PHYTOCHEMICAL INVESTIGATION OF COMPOUNDS FROM *VEPRIS GLANDULOSA* LEAF EXTRACTS AND THEIR ANTIBACTERIAL ACTIVITIES

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DECLARATION AND APPROVAL

Declaration

I declare that this thesis is my original work and has not been presented before for any award of a diploma or conferment of a degree in this or any other university.

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DEDICATION

To

My parents Dr. Joel Chirchir and Racheal Chirchir and my siblings; Dr. Collins, Patience and Rogers.

ACKNOWLEDGEMENT

I would like to give my first appreciation to God for giving me the strength and good health that enabled me to come this far. Special thanks to my supervisors; Prof. Joyce J. Kiplimo and Dr. Denis K. Chirchir for their guidance throughout the research period. Their dedication and commitment to ensuring the successful completion of this work was very much appreciated. I would like to thank the staff at Muguga South forest reserve and the University of Surrey for their invaluable facilitation. I would also like to thank my colleagues, other teaching and non-teaching staff from the University of Kabianga who contributed in various capacities to make this work a success.

ABSTRACT

Historically mankind has relied on plants for curing various bacterial diseases with great success but with modernization mankind turned to synthetic drugs which had great success initially but overtime began facing challenges such as resistance, toxicity and low drug discovery in recent times. Mankind had no option but to turn to plants for medicine because plants have an abundance of natural products which if exploited could be used for man's benefit. Plants from the genus *Vepris* are known to be good sources of medicines curing a variety of bacterial diseases such as syphilis, gonorrhea and brucellosis. *Vepris glandulosa* is a tree that is endemic to Kenya was collected at Muguga South Forest Reserve. It is known ethno botanically to cure complications such as malaria, pains, fever and brucellosis. There are no known documented phytochemical and antibacterial assays done on *Vepris glandulosa* and the study helped to bridge that gap. The crude leaf extract of *V. glandulosa* obtained after soaking in hexane was subjected to phytochemical qualitative analysis which showed presence of saponins, tannins, phenol, flavonoids, alkaloids and terpenoids. The crude extract was subjected to repeated column and thin layer chromatography for isolation and purification. Compound 1 was as a white threadlike crystal isolated at 10 % ethyl acetate, 90 % hexane. Compound 1 was subjected to ^{1}H NMR, ^{13}C NMR, COSY, DEPT, HSQC and HMBC analyses. Spectral data from these spectroscopic techniques showed compound 1 as a Lupane type pentacyclic triterpene characteristics with 6 quaternary, 6 methine, 7 methyl and 11 methylene carbons. There were olefinic carbon resonance of exocyclic double bond that appeared at δc 79.01 ppm for carbon bonded to an OH group, δc 150.95 ppm for the quaternary carbon and δc 109.3 ppm for the methylene carbon which had olefinic of δ_H 4.57 and δ_H 4.68. The ^{13}C NMR showed thirty carbon signals and ^{1}H NMR integration values gave 50 proton signals with a molecular formula of $C_{30}H_{50.}$ Compound 1 was confirmed to be Lupeol after literature comparison. Zone of inhibition assay on the crude extract showed moderate sensitivity against *Staphylococcus aureus* with 13.66 mm while resistance was observed for *Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa* with zone of inhibition values falling below 13 mm. Minimum Inhibitory Concentration values were 6 µg/ml for *P. aeruginosa* and *4* µg/ml for *S. aureus.* The Minimum Bactericidal Concentration values were 8 µg/ml for both *S. aureus* and *P. aeruginosa.* These values showed that the crude extract has bactericidal activity against both *P. aeruginosa* and *S. aureus.* The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration assays are sufficient in concluding that the crude extract is a viable antibacterial agent because clinicians use their results to determine choice of chemotherapy. It is recommended that further phytochemical and antibacterial assays be done on *Vepris glandulosa* leaf extracts and also stem bark and root extracts.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADMET	Absorption Distribution Metabolism Excretion Toxicity
AMR	Antimicrobial Resistance
ATCC	American-Type Culture Collection
CFU	Colony Forming Units
DEPT	Distortionless Enhancement by Polarisation Transfer
DMAPP	Dimethylallyl Diphosphate
FPP	Farnesyl Pyrophosphate
GC-MS	Gas Chromatography- Mass Spectrometry
GGCP	Geranyl Pyrophosphate
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HMG-CoA	Hydroxy methyl glutaryl Coenzyme A
HPV	Human Papilloma Virus
IPP	Isopentenyl Diphosphate
IR	Infrared
MBC	Minimum Bactericidal Concentration
MEV	Mevalonate
MIC	Minimum Inhibitory Concentration
NCTC	National Collection Type Cultures
NDPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
SDGs	Sustainable Development Goals
TLC	Thin Layer Chromatography

DEFINITION OF TERMS

Phytochemistry- This is a branch of chemistry that is focused on plants and plant products particularly secondary metabolites. It takes into account the structural composition, biosynthetic pathway, and medicinal and commercial applications of these metabolites.

Sustainable Development goals- These are a set of 17 interlinked goals adopted by the United Nations in 2015 to help solve global challenges so as to attain a more sustainable future by 2030.

Antimicrobial resistance- The condition in which microorganisms evolve and cease to be affected by antimicrobials. Intrinsic resistance is where the bacteria are naturally unaffected by an antimicrobial due to adaptation and acquired resistance main method of microbial resistance is due to the evolutionary need to form a defense mechanism

CHAPTER ONE

INTRODUCTION

1.1. Introduction

This chapter gives a brief background of the study and the challenges that warranted the study. It also presents the problem statement, the justification for the study, the objectives of the study, significance, scope, limitations and hypothesis of the study.

1.2. Background of the study

The discovery of penicillin by Alexander Fleming ushered in the golden age of exploration of new antimicrobials from natural products and their synthetic derivatives. Most microbial diseases became treatable after the commercialization of the antimicrobials (Sakai & Morimoto, 2022).

Even though there was a significant win in the battle against microbial diseases with the use of modern antimicrobials, there were signs after experimental analysis that suggested the possibility of resistance even before the widespread use of antimicrobials such as penicillin. Antimicrobial resistance is the biggest challenge facing use of antimicrobials among other challenges (Aminov, 2010).

The ability of microorganisms to resist clinical level concentrations of antimicrobials is a major health concern because it results in prolonged morbidity and high mortality (Nwobodo et al., 2022). Reported cases of AMR have grown exponentially over the

years pushing to number one death cases due to microbial infections. The potential dangers of microbial diseases paint a grim future such that projections of impacts of AMR is 10 million annual human deaths globally by the year 2050 (Ministry of Health: Kenya National Policy on Prevention and Containment of Antimicrobial Resistance, 2017).

Other challenges facing antimicrobials include high levels of toxicity especially with synthetic drugs which tend to have varying side effects such as nausea, diarrhea and loss of hair which is experienced with use of synthetic antineoplastic drugs. These makes most synthetic drugs fail in Absorption Distribution Metabolism Excretion Toxicity (ADMET) test and therefore less desired (Solowey et al., 2014).

It is not enough for a drug to have antimicrobial potency but it also has to meet the ADMET expectations (Bishnu et al., 2009). There is currently a colossal demand for new drugs because during the last decade semisynthetic and synthetic chemotherapeutic agents have not satisfied expectations despite the considerable resources utilized for their development. Synthetic drugs fail in ADMET, are expensive and synthetic drug discovery has been unreliable in the last decade (Bishnu et al., 2009).

Man has therefore no option but to turn to nature as a source of medicine which has the best alternative to fulfill the demand for new drugs because natural medicine mostly satisfy the ADMET, are significantly less expensive and there is virtually an unlimited potential for new discovery (Bishnu et al., 2009).

Mankind has since earliest known records used plants for their healing potential because they are readily available, affordable and non-toxic (Javid et al., 2015). According to the World Health Organization it is estimated that 80% of the earth's population relies on plants as source of medicine for most of their ailments. The heavy dependence on plants as medicinal source shows their unmatched reliability (Kilonzo & Munisi, 2021). In addition, successful advancements in phytochemistry has renewed mankind's interest in herbal medicine (Bishnu et al., 2009).

In the plant kingdom, the Rutaceae family have been used ethno botanically for traditional medicine, perfumery and gastronomy (Tangjitjareonkun & Supabphol, 2014). Rutaceae have a wide range of secondary metabolites which exhibit antibacterial, anti-inflammatory, antifungal, antioxidant and antiviral, activities (Imbenzi et al., 2014). The genus Vepris which is a member of the Rutaceae family has roughly 90 African species with examples such as *Vepris nobilis*, *Vepris grandifolia, Vepris hanangensis* and *Vepris simplicifolia* (Omujal et al., 2020).

The *Vepris* genus has had great success as an ethno medicinal source in the treatment of pneumonia, malaria, coughs, colds, infertility and rheumatism among others (Imbenzi et al., 2014). The medicinal efficacy of plant materials mostly result from the secondary metabolites such as lupeols, flavonoids, phenols, saponins, tannins and alkaloids (Sivasakthi & Saranraj, 2014).

The species in this genus are used in management of a wide variety of ailments and this makes *V. glandulosa* have great potential as a medicinal source. *Vepris glandulosa* has been known ethono botanically to treat a variety of complications such as pains, dental caries, gonorrhea and brucellosis (Imbenzi et al., 2014).

1.3. Statement of the problem

The reality is a lot of the available antimicrobials are increasingly facing resistance from emerging bacterial diseases and the biggest is the development of resistant strains that were previously controlled by the available antibacterials. The pathogenic bacteria have adapted and evolved to produce progeny resistant to available drugs thus the available chemotherapeutics antibacterials are losing the battle with bacterial diseases. To further exacerbate the problem, synthetic drug discovery has been low and synthetic chemotherapeutics are more toxic to the body and are known to cause negative side effects such as nausea and hair loss which are common side effect of use of synthetic antineoplastic drugs. The result of the challenges facing the available antibacterials is prolonged morbidity, death and financial strain on families causing governments and healthcare providers to desperately look for solutions to mediate the problem. It is due to these great concerns that mankind has been left with no option but to search for new drugs from natural sources which have virtually unlimited potential for new drug discovery.

1.4. Objectives of the study

1.4.1. General Objective

To isolate, characterize secondary metabolites from *V. glandulosa* and assess their antimicrobial activities.

1.4.2. Specific Objectives

The specific objectives of the study were;

i. To extract phytochemicals from *V. glandulosa* leaves using methanol, hexane and ethyl acetate.
ii. To isolate pure compounds from *V. glandulosa* using thin layer and column chromatography.
iii. To determine the antibacterial activities of crude extract from *V. glandulosa* leaves.
iv. To use NMR spectroscopic technique in the elucidation of the structure of compounds isolated from *V. glandulosa.*

1.5 Research questions

i. Can the phytochemicals from *Vepris glandulosa* be extracted using various solvents?
ii. Do extracts of *Vepris glandulosa* have phytochemicals that can be extracted, identified and quantified?
iii. Do crude extracts and compounds from *Vepris glandulosa* exhibit antibacterial and anticancer activities?

1.6. Justification

Nature has been used historically as a source of medicine with great success due to its affordability, availability and effectiveness. Phytochemistry has shown that nature is blessed with an abundance of possible natural products which if exploited for medicinal use will be invaluable in solving current challenges facing available antibacterials. Plants from the Genus Vepris have been successfully used ethno botanically as antibacterials

and several phytochemical analyses of plants from this Genus have shown presence of useful natural products such as alkaloids, terpenoids, tannins and phenols. It is for this reason *V. glandulosa* was chosen because it is a member of the genus Vepris and therefore there is high possibility of presence of useful natural products. *V. glandulosa* is a plant endemic to Kenya therefore low cost of obtaining plant sample. It has been known ethno botanically to treat various complications such as brucellosis, chronic cough, pain and pneumonia. *V. glandulosa* was also chosen because there is no known data on phytochemical or antibacterial assays done on the leaf extracts. The study aimed to bridge the knowledge gap on phytochemicals present in the plant and determine antibacterial potential which would possibly make it be a new affordable antibacterial source. This is in line with SDG number 3 which is about investing in health care.

1.7. Significance of the Study

The study will highlight the availability of useful natural products in the plant *V. glandulosa* through extraction, isolation and purification and determination of antibacterial potential of the crude and pure compounds from the leaf extracts. The study could therefore be an inspiration for further research to clinical trials of new alternative and affordable antimicrobial drugs to join in the fight against antibacterial diseases.

1.8. Scope of the study

The scope of the study was to look into the phytochemical potentiality of *V. glandulosa* leaves in the development of safe and viable antibacterial.

1.9. Limitations of the study

i. The study was restricted to leaves because the plant is endangered therefore assays on roots and stem bark not done.

ii. High cost of solvents and other materials needed to perform assays.

1.19. Assumptions

i. *V. glandulosa* crude extracts and pure compounds have antibacterial properties.

ii. *V. glandulosa* will become a viable source of antibacterial.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

This chapter highlights the literature of the family Rutaceae, the genus *Vepris* and also introduces literature on *V. glandulosa*. It gives comprehensive analysis of the phytochemicals present in the genus and their antibacterial activities.

2.2. Rutaceae family

The Rutaceae family is mainly found in tropical and subtropical regions that has varied types of flora ranging from trees, climbers and evergreen shrubs all of which total 1600 species and 150 genera (Tangjitjareonkun & Supabphol, 2014).

An identifiable physical feature of the family is the presence of schizogenous secretory cavities comprising essential oils. These cavities are noticeable as pellucid dots in the leaves, but also in other parts of the plants, such as the pericarp, flowers and young axes (Appelhans et al., 2021).

It is the largest family in relation to the number of species in the order sapindales and is most famous for the genus *Citrus* which has high economic value. The genus *Citrus* L. is widely cultivated due to its juicy fruits such as lemons, oranges and grapefruits (Groppo et al., 2021).

The Rutaceae family is also an important source of aromatic oils usually from genus *Boronia* and a source of timber usually from genus *Flindersia.* The chemotherapeutic importance of the family is broad ranging from being used as an analgesic, antibacterial, anthelmintic, anticancer and antifungal (Groppo et al., 2021).

The Rutaceae family are known to have the widest array of secondary metabolites among flowering plants (Groppo et al., 2021). Phytochemical analysis of the extracts from the Rutaceae family shows a wide range of secondary metabolites characteristic of the family. Some of these characteristic natural products include terpenoids, limonoids, acetophenones, alkaloids and coumarins (Appelhans et al., 2021).

2.3. The genus *Vepris*

The genus *Vepris* belongs to the Rutaceae family and the known habitats for the genus *Vepris* are mainly in the tropical and temperate areas of Asia and Africa (Giamperi et al., 2020). There are 93 species of which 92 are native to Africa and 1 is native to Asia. Cameroon has the highest number with 24 species followed by Madagascar with 23 species (Cheek et al., 2022). Some of the species found in this genus include *V. nobilis*, *V. grandifolia, V. hanangensis, V. simplicifolia* and *V. glandulosa* (Omujal et al., 2020).

Plants in the genus *Vepris* have easily identifiable phenotypic features, their leaves are usually non-pinnate with digitate foliate and their stems are non-spiny (Cheek et al., 2022). Phytochemical analysis of compounds obtained from *Vepris* has shown essential oils distributed in the leaves, alkaloids and terpenoids distributed in the roots, bark and leaves. Plants from the genus *Vepris* have traditional been used as medicinal plants to

treat various bacterial diseases such as brucellosis, malaria, gonorrhea, leprosy and dental caries (Langat et al., 2022).

A summary of the biological properties of plants from the genus *Vepris* is presented in table 2.1 below.

