Chemical Constituents and Biological Studies of *Tagetes minuta* L. and *Rauvolfia caffra* Sond.

University of Fort Hare

Together in Excellence

This Dissertation is submitted to the Faculty of Science and Agriculture, Chemistry Department, University of Fort Hare in Partial Fulfillment of the Requirements for the Master of Science Degree in Organic Chemistry

By

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Co-Supervisor: Prof. B.N. Nkeh-Chungag

2015

DECLARATION

I, **Sithenkosi Mlala**, student number 201415099, solemnly declare that this dissertation entitled "Chemical Constituents and Biological Studies of *Tagetes minuta* L. and *Rauvolfia caffra* Sond." is my original work. All sources used or quoted in the study have been indicated and acknowledged by way of complete references.

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CONFERENCES, WORKSHOPS AND PUBLICATIONS

Conferences and Workshops Attended:

1. S Mlala, OO Oyedeji, AO Oyedeji: Chemical Composition and Toxicity Test of the Essential Oils Isolated from *Acacia mearnsii*, Poster presentation at Royal Society of Chemistry (RSC), 2nd International Symposium on Natural Products at Lagoon Beach hotel in Cape Town, South Africa, 23rd of September 2014 to 25th of September 2014.

2. S. Mlala, O.O. Oyedeji, and B.N. Nkeh-Chungag: Using Indigenous Medicinal Plants to Manage Hypertension in Rural Communities in South Africa, Oral presentation at Indigenous Knowledge System (IKS) Interface 3, Burgers Park Hotel in Pretoria, South Africa, 27th of February 2015 to 1st March 2015.

3. S. Mlala, O.O. Oyedeji, and B.N. Nkeh-Chungag: Chemical Constituents and Biological Studies of *Tagetes minuta* and *Rauvolfia caffra* Oral presentation at 1st UNIVEN-WSU International Research Conference at International Conference Centre (ICC), East London, South Africa on the 2nd of September 2015 to 4th September 2015.

4. S. Mlala, O.O. Oyedeji, and B.N. Nkeh-Chungag: Phytochemical and Biological evaluation of *Tagetes minuta* and *Rauvolfia caffra* at University of Fort Hare Postgraduate conference at Alice on the 20th October 2015.

5. S. Mlala, O.O. Oyedeji, and B.N. Nkeh-Chungag: Chemical Constituents and Biological Studies of *Tagetes minuta* and *Rauvolfia caffra*, Oral presentation at the SACI Convention at Southern Sun Elangeni and Maharani Hotel, Durban, 29th November 2015 to 4th December 2015.

Publications:

1. **S. Mlala[a],** O.O. Oyedeji*[a], B.N. Nkeh-Chungag[b], C.R. Sewani-Rusike[b] and A.O. Oyedeji[c]; 2015. Chemical composition and Antioxidant activity of *Tagetes minuta.*
2. **S. Mlala[a],** O.O. Oyedeji*[a], B.N. Nkeh-Chungag[b], C.R. Sewani-Rusike[b] and A.O. Oyedeji[c]; 2015. Isolation, characterization, antioxidant and antihypertensive studies of *Rauvolfia caffra.*

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DEDICATION

I wish to dedicate this work to my late father

Mthatheni Elfort Mlala,

My late grandmother

Nokhululekile Stofile

Who could not witness this success, but I am sure they are smiling where they are now.

And my mother

Nobongile Patience Mlala

ABSTRACT

Green plants synthesize and store a variety of biochemical compounds some of which are extractable as raw material and can be used for various scientific investigations. Different plant parts have different effects as they often contain different active chemical components. Consequently, specific parts or the whole plant can be used for various treatments. The aim of this study was to extract, isolate and characterize the biologically active volatile and non-volatile compounds from *Tagetes minuta* and *Rauvolfia caffra* respectively.

Tagetes minuta plant was considered for extraction of essential oils using hydrodistillation method. *Cis*-β-ocimene (38.03%), caryophyllene oxide (18.04%), alloocimene (25.35 %), isopropyl tetradecanoate (17.02 %), *cis*-ocimene (38.14%) and *trans*-β-ocimene (37.03%) were the major components of essential oil analyzed from fresh stem, dried stem, fresh leaf, dried leaf, fresh flower and dried flower respectively. The volatile compounds were identified by Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography (GC). Essential oil of dried stem leaf and flower parts of *T. minuta* exhibit antioxidant activity as demonstrated by the DPPH and FRAP bioassays. Crude extracts were extracted from *R. caffra* stem bark using sequence of solvents namely n-hexane, dichloromethane, ethyl acetate, methanol and ethanol. A white powder, a β-sitosterol (non-volatile compound) was isolated by column chromatography from ethyl acetate fractions of *R. caffra* stem bark and identified on various spectroscopic techniques such as FTIR and (1D and 2D) NMR. Melting point was also determined to be a sharp 129-130 ºC. DCM, EA, MetOH and EtOH fractions as well as β-Sitosterol (compound SM/01), showed antioxidant activity when tested on DPPH, FRAP, total phenolic and flavonoid bioassays. This antioxidant activity might be due to the presence of hydroxyl groups in the compound and crude fractions. On the other hand, *T. minuta*'s essential oil showed high antioxidant activity when evaluated on the DPPH and FRAP bioassays, which can be attributed to the presence of oxygenated monoterpenes and sesquiterpenes known to act as free radical scavenging and reducing agents.

The use of *R. caffra* stem bark extracts against hypertension and other diseases by traditional healers could be attributed to the presence of phytochemicals (polyphenols and flavonoids) with known health benefits. Thus, it is recommended that the plant should be exploited further using modern techniques involving separation and purification of compounds that can

be used for drug formulation. This study supports the use of *T. minuta* and *R. caffra* as the potential natural antioxidant source to manage various diseases including hypertension.

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List of Abbreviations

^{13}C-NMR: Carbon Nuclear Magnetic Resonance

1D: One Dimensional

^{1}H-NMR: Proton Nuclear Magnetic Resonance

2D: Two Dimensional

AA: Ascorbic acid

AACT: Acetoacetyl-CoA Thiolase

$AlCl_3$: Aluminium chloride

ANOVA: Analysis of Variances

BA: betulinic acid

CC: Column Chromatography

COSY: Correlated Spectroscopy

DCM: Dichloromethane

DEPT: Distortionless Enhancement of Polarization *Trans*fer

dH_2O: distilled water

DMADP: dimethylallyl diphosphate

DMAPP: Dimethylallyl Diphosphate

DPP: deoxyxylulose phosphate

DPPH: (2,2-diphenyl-1-picrylhydrazyl)

EA: Ethyl Acetate

EAC: ethyl acetate compound

ECI: Electron Capture Ionization

EI: Electron Impact

ETOH: Ethanol

EtOH: Ethanol

FDP: Farnesyl diphosphate

Fe^{3+}-TPTZ: ferric tripyridyltriazine

FID: Flame Ionization Detector

FRAP: Ferric Reducing Antioxidant Power

FRAP: Ferric reducing antioxidant power

FTIR: Fourier *Trans*formation Infra-Red

GC: Gas Chromatography

GC-MS: Gas Chromatography-Mass Spectrometry

GDP: Geranyl diphosphate

GLC: Gas Liquid Chromatography

Hex: Hexane

HMBC: Heteronuclear Multiple Bond Correlation

HMG: CoA-3-hydroxy-3-methylglutaryl

HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA

HMGR: CoA-3-hydroxy-3-methylglutaryl Reductase

HMQC: Heteronuclear Multiple Quantum Correlation

IDP: Isopentenyl diphosphate

iPD: (-)-*trans*-isopiperitenol dehydrogenase

iPI: (+)-*cis*-isopulegone isomerase

IPP: Isopentenyl Diphosphate

kg: kilogram

L: litter

L3OH: (-)-limonene-3-hydroxylase

LD_{50}: 50 % Lethality Dose

MEP: 2C-methyl-D-erythritol-4-phosphate

MEP: Methylrythritol phosphate

MetOH: Methanol

MEV: Mevalonic acid

MFS: (+)-menthofuran synthase

mg: milligram

mL: milliliter

mmHg: millimeter of mercury

MR: (-)-menthone reductase

MS: Mass Spectrometry

MVA: Mevalonate

MW: Molecular weight

nm: nanometer

NMR: Nuclear Magnetic Resonance

NOESY: Nuclear Overhauser Enhancement Spectroscopy

OA: oleanolic acid

ppm: parts per million

PR: (+)-pulegone reductase

PT: prenyl *trans*ferases

SEM: standard error of mean

TLC: Thin Layer Chromatography

TMDF: *Tagetes minuta* dried flower

TMDL: *Tagetes minuta* dried leaf

TMDS: *Tagetes minuta* dried stem

TMFF: *Tagetes minuta* fresh flower

TMFL: *Tagetes minuta* fresh leaf

TMFS: *Tagetes minuta* fresh stem

UA: ursolic acid

USA: United States of America

UV-Visible: Ultraviolet-visible

X: mean

µg/mL: micro gram per milliliter

µL: microliter

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Background of the study

Unlike human beings and animals, plants do not have a specific immune system but rather depend on the presence of a number of compounds to defend themselves against parasites and predators. Parasites that attack plants use similar biochemical pathways to those attacking human beings; therefore many compounds utilized by plants in their defense may be used to treat human parasitic diseases. Thus, use of plants by humans as medicine enables them to benefit from protective effects of these chemicals (Willcox and Gilbert, 2012).

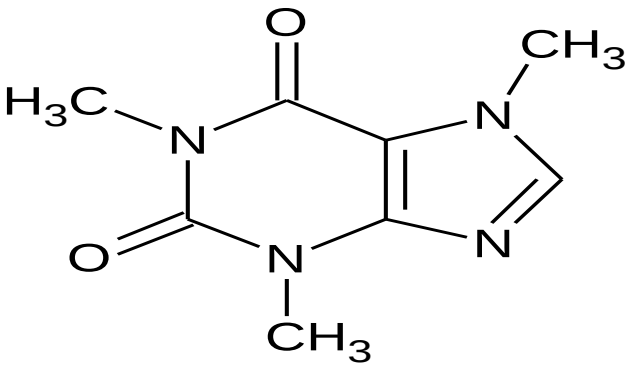
The term medicinal plants include various types of plants which are used in herbalism. These medicinal plants are considered as rich resources of ingredients which can be used in drug development and synthesis. Medicinal plants are bioactive phytochemical or bionutrient rich sources. Plants synthesize a broad range of secondary metabolites or phytochemicals, including alkaloids and terpenoids that are toxic to herbivores and pathogens (Wittstock and Gershenzon, 2002). Phytochemicals are naturally occurring biologically active compounds which may be of greater health benefits to humans than those attributed to macronutrients and micronutrients (Phillipson, 2001).

Green plants synthesize and store a variety of biochemical compounds some of which extractable as raw material and can be used for various scientific investigations (Joy et al., 1998). The Industrial Revolution and Advances in Organic Chemistry led to the preference of synthetic products for pharmacological treatment (Rates, 2001). Different plant parts may have different effects as they may often contain different active ingredients. Consequently, specific parts of the plant may be used or the whole plant for various treatments (Suliman, 2011).

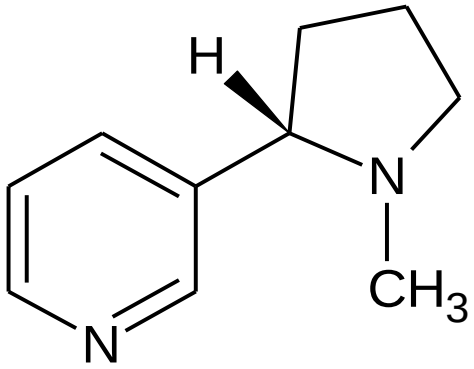
1.2 Alkaloids

Alkaloids are a unique group of low molecular weight with nitrogen-containing compounds which are mostly derived from amino acids. About 20% of all plant species contains alkaloids. Due to their powerful biological activity, approximately 12 000 known alkaloids have been utilized as pharmaceuticals i.e. as stimulants, narcotics and poisons. Plant derived alkaloids which are used clinically include codeine, morphine, colchicines, papaverine and

ajmaline. Caffeine, nicotine, cocaine and *O*-diacetylated morphine derivative heroin are other well-known alkaloids of plant origin (Crozier et al., 2006).



Caffeine



Nicotine

1.3 Terpenoids

Terpenoids are defined as hydrocarbons of plant origin with the general formula $(C_5H_8)_n$ as well as their oxygenated, hydrogenated and dehydrogenated derivatives (Yadav et al., 2014). Terpenoids and steroids are synthesized from isoprenoid (C_5) units that are directly derived from isopentenyl (3-methyl-3-ene-1-yl) pyrophosphate. These isoprenoid units are linked together in a head-to-tail manner. Terpenoids create one of the largest groups of natural products accounting for more than 40 000 compounds with many more new compounds being discovered every year. Terpenoids are found in almost all plant species and they are essential for plant growth, metabolism and development. Terpenoids are generally alicyclic compounds and isomerism is commonly found. Some of the terpenoids are synthesized by organisms such as bacteria and yeast as a part of primary or secondary metabolism; however, most terpenoids originate from plants. Terpenoids are classified into several classes; such as monoterpenoids, diterpenoids, triterpenoids and tetraterpenoids. Terpenoids have been shown to have anti-cancer, antimicrobial, anti-fungal, antiviral, anti-parasitic, immunomodulatory, anti-allergenic, anti-inflammatory, antispasmodic, and anti-hyperglycemic properties. There are several biosynthesis pathways of plant terpenoids which mainly use enzymes such as mevalonate (MVA), mevalonic acid (MEV) for cytosol, 2C-methyl-D-erythritol-4-phosphate (MEP) for plastids and deoxyxylulose phosphate (DPP) pathways (Thoppil and Bishayee, 2011; Dhanarasu, 2012; Bohlmann and Keeling, 2008).

1.3.1 Mevalonate (MVA) Pathway:

A formal type Claisen condensation reaction of two acetyl-CoA molecules produces acetoacetyl-CoA catalyzed by acetoacetyl-CoA thiolase. A type of aldol condensation addition of another acetyl-CoA molecule yields 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) catalyzed by HMG-CoA synthase. Consequent reduction of the 3-hydroxy-3-methylglutaryl-CoA to (R)-mevalonic acid by HMG-CoA reductase through the use of NADPH fragments. Formed mevalonic acid to mevalonic acid-5-diphosphate through phosphorylation process catalyzed by mevalonate and phosphomevalonate kinases. Phosphorylation assisted decarboxylation generating isopentenyl diphosphate (IPP). IPP converted to dimethylallyl diphosphate (DMAPP) through isomerization (Tesso, 2005).

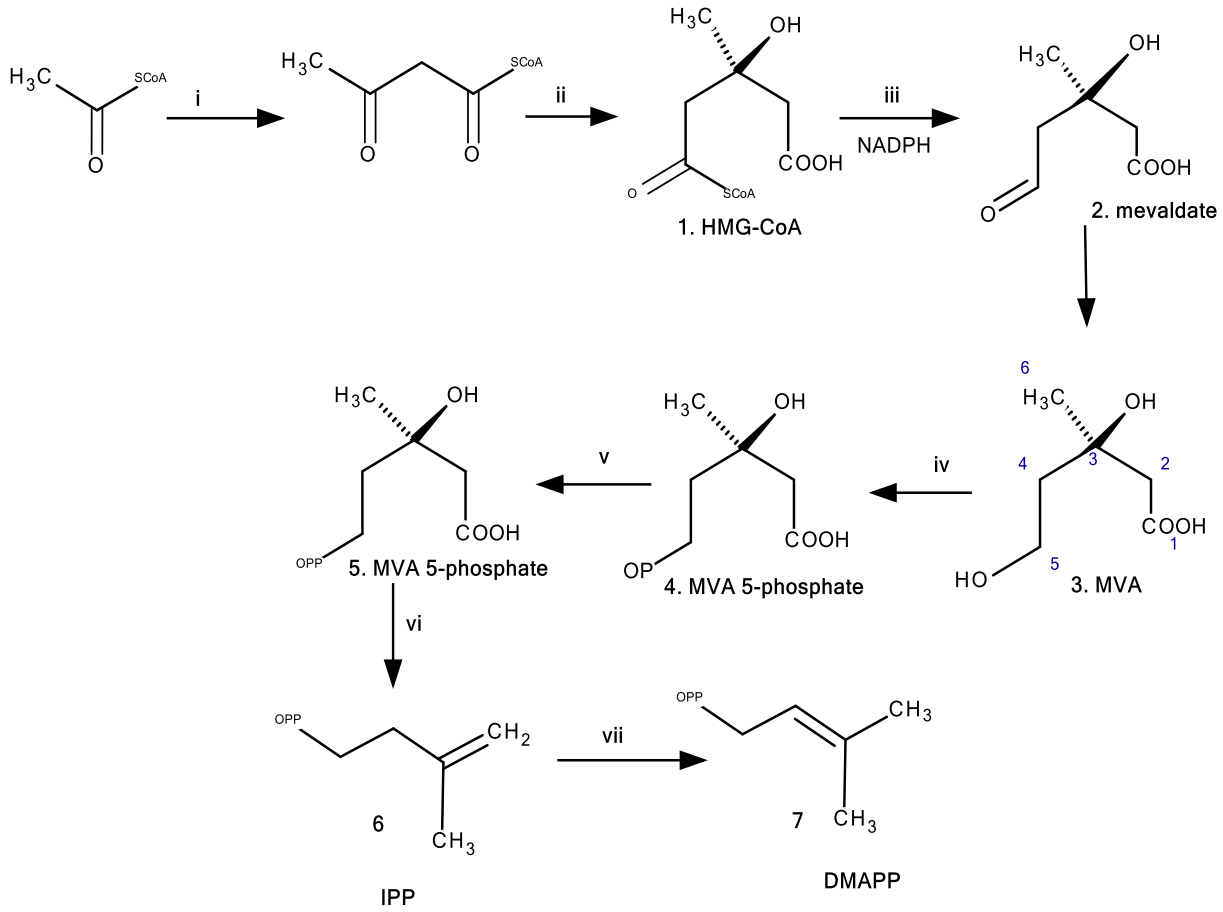


Figure 1.1: Biosynthesis of terpenoids through Mevalonate pathway

Key steps in Mevalonate pathway. Important precursors are dimethylallyl diphosphate (DMAPP), isopentenyl diphosphate (IPP) and CoA-3-hydroxy-3-methylglutaryl (HMG). Enzymes: i, acetoacetyl-CoA thiolase (AACT); ii, HMG-CoA synthase; iii, HMG-CoA

reductase (HMGR); iv, mevalonate kinase; v, phosphomevanolate kinase; vi, mevalonate 5-diphosphate decarboxylase; vii, Isopentenyl diphosphate (IPP) isomerase.

1.3.2 MEP and MEV Biosynthesis Pathway of Terpenoids in Plants

Figure 1.2 displays two biosynthesis pathways namely mevalonic acid (MEV) for cytosol and 2C-methyl-D-erythritol-4-phosphate (MEP) for plastids. Various metabolic pathways of plant terpenoids are all fixed in the formation of only two isomeric C_5 precursors namely dimethylallyl diphosphate **(**DMADP) and Isopentenyl diphosphate (IDP). Terpenoid synthase (TPS) activity can result in the establishment of the smallest plant terpenoids which are hemiterpenoids (C_5) directly from DMADP. Alternatively, prenyl *trans*ferases (PT) naturally occur in plants as both homeric and modular heteromic enzymes. Condensation of FDP and GGDP yields the classes of triterpenoids (C_{30}) and tetraterpenoids (C_{40}) (Bohlmann and Keeling, 2008).

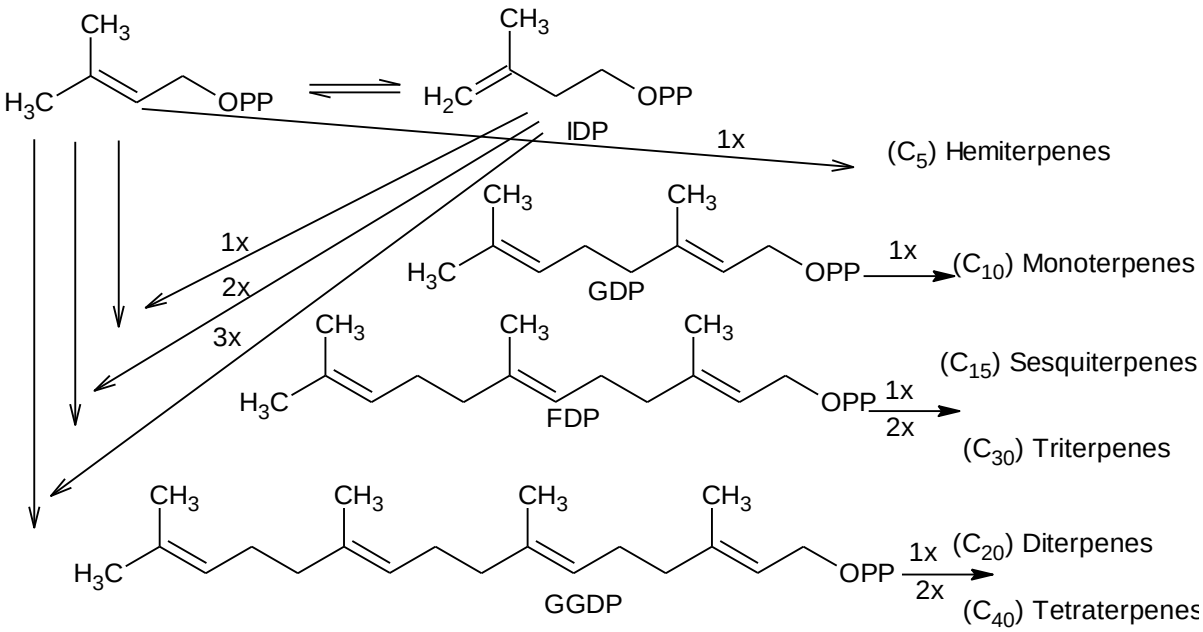


Figure 1.2: Biosynthesis pathways of plant terpenoids.

Key steps: Dimethylallyl diphosphate **(**DMADP), Farnesyl diphosphate (FDP), Geranylgerany diphosphate (GGDP), Isopentenyl diphosphate (IDP), Methylrythritol phosphate (MEP), Geranyl diphosphate (GDP)

1.3.3 Biosynthesis of (-)-menthol and other related monoterpenes

(-)-Menthol is the known major component of the essential oil from peppermint plant. Biosynthesis of (-)-menthol from GDP is achieved through a series of enzymatic reaction steps starting with acyclic monoterpene (-)-limonene formation followed by number of redox reaction modification. (-)-limonene synthase (LS) is a multiproduct monoterpene synthase from plant that stereospecifically generates (-)-limonene with minor amounts of acyclic myrcene and bicyclic (-)-α-pinene as well as (-)-β-pinene as shown in Figure 1.1. The biosynthesis mechanism from (-)-limonene to (-)-menthol involves hydroxylation by P450 (-)-limonene-3-hydroxylase (L3OH) to form (-)-*trans*-isopiperitenol, oxidation of (-)-*trans*-isopiperitenol by NAD-dependent (-)-*trans*-isopiperitenol dehydrogenase (iPD) forms (-)-isopiperitone, formation of (+)-*cis*-isopulegone by NADPH-dependent (-)-isopiperitenone reductase (iPR), formation of (+)-pulegone from of (+)-*cis*-isopulegone by (+)-*cis*-isopulegone isomerase (iPI) isomerization, (-)-menthone formation by NADPH-dependent (+)-pulegone reductase (PR) and lastly the formation of (-)-menthol by (-)-menthone reductase (MR). Metabolites such as (+)-menthofuran (by (+)-menthofuran synthase), (+)-neomenthol, (+)-isomenthol and (+)-neoisomenthol are also achieved using this biosynthesis pathway (Bohlmann and Keeling, 2008; Croteau et al., 2005).

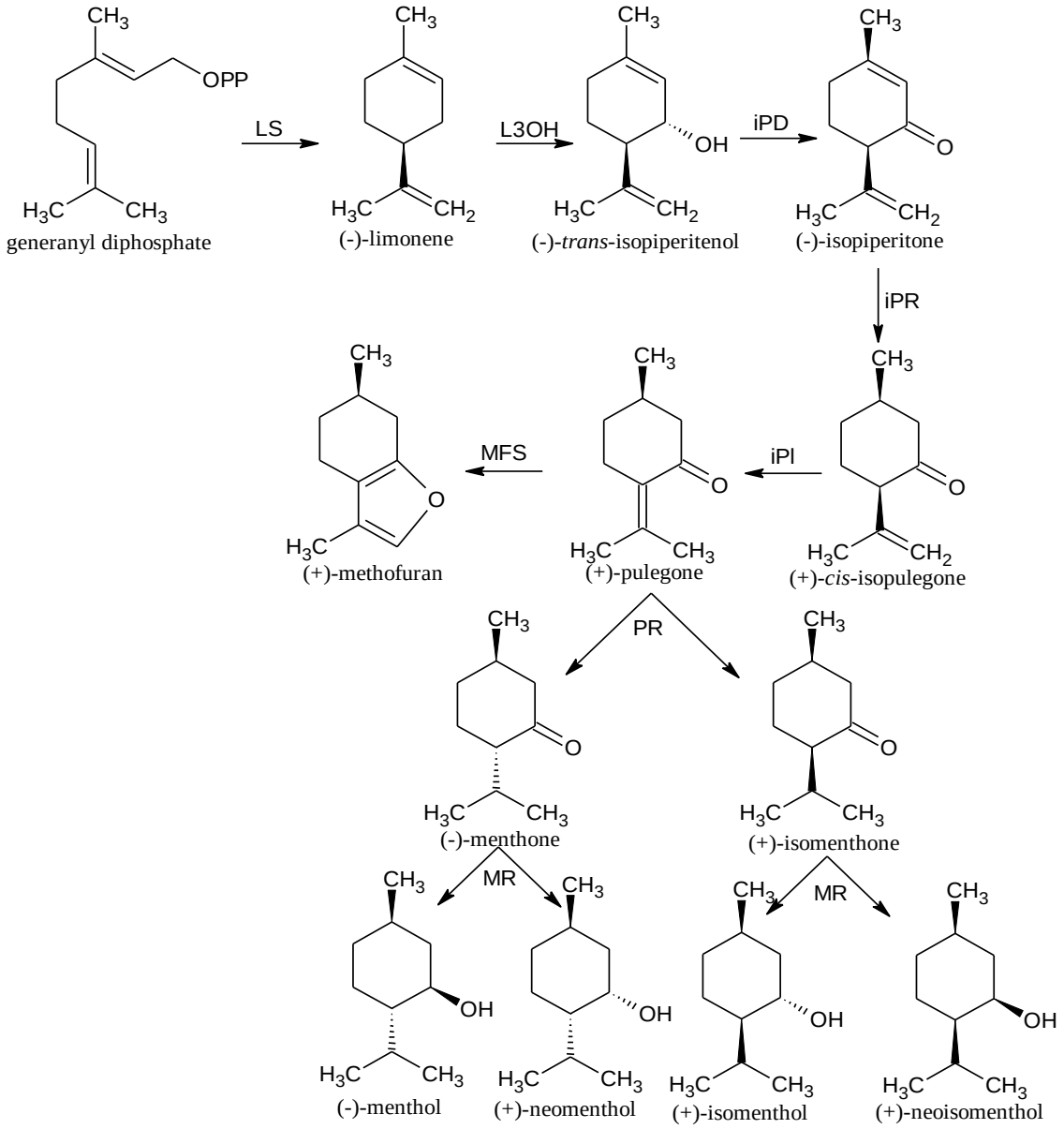


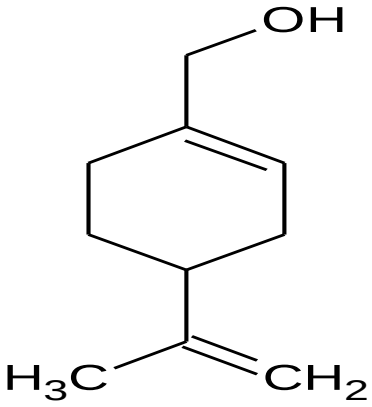
Figure 1.3: Biosynthesis of (-)-menthol and related monoterpenes from essential.

