University of Venda

ISOLATION, CHARACTERISATION AND ANTIMALARIAL ACTIVITY OF FOUR SELECTED SOUTH AFRICAN PLANTS

BY

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DECLARATION

I, Adebayo Oluwakemi Monisola, hereby declare that this dissertation for the Master of Science (MSc) degree in Chemistry submitted at the University of Venda is an original research document and has not been submitted previously for a degree at this or any other University, that it is my own work in design and in execution, and that all reference materials contained herein have been duly acknowledged.

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(Signature of Candidate) Date

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ABSTRACT

Malaria, an infectious disease affecting both human beings and other animals, is transmitted by parasitic protozoans belonging to the *Plasmodium* genus. Malaria is commonly treated with drugs such as quinine, chloroquine, and artesunate. However, the incidence of treatment failure due to drug-drug interactions and parasite resistance is increasing. Therefore, the rich medicinal potential of plants found in nature in Africa is increasingly being explored.

The traditional use of *Lippia javanica*, *Sclerocarya birrea*, *Melia azedarach* and *Capparis tomentosa* for the treatment of malaria is well-known, but the phytochemistry of these four plants is not fully known. Parts of these plants were extracted and column chromatography was used to fractionate the extracts. The antioxidant activities of the fractions were determined using free radical scavenging and reducing power assays, while the cytotoxic, antiplasmodial and antitrypanosomal activities were determined using cell toxicity assay, parasite lactate dehydrogenase (pLDH) and trypanosome assay.

The methanol stem bark extract of *Melia azedarach* (Fraction 2) had the highest phenolic content (59.39 mg GAE/g), while the methanol leaf extract of *Melia azedarach* had the highest flavonoid content of 188.65 mg QE/g. In the reducing power tests and DPPH free radical scavenging activity, the methanol stem bark extract of *Melia azedarach* had the lowest IC_{50} value of 0.1074 µg/mL and an $IC_{0.5}$ value of 0.5296 µg/mL, respectively. Furthermore, the methanol stem bark extract of *Melia azedarach* at a concentration of 50 µg/mL showed significant cytotoxicity against HeLa cells (-1.22±0.07 %). The methanol stem bark extract of *Melia azedarach* at the tested concentration (250 µg/mL) decreased the viability of *Plasmodium falciparum* to 36.38±11.96 % with an IC_{50} value of 6.5 µg/mL. Concerning the antitrypanosomal activity, the methanol stem bark extract of *Melia azedarach* affected the viability of the trypanosomes at the tested concentration (250 µg/mL), giving a viability of 14.05 ± 0.59 %, with an IC_{50} value of 0.4 µg/mL. The presence of epicatechin (**29**) and catechin (**31**) in this extract was confirmed using several spectroscopic techniques (IR, NMR, UPLC-MS and HRMS).

Keywords: Phytochemistry, anti-plasmodial, Ethnopharmacology, *Lippia javanica, Sclerocarya birrea, Melia azedarach, Capparis tomentosa.*

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SYMBOLS AND ABBREVIATIONS

% w/v	percentage of weight of solute in total volume of solution
Abs	Absorbance
ACT	Artemisinin-based Combination Therapy
AIDS	Acquired Immune Deficiency Syndrome
n-C_6H_{12}	*n*-Hexane
CH_2Cl_2	Dichloromethane
CTL	*Capparis tomentosa* leaves
CTA	Cell toxicity assay
d	doublet
dd	doublet of doublets
DEET	Diethyl meta-toluamide
DHA	Dihydroartemisinin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-1-picrylhydrazyl radical
E.M.W.	Ethyl acetate/Methanol/Water (81:11:8)
ESI	Electrospray ionization
EtOAc	Ethyl acetate
HAT	Human African Trypanosomiasis
HeLa	Human cervix adenocarcinoma
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IC_{50}	50% inhibitory concentration
IR	Infrared spectroscopy
LJL	*Lippia javanica* leaves
m	multiplet

MAL	*Melia azedarach* leaves
MAS	*Melia azedarach* stem
MS	mass spectrometry
m/z	mass to charge ratio
MeOH	methanol
mg GAE/g	milligram gallic acid equivalent per gram
mg QE/g	milligram quercetin equivalent per gram
NMR	Nuclear magnetic resonance
pLDH	Parasite lactate dehydrogenase
ppm	parts per million
s	singlet
SBL	*Sclerocarya birrea* leaves
SBS	*Sclerocarya birrea* stem
SD	Standard deviation
TC_{50}	50 % toxic concentration
TFC	Total flavonoid content
TLC	Thin layer chromatography
TPC	Total phenolic content
UPLC-MS	Ultra-performance liquid chromatography - mass spectrometry
RDT	Rapid Diagnostic Test
WHO	World Health Organization

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Malaria is a vector-borne disease that is transmitted by parasitic protozoans (a group of single-celled microorganisms) which affect humans and animals. These parasitic protozoans belong to the *Plasmodium* family. Protozoans spread to humans through the bites of contaminated female *Anopheles* mosquitoes. Malaria is endemic in the tropics and sub-tropical areas of the world, including Africa, South Asia, and South and North America, and affects more than 650 million individuals, killing about 3 million people yearly.[1] This disease typically influences and kills children in Sub-Saharan Africa, making it a challenging general health issue.[2] Malaria is an old disease that has been affecting people for over 40 000 years. The name malaria originates from medieval Italian and is translated as “bad air”. Malaria is also known as ague and marsh fever because it is associated with swamps. Charles Louis Laveran, a French army doctor who worked in Algeria, was the first to notice parasites in the red blood cells of humans where he detected that malaria was caused by this protozoan. The first effective treatment for malaria was the cinchona tree stem bark which contains quinine.[3]

Malaria is currently a global disease and new cases of malaria were estimated by the World Health Organization in 2012 as being around 214 million, while about 89 million cases resulted in death where many of these cases occur in babies and children below 6 years old and about 125 million are pregnant women who fall victim of this disease where maternal malaria causes about 150 000 infant deaths globally and this is increasing. According to UNICEF and the WHO, there were 206 million incidents of malaria all over the world in 2015.[4] As at 2012, this disease was judged to have killed about 472 000 people, many of whom were young people in Africa.[5] The WHO African region was carrying the largest proportion of the global malaria burden in 2015. In South Africa, transmission of malaria is seasonal with the highest number of cases occurring around October to May, with a significant inter-annual variation in the number of malaria cases. For example, in 2016 there were approximately 7 755 malaria cases, rising to 12 098 in 2017 according to an investigation by the Department of Health.[3]

Efforts have been made to reduce this ailment in Africa. However, these have only been moderately successful, and none has been able to get rid of this disease entirely. Malaria has been treated commonly with synthetic drugs such as quinine, chloroquine, and

artesunate. Treatment failure has led to the combination of two or more drugs to combat resistance, for example using artemisinin-based combination therapy (ACT) such as artemether + amodiaquine, artemether + lumefantrine, dihydroartemisinin. These are used currently in the treatment of malaria.

Generally, synthetic drugs have been found to be very expensive, which makes them mostly unavailable. Due to these limitations, Fabricant found that there are medicinal resources found in plants around us in Africa, as well as in the rest of the developing world, which are being used as pharmacological agents in the production of bioactive compounds which serve as leads for use as medicines.[6] Generally, plants contain phytochemicals, which makes them globally acceptable in the treatment of diseases in the rural areas of South Africa and other parts of the world, where these plants are accessible and affordable forms of treatment for communities across the continent. It is believed that these medicinal plants are less harmful to humans than synthetic drugs.[6]

1.2 Problem Statement

In recent years it has been found that herbal therapies are less damaging and thus safer to the human body than synthetic drugs. The long-term use of herbal concoctions seems to suggest that these herbs are safer. However, this is not entirely true because documented evidence of either adverse or non-adverse effects of medicinal plants is lacking.[7] Another challenge facing the use of traditional medicine is dosage; they are usually taken at the discretion of the patient, where lack of scientific evaluation of the constituent poses a danger on the use. Recently, there has been a reappearance of interest in plants as sources of medicine and new molecules for elucidation of physiological/biochemical phenomena. Ethnobotanical screening programs have been conducted on South African plants and a few studies have been adopting more direct approaches to isolate some compounds of these plants.[7] For example, traditional healers in South Africa use *Lippia javanica*, *Sclerocarya birrea*, *Melia azedarach* and *Capparis tomentosa* for the treatment of malaria because they are readily available. However, though the phytochemistry of these four plants has been investigated, there is insufficient information about the chemical constituents of these plants.

The major chemical compounds present need to be isolated, characterized and screened. Furthermore, their possible bioactivity needs to be documented. Against this backdrop, a phytochemical analysis can shed more light on the biological activity of these plants.

1.3 Aim

The aim of this study was to investigate the antimalarial activity of *Sclerocarya birrea, Lippia javanica, Melia azedarach* and *Capparis tomentosa*, by in vitro screening.

1.4 Objectives

i. To determine antimalarial activity of the crude extracts of four selected plants.
ii. To isolate, identify and determine the structures of the isolated bioactive compound(s) from extracts of plants that exhibited useful antimalarial activity.
iii. To determine the antimalarial activity of the fraction(s) and pure compounds of the plant(s) with the highest bioactivity.
iv. To determine the cytotoxicity of selected plant extracts.
v. To determine the antioxidant activity, total phenolic content and flavonoid content of the selected plant extracts.

1.5 Dissertation outline

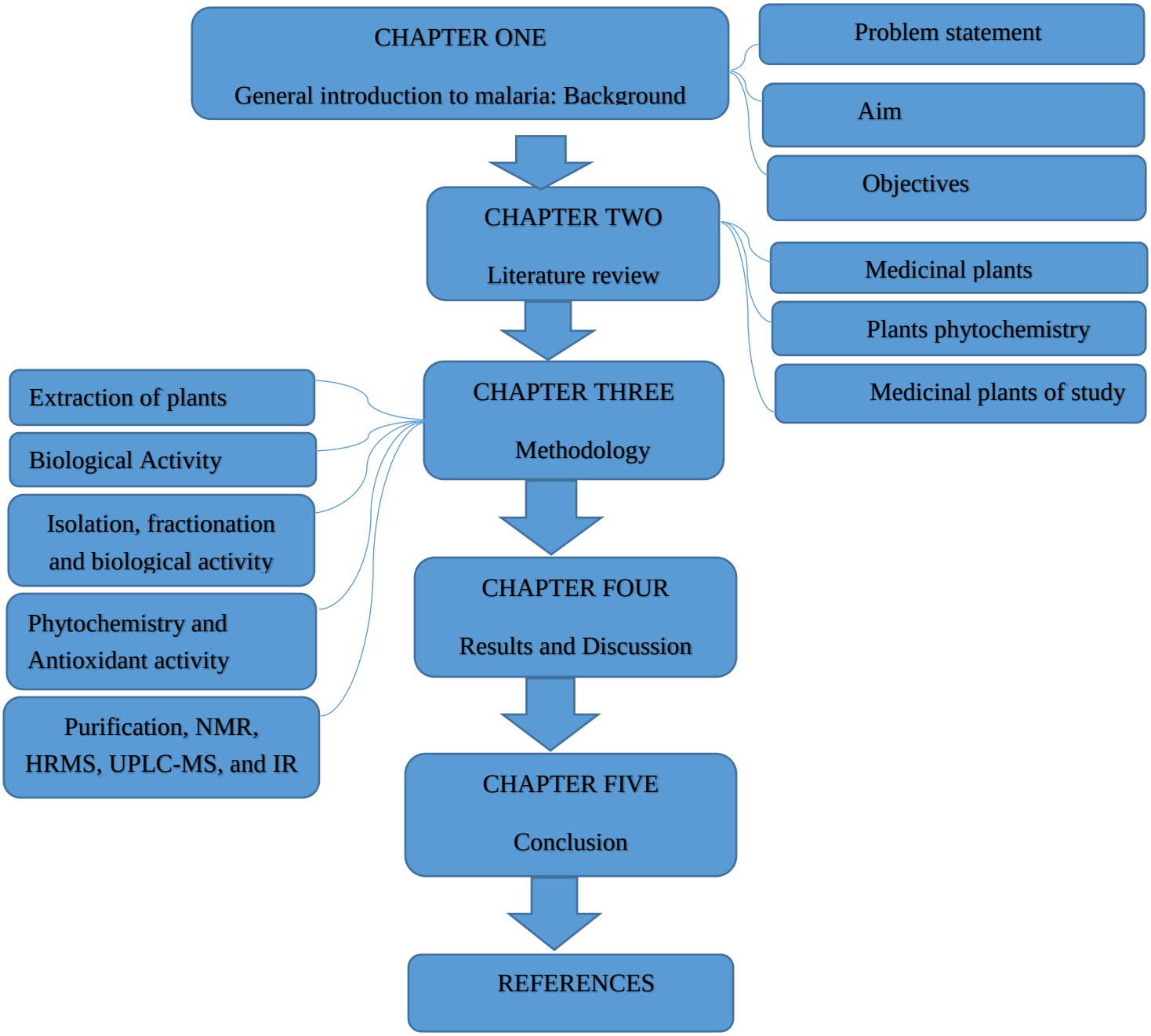


Figure 1.1: Outline of the dissertation

CHAPTER 2

LITERATURE REVIEW

2.1 Mode of transmission

Infected female *Anopheles* mosquitoes commonly transmit malaria. The parasites are introduced from the mosquito's saliva into a person's blood through a single bite; from there the parasites travel to the liver, where they mature and reproduce (Figure 2.1). The five species of *Plasmodium* that can infect and be spread by humans are *P. falciparum, P. vivax, P. ovale,* and *P. malariae*. Most deaths are caused by *P. falciparum* because *P. vivax*, *P. ovale*, and *P. malariae* generally cause a milder form of malaria, while the species *P. knowlesi* rarely causes disease in humans.[8]

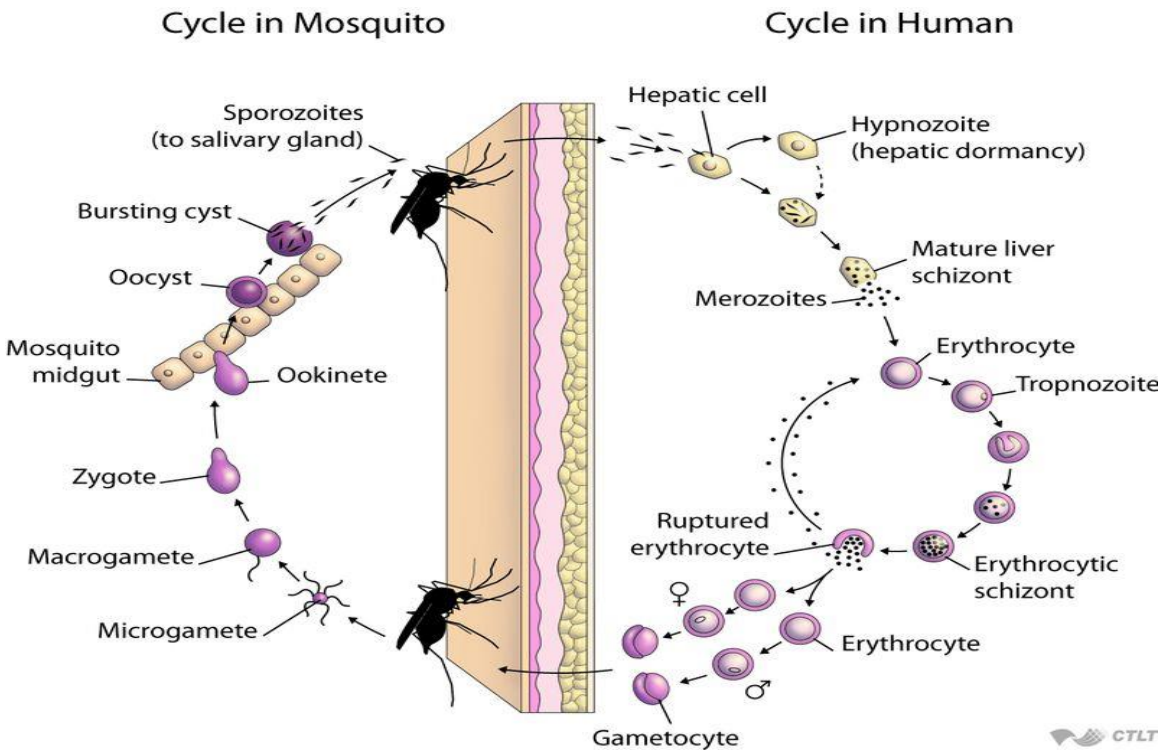


Figure 2.1: *Plasmodium* lifecycle.[9]

2.1.1 Complications

Many complications are associated with malaria, the most serious among these being the development of respiratory distress, which usually occurs in adults and children with severe *P. falciparum* malaria. Other possible complications include oedema, metabolic acidosis, respiratory compensation of metabolic acidosis, with concomitant pneumonia and severe anemia.[10] Acute respiratory distress syndrome occurs in 5-25 % of adult patients. *P. falciparum* also causes cerebral malaria, which is a severe form of malaria involving encephalopathy. This form of malaria is associated with retinal whitening, which is considered a useful clinical sign in distinguishing malaria from other types of fever. Severe headache, low blood sugar, and renal failure may also occur. Complications include spontaneous blood loss, stillbirths in pregnant women as well as death as a consequence of complications.[11]

2.1.2 Diagnosis

The microscopic examination of blood films is usually used to confirm malaria. Confirmation can also be done by antigen based rapid diagnostic tests (RDT). Typically, RDTs are used to detect if the malaria symptoms are caused by *Plasmodium falciparum* or other types of *Plasmodium* because the treatment method is different for the other types of *Plasmodium*-caused infections. The most sensitive and most common method to spot the malarial parasite is microscopy. The sensitivity of the blood film microscopy usually ranges from 75-90 % in optimum conditions to as low as 50 %. Commercially accessible RDTs are usually more accurate than blood films in determining whether malaria parasites are present.[12]

2.1.3 Prevention

Avoidance of mosquito bites is the backbone of malaria prevention. People living in areas that are malaria-endemic should use insecticide-treated bed nets in their rooms, and screened windows and doors, to create a barrier against mosquitoes.

Treatment with insecticide helps to reduce the lifespan of mosquitoes, thereby decreasing the chance of transmitting malaria from one person to another.[13] Insecticide-treated nets are meant to provide personal protection for people who sleep under them. This means that once a good number of people are using it, it will create community-wide protection, by

drastically reducing the number of mosquitoes in the community. Some communities in Limpopo and other affected areas in South Africa use *Lippia javanica* or *Thamnosma africana*, which are insect repellents, as prevention by planting them around their houses. Another preventive measure that can be taken is the use of effective repellents containing 20-50 % DEET (*N*,*N*-diethyl-*meta*-toluamide or *N*,*N*-diethyl-3-methylbenzamide). These provide long-lasting protection for both adults and children aged less than 6 months and should be encouraged. Lastly, travelers to malaria-endemic areas should use chemoprophylaxis, depending on the malaria risk assessment of the areas to be visited.[14]

2.1.4 Treatment

The methods used currently for evaluating the healing potency of antimalarial drugs are quite inaccurate and indifferent.[15,16] In severe malaria, these methods are inadequate, especially when the intention of treatment is to prevent complications and save life. In dissimilar clinical situations, antimalarial drug treatment should be considered in terms of the balance between the benefits of the antimalarial drug action and the risks of drug toxicity. In severe *falciparum* malaria, the balance is considerably different, compared with uncomplicated malaria infections.[17]

2.1.5 Medication

Combination therapy has been very effective so far and it includes at least two different blood schizonticidal drugs that have different modes of actions and different biochemical targets in the parasite. Furthermore, there are numerous rationales which have been used to support these mixed treatment uses; such as the issue of recognizing an appropriate medication for diverse epidemiological reactions, the cost viability of mix treatments, as it is more expensive than the traditional single medication treatments.[17] The currently prescribed combination treatment is categorized as artemisinin-based and non-artemisinin-based combination therapy.[18]

2.1.5.1 Mono/single therapy

Quinine (1) belongs to a group of alkaloids known as blood schizonticides and as weak gametocides when used against *P. falciparum, P. malariae* and *P. vivax*. Quinine is deposited in the food vacuoles of *Plasmodium* species and prevents hemozoin biocrystallization, consequently allowing an accumulation in the heme detoxification pathway, which enables the accumulation of free cytotoxic heme , achieving the death of the parasite.[19]

Chloroquine (2) belongs to the group of 4-aminoquinoline compounds. It reaches high concentrations in the parasite's acidic food vacuole because it is alkaline in nature; as a result, there is an increase in the internal pH. This leads to the transformation of toxic heme to hemozoin, which happens by preventing the bio-crystallization of hemozoin.This poisons the parasite by allowing increased levels of toxicity of the parasites.[20]

Amodiaquine (3) is a 4-aminoquinoline compound that acts by inhibiting the activity of the heme polymerase, resulting in the build-up of heme, which is poisonous to the parasite. It binds the free heme and prevents the parasite from transforming it to form less toxic products. The drug-heme complex is poisonous and the function of the membrane is disrupted.[21]

Pyrimethamine (4) is commonly used in chloroquine-resistant *P. falciparum* strains; it is used in combination with sulfadoxine and can be used in cases of uncomplicated malaria only.[8] The mode of action is to inhibit dihydrofolate reductase in the parasite, thereby halting the biosynthesis of purines and pyrimidines, and inhibiting DNA replication processes; this will in turn stop cell division and reproduction of the parasite. This primarily acts on the schizonts in the erythrocytic phase. This combination is currently only used with a sulphonamide or artesunate.[22]

Artesunate (5) is a hemi-succinate derivative of the active metabolite dihydroartemisinin. It is currently the most frequently used out of all the artemisinin-type drugs. The effect is mediated by a decrease in gametocyte transmission. In this process, hydrolysis of the 4-carbon ester group via plasma esterase enzyme is involved and it is postulated that the cleavage of endoperoxide bridges in the pharmacophore of DHA produces reactive oxygen species (ROS), which will then increase the oxidative stress and cause malarial protein destruction by alkylation. *Plasmodium falciparum* potently inhibits artesunate exported protein 1 (EXP1), a membrane glutathione S-transferase. The amount of glutathione in the parasite is reduced as a result of this inhibition.[23]

Artemether (6) is a derivative of artemisinin.[24] The mechanism of action for artemisinin is not known yet but one of the proposed mechanisms is that by inhibiting the antioxidant and the metabolic enzymes of the parasite, oxidative and metabolic stress will be inflicted by artemisinin on the cell; some pathways affected in the parasites concerned are glutathione and glucose metabolism. As a result, lesions and reduced growth of the parasite may occur.[18]

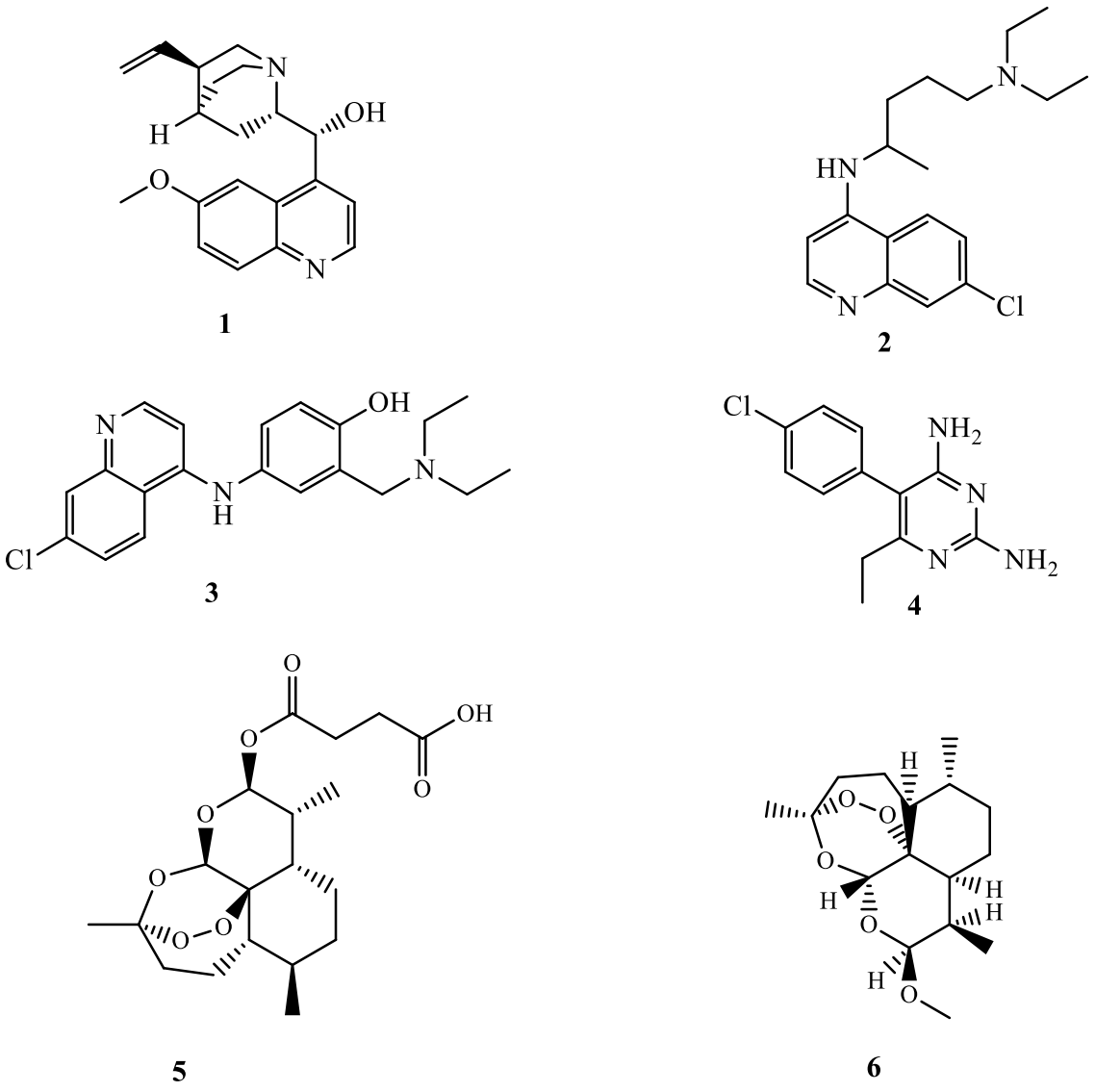


Figure 2.2: Structures of drugs used in non-artemisinin-based therapy

2.1.5.2 Artemisinin-based combination therapy (ACT)

The mode of action of artemisinin is very different when compared to the conventional antimalarial drugs. Artemisinin is a sesquiterpene lactone which contains a rare peroxide

bridge. This peroxide is responsible for the mechanism of action of the drug.[119] This causes a rapid reduction in the parasite biomass, resulting in a reduction in clinical symptoms, reducing the transmission of gametocytes, thereby reducing the potential for the spread of resistant alleles. The commonly-accepted theory is that they are activated firstly through cleavage after reacting with heme and iron (II) oxide, which results in the generation of free radicals that in turn damage susceptible proteins, resulting in the death of the parasite.[25]

Artemether (6) and lumefantrine (7): Lumefantrine is only used when combined with artemether to treat acute uncomplicated malaria. The name 'co-artemether' is mostly used to describe the combination therapy. The half-life of lumefantrine is longer than artemether, and any residual parasite that remains after combination treatment is therefore cleared. Commercial generics such as Coartem and Raiment are also available.[11]

Amodiaquine (3) and artesunate (5) are only used in combination with amodiaquine. These have been tested and proven to be very effective, as amodiaquine retains some efficacy.[122] A suggested link with neutropenia is a potential disadvantage. Commercial generics such as Coarsucam and ASAQ are also available.[26]

Sulfadoxine (8) / pyrimethamine (4) and artesunate (5) are a combination that is usually well-tolerated in the body. However, the efficacy is dependent on the body system's level of resistance to any of the components, thereby regulating its use. This is following the WHO recommendation for usage in uncomplicated *P. falciparum* malaria. Generics such as Ariplus and Amalar plus are available.[27]

Dihydroartemisinin (6) piperaquine phosphate (9): The active metabolite of all artemisinin derivatives is dihydroartemisinin (artemether, artemisinin and artesunate); this is also available as a drug on its own. The slow absorption and long half-life characteristic of piperaquine[26] make it a good partner drug for artemisinin derivatives,[29] which are fast acting. However, they have a short half-life. Generics are Duo-cotecxin and Eurartesim.[30]

Mefloquine (10) and artesunate (5): This combination is used to treat chloroquine-sensitive or resistant *Plasmodium falciparum* malaria. Remarkably, the combination with artesunate seems to reduce the adverse reactions, possibly due to the slow initiation of the action of mefloquine. Generics such as Artequine and ASMQ are also available.[21]

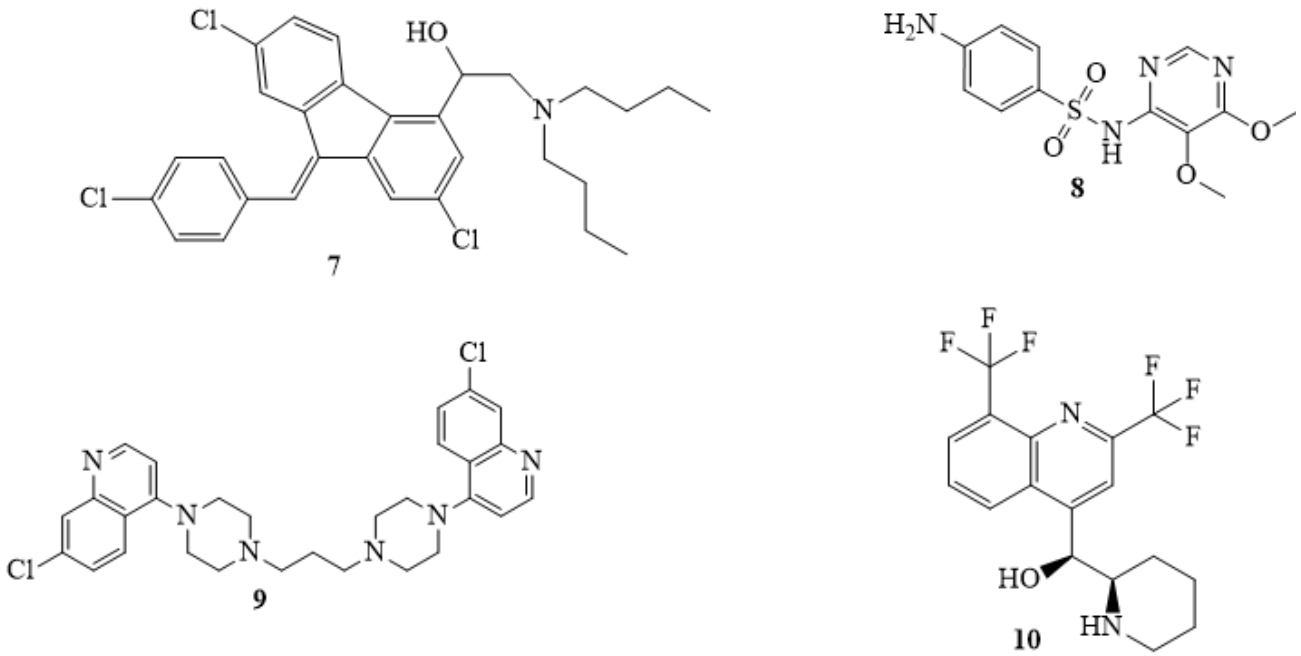


Figure 2.3: Structures of drugs used in artemisinin-based combination therapy.

