ANTIPLASMODIAL AND PHYTOCHEMICAL INVESTIGATION OF *MONANTHOTAXIS PARVIFOLIA* (OLIV.) VERDC SSP. *KENYENSIS* VERDC (ANNONACEAE)

By

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A research thesis submitted for the award of the degree of Doctor of Philosophy in Pharmaceutical Sciences in the Department of Pharmacology and Pharmacognosy, University of Nairobi.

November, 2015

Declaration

This thesis is my original work and has not been presented for award of a degree in any other university.

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Title of the work**: Antiplasmodial and Phytochemical Investigation of *Monanthotaxis parvifolia* (Oliv.) Verdc ssp. *kenyensis* Verdc (Annonaceae).**

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Dedication

This thesis is dedicated to my departed parents, my father; Samuel Mwangi Mbuki and my mother; Monicah Waithira Mwangi for their support and love for education; and to my family for their unwavering love and support.

I also dedicate it to all those who have committed their lives to conservation of forests and folklore for future prosperity.

"…..And the leaves of the tree are for the healing of the nations."

Revelation 22: 2b.

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Abbreviations and acronyms

^{1}H	Hydrogen isotope (proton)
^{13}C	Carbon 13 isotope
APAD	3-Acetylpyridine adenine dinucleotide
APADH	Reduced 3-acetylpyridine adenine dinucleotide
APT	Attached Proton Test
CC	Column Chromatography
CHO	Chinese Hamster Ovarian
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EIS	Electron ionization spray
FCS	Fetal Calf Serum
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GTT	Glucose tolerance test
HEPES	4-(2-hydroxyethyl-piperazine)-ethanesulfonic acid
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HPLC	High Performance Liquid Chromatography
ID	Internal Diameter
IR	Infra red
LDH	Lactate Dehydrogenase enzyme (host)

MMV	Medicines for Malaria Venture
MPK	*Monanthotaxis parvifolia* ssp. *kenyensis*
MS	Mass spectroscopy
MTT	Methylthiazolyltetrazolium
NBT	Nitro blue tetrazolium salt
NMR	Nuclear magnetic resonance
PDA	Photodiode array
PBS	Phosphate buffer solution
pLDH	plasmodium Lactate Dehydrogenase enzyme
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
TLC	Thin Layer Chromatography
Tris	tris (hydroxymethyl) amino- methane
U.K.	United Kingdom
UV	Ultraviolet
WHO	World Health Organization
WMR	World Malaria Report

Abstract

Title: Antiplasmodial and Phytochemical Investigation of *Monanthotaxis parvifolia* (Oliv.) Verdc ssp. *Kenyensis* Verdc (Annonaceae).

Introduction

Attention has recently shifted to ethnopharmacological study of plants as sources of alternative medicines. This approach has yielded clinically useful medicines particularly for the treatment of malaria. Upon literature survey, plants belonging to the Annonaceae family have been found to be widely used in traditional medicine for the treatment of malaria. In Kenya, particularly in the coastal region, *Monanthotaxis parvifolia* (Oliv.) ssp. *kenyensis* Verdc (Annonaceae) is used for the treatment of malaria. The aim of the present study was to investigate the antiplasmodial and phytochemical properties of this plant. This was in order to establish its ethnopharmacological basis of its use in the treatment of malaria.

Materials and Methods

The authenticated plant material consisting of the twigs and leaves was collected from Thika, Kenya. Preparation of the reagents and plant specimens for macroscopic and microscopic study was carried out as per published protocols or validated methods. Soxhlet extraction and bioassay-guided fractionation in combination with chromatographic techniques were used for the extraction, isolation and purification of the isolated compounds. Spectroscopic methods were used to carry out structure elucidation of the isolated compounds. *In vitro* antiplasmodial tests for both the crude extracts and phytochemical isolates were carried out using the chloroquine-sensitive (D10) and chloroquine-resistant (Dd2) *Plasmodium falciparum* strains. Cytotoxicity testing was done using Chinese Hamster Ovarian (CHO) cells on active crude extracts and the phytochemical isolates. Crude extract of *Monanthotaxis parvifolia* were also tested for glycaemic, analgesic and antipyretic activities.

Results and Discussion

The antiplasmodial activity against the chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strain ranged from 5.58 to 38.07 µg/ml for the

crude water and methanol extracts from the leaves and twigs. The isolated compounds (Quercetin-3-*O*-α-rutinoside, Quercetin-3-*O*-α-rarabinofuanoside) exhibited antiplasmodial activity ranging from 10.85 to 24.93 µg/ml. All tested extracts and the isolated compounds showed little or no toxicity to the Chinese Hamster Ovarian (CHO) cells. The crude extracts also exhibited antipyretic and analgesic activity.

Conclusion and Recommendation

The reported microscopic features showing the presence of paracytic stomata, oil glands and covering trichomes will be found to be useful in the authentication of this plant in future. The study has further provided information on antiplasmodial, toxicity, antipyretic, analgesic and glycaemic activities of *Monanthotaxis parvifolia*. In view of the finding of higher antiplasmodial activity in the polar crude extracts, it is recommended that further work including *in vivo* studies be carried out.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

1.1.1 Plants as sources of medicines

Plants have been reported to be the basis of many traditional medicine systems throughout the world for many years and still continue to bestow mankind with many remedies (Ortholand and Ganesan, 2004; Balunas and Kinghorn, 2005; Botsaris, 2007; Jachak and Saklani, 2007; Vaidya *et al*., 2007; Titanji *et al*., 2008; Bekalo *et al*., 2009; Ansari and Inamdar, 2010; Kuete and Efferth, 2010; Uprety *et al*., 2012; Olorunniyi and Morenikeji, 2013; Pan *et al*., 2013). The plant-based indigenous knowledge has been handed down from generation to generation in different parts of the world and has significantly contributed to the development of various traditional systems of medicines (Eisenberg *et al*., 2011; Bussmann, 2013). Further to this observation it has been reported that one approach that can be used for the identification of new medicines including antimalarials is to search for compounds that cure or prevent the disease in plants empirically used to treat the condition (Randrianarivelojosia *et al*., 2003). For instance, plant-based medicines which were formally dispensed in the form of crude medicine such as tinctures, teas, poultices, powders and other herbal formulations are being used as the foundation of novel medicine discovery (Samuelsson, 2004). The use of plants as medicines also involved the isolation of active compounds. This started with the isolation of morphine from opium in the early 19^{th} century and subsequently led to the isolation of early medicines such as cocaine, codeine, digitoxin and quinine, some of which are still in use (Newman *et al*., 2000; Butler, 2004).

The isolation and characterization of pharmacologically active compounds from medicinal plants flourish up to date (Koehn and Carter, 2005; Pan *et al*., 2013; Ntie-Kang *et al*., 2014). Indeed it has been estimated that about 80 per cent of all the world's medicines were originally derived from plant sources (Cseke *et al*., 2006; Newman and Cragg, 2007). It is projected that around 250,000 flowering plant

species occur throughout the world and about half (125,000) of these plants are found in the tropical forests. The likelihood therefore of finding new compounds from plants is huge, as up to date, only about six per cent of tropical plant species have been investigated for their pharmaceutical potential (Fabricant and Farnsworth, 2001; Gurib-Fakim, 2006; Cragg and Newman, 2013; Pan *et al*., 2013). A number of plants from different families have been explored for the antimalarial activity. Some of these families include Agavaceae, Gramineae, Leguminosae, Moraceae and Rutaceae. In Rutaceae family is the *Zanthoxylum* genus which has species used locally for the treatment of malaria (Randrianarivelojosia *et al*., 2003). Indeed there have been successful reports of medicines developed from plants for the treatment of malaria. The classical example of such a medicine is the antimalarial lead, quinine. Quinine was originally isolated from Cinchona bark of Rubiaceae family. It served as the template for the synthesis of chloroquine and mefloquine, prototype of antimalarial medicines. However, the development of resistance by the parasite to these agents prompted the search for other synthetic and natural product-based agents (Evans, 1996; Newman *et al*., 2000; Maude *et al*., 2010; Nogueira and Lopes, 2011; Wells, 2011; Cruz *et al*., 2013).

The search led to the isolation and identification of the potent antimalarial medicine artemisinin, a sesquiterpene endoperoxide lactone isolated from *Artemisia annua* (Wormwood). The plant was reported to have been used as an antimalarial agent in China for a long time (Cui and Su, 2009; Graziose *et al*., 2010; Maude *et al*., 2010; Sá *et al*., 2011). Further reviews have since shown that the compound is not only present in *A. annua* species but it is also found in other plant species as well (Cui and Su, 2009). The basic structure of artemisinin was used for the semi-synthesis of other compounds which were directed to the improved pharmacology of the base molecule. This led to the identification of artemether which was reported to be more potent than the parent molecule. Artemether is currently being widely used throughout the world. Other modifications were made to improve on the solubility and the distribution but the active constituents ended up being the same and were therefore considered to be prodrugs of dihydroartemisinin. Further studies were conducted to establish the mode of action of artemisinin. The activity was

associated with the presence of the peroxide bridge in the molecule. This led to the synthesis of many compounds containing the basic artemisinin structure. Some of these were later tested for activity and claimed to be more active than artemisinin, (Sá *et al*., 2011). **Figure 1.1** illustrates some of the well known plant-derived compounds with antimalarial activity.

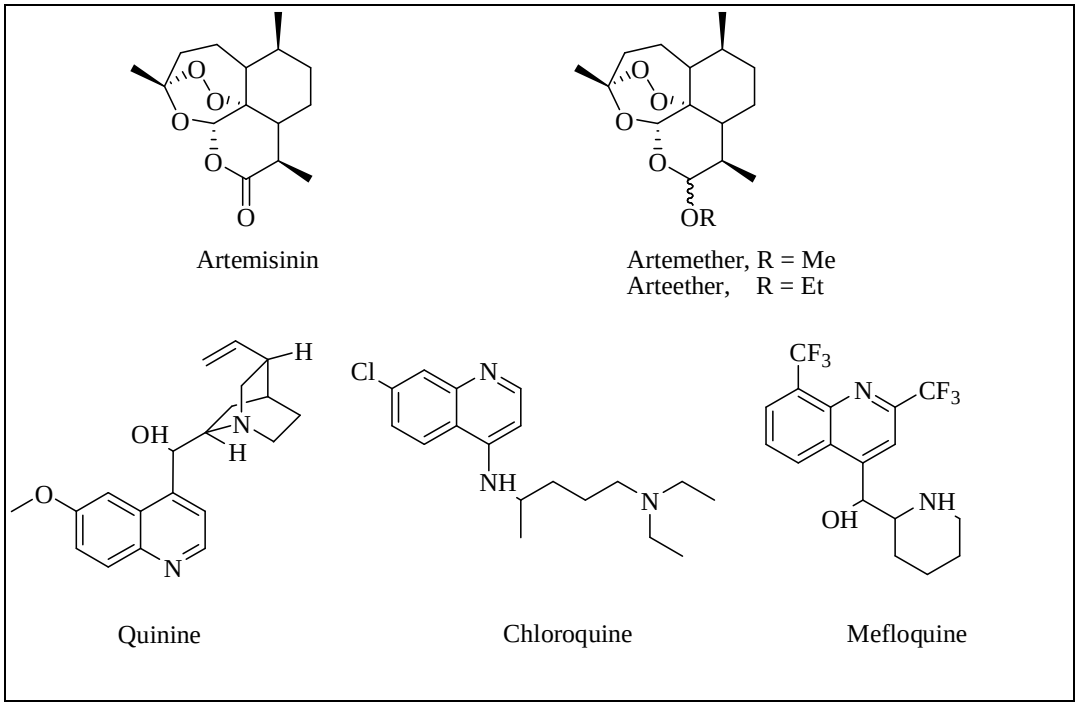

Figure 1.1: Compounds with known antimalarial activity.

The success story of the plant based compounds in the management of many diseases guided this study where it was envisaged to come up with a solution to some of the current challenges in the management of malaria.

1.2 Literature review

1.2.1 Malaria disease

Malaria is a life-threatening human parasitic disease transmitted almost exclusively through the bites of infected female anopheles mosquitoes. The parasites are from

the *Plasmodium* genera. The vectors carry sporozoites, which are the motile infective stage of the parasite, in their salivary glands. The vectors bite mainly between dusk and dawn (Hobbs and Duffy, 2011; WHO, 2013). The WHO (2015) reports goes on to indicate the four main species of the parasite that cause malaria in humans namely: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. *Plasmodium falciparum* and *Plasmodium vivax* are reported to be the most common while *Plasmodium falciparum* is the most lethal. In recent years some human cases of malaria have also been reported to occur with *Plasmodium knowlesi*, which is a species that causes malaria among monkeys and is claimed to occur in forested areas of South-East Asia (Lee *et al*., 2009; O'Connell *et al*., 2012; Singh and Singh, 2013; WHO, 2015).

1.2.1.1 Transmission of malaria

The intensity of the transmission due to the bites depends on factors related to the parasite, the vector, the human host and the environment (Snow *et al*., 2012). There are about 20 different *Anopheles* species which are economically important around the globe. They all have the common characteristic as vector species that they bite at night. The *Anopheles* mosquitoes are found to breed in water where each species is observed to have its breeding preference. Transmission is noted to be more intense in those places where the mosquito life span is longer and also where it prefers to bite humans rather than other animals.

Transmission is also observed to depend on climatic conditions that may affect the number and survival of the mosquitoes; these include factors like rainfall patterns, temperature and humidity. In many places, transmission is noted to be seasonal, with the peak being during and just after the rainy seasons (Hay *et al*., 2002; Egbendewe-Mondozozo *et al*., 2011). It has indeed been observed that malaria epidemics can occur when climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria like was the case in Kenya (Some, 1994; John *et al*., 2009). The epidemics can also occur when people with low immunity move into areas with intense malaria transmission for example in cases of refugees or travelers (Behrens *et al*., 2010).

Human immunity is also observed to be another important factor, especially among adults in areas of moderate or intense transmission conditions. It is however noted that partial immunity is developed over years of exposure and although it does not give complete protection, it is observed to reduce the risk so that malaria does not cause severe disease (WHO, 2013).

1.2.1.2 Symptoms of malaria

Malaria symptoms which are mainly fever, headache, chills and vomiting, usually appear 7-15 days after an infective mosquito bites. These symptoms are not disease specific and misdiagnosis can occur. *P.falciparum* malaria if not treated within 24 hours, progresses to severe illness often leading to death. Children with severe malaria may present with severe anaemia, respiratory distress due to metabolic acidosis or even cerebral malaria with multi-organ involvement including in adults. Partial immunity with asymptomatic infections is noted in individuals resident in endemic regions. *P. vivax* or *P. ovale* malaria is associated with delayed clinical relapses which may occur sometimes later even if they have left the malarious area.

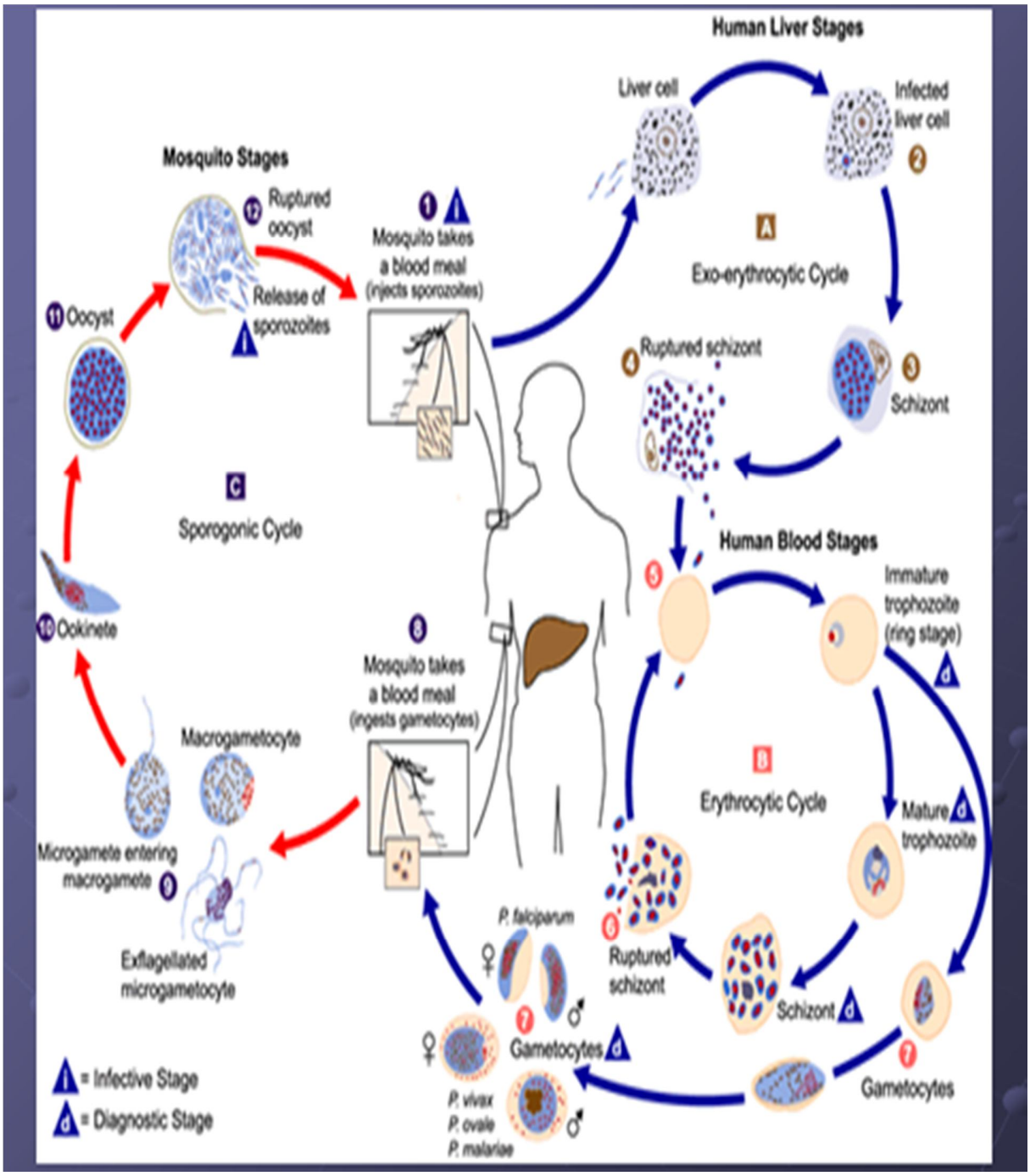

Figure 1.2: Transmission and life cycle of malaria parasite.

(Adopted from Batista *et al.*, 2009).

1.2.1.3 International perspective of malaria

The world community has been putting a lot of effort to get rid of malaria globally but these attempts have so far been unsuccessful especially in most developing countries where malaria is still one of the most serious and life threatening disease (Sachs and Malaney, 2002; Asase *et al*., 2005; Mutai *et al*., 2008; Chrubasik and Jacobson, 2010; Cohen *et al*., 2012; Snow *et al*., 2012). This is in spite of the fact that malaria is a preventable and curable disease (WMR, 2011; 2014; WHO, 2015). Indeed, according to Campbell and Steketee (2011) malaria in Africa can be eliminated. The WHO (2014) fact sheet on malaria indicates that there were about 198 million reported cases of malaria in 2013. Malaria is reckoned to kill more than one million people annually, the majority of whom are young children (Guiguemde, *et al*., 2010; Eisenstein, 2012). It is particularly important to note that ninety percent of these malaria cases in the world occur in sub Saharan Africa south of the Sahara, where the disease exerts the greatest economic burden (Nguyen-Pouplin *et al*., 2007; Eastman and Fidock, 2009; Cruz *et al*., 2013). Statistics go further to show that children under five years of age and expectant mothers are the worst affected by malaria while travelers from malaria-free areas to disease endemic areas are also vulnerable. This trend could be attributed to the huge growth of global trade and travel which have increased the speed and facility with which both infectious diseases as well as resistant microorganisms can spread between continents (WHO, 2002). This trend could however be reversed as evidenced by various reports on travel malaria (John *et al*., 2009; van Rijckevorsel *et al*., 2010; Askling *et al*., 2012; van Genderen *et al*., 2012; Smith *et al*., 2013; Fleming, 2014).

The cost of malaria to human and social well being is great. This is well illustrated by the fact that economic loss from malaria was estimated at US$ 2 billion in Africa alone in 1997. Indeed the World Health Organization and the World Bank puts malaria as the largest single component of the disease burden in Africa causing an annual loss of 35 million future life-years from disability and premature mortality. The World Malaria report (WMR, 2011) further indicated that in Africa, malaria accounts for about 20-30 % of hospital admissions and about 30-50 % of outpatient

consultations. There are also more worrying trends in malaria. For example, epidemics of malaria have frequently been reported in areas that previously were not associated with malaria (Abdulelah and Zainal-Abidin, 2007). This happened in India and Bangladesh where the number of cases increased fivefold between 1988 and 1994. Epidemics were also reported to be on the increase in highland areas where malaria was uncommon, partly due to climatic changes including high rainfall patterns. This was, for example, reported in Ethiopia where there were repeated epidemics.

In Kenya the epidemics were reported in the late 1980s and early 1990s in the high altitude areas specifically Kericho and Kisii (Some, 1994; Malakooti *et al*., 1998; WHO, 1999; Egbendewe-Mondzolo *et al*., 2011). All the foregoing would tend to indicate that malaria is no doubt a serious problem and the situation is even getting worse.

1.2.1.4 Malaria control

People have used natural products for a long time to control malaria, but since the development of modern science, alternative methodologies have been employed to contain the disease. Considering malaria to be a public health problem, use of medicines and clearing of mosquito breeding places as well as use of insecticide impregnated nets and insect repellants have so far been the major methods of controlling malaria (Cui and Su, 2009; Philipson, 1991; Sá *et al*., 2011). There have also been new challenges in control due to the threat of emerging malaria parasite that is *Plasmodium knowlesi* which was reported to be rapidly establishing itself as the fifth human-infecting malaria parasite (Lee *et al*., 2009; WHO, 2015).

According to WHO (2014), it has taken some time before an effective vaccine could be developed. This fact was further emphasized by Guiguemde *et al*. (2010) and Osorio *et al*. (2007). This is in spite of the overwhelming clinical and experimental evidence that vaccination still remains the ideal choice in the fight against parasitic diseases in both humans and animals (Knox and Redmond, 2006). However the situation is changing with regard to the malaria vaccine development as there have

been reported cases of clinical trials in seven countries in Africa (Bingham *et al*., 2012; Clemente and Corigliano, 2012; Fleming, 2014; WHO, 2015). The trials have resulted in the introduction of the vaccine although it is claimed to be only 30 % effective (Mata *et al*., 2013). There are still challenges that continue to be reported on malaria vaccine development (Lorenz *et al*., 2014). It is also important to note that according to WHO report (2015), there is no licensed malaria vaccine to date. Despite this progress in search for a malaria vaccine, prophylaxis continues to be recommended for people at risk, that is, visitors to areas with endemic malaria, expectant and nursing mothers, and children under the age of five years, anaemic people and people with HIV/AIDS. It is also important to note that some of these groups do not always have access to medicines in developing countries due to financial constraints and at times due to cultural traditions. However, in some countries such as Zambia, there were efforts to introduce Artemether-Lumefantrine (AL) combination to treat uncomplicated malaria in children less than five years by Community Health Workers (Yeboah-Antwi *et al*., 2010).

However indiscriminate use of malaria prophylaxis products has partly been blamed for the development of drug resistance among *Plasmodium falciparum* malaria parasite strain (O'Connell *et al*., 2012; Aborah *et al*., 2013). Resistance was observed against antifolates, chloroquine as well as to quinine and mefloquine (Kreidenweiss *et al*., 2006; McNamara *et al*., 2013). The situation was rather unfortunate as it caused some of the least expensive antimalarial medicines to be ineffective (Mayer *et al*., 2009). This therefore led to promotion of use of combination therapy especially those based on artemisinin which are also referred to as artemisinin-based combination therapies (ACTs). This prompted the ACTs to be adopted globally as the first line of treatment for malaria (Fidock, 2010). This approach, though noble in overcoming resistance, has raised concerns in developing countries due to cost (Kedenge *et al*., 2013). However, due to over reliance on the ACTs there are fears that resistance could already have emerged against the ACTs (Chrubasik and Jacobson, 2010; Guiguemde *et al*., 2010; Maude *et al*., 2010; Sá *et al*., 2011; Eisenstein, 2012; Onyango *et al*., 2012; Cruz *et al*., 2013; Malebo *et al*., 2013; WMR, 2014).

As a result, there is an urgent need for the development of completely new antimalarial medicines and for intensified research towards the discovery of medicines that may reverse resistance to antimalarials especially that to chloroquine (Wernsdorfer and Trigg, 1988; Wells, 2011; Cruz *et al*., 2013; Pan *et al*., 2013). This is important in view of the fact that malaria is still considered to be one of the neglected parasitic diseases (Hotez *et al*., 2006; Ndjonka *et al*., 2013). Luckily there has been intense input towards technological advances and development of new methods for the screening of natural products which may offer opportunity for natural products to serve as new leads to alleviate these challenges (Lam, 2007; Brannen *et al*., 2011; Kroll *et al*., 2011). There are reports on development of a new antimalarial with promising preventive, therapeutic and transmission-blocking activity to a number of various *Plasmodium* species (Guo *et al*., 2011; McNamara *et al*., 2013; Smith *et al*., 2013).

1.2.2 The role of plants in development of medicines for malaria

About fifteen years ago, the artemisinin derivatives such as artemether were introduced into the Kenyan market as plant derived antimalarials. This was in an effort to combat malaria treatment resistant to sulphadoxine/sulphalene-pyrimethamine (Amin *et al*., 2007; Eastman and Fidock, 2009). In an effort to combat malaria resistant to conventional treatment, a number of strategies have been tried. Synergism between chloroquine and plant derived compounds has been studied (Elford *et al.*, 1987; Al-Khayat *et al.*, 1991; Francois *et al.*, 1994). Others have studied the possible benefits of combining traditional antimalarial plant remedies with chloroquine. However, it has been noted that even with artemisinin-combination therapy it is only a matter of time before resistance develops against these antimalarials (Hobbs and Duffy, 2011; Maude *et al*., 2010; WMR, 2014).

Due to the interest malaria generates globally, antagonism between medicines used for malaria, other ailments and plant-derived antimalarial compounds were also studied (Chawira *et al*., 1987; Ekong *et al.*, 1990; Al-Khayat *et al*., 1991; Robert and White, 2004). In this respect, artemisinin showed antagonism with standard antifolates used in treatment of malaria. Plant compounds, which were active *in*

vitro but not in the standard *in vivo* mouse model were also studied (Kirby *et al*., 1995). This prompted the need of additional model systems for proper evaluation of traditional medicine. This was also important in view of the fact that methods that could be used in the study of any undesirable effects that could be associated with herbal medicines are hardly there despite their use by different cultures (Neergheen-Bhujun, 2013).

Research studies on many plants with antimalarial activity continue to be carried out in other countries as well such as Nigeria (Olorunniyi and Morenikeji, 2013); Brazil (Botsaris, 2007); South Vietnam (Nguyen-Pouplin *et al*. 2007) and Tanzania (Gessler *et al*., 1994; Nkunya, 2005; Nondo *et al*., 2015). In Kenya with collaboration with other research institutions, research on plants or herbal preparations with antimalarial activity has also been carried out and is still on-going. This is well illustrated by the publications by different authors such as Guantai, 1990; Muregi *et al*., 2004; Muthaura *et al*., 2007; Mutai *et al*., 2008; Abuga, 2009; Njogu *et al*., 2013 and Tarkang *et al*., 2014.

This kind of background thus guided the choice of the plants for this study. That, most of the plants being investigated are those that have always been used for the treatment of malaria in folklore or those which belong to families or genera with proven antimalarial properties or those with any other therapeutic value to patients with malaria. Indeed in this study plants from the Annonaceae family have been used widely in folklore for the treatment of malaria or symptoms associated with malaria such as fever and pain.

Plants are known to exhibit more than one pharmacological activity due to presence of different compounds in the plant when used in the crude form. This was, as observed, for example in the use of Cinchona bark extract for the treatment of malaria and heart ailment. This therefore led to study of *Monanthotaxis parvifolia* for some other selected pharmacological activities besides the use for treatment of malaria, particularly those that could have synergistic effect on the treatment of malaria such as analgesic and antipyretic effects. Some other pharmacological effects for which the plants in the genus are used in traditional medicine are

treatment of painful menstruation and diarrhea which were however not studied. The effect of crude extract on the glucose metabolism was tested also as diabetes is claimed to increase the risk to malaria infection (Danguah *et al.*, 2010).

1.2.2.1 Annonaceae family

The Annonaceae (Spermatophyte, Dicotyledones) are an ecologically important group of plants across the humid tropics. The individuals are identified to the family because of the distichous arrangement of the entire simple stipulate leaves on the lateral branches and the aromatic secondary compounds. The leaves are also reported to be alternate without stipules. The plants are observed to have 2-3 sepals and usually 4 petals which are in one or two whorls with many stamens. The fruits are either syncarpous or composed of many free monocarps (Beentje, 1994). The family has both the tree and the shrub genera and most of them are climbers (Johnson, 2003). The Annonaceae family was reported to be indeed indigenous to Africa (Mulholland *et al.*, 2000). Economically, the family has been appreciated as a source of the edible fruits such as “pawpaw”, “cherimoya”, “sweetsop”, “sour sop” and “custard apple” among others (Heywood, 1978). Seeds from some of the plants are reported to be useful source of edible oils (Colom *et al*., 2007).