Key steps: (-)-limonene synthase (LS); (-)-limonene-3-hydroxylase (L3OH); (-)-*trans*-isopiperitenol dehydrogenase (iPD); (-)-isopiperitenone reductase (iPR); (+)-*cis*-isopulegone isomerase (iPI); (+)-pulegone reductase (PR); (-)-menthone reductase (MR); (+)-menthofuran synthase (MFS).

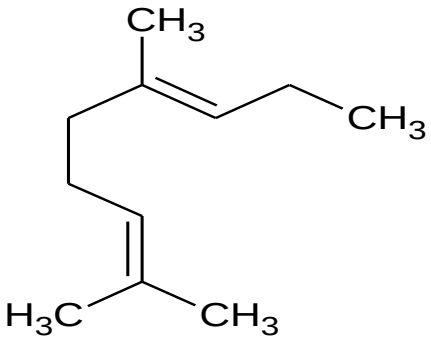
1.3.1 Monoterpenoids

Monoterpenoids (C_{10}) are formed by condensation of two isoprene units and are mainly found in the essential oils. Monoterpenes generally have a pleasant odor and they are the most common volatile compound from plants responsible for fragrances and flavour. Figure

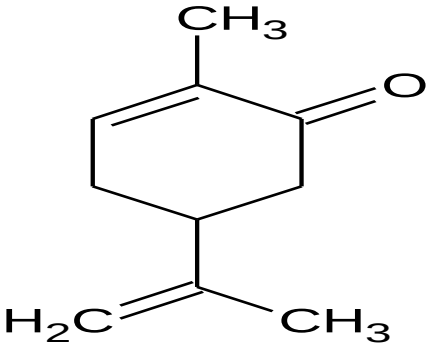
1.4 shows the examples of monoterpenoids that include perillyl alcohol, geraniol, carvone and *d*-limonene (Dhanarasu, 2012)**.**



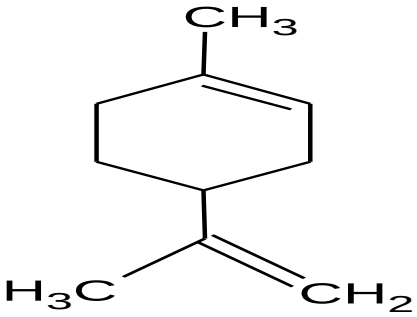
Perillyl alcohol



Geraniol



Carvone

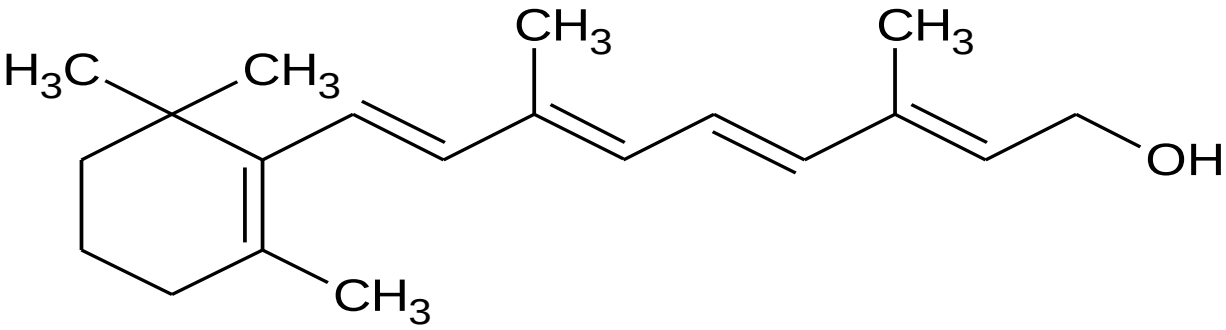


d-limonene

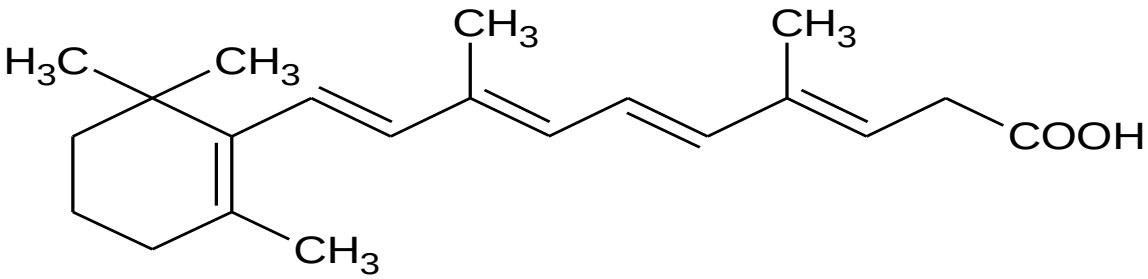
Figure 1.4: Examples of monoterpenoid compounds

1.3.2 Diterpenoids

Diterpenoids (C_{20}) are made from the condensation of four isoprenoid units. Examples of diterpenoids are retinol and *trans*-retinoic acid as displayed in Figure 1.3. Some diterpenoids are used in the treatment of various cancers such as lung, breast and ovarian cancers. Taxol is the common major diterpenoid found in the species of Pacific Yew (*Taxus brevifolia*) (Çitoğlu and Acıkara, 2010; Sar, 2006).



Retinol

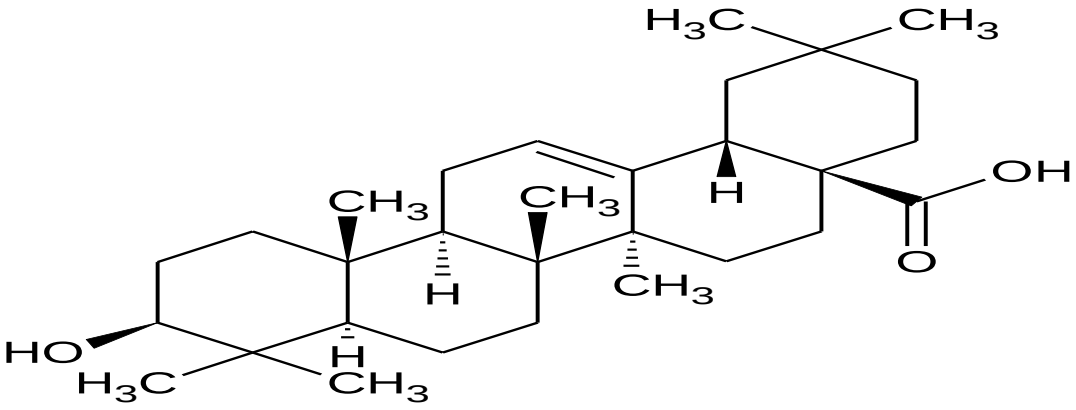


trans-retinoic acid

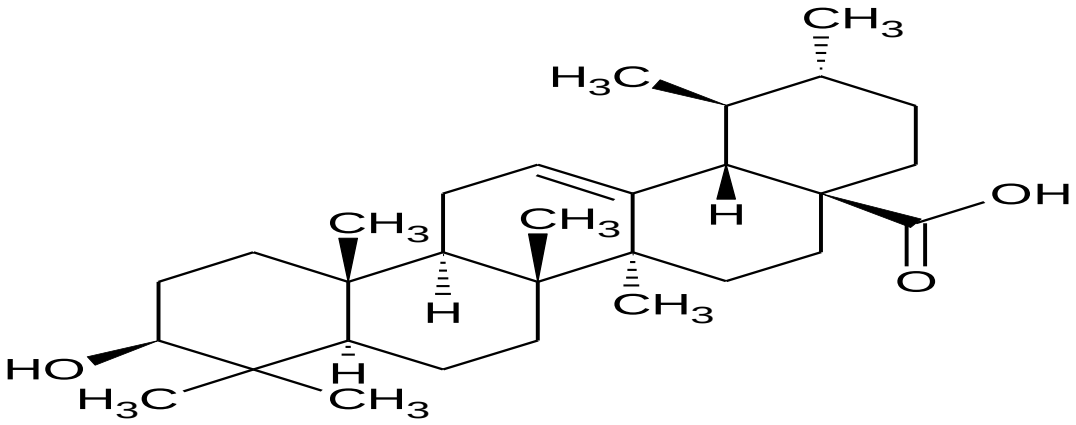
Figure 1.5: Examples of diterpenoid compounds

1.3.3 Triterpenoids

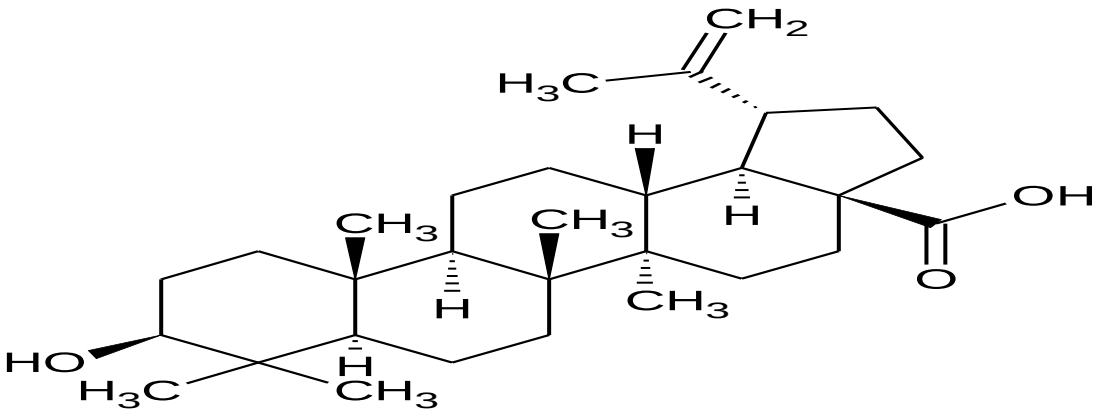
Triterpenoids (C_{30}) originate from the condensation of six isoprenoid units. Triterpenoids are widely distributed in nature that includes plants, microorganism, animals and human. Triterpenoids include oleanolic acid (OA), ursolic acid (UA), betulinic acid (BA) and lupeol as shown in Figure 1.4. Oleanolic acid and ursolic acid are isomers that are widely found in plants and in foods. Oleanolic acid and ursolic acid are used individually or in combination as hepatoprotective medication (Dhanarasu, 2012).



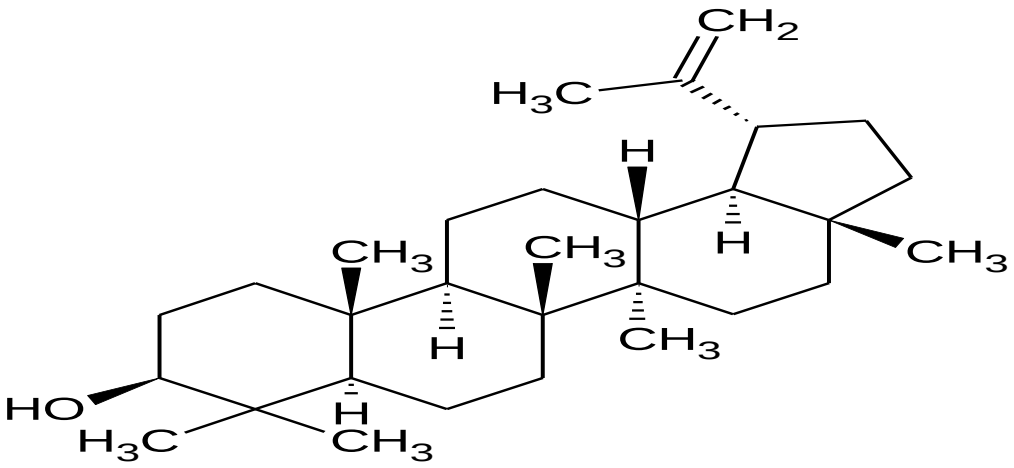
Oleanolic acid (OA)



Ursolic acid (UA)



Betulinic acid (BA)

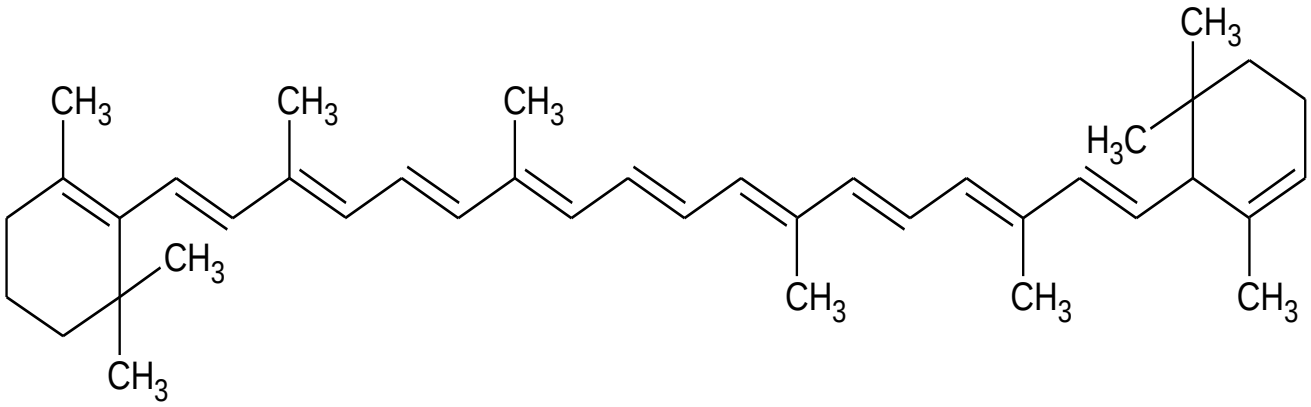


Lupeol

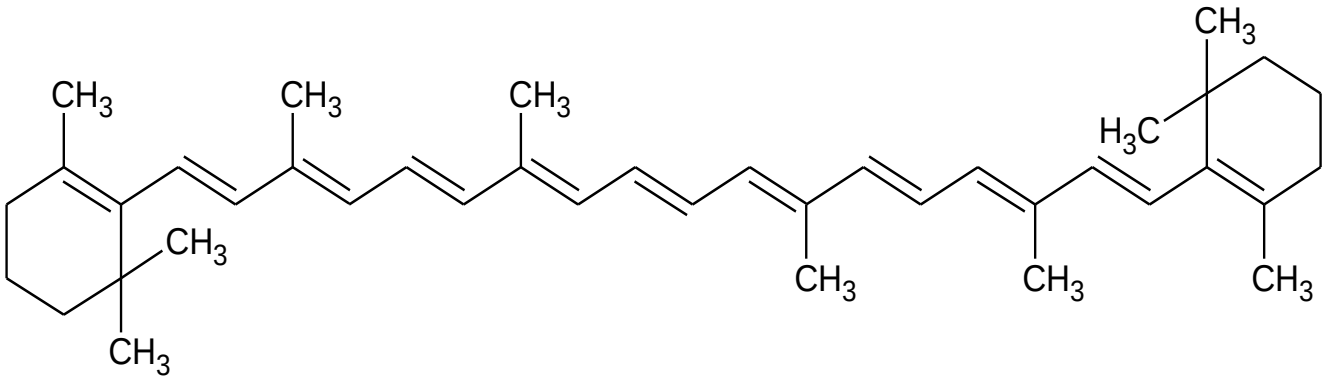
Figure 1.6: Examples of tritepenoid compounds

1.3.4 Tetraterpenoids

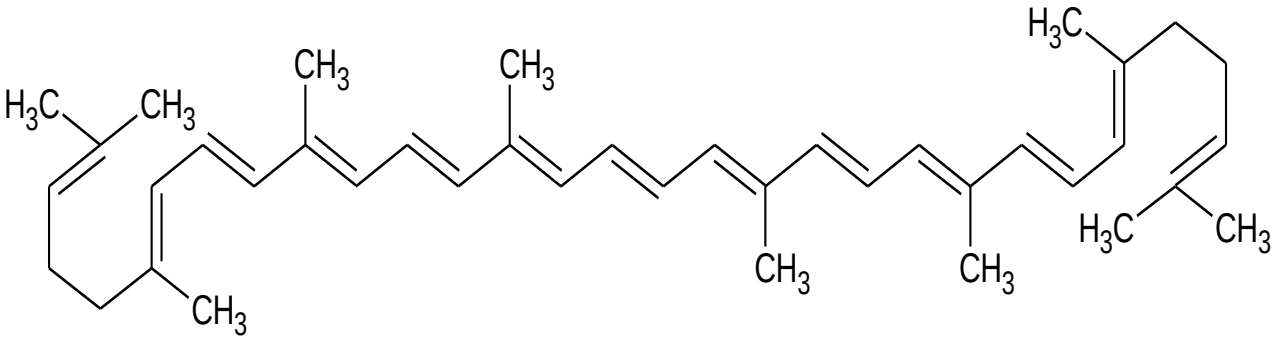
Tetraterpenoids are made from condensation of eight isoprenoid units. Tetraterpenoids are also called carotene or carotenoids due to their occurrence in carrots. Naturally occurring carotenoids are responsible for yellow, orange and purple pigments of plants, bacteria and algae. Tetraterpenoids examples are α-carotene, β-carotene, lycopene and phytoene as displayed in Figure 1.7 (Dhanarasu, 2012).



α-carotene



β-carotene



Lycopene

Figure 1.7: Shows the examples of tetraterpenoid compounds

1.4 Essential oils

Both aromatic and medicinal plants yield diverse types of hydrocarbons (aliphatic and cyclic) and their corresponding derivatives. Many plants contain volatile essential oils which are a complex mixture of volatile compounds composed of monoterpenes, sesquiterpenes and their oxygenated derivatives. The oxygenated derivatives include alcohols, aldehydes, esters, ketones, phenols and oxides. Other known volatile compounds from essential oils include phenylpropenes, sulphur-containing or nitrogen containing compounds (Bajpai et al., 2008). Many essential oils have been found to have antimicrobial activity (Haddouchi et al., 2013). Essential oils have been used traditionally as flavoring agents or preservatives of foods, or as fragrances in cosmetics and aromatherapy. Volatile essential oils can be extracted mostly by steam distillation. However, hydrodistillation is the most common extraction method to obtain essential oil from aromatic plants (Magiatis et al., 2002).

1.5 Pathophysiology of Hypertension

Hypertension is related with increase in oxidative stress. Studies show that hypertension may arise as a result of release of reactive oxygen species and that a range of antioxidant treatment reduce hypertension. Hypertensive effects of oxidative stress are typically caused by endothial dysfunction resulting from disturbances of vasodilator system, mainly degradation of nitric oxide (NO) by oxygen free radicals. Endothial dysfunction may be defined as damage that is characterized by a shift in the actions of the endothelium towards reduced vasodilation, a pro-inflammatory state and prothrombotic settings. Nitric oxide is an important signaling molecule that controls the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets collection, angiogenesis, thrombosis, vascular tone and hemodynamics. The increase in reactive oxygen species (ROS) may lead to the reduction of nitric oxide levels which could be part of the mechanism that leads to hypertension. Reactive oxygen species include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hydroxyl radical ($OH^{\cdot}$), etc. People with hypertension are at high risk for stroke, kidney failures and heart diseases. Hypertension may lead to the damage of tissues in the body through peroxidation and other oxidative mechanisms. Therefore, it is desirable to undertake studies concerning the antioxidant and antihypertensive activity of *R. caffra fractions* and *T. minuta* essential oils. Since antioxidants are able to trap reactive oxygen and this may have the ability to decrease oxidative damage and possibly hypertension (Bickers and Athar, 2006; Ceriello, 2008; Rodrigo, González and Paoletto, 2011; Uttara et al. 2009; Vaziri and Rodríguez-Iturbe, 2006).

1.6 Problem Statement

Recently, there has been a global increase in the incidences of cancer, hypertension, diabetes and other diseases that are related to oxidative stress mostly in developing countries (Milugo et al., 2013). Despite the use of the orthodox drugs, hypertension remains a major cause of death in South Africa and as well as in some other regions of the world. According to statistics, South Africa has higher number of death due to hypertension as compared to the world. About 80% of South African population relies on traditional medicine for the treatment of hypertension due to high cost and limited access to western medicine (Alamgeer et al., 2013; Byrne, Eksteen, and Crickmore, 2014). Although essential oils extracted from *Tagetes minuta* have been evaluated through several biological studies including antibacterial, antimicrobial, anti-oxidant, anticancer and antiplasmodial effect and in vitro anti-tick activities, to our knowledge there has not been any literature report on the

antihypertensive effects of essential oil from this aromatic plant (Senatore et al., 2004; Tahir and Khan, 2012; Tankeu et al., 2013; Nchu et al., 2012).

1.6.1 Justification

Terpenoids have been found to be useful in prevention and treatment of many diseases including cancer and also to have antimicrobial, antifungal, antiparasitic, antihypertensive, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory and immunomodulatory properties (Thoppil and Bishayee, 2011). *Rauvolfia caffra* parts are traditionally used in the treatment of hypertension, cough, gastrointestinal disturbances, skin infections, diarrhea, dysentery, scabies, worm infestation and malaria in many countries (Milugo et al., 2013). However, little attention has been given to determine its mode(s) of action, side effects and interactions. Hence, biological assessments (anti-hypertensive properties) of these traditional medicines are imperative to be elucidated. The search of bioactive plant-derived compounds from *R. caffra* and *T. minuta* against hypertension is therefore warranted.

1.7 Aim and Objectives

1.7.1 Aim

The aim of this study is to extract, isolate, and characterize the biologically active volatile and non-volatile compounds from *Tagetes minuta* and *Rauvolfia caffra* respectively.

1.7.2 Specific Objectives

- Collection and authentication of *Tagetes minuta* and *Rauvolfia caffra* from Eastern Cape.
- Extraction of essential oils from *Tagetes minuta* to obtain its volatile component.
- Solvent extraction of fraction extracts from *Rauvolfia caffra* using different solvents.
- Isolation of non-volatile compounds using column chromatography from fraction extract.
- Characterization of isolated compounds using different analytical techniques.
- Biological studies to determine antihypertensive and antioxidant properties of crude extracts, selected isolated compounds and essential oil of the plant.

1.8 Research Questions

- Does *Tagetes minuta* have volatile essential oil?

- Does the extract of *Rauvolfia caffra* contain pure terpenoids as non-volatile compounds?
- Do volatile essential oils, fraction extracts and non-volatile compounds have anti-hypertensive and antioxidant activity?

1.9 Hypothesis

Rauvolfia caffra contains biologically active terpenoids which can be considered for drug development and *Tagetes minuta* contains volatile constituents which can be found useful medicinally in managing life-threatening diseases including hypertension.

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CHAPTER 2

LITERATURE REVIEW

2.1 An Overview of Analytical Techniques

This chapter gives a brief explanation and background about analytical techniques that are usually employed for natural product chemistry. These techniques are divided into three namely extraction, chromatographic and spectroscopic techniques.

2.1.1 Chromatographic Techniques

Chromatographic techniques give information about thin layer chromatography, column chromatography and gas chromatography.

2.1.1.1 Thin Layer Chromatography (TLC)

Thin-Layer chromatography is a technique which solute endures distribution between stationary phase acting over adsorption and a mobile phase in the liquid form. TLC method is used to separate compounds based on their solubility in organic solvents and molecular weight. This method is used to detect the presence of compounds in the fraction extract. In TLC, the adsorbent of stationary phase is a thin uniform layer of dried finely solid powder material such as silica or alumina on an inert base such as aluminum (Milugo et al., 2013; Suliman, 2011). Thin Layer chromatography is used to visualize the chromatographic plant profile. In principle, the plant components will differ due to unique solubility and strength of their adsorption to the adsorbent. Different molecules partition differently between the free and absorbed state, such that the equilibria between two states is not the same. If a molecule is weakly absorbed, its equilibrium lies in the direction of the free state and there is a higher concentration in the mobile phase. These molecules spend more time in the mobile phase and they will be carried through the stationary phase faster and farther in a given time. On the hand, if a molecule is strongly absorbed, its equilibrium lies in the direction of the absorbed state, and has a higher concentration in the stationary phase. These molecules spend less time in the mobile phase and move slowly through stationary phase and thus do not move far in the same amount given time. Therefore, some components will be carried further than others. The polarity of the plant components of interest is the key on selecting the mobile phase. The mixture is spotted at the bottom of the TLC plate and allowed to dry. Then the TLC plate is placed in a closed container such as beaker containing solvent (mobile phase) so that the liquid level is below the level spot. The solvent ascend the TLC plate by capillary action, the

solvent filling up the spaces between the solid particles of the stationary phase. The TLC plate is removed when the solvent front approaches the top of TLC plate (Kumar, 2011; Tagbor, 2009).

2.1.1.2 Column Chromatography (CC)

Column Chromatography is one of the most useful methods for separation and purification. It is the convenient method for isolation of various terpenoids more especially sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids and sterols. This is a solid-liquid technique in which the stationary phase is a solid and mobile phase is a liquid. The usual and common adsorbent used in a column chromatography are silica, alumina, calcium phosphate, calcium carbonate, magnesia, starch, etc., and solvent selection is based on the nature of both adsorbent and the solvent.