Table 2.1
Biological activity of various species found in the genus Vepris

Plant species	Plant part	Biological activity/Diseases	Reference
Vepris nobilis	Leaves	Pain reliever, chronic cough and weight reduction	(Omujal et al*.,* 2020)
	Stem bark	Dental caries, chest disorders, gonorrhoea, cough, ulcers and syphilitic	(Omujal et al*.*, 2020)
	Root bark	Measles, rheumatism, anthelmintic, arthritis, malaria and pneumonia	(Omujal et al*.*, 2020)
Vepris glomerata	Aerial	Antioxidant	(Imbenzi et al., 2014)
Vepris simplicifolia	Fruits	Toothache	(Kilonzo & Munisi, 2021)
	Roots	Brucellosis, backache, gonorrhoea, leprosy, stomachache.	(Kilonzo & Munisi, 2021)
Vepris punctata	Aerial	Antibacterial activity	(Imbenzi et al., 2014)
Vepris ampody	Leaves	Fever tonic, antimalarial, muscle pain,	(Ngbolua, 2023)
	Stem bark	Aphrodisiac and stimulant	(Ngbolua, 2023)
	Root bark	Anticancer activity	(Imbenzi et al., 2014)
Vepris heterophylla	leaves	Insect repellant	(Ngamo et al., 2007)
Vepris macrophylla	Leaves	Antifungal and antibacterial	(Giamperi et al*.*, 2020)
Vepris uguenensis	Root bark	Antimalarial activity	(Imbenzi et al., 2014)
Vepris amaniensis	Root bark	Antibacterial activity against *Mycobacterium madagascariense* and *Mycobacterium indicus pranii*	(Erasto et al., 2013)
Vepris louisii	Stem bark	Anthelmintic	(Komtangi et al., 2014)
Vepris dainellii	Seeds	Abdominal cramps	(Denu & Desissa, 2013)
Vepris dainellii	Fruits and Stem bark	Anthelmintic, skin infections, tooth pain	(Denu & Desissa, 2013)

2.4 *Vepris glandulosa*

V. glandulosa is a shrub or tree that can grow up to 7 m in height. This lower canopy tree is confined to upland dry forests. The species grows well in the shade and reproduction is through seeds only. The species' habitat is historically Muguga South Forest Reserve,

Ragati and Limuru which have elevations of over 1550 m (Barstow, 2018). It is however threatened by the growth of local human populations which caused the expansion of agricultural spaces and urban settlements into the species range. *V. glandulosa* is classified as an endangered species (Ministry of Tourism and Wildlife, 2017).

Figure 2.1: Picture of *Vepris glandulosa* plant (Source: Researcher)

2.5 Phytochemistry review

The association of plants with humans can be traced back to the beginning of civilization. Man has relied on plants for various purposes ranging from food sources to medicinal sources. In ancient times man relied on plants as a source of chemotherapy with great success and these are the simplest early forms of phytochemistry. Phytochemistry has advanced over the past recent decades and established itself as a separate domain (Sinha et al., 2022).

It is the study of the compounds known as secondary metabolites that are made by plants. Secondary metabolites often have an ecological role in regulating the interactions

between plants and their environment. They are produced as a self-defense mechanism against pests such as insects, herbivores, microbes, excessive sunlight and other unfavorable environmental conditions. They are also known to work as attractants and pheromones (Gurupriya et al., 2018).

It takes into account the structural compositions of natural products and their chemotherapeutic and commercial applications. In-depth knowledge of phytochemistry is important for drug discovery and for the advancement of new therapeutic agents against stubborn diseases (Egbuna et al., 2018).

It is becoming evident that there is a growing preference for natural products as medicinal sources over synthetic medicine. The preference for natural chemotherapy over synthetic and semi-synthetic drugs has been reported in developed and developing countries (Sinha et al., 2022). Some of the major reasons are natural products have highly bioactive molecules which are used as lead and/or supporting structures for the development of modified derivatives and they mostly satisfy the ADMET requirements (Shwe et al., 2019).

The degree of concentration of a secondary metabolites usually varies with different sections of the plant such as seeds, stem bark, leaves, roots, flowers and fruits (Saxena et al., 2013). Through advancements in phytochemistry, the medicinal aspect of secondary metabolites derived from plant extracts has been studied extensively to establish their value and to comprehend the fundamental mechanisms of their action. These studies

involve isolation then identification chemical compounds and determining biological strength both by *in vivo* and *in vitro* studies (Saxena et al., 2013).

The largest proportion of natural products or secondary metabolites originate from plants the rest are derived from marine organisms and microorganisms (Demain, 2013). About 70, 000 out of a total of 422, 127 plant species are used as medicinal plants (Javid et al., 2015). These have been categorized by chemical, physical and functional characteristics. The pharmacological importance of these secondary metabolites ranges from anticancer, anthelmintic, antioxidants and antimicrobial just to mention a few among many chemotherapeutic uses.

Secondary metabolites with antimicrobial potency in plant material are due to the high presence of active compounds like flavonoids, phenols, saponins, terpenoids, tannins, alkaloids and steroids (Javid et al., 2015).

A summary of compounds from natural products and their antibacterial mode of action is presented in table 2.2 below (Qadri et al., 2022).

Table 2.2:

Natural products with antimicrobial potential and their mode of action

Group of compounds	Natural product	Microbe that has high potency against	Mode of action
Alkaloids	Reserpine	*Staph spp, Strepto spp*	Prevents effluxion
	Piperine	*Staph aureus*	Prevents effluxion
	Berberine	*E coli*	Prevents protein & DNA synthesis
Terpenes	menthol	*Aspergillus niger, Aspergillus fumigatus*	Destructive interference of the membrane
	Farnesol	*Staph aureus*	Destructive interference of the membrane
	Thymol	*Candida albicans, Candida glabrata*	Prevents effluxing and destructive interference of membrane
Coumarins	Galbanic Acid	Multidrug resistant isolates of *Staph aureus*	Prevents effluxing
	Asphodelin A	*Staph aureus Candida albicans*	Inhibits DNA gyrase
Phenolic compounds	Quercetin	*Staph aureus*	Inhibits effluxing
	Kaempherol	*Candida albicans*	Inhibits effluxing

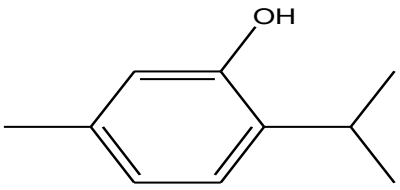

Figure 2.2: Thymol structure

2.5.1 Phytochemistry of the genus *Vepris*

Literature on phytochemical assays done on the majority of plants in the genus *Vepris* revealed a high potential for pharmacological use due to the sufficient presence of chemotherapeutic compounds (Tangjitjareonkun & Supabphol, 2014). The major compounds present in the genus *Vepris* include volatile oils, terpenoids, alkaloids and flavonoids (Ojuka et al., 2023).

Among the most notable alkaloids extracted from the African genus include; furonquinoline and Acridone alkaloids (Kiplimo et al., 2011). Furoquinoline alkaloids were obtained from *V. punctata*, *V. stolzii V. ampody* and *V. heterophylla*, whereas, Acridone alkaloids were obtained from *V. macrophylla* and *V. fitoravina* (Kiplimo et al., 2011). The most abundant alkaloid found in this genus is furoquinoline followed by acridone (Ojuka et al., 2023).

Terpenoids have been successfully extracted from various species in this genus including *V. nobilis* (Omujal et al., 2020), *V. punctata* (Imbenzi et al., 2014), *V. heterophylla* (Ngamo et al., 2007) and *V. unifoliolata* (Garcia et al., 2016) among others. The most abundant terpenoids extracted from this genus include limonoids, triterpenoids and sesquiterpenoids (Ojuka et al., 2023).Analysis of the various phytochemical test results of plants from the genus *Vepris* has shown that the most abundant natural products are alkaloids followed by terpenoids (Ojuka et al., 2023).

Table 2.3:
Compounds extracted from the various plants in the genus Vepris

Plant species	Plant part	Compound extracted	Reference
V. nobilis	Leaves	Alkaloids, sesquiterpenes, essential oils	(Omujal et al., 2020)
	Stem bark	alkaloids	(Omujal et al., 2020)
	Root bark	Alkaloids and essential oils	(Omujal et al., 2020)
V. glomerata	Aerial	Veprisinol, flavonoid, furoquinoline alkaloid	(Kiplimo et al., 2011)
V. punctate	Aerial	Furoquinoline alkaloid, flindersiamine, triterpenoids(lupeol)	(Imbenzi et al., 2014)
	Root bark	Triterpenoid (taraxerol)	(Imbenzi et al., 2014)
V. ampody	Leaves	Alkaloids, flavonoids, saponins	(Ngbolua, 2023)
V. heterophylla	Leaves	Monoterpenes, sesquiterpenes	(Ngamo et al., 2007)
V. macrophylla	Leaves	Alkaloids, essential oils (citral and citronellol)	(Giamperi et al., 2020)
V. uguenensis	Root bark	Furonquinoline alkaloid, azole and imide	(Kiplimo et al., 2011)
V. amanuensis	Root bark	Quinoline alkaloid (vesprine)	(Erasto et al., 2013)
V. unifoliolate	Leaves	Terpenes (α-pinene, myrcene and limonene)	(Garcia et al., 2016)

2.5.2 Alkaloids

Alkaloids are compounds present in plants, animals, bacteria and fungi. They have complex diverse structures with nitrogen in their structure being the common denominator that enables easier identification (Grijalva et al., 2020). Alkaloids are numerous with an estimated 12000 variations identified (O'Connor, 2010). The nitrogen atom is used as the principal requirement for classification as an alkaloid. It can be at any position in the structure but bearing in mind that this excludes nitrogen in peptide or

amide form (O'connor, 2010). Acridone and furoquinoline are the most abundant alkaloids in the genus *Vepris* (Kiplimo et al., 2011).

2.5.2.1 Acridone alkaloid

Acridone alkaloids are natural products that are commonly present in plants from the Rutaceae family (Choi et al., 2020). Acridone compounds are heterocyclic alkaloids that belong to the subclass of acridines (Kumar et al., 2012) with a carbonyl at position 9 and a nitrogen at position 10 as part of the tricyclic ring (Choi et al., 2020).

Acridines have physical characteristics that present as light-yellow needle-like crystals at standard temperature and pressure with a melting point of 110 ^{o}C and boiling point of 346 ^{o}C. Acridine is very irritable to the skin hence the name acridine which means sharp pain and its salts have fluorescence (Rupar et al., 2018).

Acridone is soluble in alcoholic potassium to give a brownish-yellow solution of its potassium salt. Acridone is however insoluble in chloroform, ether, benzene, ethanol and water (Kumar & Kumari, 2011). Acridine also has numerous biological activity due to its atypical mechanism of action and also because its molecule has a planar surface area of 38 Å, a characteristic that closely resembles its derivative Acridone (Kumar et al., 2012).

Acridone alkaloids are highly bioactive compounds that have a broad spectrum range of applications. They are used to combat cancer, viral infections, inflammations and

bacterial infections (Mandal et al., 2021). Acridones and their derivative were initially pursued for their good antimicrobial potency, but more recent efforts have been put into evaluating their anticancer potential (Sepúlveda et al., 2013).

The acridone structure can be described as having a planar structure that allows it to easily intercalate with DNA and therefore able to interfere with metabolic processes of cancer cells. The Acridone structure also allows it to bind with nucleic acids thus conferring it with the capability of being an antiviral drug (Kowalewska et al., 2017). There are various derivatives of Acridone that include thioacridones glyforine, substituted 9-aminoacridines and acronycine (Choi et al., 2020).

The most utilized method for laboratory preparation of Acridone alkaloids is the Ullman synthesis. The Ullmann method involves condensing *o*-halobenzoic acids with aniline to give *N*-phenylanthranilic acid. The cyclization of the *N*-phenylanthranilic acid under strong acid conditions produces the acridone ring (Sepúlveda et al., 2013).

The mechanism of biosynthesis of acridone in nature commences with the conversion of anthranilate into *n*-methylanthranilate with the reaction being catalyzed by the enzyme anthtranilate *n*-methyltransferase, ANMT. The next step involves the enzyme Co-enzyme A which catalyses the conversion of *n*-methylanthranilate to *n*-methylanthraniloyl-CoA.

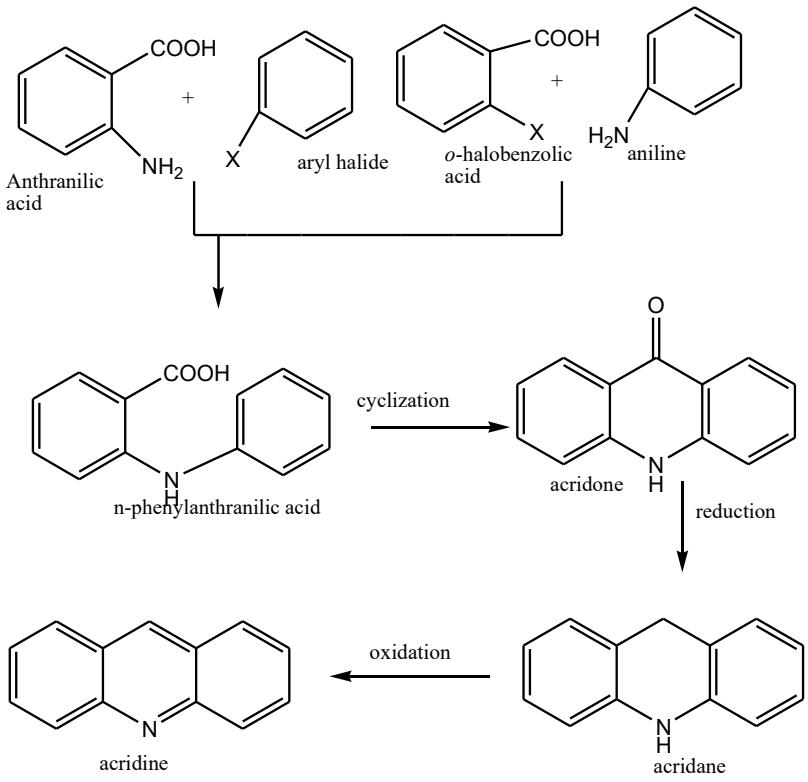

Figure 2.3: Synthesis of acridone, acridane and acridine using Ullman synthesis (Kumar et al., 2012).

The enzyme Acridone synthase a member of plant polyketide synthases then facilitates the condensation of *n*-methylanthraniloyl-CoA. This biogenesis mechanism was elucidated in the plant *Ruta graveolens* a member of the Rutaceae family (Choi et al., 2020).

2.5.3 Terpenoids

Terpenoids are a collection of compounds that are found in all living organisms. Plants have significantly higher proportions of terpenoids compared to other living organisms. (Pichersky & Raguso, 2016). Terpenoids are varied and have important contributions in the perspective of chemical ecology (Boncan et al., 2022).

Terpenes are the largest group of natural products and the basic unit of structure is five-carbon isoprene units which are linked to each other in a variety of ways (Perveen & Taweel, 2018). The name ‘terpene’ was derived from a volatile liquid extracted from pine known as terpentine (Yadava et al., 2014). Terpenoids are derivatives of terpenes or altered forms of terpenes with different functional groups, but in this thesis and works by other natural products scientists, it is used interchangeable to refer to the same thing (Masyita et al., 2022).

The alteration of terpenes has a methyl group replaced and the terpene oxidized into aldehydes alcohols and carboxylic acids. These alterations confer upon them unique biological properties. Terpenes' backbone structures make them have many possible active sites that can undergo glycosylation which further increases the range of diversity of these groups of compounds (Perveen & Taweel, 2018).

The ultimate unit of measure responsible for the structural identity of Terpenes is the isoprene unit which has the molecular formula C_5H_8 (Masyita et al., 2022). The isoprene unit is derived from isopentenyl diphosphate, IPP and its allylic isomer dimethylallyl diphosphate DMAPP as was first recognized by Leopold Rutzicka and Otto Wallach (Tholl, 2015). The number of isoprene units determines the classification of terpenes. One isoprene unit is the hemiterpene C5, two isoprene units is the monoterpene C10, three isoprene units is sesquiterpene C15, four isoprene units is diterpene C20, five is sesterpene C25, six isoprene units is triterpene C30 and 8 isoprene units is tetraterpene C40 (Masyita et al., 2022).

Since terpenoids are terpenes with an oxygen moiety plus some structural modifications, the two terms are used interchangeably, therefore the classification of terpenoids are also denoted as hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, sesterpenoids, triterpenoids, tetraterpenoids and polyterpenoids (Boncan et al., 2022).

There are only two proven methods for the biological synthesis of triterpenoids: the mevalonate pathway (MEV pathway) and the methylerythritol 4-phosphate/ deoxyxylulose 5-phosphate pathway (MEP/DOXP pathway) (Noushahi et al., 2022). This study research thesis we are focused on the first method which is the Mevalonate pathway, which is common to higher plants (Noushahi et al., 2022).

