# SCREENING FOR ANTIMICROBIAL COMPOUNDS IN *GARDENIA VOLKENSII* AND *MEYNA TETRAPHYLLA* (RUBIACEAE)

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**A Thesis Submitted to the Graduate School in partial fulfillment for the requirements of the Award of the Master of Science Degree in Chemistry of Egerton University**

**Egerton University**

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## DECLARATION AND RECOMMENDATION

### Declaration

I declare that this thesis is my original work and has not been submitted for an award in any university as by my knowledge.

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### Recommendation

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

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## DEDICATION

This thesis is dedicated to my late parents, my husband and our children.

## ACKNOWLEDGEMENT

It is with great pleasure that I extend my sincere thanks to all those who made this work a success, especially my supervisors Dr. P.K. Cheplogoi and Dr. E.M. Mwangi for their help, guidance and the constructive criticism they gave me throughout. My special thanks go to Dr. S.T. Kariuki of Botany Department for the identification of the plants, Prof. D.A Mulholland and Mr. M.K. Langat of University of Surrey, UK for both NMR and MS analysis and Ms. Esther Gicheru of Dairy department for the bioassay tests. Many thanks to the Chairman, all teaching and technical staff of Chemistry Department, Egerton University for their massive support, encouragement and great interest in my work which kept my morale high at every stage of this research. Special thanks to Research and Extension section, Egerton University for granting me the funds to accomplish this work. I would also like to thank all my friends and family members for their prayers and encouragement. Finally my special thanks to my beloved husband and our children for their moral support. Thank you for creating a favourable environment for the good performance and success of this work.

To you all I say God bless!

## COPYRIGHT

## ABSTRACT

In Africa, 80% of the population use traditional medicinal plants to treat common ailments like malaria, headache and pneumonia. This is due to the high cost of conventional drugs from developed countries. Examples of these medicinal plants include *Gardenia volkensii* and *Meyna tetraphylla* that are both used by the Pokots in Kenya to treat a variety of ailments including antimicrobial diseases. The aim of this project was to test for the antimicrobial activity of the crude extracts and isolated compounds in the above two plants. The plant samples were collected from Baringo District in Kenya. The air dried and ground plant material of *G. volkensii* fruit seeds (418 g) was sequentially extracted using hexane, dichloromethane and methanol to give 8.00 g; 14.36 g and 10.85 g of extract. The *G. volkensii* fruit covers (408.5 g) yielded 3.68 g, 6.04 g and 7.41 g of hexane, dichloromethane and methanol extracts. The sequential extraction of *M. tetraphylla* leaves (636 g) yielded 7.38 g, 11.84 g and 9.55 g of hexane, dichloromethane and methanol extracts. The *G. volkensii* stem bark (10.22 g), leaves (9.34 g) and the *M. tetraphylla* root bark (20.47 g) were extracted using methanol only. The crude extracts for each solvent was screened for antimicrobial activity. Exactly 20-40 µL of 10,000 mg/L solution of extract was spiked and its antimicrobial activities on ***Bsb***, ***SA, ST, CA*** and ***EC*** studied. The crude dichloromethane fractions from both plants were purified by step gradient isolation (dichloromethane/methanol) followed by repeated column chromatography (ethyl acetate/hexane). This gave **GV1** and **GV2** from *G. volkensii*. **GV1** (65.10 mg) was a mixture while **GV2** (34 80 mg) was pure. *M. tetraphylla* gave **MT1** (164 mg), **MT2** (48 mg), **MT3** (63 mg), **MT4** (72.20 mg) and **MT5** (88.80 mg) pure compounds after repeated column chromatography (dichloromethane/methanol). **GV2** (monoterpenoid or modified iridoid) was a novel compound. **GV1** showed antimicrobial activity on ***SA*** and ***EC*** while **GV2** showed activity on ***EC***. **MT1** and **MT5** showed activity on ***EC***, **MT2** on ***Bsb*** and ***EC***, **MT3** on ***Bsb*** while **MT4** showed no activity. There was no activity on ***CA*** and ***ST*** for all the crude extracts and pure compounds. Methanol was used as the negative control for the pure compounds while some selected antibiotics (Amoxicillin®, Tetracycline®, Doxycycline®, and Septrin®) were used as positive controls. The $\mathbf{IC_{50}}$ for *G. volkensii* stem bark and *M. tetraphylla* root bark, the most active crude extracts on *Bacillus subtilis* were determined using probit analysis (graphpad prism) and compared with the $\mathbf{IC_{50}}$ for Doxycycline® antibiotic which was 0.335 mg/mL. The $\mathbf{IC_{50}}$ for *G. volkensii* stem bark was 1166.809 mg/mL while for *M. tetraphylla* root bark was 699.842 mg/ml, 3,483 times and 2,089 times less active as compared to the Doxycycline® antibiotic respectively. This is an indication that, both plants can be used to cure microbial diseases though a higher dose is needed. Further research on their toxicity was recommended.

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## LIST OF ACRONYMS

| | |
|---|---|
| *Bsb* | *Bacillus subtilis* |
| *CA* | *Candida albican* |
| $^{13}C$ NMR | Carbon-13 Nuclear Magnetic Resonance |
| $CH_2Cl_2$ | Dichloromethane |
| $CH_3COOCH_2CH_3$ | Ethylacetate |
| $CH_3(CH_2)_4CH_3$ | Hexane |
| $CH_3OH$ | Methanol |
| COSY | Correlated Spectroscopy |
| *d* | doublet |
| DEPT | Distortionless Enhancement by Polarization Transfer |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| *EC* | *Escherichia coli* |
| FTIR | Fourier Transform Infrared Spectroscopy |
| GC | Gas Chromatography |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| HMBC | Heteronuclear Multiple Bond Connectivities |
| HSQC | Heteronuclear Single Quantum Correlation |
| HPLC | High Performance Liquid Chromatography |
| $^{1}H$ NMR | Proton Nuclear Magnetic Resonance |
| $IC_{50}$ | Inhibition Concentration that reduces the effect of microorganisms by 50% |
| IR | Infra Red Spectroscopy |
| LC | Lowest Concentration |
| *m* | multiplet |
| MIC | Minimum Inhibitory Concentration |
| MS | Mass Spectrometry |
| NMR | Nuclear Magnetic Resonance |
| NOESY | Nuclear Overhauser and Exchange Spectroscopy |
| ppm | Parts per million |
| $R_f$ | Retardation factor |

| | |
|---|---|
| *s* | singlet |
| *SA* | *Staphylococcus aureus* |
| *ST* | *Salmonella typhimurium* |
| *t* | triplet |
| TLC | Thin Layer Chromatography |
| TOF-MS | Time-of-Flight Mass Spectrometer |
| UV | Ultra Violet Spectroscopy |
| Glu | Glucose |

# CHAPTER ONE

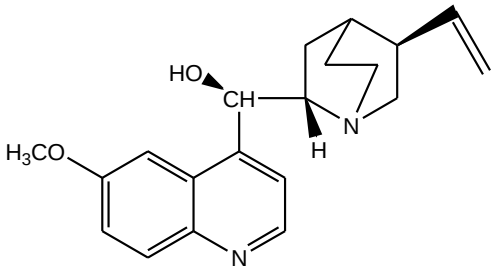
## INTRODUCTION

### 1.1. Background information

Plants are living things and share the characteristics, which we recognize as life with other living things. The earth is a plant-oriented planet and the green plant is fundamental to all other life. Were man to perish tomorrow, vines would destroy his mighty temples and grass would soon grow in the main streets of the world. This means the disappearance of man along with every other animal (Forbes and Watson, 1992).

Plants are extremely important in the lives of people throughout the world and many people depend on them to satisfy basic human needs such as food, clothing, shelter and health care. They provide all our food, either directly or indirectly (as feed for animals) and insufficient food leads to intolerable consequences (Forbes and Watson, 1992; Jules, 1974). They also provide fibres such as cotton and flax, from which much of our clothing and other woven fabrics are made. They give wood, one of our most important construction and furnishing material and still the main fuel supply of most of the world's population (Addae-Mensah, 1992). They also provide many other complex substances like dyes, tannins, waxes, resins, rubber, scents, flavours, medicines, and other drugs (MacMillan, 2006).

Many higher plants have been the source of medical agents since the earliest times and today they continue to play a dominant role in the primary health care of about 80% of the world's population (Addae-Mensah, 1992). Medicinal agents derived from plants are also an essential feature in the health care system of the remaining 20% of the population residing mainly in developing countries. Of the worlds twenty-five best selling pharmaceutical agents, twelve are derived from natural products, which continue to play an important role in drug discovery programs of the pharmaceutical industry and other research organizations. An example is *Cinchona* species whose bark was used 400 years ago to reduce fever and is still used today to make quinine **(1)**, a drug used to treat malaria and other diseases (Akerele, 1991).




Quinine **(1)**

Without plants, most medicines we take would not exist. Over 40% of medicines now prescribed in the U.S.A contain chemicals derived from plants. Historically, plant medicines were discovered by trial and error. Our ancestors noticed that aches and pains went away when they drank tea made from the bark of a willow tree. Later, scientists found that the willow bark contained salicylic acid, the active ingredient in aspirin® and this process continues even today. Throughout the world, including the wild places in the U.S.A, botanists and chemists search the plant kingdom for new medicines. For example, the native Pacific yew was burned as trash generated by logging operations in the Pacific Northwest. In 1975, a substance in its bark, taxol, was found to reduce the production of cancerous tumours (Facchini *et al*., 2000).

A comprehensive search of known plants for medicinal chemicals is an enormous task. Of the estimated 250,000 plant species on earth, only 2% have been thoroughly screened for chemicals with potential medicinal use. Many native plant habitats are destroyed almost daily and therefore many medicinally valuable plants will be gone before scientists can investigate them (Facchini *et al*., 2000).

People in Africa, Asia, North and South America, Australia and New Zealand have used concoctions prepared from a wide range of medicinal plants for treating the sick. The information on which plant and what part of the plant cures what disease was passed on from generation to generation. This rich heritage of traditional medicinal practices was looked down upon following the slicing of third World countries into fragmented pockets with European spheres of influence. It was branded as primitive although many pharmaceutical drugs and medicinal syrups administered to patients in modern hospitals are of plant origin (De Sa' Ferreira and Ferrao, 1999).

In Africa, up to 60% of the population consult one of an estimated 200,000 traditional healers (Van Wyk *et al*., 1997), especially in rural areas where these healers are more numerous and accessible than allopathic physicians. Although plant extracts have been used in the treatment of diseases, according to knowledge accumulated over centuries, scientific research has shown some secondary metabolites present in these medicinal plants to be potentially toxic and carcinogenic, thus care should be taken before use (De Sa' Ferraira and Ferrao, 1999). Secondary metabolites are molecules that are not necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. They are often involved in key interactions between plants and their abiotic and biotic environments that influence them (Facchini *et al*., 2000).

The Rubiaceae family comprises of about 637 genera and 10,700 species (Mongrand *et al*., 2005). This family is mostly used to treat malaria, headaches, asthma, epilepsy, sore eyes and as an emetic in many developing countries. *Mitragyna inermis* is used in Mali to treat malaria and fever. *Gardenia saxatilis* is used in northeastern part of Thailand to externally relieve pain and paralysis of limbs. *Gardenia volkesii* fruits and *Meyna tetraphylla* leaves and roots are used by the Pokots in Kenya to treat malaria, fever, headaches, and infected hooves of goats and camels (Catherine, 1998).

**1.2 Statement of the problem**

*Gardenia volkensii* and *Meyna tetraphylla* are used by the Pokots in Kenya to treat malaria and microbial diseases. There is little knowledge about their composition and efficacy. Studies done in Botswana showed that the compounds isolated from *Gardenia volkensii* have some biological activity. This project aimed to validate the antimicrobial activity of *Gardenia volkensii* in Kenya and on *Meyna tetraphylla.*

## 1.3 Objectives

### 1.3.1 General objective

To isolate and characterize antimicrobial compounds that are in *Gardenia volkensii* fruits and *Meyna tetrapylla* leaves.

### 1.3.2 Specific objectives

1. To extract the fruits, leaves and stem bark of *Gardenia volkensii*
2. To extract the leaves and root bark of *Meyna tetraphylla*.
3. To test for antimicrobial activities in crude extracts.
4. To screen pure compounds of *Gardenia volkensii* fruits and *Meyna tetraphylla* leaves for antimicrobial activity.
5. To elucidate the structures of the pure compounds using Mass Spectrometer (MS) and Nuclear Magnetic Resonance spectrometer (NMR).

## 1.4 Hypothesis

1. There are compounds in *Gardenia volkensii* fruits, leaves and stem bark.
2. There are compounds in *Meyna tetraphylla* leaves and root bark.
3. The antimicrobial activity of the crude extracts from *Gardenia volkensii* and *Meyna tetraphylla* is well known.
4. The compounds in *Gardenia volkensii* fruits and *Meyna tetraphylla* leaves are known.
5. The structures of the pure compounds from *Gardenia volkensii* fruits and *Meyna tetraphylla* leaves are known.

## 1.5 Justification

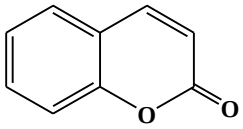
Due to the high cost of the modern medicinal drugs and syrups, many third world countries are unable to meet the high medical bills involved in the importation of medicine from the developed countries. This has made traditional medicinal practice very popular in developing countries because there is evidence that many medicinal plants cure many microbial diseases. Though no antimicrobial research has been done on *Meyna tetraphylla*, the Pokots of Kenya have been using the plant as animal fodder and medicine. The root decoction is given to the pregnant women to reduce pain and to treat infected hooves of goats and camels. Some

biological activity has been done on some few isolated compounds from *Gardenia volkensii* parts in Botswana. There was need to isolate more compounds because the Pokots of Kenya use many parts of this plant to treat microbial diseases (sore eyes, headaches, asthma, dysmenorrhoea, infertility, epilepsy, convulsions, earache, fever and as emetic). The bioactivity of *Gardenia volkensii* and *Meyna tetraphylla* crude extracts and the pure compounds would validate their use as herbal medicinal plants by the Pokots.

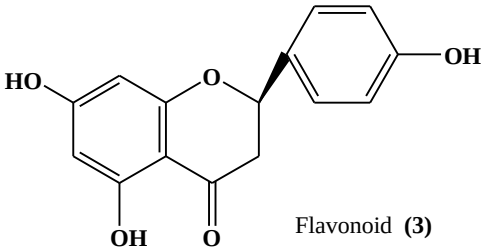
# CHAPTER TWO
# LITERATURE REVIEW

## 2.1 The genus *Gardenia*

This genus belongs to the Rubiaceae family. This family comprises of 10,700 species distributed in 637 genera (Mongrand *et al*., 2005). Most of the species are tropical but a number occur in temperate regions and there are a few Arctic ones. They are trees, shrubs or infrequently herbs and many of them are important sources of medicinal natural products, particularly coumarins such as 2H-1-benzopyran-2-one (**2**) and flavonoids such as (*R*)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-2,3-dihydrochromen-4-one (**3**). They are known to cure many diseases like malaria, fever, colic, muscular pains and for the expulsion of worms (Mongrand *et al*., 2005; Shellard and Phillipson, 1964).




2H-1-benzopyran-2-one **(2)**




Flavonoid **(3)**

The *Gardenia* genus consists of more than 250 species spread among the tropical forests of certain regions of the world. Seventeen of these species occur in Thailand. They are a popular ornamental shrub found world-wide and has been used in folkloric medicine for the remedy of a variety of diseases including malaria (Weenen *et al.*, 1990; Gakunju *et al.*, 1995). Chemical investigation of nearly twenty *Gardenia* species has yielded over one hundred chemical constituents belonging to different classes of bioactive compounds. The compounds include terpenoids, flavonoids, steroids, carbohydrates, alkaloids, aliphatic natural products and simple aromatic natural products (Gakunju *et al.*, 1995). Most of these compounds were found to have potential biological activities such as antifungal, anti-inflammatory, antiviral, antioxidant and estrogenic activities (Dictionary of Natural Products, 2007). Some examples of *Gardenia* species, their ethnomedicinal usage and compounds isolated from them are given in **Tables 1-2**.

**Table 1: Ethnomedical uses of *Gardenia* species**

| ***Gardenia* species** | **Part studied** | **Disease** | **References** |
|---|---|---|---|
| *G erubescens* | Dried aerial parts | Gonorrhea | Hussain *et al*., 1991 |
| *G. erythroclada* | Dried bark | Anti-pyretic | Mokkhasmit *et al*., 1971 |
| *G .gumnifera* | Buds | Anti-septic | Mukherjee and Namhata 1990 |
| *G. gumnifera* | Gum | Constipation | Mukherjee and Namhata 1990 |
| *G. jovis-tonantis* | Dried leaves | Anti-malarial | El Tahil *et al*., 1999 |
| *G. jovis-tonantis* | Dried roots | Epilepsy | Mathias, 1982 |
| *G. jovis-tonantis* | Fresh root bark, leaves, roots and stem bark. | Female sterility, wounds, cough, asthma and stomach-ache | Chhabra *et al*., 1991 |
| *G. lucida* | Dried flowers and leaves | Burns | Khan *et al*., 1984 |
| *G. taitensis* | Dried bark and flowers | Headache | Croft and Tuipulotu, 1980 |
| *G. ternifolia* | Roots | Fever | Gakunju *et al*., 1995 |
| *G. ternifolia* | Dried leaves and bark | Syphilis and ulcers | Ochola *et al*., 1995 |
| *G. ternifolia* | Leaves, roots and stem bark | Asthma, wound, cough and stomach-ache | Chhabra *et al*., 1991 |
| *G. trincantha* | Roots | Dysentery and diarrhoea | Le Grand, 1989 |
| *G. turgida* | Roots | Rheumatic joints | Jain, 1989 |
| *G. turgida* | Roots | Anthelminihic | Joshi, 1991 |

Some species of *Gardenia* that have been investigated for biological activity are given in the **Table 2** below.

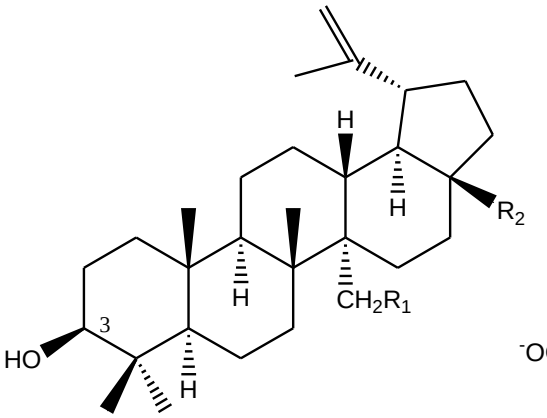
**Table 2: Bioactivity of *Gardenia* species**

| ***Gardenia* species** | **Part studied** | **Activity** | **Concentration** | **References** |
|---|---|---|---|---|
| *G. erubescens* | Dried roots | Analgesic | 50 µg/kg | Hussain *et al*., 1991 |
| *G. erubescens* | Dried roots | Diuretic | 25 mg/l | Hussain *et al*., 1991 |
| *G. erubescens* | Dried roots | Hypotensive | 800 µg/Animal | Hussain *et al*., 1991 |
| *G. gumnifera* | Florets (disc) | Insecticidal | 3.5 mg/ml | Salma and pratap, 1999 |
| *G. gumnifera* | Florets (disc) | Larvicidal | 14.56 mg/ml | Suryadevara and Khanam, 2002 |
| *G. triacantha* | Dried stem and bark | Anti-bacterial | 10 mg/ml | Laurens *et al*., 1985 |
| *G. obtusifolia* | Leaves and twigs | Anti-HIV-1 | 200 mg/ml | Tuchinda *et al*., 2002 |

### 2.2 Triterpenes from the *Gardenia* species

#### 2.2.1 Lupane Triterpenoids

Most lupane triterpenoids isolated from *Gardenia saxatilis* are known to exhibit some biological activity. Betulinic acid **(6)** in particular, is widely active and exhibits *in vitro* antimalarial (Ziegler *et al*., 2004) and anti-inflammatory effects (Mukherjee *et al*., 1997). It is highly regarded for its anti-HIV-1 activity (Hashimoto *et al*., 1997) and specific cytotoxicity against a variety of tumour cell lines. It also exhibits antineoplastic activity against malignant melanoma (mediated by the induction of apoptosis) without toxicity (Pisha *et al*., 1995) and it shows weak antibacterial activity towards Gram negative bacteria (Houghton *et al*., 1997). Lupeol **(5)** is an antineoplastic agent as well as an anti-inflammatory (trypsin and chymotrypsin inhibitor) agent (Rajic *et al*., 1999). Some of the lupane triterpenoids and their biological activities from *Gardenia saxatilis* are given in **Table 3**.




