PHYTOCHEMICAL, ELEMENTAL AND BIOLOGICAL STUDIES OF THREE *FICUS* SPECIES (MORACEAE) FOUND IN KWAZULU-NATAL, SOUTH AFRICA

By

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Phytochemical, Elemental and Biological studies of three *Ficus* species (Moraceae) found in KwaZulu-Natal, South Africa

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A thesis submitted to the school of Chemistry & Physics, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy.

This Thesis has been prepared according to **Format 4** as outlined in the guidelines from the College of Agriculture, Engineering and Science which states:

This is a thesis in which chapters are written as a set of discrete research papers, with an Overall Introduction and Final Discussion. Where one (or all) of the chapters has already been published. Typically, these chapters will have been published in internationally- recognised, peer- reviewed journals.

As the candidate's supervisor, I have approved this thesis for submission.

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Co-supervisor:

Signed: ----------Name: ----------Date:

ABSTRACT

Ficus (Moraceae), with over 800 species, is one of the understudied genera in modern pharmacognosy. Rural households depend on their fruits for food while other plant parts such as leaves and bark are utilised for medicinal purposes. Phytochemical analyses and biological activities of different plant parts, as well as the nutritional value of the edible fruits of many of the species are yet to be investigated. This study aimed at investigating three *Ficus* species (*Ficus burtt-davyi*, *Ficus sur* Forssk and *Ficus sycomorous* Linn) that produce edible fruits and are indigenous to KwaZulu-Natal, South Africa, as a source of secondary metabolites and essential dietary elements, due to their claimed medicinal and nutritional value. Plant material was subjected to chromatographic analyses and isolated compounds were identified using spectroscopic techniques and by comparison with previously reported data. Fruit and soil samples that were collected from sites within KwaZulu-Natal, were digested and analysed for macro, micro and toxic elements by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

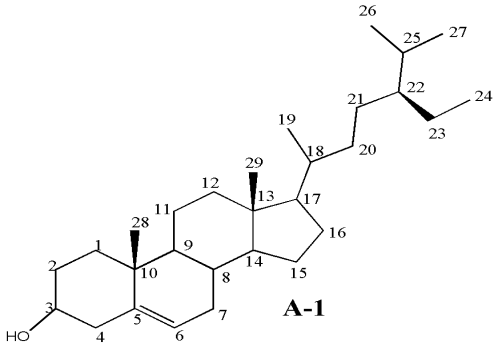
The phytochemical investigation of *F. burtt-davyi* revealed the bark to be rich in pentacyclic triterpenoids (lupeol and lupeol acetate) and the known antioxidant flavonoid ((+)-catechin) whilst the leaves were rich in sterols (β-sitosterol, campesterol, and stigmasterol). Both leaves and fruits also contained phaeophytin **a**, lutein and α-amyrin. This is the first phytochemical report on this species. The cytotoxic results indicated that lupeol and (+)-catechin, the most abundant bioactive compounds in the stem bark, were responsible for its synergistic cytotoxic effects against breast and colorectal adenocarcinoma cell lines. This study supports the use of this plant species as a substitute for antioxidant supplements and as an alternative medicine for oxidative stress related

non-communicable chronic diseases in vulnerable communities. The phytochemical analysis of *F. sur* revealed two pharmacologically active triterpenoids (lupeol and sitosterol), one pheaophytin (pheaphytin **a**) and one flavonoid (epicatechin).

The analytical results indicated that the fruits of *F. burtt-davyi*, *F. sur* and *F. sycomorous* are good sources of essential dietary elements and can contribute significantly ($p < 0.05$) to the recommended dietary allowances (RDAs) for most nutrients. The fruits of *F. sur* and *F. burtt-davyi* are good dietary sources of Se and Mn, respectively. The concentrations of As, Cd, and Pb were below the instrument detection limits in all three figs indicating that the species do not tend to accumulate these toxic elements. Data from this study showed that metal interactions in soil influenced their availability, but uptake was to a greater extent controlled by the plant. Statistical analyses revealed synergistic relationships in the plants, thereby confirming that uptake of elements is controlled to meet metabolic needs. Overall, this study validates the ethnomedicinal use of these figs and reveals the nutritional and medicinal benefits of consuming the indigenous edible fruits. It also addressed the need for analytical information on the elemental concentrations in indigenous edible fruits consumed in South Africa.

SUMMARY OF COMPOUNDS ISOLATED

Compounds in subsequent chapters are not repeated



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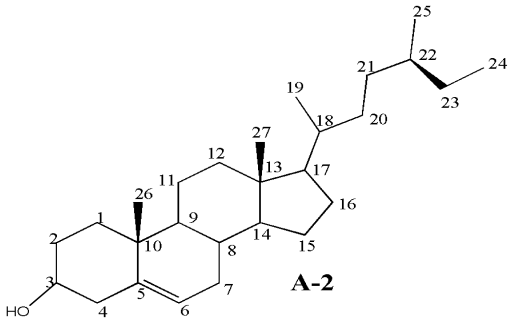

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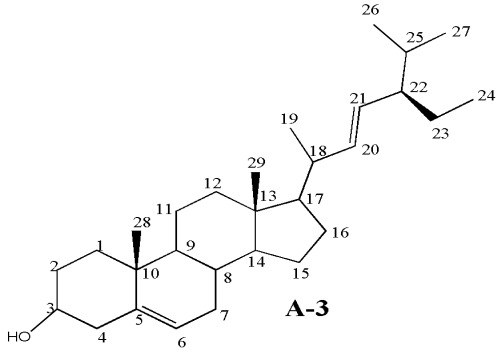

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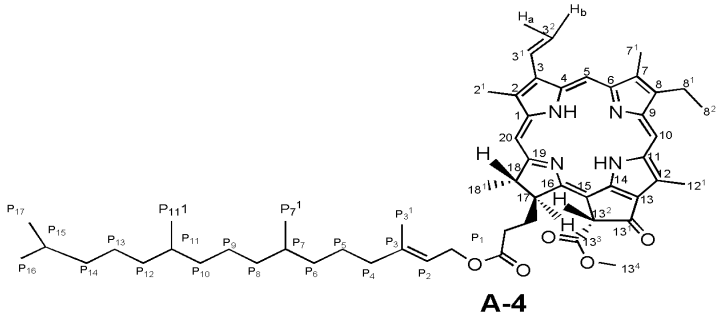

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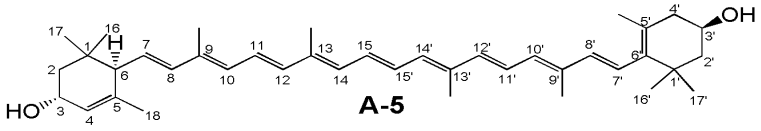

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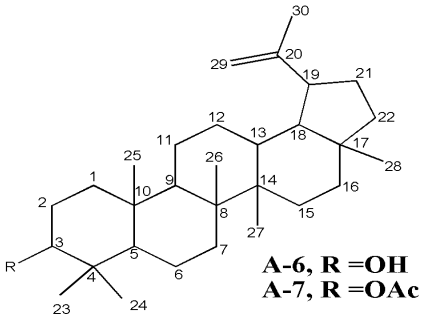
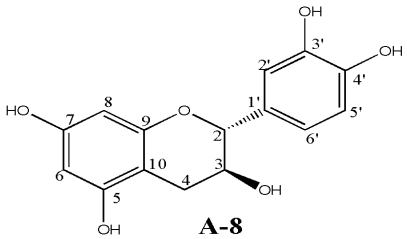


A-6, R =OH
A-7, R =OAc



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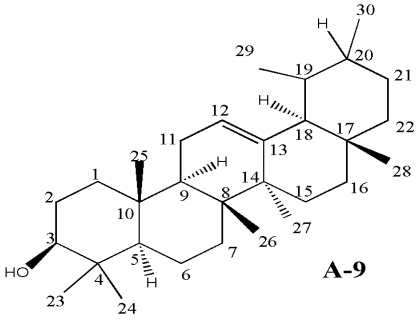

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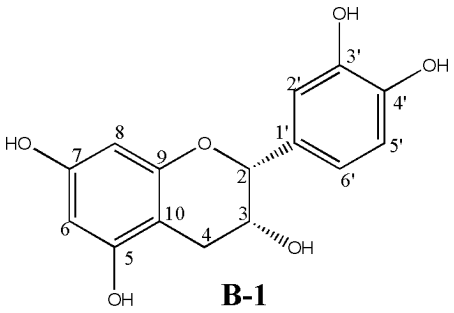

B-1

ABBREVIATIONS

Ac - acetate

ANOVA - analysis of variance

AOAC - Association of analytical communities

BAF- bioaccumulation factor

Br - broad resonance

CA- cluster analysis

cc - column chromatography

CEC- cation exchange capacity

^{13}C-NMR - C-13 nuclear magnetic resonance spectroscopy

COSY - correlated spectroscopy

CRM- certified reference material

d - doublet

dd - double doublet

DEPT - distortionless enhancement by polarisation transfer

DRI - dietary reference intake

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EDTA- ethylenediaminetetraacetic acid

EIMS – electron impact-mass spectroscopy

Ex – exchangeable

FRAP – ferric reducing antioxidant potential

GC-MS - gas chromatography-mass spectrometry

Hz - Hertz

^{1}H-NMR - proton nuclear magnetic resonance spectroscopy

HMBC - heteronuclear multiple bond coherence

HRMS – high resolution- mass spectrometry

HSQC - heteronuclear single quantum coherence

ICP-OES - inductively coupled plasma-optical emission spectrometry

IR - infrared

m - multiplet

Me - methyl

MS - mass spectrometry

NOESY - nuclear overhauser effect spectroscopy

RDA – recommended dietary allowance

RSA - radical scavenging activity

S - singlet

SD- standard deviation

SOM- soil organic matter

T - triplet

TLC - thin-layer chromatography

UL- tolerable upper intake level

UV - ultraviolet

DECLARATIONS

Declaration 1: Plagiarism

I, Ogunlaja Olumuyiwa Olufisayo declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a. Their words have been re-written but the general information attributed to them has been referenced

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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

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Declaration 2: Publications and Conferences

Publication 1

Title: Chemical constituents and *in vitro* antioxidant activity of crude extracts and compounds from leaves and stem bark of *Ficus burtt-davyi*.

Authors: Olumuyiwa O. Ogunlaja, Roshila Moodley, Himansu Baijnath, Sreekantha B. Jonnalagadda.

Journal: Acta Poloniae Pharmaceutica, **2016**, 73, 1593-1600.

Publication 2

Title: Nutritional evaluation, bioaccumulation and toxicological assessment of heavy metals in edible fruits of *Ficus sur* (Moraceae).

Authors: Olumuyiwa O. Ogunlaja, Roshila Moodley, Himansu Baijnath, Sreekantha B. Jonnalagadda.

***Journal*:** Journal of Environmental Science and Health, Part B, **2017**, 52(2): 84-91.

Publication 3

Title: Cytotoxic activity of the bioactive principles from *Ficus burtt-davyi*.

Authors: Olumuyiwa O. Ogunlaja, Roshila Moodley, Moganavelli Singh, Himansu Baijnath, Sreekantha B. Jonnalagadda.

Journal: Manuscript submitted to Journal of Environmental Science and Health, Part B on the 26th of June 2017 (Currently under review).

Publication 4

Title: Antioxidant activity of bioactive principles from edible fruits and leaves of *Ficus sur* Forssk *(*Moraceae*)* found in KwaZulu-Natal, South Africa.

Authors: Olumuyiwa O. Ogunlaja, Roshila Moodley, Himansu Baijnath, Sreekantha B. Jonnalagadda.

Journal: Manuscript submitted to Medicinal Chemistry Research on the 6th of June 2017 (Currently under review).

Conference contribution from this thesis

1. ***Title:*** College of Agriculture, Engineering and Science Postgraduate Research Day, 29th November **2016**, UKZN Howard College Campus.

   Oral: Nutritional evaluation, bioaccumulation and toxicological assessment of heavy metals in edible fruits of *Ficus sur* Forssk (Moraceae). *(Olumuyiwa O. Ogunlaja,* Roshila Moodley, Himansu Baijnath, Sreekantha B. Jonnalagadda)

2. ***Title:*** 20th International Conference of Functional Food Center (FFC) - 8th International Symposium of Academic Society of Functional Foods and Bioactive Compounds (ASFFBC) 22-23th, September **2016**, Harvard Medical School, Boston, USA.

   Poster*:* Antioxidant and cytotoxicity activities of crude extracts and compounds from leaves and stem bark of *Ficus burtt-davyi.* (*Olumuyiwa O. Ogunlaja*, Roshila Moodley, Himansu Baijnath, Moganavelli Singh, Sreekantha B. Jonnalagadda)

3. ***Title:*** College of Agriculture, Engineering and Science Research Day 22nd September **2015**, UKZN Pietermaritzburg Campus.

   Poster: Chemical constituents and *in vitro* antioxidant activity of crude extracts and isolated compounds from leaves and stem bark of *Ficus burtt-davyi* (Moraceae). *(Olumuyiwa O. Ogunlaja,* Roshila Moodley, Himansu Baijnath, Sreekantha B. Jonnalagadda)

4. ***Title:*** Royal Society of Chemistry's 2nd International Symposium on Natural Products Conference 23-25th September **2014**, Cape Town, South Africa.

   Poster: Chemical constituents of leaves and stem bark of Uluzi (*Ficus burtt-davyi*). (*Olumuyiwa O. Ogunlaja*, Roshila Moodley, Himansu Baijnath, Sreekantha B. Jonnalagadda)

In all of the publications, my role included carrying out all the experimental work and writing of the manuscripts. The co-authors contribution was that of an editorial nature and checking on the scientific content and correct interpretation.

Signed: ……..

DEDICATION

This thesis is dedicated to the loving memory of my beloved Dad, Mr. Taiwo Ogunlaja and Mum, Mrs Adebisi Ogunlaja

ACKNOWLEDGEMENTS

I would like to acknowledge God, the Almighty, for giving me the strength, sound mind and health to pursue and realise this dream. I would like to thank my supervisors, Prof. S.B. Jonnalagadda and Dr. R. Moodely. Thanks for your unshaken support and encouragement throughout my four years of study. I highly appreciate your support, passion, encouragement, guidance and above all, love during the duration of my PhD journey. My thanks also go to Prof. Himansu Baijnath for his professional expertise in the collection and identification of plant material for this study.

In addition, I wish to express my appreciation and gratitude to the many people who have in one way or another helped me over the course of study. In particular, I would like to thank the following:

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My special thanks go to my friend Dr. (Mrs) Gbonjubola Awolola who has been very close to me back home and here in UKZN.

My special thanks also go to the “Chapel of Praise” The Redeemed Christian Church of God community in Durban, especially to Pastor and Pastor (Mrs.) Gabriel Adejimi, Dr and Mrs Oyesola, Dr. and Mrs Sogun and the Sunday school group. They have been my family in Durban, especially before my immediate family joined me in Durban.

My PhD dream would not have been attainable without the continued love, support, and encouragement of my wife, Dr. (Mrs) Aemere Ogunlaja, and my lovely sister, Mrs. Oluyemisi Beckley, who has been a strong influence in all of my accomplishments. Thank you for always being there for me.

To my children, Similoluwa, Oluwarotimi and Oluwawemimo, thank you for your patience and love all these years. I am grateful to God for keeping you and bringing you here in South Africa towards the completion of this journey, your presence at this time has been a source of strength and joy. I love you very much and I am looking forward to a blissful adventure together.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Plant derived substances and traditional herbal medicine

The World Health Organisation (WHO) defines medicinal plants as any plant which has one or more of its organs consisting of substances which are capable of being used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis (WHO, 1978). Plant-derived substances have recently become of great interest due to their versatile applications and, over the years, medicinal plants have demonstrated the potential to be the richest bio-resource of herbal drugs of traditional systems, modern medicine and chemical entities for synthetic drugs (Goldfrank *et al*., 1982; Rates, 2001). Any part of the plant including the leaves, root, flowers, bark, seeds and fruits can be a source of natural plant constituents. In developing countries, more and more people rely on traditional herbal medicine for their healthcare needs (Cunningham, 1985; Shackleton *et al*., 2001). The use of plants and plant concoctions in healthcare is predominant in areas with inadequate modern health services (Saeed *et al.*, 2004). Plants for therapeutic purposes, dates back several centuries; this is well documented by the Indians, the ancient Chinese, and North African civilisations (WHO, 1978). The pattern of medicinal plant utilisation is depicted in Figure 1-1.

Studies show that 70-80% of Africans utilise and rely on traditional medicine for their healthcare needs (Goggin *et al*., 2009; Gqaleni *et al*., 2007; Mahlangeni *et al*., 2014; Olsen, 1998). In South Africa, approximately 700 indigenous plant species are used by 60% of the indigenes as traditional

medicine (Meyer and Afoloyan, 1995). It is also reported that approximately 20 000 tonnes of South African indigenous plant material are used each year with an average of 750 g being consumed by individual traditional medicine user per use, and at least 3 visits/uses annually (Mander, 1998). In addition, gathered wild fruits have been reported to make up to more than 50% of the fruits consumed by the local people in a study involving 150 rural households across three villages in the Northern province and KwaZulu-Natal in South Africa yearly (Herzog *et al*., 1993; Shackleton *et al*., 2002).

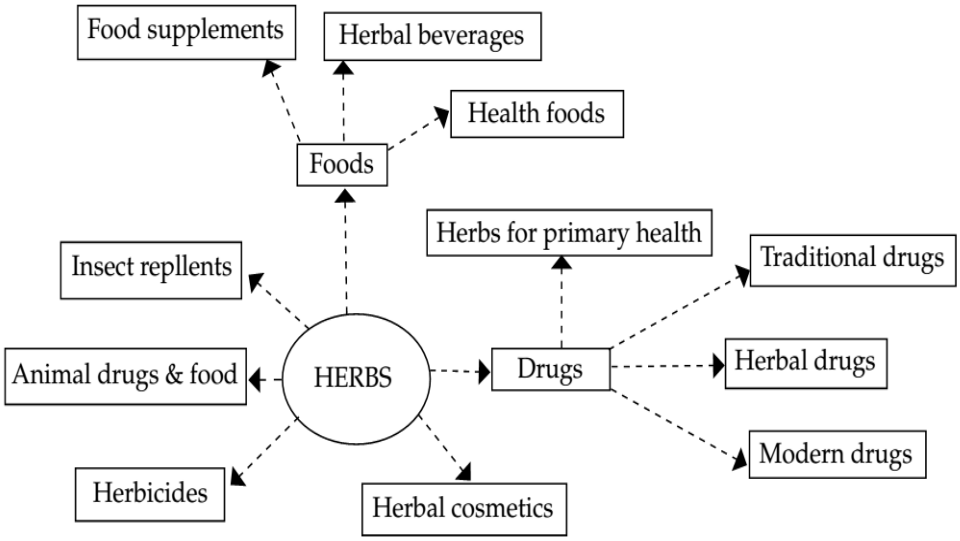

Figure 1-1: Patterns of herbal utilisation.

(Riewpaiboon, 2004)

This dependence on wild edible fruits and indigenous plants as better alternatives for healthcare is enhanced since the use of most of the plants, aside from being quite affordable, incorporates practices which are based on religious beliefs and social-cultural norms. Regardless of the reasons for seeking out traditional medicine, there is little doubt that interest in this practice has grown, and will almost certainly continue to grow around the world. Although intensive research has been

carried out on the isolation of bioactive compounds from medicinal plants, many species remain unstudied due to the large plant biodiversity. A phytochemical investigation of such medicinal plants could lead to the isolation of novel bioactive compounds with pharmacological importance.

1.2 Rationale for the study

In the rural areas of South Africa, people depend on trees growing in the wild for fruits due to accessibility and affordability, thereby inadvertently exploiting the therapeutic and nutraceutical potential of such fruits. In modern pharmacognosy, *Ficus* (Moraceae) is one of the understudied genera among more than 850 genera, despite the fruits being consumed by animals and humans and despite the leaves, bark and twigs being used as medicine. *Ficus burtt-davyi* Hutchinson, *Ficus sur* Forssk and *Ficus sycomorus* Linn are three medicinal species with edible fruits found in South Africa. Although biological studies of some of the *Ficus* species in South Africa have been conducted, phytochemical information on the parts used medicinally and nutritional information on the edible parts of most *Ficus* species has not been documented. Plant derived substances are used in both folk and modern medicine for the treatment and management of numerous human diseases. The phytochemical screening of new plants can lead to the isolation and identification of bioactive compounds that can serve as potential drug leads towards new therapies for the effective treatment and management of human diseases. In addition, heavy metals from the environment can bioaccumulate in the fruits of these indigenous figs and consumption of the fruits, if contaminated, can result in metal toxicities and adverse health effects. A nutritional evaluation through assessment of the levels of essential and toxic elements in edible fruits is imperative since human exposure to toxic metals through the agricultural food chain may occur especially when

levels of these elements are not phytotoxic. There is therefore a need to phytochemically and analytically investigate *Ficus* species to add to the base of knowledge on this genus.

1.3 Aim and objectives of the study

This study aims to phytochemically and analytically investigate three medicinal *Ficus* species that produce edible fruits and that are indigenous to KwaZulu-Natal, South Africa namely *Ficus burtt-davyi* Hutchinson, *Ficus sur* Forssk and *Ficus sycomorus* Linn, as possible sources of secondary metabolites, hence validate their ethnomedicinal use, and to determine the nutritional value of the edible fruits.

The objectives of the study were:

- To extract and isolate the secondary metabolites from various parts of the *Ficus* species under investigation.
- To identify and characterise isolated secondary metabolites using various spectroscopic techniques such as Nuclear Magnetic Resonance (NMR), Infrared Spectroscopy (IR), Ultraviolet-Visible Spectroscopy (UV-Vis), and Gas Chromatography-Mass Spectroscopy (GC-MS).
- To identify suitable bioassays, based on classification of the compounds isolated and to test the isolated compounds for their biological activities thereby promoting further use of the plants or validating their ethnomedicinal use.
- To determine the nutritional value of the fruits by comparing the elemental concentrations in the fruits to recommended dietary allowances (RDAs).

- To determine the effect of geographical location and soil quality on elemental content of fruits and hence determine their impact on elemental uptake.
- To determine and assess metal contamination in fruits and hence determine their safety for consumption.

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CHAPTER 2

LITERATURE REVIEW

2.1 Nutrients

Vitamins and minerals are classified as micronutrients (minerals required in very small amounts) and macronutrients that include minerals required in larger amounts, carbohydrates, proteins and fats (McDowell, 2003; Walker, 1990). Minerals that are essential to man include Fe, Ca, Ni, Co, Cu, Mg, Cr, Mn, Zn, and Se and most of these are essential at low concentrations. Table 2-1 shows the recommended dietary allowances (RDAs) for essential elements set to be adequate for 97 to 98% of the individuals in the defined class (FNB, 2011).

Table 2-1: Dietary Reference Intakes (DRIs) - Recommended Intakes for Individual.

Life stage	Ca (mg/d)	Cr (ug/d)	Cu (ug/d)	Fe (mg/d)	Mg (mg/d)	Mn (mg/d)	Se (ug/d)	Zn (mg/d)
Males								
14-18yrs	1,300	35	890	11	410	2.2	55	11
19-50yrs	1,000	35	900	8	400	2.3	55	11
>51yrs	1,200	30	900	8	420	2.3	55	11
Females								
14-18yrs	1,300	24	890	15	360	1.6	55	9
19-50yrs	1,000	25	900	18	310	1.8	55	8
>51yrs	1,200	20	900	8	320	1.8	55	8

Recommended intakes for individuals [a, b]

[a] Recommended Dietary Allowances (RDA)-Daily intake adequate for 97 to 98% of healthy individuals each defined class. [b] Sourced from: Food and Nutrition Board, Institute of Medicine, National Academies, 2011.

In addition, Table 2-2 shows the Acceptable Macronutrient Distribution Ranges towards the Dietary Reference Intakes for most individuals (FNB, 2011).

Table 2-2: Dietary Reference Intakes (DRIs): Acceptable Macronutrient Distribution Ranges.

Macronutrient	Children, 1–3 y %	Children, 4–18 y %	Adults %
Carbohydrate	45–65	45–65	45–65
Protein	5–20	10–30	10–35
Fat	30–40	25–35	20–35

Sourced from: Food and Nutrition Board, Institute of Medicine, National Academies, 2011.

From a nutritional perspective, fruits are foods that are lower in energy but higher in phytochemicals, micronutrients and bioactive compounds compared to other food sources. Fruits are important to the human diet because they contribute towards the components that are essential for overall wellbeing such as trace elements, vitamins, carbohydrates, minerals and proteins (Itanna, 2002). Many investigations have reported a strong correlation between fruit consumption and reduced risk of cardiovascular disease, diabetes, obesity, hypertension, chronic respiratory disease, hypercholesterolemia and non-communicable diseases (WHO/FAO, 2003; Dragsted *et al*., 2006; Esmaillzadeh *et al*., 2006; Norman *et al*., 2006; 2007; Heidemann *et al*., 2008; Stea *et al*., 2008; Mayosi *et al*., 2009). Epidemiological reports have also suggested that a diet rich in antioxidant-rich fruits significantly reduced the risk of many cancers (Bradbury *et al*., 2014;

Genkinger *et al*., 2004; Rahman, 2001) including oesophageal, lung and gender specific breast / cervical cancer in adult females, and prostate cancer in adult males (Norman *et al*., 2006). In children, the nutrition related conditions that fruit consumption can ameliorate include vitamin A deficiency, malnutrition and obesity (Bosman *et al*., 2011; Mamabolo *et al*., 2006).

Based on dose-response effect, WHO recommends consumption of 160 g and 240 g of fruits each day in children aged 0-4 years and 5-14 years, respectively (WHO/FAO, 2003; Lock *et al*., 2004). It also recommends an intake of 600 g per day of fruit and vegetables in adults. Although the Department of Health, South Africa, focuses mainly on undernutrition, they have set strategic objectives for nutrition-related chronic diseases leading to the Food-Based Dietary Guidelines (FBDGs). These guidelines recommend the consumption of plenty of fruits every day.

Table 2-3: Dietary Reference Intakes (DRIs): Tolerable Upper Intake Levels (UL).

Males/Females	**As**	**Ca**	**Cr**	**Cu**	**Fe**	**Mg**	**Mn**	**Se**	**Zn**
(Life Stages)		(g/d)		(ug/d)	(mg/d)	(mg/d)[c]	(mg/d)	(ug/d)	(mg/d)
9-13yrs	ND	2.5	ND	5,000	40	350	6	280	23
14-18yrs	ND	2.5	ND	8,000	45	350	9	400	34
19-70yrs	ND	2.5	ND	10,000	45	350	11	400	40
>70yrs	ND	2.5	ND	10,000	45	350	11	400	40

Tolerable Upper Intake Levels (UL)[a b]
[a]UL = Maximum level of daily nutrient intake that is indicative of no possible adverse effect.
[b] Food and Nutrition Board, Institute of Medicine, National Academies.
[c] Intake from pharmacological source only. ND = Not detected.

Table 2-3 provides the Tolerable Upper Intake Levels (ULs) for the essential elements. This is the maximum level of daily nutrient intake that is indicative of no possible adverse effects in humans. Elemental concentrations in food, whether essential or non-essential, is of global concern as industrialisation has negatively affected forests and agricultural land in most parts of the world which has led to elevated concentrations of nutrients in food that are proving to be toxic (Ajasa *etal*., 2004; Al-Alawi and Mandiwana, 2007; Erdemoglu and Basgel, 2006; Maksimović *et al*., 1999).

Minerals that are essential to plants include Mg, Ca, Cr, Co, Cu, Fe, Mn, Mo, Ni, Se and Zn however plants also contain toxic elements such as As, Cd and Pb. Essential and non-essential elements are found in soil, from which plants get their nutrients. The availability of metals in growth-soil, as well as the affinity of edible fruits for these metals can play a major role in their translocation and bioaccumulation. Generally, the first step in elemental bioaccumulation by plants is uptake from the soil and this depends on the absorption ability of the root surface. Elemental entry into the human food chain *via* the agricultural route by the root is governed by elemental mobility and availability (John and Leventhal, 1996). The element can either be moved through diffusion to the root or the root can grow to meet the element through a solution phase.

Factors that control elemental availability through soil-plant associations are solubility, complexation formation and chelation, and these are regulated by soil pH, organic matter content, ionic exchange and other biological processes (Violante *et al*., 2010). Additionally, redox reactions can mobilise or immobilise metals, by controlling the oxidation state which is subject to metal species and this is of great importance in controlling their mobility and toxicity; such elements include Pb, Cr, Se, As, Ni, Co and Cu (Violante *et al*., 2010). Elemental solubility in the soil–

liquid matrices determines its mobility and bioavailability. At a given time, only some of the total elemental concentration in the soil will be available in soil solution for uptake by the plant whilst the rest would be bound to the soil matrix. Table 2-4 shows the mobility of an element based on the fraction to which the element is complexed.

Table 2-4: Chemical forms of metals in soil.

	FRACTION	MOBILITY
T	In pore water (dissolved)	High
O	Weakly adsorbed (exchangeable)	High
T	Associated with carbonates	High
A	Associated with Fe, Mn oxides	Moderate
L	Complexed by organics	Moderate
	Associated with sulfide	Low
	In the mineral lattice	Low

Source: Gunn *et al*., 1988; Salomons, 1995.

Organic substances can form chelates (soluble compounds) which bind elements such as Mn, Fe, Zn and Cu thereby increasing their solubility and their availability to plants (Clemens *et al.*, 1990; Havlin *et al*., 1999). This is achieved by keeping the cation from interacting with other inorganic compounds *via* the formation of a ring around the cation in the solution, so that it diffuses easily from the soil into the root (Brady and Weil, 1999). Figure 2-1 shows the dynamics of chelation for uptake of Fe by the root of the plant. The cation-chelate complex can be absorbed by the root into its membrane before releasing the elemental cation from the complex or the cation-chelate complex liberates the elemental cation prior to absorption by the root. The two mechanisms end

up with the absorption of the elemental cation by the root and the binding of chelates to other metals after its return to the soil solution.

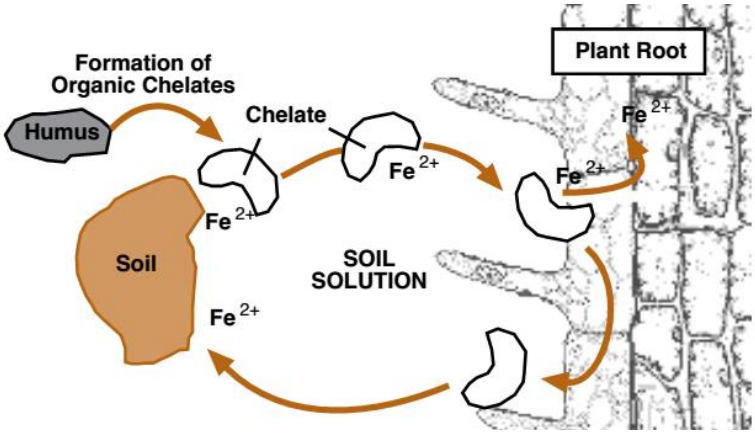


Figure 2-1: Cycling of chelated iron (Fe^{2+}) in soils.

Source: McCauley *et al*. (2009).

2.2 Soil

The Soil Science Society of America, defines soil as "the unconsolidated mineral or organic matter on the surface of the earth that has been subjected to and shows effects of inherent and environmental factors of climate (water and temperature effects), and macro and micro-organisms, conditioned by relief, acting on parent material over a period of time" (David, 2008). It serves as a natural medium for growth of land plants and as a source of nutrients for these land plants. Generally, soil consist of the following basic components: water, mineral particles, organic matter and air (Brady and Ray, 2002) and organic matter further consists of living organisms, humus, and roots (Brady and Ray, 2002). Humus is usually formed from decayed organic matter and can be described as semi-soluble organic substances having a dark colour. Figure 2-2 shows the average composition of soil in good condition for optimal plant growth (Pidwirny, 2006). Under ideal

conditions, good soil volume would be 50% soil pores and 50% solid material with the pores containing 50% air and 50% water for optimal plant moisture. (Pidwirny, 2006).

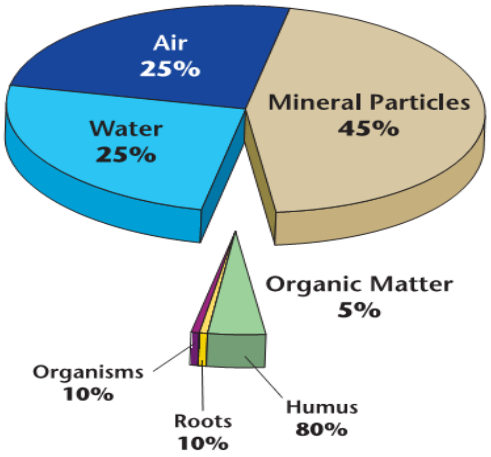


Figure 2-2: Average composition of soil.
(Pidwirny, 2006).

2.2.1 Soil formation and morphology

Soil is weathered mineral materials of rock resulting from several thousand years of chemical and physical processes, influenced by environmental factors (water, temperature and wind abrasions) and biological agents (animals and plants) whose impact physically wear the rock and minerals. Weathering due to physical influences results in the exposure of larger surface area which will ultimately weather by chemical processes through different aqueous chemical reactions in the course soil formation. Carbon dioxide, a by-product of respiration and decay of living organisms could dissolve in pore water (found in pore spaces) forming carbonic acid, which is capable of dissolving minerals. The weathering processes, both chemical and physical, occur simultaneously, thereby enhancing each other and hastening soil-forming processes. The process of soil formation

produces five visible distinct layers called horizons (O, A, B, C and R) in the soil profile (Pidwirny, 2006).

Soil horizons are characterised by the texture, colour, structure and consistency of the soil and have varying chemical composition. The topmost layer is the O horizon consisting mainly of plant litter at various degrees of decay (Pidwirny, 2006). This layer is followed by the A horizon, where it is a layer with mixture of humus, mineral particles and other organic materials. It is characterised by greyish to dark-brown to black colour and it is usually porous and light in texture. The A horizon is also known to have maximum biological activity. This horizon has a darker upper layer indicating organic accumulation, with another layer below indicating loss of material (Pidwirny, 2006). The B horizon is a soil layer consisting mostly of minerals under strong influence of illuviation as it collects and accumulates most of the iron, clay, aluminium and silicates of the soil. The C horizon is composed of weathered parent rock material. The C horizon has a varied texture and varying particles sizes from clay to boulders. The influence of translocation, organic changes and pedogenic developments do not significantly affect the C horizon (McCauley *et al*., 2009). The final layer in a typical soil profile, R horizon, if present, consists of unweathered bedrock (McCauley *et al*., 2009). Figure 2-3 shows the basic soil horizons.

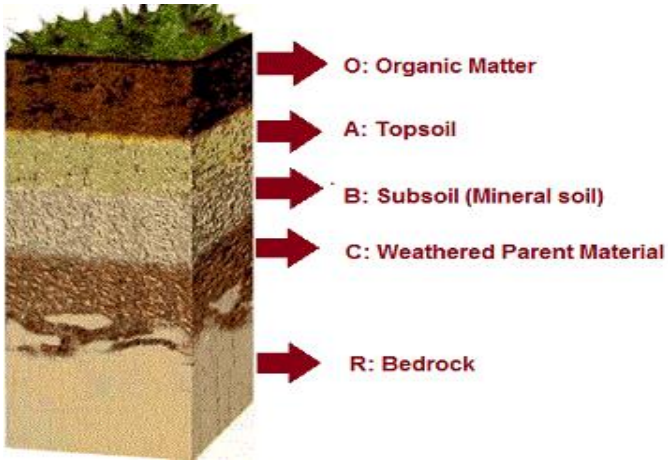


Figure 2-3: A general soil profile.
(USDA-NRCS, 2011).

2.2.2 Texture

Mineral particle sizes are distributed differently and this determines the texture of any particular soil (Pidwirny, 2006). The coarseness or fineness of these mineral particles in the soil is called the texture of the soil. The different sizes of particles and their amount relative to each other determines the texture of soil. Soil texture determines the movement of water, air and plant root penetration. Sand (0.05 to 2 mm in diameter), silt (0.002 to 0.05 mm in dimeter) and clay (< 0.002 mm in diameter) are the major classes of soil. Clay particles which have the largest surface area to volume ratio, are highly reactive and have affinity for or can hold positively charged nutrient ions. Figure 2-4 shows the 12 primary classes of soil texture based on the amount of the major mineral particles (clay, silt and sand) (Smith and Smith, 1990; USDA-NRCS, 2011).

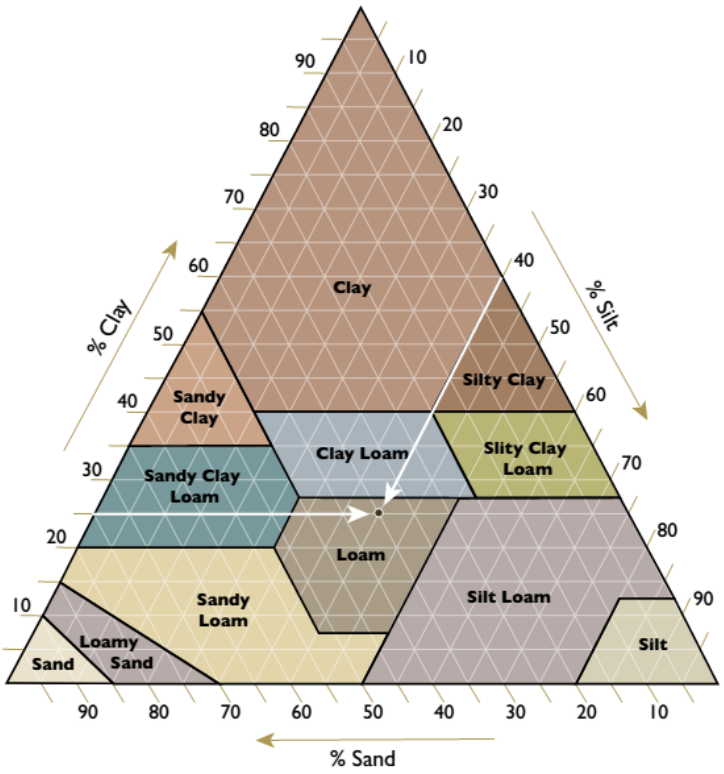


Figure 2-4: Soil textural triangle. Note that a solid with 25% clay and 40% silt is loam. (USDA-NRCS, 2011).

2.2.3 Soil properties

For plants to take up nutrients they need to be in the right form, soluble or weakly bound in soil solution. Plants are primarily able to take up the ionic form of nutrients *via* their roots and many of these nutrients are taken up as cations. The main soil parameters that govern the processes of sorption, desorption and mobility of elements in the soil are water content, pH, soil organic matter (SOM) and cation exchange capacity (CEC) (Pongrac *et al.*, 2007). A change in these soil physical and chemical conditions directly influences the availability of elements or nutrients for plant uptake.

2.2.3.1 pH

The pH of soil is considered as the main soil property that controls the biological and chemical processes in the soil environment (Vangheluwe *et al*., 2005). Soil pH affects how the nutrients react with each other, their solubility and hence the availability of nutrients and heavy metals in soil for uptake by plants (Smith, 1996). Theoretically, soil pH is defined as a measure of the hydrogen ion (H^+) concentration in solution present in soil pores which is in dynamic equilibrium with the predominantly negative charged surfaces of the soil particles (Vangheluwe *et al*., 2005). Consequently, the number of the binding sites for cations on the soil particles is mainly dependent on the soil pH. This is also known as the measure of acidity and alkalinity in soil solution. Generally, with acidic soils, there is increase in the trace metals mobility because there is a greater chance of cation exchange reactions occurring (Pakade *et al*., 2013). As the soil pH increases, the bioavailability of heavy metal cations decreases (Alloy, 1995; Kabata-Pendias and Pendias, 2001). When soils are alkaline, there is decreased solubility which hampers the cations availability; this can result in increased susceptibility to leaching or erosion losses in very acidic soils, the opposite holds for the availability of anions. The best soil pH levels for overall nutrient availability are levels near 7. These pH levels enhance soil microorganism activity and encourages crop tolerance. Chemical amendments change soil pH and they may only be effective for a relatively short period and generally lack economic viability.

At low pH, beneficial elements such as Ca and Mg become less available to plants with availability reaching highest within a pH range of 6.5 to 8 (McCauley *et al*., 2009). The availability of other elements such as Mn, Cu, Zn and Fe increases in the 5 - 7 pH range (McCauley *et al*., 2009) and increased Mn uptake may result in levels that are toxic to plants. In addition, there is decrease in

organic matter decomposers (bacterial population) and total hindrance of bacterial activity in highly acidic soil, thereby causing in build-up of organic matter and bound cation nutrients (Brady and Ray, 2008). Soil pH is greatly influenced by NH_4^+ fertilisers, organic matter decomposition, weathering of minerals and parent material, land management practices and climate (Brady and Ray, 2008; Foth, 1990).

2.2.3.2 Soil organic matter (SOM)

Soil organic matter (SOM) consists of varying degrees of decaying animal and plant remains (Moodley *et al.,* 2012). It is the main source of energy and food for soil flora and fauna which provide them with metabolic energy, enables biological processes, provides macro- and micro-nutrients and ensures the storage and adequate release of nutrients and energy (McCauley, 2005; Pidwirny, 2006). Humus is a reactive and important part of SOM, contributing to the soil`s ability to retain nutrients on the exchange sites (McCauley *et al*., 2005; 2009). SOM consists of approximately 35-50% humus (Prasad and Power, 1997). It serves as a reservoir of nutrients, trace elements and water in the soil. SOM consists of negative charges due to dissociation of organic acids, which have affinity to adsorb metal cations, reduces leaching by water thereby increasing its availability to plants (Taiz and Zeiger, 2002; Vangheluwe *et al*., 2005). In addition, SOM enhances the availability of nutrients by increasing the soil's cation exchange capacity (CEC), providing chelates, thus, increasing the solubility of some nutrients in the soil solution (McCauley, 2005). Consequently, as SOM increases, availability and uptake of nutrients increases. Soil texture, temperature, vegetation, rainfall, rate of decomposition, parent material and landscape are factors known to affect SOM (McCauley, 2009). Thus, soil organic matter increases with increasing decomposition.

2.2.3.3 Cation exchange capacity (CEC)

The cation exchange capacity (CEC) is the potential of the soil to interchange cations such as Ca^{2+}, Na^{+}, Mg^{2+}, $Al^{3+}$and K^{+} between soil solution and clay mineral or organic complexes (Chapman, 1965). Clay mineral and SOM provide the negatively charged sites (exchange sites) that positively charged ions (cations) are attracted to and where they are retained. The exchange sites are formed because of the dissociation of H^{+} ions in phenolic groups and carboxylic acid found in the organic structures in the soil (Taiz and Zeiger, 2002)**.** As soil pH increases, the number of negative charges on clay mineral and organic matter increases, which increases the CEC. Figure 2-5 shows a simplified representation of CEC based on a primarily negatively charged colloid attracting cations at the exchange sites.

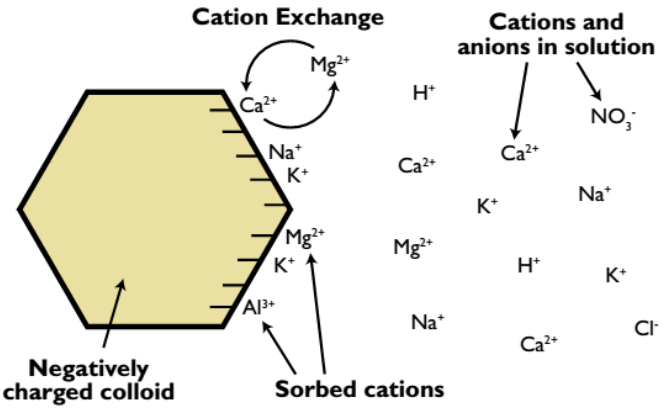


Figure 2-5: Simplified representation of exchange capacity.
(Brady and Ray, 2002).

At elevated levels, heavy metals and trace elements have the potential to be toxic, therefore monitoring soil properties is vital. In some cases, the mineral and heavy metal concentrations could be because of geographical variations, plant's genetic factors, efficiency of mineral uptake, different growth conditions, irrigation with polluted water, soil fertility, farm practices, industrial

pollution and the analytical procedure employed (Ozcan and Akgul, 1998; Rehman *et al*., 2008; Sial *et al*., 2006). Constituents of wild medicinal plants have received much research investigation and reporting, according to a literature survey (Aloskar *et al*., 1992). However, the influence of inorganic elements on the biosynthesis of the plants pharmacologically-active chemicals has been poorly investigated (Aloskar *et al*., 1992).

2.3 Functional foods and plant based sources

The idea of "functional foods" was first introduced around mid-1980s and by the 1990s, consumers began to view food from a radically different vantage point. Functional food is defined as any food or food component that may provide benefits beyond basic nutrition and may prevent disease or promote health (Hasler, 2000). Today, there is increasing demand for food that contain components with healing and protective ability. Among several factors driving this intense interest, is the unstable cost of healthcare for chronic diseases and recent scientific reports associating foods and/or food constituents to ideal health status. Such foods include fruits, grains, fortified foods, beverages, vegetables, and some dietary supplements. Currently, functional properties of many indigenous foods are undergoing studies and developement (Hasler, 2000). Waltham (1998) estimated that the market value of functional foods in the United States of America was 28.9 billion USD, but more significantly, perhaps, is that there is the possibilty of functional foods to improve health, alleviate diseases, and reduce costs for health care. Furthermore, there are emerging economic possibilities for most developing countries with more research findings on functional foods. This is because such countries have traditional know-how of the health effects of plant species indigenous to them and are naturally bestowed with species richenss and abundance. For

instance, packaged functional foods in Japan in 2015 had a retail value of $8.5 billion, an 8% increase from the $7.8 billion retail value in 2010 (Acheson, 2016).

South Africa, with over 22 000 species, representing about 10% of the world's species in biodiversity, is considered as a hotspot in the nutraceutical (Functional foods) industry, but relatively few of these plants are utilised (Coetzee *et al*., 1999; Williams *et al*., 2006). Increasing evidence from epidemiological, *in vitro*, *in vivo* as well as clinical trial data indicate that the danger of chronic diseases, mostly cancer, can be reduced by consuming a plant-rich diet (Block, l992; Block *et al*., 1992; Bradbury *et al*., 2014; Hung *et al*., 2004; Key, 2011; Marmot, 2011; Rahman and Lowe, 2006; Takachi *et al*., 2008; Wang *et al*., 2014). The current burden of disease in South Africa, coupled with the cost of modern health services makes it important to investigate and determine the functional food properties of indigenous plant-based foods such as indigenous fruits.

2.4 The genus *Ficus*

2.4.1 Phylogeny of genus *Ficus*

The genus *Ficus* belongs to the Moraceae family (Mulberry family, comprising 40 known genera) (Woodland, 1997) and one of the most populous in number of species of all plant genera because of its remarkably large variation in the habitats of its species (Herre *et al.,* 2008). Moraceae is found mainly in the tropics and the semi-tropical regions. They include a variety of shrubs, herbs and trees, characterised by the presence of laticifier and milky sap in all parenchymatous tissues (Duncan and Duncan, 1988; Everett, 1968; Godfrey, 1988). The Moraceae, of the order Urticales, class Magnoliopsida and division Magnoliophyta are dicotyledons and flowering plants. Five

tribes of Moraceae are known: Artocarpeae, Moreae, Dorstenieae, Ficeae, and Castilleae (Rohwer *et al*., 1993).

Ficeae has only one genus, *Ficus*, with more than 800 species of shrubs, woody trees, vines, epiphytes and hemi-epiphytes, largely distributed in the tropics and subtropics (Berg, 1989; Datwyler and Weiblen, 2004; Lansky and Paavilainen, 2011). They can be either monoecious with bisexual inflorescences or dioecious (Berg and Corner, 2005; Weiblen, 2000). The Asian-Australian region is the main habitat for most species where 500 species are known constituting 66% of the world`s species while Central and South America (Neotropical region) is a habitat to approximately 132 species (Burrows and Burrows, 2003). Presently, there are 112 recognised species in Africa (south of the Sahara, including Madagascar) (Afrotropical region), of which 36 are found in Southern Africa (Botswana, Namibia, Mozambique south of the Zambezi River, Zimbabwe and South Africa) (Burrows and Burrows, 2003). There are twenty five *Ficus* species in South Africa, two of which are sub-endemic, *F. burtt-davyi* Hutchinson (found in the southern part of Mozambique) and *F. tettensis* Hutchinson (found in Botswana and Zimbabwe) and one which is endemic, *F. bizanae* Hutch. and Burtt-Davy (Burrows and Burrows, 2003).

Collectively, the *Ficus* genus is commonly referred to as fig trees or figs. Edible figs occupy a significant nutritional position globally, particularly in regions with climates that sustain them (Lansky and Paavilainen, 2011).

2.4.2 Ethnobotanical uses and biological activity of *Ficus* species

For many centuries, *Ficus* species, have been used in medicine with widespread and diverse applications. Generally, they are used as sources of fruit and for several purposes in traditional

medicine. A list of the more prevalent *Ficus* species, their uses in traditional medicine and their biological activities are presented in Table 2-5.

Table 2-5: Ethnobotanical uses and biological activities of *Ficus* species

Species	Plant part	Ethnobotanical uses	Biological activity	Reference
F. asperifolia	Leaf, latex, fruits, stem bark	Cancers, tumours, colic, conjunctivitis, fever, gout, headache, haemorrhoids, abortifacient, menstruation, venereal diseases	Fibroblast stimulation, antioxidant, antimicrobial	Annan and Houghton, 2008; Ayensu, 1978; Burkill, 1985; Vasileva, 1969
F. awkeostang	Root	Liver disease	Cell cycle arrest, apoptosis, inhibition of proliferation in leukaemia cells	Chang *et al*., 2005; Yang *et al*., 1987
F. benghalensis	Stem bark, latex, fruits, root	Warts, asthma, boils, cough, diarrhoea	Antioxidant, anthelmintic, anti-tumour	Aswar *et al*., 2008; Augusti *et al*., 2005; Reddy *et al*., 1989; Shukla *et al*., 2004
F. benjamina	Leaves		Anti-inflammatory, antinociceptive, antipyretic	Farag, 2005

F. carica	Fruit, root, stem bark leaves and latex	Colic, indigestion, loss of appetite, diarrhoea, sore throat, cough, bronchial problems, anti-inflammatory, bee sting, eczema, cardiovascular remedy, haemorrhoids, head wounds	Cytotoxicity, antiplatelet, antioxidant, anticancer, haemostatic, hypoglycaemic, hypo-lipidemic, anti- human herpes simplex virus (HSV)	Burkill, 1935; Canal *et al*., 2000; Gilani *et al*., 2008; Hemmatzadeh *et al*., 2003; Perez *et al*., 2003; 1999; Ponelope, 1997; Richter *et al*., 2002; Rubnov *et al*., 2001; Solomon *et al*., 2006; Vinson *et al*., 2005; Yazicioglu and Tuzlaci, 1996; Wang *et al*., 2004
F. elastic	Latex, roots	Roundworms and tapeworms infections, ear oedema and arthritis	Anti-inflammatory	Nagaraju and Rao, 1990; Sackeyfio and Lugeleka, 1986
F. exasperate	Leaves and stem bark	Hypertension, and arthritis, ulcers, diabetes, wound healing, venereal and infectious diseases	Ant nociceptive effect, anti-inflammatory, anti-microbial, hypolipidaemic activity, antioxidant, anti-bacterial, hypotensive	Akah *et al.*, 1997; Ayinde *et al.* 2007; Buniyamin *et al*., 2007; Burkill, 1985; Nimenibo-Uadia, 2003; Odunbaku *et al.*, 2008; Umerie *et al*., 2004; Woode *et al*., 2009
F. fistulosa	Leaves	Malaria	Antimalarial	Zhang *et al*., 2002

F. glomerata	stem bark	Diabetes	Antidiabetic, antioxidant activity, hepatoprotective, antiulcer, anti-HIV- 1 integrase	Bunluepuech and Lewtrakul, 2009; Channabasavaraj et al., 2008; Rahman et al., 1994; Rao et al., 2008
F. hispida	Stem bark, fruits	Swellings, leprosy, anaemia, jaundice, diuretic, haemorrhage, lactation	Antidiarrheal activity hypoglycaemic	Acharya and Kumar, 1984; Alam, 1992; Ghosh *et al*., 2004; Nyman *et al*., 1998; Petelot, 1954
F. maxima	Leave, stem bark and wood, latex	Rheumatism, fever, gingivitis, anaemia, parasites		Barrett, 1994; Diaz *et al*., 1997; Duke and Vasquez, 1994; Lentz, 1993; Lentz *et al*., 1998
F. microcarpa	Roots, stem bark	Typhoid	Cytotoxicity, antioxidant, antimicrobial	Anis and Iqbal, 1986; Ao *et al*., 2008; Chiang *et al*., 2005
F. polita	Leaves	Malaria	Antimalarial	Etkin, 1997; Gbeassor *et al*., 1990

F. racemosa.	Leaves, fruits, stem bark, latex, root sap	Boils, blisters and measles, visceral obstruction, diarrhoea and constipation, diabetes, burns, swelling, leucorrhoea dysentery, haemorrhoids, stomach-ache, heat stroke, chronic wounds and malaria in cattle	Antidiabetic, antihyperglycaemic, hypoglycaemic, antioxidant, radio-protective, anti-inflammatory, anti-edemic, antibacterial, antitussive, antidiarrheal, wormicidal	Benny and Adithan, 2000; Bheemachari *et al*., 2007; Chandrashekhar *et al*., 2008; Deshmukh *et al*., 2007; Forestieri *et al*., 1996; Ghimire *et al*., 2000; Li *et al*., 2003; 2004; Mandal *et al*., 2000; Nadkarni *et al*., 1976; Narender *et al*., 2009; Patil *et al*., 2006; Paudyal, 2000; Rao *et al*., 2003; Sharma and Gupta, 2008; Siwakoti and Siwakoti, 2000; Sophia and Manoharan, 2007; Thapa, 2001; Tiwari, 2001; Yadav, 1999
F. reflexa	Stem bark, leaves	Gout, gastrointestinal ulcers		Gurib-Fakim *et al*., 1996
F. religiosa	Stem bark, fruits, leaves, roots,	Abortifacient, epilepsy, diarrhoea, dysentery, asthma, cough, respiratory disorders, malarial, ulcers, gonorrhoea,	Antioxidant, antidiabetic, anticonvulsant, anti-inflammatory, analgesic activities, anti-lipid,	Anis *et al*., 2000; Jain *et al*., 2004; 2005; Khanom *et al*., 2000; Kirana *et al*., 2009; Kunwar and Bussmann, 2006; Mahishi *et al*., 2005; Mousa *et al*., 1994; Panda, 2005; Pandit

	latex and seeds	scabies, infertility treatment, leprosy, laxative and purgative, rheumatism, and tuberculosis	peroxidative effect, antimicrobial and antiviral	*et al*., 2010; Samy *et al*., 2008; Sharma *et al*. 2001; Singh and Panda, 2005; Sreelekshmi *et al*., 2007; Vyawahare *et al*., 2007
F. septica	Leaves, fruits, roots	Headache, gastroenteritis, gastralgia, cephalgia, somatic pains, bacterial and fungal diseases, cough, diarrhoea, fever, urinary tract infections	Antimicrobial	Baumgartner *et al*., 1990; Holdsworth, 1980; 1993; 1992; Holdsworth *et al*., 1989; 1983
F. thonningii	Leaves, stem bark, root	Back pain, dislocated limbs, lactation, diabetes, appetite suppressant, and renal conditions	Cardioprotective, hypoglycaemic	Bhat *et al*., 1990; Kokwaro, 1976; Musabayane *et al*., 2006; 2007
F. thunbergii	Leaves	Back pain, rheumatism	None	Kitajima *et al*., 1994
F. toxicaria	Latex, leaves	Toothache, gastroenteritis		Grosvenor *et al*., 1995; Mahyar *et al*., 1991

2.4.3 A phytochemical review of the compounds from *Ficus* species

Ficus species are known to have a wide distribution of secondary metabolites in their different parts including triterpenoids, flavonoids, alkaloids sterols and coumarins.

2.4.3.1 Triterpenoids

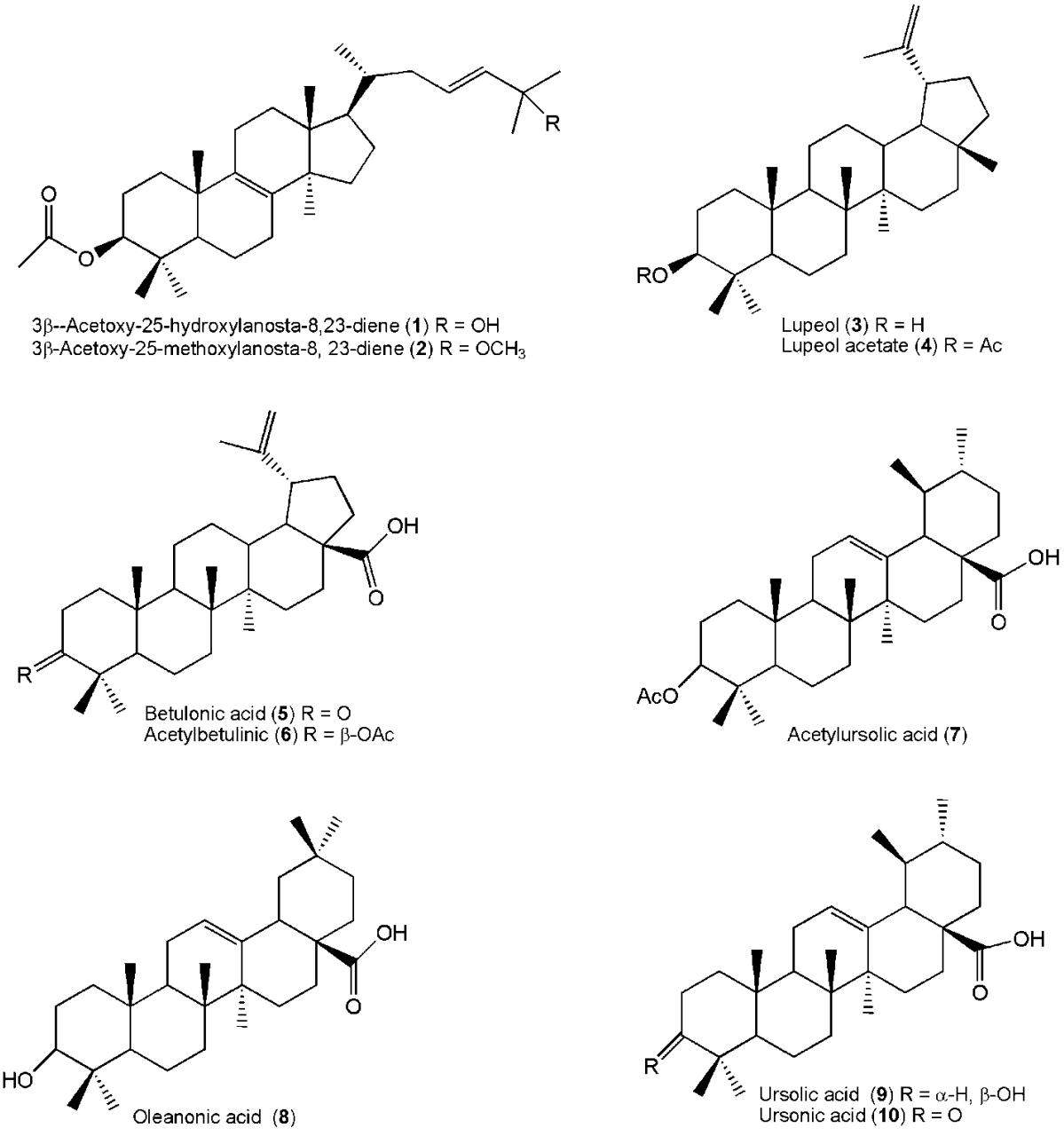


Figure 2-6: Triterpenoids from *Ficus* species.

One of the most commonly isolated class of compounds from the genus *Ficus* are triterpenoids. Chiang *et al* (2005), isolated the tetracyclic triterpenoids, 3β-acetoxy-25-hydroxylanosta-8,23-diene (**1**) and 3β-acetoxy-25-methoxylanosta-8,23-diene (**2**) from the roots of *F. microcarpa.* A number of pentacyclic triterpenoids such as lupeol (**3**) and lupeol acetate (**4**) (Chiang and Kuo, 2002), betulonic acid (**5**), acetylbetulinic acid (**6**), acetylursolic acid (**7)**, oleanonic acid (**8**), ursolic acid (**9**) and ursonic acid (**10**) have also been isolated from the aerial roots of *F. microcarpa* (Chiang *et al.,* 2005) (Figure 2-6).

2.4.3.2 Flavonoids

Another class of compounds commonly found in this genus are the flavonoids. The stem bark of *F. cordata* and the leaves of *F. septica* are reported to contain epiafzelechin (**11**), and genistein (**12**) respectively (Kuete *et al*., 2008; Wu *et al*., 2002). Also, quercetin (**13**), rutin (**14**) and kaempferitrin (**15**) have been isolated from the leaves of *F. benjamina*, *F. carica* and *F. septica*, respectively (Almahyl *et al*., 2003; El-Kholy and Shaban, 1966; Wu *et al*., 2002). Ficuisoflavone (**16**) and isolupinisoflavone E (**17**) have also been isolated from the stem bark of *F. microcarpa.* (Li and Kuo, 1997) (Figure 2-7).

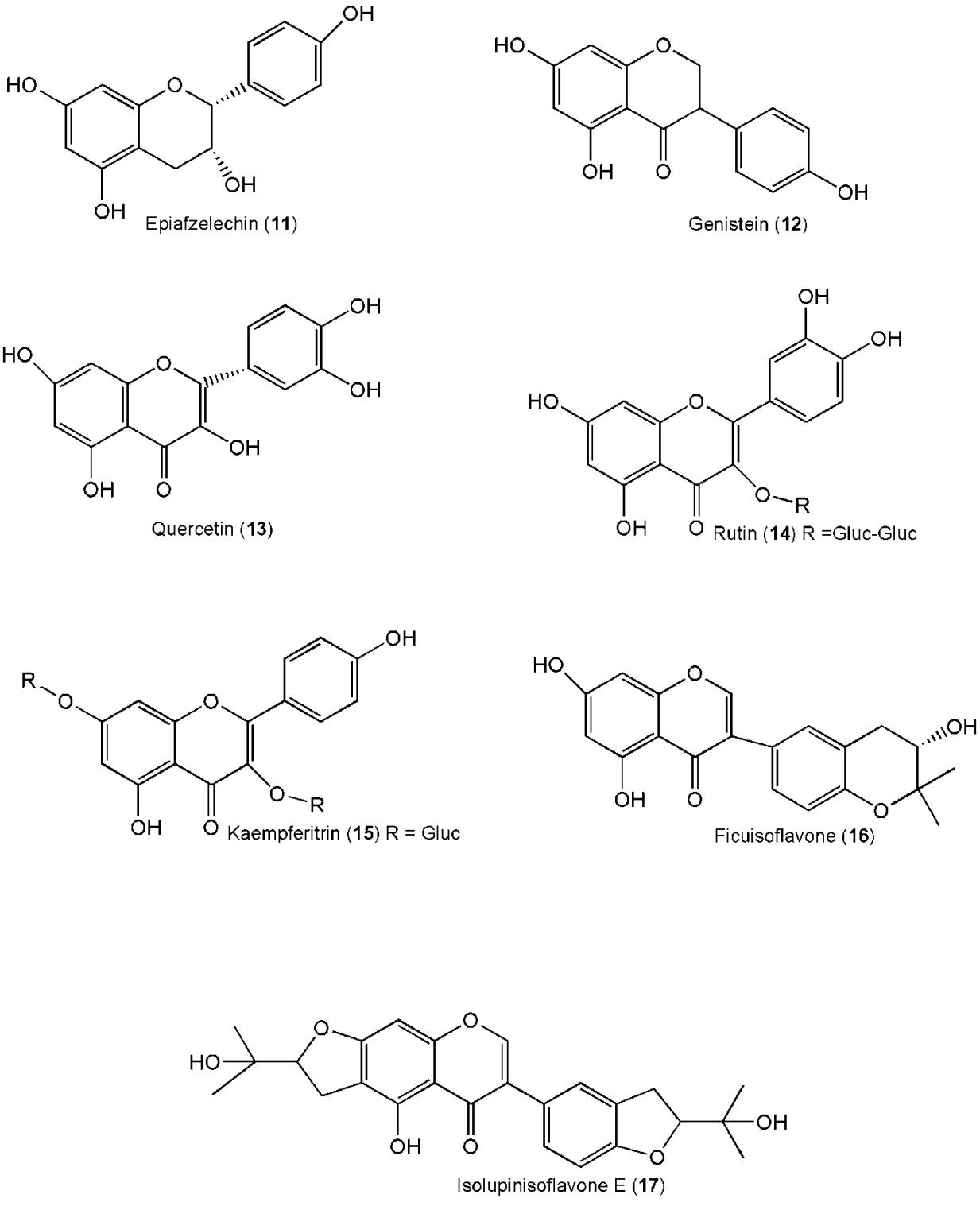
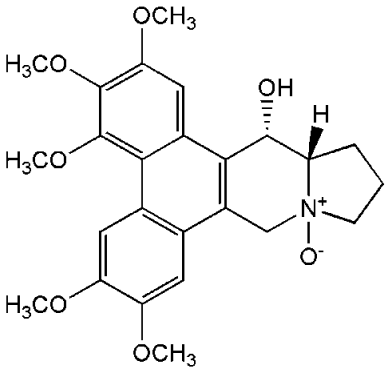


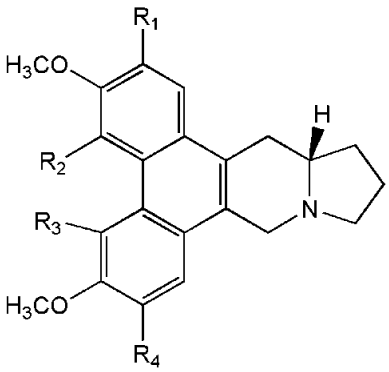
Figure 2-7: Flavonoids from *Ficus* species.

2.4.3.3 Alkaloids

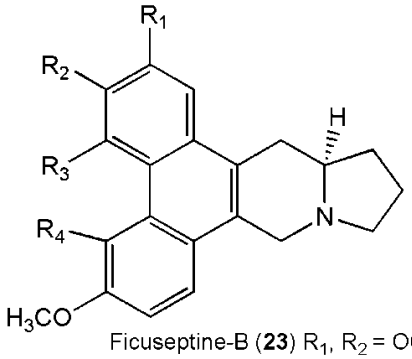
Alkaloids have been isolated from this genus. Phenanthroindolizidine alkaloids were isolated from *F. septica* and *F. hispida* (Damu *et al*., 2005; Peraza-Sánchez *et al*., 2002). Ficuseptine-A (**18**), antofine (**19**), tylophorine (**20**), tylocrebrine (**21**) and isotylocrebrine (**22**) were isolated from the leaves of *F. septica;* Ficuseptine-B (**23**), Ficuseptine-C (**24**) and Ficuseptine-D (**25**) were isolated from the stem bark (Baumgartner *et al*., 1990; Damu *et al.*, 2005; Wu *et al*., 2002; Yang *et al*., 2006). Hispidine (**26**) was isolated from *F. hispida* (Venkatachalam and Mulchandani, 1982) (Figure 2-8).



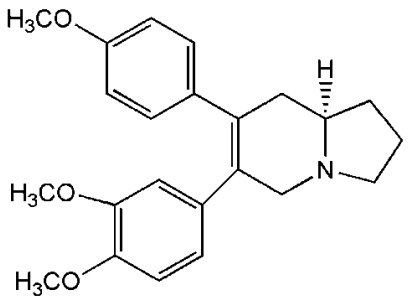
Ficuseptine-A (**18**)



Antofine (**19**) R_1= OCH_3, R_2= H, R_3= H, R_4= H
Tylophorine (**20**) R_1= OCH_3, R_2= H, R_3= H, R_4= OCH_3
Tylocrebrine (**21**) R_1= OCH_3, R_2= H, R_3= OCH_3, R_4= H
Isotylocrebrine (**22**) R_1= H, R_2= OCH_3, R_3= H, R_4= OCH_3



Ficuseptine-B (**23**) R_1, R_2 = OCH_2O, R_3 = H, R_4 = OCH_3
Ficuseptine-C (**24**) R_1, R_2 = OCH_2O, R_3, R_4 = H
Ficuseptine-D (**25**) R_1 = H, R_2, R_3 = OCH_3, R_4 = H



Hispidine (**26**)

Figure 2-8: Alkaloids from *Ficus* species.

2.4.3.4 Sterols

A number of sterols have been isolated from this genus such as β-sitosterol (**27**), β-sitosterol-β-D-glucoside (**28**), ψ-taraxasterol ester (**29**), baurenol (**30**), 24-methylenecycloartanol (**31**) and stigmasterol (**32**) (El-Kholy and Shaban, 1966; Wu *et al*., 2002) (Figure 2-9).

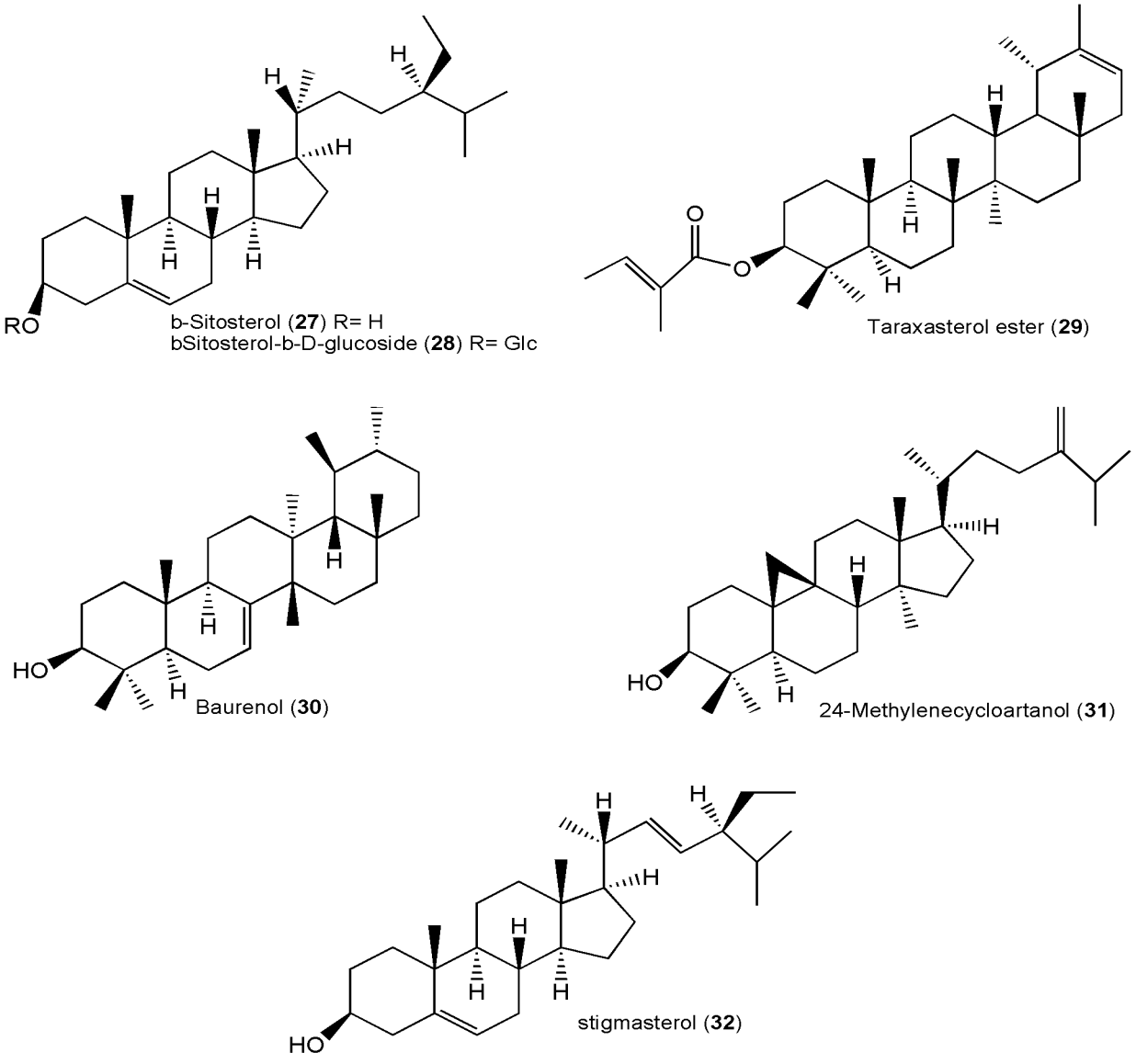


Figure 2-9: Sterols from *Ficus* species.

2.4.3.5 Coumarins

The prominentcoumarins found in fig leaves are the furocoumarins such as bergapten (**33**) and psoralen (**34**) isolated from *F. carica* (Damjanić and Akačić, 1974; Meng *et al*., 1996). In addition, marmesin (**35**) and umbelliferone (**36**) were obtained from the leaves of *F. carica* (Innocenti *et al*., 1982; Wu *et al*., 2002) (Figure 2-10).

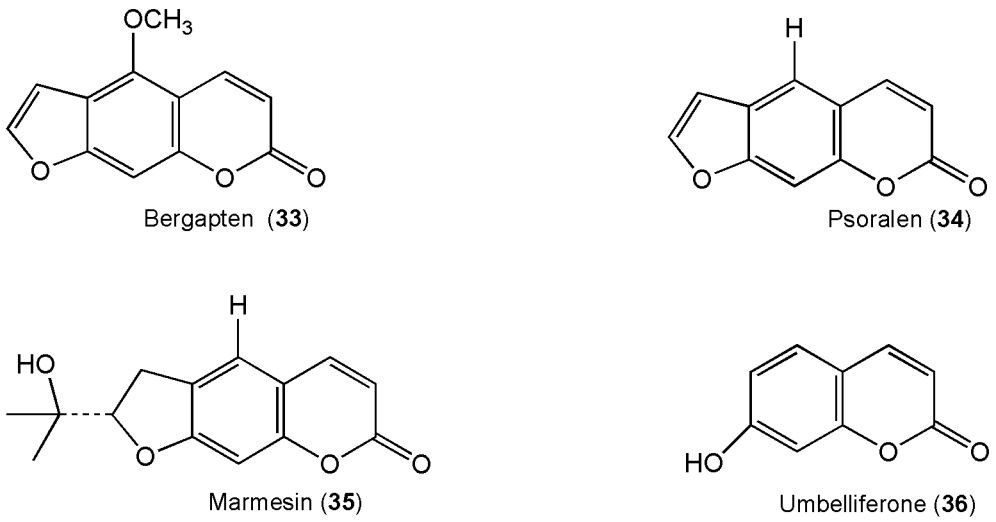


Figure 2-10: Coumarins from *Ficus* species.

2.4.4 Ethnobotanical review of the *Ficus* species selected for study

2.4.4.1 *Ficus burtt-davyi* Hutchinson

Figure 2-11: *Ficus burtt-davyi* Hutchinson.

Ficus burtt-davyi Hutchinson (Figure 2-11) is named after Joseph Burtt Davy, a South African botanist. It is a shrub, climber or small tree that grows up to about 5 m (Burrows and Burrows, 2003). They have somewhat oval shape, glossy green leaves with varying sizes ranging from approximately 2 to 10 cm in length based on its environment. The fruits are usually found paired in-between the leaves appearing green mottled with white, turning yellowish when ripe from March to November and they are approximately 10 mm in diameter. This shrub adapts easily to several habitats and can be epiphytic on trees as a strangler fig, as well as on rocks (epilithic) where their roots can split the rocks to find nutrients. The bark is pale, whitish-grey in appearance with

young velvety branches. Pollination of *F. burtt-davyi* is by *Elisabethiella baijnathi*, a tiny wasp (approximately 1.5 mm long). *Ficus burtt-davyi* is found in thicket, dune forests, coastal swamp forest, scrub, and on sand dunes, and in Savanna on rocky outcrops and cliffs normally in eastern part of Western Cape, Eastern Cape, KwaZulu-Natal, and north into Mozambique specifically along the coastal belt. The pounding and weaving of *Ficus burtt-davyi* bark for mat making has been known since early times. Fruits are eaten and used as a laxative in the Eastern Cape (Burrows and Burrows, 2003).

2.4.4.2 *Ficus sur* Forssk

Figure 2-12: *Ficus sur* Forssk.

Ficus sur Forssk (formerly *F. capensis*) (Figure 2-12) is commonly referred to as the Cape fig, broom cluster fig, bush fig or Malabar tree. It is a large tree, with spreading canopy of approximately 12 m in height, but reaching 25 to 30 m in some other areas. Its leaves form a dense,

round/large canopy, they have shiny leaves and purplish figs carried in large clusters appearing attractive usually on the lower parts of the trunk. The green leaves are ovate to broadly elliptic in shape, up to 23 x 12.8 cm. The bark is smooth at first, grey to pale grey in appearance and produces latex appearing whitish and milky which exudes when any part of the plant is injured. The fruits (3 to 4 cm in diameter) are large, heavily branched clusters on the stem and low on the main branches becoming red mottled with cream or pink when ripe (September to March). The ripe figs have a pink colouration usually attractive to several fruit-eating birds, some having visiting birds, seen only when figs are ripe. *F. sur* can be found in Eastern Cape, KwaZulu-Natal, Western Cape, Limpopo, and Mpumalanga usually alongside rivers and waterways, riverine forests and drier woodlands. *F. sur*, together with *F. burtt-davyi*, are the two most southerly occurring figs (Burrows and Burrows, 2003). Lung illness has been treated with the latex from the stems as well as administered to cows for increased milk production. Root and bark decoctions are administered for pulmonary tuberculosis, influenza and skin diseases (Hutchings *et al*., 1996; Watt and Breyer-Brandwijk, 1962). Anaemia, sexually transmitted diseases and diarrhoea are among other diseases treated with *F. sur* (Irvine, 1961; Malcom and Sofowora, 1969). The fruits of *F. sur* are edible and the most palatable of all indigenous figs in South Africa. It is much sought after by humans and all fruit-eating animals due to its large size, proportionately thick flesh and sweet taste when ripe, which can be eaten fresh or sun dried to make a sweet preserve if the insect-laden seeds are removed (Burrows and Burrows, 2003).

2.4.4.3 *Ficus sycomorus* Linn

Figure 2-13: *Ficus sycomorus* Linn.

Ficus sycomorus Linn (Figure 2-13) is a semi-deciduous savannah tree usually large forming canopy of height between 5 to 25 m and occasionally with buttress rooting system. The bark has a distinctive greenish-yellow colour with scattered grey scales usually appearing like pale brown patches at areas where scales have fallen and with heavy latex flow. The leaves are large, broadly oblong to almost circular up to 5 to 17 x 3.5 to 15 cm in size. It is dark green, rough and harsh to the touch. When considered together, the yellow bark and the harsh, rough leaves are diagnostic features. The fruits occur solitary or paired, with globose or ovoid shape and ripe ones having yellow-red to reddish-purple colouration, up to 3.5 x 5 cm, pubescent or almost glabrous. They grow on the axils of leafs on old wood branches having about 10 cm of no leaf. Flowering / fruiting occurs all year, which peaks between July and December (Orwa *et al.*, 2009). *F. sycomorus* is found occurring southwards through eastern Africa as far as KwaZulu-Natal. It is found along river banks, forming a distinctive part of the riverine thicket and also mixed woodland. Medicinal use

of the bark includes treatment of coughs, scrofula, throat and chest diseases. Dysentery treatment and superficial application for inflamed areas are also known medicinal uses of the latex; bark and milky latex mixture are also used for ringworm treatment. The leaves are acclaimed to be effective antidote for snakebite as well as good for jaundice treatment. The roots are said to be good as laxative and for anthelmintic treatments (Orwa, *et al.*, 2009). Among the wild indigenous figs, few are as palatable as *F. sycomorous*. The fruits have a good flavour; the Tonga women dry them after which they acquire a rather sultana-like taste (FAO, 1988; Palmer and Pitman, 1972).

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CHAPTER 3

Chemical constituents and *in vitro* antioxidant activity of crude extracts and compounds from leaves and stem bark of *Ficus burtt-davyi* Hutchinson

Abstract

Ficus burtt-davyi Hutchinson (Moraceae) is an endemic species of South Africa. In this study, a phytochemical analysis of the leaves and stem bark of *F. burtt-davyi* resulted in the isolation of five triterpenes (lupeol, lupeol acetate, β-sitosterol, stigmasterol and campesterol), one carotenoid (lutein), a phaeophytin (phytyl-7-ethyl-25-(methoxycarbonyl)-3,8,13,17-tetramethyl-26-oxo-12-vinyl-17,18-dihydro-2,20 ethanoporphyrin-18-propanoate or phaeophytin **a**) and one flavonoid (+)-catechin). The *in vitro* antioxidant study of the methanol extracts of leaves and stem bark, (+)-catechin and phaeophytin **a** using the 1,1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, ferric reducing antioxidant power (FRAP) assay and hydrogen peroxide (H_2O_2) assay showed significantly higher ($p < 0.05$) antioxidant activity for the methanol extract of the stem bark than the leaves, with IC_{50} values (in μg mL^{-1}) of 58.28 ± 5.05 for DPPH, 46.09 ± 0.06 for FRAP and 151.03 ± 1.60 μg mL^{-1} for H_2O_2. The results suggest that the plant can be used as a therapeutic agent in alternative medicine for oxidative stress related degenerative diseases.

Key words: phaeophytin**;** triterpenes; flavonoids; antioxidants.

3.1 Introduction

With over 850 species, *Ficus*, of the plant family Moraceae, is one of the oldest, most successful, but understudied genera in modern pharmacognosy and has extensive distribution of secondary metabolites such as triterpenoids, phenolics, flavonoids, alkaloids, coumarins and sterols (Chang *et al*., 2005; Chiang and Kuo, 2002; Lansky and Paavilainen, 2011; Lee *et al.*, 2002). *Ficus*, within their vasculatures, possesses and secrete a latex-like material, affording protection from harm and providing self-healing from physical injuries (Lansky *et al*., 2008). For many centuries, animals and humans have depended on its fruit as a source of nourishment while other plant parts such as the leaves, bark, and roots have been utilised for medicinal purposes. The latex from *Ficus* species has been investigated for their anticancer activity as far back as the 1940`s (Lansky *et al*., 2008) and extracts have demonstrated enhanced intracellular accumulation of daunomycin in K562/R7 leukemic cells as well as cytotoxic effects on the growth of multi-drug resistant human sarcoma MES-SA/Dx5 cells (Lansky *et al*., 2008). Thus, the extracts play an adjunctive role in multiple cancer prevention and cancer chemotherapy (Lansky *et al*., 2008; Simon *et al.*, 2001). The antioxidant potential of the plant has also been reported where the extracts have been shown to decrease lipid peroxidation and increase antioxidant enzymes (Shukla *et al*., 2004). Although the medicinal benefits of *Ficus* in humans are based on historical and anecdotal reports, with few modern clinical trials, ethnomedicinal uses suggest anti-neoplastic and anti-inflammatory actions (Lansky *et al*., 2008).

Ficus burtt-davyi, known as Uluzi by the Zulu people in KwaZulu-Natal, is highly adaptable to a wide variety of habitats and has even been known to grow on larger trees (epiphytic) as a strangler fig, as well as on rocks (epilithic) where the roots are able to split the rocks in their search for

nutrients (Burrows and Burrows, 2003). The fruits of the plant are edible and also used as a laxative by the locals in the Eastern Cape (Burrows and Burrows, 2003). To date, no information has been reported on the chemical composition and antioxidant activity of this plant. In this study, we report on the isolation and identification of compounds from the leaves and stem bark of *F. burtt-davyi*. In addition, we report on the antioxidant activity of the methanol (MeOH) extracts of the leaves, stem bark and selected isolated compounds using a multi-method approach due to the complexity of the mechanisms of antioxidant activity (Dejian *et al*., 2005).

3.2 Experimental

3.2.1 General experimental procedures

The ^{1}H, ^{13}C and 2D-NMR spectra were recorded on a BrukerAvance^{III} 400 MHz spectrometer at 400.22 MHz for ^{1}H and 100.63 MHz for ^{13}C. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The ^{1}H and ^{13}C chemical shifts of the deuterated chloroform ($CDCl_3$) were 7.24 and 77.0 respectively referenced to the internal standard, tetramethylsilane (TMS), respectively. Infrared (IR) spectra were recorded using a Perkin-Elmer Universal ATR spectrometer. UV spectra were obtained on a Hewlett Packard UV-3600 Spectrophotometer. Column chromatography (CC) was performed with Merck silica gel 60, (0.040–0.063 mm). Thin layer chromatography (TLC) was performed on Merck 20 cm×20 cm silica gel 60, F_{254} aluminium sheets. The spots were analysed under UV (254 nm and 366 nm), visualised using 10% H_2SO_4 in MeOH followed by heating. Solvents (analytical grade) and other chemicals used were supplied by either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA) chemical companies.

3.2.2 Plant material

The leaves and bark from *F. burtt-davyi* were collected on the Westville Campus of the University of KwaZulu-Natal (UKZN), Durban, South Africa, in June 2013. The plant was authenticated by Prof. Himansu Baijnath and a voucher specimen (Ogunlaja, O1) has been deposited in the Herbarium of the School of Life Sciences, UKZN, Westville. The stem bark and leaves of the plant were dried and ground using a mini-industrial grinder (Wiley Mill).

3.2.3 Extraction, fractionation and isolation

The air-dried, powdered leaves (970 g) of *F. burtt-davyi* was sequentially exhaustively extracted with (4 L × 2) of n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and MeOH for 24 h using an orbital shaker. The extracts were concentrated by evaporation under vacuum at controlled temperatures to obtain crude extracts of n-hexane (24.22 g), DCM (15.64 g), EtOAc (7.76 g) and MeOH (52.98 g). The n-hexane and DCM crude extracts of leaves were combined due to similar TLC profiles (similar R_f values). This combined extract (38.00 g) was subjected to CC using a gradient elution system of n-hexane: EtOAc starting with 100% n-hexane until 100% EtOAc was reached through 10% increments of EtOAc, collecting 100 mL fractions at each stage. Six main fractions (I-VI) were obtained and fraction II yielded compound A-**1** (105 mg), which was eluted with n-hexane: EtOAc (8:2). Fraction III (1.23 g) was purified further to give compound A-**2** (18.6 mg) and compound A-**3** (20.1 mg), respectively. The EtOAc extract of the leaves (7.0 g) was separated using CC in a similar manner to yield seven major fractions (I-VII) based on similar TLC profiles. Fraction II and III afforded compound A-**4** (30.5 mg) and compound A-**5** (10.5 mg), respectively.

The n-hexane and DCM crude extracts of the stem bark were combined due to similar TLC profiles (similar R_f values). This extract (24.35 g) was subjected to CC in a similar manner to that of the leaves to yield nine major fractions based on TLC profiles. Fractions II and III gave compound **A-6** (4.5 g) and compound **A-7** (4 g), respectively which were eluted with 100% DCM and re-crystallised in MeOH. The MeOH extract from the stem bark was partitioned with EtOAc and the EtOAc fraction was concentrated to yield 4 g of extract. This was separated on a 1.5 cm diameter column using CC and a gradient of n-hexane: EtOAc (8:2) to 100% EtOAc (10% increments of EtOAc, 20 mL fractions) to afford compound **A-8** (23 mg).

3.2.4 Phenolic content and *in vitro* antioxidant assays

3.2.4.1 Estimation of total phenolic content (TPC)

The total polyphenol content (TPC) of the extracts from *F. burtt-davyi* was determined as Gallic acid equivalent (GAE) according to the method described by McDonald *et al*. (2001) with slight modifications. Briefly, 200 µL of the extract (240 µg mL^{-1}) was incubated with 1 mL of ten-fold diluted Folin Ciocalteau reagent and 800 µL of 0.7 M Na_2CO_3 for 30 min at room temperature. Absorbance values were determined at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

3.2.4.2 DPPH free radical-scavenging activity assay

The capacity to scavenge the ‘‘stable’’ free radical 1,1- diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the procedure describe by Ahmad *et al*. (2011) with some modifications.

Various concentrations (7.5-500 µg mL^{-1}) of extracts and isolated compounds (3000 µL) made from a stock solution (10 µg mL^{-1}) were mixed with 2850 µL MeOH solution containing DPPH radicals. The mixture was then vortexed, and incubated for 30 min at room temperature. The scavenging activity was evidenced by a change in color from purple to yellow, which was further measured by the decrease in absorbance at 517 nm using a Shimadzu UV–Vis spectrophotometer. Ascorbic acid was used as the standard while MeOH served as the blank. The assays were done in triplicate. The difference in absorbance between a test sample and the control (DPPH + MeOH) was expressed as percentage inhibition.

$$\% \text{ inhibition} = [(A_o - A_{sample} / A_{o)} \times 100]$$

Where, A_o (control) = Absorbance of DPPH + methanol and A_{sample} = Absorbance of the sample. The IC_{50}value which is the inhibitory concentration in µg mL^{-1} of samples, or standard, necessary to reduce the initial DPPH by 50% as compared to the negative control was determined graphically by plotting the absorbance of DPPH as a function of sample concentration in µg mL^{-1} for the standard and samples.

3.2.4.3 Ferric reducing antioxidant power (FRAP) assay

The reducing power of the extracts and compounds were determined according to the method of Oyaizu (1986) as described by Behera *et al*. (2006) with some modifications. Various concentrations (7.5-500) µg mL^{-1} were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 0.1% potassium ferricyanide and the mixture was incubated at 50 ^oC for 30 min. After the addition of 2.5 mL of 10% TCA, the mixture was centrifuged at 3000 rpm for 10

min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. The methanolic extract without reagents was used as a negative control while ascorbic acid with the same concentrations was used as positive controls.

3.2.4.4 Hydrogen peroxide-scavenging activity assay

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*. (1989). A solution of hydrogen peroxide (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Samples of various concentrations (7.5-500 µg mL^{-1}) were transferred into the test tubes, and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After addition of 0.6 mL hydrogen peroxide solution, tubes were vortexed and absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. Phosphate buffer (50 mM) without hydrogen peroxide was used as a blank and ascorbic acid was used as the positive control. Hydrogen peroxide scavenging ability was calculated using the following equation:

Hydrogen peroxide scavenging activity = (1- absorbance of sample/absorbance of control) ×100

3.2.5 Statistical analyses

The experimental results were expressed as mean ± standard deviation (SD) of three replicates and IC_{50} values were calculated by linear regression. The data were subjected to one way analysis of variance (ANOVA) to determine significant differences between means ($p < 0.05$) Tukey`s test was used for post-hoc analyses. All the statistical tests were performed using graphpad prism 6.0.

3.3 Results and discussion

3.3.1 Structure elucidation of compounds from *F. burtt-davyi*

Compound **A-1**, **A-2** and **A-3** were identified by using spectral data (IR, UV, GC-MS, ^{1}H-NMR, ^{13}C- NMR and 2D NMR) and by comparison of the NMR data with those reported in literature. Compound **A-1** was identified as sitosterol (Chaturvedula and Prakash, 2012)**,** compound **A-2** as campesterol (Choi *et al*., 2007) and compound **A-3** as stigmasterol (Chaturvedula and Prakash, 2012).

Compound **A-4** was isolated as a dark green amorphous solid. The mass spectrum of compound **A-4** obtained by HR-ESI-MS (positive mode) gave *m/z* value at 893.5530 corresponding to the molecular formula ($C_{55}H_{74}N_4O_5$) $[M+Na]^+$, (calc. $C_{55}H_{74}N_4O_5$ Na 894.1999). The IR spectrum of compound **A-4** showed absorption bands at 2922, 3388, 1618 and 1376 cm^{-1} corresponding to C(sp^3), NH, CH=CH (vinyl group) and CN, respectively. The UV spectrum of compound **A-4** showed absorption bands at 414 and 669 nm. The data is consistent with the presence of a porphyrin nucleus. The ^{1}H-NMR spectrum of compound **A-4** showed characteristic peaks for chlorophyll derivatives. The ^{1}H-NMR spectrum showed an upfield shift at (δ_H -1.70) (the NH proton from the pyrrole ring), a resonance at δ_H 6.1–7.9 (vinyl group), meso-proton signals down field at δ_H 9.47 (H-10), 9.33 (H-5), and δ_H 8.53 (H-20) and δ_H 6.28 (dd, J= 1.6, 17.5 Hz, H-3a), δ_H 6.16 (dd, J= 1.5, 11.55 Hz, H-3b) for a mono-substituted vinyl group corresponding to the structure of phaeophytin **a.** The characteristic phytyl side chain resonances at δ_H 1.60 (CH), 1.25(CH_2) and 0.97(CH_3) were also observed. The ^{13}C-NMR, DEPT 90 and DEPT 135 spectra resolved fifty five carbon resonances corresponding to eleven methyl, fourteen methylene, eleven methine and

twenty-one quaternary carbons signals. These chemical shifts were in accordance with those reported in literature (Wang *et al*., 2009), thus, compound **A-4** was established as phytyl-7-ethyl-25-(methoxycarbonyl)-3,8,13,17-tetramethyl-26-oxo-12-vinyl-17,18-dihydro-2,20-ethanoporphyrin-18-propanoate (phaeophytin **a**).

Compound **A-5** was obtained as a yellow colored compound and GC-MS data showed molecular ion peak [M^+] at *m/z* 568.9 which corresponds to the molecular formula $C_{40}H_{56}O_2$. The UV spectrum of compound **A-5** showed absorption maxima at 454, 480 and 430. The ^{1}H-NMR spectrum showed resonance in the olefinic region between δ_H 5.09-6.62, methylene resonances of cyclohexene between δ_H 1.33-1.44 with a strong OH resonance at δ_H 4.23. Based on spectral data and those reported in literature, compound **A-5** was identified as lutein.

Compound **A-6** was isolated as a white solid. The IR spectrum exhibited characteristic absorption frequencies at 3326, 878 and 1637 cm^{-1} typical of the O-H, unsaturated out plane C-H and C=C bond vibrations, respectively. The absorptions bands observed at 1379 and 1452 cm^{-1} were due to the distortion vibrations of CH_3 groups and methylenic vibrations, respectively. The mass spectrum of compound **A-6** obtained by HR-ESI-MS (positive mode) gave *m/z* value at 449.3756 corresponding to the molecular formula ($C_{30}H_{50}O$) $[M+Na]^+$, (calc. $C_{30}H_{50}O$ Na 449.7174). The ^{1}H-NMR spectrum for compound **A-6** revealed the presence of seven tertiary methyl singlets protons at δ_H 0.73, 0.76, 0.80, 0.92, 0.94, 1.00 and 1.65; a multiplet at δ_H 3.18 (H-3), and characteristic olefinic protons at δ_H 4.66 (H-29 a) and 4.54 (H-29 b) and a sextet at δ_H 2.39 (19β –H). The DEPT 90 and 135 experiments resolved ten methylene, five methine and five quaternary carbons. Based on spectral information and those reported in literature, compound **A-6** was

identified as lupeol. The presence of lupeol in *F. burtt-davyi* was not reported before the current study.

