EVALUATION OF BIOLOGICAL ACTIVITY OF ALKALOIDS AND FLAVANOIDS IN *ZANTHOXYLUM GILLETII* (DE WILD WATERMAN) EXTRACTS FROM DIFFERENT GEOGRAPHICAL REGIONS IN KENYA

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REG. No. I56/10671/2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTERS OF SCIENCE (BIOTECHNOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES, KENYATTA UNIVERSITY

OCTOBER, 2011

DECLARATION

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

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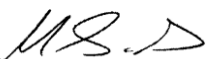

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DEDICATION

This research work is dedicated to my family and friends: my dear wife Linet and our daughters Victoria and Vollyne, my mother Mrs. Jane Atieno and my late father Mr. Albert Gaya Obilo, my brothers and sisters. To all the young people in Kenya and other parts of the world who should understand that they are the change they desire.

ACKNOWLEDGEMENTS

I acknowledge and express my warmest thanks to the following people for all their dedication, hard work and wise counsel; my supervisors Dr. J. N. Ngeranwa, Dr. Alice N. Muchugi and Professor Monique J. Simmonds for their constant teaching, encouragement and criticism, for all the advice and unrelenting attention they gave me. Without your wonderful guidance this work would not have been realized; you ensured that I understood and appreciated research as an enjoyable duty for which scientists are called. Many thanks to the Director, National Museums of Kenya, Director, Research and Collections Dr. Helida A. Oyieke for the enabling environment and facilities the Institution availed to me. I would like to thank in a very special way the Royal Botanic Gardens (RBG) Kew for their contribution in my studies and all the financial support through the Kenyan Useful Plants Project (UPP).

Many thanks to the Seeds for Life Project (SfLP) and the UPP team; Mr. Timothy R. Pearce, Dr. Tiziana Ulian, Dr. P. N. Muthoka and Mr. B. M. Kamondo for all their selfless support and encouragement; I sincerely appreciate. My gratitude also goes to the Jodrell Laboratory staff and team of brilliant scientists; in this magnificent Laboratory led by Professor Simmonds, Dr. S. Graham, Dr. K. Tetsoh, Dr. J. Kite, Dr. S. Larson and Dr. E. Porter, you are wonderful people. I appreciate the support from my colleagues in the E.A herbarium, who supported my work in many ways. Most of all I thank in a special way my family and friends; my dear wife Linet and my daughters Victoria and Vollyne who were very patient with me while I kept away from home for many days, they greatly encouraged me. My friends, who kept urging me to go on, Raphael Aoko and family, Ogolla Brown, Ogweno Ayayo and family, J.F. Kawaka, D. Miyawa, my mother Mrs. Jane Gaya, my brothers and sisters; David, Solomon, Monique, Maureen and Beatrice, for all your love support and encouragement I was able to move this far, may God reward all of you abundantly.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BAW	Butan-1-ol, Acetic acid and Water
BI	Biological interactions
CAW	Chloroform, acetic acid, water
$CHCl_3$	Chloroform
DAF-FM	4-amino-5-methylamino-2', 7'-difluorofluorescein
EC_{50}	Half maximal effective concentration
ESI	Electron spray ionization
ESI-MS	Electron spray ionization mass spectrometry
EtoAc	Ethyl acetate
FAO	Food and Agriculture Organization
FDA	Food and drug administration
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
INT	p-iodonitrotetrazolium violet (2-[4- indophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride
LC-MS	Liquid chromatography mass spectrometry
M/z	Mass - to - charge ratio
MAC	Methanol, Ammonia and Chloroform
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
NMK	National Museums of Kenya
NO	Nitrogen oxide
PDA	Photodiode array
RBG-Kew	Royal Botanic Gardens, Kew
Rf	Refractive factor
S.E	South East
SfLP	Seeds for Life Project
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNP	Sodium nitroprusside
SUG	Sustainable Use group
TLC	Thin layer chromatography
UPP	Useful Plants Project
US FDA	United States Food and Drug Administration
YEP	Yeast peptone

ABSTRACT

Plants appear to be almost an exclusive source of medicine to the worlds' gradually increasing population with substances derived from them constituting a big percentage of all prescribed conventional medicines and other products. This puts pressure on the demand for medicinal plants, most of which are sourced from the wild. *Zanthoxylum gilletii* is an African indigenous deciduous tree which is overexploited for its medicinal use among many communities. This species has its habitat encroached and information on its alkaloids and flavanoids lacking. The objectives of this study were to determine antimicrobial and antioxidant activity of plant extracts and then identify alkaloids and flavanoids in the Kenyan *Z. gilletii* root, bark and leaf samples from different geographical regions. Antimicrobial activity tests were carried out using TLC bioautographic assays and ninety six well microtitre assays. Pathogens used during the tests were *Saccharomyces cereveciae* and *Cladosporium herbarum* as fungal pathogens; *Bacillus subtilis* and *Pseudomonas syringae* as the bacterial pathogens. Efficacy was confirmed by determining the minimum inhibitory concentration (MIC) and comparing against clinically used drugs, nystatin and chloramphenical as positive controls in antifungal and antibacterial assays respectively. Antioxidant activity was tested using the nitric oxide scavenging assay. Alkaloids and flavanoids were identified using HPLC and LCMS analysis methods. Plant extracts showed antimicrobial activity against both gram positive and gram negative bacteria while tests for antioxidant activity of the plant extracts were negative. Analysis of variance (ANOVA) revealed no significant difference between extracts from different plant parts and regions (P =0.05). Extracts contained a wide range of alkaloids and two flavanoids diosmin and hesperidin with compound profiles varying among plant parts and from the different geographical regions. Good antimicrobial activity was observed in the root extracts while the root extracts did not show any antimicrobial activity. Study revealed that the compound profiles relate with antimicrobial activity and that *Z. gilletii* is a poor antioxidant.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Most of the world's population, especially in developing countries still rely on natural remedies. In the past, traditional peoples or ancient civilizations depended greatly on local flora and fauna for their survival. Although some preparations may have been dangerous, or worked by a ceremonial or placebo effect, traditional healing systems usually had a substantial active pharmacopoeia, in fact most western medicines up until the 1920s were developed similarly (Bensky et al., 2004). In developed countries, the natural products continue to be an important source of therapeutically effective medicines (Quamina, 2003). Out of the 520 new drugs approved between 1983 and 1994, 39% were natural products or derived from natural products and the proportion of antibacterial and anticancer drugs derived from natural products was more than 60% of best selling non-protein drugs in 2001 (Quamina, 2003).

Plants have always been a rich source of major compounds (e.g. morphine, cocaine, digitalis, quinine, tubocurarine, nicotine and muscarine). Many of these lead compounds are useful drugs (e.g. morphine and quinine), while others have been the basis for synthetic drugs (e.g. local anaesthetics developed from cocaine). Clinically useful drugs which have been recently isolated from plants include the anticancer agent paclitaxel (Taxol) from the yew tree, and the antimalarial agent artemisinin from *Artemisia annua*.

Medicinal plants have been used to cure a number of diseases. Though the recovery is sometimes slow, the therapeutic use of medicinal plant is becoming popular because many

have fewer side effects than single compound pharmaceutical drugs and plant derived extracts are not associated with causing antibiotic resistant microorganisms (Rawat and Uniyal, 2003).

There is a widespread belief that “green medicines” are healthier and more harmless or safer than synthetic ones (Parvathi et al., 2003). Furthermore, there are still many reasons why botanical drugs are still needed; most important of then involve the problem of resistance to antimicrobial and anticancer drugs. Unlike the conventional medicines, traditional medicinal systems of the East always rely on the belief that complex diseases such as diabetes, heart disease, cancer and psychiatric disorders are best treated with complex combinations of botanical and non-botanical remedies (Quamina, 2003). Plants have adapted similar strategy in their biochemical battle against diseases. Instead of relying on a single flavonoid to stop pathogens, plants produce families of structurally and functionally diverse antimicrobial compounds. These act together to prevent development of resistance.

Pharmacognosy provides the tools to identify select and process natural products destined for medicinal use. Usually, the natural product compound has some form of antimicrobial activity and that compound is known as the active principle. Many of today's medicines are obtained directly from a natural source (El-Shemy et al., 2007). Many countries in Africa are endowed with vast natural resources in form of medicinal plants. Majority of people in Africa have for centuries relied on plant-based traditional medicines for their care.

Zanthoxylum gilletii (De Wild Waterm) is an indeginous decidous tree growing 10-35m high, belonging to the family Rutaceae. It has an open spreading crown usually with a straight cylindrical trunk which is straight and branchless for several metres (Fig.1). The trunk and the

thicker branches are covered with conspicuous cone shaped, sharply pointed corky, woody based prickles up to 5 cm in diameter at the base. The stem wood is bright yellow, hard sweet scented and termite resistant. The trunk has a diameter of 30-90 cm (Dharani, 2002).

Fig 1: Flowering *Z. gilletii* tree (A) and stem bark of *Z. gilletii* in Kakamega forest (B)

Z. gilletii is traditionally recognized as having many uses. Phytochemical studies have identified numerous compounds with potential in medical applications such as cancer treatment, antioxidant, anticoagulant and antibacterial agents (Hisatomi et al., 2000; Xiong et al., 1995; Chen et al., 1994; Abbiw, 1990). Traditionally the communities chewed the bark of the tree and swallowed the juice for the treatment of colds, rheumatic pains, stomach ache and to alleviate tooth-ache (Beentje, 1994; Kokwaro, 1993). The bark is also used to treat the cattle disease anaplasmosis, while fresh twigs are used as a tooth brush for tooth ache and bleeding gums (Gachathi, 2007).

Zanthoxylum gilletii contains a range of compounds associated with its medicinal properties. These compounds include:- alkaloids, flavonoids, xanthophylls, phenolic acids, saponins, coumarins and hydroxycinnamic acids (Islam and Ahsan, 1997). The species is also a valuable timber species which is currently being overexploited in the Kenyan moist afromontane natural forests for its timber and medicinal value (Louppe et al., 2008; Ochieng' Obado and Odera, 1995). Regeneration of *Z. gilletii* is adversely affected by the shade in closed forest canopy, few seed sources, and insect herbivory. Phytophagous insects play a significant role in regeneration of *Z. gilletii* since they feed on the seed quickly before it can regenerate (Mbai-Opondo et al., 2000) Seeds which can be stored for upto 2 months are susceptible to insect attack (Patrick and Bo-Tengnas, 2005).

1.2 Problem Statement and justification

Zanthoxylum gilletii, a flagship species in most afromontane forests in Kenya is sparsely distributed and is rapidly dissapearing from its natural habitat in Kenya; this is due to poor harvesting methods and overexploitation by traditional medical practitioners based on its wide application in treatment and management of various diseases and use as timber (Louppe et al., 2008; Ochieng' Obado and Odera, 1995; Kokwaro, 1988). Due to low population, the harvesting of *Z. gilletii* for timber extraction in Kakamega Forest in Kenya had declined from 645 m^3/year in the 1930s to 100 m^3/year in 2000 (Louppe et al., 2008).

Currently, there is very little pharmacological data to link the traditional medicinal uses of the plant with the availability of alkaloids and flavanoids in the species in Kenya. Though widely

used in traditional medicine therapy, there is no published documentation describing the biologically active compounds occurring in the *Z. gilletii* in Kenya. The diversity and variation in abundance of active phytochemicals expressed in the species in Kenya is also not documented. Clear evidence of medicinal properties of *Z. gilletii* is lacking and there is no scientific data showing how the compounds in this particular species vary, hence research in this area would assist and support conservation strategies for the species. Some plant parts can be more sustainably harvested than others, it is therefore important to asses levels of expression of these active compounds in different plant parts. The knowledge gained will ensure possibility of targeted harvesting, conservation and utilization strategies of the species.

Plants produce alkaloids as a means of protecting themselves against predation; these are also used as natural source of insecticides and fungicides (Kayani et al., 2007). Alkaloids and flavanoids help biologically in storage of waste nitrogen, cationic balancing and protection against parasites, and their principal action is on the nervous system, that is why their accumulation in *Z. gilletii* could secure the plant from grazing animals and make them source for traditional medicine to the communities (Kayani et al., 2007). It is therefore very important to determine if the alkaloids and flavanoids produced in *Z. gilletii* have any antimicrobial activity at all. Findings from this study will be valuable in providing data that will support use of *Z. gilletti* to control economically important pathogens like *Bacillus subtilis* and *Pseudomonas syringae* among poor community members. This study investigated antimicrobial activity and variation in profiles of alkaloids and flavonoids in *Zanthoxylum gilletii* extracts collected from the three different geographical regions in Kenya.

1.3 Research questions

The research questions that guided this study include the following:

1. Do extracts of *Z. giletii* show any antimicrobial or antioxidant activity?
2. What alkaloids and flavanoids occur in *Z. gilletii*?
3. Do the identified alkaloids and flavanoids vary in different plant parts within a plant and among plants from different geographical regions?

1.4 Hypotheses

Alkaloids and flavanoids in *Z. gilletii* vary in the root, leaf and bark extracts from different geographical regions in Kenya and their profiles influence antimicrobial activity of the extracts.

1.5 General objective

To determine antimicrobial and antioxidant activity of alkaloids and flavanoids present in *Zanthoxylum gilletii* extracts collected from different geographical regions in Kenya.

1.5.1 Specific objectives

1. To determine the antimicrobial and antioxidant activity of root, bark and leaf extracts of *Z. gilletii* extracts from different geographical regions.
2. To determine the alkaloids and flavanoids present in root, bark and leaf extracts of *Z. gilletii* collected from different geographical regions in Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy and Biology of *Zanthoxylum gilletii*

Zanthoxylun gilletii (De Wild Waterm) was fomerly called *Zanthoxylum macrophyllum* (Oliver) and *Fagara macrophyllum* (Engler). The chemistry of the species is in a confused state owing to doubtful authenticity of the material examined by the different investigators and due to confused botanical synonymy of the genus (Goodson, 1921). *Zanthoxylum gilletii* sometimes referred to by synonym *Zanthoxylum zanthoxyloides* had common names in West and East Africa where it was known as candle wood; *Zanthozylum*; *Fagara* and East African satin wood in East Africa (Patrick and Bo-Tengnas, 2005; Addae, 1992; James, 1992). Different vernacular names are used by different ethnic communities in Kenya to describe the species for example, the plant is called shikuma among the Luhya, sogowait among the Kipsigis and Kalenjin, sogomaitha among the Luo and muchagatha/mumondo among the Kikuyu (Gachathi, 2007; Kokwaro, 1993; Sindiga et al., 1995).

This is a polymorphous species, closely related and often difficult to distinguish from *Z. heizii* (Aubr. Et Pellegr.) Waterman), which has crenate leaf margins, and *Z. tessmannii* (Engler) Waterman (*Fagara tessmanii Engler*), whose leaves are asymmetrical and cuneate at the base (Gachathi, 2007). The flowers are very small, reddish brown, yellow to white pink or greenish, unstalked, in large terminal panicles up to 90 cm long, the inflorescence stalk with small prickles; flowers 5-merous, isolated or in small bundles. The fruits occur in large, dense bundles, individual fruit is a small, spherical flattened capsule 5 -8 mm in diameter, reddish purple, containing one globose, blackish-blue, metallic-shinning seed of peppermint smell

(Gachathi, 2007; Neuwinger, 1996). *Zanthoxylum gilleti* is dioecious, and therefore male and female trees must be in close proximity in order for pollination to take place (Patrick and Bo-Tengnas, 2005).

2.2 Ecology and distribution of *Z. gilletii*

The genus *Zanthoxylum* is distributed worldwide from the tropics to the temperate zones. There are over 200 species from small shrubs to large trees (Leupe et al, 2007). *Z. gilletii* is widely distributed in Africa covering countries like; Sierra Leone to Kenya, Sudan, Angola, Malawi, Zambia and Zimbabwe. It is one of the main trees of the West African forests, very common in Ivory Coast (Beentje, 1994; Neuwinger, 1996; Katende, 2000). The species is found in forest Savanna mosaic of lowland forests, West Tropical African, in Ghana, Senegal, Mali, Guinea Bissau, Guinea, Nigeria, Togo, Cameroon, Zaire and Tanzania, (Maurice, 1993). It is also distributed in Sierra Leone, Gambia and Darhomey (Ayensu, 1978).

In Nigeria it is recorded as flowering in January to February, May to October and fruiting between July and November. However, intensive research is going on to show the great potential this plant has in therapy and folk medicine use (FAO, 1986). In Kenya, it can be found in a number of floral regions and the Kenyan main water towers including: Mount Kenya forest, the Mau Forest block and Kakamega forest. This timber tree grows in tropical rain forests especially at lower and medium altitudes from 1500-2500 metres (Dharani, 2002). Other *Zanthoxylum species* less commonly used but are found in Kenya include: *Z. mildbraedii, Z.chalybeum* and *Z. rubescens* which are both found in Kakamega forest (Patrick and Bo-Tengnas, 2005).

2.3 Effects of geographical variation on plant secondary compounds

Geographical variation may have some effects on the level of medicinal active compounds of plants of the same species (Sheen et al., 1994). Variation among plants of the same species may be due to age, climate, soil or season of the year. Generally, most plants show a marked seasonal and climatic variation in their antimicrobial activity (Ebuu and Sawyerr, 1994). This variation, however, does not justify genetic variation. Tropane alkaloids have been extensively studied by Australian workers. Trees from natural stands on various locations were examined and their progeny raised side-by-side in experimental plantations.

Research findings showed that proportions of any alkaloids studied varied greatly within this same species (Trease and Evans, 1996). Chemical diversity in nature is based on biological and geographical diversity, so researchers travel around the world obtaining samples to analyze and evaluate in drug discovery screens or bioassays (Cragg and Newman, 2007). Living plants contain a much greater diversity of bioactive compounds than any chemical library made by humans. Moreover, biochemical profiles of plants harvested at different times and locations may vary greatly (Quamina, 2003). In one of their studies on management of medicinal plant resources in Nyanza recommended that there should be studies to ascertain possible variations in medicinal plant active compounds in different geographical regions in order to prevent genetic erosion by planning their conservation (Ochieng' Obado and Odera, 1995). There is therefore a need to establish whether the geographical regions affect the efficacy, quality and quantity of alkaloids and flavanoids in *Z. gillettii* which contains among others the furoquinoline alkaloid, skiammianine, cinnamic acid amide, benzo phenanthridine alkaloids and chelerythrine (Adesina and Johannes, 1988).

From selected provenance lines, studies should be carried out to quantify the products of economic value and determine their efficacy, quality and quantity. Most productive lines and regions can then be identified and such information availed to farmers and traditional medical practitioners (Mukiama, 2005). Some studies done on the the tropane alkaloids (Trease and Evans, 1996) and the extenssive study done on the Pacific yew tree (Wall and Wani, 1995), it is evident that phytochemical composition of plants of the same species growing in different geographical regions can vary.

2.4 Traditional uses of *Z. gillettii*

The bark is chewed and the juice swallowed for the treatment of stomachache (Kokwaro, 1993). The stem bark decoction is commonly used for back pain and externally for all urinogenital complaints including infections. The root bark or the fruit pulp is a liniment for rheumatism and pain. A decoction of young leaves eases coughs and is said to be effective on gonorrhea and bilharzia. This species is used in Nigeria and Ghana for its antiseptic and analgesic properties. Decoction of the bark cures rheumatic troubles while the ground bark mixture is used to help heal the womb after child birth (Abbiw, 1990). Extracts of the species have shown antisickling properties *invitro*. It has also been used as an analgesic, anticancer, antihypertensive, antipyrectic, antirheumatic, antisickling, antiplasmodic, antimicrobial, and as circulatory stimulant (Addae, 1992; James, 1992).

Table 1: Summary of the uses of traditional *Zanthoxylum gilletii*

Country	Part used	Used to treat	References
Ivory Coast	Stem bark/ twig bark	Genital-urinary infections, kidney pains, rheumatism	(Weenan et al., 1990)
Kenya	Roots, Bark chewed	Toothache and stomachache, venereal infections, pneumonia	(Kokwaro, 1993)
S. E. Benin	Bark, shoots	Venereal infections, Severe palpitations	(Adesina and Johannes, 1988)
S. E. Cameroon	Shoot, root	Severe palpitations, gonorrhea, impotence	(Adesina and Johannes, 1988)
Equatorial Guinea	Leaf juice	Enlarged spleen	(Katende, 2000, Neuwinger, 1996)
Gabon	Leaf macerate	Madness, snake bite	(Katende, 2000, Neuwinger, 1996)
Zaire	Bark powder/decoction	*Mansonia altissima*, arrow poison, fish poison, internal parasites, female sterility	(Katende, 2000, Neuwinger, 1996)

Other uses include; use as an anticancer, for backache, fumigation, dysentery, fever, to manage pain after delivery and syphilis of the throat (Ayensu, 1978). *Z. gilletii* has also been used in treatment of elephantiasis and to alleviate general body weakness in combination with *Xylopia aethiopica.*

Root bark is soaked in sterile water for 12 hours; table spoonful of the resulting solution is taken 3 times daily to treat gonorrhea or as urinary antiseptic (FAO, 1986). This species has been used to manage impotence, fever, lower abdominal pains, small pox, whooping cough and wounds (Mshane and Ekper, 2000; Addae, 1992; James, 1992). Branches of the plant contain inflammable resins and are used as processional torches by the villagers in West Africa (FAO, 1986).

