PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITY OF *GIRARDINIA DIVERSIFOLIA* (LINK) FRIIS (URTICACEAE)

BY

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DECLARATION

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

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b) This thesis has been submitted for examination with our approval as university supervisors.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	- percent
$(M+1)^+$	- molecular ion
£	- sterling pounds
°C	- degrees Celsius
µg	- microgram(s)
µl	- microlitre(s)
µm	- micrometre(s)
^{13}C	- carbon-13 isotope
^{1}H	- proton
ATCC	- American type culture collections
BPH	- Benign Prostate Hyperplasia
C	- carbon atom
cm	- centimetre(s)
cm^{-1}	- frequency/wavenumber
CMR	- carbon-13 nuclear magnetic resonance
d	- doublet
EAPRM	- ethyl acetate partition of root methanol extract
EAPSM	- ethyl acetate partition of stem methanol extract
EIMS	- electron impact mass spectrometry
eV	- electron volts
g	- gram(s)
GdRC	- *Girardinia diversifolia* root chloroform extract
GdRM	- *Girardinia diversifolia* root methanol extract
GdRP	- *Girardinia diversifolia* root petroleum ether (60-80 °C) extract
GdSC	- *Girardinia diversifolia* stem chloroform extract
GdSM	- *Girardinia diversifolia* stem methanol extract
GdSP	- *Girardinia diversifolia* stem petroleum ether (60-80 °C) extract
h	- hour(s)
H	- hydrogen atom/proton
i.d.	- internal diameter
Inc.	- incorporated
IR	- infra-red

IUPAC	- International Union of Pure and Applied Chemistry
kg	- kilogram
L	- litre(s)
LSC	- liquid-solid column chromatography
Ltd.	- limited
M	- molar
m	- multiplet
m/z	- mass to charge ratio
mg	- milligram(s)
MHz	- megahertz
MIC	- minimum inhibitory concentration
min	- minute(s)
ml	- millilitre(s)
mm	- millimetre(s)
MS	- mass spectrometry
MW	- molecular weight
nm	- nanometre(s)
NMR	- nuclear magnetic resonance
Pa	- Pascals
PMR	- proton nuclear magnetic resonance
q	- quartet
R_f	- retention factor
s	- singlet
t	- triplet
TLC	- thin layer chromatography
UV	- ultraviolet
v/v	- volume by volume
w/v	- weight by volume
WHO	- World Health Organisation
λ_{max}	- wavelength of maximum absorption
ν_{max}	- frequency of maximum absorption

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ABSTRACT

Girardinia diversifolia (Link) Friis (Urticaceae) is a herb used traditionally for various ailments, including microbial infections. The objective of this study was to carry out phytochemical investigation of its chemical constituents and screen it for *in vitro* antibacterial and antifungal activities.

The plant material was collected from Kamweti Location, Kirinyaga District. Phytochemical studies showed that the roots and stems of *Girardinia diversifolia* contained saponins, tannins and cardiac glycosides but lacked alkaloids and anthracene glycosides. Extracts of the plant material were prepared by sequential Soxhlet extraction using petroleum ether (60-80 °C), chloroform and methanol. Ethyl acetate extract was prepared by partitioning the methanol extract between water and ethyl acetate. Chromatographic fractionation of the petroleum ether root extract using normal-phase silica gel yielded four crystalline compounds, three of which were identified as β-sitosterol, 3-hydroxystigmast-5-en-7-one and 7-hydroxysitosterol. This is the first time that these compounds are reported from *Girardinia diversifolia.*

Agar diffusion method was used to screen for *in vitro* antibacterial and antifungal activities. All the tested extracts exhibited varying degrees of antibacterial activity against *Bacillus pumilus*, *Staphylococcus aureus* and *Escherichia coli*. Bioautography using *Bacillus pumilus* revealed several components with antibacterial activity in all extracts. Only the ethyl acetate extract exhibited significant antifungal activity against *Aspergillus niger* and *Candida albicans*. Bioautography using *Saccharomyces cerevisiae* revealed one component in the ethyl acetate extract with remarkable antifungal activity. 7-hydroxysitosterol and the yet to be identified fourth isolate exhibited modest antibacterial activity against *Bacillus pumilus*.

The present study gives scientific credence to the traditional use of *Girardinia diversifolia* in the treatment of bacterial and fungal infections. The antimicrobial activity seems to be concentrated in the polar fractions and these should be investigated further to isolate the bioactive components. *In vivo* studies should also be carried out to establish therapeutic efficacy and toxicity of the isolates.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Plants as sources of pharmaceuticals

The pharmaceutical industry has experienced tremendous growth in recent years. Fuelled by technological advancement in medicinal and combinatorial chemistry, and increased knowledge of molecular biology, the process of drug discovery has undergone rapid evolution. Using highly automated laboratory processes, numerous drugs have been rationally designed, synthesized, clinically tested and marketed. Many of these drugs have been at the forefront in combating existing, emerging and re-emerging diseases. However, in spite of major technological advances in synthetic drug production, plants still occupy an important position in health care delivery system [1].

Herbal medicines have experienced an unprecedented renaissance in the past two decades [2]. Whereas the increase in usage in the developed world may be attributed to a desire to consume what is natural and hence avoid the deleterious effects of synthetic compounds, the economic reality of the inaccessibility of modern medication for many in the developing countries has played a major role in the broad use of herbal medicines [3]. The World Health Organization (WHO) estimates that in Africa, up to 80 % of the population uses traditional medicine for primary health care [4]. Continuous research is needed to ascertain the efficacy of these medicinal plants as attempts are made globally to integrate traditional medicine with conventional health care systems. The folklore knowledge of use of plants and other natural products will guide this process.

Plants and plant extracts have been used empirically as therapeutic agents since time immemorial. Before the advent of modern medicines, plants were solely depended on for treatment of various ailments. Over the years, through trial and error, communities all over the world have identified many plants with medicinal properties. This folklore knowledge is passed along generations. The pharmaceutical field is awash with numerous plant-based drugs that have contributed immensely to modern conventional

health care delivery system. The WHO estimates that 25 % of modern medicines are derived from plants first used traditionally [4].

On numerous occasions, the folklore use of plants in treatment of specific disease syndromes with success has provided the primary hint for industrial research that has led to development of some of the plant-based pharmaceuticals. This approach is best exemplified by the antimalarial drug, artemisinin. For more than 2,000 years, the Chinese have used an extract from wormwood plant, *Artemisia annua*, for the treatment of malarial fever. In the 1970's, bioassay-guided screening and phytochemical investigation of the plant extract led to identification and structural elucidation of the active principle against malaria as a sesquiterpene lactone peroxide, artemisinin. More active derivatives such as oil-soluble artemether and a water-soluble artesunate have been synthesized [5]. Many other valuable medicines have been obtained from plants via a similar approach.

Native Peruvians had traditionally used the stem bark of *Cinchona succirubra* as a febrifuge [6]. Scientific research led to isolation of several quinoline alkaloids, two of which are the antimalarial quinine, and its diastereomer quinidine used as an antiarrhythmic agent. Discovery of quinine revolutionized treatment of malaria and has remained the drug of choice in the management of multidrug-resistant and severe complicated malaria due to *Plasmodium falciparum.* The commercial sources of the two drugs are *C. succirubra, C. ledgeriana, C. calisaya* and their hybrids [6].

In South America, a crude extract of the stem bark of various species of the genus *Strychnos,* referred to as curare, was used as an arrow poison [6]. It was observed to cause muscle paralysis in animals without killing them. Phytochemical studies led to isolation of d-tubocurarine, an isoquinoline alkaloid. Tubocurarine is used in surgery as a skeletal muscle relaxant. Prospective studies showed higher amounts were obtainable from *Chondodendron tomentosum,* and this has remained its commercial source.

In other cases, standardized solvent extracts of medicinal plants have been developed and used clinically. A case in point is the chloroform extract of the stem bark of *Prunus africana.* In several parts of Africa, especially South Africa, the stem bark of

Prunus africana was traditionally used for the relief of micturition difficulties caused by prostatic enlargement in elderly men [6]. Scientific work demonstrated clinical benefits of the chloroform extract of its stem bark in the management of Benign Prostate Hyperplasia (BPH) and was subsequently patented by a French scientist in 1971. Although the active component responsible for prostatic therapy has not yet been identified, clinical and pharmacological experiments have consistently confirmed the efficacy of the extract in the treatment of BPH. A toxicological study on the systemic effects of chloroform, methanol and aqueous extracts of *P. africana* bark in laboratory rats revealed mild to negligible acute and chronic toxicities of the bark when administered at therapeutic doses. The chloroform extract demonstrated organ-specific activity since it had no significant effect on the seminal vesicular and testicular tissues while it caused significant reduction in weight of the prostate gland, the pathological site in BPH [7]. Solvent extracts of several other plants used indigenously in the management of prostatic enlargement-related symptoms have also been developed and commercialized. These include *Urtica dioica, U. urens, Serenoa repens* and *Cucurbita pepo.*

In several other cases, serendipity has played a part in the discovery of novel compounds of medicinal value. An example is the discovery of the vinca alkaloids. Different communities worldwide have traditionally used Madagascar periwinkle, *Catharanthus roseus*, for treatment of various ailments such as diabetes, colds, inflammation, sore throats and tumours [6]. The folklore claims of its medicinal uses aroused the interest of Western researchers. During a screening programme for plants with antidiabetic activities, scientists from the University of Western Ontario in Canada discovered that extracts of *C. roseus* from the West Indies had leukopenic activity, while a random screening programme at the Eli Lilly Laboratories in Indianapolis, USA, showed the plant has anticancer properties. Combined efforts by the two research institutes led to the isolation of the active bis-indole alkaloids vinblastine and vincristine, which were developed as commercial drugs [8]. Both are used in conventional medicine as antineoplastics. A semi-synthetic derivative, 5′-nor-anhydrovinblastine (vinorelbine), has been developed and is already in clinical use.

Numerous other plants have provided lead compounds that have acted as models for development of new drugs. For example, camptothecin, a quinoline alkaloid isolated

from *Camptotheca acuminata*. Camptothecin has high antineoplastic activity but lacks clinical utility due to its toxicity. Two anticancer analogs, topotecan and irinotecan, were derivatized and are in clinical use [6].

Diosgenin, a steroidal sapogenin, is used as a starting molecule in semisynthesis of sex hormones including oral contraceptives such as mestranol and norethisterone. It is commercially sourced from the leaves and rhizomes of certain medicinal plant species of the genus *Dioscorea* such as *D. deltoidea, D. sylvatica, D. mexicana, D. composita* and *D. prazeri.* A closely related steroidal sapogenin referred to as hecogenin acts as a precursor molecule in semisynthesis of corticosteroids such as cortisone and betamethasone. Its commercial sources are the leaves of *Agave sisalana* cultivated in the East African region for sisal, *A. rigida* found in Mexico and *Hechtia texensis* of Central America [6].

Plants have remained the primary source of several other clinically useful drugs. These include atropine, an anticholinergic from *Atropa belladonna*, codeine, a narcotic analgesic from *Papaver somniferum*, colchicine, an anti-inflammatory agent from *Colchicum autumnale*, digoxin, a cardiotonic from *Digitalis lanata*, the antineoplastic taxanes paclitaxel and docetaxel from the genus *Taxus*, and reserpine, an antihypertensive indole alkaloid from *Rauwolfia serpentina* [1].

It is estimated that the world market for drugs derived from plants is in excess of £ 450 billion [9]. Further, it is estimated that the number of higher plant species on earth is well over 250,000, of which only about 2,500, representing only 1 %, have been investigated to any depth in terms of their bioactive potentials [1]. Since most secondary metabolites are genus or species-specific, the plant kingdom, undoubtedly, still holds many species containing substances of medicinal value which have yet to be discovered. This untapped wealth has become an important research area by multinational drug companies and research institutes. Large-scale programs are ongoing to prospect for new drugs to cure diseases against which the available treatments have severe limitations such as cancer and malaria, to manage recent syndromes which are still incurable such as Acquired Immune Deficiency Syndrome (AIDS), and to replace or augment molecules rendered ineffective by the development of therapy-resistant strains such as drug-resistant *Mycobacterium tuberculosis* [6,10].

Each year over 2,000 novel molecular entities are reported from plants, up to one third of which may have some degree of biological activity [1]. Therefore, the potential for discovery of novel pharmacologically active principles from plants, and the subsequent economic commercialisation, is considerable.

Infections from bacteria and fungi are one health issue for which numerous plant-based remedies have been used traditionally. Many of these remedies have continually been used without concrete scientific evidence of their pharmacological activities. As with all other phytopharmaceuticals, modern scientific research is essential to provide scientific substantiation of folklore claims of therapeutic efficacy and safety of these remedies, and identify their active constituents.

1.2 Bacterial and fungal infections

Humans have coexisted with microbes for as long as they have lived. Some of these microbes are pathogenic. According to the latest WHO estimates, infectious diseases cause approximately 15 million deaths annually, accounting for 26 % of global mortality [11]. Bacterial and fungal infections are among these infectious diseases.

Several bacteria are culprits in pathogenesis of illnesses in humans. Gram-positive bacterial infections are attributed mostly to members of the genera *Staphylococcus, Streptococcus, Bacillus, Clostridium, Corynebacterium, Mycobacterium, Actinomyces, Nocardia* and the Spirochetes *Borrelia, Leptospira* and *Treponema*. Gram-negative bacterial infections are caused mainly by microorganisms of the genera *Neisseria*, *Vibrio, Pseudomonas*, Enterobacteriaceae such as *Salmonella, Shigella, Escherichia, Klebsiella* and *Proteus*, and the Parvobacteria *Haemophilus, Bordetella, Brucella, Pasteurella* and *Legionella* [12]. The infectivity, virulence and pathogenicity of these microorganisms vary depending on their invasiveness and the host's immunity. A few of these microorganisms infect one particular organ system, while others infect several organ systems. Generally, they are involved in respiratory tract infections, gastrointestinal tract infections, cardiovascular system infections, nervous system infections, skin infections, genito-urinary tract infections, post-surgical and post-trauma infections, and bone and joint infections [13].

