PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF THE COMPOUNDS FROM THE ROOT BARK OF *VERNONIA AURICULIFERA;* HIERN (ASTERACEAE)

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DECLARATION

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

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This thesis has been submitted for examination with our approval as University supervisors.

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Prof. Gathu Nyagah
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DEDICATION

To my loving wife, Wairimu

And our Children,

Muthoni, Wangui, Nyokabi and Waihuini

ACKNOWLEDGEMENTS

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ABBREVIATIONS

WHO	World Health Organisation
NOESY	Nuclear Overhauser Effect Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer.
NMR	Nuclear Magnetic Resonance

TLC	Thin Layer Chromatography
DCM	Dichloromethane
FT-IR	Fourier Transform Infra Red.
UV	Ultra-Violet spectroscopy
LD_{50}	Dose required to kill 50% of brine shrimp larvae
SAS	Statistical analysis system
TMS	Tetramethylsilane
AIDS	Acquired immunity deficiency syndrome
HIV	Human-immune deficiency virus
RTI	Respiratory Tract Infections
TB	Tuberculosis
TSC	Teachers Service Commission
COSY	Correlation spectroscopy
IR	Infrared spectroscopy
ppm	parts per million
MS	Mass spectroscopy
^{13}C-NMR	Carbon -13 nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
$CDCl_3$	Deuterated chloroform
SD	Standard deviation

ABSTRACT

In the present study, the root bark of *V. auriculifera* was sequentially extracted with *n*-hexane, dichloromethane (DCM) and methanol respectively. The activities of crude extracts of *n*-hexane, DCM and methanol against brine shrimp larvae were compared. As larvicides, the DCM crude extract was most active (LD_{50} = 318 ppm), followed by *n*-hexane (LD_{50} = 788 ppm), and lastly by methanol extract (LD_{50} = 931 ppm). The anti-bacterial assay showed that crude extracts were generally more active on *E. coli* than *S. aureus*. *n*-hexane and methanol extracts were found to be active against *E. coli* (Inhibition diameter > 10 mm) at 1000 ppm.

Fractionation of DCM extracts appears to have yielded compounds whose activity against *S. aureus* seems to be enhanced. Fractionation is thought to concentrate active ingredients.

The *n*-hexane extract, methanol extract and compounds **3, 4, 5, 10** and **11** with activities (inhibition diameters ≥ 10 mm) against *E. coli* can be used to control this bacterium. For *S. aureus* only compounds **5, 7, 9** and **10** which had activities ≥ 10 mm that could be used to control it.

The fungal assay using *Candida albicans* showed significant activity (Inhibition diameter > 10 mm) with *n*–hexane and DCM extracts. For compounds only **1** and **11** had good

results (inhibition diameters ≥ 10) with this fungus. Thus *n*-hexane, DCM extracts and compounds **1** and **11** could suitable candidates for control of *Candida albicans.*

Column chromatography of the *n*-hexane extracts followed by re-crystallization in methanol yielded two triterpenoids which were characterized as lupeol (**6**) and an ester of a long chain fatty acid and taraxerol (**7**) whose suggested name is α-taraxerol octanoate and is a possible novel compound. The structures of these compounds were elucidated and using ^{1}H-NMR and ^{13}C-NMR experiments and also matching their spectra with those found in the literature. Melting points, ultra-violet (UV) and infra-red (IR) absorptions of these compounds are reported.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Natural products once served man as the source of all drugs, and higher plants provided most of therapeutic agents. Today, natural products (and their derivatives and analogs) still represent over 50% of all drugs in clinical use, with higher plant – derived natural products representing 25% of the total (Balandrin *et al.*, 1993). Some of those drugs in clinical use are quinine from cinchona bark, morphine and codeine from latex of opium poppy, digitalis leaves and atropine (derived from (-) -hyoscyamine and hyoscine) from species of solanaceae. The World Health Organization (WHO) estimates that 80% of the people in developing countries rely on traditional medicine for their primary health care,

and about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Farnsworth, 1988).

In the United States, plant-derived drugs represent about 25% of the prescription drug market, and in 1991 this equated to retail value of approximately US$ 15.5 billion (Pezzuto, 1997). From 1983 to 1994, 39% of the new approved drugs were of natural origin, including original natural products, products derived semi-synthetically from natural products, and synthetic products based on natural products model (Cragg *et al.*, 1997).

Further evidence of the importance of natural products is provided by the fact that almost half of the World's 25 best selling pharmaceuticals in 1991 were either natural products or their derivatives (O'Neil *et al.,* 1993). Conservative estimates suggest that there are 250,000 to 500,000 species of higher plants existing in this planet, and only a small percentage of plants have been studied for their potential value as sources of drugs. Obviously natural products will continue to be extremely important as sources of medicinal agents. In addition to natural products which have found direct application as drug entities, many others serve as chemical models or templates for the design, synthesis and semi-synthesis of novel substances for treating human diseases.

Of all pharmaceuticals dispensed in the United States having higher plant origins, very few are intended for use as anti-microbials, since we have relied on bacterial and fungal

sources for these activities. Since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobials has been virtually non-existent.

Clinical microbiologists have two reasons to be interested in the topic of anti-microbials from plant extracts. First, it is likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians, of which several are already being tested on humans. It is reported that on average, two or three antibiotics derived from micro organisms are launched each year (Clark, 1996). After a downturn in that pace in recent decades, the pace is again quickening as scientists realize that effective life span of any antibiotic is limited. Worldwide spending on finding new effective agents is expected to increase by 60% from the spending levels in 1993 (Alper, 1998). New sources, especially plant sources, are also being investigated. Second the public is becoming increasingly aware of the problem of over prescription and misuse of traditional antibiotics. In addition, many people are interested in having autonomy over their medical care. A multitude of plant compounds (often of unreliable purity) is readily available over the counter from herbal suppliers, and self medication with these substances is a common practice. The use of plant extracts as well as alternative forms of medical treatment is enjoying great popularity in the late 90s. Earlier in the decade, approximately one third of people surveyed in the United States used at least one unconventional therapy during the previous year (Eisenberg, *et al.,* 1993). It was reported that in 1996, sales of botanical medicines increased by 37% over 1995 (Klinck, 1997). It is speculated that the American public may be reacting to over prescription of sometimes toxic drugs.

1.2 Bacterial and Fungal Infections

Most infections are caused by bacteria and fungi. These infections are usually rare or much less serious in people with healthy immune systems. Some of these infections can be treated with relatively inexpensive drugs. They include tuberculosis, pneumonia, thrush and bronchitis.

The emergence of HIV/AIDS in recent years has made control of fungal and bacterial infections difficult. This is because these micro-organisms take advantage of a weakened immune system and multiply very fast. One such disease that is causing concern is Tuberculosis (TB). It has been reported that before emergence of HIV, TB infections were limited to about 0.2% of the population (Harris, 1990). This would result in 40,000 to 60,000 new cases. Today the situation is that TB infection has reached dangerous levels. Many in-patients are normally placed in isolated wards because TB is highly infectious. TB treatment has also been complicated by emergence of multi-drug resistant strains. As such, it is now becoming a very expensive disease to treat and may put a considerable strain on the health budget.

1.3 Impact of Infections on Human Health and Life.

The most common infectious diseases in the World are the respiratory tract infections (RTI). Typical respiratory infections are common cold, influenza and influenza like infections, acute bronchitis and acute pneumonia. The first three are usually mild, self limiting viral diseases whereas pneumonia and acute bronchitis continues to be an

important cause of mortality. In the United States pneumonia is the sixth common cause of death and the most important cause of infection related mortality (US Bureau of the Census 1990). A similar epidemiological picture is seen in other countries.

Bacterial and fungal infections constitute a significant sector of the health budget especially with the rise of HIV/AIDS pandemic. These infections occur as opportunistic infections that eventually bring down people suffering from HIV/AIDS. Thus, there is need to search for drugs that can manage AIDS as well control bacterial and fungal infections.

CHAPTER 2

LITERATURE REVIEW

2.1 Control of Bacterial and Fungal Diseases.

Most infections are caused by bacterial and fungi. Before the discovery of Penicillin, many people suffered and died from bacterial infections that are no longer considered dangerous today. For instance, just hurting on a nail could eventually lead to death. The search for other antibiotics continued. These antibiotics were successfully used to control many infectious diseases.

Today, antibiotics are still the main agents used in the control of many bacterial and fungal diseases. However, bacteria resistance to antibiotics has become a health problem

(Morse *et al*., 1986). It is reported that, on average, two or three antibiotics derived from micro-organisms are launched each year (Clark, 1996). The pace is again quickening as scientists realize that the effective life span of any antibiotic is limited. Worldwide spending on finding new effective agents (including vaccines) is expected to increase 60% from spending levels in 1993 (Alper, 1998). The drugs for which most resistances have developed are β-lactam antibiotics. *Staphylococcus aureus* for instance has been resisting a lot of β-lactam antibiotics (Sabath *et al.*, 1977).

Another area where antibiotic resistance has been a problem is treatment of tuberculosis (Davidson *et al.*, 1981). *Mycobacterium avium* (MAC) infections and those of *M. Fortuitum*, *M. chelonae* and *M. tuberculosis* have increased in patients with acquired immunodeficiency syndrome (Naik and Ruck, 1989). Infection caused by these organisms especially MAC are resistant to common anti-mycobacterial agents (Dutt and Stead, 1979). The continued search for effective anti-mycobacterial drugs is therefore still very important. The search for antimicrobial agents has however continued to concentrate on lower plants namely fungi and bacteria, but this has become less profitable (Mitscher *et al.*, 1987). Due to high costs involved, the search for new antibiotics is directed to higher plants and higher animals.

Lastly, the ascendancy of the human immunodeficiency virus (HIV) has spurred intensive investigation into the plant derivatives, which may be effective, especially for use in under developed nations with little access to expensive Western medicine.

2.2 Plant Products as Anti-microbial Agents

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives (Geissman, 1963). Most of them are secondary metabolites, of which at least 12,000 have been isolated. In many cases, these substances serve as plant defensive mechanisms against predation by micro-organisms, insects and herbivores (See Table 1). Some, such as terpenoids, give plants their odours, others such as quinines and tannins are responsible for plant pigments.

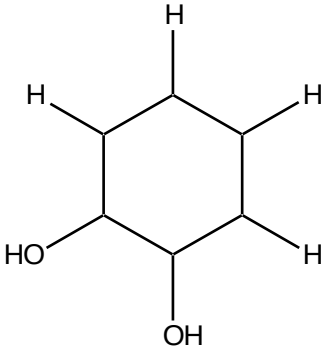
Table 1: Some plants containing anti-microbial activity

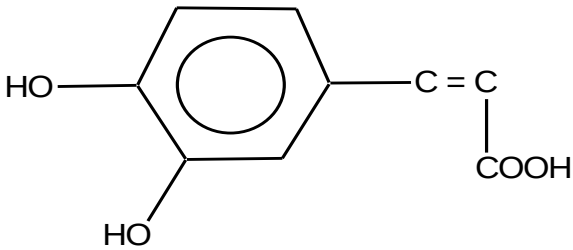
COMMON	SCIENTIFIC NAME	COMPOUND	CLASS	ACTIVITY	REFERENCES
Aloe	*Aloe barbadensis Aloe vera*	Aloin	quinone	*Corynebaterrium, Salmonella, S. aureus Streptococcus*	(Martinez *et al.*, 1996)
Apple	*Malus sylvestris*	Phlorectin	Flavanoid derivative	General	(Hanter *et al.*, 1993)
Chamomule	*Matricarin chamomilla*	Anthemic acid	Phenolic acid	*M. tuberculosis S. typhi* and *S. aureus*	(Bose, 1958, Hamburger and Hostehmann, 1991, Schee, 1972)
Chilly peppers	*Capsicum annum*	Capsaicum	Terpenoid	Bacteria	(Cichewiczc and Thorpe, 1996, Jones Luchsinger, 1986)

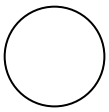
Japanese (herb)	*Rabdosia trichocarpa*	Trichorabdol A	Terpene	*Helicobacter pyroli*	(Kadota *et al.*, 1997)
Onion	*Alum cepa*	Allicin	Sulfoxide	Bacteria, candida	(Vohora *et al* 1973)
Oregon grape	*Mahonia aquifolia*	Berberine	Alkaloid	Plasmodium	(Omulokoli *et al.*, 1997)
Purple prairie clover	*Petalostemum*	Petalostemumol	Flavanol	Bacteria, fungi	(Hufford *et al.*, 1993)
Tree bard	*Podocarpus nagi*	Totaral nagilactone	Flavanol lactone	*P. acnes*, other gram positive bacteria, fungi	(Kubo *et al* 1994) (Kubo *et. al* 1992)
Pao d'arco	*Tabebuia*	Sesquiterpenes	Terpenoids	Fungi	(Cowan, 1999)

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids (**1**) are common representative of phenyl propane derived compounds. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Branther *et.al* 1996) and fungi (Duke, 1985).

Catechol (**2**) and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two -OH groups and pyrogallol has three. The site (s) and number of hydroxyl groups are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman 1963). Eugenol (**3**) another phenolic substance found in clove oil is bacteriostatic to both fungi (Duke, 1985) and bacteria (Thomson, 1978).

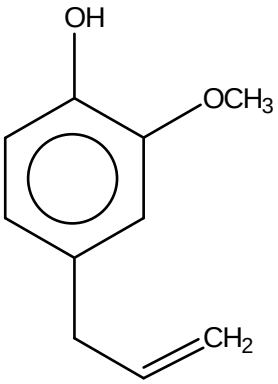






Caffeic acid **(1)**

Catechol **(2)**

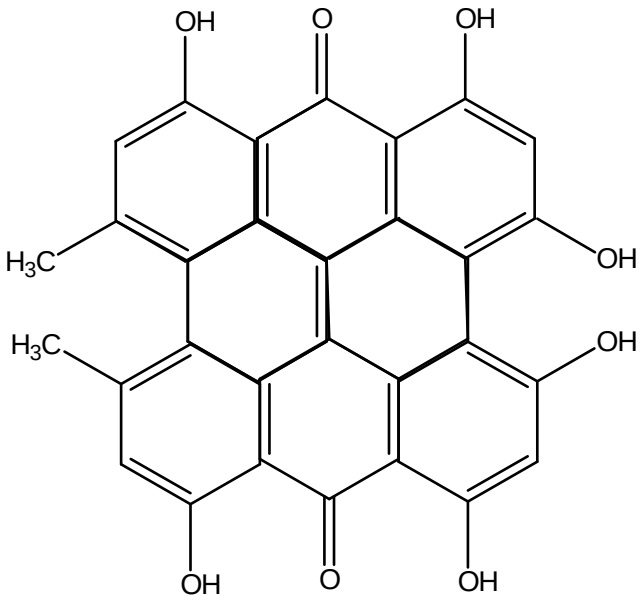


Eugenol **(3)**

Quinones is another class of compounds which are biologically active. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being coloured, are responsible for the browning reaction of cut or injured fruits and vegetables.

Quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al*., 1996) often leading to inaction. For that reason, the potential range of quinone anti-bacterial effects is great. Probable targets in the microbial cell are surface exposed adhesins, cell wall polypeptides and membrane bound enzymes. Quinones may also render substrates unavailable to micro-organisms. As with all plant derived anti-microbials, the possible toxic effects of quinones must be thoroughly examined.

Kazmi *et al.*, (1994) described an anthraquinone from *Cassia italica*, a Pakistan tree, which is bacteriostastic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum* and *Pseudomonas aeruginosa* and bactericidal to *Pseudomonas pseudomallic*. Hypericin (**4**), an anthraquinone from St John's Wort, *Hypericum perforatum*, has received much attention in the popular press lately as an anti-depressant. (Duke, 1985) reported that it had general anti- microbial activities.

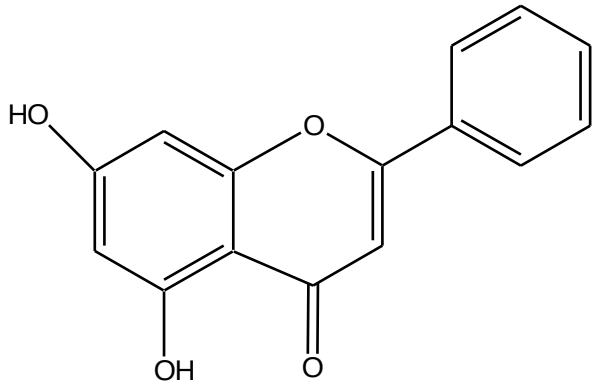


Hypericin **(4)**

Plants are known to synthesize flavonoids in response to microbial infections (Dixon, *et al.*, 1983) and hence it should not be surprising that they have been found to be effective anti- microbial substances against a wide array of micro-organisms. Their activity is probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacteria cell walls.

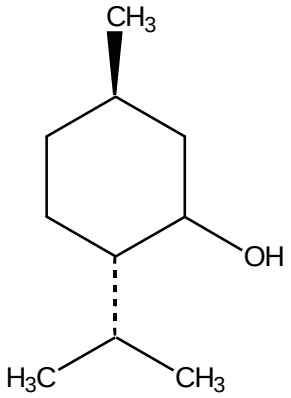
Catechins flavonoids have been extensively researched due to their occurrence in oolong green teas. It was noticed sometime ago that these teas exerted anti microbial activity (Toda, 1989) and that they contain a mixture of Catechin compounds. These compounds inhibited *in vitro Vibrio cholerae* (Borris, 1996), *Streptococcus mutans* (Sakanata *et al.* 1989, Sakanata *et al.*, 1992, Batista *et al.*, 1994, *Shigella,* Vijaya *et al.*, 1995 and Tsuchya *et al.*, 1996) and other bacteria and micro organisms (Thomson, 1978 and Sakanata, *et al*., 1992).

Flavonoid compounds also exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside (Pengsuparp, *et al.*, 1995), glycyrhizin from licorice (Watanabe, *et al.*, 1996) and Chrysin (**5**) (Critchfield *et al.*, 1996) against HIV.

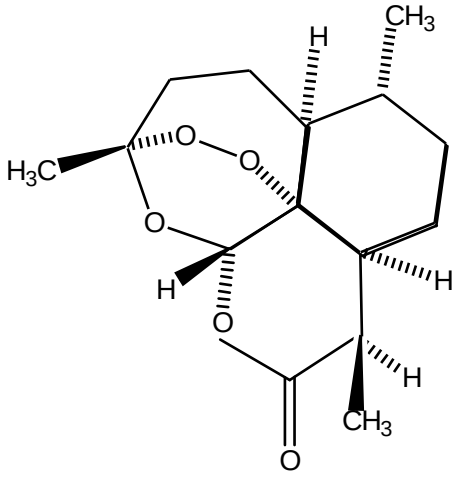


Chrysin **(5)**

The fragrances of plants is due to terpenoids. Common examples of terpenoids are menthol (**6**) and camphor (monoterpenes) farnesol and artemisinin (Sesquiterpenoid). Artemisinin (**7**) and its derivative α- artemether, also known by the name qinghaousu, find current use as antimararials (Vishwakarma, 1990). In 1985, the steering committee of the scientific working group of WHO decided to develop the latter drug as a treatment of cerebral malaria.



Menthol **(6)**



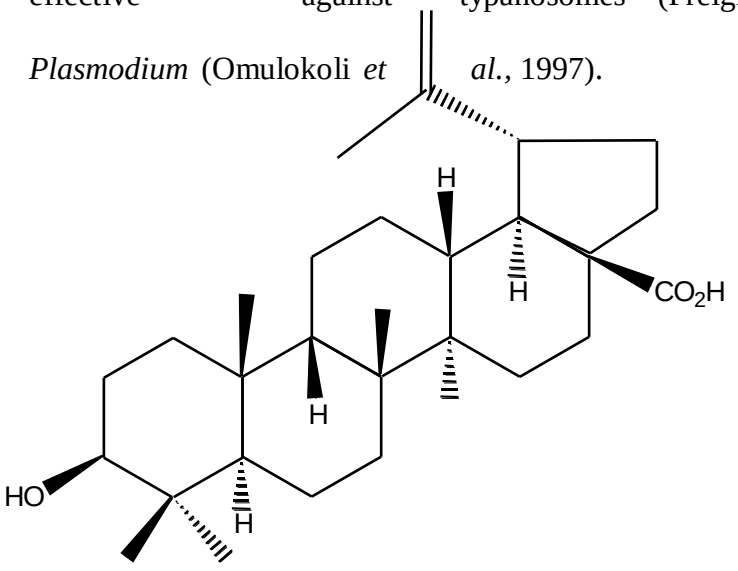
Artemisin **(7)**

Terpenoids are active against bacteria (Mendoza *et al.*, 1990; Ahmed 1993), fungi (Suresh *et al.*, 1997, Rana *et al.*, 1997) and viruses (Fujioka and Kashiwada, 1994; Hasegawa *et al.*, 1994). In 1997, it was reported that 60% of essential oil derivatives

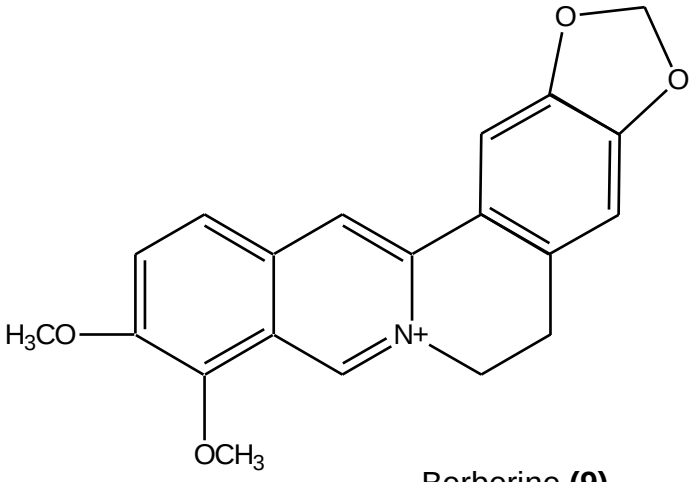
examined to date were inhibitory to fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1997). The triterpenoid betullinic acid (**8**) is just one of the several terpenoids which have been shown to inhibit HIV. The terpenoid, petalostemmumol obtained from the ethanol soluble fraction of the purple prairie clover showed excellent activity against *Bacillus subtilis* and *Staphylococus aureus* and less activity against gram negative bacteria as well as *Candida albicans* (Hufford *et al.*, 1993). Two diterpenes isolated by (Batista *et. al.* 994) were more democratic, they worked well with *S. aureus, V.cholerae, P. aeruginosa* and *candida SPP.*

Alkaloids are another group of compounds which show anti–microbial activity. The diterpenoid alkaloids from the plants of *Ranuniculaceae* or buttercup (Jones *et. al.* 1986) family (Atta-ur-Rahman and Choudhary, 1995) are commonly found to have antimicrobial activity (Omulokoli *et al.,* 1997). Solamargine, a glycoalkaloid from the berries of S*olanum khastanum* and other alkaloids may be useful against HIV infection (Sethi, 1979 and Mcmahon *et al.,* 1995) as well as intestinal infections associated with AIDS (Mcdevitt *et al.,* 1996).