Compound **A-7** was isolated as white crystalline needles. EIMS for $C_{32}H_{52}O_2$ *m/z* (rel. int.): 468 [M+] (17.2%), 453 (2.9%), 425 (1.5%), 408 (1.7%), 365 (3.9%), 189 (75.4%), 109 (73.1%), 43 (100%). The diagnostic peaks occurred at *m/z* 408 [M-AcOH], 249 [M-$C_{16}H_{27}$] and 189 [249-AcOH]. The IR spectrum of compound **A-7** exhibited characteristic absorption frequencies at 1732 cm^{-1} (C=O), 3073 cm^{-1} (exomethylene group), 2939 cm^{-1} (C-H) and 1244 cm^{-1} (C-O). The ^{1}H-NMR spectrum of compound **A-7** showed eight methyl resonances between δ_H 0.76-2.01. The ^{1}H-NMR spectrum of compound **A-7** was similar to compound **A-6** with addition of an acetate methyl resonance at δ_H 2.01 (H-32) and the proton resonance at δ_H 4.46 (H-3) which is further downfield due to presence of the acetoxy group. Based on spectral information and in comparison with that in literature (Mahato and Kundu, 1994) compound **A-7** was identified as lupeol acetate. Lupeol acetate has never been reported isolated before from *F. burtt-davyi*.

Compound **A-8** was isolated as a light brown powder. The mass spectrum of compound **A-8** obtained by HR-ESI-MS (positive mode) gave *m/z* value at 313.0685 corresponding to the molecular formula ($C_{15}H_{14}O_6$) $[M+Na]^+$, (calc. $C_{15}H_{14}O_6$ Na 313.2596). The IR spectrum showed characteristic absorption bands for the O-H group (3215 cm^{-1}), C=C group (1623 cm^{-1}), and C-O group (1145–1019 cm^{-1}). The ^{1}H-NMR spectrum of compound **A-8** showed characteristic resonances for flavonoids at δ_H 6.85 (H-2'), 6.76 (H-5') and 6.74 (H-6') from the B-ring catechol moiety as well as at δ_H 5.94 as H-8 and 5.87 as H-6 from the meta-coupled protons of the A-ring resorcinol moiety. The proton resonances at δ_H 4.59 (H-2), 3.99 (H-3), 2.87 (H-4) and 2.53 (H-4) were used to establish a flavanol skeleton for compound **A-8**. The ^{13}C-NMR spectrum showed a

diagnostic peak at δ_C 82.8 (C-2) as well as a strong H-2/H-3 correlation from the COSY experiment, thus confirming the structure of compound **A-8** to be (+)-catechin (Es-Safi *et al*., 2006). Catechin and its analogueshave been shown to possess significant bioactivity, such as anti-inflammatory, anti-allergic, anti-mutation, anti-aging, improving liver function and removal of free radicals (Liu and Chen, 2004). This is the first report of compounds A**1-A8** being reported in *F. burtt-davyi*.

3.3.2 Total phenolic content (TPC)

Table 3-1: Total phenolic content (TPC) and extraction yield (%, mg extract per g sample×100) of *F. burtt-davyi* leaves and stem bark extracts.

Extracts	Yield (%)	TPC (mg g^{-1} GAE) *
Leaves		
EtOAc	0.51^{c}	0.43 ± 0.01^{c}
MeOH	3.05^{b}	1.14 ± 0.12^{b}
Stem bark		
EtOAc	0.80^{c}	1.05 ± 0.22^{bc}
MeOH	5.46^{a}	5.90 ± 0.45^{a}

Data are presented as mean ± SD (n = 3). *GAE (Gallic Acid Equivalent).Values with different superscripts letters along a column are significantly different from each other by Tukey's HSD multiple post hoc test, ($p < 0.05$).

The extraction yields indicated that the stem bark of *F. burtt-davyi* produced the highest amount of extract. Furthermore, the total phenolic content (TPC) of the MeOH extract of the stem bark was significantly higher ($p < 0.05$) than that from other extracts (Table 3-1). Based on this result, the MeOH extracts from the leaves and stem barks were selected for *in vitro* antioxidant study.

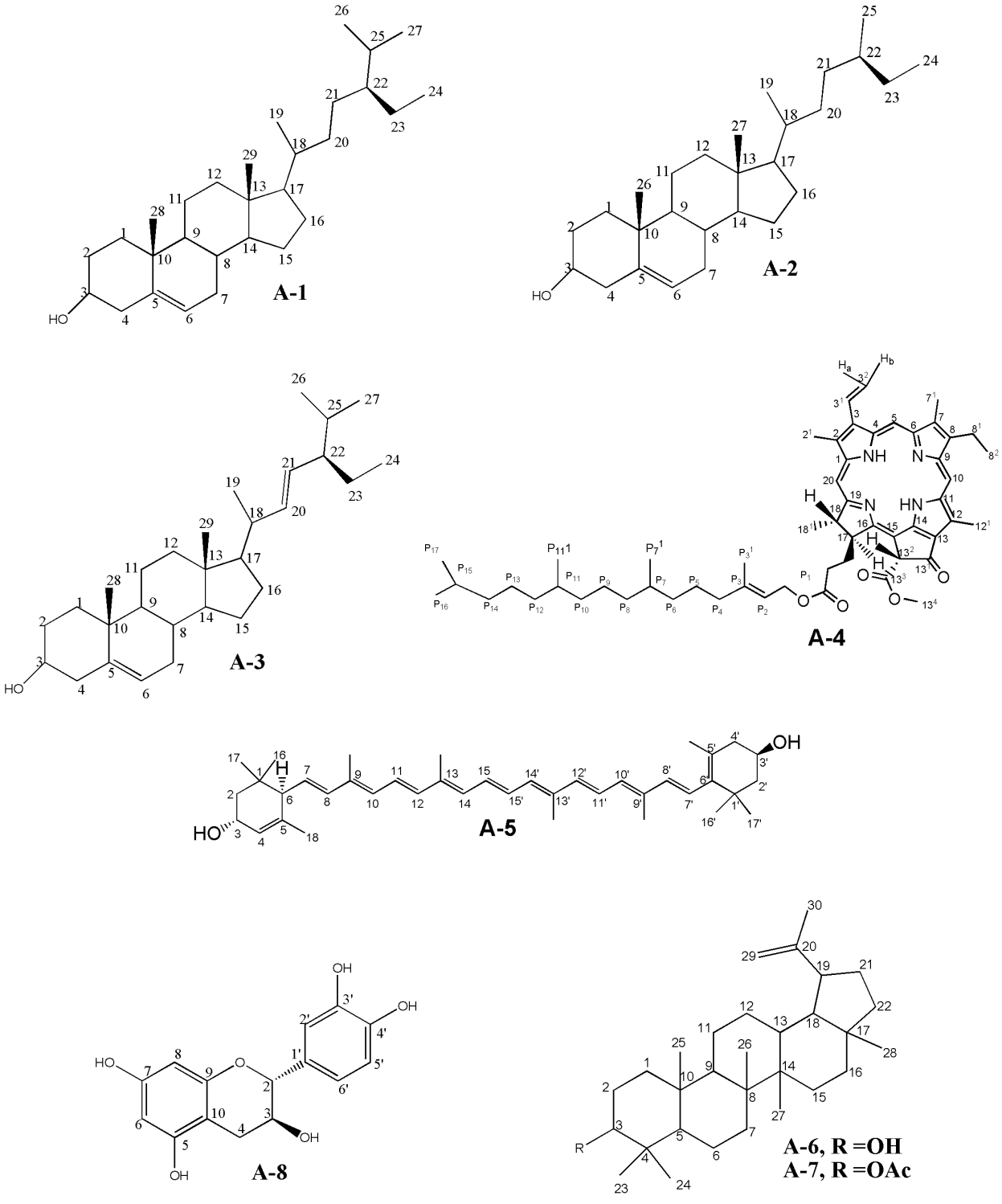

Figure 3-1: Chemical structures of compounds (A1-A8) isolated from *F. burtt-davyi*

3.3.3 DPPH free radical-scavenging activity assay

Most antioxidants possess proton radical scavenging activity which may be monitored by discoloration of the purple 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals to yellow DPPH in a dose-dependent pattern. For this study, the DPPH assay was used to detect the antioxidant activity of the extracts and compounds from *F. burtt-davyi* as shown in Figure 2-1. The results indicated that at lower concentrations (< 25 µg mL^{-1}), the activity of the extracts (stem bark & leaves) and compounds were comparable but were significantly lower than that of AA.

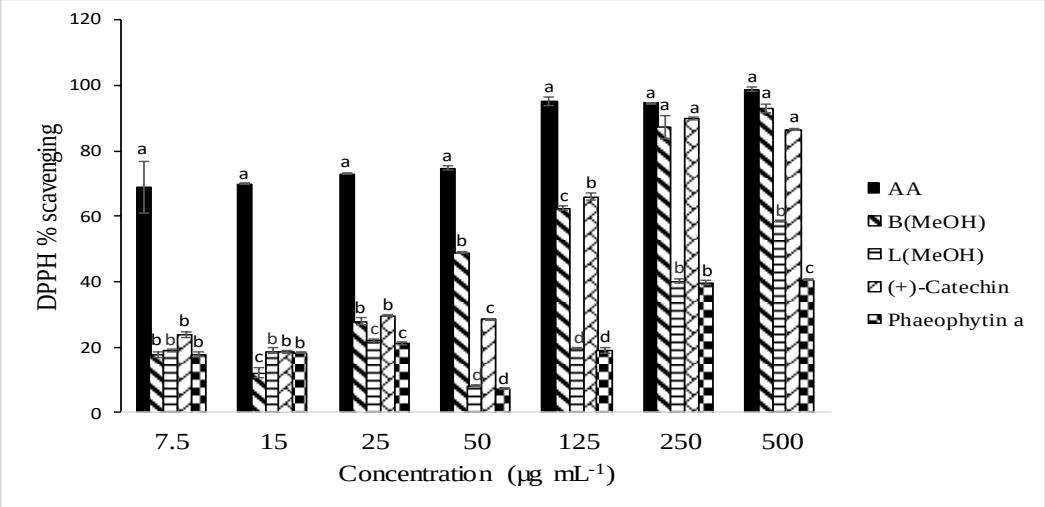

Figure 3-2: DPPH radical scavenging activity of MeOH extracts of *F. burtt-davyi* stem bark (B), leaves (L), phaeophytin a, (+)-catechin and ascorbic acid (AA). Different letters for a concentration indicate significantly different means (Tukey's post hoc comparisons, $p < 0.05$).

At higher concentrations (> 125 µg mL^{-1}), the activity of the MeOH extract from the bark, (+)-catechin and AA were not significantly different but higher than the other substances tested. The high activity of the MeOH extract of the bark could be attributed to the presence of (+)-catechin in the extract thereby suggesting that the activity of an extract may be as a result of the presence

of one bioactive compound, which, at lower concentrations, may be diminished by the antagonistic effects of other compounds or the presence of other non-active compounds.

3.3.4 Ferric reducing antioxidant power assay (FRAP assay)

Numerous studies have linked the electron donation capacity (reduction) of an antioxidant to its anti-oxidative activity (Siddhuraju *et al*., 2002). The presence of electron donating substances, such as antioxidant samples, causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The total reducing power of the extracts and compounds from *F. burtt-davyi* were compared to AA (Figure 3-3).

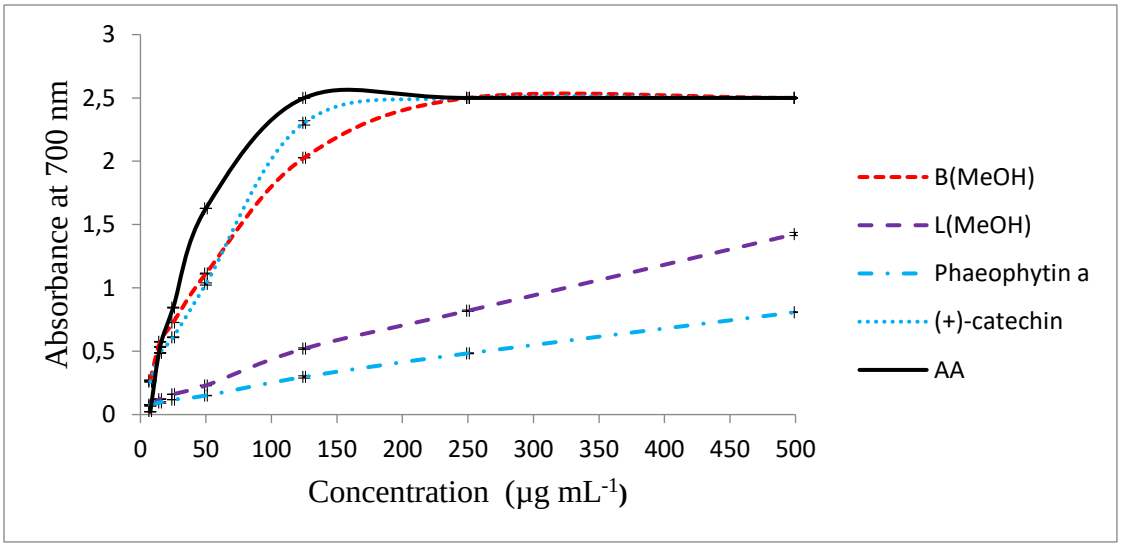

Figure 3-3: Reducing power of MeOH extracts of *F. burtt-davyi* stem bark B(MeOH), leaves L(MeOH), isolated compounds (phaeophytin a and (+)-catechin) and ascorbic acid (AA). Values represented as Mean ± SD (n = 3).

Similar to the results produced by the DPPH radical scavenging activity, the results obtained from the FRAP assay showed the reducing power of the MeOH extract from the stem bark, (+)-

catechin and AA to be comparable and significantly higher than that of the MeOH extract from leaves and phaeophytin **a.**

3.3.5 Hydrogen peroxide-scavenging activity assay

Although hydrogen peroxide is a weak oxidising agent, it can cross cell membranes rapidly and while inside the cell, form hydroxyl radicals which are primarily responsible for its toxic effects. Therefore, removal of the hydroxyl free radical is necessary to ensure a good health status. The scavenging effects of the extracts and isolated compounds in *F. burtt-davyi* were evaluated against this free radical (Figure 3-4). The antioxidant activity of the extracts and compounds using hydrogen peroxide were similar to those produced by the DPPH and FRAP assays thereby confirming the activities of all the tested substances.

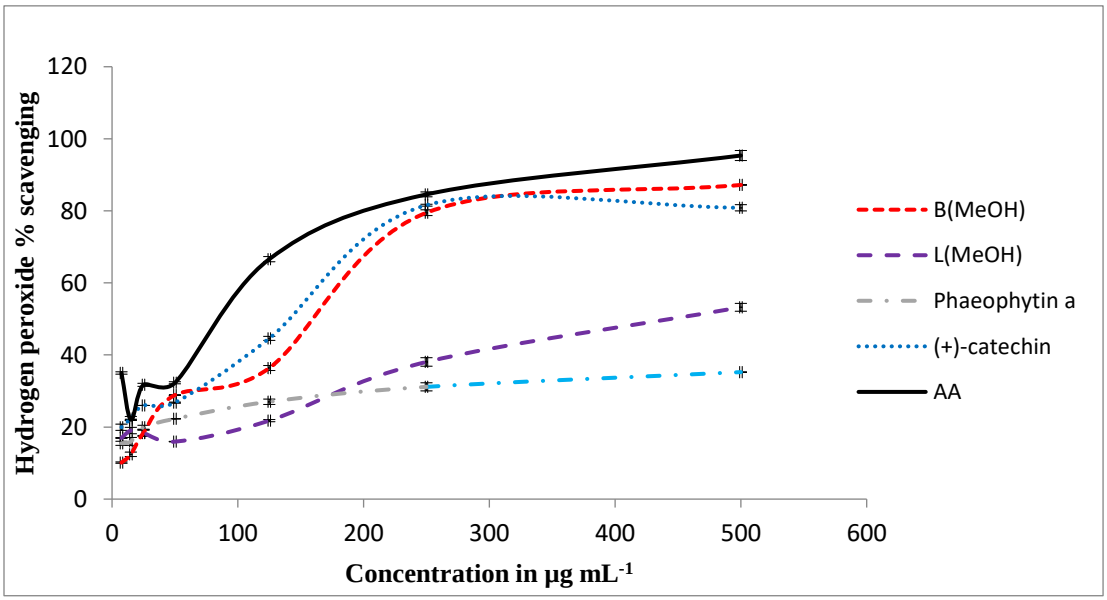

Figure 3-4: Hydrogen peroxide scavenging activity of MeOH extracts of *F. burtt-davyi* stembark B(MeOH), leaves L(MeOH), isolated compounds (phaeophytin aand (+)-catechin) and ascorbic acid (AA). Values represented as Mean ± SD (n =3).

Table 3-2 shows the IC_{50} values for the different antioxidant assays. Although compounds **A1-A8** were tested for anti-oxidant activity, the IC_{50} values for sitosterol, campesterol, stigmasterol, lutein, lupeol and lupeol acetate were extremely high (> 1000 µg $mL^{-1)}$) for all assays therefore, their results are not shown in Table 3-2. The results confirm that the MeOH extract and (+)-catechin have a significantly higher antioxidant activity ($p < 0.05$), with comparable IC_{50} values for all three antioxidant assays. However, the IC_{50} value for the standard AA was significantly lower than the plant extracts and compounds. From the TPC and antioxidant activity results, it is clear that higher TPC indicates higher antioxidant activity. This is in agreement with previous reports (Gorinstein *et al*., 2003; Maisuthisakul *et al*., 2007).

Table 3-2: IC_{50} of different extracts and compounds from *F. burtt-davyi* for various antioxidant assays.

Extracts/compound	**DPPH*(** µg mL^{-1})	**FRAP*(** µg mL^{-1})	**H_2O_2 scavenging*(** µg mL^{-1})
L(MeOH**)**	405.39 ± 20.58^{c}	420.95 ± 8.46^{c}	449.56 ± 6.66^{c}
B(MeOH)	58.28 ± 5.05^{b}	46.09 ± 0.06^{b}	151.03 ± 1.6^{b}
phaeophytin **a**	611.15 ± 10.06^{d}	777.80 ± 1.60^{d}	$>1000^{d}$
(+)-catechin	61.19 ± 0.68^{b}	46.89 ± 0.33^{b}	125.15 ± 1.16^{b}
Ascorbic acid (AA)	1.14 ± 0.08^{a}	39.32 ± 0.017^{a}	81.56 ± 0.63^{a}

Each value is represented as Mean ± SD (n = 3). $^{a-d}$ Means in the same column followed by a different letter are significantly different ($p< 0.05$). *No significant difference between assays ($p < 0.05$).

Thus, the present study has shown that the leaves and stem bark of *F. burtt-davyi* possess moderate to significantly good antioxidant activity and may contribute to the retardation of the inflammatory process mediated by reactive oxygen metabolites from phagocytic leukocytes that invade the tissues and cause injury to essential cellular components (Parfenov and Zaikov, 2000). In addition,

previous studies have shown that plant-derived sterols exhibit anti-carcinogenic effects on different types of cancers (Awad *et al*., 1998; De Stefani *et al*., 2000; La Vecchia *et al*., 1994; Von Holtz *et al*., 1998). *F burtt-davyi* could therefore be said to possess anti-cancer potential having isolated several sterols from the stem bark.

3.4 Conclusion

To the best of our knowledge, this is the first report of the phytochemical constituents of *F. burtt-davyi,* resulting in the isolation of a carotenoid which has been documented to exhibit moderate antibacterial activity and phaeophytin **a** with strong anti-HCV-NS3 protease activity with little cytotoxicity. The data from this study suggests that *F. burtt-davyi* possessed moderate to good anti-oxidative activity and can be used as a potential alternative medicine for oxidative stress related non-communicable chronic diseases.

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CHAPTER 4

Cytotoxic activity of the bioactive principles from *Ficus burtt-davyi* Hutchinson

Abstract

Ficus burtt-davyi Hutchinson (Moraceae) is a medicinal plant species indigenous to Southern Africa. In this study, a phytochemical and cytotoxic investigation on *F. burtt-davyi* was conducted to evaluate its ethnomedicinal use. The phytochemical study of the fruits yielded triterpenoids (lupeol and α-amyrin). The cytotoxic evaluation was done on the MeOH extracts from fruits and stem bark and selected compounds isolated from *F. burtt-davyi* stem bark and fruits (lupeol, α-amyrin, lupeol acetate and (+)-catechin) against two human cancer cell lines (breast adenocarcinoma (MCF-7) and colorectal adenocarcinoma (Caco-2)) and normal human embryonic kidney cells (HEK293) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. The methanol extract from the stem bark was significantly cytotoxic to MCF-7 and Caco-2 cell lines ($p < 0.05$) in a concentration-dependent manner with IC_{50} values of 6.6 and 8.1 µg mL-1, respectively relative to the control. Lupeol and (+)-catechin showed cytotoxic activity against MCF-7 cell lines with IC_{50} values of 22.6 and 29.8 µg mL^{-1}, respectively and greater cytotoxic activity against Caco-2 cell lines with IC_{50} values of 10.7 and 9.0 µg mL^{-1}, respectively. Data from this study suggests that *F. burtt-davyi* exhibits cytotoxicity with no significant inhibitory effects against HEK293. The results also indicate that (+)-catechin and lupeol, the most abundant bioactive principles in the stem bark, are responsible for the synergistic

cytotoxic effects against tested human cancer cell lines. This study provides evidence on the pharmaceutical potential of the medicinal plant, *F. burtt-davyi*, as a chemotherapeutic agent against cancer.

Keywords: breast adenocarcinoma, colorectal adenocarcinoma, catechin, lupeol, anti-cancer.

4.1 Introduction

Cancer is one of the major causes of morbidity and mortality throughout the world. Its prevalence in South Africa is also well documented and has been attributed to different factors such as smoking, occupational exposure, infections, lifestyle, and environmental pollutants (NCR, 2010). Current statistics show an average of 8.2 million deaths per year due to cancer with 12.7 million new cases each year, most of which are from developing countries (Ferlay *et al*., 2010; Stewart and Wild, 2014). It is also estimated that new cancer cases across the world will increase to 21·4 million by 2030 (Ferlay *et al*., 2010). A recent survey of underlying causes of death in South Africa showed cancer to be the second most frequent cause of death, with the first one being tuberculosis (SSA, 2014). This survey highlights the need for more effective management and treatment of cancer in South Africa and it also confirms that the search for a cure should form part of national health initiatives of all nations.

Generally, plant derived bioactive principles have gained attention in alternative therapeutic strategies to fight against different diseases, primarily because of their high therapeutic index and since medicinal plants have been used effectively in traditional medicine for centuries (Nisa *et al*.,

2011; Sharma *et al*., 2011; Shoeb, 2006). Plant-derived substances have a long history in cancer treatment with approximately 60% of the anticancer drugs currently in use being isolated from natural products sourced from plants (Conforti *et al*., 2008; Gonzales and Valerio, 2006; Jain and Jain, 2011; Khakdan and Khosro, 2013). This indicates a high possibility of indigenous medicinal plants serving as potential sources in the development of more effective anticancer agents (Caamal-Fuentes *et al.*, 2011).

Previous reports have shown that many *Ficus* species possess anticancer potential (Lansky *et al*., 2008; Pratumvinit *et al.*, 2009*;* Simon *et al*., 2001; Sirisha *et al*., 2010; Zeng *et al., 2012*). Free radical can cause oxidative stress, and it has been implicated in a number of studies as the factor responsible for a number of chronic non-communicable diseases, including cancer *via* DNA damage (Chandra *et al*., 2000). The potential anticancer activity of *Ficus* species has been credited in some reports to the secondary metabolites, such as, terpenoids and flavonoids that are capable of preventing oxidative stress-related diseases such as cancer (Elsharkawy, 2013; Sirisha *et al*., 2010). In a number of studies, the antioxidant activity has been linked to anticancer effects (Li *et al*., 2007; Sirisha *et al*., 2010). We also showed significantly high antioxidant activity of the bioactive principles from the stem bark of *Ficus burtt-davyi* (Ogunlaja *et al*., 2016).

Ficus burtt-davyi Hutch. (Moraceae) is a medicinal plant species indigenous to Southern Africa (Burrows and Burrows, 2003). *F. burtt-davyi* is highly adaptable, and can survive in wide variety of habitats and its fruits are used in traditional medicine as a laxative (Burrows and Burrows, 2003). Previously, we reported on the isolation of triterpenes, pigments and a flavonoid from the leaves and stem bark of *F. burtt-davyi* (Ogunlaja *et al*., 2016). In the present study, we report on the secondary metabolites isolated from the fruits of *F. burtt-davyi*. In addition, a cytotoxic

evaluation of the methanol extracts from the fruits and stem bark, as well as the isolated compounds from the fruits and stem bark against two human tumour cell lines (breast adenocarcinoma (MCF-7) and colorectal adenocarcinoma (Caco-2)), relative to normal human embryonic kidney (HEK293) cell, using the MTT cell viability assay was carried out.

4.2 Materials and Methods

4.2.1 Experimental

4.2.1.1 General experimental procedures

Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C, 1D and 2D) were recorded in deuterated chloroform ($CDCl_3$), referenced to the internal standard, tetramethylsilane (TMS) (Merck, Darmstadt, Germany) on a 400 MHz spectrometer (AVANCEIII, Bruker, Rheinstetten, Germany) at 400.22 MHz for ^{1}H and 100.63 MHz for ^{13}C. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The ^{1}H and ^{13}C chemical shifts of $CDCl_3$ were 7.24 and 77.0, respectively. Infrared (IR) spectra were recorded using a Perkin-Elmer Universal ATR spectrometer. UV-Vis spectra were obtained on a UV-Vis-NIR spectrophotometer (UV-3600, Shimadzu, Tokyo, Japan). Column chromatography was performed using silica gel (Kieselgel 60, 0.040–0.063mm, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on aluminum sheets (Merck silica gel 60, 20 × 20 cm, $F_{254,}$ Merck, Darmstadt, Germany). The spots were analysed under UV (254 nm and 366 nm), visualised using 10% H_2SO_4 in methanol (MeOH) followed by heating. Mass spectral data was obtained using a gas chromatography-mass spectrometer (GC-MS QP2010SE Series, Shimadzu, Tokyo, Japan) with LabSolution Software

and equipped with a DB-5SIL MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) fused silica capillary column. Helium (2 mL/min) was used as a carrier gas and MeOH or dichloromethane (DCM) was used to dissolve the samples. The injector was kept at 250 °C whilst the transfer line was at 280 °C. The column temperature was held at 50 °C for 2 min, and then ramped to 280 °C at 20°C/min where it was held for 15 min.

4.2.2 Chemicals and Reagents

All cell culture reagents and media were obtained from Lonza BioWhittaker, Walkersville, USA. 5-fluorouracil (5-FU) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was supplied by Hyclone, GE Healthcare, Utah, USA. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide, solvents (analytical grade) and other chemicals used were supplied by Merck (Darmstadt, Germany).

4.2.3 Plant material

Fresh plant material was collected between January to August 2016 from the University of KwaZulu-Natal (UKZN), Westville campus, South Africa and was authenticated by a botanist, Prof. H. Baijnath. A voucher specimen (Ogunlaja, O1) was deposited in the WARD Herbarium of the School of Life Sciences, UKZN, Westville.

4.2.4 Extraction, isolation and preparation of test samples

The air-dried, powdered fruit (820 g) of *F. burtt-davyi* was sequentially extracted exhaustively with hexane (3 × 2000 mL), DCM (3 × 2000 mL) and MeOH (3 × 2000 mL) on an orbital shaker at room temperature for two days each. The DCM extract (13.2 g) was subjected to column chromatography on a 4 cm diameter column using a gradient elution system of n-hexane: ethyl acetate (EtOAc) (v/v) starting with 100% n-hexane until 100% EtOAc was reached through 10% increments of EtOAc. Fractions of 100 mL were collected for each eluent step and fractions with similar retention factors (R_f) on TLC plates (similar TLC profiles) were combined and concentrated using a rotatory evaporator to give five major fractions (I-V). Compounds **A-6** (20.5 mg) and **A-9** (10.9 mg) were obtained from Fractions I and III, respectively.

4.2.5 Cell viability and cytotoxicity testing

Human cancer cell lines ((breast adenocarcinoma (MCF-7) and colorectal adenocarcinoma (Caco-2)) were used and normal human embryonic kidney cell lines (HEK293) were used as the control. All cell lines were originally obtained from the American Tissue Culture Collection (ATCC) (Manassas, Virginia, USA). Cells were grown as monolayers in Minimum Essential Medium, supplemented with 10% FBS and an antibiotic mixture of penicillin (100 µg mL^{-1}) and streptomycin (100 µg mL^{-1}). A volume of 100 µL of medium containing 2.0×10^4 cells were seeded in a 96-well microtiter plate and incubated at 37 °C in an atmosphere of 95% air and 5% CO_2, with 100% relative humidity. The MeOH extract from the stem bark and fruits and the four isolated compounds were subjected to an *in vitro* cytotoxicity bioassay by the mitochondrial dependent reduction of yellow MTT to purple formazan (Mosmann, 1983) against MCF-7, Caco-

2 and HEK293 cell lines. Stock solutions (20 µg mL^{-1}) of test samples (extracts and compounds) and positive control (5-FU) were prepared by dissolution in dimethyl sulfoxide (DMSO). The final concentrations of the test samples in culture were 1, 5, 25, and 50 µg mL^{-1}. After 24 h of incubation and attachment, the cells were treated with fresh media containing the various concentrations of crude extract, compounds and positive control or DMSO vehicle control. This was incubated for 48 h, thereafter, the old medium was replaced with fresh medium containing 10 µg mL^{-1} of MTT in phosphate-buffered saline (PBS) and incubated for 4 h at 37 °C. The supernatant was aspirated, MTT-formazan crystals were dissolved in 100 µL of DMSO and the absorbance was measured at 570 nm by a microplate reader (MR-96A, Mindray, Shenzhen, China). Assays were conducted in triplicate. Cell survival (growth) was calculated by comparing the absorbance of treated and untreated cells. The percentage cell inhibition was determined. The percentage viability was plotted against the extract concentrations and the 50% cell viability value (IC_{50}) was calculated from the curve (Hensen *et al*., 1989).

4.2.6 Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one-way analysis of variance (ANOVA) to determine significant differences between means. Tukey`s test was used for post-hoc analyses. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). Results with $p < 0.05$ were regarded as statistically significant.

4.3 Results and discussion

4.3.1 Structure elucidation

Previously, we reported on the phytochemical investigation, resulting in the isolation of compounds from the leaves and stem bark of *F. burtt-davyi* (Ogunlaja *et al*., 2016). These compounds were lupeol, lupeol acetate and (+)-catechin. In this study, compound **A-6** was identified using spectral data (IR, UV, GC-MS, ^{1}H-NMR, ^{13}C-NMR and 2D NMR) and by comparison of the NMR data with those reported in literature. Compound **A-6** was identified as lupeol (Figure 4-1) (Mohato and Kundu, 1994) which was previously isolated from the stem bark of *F. burtt-davyi.*

Compound **A-9** was isolated as a white powder. GC-MS data showed molecular ion peak [M+] at *m/z* 426 which corresponds to the molecular formula $C_{30}H_{50}O$. The IR spectrum showed a broad absorption band at 3337 cm^{-1} (OH group) and a band at 1555 cm^{-1} (C=C). The UV spectrum of compound **A-9** gave maximum wavelength (λmax) at 229 nm. The ^{1}H-NMR spectrum showed characteristic resonances for the oleanane-type pentacyclic triterpene at δ_H 5.09 (t, J = 3.41 Hz, H-12), 3.18 (dd, J= 5.17; 10.89 Hz, H-3) and eight methyl resonances. The ^{13}C-NMR spectrum showed diagnostic peaks at δ_C 79.08 (C-3), 124.4 (C-12) and 139.5 (C-13) indicating the presence of a Δ^{12}-double bond of the olean-12-ene-type. Based on spectral information and comparison with the literature (Dias *et al*., 2011; Fingolo *et al*., 2013; Hassan *et al*., 2015), compound **A-9** was identified as α-amyrin (Figure 4-1). This is the first report of α-amyrin from *F. burtt-davyi.*

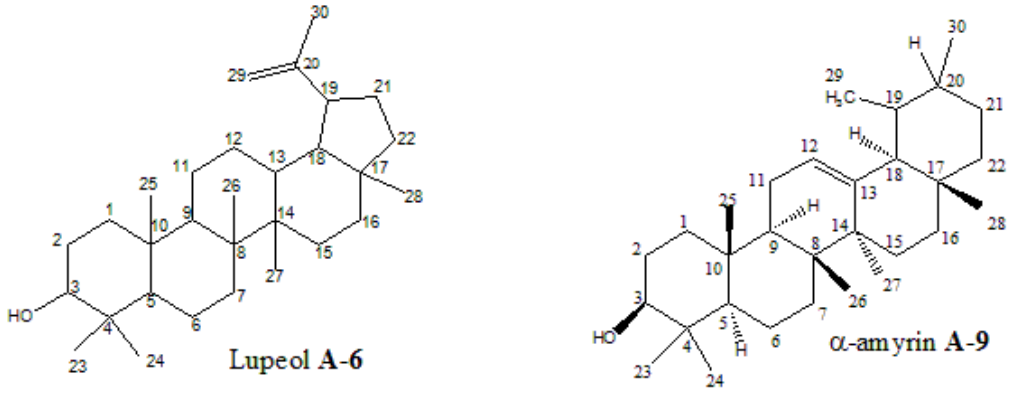

Figure 4-1: Chemical structures of compounds A-6 and A-9 isolated from the fruits of *F. burtt-davyi*.

4.3.2 *In-vitro* cytotoxicity

The cytotoxic activity of the MeOH extracts from fruits and stem bark and selected compounds previously isolated from *F. burtt-davyi* stem bark (lupeol, lupeol acetate and (+)-catechin) and fruits (lupeol and α-amyrin) was determined using the MTT cell viability assay using different cancer cell lines at various concentrations (Ogunlaja *et al*., 2016). The IC_{50} value estimated by nonlinear regression analysis was used as a parameter for cytotoxicity. The results are summarised in Table **4-1**. The results showed that the MeOH extract of the stem bark (MeOH (SB)) of *F. burtt-davyi* significantly reduced cell viability in both MCF-7 and Caco-2 cell lines, with IC_{50} values of 6.6 and 8.1 µg mL^{-1}, respectively. These values are significantly lower than the cytotoxic activity for crude extracts of ($IC_{50} \leq 30$ µg mL^{-1}) recommended by the American National Cancer Institute (Suffness and Pezzuto, 1990**)**. Furthermore, the extracts showed a significantly low cytotoxicity ($p < 0.05$) towards normal human embryonic kidney cells (HEK293), relative to the control (5-FU). This result shows the selectivity of the extract towards the cancer cells.

Lupeol and (+)-catechin showed cytotoxic activity against MCF-7 cell lines with IC_{50} values of 22.6 and 29.8 µg mL^{-1}, respectively and greater cytotoxicity against Caco-2 cell lines with IC_{50} values of 10.7 and 9.0 µg mL^{-1}, respectively. The potential of lupeol to inhibit the growth of cancer cells was also observed in previous studies (Aratanechemuge *et al*., 2004; Moriarty *et al*., 1998; Nana *et al*., 2012). Cmoch *et al*. (2008) reported the IC_{50} value for lupeol against MCF-7 to be low (< 15 µg mL^{-1}), which is corroborated by this study (22.6 µg mL^{-1}). In addition, the observed synergistic cytotoxic effects by MeOH (SB) against tested human cancer cell lines is in agreement with a previous report which suggested that lupeol, in combination with other bioactive principles, could be beneficial in the treatment of cancer (Lee *et al*., 2007). Lupeol showed strong, 2-fold cytotoxic activity against Caco-2 cell lines compared to 5-FU. Similarly, MeOH (SB) and (+)-catechin showed significantly stronger cytotoxic activity ($p < 0.05$) against Caco-2 cells by nearly 3-fold compared to 5-FU (Table 4-1). Conversely, lupeol acetate and α–amyrin exhibited low cytotoxic activity ($IC_{50} >$ 50 µg mL^{-1}) against all the tested cell lines, similar to previous reports (Hassan *et al*., 2015).

Table 4-1: Cytotoxicity of the methanol (MeOH) extract of fruits (F) and stem bark (SB) and isolated compounds (lupeol, lupeol acetate, (+)-catechin and α--amyrin from *F.burtt-davyi*,, and positive control (5-FU) against MCF-7, Caco-2 and HEK293 cell lines.

	IC_{50} µg mL^{-1}		
Plant extracts/Compounds	MCF-7	Caco-2	HEK293
MeOH (F)	$>50^{c}$	$>50^{c}$	189.5^{c}
MeOH (SB)	6.6^{a}	8.1^{a}	207.5^{d}
Lupeol	22.6^{b}	10.7^{a}	179.1^{c}
Lupeol acetate	$>50^{c}$	$>50^{c}$	162.0^{c}
(+)-catechin	29.8^{b}	9.0^{a}	120.2^{b}
α—amyrin	$>50^{c}$	$>50^{c}$	121.3^{b}
5-FU (Control)	26.2^{b}	23.1^{b}	102.0^{a}

Values with different superscript letters along a column are significantly different from each other by Tukey's HSD multiple post hoc test, ($p < 0.05$).

Figure 4-2 and 4-3 show cell viability after exposure of MCF-7 and Caco-2 cells, respectively to different concentrations of test samples. The MeOH extract of the fruits (MeOH (F)), lupeol acetate and α-amyrin showed very weak cytotoxic effects against the tested cell lines (Table 4-1) and are therefore omitted from the results. The addition of 1–50 µg mL^{-1} of MeOH (SB) and test compounds ((+)-catechin and lupeol) to both cell lines (MCF-7 and Caco-2) resulted in a dose-dependent inhibition of cell proliferation. The results of the post-hoc analyses (Tukey`s test) showed mean percentage cell viability values for MeOH (SB) and compounds (lupeol and (+)-catechin) to be significantly different ($p < 0.05$) across all concentrations relative to the vehicle

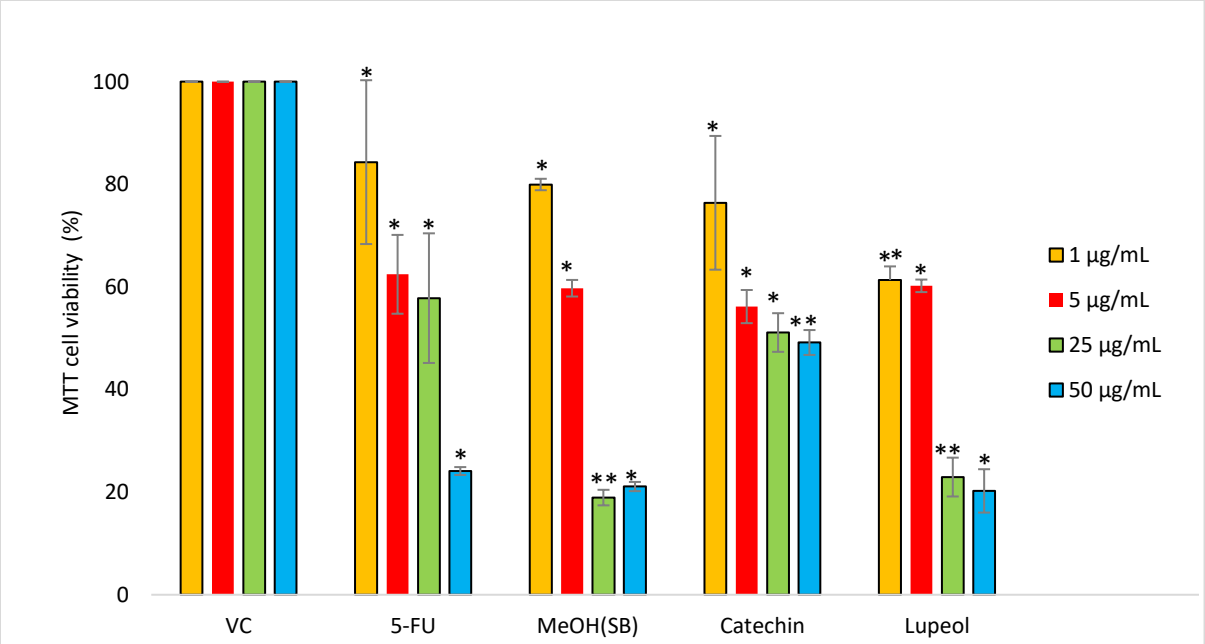

Figure 4-2: Cell viability by MTT assay after exposure of MCF-7 cells to different concentrations of *F. burtt-davyi* methanol extract (stem bark) (MeOH (SB)), isolated compounds ((+)-catechin and lupeol), DMSO vehicle control (VC) and positive control, 5- fluorouracil (5-FU). * - $p < 0.05$ from DMSO; ** - $p < 0.05$ from DMSO and 5-FU.

control (DMSO). Lupeol displayed significant cytotoxicity against MCF-7 cells at 25 and 50 µg mL^{-1} and significant cytotoxicity against Caco-2 cells across all tested concentrations markedly reducing cell viability compared to 5-FU. MeOH (SB) also showed significant cytotoxicity relative to 5-FU, at 25 and 50 µg mL^{-1} against both MCF-7 and Caco-2 cells. (+)-Catechin exhibited selectivity towards Caco-2 cells compared to MCF-7 with significant cytotoxicity at 25 and 50 µg mL^{-1} thereby reducing cell viability compared to 5-FU.

The higher cytotoxic activity of MeOH (SB) of *F. burtt-davyi* could be attributed to the presence and synergistic effect of the more active secondary metabolites, (+)-catechin and lupeol. Similarly, the lower cytotoxic activity of MeOH (F) could be as a result of the presence and of the less active

compounds, lupeol acetate and α–amyrin. This report highlights the cytotoxic activity of the extracts and pure compounds from *F. burtt-davyi* which, to the best of our knowledge, is the first such report.

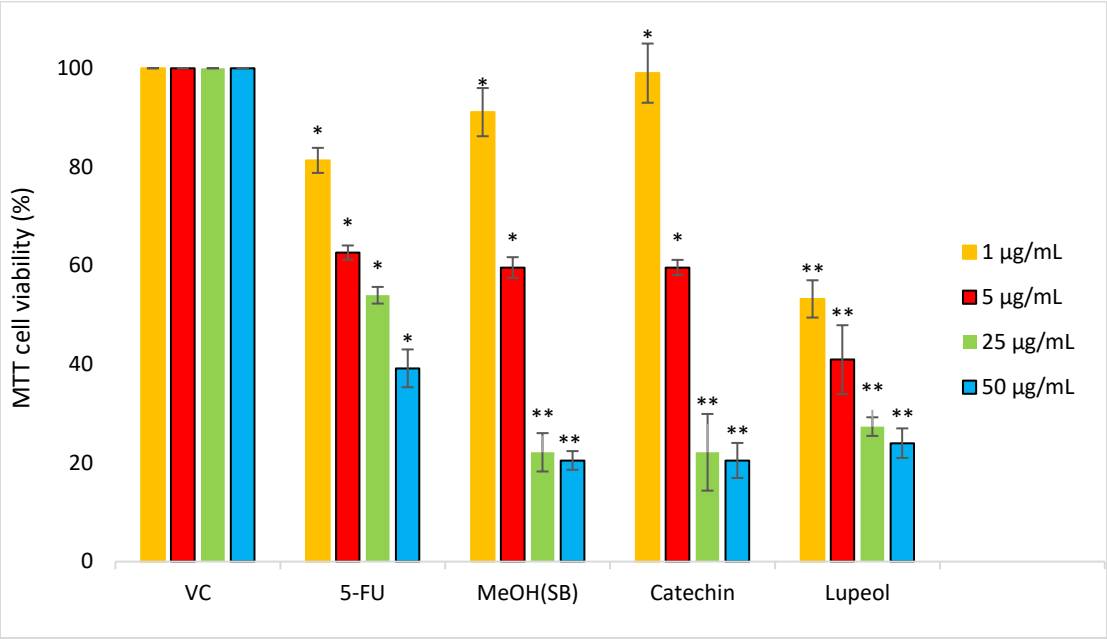

Figure 4-3: Cell viability by MTT assay after exposure of Caco-2 cells to different concentrations of *F. burtt-davyi* methanol extract (stem bark) (MeOH (SB)), isolated compounds ((+)-catechin and lupeol), DMSO vehicle control (VC) and positive control, 5- fluorouracil (5-FU). * - $p < 0.05$ from DMSO; ** - $p < 0.05$ from DMSO and 5-FU

4.4 Conclusion

The present study established the cytotoxic potential of the methanol extracts of the stem bark of *F. burtt-davyi* against MCF-7 and Caco-2 cells with no significant adverse effect on normal HEK293 cells, thereby validating its ethnomedicinal use. This selectivity to cancer cells *in vitro* warrants further *in vivo* studies. Data from this study suggest that (+)-catechin and lupeol, the most

abundant bioactive compounds in the stem bark, are responsible for the synergistic cytotoxic effect of this extract against breast and colorectal adenocarcinoma cell lines. In addition, data from this study provides evidence on the potential of *F. burtt-davyi* as a chemotherapeutic agent against cancer.

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CHAPTER 5

Elemental composition of the edible fruits of *Ficus burtt-davyi* Hutchinson from different habitats in KwaZulu-Natal, South Africa.

Abstract

Many figs are known to have medicinal value and their fruits are often consumed as food by the rural communities. *Ficus burtt-davyi* Hutchinson is an indigenous medicinal fig used as a laxative by the locals in southern Africa. In this study, the concentrations of various elements and the proximate chemical composition was determined in the edible fruit of *F. burtt- davyi* from eight different sites in KwaZulu-Natal, South Africa. The elemental distribution of essential elements in the fruits varied significantly with site ($p < 0.05$) and the concentrations were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu. The results of the proximate composition indicated that the fruit contained 5.0 ± 0.3% protein, 8.4 ± 0.4% lipids, 78.9 ± 0.6% carbohydrates, 4.0 ± 0.7% crude fibre and 3.7 ± 0.1% ash. Hierarchical cluster analysis showed variation in elemental distribution between the two distinct habitats (coastal and inland forests) representing the sampling sites. This study suggests that the fruits can serve as an alternative source of energy and may contribute positively to the diet without posing the risk of adverse health effects due to low concentrations of toxic elements. The consumption of about 20.0 g of *F. burtt-davyi* fruit can contributes up to 43.8% towards the Recommended Dietary Allowance for Mn.

Keywords: Recommended Dietary Allowance; elemental distribution; essential elements; toxicity.

5.1 Introduction

Trees have been an essential part of human survival from the earliest time, providing basic needs such as shelter, firewood, medicine and food. The use of indigenous plants as food and to treat medical conditions in humans' dates back to time immemorial. Many Southern African trees have edible and often delicious fruits, but many of them are yet to be domesticated and developed into commercial crops (Van Wyk *et al*., 2000). Wild fruit trees are important to rural people, especially rural children, because they add important nutrients to the diet and are freely available when other food may be inadequate. In addition, several epidemiological studies have shown an inverse correlation between the consumption of fruits and the incidence of several chronic diseases such as cancer (Block, l992; Block *et al*., 1992), diabetes (Ford and Mokdid, 2001) and heart disease (Rimm *et al*., 1996).

Generally, there is renewed awareness for food safety due to several reports associated with consumption of contaminated food products and their adverse effects on humans (D'Mello, 2003). This has motivated research concerning the risk associated with consumption of plant-based food products. Soil to plant transfer is a major route of contamination, thus, the occurrence of heavy metals in soils (natural and polluted) and plant-based foods have been the focus of a number of studies due to contamination issues (Gebrekidan *et al*., 2013; Nabuloa *et al*., 2010a,b; Wang *et al*., 2003). Heavy metals are among the major contaminants of plant-based food products (Zaidi *et al*., 2005). They are often linked with toxicity associated with environmental pollution due to them being non-biodegradable, having high residence time and their potential to accumulate in different organs of plants (Nabulo *et al*., 2011; Sathawara *et al*., 2004; Singh *et al*., 2010). They can also accumulate in the different organs of humans, thereby leading to undesirable and harmful effects

(Jarup, 2003). Although some metals are required by humans in very small amounts for growth and optimum performance, others such as As, Cd and Pb are toxic even at very low concentrations. These heavy metals are not only cytotoxic, but also carcinogenic and mutagenic in nature (Ivanova *et al*., 2008). Generally, metal toxicity results from alteration in the conformational structure of nucleic acids, protein or by interference with oxidative phosphorylation and osmotic balance (Yaoa *et al*., 2008).

Figs have been shown to be over 11 000 years in existence (Kislev *et al*., 2006). Edible figs have been cultivated in Asia since ancient times and featured predominantly in the diet of animals, humans and other close relatives for many centuries. *Ficus burtt-davyi* Hutch is a fig that is endemic to South Africa and belongs to the mulberry family (Moraceae) (Burrows and Burrows, 2003). It is found in a wide variety of habitats, as a shrub in coastal scrublands, scrambling amongst the surrounding vegetation (Berg, 1990). It may also occur along the beach margins. *F. burtt-davyi* also grows as a hemi-epiphyte on the stems of large forest trees. Additionally, it may grow as a rock-splitter against rock surfaces or cliffs and it is distributed from the Gouritz River in Western Cape Province of South Africa to the Escourt and Piet Retief areas of KwaZulu-Natal (Burrows and Burrows, 2003). Its fruits are reported to be eaten by the Transkeian people of the Eastern Cape Province of South Africa (Burrows and Burrows, 2003; Fox and Young 1983; Rose and Guillarmod, 1974).

Previously, we reported on the nutritional value, bioaccumulation and toxicological assessment of heavy metals in the edible fruits of *Ficus sur* (Ogunlaja *et al*., 2017). Although the fruits of *F. burtt-davyi* are eaten and used as a laxative by the locals in South Africa, information about the nutritive as well as mineral content is lacking, hence the need for this study. In this study, we

examine the edible fruits of *F. burtt-davyi* for proximate nutrient and mineral content. This work would provide necessary baseline information on the edible fruit and also provide the basis for their wider utilisation and application.

5.2 Materials and methods

5.2.1 Sample collection and Preparation

The soil and fruit samples were collected from eight different sampling sites, representing two distinct habitats (coastal and inland forests) in KwaZulu-Natal, South Africa (Figure 5-1), between February and March, 2014. Composite soil samples from six points along the drip line of each tree were collected randomly from eight designated sampling points with a plastic hand shovel at 15-20 cm depth. A thoroughly mixed representative soil sample was taken from each site and was dried overnight in an oven at 40 °C then passed through a 2 mm mesh sieve to remove organic matter and gravel. About 10 g of this soil was crushed with a mortar and pestle to reduce the particle size for microwave digestion. Samples were stored in sealed plastic bags and kept in a refrigerator until analysed.

Samples of tree-ripened fruits were randomly picked from trees, placed in sealed plastic bags and taken to the laboratory for further analysis. The average temperature during the sampling period was 27 °C with no rain or wind but sunshine. All fruit samples were washed thoroughly with double distilled water. Thereafter, fruit samples were oven-dried at 50 °C, overnight. Dried fruit samples were crushed using a food processor (Kenwood Compact Blender, BL380) and the resultant powder samples were stored in a refrigerator in sealed polyethylene bags until analysed.

5.2.2 Reagents and chemicals

All chemicals used were supplied by Merck (Kenilworth, USA) and Sigma (St. Louis, USA) Chemical Companies and were of analytical-reagent grade. Elemental calibration standards were prepared from spectroscopic grade stock standard solutions of 1000 mg L^{-1} (Sigma-Aldrich, Buchs, Switzerland).

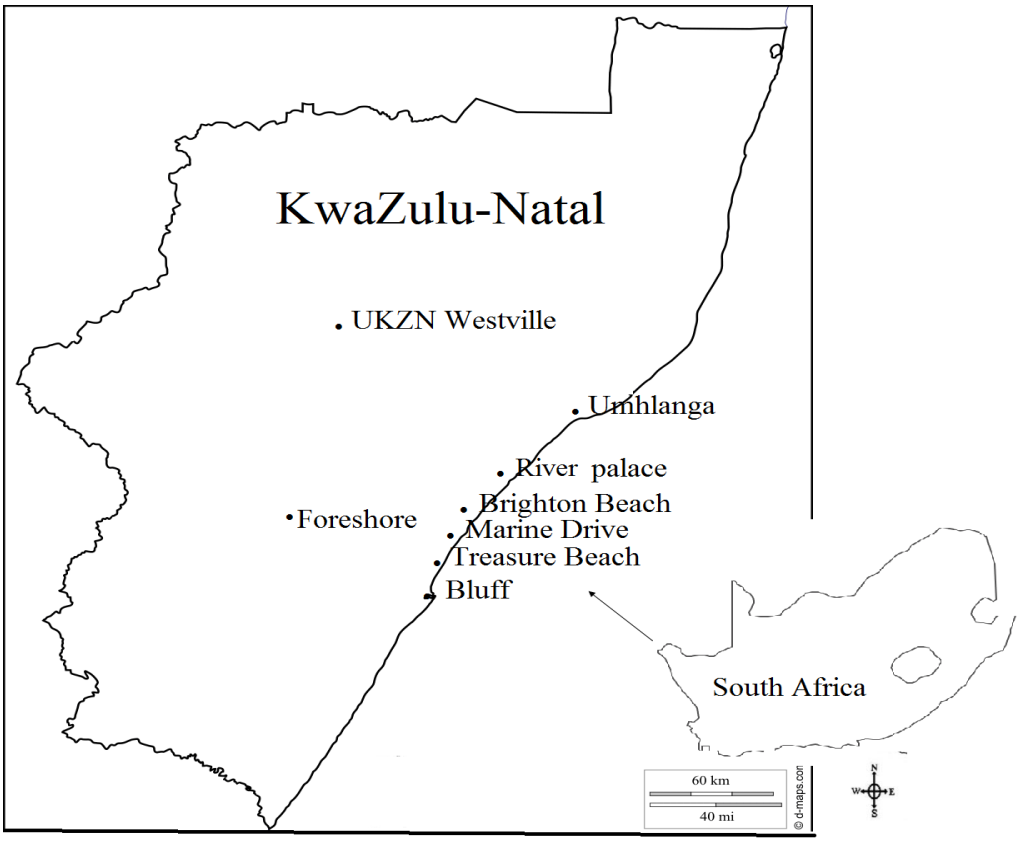

Figure 5-1: Map of selected sampling sites in KwaZulu-Natal, South Africa.

5.2.3 Analytical quality assurance and instruments

All plastic containers were washed with laboratory liquid detergent and then soaked in 1M HNO_3, overnight. Glassware and other equipment were cleaned with 6M HNO_3 and rinsed off with Millipore™ water (Billerica, MA, USA) to minimise the risk of contamination before usage. Millipore™ water was used throughout the experiments. Working standards were made up with Millipore™ water and 10 mL of 70% HNO_3 to match the sample matrix. The blank reagents and certified reference material (CRMs) for plant (BCR-402, Institute for Reference Materials and Measurement, European Commission, Joint Research Centre, Belgium) and soil (D081-540, ERA, A waters Company, Milford, MA, USA) were used to verify the accuracy, precision and efficiency of the analytical method. All digested samples were analysed within a week after digestion.

Table 5-1: Validation of the analytical method using plant (n = 8) and soil (n = 6) certified reference materials (CRM).

	BCR-402				**D081-540**			
Metals	**Measured**	**SD**	**Certified**		**Measured**		**Certified**	
	Mean		**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
As	0.091	0.011	0.093	0.010	100.5	1.20	101	5.92
Cd	-	-	-	-	139.9	1.3	143	5.6
Co	0.175	0.004	0.178	0.008	200.6	6.1	199	4.1
Cr	5.18	0.065	5.19[a]	-	90.3	2.6	86.8	6.1
Fe	245		244[a]	-	12823	20.4	12800	18.0
Mn	-	-	-	-	431.7	9.4	425	9.7
Se	6.72	0.210	6.70	0.25	129.8	2.2	127	4.5
Ni	8.25	0.591	8.25[a]	-	241.2	10.10	236	4.17
Zn	25.3	0.002	25.2[a]	-	128.9	5.6	130	11.5

*Values are in µg g^{-1} dry mass (95% confidence interval).
[a] Indicative values (without uncertainty). CRM - BCR-402 for plant and D081-540 for soil.

The measured mean values were compared with the corresponding certified values (Table 5-1). Experimental results compared well with certified values ($p < 0.05$) with recovery percentages being within acceptable limits.

5.2.4 Metal extractions

5.2.4.1 Exchangeable metals

Exchangeable metals were extracted using the extracting solution prepared by diluting 38.542 g of ammonium acetate ($NH_4CO_2CH_3$), 25 mL of acetic acid (CH_3COOH, 96%) and 37.225 g of ethylenediaminetetraacetic acid (EDTA) to 1L in double distilled water. Exactly 50 mL of extracting solution was added to 5.0 g of dry soil samples in 250 mL polyethylene bottles and shaken in a laboratory shaker for 2 h. Thereafter, solutions were filtered on Whatman No. 1 filter papers and then Millipore 0.45 µm filter membranes to permit analysis of extracted metals. All samples were stored in plastic bottles and kept in a refrigerator until analysed.

5.2.4.2 Total metals

Samples of dried, powdered fruit and soil (0.25 g each) were accurately weighed and digested by applying the optimised procedure as described by Endalamaw and Chandravanshi, (2015) with some modifications. Fruit and soil digests were transferred to 25 mL volumetric flasks, diluted to the mark with Millipore^{TM} water and stored in polyethylene bottles prior to elemental analysis.

5.2.5 Soil pH, Cation Exchange Capacity (CEC) and Soil Organic Matter (SOM)

The soil pH was determined in a soil: water suspension of 1 g in 2.5 mL of deionised water using a pH meter (Aqualytica, Model pH 17). Soil organic matter (SOM) was measured using the potassium dichromate wet oxidation by titration method as described by Walkley and Black, (1934) and was expressed as percent carbon. The cation exchange capacity (CEC) of soil was determined using the pH 7.0 ammonium acetate method (Chapman, 1965).

5.2.6 Determination of chemical composition

Fruit samples were analysed for proximate chemical composition (moisture, crude fat, fibre and protein) according to the Association of Official Analytical Chemists methods (AOAC, 2000). The carbohydrate content was obtained by difference.

5.2.7. Analytical procedure

Standard solutions of 13 elements (As, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, Co, and Zn) were prepared by diluting their corresponding 1000 mg L^{-1} stock standard solutions. Elemental analysis was by inductively coupled plasma-optical emission spectrometry (ICP-OES) due to its high dynamic linear range and sensitivity. Analytical wavelengths were selected based on minimum spectral interferences and maximum analytical performance.

5.2.8 Data analysis

5.2.8.1 Bioaccumulation factor (BAF)

Generally, elements are persistent in the environment and tend to accumulate in plant tissues. In this study elemental bioaccumulation was evaluated by comparing their concentration in the fruit against that in the growth soil.

$$BAF = [Fruit]/ [Soil]_{Exchangeable}$$

5.2.8.2 Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences, (PASW version 23, IBM Corporation, Cornell, NY, USA).

5.3 Results and Discussion

5.3.1 Elemental analysis

The fruit and the growth soil samples were analysed for thirteen elements comprising of macro-elements (Ca and Mg), micro-elements (Cu, Co, Fe, Mn, Ni, Cr, Se and Zn), and toxic elements (As, Cd, and Pb). Although, total metal concentration in the soil is a valuable indicator of soil contamination, the risk from metals to a large extent is governed by their bioavailability (Prokop *et al.*, 2003; Tokalioglu *et al.*, 2000; Van Gestel, 2008; Xiao *et al.*, 2011). The heavy metal concentration in soil (total and exchangeable) and fruit samples of *F. burtt-davyi* from eight different sites in KwaZulu-Natal are summarised in Table 5-2 for those elements in fruits that were above the instrument detection limits. The exchangeable percent (% Ex) was also calculated to

measure the percent of elements from the soil that are available for plant uptake. Arsenic, Cd, and Pd in soil and in fruit were below the detection limit of the instrument therefore they were excluded from the discussion.

Plants, through various physiological processes are able to regulate metals through storage and detoxification, in order to adapt to varied metal concentration conditions (Ciszewski *et al*., 2013; Fairbrother *et al*., 2007; Peto, 2010). Calcium in soil (total and exchangeable) ranged from 496 to 8405 μg g^{-1} and 245 to 4399 μg g^{-1}, respectively with highest concentrations of 14530 μg g^{-1} in the fruit observed at site S8 where the exchangeable Ca was 490 μg g^{-1} (BAF = 29.2). Calcium in fruits is believed to delay ripening and can contribute about 2% towards body total weight of many fruits (Valvi and Rathod, 2011). Likewise, there was accumulation of Mg at all sites with Mg in soil (total and exchangeable) and fruits ranging from 214 to 1035 μg g^{-1}, 199 to 340 μg g^{-1} and 2461 to 3728 μg g^{-1}, respectively. For Ca and Mg, soil concentrations were lower than fruit concentrations, indicating the ability of the fruits to bioconcentrate and bioaccumulate these metals.

Table 5-2: Elemental concentrations (μg g^{-1}) in fruit and soil (Total (T) and exchangeable (E) samples (Mean (standard deviation); 95% confidence interval, n = 3) and bioaccumulation factors (BAFs).

Site	Element	Fruit	Soil(T)	Soil (E)	[Fruit]/[Soil]T	[Fruit]/[Soil]E	Ex%
1	Ca	13540 (380)	4928 (1429)	3388 (2433)	2.7	4.0	68.7
2		9363 (462)	5549 (671)	4399 (116)	1.7	2.1	79.3
3		7921 (159)	975 (102)	245 (12)	8.1	32.3	25.1
4		12713 (840)	724 (464)	7171 (9)	17.6	17.7	99.0
5		8738 (145)	8405 (1756)	2115 (421)	1.0	4.1	25.2
6		13807 (862)	865 (80)	799 (78)	16.0	17.3	92.3
7		8649 (237)	863 (83)	409 (23)	10.0	21.2	47.4
8		14530 (750)	496 (322)	490 (27)	29.3	29.7	98.6
1	Cu	11.3 (4.50)	13.3 (1.98)	8.89 (0.92)	0.8	1.3	66.7
2		9.83 (5.83)	13.1 (8.05)	11.9 (1.10)	0.7	0.8	90.4
3		11.5 (2.04)	12.0 (0.90)	9.00 (0.97)	1.0	1.3	75.2
4		13.2 (1.33)	6.06 (1.55)	5.98 (0.99)	2.2	2.2	98.7
5		13.7 (2.58)	25.7 (5.37)	13.0 (1.22)	0.5	1.1	50.6
6		14.4 (0.59)	6.09 (0.97)	5.77 (0.78)	2.4	2.5	94.7
7		11.9 (5.55)	5.38 (1.41)	5.12 (1.02)	2.2	2.3	95.2
8		12.7 (1.29)	4.60 (3.77)	4.14 (0.65)	2.8	3.1	90.1
1	Fe	34.8 (7.04)	7254 (1151)	379 (11)	0	0.1	5.2
2		63.2 (5.23)	7679 (1558)	590 (41)	0	0.1	7.7
3		78.6 (33)	7556 (484)	476 (24)	0	0.2	6.3
4		15.9 (1.50)	7804 (3602)	246 3(4)	0	0.1	3.1
5		50.3 (2.88)	5908 (1589)	429 (17)	0	0.1	7.3
6		19.0 (8.37)	11680 (421)	308 (94)	0	0.1	2.6
7		18.0 (7.70)	9438 (207)	246 (28)	0	0.1	2.6
8		103 (14)	5075 (549)	395 (29)	0	0.3	7.8
1	Mg	3014 (62)	779 (191)	376 (35)	3.9	8.0	48.3
2		2673 (75)	681 (109)	265 (21)	3.9	10.1	39.0
3		2461 (123)	483 (75)	289 (41)	5.1	8.5	59.9
4		2993 (120)	296 (130)	205 (19)	10.1	14.6	69.3
5		3328 (86)	1035 (255)	399 (10)	3.2	8.3	38.5
6		3096 (313)	411 (16)	306 (21)	7.5	10.1	74.3
7		3728 (158)	572 (36)	400 (27)	6.5	9.3	70.0

8		3644 (111)	215 (47)	199 (51)	17.0	18.3	92.6
1	Mn	24.9 (1.10)	147 (19)	127 (31)	0.2	0.2	86.7
2		20.4 (1.73)	140 (30)	129	0.1	0.2	92.1
3		43.6 (2.86)	173 (7.84)	140	0.3	0.3	81.0
4		53.8 (3.21)	85.1 (44)	59.9	0.6	0.9	70.4
5		9.51 (0.20)	137 (37)	121	0.1	0.1	88.5
6		54.8 (1.92)	123 (5.77)	62.9	0.4	0.9	51.2
7		28.9 (1.36)	246 (50)	125	0.1	0.2	50.8
8		46.0 (2.55)	37.4 (3.82)	37.0	1.2	1.2	98.8
1	Zn	49.0 (3.0)	34.2 (11)	30.0	1.4	1.6	87.6
2		27.8 (7.41)	41.8 (4.97)	20.8	0.7	1.3	49.6
3		27.9 (2.80)	24.0 (2.11)	9.89	1.2	2.8	41.2
4		25.3 (1.32)	18.5 (13)	10.0	1.4	2.5	54.2
5		42.3 (2.54)	79.2 (23)	20.0	0.5	2.1	25.2
6		25.9 (1.48)	20.2 (1.84)	19.0	1.3	1.4	93.9
7		25.4 (0.68)	27.4 (5.74)	15.3	0.9	1.7	55.7
8		28.4 (2.41)	45.0 (11)	21.6	0.6	1.3	48.0

* $[F]/[S]_{T}$-$[Fruit]/[Soil]_{Total}$ ** $[F]/[S]_{A}$-$[Fruit]/[Soil]_{Exchangeable}$ *** Ex% - $[Soil]_{Exchangeable}/[Soil]_{Total}$

Iron is an essential element to humans and a very important constituent of haemoglobin. It promotes the breakdown (*via* oxidation) of carbohydrates, protein and fat in order to control body weight which is a very important risk factor in diabetes. Table 5-2 revealed that the maximum concentration of Fe in the fruit of *F. burtt-davyi* was 103 ± 14 µg g^{-1} which was observed at site 8. The BAFs (exchangeable) of the fruit for Fe were relatively low (< 0.3 at all sites), suggesting that Fe uptake is controlled by the fruit. This observation is similar to our previous report on the fruits of *F. sur* (Ogunlaja *et al*., 2017). Overall, about 5.3% of total soil Fe was in exchangeable form. About 77.4% of total soil Mn was in exchangeable form but the BAFs (exchangeable) ranged from 0.1 to1.2 with the highest occurring only at one site (S8). This may be due to the lowering of the rate of Mn uptake by plant by Mg (Kies, 1994; Maas *et al*., 1969). The fruit tends to regulate the

uptake of Mn based on metabolic requirements. The concentration of Mn in fruits ranged from 9.51 to 54.8 µg g^{-1} which are below the maximum limits of 2000 µg g^{-1} (Kabata-Pendias and Pendias, 1989).

Total soil Cu ranged from 4.60 to 25.7 µg g^{-1} while the exchangeable concentration ranged from 4.14 to 13.0 µg g^{-1} with an average of 82.7% in exchangeable form. The high exchangeable value for Cu may be due to the high stability of the Cu complex formed with EDTA (Madrid *et al*., 2008). Copper concentrations in the fruits were all above the WHO permissible limit of 10 µg g^{-1} (WHO, 2005) for plants. Elevated concentrations of Cu are known to cause anaemia (*via* Mn depletion, which leads to iron deficiency anemia), liver and kidney damage, stomach and intestinal irritation in humans (Raymond and Felix, 2011).

Generally, Zn concentration in fruits (25.3 to 49.0 µg g^{-1}) was higher than total and exchangeable soil concentrations. Although, about 56.9% of Zn was available for uptake by the fruit, the BAF was less than 1 for the studied sites. The concentration of Zn in the fruits at sites S1 (49.0 µg g^{-1}) and S5 (42.3 µg g^{-1}) were above the maximum levels for plants set by the Department of Health, South Africa, which is 40 µg g^{-1} (DoH, 2004).

Although the total concentration of the trace essential elements (Co, Cr, and Se) in the soil ranged from 1.12 to 4.77 µg g^{-1}, 1.8 to 50.2 µg g^{-1} and less than 16.5 µg g^{-1}, respectively, the concentration in the fruits were below the instrument detection limits, hence they were excluded from Table 5-2. Ni concentrations in the fruits were also found to be below the instrument detection limits. Total soil Ni was also below 0.56 µg g^{-1}. This result showed that *F. burtt-davyi* fruits tend to exclude these metals.

Data from this study showed that the elemental concentration in the fruits of *F. burtt-davyi* was the highest for Ca, followed by Mg, Fe, Mn, Zn and Cu. In addition, the concentrations of trace essential elements (Co, Cr, Ni and Se) and toxic metals (As, Cd and Pb) were found to be below the instrument's detection limits.

5.3.2 Estimated contribution of metals in fruits to the diet

Indigenes of South Africa often rely on fruits among other natural resources to meet their basic nutritional needs, but often, they are faced with dietary and nutrient deficiency diseases. It is therefore paramount to determine the levels of micronutrients in such indigenous fruits and estimate their contribution to the diet. In this study, the elemental concentration in the fruits of *F. burtt-davyi* were compared to Dietary Reference Intakes (DRIs) (Table 5-3). The results show that the fruit can contribute significantly to the health and nutrition of most individuals for most elements.

Calcium contributes between 17-22% towards the RDA for the element. Generally, malnourished children are often faced with dietary diseases due to deficiencies in Zn, Fe and Cu (Maharaj *et al*., 2003). Zinc is an essential trace element to humans and it is required for a healthy immune system; its deficiency will result in recurrent infections due to an under-performing immune system (Moscow and Jothivenkatachalam, 2012; Prasad, 1982). Iron and Cu are essential to human health and their deficiency normally occur simultaneously leading to anemia, glucose intolerance, likelihood of infections and nervous system problems (Davis *et al*., 1987; Labadarios, 2007). Consumption of about 20.0 g of fruit contributes 5.7-7.9% towards the RDA for Zn, 5.3-12.0% for Fe and 27.8% for Cu in most individuals (Table 5-3).

Table 5-3: Comparison of Dietary Reference Intake (DRI). * (Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Level (UL)) of elements for most individuals to the average concentration of elements (n = 8) in *F. burtt-davyi* fruits.

Element	Average concentration (mg / 20 g DM)	DRI (mg/ day)		Estimated contribution to RDA (%)
		RDA	UL	
Ca	223.15	1000-1300	2500	17.2-22.3
Cu	0.25	0.9	8	27.8
Fe	0.96	8-18	45	5.3-12.0
Mg	62.34	310-320	350	19.5-20.1
Mn	0.70	1.6-2.3	9	30.4-43.8
Zn	0.63	8-11	34	5.7-7.9

* Sourced from: Food and Nutrition Board, Institute of Medicine, National Academies, 2011.

Manganese rich, plant-based foods include pineapple, spinach, peanuts, sweet potato, brown rice and pecan nuts (USDA, 2009). Manganese is a component of the powerful antioxidant manganese super oxide dismutase (MnSOD) enzyme, which neutralises free radicals in the human body (Ademuyiwa *et al*., 2007; Mistry and Williams, 2011; Paynter, 1980). A diet rich in Mn may prevent cancer and other devastating diseases like arthritis, osteoporosis, diabetes and epilepsy (Ekmekcioglu *et al*., 2001; Osterode *et al*., 1996). Data from this study showed that *F. burtt davyi* fruits can serve as an alternative and cheap source of Mn, contributing 30.4-43.8% towards RDA if 20.0 g is consumed. A deficiency of Mn can lead to iron-deficiency anaemia, due to the role of Mn in Fe utilisation (Balch and Balch, 1990). Other deficiency symptoms include nausea, vomiting, impaired glucose tolerance, high cholesterol levels, skin rash, loss of hair colour, dizziness, hearing loss, and impaired reproductive function (Balch and Balch, 1990; Friedman *et*

al., 1987; Ulene, 2000). Extreme lack of Mn in infants may result in convulsions, paralysis, blindness and deafness (Lupines, 2001; Ulene, 2000).

5.3.3 Proximate chemical composition

The moisture content of the fresh fruit of *F. burtt-davyi* was 23.7%. On a dry weight basis, the protein content was 5.0 ± 0.30%, lipid 8.4 ± 0.40%, carbohydrates 78.9 ± 0.55%, crude fibre 4.0 ± 0.70% and ash 3.7 ± 0.10%. The results show the fruits to be high in energy and low in fats.

5.3.4 Soil quality parameters analysis

Soil pH ranged from 6.5 to 7.4 while SOM ranged from 3.4 to 8.2%. The CEC also ranged from 2.2 to 18.8 meq/100.

5.3.5 Statistical analysis

Principal component analysis (PCA) permitted a reduction of 12 variables (elements in soil) to four principal components (PC1, PC2, PC3 and PC4), which were extracted, based on the eigenvalue > 1. The retained principal components were interpreted using varimax rotation with Kaiser Normalisation. These four PCs described over 80% of the total variability which was enough to describe the overall elemental pattern, signifying different sources (Table 5-4).

Table 5-4: Rotated component matrix for variables in the soil samples (n = 40).

	Component			
Element	1	2	3	4
CoT	**0.97**	-0.03	0.05	0.12
CdT	**0.92**	-0.08	0.09	0.29
CrT	**0.91**	0.02	0.11	-0.26
FeT	**0.90**	-0.16	-0.04	0.32
CaT	-0.08	**0.90**	0.35	0.05
CuT	-0.10	**0.90**	-0.04	0.03
MgT	0.11	**0.87**	0.27	0.37
ZnT	-0.14	**0.74**	0.07	-0.06
SeT	0.28	0.55	-0.54	-0.41
AsT	-0.03	0.32	**0.87**	0.01
NiT	0.28	0.11	**0.84**	0.01
MnT	0.31	0.17	0.03	**0.90**
Eigenvalues	3.95	3.69	1.73	1.12
% Total variance	32.93	30.76	14.42	9.31
Cumulative %	32.93	63.69	78.12	87.43

Extraction Method: Principal Component Analysis
Rotation Method: Varimax with Kaiser Normalisation. Bold figures indicate values > 0.7

The first component explained 32.9% of the total variance; high loadings (> 0.7) in PC1 were obtained for Cd, Co, Cr and Fe (Table 5-4). The high positive loadings and close association of heavy metals Co, Cd, Cr and Fe could suggest their common anthropogenic sources (vehicular emissions). Elevated levels of these trace metals have been reported in areas with high traffic density in South Africa (Olowoyo *et al*., 2012). PC2 was strongly represented by Ca, Cu, Mg and Zn, contributing 30.8% of the total variance. A quasi-independent behaviour was also observed within the group due to Se having a loading of 0.55, which was further corroborated by a large

distance in the 3-D PCA loading plot (Figure 5-2), indicative of poor correlation and different sources (Table 5-4). PC3 contributed 14.4% to the total variance with a high loading on As and Ni, suggesting a common origin, while PC4 was dominated by Mn (0.90), accounting for 9.3% of the total variance. This was confirmed by Figure 5-2, suggesting that it came from a different source. Similar occurrence was also reported by Mahlangeni *et al*. (2016).

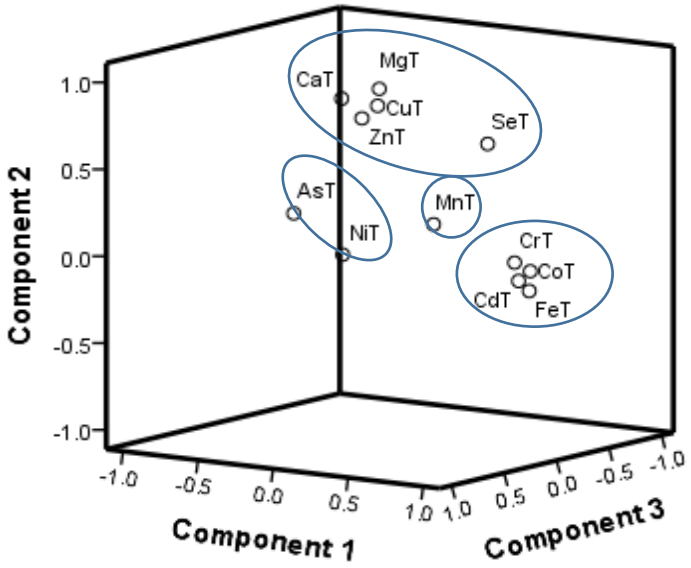

Figure 5-2: Factor loading plot (showing the four distinct principal components and their significant relationship) for 12 element in soil (constructed for 8 sampling sites).

Hierarchical cluster analysis (CA) measuring distribution patterns, based on the Euclidean distance between mean elemental concentrations of studied elements, was evaluated in *F. burtt-davyi* fruits. Figure 5-3 shows the CA results for the elemental distribution pattern as a dendrogram in the studied area. Generally, the CA revealed varied elemental distribution in the two distinct habitats (coastal and inland). Four clusters are observed (1) S3–S4-S7; (2) S6; (3) S8; (4) S1–S2–S5. However, clusters 1 and 3 are joined together at a relatively higher level implying perhaps similarity in elemental distribution. The proximity in the dendrogram between S1 and S2, and S3

and S4 show similarity in elemental distribution at the studied sites. Although, S6 and S8 were joined at a relatively higher distance, their proximity in the dendrogram show similarity in elemental distribution at sites representing the inland habitat. Hierarchical cluster analysis revealed similar distribution of elements between the inland habitats which was dissimilar to that of coastal habitat (Figure 5-3).

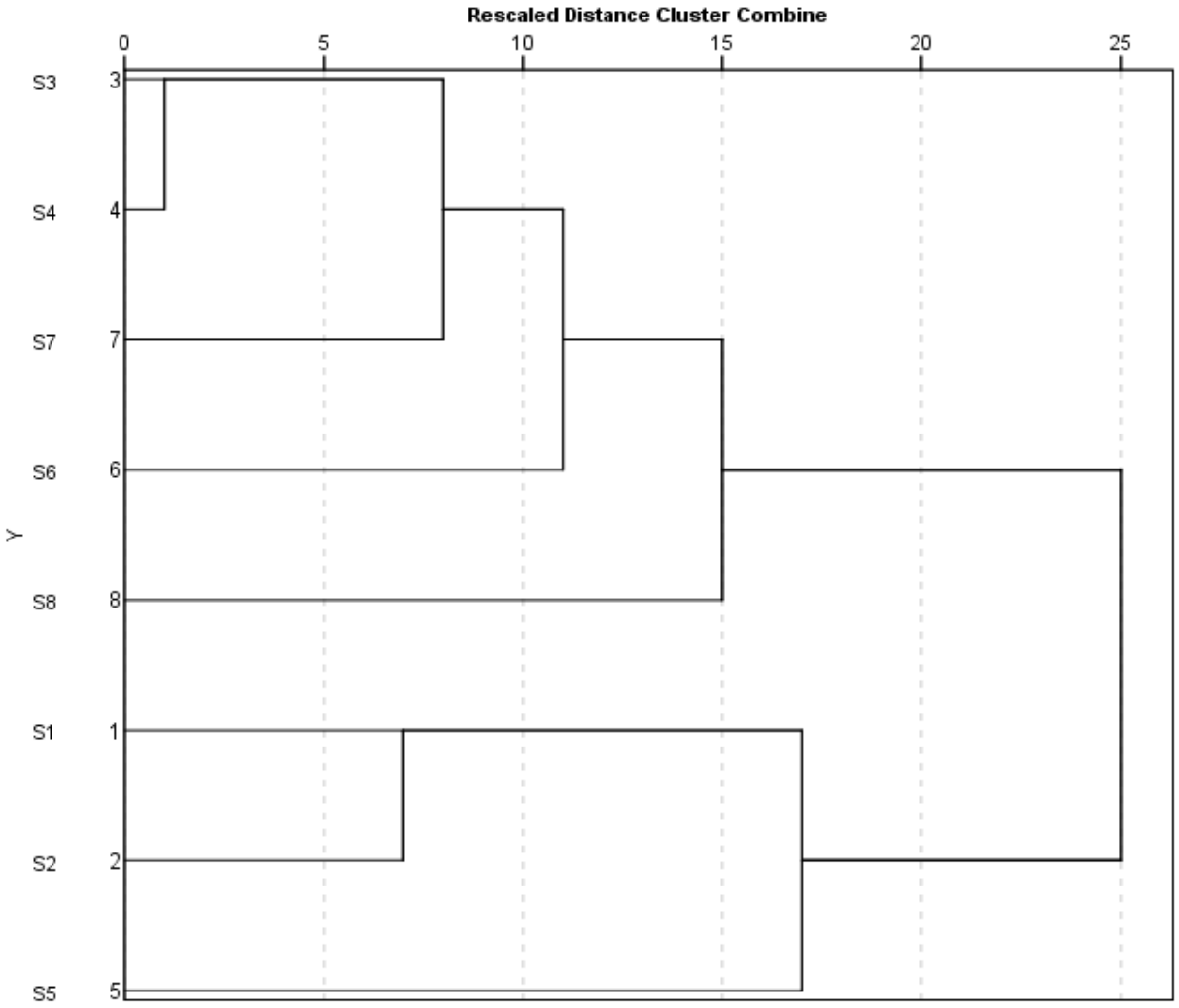

Figure 5-3: Hierarchical cluster analysis dendrogram showing the relationship between the studied sites using Ward`s method by Euclidean distance.

Sites - (S1-Bluff, S2-Treasure Beach, S3-Marine Drive. S4-Umhlanga, S5-Brighton Beach, S6-UKZN, Westville, S7-River palace and S8-Foreshore.)

Table 5-5 summarised the significant correlations between elements in the soil and fruits of *F. burtt-davyi* that have been extracted from an inter-item correlation matrix. Only the strong correlations are presented. There was a three-way synergy between Ca, Cu and Mg in the soil, indicating that these elements have a common origin as observed by PCA (Figure 5-2). There was a significantly positive correlation between total soil Ca with exchangeable Cu (r = 0.9) and total soil Mg with exchangeable Mn (r = 0.9) indicating a synergistic effect between these metals in soil. Although there was a significant correlation between exchangeable Fe with Fe in the fruit (r = 0.7), thereby indicating that uptake of Fe was dependent on soil concentrations, there was no significant correlation between soil concentrations (total and exchangeable) and fruit concentrations for the other metals studied, thereby indicating that uptake was regulated by the plant to meet physiological needs.

Table 5-5: Inter-item correlation matrix for concentrations of elements in Fruits (F) and soil (S)

	CaF	CaE	CaT	CuE	CuT	FeF	MgE	MgT	MnF
CaT	ns	**0.8****	1						
CuE	ns	**0.7***	**0.9****	1					
CuT	ns	ns	**0.8****	**0.8****	1				
FeE	ns	ns	**0.7***	**0.7***	ns	**0.7***			
MgT	ns	**0.7***	**0.9****	**0.9****	**0.8****	ns	ns	1	
MnF	ns	ns	**-0.9****	**-0.7***	**-0.7***	ns	**-0.7***	**-0.8****	1
MnE	**-0.8****	ns	ns	**0.7***	ns	ns	**0.7***	**0.9****	**-0.7***

*, ** - significant at $p \leq 0.05$ and $p \leq 0.01$, respectively. a. XF–$[X]_{Fruit}$ where X = the various elements. b. XT–$[X]_{Soil\ Total}$ where X = the various elements. c. XE–$[X]_{SoilExchangeable}$ where X = the various elements.

5.4. Conclusion

Data from this study showed accumulation of Ca and Mg in comparison to other essential metals, indicating that the plant required more of these elements to meet its metabolic needs. The concentration of toxic metals (As, Cd and Pb) in the fruits were below the instrument detection limits, which showed that it is safe for human consumption. The fruits were also found to be rich in Mn which may be beneficial in maintaining a healthy immune system, especially in the poor and vulnerable communities of South Africa that rely on these figs for nutrition. Hierarchical cluster analysis showed variation in elemental distribution among dissimilar sites.

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CHAPTER 6

Antioxidant activity of the bioactive principles from the edible fruits and leaves of *Ficus sur* Forssk. (Moraceae)

Abstract

Ficus sur Forssk. (Moraceae) is a medicinal plant species found in Africa. It is used to treat anaemia, diarrhea and sexually transmitted diseases. In this study, a phytochemical investigation of *F. sur* was conducted and the antioxidant properties of the isolates and extracts were evaluated using the ferric ion (Fe^{3+}) reducing antioxidant power (FRAP) and 1,1- diphenyl-2-picrylhydrazyl (DPPH) assays. Two pharmacologically active triterpenoids (lupeol and β-sitosterol), one pheaophytin (pheaophytin **a**) and one flavonoid (epicatechin) were isolated from the fruits and leaves. The structures of the isolated compounds were characterized by spectroscopic techniques and by comparison of the spectroscopic data with the literature values. The findings reveal significantly higher ($p < 0.05$) antioxidant activity for the methanol extract of the fruits (IC_{50} 9.06 μg mL^{-1}) which may be accounted for by the higher phenolic content and presence of epicatechin. The results show the species to be rich in pharmacologically active compounds that are documented to exhibit therapeutic and chemo-preventive beneficial effects. Therefore, their consumption would have a profound influence on nutrition and health, especially among the indigenous people of Africa.

Keywords: Flavonoid, Triterpenes, Epicatechin, Figs, Antioxidants

6.1 Introduction

Free radical and reactive oxygen species (ROS) from normal metabolic processes and from environmental pollution may cause protein and DNA damage within cells via chemical chain reactions, leading to oxidative stress (Aqil *et al.*, 2006; Stohs and Bagchi, 1995; Tiwari 2001). Oxidative stress is a major risk factor leading to a variety of chronic and degenerative disorders such as cardiovascular and neurodegenerative diseases, aging and cancer (Kao *et al*., 2013; Kiem *et al*., 2011; Pham-Huy *et al.,* 2008; Willcox *et al.,* 2004). Plant based antioxidants are well known for their anticancer, anti-inflammatory and anti-aging properties (Matés *et al*., 1999; Mayne 2003; Noguchi and Niki, 2000; Pinnell 2003). These activities are largely attributed to the presence of compounds such as flavonoids, tannins, steroids, glycosides, coumarins and pentacyclic triterpenes (Ragasa *et al*., 2009; Sirisha *et al*., 2010).

The Southern African region of the world contains a vast source of natural products, with South Africa accounting for over 22 000 plant species, representing about 10% of the world's botanical richness (Coetzee *et al*., 1999; Williams *et al*., 2006). The genus *Ficus (*Moraceae) has more than 850 species growing all over the world, with 25 of the 36 species being indigenous to Southern Africa (Namibia, Botswana, Zimbabwe, Mozambique south of the Zambezi River and South Africa) (Burrows and Burrows, 2003).

Some *Ficus* species that have received extensive phytochemical and pharmacological investigation include *F. carica* (Gilani *et al*., 2008; Rubnov *et al*., 2001), *F. racemosa* (Chandrashekhar *et al*., 2008; Li *et al*., 2004), *F. religiosa* (Pandit *et al.*, 2013; Samy *et al*., 2008), *F. microcarpa* (Ao *et al*., 2008;Chang *et al*., 2005), *F. exasperata* (Ayinde *et al*., 2007; Odunbaku *et al*., 2008), *F. glomerata* (Patil *et al* ., 2006*;* Rahman *et al*., 1994), *F. benghalensis* (Shukla *et al*., 2004) and *F.*

benjamina (Farag, 2005). Although a wide variety of compounds including phenolics, flavonoids, alkaloids, coumarins and sterols have reportedly been isolated from the genus *Ficus*, members of this genus are particularly known for their high content of triterpenoids (Chang *et al*., 2005; Chiang and Kuo, 2002; Lansky and Paavilainen, 2011; Lee *et al.*, 2002).

Ficus sur is commonly referred to as the Cape fig, broom cluster fig, bush fig or Malabar tree. It is a large spreading tree, usually about 12 m high, but reaching 25 to 30 m in some areas. The fruits of the plant are edible. The root and bark decoctions from the plant are used in traditional medicine to treat a variety of ailments including pulmonary tuberculosis, influenza and skin diseases (Eldeen *et al*., 2005; Hutchings *et al*., 1996). Previously, we reported on the antioxidant activity of *Ficus burtt-davyi* Hutch and the cytotoxicity of the bioactive principles from *F. burtt-davyi* (Ogunlaja *et al*., 2016). In this study, we report on the isolation and identification of the bioactive principles from the fruits and leaves of *F. sur*. Additionally, we report on the antioxidant activity of selected crude extracts and isolated compounds from this plant species.

6.2 Experimental

6.2.1 General experimental procedures

The ^{1}H, ^{13}C and 2D-NMR spectra were recorded using a 400 MHz spectrometer (Avance III Bruker, Rheinstetten, Germany) at 400.22 MHz for ^{1}H and 100.63 MHz for ^{13}C. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The ^{1}H and ^{13}C chemical shifts of the deuterated chloroform ($CDCl_3$) were 7.24 and 77.0, respectively, referenced to the internal standard, tetramethylsilane (TMS). Infrared (IR) spectra were recorded using a Perkin-Elmer Universal ATR spectrometer. UV spectra were obtained on a Hewlett Packard UV-3600

spectrophotometer. Column chromatography was performed with Merck silica gel 60 (0.040 - 0.063 mm). Thin layer chromatography (TLC) was performed on Merck 20 x 20 cm silica gel 60, F254 aluminum sheets. The spots were analyzed under UV (254 nm and 366 nm), visualized using 10% H_2SO_4 in MeOH followed by heating. Solvents (analytical grade) and other chemicals used were supplied by either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA) chemical companies.

6.2.2 Plant material

The plant was collected in August 2015 from the University of KwaZulu-Natal (UKZN), Westville campus, South Africa. The identity was confirmed by examining material in the WARD herbarium, School of Life Sciences, University of KwaZulu-Natal, where a species is lodged (Ogunlaja, 2).

6.2.3 Extraction and isolation

Dried, powdered fruits (800 g) and leaves (950 g) were subjected to sequential extraction with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) by continuous shaking on an orbital shaker for 48 h at room temperature for each solvent. All extracts were concentrated by evaporation under vacuum at controlled temperatures and stored in a refrigerator at 4 ^{o}C until analysed.

The crude DCM extract of fruits (9.19 g) was subjected to column chromatography using 100% n-hexane that was stepwise increased by 10% to 100% EtOAc at a flow rate of approximately 50 mL/min, collecting eight 100 mL fractions for each eluent step. Fractions 20-23 were combined

and further purification with hexane: EtOAc (1:1) to afford compound **A-1 (**150.5 mg**).** Compound **A-6** was eluted with hexane: EtOAc (8:2), and re-crystallized in MeOH to give a white powder.

The MeOH extract of fruits (13.7 g) was subjected to partitioning with an equal volume of EtOAc and DCM. The EtOAc fraction was dried with anhydrous Na_2SO_4, and the resultant concentrated extract subjected to column chromatography with fractions 9-12 yielding yellow crystals of compound **B-1** (41.0 mg)**.**

The Hex extract of leaves (14.07 g) was separated similar to the DCM extract of fruits and yielded compound **A-1** (105 mg) with hexane: EtOAc (8:2). Similarly, the crude EtOAc extract of leaves (10.44 g) was subjected to column chromatography with a hexane: EtOAc step gradient (with 10% increments every 100 mL). Compound **A-4** (41.87 mg) was eluted with hexane: EtOAc (8:2) as a dark green amorphous solid. The other extracts from the fruits and leaves did not yield compounds that could be elucidated.

6.2.4 Determination of total phenolic content (TPC)

The total polyphenol content (TPC) of *F. sur* extracts (fruits and leaves) was determined as gallic acid equivalent (GAE) according to the method as described by McDonald *et al*. (2001) with slight modification. Briefly, 200 µL of the extract (240 µg mL^{-1}) was incubated with 1 mL of ten-fold diluted Folin Ciocalteau reagent and 800 µL of 0.7 M Na_2CO_3 for 30 min at room temperature. Absorbance values were determined at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

6.2.5 Determination of total flavonoid content

The total flavonoid content of the *F. sur* extracts (fruits and leaves) was determined according to the procedure as described by Chang *et al*. (2002) with some modification. Briefly, 500 µL (240 µg mL^{-1}) of each extract was mixed with 500 µL of MeOH, 50 µL of 10% $AlCl_3$, 50 µL of 1 mol/L potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was measured at 415 nm on a Shimadzu UV mini 1240 spectrophotometer. The total flavonoid content was calculated as quercetin equivalent (QE) in µg per mg dry extract.

6.2.6 Ferric (Fe^{2+}) Reducing Antioxidant Power (FRAP) assay

The total reducing power of the MeOH extracts (fruits and leaves) and isolated compounds from *F. sur* was determined according to the Ferric Reducing Antioxidant Power (FRAP) method as described by Behera *et al*. (2006) with some modifications. Various concentrations (7.5-500 µg mL^{-1}) in DCM or MeOH were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 0.1% potassium ferricyanide and the mixture was incubated at 50 ^{o}C for 30 min. After the addition of 2.5 mL of 10% TCA, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. In this assay, the Fe^{3+}/ferricyanide complex is reduce to the ferrous form (Fe^{2+}), and the test solution colour changes from yellow to pale green or blue, depending on the reducing power of the antioxidant. MeOH without reagents was used as a negative control while ascorbic acid and butylated hydroxyanisole (E320) (BHA) with the same concentrations were used as positive controls. All procedures were performed in triplicate.

6.2.7 DPPH radical scavenging activity assay

The antioxidant activity of the extracts (fruits and leaves) and isolated compounds was measured in terms of radical scavenging ability, using the DPPH method as describe by Ahmad *et al*. (2011) with some modifications. Various concentrations (7.5-500 µg mL^{-1}) of extracts and isolated compounds (150 µL) made from stock solutions (10 µg mL^{-1}) were mixed with 2850 µL MeOH solution containing DPPH radicals. The mixture was then vortexed, and incubated for 30 min at room temperature. Thereafter, the absorbance was measured at 517 nm against MeOH as a blank using a UV-Vis spectrophotometer. The scavenging activity was evidenced by a change in colour from purple to yellow, due to proton transfer to the $DPPH^{\bullet}$ free radical by a scavenger which was further measured by the decrease in absorbance at 517 nm using a Shimadzu UV–Vis spectrophotometer. Ascorbic acid and BHA were used as standards and the procedure was done in triplicate. The difference in absorbance between the test sample and the negative control (DPPH + MeOH) was expressed as percentage inhibition. The percentage free radical scavenging activity was calculated according to the following equation:

% scavenging activity = % inhibition = $[(A_o - A_{sample} / A_o) \times 100]$

Where A_o = Absorbance of the negative control and A_{sample} = Absorbance of sample.

6.2.8 Statistical analyses

The experimental results were expressed as mean ± standard deviation (SD) of three replicates and IC_{50} values were calculated by linear regression. The data were subjected to one-way analysis of variance (ANOVA) to determine significant differences between means ($p < 0.05$). Tukey`s test was used for post-hoc analyses. All the statistical tests were performed using graphpad prism 6.0.

6.3 Results and discussion

6.3.1 Structure elucidation of compounds from *F. sur*

The DCM extract from the fruits of *F. sur* afforded two compounds **A-1** and **A-6** (Figure 6-1) which were identified as β-sitosterol and lupeol, respectively (Chaturvedula and Prakash, 2012; Mahato and Kundu, 1994). β-sitosterol was also isolated from the leaves. Previously, these triterpenes were isolated from the edible fruits of *Harpephyllum caffrum* (Moodley *et al*., 2014).