C-3 didehydro analogue of 2 Lupenone **(4)**

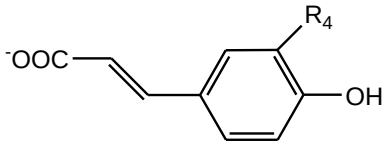
$R_1$ = H, $R_2$ = $CH_3$ Lupeol **(5)**

$R_1$ = H, $R_2$ = COOH Betulinic acid **(6)**

$R_1$ = A, $R_2$ = COOH Winchic acid **(7)**

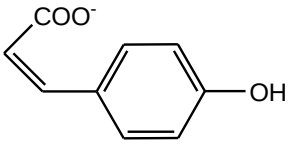
$R_1$ = B, $R_2$ = COOH Messagenic acid A **(8)**

$R_1$ = C, $R_2$ = COOH Messagenic acid B **(9)**




**A** $R_4$ = $OCH_3$

**C** $R_4$ = H



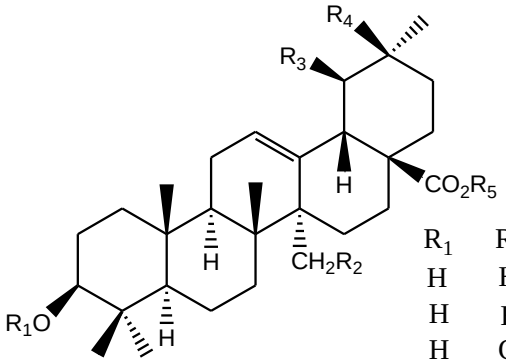

**B**

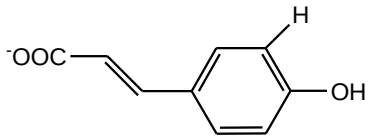
**Table 3: Lupane Triterpenoids from** *Gardenia saxatilis*

| Compounds | Activity | References |
|---|---|---|
| Lupenone **(4)** | - | Suksarnrarn *et al*., 2003 |
| Lupeol **(5)** | Anti-inflamatory, and antineoplastic - | Suksamrarn *et al*., 2003; Rajic, *et al*., 1999 |
| Betulinic acid **(6)** | Anti-malarial, anti-inflamatory, anti-HIV-1, antineoplastic and antibacterial | Ziegler, *et al*., 2004; Mukherjee, *et al*., 1997; Hashimoto, *et al*., 1997; Pisha, *et al*., 1995; Houghton, *et al*., 1997 |
| Winchic acid **(7)** | - | Suksarnrarn *et al*., 2003 |
| Messagenic acid A **(8)** | - | Suksarnrarn *et al*., 2003 |
| Messagenic acid B **(9)** | - | Suksarnrarn *et al*., 2003 |

### 2.2.2 Oleanane Triterpenoids

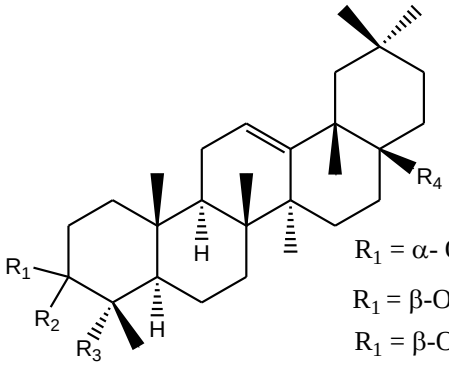
Most of the oleananes triterpenoids have been isolated from several *Gardenia* species. Oleanolic acid, **(10),** ursolic acid **(11)** and α-amyrin **(16)** showed a variety of biological activity as indicated in **Table 4.**




C

| $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | |
|---|---|---|---|---|---|
| H | H | H | $CH_3$ | H | Oleanolic acid **(10)** |
| H | H | $CH_3$ | H | H | Ursolic acid **(11)** |
| H | C | H | $CH_3$ | H | Uncarinic acid E (**12)** |
| H | C | $CH_3$ | H | H | 27-*p*-(*E*)-coumaroyl oxyursolic acid **(13)** |
| H | H | H | $CH_3$ | $CH_3$ | Oleanolic acid acetate (**14)** |
| glucoside | H | H | $CH_3$ | H | Oleanolic acid 3-*O*-Glucoside **(15)** |




$R_1$ = α- OH, $R_2$ = β-H, $R_3$ = $CH_3$, $R_4$ = $CH_3$ α-amyrin **(16)**

$R_1$ = β-OH, $R_2$ = α-H, $R_3$ = $CH_3$, $R_4$ = $CH_3$ β-amyrin **(17)**

$R_1$ = β-$OCOCH_3$, R2 = α-H, $R_3$ = $CH_3$, $R_4$ = $CH_3$ β-amyrin acetate **(18)**

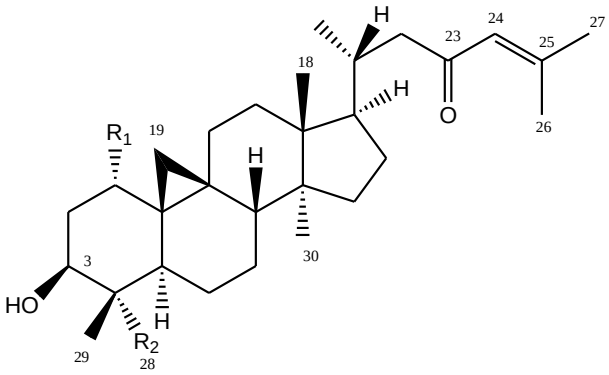
$R_1$ =α-OH, $R_2$ = β-H, R3 = α-COOH, $R_4$ = COOH Gypsogenic acid **(19)**

**Table 4: Oleanane Triterpenoids**

| Compounds | Species | Activity | References |
|---|---|---|---|
| Oleanolic acid **(10)** | *G. saxatilis, G. erubescens* | Anti-inflammatory, Anti-HIV, antiulcer | Suksarnrarn *et al*., 2003; Adelakun *et al*., 1996 |
| Ursolic acid **(11)** | *G. saxatilis, G. erubescens* | Antineoplastic, Antiulcer, Anti-HIV | Suksarnrarn *et al*., 2003; Adelakun *et al*., 1996 |
| Uncarinic acid E **(12)** | *G. saxatilis* | - | Suksarnrarn *et al*., 2003 |
| Coumaroyloxyursolic acid **(13)** | *G. saxatilis* | - | Suksarnrarn *et al*., 2003 |
| Oleanolic acid acetate **(14)** | *G. erubescens, G. jasminoides* | - | Adelakun *et al*., 1996; Wang *et al*., 1986 |
| Oleanolic acid 3-*O*-glucoside **(15),** | *G. sootepensis* | - | Wang *et al*., 1999; |
| α-amyrin **(16)**, | *G. turgida* | Antineoplastic | Joshi *et al*., 1979 |
| β-amyrin **(17)** | *G. lucida* | - | Shukla and Mukharya, 1990 |
| β –amyrin acetate **(18)** | *G. imperialis* | - | Babady-Bila and Tandu, 1987 |
| gypsogenic acid **(19)** | *G. lucida, G. turgida* | - | Shukla and Mukharya, 1990; Joshi *et al*., 1979 |

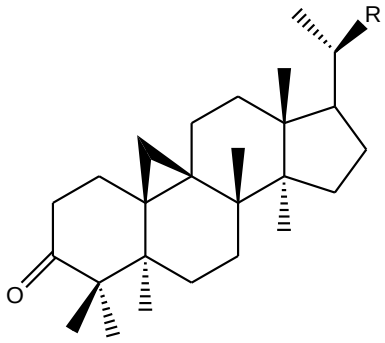
### 2.2.3 Cycloartane Triterpenoids

These are based on the parent hydrocarbon and the numbering is shown in compound **20.** A summary of these compounds and their sources are given in **Table.**



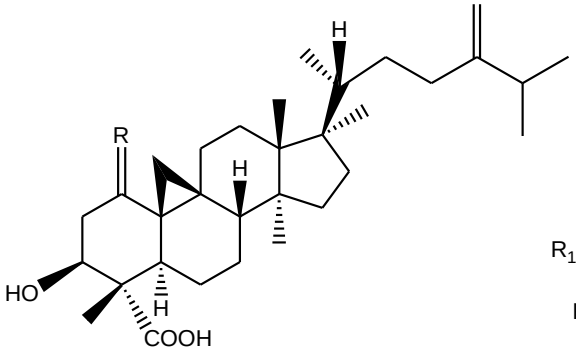

$R_1$ = OH, $R_2$ = $CH_3$ Thailandiol **(20)**

$R_1$ = H, $R_2$ = COOH Gardenolic acid **(21)**



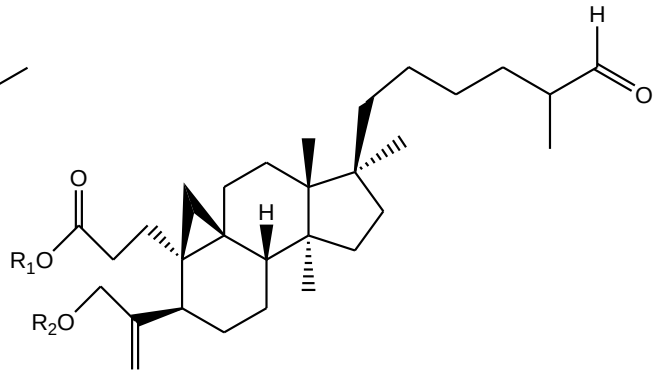

R = $CH_2$-CH=CH-CH=C($CH_3$)$_2$ Cycloartadienone **(22)**

R = $CH_2$-$CH_2$-CH=C($CH_3$)$_2$ Cycloartenone **(23)**



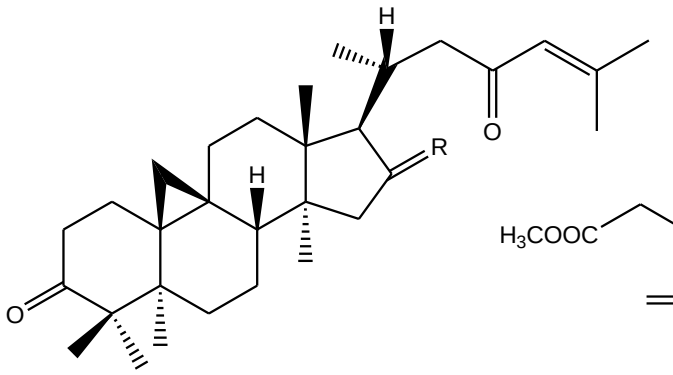

R=O Quadrangularic acid E **(24)**

R=$H_2$ 3β-hydroxy-5α-cycloart-24(31)-en-28-oic acid **(25)**



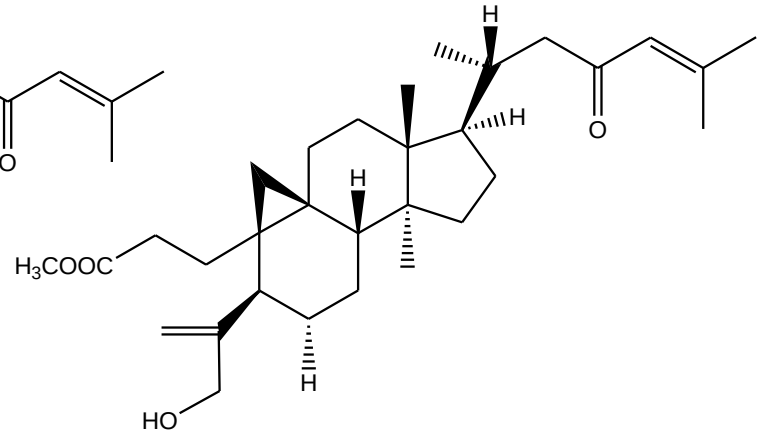

$R_1$ = H, $R_2$ = H Coronalolic acid **(26)**

$R_1$ = $CH_3$, $R_2$ = $CH_3$CO Methyl coronalolate acetate **(27)**

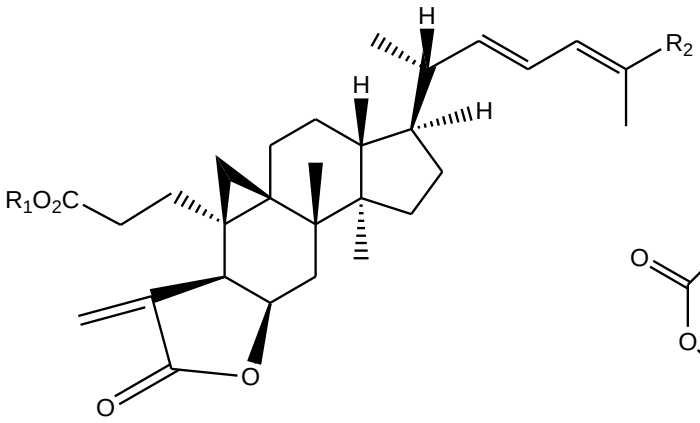



R=$H_2$ 5α-cycloart-24-ene-3,23-dione **(28)**

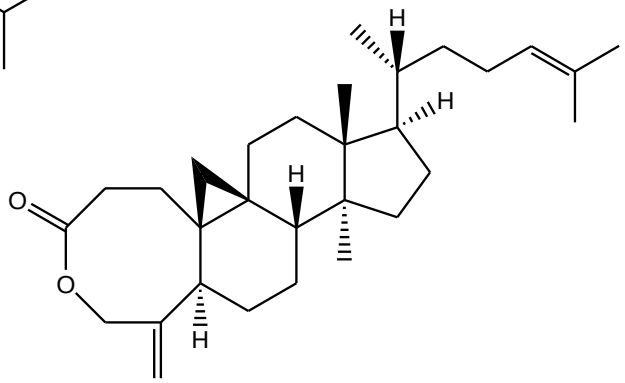
R=O 5α-cycloart-24-ene-3,16,23-trione **(29)**




Methyl-3,4-*seco*-cycloart-4(28),24-diene -29-hydroxy-23-oxo-3-oate **(30)**




| $R_1$ | $R_2$ | |
|---|---|---|
| $CH_3$ | $CH_3$ | Tubiferolide methyl ester **(31)** |
| H | CHO | Coronalolide methyl ester **(33)** |
| $CH_3$ | CHO | Tubiferaoctanolide **(34)** |




Coronalolide **(32)**

**Table 5: Cycloartane triterpenoids**

| Compound | Species | References |
|---|---|---|
| Thailandiol**,(20)** | *G. thailandica* | Tuchinda *et al*., 2004 |
| Gardenolic acid A**,(21)** | *G. thailandica*, *G. jasminoides* | Tuchinda *et al*., 2004; Qin *et al*., 1989 |
| Cycloartadienone**, (22)** and Cycloartenone **(23)** | *G. gordonii*, *G. hilli*, *G. storckii* | Davies *et al*., 1992 |
| Quadrangularic acid E **(24)** | *G. thailandica* | Tuchinda *et al*., 2004 |
| 3β-hydroxy-5α-cycloart-24(31)-en-28-oic acid**(25)** | *G. thailandica* | Tuchinda *et al*., 2004 |
| Coronalolic acid **(26)** | *G. coronaria*, *G. sootepensis* | Silva *et al*., 1997 |
| Methyl coronalolate acetate **(27)** | *G. coronaria*, *G. sootepensis* | Silva *et al*., 1997 |
| 5α-cycloart-24-ene-3,23-dione **(28)** | *G. obtusifolia* | Tuchinda *et al*., 2002 |
| 5α-cycloart-24-ene-3,16,23-trione **(29)** | *G. obtusifolia* | Tuchinda *et al*., 2002 |
| Methyl-3,4-*seco*-cycloart-4(28),24-diene-29-hydroxy-23-oxo-3-oate **(30)** | *G. obtusifolia* | Tuchinda *et al*., 2002 |
| Tubiferolide methyl ester **(31)** | *G. tubifera* | Reutrakul *et al*., 2004 |
| Coronalolide **(32)** | *G. coronaria*, *G. tubifera*, *G. sootepensis* | Reutrakul *et al*., 2004; Silva *et al*., 1997 |
| Coronalolide methyl ester **(33)** | *G. coronaria*, *G. tubifera*, *G. sootepensis* | Reutrakul *et al*., 2004; Silva *et al*., 1997 |
| Tubiferaoctanolide **(34)** | *G. tubifera* | Reutrakul *et al*., 2004 |

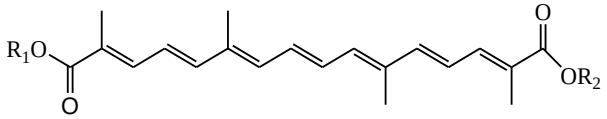
## 2.3 Other terpenoids

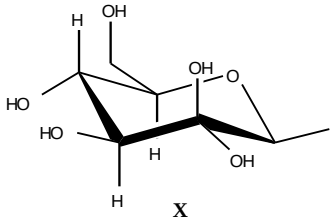
### 2.3.1 Apocarotenoid

The flowers of *Gardenia jasminoides* are used traditionally to treat hepatitis and diabetes. They have a strong scent, thus, their essential oil is used in perfumery and as contraceptives (Zhao *et al*., 1994).

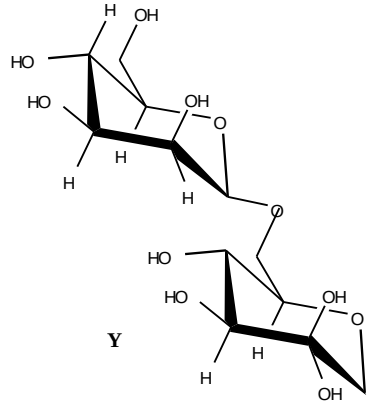
The fruits are yellow or red husks and are used as colorants for textiles and especially for food like noodle and sweets, which makes the identification of those carotenoids highly desirable (Pfister *et al.*, 1996). Recent investigation showed that the colour of these fruits is mainly due to the presence of carotenoids. On the basis of HPLC and UV/Vis data, it was postulated that crocetin glycosyl ester, especially Crocin-(Crocetin-di-(β-gentiobiosyl)-ester **(40)** are the main pigments (Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985).

The fruits of *Gardenia jasminoides* have been used for years for the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders and hypertension and as contraceptives (Tseng *et al.*, 1995). Their pharmacological actions such as protective activity against oxidative damage, cytotoxic effect, anti-inflammatory activity and fibrolytic activity have already been elucidated (Tseng *et al.*, 1995; Jagedeeswaran *et al.*, 2000). Further more, Crocetin **(35)** and Crocetin-(Crocetin-di-(β-gentiobiosyl)-ester **(40)** have shown anti-oxidant and anti-tumour effects respectively as indicated in **Table 6**.

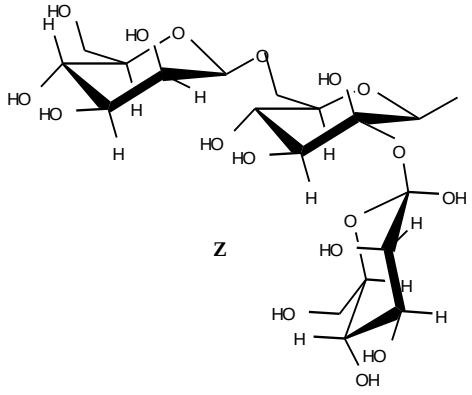



X




Y




Z

$R_1 = R_2 = H$ Crocetin, **(35)**

$R_1 = H$, $R_2 =$**X** Crocetin-mono-(β-D-glucosyl)-ester **(36)**

$R_1 = H$, $R_2 =$ **Y** Crocetin-mono-(β-gentiobiosyl)-ester **(37)**

$R_1 = R_2 =$ **X** Crocetin-di-(β-D-glucosyl)-ester **(38)**

$R_1 = X$, $R_2 =$ **Y** Crocetin-(β-D-glucosyl)-(β-gentiobiosyl)-ester **(39)**

$R_1 = R_2 =$ **Y** (Crocetin-di-(β-gentiobiosyl)-ester **(40)**

$R_1 =$ **Y**, $R_2 =$ **Z** Crocetin-(β-gentiobiosyl)-(β-neapolitanosyl)-ester **(41)**

$R_1 = Z$, $R_2 =$ **Z** Crocetin-di-(β-neapolitanosyl)-ester **(42)**

**Table 6: Apocarotenoids from *Gardenia jasminoides*** fruits

| **Compound** | **Activity** | **References** |
|---|---|---|
| Crocetin, **(35)** | Singlet oxygen quencher | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| Crocetin-mono-(β-D-glucosyl)-ester, **(36)** | - | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| Crocetin-mono-(β-gentiobiosyl)-ester **(37)** | - | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| Crocetin-di-(β-D-glucosyl)-ester **(38)** | - | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| Crocetin-(β-D-glucosyl)-(β-gentiobiosyl)-ester **(39)** | - | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| (Crocetin-di-(β-gentiobiosyl)-ester **(40)** | Anti-tumour, Choloretic agent | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| Crocetin-(β-gentiobiosyl)-(β-neapolitanosyl)-ester **(41)** | - | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |
| Crocetin-di-(β-neapolitanosyl)-ester **(42)** | - | Maria *et al*., 1997; Pfister *et al.*, 1996; Nishizawa *et al*., 1988; Kamikura and Nakazato, 1985 |

### 2.3.2 Iridoids

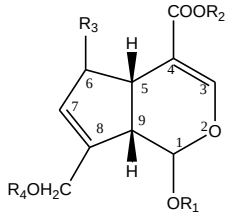
The iridane skeleton found in iridoids is monoterpenoid in origin. It contains a cyclopentane ring which is usually fused to a six membered oxygen heterocycle as shown in **Table 7** (Dewick, 2001). Various iridoids have been isolated from *Gardenia jasminoides*.

Geniposide **(43)** is the major component of the iridoids in *Gardenia jasminoides* fruits and has been reported to possess inhibitory activity on 5-Lipoxygenase (Nishizawa *et al.*, 1988), and activity against tumour promoting 12-*O*-tetradecanoylphorbol-13-acetate with an activation of protein kinase C. (Lee *et al.*, 1995). It has some inhibitory effects on ovalbumin–induced junction permeability and recovery of transepithelial electrical resistance in guinea pig trachea, showing its potential as an antiasthma therapy (Liaw and Chao, 2001). It was also shown to have radioprotective activity after sublethal irradiation in mice (Hsu *et al.*, 1997) and modulating activity on cytochrome P-450-dependent mono-oxygenase, glutathione and glutathione 5-transferase in rat liver (Kang *et al*., 1997).

Enzymatic hydrolysis of geniposide **(43).** by β-D-glucosidases in the liver and intestine yields genipin **(44)**. Genipin **(44)** has been shown to inhibit hepatocyte apoptosis induced by transforming growth factor $\beta$1 via the interference with mitochondrial permeability transition (Yamanoto *et al.*, 2001). It also protects hippocampal neurons from Alzhaimers amyloid β protein toxicity (Yamazaki *et al.*, 2001).

Since geniposide is transformed into genipin **(44)** by bacterial enzymes in the body (Akao *et al.*, 1994), it may be that genipin **(44)** mainly plays an important role in the efficacy. Thus, when geniposide **(43)** is orally administered, genipin **(44)** seems to be effectively produced in the intestine and then absorbed (Hye-Jin *et al.*, 2004).

