Phytochemistry and Anti-cancer Potential of Compounds Isolated from Kenyan Medicinal Plants, *Moringa oleifera* and *Prunus africana*

MAIYO FIONA CHEPKOECH

A thesis submitted to the school of life sciences, University of KwaZulu-Natal, Westville in fulfilment of the degree of Master of Science in Biochemistry

Supervisor

Dr. Moganavelli Singh

Co-supervisor

Dr. Roshila Moodley

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This is a thesis in which the chapters are written as discreet research papers with an overall introduction and literature and a final summary. These chapters have been submitted for publishing in internationally recognised peer-reviewed journals.

As the candidates supervisor I have approved this thesis for submission.

Supervisor: Dr. Moganavelli Singh Sign……

Co-supervisor: Dr. Roshila Moodley Sign……

This is to certify that this thesis is the original work of Miss. Maiyo Fiona

Sign…….

ABSTRACT

Cancer is one of the leading causes of death worldwide and of all the cancer related deaths occurring worldwide, 70% are in low and middle income countries. In Africa, the disease kills more people than HIV, TB and malaria combined. In Kenya, cancer continues to claim millions of lives. The poor prognosis of cancer in Kenya is due to a lack of facilities for diagnosis and treatment which is further worsened by poor access to these facilities resulting in the majority of Kenyans turning to herbal medicine for treatment. Natural products have been an invaluable source of therapeutic drugs with 60% of cancer chemotherapy in the market today being plant-derived drugs. However, challenges such as drug toxicity and resistance facing chemotherapy it has become imperative to find alternate drugs with improved specificity and efficiency. The current study focuses on two plants, *Moringa oleifera* and *Prunus africana* which are widely used in Kenya for cancer treatment and are reputed to cure various malignancies. There is need to obtain insight into the phytochemistry of these plants and their supposed activity as well as bioprospecting for potential novel anticancer drugs. In this work, the phytochemistry, *in-vitro* cytotoxicity and apoptosis induction activity of isolated phytocompounds on mammalian colorectal adenocarcinoma (Caco-2), hepatocellular carcinoma (HepG2) and the non-cancer human embryonic kidney cell (HEK293) cell lines are reported. Phytochemical studies using different chromatographic and spectroscopic techniques led to the isolation of six compounds, namely quercetin-3-O-glucoside, 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate, lutein, β-sitosterol, β-amyrin and sitosterol-3-O-glucoside. These were successfully purified and their structures were confirmed using spectroscopic techniques. GC-MS profiling of *P. africana* extract revealed 9 compounds of interest. Four of the purified compounds were tested for potential anticancer activity using *in-vitro* cytotoxic and

immunofluorescent techniques. The MTT cell proliferation assay was used to test for cytotoxic activity and dual staining using acridine orange and ethidium bromide were used to study apoptosis. A concentration dependent cytotoxicity was observed across all cell lines with low activity on the non-cancer HEK293 cell line. 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate was the most cytotoxic compound tested across all cell lines based on IC_{50} values with β-amyrin being the least cytotoxic. All compounds tested exhibited higher cytotoxic activity on the colorectal adenocarcinoma (Caco-2) cell line than the hepatocellular (HepG2) cancer cell line. Further investigation on possible mechanisms of action using immunofluorescent studies confirmed apoptosis as one of the modes of cell death which was also selective to cancer cells. Higher apoptotic indices were observed on the cancer cell lines than the non-cancer cell line. The findings in this study will contribute to the existing knowledge on natural products and their anticancer activity and will provide a basis for development of potential novel cancer drugs.

DECLARATION 1 - PLAGIARISM

I, Maiyo Fiona Chepkoech declare that

1. The research reported in this thesis is my original work except where otherwise stated.
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.
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and then source being detailed in the thesis and in the References sections.

Signed……

DECLARATION 2 – PUBLICATIONS

Publication 1

1. Maiyo FC, Moodley R, Singh M. Cytotoxicity, antioxidant and apoptosis studies of quercetin-3-O-glucoside and 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate from *Moringa oleifera.* Submitted for publication.

Publication 2

2. Maiyo FC, Moodley R, Singh M. Phytochemstry, cytotoxicity and apoptosis study of β-sitosterol-3-O-glucoside and β-amyrin from *Prunus africana.* Submitted for publication.

Conferences attended

1. Poster presentation - Cytotoxicity and antioxidant studies of quercetin-3-O-glucoside and 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate from *Moringa oleifera*- University of KwaZulu-Natal, School of Life Sciences postgraduate research day 26th May 2014.
2. Poster presentation - Cytotoxicity, antioxidant and apoptosis studies of quercetin-3-O glucoside and 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate from *Moringa oleifera*-South African Society of Biochemistry and Molecular Biology (SASBMB) congress, Worcester, western cape 6th-9th July 2014.

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DEDICATION

This thesis is dedicated to my late mother Milcah Ruto whose love, guidance and sacrifice is why I am here today.

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ABBREVIATIONS

^{13}C-NMR	C-13 nuclear magnetic resonance spectroscopy
^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy
ATCC	American tissue culture collection
BPH	Benign prostate hyperplasia
Caco-2	Colorectal adenocarcinoma cell line
$CDCl_3$	Deuterated chloroform
d	Doublet
DCM	Dichloromethane
dd	Double doublet
DEPT	Distortionless enhancement by polarisation transfer
DMSO	Dimethylsulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
FBS	Foetal bovine serum
GC-MS	Gas chromatography-mass spectrometry
HEK293	Human embryonic kidney cells cell line
HepG2	Hepatocellular carcinoma

HMBC	Heteronuclear multiple bond coherence
HPLC	High pressure liquid chromatography
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IR	Infra-red radiation
J	Coupling constant
KEFRI	Kenya forestry research institute
m	Multiplet
MEM	Minimum essential medium
MHz	Megahertz
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NIST	National institute of science and technology
PBS	Phosphate buffered saline
ppm	Parts per million
s	Singlet
t	Triplet
TLC	Thin layer chromatography

TMS Tetramethylsilane

UV Ultra-violet radiation

CHAPTER 1

1.1 Introduction

According to the World Health Organization, cancer is a leading cause of death worldwide and is fast becoming an epidemic. According to Bray *et al* (2013), about 7.6 million people worldwide died from cancer in 2008 and approximately 70% of these deaths occurred in low and middle income countries. These deaths are predicted to continue rising. Cancer may be defined as an abnormal growth of cells that invade and spread to other tissues and organs in the body by a process referred to as metastasis. Apart from a genetic predisposition to specific types of cancers such as BRCA1 breast cancer, chronic viral infections, toxic chemicals from the environment, exposure to ultra violet radiation, contamination from food such as aflatoxins from grain (Danaei *et al*., 2005; Boffetta *et al*., 2009; Hamilton *et al*., 2000; Metcalfe *et al*., 2004) can all cause cancer. The high prevalence of communicable diseases such as HIV leads to immune suppression which is a risk factor for cancer (Silverberg *et al*., 2011). Genes plays a major role in cancer in that it can be inherited. Cancer occurs across all age groups but commonly manifests in older people due to an accumulation of risk factors with time. Cancer has existed for thousands of years and, previously, the only form of treatment came from natural products. This is well documented in ancient medical literature and in several historical texts (Cragg and Newman, 2013; Balandrin *et al*., 1993).

Cancer has only become well understood in the last century and knowledge of its epidemiology continues to be studied. Cancer is a multi-stage disease that starts with a mutation in the cells' DNA. This modification in the DNA of a cell can alter or damage critical genes which are not repaired by the cells' repair mechanisms. DNA replication of these damaged genes occurs

followed by cell division making the mutation permanent. Once the damage has occurred, the cell breaks free from normal control processes and replicates profusely, growing into a mass of cells. This mass of cells can be transported through the blood stream to form cancers in other parts of the body by the process of metastasis. Because of the complexity and number of molecular players involved in the cell cycle and the development and progression of cancer, it becomes a difficult disease to control. It is a culmination of defects in the cell cycle that are part of normal cell processes that eventually lead to tumor development.

The most common cancers in Africa are cancers of the cervix, liver, breast, prostate, Kaposi sarcoma and Non-Hodgkin's lymphoma, with the latter two occurring mostly in the HIV population. The most prevalent cancer in the world is lung cancer followed by breast cancer with 1,608,000 and 1,384,000 new cases, respectively, diagnosed in 2008 as shown in Figure 1.1.