Fungi produce disease in humans in either of the two morphological forms, yeasts or moulds. Yeast-like fungi involved in human infections include *Blastomyces dermatitidis*, *Candida albicans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*, and the infective agents of chromoblastomycosis, namely, *Phialophora verrucosa*, *Fonsecaea pedrosoi*, *F. compactum* and *Cladosporium carrionii*. Pathogenic moulds include the *Aspergillus* species, the dermatophytes and fungi of the order Mucorales [12-14]. Depending on the level of penetration into the body tissues, they produce three types of mycoses. Dermatomycoses, which infect the skin and its appendages, and the mucous membranes, are usually due to the dermatophytes of the genera *Epidermophyton*, *Microsporum* and *Trichophyton*. Subcutaneous mycoses infect the dermis, connective and bone tissues. They include sporotrichosis, chromoblastomycosis, phaeohyphomycosis and maduromycosis. Systemic mycoses infect the internal organs. They include those due to true pathogens, namely, coccidioidomycosis, paracoccidioidomycosis, histoplasmosis and blastomycosis, and those due to opportunistic fungi namely cryptococcosis, aspergillosis, mucormycosis, pneumocystosis and candidiasis [12].

Fungal infections in humans have increased dramatically in incidence and severity in recent years as a detrimental effect of advances in surgery and cancer treatment, increased use of broadspectrum antibiotics and emergence of Human Immunodeficiency Virus (HIV). Three of these are of particular medical significance as opportunistic infections in immunosuppression syndromes: cryptococcal meningitis, oropharyngeal candidiasis and *Pneumocystis carinii* pneumonia [13].

The burden of disease impacted by microbial infections is enormous. Governments worldwide are spending a large proportion of their national budgets on curative health care to provide treatment to illnesses arising from bacterial and fungal infections. Governments in the developing world are faced by a twin problem of disease and poverty. In these countries, Kenya included, poverty exposes hundreds of millions of people to the hazard of infectious diseases in their everyday lives, yet these countries have limited resources to adequately address health problems of their citizenry [15,16].

1.3 Antibacterial and antifungal drugs

Since their discovery during the 20th century, antimicrobial agents have substantially reduced the threat posed by infectious diseases. The use of these drugs, combined with improvements in sanitation, housing and nutrition, and the advent of widespread immunization programmes, has led to a dramatic drop in deaths from diseases that were previously endemic, untreatable and frequently fatal. Over the years, antimicrobial agents have saved the lives and eased the suffering of millions of people. By helping to bring many serious infectious diseases under control, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century [17]. Antibacterial and antifungal drugs have played a part in realization of this global success against disease.

Several antibacterial and antifungal drugs are in current clinical use [18-20]. A number of them were first isolated from lower plants. Such include penicillins from the mould *Penicillium chrysogenum*, cephalosporins from the mould *Cephalosporium acremonium*, chloramphenicols from the bacterium *Streptomyces venezuelae*, lincosamides, macrolides, tetracyclines, rifamycins, and amphotericin B from certain *Streptomyces* species, griseofulvin from the mould *Penicillium griseofulvum*, and nystatin from *Actinomyces* species [21].

However, despite the success of these antibiotics, much of the progress achieved in recent decades towards improvement of human health is now at risk due to development of antimicrobial resistance, emerging and re-emerging microbes, and inaccessibility of more efficacious conventional antimicrobial drugs by the majority poor.

1.3.1 Antimicrobial resistance

The containment of the burden of disease attributable to bacterial and fungal infections is now seriously jeopardized by a recent development: the emergence and spread of microbes that are resistant to affordable and effective first-line drugs. Thus, the clinician's antibiotic armamentarium is being steadily eroded, and research

directed towards isolation or synthesis of new drugs is still a matter of considerable urgency [6].

The bacterial infections that contribute most to human disease are also those in which antimicrobial resistance is most evident: diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections and nosocomial infections. Some important examples include penicillin-resistant *Streptococcus pneumoniae,* vancomycin-resistant strains of the genus *Enterococcus*, methicillin-resistant *Staphylococcus aureus,* multidrug-resistant *Salmonella typhi* and *Mycobacterium tuberculosis,* and a new strain of *Escherichia coli* [17].

Resistance of microbial infections to first-line antimicrobials has necessitated introduction of second-line and, in some cases, third-line drugs. These replacement drugs, though equally efficacious, are usually more toxic and less clinically characterized than the first-line drugs. They are only considered in the case of resistance to the drugs of first choice, failure of clinical response to the conventional therapy, and if their toxicity can be expertly dealt with [18]. In addition, they are nearly always much more expensive than the first-line drugs. For example, the drugs needed to treat multidrug-resistant forms of tuberculosis are over 100 times more expensive than the first-line drugs used to treat non-resistant forms. Ironically, since the high cost of replacement drugs is prohibitive, they remain largely inaccessible in countries where the disease burden is high, with the result that some diseases can no longer be conventionally treated in areas where resistance to first-line drugs is widespread [17]. This forces patients to seek alternative traditional medicines.

1.3.2 Emerging and re-emerging microbes

In spite of concerted efforts at preventive health care, which has included well coordinated immunization programmes, global health is still under threat from existing, emerging and re-emerging infectious diseases. Emergence of new microbes causes re-emergence of previously controlled microbes. For example, the emergence of HIV has caused re-emergence of several microbes including tuberculosis-causing bacterium, *Mycobacterium tuberculosis,* and opportunistic fungi such as *Pneumocystis carinii* and *Cryptococcus neoformans*.

It is appreciated that infectious diseases, which have shaped the course of humanity and caused incalculable suffering and death, will continue to confront the society in unpredictable ways as long as humans and microbes co-exist [16]. This calls for formulation of effective counter measures including continued scientific research into affordable therapies.

1.3.3 Inaccessibility of conventional health care system

Even though most antibacterial and antifungal drugs in current clinical use are relatively inexpensive, half of the world's population lacks regular access to essential drugs [15]. A demographic survey carried out in Kenya in 2005 indicated that 56 % of the populace lives below the poverty line [22]. For this segment of the society, conventional medication is out of reach. Their lifeline has been the yet to be regulated traditional medicine practices.

There is no definitive legal framework governing traditional medicine practices and herbal products in Kenya. The current regulatory framework under Legal Notice 147 of 25th. September, 1981 provides mechanism for registration of conventional pharmaceutical products, but excludes alternative/complementary medicinal products. However, a subcommittee on drug registration of alternative and complementary medicines under the aegis of Pharmacy and Poisons Board, the drug regulatory authority, has been providing guidance on registration of formulated herbal products.

In recognition of the potential benefits of the traditional medicine in the mainstream health care delivery systems in the developing countries, the World Health Organization has established a strategic plan for traditional medicine [4]. The plan is aimed at facilitating the integration of traditional medicine into the national health care systems of its member states, and promoting proper use of safe and efficacious traditional remedies. Scientific research in medicinal plants will accelerate this process. This research project is a modest contribution towards this initiative.

1.4 Previous antibacterial and antifungal studies on plants

Several research studies have documented various plants with *in-vitro* antibacterial and antifungal activities. In a large scale screening programme investigating the bioactivity and phytochemistry of African medicinal plants, spanning over a period of 15 years, the Institute of Pharmacognosy and Phytochemistry of the University of Lausanne, Switzerland, has documented several plants with antifungal and antibacterial activities, and their bioactive constituents. More than thirty plants from various African countries exhibiting antifungal activities have been studied following a bioactivity-guided isolation procedure and many natural products with interesting antifungal properties isolated [8]. The antifungal agents include terpenoid, quinonoid, phenylpropanoid and polyphenolic types of constituents. A few of these include: monoterpenes from *Valeriana capense* and *Ocotea usambarensis*, diterpenes from *Parinari capensis*, *Clerodendrum uncinatum* and *Bobgunnia madagascariensis*, triterpene glycosides from *Rapanea melanophloeos*, *Dolichos kilimandscharicus* and *Clerodendrum wildii*, xanthones from *Hypericum roeperanum* and *Polygala nyikensis*, and anthraquinones from *Morinda lucida*. Of particular importance in this research programme, a 'quinone-methide' diterpene with potent antifungal activity has been isolated from the root bark of *Bobgunnia madagascariensis*. This compound showed strong antifungal properties towards human pathogenic fungi, in particular the yeast *Candida albicans* (MIC: 19 µg/ml), as well as against other *Candida* species. It was more potent than the reference antifungal compounds amphotericin B and fluconazole (MIC 0.5 µg/ml) and represents a promising lead for development of novel antimycotic drugs [8]. In the same research programme, the dichloromethane root extract of *Newbouldia laevis* and the dichloromethane leaf extract of *Myrica serrata* exhibited interesting antibacterial activity against *Bacillus subtilis* and *Escherichia coli*.

Elsewhere, studies on the essential oil of *Brachylaena hutchinsii* showed that it has antibacterial activity comparable to that of gentamicin against *Proteus mirabilis* [23]. It also exhibited activity against *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus luteus* and *Enterococcus faecalis*. In another research work, various solvent extracts of the stem bark of *Pterocarpus erinaus* exhibited varying

degrees of antimycobacterial activity against four *Mycobacterium* species: *M. stegmatis, M. tuberculosis, M. bovis* and *M. avium-complex* [24].

Khatune *et al.*, working on the seeds of *Psoralea corylifolia*, isolated four compounds namely psoralidin, bakuchicin, psoralen and angelicin. Pharmacological activity studies demonstrated significant antibacterial activities of the isolated compounds against a number of Gram-positive and Gram-negative bacteria [25]. Ramezani *et al.* reported antimicrobial activity of the methanol extracts of the aerial parts of four *Artemisia* species of Iran, namely: *A. diffusa, A. oliveriana, A. scoparia* and *A. turanica.* All extracts were effective against two Gram-positive bacteria, *Bacillus subtilis* and *S. aureus.* In addition *A. oliveriana* and *A. turanica* inhibited *Pseudomonas aeruginosa* while *A. scoparia* exhibited antifungal activity against *C. albicans* [26].

Ahmad and Arina reported antibacterial and antifungal activities of ethanolic extracts of 45 Indian medicinal plants against certain multi-drug resistant bacteria and a yeast, *C. albicans*, of clinical origin. Qualitative phytochemical tests, thin layer chromatography and TLC bioautography of certain active extracts demonstrated the presence of common phytocompounds in the plant extracts including phenols, tannins and flavonoids as major active constituents [27]. Elsewhere, research work conducted by Saeed and Sabir demonstrated wide ranging antibacterial activity of an essential oil, chloroform extract and seven newly isolated sesquiterpenoids from the oleo-gum-resin of *Commiphora mukul* against both Gram-positive and Gram-negative bacteria comparable to that of kanamycin used as the reference [28].

Phytochemical studies on *Ganoderma annulare* by Smania *et al.* led to isolation of, amongst others, five triterpenes, namely applanoxidic acids A, C, F, G and H. The minimum inhibitory concentration (MIC) of applanoxidic acids A, C and F against *Microsporum canis* and *Trichophyton mentagrophytes* ranged between 500 and 1000 µg/ml. Although the exhibited activity was not comparable to that of conventionally used antifungals, the complexity of their structures may encourage further modification studies directed towards increasing the observed antifungal activity [29]. In another study, ethanolic extracts of the trunk bark of some Cuban *Zanthoxylum* species, *Z. fagara, Z. elephantiasis* and *Z. martinicense*, showed activity against

different species of fungi [30]. Another study demonstrated a moderate antibacterial and a high antifungal activity of the ethanolic extract of *Erigeron breviscapus* whole plant [31].

In a study of the medicinal plants used traditionally by the Fulani ethnic group of Guinea Bissau in the treatment of several disorders including venereal diseases, ethanol extracts of *Cassia sieberiana* root, *Chamaechrista nigricans* aerial parts, *Guiera senegalensis* leaves, and *Terminalia macroptera* root were shown to have promising antibacterial activity with MIC less than 200 µg/ml against nine strains of *Neisseria gonorrhoeae*. The tested strains included penicillin-resistant and tetracycline-resistant clinical isolates. Other plants shown to have *in-vitro* antibacterial activity included: *Lippia chevalieri, Pavetta oblongifolia, Piliostigma thonningii* and *Senna podocarpa* [32].

A study conducted on plants from 20 families used in the folk medicine of the Hausa ethnic group of Northern Nigeria showed that extracts of *Annona squamosa, Calotropis procera, Trianthema pentandra* and *Tephrosia purpurea* exhibited desirable selective antibacterial activity. The extract of *A. squamosa* was strongly active against *Salmonella* species and *E. coli*, while *T. pentandra* showed strong activity against *Streptococcus* species, *Proteus vulgaris* and *S. aureus* [33]. Extracts of several plants, including *Combretum micranthum, Cucurbita maxima, Hibiscus sabdariffa, Microcarpus scaber, Pistia stratoites* and *Sclerocarya birrea*, demonstrated broad, non-specific antibacterial activity.

Working on *Psoralea grandulosa*, Erazo and co-workers described significant antibacterial activity of its petroleum ether extract against Gram-positive bacteria. The extract had a MIC of 12.5 µg/ml against *S. epidermidis, S. aureus* and *Micrococcus flavus*, while against *B. subtilis* the recorded MIC was 25 µg/ml. Using bioautographic agar overlay assay technique, two compounds were shown to be responsible for the observed antibacterial activity. One of the compounds was isolated and identified as a monoterpene, bakuchiol. It had a MIC of 10 µg/ml against all the tested Gram-positive bacteria except *S. aureus*, for which the MIC was 5 µg/ml [34]. In a similar study, antimicrobial evaluation of the differential solvent extracts of *Garcinia kola* seeds revealed that the petroleum ether, ethanol and ethyl acetate

fractions possessed antimicrobial properties. Further work revealed that the observed activity was due to the presence of a polyisoprenyl benzophenone, kolanone, in the petroleum ether extract, as well as the hydroxybiflavanonols present in the ethyl acetate fraction. One of the hydroxybiflavanonols showed significant bacteriostatic activity against both Gram-positive and Gram-negative bacteria, and fungistatic activity against *C. albicans* and *Aspergillus niger*. Its MIC against *S. aureus* was 3.1×10^{-7} µg/ml and 3.1×10^{-3} µg/ml against *E. coli* [35].