The rates at which the components of the mixture are separated rely on the activity of the adsorbent and the solvent polarity. If the activity of the adsorbent is high and the polarity of the solvent is very low, then the separation is very slow but gives good separation. Meanwhile, if the activity of the adsorbent is high and the polarity of the solvent is high, then separation is rapid but only gives a poor separation, i.e., the separated components are not 100 % pure.

The adsorbent is made into dry packing or slurry with suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through a column. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The components with greater adsorption ability will be adsorbed at the top and the one with lesser adsorption ability will be adsorbed at the bottom. Different components can be adsorbed and collected separately by adding more solvent at the top and this process is called elution and the solvent is known as eluent. The weakly adsorbent component will be eluted more rapidly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent gives the pure components (Dhanarasu, 2012).

2.1.1.3 Gas Chromatography (GC)

Gas Chromatography (GC) also known as Gas Liquid Chromatography (GLC) is a technique that separates mixtures into individual components by a process which rely on the

redistribution of the components between stationary phase also known as support material in the form of a liquid, solid or combination of both and a gaseous mobile phase.

It has been reported that many pharmacologically active components in herbal medicine are volatile chemical compounds. Consequently, the analysis of volatile compounds by GC is of paramount importance in the analysis of herbal medicine and it has a number of advantages. Firstly, GC analysis of volatile oil gives a reasonable "fingerprint'' which can be employed to identify the plant. The relative concentration and composition of organic compounds in the volatile oil are characteristic of particular plant and the impurities in the volatile essential oil can be easily detected. Secondly, method of extraction of essential oil is straightforward and can be standardize and components can be readily identified using Gas Chromatography-Mass Spectrometry (GC-MS). One of the main advantages of GC is in its high sensitivity of detection for almost all volatile chemical compounds. This is mainly witnessed in the usual Flame Ionization Detector (FID) and GC-MS. The high selectivity capillary columns enables separation of many volatile components simultaneously within relatively short time is one of the most important advantages. GC disadvantage is that it is not convenient for analysis of polar and non-polar samples (Kamboj, 2012).

2.1.2 Extraction Techniques

Extraction involves separation of portions of plant or animal tissue that are medically active from inactive or inert components by employing selective solvents in standard extraction procedures. Impure liquids, semisolids or powders that are intended for external use only are obtained as natural products from plants (Handa et al., 2008). Extraction techniques look more on hydrodistillation and solvent extraction which are mostly the main methods of extraction in natural product chemistry.

2.1.2.1 Hydrodistillation

Hydrodistillation is a simple form of steam distillation which is often used to isolate non-water soluble compounds. In order to isolate essential oil by hydrodistillation, the aromatic plant is packed in a container and sufficient amount of water is added and boiled. Due to the influence of hot boiling water, the essential oil is freed from the tissues of a plant. The vapour mixture of water and essential oil is cooled by cold water from a condenser. The condenser distillate flows into the separator (Clevenger apparatus), where the oil automatically separates from water (Handa et al., 2008).

2.1.2.2 Solvent Extraction

The principle of solid-liquid extraction is when a solid material (plant material) immersed into a liquid solvent and all the components that are soluble from solid material moves to the liquid solvent. Thus, the solvent extraction of a plant result to a mass *trans*fer of soluble active medicinal ingredients to the solvent and this takes place in a concentration gradient. The mass *trans*fer concentration from the plant to the solvent increases until it has reached the equilibrium when the concentration of soluble active ingredients in solid material and solvent are the same. When equilibrium is reached there will be no longer a mass *trans*fer from plant to the solvent (Handa et al., 2008).

2.1.3 Spectroscopic Techniques

Spectroscopic techniques focus on background theory of Nuclear Magnetic Resonance (NMR) spectroscopy (both 1D and 2D NMR), Gas Chromatography-Mass Spectrometry (GC-MS), Fourier Transformation Infra-red (FTIR) spectroscopy and Mass Spectrometry (MS).

2.1.3.1 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is a study of interaction of radiofrequency (RF) with unpaired nuclear spins in an external magnetic field to extract structural information about a given sample (Tagbor, 2009). NMR spectroscopy has become a vital tool for the characterization and elucidation of organic molecules, since the early 1960s. Recent spectrometers are much more sensitive and acquired much higher field strength, allowing the use of powerful 2D-NMR experiments. While the early spectrometers were relatively insensitive and performed simple experiments at low-field strength, but provided reliable measurements of chemical shifts and coupling constants (Kwan and Huang, 2008).

2.1.3.1.1 One Dimensional NMR

Both ^{1}H and ^{13}C-NMR give important information about qualitative and quantitative composition of an unknown compound and they also help in the determination of the molecular formula (Elyashberg, 2015).

2.1.3.1.1.1 One Dimensional -Proton NMR (^{1}H-NMR)

Proton NMR is a plot of signals emerging from absorption of radiofrequency (RF) during an NMR experiment in a compound under study as a function of chemical shifts. The position of the chemical shifts shows information regarding the chemical and electronic environment of

protons, and the splitting pattern reveals information about the number of neighboring protons. The range of the chemical shifts in proton NMR is normally 0-10 ppm.

2.1.3.1.1.2 One Dimensional -Carbon NMR (^{13}C-NMR)

^{13}C-NMR is a plot of signal resulting from different carbons as a function of chemical shift. The signal in ^{13}C-NMR experiments usually appear as singlets because of the decoupling of the attached protons. Different techniques of recording 1D ^{13}C-NMR has been developed so that it is possible to differentiate between primary, secondary, tertiary and quaternary carbons from 1D ^{13}C-NMR. The range of chemical shifts in ^{13}C-NMR is normally 0-230 ppm (Kwan and Huang, 2008).

2.1.3.1.2 Two Dimensional NMR

2D NMR look mostly on the correlation of two 1D NMR techniques namely 2D-^{1}H, ^{1}H-Correlated Spectroscopy (COSY), 2D-Nuclear Overhauser Enhancement Spectroscopy (NOESY), Heteronuclear Multiple Quantum Correlation (HMQC), Heteronuclear Multiple Bond Correlation (HMBC) and DEPT. 2D NMR provides with information and important tools available for elucidation of molecular structure, functions and dynamics. There are four steps involved in any 2D NMR experiment namely preparation, evolution, mixing and detection. Preparation step takes place when a 90º pulse excites all sample nuclei (which consist of two nuclei A and B) simultaneously. During the preparation step, nucleus A gets excited creating magnetization in x-y plane. During evolution step, chemical shift of nucleus A is measured. On the mixing step, there is a *trans*fer of magnetization from nucleus A to nucleus B through J coupling or NOE interaction. Detection is normally recording an FID and determining the frequency of nucleus BBY Fourier *trans*formation. All possible pairs in the sample for analysis go through this process of detection simultaneously. To observe the second dimension, the chemical shift of nucleus A has to be measured before it passes through its magnetization to nucleus B (Kumar et al., 2011).

2.1.3.1.2.1 ^{1}H, ^{1}H-COSY (Correlated Spectroscopy)

Correlated Spectroscopy (COSY) is used to identify the relationship between protons on adjacent carbon atoms. In the upgraded version COSY-45 experiment, each peak represents a through bond coupling between two protons (Kwan and Huang, 2008).

2.1.3.1.2.2 NOESY (Nuclear Overhauser Enhancement Spectroscopy)

Nuclear Overhauser Enhancement Spectroscopy (NOESY) method provides the basis for conformation studies especially on biomolecules. It is a homonuclear correlation via dipolar

coupling that may be due to NOE or chemical exchange. It is a powerful and most useful technique as it permits to correlate nuclei through space (distance less than 5Å) and allows the assignment of relative configuration of substituents at chiral centres (Tesso, 2005).

2.1.3.1.2.3 Heteronuclear Multiple Quantum Correlation (HMQC)

Heteronuclear Multiple Quantum Correlation (HMQC) is 2 dimensional method with one proton and one carbon axis. The position of each peak in this technique is phase sensitive. Two dimensional HSQC represents the chemical shift of every proton and its directly attached carbon (Kwan and Huang, 2008).

2.1.3.1.2.4 Heteronuclear Multiple Bond Correlation (HMBC)

Heteronuclear Multiple Bond Correlation (HMBC) is an improved version of HMQC appropriate for determining long-range chemical shifts ^{1}H-^{13}C correlations. HMBC gives information about the chemical shift of carbon atoms that are about 2-3 bonds away from the proton to which they correlate, due to the reason that it is a long-range chemical shift correlation experiment. Consequently, quaternary carbon atoms are also detected (Kumar et al., 2011)

2.1.3.1.2.5 Distortionless Enhancement of Polarization *Trans*fer (DEPT)

Distortionless Enhancement of Polarization *Trans*fer (DEPT) is a NMR method used to determine the presence of primary, secondary and tertiary carbons. In DEPT, a second *trans*mitter irradiates ^{1}H during the sequence, which affects the appearance of the ^{13}C spectrum (Tesso, 2005). This technique differentiates between CH, CH_2 and CH_3 by variation of selection angle parameter: 135º angle gives all CH and CH_3 in an opposite phase to CH_2; 90º angle gives only CH groups, while 45º angle give all carbons attached to protons (irrespective of number) in phase. The signal of quaternary and carbons with no proton attachment do not appear in DEPT (Tesso, 2005).

2.1.3.2 Fourier *Trans*formation Infrared Spectroscopy (FTIR)

The absorption in the infrared region results in the changes in both vibrational and rotational status of the molecules. The absorption frequency relies on the vibrational frequency of the molecule, whereas the absorption intensity depends on how effectively the infrared photon energy can be *trans*ferred to the molecule and it relies on the change in the dipole moment that occurs as a result of molecular vibrations. As a result, a molecule absorbs infrared light only if the absorption causes a change in dipole moment. Hence, all compounds except for elemental diatomic gases which are N_2, O_2 and H_2 have infrared spectra and most components

present in flue gas can be analyzed by their characteristic infrared absorption. If only one species from specific sample is to be analyzed, a species-specific instrument can be employed. In this case analysis is performed in a narrow wavelength where species of interest has a characteristic absorption. Nevertheless, the other species present in the sample may also absorb at the analytical wavelength, and for this case the spectrometer should be calibrated for cross sensitivities. Therefore, for quantification of many components absorbing in the mid infrared-region (400-5000 cm^{-1}), Fourier *Trans*form Infrared (FTIR) spectroscopy can be used or conventional dispersive infrared analysis. FTIR is faster and has better signal-to-noise ratio as compared to dispersive infra-red analysis, therefore FTIR is more preferable

FTIR is a non-destructive technique and it provides a pre*cis*e measurement method which does not require external calibration. It can collect a scan every second due to its increasing speed and it has greater optical throughput. It is easy to use since it is mechanically simple with only one moving part. Experimental steps of instrumental process involve the source, interferometer, sample, detector and computer. In the source the energy is emitted from a glowing black-body source. This beam passes through a hole which control amount of energy accessible to the sample. In the Interferometer, the beam enters the interferometer where spectral encoding takes place. The resulting interferogram signal then exits the interferometer. In the sample step, the beam enters the sample section where it is *trans*mitted through the surface of sample depending on the type of analysis being accomplished and this is where absorption of specific frequencies of energy which are uniquely characteristic of sample takes place. Advantages of FTIR include speed, sensitivity, mechanical simplicity and internal calibration. These advantages make FTIR measurement more accurate and reproducible (Claes and Lars-Erik, 1997).

2.1.3.3 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is both qualitative and relatively quantitative in composition and can provide fingerprint of the volatile essential oil of herbal medicine. GC-MS has two most important and significant advantages. Firstly, GC-MS has very good separation ability in general, which can produce a chemical fingerprint of high quality. Secondly, due to the coupled of mass spectrometry and corresponding mass spectral database, GC-MS could provide quantitative and relatively quantitative composition information of the herb being investigated which will be very useful for further research for elucidating the chemical constituents in herbal medicine and its pharmacology relationship in further research. Therefore, GC-MS should be

the most preferable tool for the analysis of the volatile chemical compounds in herbal medicine (Kwan and Huang, 2008).

2.1.3.4 Mass Spectrometry (MS)

Mass spectrometry is both sensitive and selective method for molecular analysis and can identify the yield on molecular weight and structure of the molecule. In MS analysis, there are methods to ionize compounds and separate ions according to mass-charge ratio. Common methods of ionization are electron impact (EI) and electron capture ionization (ECI) and are used in conjunction with gas chromatography (GC) (Tesso, 2005).

In this study, hydrodistillation, solvent extraction, TLC, CC, GC, GC-MS, ^{13}C-NMR, ^{1}H-NMR, COSY, HMBC, HMQC and FTIR analytical techniques will be employed. These techniques will be used to extract, visualize plant profile, and isolate compounds as well as structural elucidation of pure compounds.

2.2 Natural Products

2.2.1 Uses of Natural Products in Hypertension

Hypertension commonly known as high blood pressure is defined as persistent systolic blood pressure of > 140 mmHg and/or a diastolic blood pressure > 90 mmHg. High blood pressure has been found to be associated with many chronic conditions such as obesity, insulin resistance, atherosclerosis and cardiovascular diseases (Seed at et al., 2014). There are mainly two types of hypertension called essential and secondary. In the essential hypertension, pathogenesis is multifactorial and highly complex and may be caused by increase in sympathy nervous system activity, increase in the production of sodium-retaining hormones and vasoconstrictors, scarcities of vasodilators namely prostacycline, nitric oxide and unknown causes. Pathogenesis of secondary hypertension is due to chronic kidney disease, renovascular disease, cushing's syndrome, pheochromacytoma, drugs such as nonsteroidal anti-inflammatory drug and oral contraceptives (Joshi et al., 2012). Symptoms of hypertension are headaches, heart palpitation, flushed face, blurry vision, nosebleed, urinate more often, dizziness, fatigue and ringing noise in ears. According to World Health Organization (WHO), approximately 2 out of 5 deaths in South Africa are due to non-communicable diseases. This can be caused by high level of major risks such as tobacco use, alcohol abuse, and unhealthy diet, high salt intake, obesity and physical inactivity. Hypertension accounts for about 40 % early mortality among men and 29 % early mortality among women occurring before the age of 60 years (WHO, 2014). The management of

hypertension developing countries due to poor lifestyle and insufficient health care system is inadequate. It has been estimated by World Health Organization (WHO) that about 60 % of world population use one form of traditional medicine or the other while 80 % population of developing countries relies completely on traditional practices for their primary health necessities (Alamgeer et al., 2013). It is evident that medicinal plants can be potential sources of bio-active components that can be used for drug formulation against many chronic diseases such as diabetes, cancer and hypertension (Siddiqi, Mehmood, Rehman, and Gilani, 2012). Thus, this study focuses on the extraction and isolation of biologically active components from selected plants.

2.2.2 Natural Antioxidant Agents

Ever since ancient times, the essential oils/fractions from herbal medicinal plants played an important role in food, drug formulations and perfumery. Most essential oils are reported to possess antioxidant, anti-inflammatory and antimicrobial activities. Food antioxidants such as ascorbic acids, amino acids, α-tocopherol, carotenoids, proteins, flavonoids and other phenolic compounds might also play a significant role as physiological and dietary antioxidants. Natural antioxidants are known to exhibit an extensive range of other biological properties such as antibacterial, anti-inflammatory, antiallergic, antithrombotic and vasodilatory activities (Fernandes de Oliveira et al., 2012; Imelouane et al., 2010). Attractive colours of flowers, fruits and leaf are believed to be due to over 4000 varieties of flavanoids in plants. Natural antioxidants are active against oxidative stress which is associated with chronic disease such as hypertension (Petricevich et al., 2014). It is therefore important to find plants whose secondary metabolites have potent antioxidant potential and to investigate their effects on treating hypertension.

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CHAPTER 3
PHYTOCHEMICAL EVALUATION AND ANTIOXIDANT STUDIES OF *TAGETES MINUTA*

3.1 Introduction

This chapter will focus on extraction using hydrodistillation method, chemical constituents and antioxidant effects of essential oil from *Tagetes minuta* plant.

3.1.1 Origin, morphology, ecology, chemotypes and uses of Tagetes minuta

Tagetes is a genus that belongs to the Asteraceae family and this genus consists of 56 different species, of which 27 grow annually and 29 grow perennially (Senatore et al., 2004). Some of the common species of this family are *Tagetes tenuifolia, Tagetes erecta, Tagetes minuta* and *Tagetes patula* (Tahir and Khan, 2012). *T. minuta* traditionally known as *nukayo* in Xhosa is native to the temperate grassland and mountain of the southern part of South America and North America. It was introduced to Europe, Asia, Africa, India, Australia, Madagascar and Hawaii. It commonly spreads in forests roadside and usually grows adjacent to forest margin as common weed. *T. minuta* is one of the annual plants of 1-2 m in height, with upright erect stems that bear leaf of up to 15 cm in length that are glossy green in colour with 19-17 leaflets and creamy-yellow flowers of 10 mm long ad 2 mm wide in size (Dold and Cocks, 1999; Tankeu et al., 2010; Wang and Chen, 2006).

(i)

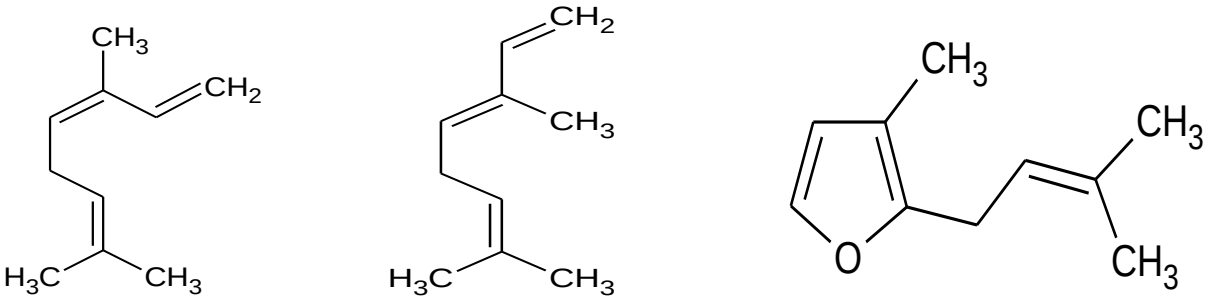
(ii)

Figure 3.1: (i) *Tagetes minuta* whole plant (ii) *T. minuta* aerial parts with flowers

T. minuta plant is used as flavouring component in candy, beverages, milk, cheese, desserts, baked foods as well as gelatins and it also finds uses in cooking (Sadia et al., 2013a; Tankeu et al., 2010). Medicinally, *T. minuta* whole plant is reported to be used as condiment, stomach

strengthener, diaphoretic, purgative and hysteria remedy. The different parts of the plant have different uses, thus its leaf are used for wound-healing, bronchodilatory, microbial, inflammatory conditions. It is also used for kidney problems, piles and muscular pain. Whereas leaf extract can be useful in earache, hemorrhoids, insecticide and as snuff. Its flowers are used for fevers, indigestion and gastritis, mild laxative and for epileptic fits (Sadia et al., 2013b). Essential oil of *T. minuta* is commercially produced in countries like South Africa, Australia and Brazil (Senatore et al., 2004; Tankeu et al., 2010).

It has been reported that *T. minuta* contains a varying number of secondary metabolites that include flavanoids, terpenoids, saponines, thiophenes, monocyclic, bicyclic and acyclic monoterpenes (Sadia et al., 2013b; Tankeu et al., 2010). Previous studies report that *T. minuta* essential oil constitutes of major components such as *cis*-ocimene (28.5 %), beta-ocimene (16.83 %) and rosefuran (11.94 %) (Nchu et al., 2003). Phytochemical analysis of these essential oils showed main constituents as limonene, dihydrotagetone, *trans*-tagetone, and *cis*-tagetone and *cis*-tagetenone (Sadia et al., 2013b).



cis-Ocimene β-Ocimene Rosefuran

Figure 3.2: Major components from *Tagetes minuta* essential previous studies

3.2 Methods and Materials

3.2.1 Collection of Plant Material and its Preparation

Tagetes minuta plant was collected on March 2014 from Komga and taken to Selmar Schonland Herbarium at Rhodes University for identification, confirmation and deposition of voucher specimen (MS/PL4) by Dr. T Dold. The fresh leaf stem and flowers of *T. minuta* plant were separated from each other. The some specific amount of T. minuta fresh parts were taken for hydrodistillation while the other amount was air dried for 5 days.

Figure 3.3: *Tagetes minuta* collection from Komga, Eastern Cape

3.2.2 Hydrodistillation

Extraction of essential oil of *T. minuta* was carried out using hydrodistillation method. 300 g of fresh and dried plant material namely fresh stem, leaf and flowers were subjected to the Hydrodistillation set-up using Clevenger apparatus as prescribed by European pharmacotia. Enough amount of distilled water was poured to fresh and dried plant material at separate times and after 4 hours of boiling the mixture; the essential oil was collected into hexane and *trans*ferred to a dark vial which was stored in the refrigerator.

3.3 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Chemical constituents were identified using Gas Chromatography-Mass Spectroscopy (GC-MS) and Gas Chromatography (GC). The following chromatographic conditions were used in the analysis of essential oils. An HP 5890 series II Gas Chromatography equipped with an Agilent mass spectrometry detector was used. GC was equipped with flame ionization detector (FID). The mass spectrometry (MS) was operated in the full scan mode from 30 to 350 amu. Helium was used as a carrier gas at constant pressure of 100 kPa, and flow of 1.2 mL/min with linear velocity 30.1 cm/sec. A 1 µL volume of sample was injected at a temperature of 280˚C. An HP-5MS fused silica column with 30 m length by 0.25 mm internal diameter and 0.25 µm film thickness capillary was used. The Oven temperature was programmed from 40˚C for 2 minutes at 10˚/min to 280˚C with spit ratio of 1:30. Wiley Library was used to identify each compound as eluted from the column. Similar conditions

were employed for GC and Kovat indexes were calculated using alkane standards. Homologous standard series of n-alkanes were run under the same conditions for determination of Kovat indices.

3.4 Biological Studies

Antioxidant activity of TMDS, TMDL and TMDF essential oils from *Tagetes minuta* were evaluated. The oil from dried parts were selected due to their high concentration of chemical constituent compounds as compared to fresh parts essential oil namely TMFS, TMFL and TMFF (Figure 3.9).

3.4.1 Antioxidant Studies of essential oil

The oils were evaluated for their antioxidant activity using FRAP and DPPH assays. All the absorptions were read from Phoenix-2000V UV Vis Spectrophotometer.

3.4.2 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay evaluates antioxidants according to their reducing activity in a redox-linked colorimetric method which is a direct indicator of total antioxidant power. Reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex which is an FRAP agent results in a ferrous complex which produces an intense blue coulor to confirm its formation. Absorption of FRAP reagent was read at 593 nm. Acetate buffer, $FeCl_3$ (20 mM) and TPTZ were all prepared to form FRAP reagent as follows. Acetate buffer was prepared by dissolving 3.1 g of sodium acetate trihydrate ($C_2H_9NaO_5$) in 500 mL dH_2O, afterwards 16 mL of glacial acetic acid was added to the mixture, and prepared mixture was made up to 1 L with distilled water (dH_2O) and stored at room temperature. $FeCl_3$ (20 mM) was prepared by dissolving 1.1 g in 200 mL dH_2O. 0.156 TPTZ was dissolved in 50 mL HCl (40 mM). FRAP reagent was prepared by combination of 100 mL acetate buffer, 10 mL TPTZ and 10 mL $FeCl_3$. Ascorbic acid (AA) stock was prepared by dissolving 0.1 g of ascorbic acid ($C_6H_8O_6$) in 100 mL dH_2O. A standard curve stock solution of 80µg/mL was prepared by adding 640 µL of AA stock and 3360 µL of dH_2O. Double diluted standard solutions of 200, 160, 80, 40, 20, 5 and 2.5 µg/mL as well as blank were prepared. 1 mg/mL of TMDS, TMDL, TMDF essential oils were prepared respectively; by dissolving 300 µL which is equivalent to 0.3 g in 0.2 mL Tween 80 and diluted to 5 mL by dH_2O.

A procedure for standard curve and sample/standard was started by pipetting 100 µL of sample into labeled test tube, 3 mL of freshly mixed FRAP reagent was added and the prepared solution was incubated in water bath at 40 ºC for 4 minutes. The absorbance was

read at 593 nm. Standard curve was drawn and an antioxidant activity of samples was extrapolated (as ascorbic acid equivalent/mg) from the standard curve (Fernandes de Oliveira et al., 2012).

3.4.3 DPPH Assay

The scavenging reaction between DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and an antioxidant (H-A) is expressed as:

$$\textbf{DPPH + (H-A)} \longrightarrow \textbf{DPPH-H + (A)}$$

(Purple) (Yellow)

This test was based on the principle that antioxidants react with DPPH, which is a stable free radical and is reduced to DPPHH as result the absorbance decreases. Standard (Ascorbic acid) curve was prepared by two steps. In first step, stock was prepared by dissolving 0.1 g of ascorbic acid in 100 mL dH_2O. In the second step, 160 µg/mL standard curve stock was prepared by diluting 640 µL of ascorbic acid with 3360 µL of dH_2O. Standard solutions were prepared by double diluting to yield 200, 160, 80, 40, 20, 10, and 5 µg/mL concentrations. 0.01 DPPH (MW= 394.32 g/mol) was dissolved in 500 mL methanol to make up 0.01 mM and the absorbance (OD) was measured to be around 1.5 nm at 517 nm. Prepared DPPH was kept in the dark covered in aluminium foil. TMDS, TMDL, TMDF essential oil stocks were prepared by dissolving 300 µL of oil which is equivalent to 0.3 g in 0.2 mL Tween 80 and diluted to 5mL by dH_2O. 120, 60, 30 and 15 µg/mL for TMDS, TMDL and TMDF were prepared in duplicate for each essential oil.

Procedure started by pipetting 1 mL sample into labeled test tube and 1 mL methanol was used as blank. 3 mL DPPH was added to the sample and the solution was incubated in the dark for 30 minutes. The absorbance of prepared duplicates for standard curve and samples was read at 517 nm. % Inhibition was calculated as:

$$\%\ \text{Inhibition} = \frac{A_B - A_S}{A_B}$$

Where A_B is blank absorbance for DPPH and A_S is sample/standard absorbance. After plotting the inhibition and standard curves, IC_{50} was determined. IC_{50} is the concentration of extract that inhibit the formation of DPPH radicals by 50 % (Fernandes de Oliveira et al., 2012).