2.1.6 Drug resistance

Treatment failure may be caused by drug resistance. However, drug resistance is not really caused by treatment failure; other factors include adherence and noncompliance, poor drug quality, drug-drug interactions, malabsorption, wrong diagnosis and giving of incorrect doses of drugs. These biological influences on the survival ability of the parasite to the existence of an antimalarial allow the potential for further transmission and persistence of resistance. Even with medication, therefore, any factor that acts by reducing or removing parasites might enable the progress of drug resistance. This could explain the poor response associated with individuals whose immune systems are compromised. The use of antimalarial products with the same chemical compound also increases resistance rates.[31]

Antimalarial resistance is sometimes increased by processes which occur in some species of *Plasmodium*. Therefore, when using combination therapy, the pharmacokinetics of the chosen antimalarial is important: the choice between a drug with a long half-life and one that is metabolized rapidly may be very complex and still remains unclear. Furthermore, when a drug combination is mismatched; for example, having an 'unprotected' period,

where only one drug prevails, can increase the likelihood that parasites become resistant. Therefore, the treatment regimen is an important factor.[32,33]

2.1.7 Limitations of synthetic drugs

Resistance to chloroquine was the first to be discovered. The development of this resistance was later found to be due to the evolution of an efflux mechanism that expels chloroquine from the parasite before the required level is reached to inhibit effectively the process of heme polymerization.[126] Resistance to other quinolone antimalarials, such as halofantrine, amodiaquine, quinine, and mefloquine, are believed to have the same mechanisms.[33] *Plasmodium* species have developed resistance to antifolate combination drugs as sulfadoxine and pyrimethamine are commonly used. There are two gene mutations which are assumed to be responsible for allowing the synergistic blockage of the two enzymes that are involved in the folate synthesis. Atovaquone is only used when combined with another antimalarial compound, as the selection of resistant parasites occurs very rapidly when it is used as a monotherapy.[21,34] Resistance is now common against all classes of antimalarial drugs, even artemisinin. The treatment of resistant strains is increasingly dependent on this class of drugs. However, resistance to artemisinin has been detected in South Africa, too.

Before the emergence of modern medicinal chemistry, natural products derived from plant and animal materials were used by mankind to treat diseases.[35] There exists a long history of Chinese, Sumerian, Indian and Egyptian pharmacopoeias describing the application of natural substances to cure diseases. As modern science developed, natural products served as a point of departure for pharmacological development. However, the field of natural products remains under-explored. As a result, some 99 % of bacterial species in our environment have not yet been properly investigated, and only about 4 % of the Earth's 250 000 plant species have been screened for some kind of biological activity.[35]

2.2 Medicinal plants for pharmacological purposes

Worldwide, medicinal plants have been used historically by rural people as their main remedies for many illnesses. Medicinal use of herbs and plants can be traced as far back as the Sumerian and Akkadian human civilizations of around three thousand years BC.[36] The ancient Greek author Hippocrates (ca. 460–377 BC) described medicinal products of plant and animal origin, and listed about 400 different medicinal plant species. Natural

products have always been an essential part of traditional medicine systems, such as the Chinese, Ayurvedic, and Egyptian traditions.[37,38,39]

Around 88 % of the world's population depends primarily on traditional medicine for their primary health care.[40] The World Health Organization defines a medicinal plant as any plant that contains a substance/s in at least one of its parts, which can be utilized therapeutically, or are precursors for chemical semi-synthesis.[41] These include fruit, leaves, roots, rhizomes, stems, bark, flowers, grains or seeds, used to control or treat a disease condition. Such non-food plant components or bioactive parts are called phytochemicals (from Greek 'phyto-' signifying 'plant') and are generally responsible for protection of the plant against microbial infection or invasion.[6,42]

Phytochemicals have also been associated with a range of fruit; for example, grapes and apples; vegetables, such as broccoli and onion; flavors like ginger and turmeric; and drinks such as green tea and red wine[40]. The investigation and application of these indigenous or local remedies is currently called ethno-pharmacology. Ethnopharmacology has been an important part of scientific investigation of traditional medicines and is beginning to be incorporated into mainstream medicine. As a result, ancient catalogues such as *De Materia Medica*, *Historia Plantarum*, and *Species Plantarum*, have been re-published in endeavours to provide new scientific insights on the medicinal uses of plants.[6,43]

Medicinal plant-based treatments are accepted more and more by urban dwellers, perhaps because of the growing inefficacy of modern medication used to manage infections such as malaria, typhoid fever, gonorrhea, and tuberculosis, due to the growing resistance of several microbes to different medications. The growing cost of synthetic medication has also become an important contributor to the developing traditional medicines market.[44] The rapid population growth has made it almost impossible for present-day health facilities to satisfy the world's growing health needs, thus increasing the demand for natural health remedies. Challenges with the use of antibiotics, such as the increase in multi-drug resistant (MDR) strains of pathogenic bacteria, such as methicillin resistant *Staphylococcus aureus*, *Helicobacter pylori*, and MDR *Klebsiella pneumoniae,* have led to growing interest in plants with antimicrobial properties. Furthermore, the rising rate of opportunistic infections concurrent with HIV infections and people on immunosuppressive chemotherapy, as well as the relative toxicity of many antifungal and antiviral drugs, have increased the pressure on medicinal and pharmaceutical companies to investigate new options and novel sources of medication.[45,46] The plant products are applied in various forms; for example poultices, concoctions of different plant blends, extracts as teas or tinctures, or as component

mixtures in porridges and soups, administered in various ways, including oral, nasal (smoking, sniffing or steaming), topical (salves, oils or creams), bathing or rectal (bowel purges).[47-49]

Rich sources of crude materials for primary health care in Africa and different parts of the developing world are presented by medicinal plants.[50,51] The *Vinca* alkaloids, tubocurarine, and reserpine are some important examples.[52]

2.3 Cytotoxicity of medicinal plants

A number of plants are being collected and utilized for the treatment of numerous diseases, while nothing or very little is known by the users about their harmful effects or how toxic the plants could be. Before these plants are used for sickness treatment purposes, appropriate measures must be taken to identify those plants that are really valuable for treatment of different diseases, while some will cause unfavourable effects on use, which could even lead to death. Unfortunately, most investigators who are studying plant activities are not looking deeply into the toxicities of these plant parts.[53] Cell culture assay study is very important to assess the cytotoxicity of plant materials, which helps to evaluate plants in vitro to understand whether it contains poisonous compounds in quantities large enough to kill cells either directly or indirectly through the inhibition of cell metabolic pathways.

There are 3 important parameters on which cytotoxicity estimations are done; namely, determining cell membrane integrity, which is one of the most widely recognized methods of estimating cell viability and cytotoxic effects. Compounds that have cytotoxic effects frequently trade off cell membrane integrity. Fundamental dyes, for example, Trypan-blue, are regularly rejected from within healthy cells; nonetheless, if the cell membrane has been compromised, they cross the membrane and stain intracellular components.[54]

Cytotoxicity can be determined by means of the metabolic MMT or MTS assay. This assay estimates the metabolic capacity by estimating cellular ATP levels or mitochondrial action. Receptive cells will reduce the MTS reagent to a coloured formazan compound. The third sort of assay is the direct counting of cell number, as dead cells ordinarily disconnect from a culture plate, and are washed away in the medium. Cell number can be estimated by direct cell counting. TC_{50} is the concentration causing a 50 % decrease in living cells.[45]

2.4 Antioxidant activities of medicinal plants

Antioxidants protect cells against the harmful effects of reactive oxygen species, generally called free radicals; for example singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite, resulting in oxidative stress causing cellular damage.[55] Natural antioxidants play a key role in the health maintenance and prevention of chronic and degenerative diseases; for example, atherosclerosis, cardiac and cerebral ischemia, carcinogenesis, neurodegenerative disorders, diabetes during pregnancy, rheumatic disease, DNA damage and ageing.[56] Antioxidants act by scavenging the 'free oxygen radicals,' producing a relatively 'stable radical'. The free radicals are metastable chemical species, which tend to trap electrons from available molecules. If these radicals are not scavenged effectively, they may damage key biomolecules such as lipids, proteins (including those present in all membranes), mitochondria, and DNA, causing abnormalities and leading to disease.[57]

It has been reported that free radicals are associated with a number of diseases, including tumor inflammation, hemorrhagic shock, atherosclerosis, diabetes, gastrointestinal ulcerogenesis, asthma, rheumatoid arthritis, cardiac disorders, cystic fibrosis, neurodegenerative diseases (such as Parkinsonism and Alzheimer's disease), AIDS and even early ageing.[58] The human body produces its own antioxidants; for example, glutathione and catalases, which help to eradicate the free radicals produced in the body. However, they are insufficient in the prevention of oxidative stress. Consequently, this deficiency must be compensated by intake of common exogenous antioxidants; for example, vitamin C, vitamin E, flavones, β-carotene, and other typical substances found in plants. Plants contain a wide range of free radical scavenging molecules, including phenols, flavonoids, and terpenoids. These have good antioxidant activities. Numerous plants, citrus products and soil vegetables contain ascorbic acid, vitamin E, carotenoids, flavonols and phenolics, which have the capacity to search out and neutralize free radicals in the human body. Noteworthy, antioxidant properties have been recorded in phytochemicals that are important for reduction in the event of numerous diseases.[53]

Many dietary polyphenolic constituents obtained from plants are more viable antioxidants in vitro than vitamins E or C. In this way the constituents may contribute altogether to the defensive effects in vivo. Methanol extract of cinnamon contains a number of antioxidant compounds that can effectively scavenge reactive oxygen species, including superoxide anions and hydroxyl radicals as well as other free radicals in vitro.[39]

The fruit of cinnamon, an under-utilised and unpredictable piece of the plant, contains a good measure of phenolic antioxidant agents to fight the harming effects of free radicals and may protect against mutagenesis.[56] Antioxidants are often added to food to prevent oxidation, and they act by inhibiting the initiation and propagation steps leading to chain termination, thereby delaying the oxidation procedure.[60]

2.5 Phytochemicals of medicinal plants

Phytochemicals are plant chemicals that are not nutritive but rather have disease-preventing properties. They are redundant supplements, meaning they are not required by the human body to continue living. Plants produce these chemicals to protect themselves and can also protect humans against diseases. There are many known classes of phytochemicals. However, the common phytochemical classes are flavonoids, alkaloids, steroids, terpenoids, tannins, saponins, phenolics, and glycosides, which have been found in many plants; for example, lycopene in tomatoes, isoflavones in soy and flavonoids in flowering plants.[61] Various mechanisms of action of phytochemicals have been proposed. For example the, phytochemicals may inhibit microorganisms, interfere with some metabolic processes, or modulate gene expression and signalling pathways. Phytochemicals can be used as chemotherapeutic or chemo-preventive agents. Chemo-preventive phytochemicals can therefore be taken to prevent cancer. Plant components might be used for both chemoprevention and malignant growth treatment.[41]

Phytochemicals also act as antioxidants, hormones, and enzyme stimulants. In addition, they can interfere with DNA replication and act as antiplasmodial and anti-bacterial agents. Research has shown that a wide assortment of secondary metabolites, for example, alkaloids, flavonoids, terpenoids, and tannins, are usually found in plants with antiplasmodial properties.[62] A brief review of some important phytochemicals follows below.

2.5.1 Flavonoids

Flavonoids are important groups of polyphenols generally found among the flora. Structurally, they consist of more than one benzene ring in their structure (producing a range of fragrant C_{15} compounds) and their utilization as malaria prevention agents or free radical scavengers has been ascertained.[56,60] The structures are based on parent compounds, known as flavans. It is believed that flavonoids act by inhibiting the fatty acid biosynthesis (FAS II) of the parasite.[3] Flavonoids have also been shown to inhibit the influx

of L-glutamine and myoinositol into infected erythrocytes, and are known as pigments in higher plants. Several of them have been found to be active as anti-malarial flavonoids. Quercetin, kaempferol and quercitrin are common flavonoids, found in almost 90 % of flowering plants. The different groups of flavonoids include flavones, dihydroflavones, flavans, flavonols, and anthocyanidins (Fig. 2.4), proanthocyanidins, chalcones and catechin and leucoanthocyanidins. The basic subgroups of the flavonoids include flavones **11**, flavonols **12**, flavanones **13**, chalcones **14**, catechins **15**, anthocyanins **16**, and isoflavonoids **17**. Flavonoids occur abundantly in grain, roots, vegetables, bark, stems, leaves, drinks and thus form an important component of the human diet while also fighting some disease-causing microorganisms and infections in general.[63]

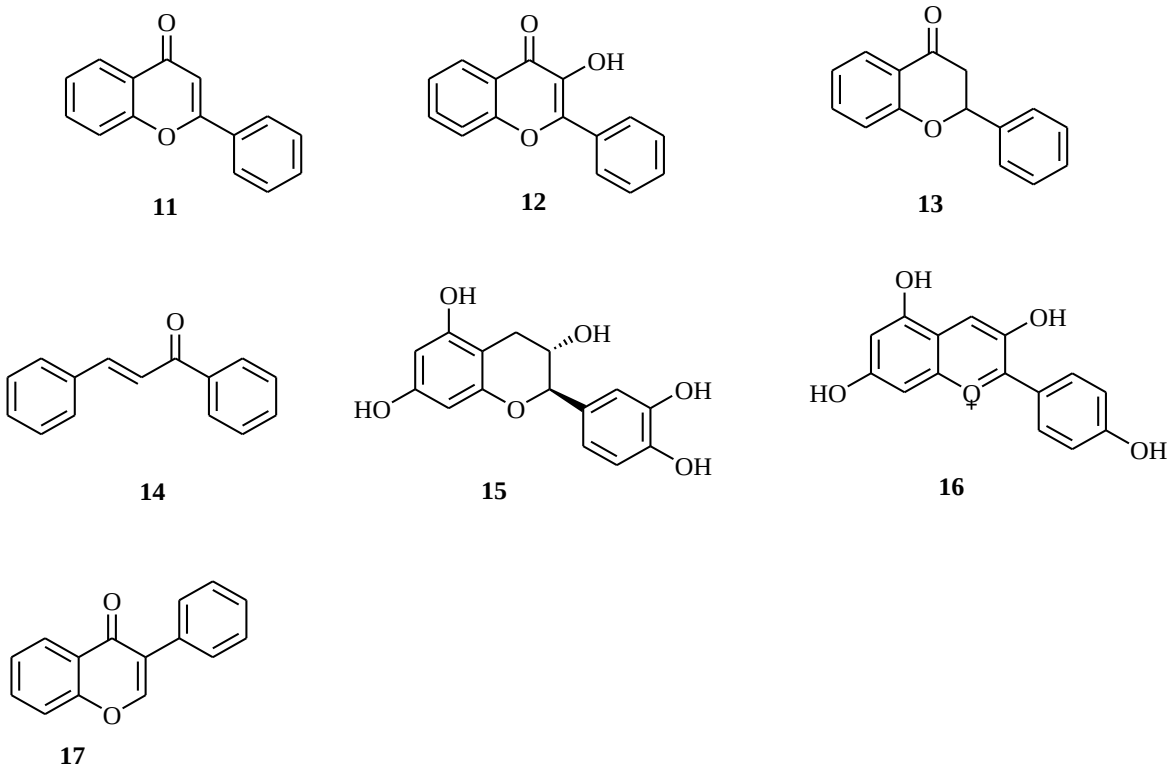


Figure 2.4: Examples of flavonoids

2.5.2 Tannins

Tannins are a class of astringent, polyphenolic biomolecules that bind to and precipitate proteins and various other organic compounds, including amino acids and alkaloids. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissues. Tannins have the characteristic feature to tan, that is, they convert skin to leather. They are acidic because of the presence of phenolic and carboxylic groups.[3]

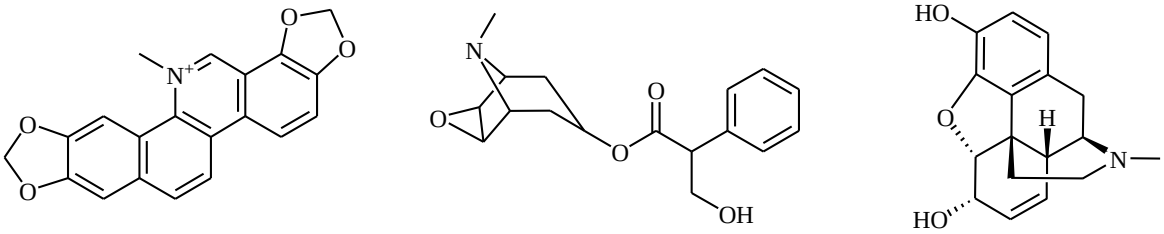
Tannins form complexes with proteins, sugars, gelatin and alkaloids. Furthermore, they are categorized as water soluble tannins and condensed tannins. Water soluble tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending upon the sort of acids created, the water soluble tannins are called gallotannins or ellagitannins; they form pyrogallic acid on heating. Tannins can be used as an antiseptic due to the presence of the phenolic groups. Typical hydrolysable tannins include the aflavins (from tea), daidezein, genistein and glycitein.[37]

Tannin-rich medicinal plants (such as pomegranate, *Punica granatum*) have been found to produce gallagic acid and punicalagin from ellagitannin, which exhibit high antiplasmodial activity against *Plasmodium falciparum* strains D6 and W2 and are used to treat a number of diseases without any toxic effects. In Ayurveda, formulations based on tannin-rich plants have been used to treat diseases such as malaria, leucorrhoea and rhinorrhea.[38,39]

2.5.3 Alkaloids

This is the largest group of secondary metabolites, consisting of nitrogen-containing organic compounds derived from amino acid building blocks, with different organic groups attached to the peptide chain. The alkaloids are organic bases because of the lone pair of electrons associated with the nitrogen atom, and may be primary, secondary or tertiary amines.[64]They react with acids to form crystalline ammonium salts. Most alkaloids exist as solids, for example atropine **19**. Furthermore, most alkaloids are insoluble in water, whereas their salts are water-soluble.[65] In nature, the alkaloids exist widely in the seeds and roots of plants and regularly as mixtures with plant acids. Alkaloids have pharmacological applications as anesthetics and CNS stimulants.[118] More than 12 000 alkaloids have been found in about 20 % of the plant species and just a few have been investigated for medicinal purposes.[65]

Alkaloids in clinical use include analgesics morphine **20** and codeine, the muscle relaxant (+)-tubocurarine, antimicrobial and antiplasmodial compounds sanguinarine **18** and berberine, the anticancer agent vinblastine, the antiarrhythmic ajmaline, the pupil dilator atropine **19**, and the narcotic scopolamine. Other important plant parts are leaves, seeds and root alkaloids addictive stimulants caffeine, nicotine, codeine, atropine **19**, morphine **20**, ergotamine, cocaine, nicotine and ephedrine (Fig. 2.5). Amino acids are precursors for alkaloid biosynthesis with ornithine and lysine commonly used as starting materials.[66]



18 **19** **20**

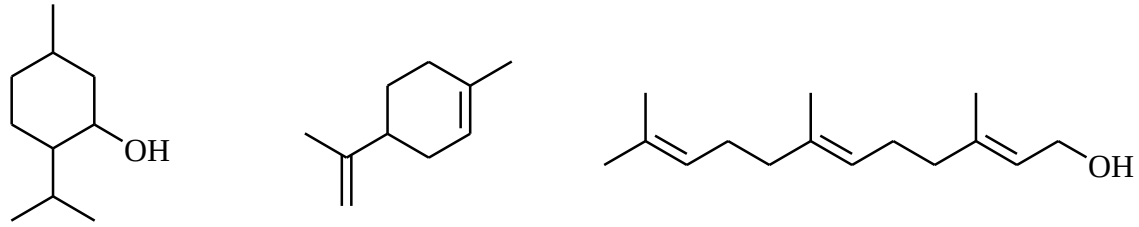
Figure 2.5: Examples of alkaloids

2.5.4 Terpenes

Terpenes are among the most extensive and chemically varied groups of compounds. They are unsaturated hydrocarbons existing in liquid systems, such as essential oils, juices or oleoresins.[65] Simple terpenoids are based on multiples of isoprene, with a typical formula $(C_5H_8)_n$. They are classified by the number of isoprene units associated with the development of these compounds and exist as monoterpenes, diterpenes, triterpenes, hemiterpenes, sesquiterpenes, and polyterpenes (e.g., rubber) (Fig. 2.6). Examples of monoterpenes (C_{10} compounds) are terpinen-4-ol, thujone, camphor, eugenol, terpineol, menthol **21** and limonene **22**. Diterpenes (C_{20} compounds), are traditionally found in latexes, and the anticancer agent Taxol is a good example. The triterpenes (C_{30} compounds), include steroids, sterols, and heart or cardiac glycosides with mitigating, soothing, insecticidal or cytotoxic activity.[67]

Some common triterpenes are amyrins, ursolic acids and oleano lic acid. Sesquiterpenes (C_{15} compounds), like monoterpenes, are found in many essential oils (e.g., humulene, farnesenes and farnesol **23**. A large number of sesquiterpene lactones has been described and broadly they have antimicrobial (especially antiprotozoal) and neurotoxic activities.

The sesquiterpene lactone, palasonin, isolated from *Butea monosperma*, has anthelmintic activity, inhibits glucose absorption and lowers the glycogen content in *Ascaridia galli*.[68]



21 **22** **23**

Figure 2.6: Examples of terpenes

2.5.5 Glycosides

Glycosides are colourless organic, water-soluble phytoconstituents, found in the cell sap of plants. Chemically, glycosides contain a sugar (such as glucose) and a non-carbohydrate part (aglycone or genin).[65] The aglycones are mostly alcohols, glycerol or phenols. Glycosides are neutral in reaction and can be quickly hydrolyzed into their components with mineral acids. Glycosides are characterized based on the kind of sugar, chemical nature of the aglycone or their pharmacological action. The older or trivial names of glycosides typically have the suffix 'in' and typically contain the plant name, such as strophanthidin from *Strophanthus*, digitoxin from *Digitalis*, barbaloin from *Aloe barbadensis*, salicin from *Salix*, cantharidin from *Cantharides,* and prunasin from *Prunus.* More typically, however, the precise names are obtained by replacing the "ose" postfix of the parent sugar with "oside", for example vermonioside, kaempferol-3-*O*-β-rutinoside, kaempferol-3-L-rhamno-D-glucoside and quercetin-3-*O*-β-rutinoside.

Extracts of plants containing cyanogenic glycosides are utilized as flavouring agents in many pharmaceutical preparations. Amygdalin has been used in the treatment of cancer and as a cough suppressant in different preparations. However, excessive ingestion of cyanogenic glycosides can be lethal. Therefore, a few foodstuffs containing cyanogenic glycosides cause poisoning (extreme gastric irritation and damage) if they are not properly prepared.[69]

2.5.6 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant that possesses large amounts of saponins that were once utilized as a cleanser. Saponins behave 'soaplike' in water; for example, they produce foam. On hydrolysis, an aglycone is created, called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal.[70]

Sapogenins are classified into (i) phenolic acids, (ii) flavonoid polyphenolics (flavanones, flavones, xanthones and catechins), and (iii) non-flavonoid polyphenolics. Caffeic acid is the most widely-occurring phenolic compound in the floral world, followed by chlorogenic acid, which is known to cause contact dermatitis among humans. Lastly, phenolic compounds were found to be the major antimalarial components of *Parkia biglobosa*.[71]

2.5.7 Phenolics

Phenolics (Figure 2.7) represent a large group of common antioxidants found in apples, green tea, and red wine. They are known as nutraceuticals due to the capacity of some to stop malignant growth and to some precursors are flavones, rutin, naringin, hesperidin and chlorogenic acid. They also can be classified chemically as hydroxybenzoic acid derivatives; for example, gallic acid **24** and derivatives of hydroxycinnamic acid, for example, coumaric, caffeic **25**, ferulic **26** and sinapic acids **27**.[50]

2.5.8 Steroids

Plant steroids (or steroid glycosides), also called 'heart' or cardiotoxic glucosides are amongst the most prominent plant phytoconstituents that are used therapeutically as cardiac medication.[65] The steroid, ergosterol-5,8-endoperoxide isolated from the aerial parts of *Ajuga remota*, exhibited high antiplasmodial activity against the chloroquine-sensitive *P. falciparum* FCA20/GHA strain.[72] Steroidal saponins with anti-malarial activity have also been isolated from the leaves of *Vernonia amygdalina*.[73] Ohigashi *et al*. (1994) reported the isolation of vernonioside A1, which also has high antiplasmodial activity against *P. falciparum*. Diosgenin and cevadine (from *Veratrum viride*) are instances of plant steroids.[72]

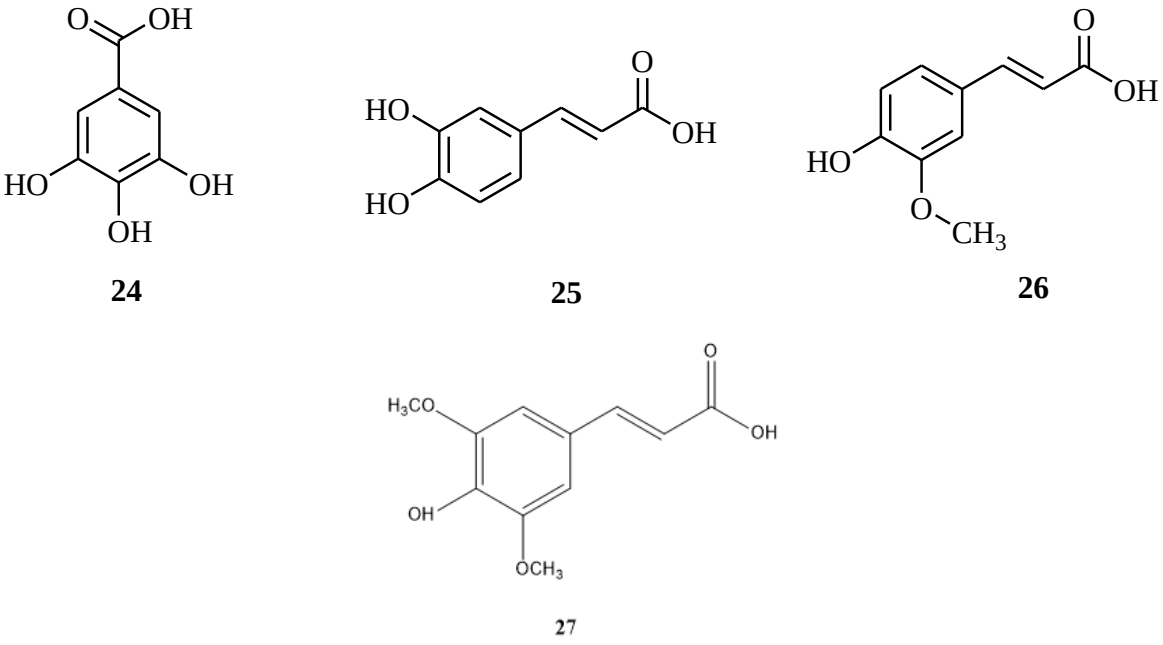


Figure 2.7: Examples of phenolic compounds

2.6 Medicinal plants in this study

Malaria causes suffering, death and economic loss in endemic areas, especially in sub-Saharan Africa. Thus, this calls for extensive research and development of affordable, effective and safe antimalarial drugs. When antimalarial resistance is again increasing and the majority of African households do not have access to affordable and effective antimalarial drugs, development and promotion of phytomedicines is a promising and sustainable solution to malaria treatment. Moreover, herbal medicines are widely reputed to be safe and effective. Most modern synthetic drugs used have been derived from higher plants, using leads from traditional knowledge. Examples of some of the most successful antimalarial agents developed from plants, relying on traditional knowledge leads, include quinolines and artemisinin derivatives.[58]

Many surveys carried out have revealed that traditional healers and local communities in the malaria endemic areas (mostly rural) of South Africa believed in the efficacy of some selected plants for herbal medicines, to treat malaria. These include *Adansonia digitata, Capparis tomentosa, Sclerocarya birrea, Albizia amara, Lippia javanica, Melia azedarach, Dalbergia nitidula, Lannea discolor and Vangueria infausta,* which are all considered as sources of antimalarial agents. These reports influenced our decision to select four plants from these commonly used plants for our studies. This was because these four selected

plants are used so widely ethnomedicinally. However, their antiplasmodial activities and efficacy as an antimalarial medication still need to be clarified.[74]

2.6.1 *Lippia javanica*

Lippia javanica (Figure 2.8) is a member of the family *Verbenaceae*, a group of shrubs, herbs or small trees with leaves that are fragrant; it typically occurs in central, eastern and southern Africa. These plants are widely available through parts of South Africa, besides the Western Cape. *L. javanica* is found from the Eastern Cape, northwards, extending into tropical Africa, including Botswana, Malawi, Tanzania, Kenya, Swaziland, Mozambique and Zambia.[61]

Figure 2.8: *Lippia javanica* plant
(www.bing.com.images *16/02/2017*)

Lippia javanica is used as a natural tea and has ethnomedicinal applications. Diverse classes of phytochemicals such as flavonoids, alkaloids, amino acids, triterpenes, and iridoids have been documented from *L. javanica*. Analyses show that it has a wide range of

pharmacological activities which relate to antimalarial,[75] anticancer, malignant growth anticipation operator, antidiabetic, antimicrobial and antiplasmodial effects.[76]

Many of the traditional uses of *L. javanica* have been supported by phytochemical and pharmacological investigations. However, phytochemical examination of *L. javanica* prompted the isolation of eight major compounds, 4-ethylnonacosane, (*E*)-2(3)-tagetenone epoxide, myrcenone, piperitenone,[77] apigenin, cirsimaritin, 6-methoxyluteolin 4'-methyl ether and 6-methoxyluteolin 3',4',7-trimethyl ether, tested against *Mycobacterium tuberculosis* and HIV-1 reverse transcriptase, for bioactivity. Lippialactone, obtained from the ethyl acetate extract of aerial parts of *Lippia javanica*, collected from South Africa, appeared to be active against the chloroquine sensitive D10 strain of *P. falciparum*.[75]

2.6.1.1 Insect repellent activity of *L. javanica*

Lippia javanica is commonly used as a mosquito repellent by local people in South Africa. In addition, past investigations have shown that essential oils from the species have solid and lasting repellent activity against the female *A. arabiensis*. The topical use of 5 mg/cm^2 of *L. javanica* also provided 100 % protection against *Anopheles aegypti* for 8 hours.[61,78] Ethanol leaf extracts of *L. javanica* are used for adulticidal action against *A. arabiensis*. Dichloromethane and ethanol extracts showed 45 % and 55 % mosquito mortality, respectively. These discoveries enhance the view that *L. javanica* is a potential antimalarial and to some degree support the traditional use of the plant species as a mosquito repellent in India,[79] South Africa,[79] and Zimbabwe.[80] Research has shown that the topical utilization of 5 mg/cm^2 of *L. javanica* extract provides 100 % protection against *Anopheles* mosquito for 8 hours.[13]

2.6.2 *Sclerocarya birrea*

Sclerocarya birrea (Figure 2.9), also called the *Marula*, with *Sclero* ("hard"), and *Carya* ("nut"), alluding to the plump fruit, contains a hard nut inside. The *marula* belongs to the *Anacardiaceae* family, which is normally a single-stemmed medium-sized tree with wide spreading crown, endemic to South Africa.