The methanol extract of *Memecylon malabaricum* leaves showed strong to moderate inhibitory activity against Gram-positive and Gram-negative bacteria, and fungi. The screened microorganisms included *E. coli, Salmonella typhi, B. subtilis, P. aeruginosa, Aspergillus niger, A. flavus, A. versicolor* and *A. flaviceps* [36]. In a similar work carried out on *Euroschinus papuanus*, the methanolic extracts of the leaves, stem bark, stem heart wood, root bark, root heart wood, and the fractions obtained on partitioning with petroleum ether, dichloromethane, ethyl acetate and butanol, demonstrated a broad spectrum antibacterial activity against a total of 13 Gram-positive bacteria, 12 Gram-negative bacteria, a protozoa and 10 fungi [37].

Several studies have reported plants with broad spectrum antibacterial activities, including: ethanolic extracts of *Acanthospermum hispidum* leaves and flowering tops [38], ethanolic extracts of the leaves and seeds of *Bixa orellana* [39], benzene extract of *Vernonia cinerea* [40], various parts of *Derris elliptica, D. indica* and *D. trifoliata* [41], and the ethanolic extracts of the dry fruits of *Caesalpinia pulcherrima*, aerial parts of *Euphorbia hirta* and the flowers of *Asystasia gangetum* [42].

Other studies have reported combined antibacterial and antifungal activities of *Terminalia catappa, Swietenia mahagoni, Phyllanthus acuminatus, Ipomoea* species, *Tylophora asthmatica, Hyptis brevipes* [43], and *Trewia polycarpa* [44]. Two diterpenoids, 16α-hydroxy-cleroda-3,13(14)-Z-diene-15,16-olide and 16-oxo-cleroda-3,13(14)-E-diene-15-oic acid, isolated from the seeds of *Polyalthia longifolia* demonstrated significant antibacterial and antifungal activities [45]. In another research study, bioactivity guided fractionation of the solvent extracts of the aerial parts of *Lythrum salicaria* led to isolation of two antifungal triterpenoids, oleanolic

and ursolic acids, from the dichloromethane extract, and an antibacterial hexahydroxydiphenoyl ester, vescalagin, from the methanol extract [46].

Some studies have reported plants with selective antifungal activity. Three limonoids isolated from the fruits of *Khaya senegalensis* exhibited moderate antifungal activity [47], while the chloroform partition of the methanol extract of *Alianthus excelsa* stem barks showed fungistatic and fungicidal activity against *A. niger, A. fumigatus, Penicillium frequentence, P. notatum* and *Botrytis cinerea* [48].

These studies indicate that many plants have potential antibacterial and antifungal activities which should be investigated for possible isolation of novel bioactive compounds.

1.5 The genus *Girardinia* Gaudich.

1.5.1 General description

The genus *Girardinia* Gaudich. belongs to the stinging nettles tribe, Urticeae, of the family Urticaceae. Urticeae is usually characterized by distinctive stinging hairs [49]. Before the work of Hutchinson, more than 25 species of *Girardinia* had been recorded [50]. This was mainly due to taxonomic study based on anatomy and morphology. Plants in this genus differ greatly in leaf morphology. However, a study of their amino acid content revealed only two distinct species: *Girardinia bullosa* and *G. diversifolia.* The two species grow widely in Kenya [51].

While *G. bullosa* is consistent in morphology irrespective of geographical and ecological location, *G. diversifolia* shows high diversity and variants were considered as ecotypes. This diversity is so great that variations have been noted on similar plant individuals occurring at different habitats and on the same plant individual at immature and mature stages [50]. In spite of considerable variation, the following formerly accepted distinct taxa have now been reclassified as synonyms of *G. diversifolia*: *G. armata* Kunth, *G. chingianae, G. condensata* (Steud) Weddel, *G. cuspidata* Weddel, *G. erosa* Deen, *G. formosana* Yamemoto, *G. furialis* Blume, *G. heterophylla* Deen, *G. javanica* Weddel, *G. leschenaultiana* Deen, *G. palmata*

Hooker, *G. vitifolia* Franch, *G. zeylanica* Deen, *Urtica acerifolia* Zenker, *U. adoensis* Steud, *U. diversifolia* Link, *U. ferocissima* Sweet, *U. heterophylla* Vahl, *U. horrida* Link, *U. linkiana* Heynh and *U. palmata* Forsk [50].

Morphologically, *G. bullosa* is a tall perennial herb reaching an average height of 250 cm in Kenyan Highlands. Its leaves are broadly ovate to subcordate, usually more than 15 cm long and about 25 cm wide, bullate, with doubly serrate margin. Mature stems reach more than 2 cm in diameter. The plant is monoecious with long axillary inflorescences reaching up to 10 cm long. The leaves, stems and flowers are covered with long, slender stinging trichomes. It grows only in the East African region including such countries as Ethiopia, Kenya, Tanzania and Burundi [50].

1.5.2 *Girardinia diversifolia* (Link) Friis (Urticaceae)

1.5.2.1 Description and occurrence

Girardinia diversifolia (Link) Friis (Urticaceae) is also referred to as giant nettle. It is an erect, annual herb reaching 75-300 cm in height depending on soil nutrients and rainfall. It has large, up to 8 cm wide, lobed, coarsely dentate, alternate leaves. The stem grows up to 4 cm in diameter at the base. The flowers are small and greenish in colour, occurring as unisexual, axillary inflorescences, while the roots are fibrous [50,51]. As in *G. bullosa*, the leaves, stems and flowers are covered with long, slender stinging trichomes as captured in Figure 1.

Ecologically, it grows naturally in forests, shady moist places, along streams, near villages, and on moist fertile soils rich in humus and organic matter at altitudes ranging between 1200 and 3000 m above sea level. Geographically, it flourishes in sub-tropical and temperate regions including the Himalayas, Nepal, India, Sri Lanka, Thailand, North Korea, Taiwan, Yemen, Ethiopia, Uganda, Tanzania, Kenya, Zimbabwe, Malawi, Madagascar, Zambia and South Africa. In Kenya, it grows in wet montane forest zones within 1200-2100 m above sea level. These wet montane forests are found in Mt. Kenya, Aberdares Ranges, Mau Hills, Chyulu Hills, Mt. Elgon, Suswa Crater, Kakamega Forest and Lake Nakuru [50].

1.5.2.2 Medicinal uses

Various morphological parts of *G. diversifolia* have been used medicinally by different communities worldwide [49,52]. The leaf is used externally as an astringent and in treatment of scrofula, the tuberculosis of bone and lymph gland [53]. A decoction of the roots and basal stems is used orally as a cure for malignant boils, gastric problems and constipation [54]. The root is applied externally for treatment of cuts and wounds, headaches and swollen joints [55]. A decoction of the plant is used to treat fevers while its ashes are applied externally in the treatment of ringworm and eczema.

Its folklore use in the management of malignant boils, scrofula, eczema, cuts and wounds indicates potential antibacterial activity, while its use in the treatment of ringworm suggests potential antifungal activity. Previous studies done on the plant leaf extract showed the presence of alkaloids, sterols, saponins, flavonoids and carotenoids [50]. However, no compounds were isolated.

1.5.2.3 Non-medicinal uses

In the regions neighbouring the Himalayas, especially Nepal, stems of *G. diversifolia* have been used in production of bast fibres. The local people use these fibres for weaving such items as sacks, bags, fishing nets, ropes, and fabrics. The leaves are used as pot vegetables [49].

In a study involving three geographical regions in Kenya, Kariuki investigated and established potential utility of *G. diversifolia* stems as a source of bast fibre for use in weaving and paper-making, and leaves as a foliar fertilizer/pesticide in organic farming [50]. The plant has since been domesticated in some areas surrounding Mt. Kenya Forest and Mau Forest.

Fig. 1: A mature *Girardinia diversifolia* plant. Note the broad dentate leaves and numerous trichomes covering all aerial parts of the plant.

1.6 Principles of fractionation, isolation and identification of compounds

1.6.1 Fractionation and isolation of compounds from plant extracts

Chromatographic fractionation of plant extracts is largely achieved by use of liquid-solid column chromatography (LSC). In this procedure, the principle underlying the separation of compounds is adsorption at the solid-liquid interface. Solid adsorbents such as silica gel and alumina are used as stationary phases while various organic solvents are used as mobile phases. Since adsorption is caused by the attraction of sample components to the polar sites on the stationary phase, increasing the polarity of the mobile phase increases the rate of movement of compounds down the column [56]. There are two methods by which sample components are eluted: isocratic elution and gradient elution.

In isocratic elution, the mobile phase composition remains constant during the entire chromatographic run. Although continuous passage of a single eluting solvent through the column may eventually provide separation, isocratic elution is only preferred for simple mixtures whose components have retention factors within a narrow range. To adequately handle complex mixtures that have both weakly and strongly retained components like crude plant extracts, gradient elution is employed. This technique, also referred to as solvent programming, involves changing the mobile phase composition either stepwise or continuously as elution proceeds [57]. Initially, the mobile phase is composed entirely or mostly of the less polar solvent which facilitates separation of low-polarity components. During development of the chromatogram, the relative amount of the more polar solvent(s) is gradually increased thus increasing the eluent strength of the mobile phase enabling elution of the more strongly adsorbed sample components [57,58].

The eluate is collected as a series of fractions which are then examined appropriately for the presence of compounds. A commonly used method is thin layer chromatography (TLC). Those fractions showing similar profile are pooled and subjected to laboratory manipulation aimed at isolation of pure compounds. Crystalline compounds are usually purified by recrystallization from a suitable solvent or mixture of solvents [59].

1.6.2 Techniques in structure elucidation and identification

Elucidation of the molecular structure of unknown compounds is accomplished by use of a combination of the following spectroscopic techniques: nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and infrared (IR) spectrophotometry [56].

In nuclear magnetic resonance spectroscopy, the characteristic absorption of radiofrequency energy by certain spinning nuclei in a strong magnetic field permits identification of atomic configurations in molecules [58]. The precise frequency from which energy is absorbed gives an indication of how an atom is bound to, or located spatially with respect to, other atoms. Thus, NMR offers an excellent physical means of investigating molecular structure and molecular interactions [56]. Two nuclei are mostly used in structural investigation of organic compounds by NMR: ^{1}H and ^{13}C, hence proton-nmr (PMR) and ^{13}C-nmr (CMR) respectively.

The utility of infrared spectroscopy in structure elucidation is based on the presence of characteristic absorption bands of specific functional groups in a molecule. When a polyatomic molecule interacts with infrared radiation, portions of the incident radiation are absorbed at specific wavelengths due to stretching and bending vibrations [57,58]. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum that is uniquely characteristic of functional groups that make up the molecule and of the overall configuration of the molecule as well [57].

Mass spectrometry is used in structure elucidation in two principal ways. First, it gives molecular weight of the parent ion, enabling determination of the molecular formula of the compound. Secondly, characteristic spectral features in the mass spectrum indicate presence of certain structural units, a reconstruction of which gives probable structure of the compound [60].

Other techniques used as confirmatory tests of identity include ultraviolet-visible spectrophotometry, melting point determination, thin-layer chromatography, optical rotation measurement and characteristic colour reactions [56].

1.7 Principles of antibacterial and antifungal activity testing

1.7.1 Screening for antibacterial and antifungal activity

Progress in fundamental and applied research has made it possible to cultivate bacteria, fungi, viruses and protozoa in the laboratory, thus enabling *in-vitro* screening of antibacterial, antifungal, antiviral and antiprotozoal agents. When a suitable culture of microorganisms is placed in an environment in which it would normally reproduce, it responds to the presence of an antimicrobial agent to which it is sensitive by having reduced rate or complete inhibition of growth [61]. The inhibition of the growth of the cultured microorganism in presence of test plant extracts is indicative of antimicrobial activity [10]. The observed activity is then compared to that of a reference antimicrobial agent whose activity is known. Two major practical techniques are widely used in the screening of antibacterial and antifungal activities: agar diffusion assay and tube assay.

In the agar diffusion assay, a nutrient agar containing nutrients to support the growth and multiplication of microorganisms is inoculated uniformly with a sensitive micro-organism. An aqueous solution of the test sample is placed in a suitable reservoir on the surface of the solidified agar from which it diffuses radially. Upon incubation, multiplication of the microorganism commences and continues until it is inhibited by contact with the test solution, creating circular areas in which microorganisms do not grow, referred to as zones of inhibition [61,62]. The size of the zones of inhibition is dependent on the activity of the test sample. It provides the quantitative basis of comparison of the antimicrobial activity of the test sample to that of the reference standard whose potency is known.

In the tube assay, chilled nutrient broth, inoculated uniformly with a suitable sensitive microorganism, is added into a tube containing test sample solution and an equal volume added to another tube containing reference antibiotic solution. On incubation, microbial growth produces turbidity which is determined visually or spectrophotometrically at 530 nm. Since the growth of the microorganism is reduced to an extent dependent on the antimicrobial activity of the test solution, comparison of the degree of turbidity in the sample tube to that in the standard tube provides a

quantitative estimate of the antimicrobial activity of the sample extract against a reference antibiotic of known potency [61,62].

1.7.2 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of plant extracts, chromatographic fractions and isolates exhibiting antimicrobial activity in the primary screening is studied on observed sensitive microorganisms. The MIC indicates the effectiveness of a sample as an antimicrobial agent [62]. Two methods are commonly used: broth and agar dilution. In the broth dilution method, a convenient stock solution of the sample is prepared in a suitable liquid nutrient medium and two-fold dilutions in broth prepared serially in tubes. Equal volumes of a standardized inoculum of the test microorganism are added into each tube and then incubated. Microbial growth is indicated by turbidity. The lowest concentration of the sample inhibiting growth is taken as the MIC of the test microorganism.