3.5 Statistical Analysis

The data was analyzed using Graphpad Prism (v5). Analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to compare the differences between fractions of *T. minuta*. Data was expressed as mean ± standard error of mean. P values lower than 0.05 were considered significant.

3.6 Results and Discussion

Hydrodistillation of *Tagetes minuta* parts (stem, leaf and flowers) yielded yellow liquid essential oil with a pleasant odor which presents characteristic of the plant (Table 3-1). The essential oils yield from both fresh and dried parts of plant material was displayed below (Table 3-2).

Table 3-1: Physical properties of *T. minuta* essential oils

Essential oil	**Colours**	**Odor**
TMFS	Yellow	Intense aromatic
TMDS	Light Yellow	Intense aromatic
TMFL	Yellow	Intense aromatic
TMDL	Yellow	Intense aromatic
TMFF	Yellow	Intense aromatic
TMDF	Yellow	Intense aromatic

Table 3-2: Percentage yield (w/w) of essential oil extracted from *Tagetes minuta*

	Stem	**Leaf**	**Flowers**
Fresh	0.20 %	0.65 %	1.12 %
Dried	0.20 %	0.91 %	1.10 %

3.6.1 Essential oil Composition

Essential oil composition from parts (stem, leaf and flowers) of *Tagetes minuta* plant is displayed in the following tables.

Table 3-3: Chemical composition of *Tagetes minuta* fresh stem (TMFS) essential oils

S/NO	Compound	%	Formula	Kovat Index	Method of Identification
Monoterpene Hydrocarbons					
1.	Limonene	4.79	$C_{10}H_{16}$	1031	GC-MS, GC
2.	*cis*-β–Ocimene	38.03	$C_{10}H_{16}$	1040	GC-MS, GC
3.	*trans*-β–Ocimene	3.26	$C_{10}H_{16}$	1043	GC-MS, GC
4.	Alloocimene	12.32	$C_{10}H_{16}$	1128	GC-MS, GC
Oxygenated Monoterpenes					
5.	*cis*-Tagetone	3.48	$C_{10}H_{16}O$	1149	GC-MS, GC
Oxygenated Sesquiterpenes					
6.	*trans*-Caryophyllene	5.46	$C_{15}H_{24}$	1417	GC-MS, GC
7.	γ-Elemene	4.20	$C_{15}H_{24}$	1430	GC-MS, GC
8.	β-Caryophyllene	3.96	$C_{15}H_{24}$	1431	GC-MS, GC
9.	α-Caryophyllene	3.10	$C_{15}H_{24}$	1467	GC-MS, GC
10.	Elixene	2.57	$C_{15}H_{24}$	1514	GC-MS, GC
Oxygenated Sesquiterpenes					
11.	Spathulenol	12.10	$C_{15}H_{24}O$	1640	GC-MS, GC
12.	α –Cadinol	5.89	$C_{15}H_{26}O$	1647	GC-MS, GC
Total identified = 99.16 %					

Table 3-4: Chemical composition of *Tagetes minuta* dried stem (TMDS) essential oils

S/NO	Compound	%	Formula	Kovat Index	Method of Identification
Monoterpene Hydrocarbons					
1.	α–Pinene	1.41	$C_{10}H_{16}$	939	GC-MS, GC
2.	Sabinene	0.28	$C_{10}H_{16}$	969	GC-MS, GC
3.	β-Pinene	3.38	$C_{10}H_{16}$	974	GC-MS, GC
4.	p-Cymene	0.53	$C_{10}H_{16}$	1026	GC-MS, GC
5.	Limonene	3.67	$C_{10}H_{16}$	1031	GC-MS, GC
6.	*trans*-β-Ocimene	2.06	$C_{10}H_{16}$	1097	GC-MS, GC
7.	Neo-Alloocimene	0.66	$C_{10}H_{16}$	1129	GC-MS, GC
Oxygenated Monoterpenes					
8.	*cis*-Tagetone	1.09	$C_{10}H_{16}O$	1146	GC-MS, GC
9.	Iso-Borneol	0.57	$C_{10}H_{18}O$	1155	GC-MS, GC
10.	Terpin-4-ol	0.70	$C_{10}H_{18}O$	1177	GC-MS, GC
11.	L-Camphor	1.90	$C_{10}H_{16}O$	1192	GC-MS, GC
12.	α-Terpinol	2.11	$C_{10}H_{18}O$	1198	GC-MS, GC
13	Verbinone	2.33	$C_{10}H_{14}O$	1204	GC-MS, GC
Ester					
14.	(-)-Bornyl Acetate	1.56	$C_{12}H_{20}O_2$	1284	GC-MS, GC
Sesquiterpene Hydrocarbons					
15.	α-Gurjunene	2.77	$C_{15}H_{24}$	1412	GC-MS, GC

16.	β-Caryophyllene	8.38	$C_{15}H_{24}$	1418	GC-MS, GC
17.	α-Guaiene	0.84	$C_{15}H_{24}$	1439	GC-MS, GC
18.	α-Humelene	6.58	$C_{15}H_{24}$	1467	GC-MS, GC
19.	α-Selinene	2.73	$C_{15}H_{24}$	1493	GC-MS, GC
Oxygenated Sesquiterpenes					
20.	Elemol	9.31	$C_{15}H_{26}O$	1550	GC-MS, GC
21.	*trans*-Nerolidol	3.33	$C_{15}H_{26}O$	1564	GC-MS, GC
22.	Caryophyllene Oxide	18.04	$C_{15}H_{24}O$	1573	GC-MS, GC
23.	Zierone	1.01	$C_{15}H_{22}O$	1574	GC-MS, GC
24.	Spathulenol	10.67	$C_{15}H_{24}O$	1576	GC-MS, GC
25.	Humelene Epoxide II	3.92	$C_{15}H_{24}O$	1606	GC-MS, GC
26.	Alloaromadendrene Epoxide	1.21	$C_{15}H_{24}O$	1639	GC-MS, GC
27.	α –Eudesmol	2.90	$C_{15}H_{26}O$	1652	GC-MS, GC
28.	Ledol	1.00	$C_{15}H_{26}O$	1665	GC-MS, GC
29.	*cis*-α-Santalol	1.07	$C_{15}H_{24}O$	1678	GC-MS, GC
30.	13-Hydroxy - valencene	1.02	$C_{15}H_{24}O$	1767	GC-MS, GC
Others					
31.	Octadecene	1.04	$C_{18}H_{36}$	1790	GC-MS, GC
Total % identified = 98.07 %					

Table 3-5: Chemical composition of *Tagetes minuta* fresh leaf (TMFL) essential oils

S/NO.	Compound	%	Formula	Kovat Index	Method of Identification
Monoterpene Hydrocarbons					
1	Limonene	4.94	$C_{10}H_{16}$	1031	GC-MS, GC
2	*trans-β*-Ocimene	7.39	$C_{10}H_{16}$	1043	GC-MS, GC
3	Alloocimene	25.35	$C_{10}H_{16}$	1128	GC-MS, GC
Oxygenated Monoterpenes					
4	*cis*-Tagetone	2.53	$C_{10}H_{16}O$	1156	GC-MS, GC
Sesquiterpene Hydrocarbons					
5	β-Caryophyllene	22.87	$C_{15}H_{24}$	1418	GC-MS, GC
Oxygenated Sesquiterpenes					
6	Caryophyllene oxide	15.81	$C_{15}H_{24}O$	1573	GC-MS, GC
7	Spathulenol	4.64	$C_{15}H_{24}O$	1576	GC-MS, GC
8	Octadecanoic acid	5.88	$C_{18}H_{36}O_2$	2172	GC-MS, GC
Diterpene Hydrocarbons					
9	Docosane	3.65	$C_{22}H_{46}$	2200	GC-MS, GC
10	Tricosane	5.80	$C_{23}H_{48}$	2300	GC-MS, GC
Total % identified = 98.86 %					

Table 3-6: Chemical composition of *Tagetes minuta* dried leaf (TMDL) essential oils

S/NO.	Compound	%	Formula	Kovat Index	Method of Identification
Monoterpene Hydrocarbons					
1.	p-Cymene	0.38	$C_{10}H_{16}$	1026	GC-MS, GC
2.	Limonene	2.74	$C_{10}H_{16}$	1031	GC-MS, GC
3.	*cis*-β-Ocimene	6.73	$C_{10}H_{16}$	1040	GC-MS, GC
4.	*trans*-β-Ocimene	2.47	$C_{10}H_{16}$	1049	GC-MS, GC
Oxygenated Monoterpenes					
5.	Dihydrotagetone	4.90	$C_{10}H_{18}O$	1061	GC-MS, GC
6.	Diethyl-Carbitol	2.58	$C_8H_{18}O_3$	1081	GC-MS, GC
7.	Methyl benzoate	1.93	$C_8H_8O_2$	1091	GC-MS, GC
8.	Nonanal	1.45	$C_9H_{18}O$	1107	GC-MS, GC
9.	*cis*-Tagetone	7.01	$C_{10}H_{16}O$	1149	GC-MS, GC
10.	1-Nonanol	0.27	$C_9H_{20}O$	1171	GC-MS, GC
11.	Terpinen-4-ol	10.17	$C_{10}H_{18}O$	1177	GC-MS, GC
12.	Octanoic acid	2.82	$C_8H_{16}O_2$	1179	GC-MS, GC
13.	L-Camphor	2.19	$C_{10}H_{16}O$	1192	GC-MS, GC
14.	α-Terpinol	0.44	$C_{10}H_{18}O$	1198	GC-MS, GC
15.	Decanal	1.81	$C_9H_{19}O$	1204	GC-MS, GC
16.	Nerol	1.20	$C_9H_{18}O$	1228	GC-MS, GC
17.	2-Phenoxyethanol	0.37	$C_9H_{18}O_2$	1245	GC-MS, GC

Sesquiterpene Hydrocarbons					
18.	α-Gurjunene	2.10	$C_{15}H_{24}$	1412	GC-MS, GC
19.	β-Caryophyllene	3.26	$C_{15}H_{24}$	1418	GC-MS, GC
20.	δ-Elemene	0.60	$C_{15}H_{24}$	1430	GC-MS, GC
21.	α-Humelene	0.17	$C_{15}H_{24}$	1454	GC-MS, GC
22.	α-Caryophyllene	2.11	$C_{15}H_{24}$	1467	GC-MS, GC
23.	Germacrene-D	0.31	$C_{15}H_{24}$	1480	GC-MS, GC
24.	Bicyclogermacrene	0.24	$C_{15}H_{24}$	1494	GC-MS, GC
Oxygenated Sesquiterpenes					
25.	Tridecanal	1.56	$C_{13}H_{26}O$	1511	GC-MS, GC
26.	*trans*-Nerolidol	1.88	$C_{15}H_{26}O$	1564	GC-MS, GC
27.	Spathulenol	0.69	$C_{15}H_{24}O$	1576	GC-MS, GC
28.	Caryophyllene Oxide	1.28	$C_{15}H_{24}O$	1581	GC-MS, GC
29.	Humelene epoxide II	0.35	$C_{15}H_{24}O$	1606	GC-MS, GC
30.	Tetradecanal	3.38	$C_{14}H_{28}O$	1611	GC-MS, GC
31.	Benzophenone	0.67	$C_{13}H_{10}O$	1621	GC-MS, GC
32.	Ledol	3.66	$C_{15}H_{26}O$	1665	GC-MS, GC
33.	Isopropyl tetradecanoate	17.02	$C_{17}H_{34}O_2$	1824	GC-MS, GC
34.	Benzyl benzoate	2.14	$C_{14}H_{12}O_2$	1762	GC-MS, GC
35.	Tetradecanoic acid	2.25	$C_{14}H_{28}O_2$	1780	GC-MS, GC
36.	Methyl Hexadecanoate	0.44	$C_{17}H_{34}O_2$	1926	GC-MS, GC
37.	1-Octadecanol	4.03	$C_{18}H_{38}O$	2082	GC-MS, GC

38.	Methyl stearate	0.70	$C_{19}H_{38}O_2$	2128	GC-MS, GC
Total % identified = 98.3 %					

Table 3-7: Chemical composition of *Tagetes minuta* fresh flower (TMFF) essential oil

S/NO	Compounds	%	Formula	Kovat Index	Method of Identification
Monoterpene Hydrocarbons					
1.	Limonene	19.06	$C_{10}H_{16}$	1031	GC-MS, GC
2.	*cis*-Ocimene	38.14	$C_{10}H_{16}$	1040	GC-MS, GC
Others					
3.	Valeric acid	30.90	$C_5H_{10}O_2$	1084	GC-MS, GC
Oxygenated Monoterpenes					
4.	Linalool	11.90	$C_{10}H_{18}O$	1100	GC-MS, GC
Total % identified = 100 %					

Table 3-8: Chemical composition of *Tagetes minuta* dried flower (TMDF) essential oils

S/NO	Compound	%	Formula	Kovat Index	Method of Identification
Ester					
1.	2-Acetylfuran	1.12	$C_6H_6O_2$	910	GC-MS, GC
Monoterpene Hydrocarbons					
2.	α-Pinene	0.11	$C_{10}H_{16}$	939	GC-MS, GC

3.	Camphene	0.10	$C_{10}H_{16}$	953	GC-MS, GC
4.	Sabinene	0.56	$C_{10}H_{16}$	972	GC-MS, GC
5.	β-Pinene	3.08	$C_{10}H_{16}$	981	GC-MS, GC
6.	Myrcene	0.16	$C_{10}H_{16}$	991	GC-MS, GC
7.	α-Phellandrene	0.20	$C_{10}H_{16}$	1005	GC-MS, GC
8.	N-Octanal	0.15	$C_{10}H_{16}$	1023	GC-MS, GC
9.	Limonene	4.75	$C_{10}H_{16}$	1031	GC-MS, GC
10.	*cis*-β-Ocimene	4.41	$C_{10}H_{16}$	1032	GC-MS, GC
11.	Epoxy-Ocimene	2.12	$C_{10}H_{16}$	1040	GC-MS, GC
12.	*trans*-β-Ocimene	37.03	$C_{10}H_{16}$	1049	GC-MS, GC
13.	α-Ocimene	0.72	$C_{10}H_{16}$	1056	GC-MS, GC
Oxygenated Monoterpenes					
14.	Dihydrotagetone	5.11	$C_{10}H_{18}O$	1061	GC-MS, GC
15.	Filifolone	0.10	$C_{10}H_{14}O$	1108	GC-MS, GC
16.	Thujone	0.32	$C_{10}H_{16}O$	1116	GC-MS, GC
18.	Isophorone	1.80	$C_9H_{14}O$	1118	GC-MS, GC
19.	Chrysanthenone	0.22	$C_{10}H_{16}O$	1125	GC-MS, GC
20.	Alloocimene	12.29	$C_{10}H_{16}$	1142	GC-MS, GC
21.	*cis*-Tagetone	1.52	$C_{10}H_{16}O$	1156	GC-MS, GC
22.	β-Phellandren-8-ol	0.13	$C_{10}H_{16}O$	1163	GC-MS, GC
23.	Endo-Borneol	0.10	$C_{10}H_{18}O$	1165	GC-MS, GC
24.	Terpineol-4	0.11	$C_{10}H_{18}O$	1177	GC-MS, GC

25.	Dehydroelshotizia ketone	0.12	$C_{10}H_{14}O_2$	1180	GC-MS, GC
26.	α-Terpineol	3.10	$C_{10}H_{18}O$	1189	GC-MS, GC
27.	Myrtenol	2.10	$C_{10}H_{16}O$	1194	GC-MS, GC
28.	N-Decanal	0.11	$C_{10}H_{20}O$	1201	GC-MS, GC
29.	Verbenone	0.12	$C_{10}H_{14}O$	1204	GC-MS, GC
30.	m-tert-Butylphenol	5.94	$C_{10}H_{14}O$	1228	GC-MS, GC
31.	*trans*-Ocimenone	3.11	$C_{10}H_{14}O$	1235	GC-MS, GC
32.	m-Thymol	0.29	$C_{10}H_{14}O$	1290	GC-MS, GC
Sesquiterpene Hydrocarbons					
33.	Bicycloelemene	0.29	$C_{15}H_{24}$	1337	GC-MS, GC
34.	β-Isocomene	0.10	$C_{15}H_{24}$	1407	GC-MS, GC
35.	α-Gurjunene	0.17	$C_{15}H_{24}$	1409	GC-MS, GC
36.	β-Caryophyllene	2.79	$C_{15}H_{24}$	1418	GC-MS, GC
37.	α-Humelene	1.39	$C_{15}H_{24}$	1454	GC-MS, GC
38.	Germacrene-D	0.17	$C_{15}H_{24}$	1480	GC-MS, GC
39.	Bicyclogemacrene	0.45	$C_{15}H_{24}$	1494	GC-MS, GC
Oxygenated Sesquiterpenes					
40.	Spathulenol	1.08	$C_{15}H_{24}O$	1576	GC-MS, GC
41.	(-)-Humelene epoxide	0.12	$C_{15}H_{24}O$	1606	GC-MS, GC
Total % identified = 97.64 %					

Table 3-9: Summary of GC-MS results of essential oil from *Tagetes minuta.*

Essential oils	Total % identified	No. of compounds identified	Major Components
Fresh stem (TMFS)	99.16	12	*Cis*-β-ocimene (38.03 %), alloocimene (12.32 %), spathulenol (12.10 %), α-cadinol (5.89 %) and *trans*-caryophyllene (5.46)
Dried stem (TMDS)	98.07	31	Caryophyllene oxide (18.04 %), spathulenol (10.67 %), elemol (9.31 %), β-caryophyllene (8.38 %) and α-humelene (6.58 %)
Fresh leaf (TMFL)	98.86	10	Alloocimene (25.35 %), β-caryophyllene (22.87 %), caryophyllene oxide (15.81 %), *trans-β*-ocimene (7.39 %), octadecanoic acid (5.88 %) and Tricosane (5.80 %)
Dried leaf (TMDL)	98.3	38	Isopropyl tetradecanoate (17.02 %), terpinen-4-ol (10.17 %), *cis*-tagetone (7.01 %), *cis*-β-ocimene (6.73 %)
Fresh flower (TMFF)	100	4	*Cis*-β-ocimene (38.14 %), valeric acid (30.90 %), limonene (19.06 %) and linalool (11.90 %).
Dried flower (TMDF)	97.64	41	*Trans*-β-ocimene (37.03 %), alloocimene (12.29 %), m-tert-butylphenol (5.94 %) and dihydrotagetone (5.11 %).

GC and GCMS were used to identify components from *T. minuta* essential oils. GC: Co-injection of known standard was used in order to calculate Kovat Index (KI) values using the equation:

$$KI = 100 \times (n + (N - n)(\frac{t_c - t_n}{t_N - t_n}))$$

Where KI= Kovat index for volatile compounds

n= initial number of carbons of alkane standard

N=next number of carbons of alkane standard

t_c= retention time of unknown compound

t_n= retention time of initial alkane

t_N= retention time of next alkane.

A total of twelve compounds (Table 3-3, Appendix 1a and 1b) which constitutes 99.16 % were identified from *T. minuta* fresh stem (TMFS) essential oil with *cis*-β-ocimene (38.03 %) being the major component and other significant compounds are Alloocimene (12.32 %), spathulenol (12.10 %), α-cadinol (5.89 %) and *trans*-caryophyllene (5.46 %) as shown in Table 3-9.

A total of thirty one compounds which constitutes 98.07 % (Table 3-4, Appendix 2a and 2b) were identified from *T. minuta* dried stem (TMDS) essential oil. Five major components (Table 3-9) were identified to be caryophyllene oxide (18.04 %), spathulenol (10.67 %), elemol (9.31 %), β-caryophyllene (8.38 %) and α-humelene (6.58 %).

A total of 10 compounds (Table 3-5, Appendix 3a and 3b) representing 96.8 % *T. minuta* fresh leaf essential oil were identified constituting of major components such as *trans*-β-ocimene (7.39 %), Alloocimene (25.35 %), β-caryophyllene (22.87 %), caryophyllene oxide (15.81 %), octadecanoic acid (5.88 %) and tricosane (5.80 %) as displayed in Table 3-9.

A total of 38 compounds (Table 3-6, Appendix 4a and 4b) representing 98.3 % of *T. minuta* dried leaf essential oil was identified high percentage *cis*-β-ocimene (6.73 %), *cis*-tagetone (7.01 %), terpinen-4-ol (10.17 %), as well as isopropyl tetradecanoate (17.02 %).

A total of four compounds (Table 3-7, Appendix 5a and 5b) were identified which constitutes 100 % were identified from *T. minuta* essential oil. Three compounds were identified to be major compounds which are *cis*-ocimene (38.14 %), valeric acid (30.90 %), limonene (19.06) and other significant compound of linalool (11.90 %).

Forty one compounds in total (Table 3-8, Appendix 6a and 6b) which constitutes 97.64 % were identified from *T. minuta* dried flower essential oils. Major component was identified to be *trans*-β-ocimene (37.03 %) and other significant compounds which were alloocimene (12.29 %), m-tert-butylphenol (5.94 %) and dihydrotagetone (5.11 %) as demonstrated in Table 3-9.

Table 3-10: Area percentage of terpenes and their oxygenated derivatives in the chemical composition of essential oil from fresh and dry parts (stem, leaves and flowers) of *Tagetes minuta*

Essential Oil	**Area percentage of terpenes and their oxygenated derivatives**						
	MH	**OM**	**SH**	**OS**	**DH**	**E**	**O**
TMFS	54.8	3.48	19.29	17.99	-	-	-
TMDS	11.99	8.7	21.3	53.48	-	1.56	1.04
TMFL	37.68	2.53	22.87	26.33	9.45	-	-
TMDL	12.32	37.14	8.79	40.05	-	-	-
TMFF	57.2	11.9	-	-	-	-	30.9
TMDF	53.39	36.57	5.36	1.2	-	1.12	-

MH= Monoterpene Hydrocarbon, OM= Oxygenated Monoterpenes, SH= Sesquiterpene Hydrocarbon, OS= Oxygenated Sesquiterpenes, DH= Diterpene Hydrocarbon, E= Esters, O = Others, TMFS= *T. minuta* fresh stem, TMDS= *T. minuta* dried stem, TMFL= *T. minuta* fresh leaf, TMDL= *T. minuta* dried leaf, TMFF= *T. minuta* fresh flower and TMDF= *T. minuta* dried flower.

It has been observed (Table 3-10) that monoterpene hydrocarbons are more abundant in the *T. minuta* essential oil and this was confirmed by the high percentage from different parts essential oil such as fresh stem (TMFS) which contributes 58.8 %, 57.2 % for fresh flower (TMFF), 53.39 % for dried flower (TMDF), fresh leaf (TMFL) being 37.68 % as well dried stem (TMDS). Whereas the remaining percentage represent oxygenated sesquiterpenes, oxygenated monoterpenes, sesquiterpene hydrocarbons, others, diterpene hydrocarbons and esters.

3.7 Antioxidant Activity of Essential Oils

Means of essential oils from *T. minuta* namely TMDS, TMDL and TMDF were compared amongst each other and their significance is displayed in Table 3-11.

3.7.1 DPPH Assay

The free radical scavenging ability of TMDS, TMDL and TMDF essential oil from *T. minuta* was determined from reduction absorbances of DPPH radical at 517 nm. The capacity of natural products to donate electrons can be measured by DPPH radical colour change from purple to form yellow DPPHH. In this assay, the colour change was observed indicating reduction from purple DPPH colour to yellow DPPHH signifying that the tested essential oils have antioxidant activity. Table 3-11 shows that TMDF (IC_{50}=18.7±0.35) showed highest radical scavenging ability as compared to the other essential oils in the order: TMDF> TMDL> TMDS. Antioxidant activity of TMDF ($p< 0.001$) was recorded to be significantly better as compared to the others essential oils. The antioxidant activity may be due to the presence of hydroxyl groups that might come from oxygenated terpenes (Table 3-10) from dried part essential oil (Saeed, et al., 2012).

3.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity *T. minuta* essential oil such as TMDS, TMDL and TMDF was further established through their reducing power. In this assay, the reducing ability ($Fe^{3+}\rightarrow Fe^{2+}$) was established by the resulting noticeable intense blue colour indicating the formation of ferrous complex which also mean that *T. minuta* essential oil antioxidant activity. Table 3-11 shows that TMDF (IC_{50}=113.7±1.2) has high reducing power and differ significantly ($p< 0.001$) in comparison with the other essential oils in the activity order: TMDF> TMDL> TMDS. In general the antioxidant activity of essential oils depends on the phenolic compounds content and the reaction of phenol compounds against chain-carrying peroxyl radicals (Ćavar et al., 2012).

The essential oil (TMDS, TMDL and TMDF) from *Tagetes minuta* showed antioxidant properties with varying activity depending on the part of the plant. Therefore, this plant could be a potential source of natural antioxidants which are more preferable as compared to the synthetic antioxidants.

Table 3-11: Antioxidant activity of *Tagetes minuta* essential oils by DPPH and FRAP assays

	TMDS	**TMDL**	**TMDF**
DPPH (AAE/ mg extract)	6.74 ± 0.27	14.4 ± 0.17***	18.7 ± 0.35***
FRAP (AAE/ mg extract)	26.30 ± 0.41	63.56 ± 1.46	113.7 ± 1.2***

AAE= Ascorbic acid equivalent

Reported as mean ± standard error of mean (X ± SEM); **p< 0.01, p***< 0.001= statistically difference when compared with TMDL

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CHAPTER 4
PHYTOCHEMICAL ASSESSMENT AND BIOLOGICAL APPLICATION OF *RAUVOLFIA CAFFRA*

4.1 Introduction

This chapter will look at the extraction of crude fractions from *R. caffra* using different organic solvents, isolation of pure compounds from fraction extracts using column chromatography and biological assays namely antioxidant and antihypertensive effects of R. caffra extracts.