The tree is characterized by a faint dappled bark, while the fruit consists of a white flesh that is rich in ascorbic acid, containing several times the amount found in an orange. In

Africa, the fruit is used traditionally as a food and has some economic importance. The juice yields at least 34 sesquiterpenes. The nut is edible, containing 10.0 %, 55.0 %, and 30.0 % Protein, oil, and starch, respectively.[81] The oil contains oleic, palmitic, myristic, and stearic acids, while the kernel protein contains amino acids, with a predominance of glutamic acid and arginine. The gum is rich in tannin (0.4 %). Flavonoids and tannins are present in the leaves, but no alkaloids or steroids have been detected.[74] The fruit pulp contains citric and malic acids, ascorbic acid, and saccharides. The extracts of different plant parts have high total phenolic contents, radical scavenging ability, and antioxidant activity. *Sclerocarya birrea* has been widely studied with regard to its antidiabetic, anti-inflammatory, analgesic, antiparasitic, antimicrobial, and antihypertensive activities. Studies have also shown that the leaves contain compounds like terpinen-4-ol, being the most abundant compound (35.83 %), followed by pyrrolidine (32.15 %), aromadendrene (13.63 %) and α-gurjunene (8.77 %).[68]

Figure 2.9: *Sclerocarya birrea* tree

The gum from the tree is rich in tannin and is now and then utilized in making an ink substitute. Biological studies of *Sclerocarya birrea* have been done extensively and have demonstrated antiplasmodial and antimalarial, antidiabetic, and cell reinforcement activity.[82]

Sclerocarya birrea has been tested for in vivo and in vitro antimalarial activity, where the methanol extracts are more active than an aqueous extract against the *Plasmodium falciparum* strain D6 when tested in mice.[63]

2.6.3 *Melia azedarach*

Melia azedarach Linn (Fig. 2.10) is an invasive, deciduous tree which belongs to the family *Meliaceae*. The generic name is derived from the Greek word *'meli'* meaning ‘honey’ and the species name originates from the Persian word *'azzadirackt'* signifying "respectable tree".[85] It is also known as syringa, berry tree, Cape lilac, China berry, and China tree. It is widely distributed in Pakistan, Australia, Indonesia, India, Persia, Southeast Asia, South Korea and Japan. It is also widespread throughout all provinces in South Africa. It is also cultivated in Brazil, Philippines, Argentine and United States of America.[6]

Figure 2.10: *Melia azedarach* tree

2.6.3.1 The genus *Melia*

The genus name *Melia* was derived from *meli* the Greek word used by Theophrastus (c. 371-c. 287 BC) the Greek father of botany, for the ash tree, derived from "*meli*", the word for honey. Examples of species in this genus are *M. volkensii, M. toosendan, M. indica* and *M. dubia*.[83]

2.6.3.2 Taxonomy

Melia azedarach is known by many names, such as white cedar (English), umsilinga (isiZulu), bessieboom or maksering (Afrikaans); its full botanical classification is as follows:[84]

- **Kingdom** : plantae
- **Division** : Magnoliophyta
- **Class** : magnoliopsida
- **Order** : sapindales
- **Family** : *Meliaceae*
- **Genus** : *Melia*
- **Species**: *M. azedarach*

2.6.3.3 Ecology and physiology

Melia azedarach is a fast-growing deciduous tree that reaches a height of 4.5 to 10 m and has a canopy that is typically 6.5 m in width. The tree is frequently composed of a few smaller trunks because it can easily grow from the roots. Stems can vary in colour from olive-green and dark colored to a purplish red. Leaf scars from dropped leaves are three lobed and perceptible.[88] The bark has a dappled shading and is typically a dull dark-colored or ruddy, changing to light-dark colored spots. The leaves are interchanged and compound and ordinarily bi-pinnately; with lengths of 30-60 cm and width 23-40 cm. The leaves discharge a musky scent when pounded. The leaflets are spear-formed and decrease towards the tip with a dim green upper surface and a lighter green under surface. The blossoms are shorter, pink to lavender, star-molded and fragrant. Round yellowish-tan

berries are formed from the flowers. However, held together in groups; with each berry containing 1-6 seeds may stay on the tree after the leaves fall[89] (Figure 2.11).[85]

Figure 2.11: *Melia azedarach* leaves, flowers and fruits (https://en.wikipedia.org/wiki/*Melia_azedarach*, accessed on 16/02/2017).

2.6.3.4 Ethnomedicinal uses of *Melia azedarach*

The plant is used medicinally in the treatment of inflammation, leprosy, and cardiovascular disorders.[86] The organic extracts of *M. azedarach* have larvicidal and ovicidal effects, while the leave extracts additionally have antiviral and ovicidal activity. Medicinally, certain parts, for example, leave decoctions, are used by the Vhavenda people for treating malaria, leprosy, chicken pox, smallpox, and as blood purifier. In addition, the seeds are used to treat rheumatism, as anthelmintic, antileprotic, antipoisonous, and also are used in making mosquito coils. Young branches have been utilized for the treatment of intestinal sickness, diabetes, cough, and skin diseases. Exploratory and clinical studies have demonstrated that it has antimicrobial, mitigating, cell reinforcement, cardiodefensive, pain-relieving, antiplasmodial, antiulcer, antipyretic, and prophylactic properties.[91,132] On the other hand, the bark is used to produce anthelmintic, it is also used as anticancer agents,

antispasmodic, antiviral, gonorrhea, cough, fever, loss of appetite, vomiting, skin diseases and excessive thirst. The flower is an astringent as well.[38]

The young branch is used in treating tooth diseases. The seed contains oil that is used as antiseptic for sores and ulcers, rheumatism and skin diseases such as ringworm and scabies, malaria fever and leprosy, antibacterial, central nervous system depressant, mild analgesic, aromatherapy.[87]

Apart from the medicinal uses of *M. azedarach*, the fruit stones make ideal beads and are used in making necklaces and rosaries. The wood is also used to make household utensils, drums, fruit boxes, kitchen furniture and shelving. In Swaziland and South Africa the thickened latex is used as a bird lime, and poles are used in hut building as well as making bee hives. *Melia azedarach* is also used as a shade tree in coffee plantations, and the tree is a good source of fuel wood.[85]

2.6.3.5 Phytochemistry of the genus *Melia*

Many fungal metabolites, including two new alkaloids, 12β-hydroxy-13α-methoxyverruculogen TR-2 and 3-hydroxyfumiquinazoline A were isolated from the fermentation broth of *M. azedarach* and LN-4, an endophytic fungus isolated from the stem bark of *Melia azedarach*.[73] Sixteen compounds showed potent antifungal activities against phytopathogenic fungi (*Botrytis cinerea*, *Alternaria solani*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium solani*, *Fusarium oxysporum* f. sp. niveum, *Fusarium oxysporum* f. sp. vasinfectum, and *Gibberella saubinettii*), and four of them, 12β-hydroxy-13α-methoxyverruculogen TR-2, fumitremorgin B, verruculogen, and helvolic acid, exhibited antifungal activities.[95]

The limonoid 1-cinnamoyl-3,11-dihydroxymeliacarpin, meliacin and nimbinene, have also been isolated from the ethyl acetate extract of the leaves of *M. azedarach* with anti-viral activities.[88] Melianoninol, melianol, melianone, meliandiol, vanillin and vanillic acid were isolated from the fruits of *Melia azedarach*[89] and melianoninol, a new compound which possesses some anti-feeding properties against the imported cabbage worm (*Pieris rarae L.*). Kaempferol- 3-*O*-β-rutinoside, rutin and kaempferol-3-L-rhamno-D-glucoside.[54] A hot methanolic extract of *Melia azedarach* leaves contained the dipentadecyl ketone, glycerol 1,3-bisundec-9-enoate 2-dodec-9-enoate and glycerol tris-tridec-9-enoate,[90] quercetrin, and quercetin-3-*O*-β-rutinoside. In this present work leaves and stem bark of *Melia*

azedarach extracted in methanol, were investigated for their phytochemical content and antioxidant activity, respectively.[91]

2.6.3.6 Antioxidant activity

Many studies have been carried out on the antioxidant activities of *Melia azedarach*. The stem bark methanolic extract shows a strong dose-dependent radical scavenging activity (RSA) against DPPH.[99] The % RSA of *Melia azedarach* stem bark extracts was 91.75 % at 0.1 mg/mL, while that of the standard quercetin was 96.39 % at 0.1 mg/mL. This means that the stem bark of *Melia azedarach* is a strong antioxidant. However, Adil *et al.*[92] reported that the stem bark extracts of *M*. *azedarach* had a free radical inhibition found to be in the range of 55.43-63.86 % and the leaves 35.57-52.11 %, respectively. Statistically, there was no major difference between the free radical scavenging activity of *M. azedarach* stem bark extracts and the standard quercetin ($p > 0.05$). Lastly, the activity of the standard quercetin was higher but similar to that of the extract of *M*. *azedarach.*[92]

2.6.3.6 Antimalarial activity

Charturvedi and Raseroka found that *Melia* species have antimalarial properties.[93] The methanol extracts of seeds, bark and leaves of *Melia azedarach* have been tested in mice to survey their antimalarial impact against the intestinal sickness parasite *Plasmodium berghei.*[91] However, the results were less significant than chloroquine.[93]

2.6.4 *Capparis tomentosa*

Capparis tomentosa (Figure 2.12) belongs to the family *Capparaceae* and occurs in Senegal, Eastern Gambia, Mauritania, Swaziland and South Africa. Few parts of *Capparis tomentosa* have been shown to be lethal, particularly the roots.[50] They are generally utilized in traditional medicines where the root powder decoctions are valuable in treating stiffness, coughing, cold, stomach pain, jungle fever, loose bowels, tuberculosis, circulatory trouble, gonorrhoea, syphilis, and endometritis. The pulverized roots are utilized to treat wounds, snakebites, swellings, cerebral pain, skin diseases and headache, while the smoke of burning bark powder can be inhaled to treat tuberculosis, bronchitis and chest pain. Leaf decoctions are used to treat skin diseases and wounds. *Capparis tomentosa* is a plant

believed to have magico-medicinal properties, and it is therefore normally utilized in customs and rituals.[103] The leaves are cooked and eaten as a vegetable when there is a lack of food. The foliage is perused by goats and camels, in spite of the fact that it has been recorded as harmful to domesticated animals.

Figure 2.12: Leaves of *Capparis tomentosa.*

(www.bing.com/images 02/03/2017)

The fruit has been described as edible, but has also been reported as harmful to humans and numerous other animals. Cattle also benefit from the pressed cake. The stems are generally utilized as kindling, cut branches are sometimes planted to make a fence, and this plant additionally acts as an attractive greenhouse plant.[94]

2.6.4.1 Antimalarial activity of *Capparis tomentosa*

No antimalarial activity has been detected. However, past studies have shown the antimicrobial, cell reinforcement, antiplaque, anthelmintic and antibacterial actions of this plant.[95]

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Chemicals and solvents

All the chemicals used were of analytical grade purchased from Merck (Germany).

3.2 Plant Materials and preparation

Fresh *Lippia javanica* leaves, fresh *Sclerocarya birrea* leaves and stem bark, fresh *Melia azedarach* leaves and stem bark, as well as fresh *Capparis tomentosa* leaves, were collected at the University of Venda grounds in December 2017. Plants were identified by the Botany Department, University of Venda. Fresh green leaves of *Lippia javanica, Sclerocarya birrea, Melia azedarach,* and *Capparis tomentosa,* fresh stem bark of *Sclerocarya birrea* and fresh stem bark of *Melia azedarach* were used in this study.

3.2.1 Sample preparation

The plant leaves were removed from their stems and spread to air-dry at room temperature (20-28°C) for 28 days. The plant materials were milled and ground to a fine powder. These powders were carefully labelled. After grinding, they were weighed and stored in water/air-proof containers until use. The weights are recorded in Table 1.

3.2.2 Sample extraction

The different ground plant samples were each soaked for 48 h in 2 L methanol (Merck, Germany, 99.8 %) in a 5 L amber bottle, with occasional stirring and shaking. After 48 h, a Whatman No.1 filter paper was used to filter the leave samples, while the bark samples were filtered using a suction pump. Filtration was repeated three times after suspending the filter cake in a solvent, and the filtrates were combined. Each filtrate was then concentrated in a rotary evaporator (Büchi, Switzerland) and the methanol was collected and recycled to extract all the plant samples.

Table 1: Plant samples and their dry weights (g).

PLANT SAMPLE	YIELD (g)
Sclerocarya birrea leaves (SBL)	304.8
Lippia javanica leaves (LJL)	294.9
Capparis tomentosa leaves (CTL)	203.9
Melia azedarach leaves (MAL)	363.4
Melia azedarach stem (MAS)	282.9
Sclerocarya birrea stem (SBS)	855.9

3.3 Bioactivities of the crude plant extracts

Fifty mg of each of the crude methanol extracts was stored in a No 4 sample vial, labelled and sent to the Microbiology Department at Rhodes University for biological activity testing, as described in the following sections.

3.3.1 Antiplasmodial activity (pLDH) – Single Concentration Screen

Malaria parasites (*Plasmodium falciparum* CQ sensitive strain 3D7) were maintained in an RPMI 1640 medium containing 2 mM L-glutamine and 25 mM Hepes (Lonza). The medium was supplemented with 5 % Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 µg/mL gentamycin and 2 - 4 % hematocrit human red platelets. The parasites were cultured at 37^{o}C under atmosphere of 5 % CO_2, 5 % O_2, 90 %, N_2 in T75 culture flasks.

Single concentration screening was executed using crude mixtures at 25 µg/mL concentration for the portion of the plant which displayed the highest bioactivity (*Melia azedarach* stem bark) with fractions MASF1, MASF2, MASF3, MASF4, and MASF5 added to parasite colonies in 96-well, clear plates and incubated for 48 h in a 37 °C CO_2 incubator. After 48 h, 20 µL culture was removed from each well and added to 125 µL of a mixture of

Malstat and NBT/PES solutions in a fresh 96-well plate. These solutions were used to measure the activity of the parasite lactate dehydrogenase (pLDH). A purple product was formed in the presence of pLDH, and this product was quantified by means of a Spectramax M3 microplate reader at 620 nm (Abs_{620}).

An Abs_{620} reading in each well is an indication of pLDH activity, and thus, the number of parasites present. For each compound concentration, the % parasite viability (the pLDH activity in compound-filled wells relative to untreated controls) was determined. The samples were tested in duplicate. (This test was carried at Rhodes University, Biochemistry Department).

3.3.2 Antitrypanosomal activity – Single Concentration Screen

Trypanosoma brucei (*T.b.*) parasites are the causative agent of African sleeping sickness (human African trypanosomiasis) in people and Nagana (animal African trypanosomiasis) in cattle. The subspecies responsible for Nagana - *Trypanosoma brucei (T. brucei)* - is not infective to people and is normally used for drug screening.

To evaluate trypanocidal activity, samples were added to cultures of *T. brucei* in 96-well plates at fixed concentrations of 20 µg/L for the crude extracts of the selected plant samples (SBS, LJL, CTL, SBL, MAL, MAS), at 25 µg/mL for normal extracts (except if generally expressed).

After 48 h incubation, parasites surviving treatment were counted by the addition of a resazurin based reagent. Resazurin is reduced to resorufin (a fluorophore (Exc_{560}/Em_{590})) in suitable cells. In this manner it was evaluated in a Spectramax M3 microplate reader (This test was conducted at Rhodes University, Biochemistry Department).

3.3.3 Antitrypanosoma brucei Assay – Dose-response (IC_{50})

Trypanosoma brucei (T.b.) parasites are the causative agents of African sleeping sickness (human African trypanosomiasis) in individuals and Nagana (animal African trypanosomiasis) in cattle. The subspecies responsible for Nagana - *Trypanosoma brucei* (*T. brucei*) is not infective on humans and is generally used to determine the trypanocidal activity of test samples. Serial dilutions of the solutions were added to in vitro cultures of *T. brucei* in 96-well plates with 25 µg/mL of the selected plant extracts (SBS, LJL, CTL,

SBL, MAL, MAS). After 48 h incubation, parasites surviving treatment were determined by addition of a resazurin-based reagent. Resazurin is reduced to resorufin (a fluorophore with Exc_{560}/Em_{590}) in sensitive cells and was valued in a Spectramax M3 microplate reader.

For each compound, % parasite viability – the resorufin fluorescence in compound-treated wells relative to untreated controls – was determined. Compounds were tested in duplicate. For each compound, percentage viability was plotted against log (compound concentration) and the IC_{50} (50 % inhibitory concentration) values were obtained from the resulting dose-response curve by non-linear regression. For comparison, pentamidine (a current prescription drug for trypanosomiasis) was used as a control for screening, with IC_{50} 0.012 µg/mL (This test was conducted at Rhodes University, Biochemistry Department).

3.3.4 pLDH assay – Dose-response relationships

Malaria parasites (*Plasmodium falciparum* CQ sensitive strain 3D7) were maintained in an RPMI 1640 medium containing 2 mM L-glutamine and 25 mM Hepes (Lonza). The medium was supplemented with 5 % Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 µg/mL gentamycin and 2-4 % hematocrit human red blood cells. The parasites were cultured at 37°C under an atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2 in sealed T75 culture flasks.

To determine the parasitocidal potency of test compounds, serial dilutions of the compounds were added to in vitro cultures of *P. falciparum* (CQ sensitive strain 3D7), 25 µg/mL of extracts of SBS, LJL, CTL, SBL, MAL, and MAS, for the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach* stem bark), with fractions MASF1, MASF2, MASF3, MASF4, and MASF5, in 96-well plates. After 48 hours, 20 µL of culture was removed from each well and mixed with 125 µL of a mixture of Malstat and NBT/PES solutions in a new 96-well plate. These solutions were used to measure the activity of the parasite lactate dehydrogenase (pLDH) enzyme in the cultures. A purple product is formed when pLDH is present, and this product was quantified using a Spectramax M3 microplate reader (Abs_{620}). The Abs_{620} reading in each well is an indication of the pLDH activity and thus the number of parasites present.

Compounds and fractions were tested in a range, starting from 100 µM (or µg/mL) and expanded using a 3-fold sequential dilution. For each fraction, the % parasite viability (the pLDH activity in compound-treated wells relative to untreated controls) was calculated. Compounds were tested in duplicate. For each compound or fraction, the percentage

viability was plotted against log (compound concentration) and the IC_{50} (50 % inhibitory concentration) value was obtained from the resulting dose-response curve through non-linear regression. Similarly, chloroquine (a standard antimalarial drug) was used as a standard (IC_{50} values from 0.01-0.05 µM) (This test was conducted at Rhodes University, Biochemistry Department).

3.3.5 Cytotoxicity Assay – Single Concentration Screen

To evaluate the cytotoxicity of the compounds, they were incubated at a concentration of 50 µg/mL for the crude extracts of the selected plants (SBS, LJL, CTL, SBL, MAL, MAS), for the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach* stem bark), with fractions MASF1, MASF2, MASF3, MASF4 and MASF5, and 50 µg/mL for extracts in 96-well plates containing HeLa (human cervix adenocarcinoma) cells for 48 hours. The number of cells surviving treatment was determined using the resazurin-based reagent and reading resorufin fluorescence in a Spectramax M3 microplate reader (All these experiments were conducted at Rhodes University, Biochemistry Department).

3.3.6 Statistical Interpretations

An Excel spreadsheet was used to process all the data collected and statistical analysis was done using the SPSS package, where the reduced concentrations and the absorbance were utilized in calculating the linear regressions. Anova (One-way analysis of variance) was used to compare the mean values of the crude extracts, the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach* stem bark), with fractions MASF1, MASF2, MASF3, MASF4 and MASF5, obtained from each of the tests (polyphenolic and antioxidant contents), where $p < 0.05$ was considered statistically significant.

3.4 Isolation and purification of fractions of *Melia azedarach* stem bark

3.4.1 Isolation of *Melia azedarach* stem bark using column chromatography

The crude methanol extract of *Melia azedarach* stem bark (282.9 g) was subjected to column chromatography over silica gel. The extract was eluted initially with hexane and the polarity was gradually increased with ethyl acetate and finally methanol, yielding 14 fractions.

TLC was run on the fractions and fractions with the same TLC profile were combined and concentrated to dryness on a rotary evaporator, giving a total of 6 fractions coded as A1-A6. Fraction A1 was obtained with ethyl acetate (100 %), Fraction A2 was obtained with ethyl acetate/methanol (90:10), Fractions A3-A4 were obtained with ethyl acetate/methanol (70: 30), Fraction A5 was obtained with methanol/ethyl acetate (30:70), and A6 was obtained with methanol (100 %). The collected fractions were monitored on TLC plates. Fractions A1, A2, A3, A4, and A5 (Figure 3.2) were further fractionated using column chromatography because they contained a large amount of material and more bioactive than Fraction A6, which was not found to be active and was kept separately for future investigation.

3.4.1.1 Fractionation of isolated fractions using column chromatography

Fraction A1 (6.3 g) was subjected to silica gel column chromatography; the column was eluted with CH_2Cl_2/EtOAc (90:10), followed by an increasing gradient of EtOAc (up to 100 %), to obtain fraction MASF1 (2.55 g). Fraction A2 (6.83 g) was subjected to silica gel column chromatography; the column was eluted using EtOAc/MeOH (90:10), followed by an increasing gradient of EtOAc/MeOH (up to 70:30), to obtain fractions MASF2 (2.18 g). Fraction A3 (10.41 g) was also subjected to silica gel column chromatography and the column was eluted using EtOAc/MeOH (90:10), followed by an increasing gradient of *n*-C_6H_{12}/EtOAc (up to 30:70), to obtain MASF3 (7.37 g). Fraction A4 (17.20 g) was subjected to silica gel column chromatography; the column was eluted using EtOAc/MeOH (90:10) followed by an increasing gradient of EtOAc/MeOH (up to 70:30), to obtain fraction MASF4 (13.05 g), and fraction A5 (4.45 g) was subjected to silica gel column chromatography; the column was eluted with EtOAc/MeOH (80:20), followed by an increasing gradient of MeOH (up to 100 %), to obtain fraction MASF5 (1.70 g). TLC plates were used to monitor the fractions gathered. Thin layer chromatograms were developed using ethyl acetate/methanol/water (EMW 81:11:8). A characteristic stain containing 1g diphenylboric acid ethylamino ester in 100 mL methanol, and 5 mL PEG 4000 and 95 mL ethanol was used to view compounds on a TLC plate. The procedure for the isolation of fractions is summarized in Figure 3.2.

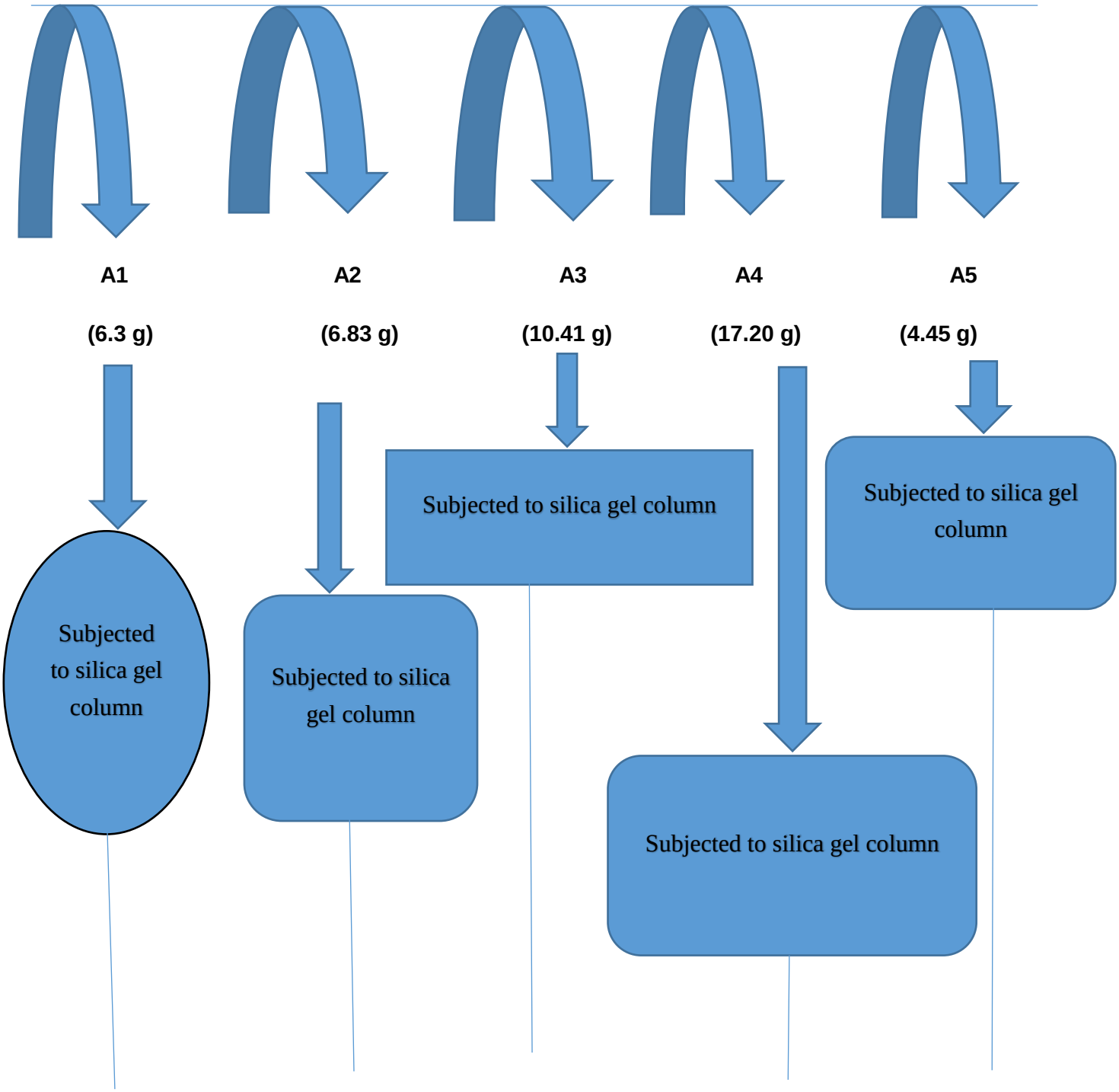


Figure 3.1: Isolation and fractionation of *M. azedarach* using column chromatography.

