ANTI-OXIDANT AND MONOAMINE OXIDASE INHIBITION STUDIES ON THE SURFACE EXUDATES OF *GARDENIA TERNIFOLIA*

BY

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DECLARATION

This thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people´s work, or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi´s requirements.

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DEDICATION

This thesis is dedicated to my family.

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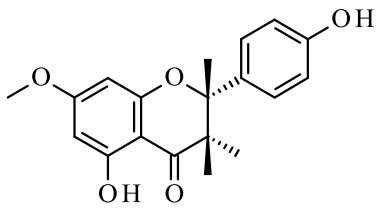
ABSTRACT

Numerous scientific studies point to oxidative stress (OS) as an important etiological factor in common diseases such as artherosclerosis, rheumatoid arthritis, tumors, cancer, diabetes, hypertension, cardiovascular and neurological diseases. Enzymes in the brain such as Monoamine Oxidases A and B work synergistically with Reactive Oxygen Species in the oxidative deamination of neurotransmitters enhancing the occurrences of neurological disorders such as depression, Alzheimer and parkinson's diseases. Synthetic Monoamine Oxidase Inhibitors (MAOIs) have been developed to prevent this from happening towards improving and maintaining brain cell effective communication but the problem has persisted.The usage of these synthetic MAOIs has been reported to have many side effects associated with them just like the synthetic anti-oxidants. Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and *tert*-Butylhydroquinone (TBHQ). These include: blood pressure changes, headache, drowsiness and insomnia among others. Scientific evidences point to natural anti-oxidant molecules in plants as effective tools for the control of oxidative stress, and flavonoids are one of the major groups of anti-oxidants in plants most of which occur in their surface exudates. *Gardenia ternifolia* leaves are used to manage mental illness connected with neurotransmission in some countries like Uganda, Congo Brazaville among others. This study therefore, sought to investigate the anti-oxidant and MAO-Inhibition activities of the compounds found on the surface exudates of *Gardenia ternifolia* so as to improve the knowledge base and also in finding dietary supplements for MAO inhibition that could improve neurological conditions.

Surface exudates of *Gardenia ternifolia* fresh leaves from Kagundo, Machakos County were extracted through consecutive dipping in fresh amounts of acetone for 15 seconds to get the crude extract. Chromatographic separation of the crude extract gave a total of six compounds which were successfully characterized using spectroscopic techniques and identified as 5,4'-dihydroxy-7-methoxyflavanone (**164**), 3,5,4'-trihydroxy-7-methoxyflavone (**165**), 5,7-dihydroxy-3,4'-dimethoxyflavone (**166**), 3,3',5-trihydroxy-4',7-dimethoxyflavone (**168**), β-sitosterol(**169**) and stigmasterol (**170**). Compound **166** was transformed to **167** through acetylation. All the seven compounds were tested for their anti-oxidant and MAO Inhibition activity. The activity of the flavonoids showed that flavonols had better anti-oxidant activity as compared to 3-methoxyflavones isolated from the surface exudates of *Gardenia ternifolia*. The most active compound was 3, 3', 5-trihydroxy-4', 7-dimethoxyflavone (**168)** with IC_{50} of 40.3μM. The MAO-A and B inhibition activities revealed that 3, 4'-methoxyflavones had better activity as compared to hydroxylated flavonoids in these positions. Compound **166** was the most active and more selective to MAO-A (IC_{50} = 0.033 μM), showing binding affinity of (Ki) 0.0379 μM compared to MAO-B (IC_{50} = 4.133μM). It also showed competitive reversible type of inhibition for the enzymatic active site. Acetylating the hydroxyl groups at C-5 and C-7 of compound **166** to **167** led to a decrease in MAO-A and B activities exhibiting IC_{50} =10.40 and 42.00 μM, respectively.The anti-oxidant and selective MAO-A inhibitory properties observed for the flavonoids isolated from the surface exudates of *Gardenia ternifolia* suggest their possibility in generating selective pharmacological influence that might be useful for the prevention of numerous free radical based diseases as well as in the management of depression and other related neurological disorders.



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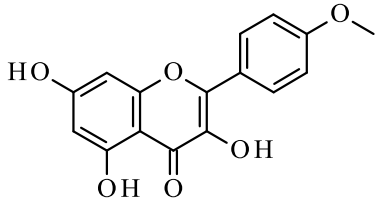

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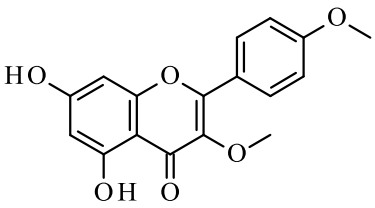

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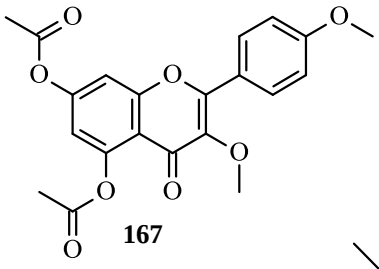

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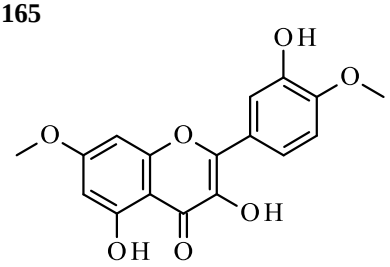

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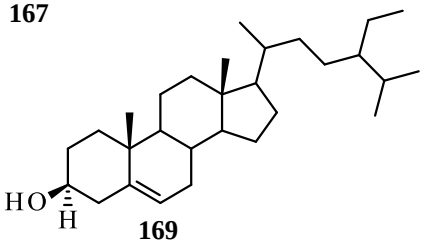

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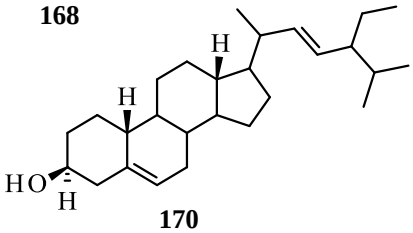

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LIST OF ABBREVIATIONS

BHA	Butylated Hydroxyanisole
BHT	Butylated hydroxytoluene
CC	Column Chromatography
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
^{13}C-NMR	Carbon-13 NMR
COSY	COrrelation Spectroscopy
d	Doublet
CH_2Cl_2	Dichloromethane
dd	Doublet of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMAPP	Dimethyl Allyl Diphosphate
DMSO	Dimethyl sulfoxide
DPPH	Diphenyl picryl hydrazine
EIMS	Electron Impact Mass Spectrometry
FAD	Flavin Adenine Denucleotide
FPP	Farnesyl Diphosphate
GGPP	Geranyl Geranyl Diphosphate
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
Hz	Hertz
IC_{50}	Inhibition concentration of substance that produce 50% inhibition of certain process
IPP	Isopentyl diphosphate
Km	Michaelis constant
m	Multiplet
m/z	Mass to charge ratio

[M] +.	Molecular ion
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MeOH	Methanol
MEP	Methylerithyritol phosphate
MS	Mass Spectrometry
MVA	Mevalonate
NMR	Nuclear Magnetic Resonance
^{1}H-NMR	Proton NMR
R_f	Retention factor
ROS	Reactive oxygen species
S	Singlet
SAR	Structure Activity Relationship
SD	Standard Deviation
SOD	Superoxide dismutases
SPSS	Statistical Package for Social Sciences
TBHQ	*Tert* Butyl hydroquinone
TLC	Thin layer chromatography
UV	Ultra violet
Vmax	Maximum velocity

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CHAPTER ONE

INTRODUCTION

1.1 Background

Plants in general are rich sources of nutrients for many organisms like insects, fungi, bacteria, protists and vertebrates. They lack an immune system as opposed to animals but they have developed a lot of features to enable them thrive well in their various ecological conditions. These features range from structural, chemical, and protein based defense mechanism (Brian and Gwyn, 2008) well developed in such a way that they are able to detect and respond to the invading organisms. The response to the invading organism is to stop them from causing damage to the plant. Glanded epidermal appendages such as trichomes exist in most plants. They produce surface exudates containing hydrophobic isoprenoids and phenylpropanoids-including flavonoids, tannins and other phenolic compounds (Wagner *et al*., 2004). Sugar esters bearing both hydrophilic and hydrophobic parts also exist in glandular trichome exudates as reported by Wagner *et al* (2004). These compounds have been linked in several entomological studies and have revealed that they offer insect resistance in several plants (Spring, 2000) as seen in potato (Solanaceae) inhibiting aphid infestation (Goffreda *et al*., 1990; Wang *et al*., 2004) helping plants to thrive well in various habitats. Plants are increasingly being exploited for drug development in addition to increased need in validating traditional medicines which has been practiced for years (Wagner, 1991).

The phytochemicals under investigations include surface exudates found in the leaves. Generally, surface structures create a barrier between invaders and the interior of the leaf and because of this, they are mechanical deterrents to pathogens (Bennet and Wallsgrove, 1994). Surface exudates constituents are reported in studies to strongly absorb the ultra violet (UV) radiation, acting as

sunscreens thus demonstrating a photo protective role to the higher plants against the damaging radiations (290-400 nm) (Caldwell, 1971). Monoterpenes, sesquiterpenes and diterpenes are the most commonly found terpenoids in surface exudates (Kellogg, 2001). In some plants, productions of terpenophenolic cannabinoids like in Cannabis occur (Mahlberg and Kim, 1992). Flavonoids in most cases are deposited as methoxylated aglycones in the surface exudates of many plant species (Wang *et al*., 2004). Studies have indicated that the aglycones are deposited externally on the surface of the leaves as constituents of resinous excretions (Wollenweber and Dietz, 1982) while glycosides occur in vacuoles and chromoplasts in many plants. The presence of phenolic compounds in plants has been linked to the anti-oxidant activities terminating free radicals *via* various processes (Cook and Samman, 1996). Flavonoids are amongst these groups reported as having a range of activities like: anti-inflammatory action, radical scavenging, anti- cancer and inhibitors of oxidative and hydrolytic enzymes (Frankel, 1995).

The plant *Gardenia ternifolia* has been known to contain many phenolic compounds. Among other classes of compounds, it contains flavonoids majorly deposited on the leaves some of which could be having anti-oxidant activities. *Gardenia ternifolia* leaves also are used to manage stress, depression, traumatic disorders and other mental illness cases in countries such as Uganda (Tabuti *et al*., 2003) and Congo Brazaville (Diafouka, 1997). These have been linked to abnormal functioning of nerve cell circuits or pathways that connect particular brain regions. Nerve cells within these brain circuits communicate through chemicals called neurotransmitters. Lack of essential neurotransmitters in the brain such as serotonin, dopamine and adrenaline whose breakdown is facilitated by Monoamine Oxidase enzyme in the brain, contributes majorly to this abnormality. This study therefore aimed at investigating phytochemicals found in the surface exudates of *Gardenia ternifolia* for their anti-oxidant and Monoamine Oxidase (MAO) inhibition

activity. The active constituents identified as having good antioxidant and MAOI activity may be of greater value in finding the new drug design maps in the treatment strategies employed for these disorders associated with reactive oxygen species

1.2 Reactive Oxygen Species (ROS) and Oxidative Stress

ROS are molecules which are chemically reactive containing oxygen. Examples include super oxide anion, hydrogen peroxides, nitric oxide and hypochlorite (Moslen, 1994). They are produced in the body from different cell physiological processes to protect it from foreign substances like micro-organisms ((Moslen, 1994). ROS can also be generated by several conditions like environmental phenomena which include exposure to ultra-violet light, heat and even ionizing radiation (Feinendegen, 1999). Production of ROS in good amounts leads to body defense against invading foreign substances but a higher production is lethal to the body cells, tissues, organs and organ systems due to the high risk of attack and sebsequent damages. When the anti-oxidants are not enough in the body, the levels of ROS increases and react with the cell components such as the DNA, proteins, amino acids as well as the lipids causing cell death (Kannan & Jain, 2000). The oxidative damage also increases in human tissues with age and has major contributions towards the functional decline which is a characteristic of aging such as development of wrinkles, dull skin, menopausal problems as well as cancer (Quiroga *et al*., 1993).

The resultant oxidative stress also has been associated with several degenerative diseases causing lipid peroxidation like ischemia-reperfusion injury, hemoglobinopathy, drug-induced anaemia, disseminated intravascular coagulation (Omar *et al.*, 1991), tumors, cancer, cardiological disorders such as coronary artherosclerosis (Jackton *et al*., 1993) diabetes mellitus (Sugawara *et al*., 1992)

and even neurological disorders such as depression, alzheimer´s and parkinson diseases, cerebral edema, traumatic epilepsy and spinal injury (Simonian and Coyle, 1996).

Several studies have also been conducted in finding out the effects of ROS on amino acids found in the body of animals as well. Suto *et al* (2006) conducted a study in which essential amino acids like methionine, histidine, tryptophan and non essential amino acid tyrosine were subjected to different ROS conditions such as singlet oxygen, superoxide and hydroxyl radicals. In each case, oxidative products were obtained. Since amino acids are the basic precussors in the formation brain neurotransmitters, oxidation of amino acids by ROS leads to fatal body conditions affecting neurological, cardiological, urological as well as physiological processes (Tasman *et al*., 1997). Enzymes in the brain such as Monoamine Oxidase B (MAOs) also act synergistically with ROS contributing to the oxidation of these important chemical messengers of the brain (Tipton *et al*., 2004). Some conditions associated with the lack of these neurotransmitters in the body are: obesity, depression, anxiety, chronic fatigue, insomnia, attention deficit, panic attacks and digestive complaints and spinal cord nerve injury. Disorders such as schizophrenia, Parkisons disease and Alzheimer´s are also oxidative stress related diseases (Tasman *et al*., 1997). The above presented information show clearly that increase in the ROS levels in the body leads to several ailment conditions. To prevent or delay the oxidation process, the body produces enzymes such as superoxide dismutases (SOD*)* which eliminates excess from the body (Carlson, 2005). However, SOD energy level differs from one person to the next and also declines with age.

Consuming anti-oxidants as food suppliments with the same properties as SOD enzymes is one way of boosting the lowered energy level of SOD enzymes (Carlson, 2005). Although commercial anti-oxidants such as BHT, BHA, TBHQ and gallic acid esters are usually used in foods, their health and safety is amajor concern and seriously questioned (Branen, 1975; Ito *et al*., 1983). Synthetic

anti-oxidants have been shown to be involved in carcinogenesis as well as liver damage in laboratory animals (Gartner *et al*., 1997). Other reports also indicate that their solubility is low with moderate anti-oxidant activities (Branen, 1975; Barlow, 1990). Stringent measures on their applications have been put in place which has led to the rise of interest in the search for natural scavengers of free radicals as their substitutes (Lim *et al*., 2002; Kayano *et al*., 2002; Gyamfi and Aniya, 2002).

1.3 Statement of the Problem

Oxidative stress is a factor in the formation and development of many diseases such as cardiovascular and neurological conditions like Parkinson's, depression, alzheimer's, disease, aging and rheumatoid arthritis (Quiroga *et al*., 1993). In preventing or delaying the oxidation process in foods, commercial anti-oxidants such as BHT, BHA are added. The safety concern of these synthetic anti-oxidants is attracting many reports such as being tumor promoters in the stomach, liver as well as lung related problems (Branen, 1975; Ito *et al*., 1983). Enzymes in the brain such as Monoamine oxidases (MAOs) are involved in the oxidative deamination of important neurotransmitters such as adrenaline, norepinephrine, serotonin and dopamine from the brain (Tipton *et al*., 2004). Synthetic Mono Amine Oxidase Inhibitors (MAOIs) have been developed to prevent this from happening in improving and maintaining brain cell communication but the problem has persisted. The usage of these synthetic MAOIs is reported to have many side effects associated with them just like the synthetic antioxidants such as sudden drop in blood pressure upon standing up (Grady *et al*., 2012), dry mouth, nausea, diarrhea or constipation, headache, drowsiness, Insomnia, skin reaction at the patch site, dizziness, increase in body weight, difficulty in passing urine, painful muscles and other fatal drug interactions (Mosher *et al*., 2007; Rang *et al*., 2003).

Identification of plant's phytochemicals for their anti-oxidant potentials and monoamine oxidase inhibitory activities is of enomous interest because of possible application in dietary supplements to improve the anti-oxidant capacity of the body as well as neurophysiological functions.

1.4 Objectives of the Study

1.4.1 General Objective

The general objective of the study was to evaluate secondary metabolites from the surface exudates of the aerial parts of *Gardenia ternifolia* for their anti-oxidant and MAO Inhibitory constituents in reducing oxidative stress and neurological disorders such as depression.

1.4.2 Specific Objectives

The specific objectives of this study were:

i. To isolate and elucidate the structures of secondary metabolites from *G. ternifolia* surface exudates.
ii. To establish the antioxidant and MAO inhibitory activities of the isolated compounds.
iii. To improve the lipophilicity of the most potent compound against MAO through structural modification.

1.5 Justification and Significance of the Study

Epidemiological studies have shown a lower incidence of illnesses in people consuming plant's phytochemicals especially flavonoid rich diets (Lemeshow *et al*., 1998). *Gardenia ternifolia* leaves are rich in flavonoid aglycones and are also used to manage mental illness and insanity cases in countries such as Uganda (Tabuti *et al*., 2003) , Congo Brazaville (Diafouka, 1997) and Ethiopia.

This study is therefore is very important in identifying the compounds present on the leaf surface exudates of this plant responsible for the therapeutic potential, which may also act as new chemical lead compounds to be used towards the search for the cure of ROS related diseases as well as the management of the neurological conditions associated with the lack of neurotransmitters.

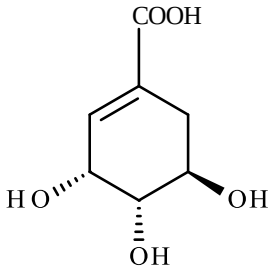
CHAPTER TWO

LITERATURE REVIEW

2.1 Flavonoids and their Occurance

Flavonoids are phenolic secondary metabolites found in plants. They are distributed in both vascular and about half of all non-vascular plant species. Over 6000 chemical structures of flavonoids have been identified in research studies (Anna and Lasse, 2011).

Flavonoids can be grouped into several categories depending on the extent of oxidation and the substituents. The biosynthetic pathway is through the shikimic acid (**1**) and acetate pathways, producing C6-C3-C6 basic skeleton with a heterocyclic benzopyran ring containing oxygen (C3) and two aromatic rings C6 (Michael *et al*., 2001).



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The ring that originates from the acetate pathway is generally labeled ring A, while the ring derived from the shikimic acid pathway is generally labeled ring B, **Figure 2.1**.

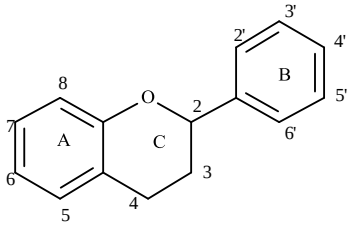


Figure 2.1:Basic skeleton of flavonoid

Based on the oxidation state of ring C and the substituents, the different categories which they are grouped into are: flavones, flavanone, flavonols, isoflavones, anthocyanidins and flavan-3-ol as shown in **Figure 2.2** below.

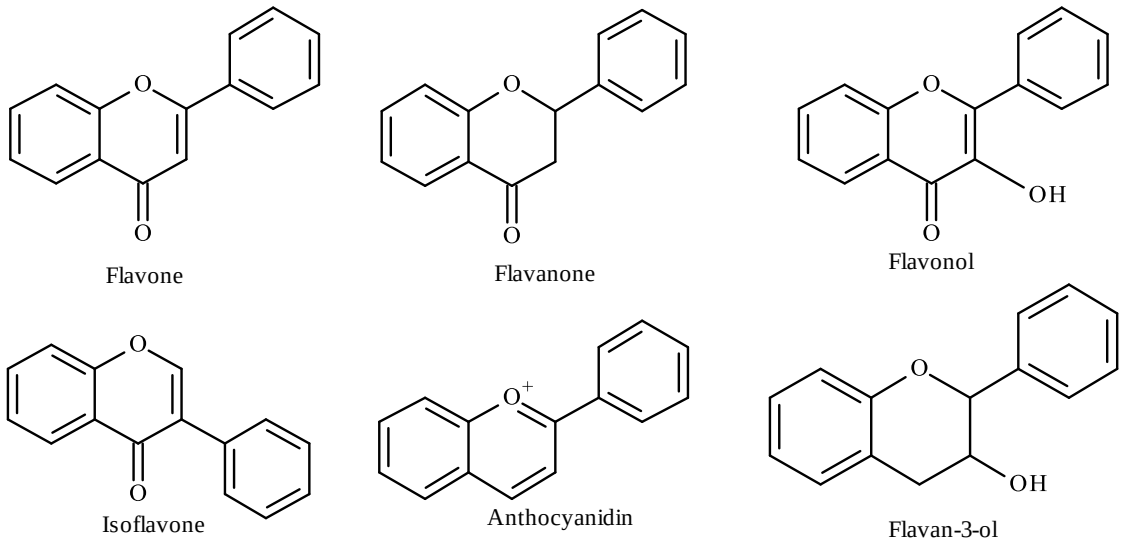
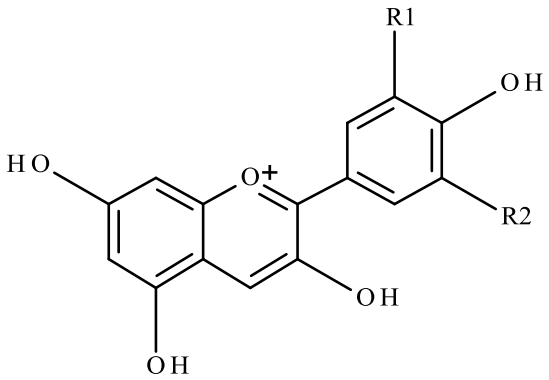


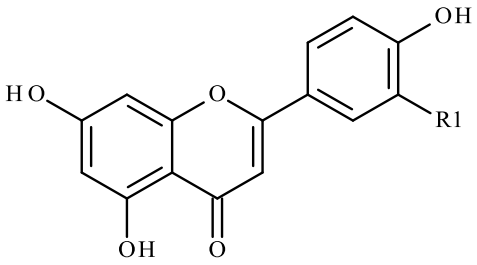
Figure 2.2: Different groups of Flavonoids

According to the report prepared by Seema *et al* (2013) on updated US Department of Agriculture (USDA) database for the flavonoid content of selected foods, the various classes have several examples. Anthocyanidins are the most popular and plentiful type of flavonoids which give plants their blue, red or purple coloration seen in most fruits such as blueberries, cherries, grapes and blackberries. Examples of these include compounds **2-7**.



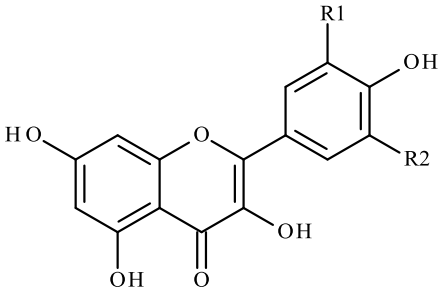
	Anthocyanidin	**R_1**	**R_2**
2	Cyanidin	H	OH
3	Delphinidin	OH	OH
4	Malvidin	OCH_3	OCH_3
5	Pelargonidin	H	H
6	Peonidin	H	OCH_3
7	Petunidin	OH	OCH_3

Flavones are not as common and widespread as flavonols but they can be found in some herbs and plants (Seema *et al*., 2013). Examples of flavones include apigenin (**8**) and luteolin (**9**).



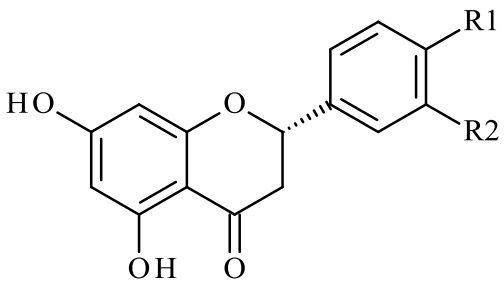
	Flavone	R_1
8	Apigenin	H
9	Luteolin	OH

The most commonly found type of flavonoids is flavonols which are widely distributed in nature and commonly found in many plant leaves and other pigments. Examples include compounds **10-13**:



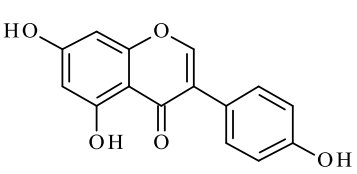
	Flavonols	R_1	R_2
10	Quercetin	OH	H
11	Isorhamnetin	OCH_3	H
12	Kaempferol	H	H
13	Myricetin	OH	OH

The report also stated that flavanones are mostly found in many fruits and herbs, but considering the whole plant kingdom, they are mostly found in the citrus species especially their fruits and juices: Oranges, lemons and grapes. Common examples of flavanones include naringenin (**14**), hesperetin (**15**) and eriodictyol (**16**).



	Flavanone	R_1	R_2
14	Naringenin	OH	H
15	Hesperetin	OCH_3	OH
16	Eriodictyol	OH	OH

Isoflavones on the other hand are mostly present in the Leguminosae family like the soybeans, soy foods and examples include genistein (**17**), daizein (**18**) and glycitein (**19**)



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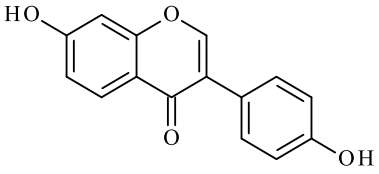

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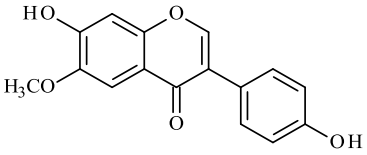

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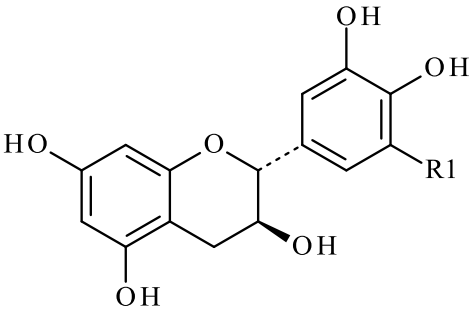
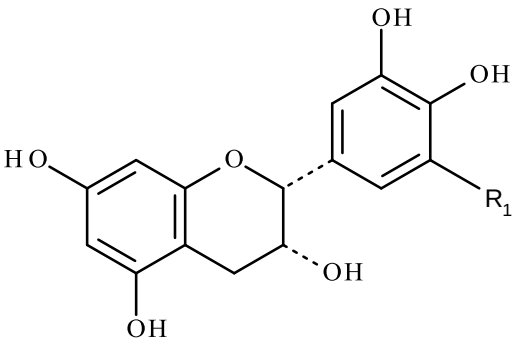
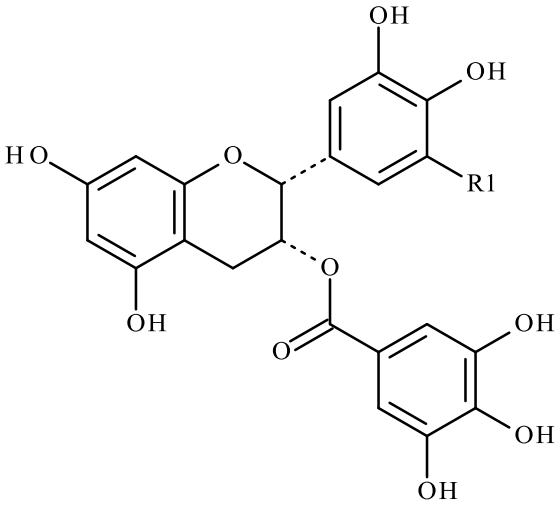
The updated data also gives flavanol monomers-the catechins mainly found in teas, chocolate, apples, berries, and grapes .The report also includes dimers and polymers.



	Flavanol	R_1
20	(+)-Catechin	H
21	(+)-Gallocatechin	OH



	Flavanol	**R_1**
22	(-)-Epicatechin	H
23	(-)-Epigallocatechin	OH



24	(-)-Epicatechin gallate	H
25	(-)-Epigallocatechin	OH

Due to the greater importance of flavonoids, several food sources exist as summarized in **Table 2.1**.

Table 2.1: Common dietary flavonoids (Seema et al., 2013)

FLAVONOIDS		
SUBCLASS	**DIETARY FLAVONOIDS**	**COMMON FOOD SOURCES**
ANTHCYANIDINS	Cyanidin (**2**) Delphinidin **(3**) Malvidin (**4**) Pelargonidin (**5**) Peonidin (**6**) Petunidin (**7**)	Red, blue, and purple berries; red and purple grapes, redwine, cherries, strawberries, tea, cabbage, bananas, pears, plums
FLAVANOLS	**Monomers (Catechins)** Catechin (**20**) Epicatechin (**22**) Epigallocatechin (**23**) Epicatechin gallate (**24**) **Dimers and Polymers** Theaflavins, Thearubigins Proanthocyanidins	**Catechins**: Teas (particularly green and white), chocolate, grapes, berries, apples Theaflavins, Thearubigins: BlackTea **Proanthocyanidins**: Chocolate, apples, berries, red grapes, red wine
FLAVANONES	Hesperetin (**15**) Naringenin (**14**) Eriodictyol (**16**)	Citrus fruits and juices, e.g., oranges, grapefruits, lemons and tomatoes
FLAVONOLS	Quercetin (**10**) Kaempferol (**12**) Myricetin (**13**) Isorhamnetin (**11**)	Yellow onions, scallions, kale, broccoli, apples, berries, teas, lettuce, olives, grapes fruit peels, berries, sweet potatoes.

FLAVONOIDS		
SUBCLASS	**DIETARY FLAVONOIDS**	**COMMON FOOD SOURCES**
FLAVONES	Apigenin (**8**) Luteolin (**9**)	Parsley, thyme, celery, hot peppers, apple skin, oranges, apples watermelon, carrots, lettuce
ISOFLAVONES	Daidzein (**18**) Genistein (**17**) Glycitein (**19**)	Soybeans, soy foods, legumes

In nature, flavonoids mostly occur as polymers and to some extent dimers are reported to be the most abundant form. The linkage is majorly through Carbon-Carbon or Carbon-Oxygen-Carbon bonds (Bruneton, 1999). Flavonoids in food and those in medicinal plants are assimilated in the body and relayed to the various parts of the body including the brain, passing the BBB before acting on the central nervous system (Bruneton, 1999).

Flavonoids possess a variety of functions in plants due to the wide range of colour production such as yellow, blue, red and purple colours in flowers and fruits (de Groot and Rauen, 1998) and carotenoids together with chlorophylls are also produced (Brouillard and Dangles, 1994).These help in plant pollination and seed dispersal by attracting birds and insects-the pollinating and dispersing agents thus ensuring plant species´ continuity and sustainability in the various natural ecosystems.

Apart from these important roles flavonoids play in plants in their various ecological conditions, they also exhibit physiological functions that help in increasing the plant productivity and adaptability in the various habitats. They help in protecting the plants against ultraviolet light

damage thus acting as sunscreens through absorbing the dangerous UV radiation as demonstrated by the study conducted by Caldwell in 1971.

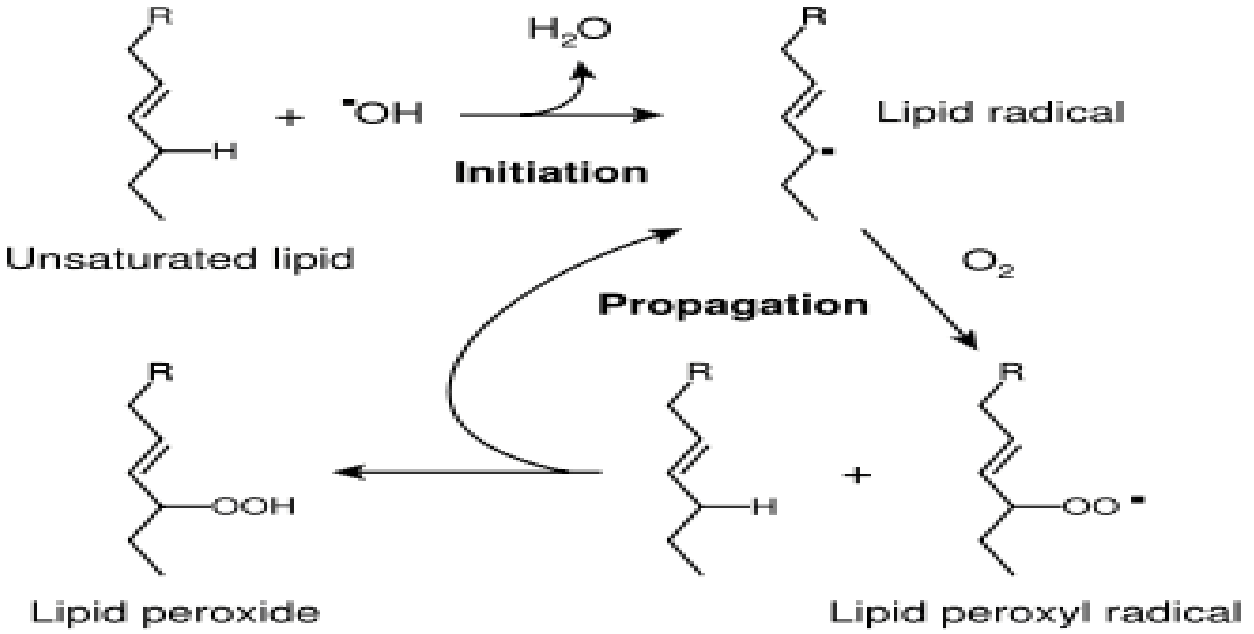
2.2 Flavonoids and their Antioxidant Potential

Many flavonoids can be effective in reducing free radical damage to cells and other various components in body tissues and organs by providing anti-oxidant benefits. The anti-oxidant capacity of almost every group of flavonoids is fully studied (Korkina and Afanas, 1997). Amongst the various sub-classes, (Figure 2.2)**,** the flavones and catechins (Tapas *et al*., 2008) are the most useful groups of flavonoids in protecting the body against ROS. Various tissues and cells in the body are constantly endangered by the damages generated by reactive oxygen species and free radicals produced during normal the normal metabolism of oxygen or caused by exogenous damage through toxins as reported by de Groot (1992) and Grace (1994).These processes are summarized in Table 2.2.

Table 2.2: Different mechanisms of production of ROS in the body (Manach *et al*., 1995)

Reactive Oxygen Species	**Mechanism of production**
Superoxide anion (O_2).	O_2 (One electron reduction)- heme proteins
Hydrogen peroxide (H_2O_2)	O_2 (Two electron reduction)
HO_2	proton addition to O_2
OH (Hydroxy radical)	Produced from Fentons reaction (3 electron reduction of O_2)
$^1O_2$	Singlet Oxygen production
ROO (Peroxy/ Lipid Peroxy radical)	proton hydrogen abstraction
RO (Alkoxy radical)	organic hydroperoxide

Many studies done have shown various mechanisms through which free radicals interfere with the nomal body physiological processes like lipid peroxidation of the cell membrane which causes changes in the cell net charge, leading to changes in the osmotic pressure and making it to swell and finally cell death (Devasena *et al*., 2014).



Study conducted by Halliwell (1995) showed various ways in which different organisms have developed to protect themselves from ROS as revealed by the presence of anti-oxidant defense factors of the body including several groups of enzymes like superoxide dismutase (SOD*)*, catalase, and glutathione peroxidase as well as glutathione, ascorbic acid, and *alpha*-tocopherol. All these are classified as endogenous antioxidants (Durga *et al*., 2014). High production of the free radicals in the body leads to depletion of endogenous anti-oxidants. According to Nijveldt *et al* (2001), flavonoids can act as anti-oxidants in two ways. The first one, they can increase the ability of endogenous anti-oxidants thus increasing the level of action of endogenous anti-oxidants. The

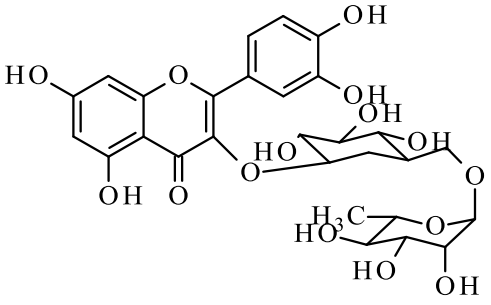
second way is by interfering with many different systems in the human body generating free radicals as discussed below.

2.2.1 Direct Radical Scavenging

Korkina and Afanas (1997) reported that flavonoids react with radicals giving a product which is more stable and less reactive. This is postulated to be through the hydroxyl group of the flavonoid.

Flavonoid (OH) + R• = flavonoid (O•) + RH (Middleton, 1998)

R• is a free radical and O• is an oxygen free radical. Certain flavonoids have been reported to directly scavenge superoxides forms while others scavenge peroxynitrite (Nijveldt *et al*., 2001) (Table 2.2) above. Flavonoids such as epicatechin (**22**) and rutin (**26**) are also reported to be potential radical scavengers (Hanasaki *et al*., 1994). Since flavonoids have the capacity to scavenge the free radicals, they can also prevent low density lipoprotein from being oxidized (Kerry and Abbey, 1997). This prevents free radical formation which may contribute to the pathogenesis of atherosclerosis.



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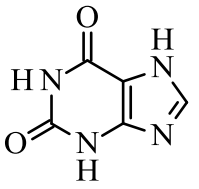
2.2.2 Xanthine Oxidase Pathway

Another proposed way by which flavonoids acts as anti-oxidants is by interfering with the xanthine oxidase pathway. This is a super oxide producing enzyme with a general low specificity (Bonini *et al*., 2004) and its route is linked to be very important in the oxidative injury to cells and tissues

mostly after ischemia-reperfusion (Sanhueza *et al*., 1992). Xanthine dehydrogenase and oxidase catalyzes the metabolism of xanthine (**27**) to uric acid (**28**). The former is the form present under physiological conditions and it is changed to the latter during ischemic conditions (Nijveldt *et al*., 2001).



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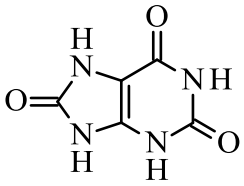

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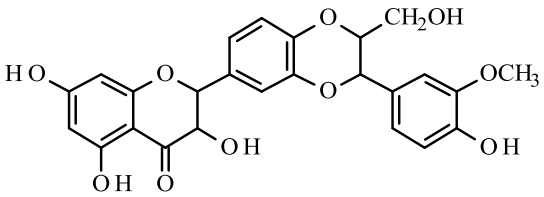
Scientific studies have shown that flavonoids like quercetin (**10**) and silibinin (**29**), inhibit xanthine oxidase activity (Shoskes, 1998; Lio *et al*., 1986) thereby resulting in decreased oxidative injury. Cos *et al* (1998) did structure activity relationship study on flavonoids and classified them as superoxide scavengers and xanthine oxidase inhibitors. The study revealed that luteolin (**9**) was the most potent.



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2.2.3 Nitrogen Monoxide (NO)

Scientitific reports indicate that quercetin (**10**) reduces ischemia-reperfusion injury by hindering inducible nitric-oxide synthase action (Shoskes, 1998). The production of Nitrogen monoxide in the body is through different cell types involving macrophages and endothelial cells. Nitrogen monoxide is used by the inner linings of blood vessels in signalling smooth muscles, producing

relaxation and thereby increasing the volume of blood flowing in them. Nitrogen monoxide is very reactive but can diffuse freely from one membrane to the next (Stryer, 1995). Initial release of nitrogen monoxide through nitric-oxide synthase is crucial in keeping blood vessels dilated but a higher concentrations of it is fatal in which production of both nitric oxide and superoxide anions is enhanced (Devasena *et al*., 2014). Nitrogen monoxide reacts with free radicals due to its high reactivity, and thereby producing highly damaging peroxynitrite ($ONOO^-$). Because of its oxidizing properties, peroxynitrite can damage a wide array of molecules in cells like the DNA and the proteins irreversibly. Nitrogen monoxide injury takes place mostly via peroxynitrite ($ONOO^-$) pathway since peroxynitrite can directly oxidize low density lipoproteins generating an irreversible fatal damage to the cell membrane (Nijveldt *et al*., 2001). The use of flavonoids leads to the scavenging of free radicals hence limiting the reaction with nitrogen monoxide leading to less damage to the lipid cell membranes (Shutenko *et al*., 1999).

2.2.4 White Blood Cells Immobilization

The primary role of white blood cells is to protect the body from any infectious disease along side foreign invaders. They are produced from a gene activation potential cell in the bone marrow called hematopoietic stem cell (Maton *et al*., 1997). White blood cells are found throughout the body including the lymphatic system and the blood. The deactivation and strong fixing of white blood cells to the inner walls in the surface of blood vessels also contributes to the formation of oxygen-derived free radicals, the release of inflammatory mediator's and cytotoxic oxidants which activates the complement system (Nijveldt *et al*., 2001). Under normal physiological conditions, white blood cells localized freely along these linings but during ischemic conditions and inflammations, endothelium derived mediators and other complementary factors causes them to stick on the cell

linnings making them immobile hence facilitating the release of neutrophils (Devasena *et al*., 2014).