4.1.1 *Rauvolfia* Genus

Rauvolfia is an evergreen genus consists of trees and shrubs that belong to the family of Apocynaceae (Milugo et al., 2013). This genus has approximately 85 species that include *R. serpentina, R. tetraphylla, and R. grandiflora* Mart as well as *R. caffra*. It is mainly distributed in tropical regions. Rauvolfia is widely distributed in Africa, America, Asia and Oceania and is well-known as a rich source of unique heterocyclic alkaloids with skeletons of monoterpene indole. Alkaloids have gained a lot of interest, due to their anticancer, antimarial and sedative properties and Ravoulfia species are mainly known for its phytochemical reserpine, which were widely employed as an antihypertensive drug (Cancelieri et al., 2002; Gao et al., 2006; Kumar, 2011; Njau et al., 2014)

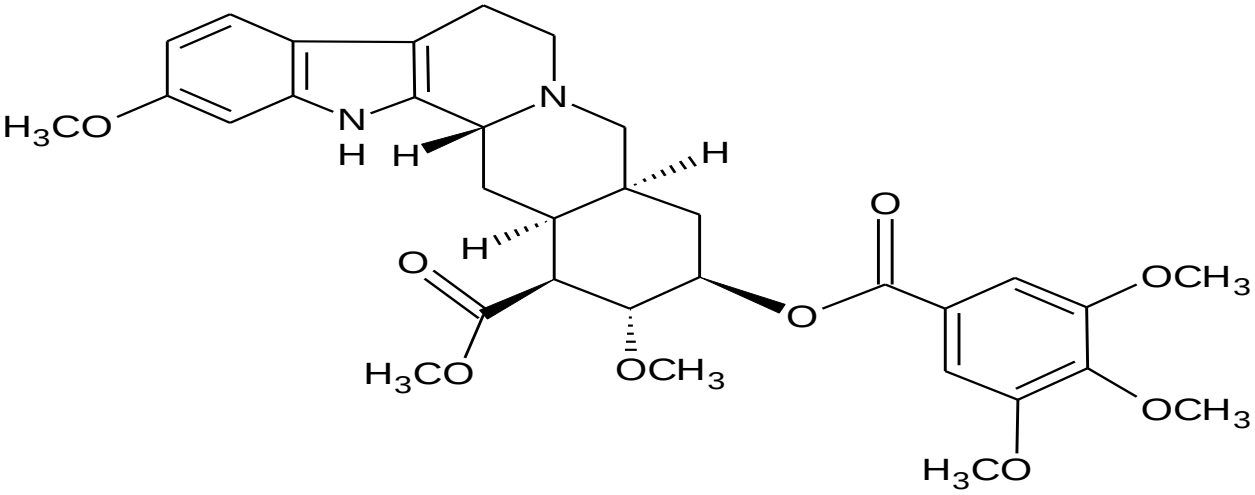
4.1.2 Description and uses of *Rauvolfia caffra*

Rauvolfia caffra is widespread in tropical Africa from West Africa and south of Sudan to South Africa. It mainly grows in rainforest, montane forest and secondary forest, from sea level up to 2500 m altitude (Adeshina et al., 2010). It is commonly known as quinine tree and is traditionally used to manage tumors and other diseases that are associated with oxidative stress in many countries (Milugo et al., 2013). The pounded stem bark from R. caffra is applied direct to skin lessions and rashes. Stem bark decoction are taken as an astringent, purgative or emetic to treat fever, swelling, abscesses, gastrointestinal disturbance, hypertension, hepatitis, worm infections, malaria, scabies, dysentery and pneumonia (Njau et al., 2014).

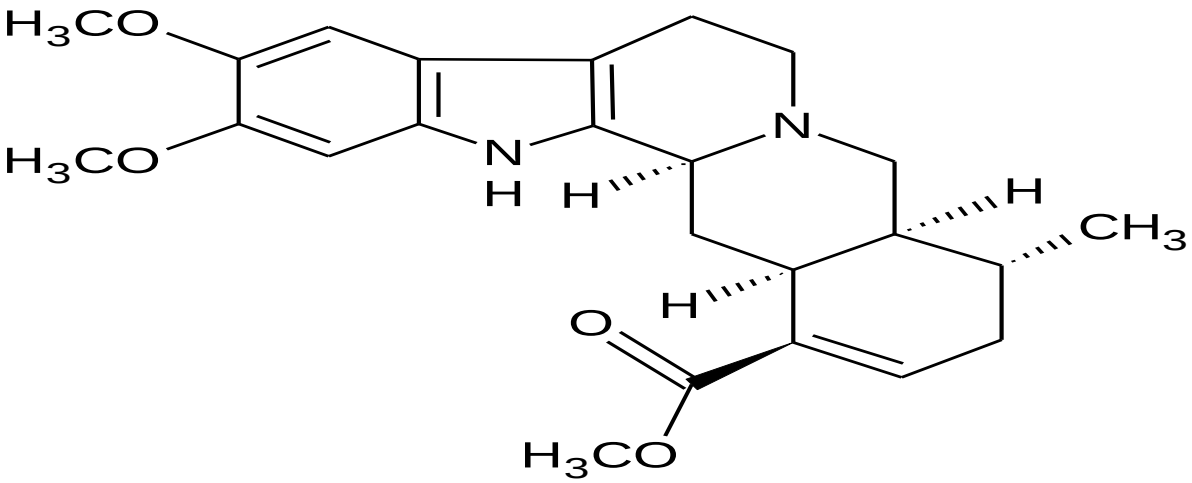
Rauvolfia caffra is a rich source of alkaloids, most of which have been isolated and identified. These alkaloids found uses in pharmacological industries as antimalarial, antitumor and antidiabetes agents. Many phytochemical screening tests have been done in the fraction extract of *R. caffra* to test the presence of alkaloids, steroids, flavanoids, saponines,

tannins, terpenoids, Anthraquinones, anthocynoside, reducing sugars, coumarins, cardiac glycosides and phenols. Fraction extracts that were used for screening are hexane, dichloromethane (DCM), methanol and distilled water from *R. caffra* leaf and stem bark (Milugo et al., 2013; Njau et al., 2014).

According to Milugo et al., *R. caffra* has high antioxidant activity. These authors also reported the presence of alkaloids (63%), saponins (15%); terpenoids, cardiac glycosides and steroids (82%) (Milugo et al., 2013). About 28 alkaloids have been identified in *R. caffra* and these includes corynane, strictamine, sarpagan, akuammicine, pleiocarpamine, indolenine, dihydroindole, peraksine, heterohimbine, ajmalicine, ajmaline, rescinnamine, reserpiline, reserpine, hydroxyheteroyohimbine as well as norajinaline and two were isolated; which are reserpine **(4a)** and reserpiline **(4b)** (Nasser and Court, 1984).



Reserpine



Reserpiline

Figure 4.1: Isolated alkaloids from *Rauvolfia caffra*

Figure 4.2: *Rauvolfia caffra* tree

4.2 Materials and Methods

4.2.1 Collection

Rauvolfia caffra was collected from Elliotdale, Eastern Cape, South Africa. *R. caffra* was identified and voucher specimen (MS/PL5) deposition was done by Dr. A. Mahoyi at Department of Botany Herbarium, University of Fort Hare.

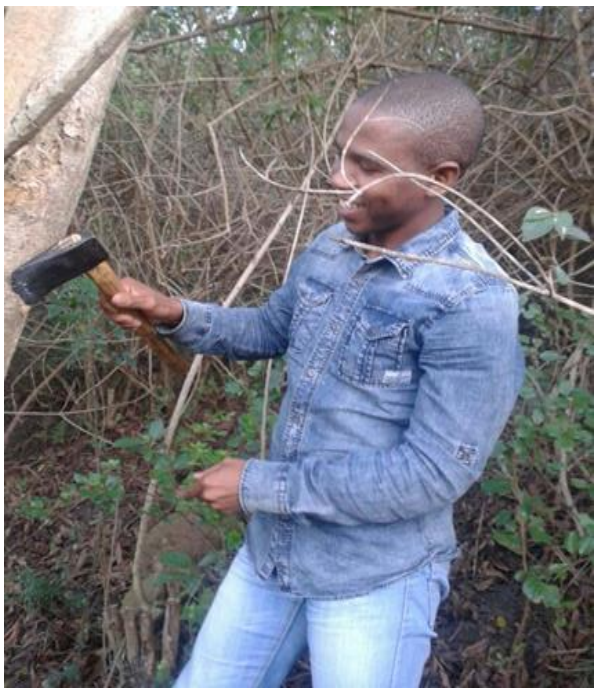


Figure 4.3: *Rauvolfia caffra* bark collection from Elliotdale, Eastern Cape

4.2.2 Sequential Solvent Extraction

Extractions of non-volatile compounds in the form of fraction extracts from *R. caffra* were done using different solvent. Plant material was air dried and grounded into powder using electric blender. Weighed powdered material was fed into 5000 mL Erlenmeyer flask and extraction of constituents was done using organic solvent namely hexane, dichloromethane (DCM), ethyl acetate and methanol respectively according to their order of polarity. This extraction was done by continuous shaking on a shaker for a period of 48 hours twice for each solvent. After this period, the resulting extracts were filtered through a Whatman filter paper (size: 32.0 cm), concentrated through a reduced pressure rotavapor and the remaining solvent was allowed to evaporate at room temperature to obtain dried crude extracts (Mungege et al., 2014).

4.2.3 Isolation

Thin Layer Chromatography (TLC) was done for each fraction extract using different solvent ratio (hexane: ethyl acetate = 9:1, 8:2 and 7:3) in order to identify better separable fraction extract. Therefore, 3.291g ethyl acetate fraction was subjected into a column chromatography to isolate individual compounds at ratios 9:1, 8:2, 7:3, 6:4 and 4:6 of hexane: ethyl acetate. One compound (0.054 g compound SM/01) was isolated from column chromatography packed with silica gel 60 (0.063-0.200mm) and visualized by TLC plate with anisaldehyde/sulphuric acid spray reagent.

4.2.4 Characterization

A compound isolated from column chromatography was characterized using spectroscopic techniques such as:-

- Fourier *Trans*formation Infrared Spectroscopy (FT-IR)
- Nuclear Magnetic Resonance Spectroscopy (NMR)
- Mass Spectroscopy (MS)
- UV-Visible Spectrophotometer

4.3 Biological Studies

Ethical approval for this study was obtained from Walter Sisulu University Ethics Committee (DRD/SREC: Ref. No.: 31). Statistical analysis was similar to that of Chapter 3.

4.3.1 Experimental Animals

Spontaneously hypertensive rats (250-350 g) and Swiss mice (20-40 g) were used. Animals were produced from South African Vaccine program, Johannesburg. The animals were

housed and allowed to equilibrate for two (2) weeks in the Department of Biological and Environmental Animal holding facility. Rats were kept under standard laboratory conditions. Animal lighting was by day light only temperature was maintained at 24˚C. Wood shavings mixed with shredded paper were used as bedding. Animals had free access to rat pellets (Epol SA: protein-180 g/kg, Moisture-120 g/kg, Fat-25 g/kg, Fibre-60 g/kg, Calcium-18 g/kg and Phosphorus-7 g/kg) and water *ad libitum*. The cages were cleaned with water and disinfected weekly while bedding was replaced 2 times per week. All experiments were in strict compliance with international protocols.

4.3.2 Acute Toxicity of plant extract

Locke's method for acute toxicity test was used to determine the toxicity profile of the extracts which consists of 2 phases. In phase 1, 9 mice were allocated to 3 groups of 3 mice each and treated with indicated doses of extract (10, 100 and 1000 mg/kg). In the second phase, 3 mice were used, each receiving an independent dose of extract (1600, 2900 and 5000 mg/kg). Each group was monitored for mortality after 30 minutes, 4 hours and 24 hours respectively. The acute toxicity of hexane, ethyl acetate, dichloromethane (DCM), methanol and ethanol were evaluated and the LD_{50} was calculated as follows.

$$LD_{50} = \sqrt{D_1 \times D_2}$$

Where D_1 is the lowest dose that kills mice and D_2 is the highest dose that does not kills mice. Mice weighing between 25 and 40 g were employed and orally treated with indicated doses of plant extracts namely Hexane, DCM, EA, MetOH and EtOH. They were monitored for 30 minutes, 4 and 24 hours for mortality (Lorke, 1983).

4.3.3 Anti-hypertensive studies of fraction extracts and isolated compound of *R. caffra*

Baseline blood pressure was obtained for all animals at start of experiment after which animals received oral treatments as follows and as randomly assigned per group.

Group 1 was treated with 500 mg/kg of dichloromethane (DCM).

Group 2 was treated with 500 mg/kg of methanol (MetOH).

Group 3 was treated with 300 mg/kg of ethyl acetate (EA).

Group 4 was treated with 15 mg/kg of β-sitosterol (SM/01).

Group 5 was treated with 10 mg/kg of furosemide.

Blood pressure (Systolic and Diastolic) was measured 2, 4 and 6 hours after extract administration. The CODA 8 non-invasive Blood pressure machine (Kent Scientific Corporation, USA) was used to measure blood pressure as per manufacturer's instructions (Malkoff, 2005).

4.3.4 Antioxidant studies of *R. caffra* fractions

Similar antioxidant assays as in chapter 3 such as DPPH and FRAP were used but the fraction were dissolves in tween 80, DMSO and dH_2O for both assays. The determination of total phenolic and total flavonoid antioxidant content were further considered for *R. caffra* stem bark fractions namely DCM, EA, MetOH and EtOH in which hexane fraction and β-sitosterol compound (SM/01) were not studied due to the insufficient yields (Politeo, Juki, and Milo, 2006).

4.3.4.1 Folin Ciocalteau total phenolic content

Total phenolic content was evaluated using modified Henríquez et al., 2010 method. Folin Ciocalteau reagent was prepared by diluting 1 mL of Folin Ciocalteau solution with 9 mL of dH_2O. 75 g/L Na_2CO_3 was prepared by dissolving 7.5 g with dH_2O to make up 100 mL. Gallic acid stock was prepared by dissolving 0.1 g gallic acid in 10 mL ethanol and then diluted to make up 100 mL with dH_2O. To prepare garlic acid stock, 100 µL of gallic acid was diluted with 900 µL of dH_2O. For standard curve, prepared gallic stock solution was double diluted to 100; 50; 25; 12.5; 6.25; 3.12, 1.56, 0.078 and 0 µg/mL (blank). 1 mg/ml of plant fraction extracts such as DCM, EA, MetOH and EtOH were prepared by adding 400 µL of plant extract and 3600 µL of dH_2O. Procedure for standard and sample started by mixing 0.5 mL of prepared plant/standard and 2.5 mL of diluted Folin Ciocalteau reagent. After allowing the mixture to stand for 5 minutes, 2 mL of Na_2CO_3 solution was added and the mixture was incubated at room temperature for 60 minutes. Afterwards the absorbance for sample and standards was read at 765 nm. Standard curve was plotted in order to extrapolate the active concentration for total phenols (Henríquez et al., 2010).

4.3.4.2 Total flavonoids content determination

3 mL of plant extracts such as DCM, EA, MetOH and EtOH were mixed with 1 mL of $AlCl_3$ (prepared by dissolving 0.4 g crystalline $AlCl_3$ and 1.2 g crystalline sodium acetate in 300 mL distilled water). On the other hand, quercetin was prepared 100 mg in DMSO and then was made up to 100 mL with ethanol to prepare stock. The mixture was incubated for 20

minutes. 100 μg/mL of quercetin standard curve was prepared and the absorbance read at 430 nm (Ghasemzadeh et al., 2010).

4.4 Results and Discussion

Table 4-1 shows the % yields as well as the colour of the different fraction extract from *Rauvolfia caffra*.

Table 4-1: Properties of fractions extracted from *Ravoulfia caffra.*

Fraction	**% Yield**	**Colour**
Hexane	3.02 %	Yellow
Dichloromethane	3.94 %	Dark green
Ethyl Acetate	0.8 %	Green
Methanol	0.20 %	Brown
Ethanol	3.56 %	Green

4.4.1 Thin Layer Chromatography (TLC) Analysis of Fraction

TLC analysis was done with Figure 4.4, 4.5, 4.6 and 4.7 representing visualized fingerprints for hexane, DCM, EA and MetOH fraction extracts respectively. TLC isolation showed different fingerprints that are due to the compound contained in the different fraction extracts of *R. caffra*. However, ethyl acetate was taken for isolation due to promising fingerprint for better separation and cyan blue colour on the TLC which may be due to the presence of triterpenoids that are the secondary metabolites of interest. EA fraction showed better separable mixture from TLC isolation (Figure 4.6) and it was packed in a column chromatography for isolation.

Hexane Fraction

Hex:EA=7:3 Hex:EA=8:2 Hex:EA=9:1

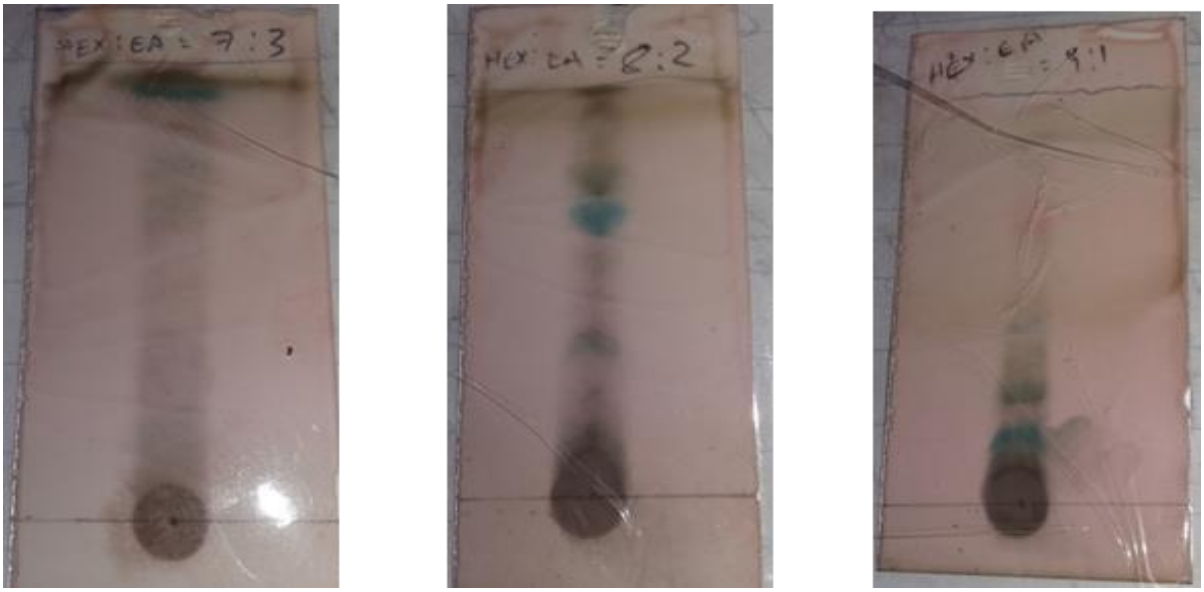
Figure 4.4: TLC plate showing R_f bands for different compounds contained in hexane fraction of *Rauvolfia caffra* at different ratios (mobile phase)

Dichloromethane (DCM) Fraction

Hex:EA=7:3 Hex:EA=8:2 Hex:EA=9:1

Figure 4.5: TLC plate showing R_f bands for different compounds contained in DCM fraction of *Rauvolfia caffra* at different ratios (mobile phase)

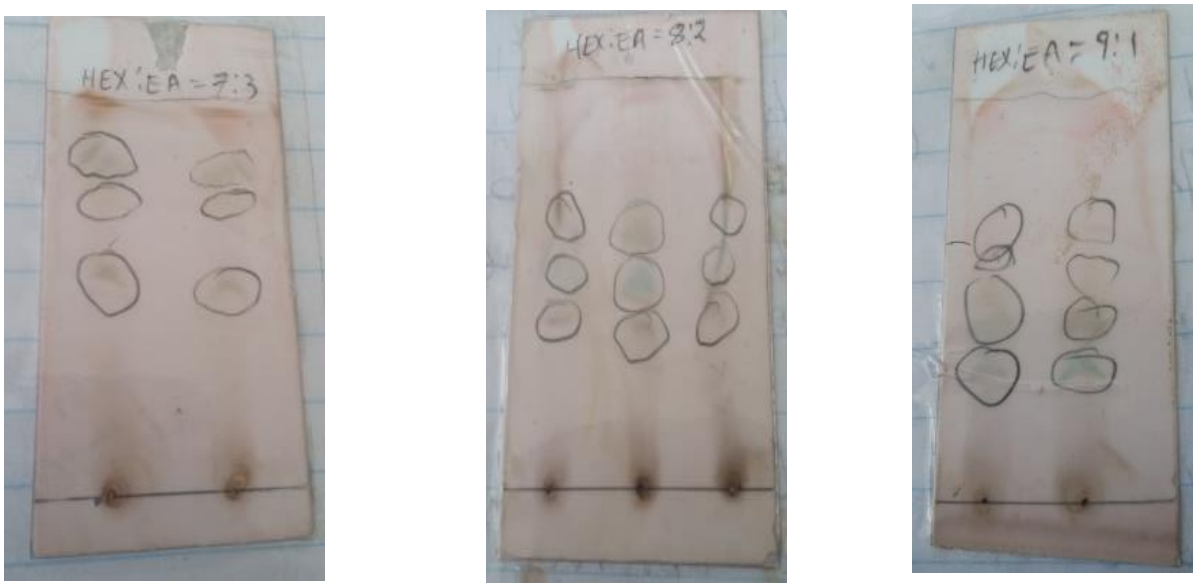
Ethyl Acetate (EA) Fraction



Hex:EA=7:3 Hex:EA=8:2 Hex:EA=9:1

Figure 4.6: TLC plate showing R_f bands for different compounds contained in ethyl acetate fraction of *Rauvolfia caffra* at different ratios (mobile phase)

Methanol Fraction

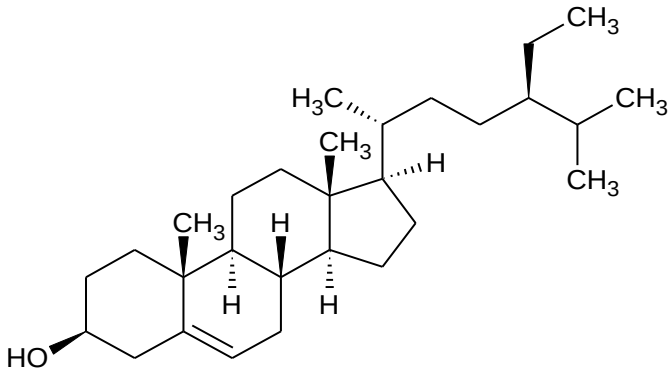


Hex:EA=7:3 Hex:EA=8:2 Hex:EA=9:1

Figure 4.7: TLC plate showing R_f bands for different compounds contained in methanol fraction of *Rauvolfia caffra* at different ratios (mobile phase)

4.5 Compound SM/01

Column chromatography led to an isolation of one pure compound from ethyl acetate fraction extract of *R. caffra* stem bark. Compound SM/01 was identified by various spectroscopic techniques such as NMR, FTIR and melting point. Table 4-2 and Table 4-3 display physical data and FTIR absorption of the compound SM/01 β-sitosterol respectively. The FTIR spectrum (Figure 1c, Appendix C) of β-sitosterol with molecular mass of 414.71 g/mol had major absorption band at 3000 (C-OH), 1771 (C=C), 1378 (C-H) as shown in Table 4-4. Proton NMR of SM/01 compound reveals the presence of 6 methyl signals appeared as two singlets at δ 0.68 and 1.02.; three methyl doublets at δ 0.8, 0.81, 0.94; triplet appeared at δ 0.83. It is shown from ^{13}C NMR that the compound SM/01 is composed of 29 carbons (Table 4-4). The lack of protons corresponding to the double bond between C20 and C21 in compound SM/01 proposed the presence of a tri-substituted double bond at C5 and C6 in the structure. The ^{1}H NMR spectrum of compound SM/01 displayed a proton consistent to the proton linked to the C-3 hydroxyl group which seemed as a triplet of doublet at δ 3.53. ^{13}C, ^{1}H, COSY, HMBC, HSQC all showed the presence of 29 carbons with 6 methyls, 11 methylenes, and 4 quaternary carbons suggesting the skeletal structure of β-sitosterol with chemical formula (Figure 4.9) of $C_{29}H_{50}O$ and this was in agreement with literature (Chaturvedula and Prakash, 2012).



β-Sitosterol

Figure 4.8: Chemical structure of an isolated compound from ethyl acetate fraction

Table 4-2: Physical data of compound SM/01

Compound	Description	% Yield	Mp
SM/01	White powder	1.64%	128-130 ºC

Table 4-3: Major FTIR absorption bands of compound SM/01

Compounds	**Absorption bands**		
	ν_{C-OH}	**$\nu_{C=C}$**	**ν_{C-H}**
SM/01	3000	1771	1378

Table 4-4: ^{13}C and ^{1}H NMR chemical shift bands of compound SM/01

Position	**^{13}C**	**^{1}H**
1	37.5	
2	31. 6	
3	72	3.5
4	42.4	
5	141	
6	122	
7	32	
8	31.8	
9	50	
10	36.5	
11	21	
12	40	
13	42	
14	57	
15	24.5	
16	28	
17	56.2	
18	36.2	
19	19.1	0.94
20	34	
21	26	
22	45.5	
23	23	
24	12.3	0.83
25	29	
26	15.6	0.8
27	19.7	0.81
28	19	0.68
29	12	1.02

4.6.1 Acute Toxicity of *R. caffra* fraction extracts

Acute toxicity results showed that only 2 mice died after 30 minutes at 2900 and 5000 mg/kg for MetOH extract and LD_{50} calculated to be 2154.1 mg/kg p.o. while no mice died when

treated with other fractions of n-hexane, DCM, EA, EtOH and LD_{50} was estimated at ≥ 5000 mg/kg p.o. The mice died at higher concentrations for MetOH fraction which therefore suggests that some *R. caffra* fractions are toxic at higher concentrations. However, the other fractions tested have LD_{50} greater than 5000 mg/kg p.o. which is suggested to be safe by D. Lorke. Therefore the absence of death among mice in other extracts support the above claim (Bulus, Atawodi, and Mamman, 2011; Lorke, 1983).

4.6.2 Antioxidant Activity of *R. caffra* Stem Bark Fraction Extracts

Antioxidant capacity of *R. caffra* fractions namely dichloromethane (DCM), ethyl acetate (EA), methanol (MetOH) and ethanol (EtOH) was examined using four different assays including total phenolic content, total flavonoid determination, DPPH and FRAP.

4.6.2.1 Total Phenolic Content

Total phenolic content in DCM, EA, MetOH and EtOH fraction extracts was identified using the Folin-ciocalteu´s reagent. Table 4-6 shows that the MetOH (8.42 ± 1.2 µg/mL) and EtOH (5.51 ± 0.11 µg/mL) have high phenolic content as compared to the other fraction extract. The antioxidant potency of these fractions is in this order: MetOH> EtOH> EA> DCM. Methanolic extract (MetOH) showed to be significantly different when compared to ethanolic extract (EtOH) as displayed (Table 4-6).

