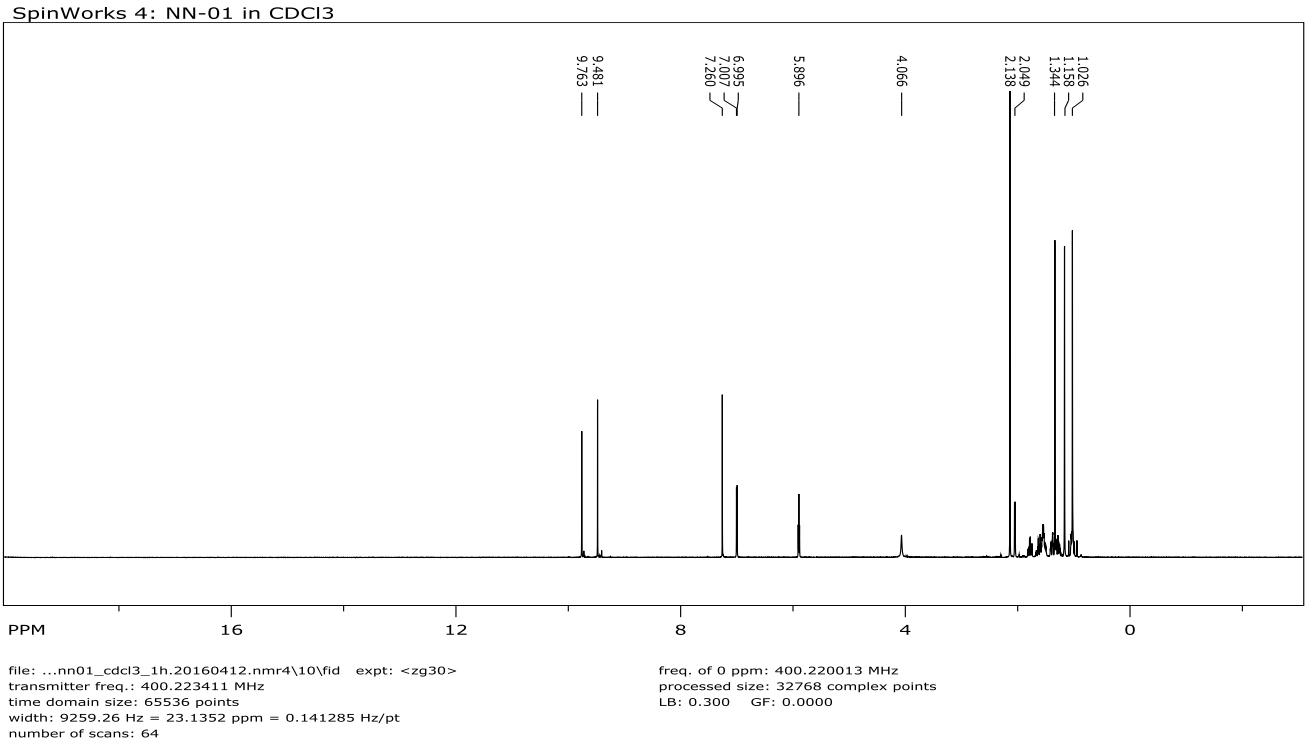

Figure 1: Proton NMR of NN-01

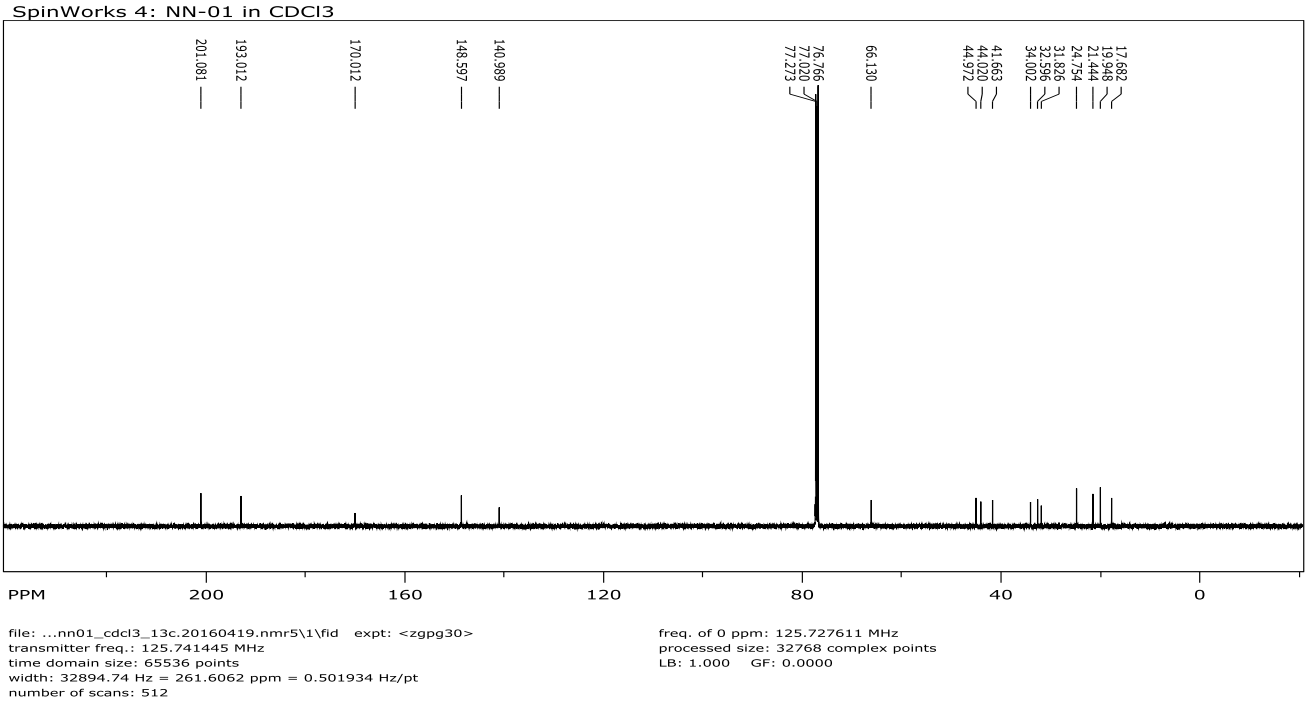

Figure 2: Carbon 13 NMR spectra