The MeOH extract of the fruits yielded compound **B-1**. The ^{1}H-NMR spectrum for compound **B-1** showed characteristic resonances for flavonoids at δ_H 6.88 (H-2', d, J=1.60 Hz), δ_H 6.65 (H-5', d, J=8.2 Hz) and δH 6.64 (H-6', dd, J=1.60, 8.2 Hz) from the B-ring catechol moiety as well as at δ_H 5.88 (H-6, d, J=2.24 Hz) and δ_H 5.70 (H-8, J=2.24 Hz) from the meta-coupled protons of the A-ring resorcinol moiety. The isomers catechin and epicatechin may be differentiated by the chemical shift of C-2 in the ^{13}C-NMR spectrum which is approximately δ_C 78.0 for epicatechin and δ_C 82.2 for catechin, and by correlations between H-2 and H-3 in the COSY experiment which is strong for catechin and weak for epicatechin because of the difference in the dihedral angle (Es-Safi *et al*., 2006). Based on the resonance for C-2 at δ_C 78.0, a weak H-2/H-3 correlation in the COSY experiment, ^{1}H-NMR, ^{13}C-NMR, and data in literature (Kiem *et al*., 2011; Ragab *et al*., 2013) compound **B-1** was identified as epicatechin. This was further confirmed by GC-MS data, IR and UV-Vis spectroscopy. Epicatechin has previously been isolated from other *Ficus* species (Awolola *et al*., 2014; Kiem *et al*., 2011; Ragab *et al*., 2013).

The EtOAc extract from the leaves afforded compound **A-4**, which was a dark green amorphous pigment (phaeophytin **a**). The spectral data for compound **A-4** compared well with our data on

phaeophytin **a** which was previously isolated from *F. burtt-davyi* (Ogunlaja *et al*., 2016), confirming compound **A-4** to be phaeophytin **a**.

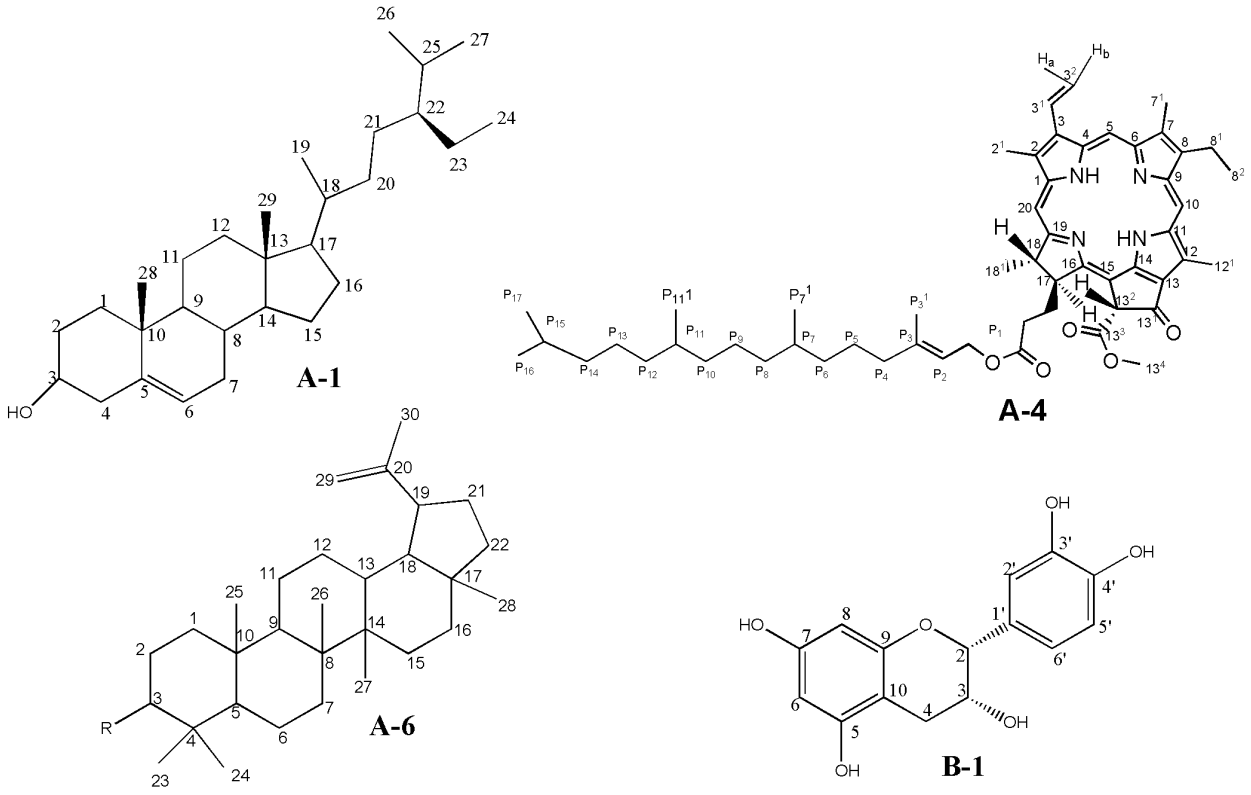

Figure 6-1. Chemical structures of the compounds A-1 (β-sitosterol), A-4 (phaeophytin a), A-6 (lupeol), B-1 (epicatechin) isolated from *F. sur*.

6.3.2 Phenolic content, total flavonoid content and *in vitro* antioxidant assays

Preliminary testing was done on the extracts of fruits and leaves to determine the different classes of compounds present. Table 6-1 shows the percentage yield, total phenolic content (TPC) and total flavonoid content of various parts of *F. sur*. TPC and total flavonoid content of the Hex and DCM extracts of both fruits and leaves were extremely low and are therefore omitted from Table

6-1. The MeOH extract from both fruits and leaves showed a higher yield compared to the other extracts. In addition, the EtOAc extract of fruits was the least recovered. The MeOH extract of the fruits showed a significantly higher ($p < 0.05$) TPC, and flavonoids were only detected in the fruits.

Table 6-1. Percentage yield (mg extract per g sample × 100), total phenolic content (TPC) and total flavonoid content of the extracts of *F. sur* (fruits and leaves).

Extracts	Yield (%)	TPC (mg g^{-1} GAE) *	Total flavonoids content (mg g^{-1} QE)**
Fruit			
Ethyl acetate	0.63	0.43 ± 0.13^{bc}	0.11 ± 0.06^{b}
Methanol	1.72	1.20 ± 0.09^{a}	0.59 ± 0.12^{a}
Leaf			
Ethyl acetate	1.10	0.38 ± 0.04^{c}	ND
Methanol	2.16	0.51 ± 0.17^{b}	ND

Data are presented as mean ± SD (n = 3). *GAE (gallic acid equivalent) and **QE (quercetin equivalent). Values with different superscript letters along a column are significantly different from each other by Tukey's HSD multiple post hoc test, ($p < 0.05$). ND – Not detected.

6.3.3 *In vitro* antioxidant assays

The functional component in food is widely assessed by their antioxidant capacity, hence, in this study, the extracts of fruits and leaves and isolated compounds were evaluated for their *in vitro* antioxidant potential using the FRAP and DPPH assays, relative to the positive controls (ascorbic

acid and BHA). Except for epicatechin and the MeOH extracts from fruits and leaves, other isolated compounds and extracts showed very weak antioxidant activity for both assays and are therefore omitted from the results.

Both the DPPH and FRAP assays show the antioxidant activity of the extracts and tested compounds to be concentration dependent (Figure 6-2 and Figure 6-3). The MeOH extracts of the fruits displayed significantly higher ($p < 0.05$) radical scavenging activity than other extracts especially at higher concentrations (50-500 μg mL^{-1}), and the difference was not significant ($p > 0.05$) with the standard antioxidants in some cases (250 and 500 μg mL^{-1}).

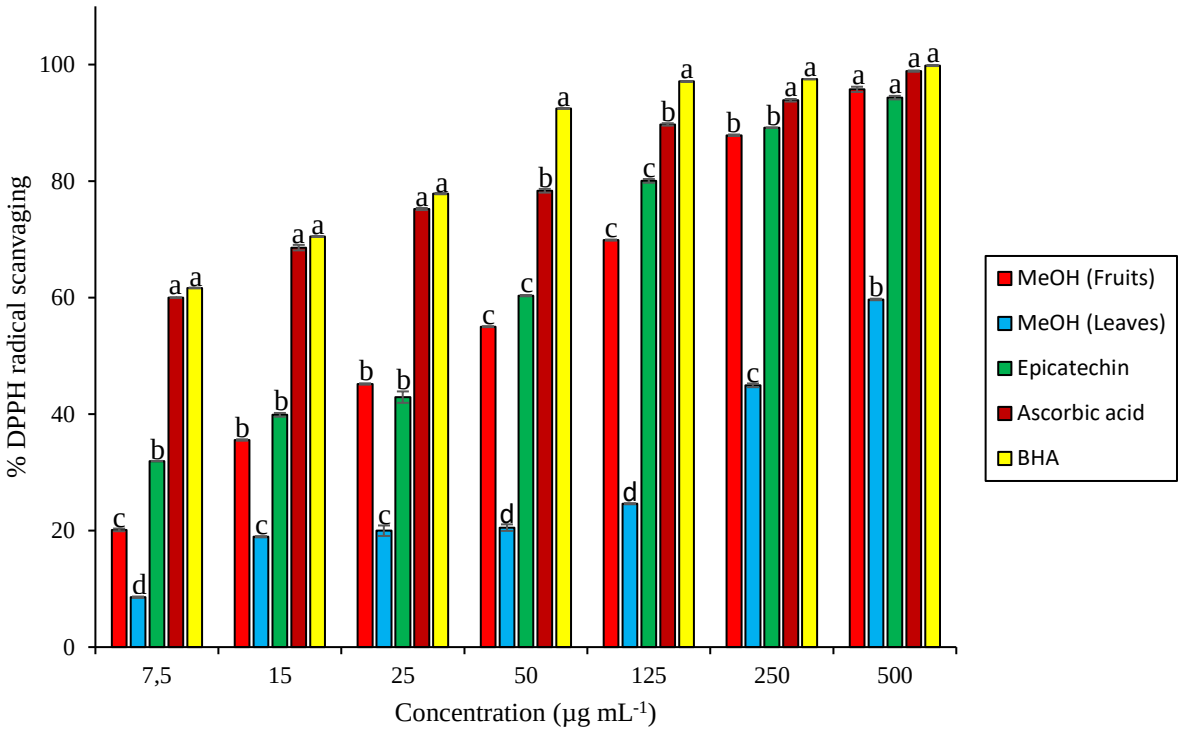

Figure 6-2. DPPH radical scavenging activity (%) of MeOH extract of *F. sur* (fruits and leaves), epicatechin and positive controls (ascorbic acid and BHA). Data are presented as mean ± SD, n=3. [a-d] Values with different letters over the bars for a given concentration of each extract and compound are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

Similarly, the results produced by the FRAP assay showed the reducing power of the MeOH extract of fruits to be comparable to that of the positive controls (Figure 6-3). The antioxidant activity of the MeOH extract of leaves is significantly lower than the other tested samples. The DPPH radical scavenging activity was found to be in the order of BHA > ascorbic acid > MeOH (fruits) > epicatechin > MeOH (leaves). The ferric reducing antioxidant power was found to be in the order of BHA > ascorbic acid > epicatechin > MeOH (fruits) > MeOH (leaves).

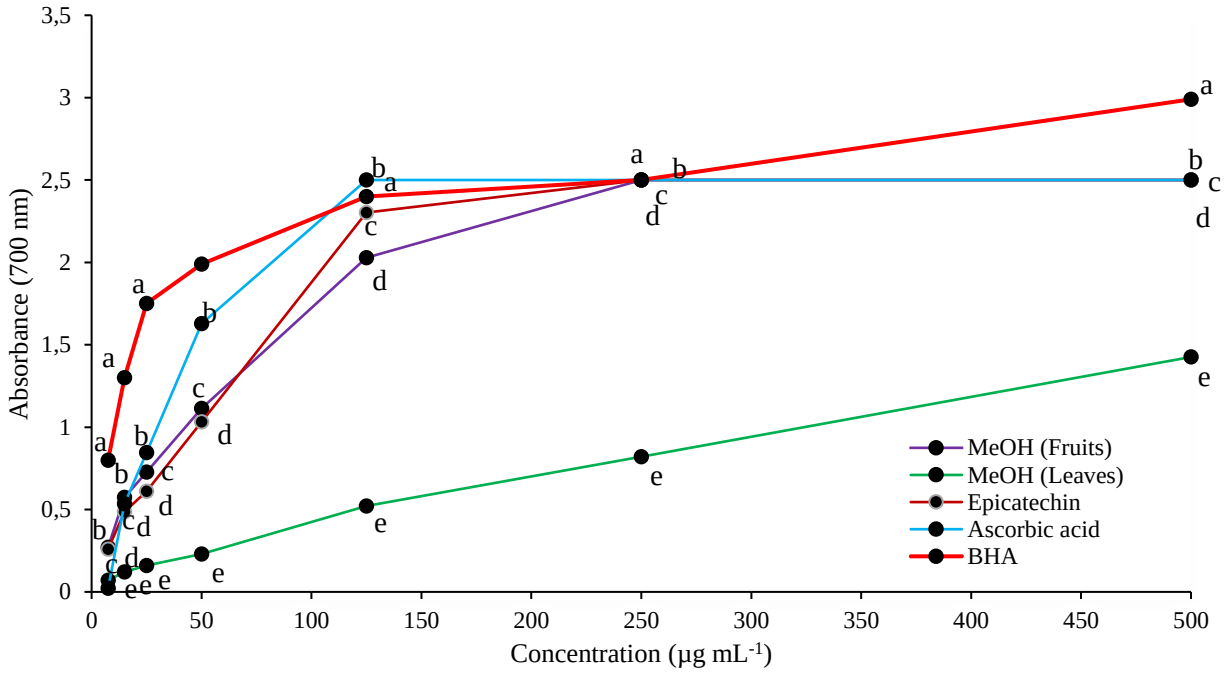

Figure 6-3. Reducing power of the MeOH extract of *F. sur* Forssk. (fruits and leaves), epicatechin, ascorbic acid and BHA. Data are presented as mean ± SD of triplicate determinations. [a-e] Values with different letters over the lines for a given concentration of each extract and compound are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

Table 6-2 compares the IC_{50} values obtained for the extracts and compounds for the DPPH assay. The results showed that the extract of the fruits had an IC_{50} value comparable to the controls which was significantly higher ($p < 0.05$) than the leaves. As polyphenolic compounds, epicatechin and catechin have the ability to act as antioxidants via a free radical scavenging mechanism with the formation of the less reactive flavonoid phenoxyl radical. The high potential of flavonoid compounds to scavenge free radicals may be explained by their ability to donate a hydrogen atom from their hydroxyl group and thereby scavenge the free radicals. Data from this study and our previous report (Ogunlaja *et al*., 2016), suggests that the antioxidant activity of the MeOH extract of the stem bark of *F. burtt-davyi* and MeOH extracts of fruits of *F. sur* is due to the presence of catechin and epicatechin, respectively.

Table 6-2. IC_{50} of different extracts and compounds from *F. sur* for the DPPH assay.

Extracts/compound	DPPH*(μg mL^{-1})
MeOH (leaves)	369.19 ± 12.04^{d}
MeOH (fruits)	$9.06 \pm 2..21^{b}$
Epicatechin	26.75 ± 4.11^{c}
Ascorbic acid	2.03 ± 0.01^{a}
BHA	1.93 ± 0.11^{a}

Each value is represented as Mean ± SD (n = 3). $^{a-d}$ Means in the same column followed by a different letter are significantly different ($p < 0.05$). *No significant difference between assays ($p < 0.05$).

6.4 Conclusion

The phytochemical investigation shows *F sur* fruits to be rich in β-sitosterol, lupeol and epicatechin and leaves to be rich in pheaophytin **a** and β-sitosterol. The MeOH extracts of the fruits show significant antioxidant activity, which may be accounted for by the higher phenolic content and presence of epicatechin. These results highlight the medicinal benefits associated with consumption of the edible fruits of *F sur*, emphasizing its importance in South Africa, where reliance on medicinal wild foods (fruits) is on the rise due to availability and accessibility. This study lends scientific credence and validity to the ethnomedicinal use of *F. sur* whilst underpinning the benefits of consuming the indigenous edible fruits.

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CHAPTER 7

Nutritional evaluation, bioaccumulation and toxicological assessment of heavy metals in edible fruits of *Ficus sur* Forssk (Moraceae)

Abstract

Ficus sur Forssk (Moraceae) is an indigenous medicinal plant with a wide distribution in Africa. In this study, the nutritional potential of this indigenous plant to meet domestic food demands and reduce food insecurity in KwaZulu-Natal, South Africa, was investigated. The proximate composition and concentrations of metals in the edible fruits collected from eight different sites in KwaZulu-Natal were determined to assess for nutritional value and the concentrations of metals in the growth soil was determined to evaluate the impact of soil quality on elemental uptake. The fruits contained high levels of moisture (88.8%) and carbohydrates (65.6%). The concentrations of elements in the fruits were found to be in decreasing order of Ca >Mg >Fe >Zn>Cu >Mn > Se with low levels of toxic metals (As, Cd, Co and Pb). This study shows that the consumption of the fruits of *F. sur* can contribute positively to the nutritional needs of rural communities in South Africa for most essential nutrients without posing the risk of adverse health effects.

Keywords: Elemental distribution, nutrition, toxicity, soil quality.

7.1 Introduction

Undernutrition is a common health problem amongst rural South Africans, and its effects on growth and development has detrimental outcomes, the most serious one being its ability to potentiate the development of non-communicable diseases such as cardiovascular disease, cancer and diabetes (Mayosi *et al*., 2009; Norman *et al*., 2007). Evidence from scientific health reports have indicated that the consumption of fruits can reduce the risk of many of the nutrition-related diseases and risk factors that contribute substantially to the burden of disease in South Africa (Bosman *et al*., 2011). Indigenous plants have been identified as having the potential to meet domestic food demands thereby reducing food insecurity, especially in rural societies and this has increased interest in the exploitation, quantification and utilisation of such food plants (Guinand and Dechassa, 2000; Kebu and Fassil, 2006).

In the rural areas of many African countries, people depend on trees growing in the wild for fruit due to accessibility and affordability, thereby inadvertently exploiting the therapeutic and nutraceutical potential of such fruits. In some cases, indigenous fruits with very little or no documentation of their chemical composition are the only fruits consumed (Mahapatra *et al*., 2012). On average, indigenous fruits contribute 42% towards the food basket for most rural people in Southern Africa (Akinnifesi *et al*., 2006). A daily consumption of indigenous food plants in sufficient quantities can help prevent numerous diseases, improve the nutrition and health of children and the elderly and boost the immune system of HIV/AIDS patients (Barany *et al*., 2001; WHO, 2005a). Although rural households in South Africa frequently turn to indigenous fruits for food, they are often faced with dietary and nutrient deficiency diseases, a situation which children

and lactating women are most vulnerable to (Herzog *et al*., 1993; Moodley *et al*., 2012; Shackleton *et al*., 2004).

The total wellbeing of humanity depends largely on the regular daily intake of macro and micro-elements in the diet, soil being the primary source. Generally, plants depend directly on soil for support and nutrients and metals are mobilised from soil to different parts of plants depending on the nature and quality of the soil matrix (Moodley *et al*., 2012). Excessive levels of heavy metals and metalloids may occur in soil as a result of normal geological occurrences and anthropogenic inputs such as the application of pesticides, waste disposal (industrial, agricultural and domestic), waste incineration, urban effluent and vehicle exhausts (Cui *et al*., 2004; Pakade *et al*., 2013). In addition, the affinity of different plant parts for these metals can also play a major role in the transfer and eventual bioaccumulation of heavy metals. Heavy metals are known to have long biological half-lives and may act as cumulative slow poisons, directly influencing public health because humans do not have an effective mechanism for their removal from the body (Cui *et al*., 2004). In recent years, studies on the impact of heavy metals have increased significantly, especially in the areas of toxicology, due to their non-biodegradable nature and implication in abnormal cell functioning which is linked to certain types of cancers (Banas *et al*., 2010; Mathee *et al*., 2002; Tu¨rkdog˘an *et al*., 2002).

Ficus sur Forssk, of the plant family Moraceae, is an indigenous medicinal plant found in KwaZulu-Natal, South Africa (Burrows and Burrows, 2003). It occurs from the Southern Cape northwards throughout eastern South Africa, to tropical Africa, to Senegal and Cape Verde Islands in the west, Ethiopia in the north and Yemen in the east (Burrows and Burrows, 2003). It is known as Umkhiwane in isiZulu and serves as an immediate source of food both to animals and humans,

hence playing a key role in their everyday survival. An evaluation of the nutritional benefits and concentration of essential and toxic heavy metals in the edible fruits is imperative since metal toxicity in humans, through the agricultural food chain, at plant tissue concentrations are not necessarily phytotoxic.

Previously, the nutritional value of the fruit of indigenous medicinal plants and the impact of soil quality on elemental uptake was reported (Mlambo *et al*., 2016; Moodley *et al*., 2012; 2013). This study aimed to investigate the elemental distribution and concentration of 13 elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) in the edible fruits of *F. sur* and to assess for nutritional value and potential metal toxicity. Soil quality parameters were also investigated to evaluate their impact on elemental uptake.

7.2 Materials and Methods

7.2.1 Sample collection and preparation

F. sur fruit and soil samples from below the tree were collected from eight different sampling sites in KwaZulu-Natal, South Africa; 1-Reservior Hills, 2 - Overport, 3 – Burman Bush, 4 – Pigeon Valley, 5 – UKZN, Howard Campus, S6 - Hibberdene, 7 – Pietermaritzburg and 8 -Byrne (Figure 7-1) between February and March, 2014. Sites were flat with varied yet verdant climate (humid and subtropical). The topography was diverse with loamy soil type and an annual rainfall of 1009 mm. The average daily temperature ranged from 20.6 to 28.6 °C. Samples of tree-ripened fruits were randomly picked from trees, placed in sealed plastic bags and taken to the laboratory for further analyses. The plant was identified and classified by a taxonomist at the School of Life

Sciences, University of KwaZulu-Natal, South Africa and a voucher specimen deposited in the ward Herbarium. Fruit samples were washed thoroughly with double distilled water to remove extraneous matter and then chopped into smaller pieces with a stainless steel knife. Thereafter, fruit samples were oven-dried at 50 ^{o}C overnight to ensure complete removal of moisture. Dried fruit samples were crushed using a food processor (Kenwood Compact Blender, BL380) then stored in a refrigerator in sealed plastic bags until analysed.

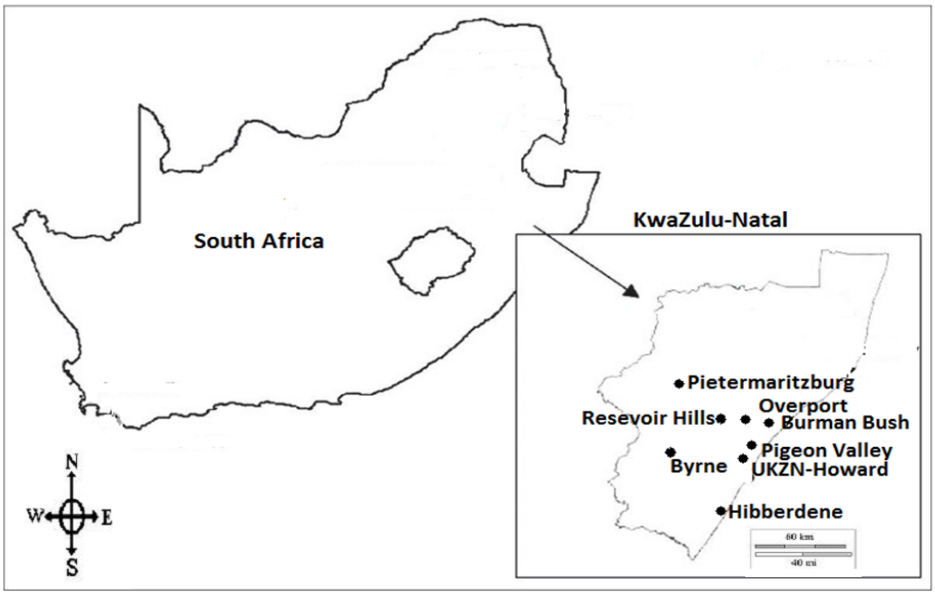

Figure 7-1: Map of selected sampling sites in KwaZulu-Natal, South Africa.

Soil samples were collected from six random points along the drip line of each tree, at a depth of 15-20 cm, with the use of a plastic hand shovel. These were thoroughly mixed in a clean plastic bucket to achieve homogeneity, thus forming the composite sample. A representative soil sample was taken from each site and was dried overnight in an oven at 40 °C then passed through a 2 mm mesh sieve to remove organic matter and gravel. Some of this soil (10 g) was crushed with a mortar

and pestle to reduce the particle size for microwave digestion. Samples were stored in sealed plastic bags and kept in a refrigerator until analysed within 2 weeks.

7.2.2 Analytical quality assurance, chemicals and instruments

All plastic containers were washed with double distilled water and then soaked overnight in 1M HNO_3. Glassware and other equipment were cleaned with 6M HNO_3 and rinsed off with double distilled water to prevent contamination before usage. Millipore™ water (Billerica, MA, USA) was used throughout the experiments. All chemicals used were supplied by Merck (Kenilworth, USA) and Sigma (St. Louis, USA) Chemical Companies and were of analytical-reagent grade. Elemental calibration standards were prepared from spectroscopic grade stock standard solutions of 1000 mg L^{-1}. All digested samples were analysed within a week after digestion.

The Microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, NC, USA) with patented Xpress technology that consists of MARSXpress™ vessels and IR temperature sensors, was used for digestion. Each digestion vessel comprises liners (Teflon PFA, Dupont, Wilmington, DE, USA), caps, and composite sleeves that have a self-regulating pressure control.

Fruit and soil samples (0.25 g each) were weighed into the 50 mL liners, to which, 10 mL of HNO_3 was added. Fruit samples were pre-digested for 1 h prior to microwave digestion. Liners were capped, placed into the sleeves, loaded onto the 40-place carousel, and placed into the microwave. The appropriate method was loaded and the system started. The power was set to 100% at 1600 W and the temperature was ramped to 180 °C (for fruit samples) and 200 °C (for soil samples) for 15 min where it was held for 15 min. Fruit and soil digests were transferred to 50 mL volumetric

flasks, diluted to the mark with double distilled water and stored in polyethylene bottles prior to elemental analysis.

7.2.3 Extraction of exchangeable metals

The extracting solution was prepared by diluting 38.542 g ammonium acetate ($NH_4CO_2CH_3$), 25 mL acetic acid (CH_3COOH, 96%) and 37.225 g ethylenediaminetetraacetic acid (EDTA) to 1L in double distilled water. Exactly 50 mL of extracting solution was added to 5.0 g of dry soil samples in 250 mL polyethylene bottles and shaken in a laboratory shaker for 2 h. Thereafter, solutions were filtered through Whatman No. 1 filter papers and then Millipore 0.45 µm filter membranes to permit analysis of extracted metals. All samples were stored in plastic bottles and kept in a refrigerator until analysed.

7.2.4 Soil pH, Cation Exchange Capacity (CEC) and Soil Organic Matter (SOM)

The pH of soil was determined by measuring the pH of the solution, 1:2.5, dry wt/v using a Fisher Scientific™ FE150 pH meter, fitted with a glass electrode (Khan *et al*., 2008). Cation exchange capacity (CEC) of soil was determined using the pH 7.0 ammonium acetate method (Chapman, 1965) while soil organic matter (SOM) was measured according to the procedure adopted from Walkley and Black, (1934).

7.2.5 Proximate composition determination

Moisture, crude protein, fat, ash and crude fibre content of *F. sur* fruits were determined according to the Association of Official Analytical Chemists method (AOAC, 2000). Total carbohydrate content was estimated by difference.

7.2.6 Elemental analysis

All extracted and digested samples (soil and fruit) were analysed for As, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, Co, and Zn by inductively coupled plasma-optical emission spectrometry (ICP-OES) due to its multi-element determination capability, high dynamic linear range and sensitivity (Perkin Elmer, 5300DV). Analytical wavelengths were selected based on minimum spectral interferences and maximum analytical performance. Initially, the three most sensitive lines were chosen. From these lines, the line with no interfering elements was selected. The accuracy of analytical procedures was checked by analysing certified reference materials (CRMs).

7.2.7 Bioaccumulation factor (BAF)

The Bioaccumulation factor (BAF) expresses the ability of a metal species in its different forms to migrate from the soil through the plant parts and make itself available for consumption (Cui *et al*., 2004).

$$BAF = [Fruit] / [Soil]_{Exchangeable}$$

7.2.8 Statistical analysis

The difference in heavy metal concentrations between the eight sites was investigated using discriminant analysis. The results of this analysis were assessed by examining the canonical correlation statistics, the Wilk's lambda statistics, the significance levels and the percentage of original group cases correctly classified. In addition, a Pearson's correlation analysis was applied to the dataset to quantitatively analyse and confirm the relationship between soil quality parameters (pH, SOM and CEC) and heavy metal concentrations. Principal component analysis (PCA) was carried out to identify patterns in the elemental data by identifying the different groups of metals that correlate and thus can be considered as having a similar behaviour and common origin. All statistical analyses were performed using the Statistical Package for the Social Sciences, (PASW version 23, IBM Corporation, Cornell, NY, USA).

7.3 Results and Discussion

7.3.1 Proximate chemical composition

The results of the proximate analysis showed *F. sur* fruits to contain 88.8 ± 0.2% moisture. On dry mass basis, the carbohydrate content was 65.6 ± 0.03%, protein 5.2 ± 0.1%, fat 4.7 ± 0.2%, crude fibre 7.7 ± 0.02% and ash 17.67 ± 1.3%. The high level of carbohydrates in the fruits indicates that they can serve as an immediate and alternative source of energy. These values were similar to those reported by Wilson and Downs (2012).

7.3.2 Soil quality parameters analysis

Soil pH ranged from 5.3 to 8.2 while SOM ranged from 11.0 to 22.3%. Higher pH values were observed at site 4 (7.6 ± 0.09), site 6 (7.3 ± 0.03) and site 7 (8.2 ± 0.08) and may be connected with the higher concentration of Ca in the soil compared with other studied sites. The CEC ranged from 2.30 to 3.70 meq/100.

7.3.3 Elemental analysis

Method validation for the analytical procedure was carried out by measuring CRMs, BCR-402 (Institute for Reference Materials and Measurement, European Commission, Joint Research Centre, Belgium) for fruits samples and D081-540 (ERA, A waters Company, USA) for soil samples. Experimental results were compared with certified results (Table 7-1). Measured values compared well to the certified values ($p < 0.05$) with recovery percentages being within acceptable limits.

Table 7-1: Validation of the analytical method using plant and soil certified reference materials (BCR-402 for plant and D081-540 for soil).

Element	Concentration in BCR-402 (µg g^{-1})		Concentration in D081-540 (µg g^{-1})		
	Measured*	Certified	Measured	Certified	Acceptable limit
As	0.10 ± 0.014	0.093 ± 0.010	83.2 ± 3.36	101 ± 5.92	61.0 - 116
Cd	-	-	156.0 ± 4.6	143 ± 5.6	116 - 159
Co	0.174 ± 0.006	0.178 ± 0.008	238.5 ± 7.8	199 ± 4.1	166 - 233
Cr	4.98 ± 0.248	5.19 [a]	102.6 ± 2.3	86.8 ± 6.1	69.3 - 104
Fe	240 ± 6.75	244[a]	13119 ± 608	12800 ± 18.0	5380 - 20100
Mn	-	-	448.5 ± 18.7	425 ± 9.7	347 - 502
Se	6.80 ± 0.16	6.70 ± 0.25	161.5 ± 5.9	127 ± 4.5	98.4 - 156
Ni	8.23 ± 0.46	8.25[a]	212 ± 21.70	236 ± 4.17	175 - 302
Zn	25.40 ± 0.68	25.02[a]	136.9 ± 7.9	130 ± 11.5	113 - 184

*Values are in µg g^{-1}dry mass (mean ± standard deviation, 95% confidence interval, n = 3).
[a] Indicative values (without uncertainty). BCR-402 for plant and D081-540 for soil.

The concentrations of heavy metals in soil (total and exchangeable) and fruits are summarised in Table7-2 for those elements in fruits that were above the instrument detection limits. Although, total soil Pb (21.5-78.4 µg g^{-1}) was above the South African maximum permissible level of 6.6 µg g^{-1} in soils at all sites, the concentrations of Pb in all fruit samples were found to be below the instrument detection limit. In addition, concentrations of the other toxic elements, As, Cd and Co in all fruit samples were below the instrument detection limit. Selenium was found in fruit samples from site 1 (0.86 µg g^{-1}) and site 2 (2.16 µg g^{-1}) only and Ni was found in the fruit sample from site 8 (10.30 ± 1.44 µg g^{-1}) only.

Table 7-2: Elemental concentrations (µg g^{-1}) in fruit and soil (Total (T) and exchangeable (Ex)) samples (Mean ± standard deviation; 95% confidence interval, n=3) and bioaccumulation factors (BAFs).

		Concentration (µg g^{-1})			BAF		
	Element	Fruit	Soil (T)	Soil (Ex)	$[F]/[S]_T$*	$[F]/[S]_{Ex}$**	Ex%***
1	Ca	3313 ± 21	630 ± 37	454 ± 19	5.3	7.3	72.1
2		2270 ± 152	1114 ± 29	1043 ± 34	2	2.2	93.7
3		2355 ± 40	394 ± 5.58	250 ± 3.91	6	9.4	63.5
4		2613 ± 24	2528 ± 333	2085 ±7 1	1	1.3	82.5
5		19467 ± 35	1180 ± 41	1033 ± 27	1.7	1.9	87.5
6		3310 ± 74	1476 ± 75	1433 ± 31	2.2	2.3	97.1
7		2792 ± 49	4671 ± 9.9	4089 ±3 7	0.6	0.7	87.6
8		7746 ± 232	2173 ± 182	786 ± 50	3.6	9.9	36.2
1	Cu	4.31 ± 0.30	7.78 ± 0.52	2.36 ± 0.30	0.6	1.8	30.3
2		4.31 ± 0.97	7.76 ± 0.71	6.90 ± 0.30	0.6	0.6	88.9
3		5.01 ± 1.37	3.06 ± 1.32	2.78 ± 0.12	1.6	1.8	90.6
4		2.49 ± 0.39	18.2 ± 2.51	10.7 ± 0.30	0.1	0.2	58.9
5		3.41 ± 0.28	8.54 ± 0.60	6.15 ± 0.12	0.4	0.6	72
6		2.23 ± 0.55	6.79 ± 1.05	6.25 ± 0.33	0.3	0.4	92
7		5.11 ± 0.68	23.6 ± 1.20	7.65 ± 0.16	0.2	0.7	32.4
8		17.4 ± 2.07	8.82 ± 1.02	1.63 ± 0.26	2	10.6	18.5
1	Fe	61.5 ± 2.56	31536 ± 95	327 ± 28	0	0.2	1
2		28.0 ± 1.56	20279 ± 104	261 ± 8.59	0	0.1	1.3
3		21.6 ± 1.61	18391 ± 601	186 ± 4.73	0	0.1	1
4		29.9 ± 2.95	18534 ± 33	475 ± 18	0	0.1	2.6
5		62.7 ± 1.80	15302 ± 118	710 ± 13	0	0.1	4.6
6		35.4 ± 5.10	17534 ± 394	182 ± 4.13	0	0.2	1
7		105 ± 9.56	67040 ± 352	447 ± 2.51	0	0.2	0.7
8		129 ± 3.90	15171 ± 553	96.9 ± 7.58	0	1.3	0.6
1	Mg	1798 ± 162	2534 ± 84	19.6 ± 2.45	0.7	91.5	7.8
2		1233 ± 40	550 ± 31	73.1 ± 26	2.2	16.9	13.3
3		1255 ± 4.89	251 ± 6.71	73.2 ± 0.63	5	17.2	29.17
4		1022 ± 6.56	1189 ± 73	48.7 ± 1.64	0.9	21	4.09
5		1487 ± 24	874 ± 11	27.6 ± 3.08	1.7	53.8	3.16
6		1284 ± 36	492 ± 32	33.3 ± 2.23	2.61	38.5	6.77
7		1613 ± 34	1549 ± 62	26.3 ± 3.01	1.04	61.3	1.7
8		1766 ± 179	23880 ± 341	83.8 ± 22	0.07	21.1	0.35
1	Mn	0.47 ± 0.01	16.3 ± 0 .74	4.09 ± 0.26	0	0.1	0.3

2		0.52 ± 0.05	18.1 ± 1.39	14.0 ± 0.42	0	0	0.8
3		1.83 ± 0.02	37.5 ± 1.07	32.5 ± 0.71	0.1	0.1	0.9
4		0.14 ± 0.01	23.0 ± 2 .08	12.6 ± 0.32	0	0	0.6
5		0.04 ± 0.01	16.1 ± 0.48	9.90 ± 0.22	0	0	0.6
6		1.17 ± 0.03	19.9 ± 0.50	13.8 ± 0.15	0.1	0.1	0.7
7		0.92 ± 0.24	237 ± 26	75.0 ± 1.10	0	0	0.3
8		20.2 ± 1.25	35.3 ± 0.40	11.5 ± 1.36	0.6	1.8	0.3
1	Zn	13.7 ± 4.63	27.3 ± 2.77	6.22 ± 0.73	0.5	2.2	22.8
2		14.6 ± 1.91	24.7 ± 2.51	18.1 ± 0.87	0.6	0.8	73.4
3		13.5 ± 1.89	9.42 ± 0.33	5.81 ± 0.13	1.4	2.3	61.7
4		6.90 ± 1.17	54.2 ± 3.60	39.1 ± 5.14	0.1	0.1	72.2
5		13.4 ± 1.03	23.5 ± 1.19	15.1 ± 0.40	0.6	0.9	64.3
6		4.35 ± 0.29	21.9 ± 0.48	19.4 ± 0.34	0.2	0.2	88.8
7		18.1 ± 4.89	37.8 ± 1.36	15.3 ± 0.52	0.5	1.2	40.5
8		22.8 ± 2.17	22.8 ± 0 .70	4.58 ± 0.61	0.1	5	20.1

* $[F]/[S]_T$-$[Fruit]/[Soil]_{Total}$

** $[F]/[S]_A$-$[Fruit]/[Soil]_{Exchangeable}$

*** Ex% - $[Soil]_{Exchangeable}/[Soil]_{Total}$

The fruit tended to accumulate Mg producing BAFs (Exchangeable) between 16.9 and 91.5 even though, Mg mobility was low (0.4 to 29.2%). This is seen at site 1, where Mg in the fruit was more than ninety times that which was exchangeable (Table 7-2). Calcium concentrations in fruits ranged from 2270 to 7746 µg g^{-1} with BAFs (Total) between 0.6 and 6.0 indicating the tendency of the plant to accumulate this metal. Copper is an essential micronutrient in humans for the production of blood hemoglobin. High doses of Cu can result in anemia, liver and kidney damage, and stomach and intestinal irritation (Raymond and Felix, 2011). Total soil Cu was relatively low (< 24 µg g^{-1}) and on average 56% was in exchangeable form. Copper concentrations in the fruits were within a small range of variation (1.43-17.4 µg g^{-1}) however at site 7, Cu in the fruits exceeded the WHO permissible limit of 10 µg g^{-1} for plants (WHO, 2005b).

Zinc is a trace element that is essential for human health, its shortages can cause birth defects. Zinc at elevated levels in soil can negatively interrupt the activity of microorganisms and earthworms, thus retarding the breakdown of organic matter (Raymond and Felix, 2011). The concentration of Zn in the fruit ranged from 4.35 to 18.1 µg g^{-1}. More than 50% of the sites tended to accumulate Zn in the fruits with BAFs (Exchangeable) > 1. The concentrations of Cu and Zn in the fruits exhibited safe levels relative to the maximum levels for fruit set by the Department of Health, South Africa, which is 30 and 40 µg g^{-1}, respectively (DOH, 2004).

Site 8 was observed to have the lowest exchangeable Fe (96.9 µg g^{-1}) but the highest concentration of Cu (17.4 µg g^{-1}), Mn (20.2 µg g^{-1}) and Zn (22.8 µg g^{-1}) in the fruits. This three-way synergy between Cu, Zn and Mn is as a result of the reduction of soil retention capacity of one metal due to increase in concentration of a contending metal ion. This observation is in agreement with reports from literature (Moodley *et al*., 2012). Although, total soil Fe was relatively high at all sites (15171 to 67040 µg g^{-1}), BAFs (Exchangeable) were relatively low suggesting the plants control on uptake.

Manganese is an essential trace metal. Its deficiency produces severe skeletal and reproductive abnormalities in mammals and its toxicity symptoms are lung and brain damage (Takeda, 2003). The concentration of Mn in fruits ranged from 0.04 to 20.17 µg g^{-1} which are below the maximum limits of 2000 µg g^{-1} (Kabata-Pendias and Pendias, 1992). This study shows that for the essential elements Ca, Mg, Fe, Cu, Mn and Zn, the fruit tends to exclude the element when soil concentrations are high but accumulated them when soil concentrations are low, in accordance with metabolic requirements.

7.3.4 Estimated contribution of metals in fruits to the diet

The elemental distribution in the edible fruits of *F. sur* was compared to Dietary Reference Intakes (DRIs) (Table 7-3) (IMFNB, 2001).

Table 7-3: Comparison of Dietary Reference Intake (DRI) (Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Level (UL)) of elements for most individuals to the average concentration of elements (n = 3) in *F. sur* fruits.

Element	Average concentration (mg / 20 g DM)	DRI (mg/ day) RDA	UL	Estimated contribution to RDA (%)
Ca	109.67	1000-1300	2500	8.4-11.0
Cu	0.11425	0.9	8	12.7
Fe	1.18275	8-18	45	6.6-14.8
Mg	28.645	310-320	350	9.0-9.2
Mn	0.06315	1.6-2.3	9	2.7-3.9
Se	0.0302	0.055	0.4	54.9
Zn	0.268375	8-11	34	2.4-3.4

In South Africa, fortification of wheat and maize flour has improved the Fe status of children in the country but 24% of children still remain anaemic (Faber *et al*., 2005; WHO, 2008). The consumption of 20.0 g of fruit (which is the average weight of a handful of fruits) contributes between 6.6-14.8% towards the RDA for Fe for most adults. Copper deficiency is normally due to decreased Cu at birth, insufficient dietary Cu intake and poor absorption. Consumption of 20.0 g of fruit contributes about 12.7% towards the RDA for Cu for most adults. Selenium is reported to help with the prevention of heart disease and cancer, however if in excess, it can produce toxicity effects such as depression, gastrointestinal disturbances and excessive tooth decay (Tank and

Strovick, 1960). About 20 g of *F. sur* fruits may contribute more than 54% towards the RDA for Se; however, this amount does not exceed the UL so it would be considered safe.

Calcium and Mg are macro-elements required for bone formation, the development of strong teeth, muscle regulation and control of blood pressure. A common nutritional problem among South Africans is hypocalcemia which is due to a deficiency in Ca. In a study done on a randomly chosen rural South African community, 13.2% of children were found to have abnormally low levels of Ca in their blood (Pettifor *et al*., 1979). Consumption of 20.0 g of *F. sur* fruits may contribute between 8.4 -11.0% towards the RDA for Ca. A report by WHO showed that an average adult in South Africa consumes only half their RDA for Mg (WHO, 2009). An intake of 310-320 mg of Mg per day is recommended and the consumption of approximately 20.0 g of *F. sur* fruits, may contribute approximately 28.6 mg (9.0-9.2%) towards its RDA.

7.3.5 Statistical analysis

The result of discriminant analysis showed that heavy metal concentrations exhibited discrimination between studied sites (Table 7-4). Generally, the larger the canonical correlation statistic, the greater is the between groups variation as a proportion of the total variation, and the larger the Wilk's lambda statistic, the greater is the within-group variation as a proportion of the total variation. There was a high degree of between site variations for As, Ca, Cr, Cu, Co, Mg, Mn, Pb, Se and Zn; canonical correlation values ranging from 0.699 to 0.997 and a few lower degrees of within-group site variations; Wilk`s lambda statistic ranged from 0.005 to 0.511. The statistically significant results also demonstrate that the originally grouped cases have high percentage (28.6% to 88.6%).

Table 7-4: Discriminant analyses results for the studied sites.

Variation	Canonical correlation	Wilk`s Lambda statistic	Chi-squares	D.F	Sign.	% of grouped cases correctly classified
As	0.777	0.396	27.824	6	0	28.6
Ca	0.699	0.511	20.154	6	0.003	40.0
Cr	0.988	0.024	112.346	6	0	60.0
Cu	0.967	0.065	81.967	6	0	60.0
Co	0.985	0.029	106.087	6	0	45.7
Mg	0.997	0.005	157.781	6	0	88.6
Mn	0.992	0.015	125.429	6	0	60.0
Pb	0.922	0.151	56.761	6	0	60.0
Se	0.821	0.326	33.657	6	0	31.4
Zn	0.759	0.423	25.795	6	0	57.1

D.F- Degree of freedom; Sign-Significance.

The main soil parameters that govern the processes of sorption and desorption of trace elements are pH, CEC and SOM. Table 7-5 shows the inter-item correlation matrix for significant correlations in the soil and fruits of *F. sur*. Soil pH correlated significantly to exchangeable Cu (r = 0.8) and Ca (r = 0.9) indicating that pH was a significant parameter for controlling availability of these metals and not SOM and CEC. There was a significant 3-way positive correlation between Fe, Mn and Ni in the soil which is an indication of the same geological parent material. Exchangeable Zn and Mn correlated positively to their total soil concentrations, suggesting that total soil concentrations may be used to predict the availability of these elements, similar to previous studies (Sauv`ie *et al*., 2000). For concentrations in the fruit, there was a significant 4-way positive correlation between Ca, Cr, Cu and Mn suggesting that the plant requires proportional amounts of these elements for metabolic processes.

Table 7-5: Inter-item correlation matrix for concentration of elements in fruit (F) and soil (S).

	CaF	CrF	CuF	CuT	FeT	MgT	MnF	MnT	MnE	NiT	ZnT	ZnE
CaF												
CrF	1.0**											
CuF	0.9*	1.0**										
CuT	-0.1	-0.1	-0.1									
FeT	-0.2	-0.2	-0.1	0.7*								
MgT	1.0**	1.0**	1.0**	-0.1	-0.2							
MnF	1.0**	1.0**	1.0**	-0.1	-0.2	1.0*						
MnT	-0.1	-0.1	0.0	0.7*	0.9**	-0.1	-0.1					
MnE	-0.2	-0.2	-0.1	0.6	0.9**	-0.2	-0.1	1.0**				
NiT	0.0	0.0	0.1	0.7*	0.8*	0.0	0.0	0.9**	0.7*			
ZnT	-0.1	-0.1	-0.2	0.8*	0.3	-0.1	-0.2	0.3	0.1	0.3		
ZnE	-0.4	-0.4	-0.5	0.5	-0.1	-0.4	-0.4	0.0	-0.1	0.0	0.8*	

*, ** - significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.
$XF–[X]_{Fruit}$, $XT–[X]_{Soil\ Total}$, $XE–[X]_{SoilExchangeable}$ where X = the various elements.

In order to further identify the relationships between different metals in soil and their corresponding origins, principal component analysis (PCA) was conducted (Table 7-6). Figure 7-2 represents the component plot in rotated space (3-D plot) showing the relationships among the twelve heavy metals. Table 7-6 shows that the elements are dominated by three principal components which accounted for 73.1% of the total variance. Factor 1 was best represented by As, Cr, Co, Mn. Ni and Se, accounting for 49.0% of the total variance. The Cu loading (0.58) is not as high as that of other elements of the group, suggesting a quasi-independent behaviour within the group.

Table 7-6: Rotated component matrix for variables in the soil samples (n = 40).

	Component		
Element	1	2	3
As	**0.77**	0.28	0.11
Ca	0.34	**0.62**	0.37
Cr	**0.92**	0.20	0.17
Cu	0.58	**0.77**	0.09
Co	**0.88**	0.24	0.31
Fe	0.20	0.01	**0.87**
Mg	0.02	0.49	0.28
Mn	**0.87**	0.23	0.32
Ni	**0.64**	0.09	0.12
Pb	0.40	**0.80**	-0.11
Se	**0.71**	-0.10	-0.25
Zn	-0.10	**0.90**	-0.13
Eigenvalues	5.89	1.85	1.03
% Total variance	49.04	15.43	8.61
Cumulative %	49.04	64.47	73.08

Extraction Method: Principal Component Analysis. Rotation Method: Varimax with Kaiser Normalisation. Bold figures indicate values ≥ 0.5.

Factor 2 contributed 15.4% to the total variance with a high loading on Ca, Cu, Pb and Zn. Factor 3 is dominated by Fe (0.87), accounting for 8.6% of the total variance. These associations strongly suggest that the elements clustered together have a similar source (Li *et al*., 2000; Niu *et al*., 2009; Zhou *et al*., 2007).

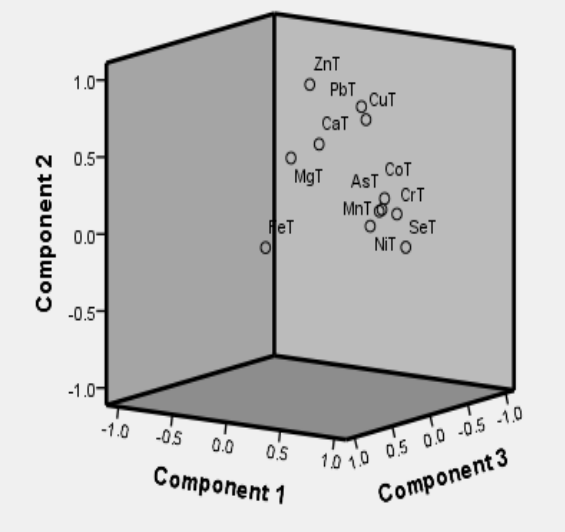

Figure 7-2: PCA analysis loading 3-D plot (PC1 vs PC2 vs PC3) for 12 heavy metals.

7.4 Conclusion

The concentrations of elements in the fruits were found to be in decreasing order of Ca >Mg >Fe >Zn>Cu >Mn >Se while the concentrations of toxic metals (As, Cd, Co, and Pb) were found to be below the instrument's detection limits. This study shows that the fruits of *F. sur*conform to the RDAs for the elements in focus and that their consumption can contribute significantly towards a balanced diet without posing the risk of adverse health effects. Statistical analysis revealed synergistic relationships in the plant thereby confirming that uptake of elements is controlled to meet metabolic needs.

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CHAPTER 8

Elemental distribution and nutritional value of the edible fruits of *Ficus sycomorous* Linn

Abstract

Ficus sycomorous Linn is a medicinal plant commonly distributed in KwaZulu-Natal, South Africa. Its fruits are usually consumed by the indigenous communities. Elemental concentrations in the edible fruits of *F. sycomorous* was investigated to assess for nutritional value by comparison with recommended dietary allowances (RDAs) and the possible risk associated with its consumption. The elemental concentrations in the fruits, as well as the growth soil acquired from eight different sites were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) so as to assess the impact of soil quality on the distribution of element and their uptake. Proximate chemical composition results showed high levels of moisture (55.8 ± 0.3%), carbohydrates (25.3 ± 1.1%), protein (5.6 ± 0.2%), fat (8.9 ± 0.5%), crude fibre (55.8 ± 0.9%) and ash (4.4 ± 0.4%). The elemental concentrations in the fruits conformed with the RDAs and decreased if the order of Ca > Mg > Fe > Zn > Mn > Cu > Cr with concentrations of toxic metals (As, Cd, Co and Pb) being below the instrument detection limits. The intake of the fruits by humans can improve the health, and meet the nutritional demands of indigenous people without posing the risk of adverse effects. Statistical analyses of soil showed similarity for Cd, Co, Cr, Ni and Pb, suggesting the same source whilst As, Cu and Zn have a common origin. Data from this study indicated that elemental interactions in soil significantly influenced their availability, but uptake was to a greater extent controlled by the plant.

Keywords: Elemental uptake; edible fruit; soil quality; recommended dietary allowances.

8.1 Introduction

The burden of disease related to non-communicable diseases is becoming increasingly harmful and it is projected to increase significantly in South Africa over the next decades, if necessary measures are not put in place to curtail the trend (Abegunde *et al*., 2007). An insight into the magnitude, and risk factors for non-communicable diseases in South Africa is important for effective action. Among the risk factors associated with non-communicable diseases is unhealthy diet, with a potential to translate to cardiovascular disease, diabetes, and cancer (Mayosi *et al*., 2009).

Nutrition plays a critical function in wellness. It provides not only essential nutrients, but also promotes good health and prevention of diseases (Willet, 1994). Every individual requires food for proper development and sound health, but the quality as well as the quantity of food is also important. The quality of food depends on the relative composition of various nutrients such as proteins, fat, carbohydrate, vitamins and mineral. Fruits are vital to the human diet and are known to contain components with several types of health promoting action, such as vitamins, essential minerals, antioxidants and prebiotics (fibres) (Itanna, 2002).

Heavy metals are ever-present in the environment as a result of both natural geological occurrences and anthropogenic inputs, and humans are exposed to them through various pathways (Wilson and Pyatt, 2007). At elevated concentrations, essential elements such as chromium, copper, iron, manganese and zinc may lead to metal toxicity symptoms. Other elements such as arsenic, lead, cadmium and nickel are most often found to be responsible for harmful damage to humans. In

South Africa, rural people traditionally harvest a wide range of fruits from the wild because of its taste, and as food supplements to tide over food shortage. Indigenous wild fruits form a major portion of the diet of low-income and middle-income people of South Africa, hence their nutritive value is important. Although the efficacy of medicinal plants as a therapeutic agent is often accounted for by the phytochemical constituents, prolonged ingestion of medicinal plants can result in elemental accumulation at harmful levels in humans (Sharma *et al*., 2009; WHO, 1992). Based on this, elemental screening of medicinal plants is paramount for quality control and safety (Arceusz *et al*., 2010; Liang *et al*., 2004).

Ficus sycomorous Linn, (Moraceae), also called the sycamore fig is native to South Africa and it is commonly found in the province of KwaZulu-Natal. Its dried fruits are taken orally by adults in Venda (Vhavenda) for the management of tuberculosis (Arnold and Gulumian, 1984). The Vhavenda people also enjoy eating the ripe fruits, especially the smaller, pinkish ones (thole) which are sweeter than the larger ones (mahuyu) (Burrows and Burrows, 2003; Netshiungani, 1981). In addition, fresh fruits are boiled and used as a pressing for the teats of cattle and goats to encourage milk production (lactation) (Arnold and Gulumian, 1984; Mabogo, 1990). Although biological and phytochemical studies of *F. sycomorous* are documented (Al-Matani *et al*., 2015; El-Sayed *et al*., 2010; Ramde-TIendrebeogo *et al*., 2012; Romeh, 2013), information on the nutritional value of the edible fruits are not reported, hence the need to analytically investigate the fruits. In addition, heavy metals from the soil may bioaccumulate in the fruits and eating such fruits can result in adverse health effects and metal toxicities. The evaluation of the nutritional benefits and concentration of essential and toxic heavy metals in the edible fruits is imperative since harmful metal reactions in humans, occur at concentrations that are not necessarily phytotoxic.

Previously, we reported on the nutritional evaluation, bioaccumulation and toxicological assessment of heavy metals in the indigenous medicinal fruits of *F. sur* (Ogunlaja *et al*., 2017). This study investigated the elemental concentrations of As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn in the fruits as well as the growth soil in order to assess the nutritional value and evaluate the elemental uptake by edible fruits of *F. sycomorous.*

8.2 Materials and methods

8.2.1 Sampling

Tree-ripened fruit samples, randomly picked from eight different sampling locations within KwaZulu-Natal, South Africa was used for this investigation (Figure 8-1). Samples of fruits were placed in different sealed plastic bags based on location, and taken to the laboratory for further analyses. The plant was identified and classified by a taxonomist at the School of Life Sciences, University of KwaZulu-Natal, South Africa and a voucher specimen was deposited in the Herbarium. Soil samples at a depth of 15-20 cm were collected beneath the trees from the eight different sampling sites from which the fruits were picked.

8.2.2 Reagents, Analytical quality assurance and Standards

All the chemicals used for this investigation were supplied by Sigma Aldrich (St. Louis, USA) and Merck (Kenilworth, USA) chemical companies. Analytical reagent grade chemicals were used for samples and spectroscopic grade were used for standards. Elemental calibration standards were prepared from spectroscopic grade stock standard solutions of 1000 mg L^{-1}. Glassware and other

equipment were cleaned with 6M HNO_3 and rinsed off with double distilled water to prevent contamination before usage. Millipore™ water (Billerica, MA, USA) was used throughout the experiments. All plastic containers were washed with double distilled water and then soaked overnight in 1M HNO_3. All digested samples were analysed within a week.

Figure 8-1: Map of selected sampling sites in KwaZulu-Natal, South Africa.

8.2.3 Sample Preparation

Fruits were washed with double distilled water, and dried in an oven at 50 °C, for 72 h. Dried fruits were then finely grounded using a mill and stored in plastic bags until analysed. A representative soil sample from each site was dried overnight in an oven at 40 °C then passed through a 2 mm mesh sieve to remove organic matter and gravel. Thereafter, soil samples (10 g) was crushed with a mortar and pestle to reduce the particle size for digestion. Samples were stored in sealed plastic bags and preserved in a refrigerator until analysed.

8.2.4 Digestion and elemental analysis of samples

Samples of dried, powdered fruit and soil were accurately weighed and digested by applying the optimised procedure as described by Endalamaw and Chandravanshi, (2015) with some modification. Approximately 0.25 g each of certified reference material (CRM), dried fruit and soil samples were placed in the 50 mL beakers to which 10 mL of HNO_3 (70%) was added and allowed to pre-digest for 1 h (Ogunlaja *et al*., 2017). The samples were properly wetted and digested on the hot plate for a minimum of 45 min at 160 °C, while swirling. It was removed from the hot plate before becoming dry and cooled. The fruit and soil digests were transferred into a 25 mL volumetric flask, diluted to the mark with Millipore^{TM} water and stored in polyethylene bottles prior to elemental analysis.

8.2.5 Extraction of exchangeable metals

The extracting solution was prepared by mixiing 38.542 g of ammonium acetate ($NH_4CO_2CH_3$), 25 mL of acetic acid (CH_3COOH, 96%) and 37.225 g of ethylenediaminetetraacetic acid (EDTA) to 1 L in double distilled water. Exactly 50 mL of extracting solution was added to 5.0 g of dry soil samples in 250 mL polyethylene bottles and shaken in a laboratory shaker for 2 h. Thereafter, solutions were filtered through Whatman No. 1 filter papers and then Millipore 0.45 µm filter membranes to permit analysis of extracted metals. All samples were stored in plastic bottles and kept in a refrigerator until analysed.

8.2.6 Soil pH, Cation Exchange Capacity (CEC) and Soil Organic Matter (SOM)

The pH of soil was determined by measuring the pH of the solution, 1:2.5, dry wt/v using a pH meter (Aqualytica, Model pH 17) fitted with a glass electrode. Cation exchange capacity (CEC) of soil was determined using the pH 7.0 ammonium acetate method (Chapman, 1965) while soil organic matter (SOM) was measured according to the procedure adopted from Walkley and Black (Walkley and Black, 1934).

8.2.7 Determination of proximate chemical composition

The proximate chemical composition of *F. sycomorus* fruits (moisture, crude protein, fat, fibre and crude ash) was determined in triplicate according to the standard methods of analysis, as described by the Association of Official Analytical Chemists (AOAC, 2000). Total carbohydrate content was obtained by difference.

8.2.8 Elemental analysis

All extracted and digested samples (soil and fruit) were analysed for As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) due to its multi-element determination capability, dynamic linear range and low detection limits. Measurements were carried out using the Perkin Elmer® Optima™ 5300 Dual View ICP-OES (Billerica, Massachusetts, USA) with axial plasma observation. Analytical wavelengths were selected based on minimum spectral interferences and maximum analytical performance. Initially, the three most sensitive lines were chosen. From these lines, the line with no interfering elements was selected. The accuracy of analytical procedures was checked by analysing certified reference materials (CRMs) for plant (BCR-402, Institute for Reference Materials and Measurement, European Commission, Joint Research Centre, Belgium) and soil (D081-540, ERA, A waters Company, Milford, MA, USA). The experimental means were compared with the corresponding certified values. The elemental composition of the certified reference materials, CRMs (BCR 402 for plant and D081-540 for soil) was used to ensure accuracy of the method of determination and the results are represented in Table 8-1. The values for As, Co and Se are certified whilst those for Cr, Fe, Ni and Zn are indicative so no uncertainties were ascribed to them. The measured values compared well with certified results at 95% confidence interval.

8.2.9 Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences, (PASW version 24, IBM Corporation, Cornell, NY, USA). Pearson's correlation analysis was applied to the dataset to quantitatively analyse and confirm the relationship between soil quality

parameters (pH, SOM and CEC) and heavy metal concentrations. In addition, principal component analysis (PCA) and cluster analysis (CA) the commonly used multivariate statistical methods in environmental studies (Dı´az *et al*., 2002) were carried out. PCA was used to reduce data and extract a small number of principal components for analysing relationships among the elemental soil concentrations. Furthermore, hierarchical CA was carried out to classify the elements on the basis of the similarities of their chemical properties. A dendrogram was also created to assess the cohesiveness of the clusters formed, in which correlations among elements can readily be detected.

8.3 Results and Discussion

8.3.1 Proximate and elemental analyses

The proximate chemical composition of *F. sycomorus* fruits showed high levels of moisture (55.8 ± 0.3%) and carbohydrates (25.3 ± 1.1%) yet lower than that of *Ficus sur* fruits with 88.8% moisture and 65.6% carbohydrates (Ogunlaja *et al*., 2017). The fruits also contained 5.6 ± 0.2% protein, 8.9 ± 0.5% fat, 55.8 ± 0.9% crude fibre and 4.4 ± 0.4% ash. The values for protein and fat were similar to those reported by Wilson and Downs, (2012). The fruits of *F. sycomorus* contained higher crude fibre content compared to *F. sur* fruits (7.7%) (Ogunlaja *et al*., 2017). Based on the intake level observed to protect against coronary heart disease, the American Heart Association (AHA) set the Adequate Intake (AI) for crude fibre in foodstuffs at 38 and 25 g per day for young men and women, respectively (AHA, 1983). Fibres from the diet have been reported as a significant modifier of the immune system, thereby, reducing the risk factors that leads to cardiovascular disease, diabetes, cancer, and obesity (Cho *et al*., 2013; Chuang *et al*., 2012;

Streppel *et al*., 2008). The proximate chemical data showed that the fruits of *F. sycomorous* can contribute significantly towards the AI for crude fibre.

Soil pH ranged from 5.6 to 7.3 while SOM ranged from 10.8 to 21.9%. The CEC also ranged from 2.0 to 3.8 meq/100.

Table 8-1: Validation of the analytical method using plant (n = 8) and soil (n =6) certified reference materials (CRMs).

Metals	BCR-402 Measured Mean	SD	Certified Mean	SD	D081-540 Measured Mean	SD	Certified Mean	SD
As	0.091	0.011	0.093	0.010	100.5	1.2	101	5.92
Cd	-	-	-	-	139.9	1.3	143	5.6
Co	0.175	0.004	0.178	0.008	200.6	6.1	199	4.1
Cr	5.18	0.065	5.19[a]	-	90.3	2.6	86.8	6.1
Fe	245		244[a]	-	12823	20.4	12800	18.0
Mn	-	-	-	-	431.7	9.4	425	9.7
Se	6.72	0.210	6.70	0.25	129.8	2.2	127	4.5
Ni	8.25	0.591	8.25[a]	-	241.2	10.1	236	4.17
Zn	25.3	0.002	25.2[a]	-	128.9	5.6	130	11.5

*Values are in µg g^{-1} dry mass (95% confidence interval).
[a] Indicative values (without uncertainty). CRM- BCR-402 for plant and D081-540 for soil.

The elemental concentrations in the soil (total and exchangeable) and fruits samples is summarised in Table 8-2. Lead is a non-essential, toxic metal. Continuous low intake in humans causes Pb accumulation in the body and can damage nervous system and kidney. The mean total soil concentrations for Pb ranged from 4.8-44.0 µg g^{-1} across the study sites. This elevated concentration is above the South African maximum permissible level of 6.6 µg g^{-1} for agricultural soil at most sites (WRC, 1997). Previously, Ogunlaja *et al*. (2017) also reported similar results for

soil Pb, which may be due to vehicular emissions. At all study sites, the concentration of Pb in the fruit samples were below the instrument detection limits. Although, total soil As, Cd, Co and Ni ranged from 7.4-8.9 µg g^{-1}, 0.9-6.7 µg g^{-1}, 1.8-27.6 µg g^{-1} and 4.5-12.1 µg g^{-1}, respectively, concentrations in the fruits were found to be below the instrument detection limits and are therefore omitted from Table 8-2. This showed that the fruits of *F. sycomorous* does not accumulate these toxic metals.

The bioaccumulation factor (BAFs exchangeable) for the major elements Ca and Mg ranged from 6.7-49.0 and 3.5-23.4, respectively, indicating the tendency of the plant to accumulate these metals. Total soil Cu concentration ranged from 5.4-44.0 µg g^{-1} which exceeded the South African maximum permissible level of 6.6 µg g^{-1} for agricultural soil at most sites (WRC, 1997). Except for sites **1**, **4** and **5**, Cu concentration in the fruits exceeded the WHO permissible limit of 10 µg g^{-1} for plants (WHO, 2005), although the fruits did not tend to bioaccumulate Cu (BAF < 1 .0 for most sites) (Table 8-2). Copper is essential for humans and it is necessary for the formation of haemoglobin and red blood cells (Davis and Mertz, 1987). Elevated levels in the food chain can result in diarrhoea, vomiting, liver damage, fatigue and depression.

Table 8-2: Elemental concentrations (µg g^{-1}) in fruit and soil (Total (T) and exchangeable (Ex)) samples (Mean ± standard deviation; 95% confidence interval, n = 3) and bioaccumulation factors (BAFs).

Site	Element	Fruit	Soil(T)	Soil (E)	$[F]/[S]_T$*	$[F]/[S]_{Ex}$**	Ex%***
1	Ca	6963 ± 227	2944 ± 57	299 ± 38	2.4	23.3	10.2
2		6089 ± 197	741 ± 35	124 ± 27	8.2	49	16.8
3		6882 ± 115	6205 ± 115	410 ± 19	1.1	16.8	6.6
4		6076 ± 817	7579 ± 385	446 ± 32	0.8	13.6	5.9

5		6917 ± 615	12155 ± 771	465 ± 18	0.6	14.9	3.8
6		5446 ± 519	5387 ± 262	510 ± 19	1	10.7	9.5
7		4447 ±77	2288 ± 49	566 ± 22	1.9	7.9	24.7
8		5092 ± 87	2214 ±154	757 ± 53	2.3	6.7	34.2
1	Cr	0.10 ± 0.13	49.7 ± 0.39	0.76 ± 0.11	0	0.1	1.5
2		0.33 ± 0.26	44.2 ± 6.54	0.39 ± 0.12	0.01	0.8	0.9
3		0.17 ± 0.04	110 ± 25.27	0.52 ± 0.01	0	0.3	0.5
4		0.39 ± 0.01	35.9 ± 4.48	0.55 ± 0.01	0.01	0.7	1.5
5		0.20 ± 0.10	40.5 ± 3.78	0.57 ± 0.01	0	0.3	1.4
6		0.12 ± 0.08	36.0 ± 3.42	0.59 ± 0.01	0	0.2	1.6
7		0.04 ± 0.03	31.8 ± 2.25	0.61 ± 0.01	0	0.07	1.9
8		0.35 ± 0.05	57.7 ± 5.42	0.63 ± 0.01	0.01	0.6	1
1	Cu	8.1 ± 0.59	12.4 ± 0.19	11.8 ± 0.16	0.6	0.7	95.2
2		10.7 ± 1.31	5.4 ± 0.48	5.1 ± 0.05	2	2.1	94.1
3		10.2 ± 0.26	40.5 ± 1.00	15.4 ± 0.32	0.3	0.7	38.1
4		9.3 ± 1.17	44.0 ± 4.62	16.0 ± 0.24	0.2	0.6	36.4
5		9.9 ± 0.63	19.2 ± 1.90	16.4 ± 0.15	0.5	0.6	85.5
6		10.5 ± 0.80	22.7 ± 0.87	16.8 ± 0.20	0.5	0.6	74.0
7		10.8 ± 1.75	15.1 ± 0.58	12.4 ± 2.23	0.7	0.9	82.6
8		10.8 ± 0.11	12.3 ± 1.82	11.7 ± 0.26	0.9	0.9	95
1	Fe	18.6 ± 14.95	11695 ± 121	234 ± 26.74	0	0.08	2
2		65.4 ± 16.19	8849 ± 844	418 ± 359	0.01	0.2	4.7
3		45.1 ± 8.44	36700 ± 1900	437 ± 53.42	0.	0.1	1.2
4		55.7± 9.97	9194 ± 1319	438 ± 64.91	0.01	0.1	4.8
5		64.1 ± 17.74	12159 ± 1046	436 ± 17.46	0.01	0.1	3.6
6		13.3 ± 3.25	14416 ± 1322	399 ± 58.49	0	0.03	2.8
7		22.0 ± 12.56	9302 ± 310	298 ± 92.88	0	0.07	3.2
8		9.4± 1.15	13715 ± 1980	324 ± 146	0	0.03	2.4
1	Mg	1766± 42.43	575 ± 11.18	131 ± 29.22	3.1	13.5	22.7
2		1998 ± 48.86	277 ± 28.97	85.2 ± 26.79	7.2	23.4	30.8

3		2018 ± 81.29	1381 ± 8.22	162 ± 29.90	1.5	12.5	11.7
4		1982 ± 122	1650 ± 189	161 ± 17.76	1.2	12.3	9.7
5		2060 ± 30.78	2672 ± 366	133 ± 27.39	0.8	15.4	5.0
6		2676 ± 141	2748 ± 281	118 ± 15.04	1.0	22.6	4.3
7		2199 ± 22.23	1053 ± 39.93	574 ± 56.87	2.1	3.8	54.5
8		1815 ± 26.71	602 ± 107	517 ± 70	3.0	3.5	85.9
1	Mn	15.4 ± 0.41	213 ± 4.35	184 ± 21.52	0.01	0.08	86
2		65.7 ± 1.94	55.8 ± 5.98	33.9 ± 3.02	1.2	1.9	60.7
3		14.4 ± 0.53	1615 ± 114	207 ± 3.45	0.01	0.07	12.8
4		6.0 ± 0.33	293 ± 44.33	20.8 ± 0.33	0.02	0.3	7.1
5		6.5 ± 0.16	278 ± 27.14	20.8 ± 0.12	0.02	0.3	7.5
6		14.9 ± 0.98	346 ±36.14	20.6 ± 0.14	0.04	0.7	6.0
7		3.3 ± 0.10	179 ±12.29	20.1 ± 0.50	0.02	0.2	11.2
8		9.7 ± 0.23	198 ± 29.20	16.3 ± 0.44	0.05	0.6	8.2
1	Zn	42.5 ± 21.11	62.7 ± 12	36.7 ± 0.31	0.7	1.2	58.5
2		17.7 ± 1.71	54.6 ± 58.28	29.6 ± 26.33	0.3	0.6	54.1
3		41.6 ± 8.93	134 ± 38.68	57.4 ± 7.07	0.3	0.7	42.9
4		35.5 ± 12.48	170 ± 28.95	36.2 ± 11.23	0.2	1.0	21.3
5		24.7 ± 3.18	90.4 ± 43.17	22.9 ± 4.93	0.3	1.1	25.4
6		35.5 ± 13.72	62.6 ± 19.35	16.7 ± 4.60	0.6	2.1	26.7
7		44.8 ± 9.32	80.1 ± 30.80	19.3 ± 7.93	0.6	2.3	24.1
8		35.2 ± 0.11	55.2 ± 14.50	15.2 ± 1.04	0.6	2.3	27.5

* $[F]/[S]_T$-$[Fruit]/[Soil]_{Total}$
** $[F]/[S]_A$-$[Fruit]/[Soil]_{Exchangeable}$
*** Ex% - $[Soil]_{Exchangeable}/[Soil]_{Total}$

Total soil Cr ranged from 31.8-110 µg g^{-1} and < 1.9% was in mobile form. The concentration in the fruits were within a small range of variation (0.04-0.39 µg g^{-1}) suggesting the plants control Cr uptake. This is in agreement with other reports (Adriano, 2001; Golovatyj & Bogatyreva, 1999). The available Cr ranged from 0.39-0.76 µg g^{-1}, which is less than the phytotoxicity range of 1-5

µg g^{-1} for available Cr in soil (Adriano, 2001). Chromium is an essential element to humans and its deficiency includes impaired glucose tolerance, elevation in serum insulin, glycosoria, impaired growth and altered immune function. In this study, the elemental concentrations in the fruits were in the decreasing order of Ca > Mg > Fe > Zn > Mn > Cu > Cr and the toxic metals (As, Cd, Co and Pb) were below the instrument detection limits.

8.3.2 Estimated contribution of metals to the diet

Fruits play a very important role in the diet of humans, as they provide micronutrients which can contribute beneficially to Recommended Dietary Allowances (RDAs), and may help meet the nutritional needs of impoverished rural communities where nutritionally deficient diseases are common. Table 8-3 shows the comparison of the mean elemental concentrations in 20.0 g of *F. sycomorous* fruits with the Dietary Reference Intake (DRI) for most individuals. The results showed that the fruits contained most essential elements, and intake of 20.0 g can adequately contribute to the diet without exceeding the tolerable upper intake levels (ULs) for most elements (Table 8-3). Depletion of Ca and Mg in the growth soil are often linked to deficiencies in diet, which can be managed by the use of Ca and Mg supplements. These enhancement drugs may be taken to strengthen bones, regulate muscle and nerve function and control blood pressure. The consumption of 20.0 g of fruits may contribute between 9-12% and 13% to the RDA for Ca and Mg, respectively. Consumption of 20.0 g of fruits would contribute about 4.5-10.1% towards the RDA for Fe and >22.0% towards the RDAs for Cu and Mn. *F. sycomorous* fruits were richer in the nutrients Ca, Mg, Mn and Zn compared to fruits of *F. sur* (11%, 9%, 4% and 3%, respectively, towards the RDA) (Ogunlaja *et al.*, 2017).

Table 8-3: Comparison of Dietary Reference Intake (DRI). *(Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Level (UL)) of elements for most individuals to the average concentration of elements (n = 3) in *F. sycomorous* fruits.

Element	Average concentration (mg / 20 g DM)	DRI (mg/ day)		Estimated contribution to RDA (%)
		RDA	UL	
Ca	122.35	1000-1300	2500	12
Cr	0.0043	0.024-0.035	ND	20
Cu	0.20	0.9	8	22
Fe	0.81	8-18	45	10
Mg	42.00	310-320	350	14
Mn	0.36	1.6-2.3	9	23
Zn	0.69	8-11	34	9

*Sourced from: Food and Nutrition Board, Institute of Medicine, National Academies, 2011.

Chromium is known to improve the efficiency of insulin and it is needed in the metabolism of protein, fat and carbohydrate (Chowdhury *et al*., 2003). Its deficiency includes diabetes and cardiovascular disease. For Cr, a daily intake range of 0.024-0.035 mg is endorsed and eating of approximately 20.0 g of *F. sycomorous* fruits, may contribute approximately 0.0043 mg (12.3-19.9%) towards its RDA.

8.3.3 Statistical result

To further identify the elemental association in the soil, principal component analysis (PCA) was used for elemental source identification. Table 8-4 shows the factor loadings with a VARIMAX rotation, as well as the eigenvalues. Three principal components (factors) were obtained describing the 86.3% of the total variance. Factor 1 is strongly dominated by Cd (0.98), Co (0.95), Cr (0.95),

Fe (0.97), Mn (0.96), Ni (0.90) and Pb (0.81) accounting for 56.6% of the total variance. The high loadings and close association suggest their common anthropogenic inputs. Previously, we reported similar patterns in soils from KwaZulu-Natal (Mahlangeni *et al*., 2016).

Table 8-4: Rotated component matrix for variables in the soil samples (n = 40)

	Component		
Element	1	2	3
Cd	0.98		
Fe	0.97		
Mn	0.96	0.17	0.16
Co	0.95	0.17	0.12
Cr	0.95	-0.12	
Ni	0.90	0.31	-0.11
Pb	0.81	-0.11	
Ca	0.12	0.94	0.14
Mg		0.91	0.19
As	-0.31		0.81
Cu	0.48	0.41	0.71
Zn	0.29	0.32	0.64
Eigenvalues	6.79	2.48	1.08
% Total variance	56.62	20.66	8.99
Cumulative %	56.62	77.28	86.28

Extraction Method: Principal Component Analysis. Rotation Method: Varimax with Kaiser Normalisation.

The strong association between Ca and Mg in Factor 2 accounts for 20.7% of the total variance indicating that the element originated from soil mineral forming processes. Factor 3 is represented by As, Cu, and Zn accounting for 9.0% of the total variance. Figure 8-2 shows a 3-D plot of the PCA loadings, and the relationships among the twelve metals are distinct.

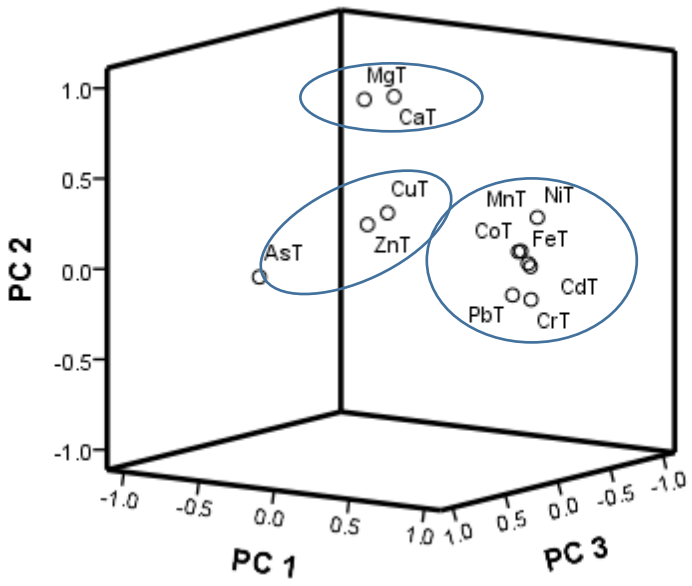

Figure 8-2: 3-D PCA analysis loading plot (PC1 vs PC2 vs PC3) for 12 elements in soil.

As expected, Cd, Co, Cr, Fe, Mn, Ni and Pb show a more significant positive association than other members of the PC, suggesting that they have a similar source (Figure 8-2). Similarly, a strong association existed between Ca and Mg, confirming their natural geological origin.

Furthermore, hierarchical cluster analysis (CA) was applied to standardised elemental concentrations and identify homogenous groups by Ward's method with Euclidean distance as the criterion for forming clusters of elements. Figure 8-3 shows that the elements were clustered into two main clusters (A and B).

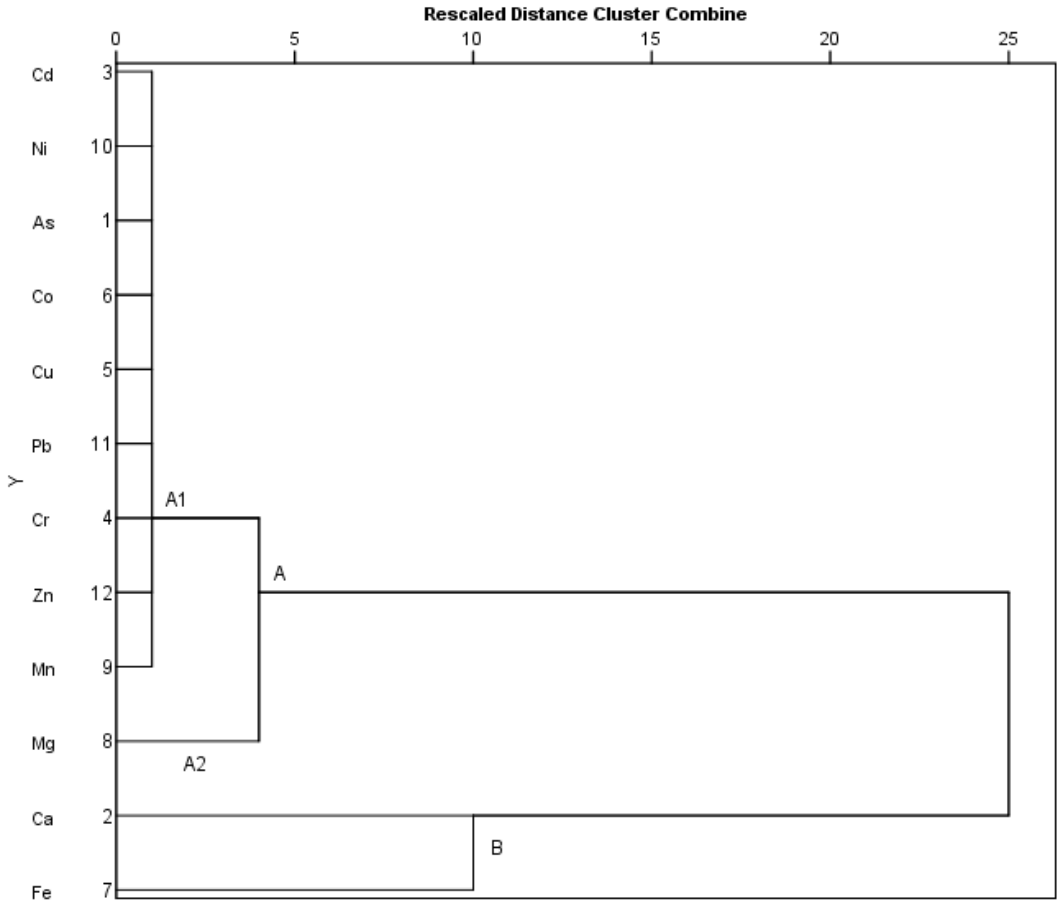

Figure 8-3: Hierarchical cluster analysis dendrogram for 12 elements in soil using Ward`s method by Euclidean distance (the distances reflect the degree of correlation between different elements).

Cluster A contains two lower clusters, A1 (Cd, Ni, As, Cr, Cu, Pb and Cr) and a stand-alone cluster A2 (Mg). Cluster B consisted of Ca and Fe. However, cluster A and B are joined at a relatively higher level, signifying a common source. The proximity in the dendrogram between Mg and cluster A suggests a form of similarity in distribution patterns in the soil. This association is stronger than the association between Mg and cluster B, occurring at a higher level. In addition, the PCA and CA produced similar results for heavy metals (Cd, Co, Cr, Ni and Pb), indicating a common source. PCA and CA also showed significant correlation between As, Cu and Zn,

suggesting another common source. The inter-item correlation matrix for concentrations in the soil and fruits of *F. sycomorous* showed no significant correlation between soil concentrations (total and available) and plant concentrations. This indicates that elemental uptake by the fruits was not dependent on soil concentrations, but the plant controlled uptake to meet metabolic needs. In soil, significant synergistic relationships existed between Mg and Ca ($r = 0.8$) and Zn and Cu ($r = 0.8$). Also, a three-way synergy existed between Mn, Fe and Cr ($r \geq 0.9$). These relationships were also seen in PCA and CA.

8.4 Conclusion

Data from this study provides information on the nutritional significance of the fruits of *F. sycomorous* which suggested that it is good for health and does not have a tendency to accumulate toxic elements (As, Cd, and Pb). The consumption of 20.0 g of fruits may contribute 22% and 23% towards the RDA for Cu and Mn, respectively. The elemental concentrations in the fruits were in decreasing order of Ca > Mg > Fe > Zn > Mn > Cu > Cr. Although, sites had effect on elemental distribution, statistical analyses showed that uptake of elements by the fruits is controlled to meet physiological needs as evidenced by the bioaccumulation factors.

Acknowledgements

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CHAPTER 9

SUMMARY AND CONCLUSION

9.1 Summary

This work focused on three *Ficus* (Moraceae) species which are indigenous to South Africa and produce edible fruits namely *Ficus burtt-davyi* Hutchinson, *Ficus sur* Forssk and *Ficus sycomorus* Linn. For many centuries, indigenous communities in KwaZulu-Natal, South Africa have used their edible fruits for food, however, other parts such as leaves, bark, and roots are utilised as medicine, in the management, treatment and control of different human diseases such as sore throat, diarrhoea, anaemia, infertility, chest infections, coughs, respiratory and liver problems, ring worms, toothache, scrofula, pulmonary tuberculosis, influenza, skin diseases, dysentery as well as sexually transmitted diseases. Numerous studies have shown that *Ficus* species are a good source of bioactive compounds and essential nutrients. Previous studies on the *Ficus* genus also indicated moderate to good antioxidant and cytotoxic activities, but information on the nutritional and medicinal potential of some *Ficus* species found in KwaZulu-Natal is still lacking. This study described the phytochemistry and biological activities of *F. burtt-davyi and F. sur*. In addition, it focused on the proximate chemical composition (carbohydrate, protein, lipid, crude fibre and ash content), as well as the elemental distribution in the edible fruits of *F. burtt-davyi, F. sur* and *F. sycomorus* in order to assess for potential toxicities. This was done by the determination of the elemental concentrations in corresponding growth soil, sampled from eight different sites in KwaZulu-Natal. The nutritional value of the three edibles figs was also determined by comparing their elemental concentrations to recommended dietary allowances (RDAs).

9.2 Findings from *F. burtt-davyi* Hutchinson

Previously, *F. burtt-davyi* was only reported in the treatment and management of stomach-aches. A phytochemical investigation of the leaves, fruits and stem bark resulted in the isolation of six triterpenes (lupeol, lupeol acetate, α-armyrin, β-sitosterol, stigmasterol and campesterol), one carotenoid (lutein), a phaeophytin (phaeophytin **a**) and one flavonoid (+)-catechin). The compound, (+)-catechin demonstrated antioxidant as well as cytotoxic activities. Phaeophytin **a** was previously shown to possess activity against the hepatitis C virus as well as antioxidant, antibacterial and anticancer activities. Lutein has also been documented to exhibit moderate antibacterial activity. The data from this study suggests that *F. burtt-davyi* possessed moderate to good anti-oxidative activity and can be used in alternative medicine for oxidative stress related non-communicable chronic diseases. The phytochemical, antioxidant activity and cytotoxic results from this study also indicated that (+)-catechin and lupeol, the most abundant bioactive compounds in the stem bark are responsible for the synergistic cytotoxic effect of this extract against breast and colorectal adenocarcinoma cell lines.

The elemental distribution of essential elements in the fruits were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu. The fruits were found to be rich in Mn which make them a cheaper alternative to Mn supplements for boosting the immune system. The result of the proximate composition indicated that the fruits are rich in carbohydrates and low in fat content. This study shows that consumption of the fruits can contribute positively towards a balanced diet, and hence ensure food and health safety. This study showed that the edible fruits and stem bark are rich sources of triterpenes. It also demonstrated the immune boosting properties of these bioactive principles from the edible fruits and stem bark, which are especially important in rural

areas of South Africa where immune system enhancing drugs are very expensive, and generally beyond the purchasing power of most people with burden of high incidences of HIV and hepatitis.

9.3 Findings from *F. sur* Forssk

Our study revealed the leaves to contain a dark green amorphous pigment, phaeophytin **a.** In addition, two triterpenoids (β-sitosterol and lupeol) were isolated from the leaves and fruit, respectively. These compounds have been reported to show anti-inflammatory activity, as well as cholesterol-lowering effects. The fruit also afforded a strong antioxidant flavonoid, epicatechin, which has been shown to have cardio-protective benefits**.** Data from the antioxidant study of the crude extracts and compounds showed that *F.sur* can be used in traditional medicine, as an immune system enhancing alternative, for the improvement of the immunity of HIV and hepatitis patients in South Africa. Epicatechin exhibited a significantly stronger antioxidant activity than the known antioxidant, ascorbic acid.

The elemental investigation showed that the fruits conform to the Recommended Dietary Allowances (RDAs) for most elements investigated, and when eaten, may considerably improve the diet. Consumption of the fruits may lead to significant beneficial increase in Se, an essential trace element, known for its strong antioxidant and immune boosting activities which can in turn help to prevent chronic non-communicable diseases and boost the immune system of patients suffering from diseases such as HIV which is predominant in rural communities in South Africa. The results showed that fruits tended to exclude toxic metals such as As, Cd, Co and Pb and accumulated essential macro elements such as Ca and Mg. Data from this study indicates that the

fruits can serve as an immediate and alternative source of energy due to high level of carbohydrates.

This study shows that the consumption of the fruits of *F. sur* which are freely and readily available can help to prevent chronic non-communicable diseases, improve the diet as well as the health of rural communities in South Africa for most essential nutrients without posing the risk of adverse health effects.

9.4 Findings from *F. sycomorus* Linn

Proximate chemical composition showed *F. sycomorus* to be rich in fibre. The concentrations of elements in the fruits were found to be in decreasing order of Ca > Mg > Fe > Zn > Mn > Cu > Cr and toxic metals (As, Cd, Co and Pb) were below the instrument detection limit indicating that the intake of the can beneficially improve the health and nutritional needs of indigenous communities in South Africa. The Principal component (PCA) and Hierarchical cluster analyses (CA) showed that Cd, Co, Cr, Ni and Pb in the soil came from the same source whilst As, Cu and Zn have a common origin. Data from this study showed that elemental availability was dependant on the metal interactions in soil, but uptake was considerably controlled by the plant.

9.5 Overall conclusion

In general, we have successfully carried out the phytochemical investigation of some *Ficus* species and some of the isolated compounds exhibited antioxidant and cytotoxicity activities which supported their use in traditional medicine in South Africa. This study also showed that the edible

fruits are rich in phytocompounds with immune boosting properties, which are needed by most indigenous communities in South Africa. In addition, elemental analysis showed that the consumption of the fruits (*F. burtt-davyi*, *F. sur* and *F. sycomorus*) can to a great extent improve the health and nutrition of humans for the elements investigated. Data from this study lends scientific credence and validates the ethnomedicinal use of the plants. It also gives insight into the nutritional and medicinal benefits of consuming the indigenous edible fruits.

9.6 Recommendation and further work

In vitro activity was carried out in all the bioassays undertaken in this work. *In vivo* experiments need to be carried out on the bioactive extracts and compounds to further determine whether or not they have the potential to be developed into drugs.

Although the MTT assay used for cytotoxicity evaluation is fast and reliable, it does not differentiate whether these compounds kill cells or merely inhibit their growth. The mode of action of these compounds could be determined by clonogenic assays.

Isolation and identification of the phytocompounds in the other parts such as roots and twigs of the investigated plants is also recommended.