2.5 Propagation of *Z. gillettii*

Seed of numerous of *Zanthoxylum* have been found to have low germination rates (World Agroforestry Centre, 2005; Frances, 2004; Francis, 2000). *Zanthoxylum gilletii* has shown poor seed germination due to presence of chemicals within the seeds, which inhibits germination. The seeds are classified as either intermediate or orthodox in their storage behavior (Michael et al., 2007). Low viability of the seeds can be associated with empty seed, seed predation by insects and an oily seed coat (Reinartz and Popp, 1987). The red-brown fruit are picked from the tree before the capsules open and dried in the sun for 1-2 day, and then shaken to remove the seed. Propagation is by seed and wildings. It is fast growing and can be planted as pure stand and in enrichment planting with other trees like *Aningeria* and *Entandophragma* species where natural forest has been felled on Mt. Elgon (Katende, 2000).

2.6 Phytochemistry

Phytochemical investigations carried out on a related species, *Z. chalybeum* activity have yielded pure crystalline alkaloids (Olila and Opuda, 2001). Chemical investigations of *Z. gilletii* from Nigeria described the isolation of the furoquinoline alkaloid, skimmianine, the cinnamic acid amide, fagaramide and benzo phenanthridine alkaloids, nitidine, dihydrochelerythrine and chelerythrine alkaloids (Adesina and Johannes, 1988). Other constituent compounds include volatile oils, vanillic acid, and hydroxyl benzoic acid (Maurice, 1993). *Z. gilleti* from Nigeria appears to be very rich in aromatic amide (Adesina, 2005). The chemical composition and antimicrobial activity of Zanthoxylum from Ghana and Ivory Coast was also studied to verify the use of the species as an antibacterial agent. This

study showed that Z. *gilletii* contains fagaramide and lupeol (Mshane and Ekper, 2000; James, 1992; Mensah and Torto, 1970; Goodson, 1921). Antimicrobial activity has been demonstrated in *Z. zanthoxyloides*, a species that is closely relates to *Z. chalybeum* and *Z. gilletii* (Mai et al., 2001). The species of *Zanthoxylum* so far studied, vary greatly in their chemistry and their taxonomy is in some cases obscure.

This is because; apart from the inter-specific variation in chemistry, the extent of variations of active constituents within a species (particularly from different geographical regions) is still not clear (Olila and Opuda, 2001). Other chemical compounds recorded in Ghanian *Z. gilletii* include fagarimide, berberine, skimmiamine, benzoic acid derivatives, essential oils, tannins and saponins, (James, 1992; Mensah and Torto, 1970). An *invitro* study of the extracts of *Z. gilletii* showed antifungal and antibacterial activity and inhibited *invitro* growth of *Candida albicans, Cryptococcus neoformans* and other filamentous fungi (Addae, 1992; James, 1992; Mshane and Ekper, 2000). The species which has been used as an antileukemia, anticancer and an antiviral has shown activity against *Mycobacterium smegmatis, Klebisiela pneumonia* and *Candida albicans. Z. gilletii* has also been used to reduce pain in sickle cell patients and in the treatment of sickle cell anemia in Nigeria (Maurice, 1993). Ethyl acetate extracts of *Z. gilletii* showed antimicrobial activity against tumor cells in research carried out in University of Nantes (France) and extracts showed moderate activity against HIV virus (Hostettmann et al., 1996).

According to a study by Weenen et al., (1990), the most active constituents isolated from root bark of *Z. gilletii* was N-isobutyldeca-2, 4 dienamide which was shown to inhibit the growth

of *Plasmodium falciparum* (Weenen et al., 1990). The activity of this compound was mainly due to the presence of α, β-unsaturated carbonyl moiety. Another study confirming use of *Z. gilletii* as an effective antimalarial agent was carried out by (Zirihi et al., 2007) . The results of this study indicated that bark extracts of several plants of *Fagara macrophylla* （Oliv. Engl.), have very good antiplasmodial activities and a very low cytotoxicity. Among the active molecules, nitidine was the principal alkaloid of *Z. giletii*. The value of the IC_{50} of molecule identified as nitidine (0.16 μg/ml) is of the same order as that of chloroquine (0.15 μg/ml); the bio-guided fractionation of *Fagara macrophylla* thus made it possible to isolate the compound nitidine which had very high interesting antiplasmodial activity. Nitidine was isolated for the first time from *Toddalia asiatica*, a plant used in Kenya to treat fevers and malaria (Zirihi et al., 2007).

Nitric oxide (NO) plays a critical role as a molecular mediator of a variety of physiological processes, including blood-pressure regulation and neurotransmission. In endothelial cells, as well as in neurons and astrocytes, NO is synthesized from L-arginine in a reaction catalyzed by nitric oxide synthase (Itoh et al., 2000). The potential spatial and temporal control of nitric oxide release is made possible by photolysis of NO precursors which makes this an attractive approach for generating NO in experimental systems. SNAP (*S*-nitroso-*N*-acetylpenicillamine) has been shown to release NO in response to light stimulation in both aqueous and isopropyl alcohol solutions. The nitric oxide (NO) radical is very unstable, and no equilibrium indicators for NO are known. However, NO is readily oxidized to the nitrosonium cation (NO^+), which is moderately stable in aqueous solutions but highly reactive with nucleophiles or other nitrogen oxides. DAF-FM (4-amino-5-methylamino-2',7'-

difluorofluorescein and its diacetate derivative (DAF-FM diacetate) have significant utility for measuring nitric oxide production in living cells or solutions (Kojima et al., 1993). The fluorescence quantum yield of DAF-FM is reported to be 0.005 but increases 160 fold to 0.81 after reacting with NO. DAF-FM reagent has some important advantages over DAF-2 (Nagata et al., 1999).

2.7 Phytochemical analysis

Phytochemical analysis of all extracts was done through thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS)

2.7.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) gives a quick review of the number of components in a mixture and has been used to support the identity of compounds in a mixture when the Rf of the compounds are compared with the Rf of a known compound (both run on the same TLC plate) (Svendsen and Verpoorte, 1983). TLC is a rapid analytical method which uses small amounts of sample material. TLC relies on separation of compounds on solid phase sorbent mounted on a glass, aluminium or plastic plate using a liquid mobile phase. Various sorbents can be used depending on the solvent system for the mobile phase, e.g. silica, cellulose, polyamide are the main types available and are either attached to plastic, glass or aluminium plates. These can come with florescent indicators F254 in the stationary phase or without this marker. For observing under UV light and for spraying with some detection agents, plastic backed plates can be very useful.

2.7.2 High performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) methods

HPLC and LC-MS utilize a thin layer of adsorbent material which holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that indicates UV absorbance and/or mass spectra of compounds present in analysed samples along with the retention times of the compounds present. Retention times vary depending on the interactions between the stationary phase and the molecules being analyzed. Mass spectrometry, often used to aid structural determination, is a method in which individual compounds can be identified based on their mass/charge (m/z) ratio, after ionization. Chemical compounds exist in nature as mixtures, so the combination of liquid chromatography and mass spectrometry (LC-MS) is often used to separate the individual chemicals. Identification of compounds, based on mass fragmentation, molecular weight and accurate mass is aided by a database of known compounds.

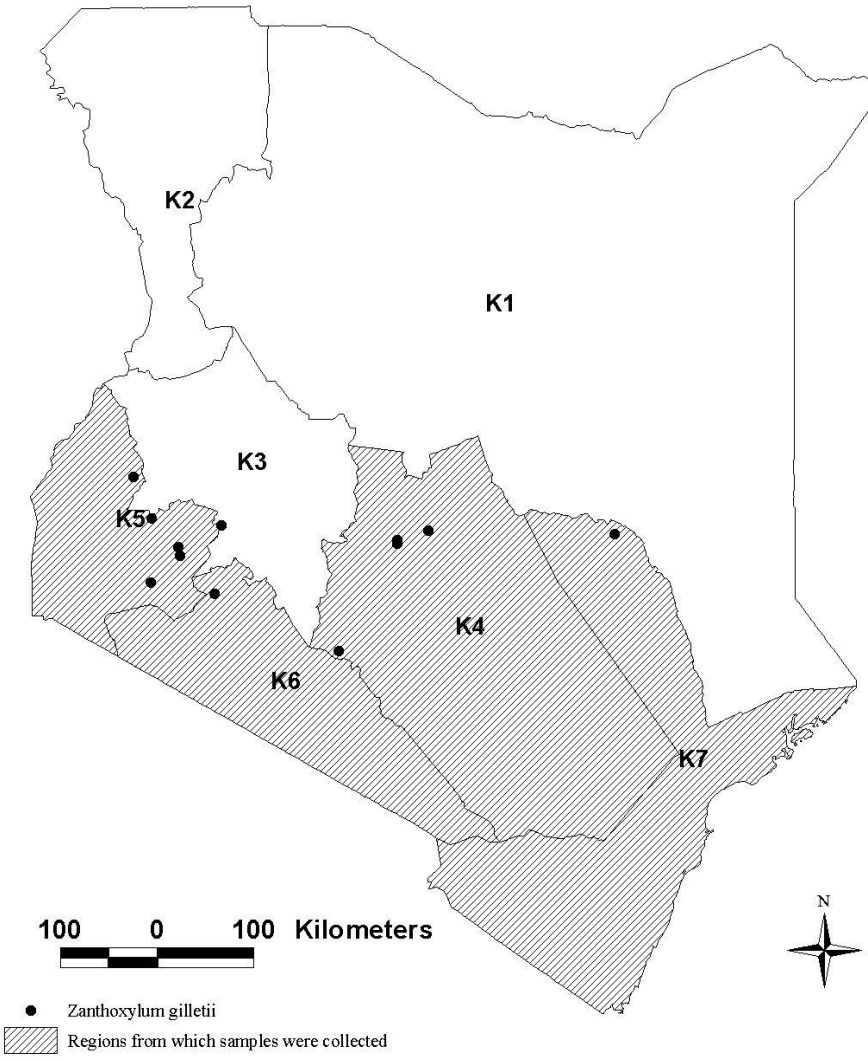
CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and preparation

Plant samples (leaf, root and bark) were collected randomly from trees growing in the Kakamega forest, Southern Mau forest block and Mount Kenya region at Chogoria forest station; K4, K5 and K6 respectively (Fig 2). Samples collected together with their voucher specimen were authenticated in the field, transported and kept at the East African Herbarium at the National Museums of Kenya (NMK) where the voucher specimen were deposited (Voucher numbers, Table 2).

These regions (K4, K5, K6 and K7) represent the distribution of *Z. gilletii* in Kenya. Samples were collected from different plants during the day between 1000 hours and 1300 hours. Samples from Kakamega forest were collected from an area with an altitude of 1649 metres above sea level with a GPS location of 00° 20’ 195 N ; 034º 52’ 607 E. Samples from Mau forest block were collected from an area with an altitude of 2340 metres above sea level and with a GPS location of 00° 47’ 357 S ; 035º 34’ 831 E, Rift valley Province, Sogoo - Sitotuet village, Mulot Division of Narok District. Samples from Mount Kenya region were collected from an area with an altitude of 1768 metres above sea level at Chogoria forest station of Central Province with the GPS location of 00° 14’ 115 S ; 037º 35’ 031 E. The records for distribution were retrieved from the National Museums of Kenya database and from the available voucher specimens at the National Museums of Kenya. Species distribution is illustrated in the distribution map shown in Fig 2. Samples were pretrated by cleaning with a hard brush to remove dirt and soil particles in root and barks.



K1 - North Eastern Kenya.
K2 – West of Lake Turkana, Lokichogi, Lodwar
K3 - Central Rift valley, Kitale Maralal.
K4 – Mt Kenya, Nairobi and Central Kenya.
K5 – Western Rift Valley, Kakamega, Kericho.
K6 – Southern Rift Valley, Mau, Narok.
K7 – Coastal Kenya and Eastern.

Fig 2: Distribution of *Z. gilletii* in different geographical regions in Kenya (Map source: Seeds for Life Project, NMK database extract).

These were then chopped into very small pieces and and dried on laboratory tables at room temperature. Samples were then ground into fine powder in a grinder and sieved to give particle size of 50–150 mm. these were kept in air tight paythene bags. The plant powder was then stored in labeled zip locked polythene bags at room temperature before they were transported to the UK where extraction and phytochemical analysis on the 9 samples was carried out at the Jodrell Laboratory. Here the samples were documented in the laboratory database on arrival. All samples were given an accession database number in the Biological interactions' (BI) accession database (Table 2).

Table 2: The Voucher specimen and the BI accession database numbers for *Z. gilletii* samples from Kenya:

Voucher numbers	**Database no.**	**Plant part**	**Collection region**
NMK-125/09 (a) - (Gaya and Daniel, 2009)	BI 18826	Root	Kakamega Forest
NMK-125/09 (b) - (Gaya and Daniel, 2009)	BI 18827	Leaf	Kakamega Forest
NMK-126/09 - (Gaya and Daniel, 2009)	BI 18834	Bark	Kakamega Forest
NMK-128/09 - (Gaya and Daniel, 2009)	BI 18828	Root	Mt. Kenya, Chogoria
NMK-128/09 - (Gaya and Daniel, 2009)	BI 18829	Bark	Mt. Kenya, Chogoria
NMK-128/09 - (Gaya and Daniel, 2009)	BI 18830	Leaf	Mt. Kenya, Chogoria
NMK-127/09 -(Gaya and Daniel, 2009)	BI 18831	Bark	Mau Forest, Narok
NMK-127/09 - (Gaya and Daniel, 2009)	BI 18832	Root	Mau Forest, Narok
NMK-127/09 - (Gaya and Daniel, 2009)	BI 18833	Leaf	Mau Forest, Narok

3.2 Extraction of plant material for antimicrobial and antioxidant activity tests

Plant material tested against *Saccharomyces cereviciae* was extracted in 3 different solvents; water, methanol and ethyl acetate (EtoAc) to test for the most suitable solvent for extracting the samples. Plant material, 50 mg (0.05 g) was extracted in 1 ml of each of the three solvents listed above. These were left to extract for about 2 hours. The extracts were then centrifuged for 3 minutes and filtered into clean vials labelled with their respective BI numbers and solvent.

The samples tested against other fungal and bacterial pathogens *Cladosporum herbarum*, gram positive *Bacillus subtilis* and gram negative *Pseudomonas syringae* was each extracted in both chloroform ($CHCL_3$) and 80% aqueous methanol (MeOH) for selective extraction of polar and non polar compounds respectively.

3.2.1 Extraction of plant material for TLC, HPLC and LC-MS analysis

Nine bottles were labeled with BI accession database numbers (Table 2). Into each of the labeled sample bottles, 0.5 g of dry powdered samples were weighed. To each vial, 5 ml of 100% methanol was added and shaken well. These were left to stand in the fume cupboard for 3 days to extract. The extracts were filtered to remove the plant material and stored at room temperature prior to use. The extracts were later analysed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and by liquid chromatography mass-spectrometry (LC-MS).

3.3 Testing antimicrobial activity of *Z. gilletii* extracts

Test microorganisms were obtained from the stock culture maintained at the Jodrell Laboratory. Test organisms were: *Saccharomyces cereviciae* and *Cladosporum herbarum* (IMI 300461); the gram positive *Bacillus subtilis* (IMI 347329) and gram negative *Pseudomonas syringae* (IMI 347448) bacteria. Ninety six well plates were used for the different bioassays in combination with TLC bioautographic assays for the antimicrobial tests.

3.3.1 Efficacy test of *Z. gilletii* extracts on *Saccharomyces cereviciae*

Plant materials were extracted in 3 different solvents; water, methanol and ethyl acetate (EtoAc) to test for the most suitable solvent for extracting the samples. Plant material, 50 mg (0.05 g) was extracted in 1 ml of each of the three solvents listed above. These were left to extract for about 2 hours. The extracts were then centrifuged for 3 minutes and filtered into clean vials labelled with their respective BI numbers and solvent.

3.3.1.1 Broth preparation

YPD powder broth (10 g) was dissolved in 200 ml of distilled water to make broth. All bottles to be used in the experimental procedure were cleaned well; then together with the pipette tips and prepared broth, all were autoclaved for 2 hours. The autoclaved broth was poured into the smaller autoclaved clean bottles. Yeast *Saccharomyces cereviciae* (TSC Bioscience Lab) was used in the experiment. The yeast was placed into a Petri dish and incubated overnight. Hands were sterilized using 70% ethanol. All operations were carried out in the lamina flow hood, taking care to avoid any contamination. A ninety six well plate was used to run the bioassay

To test the three different extracts, only 3 samples were used and the experimental set up was in duplicates; 72 of the 96 wells were used. Using sterilized pipette tips, 50 µl of broth was put into all 72 wells and then 49 µl of broth was added into the first row to make a total of volume 99 µl of broth in that row. Sample extracts (1 µl), were added to the first row in duplicate beginning with water extracts then MeOH and EtAc respectively, making a total volume of 100 µl of 1% extract in the first row.

3.3.1.2 Serial dilution of extracts

Using a multi channel pipette, 50 µl of sample and broth from the first row; extracts in the wells were serially diluted using the 50 µl from the first row. This 50 µl of sample and broth was then moved from the first row into the second and the same mixing process repeated at the same time reducing the concentration by half in all wells in the row. This process was repeated for all rows, effectively lowering concentration by half each time. After diluting the last row; the 50 µl of sample and broth used for dilution was withdrawn from last row and discarded, leaving all the wells with a uniform volume of 50 µl and a decreasing concentration as shown below:

Row (B2-7) = 1%	Row (C2-7) = 0.5%
Row (D2-7) = 0.25%	Row (E2-7) = 0.125%
Row (F2-7) = 0.0625%	Row (G2-7) = 0.03125%

3.3.1.3 Controls, cell preparation, inoculation and incubation

Duplicate wells were used for both positive and negative controls. Antifungal drug nystatin was used as positive control while blank wells were used as negative control. Two duplicate

wells used as positive controls had 50 µl of broth introduced into the wells. The other set of duplicate wells used as negative controls had 100 µl of broth introduced into them. *Saccharomyces cereviciae* was incubated overnight. Three cuvettes were needed to measure the cell volume: 1000 µl of broth was pipetted into 2 cuvettes to be used as a control. A small quantity of yeast cells were removed from the Petri dish and smeared on the inner wall of a bottle containing broth, this was shaken well. From the resulting cell suspension, 1000 µl was put into the third cuvette. Cell volume was determined using a 1601 Shimadzu UV- visible spectrophotometer. Operated as per standard operation procedure detailed below: Power was turned on, wavelength set at 600 nm; two cuvettes with broth placed in the 2 holes with transparent clear side facing the origin of the parallel UV rays. Machine was then auto zeroed to calibrate with the broth cuvettes inside; One of the cuvette was removed and replaced with the one containing cells. A reading was taken (0.432). Required cell volume should be about (0.100). Remaining cells in the bottle were diluted down three times (X 3) to attain 0.1 cell volume as standard requirement. About 3 ml of broth was added to the cell in the bottle to obtain this concentration.

Using a multi chanel pipette, 50 µl of inoculum was added to all the 72 wells and positive controls. The negative control wells had no yeast cells introduced into them. Inoculation was achieved beginning from the lowest concentration (0.03125%) to the highest concentration (1%). The plate was then covered and placed into an incubator with the temperature set at 30 ^{o}C and left to incubate for 24 hours.

3.3.2 Testing for antifungal activity of *Z. gilletii* extracts

Ground plant material (BI 18826 – 18834) was each extracted in both chloroform ($CHCL_3$) and 80% aqueous methanol (MeOH) for selective extraction of polar and non polar compounds respectively. Eighteen rotational test tubes were labelled with BI numbers appropriately for the extracts in the two sets of solvents. Approximately 1 g of plant material was placed into each of the samples in the test tubes; 5 ml of respective extracting solvents were added. The samples were agitated for two hours. After two hours of extraction, samples were centrifuged at 1000 rpm for 3 minutes. Using a pipette, the MeOH and $CHCl_3$ extracts were transferred into pre-weighed labelled vials. Care was taken to avoid taking up any solid particles in the clear extracts. Extracts were dried in heat block at 40 °C in the fume cupboard and re-weighed after drying.

3.3.2.1 Silica gel TLC bioautograhic assay

Two solvent systems were used in the separation of plant extracts. A mixture of chloroform and acetone (9:1, v/v) was used for separation of the chloroform extracts; whilst a mixture of chloroform, methanol and water (73:24:3, v/v/v) was used for the aqueous methanol extract. Two aluminum backed TLC plates (″10 x 20″) were used. Plates were labeled and marked with extract numbers for both the methanol and chloroform extracts. Using very fine (200 µl) capillary pipette tips, an even line of 1 cm was drawn with extracts of each of the samples. Two identical plates were prepared for the purposes of comparison. Two plates each were placed into each of the two different solvent systems to develop. Plates were then removed and solvent front quickly marked using a pencil. The plates were then left to dry in the fume

cupboard for 30 minutes. After which, the plates were observed under UV light in which short wavelength UV absorbing compounds were observed at 254 nm whilst the long wavelength UV- absorbing compounds were viewed at 366 nm; bands observed at the two different wavelengths exhibited different colours. For one of the duplicate plates, observations were made under the UV light and the points at which different colours fluoresced were marked out using a pencil and their respective Rf values calculated using the formula as detailed in section 2.1.4.

3.3.2.2 Fungal spray

Fungal spore suspension was prepared by adding spores of fungus, *Cladosporium herbarum* to a malt extract liquid medium (Oxoid L39, 2%) using a sterilized spatula end. The spore suspension was then sprayed evenly over one of each of the duplicate plates for samples extracted in aqueous methanol and chloroform. The sprayed plates were placed over plate holders with a little water at the bottom to create a humid environment for fungal growth and the covers replaced. The two sets were then incubated at 25 °C over 72 hours.