3.4.1.2 Purification of isolated fractions using column chromatography

Isolated fractions MASF1, MASF2, MASF3, MASF4 and MASF5 were tested for their biological activity. Fraction MASF1, which was eluted with 100 % EtOAc, was found to have the highest bioactivity and was further purified. Four compounds (**28**, **29**, **30** and **31**) were isolated from fraction MASF1, and the process is as shown in Figure 3.3. These compounds were further tested for their biological activities against *P. falciparum* and the structures were elucidated using NMR, IR, UPLC-MS, and HRMS.

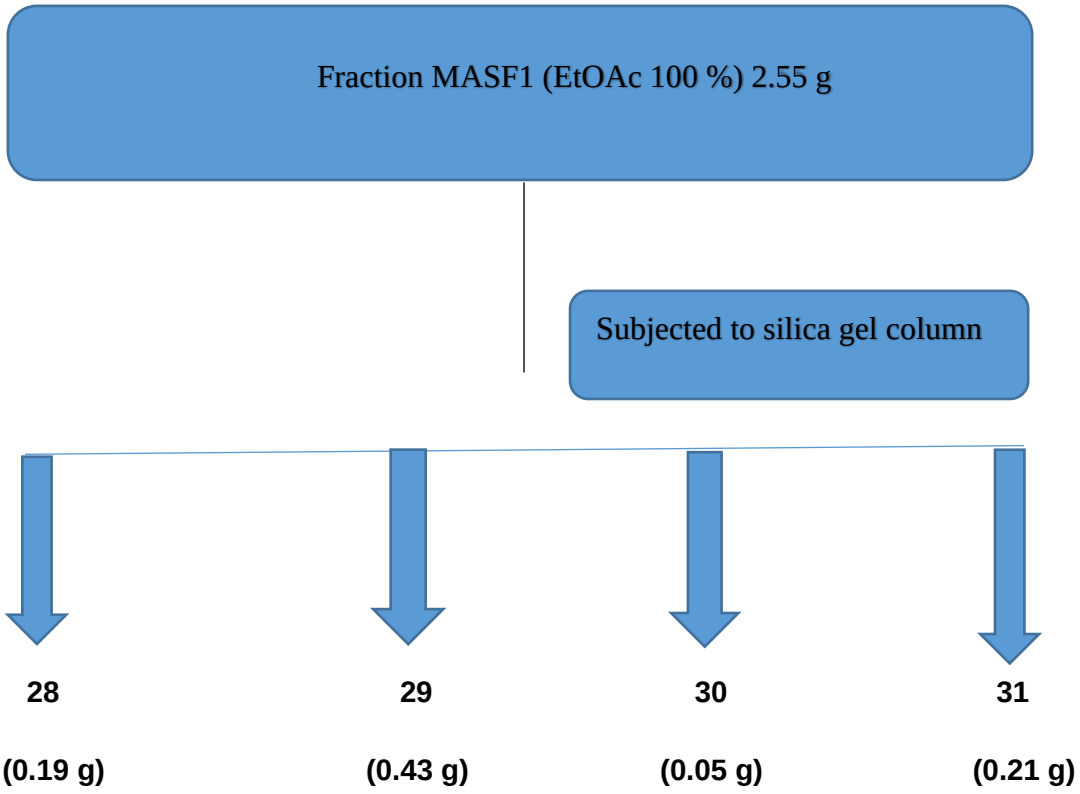


Figure 3.2: Purification of isolated compounds

3.5 Structure elucidation of isolated compounds

Identifying compounds requires many methods, for example, high resolution mass spectrometry (HRMS), nuclear magnetic resonance spectroscopy (NMR), ultra-pressure liquid chromatography-mass spectrometry (UPLC-MS) and infrared spectroscopy (IR); however, for the purpose of this study, all the aforementioned methods were used for

structure elucidation of the isolated compounds **(28, 29, 30 and 31).** The structures of compounds **29** and **31** were confirmed while compounds **28** and **30** could not be identified due to the large number of impurities which were difficult to remove (UPLC instrument: Waters Synapt).

A Bruker Avance 400 MHz NMR spectrometer was used to perform the one-dimensional and two-dimensional NMR (^{13}C-NMR and ^{1}H-NMR) experiments. DMSO-d_6 was used as NMR solvent. Residual protonated solvent was used as a calibration standard. The multiplicities are presented below. Two-dimensional spectra were used to obtain the main C-H and C-C connectivities. Heteronuclear multiple bond correlation (HMBC) experiments gave information on the direct ^{13}C and ^{1}H heteronuclear bonding.

A Bruker Alpha Fourier Transform IR spectrometer was used to record FTIR spectra, with absorption maxima detailed as wavenumbers (cm^{-1}).

High-resolution mass spectra (HRMS) were obtained on a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) associated with a Waters Acuity ultra-performance liquid chromatograph ESI probe, ESI Pos, Cone Voltage 15 V. Column chromatography was set up using Silica Gel 60F, 90-180 mesh. Analytical Thin Layer Chromatography (TLC) was performed on commercial aluminium-supported silica gel 60 pre-covered TLC sheets.

3.5.1 Spectroscopic data of epicatechin (29) and catechin (31)

3,3',4',5',7-Pentahydroxyflavan (29)

Epicatechin (**29**): brown fine powder

^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 9.24 (s,1H, 5-OH), 8.99 (s, 1H, 7-OH), 8.92 (s, 1H, 4'-OH), 8.87(s,1H, 3'-OH), 6.71-6.65 (d, *J* = 8 Hz, 1H, 5'-H), 6.67 (d, *J* = 8 Hz, 1H, 6'-H), 6.60 (d, *J* = 8 Hz,1H, 2'-H), 5.88 (d, *J* = 0.8 Hz, 1H, 6-H), 5.68 (d, *J* = 0.8 Hz, 1H, 8-H), 5.30 (s,1H, 3-OH), 4.91 (m, 1H, 3-H), 4.48 (d, *J* = 8 Hz, 1H, 2-H), 2.62 (dd, *J* = 16 Hz and *J* = 48 Hz,1H, 4a-H), 2.37(dd, *J* = 8 Hz and *J* = 16 Hz, 1H, 4b-H) ppm.

^{13}C-NMR (100 MHz, DMSO-d_6): δ_C 156.53 (C-5), 156.26 (C-7),155.43 (C-9), 144.92 (C-4' & 3'), 130.65 (C-1'), 118.52 (C-6'), 115.14 (C-5'), 114.57 (C-2'), 99.13 (C-10), 95.16 (C-6), 93.90 (C-8), 81.07 (C-2), 66.38 (C-3), 29.17 (C-4) ppm.

HRMS: *m/z* 291.0856 $[M+H]^+$. Calcd. for $C_{15}H_{14}O_6$+H: 291.08239.

IR: ν_{max} (ATR): 3332.42 (O-H stretch), 2941.93 (C-H stretch), 2833.27 (C-H stretch) cm^{-1}.

3,3',4',5',7-Pentahydroxyflavan (31)

Catechin (**31**): chocolate-coloured particles

^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 9.26 (s, 1H, 5-OH), 9.01 (s, 1H, 7-OH), 8.93 (s, 1H, 4'-OH), 8.89 (s, 1H, 3'-OH), 6.71-6.67 (m, 2H, 5' & 6'-H), 6.59 (d, *J* = 8 Hz, 1H, 2'-H), 5.88 (s, 1H, 6-H), 5.68 (s, 1H, 8-H), 5.30 (s, 1H, 3-OH), 4.98 (m, 1H, 3-H), 4.47 (d, *J* = 4 Hz, 1H, 2-H), 2.67 (dd, *J* = 4.8 Hz and *J* = 8 Hz, 1H, 4a-H), 2.36 (dd, *J* = 16 Hz and *J* = 8 Hz, 1H, 4b-H) ppm.

^{13}C-NMR (100 MHz, DMSO-d_6): δ_C 156.54 (C-5), 156.27 (C-7), 155.44 (C-9), 144.93 (C-4, C-3'), 120.41 (C-1'), 118.54 (C-6'), 115.15 (C-5'),114.58 (C-2'), 99.14 (C-10), 95.17 (C-6), 94.33 (C-8), 81.07 (C-2), 72.08 (C-3), 28.84 (C-4) ppm.

HRMS: *m/z* 289.0701 $[M-H]^+$. Calcd. for $C_{15}H_{14}O_6$-H: 289.07121.

IR: ν_{max} (ATR): 3331.82 (O-H stretch), 2943.98 (C-H stretch), 2832.48 (C-H stretch) cm^{-1}.

3.6 Quantitative phytochemical analysis

From the crude extract and fractions of *Melia azedarach* stem bark (MASF1, MASF2, MASF3, MASF4 and MASF5) and methanol crude extract and fractions of the leaves (LF1, LF2, LF5 and LF6), a 1 mg/mL solution of each extract in MeOH/water (80:20) was prepared for analysis.

3.6.1 Quantitation of total phenolic contents (TPC)

The TPC quantitation was conducted using Folin-Ciocalteau reagent (FCR), (Sigma Aldrich, St. Louis, MO, USA), with some modifications, according to Anokwuru et al.[40]

Each well of the 96-well plates were filled with 80 µL of distilled water, followed by 100 µL of the methanol crude extracts (CRS) of the plant which exhibited the highest bioactivity (*Melia azedarach*), with stem bark fractions (MASF1, MASF2, MASF3, MASF4 and

MASF5), and the methanol crude extract (CLF) and fractions of the leaves (LF1, LF2, LF5 and LF6).

Plant sample (1 mg/mL) was added in triplicate. A volume of 60 µL of 10 % Folin-Ciocalteau reagent (10 mL FCR diluted with 90 mL distilled water) and 60 µL of 7 % sodium carbonate (Na_2CO_3) (Minema Chemicals, Randpark Ridge, Gauteng, RSA) solution was added to the mixture. The mixture was allowed to stand for 30 min after which absorbance was read at 760 nm using a VersaMax™ tuneable microplate reader. The total phenolic content was expressed as gallic acid equivalent (mg GAE/g sample), by means of the calibration curve of gallic acid (Sigma-Aldrich), which was used to convert the absorbance of the sample to total phenolic content, expressed in milligram gallic acid equivalent to extract per gram of the crude extract and fractions (mg GAE/G). Linearity range of the calibration graph was 10 to 1000 µg/mL.

3.6.2 Quantitation of total flavonoid contents (TFC)

The total flavonoid content was estimated using quercetin (Sigma-Aldrich) as a standard.[5] About 100 µL was used of methanol crude extract of the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach*), with stem bark fractions CRS, MASCR, MASF1, MASF2, MASF3, MASF4 and MASF5, as well as methanol crude extract and fractions of the leaves (CRS, LF1, LF2, LF5 and LF6). A 1 mg/mL sample was mixed with 80 µL distilled water in each well of the 96-well plates in quadruplicate. A volume of 100 µL aluminium chloride (Minema Chemicals, Randpark Ridge, Gauteng, RSA) solution (2 %) was added to the previous mixture. The plate was allowed to stand for 30 min and then the absorbance was measured at 420 nm. The TFC was expressed as quercetin equivalents (mg QE/g of sample). The linearity range of the quercetin calibration curve was 10 – 1000 µg/mL.

3.7 Antioxidant activity of *Melia azedarach*

3.7.1 Quantitative test for total antioxidant contents

The antioxidant content of *Melia azedarach* stem bark and leaves was determined using the radical scavenging DPPH assay and reducing power assay.

Reagent preparation for DPPH and reducing power assays are as follows: A solution of 0.125 M DPPH (Sigma-Aldrich) was prepared by dissolving 9.85 mg of DPPH in 200 mL of ethanol.

The phosphate buffer (pH 6.6, 0.2 M) was prepared by mixing 37.5 mL dibasic sodium phosphate solution (0.2 M) (NaH_2PO_4) (Merck chemicals) and 62.5 mL of monobasic sodium phosphate solution (0.2 M) (Na_2HPO_4).

The potassium hexacyanoferrate 1 % was prepared with 1 g potassium ferricyanide in 100 mL distilled water. The trichloroacetic acid 10 % was prepared with 10 g trichloroacetic acid in 100 mL distilled water. Ferric chloride 0.1 % (w/v) was obtained by dissolving 0.1 g ferric chloride hexahydrate (Merck) in 100 mL distilled water.[5]

3.7.2 Radical scavenging activity DPPH assay

The DPPH assay was run as described by Alkan *et al.,*[108] with a few modifications. Briefly, into a 96-well plate, 100 µL distilled water and 100 µL of methanol crude extract (CRS) and the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach*), with stem bark fractions MASF1, MASF2, MASF3, MASF4 and MASF5, and the methanol crude extract (CLF) of the leaves (LF1, LF2, LF5 and LF6), and the controls, were added in triplicate. Then serial dilutions of the samples were realized, giving the following concentrations in µg/mL: 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078. A volume of 200 µL of DPPH solution was added to each well and the plates were incubated for 30 min in the dark. After incubation, the absorbance of each well was read using a microplate reader (Versamax) at 517 nm. The percentage inhibition of DPPH or the decolouration of the wells was calculated using the following formula:

$$\% \text{ inhibition} = \frac{(A_{DPPH} - A_{sample}) \times 100}{A_{DPPH}}$$

where *A* stands for the measured absorbance.

This percentage inhibition allowed us to determine graphically the 50% inhibitory concentration (IC_{50}), which is the concentration of extract or control that causes a 50 % reduction in the DPPH colour.[108]

3.7.3 Reducing power assay

The assay was run according to the method of Oyaizu,[109] with slight modifications. Briefly, after adding 50 µL of sodium phosphate buffer (pH 6.6, 0.2 M) to each well, 50 µL of 1 mg/mL of methanol for the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach*), with stem bark fractions CRS, MASF1, MASF2, MASF3, MASF4 and MASF5, leaf fractions (CLF, LF1, LF2, LF5 and LF6), and controls were added in triplicate. The extracts and control were set and serial dilution was done, giving the following concentrations in µg/ml: 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, and 0.0078. Thereafter, 50 µL potassium hexacyanoferrate (1 %) was added to each well, which was then incubated for 30 min at 50°C. Fifty µL trichloroacetic acid 10 % solution was introduced into each well and 80 µL of the content of each well was transferred to new 96-well plates.

A volume of 80 µL distilled water was added to the wells of the new plates, followed by 16 µL ferric chloride 0.1 % solution. The plates were then read at 690 nm using a microplate reader (VersaMax). In this assay, we are looking for the concentration that gives 0.5 % ($EC_{0.5}$) of the absorbance at 690 nm. The EC_{50} was determined graphically, by plotting the measured absorbance against the concentration.[109]

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Biological activities of the crude extracts

4.1.1 Antiplasmodial activity of the selected plant samples

Crude extracts of *Sclerocarya birrea* (leaves, stem bark), *Lippia javanica* (leaves), *Capparis tomentosa* (leaves) and *Melia azedarach* (leaves and stem bark) were tested for their antiplasmodial activities. The single dose results and IC_{50} are presented in Figure 4.1.

The antimalarial activity of the methanol crude extract of (*Sclerocarya birrea* stem bark*, Lippia javanica* leaves*, Capparis tomentosa* leaves*, Sclerocarya birrea* leaves*, Melia azedarach* leaves*, Melia azedarach* stem bark) against *Plasmodium falciparum* CQ sensitive strain 3D7, was determined using parasite lactate dehydrogenase (pLDH) assay. *Melia azedarach* stem bark, caused a significant decrease of 36.22±11.96 % at the tested concentration of 50 µg/mL, followed by *Melia azedarach* leaves which caused a significant decrease in the pLDH, with 36.28±13.59 % and *Sclerocarya birrea* stem bark, with 40.86±15.05 %, with a tested concentration of 50 µg/mL, while *Sclerocarya birrea* leaves, *Lippia javanica*, and *Capparis tomentosa* gave 65.20±0.07 % , 70.40±6.72 % , and 82.38±18.98 % respectively .

To further support this result, *Melia azedarach* stem bark was not found to be cytotoxic; this was further confirmed with the pLDH IC_{50} assay because it could decrease pLDH activity to less than 20 %.

For comparison, chloroquine (a standard anti-malarial drug) was used as a standard (IC_{50} values range from 0.01-0.05 µM), the concentration used was 20 µg/mL

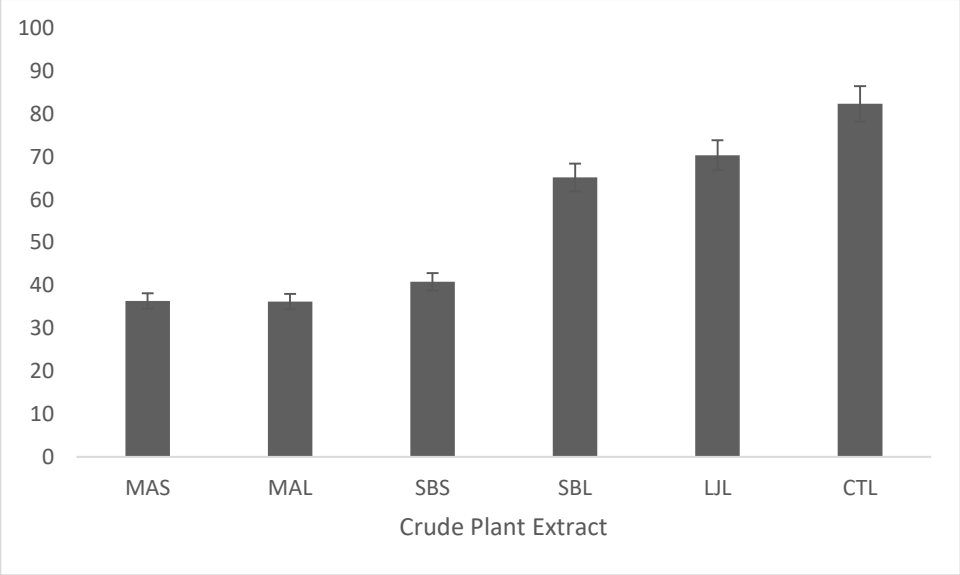


Figure 4.1: pLDH antiplasmodial Assay – single concentration screen for plants.

4.1.2 Single Concentration Antitrypanosomal Screen of selected plant samples

Following the plasmodial study of the methanol crude extracts, single concentration antitrypanosomal screening of the selected plant materials (*Sclerocarya birrea* stem bark, *Lippia javanica* leaves, *Capparis tomentosa* leaves, *Sclerocarya birrea* leaves, *Melia azedarach* leaves, *Melia azedarach* stem bark), was carried out. The results were expressed as a % parasite viability (the resorufin fluorescence in compound-treated wells relative to untreated controls). Extracts that reduced parasite viability to less than 20 % are considered for further testing (dose-response and cytotoxicity assays). Pentamidine (an existing drug treatment for trypanosomiasis) was used as a control drug standard.

The *Melia azedarach* leaves extract was able to reduce the % viability of *Trypanosoma brucei* by 11.62± 2.50 %, followed by *Melia azedarach* stem bark, with 14.05±0.59 %, while *Sclerocarya birrea* leaves & stem, *Capparis tomentosa* and *Lippia javanica* leaves were 84.96±7.65 %, 100.07±10.37 % and 100.88±2.52 % and 100.73±0.00 % respectively (Figure 4.2). The crude methanol extract of *Melia azedarach* leaves had the highest activity which confirmed that we could continue our survey of this particular plant and its fractions. *Melia azedarach* leaves and *Melia azedarach* stem reduced the parasite viability to < 20 % and was further subjected to IC_{50} testing.

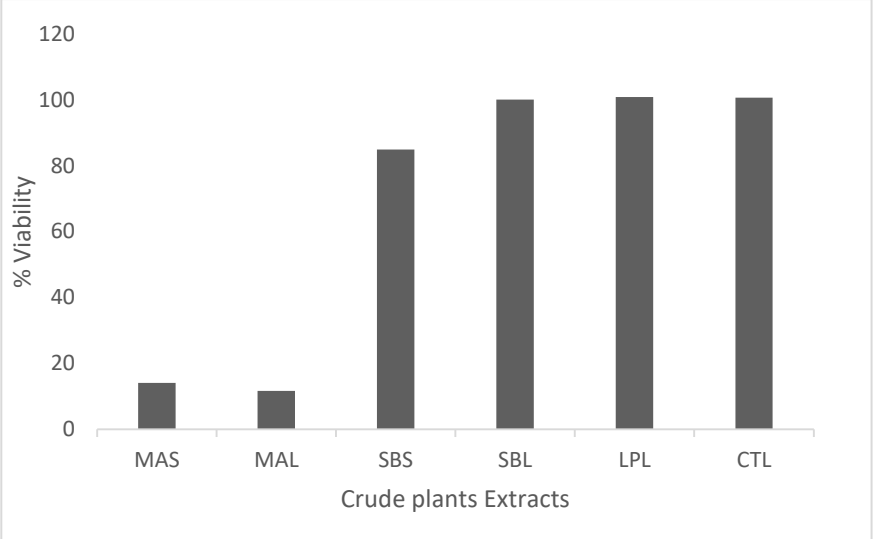


Figure 4.2: Antitrypanosoma activity of methanol crude extracts expressed as parasite % viability ±SD for individual plant samples.

4.1.3 *Trypanosoma brucei* – Dose-response curves (IC_{50}) of the selected plant samples

The crude methanol extract of *Melia azedarach* stem bark decreased the viability of the *trypanosomes* at 250 µg/mL concentration (Table 3) with IC_{50} values of 0.4 µg/mL above *Melia azedarach* leaves decreased with IC_{50} values of 10.4 µg/mL, therefore displaying an antitrypanosomal activity. *Sclerocarya birrea* leaves and stem bark, *Lippia javanica* and *Capparis tomentosa* did not show any significant value and so it can be concluded that they were not able to decrease the parasite viability at all.

Drug standard and yields IC_{50} values were in the range 0.001-0.05 µM. The concentration used was the following: 3-fold dilutions at 100 µg/mL starting concentration. Table 2 shows the IC_{50} values obtained for two extracts, followed by the dose-response plots and % viability ±SD data used to prepare the graphs**.** Pentamidine standard: IC_{50} – 0.012 µg/mL.

The pentamidine used as reference drug showed an IC_{50} value of 0.003114 µM. In literature, the antitrypanosomal activity and bioactive constituents from *Melia azedarach* has not been undertaken. Therefore, that makes our study sought to detect important antitrypanosomal activity of *M. azedarach*.

Table 2 *Trypanosoma brucei* Assay – IC_{50}

Plant extracts	IC_{50} (µg/mL)
Melia azedarach stem bark (MAS)	0.4
Melia azedarach leaves (MAL)	10.4

4.1.4 Antimalarial Dose-response assay – IC_{50} for the selected plant samples

Methanol crude extract of *Melia azedarach* stem bark gave the IC_{50} values obtained for each plants sample (Table 4), and *Melia azedarach* stem bark revealed that at a concentration of 100 µg/mL it decreased the viability of *Plasmodium falciparum* with an IC_{50} value of 6.5 µg/mL, while *Sclerocarya birrea* leaves and stem bark gave 19.4 µg/mL and 9.3 µg/mL respectively. Chloroquine standard @ IC_{50} – 29 µM. *Melia azedarach* leaves and stem bark, *Lippia javanica* and *Capparis tomentosa* did not show any significant value and so it can be said that they are not able to decrease the parasite viability at all.[128] This further authenticate our studies to continue only with *Melia azedarach* stem bark in our study.

Table 3 pLDH (Malaria) assay – IC_{50}

Fraction	IC_{50} (µg/mL)
MAS	6.5
SBS	19.4
SBL	9.3

4.1.5 Single Concentration Cytotoxicity Screen for the selected plant samples

A cytotoxicity assay was carried out on the selected plant samples, *Melia azedarach* leaves, *Sclerocarya birrea* leaves, *Melia azedarach* stem bark, *Sclerocarya birrea* stem bark, *Capparis tomentosa* leaves and *Lippia javanica* leaves. *Melia azedarach* stem bark was able to reduce the % viability of *Trypanosoma brucei* by -1.22± 0.07 % followed by *Sclerocarya birrea* stem bark, with 61.95±7.47 %, while *Melia azedarach* leaves, *Sclerocarya birrea* leaves & stem, *Capparis tomentosa* and *Lippia javanica* leaves were 90.32±2.47 %, 77.95±2.47 %, 73.88±5.68 % and 69.91±2.07 %, respectively, with a very wide margin. This further confirmed *MA*S as a plant of consideration.

Figure 4.3 shows the % HeLa cell viability ±SD obtained for each sample. The concentration used was 20 µg/mL, and the plant sample *Melia azedarach* stem bark caused significant cytotoxic effects (reduced the viability of HeLa cells to below 50 %). To further support this result, *Melia azedarach* stem bark was not found to be cytotoxic; this was further confirmed with the pLDH IC_{50} assay because it could decrease the pLDH activity to less than 20 %.

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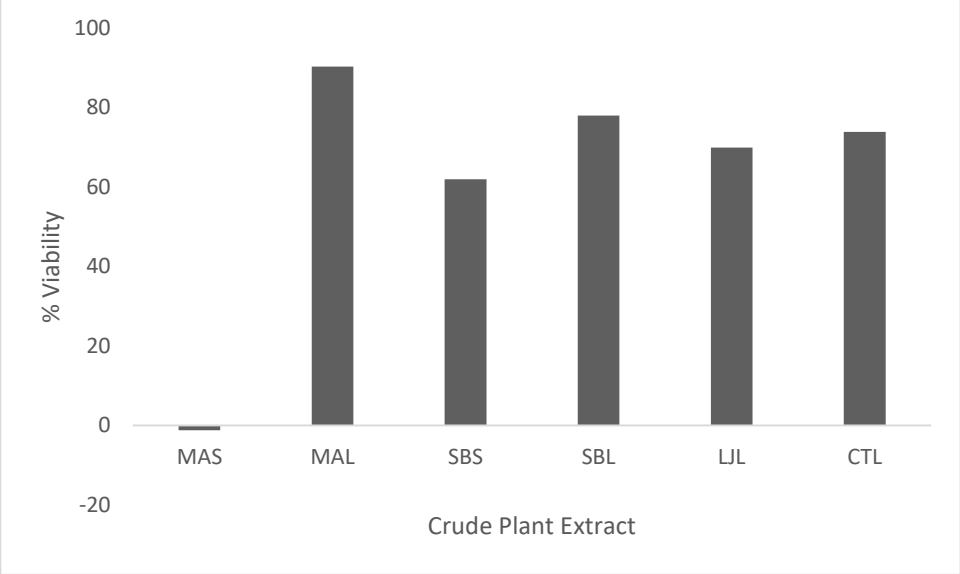

Figure 4.3: Cytotoxicity Assay – Single Concentration Screen of all the plant Samples.

4.2 Biological activities of *Melia azedarach* stem bark fractions

4.2.1 Antiplasmodial activity of fractions of *Melia azedarach*

The antimalarial activity of the fraction of the plant which exhibited the highest bioactivity (*Melia azedarach* stem bark) with MASF1, MASF2, MASF3, MASF4 and MASF5 against *Plasmodium falciparum* CQ sensitive strain 3D7 was determined using parasite lactate dehydrogenase (pLDH) assay. Fraction MASF1 caused a significant decrease with the % viability of 4.92±3.09 %, with a tested concentration of 50 µg/mL, while fractions MASF2, MASF3, MASF4 and MASF5 showed 73.049±9.16 %, 46.10±1.31 %, 48.47±7.36 % and 60.23±2.94 % viability, respectively, causing a significant decrease in the pLDH with the tested concentration (50 µg/mL). To further support this result, *Melia azedarach* was found to be non-cytotoxic; this was further confirmed with the pLDH IC_{50} assay because it decreased pLDH activity to less than 20 %. Fraction SF1 was found to be more active than MASF2, MASF3, MASF4, and MASF5 at the same concentration. In studies, the antiplasmodial activity of phenolics from *Melia azedarach* was confirmed with our results (Figure 4.4).[6]

For comparison, chloroquine (a standard anti-malarial drug) was used as a standard (with IC_{50} values ranging from 0.01-0.05 µM). The concentration used was 20 µg/mL.

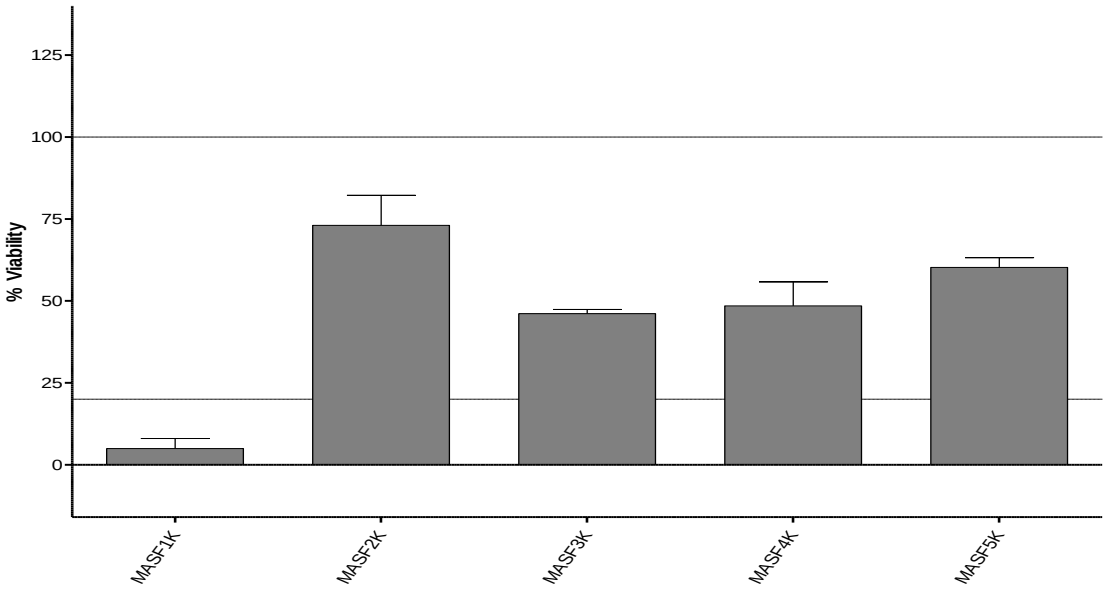

Figure 4.4: Antiplasmodial activity for fractions % viability against plant samples

4.2.2 Single Concentration Antitrypanosomal Screen of *Melia azedarach* stem bark fractions

The fraction of the plant which exhibited the highest bioactivity (*Melia azedarach* stem bark) with MASF1, MASF2, MASF3, MASF4, and MASF5, was tested using the trypanosome assay against *Trypanosoma brucei* (*T.b.*) parasites. The fraction MASF1 showed the highest activity of 2.48±2.66 % at concentration 50 (Figure 4.5), followed by MASF2, MASF3, MASF4 and MASF5 with 85.87±0.18 %, 98.91±6.16 %, 96.59±4.21 % and 101.62±13.25 %, respectively, which appears to have a very low activity. Results were expressed as % parasite viability (the resorufin fluorescence in compound-treated wells relative to untreated controls). Fractions and compounds were tested in duplicate. Compounds/extracts that reduced parasite viability to less than 20 % are considered for further testing (dose-response and cytotoxicity assays). Pentamidine was used as a control drug standard. Fraction SF1 of *Melia azedarach* reduced the parasite viability to < 20 % and was used for further IC_{50} determination.