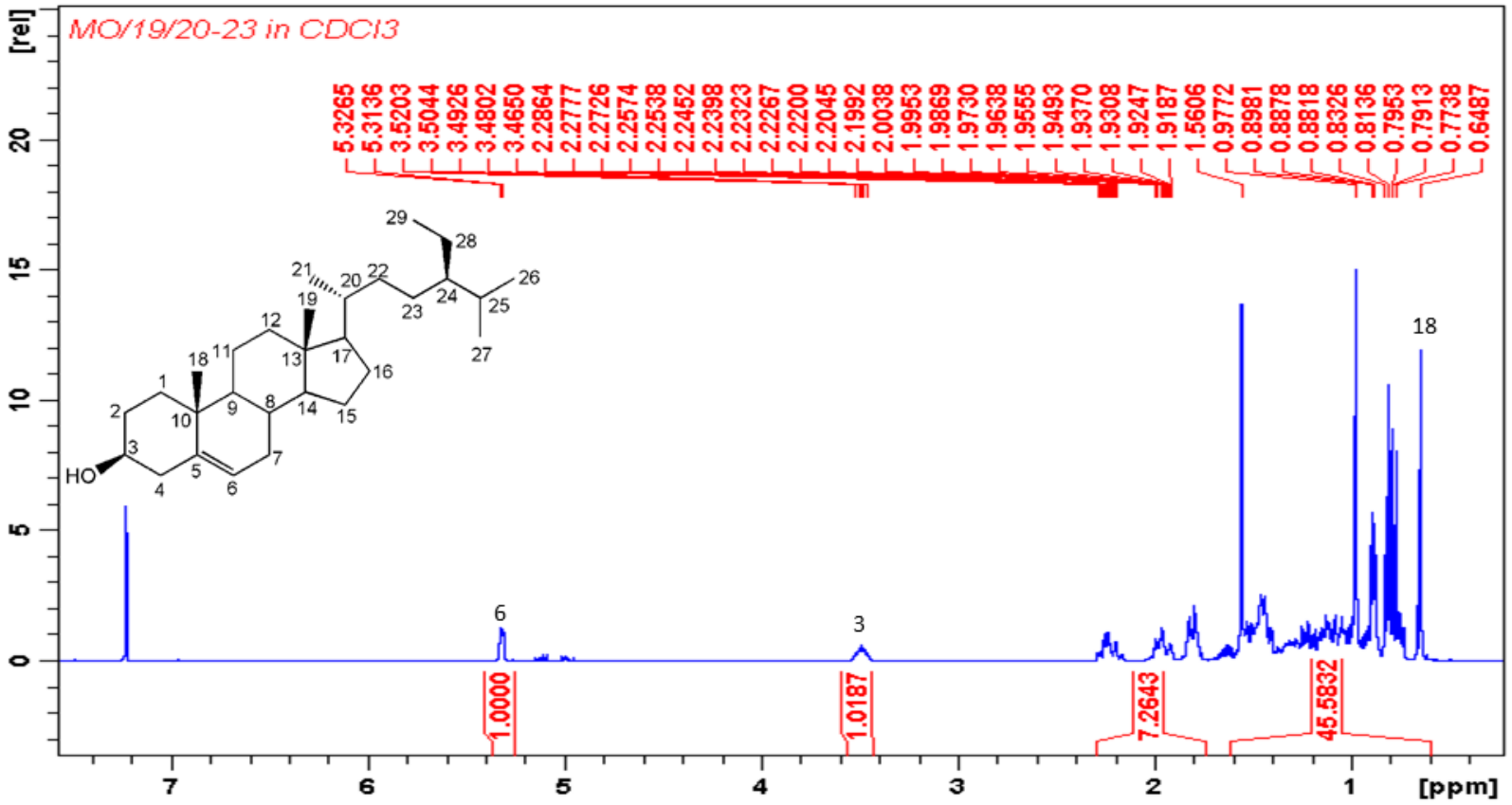
Phytochemical, Elemental and Biological studies of three *Ficus* Species (Moraceae) found in Kwazulu-Natal, South Africa

Appendix 1

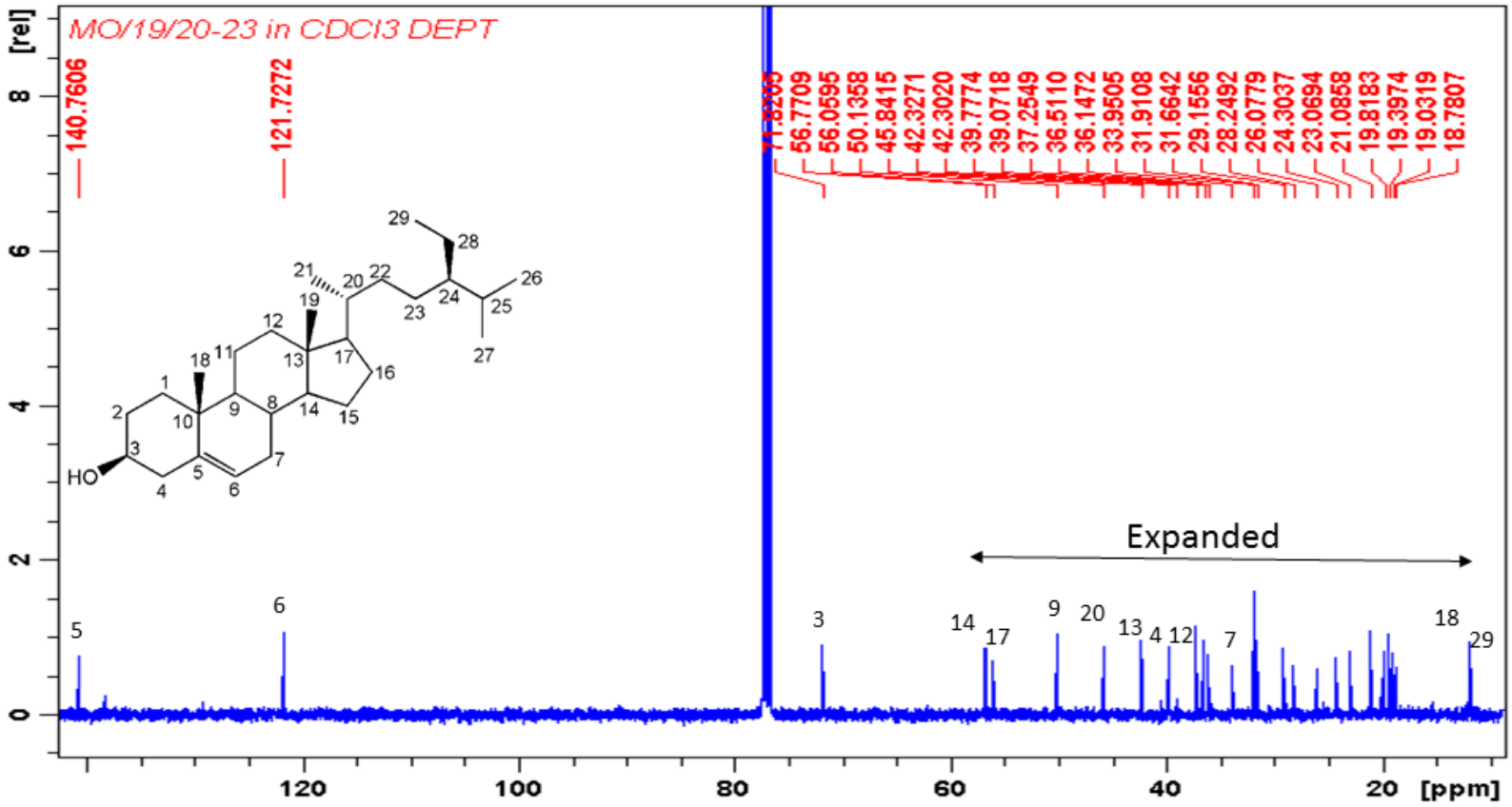
(Supporting information includes NMR, IR, UV, and MS spectra)

OLUMUYIWA OLUFISAYO OGUNLAJA

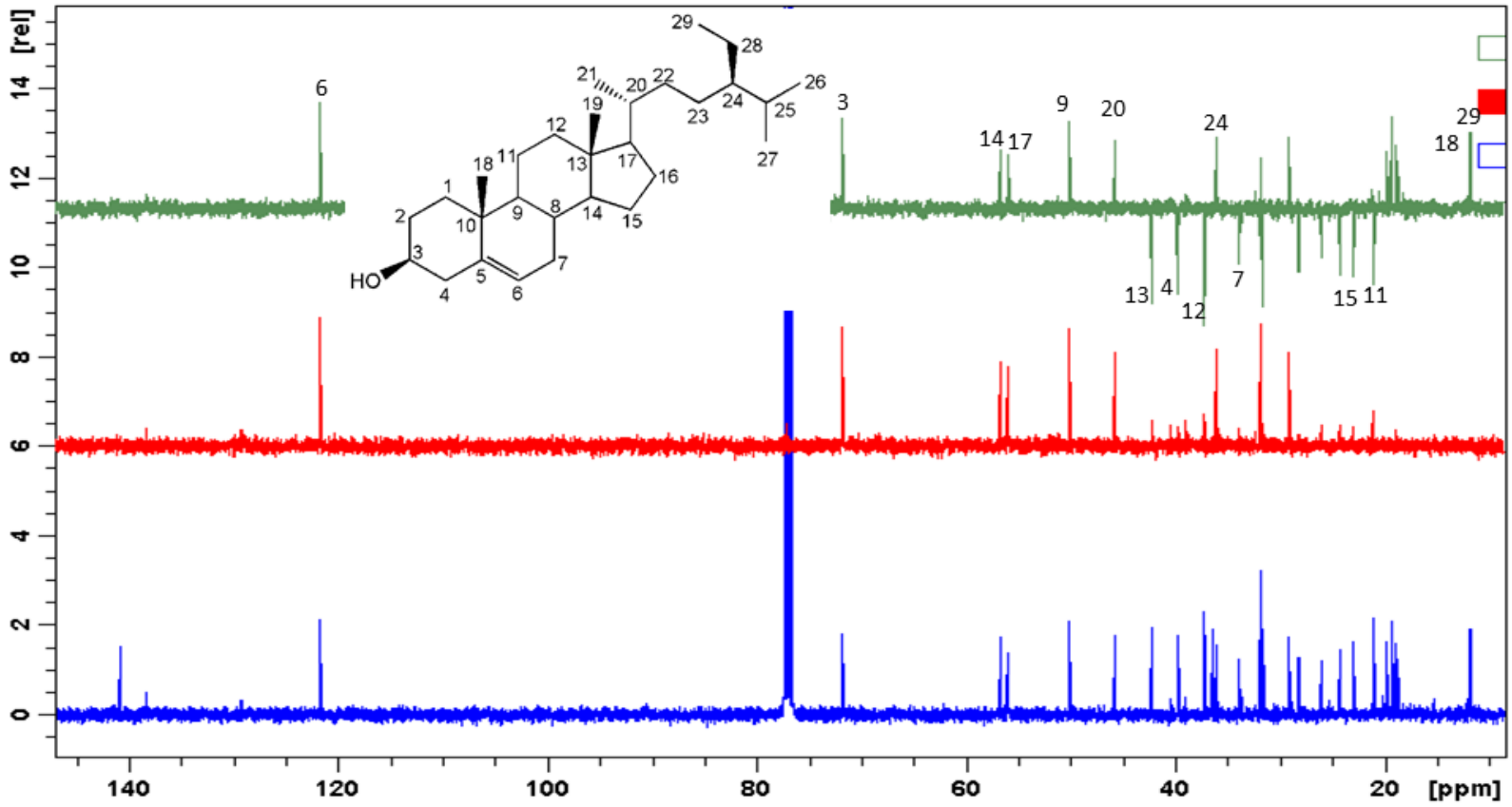
2017



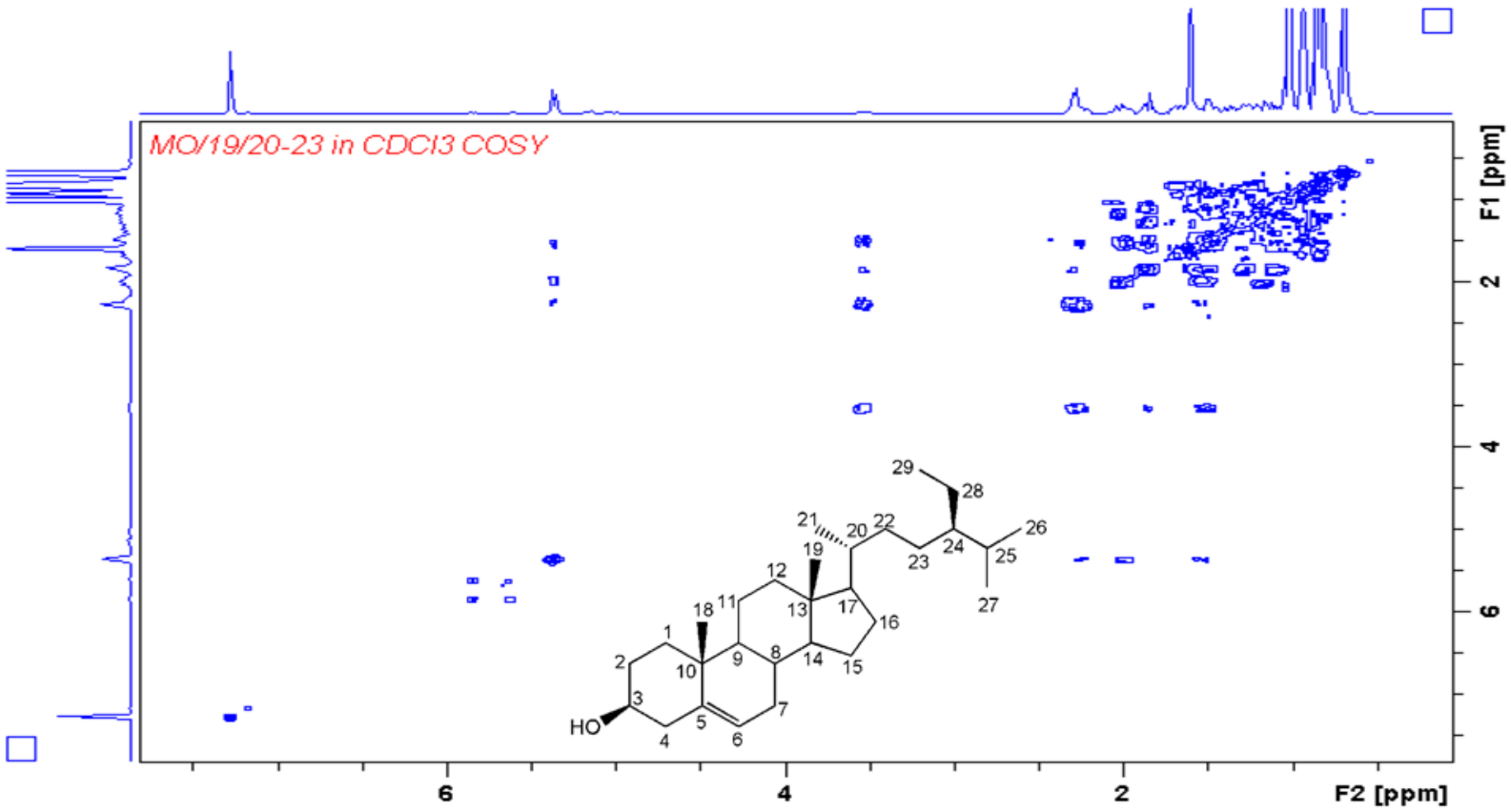
^{1}H NMR spectrum of sitosterol A-1



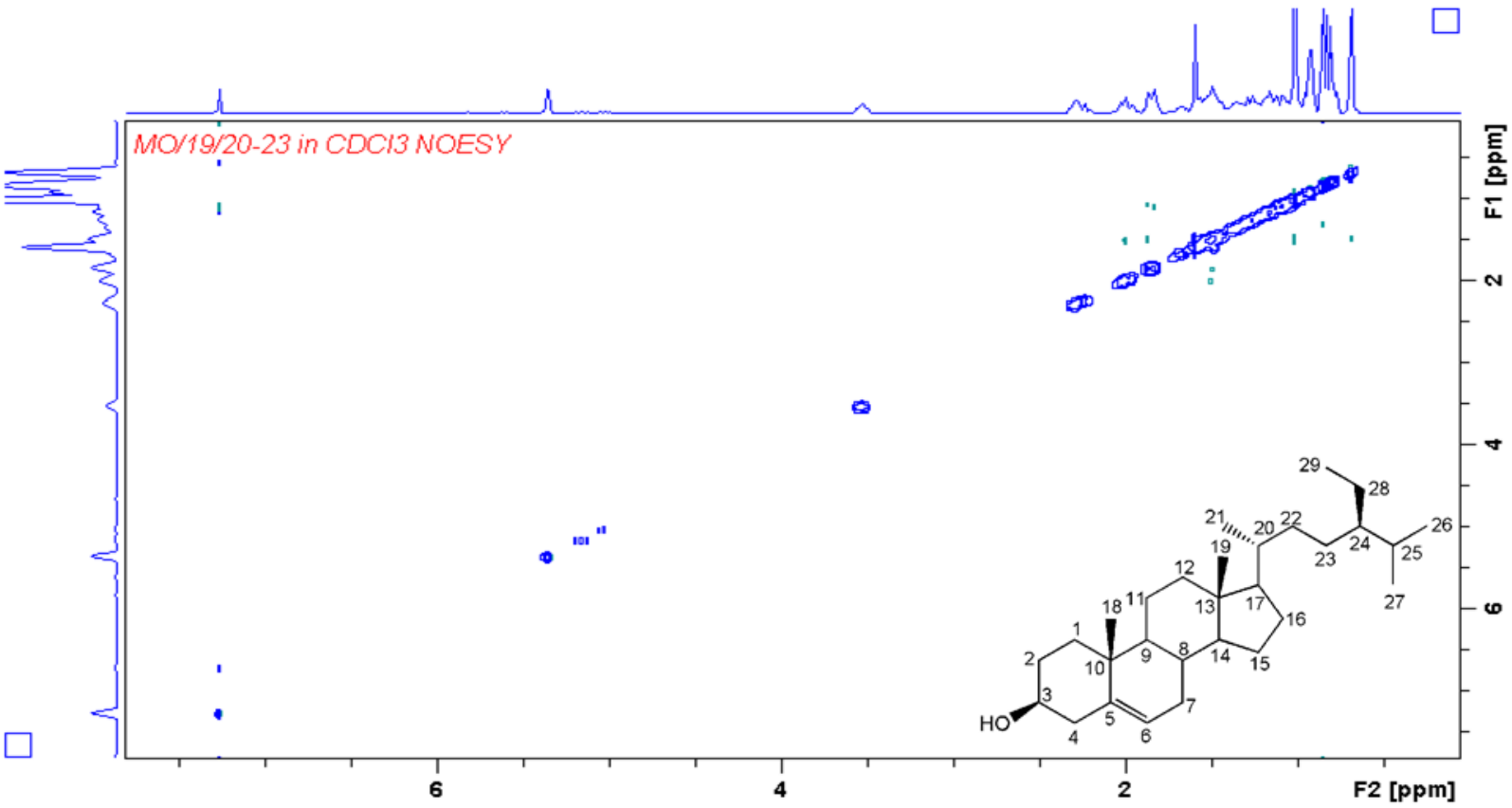
^{13}C NMR spectrum of sitosterol A-1



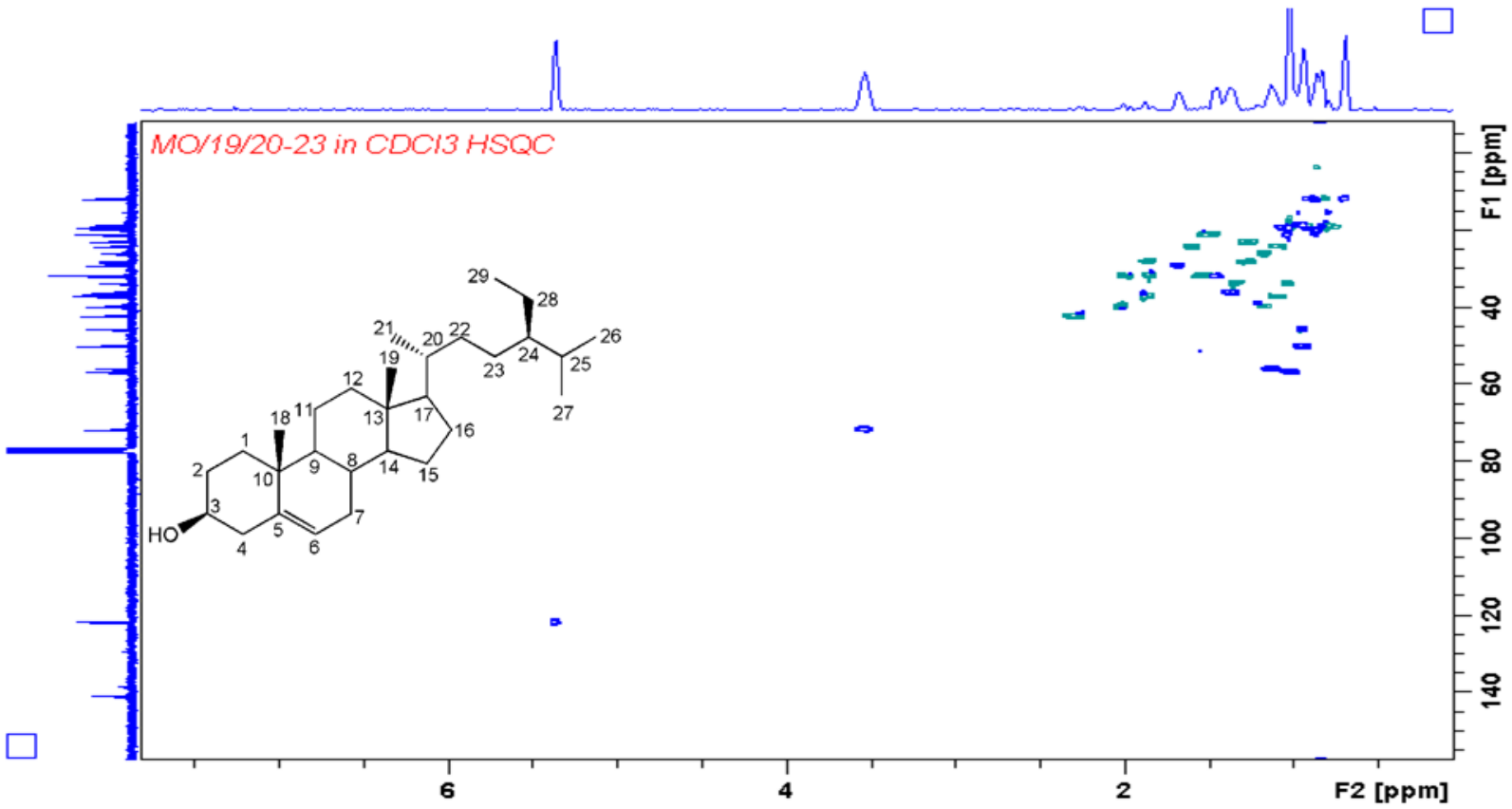
DEPT spectrum of sitosterol A-1



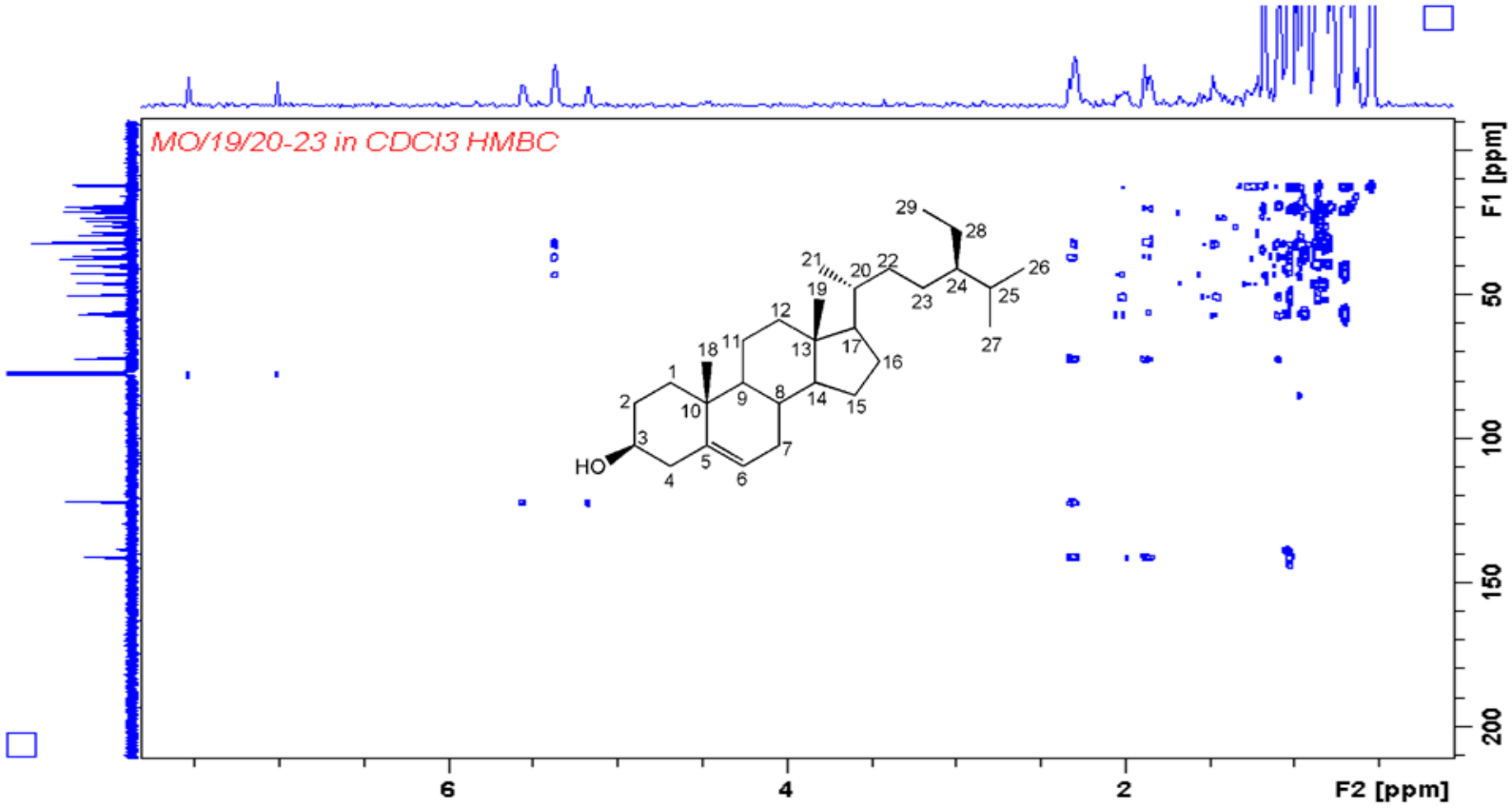
COSY spectrum of sitosterol A-1



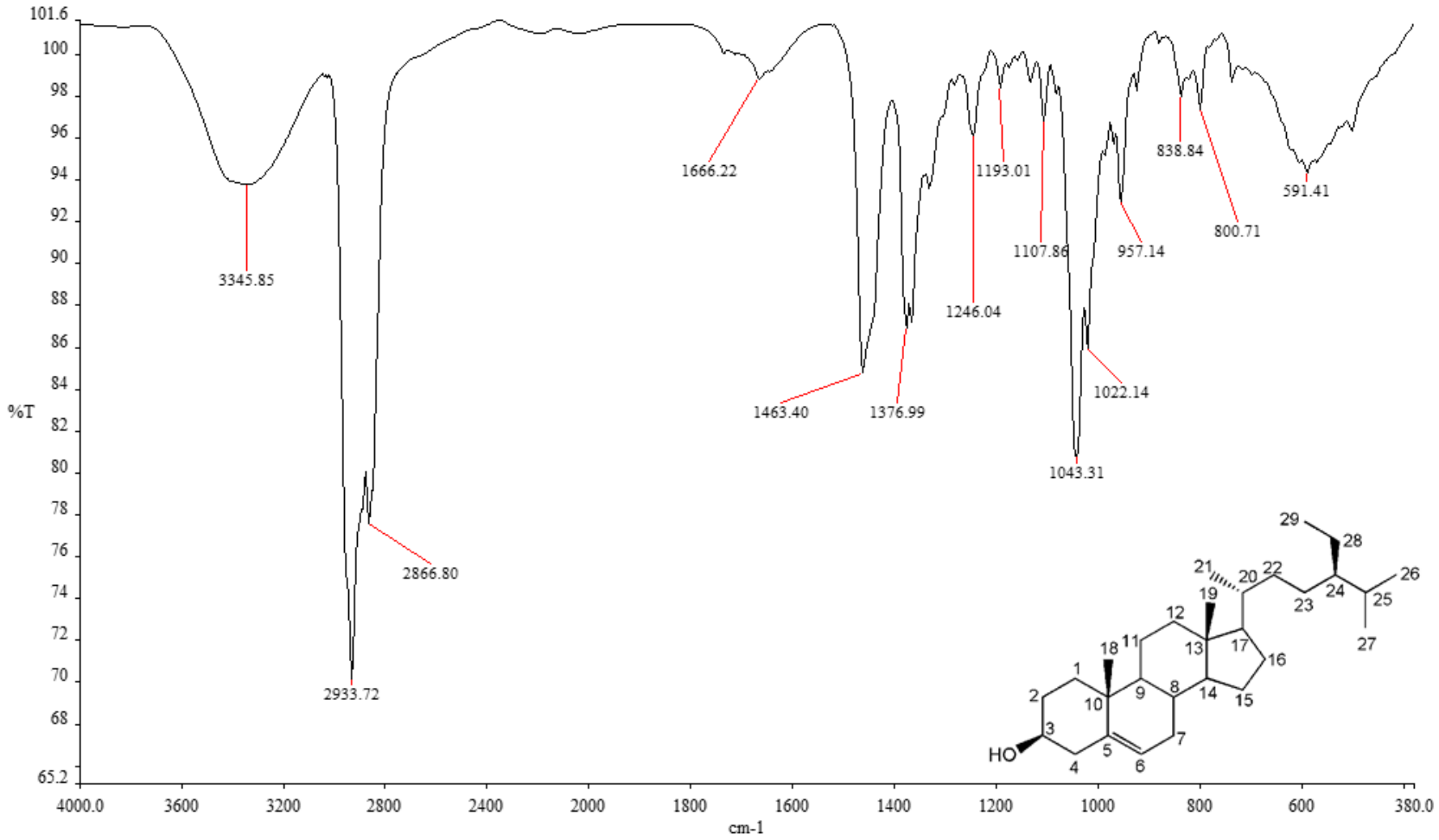
NOESY spectrum of sitosterol A-1



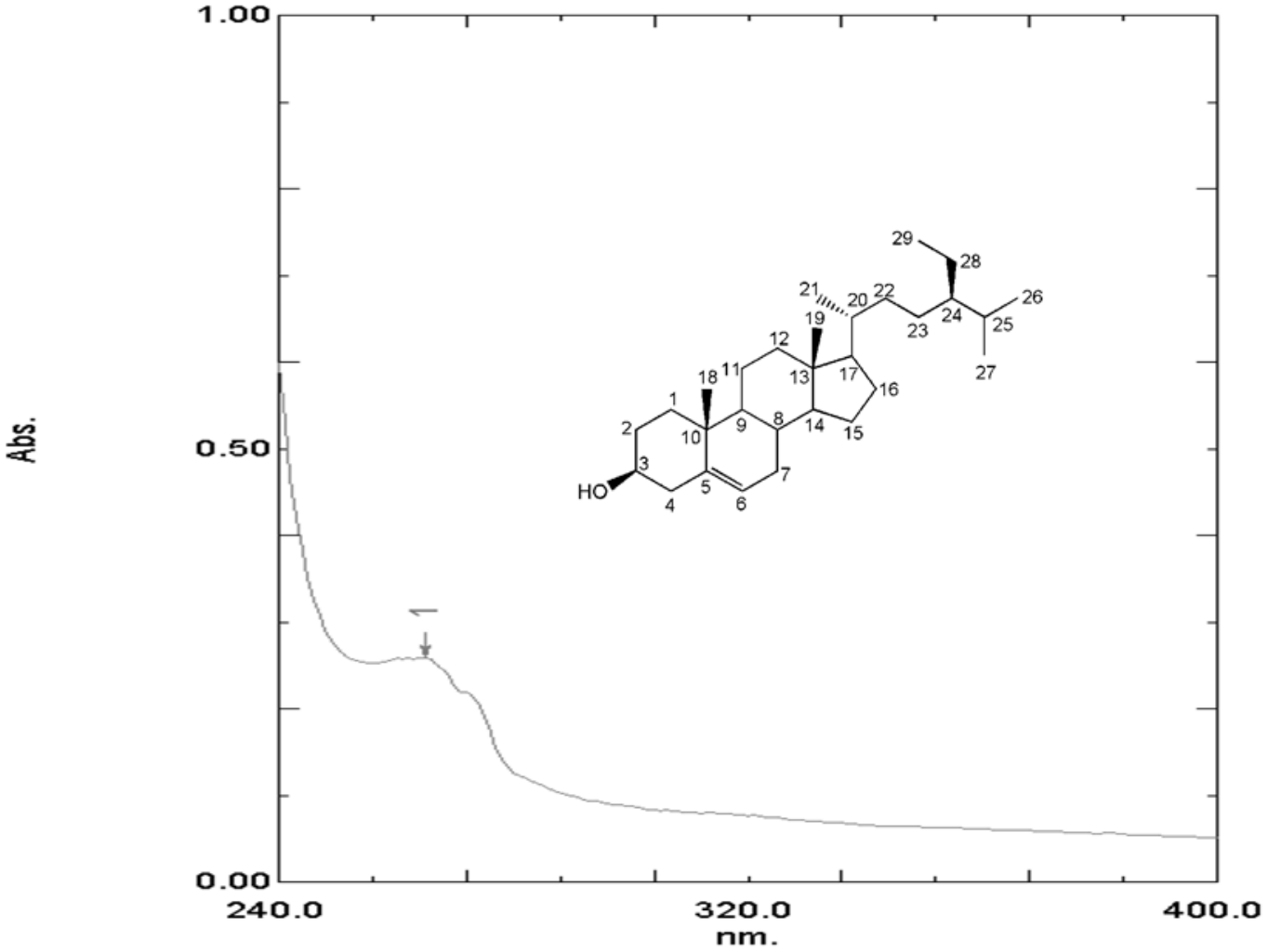
HSQC spectrum of sitosterol A-1



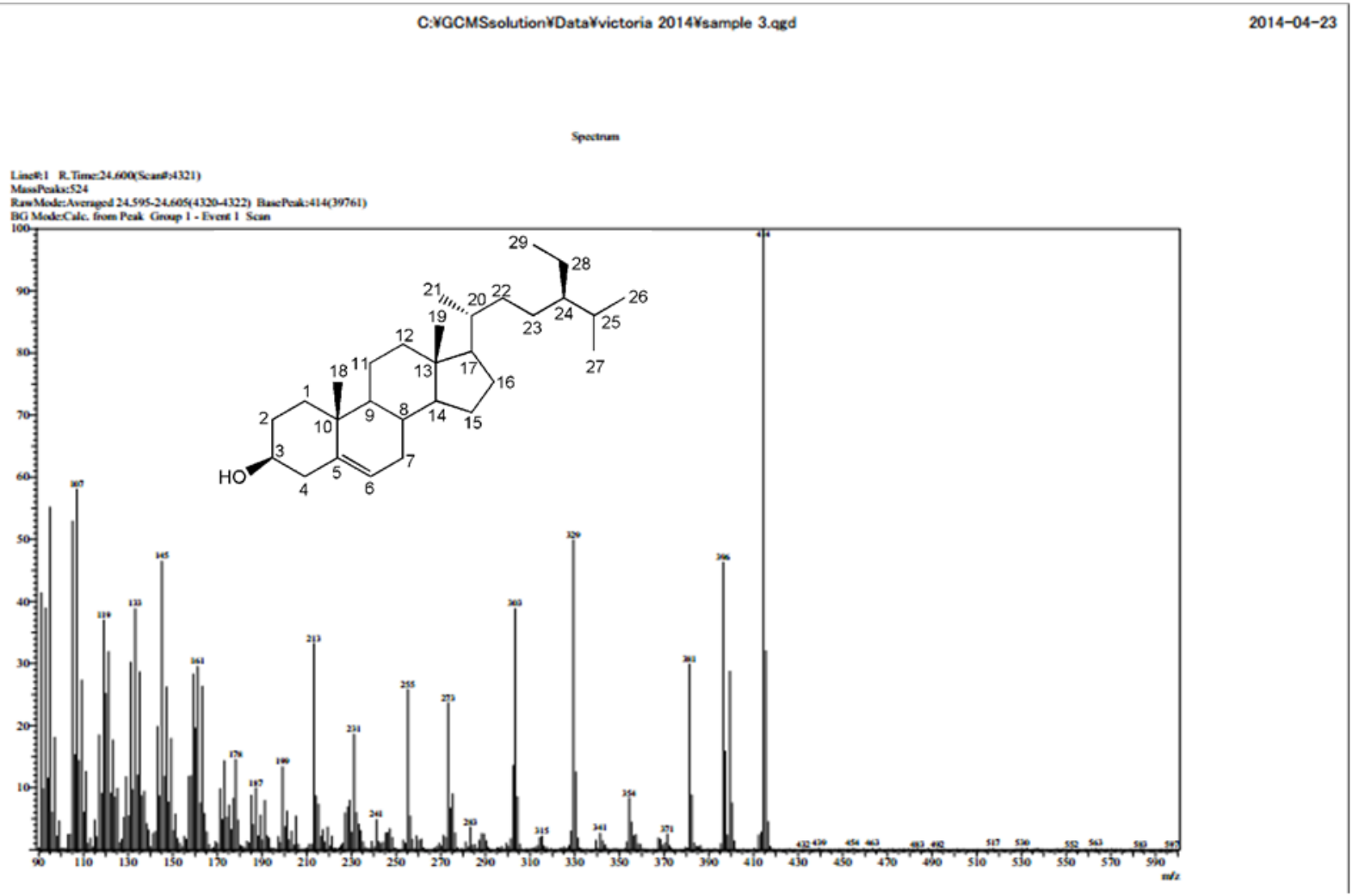
HMBC spectrum of sitosterol A-1



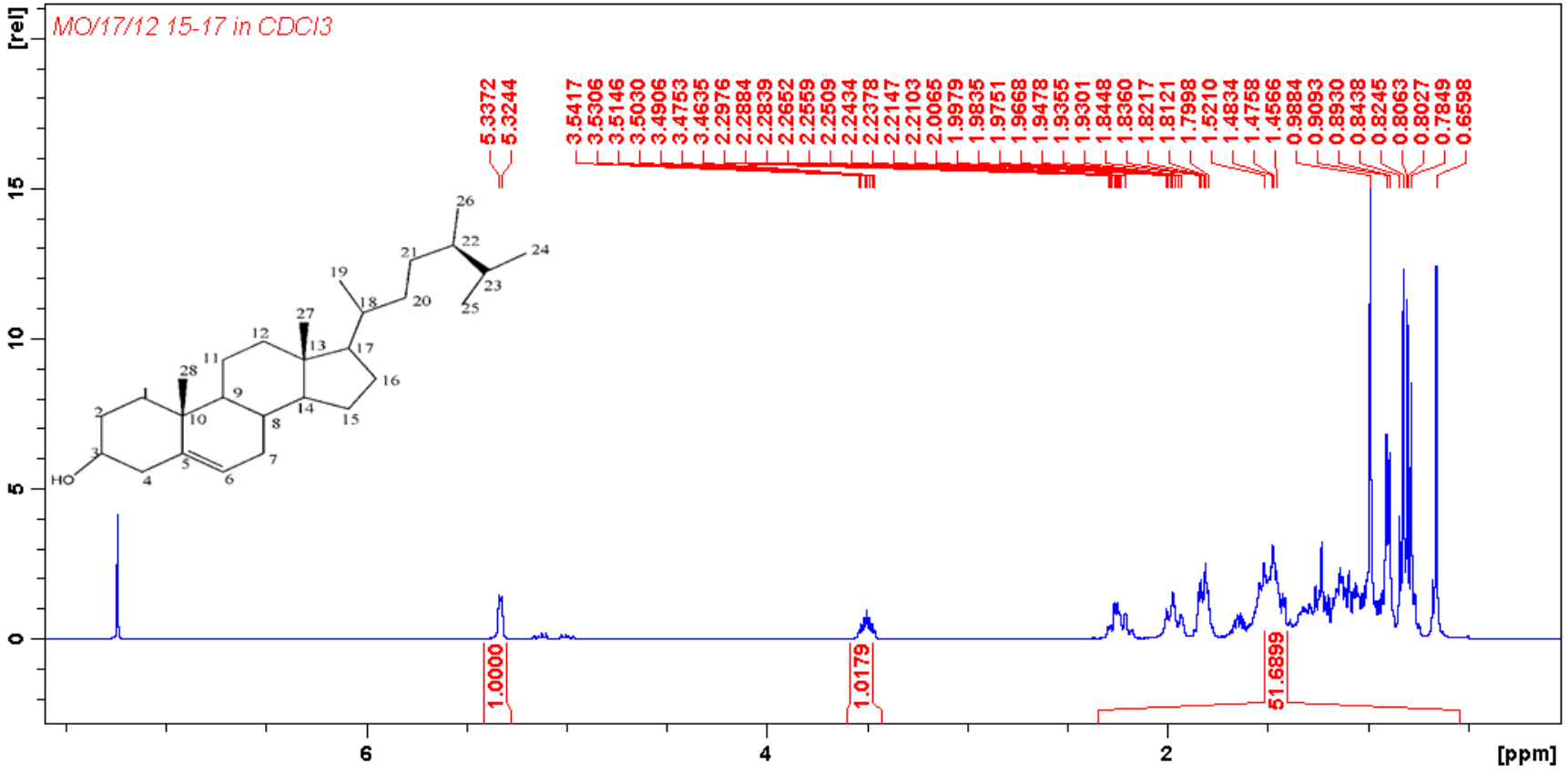
IR spectrum of sitosterol A-1



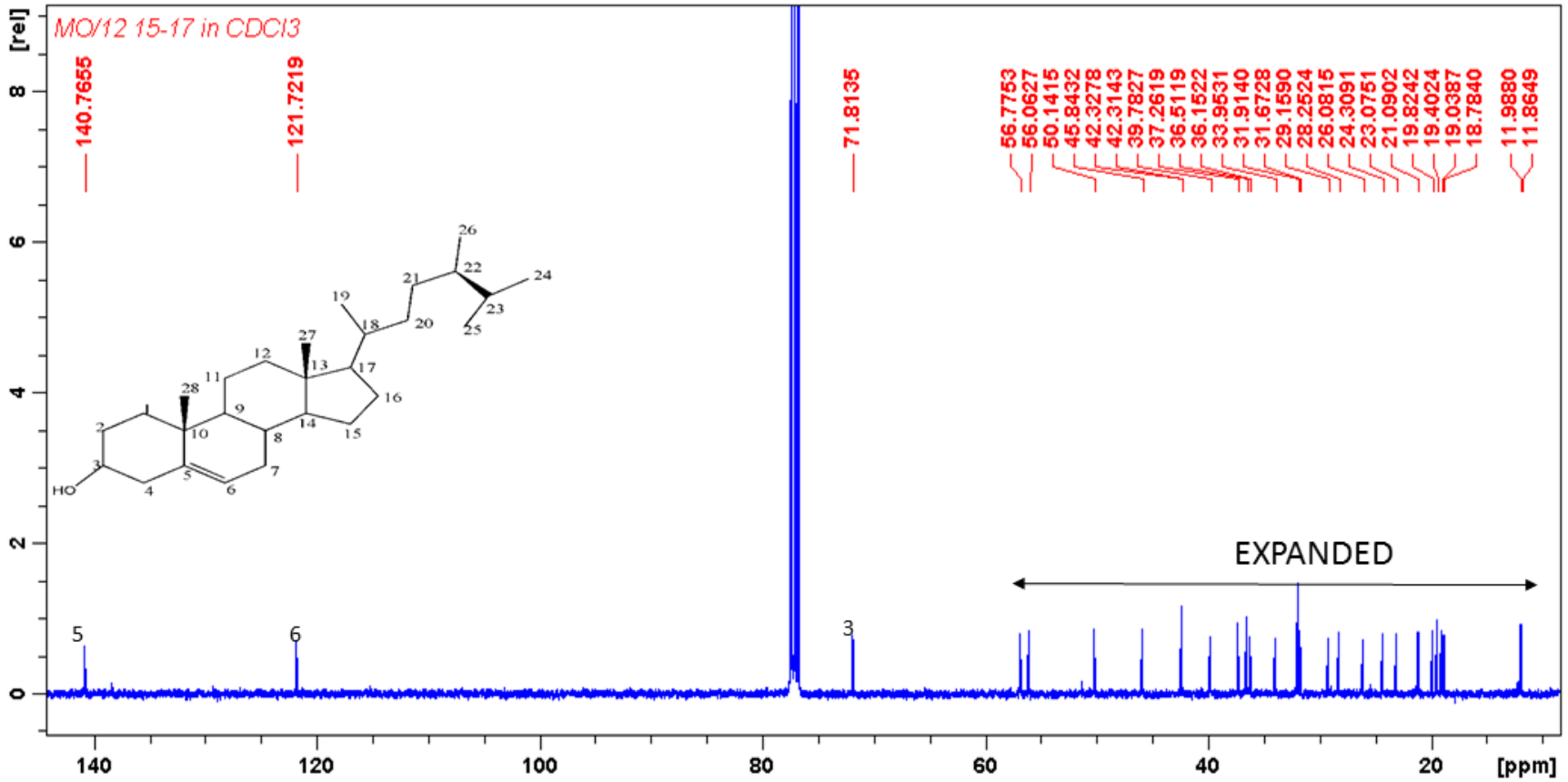
UV spectrum of sitosterol A-1



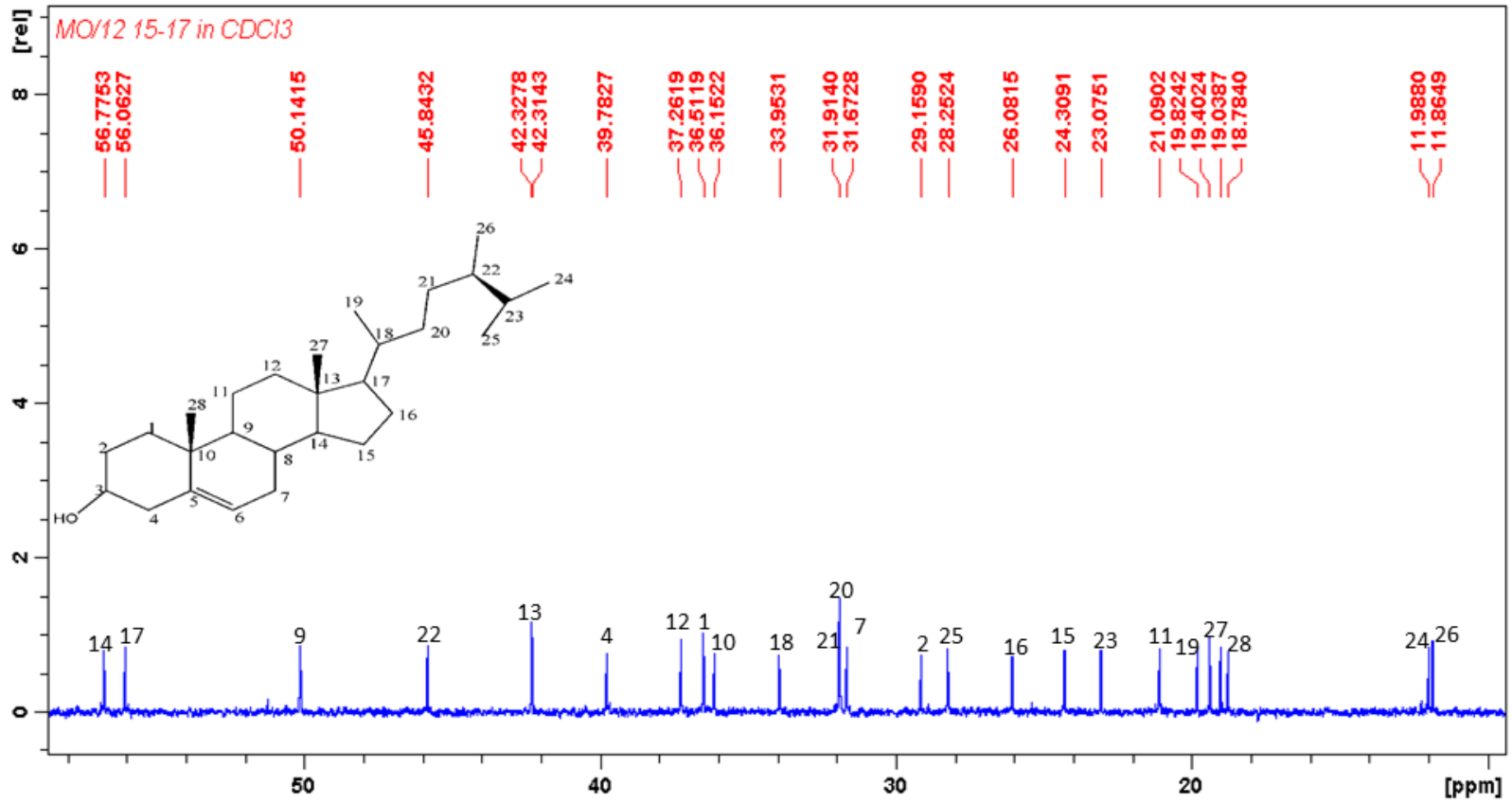
Mass spectrum of sitosterol A-1



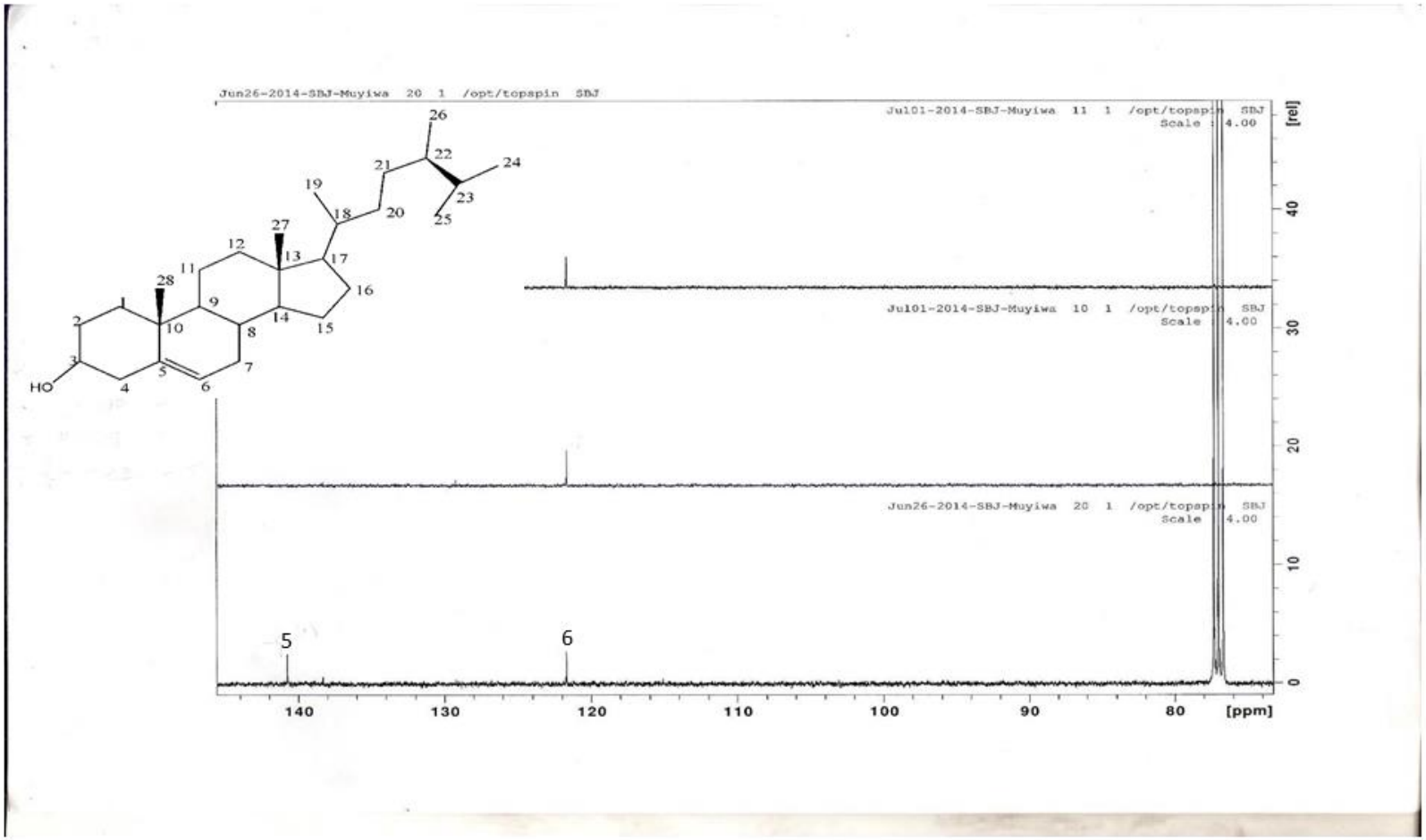
^{1}H NMR spectrum of campesterol A-2



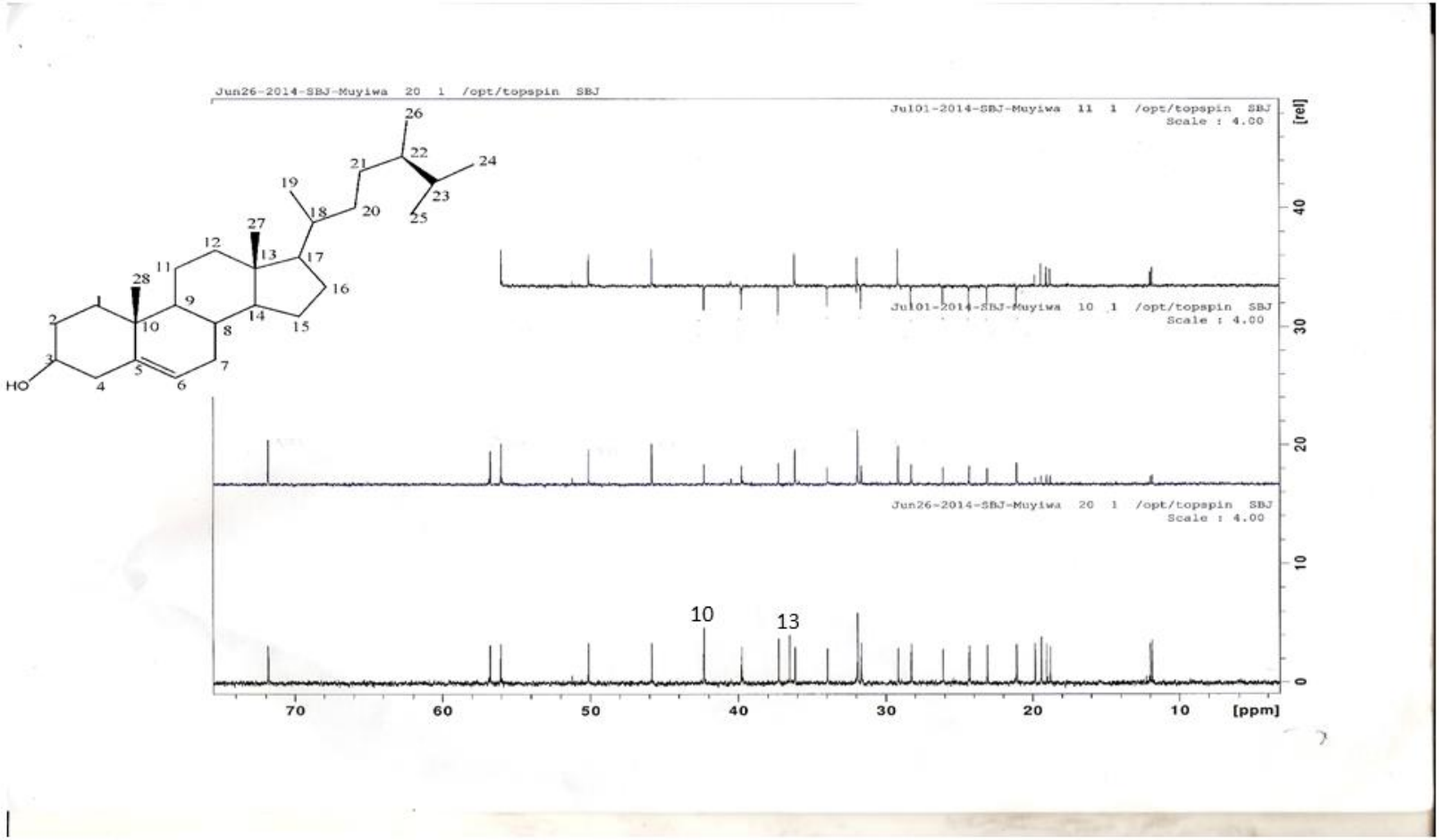
^{13}C NMR spectrum of campesterol A-2



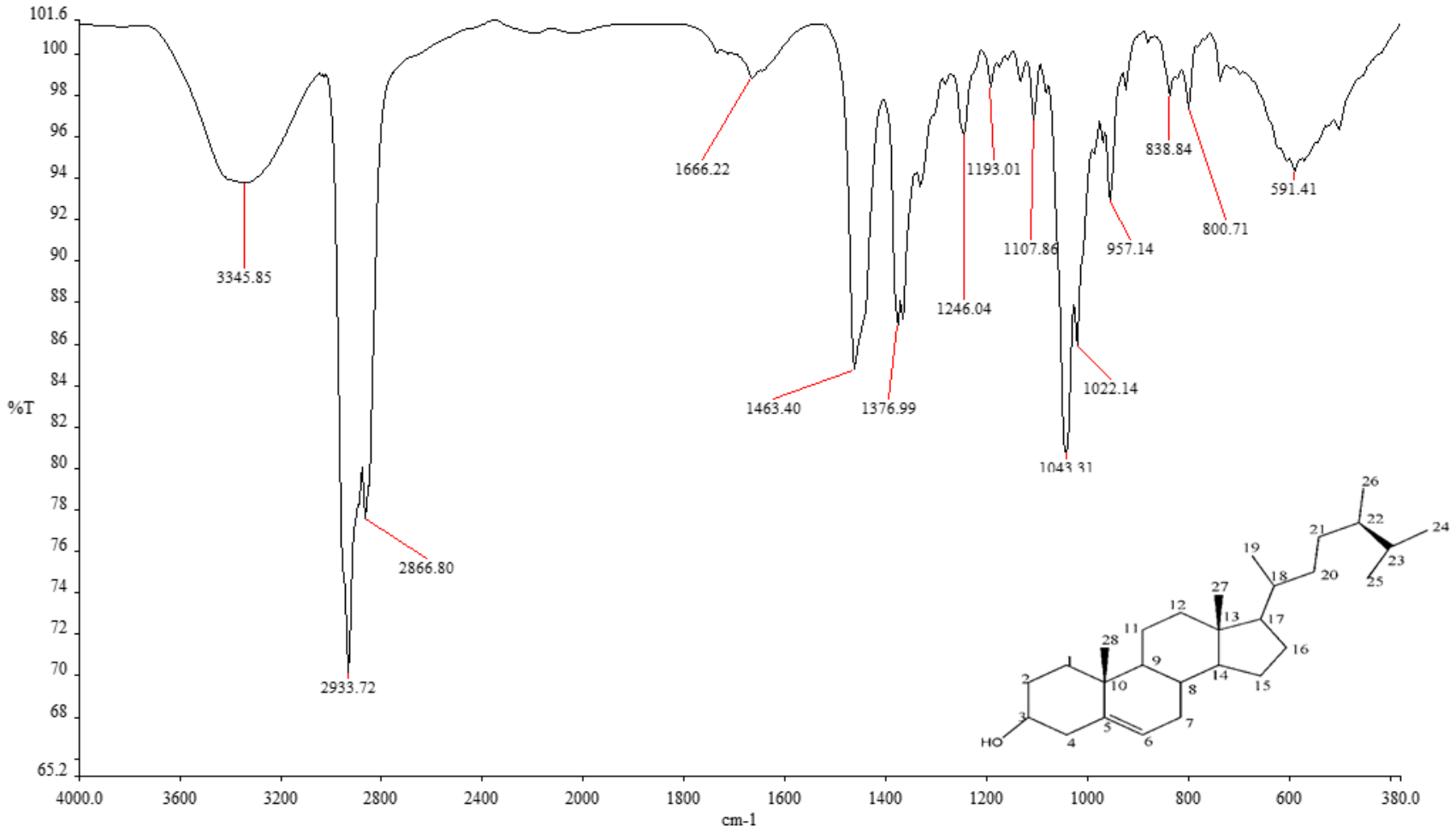
Expanded ^{13}C NMR spectrum of campesterol A-2



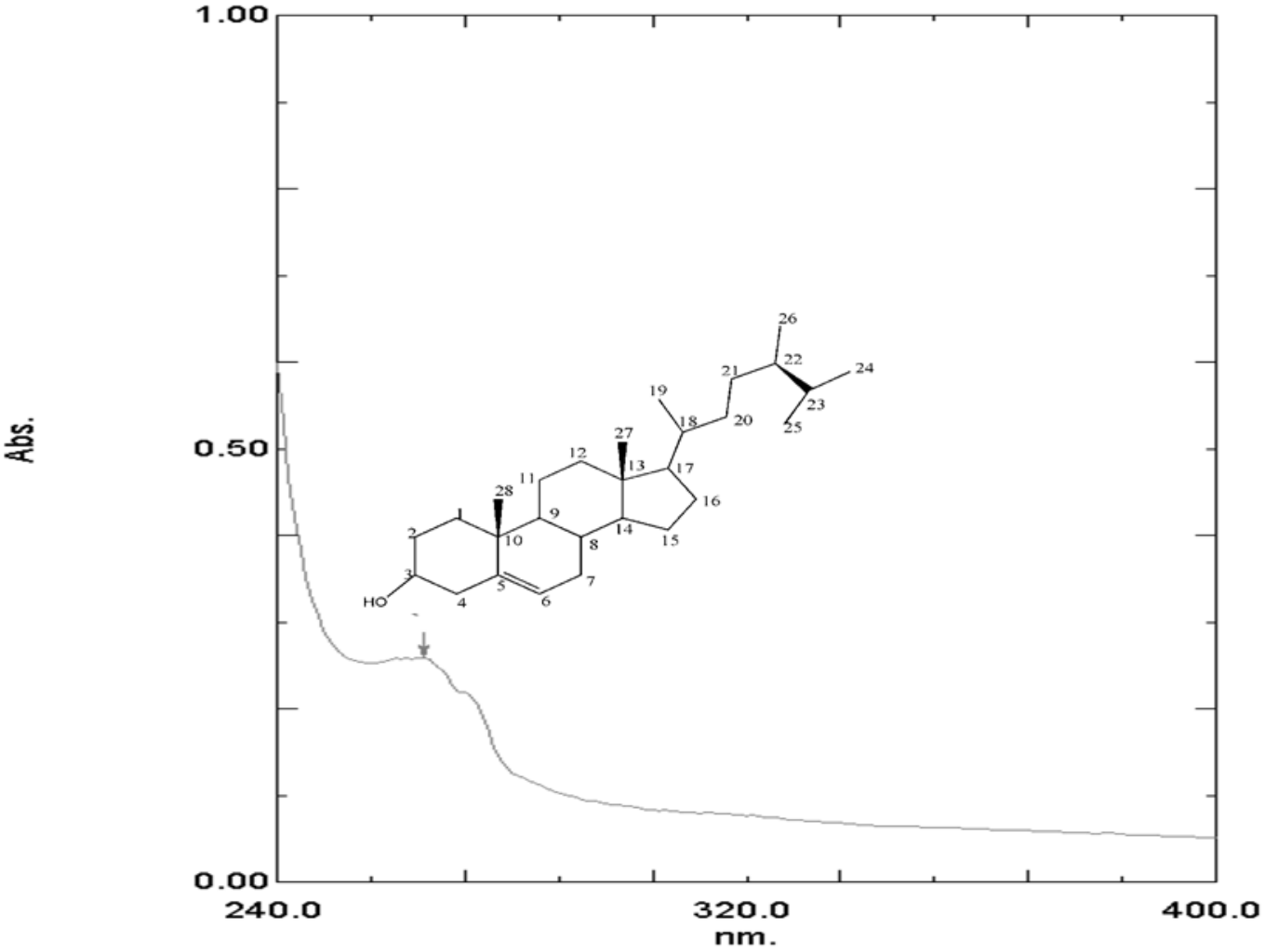
DEPT spectrum of campesterol A-2



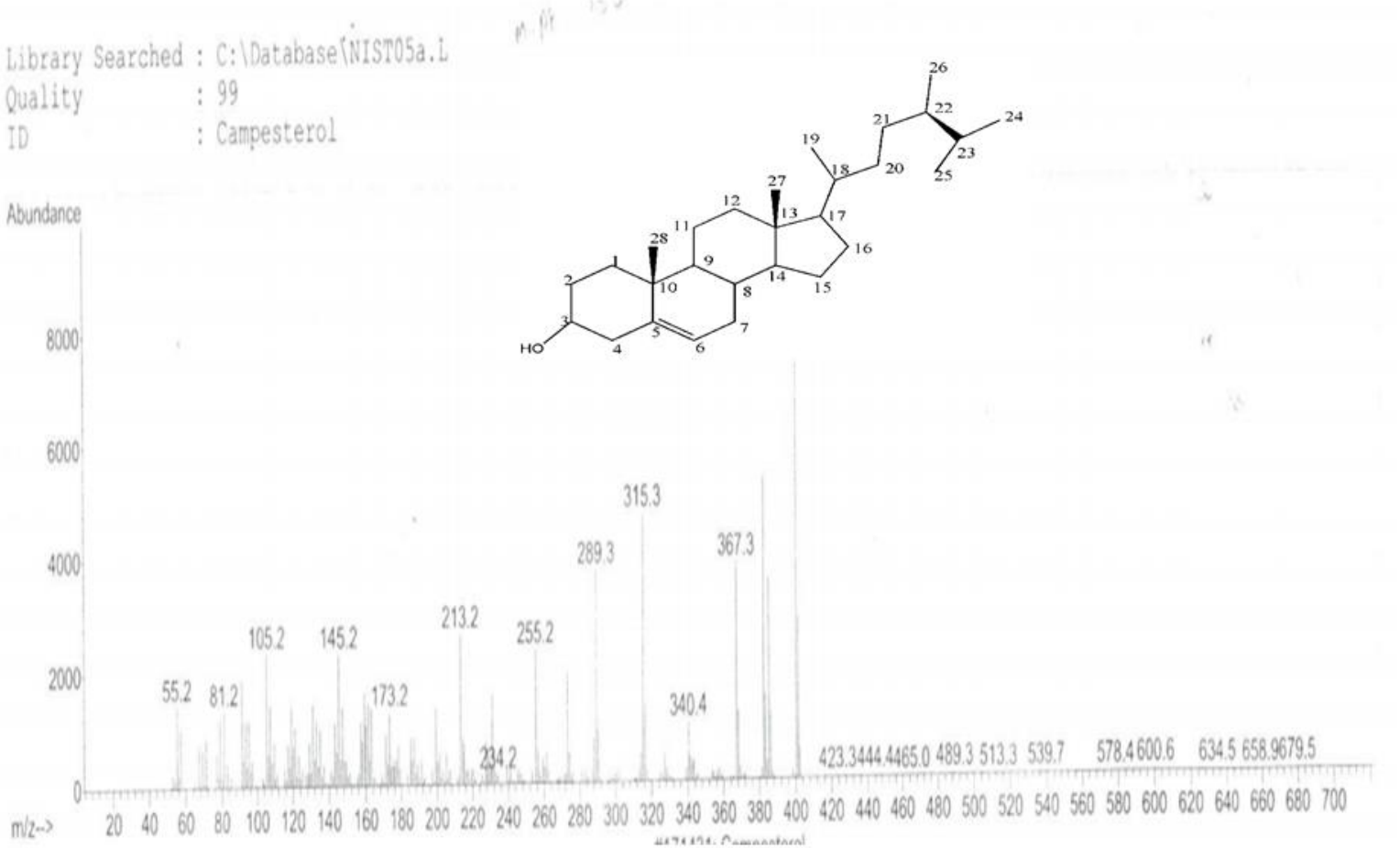
Expanded DEPT spectrum of campesterol A-2



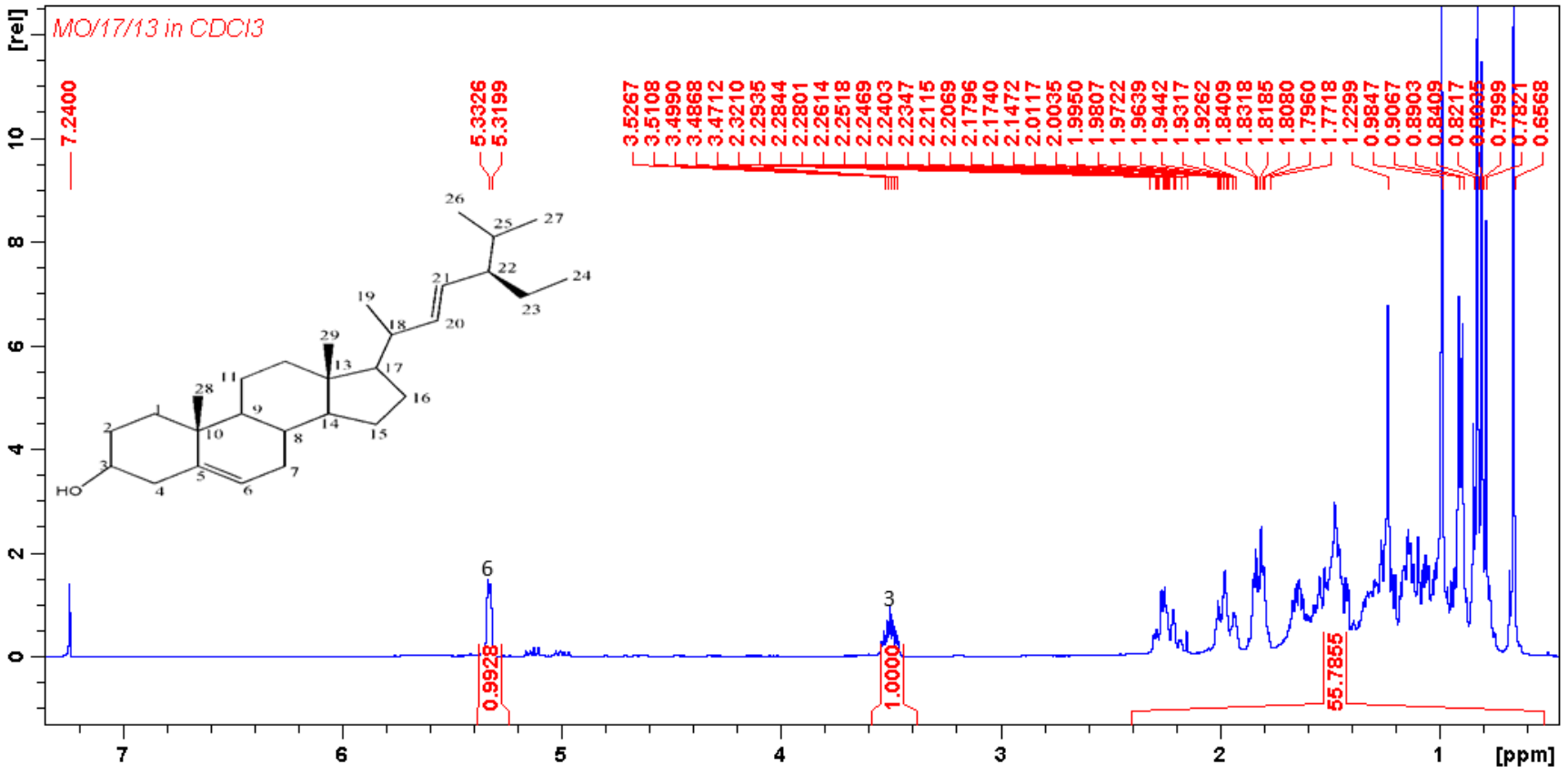
IR spectrum of campesterol A-2



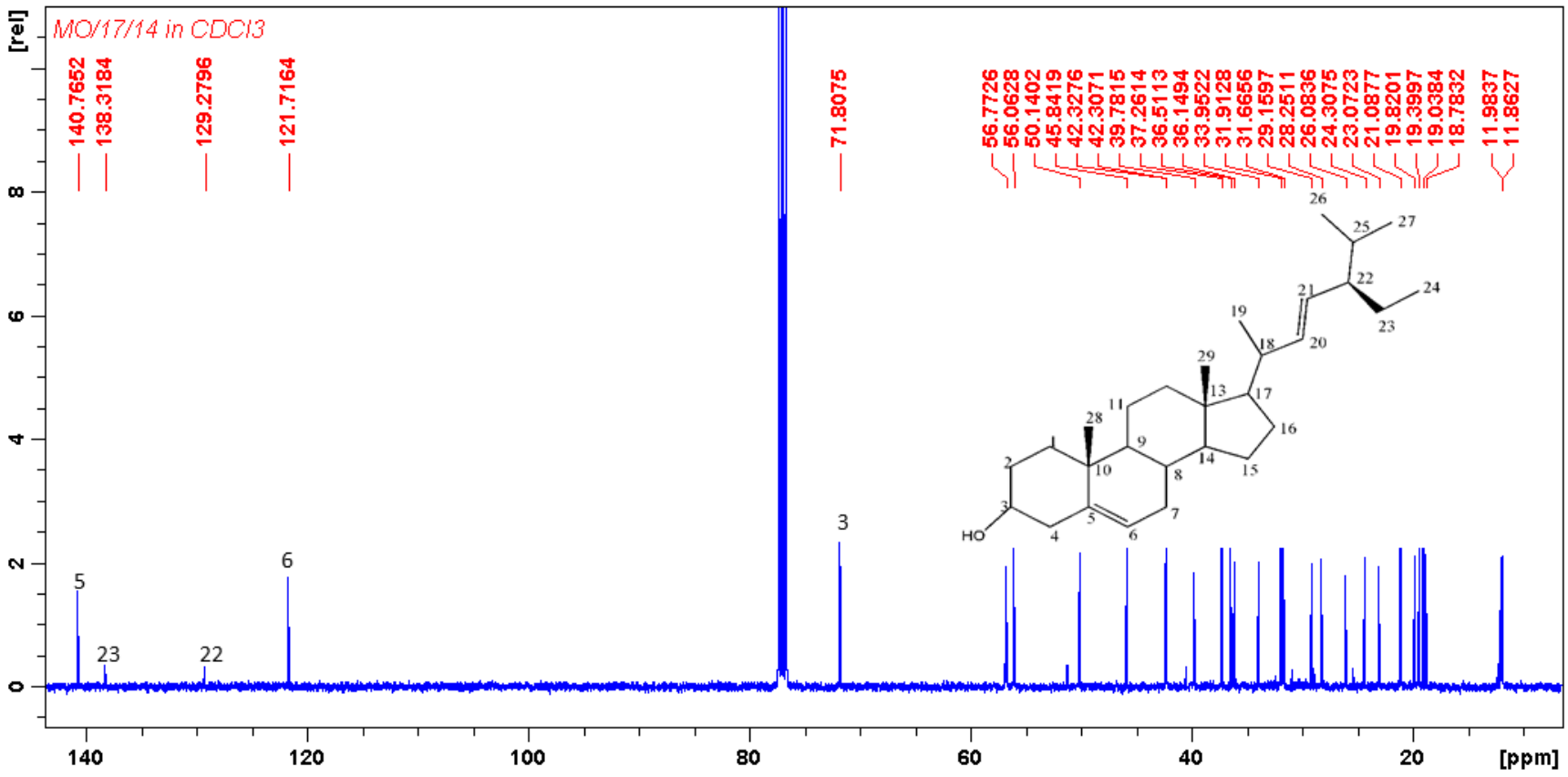
UV spectrum of campesterol A-2



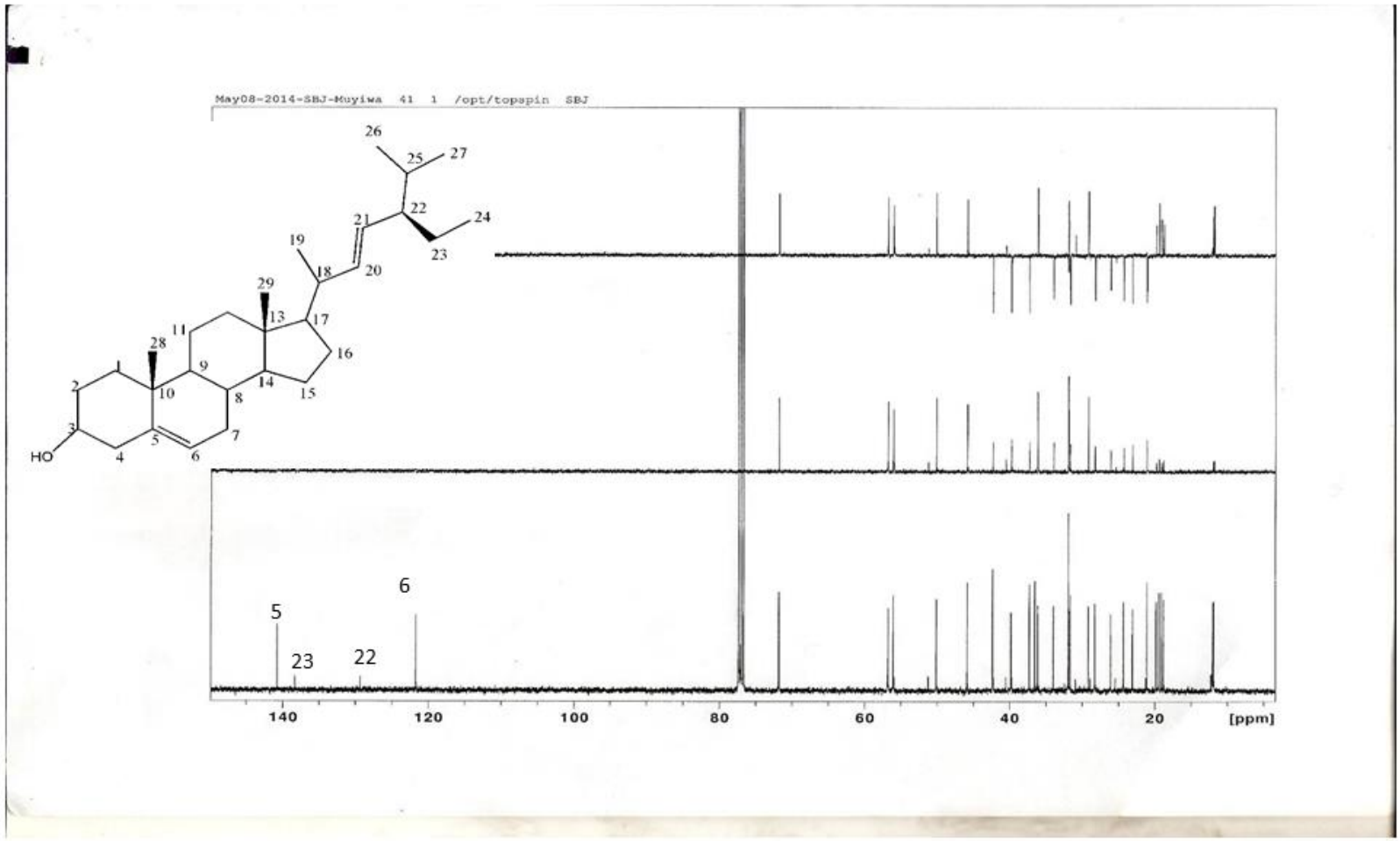
Mass spectrum ofcampesterol A-2



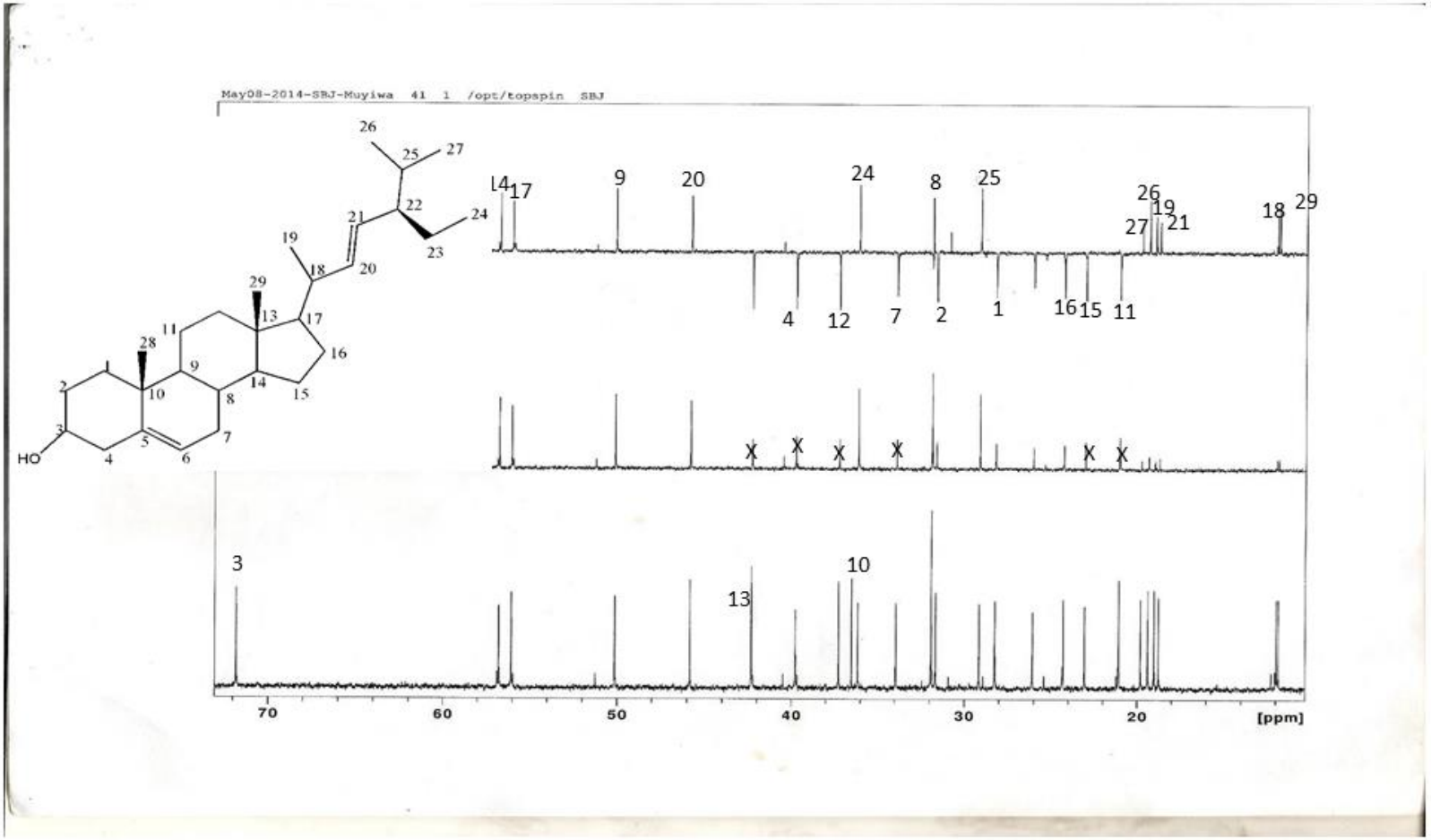
^{1}H NMR spectrum of stigmasterol A-3



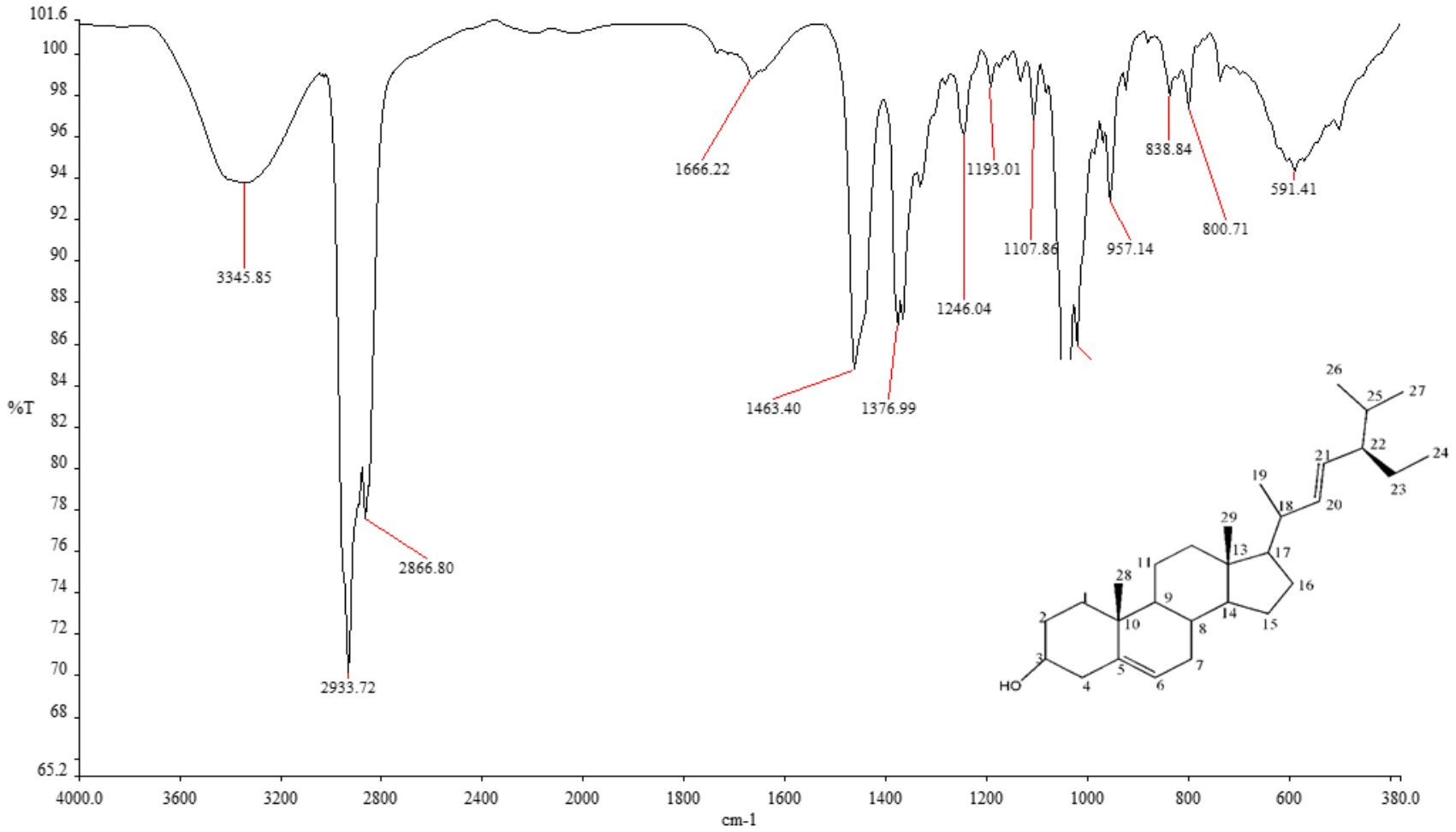
^{13}C NMR spectrum of stigmasterol A-3



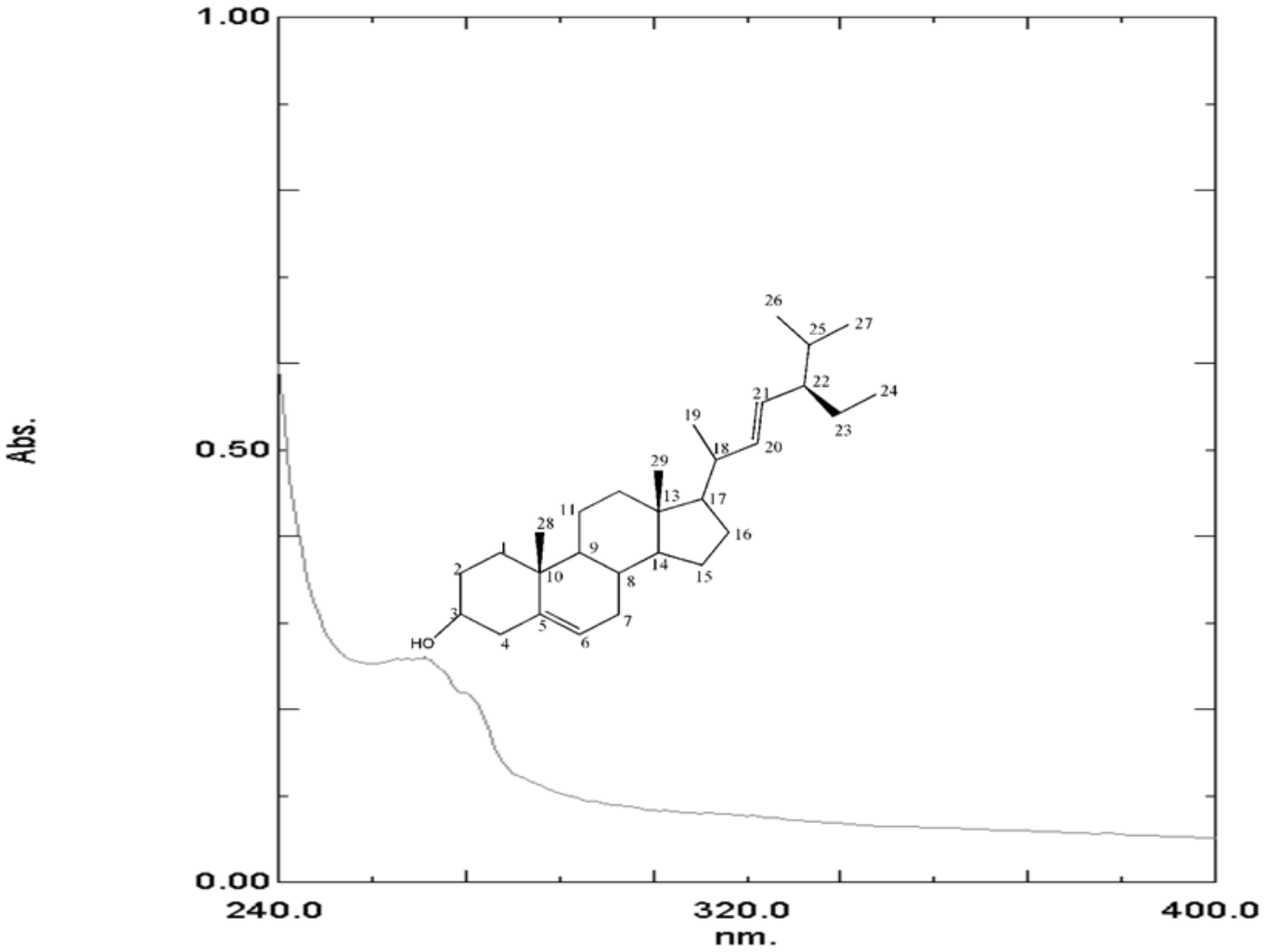
DEPT spectrum of stigmasterol A-3



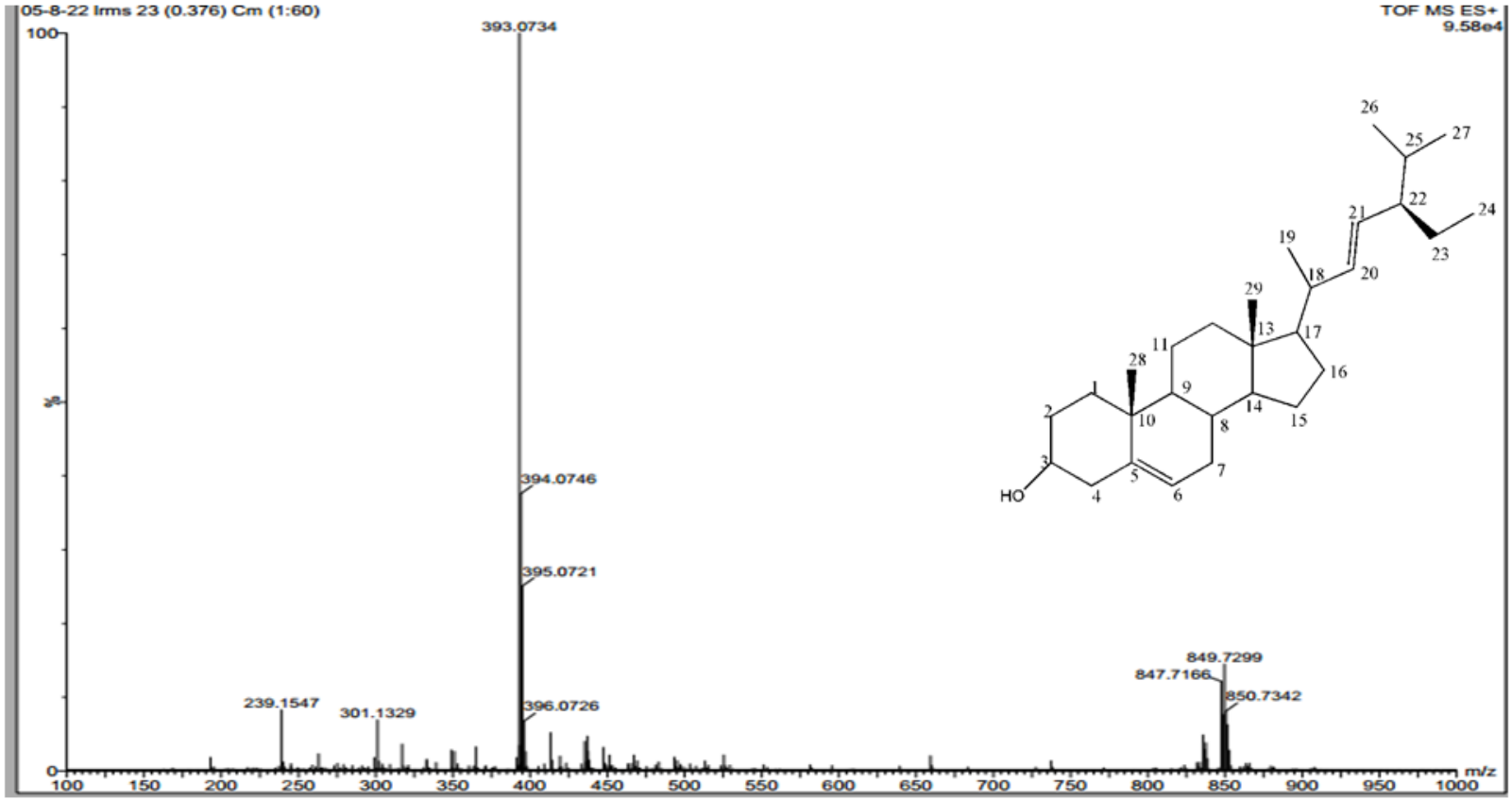
Expanded DEPT spectrum of stigmasterol A-3