4.6.2.2 Total Flavanoids content

Flavonoids are defined as polyphenol compounds with lower molecular mass, obtained in plant parts such as leguminous, fruits, leaf and flowers (Fernandes de Oliveira et al., 2012). The total flavonoid contents varied from 1.87±0.06 to 18.11±0.165 µg/mL with the descending order: DCM> EA> MetOH> EtOH (Table 4-6). So the assay test resulted in the highest flavonoid content of DCM while the ethanolic extract had a small content in comparison with the other solvent fractions.

4.6.2.3 DPPH Radical Scavenging Activity

In this assay, the colour change was observed indicating reduction from purple DPPH colour to yellow DPPHH signifying that the fraction extracts have antioxidant activity. IC_{50} were reported to be 15.34 ± 0.25, 9.16 ± 0.78, 42.56 ± 0.57 and 46.22 ± 0.08 µg/mL for DCM, EA, MetOH and EtOH respectively. Table 4-6 displays that MetOH has higher scavenging capacity as compared to other extracts as shown in the order: EtOH> MetOH> DCM> EA. It was showed that the *R. caffra* fraction extracts exhibit antioxidants activity and can be

considered as potent antioxidants. This may be due to the presence of hydroxyl groups in the evaluated fraction extracts because the higher the number of hydroxyl, the better the radical scavenging ability (Pellati et al., 2004).

4.6.2.4 FRAP Assay

The antioxidant activity *R. caffra* stem bark fraction extracts was further established through their reducing power as demonstrated. In this assay, the reducing ability ($Fe^{3+} \rightarrow Fe^{2+}$) was established by the resulting noticeable intense blue colour indicating the formation of ferrous complex which also mean that *R. caffra* stem bark fraction extract preserve antioxidant activity. Table 4-5 shows that MetOH (204 ± 8.07 µg/mL) fraction had higher IC_{50} as compared to other extracts as shown in the order: MetOH> EtOH> DCM> EA (Table 4-6). MetOH extract showed to exhibit high antioxidant activity in FRAP assay and total phenolic content. This antioxidant activity is in agreement Njau et al., which reported that *R. caffra* stem bark methanolic extract has high antioxidant activity. Number of phytochemicals such as flavonoids, phenylpropanoids and phenolic extracts are known to be responsible for antioxidant activity of fruits vegetable and medicinal plants. The plants with antioxidant activity are mostly used in food industry to improve the quality and nutritional value of food (Njau et al., 2014; Pellati et al., 2004; Saeed et al., 2012).

Table 4-5: Antioxidant activity of DCM, EA, MetOH and EtOH extracts by total phenolic and flavonoids, DPPH as well as FRAP

	DCM	**EA**	**MetOH**	**EtOH**
Total Phenolic Content (GAE/ mg extract)	2.69 ± 0.19	3.12 ± 0.53	8.42 ± 1.2**	5.51 ± 0.11
Total Flavonoids (QE /mg extract)	18.11 ± 0.165***, ###	10.82 ± 0.05***, ###	9.71 ± 0.15###	1.87 ± 0.06
DPPH (AAE/ mg extract)	15.34±0.25	9.16±0.78	42.56±0.57***	46.22±0.08###
FRAP (AAE/ mg extract)	44.32 ± 1.1	44.07 ± 1.51	204 ± 8.07***	173.6 ± 2.53###

Reported as mean ± standard error of mean (X±SEM); ** or ##p<0.01, *** or ###p<0.001= statistically difference of two *R. caffra* fractions comparison

*- comparing with MetOH, #-comparing with EtOH

GAE- Gallic acid equivalent

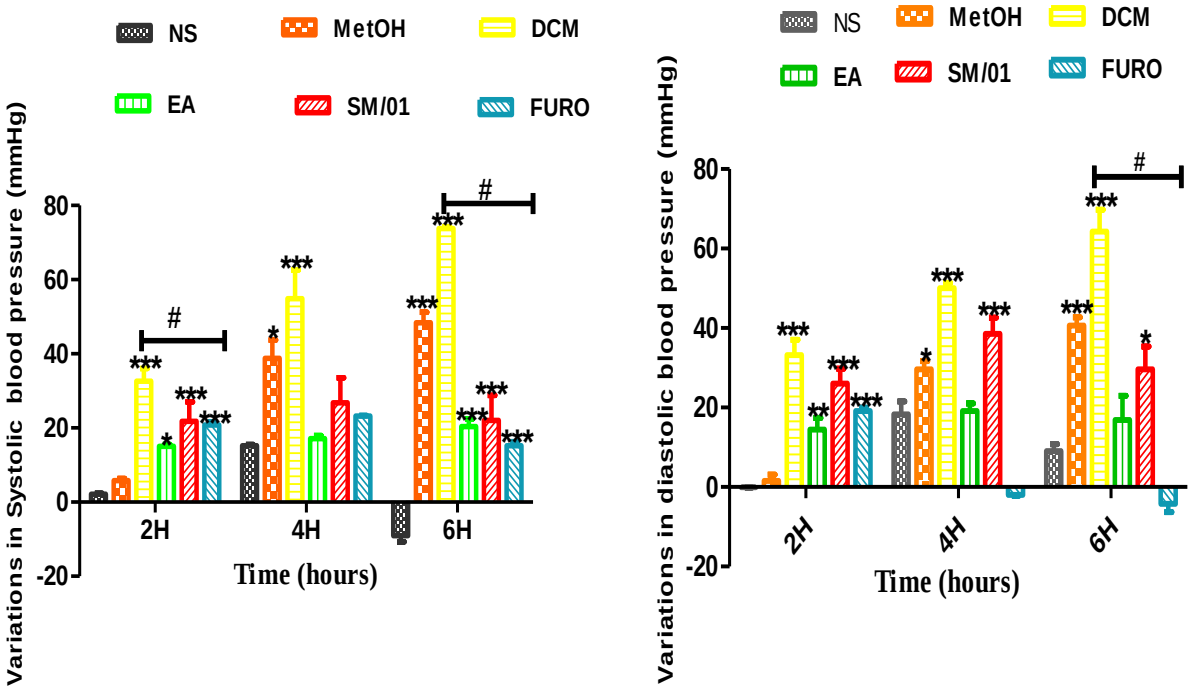
QE- Quercetin equivalent

AAE- Ascorbic acid equivalent

This present study shows that MetOH has the highest antioxidant activity on FRAP and total phenolic assays, while DCM and EtOH has antioxidant ability on total flavonoids and FRAP respectively. This suggests that these *R. caffra* antioxidant activities are method dependent.

4.7 Antihypertensive Studies

Figure 4.9 shows the hypotensive effect of *Rauvolfia caffra* fractions on systolic and diastolic blood pressure compared to normal saline (NS) control. Methanolic, DCM and EA extracts as well as EAC showed a significant decrease in the systolic and diastolic blood pressure of spontaneously hypertensive rats at the doses 500, 300 and 15 mg/kg. The effect of DCM ($p<0.001$) increased with time with the best effect at 6 hours on both systolic and diastolic blood pressure. The entire tested fraction fractions and a compound were significantly better than furosemide except for ethyl acetate extract at 2 and 4 hour for both blood pressures (Figure 4.9 (a) and (b)). The β-sitosterol compound (SM/01) showed high significance difference as compare to ethyl acetate fraction which it was isolated from, at 2 and 6 hours for systolic and 2, 4 and 6 hours for diastolic blood pressure (Figure 4.9 a and b). This suggests that the compound is more active at its pure state than as a mixture.



(a) **(b)**

Figure 4.9: (a) Shows the hypotensive effect of *R. caffra* fractions on Systolic blood pressure. **(b)** Diastolic blood pressure. **Key:** NS= normal saline (control), FURO= furosemide (standard drug), MetOH= methanol extract, DCM= dichloromethane, EA= ethyl acetate and SM/01= β-sitosterol. Data are expressed as Mean ± SEM for 6 animals. #= maximum significance difference when compared to standard drugs, **p< 0.01 and p***< 000.1 = statistically different with comparison of tested fractions and compound against control group (NS) and analysis of variances (ANOVA, Dunnett's).

The high significance difference of DCM and MetOH in systolic and diastolic blood pressure may be due to the good antioxidant capacity which showed in the present study. This is due to a reason that the many studies reported that hypertension results due to the increase in oxidative stress. However, antioxidants play a significant role on reducing the oxidative stress and thus decreasing hypertension (Beg, Sharma, Akhtar, Gupta, and Mohd, 2011). The decrease in blood pressure can be associated with a number of chemical mechanisms. Most of experimental studies reported that the glucose and fructose intake results in the increase in blood pressure. The rise in glucose level in the body can be associated with the reduction of nitrite oxide (NO) levels eventually resulting in the rise of blood pressure. It has been reported that plant extracts which are rich with polyphenols having antioxidant effects that elevate the endothelial dysfunction by increasing NO formation (Alamgeer et al., 2013;

Siddiqi et al., 2012). Therefore, from present study Rauvolfia caffra may be considered as potential natural antioxidant source which can play a significant in the treatment of hypertension.

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CHAPTER 5

5. Conclusion and Recommendations

In conclusion, two plants namely *Tagetes minuta* and *Rauvolfia caffra* were considered for the isolation of volatile components and non-volatile compounds respectively. The extraction of essential oils (volatile compounds), fraction extracts as well as the isolated compound, identification and biological studies were evaluated on *R. caffra* fraction fractions from stem bark which showed antioxidant activity which may be due to the presence of hydroxyl groups in the compound mixture or in the pure isolated compound. The use of *R. caffra* stem bark extracts in managing hypertension and other diseases by traditional healers can be scientific supported with the presence of phytochemicals (polyphenols and flavonoids) well-known health benefits. Thus, it is recommended that the plant should be exploited further and developed using modern biotechnology which involves separation and purification of compounds under controlled environment to be considered for pharmaceutical formulation.

Tagetes minuta essential oil (TMDS, TMDL and TMDF) showed high antioxidant activity on DPPH and FRAP assays. This activity can be attributed to the presence of oxygenated monoterpenes and sesquiterpenes which act as free radical scavenging and reducing agents. It is therefore recommended that *T. minuta* and *R. caffra* may serve as alternative natural antioxidant sources to treat various diseases including hypertension.