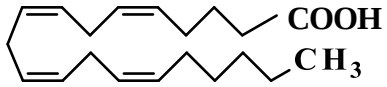
Oxidants and inflammatory mediators are also released as a result leading to tissue injury. Reports by Friesenecker *et al* (1994) indicated that oral administration of a dose of purely micronized fraction of flavonoids reduces the white blood cells immobilization during the re-oxygenation of the tissue (reperfusion). Some flavonoids have also been reported to be having the inhibitory capacities for the degranulation of neutrophils (type of white blood cells) without affecting superoxide production (Ferrandiz *et al*., 1996).

2.2.5 Interaction with Other Enzymes

This is also another important way through which flavonoids work in elimination of reactive oxygen species. Studies have shown that lipid peroxidation is evident when ROS are in the company of iron (Bennett *et al*., 1981). Certain flavonoids such as quercetin (**10**) are known to chelate with iron (Ferrali *et al.,* 1997) hence preventing the productiont of free radicals. Studies done with quercetin (**10**) and rutin (**26**) reported that quercetin (**10**) has a good iron-chelating and iron-stabilizing properties and this has been attributed to its *ortho*-dihydroxy substitution.

Flavonoids also reduces complement initiations hence reducing firm adhesion of inflammatory cells to the endothelium as seen in the case of white blood cells (Friesenecker *et al*., 1994) thus generally resulting in reduced inflammatory response. Flavonoids also contribute to the reduction of the release of peroxidase enzyme. The reduction hinders the production of ROS by neutrophils-type of white blood cells by interfering with alpha1-antitrypsin activation. This protease inhibitor inhibits a several enzymes (proteases) thus protecting several tissues (Wu and Foreman, 1991). Flavonoids also interfere with the enzymatic systems by reducing the metabolism of arachidonic acid (**30**). The

production of arachidonic acid (**30**) commences an inflammatory response in the cell and the neutrophils with lipoxygenase create certain chemotactic compounds from arachidonic acid (**30**) (Ferrandiz and Alcaraz, 1991).



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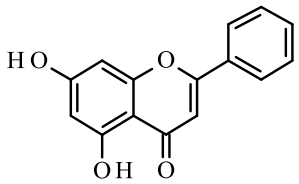
The inhibitory property of flavonoids to several enzymatic systems is of great interest and is currently being exploited in studies including brain related disorders caused by the depletion of the neurotransmitters by the monoamine oxidase(s) enzyme. Lots of research work has been published in this area in search of a potent natural MAO inhibitor with dual capacity i.e (Inhibition of MAO enzymes together with the reduction of ROS). Accumulation of excessive ROS levels in the body leads to the depletion of essential amino acids used in the synthesis of monoamines in the brain. This is due to their oxidative deamination to the corresponding oxidative products (Suto *et al*., 2006).

2.3 Mono Amines

Monoamine describes neurotransmitters transmitting signals of a nerve to a cell. They contain a single amino group linked aromatic ring through -CH_2-CH_2- bond acting as abridge. They are obtained through the action of aromatic amino acid decarboxylase on aromatic amino acids phenylalanine (**33**), tyrosine (**34**), tryptophan (**35**), and also thyroid hormones-triidothyronine (Mele *et al.*, 2010).



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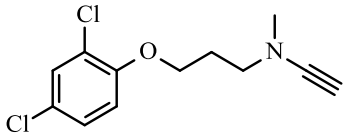

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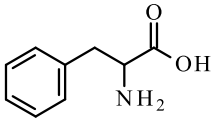

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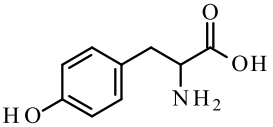

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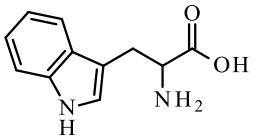

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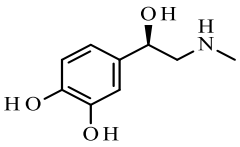
Monoamines are grouped into two classes upon which several neurotransmitters are classified. The classes include catecholamines and indolamines. The former include epinephrine (**36**), norepinephrine (**37**) and dopamine (**38**), while the latter is composed of serotonin (**39**) and melatonin (**40**) (Mele *et al.*, 2010).



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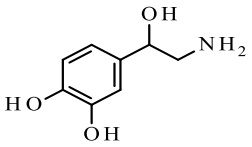

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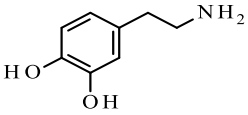

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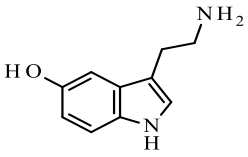

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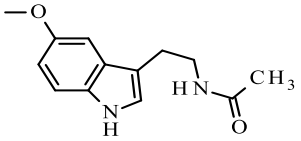

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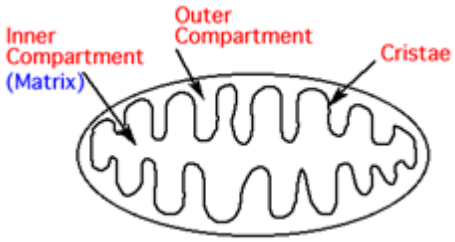
Drugs used to reduce or elevate the effects of monoamines are used to treat patients suffering from psychiatric disorders including depression, anxiety, and schizophrenia (Kurian *et al.*, 2011).

2.3.1 Mono Amine Oxidase (MAO)

MAO is agroup of enzymes which speeds up the breakdown of neurotransmitters (Tipton *et al.*, 2004), involved in controlling neurological conditions such as mood. MAOs are associated with the outer membrane of mitochondria (**41**) where they bind.



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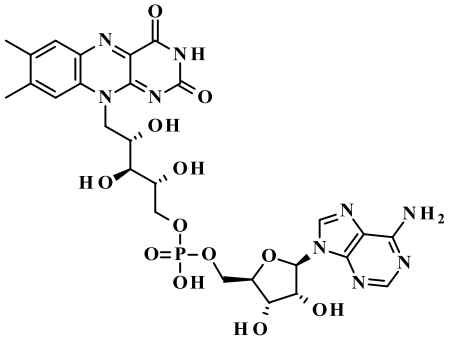
This enzyme was initially uncovered in the liver in 1928 by Mary Bernheim, a British biochemist and named tyramine oxidase (Hare, 1928). It was later renamed monoamine oxidase in 1999 (Slotkin, 1999). It is a protein that contains FAD (**42**) thus, classified as flavoproteins -proteins that contain a nucleic acid derivative of riboflavin (**43**).



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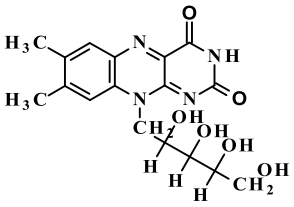

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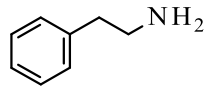
In human beings, two types of this enzyme exist: MAO-A and B (Shih and Chen, 2004). All are found in human git, astroglia and neurons as well as outside the CNS. Monomine oxidase A is also

available in the placenta, liver and apulmonary vascular endothelium while MAO-B is mostly found in blood thrombocytes. These enzymes (MAO-A and B) catalyze the breakdown of monoamines in removing an amine group from agiven molecule with the use of oxygen, resulting in other corresponding by-products such as aldehyde and ammonia (Shih and Chen, 2004).

MAO-A is specifically very crucial in the breakdown of monoamines taken in food (Kalgutkar *et al*., 2001). Adrenaline (**36**), noradrenaline (**37**), serotonin (**39**) and melatonin (**40**) are deaminated by MAO-A whle phenethylamine (**44**) and benzylamine (**45**) are broken down by MAO-B. Dopamine (**38**), tyramine (**46**) and tryptamine (**47**) are equally brocken down by both. Certain specific reactions catalyzed by MAO include conversion of adrenaline (**36**) or noradrenaline (**37**) to 3, 4-dihydroxymandelic acid (**48**) (Ley *et al*., 2002), metanephrine (**49**) or normetanephrine (**50**) to vanillylmandelic acid (VMA) (**51**) (Fatiadi and Schaffer, 1974), dopamine (**38**) to dihydroxyphenylacetic acid (**52**), and 3-methoxytyramine (**53**) to homovanillic acid (**54) (**Lambert *et al*., 1993).



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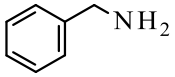

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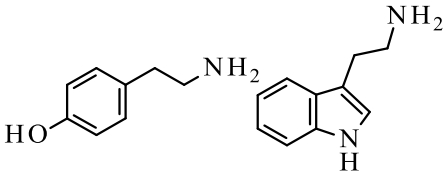

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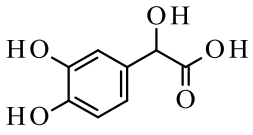

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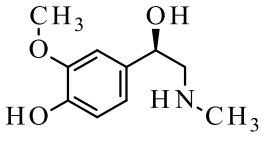

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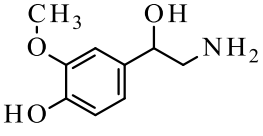

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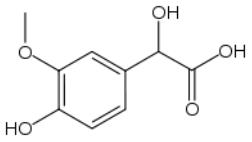

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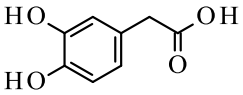

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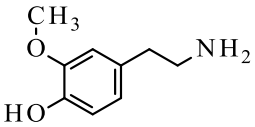

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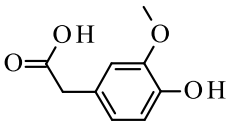

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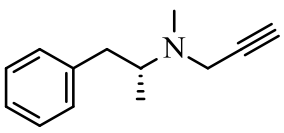
2.3.2 MAO Inhibitors (MAOI)

Monoamine oxidase inhibitors (MAOIs) are drugs which prevent the action of the monoamine oxidase. They have been recommended in depression management for many ages and are particularly effective in treating depression (Mario *et al*, 2012). They are also employed in the management of Parkinson's disease and other several neurological disorders. Scientific reports have indicated that MAOIs have dangerous dietary and drug interactions and thus they have been for many ages kept as a final resort of treatment used when other drugs like serotonin (**39**) (SRIs) and tricyclic antidepressants (TCAs) have failed. Drugs like selegiline (**55**) and moclobemide (**56**) have been developed as new MAOIs and are considered to provide a safer alternative (Liebowitz *et al*., 1990) thus are sometimes now considered to be used as first-line therapy but the side effects remains still a big challenge in the medical field.



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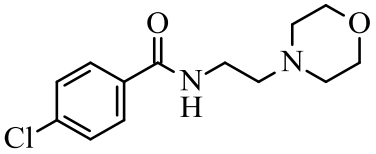

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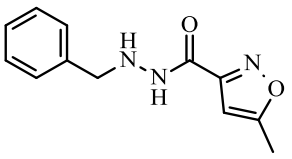
Monoamine oxidase inhibitors have proven to be effective in the treatment of several disorders such as panic disorder with agoraphobia (Buigues and Vallejo, 1987) social phobia, (Liebowitz *et al*., 1992; Versiani *et al*., 1992; Heimberg *et al*., 1989), bulimia (Rothschild *et al.*, 1994), typical depression (Jarret *et al*, 1999), mixed anxiety with depression, post-traumatic stress disorder (Davidson *et al*., 1987) as well as borderline personality disorder (Solloff *et al*., 1993).

In clinical depression and anxiety treatment, inhibition of both MAO-A and B is used. MAOIs are specifically indicated for outpatients with neurotic depression like conditions convoluted with panic disorders involving repeated episodes of depressed mood in a person, in responding to a feeling of rejection (Dowson, 1987). On February 28, 2006, FDA approved some monoamine oxidase inhibitors to be used in the treatment of depression such as: isocarboxazid-(Marplan) (**57**), isoniazid (laniazid) (**58**), nialamide(niamid) (**59**), phenelzine (nardil, nardelzine)(**60**), procarbazine(**61**), hydracarbazine(**62**), tranylcypromine (**63**). Some selective MAO-A Inhibitors approved to be used were: Moclobemide (aurorix, manerix) **(56**), pirlindole (pirazidol) (**64**) and toloxatone (humoryl) (**65**). Selective MAO B inhibitors such as Rasagiline (azilect) (**66**) and Selegiline (Deprenyl, Eldepryl, Emsam) (**55**) were also approved to be used in the management of depression.



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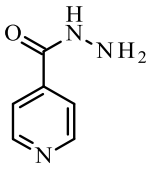

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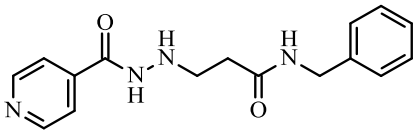

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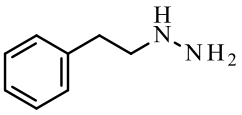

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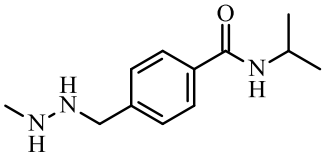

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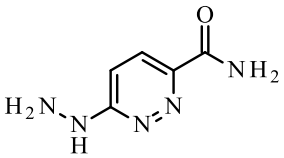

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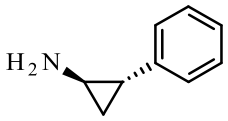
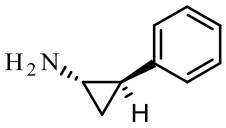


(1R,2S)-tranylcypromine



(1S,2R)-tranylcypromine

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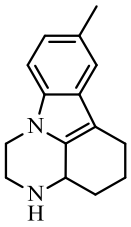

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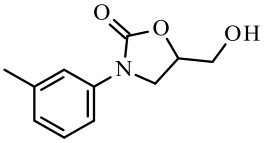

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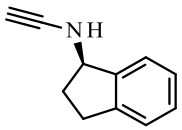

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2.3.3 Identification and Mechanism of Action of Monoamine Oxidase Inhibitors

Determination of monoamine oxidse inhibitors is of more attention as far as drug discovery is concerned (Shulman *et al*., 2013). Recent efforts being carried out by the researchers towards the discovery of monoamine oxidase inhibitors are concentrated on selective MAO-A and B. Selective Inhibitors that show selectivity towards MAO-A have shown effectivity in the management of depression (Kahn *et al*., 1989) while MAO-BIs are used for Alzheimer and Parkinson's disease management (Youdim *et al*., 2006).

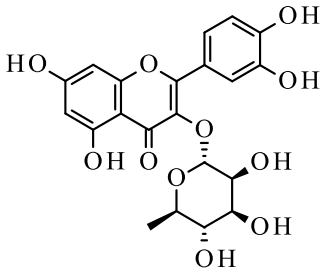
The action of MAOIs involves inhibition of the activity of monoamine oxidase enzyme hence the breakdowns of monoamines are prevented thereby increasing their availability in the brain for the various nerve impulse transmissions. Early monoamine oxidase inhibitors were reported to covalently bind to the monoamine oxidase enzymes hence interfering with the enzymatic activity

until new enzymes are made by the cell. Newer monoamine oxidase inhibitors like moclobemide (**56**) are reversible (Fulton and Benfield, 1996) and their action depends on the level of concentration of both the neurotransmitters and the monoamine oxidase inhibitor. Monoamine oxidase inhibitors differ by their selectivity for the substrates. Some prevent the action of both MAO-A and B equally while others are selective. Inhibition of MAO-A majorly affects neurotransmitters essential in mood or anxiety disorders. The usage of MAO-B inhibitors has been found to increase the basal dopamine levels and more recently, the inhibitors have been included in the list of drugs used for the management and treatment of anxiety disorders and Alzheimer's disease (AD) (Yamada and Yasuhara, 2004). Scientific search has shown that inhibition of MAO-B is of greater importance to the brain neurons due to the elimination of ROS producing step during the oxidative deamination process catalysed by the enzyme (Yamada and Yasuhara, 2004).

2.3.4 Interactions between Flavonoids and Monoamine Oxidase

A lot of research has been done using plants phytochemicals in the investigation of the inhibitory effects for both MAO-A and B. A number of flavonoids have been isolated and identified as inhibitors of both the isoforms. These include flavonols such as kaempferol (**12**), quercetin (**10**) and flavones; apigenin (**8**) and chrysin (**31**) from *Gingko biloba* (*Ginkgoaceae*) extract (Sloley *et al*., 2000). MAO-A inhibition activities from these flavonoids in this study were as follows with IC_{50} values: chrysin (2µM), apigenin (1 µM), kaempferol (0.7 µM) and quercetin (5 µM) in which phenelzine (**60**) the standard was (0.04 µM). Quercetin (**10**) from (*Calluna vulgaris* (Ericaceae) demonstrated MAO-A inhibitory activity of IC_{50} = 18 µM in a study by Saaby *et al* (2009) in which clorgylin (**32**) an irreversible and a selective MAO-A inhibitor, used as the standard showed IC_{50} = 0.2 µM. Chimenti *et al*., 2006 in a study reported quercetin (**10**) showing high inhibition activity towards MAO-A, IC_{50} = 0.01 µM while inhibition activity for MAO-B was IC_{50} = 20 µM.

Quercetrin-3-glucoside (isoquercetrin) (**67**), rutin (**68**) and quercetin (**10**) isolated from *Melastoma candidum* (Melastomataceae) also inhibited MAO-B with IC_{50} = 19, 12, 4, 11 µM respectively in which selegiline (**55**), used as the standard had IC_{50} = 19 µM (Lee *et al*., 2001). The flavan-3-ols, (+)-catechin-**20** and (-)-epicatechin-**22** also from *Uncaria rhynchophylla* (Rubiaceae) showed MAO-B activity of IC_{50} = 89 and 59 µM respectively where IC_{50} for selegiline (**55**) was 0.3 µM as reported by Hou *et al* (2005).



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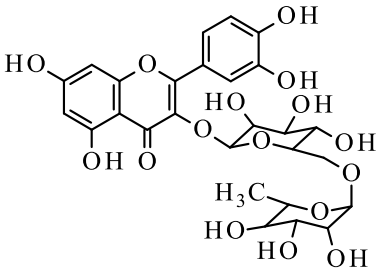

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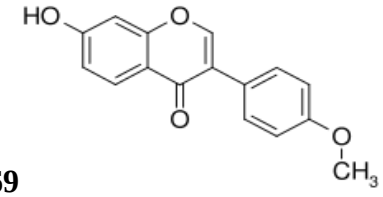
Formononetin (**69**), an isoflavone isolated from the roots of *Sophora flavescens* (Fabaceae), showed MAO-A inhibitory activity with an IC_{50} = 21 µM and 11 µM for MAO B. Kushenol (F)-(**70)** also isolated from the same plant exhibited IC_{50} = 104 µM (MAO-A) and 63 µM (MAO-B) according to the report published by Hwang *et al* (2005).



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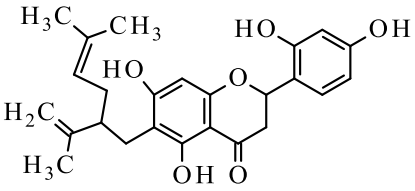

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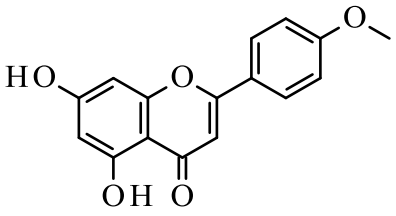
Naringenin (**14**) from *Mentha aquatic* (Lamiaceae) (Olsen *et al*., 2008) had inhibition of IC_{50} = 955 µM (MAO-A) and 288 µM (MAO-B) while C_{50} value for clorgylin was 0.003 µM and deprenyl showed IC_{50} = 0.1 µM. On the basis of the reported literature of the ability of naringenin in

crossing the blood brain barrier, they concluded that the isolated compound can enter into the CNS and be used in the treatment of depression-like conditions (Youdim *et al*.,2004). Investigations of pure anthocyanidins inhibitory activity on MAO-A and B have also been done (Dreiseitel *et al*., 2009) in which IC_{50} values were determined: delphinidin-**3** (35 µM and 31 µM) malvidin-**4** (22 µM and 19 µM), cyanidin-**2** (30 µM and 32 µM), pelargonidin-**5** (27 µM and 43 µM), petunidin-**7** (32 µM and 43 µM) and peonidin-**6** (31 µM and 22 µM). The same study also conducted the inhibitory activities of different glycosides and diglycosides of the anthocyanidins and IC_{50} ranged from 29-117 µM for MAO-A and 31-242 µM for MAO-B.

Han *et al* (1987) isolated flavonoids such as apigenin (**8**), eriodictyol (**16**) acacetin (**71**), luteolin (**9**) and diosmetin (**72**) from *Chrysanthemum indicum* and screened them for their MAO B inhibitory activities.



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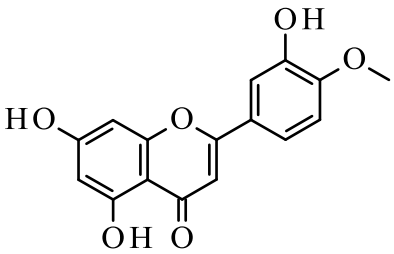

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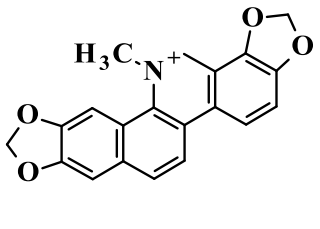
The research revealed that acacetin (**71**) and diosmetin (**72**) showed good inhibitory activity towards rat liver mitochondrial monoamine oxidase MAO-B with an IC_{50} value of 2.46 and 2.11mM respectively. A study by Ryu *et al* (1988) led to the isolation of apigenin (**8**) and kaempferol (**12**) from *Sophorae flos* (Fabaceae) and the inhibitory effects towards rat brain mitochondrial monoamine oxidase MAO-A with an IC_{50} value of 10µM each. This research revealed that both compounds did not inhibit MAO-B. Haraguchi *et al* (2004) isolated 5-hydroxyflavanone from *Gentiana lutea* which showed an IC_{50} value of 39.6 and 3.8 µM towards

both rat brain mitochondrial monoamine oxidase MAO-A and B. A study conducted by Han *et al* (2007) on *Cayratia japonica* (vitaceae) led to the isolation of flavonoids from the plant and the compounds were tested for their MAO-A and the inhibitory potency against was: flavone> flavonol> flavone glycoside> flavanonol. According to the series given, apigenin (**8**) showed inhibitory effects of IC_{50} 1.17µM. They also observed that the level of MAO inhibitory activity decreased as the number of OH groups increased in ring B of the flavone.

2.3.5 Interactions between Monoamine Oxidase and Alkaloids

A part from flavonoids, several studies have been done also with alkaloids as an alternative source for MAO inhibitory activity. A study conducted by Lee *et al* (2001) in the investigation of MAO inhibition activity in mouse brain from alkaloids, such as sanguinarine (**73**) and chelidonine (**74**) isolated from the herb *Chelidonium majus* revealed that sanguinarine (**73**) showed MAO-A activity of IC_{50} = 24.5 µM but chelidonine (**74**) did not inhibit MAO-Activity. Another study conducted by Kong *et al* (2001) isolated three alkaloids: jatrorrhizine (**75**), berberine (**76**) and palamatine (**77**) from Coptis (chinensis rhizome) and evaluated their MAO inhibitory activity. Jatrorrhizine (**75**) showed a non-competitive inhibition of the isoenzymes obtained from brain mitochondria with IC_{50} values of 4 and 62 mM. On the other hand, berberine (**76**) competitively inhibited MAO-A with an IC_{50} values of 126 mM. Palamatine (**77**) did not show any inhibitory action up to 200µM towards both MAO-A and B.

They also evaluated the rat brain mitochondrial MAO-A and B inhibitory action of piperine (**78**) and found IC_{50} value 49.3 and 91.3 µM respectively. Based on the inhibitory results obtained, they proposed that it could be due to the initiation by the hydrogen bonding of its naked amide with –NH–, –OH and –SH protons.



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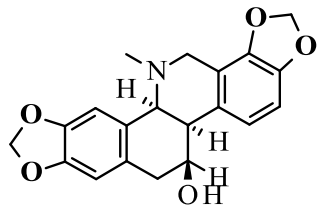

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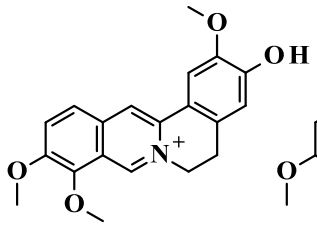

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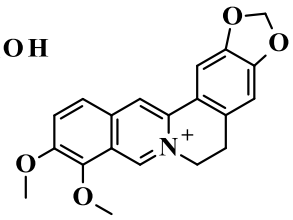

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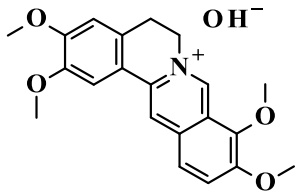

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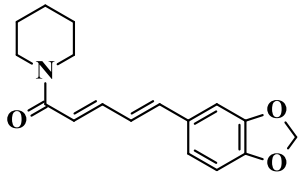

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The history of traditional health care dates back to the ages and a large majority of the population still use it to the present. It has been reported by many researchers that almost 80% of the total population in southern Africa depends on this healthcare provision. In South Africa, around 27 million people use traditional herbal medicines from aound 1,020 plant species as reported by Fennell *et al* (2004); Dauskardt,(1990) and Williams (1996). The majority of these are for the treatment for mental health related problem such as depression and other psychiatric related illness.

Large proportion of the treatment used by these traditional medicine practitioners comprises a lot of herbal preparations administered to the victims. The African traditional healers recognize and manage several mental disorders and illnesses of the central nervous system like depression, depilepsy (Van Wyk *et al*., 1997), nightmares, hysteria, anxiety, convulsions, and other mental related conditions with indigenous plants (Gelfand *et al*., 1985; Hutchings *et al*., 1996; Sobiecki, 2002).

In South Africa, these plants have been screened for their MAO inhibition activities (Stafford *et al*., 2007). *Ruta graveolens* showed a good MAO-A inhibitory activity (ethyl acetate leaf extract = IC_{50} 5µg/ml, petroleum ether extract= 3 µg/ml) MAO-B inhibition (ethyl acetate leaf extract IC_{50} = 7µg/ml petroleum ether extract = 3µg/ml) (Stafford *et al*., 2007).

According to these findings, the usage of indigenous plants by the traditional medicine practitioners is supported and may act as a road map leading to the discovery of novel MAO inhibitors. This study therefore focuses on the genus *Gardenia* due to its wide range usage by many communities across the world majorly in the treatment of mental or physiological related illness (Diafouka, 1997).

2.4 The genus *Gardenia*

The genus *Gardenia* belongs to the family Rubiaceae, characterized by leaves being opposite and sometimes whorled, with interpetiolar stipules with 140 species known to date. Generally this family has flowers with tubes and lobes, and the fruits may be a capsule berry or drupe (Beentje, 1994). Their fruits have thick walls, becoming pulp with a persistent carlyx except in two species (*G.ternifolia* and *G.volkensii*) (Beentje, 1994).

2.4.1 Folklore Information of the *Genus*

Gardenia species have been reported to be used medicinally in so many ways. The fruits of *Gardenia jasminoides* have been used widely in Japan and China and are reported to have laxative, anti-inflammatory, antipyretic, antihepatitis, diuretic, cholagolic and hemostatic effects (Yamauchi *et al*., 1974). Kim and Chang (1995) reported that the extract of the fruit reduces cholesterol level in rat´s serum. Certain regions in South East Asia use the leaves in poultices and the roots of the plant is used in the treatment of the nervous disorders and indigestion as reported in the

Publications & Information Directorate (PID*)* (1956). The report also includes information that the resinous exudates of *Gardenia gummifera* and *Gardenia lucida* have expectorant, anthelmintic, antiplasmodic and diaphoretic effects. Ether extract of the leaves of *Gardenia lucida* showed antibiotic activity against *Escherichia coli* and *Staphylococcus aureus* as reported by Joshi and Magar (1952). Hussain *et al* (1991) also reported that the methanol extract of *Gardenia erubescens*, a local medicinal plant found in Nigeria, showed hypotensive, sedative, analgesic as well as diuretic effects *in vivo* on mice, cats and rats. In Northern Nigeria, water decoction of the aerial parts of this species is used for the treatment of loss of apetite, insomnia as well as abdominal disorders (Dalziel, 1955). The Santals, an Indian tribe uses a root decoction of *Gardenia turgida* as a remedy for indigestion in children. Fruit juices of the same species are also used in the treatment of mammary gland infections. When the roots are crushed, it forms lather with water which is used in the treatment of headache (PID, 1956).

In Kenya, this genus is represented by four major species according to Beentje 1994. These are *Gardenia fiorii, Gardenia posoqueriodes, Gardenia volkensii* and *Gardenia ternifolia.* The species *Gardenia fiorii* occurs mainly in the dry open bush land areas of North Eastern Province of Kenya (Beentje, 1994). It is used by the residents in making spears due to its hard wood nature. *Gardenia posoquerioides* is a shrub growing to a height of about 3.5 meters and is found in moist forest such as shimba Hills, Buda and Rabairegions of the Coastal province of Kenya (Beentje, 1994). The root decoction of the plant is used by the Digo people as a remedy against syphilis (Kokwaro, 1976; Beentje, 1994). *Gardenia volkensii* is also a shrub growing to a height of about 7.5 meters with smooth and silvery grey back. In the Kenyan Coastline, it is found in the riverines woodland or wooded grassland (Beentje, 1994). Folklore information reports that many communities in Kenya and other African countries use the fruits of this plant as emetics and purgative (Kokwaro, 1976).

2.4.2 *Gardenia ternifolia* (Schum and Thonn)

Gardenia ternifolia (Schum and Thonn) is a shrub or tree which grows to a height of about 10 meters with a greenish grey back (Beentje, 1994). The leaves normally occur in triplets with an elliptic or slightly obvate shape and the base angles being attenuate to obtuse. The leave´s apex is rounded (Beentje, 1994). The fruits surfaces are smooth and they are yellow and sometimes reddish with an ellipsoid shape (Beentje, 1994). This species occurs mainly in the woodland or riverine woodlands but in Kenya, it occurs in Nyanza, Central, Eastern, Coast and the Western provinces.

2.4.3 Folklore Information on *Gardenia ternifolia*

Gardenia ternifolia is known among Kenyan communities, where it occurs with the following names siuma (luhya), kurkoi (Boni), onduongi or rayadhi (Luo), geninyet (Maasai), kimwemwe (Swahili) (Kokwaro, 1976; Beentje, 1994). This wide spread recognition suggests the ethno-medical value of this plant. For example the ethno-medical usages of the bark of *Gardenia ternifolia* suggest that it has emetic properties and the fruit is used as an eye medicine (Beentje, 1994). The infusion also from the roots is administered after snake bite as an antidote and it causes the victim to vomit heavily that counteracts the effects of the poison (Kokwaro, 1976). A decoction from the fruits is also used to treat malaria and also taken as a purgative (Kokwaro, 1976). **Table** (**2.3**) gives a summary of ethno-medical usages of different parts of the plant in Kenya and other countries in Africa.

Table 2.3: Ethno-medical information on *Gardenia ternifolia*

Gardenia ternifolia			
Country	**Traditional Uses**	**Plant Part**	**Reference**
Kenya	An infusion taken to treat malaria fevers, decoction taken orally to dress ulcers, as purgative and astringent and applied as antisnake venom	Root bark	Gakunjuet *et al*, 1995; Achola *et al.*, 1995
Guinea- Bissau	Used to treat jaundice	Root bark	Silvia *et al*., 1996
Western Sudan	Used to treat jaundice		
Tanzania	Used as aphrodisiac and against snake bite, cough, malaria and as a laxative	Roots	Chhabra *et al.*, 1991
Uganda	Decoction used in treatment of insanity	Roots	Tabuti *et al*., 2003
Kenya	Used to treat malaria	Dried fruit	Chhabra *et al*., 1991; Achola *et al*., 1995
French Guinea	Decoction used to treat syphilis infection	Dried leaves	Achola *et al*., 1995
Rwanda	Decoction used to treat malaria	Dried leaves	Hakizamungu *et al*., 1992
Tanzania	Decoction used to manage fevers and to treat wounds	Dried leaves	Chhabra *et al*, 1991
Tanzania	Decoction used to treat stomachache, arthritis and asthma	Stem Bark	Chhabra *et al*, 1991
Congo Brazaville	Decoction used to treat epilepsy and other mental problems	Stem Bark	Diafouka, 1997
Ethiopia-Western region	Decoction taken orally in treatment of paludism (malaria symptoms with high fever and chills)	Stem Bark	Gidey *et al*., 2009

2.4.4 Biological Activities for the Extracts of *Gardenia ternifolia*

The dried leaves of this plant are reported to show anthelmintic activity in controlling internal parasites (endoparasites) (Ibrahim, 1992). The methanol extract of dried roots were investigated for molluscidal activity against *Bulinus globosus* and was found to be inactive at a concentration of 100 ppm (Sofowara and Adewunmi, 1980). Achola *et al* (1995) also reported that 0.05 µg/ml of 70% methanol extract of dried leaves of *Gardenia ternifolia* showed bronchodilator activity on guinea pig trachea.

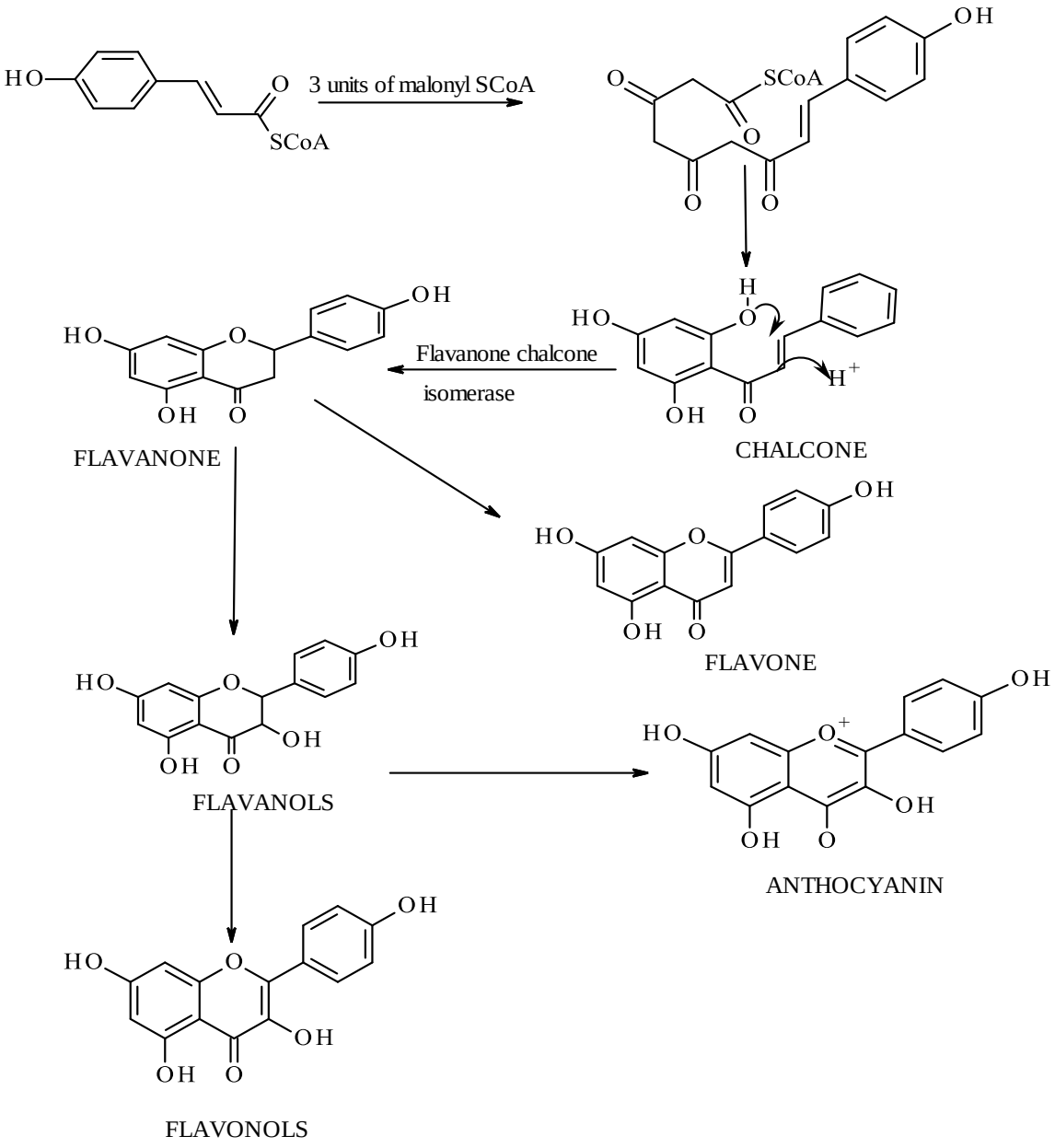
Investigations of hypotensive effects of 70% methanol extract on a rat isolated perfused hepatocytes by Achola *et al* (1995) found a dose of 40 µg/animal to be active but in Guinea-Bissau, antiviral activity was investigated using ethanol extracts and the extracts were active at 25 µg/ml when the cell culture experiment was performed with the virus for Africa swine fever. *Gardenia spp* belongs to the plant family Rubiaciae which has several classes of compounds ranging from flavonoids, alkaloids, iridoids and even terpenoids. Previous studies on the extracts of several species in this genus have led to the isolation of flavones, flavonols, flavanones, iridoids, acids and even esters.

2.4.4.1 Flavonoids of the genus *Gardenia*

They comprise an enomous class of plants phytochemicals deriving part of their structure from shikimate and part from the polyketide pathway. They are usually found as conjugates with various carbohydrates (i.e. glycosides). However, flavonoids isolated from the genus *Gardenia* mostly are encounted as free aglycones of the subclasses flavones and flavonols and few cases of flavanones have been reported (Virinder *et al*., 2000).

Scheme 2.1 summarizes the biosynthetic pathway of flavonoids which proceeds through the condensation of the Coenzyme A ester of 4-hydroxycinnamic acid and a triketide, through the

action of chalcone synthase enzyme. The chalcone then cyclyses to form flavanone structure. The process is catalyzed by the enzyme chalcone-flavanone isomerase. Different oxygenation patterns of the aromatic rings are evident from the polyketide pathway (meta-deposition) and the shikimate pathway (Dewick, 1988).



Scheme 2.1: Biosynthesis of Flavonoids (Dewick, 1988)

Several flavonoids have been isolated from different species of *Gardenia* as summarized in Table 2.4.