In the agar dilution method, serial dilution of the sample is prepared in molten nutrient agar capable of supporting growth of different microorganisms. The agar is then poured into plates and left to solidify. Equal volumes of standardized inocula of the test microorganisms are streaked or poured into wells cut on the surface of the solidified agar. After incubation at 37 °C for 18 h, the lowest concentration with no growth or with only a few discrete colonies is recorded as the MIC of the particular strain under test. Hence, agar dilution method allows several microorganisms to be tested simultaneously on the same agar plate.

1.7.3 Bioautography

For the isolation of active compounds by activity-guided fractionation, bioautography is the method of choice. This autobioassay method combines thin layer chromatography with a bioassay *in situ* and allows localization of bioactive constituents in plant extracts [8]. The technique relies on the transfer of active compounds from the chromatographic stationary phase into the agar layer inoculated with the test microorganism by a diffusion process. A suspension of the test microorganism in a suitable broth is applied to a developed TLC plate. Incubation at

optimum conditions permits growth of the microorganism. The plate is then sprayed with a dehydrogenase-activity detecting reagent such as a tetrazolium salt. Dehydrogenases in metabolically active microorganisms convert the tetrazolium salt into the corresponding intensely colored formazan. Bioactive components inhibit growth of the microorganisms forming zones of inhibition which appear as clear haloes against a purple background [63]. The diameter of the halo increases in proportion to the growth inhibition and acts as an indicator of the degree of antimicrobial activity [64].

1.8 Rationale of the research project

Universal accessibility to affordable health care is one of the Millennium Development Goals set by the United Nations at the turn of this century to be achieved by the year 2015. In Kenya, efforts towards provision of affordable health care have been hindered by lack of drugs in the public health care facilities which cater for the health needs of the majority of the citizenry. There is therefore a need to devise cost-effective solutions to improve delivery of health care services. One such solution is the integration of affordable, safe and efficacious traditional medicine with the conventional health care system. Whereas many of the herbs used medicinally have a traditional reputation for their uses, there is little scientific documentation of their pharmacological actions and active constituents [65]. Yet, for successful integration of phytomedicines in the conventional health care system, scientific evidence of their pharmacological activities will be required.

The medicinal properties of plants are normally dependent upon the presence of certain active principles. Isolation of these compounds may provide potential therapeutic agents or provide lead compounds for chemical derivatization of better analogs. *Girardinia diversifolia* has been used traditionally for numerous ailments, including bacterial and fungal infections [52-55,66]. However, literature search shows neither biological activity studies nor isolated compounds have been reported from this plant. This justifies research in this plant.

1.8.1 Hypotheses

The objectives of this study were based on the following hypotheses:

1. One or more extract(s) of the stems and roots of *Girardinia diversifolia* possess antibacterial and/or antifungal activity responsible for the reported ethnomedicinal uses.

2. The observed antibacterial and/or antifungal activity is attributable to one or more chemical principle(s).

1.8.2 Objectives

1.8.2.1 General objective

The major objective of this study was to investigate phytochemical composition and antimicrobial activity of *Girardinia diversifolia.*

1.8.2.2 Specific objectives

1. To investigate the antibacterial and antifungal activities of the petroleum ether (60-80 °C), chloroform, methanol and ethyl acetate root and stem extracts of *Girardinia diversifolia.*

2. To isolate phytochemical constituents of *Girardinia diversifolia* by chromatographic fractionation and characterize them by spectroscopic techniques.

3. To investigate the antibacterial and antifungal activities of the isolated compounds.

CHAPTER 2: EXPERIMENTAL

2.1 Solvents, materials and reagents

2.1.1 Solvents

Extracts of the root and stem powders of *Girardinia diversifolia* were prepared by sequential Soxhlet extraction using general purpose reagent grade solvents, namely, petroleum ether (60-80 °C), chloroform, methanol and ethyl acetate (Sigma-Aldrich GmbH, Seelze, Germany). The solvents were distilled using a laboratory distiller before use. Analytical grade acetone and benzene (Sigma-Aldrich GmbH, Seelze, Germany) were used for recrystallization and fractionation respectively. Analytical grade methanol and hexane (Sigma-Aldrich GmbH, Seelze, Germany) were used to prepare solutions for ultraviolet spectrophotometry. Dimethyl sulfoxide (Fisher Scientific, Loughborough, United Kingdom) was used to prepare aqueous suspensions of extracts for antimicrobial activity screening.

2.1.2 Materials

All filtrations to remove particulate matter from crude solvent extracts, solutions of pooled fractions and solutions of isolated compounds were done using Whatman filter paper No. 1 (Whatman International Ltd, Maidstone, England). Crystallized isolates were recovered from their mother liquors by filtration under vacuum using sintered glass filter funnel No. 4 (Schott Duran GmbH & Co., Wertheim/Main, Germany).

Open column chromatography on normal phase silica gel powder porosity 32-63 μm (Sigma-Aldrich GmbH & Co., Seelze, Germany) was used for fractionation of the crude solvent extracts. Thin layer chromatography on aluminium plates precoated with 0.2 mm thick layer of normal phase silica gel 60 GF_{254} (Sigma-Aldrich GmbH & Co., Seelze, Germany) was used for qualitative screening of the extracts, and monitoring of the chromatographic fractionation of the extracts and purification of the isolates.

Master cultures of the microorganisms used in the antibacterial and antifungal activities studies were maintained in the Microbiology Laboratory of the Drug Analysis and Research Unit (DARU) of the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Nairobi. The microorganisms used for antibacterial activity studies included *Escherichia coli* (ATCC 10536), *Staphylococcus aureus* (ATCC 29737) and *Bacillus pumilus* (ATCC 11204) while *Aspergillus niger* (environmental isolate), *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763) were used for antifungal activity studies. Tryptone soy agar and Sabouraud's dextrose agar (Oxoid, Hampshire, England) were used as nutrient media for the growth of bacteria and fungi respectively. The following antibiotics were used as reference standards: gentamicin and benzylpenicillin (Troje Medical GmbH, Hamburg, Germany) for antibacterial activity, and nystatin (Laboratory and Allied Pharmaceuticals, Nairobi, Kenya) for antifungal activity.

Bioautography was performed on 20 × 20 cm glass-backed TLC plates precoated with a 0.2 mm thick layer of normal-phase silica gel 60 GF_{254} (Merck GmbH, Darmstadt, Germany). The solutions of extracts and reference antibiotics were applied on the plates using a calibrated syringe.

2.1.3 Reagents

A 1 % w/v vanillin in concentrated sulphuric acid was prepared using vanillin (BDH Chemicals Ltd., Poole, England) while iodine vapour was prepared using iodine resublimed general reagent (Merck, Damstadt, Germany).

A 2.5 mg/ml aqueous solution of methylthiazolyl tetrazolium bromide (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a dehydrogenase-activity indicator to visualize bioactive components on the bioautograms.

2.2 Equipment

The root and stem powders of *G. diversifolia* were extracted using a Soxhlet apparatus (Quickfit, Birmingham, United Kingdom) mounted on an electrically-heated

electrothermal isomantle. Methanol extracts were reduced to dryness using a Heidolph VV2000® rotary vacuum evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a Julabo EM-F18® refrigerated bath circulator (Julabo Labortechnik GmbH, Seelbach, Germany), a WB2000® water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) and an RV® rotary vane pump (Edwards High Vacuum International, West Sussex, United Kingdom) while all the other solvent extracts were reduced to dryness using a Heidolph VV2000® rotary vacuum evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a Polyscience cooler (Polyscience, Niles, USA), a WB2000® water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) and a N820.3FT Laboport® diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany). Extracts requiring storage at low temperature and solutions of isolated compounds for recrystallization were kept in a Samsung refrigerator (Samsung Electronics, Seoul, South Korea) maintained at 2-8 °C.

The extracts were fractionated in glass columns whose dimensions were: 2 cm internal diameter (i.d.) by 50 cm length, 2 cm i.d. by 100 cm length, and 1 cm i.d. by 100 cm length. The chromatographic fractions were collected using a SuperFrac™ automatic fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). Ultraviolet light quenching and fluorescing spots on thin layer chromatograms were visualized using Min UVIS® ultraviolet light lamp (Desaga GmbH, Heidelberg, Germany).

All the glassware used in the antimicrobial activity studies were sterilized in a Memmert® universal oven (Memmert GmbH & Co. KG, Schwabach, Germany) using dry heat at 150 °C for 1 h, while the nutrient media and distilled water were sterilized using a portable autoclave (Dixon's Surgical Instruments Ltd., Essex, United Kingdom) at 121 °C for 15 min before use. The bacteriological wire loop and the cork borer were sterilized by flaming using a Bunsen burner flame. The diameters of the zones of inhibition were measured using a hand-held electronic digital vernier caliper with a precision of 0.1 mm. All bench work involving use of microorganisms was carried out in a Bioflow® laminar flow cabinet (Vermeulen L. J. BVBA, Westmalle, Belgium), while Freez I® incubator (Analis, Suarlee, Belgium) was used for incubation of the microorganisms.

The melting points of the isolates were determined using Gallenkamp melting point apparatus (A. Gallenkamp & Co. Ltd, London, England) using capillary tubes as the sample holders. The nuclear magnetic resonance spectroscopic data was obtained using a Varian-Mercury 200 MHz spectrometer (Varian Inc, Palo Alto, USA). The data was acquired using an on-line computer (Sun Micro-Systems, California, USA) and analyzed using Varian software. Mass spectrometric data was obtained by Electron Impact Mass Spectrometry (EIMS) using 70 and 20 or 30 eV ionizing energies on a VG 12-250™ quadruple mass spectrometer (VG Analytical, Manchester, United Kingdom) operated at 8×10^{-6} Pa vacuum pressure, 180 °C ion source temperature and 400 °C heated solid probe temperature. The mass spectra were acquired by an on-line computer and processed using MassLynx® software (Microsoft Corporation, Redmond, USA). Infrared spectra were acquired using IRPrestige-21® fourier transform infrared spectrophotometer (Shimadzu corporation, Kyoto, Japan) while the ultraviolet-visible spectrophotometric absorption spectra were obtained using a PU8750™ UV/Visible scanning spectrophotometer (Philips Scientific, Cambridge, Great Britain). Samples were weighed using AUW220D® analytical balance (Shimadzu corporation, Kyoto, Japan) with a 0.01 mg precision for samples up to 500 mg, and a PB3002 DeltaRange® top loading balance (Mettler Toledo AG, Greifensee, Switzerland) with a 100 mg precision for samples weighing more than 500 mg.

2.3 Procedures

2.3.1 Plant collection, identification and preservation

The roots and stems of *Girardinia diversifolia* were collected from Kamweti Location neighbouring Mt. Kenya Forest, Kirinyaga District, in September 2006. The plant was identified and authenticated at site by a taxonomist. A voucher specimen of the plant is deposited in the herbarium of the School of Pharmacy, University of Nairobi.

The collected roots and stems of *G. diversifolia* were cut into small pieces and dried at room temperature. The dried roots and stems were then ground into fine powder and kept in labelled plastic containers until used.

2.3.2 Preparation of extracts

Six hundred grams of the root powder of *G. diversifolia* were Soxhlet extracted using 5 L of petroleum ether (60-80 °C) for 48 h. The solution was passed through a plug of cotton wool, filtered, and reduced to dryness to give a dark yellow solid material. The percentage yield was determined and the extract labelled GdRP (*Girardinia diversifolia* root petroleum ether extract). The procedure was repeated using stem powder and the obtained dark green solid extract labelled GdSP (*Girardinia diversifolia* stem petroleum ether extract). The root and stem powders previously extracted with petroleum ether were further sequentially Soxhlet extracted with chloroform and methanol, and the respective percentage yields determined.

A 35 g portion of root methanol extract was dissolved in 50 ml of methanol with gentle heating and approximately 300 ml of distilled water added. The mixture was vigorously shaken to disperse and successively partitioned with three 300 ml portions of ethyl acetate. The three ethyl acetate partitions were combined, reduced to dryness and the yield determined. Stem ethyl acetate extract was prepared similarly using 27 g of stem methanol extract.

All extracts were kept in aluminium foil-sealed containers at room temperature except the methanol extracts which were kept refrigerated at 2-8 °C until used. Table 1 gives the percentage yield of each extract.

Table 1: Yields of *Girardinia diversifolia* root and stem solvent extracts

Extract	Colour	Yield (%)
Girardinia diversifolia root petroleum ether (GdRP)	Dark yellow	0.61
Girardinia diversifolia stem petroleum ether (GdSP)	Dark green	0.58
Girardinia diversifolia root chloroform (GdRC)	Green	0.80
Girardinia diversifolia stem chloroform (GdSC)	Black	0.82
Girardinia diversifolia root methanol (GdRM)	Brown	8.26
Girardinia diversifolia stem methanol (GdSM)	Dark brown	6.22
Ethyl acetate partition of root methanol (EAPRM)	Brown	0.82
Ethyl acetate partition of stem methanol (EAPSM)	Dark brown	0.67

2.3.3 Preparation of reagents

2.3.3.1 Chromatographic visualizing reagents

Iodine vapour was prepared by placing 10 g of iodine resublimed general reagent at the bottom of a chromatographic tank. Sublimation of the solid iodine saturated the chamber with iodine vapour. Developed TLC plates were placed inside the closed tank for approximately 5 min. Yellow-brown colouration indicates compounds containing conjugated double bonds [67].

A solution of 1 % w/v vanillin in concentrated sulphuric acid was prepared by dissolving 0.5 g of vanillin in 50 ml concentrated sulphuric acid. The solution was used as a spray on chromatograms which were then heated at 100 °C for 10 min. Terpenoids, lignans and cucurbitacins develop varied colours with this reagent [67].

2.3.3.2 Reagents for phytochemical tests

Mayer's reagent was prepared using mercuric chloride and potassium iodide. Separate solutions of 0.27 g of mercuric chloride and 1 g of potassium iodide were prepared in 12 ml and 2 ml of water respectively. The two solutions were then mixed and made to 20 ml.