3.3.2.3 Quantitative antifungal assay of active extracts

Based on the results from section 2.3.3, four extracts (BI 18826, BI 18834, BI 18828 and BI 18832), were active. Therefore, four clean sample bottles were labeled (Table 6). The extracts which were dissolved in acetone were centrifuged and transferred into the clean, labelled and pre-weighed empty bottles; these were air dried.

Table 3: Calculated dry weight of the extracts active against fungus *C. herbarum*

Samples	Bottle + sample (g)	Empty bottle (g)	Extract wt (g)
BI 18826	10.6795	10.6778	0.0017
BI 18834	10.6511	10.6357	0.0154
BI 18828	10.5150	10.4784	0.0366
BI 18832	10.5463	10.5210	0.0253

3.3.2.4 Microtitre plate assay

Samples were set up on a ninety six well plate. Each of the experimental samples on the plate was introduced in duplicates. Solvent used throughout the experimental set-up was deionised water, which was also used as the negative control. Positive control was the antifungal drug nystatin (800 µg/ml). The stock solution for the positive control was prepared by dissolving 0.8 mg of nystatin in 1 ml of water. The fungus used in the experiment was *Cladosporium herbarum* (IMI 300461). Fungal spore suspension was prepared by adding fungal spores to malt extract liquid medium (Oxoid L39, 2%), using a sterilized spatula end. Stock solutions were made of all the experimental samples; BI 18826, BI 18834, BI 18828, and BI 18832 (800 µg/ml).

3.3.2.5 Plate preparation

All ninety six wells in the plate were used. The first two columns were used as positive control wells containing nystatin and inoculum. The last two columns were used as negative control wells containing water and inoculum only. Into the first two wells of each of the first two rows, 100 µl of 800 µg/ml of stock solution for extracts and positive control drug were

added. Into the second wells of the second row, already containing stock solutions, 100 µl of water was added uniformly. The same volume of water was added to all remaining wells from second well in second row up to the eighth well in the eighth row. All the wells had a volume of 100 µl, except all wells in row number B which had a volume of 200 µl. Into rows A and B, columns 1, 2 (used as positive control), 100 µl of 800 µg/ml 100 µl positive control was added.

Into the entire columns 11 and 12 (used as the negative control); 100 µl of water was added making the total volume of the negative control 100 µl. Into all other wells in the plate; 100 µl of extract stock was added with BI 18826 into columns 3, 4; BI 18834 into columns 5, 6; BI 18828 into columns 7, 8; and BI 18832 into columns 9 and 10. Each of the experimental samples was applied in duplicates as shown (Figure 3).

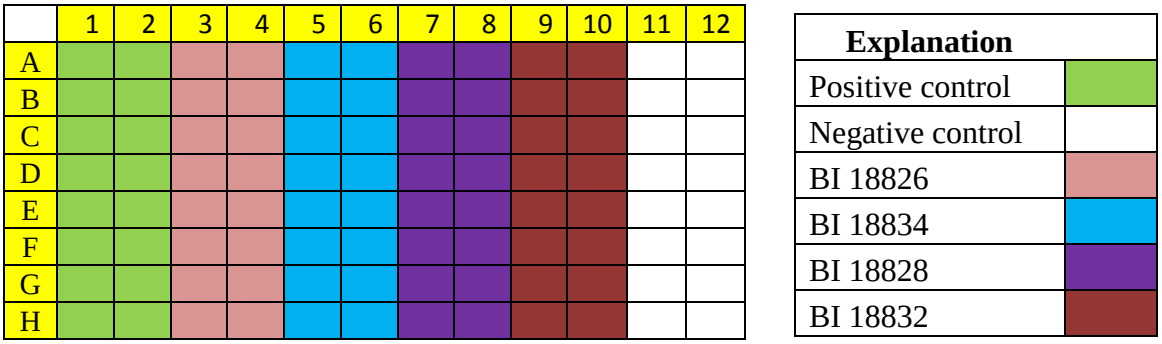


Fig 3: Distribution of extracts on the in the 96-well microtitre plate.

3.3.2.6 Serial dilution of extracts

Using a multi channel pipette, 100 µl of samples from row B were mixed up by continuously drawing it in and out of the well seven times before the same volume was moved to the next row (C) and the same process repeated until the last row (H). After the last column (A), the 100 µl volume of the sample used in the dilution was discarded; leaving all the ninety six wells uniform with 100 µl of sample and serially diluted wells which had the following concentration after inoculation:-

Row A = 400 µg /ml
Row B = 200 µg/ml
Row C = 100 µg/ml
Row D = 50 µg/ml
Row E = 25 µg/ml
Row F = 12.5 µg/ml
Row G = 6.25 µg/ml
Row H = 3.125 µg/ml

3.3.2.7 Inoculation with fungal spores

Fungal spore suspension in malt extract liquid medium (oxoid, L39, 2%) was poured into the sample tray. Using a multi channel pipette, 100 µl of fungal spore suspension was introduced to all ninety six wells, beginning with row containing the lowest concentration (H = 3.125 µg/ml). The plate was covered and placed into a zip locked polythene bag which was then sealed. This was then incubated at 25 °C over 48 hours. The antifungal activity of the extracts were observed as clear wells without fungal growth and recorded as the minimum inhibitory concentration (MIC) for the test samples.

3.3.3 Testing for antibacterial activity of *Z. gilletii* extracts

3.3.3.1 Preparation of plant extract and silica gel TLC analysis of extracts

Extract preparation was the same as for the antifungal test (sections 2.2.1and 2.4). However, there were modifications in the final development of the TLC autobiographic assay as follows: The Gram positive *Bacillus subtilis* (IMI 347329) and Gram negative *Pseudomonas syringae* (IMI 347448) were used as bacterial organisms. chloramphenicol, an antibacterial drug (800 µg/lm) was used as a positive control while the negative controls were the blank wells. Pre- coated aluminium backed TLC plates (20 x 20 cm), silica gel, (F_{254}, 5334) were cut into two and used; while p-iodonitrotetrazolium violet (2-[4- indophenyl]-3-[4-nitrophenyl]-5- phenyltetrazolium chloride (INT) was used as dye which shows a pink colour in the presence of bacteria; while inhibition of bacterial growth is shown by a lack of pink colour.

3.3.3.2 TLC bioautographic assay

The TLC plates were developed in duplicates for both MeOH and $CHCl_3$ extracts with the same solvent system as in antifungal assay being used. Plates were dried and observed under the UV light (254 and 366 nm). Yeast extract peptone (YEP) was prepared by dissolving nutrient agar (2.8 g in 100 ml) in deionised water which was heated to 40 °C and constantly shaken. *Bacillus subtilis* and *P. syringae* were picked up with a glass rod from colony on agar plate and dispersed in their respective bottles of YEP medium which was already cooled down to 30 °C to form bacterial suspension in medium. The seeded medium was then poured onto the developed plates and allowed to set. The TLC plates were then put in zip locked

polythene bags and incubated overnight at 37 °C. After incubation, the plates were treated with INT (10 µg/ml) which was prepared by dissolving INT powder (0.0005 g in 50 ml of 10% aqueous EtOH). The plate was further incubated for 24 hours. Clear inhibition zones could be observed as white patches against a pink background. Following the outcome of the bioautographic assay, extracts were further evaluated by 96 well microtitre assay to determine minimum inhibitory concentration (MIC) of active extracts.

3.3.3.3 Microtitre plate assay

Extracts in MeOH and $CHCl_3$ on different sets of plates were both used in this assay. Yields of extracts were determined (results section) and dry extracts were dissolved in the same solvents as were used during the TLC bioautographic assay (section 2.6.4) to make stock solution of extracts (800 µg/ml); these were diluted to give six concentrations (400, 200, 100, 50, 25, and 12.5 µg/ml). Chloramphenical (800 µg/ml) dissolved in respective solvents (MeOH for methanol extracts and propan 2-ol for $CHCl_3$ extracts) for each extract was used as positive control, while blank wells were used as negative control for each plate. Extracts and the positive control were put into wells in duplicates and serially diluted to achieve the six concentrations above. Plates were then placed in the drying block in the fume cupboard to evaporate the solvents.

YEP medium was prepared by dissolving yeast extract (0.3 g), bacterial peptone (1.0 g) and NaCl (0.5 g) in deionised water (100 ml). The medium in two different containers (for *B. subtilis* and *P. syringae*) were seeded with the two different bacteria in the respective containers. An aliquot of seeded YEP (200 µl) was placed in each well and left on the rotary shaker (120 rpm) for one hour to dissolve samples in medium. The final concentrations after

addition of seeded YEP were 200, 100, 50, 25, 12.5 and 6.25 µg/ml. The plates were incubated at 37 °C for twenty four hours; after which 20 µl of INT solution (50 µg/ml) was added to each well and incubated for a further 24 hours. Inhibition of bacterial growth was observed as a failure to develop a pink colour.

3.4 Fractionation of active extracts by HPLC and LC MS

The extracts identified as active against *Bacillus subtilis, Pseudomonas syringae* and *Cladosporium herbarum* (Table 11) were prepared for HPLC fractionation and a re-run in the LC MS to identify the specific compounds responsible for the activity. Concentrations similar to those observed as the minimum inhibitory concentration (MIC) (Table 11) were prepared for each of the active extracts.

The extracts were then fractionated over the HPLC and the LC MS columns with solvent system similar to those shown in (Tables 6 and 7). Fractions were collected automatically into ninety six well microtitre plates. From the prepared extracts, an injection of 40 µl was made. Fractions were then collected into the microtitre plate at intervals of 15 seconds each with a ten minute allowance between the injections to allow for column wash. Fractions were collected at very close intervals to achieve more precise results. Fractions collected in the first four minutes were not considered. A total of eighteen plates were collected.

After collecting the fractions, the microtitre plates were put in the fume cupboard to evaporate the solvents and dry out over 24 hours. After which, 200 µl of inoculum were added to all the wells of the different labelled plates for each of the different test (*B. subtilis, P. syringae* and *C. herbarum*). The wells containing fractions collected in the initial six minutes for all plates

were treated with chloramphenical (50 µl) and nystatin (50 µl) as the antibacterial and antifungal positive controls respectively. The plates containing medium seeded with bacteria were incubated at 37 °C for twenty four hours before being treated with INT. Those plates containing medium seeded with fungal spores were incubated at 25 °C for forty eight hours. Observations were then made and time at which fractions containing the active compounds eluted were recorded as clear wells similar to the positive control wells.

3.5 Testing for antioxidant activity

The *Z. gilletii* ethanol extracts were screened for their antioxidant activity using the Nitric Oxide (NO) scavenging activity assay; good antioxidant activity would be acceptable if the NO scavenging activity is in the range of (50 – 100%) (Balakrishnan *et al*., 2009). Nitric oxide scavenging assay measures the production of nitric oxide by its reaction with non-fluorescent substrate to yield a fluorescent compound.

3.5.1 Preparation of stock solutions

Stock solution of the control was prepared by dissolving 10 mg of carboxy-PTIO potassium salt in 0.833 ml of DMSO to make a solution with concentration of 40 mM which was stored at -20 °C before use. The substrate (DAF-FM) was prepared by dissolving 1 mg DMSO to form stock solution with concentration of 6 mM which was also stored at -20 °C prior to use. For the assay, 2.5 µl of DAF –FM stock was diluted in 4.998 ml of water (0.020 mM) just prior to use. A 10 ml solution of sodium nitroprusside (SNP) with a concentration of 20 mM was prepared fresh on the day of use by dissolving 59.59 mg in 10 ml of water; this was protected from direct light.

3.5.2 Preparation of the 96 well plate and running of the assay

Black coloured 96 well plate was used in this assay to avoid any "leakage" of fluorescence between neighbouring wells and to increase efficiency in the reading of absorbance. Into all wells 48 µl of water was added, then 2 µl of DMSO, carboxy-PTO or the extracts under investigation (BI 18826 – BI 18834) as per the planned plate lay out (Table 4). Into each of the wells 50 µl of DAF-FM solution was added; then into the control and extract wells, 100 µl of SNP was added before adding 100 µl of water into blank wells. The plate was then incubated for 11 minutes at room temperature in full light before being placed into the plate reader. Fluorescence reading was done with an excitation at 485 nm and emission at 538 nm.

Table 4: Arrangement of samples on the 96 well plate during the NO assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	18826	18826	18827	18827	18834	18834	18828	18828	18833		PTIO +SNP	PTIO +H_2O_2
B	18826	18826	18827	18827	18834	18834	18828	18828	18833		PTIO +SNP	PTIO +H_2O_2
C	18826	18826	18827	18827	18834	18834	18828	18828	18833		PTIO +SNP	PTIO +H_2O_2
D	18826	18826	18827	18827	18834	18834	18828	18828	18833		PTIO +SNP	PTIO +H_2O_2
E	18829	18829	18830	18830	18831	18831	18832	18832	18833		SNP+ H_2O_2	EtOH + H_2O_2
F	18829	18829	18830	18830	18831	18831	18832	18832	18833		SNP+ H_2O_2	EtOH + H_2O_2
G	18829	18829	18830	18830	18831	18831	18832	18832	18833		SNP+ H_2O_2	EtOH + H_2O_2
H	18829	18829	18830	18830	18831	18831	18832	18832	18833		SNP+ H_2O_2	EtOH + H_2O_2

3. 6 Analysis of samples by thin layer chromatography (TLC)

In this research, solid phase sorbent was mounted on aluminium backed silica plates without florescent indicators F254 (sigma code 5334). Approximately 20 µl of each methanol extract was applied to the selected TLC plates (10 x 20 cm, half width of normal plates). Plates were allowed to dry between each 5 µl application. Standard of papaverine chloride (in 100% methanol) at a concentration of 2 mg/ml was then applied (5 µl), to the plate. The plates were developed in glass tanks in the solvent systems listed in Table 5. Papaverine chloride was selected as a standard because of the alkaloid's structural similarity to some of the alkaloids known to occur in *Z. gilletii*; and this was confirmed by the fact that its Rf values after the TLC analysis were very close to those of the identified alkaloids in this investigation (Svendsen and Verpoorte, 1983).

The solvent front was marked on the plate in pencil immediately on removal from tank. The plates were then allowed to air dry in a fume cupboard. After which, they were observed under UV light and the UV absorbing spots of varying colours were circled in pencil. Photographic illustrations of the observed UV spectra are shown in (Fig 6). TLC-UV spectra were observed under the UV spectrophotometer - model UV- Desage at wavelengths of 254 and 366 nm.

Table 5: Solvent systems used in TLC of extracts of *Z. gilletii*

Solvent System	**BAW** - 40 ml Butan-1-ol: 10 ml Acetic acid: 50 ml Water	**MAC*** - 10 ml Methanol: 1 ml Ammonia: 89 ml Chloroform:
Plates	Silica 5553	Silica 5577
Standard(s)	Papaverive Chloride	Papaverine Chloride
Spray(s) used	Dragendorffs' reagent	Dragendorffs' reagent

BAW = Butan-1-ol, Acetic acid and Water **MAC** = Methanol, Ammonia and Chloroform

The plates were further developed by spraying with the dragendorffs' reagent and the retention factor (Rf values) of each compound and the standards were calculated using standard formula (Svendsen and Verpoorte, 1983). The Rf values were calculated using the formula below:

$$Rf = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent front}}$$

After viewing plates under the UV light, and before spraying with any reagents, the plates were scanned using a Camag TLC Scanner 3 model under a range of wavelengths from 254 – 350 nm. Resulting data for different colour peaks, heights and bases of the peaks observed at 350 nm were analysed and interpreted as shown in (Apendix 1). The appearance of different colours in the TLC plate and formation of the various peaks viewed after the TLC scan represents the separation of the different groups of compounds present in the extracts of *Z. gilletii*.

3.6.1 Alkaloid analysis

The sample was filtered, dried then rehydrated in 10 ml of 0.5 M hydrochloric acid. To this acidified extract was added an equal volume (10 ml) of chloroform in a separating funnel, this was shaken and left to settle. The bottom layer (acidified chloroform) was drained into a labeled bottle and a second equal volume (10 ml) of chloroform was added to the aqueous extract, this was shaken again and left to settle. The chloroform layer was drained out and added to the first collection. The acidified chloroform layer was then air dried in the fume cup board. The remaining acidified aqueous layer was basified with concentrated ammonia approximately 1-2 mls and the pH checked to confirm that it had changed to alkaline using pH paper (Sigma). An equal volume of chloroform (10 mls) was then added to the basified sample in separating funnel, shaken and left to settle. The bottom layer (basified chloroform) was then drained out into a labeled vial and a second equal volume of chloroform added to the aqueous extract. This was shaken again and left to settle before the chloroform layer was drained again and added to the first collection. The basified chloroform layers were placed to dry in the fume cupboard. The remaining aqueous layer was finally dried in a heat block at 40°C. The two chloroform layers (acid + basic) were rehydrated in 100% methanol. The dry aqueous layer was re-hydrated in 100% water. All samples were kept in the cold room prior to analysis. The alkaloid partition fractions were run in confirmatory alkaloid tests using TLC and LC-MS analyses. For the TLC analysis, the same solvent systems shown in the Table 2.0 were used.

3.7 Analysis of samples by HPLC and LC-MS

Plant material was extracted in 100% methanol (100 mg/ml) for three days. The extracts were then centrifuged at 10,000 rpm for 2 min and using a glass pipette the clarified extracts were transferred into small clean labelled vials (BI 18826 – 18834 stock solutions). Centrifugation was undertaken to remove all particulates from the extracts, thus preventing the possibility of column blockages. From the stock solution 1ml was placed into an HPLC vial (Chromacol) and 300 µl into a smaller LC-MS vial (Chromacol).

HPLC analyses were carried out on a Waters 600 pump with a 600E controller, Waters 717plus autosampler coupled to a Waters 996 photodiode array detector; this was all controlled through a PC workstation running Empower software. Detection was achieved by scanning through 200-550 nm scans per second. Data were collected for 30 minutes and there was a delay of 10 minutes between injections to ensure column equilibrium between samples. The column used was a Phenomenex Luna C18 capillary column 250 mm x 4 mm i.d. x 5 µm. Extracts were analysed using gradient solvent programmes as detailed in (Table 6) with a flow rate of 1 ml/min with in-line degassing and an injection volume of 20 µl.

Table 6: Gradient conditions for HPLC analyses of *Zanthoxylum* extracts

Time	A	B	C
0	15	75	10
20	90	0	10
25	90	0	10
27	15	75	10
30	15	75	10

Gradient solvent programme:
A- Methanol
B- Water
C- 5% Acetic acid in methanol

Initial LC-MS analyses were carried out on a Waters Alliance 2695 separations module, mass detection was achieved with a Waters Micromass ZQ MS with an Electrospray source (ESI) operating in positive and negative mode. Ultraviolent detection was achieved with a 2996 PDA scanning through 200-500 nm, all instruments were controlled through a PC running MassLynx software. Chromatography was achieved on a Phenomenex Luna C18 column 150 mm x 4.6 mm i.d. x 3 µm using a gradient as detailed in (Table 7), with a 0.5 ml/min flow rate and injection volume of 10µl.

Table 7: Gradient conditions for LC-MS analyses of *Z. gilletii* extracts

Time	A	B	C
0	0	90	10
20	90	0	10
25	90	0	10
27	0	90	10
37	0	90	10

Gradient solvent programme:
A- Methanol
B- Water
C- 1% Formic acid in acetonitrile

Accurate mass LC-MS analyses were carried out on a ThermoScientific LTQ Orbitrap XL with an Electrospray source (ESI) operating in positive or negative mode, the LC system was an Accela system; all data was analysed using XCalibar software. Chromatography was achieved on a Phenomenex Luna C18 column 150 mm x 3 mm i.d. x 3 µm. With a gradient the same as for ZQ analyses (Table 2.2) but with a 0.4 ml/min flow rate and a 5µl injection volume. Samples were run in both positive and negative mode in full ms scan mode to allow

data to be recorded and to allow accurate mass determination. Compounds present in the extracts were identified on their UV spectra and retention times (HPLC analysis) and for LC-MS analyses it was based on their accurate mass, molecular formula and also mass fragmentation pattern (μs/μs) compared with known compounds reported in the literature.

3.8 Data analysis

Data resulting from the minimum inhibitory concentration (MIC) assays carried on the identified biologically active extracts were entered in Excel. Analysis of Variance (ANOVA) was used to answer questions on probability that the variation among group of sample means occurred as a result of randomly collected samples from a common population. The quantitative data (Table 11) was summarized using descriptive statistics namely; mean, standard deviation, averages and variance. T-test was used to separate means. The F values, F- critical values and P- values were also calculated to determine significant difference at 95% confidence level.

CHAPTER FOUR

RESULTS

4.1 Antimicrobial activity of *Z. gilletii* extracts

4.1.1 Antifungal activity

There was no antimicrobial activity observed from all the nine MeOH and $CHCl_3$ extracts of *Z. gilletii* against the growth of yeast cells- *Saccharomyces cerviciae*. The extracts did not inhibit the growth of yeast cells (*S. cereviciae*). The results also indicate that the extracts were not cytotoxic to the yeast cells. Because of the lack of activity, no further extracts were tested against these yeast cells.

Investigations carried out to test the activity of MeOH and $CHCl_3$ extracts of *Z. gilletii* against *Cladosporium herbarum* using TLC bioautographic assays revealed that the plate with the $CHCl_3$ extracts had clear white zones against a dark blue background. This was observed in the three root extracts from Kakamega, Mt. Kenya and Mau (BI 18826, 18828 and BI 18832) respectively and a bark extract (BI 18834) from Kakamega. The rest of the extracts as well as plates with MeOH extracts were covered uniformly with fungal growth, therefore not studied further in this assay.