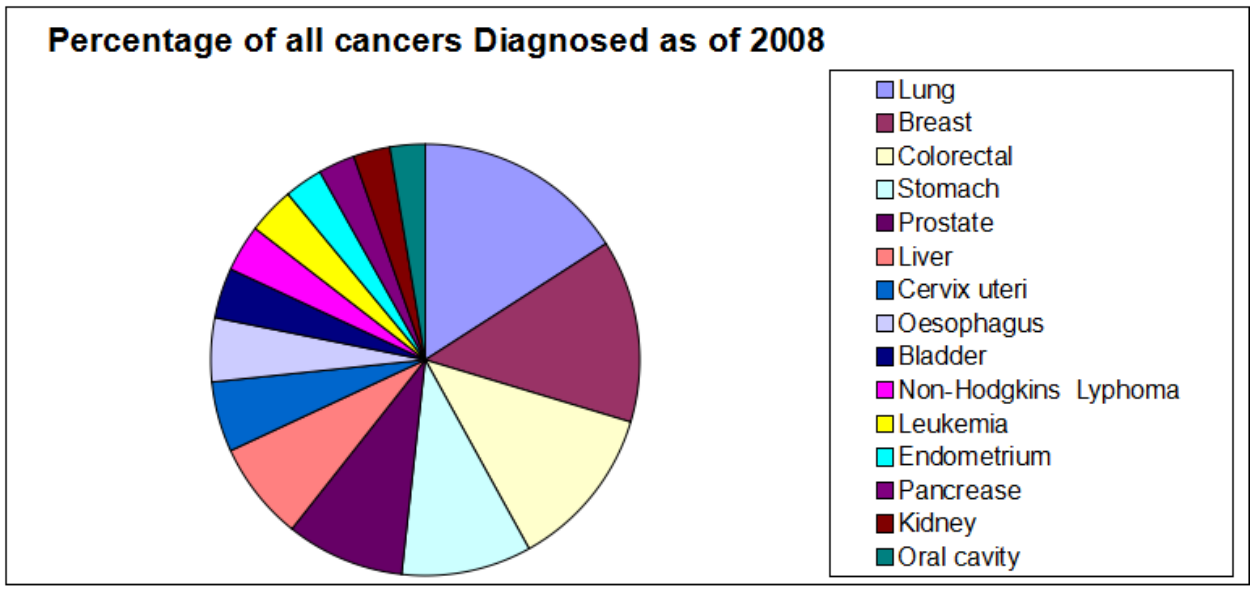


Figure 1.1: Percentage of all cancers diagnosed as of 2008 (American Cancer Society, 2011).

In Africa, non-communicable diseases like cancer, diabetes and hypertension have become a major public health concern. According to the International Agency for Research on Cancer, 715,000 new cancer cases and 542,000 cancer deaths were reported in Africa in 2008. However, this continues to be given a low public health priority in the continent due to the burden of infectious diseases agenda taking center stage and the lack of proper awareness. This has been further influenced by the fact that the continent is still dealing with other pressing healthcare problems such as HIV and TB (Parkin *et al*., 2003).

The incidence of cancer in Kenya alone has increased in recent years, being the third most common cause of death after infectious and cardiovascular diseases (Torrorey *et al*., 2014). Cancers commonly prevalent in developed countries are now on the rise in other countries. This is attributed to the changing lifestyle in these areas as people continue to embrace westernisation especially when it comes to nutrition. This adoption of western cultures has seen an increase in colorectal/stomach, liver and breast cancer (Jamison *et al*., 2006). In Kenya, cervical and breast cancer was the leading cause of death in women in 2008 and Kaposi's sarcoma and prostate cancer in men (National guidelines for cancer management Kenya, 2013). Kenyans in rural areas have poor access to health care facilities and are unable to afford modern medicine, resulting in them turning to traditional herbal medicine and herbalists for treatment (Wagate *et al*., 2008; Kigen *et al*., 2013). In Kenya, the majority of people in the rural and urban areas use traditional medicine to treat illnesses ranging from stomach ache to HIV and cancer. This herbal medicine is usually sold in the form of dry, ground plant material or as herbal tonics. Herbalists prepare different concoctions of the bark, root and leaves from selected plants that are reputed to have healing properties. Herbal medicine is generally bought from traditional medicine men in rural areas as ground plant material or on the streets as herbal tonics. Two of the most commonly used

plants for treatment of cancer and other malignancies in Kenya are *Prunus africana* and *Moringa oleifera* (Ochwang'i *et al*., 2014; Muriuki *et al*., 2012). These trees are widely grown in the country.

Cancer treatment includes a combination of surgery, radiation and chemotherapy. Treatments can be successful when the cancer is still at its early stage. Medicinal plants have been used for thousands of years to treat and prevent a number of diseases in many cultures across the globe, including cancer. Knowledge of traditional medicine is passed down from one generation to another and most, if not all, traditional doctors have no knowledge of the bioactive compounds present in the plants responsible for its activity.

The activity of most plants against diseases is due to bioactive compounds found within the plants. A large number of plant bioactive compounds have been isolated and are commercially available as drugs. The study of plant bioactive compounds especially with regards to anti-cancer agents started in the early 1950s. Some examples of plant-derived compounds that are currently in use as cancer drugs are vinblastine, vincristine and campothecin derivatives which are used in combination with other drugs against a variety of cancers (Cragg and Newman, 2013). The National Cancer Institute has collected around 35,000 plant samples from 20 countries and has screened 114,000 extracts for anticancer activity (Shoeb, 2006). Among the widely used anticancer drugs between 1983 and 1994, 60% are of natural origin, as either derivatives of natural products or synthetics based on natural products (Bhanot *et al*., 2011). These natural products include plants, marine organisms and micro-organisms (Cragg and Newman, 2013).

Natural compounds are extracted from plants using organic solvents of varying degrees of polarity, with non-polar compounds being extracted by non-polar solvents, e.g. hexane and

dichloromethane, and the more polar compounds being extracted by polar solvents e.g. methanol, ethyl acetate or water. Purification of compounds is carried out by various chromatographic techniques and identification is done by extensive spectroscopic analyses using gas chromatography-mass spectroscopy (GC-MS), Nuclear Magnetic Resonance (NMR), Ultra-Visible spectroscopy (UV-Vis) and Infra-Red spectroscopy (IR).

Cancer chemotherapy is faced with many challenges among which are non-specificity, drug toxicity and multi-drug resistance. A large number of drugs (Table 1.1) approved by the Food and Drug Administration (FDA) are effective but many lack specificity leading to significant toxicity and side effects that culminate in the patient dying from these effects rather than from the tumour itself. This has led researchers to focus on finding alternative drugs and ways of improving on existing drugs especially with regards to the drugs toxicity. Nature's biological and chemical diversity have provided the opportunities to discover novel compounds with potential therapeutic uses.

Table 1.1: Some chemotherapy drugs and their modes of action (Payne and Miles, 2008)

TYPE	**MODE OF ACTION**	**EXAMPLE**
Alkylating agents	Damages the DNA	Chlorambucil, Busulfan,
Antimetabolites	Damages DNA and RNA	5-Fluorouracil,
Anthracyclines	Interfere with enzymes during DNA replication	Doxorubicin, Idarubicin
Topoisomerase inhibitors	Inhibits the activity of topoisomerase	Etoposide, Topotecan
Platinum complexes	Inhibits DNA, RNA and protein synthesis.	Oxaliplatin, Cisplatin
Taxanes	Binds to tubulin and inhibits depolymerisation	Paclitaxel, Docetaxel
Tubulin interactive agents	Binds to tubulin preventing polymerisation and microtubule formation leading to apoptosis.	Vincristine, Vinblastine

It is thus important to study the phytochemistry of these plants and to ascertain whether compounds present in these plants show promise as anticancer agents that can be developed into new anticancer drugs.

1.2 Apoptosis as a target for chemotherapy

The uncontrolled cell division in cancer is due to interference in the cell cycle. This link between the cell cycle and cancer has led to the idea that cancer is a cell cycle disease, since the over-proliferation of cells is due to a reduction in sensitivity to signals (proto-oncogenes / tumour suppressor genes) controlling normal cell proliferation. This can be due to internal or external factors which can cause loss of function or gain of function mutations (Collins *et al*., 1997). If DNA is damaged at any stage of the cell cycle the damage is repaired by the cell's repair mechanisms or the cell undergoes apoptosis. Otherwise the mutation is passed on to daughter cells which may grow to become cancer cells (Boik, 2001).

Apoptosis is programmed cell death and is vital for maintaining cell populations and tissue homeostasis. Unlike necrosis, apoptosis acts on individual cells without causing rupture that could lead to inflammation, (Jurisic and Bumbasirevic, 2008). Apoptotic cells are rapidly phagocytosed and cause no inflammation unlike necrotic cells which lose membrane integrity and release the cytoplasmic contents into the surrounding tissue, causing inflammation (Elmore, 2007). Apoptosis can be activated through the extrinsic pathway (death receptor pathway) or the intrinsic (mitochondrial mediated) pathway (Arunakaran *et al*., 2012). The extrinsic pathway is activated when a ligand binds to tumour necrosis factor (TNF)-related apoptosis inducing ligand or apoptosis-mediating surface antigen (FAS) receptors which lead to activation of the initiator caspases. Initiator caspases 8, 9 and 10 lead to activation of the effector caspase 3, which results

in activation of the Bcl-2 family proteins. Bcl-2 family proteins consist of the pro-apoptotic (Bax, Bak, and Bid) and anti-apoptotic (Bcl-2, Bcl-XL) proteins (Cerella *et al*., 2014). The mitochondrial pathway is activated by external stimuli, including toxins and radiation, that lead to DNA damage causing activation of the Bcl-2 family proteins, in particular, Bid, which leads to the formation of the apoptosomes and activation of caspase 9. Cytotoxic agents, including natural products are suggested to induce apoptosis through the mitochondrial pathway (Pedersen *et al*., 2002). The tumour suppressor gene *p53* regulates the expression and its mutation in most cancers and is linked to their resistance to apoptosis. Natural compounds are known to induce apoptosis by up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins. Some chemotherapy drugs in the market act by inducing apoptosis in cancer cells through various mechanisms.

1.3 Use of traditional medicine for cancer treatment

Despite the many advanced forms of cancer therapy in use today, traditional medicine continues to take center stage in the fight against cancer (Butler, 2004). Herbal medicine is used worldwide by millions of people who use it in different ways for prevention and treatment of a plethora of illnesses. In Africa, it is widely used due to poverty, poor access to health facilities and cultural beliefs. In Kenya, 90% of the population use herbal medicine in different forms (Kareru *et al*., 2007). This is mainly due to poor access to health care facilities and their further influence by cultural beliefs. However, the majority of Kenyans also believe in the effectiveness of traditional medicine and use it in combination with prescribed drugs to treat chronic illnesses. Herbal medicine has been recommended by medical practitioners for patients who have become resistant to chemotherapy and radiation, and to the weak and elderly who may not be able to

handle the chemotherapy side effects (Singh *et al*., 2003). Using herbal medicine in combination with modern chemotherapy may lead to drug interactions, making the conventional drug more or less effective. It is thus important to study the chemical constituents of medicinal plants used and how they may interact with conventional medicine. In Kenya, illnesses such as diabetes, hypertension, cancer, HIV/AIDS and infertility (Kigen *et al*., 2013) are treated using herbal medicine, since it can be easily accessed from herbalists who also provide advice on its use and dosage (Figure 1.2).