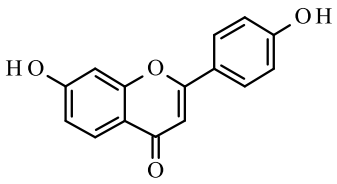
Table 2.4: Flavonoids from the Genus *Gardenia*

COMPOUND	**PLANT SOURCE**	**REFERENCE**
7,4´-Dihydroxy flavones **(79)**	*Gardenia sootepensis* (F)	Liang *et al*., 1991
5,3´-Dihydroxy-3,6,7,4´,5´-pentamethoxyflavone **(80)**	*Gardenia fosbergii* (BE)	Gunatilaka *et al*., 1979
5,5´-Dihyroxy-3,6,7,3´,4´-pentamthoxyflavone **(81)**	*Gardenia cramerii* (BE)	Gunatilaka *et al*., 1982
5,4´-Dihydroxy-3,6,7,8-tetramethoxyflavone **(82)**	*Gardenia fosbergii* (BE)	Gunatilaka *et al*., 1982
5,5´-Dihydroxy-6,7,2´,3´-tetramethoxyflavone **(83)**	*Gardenia fosbergii* and *Gardenia cramerii* (BE)	
5,4´-Dihydroxy-3,6,7-trimethoxyflavone **(84)**	*Gardenia fosbergii* (BE)	Gunatilaka *et al*., 1979
3´,4´-Dihydroxywogonin **(85)**	*Gardenia resinifera* (Gum)	Chhabra *et al*., 1976
3´,4´-Dimethoxywogonin **(86)**	*Gardenia resinifera* (Gum)	Gupta *et al.,*1975 and Chhabra *et al*., 1976
Gardenin A **(87)**	*Gardenia resinifera* (Gum) Gardenia turgida (R) *Gardenia lucida*	Stenhouse and Groves, 1877; Gupta *et al*., 1975; Krishnamurti *et al*., 1972; Joshi *et al*., 1979.
Gardenin B **(88)**	*Gardenia resinifera* (Gum) Gardenia turgida (R) *Gardenia lucida*	Gupta *et al*.,1975; Rao and Venkataraman, 1970; Joshi *et al*., 1979
Gardenin C **(89)**	*Gardenia lucida*	Rao and Venkataraman, 1970; Gupta *et al*., 1975.
Gardenin D **(90)**	*Gardenia lucida*	
Gardenin E **(91)**	*Gardenia resinifera* (Gum)	

COMPOUND	PLANT SOURCE	REFERENCE
Gardenin A-5-•-D-glucopyranoside **(92)**	*Gardenia florida* (S)	Tewari and Mukharya, 1988
5-Hydroxy-7,4´-dimethoxyflavanone **(93)**	*Gardenia erubescens* (ST)	Adelakun and Okogun, 1996
5-Hydroxy-7,4´-dimethoxy flavones **(94)**		
5-Hydroxy-3,6,7,3´,4´,5´-hexamethoxyflavone **(95)**	*Gardenia fosbergii* (BE)	Gunatilaka *et al*., 1979
5-Hydroxy-6,7,3´,4´,5´-pentamethoxyflavone **(96)**	*Gardenia fosbergii* (BE) *Gardenia Cramerii* (BE)	Gunatilaka *et al*., 1982
5-Hydroxy-7,3´,4´-trimethoxyflavanone **(97)**	*Gardenia erubescens* (ST)	Adelakun and Okogun, 1996
4´-Hydroxywogonin **(98)**	*Gardenia resinifera* (Gum)	Gupta *et al*., 1975
3-*O*-Methylkaempferol **(99)**	*G. gordonii (BE)* *G. grieve (BE)* *G. hillii (BE)* *G. storkii (BE)*	Miller *et al*., 1989
Nevadensin (**100)**	*Gardenia resinifera* (Gum)	Gupta *et al*., 1975; Krishnamurti *et al*, 1972
5,6,7,4´-Tetrahydroxy-3,3´,5´-trimethoxyflavone (**101)**	*Gardenia fosbergii* (BE)	Gunatilaka *et al*., 1979
5,7,3´,4´-Tetrahydoxy-6,8-dimethoxyflavone (**102)**	*Gardenia gum*	Chhabra *et al*., 1977
5,7,3´,5´-Tetrahydoxy-8,4´-dimethoxyflavone (**103)**	*Gardenia resinifera* (Gum)	
5,7,4´-Trihydroxy-3,6-dimethoxyflavone (**104)**	*Gardenia gordonii* (BE) *Gardenia grieve (BE)* *Gardenia storckii (BE)* *Gardenia tailensis*	Miller *et al*., 1989
5,7,4´-Trihydroxy-6,8-	*Gardenia resinifera* (Gum)	Gupta *et al*., 1975

COMPOUND	PLANT SOURCE	REFERENCE
dimethoxyflavone (**105)**		
5,3´,5´-Trihydroxy-3,6,7,4-tetramethoxyflavone (**106)**	*Gardenia fosbergii* (BE)	Gunatilaka *et al*., 1982
3´,4´,5´-Trihydroxywogonin (**107)**	*Gardenia resinifera* (Gum)	Chhabra *et al*., 1976
3´,4´,5´-Trimethoxywogonin (**108)**		Gupta *et al*., 1975; Krishnamurti *et al*, 1972
5,7,3´-Trihydroxy-6,8,4´-trimethoxyflavone (**109)**		Gupta *et al*., 1975
5,7,4´-Trihydroxyflavone (**110)**		

RT=Roots, **GE**=Gum Exudates, **LF**=Leaf, **BE**=Bud Exudates**ST=**Stem



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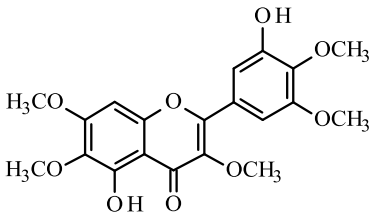

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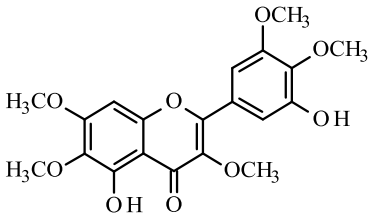

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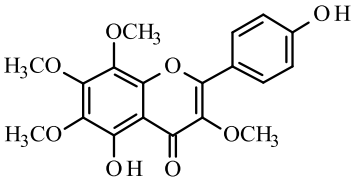

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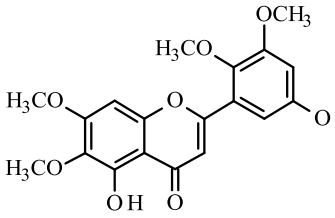

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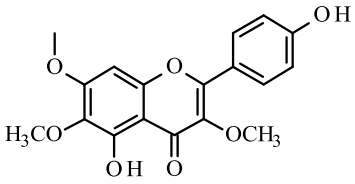

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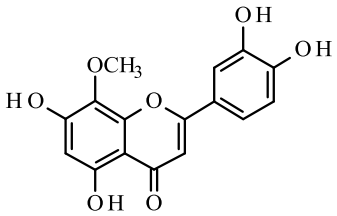

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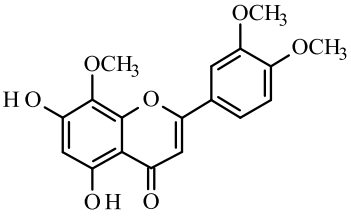

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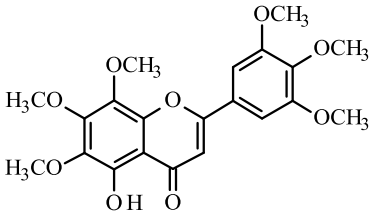

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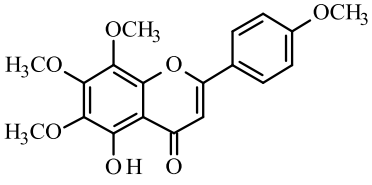

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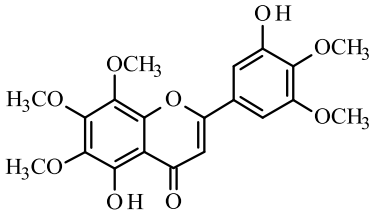

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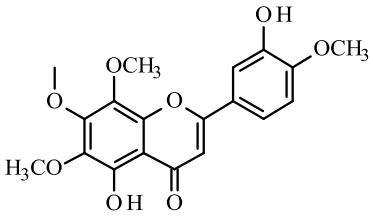

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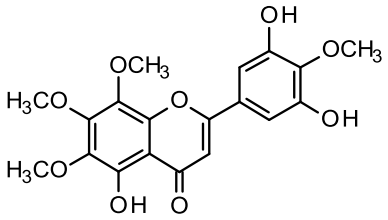

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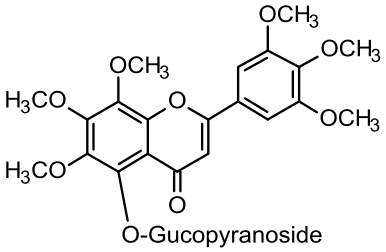

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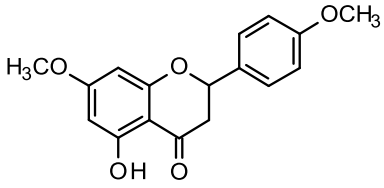

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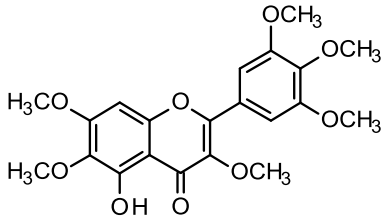

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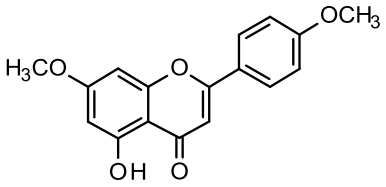

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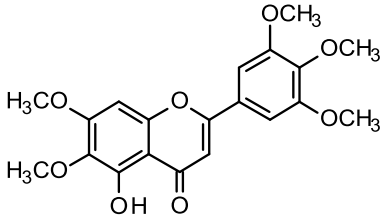

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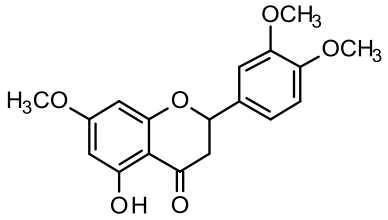

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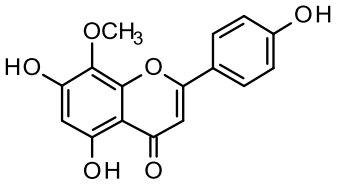

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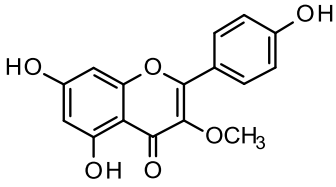

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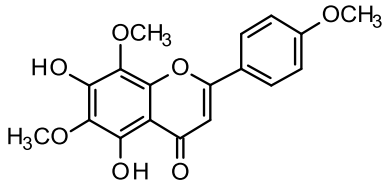

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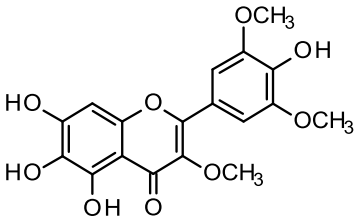

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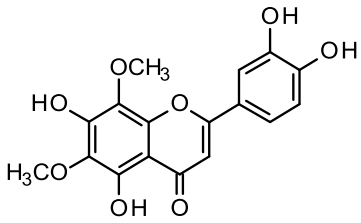

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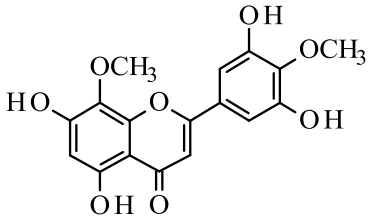

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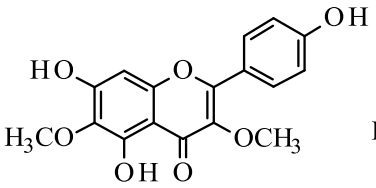

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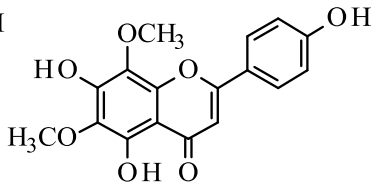

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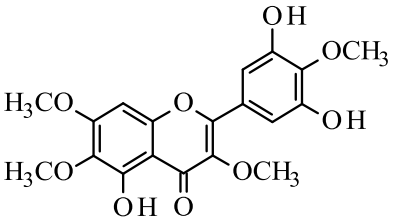

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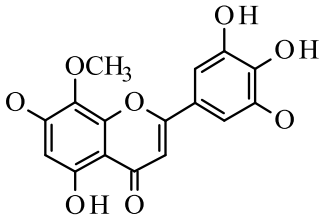

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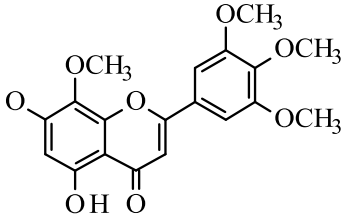

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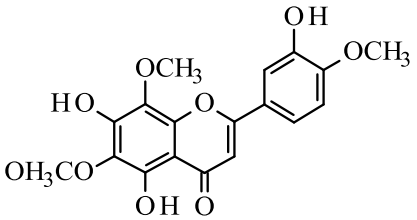

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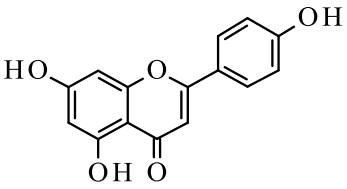

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2.4.4.2 Terpeniods of the genus *Gardenia*

Terpenoids forms the biggest category of plants secondary metabolites over 40,000 is known so far (Sacchettini and Poulter, 1997; Dewick, 2002; Roberts, 2007). They play very important tasks in primal metabolism and ecological intercommunications in both plants and animals which they are found such as defense against predators, pathogens and against competitors (Gershenzon & Dudareva, 2007). They also show economically and medically valued characteristics (Sacchettini and Poulter, 1997) like certain sesquiterpenes and monoterpenes being volatile organic compounds are useful in perfume and food industries (Schulz and Dickschat, 2007). Certain terpenoids from

microbes have phytotoxic metabolites which have remarkable influence on agriculture (Collado *et al*., 2007).

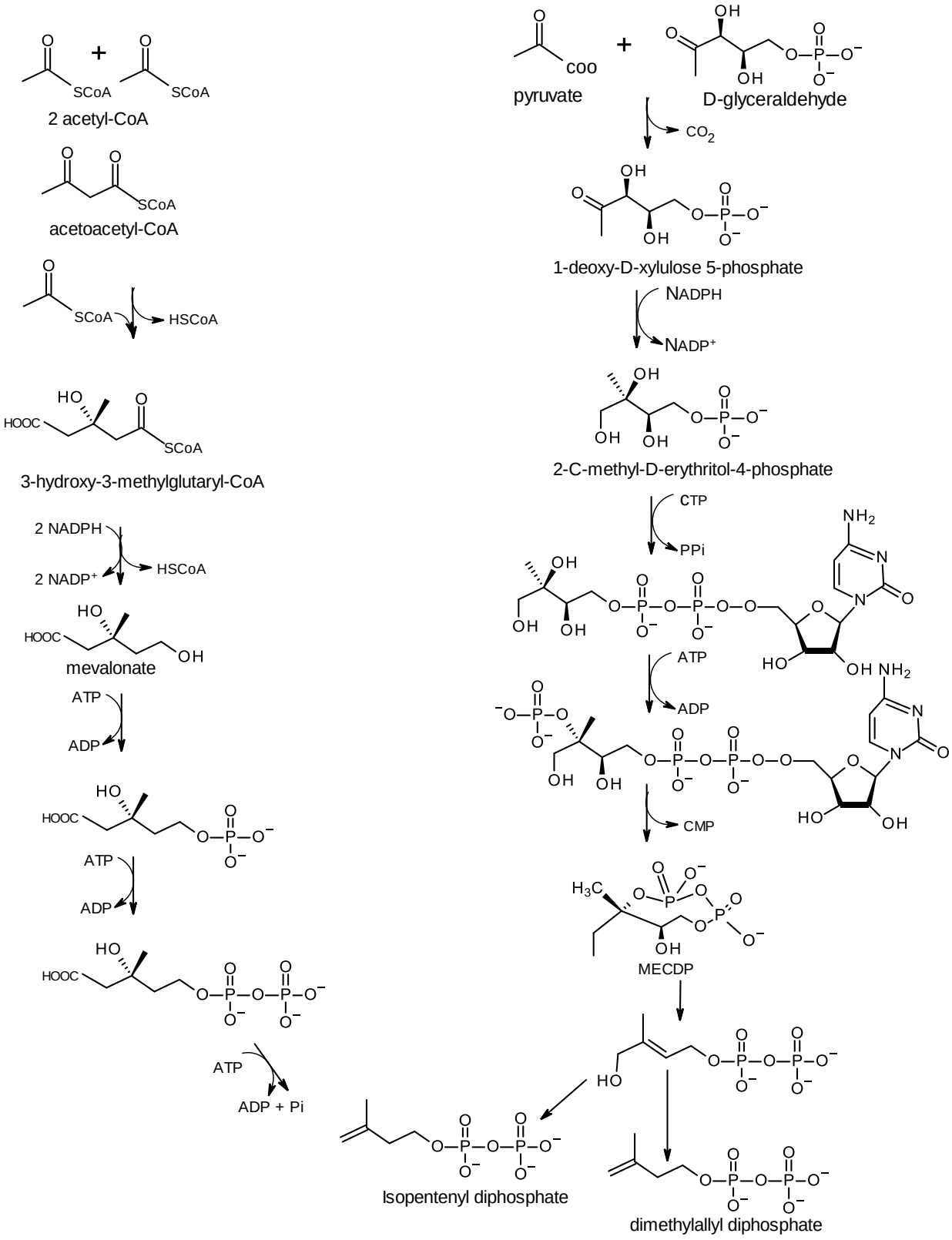
Terpenes represent a large group of volatile floral and fruit scents-adaptations that plants have evolved to in order to attract pollinators and seed disperser´s agents to maximize their fertilization and survival rates. The presence or absence of a scent may have a substantial impact on the yield important crops (Dudareva & Pichersky, 2000). Terpenoids are grouped as hemiterpenes C_5, monoterpenes C_{10}, sesquiterpenes C_{15} , diterpenes C_{20}, tri-terpenes C_{30} , tetraterpenes C_{40}, and polyterpenes $(C_5)_n$ where ´n´ is 9–30,000 (McGarvey and Croteau, 1995) The groups are categorized using the number of isoprene units linking together during their formations. Monoterpenes and sesquiterpenes are the ingridients of essential oils that are very important fragrance and flavouring agents in soaps, cosmetics, foods, beverages, perfumes as well as household detergents and waxes (Singh *et al.,* 1989; Mahmoud and Croteau, 2002).

All terpenoids derive from the 5-carbon building block isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)-isoprene units. The biosynthesis of isoprenoids constitutes: C_5 isoprenoid unit formation, C_5 units condensation- chain elongation and finally the cyclization and modification of straight isoprenoid precursors (Lorenzo & Eugenio, 2011). IPP is produced by two pathways in plants. The first pathway is the mevalonate (MVA) pathway while the second is the methylerythritol phosphate (MEP). The mevalonate pathway (Scheme: 2.2 A) commences with the molecules of the acetate initiated as acetyl-CoA, giving 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and finally transformed into mevalonate. This is initiated through three phosphorylation reactions then decarboxylation and elimination giving IPP. DMAPP is eventually produced from IPP through equilibration reaction catalyzed by the enzyme, IPP-DMAPP isomerase which utilizes a protonation-deprotonation mechanism (Leyes, *et al.,* 1999).

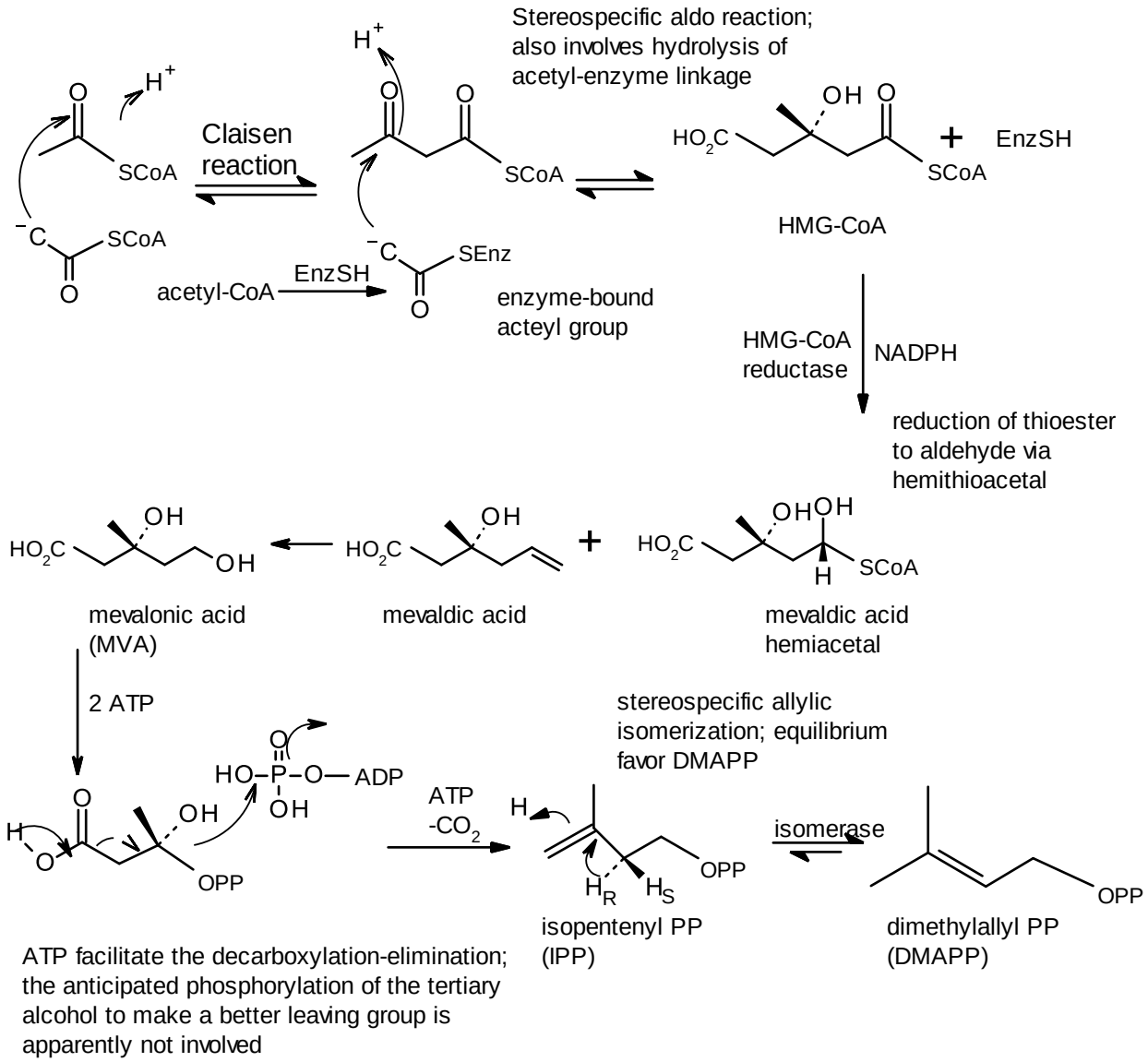
A lot of research in this area has led to the generation of the statins-HMG-CoA reductase inhibitors, widely used as preventive medicine for human cardiovascular diseases by lowering blood cholesterol levels in the body (Stancu and Sima, 2001).

Another individualistic mevalonate pathway; MEP or deoxyxylulose 5-phosphate pathway (Scheme 2.2 B) discovered in some bacteria and the plastids of both lower and higher plants in the 1990´s (Rohmer, 1999; Eisenreich, *et al.,* 2000), begins with the condensation of pyruvate and glyceraldehyde-3-phosphate forming 1-deoxyxylose-5-phosphate which undergoes a rearrangement coupled with a reduction to form MEP.

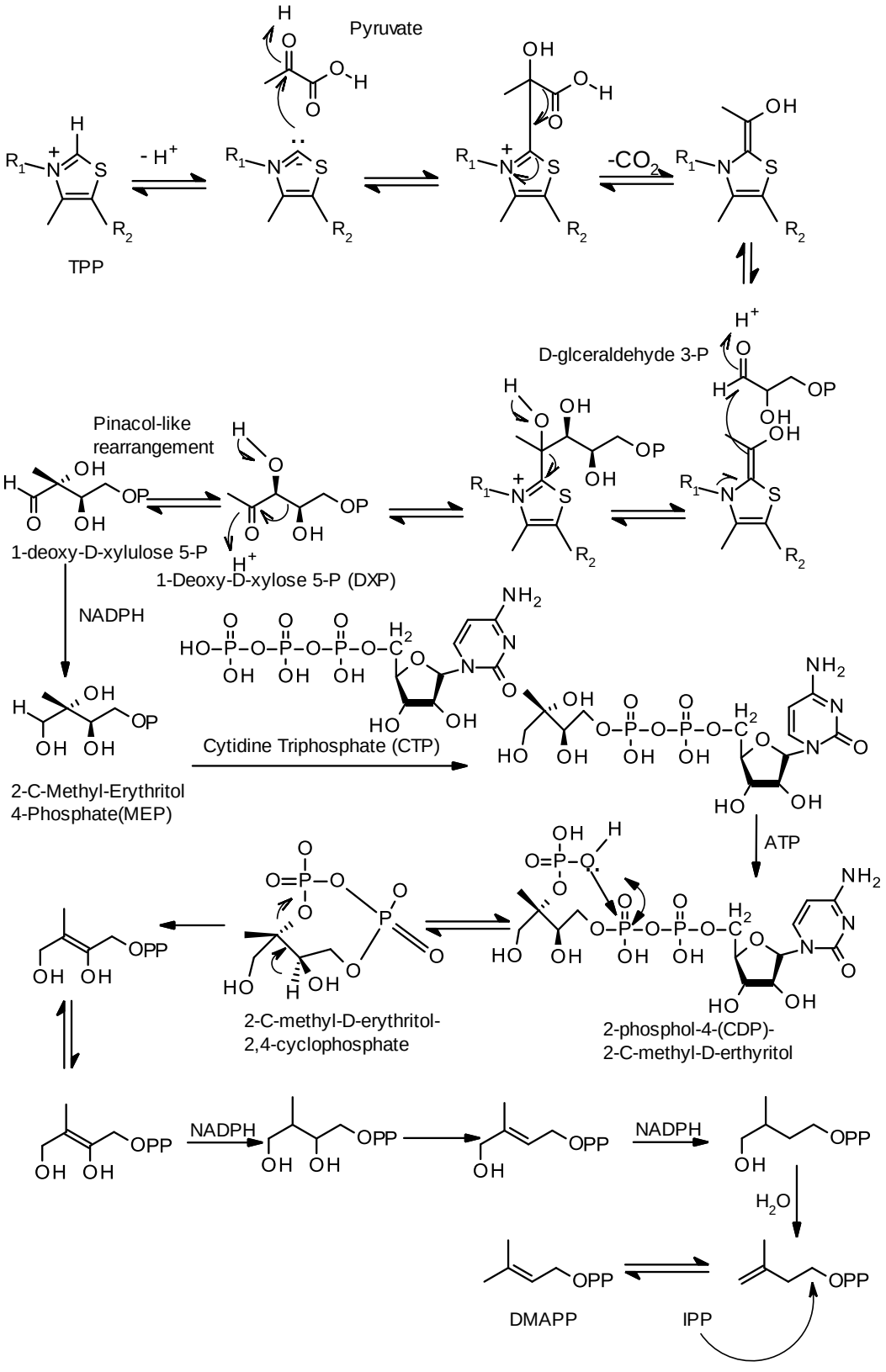
This is then transformed into its cyclic diphosphate, 2-*C*-methyl-D-erythritol-2, 4-cyclodiphosphate (MECDP), by sequential phosphocytidyl transfer and phosphorylation. MECDP then undergoes twice reduction steps to give IPP and DMAPP (Fig: 2.14 B and 2.15) (Eisenreich, *et al.*, 2004).



Scheme 2.2: Two independent pathways for biosynthesis of IPP and DMAPP in plants



Scheme 2.3: The mechanistic details of mevalonic acid pathway.



Scheme 2.4: Mechanistic details of Deoxyxylulose phosphate pathway

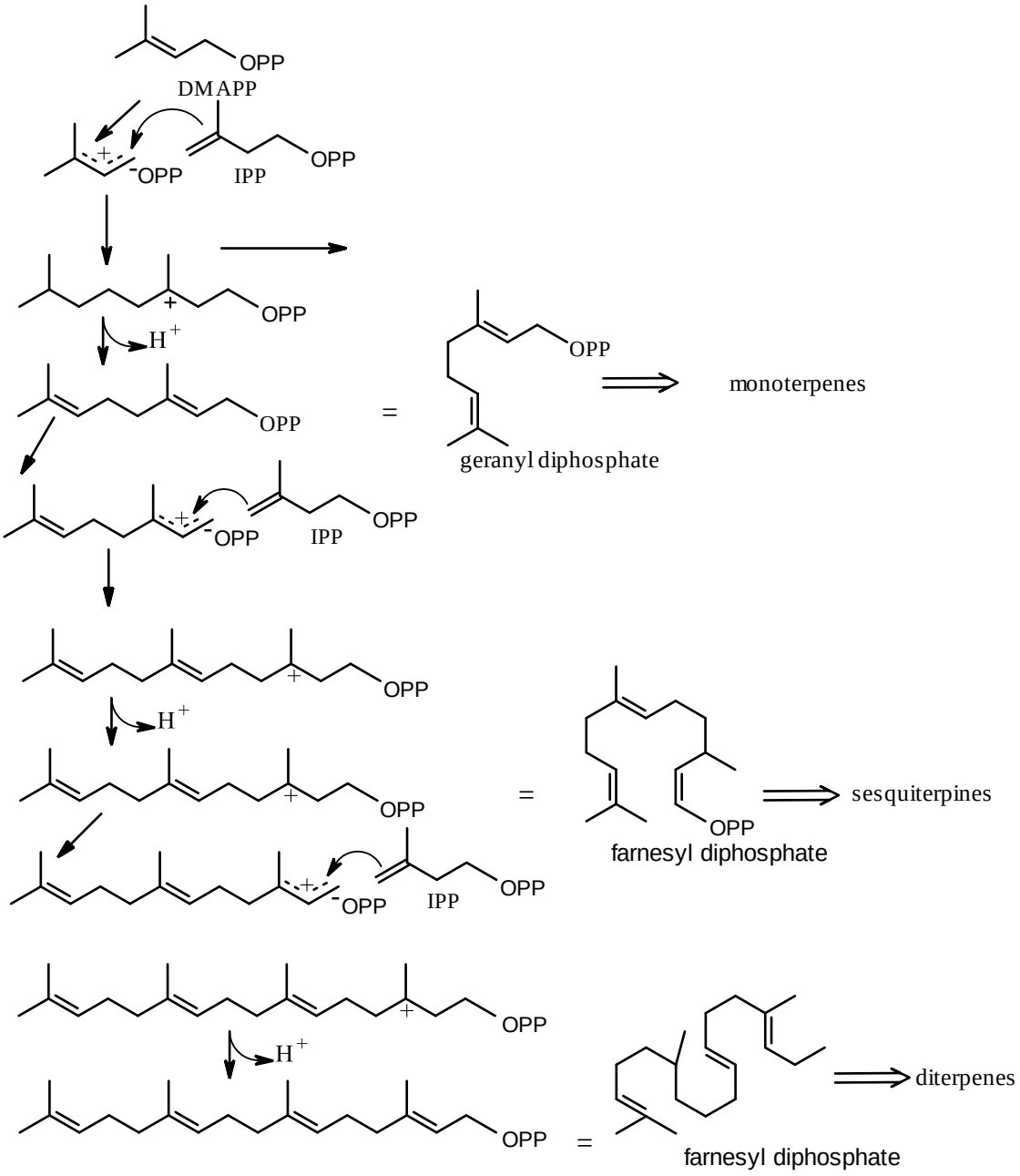
Carbon skeletons of straight isoprenoid units are put together through head-to-tail repetitive condensation of IPP with DMAPP via catalysis of prenyltransferases (a group of prenyl chain elongating enzymes) (Takahashi and Koyama, 2006).

Combining IPP with DMAPP by geranyl diphosphate synthase enzyme produces geranyl diphosphate (GPP, C_{10}) while addition of IPP to GPP by farnesyl diphosphate synthase produces farnesyl diphosphate (FPP, C_{15}), which can link to IPP by geranylgeranyl diphosphate synthase to result in to geranylgeranyl diphosphate (GGPP, C_{20}), although FPP synthase and GGPP synthase can also utilize only IPP and DMAPP as the initial substrates in multistep elongation sequences via bound intermediates (Poulter and Rilling, 1981).

The reaction progresses via an ionization-condensation-elimination mechanism involving an initial ionization of the allylic diphosphate ester forming a charge delocalized carbocation undergoing C4´-C1 linking through the terminal double bond of IPP producing a tertiary carbocation then deprotonation completing the process (Poulter and Rilling, 1978). The acyclic intermediates resulting are primary precursors where monoterpenes (from GPP), sesquiterpenes (from FPP) and diterpenes (from GGPP) are obtained. Terpenoid synthases / cyclases - their reaction products are most often cyclic; are involved in the conversion of the three acyclic branch point intermediates to the parent skeletons of the various monoterpene, sesquiterpene and diterpene types extensively contributing to the wider structural diversity experiencedin this group of compounds (Davis and Croteau, 2000).

Reaction of this family of enzymes may be seen as intramolecular form of intermolecular electrophilic linking reaction facilitated by prenyltransferases (Davisson *et al.,* 1985) with their differences in mechanistic pathways.

Terpenoid cyclases, responsible for catalyzing some of the most complex chemical reactions occurring in nature exhibits Michaelis constants for the prenyl substrate that barely exceed 10 μM and turnover numbers typically ranging from 0.03 to 0.3 s^{-1}(Davisson *et al.*, 1985). Nearly two-thirds of atoms of carbon of a linear terpenoid substrate undergo bonding changes, configuration or hybridization during cyclization processes (Christianson, 2006).



Scheme 2.5: Chain elongations by prenyltransferases

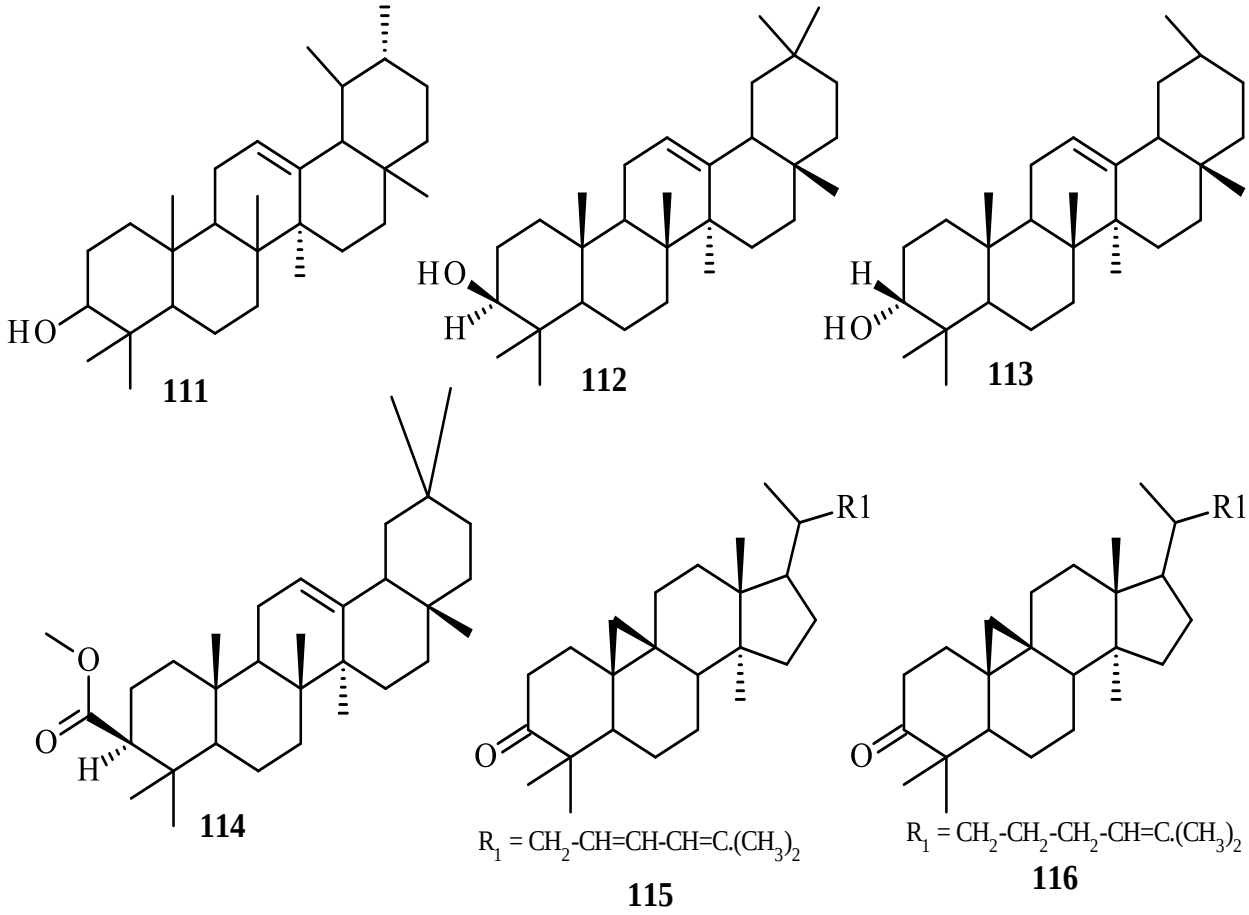
Examples of terponoids and steroids reported from *Gardenia* species are summarized below.