The isoprenoid pathway reactions are responsible for several primary and secondary metabolites and in this case, result in the C5 building blocks of dimethylallyl diphosphate and isopentenyl diphosphate. The MEV pathway which results in terpenoids and for higher plants is localized in the cytosol and endoplasmic reticulum (Boncan et al., 2022).

2.5.3.1 Triterpenoids

Triterpenoids are a group of natural product compounds that have a wide range of diverse structures with 30 carbon atoms. Triterpenoids are vast with more than 20,000 variations recorded. Most of the triterpenoids are found naturally in the plant kingdom with a small percentage emanating from the other groups (Thimmappa et al., 2014).

Triterpenoids are further subdivided into several groups based on their structures and this include acyclic, monocyclcic, bicyclic, tricyclic, tetracyclic, pentacyclic and hexacyclic

all of which have been successfully obtained naturally. Pentacyclic and tetracyclic are however the two main groups (Thimmappa et al., 2014).

Examples from the two main groups include oleandrin, euphol and cucubitacins for tetracyclic and by far the largest group which is the pentacyclic triterprnoids has lupane, oleane, taraxastane, ursane, friedelane, and serratane (Banerjee et al., 2019).

2.5.3.2 Pentacyclic triterpenoids

Pentacyclic triterpenes are secondary plant metabolites which arise from the cyclization of squalene (Laszczyk, 2009). Pentacyclic triterpenoids have mostly the conformation 6-6-6-6-5 and 6-6-6-6-6 (Thimmappa et al., 2014). Pentacyclic triterpenoids just like other triterpenoids have 30 carbon skeleton atoms but are distinguished by 5 rings structure that are interconnected (Nistor et al., 2023).

Pentacyclic triterpenes are divided into different groups which include ursane, oleanane and Lupane (Fig 2.4(1)) depending on the structure of each. Ursane has constituents such as uvaol, α-amyrin and ursolic acid, Oleanane which contains erythrodiol oleanolic acid and ß-amyrin (Fig 2.4(2)) and the most common lupane which contains betulinic acid, betulin and lupeol (Nistor et al., 2023).

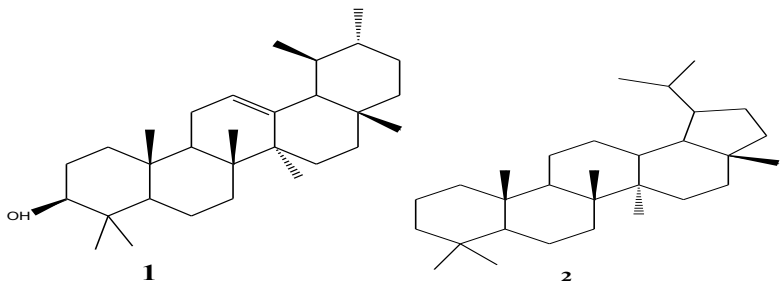

Figure 2.4: (1) α-Amyrin and (2) Lupane structures (Nistor et al., 2023).

2.5.3.3 Lupeol biosynthesis

Lupeols just like other triterpenoids are made up of the C5 units which are the foundation of the Lupeol structure. These C5 units stem from, isopentenyl diphosphate and dimethylallyl diphosphate that undergoes a biosynthetic pathway known as the Mevalonate pathway (Sawai & Saito, 2011).

The condensation of two IPP molecules with DMAPP results in the formation of FPP. Two FPP molecules are condensed to a C30 compound structure squalene that is then epoxidised to 2,3-oxidosqualene (Valenzuela et al., 2017). The next step is the cyclization reaction of 2,3-oxidosqualene which is catalyzed by the enzyme oxidosqualene cyclase, OSC and this reaction occurs in plants, fungi and animals *(*Thimmapp*a* et al., 2014).

Some of the classes of oxidosqualene cyclases are multifunctional and their involvement in the cyclization reaction of the 2,3-oxidosqualene gives rise to a wide range of triterpene scaffold structures *(*Thimmapp*a* et al., 2014). In the case of lupeol the enzyme responsible for its formation is known as Lupeol synthase which is a member of the oxidosqualene cyclases and it converts the 2,3-oxidosqualene via the chair-chair-chair substrate conformation (Gallo & Sarachine, 2009).

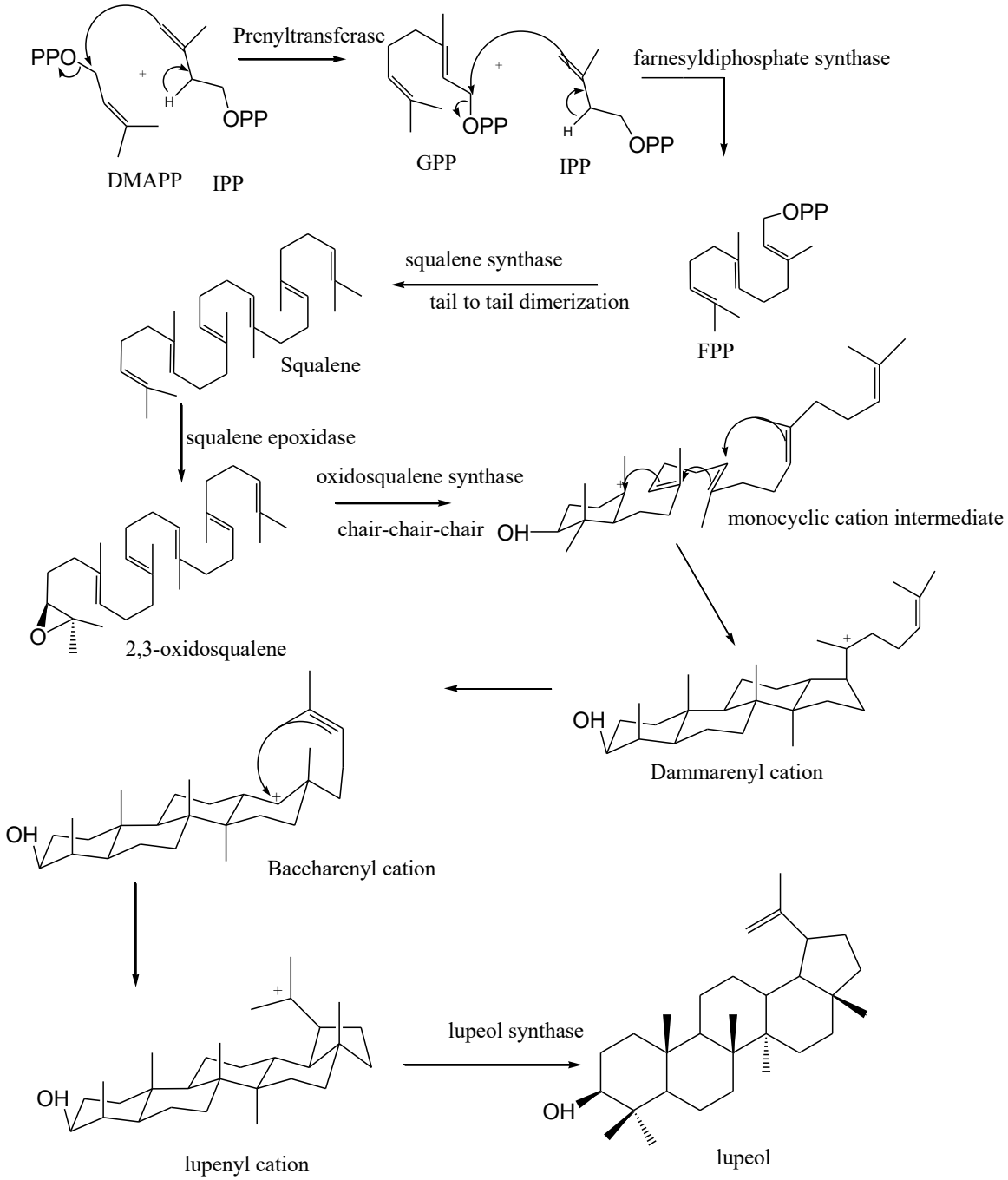

Figure 2.5: Mechanism of lupeol biosynthesis (Gallo & Sarachine, 2009).

The process of lupeol synthase-catalyzed cyclization of 2,3-oxidosqualene involves several steps beginning with substrate folding and then consecutive electrophilic additions that result in intermediate dammarenyl cation (Fig 4.4). The dammarenyl cation then undergoes reorganization that encourages ring enlargement to facilitate the formation of the baccharenyl cation intermediate. Electrophilic addition reaction on the baccharenyl cation yields the lupenyl cation. The lupenyl cation intermediate undergoes deprotonation of the methyl group at position 29 and this transforms it into the final product lupeol (Gallo & Sarachine, 2009).

2.6.1 Biological importance of terpenoids

One of the most important characteristics that entices researchers into extensive investigation of natural products is the prospective contribution to human ecology (Pichersky & Raguso, 2016).

Terpenoids have alterations in their chemical structures that make them have potent biological activity and therefore good commercial viability. These alterations make them useful as anticancer, anti-inflammatory, antifungal, antibacterial and antihypertension agents among others (Perveen & Taweel, 2018).

The altered structures also make them useful in other applications such as food in flavourings, pigments, cosmetics, pesticides and herbicides (Perveen & Taweel, 2018). It is widely known that terpenoids are known to be part of or major contributors to the production of flavours and scents in the plants that synthesize them. The monoterpene

limonene is responsible for the scent of lemons while the scent of pine trees is due to the emission of the monoterpene pinene (Brian, 2023).

Terpenoids also perform their duty as deterrents against animals that may want to consume the plant. The bitter taste and strong odour that discourage animals from consuming plants like in the case of oregano and thyme are due to terpenoids (Brian, 2023). Another form of defense executed by terpenoids is allelopathic action where the terpenoid is released into the environment around the plant creating unfavorable conditions for rival plants. Exploiting allelopathic properties of terpenoids could result in creating environmentally friendly herbicides (Perveen & Taweel, 2018).

One of the most recognisable drugs in sub-Saharan Africa that has had great success in malaria treatment is sesquiterpene lactone. This sesquiterpene is extracted from the plant *Artemisia annua* and it is known as artemisinin. The various modified forms of artemisinin have been very effective in the control of malaria because of their strong antimalarial capacity (Perveen & Taweel, 2018).

The diterpene taxol which is extracted from the plant *Taxus baccata* has been a good natural antineoplastic due to its ability to inhibit cell division. Aromatherapy is gaining popularity because of promotes physiological health and terpenoids are the compounds of choice in that field (Brian, 2023).The most common application of terpenoids by man is through oral consumption of plants rich in terpenoids. Terpenoid carotene present in

carrots are instigators in the synthesis of vitamin A that improves eyesight (Pichersky & Raguso, 2016).

2.6.2 Structure vs antimicrobial activity relationship of pentacyclic triterpenes

Pentacyclic triterpenes are currently a very resourceful class of natural products because they contain an abundance of compounds that are important in new drug development (Dzhemileva et al., 2023). Pentacyclic triterpenes have a high potential for conversion into synthetic or semi-synthetic drugs due to the presence of readily transformable functional groups (Dzhemileva et al., 2023). They have the natural chemotherapeutic characteristic of low toxicity and the 5-ring backbone which is lipophilic and may have magnified activity and accurate selectivity depending on substituents attached to it (Mallavadhani et al., 2004). Due to the good ADMET, pentacyclic triterpenoids are considered leading natural compounds with regard to new drug development (Mallavadhani et al., 2004).

Figure 2.6 below shows 3 possible variations of lupane derivatives with interchangeable functional groups at sites labelled R, R1 and R2.

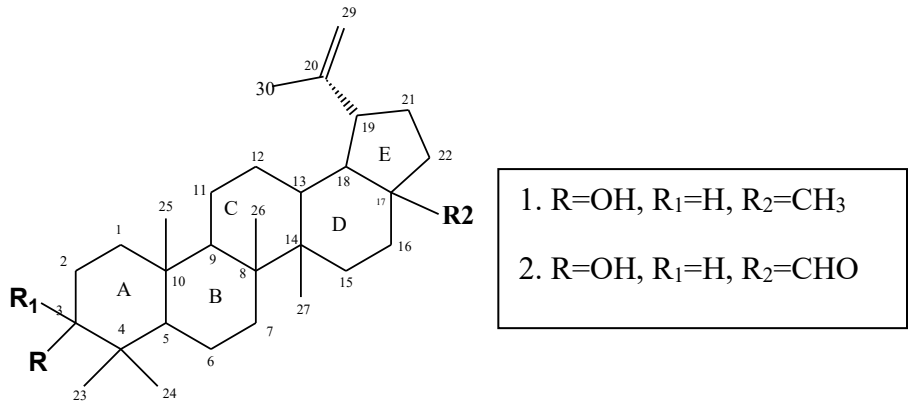

Figure 2.6: Lupane derivatives. (Mambu et al., 2006).

The structure-activity relationship of pentacyclic triterpenes showed the oxygen moiety at position C3 inferred bactericidal activity against both gram-positive and gram-negative bacteria. The oxygenated group at C3 could be any of the known functional groups such as glycosido, hydroxyl, ester, or carbonyl. A carboxyl group at C17 or C20 gives around 80 % antibacterial activity (Pacheco et al., 2012).

The presence of hydroxyl and its lipophilicity also improves the bacterial function because it enables the compounds attack the polar heads and the lipophilic tails of microbe membrane and this negatively affects the polar transmembrane passageways and lipodial intermembrane (Mahizan et al., 2019).

A large portion of biologically active triterpenes have π-bonding at carbon positions 5, 6, 9, 11, 12 and 13. A small portion have π-bonding at carbon positions 20, 21 and 30. Studies have shown that the absence of π-bonding has an impact on the antimicrobial potential of a triterpene because very few biologically active triterpenes lack π-bonding (Pacheco et al., 2012). It has been observed that the functional groups of the biologically active triterpenes are located in the A and E rings and with π-bonding. All these have an impact on the degree of antimicrobial potential of the triterpene (Pacheco et al., 2012).

2.7.1 Descriptions of bacteria that were used in the study

Staphylococcus aureus is a gram-positive bacterium that has a spherical shape, non-spore-forming and is non-motile with the colonies having a characteristic golden-yellow appearance. *Staphylococcus aureus* is commensal and it is estimated to inhabit roughly 30 % of a healthy person's various body areas. *Staphylococcus aureus* is responsible for

post-surgery pyogenic infections and this is due to possessing virulent genes (Rasheed & Hussein, 2021).

Staphylococcus aureus has been observed to have antibiotic-resistant strains most notable examples being Vancomycin Resistant *S. aureus* (VRSA) and Methicillin Resistant *Staphylococcus aureus* (MRSA). One of the biggest challenges facing the epidemiology of *S. aureus* is the circulation of this bacteria within healthcare centers (Rasheed & Hussein, 2021).

Klebsiella pneumoniae is an encapsulated non-motile gram-negative bacterium that is usually found in the soil, on medical equipment and water surfaces. In humans, it usually colonizes the mucosal linings, oropharynx and gastrointestinal lining. They use these locations as starting points to invade other tissues in the human body (Effah et al., 2020).

Klebsiella pneumoniae is responsible for a wide range of illnesses such as pneumonia, urinary tract infections, endocarditis, cystitis, pyogenic liver abscesses and septicemia (Paczosa & Mecsas, 2016). In the last decade, there has been a rise in resistance to strains from *K. pneumoniae* such that its infections have become life-threatening. The mechanism of resistance involves the production of enzymes that deactivate the antibiotics making the bacteria resistant (Effah et al., 2020).

Pseudomonas aeruginosa is a gram-negative rod-shaped anaerobic bacterium that has green-blue-coloured colonies. *Pseudomonas aeruginosa* is found in water, animals, soils and plants. It has a preference for moist or humid environments and in hospitals it is found in respiratory equipment, sinks, and mops, and can be brought to health facilities by visitors who bring fruits and vegetables (Lodise et al., 2019).

Pseudomonas aeruginosa is not commonly found in the healthy human body with presence far below 10 %. Persons having greater than 10 % presence in areas such as nasal cavity, throat and skin infections by highly virulent strains are associated with high morbidity and death. The resistance mechanism involves outer membrane permeability alteration, efflux pumping, antibiotic deactivation enzyme production and target site alteration (Lodise et al., 2019).