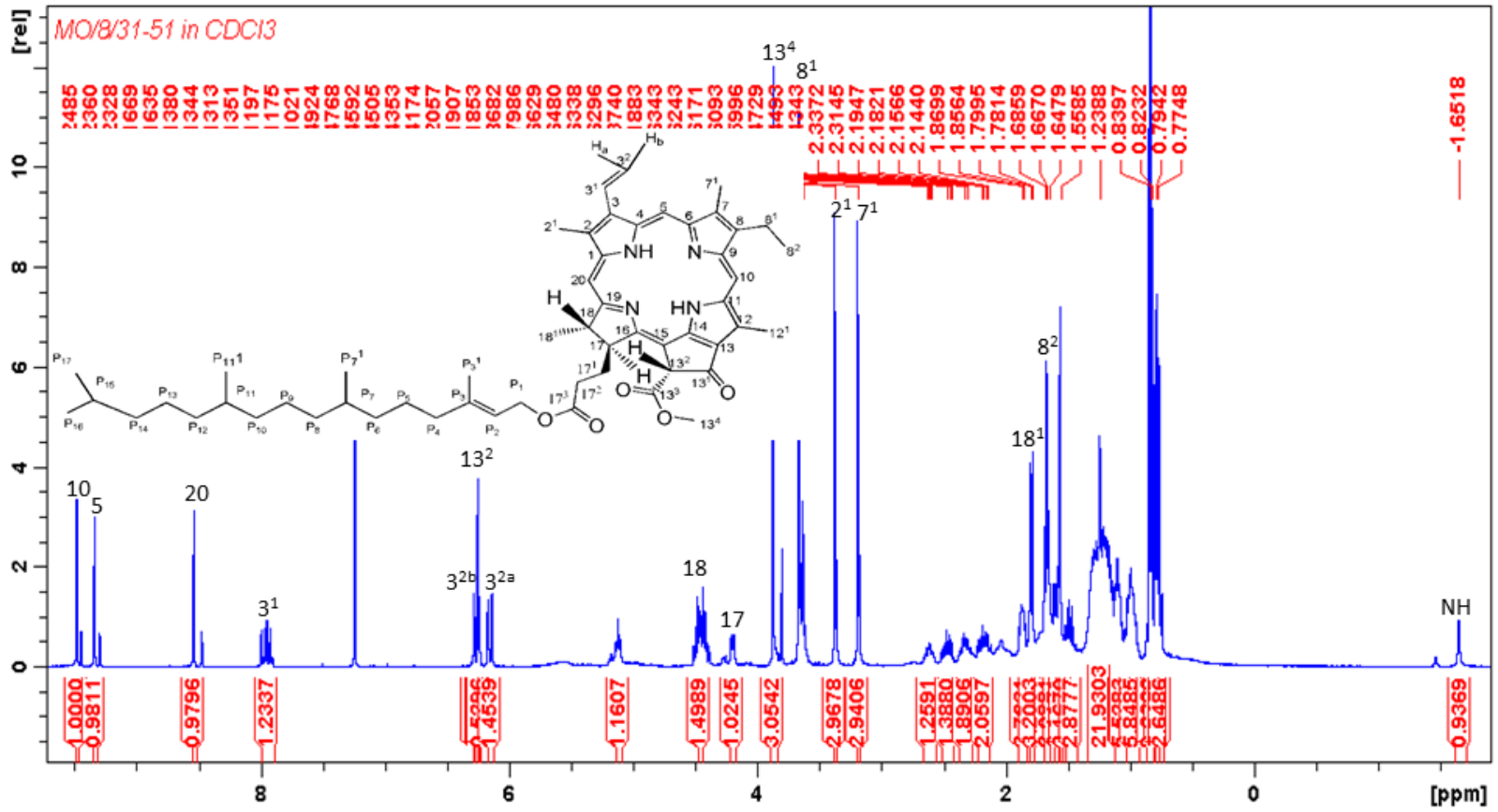
IR spectrum of stigmasterol A-3



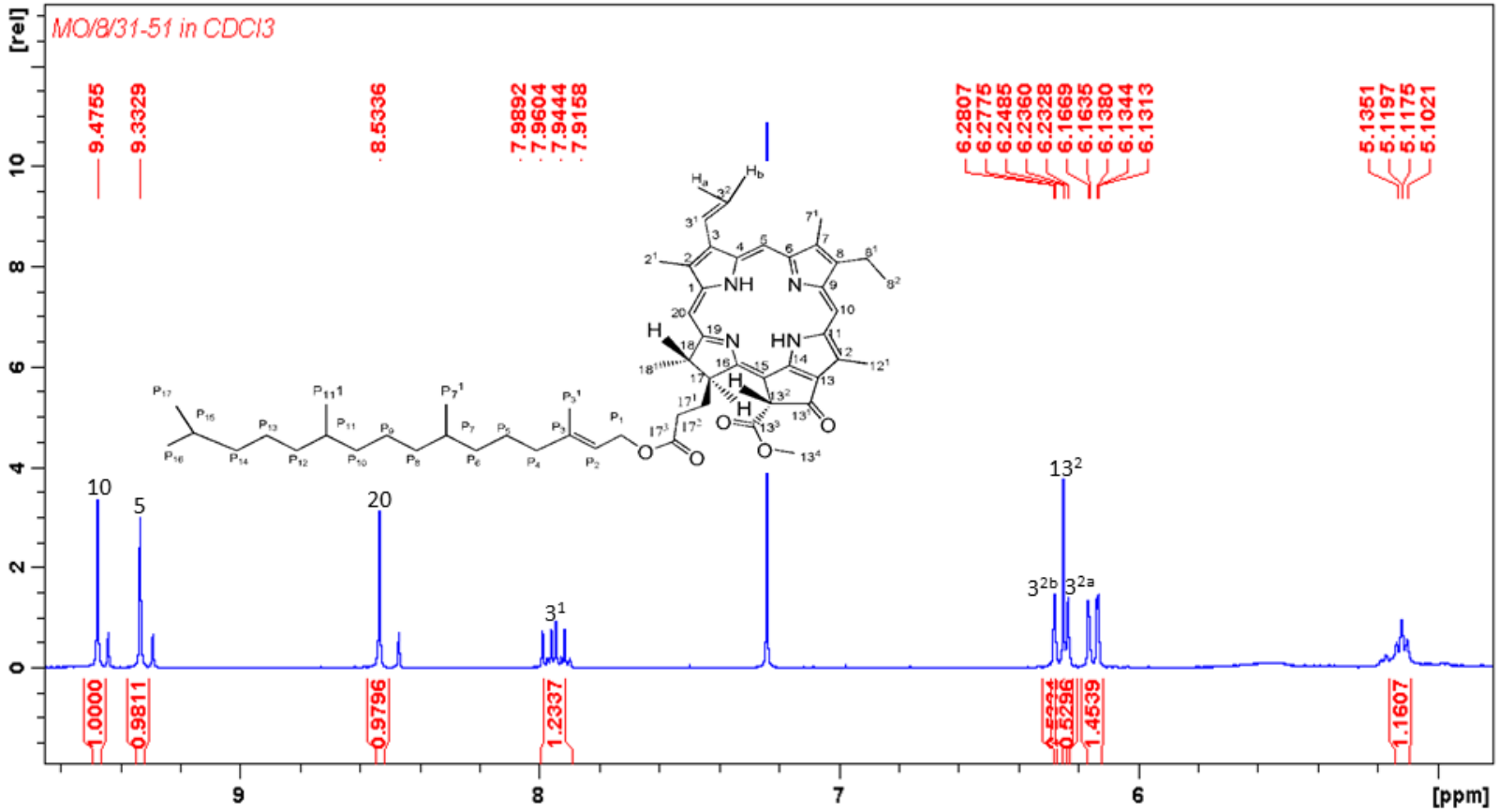
UV spectrum of stigmasterol A-3



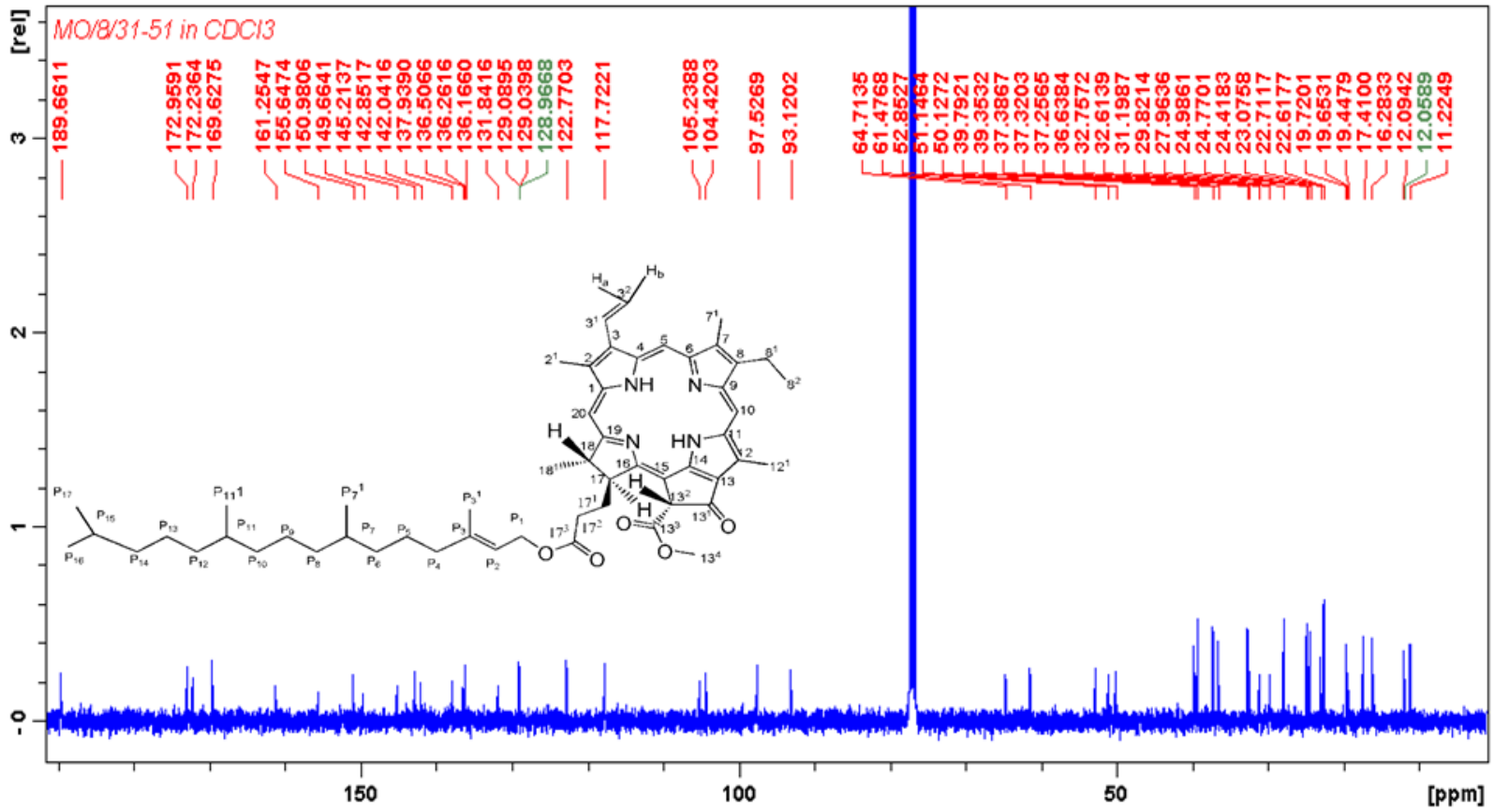
Mass spectrum of stigmasterol A-3



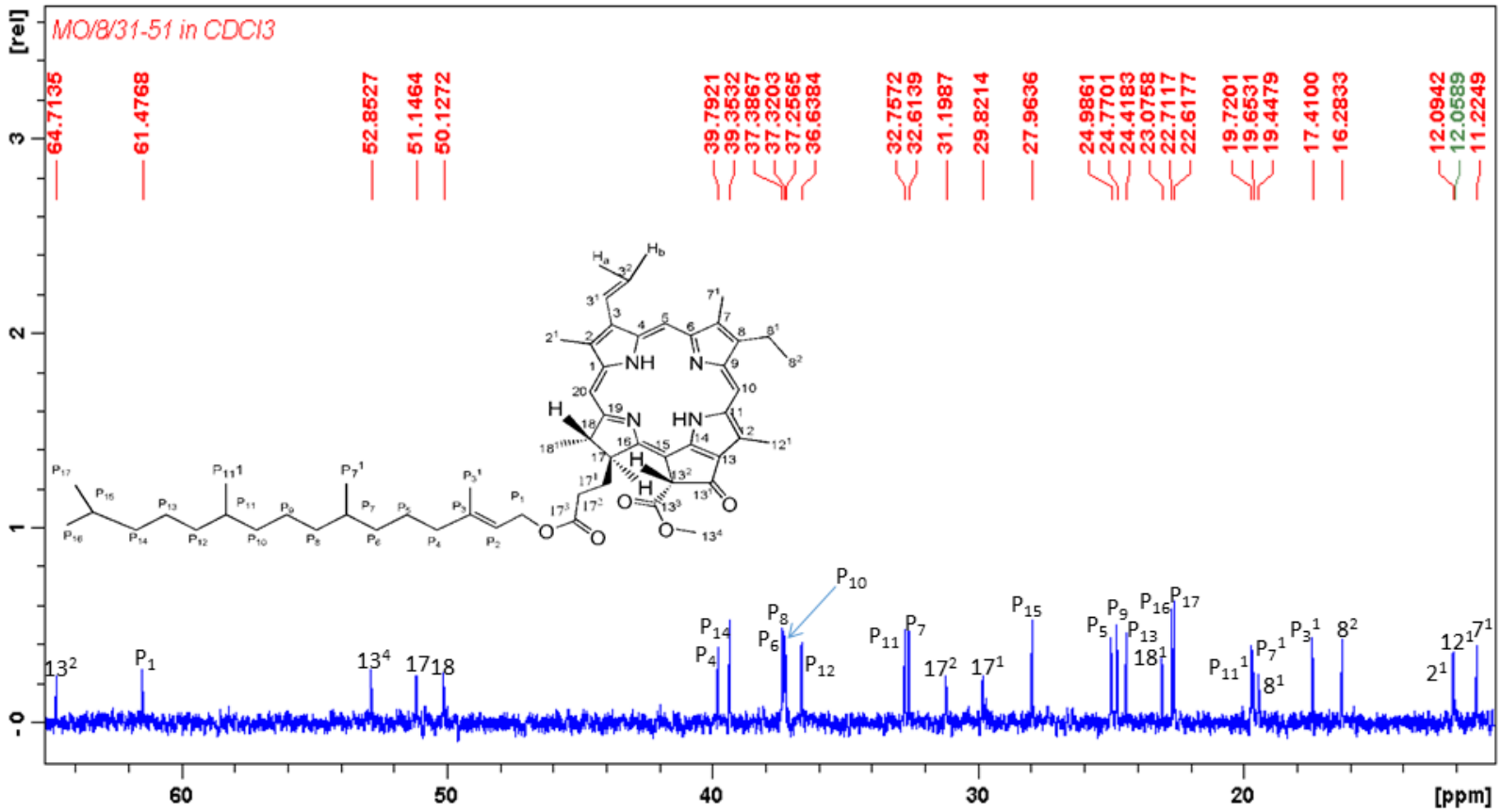
^{1}H NMR spectrum of phaeophytin **a** A-4



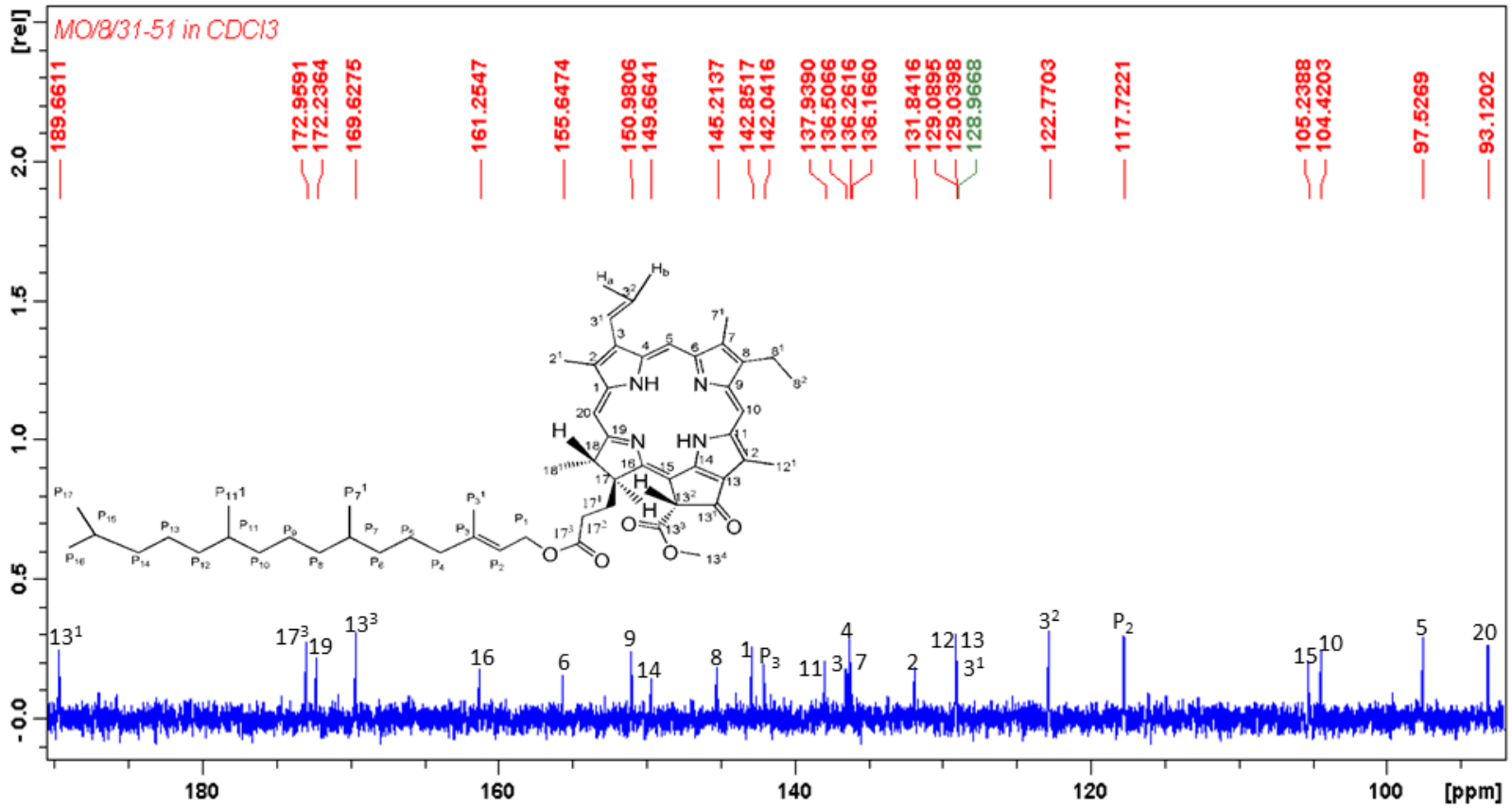
Expanded ^{1}H NMR spectrum of phaeophytin **a** A-4



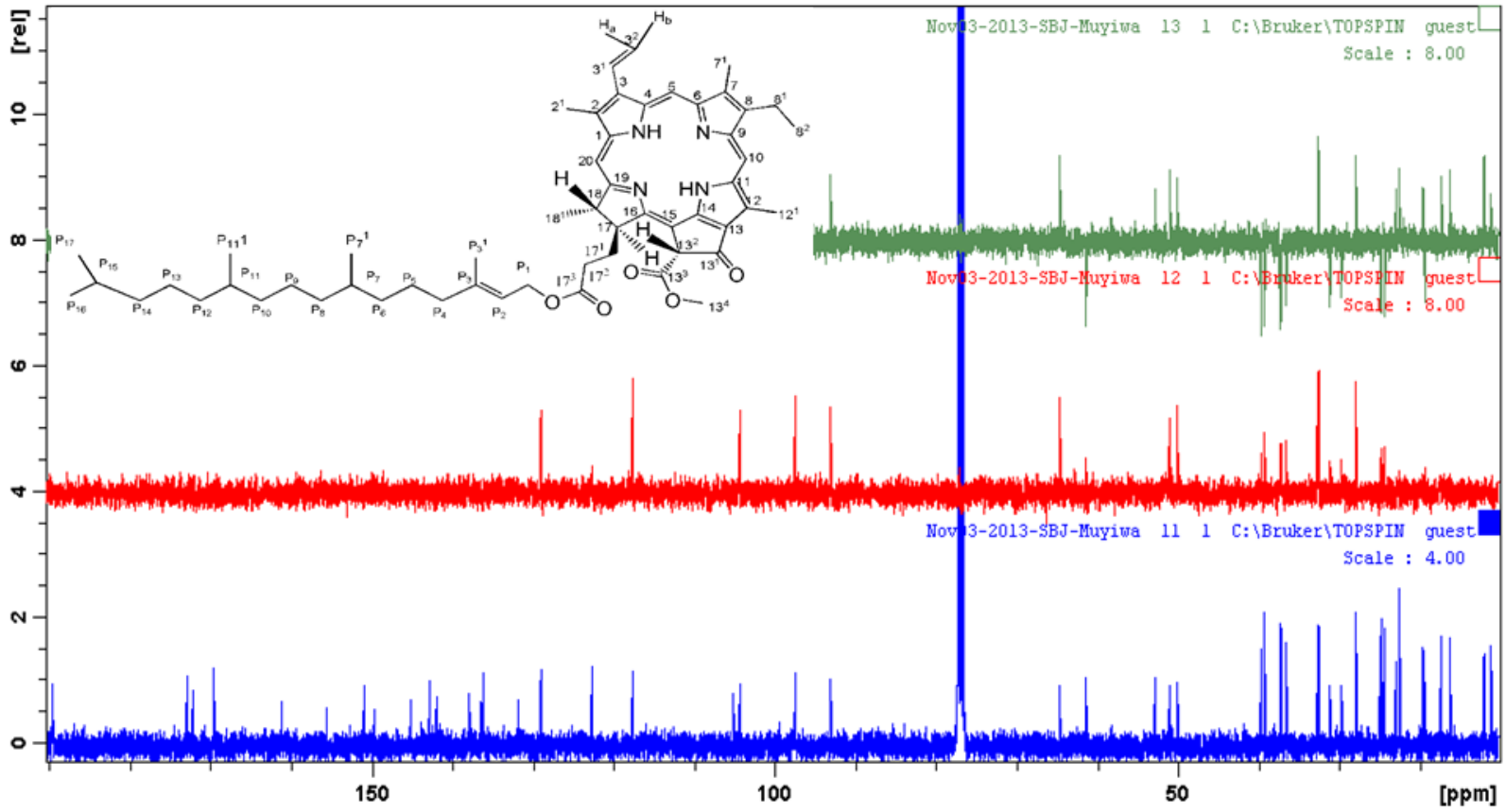
^{13}C NMR spectrum of phaeophytin **a** A-4



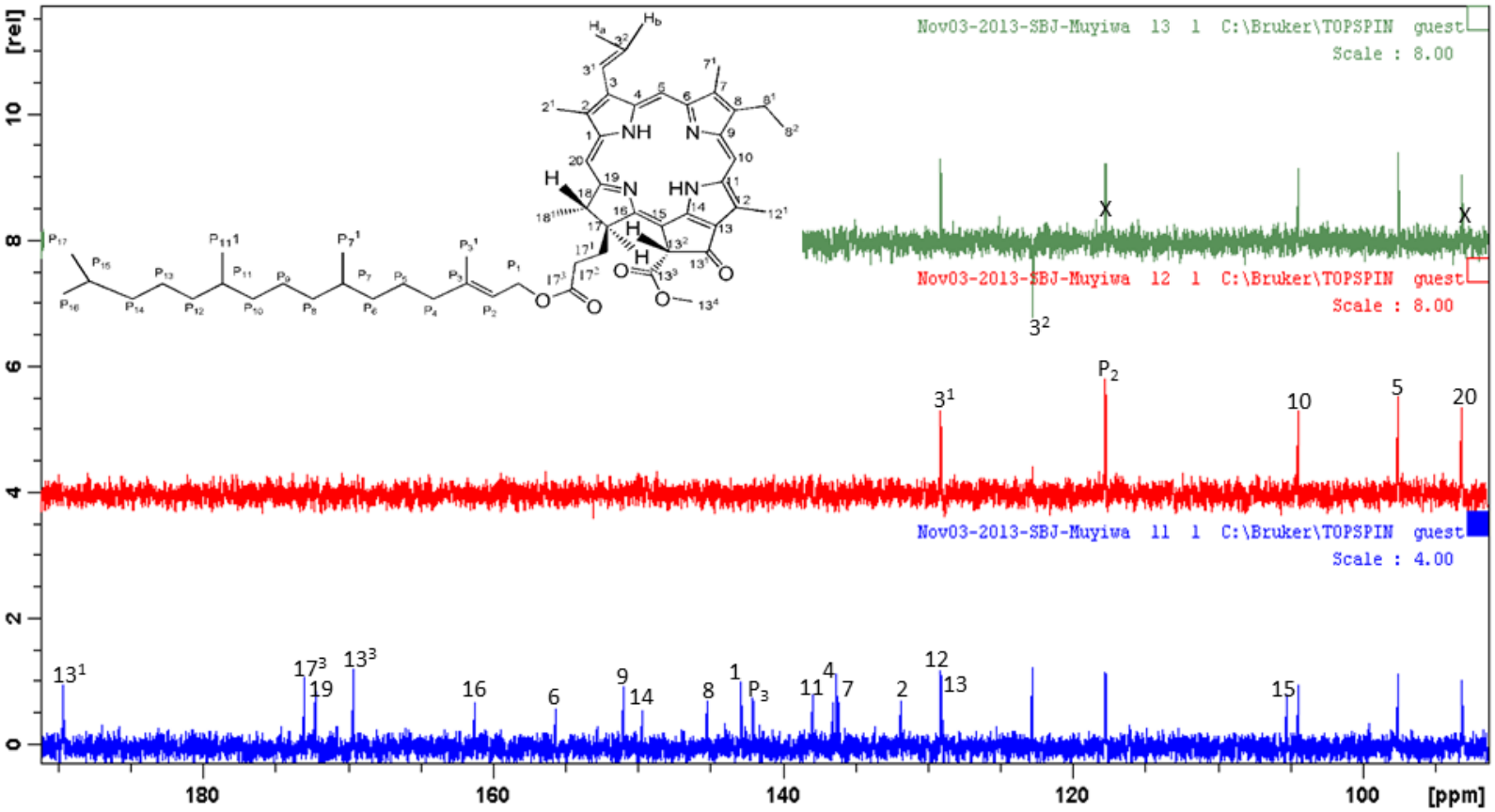
Expanded ^{13}C NMR spectrum of phaeophytin **a** A-4



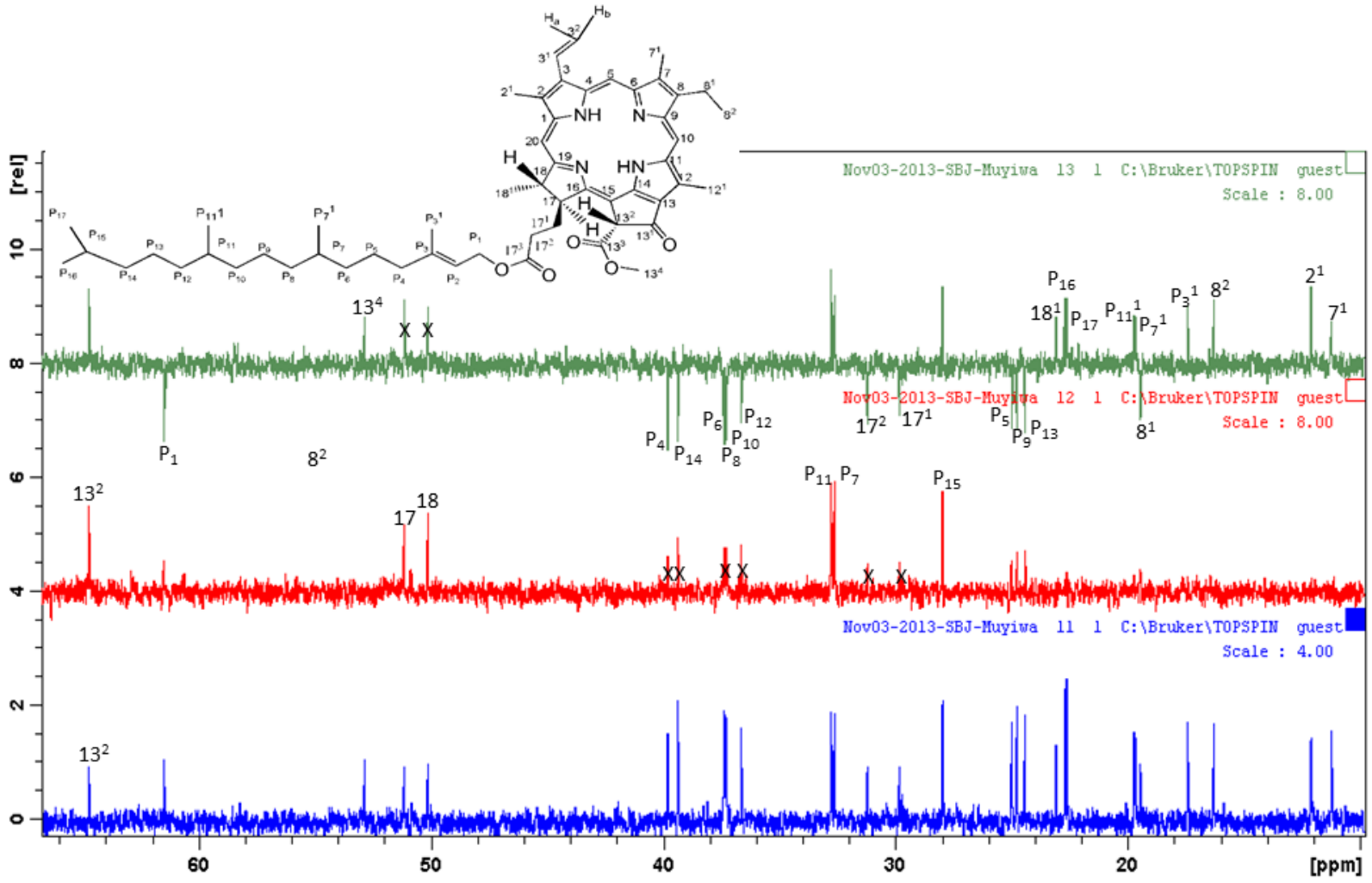
Expanded ^{13}C NMR spectrum of phaeophytin **a** A-4



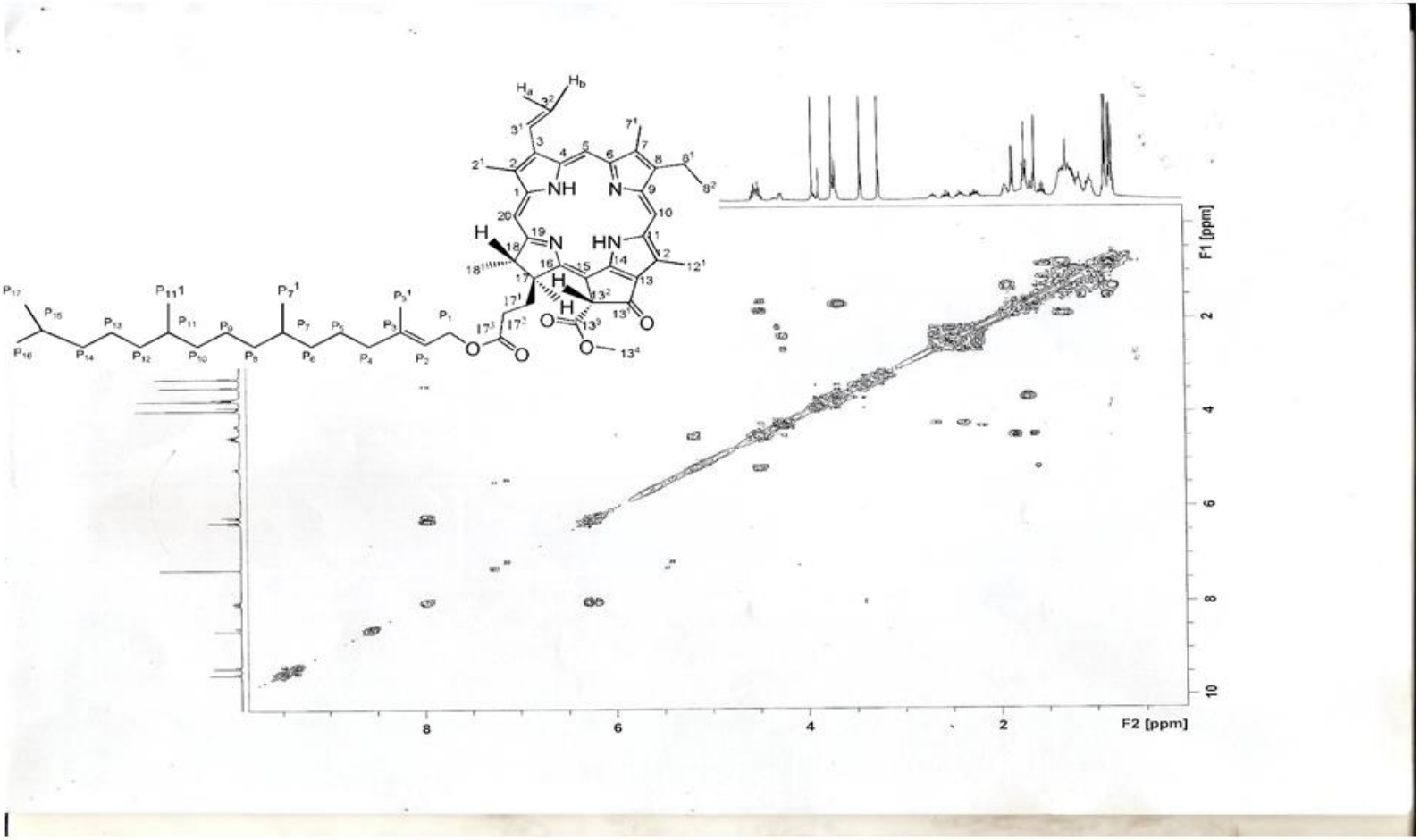
DEPT spectrum of phaeophytin **a** A-4



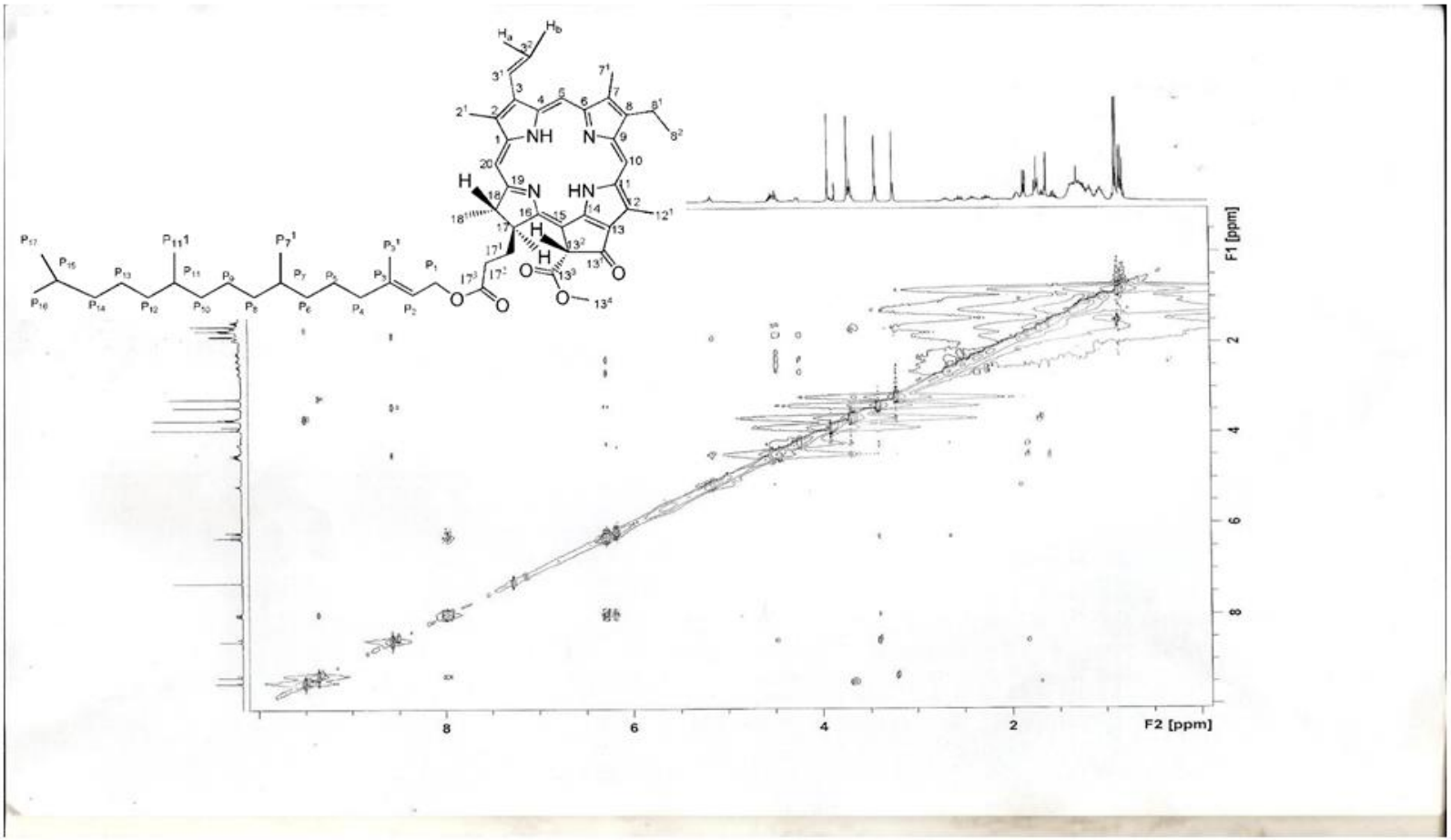
Expanded DEPT spectrum of phaeophytin **a** A-4



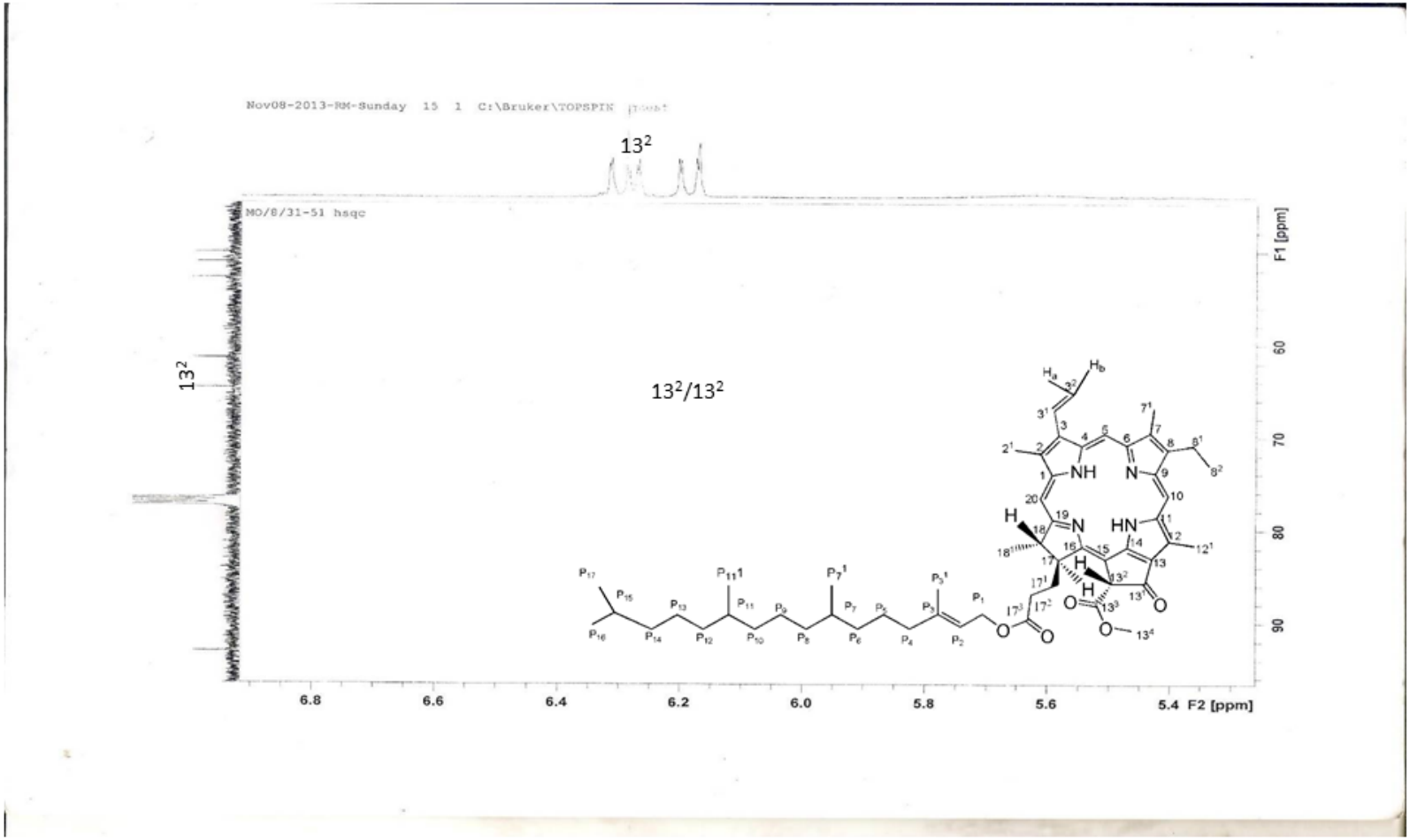
Expanded DEPT spectrum of phaeophytin **a** A-4



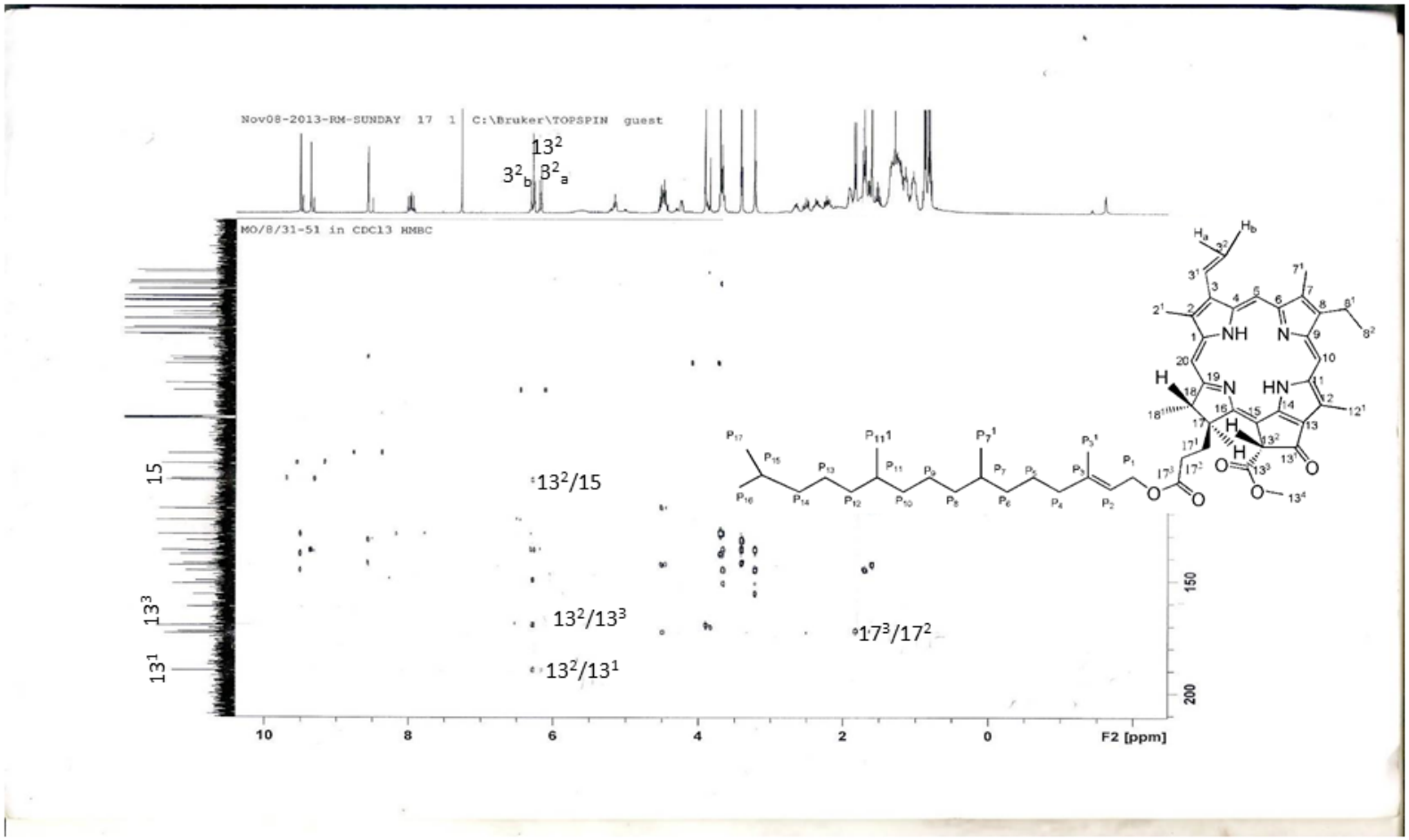
COSY spectrum of phaeophytin **a** A-4



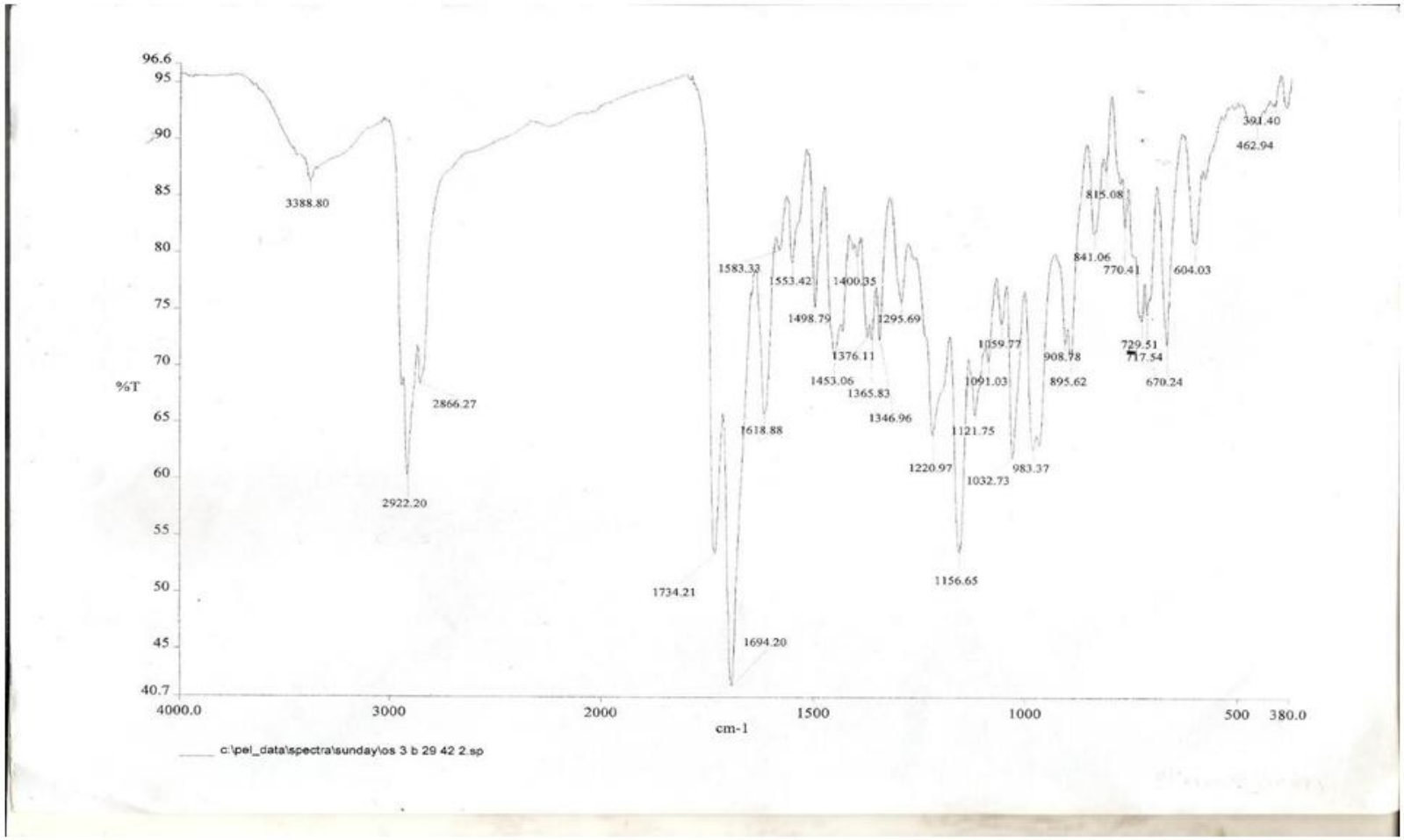
NOESY spectrum of phaeophytin **a** A-4



HSQC spectrum of phaeophytin **a** A-4



HMBC spectrum of phaeophytin **a** A-4

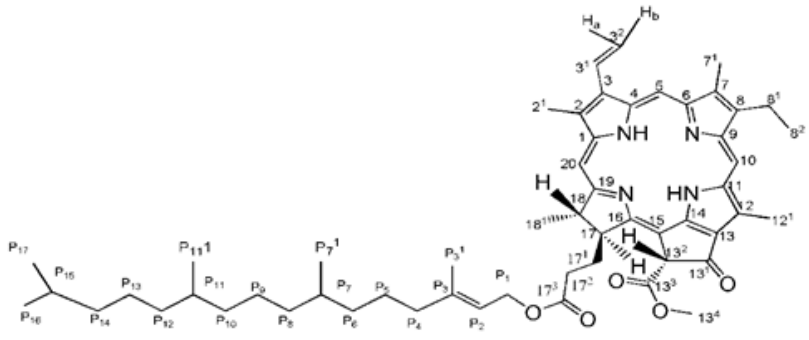


IR spectrum of phaeophytin **a** A-4

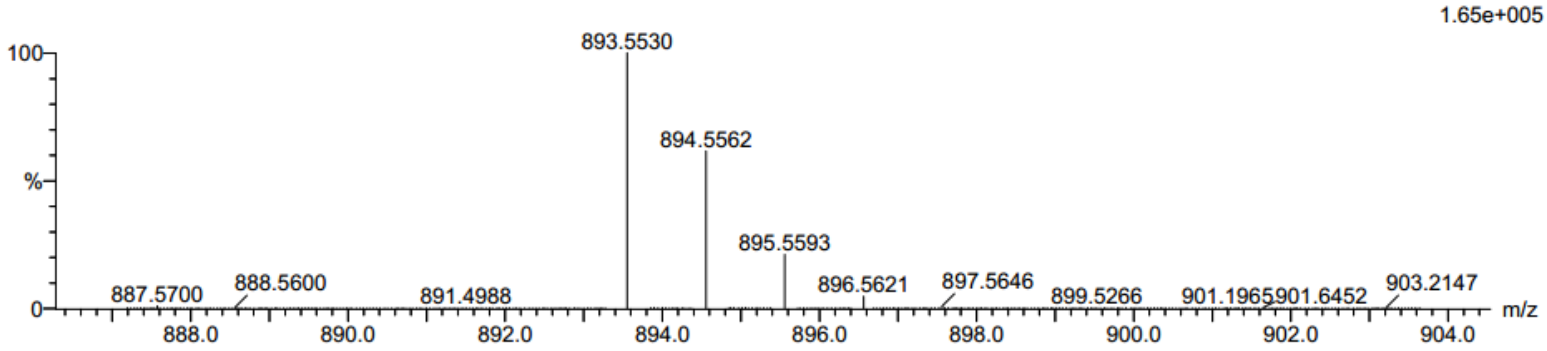
Elemental Composition Report

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

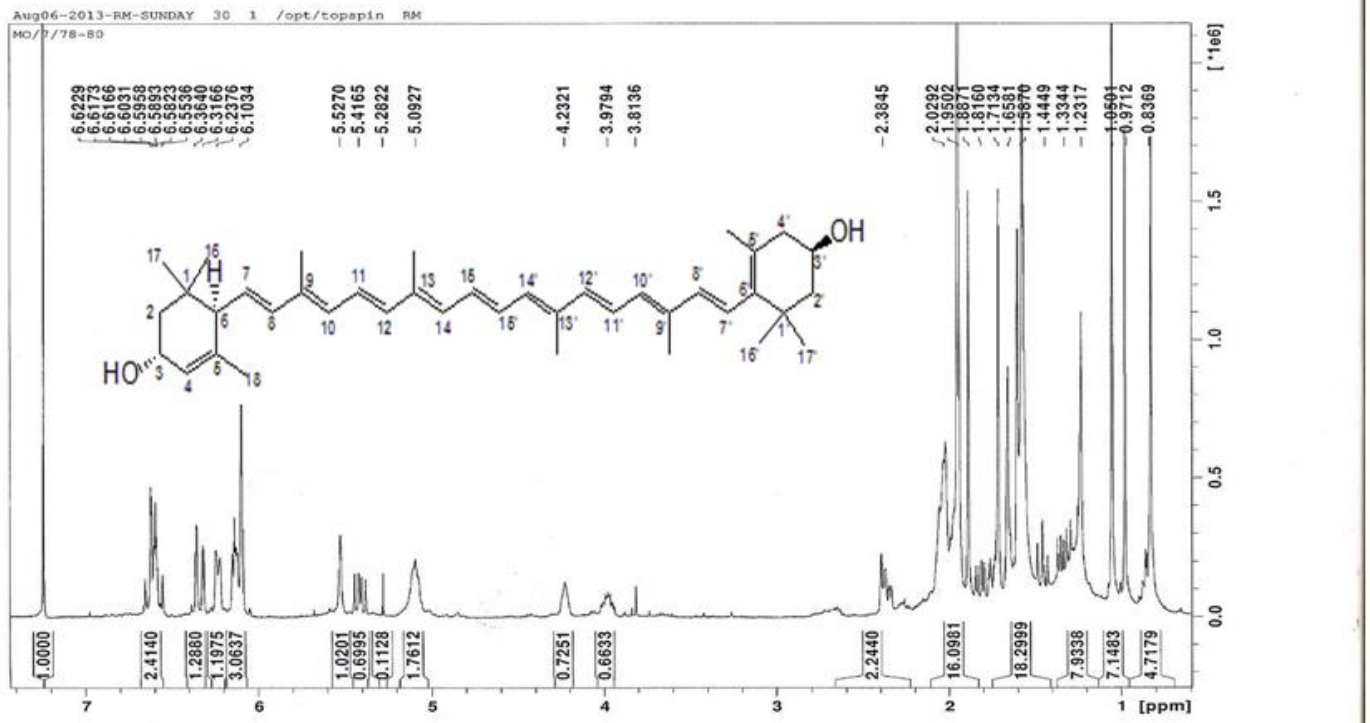


Monoisotopic Mass, Even Electron Ions
13 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 50-55 H: 70-75 N: 0-5 O: 0-5 Na: 0-1
05-3-B-29-42 53 (1.755) Cm (1:61)
TOF MS ES+

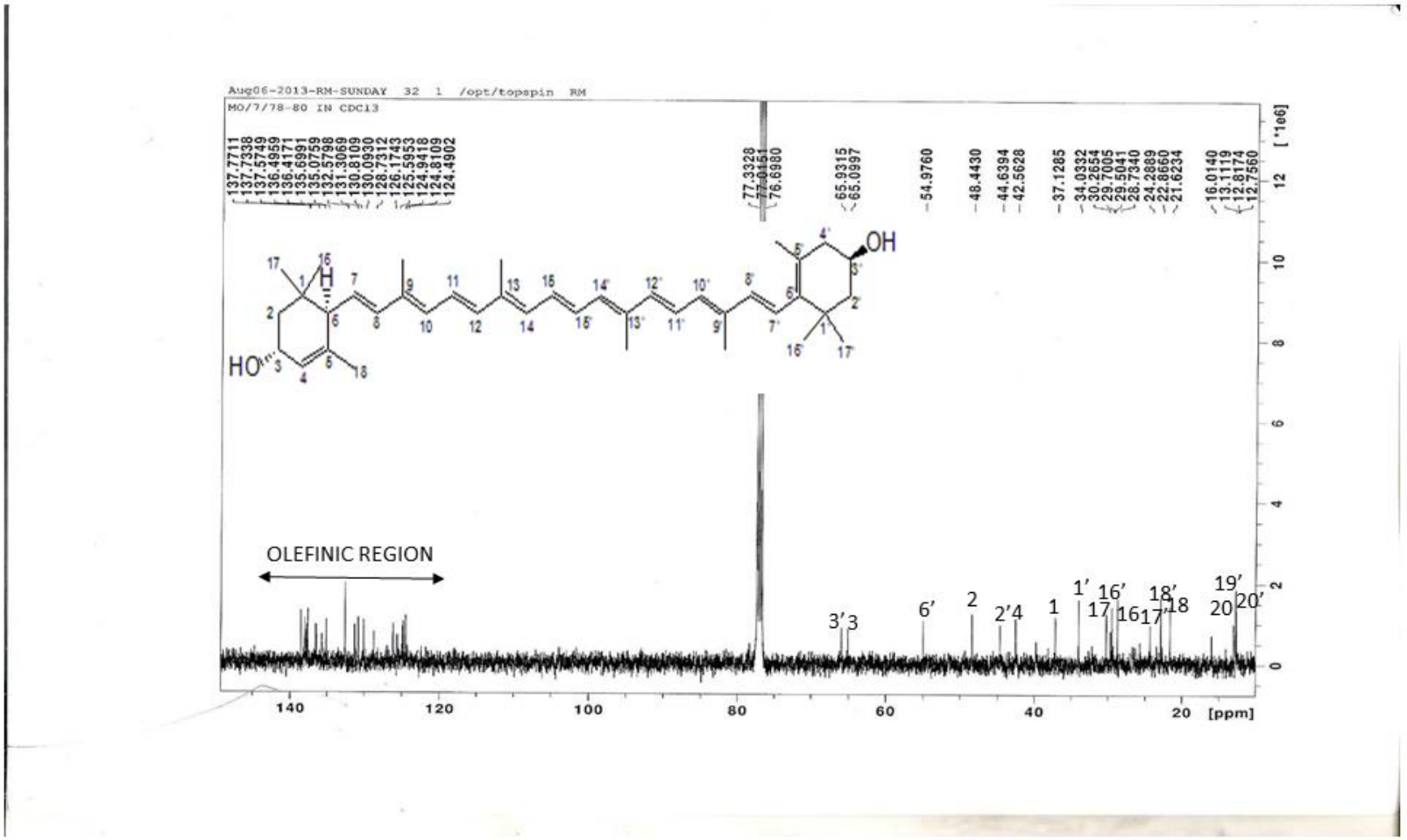


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
893.5530	893.5557	-2.7	-3.0	20.5	361.3	0.0	C55 H74 N4 O5 Na

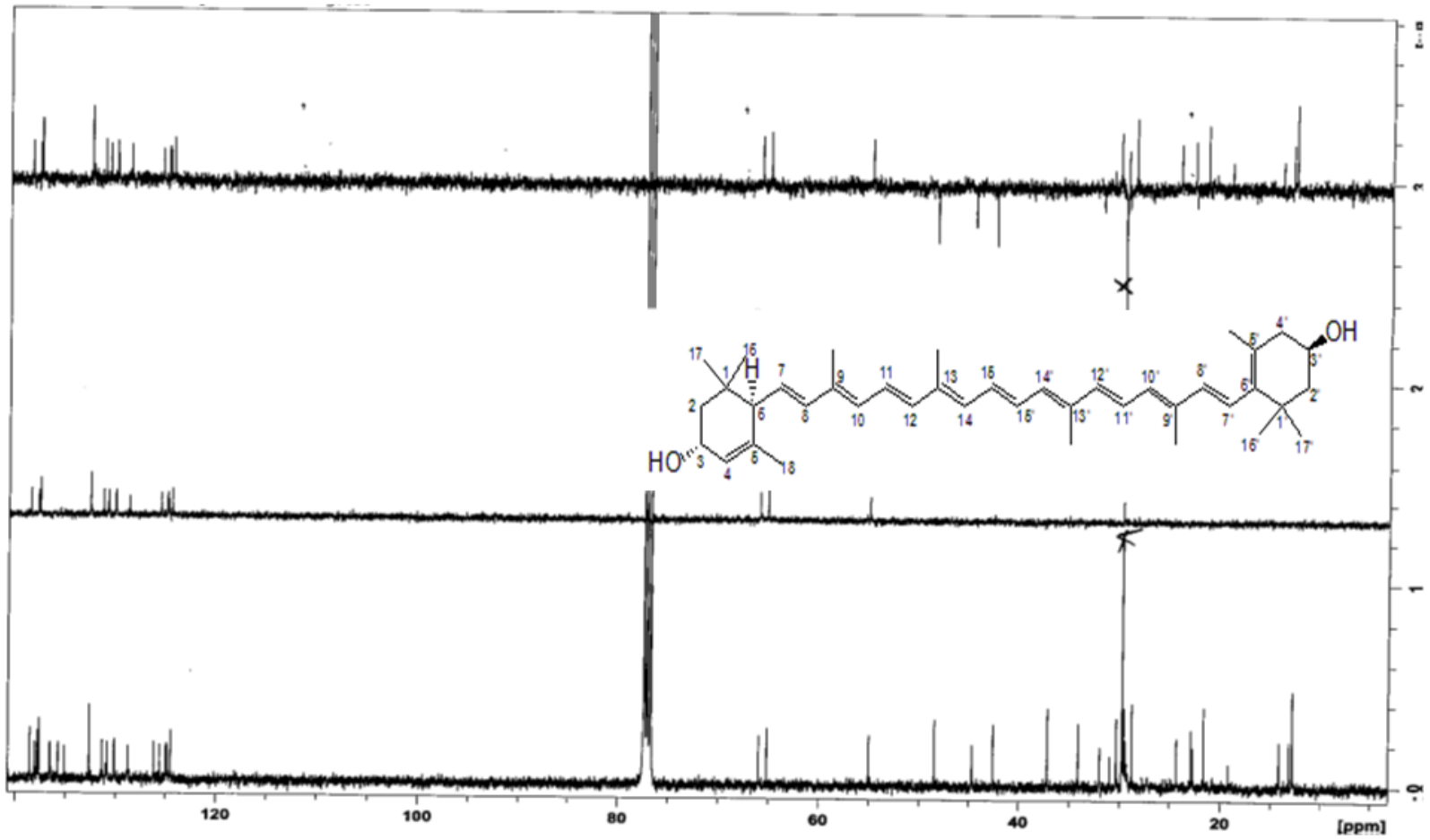
Mass spectrum of phaeophytin **a** **A**-4



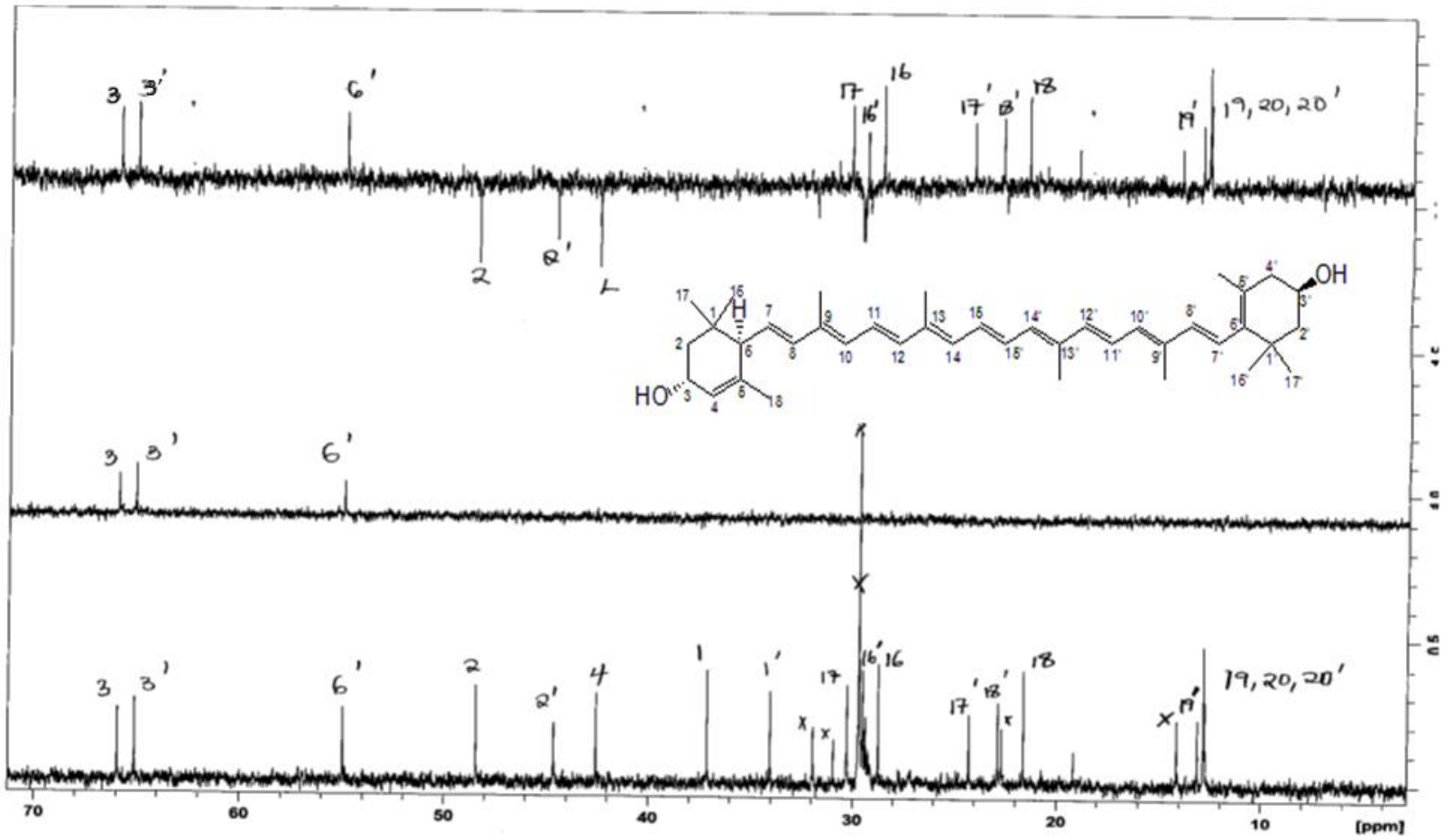
^{1}H NMR spectrum of lutein A-5



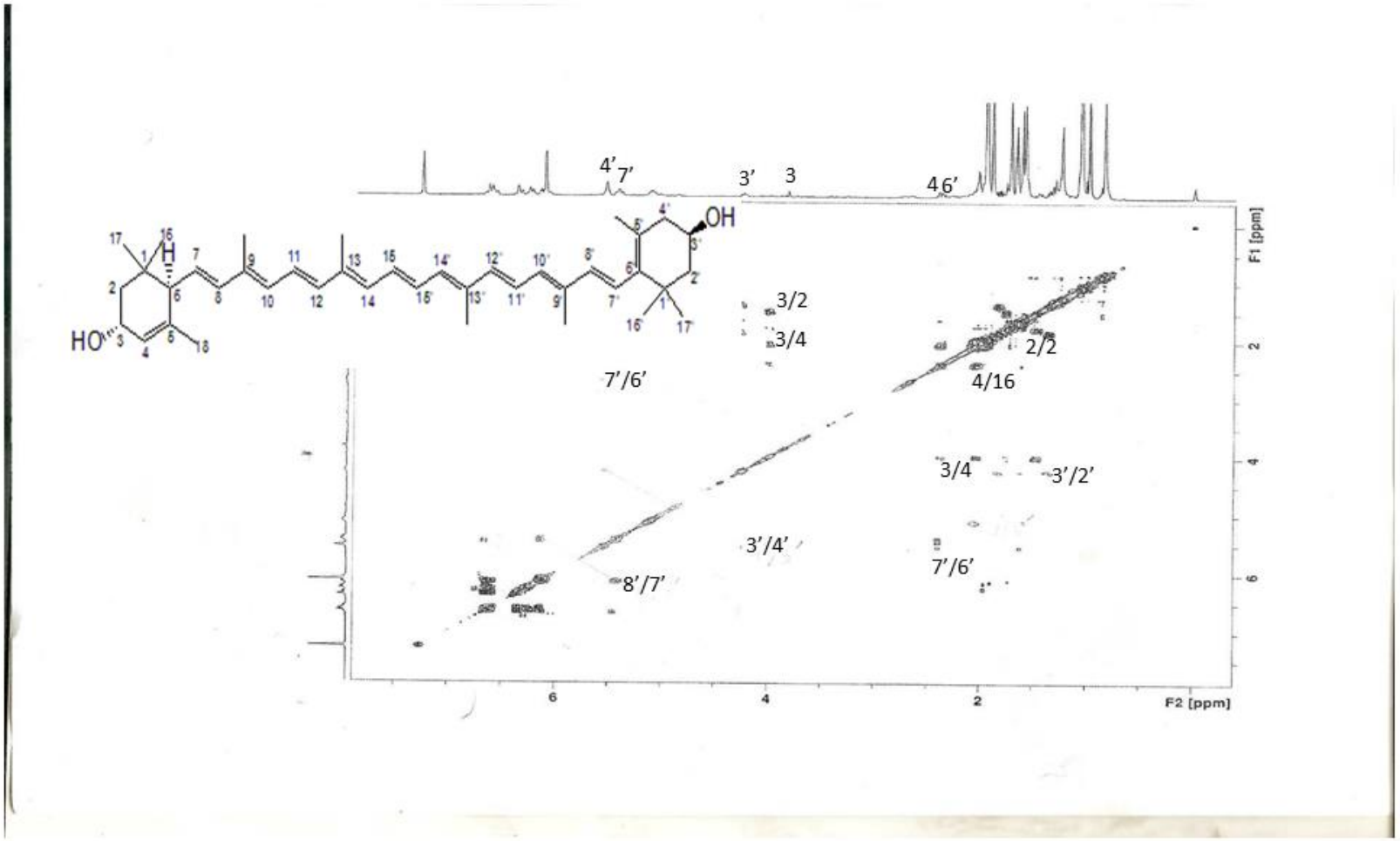
^{13}C NMR spectrum of lutein A-5



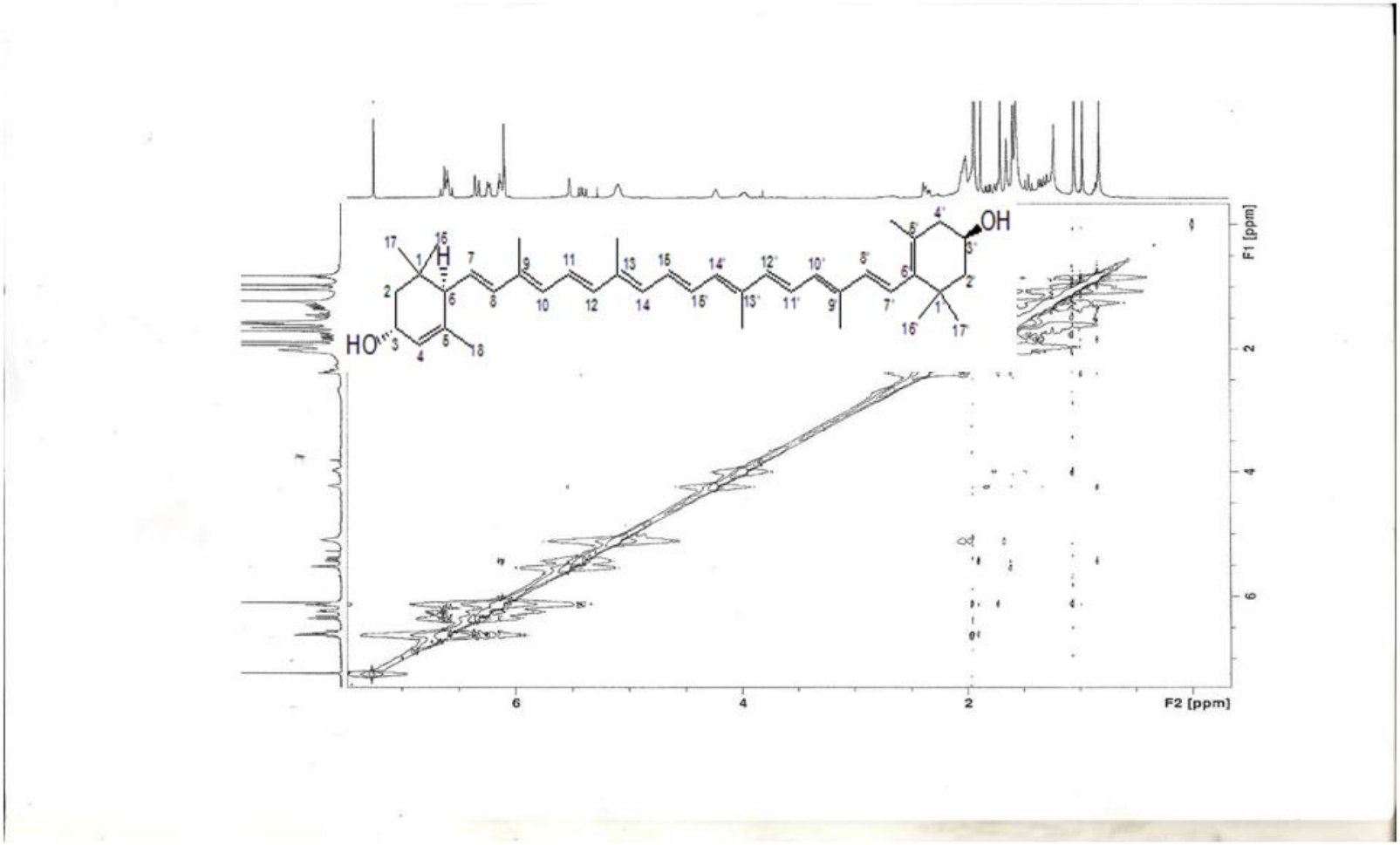
DEPT spectrum of lutein A-5



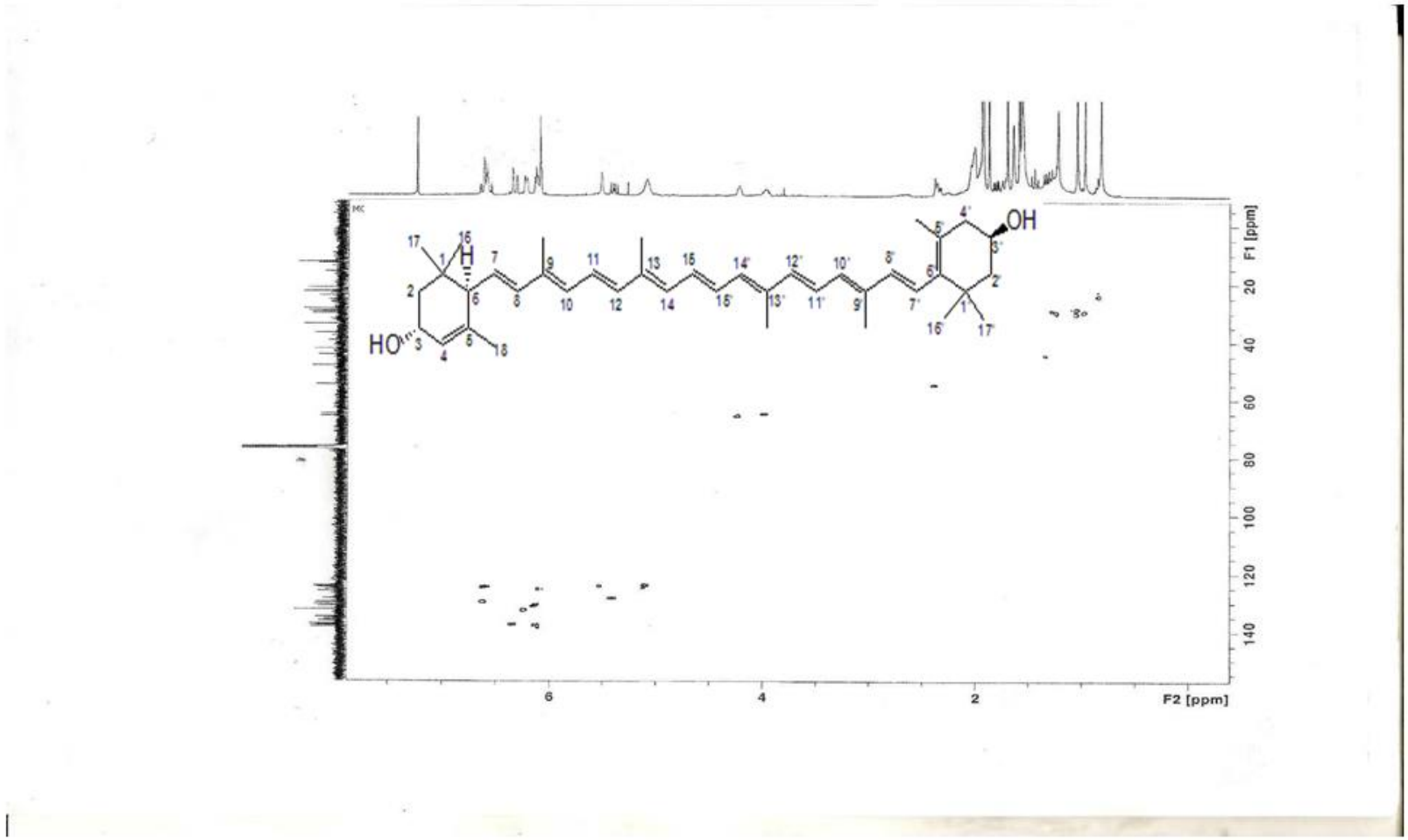
Expanded DEPT spectrum of lutein A-5



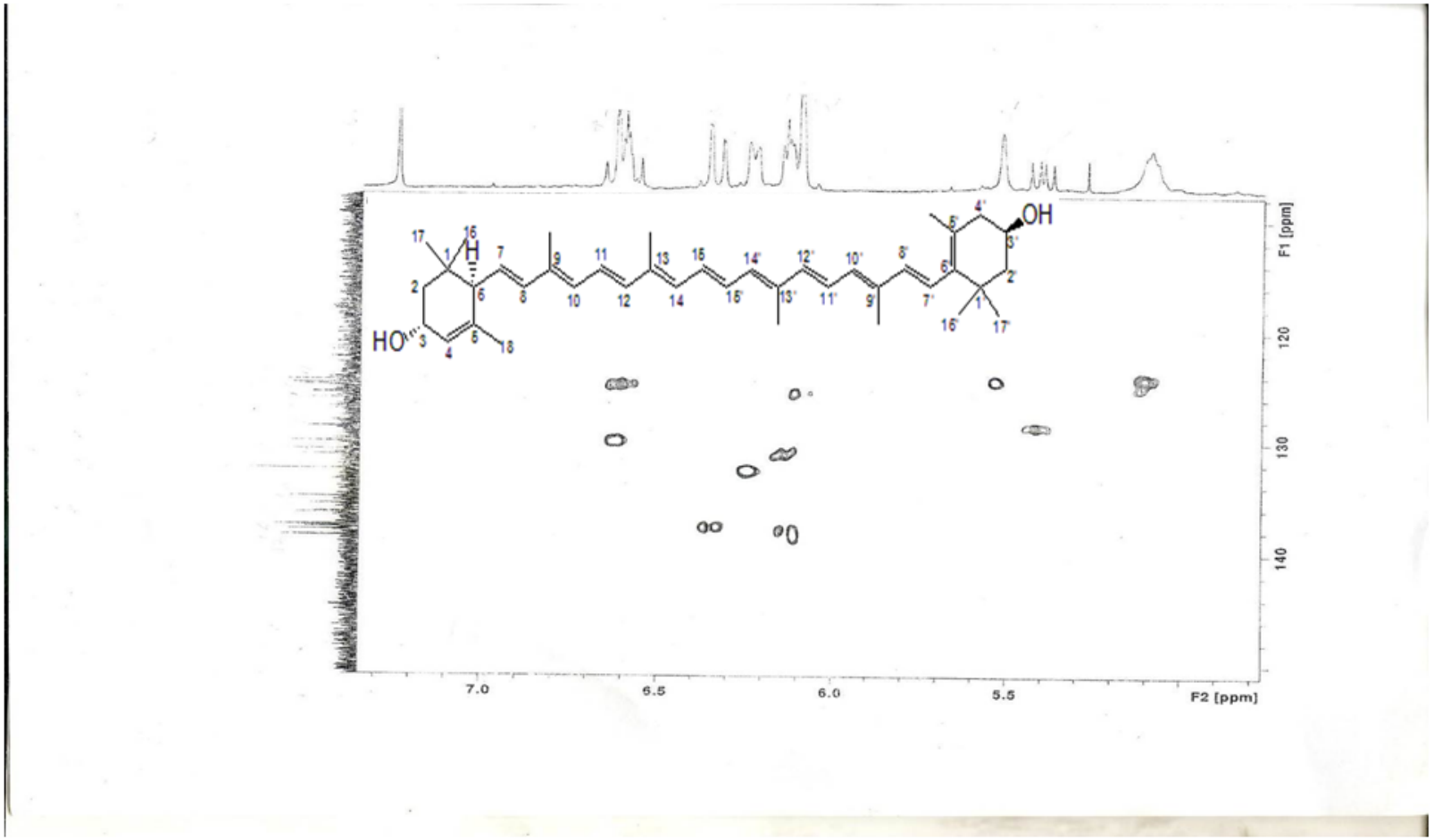
COSY spectrum of lutein A-5



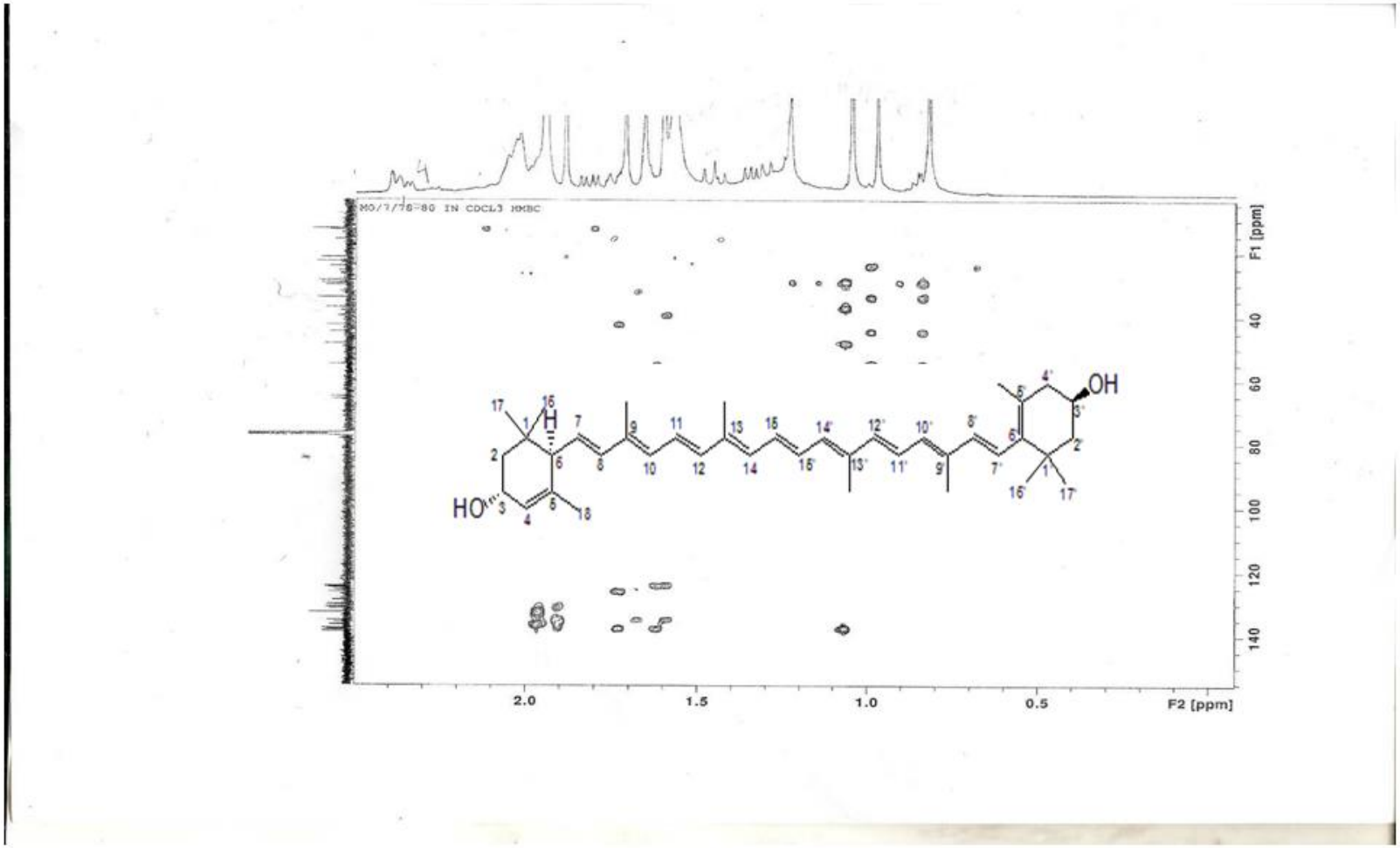
NOESY spectrum of lutein A-5



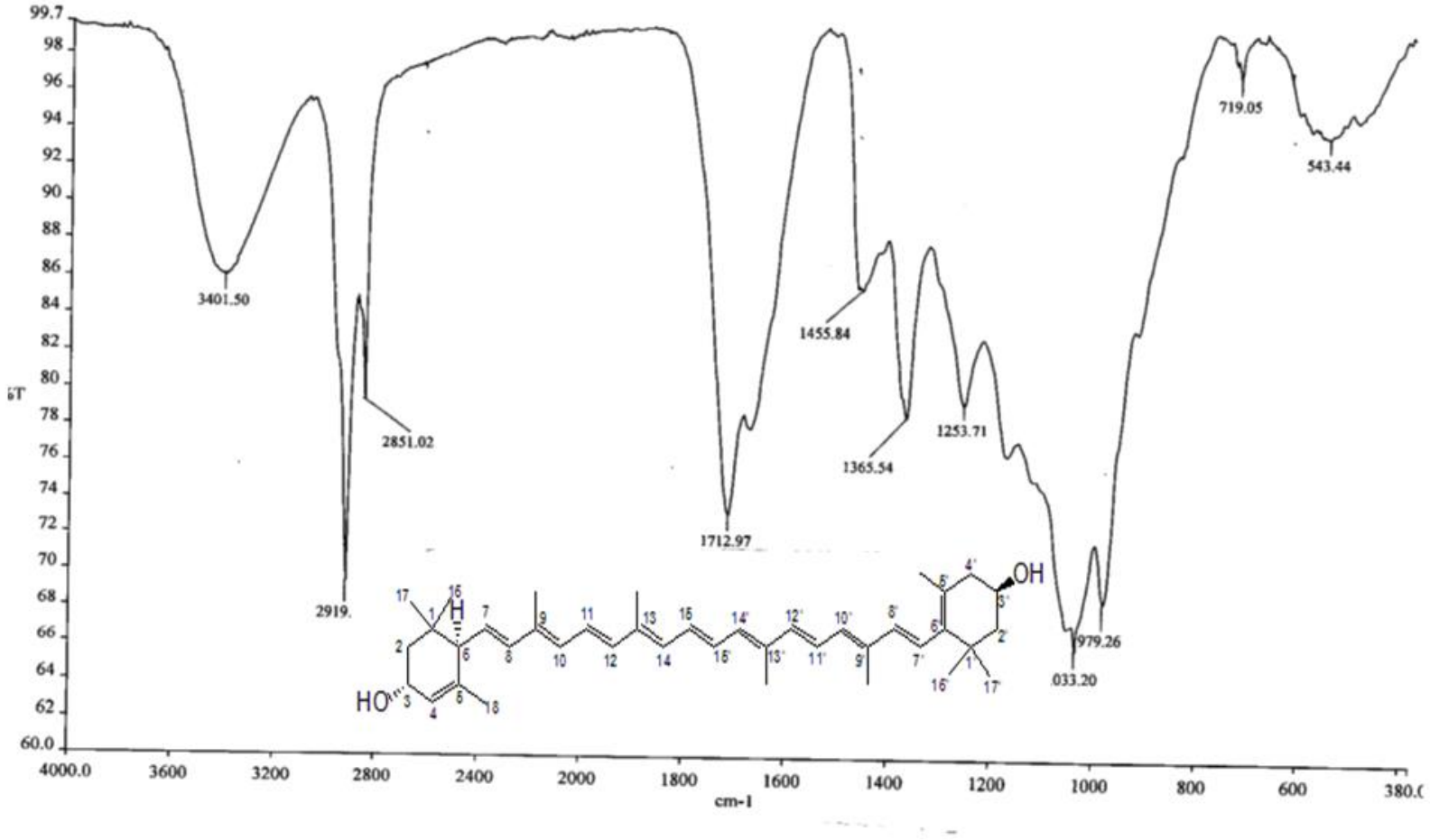
HSQC spectrum of lutein A-5



Expanded HSQC spectrum of lutein A-5



HMBC spectrum of lutein A-5

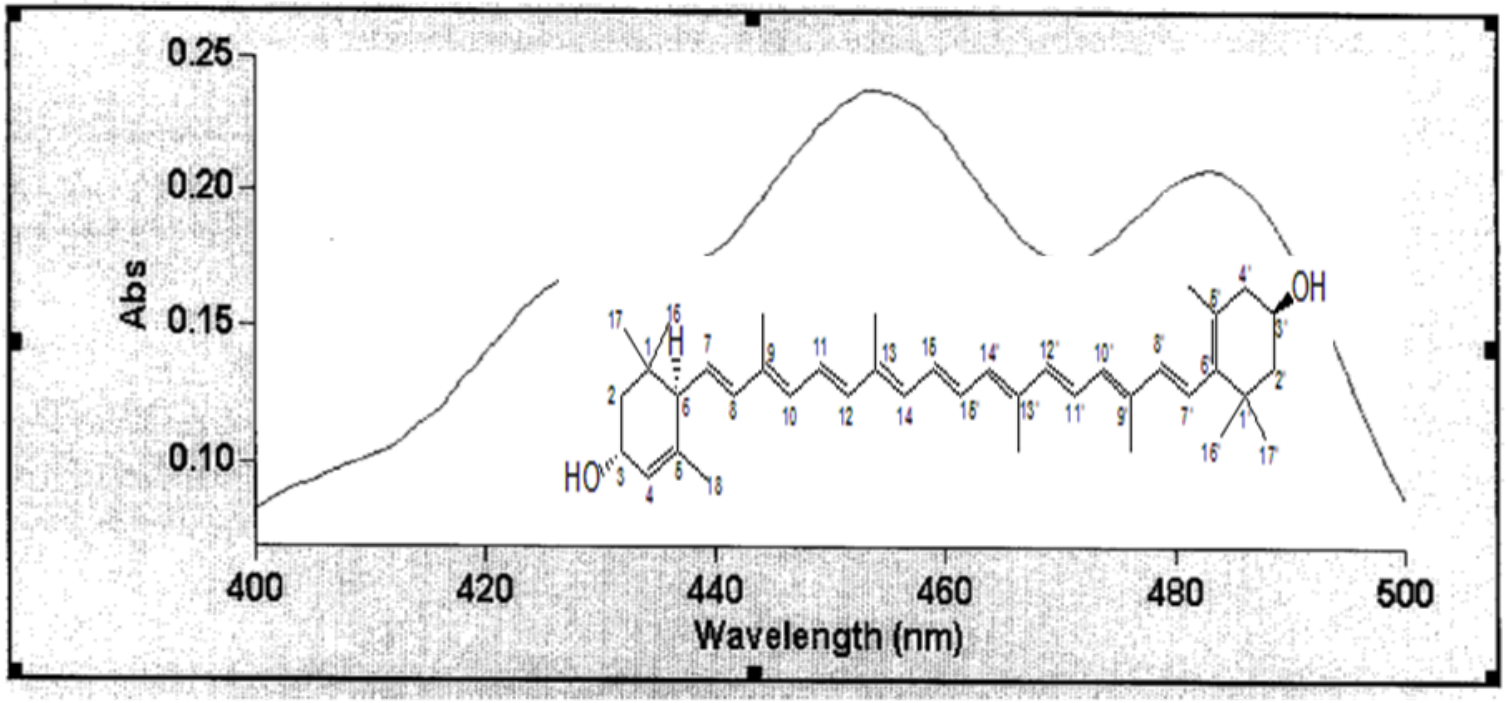


IR spectrum of lutein A-5

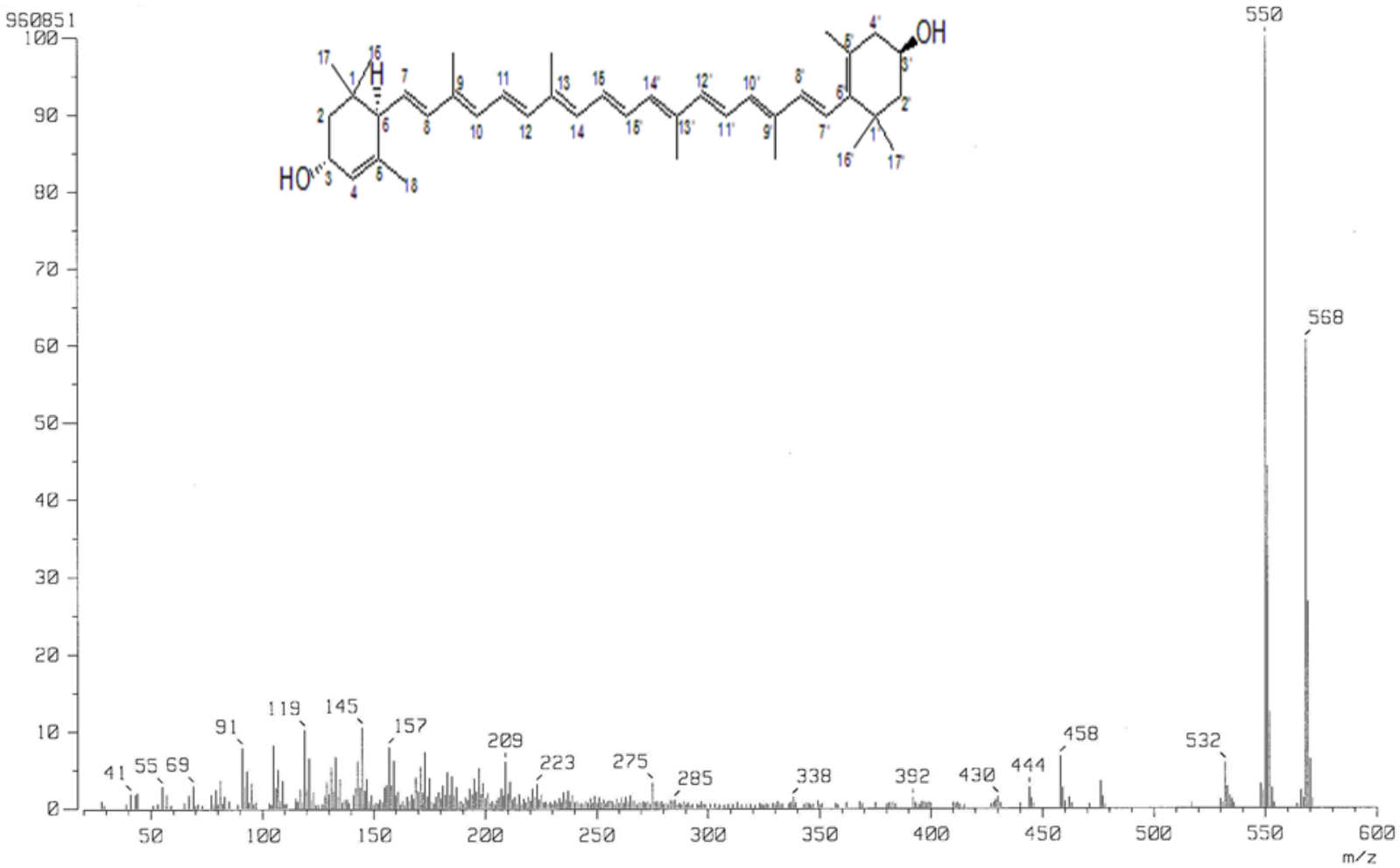
Peak Table
Peak Style
Peak Threshold
Range