Berberine (**9**) is an important representative of the alkaloid group. It is potentially effective against typanosomes (Freighburghaus *et al.,* 1996) and *Plasmodium* (Omulokoli *et al.,* 1997).



Betulinic acid **(8)**



Berberine **(9)**

Although some secondary metabolites may not be associated with a pronounced activity on any organism, it may be working synergistically with other chemicals in the plant to provide the much needed protection for the plant to survive, Bell E.A. (1980).

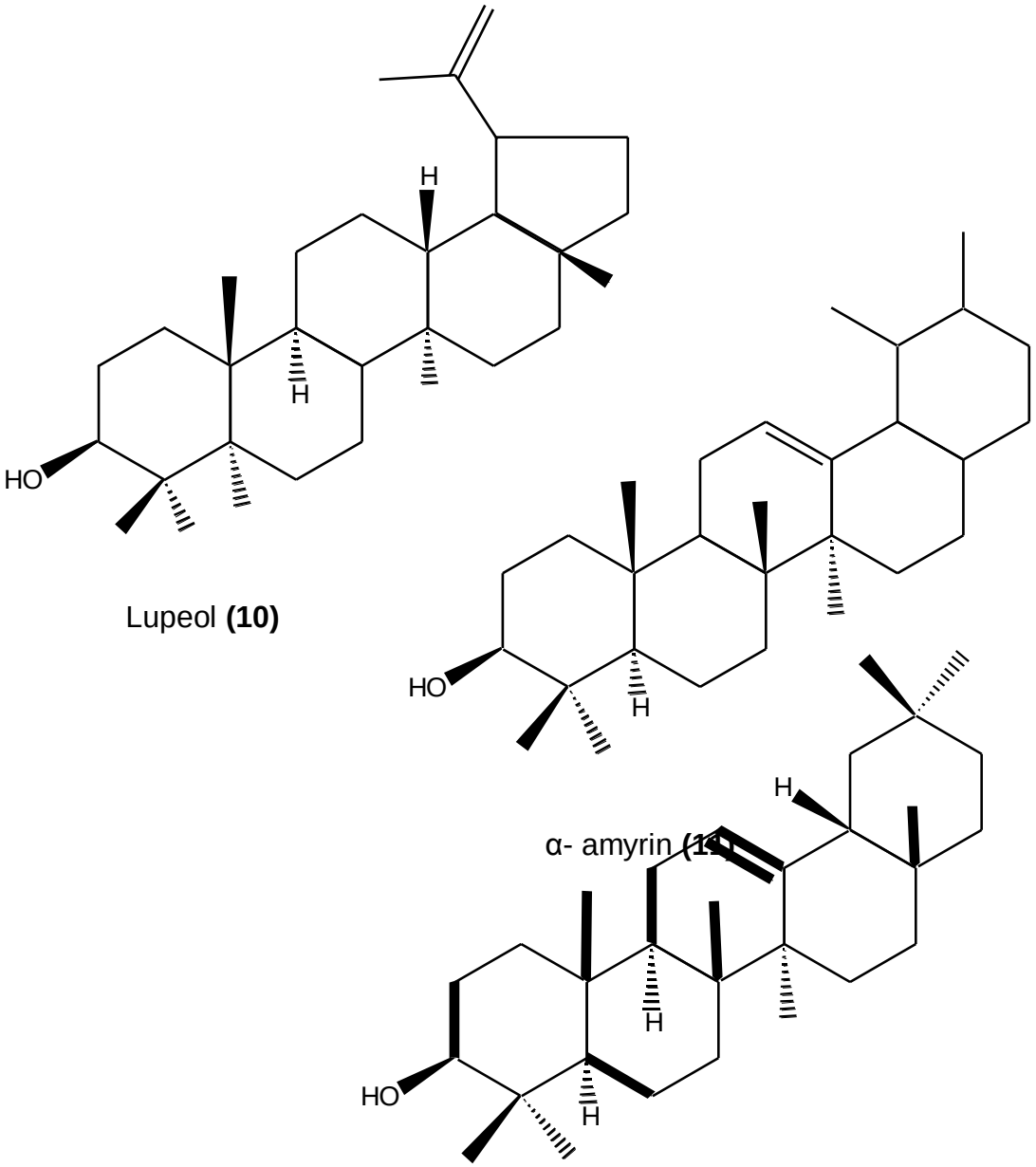
2.3 The Genus Vernonia

Many species of this large genus (Ca 1000 species) have been studied chemically (Bohlman and Jakupovic, 1990). The most characteristic chemical constituents found in them are triterpenes, steroids and lignoids, but more frequent compounds are sesquiterpene lactones and flavonoids (Costa and Mestrado, 1996). These frequent compounds are considered as taxonomic markers of this genus.

The literature has presented many publications of pharmacological activities of sesquiterpenoids and flavonoids from Vernonia species (Kiesel, 1975; Bruneton 1995 and Costa and Mestrado, 1996) but very little on triterpenoids. Although, triterpenoids were

considered to be relatively innocuous plant constituents, several have been established to have pronounced physiological activity. Whereas phytosteroids are requirement for insect and fungal growth, triterpenoid compounds interfere with this process and hence exhibit anti- herbivore and anti-fungal effects (Croteau and Johnson, 1985). They have also been found to exhibit mild anti-bacterial activity (Bittner *et al.,* 2002).

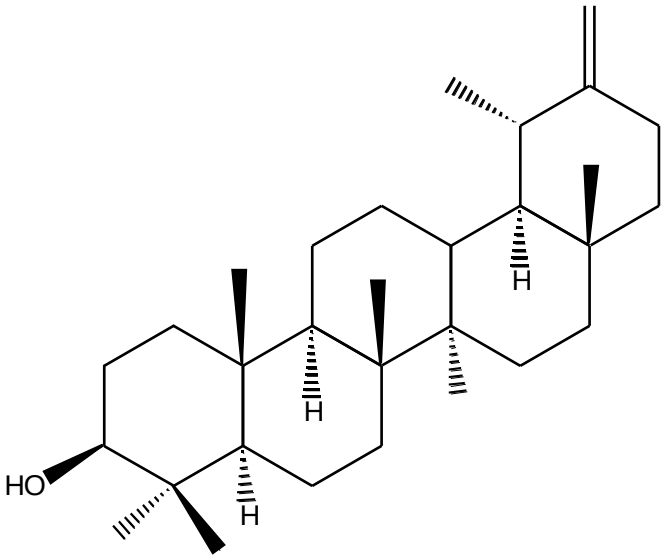
A number of triterpenoids have been isolated from Vernonia species. Among the most common triterpenes are lupeol (**10**) and its derivatives, α- and β-amyrim, (**11** and **12**) taraxasterol (**13**), taraxerol (**14**), Germanicol (**15**) and Friedelin (**16**).



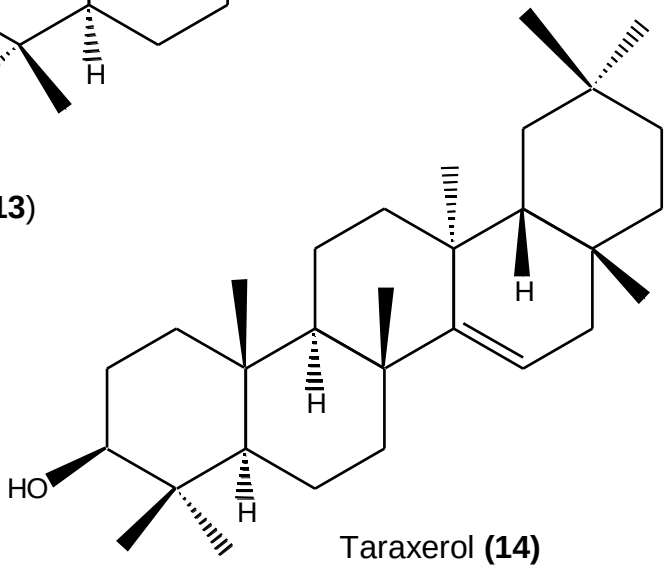
Lupeol **(10)**

α- amyrin **(11)**

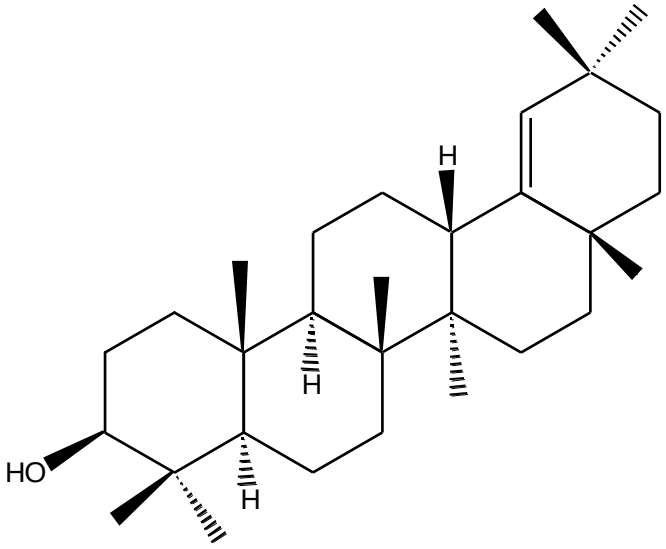
β-amyrin(**12**)



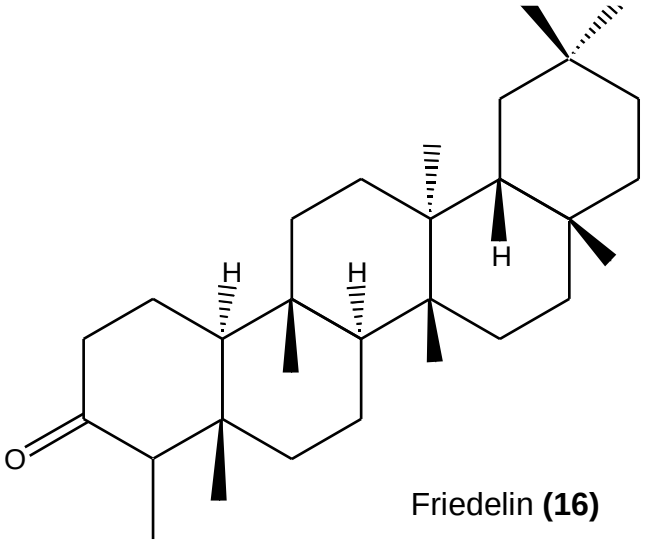
Taraxasterol (**13**)



Taraxerol **(14)**



Germanicol **(15)**



Friedelin **(16)**

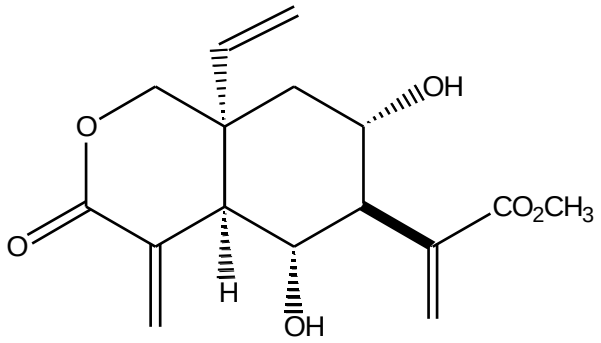
The fact that they have been found in a number of plants of this genus suggests that they have a significant role in plant defence. The more oxygenated the triterpenoids the higher the potency (Seigler, 1983).

2.4 The Species *Vernonia Auriculifera.*

Plate 1: *Vernonia Auriculifera*

The plant found in high altitude areas such as Kericho, Laikipia and Nyeri districts. It is a shrub growing up to a height of about 2 ½ metres. Traditionally it has been used as a medicine for a long time and has uses similar to *V. amygdalina* (Kokowaro, 1976).

Unlike other species of vernonia this plant has not been thoroughly investigated. To date work done on the aerial part of the plant yielded the known 8-desacylvernodalol (**17**) (Keriko *et. al.* 1995a), a sesquiterpenoid with the elemane skeletal feature. This makes it taxonomically to fit in the old world species. The other isolates of this plant is a mixture of hydroperoxides of methyl acid esters from its leaves. The mixture has been found to be very lethal to brine shrimps (Keriko *et. al.* 1995b). These hydroperoxides could be part of the plant strategy to defend itself from predators and other enemies. In fact the strong lethal effects of lipid peroxides against human cells is well documented (Yagi, 1993).



8-Desacylvernodalol **(17)**

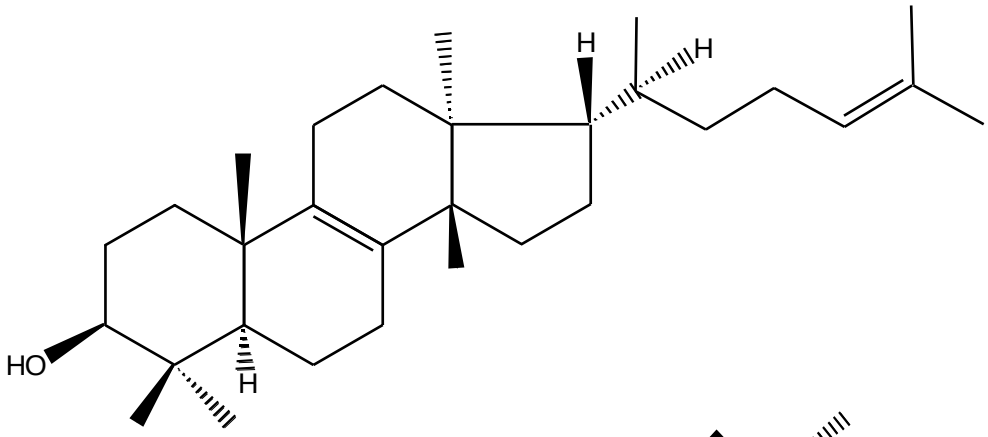
2.5 Biogenesis of Triterpenoids

Triterpenoids are considered to arise from squalene -2,3 -expoxide (Scheme 1). According to the proposed Scheme (Coates, 1976) cyclization of squalene, 2,3- epoxide (**18**) via a series of chair-chair-chair- boat transition state gives rise to many of the most common triterpenoids. This cyclization is thought to occur easily by opening the expoxide through acid catalysis which receives anchimeric assistance from adjacent double bonds (Van Tamelen *et al.,* 1966 and Van Tamelen 1968), through methyl and hydride shifts along with skeletal rearrangements, the final pentacyclic triterpenoid structures are achieved. In practice most of these intermediates have not been substantiated.

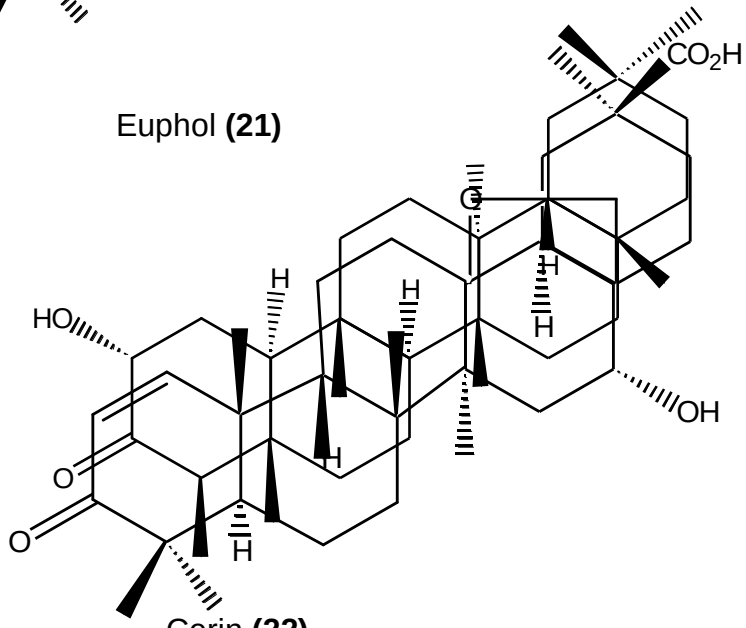
Examples of the common plant pentacyclic triterpenoids are betulin (**8**), lupeol (**10**), α- and β- anyrin (**11** & **12)**, taraxasterol (**13**), taraxerol (**14),** Fredelin (**16**), oleanolic (**17**), Ursolic acid (**19**) and euphol (**21**). These triterpenoid constitute several percent of dry weight of many plants. The bark of trees such the cork oak (*Quercus suber)* and the white

birch (*Betula alba)* contain large amounts of triterpenoids. Friedelin (**16**) and cerin (**22**) are the main compounds of cork (Croteau and Johnson, 1985).

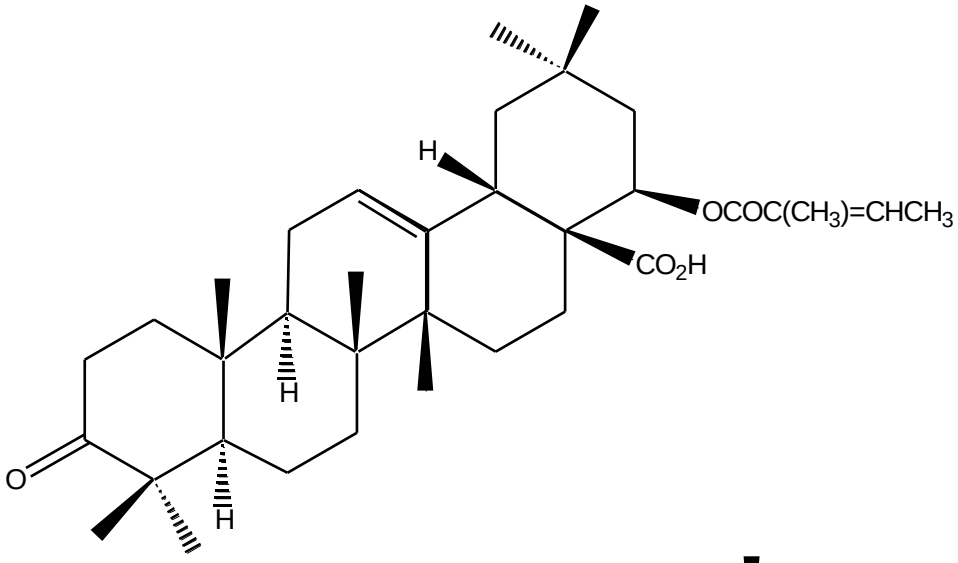
Having formed the basic triterpenoid skeleton, a variety of oxidations and skeletal rearrangements may occur leading to synthesis of biologically active triterpenoids, Examples of such highly oxygenated and bioactive triterpenoids are Jaquinonic acid (**23**) lantadene A (**24**), 22- hydroxytingenone (**25**) soyasapogenol B (**26**), tingenone (**27**) and papyriferic acid (**28**).