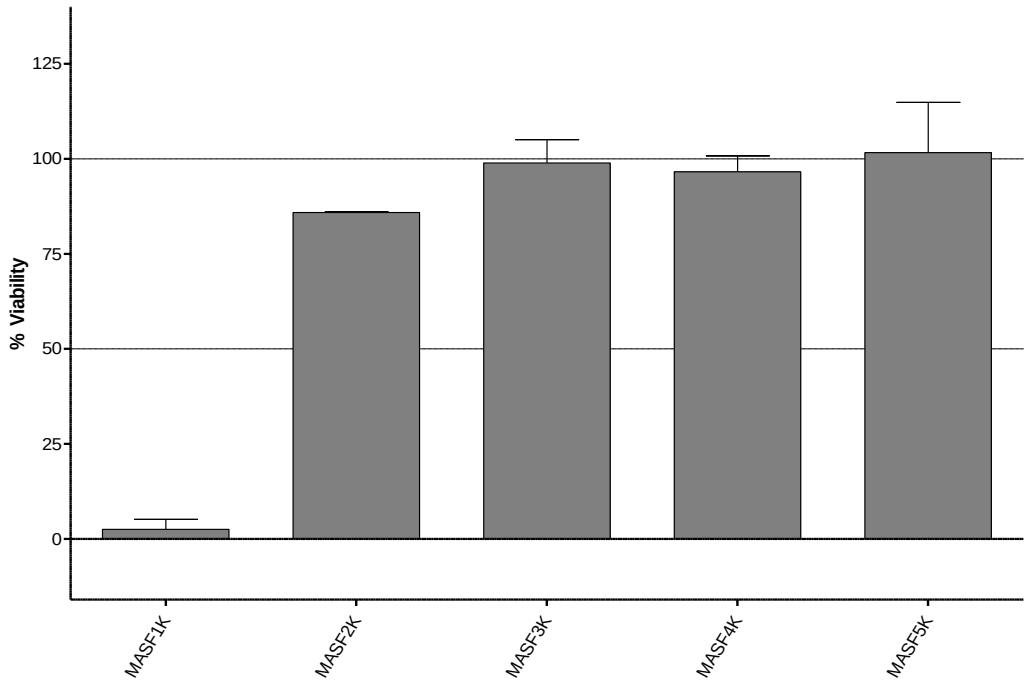

Figure 4.5: Antitrypanosoma activity of fractions of *M. azedarach* stem bark expressed in parasite % viability ±SD for individual fractions

4.2.3 Dose-response Antitrypanosoma brucei Assay (IC_{50}) of *Melia azedarach* fractions

Only fraction MASF1 of *Melia azedarach* stem bark affected the growth of the trypanosomes at a 250 µg/mL concentration with an IC_{50} value of 0.44 µg/mL (Figure 4.6). Fraction MASF1 apparently decreases the viability of *T. brucei* at this concentration, therefore displaying antitrypanosomal activity. The fractions were tested in duplicate and percentage viability was plotted against Log (Fraction concentration): the IC_{50} (50% inhibitory concentration) was obtained from the resulting dose-response curve by non-linear regression. For comparison, pentamidine (an existing drug for treatment of trypanosomiasis) was used as a drug standard and IC_{50} values in the range 0.001-0.05 µM were found. This is the first reported study of the antitrypanosomal activity of fractions of *Melia azedarach*.

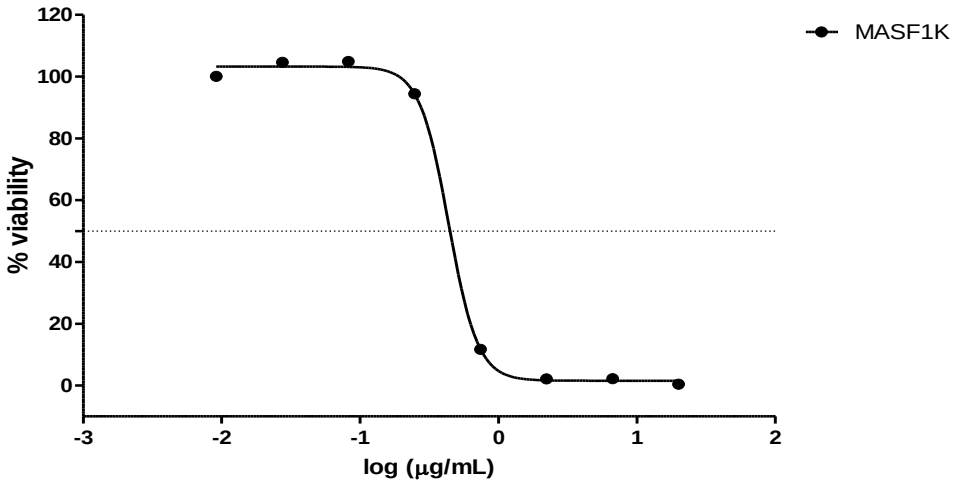

Figure 4.6: Dose-response curve for *Trypanosoma* assay

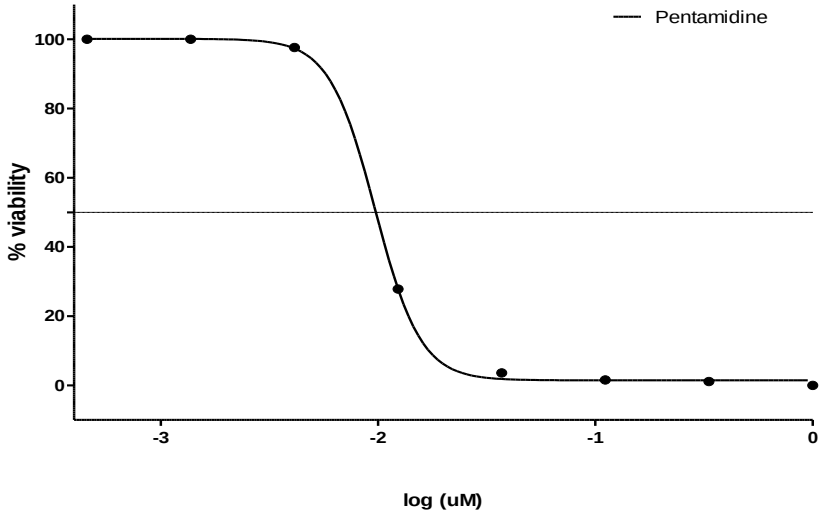

Figure 4.7: Dose-response curve for pentamidine standard: IC_{50} 0.09 µM

4.2.4 Dose-response antimalarial assay – IC_{50} for the fractions of *Melia azedarach* stem bark

Fractions MASF1, MASF2, MASF3, MASF4 and MASF5 were tested in a range, starting from 100 µg/mL and extended using a 3-fold serial dilution. For each fraction, % parasite viability (the pLDH activity in compound-treated wells relative to untreated controls) was calculated. Fractions were tested in duplicate. For each Fraction, percentage viability was plotted against Log (compound concentration) and the IC_{50} (50% inhibitory concentration) obtained from the resulting dose-response curve by non-linear regression. For comparison, chloroquine was used as a standard (IC_{50} values range from 0.01-0.05 µM.). The IC_{50} values obtained for fraction MASF1 showed at concentration 100 µg/mL decreased the viability of *Plasmodium falciparum* with IC_{50} value of 13 µg/mL. The chloroquine standard had IC_{50} 50 µM.

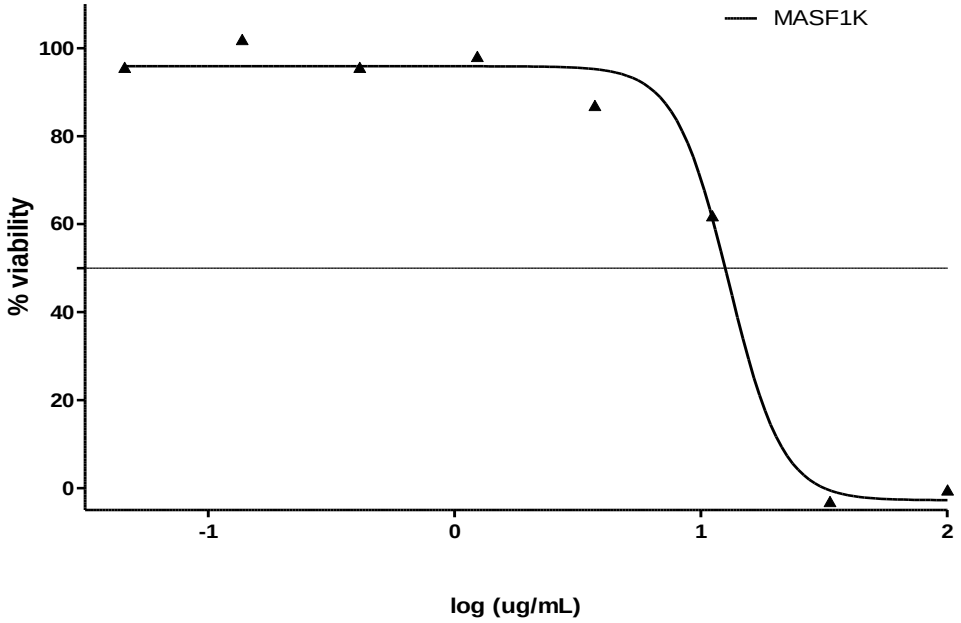

Figure 4.8: Dose-response curve for antimalarial assay: % viability against log (concentration)

4.2.5 Single Concentration cytotoxicity assay for *Melia azedarach* stem bark fractions

The fractions of the plant that exhibited the highest cytotoxicity (*Melia azedarach* stem bark) were labelled MASF1, MASF2, MASF3, MASF4 and MASF5 and subjected to the trypanosome assay against *Trypanosoma brucei* (*T.b.*) parasites. Fraction MASF1 showed a relatively high activity at concentration 50 % (Table 4). Table 4 shows the % HeLa cell viability ±SD obtained for each fraction. Concentration used: 20 µg/mL, the fraction MASF1 caused significant cytotoxic effects (reduced the viability of HeLa cells to below 50 %) by 102.77± 1.62, followed by MASF4 with 102.92± 0.05 followed by MASF2 with 106.10±0.27 followed by MASF3 with 107.15±2.49 and then MASF5 with 108.38±0.51. To further support this result, *Melia azedarach* stem bark was found to be cytotoxic against *Plasmodium falciparum*, its fraction MASF1 also had a high activity against cancer cells and *Trypanosoma brucei*. This was further confirmed with the pLDH IC_{50} assay because it could decrease pLDH activity to less than 20 %.

Table 4: Cytotoxicity of the fractions obtained from fraction A1 of *Melia azedarach* stem bark and their % viability and standard deviations

Fraction	% Viability ± SD
MASF1	102.77±1.62
MASF2	106.10±0.27
MASF3	107.15±2.49
MASF4	102.92±0.05
MASF5	108.38±0.51

4.3 Phytochemical analysis of *Melia azedarach*

4.3.1 Total phenolic content (TPC).

Phenolic compounds are well-known phytochemicals found in all plants. They consist of simple phenols, benzoic and cinnamic acid, coumarins, tannins, lignins, lignans and flavonoid.[96] Plant foods are rich sources of phenolic, which are molecules that can act as antioxidants, to prevent heart disease. Chemical procedures are used to detect the presence of total phenolic, while spectrophotometric and chromatographic techniques are utilized to identify and quantify individual phenolic compounds.[78]

In this study the determination of total phenolic content of the leaves and stem bark of *Melia azedarach* was crucial in supporting the belief that the stem bark of *Melia azedarach* has untapped potential for disease prevention compared to the leaves, for which this research shows the fractions and their respective total phenolic content value (Table 6). Therefore, the total phenolic content in crude extract and fractions of the leaves and stem bark of *Melia azedarach* were estimated using the Folin-Ciocalteau reagent. The total phenolic content of the methanol crude extract *Melia azedarach* stem bark and leaves and the fractions were calculated and expressed as GAE using the regression equation of gallic acid calibration curve ($Y= 2.0676 X + 0.6464$, $R^2 = 0.6268$, see Figure 4.9).

The results of this assay ranged from 59.39±0.179 mg GAE/g to 1.79±0.94 mg GAE/g. Fraction MASF2 was the most interesting one, with the highest phenolic content (59.39±0.179 mg GAE/g), followed by the crude extract of the stem bark (56.67±1.013 mg GAE/g), while for the leaves, the LF3 had the highest phenolic content with (9.81±1.31 mg GAE/g) fraction LF6 had the lowest content (1.16±0.94 mg GAE/g), as shown (Figure 4.9). This shows that Fraction MASF2 from the stem bark of *Melia azedarach* has more polyphenolic compounds than the other tested fractions and those of the leaves. Our results also show that polyphenolic compounds are responsible for the phenolic activities of *Melia azedarach.* This is similar to the findings by Katsube *et al*.[97]

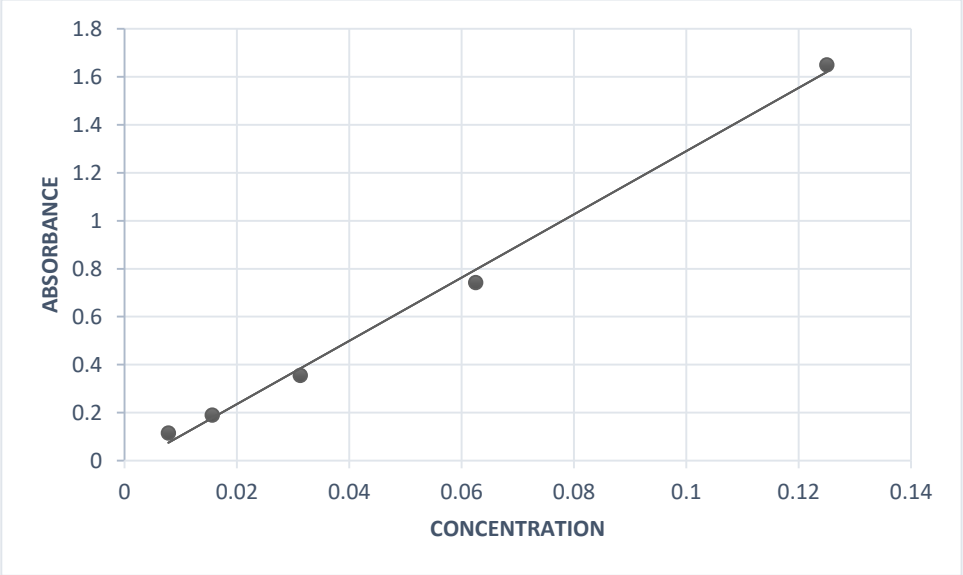

Figure 4.9: Regression equation of gallic acid calibration curve for total phenolics

4.3.2 Total flavonoid content (TFC) of *Melia azedarach*

Flavonoids are part of the polyphenol class of phytonutrients, where the polyphenols have historically been used in traditional medicines, according to the literature, and they are associated with skin protection, brain function, blood sugar and blood pressure regulation, in addition to their antioxidant activity. The physicochemical properties of flavonoids influence their metabolic fate; that is, their digestion, absorption, and biotransformation. The bioactivity of these polyphenols *in vivo* is a major determinant in their ability to exert biological activities important to human health.

There are several significant groups of flavonoids. These include anthocyanidins, flavanols, flavones, flavonols, flavanones and isoflavones. Within the flavanol subgroup, there are still more subgroups. Each of these subgroups and each type of flavonoid carries its own distinct set of actions, benefits and originating foods. Flavonoids have a large family of over 5000 compounds of hydroxylated polyphenols that carry out important functions in plants, including attracting pollinating insects. Environmental stresses such as microbial infections are also resisted. Their biological activity in humans appears to be strongly influenced by their chemical nature.[98] Total flavonoid content of the methanol crude extracts of the leaf fractions and stem bark fractions of *Melia azedarach* were determined using the aluminium chloride ($AlCl_3$) colorimetric method, with quercetin being used as a standard. We constructed the standard curve of equation Y= 3.0803 X - 0.093, where R^2 = 0.9516 (see Figure 4.10). The total flavonoid content was found to vary from 88.65±5.054 mg QE/g to 8.64±0.01 mg QE/g.

Therefore, the assay showed the highest flavonoid content of Fraction LF5 as 88.65±5.054 mg QE/g while Fraction SF3 8.64±0.0.81 mg QE/g had the lowest content in comparison with the other fractions. This shows that there are more flavonoid compounds distributed in the leaf fractions compared to the stem bark fractions of *Melia azedarach*.

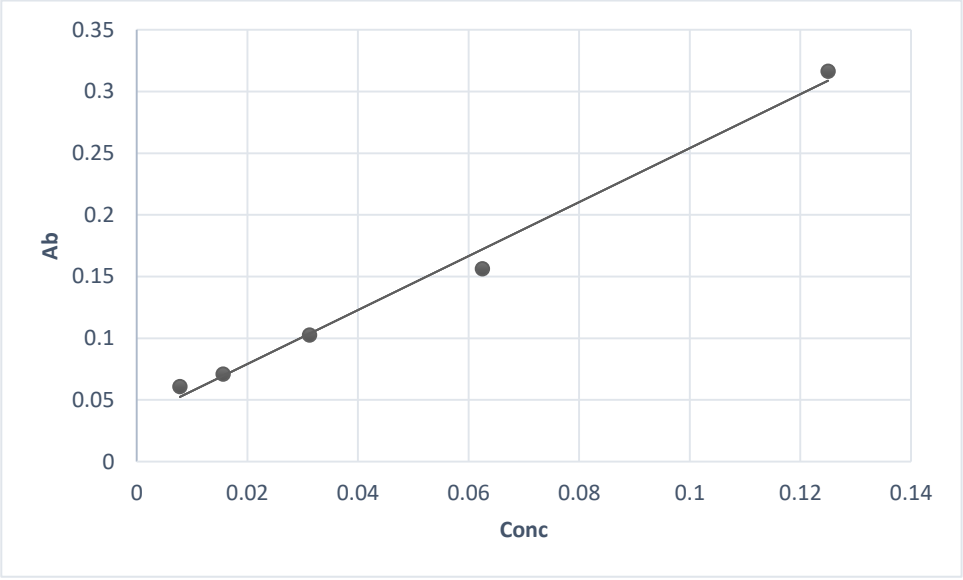

Figure 4.10: Regression equation of quercetin calibration curve for total flavonoids

4.3.3 Comparison of total phenolics and total flavonoids of *Melia azedarach*

Table 5 Total flavonoids and total phenolics of *Melia azedarach* in comparison to the stem bark and leaves.

FRACTION	TOTAL PHENOLICS (mg GAE/g)	TOTAL FLAVONOIDS (mg QE/g)
CRS	56.67	17.32
MASF1	41.40	17.11
MASF2	59.39	9.10
MASF3	55.16	8.64
MASF4	53.83	9.83
MASF5	53.87	10.91
CRL	51.59	77.81
LF2	1.79	69.91
LF3	9.81	41.96
LF5	5.88	88.65
LF6	1.16	8.49

Quantitative phytochemical evaluation specifies that the leaves of *Melia azedarach* contain a significantly high amount of flavonoids (Table 5). With LF5 found in the range of 88.65 ± 5.54 mg QE/g and LF6 8.49 ± 0.05 mg QE/g, while flavonoid content observed in the stem bark fractions of this same plant is relatively low for CRS (17.35±0.335 mg QE/g) to MASF3 8.64±0.04 mg QE/g, compared to the leaf fractions. Comparatively, all the stem fractions exhibited a good quantity of phenols, starting from MASF2 (59.39±0.179 mg GAE/g) to MASF1 (44.40±0..94 mg GAE/g) and , LF3 had the highest phenolic content of 9.81±1.31 mg GAE/g fraction while LF6 had the lowest phenolic content (1.16±0.94 mg GAE/g) in

Melia azedarach. An earlier report on the preliminary analysis also confirms that *Melia azedarach* stem bark is rich in phenolics and slightly rich in flavonoids, while the leaf is only rich in flavonoids. Amongst all the varieties analysed, therefore, the stem bark was found to be the most promising one in this study, which prompted us to project only *Melia azedarach* stem bark for further studies. LF3 had the highest phenolic content, with (9.81±1.31 mg GAE/g), while fraction LF6 had the lowest content (1.16±0.94 mg GAE/g).

4.4 Antioxidant activity of *Melia azedarach* stem bark

The DPPH free radical scavenging assay was used to measure the antioxidant activity of fractions and crude extract on the basis of free radical scavenging efficacy, while reducing power was measured on the basis of reduction potential. The results were expressed as an IC_{50} or $IC_{0.5}$ (µg/mL) value, which indicates the total amount of antioxidants in the crude extract, fractions MASF1, MASF2, MASF3, MASF4 and MASF5, that is required to reduce the initial concentration of DPPH by half (50 %). This study revealed that the crude extract and fractions all have free radical scavenging ability, reductive capability and contain a good amount of phenolic compounds. The DPPH radical reacts with antioxidants which have the ability to donate protons to free radicals. In this assay, a colour change was observed, indicating reduction from purple DPPH to yellow reduced DPPH, indicating that the tested compounds have antioxidant activity.

The highest IC_{50} value indicates the lowest antioxidant activity, while the lowest IC_{50} value implies the highest activity. In the DPPH free radical scavenging activity test, positive results were obtained from this assay and generally from the stem bark fraction of *Melia azedarach*, with Fraction MASF1 exhibiting the lowest IC_{50} value of (0.1074±1.2969 µg/mL). This was followed by Fraction MASF4 (0.1605±5.5217 µg/mL), and leaf fraction LFCR showed the highest IC_{50} value of (57.69±2.0811 µg/mL), as shown in Table 7. The DPPH free radical scavenging activity of fraction MASF1 (0.1074±1.2969 µg/mL) is greater than all the other fractions.

The antioxidant activity of the crude extract, fraction for the leaves and stem bark was also evaluated by ferric-reducing power assay. In this method, the yellow test solution changed to various shades of green and blue when the existence of reducers transformed the Fe^{3+} ferricyanide complex to its ferrous form (Fe^{2+}). As a result, Fraction MASFR1 showed the lowest $IC_{0.5}$ value of 0.5296 ± 0.2955 µg/mL for the reducing power. Therefore, Fraction

MASFR1 exhibited the highest antioxidant activity. The antioxidant activity may be due to the presence of hydroxyl groups present in them.

Table 6 Antioxidant activity of crude extract, fractions, pure compound and control (gallic acid) against DPPH. Values in the same column followed by the same letter are significantly different ($p < 0.05$)

SAMPLE CODE	DPPH IC_{50} (mg/mL)	REDUCING POWER IC_{50} (mg/mL)
STCR	0.1570 ± 2.4527	2.0018 ± 0.0399
SFR1	0.1074 ± 1.2969	0.5296 ± 0.2955
SFR2	0.3869 ± 5.7425	0.6427 ± 0.4248
SFR3	0.5142 ± 11.9181	0.9465 ± 0.2095
SFR4	0.1605 ± 5.5217	0.9349 ± 0.4677
SFR5	0.4992 ± 0.9945	1.7291 ± 0.1369
LFCR	57.69 ± 2.0811	2.8201 ± 0.0414
LFR2	3.3829 ± 0.3225	11.5297 ± 0.0254
LFR3	38.529 ± 2.4527	33.0839 ± 0.0110
LFR5	1.5003 ± 6.6188	19.2844 ± 0.0167
LFR6	5.7078 ± 2.5559	7.1598 ± 0.0801
G.A.	0.1076 ± 0.0202	0.1052 ± 0.1227

The results obtained are expected, considering previous research, Gayatri and Rajani[99] and Nahak and Sahu[30] showed that polyphenols from the stem bark of *Melia azedarach* contain comparatively strong antioxidants. Furthermore, they also exhibited good electron-donating ability, which implies that polyphenolics reduce the level of free radicals. These results

suggest that the free radical scavenging compounds are highly polar, while the reducing compounds are less polar.

4.5 Ultra-performance liquid chromatography-mass spectrometry

The chemical profile shown in Figure 4.11 of the crude methanol extract of *Melia azedarach* was obtained using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). From fraction MASF 1 (Figure 4.12) we can say that it consists of the major compounds of *Melia azedarach,* as shown in the profile (*m/z* 289.07). MASF1 was further purified by column chromatography, where two compounds were isolated, as epicatechin (**29**) and catechin (**31**) both being a polyphenolic compounds and antioxidant.

The crude extract UPLC-MS profile exposed many major peaks but the peak labelled 30 with molecular ion $[M+H]^+$ at *m/z* 291.09 was identified as epicatechin (**29**) and/or catechin (**31**). They are both well-known polyphenolic compounds. Other compounds were not identified due to challenges identified during the purification of lingering impurities present in them. The crude profile indicated with an arrow shows a peak with molecular ion at *m/z* 291.09 for $[M+H]^+$, while the HRMS shows a molecular ion at *m/z* 289.0701 for $[M-H]^+$. Together with the NMR data, this confirms that these two compounds are major constituents of this plant, as considered in the UPLC-MS of fraction MASF1 and the crude extract of *Melia azedarach* stem bark.