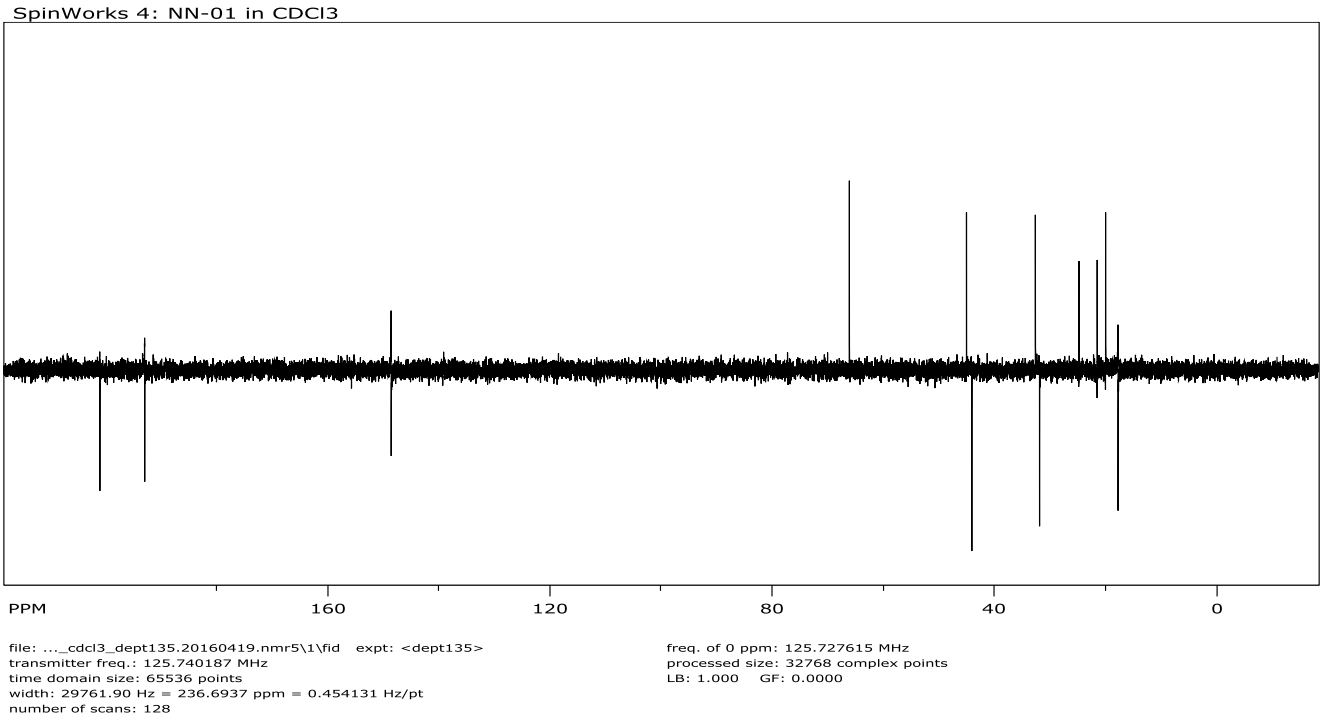

Figure 3: dept NMR spectra for NN-01

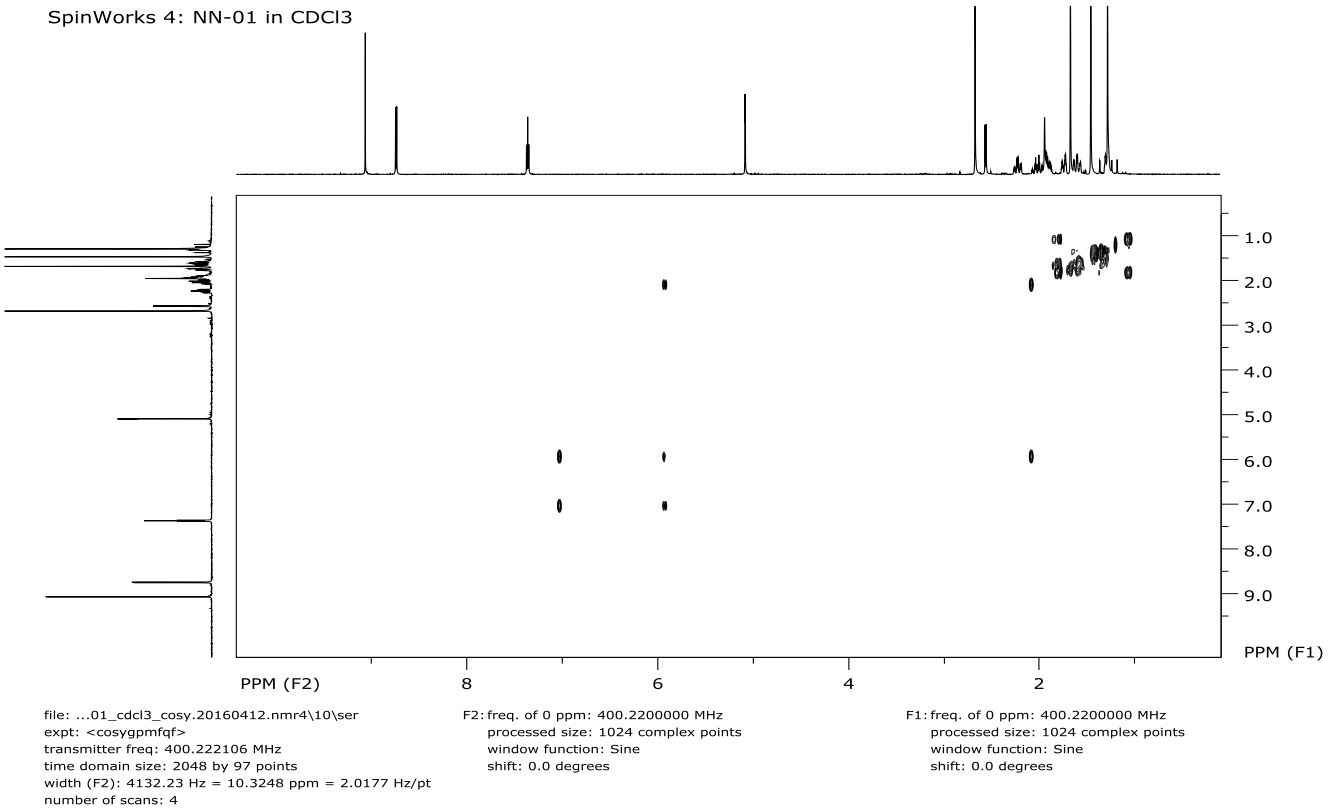