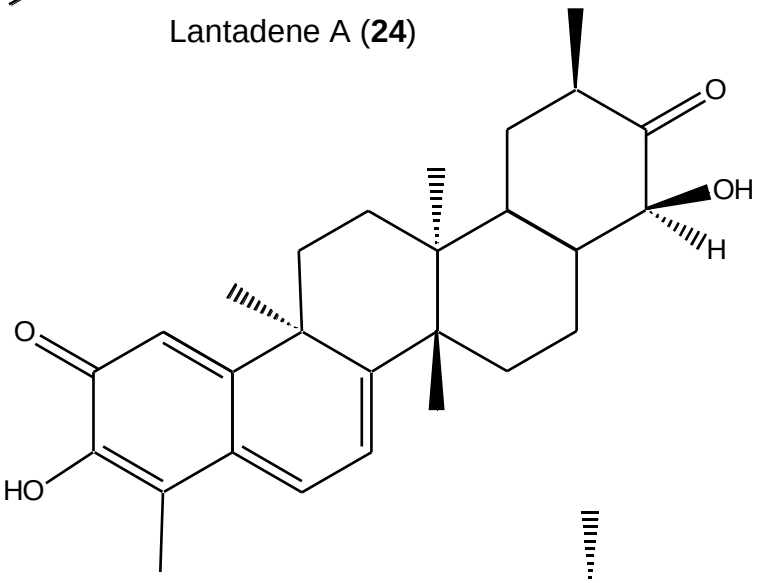
Euphol **(21)**



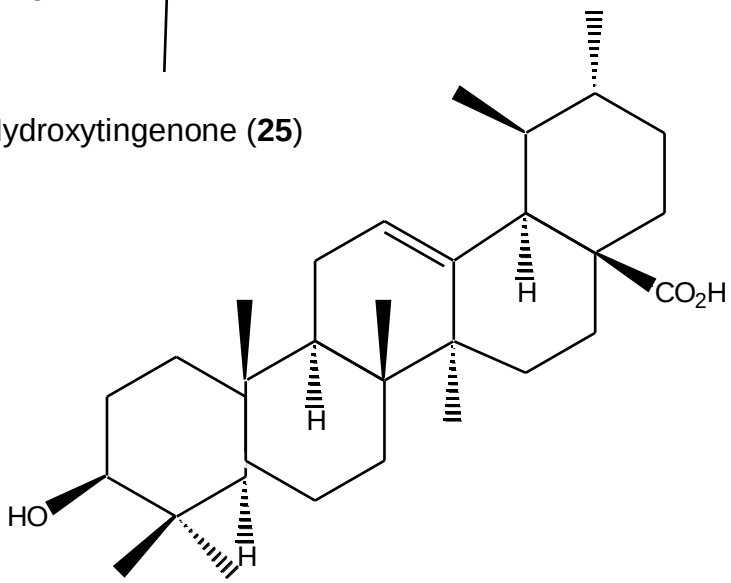
Cerin **(22)**



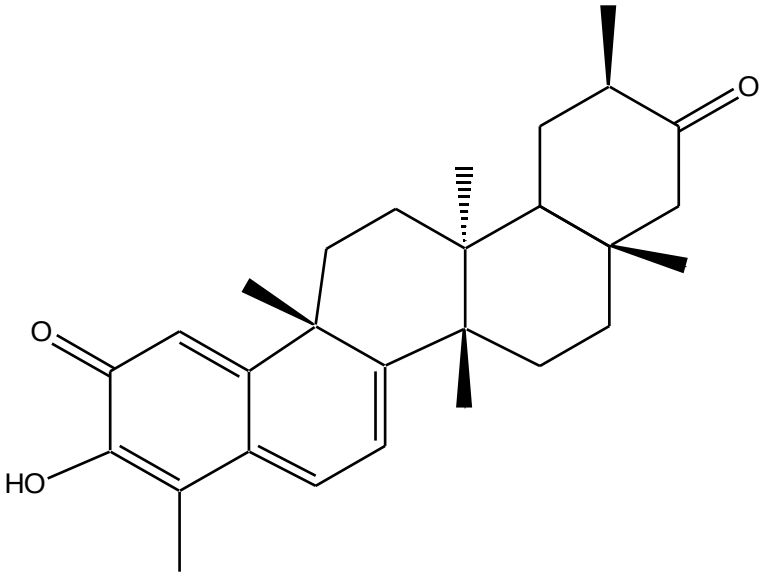
Lantadene A (**24**)



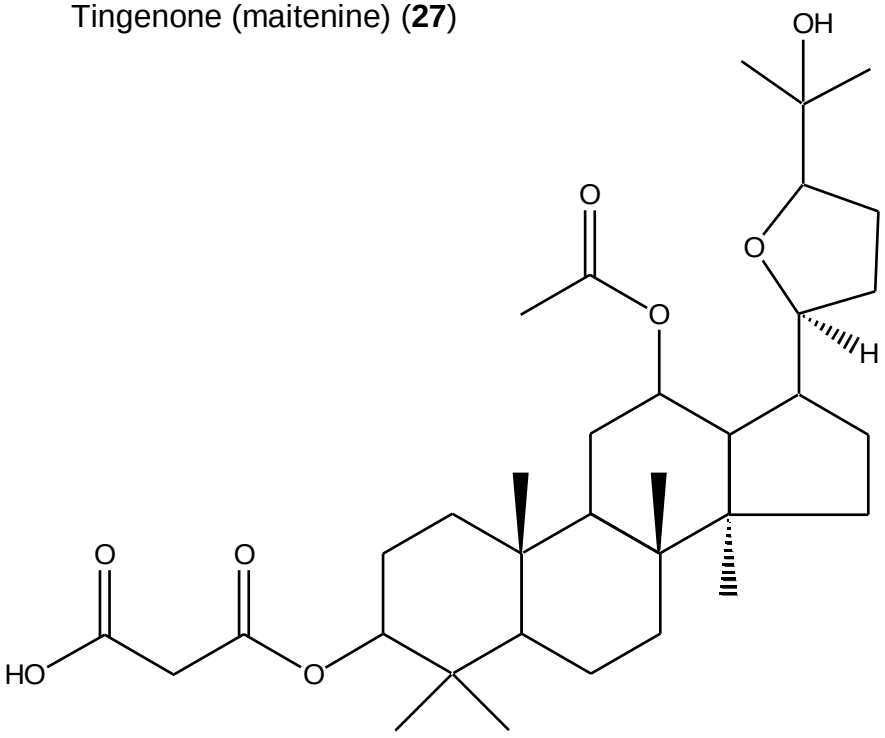
22- Hydroxytingenone (**25**)



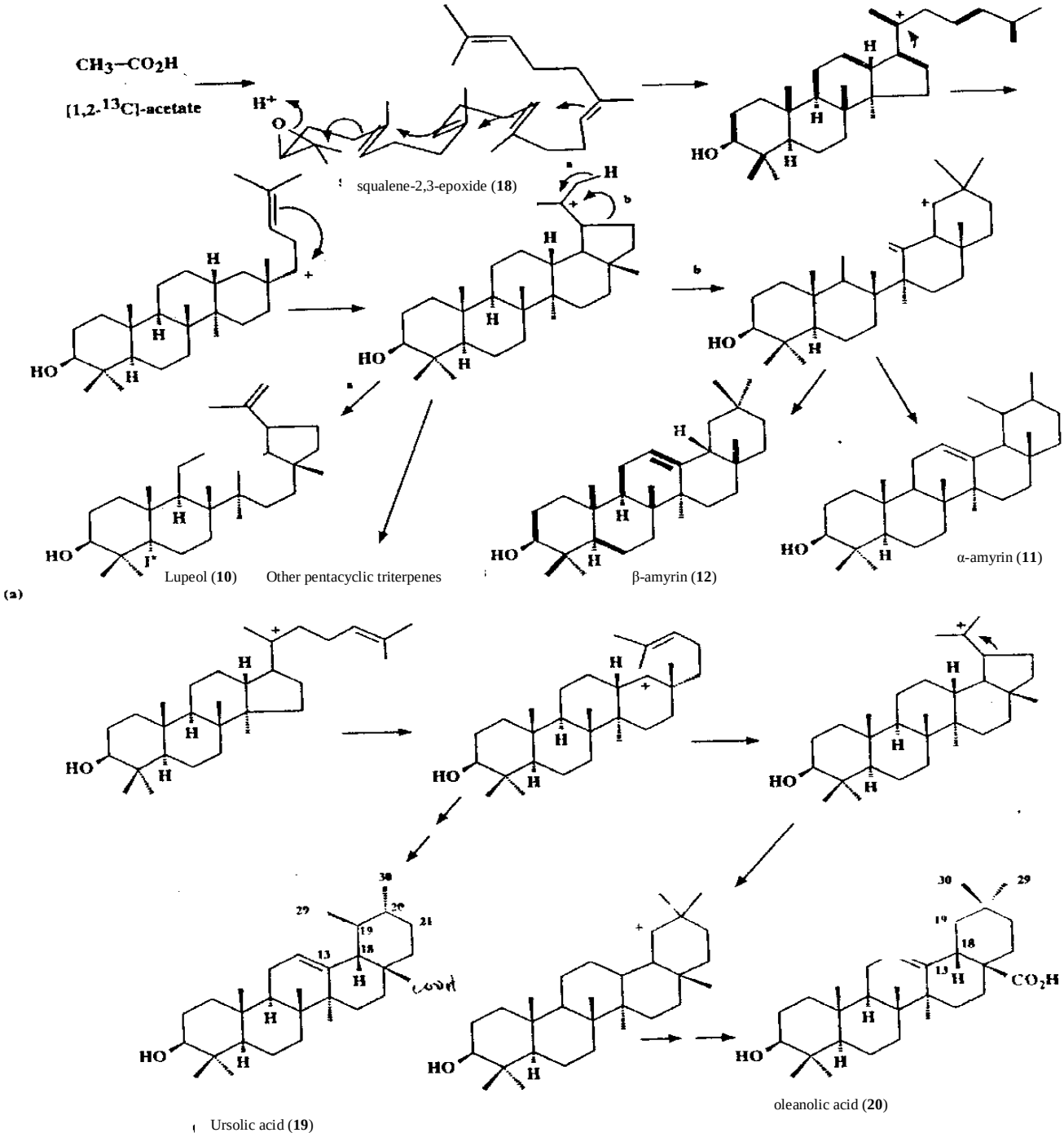
Soyasapogenol B (**26**)



Tingenone (maitenine) (**27**)



Papyriferic acid (**28**)



SCHEME 1: Biogenesis of pentacyclic triterpenes from chair-chair-chair-boat conformation of squalene-2, 3-epoxide

2.6 Biological Activity of Triterpenoids

Triterpenoids may be found in all tissues of the plant, but different organs within an individual plant may produce different types of triterpenoids. For instance, the Juvenile growth phase internodes of the Alaska paper birch (*Betula resinfera*) are made unpalatable to the snowshoe hare (*Lepus americanus*) by extremely high concentration of the triterpene papyriferic acid (**27**) (Harborne, 1996). This is more than 25 times the level in mature internodes.

Although triterpenes have been considered to be relatively innocuous plant constituents, several have been established to have pronounced physiological activity. Although, phytosteroids are required for insect and fungal growth, triterpenoids compounds may interfere with this process and exhibit anti-herbivore and anti-fungal effects (Croteau and Johnson, 1985). In general those that are highly oxygenated appear to be more active (Seigler, 1983). Indications that some triterpenoids may also have anti-bacterial activity (Bittner *et al*., 2002) suggests these compounds could have a broader role in plant defence. If this is so, one might expect to find an evolutionary trend of increasing oxidation and rearrangement to correlate with increasing activity against herbivorary and other potential plant enemies.

2.7 Statement of the Problem

Infectious diseases have been and are still a health problem to man. Some bacteria and fungi have become resistant to modern day antibiotics. The situation has been worsened

by HIV/AIDS epidemic which lowers the immunity of victims making them susceptible to opportunistic infections by bacteria and fungi. This has created the need to search for new antibiotics, perhaps with different structures to those of known antibiotics. The search for such compounds is directed to the plant kingdom. The present research was undertaken to investigate the antibiotic potential of the root-bark of *Vernonia auriculifera.*

2.8 Rationale and Justification

Vernonia species are known to yield extracts and compounds that exhibit biological activity against bacteria, fungi, protozoa and tumour cells. This implies that they can be potential practical sources of antibacterial, antifungal, anti-protozoa and anti-tumour drugs. Such drugs sourced from plants have added advantages (www.telmedpak.com/agriculture) since they have minimal side effects and that target organisms do not develop any clinical significant resistance to them. Hence there is need to look for alternative drugs of plant origin which have vast reserves of biologically active compounds. Furthermore, a lot of foreign exchange earnings that go to import conventional medicine can be saved by developing medicine from plant sources.

2.9 Hypotheses

1. *Vernonia auriculifera* **does not** contain triterpenoids that have anti-bacterial and anti-fungal activities.

2. Triterpenoids from *Vernonia auriculifera* **have** anti-bacterial and anti-fungal activities

2.10 Objectives of Study

2.10.1 General objective:

To screen for, isolate and characterize anti-bacterial and anti-fungal compounds from *V. auriculifera.*

2.10.2 Specific objectives

i. To screen for biologically activity in the *n*-hexane, dichloromethane and methanol extracts of *V. auriculifera*.
ii. To carry out chromatographic separation of the extracts.
iii. To identify the compounds by spectroscopic methods (IR, UV, MS and NMR)
iv. To carry out anti-bacterial and anti-fungal tests on isolated compounds.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 General Experimental Procedures

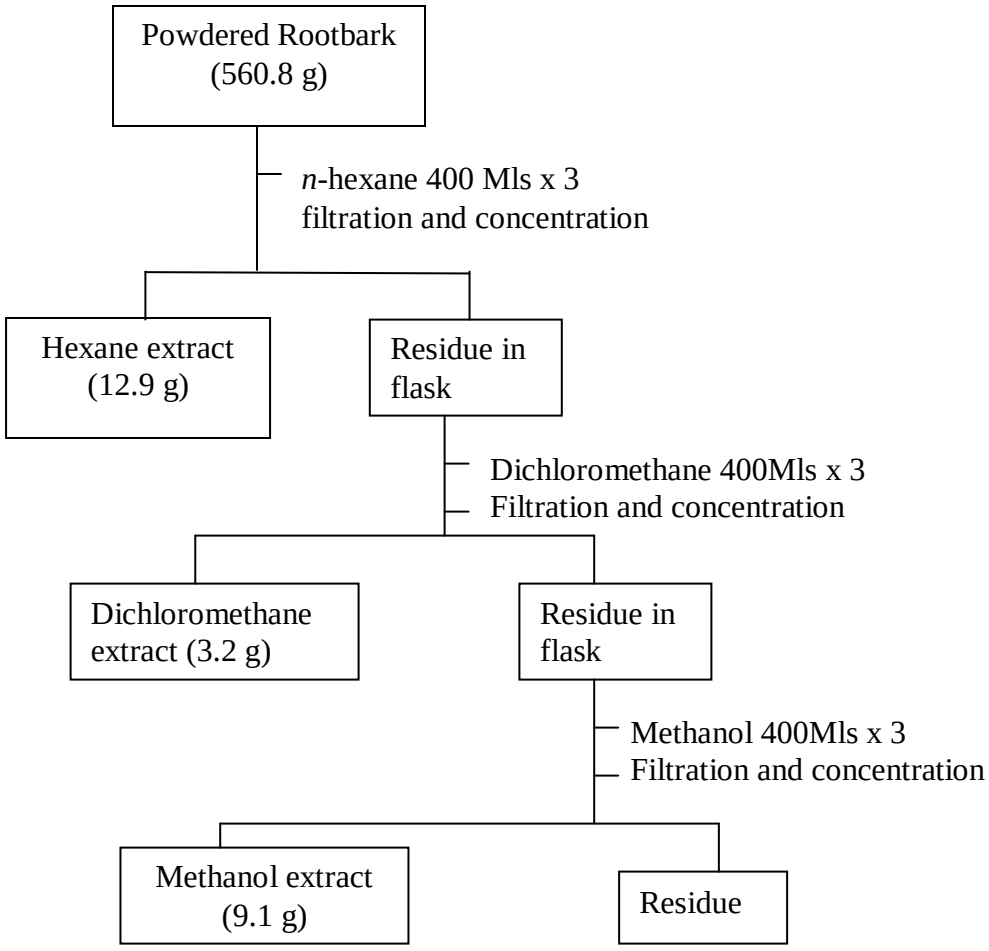
All recyclable glassware were soaked in chromic acid, washed with soap and rinsed with water. The glassware were then dried at 120° C for at least one hour. All solvents, reagents and TLC plates were obtained from Kobian Kenya Ltd. The solvents were distilled before use.

3.2 Plant Material

The root barks of the plant, *Vernonia auriculifera,* were collected from Kericho, Laikipia and Nyeri Districts with the help of a plant taxonomist. Voucher specimens have been kept in the herbarium of Botany Department, JKUAT, Nairobi, Kenya.

3.3 Solvent Extraction

The collected root bark were dried in a shade and thereafter grounded into powder. Approximately 564g of the powered root bark were soaked in *n*-hexane for 3 days. The mixtures were then filtered and the filtrate concentrated in vacuo to yield a crude extract. Further extraction using *n*-hexane was done with a repeat of two more times using the same procedure as in the first case. A total of 13 g of extract were obtained. To the residue remaining in the extraction flask after exhaustive extraction using *n*-hexane, DCM was added and a similar procedure was followed. A total of three extractions were done out of which 3.2 g of extract were obtained. Lastly, methanol was added to the residue in the flask and three extractions were carried out, yielding 9 g of the extract.



Key
Fr - Fraction

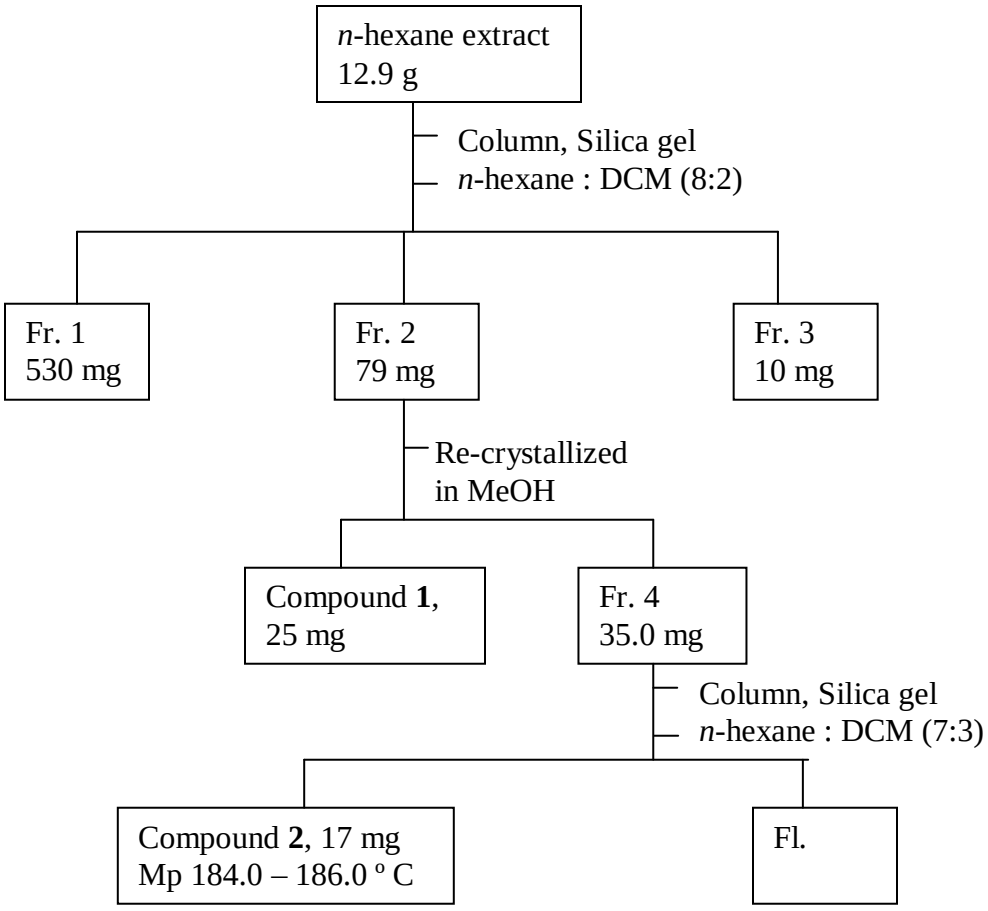
Scheme 2. Scheme showing successive extractions of crude extracts using *n*-hexane, dichloromethane and methanol.

3.4 Chromatographic Isolation of Compounds from *n*-hexane and dichloromethane extracts.

The TLC profile of each crude extract were carried out using various solvent systems to determine which solvent systems was appropriate for use in column chromatography. Each crude extract was then chromatographed on silica gel 60 (230-400 mesh) and several fractions were obtained. The fractions were fractionated further to yield pure products. Below are schemes showing chromatographic isolation of compounds from n-hexane and dichloromethane extracts respectively.

3.4.1 Isolation of Compound 1 and 2

The yellow paste of *n*-hexane extract (12.9 g) was subjected to column chromatography using *n*-hexane: DCM (8:2) as the eluting system. Four fractions (1,2,3 and 4) were obtained. Re-crystallisation of fraction 2 (79.0 mg) in methanol yielded compound **1** (45 mg). Further fractionation of fraction 4 (35.0 mg) on silica gel column using *n*-hexane: DCM (7:3) gave compound **2** (17 mg).