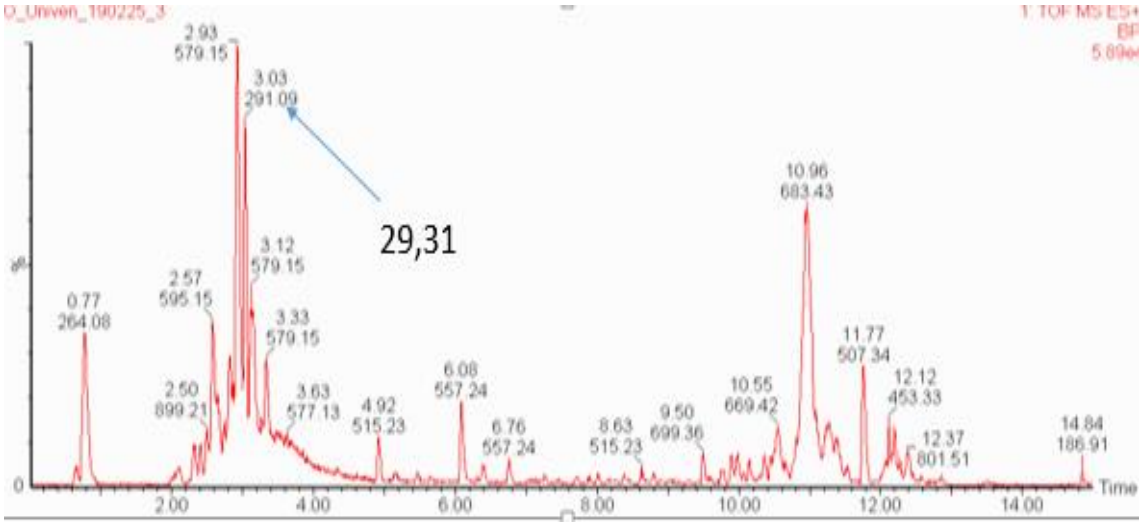

Figure 4.11: UPLC-MS of *Melia azedarach* crude extract

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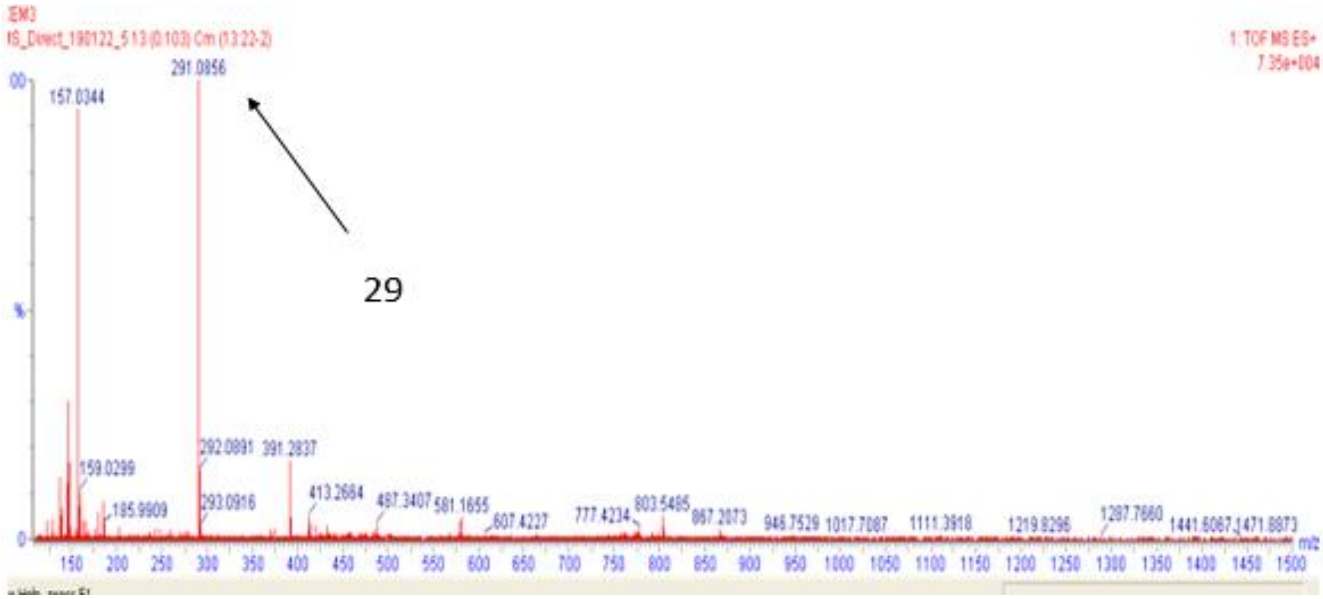


Figure 4.12: UPLC-MS of Fraction MASF1 (**29**)

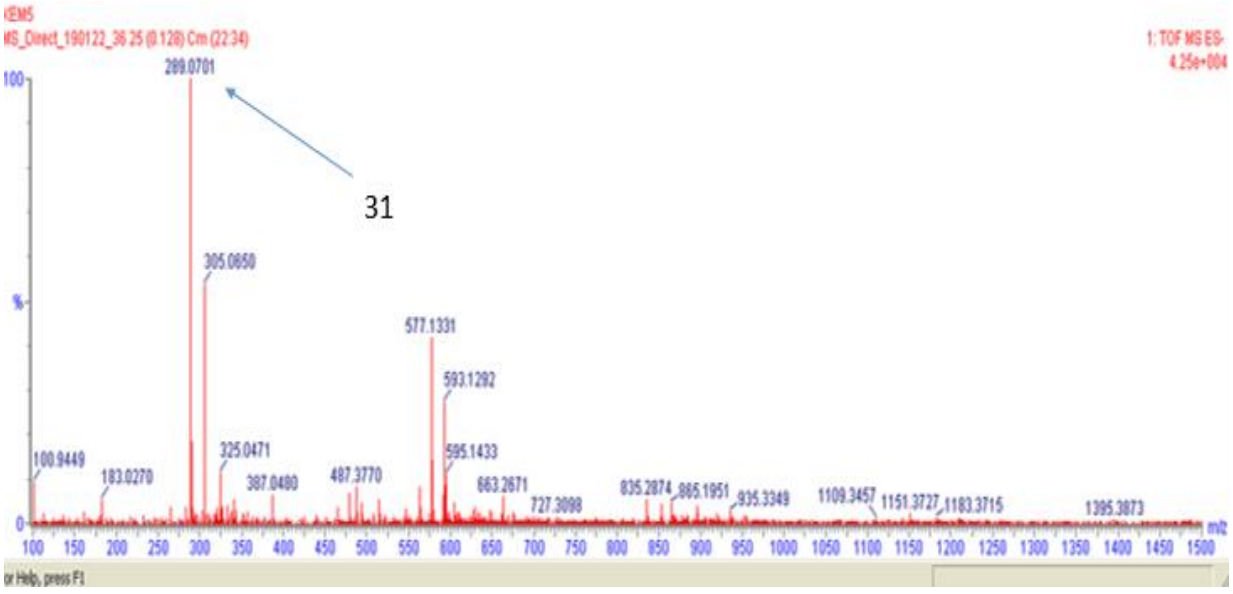
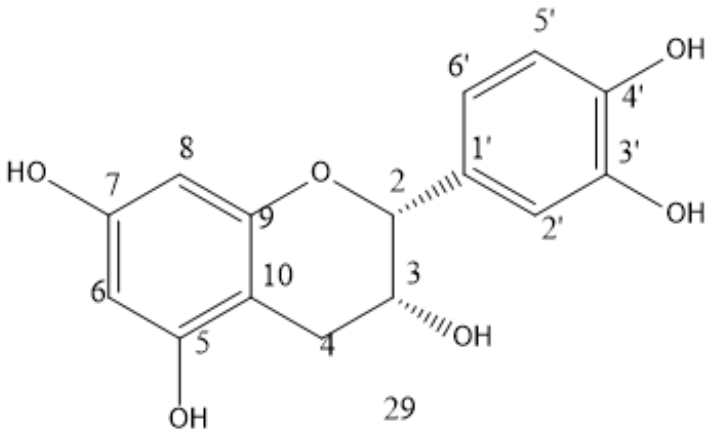


Figure 4.13: HRMS of Fraction MASF1 (**31**)

4.6 Structure Elucidation of compounds extracted from *Melia azedarach*

4.6.1 3,3',4',5',7-Pentahydroxyflavan (epicatechin 29)



The spectral data are summarized in Table 7.

The HRMS spectrum (Appendix 2) revealed a $[M+H]^+$ ion peak at *m/z* 291.0856 corresponding to the molecular formula $C_{15}H_{14}O_6$, while the IR spectrum (Appendix 1) of epicatechin showed a broad band at 3332.42 cm^{-1}, which corresponds to a phenolic and alcoholic O-H stretching; the other bands at 2941.93 cm^{-1} and 2833.27 cm^{-1} are due to the presence of saturated C-H stretching.[106]

In addition, the UV spectrum showed absorption maxima at 230 and 279 nm, consistent with conjugated π-π* transitions arising from the aromatic rings; this is in agreement with literature reports.[100]

The ^{1}H-NMR spectrum (Appendix 3) of epicatechin (**29**) revealed that proton 3-H was split by 2-H and 4-H, resulting in a multiplet , doublet of doublet of doublets with $J_{2,3} < 1$ HZ, $J_{3,4a} = 16$ Hz and $J_{3,4b} = 8$ Hz at δ_H 4.91 ppm. The protons at 4-H were split by 3-H resulting in a doublet of doublets at 2.37 and 2.62 ppm. The chemical shift of 2-H, a doublet at 4.48 ppm, with coupling constant ($J_{2,3,} < 1$ Hz), the two C-4 protons 4a-H and 4b-H are diastereotopic and are split by 3-H resulting in a doublet of doublets at 2.62 and 2.37 ppm respectively, with $J_{4a,4b} = 16$ Hz, suggesting that the flavan structure possess the *cis*-2,3 stereochemistry.[106,117]

Special signals of the B-ring (aromatic protons) are of the ABX-type, where the methine proton 2'-H is split by 6'-H in a *meta* position, giving a doublet at δ_H 6.60 (d, $J = 8$ Hz). 5'-H is split by 6'-H in an *ortho* position, giving a doublet, 6'-H is split by 5'-H and 2'-H, giving a

doublet at δ_H 6.67 ppm (d, *J* = 8 Hz). The aromatic ring A revealed the 6-H and 8-H are *meta* coupling, giving two doublets at δ_H 5.88 and 5.68 ppm (*J* = 0.8 Hz), therefore, the basic structure of this compound is deduced as 3,3',4',5',7-pentahydroxyflavan.[101]

The structure assignment of epicatechin (**29**) was further confirmed using the ^{13}C-NMR (Appendix 4) and DEPT135 spectra (Appendix 5),both showing fifteen carbon signals, consisting of one methylene at δ_C 29.17 ppm (C-4), seven quaternary carbons at δ_C 156.53 (C-5), 156.26 (C-7),155.43 (C-9), 144.92 (C-3' & C-4'), 120.41 (C-1'), and 99.13 (C-10) ppm, and seven methine carbons at δ_C 118.52 (C-6'), 115.14 (C-5'),114.57 (C-2'), 95.16(C-6), 93.90 (C-8), 81.07 (C-2) and 66.38 (C-3) ppm.

For the ^{1}H-^{1}H COSY spectrum of epicatechin (**29**) (Appendix 7), the correlations show the coupling between 3-H and 2-H at δ_H 4.48 and 4.91 ppm also between 3-H and 4-H at δ_H 2.3 ppm and 2.3 ppm. The 6-H and 8-H methine signals can be seen at δ_H 5.6 ppm and 5.6 ppm. All these positions were further confirmed by long-range coupling, as seen in the HMBC (Appendix 6).

The methyl proton peaks at 4a-H (δ_H 2.4 ppm) also show correlations with H-4b (δ_C 2.4 ppm). The 7-OH at (δ_H 8.9 ppm) correlates with 5-OH at (δ_H 8.9 ppm). This correlates with published studies.[43,102]

The HSQC spectrum (Appendix 8) shows seven quaternary carbons at δ_C 156.53 (C-5), 156.26 (C-7),155.43 (C-9), 144.92 (C-3' & C-4'), 120.41 (C-1') and 99.13 (C-10) ppm and the 5-OH (δ_H 9.24), 7-OH (δ_H 8.99), 4'-OH (δ_H 8.92), 3'-H (δ_H 8.87) and 3-OH (δ_H 5.30) ppm groups are not coupling to C or H, which is in agreement with our DEPT135 (Appendix 5). The ^{1}H and ^{13}C chemical shifts correlate with those reported in the literature.[103]

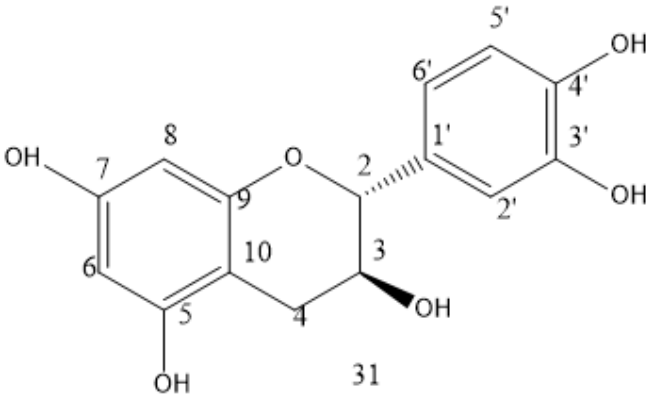
Elemental analysis: Calcd. for $C_{15}H_{14}O_6$: C 62.07; H 4.86; O 33.7. Found: C 63.44; H 7.73; O 28.83.

The relative stereochemistry of epicatechin was determined by comparison with the literature, confirming the isolation of epicatechin (**29**).[104] It is polyphenolic and was previously isolated from *Trichilia emetica* seeds (as shown in Table 7) by Usman *et al.*[31] and Iwan *et al*.[7] and investigated as an antimalarial.[45] Epicatechin is not a novel compound but our study is the first to confirm that it has antimalarial activity and also to confirm this from *Melia azedarach* stem bark.

Table 7: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra data of epicatechin (**29**) in DMSO-d_6 (δ in ppm, *J* in Hz).

Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)	^{1}H-NMR (δ ppm) (Usman *et al.*)[31]
2	4.48 (d, *J* = 8 Hz, 1H)	81.07 (CH)	4.83 (s, br)
3	4.91 (m, 1H, 3-H)	66.38 (CH)	4.19 (m, 1H)
4	2.62 (dd, *J* = 16 H_Z and 48 Hz,1H, 4a-H) & 2.37 (dd, *J* = 8 and 16 Hz, 1H, 4b-H)	29.17 (CH_2)	2.86 (dd, *J* = 4.8, 16.80 Hz & 2.73 (dd, *J* = 2.7, 16.8 Hz, 1H)
5	-	156.53 (C)	
6	5.88 (d, *J* = 0.8 Hz, 1H, 6H)	95.16 (CH)	5.93 (d, *J* = 2.3 Hz, 1H)
7	-	156.26 (C)	
8	5.68 (d, *J* = 0.8 Hz, 1H, 8H)	93.90 (CH)	5.96 (1H, d, *J* = 2.3 Hz)
9	-	155.43 (C)	
10	-	99.13 (C)	
1’	-	120.41 (C)	
2’	6.60 (d, *J* = 8 Hz,1H)	114.57 (CH)	6.99 (*J* =1.7 Hz, 1H)
3’	-	144.92 (C)	
4’	-	144.92 (C)	
5’	6.71-6.65 (d, *J* = 8 Hz, 1H)	115.14 (CH)	6.77 (d, *J* = 8.2 Hz)
6’	6.67 (d, *J* = 8 Hz, 1H)	118.52 (CH)	6.81 (dd,*J* =1.7, 8.2 Hz)

4.6.2 3,3',4',5',7-Pentahydroxyflavan (catechin 31)



The spectral data are summarized in Table 8.

The HRMS spectrum (Appendix 10) revealed a $[M-H]^+$ ion peak at *m/z* 289.0701 matched with the compound's molecular formula $C_{15}H_{14}O_6$, while the IR spectrum (Appendix 9) of catechin showed a broad band at 3331.82 cm^{-1}, which corresponds to phenolic and alcoholic O-H stretching, while the other bands at 2943.98 cm^{-1} and 2832.48 cm^{-1} are due to the presence of saturated C-H stretching.[106,134]

These results are supported by the UV spectrum, showing absorption maxima at 230 and 279 nm for conjugated π-π* transition, this can be said to arise from the aromatic rings. This result is consistent with literature reports.[100]

The ^{1}H-NMR spectrum (Appendix 12) of catechin (**31**) revealed that there is only one methylene group with δ_H 2.63 (4a-H) and 2.36 (4b-H) and seven methine signals at δ_H 6.71 (H- 5'), 6.67 (6'-H), 6.59 (2'-H), 5.88 (6-H), 5.68 (8-H), 4.98 (3-H) and 4.47 ppm (2-H). Proton 3-H (δ_H 4.98) was split by 2-H (δ_H 4.47) and 4a-H (δ_H 2.27) resulting in a multiplet at δ_H 4.98 ppm. The protons at 4-H were split by 3-H, which gives a doublet of doublets at δ_H 2.27 and δ_H 2.38 ppm. The geminal coupling observed between 4a-H and 4b-H, viz., $J_{4a,4b}$ = 8 Hz suggests that the flavan structure possesses the *trans*-2,3 relative stereochemistry.[101,105]

Special signals of the B-ring (aromatic protons) are of the ABX-type where the methine proton 2'-H was split by 6'-H in a *meta* position giving rise to a doublet at δ_H 6.67 (d, J = 8 Hz). 5'-H was split by 6'-H in an *ortho* position giving a doublet, 6'-H was split by 5'-H and 2'-H, giving a doublet at δ_H 6.81(d, J = 0.02 Hz).The aromatic ring A also revealed that 3'-H and 4'-H are coupling, giving rise to a doublet at δ_H 9.01 and δ_H 8.89 ppm. Therefore, the structure of this compound is deduced as 3,3',4',5',7-pentahydroxyflavan.

The structural assignment of catechin (**31**) was further confirmed using the ^{13}C-NMR (Appendix 11) and DEPT135 spectra (Appendix 14) and they both showed fifteen carbon signals of δ_C 156.54 (C-5), 156.27 (C-7), 155.44 (C-9), 144.93 (C-4' & C-3'), 130.65 (C-1'), 118.54 (C-6'), 115.15 (C-5'),114.58 (C-2'), 99.14 (C-10), 95.17 (C-6), 94.33 (C-8), 81.07 (C-2), 72.08 (C-3) and 28.84 (C-4) ppm, which consists of one methylene at δ_C 29.17 (C-4) ppm and seven quaternary carbons at δ_C 156.54 (C-5), 156.26 (C-7), 155.43 (C-9), 144.93 (C-3' & C-4'), 130.65 (C-1') and 99.13 (C-10) ppm and seven methine carbons at δ_C 118.54 (C-6'), 115.15 (C-5'),114.58 (C-2'), 95.17 (C-6), 94.33 (C-8), 81.07 (C-2), 72.08 (C-3) and 28.84 (C-4) ppm.

For the ^{1}H-^{1}H COSY of catechin (**31**) correlations (Appendix 13) the correlations show that there is coupling between 3-H and 2-H at δ_H 4.8 ppm and 4.8 ppm (and also between 3-H and 4-H at δ_H 2.3 ppm and 2.3 ppm). Furthermore, methine signals can be seen for 6-H and 8-H at δ_H 5.3 ppm and 5.3 ppm. All these positions were further confirmed by long-range couplings seen in the HMBC.

In the HMBC spectrum (Appendix 16) of catechin (**31**), the methyl proton peak at 6-H (δ_H 5.8) ppm is correlating with C-8 (δ_C 90.8 ppm), 7-OH (δ_C 6.8) correlates with C-4' (δ_C 140.7), while all the quaternary carbons do not correlate with any hydrogen, correlating with other studies.[100]

The HSQC spectrum (Appendix 16) shows seven quaternary carbons at δ_C 156.54 (C-5), 156.27 (C-7), 155.44 (C-9), 144.93 (C-4' & C-3'), 130.65 (C-1'), 99.14 (C-10) and also the 5-OH (δ_H 9.26), 7-OH (δ_H 9.01), 4'-OH (δ_H 8.93), 3'-OH (δ_H 8.89), 3-OH (δ_H 5.30 ppm) groups which is in agreement with DEPT135 (Appendix 14). See Table 8 for data for the NMR which reveals the ^{1}H and the ^{13}C values reported in the literature by Michael *et al*.[103]

Elemental analysis: Calcd. for $C_{15}H_{14}O_6$: C 62.07; H 4.86; O 33.07. Found: C 61.83; H 7.41; O 30.76.

Table 8: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra data of catechin (**31**) in DMSO-d_6 (δ in ppm, *J* in Hz).

Position	^{1}H-NMR (δ ppm)	^{13}C-NMR (δ ppm)	^{13}C-NMR (δ ppm) (Toure *et al.*)[102]
2	4.47 (d, *J* = 4 Hz, 1H)	81.07 (CH)	82 (CH)
3	4.98 (m, 1H, 3-H)	66.38 (CH)	68.1(CH)
4	2.63 (dd, *J* = 4.8 Hz and *J* = 8 Hz, 1H, 4a-H) & 2.36 (dd, *J* = 16 and *J* = 8 Hz, 1H, 4b-H)	28.84 (CH_2)	29.3 (CH_2)
5	-	156.54 (C)	157.1 (C)
6	5.88 (s, 1H, 6H)	95.17 (CH)	96.5 (CH)
7	-	156.27 (C)	158.1(C)
8	5.68 (s, 1H, 5H)	94.33 (CH)	95 (CH)
9	-	155.43 (C)	158 (C)
10	-	99.14 (C)	100.8 (C)
1’	-	130.65 (C)	132.0 (C)
2’	6.59 (d, *J* = 8 Hz, 1H)	114.58 (CH)	115.8 (CH)
3’	-	144.93 (C)	146.9 (C)
4’	-	144.93 (C)	146.8 (C)
5’	6.71-6.65 (d, *J* = 8 Hz, 1H)	115.15 (CH)	116.2 (CH)
6’	6.67 (d, *J* = 8 Hz, 6’-H),	118.54 (CH)	119.5 (CH)

CHAPTER 5

CONCLUSION

The main objectives of this study were accomplished to a great level, which were to investigate the antimalarial activity of four selected South African plants *Melia azedarach* leaves and stem bark, (*Sclerocarya birrea* stem bark and leaves, *Capparis tomentosa* leaves and *Lippia javanica)*. These plants are commonly used by traditional healers and local people in the rural areas of South Africa to treat malaria. The study also sought to isolate and characterize the bioactive compound(s) from the plant with the highest bioactivity (*Melia azedarach* stem bark) as well as testing for the antioxidant activity of the fractions, identifying the phytochemical constituents of the fractions (MASF1, MASF2, MASF3, MASF4 AND MASF5), and the crude extract (A1) of the plant that is bioactive. *Melia azedarach* stem bark contains polyphenolic compounds, compared to its leaves from our results, and also from testing for the antiplasmodial, antitrypanosomal, antiparasitic and cytotoxic activities of these fractions.

Four compounds (**28**, **29**, **30** and **31**), were isolated from the stem bark methanol extract of *Melia azedarach* where compounds **29** and **31** were identified as epicatechin and catechin, respectively, which are well known polyphenolic compounds from *Melia azedarach*. This is the first report comparing these commonly-used plant's stem bark and leaves of *Melia azedarach* for antimalarial purposes. Furthermore, only compounds **29** and **31** were characterized and identified using a combination of various spectroscopic techniques, such as nuclear magnetic resonance (NMR), infra-red (IR) spectroscopy and high resolution mass spectroscopy (HRMS) as epicatechin (**29**) and catechin (**31**) which have been isolated before from various medicinal plants.

The structures of compounds **29** and **31** were confirmed to our satisfaction. However, the structure elucidation of compounds **28** and **30** was not possible due to the lingering presence of impurities in the samples (Appendix 17-27). As a result, it is recommended that further chemical investigation should be conducted.

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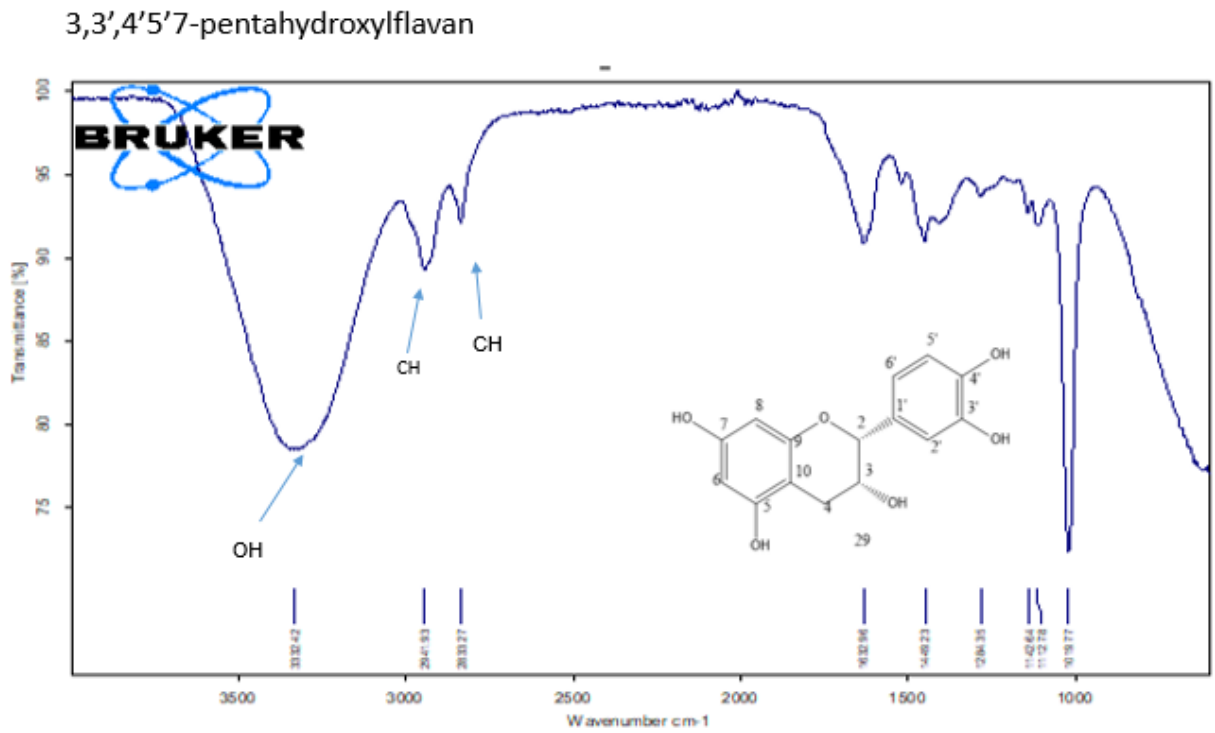
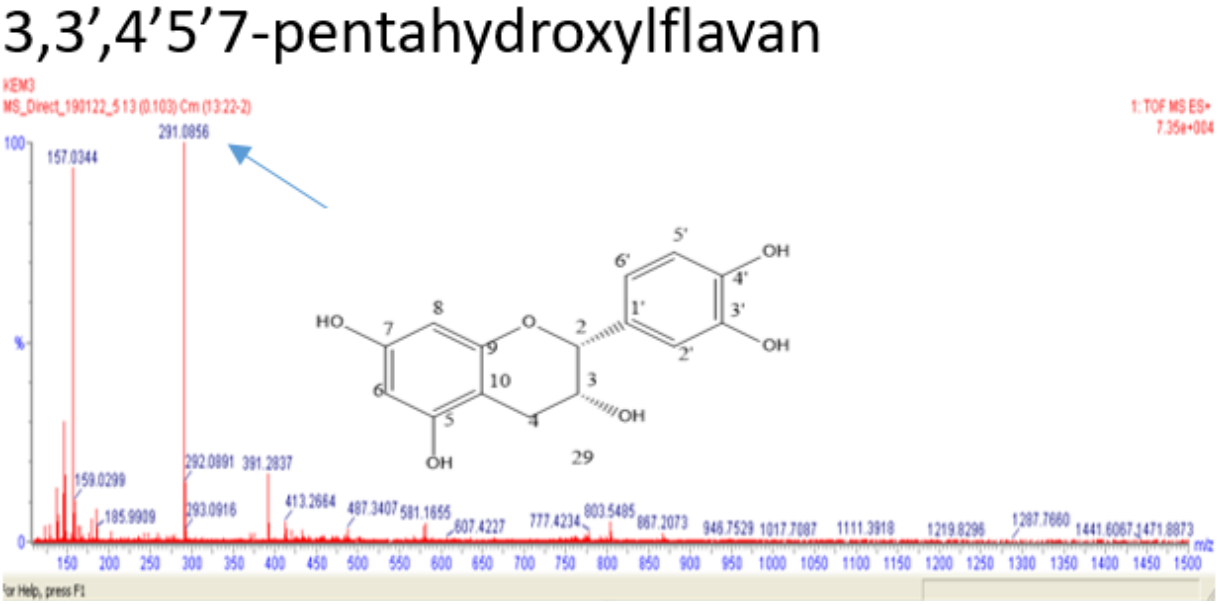
Appendix

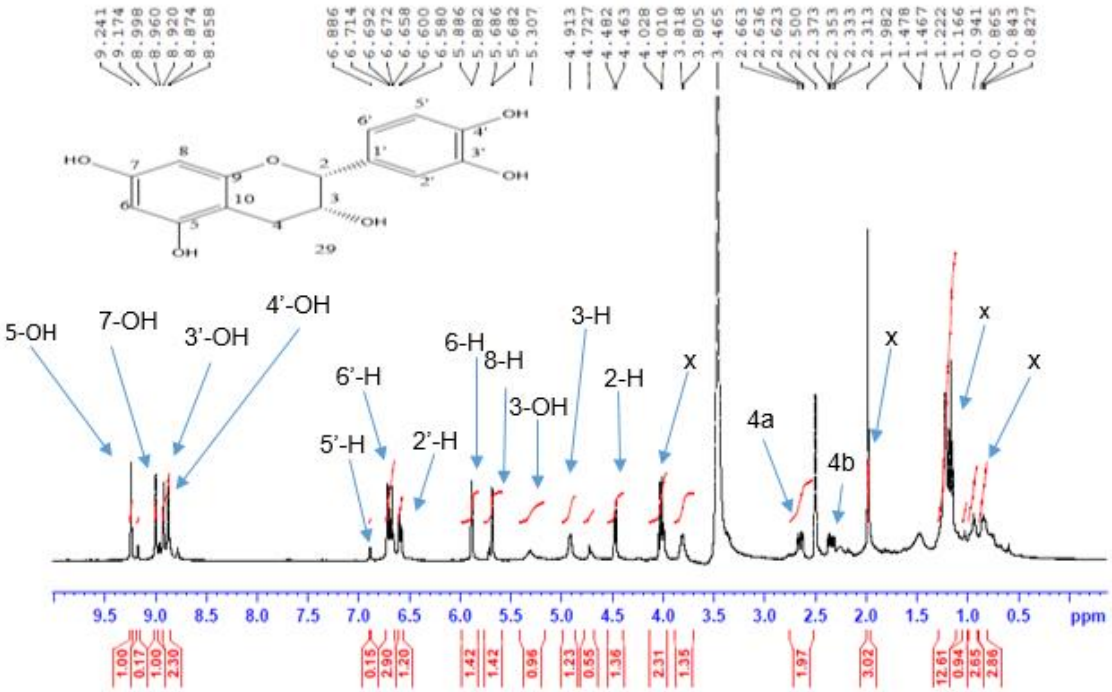


Appendix 1: IR spectrum of epicatechin (**29**)



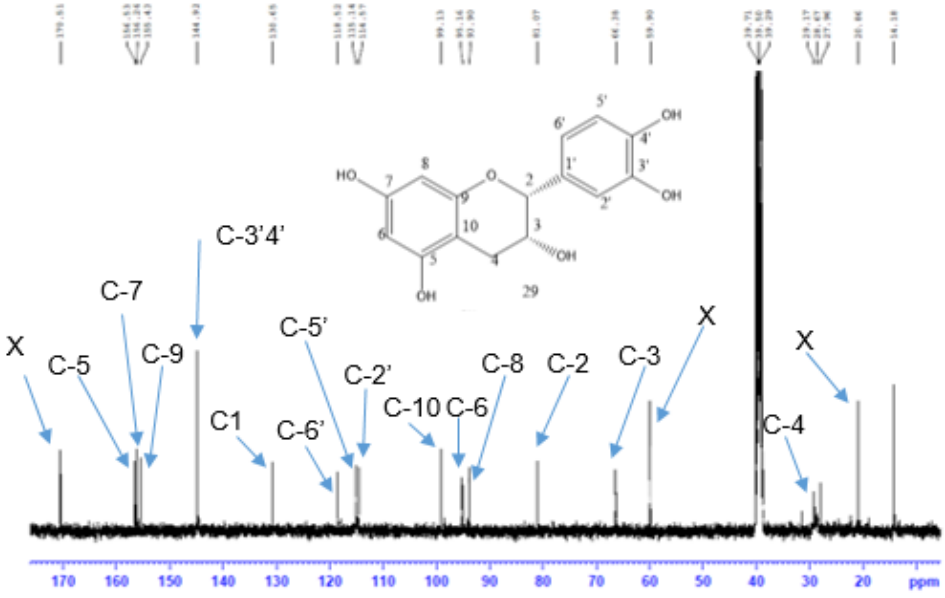
Appendix 2: HRMS spectrum of epicatechin (**29**)

3,3',4'5'7-pentahydroxylflavan

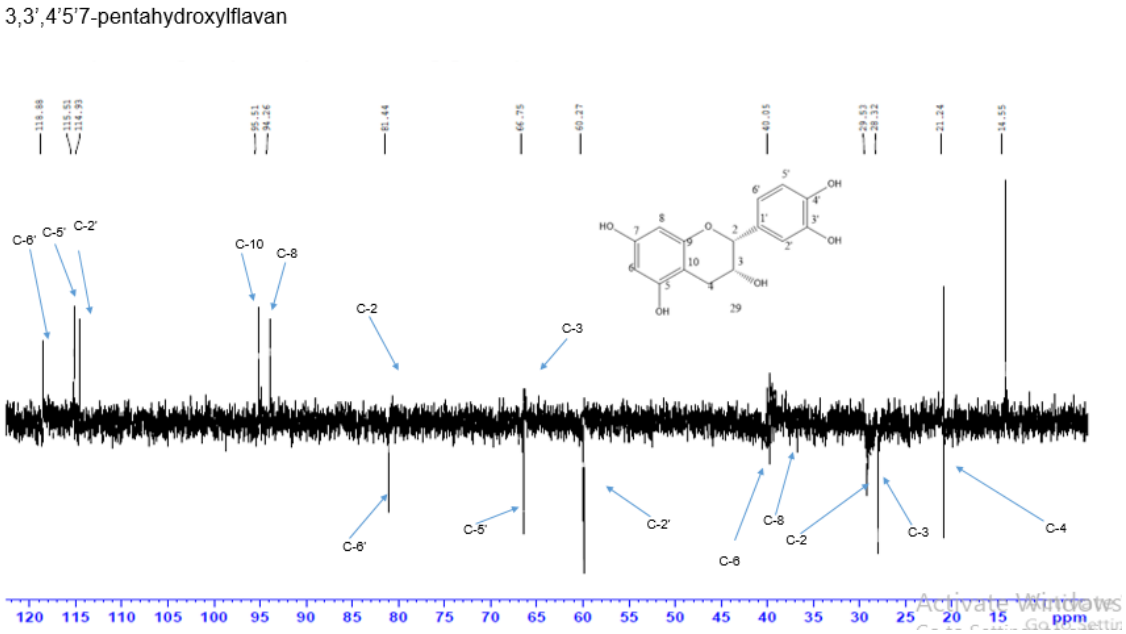


Appendix 3: Expanded ^{1}H-NMR spectrum of epicatechin (**29**).