Dragendorff's reagent was prepared thus: approximately 0.21 g of basic bismuth nitrate was dissolved in a mixture of 2.5 ml acetic acid and 10 ml water to produce solution A. Approximately 2 g of potassium iodide were dissolved in 5 ml water to produce solution B. To 4 ml acetic acid was added 1 ml of each of the two solutions and the mixture made to 20 ml with water.

Kedde reagent was prepared by mixing 5 ml of 3 % ethanolic 3,5-dinitrobenzoic acid with 5 ml of 2 M NaOH.

2.3.3.3 Reagent for visualizing bioactive components

A 2.5 mg/ml solution of methylthiazolyl tetrazolium bromide was prepared by dissolving 25 mg of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide in

10 ml of water. The solution was used as a spray on bioautograms. Bioactive components appeared as clear haloes against a purple background.

2.3.4 Phytochemical tests

2.3.4.1 Tests for alkaloids

Approximately 1 g of *G. diversifolia* root powder was mixed with 5 ml of 10 % H_2SO_4, warmed in a water bath for 2 min and filtered. Two drops of Mayer's reagent were added to 1 ml of the filtrate and observations made. The rest of the filtrate was alkalinized using dilute ammonia and extracted with 2 ml of chloroform. The chloroform was evaporated off to leave a solid residue which was dissolved in 0.2 ml of 10 % H_2SO_4 and divided into two portions. To one portion was added a drop of Mayer's reagent while to the other a drop of Dragendorff's reagent. A white to buff precipitate with Mayer's reagent and an orange-red precipitate with Dragendorff's reagent suggests presence of alkaloids or nitrogen-containing compounds [6,67].

2.3.4.2 Tests for cardiac glycosides

Approximately 1 g of *G. diversifolia* root powder was mixed with 10 ml of 70 % alcohol and heated in a water bath at 70 °C for 2 min to extract, and then filtered. To the filtrate, 10 ml of water and 5 drops of a strong solution of lead subacetate were added and the solution filtered. To the filtrate, 10 % H_2SO_4 was added dropwise until no further precipitation occurred. The resulting solution was filtered and extracted with two successive 5 ml portions of chloroform. The two chloroform extracts were combined, washed with 1 ml of water, filtered, divided into two equal portions in petridishes and evaporated to dryness. The dry extracts were subjected to tests I and II.

Test I: Kedde test

Two drops of Kedde reagent were added to one of the dry extracts. Purple colour indicates presence of cardiac glycosides whose aglycone moiety has unsaturated lactone ring [67].

Test II: Keller-Kiliani test

To the second extract, 0.4 ml of glacial acetic acid containing trace ferric chloride was added, shaken gently to dissolve and 0.5 ml of concentrated H_2SO_4 carefully added. A reddish-brown colour at the interphase, which gradually turns blue-green, indicates presence of deoxysugars [6].

2.3.4.3 Tests for saponins

Test I

A little amount of *G. diversifolia* root powder was placed in a test tube and water added. The mixture was shaken and left to stand. Persistent frothing suggests presence of saponins [6].

Test II

Approximately 0.5 g of *G. diversifolia* root powder was extracted with 20 ml of water heated in a water-bath at 70 °C for 5 min and filtered. Two ml of 1.8 % w/v NaCl solution were placed in each of two test tubes. To one test tube was added 2 ml of water while to the other was added 2 ml of the extract. To each tube was added a drop of blood and the tubes inverted gently to mix. Haemolysis in the tube containing the extract and not in the tube containing water indicates presence of saponins [6].

2.3.4.4 Tests for anthracene glycosides

Test I: Borntrager test

Approximately 0.5 g of the powdered *G. diversifolia* root was boiled with 5 ml of dilute H_2SO_4 for 5 min. The extract was filtered whilst hot, cooled and the filtrate shaken with an equal volume of carbon tetrachloride. The organic layer was separated and shaken with a few drops of dilute ammonia. A rose pink to red colour in the ammoniacal layer indicates presence of anthracene aglycones in the oxidized state [6].

Test II: Modified Borntrager test

Approximately 0.5 g of the powdered *G. diversifolia* root was treated in a similar manner as in Test I except that a few drops of 5 % $FeCl_3$ were added during extraction

with dilute ammonia. A rose pink to red colour in the ammoniacal layer indicates presence of anthracene aglycones in the reduced state [6].

2.3.4.5 Tests for tannins

Approximately 1 g of *G. diversifolia* root powder was mixed with 10 ml of water and the mixture boiled in a water-bath to extract, and filtered. To a 2 ml portion of the filtrate was added three drops of ferric chloride solution while to another 2 ml portion was added 1 ml of lead subacetate. Development of a brown-green precipitate with ferric chloride and a creamy-brown precipitate with lead subacetate suggests presence of tannins [6].

All the above tests were repeated using *G. diversifolia* stem powder.

2.3.5 Isolation of compounds

2.3.5.1 Fractionation of petroleum ether root extract

Two chromatographic columns were run. In the first chromatographic procedure, 6 g of *Girardinia diversifolia* root petroleum ether extract (GdRP) were fractionated using 65 g of silica gel by isocratic elution using 1 L chloroform. In the second procedure, 10.3 g of the GdRP extract were fractionated using 110 g of silica gel by gradient elution using 400 ml of 100 % benzene, 500 ml of 50 % benzene: 50 % chloroform mixture, and 1.4 L of 100 % chloroform. In both chromatographic procedures, the eluate was collected in 4 ml fractions. Appendix 1 shows GdRP extract undergoing chromatographic fractionation.

Every other third tube of the fractions was profiled by thin-layer chromatography using 98:2 % v/v chloroform:methanol. The GdRP extract was included as a reference. The spots in the developed chromatograms were located by visualizing in ordinary light, observing under both short and long ultraviolet light (short UV and long UV at 254 nm and 366 nm respectively), exposure to iodine and spraying with 1 % w/v vanillin, in that order.

The chromatograms were analyzed and those fractions showing similar profile pooled. From the first column, ten pooled fractions labelled F1–F10 were obtained. The second column gave eleven pooled fractions labelled ^{2}F1–^{2}F11. The TLC profile of the pooled fractions was then obtained. The TLC profiles were used to indicate major isolable components in each fraction. These were targeted for isolation. The pooled fractions were reduced to dryness and their yields determined. Each pooled fraction was then dissolved in minimum amount of an appropriate crystallizing solvent and kept in the refrigerator.

2.3.5.2 Isolation of compounds from the fractions of petroleum ether root extract

2.3.5.2.1 Isolation of PMN-01

Compound PMN-01 was isolated from pooled fractions F4 of the first column and ^{2}F8 of the second column. The dry pooled fraction F4 was dissolved in acetone with warming in a water bath at 50 °C, the resultant solution filtered, reduced to a minimum in a water bath at 50 °C and kept in the refrigerator. Colourless platy crystals formed after 24 h. The purity of the crystals was improved by successive recrystallisation from acetone, using TLC to monitor, until a pure compound was obtained. Pooled fraction ^{2}F8 of the second column was similarly treated to yield colourless platy crystals. A thin layer chromatogram of F4 and ^{2}F8 crystals using a 98:2 % v/v chloroform:methanol mixture gave a single spot with R_f value of 0.5. It stained faint-yellow on exposure to iodine vapour, and purple when sprayed with 1 % w/v vanillin. The purple colouration turned green after some time. The two crystalline powders were kept in capped sample vials and labelled PMN-01.

2.3.5.2.2 Isolation of PMN-02

Compound PMN-02 was isolated from pooled fraction ^{2}F9 of the second column. Thin layer chromatogram of ^{2}F9 developed using a 98:2 % v/v chloroform:methanol mixture revealed three major spots. The first one had R_f value of 0.5, stained faint-yellow on exposure to iodine vapour, and developed purple colouration, which later turned green, when sprayed with 1 % w/v vanillin. This indicated that it was identical to the earlier isolated PMN-01 crystals. The second spot had R_f value of 0.3, stained faint-yellow on exposure to iodine vapour and violet when sprayed with 1 % w/v

vanillin. The third spot had R_f value of 0.2, appeared as a black spot against a green background when viewed under short ultraviolet light, and stained yellow-brown on exposure to iodine. It was the most prominent and the responsible component targeted for isolation by further column chromatographic fractionation of the pooled fraction ^{2}F9.

Approximately 1.2 g of the pooled fraction ^{2}F9 was fractionated using 20 g of silica gel by isocratic elution using 500 ml chloroform. The eluate was collected in 2 ml fractions and every other third fraction profiled by TLC using 98:2 % v/v chloroform:methanol mixture. Six pooled subfractions labelled ^{2}F9a–^{2}F9f were obtained. The spot with R_f value of 0.5 was largely in ^{2}F9a, that with R_f value of 0.3 was largely in ^{2}F9b, ^{2}F9c, ^{2}F9d, while the component with R_f value of 0.2 was prominent in ^{2}F9e and in minute quantities in ^{2}F9f.

Pooled subfractions ^{2}F9e and ^{2}F9f were combined, reduced to dryness, dissolved in minimum acetone with gentle warming, filtered whilst hot and kept in the refrigerator. Colourless needle-like crystals formed after approximately five days. The crystals were purified by successive recrystallisation from acetone using TLC to monitor. The recrystallized pure compound was kept in a capped sample vial and labelled PMN-02.

2.3.5.2.3 Isolation of PMN-03

Compound PMN-03 was isolated from pooled fraction ^{2}F10 of the second column. The dry pooled fraction ^{2}F10 was dissolved in minimum acetone with gentle warming in a water bath at 50 °C, filtered whilst hot and kept in the refrigerator. Small colourless platy crystals formed after approximately seven days. Purification of the crystals was achieved by successive recrystallisation from acetone until a pure compound was obtained as indicated by the TLC. A sample solution of the obtained crystals gave a single spot at R_f value of 0.1 when chromatographed using 98:2 % v/v chloroform:methanol mixture. The spot stained faint yellow on exposure to iodine vapour and blue when sprayed with 1 % w/v vanillin. The crystals were put in a capped sample vial and labelled PMN-03.

2.3.5.2.4 Isolation of PMN-04

Compound PMN-04 was isolated from the pooled subfractions ^{2}F9b, ^{2}F9c and ^{2}F9d of the refractionated pooled fraction ^{2}F9 of the second column. These subfractions contained a major component with R_f value of 0.3 in a freshly prepared 98:2 % v/v chloroform:methanol mobile phase system, stained faint-yellow on exposure to iodine vapour and violet when sprayed with 1 % w/v vanillin. The three subfractions were combined, reduced to dryness, dissolved in minimum amount of methanol and kept in the refrigerator. Small colourless and feathery crystals formed after two weeks. The crystals were purified by recrystallization from methanol until a single spot was obtained, and labelled PMN-04.

Table 2 gives weight by weight yields of the isolated compounds per kilogram of root powder.

Table 2: Yields of the isolated compounds from the petroleum ether root extract

Compound	Yield per kg of root powder (mg)	Percentage yield (% w/w)
PMN-01	100	0.01
PMN-02	5	0.0005
PMN-03	4	0.0004
PMN-04	8	0.0008

2.3.6 Investigation of antimicrobial activity of *Girardinia diversifolia*

2.3.6.1 Screening for antibacterial and antifungal activities

The agar diffusion assay method was used. The test microorganisms were subcultured overnight for 18 h in their prescribed nutrient media to obtain working cultures. A 50 mg/ml solution of each extract was prepared in dimethyl sulfoxide. Test solutions of standard antibiotics were also prepared thus: gentamicin 10 µg/ml, benzylpenicillin 1 IU/ml and nystatin 150 µg/ml according to Hewitt and Vincent protocol [62].

Nutrient media for growth of the test microorganisms were prepared as per the manufacturer's instructions, sterilized and left to cool to around 50 °C. Each of the cultured microorganisms was suspended in 5 ml of sterilized distilled water and approximately 5 ml of suspension inoculated into respective growth medium so as to produce inoculated agar with approximately 1×10^6 colony forming units per millilitre. The inoculated nutrient media were then rapidly but carefully poured into petridishes using a 100 ml measuring cylinder in such a manner as to deliver 20 ml of agar with a uniform thickness of 3 mm in each petridish. The layered agar was allowed to cool so as to set into a firm gel suitable for plating-out operation.

Using a symmetric paper template with six circles drawn in a hexagonal array, six cylindrical wells were punched into the layered media using a cork borer. The sample extracts were applied into the wells using a fixed-volume micropipette set to deliver 50 µl per well. Each extract was applied in duplicate. Dimethyl sulfoxide and standard antibiotics as appropriate were included in each petridish as negative and positive controls respectively.

A prediffusion period of 1 h was allowed to facilitate diffusion of the applied solutions into the inoculated media before the petridishes were incubated for 18 h at 37 °C. The diameters of the zones of inhibition were then measured and preserved by photography.

2.3.6.2 Bioautography

The test microorganisms, *Bacillus pumilus* and *Saccharomyces cerevisiae,* were incubated for 18 h at 37 °C. Tryptone soy agar and Sabouraud's dextrose agar nutrient media were prepared as per the manufacturer's instructions. A 20 mg/ml solution of each extract was prepared by dissolving 100 mg of petroleum ether, chloroform and ethyl acetate extracts in 5 ml of chloroform, and methanol extracts in 5 ml of methanol. A 100 µl aliquot of each solution was then spotted on each of the two glass-backed TLC plates, and the plates developed using a $CHCl_3$:MeOH, 90:10 % v/v mobile phase. A 100 µl aliquot of 10 µg/ml gentamicin solution was applied on one plate, while to the other was applied 100 µl of 150 µg/ml nystatin solution. The two standard antibiotics acted as positive controls.

A standardized inoculum of *B. pumilus* was inoculated uniformly into the prepared tryptone soy agar at 50 °C, and the inoculated agar rapidly but carefully layered over the developed TLC plate so as to produce a uniform 1 mm thick agar layer. This procedure was repeated using nystatin-containing TLC plate and Sabouraud's dextrose agar inoculated with *S. cerevisiae.* A prediffusion period was allowed by leaving the plates standing for 1 h at room temperature before incubation at 37 °C for 18 h.