The extracts showing clear inhibition zones were considered for further investigation in the 96 well microtitre assay as described earlier. The microtire assay showed that the bark extract from Kakamega (BI 18834) was the most active against *C. herbarum* with MIC of 50 µg/ml, followed by root extracts from Mt. Kenya and Mau (BI 18828 and 18832) which had MIC values of 100 µg/ml each after incubation period of 48 hours, while the positive control

nystatin inhibited bacterial growth even at the lowest concentration of 3.125 µg/ml. Poor antimicrobial activity was witnessed in root extract from Kakamega (BI 18826) which only showed slight activity at the highest concentration (200 µg/ml). It was, however, notable that all the plant extracts lost activity against the fungus after longer incubation periods (72 hours) while nystatin still maintained activity at 6.25 µg/ml after the same prolonged incubation period (Table 11).

4.1.2 Antibacterial activity of *Z. gilletii*

To test antibacterial activity TLC bioautographic assays were carried to determine antimicrobial activity. Afterwards, the extracts which showed antimicrobial activity were analysed using the 96 well microtitre assay to determine MIC of the extracts.

4.1.3 TLC bioautographic assays

Results from the TLC bioautographic assays for MeOH and $CHCl_3$ extracts of *Z. gilletii* showed clear white patches against a pink background in some extracts. The plates with MeOH extracts of roots from Kakamega, Mt. Kenya and Mau (BI 18826, 18832 and 18828), leaf from Mau (BI 18833) and bark from Kakamega (BI 18834) when sprayed with *B. subtilis* had white patches against a pink background; while a different set of plates with MeOH extracts of root, leaf and bark from Kakamega (BI 18826, 18827, 18834); root from Mt. Kenya (BI 18828) and a root extract from Mau (BI 18832) when sprayed with *P. syringae* had white patches against a pink background after 24 hours of incubation. These MeOH extracts were considered for further quantitative analysis in the 96 well microtitre assay to determine

the level of their antibacterial activity. On the other hand, plates run with $CHCl_3$ extracts of roots from the three regions (BI 18826, 18828, 18832), barks from Kakamega and Mt. Kenya (BI18834, 18829) when sprayed with *B. subtilis* had white patches against a pink background while those plates with extracts of roots from all three regions (BI 18826, 18828, 18832), bark from Kakamega (BI 18834) and Leaves from Kakamega and Mau (BI 18827, and 18833) when sprayed with *P. syringae* had clear white spots observed in them. These extracts showing antibacterial activity were also considered for a quantitative microtitre assay to determine the MIC associated with the observed activity.

4.1.4 Microtitre assays for MeOH extracts

The MeOH extract showing antibacterial activity against *B. subtilis* and *P syringae* in the TLC bioautographic assays were subjected to microtitre assays in triplicates. The root extracts from Mt. Kenya (BI 18828) was the most active against *B. subtilis* with MIC of 50 µg/ml followed by the root extracts from Kakamega (BI 18832) which had MIC of 100 µg/ml and the bark extracts from Kakamega (BI 18834) was weakly active at the highest concentration (200 µg/ml). The extracts of root from Kakamega and leaf from Mau (BI 18826 and BI 18833) did not show any activity even at the highest concentration after 25 hours of incubation (Appendix II). The positive control chloramphenical maintained activity even at the lowest concentration (3.125 µg/ml). The MeOH extracts tested had the MeOH root extracts from Mt. Kenya (BI 18828) as the most active against *P. syringae* with MIC of 50 µg/ml, while the root extract from Mau (BI 18832) had a MIC of 100 µg/ml. The bark extract from Kakamega (BI 18834) only inhibited growth of *P. syringae* at the highest concentration (200 µg/ml), while the root and bark extracts from the same region (BI 18826 and BI 18827)

did not show any activity even at the highest concentration (Appendix II) after 25 hours of incubation. The positive control wells remained active even at the lowest concentration (3.125 µg/ml) after the same period of incubation.

Differences among MeOH extracts from different plant parts and regions, tested against *B. subtilis* and *P syringae* was assessed using single factor analysis of variance (ANOVA) which revealed no significant difference among the root and bark extracts from Kakamega, Mount Kenya and Mau (P=0.05). (Appendix IV). Since there was no significant difference at 95% confidence level, no further analysis was necessary.

4.1.5 Microtitre assays for $CHCl_3$ extracts

The $CHCl_3$ root extracts from Kakamega (BI 18826) had the greatest activity against *B. subtilis* with a MIC of 50 µg/ml. Both bark and root extracts from Kakamega and Mau (BI 18834 and 18832) had MIC of 100 µg/ml, while root extracts from Mt. Kenya (BI 18828) only had weak activity at the highest concentration (200 µg/ml) after 25 hours of incubation (Tables 8). The positive control, chloramphenical maintained activity even at the lowest concentration (3.125 µg/ml) while bark from Mt. Kenya (BI 18829) did not show any activity even at the highest concentration (200 µg/ml).

The root extracts from Mau (BI 18832) was the most active against *P. syringae* with a MIC of 25 µg/ml, the most potent in this investigation. This was followed by root extract from Kakamega (BI 18826) with a MIC of 50 µg/ml, while both bark and root extracts from Kakamega and Mt. Kenya (BI 18834 and 18828) had MIC of 100 µg/ml each. The positive

control (chloramphenical) maintained activity at the lowest concentration (3.125 µg/ml) while leaf extracts from Kakamega and Mau (BI 18827 and 18833) showed no activity, even at the highest concentration (200 µg/ml) after 25 hours of incubation (Appendix II).

ANOVA analysis of activity of $CHCl_3$ extracts from different plant parts and regions tested against *B. subtilis* and *P syringae* revealed no significant difference (P= 0.05) among root and bark extracts from the different geographical regions. Other analysis comparing both chloroform and methanol extracts against both *B. subtilis* and *P syringae* also revealed no significant difference (P= 0.05) among root and bark extracts from the different geographical regions (Appendix IV). Results were compared for extracts from the different geographical regions and from different plant parts. There being no significant difference among extracts from different regions, post ANOVA analysis was therefore not necessary.

Table 8: Summary of microbial activity test showing specific MIC values for different Extracts

Extract ID	**Extract Type**	**Microorganism Tested**	**MIC** (µg/ml)
BI 18826 (root, Kakamega)	$CHCl_3$	*B. subtilis*	50
BI 18828 (root, Mt. Kenya)			200
BI 18832 (root, Mau)			100
BI 18834 (bark, Kakamega)			100
BI 18828 (root, Mt. Kenya)	MeOH	*B. subtilis*	50
BI 18832 (root, Mau)			100
BI 18834(bark, Kakamega)			200
BI 18826 (root, Kakamega)	$CHCl_3$	*P. syringae*	50
BI 18828 (root, Mt. Kenya)			100
BI 18832 (root, Mau)			25
BI 18834 (bark, Kakamega)			100
BI 18828 (root, Mt. Kenya)	MeOH	*P. syringae*	50
BI 18832 (root, Mau)			100
BI 18834(bark, Kakamega)			200
BI 18826 (root, Kakamega)	$CHCl_3$	*C. herbarum*	200
BI 18828 (root, Mt. Kenya)			100
BI 18832 (root, Mau)			100
BI 18834 (bark, Kakamega)			50

4.2 Sample bioassay guided fractionation

In order to determine the specific compounds responsible for the observed antimicrobial activity, further investigations were carried out through a bioassay guided fractionation of active extracts as described earlier (Table 9)

Table 9: Fractionation data, showing the retention time (minutes) at which the active compounds were detected.

Extract ID	Microbial tested	Extract type	Retention time(minutes) at which active compound eluted
BI 18826	*B. subtilis*	$CHCl_3$	(8-9), 9.30, (10.45-11), 12, 12.30, 13.30,14, 15.30, 16, 16.45, 18.45, (19.30-19.45), 20.45, 23-23.15.
BI 18834			9.15, (11.45-12.15), (13.30-13.45), (14.30-14.45), 16.30, 21, 21.45, 23.15, 23.45.
BI 18828			6.45, 9.45, 11, 12.45, (13.15-13.45), (14.30-14.45), (18.45-19.15), 21.45, 22.30.
BI 18832			7.15, 9.45, 10.15, 11.45, 15.45, (16.30-17)20.45, 22.45, (23.30-23.45)
BI 18826	*P. syringae*	$CHCl_3$	(6.15-13.30), 14.15, (14.45-15), (19.45-20), (20.45-21), 21.45, (23.30-23.45).
BI 18834			6.15, 6.45, 10.15, 13.45, (16.15- 16.30), (17.45 -18), (18.15-18.30), (20.15 -24)
BI 18828			8.15, (11.45-12), 13.30, 14.15, 14.45, 15.30, 17.30, 19.45, 22.15
BI 18832			7.30, (7.45-8), 9.15, (9.45-10), (10.45-11), 13.30, (20.30-21.15), (21.45-22.15), 22.15.
BI 18834	*B. subtilis*	MeOH	7.15, (7.45-8), 13.30, (13.45-14), 15.15, 18.30, (22.30-23), (23.45-24).
BI 18828			8.45, 10.30, 11.15, (13.45-14), 15.30, 18.30, 19.30, 23.30
BI 18832			6.30, (7.45-8), 8.45, 12.45, 13.30, (15.45-16), (16.45-17), (19.30-20), 22.15.
BI 18834	*P. syringae*	MeOH	6.30, 7.30, 8.15, 9.45,10.45, (13.45-14), (14.45-15), 16.15, (16.45-17), 18.45, (19.45-20), (20.45-21), 21.30
BI 18828			(6.45-7), (8.30-8.45), (9.45-10.30), 12.30, (12.45-13.15), 13.45, 14.30, (14.45-15), 16.45, 17.45, (18.45-19), 23.30, 24.
BI 18832			8.15, 10.15, 10.45, 11.30, (16.45-17), 19.45-20), 21.15, (23.30-24)
BI 18826	*C. herbarum*	$CHCl_3$	8.30, (11.45-12), 12.30, (13.45-14), 16.15
BI 18834			(6.45-7), 7.30, 8.15, 9.30, (10.45-11), 11.45, 12.30, 12.45, 13.45
BI 18828			(8.45-9), (10.30 -10.45), 11.30, (17.15-17.45), 18.15, 20.30
BI 18832			(6.15-6.30), 8.15, (12.45-13.15), 19.45 -20), (23.15-23.30).

4.3 Test for antioxidant activity

The results of the extracts of *Z. gilletii* (BI 18826 – BI 18834) in the antioxidant activity test showed that the extracts have no significant antioxidant activity. The NO scavenging activity ranging from -0.65 to 7.8 % (Fig 4) compared to the positive control ascorbic acid which is a standard antioxidant with NO scavenging activity ranging from 30 – 90% (Fig 5).

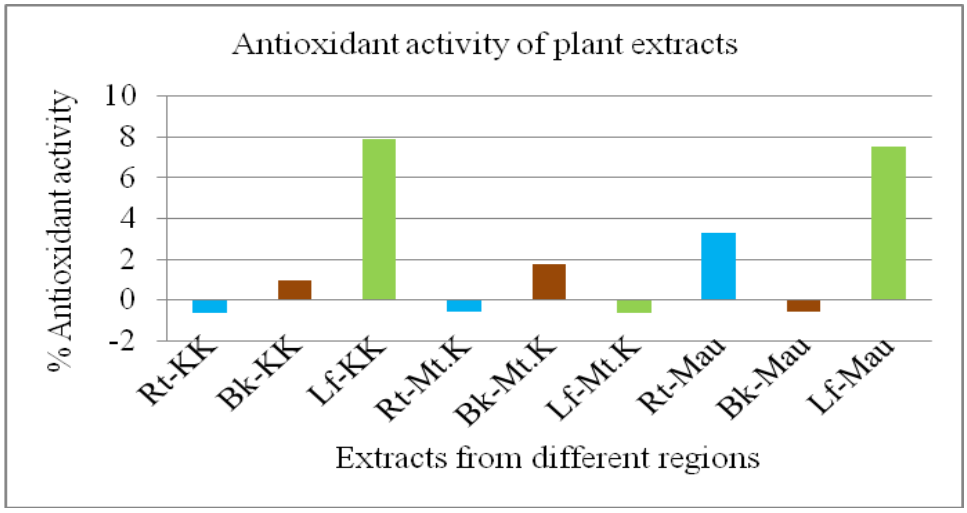


Fig 4: Percentage antioxidant activity for all plant extracts analysed.

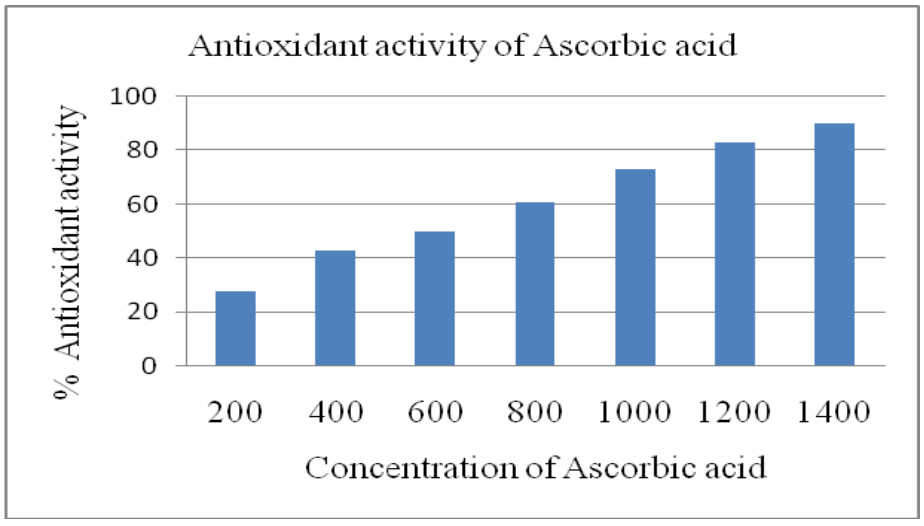


Fig 5: The percentage antioxidant activity of standard ascorbic acid.

4.4 TLC alkaloid test and UV light scan

The plates were developed in the BAW- solvent system which showed the best separation of the components of the extracts compared to those developed in CAW solvent system (Table 2). Colour displays from the origin towards solvent front were; blue, purple, and red for most leaf samples then yellow, purple, orange and blue in many root and bark samples; as viewed in the photographic illustration under the UV-light at 366 nm before treatment with Dragendorff's reagent (Fig 6).

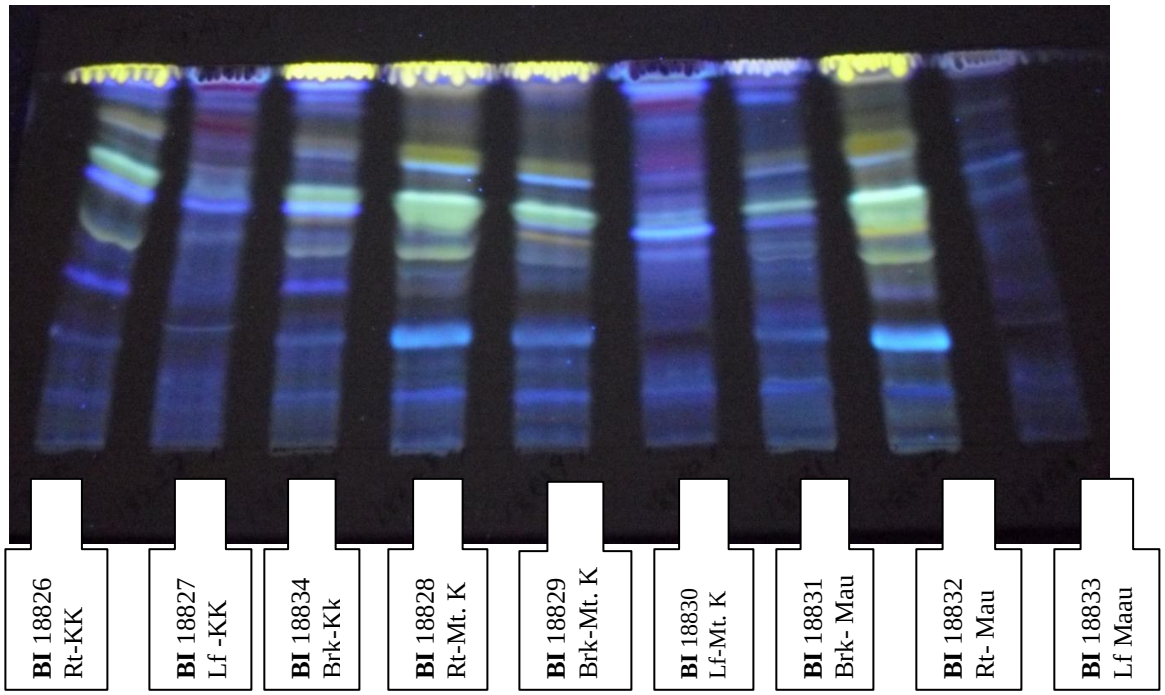


Fig 6: Photographic illustration of the TLC plate as visualised under UV- light at 366 nm.

Extract Identification codes: BI 18826 (root Kakamega), **BI 18827** (leaf Kakamega), **BI 18828** (root, Mt. Kenya), **BI 18829** (bark, Mt. Kenya), **BI 18830** (leaf. Mt. Kenya), **BI 18831** (bark, Mau), **BI 18832** (root, Mau), **BI 18833** (leaf, Mau), **BI 18834** (bark, Kakamega)

Solvent system: Butanol: Acetic acid: Water (BAW)

After the plates were sprayed with Dragendorff's reagent; there were distinct orange coloration showing the presence of alkaloids in the extracts as well as the standard papaverine chloride (STD) (Fig 6). Orange colour developed in different bands, indicating that there were different alkaloids with different retention times in the *Z. gilletii* extracts. The observed alkaloids were present in root, leaf and bark samples but separating at very different points on the plate sprayed with Dragendorff's reagent. After the spray, bands containing different alkaloids were observed as orange spots marked X, (Fig 7).

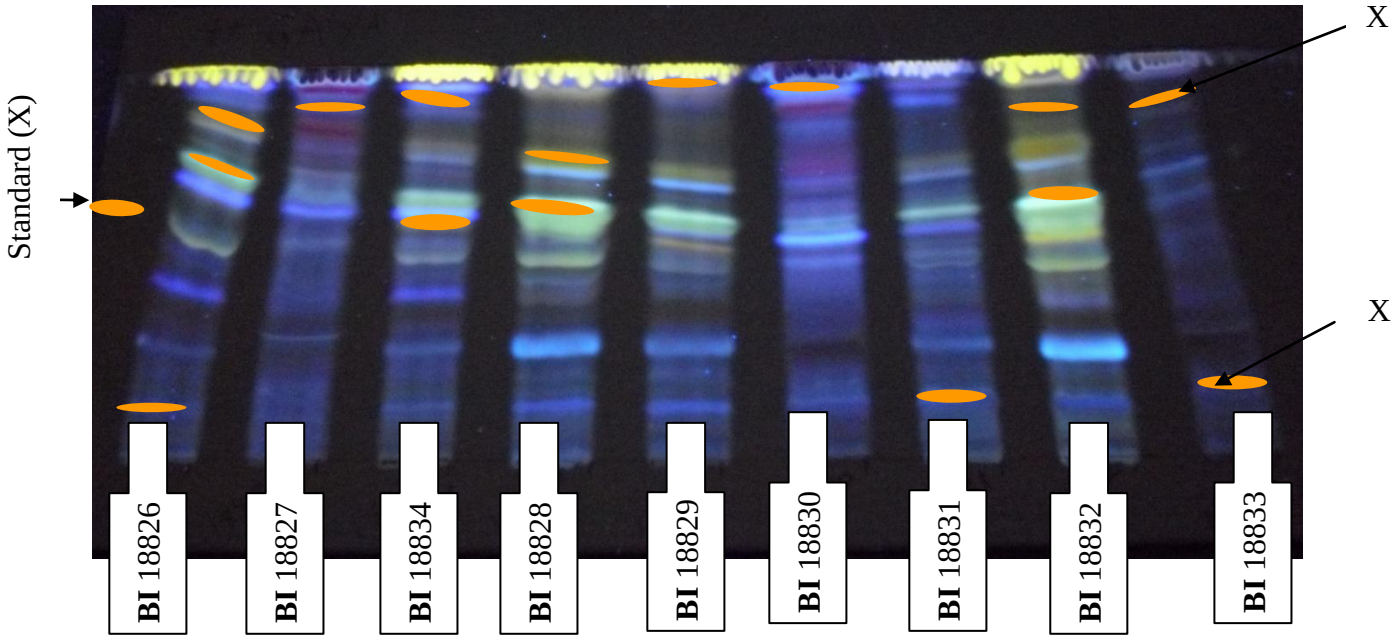


Fig 7: Detection of alkaloids with Dragendorff's reagent (Orange colour denote the alkaloid bands). (**X** = points at which orange colour developed in the TLC plate; **Standard** = papaverine bands)

Solvent system: Butanol: Acetic acid: Water (BAW)

Extract Identification codes:
BI 18826 (root Kakamega), **BI 18827** (leaf Kakamega), **BI 18828** (root, Mt. Kenya), **BI 18829** (bark, Mt. Kenya), **BI 18830** (leaf. Mt. Kenya), **BI 18831** (bark, Mau), **BI 18832** (root, Mau), **BI 18833** (leaf, Mau), **BI 18834** (bark, Kakamega)

The TLC plates were then scanned just before being sprayed with Dragendorff's reagent to have an analysis of component separation in every extract on the plate. TLC scan chromatograms in Appendix I, illustrate varying peaks which demonstrate the abundance and diversity of compounds among the extracts from different plant parts and from different regions, although some contained compounds with the same Rf values. The presence of different alkaloids is evidenced by the different retention times, peak patterns heights and areas which are different in every extract as illustrated in Appendix I. Chromatograms in Appendix I, show heights of the different peaks which represent the abundance of the compounds associated with the bands for the different extracts; root, bark and leaf extracts from all three regions. The findings from the TLC analysis and data encouraged further investigations to find out the compound profile in the extracts by means of HPLC and LC-MS.