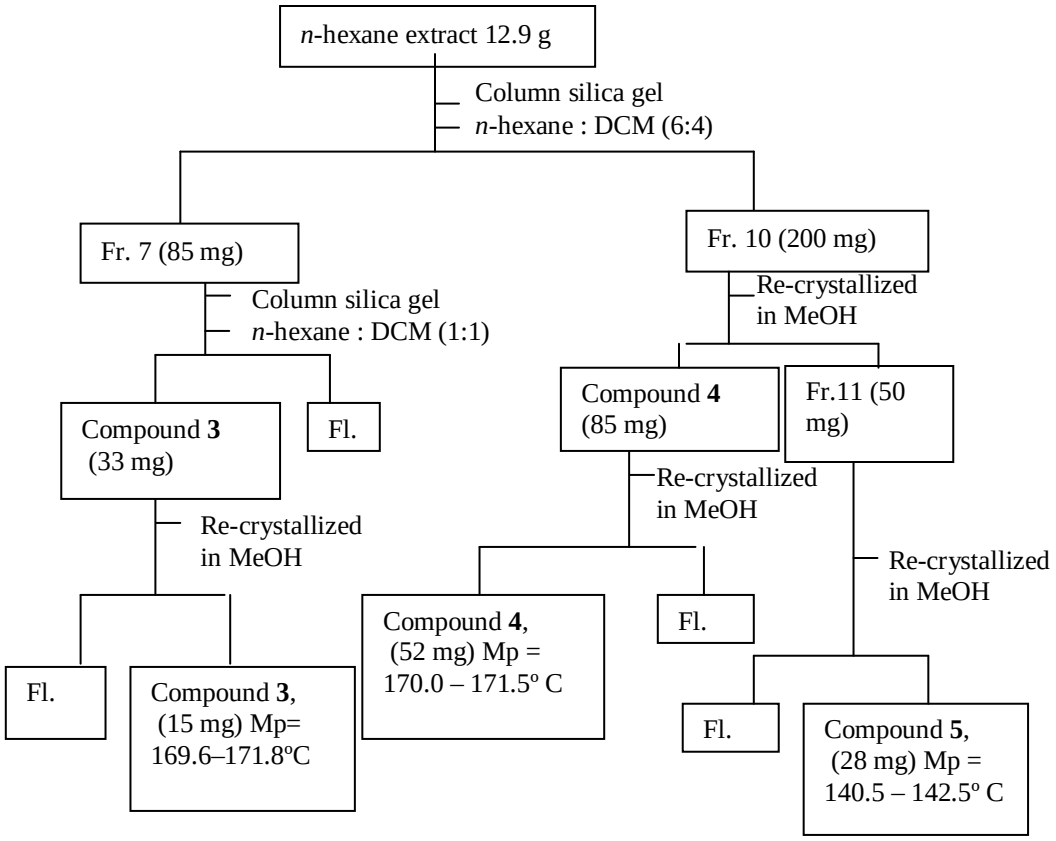
Key

Fr. – Fraction, Fl. – Filtrate, Mp – Melting point

Scheme 3. Scheme showing isolation of compounds **1** and **2** from *n*-hexane extract.

3.4.2 Compounds 3, 4 and 5

Elution of *n*-hexane extract with *n*-hexane: DCM (6:4) gave fractions 7 (85 mg) and 10 (200 mg). Further fractionation of fraction 7 on silica gel column using *n*-hexane: DCM (1:1) gave compound **3** (33 mg). Compounds **4** and **5** were obtained by recystallising fractions 10 and 11 in methanol respectively.



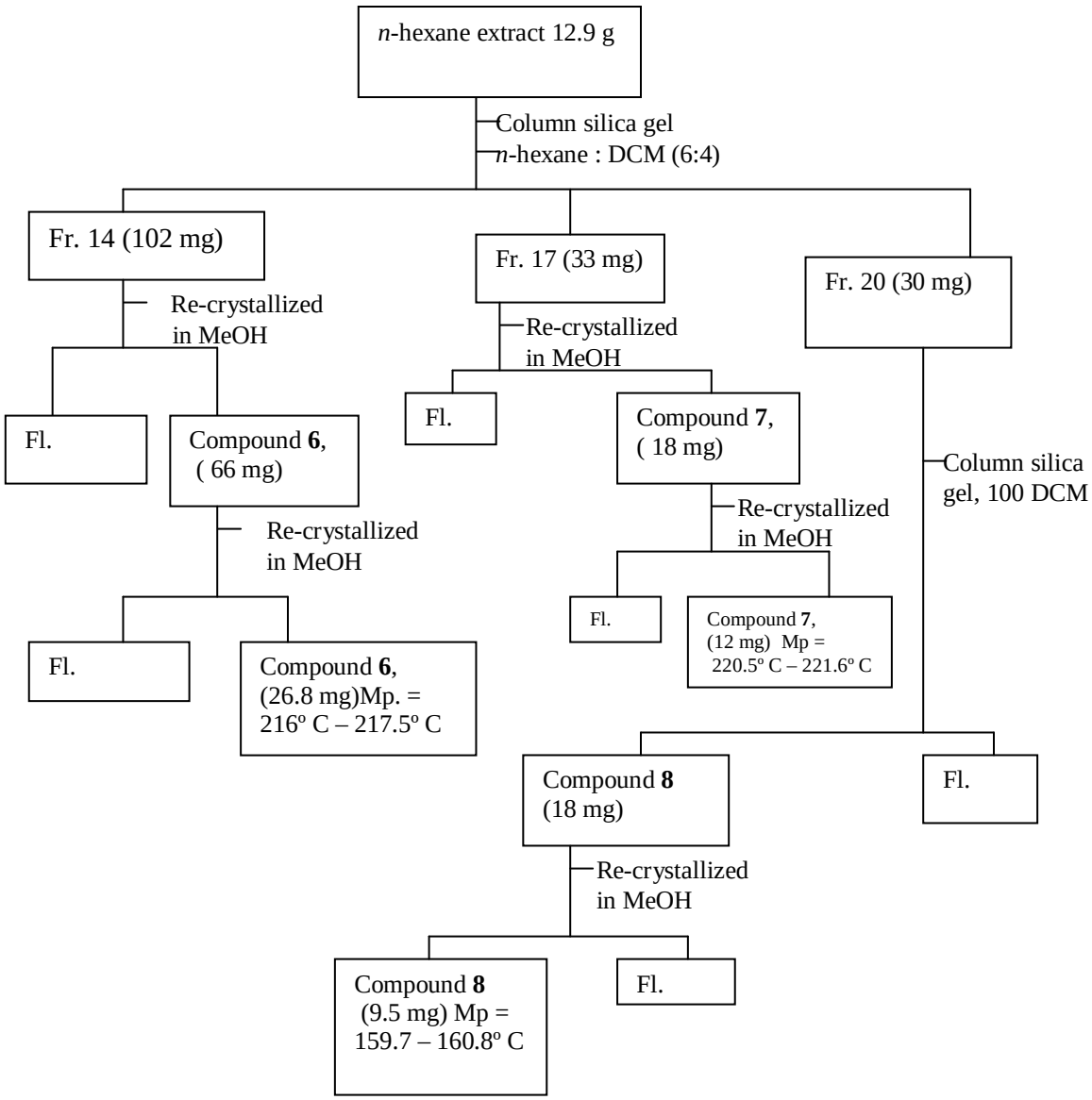
Key

Fr. – Fraction, Fl. – Filtrate, Mp – Melting point

Scheme 4. Isolation of compounds **3, 4 and 5**

3.4.3 Compounds 6, 7 and 8

Further elution of *n*-hexane extract with *n*-hexane : DCM (6:4) solvent system afforded fractions 14 (102 gm), 17 (33 mg) and 20 (30 mg). Fractions 14 and 17 were each re-crystallised in methanol to give compound **6** (26.8 mg) and **7** (12 mg) respectively. Fraction 20 (30 mg) was further fractionated with 100% DCM to yield compound **8** (9.5 mg).



Key

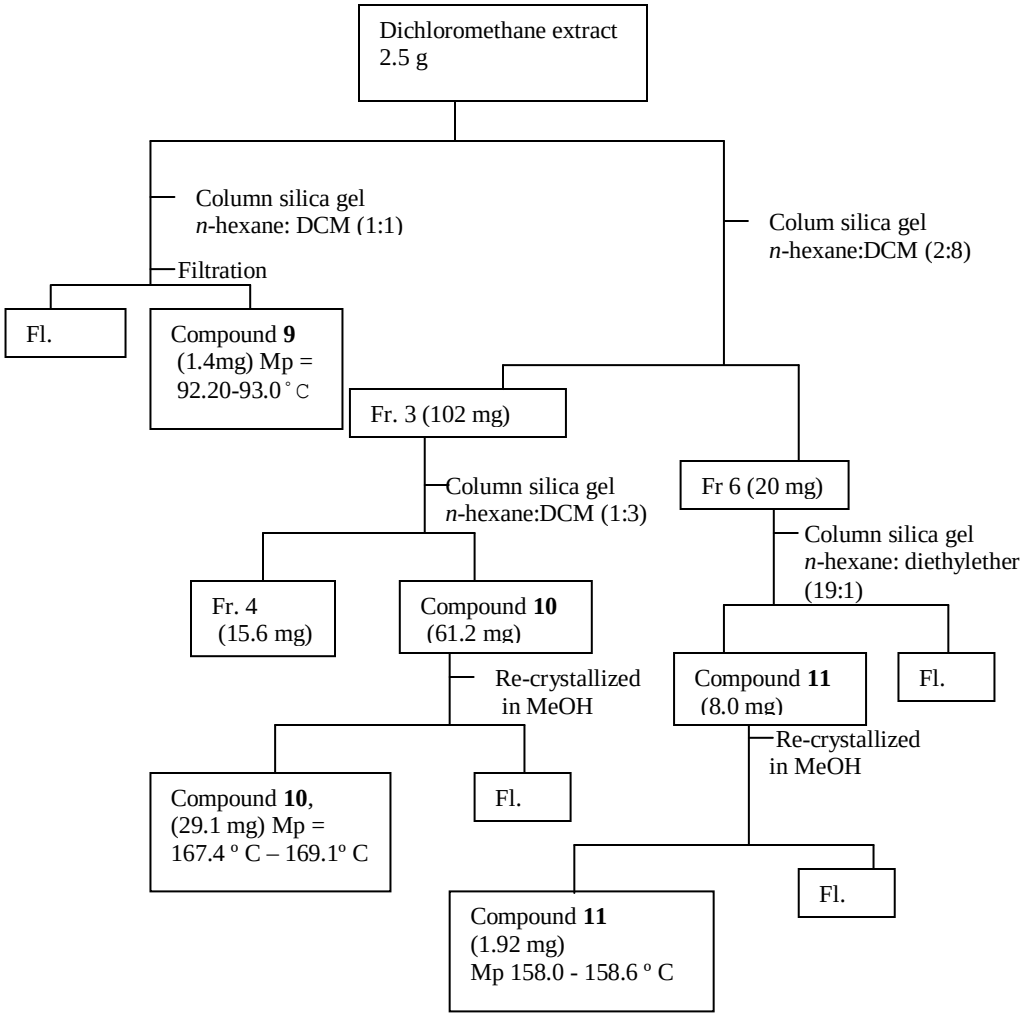
Fr. – Fraction, Fl. – Filtrate, Mp – Melting point

Scheme 5. Isolation of compounds **6, 7** and **8.**

3.4.4 Isolation of Compounds 9, 10 and 11

The dark brown paste of DCM was subjected to silica gel column chromatography.

Silvery-grey suspended in *n*-hexane: DCM (1:1) solvent system were obtained. Filtration was done and compound **9** (1.4 mg) was obtained. Elution of the column with *n*-hexane: DCM (2:8) system yielded fractions 3 and 6. Fraction 3 was further fractionated with *n*-hexane: DCM (1:3) to yield compound **10** (61.2 mg) which was purified by re-crystallizing in methanol. Compound **11** (1.9 mg) was obtained by further fractionation of fraction 6 using DCM: diethyl ether (19:1) followed by re-crystallization in methanol.



Key

Fr. – Fraction, Fl. – Filtrate, Mp – Melting point

Scheme 6. Scheme showing the isolation of compounds **9, 10** and **11.**

3.5. Biological Activity Tests

3.5.1 Brine shrimp Toxicity Bioassay

Brine shrimp (*Antemia salina*) toxicity bioassay (Alkohafi *et al.*,1989), with some modification (Keriko, 1995), was conducted as follows. Artificial sea water was prepared dissolving 38 g of sea salt (Five plan type) in distilled water (I L).

Brine shrimp eggs were added to about 80 ml of artificial sea water in a 100 ml beaker to give a large number of brine shrimp larvae (*Nauplii*) after an incubation period of 48 hours at 24º C. With a few millimetres of artificial sea water in 10 ml calibrated containers, brine shrimps 10 per container were introduced followed by addition of pre-prepared samples in Dimethyl sulphoxide (DMSO) to give the desired concentrations in parts per million (ppm). Finally artificial sea water was added to make up to the mark. A container into which DMSO alone was added served as a control. All experiments were done in triplicate. All the containers were covered and transferred into an incubator at 28ºc for a period of 24 hours. After this period of incubation, the numbers of dead and surviving brine shrimps were recorded and the values converted into percentages relative to the control (% mortality).

Effects of various concentrations (900, 800, 600, 400, 200 and 100 ppm) of different extracts on brine shrimps were quantified in terms of percentage mortality (%). The following formula was used to compute mortality.

$$\% \text{ Mortality} = \frac{(\text{No alive in control} - \text{No alive in treatment}) \times 100}{\text{No. Alive in control}}$$

$$= \frac{\text{Dead in test} \times 100}{\text{No Alive in control}}$$

The mean percentages (%) and lethal doses (LD_{50}) were computed using Statistical Analysis System (SAS), version 8.2.

3.5.2 Anti-Bacterial Bioassay

In this assay, paper disc methods was adopted (Brookes, *et al.*, 1991). It involved sub-culturing gram positive *S. aureus* and gram negative *E. coli* bacteria into sterilized nutrient broth for 24 h and then inoculating them into sterilized nutrients agar contained in a Petri-dish. A blotting or filter paper cut out so as to have protruding parts was placed carefully into the Petri-dish. Around the tips of the protruding parts of the paper 20 µl of the desired concentration of each extract or compound in a suitable solvent was uniformly applied together with a control into which 20 µl of the solvent was used alone. All experiments were done in triplicate. The set-ups were run at 37º C and the results obtained after 24 h. Growth inhibition of bacterial around and close to the tips of the filter paper was an indication of positive results while negative results were observed when there was growth around these regions as compared to the corresponding control. The inhibition zone was determined by measuring the diameter in millimetres (mm) of the circular region around each paper (disc) using a ruler.

3.5.3 Anti- Fungal Assay Test

A similar experimental procedure for anti-fungal test, using *candida albicans*, was conducted like that of anti-bacterial tests above. The difference being on the growth medium. In this case, potato dextrose agar was used and the experiments took 48 hours.

The inhibition of bacterial and fungal growth was done by comparing the mean inhibition diameters at various concentrations and relating them to the control.

The mean and the standard deviations were computed using Microsoft Excel statistical programme.

3.6 STRUCTURAL ELUCIDATION EXPERIMENTS

3.6.1 Chromatography

Column chromatography was performed on silica gel 60 (0.040-0.063 mm, 230-400 mesh) and thin layer Chromatography (TLC) on pre-coated silica gel 60 F_{254} plates (0.2 mm thickness, Merck).

3.6.2 Ultraviolet Spectroscopy (UV)

The ultra-violet (UV) spectra of purified substances was determined using a shimadzu 1601 PC spectrophotometer UV maximum was determined in dichloromethane.

3.6.3 Fourier Transform Infra-red Spectroscopy (FT-IR)

The IR spectra of the compounds were recorded using Fourier transform infrared spectrophotometer shimadzu (FT IR-8400CE). The samples were prepared using spectral grade KBr and made into pellets. The spectrum were recorded in the range between 4000 and 500 cm^{-1}

3.6.4 Nuclear Magnetic Resonance (NMR) Experiments

NMR was recorded in Gemini 300 MHz and Varian XL-200 MHz spectrometers at room temperature using $CDCl_3$ as the solvent. Chemical shifts (δ) for ^{1}H-NMR and ^{13}C-NMR were recorded in parts per millions (ppm) relative to tetramethyl silane (TMS). ^{13}C-NMR were observed at 100 MHZ and 50 MHZ respectively.

3.6.5 Physical and Spectroscopic Data of Compounds

Compound (**1).** White Amorphous solids Mp 199.0-200.2 º C, (uncorrected) IR υ max (KBr disc, cm^{-1}) 3446, 2855, 1730, 1654, 1458, 1366, 1250, 1202, 1146, 1002, 935, 810, 658, 607, UV_{λ} max CH_2CL_2 227 nm.

Compound (**2).** White Amorphous solids; Mp 184.0 – 186.0 º C; (uncorrected) IR υ max (KBr disc, cm^{-1}) 3423, 2925, 1734, 1654, 1456, 1367, 1245, 1145, 10003, 901, 806, 651, 471, UV_{λ} max CH_2CL_2 227 nm.

Compound (**3).** White crystals; Mp 169.6 – 171.8 º C; (uncorrected) IR υ max (KBr disc, cm^{-1}) 3421, 2857, 2372, 1652, 1458, 1381, 1247, 1136, 1037, 996, 807, 660, 468, UV_{λ} max CH_2CL_2 227 nm.

Compound (**4).** White Amorphous solids; Mp 169.5 – 170.8 º C; (uncorrected) IR υ max (KBr disc, cm^{-1}) 3425, 2936, 2362, 1535, 483, UV_{λ} max CH_2CL_2 220 nm.

Compound (**5).** White Amorphous solids; Mp 140.5 – 142.5 º C; (uncorrected) IR υ max (KBr disc, cm^{-1}) 3400, 2854, 1732, 1653, 1459, 1381, 1251, 1182, 1038, 994, 920, 879, 822, 776, 657, UV_{λ} max CH_2CL_2 220 nm.

Lupeol (6) (C_{3O} H_{5O} O). Amorphous solid, Mp. 216– 217.5º C; (uncorrected) IR υ max cm^{-1}, 3422, 2938, 1637, 1509, 1457, 1382, 1252, 1185, 1039, 875, 632, UV_{λ} max CH_2CL_2 220 nm. ^{13}C-NMR spectral data as shown in Table 7.

α- Taraxerol octanoate (7) (C_{39} H_{66} O_2).. White amorphous solid, Mp. 220.5 – 221.0 º C; (uncorrected) IR max. cm^{-1}, 2919, 1725, 1651, 1511, 1464, 1373, 1261, 1096, 1022, 802, 719, 669; UV_{λ} max CH_2CL_2 220 nm. This compound was partially elucidated. ^{13}C-NMR spectral data shown in Table 8 and ^{1}H-NMR data shown in figure 9 in the appendix.

Compound (**8).** White Amorphous solids; Mp 159.7 – 160.0 º C; (uncorrected) IR υ max cm^{-1}, 3423, 2936, 1652, 1460, 1378, 1054, 967, UV_{λ} max CH_2CL_2 227 nm.

Compound (**9).** Grey crystals; Mp 92.2 – 93.0 º C; (uncorrected) IR υ max cm^{-1}, 3401, 2918, 2849, 1735, 1652, 1560, 1460, UV_{λ} max CH_2CL_2 220 nm.

Compound (**10).** White Amorphous solids; Mp 167.4 – 169.1 º C; (uncorrected) IR υ max cm^{-1}, 3421, 2937, 1651, 1512, 1458, 1381, 1038, UV_{λ} max CH_2CL_2 227 nm.

Compound (**11).** White Amorphous solids; Mp 158.0 – 159.5 º C; (uncorrected) IR υ max cm^{-1}, 3421, 2935, 1651, 1459, 1378, 1056, UV_{λ} max CH_2CL_2 227 nm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 General Chapter Overview

In this chapter, details of biological assays on crude extract and isolated compounds are reported. Spectral characterisation of the triterpenoids isolated from the root bark of *V. auriculifera* are reported.

4.2 Brine shrimp bioassays for crude extracts

There was an indication of toxic compounds in all the three extracts of the plant rootbark. The activities of the extracts are given in Table 2.

Table 2: Mean percentage toxicity activity ± standard deviation of extracts of *Vernonia auriculfera* against Brine shrimps (*Artemia salina).*

EXTRACT	DOSE (PPM)						LD50
	100	200	400	600	800	900	
n- hexane	Be 0.0 ± 0.0	Bde 10.0 ± 0.0	Bcd 26.7 ± 3.3	Bbc 33.3 ± 8.8	Bab 46.7 ± 3.3	Ba 60.0 ± 0.0	788.2
dichloromethane	Ac 20.0 ± 5.8	Acb 30.0 ± 0.0	Ab 50.0 ± 5.8	Aa 80.0 ± 0.0	Aa 80.0 ± 5.8	Aa 80.0 ± 5.8	318.1
methanol	Bc 0.0 ± 0.0	Cc 0.0 ± 0.0	Cc 0.0 ± 0.0	Ccb 6.7 ± 3.3	Cb 20.0 ± 5.8	Ba 46.7 ± 3.3	936.8

Mean values with the same letters (Capital) within the same dose are not significantly different at 95% confidence limit.

Mean values with the same letters (Small) within the same extract are not significantly different at 95% confidence limit.

The results showed that larvicidal activity generally increased with increasing doses. However, dose response showed that the methanol extract had no activity between 100 and 400 ppm. After 400 ppm, its activity only increased marginally reaching a mortality of 47% at 900 ppm.

The *n*-hexane extract had enough activity to cause mortality at concentration above100 ppm. The larvicidal activity is dose dependent and reaches a maximum mortality of 60% at 900 ppm. The most lethal extract is the DCM, which has a mortality of 20% at 100 ppm. The dose dependent relationship is clearly evident and a mortality of 80% is achieved at 600 ppm. Beyond this concentration mortality does not change. The toxic compounds in DCM extract had reached a maximum and could kill no more brine shrimps beyond the concentration of 600 ppm.

Thus, DCM extract with an LD_{50} of 318.8 was most active of all the extracts. It's toxicity being three fold when compared with the methanol extract whose LD_{50} is 937. Similar findings were reported Keriko, *et al.,* (1995). Although this test is used to predict anti-tumour activity, it can nevertheless be useful in searching for anti-biotic principles.

4.3 Anti-bacterial Assay

The anti-bacterial tests indicate the presence of anti-bacterial components in both crude extracts and isolated compounds. Activity was found to be higher against *E. coli* than on *S. aureus* particularly with *n*-hexane extract and methanol extract. Activity of DCM

extract on both bacterial was low for all concentrations and nearly the same in both cases. These activities are given on Table 3.