Maximum Peak
0.0100
500.00nm to 190.00nm

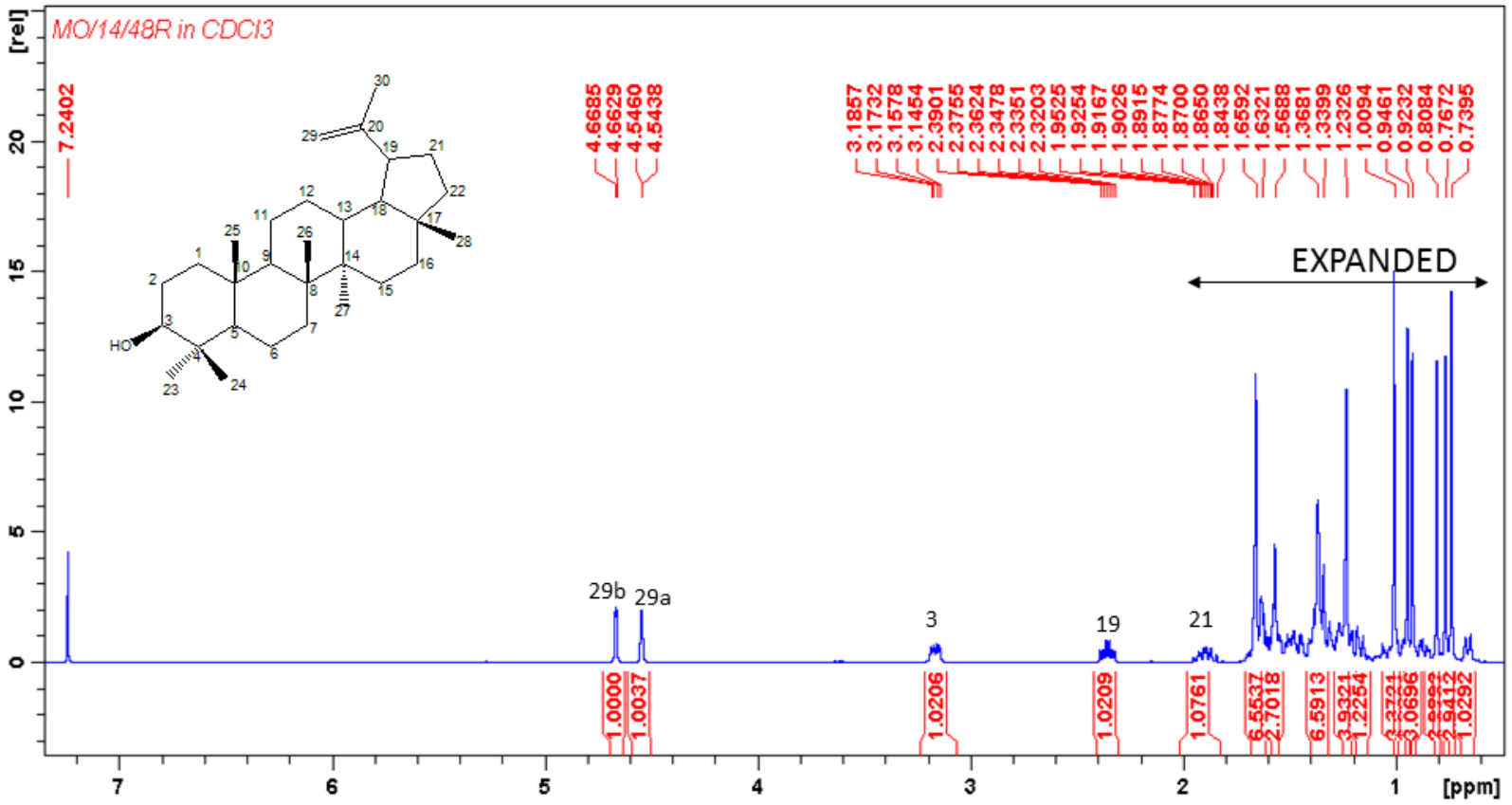
Wavelength (nm)	Abs
454.00	0.2372



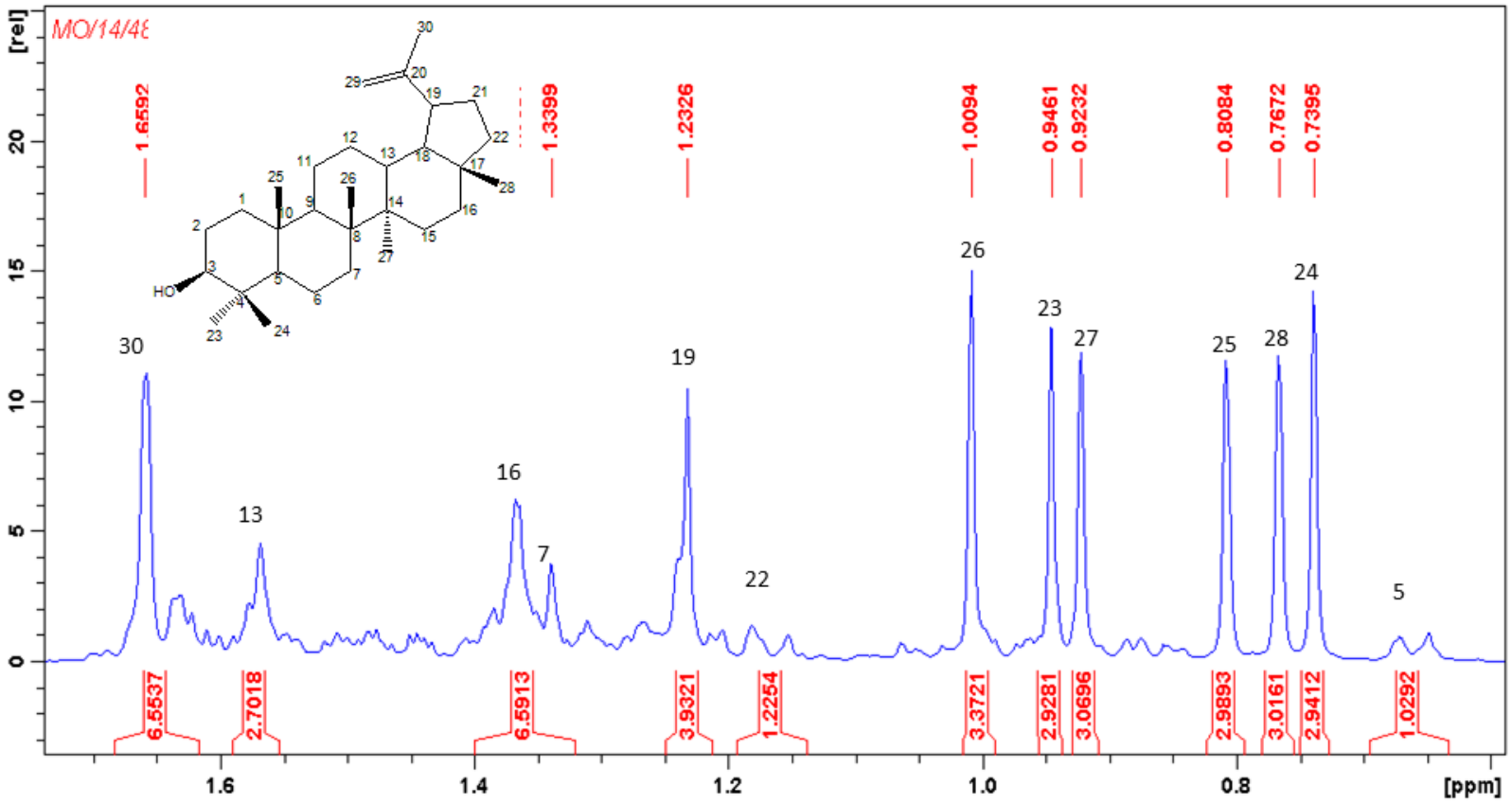
UV spectrum of lutein A-5



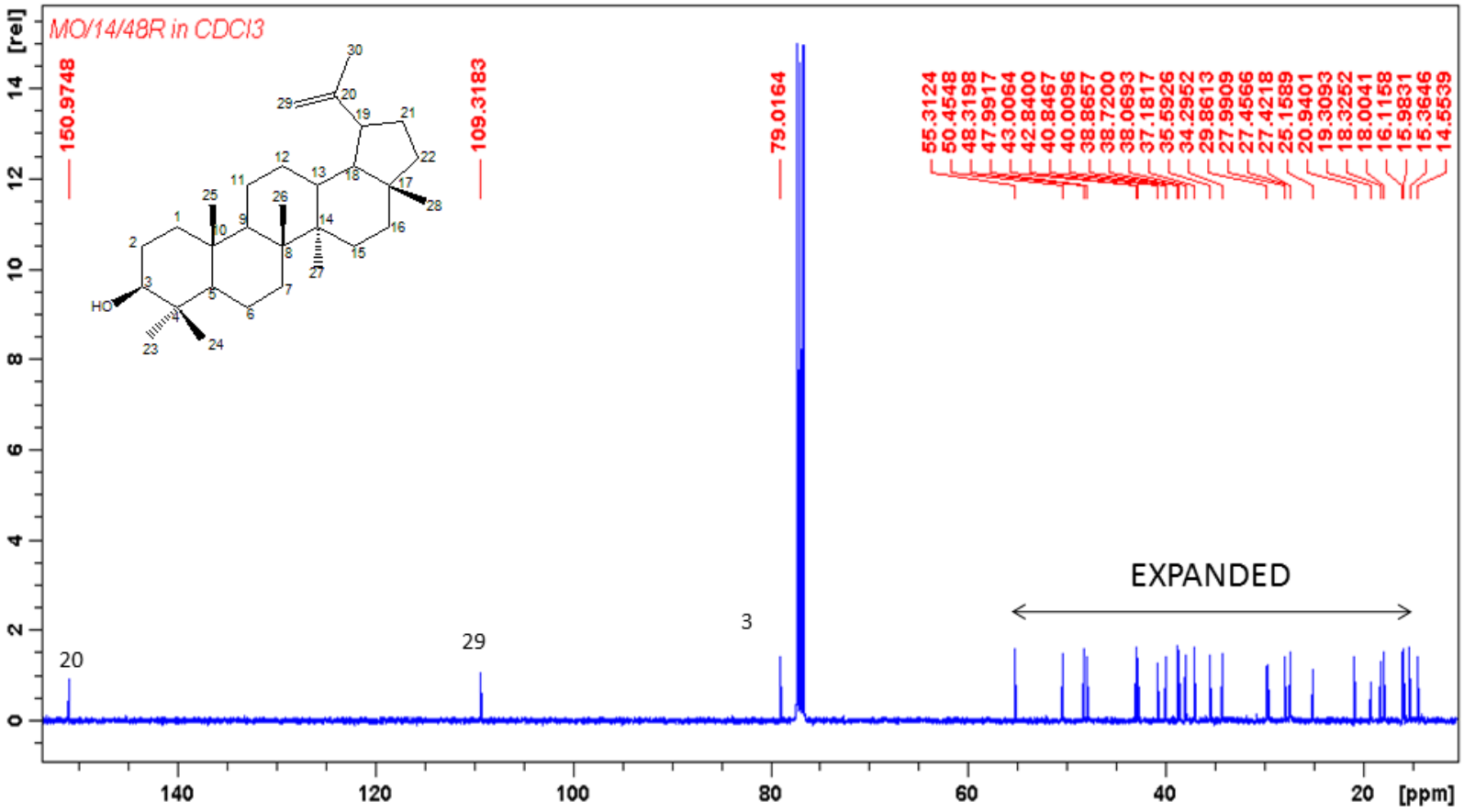
Mass spectrum of lutein A-5



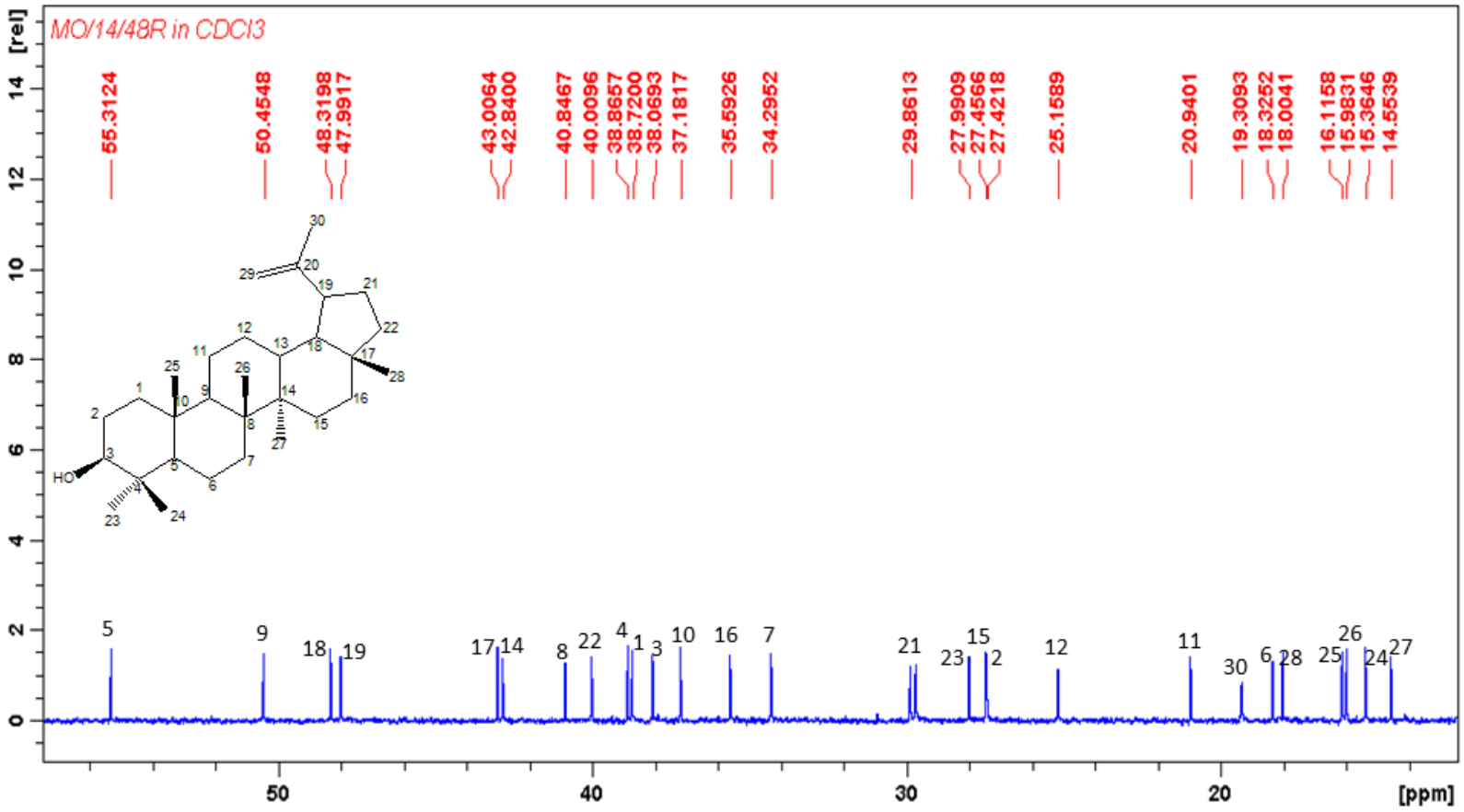
^{1}H NMR spectrum of lupeol A-6



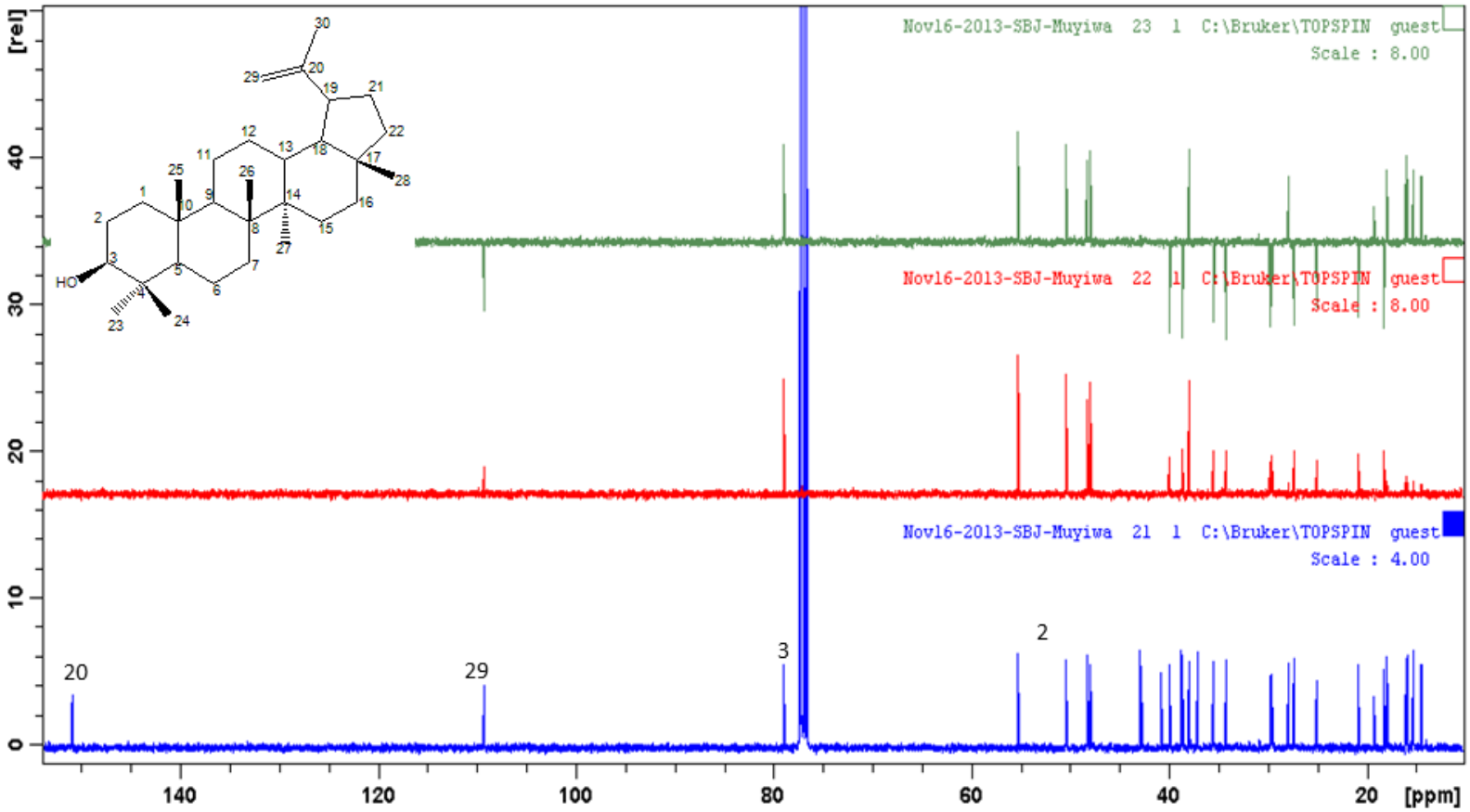
Expanded ^{1}H NMR spectrum of lupeol A-6



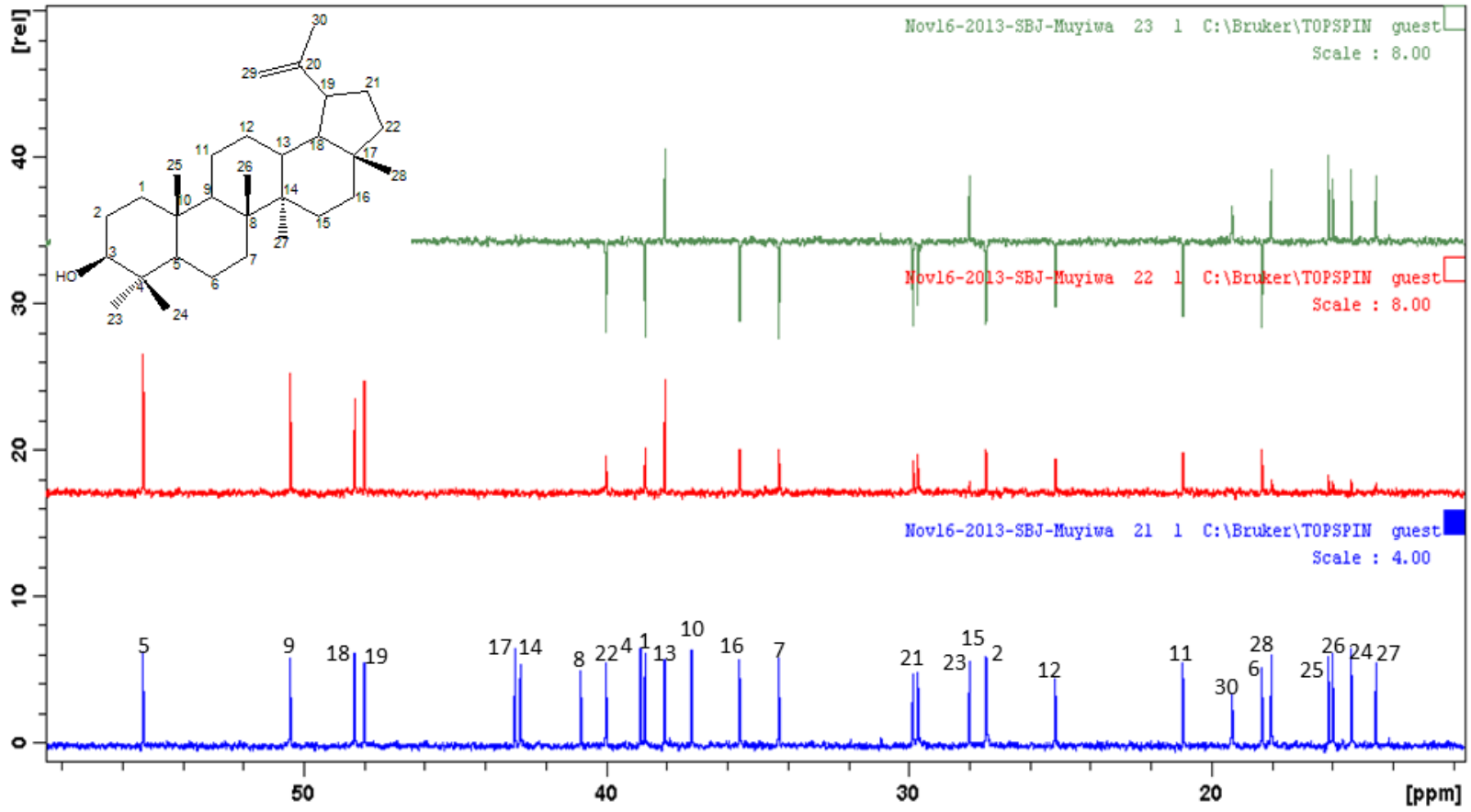
^{13}C NMR spectrum of lupeol A-6



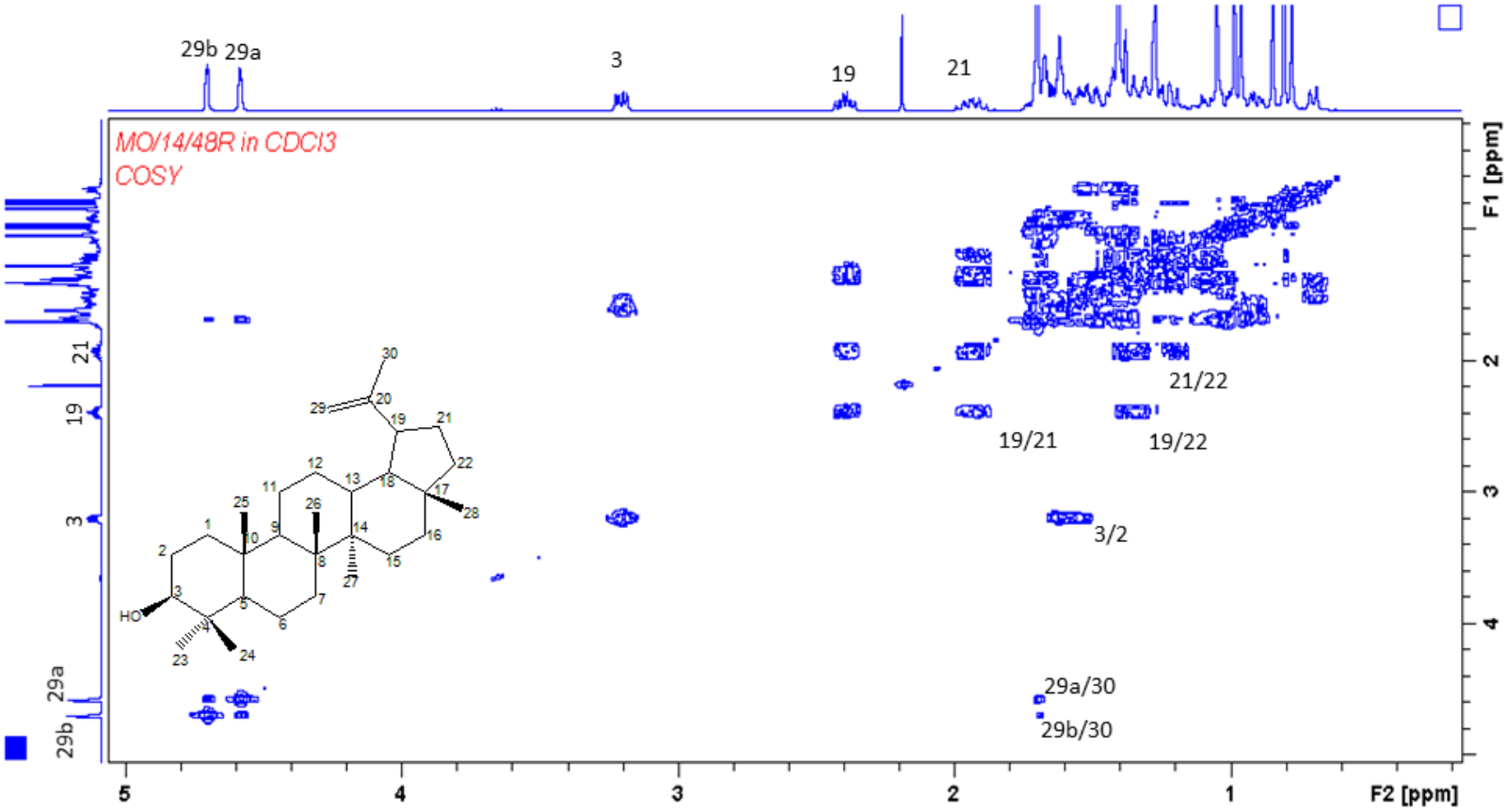
Expanded ^{13}C NMR spectrum of lupeol A-6



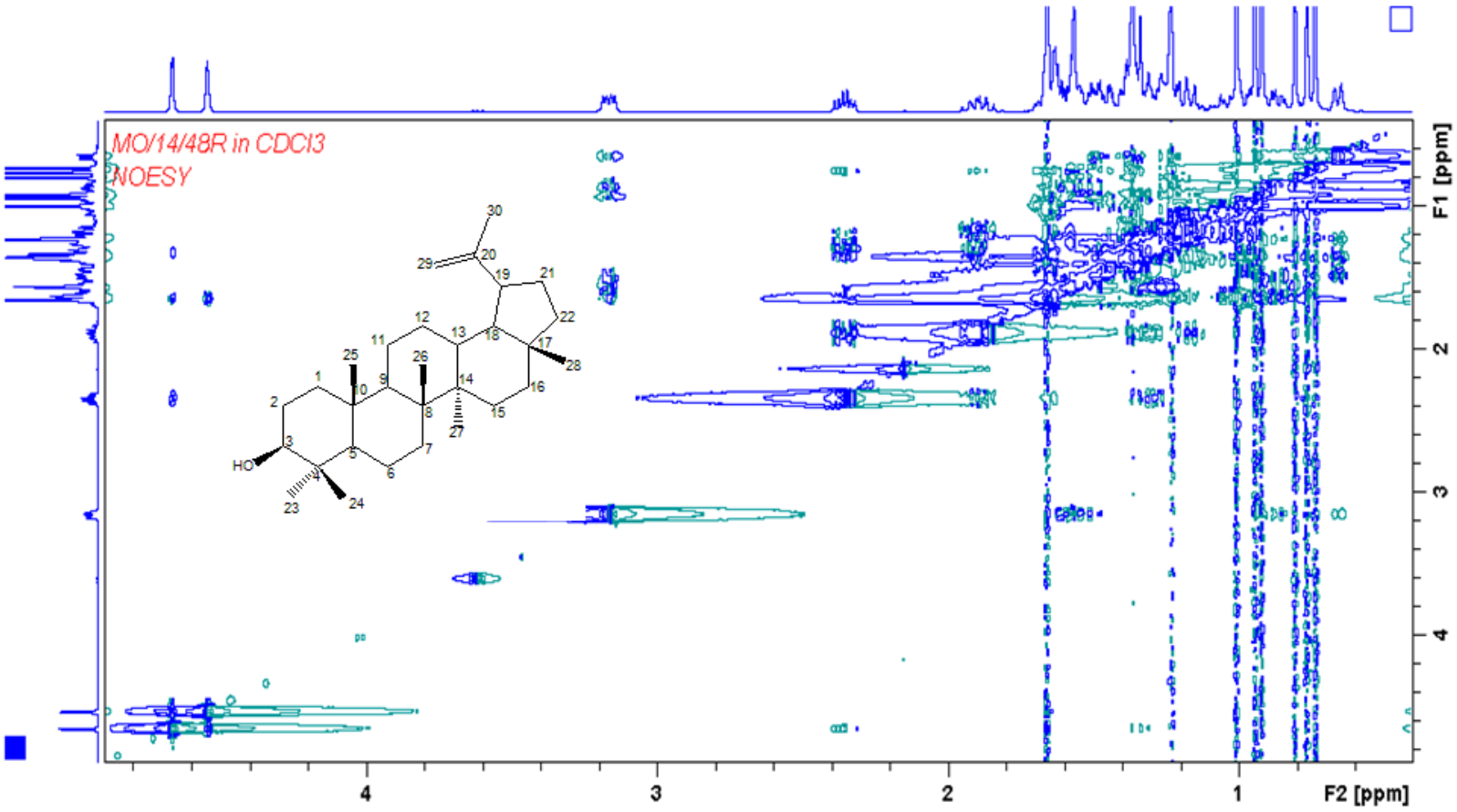
DEPT NMR spectrum of lupeol A-6



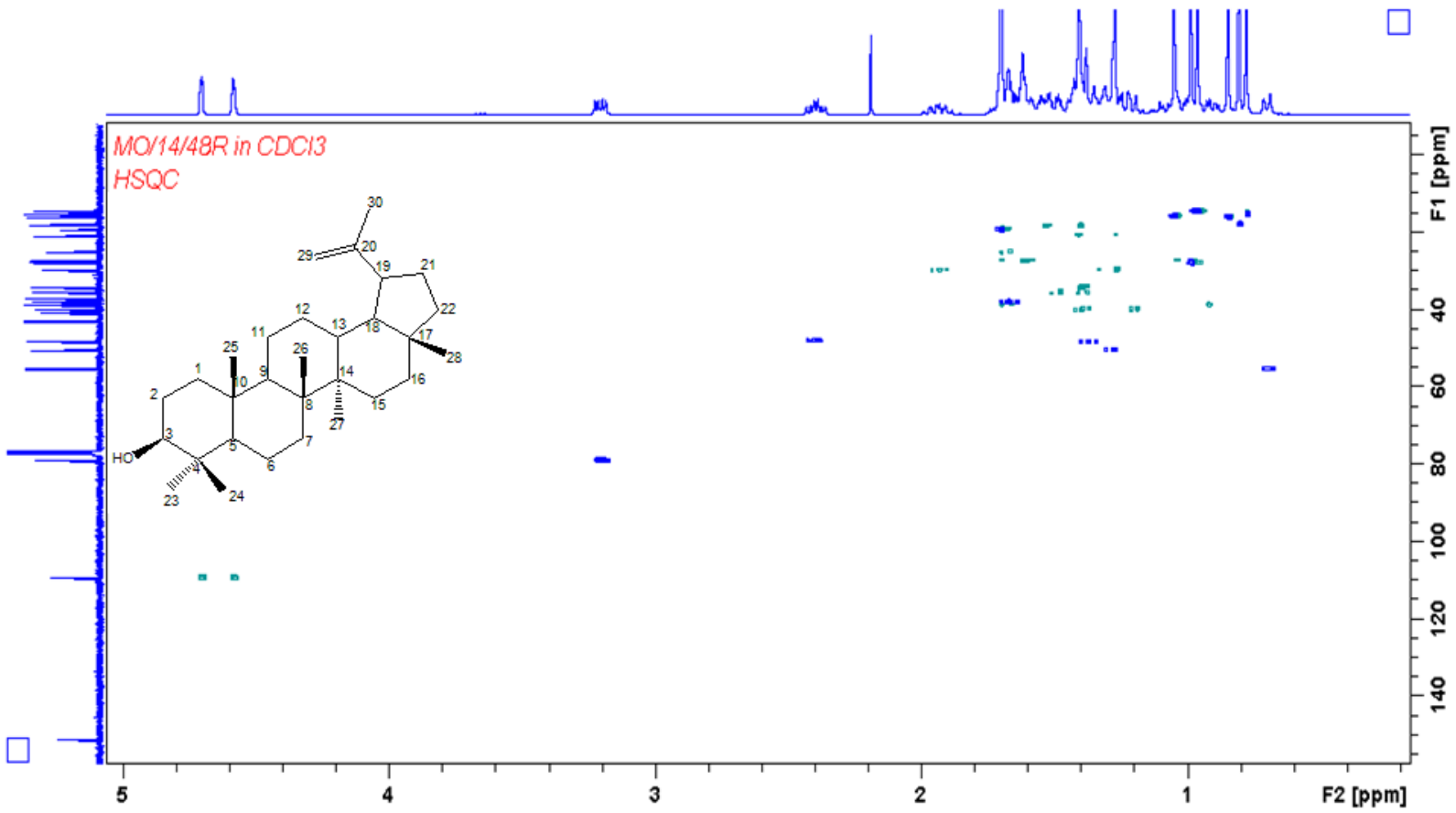
Expanded DEPT NMR spectrum of lupeol A-6



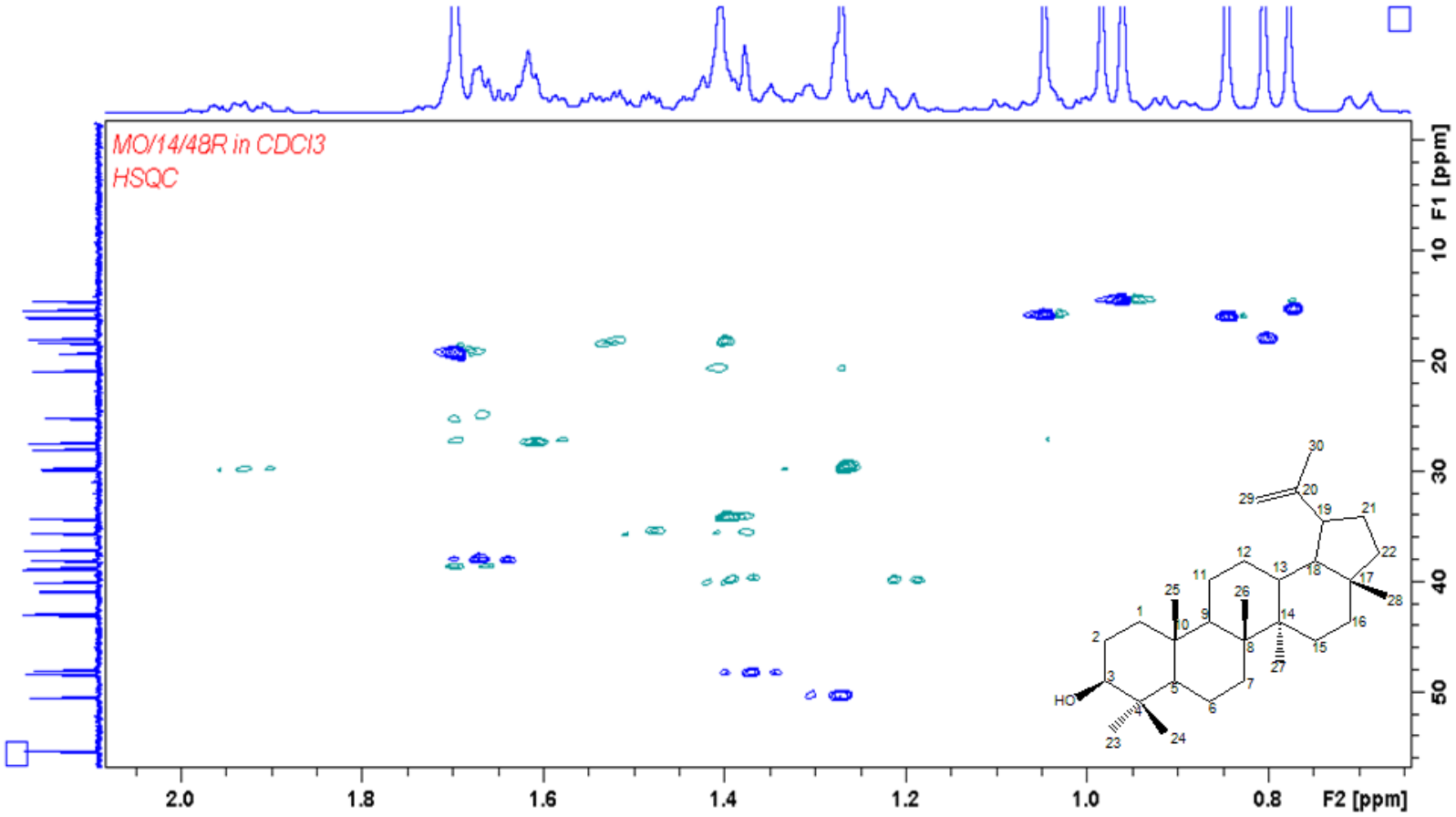
COSY spectrum of lupeol A-6



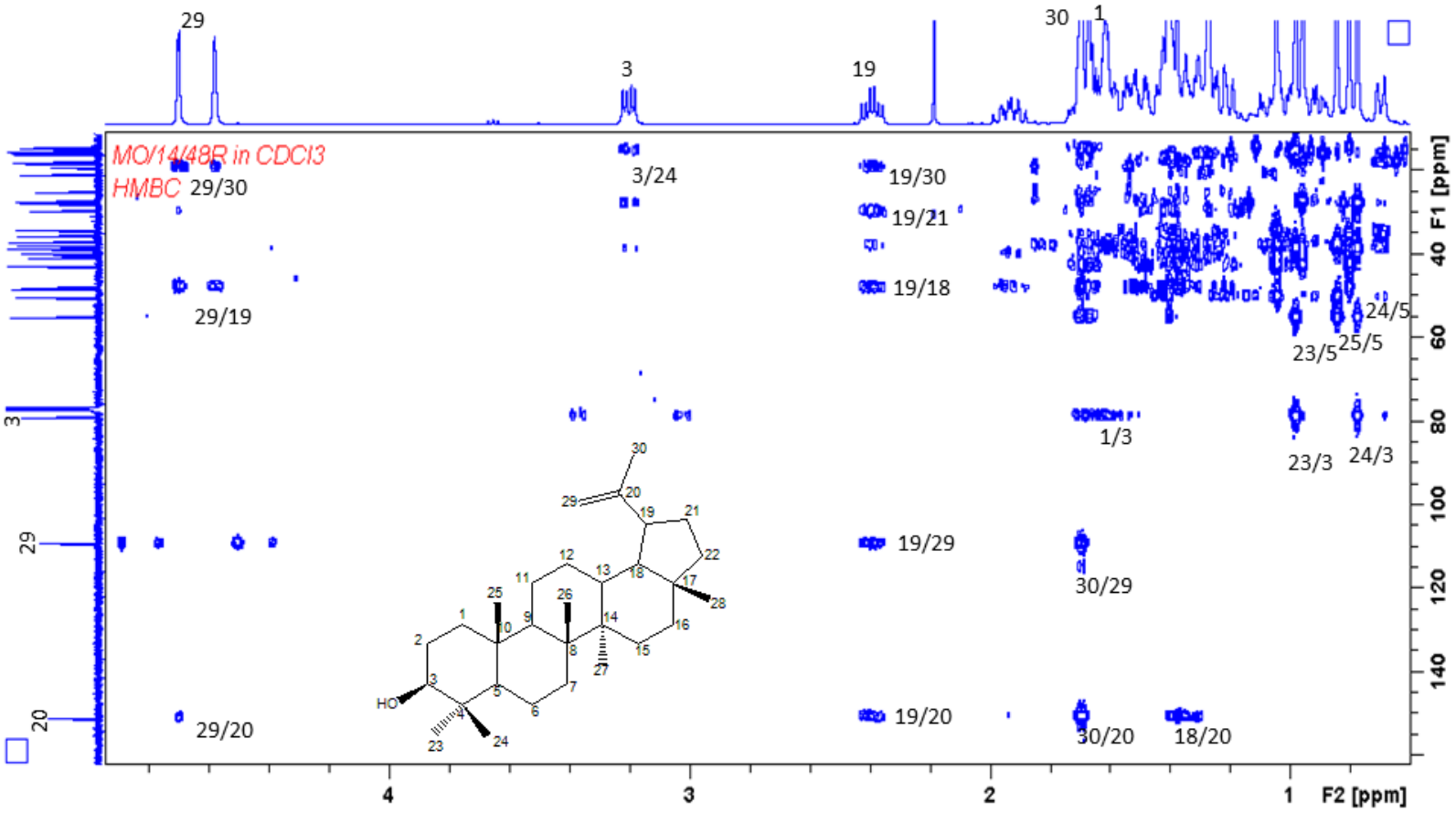
NOESY spectrum of lupeol A-6



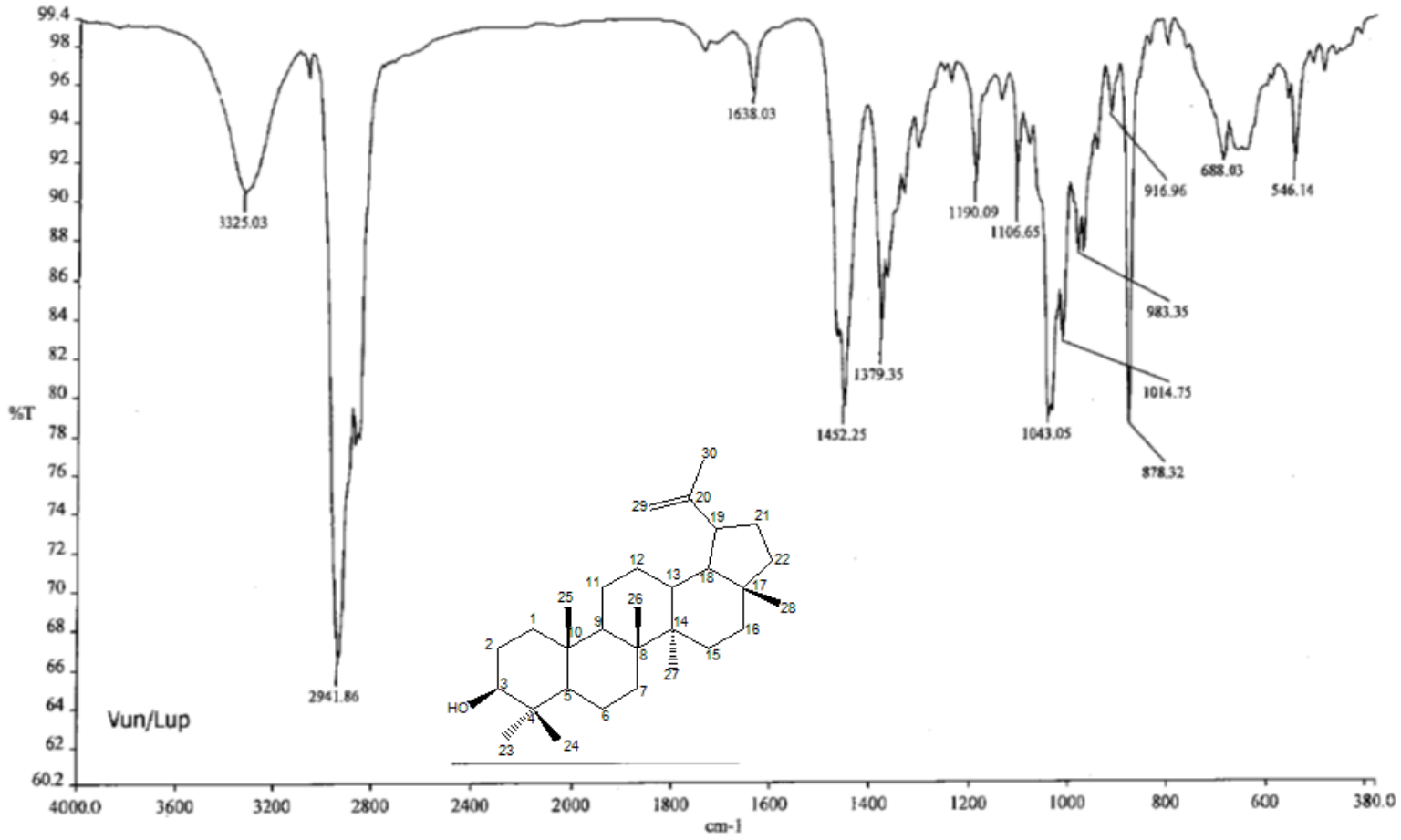
HSQC spectrum of lupeol A-6



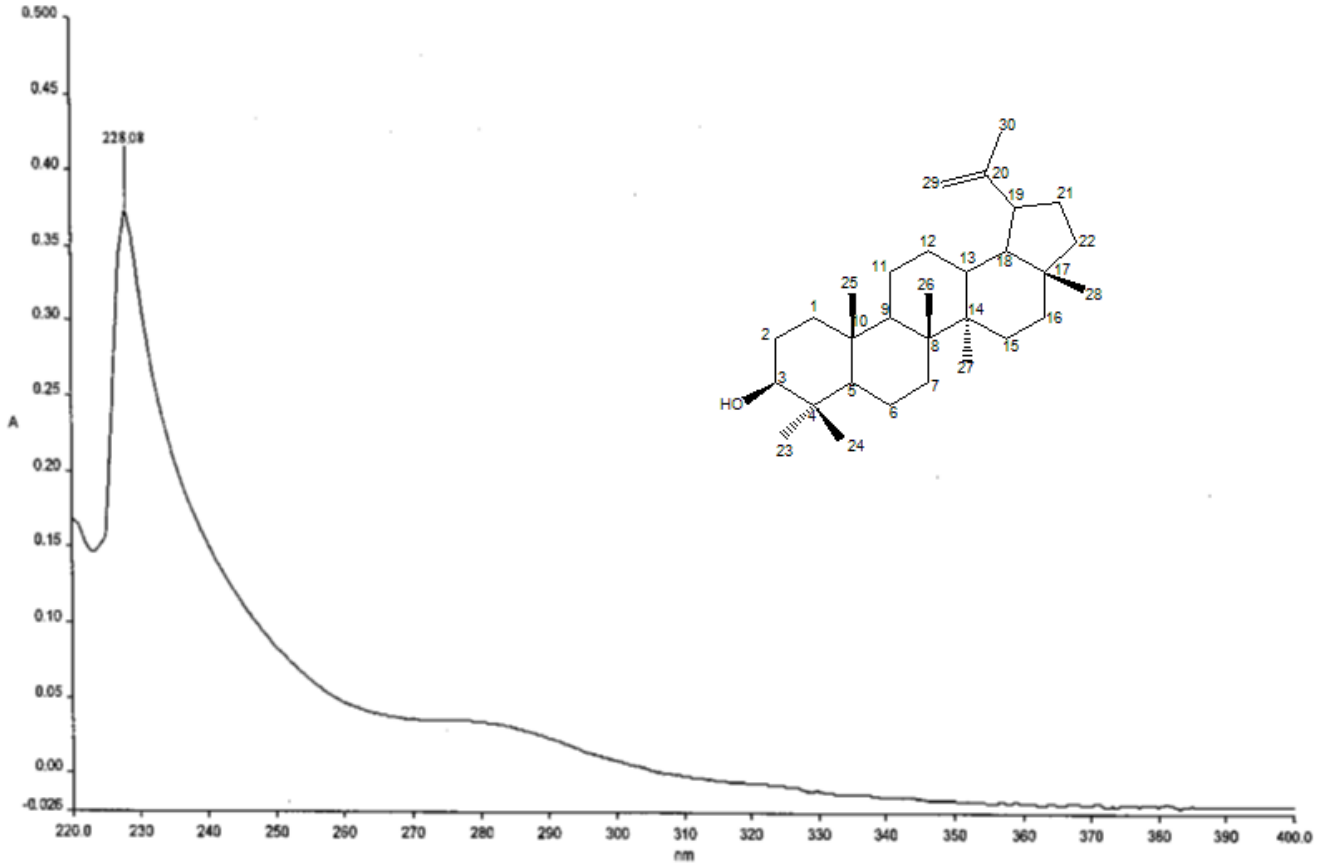
Expanded HSQC spectrum of lupeol A-6



Expanded HMBC spectrum of lupeol A-6



IR spectrum of lupeol A-6

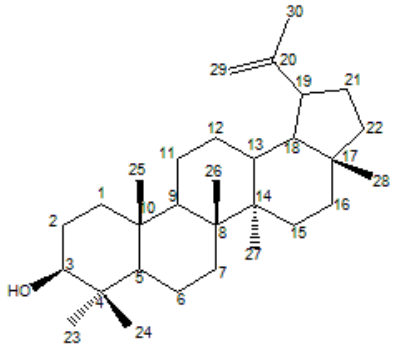


UV spectrum of lupeol A-6

Elemental Composition Report

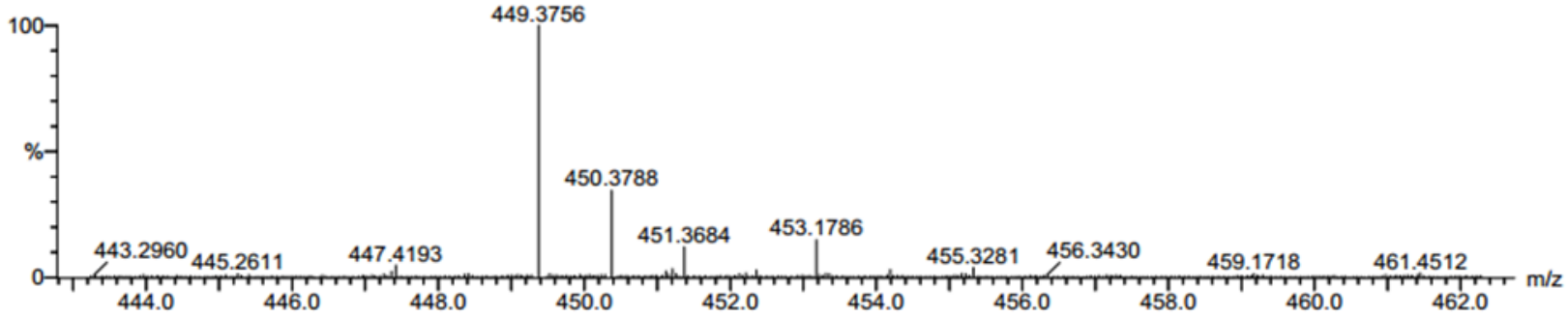
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3



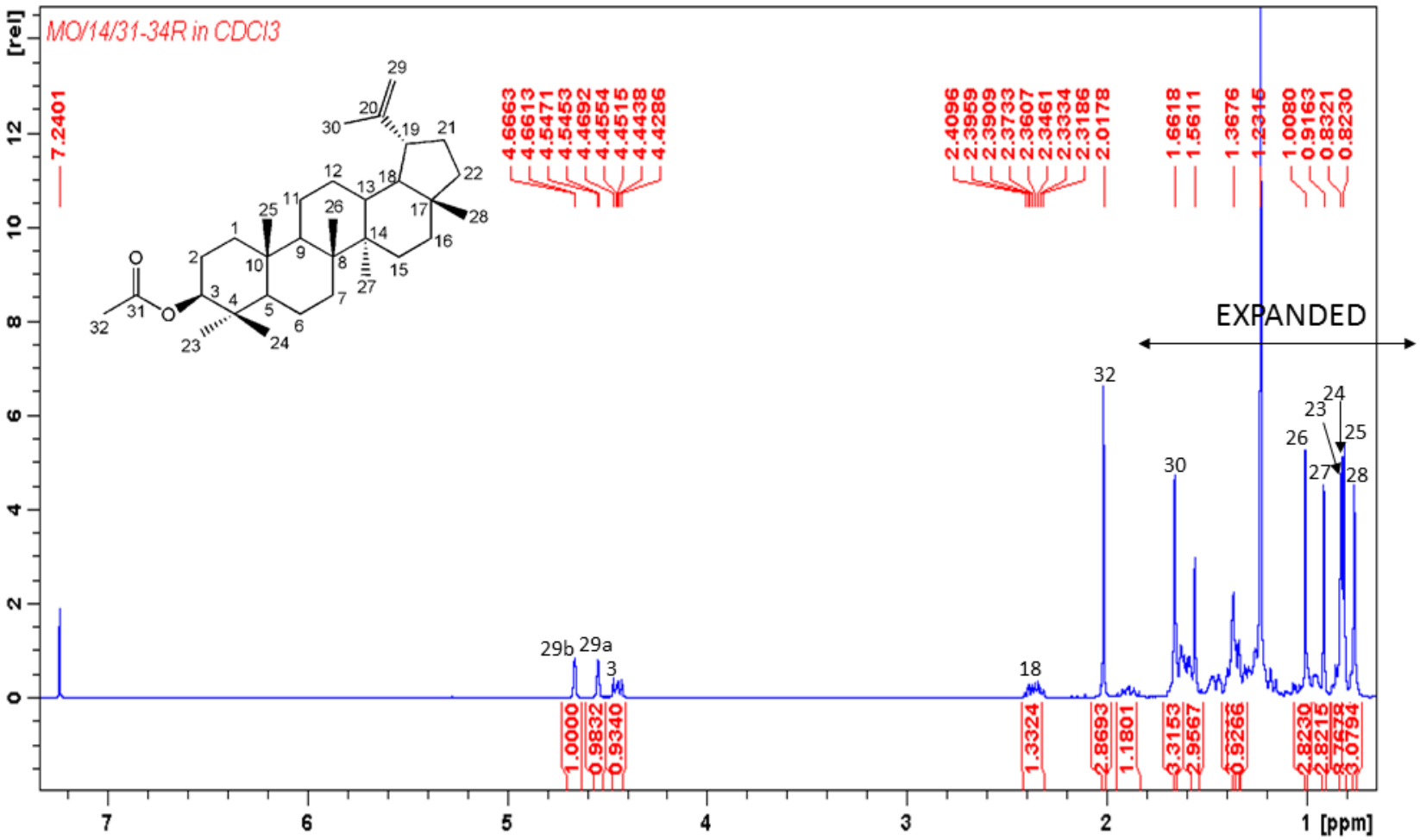
Monoisotopic Mass, Even Electron Ions
8 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 20-35 H: 40-55 O: 0-5 Na: 1-1
05-8-17-18 31 (0.512) Cm (1:60)
TOF MS ES+

9.72e+003

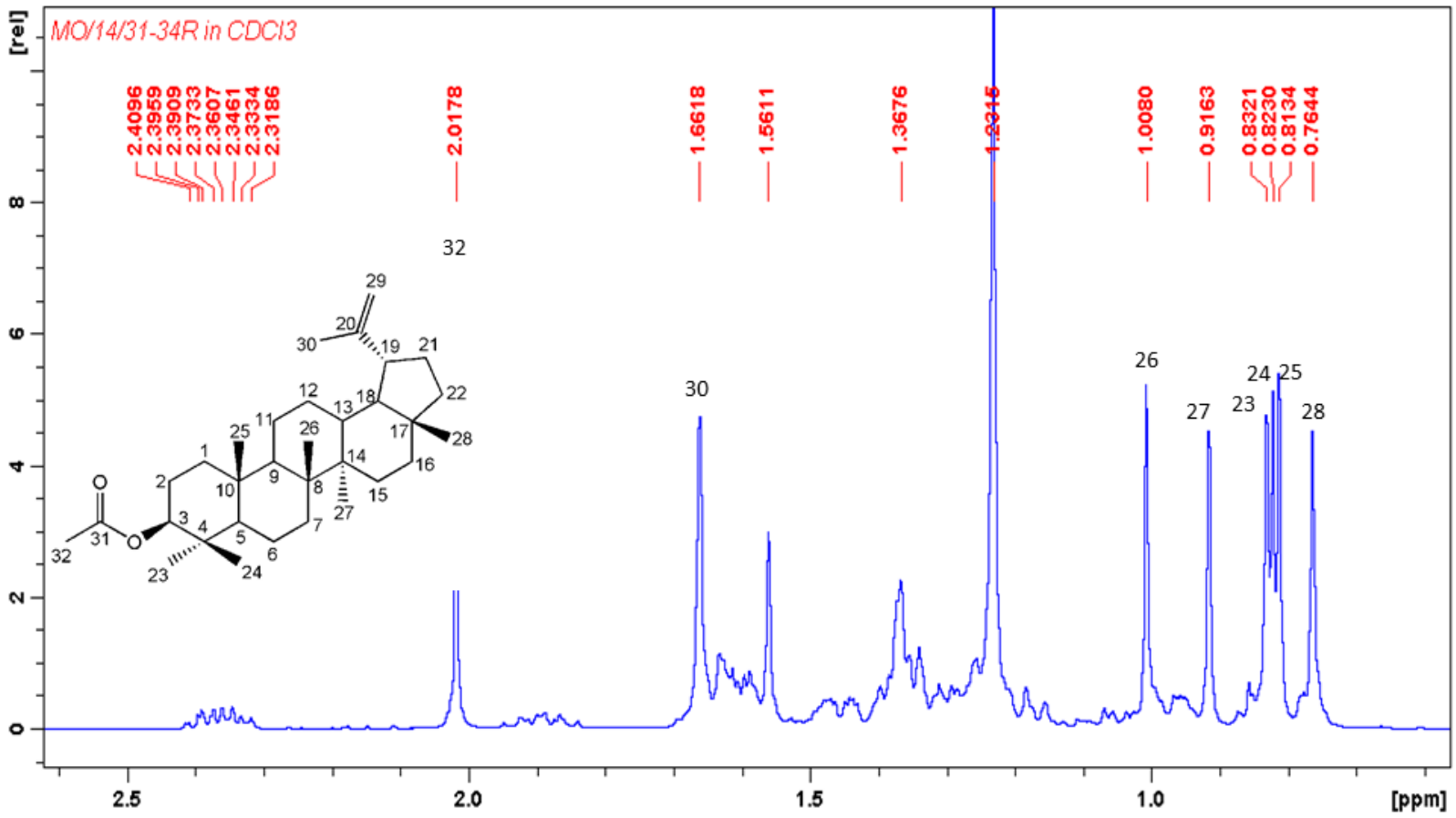


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
449.3756	449.3759	-0.3	-0.7	5.5	462.3	0.0	C30 H50 O Na

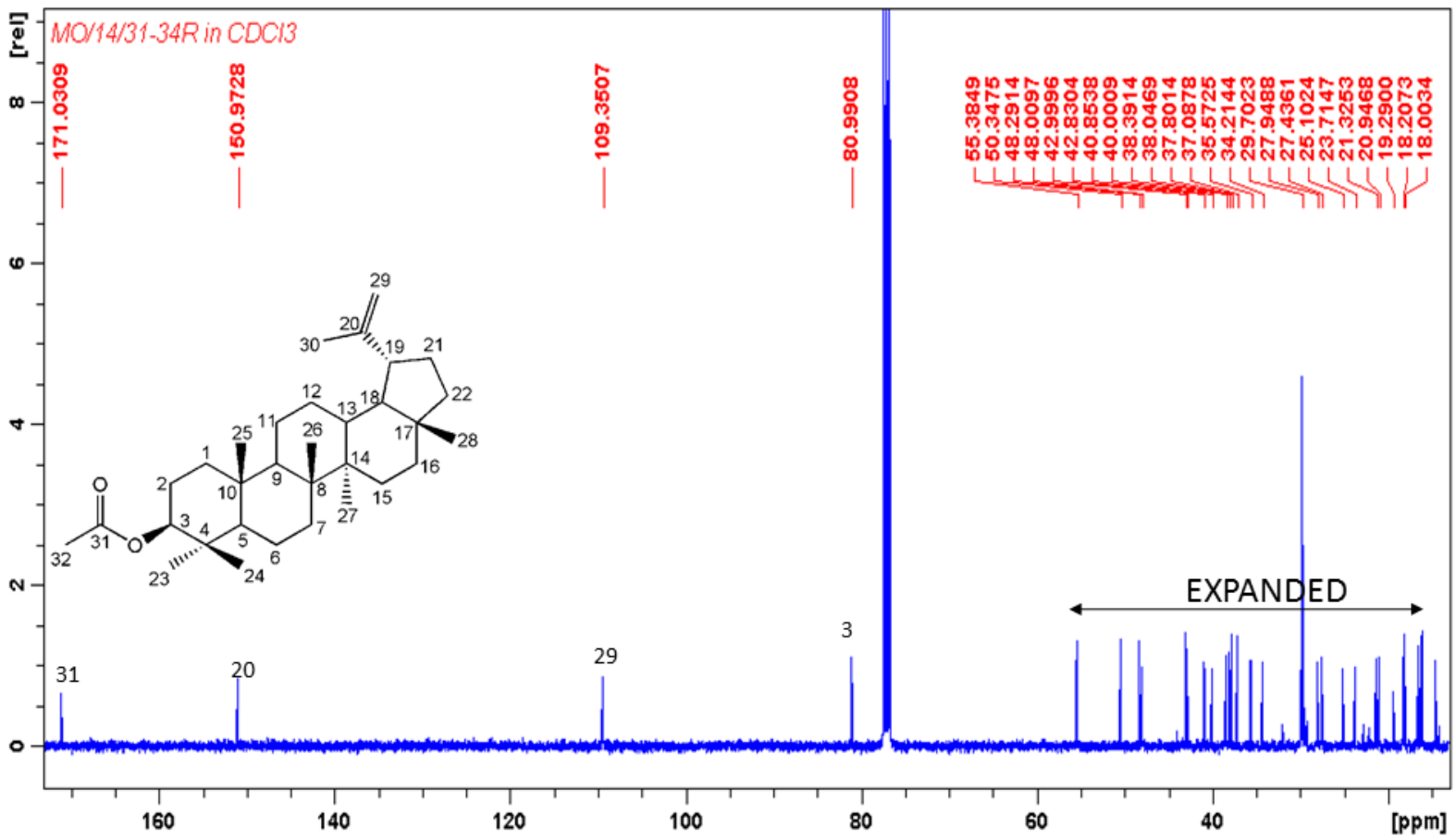
Mass spectrum of lupeol A-6



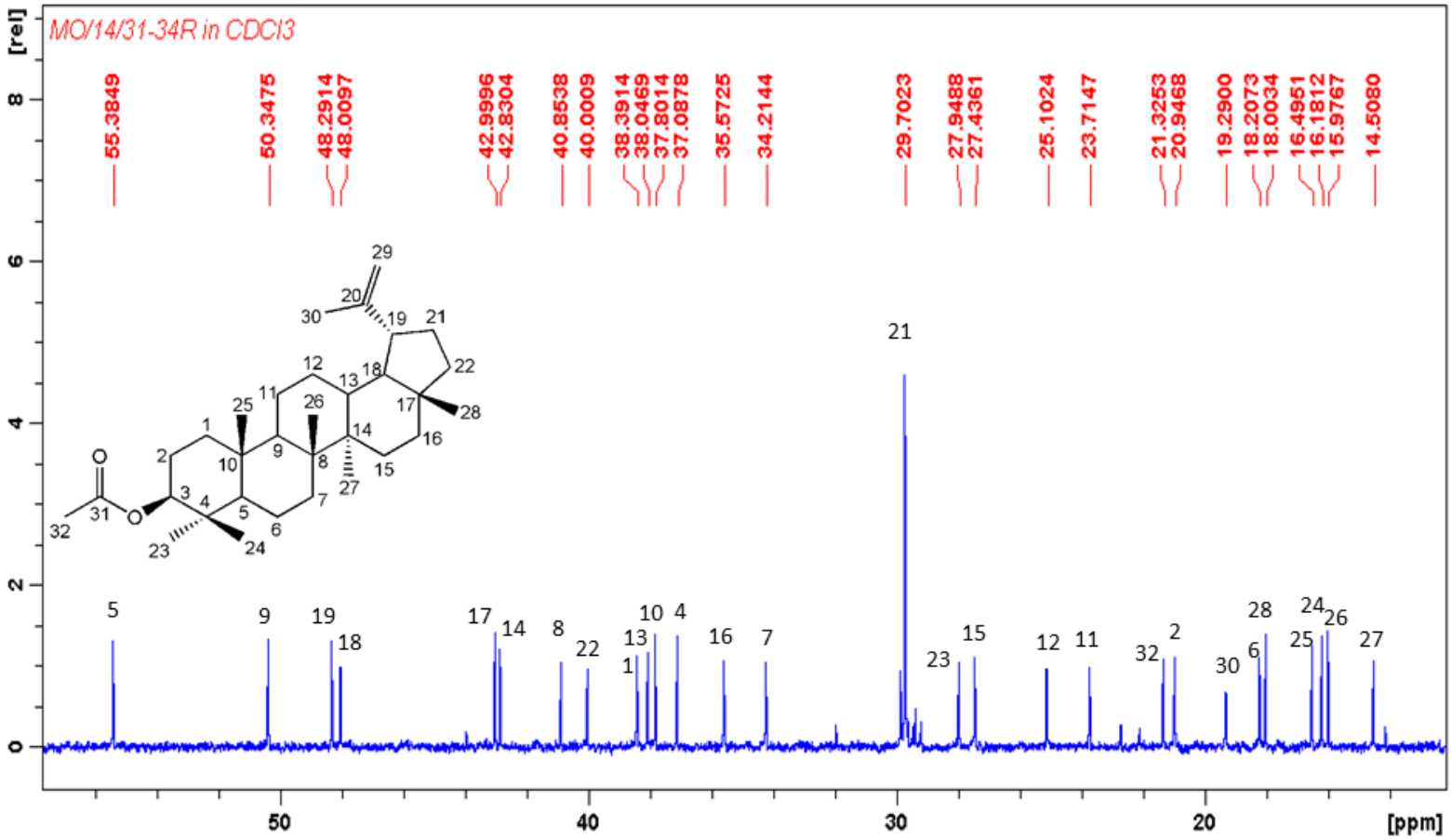
^{1}H NMR spectrum of lupeol acetate A-7



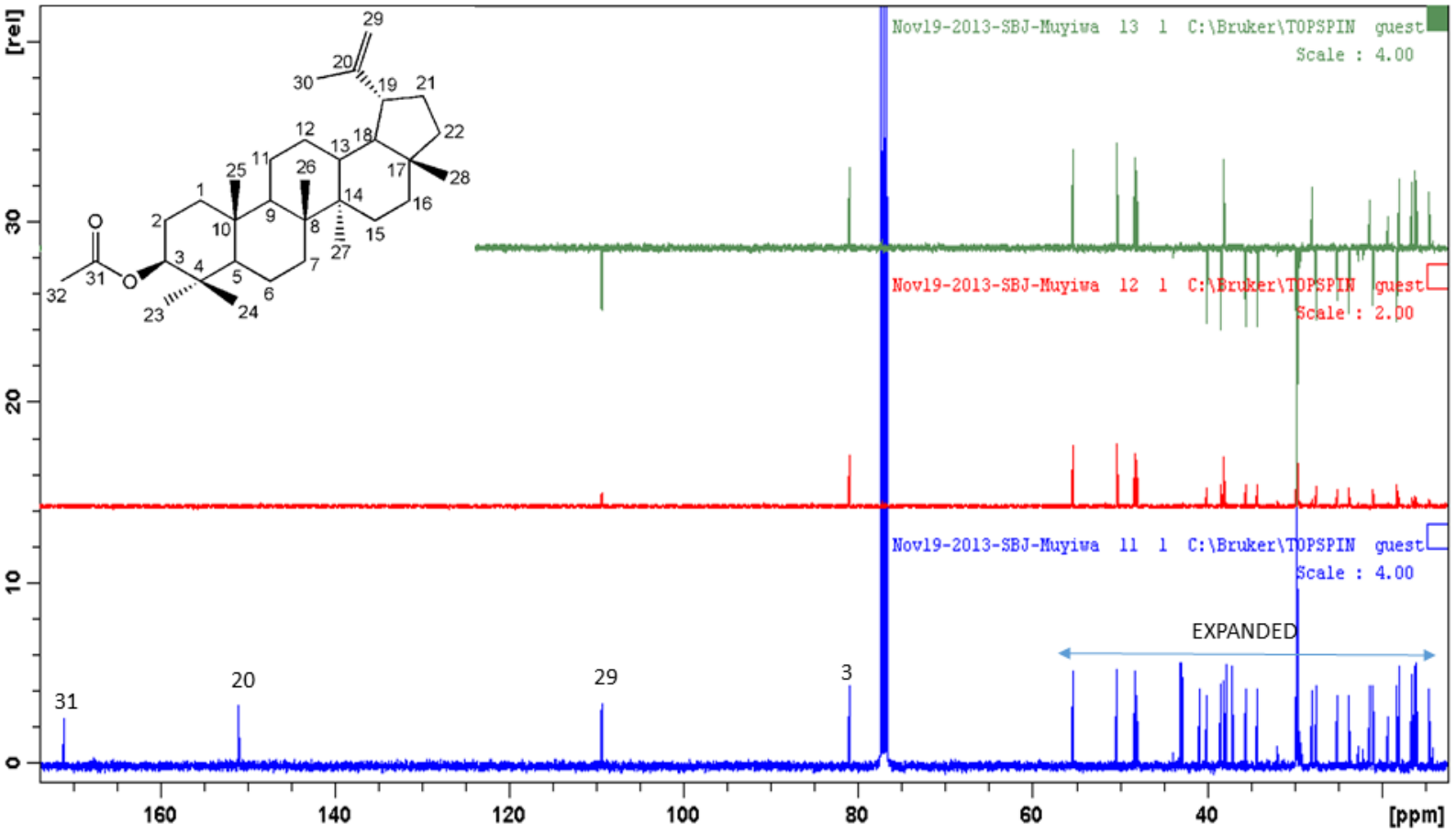
Expanded ^{1}H NMR spectrum of lupeol acetate A-7



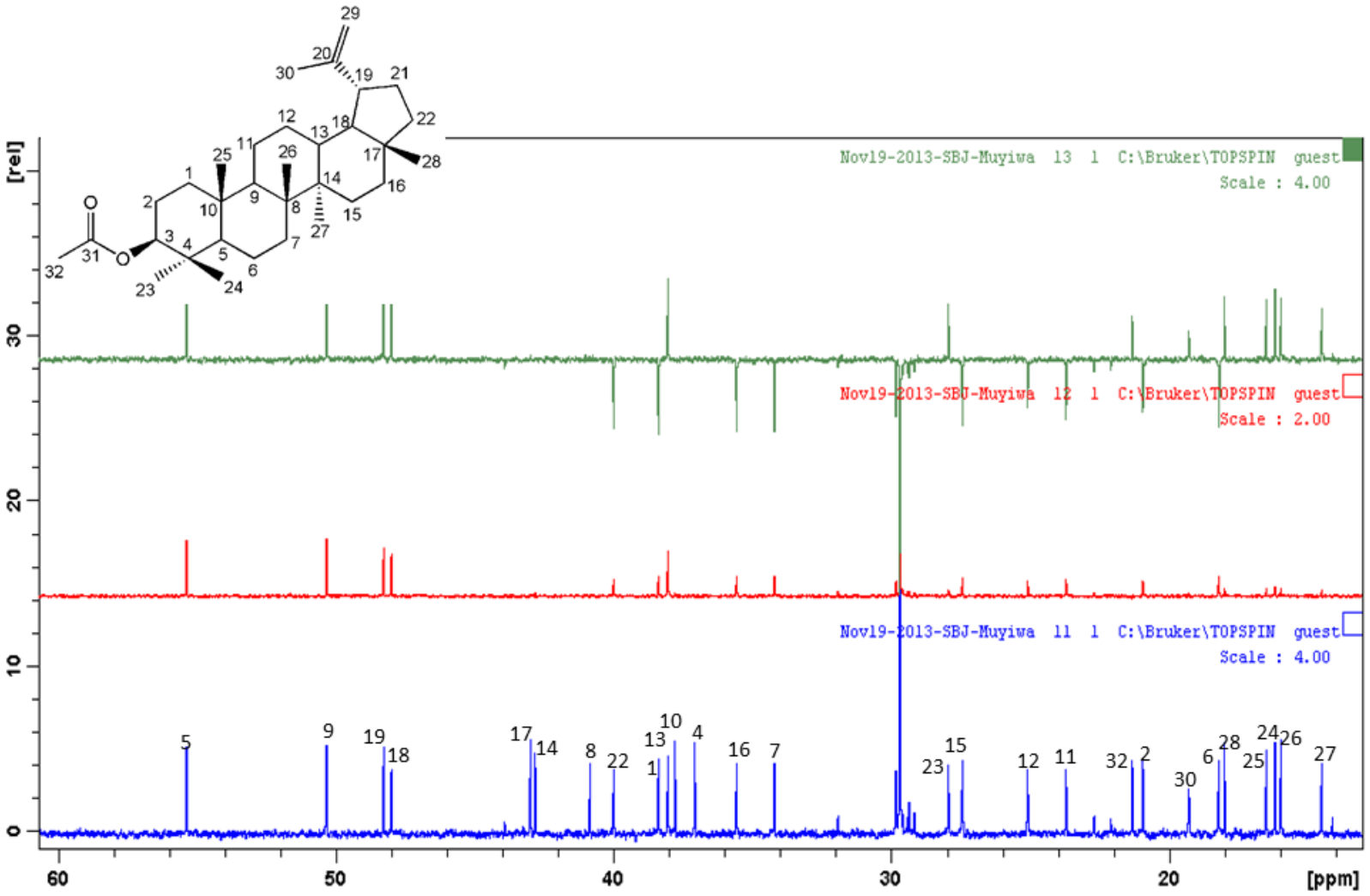
^{13}C NMR spectrum of lupeol acetate A-7



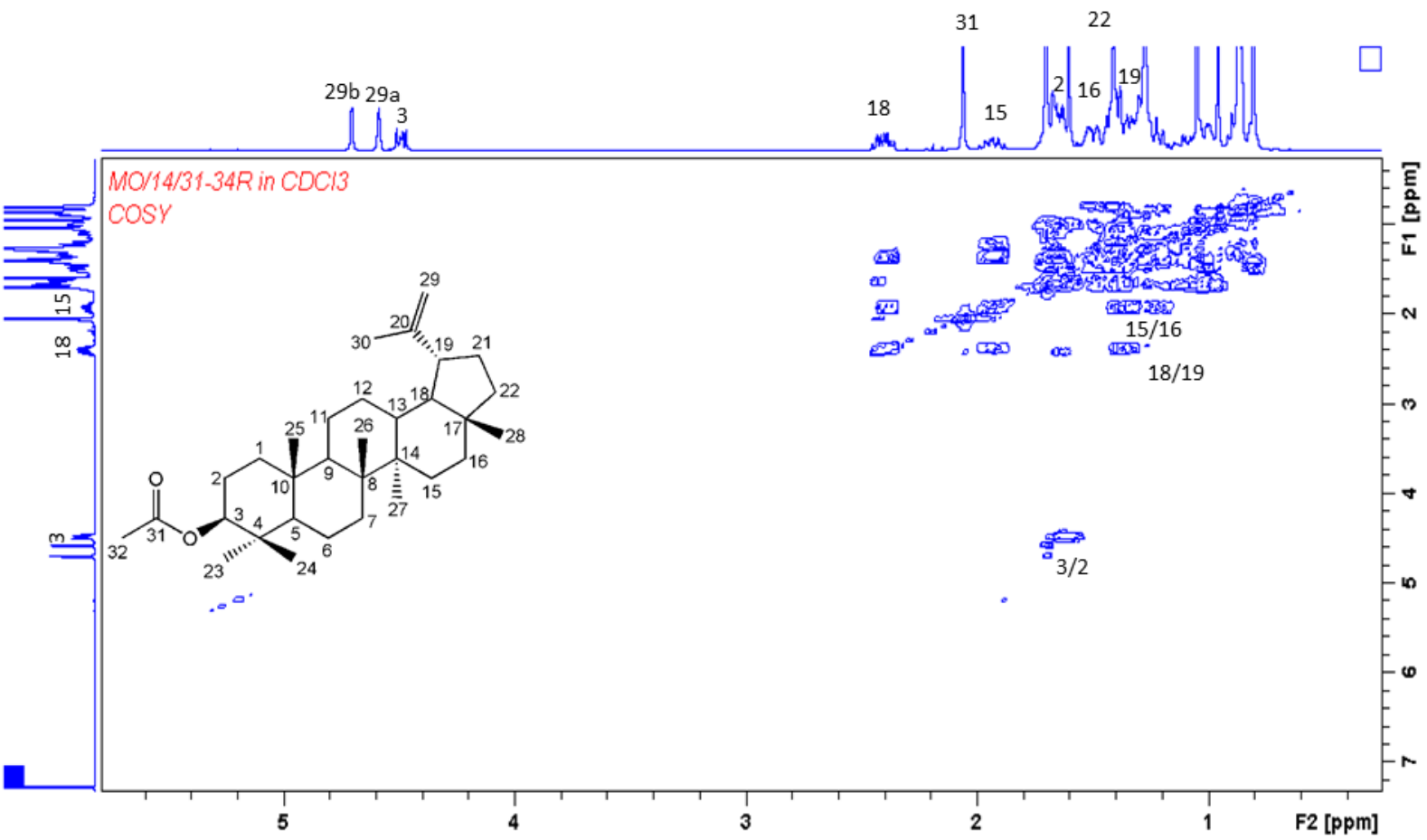
Expanded ^{13}C NMR spectrum of lupeol acetate A-7



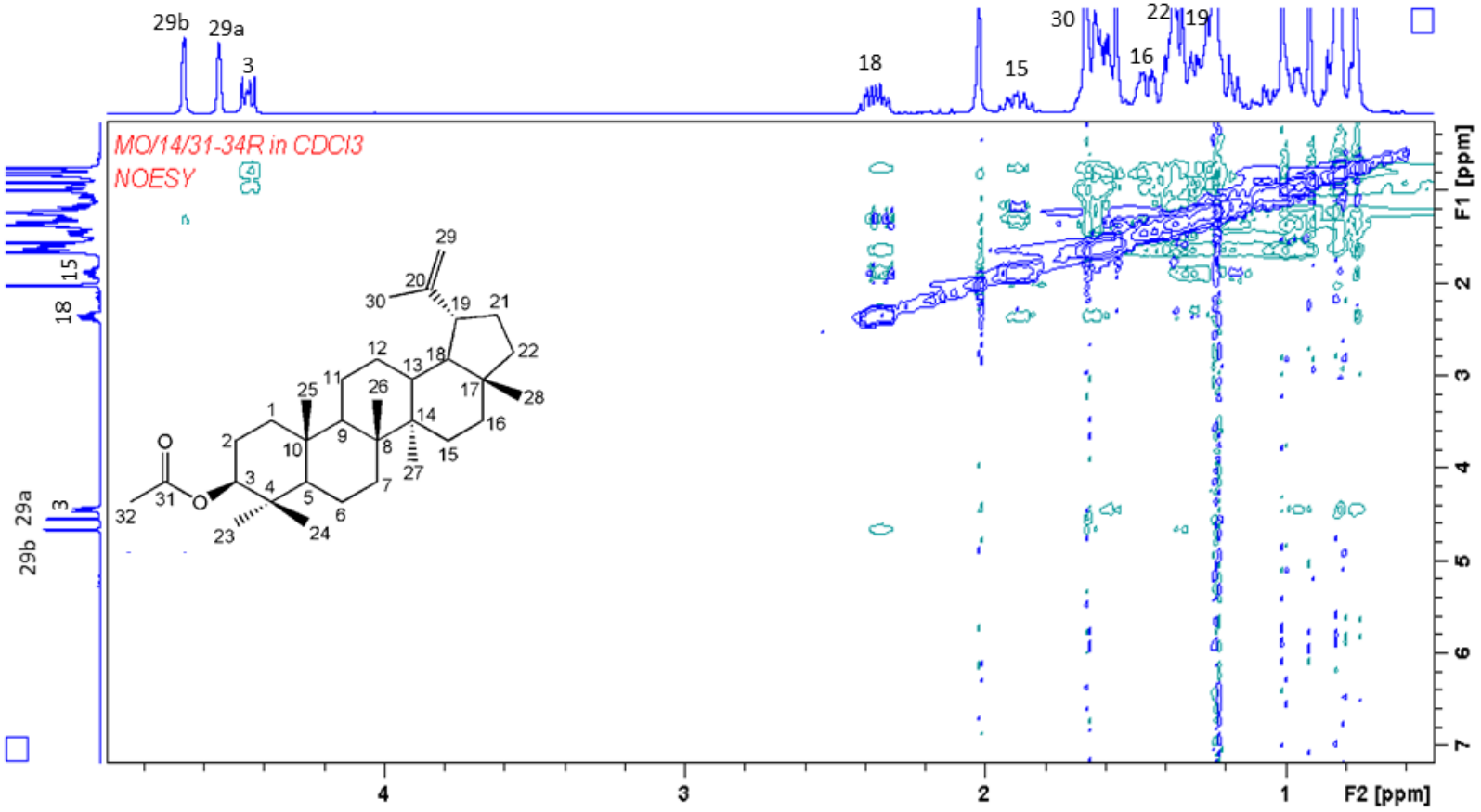
DEPT NMR spectrum of lupeol acetate A-7



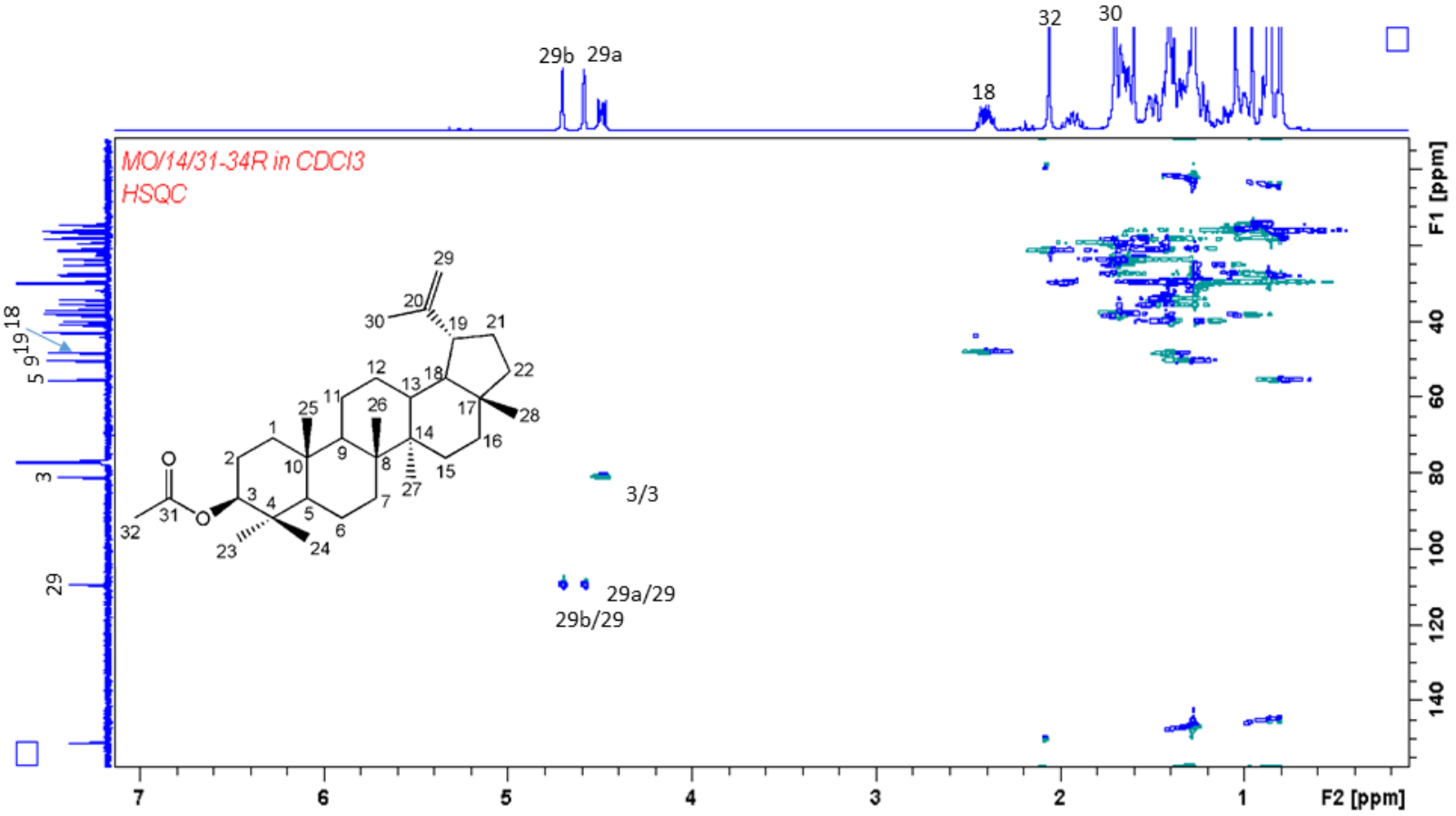
Expanded DEPT NMR spectrum of lupeol acetate A-7



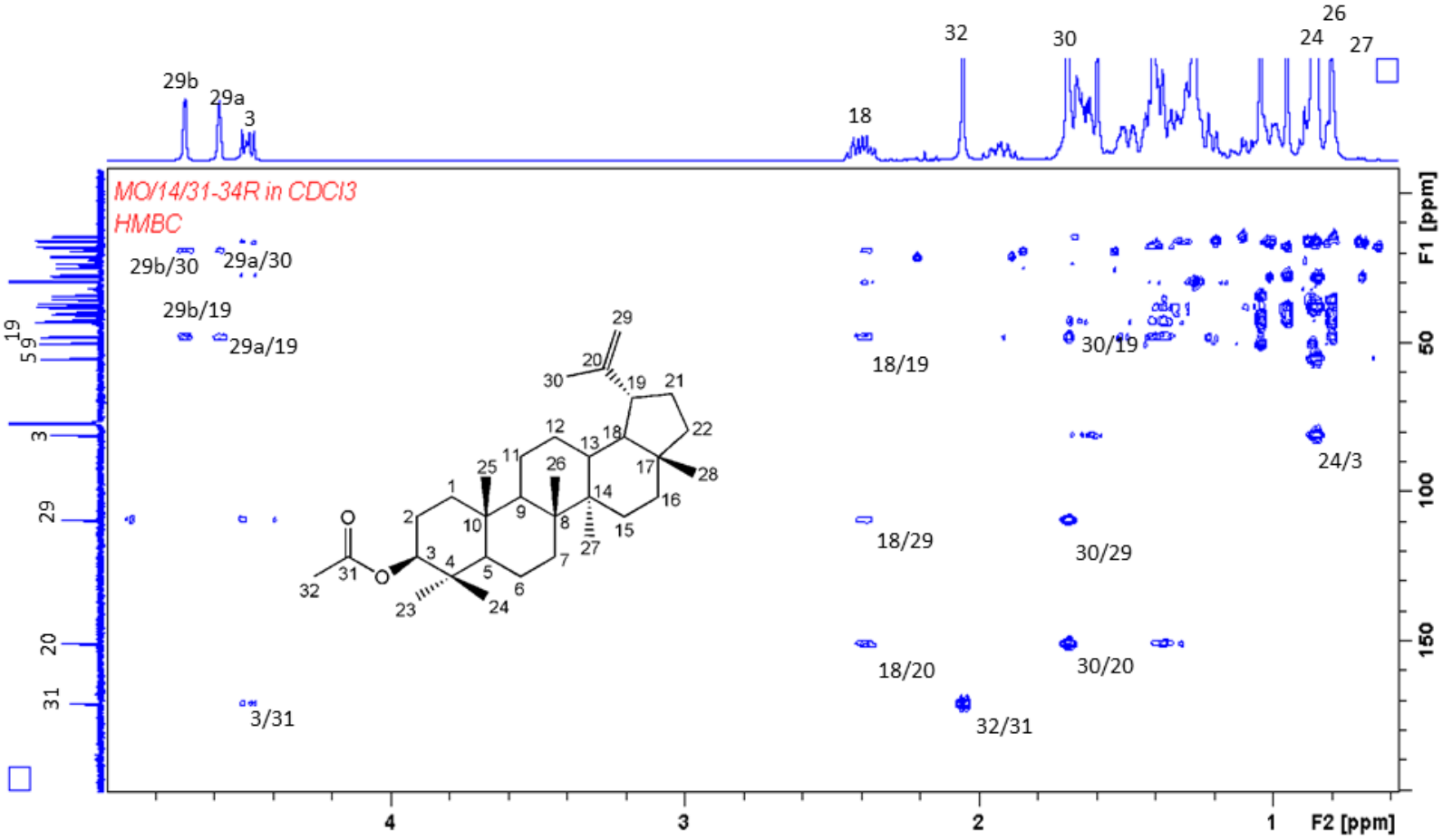
COSY spectrum of lupeol acetate A-7



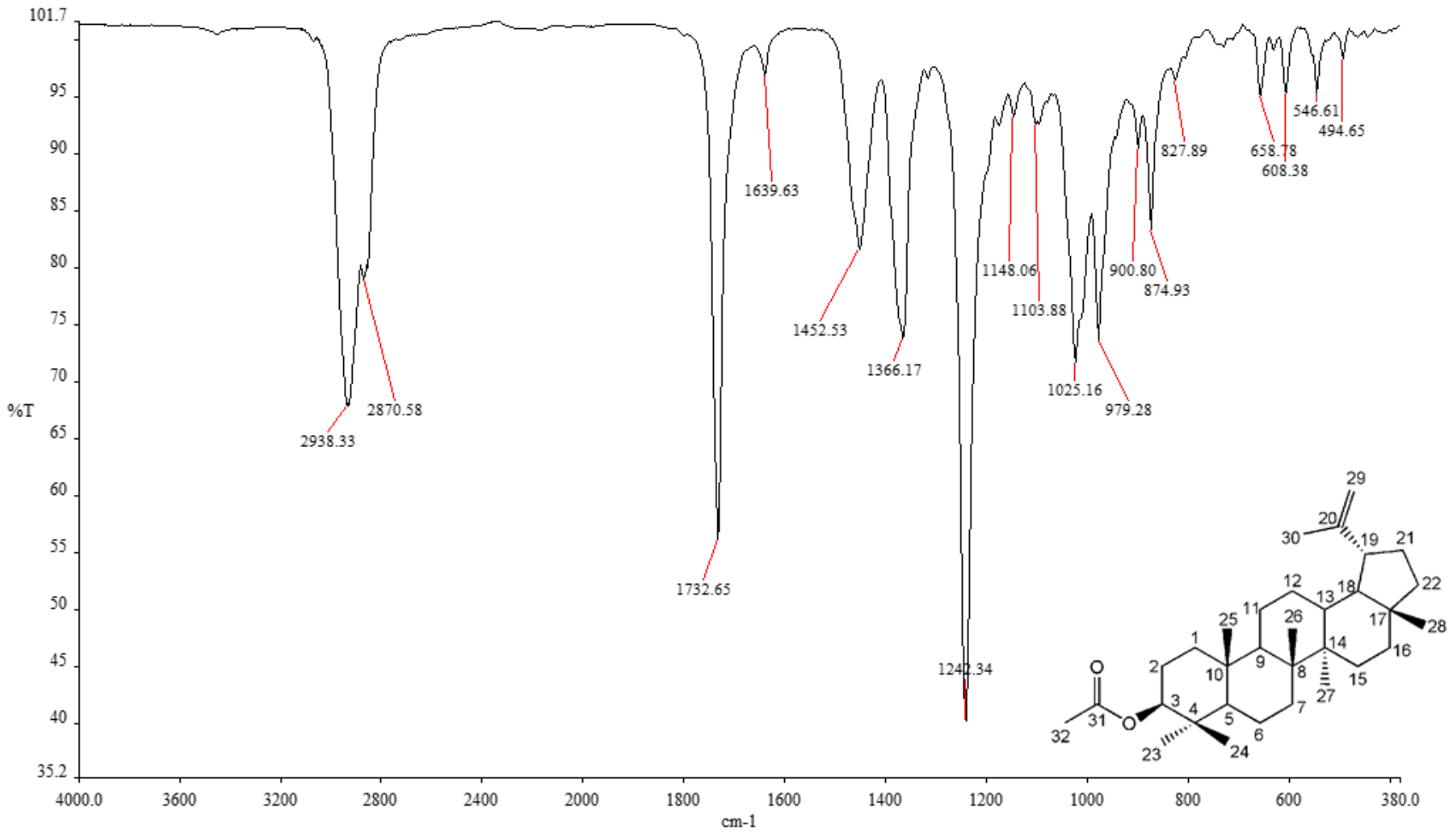
NOESY spectrum of lupeol acetate A-7



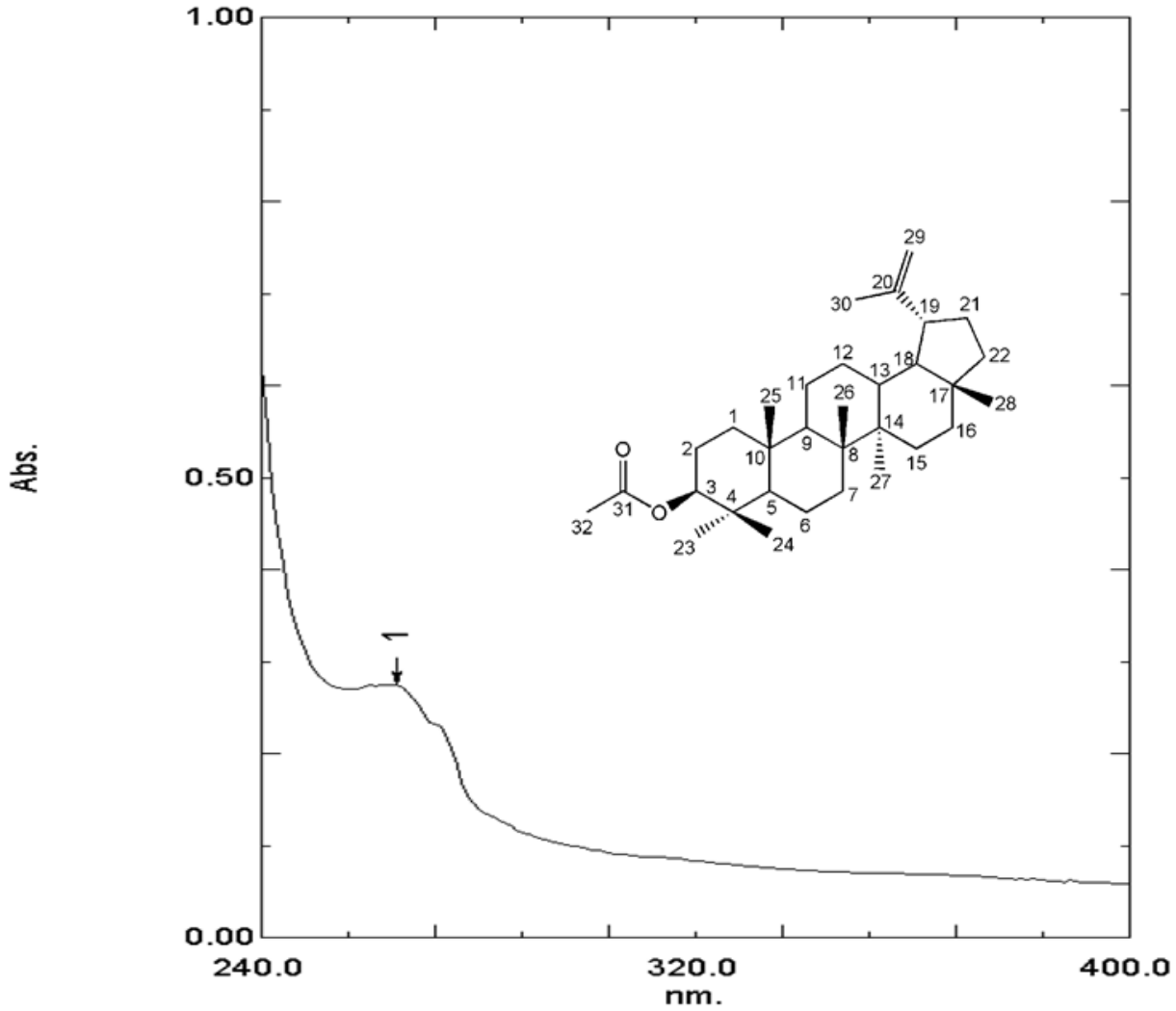
HSQC spectrum of lupeol acetate A-7



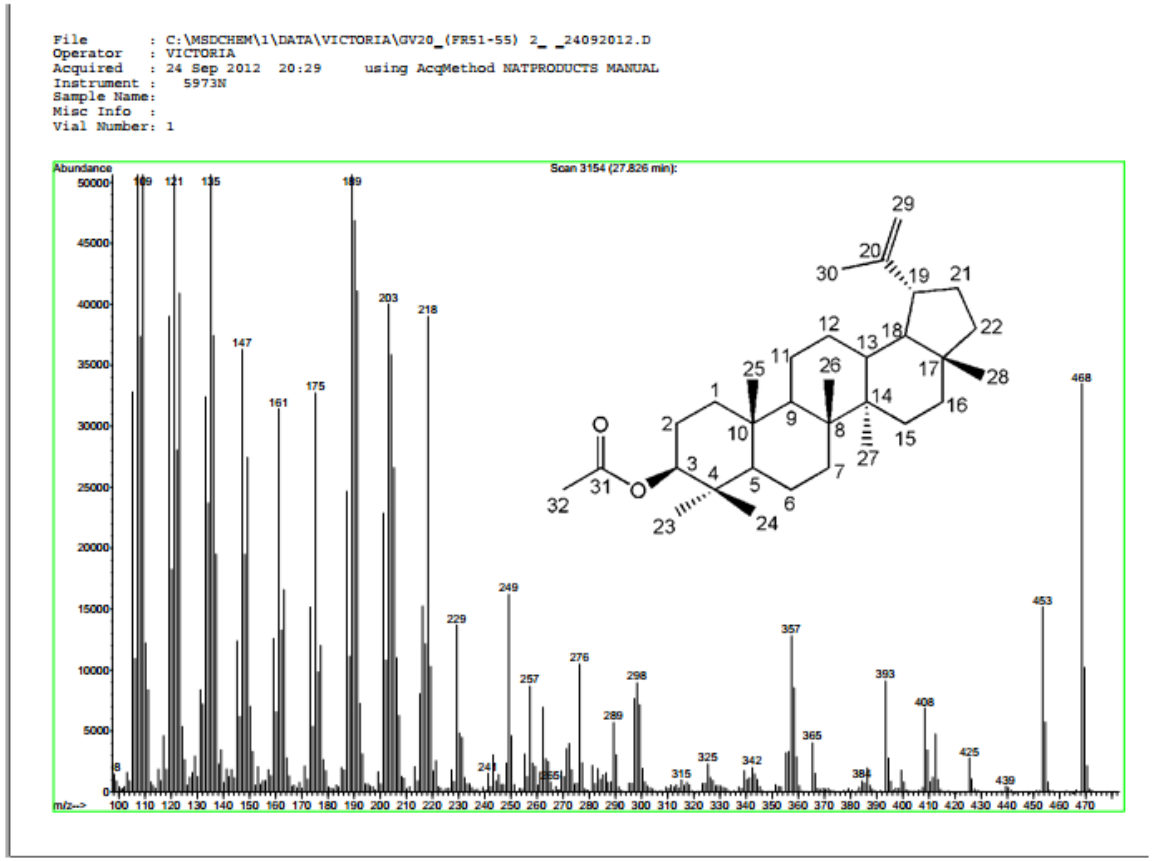
Expanded HMBC spectrum of lupeol acetate A-7