Plants in Annonaceae family have found several uses in traditional medicine (Kokwaro, 1993; Nkunya, 2005; Osorio *et al*., 2007; Murphy *et al*., 2008; Aminimoghadamfarouja *et al*, 2011; Malebo *et al*., 2013; Araujo *et al*., 2014). In Kenya for example, the decoction from *Monanthotaxis trichantha* and *Monanthotaxis trichocarpa* are reported to be used for treating headaches by the Digo community (Kokwaro, 1993; Beentje, 1994; Muthaura *et al*., 2007; Nguta *et al*., 2011). On the other hand, a decoction from *Uvaria acuminata* is used for treating dysentery, painful menstruation in women, snakes bites and pectoral diseases by the Digo and Sukuma communities. *Uvaria leptocladon* decoction from the root is used by the Digo and Somua communities as a diuretic, for gonorrhea and also as a remedy for diarrhea. The Kamba community uses the root bark decoction from *Uvaria scheffleri* which they locally refer to as *Mugugumа* as a cure for fever while the Marakwet refer to it as *Murguiyo*, the Pokot as *Tamrenwo* and

the Tugen as *Tamingweto* use the root bark to treat malaria (Beentje, 1994). A study carried out on traditional herbal medicines for malaria by Willcox and Bodecker (2004), reported use of *Annona muricata* from the family in three of the world's continents. *Uvaria species*, for example, have been used to treat fever and dysentery while *Artabotrys* and *Monanthotaxis* species have been used to treat vomiting and headache respectively (Fowler, 2006; Boyom *et al*., 2011). These symptoms may be considered to be indicators of malaria infection. Indeed according to Fowler (2006) all fever symptoms turn out to be malaria. In Ghana, the leaves of *Monanthotaxis diclina* were reported to be used as a foot ointment while in Madagascar, *Monanthotaxis heterantha* leaves are boiled and used as medicine against malaria (Fournier *et al*., 1997).

Studies carried out in China so far indicate that the *Artabotrys hexapetalus* contains antimalarial sesquiterpenoids, yingzhaosu A, B, C and D. Yinghzhaosu A and C were indeed found to contain an endoperoxide moiety similar to that in artemisinin. The antimalarial activity of artemisinin is known to be due to this functionality. The compounds obtained from *Artabotrys hexapetalus* proved to be effective in treating malaria resistant to conventional medicines (Qinghaosu, 1979; Cui and Su, 2009).

There are three *Artabotrys* species in Kenya (Kokwaro, 1993) yet no scientific work has been reported on them, while in Tanzania; *Artabotrys brachypetalum* was shown to have promising antimalarial activity. Preliminary work on root extracts of *Uvaria acuminata* from Tanzania indicated that it had high activity against the brine shrimp (Nkunya, 2005). Besides being used for the antimalarial activity, plants in the family were also employed for other pharmacological activities in folklore. Further research carried out on some species of the Annonaceae family from Thailand indicated that some of the isolated compounds show anti-tumour activity. These compounds which were isolated from the stem bark of *Ganiothalamus gigantus* were shown to be strypyrones mainly goniothalenol and ganiothelamin. Goniothalenol was reported to show good cytotoxicity but little selectivity. In other pharmacological screening assays, goniothalenol exhibited fairly prominent activity in attenuating stress and alcohol induced gastric ulceration (McLaughlin *et al*.,

1991). Similarly, compounds isolated from the bark and seeds of the North American pawpaw (*Asimina triloba)* exhibited potent pesticidal actions. The pesticidal activity was associated with acetogenins. Indeed one of these acetogenins, asimicin has been patented in America. Asimicin exhibited antimalarial activity. Annonacin, another compound isolated from the bark extract of *Annona densicoma* at concentrations of 100 µg/mL was also reported to have insecticidal and antimalarial activity (McLaughlin *et al*., 1991). Further pesticidal activity due to acetogenins was also reported in *Annona cherimolia* species of Annonaceae family in Argentina (Colom *et al*., 2007).

Monanthotaxis species from the same family have also been studied, where a number of compounds had been isolated. Crotepoxide for example was isolated from *M. caffra* as well as *M. buschananii* and flavonoids from *M. cauliflora* (Mulholland *et al*., 2000). S-Corytenchine an oxygenated protoberberine was reported to be present in *M. fornicata*.

In traditional medicine the plants are employed for more than one condition (Evans, 1996; Tarkang, 2014). Search in literature has pointed to the use of the plants in Annonaceae family for other pharmacological conditions in folklore. An example, is the use of *Monanthotaxis trichantha* water infusion as a remedy for headaches by the Digo community in Kenya (Kokwaro, 1993).

1.2.2.2 *Monanthotaxis* genus in Kenya

There are six species of *Monanthotaxis* found in Kenya (Beentje, 1994). These are namely; *Monanthotaxis buchananii* (Engl.) Verdc; *Monanthotaxis faulknerae* Verdc; *Monanthotaxis fornicata* (Baill.) Verdc; *Monanthotaxis parvifolia* (Oliv.) Verdc. Ssp. *kenyensis* Verdc; *Monanthotaxis schweinfurthii* (Engl. & Diels) Verdc. Var. *schweinfurthii* and *Monanthotaxis trichocarpa* (Engl. & Diels) Verdc. They are found growing along the coast in isolated places like in the evergreen forest in Mrima, Shimba Mwarakaya and Pangani. In the inland they grow at an altitude of between 500 to 1000 m above sea level. The exact description of the plants varies from one species to the other but they are all characterized by flowers in extra-

axillary cymes while the fruits are monocarps which have 1-8 seeds. The young branches have either spreading reddish hairs or appressed brown hairs. The leaves are glabrous in appearance on the surface with a leaf base which is either rounded or cordate and arranged opposite to each other. The leaf apex varies from obtuse to emarginate or from acute to acuminate depending on the species. The size of the petals ranges from 4 to 9 mm in length with the biggest size of petals being found in *Monanthotaxis parvifolia* where their length ranges from between 8 to 9 mm. A summary of *Monanthotaxis* species found in Kenya is shown in Table 1.1.

Table 1.1: Summary of *Monanthotaxis* species found in Kenya.

Name of the Species	Synonyms	Local name(s)	Distribution
Monanthotaxis buchananii (Endl.) Verdc	*Popowia djurensis* Engl, *P. buchananii* (Engl.) Engl,& Diels	-	Coast & Western; evergreen forest and thickets on rocky hills
Monanthotaxis faulknerae Verdc	-	Mukomikila (Giriama)	Coast
Monanthotaxis fornicata (Baill.) Verdc	*Eannestemon fornicates* (Baill.) Excell	Mguku, Mgweni (Kiswahili), Moukatwa (Baju), Longhi (Bonju), Mbulishi (Giriama)	Coast; in the forest, woodland or thicket
Monanthotaxis parvifolia (Oliv.) Verdc ssp. *kenyensis* Verdc	*Popowia* sp. Aff. *oliverana*	Mu-unkuma (Meru)	Central; along the riverine or thickets
Monanthotaxis schweinfurthii (Engl. & Diels) Verdc. var. *schweinfurthii*	*Enneastemon schweinfurthii* (Engl. & Diels) Robys & Ghesq.)	Muganjuki (Meru), Lelbujan (Nandi)	Central and Western; scarcely distributed in the evergreen forest
Monanthotaxis trichocapa (Engl. & Diels) Verdc.	*Popowia trichocarpa* (Engl. & Diels)	Mudazala (Digo)	Coast; evergreen forest

(adopted from Beentje, 1994).

1.2.2.3 *Monanthotaxis parvifolia* ssp. *kenyensis*

This species is named taxonomically as *Monanthotaxis parvifolia* (Oliv.) Verdc ssp. *kenyensis* Verdc, synonym (*Popowia* spp. Aff. *oliverana*) of Kenya Trees and Shrubs (Beentje,1994). The plant is hereafter referred to as *Monanthotaxis parvifolia* without the supspecies. *Monanthotaxis parvifolia* is a rare species and is sparsely distributed. The plant is found growing along the riverine and thickets on well drained red soil where the land has never been cleared for cultivation. In Central Kenya, the plant is found in isolated places such as along river Chania near Thika town. As a result of high demand of land for agricultural activities, most of the traditional medical plants where *Monanthotaxis parvifolia* is not an exception, are nearly becoming extinct in this area before most of their uses could be documented for future generations (Cragg and Newman, 2013). The distribution of *Monanthotaxis parvifolia* (Beentje, 1994) is shown in Figure 1.3 below.

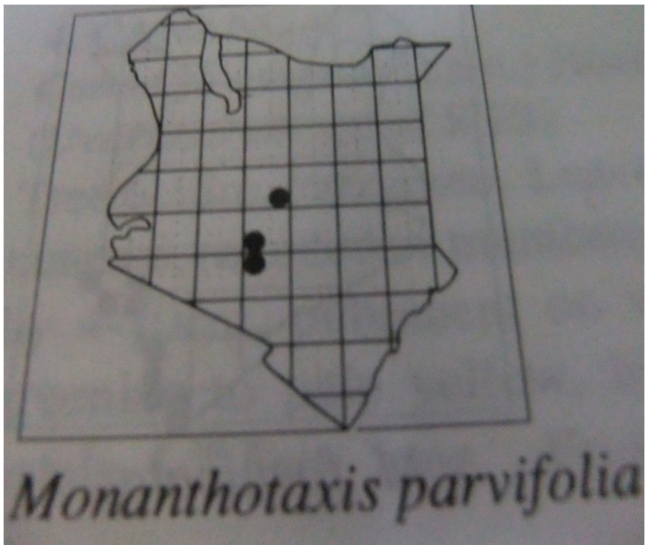

Figure1.3: Distribution of *Monanthotaxis parvifolia* plant in Kenya.

The peak season for collecting the plant is around May to June immediately after the long rainy season. *Monanthotaxis parvifolia* is mainly a climber and it grows to a height of 1-6 m. The leaves are slightly obovate while the base varied from between rounded to subcordate and the shape of the apex ranged from rounded to emarginate. On the other hand, the leaf blade is reported to be pubescent on the top and glaucous on the lower side. The size of the leaf varies from between 4-14 cm in

length to 1.5-7 cm in width. The leaves are arranged opposite each other. The flowers are yellow-green in colour, characterized with large petals of 8-9 mm in length. Like in all the species, the fruits are monocarps, red in colour and bearing 1-3 articles which were ellipsoid in shape (Beentje, 1994). Figure 1.4 below shows a *Monanthotaxis parvifolia* seedling.

Figure 1.4: A *Monanthotaxis parvifolia* seedling.

(NNM15)

1.3 Study justification

Malaria remains one of the major killer diseases in Kenya and the world at large. This can be attributed to many factors, among them resistance of the malaria parasites to the antimalarial medicines in clinical use (Chrubasik and Jacobson, 2010). A new compound/s for which malaria does not have resistancewould be very useful.

The *Monanthotaxis species* and other plants in Annonaceae family are used in traditional herbal medicines for the treatment of malaria. Results from the present work will lay on scientific basis the traditional use of *M. parvifolia*. Some extracts and molecules isolated from some plants in Annonaceae family such as *Artabotrys species* have been found to have antimalarial activity against resistant *Plasmodium* strains. Such work has not been carried out on *M. parvifolia*. This research could give a template or lead molecules that could be used to develop more active antimalarial medicines through semi-synthesis and/or structural modifications.

1.4 Study objectives

1.4.1 General objective

To investigate antiplasmodial and phytochemical properties of *Monanthotaxis parvifolia* ssp. *kenyensis* in an effort to rationalize its use in traditional medicine.

1.4.2 Specific objectives

The specific objectives of the present work were:

I. Study the macroscopic features and microscopic characteristics of *Monanthotaxis parvifolia*.

II. Isolate and characterize the phytochemical constituents of *Monanthotaxis parvifolia*.

III. Screen the crude extracts and phytochemical isolates of *Monanthotaxis parvifolia* for antiplasmodial activity.

IV. Determine cytotoxicity of *Monanthotaxis parvifolia* extracts and the

phytochemical isolates.

V. Investigate *Monanthotaxis parvifolia* extracts for glycaemic, analgesic and antipyretic effects.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Materials, chemicals and equipment

2.1.1 Solvents

Acetone, acetonitrile, acetic anhydride, chloroform, dichloromethane, ethanol, ethyl acetate, hexane, methanol and petroleum ether were of general purpose grade. The solvents for the extraction, partitioning and developing chromatograms in the thin layer chromatography (TLC) were distilled in glass apparatus prior to use. The solvents used in preparation of mobile phases for the column chromatography (CC) and high performance liquid chromatography (HPLC) were of analytical grade unless where specified otherwise in the text. They were obtained from Alpha Chemicals Ltd, Kobian Kenya Ltd and Kanha Laboratory Supplies (Nairobi, Kenya). Water used for partitioning of extracts, preparation of standard drug and physiological solutions was freshly distilled by use of distillation apparatus (Gesedschatt fur Labortechnik GmbH, Burgwedel, Germany) before use. Water used for the culturing procedures was purified by a Millipore Synergy water purification system purchased from Microsep, Tygervalley, South Africa.

2.1.2 Chromatographic materials

Aluminium pre-coated 0.25 mm thick analytical TLC plates of silica gel 60 GF_{254} and normal silica gel 60 (0.063-0.2 mm mesh) for open column chromatography (Sigma-Aldrich GmbH & Co.,Darmstadt, Germany) were employed. Whatman paper (Whatman International Ltd., Maidstone, England) was used for filtration.

2.1.3 Chemicals and standards

Chloroquine diphosphate and emetine dihydrochloride (Sigma-Aldrich) were of analytical grade and were used as t reference medicine for the antiplasmodial and cytotoxicity assays, respectively. D-Glucose and D-sorbitol (Sigma-Aldrich) were used in preparing the respective solutions for washing the blood pellets during

synchronization of the malaria parasites. Calcium chloride, sodium chloride, potassium chloride (RFCL Ltd, New Delhi, India), sodium dihydro-orthophosphate, magnesium sulphate, magnesium chloride and sodium bicarbonate (BDH Ltd, Poole, England) used for the preparation of physiological solutions were of analytical grade.

2.1.4 Reagents

2.1.4.1 Clearing and staining reagents in microscopic work

Chloral hydrate (BDH Chemicals Ltd., Poole, England) and hydrochloric acid were used for clearing the plant sections of chlorophyll before mounting on the microscope. Phloroglucinol (BDH Chemicals Ltd., Poole, England) and concentrated sulphuric acid were used for detecting lignifications in the plant tissues.

2.1.4.2 Reagents for testing of chemical constituents

A dilute solution of ferric chloride solution was used to test for the presence of tannins. Dilute ammonia solution in presence of concentrated sulphuric acid was used to test for the presence of flavonoids while that of steroids was carried out by the addition of acetic anhydride in sulphuric acid. Glacial acetic acid and ferric chloride were used to prepare the reagent for testing of glycosides.

2.1.4.3 Spray reagents for detecting separated components

Vanillin (BDH Chemicals Ltd., Poole, England) and concentrated sulphuric acid (Sigma-Aldrich) were used to prepare vanillin in sulphuric acid reagent which together with anisaldehyde in dilute alcohol was used as detecting spray reagents of separated components in developed TLC plates.

2.1.4.4 Colour developing reagents for the malaria test

Nitro blue tetrazolium (NBT) (Sigma Chemical Company, St. Louis, MO, USA) was used for colour development during the malaria assay.

2.1.4.5 Cytotoxicity assay reagent

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) obtained from (Highveld Biological, Cape Town, South Africa) was employed as such for colour formation during the cytotoxicity assays.

2.1.4.6 Giemsa stain

The Giemsa stain (Sigma) was formulated in glycerol and methanol as indicated by the supplier. A phosphate buffer, containing 9.5 g/l sodium dihydrogen phosphate at a pH of between 6.8 and 7.2 was used to dilute Giemsa solution. Two drops of Giemsa solution were added for every 1 ml of phosphate buffer. The Giemsa solution was used to stain malarial parasite's deoxyribonucleic acid (DNA).

2.1.4.7 Malstat reagent

Malstat comprised of 100 mM Tris–HCl, 0.2 M L-lactic acid, 0.2% (v/v) Triton X-100 at pH 9.1 and was Stored at 4°C until use.

2.1.4.8 Nitro blue tetrazolium salt stock solution

An aqueous solution of 100 mg nitro blue tetrazolium salt was prepared in a 50 ml plastic centrifuge tube externally lined with aluminium foil with the aid of vortexing. The NBT solution was stored until use.

2.1.4.9 PES stock solution

About 5 mg of PES were dissolved in 50 ml distilled water in a 50 ml centrifuge tube. This solution was dispensed in 5 × 15 ml centrifuge tubes and stored at -20°C until use.

2.1.5 Nutrient media

2.1.5.1 Culture medium

The RPMI-1640 (Roswell Park Memorial Institute) was the medium for the maintenance of parasite cultures and it was obtained from Gibco/BRL Life Technologies (Gaithersburg, MD, USA). Dulbeco's Modified Eagles medium (DMEM) was used as the mammalian cell culture. It consisted of Harns F-12 supplemented with 10% heat inactivated fetal calf serum (FCS) and gentamycin

(0.05 g/l) (Highveld Biological, South Africa) and it was used to maintain the Chinese Hamster Ovarian (CHO) cell-line in culture for cytotoxicity assays.

2.1.5.2 The wash medium

The wash medium for human erythrocytes was made up of 10.4 g RPMI-1640 (Roswell Park Memorial Institute) containing L-glutamine, 5.94 g HEPES, 4.0 g D-glucose, 44 mg hypoxanthine, 5 % sodium bicarbonate and 4 mg of gentamycin dissolved in 900 ml of deionized sterile water. The culture medium was prepared by supplementing the wash medium with 5 % Albumex II.

2.1.5.3 The erythrocytes

Erythrocytes for maintenance of the parasite cultures were obtained from the whole blood of consenting O positive blood group donors who had been screened for medicines prior to donating the blood.

2.1.6 Equipment

A locally assembled grinding mill was used to grind the dried plant material. Sartorius top loading balance AUY 120 (Aubagne, France) was used to weigh samples above 500 mg while analytical balance (AUW220D Shimadzu Corporation, Kyoto, Japan) with 0.01 mg precision was used to weigh samples below 500 mg.

A glass desiccator and a furnace (Carbolite Furnaces, Bamford, England) were used to determine the ash value of *Monanthotaxis parvifolia* powdered leaf. Organic solvent extracts of the dried and powdered plant materials were obtained using Soxhlet apparatus connected to a Vel electrothemal (Vel, Germany) heating mantle.

A rotary vacuum evaporator VV220 with a water bath WB2000® (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a cooler (Polyscience, Niles, USA), and a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) system was used to reduce the organic solvent extracts to dryness. Water extracts were dried by use of a lyophilizer assembly (Julabo VC, Julabo F18) which was connected to an Edward-5 vacuum pump system (Edwards's High Vacuum International, Manor Royal, Crawley, West Sussex, England). A genevac HT-12 HCl evaporator (Genevac Inc, Stone Ridge, New York) was used to concentrate or

to completely dry the fractions from HPLC.

A locally made glass column (80 cm × 3.0 cm internal diameter) was used for column chromatography. A Voss vibrating machine (Maldon, Essex, U.K.) was used to facilitate in the packing of the slurry onto the chromatographic column. An automatic Superfrac fraction collector (Pharmacia Biotech, Sweden) was used for collecting the fractions from the column. An UV chamber (Uvitec, Cambridge, UK) was used for visualizing separated components on TLC plates under either visible 254 nm or UV 365 nm light.

The Infrared (IR) spectra were recorded in a FT IR 8400 S spectrophotometer operating in a transmission mode (Shimadzu Europa GmbH, Duisburg, Germany) using 1% KBr disks. The system was supported by IR-solutions® software capable of peak integration, display of spectra suitable manipulations and print out. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ and MeOD operating at 500 MHz and 125 MHz, respectively, on a Bruker spectrometer (Bruker Daltonik GmbH, Bremen, Germany). A Waters 2424 model photodiode array (PDA) detector (Waters Corporation, Milford, USA) was used to obtain UV spectra of fractions separated from preparative- HPLC. Mass spectrometry analyses were done using a Waters Synapt G2 Mass spectrometer (Matieland, South Africa) coupled to a Mass lynx Data analyzer (Waters Corporation, Milford, USA).

An IEC Centra CLD centrifuge (International Equipment Co., Staffordshire, UK) was used to carry out the centrifugation of biological samples. A cell house 170 incubator (RS Biotech, Irvine, Scotland) was employed in the incubation of parasite cultures and test preparations.

The Nikon-model LABOPHOT-2A (Japan) fitted with a 6V, 30 W halogen lamp microscopes at X100 objective lens was used to monitor growth of the parasite cultures. The microscopy study of the plant specimens was conducted by use of a Leica microscope (Switcherland Ltd, Heerbrugg, China) connected to a computer with a Leica application software version 3.0.

All aseptic and microbiological procedures were carried out under bioflow laminar

flow equipment (Vermeulen, L.J. BVBA, Westmalle, Belgium).

Tail-flick Ugo basile analgesiometer was used to measure the analgesic response in mice to a thermal stimulus (Orchid Scientific and Innovatives, Maharashtra, India). The Non-Contact Infrared Thermometer (Hubdic Co Ltd., Korea) was used to record the temperature of the mice in the antipyretic test.

2.1.7 Malaria parasites

Two *Plasmodium falciparum* malaria parasite strains were used in this study. The chloroquine-sensitive (D_{10}) strain and the chloroquine-resistant (Dd2) strain of Indochina origin were a kind donation from Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. The asexual erythrocytic stages of these parasites were kept in a continuous culture according to the method of Trager and Jensen (1976). A gas mixture comprising of 93 % N_2, 3 % O_2 and 4 % CO_2 (Afromax, Cape Town, SA) was used to support growth of parasite cultures in the incubator.

2.1.8 Animals

The *Mus musculus* Swiss mice and Wistar rats were housed in disinfected polypropylene cages padded with saw dust, placed on metal shelves in a well-ventilated room at a controlled temperature (25 ± 2 °C) and maintained in a 12 h dark-light cycle. The animals had feed and clean tap water *ad libitum.* The drinkers containing clean tap water for the animals were washed and refilled daily. The animals were kept on an overnight fast prior to the experiment.

2.2 Methods

2.2.1 Preparation of detecting and staining reagents

Various reagents were prepared for clearing the plant specimen of chlorophyll, before staining and viewing under the microscope and for visualizing of separated components on TLC and for testing of chemical constituents.

Chloral hydrate solution for clearing plant specimens for mounting on the microscope was prepared by weighing approximately 50 g of chloral hydrate crystals which were dissolved in 50 ml of distilled water using gentle heat.

Phloroglucinol solution was prepared by dissolving about 1 g of phloroglucinol powder in 100 ml of 95 % of alcohol. The solutions were kept in well closed containers until use.

About 1 g vanillin was dissolved in 10 ml concentrated sulphuric acid to obtain the 1 % solution vanillin in sulphuric acid spray reagent.

Anisaldehyde was prepared by dissolving 6 g in 100 ml of 70% alcohol which contained 2.5 ml of concentrated sulphuric acid.

Iodine chamber was prepared by placing about 1g of iodine crystals at the bottom of 100 ml chromatographic tank which was covered with a glass or metal lid in order to generate iodine fumes used for detecting unsaturated compounds.

The methylthiazolyltetrazolium bromide (MTT) used for the cytotoxicity assays was prepared as a 0.2 mg/ml solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide by dissolving 1 mg MTT in 5 ml of distilled water just before use.

Meyer's reagent used to test for presence of the alkaloids was prepared as a potassiomercuric iodide solution. The reagent was made of 1.355 g of mercuric chloride dissolved in 60 ml of water and 5 g of potassium iodide in 20 ml of water. The two solutions were mixed and made to 100 ml with water. The reagent was kept at -4 ° C until use.

2.2.2 Collection and preparation of plant materials

2.2.2.1 Identification of the plant

Monanthotaxis parvifolia was collected when fresh, in flower and fruit from its natural habitat in Thika, Kiambu County, Kenya, in June, 2006. A voucher specimen of the sample was prepared by pressing. The plant was identified at the National Herbarium, National Museums of Kenya (NMK/BOT/CTX/1/2). A similar voucher specimen is deposited in the Department of Pharmacology and Pharmacognosy, School of Pharmacy, University of Nairobi.

2.2.2.2 Preparation of specimens for macroscopic and microscopic work

Macroscopic observations of *Monanthotaxis parvifolia* were conducted on the leaf and small twigs. The leaves and the twigs were arranged on a plain background and photographs on shape and size of the leaves as well as arrangement of the leaves on the branches were taken and recorded. The leaf was also evaluated for the organoleptic characteristics where the colour, smell and taste were observed and recorded.

The specimens for mounting on the microscope were prepared by free-hand cutting thin sections around the midrib of the leaf. The leaf was held in position while cutting by placing it between a thin slit in polystrene pith. The leaf section was placed on a microscope slide; a few drops of chloral hydrate solution were added on the microscopic slide and a cover slip placed on top. The microscope slide was gently heated by a methylated spirit lamp flame to clear chlorophyll from the leaf section. The slide was removed from the flame, allowed to cool and placed aside on the bench till when mounted on the microscope.

In order to detect the location of different constituents that might be present in various sites of *Monanthotaxis parvifolia* leaf, histochemical tests were carried out. Sections of the leaf were treated with different reagents and the observations recorded. Detection of the presence of stored starch was done by use of iodine solution while that of tannins was carried out by use of alcoholic ferric chloride. Lignification of tissues was established by adding a few drops of phloroglucinol and

hydrochloric acid solution on a leaf section and observing the reaction under the microscope. The microphotographs were recorded at X100, X200 or X400 magnifications using a camera fitted into the Leica microscope and the data collected onto the computer.

2.2.2.3 Drying and grinding of *Monanthotaxis parvifolia*

The leaves and young twigs of *Monanthotaxis parvifolia* were collected and put in jute bags. The plant material was separated into leaves and twigs immediately but not exceeding twenty four hours after collection from the field to prevent spoilage of the material. The twigs were cut into small pieces. The leaves and the chopped twigs were separately and thinly spread out on jute bags. They were left to dry indoors with periodic turning for a period of ten to fourteen days at an average room temperature of 25 °C. The dried leaves and the twigs were separately ground to powder and stored at room temperature in labeled clear polythene bags and kept away from light until use.

2.2.2.4 Screening for phytochemical constituents in *Monanthotaxis parvifolia*

A typical screening for different classes of phytochemical constituents in *Monanthotaxis parvifolia* was carried out on aqueous extract and on the powdered leaf as per published protocols (Edeoga *et al*., 2005).

To test for tannins, about 0.5 g of dried powdered leaf was boiled in 20 ml of water in a test tube and then filtered. To the filtrate, 3-5 drops of ferric chloride solution were added. Any colour change was noted and recorded.

Two methods were used to determine the presence of flavonoids. In the first method, about 5 ml of ammonia solution was added to 10 ml of aqueous extract and to this mixture about 3 drops of concentrated sulphuric acid was added. The second method was carried out by boiling 0.5 g of powdered leaf in 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of filtrate shaken with 1 ml of dilute ammonia solution. In both methods, observations were noted and recorded.

Keller-Killani test was carried out to test for the presence of glycosides. To 5 ml of aqueous extract, 2 ml of glacial acetic acid with one drop of ferric chloride solution was added. To this mixture 1 ml of concentrated sulphuric acid was added. All the observations were noted and recorded.

A general test for alkaloids was done using Meyer's reagent. To about 0.5 g of powdered leaf was added 5 ml of 10 % sulphuric acid, boiled for about 5 min in a water bath. The mixture was filtered and to the filtrate a few drops of Meyer's reagent was added. The reaction was observed and any changes recorded.

2.2.2.5 Determination of the ash value of *Monanthotaxis parvifolia*

A typical determination of the ash value was done as per the protocol in British Pharmacopoeia (B.P., 2008). About 1 g of leaf powder was weighed into a pre-weighed platinum dish. It was moistened with sulphuric acid and placed into the furnace and gently ignited at 800 °C for 15 min. The dish was removed from the furnace, allowed to cool in the desiccator and weighed. This procedure was repeated until two successive weights did not differ by more than 0.5 mg. The results were expressed as percentage w/w.

2.2.2.6 Extraction of the plant material

In principle continuous Soxhlet extraction using mainly organic solvents is employed to ensure exhaustive extraction of the plant material. This gives an extract which is reduced under pressure to yield a solid or semi-solid residue. The crude residue is then subjected to fractionation by different chromatographic methods in order to isolate and purify separated constituents. In this study classic column chromatography (CC), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) techniques will be employed (Ikan, 1991; Evans; 1996; Harborne, 1998).

A typical extraction of the plant material is described. About 800 g of the ground material were weighed and packed into a cotton cloth bag and placed in a 2L Soxhlet extractor. The powdered material was extracted under reflux for 48 h at 40 °C with petroleum ether (60-80 °C). The extracted material was removed and dried at room temperature on a stainless steel tray. The extraction process was repeated

sequentially with chloroform and methanol. Each solvent extract was cooled and filtered using Whatman filter paper 1, concentrated to dryness by use of a Heildolph rotary evaporator at 40-45 °C and the extract residue stored at 4°C until use. The yield from each solvent extraction was recorded. The extraction procedure was as shown in Figure 2.1.

Water extracts from the leaves and twigs were prepared by separately boiling 50 g of the powdered material in 500 ml of water for 5-10 min. The extract was filtered while hot, allowed to cool, dispensed into 15 ml centrifuge tubes and centrifuged at 2000 rpm for 5 minutes. The supernatant was aspirated out into 100 ml conical flask. The supernatant was filtered through Whatman filter paper no. 1 and the filtrate lyophilized. The lyophilized extract was packed in airtight containers and stored in the fridge at -4 °C until use.

2.2.2.7 TLC fingerprinting *of Monanthotaxis parvifolia*

Thin layer chromatography fingerprinting of *Monanthotaxis parvifolia* was carried out as per the method described by Liang *et al.*, 2004. Crude plant extracts were loaded on Analytical thin layer chromatographic plates. The plate was developed using chloroform: methanol (9:1 v/v). The results were noted and documented in form of a photograph.