Table 3: Mean Inhibition Diameters (mm) ± S.D of extracts of *V. auriculifera* on *E. coli* and *S. aureus*

		E. coli	*S. aureus*
EXTRACTS	Conc. (ppm)	Mean inhibition diameter (mm) ± S.D	Mean inhibition diameter (mm) ± S.D
n-Hexane	1000	10.3 ± 0.6	8.7 ± 0.6
n-Hexane	500	9.7 ± 0.6	8.7 ± 0.6
n-Hexane	250	8.0 ± 0.0	7.0 ± 0.0
DCM	1000	8.8 ± 0.3	8.5 ± 0.5
DCM	500	8.3 ± 0.6	7.7 ± 0.6
DCM	250	8.0 ± 0.0	7.8 ± 0.3
MeOH	1000	11.9 ± 0.2	8.7 ± 0.3
MeOH	500	10.3 ± 0.6	8.5 ± 0.5
MeOH	250	8.3 ± 0.6	8.2 ± 0.3
Control		6.0 ± 0.0	6.0 ± 0.0

The generally low activity of extracts on *S. aureus* is attributed to its high resistance to antibiotics. Both n-hexane and methanol extracts were significantly active against *E. coli* at concentration of 500 ppm and above and had inhibition zones of about 10 mm. Methanol extract in particular had the highest inhibition diameter of 11.9 mm. The explanation of this activity could be due to high concentration of anti-bacterial compounds in the two extracts and also perhaps those compounds could enhance this activity through synergistic action. At 250 ppm activity of both *n*-hexane and methanol

extracts on *E. coli* had only mild effects. This may be due to low concentration of anti-bacterial compounds.

Fractionation of the extracts yielded 11 compounds. From *n*-hexane extract compounds **1-8** were isolated and DCM extracts **9-11**. When bacterial assays were done on the compounds, the compound appeared to have generally more activity on *S. aureus* than the original extracts from which they were derived. The results are summarised in Table 4.

Table 4: Mean Inhibition Diameters (mm) ± Sd of the Compounds of *V. auriculifera* on *E. coli* and *S. aureus.*

	E.coli	*S.aureus*
Compounds (1,000ppm)	Mean inhibition diameter (mm) ± S.D	Mean inhibition diameter (mm) ± S.D
1	9.7 ± 0.6	9.3 ± 0.6
2	9.2 ± 0.8	8.3 ± 0.6
3	10.7 ± 0.6	9.0 ± 0.0
4	10.0 ± 0.0	8.3 ± 0.6
5	8.0 ± 0.0	10.3 ± 0.6
6	9.3 ± 0.6	9.0 ± 0.0
7	9.0 ± 0.0	11.3 ± 0.6
8	8.7 ± 0.6	9.0 ± 0.0
9	10.3 ± 0.6	10.7 ± 0.6
10	10.0 ± 1.0	10.0 ± 1.0
11	12.0 ± 0.0	9.0 ± 1.0
Control	6.00 ± 0.0	6.0 ± 0.0

It was noted that DCM extract which had very low activity against both organisms, *E. coli* and *S. aureus,* gave products which had very high activity on both bacteria.

Fractionation in this case appear to increase concentration of the anti-bacterial components and hence increased activity.

When inhibition diameters of compounds **6** and **7** were compared on both bacteria, both were found to affect *E. coli* in nearly the same way. However compound **7** was found to be more active on *S. aureus* (11.33 mm) than *E. coli* (9.00 mm). The fact that compound **7** was reasonably active against *S. aureus* makes it a suitable candidate for further investigation in control of this multi-drug resistant bacteria. Perhaps it could act synergistically with other anti-biotics and hence help in taming this bacteria.

In general the two triterpenoids exhibited only mild activity against the two bacteria. This is not strange because bridged triterpenoids of oleanane type isolated from stem bark of *Embelia schimperi* were found to exhibit similar activities against gram positive strain of *rhodococcus* species (Bittner *et al.,* 2002). Perhaps in their defensive role against plants enemies, triterpenoids may be acting synergistically with other classes of compounds to ward off potential predators.

4.4 Anti-Fungal Bioassay

Of the three extracts only *n*-hexane and DCM had significant activity against *Candida albicans* at 1000 ppm (Inhibition diameter 10.7 mm). There was little activity below this concentration, see Table 5.

Table 5: Mean Inhibition Diameters (mm) ± Sd of extracts of *V. auriculifera* on *C. albicans*

C. albicans		
Extracts	Conc. (ppm)	Mean inhibition diameter (mm) ± S.D
n-Hexane	1000	10.7 ± 0.6
n-Hexane	500	7.7 ± 0.6
n-Hexane	250	8.3 ± 0.6
DCM	1000	10.7 ± 0.6
DCM	500	8.8 ± 0.3
DCM	250	8.7 ± 0.6
MeOH	1000	8.3 ± 0.6
MeOH	500	7.8 ± 0.8
MeOH	250	8.0 ± 0.0
Control		6.0 ± 0.0

On fractionation the only products with significant activity were compound **1**, which was derived from *n*-hexane extracts and compound **11**, derived from DCM extract. Comparison of inhibition diameter of compound **1** and *n*-hexane extract show that their diameters are nearly the same see Table 6.

Table 6: Mean inhibition diameters (mm) ± S.D of the compounds of *V. aurculifera* on *C. albicans*

C. albicans	
Compounds (1000 ppm)	Mean inhibition diameter (mm) ± S.D
1	10.7 ± 0.6
2	7.0 ± 0.0
3	8.0± 0.0
4	9.3 ± 0.6
5	9.3 ± 0.6
6	8.3 ± 0.6
7	8.0 ± 0.0
8	9.2 ± 1.0
9	8.7 ± 1.2
10	7.0 ± 0.0
11	10.0 ± 0.0
Control	6.0 ± 0.0

This suggests that the total blend of compounds in the extract had no significant synergistic effect. Thus, the activity can be ascribable to product 1. A similar situation is observed with DCM extract and compound **11**. Again the activity can be attributed to compound **11**.

4.5 Structure Elucidation of isolated compounds.

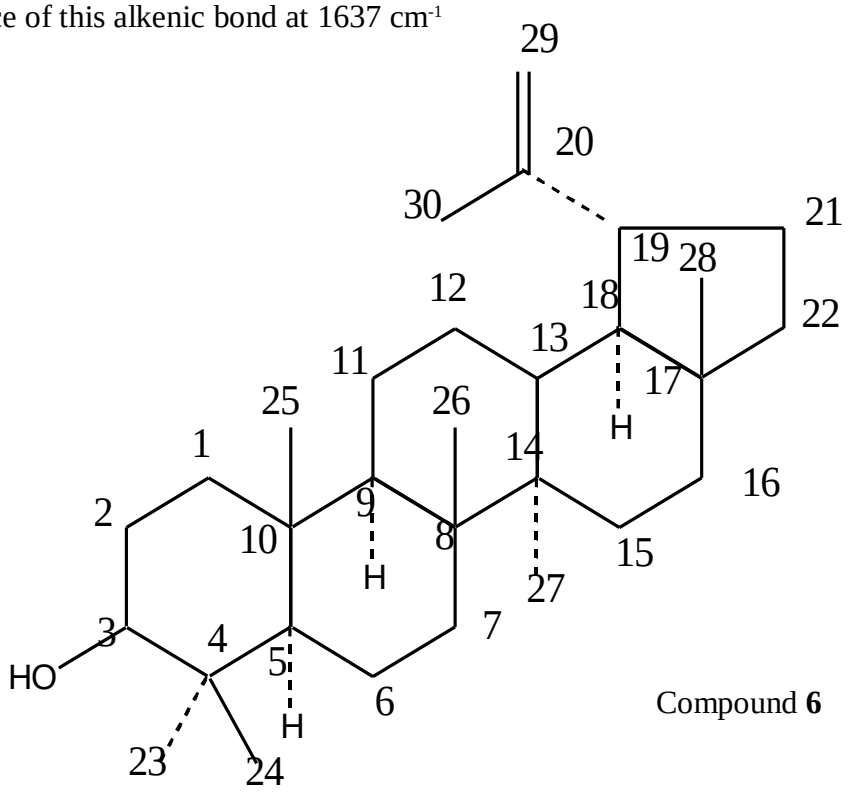
All the 11 possible compounds were obtained from silica gel column chromatography. They were later re-crystallised in methanol to yield white powders. It was only possible to elucidate compounds **6** and **7** through analysis of their NMR, IR and UV spectral data. However, appearance, melting points and FT-IR Spectral data for compounds **1, 2, 3, 4, 5, 8, 9 10,** and **11** are reported.

4.5.1 Compound 6

Compound **6**, with a molecular formulae $C_{30}H_{50}O$ was identified as Lupeol by comparison of its ^{13}C-NMR spectral data with that of Lupeol in Table 7 (Mahato and Kundu, 1994).

The signal at δ 79.2 is evidence of a carbon attached to an oxygen atom. This carbon is attached to an OH group and this is reinforced by an infra red absorption at 3422 cm^{-1}. The methine proton from this carbon was observed at δ 3.2

The exocyclic double bond (bonds outside the ring system) between C-20 and C-29 is supported by a quarternary chemical shift at δ 150.9 and the olefinic methylenic carbon at δ 109.2 at C.29 which bears two protons. The existence of two olefinic protons is confirmed by ^{1}H-NMR which occur at δ 4.68 and δ 4.56. These protons are different due to hindered rotation by the double bond. The infra red spectroscopy also shows the presence of this alkenic bond at 1637 cm^{-1}



Compound **6**

Table 7: Compares the ^{13}C-NMR data for Lupeol and compound **6.**

^{13}CNMR SPECTRUM FOR LUPEOL AND COMPOUND **6**			
CARBON	LUPEOL	COMPOUND **6**	TYPE OF CARBON
1	38.7	38.9	CH_2
2	27.4	27.6	CH_2
3	79.0	79.2	CH
4	38.3	38.8	C_q
5	55.3	53.4	CH
6	18.3	18.4	CH_2
7	34.2	34.3	CH_2
8	40.8	40.9	C_q
9	50.4	50.5	CH
10	37.1	37.2	C_q
11	20.9	21.2	CH_2
12	25.1	25.3	CH_2
13	38.0	38.1	CH_2
14	42.8	42.9	CH
15	27.4	27.5	CH_2
16	35.5	35.7	CH_2
17	43.0	43.1	C_q
18	48.2	48.4	CH
19	47.9	48.0	C_q
20	150.9	150. 9	C_q
21	29.8	29.9	CH_2
22	40.0	40.1	CH_2
23	28.0	28.1	CH_3
24	15.4	15.5	CH_3
25	16.1	16.2	CH_3
26	15.9	16.1	CH_3
27	14.5	14.7	CH_3
28	18.0	18.0	CH_3
29	109.3	109.3	CH_2
30	19.3	19.4	CH_3

Key

C_q Quarternary carbon

4.5.2. Compound 7

Compound **7** was partially elucidated as an ester of a fatty acid and a triterpenol. Evidence from ^{13}C-NMR indicated thirty eight carbon atoms. This implied a triterpene (thirty carbon atoms) attached to a long chain fatty acid.

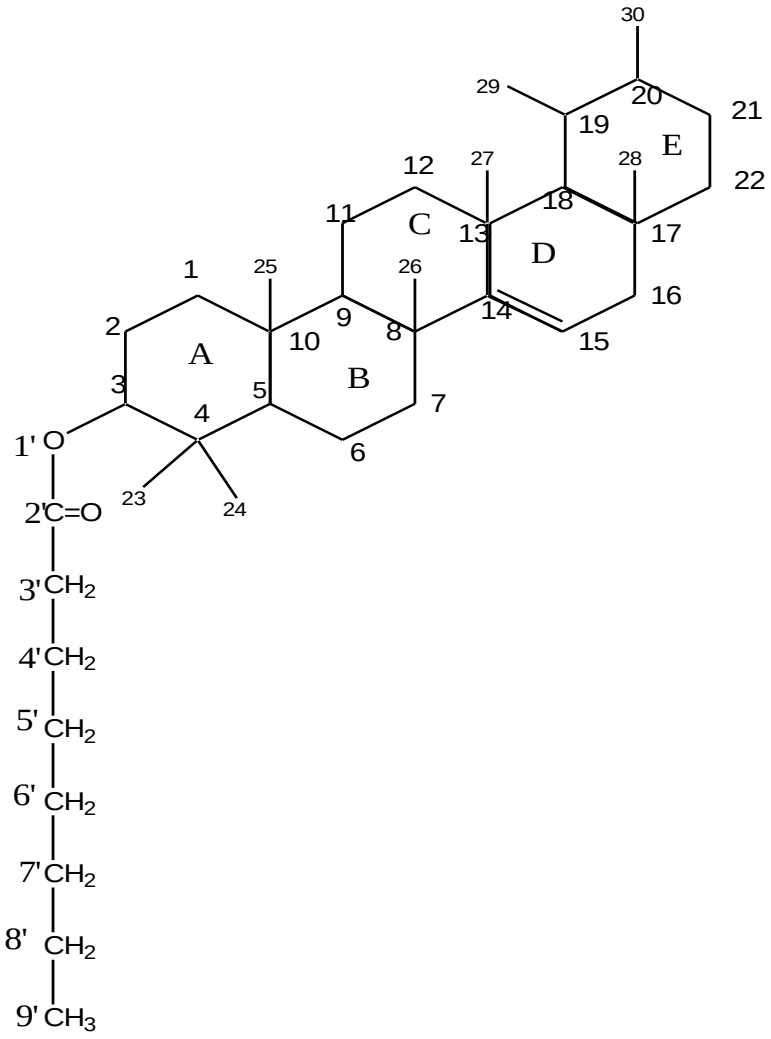
The presence of an ester linkage can be deduced from a chemical shift for a carbon attached to an oxygen atom at δ 79.2 and a carbonyl carbon atom at δ 172.6. This was further supported by Infra-red absorption at 1725 cm^{-1}

A double bond involving a quaternary carbon was evident from the signals at δ 155.9 and 117 .0. The presence of this bond was reinforced by an olefinic proton at δ 5.5 and infra red absorption at 1651 cm^{-1}. The ^{13}C-NMR shifts of the double bond of this compound is in close agreement with those of taraxerol which had a tertiary carbon at δ 117.0 at position C-15 and a quarternary carbon at δ 158.1 at position 14 (Mahato and Kundu, 1994). This suggested that the triterpenoid component had a partial taraxerol structure. However, α-amyrin provided some suggestion on the ^{13}C-NMR signals that were deviating from taraxerol. Therefore the methine signals appearing at 50.9 and 52.4 were assigned to C-19 and C-20 respectively but interchangeable. Presence of the double bond comparable to that of taraxerol in the D-ring can account for the shift to lower field of the two methine signals when compared to that of α-amyrin.

The long hydrocarbon chain is supported by the DEPT analysis. This indicated 9 methyl groups and 7 methine carbons but it was not possible to estimate the number of

methylene carbons because there was a dense overlap of shifts belonging to methylene groups at about a δ 30. This is attributed to overlap of the $–CH_2$ carbons of both the pentacyclic triterpene and the long chain hydrocarbon.

The proposed structure of pentacyclic triterpenoid (PCTT) closely compared to that of taraxerol. This arrangement is similar to that of α-amyrin where two extra methine carbons can be accounted for as observed in the DEPT experiment. The DEPT experiment further supports the existence of side chain due to the additional methyl carbon at δ 13.1. It is further proposed that the 8 extra carbon atoms can be used to estimate the CH_2 groups. This would result in 6 CH_2 groups after removing the methyl group and the ester carbon atom. This would make the number of methylenic carbons of the side chain to be 6.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15
A
3
5
B
7
4
1' O
6
23
24
2' C=O
3' CH2
4' CH2
5' CH2
6' CH2
7' CH2
8' CH2
9' CH3

Compound **7**

Table 8: Compares the ^{13}C-NMR data for compound **7**, taraxerol and α-amyrin.

C No.	Compound **7** (δ)	Taraxerol (δ)	α-amyrin (δ)
1	37.8	38.1	38.7
2	27.7	27.3	27.2
3	79.2	79.2	78.3
4	39.1	39.1	38.7
5	53.5	55.7	55.2
6	18.4	19.0	18.3
7	34.3	35.3	32.9
8	36.6	38.9	40.0
9	47.0	48.9	47.7
10	35.4	37.9	36.9
11	17.7	17.7	23.3
12	34.2	35.9	124.3
13	36.4	37.9	139.3
14	155.9	158.1	42.0
15	117.0	117.0	28.7
16	36.8	36.9	26.6
17	37.1	38.1	33.7
18	57.1	49.4	58.9
19	50.9[a]	41.4	39.6
20	52.4[a]	29.0	39.6
21	33.7	33.9	31.2
22	32.6	33.2	41.5
23	28.1	28.1	28.1
24	15.6	15.6	15.6
25	15.9	15.6	15.6
26	29.2	30.1	16.8
27	26.0	26.0	23.3
28	30.9	30.1	28.1
29	32.0	33.5	17.4
30	21.8	21.5	21.3
2’	172.9		
3’ – 8’	(28.6, 28.5, 28.4, 28.2, 26.9, 24.1)[b]		
9’	13.1		

Key

a and b are interchangeable

Similar compounds have been isolated from plants of this genus. For instance, Lupeol palmitate has been isolated from *V. acunae* (Perez, 1984) and Lupeol palmitate and α-amyrin palmitate (Tandon *et al.*, 1998) from *V. cinerea.*

Further NMR characterization to fully assign positions of carbon atoms using 2 dimensional correlation spectroscopy COSY experiments as well as the stereochemical aspects through nuclear overhauser spectroscopy NOESY need to be done.

From the arguments advanced, it would appear that this compound is a novel triterpenoid. Furthermore there is no spectral data in the literature at the moment that is consistent with it.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

a) This is the first time that the root bark of *Vernonia auriculifera* is being studied phytochemically.

b) Eleven (11) possible compounds were isolated but only two were structurally elucidated and were classified as triterpenoid types of compounds. It was not possible to characterize the other 9 compounds because of lack of NMR facilities.

c) The two triterpenoids only showed mild activity against bacteria and fungi.

d) The *n*-hexane, DCM and methanol extract showed dose dependent larvicidal activity against brine shrimp (*artemia salina)*.

e) Compound **7** could be a possible novel ester of a fatty acid and taraxerol (triterpenoid).

5.2 RECOMMENDATIONS

a) Further work could be done to fully characterise the constituents of the plant root bark extracts and bioassays carried out to establish all the active compounds against a variety of bacteria and fungi.

b) An assessment of the suitability of the extracts and constituents as cheaper natural alternatives to the available antibiotics currently in use should be done.

c) Confirmation of compound **7** using spectra measurements should be done with a view of establishing whether it is a novel ester of a long chain fatty acid and taraxerol (triterpenoid).

d) More bacteria and fungi could be used to asses the full potential of the extracts and isolated compounds.

REFERENCES

Ahmed, A. A., Mahmoud,A. A., Williams,H. J Scott, A. I., Rerbenspies, J. T and Mabry, T. J (1993). A new sesquiterpene α-methylene lactones from the Egyptian plant *Jasonia candicans* J. Nat, Prod. 56: 1276 – 1280.

Alkofahi, A., Rupprecht, J. K., Anderson, J. E., Mclaughlin, J. L, Mikoljczak,K. l., and Benard A. S. (1989). Search for new pesticides of plant origin (J. T. Arnason, J. R. Philogene and P. Morand eds.) Americam chemical society Washington D.C pp 25-40.

Alper, J. (1998). Effort to combat microbial resistance lags. ASM news 64: 440-441.

Atta –ur-Rahman and Choudhary, M. I. (1995). Diterpenoid and steroidal alkaloids. Nat. Prod. Rep. 12:361-379.

Balandrin, N. F., Kinghorn, A. D., Farnsworth, N. R. (1993) In human medicinal agents from plants .Kinghorn, A. D., Baladrin, N. F., Eds., ACS symposium series 534, (1993) pp 2-12.

Banthorpe, D. V. and B. V. Charwood (1980). The terpenoids, in secondary plant products (E. A Bell and B.V Charlwood, eds), 185-220, springer – verlag, Berlin.

Bardon, A., Catalan, C. A. N., Gutierrez, A. B. and Herz, W. (1988). Piptocarphol esters and other constituents from V. Cognata. Phyto. 27 9: 2989-2990

Batista, O., Duarte, A., Nascimento, J and Simones, M. F (1994). Structure and anti-microbial activity of diterpenes from the roots of *plectranthus lereroensis.* J.Nat. Prod. 57:858-861.

Bell, E.A. (1980). The possible significance of secondary compounds in plants. In: Secondary Plant Products (eds). E.A. Bell and B.V. Carlwood pp 11-21. Springer-Veriag, New York.

Bittner, S. Machocho, A. K, Kiprono, P. C and Grinberg S. (2003). Pentacylic triterpenoids from *Embelia Schimperi*. Phyto. 62: 573-577.

Boar, R. B. and J. Allen, (1973). β-Amyrin triterpenoids, phyto. 12, 2571-2578.

Bohlmann, F. and Jakupovic, J. (1990). Progress in the chemistry of vernoniceae (Compositae) P.L. Syst Evol. (Supp 4), pp 3-43.

Bohlmann, F., Muller, L., Gupta, R. K, King, R. M. and Robinson, H. (1981). Naturally occurring terpene derivatives part 362. Hirsutinolides from vernonia species. Phyto. 20: 2233-2237.

Borris, R. P. (1996). Natural products research: perspectives from a major pharmaceutical company. J.Ethnopharmacol 51: 367-375

Bose, P. K (1958). On some biochemical properties of natural coumarins. J. Indian Chem. Soc 58: 367-375.