A number of plants used as herbal medicine have provided useful drugs in medicine. *Atemesia annua* used traditionally in the treatment of malaria has been studied and the compound atemesin was found as the active compound. The rhizome of *Hypoxis hemerocallidea* in southern Africa is used in the treatment of urinary tract infection. It contains a diglucoside compound called hypoxoside which, upon hydrolysis, produces the aglycone rooperol which is cytotoxic to certain cancer cells and is currently undergoing clinical trials (Drewes, 2012).

Figure 1.2: A typical herbal shop in Nairobi, Kenya advertising herbal remedies for the different disorders. (http://www.nextcity.org)

1.4 Natural products in drug discovery

The need to study natural products as possible anticancer agents is important since they have been used for thousands of years for the treatment and prevention of many diseases and many have been proven to be effective. Moreover natural products have been and continue to be invaluable sources of drug leads and models for the design and synthesis of novel drugs. Natural products and their derivatives represent 60% of all drugs currently in clinical use (Cragg and Newman, 2005). These products, i.e. herbs, spices, fruit and vegetables, are used in various formulations, viz. tonics, powder, extracts or cooked as food to prevent, treat or cure diseases. Recently, the use of plants as therapeutic agents has involved the isolation of active compounds. Drug discovery is an extensive and complex process that is multidisciplinary. The development of a drug from the laboratory to the patient requires the involvement of ethnobotanists, natural product chemists, pharmacologists and clinicians. This process commences with the identification of a plant of interest followed by the isolation and purification of the active compounds through bioassay-guided fractionation. *In vitro,* followed by *in vivo* studies, are carried out, and those compounds that successfully pass this stage move on to clinical trials. This process could take 10 years or more. Despite the recent shift of drug discovery to molecular modelling and combinatorial chemistry, natural compounds remain the single most important source of drug leads and serve as starting points for semi-synthetic drugs (Balunas and Kinghorn, 2005). In the continued fight against cancer, there is a need for more efficient drugs with reduced toxicity, which is a major stumbling block in chemotherapy. The study of the phytochemical composition of these plants is, therefore, important to enlighten the public on the safety and efficacy of herbal medicine, and also for researchers to develop new and improved therapeutic agents. This can be accomplished through the isolation of the active compounds

directly from natural compounds, synthesis of compounds based on the structure of natural compounds or through synthesis of semi-synthetic compounds from natural compounds. Isolation of bioactive compounds began in the early 19th century and since then hundreds of clinically active compounds have been isolated. The plant kingdom has provided us with compounds, mainly secondary metabolites with diverse chemical structures, some of which serve as starting points for synthetic and semi-synthetic drugs. Some of these will be discussed below.

1.5 Plant secondary metabolites

Plant secondary metabolites (PSM) are phytochemicals produced mainly for chemical defense against attack by animals and insects, microorganisms (fungi and bacteria) and defense against other plants competing for nutrients and light but can also serve as a form of chemical store for the plant. These metabolites may or may not be produced by elicitation. An example is the case of vinca alkaloids where fungal proteins trigger increased synthesis and accumulation of the alkaloids (Nagle *et al*., 2006). PSM may have nutritional value but many are anti-nutritional (Acamovic & Brooker, 2005). PSMs are grouped into many classes and can be divided into two main groups i.e. those with nitrogen (N) and those without nitrogen as shown in Table 1.2 below.

Table 1.2: Classification of plant secondary metabolites (Acamovic and Brooker, 2005)

Natural compounds with Nitrogen	**Natural compounds without Nitrogen**
Alkaloids	Terpenoids
Cyanogenic glycosides	Flavonoids
Glucosinolates	Other Polyphenols

1.5.1 Natural compounds containing Nitrogen

1.5.1.1 Alkaloids

Alkaloids are low molecular weight nitrogenous compounds that are produced as the plants defense against pathogens and herbivores. An example is nicotine from tobacco which was one of the first insecticides used. Alkaloids have been used for 1000s of years in medicine and are widely distributed in the plant kingdom (Roberts and Wink, 1998). In 1200 BC in the Middle East, they used Opium latex (*Papaver somniferum*) as a painkiller. This latex contains the alkaloids morphine and codeine (Figure 1.3) which are used presently in medicine as strong analgesics (Balandrin *et al*., 1993).

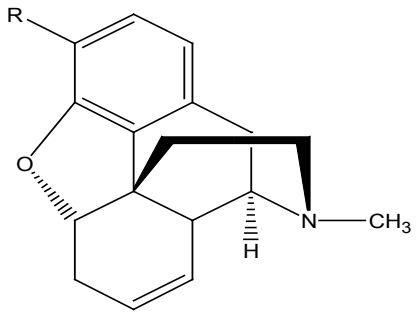


Figure 1.3: Structure of morphine and codeine (Morphine R=OH, Codeine R=OCH_3)

Alkaloids are not only found in plants, but also in frogs, ants, bacteria, and fungi. Quinine is an antimalarial drug that was isolated from *Cinchona officialis* but can also be found in other plants. Sanguinarine (Figure 1.4) is an antibacterial and antiplaque chemical used in toothpaste and was isolated from *Sanguinaria canadensis*. It has been reported that this compound exhibits anticancer potential by inducing apoptosis and cell cycle arrest at different phases, in a number of cancer cell lines. Other alkaloids with similar effects are piperine from *Piper nigrum* and berberine from *Rhizoma coptidis* (Lu *et al*., 2012).

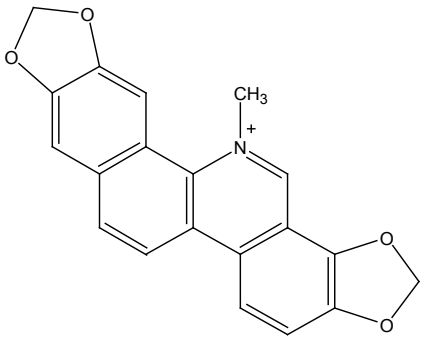


Figure 1.4: Structure of sanguinarine

Vinca alkaloids, vincristine and vinblastine, were among the first natural compounds to be used clinically as anticancer drugs (Cragg and Newman, 2005) and have become lead compounds for anti-cancer drug development.

1.5.1.2 Glucosinolates and Isothiocyanates

Glucosinolates are compounds commonly found in the family Cruciferae with over 100 being identified (Wu *et al*., 2009). The basic structure of a glucosinolate comprises of a β-D-thioglucose group, a side chain and a sulphonated oxime (R-C(=N-O-SO_3^-)-S-β-D-Glucose). They are hydrolysed by the enzyme myrosinase to form thiocyanates, isothiocyanates (R-S-C≡N) or nitriles (Verhoeven *et al*., 1997) (Figure 1.5). This enzyme occurs mostly outside the plant cell wall but also in the intestinal micro flora, and only comes into contact with glucosinolates during processing, viz. cutting, chewing and heating (Wu *et al*., 2009)

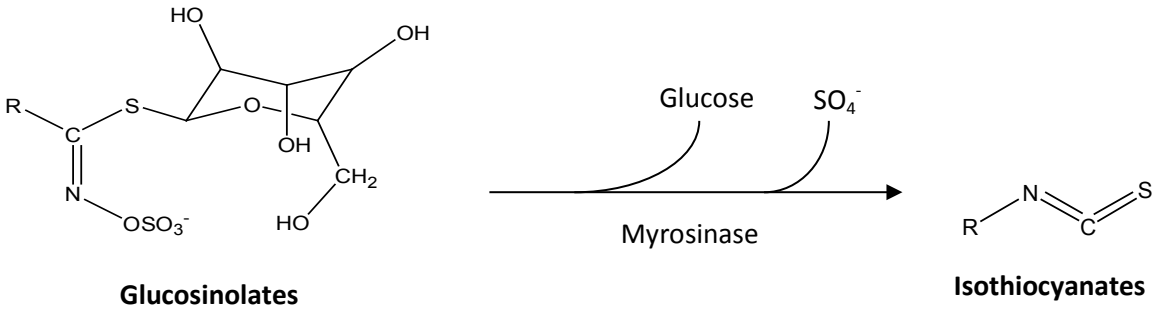


Figure 1.5: Hydrolysis of Glucosinolates to isothiocyanates by myrosinase

Studies suggest that isothiocyanates have the ability to prevent carcinogenesis through modulation of biotransformation enzymes (Zhang, 2011; Royson *et al.,* 2015). DNA can be damaged by many agents such as reactive oxygen species and electrophiles which are activated by phase 1 enzymes and inactivated by phase 2 enzymes. The primary mode of action is suggested to be inactivation of phase 1 (cytochrome P_{450}) enzymes by competitive inhibition or covalent modification (Verhoeven *et al*., 1997) and induction of phase 2 enzymes.

1.5.1.3 Cyanogenic glycosides

These compounds are the most commonly found naturally occurring nitriles and are widely distributed in higher plants, insects, bacteria and fungi (Vetter, 2000). Plants that have cyanogenic glycosides (Figure 1.6) include sorghum, cassava, passion fruit, lotus and plants of the *Prunus* species. Their role in plants is to serve as protection from predators and also as a nitrogen reserve (Fleming, 1999).