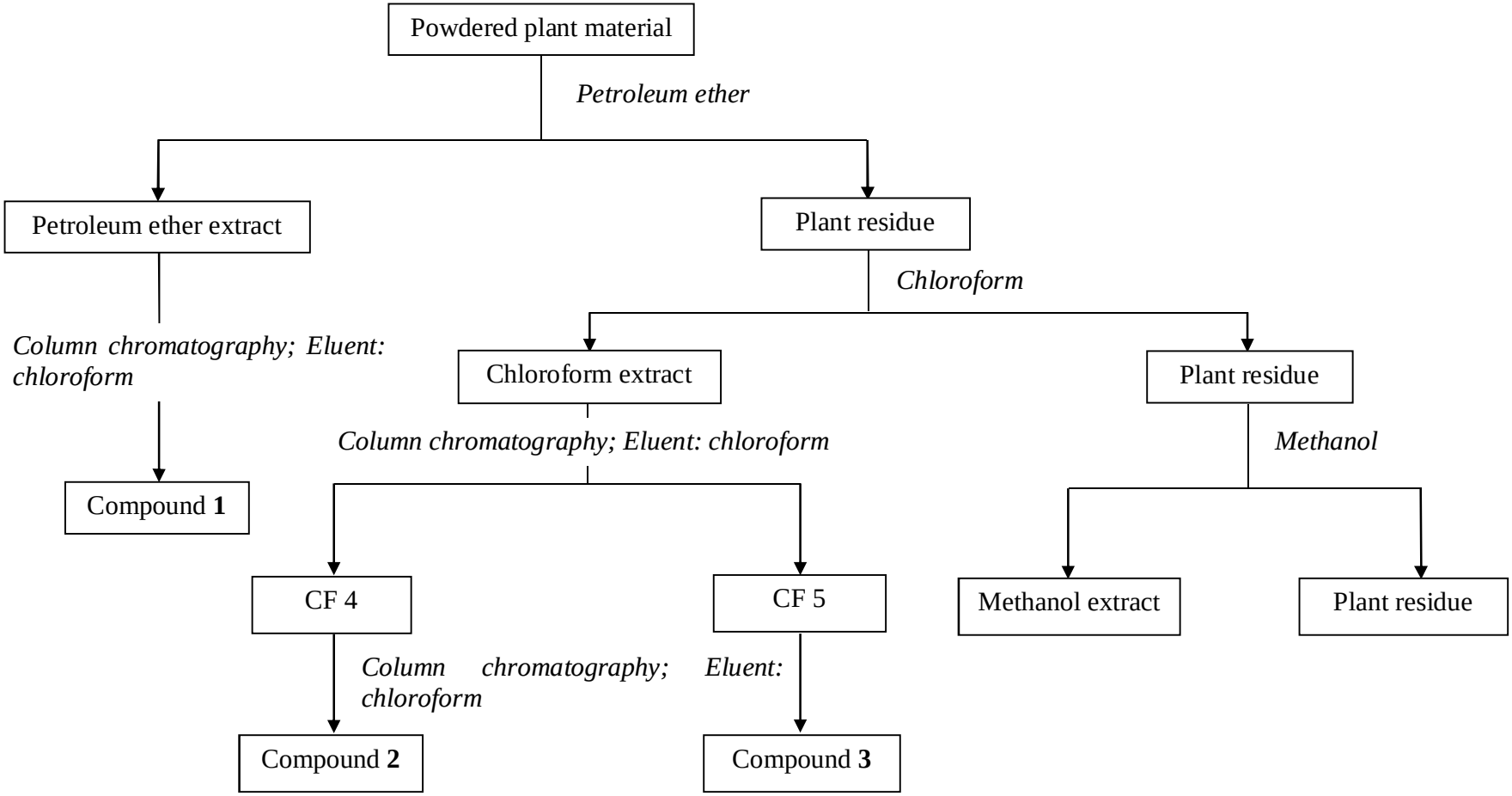

Figure.2.1: A scheme of extraction and isolation of compounds from *Monanthotaxis parvifolia*.

2.2.3 Preparation of phytochemical isolates for spectroscopy

2.2.3.1 Preparation of isolates for infrared analysis

About 2 mg of the samples were weighed by difference and triturated with 200 mg KBr to obtain a 1% w/w mixture which was pressed into thin discs. The discs were subjected to IR analysis.

2.2.3.2 Preparation of isolates for nuclear magnetic resonance analysis

The solvent evaporated samples isolated from preparative HPLC were dissolved in 500-1000 µl of deuterated methanol (CD_3OD) or deuterated chloroform ($CDCl_3$). To facilitate the removal of any particulate matter, the sample was filtered through a cotton wool plug in a Pasteur pipette. The sample was then transferred into the NMR tube for the NMR analysis. Both 1- and 2-D ^{1}H NMR were run at 400 MHz while the ^{13}C NMR was run at 100 MHz.

2.2.3.3 Preparation of isolates for mass spectrometry analysis

Small samples of approximately 2 mg of the isolated dried compounds were placed into a 2 ml glass vial dissolved in methanol/water (9:1, v/v) to yield a tentative concentration of 500 ηg/mL. The solution was infused into the mass spectrometer which was either in the positive or negative mode and the spectrum run at a flow rate of 5 µL/min.

2.2.4. Preparation of culture media

2.2.4.1 Preparation of stock culture medium

Culture medium solution was prepared by use of 5.94 g of 4-(2-hydroxyethyl piperazine)-ethanesulfonic acid (HEPES), together with 2 g glucose and 10.4 g Roswell Park Memorial Institute-1640 (RPMI-1640) powder which were transferred into a flat bottomed flask and dissolved in 1 L of water with the aid of magnetic stirring before making the volume to 1130 ml with water. The pH of the medium was adjusted to 7.2 using 20 % w/v sodium hydroxide.

2.2.4.2 Washing of the erythrocytes

Erythrocytes for maintenance of the parasite cultures were obtained from the whole blood of consenting O^+ positive blood group human donors then put in sterile clear polythene bags. The blood was withdrawn from sterile blood bags containing acid-citrate-dextrose anticoagulant comprising of tri-sodium citrate 22 g, citric acid 8 g, dextrose 24.5 g per litre of solution. It was placed upright in the refrigerator at 4 °C overnight for the blood cells to settle. The plasma was decanted out and 5 ml aliquots of the blood cell transferred into 15 ml centrifuge tubes and centrifuged at 2000 rpm for 5 min. The plasma and buffy coat was aspirated out. The erythrocytes were then re-suspended in wash medium and centrifuged at 500 g for 5 min. Washing of the erythrocytes was repeated two more times. The washed erythrocytes were then stored in 10 ml wash medium at 4 °C and used within 2 weeks.

2.2.4.3 Culturing of the *Plasmodium falciparum*

The malaria parasites were grown continuously in stock cultures according to the method of Trager and Jensen (1976). The parasites comprised the *Plasmodium falciparum* chloroquine-sensitive strain (CQS) D10 and the chloroquine-resistant strain (CQR) Dd2 as described in section 2.1.6.

The frozen malaria-isolates (≈ 5 % parasitaemia) in cryotubes stored in liquid nitrogen were quickly thawed in a water bath at 37 °C for 5 min. The content of the cryotube (≈ 1ml) was transferred under sterile conditions to a 10 ml centrifuge tube and 0.2 ml sterile 12 % NaCl was slowly added to the thawed culture. It was then mixed well by use of Vortex for (10-20) sec after that a sterile 1.6 % NaCl solution was slowly added and mixed by Vortex for another (10-20) seconds. The solution was centrifuged at 2500 rpm for 5 min and the supernatant aspirated out. The parasite pellet was then re-suspended in a 75 ml culture flask containing 10 ml culture medium and supplemented with fresh uninfected human erythrocytes from the O^+ blood group.

The culture was maintained at a 5-10 % haematocrit by sustaining the culture medium with the wash medium supplemented with Albumax II as described in section 2.1.5. The parasitaemia was maintained below 10 % by the addition of uninfected O^+ human red blood cells which were washed with the medium. The parasitaemia was checked on daily basis by use of Giemsa stained thin blood smear preparation. Changing the medium was done on daily basis. This was carried out by carefully aspirating off the media without touching the cells. In order to sustain optimal growth of the cells, fresh 5 ml of RPMI was added to the cells; the flask was aerated and quickly tightly closed. The cultures were kept at 37 °C in an incubator with a gas mixture atmosphere of 93 % N_2, 3 % O_2 and 4 % CO_2 (Clarkson, 2003). To maintain the parasites at one phase of growth usually the ring-phase; they were synchronized by use of D-sorbitol (Lambros and Venderberg, 1979).

2.2.4.4 Preparation of giemsa-stained thin blood smear

A smear of parasitic culture was made by placing a drop of about 12 µl parasitic culture at one end of a slide. Using a second slide at an angle, the drop was evenly spread along the first slide and then allowed to dry. Analytical grade methanol was used to fix the blood smear by allowing the slide with the blood smear to stand for 1 min flooded with methanol, after which the methanol was removed by decanting. The slide was covered with dilute Giemsa solution for 5 min, rinsed using running water and then allowed to dry in the open air for 3 - 5 min. A drop of microscope oil immersion was placed on the slide and the thin-blood smear viewed using an X100 oil objective of a Nikon phase contrast microscope.

2.2.4.5 *In vitro* synchronization of malaria parasites

Synchronizations were carried on malaria cultures consisting of 80% ring-phase parasites. The cultures were transferred from the culture flask to a 50 ml centrifuge tube, centrifuged at 500 rpm for 5 min and the supernatant removed. The pellet volume was about 0.5 ml when 10 ml of a parasitic culture (5 % haematocrit) was centrifuged. Then 4 ml of a 15 % D-sorbitol solution was added to every 0.5 ml of parasite pellet. After careful mixing, by inversion, the solution was incubated at 37 °C for 5 min, thereafter, 8 ml of a 0.1 % D-glucose solution was added per 0.5 ml of

the parasite pellet, followed by mixing by tube inversion. The parasite solution was again incubated at 37 °C for 5 min, centrifuged at 500 rpm for 5 min and the supernatant containing lysate erythrocytes, was removed. The pellet ring-phase infected and uninfected erythrocytes were re-suspended in 10 ml of the culture medium and returned to the culture flasks. The haematocrit was adjusted to about 5 % by adding washed erythrocytes, filled with the incubating gas mixture and returned to the incubator. The procedure was repeated until the cultures consisted of about 90 % ring-phase parasites.

2.2.4.6 Lactate dehydrogenase assay

Antiplasmodial activity was determined by use of viable synchronized parasites. This was done using the parasite lactate dehydrogenase activity according to a method described by (Clarkson *et al.,* 2003; Makler *et al.,* 1993). Chloroquine diphosphate salt (Sigma) was used as the reference drug in all the experiments. Chloroquine was prepared as a stock solution of 2 mg/ml in water and stored at -20 °C. Chloroquine activity was tested as a starting concentration of 100 ηg/ml. This was serially diluted two fold in the supplemented Rose Park Memorial Institute-1640 (RPMI -1640) medium to give ten concentrations. The test samples (crude extracts, fractions or pure compounds) were dissolved in either 10 % methanol or dimethylsulphoxide, sonicated for 20-30 min and made to volume with water to a stock solution of 2 mg/ml and stored at -20 °C over night. The stock solutions were then diluted with the culture medium on the time of the experiment. The test samples (crude extracts, fractions and pure compounds) were tested at a starting concentration of 100 µg/ml, which was serially diluted two-fold in the culture medium to give nine concentrations. The highest concentration of methanol, 0.5%, to which the parasites were exposed had no significant effect on the parasite survival (Clarkson *et al*., 2003). The cell suspension was distributed into flat-bottomed well plates containing serial concentrations of the test samples (in triplicate) under an optimum volume of 0.1 ml, together with untreated controls. This was incubated for 48 h at 37 °C in a special gas mixture atmosphere comprising of 4 % CO_2, 3 % O_2 and 93 % N_2 (Afromax, Cape Town, SA), where

the parasites matured into trophozoites.

After the culture the contents of the well were then frozen at -20 °C until they were assayed for the inhibition of the parasite growth when they were thawed by placing the plates in an incubator at 37 °C for 2 h. The process facilitated the lysing of the cells and hence releasing of the cell content into the medium.

About 100 µl of the Malstat® reagent solution was added to a separate microtitre plate by use of a Combi tube dispenser. After incubation, the test plates were removed and the parasites re-suspended within the same original test plates. Then 20 µl of the suspension containing the parasites was transferred by use of a multi-channel pipette into the corresponding well of the plate containing the Malstat® reagent. The mixture was incubated for 10-15 min at room temperature before adding NBT solution. After incubation, 25 µl of the NBT solution was added to the plates. All the air bubbles were removed by use of a blow drier and the plate was placed in a dark cupboard for 10-15 min for the colour reaction to take place. The absorbance of the formed formazan product was measured at a wavelength of 600 nm using a 7520 micro-plate reader (Cambridge Technology) (Makler *et al*., 1998). The 50% inhibitory concentration (IC_{50}) was determined from a non-linear dose response curve fitting analysis using Graph Pad Prism v.4.00 software (San Diego, California, USA). IC_{50}-values are given as a mean value with standard deviations of 2 independent experiments performed in duplicates. The absorbance data was converted into percentage viability using an Excel® (Microsoft) spreadsheet and a non-linear regression analysis model in Graph Pad Prism® 4 (San Diego, California, USA). This was used to determine the 50 % inhibitory concentration (IC_{50}) of the extracts or compounds against the parasites (Clarkson, 2002; Lategan *et al*., 2009). Confidence limits values were given in triplicates as IC_{50} and IC_{90} by normal statistical procedures and are indicative of the sensitivity level of the parasites against the tested plant extract. For comparison and control, tests with chloroquine were also included.

2.2.5 Cytotoxicity Assay

2.2.5.1 Cell culture

The cytotoxicity assay employed in this study was the method described by Mosmann *et al.* (1983). This is a quick colorimetric assay method for determining cellular growth and chemo sensitivity. The technique makes use of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) salt. The mammalian Chinese Hamster Ovarian (CHO) cell lines were routinely maintained as adherent monolayer cells in 75 cm^3 culture flasks (Falcon, USA) in complete medium composed of 1:1 ratio of Dulbecco's Modified Eagles Medium (DMEM) (Highveld Biologicals, Lyndhurst, South Africa) and Hams F-12 medium (Sigma, St Louis, MO, USA) which was supplemented with 10 % heat inactivated fetal bovine serum. The cells were incubated in a 5 % CO_2 and 95 %-air humidified atmosphere at 37 °C. The culture medium was changed every three days and the cells sub-cultured, which involved digestion of the cellular matrix with 1 % trypsin solution (Clarkson, 2002).

2.2.5.2 Preparation of methylthiazolyltetrazolium reagent

The test samples (both the isolated compounds and the crude extracts) were dissolved in water and further diluted in culture medium and incubated for 48 h in a 96-well microtiter plate at 6 fold dilution, beginning at 100 µg/ml. After the initial 48 h incubation, 25 µl of 5 mg/ml solution of MTT in phosphate buffer solution (PBS) were added to each well. The plates were incubated again for 4 h at 37 ^oC. The plates were then removed from the incubator and centrifuged for 10 min at a speed of 2050 rpm. The supernatant was aspirated from the wells without disturbing the crystals. A hundred micro litres (100 µl) of DMSO was added to each well and the plate was shaken for 5 min to dissolve the crystals. The optical absorbance of the formazan product was measured at 560 nm by a microplate reader. The absorbance data was transformed into percentage cell viability by use of an Excel® spreadsheet and a non-linear regression analysis model in Graph Pad Prism® version 4 software which was then used to draw dose-response curves and subsequently determine IC_{50} values. This was employed to determine the percent inhibition of growth and to

establish the fifty percent inhibitory concentration (IC_{50}) of the test samples against the cells.

The experiments were done in triplicate and repeated two times on separate days. Emetine was used as a positive quality control standard to monitor the experimental conditions and showed IC_{50} values within the acceptable (40-60 ηg/ml**).** The initial concentration was 100 µg/ ml and was serially diluted tenfold with the complete medium to give six dilutions the final concentration being 0.001µg/ml (Chukwujekwu *et al*., 2009).

The percentage of the cell growth in each well containing the test extract or isolate was calculated by means of the following formula:

$$\%\, Viability = \frac{Optical\ absorbance\ of\ treated\ cells}{Optical\ absorbance\ of\ untreated\ cells} X100$$

2.2.6 Pharmacological tests

2.2.6.1 Preparation of extracts for pharmacological tests

The plant extracts, mainly methanol extract, were weighed into a pestle and mortar. To prepare the stock solutions, the weighed extracts were triturated with minimum volume of dimethylsulphoxide (DMSO) and made to volume with distilled water to attain the required concentration and stored at -4 °C until use.

2.2.6.2 Testing for glycaemic activity

The effect of *Monanthotaxis parvifolia* leaf methanol extract on glucose metabolism was investigated on the Wistar rats. A total of 20 Wistar rats were used which had been starved 24 h prior to the experiment. The rats weighed on average between 190 to 250 g per body weight. They were divided into four groups which were designated as Group; I, II, III and IV, of five rats each. Group I and IV served as the negative control and the positive control respectively. Group I received only normal saline at a dose of 45 mg/Kg of body weight while animals in Group IV were treated to a dose of 500 mg/Kg of metformin per body weight. Animals in Group II and III served as the test groups and were treated to 500 mg/Kg and 1000 mg/Kg of

Monanthotaxis parvifolia methanol leaf extract respectively. The treatments were administered orally. The dextrose load of 50 % w/v was orally administered in mg/Kg to all the rats except those in the negative control. Blood samples were taken one hour prior to treatment and at time zero (t_0) that is, immediately the dextrose load was orally administered. Thereafter, blood sugar levels were assessed by withdrawing blood from the rat's tail vein every 30 min for a period of 2 h and thereafter 4 h. The concentration of dextrose in the blood samples was determined by use of the glucometer Expeeed kit®.

2.2.6.3 Screening for analgesic activity

The tail-flick test was used as a model to test *Monanthotaxis parvifolia* methanol leaf extract for analgesic effect according to method described by Bannon and Malberg, 2007; Okonon *et al*., 2012. The tail-flick test is a test of acute nociception in which a high-intensity thermal stimulus is directed to the tail of a mouse or a rat. The time from onset of stimulation to a rapid flick or withdrawal of the tail from heat source is recorded.

The animals were randomly assigned to five groups. Group I served as the positive control and was treated with the reference analgesic morphine that was administered subcutaneously at a dose of 10 mg/kg. Group II, III and IV was each treated with a dose of 200 mg/kg of methanol leaf, chloroform leaf and methanol stem extracts respectively. Group V received normal saline and served as the negative control. In order to have gentle restraint of the animals a Plexiglass tube was used. Baseline latencies of the animals were recorded. The animals' tail-flick response was tested using a tail-flick Ugo basile tail flick analgesiometer (Orchid Scientifics and Innovatives, Maharashtra, India) and the intensity of the heat source adjusted to produce tail-flick latencies of 3 to 4 sec. The light beam was focused about 15 mm from the tip of the tail. In the absence of a withdrawal reflex, the stimulus cutoff was set to 10 sec to avoid possible tissue damage to the animal. The injection volume was based on a dose of 10 ml/kg for mice.

2.2.6.4 Testing for antipyretic activity

The antipyretic effect of leaf methanol extract of *Monanthotaxis parvifolia* was also studied. The Brewer's yeast-induced pyrexia in mice was used as the experimental model. The initial temperature of mice was recorded 18 h before inducing pyrexia. Only animals which developed pyrexia of 0.5 °C to 1 °C were used for the test. The animals were randomly assigned into three groups of six animals each. Group I served as the control and received 25 µL of normal saline, Group II was injected subcutaneously with 200 mg/kg of the methanol leaf extract and Group III was injected with 150 mg/kg of aspirin and was the positive control.

Fever was induced by injecting 20 ml/kg subcutaneously of 20 % aqueous suspension of Brewer's yeast in normal saline in the nurque of the neck of mice. Body temperature was recorded by the Non-Contact Infrared Thermometer immediately before (-18 h) and 18 h after (0 h) Brewer's yeast injection. Aspirin at a dose of 300 mg/kg was injected intraperitoneally. Aspirin was used as a positive control for comparing the antipyretic action of methanol extract of *M. parvifolia* (Taiwe *et al.*, 2011; Saini and Singhal, 2012).

For each of the doses used, the volume injected to the animals was calculated according to the following formula:

$$V\,(ml) = \frac{D\left(\frac{g}{kg}\right) * P\,(kg)}{C\left(\frac{g}{ml}\right)}$$

V-volume of the medicine injected to the animal, D- dose of the drug in g per kg, p- body weight of the animal in Kg, C- concentration of the medicine in g per ml

2.2.4.5 Statistical data analysis

The results are presented as mean ± Standard Error of the Mean (SEM). Statistical significance for the pharmacological tests was analyzed using one-way ANOVA followed by Tukey's *post hoc* test. P values of less than 0.05 were considered to be statistically significant.

CHAPTER THREE

3.0 RESULTS ON MORPHOLOGICAL AND PHYTOCHEMICAL PROPERTIES OF *MONANTHOTAXIS PARVIFOLIA* SSP. *KENYENSIS*

3.1 Macro and microscopic study of *Monanthotaxis parvifolia*

The macroscopic and microscopic studies were carried on the *Monanthotaxis parvifolia* ssp. *kenyensis* Verdc and the results are as reported.

3.2 Distribution and growing environment

Monanthotaxis parvifolia was found to be sparsely distributed. It grows along river banks on well drained red soil in places which have never been cultivated. It was found to be in flower and fruit (Figure 3.1, 3.2) soon after the long rains that is around May, June and July although according to literature the peak season was indicated to be January, February, May and November (Beentje, 1994). *Monanthotaxis parvifolia* was to be growing along the riverine which had previously never been cultivated (Figure 3.3).

Figure 3.1 : *Monanthotaxis parvifolia* plant in flower and fruit in the field.

(NNM15)

Figure 3.2: Fresh specimen of *Monanthotaxis parvifolia* in flower and with fruits.

(NNM15)

Figure 3.3: *Monanthotaxis parvifolia* stumps on drained soil.

(NNM15)

3.3 Macroscopic characteristics

The macroscopic findings were in conformity with what is reported in literature. The leaf is simple and alternately arranged on the twig. The leaf has a characteristic aromatic smell. The leaves were observed to have a green colour on top and a characteristic bluish colour on the lower side. This fits the description in literature that is, a glaucous surface beneath. The shape of the leaf was observed to be obovate

with an acute apex and a rounded base. The leaf margin is entire and smooth. The size of the leaf varied from 4.0 to 9.4 cm in length and from 1.9 to 3.7 cm in width. The size of the leaf tended to grow larger as the distance from the stem increased. The length of the petiole had a big variation but with lengths varying from 0.5 to 0.7 cm. These features are shown in Figure 3.4. This conforms to what is reported in literature (Beentje, 1994; Evans, 1996; Wallis, 2005).

The flowers were small and yellow in colour and located on the lower side of the leaf (Figure 3.7). The fruits were also found on the lower side of the twigs and at the tip of the twig. They were brown in colour after drying, with one, two or three articled monocarps. This tallies with what was illustrated in the literature which previously had only afforded a sketch as shown in Figure 3.8 to 3.10. However, the location of the flowers and the fruits on the underside of the young branches makes them inconspicuous and can easily be missed out. This is despite the fact that these are key identification features for any plant. The features stated in the discussion are of importance in differentiating the different species of *Monanthotaxis* genus and in identifying family members of Annonaceae. The report on these features in form of photographs makes it easy to identify the plant and it is reported for the first time.

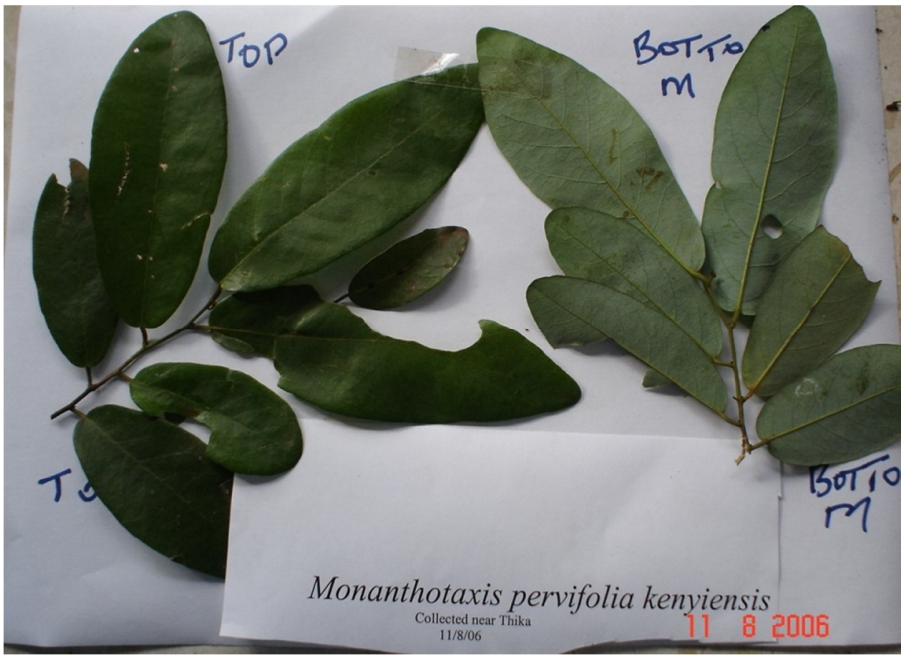

Figure 3.4: Morphological features of *Monanthotaxis parvifolia* leaves.

The figure demonstrates alternate leaf arrangement, shape and size of the leaves. (NNM14)

Figure3.5: *Monanthotaxis parvifolia* plant in the field.

(NNM15)

Figure 3.4 displays the upper and lower part of the plant in the field demonstrating the characteristic bluish under side and dark green upper sides of the leaves as observed in the field.

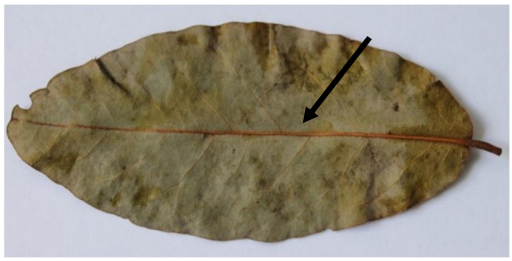

Figure 3.6

Figure 3.7

Figure 3.6: Pressed specimen of *Monanthotaxis parvifolia* leaf.

The specimen shows the lower side of the leaf. The arrow points at the net venation. (NNM14)

Figure 3.7: Pressed specimen of *Monanthotaxis parvifolia* with flower.

The Arrow points at the yellowish flower. (NNM14)

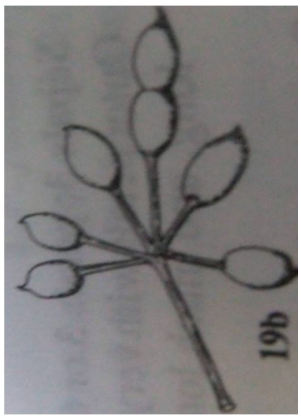

Figure 3.8 **Figure 3.9**

Figure 3.8 : Pressed *Monanthotaxis parvifolia* fruits.

The photograph shows arrangement of monocarps of *Monanthotaxis parvifolia* fruits, of one, two and three carps. (NNM14)

Figure 3.9 : Sketch of the arrangement of *Monanthotaxis parvifolia* fruits.

An illustration of fruits of *Monanthotaxis parvifolia* composed of free monocarps (Beentje, 1994)

Figure 3.10: Pressed specimen of the plant showing the fruit attached to the twig.

The arrow points at the characteristic arrangement of the fruits of Monanthotaxis parvifolia**.** (From NNM14)

3.4 Microscopic features upshots

The microscopic features of the leaf were observed under the microscope and are shown in Figure 3.11 to 3.14. Covering trichomes were found to be present as shown in Figure 3.11. These were found to be present mainly on the lower surface of the leaf. They were in appearance multicellular. The observation of the presence

of the covering trichomes tends to correspond with the description available in literature where the leaf is reported to be pubescent with fine short hair on the lower side (Beentje, 1994).

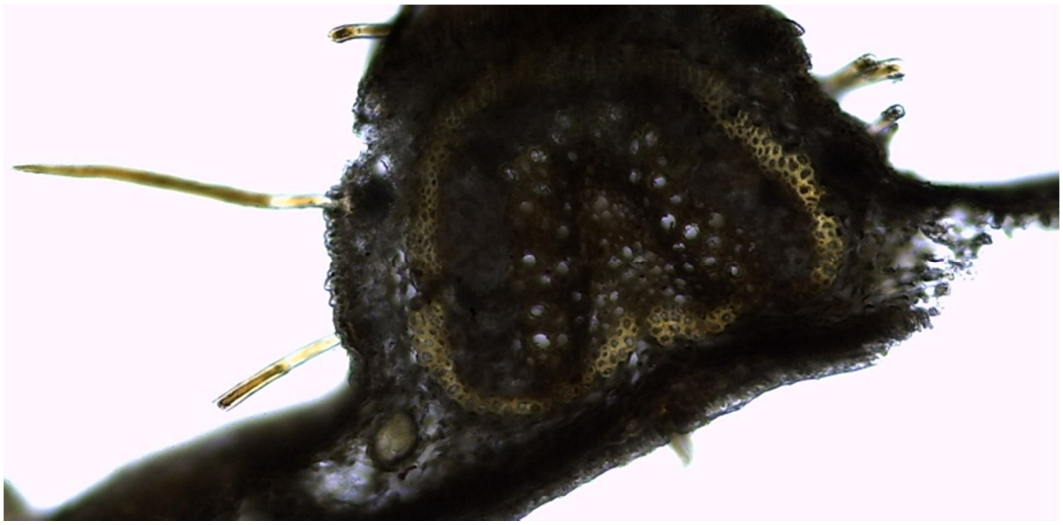

Figure 3.11: Transverse section of the leaf showing the trichomes.