Branther, A., Males, Z., Pepeljnjak and Antolic, A. (1996). Antimicrobial activity *Paliurus spina Christi mill*, J. Ethnopharmacol 52: 119-122.

Brookes, G. F., Butel, J. S., Ornaston, L. N and Adelburg, E. A. (1991). Medical Microbiology, Prentice Hall International Inc. Connecticut U.S.A.

Bruneton, J. Pharmocognosy, Phyto., medicinal plants; Lavoisier Tec & Doc; N.Y. 3rd Ed, 1995, p 267 and 501.

Chaurasia, S. C. and Vyas K. K. (1997). In vitro effect of some volatile oil against *phytophthora parasitica* var. Piperina. J. Res. Indian Med. Yoga Homeopath. 1977: 24-26.

Cichewicz, R. H. and Thorpe, P. A (1996). The anti-microbial properties of chilli peppers (capsicum species) and their uses in Mayan medicine. J. Ethno- pharmacol. 52: 61-70.

Clark, A. M. (1996) Natural products as a source for new drugs. Pharm Res 13.

Coates, R. M., (1976). Biogenetic type rearrangements of triterpenes, Fortschr, chem. Org. Naturst, 33: 73-230.

Costa, P. M. and Tese de Mestrado, (1996). Departmento de Quimica; Universidade federal Rural do Rio de Janeiro; Brazil, p 5-20.

Cowan, M.M. (1999). Clinical microbiology review, American Society for Microbiology; Vol. 12 No. 4 : 567

Cragg, G. M., Newman, D. J., Snader, K. M. J. (1997) Nat Prod. 60: 52-60.

Critchfield, J. W., Butera, S. T and folks, T. M (1996). Inhibition of HIV activation in latently infected cells by flavanoid compounds. AIDS Res. Hum. Retroviruses 12:39.

Croteau, R. and M. A. Johnson, (1985). Biosynthesis of terpenoid Wood extractives, in Biosynthesis and Biodegradation of wood compounds (T. Higuchi, ed.), 379-439, Academic press, Orlando FL.

Davidson, P. T., Khanijo V. I, Goble, M. and Moulding, T. S. (1981). Treatment of disease due to *mycrobaterium Intracellulare*. Review of infectious Disease 3: 1051-1059.

Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983). Phytoalexins: enzymology and molecular biology. Adv. Enzymol. 55: 1-69.

Duke, J. A. (1985). Handbook of medicinal herbs. CRC press, Inc. Boca Raton, Fla.

Dutt, A. K. and Stead, W. W. (1979). Long term results of medicinal treatment in *mycobacterium intracellulare* injection. American Journal of medicine, 67: 449-453.

Eisenberg, D. M., Kessler, R. C., Foster, C., Noclock, F. E. Calkins, D. R., Dehanco, T. L. (1993). Unconventional medicine in the United States: Prevalence, costs and patterns of use N. Eng. J. Med 328: 246-252.

Farnsworth, N. R. in Biodiversity Wilson, E. O. (1988). Ed., National academy Press Washington DC, pp 83-97.

Freiburghaus, F., Kaminsky, R., Nkunya, H.H and Brun, R. (1996). Evaluation of African medicinal plants for their in vitro trypanocidal activities .J. Ethnopharmacol. 55: 1-11.

Fujioka, T. and Kashiwanda, Y. (1994). Anti- AIDS agents. Betullinic acid and platonic acid as anti-HIV principles from *syzigum claviflorum*, and the anti-HIV activity of structurally related triterpenoids. J. Nat. Prod. 57:243-247.

Geissman, T. A (1963). Flavanoid compounds, Tanins, lignins and related compounds p. 265. In M. Florkin and E.H. stotz (ed), pyrrole pigments, isoprenoid compounds and phenolic plant constituents, Vol.9 Elsevier New York, N.Y.

Hamburger, H., and Hostehmann, K. (1991). The link between phyto. and medicine. Phyto. 30: 3864-3874.

Harbone, J. B., (1986). Recent advances in chemical ecology, Nat. Prod. Rep., 4. 323-344.

Harries, A. D (1990). Tuberculosis and human immunodeficiency virus infection in developing countries Lancet 335: 387-390.

Harrison, D. M. (1985). The biosythensis of triterpenes and steroids. Nat. Prod. Rep., 2: 525-560.

Hasegawa, H. S. Matsumiya, S., Uchiyama, M. Kurokawa, T. Inouye, Y., Kasai, R., Ishibashi , S. and Yamasaki, K. (1994). Inhibitory effect of some triterpenoids saponins on glucose transport in tumour cells and its application to in vitro cytotoxic and antiviral activities planta. med. 6:240-243.

Hufford, C. D., Jia Y., Croom, E. M, Muhammed. J. Iokunade, A. L, Clark, A. M and Roger, R.D. (1993). Anti-microbial compounds from *Petalostamum purpureum*. J. Nat. Prod. 56:1878-1889.

Hunter, M. D. and Hull, L. A. (1993). Variation in concentration of phloridzin and phloretin in apple foliage. Phyto. 34: 1251-1254.

Jones S. B., Jr. and A. E Luchsinger. (1986). plant systematics Mcgraw Hill book Co, New York, N.Y.

Kadota, S., Basnet, Ishii, E., Tanura, T. and Namba, T. (1997). Anti-bacterial activity of trichorabdal from Rabdosia trichocarpa against *Helicobacter pyroli* Zentbl Bakteriol 286.63-67.

Kazmi, M. H., Malik, A., Hameed, S., Akhtar, N and Noor Ali, S. (1994). An anthraquinone derivative from *Cassia italica*. Phyto. 36: 761-763.

Keriko, J. M., Nakajima, S., Baba, N., Isozaki, Y., Iwasa, J. and Karanfa, P. N. (1995a). 8-Desaylvernodalol a plant growth regulator from *Vernonia auriculifera*. Z. Natureforsch 50c, 455-458.

Keriko , J. M., Nakajima, S., Baba, N., Isozaki, Y. and Alam Md.k. (1995b). Hydroperxides of unsaturated fatty acid methyl esters from *Vernonia auricufera*. J. Jpn chem. Soc. (YAKUGAKU) 4: 338-340.

Keriko , J. M. (1995). Ph. D Thesis.

Kiesel, W. (1975). Pol. J. *Pharmacol pharm.*, 27, 461.

Klink, B. (1997). Alternative medicines: Is natural really better? Drug TOP 141: 99-100.

Kokwaro, J. O.(1976). Medicinal plants of East Africa. East African Literature Bureau, Nairobi, p72.

Kubo L., Murol, H. and Himejina, M. (1992).Antibacterial activity of totarol and potentiation. J. Nat. Prod 55: 1436-1440.

Kubo, L., Muroi, H and Kubo, A. (1994). Naturally occurring anti-acne agents. J. Nat prod. 57: 9-17

Mahato, S.B. and Kundu, A.P. (1994). Review article number 98, ^{13}C-NMR spectra of pentacyclic triterpenoids a compilation and salient features. Phyto. 37: 1517-1575

Martinez, M. J. J., Betancourt, N., Alonzo-Gonzalez and Jauregi, A (1996). Screening of some Cuban medicinal plants for antimicrobial activity. J. Ethnopharmacol. 52:171-174.

Mcdevit, J. T., D. M Schneider, S. K. Katiyar and T. D. Edlind. (1996). Berberine a candidate for treatment of diarrhoea in AIDS patients abstract 175. In program and abstracts of the 36 inter-science conference on antimicrobial agents and chemotherapy. American society for microbiology Washington D.C.

Mcmahon, J.B., M.J Currens, R.J Gulakowski R.W.J Buckheit, C. lackman- Smith, Y.F Hallock and M.R. Boyd. (1995). Michellamine B, a novel plant alkaloid, inhibits human immunodeficiency virus induced cell killing by at least two distinct mechanisms. Anti-microbial agents chemotherapy 39: 484-488.

Mendoza, L., Wilkens, M. and Urzua, A. (1997). Anti-microbial study of resinous exudates and of diterpenoids and flavonoids isolated from some Chilean pseudognaphalium (Asteraceae). J Ethnopharmacol. 58: 85-88.

Mitscher, L. A., Drake , S. Collapudi, S. and Okwute, S. K. (1987). A modern look at folkoric use of anti- effective agents. Journal of natural products, 50: 1025.

Morse, S. A., Johnson, R. A., Biddle, J.W. and Robert, M.C. (1986). High level Letracycline resistance in *Neisseria gonorrhoea*. Journal of anti-microbial chemotherapy 30 (5): 664-667.

Naik, S., Ruck, R., (1989). In-vitro activities of several macrolide antibiotics against *Mycobacterium avium* complex. Anti-microbial Agents and chemotheraphy 33: 1614-1616.

Omulokoli, E., Khan, B. and Chabra, S. C, (1997). Anti-plasmodial activity of four Kenyan medicinal plants. J. Ethnopharmacol. 56:133-137.

O'Neil, M. J Lewis, J. A., (1993). In Human Medicinal agent from plants Kinghorn A. D., Balandrin, M. F., Eds., ACS symposium series 534, pp 48-55.

Perez Sonto, N. (1984) Phyto. study of Cuban species of the genus vernonia. Rev. Cubana Farm. 18 3:336-340.

Pezzuto, J. M (1997). Biochemical Pharmacology 53, 121-133.

Rana, B. K., Singh, U. P. and Taneja V. (1997). Antifungal activity and Kinetics of inhibition by essential oil isolated from leaves *Aegle marmelas*. J. Ethnopharmacol 57:29-34.

Sabath, L. D., Wheeler, N., Laverdiere, M., Blazerie, D., and Wilkinson, B. J. (1997). A new type of penicillin resistant to *staphylococcus aureus*. Lancet 1: 443-447.

Sakanaka, S., Kim. M., Tanguchi, M. and Yammato T. (1998). Antibacteria substances in Japanese green tea extract against *streptococcus mutans*, against cariogenic bacterium. Agri. Bio. Chem. 53:2307-2311.

Sakanaka, S., Shimura, N., Aizawa, Kim, M. and Yamamoto, T. (1992). Preventive effect of green tea polyphenols against dental caries in conventional rats. Biosci. Biotechno., Biochem. 56:592-594.

Scortichini, M. and Pia Rossi, M. (1991). preliminary in vitrio evaluation of antimicrobial activity of terpenes and terpenoids towards *Erwinia amylovora* (Burri) Winslow et. al. J. Appl. Bacteriol. 71:109-112.

Seigler D. S. (1983). The role of lipids in plants resistance to insects, (P. A. Hedin, ed.), ACS symposium series 208, 303-327, American chemical society, Washington D. C.

Seth, M. L., (1979). Inhibition reverse transcriptase activity by benzophenanthridine alkalids. J. Nat. Prod. 42: 187-196.

Sign, R. K., Singh, P. and Joshi, K. C. (1987). Glaucolide A and B from *V. Elaegnifolia*. Fitoterapia 58 2: 127-128.

Stern, J. L., Hagerman, A.F., Steinberg, P. D. and P. Mason, P. K. (1996). Phlorotannin – protein interactions. J. Chem. Ecol. 22:1887-1899

Suresh, B., Sriram, S., Dhanaraj, S. A., Elango, K. and Chinnaswamy, K.(1997). Anti-candidal activity of sactolina Chinnaswamy volatile oil J. Ethnopharmacol. 55: 151-159.

Tandon, M. and Shukla, Y. N. (1995). Some chemical constituents from *V. Cinerea*. Indian drugs 32 3:132-133.

Tassou, C. C., Drosinos, E. H. and Nychas, G. J. E. (1995). Effects of essential oil from mint (*mentha piperta*) on *Salmonella enteritidis* and *Listeria monocytogenes* in model food systems at 4º C and 10º C. J. Appl. Bacterio 78: 593-600

Thomson,W. A. R (ed.) (1978). Medicines from the Earth Mcgraw- Hill Book Co., Maidenhead, United kingdom.

Toda, M., Okubo, S., Ohnishi, R. and Shimamura, T. (1989). Anti-bacterial and bactericidal activities of Japanese green tea. Jpn. J. Bacteriol. 45:561-566

Tsuchiya, H., Sato, M. Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T. and Linuma, M. (1996). Comparative study on the antibacterial activity of Phyto. flavours against methicillin – resistant *Staphylococcus aureus*. J. Ethnopharmacol. 50: 27-34.

U.S Bereau of the Census. Statistical Abstract of United States, 104 ed. U.S. Government Printing office, Washington D.C., 1990.

Van Tamelen, E. E., J. Willet, M. Schwartz and R. Nadeau, (1966). Nonenzymic laboratory cyclization of squalene-2, 3- oxide, J. Am. Chem. Soc., 88, 5937-5938.

Van Tamelen, E. E., (1968). Biorganic Chemistry: Sterols and cyclic terminal epoxides, Acc. Chem. Res., l, 111-120.

Vijaya, K., Anathan, S. and Nalini, R. (1995). Anti-bacterial effect of theaflavin, polyphenon 60 (*Camellia sinensis*) and *Euphobia hirta* on shigella species – a cell culture study. J. Ethnopharmacol. 49: 115-118.

Vishwakarma, R. (1990). Stereoselective synthesis from α-artemether from artemisinin. J. Nat. Prod. 53: 216-217.

Vohora, S. B., Rizwan, M. and Khan, J.A. (1973). Medicinal uses of common Indian vegetables. Planta Med. 23: 481-393.

Watanbe, H., Miyaji, C., Makino, M. and Abo, T. (1996). Therauptic effects of glycyrrhizin in mice infected with LP-BM5 murine rebrovirus and mechamisms involved in the prevention of disease progression. Biotherapy 9:209-220.

Wild, R. (ed.) (1994). The complete book of natural and medical cures. Rodale press, Inc. Emmaus, Pa.

www.telmedpak.com/agricultures

Yagi, K. ed., (1993). Active Oxygen, Lipid peroxides and anti-oxidants, Japan Scientific Societies Press, Tokyo.