Streptococcus pyogenes are non-motile gram-positive spherical or ovoid in shape and are facultatively anaerobic and grow in grey-white-coloured colonies (Nizet & Arnold, 2018). They can be found widespread in the human body and usually, these are commensal. There are however virulent strains of *S. pyogenes* that are responsible for diseases like meningitis, dental carries, necrotizing fasciitis and pharyngitis. Meningitis and necrotizing fasciitis are life-threatening and hence need greater attention and need wide range of possible chemotherapeutic remedies (Ghasemi et al., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

This section involves the practical process of the research and commenced from sample collection to compound extraction then isolation and purification of the crude extract followed by structure elucidation and antimicrobial activity tests. The crude extract also underwent qualitative analysis.

3.2 Sample Collection and Preparation

The fresh leaves of *V. glandulosa* were collected from Muguga South Forest Reserve (-1.2154652620858173, 36.63341878695118) (Fig 3.1). They were carefully packed and transported to the Chemistry laboratory at the University of Kabianga for storage. Authentication of the plant sample was done by a taxonomist at the University of Kabianga.

The plant sample was then cleaned in running tap water, chopped into smaller pieces, and air-dried at room temperature for two weeks. The sample was ground to powder using an electric grinder at the Chemistry laboratory and stored in airtight containers awaiting extraction.

Figure 3.1: A map showing the collection site

3.3 Cold Extraction of the Crude Leaf Extract

Cold extraction was used in obtaining the crude extract where; leaves were crushed using electric grinder then 1.4 kg of the crushed leaves was weighed and then soaked with 1.5 litres of methanol for 3 days at standard temperature and pressure with gyration every 12 hours. Decantation using filter paper (Whatman No.1) was done to obtain crude methanol extract.

The resulting filtrate was concentrated under reduced pressure to a minimum volume using a rotary evaporator and left to dry to a constant weight at standard temperature and pressure in a pre-weighed beaker. Weighing was done using an analytical balance. The weight of the extract was determined by calculation of the difference in weight of the beaker with extract minus the weight of the empty beaker.

3.4 Qualitative phytochemical analysis of *Vepris glandulosa* crude leaf extract

Various qualitative tests were done on the crude extracts to determine the phytochemical make up. Assays were done to determine presence of saponins, tannins, flavonoids, alkaloids, phenol and terpenoids.

3.4.1 Test for Saponin

Using a water bath, 5 ml of prepared crude extract was boiled in 20 ml of distilled water. After filtration, 10 ml of the filtrate obtained was mixed with about 5 ml of distilled water and strongly gyrated to obtain a steady consistent froth, 3 drops of olive oil were introduced to the froth and shaking was done vigorously (Murtiningsih et al., 2023).

3.4.2 Test for Flavonoids

To 5 ml of crude extracts, 3 ml of 1 % Aluminium chloride solution was added. To further confirm the presence of flavonoids, 5 ml of dilute ammonia solution was added followed by concentrated H_2SO_4 (Suyatno et al., 2021).

3.4.3 Phenol

To 10 ml of distilled water was put into a 30 ml test tube followed by about 5 ml of the crude extract. The test tube remained undisturbed for ½ hour after the addition of 2 ml of ammonium hydroxide and concentrated amyl alcohol 5 ml. The time was to allow reactions to occur to completion (Shaikh & Patil, 2020).

3.4.4 Alkaloids

To 1 ml of crude extract was measured and mixed with 5 ml of 1 % aqueous hydrochloric acid in a steam bath. It was filtered hot with the addition of distilled water to the residue. A few drops of Wagner's reagent was added to 1 millimeter of the filtrate (Shaikh & Patil, 2020).

3.4.5 Test for Terpenoids

To 5 ml of the crude extract, 2 millimeters of chloroform and 3 millimeters of concentrated Sulphur acid were carefully added to form a layer (Malik et al., 2017).

3.4.6 Test for Tannins

Exactly 2 ml of crude extract was measured and put into a clean test tube. A few drops of ferric chloride were added then the test tube contents were shaken vigorously (Shaikh & Patil, 2020).

3.5 Isolation and purification of compounds from *V. glandulosa* leaves

Isolation and purification of the crude extracts was done using repeated column and thin layer chromatography with solvent system of ethyl acetate /hexane.

3.5.1 Column Chromatography

A column was thoroughly cleaned and air dried for 24 hrs then a small chunk of cotton was inserted and pushed with a clean glass rod to point near the tap opening. A suitable stable tripod stand was identified then the column was clamped vertically. Silica gel of mesh 70-230 and 0.063-0.2 mm particle size was chosen and 400 g of it was put in a beaker. Hexane was added to the beaker with 400g silica gel with constant stirring to form a slurry that had no locked air bubbles.

This slurry was carefully packed in the column and degassed by running hexane repeatedly until the silica in the column stabilized. The crude extract was carefully poured into the column and it formed a green layer just above the silica gel. The commencing mobile phase was prepared at a concentration of 2 % ethyl acetate in 98 % hexane.

Calculation of preparation of 2 % Ethyl acetate:98 % Hexane solvent system.

An arbitrary starting total solvent system required was chosen to be 150 ml, therefore calculating 2 % of the target total.

i. 2% of 150 ml

$$= \frac{2}{100} \text{x } 150\text{ml} = \text{ 3 ml of ethyl acetate}$$

ii. volume of hexane required

$$\text{total volume required} = 150\text{ml}$$

$$\text{calculated volume of ethyl acetate} = 3\text{ml}$$

$$\text{volume of hexane} = 147\text{ml}$$

3 ml of the Ethyl acetate was obtained in a measuring cylinder and then carefully poured into a volumetric flask, to make the 150 ml, 147 ml of hexane was added.

The 147 ml of hexane was also carefully poured into the volumetric flask containing the 3 ml Ethyl acetate and the contents gyrated to ensure proper mixing of the solvent system. The same procedure was repeated for subsequent solvent systems with increasing polarity.

The solvent system constitutes the mobile phase and the packed silica gel in the column is the stationary phase. The column was eluded until constant clear eluent was observed and at this point, the column was allowed to run until nearing the stationary phase to exhaust the existing solvent system. The next solvent system with a different composition was then introduced and for uniformity, the 1st two 250 ml eluents were considered part of the preceding solvent system.

The 250 ml eluents were assembled and put in a fume hood overnight so that the solvents were distilled. TLC analysis was performed to the concentrated fractions and this guided the subsequent steps that followed. This procedure was repeated until the conclusion of

the column chromatography. The procedure was stopped at 40 % Ethyl acetate to 60 % Hexane because there were no more observable colour changes.

3.5.2 Thin Layer Chromatography (TLC)

Silica gel plates (Merck 0.20 mm) of dimensions 20 cm by 5 cm were drawn with a baseline of 0.5 cm. Spots were drawn at equidistance spacing of a minimum of 1 cm to allow clear reading of results. The different fractions were carefully spotted on the baseline using a capillary tube. A clean developing tank was saturated with 10 ml of the prepared solvent system by swirling thoroughly when covered with a lid and then allowed to stand for a few minutes.

The plates were placed in the developing tank with the baseline just above the solvent level and then allowed to develop until the solvent reached the solvent front. The developed TLC plates were visualized by a UV lamp set at wavelengths of 254 nm and 365 nm and then sprayed with recently prepared *p*-anisaldehyde solution before heating in an oven at 115 °C. Fractions with similar retardation factor, R_f, were pooled.

The fractions were further pooled with guidance from TLC analysis, this was for bulking or getting a usable quantity of sample. The vials containing the pooled pure samples were clearly labelled and left to dry in a fume cupboard.

3.6 Structural elucidation of compounds isolated from *Vepris glandulosa* leaves

Nuclear Magnetic Resonance test was carried out to propose the chemical structure of the isolated compound.

3.6.1 NMR spectroscopy

^{1}H NMR, ^{13}C NMR as well as 2D NMR were performed at the University of Surrey laboratories. The deuterated solvent was used to dissolve the sample pure sample and transferred to properly labelled sample tubes for easy identification. The sample tubes were put in a sample holder and fitted in a depth gauge to confirm the appropriate depth in the probe.

The sample tube was wiped on the outer surface to remove any contamination that would get into the probe. On the computer, the compressed air was switched on to momentarily suspend the tube and then switched off to allow automatic lowering into the centre of the 400 MHz NMR magnet. Once the sample was inside, the parameters were set on the computer. The spectra obtained were interpreted and the carbon skeleton as well as the nature of protons obtained were used in structure elucidation.

3.7 Determination of antibacterial activities of crude extracts

Three antibacterial assays were done including zone of inhibition, minimum inhibitory concentration and minimum bactericidal concentration to determine the potential as an antibacterial. Observations for the two assays were done after 24 hours and the results from the Minimum Inhibitory Concentration were used to set control parameters for the Minimum Bactericidal Concentration test.

3.7.1 Zone of inhibition test

The four bacteria available at the University of Kabianga Microbiology laboratory were American Type Culture Collection (ATCC) 29213 *Staphylococcus aureus,* National Collection of Type Cultures (NCTC) 13368 *Klebsiella pneumoniae, Streptococcus pyogenes* and *Pseudomonas aeruginosa.* The control was commercially available amoxillin. The assay began with sub culturing of these bacterial colonies at 37^{o} C for 24 hrs to have sufficient bacterial colonies for the subsequent experiments.

Mueller hinton agar was prepared following the manufactures instruction by weighing 7.6 g of the agar in a conical flask then 200 ml of distilled water was added. The conical flask was covered with cotton wool and was then put on a magnetic stirrer set at 380 ^{o}C and 600 revolutions per minute for homogenous mixing after which it was placed in an autoclave at a temperature of 121 ^{o}C for 15 minutes then allowed to cool for 20 minutes. It was then placed inside a laminar flow chamber together with 16 sterile Petri dish. The molten agar was then poured on each dish inside the laminar flow chamber then allowed to cool and solidify under uv radiation inside the chamber to reduce chances of contamination.

The 16 petri dish were each streaked with bacteria with 4 dish for each of the 4 bacterial colonies of ATCC 29213 *Staphylococcus aureus,* NCTC 13368 *Klebsiella pneumoniae, Streptococcus pyogenes* and *Pseudomonas aeruginosa.*

The crude extract was prepared for impregnation by mixing with small amount of methanol then shaken vigorously to form a solution. The 6 mm disks were then soaked in the solution and then placed onto the prepared petri dish with the Mueller Hinton agar. The disks with the crude extracts were placed on 12 of the petri dish meaning each of the 4 chosen bacterial colonies had 3 crude extract disks. The remaining four disks were for the Amoxillin that was placed one for each bacterial colony. The 16 petri dish were put inside incubation chamber set at 37 ^{o}C and measurements were recorded after 24 hours and 48 hours respectively.

3.7.2 Minimum Inhibitory Concentration Test

The procedure began with assembly of 10 clean sterile test tubes which were placed on 2 test tube racks each with five test tubes for each rack. Five test tubes were used for *Pseudomonas aeruginosa* and the remaining five for *Staphylococcus aureus.* The other bacteria were of *Klebsiella pneumoniae* and *Streptococcus pyogenes* were out of stock at the time of the procedure. All the ten test tubes were put 10 ml of distilled water but then addition of liquid crude extract with varying concentration was done on 8 out of the 10 test tubes. Of the 8 test tubes, two test tubes were put 2 µ/ml, another two 4 µ/ml, another two 6 µ/ml and the last two 8 µ/ml.

The ten of test tubes were each inoculated with bacteria, five for *Pseudomonas aeruginosa* and five for *Staphylococcus aureus* then allowed time to grow. The 2 test tubes out of ten with distilled water only were the controls for the experiment. Observations were made after 24 hours. The level of turbidity of the test tubes compared

to the positive control indicated bacteriostatic strength of the concentration of crude extract and complete absence was indicative of bacteriostatic action.

3.7.3. Minimum Bactericidal Concentration

Results from the Minimum inhibitory concentration test were used to determine the parameters for the minimum bactericidal concentration test. The concentration preceding the minimum inhibitory concentration was chosen as the control for the assay and 2 µ/ml for *S. aureus* and 4 µ/ml for *P. aeruginosa*. 8 sterile petri dish were swabbed with bacteria used in the minimum inhibitory concentration test.

Four petri dish were streaked with *Staphylococcus aureus* and another four with *Pseudomonas aeruginosa.* The streaked petri dishes were impregnated with the varying concentration of the crude extracts then put in the incubator for 24 hrs to allow bacterial growth. The turbidity of the petri dish indicated bacterial growth and absence indicated bactericidal effect of the concentration of crude extract.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Introduction

In this chapter, all the results obtained from chapter three were tabulated and analyzed through comparison with other related literature obtained by other researchers. The analysis gave better insight into the significance of the research after comprehensive discussion of the results.

4.2 Cold Extraction Results

Crude was extract obtained in line with objective one and below are calculations of the quantity obtained.

Weight of the beaker with extract	186.15 g
Weight of the empty beaker	28.92 g
Weight of the crude extract	157.23 g

The crude extract was then divided into two in readiness for the different assays to follow. The sample for qualitative analysis weighed 82.35 g while for isolation, purification, structural identification and antimicrobial assay weighed 74.35 g.

4.3 Qualitative analysis test

Below are the qualitative results carried out on the crude leaf extracts for *Vepris glandulosa* completing the requirements for satisfying objective one.

The qualitative analysis results showed that the leaves of the *Vepris glandulosa* are rich in in natural products and this is in line with the justification that because it belongs to the Genus *Vepris*, the probability of presence of large number of natural products is high.

Table 4.1:

Results for qualitative analysis of the crude leaf extracts of Vepris glandulosa

Group of compounds	Observation	Conclusion
Saponins	Formation of constant froth consistent with the presence of saponins and an emulsion after olive oil drops introduced	Presence of saponins
Tannins	The dark green precipitate formed after the addition of ferric chloride.	Presence of tannins
Alkaloid	There was the formation of reddish-brown colour	Presence of alkaloids
Flavonoid	Yellow colouration was seen consistent with the presence of flavonoid, then the disappearance of yellow colour after allowing time to stand following the addition of ammonia solution and concentrated Sulphur acid.	Presence of flavonoid
Terpenoids	Reddish-brown coloration of the interface	presence of Terpenoid

The results showed presence of Saponins, tannins, phenol, alkaloid, flavonoids and terpenoids are in agreement with several literature for example according to (Omujal et al., 2020) *V. nobilis* leaves contain alkaloids and terpenoids, according (Ngbolua, 2023) *V. ampody* leaves contain alkaloids, flavonoids and saponins.

The leaves of *V. nobilis* have antibacterial properties according to (Omujal et al., 2020) and also the leaves of *V. ampody* have antibacterial properties according to (Ngbolua, 2023), therefore because of *V. glandulosa* having almost similar compounds with the two, it shows why it is used as an antibacterial.

4.4 Isolation and purification results

Isolation and purification was done using the repeated column and thin layer chromatography. Chromatography is a useful separation technique that relies on the differential adsorption of different components in a mixture to the stationary phase (Coskun, 2016).

Figure 4.1: Picture of column chromatography in progress

The figure above shows the column chromatography in process at 10 % ethyl acetate/90 % hexane. Below are the column chromatography results obtained after repeated thin layer and column chromatography.

Table 4.2
Column chromatography results

Fractions	Solvent system Ethyl Acetate: Hexane	Pooled fraction
1 – 2	2 %	CFA
3- 4	2 %	CFB
5- 8	2 %	CFC
9	5 %	CFD
10- 16	5 %	CFE
18- 20	10 %	CFF
21- 23	10 %	CFG
24- 30	10 %	CFH
31- 44	15 %	CFI
45-47	15 %	CFK
48- 49	15 %	CFL
50- 53	18 %	CFE2
54- 57	18 %	CFF2
58- 69	20 %	CFM
70- 71	20 %	CFN
72- 73	20 %	CFO
74- 76	20 %	CFP
77- 80	30 %	CFQ
81- 83	30 %	CFR
84	30 %	CFS
85-88	40 %	CFT
89- 92, 94- 102	40 %	CFU
93 & 103	40 %	CFV

Table 4.2 shows the various eluents from column chromatography. The eluents are roughly viewed to be different based on the fact they were extracted using different solvent systems of increasing polarity and colour differences. The eluents were then subjected to thin-layer chromatography which gave a clear picture of exactly which eluents had similar compounds that could be pooled together.