Figure 4: NN-01 cosy NMR spectra

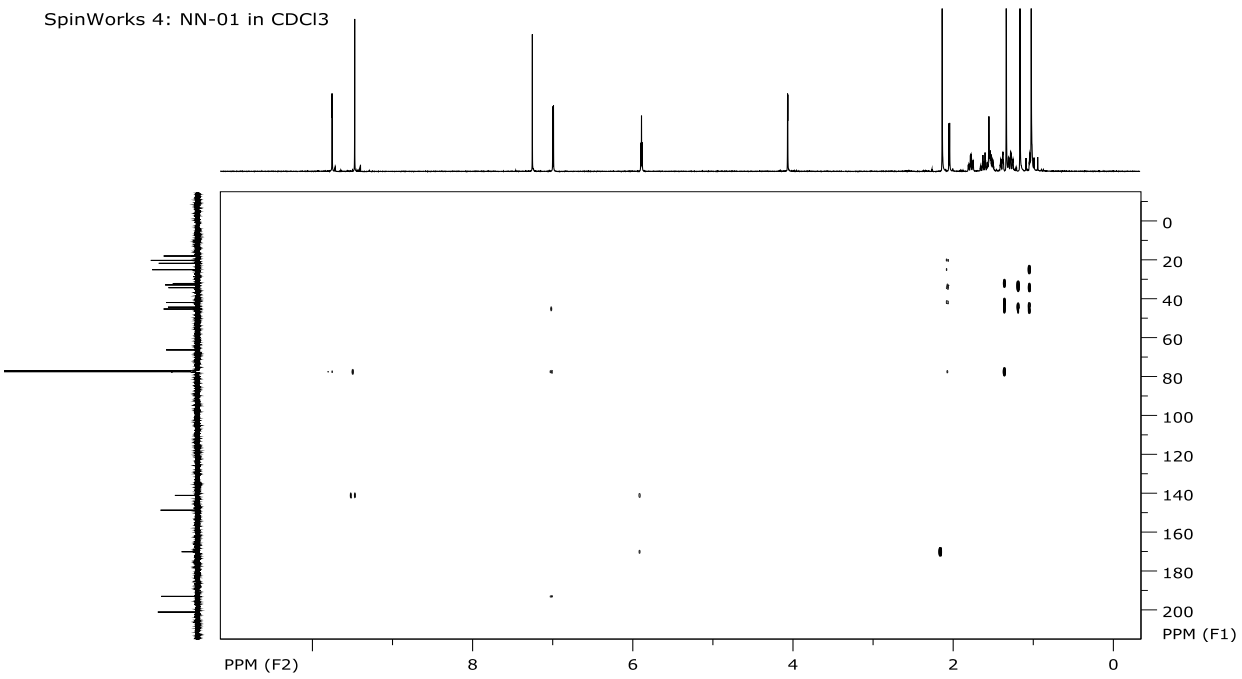

Figure 5: NN-01 hmbc spectra for NN-01