4.5 HPLC and LC-MS analysis

The HPLC and LC-MS analyses of methanol extracts of *Z. gilletii* revealed the presence of sixteen different compounds. Out of these, five were confirmed as alkaloids (dihydronitidine ($C_{21}H_{19}NO_4$), trans-fagaramide ($C_{14}H_{17}NO_3$), sanguinarine ($C_{20}H_{14}NO_4$) and 8-methylnorchelerythrine ($C_{21}H_{17}NO_4$)) while ten others are identified as alkaloids but their identification needs to be confirmed. Also identified as present in the extracts are flavonoids hesperidine ($C_{28}H_{34}O_{15}$) and diosmin ($C_{28}H_{32}O_{15}$) (Table 10), these two flavanoids have not been previously reported to occur in *Z. gilletii*. Figures 8 – 16 show the electrospray tandem mass spectrometry (ESI) positive mode LC-MS analysis of different peak chromatograms from which compounds were identified in Z. *gilleti*. The figures also show details of retention times (minutes) of compounds and given peaks as well as their molecular weights (M+1).

Validation of the five alkaloids and the two flavanoids was confirmed after the study of the MS spectra of the extracts in the positive ion mode. This involved consideration of the retention times (t_R), MS and MS^2 fragmentation ions (Liang et al., 2006). In MS spectra, the five alkaloids and the two flavanoids exhibited their quasi molecular ion $[M]^+$ status or $[M+H]^+$. In MS^2 spectra, the fragmentation ions of losing CH_3, H and CO as neutral fragment were observed (Liang et al., 2006). Their fragmentation patterns were then matched with their chemical structures, after comparison with the list of chemicals available in the in-house chemical database at the Jodrell as well as the previously identified chemicals in literature. The fragmentation data for the identified compounds is presented in Appendix V. Analysis of the LC-MS data and ion fragmentation in the positive mode to help identify different compounds resulted in the chromatograms presented in Figure 8 to Figure 16. All the chromatograms in the figures below relate to the list of compounds shown in Table 10, coded

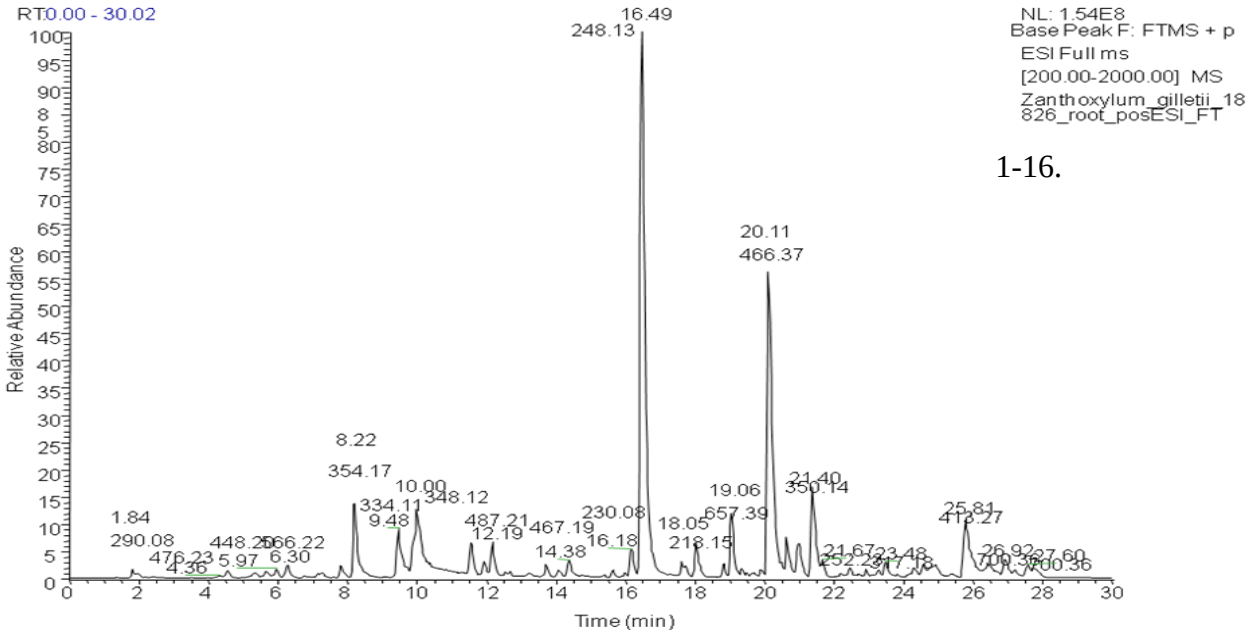


Fig 8: ESI – MS detector, positive mode chromatogram of extracts of root from Kakamega forest (BI 18826).

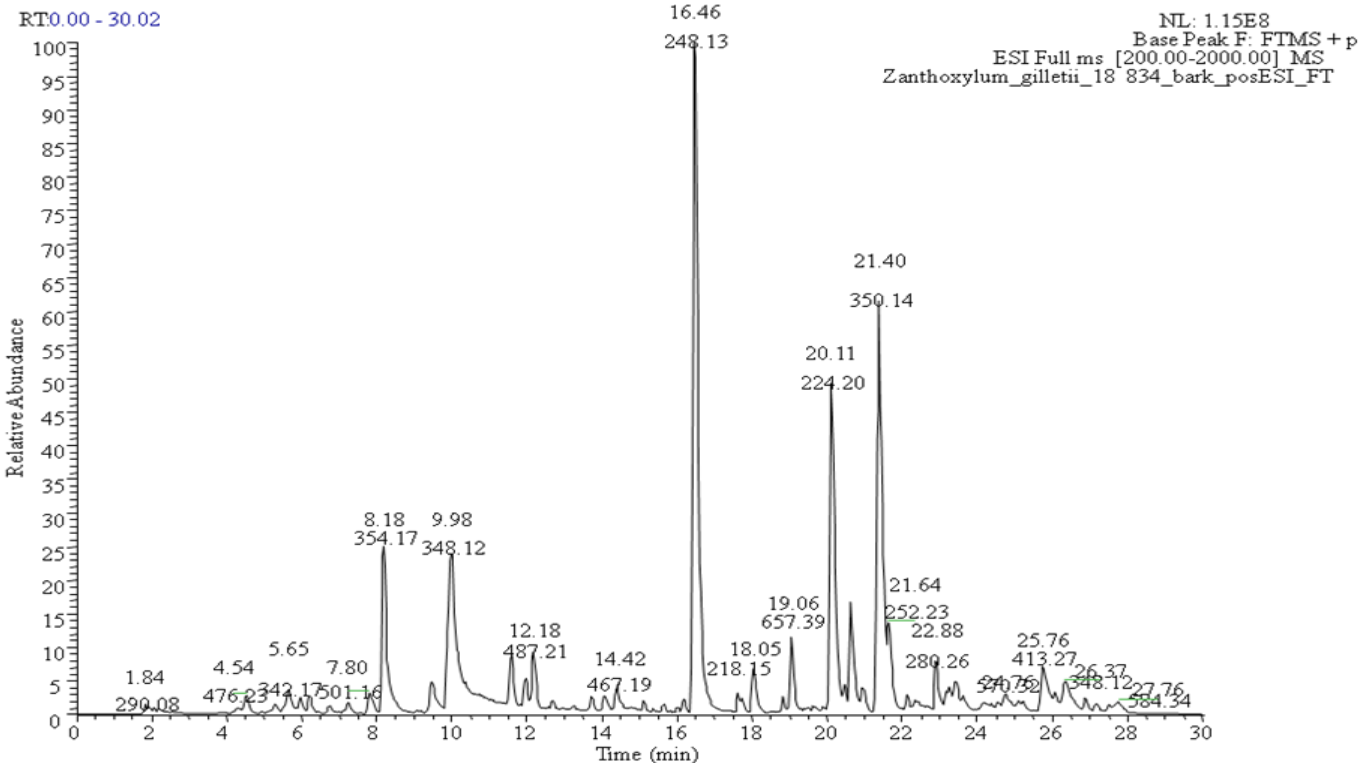


Fig 9: ESI – MS detector, positive mode chromatogram of bark extracts from Kakamega forest **(**BI 18834).

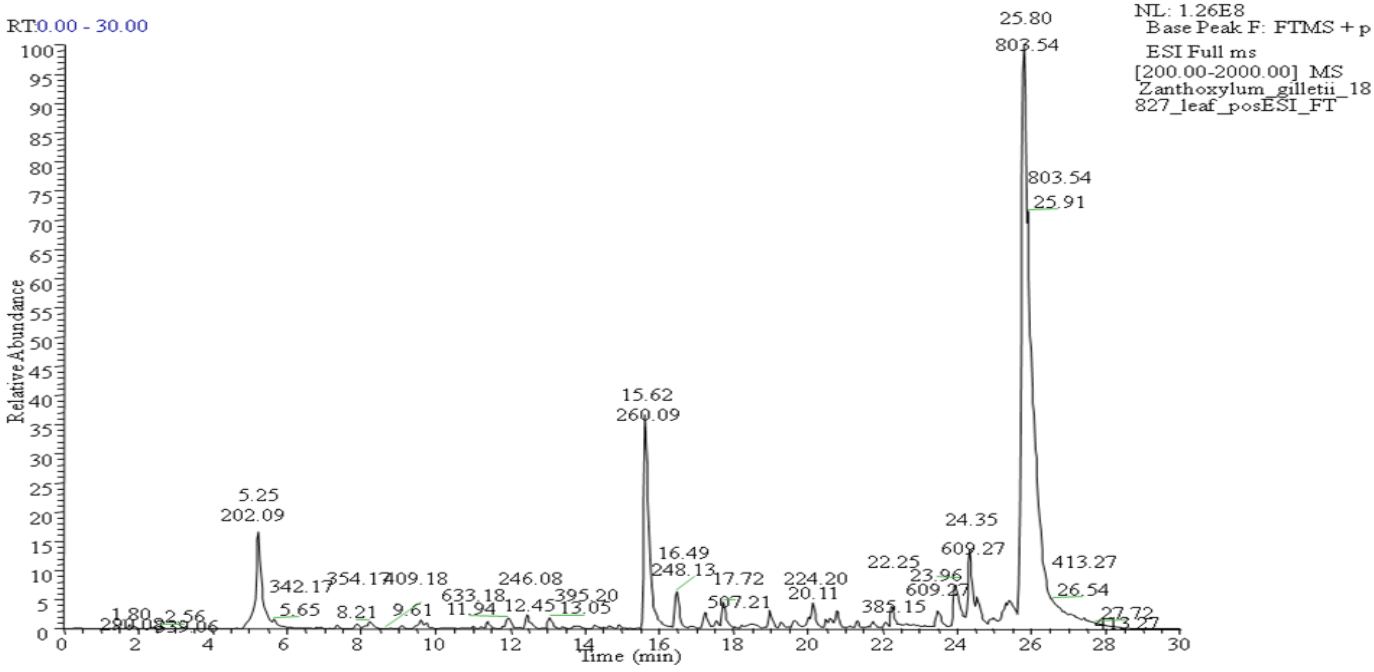


Fig 10: ESI – MS detector, positive mode chromatogram of extracts of leaves from Kakamega forest (BI 18827).

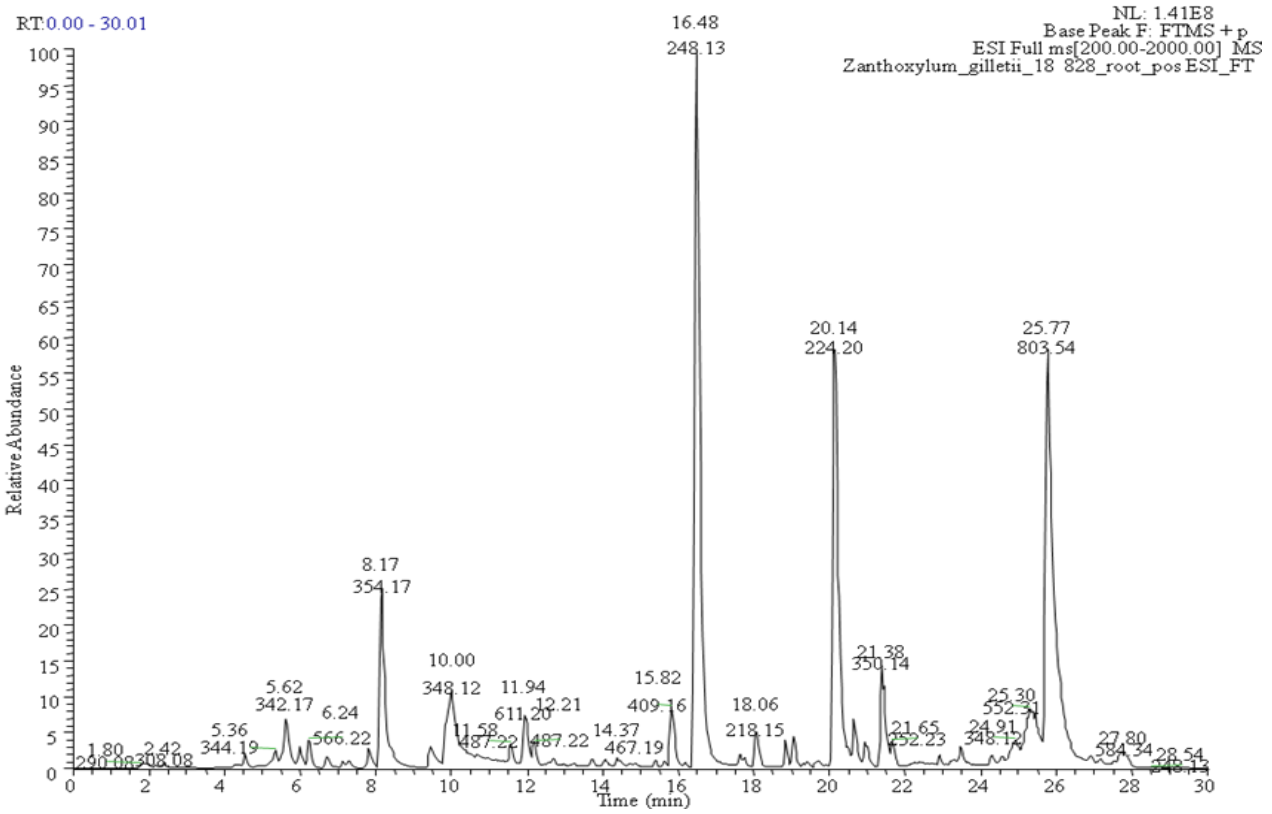


Fig 11: ESI – MS detector, positive mode chromatogram of root extracts from Mt. Kenya (BI 18828).

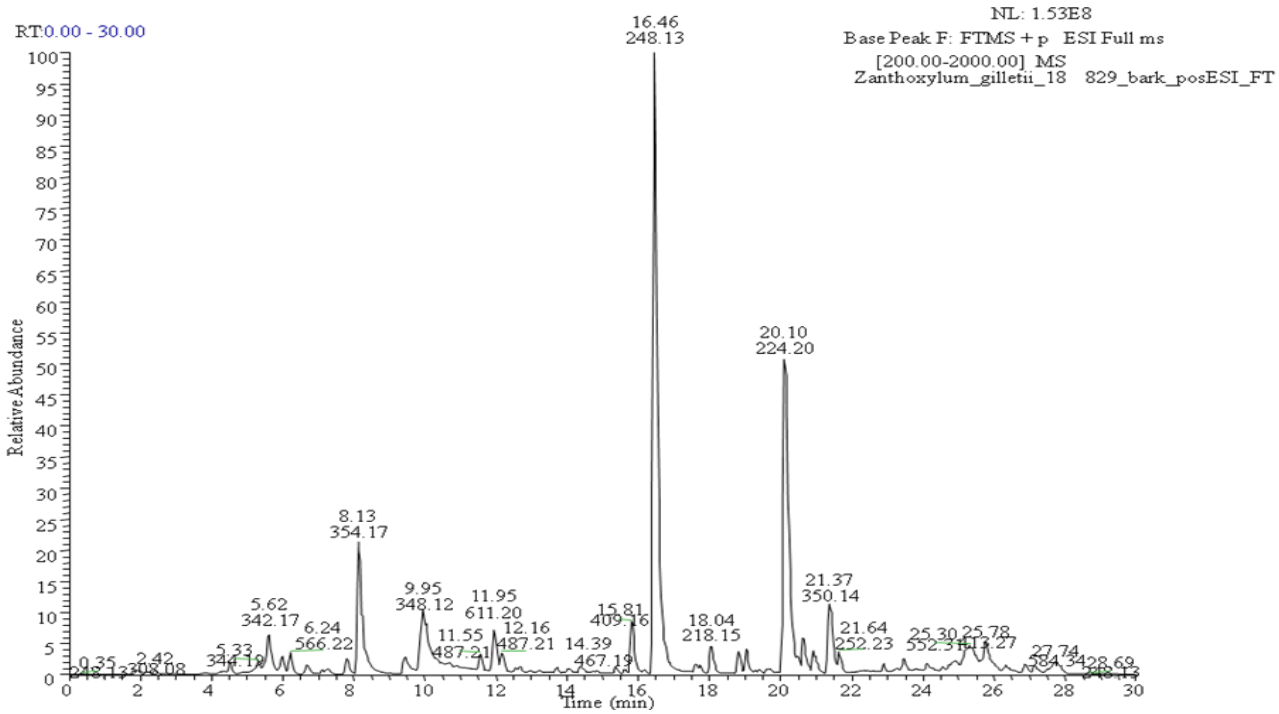


Fig 12: ESI – MS detector, positive mode chromatogram of bark extracts from Mt. Kenya (BI 18829).

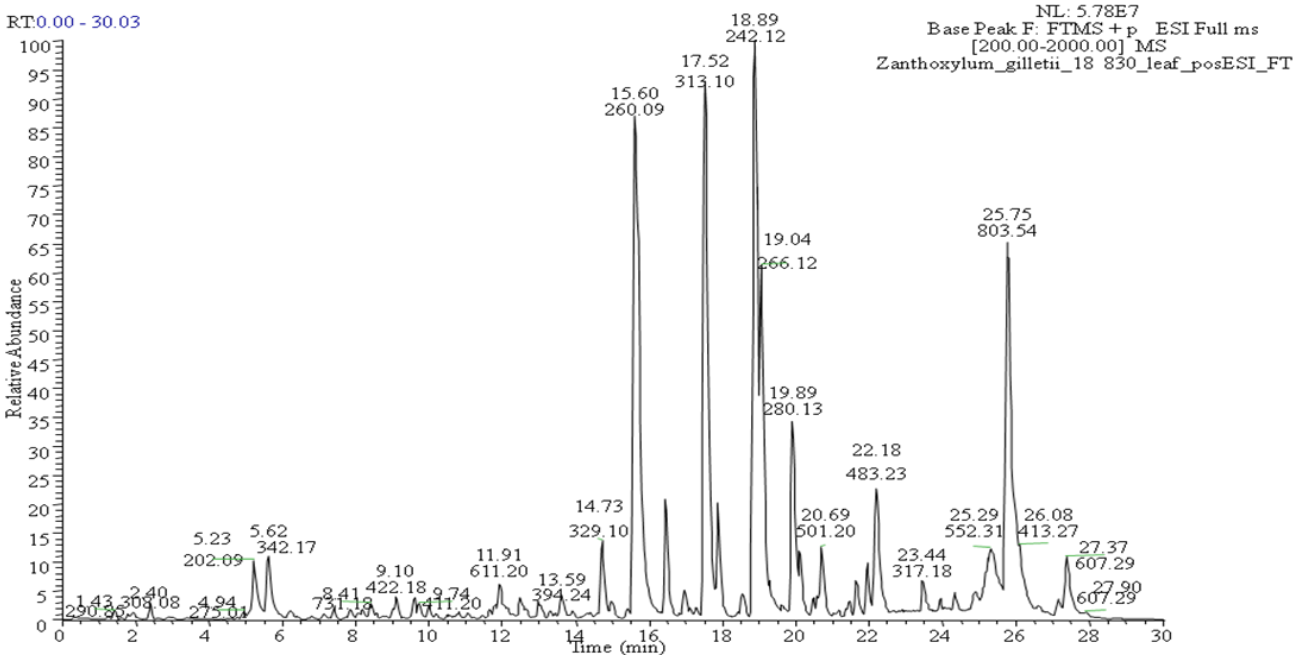


Fig 13: ESI – MS detector, positive mode chromatogram of leaf extracts from Mt. Kenya (BI 18830).