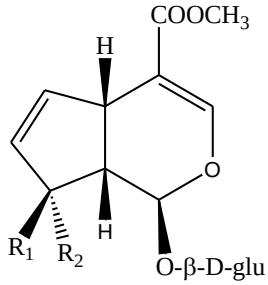
**Table 7: Iridoids from *Gardenia jasminoides* and *Gardenia. sootepensis***



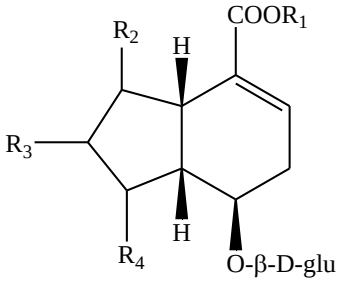

| **Compound** | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **Activity** | **References** |
|---|---|---|---|---|---|---|
| Geniposide **(43)** | β-D-Glu | $CH_3$ | H | H | Anti-asthma, anti-inflammatory, hepatoprotective, anti-tumour | Liaw and Chao, 2001; Nishizawa *et al.*, 1988; Lee *et al.*, 1995 |
| Genipin **(44)** | H | $CH_3$ | H | H | Hepatoprotective, Neuroprotective | Yamanoto *et al.*, 2001; Yamazaki *et al.*, 2001 |
| Acetyl geniposide **(45)** | β-D-Glu | $CH_3$ | H | Ac | - | Iida *et al.*, 1991, Tsai *et al.*, 1994, Miyagoshi *et al.*, 1986 |
| Choleretic geniposide acid aglycon **(46)** | H | H | H | H | - | Iida *et al.*, 1991, Tsai *et al.*, 1994, Miyagoshi *et al.*, 1986 |
| Deacetyl aspelurosidic acid **(47)** | β-D-Glu | H | α-OH | H | - | Iida *et al.*, 1991, Tsai *et al.*, 1994, Miyagoshi *et al.*, 1986 |
| Genipin gentiobioside **(48)** | 6''-*cis-p*-gentiobioside | $CH_3$ | H | H | Anti-tumour | Iida *et al.*, 1991, Tsai *et al.*, 1994, Miyagoshi *et al.*, 1986 |
| Geniposidic acid **(49)** | β-D-Glu | H | H | H | - | Iida *et al.*, 1991, Tsai *et al.*, 1994, Miyagoshi *et al.*, 1986 |
| Aspelirosidate **(50)** | β-D-Glu | $CH_3$ | α-OH | H | - | Iida *et al.*, 1991, Tsai *et al.*, 1994, Miyagoshi *et al.*, 1986 |
| Scandoside methyl ester **(51)** | β-D-Glu | $CH_3$ | β-OH | H | - | Iida *et al.*, 1991, Miyagoshi *et al.*, 1986, Wang *et al.*, 1999 |

### 2.3.3 Other Iridoids from *Gardenia* species

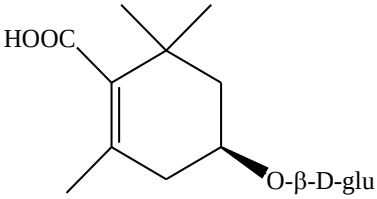
All of these compounds were isolated from *Gardenia jasminoides.* A summary of these iridoids is shown in **Table 8**.



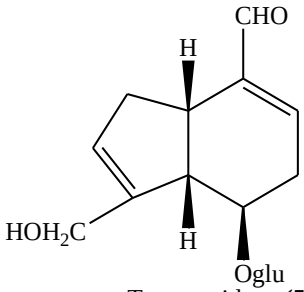

| $R_1$ | $R_2$ | | |
|---|---|---|---|
| OH | $CH_2OH$ | Gardenoside | **(52)** |
| $CH_2OH$ | OH | Monotropein methyl ester | **(53)** |



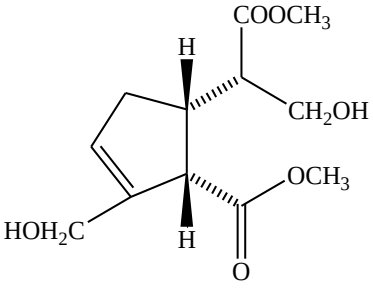

| $R_1$ | $R_2$ | $R_3$ | $R_4$ | | |
|---|---|---|---|---|---|
| $CH_3$ | H | β-OH | $=CH_2$ | Gardoside | **(54)** |
| H | β-H | H | α-$CH_3$, β-OH | Shanzhiside | **(55)** |



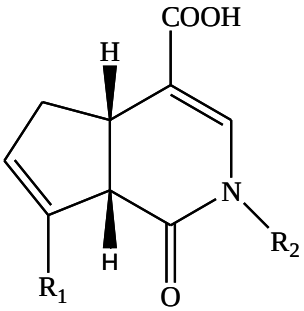

Picrocrocinic acid **(56)**



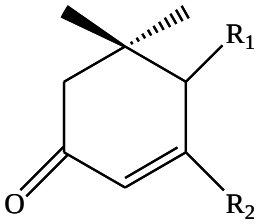

Tarennoside **(57)**



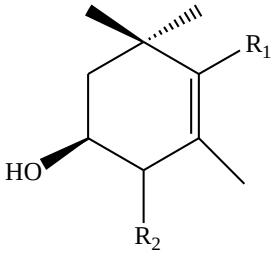

Gardenate **(58)**



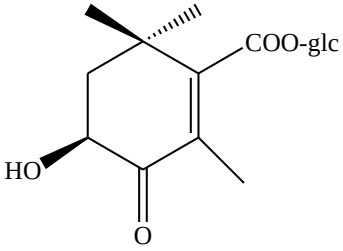

| $R_1$ | $R_2$ | | |
|---|---|---|---|
| $CH_2OH$ | H | Gardenamide A | **(59)** |
| $CH_2OH$ | $CH_2CH_2OH$ | 2-Hydroxyethyl gardenamide | **(60)** |



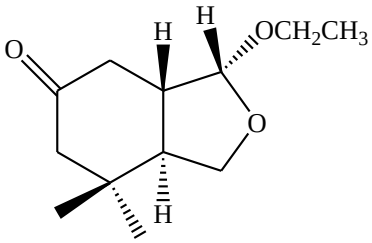

| $R_1$ | $R_2$ | | |
|---|---|---|---|
| β-$CH_2$-O-glu | $CH_3$ | Jasminoside A | **(61)** |
| α-$CH_2$-O-glu | $CH_3$ | *Epi*jasminoside A | **(62)** |
| β-$CH_2OH$ | $CH_2$-O-glu | Jasminoside E | **(63)** |
| $=CH_2$ | $CH_2$-O-glu | Jasminoside F | **(64)** |



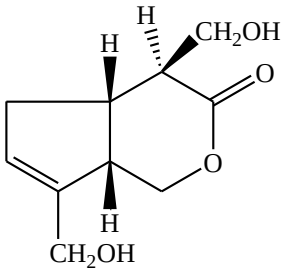

| $R_1$ | $R_2$ | |
|---|---|---|
| CHO | α-glc | Jasminoside B **(65)** |
| H | COO-glc | Jasminoside D **(66)** |




Jasminoside C **(67)**




Gardenone **(68)**




Gardendiol **(69)**

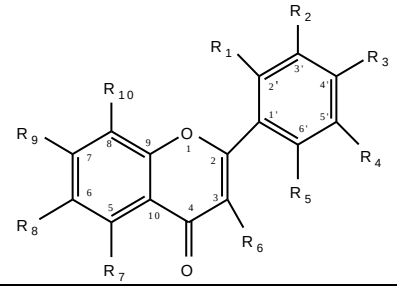
**Table 8: Other Iridoids from *Gardenia jasminoides***

| **Compound** | **References** |
|---|---|
| Gardenoside **(52)** | Machida *et al*., 2000 |
| Monotropein methyl ester **(53)** | Machida *et al*., 2000 |
| Gardoside **(54)** | Inouye *et al.*,1974 |
| Shanzhiside **(55)** | Takeda *et al*., 1976 |
| Picrocrocinic acid **(56)** | Takeda *et al*., 1976 |
| Tarennoside **(57)** | Machida *et al*., 2000 |
| Gardenate **(58)** | Machida *et al*., 2000 |
| Gardenamide A **(59)** | Machida *et al*., 2000 |
| 2-Hydroxyethyl gardenamide **(60)** | Machida *et al*., 2000 |
| Jasminoside A **(61)** | Machida *et al*., 1998 |
| Epijasminoside A **(62)** | Machida *et al*., 1998 |
| Jasminoside E **(63)** | Machida *et al*., 1998 |
| Jasminoside F **(64)** | Machida *et al*., 1998 |
| Jasminoside B **(65)** | Machida *et al*., 1998 |
| Jasminoside D **(66)** | Machida *et al*., 1998 |
| Jasmonoside C **(67)** | Machida *et al*., 2000 |
| Gardenone **(68)** | Zhao *et al.*, 1994 |
| Gardendiol **(69)** | Zhao *et al.*, 1994 |

## 2.4 Flavonoids

Flavonoids are molecules in which a suitable cinnamoyl-CoA $C_6C_3$ precursor from the shikimate pathway has acted as a starter group (Dewick, 2001). More than thirty flavonoids have been isolated from the *Gardenia* species and about six of them were found to have some biological activity. The 5,4'-dihydroxy-3,6,7,8-tetramethoxyflavone **(72,)** is anti-HIV (Tuchinda *et al*., 2002), while 3',5',5-trihydroxy-7,4'-dimethoxyflavone **(74)** is cytotoxic and anti-HIV (Reutrakul *et al*., 2004). The 3',5,7-trihydroxy-4'6',8-trimethoxyflavone **(94)** and acerosin **(98)** are estrogenic (Gupta *et al*., 1975). 5,7-dihydroxy-4',6,8-trimethoxyflavone **(95)** is anti-tubercular while 4',5,7-trihydroxy-8-methoxyflavone **(97)** is an enzyme inhibitor (Chhabra *et al*., 1977). A summary of the flavonoids isolated from *Gardenia* species is given in **Table 9.**

**Table 9: Flavonoids**




| Compound | Substitution | | | | | | | | | | Species | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **$R_5$** | **$R_6$** | **$R_7$** | **$R_8$** | **$R_9$** | **$R_{10}$** | | |
| 7,4'-Dihydroxyflavone **(70)** | H | H | OH | H | H | H | H | H | OH | H | *G. sootepensis* | Liang *et al*., 1991 |
| 5,5'-Dihydroxy-3,3',4',6,7-pentamethoxyflavone **(71)** | H | $OCH_3$ | $OCH_3$ | OH | H | $OCH_3$ | OH | $OCH_3$ | $OCH_3$ | H | *G. cramerii, G. fosbergii* | Gunatilaka *et al*., 1982 |
| 5,4'-Dihydroxy-3,6,7,8-tetramethxyflavone **(72)** | H | H | OH | H | H | $OCH_3$ | OH | $OCH_3$ | $OCH_3$ | $OCH_3$ | *G. obtusifolia, G. fosbergii* | Tuchinda *et al*., 2002, Gunatilaka *et al*., 1979 |
| 4',5,7-Trihydroxy-6-methoxyflavone **(73)** | H | H | OH | H | H | H | OH | $OCH_3$ | OH | H | *G. sootepensis* | Rukachasirikul *et al*., 1998 |

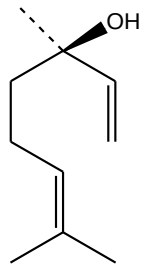
| Compounds | Substitution | | | | | | | | | | Species | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **$R_5$** | **$R_6$** | **$R_7$** | **$R_8$** | **$R_9$** | **$R_{10}$** | | |
| 3',5',5-Trihydroxy-7,4'-dimethoxyflavone **(74)** | H | OH | $OCH_3$ | OH | H | H | OH | H | $OCH_3$ | H | *G. tubifera* | Reutrakul *et al*., 2004 |
| 5,7-Dihydroxy-2',3',4',5'6'-pentamethoxyflavone **(75)** | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | OH | H | OH | H | *G. thailandica* | Tuchinda *et al*., 2004 |
| 5,7-Dihydroxy-2',3',4',5'-tetramethoxyflavone **(76)** | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | H | OH | H | OH | H | *G. thailandica* | Tuchinda *et al*., 2004 |
| 5-Hydroxy-4',7-dimethoxyflavone **(77)** | H | H | $OCH_3$ | H | H | H | OH | H | $OCH_3$ | H | *G. erubescens* | Adelakun *et al*., 1996 |
| 5-Hydroxy-2',3',4',5',7-pentamethoxyflavone **(78)** | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | H | OH | H | $OCH_3$ | H | *G. thailandica* | Tuchinda *et al*., 2004 |
| 5-Hydroxy-2',3',4',5',6',7-hexamethoxyflavone **(79)** | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | OH | H | $OCH_3$ | H | *G. thailandica* | Tuchinda *et al*., 2004 |

| **Compounds** | **Substitution** | | | | | | | | | | **Species** | **Reference**: |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | $R_8$ | $R_9$ | $R_{10}$ | | |
| 4’,5,7-Trihydroxy-3,8-dimethoxyflavone **(80)** | H | H | OH | H | H | $OCH_3$ | OH | H | OH | $OCH_3$ | *G. obtusifolia* | Tuchinda *et al*., 2002 |
| 4’,5,7-Trihydroxy-3’,3,8-trimethoxyflavone **(81)** | H | $OCH_3$ | OH | H | H | $OCH_3$ | OH | H | OH | $OCH_3$ | *G. obtusifolia* | Tuchinda *et al*., 2002 |
| 4’,5,7-Trihydroxy-3,6,8-trimethoxyflavone **(82)** | H | H | OH | H | H | $OCH_3$ | OH | $OCH_3$ | OH | $OCH_3$ | *G. obtusifolia* | Tuchinda *et al*., 2002 |
| 4’,5,7-Trihydroxy-3,6-dimethoxyflavone **(83)** | H | H | OH | H | H | $OCH_3$ | OH | $OCH_3$ | OH | H | *G. gordonii, G. taitenisis* | Miller *et al*., 1989 |
| 5,5’-Dihydroxy-2’,3’,6,7-tetramethoxyflavone **(84)** | $OCH_3$ | $OCH_3$ | H | OH | H | H | OH | $OCH_3$ | $OCH_3$ | H | *G. cramerii, G. fosbergii* | Gunatilaka *et al*., 1982 |
| 3’5,5’-Trihydroxy-3,4’,6,7-tetramethoxyflavone **(85)** | H | OH | $OCH_3$ | OH | H | $OCH_3$ | OH | $OCH_3$ | $OCH_3$ | H | *G. cramerii, G. fosbergii* | Gunatilaka *et al*., 1982 |
| Gardeanin-A-5-*O*-β-D-glucopyranoside **(86)** | H | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | H | O-gluco pyranosyl | $OCH_3$ | $OCH_3$ | $OCH_3$ | *G. florida* | Tewari and Mukharya, 1988 |
| 4’,5,7,8-tetramethoxyflavone **(87)** | H | H | $OCH_3$ | H | H | H | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | *G. lucida* | Kumari, 1989 |

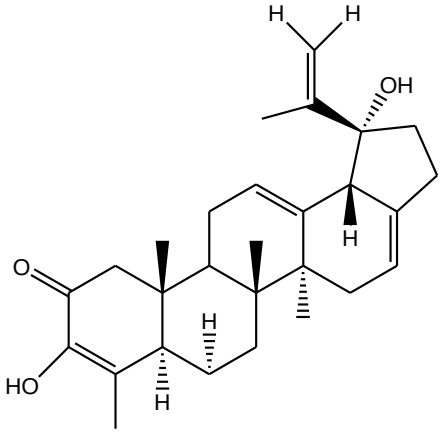
| **Compounds** | **Substitution** | | | | | | | | | | **Species** | **Reference**: |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | $R_8$ | $R_9$ | $R_{10}$ | | |
| 3’5-Dihydroxy-3,4’,5’,6,7-pentamethoxyflavone **(88)** | H | OH | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | OH | $OCH_3$ | $OCH_3$ | H | *G. fosbergii* | Gunatilaka *et al*., 1979 |
| 5-Hydroxy-3,3’,4’,6-tetramethoxyflavone **(89)** | H | $OCH_3$ | $OCH_3$ | H | H | $OCH_3$ | OH | $OCH_3$ | H | H | *G. fosbergii* | Gunatilaka *et al*., 1982 |
| 5-Hydroxy-3,3’,4’,5’,6,7-hexamethoxyflavone **(90)** | H | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | OH | $OCH_3$ | $OCH_3$ | H | *G. fosbergii* | Gunatilaka *et al*., 1979 |
| 5-Hydroxy-3’,4’,5’,6,7-pentamethoxyflavone **(91)** | H | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | H | OH | $OCH_3$ | $OCH_3$ | H | *G. cramerii, G. fosbergii* | Gunatilaka *et al*., 1982 |
| 4’,5,6,7-Tetrahydroxy-3,3’,5’-trimethoxyflavone **(92)** | H | $OCH_3$ | OH | $OCH_3$ | H | $OCH_3$ | OH | OH | OH | H | *G. fosbergii* | Gunatilaka *et al*., 1979 |
| 3’5-Dihydroxy-3,4’,6,7,8-pentamethoxyflavone **(93)** | H | OH | $OCH_3$ | H | H | $OCH_3$ | OH | $OCH_3$ | $OCH_3$ | $OCH_3$ | *G. obtusifolia* | Tichinda *et al*., 2002 |
| 3’,5,7-Trihydroxy-4’6’,8-trimethoxyflavone **(94)** | H | OH | $OCH_3$ | H | H | H | OH | $OCH_3$ | OH | $OCH_3$ | *G. species* | Gupta *et al.*, 1975 |
| 5,7-Dihydroxy-4’,6,8-timethoxyflavone **(95)** | H | H | $OCH_3$ | H | H | H | OH | $OCH_3$ | OH | $OCH_3$ | *G. species* | Chhabra *et al*., 1977 |
| 4’,5,8-Trihydroxy-6,7-dimethoxyflavone **(96)** | H | H | OH | H | H | H | OH | $OCH_3$ | $OCH_3$ | OH | *G. species* | Chhabra *et al*., 1977 |
| 4’,5,7-Trihydroxy-8-methoxyflavone **(97)** | H | H | OH | H | H | H | OH | H | OH | $OCH_3$ | *G. species* | Chhabra *et al*., 1977 |
| Acerosin **(98)** | H | OH | $OCH_3$ | H | H | H | OH | $OCH_3$ | OH | $OCH_3$ | *G. species* | Gupta *et al*., 1975 |

## 2.5 Other compounds from *Gardenia* species

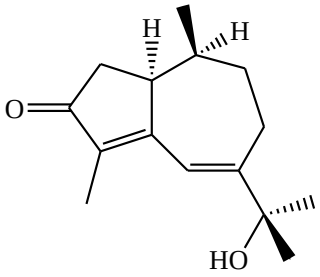
Linalool **(99)** is used extensively in perfumery industry, as a flavouring agent and aromatherapy (Dewick, 2001). It is anti-microbial and anti-convulsant while β-sitosterol is anti-bacterial and anti-fungal (Davies *et al.*, 1992). B-sitosterol **(102)** is produced commercially from soya beans as a raw material. It is antibacterial and anti-fungal (Vatcharin *et al*., 1998). A summary on the literature for compounds **99-103** is found in **Table 10**.



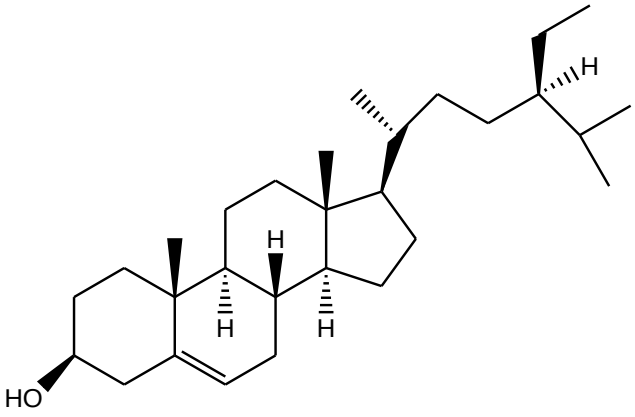

Linalool **(99)**



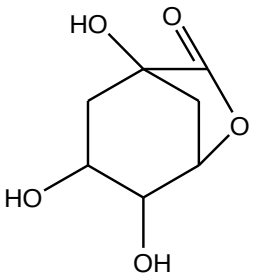

Erubescenone **(100)**




Sootepdienone **(101)**




β-sitosterol **(102)**




Quinide **(103)**

**Table 10: Other compounds from *Gardenia* species**

| Compound | Type of compound | Species | Activity | References |
|---|---|---|---|---|
| Linalool **(99)** | Linear monoterpene | *G. taitensis* | Antimicrobial, anticonvulsant | Davies *et al.*, 1992; Dewick, (2001). |
| Erubescenone **(100)** | *Nor*-,Friedo and Secolupane triterpenoids | *G. erubescens* | - | Adelakun *et al*., 1997 |
| Sootepdienone **(101),** | Guainane sesquiterpenoid | *G. sootepensis* | - | Vatcharin *et al*., 1998, |
| Sitosterol **(102),** | Stigmastane steroids | *G. sootepensis* | Antibacterial, antifungal | Vatcharin *et al*., 1998; Dewick, (2001). |
| Quinide **(103)** | Quinic acid lactone | *G. sootepensis* | - | Vatcharin *et al*., 1998, |

### 2.6 *Gardenia volkensii*

This plant is known as "The wild Gardenia", *oltakurukuriet* (Maasai), *mukumuti* (Kikamba), *Ngenenet* (Kipsigis) and *Rayudhi* (Luo). It is a deciduous tree with a single grooved stem. It has a dense spreading, roundish, twiggy crown and it could be a challenge to train because of their branching habits. A delicate fragrance fills the air in Spring when it blooms. Masses of large, beautiful starry white flowers are displayed above the glossy dark-green foliage. Flowers are followed by rather unusual oval ribbed grey-green fruits. Very good *bonsai* have been produced using this tree. It is a shrub or tree, 0.5-7.5 m with a smooth bark, which is silvery green (Bussmann *et al*., 2006). Pictures of *Gardenia volkensii* are shown on **Figures 1** and **2**.

**Figure 1: Whole plant of *Gardenia volkensii***

**Figure 2: Fruits, leaves and stems of *Gardenia volkensii***

### 2.6.1 Medicinal Usage

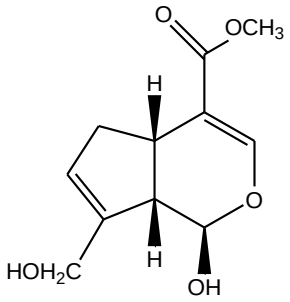
The fruits and roots are used in South Africa to treat sore eyes, headaches, asthma, dysmenorrhoea, infertility, epilepsy, convulsions, earache and as an emetic. Another indication of its nutritional and possible medicinal value is borne by the fact that fruits of this plant are eaten by elephants, kudu, velvet monkeys and baboons. The ash from burnt roots is placed on the chest as a treatment for pneumonia and some local people also believe that this shrub will protect them from lightning (Armstrong, 1986). The burnt ashes from the roots are dripped into the eyes to treat headache and drops are put into the ear to treat earache. Epilepsy is treated by taking the decoction orally (Beentje, 1994). The bark and leaves of the tree are used to treat a cut that is caused by a bewitching. Fruits are used as an emetic by the indigenous people (Catherine, 1998).