(NNM14)

Figure 3.12 represents the transverse section through the midrib and lamina at higher power (X200). The section through the midrib revealed presence of a characteristic yellow coloured wavy cuticle. It also shows irregular shaped single layered epidermal cells. This was followed by the large thin walled parenchyma cells as pointed at by arrow c in Figure 3.12. Lignified fibres are present around the midrib which was confirmed by the red colouring with phloroglucinol in hydrochloric acid reagent. Collenchyma layer was found at the lower and upper midrib. A single layer of small palisade cells was observed on the upper part of the lamina.

The oil glands were observed on the leaf lamina. This would explain the strong characteristic smell associated with the plant. This kind of work on *Monanthotaxis parvifolia* is being reported for the first time. This is a key contribution to knowledge considering that microscopic characters are well established criteria in the identification of plants of medicinal value especially the powdered drugs (Jackson and Snowdon, 1974). These pharmacognostical results are in tandem with

similar studies of other plants in the Annonaceae family mainly *Annona squamosa* Linn. and *Annona reticulata* Linn. (Agrawal *et al*., 2011; Switu *et al*., 2012).

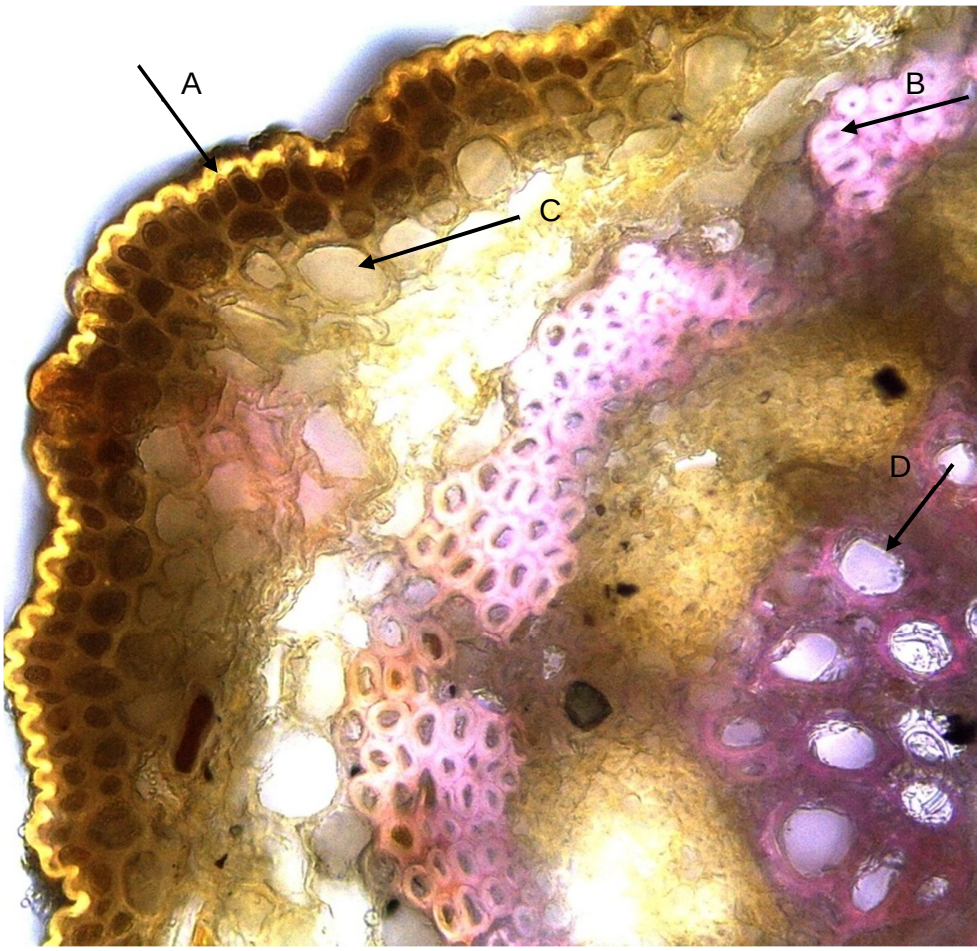

Figure 3.12: Detailed transverse section of the leaf.

The section was stained by use of phloroglucinol reagent. Arrows refer to; a- yellow and wavy cuticle; b- lignified fibres with the characteristic narrow lumen; C- parenchyma cells; d-xylem vessels. (NNM14)

The key features observed on the surface preparation were the presence of stomata with what appears to be paracytic arrangement of the subsidiary cells around the guard cells (Figure 3.13)**.** This characteristic arrangement of stomata corresponds with what was reported in other plants in the Annonaceae family (Agrawal *et al*., 2011; Switu *et al*., 2012).

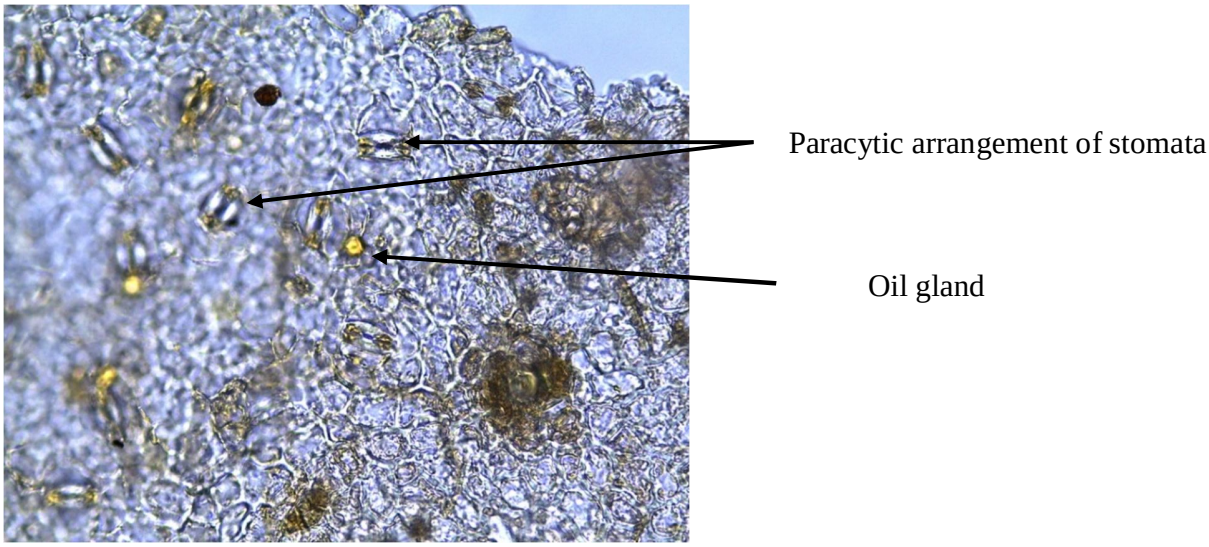

Figure 3.13: Surface preparation of the leaf.

The preparation illustrates presence of stomata, characteristic guard cells and oil glands. (NNM14)

The prominent reticulate venation system in the leaf is shown in Figure 3.14. The reticulate venation of the leaves in *Monanthotaxis parvifolia* is common in the class of Angiosperms, order of Magnoliales and family of Annonaceae (Evans, 1996).

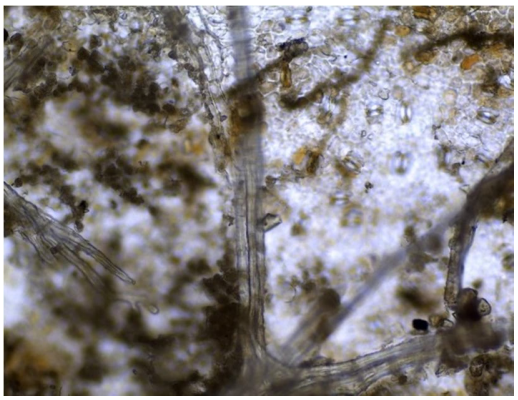

Figure 3.14: Surface preparation of the leaf showing venation.

The preparation shows the venation system and the stomata cells**.** (NNM14)

3.5 Micro chemical tests

Micro chemical tests on the leaf sections showed presence of various features in the cells as shown in Table 3.1.

Table 3.1: Micro chemical tests of *Monanthotaxis parvifolia* leaves

Test	Observation	Inference
Phloroglucinol + conc. Hydrochloric acid	Red colour on fibres	Lignified tissues
Alcoholic Ferric chloride solution	Bluish colouration on the epidermis and parenchyma	Presence of tannins
Sudan III test	Yellow colour between the parenchyma cells and vascular bundles and on the lamina	Presence of oil glands

The histochemical results correlate well with similar work done on *Annona squamosa* and *Annona reticulata* which are plants in the same family as *Monanthotaxis parvifolia*. Presence of lignified tissues, tannins and oil glands was reported in these plants. Presence of oil glands seems to be a common feature not only for plants in this genus but even in plants in other genera of Magnoliale order (Evans, 1996).

Microscopy of the powdered leaf showed presence of a uniseriate trichome. This would imply that *Monanthotaxis parvifolia* leaf has a mixture of both uniseriate multicellular trichomes and unicellular trichomes.

3.6 Ash- value

The insoluble acid value was determined as one of the physical parameters of evaluating the quality of herbal medicine. Acid insoluble value of the leaf powder of *Monanthotaxis parvifolia* was 0.7 % which was lower than that reported in literature for *Annona squamosa* which was 1.3 % (Agrawal *et al*., 2011).

3.7 Thin layer chromatographic fingerprinting profile

Thin layer chromatography profile of *Monanthotaxis parvifolia* was carried out and the results are as depicted in Figure 3.15. The thin layer chromatography profiles of the chloroform extracts of the leaf and the stem are very similar. The same was observed for methanol extracts (Figure 3.15). The main difference observed being the relatively higher concentration of the green pigment in leaf chloroform extract (CL) in comparison to the leaf methanol extract (ML). The spot is marked as visible in Figure 3.16. The results would be important when testing for the adulteration of crude drugs particularly those from closely related species (Essiet *et al*., 2011). The methanol extracts (ML and MS) leave behind a big spot on the base line. The observation indicates a lot of components in the extract did not develop with the mobile phase used in this case, Chloroform: Methanol (9:1) v/v). This necessitated the use of reverse phase- high performance liquid chromatography column system to separate the components in the methanol extract. In this study preparative HPLC was employed to achieve the separation.

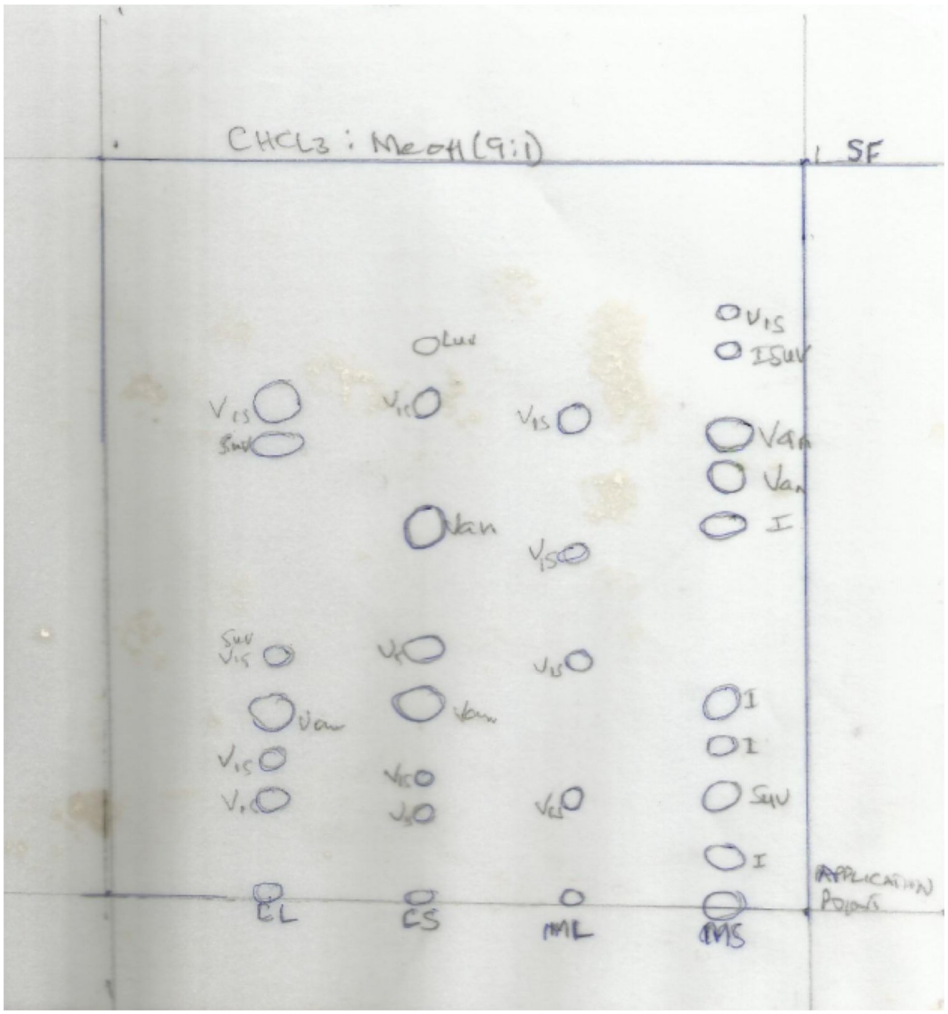

Figure 3.15: Thin layer chromatogram of *Monanthotaxis parvifolia.*

The chromatogram illustrates thin layer profiles of crude leaf and stem chloroform and methanol extracts. (NNM14)

CL and CS- chloroform leaf and stem crude extracts respectively; ML and MS – methanol; leaf and stem crude extracts respectively; Mobile phase used: Chloroform: methanol ((9: 1); I- spots viewed with iodine; van- spots detected with vanillin reagent; vis- spots detected under ordinary light; luv and suv- spots viewed under long and short UV light respectively; SF- solvent front which was 15 cm.

According to WHO-guidelines for herbal drug standardization and evaluation, thin layer chromatography fingerprinting is classified as physicochemical method of characterizing the drug (Gautum *et al*., 2010; Patil *et al*., 2012). Thin layer chromatography is the preferred finger print method for herbal drug analysis due to

its being simple, rapid and economical (Yongyu *et al*., 2011). Specifically the fingerprints of herbal medicines refer to the profiles which can illustrate the specific properties of the analyte. The chromatographic finger print analysis can either be 'Component-oriented approach' where a marker is required or 'Pattern-oriented approach' which show the chromatographic pattern of the extract (Yongyu *et al*., 2011). In this study 'Pattern-oriented approach' was applied whereby the finger print obtained from the standard extraction is considered as a standard finger print.

3.8 Yield of the *Monanthotaxis parvifolia* crude extracts

Percentage yields from each of the solvents extracts were determined. The yields were: methanol, 9.03 %; chloroform, 3.9 %; and petroleum, 3.05 % w/w. The high yield in methanol was used not only for the isolation of the compounds but also in pharmacological tests. The high yield of methanol extract would point to presence of more polar compounds in the plant.

3.9 Phytochemical constituents

The tests carried out on the leaf extracts and the powdered leaf revealed presence of different phytochemical constituents in *Monanthotaxis parvifolia*. The results are as tabulated in Table 3.2. The phytochemical characteristics of *Monanthotaxis parvifolia* indicate presence of tannins, flavonoids and glycosides but absence of alkaloids. The findings correlate with the work done on related plants in Annonaceae family, that is in *Annona squamosa* and *Annona reticulata* where presence of tannins in the leaves was reported (Switu *et al*., 2012). The phytochemical screening falls under physicochemical evaluation of a herbal drug as guided by WHO-guidelines for herbal drug standardization and evaluation (Gautum *et al*., 2010).

Table 3.2: Phytochemical screening for chemical classes of *Monanthotaxis parvifolia* leaf

Test	Observations	Inference
Tannins	A strong blue-black colour formed	Heavy presence of tannins
Flavonoids-method-1	A yellow colour was formed which faded on standing	Heavy presence of flavonoids
Flavonoids- method-2	A yellow solution was formed,	Heavy presence of flavonoids
Glycosides	A brown ring was formed at the interface of aqueous and organic layer	Presence of glycosides
Alkaloids	There was no precipitate formed with the Meyer's reagent	May suggest alkaloids not to be present

Table 3.3: Summary of pharmacognostic and phytochemical findings of *Monanthotaxis parvifolia* leaves

Method	Evaluation Parameters	Experimental	Literature	Remarks
Authentication	Part of plant collected	Twigs and leaves	As reported in literature	Beentje, 1994
	Location	Near Thika along river line		
	Family	Annonaceae		
Morphology and organoleptic evaluation	Colour	Green on top ,bluish below	As reported	
	Taste	Slightly bitter	Not reported	Reported for the first time
	Scent	Aromatic	Not reported	
	Leaf size	4-9 x1.9-3.7 cm	Within reported range	
	Phyllotaxis	alternate	opposite	
	Fruit	Monocarps,brown,1-3 articled	As reported	
Microscopic evaluation	Trichomes	Unicellular and multicellular	Not reported	Reported for first time
	Stomata	Paracytic	In other species	Switu *et al.*, 2012
	Lignification	Lignified fibres	Not reported	Reported for the time
	Oil glands	Present in lamina	Not reported	″″
	Cuticle	Wavy cuticle	Not reported before	″″
Physical evaluation	Acid ash value	0.7 %	Not reported before	″
	TLC finger print	As shown in Figure 3.15	Not reported before	″
Phytochemical constituents	Shown in Table 3.2	Results as captured in Table 3.2	Not reported before	″″
Biological activity	Reported in Chap 6	Results as shown in Chap 6	Not reported before except use in traditional medicine	″″

3.10 Conclusion

Since there is no pharmacognostic-anatomical work and phytochemical tests reported previously on *Monanthotaxis parvifolia* ssp. *kenyensis*, the present work could be used in quality control of the material. Macroscopic features, microscopic characteristics, thin layer chromatographic profiles and phytochemical screening analysis discussed here can be considered as referential identifying parameters to

substantiate and authenticate herbal preparations of *Monanthotaxis parvifolia*. The information could also be useful in the preparation of herbal monograph for its evaluation.

CHAPTER FOUR

4.0 ISOLATION AND PURIFICATION OF COMPOUNDS FROM *MONANTHOTAXIS PARVIFOLIA*

4.1 Isolation of compounds from extracts of *Monanthotaxis parvifolia*

Various separation techniques have been described in the literature for the isolation of compounds from complex plant material. The procedures applied in this study are mainly those described by Harborne (1998) and Hostettmann and Marston, (2002).

4.2 Thin layer chromatography

The phytochemical investigation of each plant extract was started by detecting for the separation of extracts by use of thin layer chromatography (TLC). A number of solvent systems were tried out until an optimal solvent system for the separation of the different components was obtained for each of the extracts. The detection of separated components was typically done by use of combined physical and chemical methods. The physical methods applied were; by viewing the separated components under long (366 nm) and short (254 nm) UV light in a UV chamber followed by exposure of the plates to iodine vapour in an iodine chamber where compounds with unsaturated bands show brown spots. After the iodine vapourizes and no brown spots are observed on the plate, the chemical detection was done by spraying the same plates with 1 % vanillin spray reagent and the plate placed in the pre- heated oven at 110 °C for 5-10 minutes for the colour to develop. Anisaldehyde in 70 % alcohol solution was also used for chemical detection where necessary.

4.3 Column chromatography

A locally made glass column of 100 cm × 4.0 cm I.D. was used. This was packed by use of wet packing using normal silica gel 60. About 300 g of the silica gel was suspended in chloroform to make a slurry. The slurry was packed into the column under vibration with the help of a Voss vibrating machine (Maldon, Essex, U.K.). Column chromatography was used to separate different extracts into fractions. The crude extracts of about 30 gm were dissolved in a small volume of solvent of approximately 50 ml and loaded onto the column by use of a glass pipette. About 2

ml fractions of eluent were collected on an automatic Superfrac fraction collector which was set at a flow rate of 15 drops per minute. Alternatively about 5 ml of the fraction was collected manually.

The elution was carried out by use of 100 % chloroform as the mobile phase. A mobile phase of ethyl acetate: methanol (4:1, v/v) was used to develop the ethyl acetate fraction previously partitioned from the methanol/water mixture.

TLC analyses were done on every 5^{th} fraction. Similar fractions were pooled to give several fractions. The combined fractions were dried *in vacuo* by use of rotary evaporation at 40-45 °C. The dried fractions were packed in small air-tight containers and stored at -4 °C until use.

4.4 Partitioning of methanol extract

About 80 g of the dried methanol leaf extract (previously obtained as described in 2.2.2.6) was weighed into 500 ml conical flask dissolved in 100 ml methanol and transferred into a 1L separatory funnel. About 300 ml of distilled water was added to form a suspension. This was partitioned sequentially with 4 × 250 ml portions of diethyl ether, chloroform and ethyl acetate. The aqueous and the organic layers were thoroughly mixed by constant shaking with a magnetic stirrer for 30 minutes then left to stand for 5 hours in a 1000 ml separating flask. The separated organic fractions from each solvent were combined and the solvent removed by reducing on a rotary evaporator at 40-45 °C and the yield from each solvent extraction recorded. The yields from diethyl ether and chloroform fractions were negligible and were kept aside. The ethyl acetate fraction on reducing with the rotary vapour resulted in a 31.3 percent yield. The ethyl acetate fraction was chosen for further follow up as guided by the antiplasmodial activity results. The extraction process is as illustrated in Figure 2.1 and partition process of methanol and fractionation on a HPLC column (Figures 4.1 and 4.3).

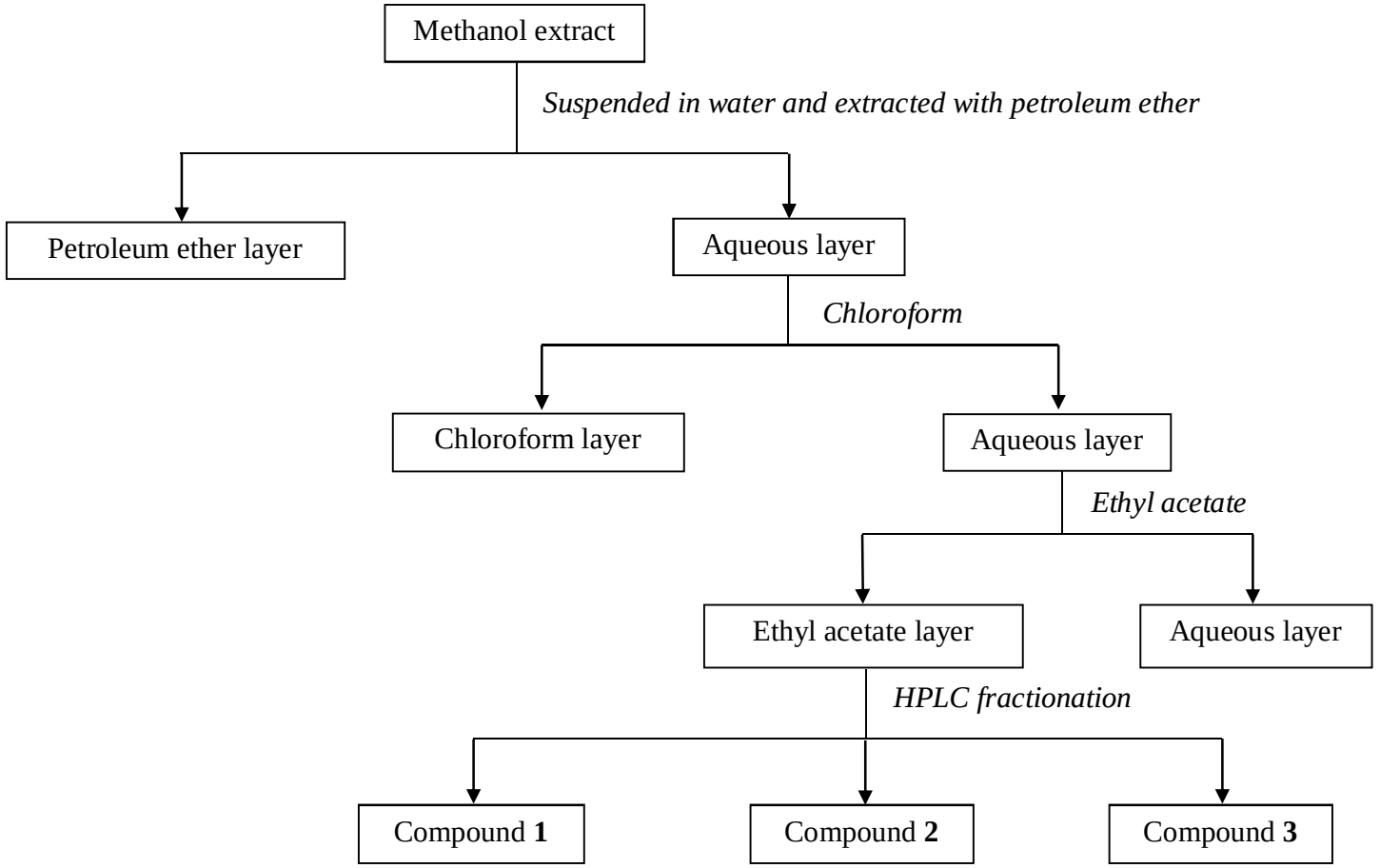

Figure.4.1: Partitioning of *Monanthotaxis parvifolia* methanol leaf extract.

4.5 Analytical high performance liquid chromatography

An automated preparative Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) sampling and fraction collection system was used to analyze and fractionate the *Monanthotaxis parvifolia* leaf methanol extracts according to a modified method by Dugo *et al*. (2009). For this study both analytical and preparative HPLC were performed on a modular Waters HPLC system (Microsep, Tygervalley, South Africa). This was fitted with a 2767 sample manager, a 2545 quaternary gradient pump, a 1500 series column heater and a 2998 photodiode array detector (PDA) which had a 2998 flow cell. MassLynx™ and FractionLynx® software version 4.1 (Waters Corporation, Milford, USA) were connected to HPLC to control the instrument, acquire data and monitor the collection of the fractions from the column.

The organic solvents used namely acetonitrile, methanol and formic acid (98-100 %), were of HPLC grade and were purchased from Sigma-Aldrich, Kempton Park, South Africa.

In order to establish the applicability of the preparative HPLC method, analysis of the dry methanol extract was carried out on analytical RP-HPLC column (Waters XBridge™ C_{18} 4.6 × 150 mm, 5 µm) fitted with a 4.6 × 20 mm guard cartridge for protection against clogging. Dry methanol leaf extract of *Monanthotaxis parvifolia* was dissolved in acetonitrile-water (25:75) to prepare a 1 mg/mL solution and a 10 µL aliquot injected into the column. The sample was separated by use of mobile phase mixture, where mobile phase A was an aqueous solution of 0.1% formic acid which was mixed on-line with mobile phase B, a 0.1% formic acid solution in methanol. This was run at a linear gradient of A: B at a flow rate of 1.2 ml/min and the chromatogram recorded at 355 nm.

4.6 Preparative high performance liquid chromatography

The analytical method was scaled up to the preparative RP-HPLC column (XBridge™ C_{18} 19 × 250 mm, 5 µm) which was fitted with a 19 × 10 mm guard cartridge (Waters Corporation, Milford, USA) in order to isolate larger quantities of

the isolates. In this case, sample solutions for fractionation and purification were prepared at a concentration of 100 mg/ml of the extract in 50% acetonitrile in water. The injection volume ranged from 50 µL to 1000 µL of the solution per run depending on the sample. The mobile phase flow rate was 20 µl/min for all purifications and the column heater was set at 30 °C. Several runs of the sample were carried out generating 7 different fractions in addition to fraction 8 as the wash out (Figure 3.2). The fractions were collected in correspondence to each peak as monitored by FractionLynx® software. Each of the fractions was evaporated on a Genevac HT-12 HCI evaporator (Genevac Inc, Stone Ridge, New York) to obtain seven semi- pure compounds. Each of the compounds was further subjected to MS and NMR analyses for structure elucidation. Six of the isolated compounds were tested for the antiplasmodial activity while only four were assayed for cytotoxicity on the Chinese Hamster Ovary (CHO) cell lines.

Although partitioning of the methanol leaf extract helped to further purify the compounds isolated through fractionation process using the preparative HPLC, this process was abandoned. This was mainly due to the partitioning process, which was found to be laborious and did not yield more pure compounds when compared to when the methanol leaf extract was injected directly into the column without subjecting the extract to the partition process as illustrated in Figure 4.3.

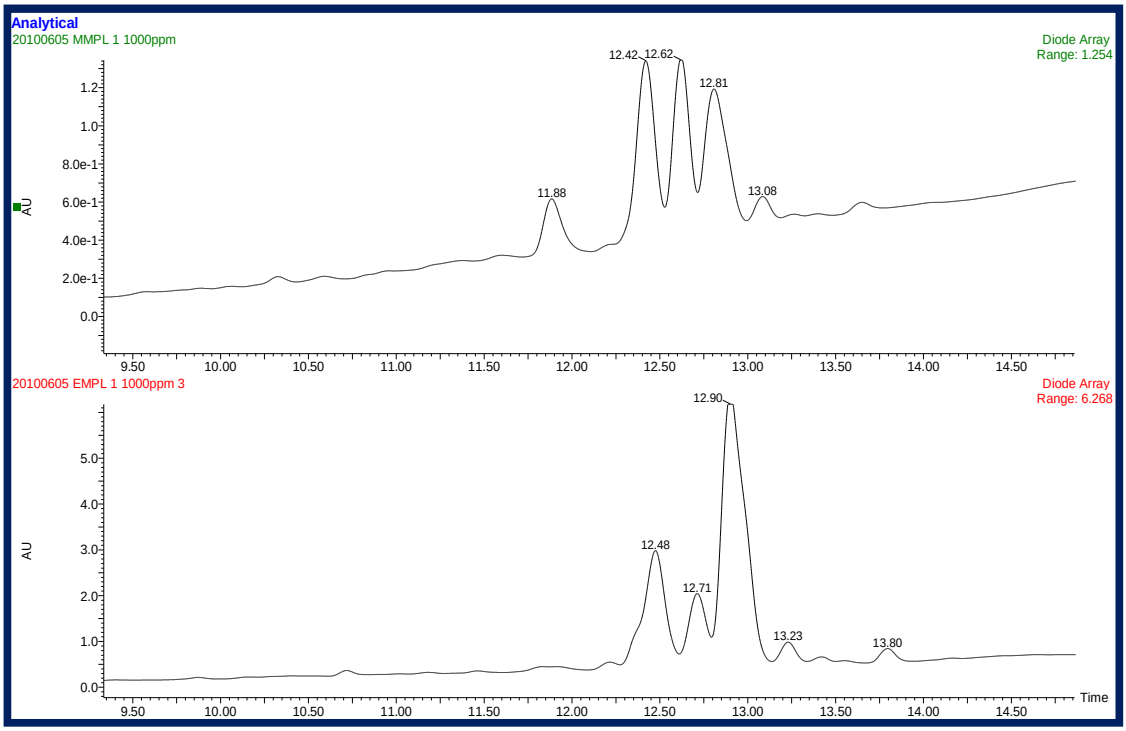

Figure 4.2: High performance liquid chromatography Chromatograms for the leaf methanol extract and Ethyl acetate fraction.