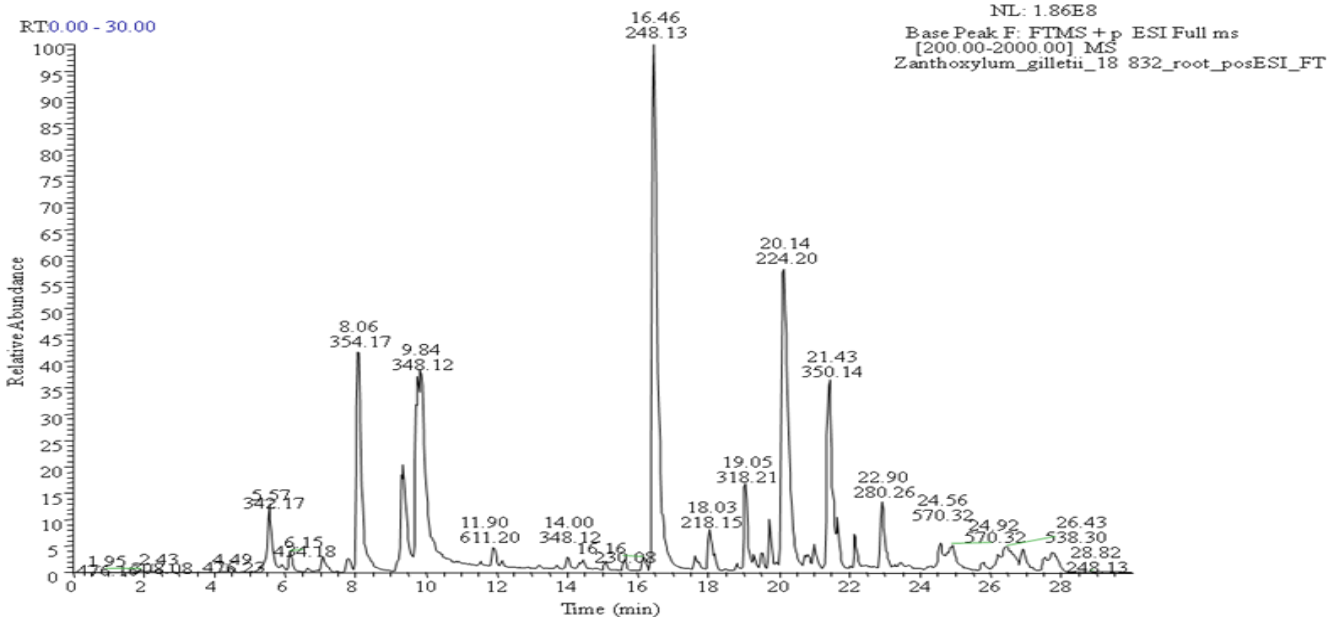


Fig 14: ESI – MS detector, positive mode chromatogram of root extracts from Mau (BI 18832).

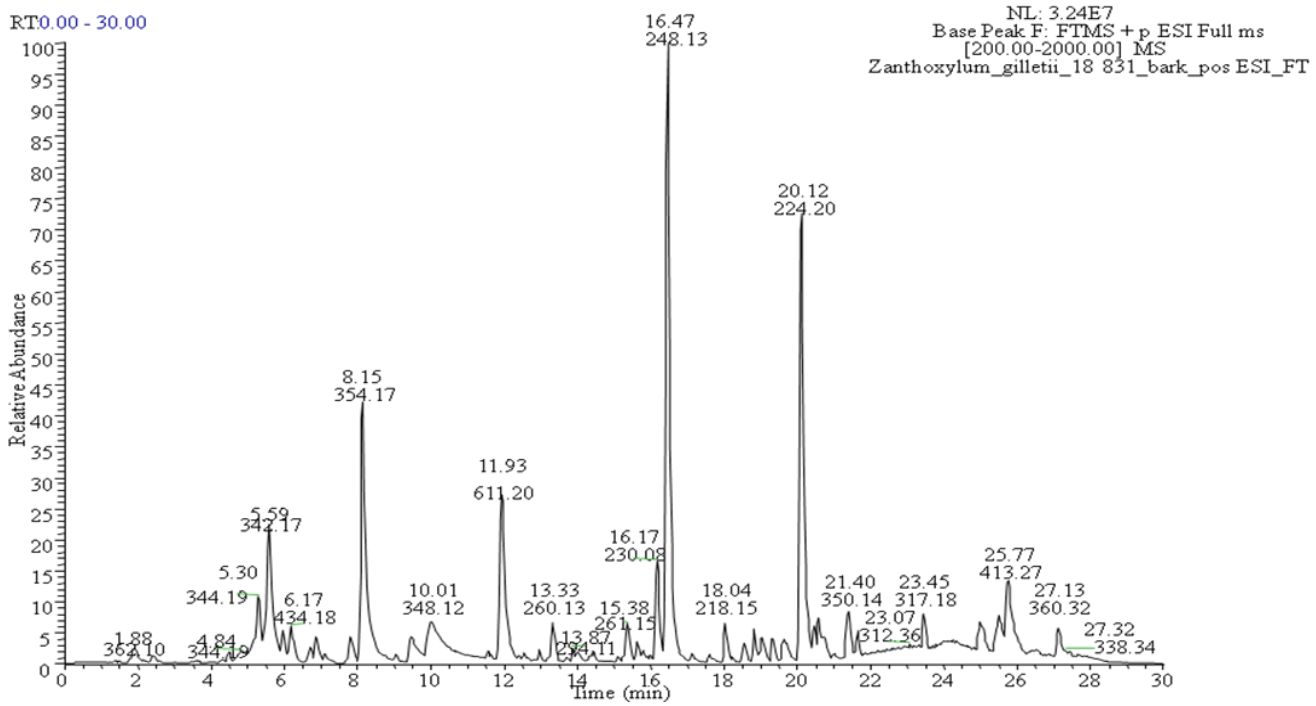


Fig 15: ESI – MS detector, positive mode chromatogram of bark extracts from Mau (BI 18831).

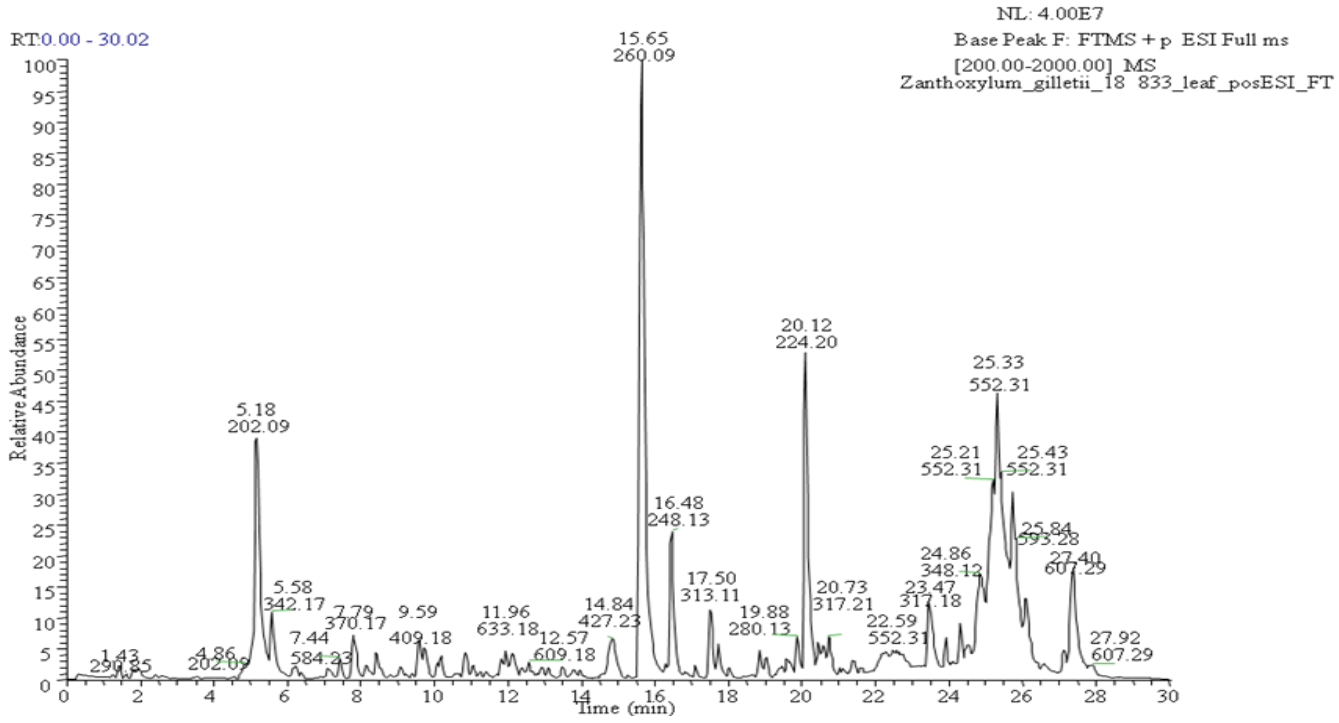


Fig 16: ESI – MS detector, positive mode chromatogram of leaf extracts from Mau (BI 18833).

A summary of the list of compounds, their molecular weights and chemical formula as identified in the extracts of *Z. gilletii* collected from the different geographical regions in Kenya is given in Table 10; made from the analysis of the LC-MS chromatograms (Figs 8-16) showing molecular weights and retention times of the various compounds detected in the various extracts.

Table 10: Molecular weights and chemical formula of some of the isolated compounds identified to be present in *Z. gilletii* extracts analysed:

Code	Rt	MW	Formula	Compound
Alkaloids				
1	4.36	475	$C_{25}H_{33}NO_8$	1 alkaloid not known to occur in *Z. gilletii*
2	5.60	341	$C_{20}H_{23}NO_4$	peroxysimulenoline (or 103 other alkaloids not in this genus)
3	6.30	565	$C_{27}H_{35}NO_{12}$	3 alkaloid with this formula
4	7.26	367	$C_{21}H_{21}NO_5$	27 alkaloids with this formula none previously reported in this genus
5	7.60	369	$C_{21}H_{23}NO_5$	fagarine I
6	8.22	353	$C_{21}H_{23}NO_4$	35 alkaloids with formula none previously reported in this genus
7	9.30	332	$C_{20}H_{14}NO_4$	Sanguinarine
8	9.48	333	$C_{20}H_{15}NO_4$	could be norchelerythrine, dihydroavicine or demethylnitidine
9	10.00	347	$C_{21}H_{17}NO_4$	8-methylnorchelerythrine
10	15.60	259	$C_{14}H_{13}NO_4$	39 alkaloids with this formula in Rutaceae
11	16.18	229	$C_{13}H_{11}NO_3$	84 alkaloids with this formula in the Rutaceae
12	16.49	247	$C_{14}H_{17}NO_3$	trans-fagaramide
13	20.11	223	$C_{14}H_{25}NO$	alkaloid matches in the genus
14	21.40	349	$C_{21}H_{19}NO_4$	dihydronitidine
Flavonoids				
15	11.95	610	$C_{28}H_{34}O_{15}$	Hesperidin (flavanone)
16	12.48	608	$C_{28}H_{32}O_{15}$	Diosmin (flavonoid)

(**Codes** 1-16; compound identity, **Rt**; retention time, **MW;** molecular weight and **Formula;** chemical formula of compound).

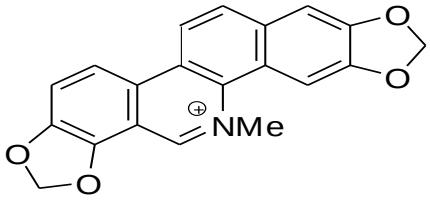
From the findings above, the compounds present in *Z. gilletii* were given codes ranging from 1-16 and sorted out according to their occurrence in the extracts obtained from samples originating from the three different geographical regions or occurring in any of the plant parts (root, leaf or bark), to give a summary of compound profile from the three geographical regions and plant parts as shown in Table 11; while Figure 14 shows the different structures of the identified compounds.

Table 11: Profile of compounds in the extracts of *Z. gilletii* from the different regions and plant parts.

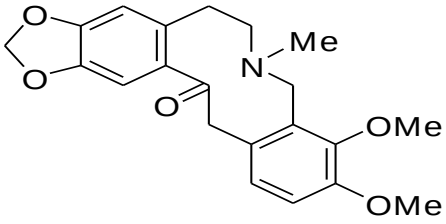
Extract	**18826**	**18828**	**18832**	**18834**	**18829**	**18831**	**18830**	**18827**	**18833**
Region	Kakamega Forest	Mt. Kenya	Mau Forest	Kakamega Forest	Mt. Kenya	Mau Forest	Kakamega Forest	Mt. Kenya	Mau Forest
Code	**R**	**R**	**R**	**B**	**B**	**B**	**L**	**L**	**L**
1	√	√	√	√	√	√	X	√	√
2	√	√	√	√	√	√	X	√	√
3	√	√	X	√	√	X	X	X	X
4	√	√	√	√	√	√	X	X	X
5	√	√	√	√	√	√	X	√	√
6	√	√	√	√	√	√	√	√	X
7	√	X	√	√	√	X	X	X	X
8	√	√	√	√	√	√	X	X	X
9	√	√	√	√	√	√	√	√	X
10	√	X	X	X	X	X	√	√	√
11	√	√	√	√	√	X	X	X	X
12	√	√	√	√	√	√	X	√	√
13	√	√	√	√	√	√	√	X	√
14	√	√	√	√	√	√	X	X	X
15	√	√	√	√	√	√	√	√	√
16	√	√	√	√	√	√	√	√	√

(Where: **R** = root; **B** = bark; **L** = Leaf; √ = compound represented by code 1-16 is present; **X** = compound represented by code 1-16 is absent; **Code** 1-16 = Table 8).

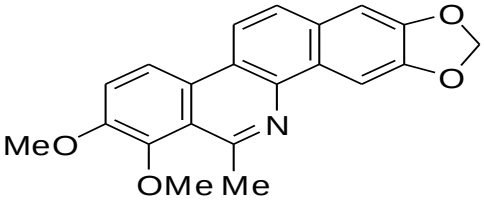
The following are the different structures of the identified compounds, Figure 14.



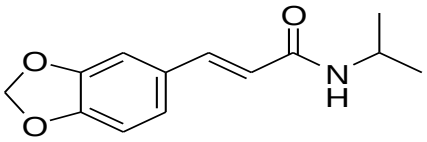
Sanguinarine



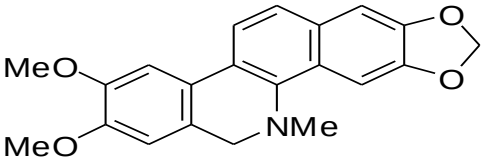
Fagarine I



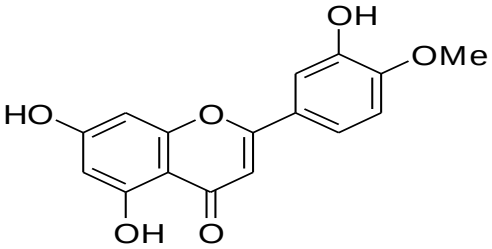
8-Methylnorchelerythrine



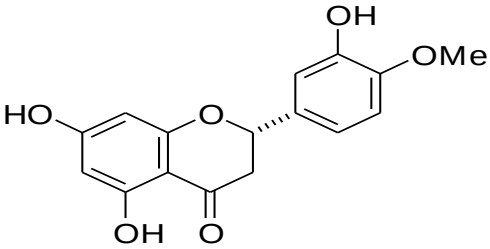
trans-Fagaramide



Dihydronitidine



Diosmin



Hesperidin

Where: Me = CH_3;

Fig 17: The Structural presentation of the compounds identified in *Z. gilletii* extracts**.**

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Zanthoxylum gilletii extracts showed some antimicrobial activity against tested microorganisms. Root extracts from all the three regions sampled, showed moderate antimicrobial activity with the extracts from Mau showing highest activity (25 µg/ml) against *P. syringae.* Bark and leaf extracts from all the three geographical regions did not show any substantial antimicrobial activity against all test pathogens with only minimal antimicrobial activity being recorded from bark extracts from Kakamega forest. In this study however, the observed antimicrobial activity was due to the alkaloids present in the extracts, although this activity could not be related to a specific alkaloid or compound found in the extracts.

Compared to previous studies on the species, (Obebiya and Sofowora, 1979) in their study on Nigerian chewing sticks showed *Z. gilletii* to be an antibacterial containing alkaloids such as canthin-6-one (220 $C_{14}H_8N_2O$) and 4, 5dihydrocanthin-6-one (222 $C_{14}H_{10}N_2O$) which could have been responsible for the antimicrobial activity. In another study carried out by Gakunji et al. (1995), it was found that the bio-guided fractionation of the ethanolic extract of *Z. gilletii*, showed antibacterial activity in the range of MFA, IC_{50}-value = 2.3 µg/ml. An alkaloid; chelerythrine also showed analgesic effect and antibacterial, antifungal and antihelmintic activities (Louppe et al., 2008; Katende, 2000). *In-vitro* study of the extracts of *Z. gilletii* showed antifungal and antibacterial activities and inhibited *in-vitro* growth of *Candida albicans, Cryptococcus neoformans* and other filamentous fungi (Mshane and Ekper, 2000; Addae, 1992; James, 1992). Other studies have showed that *Z. gilletii* can be used as

antileukemia, anticancer and as an antiviral since its extracts have shown activity against *Mycobacterium smegmatis, Klebisiela pneumonia* and *Candida albicans.*

This study, like previous studies carried out on the species from West Africa confirms that *Z. gilletii* from Kenya has antibacterial and antifungal activity. There are higher numbers of compounds in the stem and root barks compared to leaves from all the geographical regions and the recorded antimicrobial activity relate to the compound profiles. The leaf extracts which also had fewer compounds, showed no antimicrobial activity at all. The study has also shown that there are observable variations in the antimicrobial activity of extracts of *Z. gilletii* from the three geographical regions and from the different plant parts. For example; the bark extracts from Kakamega showed some antimicrobial activity when bark extracts from Mau and Mount Kenya did not show any activity at all. The root extracts from all the three regions had substantial antimicrobial activity while the leaf extracts from all regions did not have any activity at all. The antimicrobial activity in *Z. gilletii* extracts observed in this study is therefore possibly, due to some of the alkaloids present in the extracts studied.

The antioxidant activity of Z. *gilletii* extracts was between - 0.65 - 7.8 % which was too low according to recommended standards (Balakrishnan et al., 2009). Studies have shown that the acceptable antioxidant activity is pegged in the range of 50 – 100 %, like in the standard ascorbic acid (Balakrishnan et al., 2009). The plant extracts were found to contain two flavanoids hesperidine and diosmin, known antioxidants (James, 1992). The Flavanoid diosmin has been shown to have anti-inflamatory, anticancer activity as well as inhibitory activity on multidrug resistant (MDR) bacteria (Cragg and Newman, 2007). Hesperidin on the

other hand has been used as an antioxidant, anti-RNA, antistomatitic and antiviral agent (James, 1992). In this study however, the antioxidant activity of the extracts were found to be negligible. *Zanthoxylum gilletii* has been used as antioxidant to reduce pain in sickle cell patients and in the treatment of sickle cell anemia in Nigeria (Maurice, 1993). According to James (1992), the alkaloids sanguinarine, dihydronitidine and fagarine I, have been used as antihypertensive agents, cardiac inhibitor, respiratory stimulant, relaxant, and as DNA intercalators. This study therefore finds the use of *Z. gilletii* from Kenya as an antioxidant unjustified contrary to other studies carried out on the species from West African countries.

This study has shown that *Zanthoxyllum gilletii* harvested from three different geographical regions in Kenya contain many different alkaloids as well as some two flavanoids. The alkaloids identified were similar to those described in previous studies from extracts of *Z. gilletii* collected from West African countries, Ghana and Nigeria but for the first time in the Kenyan species. The two flavanoids hesperidine and diosmin, described in this study are new for the Kenyan species as the only previous studies (Michael et al., 2007) were on seed germination and physiology.

Sixteen different compounds were detected in the chloroform and methanol extracts of *Z. gilletii*. The study has shown that, out of the sixteen compounds, fourteen are alkaloids. Five of out of the fourteen alkaloids were fully identified and their structures given. The five identified alkaloids in this study were; 8-methylnorchelerythrine ($C_{17}H_{21}NO_4$), fagarine I ($C_{21}H_{23}NO_5$), sanguinarine ($C_{20}H_{14}NO_3$), trans-fagaramide ($C_{14}H_{17}NO_3$) and dihydronitidine ($C_{21}H_{19}NO_4$). All the identified alkaloids are similar to those previously described in other

species in different parts of the world. There are many alkaloids reported in *Z. gilletii* from other parts of the world but not found in the Kenyan species. Phytochemical studies in *Z. gilletii* have reported the richness of this plant in alkaloids (Gakunju et al., 1995; Mensah and Torto, 1970). Pure crystalline alkaloids such as fagaramide, nitidine, and dihydrochelerythrine have been isolated from the species (Olila and Opuda, 2001). In Nigeria, alkaloids such as zanthosimuline ($C_{20}H_{23}NO_2$), rhoifoline A and oxyavicine ($C_{20}H_{13}NO_5$); have been reported in *Z. gilletii* (Adesina, 2005). From the Ghanian species, berberine, skimmiamine, benzoic acid derivatives, essential oils and saponins have been reported (James, 1992, Mensah and Torto, 1970). Goodson (1921) isolated lupeol as well as (iso)arnottianamide (381 $C_{21}H_{19}NO_6$), integrinamide ($C_{20}H_{15}NO_6$) and dictamnine ($C_{12}H_9NO_2$) (Goodson, 1921) from the methanol bark extracts in *Z. gilletii*. Decarine ($C_{19}H_{13}NO_4$), methoxydihydrochelerythrine ($C_{22}H_{21}NO_5$), norchelerythrine ($C_{20}H_{15}NO_4$) and iriodeine ($C_{17}H_9NO_3$) are other alkaloids isolated in *Z. gilletii* (Neuwinger, 1996, James, 1992). Most of these compounds previously reported mostly from the West African species were not found in the Kenyan species in the present study.