Table 2.5: Terpenoids from the Genus *Gardenia*

COMPOUND	**SOURCE PLANT**	**REFERENCE**
α –Amyrin **(111)**	*G. turgid*	Joshi *et al*., 1979
β –Amyrin **(112)**	*G. lucida*	Shukla & Mukharya, 1990
	G.lutea	Ahmed *et al*., 1985
3-epi-β –Amyrin **(113)**	*G. imperialis*	Babady-Bila & Tandu, 1988
β –Amyrin acetate **(114)**	*G. imperialis*	Babady-Bila & Tandu, 1988
Cycloartadienone **(115)**	*G. gordonii* *G. hilli* *G. stockii*	Davies *et al*., 1992
Cycloaartenone **(116)**	*G. gordonii* *G. grievei* *G. hilli* *G. storckii*	
9,19-Cyclolanost-24-ene-3,23-diene (**117)**	*G. gordonii* *G. hilli* *G. storckii*	
9,19-Cyclolanostane-3,23-dione **(118)**	*G. taitensis*	
9,19-Cyclolanostane-3,24-dione **(119)**	*G. godornii*	
	G. grieve	
	G. hilli	
	G. storckii	
3α, 19α–Dihydroxy-olean-12-ene-28-oic acid **(120)**	*G. latifolia*	Reddy *et al*., 1975
Erubescenone **(121)**	*G. erubescens*	Adelakun *et al*., 1996
Erythrodiol **(122)**	*G. gummifera*	Reddy *et al*., 1977
Gardenolic acid **(123)**	*G. jasminoides*	Xu *et al.,* 1987

COMPOUND	SOURCE PLANT	REFERENCE
		Qin *et al*., 1989
Gypsogenic acid **(124)**	*G. turgid*	Reddy *et al*., 1973
Hederagenin **(125)**	*G. latifolia*	Reddy *et al*., 1975
19-β-Hydroerythrodiol **(126)**	*G. gummifera*	Reddy *et al*., 1977
Linalool **(127)**	*G. tahitensis*	Bessiere *et al*., 1985
4-Nor-9,19-cyclolanost-24-ene-3,23-dione **(128)**	*G. gordinii*	Reddy *et al*., 1973
	G. hilli	Davies *et al.*, 1992
	G. storckii	
Oleanolic acid **(129)**	*G. erubescence*	Adelakun *et al*., 1996
	G. ltifolia	Reddy *et al*., 1975
	G. sootepensis	Liang *et al*., 1991
	G. turgid	Joshi *et al*., 1979 Reddy *et al.*, 1973
Oleanolic acid acetate **(130)**	*G. erubescens*	Adelakun *et al*., 1996
	G. jasminoides	Wang *et al.*, 1986
Oleanolic acid 3-*O*-glucoside **(131)**	*G. lutea*	Ahmed *et al*., 1985
Oleanolic aldehyde **(132)**	*G. gummifera*	Reddy *et al.*, 1977
Spinosic acid (**133)**	*G. latifolia*	Reddy *et al.*, 1975
β- sitosterol **(134)**	*G. gummifera*	Reddy *et al*., 1977
	G. jasmioides	Yung, 1964
	G. latifolia	Reddy *et al.*, 1975
	G. lucida	Shukla & Mukharya, 1990
	G. lutea	Ahmed *et al.*, 1985
	G. sootepensis	Liang *et al*., 1991 Wang *et al*., 1999
	G. turgid	Joshi *et al*., 1979 Reddy *et al*., 1973

COMPOUND	SOURCE PLANT	REFERENCE
β–Sitosterol-3-*O*-β-D-glucopyranosyl (1-4)-*O*-α L-rhamno-pyranoside **(135)**	*G. lucida*	Shukla & Mukharya, 1990
Stigmasterol **(136)**	*G. erubescence*	Adelakun *et al*., 1996
	G. jasminoides	Wang *et al,* 1986
	G. lutea	Ahmed *et al*., 1985
Ursolic acid **(137)**	*G. erubescens*	Adelakun *et al*., 1996



111 **112** **113**

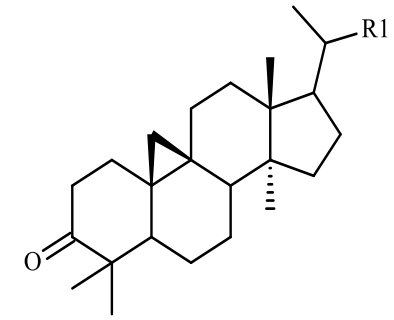
114

$R_1 = CH_2\text{-}CH{=}CH\text{-}CH{=}C.(CH_3)_2$

115

$R_1 = CH_2\text{-}CH_2\text{-}CH_2\text{-}CH{=}C.(CH_3)_2$

116



$R_1 = CH_2\text{-}CH_2\text{-}(CO)\text{-}CH{=}C.(CH_3)_2$

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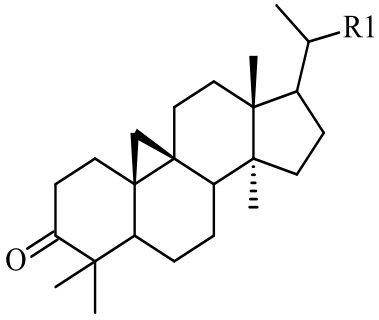

$R_1 = CH_2\text{-}CH_2\text{-}(CO)\text{-}CH_2{=}CH(CH_3)_2$

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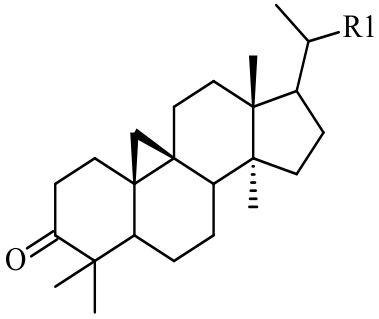

$R_1 = CH_2\text{-}CH_2\text{-}CH_2\text{-}(CO)\text{-}CH(CH_3)_2$

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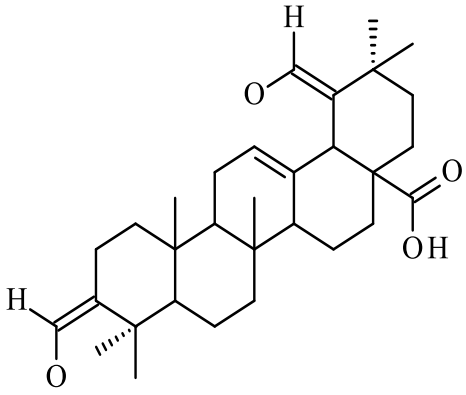

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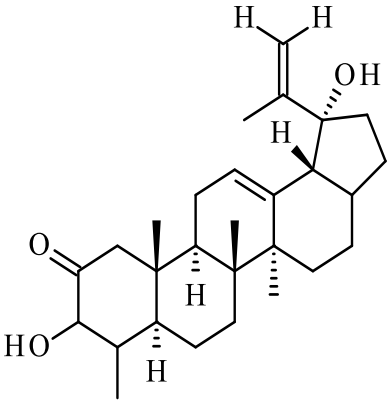

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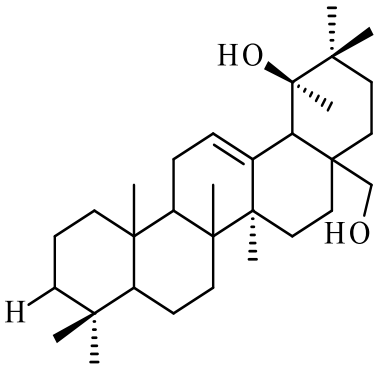

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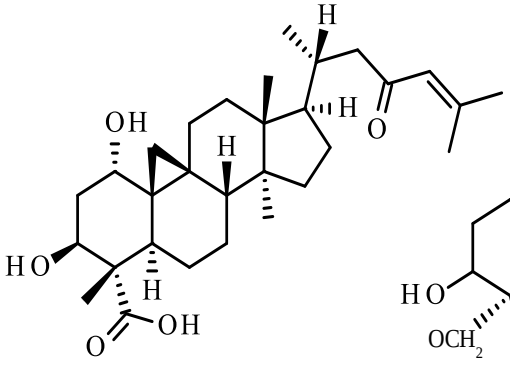

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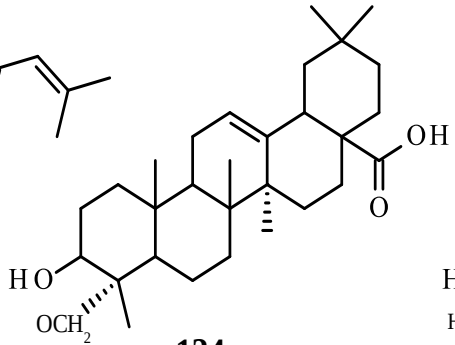

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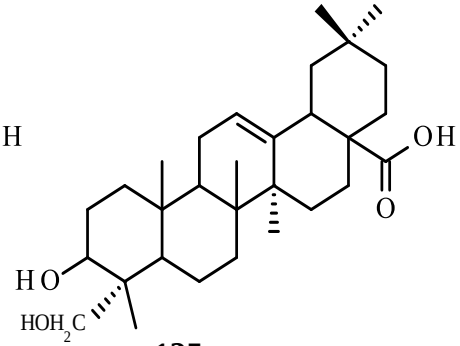

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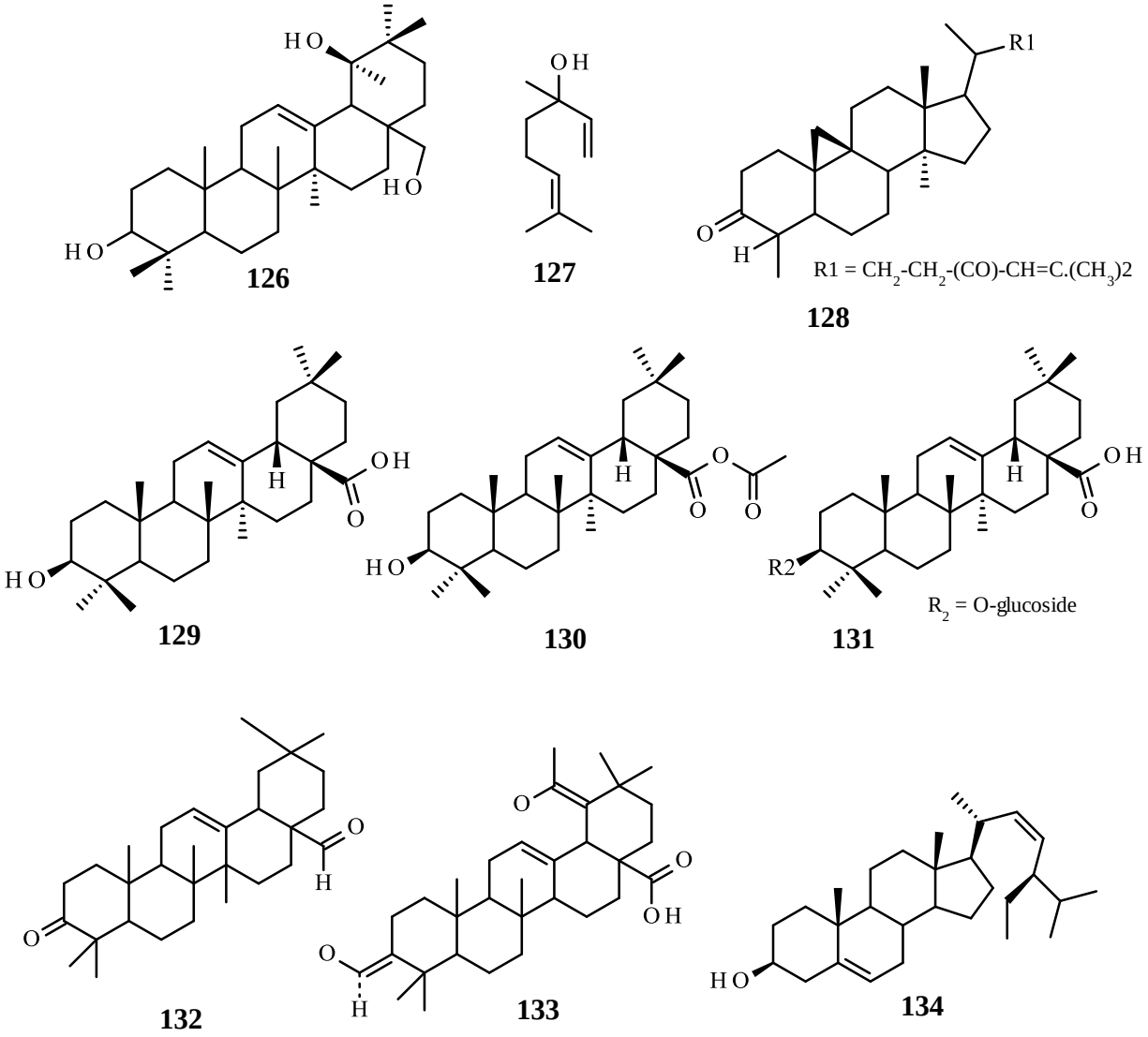


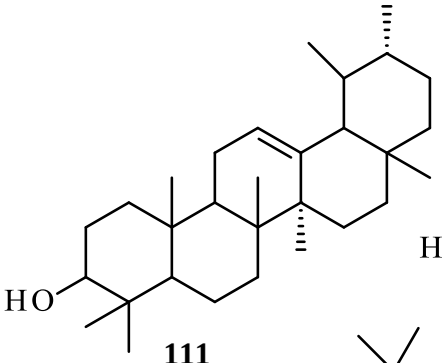
2.4.4.3 Other Compounds from Gardenia Species

Besides the above mentioned groups of compounds (Table 2.4 and 2.5), several acids, esters, alkanes and glucosides have been isolated from Gardenia species as summarized in Table 2.6 below**.**

Table 2.6: Other compounds from the Genus *Gardenia*

COMPOUND	**SOURCE PLANT**	**REFERENCE**
Bornyl-6-*O*-β –D-xylopyranosyl-β –D –glucopyranoside (**138)**	*G. jasminoides*	Watanabe *et al*., 1994
3-*O*-Caffeoyl-4-*O*-sinapoyl-quinic acid (**139)**	*G. jasminoides fruit*	Nishizawa *et al.*, 1987 ,,1988
Chlorogenic acid (**140)**		Nishizawa *et al.*, 1988
Crocetin (**141)**	*G. grandiflora*	Kahn *et al*., 1928
3,4-Di-*O*-caffeoylquinic acid (**142)**	*G. jasminoides fruit*	Wang *et al.*, 1996
Crocetin mono (β -gentiobiosyl) (**143)**	*G. jasminoides*	Pfister et al., 1996
Crocin (**144)**		Oka *et al*., 1995
3,4-Di-caffeoyl-5-(3-hydroxy-3-methyl glutaroylquinic acid (**145)**	*G. jasminoides fruit*	Nishizawa & Fujimoto, 1986
3,5-Di-*O*-caffeoyl-4-O-(3-hydroxy-3-methyl) glutaroylquinic acid (**146)**		Nishizawa *et al*., 1988
6,7-Dimethylaesculetin (**147)**		Aburada *et al.*, 1976 Miyagoshi *et al.*, 1986
Eugenol (**148)**	*G. jasminoides*	Wang,1979 and 1980
Heneicosane (**149)**	*G. sootepensis*	Liang *et al*.,1991
Heptacosanol (**150)**	*G. sootepensis*	
Hexacosyl- *p* coumarate (**151)**	*Dikamali (G)*	Chatterjee *et al*., 1980
Cis-3-Hexenyl benzoate-7-Keto-octadec-cis-11-enoic acid (**152)**	*G. tahitensis*	Dutta *et al*., 1966
Linoleic acid (**153)**	*G. lutea*	Ahmed *et al*., 1985
Linolenic acid (**154)**	*G. lucida*	Shukla & Mukharya,1990
D-Mannitol (**155)**	*G. florida*	Asai & Nakamura, 1920
	G. gummifera	Reddy *et al*., 1977

COMPOUND	SOURCE PLANT	REFERENCE
	G. latifolia	Reddy *et al*., 1975
Nonacosane (**156)**	*G. jasminoides*	Yung,1964
	G. sootepensis	Liang *et al*., 1991
Oleic acid (**157)**	*G. lucida*	Shukla & Mukharya,1990
Palmitic acid (**158)**		
	G. lutea	Ahmed *et al*., 1985
Pentacosanol (**159)**	*G.sootepensis*	Liang *et al.*, 1991
Phenethyl benzoate (**160)**	*G. tahitensis*	Bessiere *et al.*, 1985
Quinide (**161)**	*G. sooepensis*	Wang *et al*., 1996
Stearic acid (**162)**	*G. lucida*	Shukla & Mukharya,1990
Triacontane (**163)**	*G. sootepensis*	Liang *et al.*, 1991



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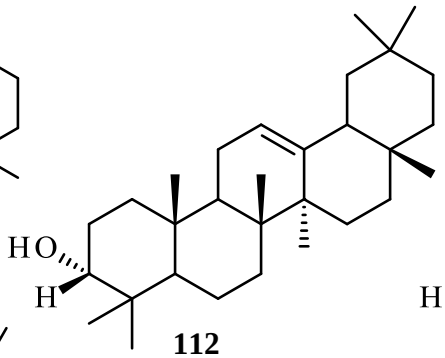

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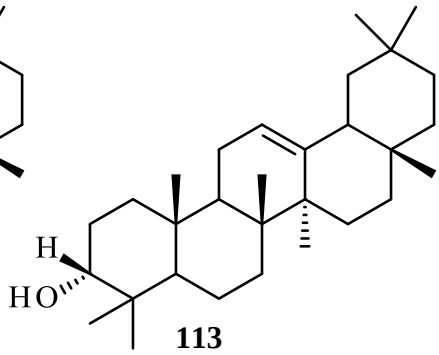

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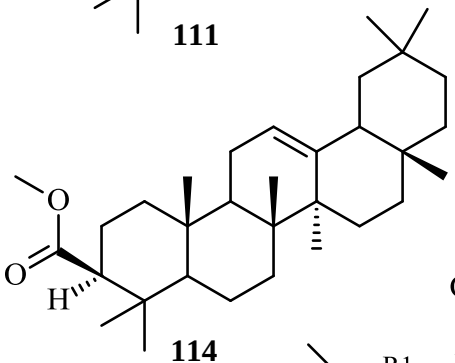

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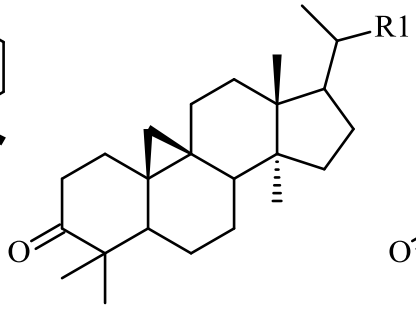

$R_1 = CH_2\text{-}CH\text{=}CH\text{-}CH\text{=}C.(CH_3)_2$

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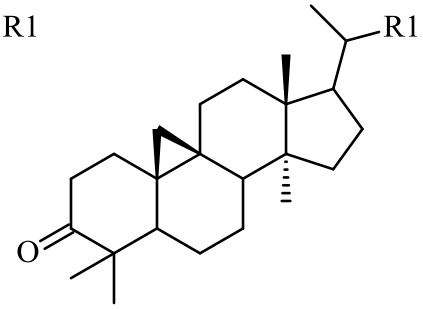

$R_1 = CH_2\text{-}CH_2\text{-}CH_2\text{-}CH\text{=}C.(CH_3)_2$

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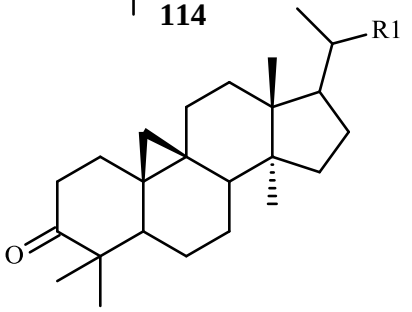

$R_1 = CH_2\text{-}CH_2\text{-}(CO)\text{-}CH\text{=}C.(CH_3)_2$

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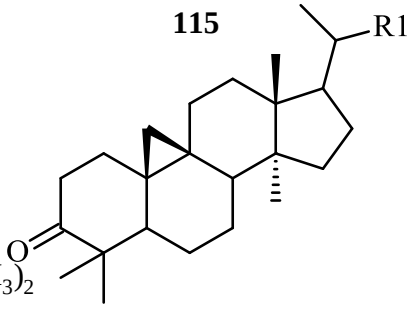

$R_1 = CH_2\text{-}CH_2\text{-}(CO)\text{-}CH_2\text{=}CH(CH_3)_2$

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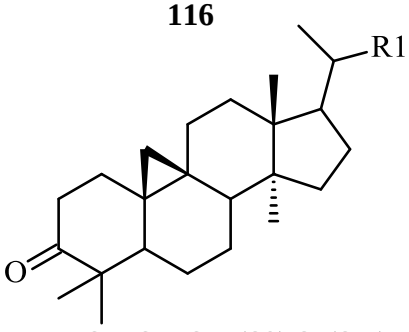

$R_1 = CH_2\text{-}CH_2\text{-}CH_2\text{-}(CO)\text{-}CH(CH_3)_2$

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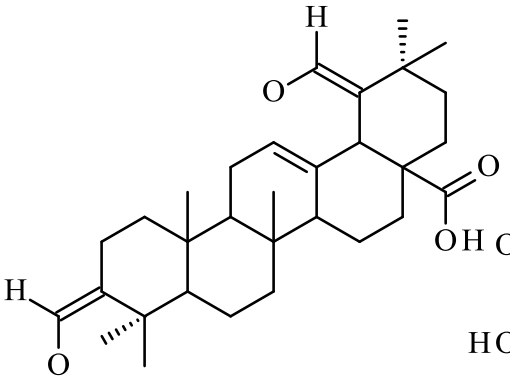

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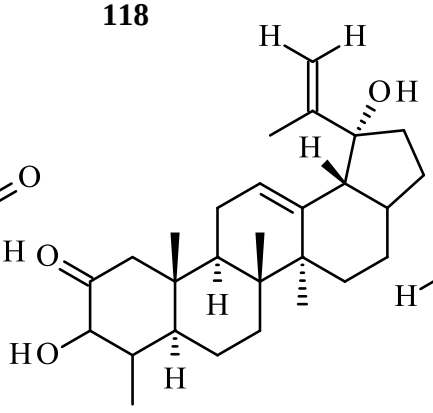

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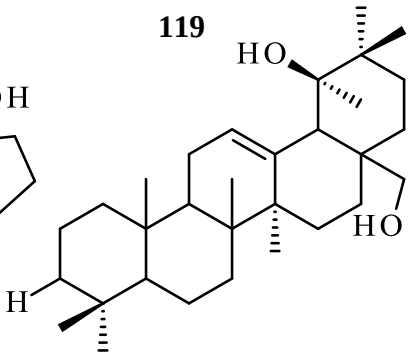

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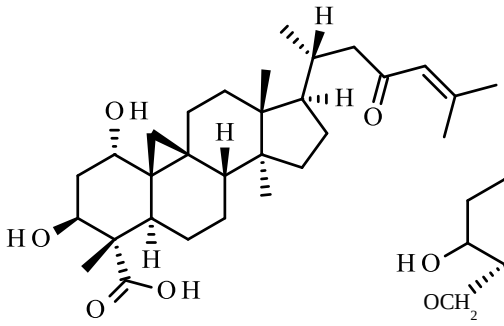

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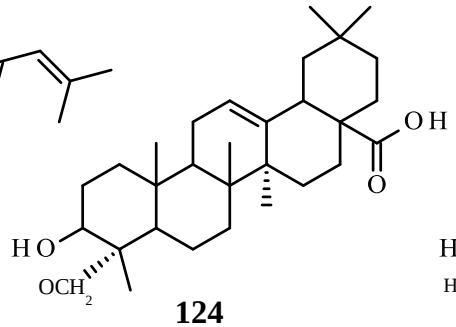

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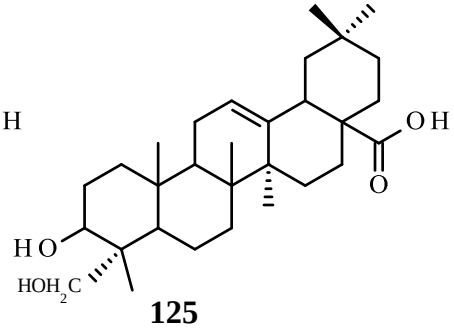

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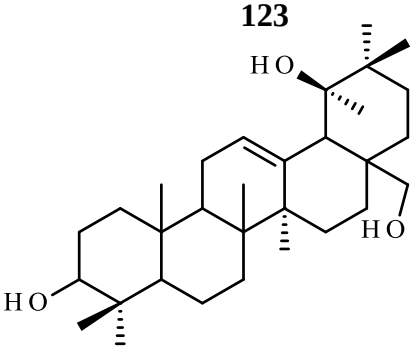

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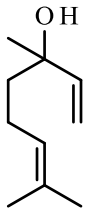

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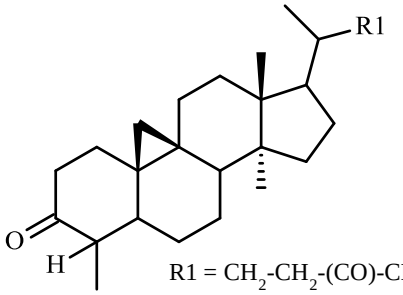

R1 = CH_2-CH_2-(CO)-CH=C.(CH_3)2

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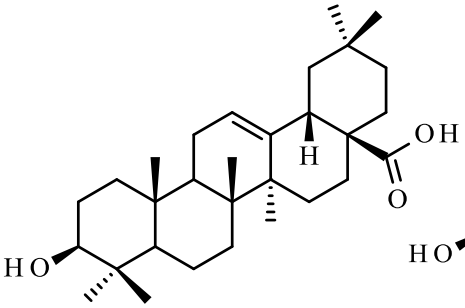

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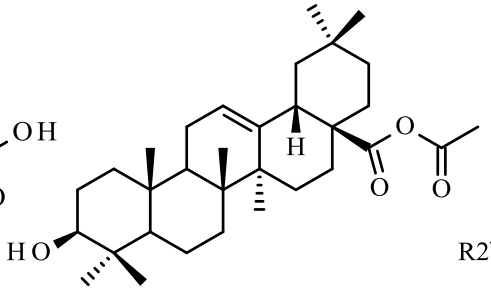

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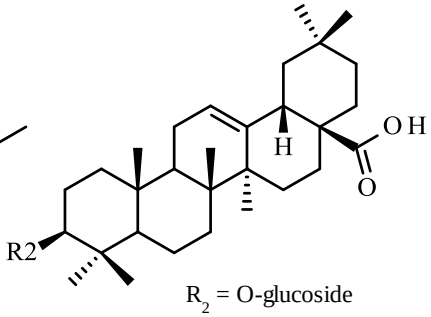

R_2 = O-glucoside

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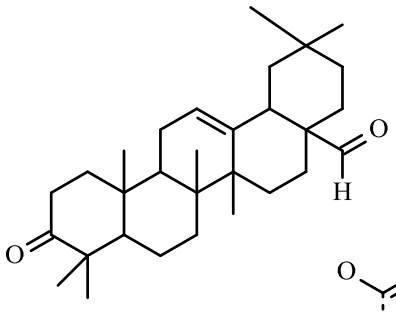

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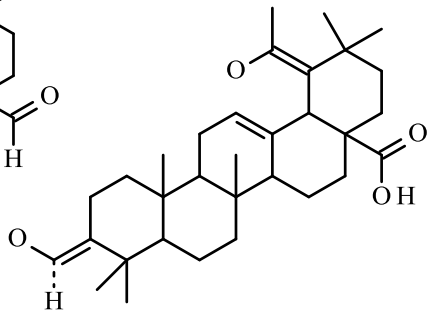

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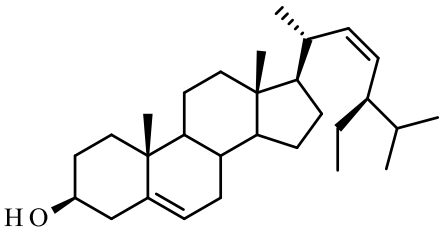

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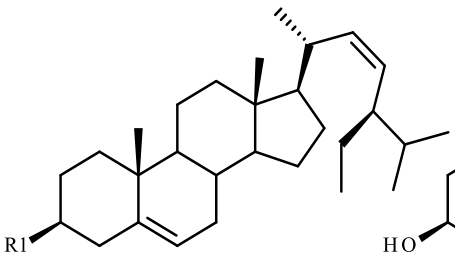
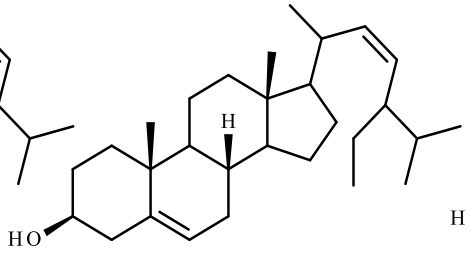


R_1 = β-O-glucopyranosyl(1---->4)
O-α-L-rhamnopyranosyl

135



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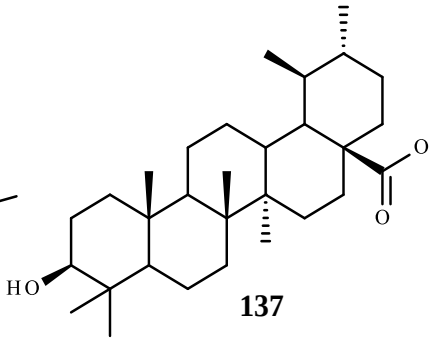

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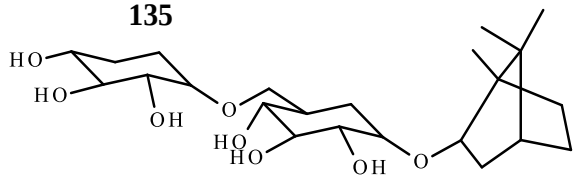

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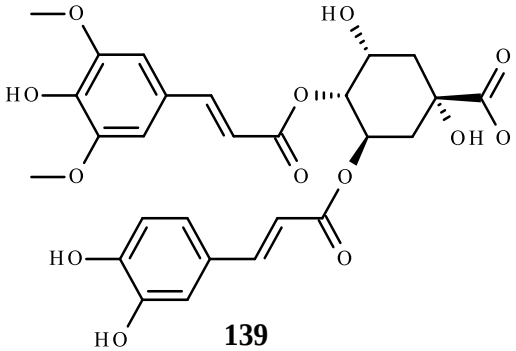

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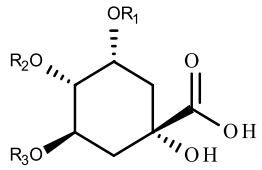
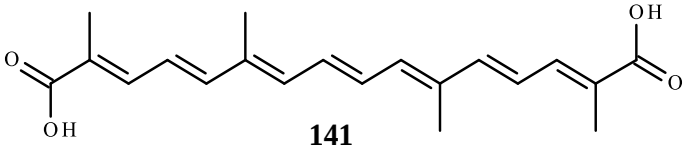


R_1 = caffeoyl

140



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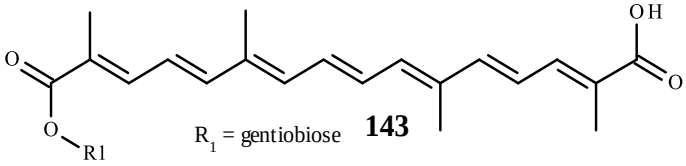
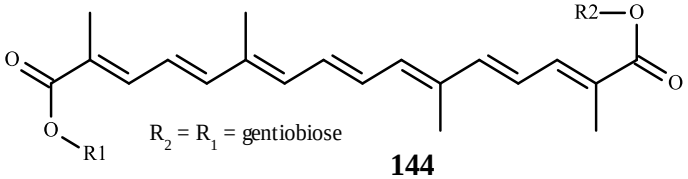


R_1 = gentiobiose **143**



$R_2 = R_1$ = gentiobiose

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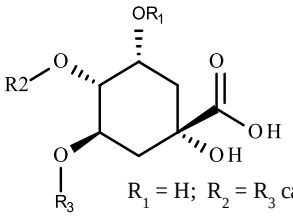
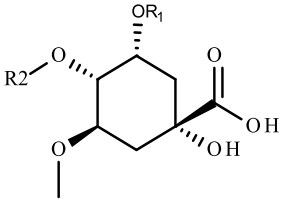


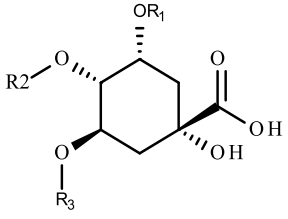
R_1 = H; $R_2 = R_3$ caffeoyl

142



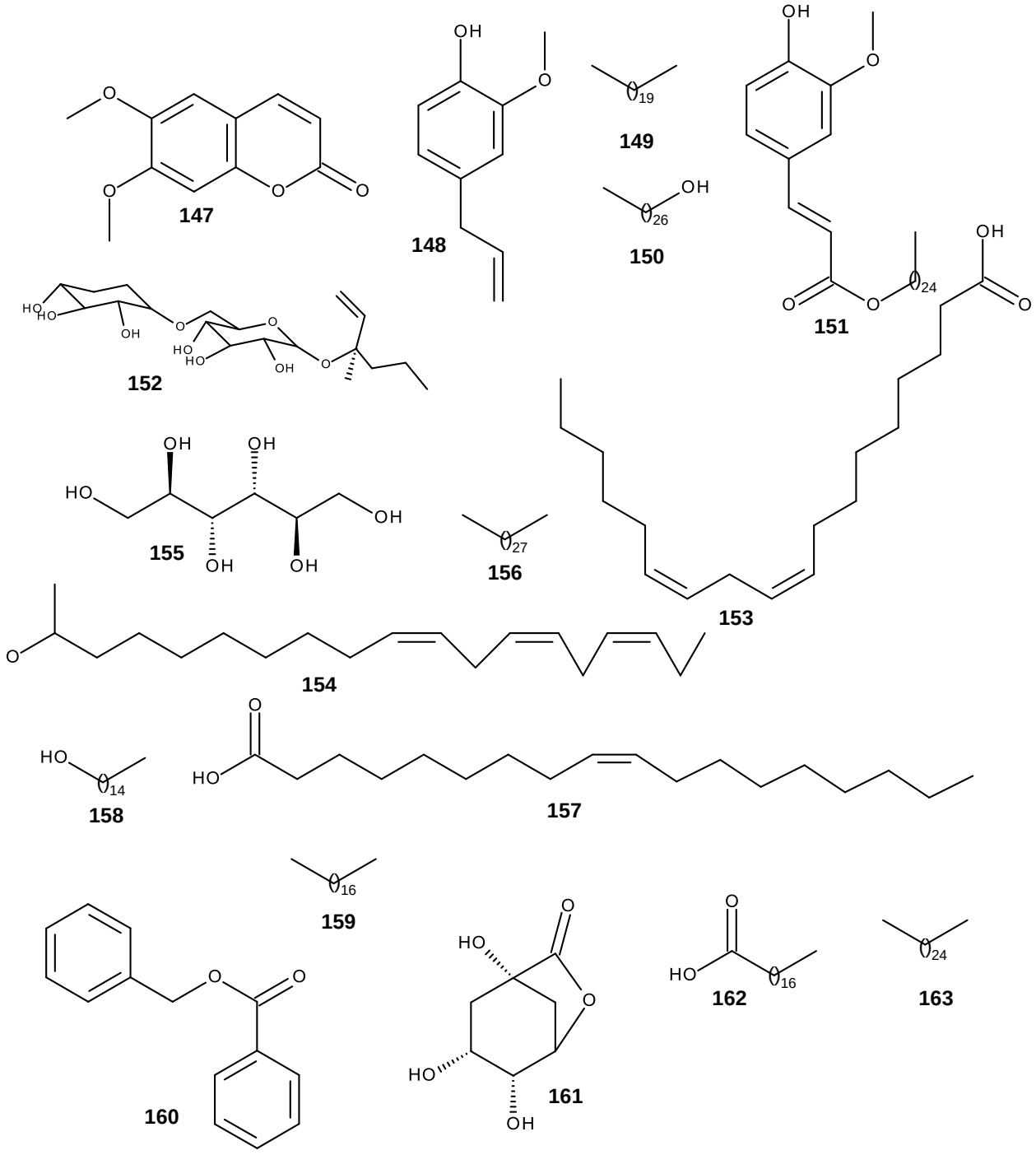
$R_1 = R_2$ = glutaroyl; $R_2 = R_3$caffeoyl

145



$R_1 = R_2$ = caffeoyl; R_3 = glutaroyl

146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CHAPTER THREE

MATERIALS AND METHODS

3.1 General

The ^{1}H NMR and ^{13}C NMR spectra were acquired on a Varian 400 MHz spectrometer and the chemical shifts values were obtained in ppm (δ) with TMS as the internal standard. The compounds were seen under UV light at 254 and 366 nm as well as exposure to iodine and ammonia fumes. Quantitative isolation of the compounds was achieved by normal phase liquid column chromatography on silica gel (60-120 mesh) with gradient elution while Kieselgel 60 H was used to make the preparative thin layer chromatography (TLC) analysis. The solvents which were used for the extraction of the surface exudates and column chromatography were purified by fractional distillation. (PTLC) plates were prepared using glass plates (20 cm by 20 cm). This was done by measuring 80 g powder of preparative silica gel and mixing with 200 ml of clean water for six plates. The resultant slurry (40 ml) was measured and poured onto each plate which was then spread uniformly using a flat spatula and left overnight to dry resulting to plates of 2 mm thickness. The plates were eventually activated in an oven at 110 °C for 30 minutes and allowed to cool before they were used for the separation process.

3.2 Plant Material

The aerial parts of *Gardenia ternifolia* were collected from Kagundo, Machakos County in Kenya in December 2012. The plant materials were located by Mr. Patrick Mutiso from the University of Nairobi herbarium, School of Biological Sciences, where a specimen (number EOA 2012/UoN is deposited).

3.3 Extraction and Isolation

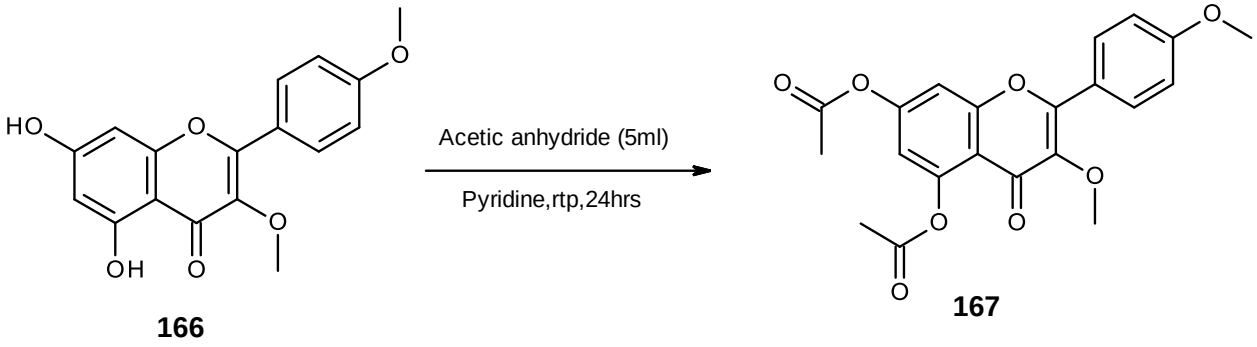
Crude leaf surface extract was obtained by dipping about 1.5 kg of fresh leaves branch by branch into about one litre of acetone as the solvent for a short period of 15 seconds hence avoiding the extraction of internal tissue components (Midiwo *et al*., 1990). This process was repeated until the colour of acetone changed to yellowish at which another fresh portion of acetone was used for the subsequent extraction. The surface exudates extract was filtered evaporated in *vacuo* using a rotary evaporator under reduced pressure giving a yellowish black gummy extract of (85 g). Acetone was used as the solvent for extraction since it would give exhaustive wash of the exudates. The portion of the extract (70 g) was adsorbed onto 70 g of silica gel, dried *in vacuo*, ground into fine powder and loaded onto a column packed with 700 g of silica gel under *n*-hexane. This was subjected to column chromatography by gradient elution initially with *n*-hexane with increasing amounts of CH_2Cl_2 up to 100 % followed by 1 and 2 % MeOH in CH_2Cl_2. This yielded (110) fractions of 250 ml each which were combined based on the similarities of their TLC profiles then dried and weighed resulting to only eleven fractions (A-K).

Fraction A which was eluted with CH_2Cl_2/*n*-hexane mixtures (1:9) yielded β-sitosterol (**169**) (65 mg) which crystallized from CH_2Cl_2/*n*-hexane mixture. It had R_f value of 0.69 in 1:1 CH_2Cl_2/*n*-hexane system. Fraction B eluted with CH_2Cl_2/*n*-hexane (1:4) showed the presence of one spot on TLC plate exposed to iodine, and was crystallized from CH_2Cl_2/*n*-hexane mixture and a white crystalline solid was obtained in 50 mg amount and the analysis revealed it to be stigmasterol (**170**) with Rf. Value of 0.5 (in 1:1 CH_2Cl_2/*n*-hexane). This compound was co-spotted with the compound isolated from fraction A and the R_f values were different in 1:1 CH_2Cl_2/*n*-hexane solvent system i.e. 0.69 and 0.5 respectively indicating that they were different compounds.

Fractions C, D and E eluted with up to 1:1 CH_2Cl_2/*n*-hexane were further combined due to the presence of one major trace of compound and were purified through a small column packed with 10g of silica gel. The resultant TLC profile on the collected single fraction showed one spot under UV 254nm which on exposure to ammonia fumes, the spot became intense with R_f value of 0.3 in 100% CH_2Cl_2. These combined fractions gave a white solid compound 5, 4'-Dihydroxy-7-methoxyflavanone (**164**) (80 mg) obtained after crystallization of the fraction from CH_2Cl_2/*n*-hexane mixture overnight. Fractions F, G and H (60 mg) eluted with up to 7:3 CH_2Cl_2/hexane, on the TLC analysis (exposed to both UV 254nm and ammonia) showed the presence of two spots which were very intense and with close R_f Values. Separation was achieved by subjecting the fractions (46 mg) to preparative-TLC (100% CH_2Cl_2) and the two bands which were visualized under UV 254nm were scrapped off carefully one after the other. The first band afforded 25 mg of 5, 7-dihydroxy-3, 4'-methoxyflavone (**166**) while the second band gave 15 mg of 3, 5, 3'-trihydroxy-7, 4'-dimethoxyflavone **(168**).

The remaining three fractions I, G and K (150 mg) eluted with up to 1% MeOH/ CH_2Cl_2, showed two spots with close R_f values. The major spot was yellow and intensified on exposure to both ammonia fumes and iodine. Separation of the major compound was achieved by subjecting the combined fraction to preparative-TLC (2% MeOH/CH_2Cl_2). This afforded 3, 5, 7-trihydroxy-4'-methoxyflavone **(165**) (120 mg) with R_f value of 0.1 in CH_2Cl_2 after recrystallization in CH_2Cl_2/hexane mixture.

3.4 Structural Modification



166 **167**

Compound (**167**) was obtained by acetylating 10 mg of 5, 7-dihydroxy-3, 4'-dimethoxyflavone (**166**) using 1ml of pyridine and 5ml of acetic anhydride. The reaction mixture was left at room temperature for 24 hours and then poured in ice cold water in 250 ml beaker and then stirred for two minutes and left to settle for 20 minutes and white precipitate formed. This was washed with cold water, filtered and dried affording 12.67 mg of the product which was white in colour.

3.5 Anti-oxidant Test

A preliminary antioxidant test was achieved by spotting the isolated compounds on a TLC plate then sprayed with (0.2 mg/ml) DPPH solution to view the active compounds which displayed white or yellowish sports on a purple background. The active compounds were analysed based on UV-VIS spectroscopic method acquired from Hou *et al* (2002). The concentration of each of the sample compound was varied by serial dilutions to give; 160, 80, 40, 20, 10, 5.0, 2.5, 1.25 µM concentrations while the concentration of DPPH was kept constant at 100 µM. The reaction mixture consisted of adding 0.5 ml of sample, 3 ml of absolute ethanol and 0.3 ml of 100µM/ml DPPH radical solution in ethanol. The solutions were measured for UV-VIS absorbance at DPPH absorbing wavelength of (517 nm) half an hour after adding the DPPH.

The absorbance measured at each of these intervals was converted into percentages of scavenged DPPH radicals using the following equation. In all the cases, the mean values were used from triplicate experiment.

$$\% \text{ of scavenged DPPH} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right) \times 100\%$$

Where A_{blank} is the absorbance of DPPH solution without sample, A_{sample} is the absorbance of the sample. The percentages of scavenged DPPH were then plotted against concentration of the compound to give graphs from which effective concentrations (μM) at half inhibition (EC_{50}) were determined. The tests were done in triplicate and analysed with statistical package for the social sciences (SPSS).

3.6 MAO Inhibition Assays

This was done at the University of Mississipi (USA) and the data was submitted to me for analysis. To determine the effect of the isolated compounds on MAO-A and MAO-B, the kynuramine deamination assay was adapted for 96-well plates as described by (Parikh *et al*., 2002). A fixed substrate concentration and varying inhibitor concentrations were used to determine the IC_{50} value at the point where 50 % inhibition of the catalytic activity of the enzyme occurred. For MAO-A, the substrate concentration of 80 μM kynuramine was chosen because the apparent Km value for substrate binding reported previously was approximately 40 μM (Parikh *et al*., 2002).

Since Km is the substrate concentration at half Vmax, therefore, 2 x Km (2 x 40 = 80 μM), was chosen in getting IC_{50} values. Similarly, for MAO-B, substrate concentration of 50μM kynuramine was used. The assay was done with the addition of inhibitor. Inhibition was calculated as percent of product formation compared to the corresponding control (enzyme-substrate reaction) without the inhibitors. The reactions were carried out in 0.1 M potassium phosphate buffer at pH 7.4.

Incubation mixtures contained 5 µg/mL of MAO-A (50 µL in buffer) and 10 µg/mL of MAOB (50 µL in buffer). The inhibitor was dissolved in DMSO or in buffer (if not dissolved in DMSO).

The total reaction volume was 200 µL yielding a final DMSO concentration of 1.0% in the reaction mixture. The reaction mixtures were pre-incubated for 10 min at 37 °C followed by the addition of MAO-A/MAO-B to initiate the reactions. Reactions were incubated for 20 min at 37 °C and were stopped immediately by the addition of 75µL of 2N NaOH. The formation of 4-hydroxyquinoline was determined fluorometrically by SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with an excitation and emission wavelength of 320 nm and 380 nm, respectively, using the Soft Max Pro program.

3.6.1 Determination of IC_{50} Values

IC_{50} values for inhibition of MAO-A and B by the selected constituents were determined using fixed concentrations of substrate and varying concentration of inhibitor/ test compounds (0.001 µM to 100 µM), clorgyline (0.10 nM to 100 nM) and deprenyl (0.01 µM to 100 µM) for MAO-A and B were used to determine the IC_{50} value at the point where 50% inhibition of the catalytic activity of the enzyme occurred.