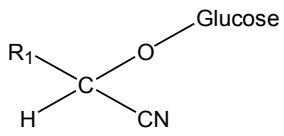


Figure 1.6: Basic structure of a cyanogenic glycoside (R_1- Aliphatic or aromatic group)

These plants can be toxic to humans if the concentration of the glycoside is high. This is because cyanogenic glycosides release HCN on treatment with dilute acids or hydrolytic enzymes (Conn, 1969). HCN is toxic to many organisms and acts by inhibiting the activity of many enzymes by binding to the metal functional group. Amygdalin, a common cyanogenic glycoside, is found in members of the *Prunus* species. Chang and co-workers (2006) established that amygdalin (Figure 1.7) induced apoptosis in LNCaP prostate cancer cells through caspase-3 activation.

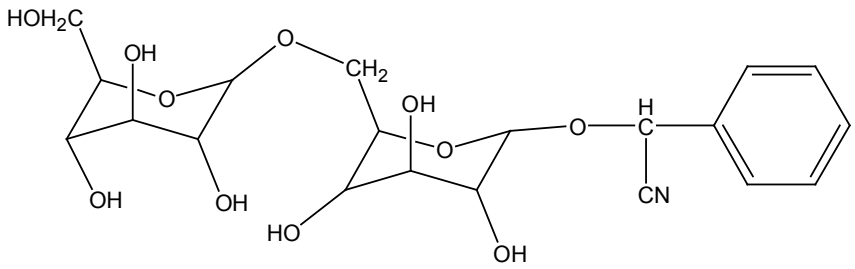


Figure 1.7: Chemical structure of Amygdalin

1.5.2 Natural compounds not containing Nitrogen

1.5.2.1 Terpenoids

Terpenoids belong to the large group of phytochemicals commonly found in plant essential oils forming the largest class of plant natural products. They are also known as isoprenoids because their structure is based on a number of isoprene units (Figure 1.8). They are further classified into different groups according to the number of isoprene units and carbon atoms. Table 1.3 reflects the different classes of triterpenoids.

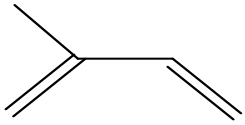


Figure 1.8: Chemical structure of an isoprene unit

Table 1.3: Classes of Triterpenoids

Terpene	Isoprene	No. of carbon
Monoterpene	2	10
Sesquiterpene	3	15
Diterpene	4	20
Sesterterpene	5	25
Triterpene	6	30
Carotenoids	8	40
Rubber	100	500

Some monoterpenes such as thymol and carveol are reported to possess antitumour activity against animal and cell models viz. MCF-7 (human breast adenocarcinoma) and P-815 (murine mastocytoma) cells (Jaafari *et al*., 2012). Phytosterols have been reported to inhibit tumour growth and control common cancers i.e. breast, colon and prostate (Bradford and Awad, 2007).

1.5.2.2 Polyphenols

1.5.2.2.1 Flavonoids

Flavonoids are polyphenolic compounds that form an important part of the human diet. They are present in many dietary plants especially in the fruits and leaves. Flavonoids are made up of a common phenylbenzopyrone structure (C6-C3-C6) (Figure 1.9).

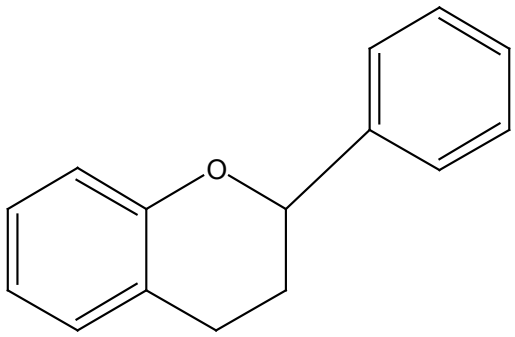


Figure 1.9: Basic chemical structure of a flavonoid

Flavonoids are classified according to their chemical structure and the substitutions and opening of the pyran ring. The major classes include: flavanols, flavonols, flavones, flavonones, anthocyanidins (Figure 1.10), isoflavones, dihydroflavonols and chalcones. Over 4000 different types of flavonoid compounds have been identified and more continue to be identified (Cook and Samman, 1995).

Flavonoids exist mainly as glycosides as this increases their polarity which is necessary for their storage in the plant cell vacuoles.

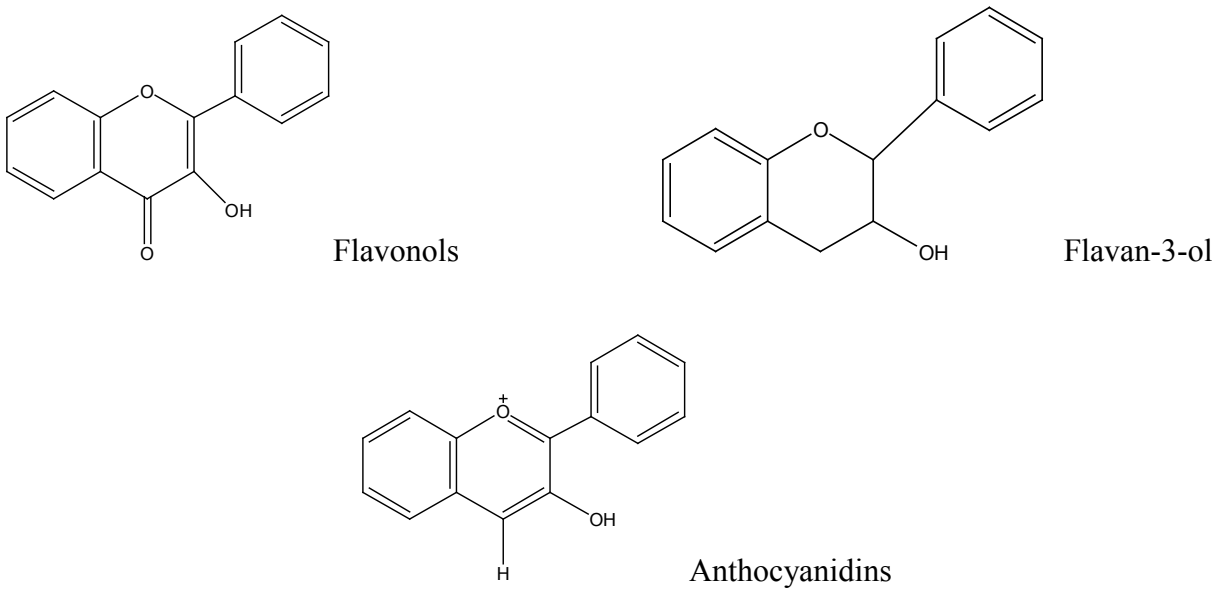


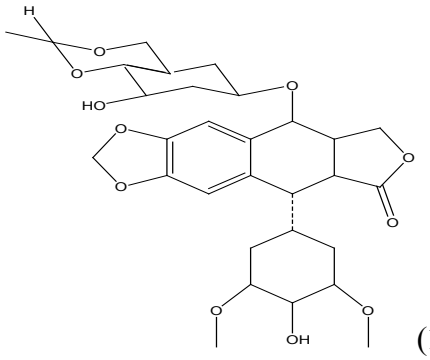
Figure 1.10: Some examples of flavonoids

Flavonoids have been found to reduce the risk and proliferation of cancer. Studies have shown that people with high intake of plant food containing flavonoids have a reduced risk of certain cancer types (Kanadaswami *et al*., 2005). *In vitro* studies have demonstrated the cytotoxicity of various flavonoids to different human cancer cell lines including MCF-7 , HL-60 (leukemia), HT-29 (colon adenocarcinoma) and A-549 (lung carcinoma) (Ren *et al*., 2003; Russo *et al*., 2012). Research has shown that plant flavonoids induce p21 in A-549 cells, resulting in growth arrest and apoptosis. Its antioxidant activity induces transcription of the p21 protein without the need of p53, a common tumour suppressor protein, which is inactivated in 50% of tumours (Galati and O'Brien, 2004).

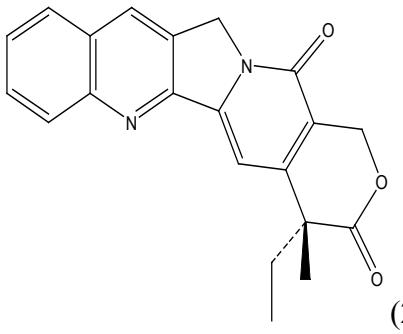
1.6 Plant-derived anticancer drugs and their mechanism of action

Since 1961, several phytocompounds have been approved as anti-cancer drugs (Demain and Vaishnav, 2010). Approximately 60% of anticancer drugs in the market are from natural

compounds or derivatives of it (Cragg and Newman, 2005). With the discovery and development of the vinca alkaloids vincristine and vinblastine from *Catharanthus roseus*, the National Cancer Institute (NCI) launched a collection and screening programme in 1960. This led to the isolation of new cancer drugs viz. taxanes and campothecins which were effective in killing a wide range of cancer cells. Taxol (paclitaxel) was isolated from the bark of *Taxus brevifolia* and is used in treatment of breast, ovarian and lung cancer. Clinically active agents that originated from campothecin (Figure 1.11) and isolated from *Campotheca aciminate* are Topotecan and Ironotecan (Saklani and Kutty, 2008). These are used in the treatment of lung, ovarian and colorectal cancers. Etoposide (Figure 1.11) and tenoposide used in the treatment of bronchial and testicular cancers are semi-synthetic derivatives of epipodophyllotoxin which was isolated from the roots of *Podophyllum peltatum* and *Podophyllum emodi*. Others include Homoharringtonine, a clinically active agent against leukemia isolated from *Cephalotaxus harringtonia* (Chin *et al*., 2006) and Elliptinium from *Bleekeria vitensis* which is used in the treatment of breast cancer (Shoeb, 2006; Cragg and Newman, 2005).