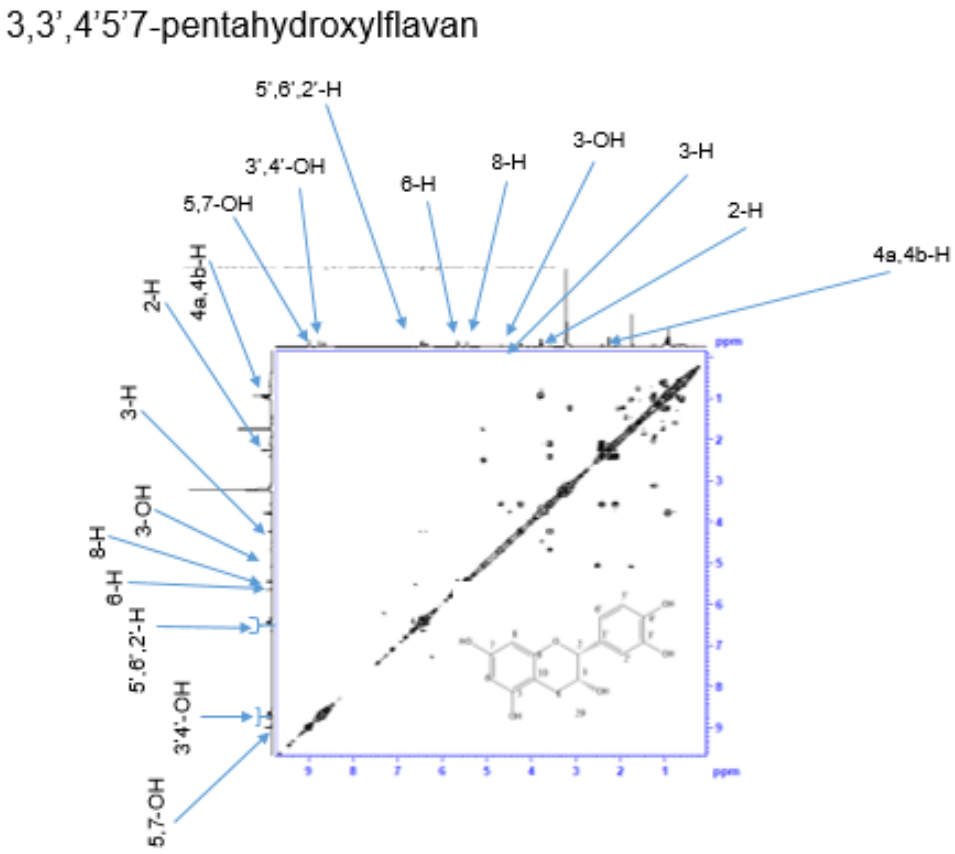
3,3',4'5'7-pentahydroxylflavan



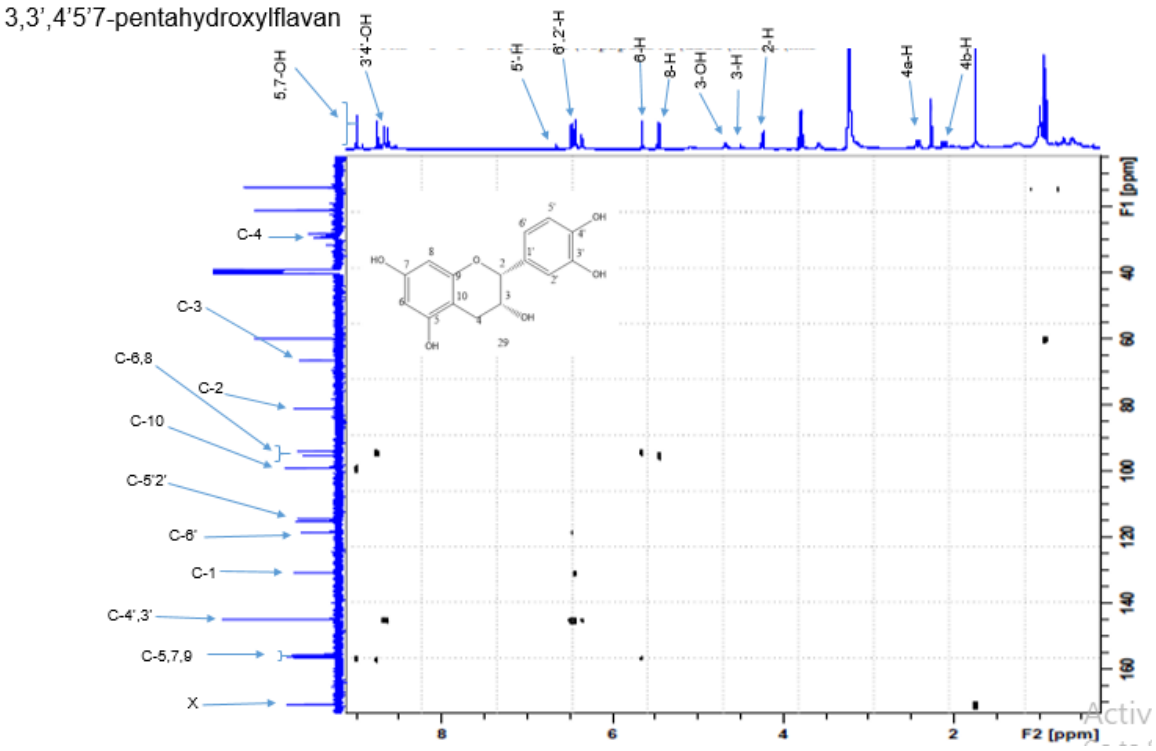
Appendix 4: Expanded ^{13}C-NMR spectrum of epicatechin (**29**).



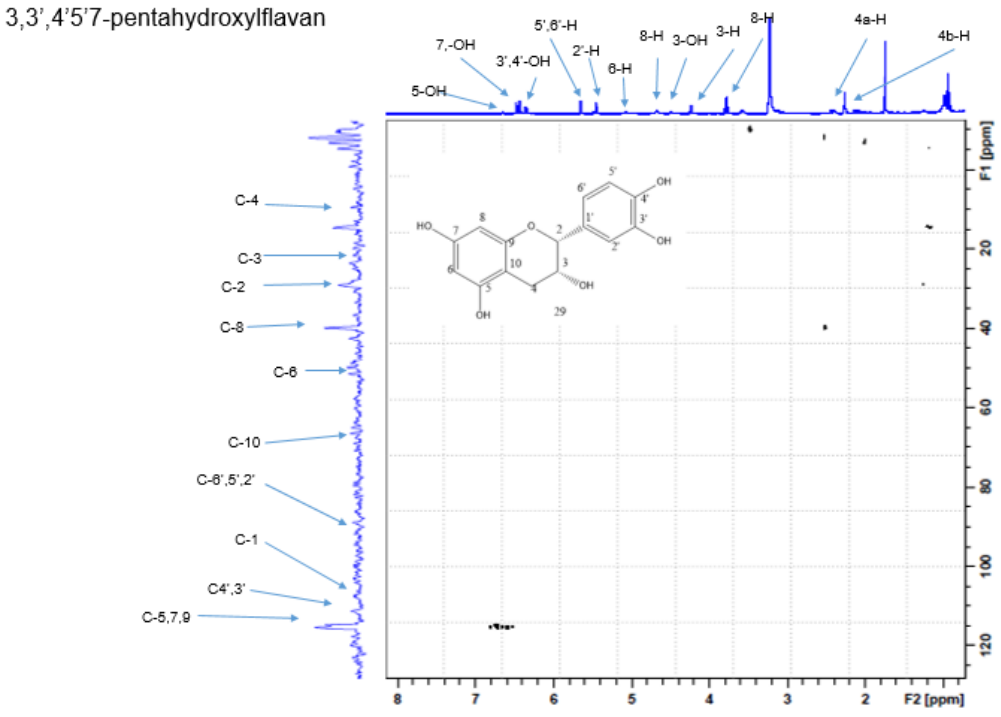
Appendix 5: DEPT135 spectrum of epicatechin (**29**).



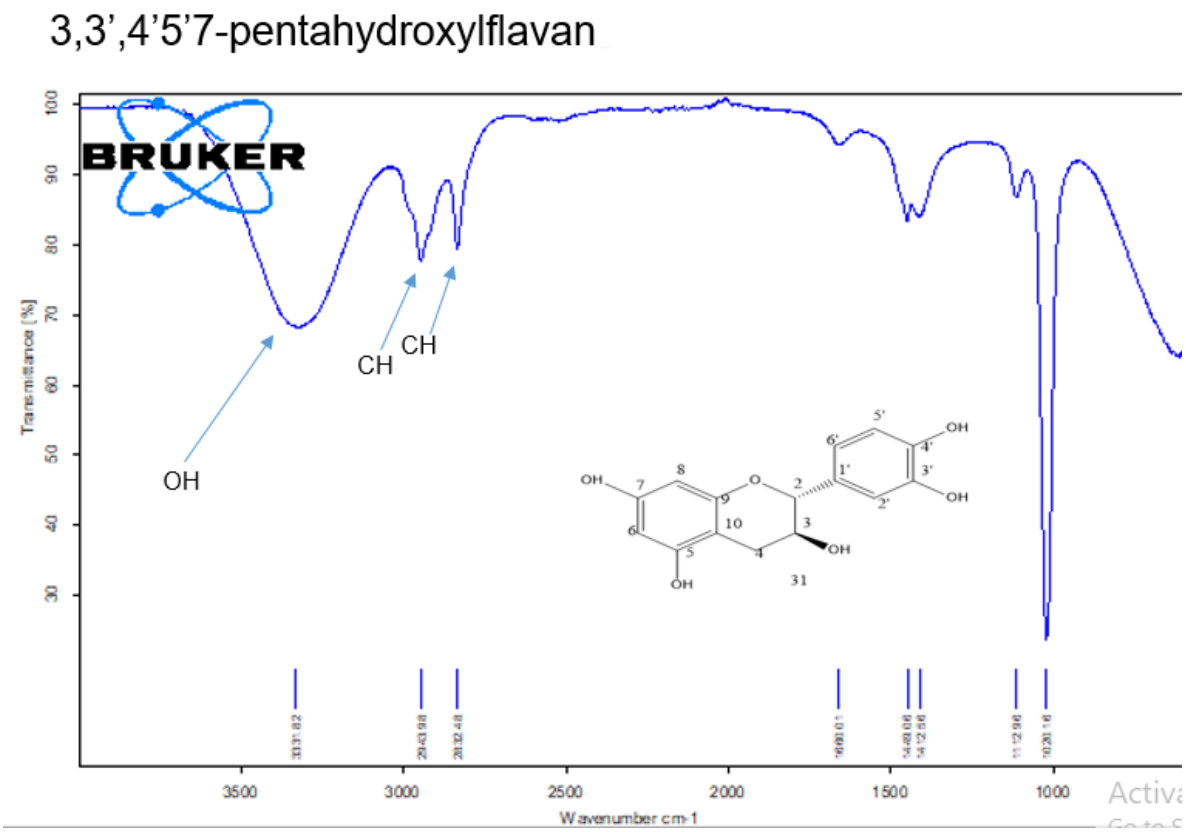
Appendix 6: COSY spectrum of epicatechin (**29**).



Appendix 7: HMBC spectrum for epicatechin (**29**).

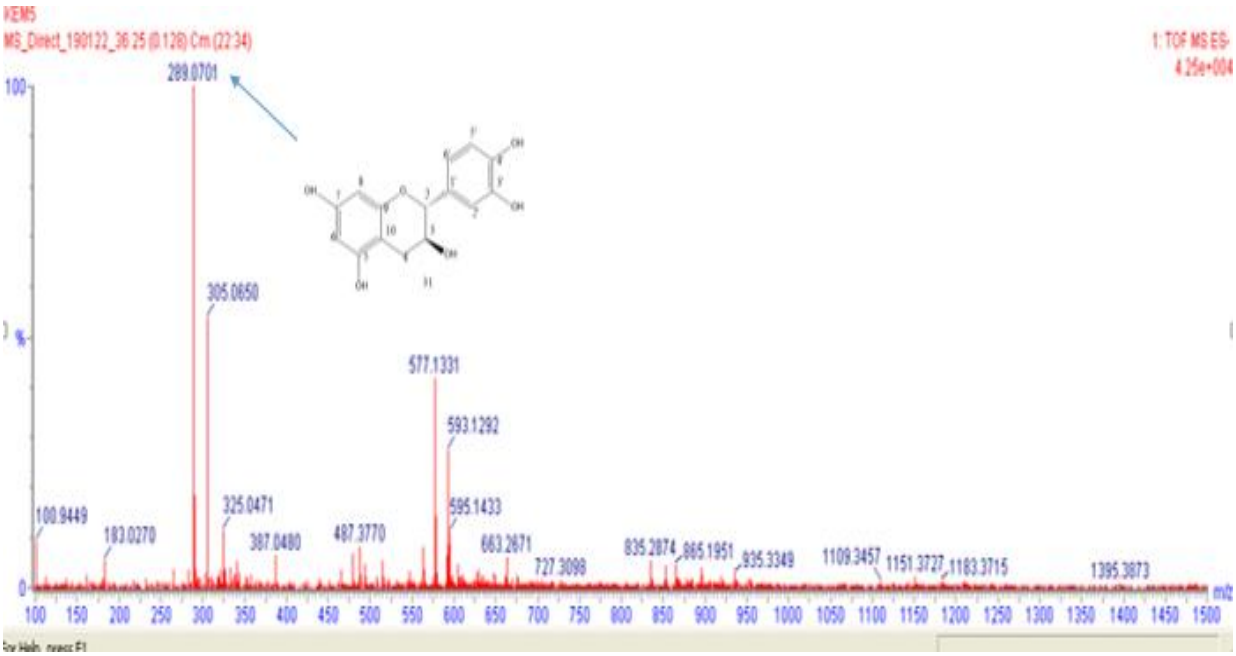


Appendix 8: HSQC spectrum of epicatechin (**29**).

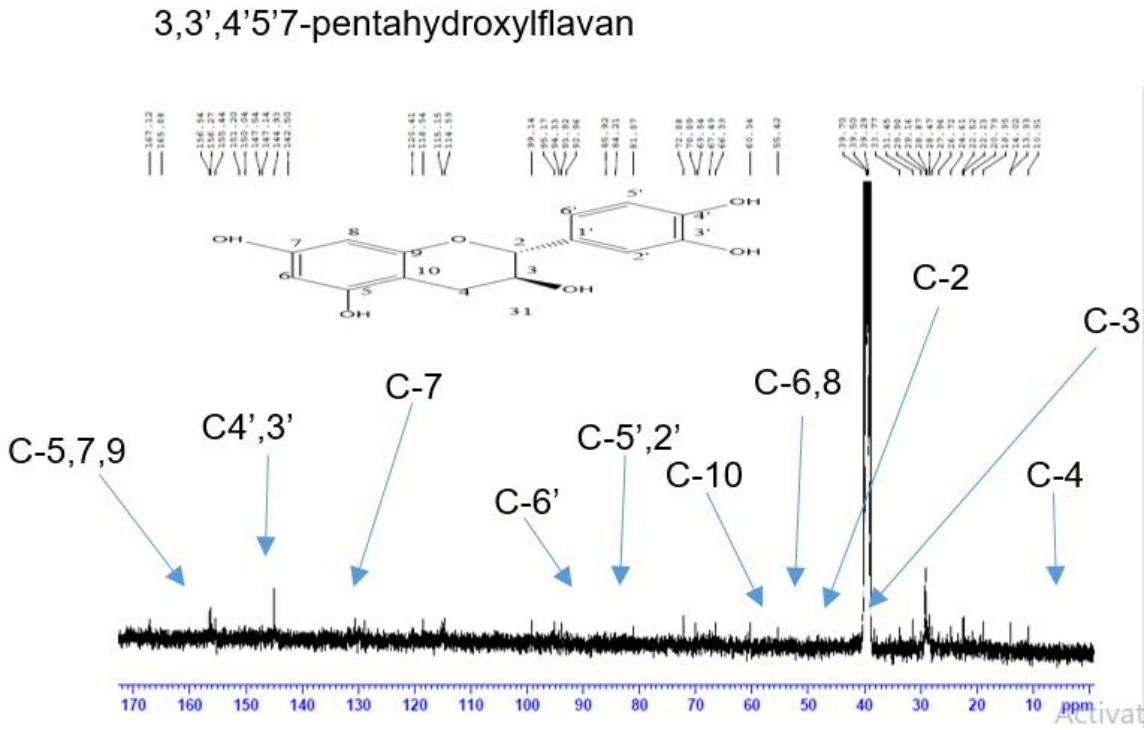


Appendix 9: IR spectrum of catechin (**31**)

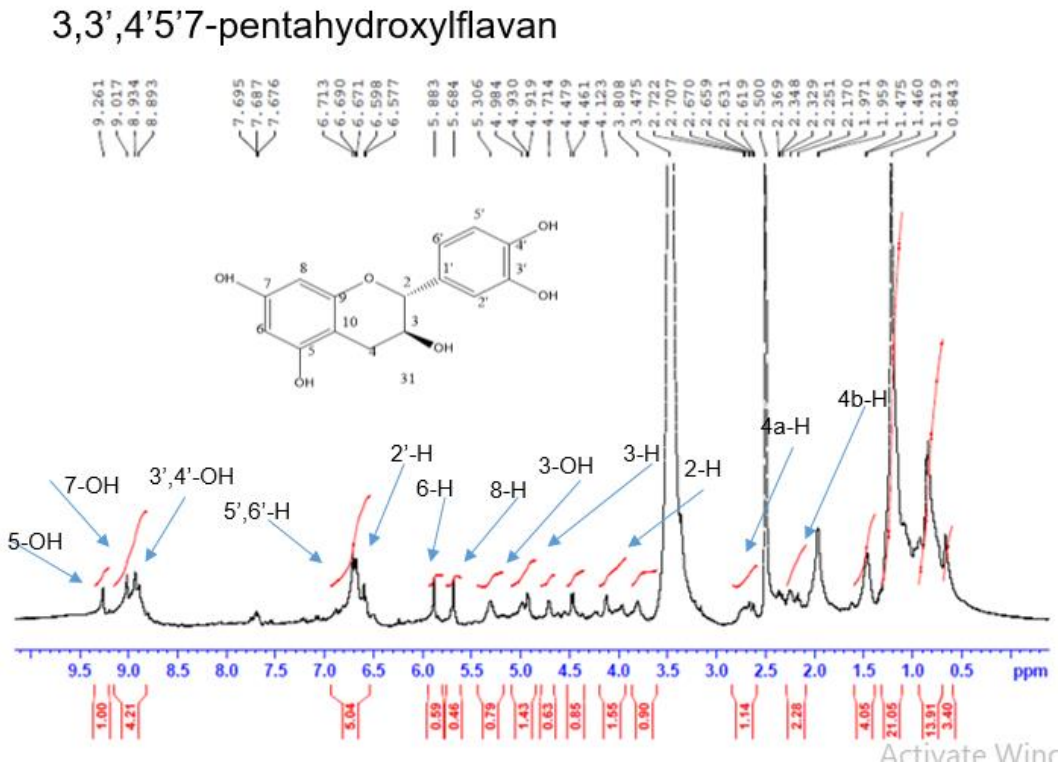
3,3',4'5'7-pentahydroxyflavan



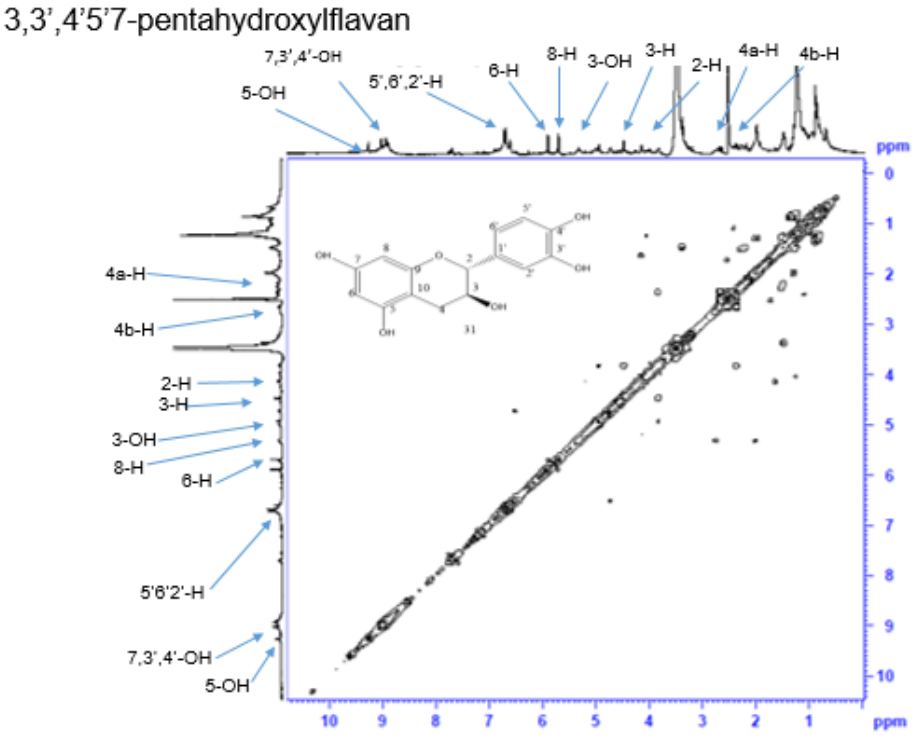
Appendix 10: Mass spectrum of catechin (**31**)



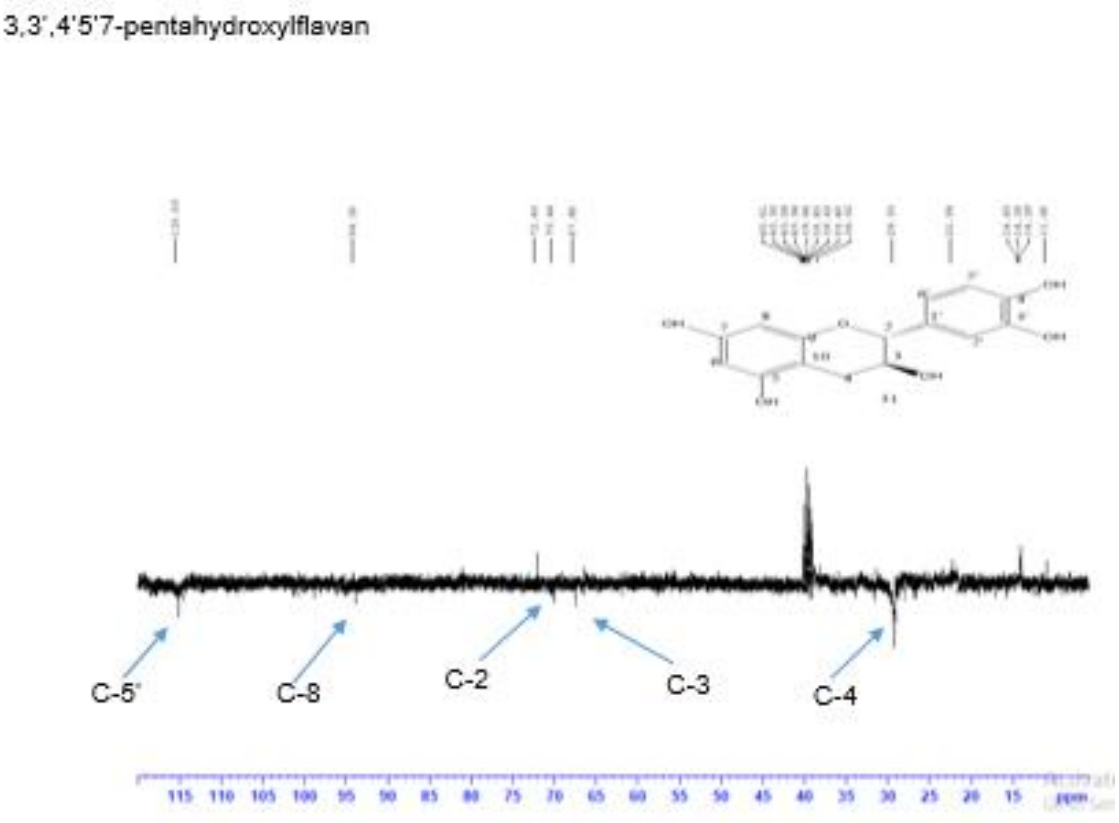
Appendix 11: Expanded ^{13}C-NMR spectrum of catechin (**31**)



Appendix 12: ^{1}H-NMR spectrum of catechin (**31**).

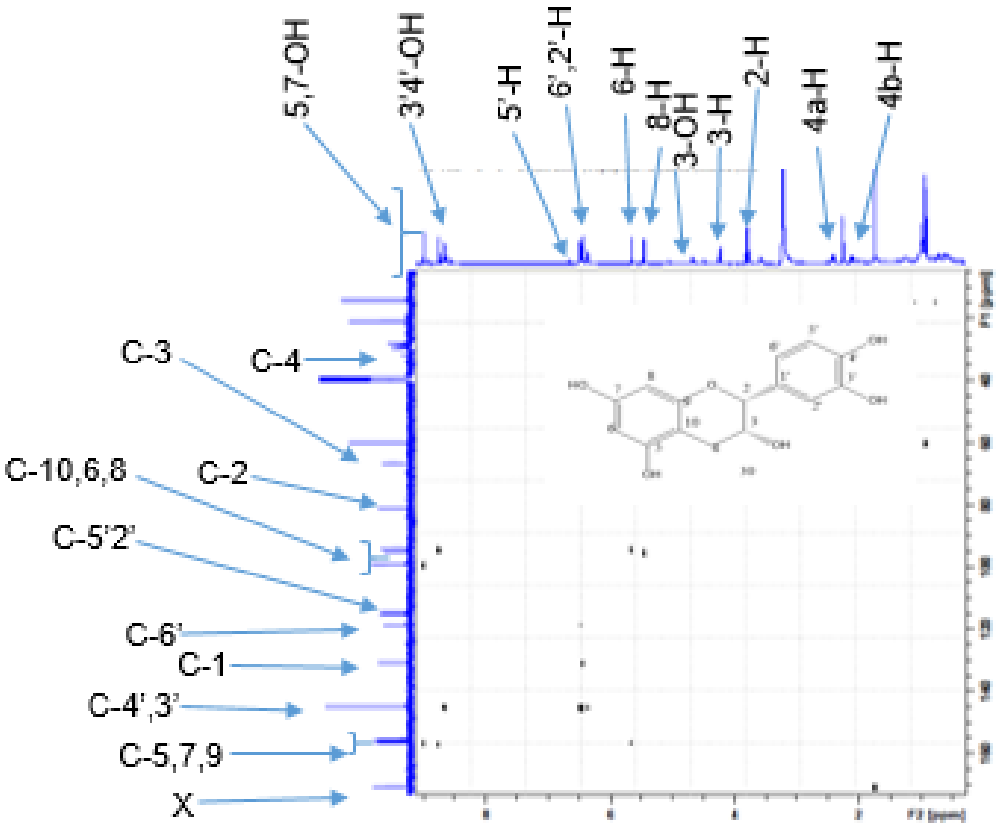


Appendix 13: COSY spectrum for catechin (**31**).