Appendix A

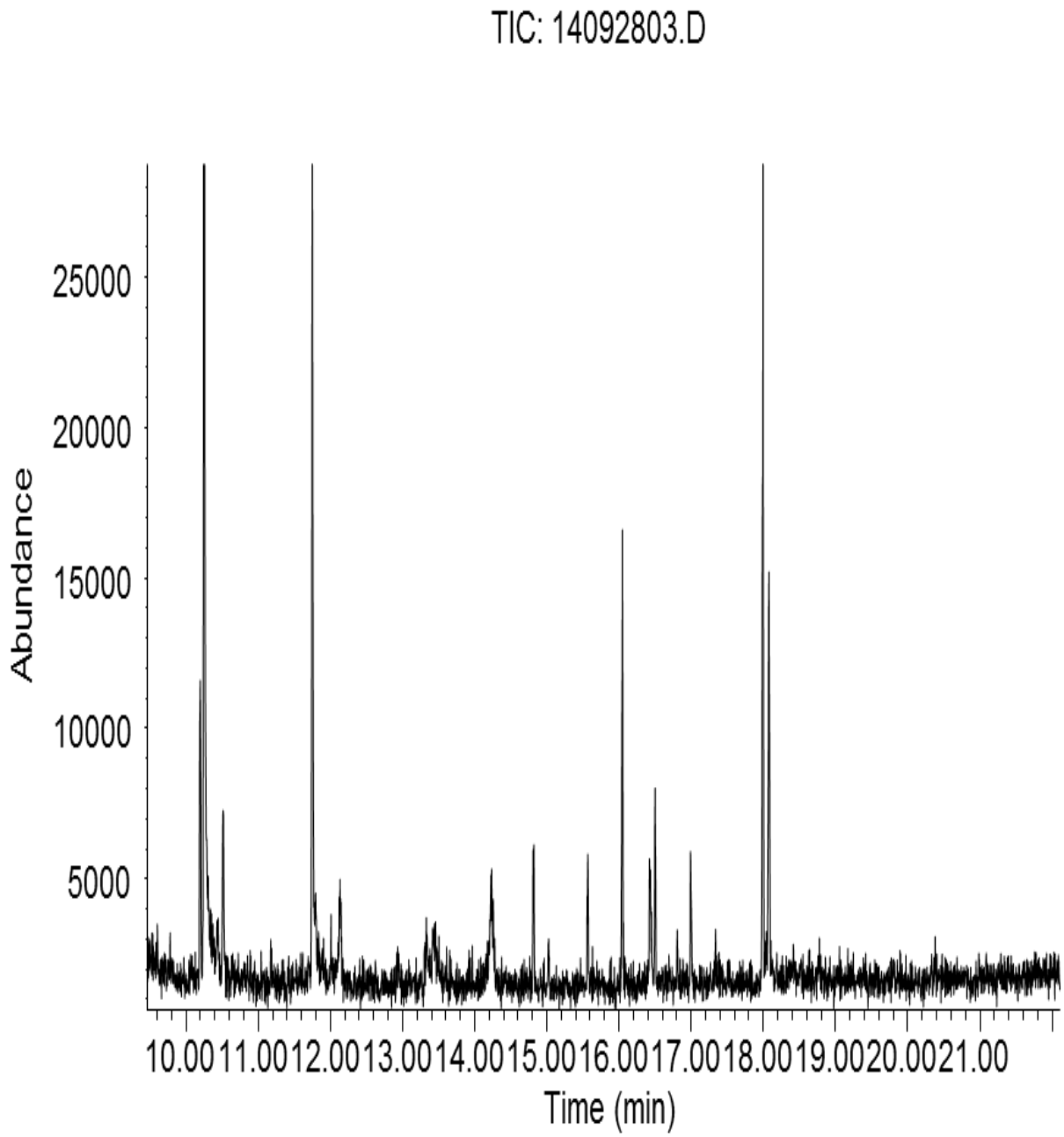

Figure 1a: *Tagetes minuta* fresh stem (TMFS) essential oil GCMS chromatogram

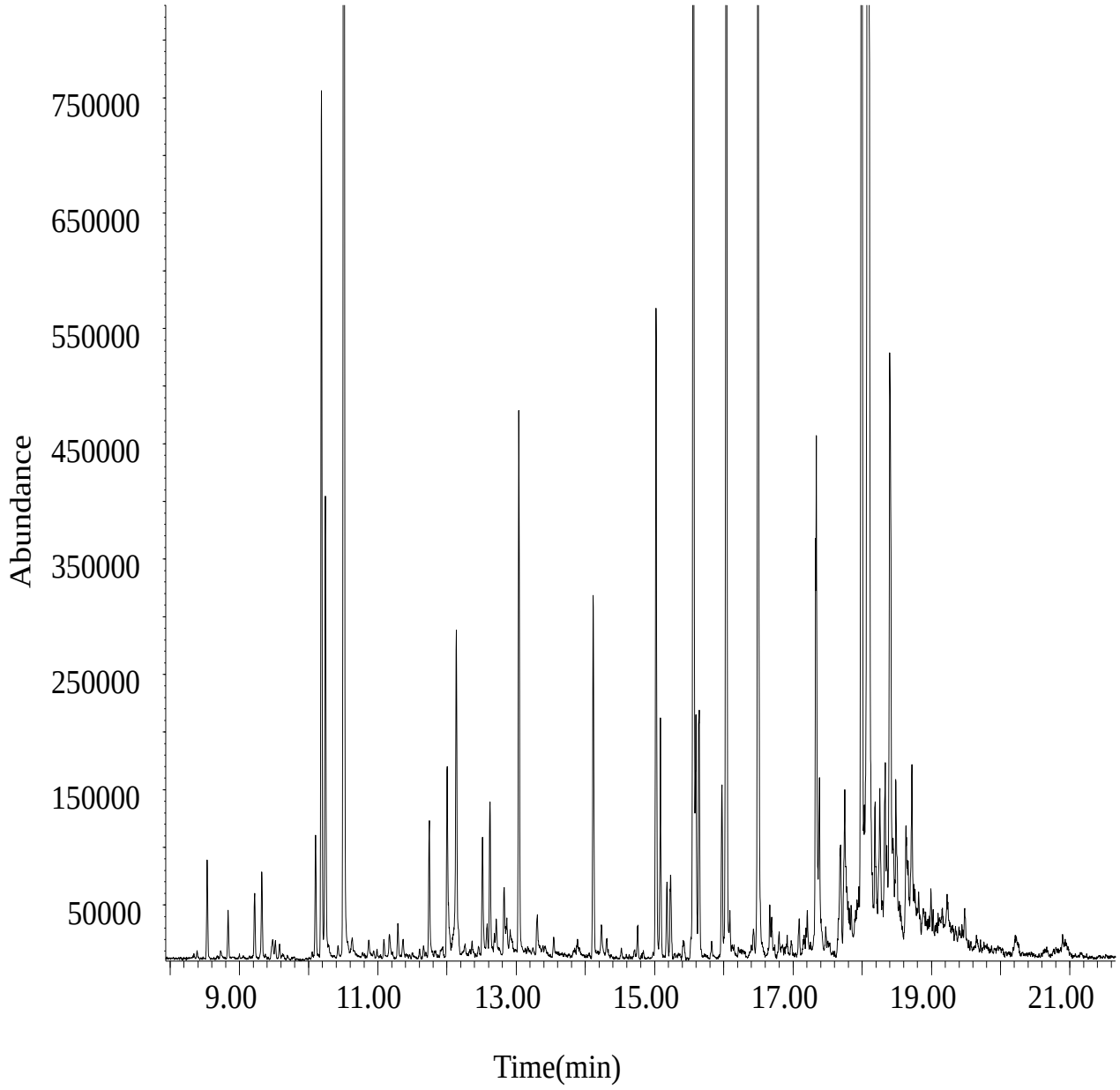

Figure 2a: *Tagetes minuta* dried stem (TMDS) essential oil GCMS chromatogram

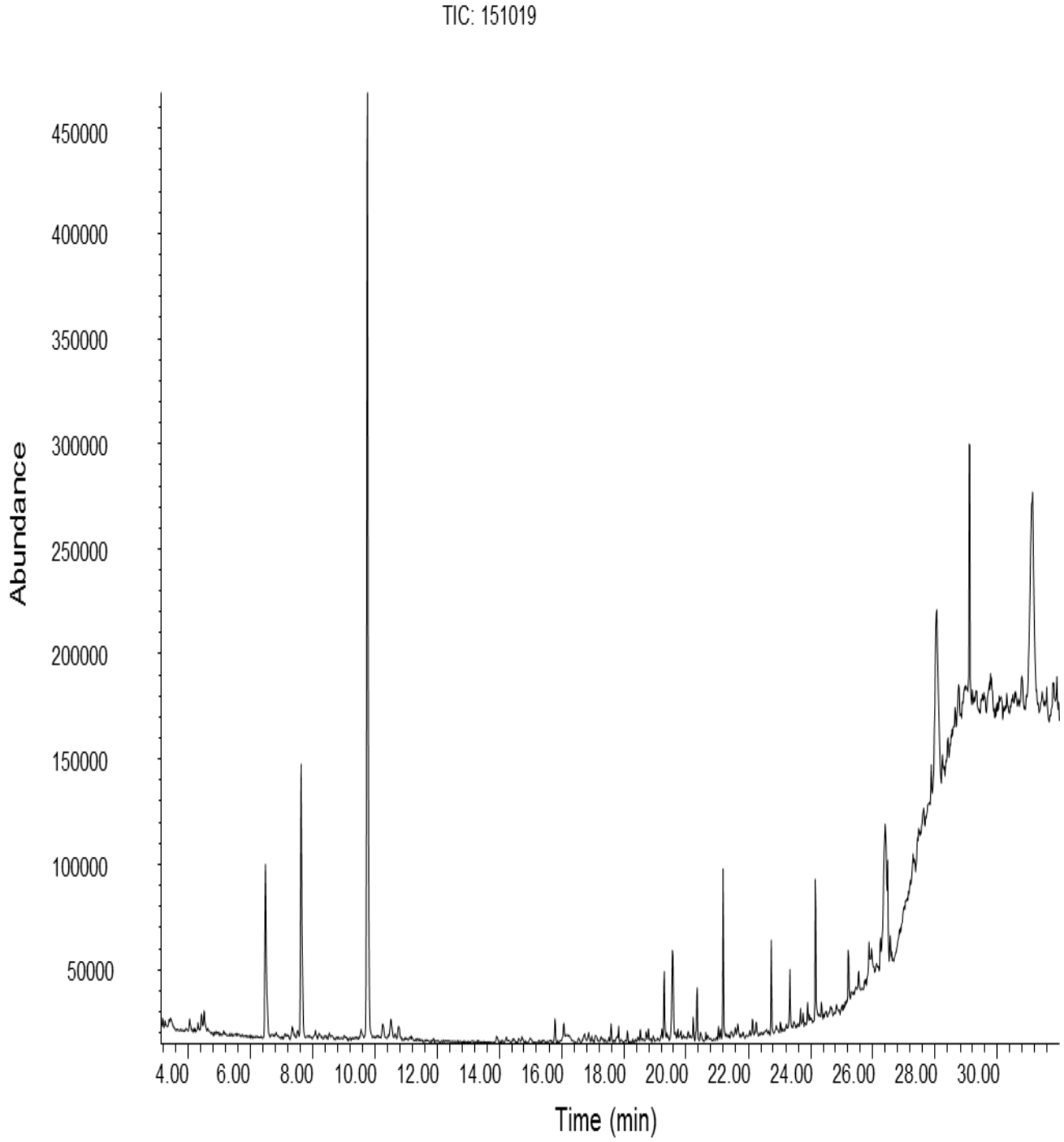

Figure 3a: *Tagetes minuta* fresh leaf (TMFL) essential oil GC-MS chromatogram

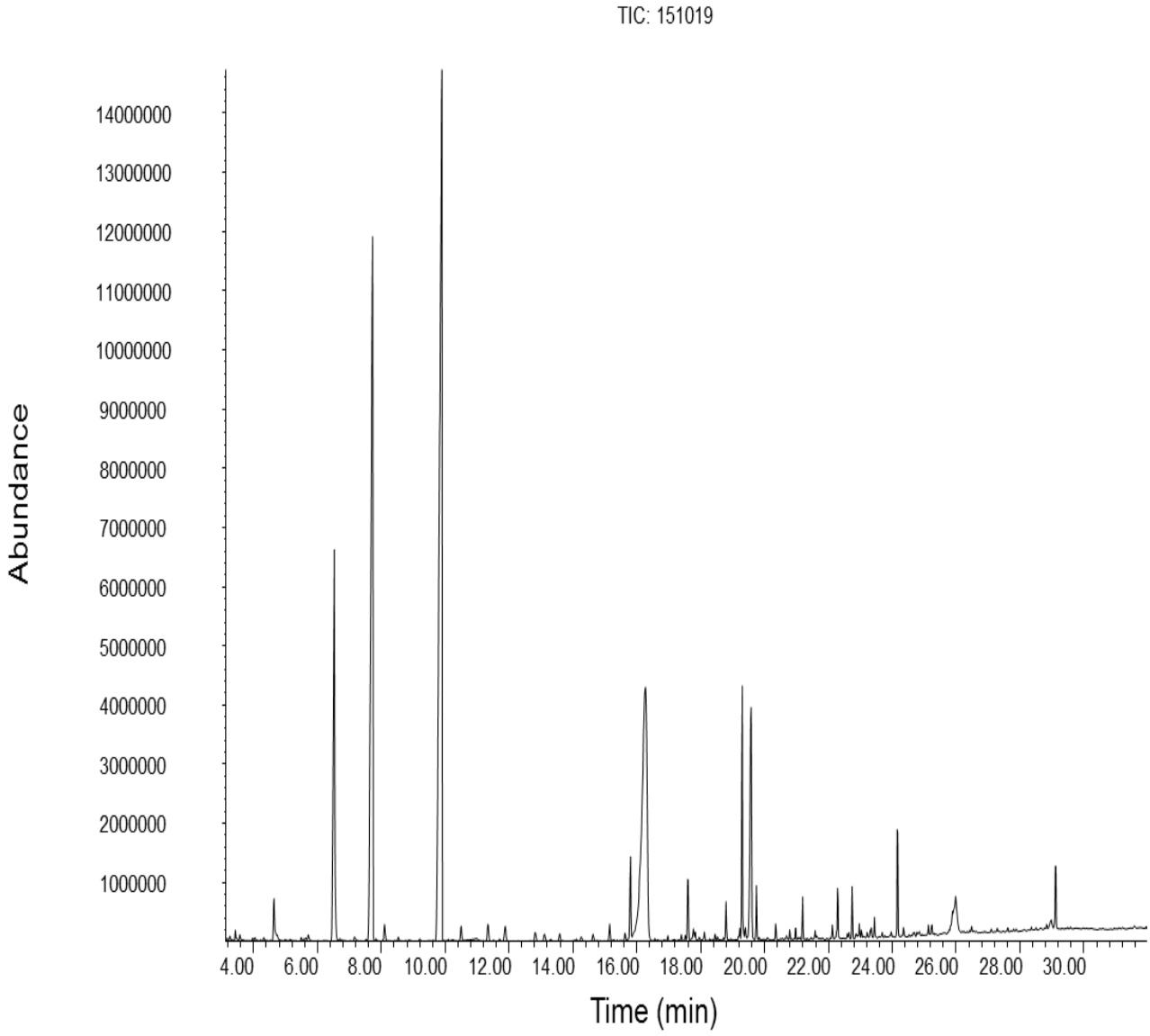

Figure 4a: *Tagetes minuta* dried leaf (TMDL) essential oil GC-MS chromatogram

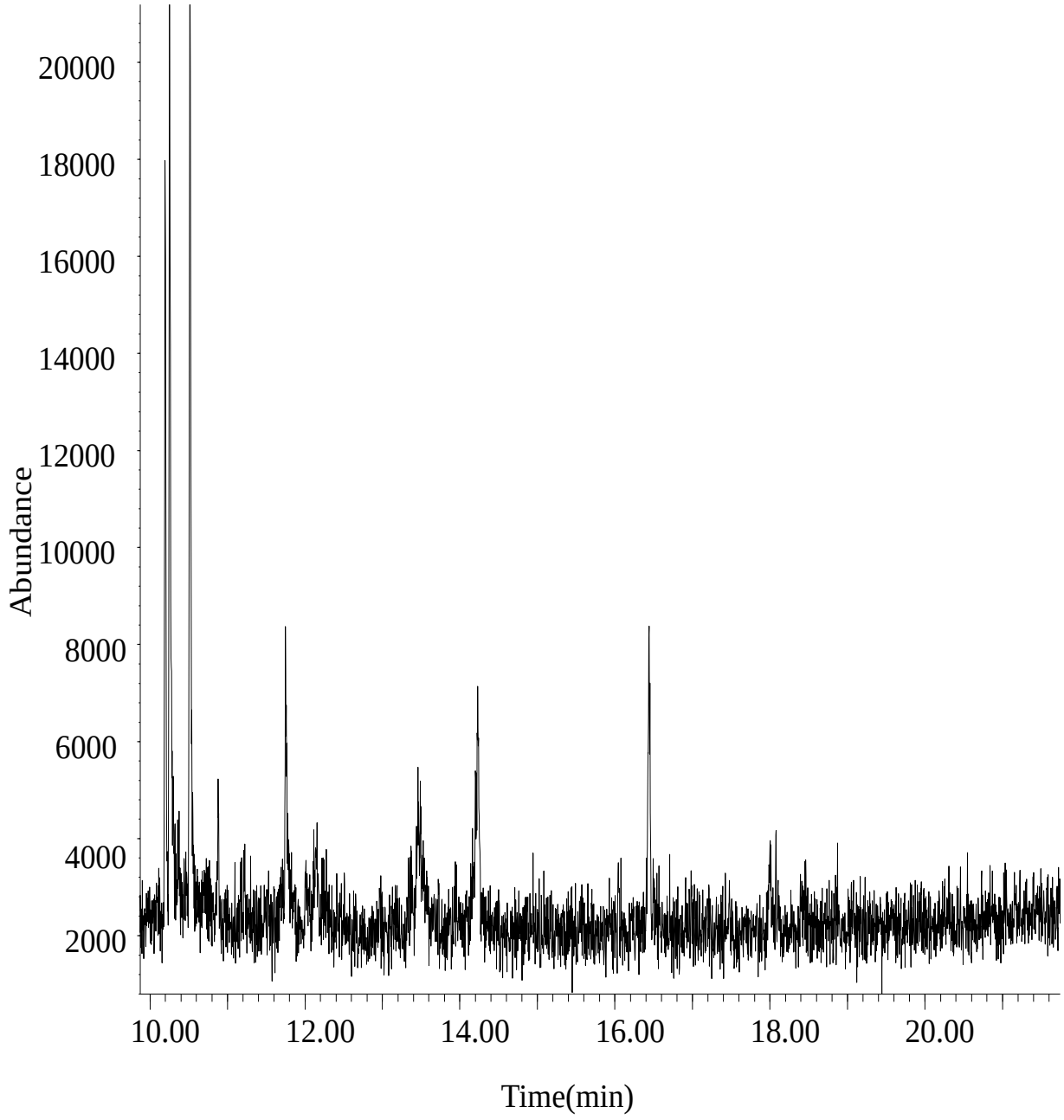

Figure 5a: *Tagetes minuta* fresh flower (TMFF) essential oil GC-MS chromatogram

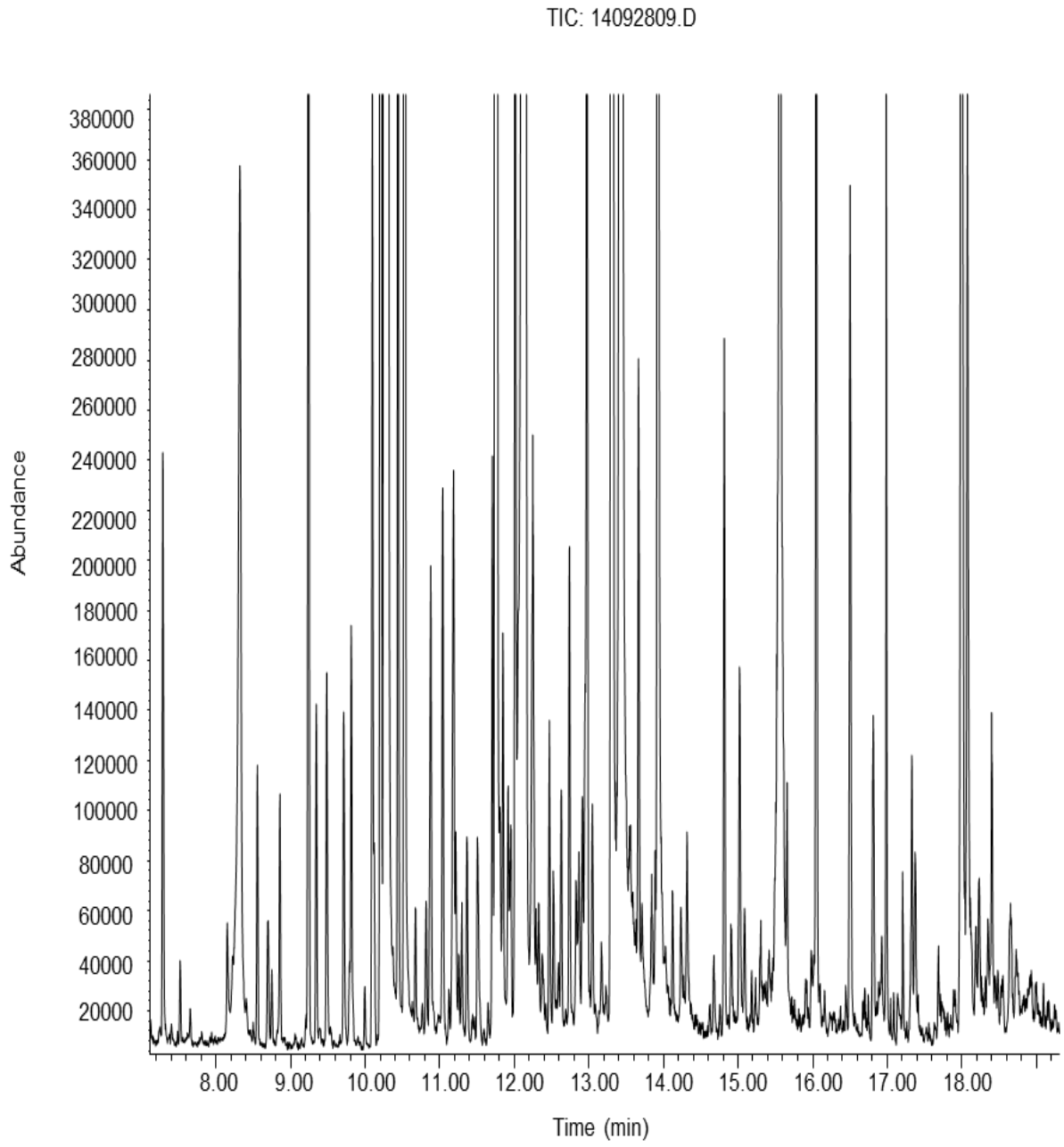

Figure 6a: *Tagetes minuta* dried flower (TMDF) essential oil GC-MS chromatogram

Appendix B

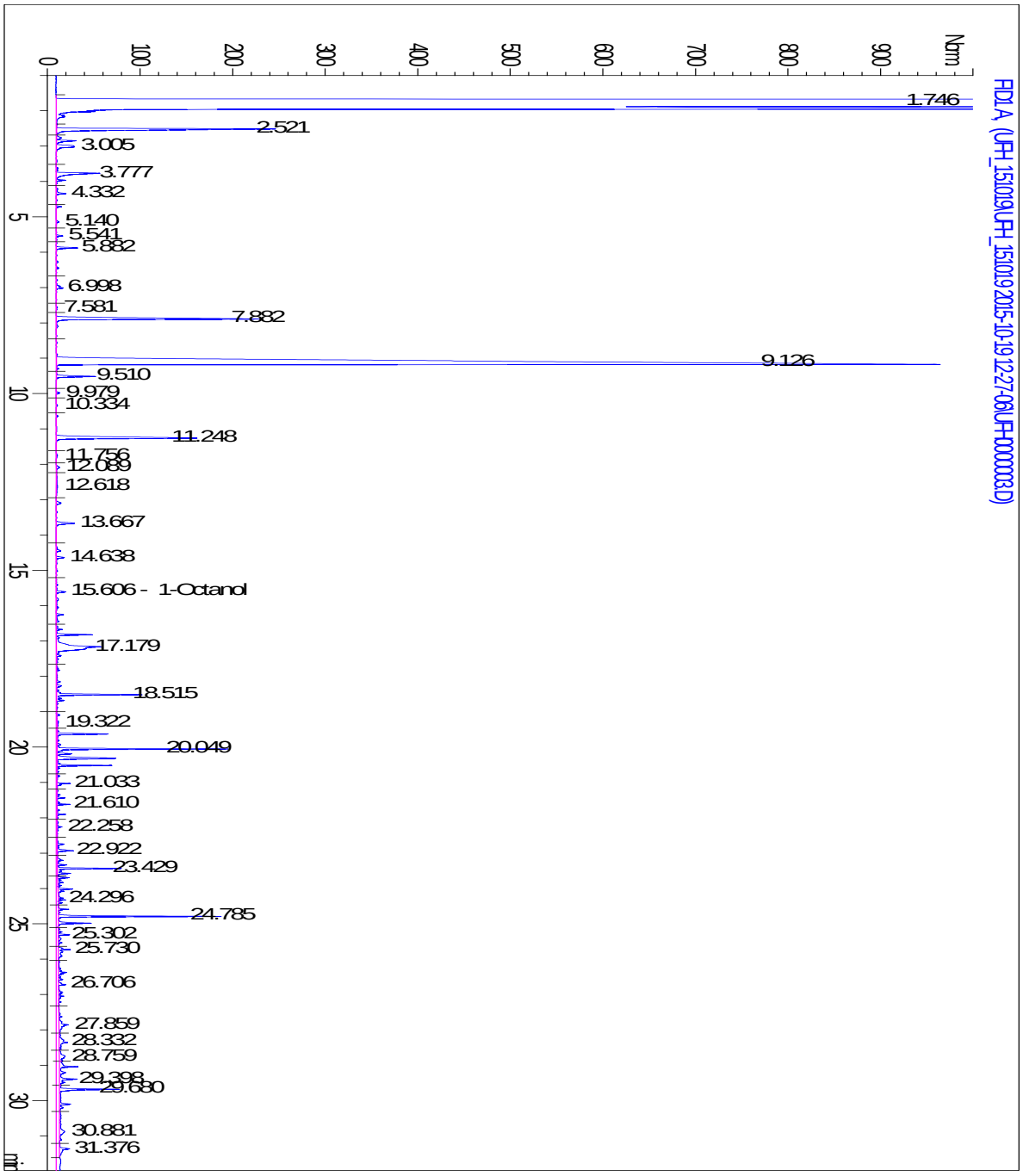

Figure 1b: *Tagetes minuta* fresh stem (TMFS) essential oil GC chromatogram

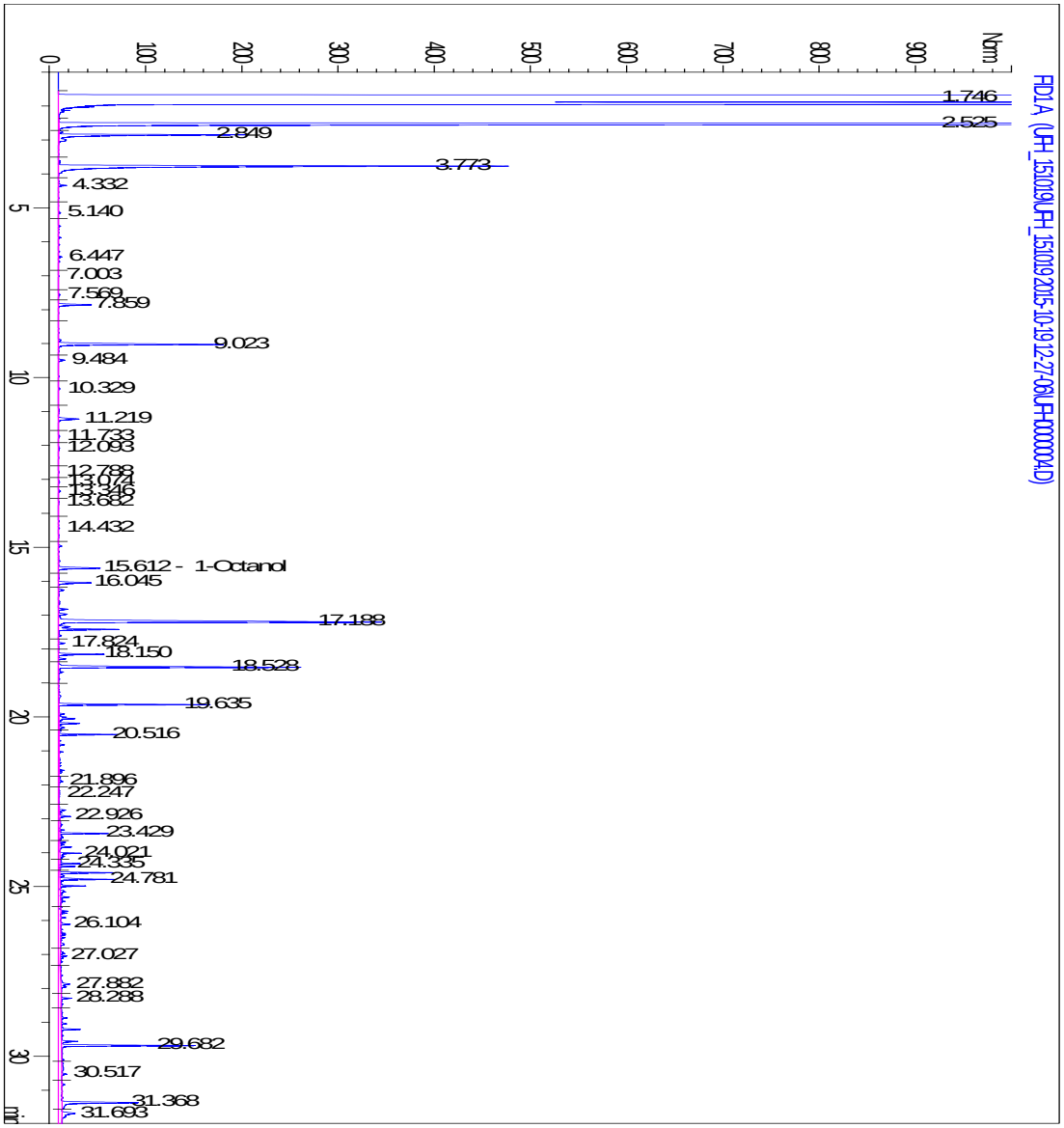

Figure 2b: *Tagetes minuta* dried stem (TMDS) essential oil GC chromatogram