MMPL- Methanol extract of *Monanthotaxis parvifolia* leaves and EMPL-Ethyl acetate partition. Chromatographic conditions: Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in MeOH; Flow rate: 20 ml/min; Injection volume: 700 µl; Gradient: 40 – 50 % B in 10 min, 50 – 95% B in 3 min, back to 40 % to re-equilibrate. Figure 4.3 shows the chromatographic peaks of the methanol extract before partioning and that of ethyl acetate fraction after partitioning..

The methanol leaf extract from *Monanthotaxis parvifolia* (MMPL) was fractionated without first undergoing partitioning which resulted in a typical preparative HPLC chromatogram (Figure 4. 3).

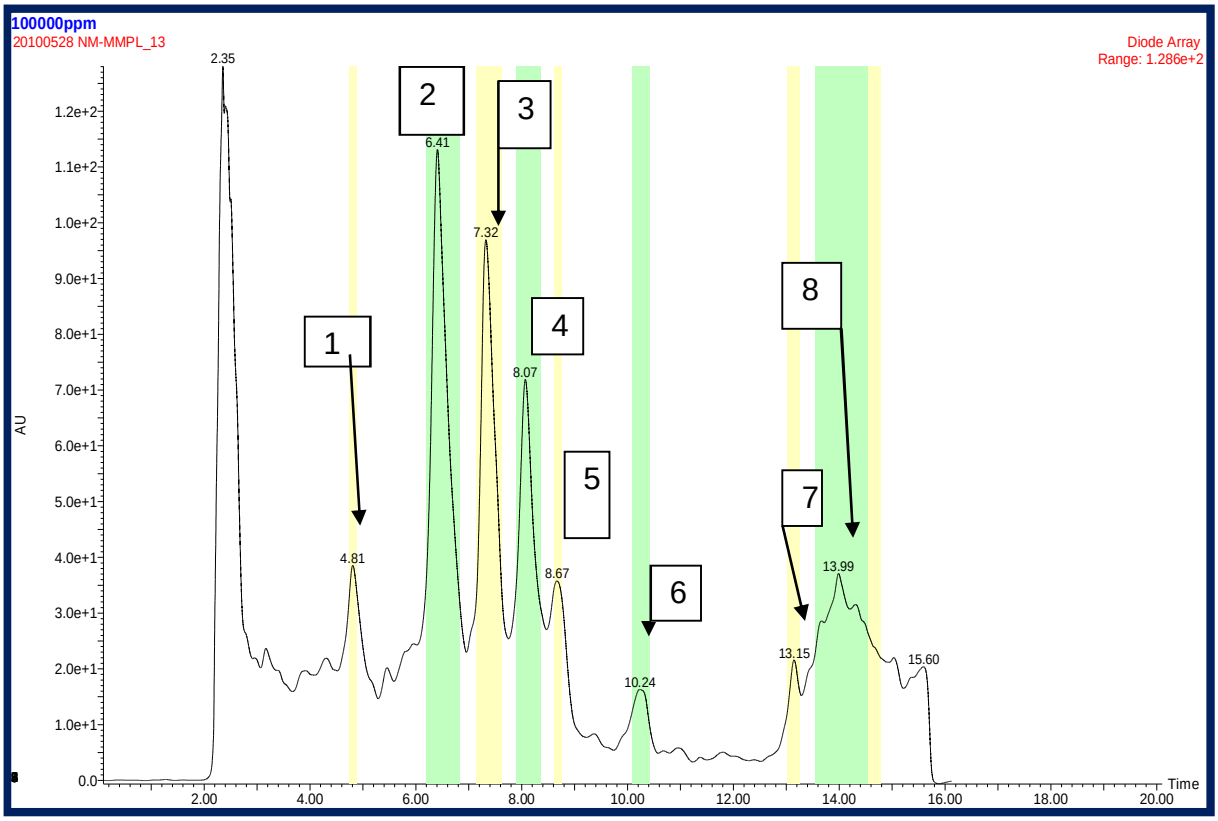

Figure 4.3: Preparative HPLC Chromatogram of *Monanthotaxis parvifolia* methanol leaf extract.

Column C_{18} 19 × 250 mm; Mobile phase A: 0.1 % formic acid in water; Mobile Phase B: 0.1 % formic acid in MeOH. Flow rate: 20 ml/min; Injection volume: 700 µl; Gradient: 40 – 50 % B in 10 min, 50 – 95 % B in 3 min, back to 40 % to re-equilibrate. The numbers in figure indicates the pooling of fractions.

The combined fractions were dried and used as such without further purification as they were found to be clean enough to elicit the necessary information on the resolving of the structures of the respective compounds by subjecting them to both nuclear magnetic resonance and mass spectrometry analyses. Fractions 2-7 produced compounds 2-7 as fraction 8 was the methanol wash out (Figure 4.3).

Due to the method of isolation used, quanties for fractions; 1, 5, 6 and 7 were not enough to generate the data for structure elucidation.

Although a 100 % purity of fraction 2 from the methanol leaf extract could not be obtained, it was used as it was to give information to resolve the structure of

compound 2 (Figure 4.4).

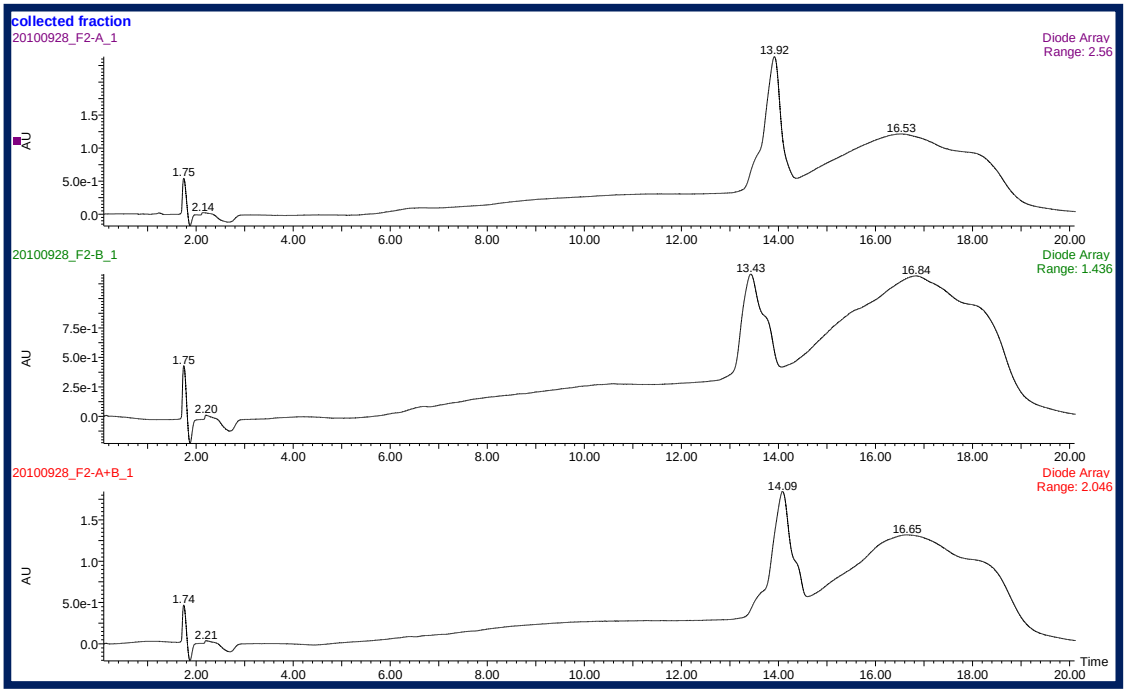

Figure 4.4: Checking the purity of Fraction 2.

Purity check of fraction 2 from methanol extract of *Monanthotaxis parvifolia* leaves

The fraction two purity checks are indicated as F2-A, F2-B and the combined fractions F2A +B.

CHAPTER FIVE

5.0 RESULTS ON STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

5.1 Introduction

Spectroscopic methods used to resolve the structures of phytochemical isolates include: - High resolution mass spectrometry which is used to determine the molecular ion and the fragmentation pattern of the molecules (Silverstein *et al*., 1991; Morales-Escobar *et al*., 2007; Kyriakou *et al*., 2012). A lot of studies have been carried out using mass spectrometry (MS) to study structures of flavonoids among other plant constituents such as phenols, tannins and glycosides (Tokusoglu *et al*., 2003; Cuyckens and Claeys, 2004; Prasain *et al*., 2004).

The Nuclear Magnetic Resonance (NMR) spectroscopy (1D and 2D) is used to identify all the proton and carbon atoms of the molecules (Shafagat, 2008; Karioti *et al*., 2009; Merkley *et al*., 2013; dos Santos *et al*., 2014; Petrica *et al*., 2014). The NMR spectrum has been shown to be of great value in structure elucidation because the properties it displays can be related to the analyst's perception of molecular structure. This includes for example the number of carbon and hydrogen atoms in a structure and the function groups attached to them as well as how they are positioned on the carbons. This way the chemical shift of a particular nucleus can be correlated with its chemical environment, the scalar coupling (or *J* coupling) indicates an indirect interaction between individual nuclei, mediated by electrons in a chemical bond and under suitable conditions; the integration of a signal is related to the number of nuclei giving rise to it. Assigning specific nuclei to a spectrum help to identify a chemical structure that is consistent with the spectrum. This may therefore give a fairly good idea of what this structure is likely to be. There are a number of techniques in common use that help with this aim. Among this is the 1D ^{1}H NMR where the ^{1}H nucleus is the most commonly observed nucleus in NMR spectroscopy. This is because hydrogen is found in almost all organic molecules. The proton also has high intrinsic sensitivity and is almost 100 % abundant in

nature. The proton NMR spectra therefore provide useful information on chemical shifts and coupling constants which is useful in determining the structure of a compound. The coupling constant *J*, further provide useful information on conformational and stereochemical position of the proton and hence give relevant geometric information of the molecule. The 1D ^{13}C-NMR generate the ^{13}C spectrum which offers further characterization of the molecule as it relates directly to the carbon skeleton. Unfortunately, ^{13}C has a lower intrinsic sensitivity than the proton and has only 1.1 % natural abundance. Typically one line is observed for each carbon atom in the molecule, as resonance overlap is rare. The chemical shift of each resonance depends on the environment, and it is possible to identify certain functional groups for which there is no direct evidence in the proton spectrum such as the carbonyls. This is particularly so when for example distortionless enhancement by polarization transfer (DEPT) experiment is done on the carbon atoms. In such a scenario the ^{13}C spectrum is designed to display separate sub spectra for CH, CH_2 and CH_3 carbon signals where the signal intensity are by polarization transfer.

In addition to mass spectrometry and nuclear magnetic resonance spectroscopy, this study utilized ultraviolet spectroscopy which gives information on presence of chromophores due to the presence of unsaturated conjugated system. The infrared spectroscopy provides information on key function groups which could be present in the molecule. The information gathered from each of these techniques was then used in trying to resolve the structures of the phytochemical isolates in this study.

5.2. Structure determination of compound 2

The compound was obtained from leaf methanol extract of *Monanthotaxis parvifolia* as a yellow amorphous powder. Phytochemical tests on the phytochemical isolate were positive for phenolic group and glycoside.

The compound gave the following spectroscopic data:

UV (MeoH) λ_{max}, (nm): 286, 365.

IR (KBr) ν_{max} (cm^{-1}): 3300(OH), 2900, 1660(C=O), 1600-1500 (.

^{1}H -NMR (400 MHz, MeOD) δ, (ppm) 7.77 (s, 1H), 7.54 (d, *J* = 8.4 Hz, 1H), 6.83 (d, *J* = 8.5 Hz, 1H), 6.36 (s, 1H), 6.17 (s, 1H), 5.07 (d, *J* = 7.7 Hz, 1H), 3.84 – 3.74 (m, 2H), 3.66 – 3.56 (m, 1H), 3.56 – 3.50 (m, 2H), 3.46 – 3.40 (m, 1H), 3.27 (m, 4H), 2.60 (s, 1H).

^{13}C-NMR (101 MHz, MeOD) δ, (ppm) 178.13(C-4), 164.78(C-7), 161.59(C-5), 157.44(C-9), 157.06(C-2), 148.54(C-4'), 144.41(C-3'), 134.40(C-3), 127.81, 121.58(C-1'), 119.09(C-2'), 116.41(C-5'), 114.70(C-6'), 104.06(C-10), 100.75(C-1"), 98.57(C-6), 94.92, 93.38(C-8), 82.54(C-3"), 77.19(C-5"), 75.78(C-2"), 73.72, 71.81(C-4"), 70.80, 68.64, 61.19, 60.57(C-4",-OCH3), 16.43(C-6").

MS m/z (M+H): 436.09 (M+H), 435.09, 419.09, 391, 303.05 (100 %), 304.05, 287.05, 241.07, 229.14, 217.10, 186.22, 149.01, 124.08**.**

A broad band observed in the IR data at 3300 cm^{-1} suggest presence of a hydroxyl (OH) bond, while the band at 2900 cm^{-1} is indicative of an aliphatic –C-H function. The band at 1660 cm^{-1} suggests presence of a conjugated carbonyl function, C=O while the bands within the range 1600-1500 cm^{-1} indicates presence of aromatic C=C skeleton.

The colour of the compound and its UV absorbance at λ_{max} 286 and 365 nm is indicative of presence of a chromophore in the structure which is suggestive of a highly conjugated aromatic nature. The UV bands of compound 2 at around 350 nm and 266 nm correspond with those reported in literature for Flavonoids (Harborne *et al.*, 1975; Takemura *et al.*, 2005; Sokolova *et al.*, 2012). This observation was further supported by the physical observation that compound 2 was a yellow coloured amorphous powder which is indicative of an aromatic compound. These observations further correlates with the results on phytochemical tests which indicated presence of Flavonoids and tannins in the plant.

Analysis of the ^{1}H-nuclear magnetic resonance at δ 7.77 to 7.55 ppm is indicative of the presence of phenolic aromatic protons. This is further supported by positive

chemical test for presence of phenolic groups and IR absorbance of a broad band at 3300 cm^{-1} suggesting presence of a hydroxyl (OH) bond. This is further supported by the fact that among the most significant and constantly encountered functional group in natural products are the phenol type –OH groups (Charisiadis *et al*., 2010). Resonance signals in $^{13}C_7$ at δ164.78 and $^{13}C_5$ at 161.59 ppm also suggest multiple aromatic C-OH bonds. In nature most phenolic compounds isolated from plants tend to be tannins. The observed molecular ion for the compound was m/z 435.09. This molecular weight is much lower than the generally accepted weight for tannins which are m/z 1000 or higher. Flavonoids are the other phenolic compounds occurring in nature which are yellow coloured. These observations are all indicative of presence of a flavonoid. This was further supported by the positive chemical test for the presence of a flavonoid. Search in literature for phenolic compounds with such molecular weight suggest quercetin and related compounds which show a distinctive mass fragment of quercetin, (M+1) of (m/z 303). Other chemical tests indicated presence of a glycosidic group. It is therefore possible that compound 2 is a glycoside derivative of quercetin.

This was also supported by the ^{1}H-NMR resonance of aromatic protons at δ 6.92 to 6.15 ppm consistent with *meta* protons H-6 and H-8 which are equivalent to those on A-ring of quercetin nucleus. This would also be indicative of the presence of a 5, 7-dihydroxyl A ring system in flavonol such as quercetin. Further analysis of the ^{1}H-nuclear magnetic resonance at δ 7.77 to 7.55 ppm could be indicative of the presence of a 3', 4'-dihydroxy functional groups in B ring in flavonol which is similar to phenolic aromatic protons in a quercetin B ring. This is further supported by positive chemical test for presence of phenolic groups and IR absorbance of a broad band at 3300 cm^{-1} suggesting presence of a hydroxyl (OH) bond. This is further supported by the fact that among the most significant and constantly encountered functional group in natural products are the phenol type –OH groups (Charisiadis *et al*., 2010; Matsuzaki *et al*., 2010). The ^{13}C-NMR chemical shifts at 164.78 and 161.59 ppm also suggest multiple aromatic C-OH bonds. In nature most phenolic compounds isolated from plants tend to be flavonoids and tannins.

Flavonoids in nature are usually yellow coloured. In the MS spectrum, m/z 435 value observed supports the identity of the compound as a flavonoid. Search in literature for phenolic compounds with such molecular weight suggest quercetin and related compounds which show a distinctive mass fragment of quercetin, (M+1) of (m/z 303). Furthermore, the observed ^{13}C-NMR chemical shifts are indicative of presence of a flavonoid. The compound gave a positive chemical test for flavonoids. Other chemical tests indicated presence of a glycosidic group. It is therefore possible that compound 2 is a glycoside derivative of quercetin.

The signal pattern in the aromatic region of the ^{13}C-NMR spectrum is indicative of three aromatic rings. In addition, the ^{13}C-NMR and APT spectra showed an anomeric carbon (δ_C 100.5), four oxymethines (δ_C 75.59, 73.72, 70.81 and 71.80) and an oxymethylene (δ_C 61.01). This was in addition to the carbonyl carbon at δC 178.13. All these data support three aromatic ring systems and presence of a sugar moiety attached to the skeleton. Comparison of ^{13}C-NMR of quercetin and that of compound 2 indicates the aglycone structure of compound 2 to be similar to that of quercetin (Tables 5.1 and 5.2). This was further supported by analysis of ^{13}C-NMR of the aglycone of a quercetin glycoside derivative from literature (Boligon *et al*., 2009; Matsuzaki *et al.*, 2010). The ^{1}H-nuclear magnetic resonance at δ 3.84 to 3.24 ppm could further suggest an aliphatic moiety which could be attributed to the protons on the glycoside. Search in literature confirms this inference (Kyriakou *et al.*, 2012). Further comparison of mass fragmentation pattern of compound 2 and quercetin indicate that the compound is a derivative of quercetin due to the presence of a base peak at m/z 303. The NMR and MS of quercetin and that of the suggested compound 2 are given in Table 5.1 and Table 5.2, respectively.

Table.5.1: ^{13}C-NMR of Quercetin, Compound 2 and Quercetin-3-*O*-α-L-rhamnopyranosylpyranoside

Carbon Atoms	Quercetin glycoside in δ (ppm) (Han *et al.*, 2004)	Compound 2 δ (ppm)	Quercetin-3-*O*-4-methyl-rhamnoside (sugar moiety) (Alfold *et al.*, 1980)
C_2	158.2	157.06	-
C_3	135.5	134.40	-
C_4	179.5	178.13	-
C_5	162.6	161.59	-
C_6	99.2	98.58	-
C_7	165.7	164.78	-
C_8	94.8	93.38	-
C_9	158.2	157.44	-
C_{10}	105.7	104.06	-
$C_{1'}$	123.4	121.52	-
$C_{2'}$	115.9	116.41	-
$C_{3'}$	145.6	144.41	-
$C_{4'}$	149.6	148.54	-
$C_{5'}$	117.6	114.70	-
$C_{6'}$	122.9	121.58	-
$C_{1''}$	104.7	100.75	-
$C_{2''}$	75.6	75.75	-
$C_{3''}$	74.9	73.72	-
$C_{4''}$	73.8	70.3	-
$C_{5''}$	77.0	77.19	-
$C_{6''}$	68.5	63.71	-
$C_{1'''}$	102.2	94.59	100.71
$C_{2'''}$	72.0	71.80	71.31
$C_{3'''}$	72.1	71.40	71.07
$C_{4'''}$	71.2	82.54	83.25
$C_{5'''}$	69.6	68.64	67.09
$C^{4'''}$ - OCH3	--	60.57	60.67
$C_{6'''}$	17.9	16.54	17.87

The ^{13}C-NMR shown in Table 5.1 illustrates that the aglycone carbon skeleton of quercetin is very similar to that of the isolated compound 2 (Han *et al*, 2004; Matsuzaki *et al*., 2010). Compound 2 was demonstrated to have a closely related structure to a quercetin glycoside previously isolated from *Japecanga* (*Smilax fluminensis*) leaves (Petrica *et al*., 2014) and from *Bauhinia longifolia* (dos Santos *et al*., 2014). These observations further support the proposed structure in to be a

flavonoid derived from quercetin.

The ^{13}C-NMR resonance at δ (ppm) 100.75 could be indicative of an anomeric carbon of a glucose moiety and was assigned to C_1". This is the carbon atom linking the sugar to the aglycone through the C-O-C bond at C_3. This was found to correlate well with similar assignment structures reported in literature (Kerhoas *et al.*, 2006; Boligon *et al.*, 2009; dos Santos *et al.*, 2014; Petrica *et al.*, 2014). However, ^{13}C-NMR and ^{13}C-APT spectra of compound 2 revealed more carbon atoms to be present. This led therefore to the conclusion that compound 2 had two sugar moieties attached to the aglycone. The 13C-NMR spectrum of this compound displayed a chemical shift at δ (ppm) at 193 which was not reflected in the 13C-APT spectrum of the same compound and was therefore considered an impurity which could have been due to a functionsl group in any of the sugar units.

The chemical shifts at δ (ppm) at 100.75 may suggest the presence of anomeric carbon of a glucose unit. The resonance at δ (ppm) 94.92 would also suggest presence of a second anomeric carbon linking the second sugar unit. The chemical shift up field at δ (ppm) 16.54 would be indicative of a methyl group in the sugar unit. This would be suggestive of a rhamnose sugar unit. The rhamnose unit could be further supported by the ^{1}H-NMR resonances at δ (ppm) 1.30-1.01. The chemical shift at δ (ppm) 60.57 would strongly suggest presence of a methoxy moiety attached to one of the sugar units. This was also supported by ^{1}H-NMR resonance at δ 3.80. In compound 2 it was proposed to be attached to C-4'" of the rhamnose sugar unit. The observations were hence indicative of presence of a disaccharide sugar attached to the aglycone made of glucose and rhamnose. The slightly up field resonances observed at the C4' of the glucose sugar unit could be associated with an α-orientation of the hydroxyl groups in the sugar moiety. On the other hand the lower chemical shift observed at C6" (63.71 ppm) could be as a result of β orientation in the molecule. The carbon resonances of the glucose and rhamnose moieties were assigned according to that of Alföldi *et al.*, 1980 and Han *et al.*, 2004, respectfully (Table 5.2) which was independent of the aglycone. The proposed structure would be as shown in. The proposed name would be quercetin-3-O-α-rhmnopyranosyl-4-methoxy (1→6)-β-D-glucopyranoside, trivial name rutin.

Table 5.2: ^{1}H and ^{13}C-NMR spectra data for quercetin, compound 2 and rutin

	Quercetin		Compound 2		Quercetin 3-*O*-β-rutinoside	
	Δ^{13}C	Δ^{1}H (J in Hz) (Guvenalp and Demirezer, 2004)	Δ^{13}C	Δ^{1}H (J in Hz)	Δ^{13}C	Δ^{1}H (J in Hz) (Han et al., 2004)
Aglycone						
2	157.3		157.06		158.2	
3	133.0		134.40		135.5	
4	177.4		178.13		179.5	
5	161.9		161.59		162.6	
6	98.5	6.17 (1H, *d*, J = 2.0)	98.58	6.17(1H, s)	99.8	6.10 (1H, *d*, J = 2.0)
7	164.1		164.78		165.7	
8	93.5	6.37 (1H, *d*, J = 2.0)	93.38	6.36 (1H, s)	948	6.26 (1H, *d*, J = 2.0)
9	156.3		157.44		159.1	
10	103.9		104.06		105.5	
1'	120.8		121.52		123.4	
2'	115.6	7.73 (1H, *d*, J = 2.0)	116.41	7.77 (1H, s)	115.9	7.70 (1H, *d*, J = 2.0)
3'	144.9		144.41		145.6	
4'	148.3		148.54		149.6	
5'	115.3	6.87 (1H, *d*, J = 8)	114.70	6.8 (1H, J = 8.5, d)	117.6	6.85 (1H, *d*, J = 8.0)
6'	121.3	7.62 (1H, *dd*, J = 2.0, 7.5)	121.58	7.61 (1H, m)	122.9	7.57 (1H, *d*, J=2.0)
1"			100.75	5.06 (1H, *J*=7.7, d)	104.7	5.10 (1H, *d*, J = 7.7)
2"			75.78		75.6	
3"			82.54		78.0	
4"			70.3		73.8	
5"			77.10		77.0	
6"			63.71		68.5	

Table 5.2 Shows the similarities of ^{1}H-NMR of compound 2 to that of quercetin and previously isolated quercetin-3-*O*-β-rutinoside.

The chemical shift of ^{13}C-NMR spectrum downfield at δ 178.13 ppm is indicative of C=O bond. This observation is further supported by the absence of a signal around

the same region in the APT experiment of ^{13}C (Appendix 4). Chemical shifts at δ 164.78 and 161.59 suggest multiple aromatic C-OH bonds (C7 and C5) which are indicative of presence of a flavonoid compound as shown in the proposed structure in. Chemical shifts ranging from 100.75 to 178.13 ppm are suggestive of a flavone type skeleton. Further examination of the mass spectrum indicates a proposed molecular formula of $C_{15}H_{11}O_7$, which is indicative of (M+1) fragment peak of quercetin. A fragmentation scheme of the compound gives a suggested sub-structure as shown in. The proposed sub-structure has close structural resemblance to a glycoside of quercetin (Figure 5.1).

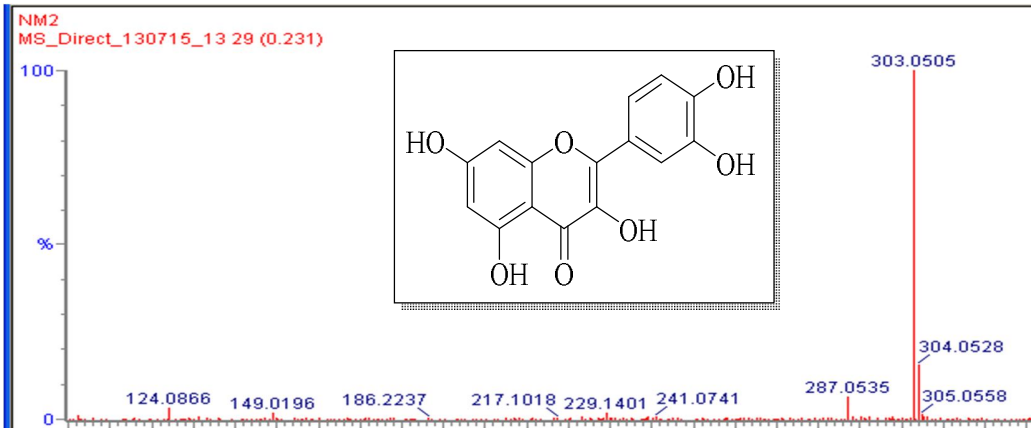

Figure 5.1 : Structure of quercetin as the base peak of fragmentation.

Combining the NMR and MS data strongly indicates that Compound **2** is probably the flavonoid glycoside rutin (quercetin-3-O-α-rhmnopyranosyl-4-methoxy (1→6)-β-D-glucopyranoside) whose chemical structure is shown in Figure 5.2.

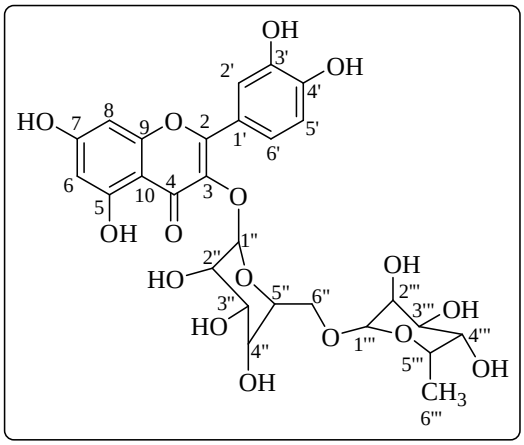

Figure 5.2: Proposed structure of Compound 2.

Search from literature indicated that a similar compound was isolated from *Bauhinia longifolia* (Bong) Steud where it is also known as guaijaverin but without the second sugar unit. The compound was reported to exhibit antivirus activity against alphavirus (dos Santos *et al*., 2014)**.** A structure very similar to compound 2 was earlier isolated from the leaves of *Morinda citrifolia* (Sang *at al*., 2001).

The mass spectrum of Compound 2 showed the fragment with the highest m/z 435.0947 which does not correspond to molecular formula. However, the molecular formula of the elucidated structure is $C_{27}H_{30}O_{16.}$ The discrepancy between m/z 435.0947 and the expected molecular ion suggests that the glycoside being highly labile may have undergone rapid fragmentation with loss of the outer sugar unit. Further fragmentation yielded the characteristic base peak of quercetin glycosides which is m/z 303.05. The proposed fragmentation pattern of quercetin glycoside was earlier reported in literature (Kerhoas *et al*., 2006). This was as demonstrated in Figure 5.3.