Thin layer chromatography is a separation technique that involves the liquid or mobile phase travelling against gravity along a solid stationary phase which is coated with silica gel (Ebere et al., 2019). Thin-layer chromatography is a useful separation technique that

shows the number of components present in a mixture and guides the isolation of different constituents in it (Cai, 2014).

4.4.1. Pooled chromatography fractions

After repeated thin layer chromatography to determine similar compounds, pooling was done to increase the mass of the compounds which important to have enough of pure compound for further assays. Below is the table of results and followed by descriptions of the various fractions.

Table 4.3:
Pooled column chromatography results

Code	Source	Solvent system
DVG1	CFC	Extracted with 2 %
DVG2	CFE2,CFF2	Extracted with 18 %
DVG3	CFE, CFF, CFL2	Extracted with 10 %
DVG4	CFI, CFG	Extracted with 15 %
DVG5	CFH	Extracted with 10 %
DVG6	CFM, CFS, CFJ, CFN, CFO, CFP, CFQ, CFR, CFT	Extracted with 20 %
DVG7	CFU	Extracted with 40 %

Combined fraction CFA had fractions 1 and 2 which were determined to contain similar compounds based on thin-layer chromatography results. After the use of a rotary evaporator and allowed time to dry, the contents of the vial were observed to be sticking to the bottom of the vial, it was light yellow. It looked like a stick gum with some white-looking crystals suspended in its matrix.

CFB was constituted of eluents 3 and 4. After rotary evaporation and allowed time to dry, the contents of the vial were observed to be light green with similar gum-like features that had white crystal suspended in its matrix.

The vial labeled CFC constituted eluents 5-8 which were also determined to have similar chemical compounds based on thin layer chromatography results. The contents of the vial labelled CFC were observed to have a dark brown colour with gum-like characteristics. It had very little visible white crystal particles suspended on its matrix.

The vial labelled CFD contained eluent 9. The contents had all similar visible characteristics with the only difference being that it had a lighter shade of brown. Combined fraction CFE had fractions 10 to 16 that were determined to have similar chemical constitutions and it was the first vial with very interesting visible physical characteristics.

The contents of vial CFE had diverse physical characteristics ranging from a solid crystal-like substance to a thick gum-like liquid. The thick gum-like suspension remained suspended on the upper portion of the vial contents and it was stuck on the glass vial wall. This gum-like yellow suspension had white crystal-like particles in its matrix. At the bottom of the vial, there were only the white thread-like crystals.

Vial labelled CFF contained eluents 18 to 20 which were determined to have similar chemical constituents after thin layer chromatography analysis. The visible characteristics were almost similar to that of vial CFE but with very little yellow gum-like liquid stuck on the walls of the glass vial and more of the white threadlike crystals at the bottom of the glass vial with some white powder.

The vial labelled CFG contained pooled eluents 21 to 23. The observed characteristic was an increase in the yellow gum-like liquid stuck to the vial wall compared to vial CFF. The was a visible decrease in the quantity of white crystal-like substances. The shape of the crystals looked different they were short with less clustering with the presence of white powder mixed with the crystals, the crystals were amorphous in shape.

The vial labelled CFH contained fractions number 24 to 30. The vial contents were observed to be white sugar-like crystals with very little visible white powder present.
The vial labelled CFI contained fractions 31-44 all of which were observed to have dark brown-coloured gum-like suspension sticking to the bottom of the glass vial. This gum-like suspension had white-looking sugar-like crystals within its matrix.

The vial labelled CFJ had fractions number 45 to 47 and was observed to have a dark green paste-like sticky substance with no visible white crystal-like or powder substances present. The subsequent vials labelled CFK, CFL, CFM, CFN, CFO, CFP, CFQ, CFR, CFS and CFT all had similar visible characteristics to CFJ.

The process of column chromatography was stopped at 40 % ethyl acetate and 60 % hexane because there were no more colour visible colour changes from the eluents. The decision to stop the process was also supported by thin-layer chromatography results that showed all the latter eluents had similar chemical constituents.

The results from Table 4.3 showed that several of the seemingly different chemical constituents from the different vials had similar compounds based on similar thin-layer chromatography results. The vial labelled DVG6 had the most pooled vials that is 9 vials pooled together. Vials labelled DVG1, DVG5 and DVG7 had only one vial and no pooling was done to them. The vials labelled DVG2 and DVG4 had only two vials pooled while the vial labelled DVG3 had 3 vials pooled in it. DVG3 which had white thread like crystal substance proceeded to the next stage in of analysis and it labeled as compound 1.

Below are some of the pictures 1 and 2 showing vials containing CFE and CFF respectively. The physical characteristics of the contents in the vials are white crystal threadlike compounds sticking to the glass. Picture 3 shows the results from thin layer chromatography analysis that clearly showed CFE, CFF, and CFL2 are similar compounds and they should be pooled.

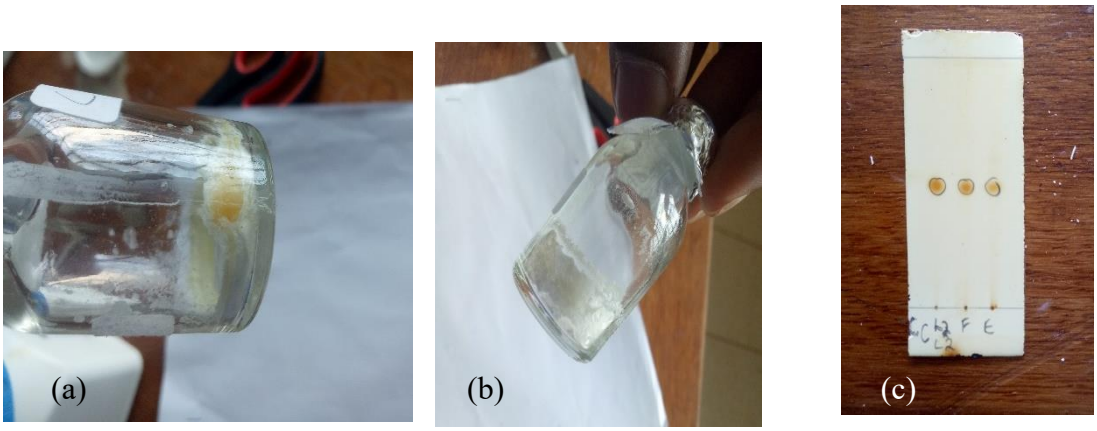

Figure 4.2: Images of isolated compounds (a) CFE & (b) CFF and Thin layer chromatography results for (c) CFF, CFE, CFL 2.

The pooled compound DVG3 which contained CFF, CFE, and CFL2 extracted at 10 % Ethyl acetate/ 90 % Hexane was denoted compound 1**.** Compound 1 was subjected to ^{13}C NMR ($CDCl_3$, 400 MHz) and ^{1}H NMR ($CDCl_3$, 400 MHz) and 2D NMR analysis.

4.5 Nuclear Magnetic Resonance Results and Discussion

The ^{1}H NMR ($CDCl_3$, 400 MHz) δ:4.57 (s, H-29a), 4.68(s, H-29b), 3.20 (1H, dd, J= 5.12; 11.27 Hz, H-3), 1.68, 1.02, 0.98, 0.96, 0.82, 0.76, 0.70 (21 H, 7 s, 7 CH3).The ^{1}H NMR spectrum showed seven methyl singlets at δ_H 0.70, 0.76, 0.82, 0.96, 0.98, 1.02, 1.68, and one secondary hydroxyl group as a doublet of doublets at δ_H 3.20.

It also showed two olefinic protons at δ_H 4.57 and 4.68 representing the exocyclic double bond. Integration showed a total of 50 Hydrogen atoms in compound 1.

The ^{13}C NMR ($CDCl_3$, 400 MHz) results were as follows δ: 37.16 (C-1), 27.40 (C-2), 79.01 (C-3), 38.71 (C-4), 55.29 (C-5), 18.00 (C-6), 34.28 (C-7), 42.82 (C-8), 50.43 (C-9), 35.58 (C-10), 20.93 (C-11), 25.13 (C-12), 38.05 (C-13), 43.00 (C-14), 27.44 (C-15), 40.00 (C-16), 40.83 (C-17), 47.98 (C-18), 48.30 (C-19), 150.95 (C-20), 20.84 (C-21), 38.85 (C-22), 27.99 (C-23), 15.37 (C-24), 16.12 (C-25), 15.97 (C-26), 14.55 (C-27), 18.32 (C-28), 109.33 (C-29), 19.31 (C-30).

The ^{13}C-NMR spectrum of the compound showed that there were 30 carbon signals.

The structure has a terpenoid of lupane skeleton that has a carbon bonded to the OH group at the C-3 position appearing at δ_C 79.01 ppm and at this point it was proposed that the molecular formula is $C_{30}H_{50}O$.

The olefinic carbons of the exocyclic double bond appeared at δ_C 150.95 ppm as a quaternary carbon and δ_C 109.33 ppm as methylene carbon which were assigned as C-20 and C-29 double bond of the lupane type triterpenoid compound.

The molecular formula of compound 1 was found to be $C_{30}H_{50}O$ hence a lupane type pentacyclic triterpenoid features, the results of the chemical shifts of compound 1 were compared against known chemical shift of lupeol in table 4.4.

The 30 signals were identified as 11 methylene, 7 methyl, 6 quaternaries and 6 methine carbons using DEPT spectrum data. The figure 4.3 shows the spectrum of ^{13}C NMR placed at lower part in comparison with DEPT 135 spectrum on upper part of the spectra. The DEPT 135 spectrum identified the 11 methylene which were the signals facing downwards while the methyl and the methine signals only were facing upwards. The quaternary signals did not appear on the DEPT 135 but through comparison with ^{13}C NMR spectrum they were identified as 6.

DEPT 90 data showed only 6 methine signals and these compared with DEPT 135 signals helped to identify the 7 methyl signals. All these are characteristic of lupane type pentacyclic triterpenoid. Further assignment of all protons and carbons was confirmed by ^{1}H-^{1}H COSY and long range HMBC Spectrum data. Therefore, compound 1 was assigned the IUPAC name 3-lup-20(29)-en-3-ol or lupeol, molecular formula $C_{30}H_{50}O$ and molecular weight of 426.7 gm/mol.

Table 4.4:
Results for the chemical shifts of ^{13}C NMR done at 400MHz in $CDCl_3$ for compound 1 compared with known ^{13}C NMR of Lupeol

S/NO	Chemical shift of compound 1 δ_C	Chemical shift of Lupeol (Shwe et al., 2019) δ_C
C-1	37.16	38.85
C-2	27.40	27.40
C-3	79.01	79.01
C-4	38.71	38.69
C-5	55.29	55.28
C-6	18.00	17.99
C-7	34.28	34.26
C-8	42.82	40.82
C-9	50.43	50.42
C-10	35.58	37.16
C-11	20.93	20.92
C-12	25.13	25.12
C-13	38.05	38.04
C-14	43.00	42.82
C-15	27.44	27.40
C-16	40.00	35.57
C-17	40.83	47.98
C-18	47.98	48.29
C-19	48.30	47.98
C-20	150.95	150.99
C-21	20.84	29.83
C-22	38.85	39.99
C-23	27.99	27.98
C-24	15.37	15.36
C-25	16.12	16.11
C-26	15.97	15.96
C-27	14.55	14.54
C-28	18.32	18.31
C-29	109.33	109.31
C-30	19.31	19.29

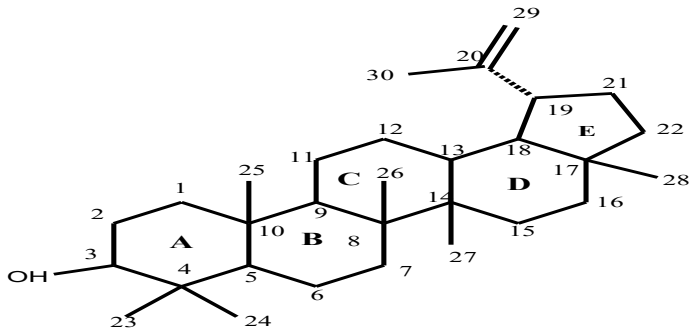

Figure 4.3: Proposed structure for compound 1 identified as Lupeol

4.5.1. Lupeol

Lupeol is a derivative of Lupane under the class of pentacyclic triterpenes. It is a common constituent of grape, hazelnut and olive oils, cocoa butter, mango pulp, white cabbage, and a variety of therapeutic plants (Son, 2022). It is usually easily identifiable by proton nuclear magnetic resonance and carbon magnetic resonance data that shows olefinic protons at 4.68 and 4.57 for the ^{1}H NMR and olefinic carbons 109.6 and 151.1 for the ^{13}C NMR (Gallo & Sarachine, 2009).

The results from the ^{1}H NMR and ^{13}C NMR are characteristic values of Lupane-type pentacyclic triterpene. The hydroxyl proton at 3.9 and carbon at 79.0 and the seven singlets attached to the tertiary groups at 0.77, 0.80, 0.84, 0.95, 0.97, 1.03, 1.20 for the protons values and 28.4, 15.8, 16.5,16.3, 14.9, 18.9, 19.7 for the ^{13}C NMR values all point to Lupeol being characteristic pentacyclic triterpene of Lupane type (Gallo & Sarachine, 2009).

Lupeol has been successfully isolated from *Zanthoxylum gilletii* a member of the Rutaceae family and used for conducting anticancer assays (Nyaboke et al., 2018). Lupeol has also been extracted from the plant *Vepris amanuensis* which is in the same genus as *V. glandulosa* (Magadula et al., 2008).

Lupeol has been seen to have biological activity as an antimicrobial, anticancer, antioxidant, antidiabetic and anti-cardiovascular remedies (Rao et al., 2016). A research analysis carried out by Gallo and Sarachine on the antiprotozoal potential of Lupeol revealed it had good activity against *Plasmodium falciparum* in vitro. The in vitro antiplasmodial activity was suggested by Gallo and Saccharine as being due to the ease of C28 hydrogen donation (Gallo & Sarachine, 2009). It has been used to augment antimicrobial activity of other antibiotics against the methicillin-resistant Staphylococcus aureus (MRSA) (Sharma et al., 2020). Lupeol has also shown that it has antiviral activity against herpes and dengue diseases (Sharma et al., 2020).

4.6. Zone of inhibition assay results

The zone of inhibition test is also known as the Kirby-Bauer test. The zone of inhibition test can also be described as a disc diffusion antibacterial sensitivity test and is used in medical healthcare facilities to test whether an antibiotic is effective against a bacterial pathogen affecting a patient (Bhargav et al*.,* 2016).

After 24 hrs the it was observed that the crude extract had an average of 11.00 mm zone of inhibition which was greater than the average of 8.00 mm for amoxillin for *K.*

pneumoniae while for *S. pyogenes* the crude extract fell short with 12.00 mm average for crude extract while 30.00 mm for amoxillin. Amoxillin performed significantly better than the crude extract for *P. aeruginosa* with 40.00 mm average against the average 11.00 mm for the crude extract. For the *S. aureus* the crude extract and amoxillin and the crude extract produced relatively similar results after 24hrs with zone of inhibition of 12.33 mm and 13.00 mm respectively.