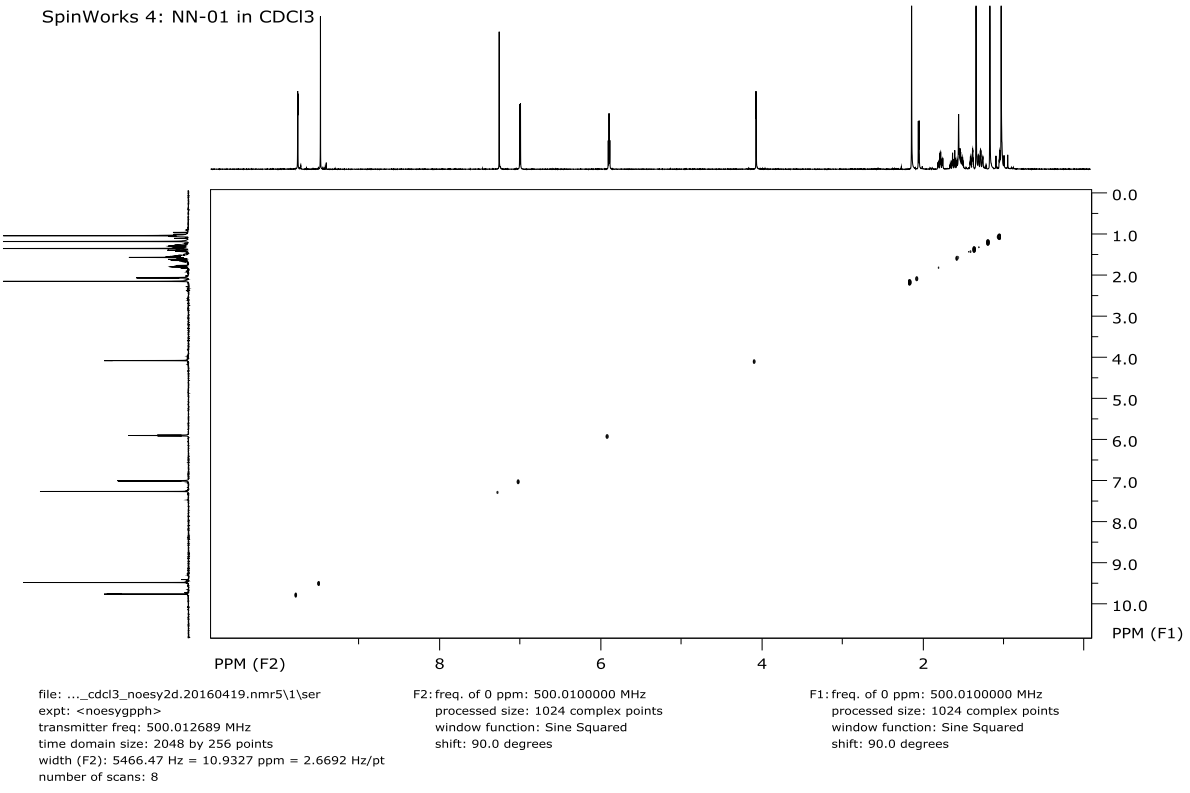

Figure 6: NN-01 noesy spectra

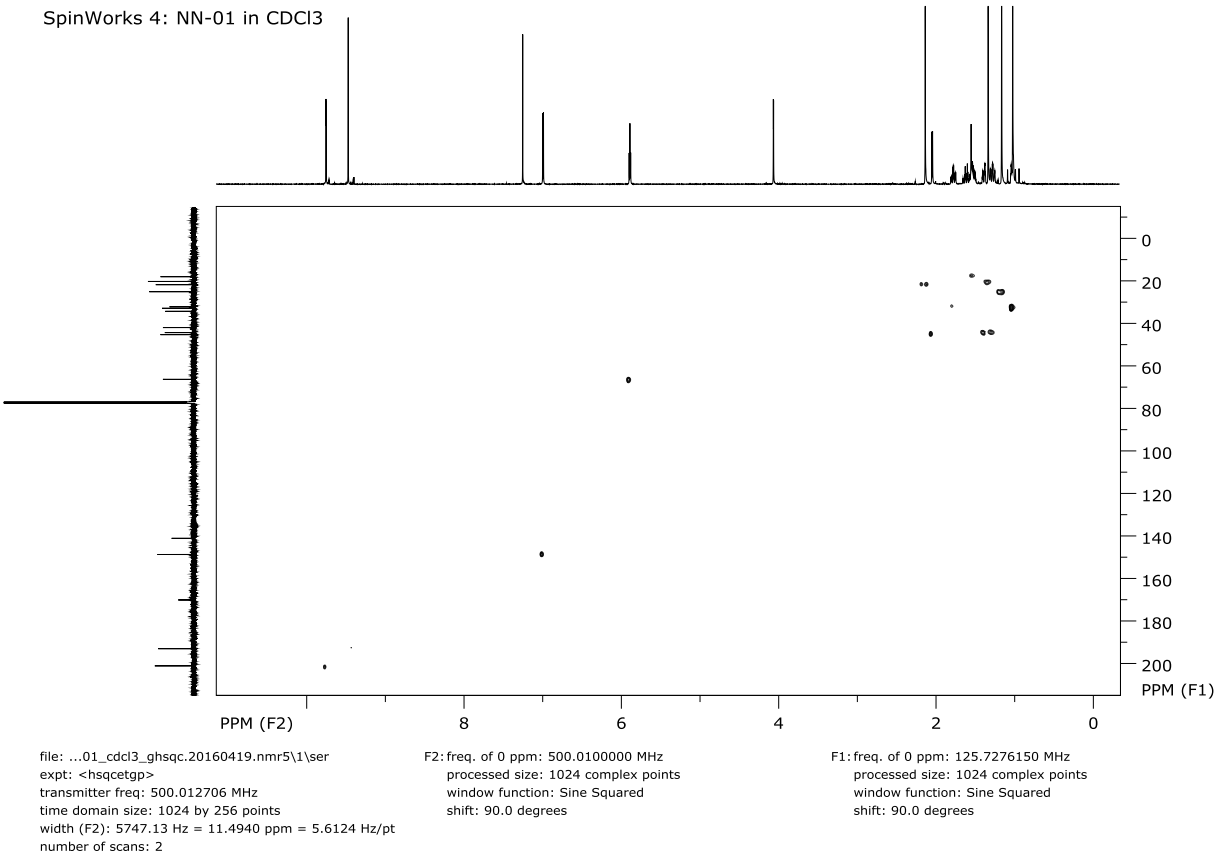

Figure 7: hsqc spectra for NN-01