APPENDICES

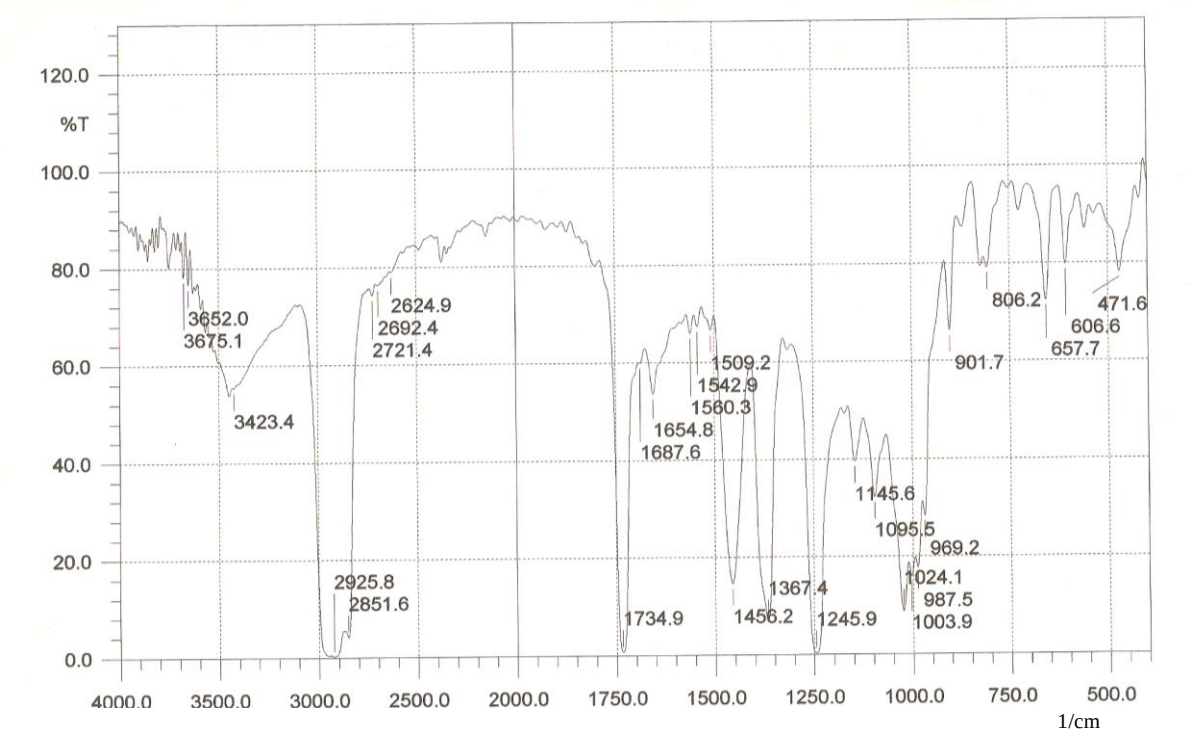

Figure 1. FT-IR spectrum of compound **1**

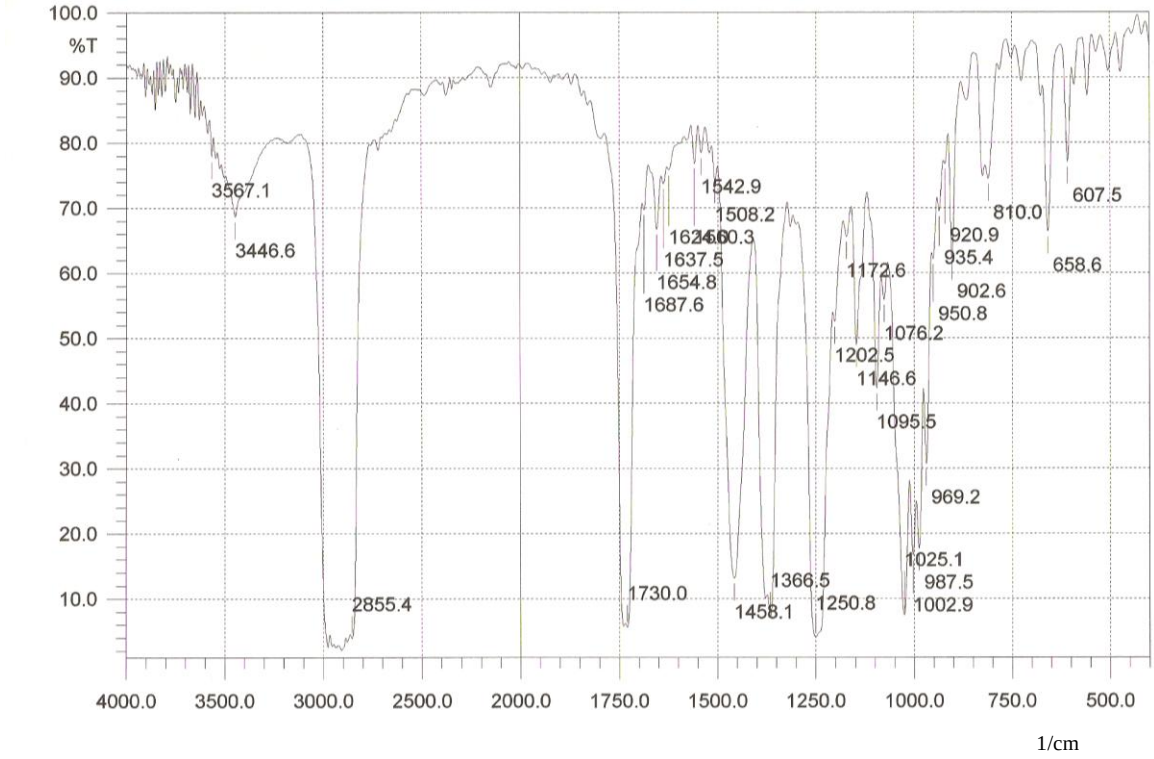

Figure 2. FT-IR spectrum of compound **2**

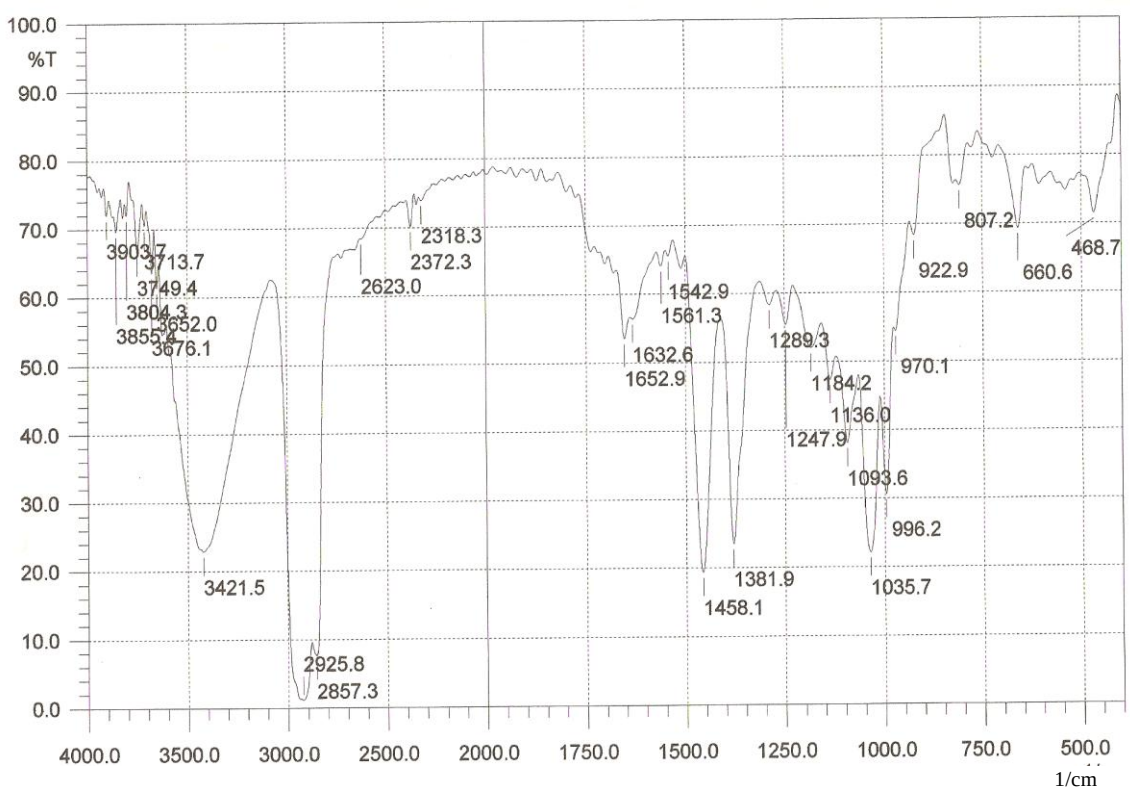

Figure 3. FT-IR spectrum of compound **3**

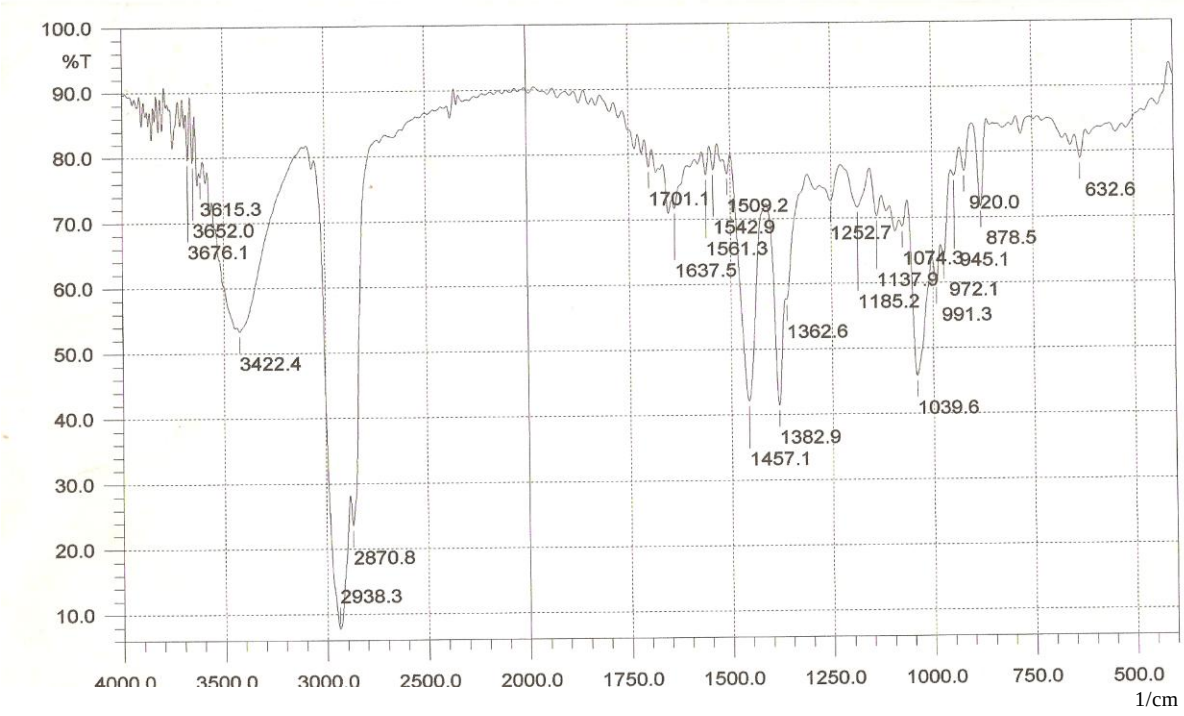

Figure 4. FT-IR spectrum of compound **4**

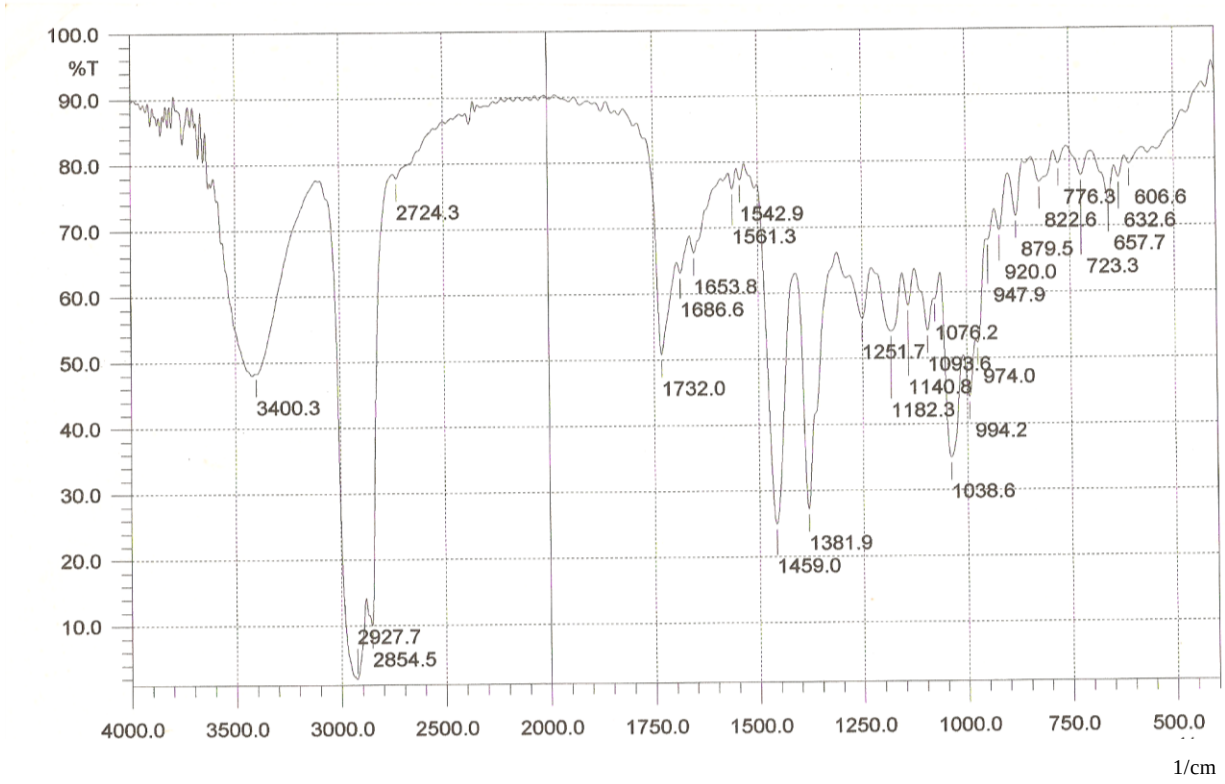

Figure 5. FT-IR spectrum of compound **5**

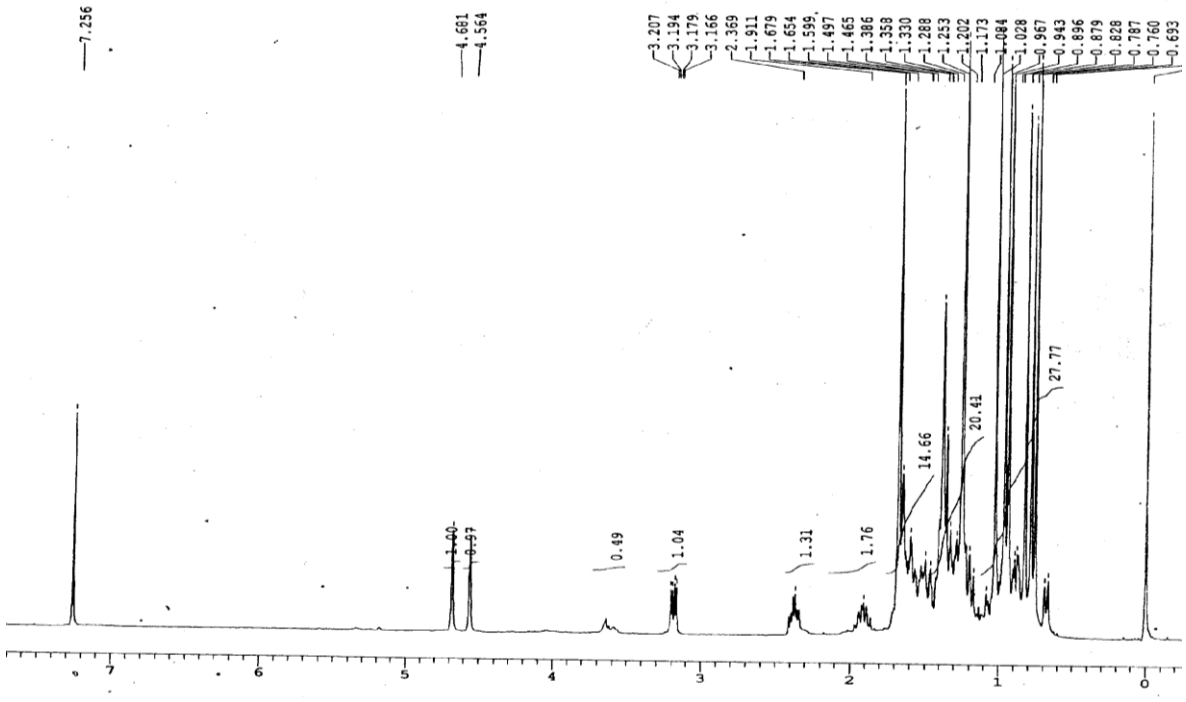

Figure 6. 300 MHz ^{1}H-NMR spectrum of compound **6**

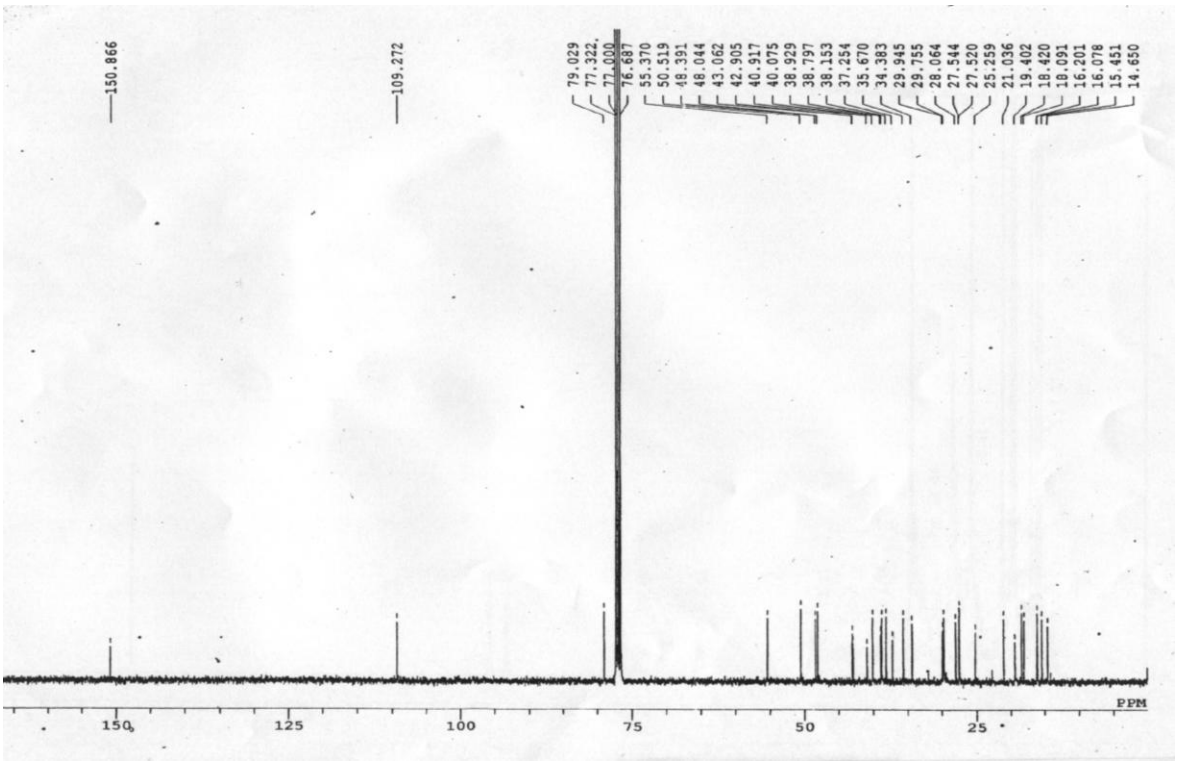

Figure 7. 100MHZ ^{13}C-NMR spectrum of compound **6**

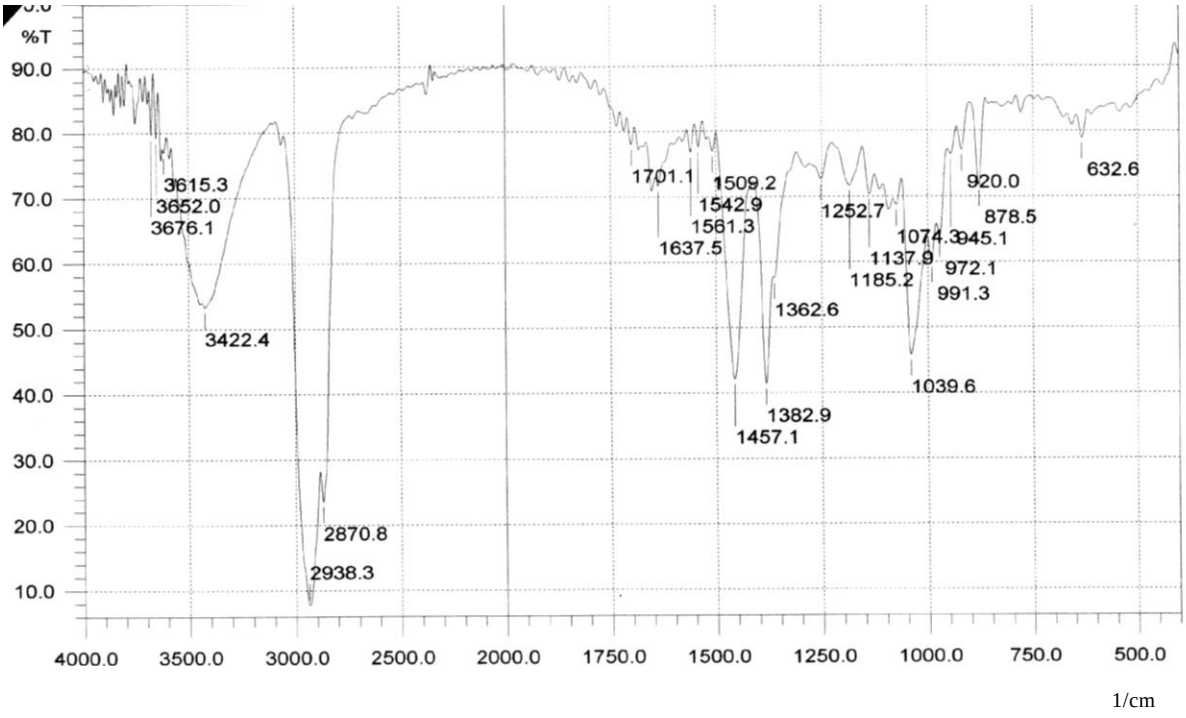

Figure 8. FT-IR Spectrum of compound **6**

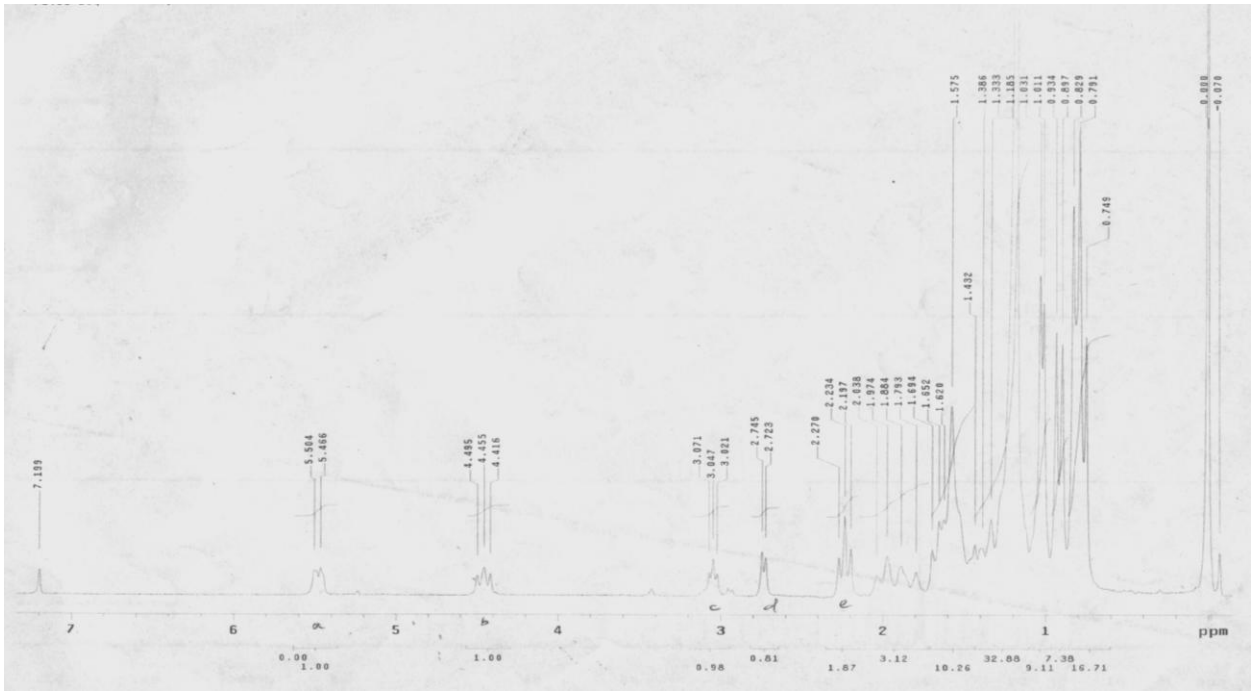

Figure 9. 200 MHz ^{1}H-NMR spectrum of compound **7**