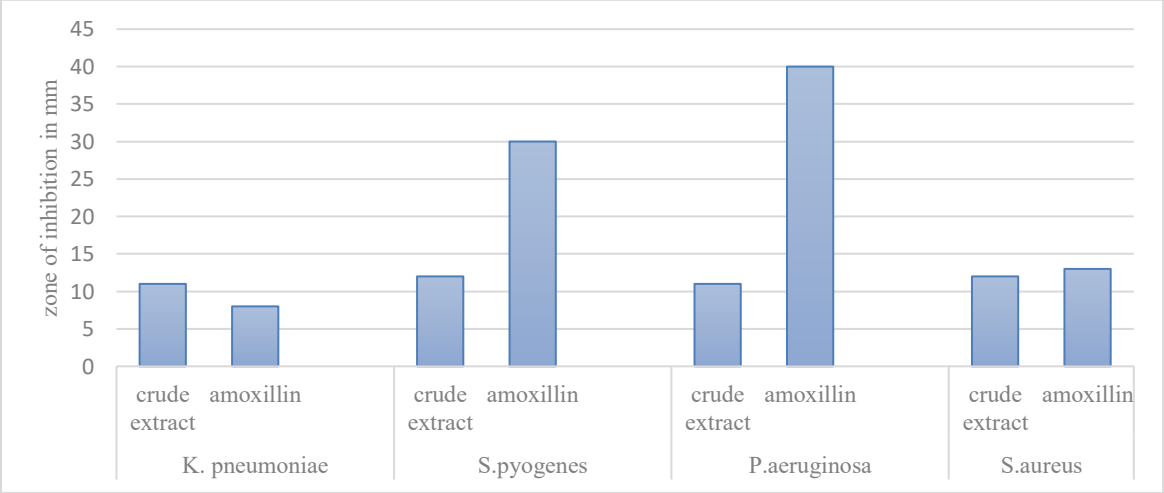

Figure 4.4: Zone of inhibition assay results after 24 hours

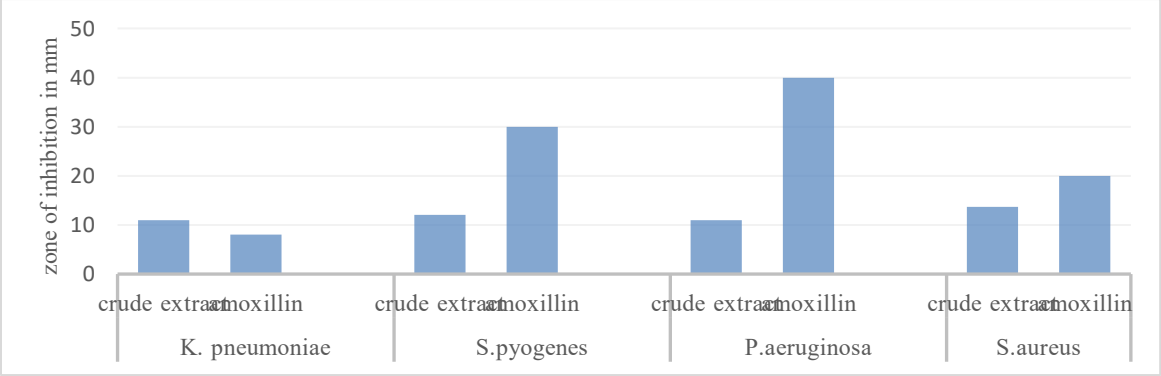

Figure 4.5: Zone of inhibition assay results after 48 hrs

After 48 hrs all the zone of inhibition values remained the same for *K. pneumoniae, S. pyogenes* and *P. aeruginosa.* The results for *S. aureus* were the only ones with changes in average disk diffusion values for both the crude extract and amoxillin. The zone of inhibition results for the crude extract changed from an average of 12.33 mm in 24 hrs to 13.66 mm in 48hrs while that of amoxillin changed from 13.00 mm in 24 hrs to 20.00 mm in 48 hrs.

Comparison with the known standards chart of the zone of inhibition of various antimicrobials helps to give a clear picture of the degree or level of potency of the compound concerning known commercial antibiotics and data can be used to determine the commercial viability of the compound.

Table 4.5 shows the standard table used to show antibiotic sensitivity and resistance status of various known antibiotics. In Table 4.5, the average zone of inhibitions from the triplicate assays was put for comparison with the commercial antibiotics. Zone diameter interpretive standards chart for the determination of antibiotic sensitivity and resistance status by the zone of inhibition method. From Table 4.5, the commercial amoxillin had better antimicrobial potency than the crude extract from the leaves of *V. glandulosa*. Commercial amoxillin showed sensitivity against *S. aureus, S. pyogenes* and *P. aeruginosa* with the zone of inhibition results of 20.00 mm, 30.00 mm and 40.00 mm respectively.

According to the table, it is implied that commercial amoxillin is best suited for the treatment of infections arising from *P. aeruginosa* because it had the highest zone of inhibition results of 40.00 mm while resistance was observed with *K. pneumoniae* that had a zone of inhibition of 8.00 mm and this implies that it would not be advisable to use commercial amoxillin as a chemotherapeutic drug against *K. pneumoniae.*

Table 4.5:

Comparison of zone of inhibition results of the crude extract and amoxillin after 48hrs with amoxillin standard values (Sarker et al., 2014).

Antimicrobial	Zone of inhibition in millimeters (mm)		
	Resistant	Moderately sensitive	sensitive
Amoxillin zone of inhibition ranges (Sarker et al., 2014)	≤ 13	14-17	≥ 18
Amoxillin against *S. aureus*			20.00
Crude extract against *S. aureus*		13.66	
Amoxillin against *K. pneumoniae*	8.00		
Crude extract against *K. pneumoniae*	11.00		
Amoxillin against *S. pyogenes*			30.00
Crude extract against *S. pyogenes*	12 .00		
Amoxillin against *P. aeruginosa*			40.00
Crude extract against *P. aeruginosa*	11.00		

The crude extract from *V. glandulosa* leaves performed better than amoxillin against *K. pneumoniae* producing a zone of inhibition of 11.00 mm (Table 4.5) while the commercial amoxillin produced an 8.00 mm zone of inhibition. The crude extract was moderately sensitive against *S. aureus* based on the literature from the table. It was

however noted that the crude extract showed resistance when introduced against *K. pneumoniae, S. pyogenes* and *P. aeruginosa.*

(a) (b)

Figure 4.6: (a) Image of petri dish with *K.pneumoniae* after 48 hours and (b) Image of *S. aureus* after 48 hrs

4.7. Minimum inhibitory concentration

Minimum inhibitory concentration is the least antibiotic concentration that will stop the visible growth of a microbe after overnight incubation (Andrews, 2001). From table 4.6 which has the Minimum Inhibitory Concentration results, for the *P. aeruginosa* the 2 µg/ml concentration of crude extract was insufficient as an inhibitor, the 4 µg/ml concentration was insufficient as well because both these test tubes were turbid indicating bacterial growth.

The observation of the 6 µg/ml concentration test tube showed it was clear indicating no bacterial colonies had grown. This therefore implied that 6 µg/ml was the minimum

inhibitory concentration. The 8 µg/ml concentrations of the crude extract had similar observable characteristics with the 6 µg/ml.

The *S. aureus* results showed that 2µg /ml had visible presence of bacterial colonies due to turbidity and thus 2 µg/ml concentration of crude extract was insufficient as a growth inhibitor. The 4 µg/ml concentration test tube had no observable bacterial colony present. The 4 µg/ml was thus noted as the minimum inhibitory concentration. The higher concentrations of 8 µg/ml and 6 µg/ml were by default sufficient to hinder growth and thus had similar visible characteristics as the control test tube solution.

Figure 4.7: Image of test tube racks with test tubes undergoing MIC

The table below are results from the Minimum Inhibitory Concentration assay taken after 24 hours.

Table 4.6:

Minimum inhibitory concentration results

Bacterial strain	concentration of crude extract µg /ml	Observation	Conclusion
P. aeruginosa	2	Growth	Insufficient as a bacterial inhibitor
	4	Growth	Insufficient a bacterial inhibitor
	6	No growth	Minimum inhibitory concentration
	8	No growth	Sufficient as a bacterial inhibitor
	0 (Distilled water)	Growth	Insufficient as a bacterial inhibitor
S. aureus	2	Growth	Insufficient as a bacterial inhibitor
	4	No growth	Minimum inhibitory concentration
	6	No growth	Sufficient as a bacterial inhibitor
	8	No growth	Sufficient as a bacterial inhibitor
	0 (Distilled water)	Growth	Insufficient as a bacterial inhibitor

4.8. Minimum bactericidal concentration

The minimum inhibitory concentration is the lowest concentration of an antimicrobial required to inhibit growth so therefore it is used to determine the concentration needed for bacteriostatic activity.

The minimum bactericidal concentration test is used to determine the lowest concentration that can eradicate bacteria completely and therefore this test will show the

lowest concentration for bactericidal activity (Owuama, 2017). The table below shows results for the Minimum Bactericidal Concentration assay taken after 24 hrs.

Table 4.7:

Minimum bactericidal concentration results

Bacteria	**Concentration of crude extract µg /ml**	**Observation**	**conclusion**
S. aureus	2	Bacterial growth	Insufficient as a bactericide
	4 (mic)	Bacterial growth	Insufficient as a bactericide
	6	Bacterial growth	Insufficient as a bactericide
	8	No bacterial growth	Minimum bactericidal concentration
P aeruginosa	4	Bacterial growth	Insufficient as a bactericide
	6(mic)	Bacterial growth	Insufficient as a bactericide
	8	No growth	Minimum bactericidal concentration

From Table 4.7 the results for the *S. aureus* showed bacterial growth in the 2µg/ml crude extract concentration as expected because it was chosen as the control based on the results from the minimum inhibitory tests. The 4µg/ml crude extract which was the minimum inhibitory concentration had bacterial growth on the petri dish showing there was still living bacteria in the solution.

The 6µg/ml crude extract concentration had bacterial growth on the plates thus it was concluded as not sufficient to cause bactericidal activity. It is important to note that from visible observation the area covered by the bacterial colonies decreased as the concentration of crude extract increased.

The visible area covered by the bacterial colonies decreased in the order 2 µg/ml> 4 µg/ml >6 µg/ml. The 8 µg /ml crude extract concentration had no bacterial growth on the petri dish and had similar visible characteristics to the control petri dish that did not have any bacteria streaked on its nutrient agar. This therefore implied that 8 µg/ml crude extract concentration of *V. glandulosa* leaf extract is the Minimum Bacterial Concentration for *S. aureus.*

The test results for *P. aeruginosa* showed visible bacterial growth in the plate having 4µg/ml crude extract concentration which was the positive control chosen based on the results from the minimum inhibitory test. The plate with the 6µg/ml crude extract concentration which was the minimum inhibitory concentration had visible bacterial growth.

The visible bacterial growth on the 6 µg/ml plates was seen to be much less than those observed on the 4 µg/ml plates. The 8 µg/ml plate had no visible bacterial growth. The 8µg/ml concentration of the crude leaf extract was noted as the minimum bacterial concentration for *P. aeruginosa.*

The zone of inhibition assay though valuable in determining sensitivity of a drug to particular pathogens, it still has to be audited by MIC and MBC assays. This is because clinical microbiologists consider this procedure an effective tool in determining the performance of other susceptibility test procedures. The minimum inhibitory test

procedure is a quantitative assay and this can show a clinical microbiologist the exact concentration of antimicrobial needed to inhibit pathogen growth (Andrews, 2001).

It is therefore imperative to carry an effective and accurate Minimum inhibitory concentration test because it will greatly affect the choice of therapeutic approach (Krochmal & Wicher, 2021).

The MBC and the MIC assays therefore are conclusive enough to show that the *V. glandulosa* leaf extract is a viable antibacterial agent and should be considered for further assays leading to commercialization as an antibacterial.

CHAPTER FIVE

SUMMARY, CONCLUSIONS & RECOMMENDATIONS

5.1. Introduction

In this chapter conclusions on qualitative phytochemical assay, isolation and purification, structure elucidation and antibacterial assay are presented in one summary. Recommendations for further research are made based on the results and discussion of results done in chapter four.

5.2. Summary

The results of the chemical assay on crude extract of *V. glandulosa* leaves showed presence of terpenoids, alkaloids, tannins, phenol, flavonoids and saponins. Spectroscopic assays and analysis showed that the unknown pure compound DVG3 extracted at 10 % Ethyl acetate/ 90 % Hexane was Lupeol. The biological assays showed that the crude leaf extracts had antibacterial activity against different types of bacterial colonies.

5.3. Conclusions

The procedure was successful in obtaining crude extracts from the grounded leaves of *V. glandulosa* using hexane. The crude extract was subjected to qualitative phytochemical assays and the compounds found to be present were terpenoids, alkaloids, tannins, phenol, flavonoids and saponins. This showed that the *V. glandulosa* leaves are rich in natural products. The crude extract was subjected to repeated column and thin layer chromatography and compound 1 which was a white thread like crystal was isolated at 10 % Ethyl acetate/ 90 % Hexane. Compound 1 was analysed using ^{13}C NMR, ^{1}H NMR, ^{1}H COSY, HMBC and DEPT spectroscopic analysis. The data from these spectroscopic assays showed the compound 1 had a molecular formula of $C_{30}H_{50}O$ and the spectra

identifiable characteristics confirmed that compound 1 is Lupeol, it is a pentacyclic triterpenoid and has a molecular weight of 426.7 g/mol. The antimicrobial activity of the crude extract from the leaves of *V. glandulosa* was evident. The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration test results implied that *V. glandulosa* is a potential option as an antimicrobial drug because of having bacteriostatic activity at concentration of 4 µg /ml and bactericidal activity at concentration of 8 µg /ml for *S. aureus* and 6 µg /ml bacteriostatic and 8 µg /ml bactericidal concentration for *P. aeruginosa*. It is therefore important that further research be carried out on *V. glandulosa* to further emphasize its antimicrobial capability for possible future consideration for clinical trials.

5.4. Recommendations

Based on the objectives of the study and the findings, the following are the recommendations

i. The pure compounds were successfully extracted, isolated, identified and their biological activity evaluated, the findings indicate that these compounds could be used as lead compounds for drug development.
ii. Antibacterial assays done on pure extracts from *V. glandulosa* identified using various spectroscopic methods showed antibacterial activity and therefore it is recommended that conservation measures be enforced by the relevant authorities to prevent *V. galndulosa* from going to extinction.

5.5. Suggestions for further research

Further phytochemical assays are to be carried out on not just the leaves, but stem bark and roots of *V. glandulosa* to discover new antimicrobials that may be in the other plant parts. The results of phytochemical analysis of various plant parts should inform the decision of potential for clinical trials.