### 2.6.2 Mutagenic and antimutagenic effects of G*ardenia volkensii*

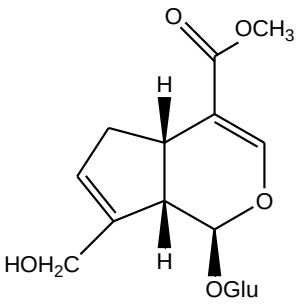
The dichloromethane extracts from different parts of *Gardenia volkensii* were investigated for mutagenic and antimutagenic effects in *salmonella*/microsome and micronucleus tests. The extract did not induce mutations neither did it modify the effect of the mutagen 4-nitro quinoline-oxide but it was genotoxic in the micronucleus test. This preliminary investigation showed that plant extracts used in traditional medicine may have particular effects with regard to mutagenicity and antimutagenicity indicating careful use in some instances and the need to isolate their active principle for further research (Verschaeve *et al*., 2004).

### 2.6.3 Compounds isolated from *Gardenia volkensii*

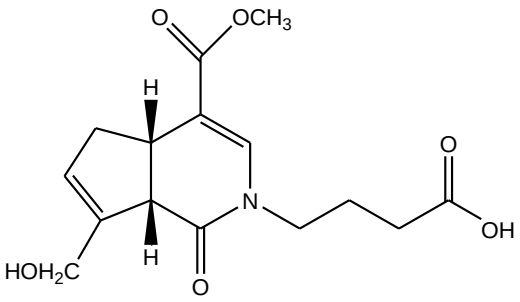
Chloroform and methanol extracts from the stem bark, twigs and seeds have so far yielded three iridoids **(44, 48, 104)**, six benzenoids **(105-110)** and two cinnamates, **(111-112).** Further isolation gave two aldehydes **(113-114)** and three flavonoids, **(115-117)** (Bernard and Runner, 2007). Some of these compounds have some biological activity as indicated in **Table 11** (page 32).



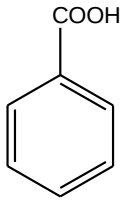

Genipin **(44)**



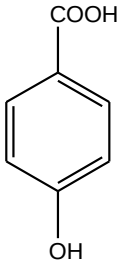

Genipin gentiobioside **(48)**



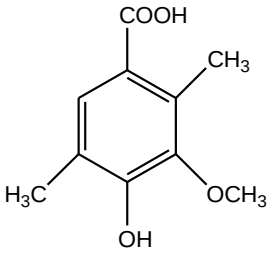

4-(2N-gardenamyl)- n-butanoic acid **(104)**



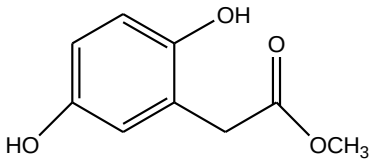

Benzoic acid **(105)**



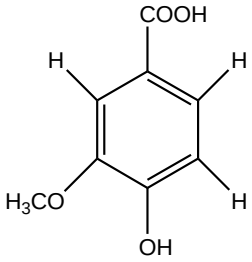

4-hydroxybenzoic acid **(106)**



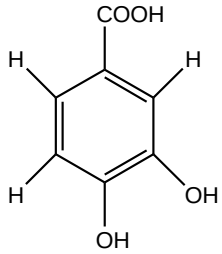

4-hydroxy-3-methoxy-2,5-dimethylbenzoic acid **(107)**



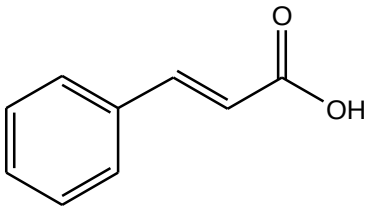

Methyl 2-(2,5-dihydroxyphenyl) acetate **(108)**



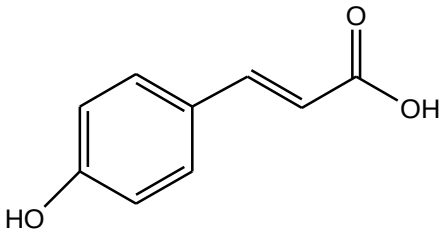

4-hydroxy-3-methoxybenzoic acid **(109)**



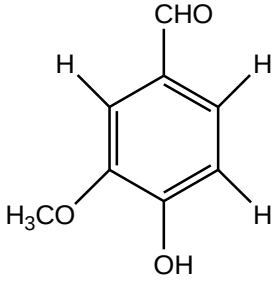

3,4-dihydroxybenzoic acid **(110)**



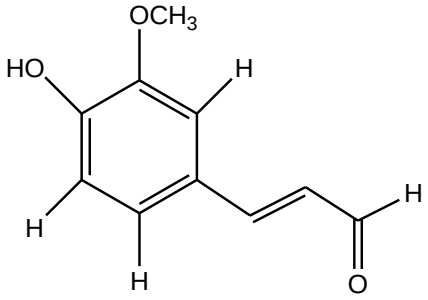

Cinnamic acid **(111)**



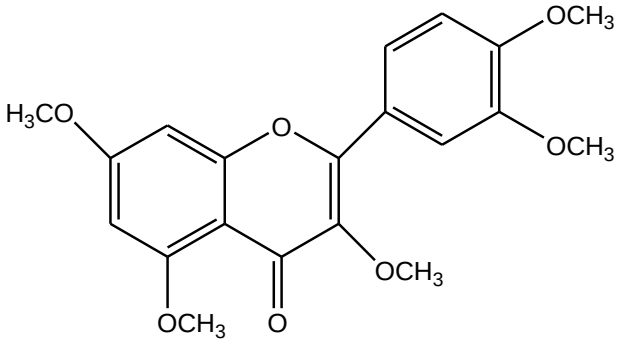

*p*-coumaric acid **(112)**



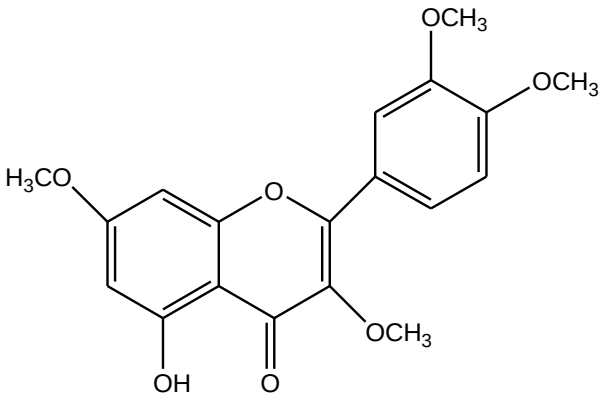

Vanillin **(113)**



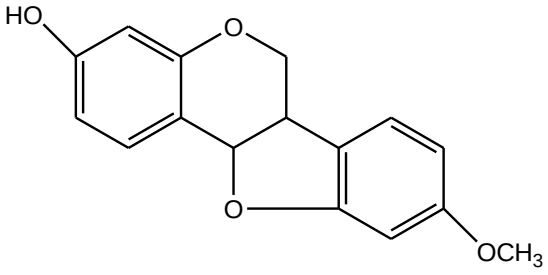

coniferaldehyde **(114)**




3,3',4',5,7-pentamethoxyflavone **(115)**




5-Hydroxy-3,3',4',7-tetramethoxyflavone **(116)**




Pterocarpan **(117)**

**Table 11 Bioactive compounds isolated from *Gardenia volkensii***

| Compound | Biological activity | Reference |
|---|---|---|
| Benzoic acid **(105),** | Antiseptic, expectorant, antifungal, antipyretic, keratolytic agent, phytotoxic, acaricide, antifouling agent | Martindale, 1996 |
| 4-hydroxybenzoic acid **(106)** | Antifungal | Ma *et al*., 1999 |
| 4-hydroxy-3-methoxybenzoic acid **(109)** | Antibaterial | Peter, 2004 |
| 3,4-dihydroxybenzoic acid **(110)** | Antioxidant, free radical scavenger, dietary chemopreventive agent (inhibits development of neoplasms in animal models), inhibits LDL oxidation, platelet aggregation inhibitor, anti-bacterial. | Garcia, *et al*.,1998; Peter, 2004 |
| *p*-coumaric acid (**112)** | Antibacterial, antifungal | Peter, 2004 |
| Vanillin **(113)** | Pharmaceutical excipient, Antioxidant, inhibits lipid peroxidation | Walton, *et al*., 2003 |
| 5-hydroxy-3,3',4',7-tetramethoxyflavone **(116)** | Antibacterial | Peter, 2004 |

## 2.7 The genus *Meyna*

*Meyna* genus of the family Rubiaceae consists of about 12 species found in Africa and the Indian Ocean islands to the South East Asia. Many of the members of the closely related genera *Keetia*, *Ps*ydrax and *Multidentia* have edible fruits too (Patrick and Bo, 2005). No phytochemical research has been done on this genus so far.

### 2.7.1 *Meyna tetraphylla*

This plant is called *Tiling'wo* (Pokot) and *Mutunguru* (Kikuyu). The plants are armed with pained spines above the nodes and the leaves appear to be in fours, actually in pairs on very short spurs at each node. The flowers are in short fascicles on these spurs, corolla lobes 4-5 and the fruit is a berry. It is a shrub or tree, which is 5-6 m long. It has white or green flowers and

its fruits are bluntly 5-angled, 13-17 by 16-20 mm. The buds are sparsely hairy, pedicels densely hairy (Beentje, 1994; Waliaula, 1988).

Crushed leaves are put between the infected hooves of goats and camels by the Pokots. It is also used as an animal fodder and the root decoction is given to the pregnant women to alleviate pain (Beentje, 1994; Waliaula, 1988). A picture of *Meyna tetraphylla* is shown on **figure 3** below.

**Figure 3: Whole plant of *Meyna tetraphylla***

**2.8 Microbial diseases**

Microbial diseases have been a problem to man for many years (Mead *et al*., 1999). Each year, more than 200 known microbial diseases are transmitted through air, food and water. They cause about fourteen million illnesses, sixty thousand hospitalizations, and one thousand eight hundred deaths every year. Examples are pneumonia, tuberculosis and cholera, which are caused by micro-organisms like *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus* bacteria (Mead *et al*., 1999).

## 2.9 Micro-organisms

These are organisms which are so small that they can only be seen under the microscope. They include bacteria, yeasts, fungi and moulds. They live almost everywhere on earth where there is liquid water, including hot springs on the ocean floor and deep inside rocks in the earth's crust. They are vital to humans and the environment, as they participate in the Earth's element cycles such as the carbon and nitrogen cycles as well as fulfilling other vital roles in virtually all ecosystems, such as recycling other organism's dead remains and waste products through decomposition. They cause many diseases like diarrhoea and cancer (Barea *et al*., 2005; Wolska, 2003). In this research project, some selected micro-organisms (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus* and *Candidas albicans*) were used for the bioassay test.

### 2.9.1 *Escherichia coli*

They are Gram-negative bacteria that commonly inhabits in the human intestine. They also live in the intestine of many other animals, wild as well as domestic. They cause severe and life-threatening diarrhoea (Barea *et al*., 2005).

### 2.9.2 *Salmonella typhimurium*

They are Gram-negative bacteria that multiply in the gastrointestinal tract of many animal species where they usually cause no disease. In humans their growth causes gastroenteritis. Six to forty eight hours after ingestion of contaminated water or food (usually poultry or beef), illness may begin with nausea and vomiting, often followed by diarrhoea. Isolations of *Salmonella* causing gastroenteritis in humans have increased in recent years in developed countries, primarily because modern methods of animal husbandry, food preparation, and distribution encourage the spread of *Salmonella* (Menichetti, 2005).

### 2.9.3 *Staphylococcus aureus,*

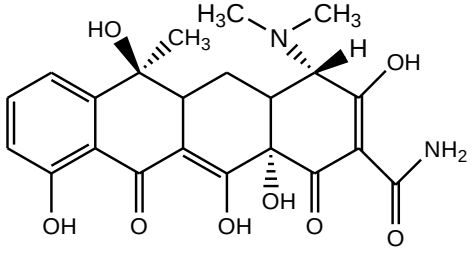
They are Gram-positive coccus that requires anaerobic conditions for growth. They live on the skin or in the nose of a person and cause a range of illnesses like skin infections such as pimples, boils, and cellulites. They also cause abscesses to life-threatening diseases such as pneumonia, meningitis, endocarditis, Toxic shock syndrome (TSS), and septicemia (Menichetti, 2005).

### 2.9.4 *Candidas albicans*

They are diploid asexual fungus and a causal agent of opportunistic oral and genital infections in humans. Systemic fungal infections (fungemias) have emerged as important causes of morbidity and mortality in immuno-compromised patients (*e.g.*, AIDS, cancer chemotherapy, organ or bone marrow transplantation). *Candidas albicans* are among the gut flora, the many organisms that live in the human mouth and gastrointestinal tract. Under normal circumstances, *they* live in 80% of the human population with no harmful effects, although overgrowth results in candidiasis (Jones *et al*., 2004).

## 2.10 Antibiotics

Antibiotics are compounds that are produced by living cells and they inhibit in very low concentrations, the growth of micro-organisms such as bacteria, fungi or protozoan. Examples are Amoxicillin®, Tetracycline®, Doxycycline® and Septrin® which were used as positive controls in the antibiotic assays in comparison with the plants crude extracts. Tetracycline® **(118)** is produced by the *Streptomyces* bacterium and is used against many bacterial infections. Most of them have activities against both Gram-negative and Gram-positive bacteria (Alfonso, 2004).




Tetracycline **(118)**

## 2.11 Spectroscopy

### 2.11.1 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy is the absorption and emission of electromagnetic radiation by the nuclei of certain atoms when they are placed in a magnetic field. In order to absorb electromagnetic radiation, nuclei must possess a non-zero magnetic moment. Samples for NMR spectroscopy are typically liquids (or solutions) and solids. The basic components of NMR include a strong magnet into which the sample is placed, a radiofrequency

transmitter and a receiver system connected to some type of data display or storage device (Phillip *et al*., 1998; Field and Sternhell, 1989).

The $^{1}H$ nucleus (proton) is the most commonly studied nucleus by NMR because of the ease of observation, its high natural abundance and the fact that it is invariably present in the majority of samples. Despite its low natural abundance (1.1%), $^{13}C$ is also an important nucleus because carbon forms the backbone of all organic compounds and structural information can be obtained by NMR spectroscopy. With modern instrumentation, NMR spectra can be obtained routinely on most isotopes. An NMR spectrum is normally presented as a graph of absorption intensity against the frequency of radiation absorbed by the nuclei in a sample. This method is quantitative in that the integrated intensity of a signal is proportional to the concentration of nuclei giving rise to it and for this reason NMR spectroscopy is a powerful technique for establishing the relative concentrations of components in mixtures (Phillip *et al*., 1998; Field and Sternhell, 1989).

Often one dimensional (1-D) NMR data obtained at the highest available magnetic field do not provide enough information to complete a structure analysis or to assign the resonances in a complex spectrum. Today a variety of multipulse sequences is applied in investigations of complex molecules and such techniques are available on most new NMR instruments. The most important benefit of these methods is that individual chemical shifts and all coupling constants can be measured unequivocally even when multiplets are overlapping. Many two dimensional (2-D) experiments are made up of some basic building blocks, for example: COSY, NOESY HMBC and DEPT which were used in this project (Phillip *et al*., 1998).

#### 2.11.1.1 COSY (Correlation Spectroscopy)

It is a two dimensional experiment in NMR that is used to identify nuclei that share a scalar (*J*) coupling. The presence of off-diagonal peaks (cross-peaks) in the spectrum directly correlates the coupled partners. Most often used to analyse coupling relationships between protons (Phillip *et al*., 1998).

#### 2.11.1.2 NOESY (Nuclear Overhauser Effect Spectroscopy)

This is a two dimensional method that is used to map NOE correlations between protons within a molecule. Most popular with, and best suited to, the study of very large molecules such as bio-polymers, although it still has a place in small molecule work. The spectrum has a layout similar to COSY but cross peaks now indicates NOEs between the correlated protons (Phillip *et al*., 1998).

#### 2.11.1.3 HMBC (Heteronuclear Multiple-Bond Correlation)

It is a two dimensional experiment that is used to identify long-range couplings (two to three bonds) between protons and carbons. It has good sensitivity because it utilises proton detection and it is an extremely powerful tool for piecing together organic structures (Phillip *et al*., 1998).

#### 2.11.1.4 DEPT (Distortionless Enhancement by Polarisation Transfer)

It is a one dimensional experiment that is used for enhancing the sensitivity of carbon observation and editing of $^{13}C$ spectra. The sensitivity gain comes from starting the experiment with proton excitation and subsequently transferring the magnetization onto carbon (the process known as polarisation transfer). The editing feature alters the amplitude and sign of the carbon resonances according to the number of directly attached protons, allowing the identification of carbon multiplicities. The experiment is typically run using different final proton pulse angle, resulting in differing signs (+ve or –ve) for various carbon resonances (Phillip *et al*., 1998; Field and Sternhell, 1989).

### 2.11.2 Mass spectrometry

In a mass spectrometer, molecules in the gaseous state under low pressure are bombarded with a beam of high-energy electrons which dislodges one of the electrons off the molecule to produce a positively charged ion called the *molecular ion*. An electron beam also fragments the molecular ion and this takes place in a variety of ways depending on the nature of the particular ion (Phillip *et al*., 1998; Solomons, 1978).

$$M + e^- \longrightarrow M^+ + 2e^-$$

Molecule  High-energy electron  Molecular ion

Once ions have been produced in a mass spectrometer, they can be further manipulated in many ways. The ions enter a flight tube, normally under high vacuum, and they are eventually separated and detected. The large arrays of ions are separated according to mass-to-charge ratio (*m/z*) and subsequently analyzed according to this ratio. The actual spectrum is a linear plot of *m/z* compared with relative abundance (or current). The type of mass analyzers in common use includes magnetic sector, quadrupole, ion trap, time-of-flight and Fourier transform-ion cyclotron resonance (FTMS) (Phillip *et al*., 1998). In this research project, time-of-flight mass spectrometry (TOF-MS) was used.

Time-of-flight mass spectrometry (TOF-MS) is a method in which ions are accelerated by an electric field of known strength. This acceleration results in an ion having the same kinetic energy as any other ion that has the same charge. The velocity of the ion depends on the mass-to-charge ratio. The time that it subsequently takes for the particle to reach a detector at a known distance is measured. This time will depend on the mass-to-charge ratio of the particle (heavier particles reach lower speeds). From this time and the known experimental parameters one can find the mass-to-charge ratio of the ion (Chernushevich *et al*., 2001).

## CHAPTER THREE

## MATERIALS AND METHODS

Below is a summary of the experimental procedures.

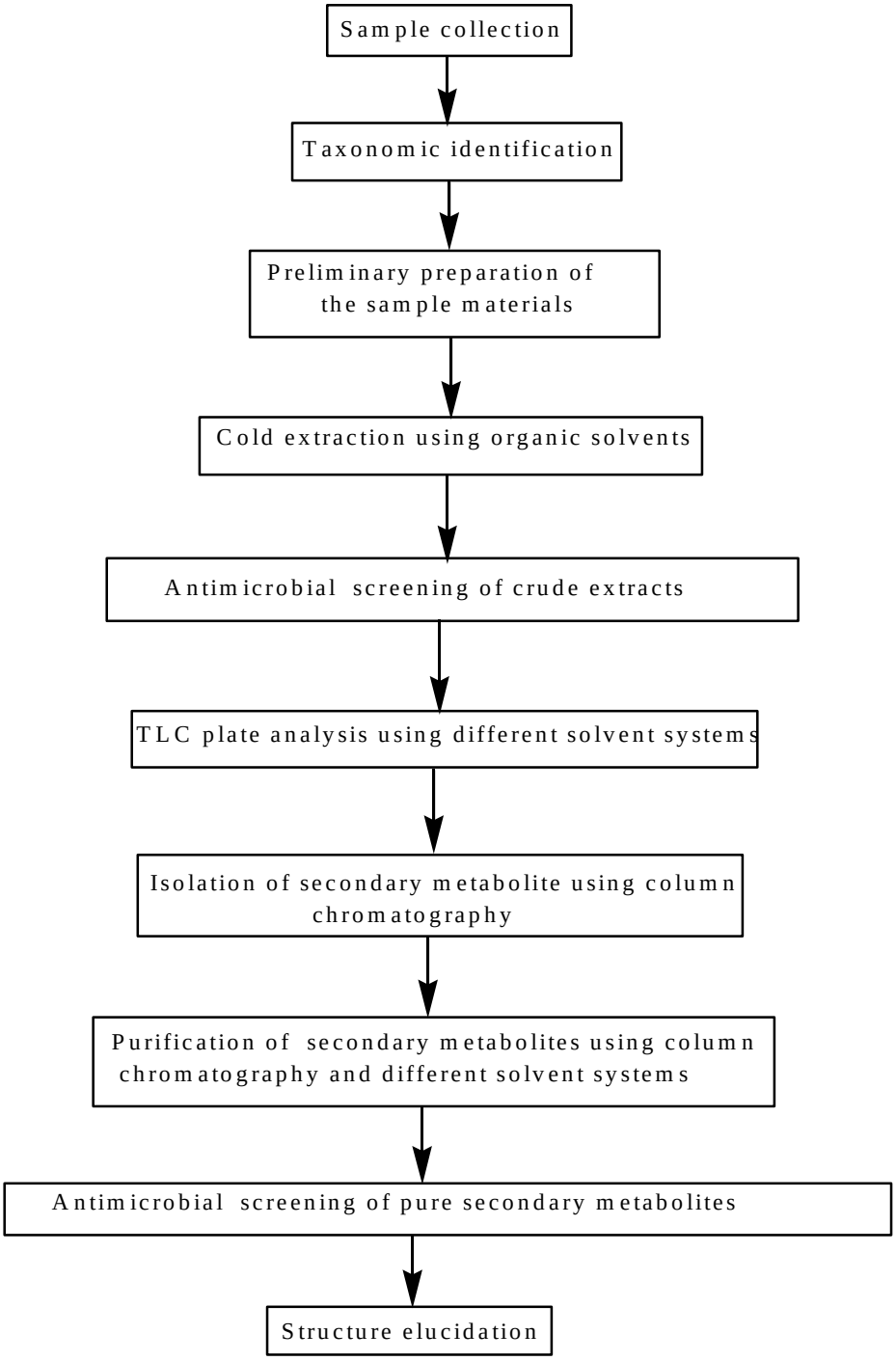



**Figure 4 Methodology**

### 3.1 Forward to Experimental

This section describes the general analytical and instrumental techniques employed throughout this work.