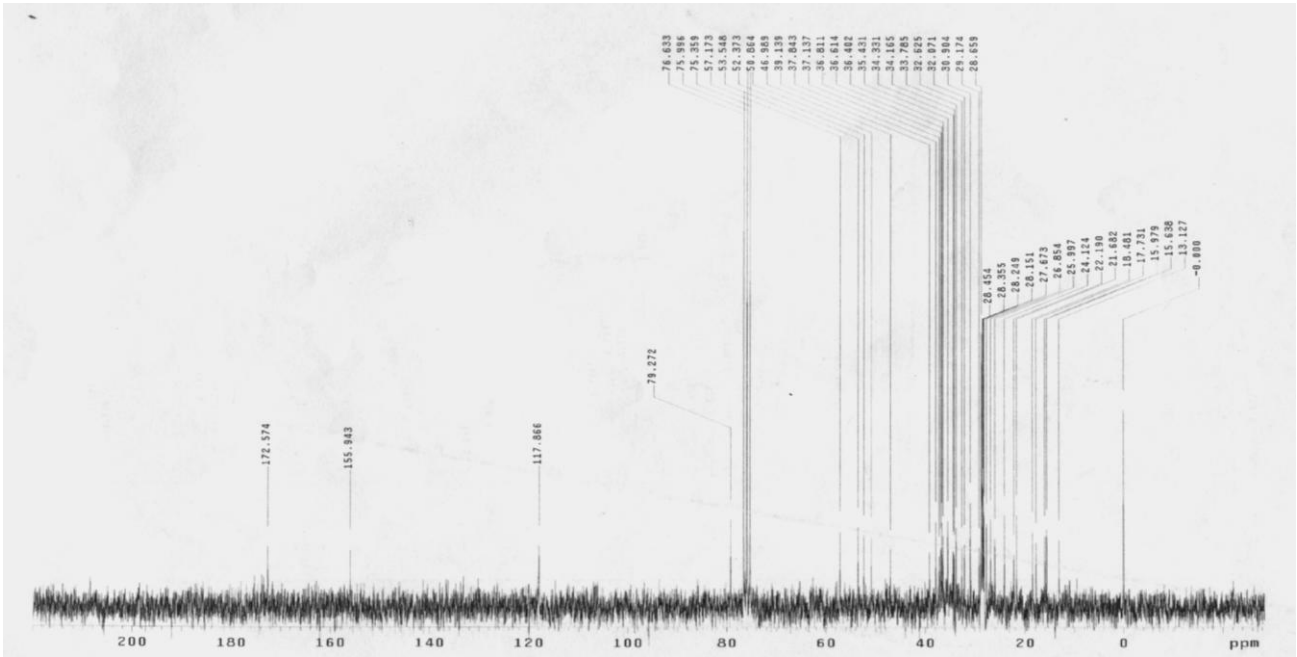

Figure 10. 50 MHZ ^{13}C-NMR spectrum of compound **7**

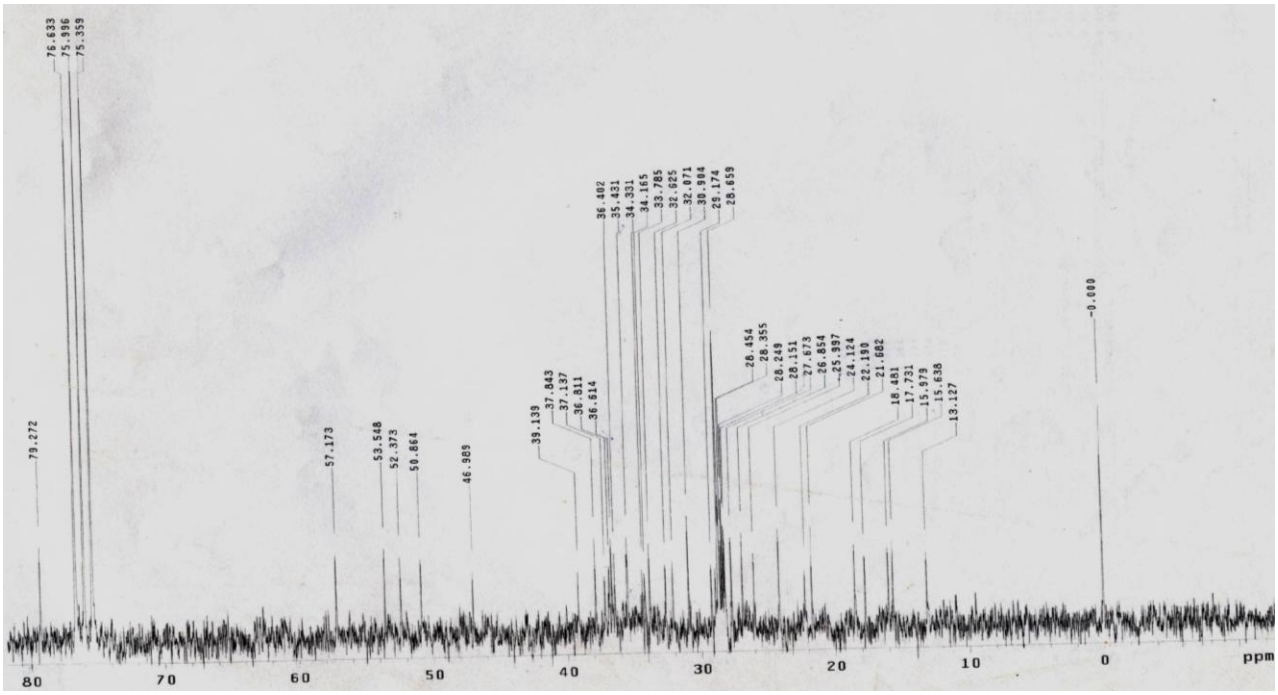

Figure 11. Section of 100 MHZ ^{13}C-NMR spectrum of compound **7**

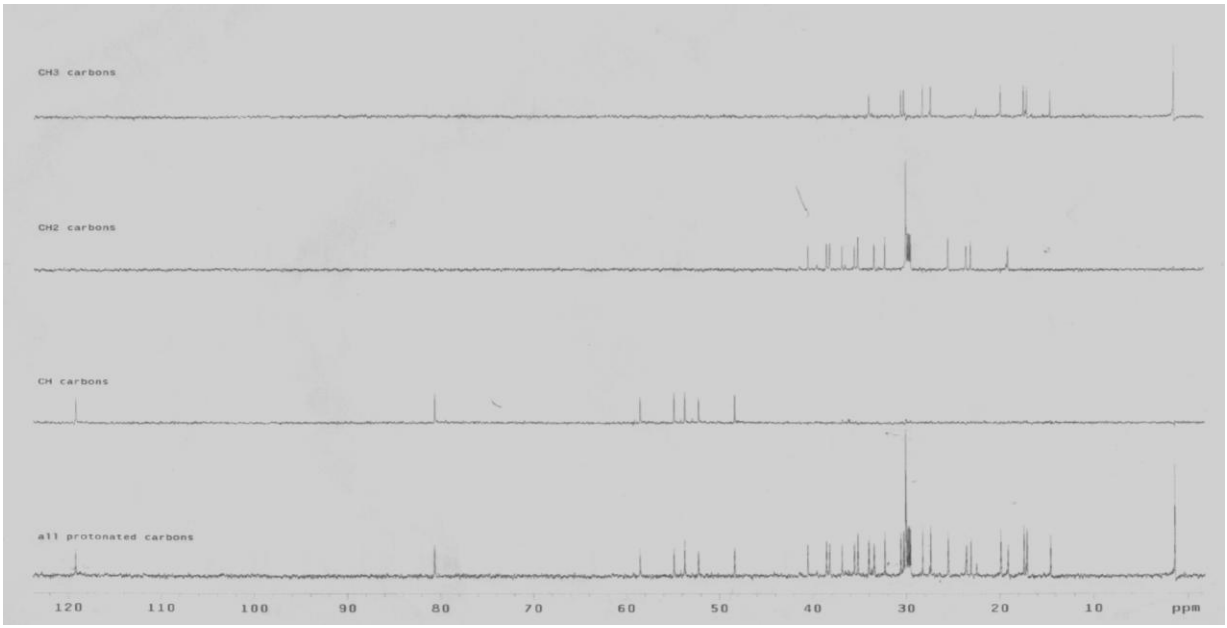

Figure 12. ^{13}C-NMR DEPT of compound **7**

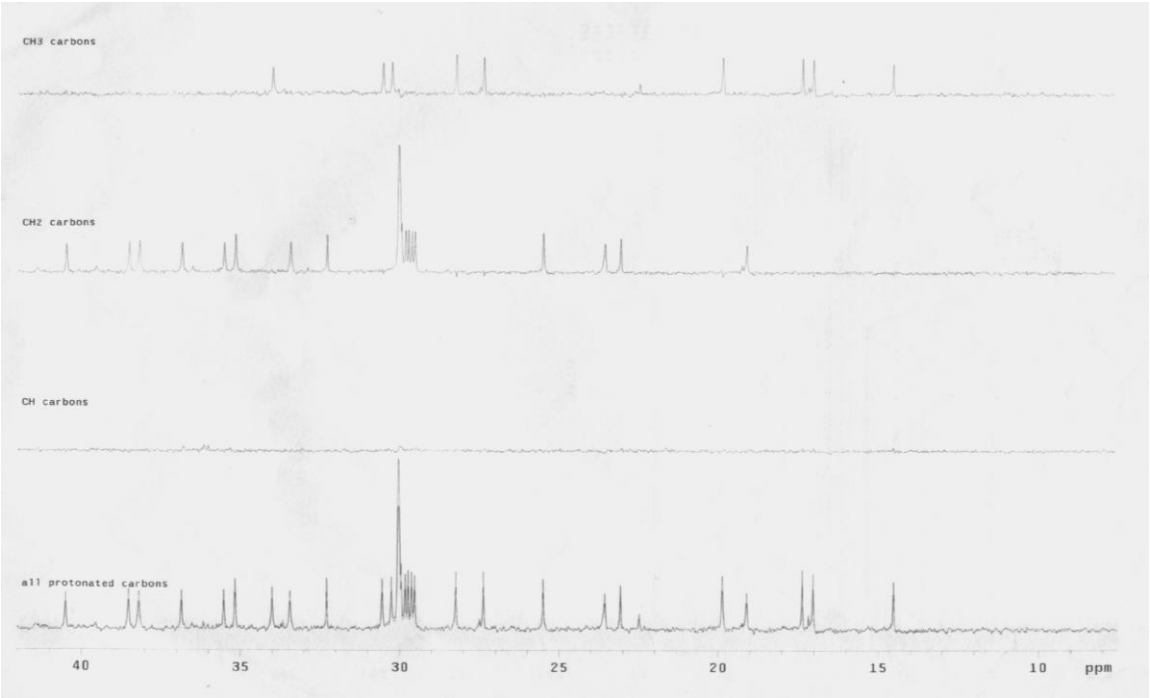

Figure 13. Section of ^{13}C-NMR DEPT of compound **7**

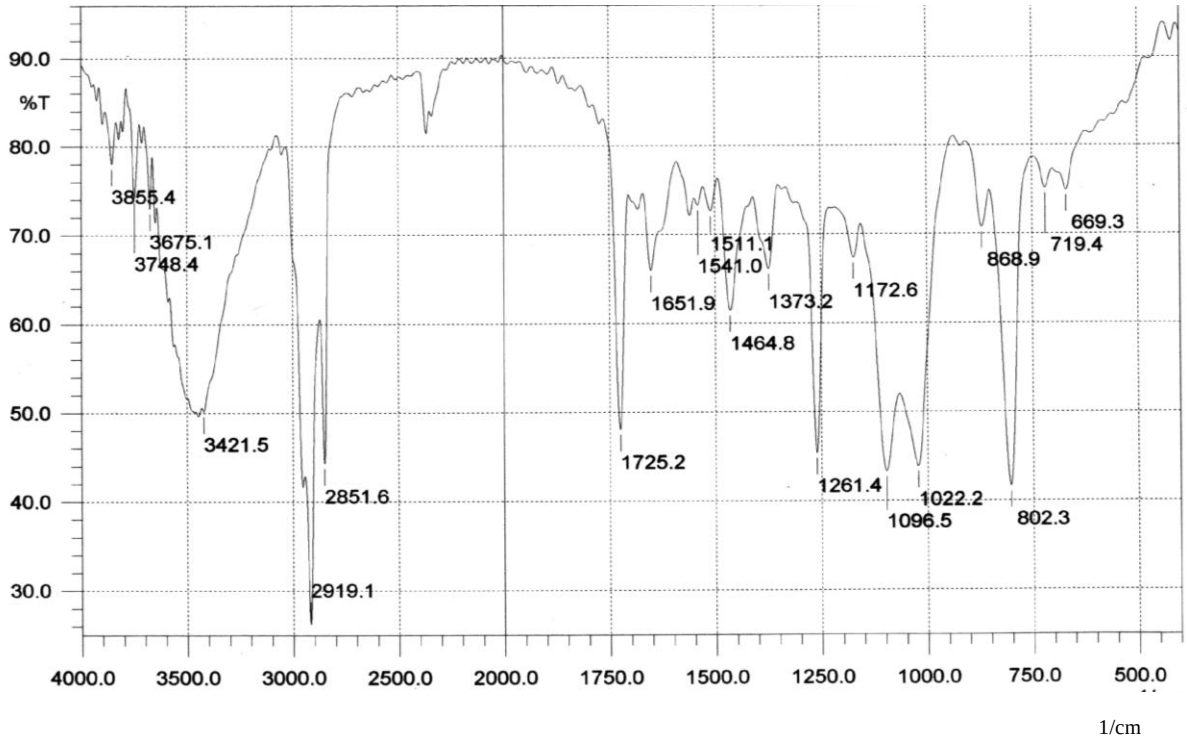

Figure 14. FT-IR spectrum of compound **7**

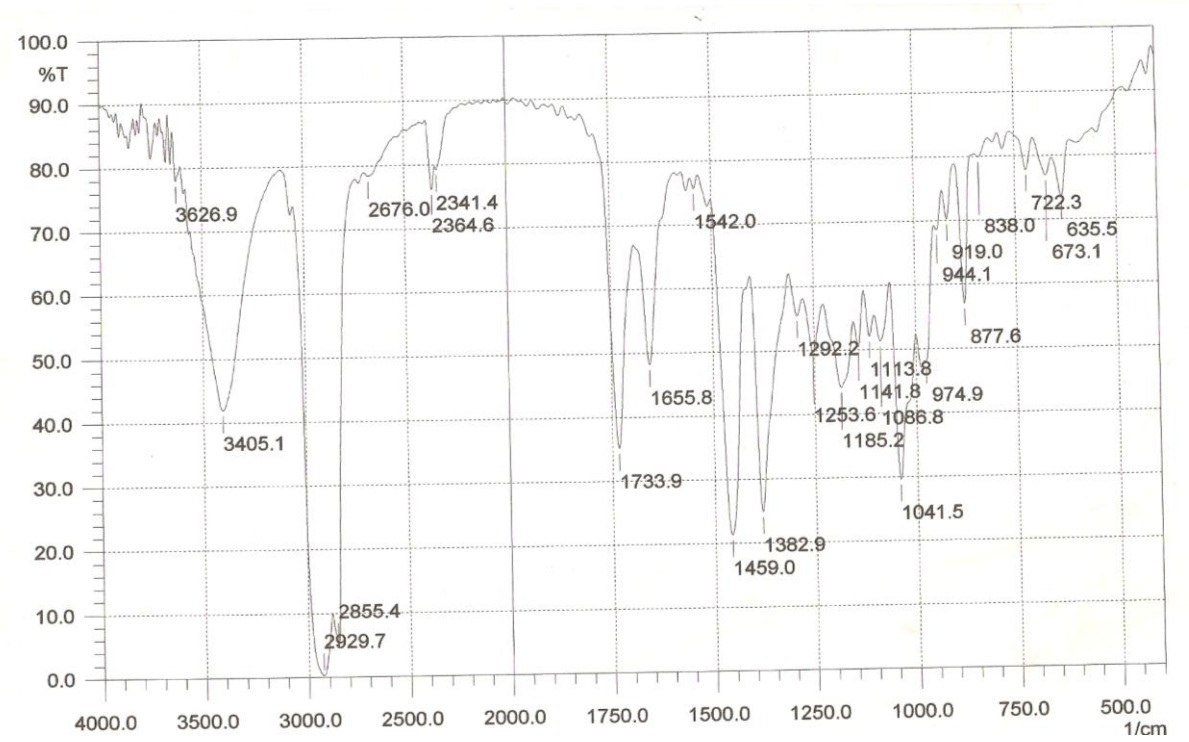

Figure 15. FT-IR spectrum of compound **8**

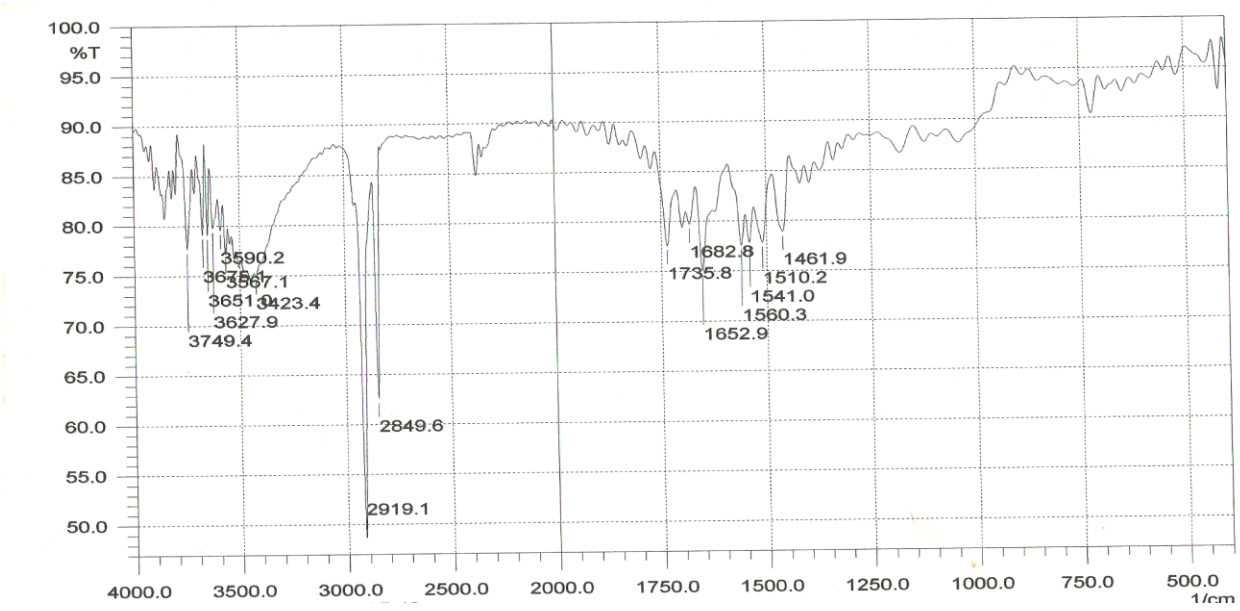

Figure 16. FT-IR spectrum of compound **9**

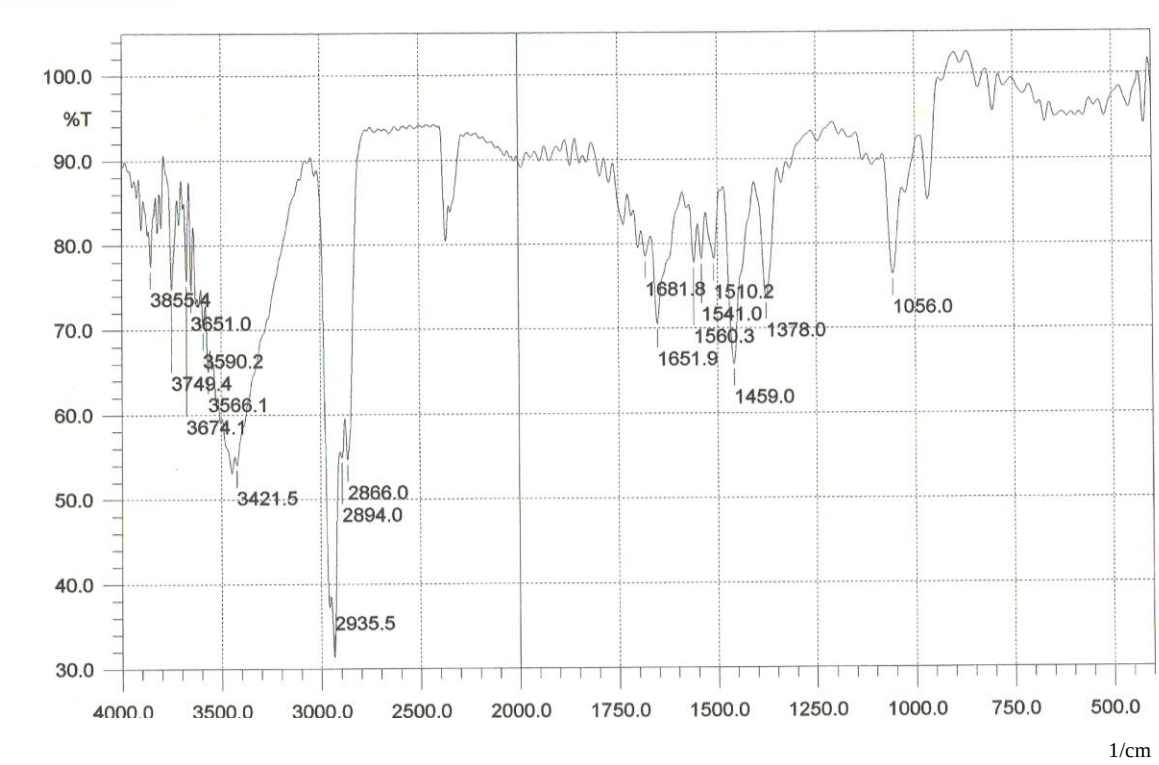

Figure 17. FT-IR spectrum of compound **10**

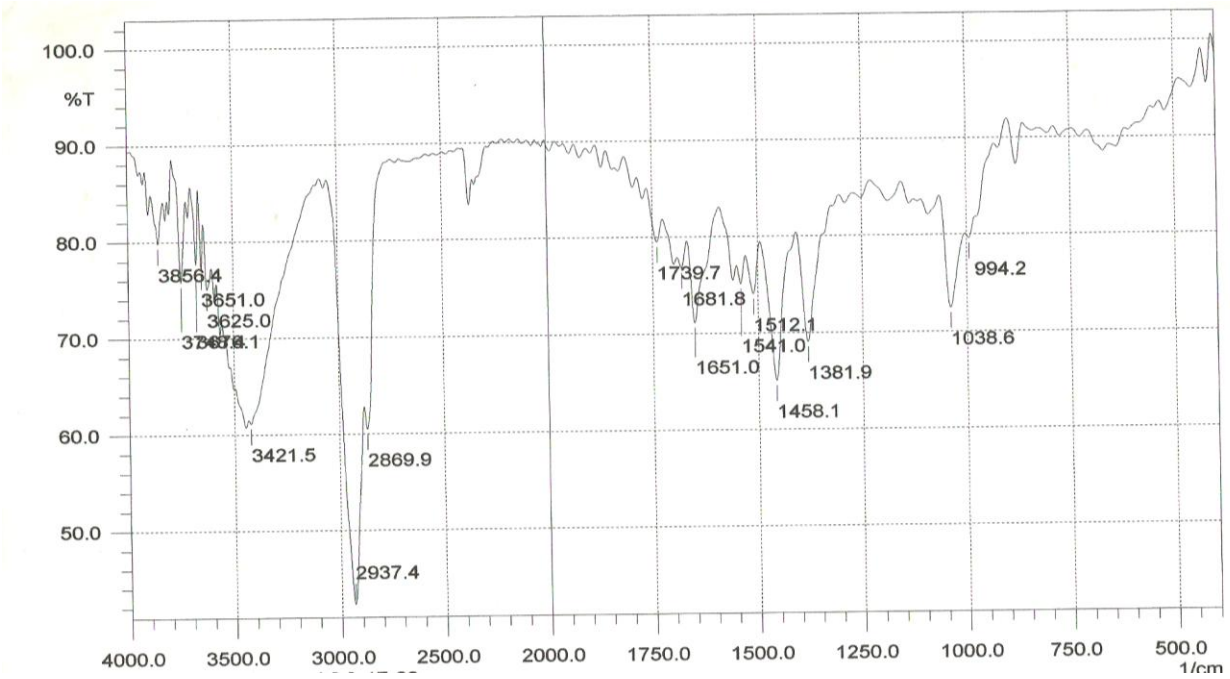

Figure 18. FT-IR spectrum of compound **11**