3.6.2 Kinetic Studies of Compounds using MAO -A and B

For determination of binding affinity of the inhibitor (Ki) with MAO-A or MAO-B the enzyme assays were carried out at different concentration of kynuramine substrate (1.90 µM to 500 µM) and at least two fixed concentrations (one conc. Nearby the IC_{50} value and second above the IC_{50} values) of the inhibitors/compound for MAO-A and B were used to determine the Km and Vmax values in presence of the inhibitor. The controls without inhibitor were also

run simultaneously. The results were analyzed by standard double reciprocal Line-Weaver Burk plots for computing Km and Vmax values, which were further analyzed to determine Ki values.

3.6.3 Analysis of Reversibility and Binding of Inhibitor with MAO-A and B

Most of the inhibitors produce inhibition of the target enzyme through formation of an enzyme-inhibitor complex. Formation of the enzyme-inhibitor complex may be accelerated in presence of high concentration of the inhibitor. The reversibility of binding of MAO-A and B with inhibitors was determined by formation of the complex by incubating the enzyme with high concentration (10x of IC_{50} value) of the inhibitor followed by extensive dialysis of the enzyme-inhibitor complex and recovery of catalytic activity of the enzymes.

MAO-A (0.2 mg/mL protein) enzyme was incubated with each inhibitor/compound, and clorgyline (50 nM) in a total volume 1 mL, 100 mM potassium phosphate buffer (pH 7.4). After 20 min of incubation at 37 °C, the reaction was stopped by chilling on the ice bath. All the samples were dialyzed against potassium phosphate buffer (25 mM; pH 7.4) at 4 °C for 13-14 hrs (Three times buffer changes). Control enzyme (without inhibitor) was also run through the same procedure and activity of the enzyme was determined before and after the dialysis.

3.6.4 Analysis of Time-Dependent Enzyme Inhibition

The binding of the inhibitors with MAO-A and B, to produce inhibition, was not time dependent. The enzyme was pre-incubated for different time periods (0-15 min) with the inhibitor at a concentration, which produces approximately 70-80% inhibition. The inhibitor concentrations (2x and 4x of IC_{50} value) used to test time-dependent inhibition were compounds and clorgyline (7.5 nM) with MAO-A (12.5 µg/mL) and deprenyl (50 nM) with MAO B (12.5 µg/mL). The controls without inhibitors were also run simultaneously. Activities of the enzymes

were determined as described above and percentage of enzyme activity remaining was plotted against the pre-incubation time to determine time-dependent inhibition.

CHAPTER FOUR

RESULTS AND DISCUSSION

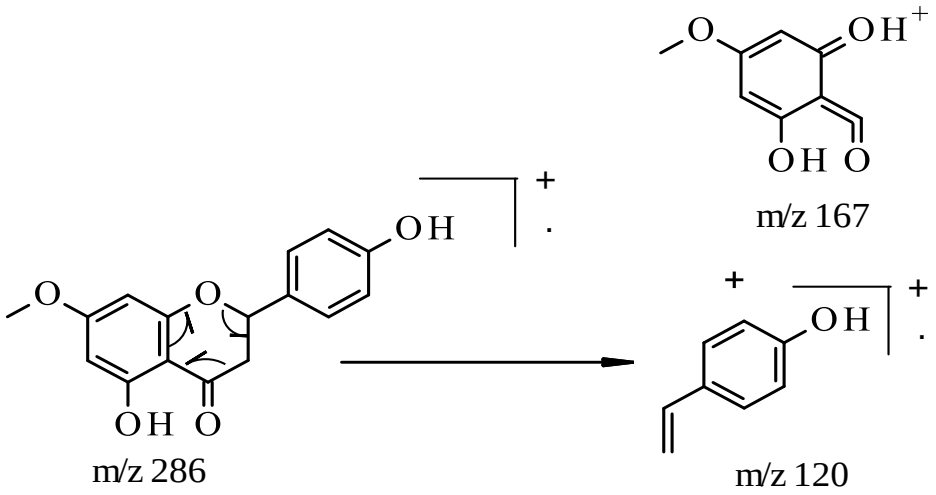
Chromatographic separation of the surface exudates of *Gardenia ternifolia* led to the isolation and characterization of six compounds: Four flavonoids and two phytosterols. One acetylated product **167** was also obtained by transforming compound **166**. Characterizations of the compounds are discussed as follows:

4.1 Flavonoids

4.1.1 5, 4'-Dihydroxy-7-methoxyflavanone (164)

Compound **164** was isolated as white crystals (80 mg) with R_f value of 0.3 (100% CH_2Cl_2) and melting point of 152-154°C. This compound was soluble in CH_2Cl_2 and also UV 254 nm active. It readily formed solids after eluting it from the column. The EI-MS showed a molecular ion peak at *m/z* 286. The presence of a flavanone skeleton was revealed from the ^{1}H-NMR signal at δ 5.35 ppm (*dd*, *J* = 12 Hz, 4 Hz) for proton H-2; δ 2.79 ppm (1H, *dd*, *J*= -16, 4 Hz) and another shift at δ 3.09 ppm (1H) (*dd*, *J* = -16, 12 Hz) for methylene protons at C-3. This data is supported by the ^{13}C-NMR signal at δ 78.97 for C-2, 43.14 for C-3 and δ 196.15 for C-4. The ^{1}H-NMR spectrum showed a geminal coupling of (-16 Hz) between the two protons attached to C-3 at δ (2.79 ppm and 3.09 ppm and also an *axial* coupling is observed of (*J* =12 Hz) between protons H-2 and one of the H-3 observed at δ 3.09 ppm. An *equatorial axial* coupling of *J* = 4 Hz is also observed between H-2 and H-3 (δ 2.79 ppm). H-2 is axial hence ring be lies equatorial. The presence of two *meta* oriented aromatic protons at δ 6.07 ppm and δ 6.04 ppm as broad singlet is consistent with the biogenetically expected oxygenation at C-5 and C-7 of ring A. Allowing the assignment of these signals to H-6 and H-8, this substitution pattern was confirmed with the presence of a chelated hydroxyl signal at δ 12.02 ppm for OH-5. The presence of a singlet peak at δ 3.80 ppm for (3H)

suggested the presence of a methoxyl group at either ring A or B. The presence of an AA'XX' spin system observed for the aromatic protons resonating at δ 6.88 ppm and δ 7.32 ppm intense doublet peaks, suggested the oxygenation on ring B to be at C-4'. Signals at δ 6.88 ppm *d* (*J*= 8.0 Hz) were due to H- 3'/5' and 2'/6' respectively. The upfield resonance observed for protons at C- 3'/5' compared to proton at C- 2'/6' was due to the effects of the oxygen substituent located at the *ortho* position. The broad singlet at δ 5.58 ppm intergrating for one proton (1H) could be due to 4'-hydroxyl proton in ring B. The specific optical activity of this compound obtained in Methanol solution showed the compound to be optically active at $[\alpha]_D^{25} = 58^{\circ}$ confirming an R- confugaration at C-2. From the EIMS data (M^+ -H=285) and a fragment ion at *m/z* 167 resulting from retro-Diels-Alder cleavage of ring C resulted in the presence and placement of methoxyl group at C-7. This was also supported by HMBC correlations between the protons at δ 3.80 with C-7. The resonance at δ 55.7 was also typical for an isolated substituted methoxyl group.



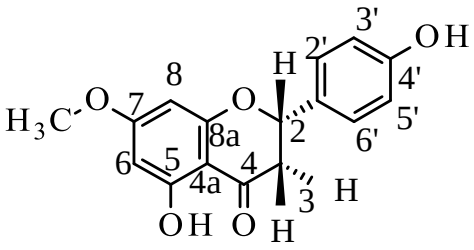
Scheme 4.1: Fragmentation pattern of 5, 4'-dihydroxy-7-methoxyflavanone (**164**)

The confirmation of the substitution pattern of ring B resulted from the ^{13}C-NMR displaying two intense peaks at δ 128.0 ppm and δ115.7 ppm (Table 4.1) which suggested two pairs of chemically

equivalent aromatic carbons with signals that overlap making them intensive for carbons 2'/6' and 3'/5' respectively. C-4' absorbed at δ 156.2 ppm, an aromatic carbon without *ortho/para* oxygenation. It was also evident from ^{13}C-NMR, δ 55.7 ppm for an isolated methoxyl substituent. From Heteronuclear polarization transfer experiment (DEPT) (Table 4.1), the signal at δ 43.1 ppm revealed the presence of a single carbon attached to two protons (CH_2) for C-3. The chemical shift values at δ 55.9 ppm, 80.0 ppm, 94.3 ppm, 95.1 ppm, 115.7 ppm, 128.0 ppm revealed the presence of protonated carbons (C-H) with the last two signals confirming the AA'XX' spin system observed in ring B. Therefore the compound was characterized as 5, 4'-dihydroxy-7-methoxyflavanone and had been previously reported from *Gardenia ternifolia* (Ochieng *et al*., 2010) and *Prunus yedoensis* (DNP, 2002) and *Dodonaea iscose* (Mata *et al*., 1991).

Table 4.1: 1H-NMR and 13C-NMR chemical shift together with DEPT and HMBC for compound 164.

Atom	**^{1}H δ (ppm)**	**J (Hz)**	**^{13}Cδ (ppm)**	**DEPT Spectrum**	**HMBC-^{2}*J***	**HMBC-^{3}*J***
2	5.35	*dd* 12,4	80.0	80.0	C-1′	C-3′, C-4
3ax	3.09	*dd* 16,12	43.1	43.1	C-2, C-4	C-1
3eq	2.79	*dd* 16,4				C-10
4			196.2			
4a			103.1			
5			162.9		C-5,	C-6, C-10
6	6.07	s	95.1	95.1		
7			164.4			
8	6.04	s	94.3	94.3	C-9, C-7	C-6, C-10
8a			168.0			
1'			130.5			
2'/6'	7.32	*d* 8	128.0	128.0		
3'/5'	6.88	*d* 8	115.7	115.7		
4'			156.2			
OCH_3	3.80	s	55.7	55.7		C-7
5-OH	12.02	s				
SOLVENT	**DMSO-d6**					



164

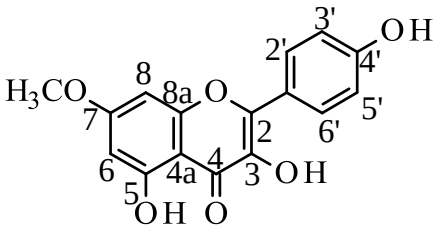
4.1.2 3, 5, 4'-Trihydroxy-7-methoxyflavone (165)

Compound **165** was eluted with 1% MeOH in CH_2Cl_2 and appeared as a yellow spot on the TLC plate with R_f 0.1 in CH_2Cl_2. It was visible under UV $_{254\text{-}366nm}$ and, on exposure to ammonia vapour, the yellow spot intensified indicating that it is phenolic. It occurred as yellow needle like crystals after recrystalization in CH_2Cl_2/*n*-hexane mixture. The MS showed a molecular ion peak at *m/z* 300. The NMR spectra (Table 4.2) showed the presence of flavonol skeleton with asingle methoxyl group and three hydroxyl substituents. From the MS and NMR data, the molecular formular $C_{16}H_{12}O_6$ was proposed. Analysis of ^{13}C-NMR (Table 4.2) revealed resonance at δ 136.4 ppm attributed to C-3 of a flavonol. Lack of a singlet peak at δ 6.88 ppm is in agreement that the compound is oxygenated at C-3 position which supports a flavonol skeleton rather than flavone. According to Agrawal (1989), C-3 oxygenation leads to lower chemical shift signals for the carbonyl carbon (C-4 and C-2) of about δ 178 and δ 155 ppm, respectively. The ^{13}C-NMR shows a peak at δ 176.4 ppm attributed to the carbonyl (C-4) and δ 147.6 ppm for the C-2 of flavonol. The presence of a chelated *peri*-hydroxyl group at C-5 was revealed from the ^{1}H-NMR spectrum which showed a peak at δ 12.43 ppm common for highly deshielded hydroxyl protons arising from chelation with the C-4 keto group. Two sets of intense broad singlets were observed at δ 6.63 ppm and δ 6.27 ppm assigned to proton at position C-6 and C-8, in ring A respecively. The broad singlet observed at around δ 9.95 ppm revealed the presence of two hydroxyl groups. An AA'XX' spin

system observed at δ 8.05 ppm doublet (J = 8 Hz) and δ 6.92 ppm doublet (J = 8 Hz) for protons 2'/6' and 3'/5' respectively in ring B supported the placement of one OH group at C-4'. The placement of methoxyl group at C-7 was supported by the published data of the same compound showing C-7 with a methoxyl substituent peak at around δ 165 ppm. The ^{13}C-NMR spectrum showed apeak at δ 165.2 ppm (Eunjung *et al*, 2008). The *m/z* peak at 167 resulting from retro-Diels-Alder cleavage $C_8H_6O_4$ also affirmed this. Further information from DEPT spectrum revealed the presence of 7 protonated carbons attributed to a single methoxyl group (OCH_3) at δ 56.34 ppm, methane protons at C-6 and C-8 in ring A (δ 97.8 ppm and δ 92.3 ppm repectively) and intense peaks at δ 130 ppm and δ 115.8 ppm for protons at 2'/6' and 3'/5' supporting the AA'XX' spin system observed in ring B. The compound therefore was characterized as 3, 5,4'-trihydroxy-7-methoxyflavone which had been previously isolated from *Gardenia ternifolia* (Ochieng *et al*., 2010) and *Dodonea viscose* (Latha and Daniel, 2001).

Table 4.2 ^{1}H and ^{13}C NMR chemical shift data, together with DEPT for compound **165**.

Atom	**δ^{1}H-NMR (ppm)**	**J (Hz)**	**δ ^{13}C-NMR (ppm)**	**DEPT**
2			147.6	
3	9.95	s	136.4	
4			176.4	
4a			104.4	
5			160.8	
6	6.63	s	97.8	97.8
7	9.95	s	165.2	
8	6.27	s	92.3	92.3
8a			156.4	
1'			122.0	
2'/6'	8.05	d (8)	130	130
3'/5'	6.92	d (8)	115.8	115.8
4'-OH			159.7	
OCH_3	3.82	s	56.3	56.3
5-OH	12.43	s		
SOLVENT	**DMSO-d6**			



165

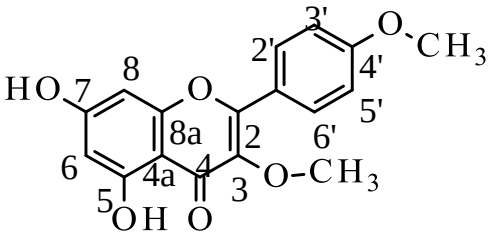
4.1.3 5, 7-Dihydroxy-3, 4'-dimethoxyflavone (166)

Compound **166** was isolated as yellow crystals (melting point of 233-235°C) which appeared as a yellow spot on the TLC plate under UV light (366 nm) with an R_f of 0.31 developed under 20% CH_2Cl_2 in n-hexane.When the spot was exposed to ammonia vapour, it intensified which indicated that it is a phenolic derivative. The EI-MS indicated a molecular peak at *m/z* 314, this together with NMR spectra, the formula $C_{16}H_{14}O_6$ was proposed. The ^{1}H-NMR (Table 4.3) showed the presence of two *meta* oriented aromatic protons at δ 6.26 ppm and δ 6.49 ppm as broad singlets.These were assigned to ring A protons of the flavone skeleton. An AA'XX' spin system was also observed at δ 8.08 and 7.10 (*d*, *J*= 8 Hz)-*ortho* coupling pattern assigned to ring B protons with C-4' substituted. Biogenetically, oxygenations are expected at C-5 and C-7 thus leading to the assignment of the *meta* oriented protons to H-6 and H-8 respectively. The presence of chelated hydroxyl proton at δ 12.77 ppm was also evident resulting from hydrogen bonding between the 5-OH and the keto group at C-4. The ^{13}C NMR spectrum showed two intense peaks at δ 130.2 and 114.0 ppm indicating chemically equivalent nuclei resonating in the same chemical environment thus confirmimg the assignment of C-2'/C-6' and C-3'/C-5'. It also displayed two peaks for the methoxyl groups at (δ_H = 3.87, δ_C = 54.9 and δ_H = 3.90, δ_C = 59.4) ppm where the NOE interaction of OCH_3 (δ_H = 3.87) with the protons 3'/5' of ring B led to the placement of this group at C-4'. The methoxyl group at δ 59.4 ppm was assigned at C-3 being typical of a methoxyl group that is di-*ortho* substituted. The

DEPT spectrum also showed the presence of eight protonated carbons at (C-6, C-8, 2OCH_3, 2'/6' and 3'/5'). Based on this spectroscopic data and comparison with the literature values, compound **166** was characterized as 5, 7-dihydroxy-3, 4'-dimethoxyflavone, a compound which had been previously reported from *Dodonaea viscosa* (Wollenweber *et al*., 1986) and *Haplopappus onorensis* (Encarnacion *et al*., 1999).

Table 4.3: ^{1}H and ^{13}C NMR chemical shift data, with DEPT and HMBC for compound **166**

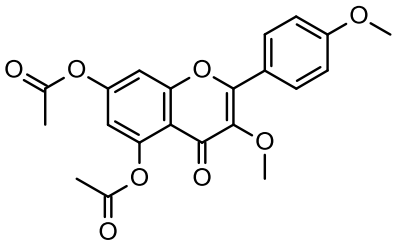
Atom	**δ^{1}H-NMR (ppm)**	**M,J (Hz)**	**δ ^{13}C-NMR (ppm)**	**DEPT**	**HMBC ^{3}J**
2			155.2		
3			138.5		
4			178.6		
5			157.0		
6	6.26	Broad (s)	98.5	98.5	
7			164.0		
8	6.49	Broad (s)	93.6	93.6	
8a			162.3		
4a			105.0		
1'			122.8		
2'/6'	8.08	d (J = 8)	130.1	130.1	C-6', C-4'
3'/5'	7.10	d (J = 8)	114.0	114.0	C-5',C-1'
4'			161.8		
OCH_3	3.87	s	54.9	54.9	C-4'
OCH_3	3.90	s	59.4	59.4	C-3
5-OH	12.77	s			
SOLVENT	**DMSO-d6**				



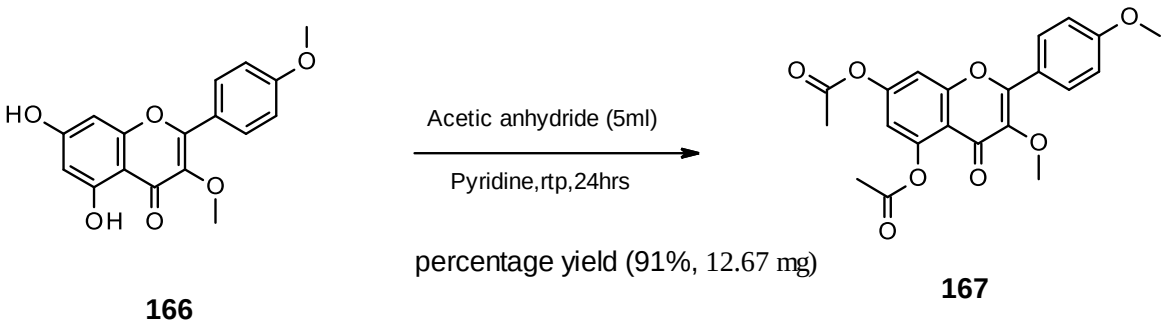
166

4.1.4 5, 7-Diacetyl-3, 4'-dimethoxyflavone (167)

Compound **167** was obtained by acetylating 5, 7-dihydroxy-3, 4'-dimethoxyflavone (**166**) (10 mg) with acetic anhydride in the presence of pyridine affording 12.67 mg (91% yield*)* product as a white solid.



167



166

percentage yield (91%, 12.67 mg)

167

The ^{1}H-NMR spectrum of this product (**167**) showed the presence of two intense acetate signals at δ 2.30 and 2.44 ppm each intergrating for three protons denoting the presence of a methyl of an acetate. This was further confirmed by ^{13}C-NMR at δ 21.1 and 21.1 ppm typical of a methyl group resonance. Futher signals at δ 168.0, 169.4 and 173.1 indicated the presence of three carbonyl carbons. The highest resonance at δ 173.11 was assigned to the carbonyl carbon C-4 of the original compound (**166**) while the remaining two are the acetate carbonyl groups. The success of the acetyltion reaction was also confirmed by the disappearance of a chelated proton at C-5 originally

observed in the starting compound (**166**) at δ 12.77. The two acetyl groups observed in this compound replaced the protons at C-5 and C-7 of the parent molecule (**166**).

Table 4.4: ^{1}H and ^{13}C NMR chemical shift data, together with DEPT for compound **167**

Atom	**δ^{1}H-NMR (ppm)**	***M, J* (Hz)**	**δ ^{13}C-NMR (ppm)**	**DEPT**
2			150.9	
3			140.9	
4			173.1	
5			154.9	
6	6.78	*d* (*J* = 4)	113.1	113.1
7			161.6	
8	7.25	*d* (*J* = 4)	108.8	108.9
8a			156.4	
4a			105.0	
1'			122.4	
2'/6'	8.01	*d* (*J* = 8)	130.0	130.1
3'/5'	6.95	*d* (*J* = 8)	114.0	114.0
4´			153.6	
OCH_3	3.76	s	55.4	55.4
OCH_3	3.83	s	59.9	60.0
CH_3	2.30	s	21.1	21.1
CH_3	2.44	s	21.1	21.1
C=O			169.4	
C=O			168.0	
SOLVENT	**DMSO-d6**			

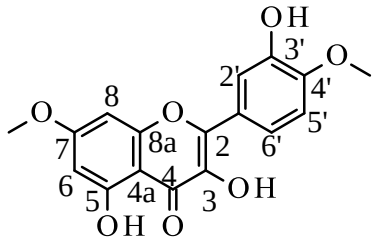
4.1.5 3, 5, 3'-Trihydroxy-7, 4'-dimethoxyflavone (168)

Compound **168** was isolated as a yellow solid with a melting point of 220-222 °C, which appeared as yellow spot that intensified in color on exposure to ammonia R_f value of 0.5 in CH_2Cl_2. A molecular ion peak at *m/z* 330 together with NMR data (Table. 4.5) proposed the molecular formular to be $C_{17}H_{14}O_7$. The ^{1}H-NMR showed the presence of a *meta* oriented aromatic protons at δ 6.39 ppm (*d, J* = 2.1 H_Z) and δ 6.50 ppm (*d, J*= 2.1 Hz). The peak at δ_H 11.72 ppm revealed the presence of a chelated hydroxyl proton attributed to OH at C-5. In consistency with the biogenetic process, the suggested oxygenation pattern for ring A is again at C-5 and C-7. The signals observed

in the aromatic region in ring B at δ 7.81 ppm (*d, J*= 2.0 Hz) were assigned to H-2', δ 7.06 ppm (*dd, J* = 8.7 Hz) for proton H-5' and δ 7.77 ppm (*dd, J* = 8.5, 2.0 Hz) for poton H-6'. This revealed the presence of an AXY spin system on ring B in agreement to the literature reports for such substituted ring B of flavonols (Midiwo *et al*., 1994; Wollenweber *et al*., 1987 and Wang *et al*., 1989). The ^{1}H-NMR signals at δ 3.90 and 4.01 ppm each intergrating for three protons were attributed to methoxy signals correlated to ^{13}C-NMR signals at δ 55.9 and 56.1 ppm. The two chemical shifts for these two methoxyl groups were attributed for an isolated methoxy group. The absence of a signal at around δ 60 ppm ruled out attachment at C-3. The placement of one of the methoxyl groups at C-7 was established from its HMBC correlation with C-7 (δ 167.0 ppm). This compound was therefore characterized as 3, 5, 3'-trihydroxy-7, 4'-dimethoxyflavone, previously isolated from *Gardenia ternifolia* (Ochieng *et al*., 2010), *Psiadia trinervia* (Wang *et al*., 1989) and *Ostrya japonica* (Wollenweber and Diertz, 1982).

Table 4.5: ^{1}H and ^{13}C NMR chemical shift data, with HMBC for compound **168**

Atom	**δ^{1}H-NMR (ppm)**	**m, J (Hz)**	**δ ^{13}C-NMR (ppm)**	**HMBC ^{3}J**
2			156.8	
3			135.6	
4			176.2	
5			160.9	C-6
6	6.39	*d* (*J* = 2.1)	97.9	
7			167.0	OMe
8	6.50	*d* (*J* = 2.1)	92.3	
8a			146.5	
4a			105.0	
1'			122.3	H-5'
2'	7.80	*d* (*J* = 2.0)	110.3	
3'			146.5	
4'			147.8	OMe
5'	7.06	*d* (*J* = 8.71)	114.6	
6'	7.77	*dd* (8.5,2.0)	121.8	
OCH_3	3.90	s	55.6	C-7
OCH_3	4.01	s	56.1	C-4'
5-OH	11.72	s		
SOLVENT	**DMSO-d6**			



168

4.2 Terpenoids

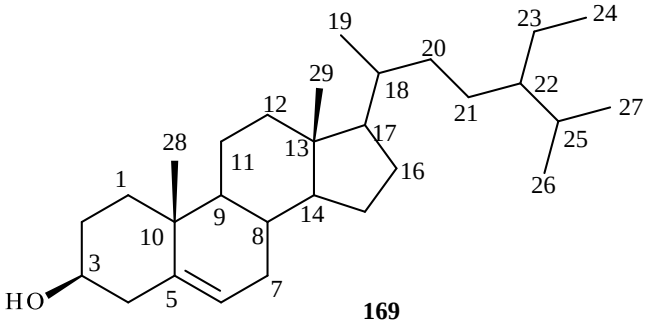
4.2.1 β-Sitosterol (169)

Fraction A eluted with CH_2Cl_2/*n*-hexane mixtures (1:9) respectively yielded β-sitosterol (1**69**) (65 mg) which crystallized in CH_2Cl_2/*n*-hexanemixture. It had an R_f value of 0.69 in 1:1 CH_2Cl_2/*n*-hexane system as opposed to stigmasterol which had an R_f value of 0.5. It was identified using the TLC plate after spotting with the eluent from the combined fraction (A) and exposing to iodine and spraying with concentrated sulphuric acid.The result was a brown/orange spot. In spraying with acidified vanillin, it showed abluish purple colour on the TLC plate characteristic of terpenoid or steroid. The difference in these two compounds was evident ^{1}H-NMR and ^{13}C-NMR chemical shift as well as the melting points.

This compound exhibited a melting point in the range of 134-136 $^{\circ}$C with a molecular ion peak at *m/z* 414. This along with NMR data corresponds to the molecular formula $C_{29}H_{50}O$. The loss of water from the molecular ion corresponded to the *m/z* peak at 329. The ^{13}C-NMR showed a total of 29 peaks attributed to nine methynes, eleven methylene and six methyls groups alongside three tertiary carbons. The ^{1} H-NMR displayed a multiplet at δ 5.36 ppm which was assigned to the vinylic protons at C-6. A multiplet again at δ 3.51 ppm was evident and was assigned to the C-3 hydroxymethine proton. The proton spectrum also displayed two intense singlets each intergrating for three protons at δ 0.68 ppm and δ 0.87 ppm which were assigned to the two tertiary methyl

groups at C-18 and C-19, respectively. The signals at δ 0.92 ppm, there was an overlap of doublets which were assigned to the secondary methyl protons attached at C-21. A dublet at δ0.84 ppm with coupling constant of 7 Hz were assigned to two equivalent methyl protons at C-26 and C-27.

The appearance of a characteristic peak multiplet at δ 5.31 ppm was supported by the occurance of vinylic carbon signals at δ 121.9 ppm and 140.0 ppm in the ^{13}C-NMR spectrum. The compound was identified as β- sitosterol and had been previously reported from *Gardenia ternifolia* (Ochieng *et al*., 2010).



169

Table 4.6: ^{1}H and ^{13}C NMR chemical shift data for compound **169**

Atom	**^{1}H-NMR ppm**	**^{13}C-NMR ppm**	**Atom**	**^{1}H-NMR ppm**	**^{13}C-NMR ppm**
1	1.88 (m)	37.5	**16**		28.5
2	1.83 (m)	31.9	**17**		56.3
3	3.51 (m)	72.0	**18**	0.68 (s)	36.3
4	2.27 (m)	42.6	**19**	0.87 (d)	19.3
5	5.36 (t)	141	**20**		34.2
6	5.36 (m)	121.9	**21**	0.92 (d)	26.3
7	1.54 (m)	31.9	**22**		46.1
8	1.99 (m)	32.1	**23**		23.3
9		50.4	**24**	0.85 (t)	12.2
10	1.51 (m)	36.7	**25**	0.84 (d)	32.2
11		21.4	**26**	0.81 (d)	19.0
12		40.0	**27**	0.81 (d)	19.6
13		42.6	**28**	0.85 (s)	19.0
14		57.0	**29**	1.01 (s)	12.2
15		24.5			
SOLVENT	**$CDCl_3$**				

Confirmation of the identity of this compound was accomplished by comparison with spectroscopic data posted in the literature (Waller, 1972).

4.2.2 Stigmasterol (170)

This compound was isolated as white needle-like crystals with melting point of 160-164 ^{o}C and R_f value of 0.5 in 1:1 hexane/ CH_2Cl_2. This compound was not sensitive to UV light (254 nm) and therefore was visualized using iodine vapour. The ^{1}H NMR spectrum exhibited olefinic protons, a hydroxymethine proton and a vinylic proton at δ 5.05 (*m*), 5.18 (*m*, 1H), 3.53 and 5.35 (*t*) assigned to protons at H-20, 21, 3 and 6 positions, respectively. The presence of these groups was confirmed from the ^{13}C NMR spectrum which showed peaks at δ 129.2 and 138.3 for olefinic carbons, 71.8 for hydroxymethine carbon and 121.9 for the vinyl carbon assigned to carbons at C-20, 21, 3 and 6 positions respectively. Methyl protons were observed at δ_H 1.01 (*s)* 3H, δ 0.93 (*m*) 3H, δ 0.85 (*m*) 3H, δ 0.82 (*m*) 3H and δ 0.80 (*m*) 3H. The ^{13}C NMR (Table 4.7) indicated presence of a quaternary carbon δ140.9 (C-5) and six methyls; δ 12.0 (C-29), 12.2 (C-24), 19.0 (C-28), 19.6 (C-27), 20.1 (C-26). Based on 1D and 2D spectra as well as correlation with literature (Prakash and Prakash, 2012) compound **170** was identified as stigmasterol. The mass spectrum of this compound showed a molecular ion peak at *m/z* 412 corresponding to molecular formular $C_{29}H_{48}O$. The *m/z* ion peak at 394 was attributed to loss of water from the molecular ion. This compound had previously been isolated from many plants including *Rubus suavissimus* (Prakash and Prakash, 2012), *Phaseolus vulgaris* (Ottand Ball, 1944), *Spillanthes acmella* (Isah *et al*., 2012) and *Gardenia ternifolia* (Ochieng *et al*., 2010).

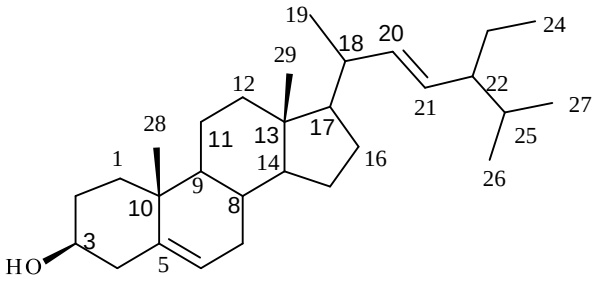


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Table 4.7: ^{1}H and ^{13}C NMR chemical shift data, with HMBC correlations for compound **170**

Atom Number	**^{1}H NMR δ, ppm**	**^{13}C NMR δ, ppm**	**HMQC δ, ppm**	**HMBC ^{2}J**	**HMBC ^{3}J**
1	1.85 (*m*)	37.5	37.5	C-2, C-10	C-3, C-5
2		31.9			
3	3.53 (*m*)	71.8	71.8		
4	2.29	42.4	42.5	C-3, C-5	C-6
5		140.9			
6	5.35 (*t*)	121.9	121.7		C-4, C-8, C-10
7	1.99 (*m*)	32.1	32.1	C-6	C-5, C-9
8	1.54	32.1	32.1	C-7, C-9	
9		50.3			
10		36.7			
11		21.3			
12		39.9			
13		42.6			
14		56.9			
15		26.3			
16		28.5			
17		56.3			
18		36.3			
19	0.93 (d)	19.2	19.2	C-18	C-17, C-20
20	5.05 (m)	129.2	138.3		
21	5.18 (m)	138.3	129.2		
22		46.1			
23		23.3			
24	0.85 (dd)	12.2	12.2		
25		29.4			
26	0.82 (d)	20.1	20.1		
27	0.80 (d)	19.6	19.6		
28	0.67(s)	19.0			
29	1.01 (s)	12.0	12.0	C-13	C-17
SOLVENT	**$CDCl_3$**				

4.3 Bioassays

All the compounds obtained were tested for their anti-oxidant and Monoamine oxidase (A and B) inhibitory activity.

4.3.1 Antioxidant Activities of the Compounds

All the compounds isolated were tested for radical scavenging activity where DPPH was used**.** The compounds were first subjected to preliminary qualitative testing on a TLC, out of which compounds 1**69** and **170** (steroids) were inactive, while the flavanoids **164, 165, 166, 167** and **168** were active. Compounds which showed activity on TLC assays were quantitavely analysed by UV-VIS absorption measurements (517 nm) on their solutions on reaction with DPPH (Figure 4.8) and the results are presented in Table **4.8.**

Figure 4.1: Samples with varied concentration of compounds but constant concentration of DPPH.

Table 4.8: Antioxidant activity of flavonoids in the presence of DPPH

Compound	**TLC assay results**	**IC_{50} (µM)**
5,4'-Dihydroxy-7-methoxyflavanone (**164**)	+	94± 0.11
3,5,4'-Trihydroxy-7-methoxyflavone (**165**)	+	75.5± 1.75
5,7-Dihydroxy-3,4'-dimethoxyflavone (**166**)	+	89± 0.22

Compound	TLC assay results	IC_{50} (µM)
3,4'-Dimethoxy-5,7-diacetylflavone (**167**)	+	> 100
3,3',5-Trihydroxy-7,4'-dimethoxyflavone (**168**)	+	40.3± 1.55
Quercetin (standard)	+	20.1± 1.34

The results obtained in this study indicated different DPPH radical scavenging rate of tested flavonoids. The most active compound was 3, 3', 5,-trihydroxy-7,4'-dimethoxyflavone **(168)** with radical scavenging activity of (40.3± 1.55) which was less than that of the standard used quercetin (20.1± 1.34 µM).

All of the compounds found to have radical scavenging activities had phenyl moiety oxygenated at either *ortho* or *para* positions, explained by the ability of hydroxyl groups at these positions to reduce radicals and form quinones. The weaker activity exhibited by the rest of the compounds could be linked to the methylation of some of their phenolic-OH groups which led to the reduction in their anti-oxidant activity (Op de Beck *et al*., 2003).

4.3.2 MAO-A and B Inhibition Activities

4.3.2.1 Determination of Inhibitory Effect of Compounds of *Gardenia ternifolia* on MAO-A and B

Recombinant human MAO-A and B were used for the evaluation of inhibitory effects of the flavonoids. Inhibition of MAO-A and B was tested with spectrophotometric kynuramine assay. The binding affinities of the inhibitors **(Ki**) with MAO-A and B were determined by substrate kinetics assays while the nature of the binding was determined by equilibrium dialysis and dissociation analysis. Compounds **164, 165, 166, 167** and **168** were evaluated *in vitro* against recombinant

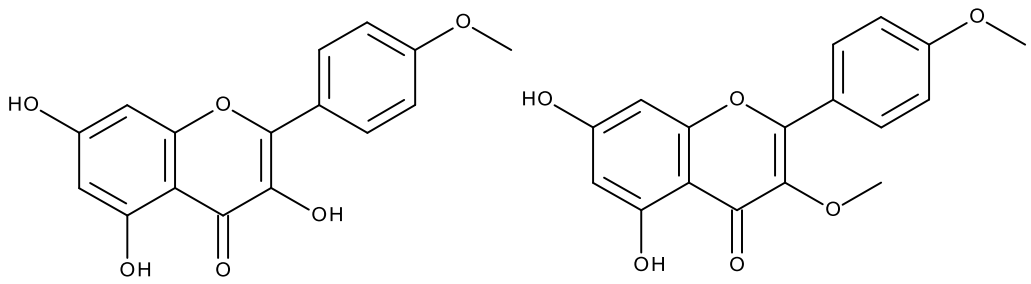
human MAO-A and B. Compound **166** (5, 7-dihydroxy-3,4'-dimethoxyflavone) demonstrated the most potent MAO-A and B inhibitory activities (**Table 4.9**). The inhibition of MAO-A by compound **166** was about 125-fold more potent (IC_{50} 0.033 μM) compared to the inhibition of MAO-B (IC_{50} 4.133 μM) but less potent than clorgyline (IC_{50} 0.0065 μM) used as the standard. The other compounds tested for the MAO inhibitory action showed marginal activities compared to the standard as summarized in **Table 4.9**. The inhibition of MAO-A and B by the other flavonoids were significantly less than those of compounds **165** and **166**. Therefore, compounds **166** and **165** were investigated in more details for kinetics and mechanisms of inhibition of MAO-A and B.

Table 4.9: *In vitro* inhibition of human recombinant MAO-A and B enzymes with compounds **164, 165, 166, 167** and **168.**

TESTS			**MAO-A**		**MAOB**	
Si No	**Highest Test conc.**	**Serial Dilution(μM)**	**IC_{50}**	**IC_{90}**	**IC_{50}**	**IC_{90}**
164	100	10	20.00	1.380	4.00	0.860
165	**100**	**10**	**1.00**	**0.510**	**17.00**	**1.340**
166	**100**	**10**	**0.033**	**0.004**	**4.133**	**0.757**
167	100	10	10.40	2.160	42.00	2.09
168	100	10	-	-	>100	-
Clorgyline	0.1	10	0.0065	0.00071	-	-
Deprenyl	1	10	-	-	0.043	0.0115

4.3.2.2 Evaluation of Inhibition Mechanisms and Kinetics

Detailed *in vitro* studies were conducted inorder to understanding the kinetics and mechanisms of inhibition of recombinant human MAO-A and B by compounds **165** and **166.** Both compounds were tested against MAO-A and B at varying concentrations of kynuramine (a non-selective substrate) in investigating the nature of inhibition of the enzymes by the two compounds



165 **166**

Figure 4.2: Structures of compound **165** and **166**

Based on dose-response inhibition, two concentrations of the inhibitors were selected (one below and another above the IC_{50}) for the inhibition experiment. In each experiment, three sets of assays were done at varying concentrations of the substrate, one control without inhibitors and two concentrations of the inhibitors. The enzyme kinetics data were presented as double reciprocal LineweaverBurk plots (Figure 4.11).

The Ki (inhibition constant/binding affinity) values and other enzyme kinetics parameters were computed with SigmaPlot 12.3 enzyme module. The binding of the two compounds (**165** and **166**) with human MAO-A increased the K_M value (the Michaelis–Menten constant) with no obvious effect on the V_{max} (maximum enzyme activity), suggesting that the inhibition of MAO-A was competitive by the two compounds, and substrate-inhibitor binding with the common enzyme active site (Table 4.10 and 4.11) and Figure 4.11.

Table 4.10: Km and Vmax of *Gardenia* compounds analyzed using Sigma plot software.

MAO-A					
SI No	Compounds Conc. (µM)	Km (µM)	Vmax (nmoles/min/mg protein)	Ki (µM)	Inhibition Types
166	0.000	27.027	25.000	0.0379	Competitive / Reversible
	0.080	83.333	23.256		
	0.160	142.857	23.256		
165	0.000	33.333	29.412	7.269	Competitive / Irreversible
	5.000	58.824	25.000		
	16.000	100.000	25.000		

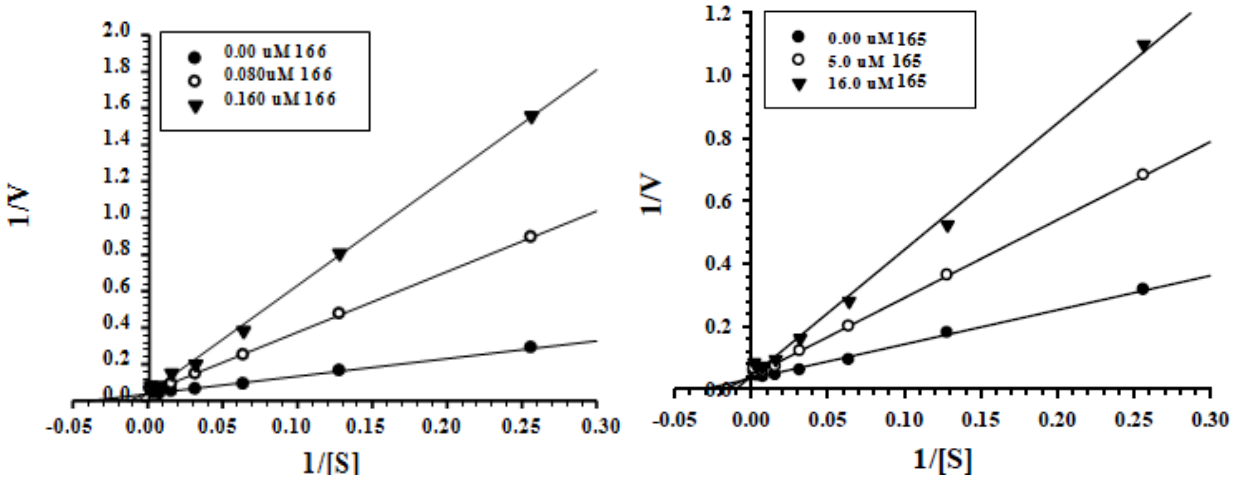


Figure 4.3: Kinetic characteristic of inhibition of human recombinant MAO-A with *Gardenia* flavanoids .The double reciprocal Line-weaver Burk plots were computed from the mean values.