(**1**)



(**2**)

Figure 1.11: Chemical structures of Etoposide (**1**) and Campothecin (**2**)

Several plant compounds have been isolated in recent years and these together with their synthetic derivatives are currently in clinical and pre-clinical development.

Flavopiridol (Figure 1.12), a synthetic flavone, is in phase 1 and 2 clinical trials in combination with other chemotherapy drugs for the treatment of a wide range of tumours. Its structure is based on an alkaloid, rohitukine, which was isolated from *Dyxoxylum binectariferum* (Shoeb, 2006; Cragg and Newman 2005; Galati and O'Brien, 2004).

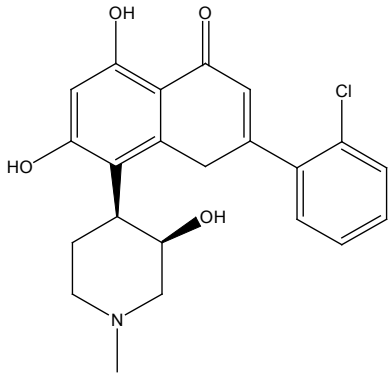


Figure 1.12: Chemical Structure of Flavopiridol

Combrestatins isolated from the South African *Combretum caffrum* are in phase 1 clinical trials (Nathan *et al*., 2012). Several derivatives have been developed from this compound e.g. combrestatin A-4, which has been reported to damage the vasculature of cancerous tumours.

A pentacyclic triterpine betulinic acid isolated from the *Betula* species is reported to have anti-inflammatory and anti-retroviral activity and has shown potential anticancer activity as well (Shoeb *et al.,* 2006). It is still in pre-clinical trials. Triterpenes, oleanolic and ursolic acid, commonly found in many plants, have been reported to possess weak anti-tumour activity. Analogs have been synthesised from these compounds leading to the development of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) (Figure 1.13) which has shown activity over a wide range of tumours (Cragg and Newman, 2005). Others include ixabepilone from *Soragium cellulosam* which is still undergoing clinical trials, and romidepsin from *Chromobacterium*

violaceum which has just been introduced into the market for the treatment of non-Hodgkins T-cell lymphoma (Gordaliza, 2007).

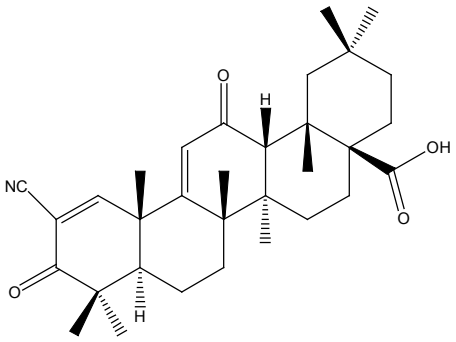


Figure 1.13: Chemical structure of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO)

Most cancer drugs including some natural compounds act at a certain point in the cell cycle. These compounds have different modes of action:

I. **Tubulin binding agents:** Vincristine and vinblastine kill the cells by binding to tubulin, thereby preventing the assembly of the mitotic spindle during metaphase. This inhibits polymerisation of β-tubulin with α-tubulin and stops mitosis (Payne and Miles, 2008). Cell division becomes impossible and the cell dies. Docetaxel and paclitaxel also bind to tubulin (3-subunit) but rather than prevent assembly they stabilise and promote the assembly of microtubules. This promotes polymerisation (Nobili *et al*., 2009).

II. **Topoisomerase inhibitors:** Topoisomerase inhibitors inhibit the unwinding of DNA, thereby preventing replication, repair and transcription (Boik, 2001). Plant-derived chemotherapy drugs that use this mechanism include campothecins (Irinotecan and Topotecan) and epidophyllotoxins (Etoposide and Tenoposide). Campothecins inhibit the activity of topoisomerase 1 by binding to the DNA-enzyme complex and prevents replication by stabilising the covalent bond (Gordaliza, 2007). Etoposide and Tenoposide, on

the other hand, bind to the DNA-topoisomerase 2 complex causing DNA strand breaks and consequently cell death.

1.7 Plants used in this study

1.7.1 *Moringa oleifera*

Moringa oleifera is also known as the drumstick tree or horseradish tree. It is a small to medium-sized rapidly growing tree that is widely cultivated in tropical areas. It is native to India and is found growing wild in the sub-Himalayan regions of northern India. It also flourishes in other regions of Asia, South America and tropical Africa (Pandey *et al*., 2012). In Kenya, it was introduced in the early 1900s by Asians building the railway as recorded in Mohammed Sadiq Cockar's 'Memoirs of the Mombasa to Kisumu Railway' (Odee *et al*., 2001). It is found in the Coastal, Eastern and Western parts of the country. The tree belongs to the family Moringaceace with 13 other species but *M.oleifera* is the most widely cultivated of the family. Other species found in Kenya are *Moringa stenopetala, Moringa arbonea, Moringa borziana, Moringa longituba, Moringa rivae and Moringa ruspoliana.*

Figure 1.14: The *Moringa* tree (Maiyo, 2012)

The *Moringa* tree has been referred to as the miracle tree because of its many uses, especially its use as herbal medicine and a source of food. This is due to the presence of phytochemicals, minerals and proteins in the plant. All parts of this tree are edible and are consumed in different ways. The plant is a rich source of nutrients as it contains high concentrations of vitamins A and C, calcium and iron. The leaves are used as animal feed, in biogas production, as manure and also as a biopesticide because of its antifungal activity (Wadhwa *et al*., 2013; Mishra *et al.,* 2011). The seeds are also used as fertilizer and in water purification and treatment (Emmanuel *et al*., 2011; Mangale *et al.,* 2012). This is due to the active coagulating agent found in the seed and its antimicrobial activity. This attribute has led to efforts encouraging domestication by families in low income areas who do not have access to clean water. The many uses of the *Moringa* tree are listed in Table 1.4.

Table 1.4: Traditional uses of different parts of the *Moringa* tree

Part of plant	**Traditional use**	**References**
Leaves	Biogas production, manure, biopesticide, animal feed, hypocholesterolemic, wound healing, larvicidal	Pandey *et al*., 2012; Wadhwa *et al*., 2013; Maria *et al*.,2011; Mishra *et al*.,2011; Kumbhare *et al*., 2012; Sholapur *et al*., 2013; Odee *et*
Bark	Dye, abortifacient, antifungal, antibacterial	Wadhwa *et al*., 2013; Mishra *et al*.,2011
Root	Diuretic, cardiac tonic, antiarthritic, abortifacient	Pandey *et al*., 2012; Kumbhare *et al*., 2012; Sholapur *et al*., 2013
Pods	Antipyretic, treatment of diabetes, antihelmintic	Wadhwa *et al.,* 2012; Mishra *et al*., 2011
Seeds	Fertilizer, water purification, water treatment, oil used in salads, lubrication, biodiesel, antipyretic	Pandey *et al*., 2012; Wadhwa *et al*., 2012

Most rural communities in Kenya have poor access to healthcare thus rely on medicinal plants including *Moringa*. The species *M.longituba* and *M.Ruspoliana* are used in the treatment of abdominal pains, eye and throat infections and sexually transmitted diseases (Odee *et al*., 2001). Different parts of these plants have been reported to possess anti-tumour and antioxidant activity due to the presence of phenolic compounds (Kumbhare *et al*., 2012).

1.7.1.1 Compounds previously isolated from *Moringa oleifera*

The *Moringa* tree is rich in compounds containing simple sugars. The plant is also rich in nitrile glycosides and glucosinolates which have been isolated from the leaves, seeds and stem bark (Malabed and Noel, 2013). These groups of compounds are unique to the Moringaceae family. Other compounds isolated include vitamins, sterols and carotenes which the plant is rich in. These are listed in Table 1.5 below.

Table 1.5: Compounds isolated from different vegetable parts of *Moringa oleifera*

Part of plant	**Compound isolated**	**References**
Leaf	B-sitosterol, Quercetin, Kaemferol, niazirin, niazirinin, niaziminin A, Niaziminin B, lutein, 4-[(4-O-acetyl-α-L--rhamnosyloxy) benzyl] isothiocyanate	Ragasa *et al*., 2012; Pandey *et al*., 2012; Faizi *et al*., 1994; Pakade *et al*., 2013; Mishra *et al*., 2011; Wadhwa *et al*., 2012
Stem bark	Epilupeol, β-sitosterol, moringine, moringinine, 4-(alpha-L-rhamnopyranosyloxy) benzyl glucosinolate	Maria *et al*., 2011; Mishra *et al*., 2011; Pandey *et al*., 2012; Sholapur *et al*., 2013
Seed	β-sitosterol, β-sitosterol-3-O-β-D-glucopyranoside, 4-(4'-O-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnosyloxy) benzyl isothiocyanate.	Ragasa *et al*., 2012; Oloduro *et al*., 2012; Oloduro *et al*., 2009

1.7.2 *Prunus africana*

Prunus africana, also known as the African cherry or red stinkwood, is an evergreen tree that grows in many parts of sub-Saharan Africa. It belongs to the genus *Prunus* and the family Rosaceae. It has a blackish brown rugged bark with a red brown inner surface. Other trees belonging to this family include *Prunus turfosa, Prunus serotina, Prunus lusitanica and Prunus amygdalus* (Kadu *et al*., 2012). It can be found in the regions extending from Cameroon to Madagascar and Ethiopia to South Africa. In Kenya it is found in the highland regions including Mt. Kenya, Mt. Elgon, Tugen Hills, and Aberdare Ranges (Gachie *et al*., 2012).