#### 3.1.1 General Chromatography

The crude extracts were spotted on aluminium TLC plates (20 x 20 cm Macharey Nagel Duren). The mobile phases used were varying ratios of hexane, dichloromethane, ethyl acetate and methanol (AR, Scharlau). Separations were monitored with inspection under ultraviolet light (UV lamp LF-204-LS, 354 nm and 634 nm) and by spraying the plate with anisaldehyde: sulphuric acid: methanol (1:2:97) mixture. Heating was done in an oven (ELECTROLUX STRUERS) at 70$^{o}$C for one minute. The plates with the best $R_f$ values were used to determine the best solvent system for the separation.

Crude extracts were then fractionated by gravity column chromatography on a 2 cm by 30 cm silica gel column (60-200 mesh Thomas Baker). Further purification was achieved by repeated column chromatography.

#### 3.1.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

Identification of pure compounds was achieved by $^{1}$H and $^{13}$C NMR spectroscopy. NMR spectra were recorded at room temperature on a 500 MHz Bruker AVANCE NMR spectrometer at the School of Biomedical and Molecular Sciences, University of Surrey at Guildford UK. Chemical shifts ($\delta$) are expressed in ppm relative to tetramethylsilane (TMS) as internal standard and coupling ($J$) are given in Hz.

#### 3.1 3 Mass spectrometry (MS)

The pure compounds obtained were also analysed using MS (Bruker MicroToF Mass Spectrometer) at School of Biomedical and Molecular Sciences, University of Surrey at Guildford, UK.

### 3.2 Extraction and purification of compounds from *Gardenia volkensii*

The plant material was collected from Baringo District in June 2006. The plant was identified by Dr. S. T. Kariuki and a voucher specimen deposited at the Botany Department, Egerton University.

The *Gardenia volkensii* fruits were cut into small pieces and the seeds and the outer cover separated. The seeds, outer cover leaves and the stem bark were all air-dried under shade to a constant weight. The plant materials were then ground to fine powder using a grinder at the Dairy Department, Egerton University. All the masses were taken using a STANTON electronic balance.

Dry powdered seeds (418.53 g) and outer covers (408.54 g) were successively and exhaustively extracted with 1 L hexane, 1 L dichloromethane and 1 L methanol for seventy two hours each in a 2.5 L Winchester bottle. Dry powders of leaves (232.20 g) and stem-bark (387.32 g) were extracted with methanol only. The solvents were evaporated under reduced pressure using a rotary evaporator (Büchi type R-205). The crude extracts were then weighed and kept in sample tubes.

The hexane (11.68 g), dichloromethane (20.40 g) and methanol (18.26 g) of fruit crude extracts showed almost similar spots with the dichloromethane extracts having more spots on visualizing with a UV lamp and anisaldehyde spraying reagent. The dichloromethane extract was subjected to a solvent step gradient of dichloromethane: methanol. The following compounds were isolated: **GV1** (fraction 15-16: 20% methanol in dichloromethane) and **GV2** (fraction 17-20: 23% methanol in dichloromethane). The fractions containing **GV1** (35.10 mg) and **GV2** (34 80 mg) were purified by repeated column chromatography using a solvent step gradient of 20% and 33% ethyl acetate in hexane respectively.

### 3.3 Extraction and purification of compounds from *Meyna tetraphylla*

The plant material (leaves and root-bark) was collected from Baringo District in June 2006. The plant was identified by Dr. S. T. Kariuki and a voucher specimen deposited at the

Botany Department, Egerton University. The leaves and the root-bark were air-dried under shade to a constant weight and ground to fine powder.

Dry powdered leaves (636 g) were successively and exhaustively extracted with 1L hexane, 1 L dichloromethane and 1 L methanol for seventy two hours each in a 2.5 mL Winchester bottle. The root-bark (455.65 g) was extracted with only methanol. The solvents were evaporated under reduced pressure using a rotary evaporator. The crude extracts were then weighed and kept in sample tubes.

The hexane (7.38 g), dichloromethane (11.84 g) and methanol (9.55 g) of the leaves crude extracts showed almost similar spots with the dichloromethane extracts having more spots on visualizing with a UV lamp and anisaldehyde spraying reagent. The following compounds were isolated: **MT1** (164.00 mg), **MT2** (48 mg), **MT3** (63.00 mg), **MT4** (72.20 mg) and **MT5** (88.80 mg) in fraction 13-51: 20% ethyl acetate in hexane. All the fractions were purified by repeated column chromatography using 20% methanol in dichloromethane.

**3.4 Bioassay test**

Approximately 14 gm of nutrient agar was weighed, dissolved in 250 mL of distilled water in a 500 mL Erlenmeyer conical flask and sterilized in an autoclave at 121ºC for 15 minutes. The nutrient agar was left to cool in a water bath to 40ºC, then dispensed into sterile Petri dishes and left to cool in a refrigerator at 4ºC.

About 1.5 g of nutrient broth was weighed, dissolved in 100 mL distilled water in a 250 mL Erlenmeyer flask, sterilized for 15 minutes at 121ºC in an autoclave, and then left to cool in a refrigerator.

Isolates of *Escherichia coli* (ATCC 11303), *Salmonella typhimurium* (C953), *Staphylococcus aureus* (Laboratory isolate), *Bacillus subtilis* (Laboratory isolate) and *Cadidas albican* (Laboratory isolate) were obtained from Dairy and Botany Department, Egerton University. The micro-organisms were inoculated into the nutrient broth using a thermally

sterilized wire loop, labelled accordingly and the date of preparation indicated. These were then incubated for 24 hours at 37ºC.

The resuscitated micro-organisms were removed from the incubator (CARBOLITE SEKONIC POCKETCORDER SK-50P) and the nutrient broth's turbidity was a sign that growth had occurred. The inocula were picked using a sterile wire loop, streaked onto respective media on agar plates (inoculated) followed by incubation at 37ºC for 24 hours.

Sterilized nutrient agar at 40ºC was poured into the plates containing the pure cultured micro-organisms. The surface was scrapped using a sterile loop for the micro-organisms to suspend in the media and then poured back into the conical flask containing the media. They were thoroughly mixed to obtain homogeneity and the nutrient agar seeded with micro-organisms was dispensed into the sterile agar plates. This was done for each micro-organism.

About 20-40 µL of extract solution (10 mg each of crude extracts of root bark, stem, fruits and leaves were dissolved in 1 mL of their respective organic solvents) was impregnated onto a 6 mm sterile paper disc using adjustable (analogue) volume micropipette. The paper disc was allowed to dry by letting the solvent evaporate for one hour. Selected antibiotics including Amoxicillin®, Tetracycline®, Doxycycline® and Septrin® were used as positive controls while the organic solvents (hexane, dichloromethane and methanol) were used as negative controls.

The dry paper disc was carefully placed on the surface of the test plate seeded with the micro-organisms, labelled and then incubated for 24 hours at 37ºC. The tests were done in duplicates and the inhibition zones recorded in millimetres. This procedure was repeated for the pure compounds. About 10-20 µL of antibiotics solution (10mg of antibiotic dissolved in 1 mL methanol) was impregnated onto sterile paper disc. The other procedures were done as for the crude extracts.

Determination of Minimum Inhibitory Concentration (MIC) was carried out for all the crude extracts and isolated compounds in a serial dilution assay. About 20 µL, 25 µL, 30 µL, 35 µL and 40 µL solution of crude and isolated compounds were inoculated as done before. The

tests were done in duplicates. The lowest concentration with the smallest inhibition zone was taken as the (MIC).

### 3.4.1 Inhibition Concentration at 50% ($IC_{50}$)

Different concentrations of Doxycycline® antibiotic (10,000, 4,000, 1,000, 400, 100, 40, 10 and 4 mg/L in methanol) were prepared using serial dilutions method. The **$IC_{50}$** for Doxycycline® antibiotic was determined using probit analysis software (GraphPad Prism was used to plot inhibition zone against log of concentration of Doxycycline®). The **$IC_{50}$** for the crude extracts and the pure compounds were determined in a similar way. The **$IC_{50}$** for the crude extracts and the pure compounds were then compared with the $IC_{50}$ for Doxycycline® antibiotic.

### 3.4.2 Probit Analysis

A dose-response curve was drawn using the Graphpad prism program, (GraphPad Prism, 2008). The logarithm of the concentrations of the compound or extract under test was plotted on the X-axis and the inhibition zone on the Y-axis. The **$IC_{50}$**s were determined from the dose response curve. The maximum inhibition (Top), the slope and the concentration that provoked the inhibition halfway between baseline and maximum were the **$IC_{50}$**.

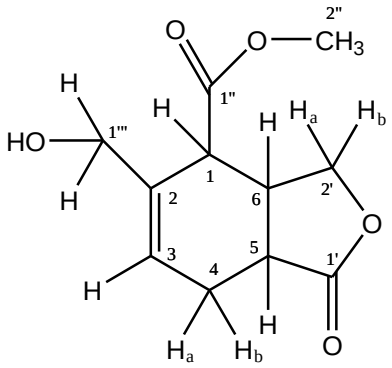
# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 *Gardenia volkensii*

The dichloromethane extract yielded **GV1** and **GV2** after repeated chromatography. **GV1** (65.10 mg) was a mixture of two compounds - light blue shinny UV active mixed with a brown UV inactive compound with an $R_f$ of 0.2 (20% ethyl acetate in hexane). A novel pure compound **GV2** (34.80 mg), UV inactive with an $R_f$ of 0.14 (33% ethyl acetate in hexane) was isolated as a light yellow powder in the yield of 0.3% of the dichloromethane crude extract.

### 4.1.1 Structure elucidation of compound GV2




**GV2**

The EI/FI-MS of **GV2** showed a molecular peak at m/z 226.08 corresponding to the molecular formula $C_{11}H_{14}O_5$. The degree of unsaturation for this compound was five. This was accounted for, by the cyclohexene, two carbonyl groups and a five membered cycloketone.

The $^1$H NMR (Appendix 2) spectrum of compound **GV2** showed one methoxy group resonance at δ 3.71 corresponding to the $^{13}$C NMR (Appendix 3) resonance at δ 52.75. The proton resonance at δ 5.81 showed a characteristic of cyclohexene double bond corresponding to the $^{13}$C NMR resonance at δ 129.44 in the HSQC-DEPT spectrum (Appendix 4). Two coupled proton NMR resonance at δ 4.50 (H-2'a) and δ 4.47 (H-2'b) indicated the presence of non-equivalent protons, characteristic of butyrolactone corresponding to the $^{13}$C NMR resonance at δ 67.87 in the HSQC-DEPT (Appendix 4). Furthermore, two proton resonances at δ 2.73 (H-4a) and δ 2.28 (H-4b) indicated the presence of two non-equivalent protons, α to the double bond of

the cyclohexene. The $^{13}C$ NMR resonance at δ 60.82 indicated the presence of an OH group next to a methylene with two proton singlets at a resonance of δ 4.29.

The eleven carbon resonances observed in the $^{13}C$ NMR spectrum were characterized by DEPT experiment (Appendix 5) which indicated that **GV2** was a monoterpenoid with a modified iridane skeleton. It consisted of one methoxy group (δ 52.75), three methylene groups (δ 38.99, δ 67.87, δ 60.82, two of them oxygenated), four methine groups (δ 49.49, δ 129.44, δ 50.72, δ 37.75, two oxygen bearing) and three quaternary (δ 140.26, δ 72.48, δ 171.60, two carbonyl) carbon signals. The chemical shift of one quaternary carbon (δ 140.26) and one methine carbon (δ 129.44) indicated the presence of a cyclohexene while the chemical shift of the other two quaternary carbons (δ 172.48 and δ 171.60) indicated the presence of two carbonyl groups.

In the COSY spectrum (Appendix 6) H-6 was correlated to H-1, H-4 and H-5. In the HMBC spectrum (Appendix 8) the correlation between C-2' and H-6, C-3 and H-5 indicated the presence of a cyclohexene. Also the correlation between C-3 and H-1'" indicated further the presence of a double bond of a cyclohexene. The NOESY spectrum (Appendix 7) confirmed the position of the methoxy group at C-1" through correlation between the methoxy proton resonance and H-1. Also the position of the double bond at C-3 and C-2 was confirmed by the NOESY spectrum through correlation between the hydroxyl proton resonance and H-3. A summary of NMR data for **GV2** is shown in **Table 12**.

A literature search based on the above molecular structure suggested that the compound was novel.

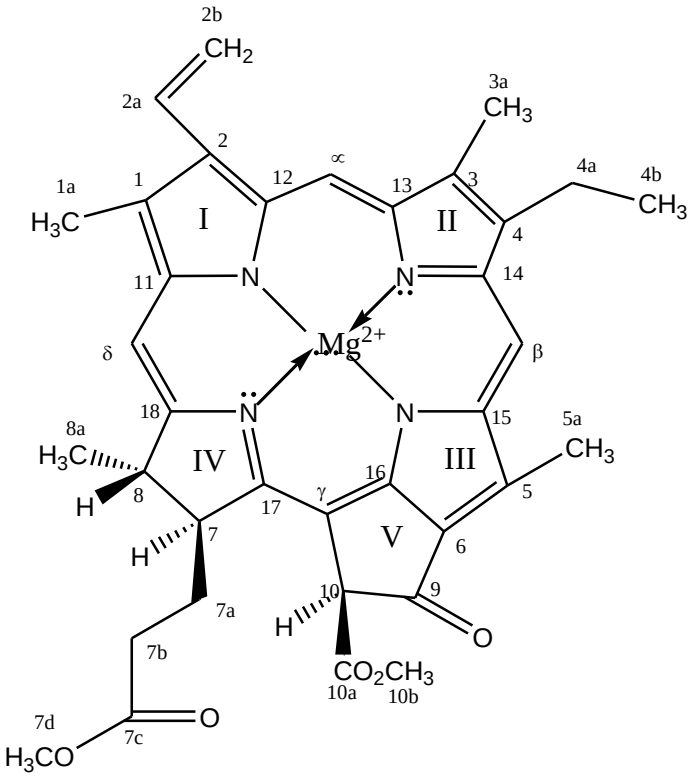
**Table 12: NMR data for compound GV2**

| Position | δ $^{1}$H ppm (J in Hz) | δ $^{13}$C (ppm) | COSY | HMBC(H→C) | NOESY |
|---|---|---|---|---|---|
| 1 | 3.74 d (13.5) | 49.49 (CH) | 6 | 3,4,6 | 1”’,2” |
| 2 | - | 14 0.26 (C) | - | - | - |
| 3 | 5.81 m | 129.44 (CH) | - | 1,4,5,1”’ | - |
| 4 | 2.73 m, 2.28 m | 38.99 ($CH_2$) | 6 | 3,5,6’ | 6 |
| 5 | 3.72 m | 50.72 (CH) | 6 | 3,4,6 | 1”’ |
| 6 | 3.18 m | 37.75 (CH) | 1,4,5 | 1,4,5,2’ | 1,2” |
| 1’ | - | 172.48 (C) | - | - | - |
| 2’ | 4.50 t (3.5)<br>4.47 t (3.0) | 67.87 ($CH_2$) | | 6 | - |
| 1” | - | 171.60 (C) | - | - | - |
| 2” | 3.71(s) | 52.75 ($CH_3$) | | - | 1”’ |
| 1”’ | 4.29(s) | 60.82 ($CH_2$) | | 3 | 3 |

### 4.2 *Meyna tetraphylla*

With repeated chromatography, five pure compounds were isolated from the dichloromethane extract using 20% methanol in dichloromethane. **MT1** (164.10 mg), UV active and green on spraying with an $R_f$ of 0.6, **MT2** (48.00 mg), UV active and blue on spraying with an $R_f$ of 0.4, **MT3** (chlorophyll derivative, 65.00 mg), UV active, green and an $R_f$ of 0.6, **MT4** (72.20 mg) UV active, grey and $R_f$ of 0.08 on spraying and **MT5** (88.80 mg) UV active, dirty green and $R_f$ of 0.42.

### 4.2.1 Structure elucidation of compound MT3




**MT3**

Compound **MT3** was isolated from the dichloromethane extract of *Meyna tetraphylla* leaves as a green powder in the yield of 0.52% of the crude extract. The data for **MT3** was assigned by 1-D and 2-D NMR spectroscopy and based on this evidence, it was proposed that **MT3** was a chlorophyll derivative lacking the phytol side chain. It could not be decided if **MT3** was a porphyrin or a metalloporphyrin as MS data was not available. The $^{13}C$ NMR data for **MT3** spectrum (Appendix 10) was compared with the literature data for chlorophyll a as shown in **Table 13** (Lotjonen and Hynninen, 1983). The difference in the chemical shifts can be accounted for by the fact that the spectra were run in different solvents. Furthermore **MT3** does not have the phyto side chain.

The degree of unsaturation was nineteen. This was accounted for by four pyrrole rings, three carbonyl groups, three vinyl groups and a cyclopentanone. The $^{13}C$ NMR spectra gave thirty six carbon resonances. Sixteen of the resonances belong to four pyrrole carbons, two methoxy carbons (δ 51.9, δ 52.0), five methyl carbons (δ 12.6, δ 11.2, δ 18.0, δ 12.6, δ 23.9), three carbonyl carbons (δ 172.56, δ 171.01, δ 189.82), one in a five membered ring), four

methylene carbons (δ 122.85, δ 19.93, δ 30.56, δ 30.05), seven methine carbons (δ 51.04, δ 51.32, δ 66.03, δ 97.61, δ 97.61, δ 93.27, δ 131.97)and fifteen quaternary carbon signals. The two carbonyl carbon signals (C-9, C-7c, and C-10a) occurred at the low field of δ 171- δ 190. All the carbon resonances were characterized by DEPT experiments (Appendix 12).

The proton resonances at δ 1.81, δ 3.67 and δ 2.52 showed a characteristic of four methyl groups attached to the pyrrole ring corresponding to the $^{13}C$ NMR resonance at δ 23.30, δ 12.26 and δ 11.28 in the HSQC-DEPT spectrum (Appendix 11). The $^{1}H$ and $^{13}C$ signals at δ 3.88 (δ 53.07) and δ 3.58 (δ 51.88) were characteristic of two methoxy groups. Three proton resonances at δ 9.51, δ 9.36 and δ 8.56 showed the presence of three methine groups between the pyrrole rings.

In the HMBC spectrum (Appendix 14), the correlation between C-7c, C-10a and the methoxy proton resonance at δ 3.58(s) and δ 3.888(s) respectively the methoxy groups at those positions.

In the COSY spectrum (Appendix 13) there was a correlation between H-8 (δ 4.46 m) and resonance at δ 1.81 d (H-8a) and δ 1.71 t (H-4b). The spectrum further showed a correlation between H-7a (δ 2.32 m) and resonance at δ 4.21 m (H-7).

**Table 13: Comparison of NMR data for chlorophyll a and compound MT3 (Chlorophyll derivative)**

| Position | δ $^{1}$H (ppm) (*J* in Hz) | δ $^{13}$C (ppm) $CDCl_3$ | δ $^{13}$C (ppm) $C_4D_8O$ (Lotjonen. and Hynninen,1983) | COSY | HMBC (H→C) |
|---|---|---|---|---|---|
| 1 | - | 136.32 | 135.5 (C) | - | - |
| 2 | - | 138.08 | 139.0 (C) | - | - |
| 3 | - | 136.61 | 134.0 (C) | - | - |
| 4 | - | 142.20 | 144.1 (C) | - | - |
| 5 | - | 131.97 | 134.20 (C) | - | - |
| 6 | - | 131.97 | 131.9 (C) | - | - |
| 7 | 4.21 m | 51.04 | 51.6 (CH) | 7a | 7b,8,8a |
| 8 | 4.46 m | 51.32 | 50.0 (CH) | 8a | 7,7b,8a, 17 |
| 9 | - | 189.82 | 189.82 (C) | - | - |
| 10 | 6.26 s | 66.03 | 66.2 (CH) | - | 6,9,10a, 16 |
| 11 | - | 155.73 | 154.4 (C) | | |
| 12 | - | 149.83 | 148.00 (C) | | |
| 13 | - | 151.07 | 151.4 (C) | | |
| 14 | - | 145.36 | 146.10 (C) | | |
| 15 | - | 149.83 | 147.7 (C) | | |
| 16 | - | 161.38 | 161.4 (C) | | |
| 17 | - | 149.8 | 155.8 (C) | | |
| 18 | - | 172.34 | 167.40 (C) | | |
| α | 9.36 s | 97.61 | 100.0 (CH) | | 2,2a,3,4 |
| β | 9.51 s | 97.61 | 107.1 (CH) | | 4,15 |
| γ | - | 105.36 | 106.2 (C) | | - |
| δ | 8.56 s | 93.27 | 92.8 (CH) | | 1,8,11 |

**Table 13: continued**

| Position | δ $^{1}$H (ppm) (*J* in Hz) | δ $^{13}$C (ppm), **(MT3)** solvent, chloroform | δ $^{13}$C (ppm) (MT3), solvent, tetrahydrofuran (literature) | COSY | HMBC (H→C) |
|---|---|---|---|---|---|
| 1a | 3.67 s | 12.26 | 12.6($CH_3$) | | 1,2,11 |
| 2a | 8.10 dd (5.0) | 131.97 | 131.5 (CH) | 2b | 1,2,2b |
| 2b | 7.26 d<br>6.19 d (11.5) | 122.85 | 118.9 ($CH_2$) | 2a | 2,12 |
| 3a | 2.52 s | 11.28 | 11.2 ($CH_3$) | | 3,4,13 |
| 4a | 3.64 m | 19.93 | 20.0 ($CH_2$) | | 4,15 |
| 4b | 1.71 t (7.5) | 19.50 | 18.0 ($CH_3$) | 1a,5a,8a | 4,4a |
| 5a | 3.67 s | 12.26 | 12.6 ($CH_3$) | 4b,8a | 15 |
| 7a | 2.32 m | 30.56 | 30.9 ($CH_2$) | 7 | 7,7b,7c, 7d,8 |
| 7b | 3.20 t (6.5) | 30.05 | 30.1 ($CH_2$) | | 7,7a,7c, 7d,8 |
| 7c | - | 172.56 | 172.56 (C) | | - |
| 7d | 3.58 s | 51.88 | 51.9 ($CH_3$) | | 7c |
| 8a | 1.81 d (7.5) | 23.30 | 23.9 ($CH_3$) | 1a,5a,8 | |
| 10a | - | 171.01 | 171.0 (C) | | |
| 10b | 3.88 s | 53.07. | 52.0 ($CH_3$) | | 10a |

### 4.3 Bioassay tests

The crude extracts from fruits, stem-bark, root-bark and leaves of both *Gardenia volkensii* and *Meyna tetraphylla* showed antimicrobial activity on ***Bsb***, ***SA*** and ***EC*** using 40 µL of 10 mg/mL extract solution with an inhibition zone of ≤12 mm but no activity on ***CA*** and ***ST*** as shown in **Table 14**. This is an indication that both plants can be used against microbial infections. All the controls showed no activity on all the microbes indicating that all the solvents evaporated in the one hour duration time that the paper disc was left to dry.