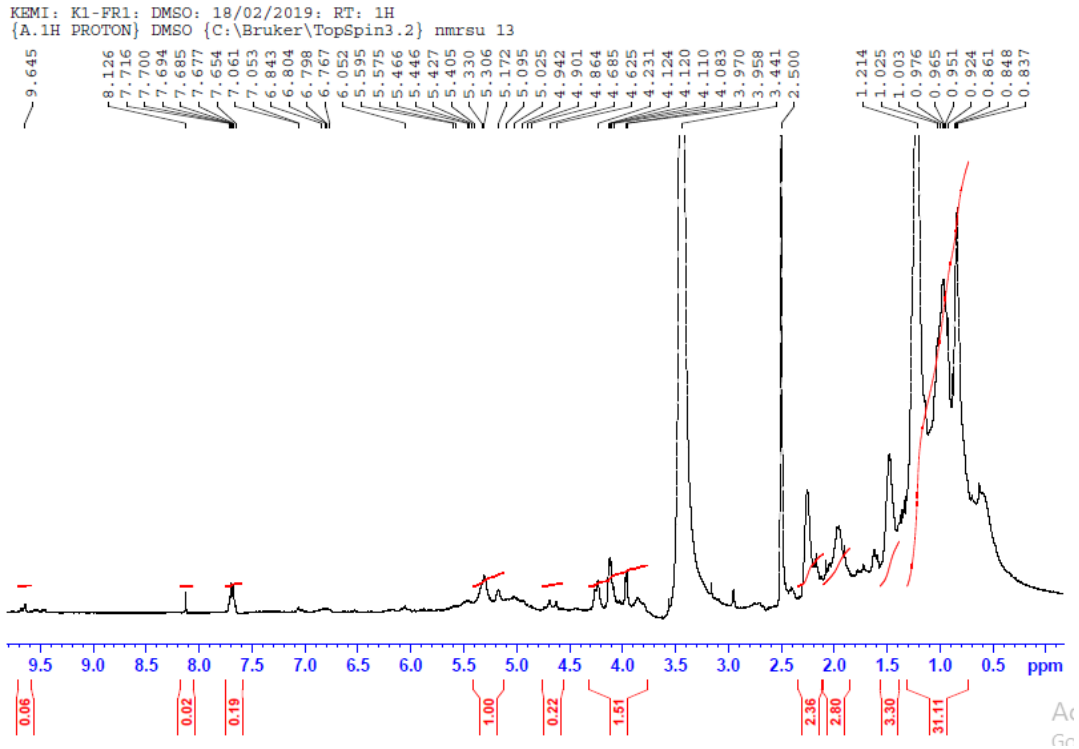


Appendix 14: DEPT135 spectrum of catechin (**31**).

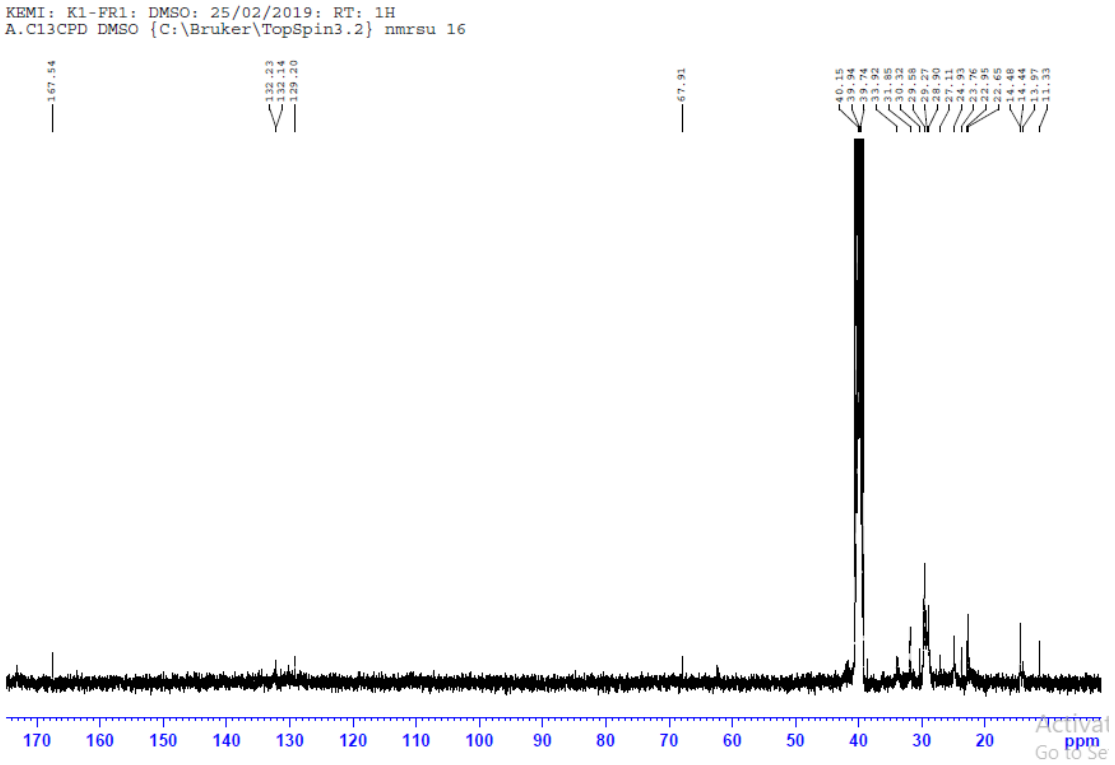
3,3',4'5'7-pentahydroxylflavan



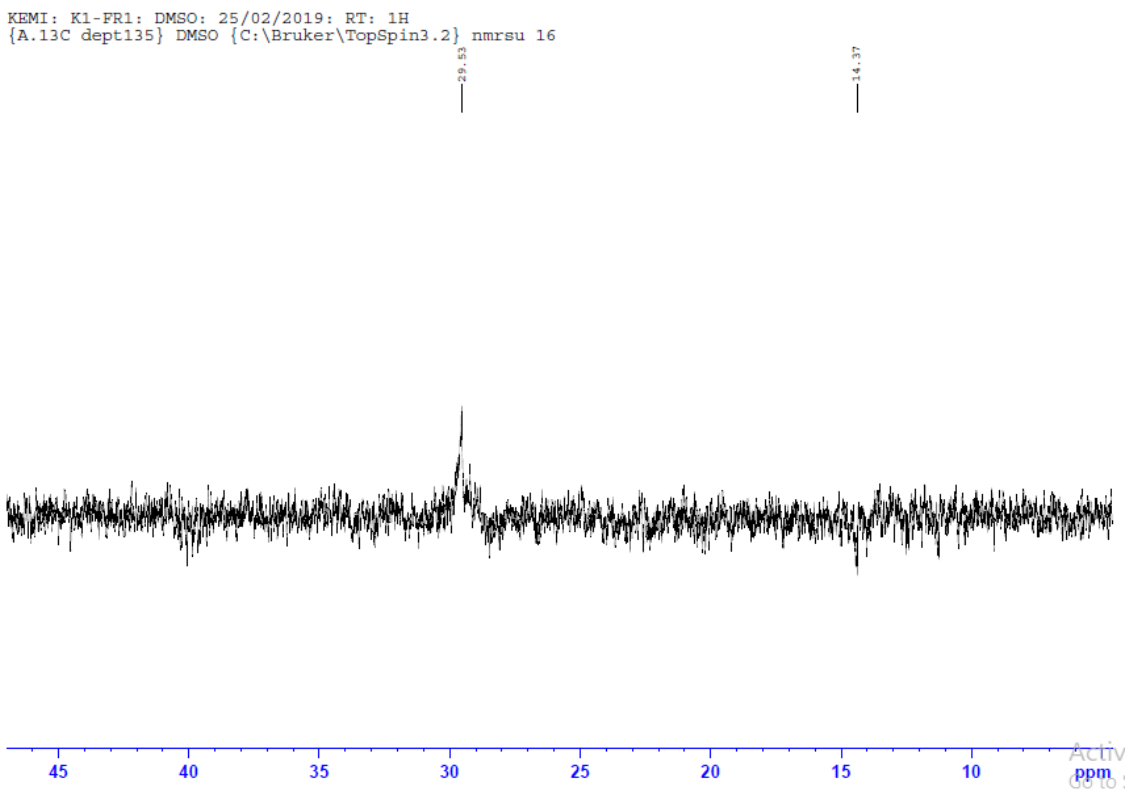
Appendix 15: HMBC spectrum for catechin (**31**)



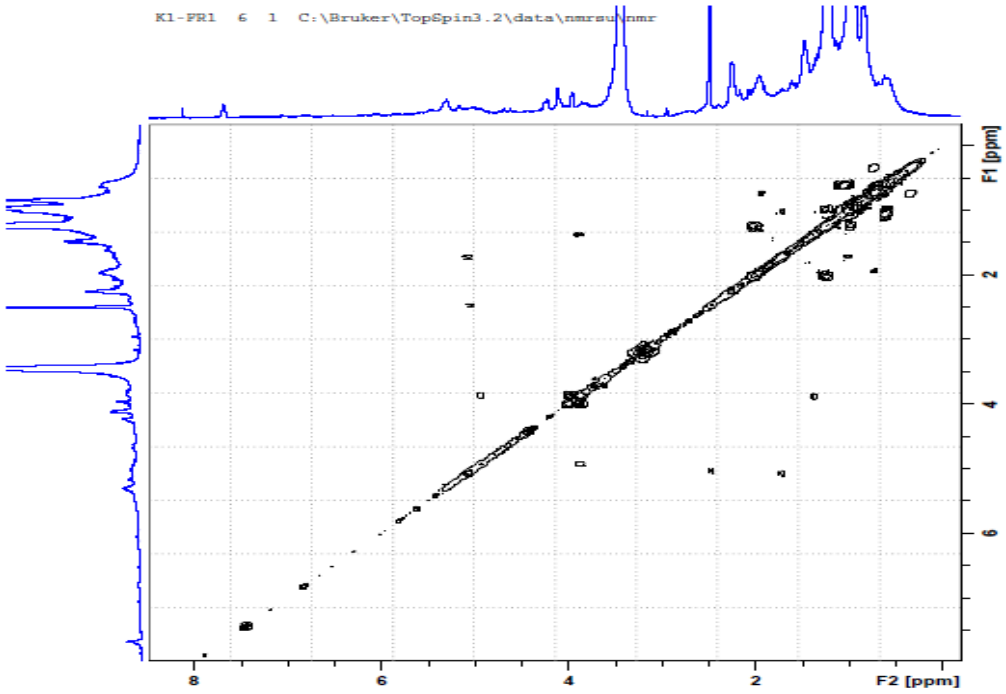
Appendix 17: ^{1}H-NMR spectrum of compound **29** (unknown)



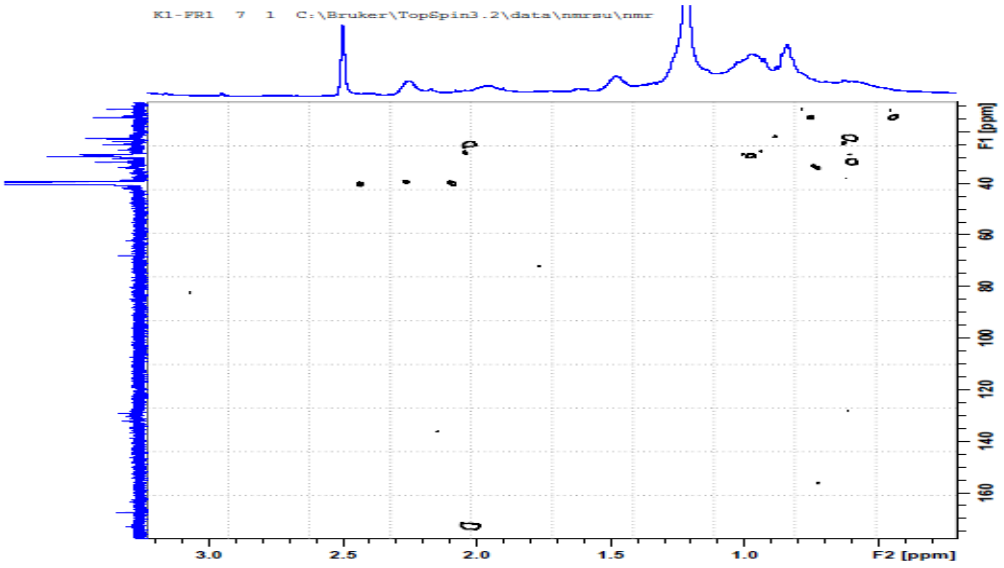
Appendix 18: ^{13}C-NMR spectrum of compound **29** (unknown).



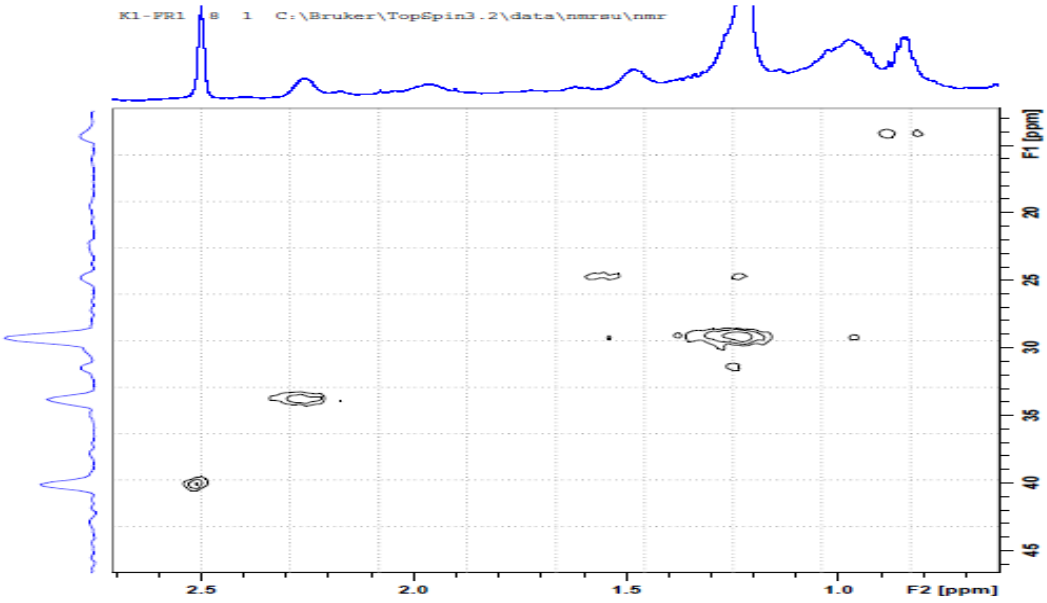
Appendix 19: DEPT135 spectrum of compound **29** (unknown).



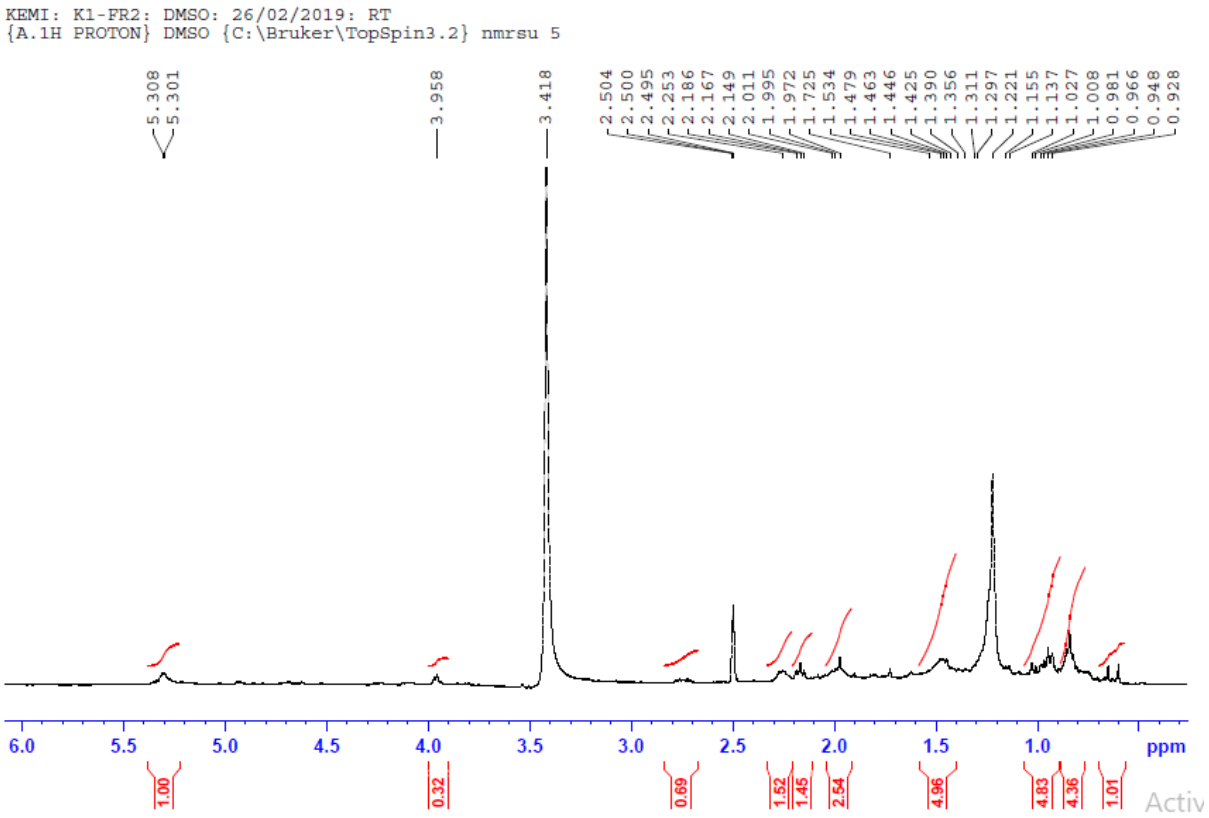
Appendix 20 : COSY spectrum for compound **29** (unknown)



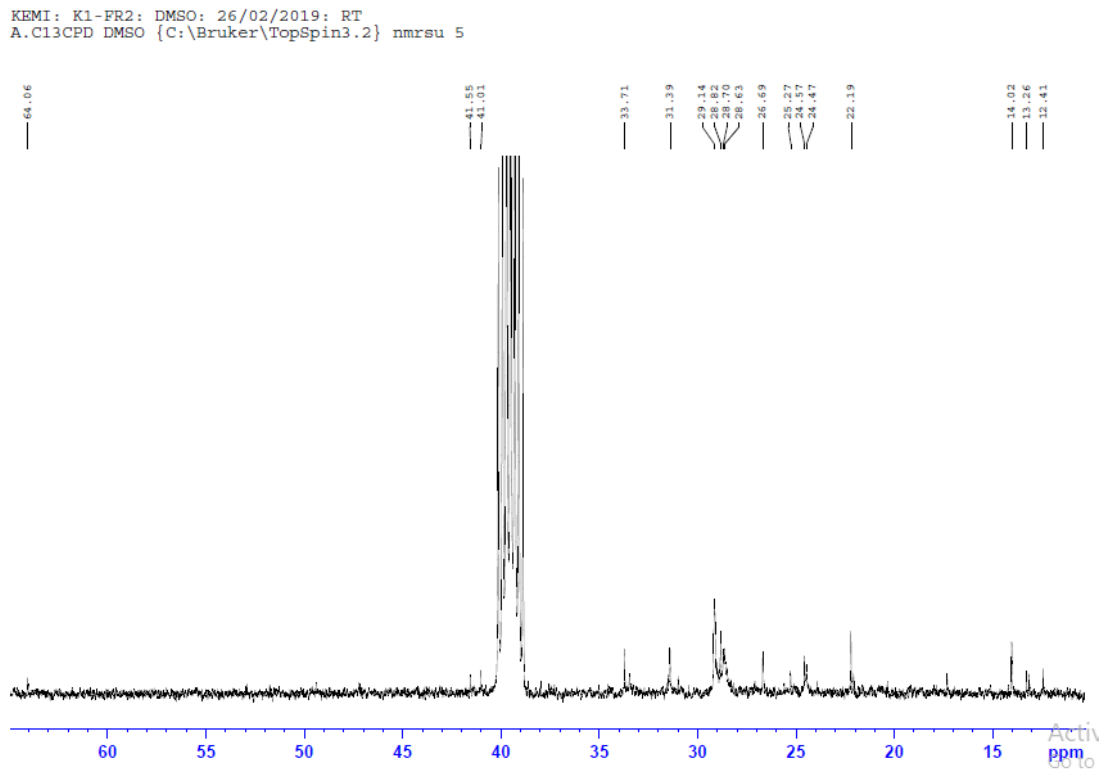
Appendix 21: HMBC spectrum for compound **29** (unknown).



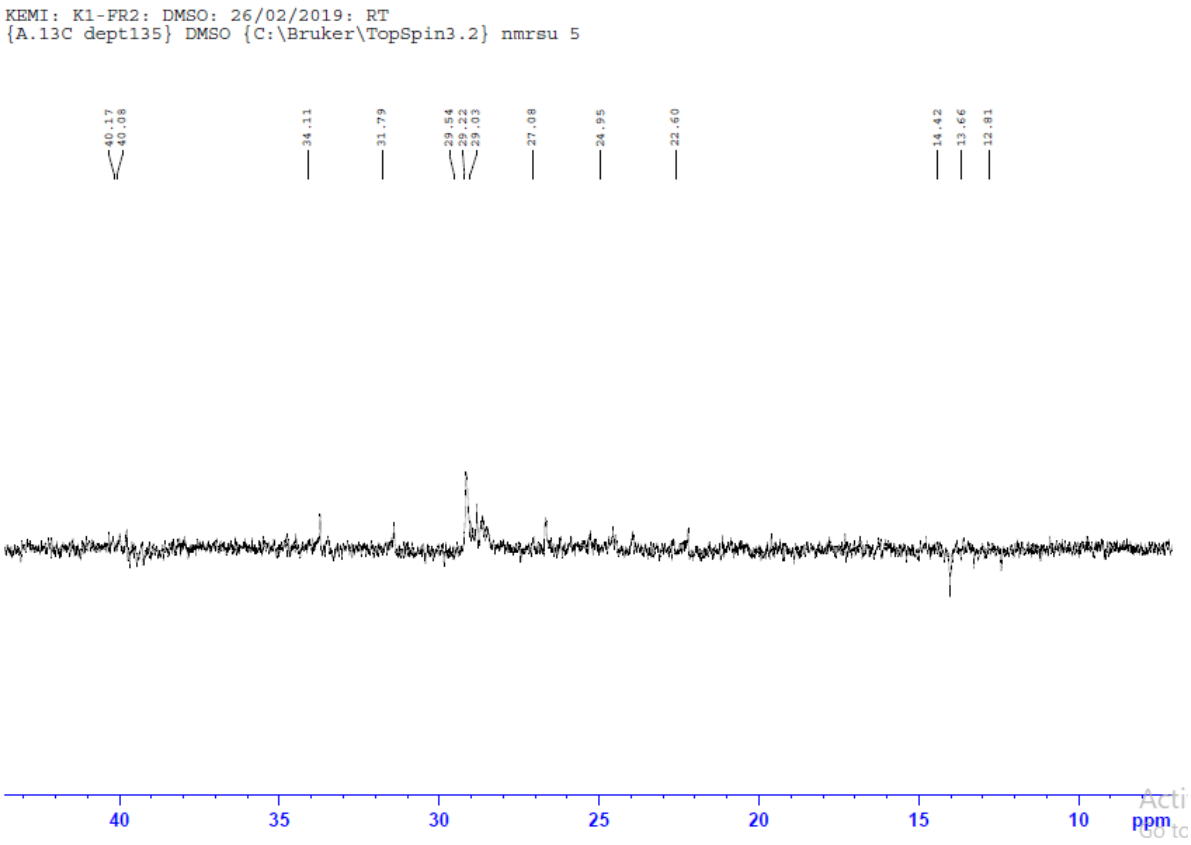
Appendix 22: HSQC spectrum of compound **30** (unknown).



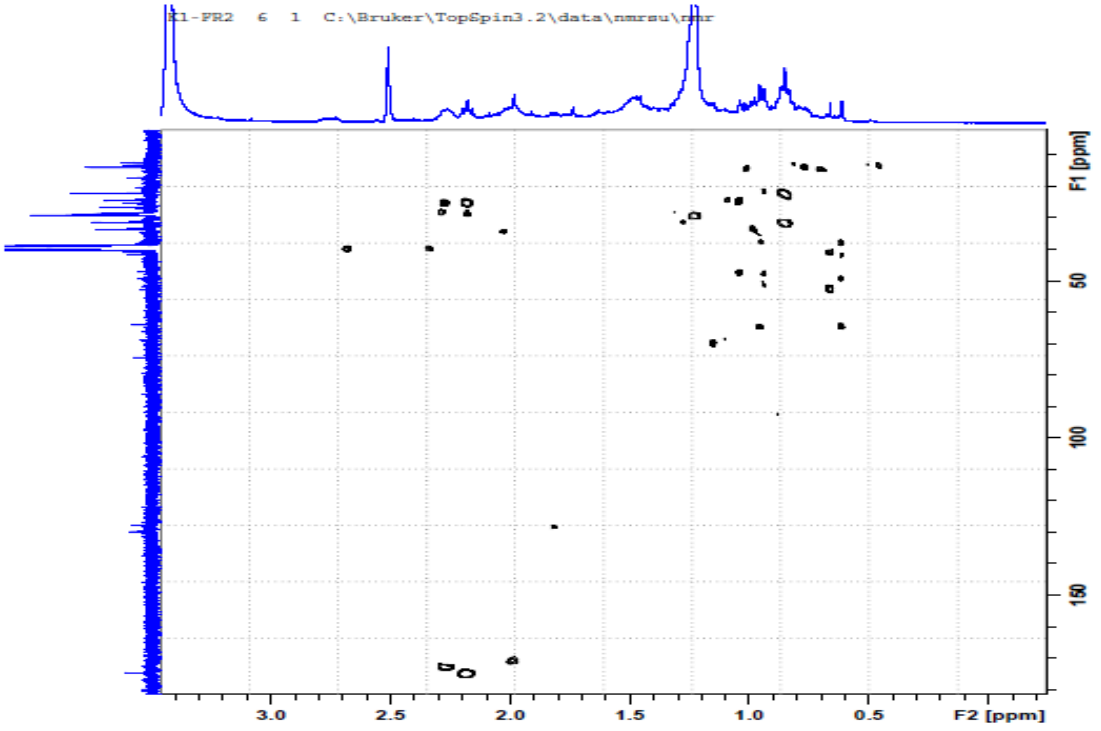
Appendix 23: ^{1}H- NMR spectrum of compound **30** (unknown).



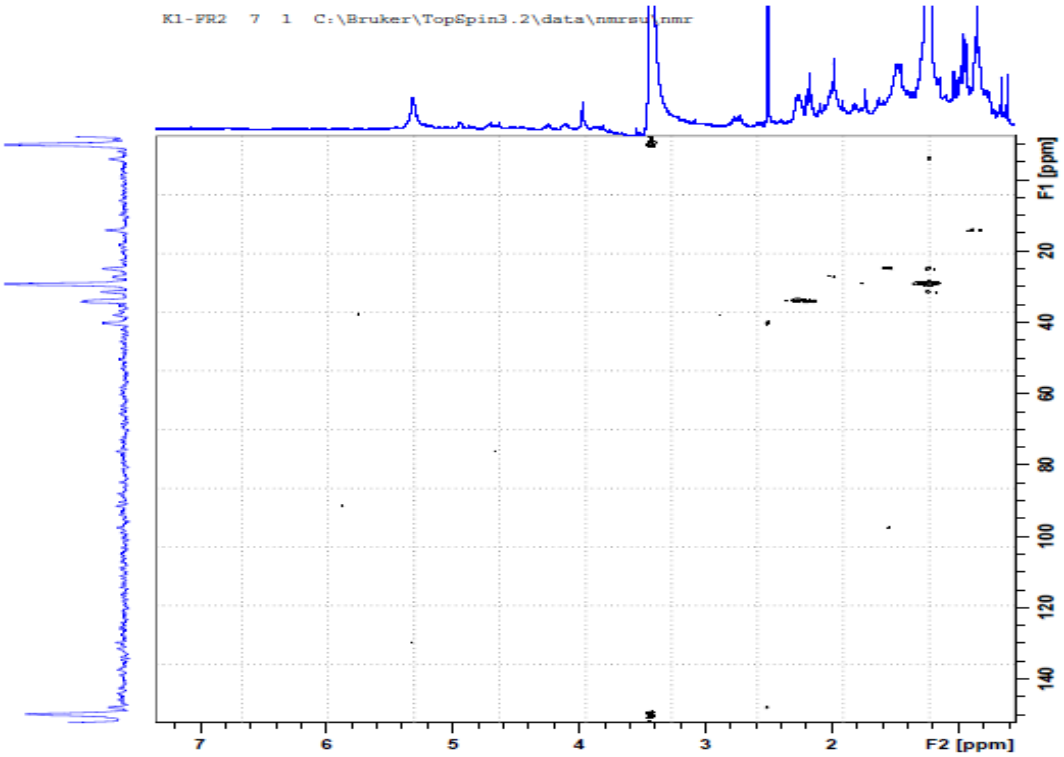
Appendix 24: ^{13}C-NMR spectrum of compound **30** (unknown).



Appendix 25: DEPT135 spectrum of compound **28** (unknown)



Appendix 26: HMBC spectrum of compound **30** (unknown)



Appendix 27: HSQC spectrum of compound **30** (unknown).