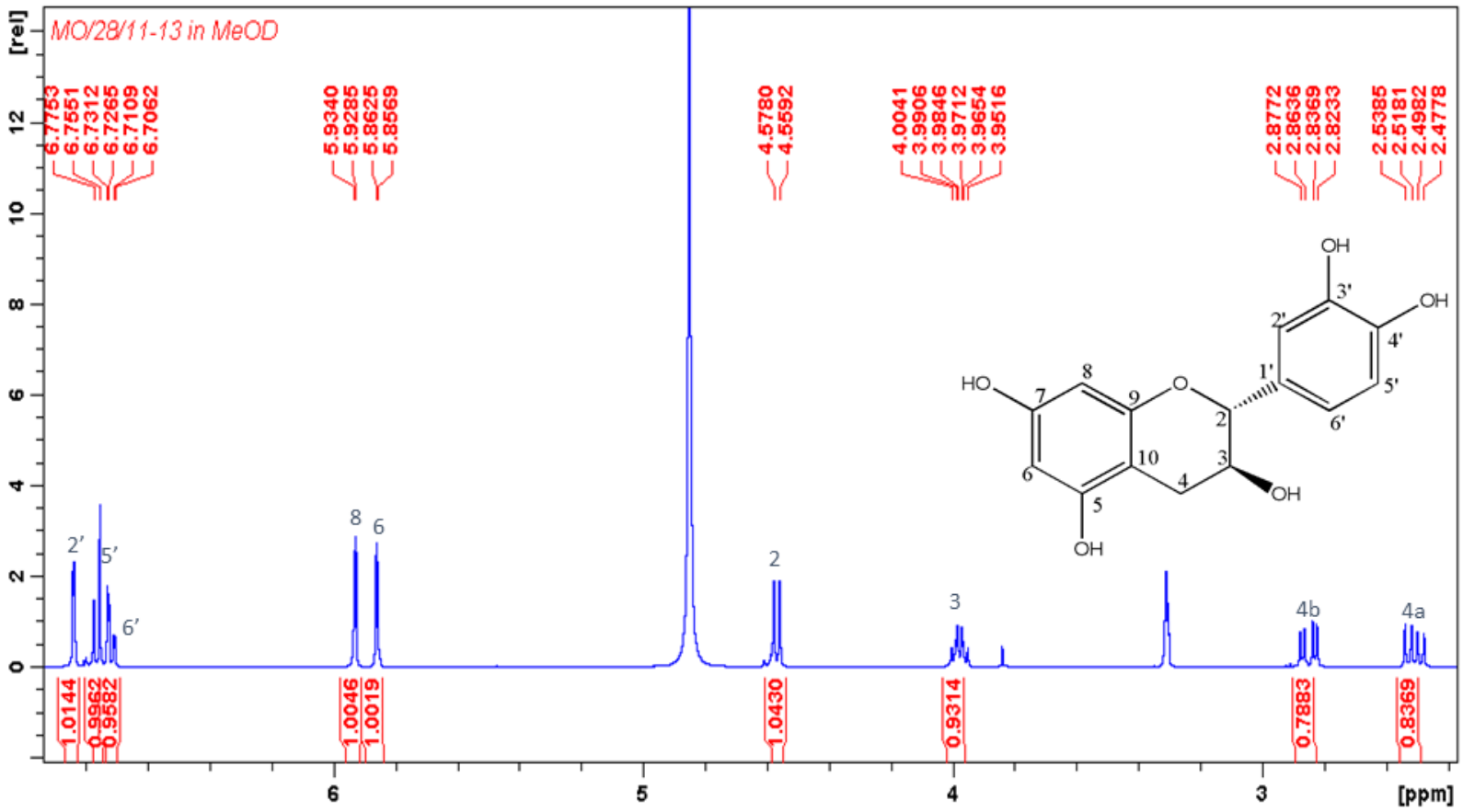
IR spectrum of lupeol acetate A-7



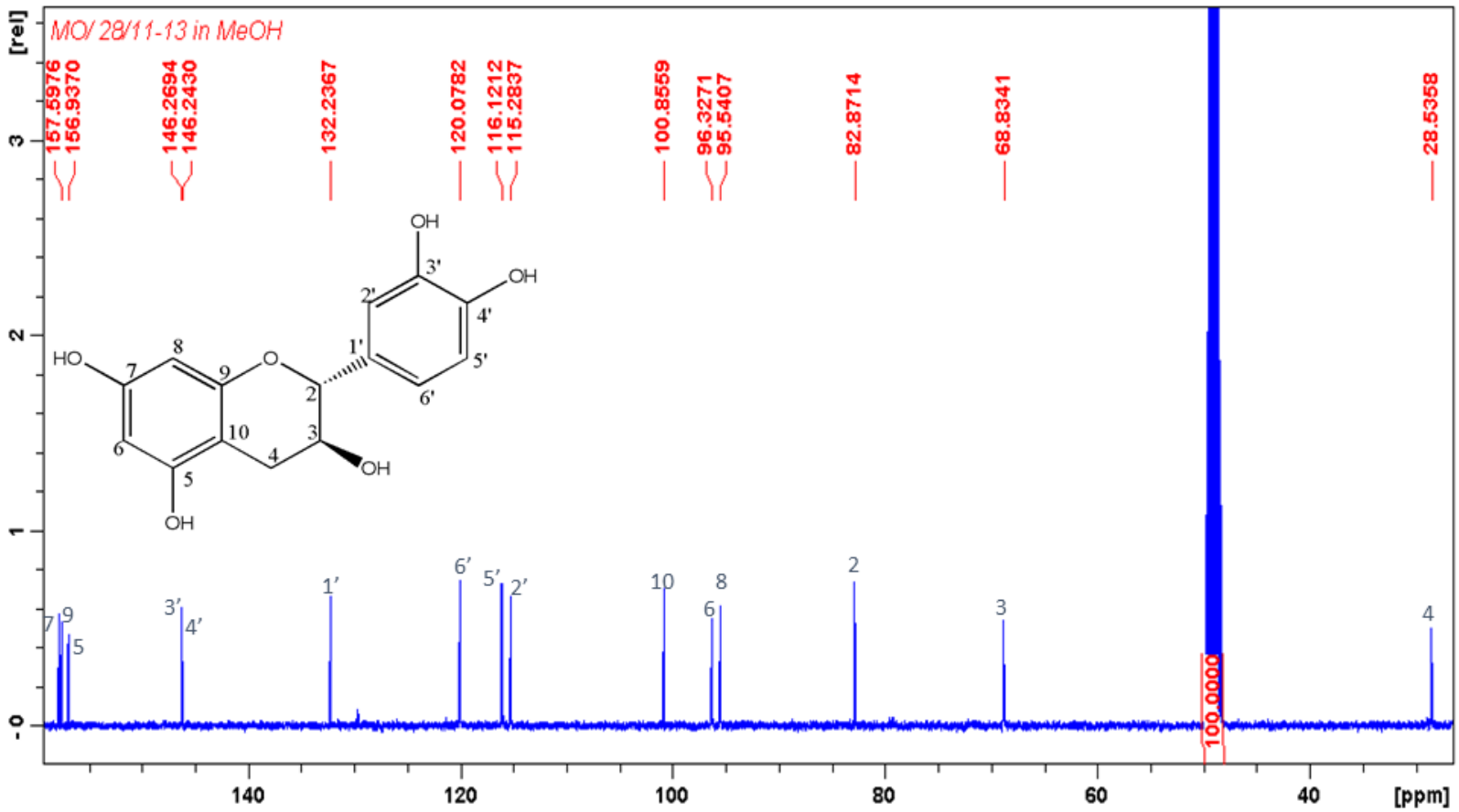
UV spectrum of lupeol acetate A-7



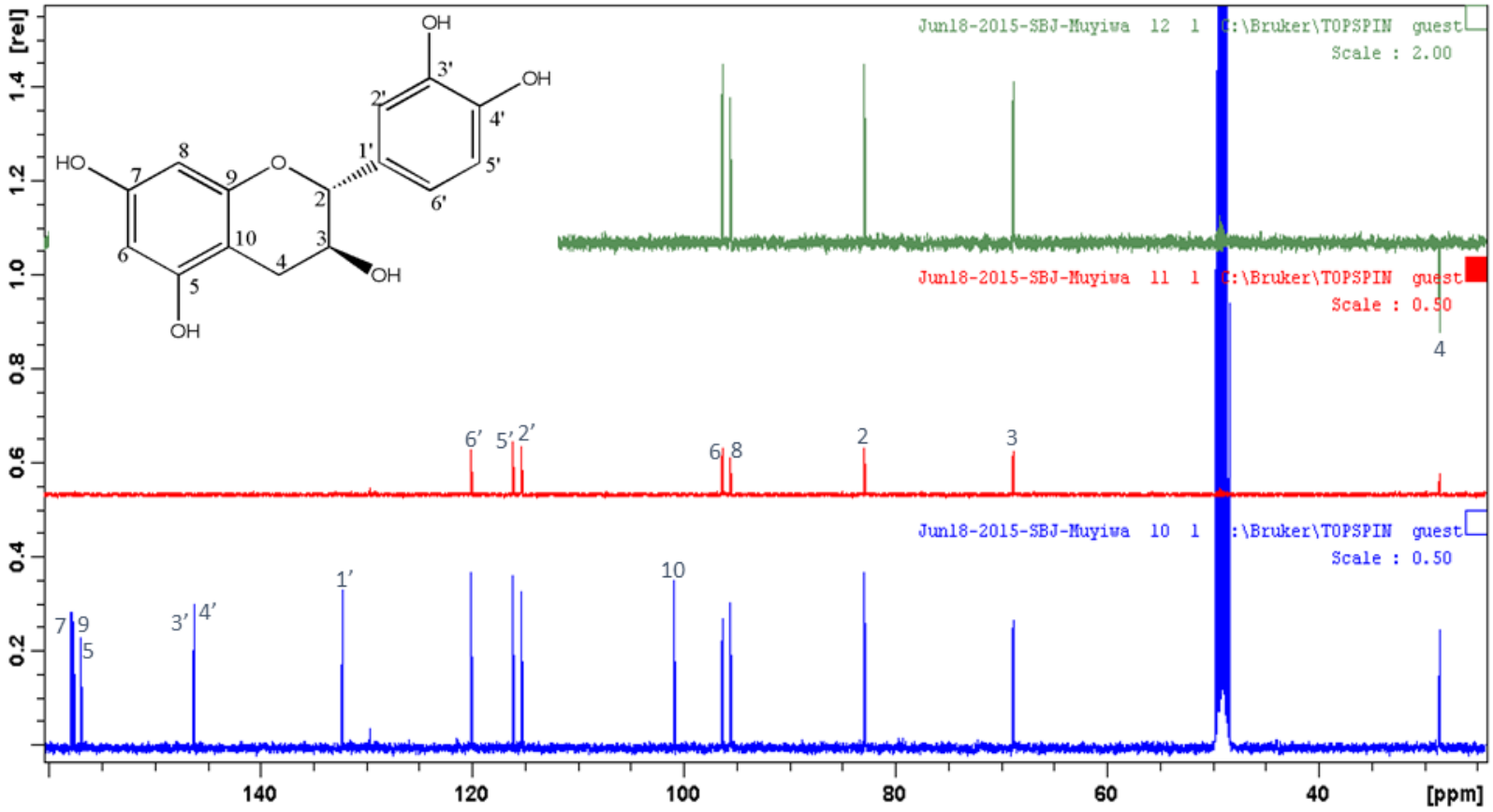
Mass spectrum of lupeol acetate A-7



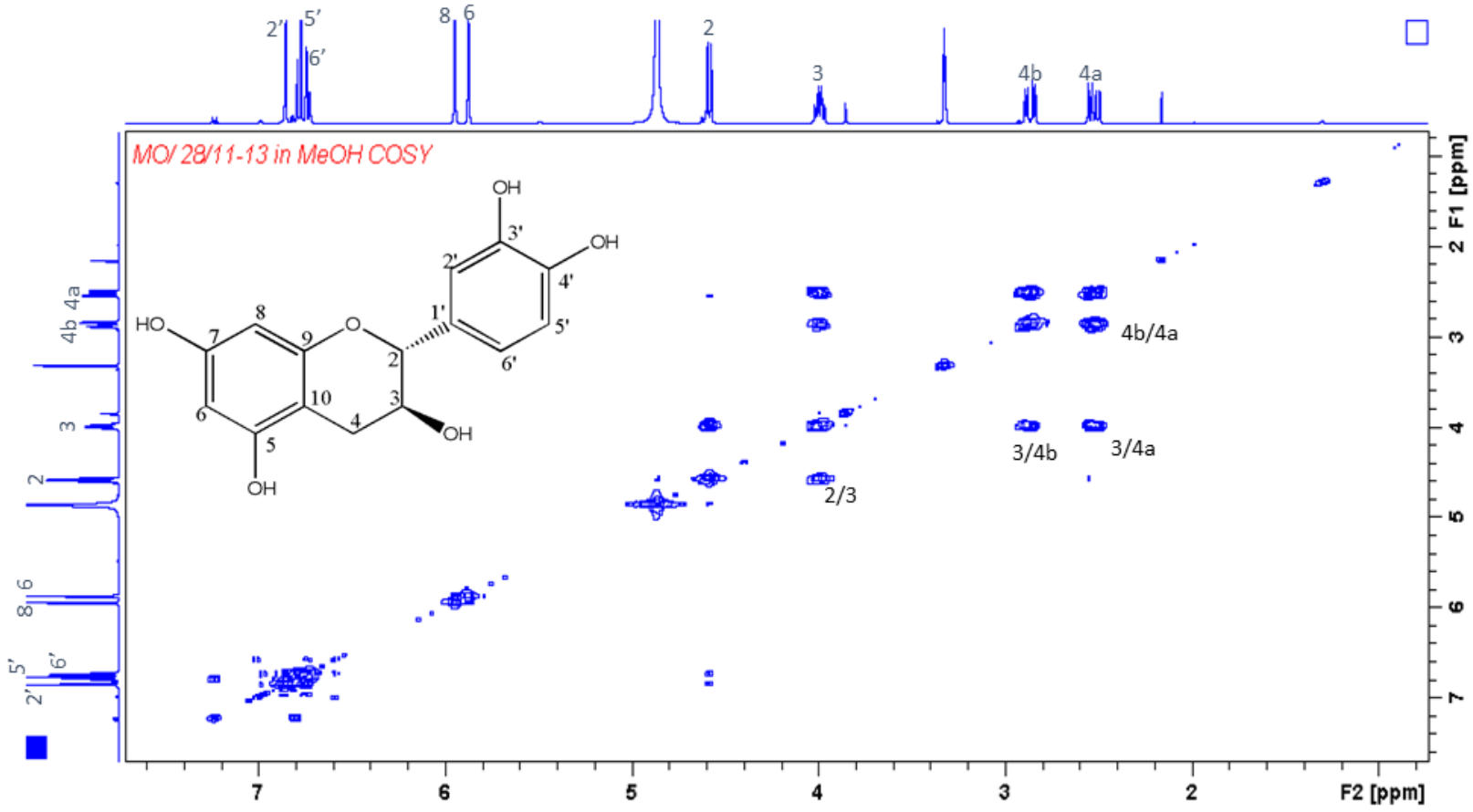
^{1}H NMR spectrum of catechin A-8



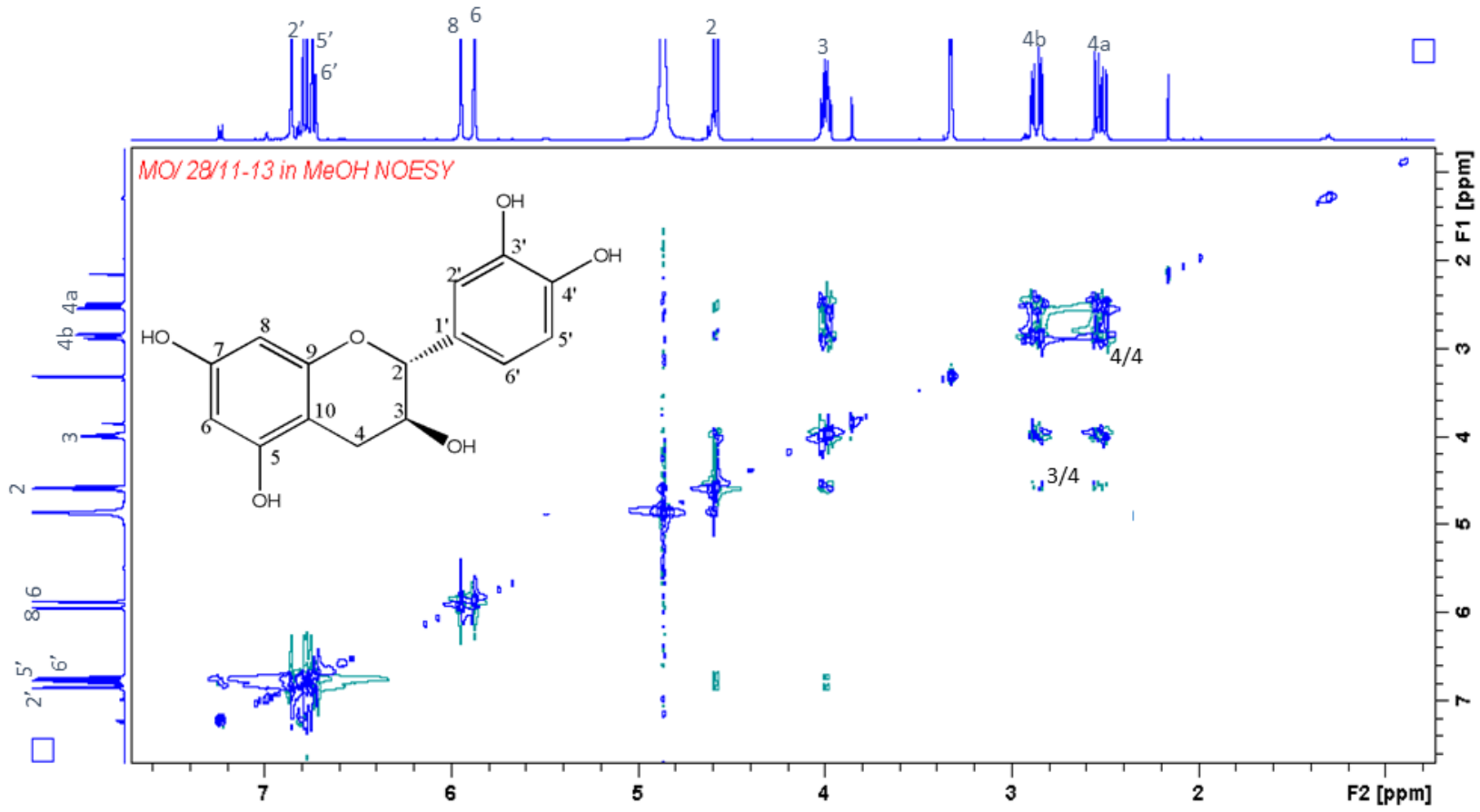
^{13}C NMR spectrum of (+)-catechin A-8



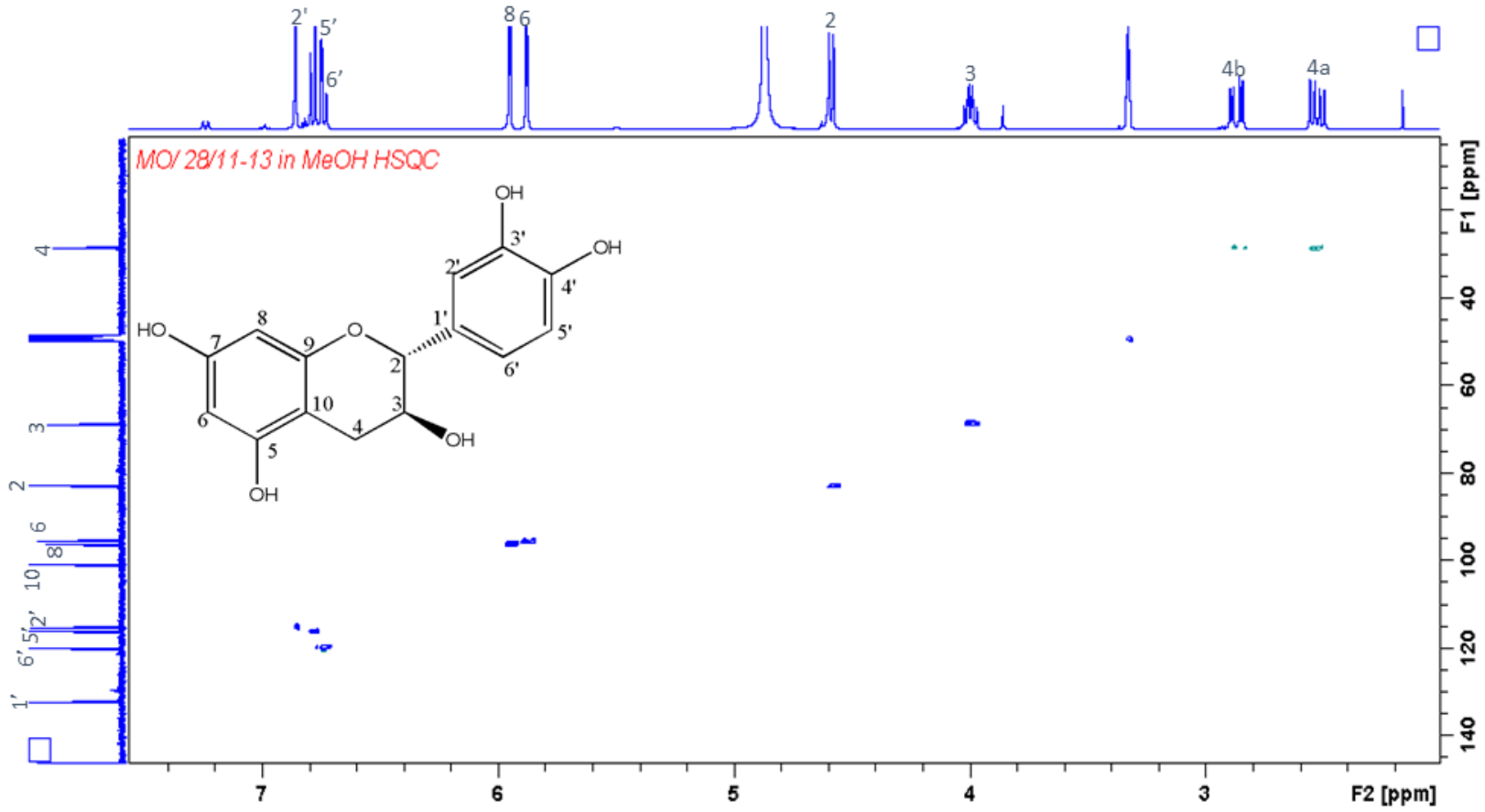
DEPT spectrum of (+)-catechin A-8



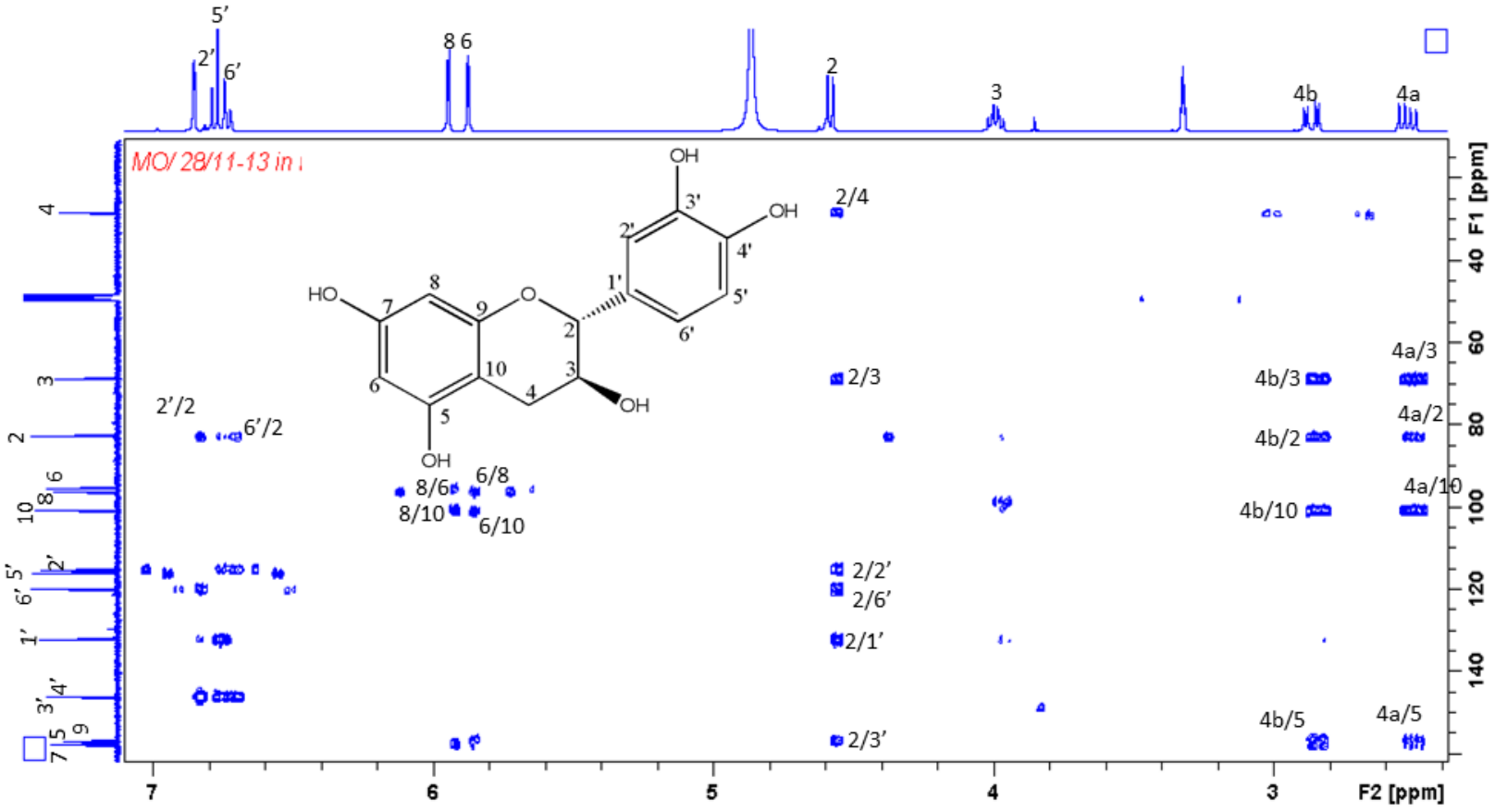
COSY spectrum of (+)-catechin A-8



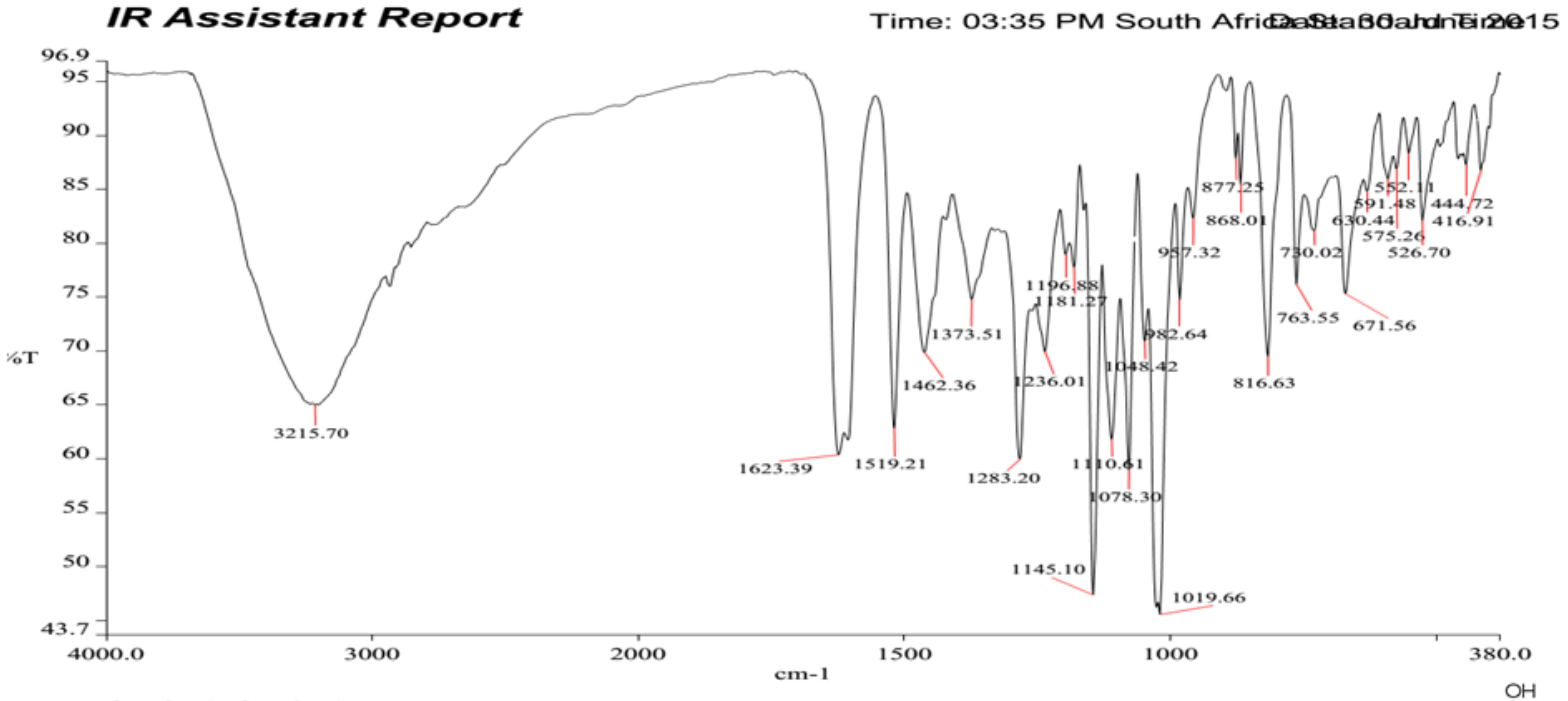
NOESY spectrum of (+)-catechin A-8



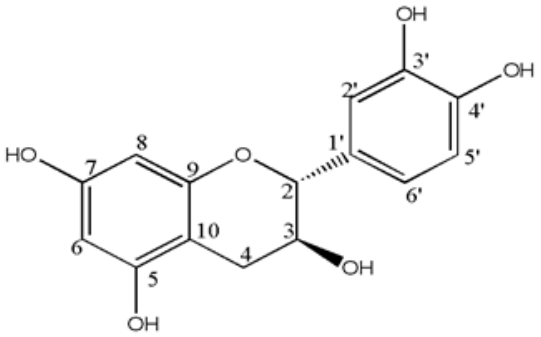
HSQC spectrum of (+)-catechin A-8

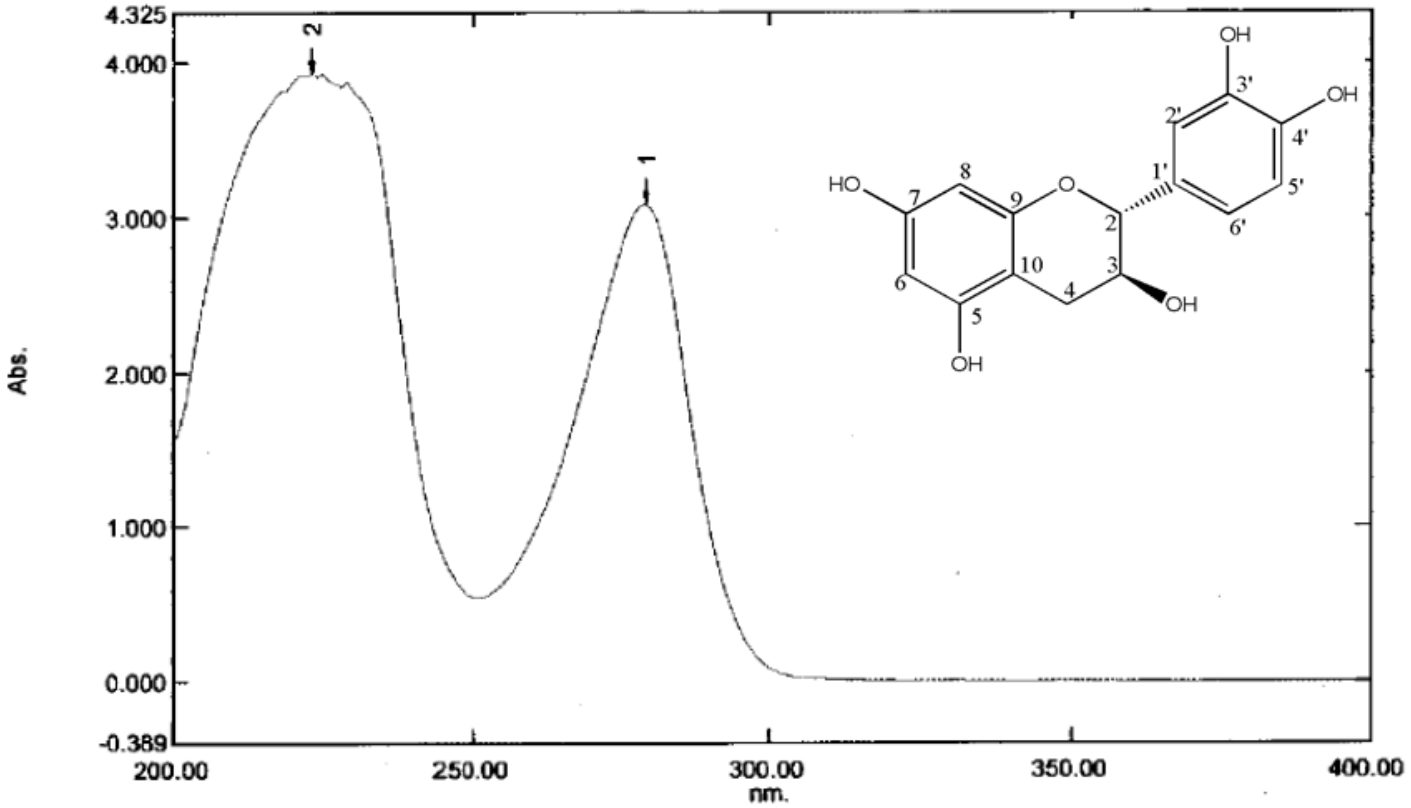


HMBC spectrum of (+)-catechin A-8



IR spectrum of catehin A-8





UV spectrum of catehin A-8

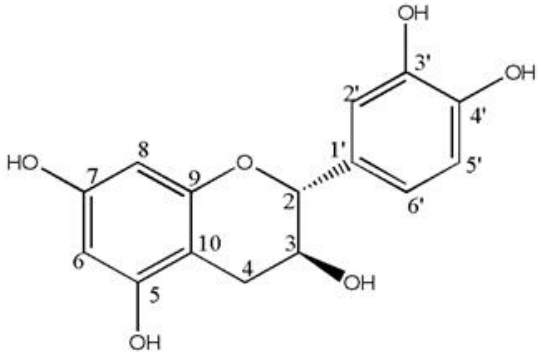
Elemental Composition Report

Single Mass Analysis

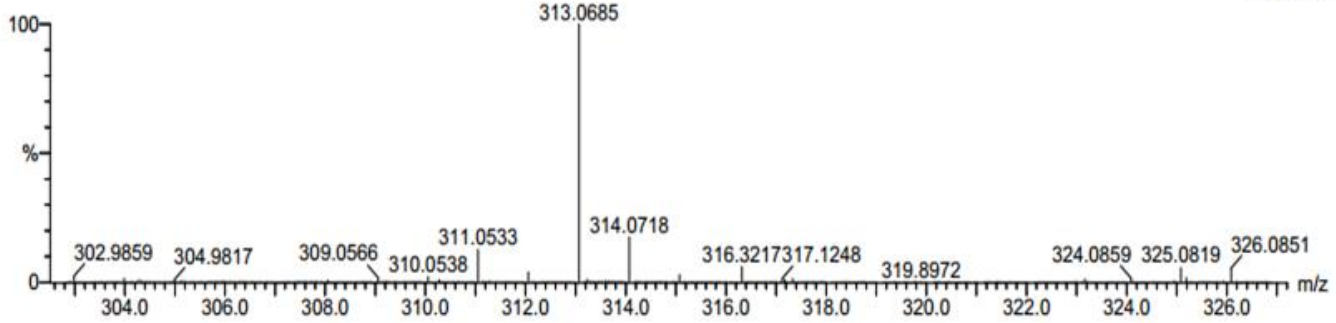
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
6 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 10-15 H: 10-15 O: 5-10 Na: 0-1

02-2-91 28 (0.911) Cm (1:61)
TOF MS ES+

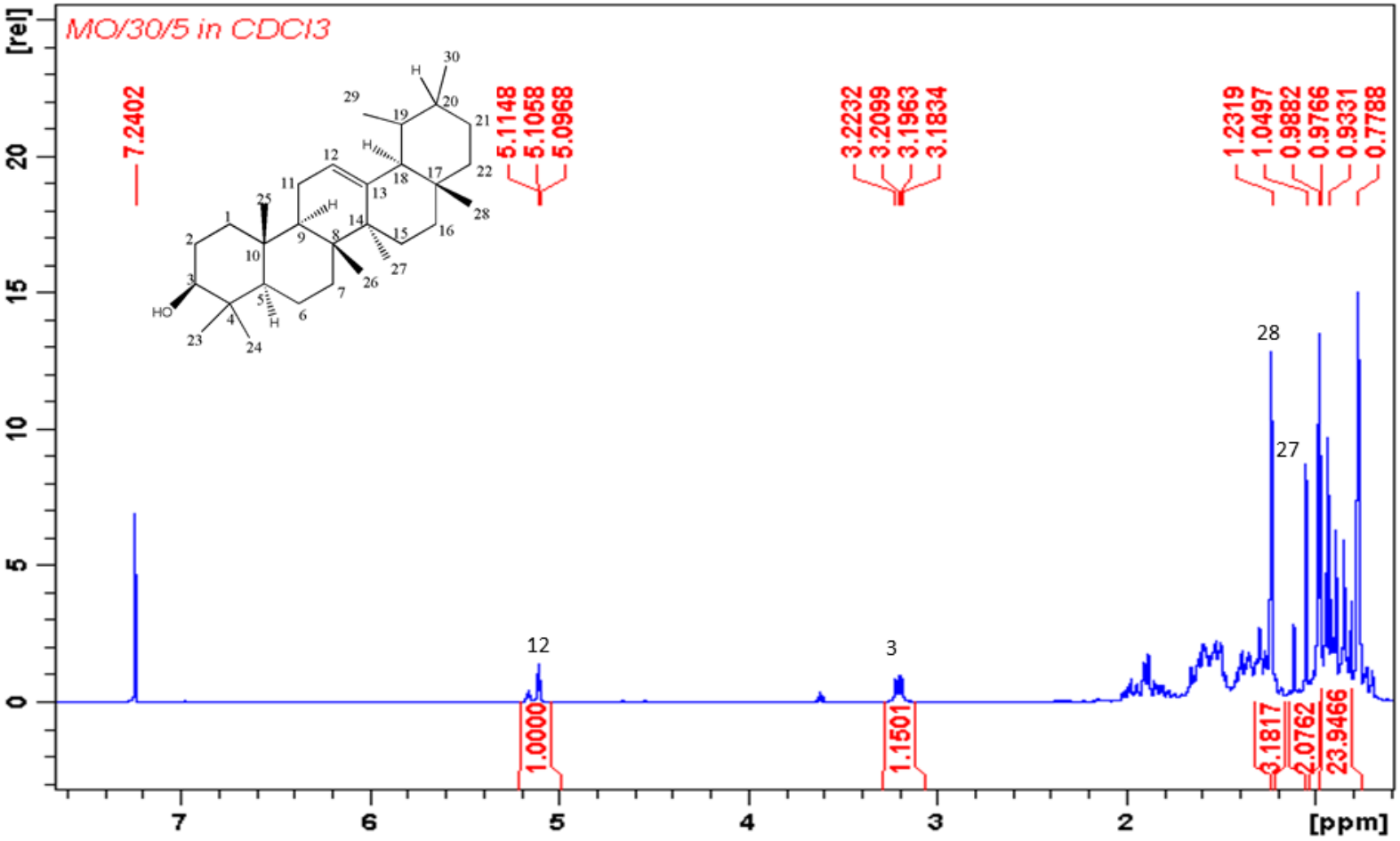


1.61e+005

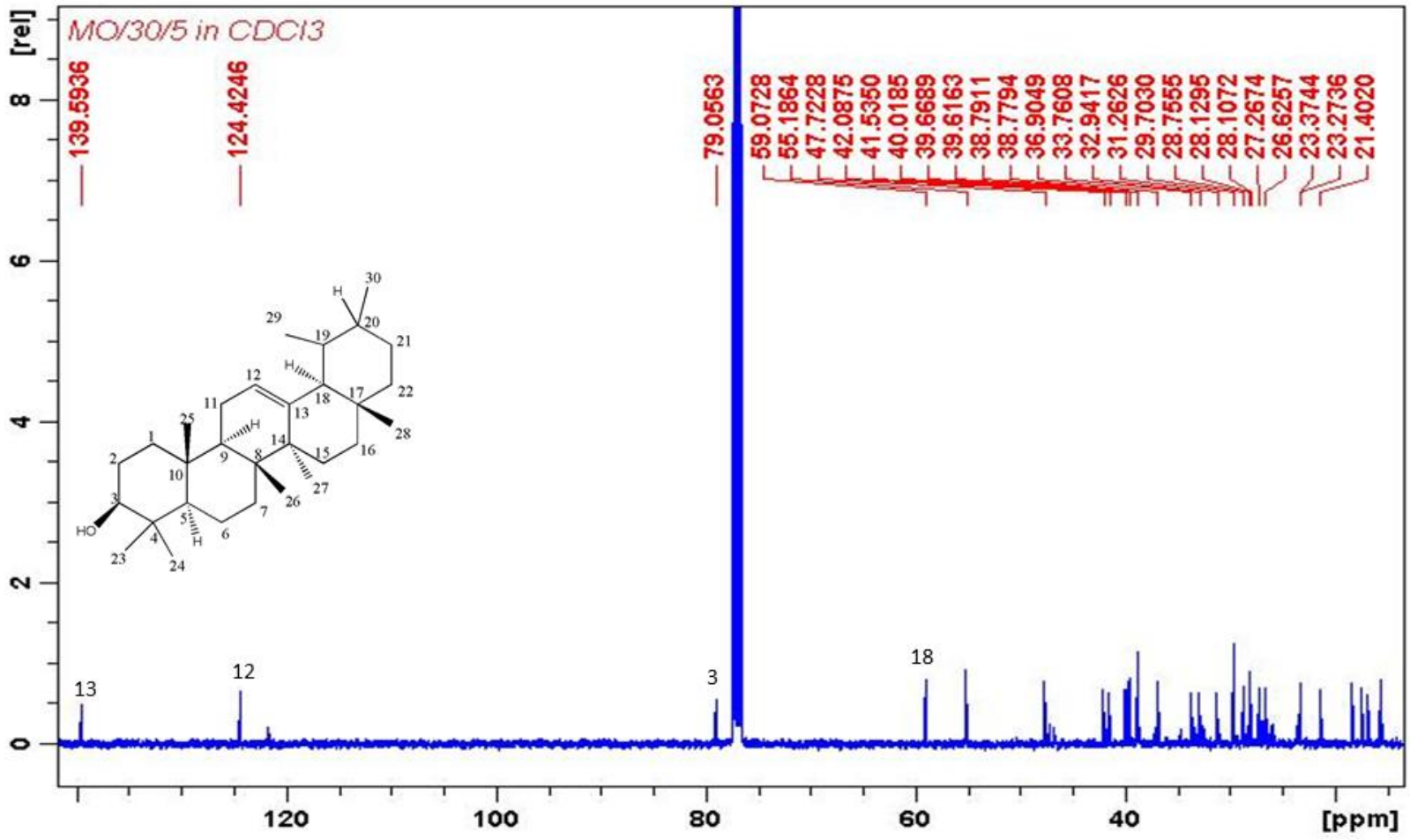


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
313.0685	313.0688	-0.3	-1.0	8.5	617.3	0.0	C15 H14 O6 Na