Table 4.11: *In vitro* inhibition of human recombinant MAO-A and B enzymes with *Gardenia* compounds.

Compounds	Monoamine oxidase-A			Monoamine oxidaseB		
	IC_{50} (μM) *,+	**Ki (μM)#**	**Type of Inhibition**	**IC_{50} (μM) *,**	**Ki (μM)#**	**Type of Inhibition**
166	0.033± 0.040	0.0379 ± 0.0008	Competitive/ Reversible	-	-	-
165	1.350 ± 0.198	7.269 ± 1.033	Mixed / Irreversible	-	-	-
Clorgyline	0.0065± 0.001	0.0013 ±0.0016	Competitive/ Irreversible	-	-	-
Deprenyl	-	-	-	0.043 ±0.011	0.0326 ±0.061	Competitive/ Irreversible

*The values presented are mean± S.D. of inhibitory effect observations.
+IC-50 values were computed from the dose response graphs.
#Ki values were computed from double reciprocal Line-weaver Burk plots.

When the binding affinities of compound **166** (Ki- 0.0379 μM) with MAO-A was compared to the standard, clorgyline (Ki-0.0013 μM), it was 29.15 fold lower. Compound **165** on the other hand exhibited mixed irreversible inhibition with MAO-A with binding affinity of (Ki-7.269 μM), 191.79 folds less than compound **166.**

4.3.2.3 Analysis of Time-Dependant Enzyme Inhibition

To analyze if the binding of compounds **165** and **166** with MAO-A to produce enzyme inhibition was time–dependent, the enzyme (MAO-A) was pre-incubated for different time periods (0-15 min) with the inhibitor at the concentrations, which produced approximately 60-80% inhibition of the enzyme. The inhibitor concentrations used to determine this time-dependant enzyme inhibitions were **165** (16.0 μM), **166** (0.20 μM) and clorgyline (0.010 μM) for MAO-A Figure 4.12**.** This was narrowed to MAO-A since no good inhibition activity (IC_{50}) as well as binding affinity (Ki) was observed towards MAO-B with the two active compounds (**165** and **166**).

The controls without inhibitors were run simultaneously and the activities of the enzyme determined. The percentage of the enzyme activity remaining was plotted against the pre-incubation time to determine time-dependent inhibition. The results showed that the binding/inhibition of MAO-A with compound **165** and **166** was not dependent on the pre-incubation time as shown in Table 4.12 and Figure 4.12.

Table 4.12: Time dependent inhibition data of *Gardenia* compounds with MAO-A (% Activity with S.D.

Time (min)	**DMSO**		**Clorgyline**		**166 (0.2µM)**		**165 (16.0µM)**	
	%	**S.D.**	**%**	**S.D.**	**%**	**S.D.**	**% Activity**	**S.D.**
0	100.00	0.00	25.35	0.37	29.07	0.50	45.80	2.22
1	87.97	0.44	10.20	0.03	26.63	0.21	37.76	0.26
2	84.87	0.50	5.86	0.33	21.79	0.93	35.19	1.60
3	81.82	1.33	3.23	0.34	21.77	0.56	28.87	0.95
5	80.32	1.69	1.81	0.48	20.46	0.19	31.86	1.58
10	77.96	3.05	1.12	0.12	20.76	0.98	31.42	2.81
15	84.71	1.28	1.40	0.63	25.97	0.27	35.80	2.93

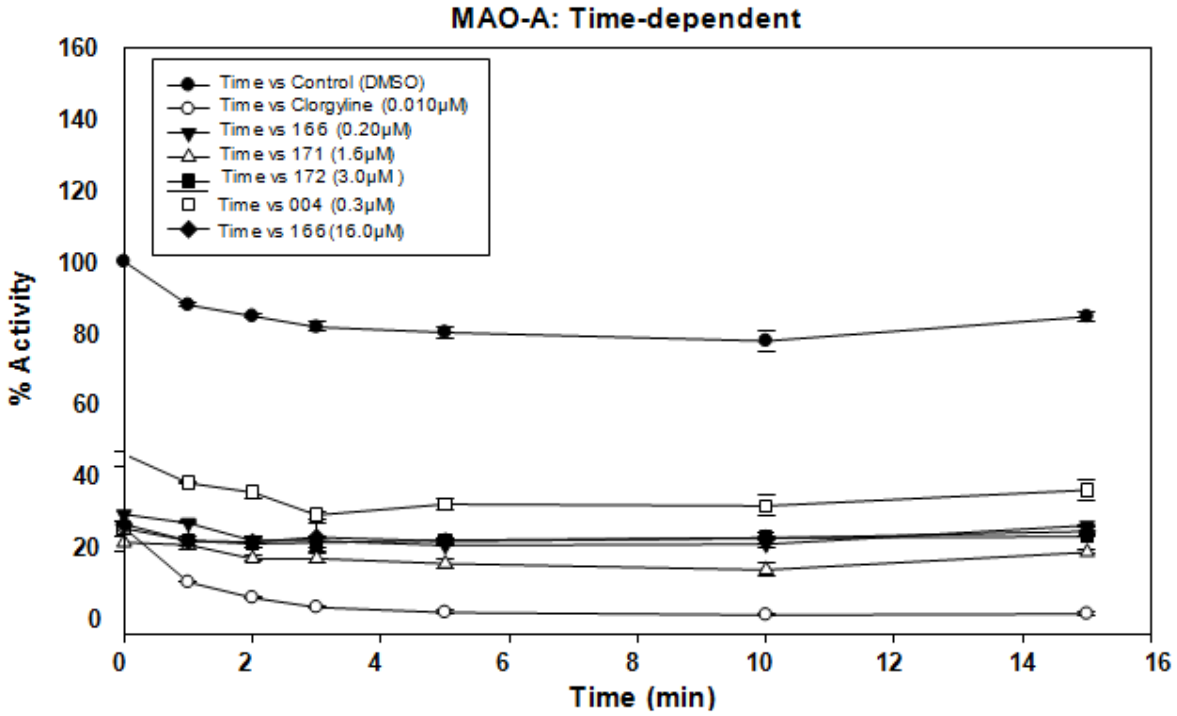


Figure 4.4: Time-dependant inhibition assay *Gardenia* compounds of recombinant human MAO-A.

4.3.2.4 Analysis of Binding of Compound 165 and 166 with MAO-A

The characteristic binding of these inhibitors (compound **165** and **166**) was also investigated by the enzyme –inhibitor complex dissociation dialysis. High concentrations of the inhibitors (Table 4.13**)** were allowed to interact with the enzyme (MAO-A) for 20 min and the resulting enzyme-inhibitor complex mixtures were dialyzed overnight against buffer solutions.The catalytic activity of the enzyme was analyzed before and after the dialysis.

Table 4.13: Reversibility /irreversibility assay data of **165** and **166** compounds with MAO-A

Compounds	**Before Dialysis**				**After Dialysis**			
	%Activity	**S.D.**	**% Inhibition**	**S.D.**	**% Activity**	**S.D.**	**% Inhibitio**	**S.D.**
Control (DMSO)	100.00	0.00	0.00	0.00	90.76	4.54	9.24	4.54
165 (100.0µM)	31.06	0.90	68.94	0.90	41.59	2.60	58.41	2.60
166 (10.0µM)	4.35	0.23	95.65	0.23	75.30	7.70	24.70	7.70
Note-After 10-14 hrs dialysis, control also lost the 10-11 % enzyme activity.								

The recombinant human MAO-A lost about 10% of the enzyme activity during the overnight dialysis. Incubation of MAO-A with 10.0 µM of compound **166** and 100 µM of ompound **165** caused more than 95% and 68% inhibition of the catalytic activity of the enzyme respectively. After the overnight dialysis, about 75% and 41% activity of the enzyme (MAO-A) was recovered from enzyme-166 and enzyme-165 incubation mixtures as summarized in **Figure 4.13**.

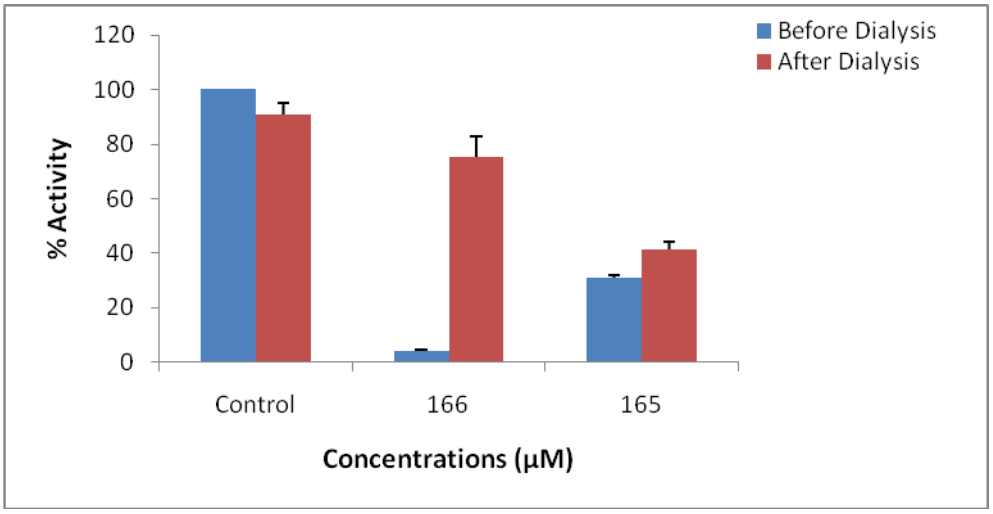


Figure 4.5: Binding of compound **165** and **166** with MAO-A

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Phytochemical investigation of *Gardenia ternifolia* surface exudates collected from Kagundo, Machakos county in Kenya was undertaken which led to the isolation and characterization of 6 compounds. Three flavones (**165, 166** and **168**), one flavanone (**164**) and two phytosterols (**169** and **170**) were obtained. Acetylation of compound **166** led to the formation of compound **167** which was also characterized.

The anti-oxidant assay revealed that flavonoids with the hydroxyl groups together with other hydroxyl or methoxy groups at either *ortho* or para positions were active. The structure activity relationship of the flavonoids showed that flavonols had better antioxidant activity as compared to methoxyflavones isolated from the surface exudates of *Gardenia ternifolia*. The most active compound was 3, 5, 3'-trihydroxy-7, 4' -dimethoxyflavone (**168**) with IC_{50} of 40.3μM. The MAO-A and B inhibition activities revealed that 3, 4'-dimethoxyflavones had better activity as compared to hydroxylated flavonoids at these positions. Compound 5, 7- dihydroxy-3, 4'-dimethoxyflavone (**166)** was the most active and more selective to MAO-A (IC_{50} = 0.033 μM), binding affinity (Ki) 0.0379 μM) more than MAO-B. It also showed competitive reversible type of inhibition for the enzymatic active site. Acetylating C-5 and C-7 positions of compound **166** led to a decrease in MAO-A and B activities of IC_{50} = 10.40 and 42.00 μM, respectively.

The anti-oxidant and selective MAO-A inhibitory properties observed from the flavonoids isolated from the surface exudates of *Gardenia ternifolia* suggest their potential in eliciting selective pharmacological effects that might be useful in administering flavonoids for the prevention of

numerous free radical based diseases or additive elements to food as well as in the treatment of depression and other neurological diseases.

5.2 Recommendations

Based on the results of this study, it is recommended that:

- Phytochemical studies of other parts of *Gardenia ternifolia* such as the stem bark, fruit, root bark and the internal tissue should be carried out inorder to isolate all the compounds in this species.
- The other three Gardenia species found in Kenya should also be phytochemically investigated in order to isolate all the phytochemicals.
- Comprehensive structure-activity relationship studies should be carried out on the active compounds **165** and **166** to determine the structural properties responsible for the MAO-A and B activities.

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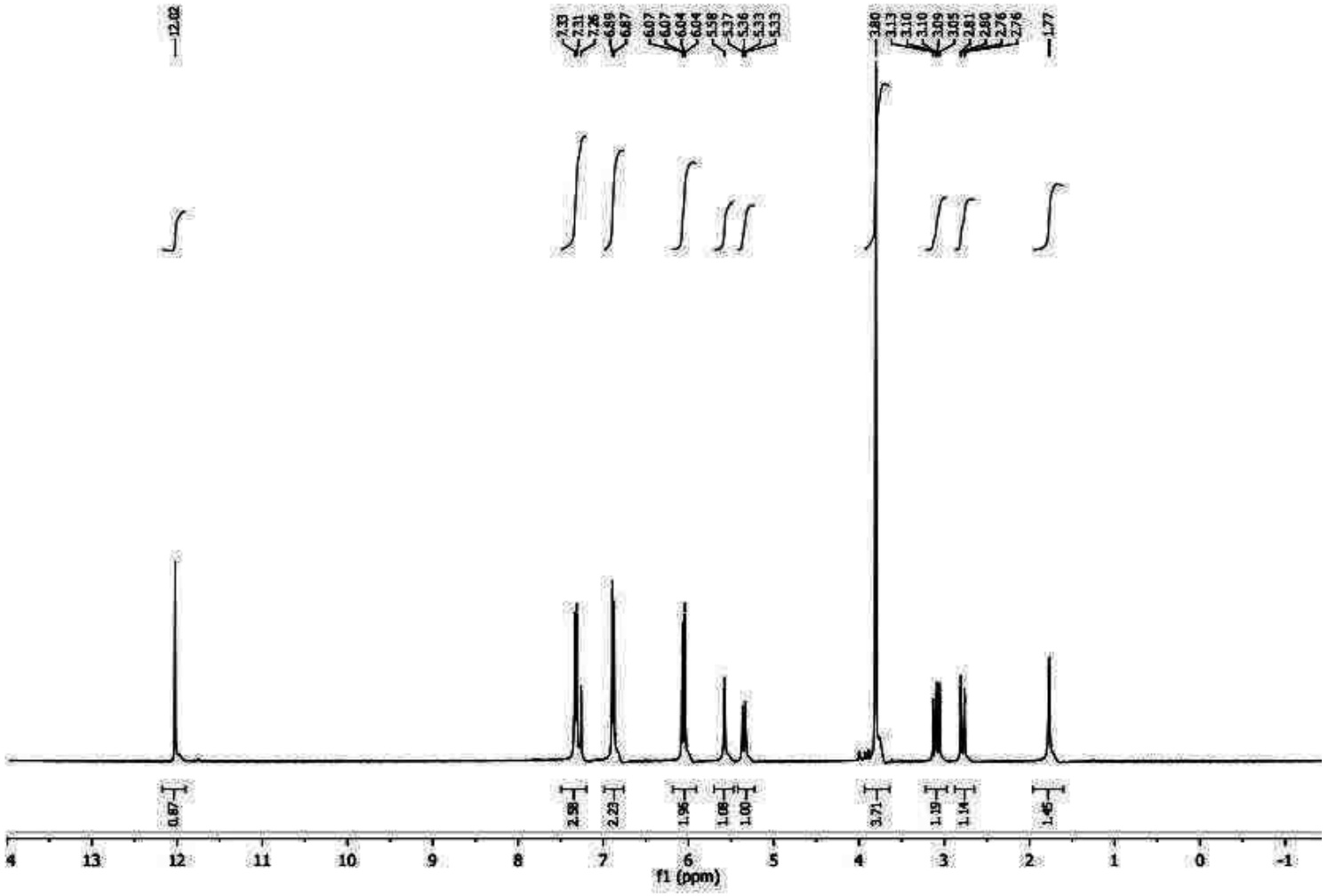
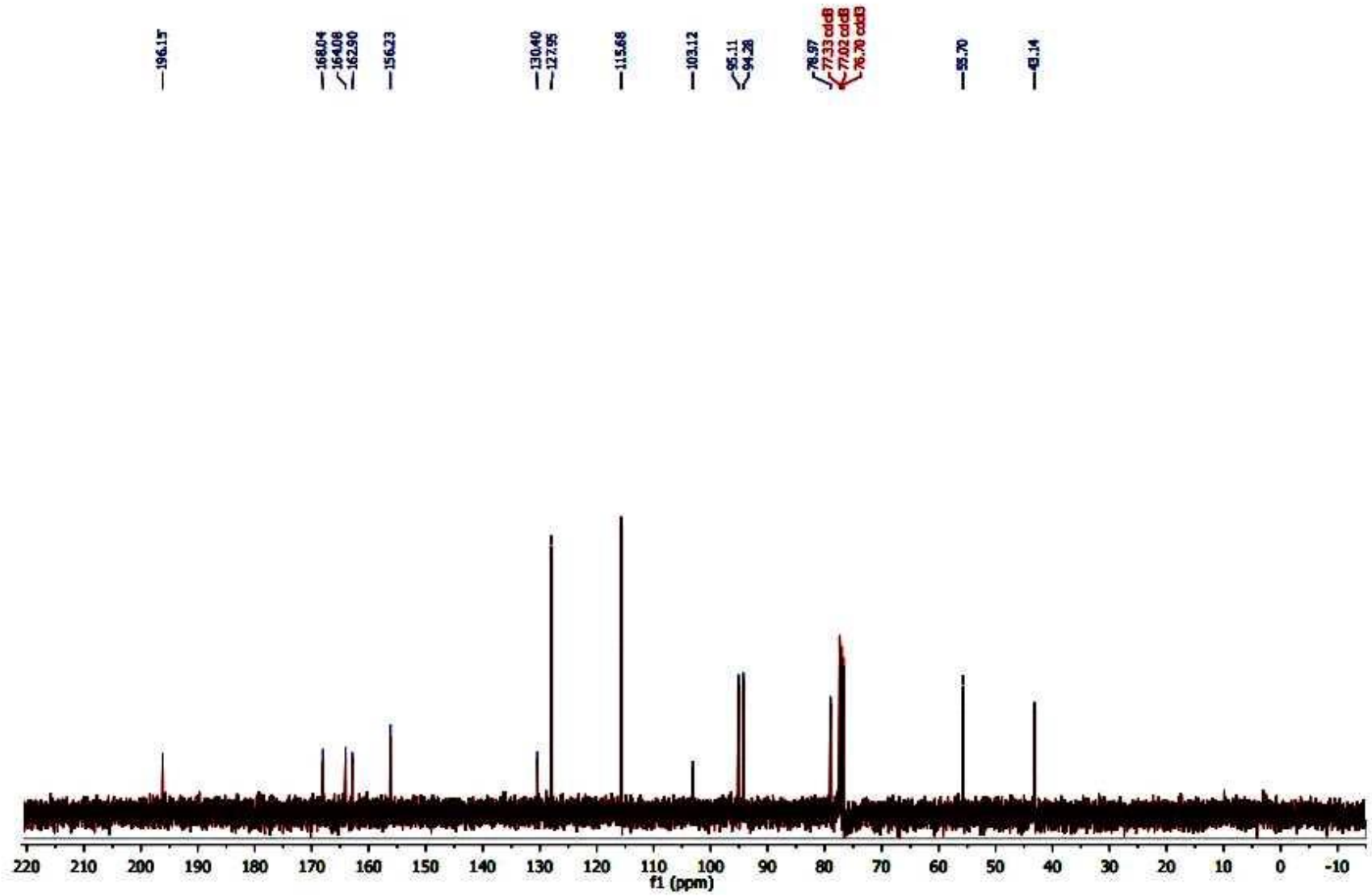
APPENDIX 1: SPECTRA FOR COMPOUND **164**