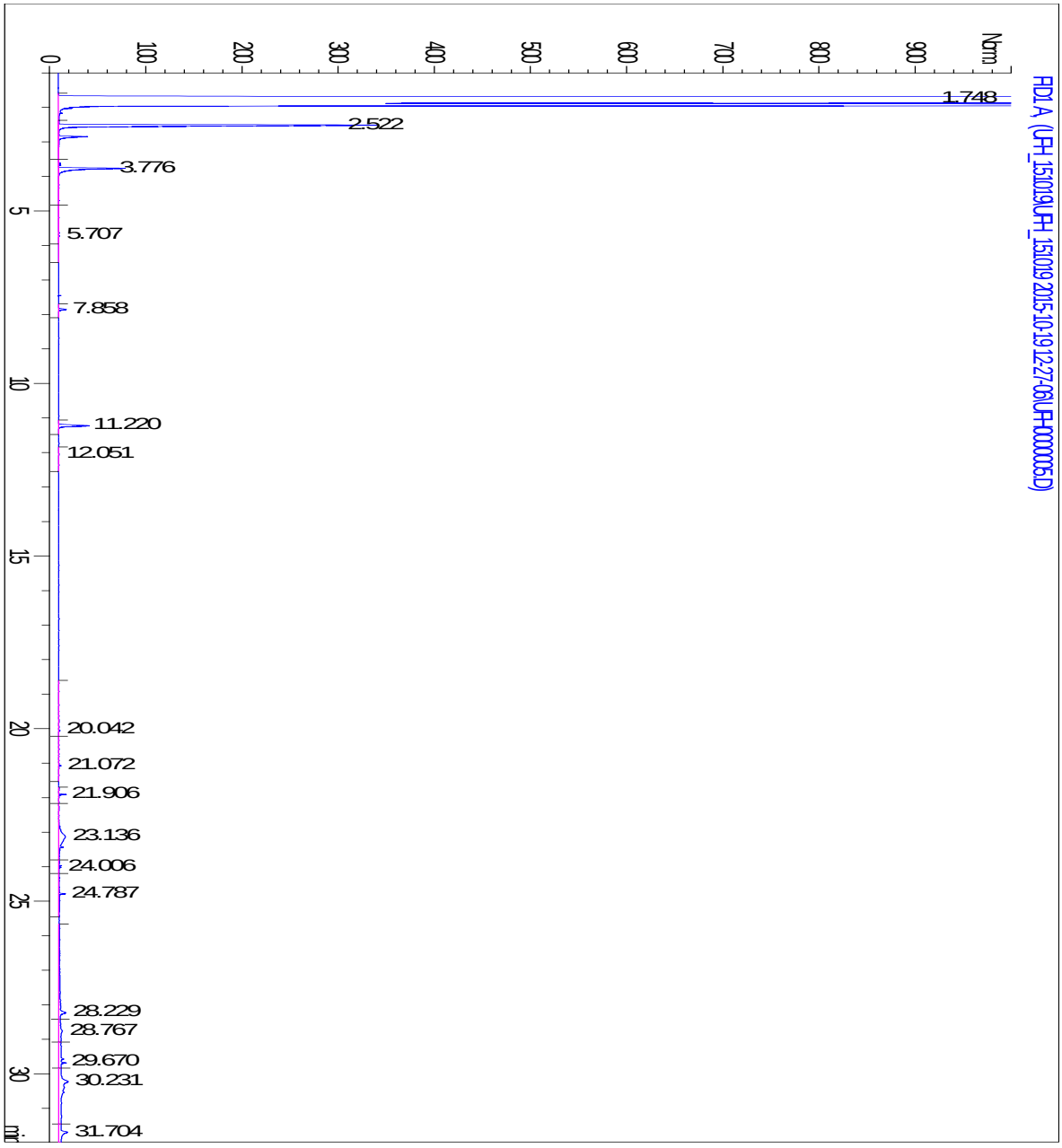

Figure 3b: *Tagetes minuta* fresh leaf (TMFL) essential oil GC chromatogram

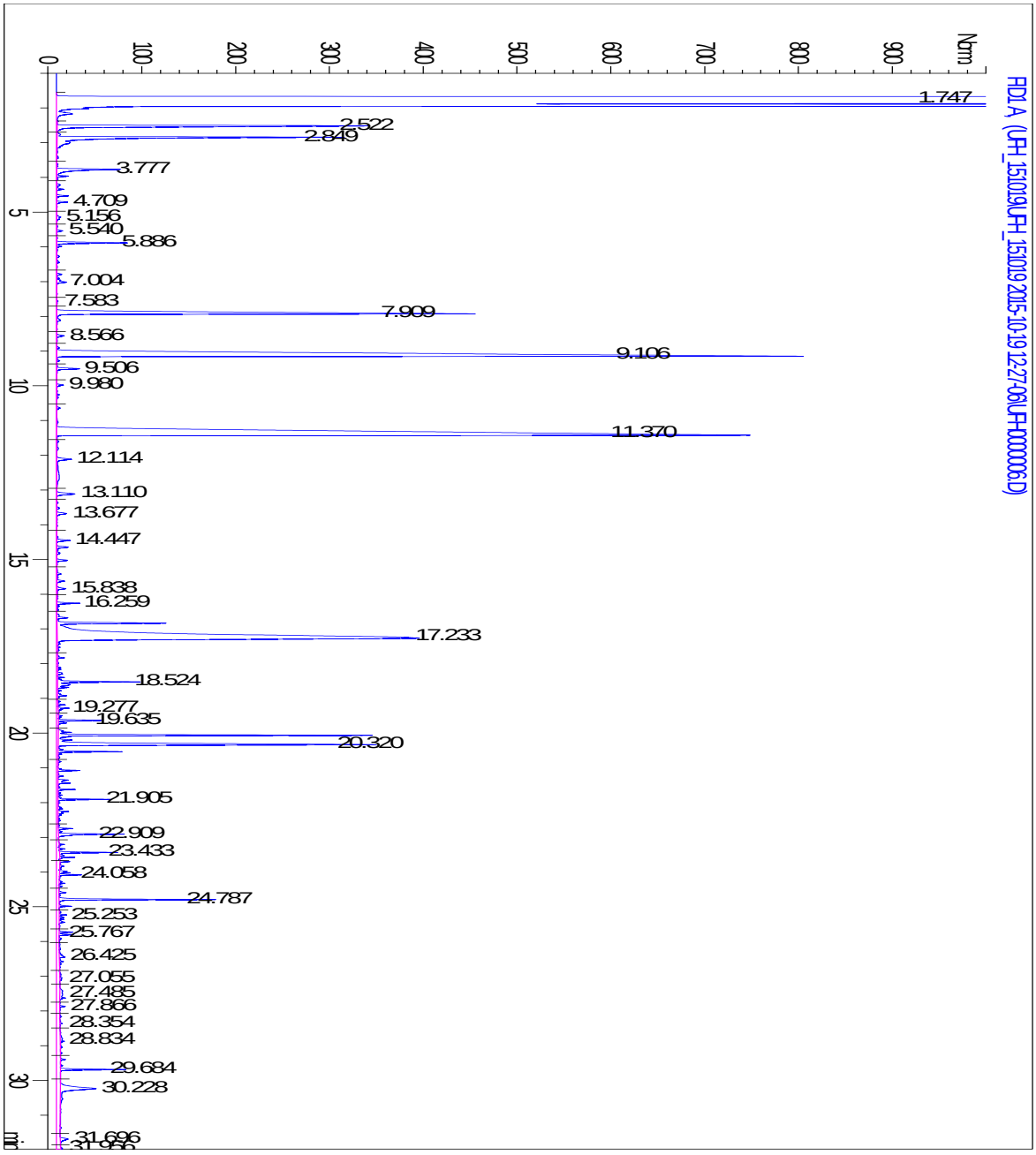

Figure 4b: *Tagetes minuta* dried leaf (TMDL) essential oil GC chromatogram

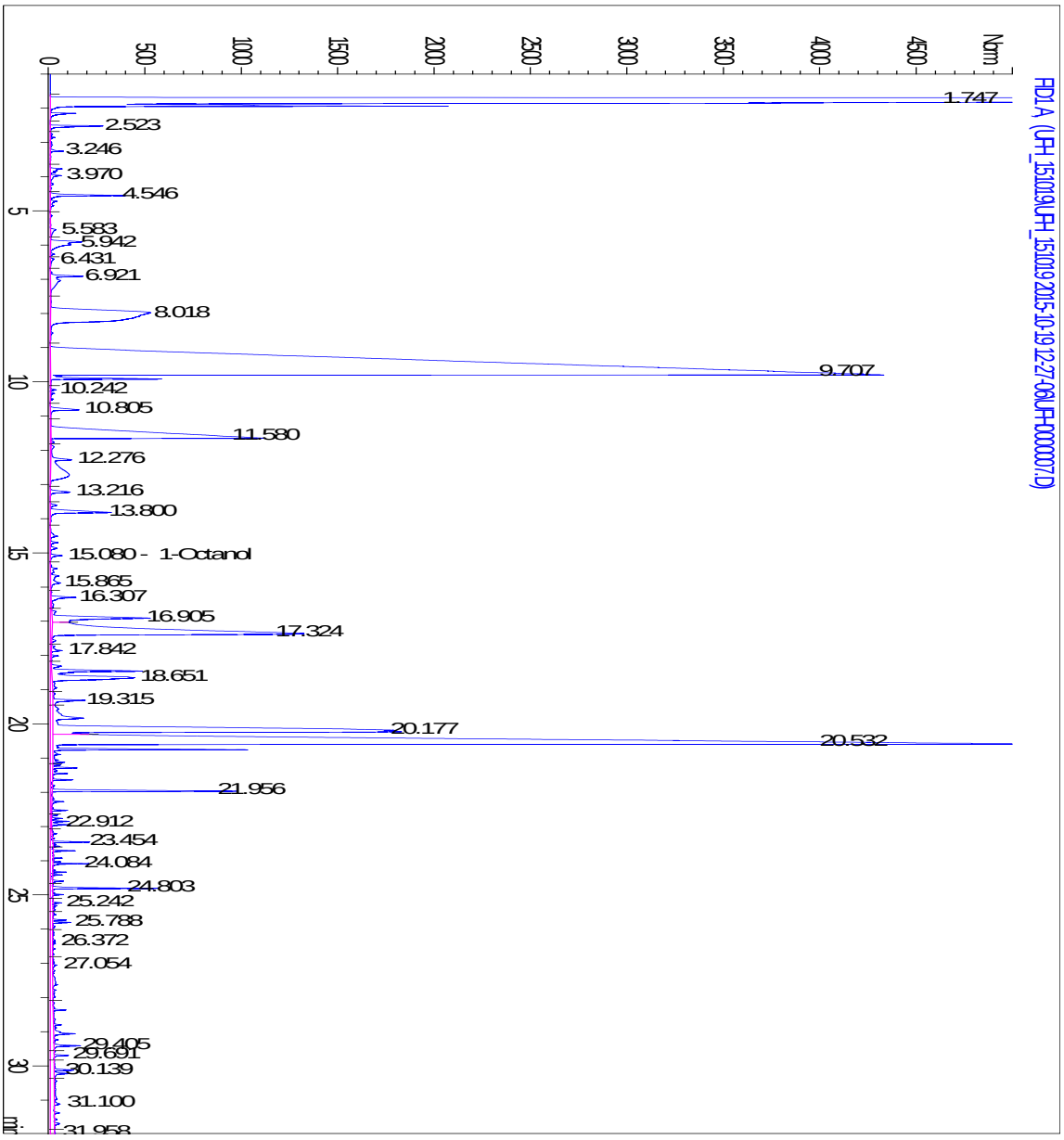

Figure 5b: *Tagetes minuta* fresh flower (TMFF) essential oil GC chromatogram

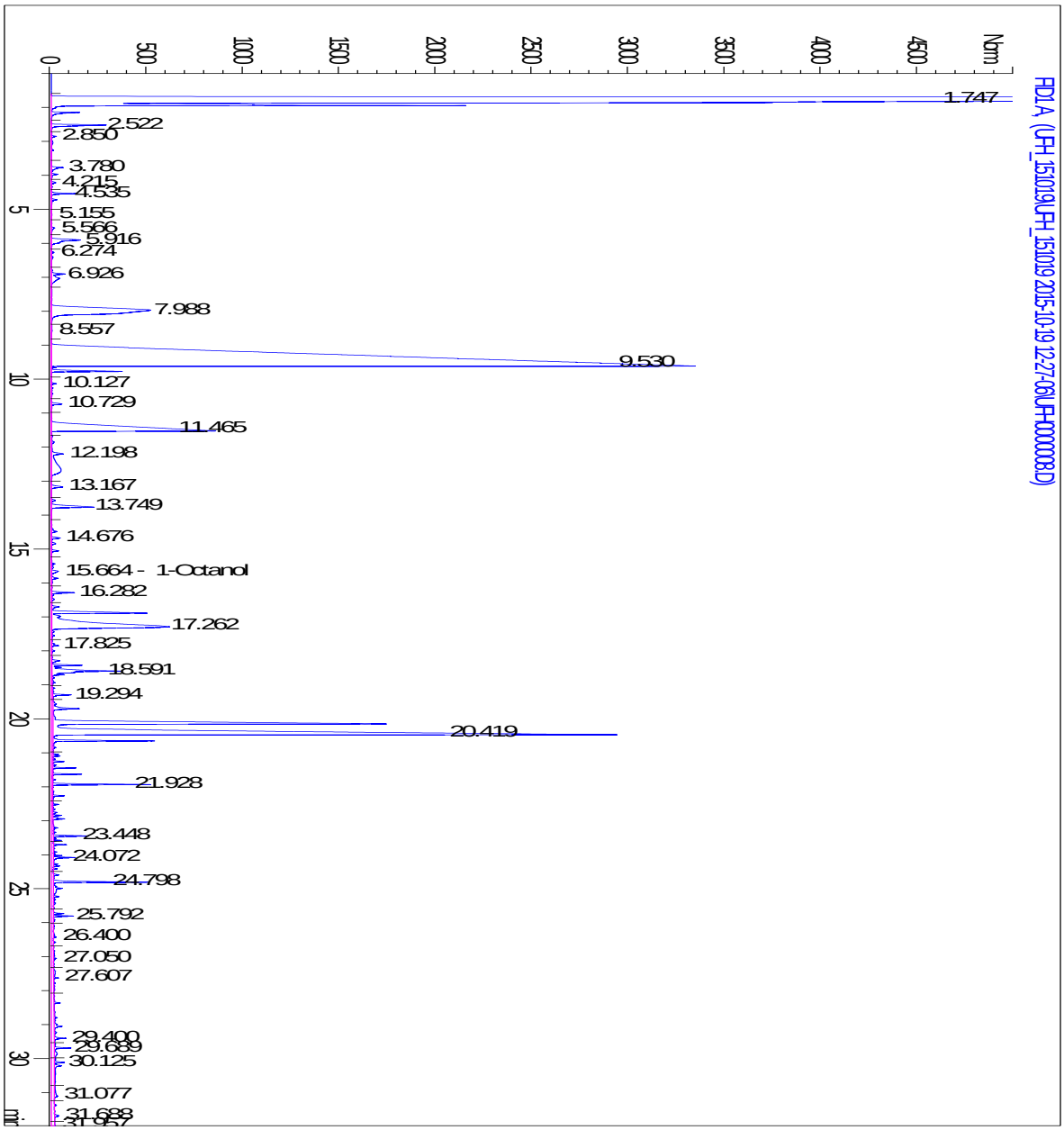

Figure 6b: *Tagetes minuta* dried flower (TMDF) essential oil GC chromatogram

Appendix C

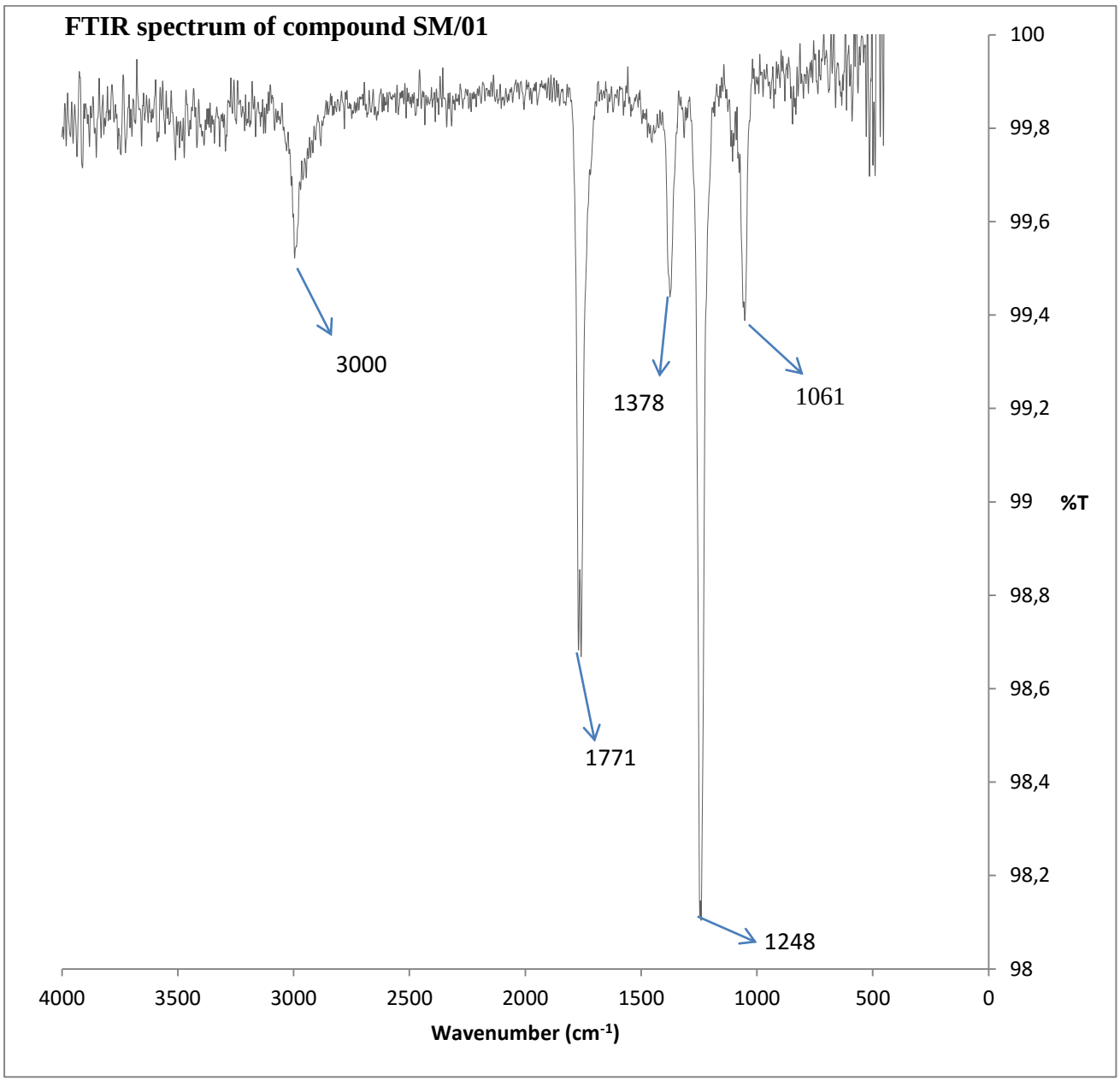

Figure 1c: FTIR spectrum of β-sitosterol

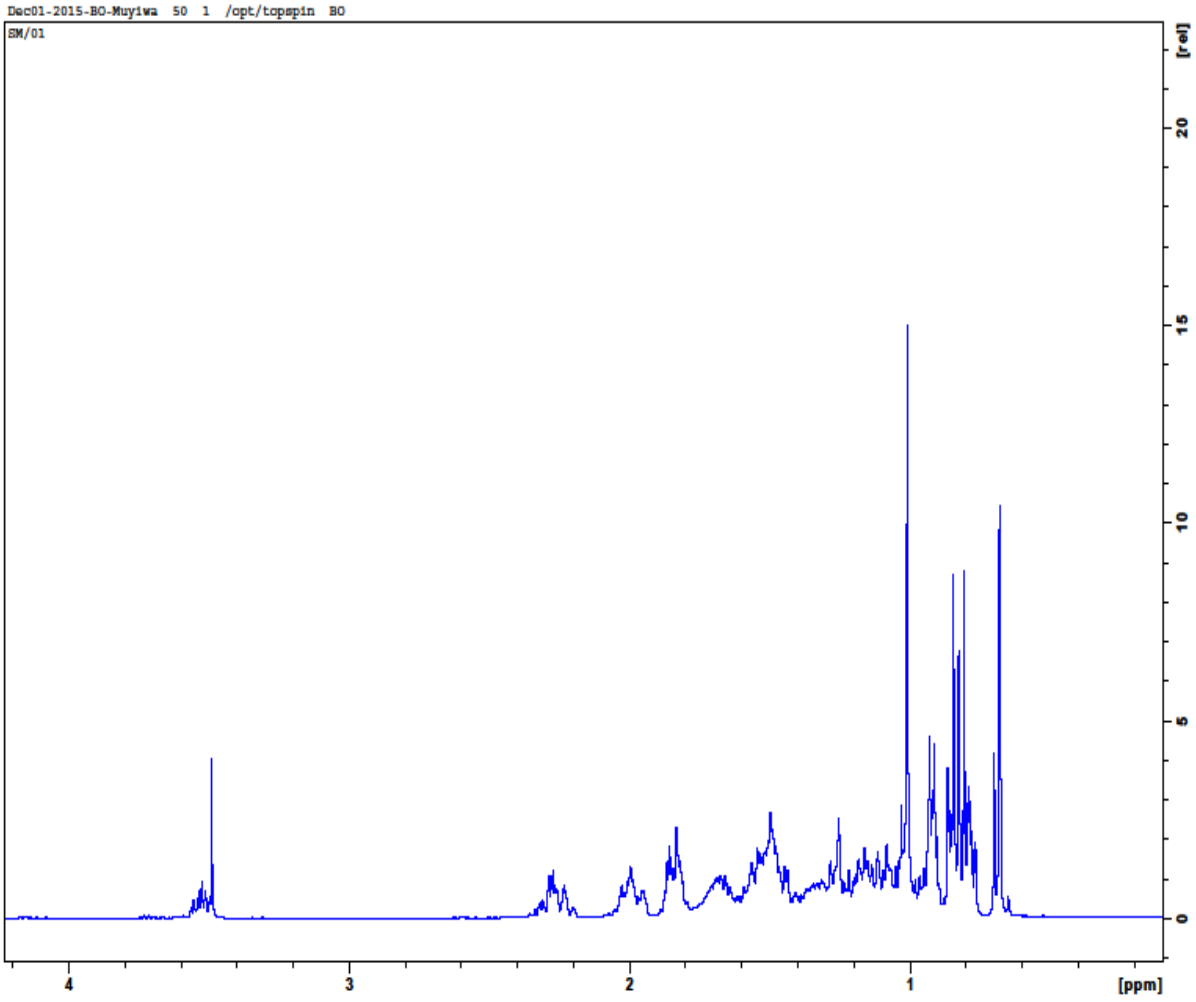

Figure 2c: Proton (^{1}H-NMR) spectrum of β-sitosterol

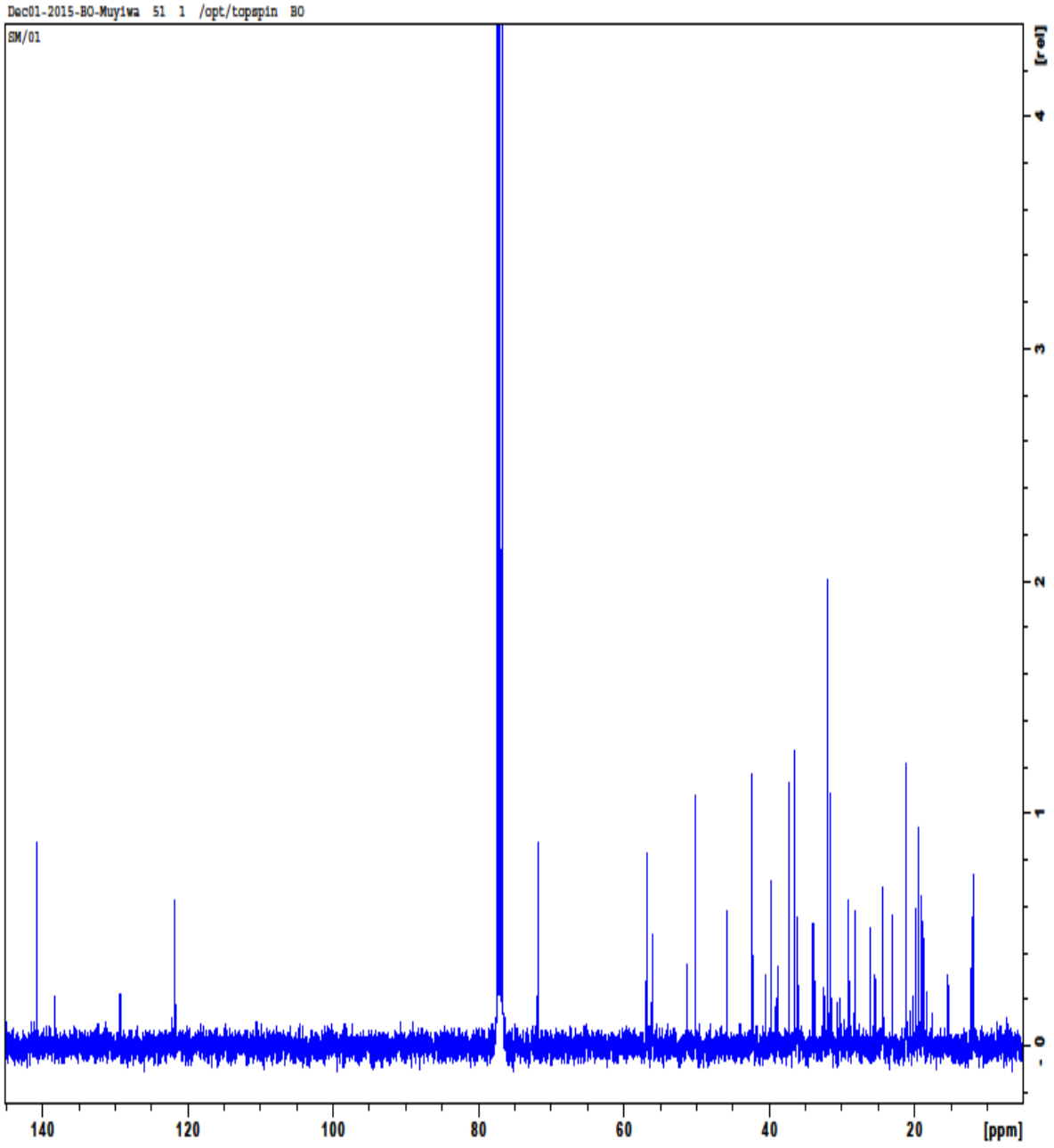

Figure 3c: Carbon (^{13}C-NMR) spectrum of β-sitosterol

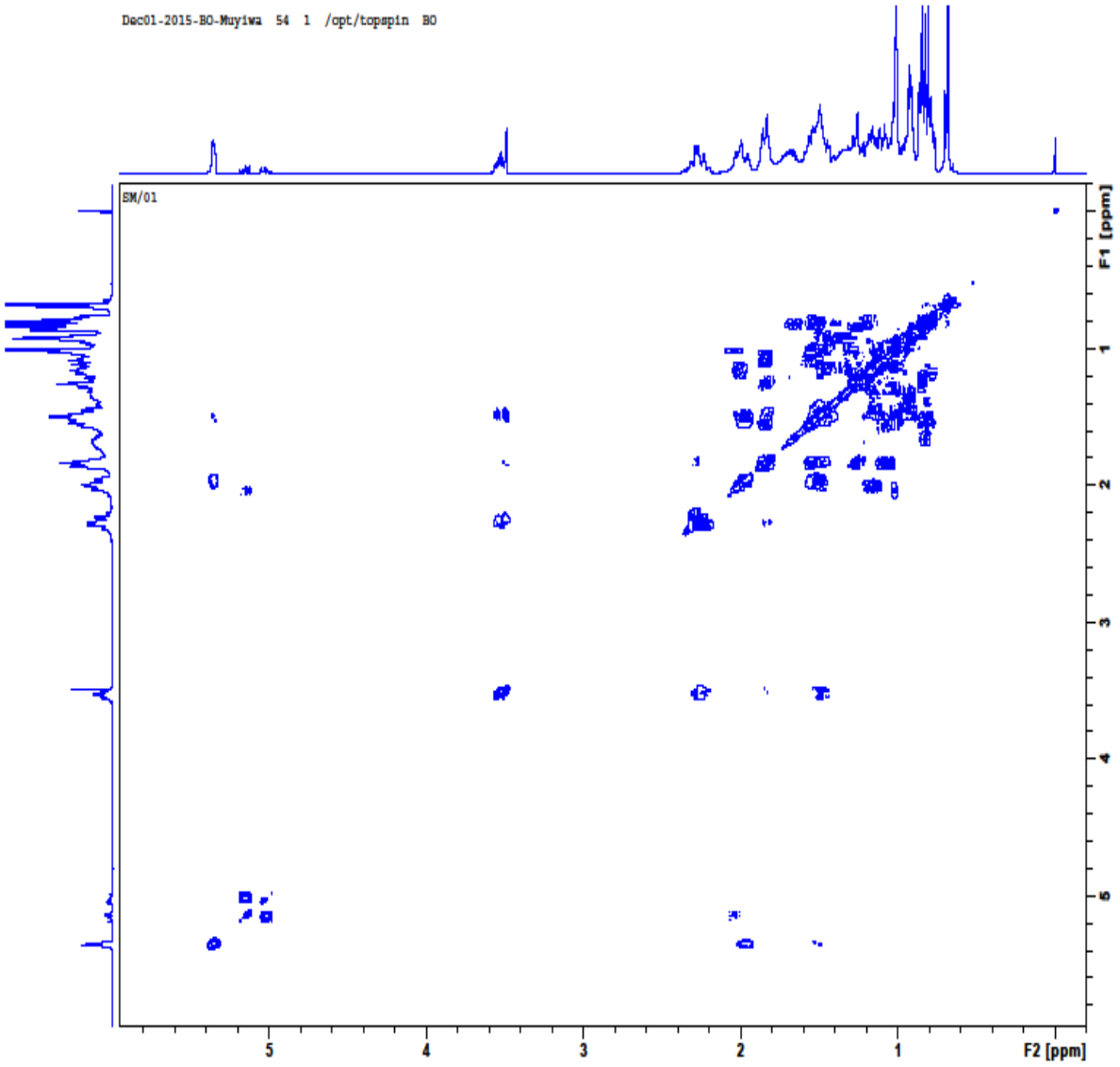

Figure 4c: COSY spectrum of β-sitosterol

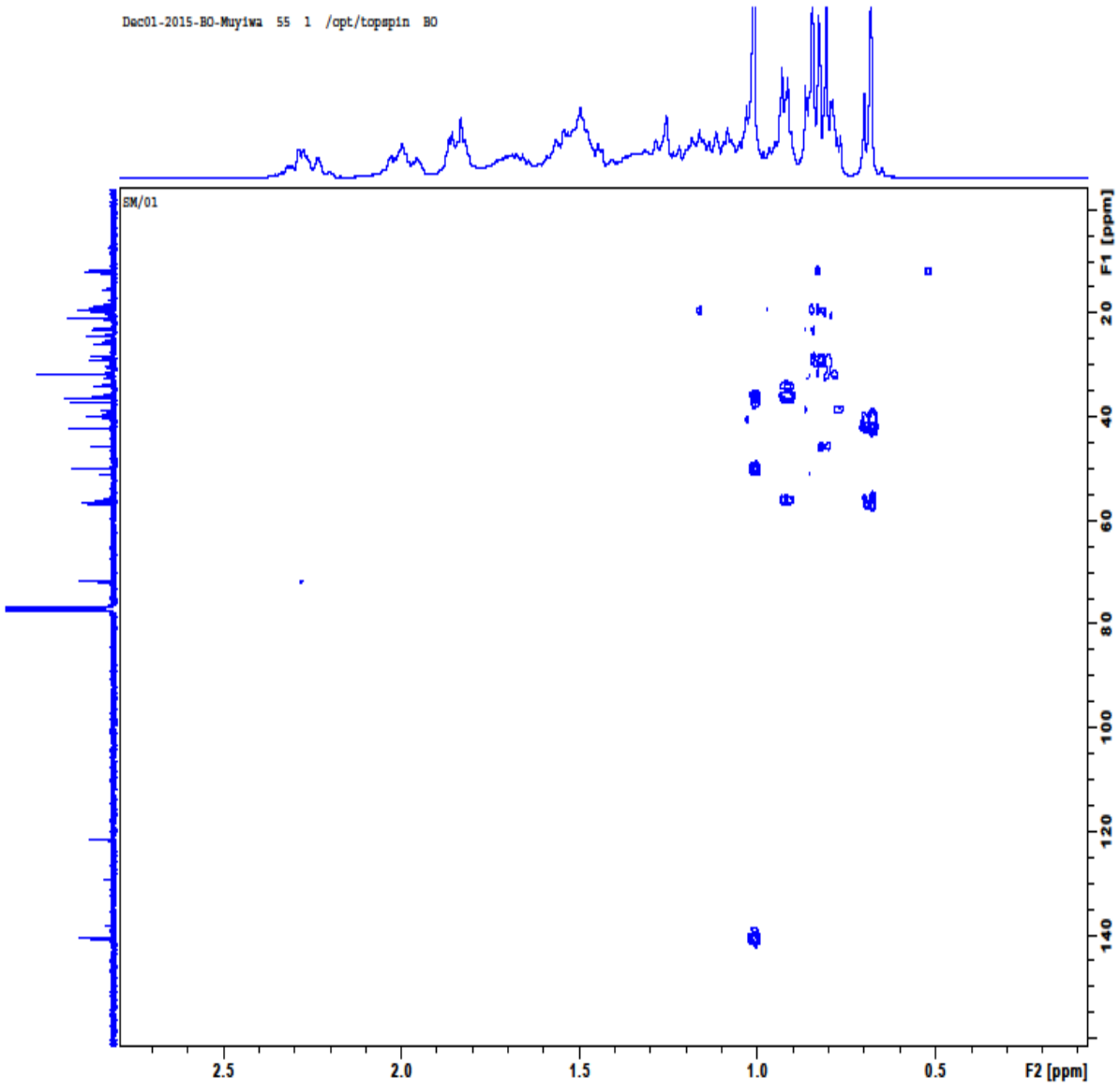

Figure 5c: HMBC spectrum of β-sitosterol

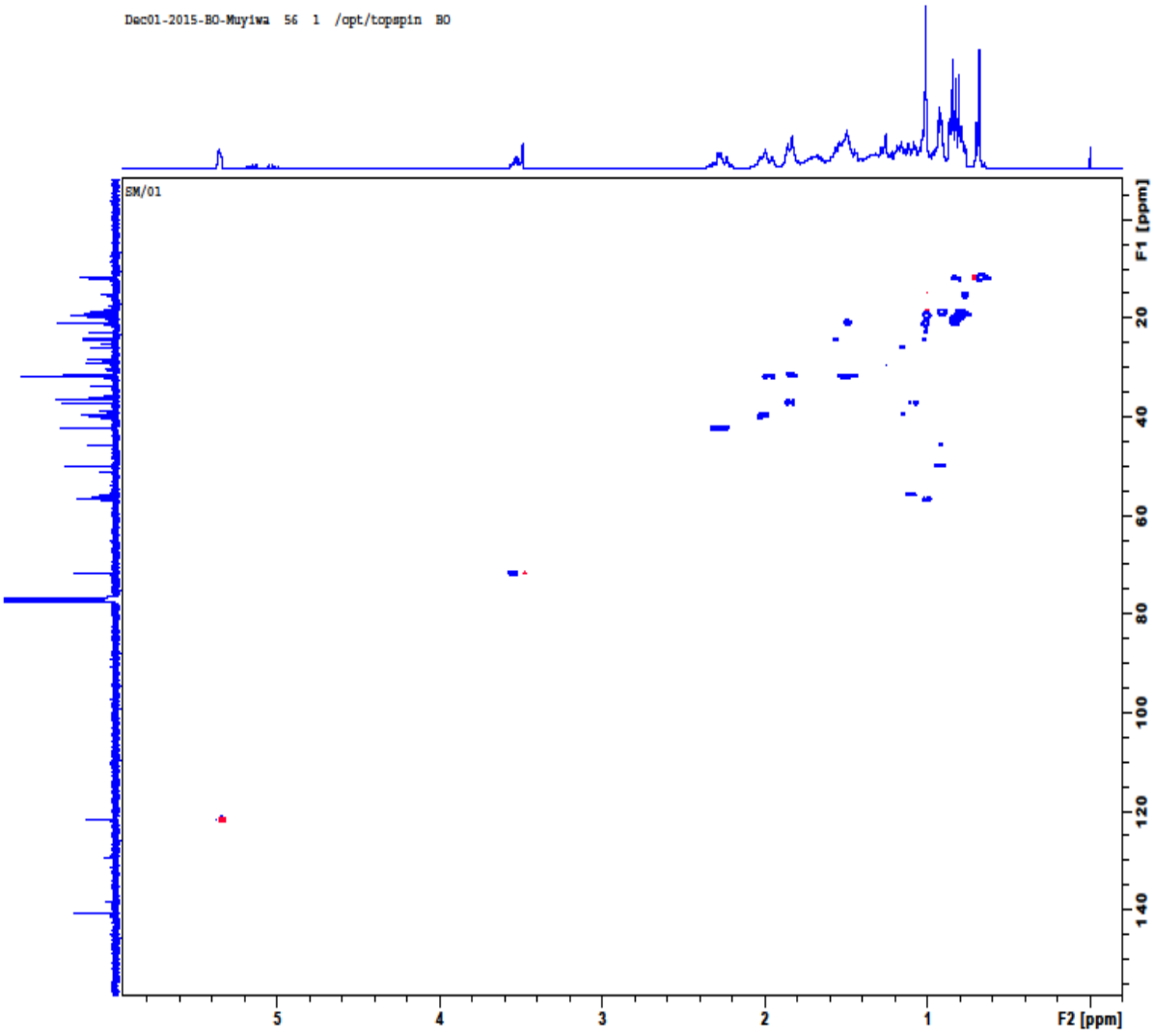

Figure 6c: HMQC spectrum of β-sitosterol