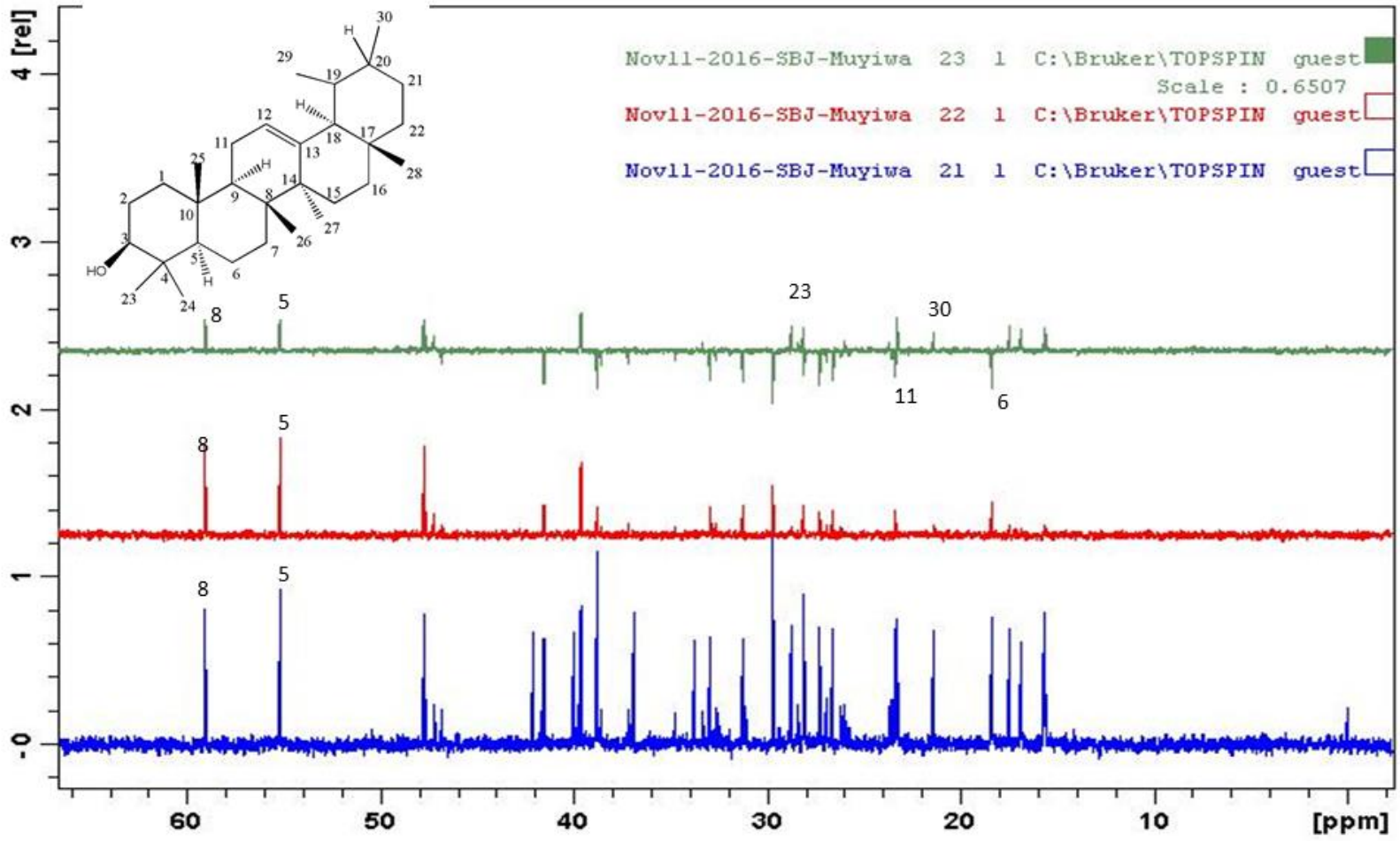
Mass spectrum of catehin A-8



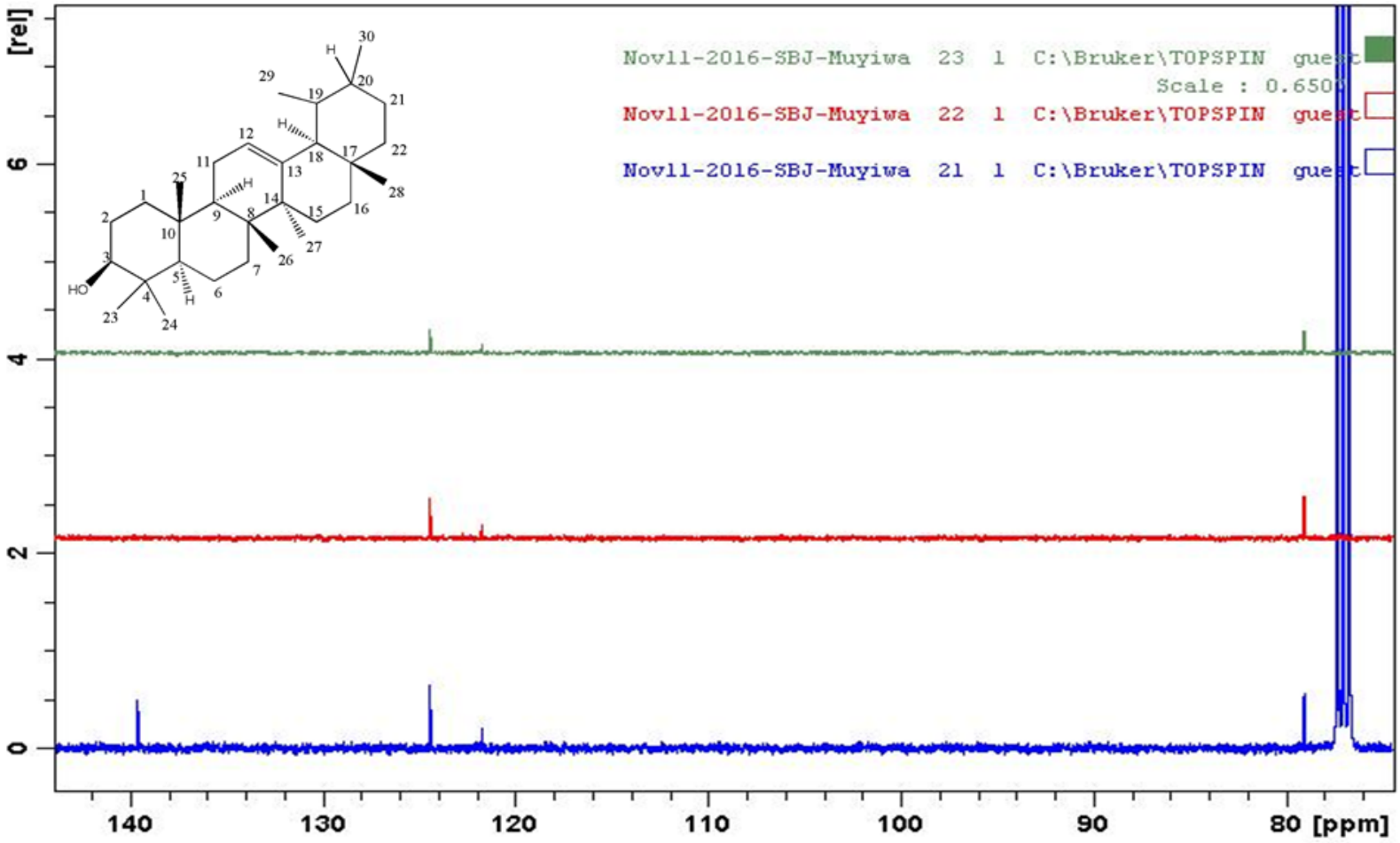
^{1}H NMR spectrum of α-amyrin A-9



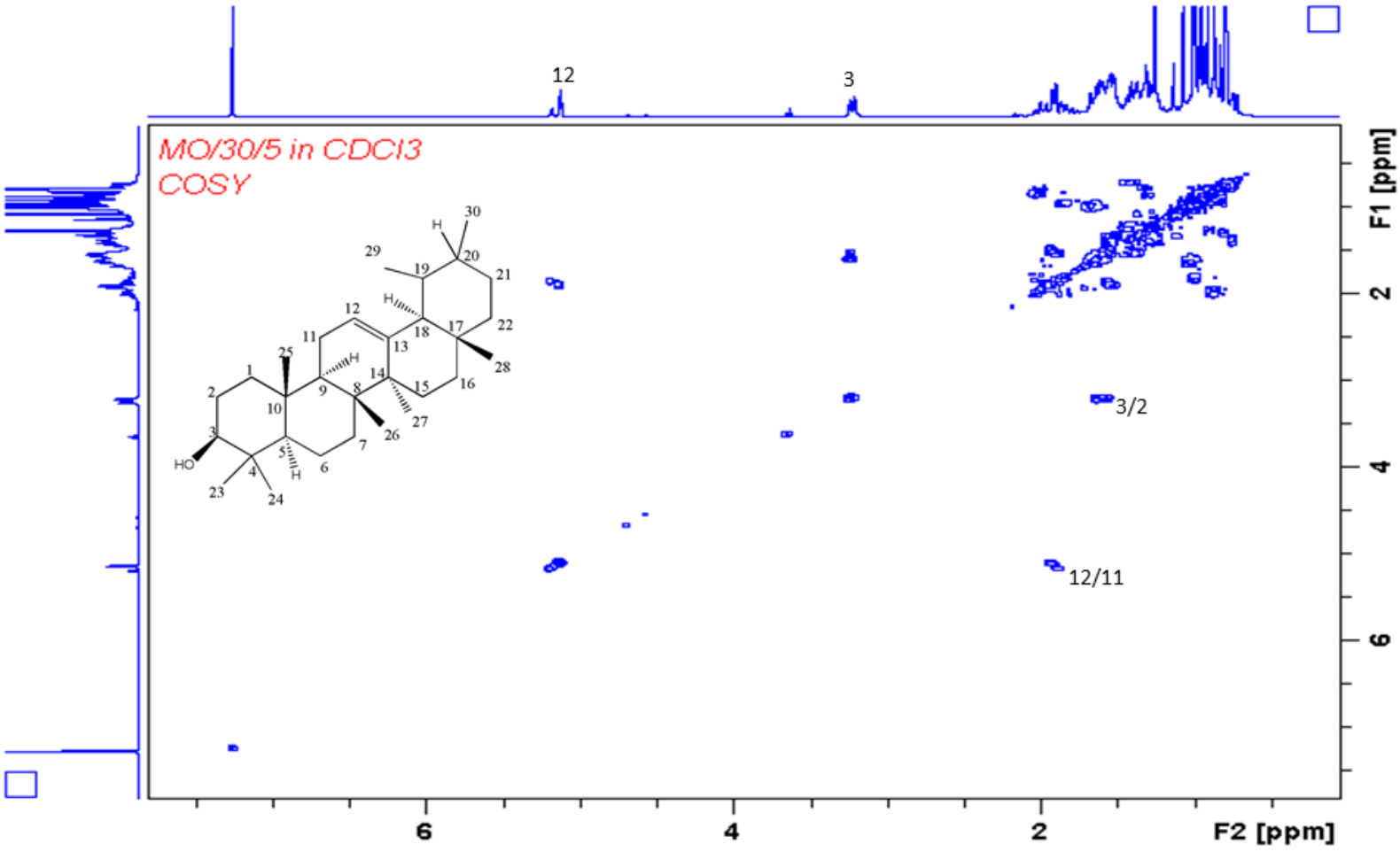
^{13}C NMR spectrum of α-amyrin A-9



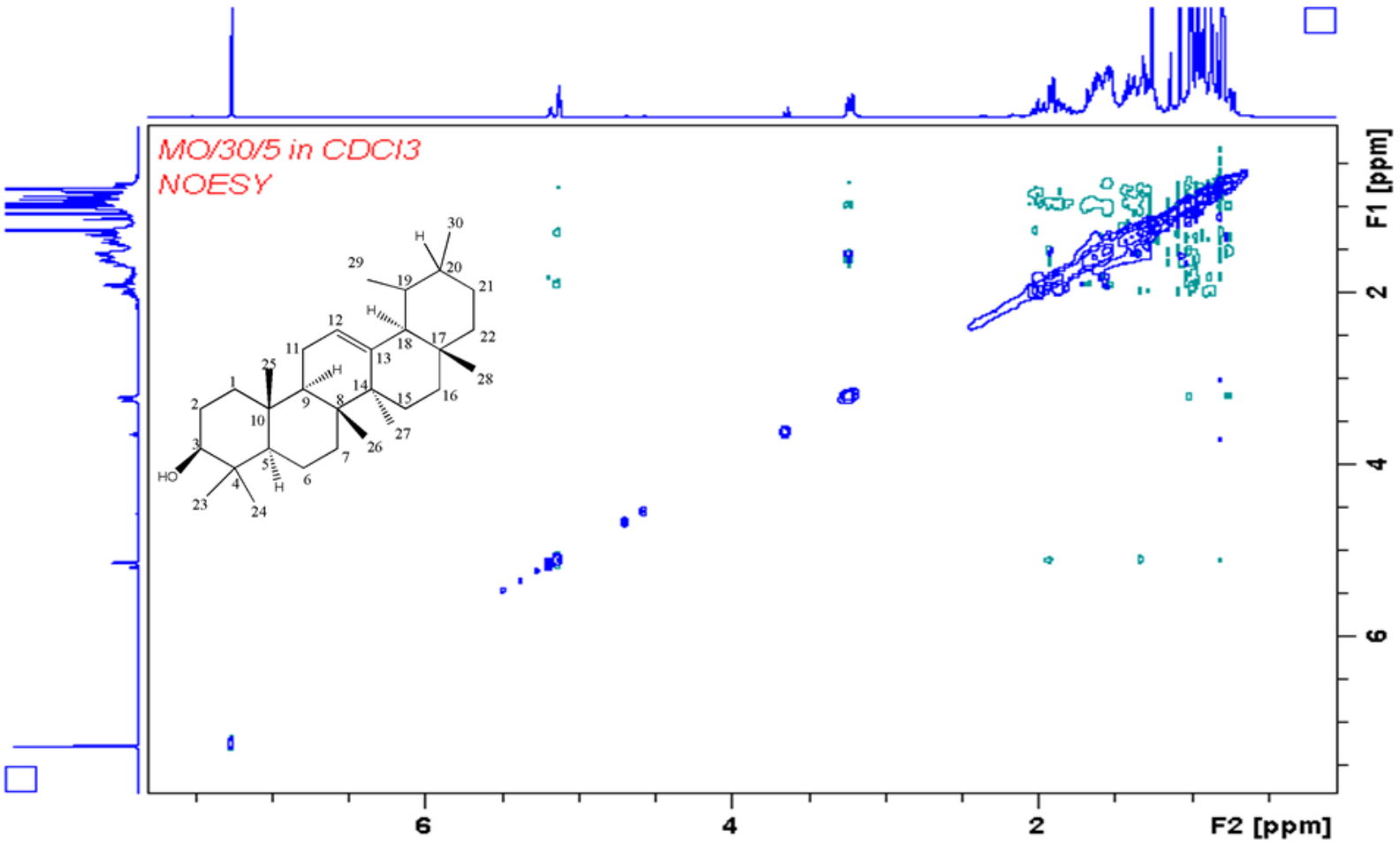
DEPT spectrum of α-amyrin A-9



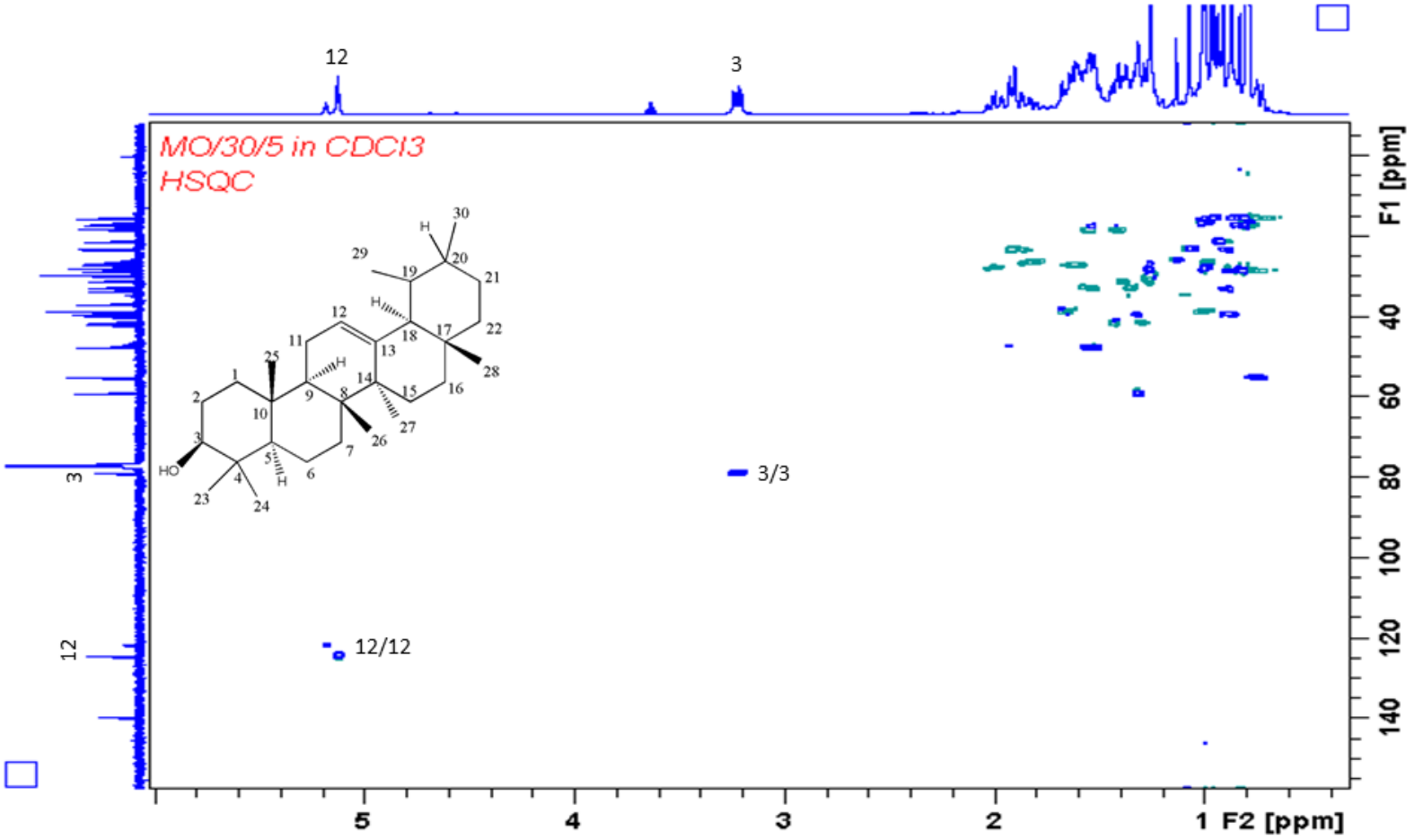
Expanded DEPT spectrum of α-amyrin A-9



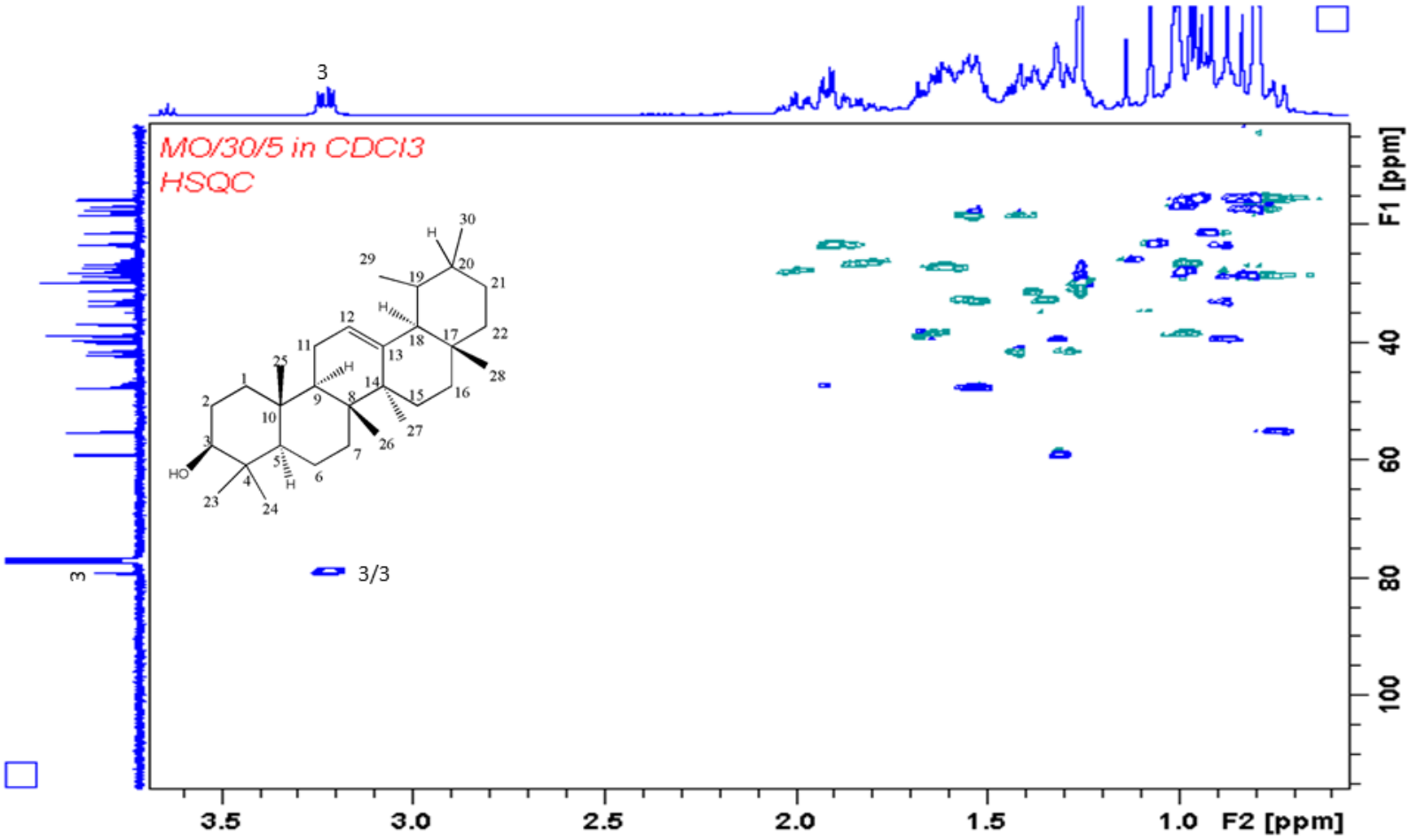
COSY spectrum of α-amyrin A-9



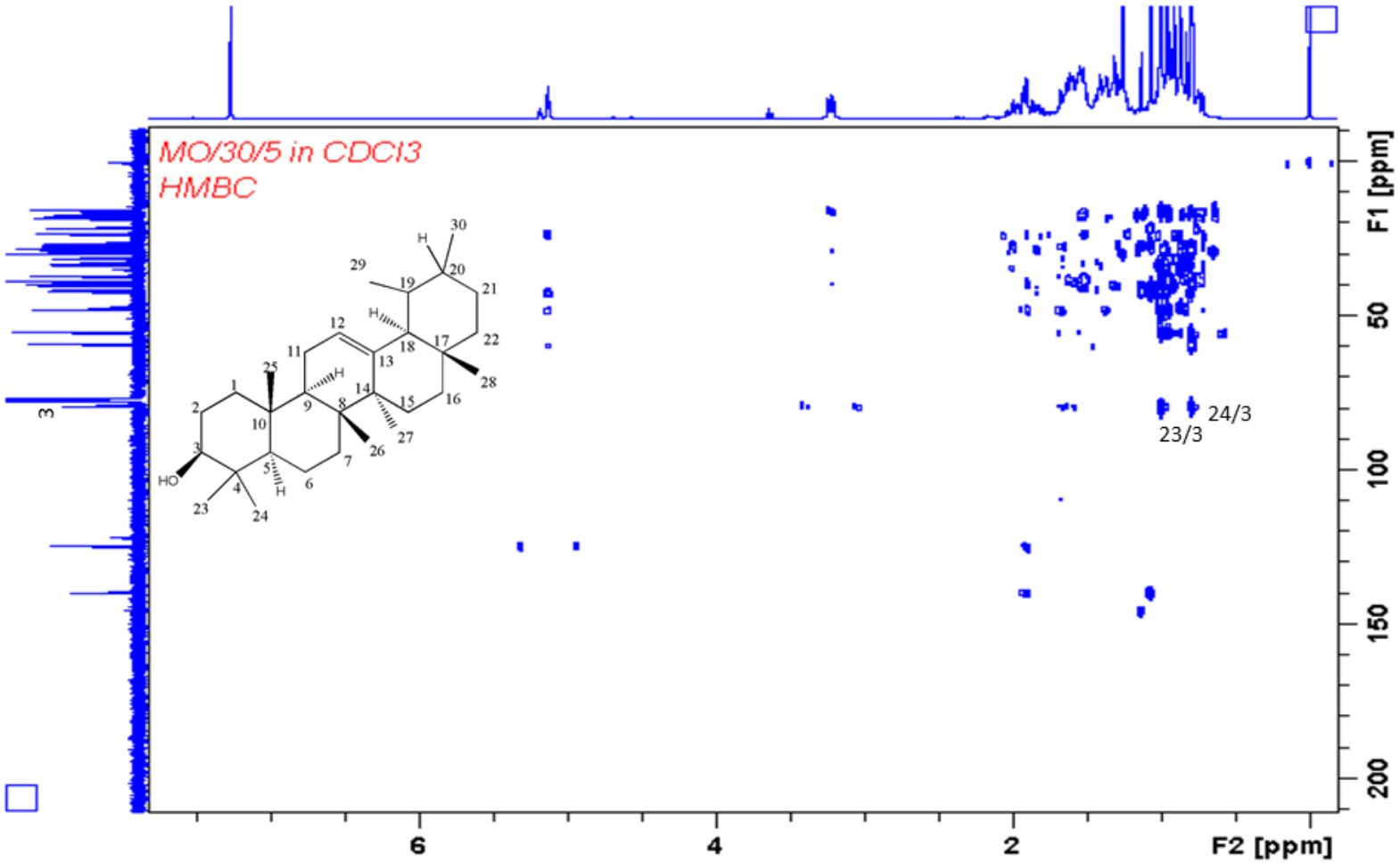
NOESY spectrum of α-amyrin A-9



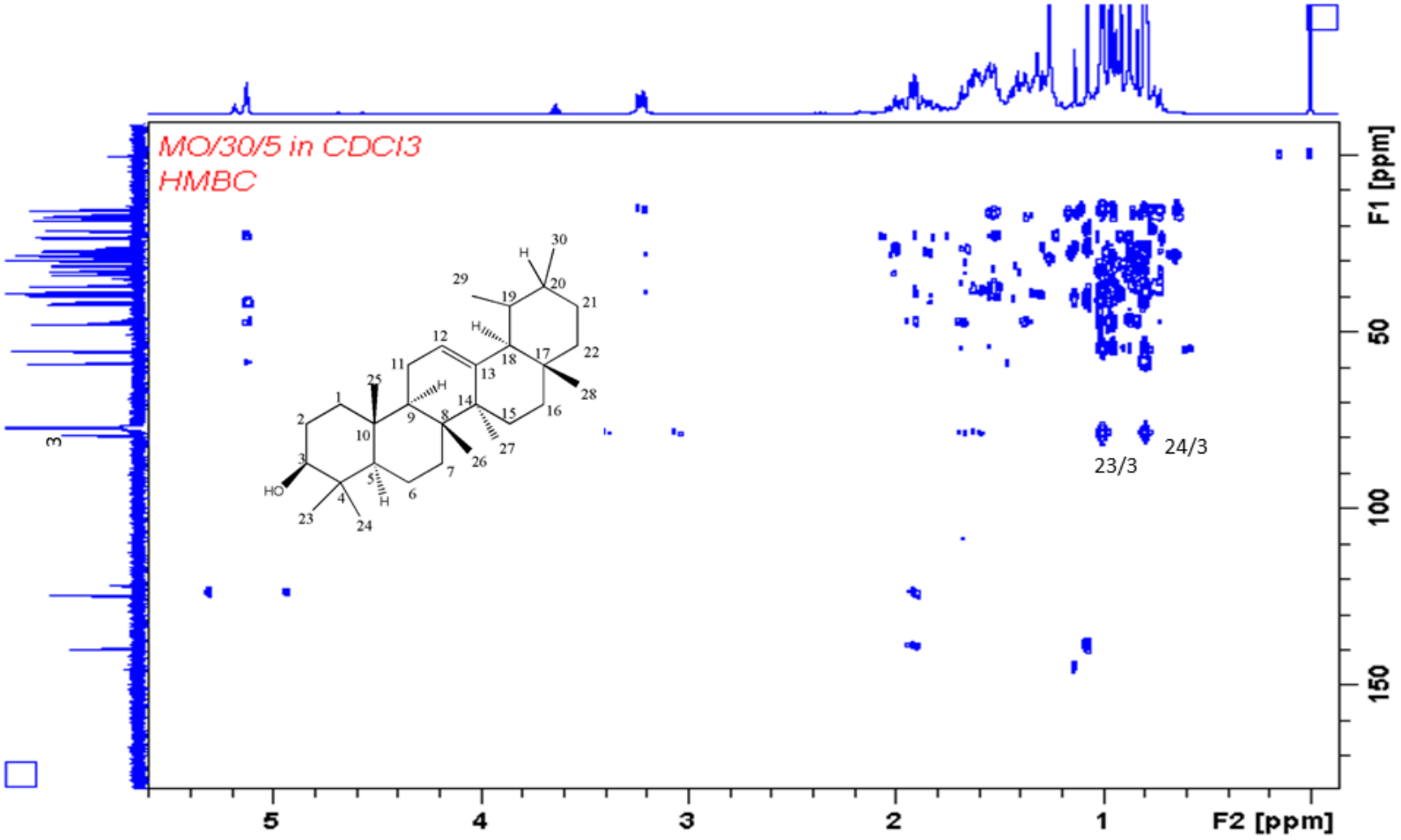
HSQC spectrum of α-amyrin A-9



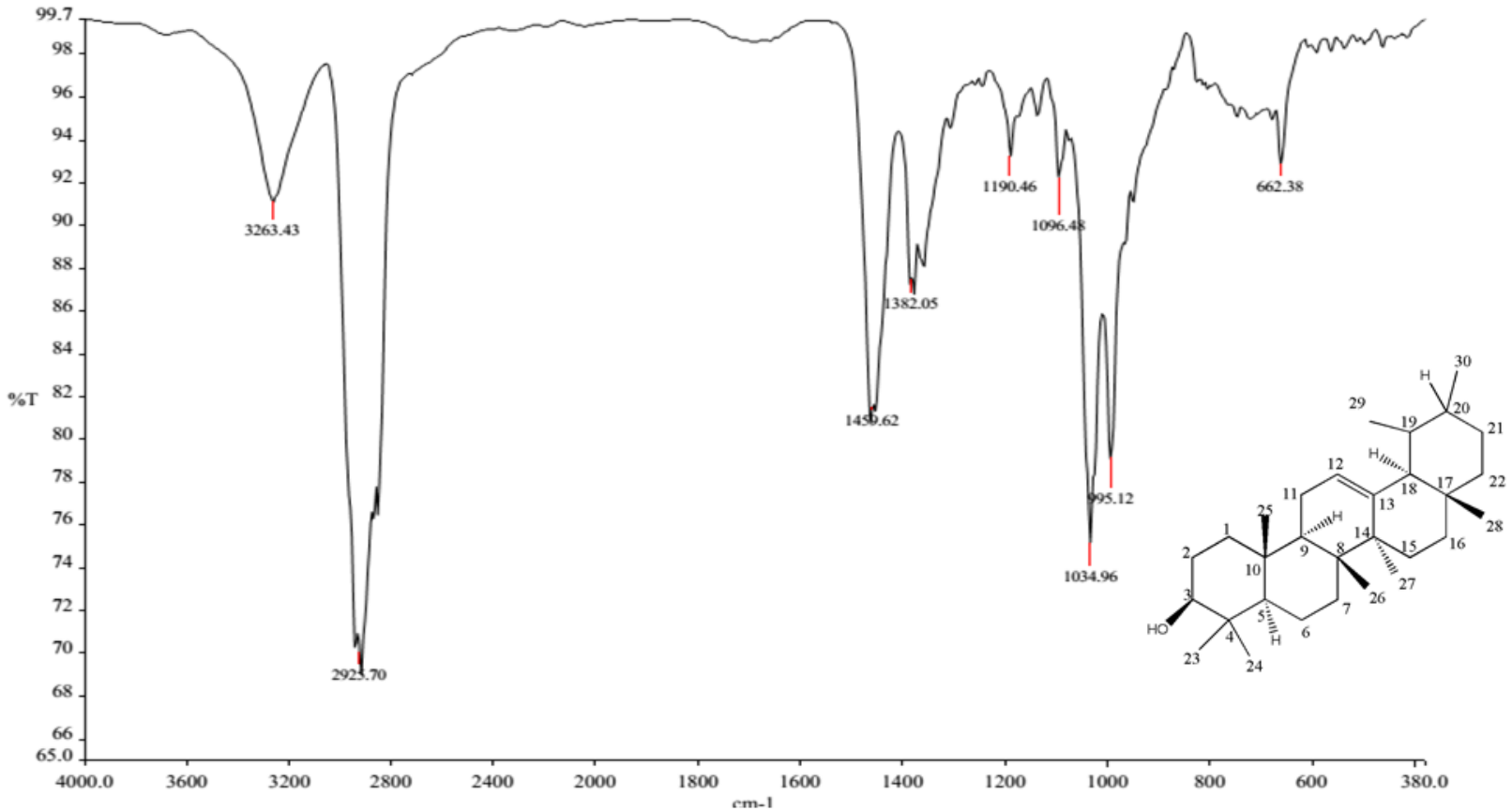
Expanded HSQC spectrum of α-amyrin A-9



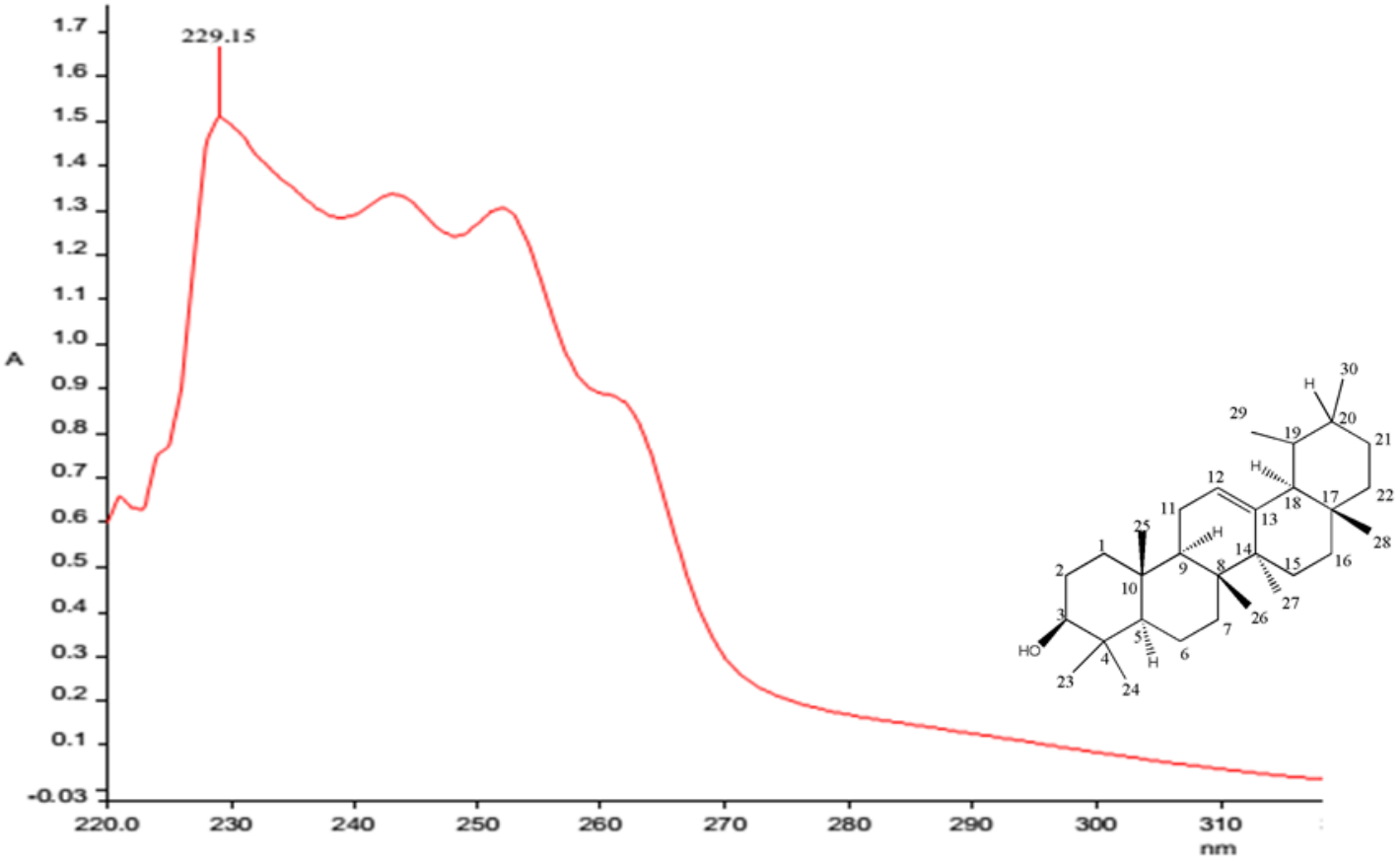
HMBC spectrum of α-amyrin A-9



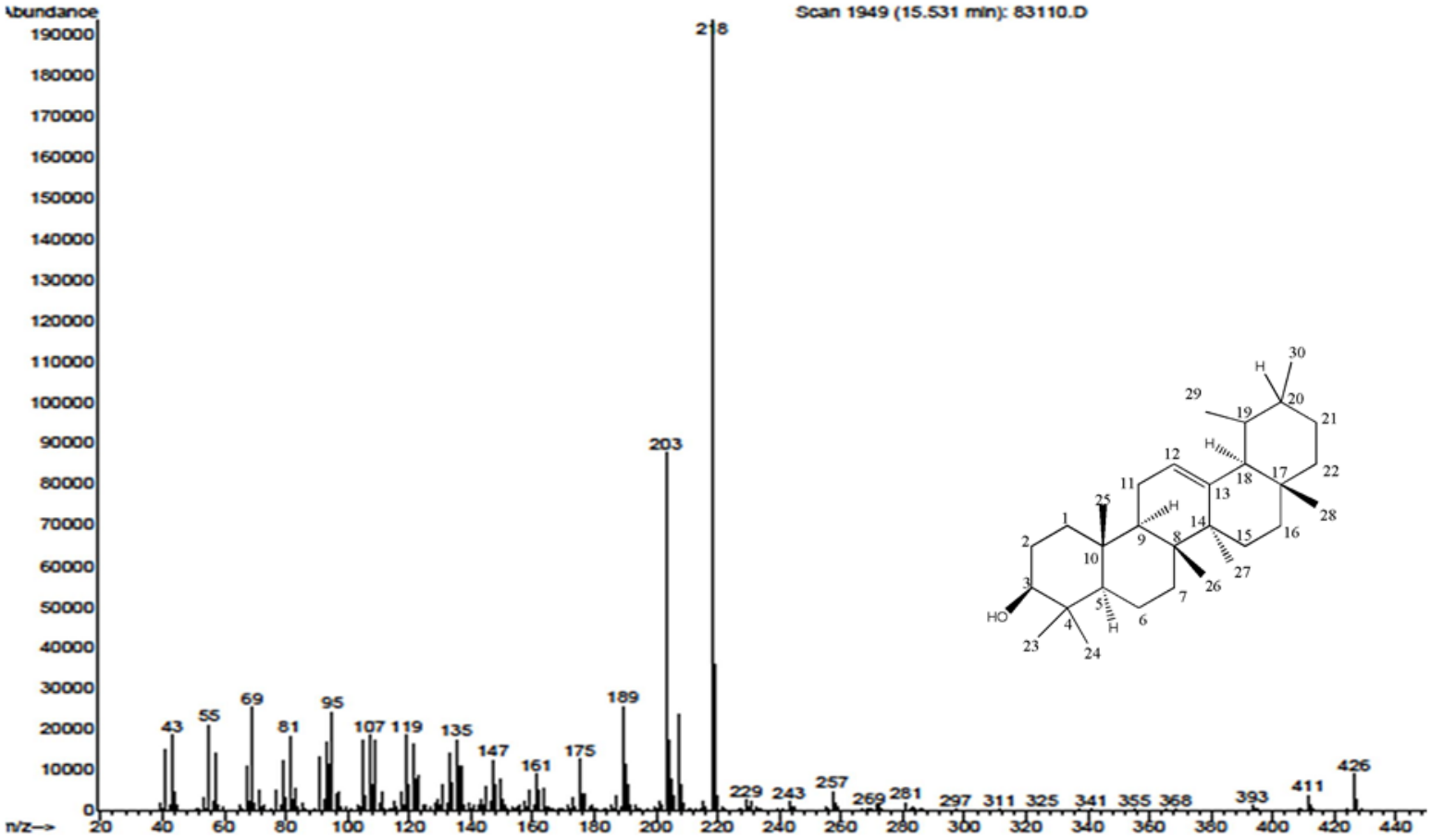
Expanded HMBC spectrum of α-amyrin A-9



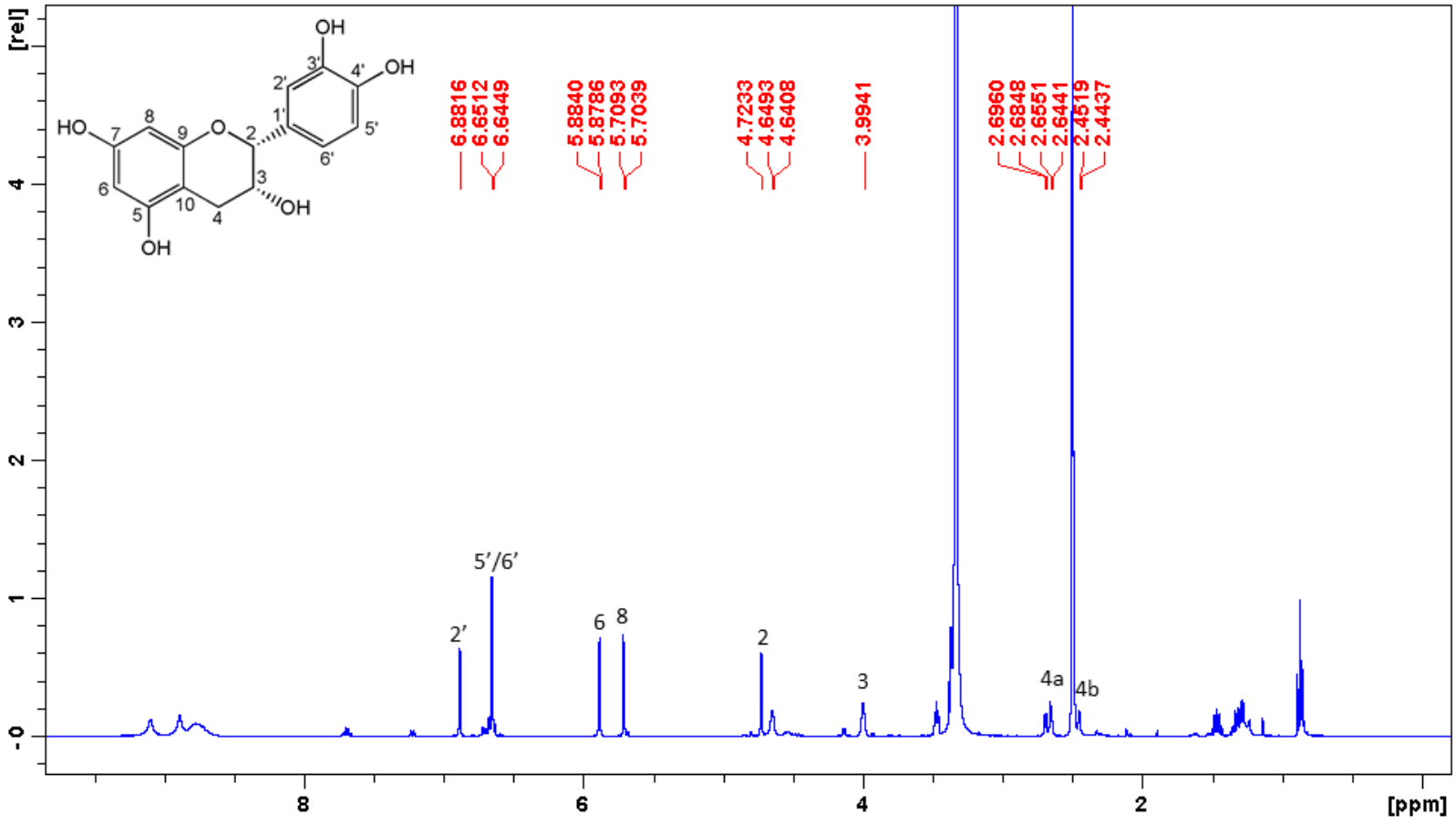
IR spectrum of α-amyrin A-9



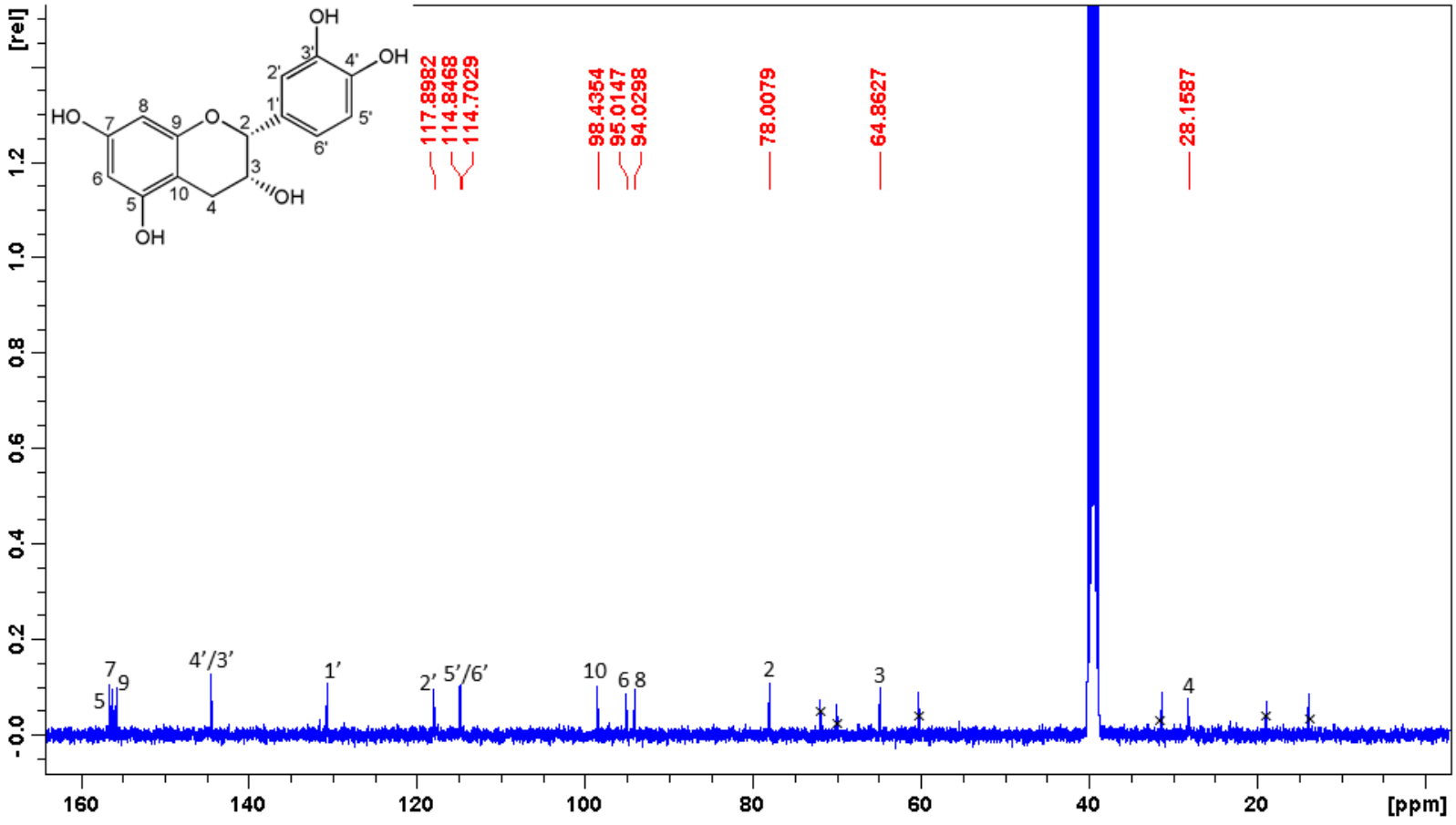
UV spectrum of α-amyrin A-9



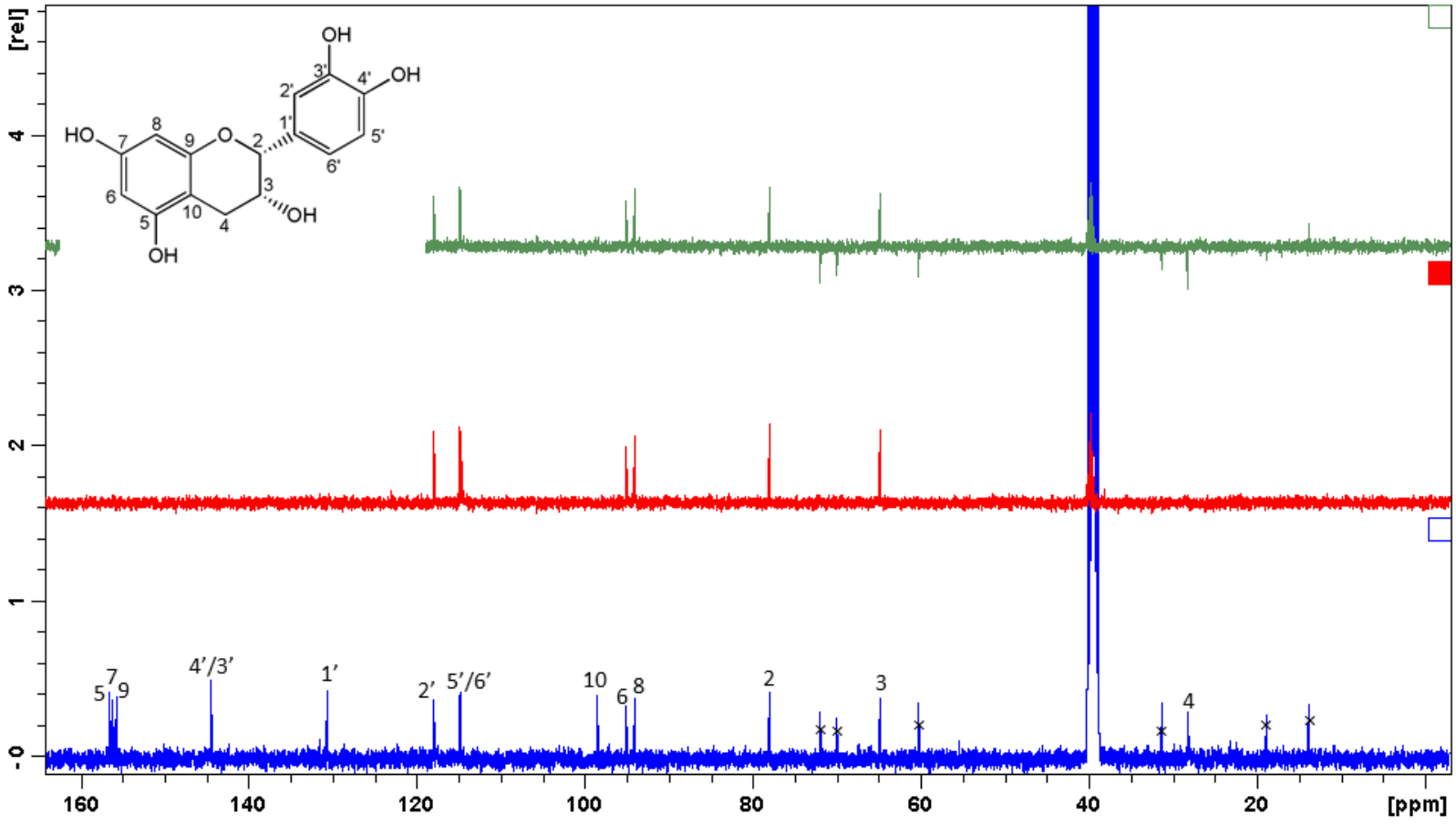
Mass spectrum of α-amyrin A-9



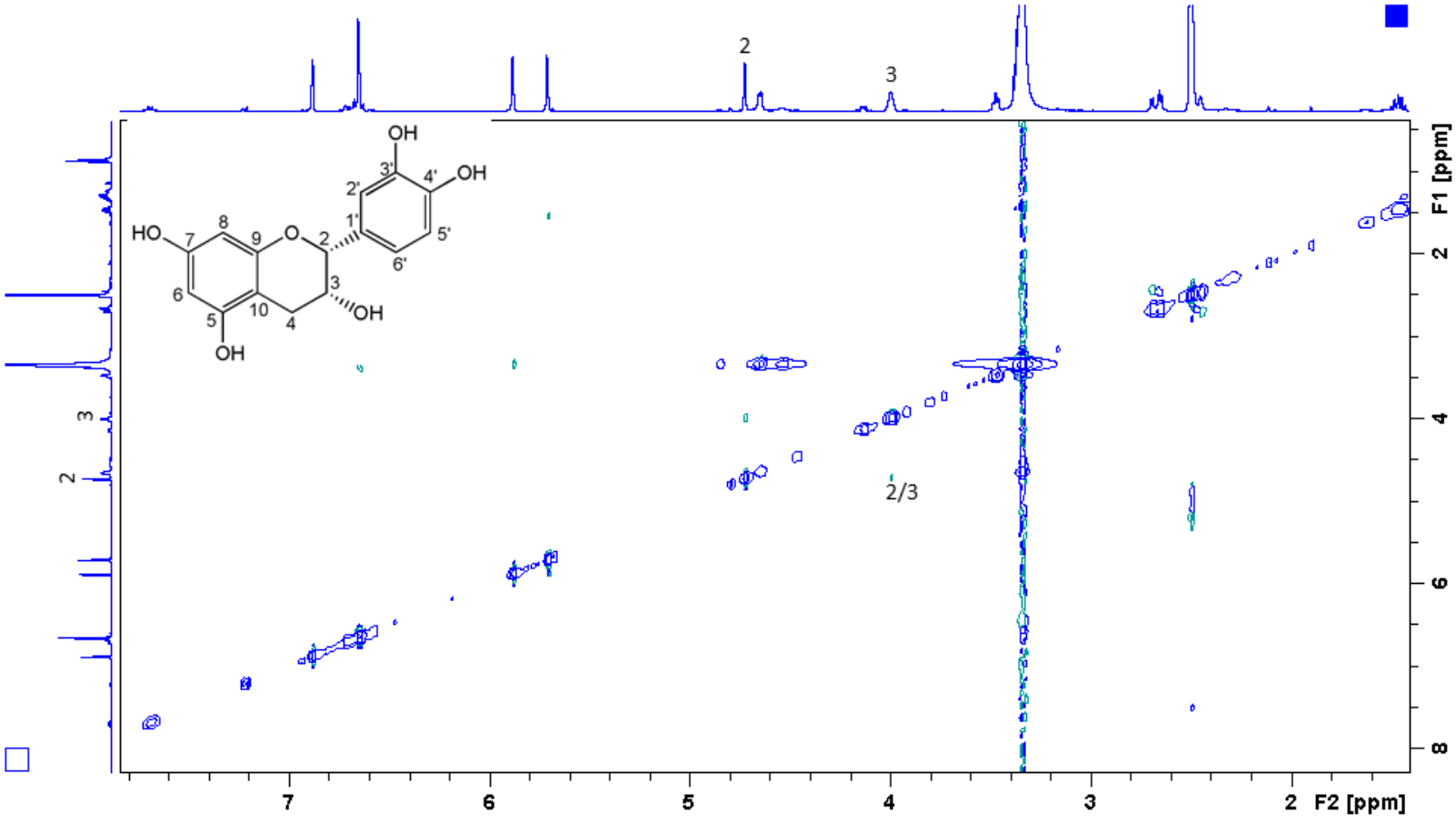
^{1}H NMR spectrum of epicatechin B-1



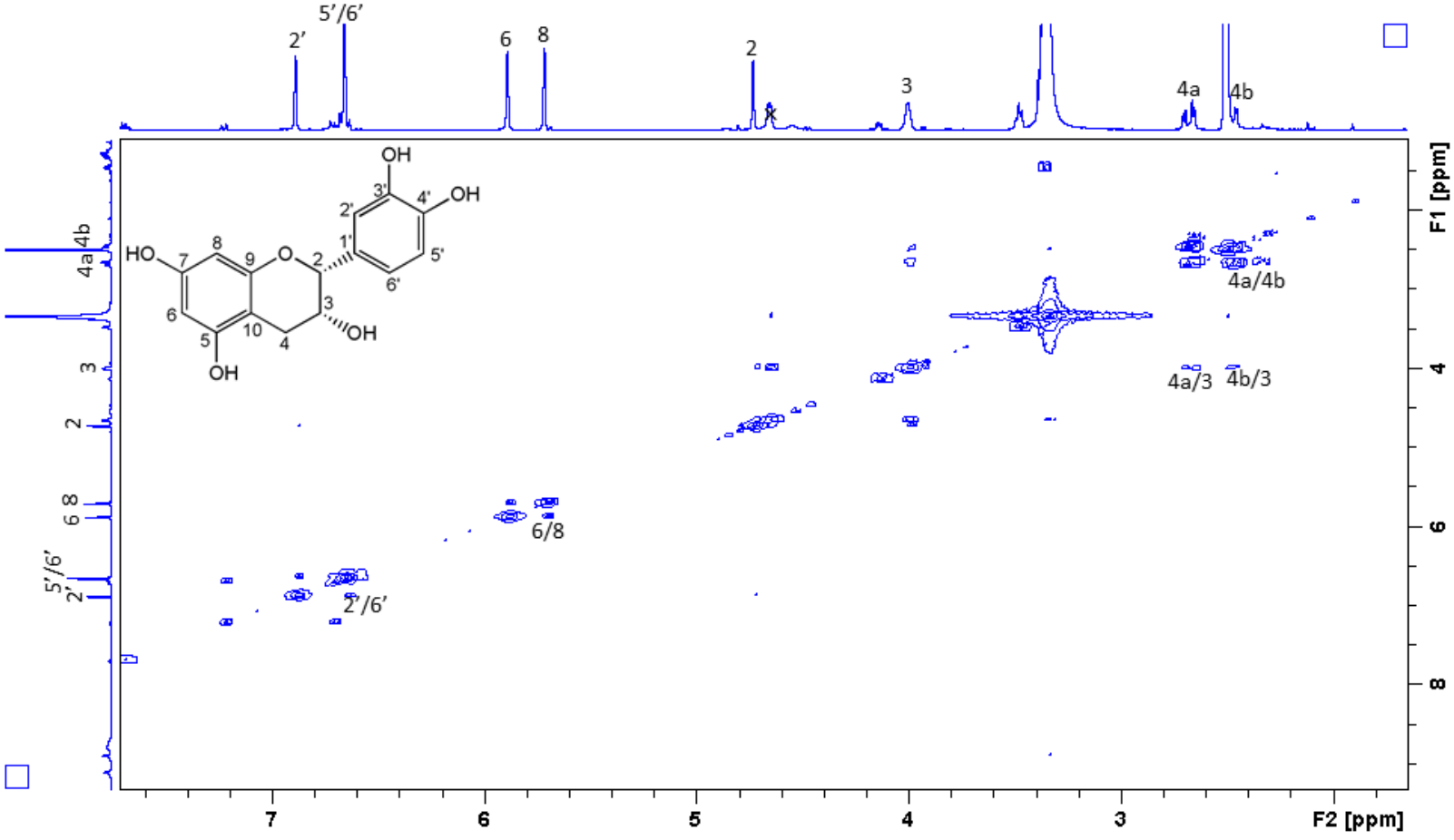
^{13}C NMR spectrum of epicatechin B-1



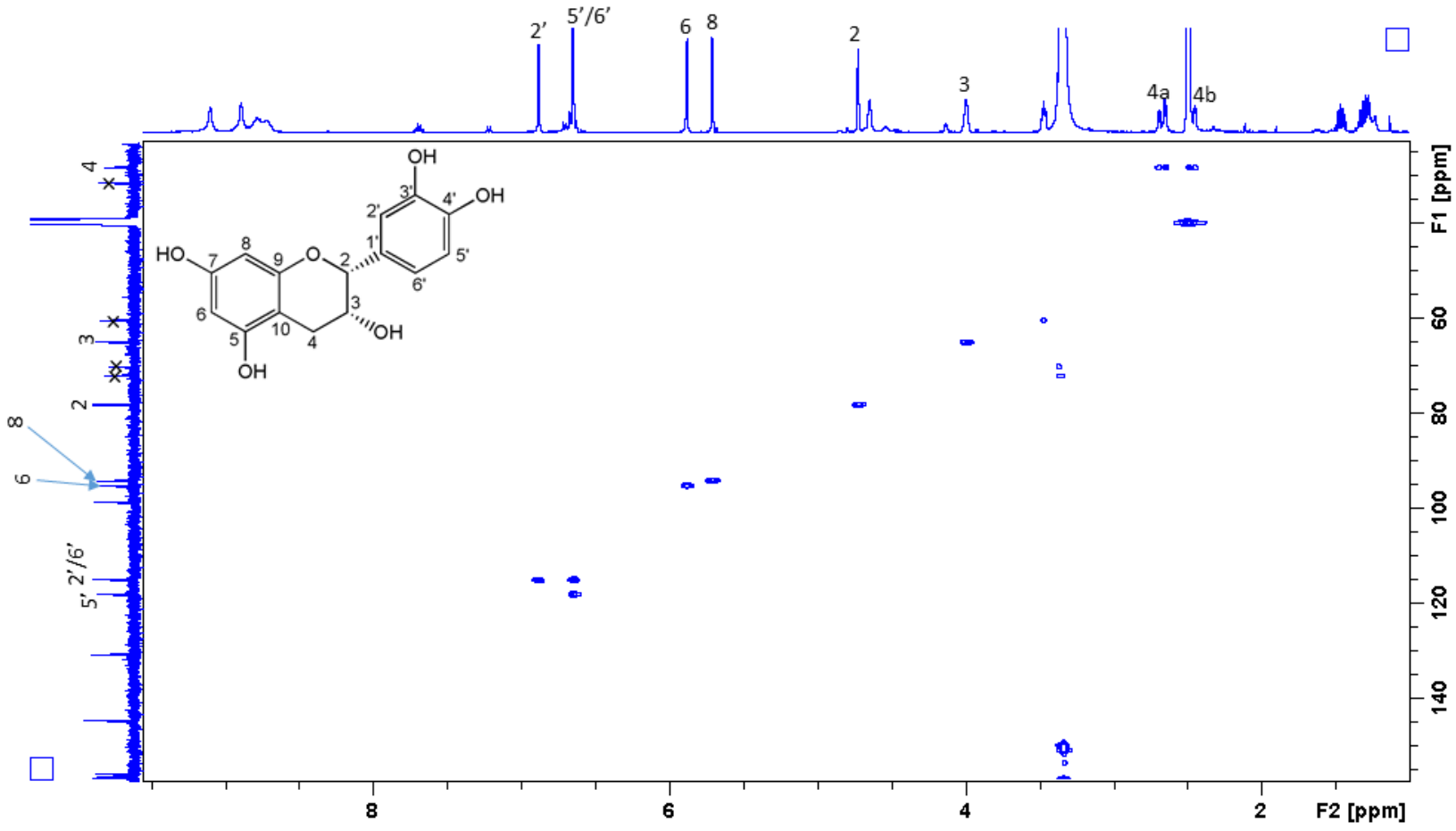
DEPT spectrum of epicatechin B-1



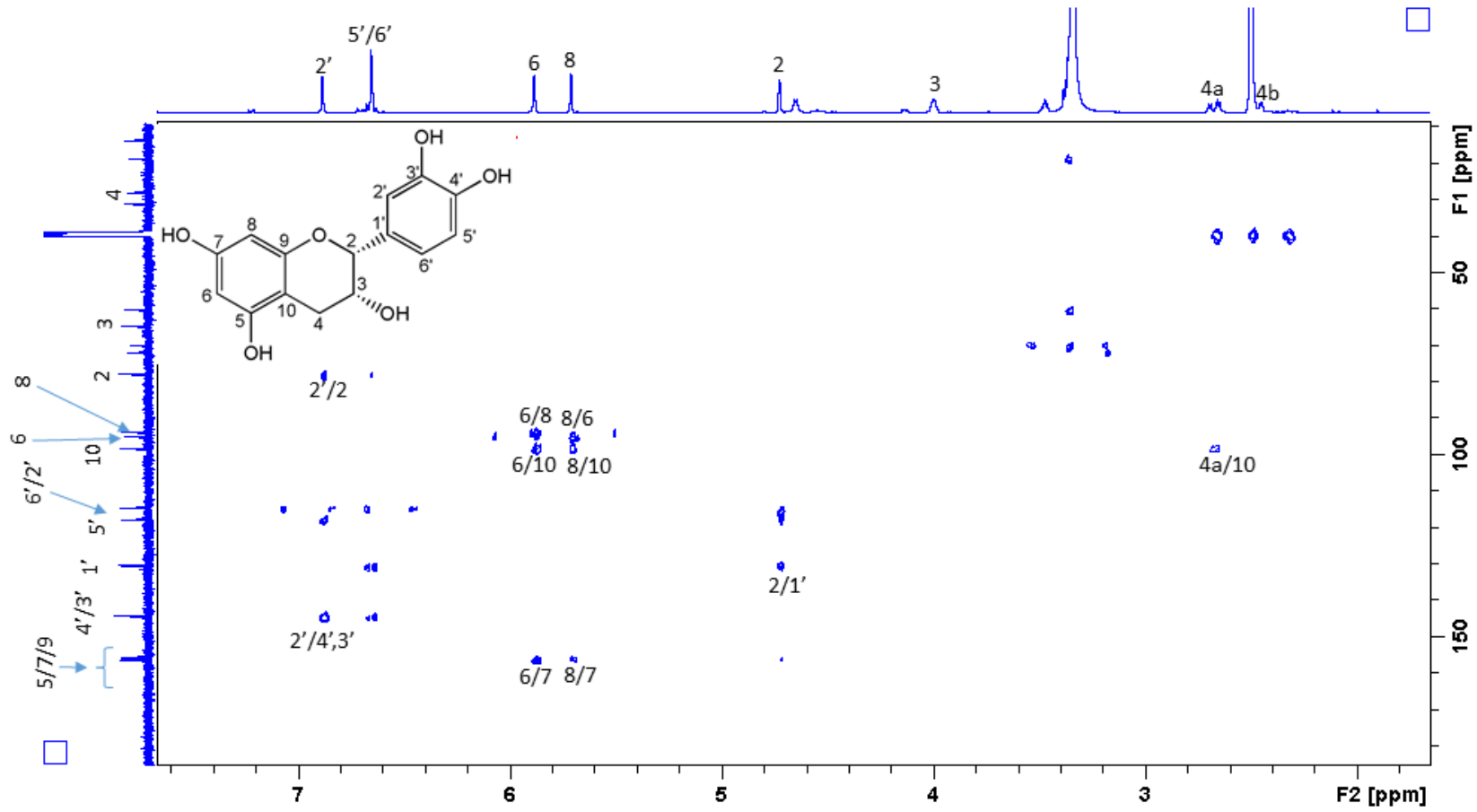
COSY spectrum of epicatechin B-1



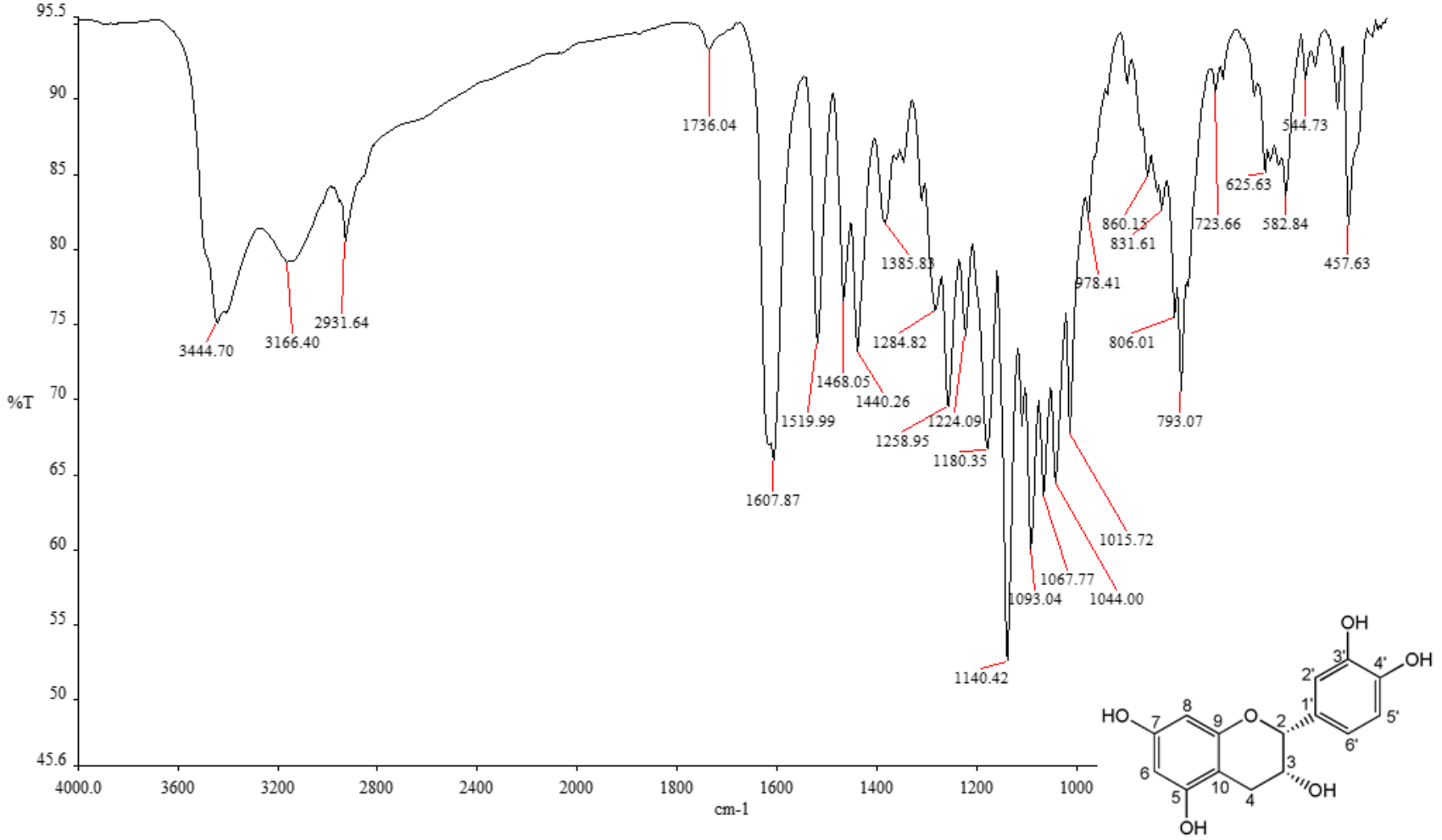
NOESY spectrum of epicatechin B-1



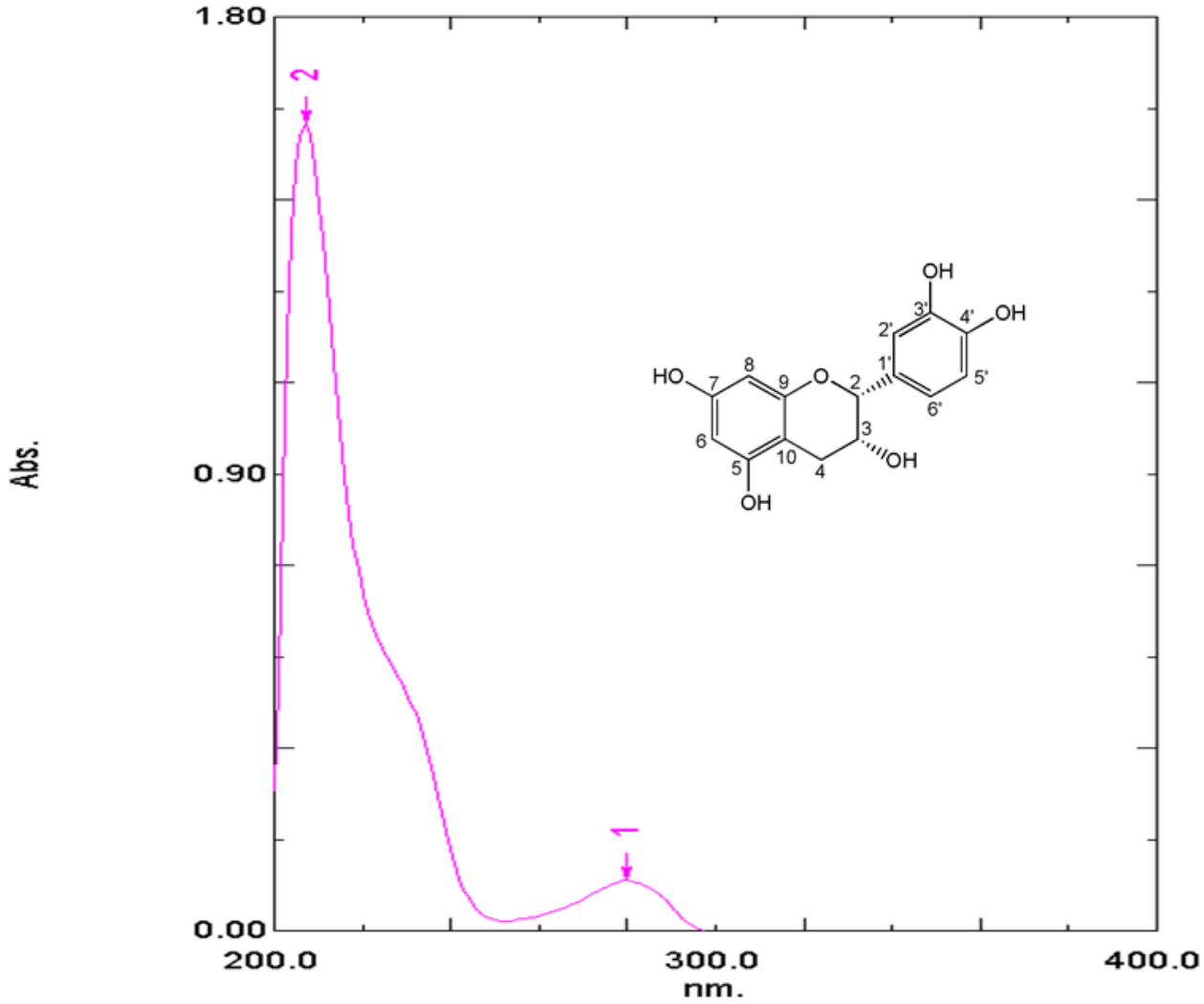
HSQC spectrum of epicatechin B-1



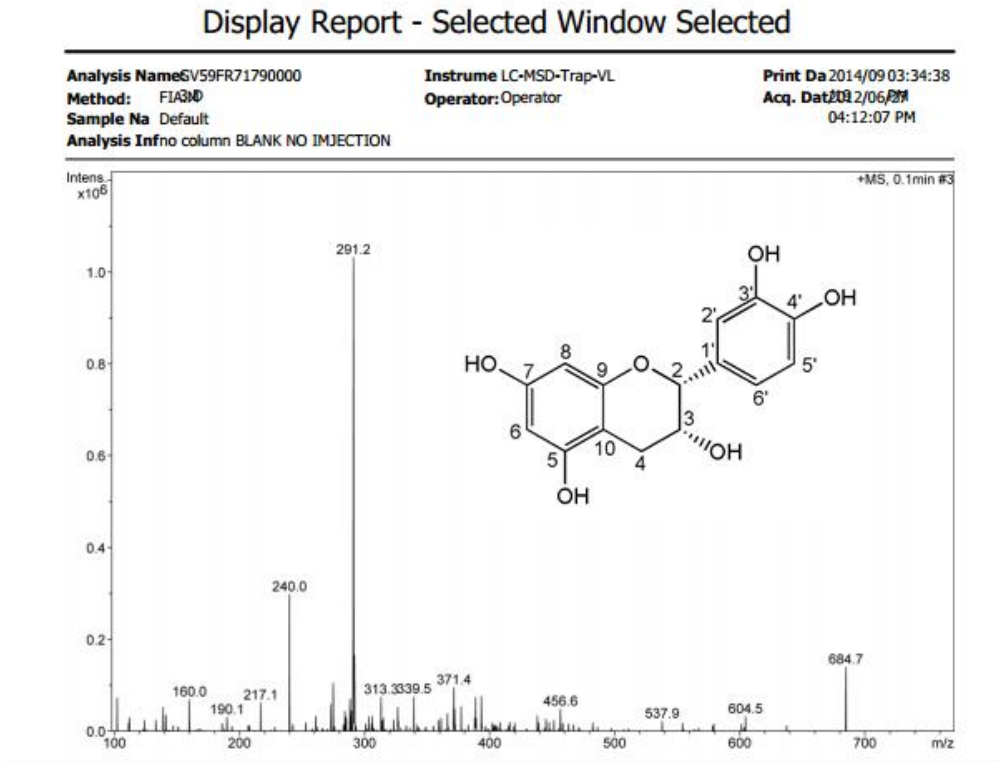
HMBC spectrum of epicatechin B-1



IR spectrum of epicatechin B-1



UV spectrum of epicatechin B-1



Mass spectrum of epicatechin B-1

Phytochemical, Elemental and Biological studies of three *Ficus* Species (Moraceae) found in Kwazulu-Natal, South Africa

Appendix 2

(Published articles in internationally recognised peer- reviewed journals)

OLUMUYIWA OLUFISAYO OGUNLAJA

2017

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CHEMICAL CONSTITUENTS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF CRUDE EXTRACTS AND COMPOUNDS FROM LEAVES AND STEM BARK OF *FICUS BURTT-DAVYI*

OLUMUYIWA O. OGUNLAJA[1], ROSHILA MOODLEY[1], HIMANSU BAIJNATH[2] and SREEKANTHA B. JONNALAGADDA[1,*]

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Abstract: *Ficus burtt-davyi, (*Moraceae), is an endemic species of South Africa. In this study, a phytochemical analysis of the leaves and stem bark of *F. burtt-davyi* resulted in the isolation of five triterpenes (lupeol, lupeol acetate, β-sitosterol, stigmasterol and campesterol), one carotenoid (lutein), a pheophytin (phytyl-7-ethyl-25-(methoxycarbonyl)-3,8,13,17-tetramethyl-26-oxo-12-vinyl-17,18-dihydro-2,20-ethanoporphyrin-18-propanoate or pheophytin **a**) and one flavonoid (+)-catechin). The *in vitro* antioxidant study of the methanol extracts of leaves and stem bark, (+)-catechin and pheophytin **a** using the 1,1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, ferric reducing antioxidant power (FRAP) assay and hydrogen peroxide (H_2O_2) assay showed significantly higher ($p < 0.05$) antioxidant activity for the methanol extract of the stem bark than the leaves, with IC_{50} values (in µg/mL) of 58.28 ± 5.05 for DPPH, 46.09 ± 0.06 for FRAP and 151.03 ± 1.60 µg/mL for H_2O_2. The results suggest that the plant can be used as a therapeutic agent in alternative medicine for oxidative stress related degenerative diseases.

Keywords: pheophytin, triterpenes, flavonoids, antioxidants

With over 850 species, *Ficus*, of the plant family Moraceae, is one of the oldest, most successful, but understudied genera in modern pharmacognosy and has extensive distribution of secondary metabolites such as triterpenoids, phenolics, flavonoids, alkaloids, coumarins and sterols (1-4). *Ficus*, within their vasculatures, possess and secrete a latex-like material, affording protection from harm and providing self-healing from physical injuries (5). For many centuries, animals and humans have depended on its fruit as a source of nourishment while other plant parts such as the leaves, bark, and roots has been utilized for medicinal purposes. The latex from *Ficus* species have been investigated for their anticancer activity as far back as the 1940's (5) and extracts have demonstrated enhanced intracellular accumulation of daunomycin in K562/R7 leukemic cells as well as cytotoxic effects on the growth of multi-drug resistant human sarcoma MES-SA/Dx5 cells (5). Thus, the extracts play an adjunctive role in multiple cancer prevention and cancer chemotherapy (5, 6). The antioxidant potential of the plant has also been reported where the extracts have been shown to decrease lipid peroxidation and increase antioxidant enzymes (7). Although the medicinal benefits of *Ficus* in humans are based on historical and anecdotal reports, with few modern clinical trials, ethno-medicinal uses suggest anti-neoplastic and anti-inflammatory actions (5).

Ficus burtt-davyi, known as Uluzi by the Zulu people in KwaZulu-Natal, is highly adaptable to a wide variety of habitats and has even been known to grow on larger trees (epiphytic) as a strangler fig, as well as on rocks (epilithic) where the roots are able to split the rocks in their search for nutrients (8). The fruits of the plant are edible and also used as a laxative by the locals in the Eastern Cape (8). To date, no information has been reported on the chemical composition and antioxidant activity of this plant. In this study, we report on the isolation and identification of compounds from the leaves and stem bark of *F. burtt-davyi*. In addition, we report on the antioxidant activity of the methanol (MeOH) extracts of the leaves, stem bark and selected isolated compounds using a multi-method approach due to the complexity of the mechanisms of antioxidant activity (9).

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EXPERIMENTAL

General experimental procedures

The ^{1}H, ^{13}C and 2D-NMR spectra were recorded on a Bruker Avance^{III} 400 MHz spectrometer at 400.22 MHz for ^{1}H and 100.63 MHz for ^{13}C. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The ^{1}H and ^{13}C chemical shifts of the deuterated chloroform ($CDCl_3$) were 7.24 and 77.0, respectively, referenced to the internal standard, tetramethylsilane (TMS). Infrared (IR) spectra were recorded using a Perkin-Elmer Universal ATR spectrometer. UV spectra were obtained on a Hewlett Packard UV-3600 spectrophotometer. Column chromatography (CC) was performed with Merck silica gel 60, (0.040–0.063 mm). Thin layer chromatography (TLC) was performed on Merck 20 × 20 cm silica gel 60, F_{254} aluminum sheets. The spots were analyzed under UV (254 nm and 366 nm), visualized using 10% H_2SO_4 in MeOH followed by heating. Solvents (analytical grade) and other chemicals used were supplied by either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA) chemical companies.

Plant material

The leaves and bark from *F. burtt-davyi* were collected on the Westville Campus of the University of KwaZulu-Natal (UKZN), Durban, South Africa, in June 2013. The plant was authenticated by Prof. Himansu Baijnath and a voucher specimen (Ogunlaja, O1) has been deposited in the Herbarium of the School of Life Sciences, UKZN, Westville. The stem bark and leaves of the plant were dried and ground using a mini-industrial grinder (Wiley Mill).

Extraction, fractionation and isolation

The air-dried, powdered leaves (970 g) of F. *burtt-davyi* were sequentially exhaustively extracted with (4 L × 2) of n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and MeOH for 24 h using an orbital shaker. The extracts were concentrated by evaporation under vacuum at controlled temperatures to obtain crude extracts of n-hexane (24.22 g), DCM (15.64 g), EtOAc (7.76 g) and MeOH (52.98 g). The n-hexane and DCM crude extracts of leaves were combined due to similar TLC profiles (similar R_f values). This combined extract (38.00 g) was subjected to column chromatography (CC) using a gradient elution system of n-hexane : EtOAc starting with 100% n-hexane until 100% EtOAc was reached through 10% increments of EtOAc, collecting 100 mL fractions at each stage. Six main fractions (I-VI) were obtained and fraction II yielded compound **1** (105 mg), which was eluted with n-hexane: EtOAc (8 : 2, v/v). Fraction III (1.23 g) was purified further to give compound **2** (18.6 mg) and compound **3** (20.1 mg), respectively. The EtOAc extract of the leaves (7.0 g) was separated using CC in a similar manner to yield seven major fractions (I-VII) based on similar TLC profiles. Fraction II and III afforded compound **4** (30.5 mg) and compound **5** (10.5 mg), respectively.

The n-hexane and DCM crude extracts of the stem bark were combined due to similar TLC profiles (similar R_f values). This extract (24.35 g) was subjected to CC in a similar manner to that of the leaves to yield nine major fractions based on TLC profiles. Fractions II and III gave compound **6** (4.5 g) and compound **7** (4 g), respectively, which were eluted with 100% DCM and recrystallized in MeOH. The MeOH extract from the stem bark was partitioned with EtOAc and the EtOAc fraction was concentrated to yield 4 g of extract. This was separated on a 1.5 cm diameter column using CC and a gradient of n-hexane: EtOAc (8 : 2, v/v) to 100% EtOAc (10% increments of EtOAc, 20 mL fractions) to afford compound **8** (23 mg).

Phenolic content and *in vitro* antioxidant assays

Estimation of total phenolic content (TPC)

The total polyphenol content (TPC) of the extracts from *F. burtt-davyi* was determined as gallic acid equivalent (GAE) according to the method described by McDonald et al. (10) with slight modifications. Briefly, 200 µL of the extract (240 µg/mL) was incubated with 1 mL of ten-fold diluted Folin-Ciocalteau reagent and 800 µL of 0.7 M Na_2CO_3 for 30 min at room temperature. Absorbance values were determined at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

DPPH free radical-scavenging activity assay

The capacity to scavenge the ''stable'' free radical 1,1-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the procedure described by Ahmad et al. (11) with some modifications. Various concentrations (7.5-500 µg/mL) of extracts and isolated compounds (3000 µL) made from a stock solution (10 µg/mL) were mixed with 2850 µL MeOH solution containing DPPH radicals. The mixture was then vortexed, and incubated for 30 min at room temperature. The scavenging activity was evidenced by a change in color from purple to yellow, which was further measured by the decrease in absorbance at 517 nm using a Shimadzu UV–Vis spectropho-

tometer. Ascorbic acid was used as the standard while MeOH served as the blank. The assays were done in triplicate. The difference in absorbance between a test sample and the control (DPPH + MeOH) was expressed as percentage inhibition.

$$\% \text{ inhibition} = [(A_o - A_{sample} / A_o) \times 100]$$

where, A_o (control) = Absorbance of DPPH + methanol.

The IC_{50} value which is the inhibitory concentration in µg/mL of samples, or standard, necessary to reduce the initial DPPH by 50% as compared to the negative control was determined graphically by plotting the absorbance of DPPH as a function of sample concentration in µg/mL for the standard and samples.

Ferric reducing antioxidant power (FRAP) assay

The reducing power of the extracts and compounds were determined according to the method of Oyaizu (1986) as described by Behera et al. (12) with some modifications. Various concentrations (7.5-500) µg/mL were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 0.1% potassium ferricyanide and the mixture was incubated at 50°C for 30 min. After the addition of 2.5 mL of 10% TCA, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. The methanolic extract without reagents was used as a negative control while ascorbic acid with the same concentrations was used as positive controls.

Hydrogen peroxide-scavenging activity assay

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (13). A solution of hydrogen peroxide (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Samples of various concentrations (7.5-500 µg/mL) were transferred into the test tubes, and their volumes were made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After addition of 0.6 mL hydrogen peroxide solution, tubes were vortexed and absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. Phosphate buffer (50 mM) without hydrogen peroxide was used as a blank and ascorbic acid was used as the positive control. Hydrogen peroxide scavenging ability was calculated using the following equation:

Hydrogen peroxide scavenging activity = (1 - absorbance of sample/absorbance of control) ×100

Statistical analyses

The experimental results were expressed as the mean ± standard deviation (SD) of three replicates and IC_{50} values were calculated by linear regression. The data were subjected to one way analysis of variance (ANOVA) to determine significant differences between means ($p < 0.05$) Tukey`s test was used for *post-hoc* analyses. All the statistical tests were performed using Graphpad prism 6.0.

RESULTS AND DISCUSSION

Structure elucidation of compounds from *F. burtt-davyi*

Compound **1**, **2** and **3** were identified by using spectral data (IR, UV, GC-MS, ^{1}H-NMR, ^{13}C-NMR and 2D NMR) and by comparison of the NMR data with those reported in the literature. Compound **1** was identified as sitosterol (14)**,** compound **2** as campesterol (15) and compound **3** as stigmasterol (14).

Compound **4** was isolated as a dark green amorphous solid. The mass spectrum of compound **4** obtained by HR-ESI-MS (positive mode) gave m/z value at 893.5530 corresponding to the molecular formula ($C_{55}H_{74}N_4O_5$) $[M + Na]^+$, (calc. $C_{55}H_{74}N_4O_5$ Na 894.1999). The IR spectrum of compound **4** showed absorption bands at 2922, 3388, 1618 and 1376 cm^{-1} corresponding to C(sp^3), NH, CH=CH (vinyl group) and CN, respectively. The UV spectrum of compound **4** showed absorption bands at 414 and 669 nm. The data are consistent with the presence of a porphyrin nucleus. The ^{1}H-NMR spectrum of compound **4** showed characteristic peaks for chlorophyll derivatives. The ^{1}H-NMR spectrum showed an upfield shift at δ_H -1.70 ppm (the NH proton from the pyrrole ring), a resonance at δ_H 6.1–7.9 ppm (vinyl group), meso-proton signals downfield at δ_H 9.47 (H-10), 9.33 (H-5) and δ_H 8.53 (H-20) ppm and δ_H 6.28 (dd, J = 1.6, 17.5 Hz, H-3a), δ_H 6.16 (dd, J = 1.5, 11.55 Hz, H-3b) ppm for a mono-substituted vinyl group corresponding to the structure of pheophytin **a**. The characteristic phytyl side chain resonances at δ_H 1.60 (CH), 1.25 (CH_2) and 0.97 (CH_3) ppm were also observed. The ^{13}C-NMR, DEPT 90 and DEPT 135 spectra resolved fifty five carbon resonances corresponding to eleven methyl, fourteen methylene, eleven methine and twenty-one quaternary carbons signals. These chemical shifts were in accordance with those reported in the literature (16), thus, compound **4** was established as phytyl-7-ethyl-25-(methoxycarbonyl)-3,8,13,17-tetramethyl-26-oxo-12-vinyl-17,18-dihydro-2,20-ethanoporphyrin-18-propanoate (pheophytin **a**).

Compound **5** was obtained as a yellow colored compound and GC-MS data showed molecular ion peak [M^+] at m/z 568.9 which corresponds to the molecular formula $C_{40}H_{56}O_2$. The UV spectrum of compound **5** showed absorption maxima at 454, 480 and 430 nm. The ^{1}H-NMR spectrum showed resonance in the olefinic region between δ_H 5.09-6.62 ppm, methylene resonances of cyclohexene between δ_H 1.33-1.44 ppm with a strong OH resonance at δ_H 4.23 ppm. Based on spectral data and those reported in the literature, compound **5** was identified as lutein.

Compound **6** was isolated as a white solid. The IR spectrum exhibited characteristic absorption frequencies at 3326, 878 and 1637 cm^{-1} typical of the O-H, unsaturated out of plane C-H and C=C bond

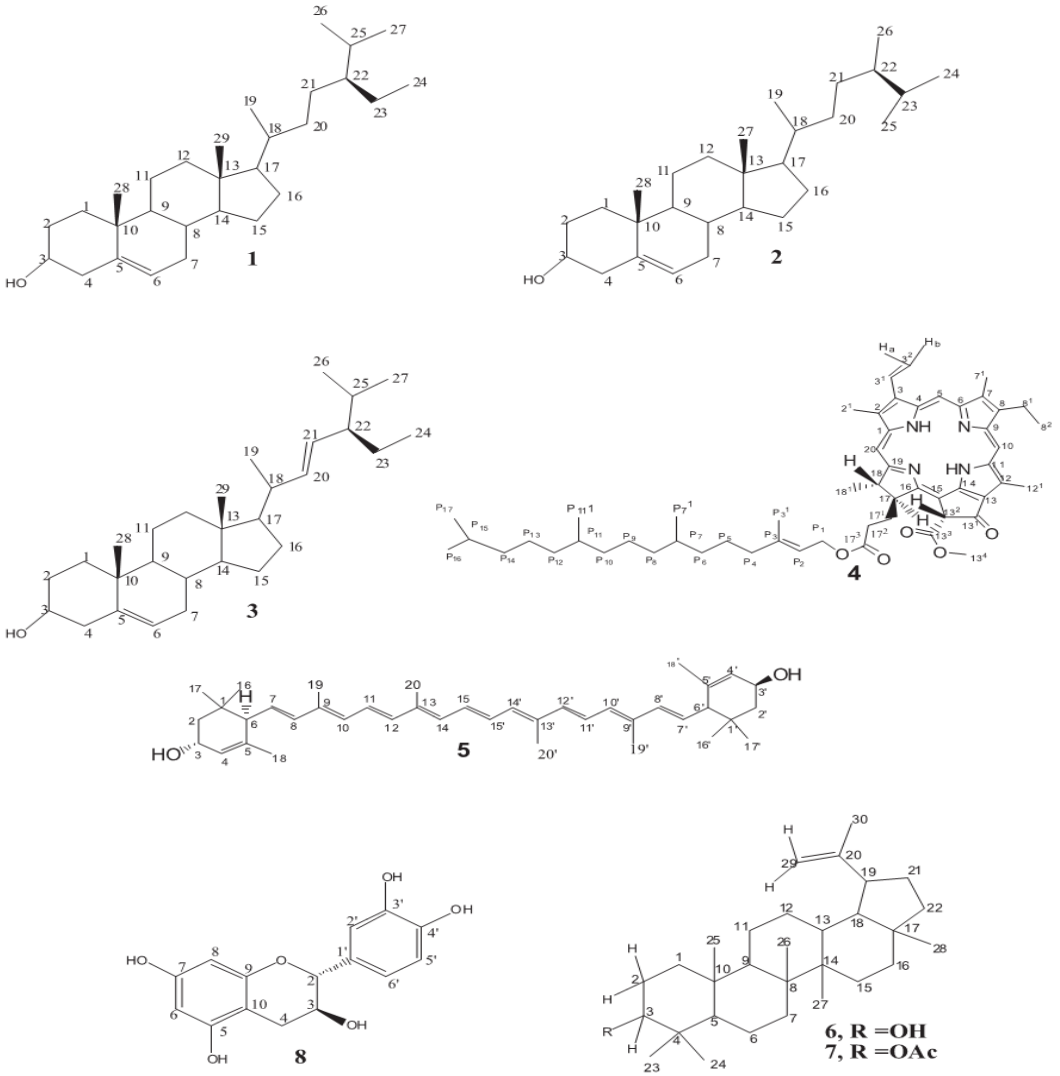

Figure 1. Chemical structures of compounds **1-8** isolated from *F. burtt-davyi*

Table 1. Total phenolic content (TPC) and extraction yield (%, mg extract per g sample × 100) of *F. burtt-davyi* leaves and stem bark extracts.

Extracts	Yield (%)	TPC (mg/g GAE) *
Leaves		
EtOAc	0.51^{c}	0.43 ± 0.01^{c}
MeOH	3.05^{b}	1.14 ± 0.12^{b}
Stem bark		
EtOAc	0.80^{c}	1.05 ± 0.22^{bc}
MeOH	5.46^{a}	5.90 ± 0.45^{a}

Data are presented as the mean ± SD (n = 3). *GAE (gallic acid equivalent).Values with different superscripts letters along a column are significantly different from each other by Tukey's HSD multiple post *hoc test*, ($p < 0.05$).

vibrations, respectively. The absorptions bands observed at 1379 and 1452 cm^{-1} were due to the distortion vibrations of CH_3 groups and methylenic vibrations, respectively. The mass spectrum of compound **6** obtained by HR-ESI-MS (positive mode) gave m/z value at 449.3756 corresponding to the molecular formula ($C_{30}H_{50}O$) $[M + Na]^+$, (calc. $C_{30}H_{50}O$ Na 449.7174). The ^{1}H-NMR spectrum for compound **6** revealed the presence of seven tertiary methyl singlets protons at δ_H 0.73, 0.76, 0.80, 0.92, 0.94, 1.00 and 1.65 ppm; a multiplet at δ_H 3.18 ppm (H-3), and characteristic olefinic protons at δ_H 4.66 (H-29 a) and 4.54 (H-29 b) ppm and a sextet at δ_H 2.39 ppm (19β -H). The DEPT 90 and 135 experiments resolved ten methylene, five methine and five quaternary carbons. Based on spectral information and those reported in the literature, compound **6** was identified as lupeol. The presence of lupeol in *F. burtt-davyi* was not reported before the current study.

Compound **7** was isolated as white crystalline needles. EIMS for $C_{32}H_{52}O_2$ m/z (rel. int.): 468 [M^+] (17.2%), 453 (2.9%), 425 (1.5%), 408 (1.7%), 365 (3.9%), 189 (75.4%), 109 (73.1%), 43 (100%). The diagnostic peaks occurred at m/z 408 [M-AcOH], 249 [M-$C_{16}H_{27}$] and 189 [249-AcOH]. The IR spectrum of compound **7** exhibited characteristic absorption frequency at 1732 cm^{-1} (C=O), 3073 cm^{-1} (exomethylene group), 2939 cm^{-1} (C-H) and 1244 cm^{-1} (C-O). The ^{1}H-NMR spectrum of compound **7** showed eight methyl resonances, seven of them between δ_H 0.76-1.66 ppm. The ^{1}H-NMR spectrum of compound **7** was similar to compound **6** with addition of an acetate methyl resonance at δ_H 2.01 ppm (H-32) and the proton resonance at δ_H 4.46 ppm (H-3) which is further downfield due to presence of the acetoxy group. Based on spectral information and in comparison with that in the literature (17) compound **7** was identified as lupeol acetate. Lupeol acetate has never been reported isolated before from *F. burtt-davyi*.

Compound **8** was isolated as a light brown powder. The mass spectrum of compound **8** obtained by HR-ESI-MS (positive mode) gave m/z value at 313.0685 corresponding to the molecular formula ($C_{15}H_{14}O_6$) $[M + Na]^+$, (calc. $C_{15}H_{14}O_6$ Na 313.2596). The IR spectrum showed characteristic absorption bands for the O-H group (3215 cm^{-1}), C=C group (1623 cm^{-1}), and C-O group (1145–1019 cm^{-1}). The ^{1}H-NMR spectrum of compound **8** showed characteristic resonances for flavonoids at δ_H 6.85 (H-2'), 6.76 (H-5') and 6.74 (H-6') ppm from the B-ring catechol moiety as well as at δ_H 5.94 (H-6) and 5.87 (H-8) ppm from the meta-coupled protons of the A-ring resorcinol moiety. The proton resonances at δ_H 4.59 (H-2), 3.99 (H-3), 2.87 (H-4) and 2.53 (H-4) ppm were used to establish a flavanol skeleton for compound **8**. The ^{13}C-NMR spectrum showed a diagnostic peak at δ_C 82.8 (C-2) ppm as well as a strong H-2/H-3 correlation from the COSY experiment, thus confirming the structure of compound **8** to be (+)-catechin (18). Catechin and its analogues have been shown to possess significant bioactivity, such as anti-inflammatory, anti-allergic, anti-mutation, antioxidant and anti-aging activity (19). This is the first report of compounds **1**-**8** being reported in *F. burtt-davyi*.

Total phenolic content (TPC)

The extraction yields indicated that the stem bark of *F. burtt-davyi* produced the highest amount of extract. Furthermore, the total phenolic content (TPC) of the MeOH extract of the stem bark was significantly higher ($p < 0.05$) than that from other extracts (Table 1). Based on this result, the MeOH extracts from the leaves and stem barks were selected for *in vitro* antioxidant study.

DPPH free radical-scavenging activity assay

Most antioxidants possess proton radical scavenging activity which may be monitored by discoloration of the purple 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals to yellow DPPH in a dose-dependent pattern. For this study, the DPPH assay was used to detect the antioxidant activity of the extracts and compounds from *F. burtt-davyi* as shown in Figure 1. The results indicated that at lower concentrations (≤ 25 µg/mL), the activity of the extracts (stem bark and leaves) and compounds were comparable but were significantly lower than that of ascorbic acid (AA).

At higher concentrations (≤ 125 µg/mL), the activity of the MeOH extract from the bark, (+)-catechin and AA were not significantly different but higher than the other substances tested. The high activity of the MeOH extract of the bark could be attributed to the presence of (+)-catechin in the extract, thereby suggesting that the activity of an extract may be as a result of the presence of one bioactive compound, which, at lower concentrations, may be diminished by the antagonistic effects of other compounds or the presence of other non-active compounds.

Ferric reducing antioxidant power assay (FRAP assay)

Numerous studies have linked the electron donation capacity (reduction) of an antioxidant to its antioxidative activity (20). The presence of electron donating substances, such as antioxidant samples, causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The total reducing power of the extracts and compounds from *F. burtt-davyi* were compared to AA (Fig. 2).

Similar to the results produced by the DPPH radical scavenging activity, the results obtained from the FRAP assay showed the reducing power of the MeOH extract from the stem bark, (+)-catechin and AA to be comparable and significantly higher than that of the MeOH extract from leaves and pheophytin **a**.

Table 2. IC_{50} of different extracts and compounds from *F. burtt-davyi* for various antioxidant assays.

Extracts/compound	DPPH* (µg mL)	FRAP* (µg/mL)	H_2O_2 scavenging* (µg/mL)
L(MeOH)	405.39 ± 20.58^{c}	420.95 ± 8.46^{c}	449.56 ± 6.66^{c}
B(MeOH)	58.28 ± 5.05^{b}	46.09 ± 0.06^{b}	151.03 ± 1.6^{b}
Pheophytin **a**	611.15 ± 10.06^{d}	777.80 ± 1.60^{d}	> 1000^{d}
(+)-Catechin	61.19 ± 0.68^{b}	46.89 ± 0.33^{b}	125.15 ± 1.16^{b}
Ascorbic acid (AA)	1.14 ± 0.08^{a}	39.32 ± 0.017^{a}	81.56 ± 0.63^{a}

Each value is represented as the mean ± SD (n = 3). $^{a-d}$Means in the same column followed by a different letter are significantly different ($p < 0.05$). *No significant difference between assays ($p > 0.05$).

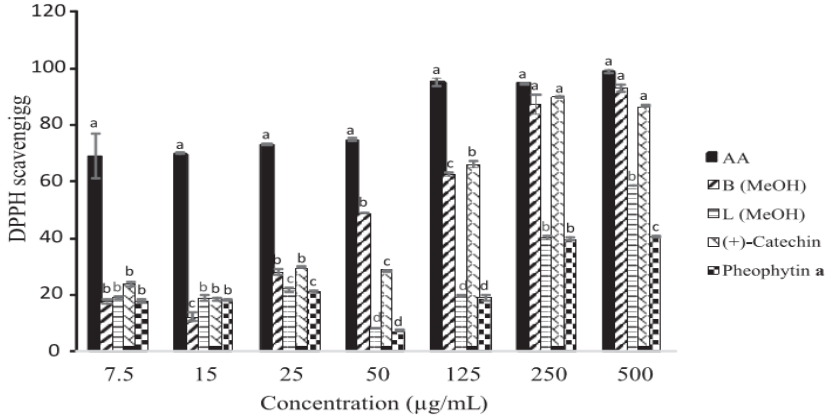

Figure 2. DPPH radical scavenging activity of MeOH extracts of *F. burtt-davyi* stem bark (B), leaves (L), pheophytin **a**, (+)-catechin and ascorbic acid (AA). Different letters for a concentration indicate significantly different means (Tukey's *post hoc* comparisons, $p < 0.05$)

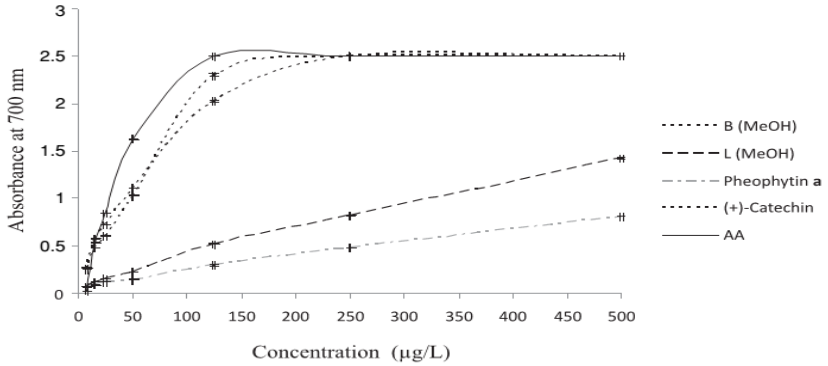

Figure 3. Reducing power of MeOH extracts of *F. burtt-davyi* stem bark B (MeOH), leaves L (MeOH), isolated compounds (pheophytin **a** and (+)-catechin) and ascorbic acid (AA). Values represented as the mean ± SD (n = 3)

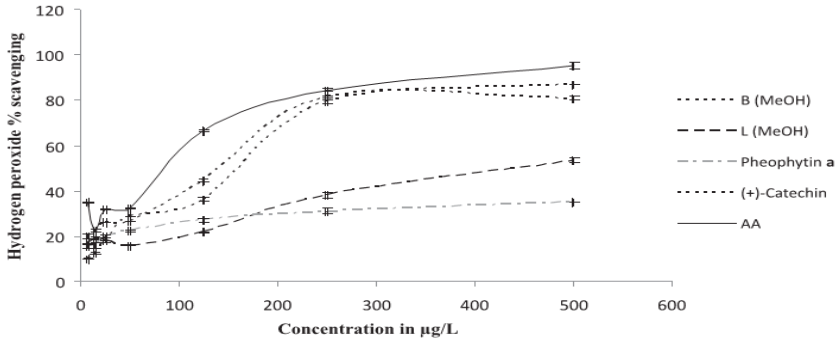

Figure 4. Hydrogen peroxide scavenging activity of MeOH extracts of *F. burtt-davyi* stem bark B (MeOH), leaves L (MeOH), isolated compounds (pheophytin **a** and (+)-catechin) and ascorbic acid (AA). Values represented as the mean ± SD (n = 3)

Hydrogen peroxide-scavenging activity assay

Although hydrogen peroxide is a weak oxidizing agent, it can cross cell membranes rapidly and while inside the cell, form hydroxyl radicals which are primarily responsible for its toxic effects. Therefore, removal of the hydroxyl free radical is necessary to ensure a good health status. The scavenging effects of the extracts and isolated compounds in *F. burtt-davyi* were evaluated against this free radical (Fig. 3). The antioxidant activity of the extracts and compounds using hydrogen peroxide were similar to those produced by the DPPH and FRAP assays thereby confirming the activities of all the tested substances.

Table 2 shows the IC_{50} values for the different antioxidant assays. Although compounds **1**-**8** were tested for antioxidant activity, the IC_{50} values for sitosterol, campesterol, stigmasterol, lutein, lupeol and lupeol acetate were extremely high (> 1000 µg/mL) for all assays, therefore, their results are not shown in Table 2. The results confirm that the MeOH extract and (+)-catechin have a significantly higher antioxidant activity ($p < 0.05$), with comparable IC_{50} values for all three antioxidant assays. However, the IC_{50} value for the standard AA was significantly lower than the plant extracts and compounds. From the TPC and antioxidant activity results, it is clear that higher TPC indicates higher antioxidant activity. This is in agreement with previous reports (21, 22).

Thus, the present study has shown that the leaves and stem bark of *F. burtt-davyi* possess moderate to significantly good antioxidant activity and may contribute to the retardation of the inflammatory process mediated by reactive oxygen metabolites from phagocytic leukocytes that invade the tissues

and cause injury to essential cellular components (23). In addition, previous studies have shown that plant-derived sterols exhibit anti-carcinogenic effects on different types of cancers (24-27). *F burtt-davyi* could therefore be said to possess anti-cancer potential having isolated several sterols from the stem bark.

CONCLUSION

To the best of our knowledge, this is the first report of the phytochemical constituents of *F. burtt-davyi,* resulting in the isolation of a carotenoid which has been documented to exhibit moderate antibacterial activity and pheophytin **a** with strong anti-HCV-NS3 protease activity with little cytotoxicity. The data from this study suggest that *F. burtt-davyi* possessed moderate to good antioxidative activity and can be used as a potential alternative medicine for oxidative stress related non-communicable chronic diseases.

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Nutritional evaluation, bioaccumulation and toxicological assessment of heavy metals in edible fruits of FicussurForssk (Moraceae)

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Nutritional evaluation, bioaccumulation and toxicological assessment of heavy metals in edible fruits of *FicussurForssk (Moraceae)*

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ABSTRACT

Ficussur (Moraceae) is an indigenous medicinal plant with a wide distribution in Africa. In this study, the nutritional potential fruit of this indigenous plant to meet domestic food demands and reduce food insecurity in KwaZulu-Natal. South Africa, was investigated. The proximate composition and concentrations of metals in the edible fruits collected from eight different sites in KwaZulu-Natal were determined to assess for nutritional value and the concentrations of metals in the growth soil was determined to evaluate the impact of soil quality on elemental uptake. The fruits contained high levels of moisture (88.8%) and carbohydrates (65.6%). The concentrations of elements in the fruits were found to be in decreasing order of Ca>Mg >Fe >Zn>Cu >Mn> Se with low levels of toxic metals (As, Cd, Co and Pb). This study shows that the consumption of the fruits of *F. sur* can contribute positively to the nutritional needs of rural communities in South Africa for most essential nutrients without posing the risk of adverse health effects.

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KEYWORDS
Elemental distribution; nutrition; toxicity; soil quality

Introduction

Undernutrition is a common health problem amongst rural South Africans, and its effects on growth and development has detrimental outcomes, the most serious one being its ability to potentiate the development of non-communicable diseases (NCDs) such as cardiovascular diseases, cancer and diabetes.[1–2] Evidence from scientific health reports have indicated that the consumption of fruits can reduce the risk of many of the nutrition-related diseases and risk factors that contribute substantially to the burden of disease in South Africa.[3] Indigenous plants have been identified as having the potential to meet domestic food demands, thereby reducing food insecurity, especially in rural societies and this has increased interest in the exploitation, quantification and utilization of such food plants.[4–5]

In the rural areas of many African countries, people depend on trees growing in the wild for fruit due to accessibility and affordability, thereby inadvertently exploiting the therapeutic and nutraceutical potential of such fruits. In some cases, indigenous fruits with very little or no documentation of their chemical composition are the only fruits consumed.[6] On average, indigenous fruits contribute 42% towards the food basket for most rural people in Southern Africa.[7] A daily consumption of indigenous food plants in sufficient quantities can help prevent numerous diseases, improve the nutrition and health of children and the elderly and boost the immune system of HIV/AIDS patients.[8–9] Although rural households in South Africa frequently turn to indigenous fruits for food, they are often faced with dietary and nutrient deficiency diseases, a situation which children and lactating women are most vulnerable to.[10–12]

The total wellbeing of humanity depends largely on the regular daily intake of macro and micro-elements in the diet, soil being the primary source. Generally, plants depend directly on soil for support and nutrients and metals are mobilized from soil to different parts of plants depending on the nature and quality of the soil matrix.[12] Excessive levels of heavy metals and metalloids may occur in soil as a result of normal geological occurrences and anthropogenic inputs such as the application of pesticides, waste disposal (industrial, agricultural and domestic), waste incineration, urban effluent and vehicle exhausts.[13–14] In addition, the affinity of different plant parts for these metals can also play a major role in the transfer and eventual bioaccumulation of heavy metals. Heavy metals are known to have long biological half-lives and may act as cumulative slow poisons, directly influencing public health because humans do not have an effective mechanism for their removal from the body.[13] In recent years, studies on the impact of heavy metals have increased significantly, especially in the areas of toxicology, due to their non-biodegradable nature and implication in abnormal cell functioning which is linked to certain types of cancers.[15–17]

Ficussur, of the plant family Moraceae, is an indigenous medicinal plant found in KwaZulu-Natal, South Africa.[18] It occurs from the Southern Cape northwards throughout eastern South Africa, to tropical Africa, to Senegal and Cape Verde

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Islands in the west, Ethiopia in the north and Yemen in the east.[18] It is known as Umkhiwane in isiZulu and serves as an immediate source of food both to animals and humans, hence playing a key role in their everyday survival. An evaluation of the nutritional benefits and concentration of essential and toxic heavy metals in the edible fruits is imperative since metal toxicity in humans, through the agricultural food chain, at plant tissue concentrations are not necessarily phytotoxic.

Previously, we reported on the nutritional value of the fruit of indigenous medicinal plants and the impact of soil quality on elemental uptake.[12,19–20] This study aimed to investigate the elemental distribution and concentration of 13 elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) in the edible fruits of *F. sur* and to assess for nutritional value and potential metal toxicity. Soil quality parameters were also investigated to evaluate their impact on elemental uptake.

Materials and methods

Sample collection and preparation

F. sur fruit and soil samples from below the tree were collected from eight different sampling sites in KwaZulu-Natal, South Africa; 1-Reservior Hills, 2 - Overport, 3 – Burman Bush, 4 – Pigeon Valley, 5 – UKZN, Howard Campus, S6 - Hibberdene, 7 – Pietermaritzburg and 8-Byrne (Fig. A1) between February and March, 2014. Sites were flat with varied yet verdant climate (humid and subtropical). The topography was diverse with loamy soil type and an annual rainfall of 1009 mm. The average daily temperature ranged from 20.6 to 28.6°C. Samples of tree-ripened fruits (Fig. A2) were randomly picked from trees, placed in sealed plastic bags and taken to the laboratory for further analyses. The plant was identified and classified by a taxonomist at the School of Life Sciences, University of KwaZulu-Natal, South Africa and a voucher specimen deposited in the ward Herbarium. Fruit samples were washed thoroughly with double distilled water to remove extraneous matter and then chopped into smaller pieces with a stainless steel knife. Thereafter, fruit samples were oven-dried at 50°C overnight to ensure complete removal of moisture. Dried fruit samples were crushed using a food processor (Kenwood Compact Blender, BL380) then stored in a refrigerator in sealed plastic bags until analyzed.

Soil samples were collected from six random points along the drip line of each tree, at a depth of 15–20 cm, with the use of a plastic hand shovel. These were thoroughly mixed in a clean plastic bucket to achieve homogeneity, thus forming the composite sample. A representative soil sample was taken from each site and was dried overnight in an oven at 40°C then passed through a 2 mm mesh sieve to remove organic matter and gravel. Some of this soil (10 g) was crushed with a mortar and pestle to reduce the particle size for microwave digestion. Samples were stored in sealed plastic bags and kept in a refrigerator until analyzed.

Analytical quality assurance, chemicals and instruments

All plastic containers were washed with double distilled water and then soaked overnight in 1M HNO_3. Glassware and other equipment were cleaned with 6M HNO_3 and rinsed off with double distilled water to prevent contamination before usage. Millipore^{TM} water (Billerica, MA, USA) was used throughout the experiments. All chemicals used were supplied by Merck (Kenilworth, NJ, USA) and Sigma (St. Louis, MO, USA) Chemical Companies and were of analytical-reagent grade. Elemental calibration standards were prepared from spectroscopic grade stock standard solutions of 1000 mg L^{-1}.

The Microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, NC, USA) with patented Xpress technology that consists of MARSXpress^{TM} vessels and IR temperature sensors, was used for digestion. Each digestion vessel comprises liners (Teflon PFA, DuPont, Wilmington, DE, USA), caps, and composite sleeves that have a self-regulating pressure control.

Fruit and soil samples (0.25 g each) were weighed into the 50 mL liners, to which, 10 mL of HNO_3 was added. Fruit samples were pre-digested for 1 h prior to microwave digestion. Liners were capped, placed into the sleeves, loaded onto the 40-place carousel, and placed into the microwave. The appropriate method was loaded and the system started. The power was set to 100% at 1600 W and the temperature was ramped to 180°C (for fruit samples) and 200°C (for soil samples) for 15 min where it was held for 15 min. Fruit and soil digests were transferred to 50 mL volumetric flasks, diluted to the mark with double distilled water and stored in polyethylene bottles prior to elemental analysis.

Extraction of exchangeable metals

The extracting solution was prepared by diluting 38.542 g ammonium acetate ($NH_4CO_2CH_3$), 25 mL acetic acid (CH_3COOH, 96%) and 37.225 g ethylenediaminetetraacetic acid (EDTA) to 1L in double distilled water. Exactly 50 mL of extracting solution was added to 5.0 g of dry soil samples in 250 mL polyethylene bottles and shaken in a laboratory shaker for 2 h. Thereafter, solutions were filtered through Whatman No. 1 filter papers and then Millipore 0.45 μm filter membranes to permit analysis of extracted metals. All samples were stored in plastic bottles and kept in a refrigerator until analyzed.

Soil pH, cation exchange capacity (CEC) and Soil Organic Matter (SOM)

The pH of soil was determined by measuring the pH of the solution, 1:2.5, dry wt/v using a pH meter fitted with a glass electrode.[21] Cation exchange capacity (CEC) of soil was determined using the pH 7.0 ammonium acetate method [22] while soil organic matter (SOM) was measured according to the procedure adopted from Walkley and Black.[23]

Proximate composition determination

Moisture, crude protein, fat, ash and crude fiber content of *F. sur* fruits were determined according to the Association of Official Analytical Chemists method (AOAC).[24] Total carbohydrate content was estimated by difference.

Elemental analysis

All extracted and digested samples (soil and fruit) were analyzed for As, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, Co, and Zn by inductively coupled plasma-optical emission spectrometry (ICP-OES) due to its multi-element determination capability, high dynamic linear range and sensitivity. Analytical wavelengths were selected based on minimum spectral interferences and maximum analytical performance. Initially, the three most sensitive lines were chosen. From these lines, the line with no interfering elements was selected. The accuracy of analytical procedures was checked by analyzing certified reference materials (CRMs).

Bioaccumulation factor (BAF)

The Bioaccumulation factor (BAF) expresses the ability of a metal species in its different forms to migrate from the soil through the plant parts and make itself available for consumption.[13]

$$BAF = \frac{[Fruit]}{[Soil]_{Exchangeable}}$$

Statistical analysis

The difference in heavy metal concentrations between the eight sites was investigated using discriminant analysis. The results of this analysis were assessed by examining the canonical correlation statistics, the Wilk's lambda statistics, the significance levels and the percentage of original group cases correctly classified. In addition, a Pearson's correlation analysis was applied to the dataset to quantitatively analyze and confirm the relationship between soil quality parameters (pH, SOM and CEC) and heavy metal concentrations. Principal component analysis (PCA) was carried out to identify patterns in the elemental data by identifying the different groups of metals that correlate and thus can be considered as having a similar behavior and common origin. All statistical analyses were performed using the Statistical Package for the Social Sciences, (PASW version 23, IBM Corporation, Cornell, NY, USA).

Results and discussion

Proximate chemical composition

The results of the proximate analysis showed *F. sur* fruits to contain 88.8 ± 0.2% moisture. On dry mass basis, the carbohydrate content was 65.6 ± 0.03%, protein 5.2 ± 0.1%, fat 4.7 ± 0.2%, crude fibre 7.7 ± 0.02% and ash 17.67 ± 1.3%. The high level of carbohydrates in the fruits indicates that they can serve as an immediate and alternative source of energy. These values were similar to those reported by Wilson and Downs.[25]

Soil quality parameters analysis

Soil pH ranged from 5.3 to 8.2 while SOM ranged from 11.0 to 22.3%. Higher pH values were observed at site 4 (7.6 ± 0.09), site 6 (7.3 ± 0.03) and site 7 (8.2 ± 0.08) and may be connected with the higher concentration of Ca in the soil compared with other studied sites. The CEC ranged from 2.30 to 3.70 meq/100.

Elemental analysis

Method validation for the analytical procedure was carried out by measuring CRMs, BCR-402 (Institute for Reference Materials and Measurement, European Commission, Joint Research Centre, Belgium) for fruits samples and D081–540 (ERA, A waters Company, Milford, MA, USA) for soil samples. Experimental results were compared with certified results (Table 1). Measured values compared well to the certified values ($P < 0.05$) with recovery percentages being within acceptable limits.

The concentrations of heavy metals in soil (total and exchangeable) and fruits are summarized in Table 2 for those elements in fruits that were above the instrument detection limits. Although, total soil Pb (21.5–78.4 $\mu g\ g^{-1}$) was above the South African maximum permissible level of 6.6 $\mu g\ g^{-1}$ in soils at all sites, the concentrations of Pb in all fruit samples were found to be below the instrument detection limit. In addition, concentrations of the other toxic elements, As, Cd and Co in all fruit samples were below the instrument detection limit. Selenium was found in fruit samples from site 1 (0.86 $\mu g\ g^{-1}$) and site 2 (2.16 $\mu g\ g^{-1}$) only and Ni was found in the fruit sample from site 8 (10.30 ± 1.44 $\mu g\ g^{-1}$).

The fruit tended to accumulate Mg producing BAFs (Exchangeable) between 16.9 and 91.5 even though, Mg mobility was low (0.4 to 29.2%). This is seen at site 1, where Mg in the fruit was more than ninety times that which was exchangeable (Table 2). Calcium concentrations in fruits ranged from 2270 to 7746 $\mu g\ g^{-1}$ with BAFs (Total) between 0.6 and 6.0 indicating the tendency of the plant to accumulate this metal.

Copper is an essential micronutrient in humans for the production of blood hemoglobin. High doses of Cu can result in anemia, liver and kidney damage, and stomach and intestinal irritation.[26] Total soil Cu was relatively low (< 24 $\mu g\ g^{-1}$) and on average 56% was in exchangeable form. Copper concentrations in the fruits were within a small range of variation (1.43–17.4 $\mu g\ g^{-1}$) however, at site 7, Cu in the fruits exceeded the WHO permissible limit of 10 $\mu g\ g^{-1}$ for plants.[27]

Zinc is a trace element that is essential for human health, its shortages can cause birth defects. Zinc at elevated levels in soil can negatively interrupt the activity of microorganisms and earthworms, thus retarding the breakdown of organic matter.[26] The concentration of Zn in the fruit ranged from 4.35 to 18.1 $\mu g\ g^{-1}$. More than 50% of the sites tended to accumulate Zn in the fruits with BAFs (Exchangeable) > 1. The concentrations of Cu and Zn in the fruits exhibited safe levels relative to the maximum levels for fruit set by the Department of Health, South Africa, which is 30 and 40 $\mu g\ g^{-1}$, respectively.[28]

Site 8 was observed to have the lowest exchangeable Fe (96.9 $\mu g\ g^{-1}$) but the highest concentration of Cu (17.4 $\mu g\ g^{-1}$), Mn (20.2 $\mu g\ g^{-1}$) and Zn (22.8 $\mu g\ g^{-1}$) in the fruits. This three-way synergy between Cu, Zn and Mn is as a result of the reduction of soil retention capacity of one metal due to increase in concentration of a contending metal ion. This observation is in agreement with reports from literature.[12] Although, total

Table 1. Validation of the analytical method using plant and soil certified reference materials (BCR-402 for plant and D081–540 for soil).

Element	Concentration in BCR-402($\mu g g^{-1}$)		Concentration in D081–540($\mu g g^{-1}$)		
	Measured*	Certified	Measured	Certified	Acceptable limit
As	0.10 ± 0.014	0.093 ± 0.010	83.2 ± 3.36	101 ± 5.92	61.0–116
Cd	—	—	156.0 ± 4.6	143 ± 5.6	116 – 159
Co	0.174 ± 0.006	0.178 ± 0.008	238.5 ± 7.8	199 ± 4.1	166 – 233
Cr	4.98 ± 0.248	5.19[a]	102.6 ± 2.3	86.8 ± 6.1	69.3 – 104
Fe	240 ± 6.75	244[a]	13119 ± 608	12800 ± 18.0	5380 – 20100
Mn	—	—	448.5 ± 18.7	425 ± 9.7	347 – 502
Se	6.80 ± 0.16	6.70 ± 0.25	161.5 ± 5.9	127 ± 4.5	98.4 – 156
Ni	8.23 ± 0.46	8.25[a]	212 ± 21.7	236 ± 4.17	175–302
Zn	25.40 ± 0.68	25.02[a]	136.9 ± 7.9	130 ± 11.5	113 – 184

*Values are in $\mu g\ g^{-1}$dry mass (mean ± standard deviation, 95% confidence interval, $n = 3$).
[a]Indicative values (without uncertainty). BCR-402 for plant and D081–540 for soil.

Table 2. Elemental concentrations ($\mu g\ g^{-1}$) in fruit and soil (Total (T) and exchangeable (Ex)) samples (Mean ± standard deviation; 95% confidence interval, $n = 3$) and bioaccumulation factors (BAFs).

		Concentration ($\mu g\ g^{-1}$)			BAF		
Site	Element	Fruit	Soil (T)	Soil (Ex)	$[F]/[S]_T$*	$[F]/[S]_{Ex}$**	Ex%***
1	Ca	3313 ± 21	630 ± 37	454 ± 19	5.3	7.3	72.1
2		2270 ± 152	1114 ± 29	1043 ± 34	2	2.2	93.7
3		2355 ± 40	394 ± 5.58	250 ± 3.91	6	9.4	63.5
4		2613 ± 24	2528 ± 333	2085 ±7 1	1	1.3	82.5
5		19467 ± 35	1180 ± 41	1033 ± 27	1.7	1.9	87.5
6		3310 ± 74	1476 ± 75	1433 ± 31	2.2	2.3	97.1
7		2792 ± 49	4671 ± 9.9	4089 ±3 7	0.6	0.7	87.6
8		7746 ± 232	2173 ± 182	786 ± 50	3.6	9.9	36.2
1	Cu	4.31 ± 0.30	7.78 ± 0.52	2.36 ± 0.30	0.6	1.8	30.3
2		4.31 ± 0.97	7.76 ± 0.71	6.90 ± 0.30	0.6	0.6	88.9
3		5.01 ± 1.37	3.06 ± 1.32	2.78 ± 0.12	1.6	1.8	90.6
4		2.49 ± 0.39	18.2 ± 2.51	10.7 ± 0.30	0.1	0.2	58.9
5		3.41 ± 0.28	8.54 ± 0.60	6.15 ± 0.12	0.4	0.6	72
6		2.23 ± 0.55	6.79 ± 1.05	6.25 ± 0.33	0.3	0.4	92
7		5.11 ± 0.68	23.6 ± 1.20	7.65 ± 0.16	0.2	0.7	32.4
8		17.4 ± 2.07	8.82 ± 1.02	1.63 ± 0.26	2	10.6	18.5
1	Fe	61.5 ± 2.56	31536 ± 95	327 ± 28	0	0.2	1
2		28.0 ± 1.56	20279 ± 104	261 ± 8.59	0	0.1	1.3
3		21.6 ± 1.61	18391 ± 601	186 ± 4.73	0	0.1	1
4		29.9 ± 2.95	18534 ± 33	475 ± 18	0	0.1	2.6
5		62.7 ± 1.80	15302 ± 118	710 ± 13	0	0.1	4.6
6		35.4 ± 5.10	17534 ± 394	182 ± 4.13	0	0.2	1
7		105 ± 9.56	67040 ± 352	447 ± 2.51	0	0.2	0.7
8		129 ± 3.90	15171 ± 553	96.9 ± 7.58	0	1.3	0.6
1	Mg	1798 ± 162	2534 ± 84	19.6 ± 2.45	0.7	91.5	7.8
2		1233 ± 40	550 ± 31	73.1 ± 26	2.2	16.9	13.3
3		1255 ± 4.89	251 ± 6.71	73.2 ± 0.63	5	17.2	29.17
4		1022 ± 6.56	1189 ± 73	48.7 ± 1.64	0.9	21	4.09
5		1487 ± 24	874 ± 11	27.6 ± 3.08	1.7	53.8	3.16
6		1284 ± 36	492 ± 32	33.3 ± 2.23	2.61	38.5	6.77
7		1613 ± 34	1549 ± 62	26.3 ± 3.01	1.04	61.3	1.7
8		1766 ± 179	23880 ± 341	83.8 ± 22	0.07	21.1	0.35
1	Mn	0.47 ± 0.01	16.3 ± 0.74	4.09 ± 0.26	0	0.1	0.3
2		0.52 ± 0.05	18.1 ± 1.39	14.0 ± 0.42	0	0	0.8
3		1.83 ± 0.02	37.5 ± 1.07	32.5 ± 0.71	0.1	0.1	0.9
4		0.14 ± 0.01	23.0 ± 2.08	12.6 ± 0.32	0	0	0.6
5		0.04 ± 0.01	16.1 ± 0.48	9.90 ± 0.22	0	0	0.6
6		1.17 ± 0.03	19.9 ± 0.50	13.8 ± 0.15	0.1	0.1	0.7
7		0.92 ± 0.24	237 ± 26	75.0 ± 1.10	0	0	0.3
8		20.2 ± 1.25	35.3 ± 0.40	11.5 ± 1.36	0.6	1.8	0.3
1	Zn	13.7 ± 4.63	27.3 ± 2.77	6.22 ± 0.73	0.5	2.2	22.8
2		14.6 ± 1.91	24.7 ± 2.51	18.1 ± 0.87	0.6	0.8	73.4
3		13.5 ± 1.89	9.42 ± 0.33	5.81 ± 0.13	1.4	2.3	61.7
4		6.90 ± 1.17	54.2 ± 3.60	39.1 ± 5.14	0.1	0.1	72.2
5		13.4 ± 1.03	23.5 ± 1.19	15.1 ± 0.40	0.6	0.9	64.3
6		4.35 ± 0.29	21.9 ± 0.48	19.4 ± 0.34	0.2	0.2	88.8
7		18.1 ± 4.89	37.8 ± 1.36	15.3 ± 0.52	0.5	1.2	40.5
8		22.8 ± 2.17	22.8 ± 0.70	4.58 ± 0.61	0.1	5	20.1

*$[F]/[S]_T$–$[Fruit]/[Soil]_{Total}$.
**$[F]/[S]_A$-$[Fruit]/[Soil]_{Exchangeable}$.
***Ex% - $[Soil]_{Exchangeable}/[Soil]_{Total}$.

Table 3. Comparison of Dietary Reference Intake (DRI) [29] (Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Level (UL)) of elements for most individuals to the average concentration of elements ($n = 3$) in *F. sur* fruits.

Element	Averageconcentration (mg / 20 g DM)	DRI (mg/day)RDAUL		Estimated contribution to RDA (%)
Ca	109.67	1000–1300	2500	8.4–11.0
Cu	0.11425	0.9	8	12.7
Fe	1.18275	8–18	45	6.6–14.8
Mg	28.645	310–320	350	9.0–9.2
Mn	0.06315	1.6–2.3	9	2.7–3.9
Se	0.0302	0.055	0.4	54.9
Zn	0.268375	8–11	34	2.4–3.4

soil Fe was relatively high at all sites (15171 to 67040 $\mu g\ g^{-1}$), BAFs (Exchangeable) were relatively low suggesting the plant control on uptake.

Manganese is an essential trace metal. Its deficiency produces severe skeletal and reproductive abnormalities in mammals and its toxicity symptoms are lung and brain damage.[29] The concentration of Mn in fruits ranged from 0.04 to 20.17 $\mu g\ g^{-1}$ which are below the maximum limits of 2000 $\mu g\ g^{-1}$.[30] This study shows that for the essential elements Ca, Mg, Fe, Cu, Mn and Zn, the fruit tends to exclude the element when soil concentrations are high but accumulated them when soil concentrations are low, in accordance to metabolic requirements.

Estimated contribution of metals in fruits to the diet

The elemental distribution in the edible fruits of *F. sur* was compared to Dietary Reference Intakes (DRIs) (Table 3).[31] The results show that the fruits can contribute significantly to the health and nutritional needs of individuals for most elements. Although fortification of wheat and maize flour has improved the Fe status of children in South Africa, rates of anemia among preschool aged children and pregnant women are 24% and 22%, respectively.[32,33] The consumption of 20.0 g of fruit (which is the average weight of a handful of fruits) contributes between 6.6–14.8% towards the RDA for Fe for most adults. Copper deficiency is normally due to decreased Cu at birth, insufficient dietary Cu intake and poor absorption. Consumption of 20.0 g of fruit contributes about 12.7% towards the RDA for Cu for most adults. Selenium is reported to help with the prevention of heart disease and cancer, however if in excess, it can produce toxicity effects such as depression, gastrointestinal disturbances and excessive tooth decay.[34] About 20 g of *F. sur* fruits may contribute more than 54% towards the RDA for Se;however this amount does not exceed the UL so it would be considered safe.

Calcium and Mg are macro-elements required for bone formation, the development of strong teeth, muscle regulation and control of blood pressure. A common nutritional problem among South Africans is hypocalcemia, which is due to a deficiency in Ca. In a study done on a randomly chosen rural South African community, 13.2% of children were found to have abnormally low levels of Ca in their blood.[35] Consumption of 20.0 g of *F. sur* fruits may contribute between 8.4–11.0% towards the RDA for Ca. A report by WHO showed that an average adult in South Africa consumes only half their RDA for Mg.[36] An intake of 310–320 mg of Mg per day is recommended and the consumption of approximately 20.0 g of *F. sur* fruits, may contribute approximately 28.6 mg (9.0–9.2%) towards its RDA.

Statistical analysis

The result of discriminant analysis showed that heavy metal concentrations exhibited discrimination between studied sites (Table 4). Generally, the larger the canonical correlation statistic, the greater is the between groups variation as a proportion of the total variation, and the larger the Wilk's lambda statistic, the greater is the within-group variation as a proportion of the total variation. There was a high degree of between site variations for As, Ca, Cr, Cu, Co, Mg, Mn, Pb, Se and Zn; canonical correlation values ranging from 0.699 to 0.997 and a few lower degree of within-group site variations; Wilk's lambda statistic ranged from 0.005 to 0.511. The statistically significant results also demonstrate that the originally grouped cases have high percentage (28.6% to 88.6%).

The main soil parameters that govern the processes of sorption and desorption of trace elements are pH, CEC and

Table 4. Discriminant analyses results for the studied sites.

Variation	Canonical correlation	Wilk's Lambda statistic	Chi-squares	D.F	Sign.	% of grouped cases correctly classified
As	0.777	0.396	27.824	6	0	28.6
Ca	0.699	0.511	20.154	6	0.003	40.0
Cr	0.988	0.024	112.346	6	0	60.0
Cu	0.967	0.065	81.967	6	0	60.0
Co	0.985	0.029	106.087	6	0	45.7
Mg	0.997	0.005	157.781	6	0	88.6
Mn	0.992	0.015	125.429	6	0	60.0
Pb	0.922	0.151	56.761	6	0	60.0
Se	0.821	0.326	33.657	6	0	31.4
Zn	0.759	0.423	25.795	6	0	57.1

D.F: Degree of freedom; Sign: Significance.

Table 5. Inter-item correlation matrix for concentration of elements in fruit (F) and soil (S).

	CaF	CrF	CuF	CuT	FeT	MgT	MnF	MnT	MnE	NiT	ZnT	ZnE
CaF												
CrF	1.0**											
CuF	0.9*	1.0**										
CuT	−0.1	−0.1	−0.1									
FeT	−0.2	−0.2	−0.1	0.7*								
MgT	1.0**	1.0**	1.0**	−0.1	−0.2							
MnF	1.0**	1.0**	1.0**	−0.1	−0.2	1.0*						
MnT	−0.1	−0.1	0.0	0.7*	0.9**	−0.1	−0.1					
MnE	−0.2	−0.2	−0.1	0.6	0.9**	−0.2	−0.1	1.0**				
NiT	0.0	0.0	0.1	0.7*	0.8*	0.0	0.0	0.9**	0.7*			
ZnT	−0.1	−0.1	−0.2	0.8*	0.3	−0.1	−0.2	0.3	0.1	0.3		
ZnE	−0.4	−0.4	−0.5	0.5	−0.1	−0.4	−0.4	0.0	−0.1	0.0	0.8*	

*,**- significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.
a.XF–$[X]_{Fruit}$, where X = the various elements.
b.XT–$[X]_{Soil\ Total}$, where X = the various elements.
c.XE–$[X]_{SoilExchangeable}$, where X = the various elements.

Table 6. Rotated component matrix for variables in the soil samples ($n = 40$).

Component			
Element	1	2	3
As	**0.77**	0.28	0.11
Ca	0.34	**0.62**	0.37
Cr	**0.92**	0.20	0.17
Cu	0.58	**0.77**	0.09
Co	**0.88**	0.24	0.31
Fe	0.20	0.01	**0.87**
Mg	0.02	0.49	0.28
Mn	**0.87**	0.23	0.32
Ni	**0.64**	0.09	0.12
Pb	0.40	**0.80**	−0.11
Se	**0.71**	−0.10	−0.25
Zn	−0.10	**0.90**	−0.13
Eigenvalues	5.89	1.85	1.03
% Total variance	49.04	15.43	8.61
Cumulative %	49.04	64.47	73.08

Extraction Method: Principal Component Analysis. Rotation Method: Varimax with Kaiser Normalization. Bold figures indicate values ≥ 0.5.

SOM. Table 5 shows the inter-item correlation matrix for significant correlations in the soil and fruits of *F. sur*. Soil pH correlated significantly toexchangeable Cu ($r = 0.8$) and Ca ($r = 0.9$) indicating that pH was a significant parameter for controlling availability of these metals and not SOM and CEC. There was a significant 3-way positive correlation between Fe, Mn and Ni in the soil, which is an indication of the same geological parent material. Exchangeable Zn and Mn correlated positively to their total soil concentrations, suggesting that total soil concentrations may be used to predict the availability of these elements, similar to previous studies.[37] For concentrations in the fruit, there was a significant 4-way positive correlation between Ca, Cr, Cu and Mn suggesting that the plant requires proportional amounts of these elements for metabolic processes.

In order to further identify the relationships between different metals in soil and their corresponding origins, principal component analysis (PCA) was conducted (Table 6). Figure 1 represents the component plot in rotated space (3-D plot) showing the relationships among the twelve heavy metals. Table 6 shows that the elements are dominated by three principal components which accounted for 73.1% of the total variance. Factor 1 was best represented by As, Cr, Co, Mn. Ni and Se, accounting for 49.0% of the total variance. The Cu loading (0.58) is not as high as that of other elements of the group, suggesting a quasi-independent behavior within the group. Factor 2 contributed 15.4% to the total variance with a high loading on Ca, Cu, Pb and Zn. Factor 3 is dominated by Fe (0.87), accounting for 8.6% of the total variance. These associations strongly suggest that the elements clustered together have a similar source.[38–40]

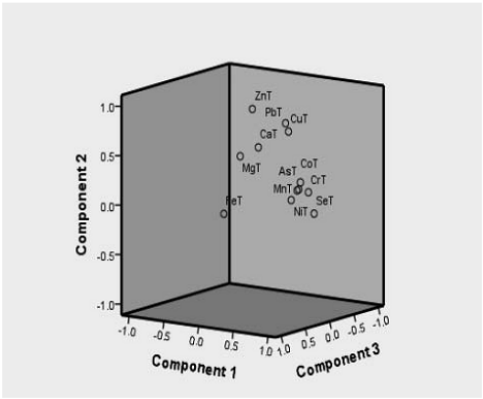

Figure 1. PCA analysis loading 3-D plot (PC1 vs PC2 vs PC3) for 12 heavy metals.

Conclusion

The concentrations of elements in the fruits were found to be in decreasing order of Ca >Mg >Fe >Zn>Cu >Mn >Se while the concentrations of toxic metals (As, Cd, Co, and Pb) were found to be below the instrument's detection limits. This study shows that the fruits of *F. sur* conform to the RDAs for the elements in focus and that their consumption can contribute significantly towards a balanced diet without posing the risk of adverse health effects. Statistical analysis revealed synergistic relationships in the plant, thereby confirming that uptake of elements is controlled to meet metabolic needs.

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Appendix

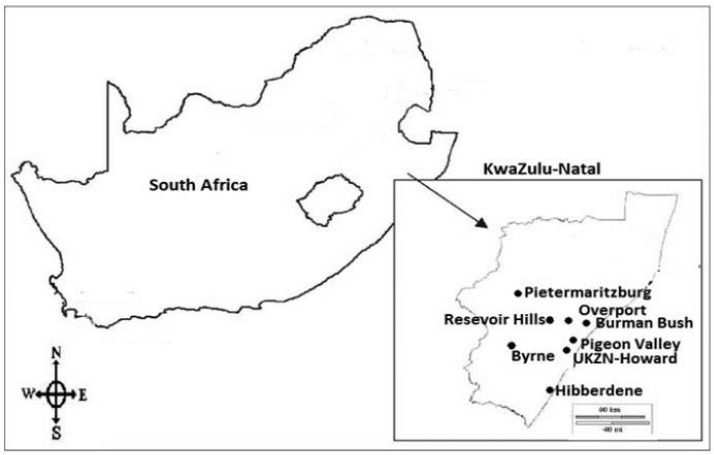

Figure A1. Map of selected sampling sites in KwaZulu-Natal, South Africa.

Figure A2. *Ficus Sur* Forssk fruits.

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CERTIFICATE OF ANALYSIS

WHITE CLOVER			
	Mass fraction based on dry mass		Number of accepted sets of data p
	Certified value [1] [mg/kg]	Uncertainty [2] [mg/kg]	
As	0.093	0.010	15
Co	0.178	0.008	7
Mo	6.93	0.19	13
Se	6.70	0.25	15

[1] Unweighted mean value of the means of p accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value is traceable to the SI.

[2] Half-width of the 95 % confidence interval of the mean defined in [1].

This certificate is valid for one year after purchase.

Sales date: 30. Juni 2014

The minimum amount of sample to be used is 100 mg.

NOTE

This material has been certified by BCR (Community Bureau of Reference, the former reference materials programme of the European Commission). The certificate has been revised under the responsibility of IRMM.

Brussels, November 1991
Revised: May 2007

Signed:

Prof. Dr. Hendrik Emons
Unit for Reference Materials
EC-JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

Additional Material Information	
	Mass fraction based on dry mass
	Value [1] [mg/kg]
Cr	5.19
Fe	244
Ni	8.25
Zn	25.2
1) The value is traceable to the SI.	

DESCRIPTION OF THE SAMPLE

The material consists of a white clover powder in a glass bottle. The bottle contains about 25 g of powder and a small PTFE ball which has been added to facilitate the homogenisation prior to use.

ANALYTICAL METHOD USED FOR CERTIFICATION

- Direct current plasma atomic emission spectrometry
- Energy dispersive X-ray fluorescence
- Electrothermal atomic absorption spectrometry
- Electrothermal atomic absorption spectrometry with Zeeman background correction
- Hydride generation atomic absorption spectrometry
- Hydride generation inductively couples plasma emission spectrometry
- Inductively coupled plasma emission spectrometry
- Inductively coupled plasma mass spectrometry
- Instrumental neutron activation analysis
- Neutron activation analysis with radiochemical separation
- Visible light or U.V. spectrometry

PARTICIPANTS

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