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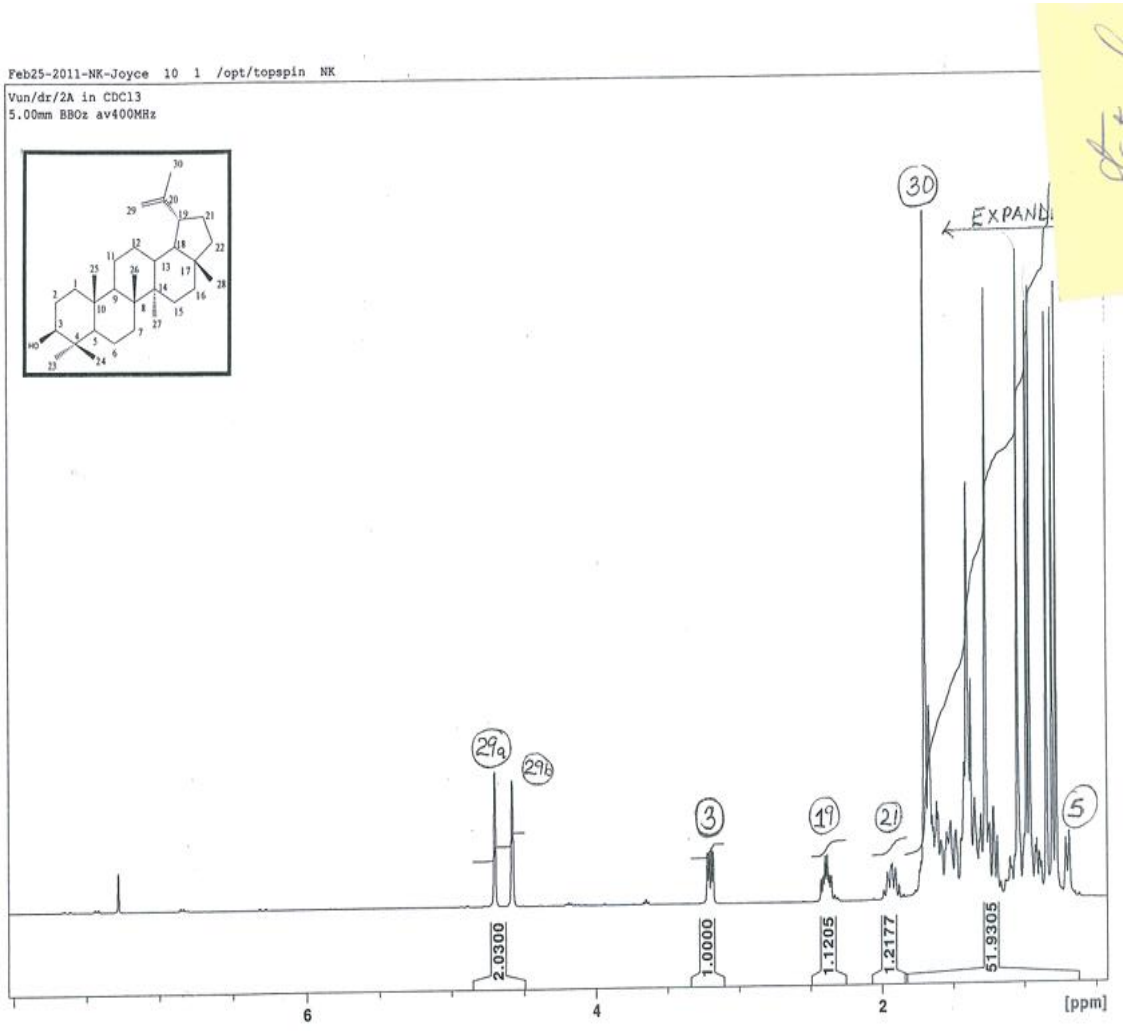
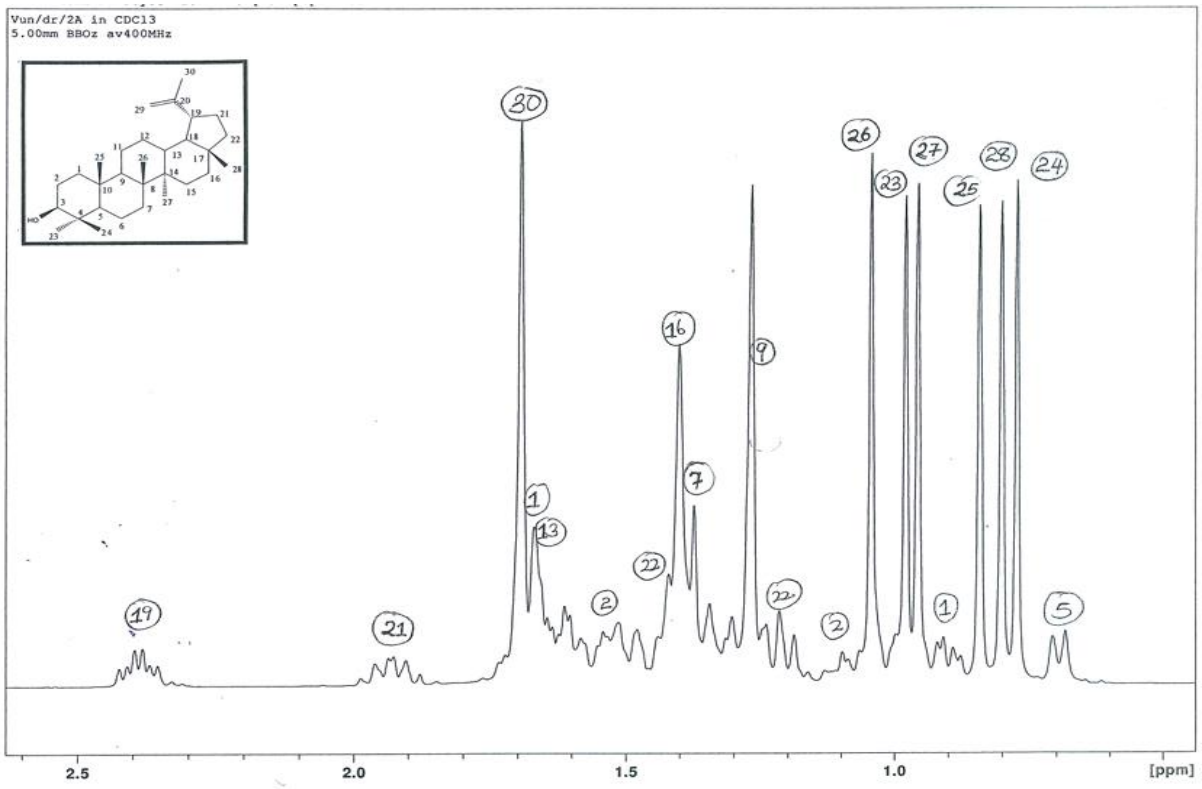
APPENDICES

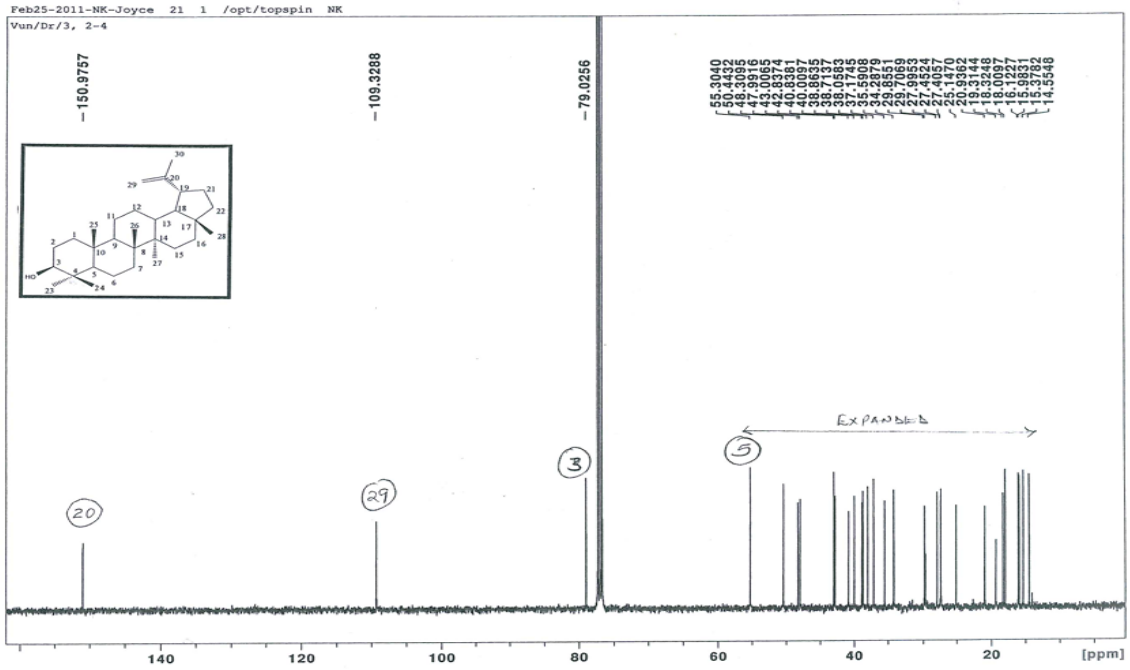
Appendix I: ^{1}H NMR Spectrum of Compound 1 (Lupeol)



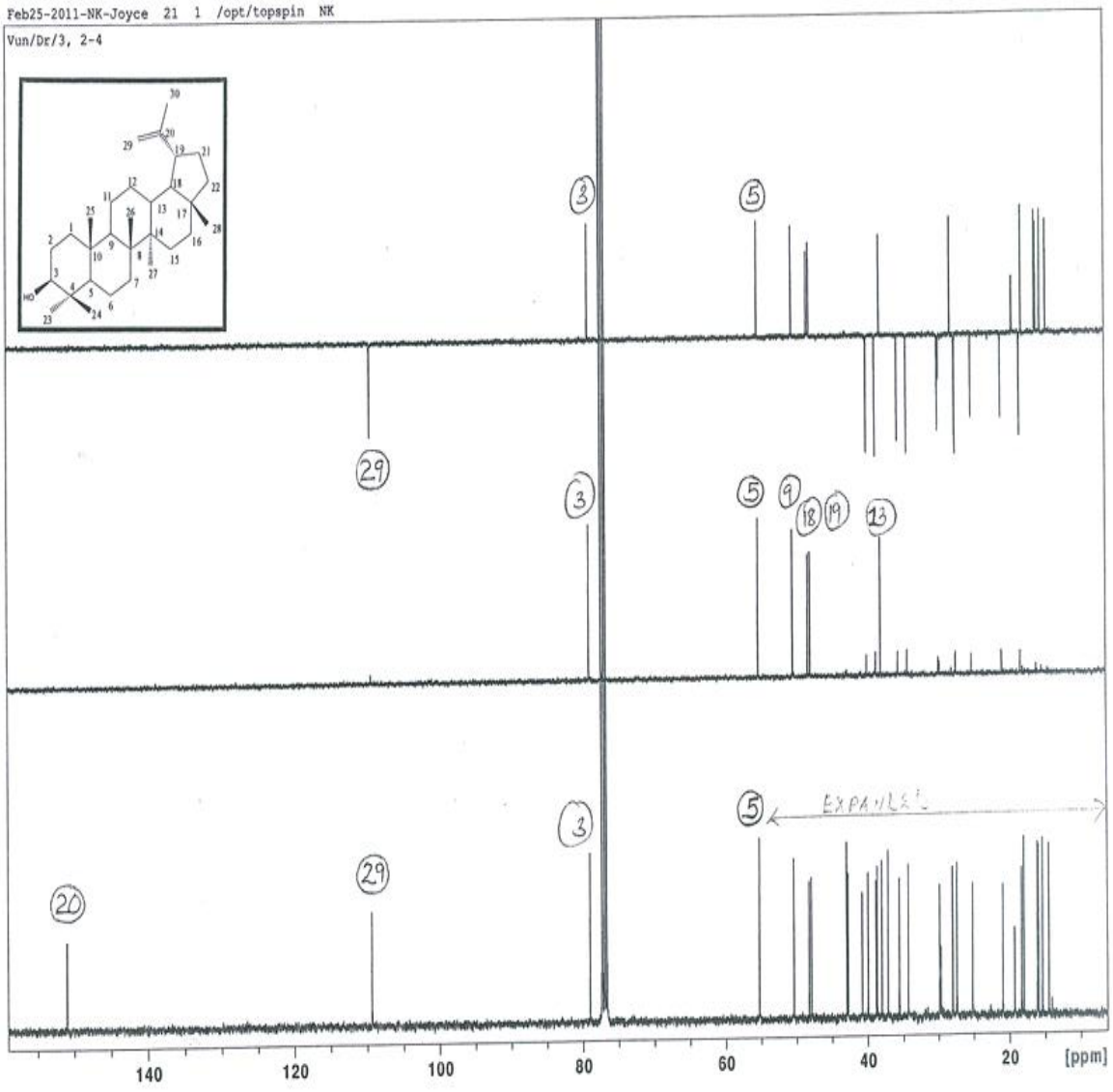
Appendix II: Expanded 1H NMR Spectrum of Compound 1 (Lupeol)



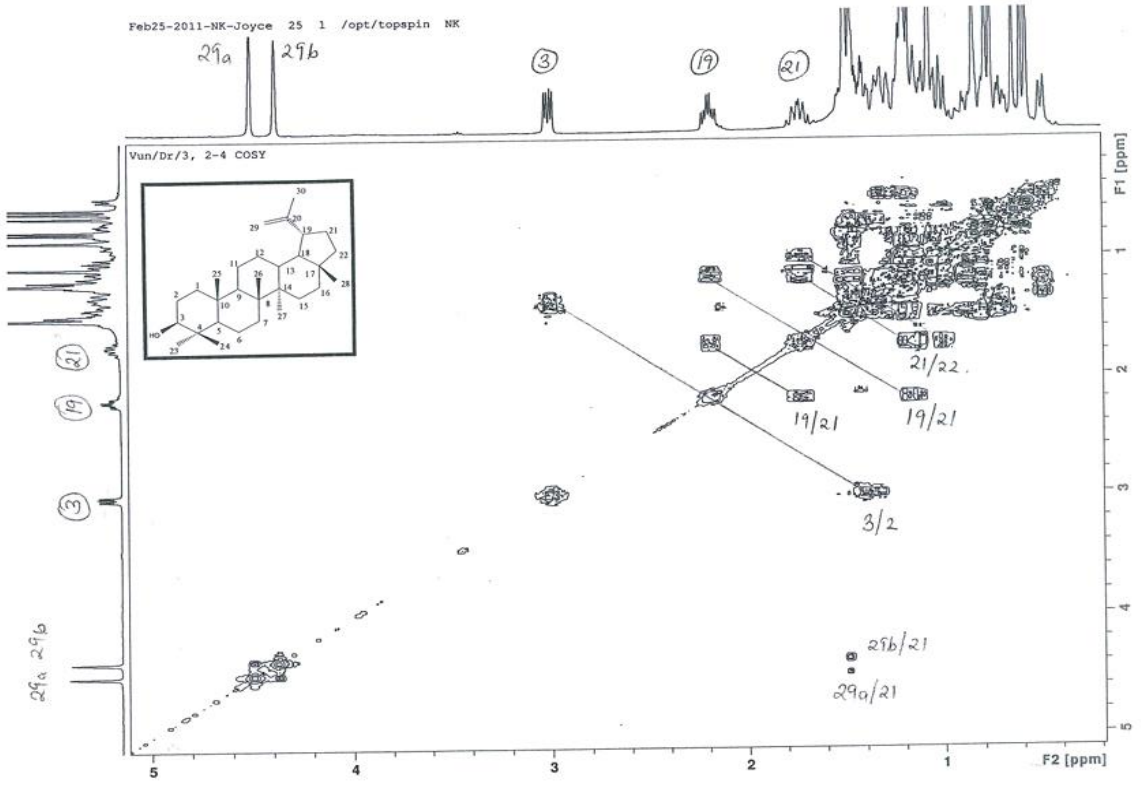
Appendix III: ^{13}C NMR Spectrum of Compound 1 (Lupeol)



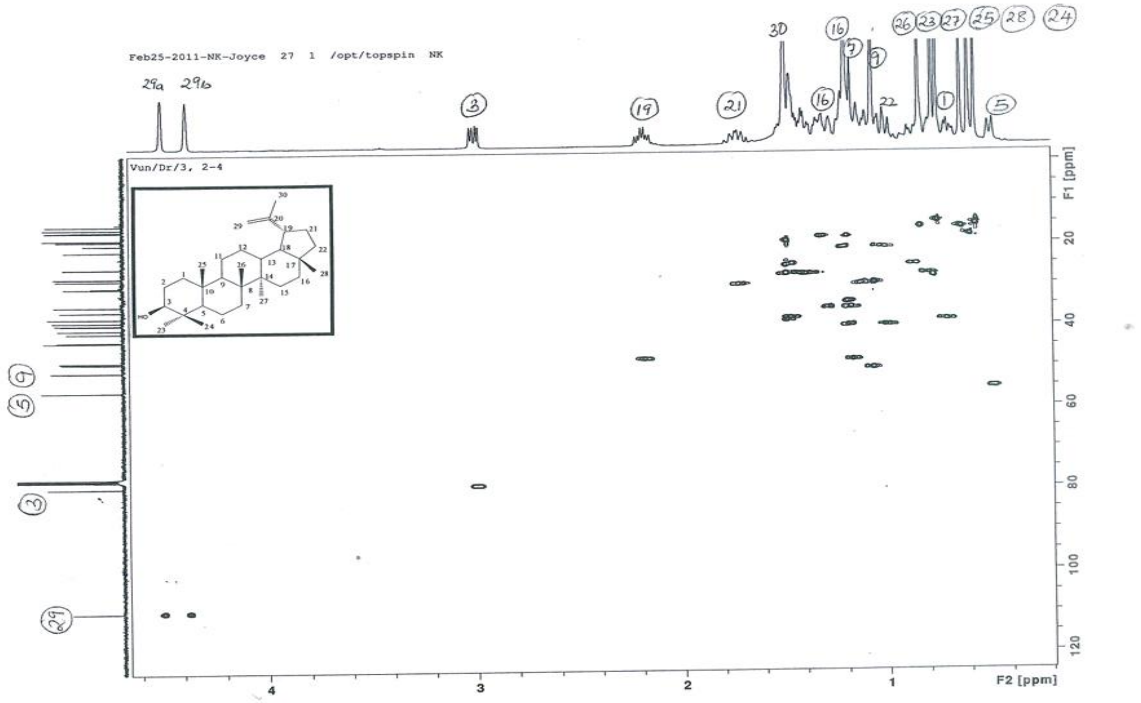
Appendix IV: DEPT Spectrum of Compound 1 (Lupeol)