The **MICs** for ***Bsb, SA*** and ***EC*** were determined using different concentrations as shown in **Tables 15**, **16** and **17**. Results on **Table 16** indicated that, for all the pure compounds isolated from both plants, only **GV1** had some activity on ***SA*** when 40 µL of 10 mg/mL solution of extract was used with an **MIC** of 35 µL. This could have been due to synergism because **GV1** contained a mixture of two compounds. **GV2**, the novel compound isolated from the same crude extract did not show any activity on ***SA*** but both compounds showed very good activities on ***EC*** with an MIC of less than 20 µL of 10 mg/ mL solution as shown in **Tables 17** and **19**. The dichloromethane fruit crude extract from which both compounds were isolated did not show any activity indicating that the concentration of the most active compounds in the crude extract were very small.

The isolated compounds from *Meyna tetraphylla*, **MT1**, **MT2** and **MT5** exhibited some good activity on ***EC*** when 40 µL to 20 µL of 10 mg/mL solution with an **MIC** of 20 µL solution used as shown in **Tables 17** and **19**. **Tables 15** and **19** indicated that **MT2** and **MT3** exhibited small activities on ***Bsb*** when 40 µL solution was used and an **MIC** of 35 µL solution. Their dichloromethane crude extracts did not show any activity on the entire test microbe. The controls, hexane, dichloromethane and methanol showed no activity as indicated in **Table 14**. Antibiotics that were used as positive controls only doxycycline® and tetracycline® showed activities when 10 µL solution was used on ***Bsb***, ***EC*** and ***SA*** as shown in **Table 18**. Amoxil® and septrin® showed no activity on ***SA*** and ***EC***. All the tests were done in duplicates.

**Table 14: Inhibition Zone Diameters (mm) of crude extracts and isolated compounds using 40 µg of 10 mg/mL solution**

| Sample | *Bsb* | *CA* | *SA* | *ST* | *EC* | Control |
|---|---|---|---|---|---|---|
| *Gardenia volkensii* stem bark ($CH_3OH$ extract) | 11 | - | 10 | - | 8 | - |
| *Gardenia volkensii* fruit seeds & fruit cover ($CH_3OH$ extract) | 7 | - | - | - | - | - |
| *Gardenia volkensii* leaves ($CH_3OH$ extract) | 10 | - | 7 | - | 9 | - |
| *Meyna tetraphylla* leaves ($CH_3OH$ extract) | 7 | - | 7 | - | 7 | - |
| *Meyna tetraphylla* root bark ($CH_3OH$ extract) | 12 | - | 10 | - | 10 | - |
| *Gardenia volkensii* fruits cover ($CH_2Cl_2$ extract) | 9 | - | 10 | - | 8 | - |
| *Gardenia volkensii* fruits seeds ($CH_2Cl_2$ extract) | 8 | - | - | - | 8 | - |
| *Meyna tetraphylla* leaves ($CH_2Cl_2$ extract) | 7 | - | - | - | - | - |
| *Gardenia volkensii* fruits cover ($CH_3(CH_2)_4CH_3$ extract) | 7 | - | 8 | - | | - |
| *Gardenia volkensii* fruits seeds ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - | - |
| GV 1 | - | - | 8 | - | 12 | - |
| GV 2 | - | - | - | - | 10 | - |
| MT 1 | - | - | - | - | 11 | - |
| MT 2 | 7 | - | - | - | 9 | - |
| MT 3 | 7 | - | - | - | - | - |
| MT 4 | - | - | - | - | - | - |
| MT 5 | - | - | - | - | 11 | - |

**Table 15: Inhibition Zone Diameters (mm) of crude extracts and isolated compounds using *Bsb* and 20 µL to 40 µL of 10 mg/mL solution**

| ***Bacillus subtilis*** | | | | | |
|---|---|---|---|---|---|
| **Sample** | **Concentrations** | | | | |
| | **40 µL** | **35 µL** | **30 µL** | **25 µL** | **20 µL** |
| *Gardenia volkensii* stem bark ($CH_3OH$ extract) | 11 | 9 | 7 | 7 | - |
| *Gardenia volkensii* fruit seeds& fruit cover ($CH_3OH$ extract) | 7 | - | - | - | - |
| *Gardenia volkensii* leaves ($CH_3OH$ extract) | 10 | 8 | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3OH$ extract) | 7 | 7 | 7 | - | - |
| *Meyna tetraphylla* root bark ($CH_3OH$ extract) | 12 | 9 | 8 | 7 | - |
| *Gardenia volkensii* fruits cover ($CH_2Cl_2$ extract) | 9 | 7 | 7 | - | - |
| *Gardenia volkensii* fruit seeds ($CH_2Cl_2$) | 8 | 7 | 7 | - | - |
| *Meyna tetraphylla* leaves ($CH_2Cl_2$ extract) | 7 | - | - | - | - |
| *Gardenia volkensii* fruits cover ($CH_3(CH_2)_4CH_3$ extract) | 7 | - | - | - | - |
| *Gardenia volkensii* fruits seeds ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| GV 1 | - | - | - | - | - |
| GV 2 | - | - | - | - | - |
| MT 1 | - | - | - | - | - |
| MT 2 | 7 | - | - | - | - |
| MT 3 | 7 | - | - | - | - |
| MT 4 | - | - | - | - | - |
| MT 5 | - | - | - | - | - |

**Table 16: Inhibition Zone Diameters (mm) of crude extracts and isolated compounds using *SA* and 20 µL to 40 µL of 10mg/mL solution**

| ***Staphylococcus aureus*** | | | | | |
|---|---|---|---|---|---|
| **Sample** | **Concentrations** | | | | |
| | **40 µL** | **35 µL** | **30 µL** | **25 µL** | **20 µL** |
| *Gardenia volkensii* stem bark ($CH_3OH$ extract) | 10 | 7 | - | - | - |
| GV fruit & cover ($CH_3OH$ extract) | - | - | - | - | - |
| GV *Gardenia volkensii* leaves ($CH_3OH$ extract) | 7 | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3OH$ extract) | 7 | - | - | - | - |
| *Meyna tetraphylla* root bark ($CH_3OH$ extract) | 10 | 7 | - | - | - |
| *Gardenia volkensii* fruits cover ($CH_2Cl_2$ extract) | 10 | - | - | - | - |
| *Gardenia volkensii* fruit cover ($CH_2Cl_2$ extract) | - | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_2Cl_2$ extract) | - | - | - | - | - |
| *Gardenia volkensii* fruits cover ($CH_3(CH_2)_4CH_3$ extract) | 8 | - | - | - | - |
| *Gardenia volkensii* fruits seeds ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| GV 1 | 8 | - | - | - | - |
| GV 2 | - | - | - | - | - |
| MT 1 | - | - | - | - | - |
| MT 2 | - | - | - | - | - |
| MT 3 | - | - | - | - | - |
| MT 4 | - | - | - | - | - |
| MT 5 | - | - | - | - | - |

**Table 17: Inhibition Zone Diameters (mm) of crude extracts and isolated compounds using *EC* and 20 µL to 40 µL of 10mg/mL solution**

| ***Escherichia coli*** | | | | | |
|---|---|---|---|---|---|
| **Sample** | **Concentrations** | | | | |
| | **40 µL** | **35 µL** | **30 µL** | **25 µL** | **20 µL** |
| *Gardenia volkensii* stem bark ($CH_3OH$ extract) | 8 | 7 | - | - | - |
| *Gardenia volkensii* fruit seeds & fruit cover ($CH_3OH$ extract) | - | - | - | - | - |
| *Gardenia volkensii* leaves ($CH_3OH$ extract) | 9 | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3OH$ extract) | 7 | - | - | - | - |
| *Meyna tetraphylla* root bark ($CH_3OH$ extract) | 9 | | - | - | - |
| *Gardenia volkensii* fruits cover ($CH_2Cl_2$ extract) | 7 | - | - | - | - |
| *Gardenia volkensii* fruit seeds ($CH_2Cl_2$ extract) | 7 | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_2Cl_2$ extract) | - | - | - | - | - |
| *Gardenia volkensii* fruits cover ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| *Gardenia volkensii* fruits seeds ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| *Meyna tetraphylla* leaves ($CH_3(CH_2)_4CH_3$ extract) | - | - | - | - | - |
| GV 1 | 12 | 11 | 9 | 9 | 9 |
| GV 2 | 10 | 9 | 8 | 8 | 8 |
| MT 1 | 11 | 10 | 10 | 9 | 9 |
| MT 2 | 9 | 9 | 7 | 7 | 7 |
| MT 3 | - | - | - | - | - |
| MT 4 | - | - | - | - | - |
| MT 5 | 11 | 9 | 9 | 8 | 7 |

**Table 18: Inhibition Zone Diameters (mm) of antibiotics using 10 µL of 10mg/mL solution**

| **Antibiotics 10 µl (0.1 mg/mL)** | ***Bsb*** | ***SA*** | ***EC*** |
|---|---|---|---|
| Amoxil® | 9 | - | - |
| Septrin® | 9 | - | - |
| Doxycycline® | 30 | 15 | 15 |
| Tetracycline® | 20 | 15 | 10 |

**Table 19: MIC (mg/mL))**

| **Crude extracts** | ***Bsb*** | ***SA*** | ***EC*** |
|---|---|---|---|
| *Gardenia volkensii* stem bark ($CH_3OH$ extract) | 0.20 | 0.30 | 0.30 |
| *Gardenia volkensii* fruit seeds & fruit cover ($CH_3OH$ extract) | 0.35 | >0.40 | >0.40 |
| *Gardenia volkensii* leaves ($CH_3OH$ extract) | 0.30 | 0.35 | 0.35 |
| *Meyna tetraphylla* leaves ($CH_3OH$ extract) | 0.25 | 0.35 | 0.35 |
| *Meyna tetraphylla* root bark ($CH_3OH$ extract) | 0.20 | 0.30 | 0.35 |
| *Gardenia volkensii* fruit cover ( $CH_2Cl_2$ extract) | 0.25 | 0.35 | 0.35 |
| *Gardenia volkensii* fruit seeds ( $CH_2Cl_2$ extract) | 0.25 | >0.40 | 0.35 |
| *Meyna tetraphylla* leaves ($CH_2Cl_2$ extract) | 0.35 | >0.40 | >0.40 |
| *Gardenia volkensii* fruits cover ($CH_3(CH_2)_4CH_3$ extract) | 0.35 | 0.35 | >0.40 |
| *Gardenia volkensii* fruits seeds ($CH_3(CH_2)_4CH_3$ extract) | >0.40 | >0.40 | >0.40 |
| *Meyna tetraphylla* leaves ($CH_3(CH_2)_4CH_3$ extract) | >0.40 | >0.40 | >0.40 |
| GV1 | >0.40 | 0.35 | <0.20 |
| GV2 | >0.40 | >0.40 | <0.20 |
| MT1 | >0.40 | >0.40 | <0.20 |
| MT2 | 0.35 | >0.40 | >0.20 |
| MT3 | 0.35 | >0.40 | >0.40 |
| MT4 | >0.40 | >0.40 | >0.40 |
| MT5 | >0.40 | >0.40 | <0.20 |

The **$IC_{50}$** for Doxycycline antibiotic on ***Bsb*** that was used for comparison with the most active crude extracts was 0.335mg/mL. This was calculated using Graphpad Prism computer program using different concentrations as shown in **Table 20** and **figure 5**. The $IC_{50}$ for *Gardenia volkensii* stem crude extract was 1166.809 mg/mL while for *Meyna teraphylla* bark crude extract was 699.842mg/ml, 3,483 and 2,089 times that of the antibiotic respectively. These concentrations were too high compared to that of Doxycycline antibiotic meaning the concoction has to be taken at higher dose to induce its activity.

**Table 20: Inhibition Zone Diameters (mm) of Doxycycline® antibiotic on *Bacillus subtilis***

| **Concentration.(µg/mL)** | 10,000 | 4,000 | 1,000 | 400 | 100 | 40 | 10 | 4 |
|---|---|---|---|---|---|---|---|---|
| **Inhibition zone (mm)** | 30 | 26 | 22 | 21 | 17 | 12 | 12 | 10 |

**A graph of $IC_{50}$ for Doxycycline® antibiotic on *Bacillus subtilis***

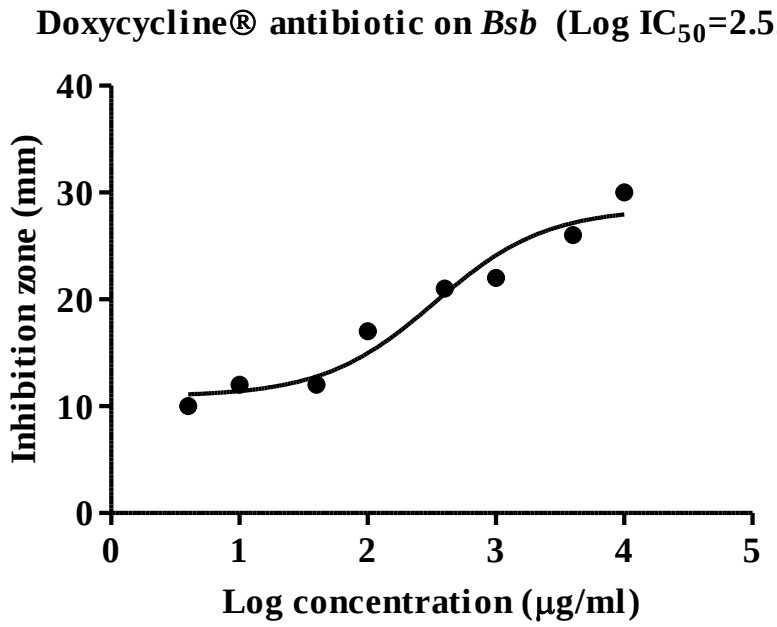



**Figure 5 A graph of $IC_{50}$ for Doxycline® antibiotic on *Bacillus subtilis***

# CHAPTER FIVE

## CONCLUSIONS AND RECOMMENDATIONS

### 5.1 Conclusion

In this project, all the crude extracts, the isolated pure **GV2** and impure **GV1** compounds from the *Gardenia volkensii* showed some antimicrobial activity at a concentration of 40 µL in 10 mg/mL. The fruit-seeds, fruit-covers, stem-bark and leaves had an inhibition of 7-11 mm **(*Bsb*),** 8-10 mm **(*SA*)** and 8-9 mm **(*EC*)**. **GV1** showed activity of 8 mm **(*SA*)** and 12 mm **(*EC*)** but non on ***Bsb*** while **GV2** showed activity of 10 mm on only ***EC*** **(Table 14)**. The **MIC** of the crude extracts was 20 µL-40 µL extract solutions **(Table 19)**. **GV1** and **GV2** had an **MIC** of 20 µL-40 µL extract solutions, an indication that this plant contained compounds that were antimicrobial. All this validates the use of these plants by the Pokots of Kenya as anti-microbial agents.

The crude extracts of the *Meyna tetraphylla* especially the root-bark and the isolated compounds showed some activity on both Gram positive and Gram negative bacteria at a concentration of 40 µL in 10 mg/mL. The root-bark and the leaves had an inhibition of 7-12 mm **(*Bsb*)**, 7-10 mm **(*SA*)** and 7-10 mm **(*EC*) Table 14**. The **MIC** of the crude extracts was 20 µL-40 µL extract solutions **(Table 19)**. **MT1** and **MT4** had no activity while **MT2** and **MT5** showed an activity of 9-10 mm on ***EC***. **MT2** and **MT3** showed an activity of 7 mm on ***Bsb.*** The **MIC** of the isolated compounds was 20 µL-40 µL extract solutions, an indication that this plant can be used as anti-microbial agents.

The $\mathbf{IC_{50}}$ for the stem-bark of *Gardenia volkensii* and the root-bark of *Meyna tetraphylla* crude extracts was too big as compared to the Doxycycline® antibiotics. Thus, the activity of the crude extracts was small and therefore a big dose of the concoction has to be administered for it to be effective.

The proposed structures of **GV2** (by NMR and MS) and **MT3** (by NMR and MS) indicated that **GV2** was a novel monoterpenoid or modified iridoid (found in almost all plants). **MT3** was identified as a chlorophyll derivative. Some chlorophyll derivatives have been shown to be cytotoxic, for example 10-Hydroxypheophytin a and 10-Hydroxyphaeophorbide a (Nakatani *et*

*al*., 1981). Compound **MT3** exhibited very small activity on ***Bsb*** at a concentration of 40 µL extract solutions. Identification of other compounds isolated from *Meyna tetraphylla* leaves, **MT1**, **MT2**, **MT4** and **MT5** was not conclusive due to lack of MS spectra.

### 5.2 Recommendations

1. Other compounds from *Gardenia volkensii* and *Meyna tetr*aphylla should be isolated and screened for antimicrobial activities.
2. The mixture of compound **GVI** should be separated and the structures elucidated.
3. Compounds **MT1**, **MT2**, **MT4** and **MT5** should be re-isolated and their MS and NMR analysed.
4. Since screening was done on only five strains of micro-organisms, (*Bacillus subtilis, Escherichia coli, Staphylococuus aureus, Cadida albicans and Salmonella typhimurium*) other strains should also be tested.
5. Toxicity of the isolated compounds should be determined for health purposes.
6. No literature on the genus *Meyna* was found thus the need to do some research on other species in this genus.

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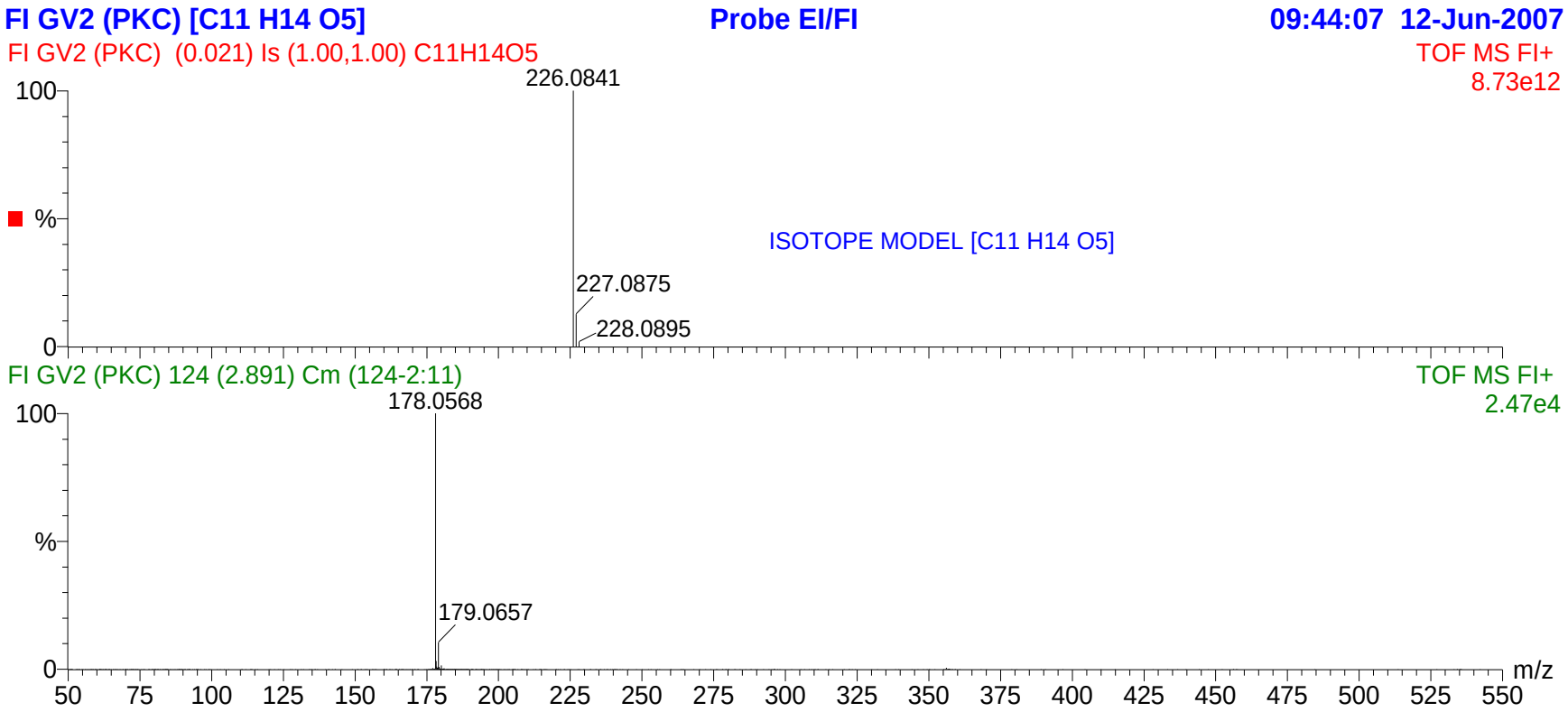
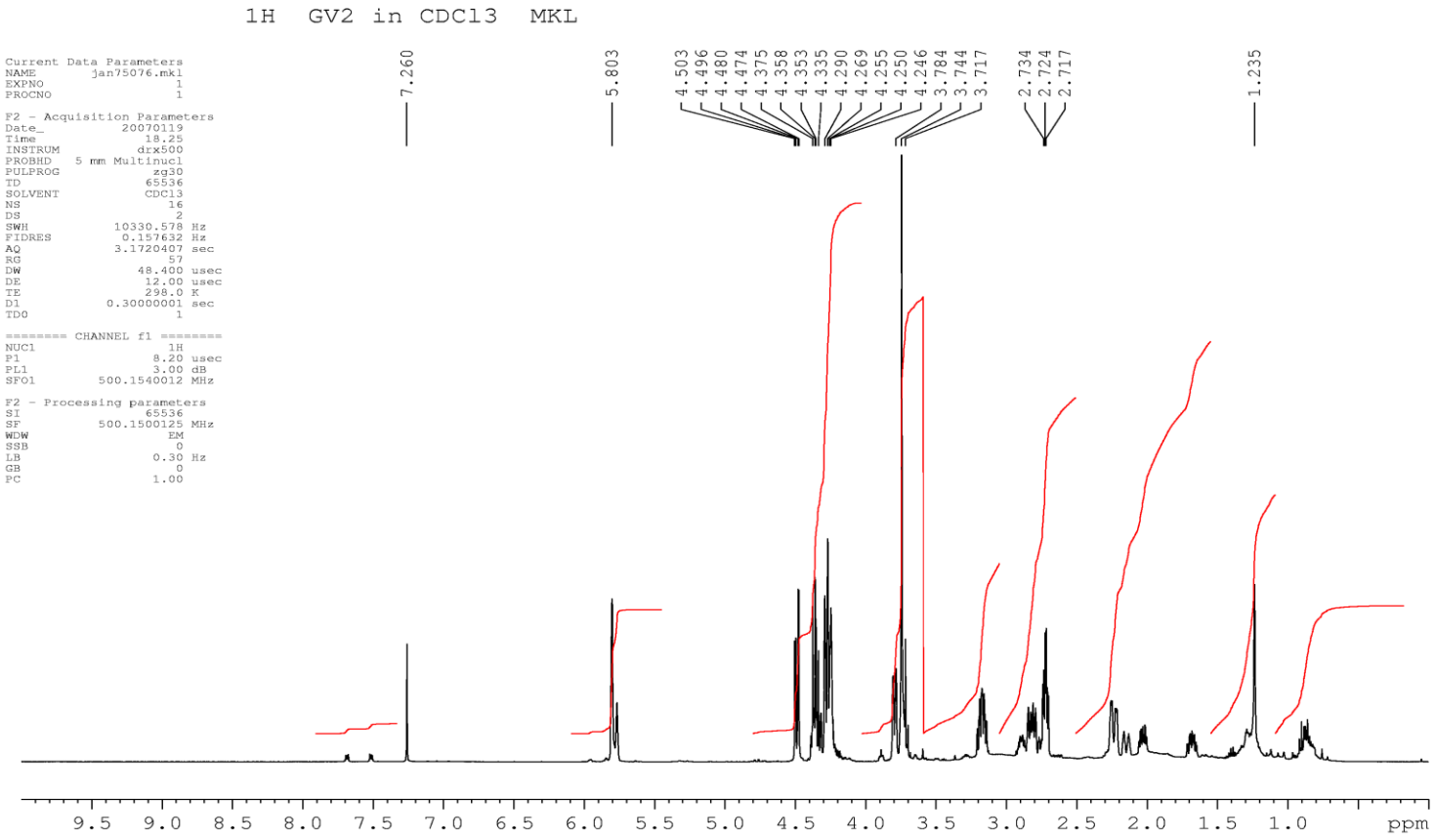
## APPENDICES