After the incubation period, the bioautograms were sprayed with a solution of methylthiazolyl tetrazolium bromide, followed by a further incubation at 37 °C for 4 h and thereafter sprayed with absolute ethanol to kill the microorganisms. The bioautograms were then observed for bioactive constituents that produced clear zones of inhibition against a purple background. The bioactive constituents were identified by their R_f values as determined using normal-phase silica gel 60 GF_{254} precoated aluminium chromatoplates developed alongside the bioautographic plates. The bioautograms were preserved by photography.

The isolated compounds, PMN-01, PMN-02, PMN-03 and PMN-04, were screened for *in vitro* antibacterial and antifungal activities by bioautography using a similar procedure.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Phytochemical composition of *Girardinia diversifolia*

The results of the phytochemical tests, as depicted in Table 3, show that the stems and roots of *G. diversifolia* contain cardiac glycosides, saponins and tannins but lack alkaloids and anthracene glycosides. These results indicate that the roots and stems may contain similar components and are in agreement with an earlier phytochemical analysis which indicated presence of saponins and sterols in the methanol leaf extract of *G. diversifolia* [50].

Table 3: Phytochemical constituents of *Girardinia diversifolia* root and stem

Phytochemical	Root	Stem
Alkaloids	Absent	Absent
Cardiac glycosides	Present	Present
Saponins	Present	Present
Anthracene glycosides	Absent	Absent
Tannins	Present	Present

Although the plant had previously been shown to contain alkaloids, this study indicated their absence. This variability could be due to existence of chemical varieties of *G. diversifolia* hence variation in the phytochemistry of members of this species in different ecological habitats. Alkaloids and anthracene glycosides are associated with numerous adverse effects of plants. Their absence is therefore significant since it indicates that the plant may be reasonably safe for use in humans, and hence its ethnomedicinal use.

3.2 Physical characteristics of the isolated compounds

Table 4 presents a summary of a few physical characteristics of the isolated compounds. Appendix 2 figuratively captures their retention behavior on normal-phase silica gel-coated TLC plates. Their retention characteristics indicate that they are closely related and may differ only by the attached functional groups. The staining characteristics of PMN-01, PMN-03 and PMN-04 with 1 % w/v vanillin support this observation.

Table 4: Physical characteristics of the isolated compounds

Characteristic	PMN-01	PMN-02	PMN-03	PMN-04
Under short UV	-	+	-	-
Iodine vapour	Faint yellow	Yellow-brown	Faint yellow	Faint yellow
1 % w/v vanillin	Purple	-	Blue	Violet
Crystallizing solvent	*Acetone*	*Acetone*	*Acetone*	*Methanol*
Crystal appearance	*Colourless plates*	*Colourless needles*	*Colourless plates*	*Colourless feathery*
$^{1}R_f$*, $^{2}R_f$*	0.48, 0.65	0.23, 0.59	0.13, 0.50	0.31, 0.62
Melting point (°C)	134-136	140-142	128-131	152-153

+ = Visualized/stained

- = Not visualized/not stained

$^{1}R_f$* was determined by TLC in a 98:2 % v/v $CHCl_3$:MeOH mobile phase system.

$^{2}R_f$* was determined by TLC in a 90:10 % v/v $CHCl_3$:MeOH mobile phase system.

3.3 Structural elucidation of the isolated compounds

3.3.1 PMN-01

Compound PMN-01 showed the following spectral data:

UV λ_{max} (MeOH) nm: 208.5.

IR ν_{max} (KBr) cm^{-1}: 3426 (O-H str), 2948 (CH_3, C-H str), 2876 (CH_2, C-H str), 1454 (CH_3, C-H bend), 1369 (=C-H bend).

MS m/z (rel. int.): 415 ($(M+1)^+$, 100), 400 (31), 397 (47), 386 (3), 382 (29), 368 (3), *330 (51), 302 (3), 274 (24), 256 (26), 214 (36), 160 (26), 145 (32), 107 (38), 95 (32),* 81 (35), 69 (28), 57 (31), 43 (82).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 5.35 (1H, d, H-6), 3.51 (1H, m, H-3), 2.27 (1H, d, H-4), 1.96 (1H, q, H-8), 1.92 (2H, t, H-1), 1.80 (2H, t, H-12), 1.74 (1H, br, s, H-25), 1.60 (2H, m, H-2), 1.57 (2H, t, H-7), 1.52 (2H, m, H-22), 1.28 (2H, q, H-16), 1.24 (2H, m, H-23), 1.19 (2H, m, H-15), 0.99 (3H, s, H-19), 0.89 (2H, m, H-11), 0.83 (6H, m, H-26, H-27), 0.81 (3H, d, H-21), 0.78 (3H, m, H-29), 0.66 (3H, s, H-18).

^{13}C-NMR ($CDCl_3$, 50 MHz): δ 37.46 (C-1), 31.85 (C-2), 72.02 (C-3), 42.49 (C-4), 140.95 (C-5), 121.95 (C-6), 32.11 (C-7), 32.11 (C-8), 50.32 (C-9), 36.71 (C-10), 21.29 (C-11), 39.98 (C-12), 42.49 (C-13), 56.97 (C-14), 24.51 (C-15), 28.47 (C-16), 56.25 (C-17), 12.19 (C-18), 19.24 (C-19), 36.36 (C-20), 18.99 (C-21), 34.14 (C-22), 26.24 (C-23), 46.02 (C-24), 29.33 (C-25), 20.05 (C-26), 19.61 (C-27), 23.26 (C-28), 12.07 (C-29).

Based on the obtained spectral data, PMN-01 was identified as β-sitosterol (Figure 2). The systematic name is stigmast-5-en-3-ol (3β,24R) while the International Union of Pure and Applied Chemistry (IUPAC) name is (3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthrene-3-ol.

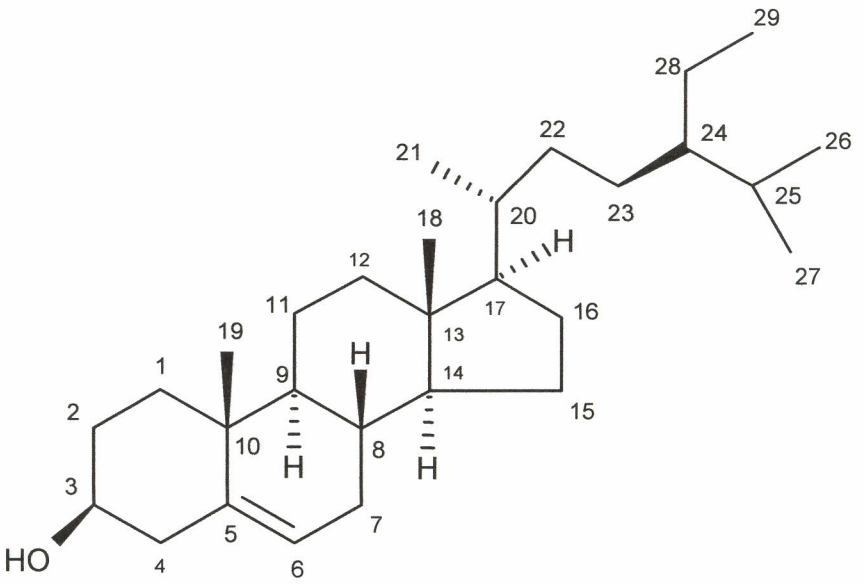


Fig. 2: Structure of β-sitosterol

Ultraviolet light spectrophotometric absorption spectrum (Appendix 3) indicated absence of conjugation with UV λ_{max} of 208.5 nm.

Infrared spectrophotometric absorption spectrum (Appendix 4) indicated presence of an aliphatic system, a hydroxyl group and a C=C double bond.

The electron impact mass spectrometry (EIMS) gave a molecular ion $(M+1)^+$ with a mass-to-charge ratio (m/z) of 415. This is consistent with the molecular formula $C_{29}H_{50}O$ (MW = 414) of β-sitosterol. The mass spectrum (Appendix 5) gave a profile consistent with the proposed fragmentation pattern for steroidal systems [68]. The fragment ion with a m/z 400 corresponds to loss of C-18 angular methyl group from the molecular ion. Fragment ion with m/z 397 corresponds to loss of a water molecule while that at m/z 386 corresponds to loss of the ethyl group at C-24 from the molecular ion. Fragmentation of the fragment ion 397 between C-17 and C-20 gave fragment ion with m/z 256 whose ring D fragmented further to give fragment ion 214. Figure 3 illustrates probable fragmentation pathway for PMN-01.

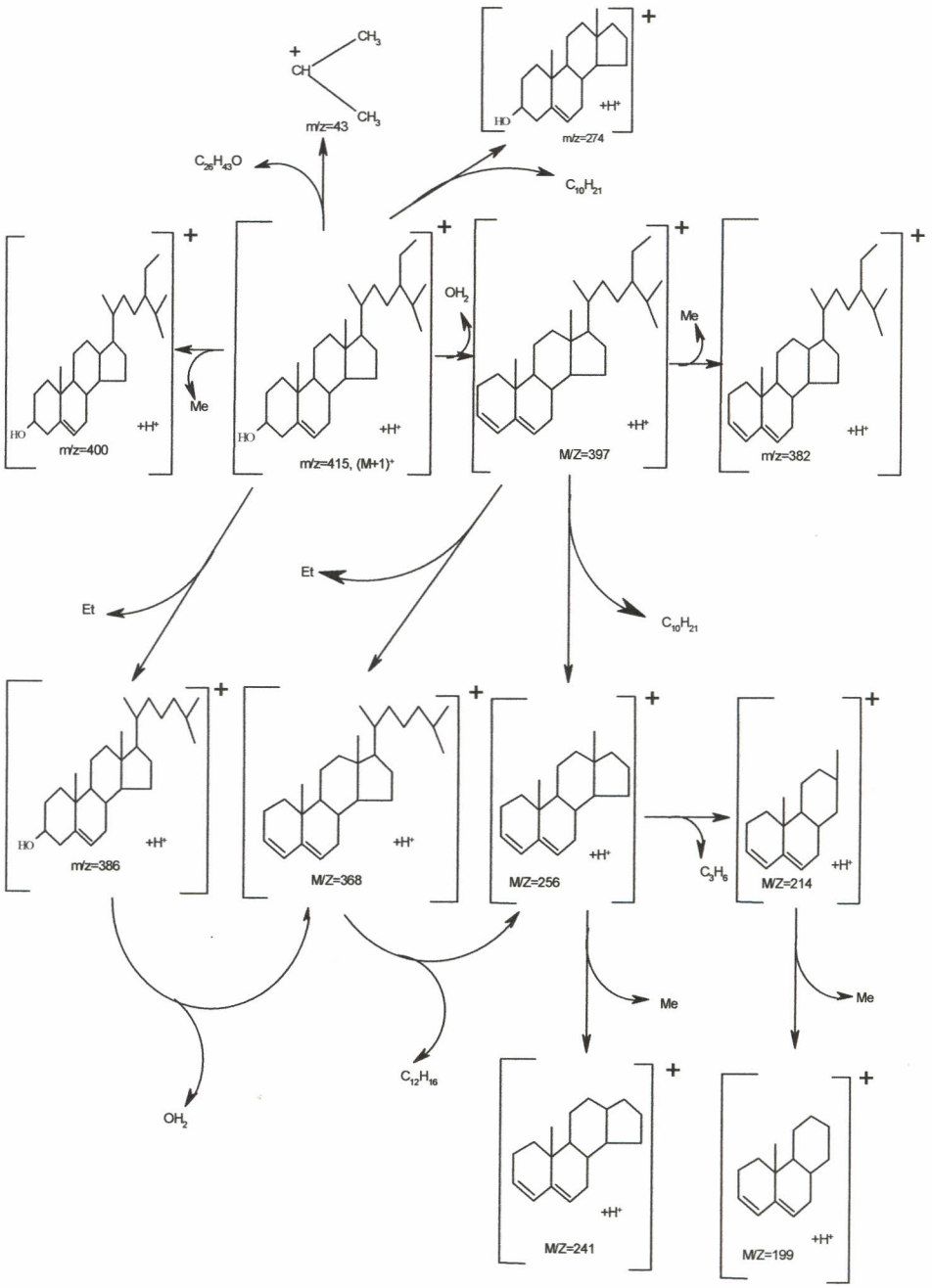


Fig. 3: Proposed fragmentation pattern of β-sitosterol

Carbon-13 NMR spectrum (Appendix 6) shows 27 resonance lines corresponding to 27 magnetically nonequivalent carbons indicating that two pairs of carbons are equivalent. According to the Distortionless Enhancement through Polarization Transfer (DEPT) spectrum (Appendix 7), the molecule contains 6 methyl carbons, 11 methylene carbons and 9 methine carbons, giving a total of 26 protonated carbons which is consistent with the proposed structure. However, the DEPT subspectrum for all protonated carbons has 25 resonance signals corresponding to 25 magnetically non-equivalent carbons, one less than expected. This is due to magnetic equivalency of a methine and a methylene carbon at δ 32.1. This signal was assigned to C-7 and C-8. The most low field signals at δ 140.9 and δ 121.9 were assigned to the sp^2 hybridised C-5 and C-6 respectively. Alkenic carbons are reasonably deshielded and resonate low field at δ range 110-150 [57,58]. The signal at δ 72.0 was due to the hydroxymethine C-3. Although tertiary carbons resonate at δ range 30-60, the electrons withdrawal effect of the hydroxyl oxygen causes deshielding of C-3, hence the observed downfield shift. Primary carbons are highly shielded and hence resonate at high fields. Therefore carbons 18, 19, 21, 26, 27 and 29 were assigned the signals upfield. The ethyl C-29 and the angular methyl C-18 are more shielded than the other methyl carbons and were assigned signals at δ 12.07 and δ 12.19, while carbons 21 and 19 were assigned signals at δ 18.99 and δ 19.24 respectively. In the terminal isopropyl group, the asymmetric C-25, a chiral centre, confers optical isomerism and causes the two methyl groups to be non-equivalent in the ^{1}H and ^{13}C NMR spectra [69]. According to the work of Nes *et al.*, the pro-S methyl group is designated as C-26 and resonates downfield of the pro-R methyl C-27 [70]. The two carbons were assigned signals at δ 20.05 and δ 19.61 respectively. In a similar manner, the other signals were assigned using literature ^{13}C chemical shifts of β-sitosterol as tabulated in Table 5.