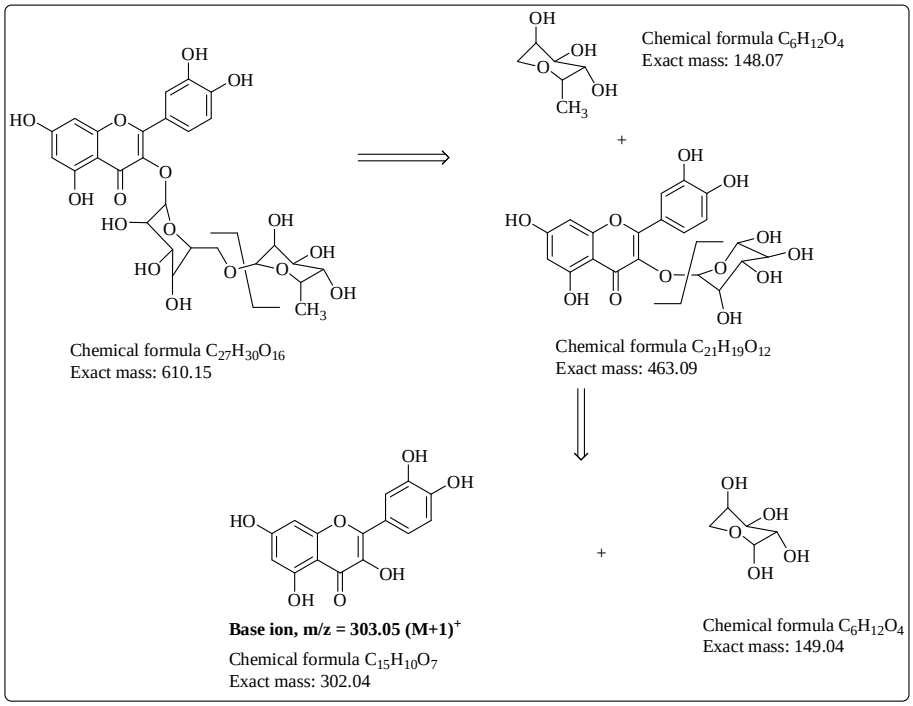

Figure 5.3: Fragmentation scheme of Compound 2 to yield the base peak ion.

In conclusion it would seem from the analyzed data for compound 2 and comparing with similar data as searched in literature, that compound 2 is a quercetin glycoside namely quercetin-3-*O*-α-rhmnopyranosyl-4-methoxy (1→6)-β-D-glucopyranoside. Further search in literature also support the above proposed fragmentation pattern with the molecule loss of a rhamnose unit (m/z 146) to give the observed peak at m/z 465 (M+1) which underwent further fragmentation by loss of a hexose unit (m/z 162) to yield the base peak m/z 303 (M+1). The base peak corresponds to that of the aglycone quercetin (Cuyckens and Claeys, 2004).

5.3 Structure elucidation of compound 3

The compound was obtained from the leaf methanol extract from preparative HPLC as a yellow amorphous compound. The compound turned ferric chloride solution green. This was therefore an indicaton that the compound was likely to be a phenolic glycoside; most likely a flavonoid.The following spectroscopic data was generated from the compound.

^{1}H-NMR (400 MHz, MeOD) δ, ppm : 7.54 (1 H, d, *J* =2.1 Hz), 7.49 (1 H, d, *J* =2.0

Hz), 7.47 (1 H, d, *J* =2.0 Hz), 6.93 (1 H, dd, *J*= 8.4, 1.9 Hz), 6.41 (1 H, d, *J* =1.8 Hz), 6.22 (1 H, d, *J* =1.8 Hz), 4.45 – 4.43 (1 H, m), 4.10 (1 H, dd, *J*= 5.6, 2.7 Hz), 3.96 – 3.90 (2 H, m), 3.83 – 3.77 (1 H, m), 3.33 (4 H, dt, *J*= 3.3, 1.6 Hz), 3.23 (1 H, d, *J* =2.0 Hz), 3.22 – 3.20 (1 H, m), 3.19 (1 H, d, *J*= 1.5 Hz), 3.18 (1 H, d, *J* =3.8 Hz).

^{13}C-NMR (101 MHz, MeOD) δ (ppm): 179.84(C-4), 165.85(C-7), 162.98(C-5), 159.08(C-2), 158.43(C-9), 149.71(C-4'), 146.2(C-3'), 134.81(C-3), 122.95(C-6'), 122.82(C-1'), 116.78(C-5'), 116.39(C-2'), 109.97, 107.99, 107.86, 105.57(C-1"), 104.73(C-10), 99.76(C-6), 94.67(C-8), 90.81(C-1'"), 88.89, 87.32(C-3'"), 78.05(C-5") 77.84(C-2'"), 77.26(C-2"), 77.19(C-5'"), 75.14(C-3"), 74.72(C-3'"), 70.96(C-4"), 66.96(C-4'"), 65.46(C-6"), 62.28(C-6'").

On close examination of the data generated for compound 3, it would appear to have the same basic nucleus carbon skeleton to that of quercetin as was observed to be the case for compounds 2 and 4. Just like compounds 2 and 4, compound 3 was obtained as yellow powder. In the ^{1}H-NMR spectrum of compound 3 of δ 7.54 (1 H, d, *J* 2.1), 7.49 (1 H, d, *J* 2.0), 7.47 (1 H, d, *J* 2.0), 6.93 (1 H, dd, *J* 8.4, 1.9) would indicate presence of a phenyl group. The signals at δ 6.41 (1 H, d, *J* 1.8), 6.22 (1 H, d, *J* 1.8) indicate presence of two tetra substituted phenyl groups. The ^{13}C-NMR spectrum indicates the presence three aromatic rings. The ^{13}C-NMR and APT spectra show presence of a carbonyl carbon (δ 178.70, C-4) and hydroxyl substituted carbons (δ 165.06(C-7), 161.83(C-5), 148.72(C-4') and 144.96(C-3') would all point to an aglycone chemical structure similar to that of quercetin. The signal at δ 105.43(C-1") tends to indicate presence of an anomeric carbon which would imply that the aglycone moiety was linked to a sugar. The presence of the sugar moiety tends to be supported by the carbon signals at δ 89.53 to 60.99. The chemical shifts could be assigned to the carbon atoms of the first sugar unit as follows; δ (ppm), 104.43, (C-1"), 75.8 (C-2"), 75.2 (C-3"), 71.17 (C-4"), 78.26 (C-5") and 62.49(C-6"). This chemical shifts could be associated to those of carbon atoms in glucose. This would be indicative of a glucose unit in the structure (Jou *et al.*, 2004). The remaining peaks could be attributed to another sugar which could most likely be glucose or a galactose moiety. This could be supported by the signal

at δ 65.67 which is indicative of an ether linkage at C-6 of the glucose. This further suggest that the sugar moiety not to be one unit but most likely to be a disaccharide made of hexose sugar units. The peaks at δ 90.81, 87.32 and 77.84 could be indicative of more than one carbon in β-orientation in the second glucose unit (Asui *et al*., 1973; Alföldi, *et al*., 1980; Hobley *et al*., 1996). The peak at δ 66.96 (C-4''') could be due to an α attachment of the function group to this carbon.

Thus from the above spectroscopic data and that presented on Table 5.4 below, the following chemical structure could be suggested for compound 3;

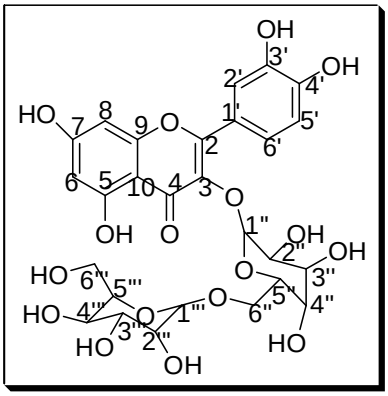

Figure 5.4: The proposed structure of compound 3.

The structure would be indicative of a diglycoside with the disaccharide sugar linked to aglycone skeleton of quercetin at C-3. Search from literature would suggest the structure as shown in Figure 5.5 to be quercetin 3-*O*-*β*-diglucoside, which was reported to be isolated from beans (Materska, 2008); pear skins (Lin and Harnly, 2008) and from jocote peels (Engels *et al*., 2012).

Table 5.3: ^{1}H-NMR and ^{13}C-NMR spectral data of compound 3.

Carbon	Quercetin glycoside, ^{13}C-NMR Jou *et al*., 2004, δ	Quercetin glycoside, ^{1}H-NMR Jou *et al*., 2004, δ	Compound 3 ^{13}C-NMR δ	Compound 3 ^{1}H-NMR δ	^{13}C-NMR of the sugar unit Usui *et al*., 1973
2	159.2	-	159.08	-	-
3	136.8	-	134.81	-	-
4	179.8	-	179.84	-	-
5	163.1	-	162.98	-	-
6	99.9	6.15 (br, s)	99.76	6.41 (s, *J* = 1.8 Hz)	-
7	165.9		165.84	-	-
8	94.8	6.26 (br, s)	94.67	6.22 (d, *J* = 1.6 Hz),	-
9	158.5	-	158.43	-	-
10	105.8	-	104.73	-	-
1'	122.9	-	122.82		-
2'	116.9	7.86 (d, J = 1.5 Hz)	116.29	7.54 (d, *J* = 2.1 Hz)	-
3'	146.4		146.2	-	-
4'	149.8		149.71	-	-
5'	116.5	6.80 (d, J = 8.4 Hz)	116.78	6.93 (dd, *J* = 8.4, 1.9 Hz)	-
6'	122.7	7.5 (dd, J = 8.4, 1.5 Hz)	122.95	7.49 (d, *J* = 2.0 Hz)	-
1"	103.9	-	105.57	-	-
2"	77.9	-	77.26	-	-
3"	75.8	-	75.14	-	-
4"	71.7	-	70.96	-	-
5"	75.5	-	78.05	-	-
6"	63.9	-	65.46	-	-
1"'	-	-	90.81	-	97.2
2"'	-	-	74.72	-	75.1
3"'	-	-	87.32	-	86.7
4"'	-	-	66.96	-	70.4
5"'	-	-	77.19	-	77.3
6"'	-	-	62.28	-	62.3

5.4. Structure elucidation of compound 4

The compound was obtained from the methanol extract of *Monanthotaxis parvifolia* leaves as a yellow amorphous powder. The compound turned ferric chloride solution green implying presence of phenolic moiety in the structure.

The compound gave the following spectroscopic data:

UV (MeOH) λ_{max}, nm: 258, 360.

IR (KBr) ν_{max} cm^{-1}: 3400(OH), 2395, 1650-1600

^{1}H NMR (400 MHz, MeOD) δ (ppm): 7.55 (d, *J* = 1.9 Hz, 1H), 7.50 (dd, *J* = 8.4, 1.9 Hz, 1H), 6.92 (d, *J* = 8.4 Hz, 1H), 6.40 (s, 1H), 6.22 (d, *J* = 1.6 Hz, 1H), 5.49 (s, 1H), 4.35 (d, *J* = 2.1 Hz, 1H), 3.97 – 3.87 (m, 2H), 3.57 – 3.49 (m, 2H).

^{13}C-NMR (101 MHz, MeOD) δ, ppm: 178.83(C-4), 164.92(C-7), 161.58(C-5), 157.99(C-2), 156.87(C-9), 148.60(C-4'), 144.72(C-3'), 133.89(C-3), 121.73(C-1'), 123.29(C-6'), 115.59 (C-2'), 115.00(C-5'), 108.17(C-1"), 104.72 (C-10), 98.51(C-6), 93.40(C-8), 86.63(C-4"), 81.99(C-2"), 77.33(C-3"), 61.17(C-5")

MS m/z: 465.19 (M+1), 445.11, 430.11, 374.97, 372.10, (371.10) (20%).355.07, 304.05, 303.05 (100%), 291.04, 252.53, 252.02 (70 %), 223.06, 186.95, 149.02, 141.95

Since compound 4 was also isolated from the leaf methanol extract of *Monanthotaxis parvifolia* similar arguments to those advanced in deriving the proposed structure of compound 2 were also applied to obtain its structure. Analysis of ^{1}H-NMR spectrum indicated a 5, 7-dihydroxylated pattern for ring A similar to that reported in literature (Morales-Escobar *et al*., 2007; Kyriakou *et al*., 2012). The ^{1}H-NMR chemical shifts at δ 7.55 to 6.23 resonances could be assigned to aromatic protons while that at δ 5.49 to 3.33 could be indicative of aliphatic protons which could be attached to a sugar unit.

The ^{13}C- NMR revealed a carbonyl carbon at δ 178.83. The chemical shifts at δ 164.92, 161.58 and 157.99 could be indicative of aromatic carbons bearing OH groups. This could be indicative of a flavone with 15-Carbon type skeleton. This has been further checked with structures reported in literature that are related to quercetin, where the molecule is reported to have several hydroxyl groups bound to the aromatic rings (Kerkoas *et al*., 2006; Matsuzaki *et al.*, 2010; Sokolova *et al*., 2012). The chemical shifts of carbon atoms in base fragment of compound 4 are compared to that reported in literature for quercetin where the carbon atoms attached to OH functional group resonate as depicted in Table 5.4**.**

Comparison of the ^{13}C-NMR of compound 4 to that reported in literature revealed presence of aromatic carbons which were assigned as follows, δ (ppm) (C8) 93.4, (C6) 98.51, (C5') 115.0, (C2') and (C6') 121.73. The chemical resonance at δ (ppm) 178.73 was assigned to C4 as a carbonyl carbon. The non-hydrogenated carbons were assigned as follows, δ (ppm) (C3) 133.89, (C5) 161.58, (C7) 164.92, (C9) 156.87, (C10) 104.72, (C1') 108.17, (C3') 144.72 and (C4') 148.60. This corresponds to similar values reported in literature for quercetin-3-*O*-α-arabinofuranoside whose ^{13}C-NMR chemical shifts are shown in Table 5.4 (Chang *et al*, 2009).

Table 5.4: ^{13}C-NMR of compound 4 compared to that of quercetin.

C	Compound 4 Experimental δ	Compound 4 ^{1}H-NMR δ	^{13}C-NMR Quercetin -3-O-α-arabinofuranoside (Chang *et al.*, 2009)	^{1}H-NMR Quercetin -3-O-α-arabinofuranoside (Chang *et al.*, 2009)
2	157.99	-	157.1	-
3	133.89	-	134.1	-
4	178.83	-	178.4	-
5	161.58	-	161.9	-
6	98.51	6.22 (1H, *J*-1.6 Hz, d)	99.4	6.20 (1H, d, J = 2.0 Hz)
7	164.92	-	164.1	-
8	93.40	6.40 (1H, s)	94.3	6.40 (1H, d, J = 2.0 Hz)
9	156.87	-	157.6	-
10	104.72	-	104.7	-
1'	121.73	-	122.4	-
2'	115.59	7.55 (1H, *J*=1.9 Hz, d)	116.2	7.48 (1H, d, J = 2.0 Hz)
3'	144.72	-	145.8	-
4'	148.60	-	149.2	-
5'	115.00	6.92 (1H, d, *J* = 8.4 Hz)	116.3	6.85 (1H, d, J = 8.0 Hz)
6'	123.29	6.9 (1H, d, *J* = 8.3 Hz)	121.7	7.57 (1H, dd, J = 2.0 and 8.4 Hz)
C_1"	108.17	-	108.6	-
C_2"	81.99	-	82.8	-
C_3"	77.33	-	77.8	-
C_4"	83.61	-	86.6	-
C_5"	61.17	-	61.4	-

The ^{1}H and ^{13}C-NMR chemical shifts for the quercetin carbon skeleton of compound 4 (Table 5.3) were comparable to those from literature as reported in the work of; G¨uvenalp and Dem_irezer, 2004; Jou *et al*., 2004; Boligon *et al*, 2009; Charisiadis *et al*., 2010; dos Santos *et al*., 2014; Petrica *et al*., 2014; where they demonstrated the –OH groups NMR spectral region in natural products. The ^{13}C-

NMR spectrum of the sugar moiety of compound 4 has only five signals (62.72, 78.87, 83.45, 88.18 and 104.98. The APT spectrum shows these signals to be due to methine carbons except for that at 62.72 which is due to a methylene carbon. The signals at 83.45 and 88.18 are typical of an arabinofuranoside. Taking the aglycone to be quercetin as identified in the preceding section, the resulting compound is quercetin-3-O-α-arabinofuranoside, also known as avicularin. This assertion is further supported by ^{1}H-NMR data. The singlet at 5.49 integrating for 1 proton corresponds to the carbon attached to the anomeric carbon. When observed using high resolution NMR spectrometers (>500 MHz), this proton would appear as a doublet (J = 1 Hz) because of splitting by H2". The 'doublet' at 4.35 is due to H3" which would be expected to appear as a triplet. H2" and H4" overlap to give the multiplet at 3.95-3.88 while the methyene protons appear at 3.54-3.52.

The mass spectrum showed a molecular ion $[M+1]^{+}$, m/z 434.19 and a proposed molecular formula of $C_{20}H_{18}O_{11}$. The base ion was similar to that of compound 2 with a mass to charge ratio (m/z) of 303.05 which had a100 % abundance. This would suggest a very similar basic carbon skeleton structure to that of quercetin, implying the presence of a C6-C3-C6 flavonoid ring structure in the compound. This was supported by the ^{13}C- NMR of quercetin and that of compound 2 and compound 4 as depicted in Table 5.1 and Table 5.4. Further analysis based mainly on the MS- data that is the molecular ion and molecular formula tentative structure was suggested as shown in Figure 5.4.

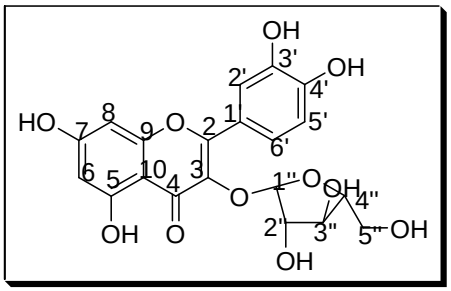

Figure 5.5: Possible structure of Compound 4.

Proposed name for compound 4: Quercetin-3-*O*-α-arabinofuranoside.

The molecular mass of the suggested compound is m/z 464. Search in literature of such compounds revealed that quercetin-3-*O*-α-arabinofuranoside flavonol is ubiquitous in nature previously isolated from *Bistorta manshuriensis* (Chang *et al*., 2009). In *Bauhinia longifolia* (Bong.) the flavonol with similar molecular mass was isolated as a mixture of isomers made up of isoquercetin which is quercetin-3-*O*-β-glucoside and hyperin which is the quercetin-3-*O*-β-galactoside (dos Santos *et al*., 2014). Further search in literature suggested molecular ion m/z 464 which is indicative of a quercetin hexose molecule (Shetty *et al.,* 2011). The ^{13}C-NMR at C-1" was comparable to that reported in literature for anomeric carbons (Alföldi *et al*., 1980). The chemical shift at C-5" was lower than that reported in literature and this could be due to the presence of a methoxy moiety attached to this carbon (Alföldi *et al*., 1980; Kim *et al*., 2004).

The UV absorption at λ_{max} 258 nm and 360 nm is indicative of presence of chromophores in the structure. This correlates well with the observation that compound 4 was coloured. The presence of yellow colour is reported to be found in flavonoids (Evans, 1996). The infrared band at 3400 cm^{-1} and a 1650-1600 cm^{-1} would suggest presence of OH and C=O functional groups respectively.

In general flavonoids are among the most numerous groups of natural products and are important to humans because most of them are physiologically active (Harborne *et al*., 1975; Evans, 1996; Riccio *et al*., 2003; Fidelis *et al*., 2012). Flavonoids exist in plants as secondary metabolites as aglycones or glycosides. A general structure of a flavonoid molecule is presented in Figure 5.6. The structure consists of 3 major rings (A, C and B). The double bonds in the flavonoids skeleton cause them to absorb visible light, and as a result, give them the yellow colour.

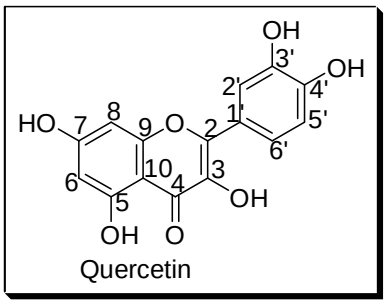

Figure 5.6 : Structure of quercetin

5.5 Structure determination of compound 5

The compound was isolated from leaf methanol extract using HPLC as a yellow amorphous compound. The compound generated the following spectroscopic data;

^{1}H NMR (400 MHz, MeOD) δ, ppm: 7.36 (s, 1H), 7.35 – 7.32 (m, 1H), 7.33 – 7.31 (m, 1H), 6.94 (s, 1H), 6.93 – 6.91 (m, 1H), 6.38 (s, 1H), 6.21 (s, 2H), 5.37 (s, 1H), 4.35 (s, 1H), 4.25 (s, 1H), 3.90 (s, 1H), 3.75 (s, 1H), 3.53 (s, 1H), 3.44 (s, 1H), 3.35 (s, 9H), 2.67 (s, 1H), 1.30 (s, 1H).

The structure of compound 5 is yet to be fully resolved. On analyzing further the ^{13}C-NMR for Carbon atoms at positions 2 to 6[1] for compounds 2, 3 and 4 as the observations presented in Table 5.4, the compound is also most likely to be a flavonoid with a common aglycone structure to that of carbon 2, 3 and 4. The small differences in δ could be attributed to the attached functional groups. It is therefore reasonable to conclude that compound 5 is most likely a flavonoid.

In general the UV spectra of flavonoids consist of two major absorption maxima bands at 240-285 nm and 300-400 nm (Harborne *et al*., 1975). Many articles have reported on the application of NMR spectroscopy in the structural elucidation of flavones. Indeed some of these have been studied to unravel the proton and carbon shifts of the isolated compounds of this class of natural products (Sahin *et al*., 2004; Moon *et al*., 2005; Park *et al*., 2006; Shafagat, 2008; Fidelis *et al*., 2012).

Hydroxyl groups are almost always present in the flavonoids and are usually attached to the B rings at positions 3' and 4' and to 5 and 7 positions of the A ring (Harborne *et al*., 1975; Moon *et al*., 2005; Shafaghat, 2008). These general characteristics were found to correlate with the characteristics of compounds 2, 3 and 4. The experimental data and the comparison of information from literature indicate the structures of the three compounds are likely to be quercetin-3-*O*-rutinoside for compound 2 and quercetrin (quercetin-3-*O*-rhamnoside) for compound 4 and quercetin-3-*O*-diglucoside for compound 3 (Figure 5.5). Compound 2 was previously isolated from *Bidens pilosa* where it was shown to have antioxidant activity (Chiang *et al*., 2004). Compound 4 was reported to be

present in the leaves of *Scutia buxifolia* Reiss (Boligon *et al*., 2009). Although the structure elucidation of the three compounds is not exhaustive, this is the first time these compounds are reported to be isolated from *Monanthotaxis parvifolia*.

In conclusion, three out of the seven compounds isolated from the methanol extract of *Monanthotaxis parvifolia* were found to be quercetin glycosides. The proposed chemical structures of compounds 2, 4 and 3 are quercetin 3-*O*-(6"–*O*-α–L-rhamnopyranosyl) -4-methoxy -β-D-glucopyranoside also known as quercetin 3-*O*-rutinoside or rutin and quercetin 3-*O*-α-L-rhamnoside and quercetin 3-*O*-dihydroglucoside respectively. Compound 3 was found to bear an aglycone moiety linked to two hexose sugars, but only the first sugar unit was tentatively identified as glucose unit. The second sugar unit was thought to be a hexose unit but the ^{13}C-NMR chemical shifts could not be assigned to it (Usui *et al*., 1973).

CHAPTER SIX

6.0 RESULTS OF ANTIPLASMODIAL, TOXICOLOGICAL AND PHARMACOLOGICAL ACTIVITIES

6.1 *In vitro* antiplasmodial activity of *Monanthotaxis parvifolia*

Antiplasmodial activity of *Monanthotaxis parvifolia* extracts and HPLC isolated compounds were tested against chloroquine-sensitive *Plasmodium falciparum* (D_{10}) strain and chloroquine-resistant *Plasmodium falciparum* (Dd_2) strain and the results are as tabulated in Table 6.1.

Table 6.1: Antiplasmodial activity of *Monanthotaxis parvifolia*.

Fraction/Isolate	IC_{50} µg/ml	
	D_{10}	**Dd_2**
Ethyl acetate fraction (L)	5.58	nd
Leaf water extract (L)	21.98	nd
Stem methanol extract (S)	4.023	nd
compound 2 leaf methanol extract	20.44	38.07
Compound 4 leaf methanol extract	24.93	29.8
Compound 5 leaf methanol extract	10.85	23.95
Chloroquine*	4.22	111.8*

*The chloroquine (CQ) is the reference standard and the concentration is expressed in ηg/ml. L-leaf; HMPL- HPLC prepared isolates of *Monanthotaxis parvifolia*; S-stem; MeOH-Methanol; nd- not determined.

The concentration at which growth of the *Plasmodium falciparum* parasites was inhibited by 50 % (IC_{50}) was estimated by interpolation from data using Graph Pad

Prism 4.0 software. The IC_{50} values ≤ 50 µg/ml for the extracts and IC_{50} values ≤ 25 µg/ml for isolates, respectively were considered active (O'Neill *et al.,* 1985; Bickii *et al.*, 2007; Osario *et al.,* 2007; Ramalhete *et al.*, 2008).

The ethyl acetate, methanol and water crude extracts of *Monanthotaxis parvifolia* exhibited high to moderate antiplasmodial activity with IC_{50} between 4.02 and 21.98 µg/ml. The fractionation of methanol leaf extract on HPLC generated the seven fractions which on further purification produced the seven compounds which were as depicted in Figure 4.2. Out of the seven compounds, three of them [compound 2 (quercetin 3-*O*-rutinoside), 4 (quercetin 3-*O*-α-L- arabinofuranoside) and 5] were tested for antiplasmodial activity. All of the three compounds exhibited moderate activity (IC_{50} < 25 µg/ml). These findings on these compounds are reported for the first time. A graphical presentation of antiplasmodial activity of chloroquin as the positive control is shown in Figure 6.1.

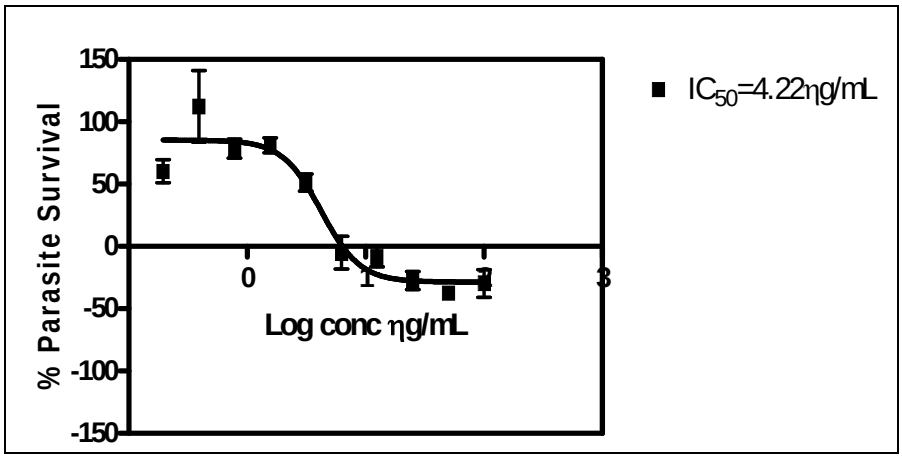

Figure 6.1: Antiplasmodial activity of chloroquine (CQ) against *Plasmodium falciparum* D10 strain.

The IC_{50} is expressed as ηg/ml.

Antiplasmodial activity of water leaf extract against the chloroquine sensitive D_{10} strain is shown in Figure 6.2. The extract exhibited moderate activity against the parasites. This finding is important in that most herbal preparations are often prepared as water decoctions (Muregi *et al.,* 2007; Mutai *et al.,* 2008; Titanji *et al.,* 2008; Nguta *et al.,* 2011).

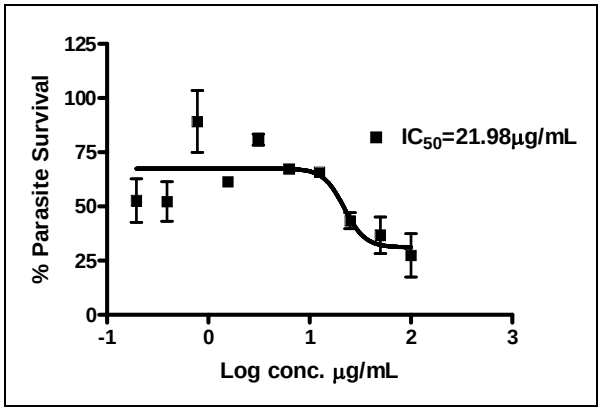

Figure 6.2: Antiplasmodial activity of water leaf extract against *Plasmodium falciparum* D10 strain.

However ,it was also noted that methanol extract from the stem had a higher activity when compared to that of water extract I(C_{50} of 4.026 µg/ml) (Figure: 6.3). This could be as a result of presence of active constituents of intermediate polarity which were more soluble in methanol. In spite of this observation, further fractionation of extracts was done on the methanol leaf extract which had a higher yield of 9.03 % and also being the part of the plant most commonly used in traditional medicine.

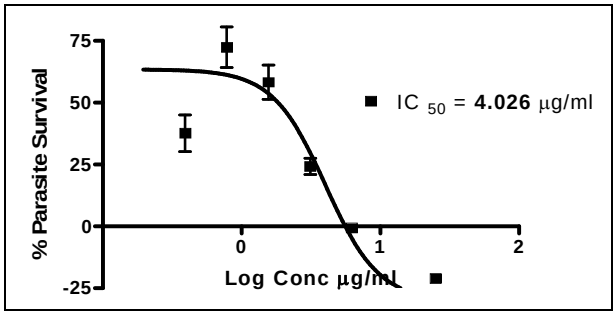

Figure 6.3 : Antiplasmodial activity of methanol stem extract against *Plasmodium falciparum* D10 strain.