The species of *Zanthoxylum* studied so far, vary greatly in their chemistry and their taxonomy in some cases is obscure, because apart from inter specific variation in chemistry, extent of variation of constituents within a species is still not clear (Olila and Opuda, 2001). This study confirms the fact that plants of the same species grown in different geographical regions can yield different products. Similarly in this study, the profile of the identified alkaloids also varied from one region to another and among the root, bark and leaf extracts. For example, the alkaloid sanguinarine ($C_{20}H_{14}NO_3$) in this study was present in root extracts from Kakamega and Mau forests but absent in root extracts from Mount Kenya forest as well as leaf extracts

from all the regions. This study has therefore demonstrated variation in the compound profiles across all geographical regions sampled and agreed with previous studies on profiles of *Z. gilletii*.

The two flavanoids, hesperidine ($C_{24}H_{34}O_{15}$) and diosmin ($C_{28}H_{32}O_{15}$) reported in this study in *Z. gilletii* extracts have never been reported in *Z. gilletii* extracts from any previous studies carried out on the species. From previous studies, diosmin is known to be anti-capillary fragility when used, mostly in women, as an antihemorrhodial and an antimetrorrhagic (James, 1992). This study showed that the two identified flavanoids were present in all extracts collected from all the three geographical regions as well as in the root, bark and leaf extracts collected from all the geographical regions. The presence of these two flavanoids in all extracts can be attributed to their instrumental role in plant life in terms of giving the plant its flavor, colour of flowers and scent among other physiological functions.

5.2 CONCLUSIONS

The extracts of *Z. gilletii* have shown moderate antimicrobial activity against gram negative bacteria *P. syringae* and gram positive *B. subtilis* while there was very low activity against the fungus *C. herbarum* and no activity at all against *S. cereveciae*. The root extracts in this study have shown the highest antimicrobial activity while the leaf extracts did not show any activity indicating that compound profiles relate directly with antimicrobial activity.

Variations in activity of extracts from different geographical regions were confirmed in this study; with root extract from Mau forest being the most active (25 µg/ml) and bark from Kakamega forest being the only bark extract to have shown activity. Samples from Mt. Kenya region had the lowest activity. Variation in antimicrobial activity was directly related with the profile of compounds in the extracts in such a way that the leaves which had very poor compound profiles did not have any antimicrobial activity while the roots was had good compound profiles showed good antimicrobial activity. Antimicrobial activity is due to the synergestic action of compounds present in the extracts.

Zanthoxyllum gilletii extracts have shown poor antioxidant activity during the nitric oxide scavenging activity test in this study.

There are many alkaloids in *Z. gilletii* extracts studied while two flavanoids diosmin and hesperidin were also identified. The two identified flavanoids were present in all extracts from all the regions. Study of compound profiles revealed that alkaloids and flavanoids varied between plant parts and across geographical regions. The root extracts had most of the

identified alkaloids and flavanoids followed by bark extracts while the leaves had the least number of alkaloids and flavanoids.

Alkaloids and flavanoids in Z. gilletii extracts vary across geographical regions and their antimicrobial activity depend on the compounds profiles.

5.3 RECOMMENDATIONS

From this study's findings, the following recommendations are suggested:-

i. *Zanthoxylum gilletii* extracts can be used as an antibacterial and antifungal but not as an antioxidant.
ii. Further antimicrobial activity tests using more pathogens is essential since there are high chances of pathogen specificity.
iii. There in need to confirm the identity of compounds responsible for the observed antimicrobial activity through further research.
iv. There is need for complete identification of the unidentified compounds from *Z. gilletii* extracts.
v. Further research to confirm possibilities of genetic variations is needed since this study has shown the profiles of compounds to be greatly varied across plant parts and regions.
vi. There is need design a strategy for conservation of all gene pools through targeted multiple germplasm collection.
vii. There is also need to design a strategy for sustainable utilization of the species since most activity has been identified in the root extracts

REFERENCES

ABBIW, K. D. 1990. Useful Plants of Ghana, West Africa: Uses of Wild and cultivated plants. *Intermidiate Technology Publication, UK***,** pg 98.

ADDAE, M. I. 1992. Towards a Rational scientific Basis for Herbal Medicine; A Phytochemists' two decade contribution. An Inaugural Lecture delivered at the University of Ghana, Legon 45. *Ghana University Press, Accra*.

ADESINA, K. S. & JOHANNES, R. 1988. Arnottianamide and Other Constituents of Zanthoxylum gilletii Root. *Journal of Natural Products,* 51**,** 601- 602.

ADESINA, S. K. 2005. The Nigerian Zanthoxylum; Chemical and Biological Values; African Journal of Traditional and Complementary and Alternative Medicines. 2, No. 3**,** 282-301.

AYENSU, E. S. 1978. Medicinal Plants of West Africa. *Reference publications, 218 St, Clair River Drive, Algonac, Michigan 48001*.

BALAKRISHNAN, N., PANDA, A. B., RAJ, N. R., SHRIVASTAVA, A. & PRATHANI, R. 2009. The Evaluation of Nitric Oxide Scavenging Activity of Acalypha indica Linn Root. *Asian Journal of Research Chemistry,* 2, 148-150.

BEENTJE, J. H. 1994. Kenya Trees,Shrubs and Lianas,National Museums of Kenya, Museum Hill, Nairobi.

BENSKY, D., STEVEN, C., ERICH, S. & ANDREW, G. 2004. Chinese Herbal Medicine. *Materia Medica,* Third Edition.

CHEN, I. S., CHEN, S. J., WU, I. L., TSA, T. S., WU, J. M., PEZZUTO, M. C., LU, H., CHAI, N. & TENG, C. M. 1994. Chemical and bioactive constituents from Zanthoxylum simulans. *Journal of Natural Products,* 57**,** 1206–1211.

CRAGG, G. M. & NEWMAN, D. J. 2007. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products,* 70**,** 461-477.

DHARANI, N. 2002. Field Guide to Common Trees and Shrubsof East Africa. *Struik Publishers, Cape town, South Africa*.

EBUU, S. & SAWYERR 1994. Medicinal Plants and practices of West Africa. *10 St. Barnabas Road, Mitcham, Surrey CR4 2DU- England*.

EL-SHEMY, H. A., ABOUL-ENEIN, A. M., ABOUL-ENEIN, K. M. & FUJITA, K. 2007. Willow Leaves' Extracts Contain Anti-Tumor Agents Effective against Three Cell Types.

FAO 1986. The medicinal forest plants of Africa and Latin America. *Forest Resources Development Branch, Forest Resources Division. FAO Forestry Department,* Forestry Paper 67.

FRANCES, A. 2004. Seed Storage Characteristics and germination of select South Florida native Plant seeds. *Fairchild Tropical Botanic Garden http://www.ftg/PDF20Files/SeedStorageBehaviourTable.pdf [accessed 26 April 2008]*.

FRANCIS, J. K. 2000. Zanthoxylum flavum Vahl. In silvics of native and exotic trees of Puerto Rico and the Caribbean Island. *(Fransis J. K, Lowe CA. eds.) United States Department of Agriculture Technical Report IITF- 15 http://www.fs.fed.us/global/iitf/Zanthoxylumfluvum.pdf [accessed 18 April 2008]*.

GACHATHI, M. 2007. Kikuyu Botanical Dictionary, A guide to Plant Names, Uses and Cultural Values. *Published by Tropical Botany, Gatuma, Kenya* Revised Second edition.

GAKUNJU, D. M., MBERU, E. K. & DOSSAJI, S. F. 1995. Potent antimalarial activity of the alkaloid nitidine from a Kenyan herbal remedy. *Antimicrobial Agent and Chemotherapy*, 39**,** 2606- 2609.

GAYA, C. H. & DANIEL, M. O. 2009. Report on collection of Orchids and other plants species from Mount Elgon and the Mau Forest, Nairobi, Kenya.

GOODSON, J. A. 1921. Constituents of the Bark of Zanthoxylum macrophylla, Oliver. *The Biochemical Journal, Biochemical Society. Cambridge University press*.

HISATOMI, E., MATSUI, M., KOBAYASHI, A. & KUBOTA, K. J. 2000. Antioxidative activity in the pericarp and seed of Japanese pepper (Xanthoxylum piperitum DC). *Agricultural Food Chemistry,* 48**,** 4924-4928.

HOSTETTMANN, K. F., CHINYANGANYA, M., MOILLARD & WOLFENDER, J. L. 1996. Chemistry, Biological and Pharmacological Properties of African Medicinal Plants. *Proceedings of the first International Organization for Chemical Sciences in Development (IOCD) Symposium, Victoria Falls, Zimbabwe. University of Zimbabwe Publications***,** 245.

ISLAM, J. K. N. & AHSAN, N. 1997. Biological activities of secondary metabolites isolated from Zieria smithii and Zanthoxylum elephantiasis on microorganisms and brine shrimps. *Physiotherapy Research,* 9**,** 64-66.

ITOH, Y., FU, H. M., HANAE, H., MICHIKO, O., KUMIKO, N., YOJIRO, U., HIROTATSU , K., TETSUO, N. & NOBORU, T. 2000. Determination and Bioimaging Method for Nitric Oxide in Biological specimens by Diaminofluorescein Fluorometry. *Analytical Biochemistry,* 237**,** 203 – 209.

JAMES, A. D. 1992. Hand book of biologically active phytochemicals and their activities. *CRC press Inc., Blvd.,N.W., Boca Raton, Florida*.

KATENDE, B. 2000. Useful Trees and Shrubs for Uganda, Technical handbook No 10, Identification, Propagation and Management for Agricultural and Pastoral Communities. *Published by Sida's Regional Land Management Unit, Nairobi*.

KAYANI, A. S., MASOOD, A., ACHAKZAI, A. K. K. & ANBREEN, S. 2007. Distribution of Secondary Metabolites in Plants of Quetta-Balochistan. *Botany,* 34**,** 1173 - 1179.

KOJIMA, H., NAOKI, N., KAZUYA, K., SHIGEMORI, K., YATAKA, K., HIROSHI, N., YASUHABU, H. & TETSUO, N. 1993. Detection and Imaging of Nitric Oxide with Novel Fluorescent Indicators: Diaminofluoresceins. *Analytical Chemistry,* 70**,** 2446 – 2454.

KOKWARO, J. O. 1988. Conservation status of the Kakamega Forest in Kenya: The Easternmost Relic of the Equatorial Rain Forests of Africa. *Monograph of Systematic Botany Missouri Botanical Gardens* 25**,** 471-488.

KOKWARO, J. O. 1993. Medicinal Plants of east Africa. *Published by Kenya Literature Bureau,* second Edition**,** 428-430.

LIANG, M., ZHANG, W., JIANG, H., LIU, R. & ZHANG, C. 2006. Simultaneous Analysis of Alkaloids from Zanthoxylum nitidum by High Performance Liquid Chromatography-diode array detector electrspray tandem mass spectrometry. *Pharmaceutical and Biomedical Ananlysis,* 42**,** 178-183.

LOUPPE, D., OTENG-AMOAKO, A. A. & BRINK, M. 2008. Plant Resources of Tropical Africa. *Timbers 1. PROTA Foundation, Wageningen, Netherlands/ Backhuys Publishers, Leiden, Netherlands/ CTA, Wageningen, Netherlands,* 7**,** 594 – 597.

MAI, H. D., VAN DUFFAT, H. T., MICHE, S., TILLEQUIN, F., BASTIEN, D. & SEVENET, T. 2001. A New Diprenyl Coumarin and Alkaloids from the Bark of Zanthoxylum dimorphophylum (Rutacea) Z. Natturforsch.

MAURICE, M. I. 1993. Handbook of African Medicinal Plants. *CRC Press, Boca Raton Ann Arbor, London*.

MBAI-OPONDO, M. L., ODHIAMBO, O. K. & NJUG'E, J. T. 2000. Some Factors Influencing Natural regeneration of Selected Tree Species in Kakamega Forest, Kenya*, Eldoret, Kenya*.

MENSAH, I. A. & TORTO, F. G. 1970. Alkaloids of Fagara macrophylla. *Phytochemistry,* 9**,** 911- 914.

MICHAEL, M. O., MUASYA, R. M., OCHUODHO, J. O. & OMONDI, W. O. 2007. Investigation on the Germination of Zanthoxylum gilletii Seeds (DeWild) Waterm (East African Satinwood) International Symposium on Innovations as Key to the Green Revolution in Africa. *Exploring the scientific Facts, Arusha, Tanzania*.

MSHANE, N. R. & EKPER, J. A. 2000. Traditional Medicine and Pharmacopoeia. *Contribution to the revision of Ethnobotanical and Floristics Studies of Ghana*.

MUKIAMA, T. K. 2005. Some Medicinal Plants of Kenya Policy Issues and Need For Commercial Development. *Department of Botany, University of Nairobi, Kenya Agricultural Research Institute (KARI), Nairobi, Kenya*.

NAGATA, N., KAZUTAKA, M. & YUKISATO I 1999. Inhibitory Effects of Catecholamines and Anti-Oxidants on the Fluorescence Reaction of 4,5-Diaminofluorescein, DAF-2, a Novel Indicator of Nitric Oxide. *Biochemistry.* 125**,** 658 – 661.

NEUWINGER, H. D. 1996. African Ethnobotany, Poisons and Drugs, Chemistry-Pharmacology-Toxicology. *Chapman and Hall, Weinheim, Bundesrepublik Deutschland*.

OBEBIYA, O. O. & SOFOWORA, E. A. 1979. Antimicrobial alkaloids from Nigerian chewing sticks. *Planta medica,* 36 204.

OCHIENG' OBADO, E. A. & ODERA, J. A. 1995. Management of medicinal plant resources in Nyanza. *Traditional Medicine in Africa. Edited by I. Sindiga, C. Nyalgotti-Chacha & M. P. Kanunah. East African Educational Publishers Ltd., Nairobi***,** 153-167.

OLILA, D. O. & OPUDA, A. J. 2001. Antibacterial and Antifungal Activities of extracts of Zanthoxylum chalybeum and Waburgia ugandensis, Ugandan Medicinal Plants. *African Health Sciences.* 1, 66-72.

PATRICK, M. & BO-TENGNAS 2005. Useful Trees and Shrubs for Kenya; World Agroforestry Centre, Nairobi, Kenya.

QUAMINA, J. 2003. Chemistry and Industry. *A Magazine of Society of Chemistry and Industry, May 5***,** Pg 13.

RAWAT, R. B. S. & UNIYAL, R. C. 2003. National Medicinal plants Board committed for overall development of the sector. 1**,** 12 – 16.

REINARTZ, J. A. & POPP, J. W. 1987. Structures of Clones of Northern Prickly Ash (Zanthoxylum americanum). *American Journal of Botany,* 74 (3)**,** 415- 428.

SINDIGA, I., CHACHA, N. C. & MARY, P. K. 1995. Traditional Medicine in Africa. *East African Educational Publishers Ltd*.

SVENDSEN, B. A. & VERPOORTE, R. 1983. Chromatography of alkaloids. *Chromatography Library,* pg 23.

TREASE & EVANS 1996. Pharmacognosy. *W. B. Sanders Company Ltd, London, Philadephia, Toronto Sydney Tokyo,* pg, 14.

WALL, M. E. & WANI, M. C. 1995. Paclitaxel: from discovery to clinic. *Taxane Anticancer Agents: basic science and current status. ACS Symposium Series 583, Washington D.C***,** pg 18-30.

WEENEN, H., NKUNYA, M. H. H., BRAY, D. H., MWASUMBI, L. B., KINABO, L. S., KILIMALI, V. A. E. B. & WIJNBERG, J. B. P. A. 1990. Antimalarial compounds contain an α,β-unsaturated carbonyl moiety from Tanzanian medicinal plants. *Planta medica CAB Direct Database, International Centre for Research in Agro-Forestry Centre, Nairobi,* 56**,** 371-373.

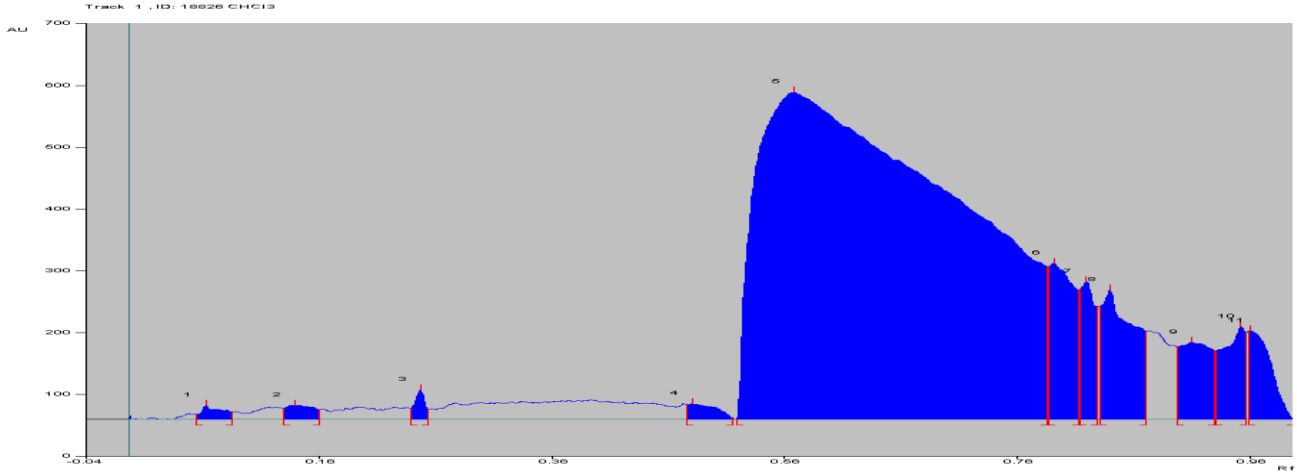
WORLD AGROFORESTRY CENTRE. 2005. Zanthoxylum Gilletii. From Agroforestry Tree Database: a tree species reference and selection guide. *www.worldagroforestry.org/sea/products/AFDbase/af/asp/SpeciesInfo.asp?splD=1788 [accessed 25 April 2008]*.

XIONG, Q., SHI, D. & M;, M. 1995. Flavonol glucosides in pericarps of Zanthoxylum bungeanum. *Phytochemistry,* 39**,** 723 – 725.

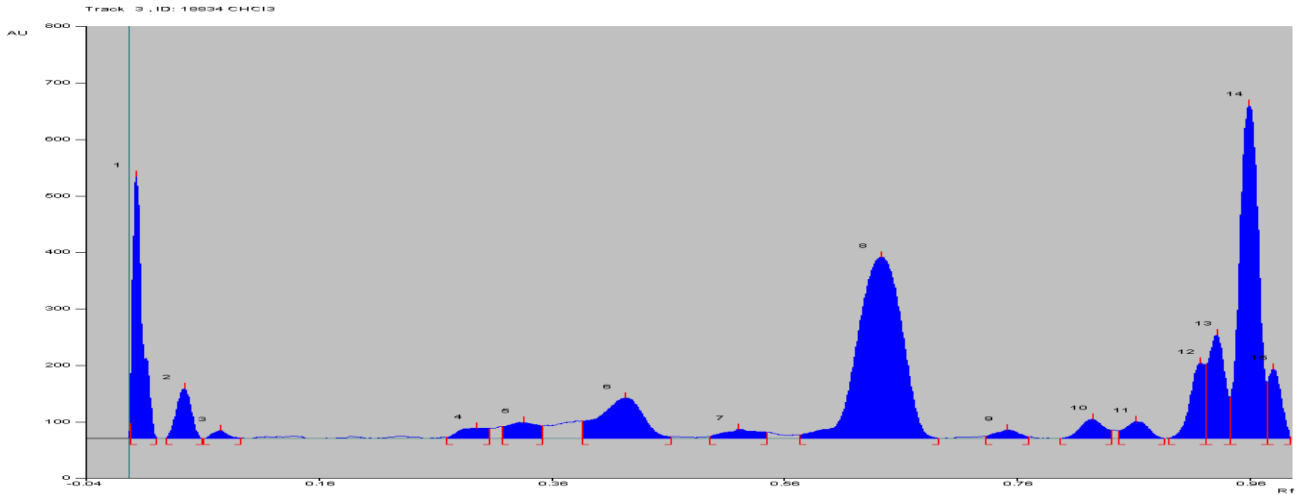
ZIRIHI, G. N., DATTE, Y., JACQUES, K. M. & GRELLIER, P. 2007. Phytochemical and pharmacological studies of alcoholic extracts of Fagara macrophylla (Oliv) Engl (Rutaveae): Chemical structure of active compound including antipaludic activity. *Journal of Chinese Clinical Medicine,* 2, 205- 210.

APPENDICES

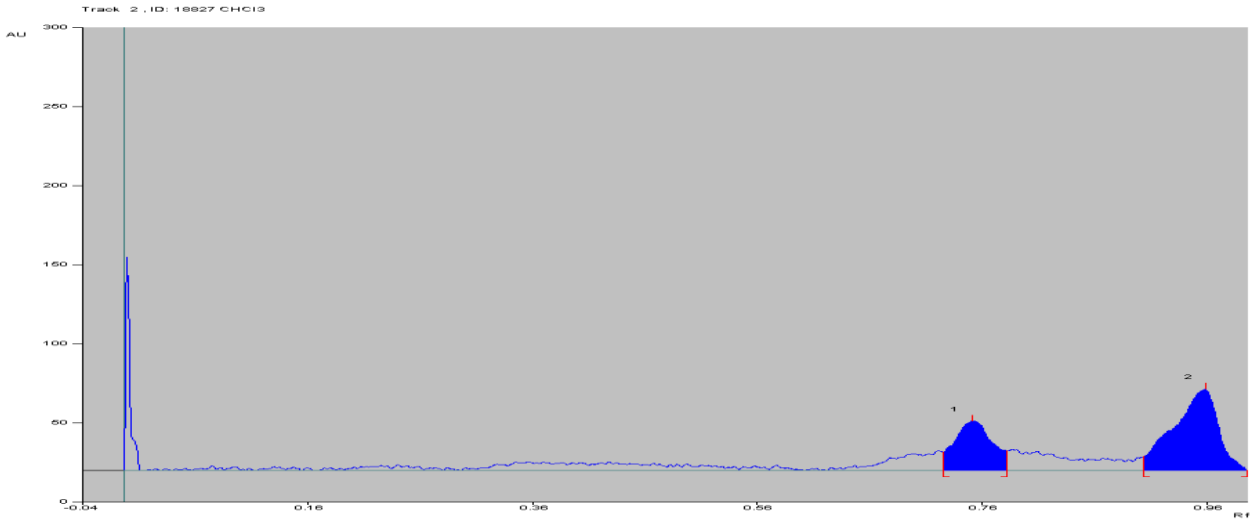
Appendix I: Chromatograms showing the interpretation of the scans of TLC plate analysed at 350 nm, showing the different peaks, peak heights and areas.