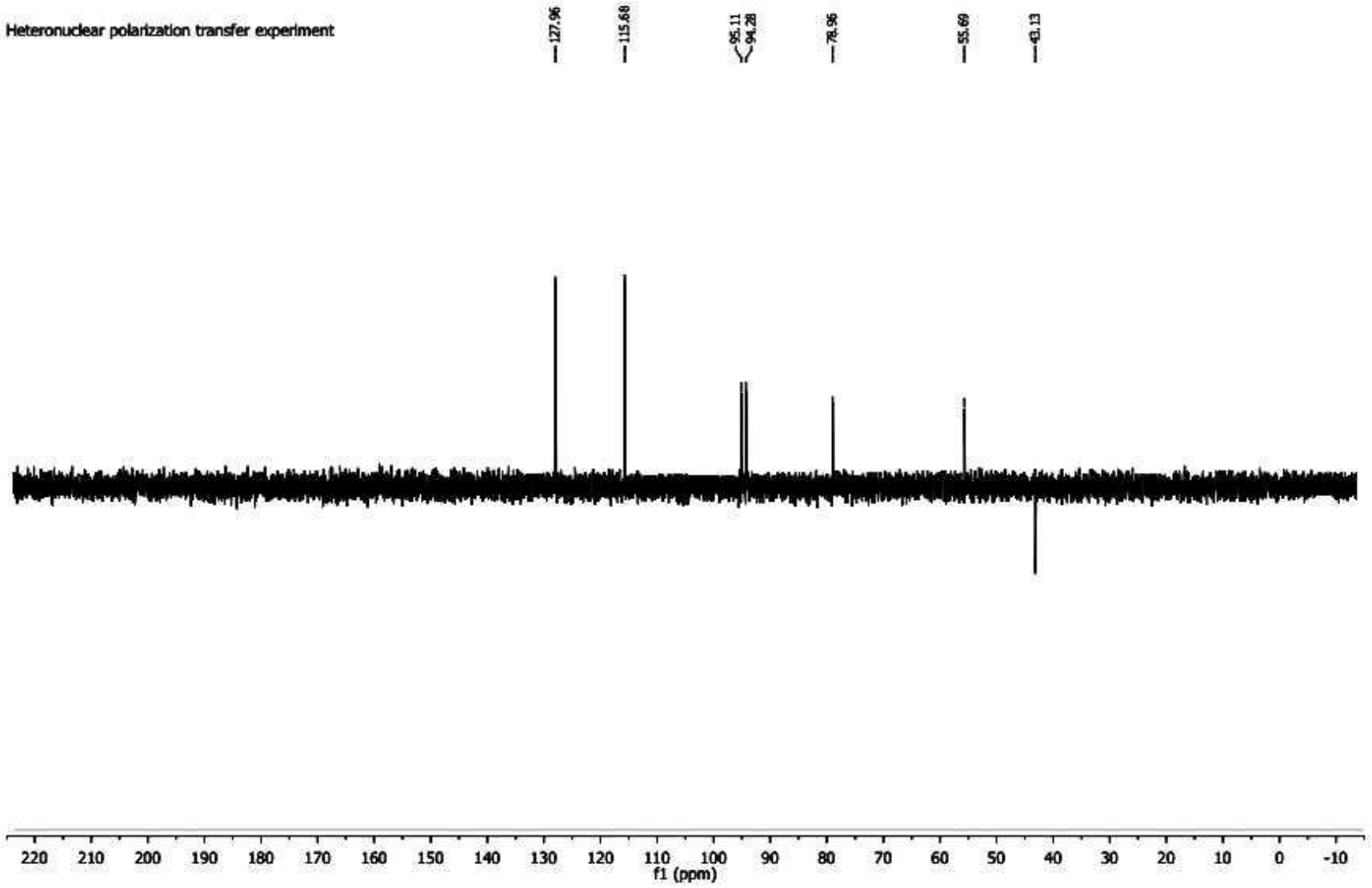
^{1}H-NMR SPECTRUM FOR COMPOUND -164



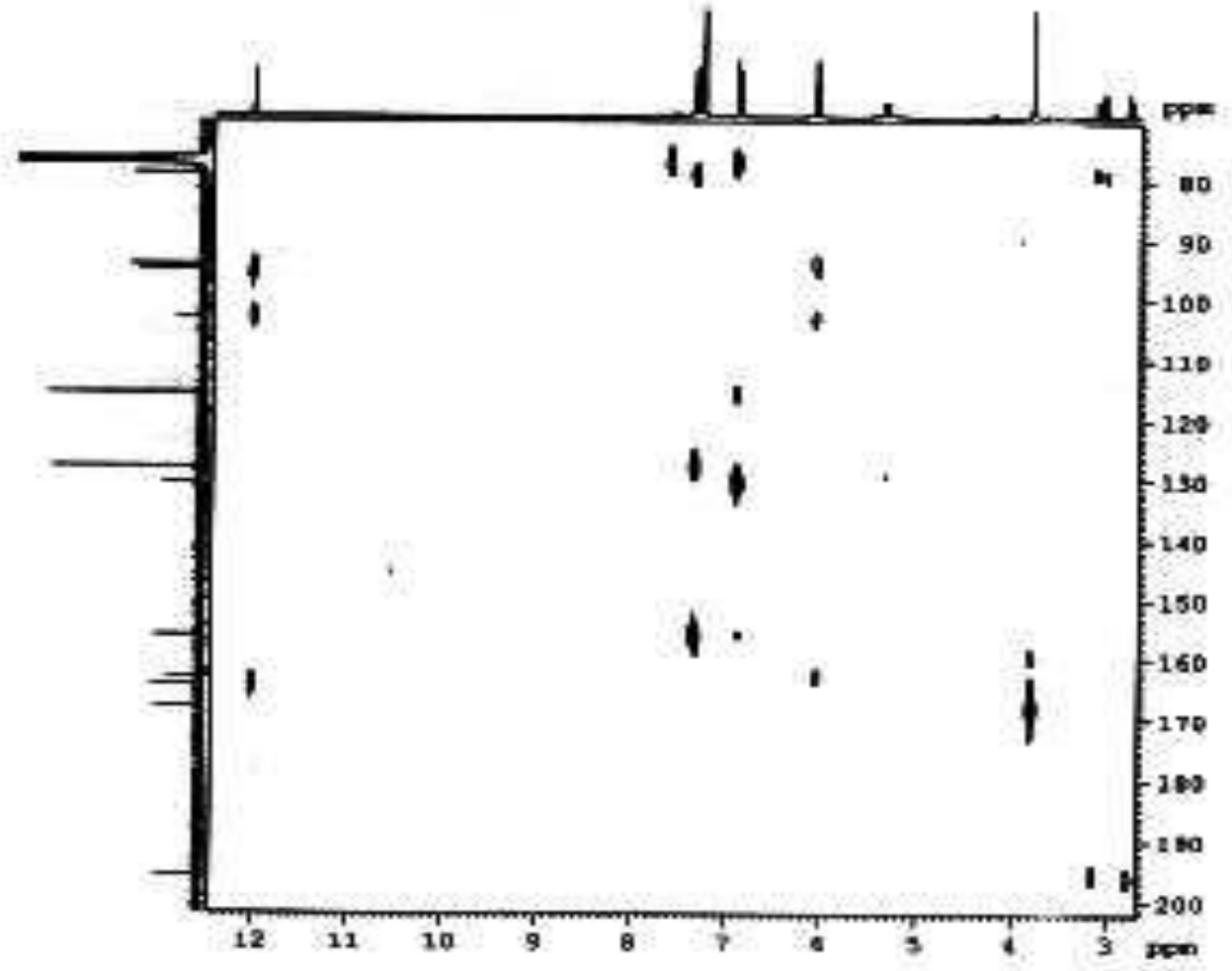
^{13}C-NMR SPECTRUM FOR COMPOUND 164



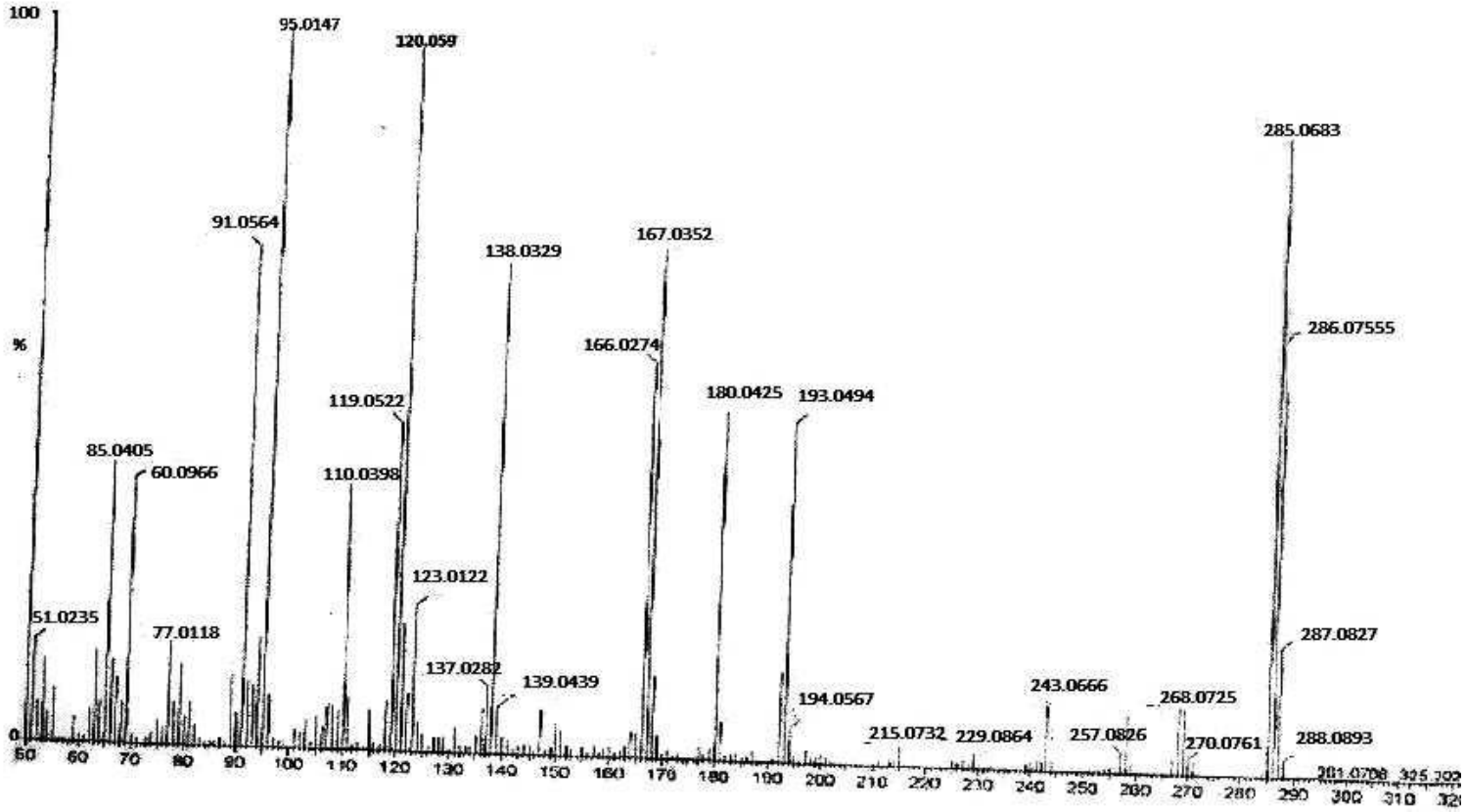
DEPT-SPECTRUM FOR COMPOUND 164



HMBC SPECTRUM FOR COMPOUND 164

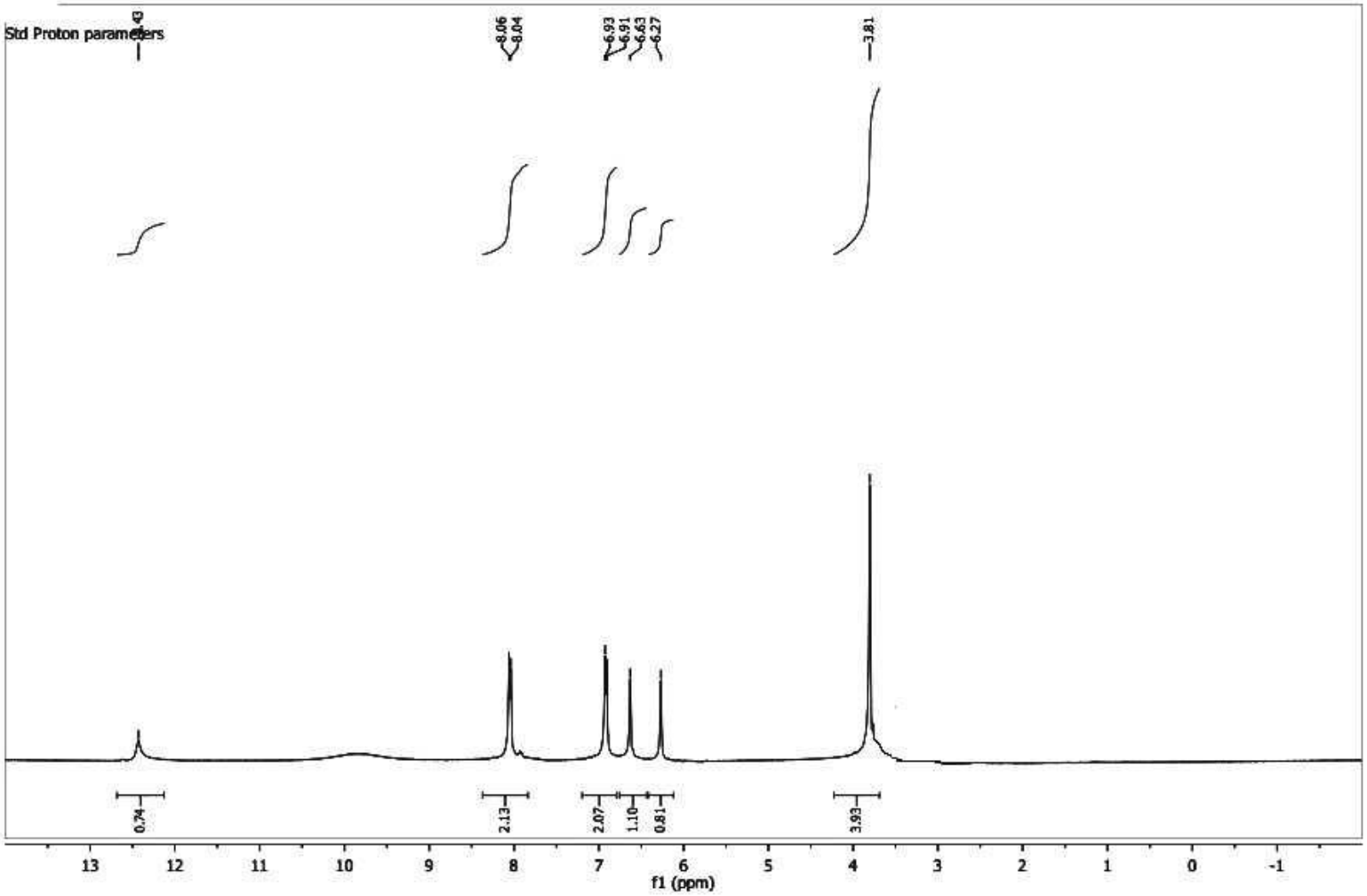


MASS SPECTRUM FOR COMPOUND 164

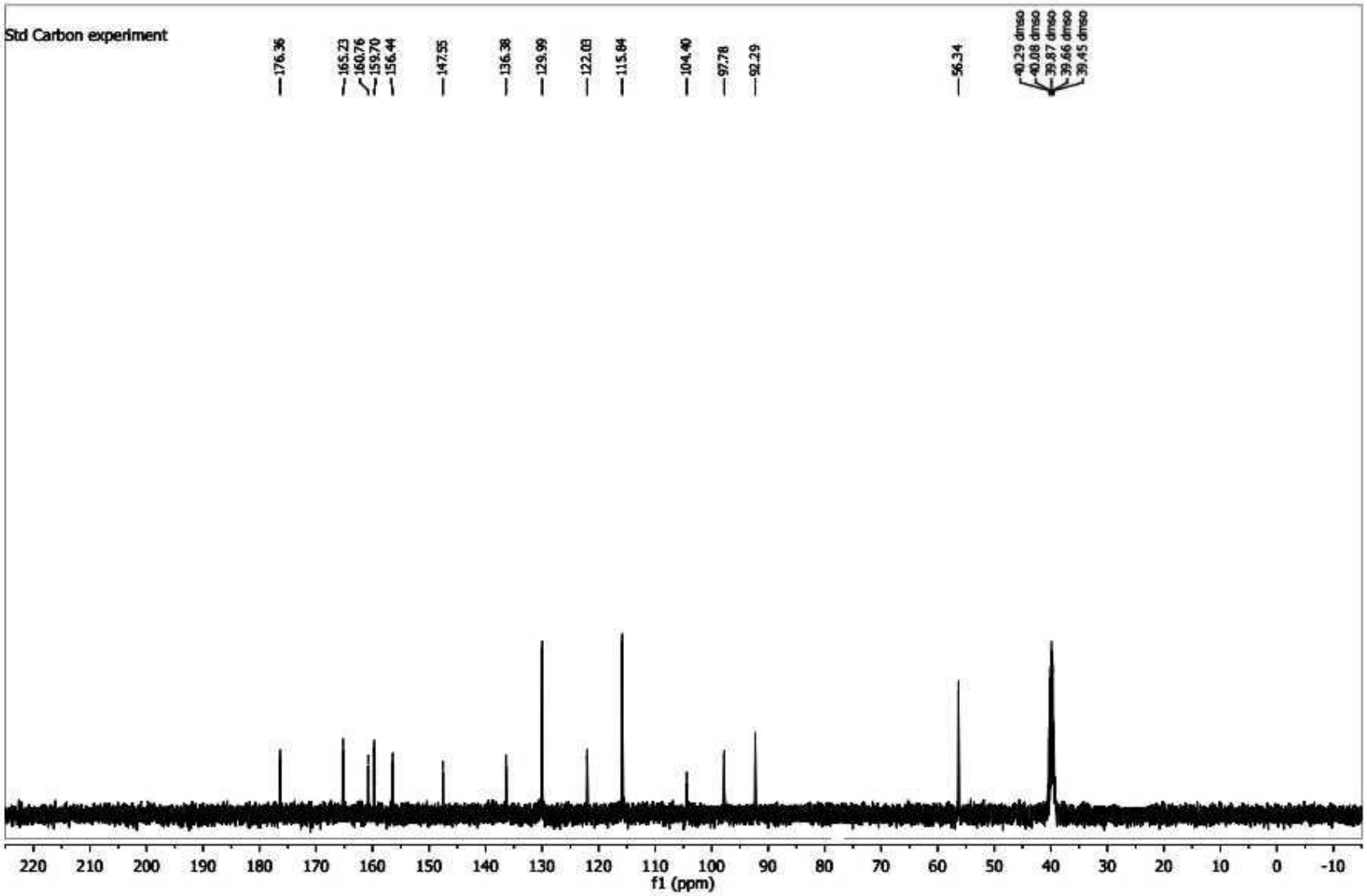


APPENDIX 2: SPECTRA FOR COMPOUND **165**

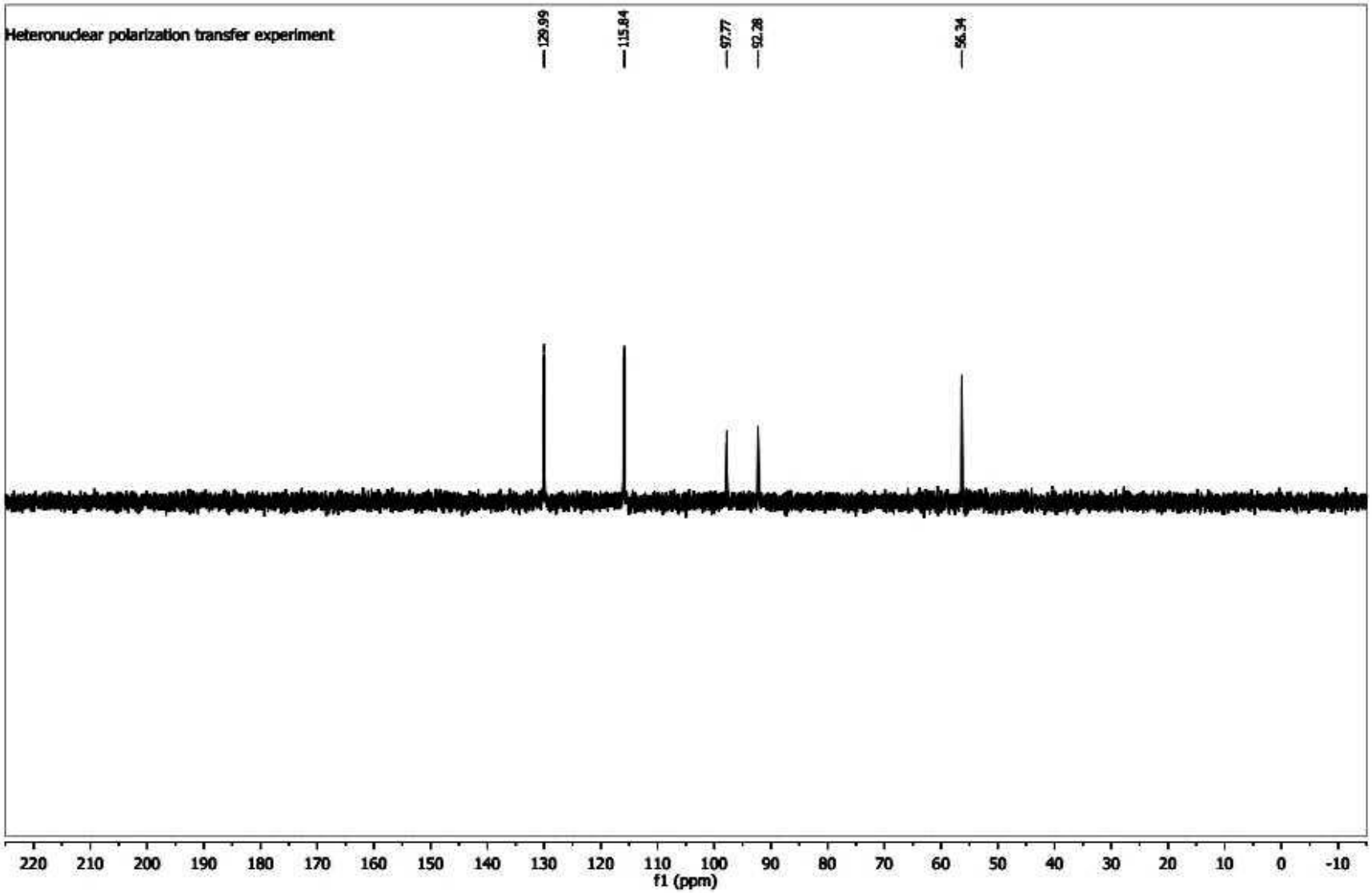
^{1}H-NMR SPECTRUM FOR COMPOUND -165



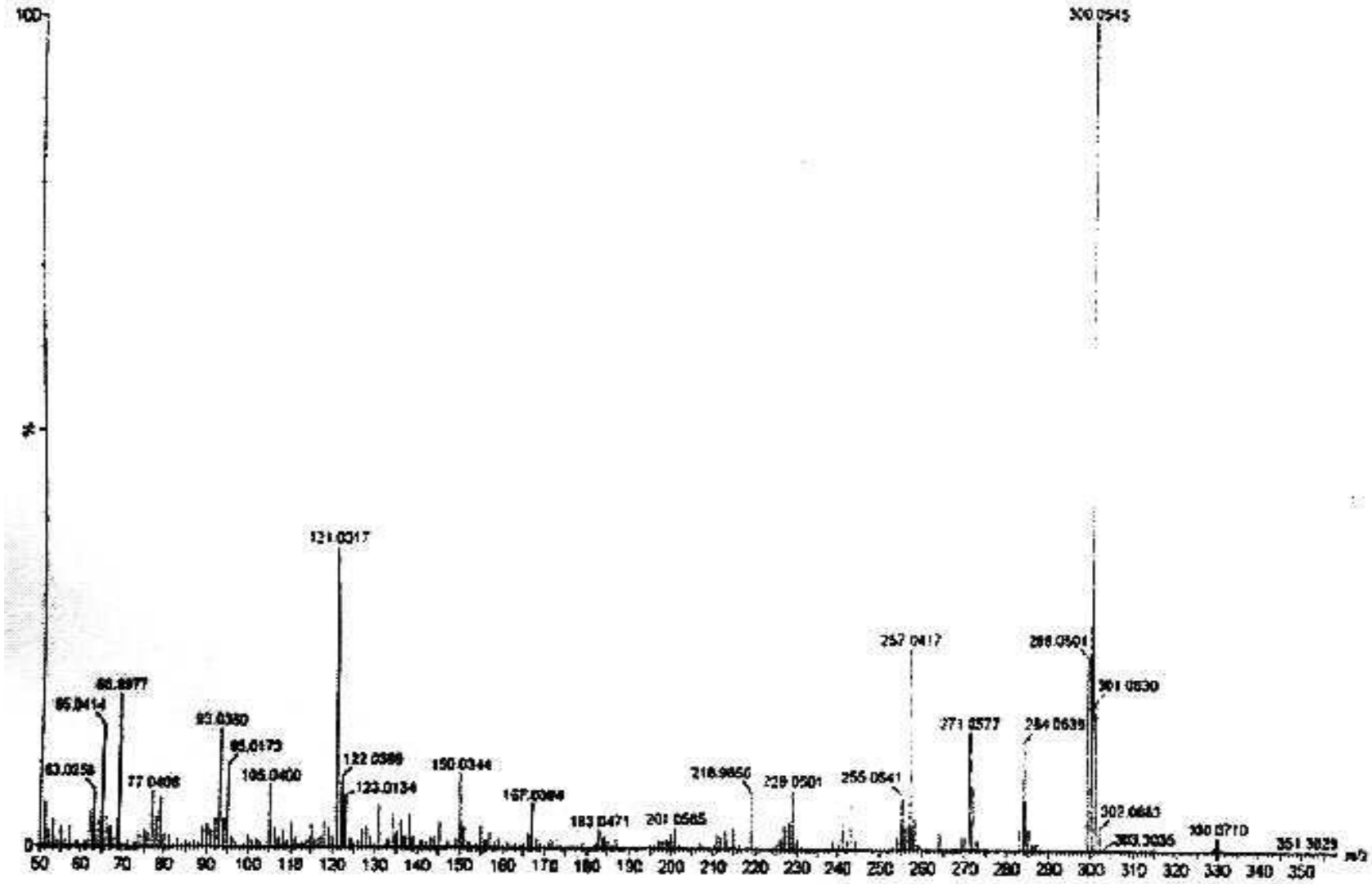
^{13}C-NMR SPECTRUM FOR COMPOUND 165



DEPT-SPECTRUM FOR COMPOUND 165

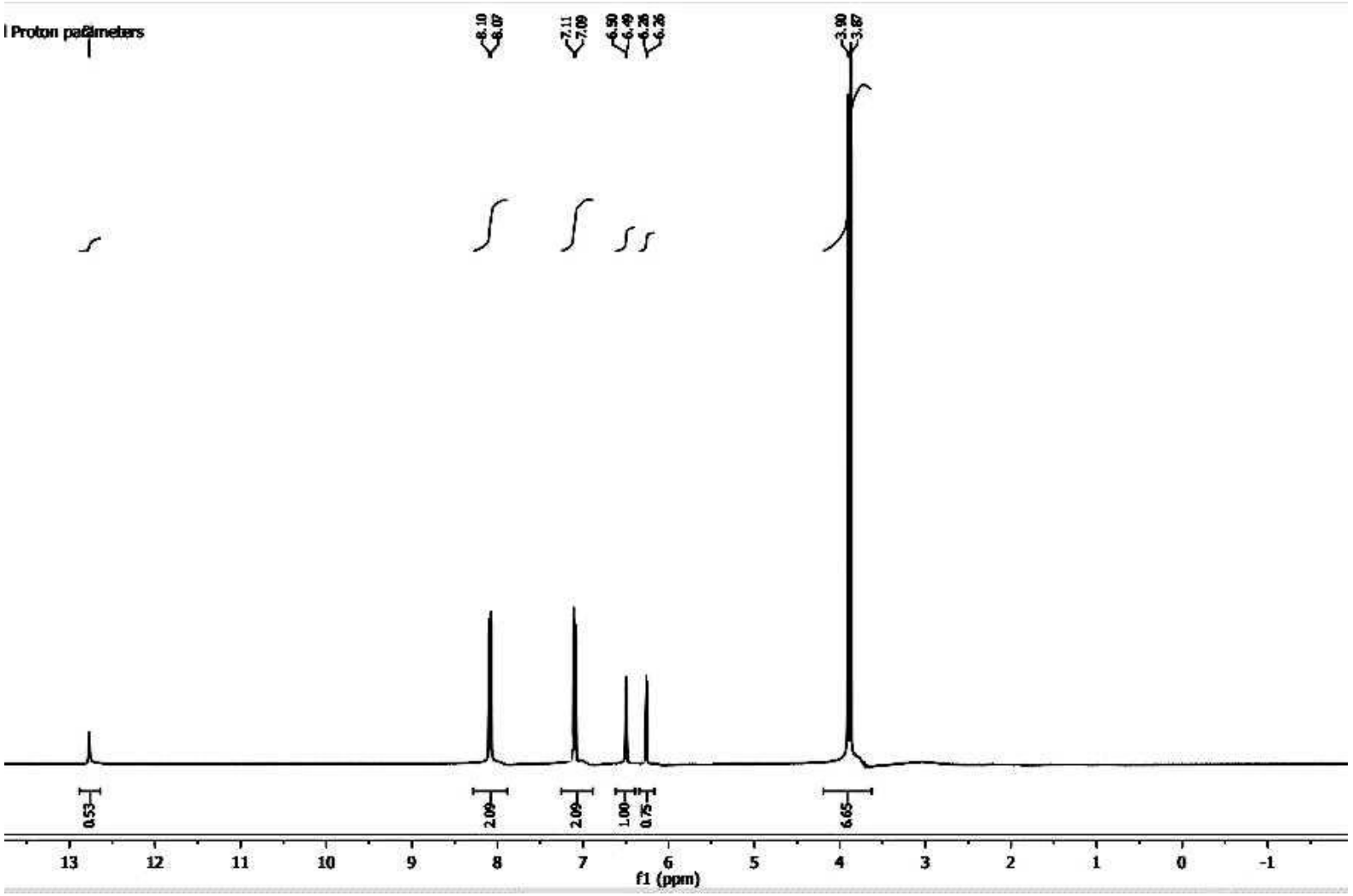


MASS SPECTRUM FOR COMPOUND 165

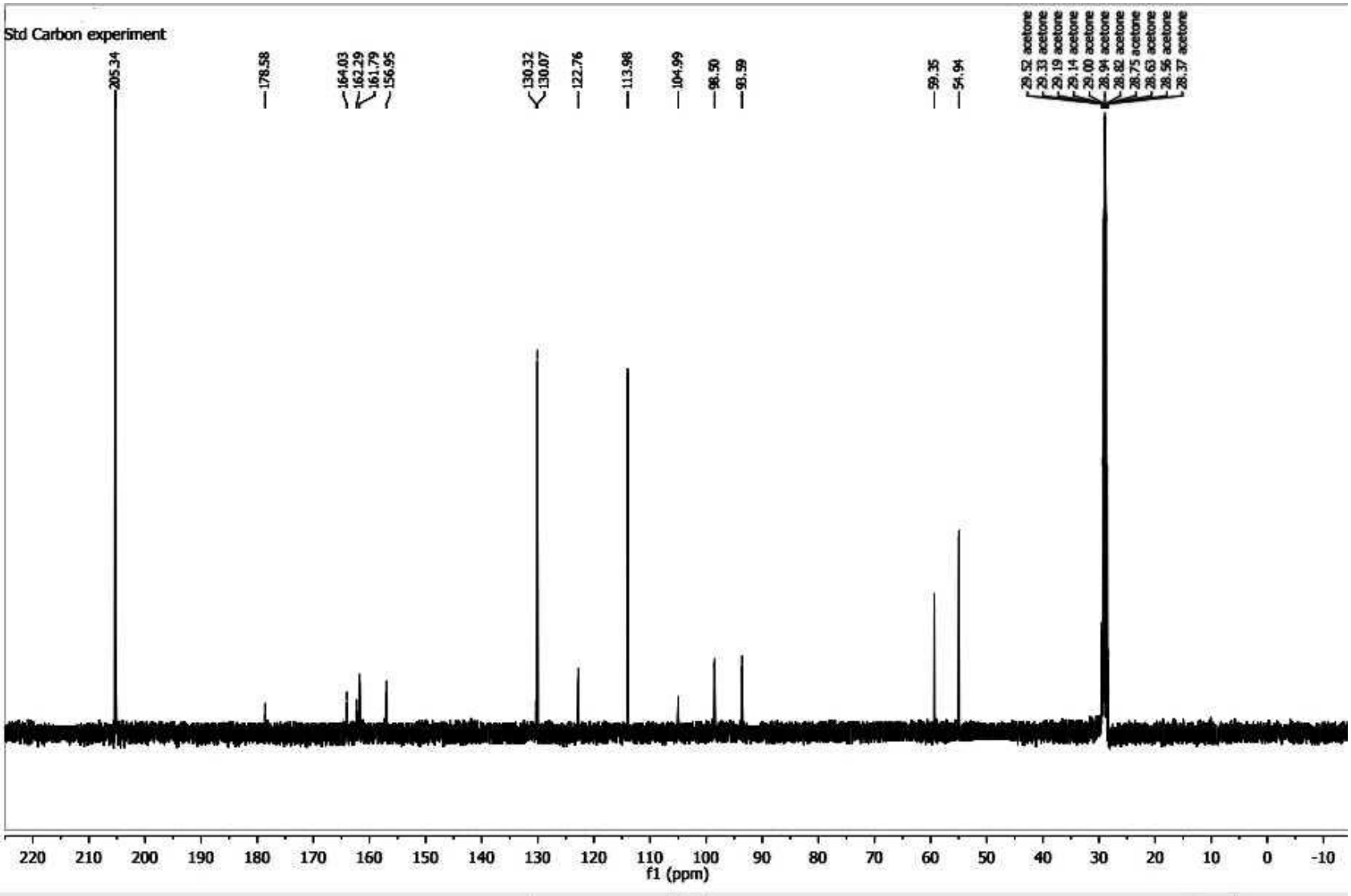


APPENDIX 3: SPECTRA FOR COMPOUND **166**

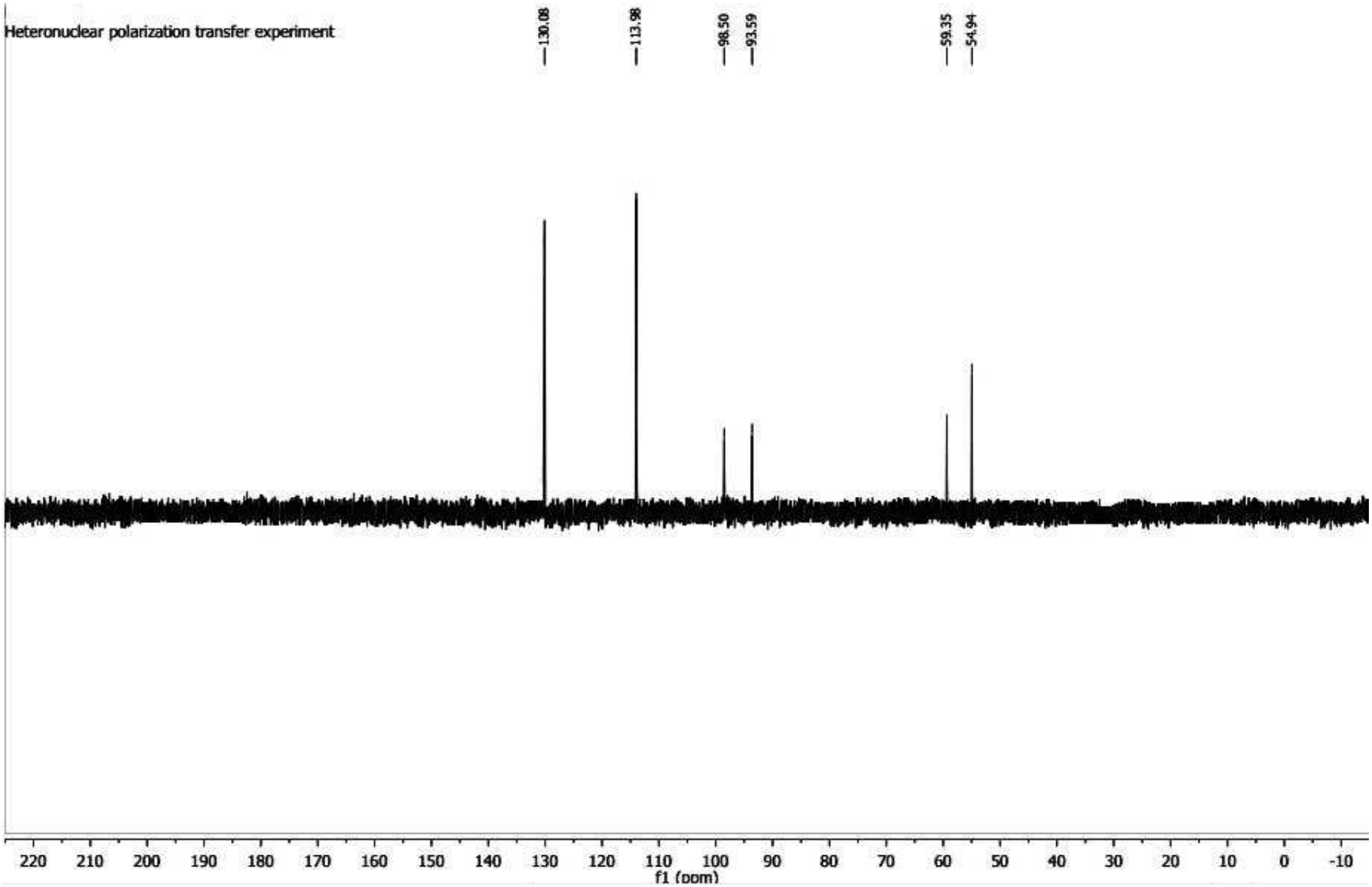
^{1}H-NMR SPECTRUM FOR COMPOUND -166



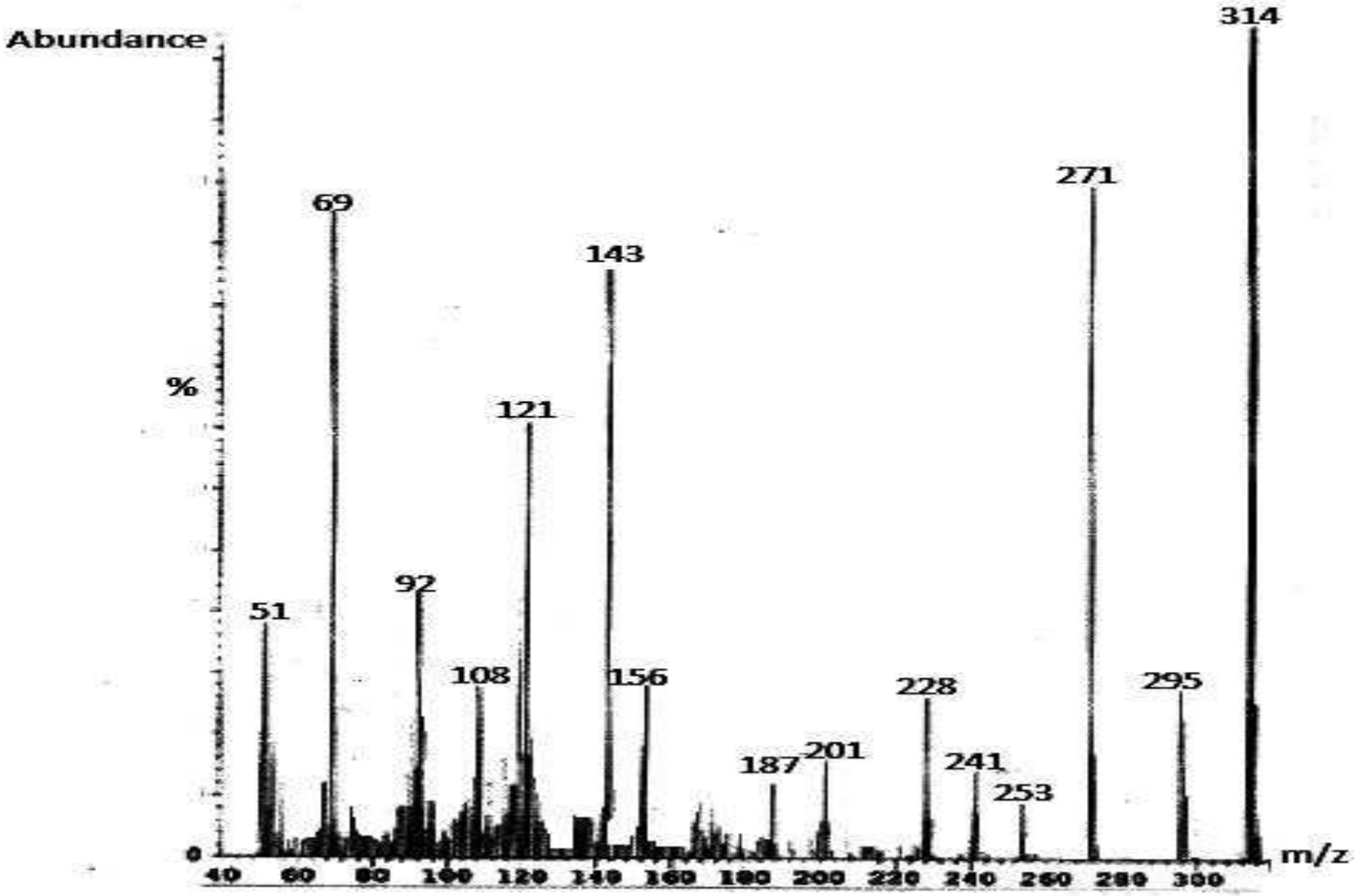
^{13}C-NMR SPECTRUM FOR COMPOUND 166



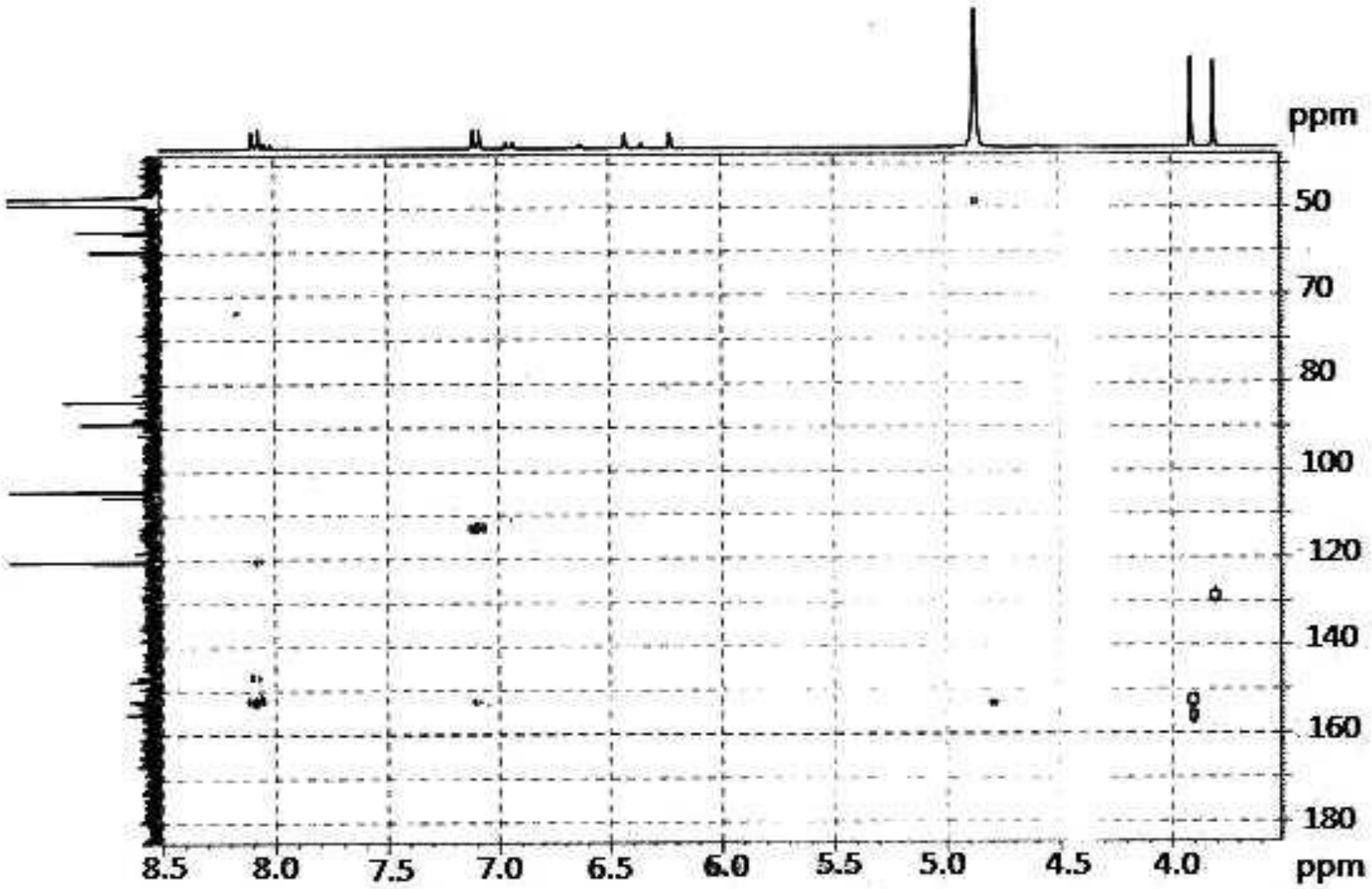
DEPT SPECTRUM FOR COMPOUND 166



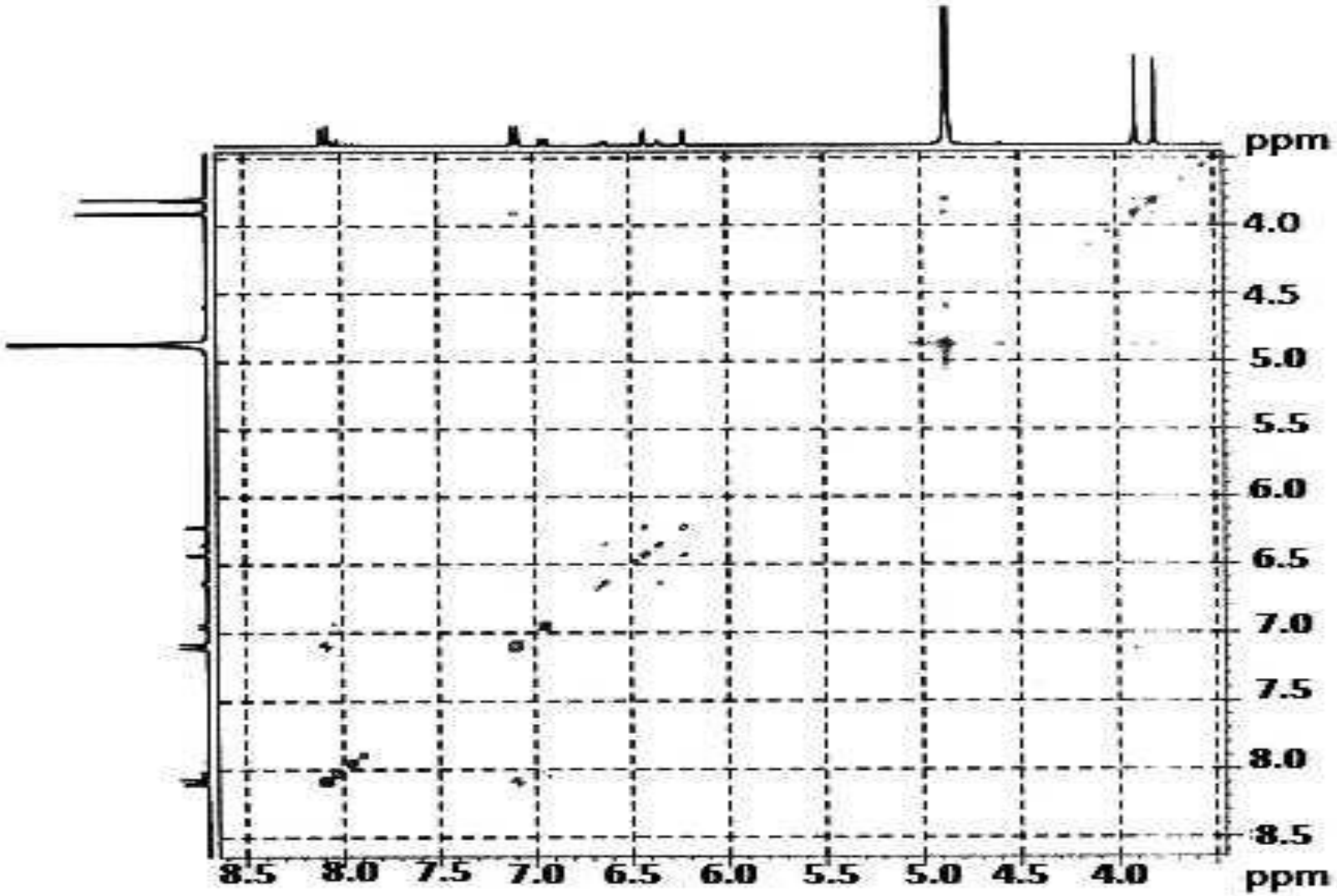
MASS SPECTRUM FOR COMPOUND 166



HMBC SPECTRUM FOR COMPOUND 166

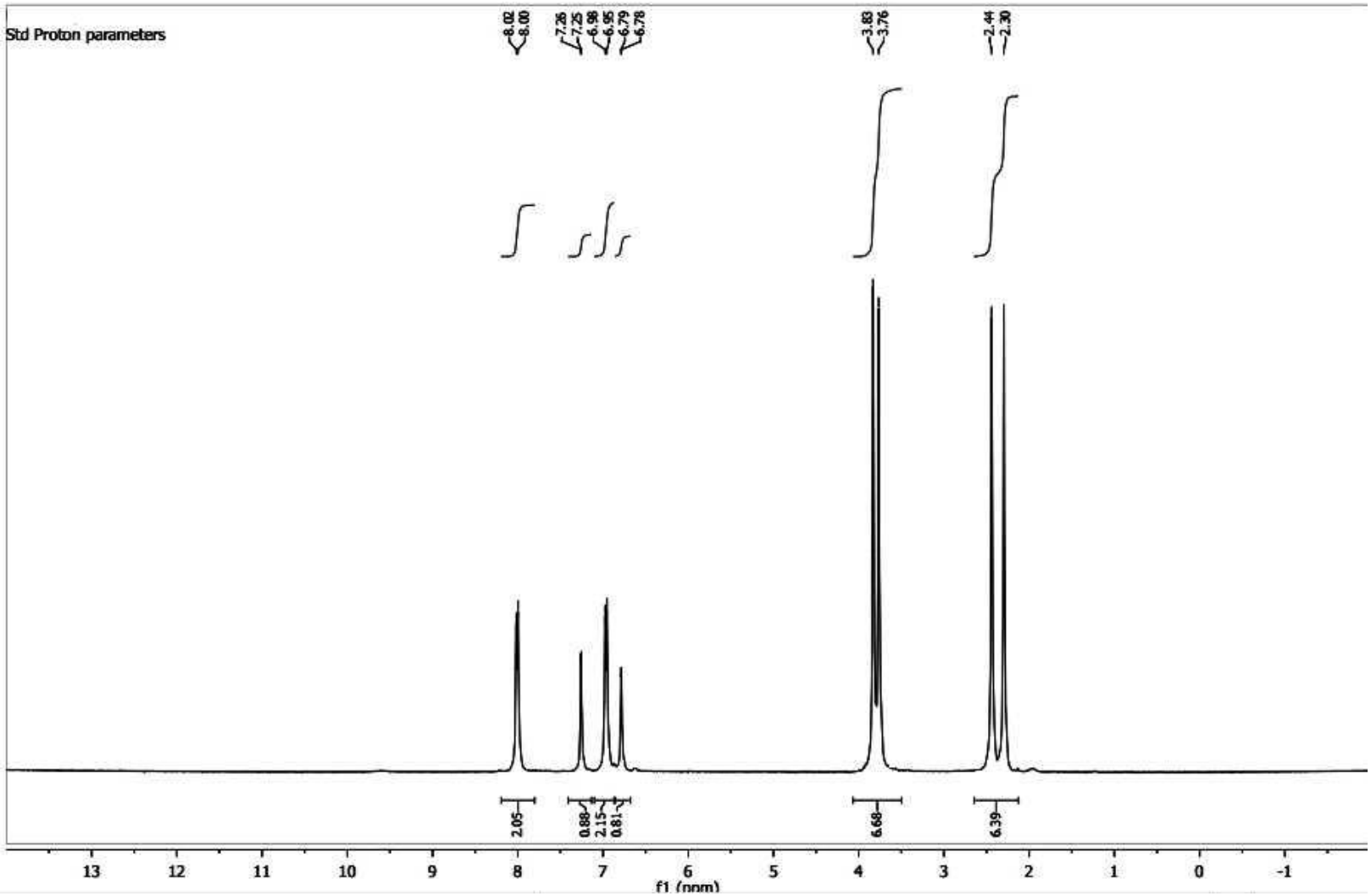


NOESY SPECTRUM FOR COMPOUND 166