On comparing the antiplasmodial activity of methanol leaf extract to that of ethyl acetate fraction partitioned from methanol extract, the ethyl acetate fraction showed slightly lower activity than that of methanol stem extract (IC_{50} of 5.58 µg/ml) (Figure 6.4). The variation in activity could be due to the presence of more constituents in methanol extract which exhibit synergism. Upon partitioning using

ethyl acetate some of those constituents are lost and hence the lower activity. This is type of synergistic activity was reported earlier in *Artemisia annua* used in traditional medicine for treatment of malaria (Cui and Su, 2009; Ferreira *et al*., 2010).

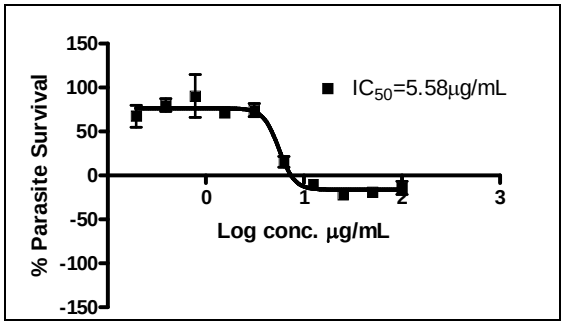

Figure 6.4: Antiplasmodial activity of ethyl acetate fraction against *Plasmodium falciparum* D10 strain.

Upon testing the phytochemical isolates for the antiplasmodial activity against the chloroquine sensitive strain, three of them showed activity less than 25 µg/ml (Table: 6.1).

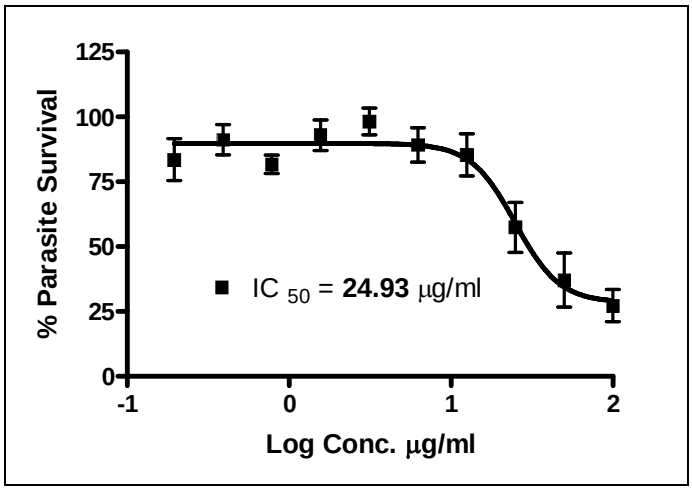

Figure 6.5: Antiplasmodial activity of compound 4 against *Plasmodium falciparum* D10 strain.

The methanol leaf isolates 2, 4 and 5 were also tested against the chloroquine

resistant *Plasmodium falciparum* (Dd2) strain. The results are presented in Table 6.1. The results clearly indicate that the antiplasmodial for both the chloroquine sensitive and resistant strains do not vary significantly. This is in contrast to the results obtained with chloroquine which differ significantly (4.03 ηg/ml versus111.8 ηg/ml). This represents almost a hundred fold difference. These findings indicate that the isolates are active both against the chloroquine sensitive and resistant strains albeit having lower activities.

Previous work on plants in the Annonaceae family had been reported to exhibit antiplasmodial activity (Sidel *et al*., 2000; Osario *et al*., 2007; Kihampa *et al*., 2009).The activity could be attributed to the presence of quercetin-based compounds. The antiplasmodial activity in plants due to the presence of quercetin-based molecules was also reported in *Artemisia annua* and other plants (Lehane and Saliba, 2008; Ferreira *et al*., 2010), however it is the first time it is reported on *Monanthotaxis parvifolia*.

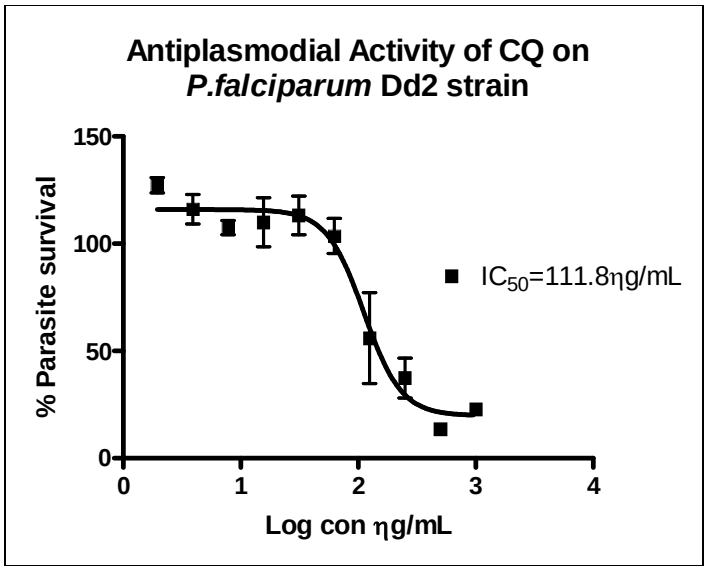

Figure 6.6: Antiplasmodial activity of chloroquine against *Plasmodium falciparum* Dd2 strain.

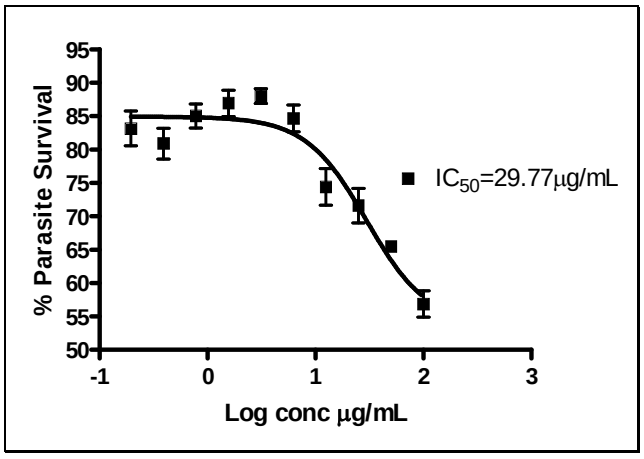

Figure 6.7: Antiplasmodial activity of compound 4 against *Plasmodium falciparum* Dd2 strain.

The antiplasmodial activity associated with the quercetin based molecules however raises some questions since quercetin and related flavonoids are known for their antioxidant activity and free radical scavenging activity. The antiplasmodial activity could be due to the fact that quercetin and other flavonoids have indirect antiplasmodial activity since they are not able to get access to the parasites' food vacuole. This could be due to their phenolic nature which renders them acidic as compared to some antiplasmodial compounds such as chloroquine which are basic in nature and therefore can easily gain access to the parasites' food vacule (Rasoanaivo *et al.*, 2011). This is further supported by the report that common dietary flavonoids inhibit the growth of the intraerythrocytic malaria parasite (Lehane and Saliba, 2008).

6.2 Cytotoxicity activity of *Monanthotaxis parvifolia* extracts

The cytotoxicity results of the isolated compounds against Chinese Hamster Ovary (CHO) cells are shown in Table 6.2. Emetine was used as a positive quality control standard to monitor the experimental conditions and showed IC_{50} values within the acceptable range of (40-60 ηg/ml**).**

Table 6.2: Cytotoxicity *of Monanthotaxis parvifolia* against Chinese Hamster Ovary cells

Fraction/ Isolate	Antiplasmodial activity (strain D_{10}, IC_{50}, µg/ml)	Cytotoxicity (IC_{50}, µg/ml)	Selectivity Index (SI)
Chloroquine	4.22	111.8	26.5
Fraction 2 leaf methanol extract	20.44	>100	>10
Fraction 4 leaf methanol extract	6.64	22.43	3.4
Fraction 5 leaf methanol extract	10.85	Nd	

Selective index (SI) has no units. Fractions 2-5 refer to fractions isolated from methanol leaf extract by use of preparative- HPLC. The fractions translated to compounds 2-5 on purification. Nd - not done.

The selective index is calculated by dividing the IC_{50} value of the CHO cell line with the IC_{50} value of the *Plasmodium falciparum.* The selective index of fraction 2 leaf methanol extract was greater than 10. Selective index values greater than 10 imply that the *Monanthotaxis parvifolia* extracts had selective inhibitory effect on the growth of the parasites and not on the normal cells. This is the first time this test has been reported on compounds from *Monanthotaxis parvifolia*. Antiplasmodial screening and cytotoxicity testing of the three isolated compounds indicated that all three compounds had good as well as selective antiplasmodial activity except for compound 4 (Tables 6.1 and 6.2) (O'Neill, 1985). Thus although the isolated compounds exhibited moderate activity against the *Plasmodium falciparum* parasite, they could be investigated further based on structure -activity- relationship modifications with the aim of improving antiplasmodial activity but retaining the low toxicity to the host cells (Lai *et al,* 2013).

6.3 Pharmacological Activity of *Monanthotaxis parvifolia*

6.3.1 Glycaemic effect of *Monanthotaxis parvifolia*

The crude methanol leaf extract of *Monanthotaxis parvifolia* was observed to have negligible effect on glucose metabolism in Wistar rats as shown in Figure 6.8. The observation that *Monanthotaxis parvifolia* extract has no effect on the glucose metabolism is very significant. This is of the observation that patients suffering from other chronic conditions such as diabetes and hypertension may also contract malaria. This would therefore imply that crude extract of *Monanthotaxis parvifolia* would not be contraindicated in the treatment of malaria in such patients. This was also an important observation as diabetes is reported to increase the risk to malaria infection (Danquah *et al*., 2010).

Monanthotaxis parvifolia is reported to be used in traditional medicine to alleviate various ailments; this is the first time this kind of work is reported on the plant. Although the effect of *Monanthotaxis parvifolia* on glucose metabolism does not seem to be directly related to the treatment of malaria, it is still important as it validates the use of the plant in traditional medicine in treating ailments related to malaria (Ramalhete *et al*., 2008).

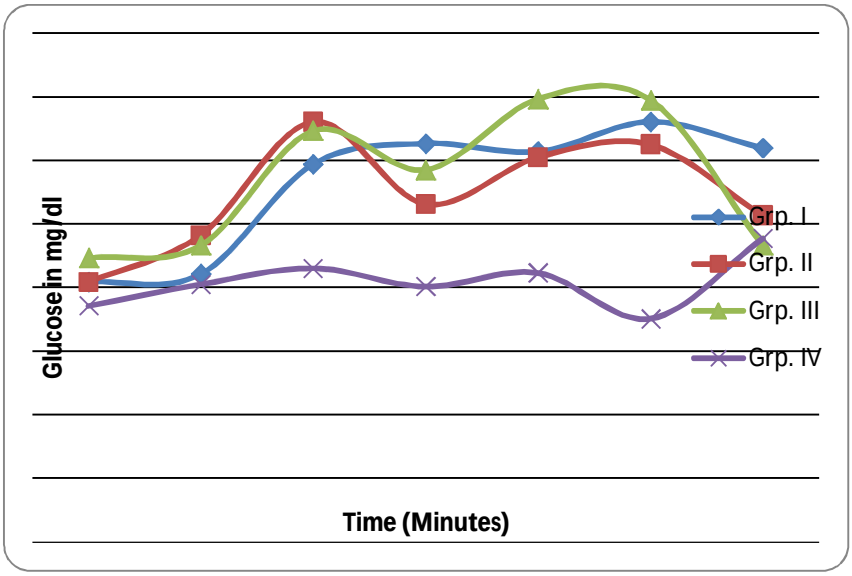

Figure.6.8: Glycaemic effect of *Monanthotaxis parvifolia* leaf extract.

Group I was negative control that received normal saline; Group II and III received test samples at doses of 500 and 1000 mg/kg body weight of *Monanthotaxis parvifolia* methanol leaf extract respectively; Group IV was the positive control which received metformin.

6.3.2 Analgesic effect of *Monanthotaxis parvifolia* leaf and stem extracts

Observations from tail-flick test showed that the leaf chloroform and methanol extracts and stem methanol extract of *Monanthotaxis parvifolia* at a dose of 200 mg/kg increased the pain threshold in mice after 30 min post treatment compared to normal saline. The effect was small compared to 10 mg/kg dose of morphine injection which served as the positive control (Table 6.3).

The tail-flick test is used for determining the antinociceptive effect of pharmacological agents (D'Amour and Smith, 1941). The tail-flick assay employs heat as the noxious stimulus. This noxious heat stimulation of the tail results in a simple nociceptive response that is a flick of the tail away from the heat source. The reflex is a spinally mediated flexion. The reflex allows the test to be carried on lightly anaesthetized animals. This test measures the complex feedback to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity (Ullah *et al*., 2014). The method is considered to be selective for the medicines acting centrally. The test is thus normally used to

test for compounds which can be used to treat acute pain. This was therefore considered to be the correct model to test for the analgesic effect of *Monanthotaxis parvifolia* for its use in traditional medicine in treatment of malaria related ailments. This is in view of the fact that pain experienced in malaria is usually of the acute form.

The results go further to validate use of plants in traditional medicines, where the plant is used to treat not only the direct ailment but other related symptoms as well (Fowler, 2006; Okokon *et al*., 2012).

Table 6.3: Analgesic effect of *Monanthotaxis parvifolia* on mice.

Treatment	Reaction time after administration		
	30 min	60 min	90 min
Normal saline	3.80±0.37	4.40±0.68	3.40±0.24
Methanol leaf extract	5.32±1.15	4.89±0.91	4.16±0.91
Chloroform leaf extract	5.10±0.56	5.21±0.75	4.33±0.52
Methanol stem extract	4.49±0.56	4.15±0.87	5.40±0.91
Morphine	12.38±0.44	11.56±0.34	10.52±0.11

Values are expressed as mean± standard error of mean (SEM) (n=6).

6.3.3 Antipyretic effect of *Monanthotaxis parvifolia* leaf extract

The antipyretic effect of *Monanthotaxis parvifolia* methanol leaf extract was tested by use of Brewer's yeast-induced hyperpyrexia in mice. Statistical analysis using general linear modeling indicated that aspirin caused a significant change in temperature compared to normal saline $p< 0.05$. The results are shown in Figure 6.9.

The antipyretic effect due to the methanol leaf fraction of *Monanthotaxis parvifolia* was not significant compared to that of aspirin, $p \geq 0.05$. However it still caused a decrease which was still lower than that of normal saline.

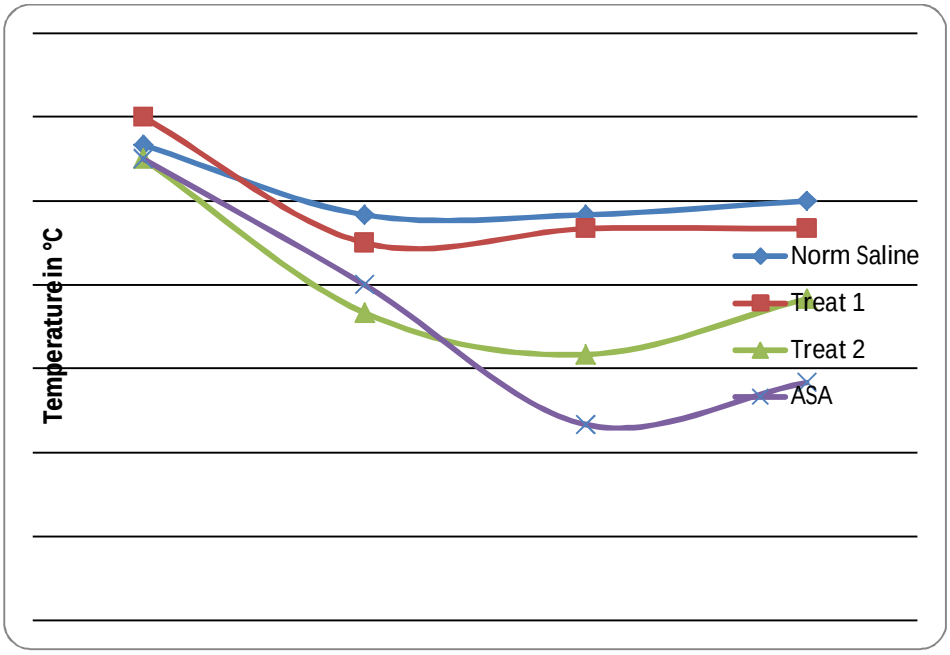

Figure 6.9 : Antipyretic effect of *Monanthotaxis parvifolia* on mice.

Normal saline, Group 1 and 2- *Monanthotaxis parvifolia* leaf methanol extract at 500 mg/ml and 1000 mg/ml dose respectively; ASA-aspirin.

Since fever is the first main symptom to appear in malaria, the observation that the *Monanthotaxis parvifolia* leaf methanol extract cause a decrease in body temperature would suggest that patients suffering from malaria get some relief when they take the decoction.

6.3.4 Conclusion

The results of this study give scientific evidence on the use of *Monanthotaxis parvifolia* traditionally for the treatment of symptoms associated with malaria. The extracts and some of the phytochemical isolates have been shown to have antiplasmodial activity *in vitro*. Three of the isolated compounds have been structurally characterized and shown to be quercetin glycosides. This would therefore indicate that the antiplasmodial activity of *Monanthotaxis parvifolia* could

be due to the presence of quercetin-based flavonoids. This is not the first time flavonoids have been shown to demonstrate antiplasmodial activity but reports from literature support the observation (Cimanga *et al*., 2008; Lehane and Saliba, 2008; Kerubo *et al*., 2013). Some of the flavonoids have been shown to potentiate the antiplasmodial activity of other compounds present in a plant extract or herbal preparation (Elford *et al*., 1987; Ferreira *et al*., 2010).

CHAPTER SEVEN

7.0 DISCUSSION, CONCLUSION AND FURTHER WORK

7.1 Discussion

Traditional medicines are a potential rich source of new medicinesagainst malaria considering natural products and their derivatives are reported to contribute more than 50 % of all medicines in modern therapeutics (Fabricant and Farnsworth, 2001; Batista *et al*., 2009; Fidock, 2010; Kingston, 2011; Cragg and Newman, 2013; Pan *et al*., 2013;14). Plants have made a tremendous contribution towards discovery of new medicines and they continue to be an important source not only for antimalarial drugs but also for other diseases (Cragg *et al*., 1997; Fabricant and Farnsworth, 2001; Cordell, 2002; Gurib-Fakim, 2006; Ansari and Inamdar, 2010; Ginsburg and Deharo, 2011; Kingston, 2011; Cragg and Newman, 2013.) History shows that plants have been an important source of medicines against many diseases and with respect to malaria. Typical and prominent examples of such medicines are quinine and artemisinin which are now the mainstay of the treatment of severe malaria worldwide and the artemisinin derivatives in combination with a second antimalarial medicine are now at the heart of the World Health Organization strategy to control malaria globally (Hobbs and Duffy, 2011; Ratsimbasoa *et al*., 2012, Wells, 2011; WMR, 2015).

The use of complementary and alternative medicine has had an upsurge in the last few years. This could be as per the argument that biologically- derived secondary metabolites and synthetic compounds derived from them work well as medicines than the randomly synthesized medicines (Ginsburg and Deharo, 2011; Willcox *et al*., 2012; Pan *et al*., 2013; van Andel and Carvalheiro, 2013).

The plant *Monanthotaxis parvifolia* was investigated for its antimalarial activity based on folklore use. Search from literature strongly suggests that the identification of medicinal plants for further research should be through ethno botanical studies. On this basis *Monanthotaxis parvifolia* was selected and investigated for its antiplasmodial activity (Newman and Cragg, 2007; Willcox *et al*., 2011).

The plant was collected when in flower and fruit and was identified and botanically authenticated at the East Africa Herbarium, National Museums of Kenya. This was important considering that proper plant identification is one of the key steps in the study of plants (Liu and Wang, 2008). The plant was studied for its general macroscopic characteristics and microscopic features particularly of the leaf. The macroscopic features of the leaf were observed to broadly correspond with the description given in literature except for the following noted features. In literature the leaves are reported to be arranged opposite each other but in this study they are noted to be arranged alternately. The leaves were also noted to have a characteristic aromatic smell which was confirmed by the presence of oil glands on a transverse section of the leaf lamina .However some *Monanthotaxis* species are reported to have essential oils (Fournier *et al.*, 1997; Boyom *et al.*, 2011). Another notable finding is that the fruits had the monocarps in a cluster displayed either as one, two or maximum three articled monocarps which were clearly ellipsoid in shape. This feature is crucial for identification of the *Monanthotaxis parvifolia* from the other species as they are very similar in morphology and can be easily confused one for the other. These morphological observations on *Monanthotaxis parvifolia* are being reported for the first time.

Microscopic study of the leaf demonstrated the presence of covering trichomes, heavy presence of lignified fibres around the midrib, the oil glands and the reticulate venation which was also lignified. The arrangement of the cells around the stomata was observed to be paracytic. These were noted to be characteristic features for this plant. This is the first time a microscopic study of *Monanthotaxis parvifolia* leaf is being reported.

The preliminary screening phytochemical tests indicated presence of phenolic compounds mainly tannins and glycosides. The results tallied well with the observation that the isolated compounds were quercetin glycosides which were postulated to be responsible tor the antiplasmodial activity of the plant. This is the first time the phytochemical results for *Monanthotaxis parvifolia* are being reported.

The crude extracts of *Monanthotaxis parvifolia* exhibited antiplasmodial activity

against the chloroquine sensitive *Plasmodium falciparum* D_{10} strain at 5.58 µg/ml. The activity exhibited by the extracts was comparable with other plants reported to have the antiplasmodial activity in the Annonaceae family. For example the extracts from *Annona muricata* and *Annona squamosa* were reported to have good antiplasmodial activity which ranged from 1 to 10 µg/ml (El Tahir *et al*., 1999; Bidla *et al*., 2004; Nguyen-Pouplin *et al*., 2007). This would support the use of the total plant extract for the treatment of malaria and related symptoms in traditional medicine.

This study provides the first report on antiplasmodial activity of isolated compounds from *Monanthotaxis parvifolia.* The antiplasmodial activity of the isolated compounds was however lower than that of the extracts. This was noted not to be similar to some reports where the isolated compounds exhibit stronger antiplasmodial activity than the plant extracts (Kuria *et al*., 2002; Chukwujekwu *et al*., 2009; Jansen *et al*., 2012). There could be many reasons advanced to explain the weak antiplasmodial activity of the isolated compounds. This could have been that the compounds exerted synergistic effect in the total extracts as for many cases reported in literature (Elford *et al*., 1987; Liu *et al*., 1989; Gathirwa *et al*., 2008; Cui and Su, 2009; Ferreira *et al*., 2010; Ginsburg and Deharo, 2011; Rasoanaivo *et al*., 2011). It could also be that the compounds which were in large quantities were less active than those in small quantities and hence masked their activity. Also compounds in large quantities have a higher chance of being extracted, isolated and purified at the expense of the compounds in small quantities. If the purified compounds are not pharmacologically active or have low activity, the plant may be reported as lacking activity. This is the more reason why total extracts must be screened as a policy.It could also be that the compounds require to be metabolized for them to exert the antiplasmodial activity. This was reported for example to have been the case in the use of *Quassia amara* leaf tea in treatment of malaria where it was found to be more active *in vivo* than *in vitro* (Bertani *at al.,* 2007).

Toxicity screening of the isolated compounds on Chinese Hamster Ovary (CHO) cells, exhibited no appreciable effect on the growth of the cells implying that phytochemical isolates have selective inhibitory effect on the malaria parasites and not on the normal cells. This provides scientific evidence of the safety of *Monanthotaxis parvifolia*. This is important as there are cases where the plant extracts or the isolated compounds are active *in vitro* but they can not be used *in vivo* because of their toxicity as in the case of quassinoids (Rasoanaivo *et al*., 2011; Verma *et al*., 2011; Wong *et al*., 2011; Bussmann, 2013; Pan *et al*., 2013).

Since malaria presents with symptoms such as joint pains and fever, medicines with analgesic and antipretic effects would significantly enhance the therapeutic outcome. *Monanthotaxis parvifolia* was therefore screened for the analgesic and antipyretic effects. The plant extracts exhibited both analgesic and antipyretic activity though weak compared to that of the respective positive controls (morphine and aspirin). The *M. parvifolia* decoctions are reported to be used in folklore to treat fever and pain (Beentje, 1994; Fowler, 2006; Muthaura *et al*., 2007; Yeboah-Antwi *et al*., 2010; Lima *et al*., 2011; Taiwe *et al*., 2011). The results therefore provide the first documented scientific rationale for the use of decoctions from *Monanthotaxis* species in alleviating pain and fever in folklore.

Monanthotaxis parvifolia was also found to have no effect on glucose metabolism implying that people with chronic illnesses such as diabetes could be treated for malaria with the *Monanthotaxis parvifolia* decoction (Danquah *et al*., 2010). This is the first time this finding is reported on the plant under the study.

The compound β-sitosterol was isolated from the leaf chloroform extract of *Monanthotaxis parvifolia* as white colourless needle-like crystals. The compound was visualized as a dark blue spot on silica gel TLC using 1% vanillin reagent. To confirm that it was β-sitosterol, the compound was co-chromatographed with a standard sample of β-sitosterol which was isolated and authenticated from our laboratory. It was not studied further since it did not demonstrate antiplasmodial activity. Nevertheless, this is the first time the presence of β-sitosterol is being reported in *Monanthotaxis parvifolia*.

Of the seven compounds isolated from methanol leaf extract, three were screened for the antiplasmodial activity but not all of them had their structures elucidated due to low isolated quantities except for compounds 2, 3, 4 and 5. The structures of compounds 2 and 4 were successfully elucidated whereas that of compound 3 was partly characterized while compound 5 was not fully structurally-elucidated as the data available was not adequate. However this is a compound of interest as it exhibited good antiplasmodial activity and should be followed up.

Compound 4 was found to have similar characteristics to that of an earlier isolated flavonol, Quercetin-3-*O*-α-L- arabinofuranoside also referred to as avicularin (Chang *at al*., 2009). Compound 2 was observed to be very closely related to compound 4 with only a methoxy group less and an extra sugar unit. Compound 2 has been proposed as quercetin-3-O-α-rhmnopyranosyl-4-methoxy (1→6)-β-D-glucopyranoside, trivial name rutin. Compound 3 has been partly characterized when it was noted to have a quercetin carbon skeleton just like compounds 2 and 4 as well as two sugar units. The structure was proposed to be a quercetin glycoside particularly quercetin diglycoside. Compound 5 was also observed to have a big part of the structure similar to that of compounds 2 and 4 based on the MS and NMR data. Thus, the compounds isolated from the methanol extract of *Monanthotaxis parvifolia* were found mainly to be flavonoids. In general, the characterization of the compounds was based on their physical characteristics such as the yellow colour and the UV, MS and NMR spectroscopic data. They showed a UV peak at the visible range of 355-360 nm implying they are coloured compounds and in nature most coloured compounds are phenols which are mainly flavonoids. Flavonoids are some of the secondary metabolites produced by plants and are known mainly for their antioxidant and free radical scavenging activities (Elford *et al*., 1987; Chen *et al*., 2008; Adewole and Ojewole, 2009; Fidelis *et al*., 2012). They are also reported to have synergistic antimalarial activity in *A. annua* (Liu et al, 1989; Ferreira *et al*., 2010). Structurally, they have C6-C3-C6 skeleton where two aromatic rings are linked through a pyran ring (Ikan, 1991; Moon *et al*., 2005). Although flavonoids had previously been isolated from *Monanthotaxis buschananii* and *Monanthotaxis cauliflora* (Mulholland *et al*., 2000), this is the first time they are being reported in

Monanthotaxis parvifolia. The structure complexity and the small quantities present in the plant makes it a challenge to determine the structure of the isolated compounds and as a result in medicine discovery (Pink *et al*., 2005).

7.2 Conclusion

In conclusion this study has provided knowledge on the macroscopic characteristics of *Monanthotaxis parvifolia* which could be of value in its authentication (Gautam *et al*., 2010). The histological features have also provided information which could be utilized for example in documentation, registration and also for quality assurance of herbal preparations containing *Monanthotaxis parvifolia*. The study has further provided information on antiplasmodial, safety, antipyretic, analgesic and glycaemic activities of *Monanthotaxis parvifolia*. Phytochemistry data confirming the presence of flavonoid related compounds is further evidence of the importance of *M. parvifolia* in traditional medicine. All these results obtained support the use of *Monanthotaxis parvifolia* for the treatment of malaria related symptoms in traditional medicine.

7.3 Further work

Monanthotaxis parvifolia ssp. *kenyensis* was collected from the wild where it grows naturally along the uncultivated river line and thickets on well drained red soil. The plant is therefore under threat of being extinct due to human encroachment on these natural habitats occasioned by the ever diminishing agricultural land (Fidock, 2010; Cragg and Newman, 2013). It would be important therefore to promote conservation of the plant through direct cultivation by use of seeds and seedlings to ensure sustained supply of the plant (Willcox *et al*., 2011; Chadwick *et al*., 2013; Pan *et al*., 2014).

This study has shown that most of the active constituents were from the polar extracts. It is therefore proposed that chromatographic methods for such compoundsbe used for isolation in order to improve on the yields of the compounds and their subsequent structure determination (Holzgrabe *et al*., 1998; Jaspers, 1999; Fidock *et al*., 2004; Wang *et al*., 2011).