1.7.2.1 Medicinal uses

In Kenya, it is traditionally used to treat malaria, stomach ache, fever, breast cancer, prostate cancer, colorectal cancer, and bovine babesiosis. It is also used as an appetite stimulant (Gachie *et al*., 2012; Kareru *et al*., 2007). Its crude extract is used worldwide in the treatment of benign prostatic hyperplasia (BPH) commonly found in older men which can go on to become life threatening cancer (Gathumbi *et al*., 2000). This is achieved through the reduction of oedema and inflammation of the prostate. This has been attributed to the presence of phytosterols, especially β-sitosterol and pentacyclic triterpenes, in the plant extract (Stewart, 2003). Phytosterols in the plants have been reported to reduce vessel permeability, hence reducing inflammation which correlates to their reported anti-inflammatory activity (Kadu *et al*., 2012). This activity is further enhanced by the presence of pentacyclic triterpenes which also have been reported to have anti-inflammatory and immunostimulatory activity. The bark contains the highest concentration of phytosterols and triterpenes. Its use in the treatment of BPH, especially in Europe and the USA, has led to massive harvesting of the bark of this tree which is sold in

local and international markets for profit (Shenouda *et al*., 2007). Pharmaceutical companies in Europe and America are manufacturing drugs for prostate illnesses using the bark of *Prunus africana* as a raw material which has led in uncontrolled harvesting of the bark for export. Due to this overexploitation, the tree is currently facing extinction because of massive debarking (Figure 1.15) and export overseas (World Conservation and Monitoring Center, 1998).

Figure 1.15: Debarking of *Prunus africana* (http://www.worldagroforestry.org)

Several important compounds have been isolated from *Prunu*s species (Table 1.6).

Table 1.6: Compounds isolated from *Prunus africana*

Part of plant	**Compounds isolated**	**References**
Leaves	β-Sitosterol, β-sitosterol-3-O-glucoside, n-docosanol,	Kadu *et al*., 2012; Mugaka *et al*., 2013.
Stem bark	Sitosterol, sitosterol-3-O-glucoside, n-docosanol, ursolic acid, ferulic acid, myristic acid, oleanolic acid, campesterol.	Kadu *et al*., 2012; Catalano *et al*., 1984; Mugaka *et al*., 2013.

1.8 Phytochemical studies

Phytochemical studies involved the use of different analytical techniques in the purification and characterisation of plant natural compounds. Chromatographic techniques are used in the purification of compounds while spectroscopic techniques are used for characterization and structural elucidation of isolated compounds. Plant compounds are extracted as crude extracts using solvents with varying degrees of polarity. Solvents used for extraction from the less polar to the most polar are hexane, dichloromethane (DCM), ethyl acetate, and methanol (MeOH) which extracts certain secondary metabolites based on their polarity. Chromatographic techniques commonly used for separation and purification are column chromatography and thin-layer chromatography (TLC). Other chromatographic techniques used for identification are Gas Chromatography-Mass Spectrometry. When a pure compound has been obtained, different spectroscopic analyses are used to elucidate the structure and to characterize the compound. These include nuclear magnetic resonance (NMR), ultraviolet-visible spectroscopy (UV-Vis) and infra-red (IR) spectroscopy. These techniques were used to achieve objectives 1 and 2 of this research and are further discussed below.

1.8.1 Chromatographic techniques.

Chromatography is a method of separation that distributes and separates components of a mixture in a stationary and a mobile phase. The stationary phase usually used is silica gel, alumina or sephadex and the mobile phase can be a liquid (organic solvents) or an inert carrier gas. The components are carried by the mobile phase through a stationary phase and are separated based on polarity, size and retention time depending on the type of chromatography used.

1.8.1.1 Column chromatography

In column chromatography, the stationary phase is packed in a glass column and the mobile phase passes through the stationary phase by moving down the column by gravity, thereby eluting different components of the mixture. Columns of different sizes are used depending on the amount of sample to be purified. The column is wet or dry packed with a stationary phase, commonly silica gel or sephadex LH20. Thereafter, the components to be separated are loaded onto the column and a suitable solvent system is added as the mobile phase to elute compounds. Solvents are used starting with the least polar (which elutes non-polar compounds) to the most polar (which elutes polar compounds). When using sephadex as the stationary phase only one polar solvent is used as separation is due to size and not polarity. This is used to purify polar compounds. Components that partition in the stationary phase stay longer in the column and are separated from those that are eluted in the mobile phase.

Figure 1.16: Columns used to purify crude extracts

1.8.1.2 Thin-layer chromatography (TLC)

In thin-layer chromatography (TLC) the stationary phase (silica gel, alumina and cellulose) is supported on a flat surface that can be glass or aluminum. The components to be separated are spotted on the surface of the stationary phase and developed in a chromatography tank. The mobile phase moves through the chromatographic plate by capillary action, thereby separating the compounds. Compounds are separated depending on their interaction with the mobile phase, similar to column chromatography.

Generally, fractions collected from column chromatography are spotted on a TLC plate and placed in a developing tank with an appropriate solvent system. Observation of the compounds is facilitated by viewing under a UV lamp and also with a spraying reagent. Compounds are identified based on their Rf values. A single spot, as shown in Figure 1.17, usually indicates a pure compound.

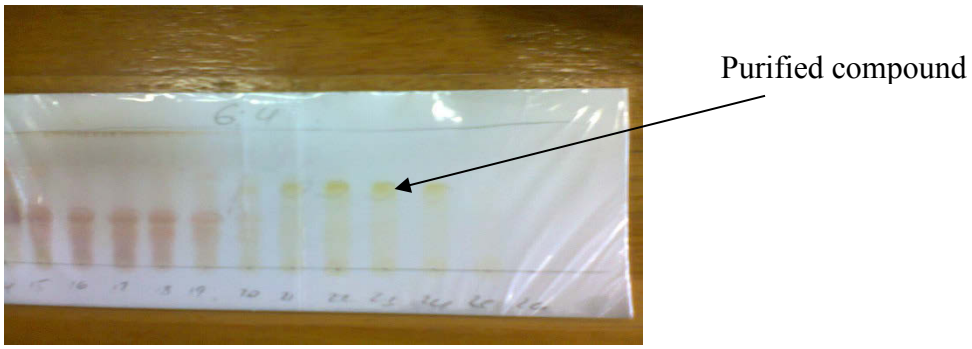


Figure 1.17: Thin layer chromatography used for identification of a pure compound.

1.8.1.3 Gas-chromatography-mass spectrometry (GC-MS)

GC-MS is a combination of two techniques, gas chromatography and mass spectroscopy. It is extensively used in drug testing, identification of environmental contaminants and in phytochemistry where it has found application in metabolic fingerprinting of plants (Jung *et al*., 2011). GC-MS is used for the separation of components in a mixture and gives a spectral output, which aids in structural elucidation and identification. The column is made of glass or stainless steel and houses the stationary phase which is made up of a polymer, mainly fused silica. The mobile phase is an inert gas e.g. helium, hydrogen, or nitrogen. The sample is injected into the column and is transported by the carrier gas through the stationary phase where different compounds interact differently with the stationary phase and are eluted at different times from the column (Figure 1.18). The time a compound takes to be eluted from the column is called the retention time. Different compounds have different retention times. Upon elution the compound goes into the ionisation chamber of the mass spectrometer and is bombarded by a string of electrons causing it to break into fragments forming a fragmentation pattern that is unique for every compound. Different compounds have different fragmentation patterns and this coupled with retention time acts as the fingerprint of a compound. GC-MS can be used to separate polar and non-polar components with high reproducibility (Lee *et al*., 2013). The combination of mass spectrometry in the presence of huge data libraries makes GC-MS an extremely efficient tool in separation with a powerful detection system. Weller (2012) referred to GC-MS as unrivalled in its efficiency and sensitivity.