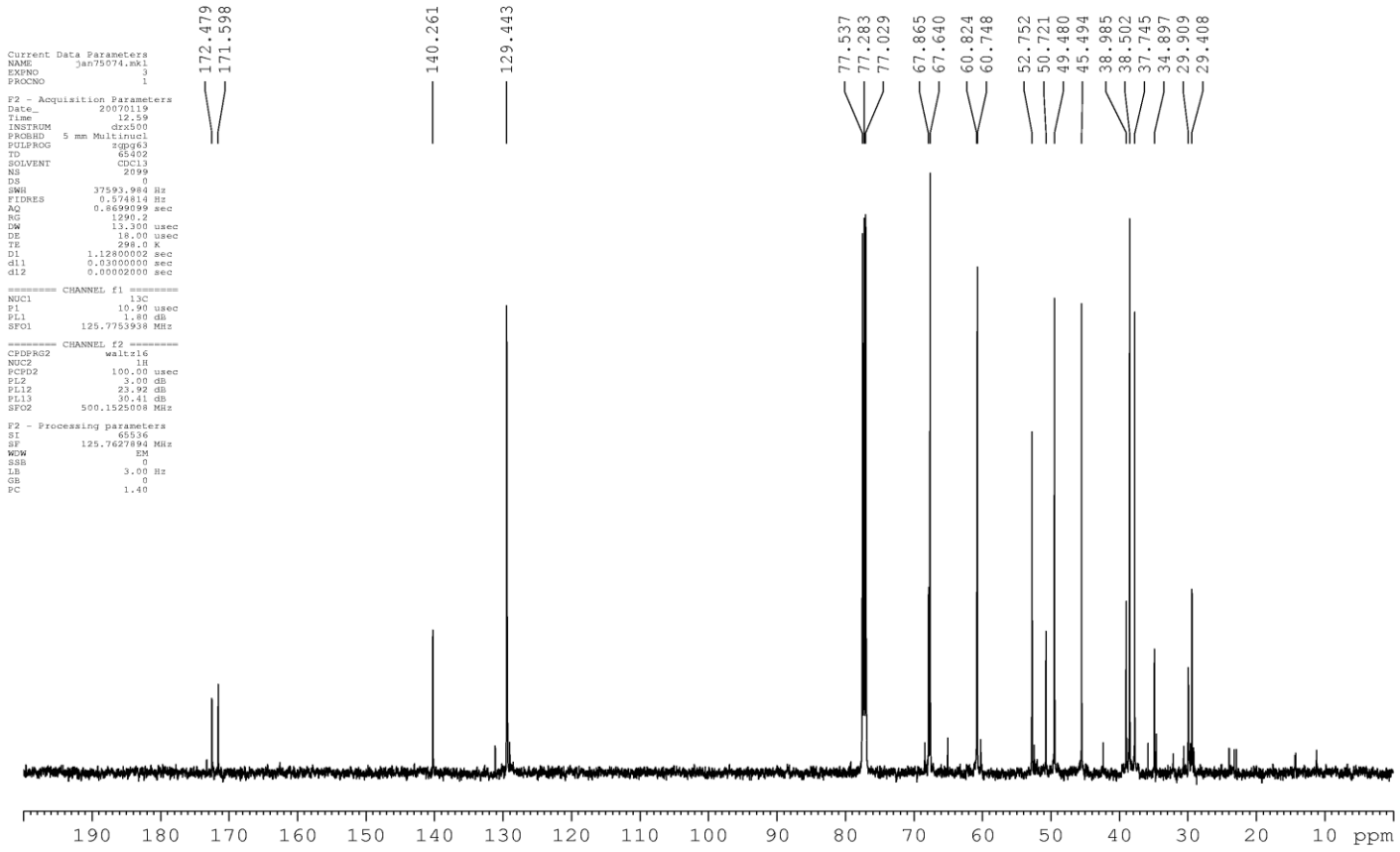
### Appendix 1: MS of GV2 (Monoterpenoid)



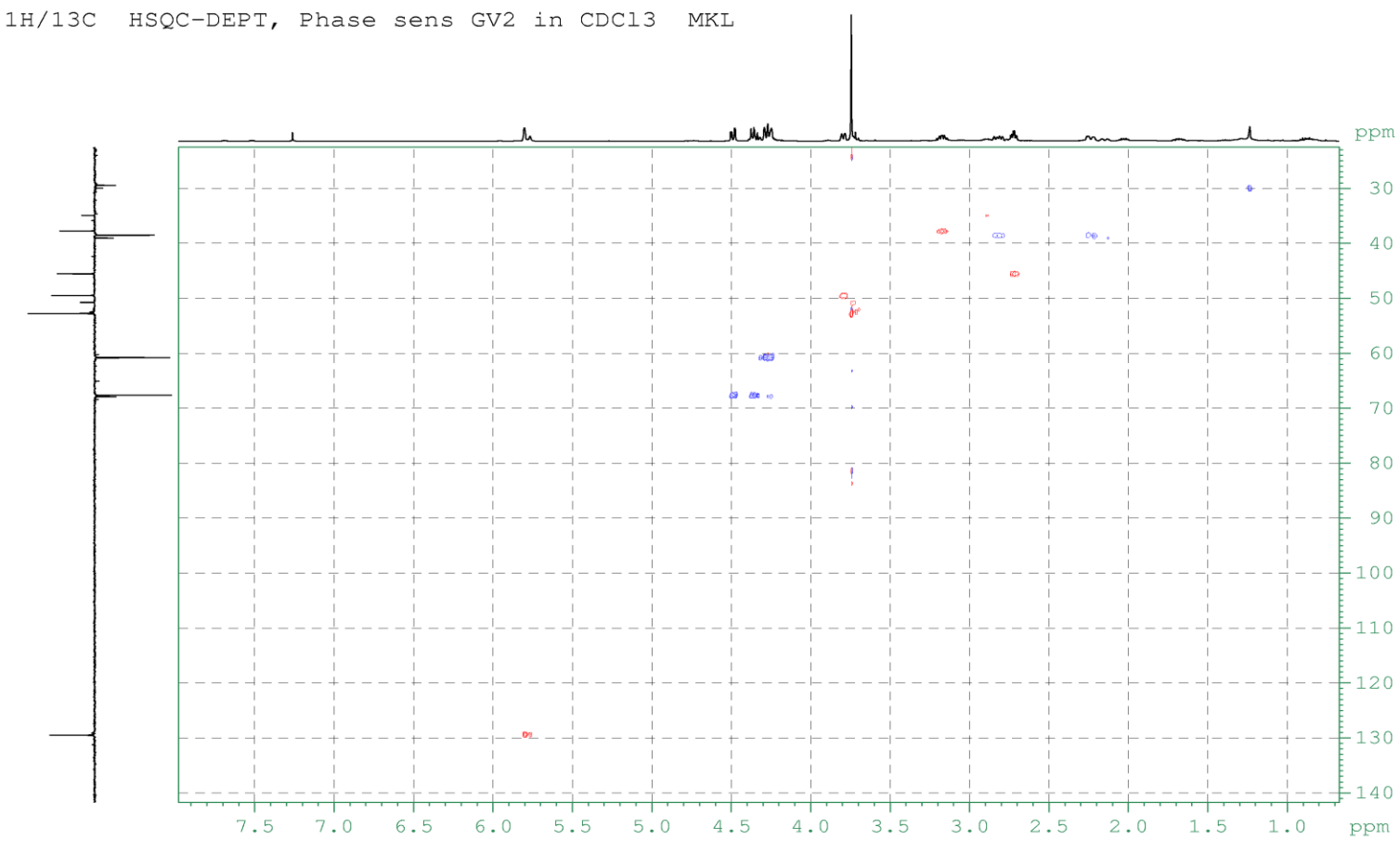
**Appendix 2: $^{1}$H NMR of GV2 (Monoterpenoid)**



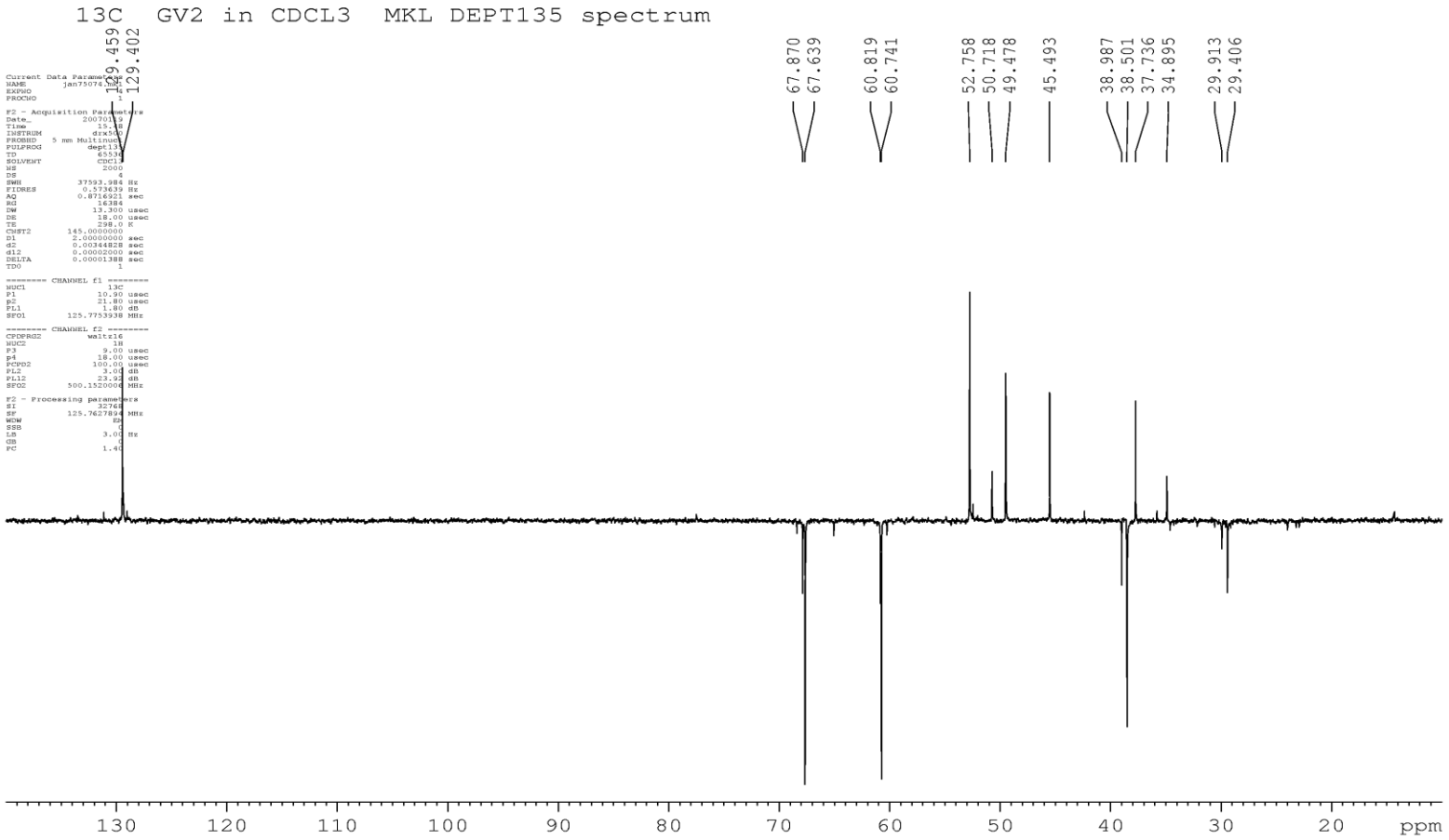
## Appendix 3: $^{13}$C NMR of GV2 (Monoterpenoid)



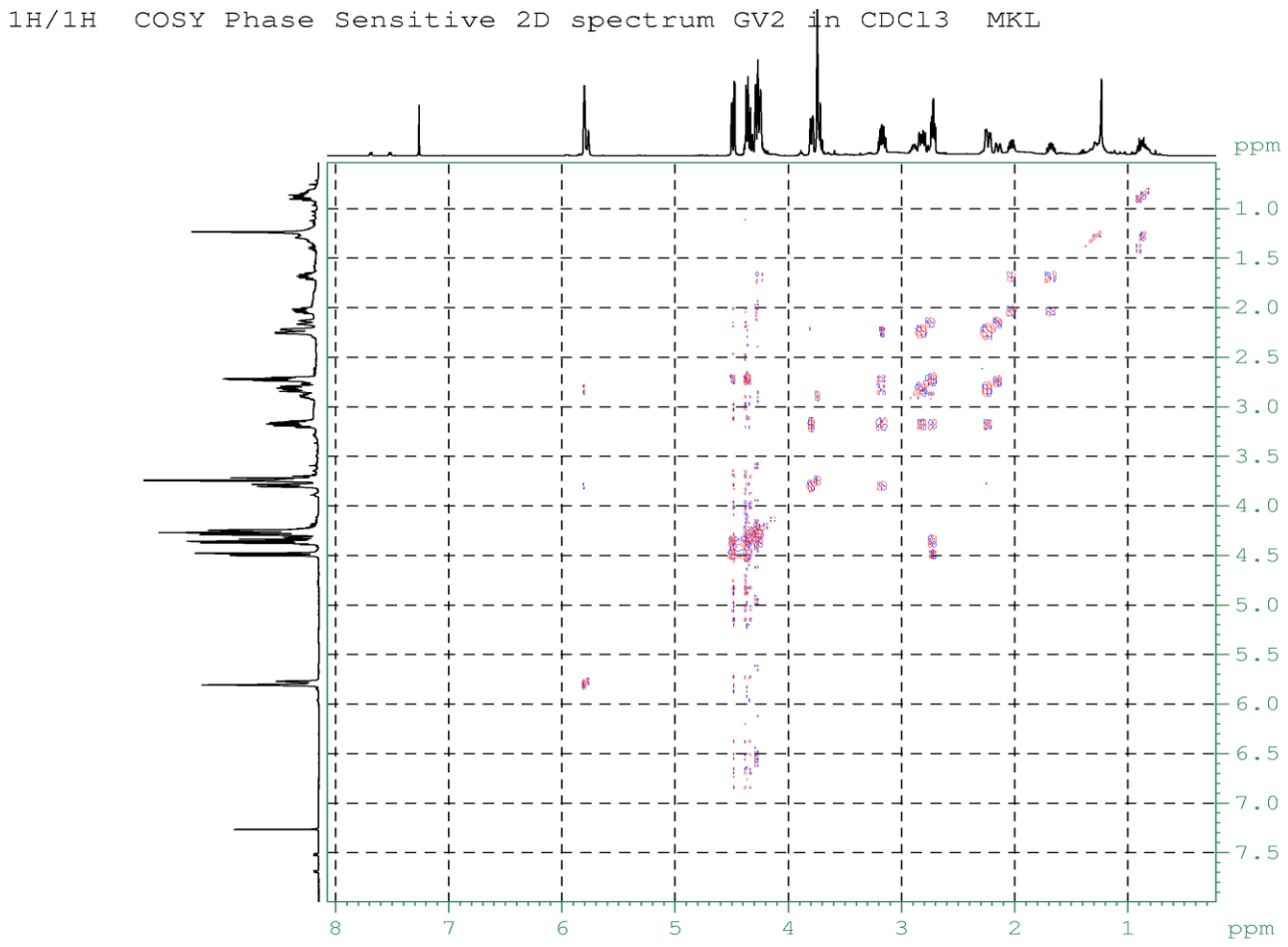
**Appendix 4: HSQC-DEPT spectrum of GV2 (Monoterpenoid)**



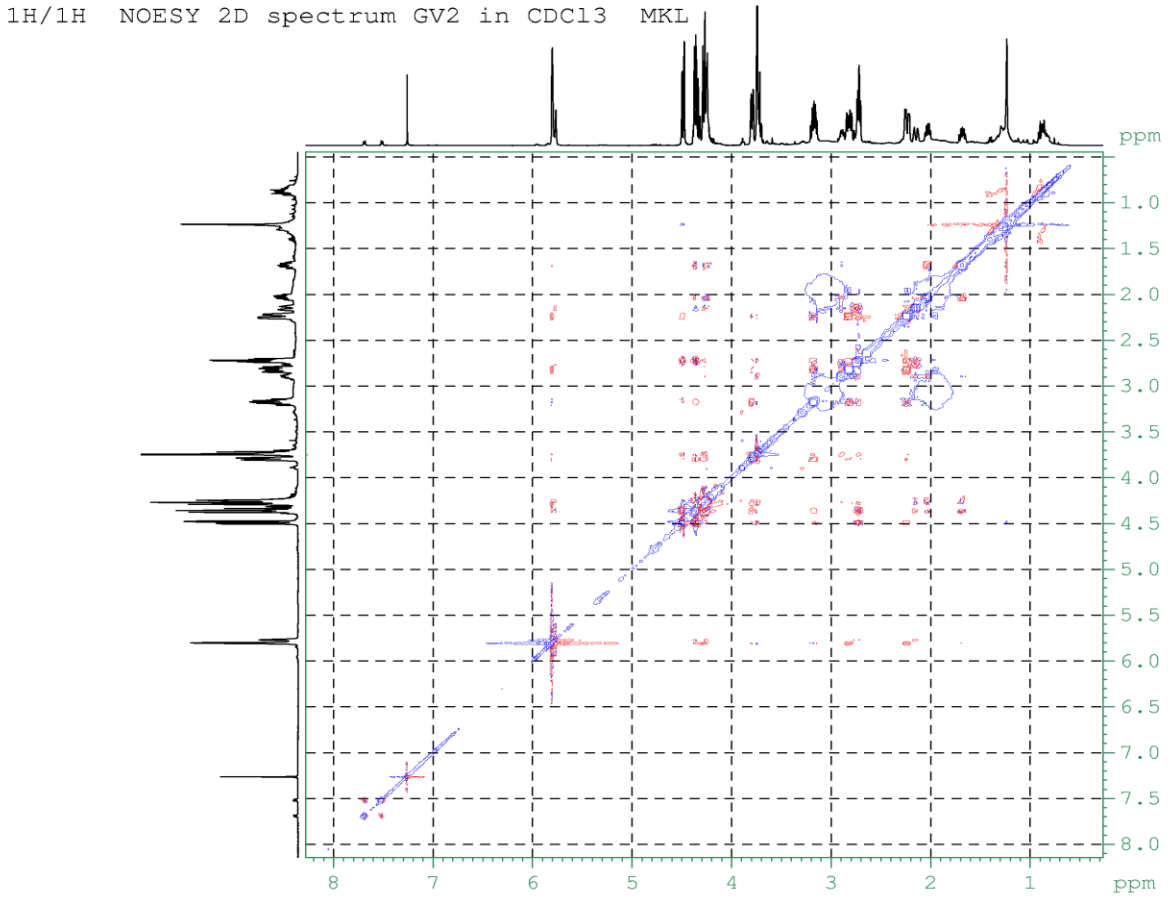
**Appendix 5: DEPT135 spectrum of GV2 (Monoterpenoid)**