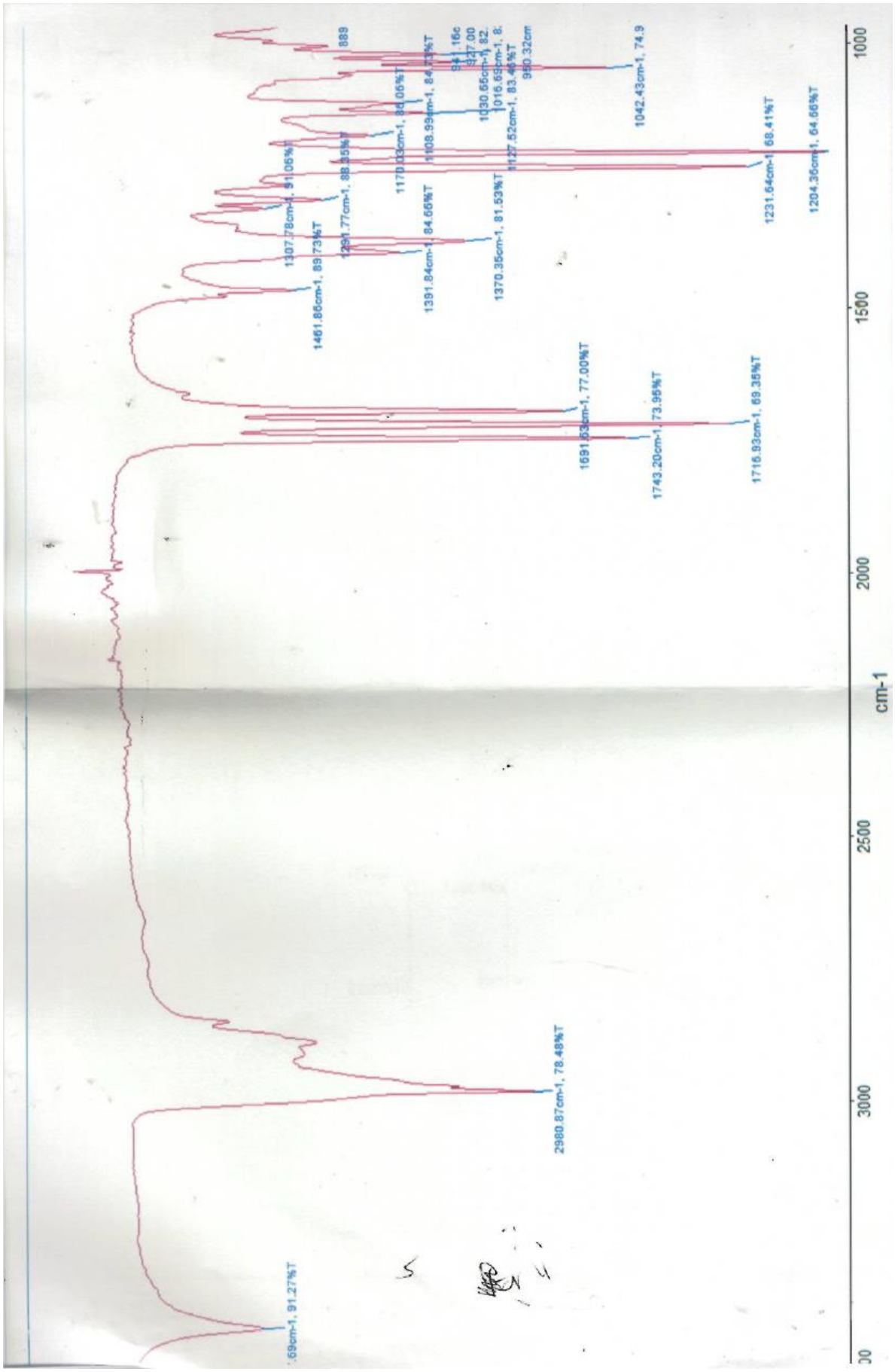

Figure 8: IR spectra for M. acetate

Appendix 4
Ethical approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

05 July 2017

Ms Zoxolo Nokulunga Nyaba (211529312)
School of Life Sciences
Pietermaritzburg Campus

Dear Ms Nyaba,

Protocol reference number: AREC/053/016M
Project title: In vivo antiplasmodial activity of *Warburgia salutaris*