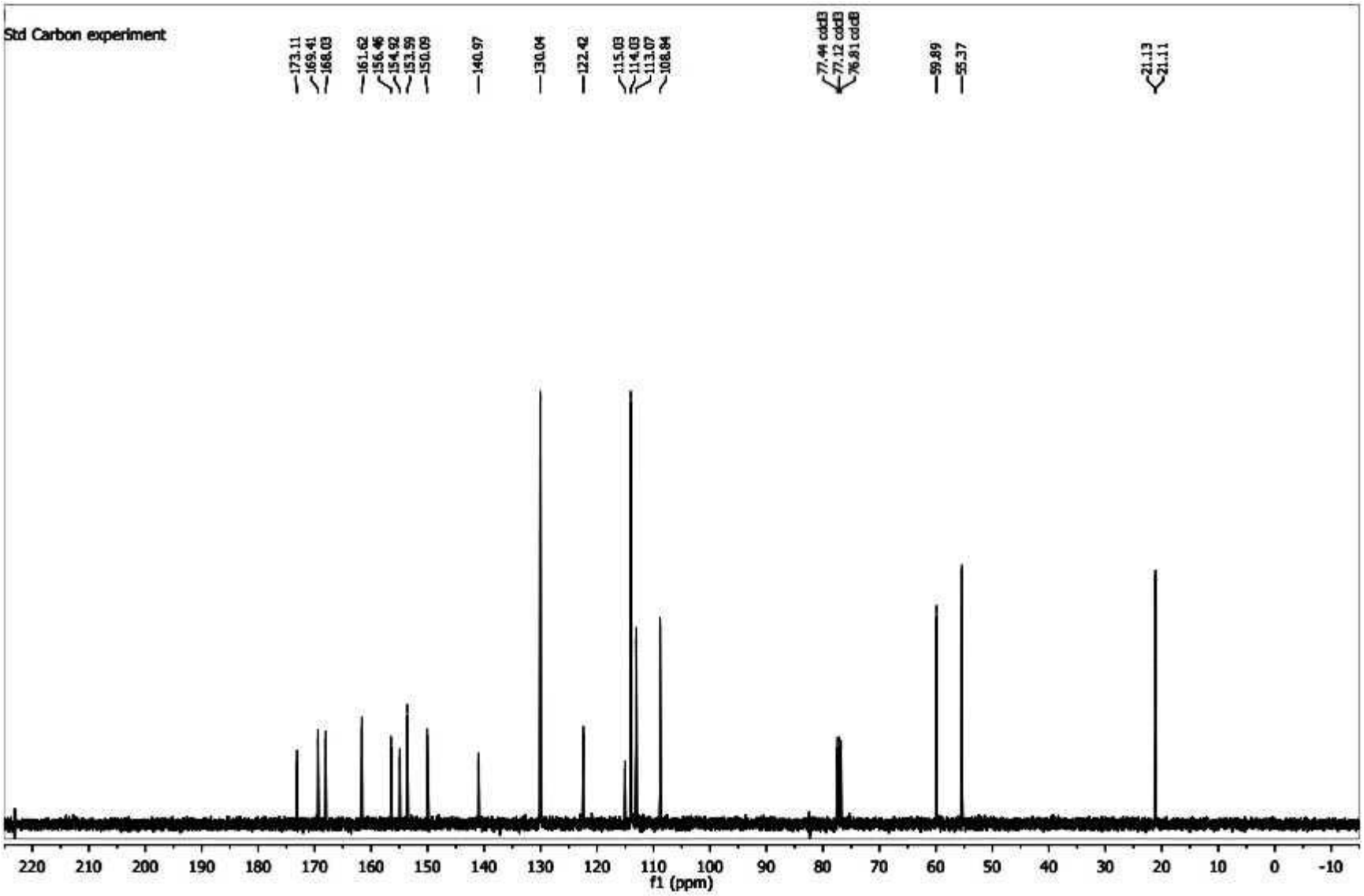


APPENDIX 4: SPECTRA FOR COMPOUND **167**

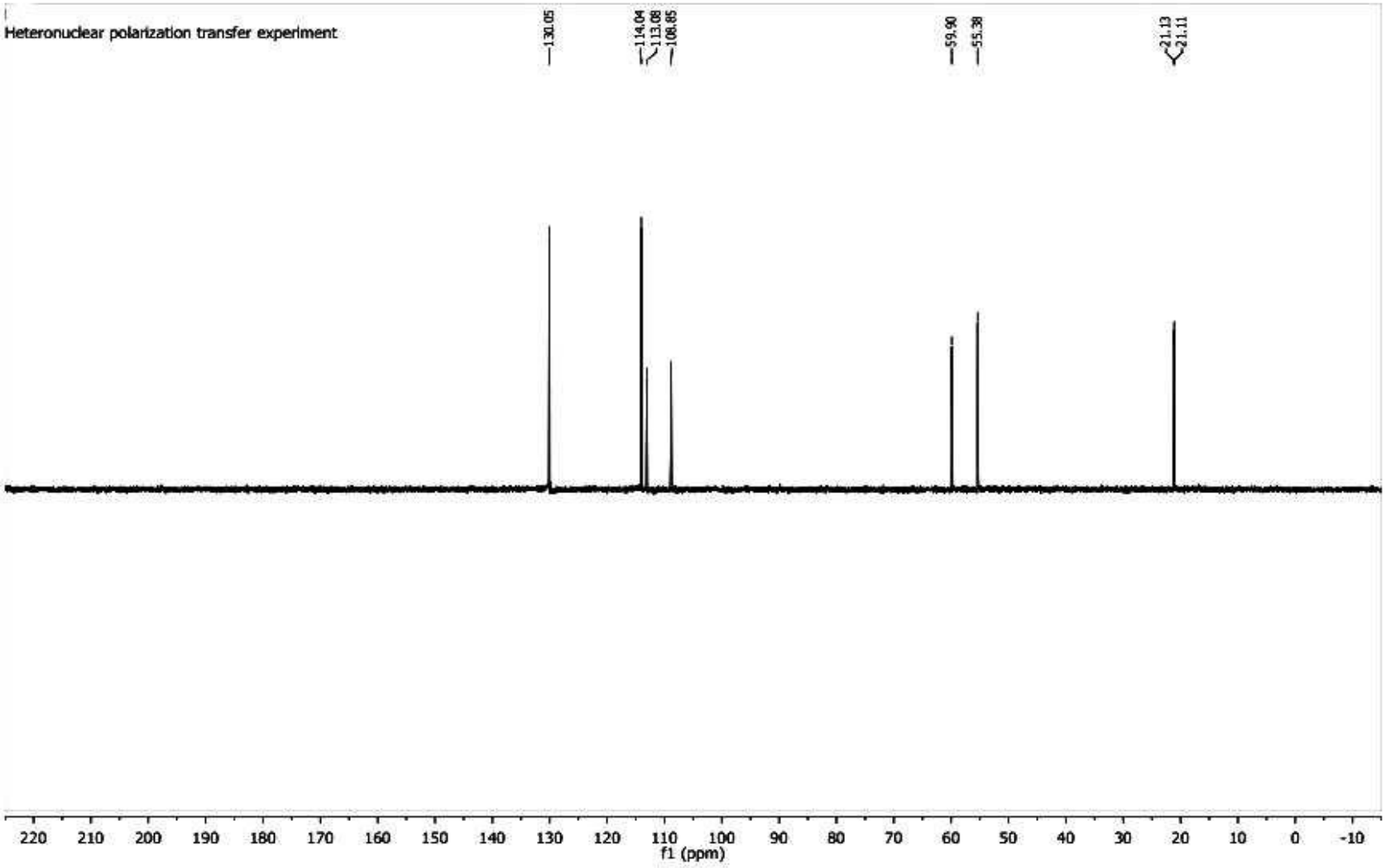
^{1}H-NMR SPECTRUM FOR COMPOUND 167



^{13}C-NMR SPECTRUM FOR COMPOUND 167

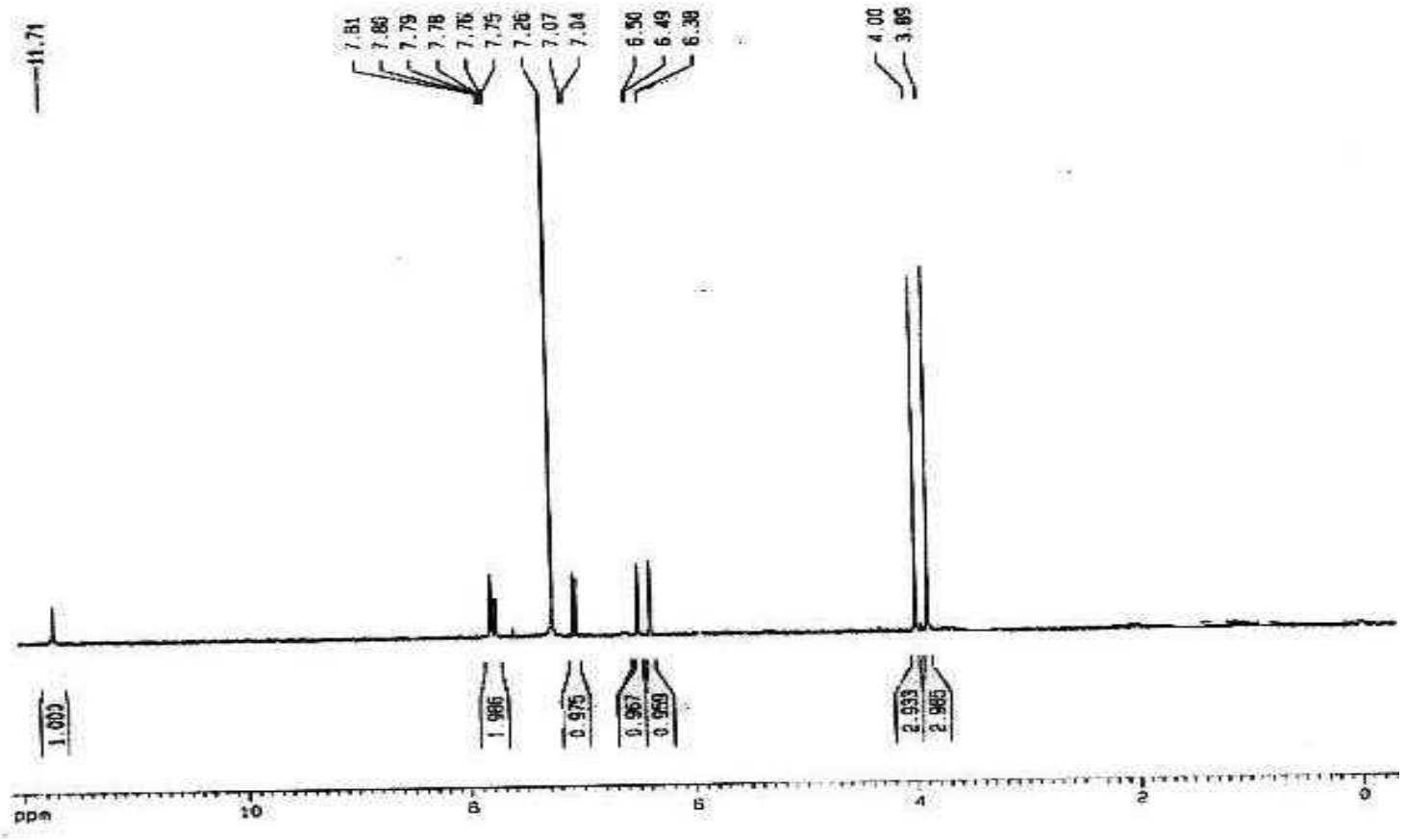


DEPT SPECTRUM FOR COMPOUND 167

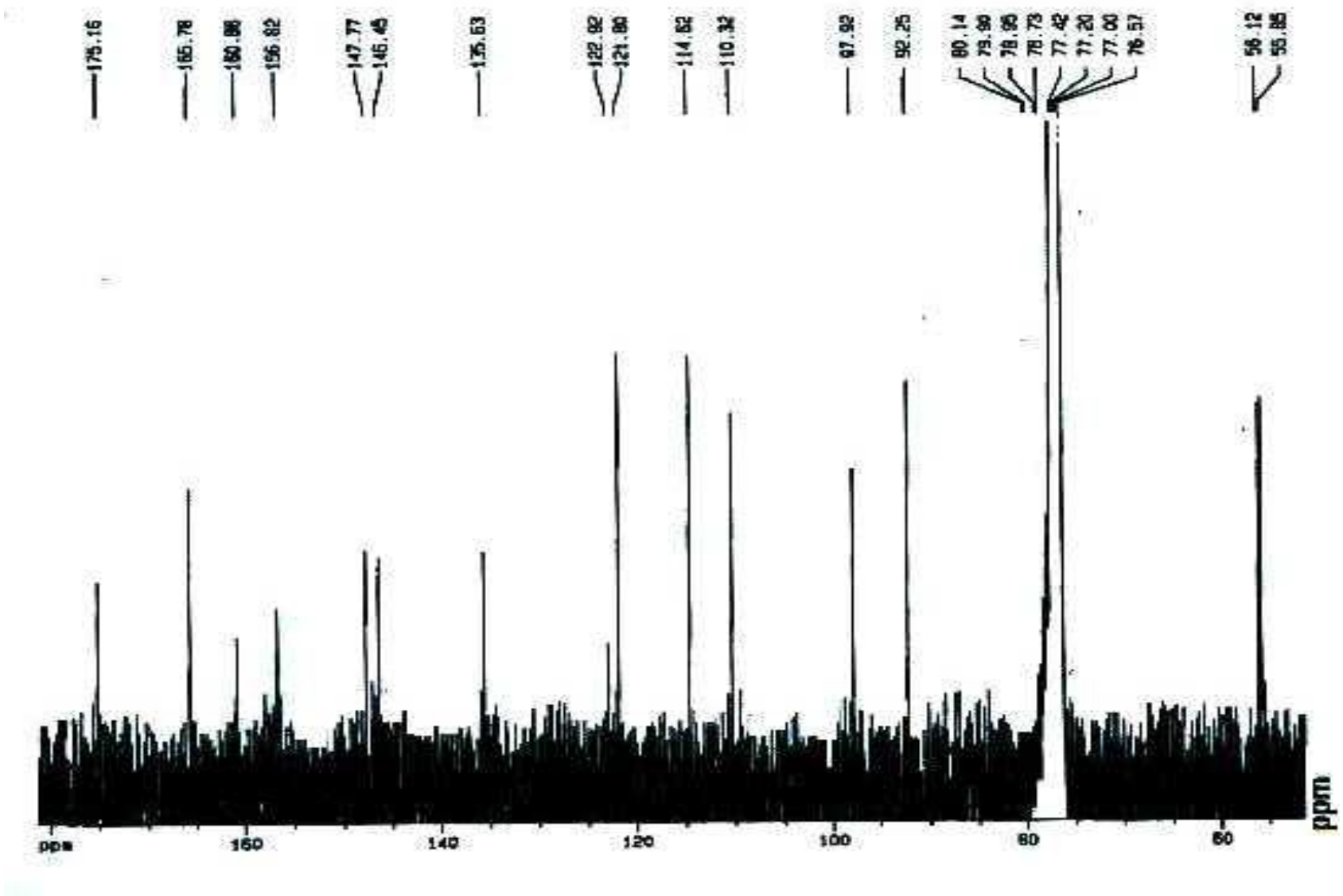


APPENDIX 5: SPECTRA FOR COMPOUND **168**

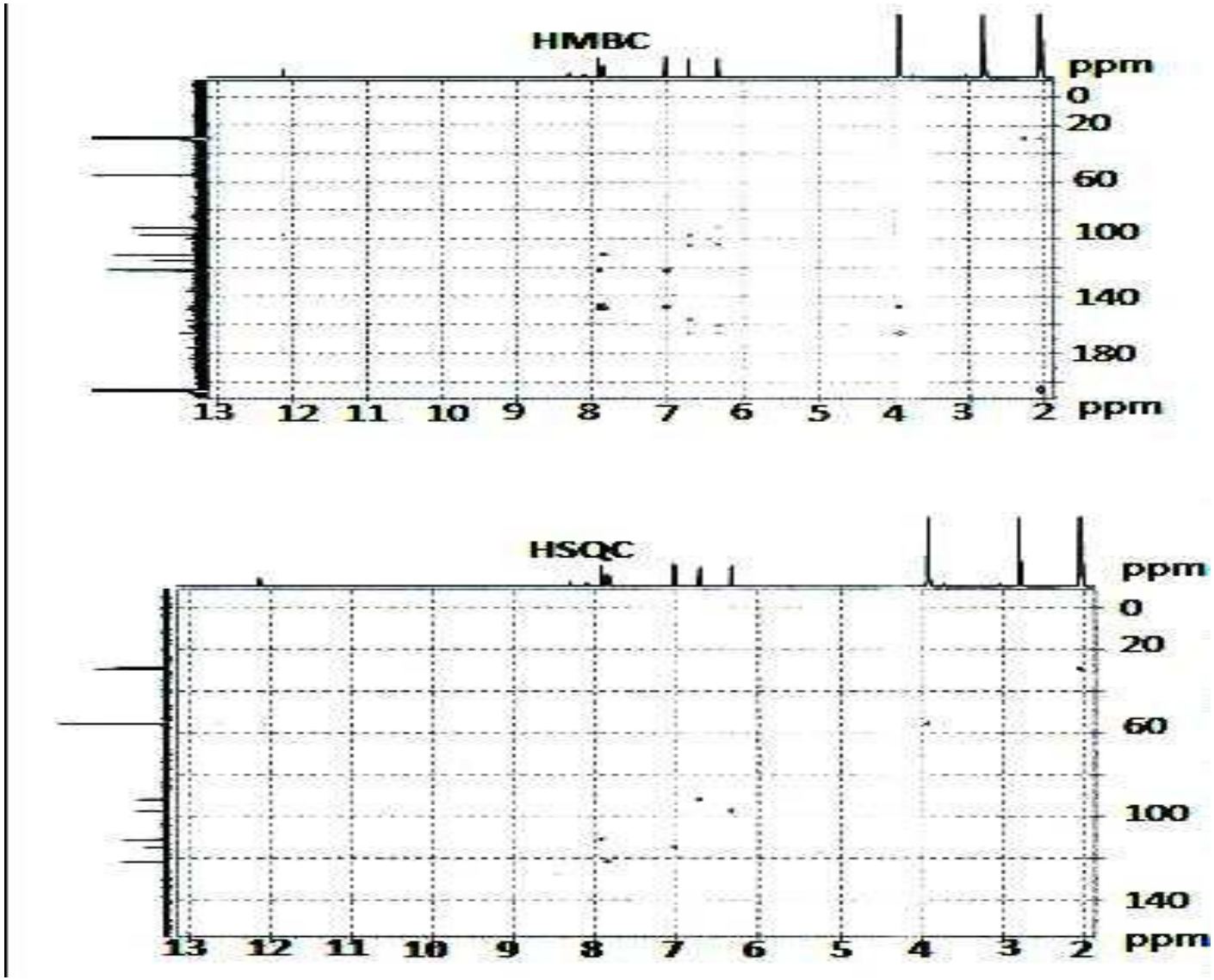
^{1}H-NMR SPECTRUM FOR COMPOUND 168



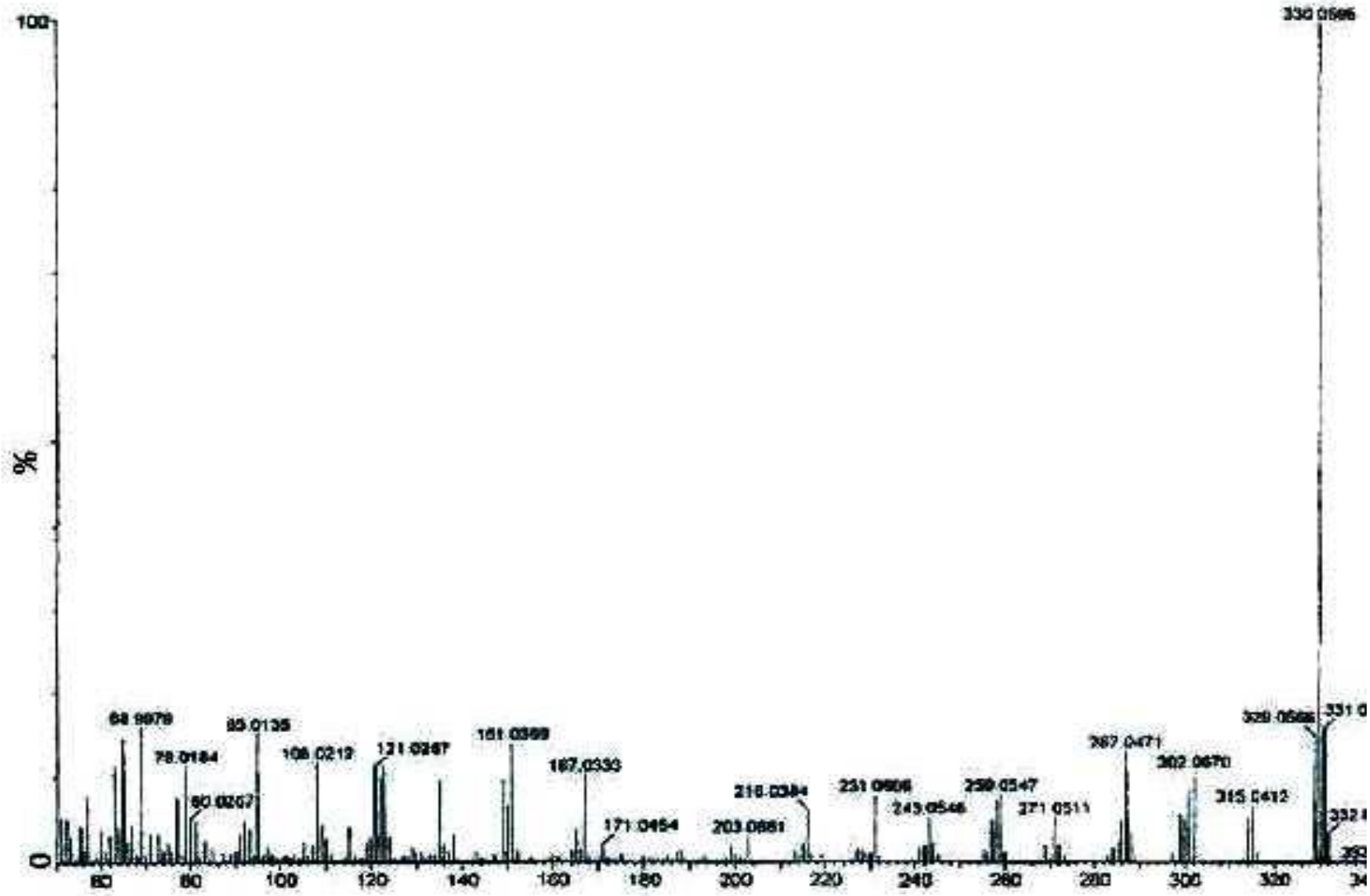
[13]CARBON SPECTRUM FOR COMPOUND168



HMQC AND HMBC SPECTRA FOR COMPOUND 168

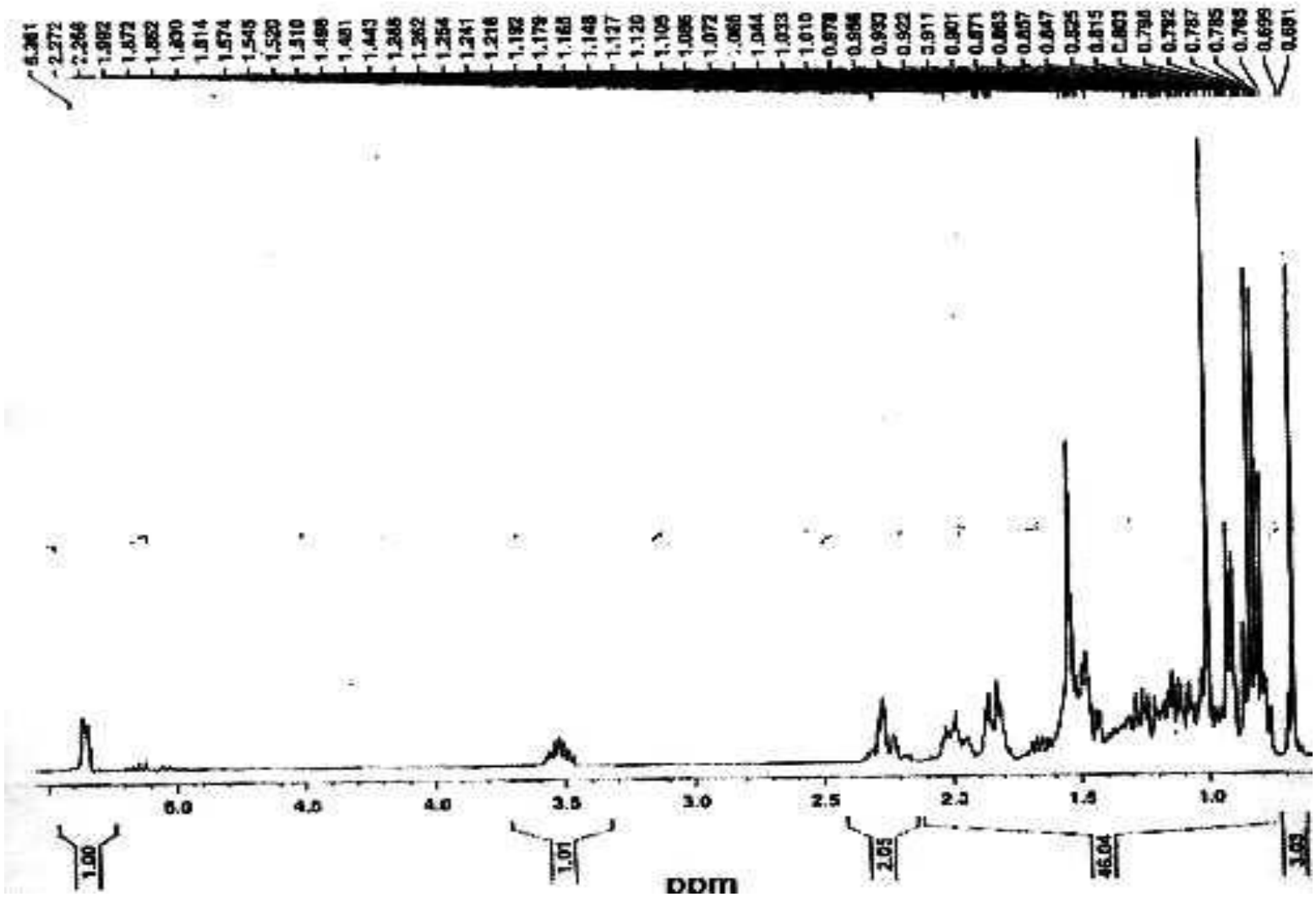


MASS SPECTRUM FOR COMPOUND 168

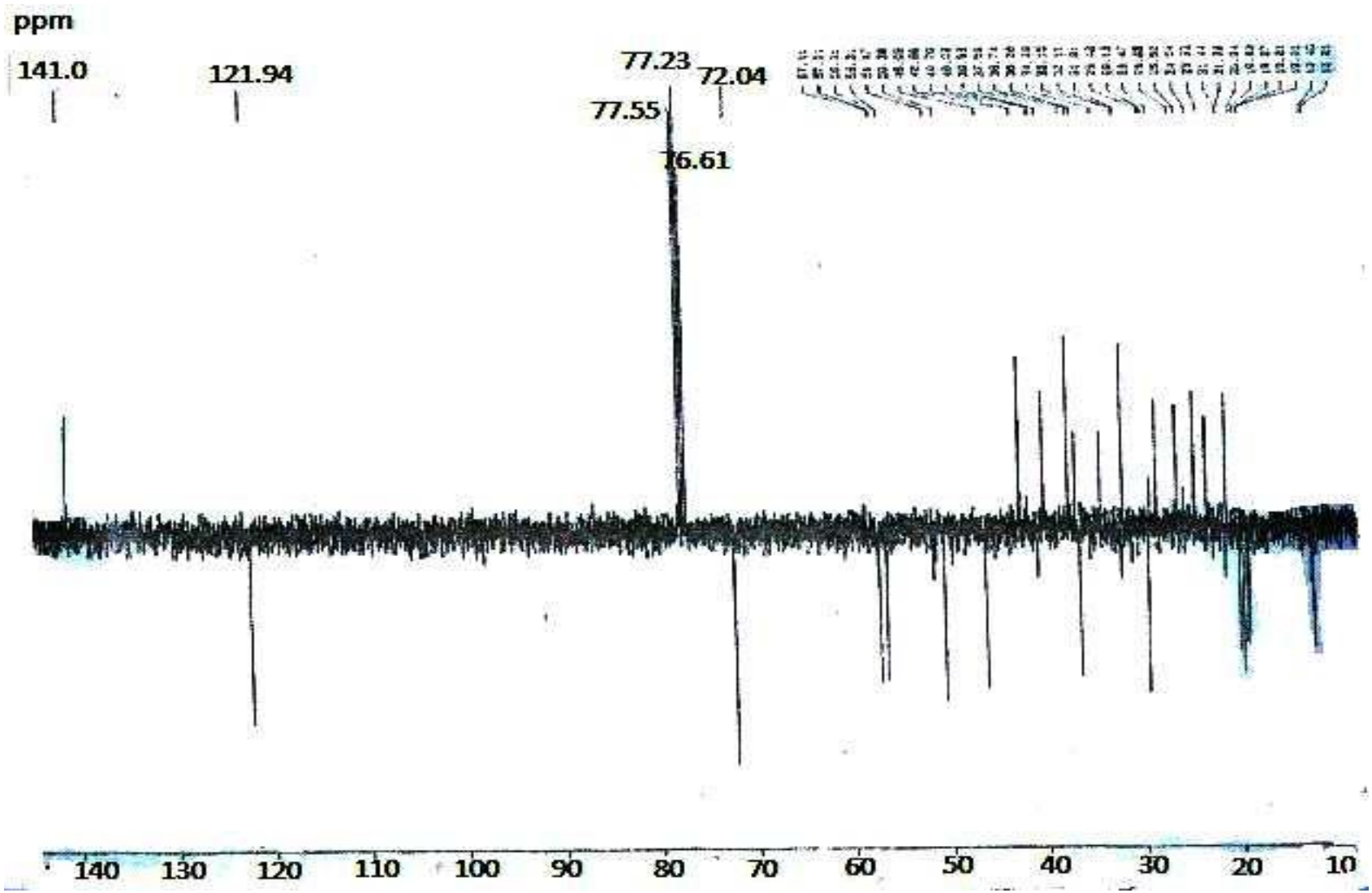


APPENDIX 6: SPECTRA FOR COMPOUND **169**

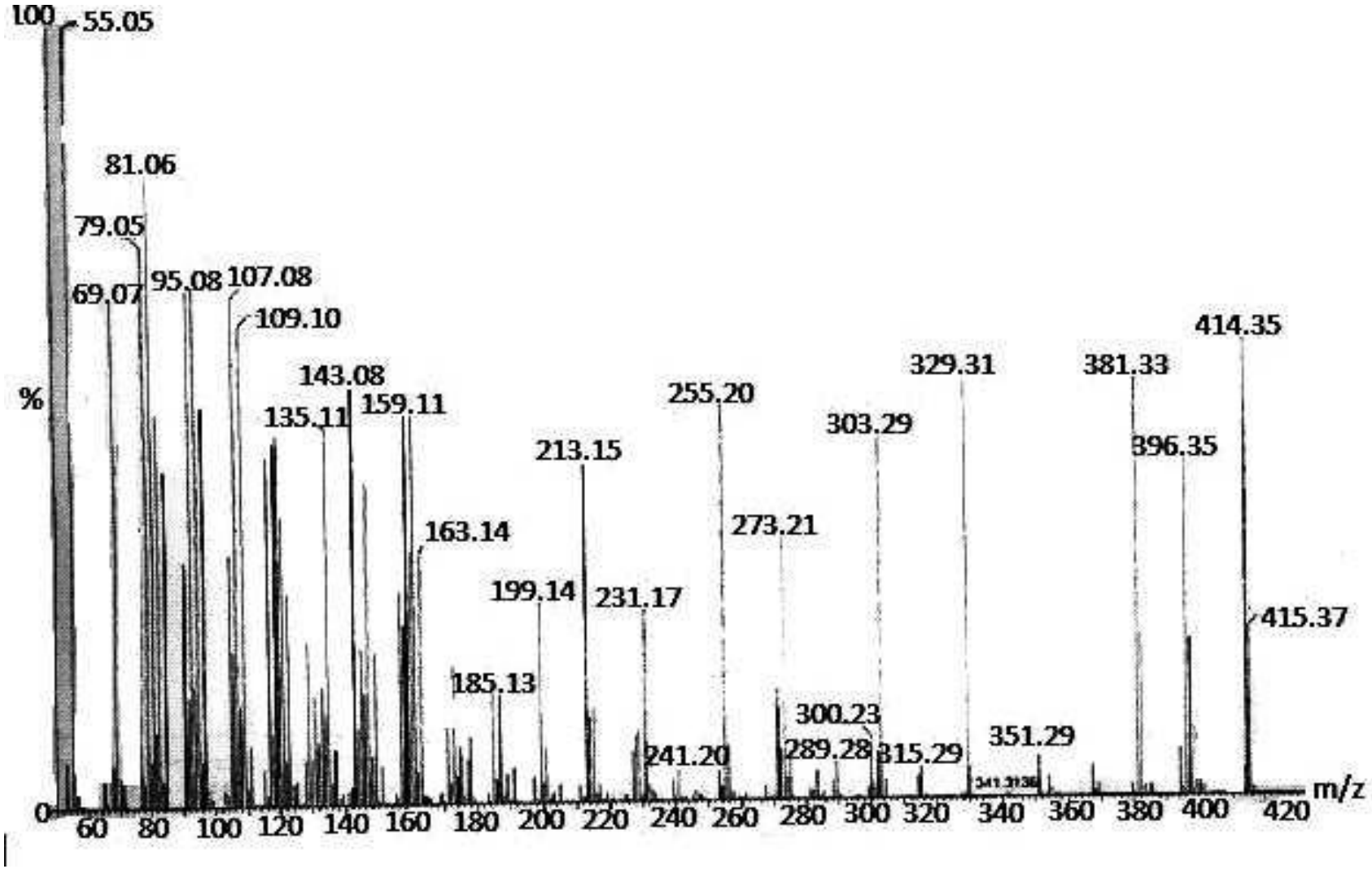
^{1}H-NMR FOR COMPOUND 169



^{13}C-NMR SPECTRUM FOR COMPOUND 169

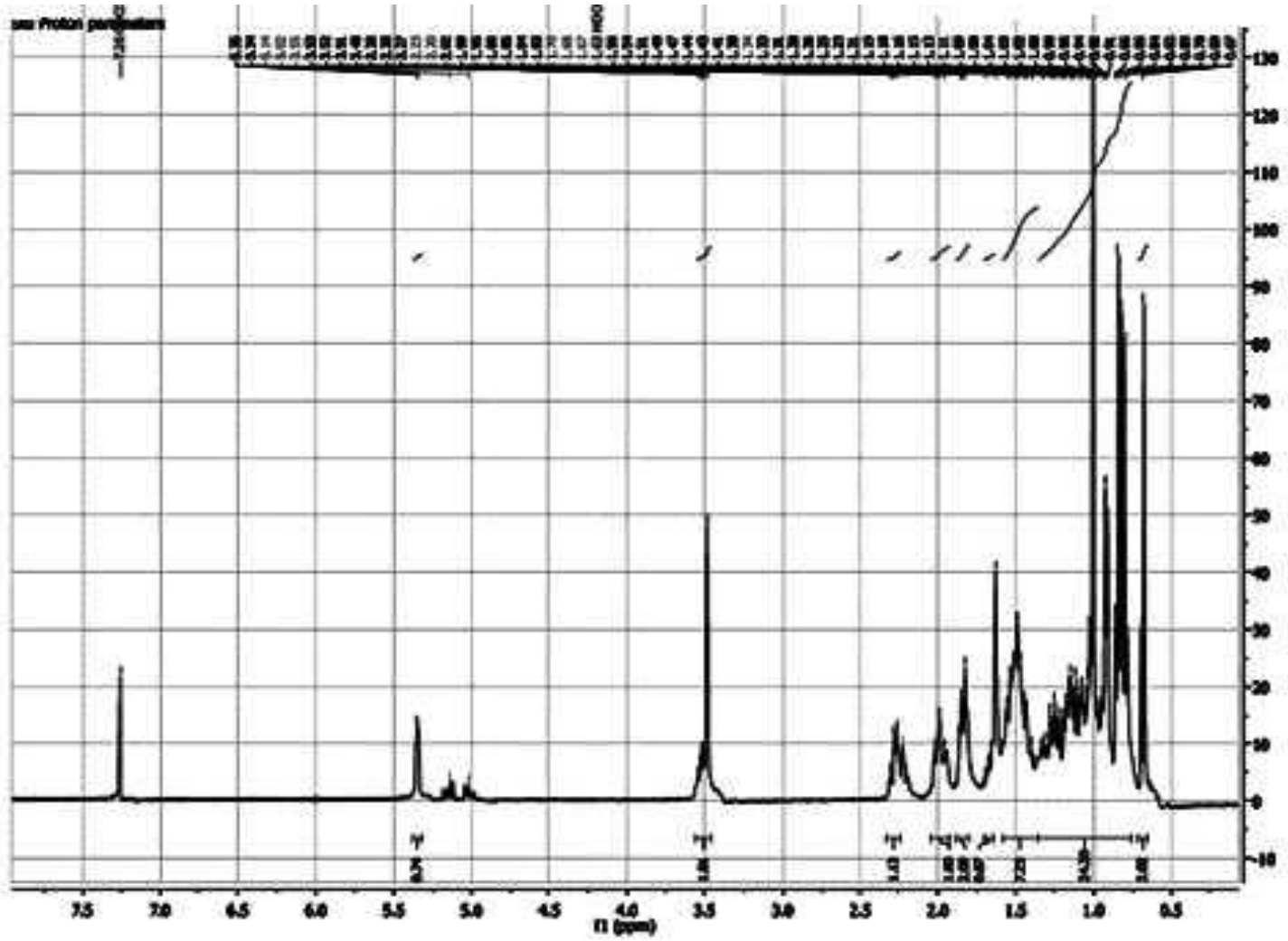


MASS SPECTRUM FOR COMPOUND 169

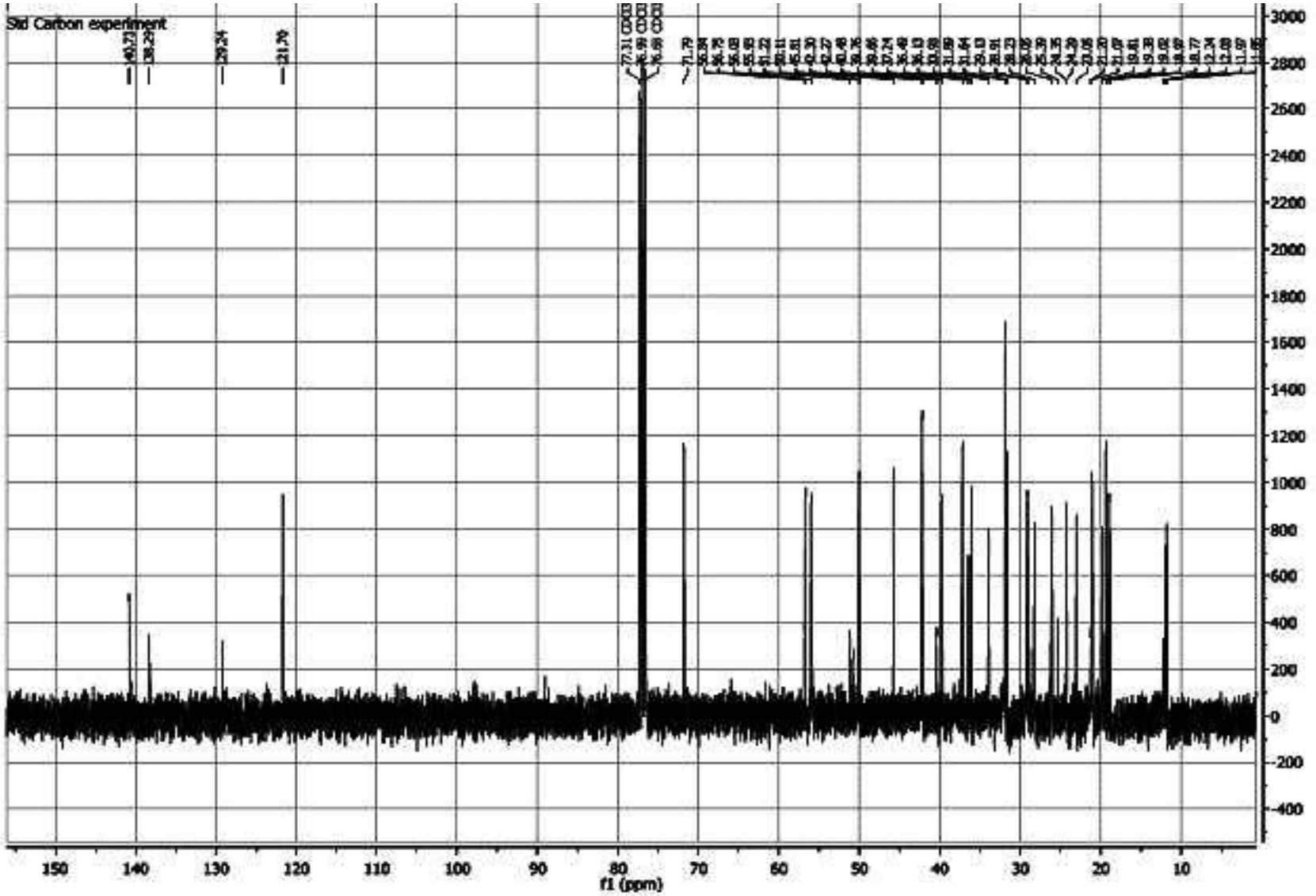


APPENDIX 7: SPECTRA FOR COMPOUND **170**

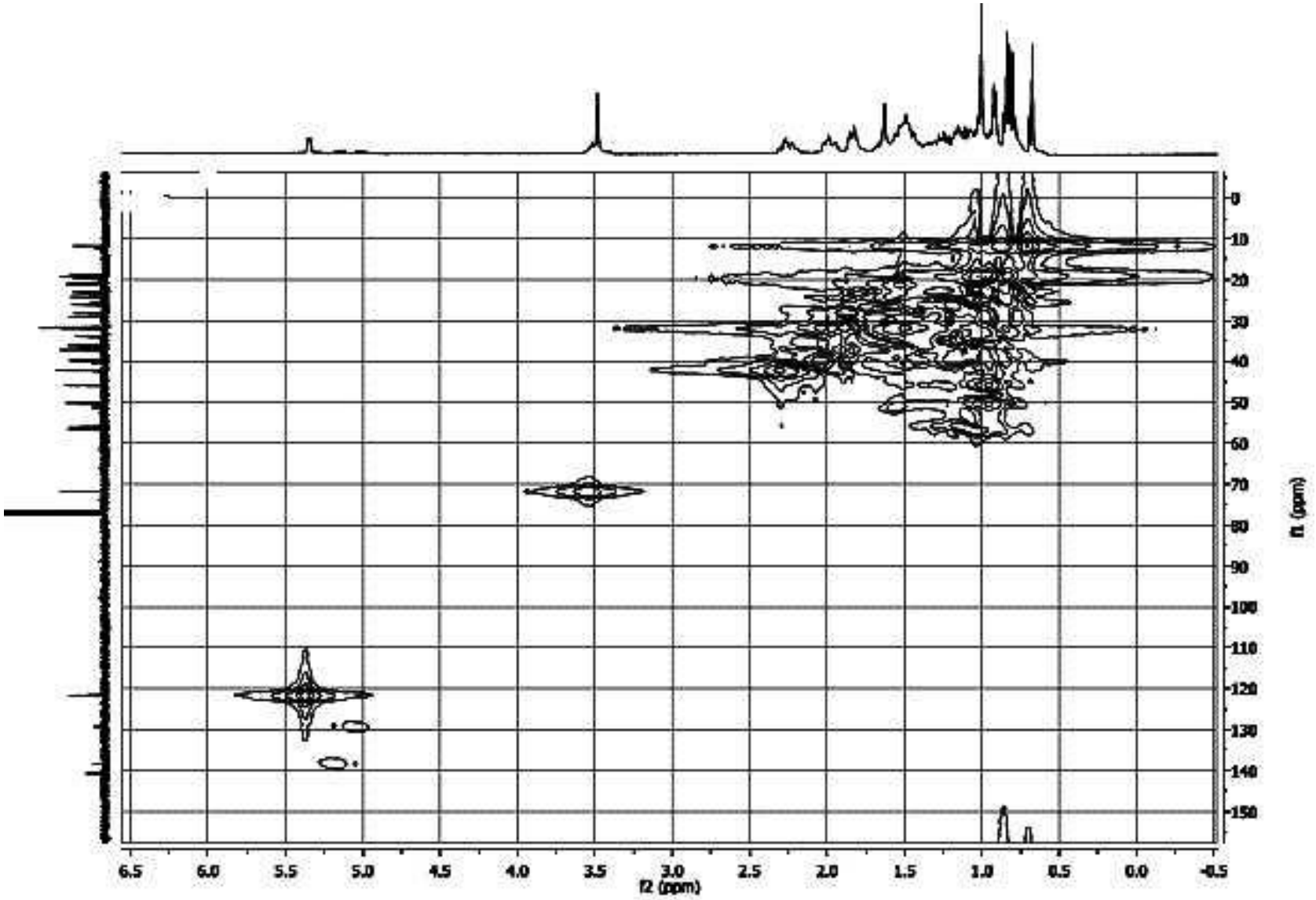
^{1}H-NMR FOR COMPOUND 170



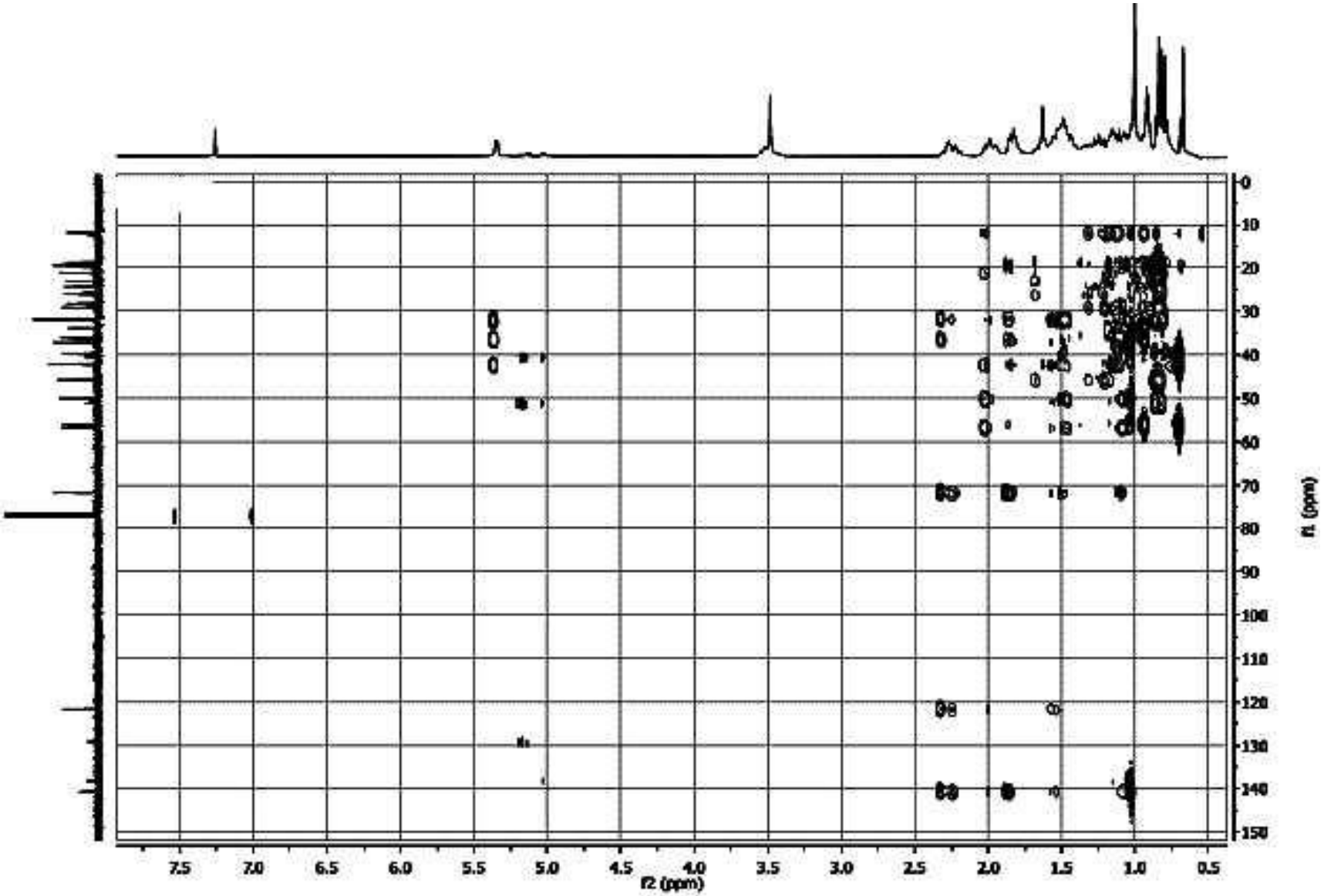
^{13}C-NMR SPECTRUM FOR COMPOUND 170



HMQC SPECTRUM FOR COMPOUND 170



HMBC SPECRUM FOR COMPOUND 170



MASS SPECTRUM FOR COMPOUND 170