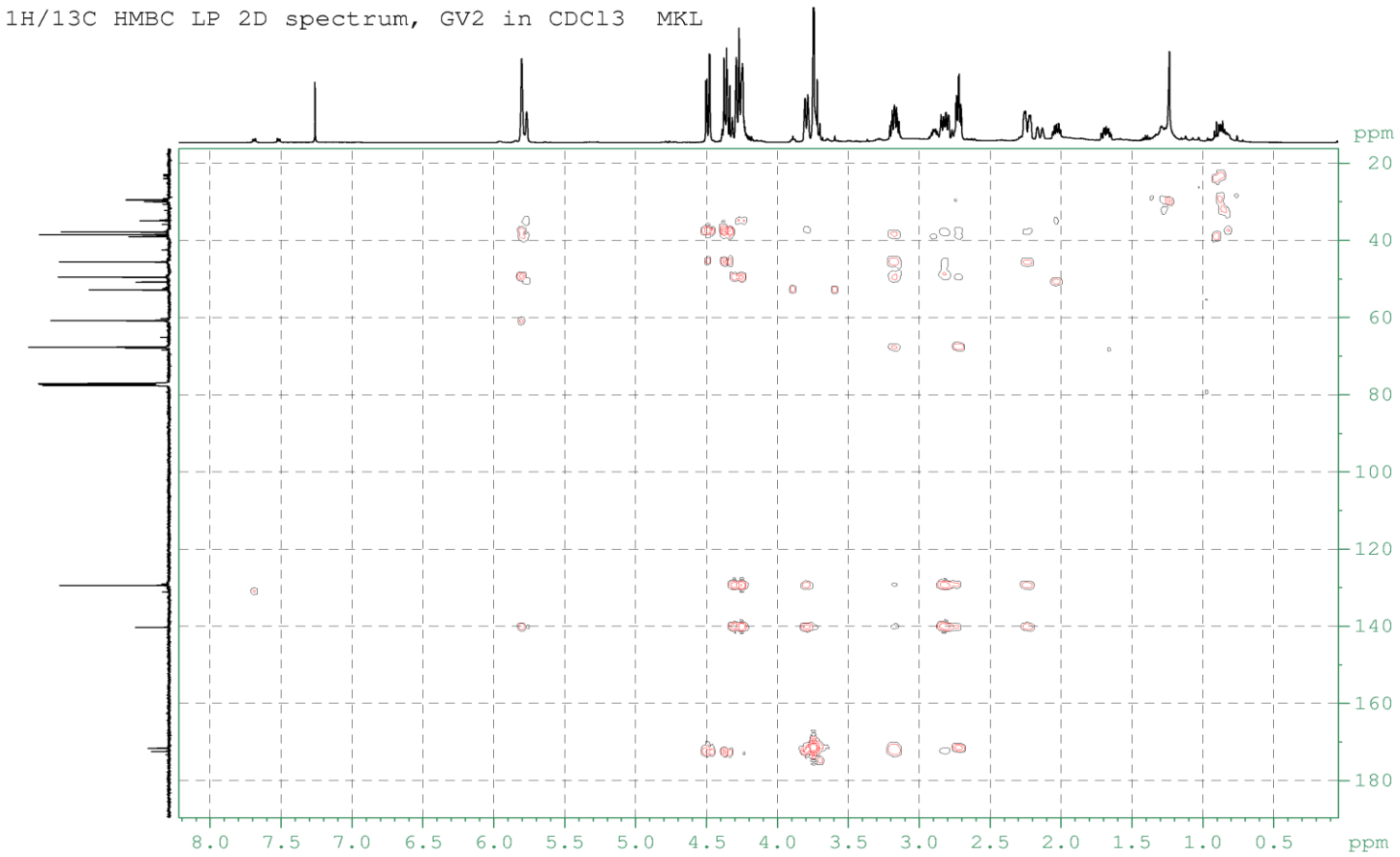
**Appendix 6: COSY of GV2 (Monoterpenoid)**



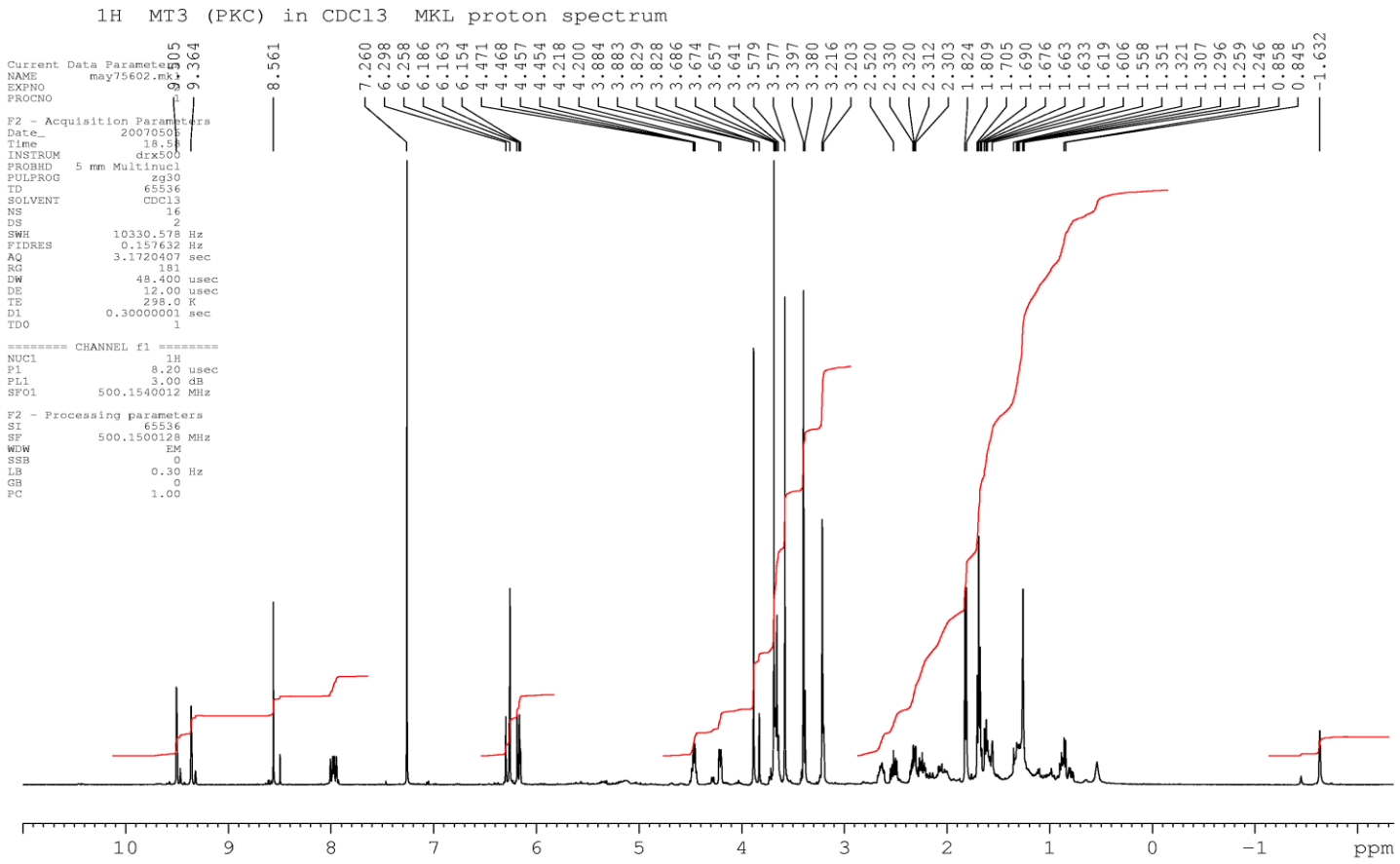
**Appendix 7: NOESY spectrum of GV2 (Monoterpenoid)**



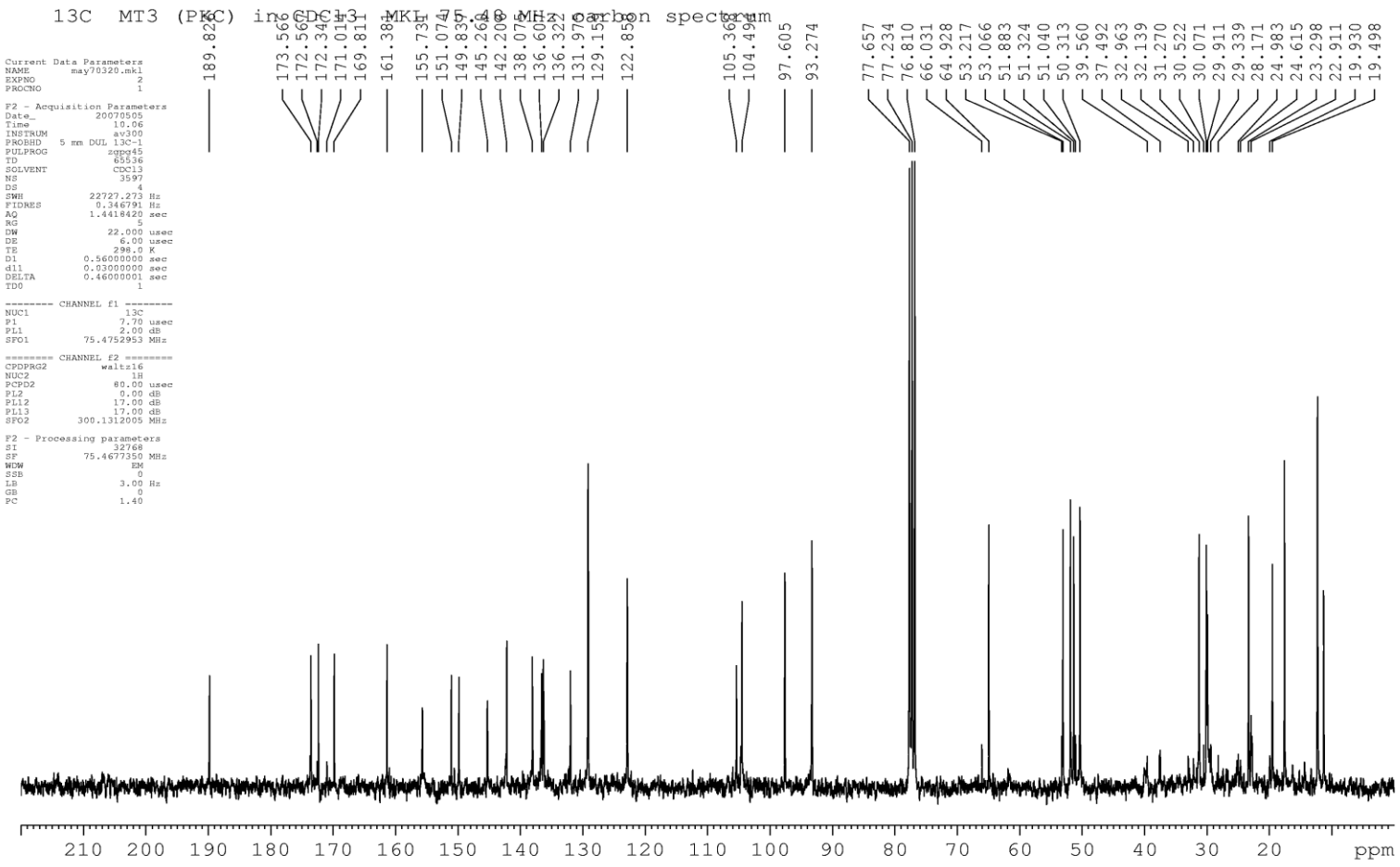
**Appendix 8: HMBC spectrum of GV2 (Monoterpenoid)**



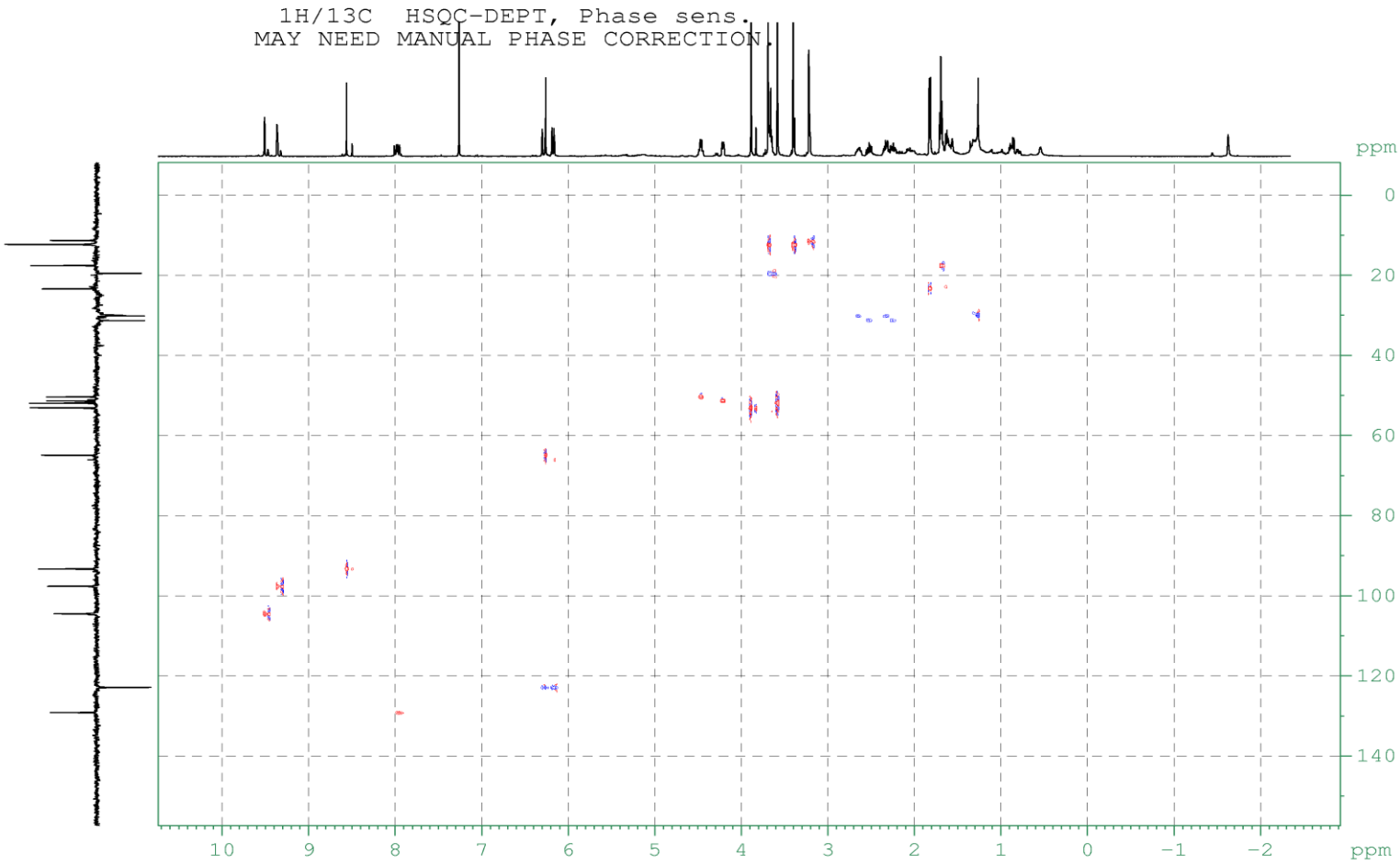
## Appendix 9: $^{1}$H NMR of MT3 (Chlorophyll derivative)



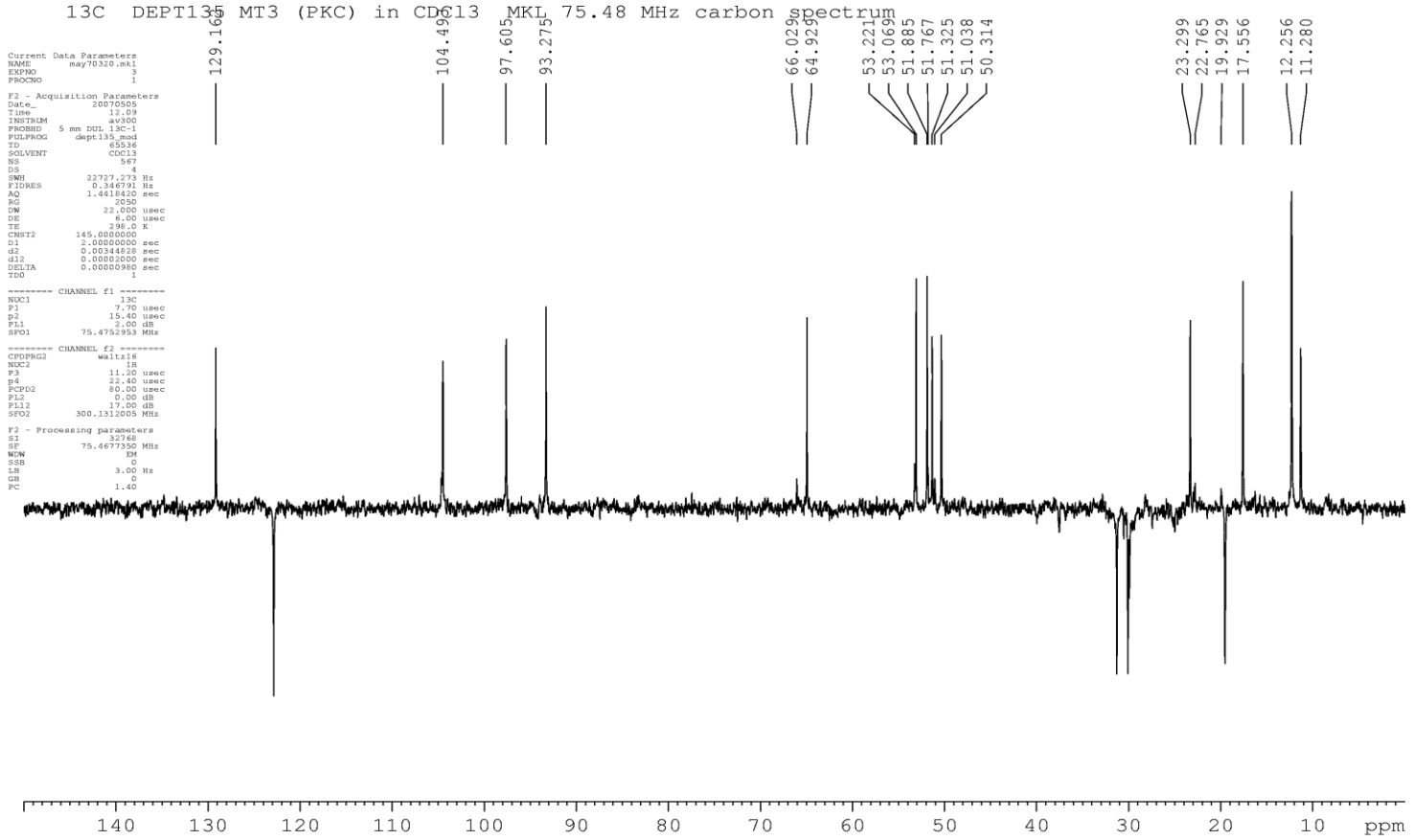
**Appendix 10: $^{13}$C NMR of MT3 (Chlorophyll derivative)**



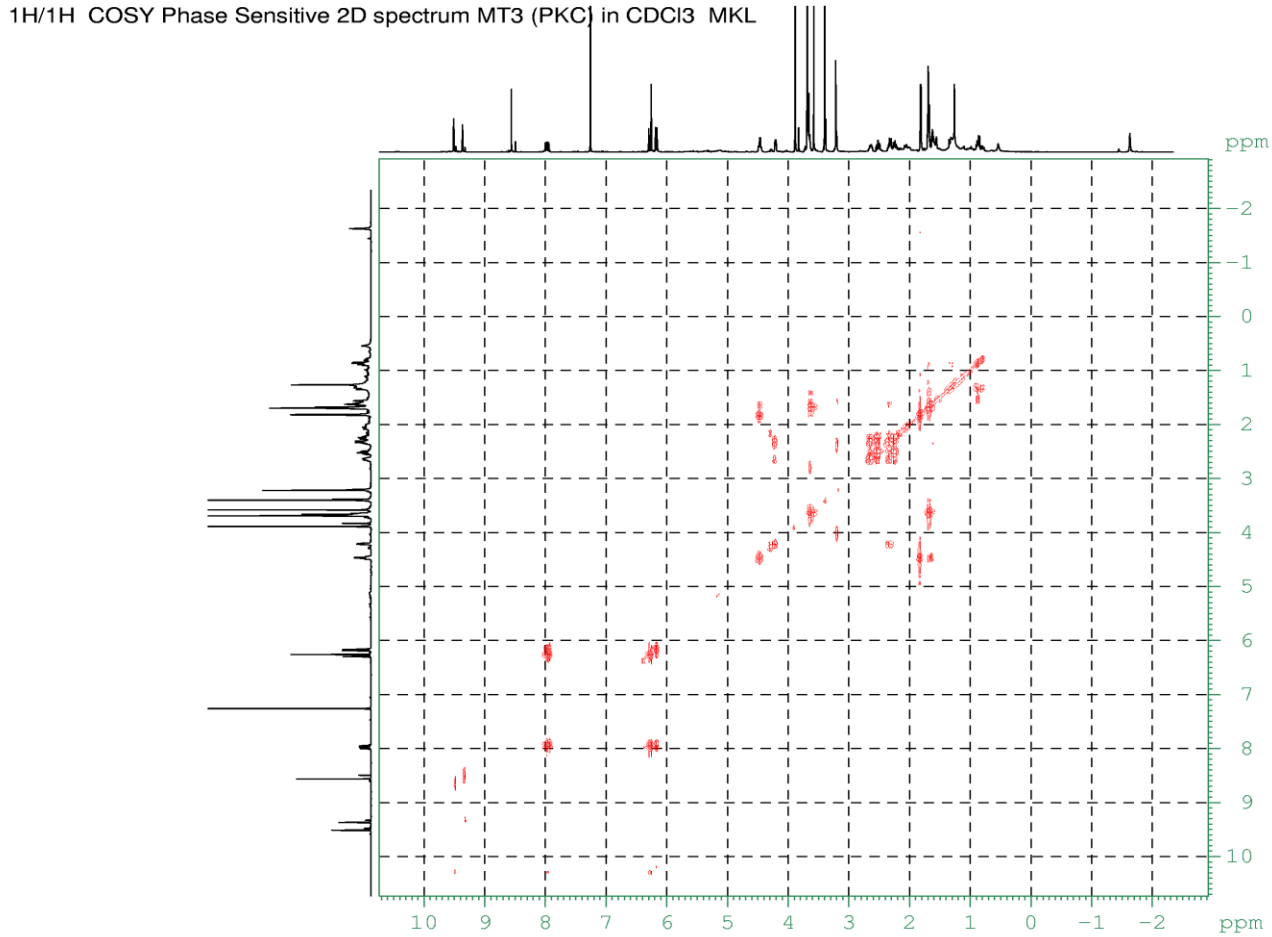
**Appendix 11: HSQC-DEPT of MT3 (Chlorophyll derivative)**



**Appendix 12: DEPT135 of MT3 (Chlorophyll derivative)**



**Appendix 13: COSY of MT3 (Chlorophyll derivative)**



**Appendix 14: HMBC of MT3 (Chlorophyll derivative)**