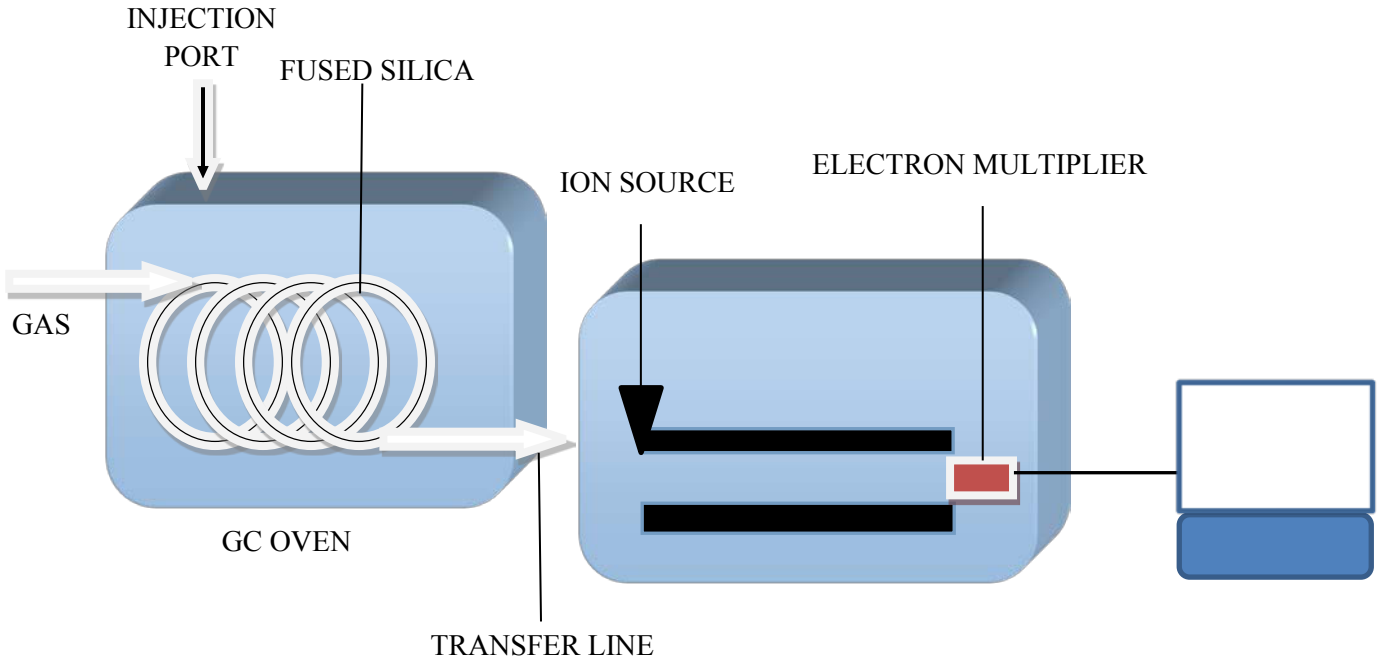


Figure 1.18: Schematic diagram of a GC-MS equipment

1.8.2 Spectroscopic analysis

1.8.2.1 Nuclear magnetic resonance (NMR) spectroscopy

This is a spectroscopic technique for determining the molecular structure of organic compounds through the analysis and interpretation of the spectrum. It is used for analysing known and unknown compounds. All atoms have nuclei which are electrically charged. When the nuclei are exposed to a magnetic field, they shift from a lower energy level to a higher energy level and back, releasing energy. This energy transfer takes place at a wavelength that corresponds to a radio frequency and the computer displays this output as an NMR spectrum. The magnetic field on a nucleus is also affected by the magnetic field on a neighbouring nucleus, causing an effect known as coupling which leads to splitting of the signal into two or more peaks. The resonance frequency of a compound depends on its chemical environment. Thus, a more electronegative

atom would result in a higher frequency. This frequency is represented as a chemical shift in parts-per-million (ppm) on the NMR spectrum. Chemical shifts in ^{1}H-NMR are in the range of 0-12 ppm and 0-220 ppm for ^{13}C-NMR.

To elucidate the structure of a compound, 1D NMR (^{1}H-NMR, ^{13}C-NMR and DEPT 90/135) is carried out. To obtain additional information especially for complex spectra that are difficult to analyse, 2D NMR is carried out. This includes ^{1}H-^{1}H Correlation spectroscopy (COSY) which shows correlations between coupled protons, and Nuclear Overhauser Enhancement Spectroscopy (NOESY) which show correlations through space, Heteronuclear Multiple Bond Correlations (HMBC) that identify H-C coupling that occurs 2-3 bonds away, and Heteronuclear Single Quantum Coherence (HSQC) that identifies H-C bonding in a molecule. A disadvantage of this chemical structural analysis tool is its lack of sensitivity (Weller, 2012).

1.8.2.2 Other spectroscopic techniques

Ultraviolet-visible light spectrometry uses radiation to identify functional groups in organic compounds. In UV-Vis spectroscopy, UV light passes through the compound and energy from the light promotes the electron to a higher energy state. It works at a range of 200 nm to 800 nm. Light absorbing molecules in a compound are known as chromophores and the wavelength of maximum absorption of a compound depends on these chromophores. Different compounds absorb light at different wavelengths depending on their functional groups present, which aid in their identification.

Infra-Red spectroscopy is a qualitative method of analyzing compounds that aid in the analysis of its structure and chemistry. This is accomplished through the recognition of characteristic

shapes and patterns that arise from bond vibration in organic compounds and by using this information together with other physico-chemical data of the sample obtained (Coates, 2000). In IR spectroscopy, the compound is exposed to infra-red frequencies and some are absorbed by the compound. The frequency absorbed causes this energy to excite bond vibrations in the molecule to a higher energy state which will be different in different compounds. The amount of frequency not absorbed and passes through is measured as percentage transmittance.

1.9 Cell culture

Cell culture is the propagation of cells *in vitro* under conditions closely resembling that of an *in vivo* system. This is a vital tool in cellular and molecular biology and is used to study the normal cell physiology and biochemistry *in vitro*. Cell culture has found wide application in the study of the effects of drugs and cytotoxic compounds on specific cell types. It is indispensable in drug screening and development on a larger scale due to its reproducibility and consistency. It is also used in the manufacture of viral vaccines and in the production of biological products such as enzymes and hormones.

In mammalian cell culture, the cells are commonly isolated from blood or soft tissue. This is done by enzymatic or mechanical means. Cells used are mostly immortalised cells, meaning they can replicate indefinitely. Immortalisation is achieved either through random mutation, e.g. in HeLa cell line or deliberate modification e.g. in the HEK293 cell line. Immortalisation enables the propagation of cells *in vitro* for an indefinite period.

Culture conditions vary for each cell type. Cells require a suitable vessel containing medium with sufficient nutrients (amino acids, carbohydrates, vitamins and minerals), growth factors and

hormones. Commonly used media in the lab include Dubelco's Minimum Essential Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM) which are commercially available and are normally supplemented with glutamine, antibiotics viz. streptomycin and penicillin as well as serum. Prepared media must be refrigerated before use at 4 ºC. Strict adherence to aseptic techniques must be observed at all times. Generally, the cells are incubated at 37 ºC temperature in CO_2 in a humidified incubator. When cells reach confluency (Figure 1.19), they are usually sub-cultured/split into other vessels to keep them at the log phase of growth or alternatively suitably plated for cell culture assays. Adherent cells can be detached enzymatically using trypsin. At this point, they can be plated and the cell numbers determined using a cell counter or a hemacytometer. Thereafter, plant extracts, or other test compounds can be added at different concentrations. Cells can be stored for future use by freezing them in a cryoprotective reagent e.g. DMSO at -130 °C. Freezing of cells is normally done gradually with a 1 ºC drop in temperature till a temperature of -80 ºC, thereby preventing formation of ice crystals and cell damage (Phelan, 1998).

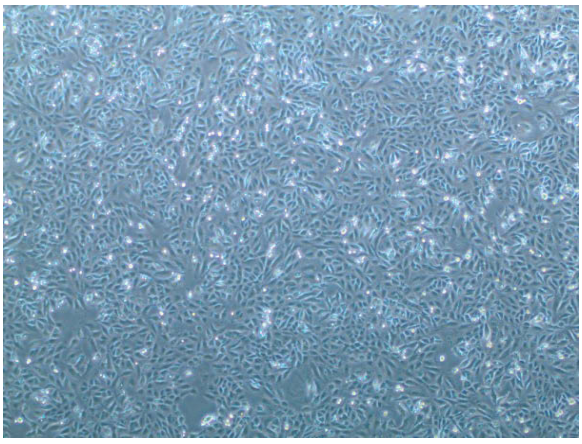


Figure 1.19: Cells at confluency (http://www.commons.wikimedia.org)

1.9.1 Cell lines used in this study

1.9.1.1 HEK293

These are human embryonic kidney cells generated 30 years ago by exposure to the human adenovirus type 5 to the cells chromosome. This modification was carried out by Graham and his team in 1977 (Thomas and Smart, 2005). This cell line is mostly used in the production of recombinant proteins but is also suitable in drug discovery studies. The greatest challenge in chemotherapy is non-selectivity of the drugs. Chemotherapy kills both cancer cells and normal actively dividing cells, including red and white blood cells. This may lead to adverse side effects including immunosuppression, which could cause severe infection, leading to death. It is thus very important to try and find selective drugs that kill cancer cells without killing normal healthy cells. In this study, HEK293 cell line has been used as the control non-cancer cell line.

1.9.1.2 Caco-2

This is a colorectal adenocarcinoma cell line obtained from a 72 year old male. Lifestyle and in particular, diet is the major risk factor for colorectal cancer. Other risk factors are genetics and old age (Hamilton *et al*., 2000). This is the third most commonly diagnosed cancer in the world with a higher occurrence in developed countries (Aggarwal *et al*., 2013). However, with the changing lifestyle, especially the adoption of western diet in developing countries, cases of people diagnosed with colorectal cancer have increased in recent years. The type of diet can either prevent or accelerate the incidence of colorectal cancer. This cell line is used as a model for colorectal cancer in this study.

1.9.1.3 HepG2

This is a hepatocellular carcinoma cell line obtained from a 15 year old adolescent. It has an epithelial morphology. It is an excellent transfection host and is widely used in studies involving Hepatitis B, hepatocellular carcinoma (HCC) and other liver disorders. It is hence indispensable in human liver cancer research and in drug delivery. Liver cancer is a leading cause of death worldwide, with the risk factors being the Hepatitis B (HBV) and Hepatitis C (HCV) viruses (El-Serag, 2012). Other risk factors include obesity, alcohol consumption and aflatoxin B intake. Occurrence is higher in men mainly due to lifestyle differences as they are more exposed to risk factors like smoking and alcohol intake (El-Serag *et al.*, 2010). The highest incidence of liver cancer is in southeast Asia and western and eastern Africa. This has been attributed to the intake of aflatoxin B in contaminated grain. The high prevalence of HIV in developing countries has also been thought to increase the incidence of HCV and HBV (Venook *et al*., 2010). In this study, the HepG2 cell line is used to study the effects of the plant compounds isolated on the cells *in vitro* as a model for liver cancer.