In the ^{1}H-NMR spectrum (Appendix 8) a doublet at δ 5.35 and a multiplet at δ 3.51, each integrating for one proton, were assigned to the vinylic proton H-6 and hydroxymethine proton H-3 respectively. A doublet at δ 2.27 integrating for two protons was assigned to H-4 while the intense singlets at δ 0.66 and δ 0.99 integrating for three protons each were assigned to the angular methyl hydrogens H-18 and H-19 respectively.

Table 5: Comparison of PMN-01 ^{13}C chemical shifts to the literature values of β-*sitosterol*

C No.	^{13}C chemical shifts		C No.	^{13}C chemical shifts	
	*β-sitosterol	PMN-01		*β-sitosterol	PMN-01
1	37.2	37.46	16	28.2	28.47
2	31.6	31.85	17	56.0	56.25
3	71.8	72.02	18	11.9	12.19
4	42.5	42.49	19	19.4	19.24
5	141	140.95	20	36.1	36.36
6	121.7	121.95	21	18.8	18.99
7	31.8	32.11	22	33.9	34.14
8	31.9	32.11	23	26.0	26.24
9	50.1	50.32	24	45.8	46.02
10	36.5	36.71	25	29.1	29.33
11	21.1	21.29	26	19.8	20.05
12	39.7	39.98	27	19.1	19.61
13	42.3	42.49	28	23.0	23.26
14	56.7	56.97	29	12.0	12.07
15	24.3	24.51			

* From reference [70]

3.3.2 PMN-02

Compound PMN-02 revealed the following spectral data:

UV λ_{max} (MeOH) nm: 238.5.

IR ν_{max} (KBr) cm^{-1}: 3413 (O-H str), 2947 (CH_3, C-H str), 2877 (CH_2, C-H str), 1661 (C=O str), 1458 (CH_3, C-H bend), 1347 (=C-H bend).

MS m/z (rel. int.): 429 ($(M+1)^+$, 100), 414 (4), 396 (21), 386 (1), 372 (3), 288 (14), 248 (9), 235 (4), 214 (3), 256 (26), 206 (11), 193 (31), 188 (16), 174 (7), 161 (24), 135 (18), 107 (11), 95 (10), 81 (10), 69 (9), 57 (10), 43 (13).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 5.7 (1H, s, H-6), 3.7 (1H, m, H-3), 3.1 (1H, t, H-8), 2.6 (2H, m, H-4), 2.5 (1H, q, H-9), 2.4 (1H, q, H-24), 2.2 (2H, d, H-17), 2.0 (2H, m, H-12), 1.60 (1H, d, H-20), 1.5 (2H, t, H-7), 1.4 (2H, m, H-22), 1.3 (1H, q, H-8), 1.2 (2H, m, H-2), 1.19 (3H, s, H-19), 0.89 (2H, m, H-11), 0.86 (6H, m, H-26, H-27), 0.84 (3H, d, H-21), 0.82 (3H, m, H-29), 0.68 (3H, s, H-18).

^{13}C-NMR ($CDCl_3$, 50 MHz): δ 36.51 (C-1), 31.31 (C-2), 70.65 (C-3), 41.98 (C-4), 165.53 (C-5), 126.24 (C-6), 202.73 (C-7), 45.58 (C-8), 45.96 (C-9), 38.47 (C-10), 21.38 (C-11), 38.85 (C-12), 43.27 (C-13), 50.07 (C-14), 26.21 (C-15), 29.26 (C-16), 54.85 (C-17), 12.16 (C-18), 17.49 (C-19), 34.10 (C-20), 17.49 (C-21), 36.26 (C-22), 26.51 (C-23), 41.98 (C-24), 28.74 (C-25), 19.20 (C-26), 19.11 (C-27), 23.20 (C-28), 12.16 (C-29).

The above spectral data enabled identification of PMN-02 as 3-hydroxystigmast-5-en-7-one (Figure 4). The systematic name is stigmast-5-en-7-one (3-hydroxy, 3β), while the IUPAC name is (3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-3-hydroxy-10,13-dimethyl-1,2,3,4,8,9,11,12,14,15,16,17-dodeca-hydrocyclopenta[a] phenanthren-7-one.

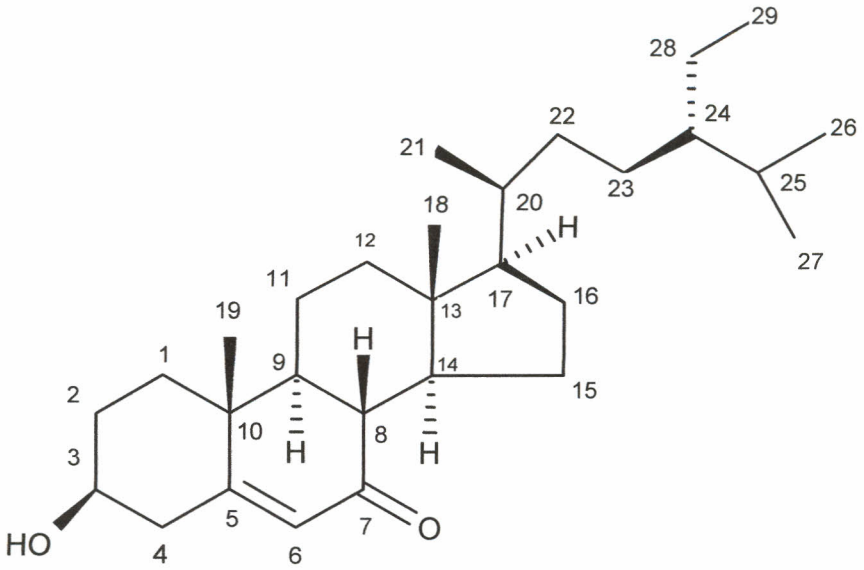


Fig. 4: Structure of 3-hydroxystigmast-5-en-7-one

The ultraviolet light spectrophotometric absorption spectrum (Appendix 9) indicated presence of α,β-unsaturated carbonyl conjugation with λ_{max} of 238.5 nm which is characteristic of enones. The calculated λ_{max} of absorption for the molecule is 239 nm. Hence λ_{max} 238.5 nm is consistent with the proposed structure.

Infrared spectrophotometric absorption spectrum (Appendix 10) showed presence of a carbonyl group, an aliphatic system, a hydroxyl group and a C=C double bond.

The EIMS gave a molecular ion $(M+1)^+$ with m/z of 429 (Appendix 11) which is consistent with the molecular formula $C_{29}H_{48}O_2$ (MW = 428) of 3-hydroxystigmast-5-en-7-one. The fragmentation pattern is indicative of a steroidal system. The fragment ions with m/z of 414 and 411 arise from loss of C-18 angular methyl group and a water molecule from the molecular ion respectively. Loss of both groups from the molecular ion forms the fragment ion 396. Figure 5 is an illustration of a proposed fragmentation pathway for PMN-02.

the most low field signal at δ 202.73. Due to the inductive and conjugative deshielding effects of the carbonyl group, C-5 experiences a down field shift compared to the chemical shift in β-sitosterol and was assigned the signal at δ 165.53. Due to the same effect, C-6 experiences deshielding and resonates a bit low field than its resonance position in β-sitosterol. It was assigned the signal at δ 126.24 while the hydroxymethine C-3 was assigned the signal at δ 70.65. Due to steric effects of the side chain, carbons 18 and 29 are highly shielded and the pair was assigned the most upfield and intense signal at δ 12.1. Carbons 19 and 21 are also shielded and were assigned the signal at δ 17.4. The terminal isopropyl methyl carbons 26 and 27 are magnetically nonequivalent as previously described and were assigned signals at δ 19.2 and δ 19.1 respectively.

In the ^{1}H-NMR spectrum (Appendix 14), the singlet signal at δ 5.7 integrating for one proton was assigned to the vinylic proton H-6. The deshielding effect of the carbonyl group is responsible for a 0.35 δ unit down field shift from its resonance position in β-sitosterol. A multiplet at δ 3.7 integrating for one proton was assigned to the hydroxymethine proton H-3. A triplet at δ 3.1 integrating for one hydrogen was assigned to H-8. Compared to its chemical shift in β-sitosterol the proton has experienced a down field shift due to the inductive effect of the carbonyl oxygen. The highly intense signals at δ 0.68 and δ 1.19 were assigned to the angular methyl protons H-18 and H-19 respectively while those at δ 0.84 and δ 0.82 were assigned to the isopropyl H-26 and H-27 respectively.

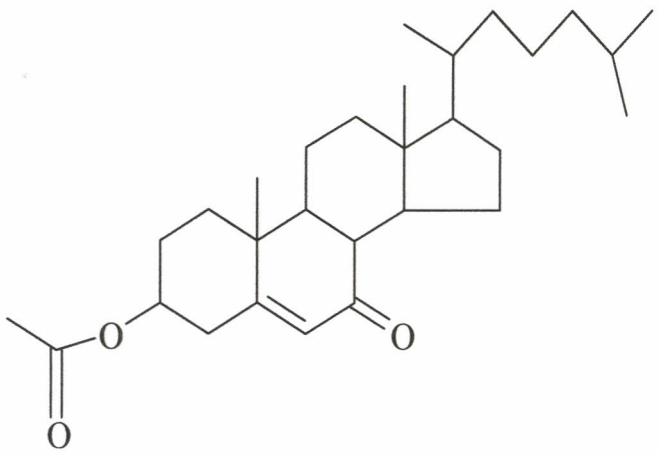


Fig. 6: Structure of cholest-5-en-7-on-3β-yl acetate

Table 6: Comparison of PMN-02 ^{13}C chemical shifts to the literature values of cholest-5-en-7-on-3β-yl acetate

C No.	^{13}C chemical shifts		C No.	^{13}C chemical shifts	
	*Cholest-5-en-7-on-3β-yl acetate	PMN-02		*Cholest-5-en-7-on-3β-yl acetate	PMN-02
1	36.1	36.51	16	39.0	38.85
2	27.5	31.31	17	55.2	54.85
3	72.3	70.65	18	11.8	12.16
4	37.7	41.98	19	16.9	17.49
5	163.2	165.53	20	35.9	34.10
6	126.7	126.24	21	18.9	17.49
7	200.1	202.73	22	36.4	36.26
8	45.3	45.58	23	24.1	26.51
9	49.9	45.96	24	39.6	41.98
10	38.5	38.47	25	28.0	28.74
11	21.3	21.38	26	22.4	19. 20
12	29.0	29.26	27	22.6	19. 11
13	43.2	43.27	28	-	23.20
14	50.2	50.07	29	-	12.16
15	26.4	26.21			

*From reference [71]

3.3.3 PMN-03

Compound PMN-03 gave the following spectral data:

UV λ_{max} (MeOH) nm: 209.5.

IR ν_{max} (KBr) cm^{-1}: 3390 (O-H str), 2944 (CH_3, C-H str), 2876 (CH_2, C-H str), 1662 (=C-H str), 1451 (CH_3, C-H bend), 1371 (=C-H bend).

MS m/z (rel. int.): 431 ($(M+1)^+$, 4), 415 (4), 414 (25), 413 (100), 400 (1), 399 (7), 395 (7), 380 (3), 212 (3), 175 (3), 159 (4), 145 (6), 143 (6), 135 (7), 119 (7), 107 (7), 95 (8), 91 (8), 81 (9), 79 (7), 69 (7), 69 (7), 57 (12), 55 (14), 43 (17).

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 5.24 (1H, s, H-6), 3.48 (1H, t, H-7), 3.21 (1H, m, H-3), 1.94 (2H, m, H-4), 1.66 (1H, m, H-8), 1.59 (1H, m, H-9), 1.51 (1H, m, H-14), 1.46 (1H, m, H-17), 1.33 (1H, d, H-20), 1.30 (1H, m, H-25), 0.62 (3H, s, H-19), 0.54 (6H, m, H-26, H-27), 0.44 (3H, m, H-29), 0.31 (3H, s, H-18).

^{13}C-NMR ($CDCl_3$, 50 MHz): δ 37.58 (C-1), 31.55 (C-2), 71.54 (C-3), 42.42 (C-4), 146.44 (C-5), 124.04 (C-6), 65.56 (C-7), 42.17 (C-8), 49.60 (C-9), 37.17 (C-10), 20.88 (C-11), 39.33 (C-12), 42.32 (C-13), 55.87 (C-14), 24.49 (C-15), 28.47 (C-16), 55.85 (C-17), 12.17 (C-18), 18.98 (C-19), 36.29 (C-20), 18.44 (C-21), 34.07 (C-22), 26.04 (C-23), 45.98 (C-24), 29.27 (C-25), 20.00 (C-26), 19.20 (C-27), 23.22 (C-28), 11.82 (C-29).

Based on the obtained spectroscopic information, isolate PMN-03 was identified as 7-hydroxysitosterol (Figure 7). The systematic name is stigmast-5-ene-3,7-diol (3β,7α) while the IUPAC name is (3S,7S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthrene-3,7-diol.

Ultraviolet light spectrophotometric absorption spectrum (Appendix 15) indicated absence of conjugation with UV λ_{max} of 209.5 nm.