It is further recommended that although the isolated compounds did not exhibit a significant antiplasmodial activity, structure activity modifications could be carried out with the aim of improving on this activity (Baladrin *et al*., 1985; Abuga, 2009; Burrows *et al*., 2013). The isolated compounds should also be tested *in vivo* using *Plasmodium berghei* in the mouse model. Due to the influence of metabolism and other pharmacokinetic factors, the compounds may have an increase or decrease in activity (Berteni *et al*., 2007). Further investigations could be carried out on the antiplasmodial activity of the isolated compounds in combination with antimalarial drugs in use currently (Mishra *et al*., 2009; Deharo and Ginsburg, 2011; Fasinu *et al*., 2012; It is further recommended that *Monanthotaxis parvifolia* be screened for alleviating persistent pain by using the formalin test model.

7.4 Dissemination of the results

The research findings of this study will be disseminated in form of publications such as a thesis, journal articles and conference or seminars presentations.

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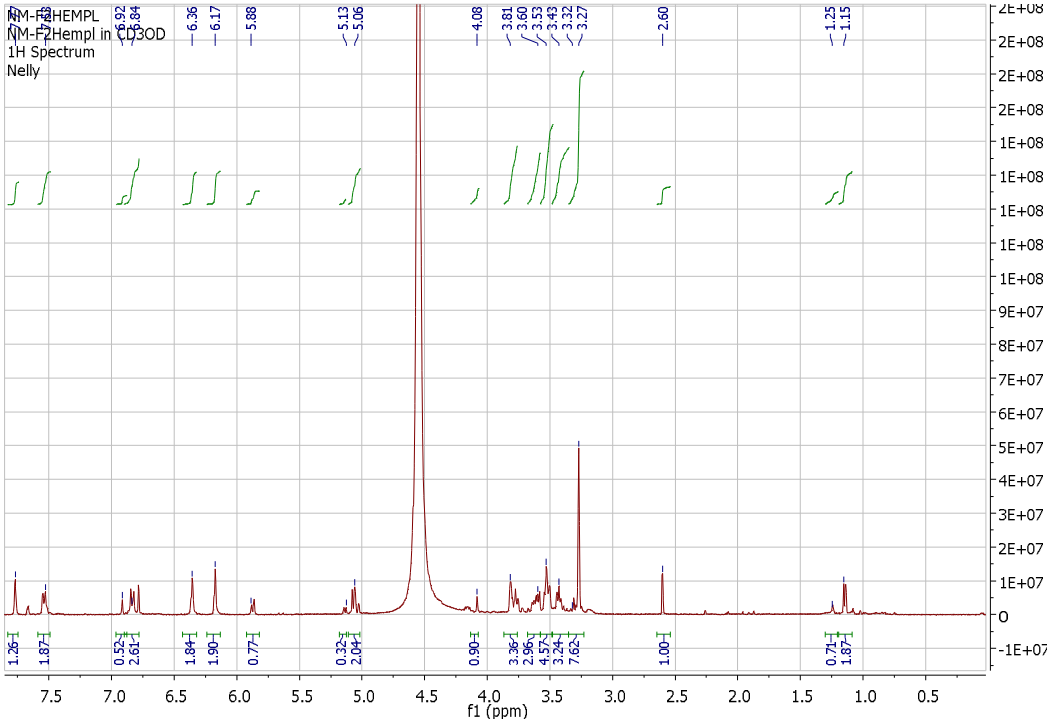
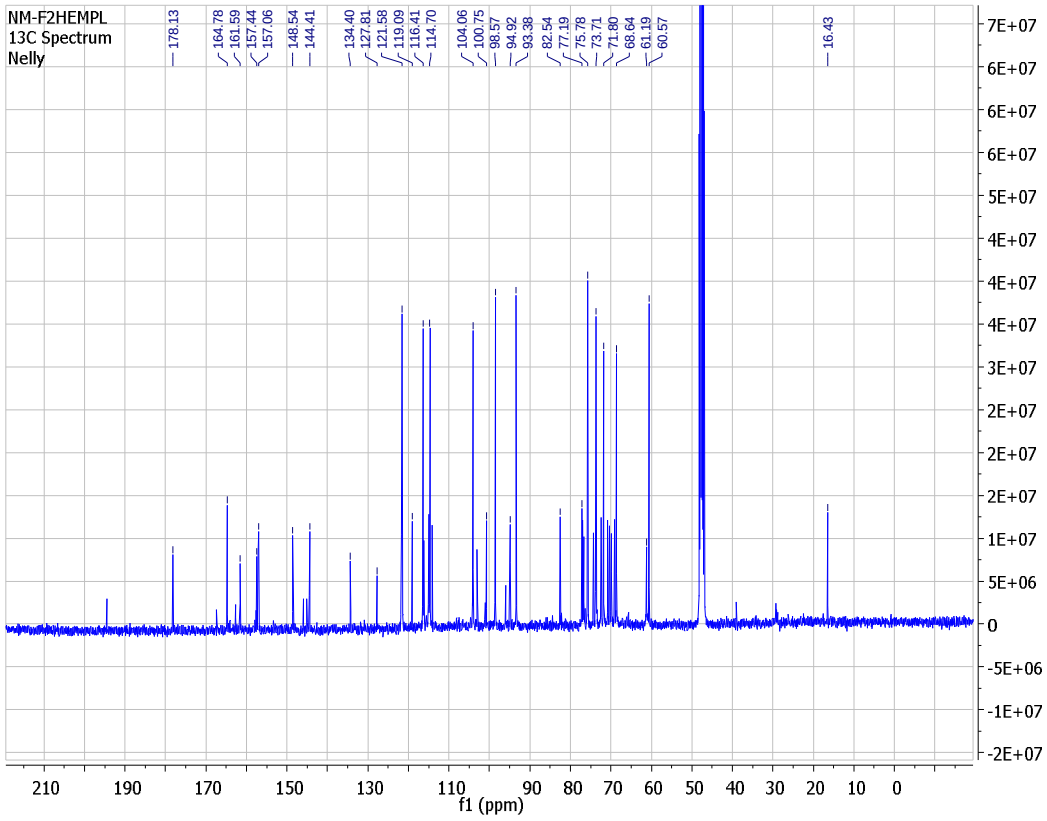
APPENDICES

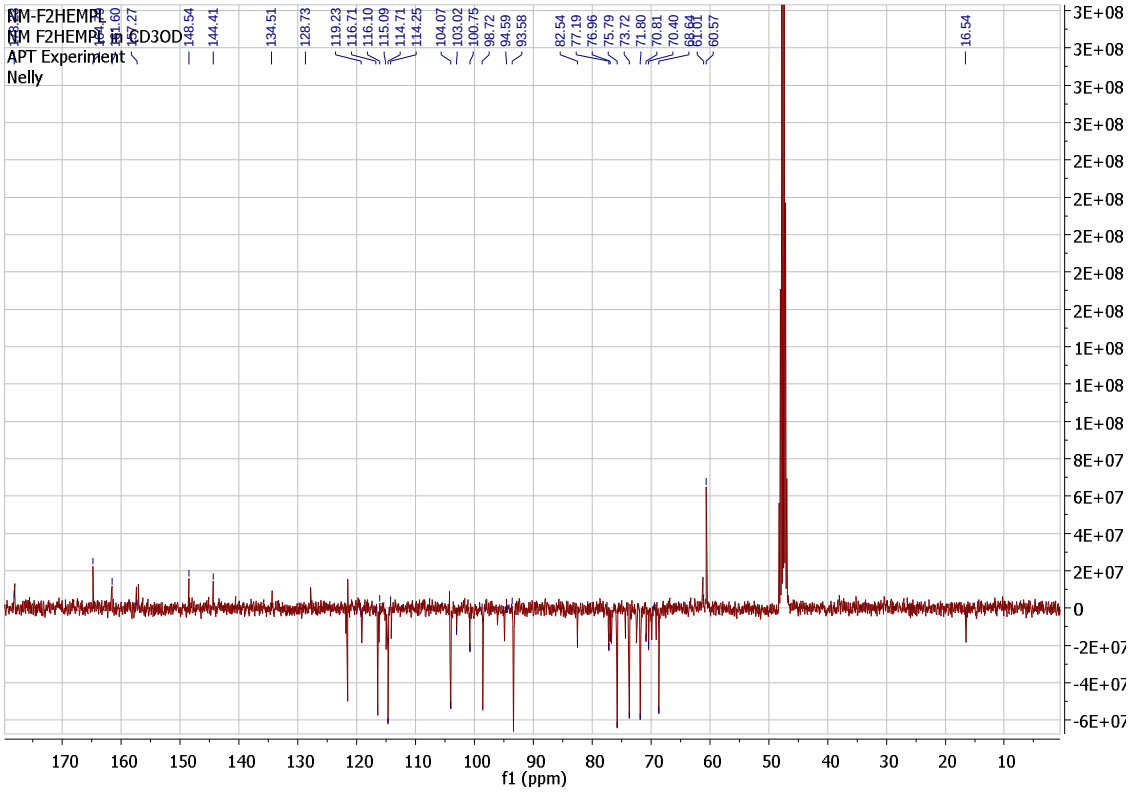
Appendix 1: ^{1}H-NMR spectrum of compound 2



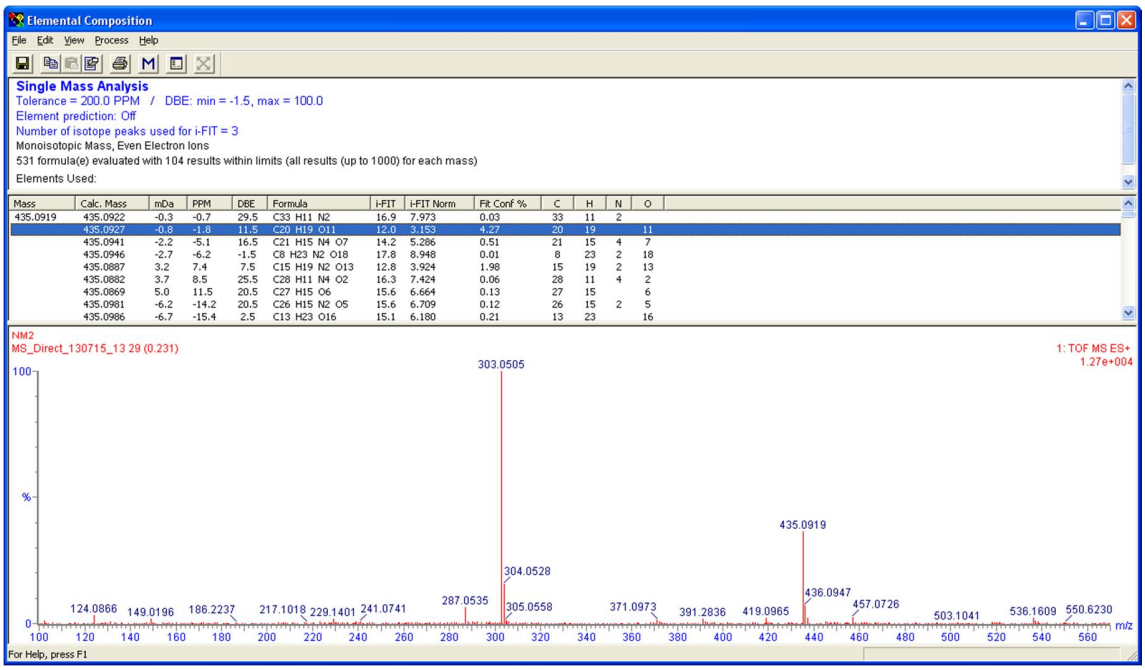
Appendix 2: The ^{13}C-NMR spectrum of compound 2



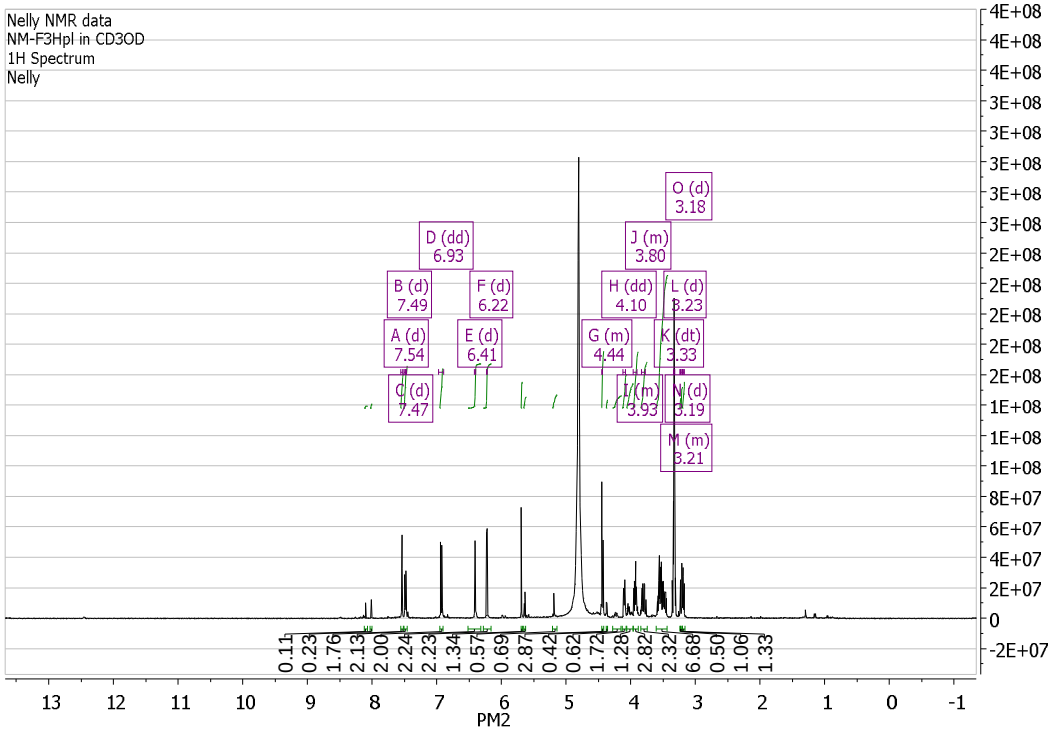
Appendix 3: ^{13}C-APT spectrum of compound 2



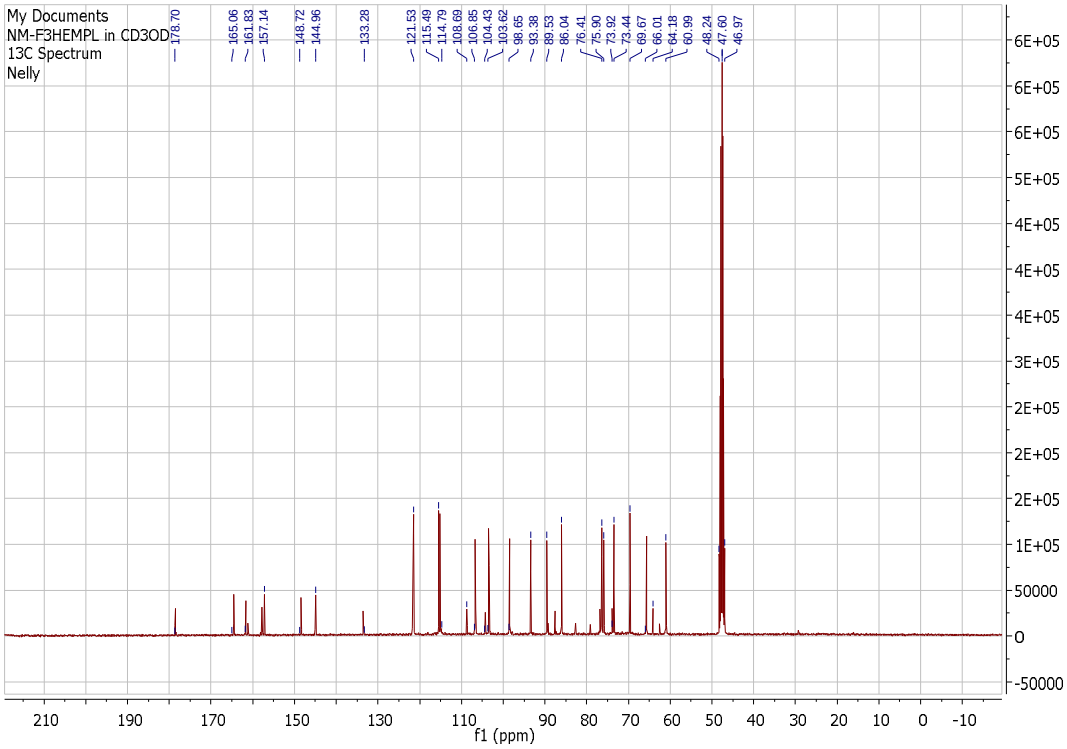
Appendix 4: High resolution mass spectrometry of compound 2



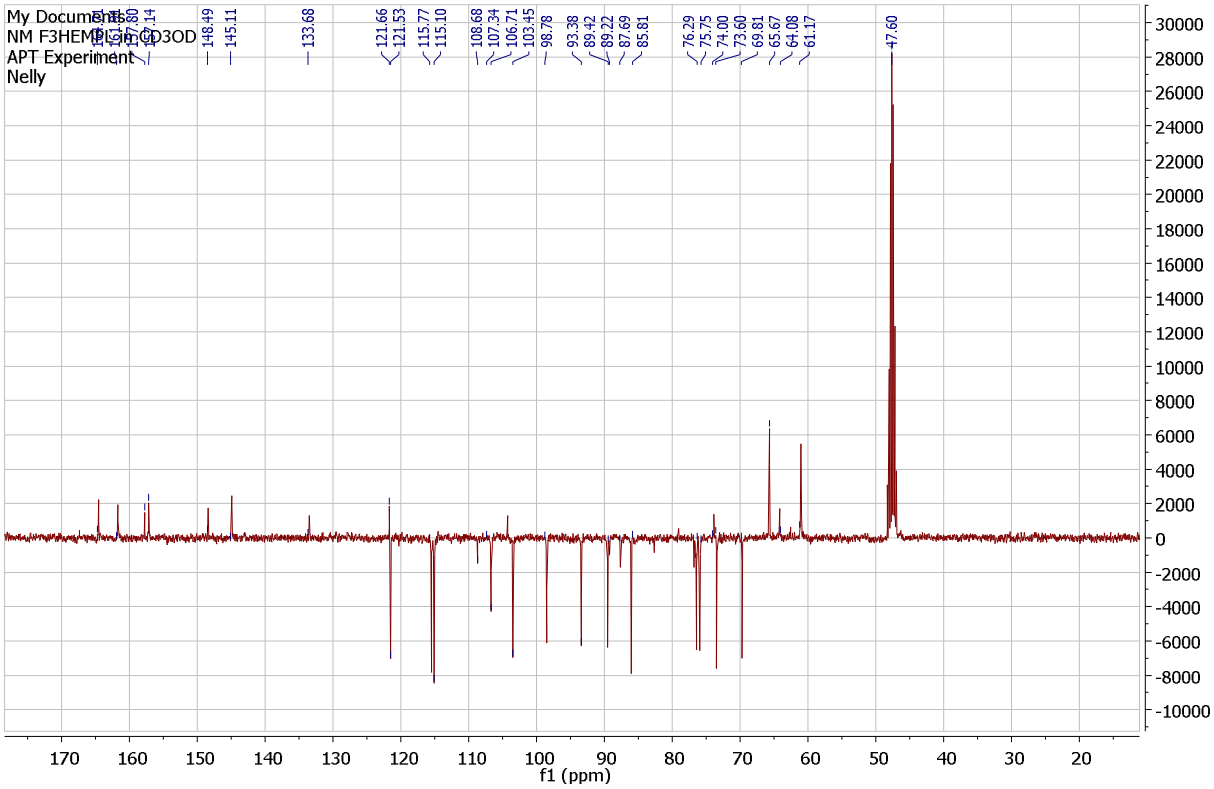
Appendix 5: ^{1}H-NMR for Compound 3 (F3HEMPL)



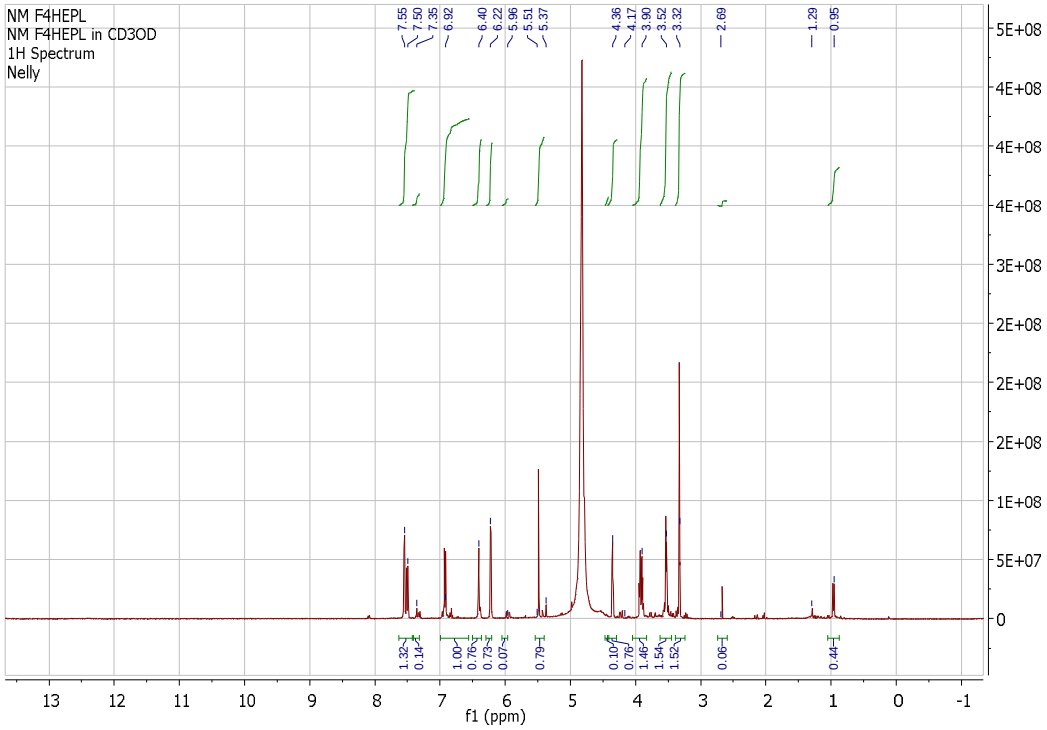
Appendix 6: ^{13}C-NMR for Compound 3 (F3HEMPL)



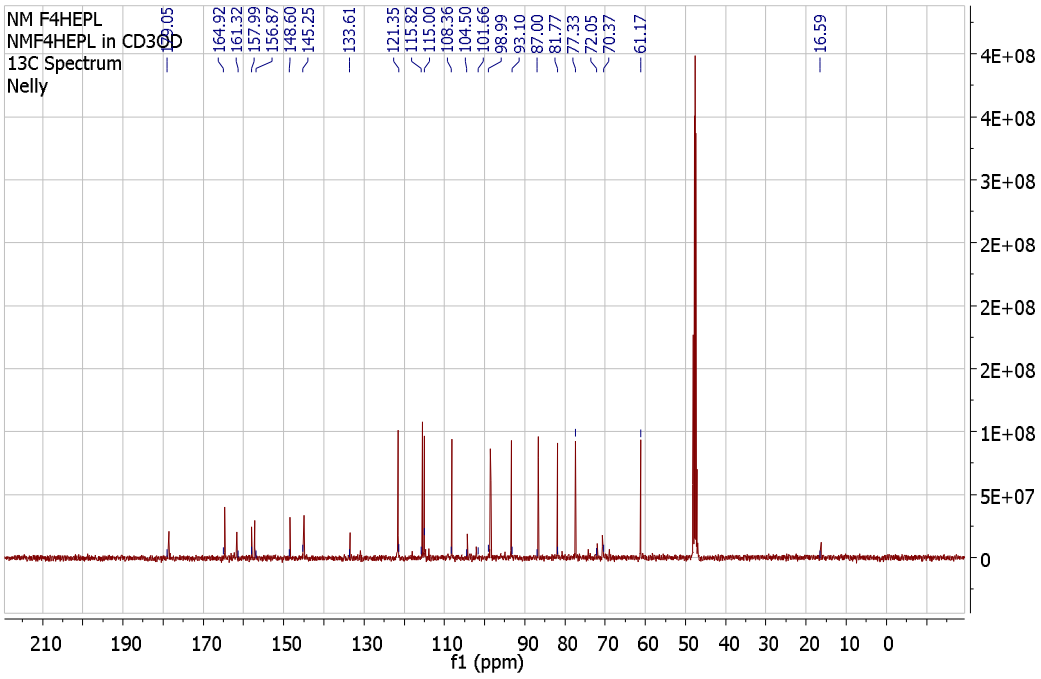
Appendix 7: ^{13}C-APT NMR for Compound 3 (F3HEMPL)



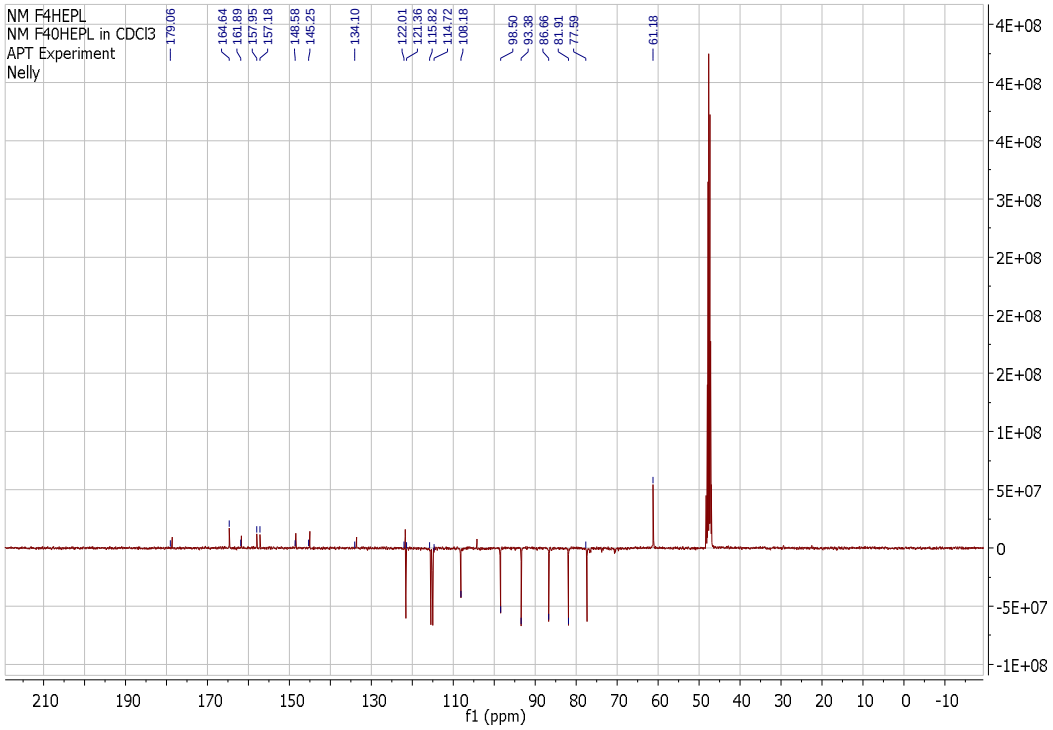
Appendix 8: ^{1}H-NMR spectrum of compound 4



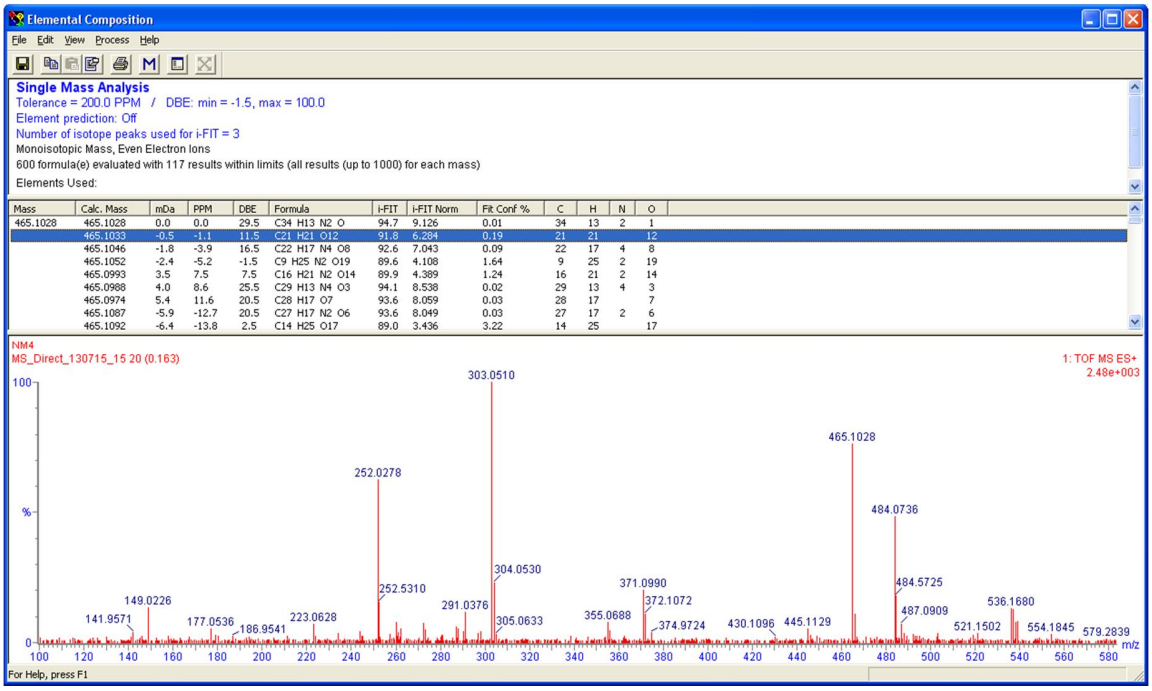
Appendix 9: ^{13}C-NMR spectrum of compound 4



Appendix 10: ^{13}C-APT spectrum of compound 4



Appendix 11: High resolution mass spectrometry of compound 4



Appendix 12: ^{1}H-NMR spectrum for Compound 5 (F5HEMPL)