Full Approval – Research Application

With regards to your report on the acute toxicity and pilot study received on 22 June 2017. The proposed plan of the main study submitted have been accepted by the Animal Research Ethics Committee and **APPROVAL** for the protocol has been granted. According to the new proposal, only 48 animals will be allowed to use for the main study.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 06 July 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Mthokozisi BC Simelane
Cc Dean & Head of School: Professor A Olaniran
Cc: NSPCA: Ms Stephanie Keulder
Cc Registrar: Mr Simon Mokoena
Cc: BRU – Dr Sanil Singh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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Appendix 5
Contribution to knowledge

Antiplasmodial activity of new sesquiterpene isolated from *Warburgia salutaris*

Z.N. Nyaba[1], P. Smith[2] and M.B.C. Simelane[1]

[1]School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, Scottville 3209, South Africa

*Email:simelanem2@ukzn.ac.za

[2]Division of Pharmacology, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

Background

- Malaria is a life-threatening disease mainly in tropical and subtropical regions. (1)
- In 2013, about 584,000 people died from malaria worldwide, with 90% of these deaths occurring in Africa.(2)
- Despite the advances that have been made in the treatment of this disease, it still remains a burden as some species have become resistant to the currently available drugs. (3)
- About 80% of rural African population mainly rely on traditional medicine to treat various illnesses. (4)
- *Warburgia salutaris* is a medicinal plant used to treat malaria in Zulu traditional medicine.(5)
- This project aims to scientifically validate the use of *W. salutaris* in traditional medicine against malaria

Materials and method

- The plant was collected from the UKZN Pietermaritzburg campus Botanical garden (29° 37′ 30″ S, 30° 24′ 14″ E).
- Extraction was done using dichloromethane.
- Isolation was done using column chromatography.
- The compound was identified and characterized by spectroscopic analysis(^{1}H NMR, ^{13}C NMR, and MS).
- The *in vitro* antiplasmodial activity was done using a chloroquine-sensitive *Plasmodium falciparum* strain (NF54).

Results and discussion

- Column chromatography led to the isolation of a new compound (NN-01).
- It is the first time this compound is isolated from *W. salutaris*.
- The compound showed good inhibition on the parasite growth.
- The cytotoxicity activity of NN-01 showed 55% and 75% cell viability for HEK293 and HEPG2 respectively.

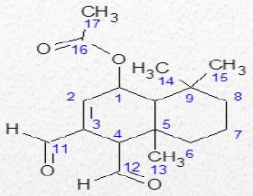

Figure 1: Structure of NN-01

Table 1: *In vitro* antiplasmodial activity of NN-01 against a chloroquine-sensitive *P. falciparum* strain (NF 54)

Sample code	*IC_{50}
NN-01	0.44±0.283
CQ	4.90±0.07▫
Art	<2▫

*IC_{50}: Inhibitory concentration; ▫=ng/ml

Conclusion

The antiplasmodial and cytotoxic activity of *W. salutaris* scientifically validates the use this plant in folk medicine.

Acknowledgements

The authors thank the University of KwaZulu-Natal Research committee and the National Research Foundation (SA) for funding this research.

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Certificate of Attendance

This is to certify that

Zoxolo Nyaba

attended the 25th SASBMB Congress

July 10-14th, 2016

East London International Convention Centre, South A

Graeme Bradley (PhD)
Chair Organising Committee

Appendix 6
Letter of edit

Bel Lettres

Editing Services

Isabel Rawlins
izzy.rawlins@gmail.com
0845058392

17 December 2017

To whom it may concern

Re: Language editing of MSc thesis: "Antiplasmodial activity of Warbugia salutaris (Bertol.F.)Chiov.(Cannelaceae)" by Ms Zoxolo Nyaba

This letter serves to confirm that I, Isabel Rawlins did language editing in the dissertation named above. I therefore grant permission for the document to be sent for external examination.

Yours truly,

Isabel Rawlins MACW (Rhodes)