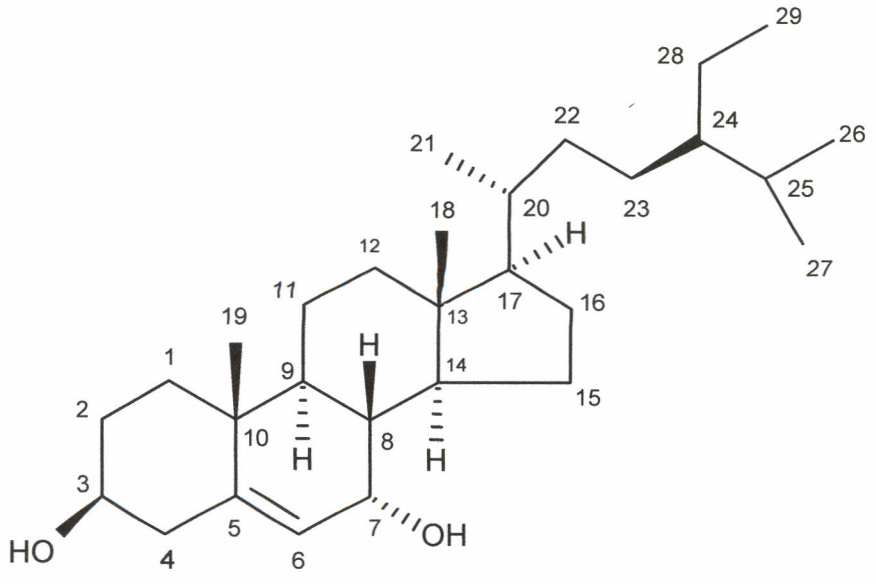


Fig. 7: Structure of 7-hydroxysitosterol

Infrared spectrophotometric absorption spectrum (Appendix 16) indicated presence of an aliphatic system, a hydroxyl group and a C=C double bond.

The EIMS spectrum (Appendix 17) shows a molecular ion $(M+1)^+$ with m/z of 431 which corresponds to the molecular formula $C_{29}H_{50}O_2$ (MW = 430) of 7-hydroxysitosterol. The base peak ion with a m/z of 413 is due to loss of water from the molecular ion. Further loss of a second water molecule gave the fragment ion with m/z of 395. Loss of C-18 angular methyl from the fragment ion 395 gave fragment ion with m/z of 380. This fragmentation is therefore consistent with steroidal systems. Figure 8 gives a proposed fragmentation pathway for PMN-03.

Carbon-13 NMR spectrum (Appendix 18) shows 29 signals corresponding to 29 carbons in the molecule which is consistent with the proposed structure. In comparison to β-sitosterol, presence of an additional OH group in the molecule is indicated by the signal at δ 65.56 in the ^{13}C-NMR spectrum. The signal is within the chemical range for hydroxymethine carbons. The assignment of the chemical shift to C-7 was based on several observations. First, the double bond between carbons 5 and 6 makes C-7 biogenically susceptible to hydroxylation. Secondly, there is an

observable downfield shift of C-5 and C-6 resonance positions in comparison to their chemical shifts in β-sitosterol. This is due to the inductive electron withdrawal effect of C-7 hydroxyl group. Thirdly, an additional signal in the ^{1}H-NMR spectrum (Appendix 19) at δ 3.21 integrating for one hydrogen, which is absent in β-sitosterol ^{1}H-NMR spectrum, is due to the hydroxymethine proton H-7.

The other signals were assigned using β-sitosterol as the reference.

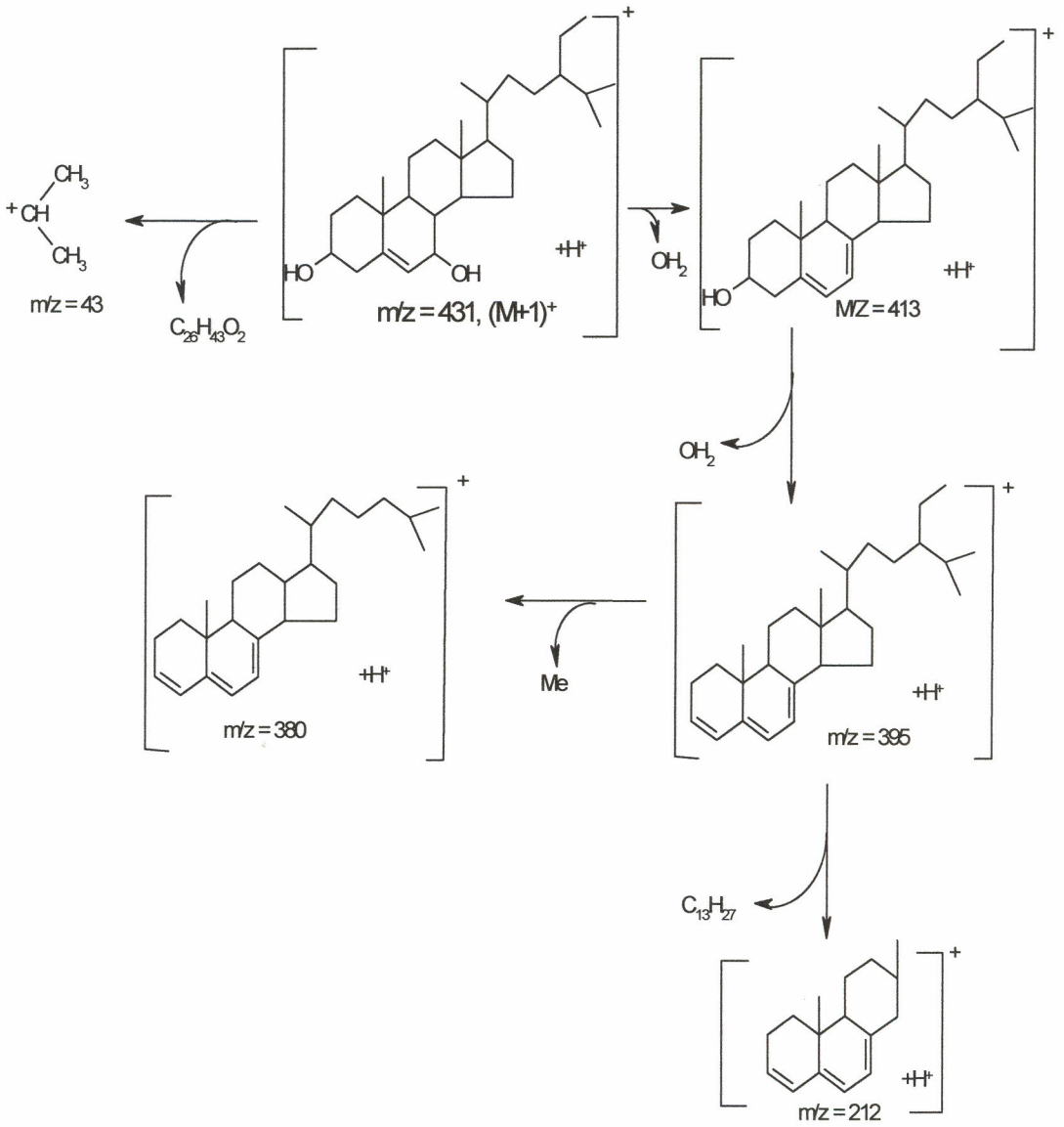


Fig. 8: Proposed fragmentation pattern of 7-hydroxysitosterol

3.4 Antibacterial and antifungal activity studies

3.4.1 Antibacterial activity

Table 7 shows the diameters of the zones of inhibition produced by the root and stem extracts of *Girardinia diversifolia* in comparison with standard antibiotics. All extracts exhibited varying degrees of antibacterial activity against test microorganisms. However, ethyl acetate extract exhibited the best antibacterial activity profile compared to the other extracts. The ethyl acetate partition of root methanol extract (EAPRM) and the ethyl acetate partition of stem methanol extract (EAPSM) had the highest activity against *Bacillus pumilus* and *Escherichia coli* while the *Girardinia diversifolia* root petroleum ether extract (GdRP) and the *Girardinia diversifolia* stem petroleum ether extract (GdSP) showed good activity against *Staphylococcus aureus* comparable to that of benzylpenicillin.

These observations are figuratively captured in Appendices 20, 21 and 22. The clarity of zone boundaries exhibited by the EAPRM and EAPSM extracts was higher when tested against *E. coli* and *B. pumilus* than against *S. aureus*. Conversely, GdRP and GdSP extracts produced better zone boundaries against *S. aureus* than against *E. coli* and *B. pumilus*. The chloroform and methanol extracts exhibited moderate antibacterial activities against the three test organisms.

3.4.2 Antifungal activity

Tested against *Candida albicans* and *Aspergillus niger*, EAPRM and EAPSM extracts exhibited higher antifungal activity than any other extract. As shown in Table 8, the diameters of the zones of inhibition produced by the two extracts against both fungi were higher than those produced by any other extract. The zone boundaries were most apparent in the ethyl acetate extracts as captured in Appendix 23. The methanol extracts demonstrated moderate antifungal activity.

Table 7: Antibacterial activity of the root and stem extracts of *Girardinia diversifolia* against *Bacillus pumilus*, *Escherichia coli* and *Staphylococcus aureus*

Test solution[a]	Diameters of the zones of inhibition[b] (mm)		
	B. pumilus	*E. coli*	*S. aureus*
GdRP	8.1	8.3	8.2
GdSP	7.8	8.3	7.9
GdRC	7.7	8.0	7.6
GdSC	7.4	7.4	7.4
GdRM	7.7	7.2	6.6
GdSM	7.6	7.1	6.6
EAPRM	8.8	8.6	7.2
EAPSM	8.8	8.4	7.1
Gentamicin	17.0	18.2	-
Benzylpenicillin	-	-	9.0

[a]Concentrations: Extracts 50 mg/ml; Gentamicin 10 µg/ml; Benzylpenicillin 1 I.U/ml

[b]Mean zone diameter from duplicate tests

- = Not tested

GdRP – *Girardinia diversifolia* root petroleum ether extract

GdSP – *Girardinia diversifolia* stem petroleum ether extract

GdRC - *Girardinia diversifolia* root chloroform extract

GdSC - *Girardinia diversifolia* stem chloroform extract

GdRM - *Girardinia diversifolia* root methanol extract

GdSM - *Girardinia diversifolia* stem methanol extract

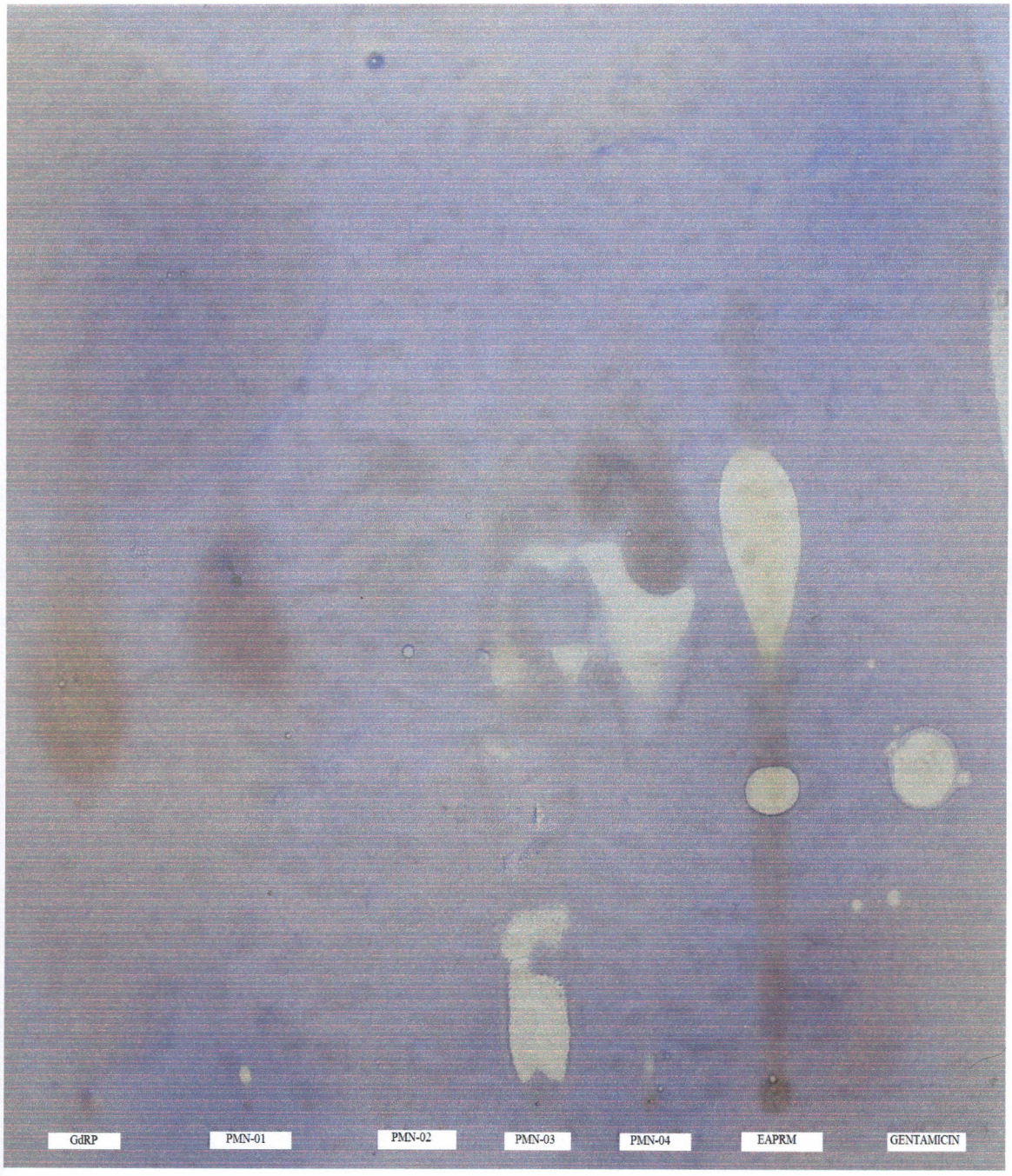
EAPRM - ethyl acetate partition of root methanol extract

EAPSM - ethyl acetate partition of stem methanol extract

APPENDICES

Appendix 1: *Girardinia diversifolia* root petroleum ether extract undergoing fractionation by open column chromatography

Appendix 26: Bioautogram illustrating bioactivity of isolated compounds against *Bacillus pumilus*



GdRP: *Girardinia diversifolia* root petroleum ether extract; EAPRM: ethyl acetate partition of root methanol extract; PMN-01, PMN-02, PMN-03, PMN-04: isolated compounds