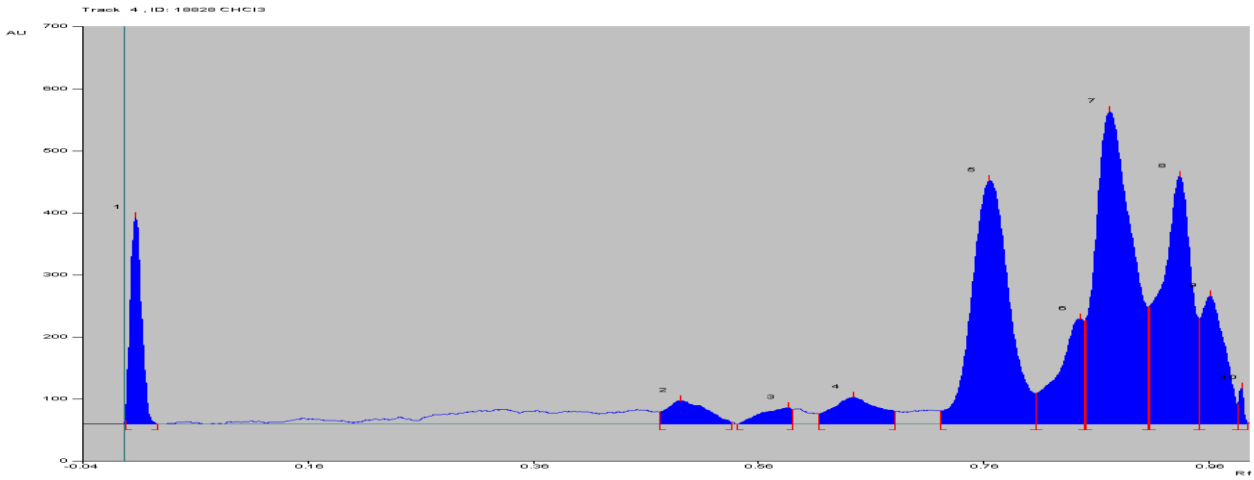
TLC scan chromatogram of BI 18826 (root, Kakameka) at 350 nm



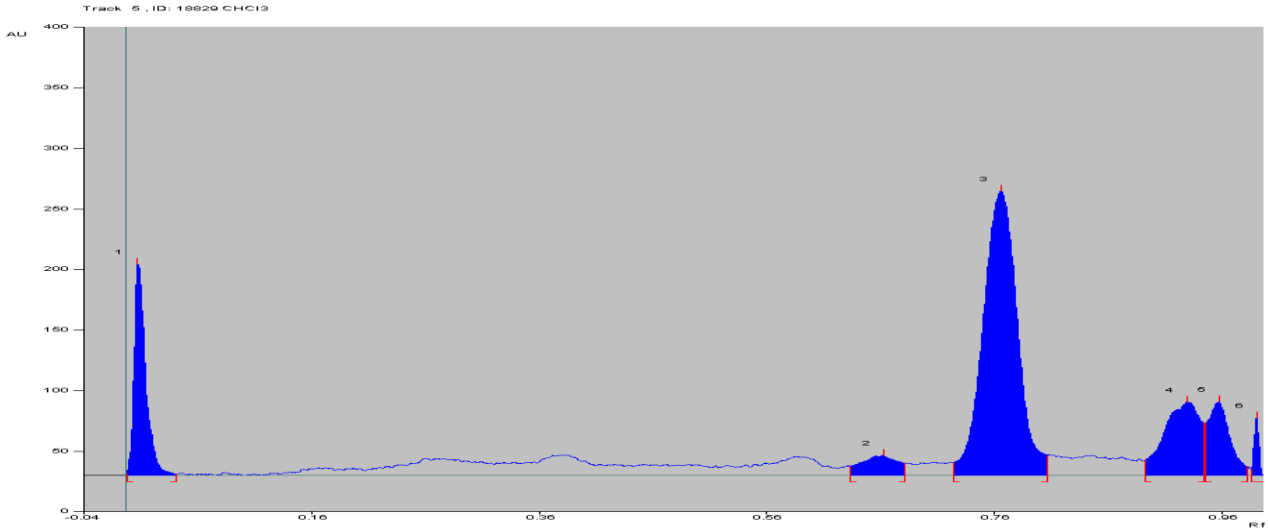
TLC scan chromatogram of BI 18834 (bark, Kakamega) at 350 nm



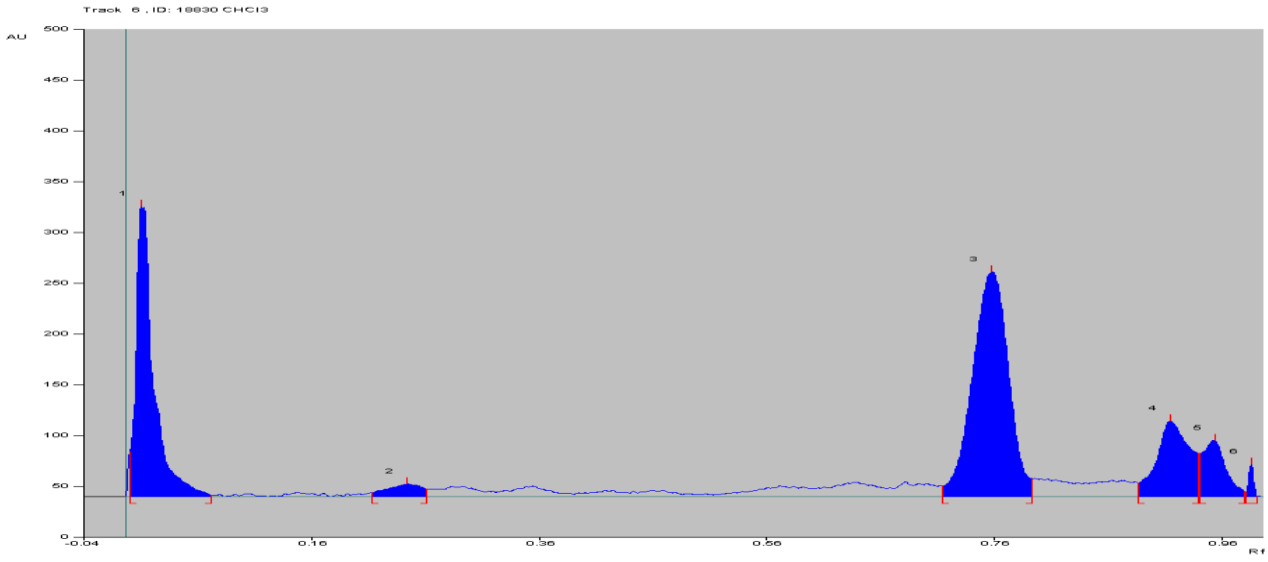
TLC scan chromatogram of BI 18827 (leaf, Kakamega) at 350 nm



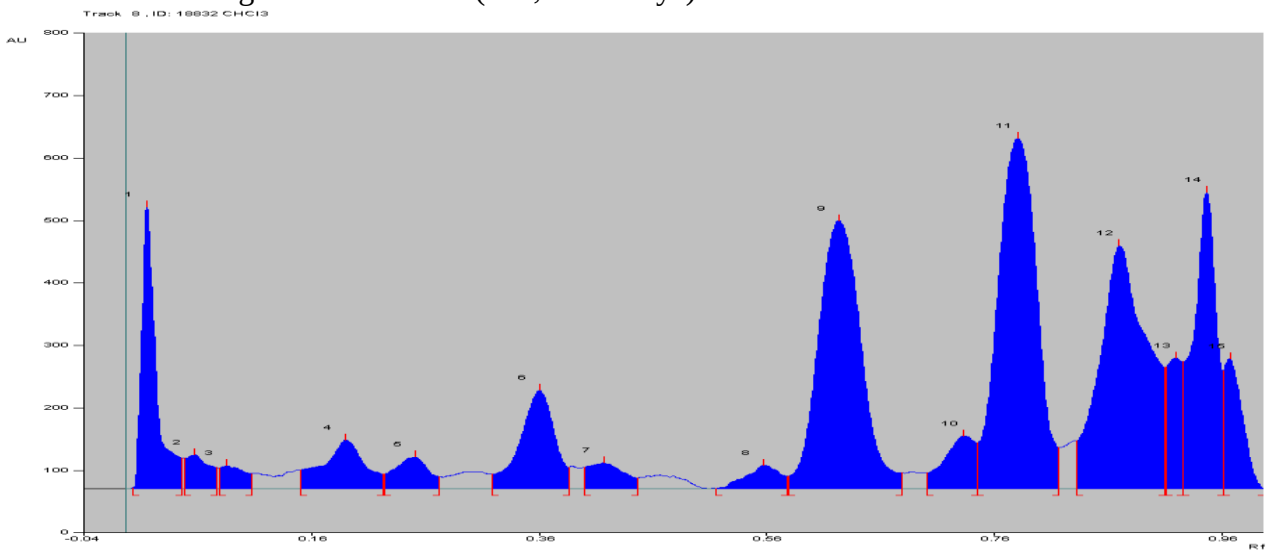
TLC scan chromatogram of BI 18828 (root, Mt. Kenya) at 350 nm



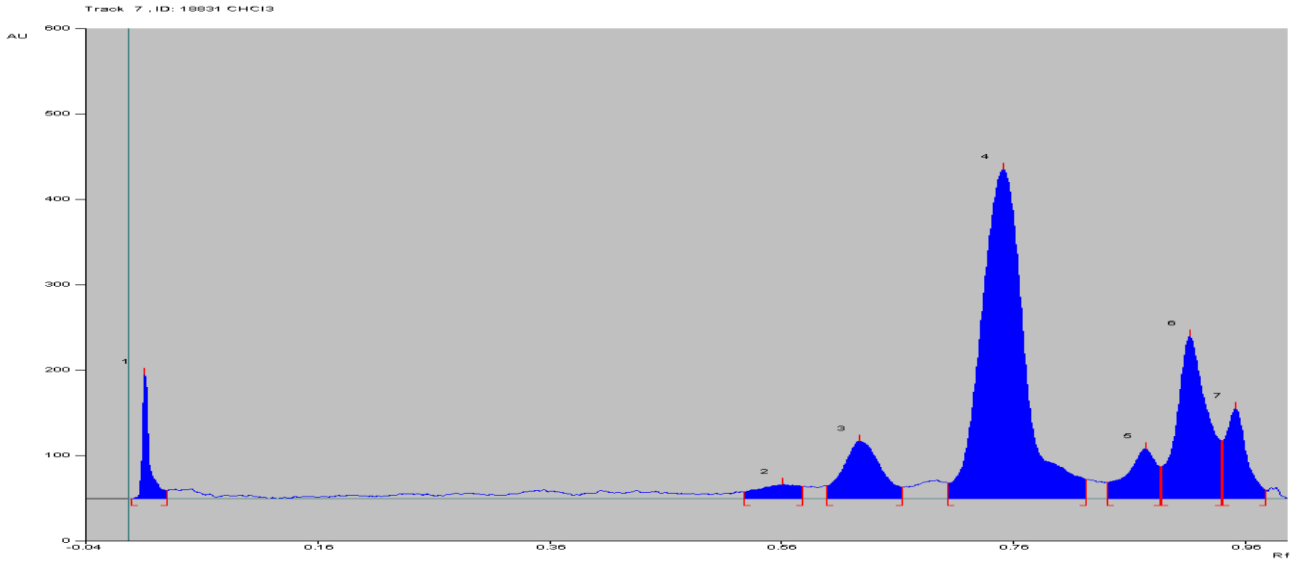
TLC scan chromatogram of BI 18829 (bark, Mt. Kenya) at 350 nm



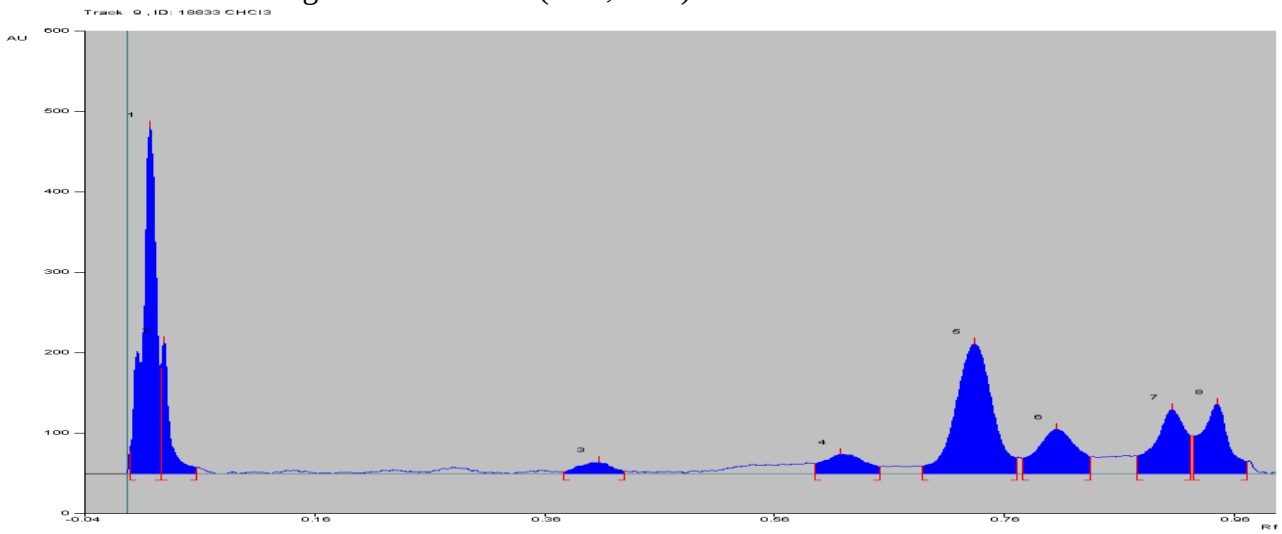
TLC scan chromatogram of BI 18830 (leaf, Mt. Kenya) at 350 nm



TLC scan chromatogram of BI 18832 (root, Mau) at 350 nm



TLC scan chromatogram of BI 18831 (bark, Mau) at 350 nm



TLC scan chromatogram of BI 18833 (leaf, Mau) at 350 nm

Appendix II: Summary of the antimicrobial activity tests carried out

plant part/region	**Extract ID**	**Tests carried out/observations**			
		S. cereviciae	*C. herbarum*	*B. subtilis*	*P. seringae*
Root, Kakamega	BI 18826	−	++	++	++
Root, Mt. Kenya	BI 18828	−	++	+	+
Root, Mau	BI 18832	−	++	++	++
Bark, Kakamega	BI 18834	−	++	+	+
Bark, Mt. Kenya	BI 18829	−	–	–	+
Bark, Mau	BI 18831	−	–	–	–
Leaf, Kakamega	BI 18827	−	–	–	–
Leaf, Mt. Kenya	BI 18830	−	–	–	–
Leaf, Mau	BI 18833	–	–	–	+
Nystatin	Nt	++	++		
Chloramphenical	Chl			++	++

(++) active; (+) mildly active; (–) not active; (≠) not tested; (Nt) nystatin, positive control antifungal assays; (Chl) chloramphenical, positive control antibacterial assays

Appendix IV: Single factor analysis of varience (ANOVA) for MICs of all extracts

Analysis of data comparing chloroform extracts of root and bark collected from Kakamega, Mount Kenya and Mau against *B. subtilis* and *P. syringae*

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Chloroform extracts of *B. subtilis*	4	450	112.5	3958.333333
Chloroform extracts of *P. syringae*	4	275	68.75	1406.25

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3828.1	1	3828.1	1.427184466	0.2773	5.987
Within Groups	16094	6	2682.3			
Total	19922	7				

Analysis of data comparing methanol extracts of root and bark collected from Kakamega, Mount Kenya and Mau against *B. subtilis* and *P. syringae*

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Methanol extracts, *B. subtilis*	3	350	116.67	5833.33
Methanol extracts, *P. syringae*	3	350	116.67	5833.33

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0	1	0	0	1	7.709
Within Groups	23333.3	4	5833.3			
Total	23333.3	5				

Analysis of data comparing both chloroform and methanol extracts of root and bark collected from Kakamega, Mount Kenya and Mau against *B. subtilis* and *P. syringae*

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Chloroform extracts of *B. subtilis*	4	450	112.5	3958.33
Chloroform extracts of *P. syringae*	4	275	68.75	1406.25
Methanol extracts, *B. subtilis*	3	350	116.67	5833.33
Methanol extracts, *P. syringae*	3	350	116.67	5833.33

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6153.3	3	2051.1	0.52022	0.6779	3.7083
Within Groups	39427	10	3942.7			
Total	45580	13				

Appendix V: HPLC ESI-MS identification; MS2 fragmentation ion (m/z $[M+H]^+$) for all the identified compounds.

Fagarine I																				
m/z	149	165.1	188.1	189	190.1	191.09	192.1	206.07	290.1	291.1	306.1	320.2	321.1	322.2	323.094	337.17	339.14	340.15	352.2	353.14
Intensity	790	556.5	10581	1598	1521	565.9	560.4	2312.8	2980	926.3	798.2	508.2	2861	1336	613.3	1109.3	1098.2	608.7	12174	2918.7
Relative	6.49	4.57	86.92	13.1	12.49	4.65	4.6	19	24.48	7.61	6.56	4.17	23.5	10.97	5.04	9.11	9.02	5	100	23.98
Sanguinarine																				
m/z	274	275.1	286.1	287	290.1	291.16	302.1	303.33	304.1	305.1	314.1	315.1	316.1	317.2	318.215	319.07	330.06	332.09	333.1	334.15
Intensity	3754	967.3	643.1	637	842.1	1707.6	7267	3638.1	21971	4874	1486	1861	1249	1339	6563.3	23890	5939.1	2132.2	560.9	822.5
Relative	15.7	4.05	2.69	2.67	3.52	7.15	30.42	15.23	91.97	20.4	6.22	7.79	5.23	5.6	27.47	100	24.86	8.93	2.35	3.44
8-methylnorchelerythrine																				
m/z	287	288	289	290	302	304	305	306	315	316	317	318	319	320	332	333	334	335	348	349
Intensity	6515	1742	519.4	450	388.3	190869	41698	1524.5	71442	23228	12316	43548	8695	1304	324130	706149	145006	1763.5	17342	3523.2
Relative	0.92	0.25	0.07	0.06	0.05	27.03	5.91	0.22	10.12	3.29	1.74	6.17	1.23	0.18	45.9	100	20.53	0.25	2.46	0.5
trans-fagaramide																				
m/z	89	91	117	119	120	126	135	145	146	147	149	150	163	164	175	176	177	192	193	206
Intensity	1294	2489	6683	7474	1279	1634.2	1907	186602	15238	2384	58436	8057	18107	1198	5136705	492165	12871	24532	3157	1090.4
Relative	0.03	0.05	0.13	0.15	0.02	0.03	0.04	3.63	0.3	0.05	1.14	0.16	0.35	0.02	100	9.58	0.25	0.48	0.06	0.02
Dihydronitidine																				
m/z	287	290.2	303.3	304	305.1	306.21	315.1	316.18	317.3	318.2	319.2	320.1	321.1	322.1	332.138	333.14	334.29	335.12	336.1	348.1
Intensity	346	323.8	611.3	8231	2091	454.1	3750	1231.4	1242	9784	53456	13918	1926	278.4	12058.9	37591	17372	117394	24155	658.6
Relative	0.29	0.28	0.52	7.01	1.78	0.39	3.19	1.05	1.06	8.33	45.54	11.86	1.64	0.24	10.27	32.02	14.8	100	20.58	0.56
Hesperidin																				
m/z	281	303.1	327.2	345	369.1	411.17	413.1	429.09	431	447.1	449	459.1	464.9	473.1	489.046	490.99	538.97	557.04	575	592.96
Intensity	810	16389	471.3	3533	2032	572.5	4971	666	8593	7355	31345	443.8	8529	825.1	2747.7	1309.7	505.5	2635.1	3792	1422.5
Relative	2.59	52.29	1.5	11.3	6.48	1.83	15.86	2.12	27.41	23.47	100	1.42	27.21	2.63	8.77	4.18	1.61	8.41	12.1	4.54
Diosmin																				
m/z	301	302.1	314.1	315	411.1	429.11	447.1	463.04	464	476.1	477.1	561	562.1	563	575.841	577.24	578.71	591	595.1	596.29
Intensity	5670	366	1130	213	102.4	79.5	256.2	32066	1826	2731	609.2	509.8	189	101.8	171.8	224.7	944.5	81.5	104.7	94
Relative	17.7	1.14	3.52	0.66	0.32	0.25	0.8	100	5.69	8.52	1.9	1.59	0.59	0.32	0.54	0.7	2.95	0.25	0.33	0.29