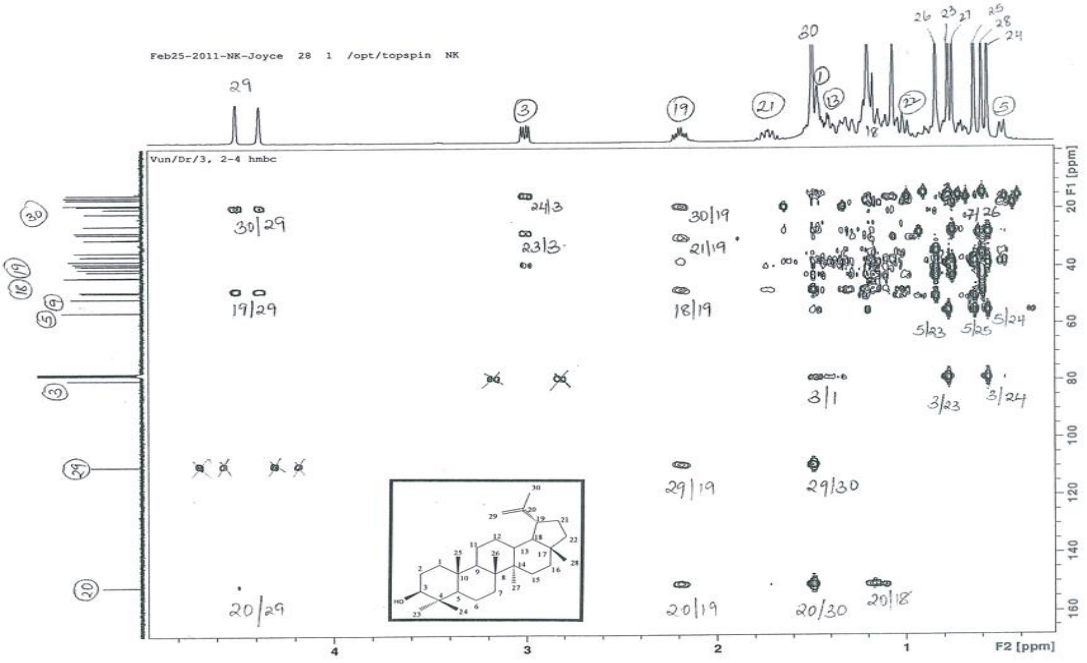
Appendix V: COSY Spectrum of Compound 1 (Lupeol)



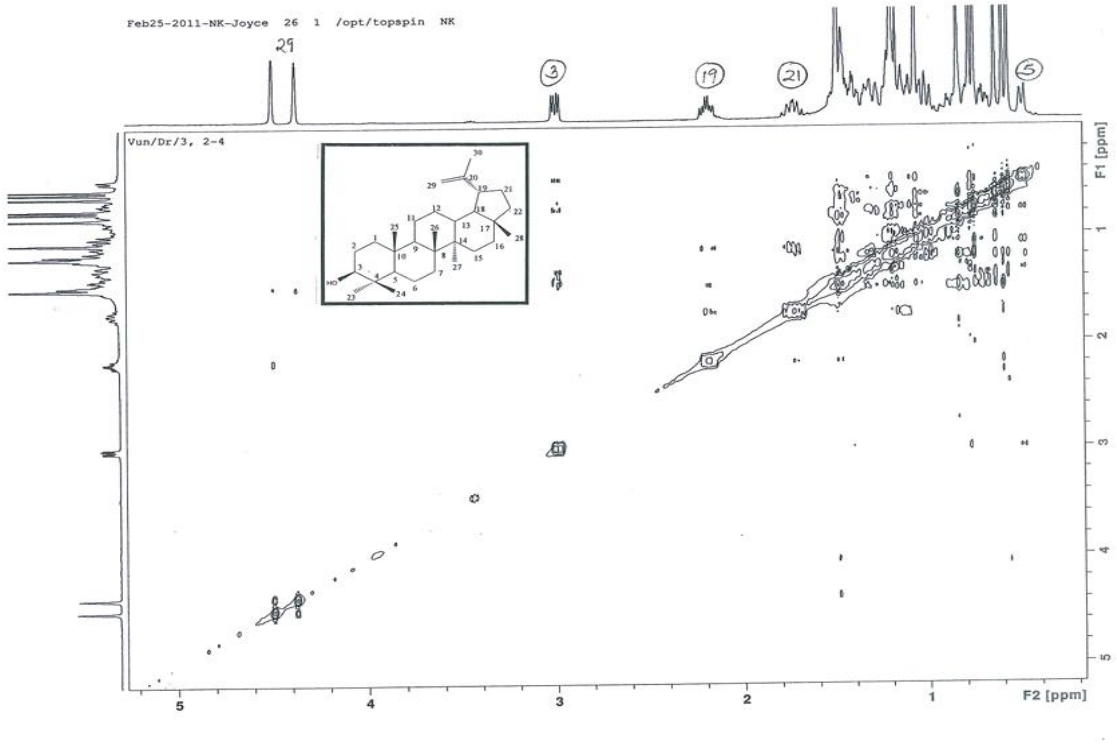
Appendix VI: HSQC Spectrum of Compound 1 (Lupeol)



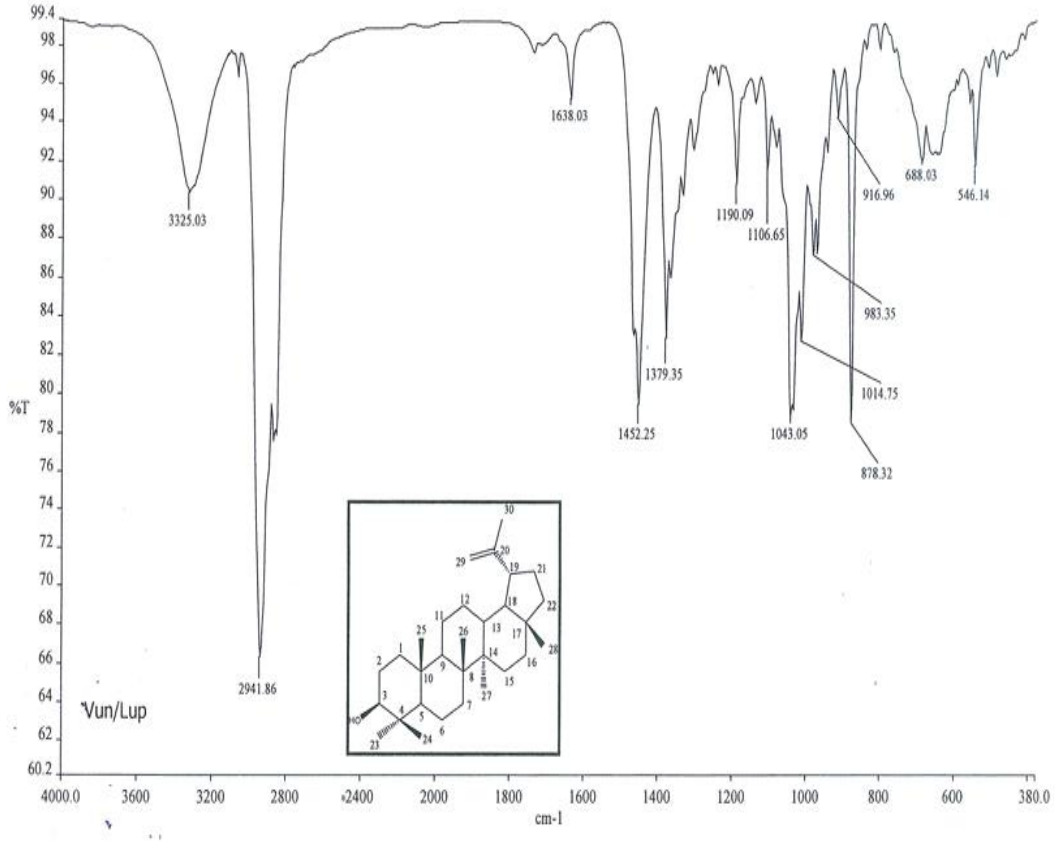
Appendix VII: HMBC Spectrum of Compound 1 (Lupeol)



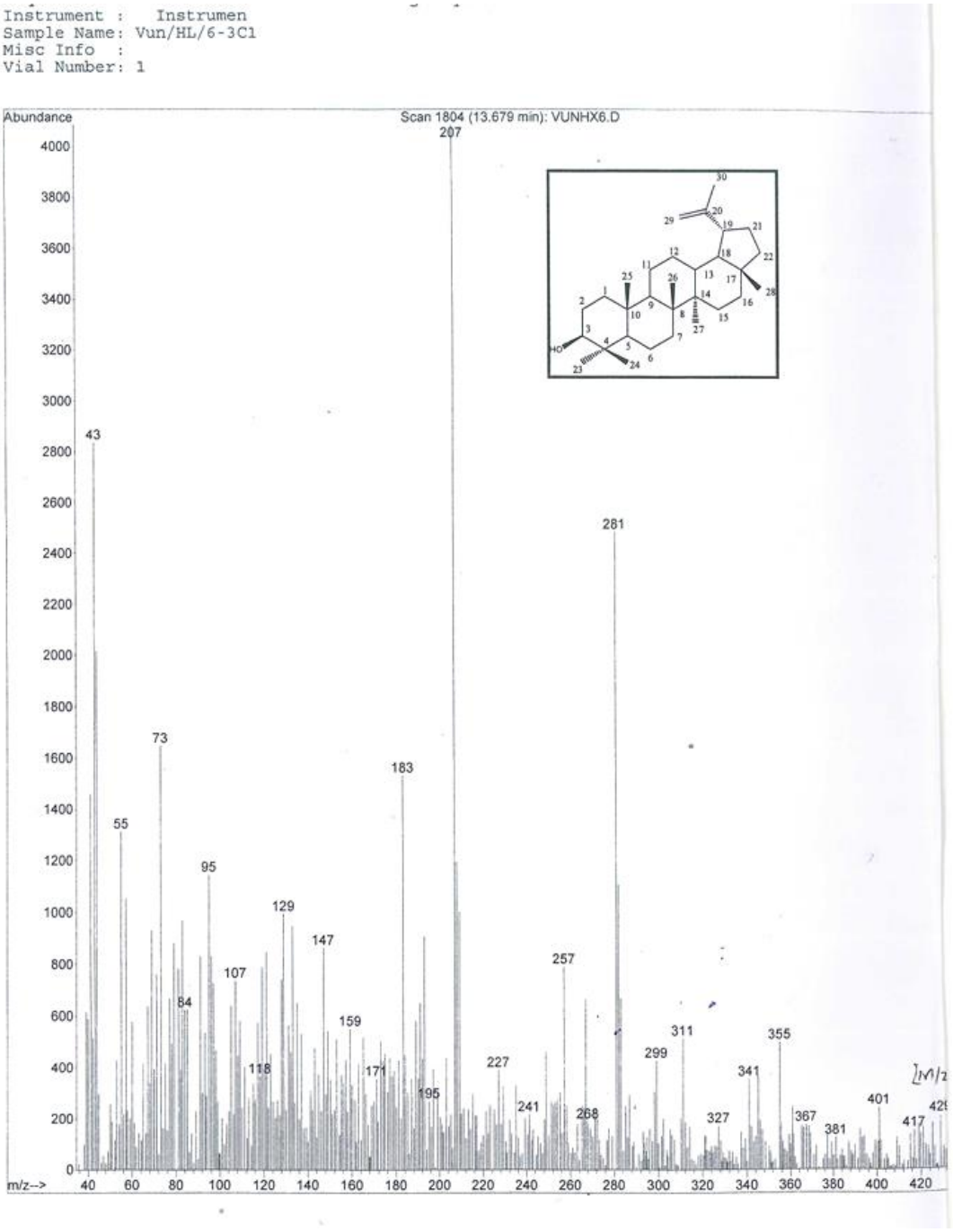
Appendix VIII: NOESY Spectrum of Compound 1 (Lupeol)



Appendix IX: IR Spectrum of Compound 1 (Lupeol)



Appendix X: GC-MS Spectrum for compound 1 (Lupeol)



APPENDIX XI: APPROVAL LETTER FOR DATA COLLECTION

UNIVERSITY OF KABIANGA
ISO 9001:2015 CERTIFIED
OFFICE OF THE DIRECTOR, BOARD OF GRADUATE STUDIES

REF: PGC/CHE/003/19 DATE: 5TH JUNE, 2024

Derrick Kibet Kirui,
MAPS Department,
University of Kabianga,
P.O Box 2030- 20200,
KERICHO.

Dear Mr. Kirui,

RE: CLEARANCE TO COMMENCE FIELD WORK/DATA COLLECTION

I am pleased to inform you that the Board of Graduate Studies has considered and approved your Masters Research proposal entitled **"Phytochemical Investigation of Compounds from *vepris glandulosa* Leaf Extracts and their Antimicrobial Activities".** Subsequently the Board has also approved the following supervisors for appointments.

1. Dr. Joyce J. Kiplimo, PhD
2. Dr. Denis K. Chirchir, PhD

You may now proceed to commence field work/data collection on condition that you obtain a research permit from NACOSTI and /or an ethical review permit from a relevant ethics review board.

You are also required to publish one (1) article in a peer reviewed journal, with all your supervisors, before your oral defense of thesis and to submit through your supervisors, and HoD, progress reports every three months, to the Director, Board of Graduate Studies.

Please note that it is the policy of the University that you complete your studies within two years from the date of registration. Do not hesitate to consult this office in case of any difficulties encountered in the course of your studies.

I wish you all the best in your research and hope that your study will yield original contribution for the betterment of humanity.

Yours Sincerely,

UNIVERSITY OF KABIANGA 5 JUNE 2024

Dr. Ronald K. Rop
DIRECTOR, BOARD OF GRADUATE STUDIES.
RKR/hk

Cc 1. Dean, SST
2. HOD, Mathematics, Actuarial & Physical Sciences
3. Supervisors

APPENDIX XII: NACOSTI RESEARCH LICENSE

REPUBLIC OF KENYA

NATIONAL COMMISSION SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **382525**

RESEARCH LICENSE

Date of **24 /Jun 2024**

This is to Certify that Mr.. Derrick Kibet Kirui of University of Kabianga, has been provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Kiambu on the topic: PHYTOCHEMICAL INVESTIGATION OF COMPOUNDS FROM VEPRIS GLANDULOSA LEAF EXTRACTS ANDTHEIR ANTIMICROBIAL ACTIVITIES for the period ending : 24/June/2025.

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APPENDIX XIII: PUBLICATION TITLE PAGE

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Original Article

Extractives from Vepris glandulosa Leaf Methanol Extract and Its Antibacterial Activity

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ABSTRACT

02 January 2025 The challenges facing currently available antimicrobials such as resistance with no recent significant discoveries are a major health concern in Kenya

characterized by prolonged hospitalization, high medical costs, and high annual

Keywords: fatalities. These challenges put great strain on healthcare systems and as such there is emphasis on new drug discoveries to mitigate the predicament. The

Vepris glandulosa, objective of the study was to determine the antibacterial activity of Vepris

Lupeol, glandulosa extracts after careful purification, isolation and structural

Antimicrobial, characterization. In this study, crude methanol extracts were obtained using the

Crude Extracts cold extraction method. Isolation of compounds was accomplished by repeated

Chromatography. column and thin-layer chromatography. One compound was obtained which was subjected to both 1D and 2D NMR spectroscopic analysis and the spectroscopic data obtained was used to propose the structure. The compound had its structure fully elucidated and was confirmed by comparing its NMR spectroscopic data with the literature reported to be

Lupeol. Lupeol showed antibacterial activity against selected gram-negative and gram-positive bacteria.

APA CITATION

Kirui, D., Kiplimo, J. & Chirchir, D. (2025). Extractives from Vepris glandulosa Leaf Methanol Extract and Its Antibacterial Activity. East African Journal of Health and Science, 8(1), 1-9. https://doi.org/10.37284/eajhs.8.1.2577.