1.9.2 *In vitro* anti-cancer screening

In vitro anti-cancer screening has been used for a long time by researchers as a rapid tool in screening natural and synthetic compounds for drug development. It is a major approach for the search of potential anti-cancer agents (Houghton *et al*., 2007). Cellular assays are the most commonly used for *in vitro* cancer screening because they are inexpensive, simple and rapid (Atta-ur-Rahman *et al*., 2005). These assays provide a way of rapidly screening a large number of compounds for potential activity. In these assays the rate of cell growth is measured by the formation of a colour change which is directly proportional to the number of viable cells. There

are a number of cellular assays that can be used, but the two most commonly used in research for anti-cancer studies are the Sulforhodamine B (SRB) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. These assays are very specific and, therefore are used often when studying the activity of plant compounds. In this study, we utilized the MTT assay in our cytotoxicity assessment of compounds isolated.

1.9.2.1 MTT assay

The MTT assay is used in many laboratories worldwide due to its efficiency and reproducibility (Rubinstein *et al*., 1990). The principle of this assay is based on the succinate-tetrazolium reductase pathway in the mitochondria of living cells (Salati *et al*., 2013). This assay measures the metabolic activity of cells by their ability to reduce the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan using the succinate-tetrazolium reductase system. Viable cells with intact mitochondria use oxidised substrates and coenzymes, including NADPH and NADH, which reduce the formazan salt from yellow to pink (Figure 1.20) (Vistica *et al*., 1991). Cells at confluency are plated on 24, 48 or 96 well plates and are incubated for 24 hours to allow for adherence of the cells to the plate. The compounds or extracts to be studied are then added and the cells incubated for 48 hours. Thereafter, MTT is added and the cells incubated for 4 hours. Formazan crystals accumulate in living cells. A suitable chemical such as DMSO is added after 4 hours to dissolves the formazan, producing a pink/violet colour which can be determined spectrophotometrically using a multiplate reader at 570 nm.

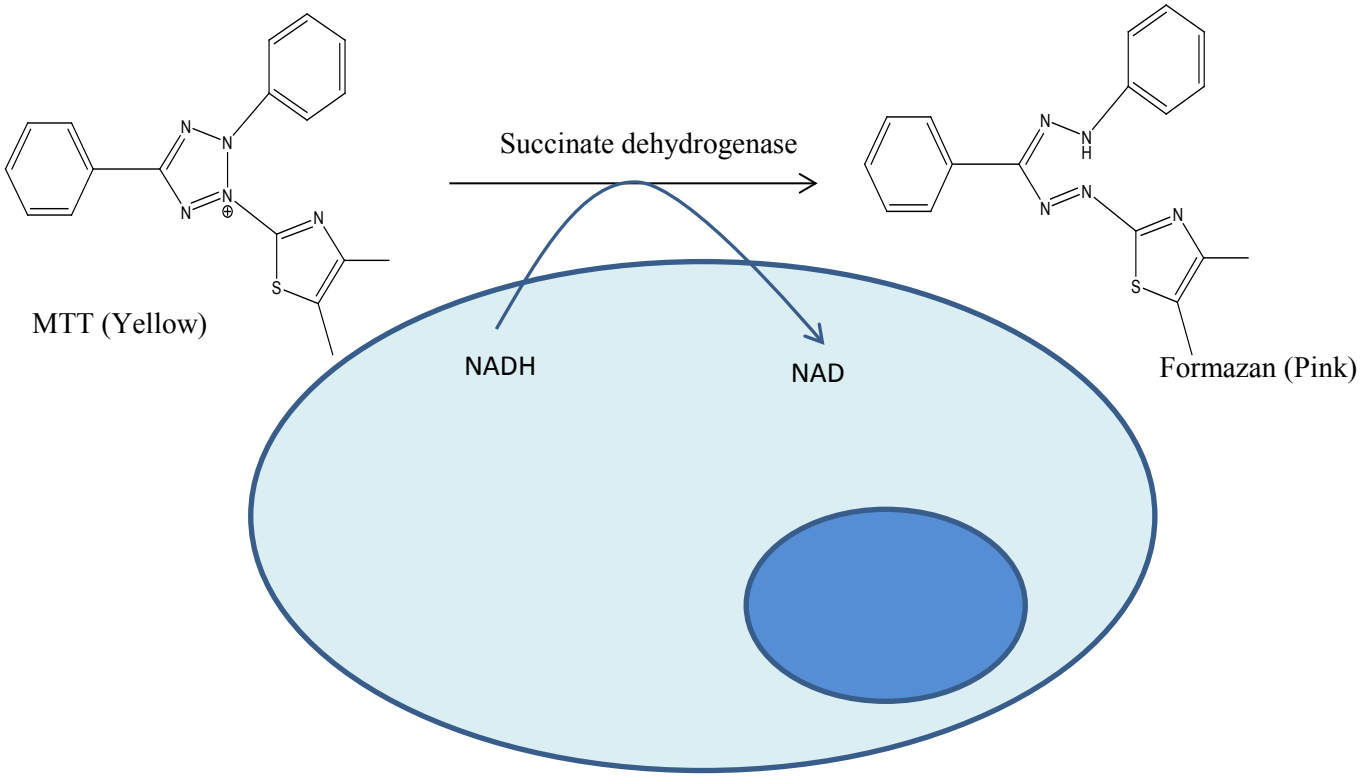


Figure 1.20: Reduction of MTT to a pink formazan salt by cellular dehydrogenases

IC_{50} value is the most commonly used parameter for measuring cytotoxicity *in vitro*. This is the inhibitory concentration described as the concentration at which the possible cytotoxic agent attains 50% death. We have represented our cytotoxicity results in form of IC_{50} values which were calculated on Microsoft Excel 2010™.

1.9.2.2 Acridine Orange/Ethidium Bromide (AO/EB) Apoptosis Assay

Tumour cells escape apoptosis because of gene mutation that causes production of proteins that inhibit apoptosis. This inhibition is a known characteristic of cancer cell growth and metastasis (Jurisic and Bumbasirevic, 2008). Induction of apoptosis is thus used as an important indicator of

the ability of chemotherapeutic agents to inhibit tumour growth and progression (Darakshan and Ghanbari, 2013). Apoptotic cells can be examined under a fluorescence microscope by staining and studying morphological changes of the cells. Morphological features of apoptotic cells include cell shrinkage, condensed chromatin, and fragmentation of the nucleus (Sumathi *et al*., 2013; Attari *et al*., 2009; Meshkini *et al*., 2009)

The AO/EB assay is used to study apoptotic cells *in vitro* by means of fluorescence. Changes in cellular and nuclear morphology, characteristic of apoptosis, are studied under a fluorescent microscope (Elmore, 2007). The assay is both qualitative and quantitative. Acridine orange and ethidium bromide bind to DNA and RNA by intercalation between adjacent base pairs causing the helical structure to stretch. This binding enhances its fluorescence intensity (Nafisi *et al*., 2006). Acridine orange stains live and dead cells while ethidium bromide stains dead cells only. Live cell appear green under the microscope and early apoptotic cells have a bright green nucleus due to chromatin condensation and nuclear fragmentation. Late apoptotic cells appear orange because of taking up ethidium bromide and necrotic cells will stain orange but with a normal nuclear morphology (Khasibhatla *et al*., 2006). The cells can be counted under the microscope and the apoptotic index calculated.

1.10 Antioxidants

Antioxidants are compounds that donate electrons to prevent electron loss from other compounds such as fatty acids and DNA to free radicals. They may also be referred to as free radical scavengers. Free radicals, also known as reactive oxygen species (ROS) contain an uneven number of electrons and gain or lose an electron to other compounds, thereby making them free radicals. The generation of free radicals in the body leads to oxidative stress which may cause

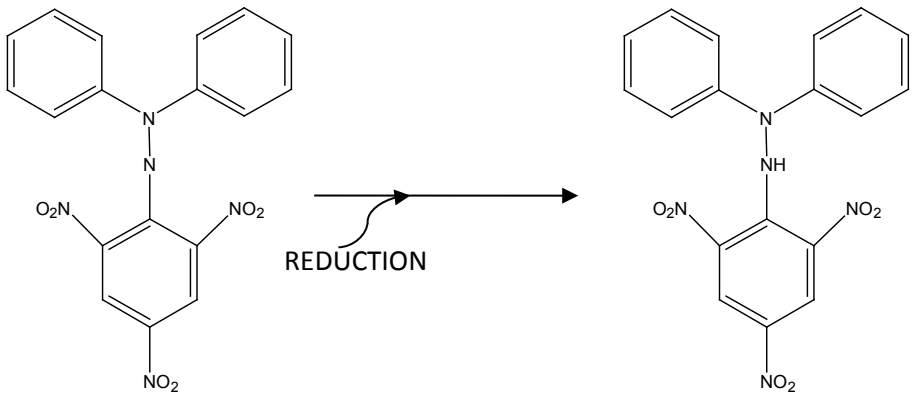
diseases like cancer, cardiovascular diseases and aging (Boik, 2001). Free radicals damage cellular DNA which may result in cancer development. This occurs frequently in the cell but is generally repaired by cellular mechanisms

ROS can be produced in different ways, including immune cell activity and cellular respiration. Environmental and social factors may also lead to the production of ROS e.g. smoking and radiation. The body has its own antioxidants like enzymes catalase and superoxide dismutase, but due to the continuous production of free radicals it must obtain enough antioxidant supply through the diet. Polyphenolic compounds, especially flavonoids, have been reported to possess high antioxidant activity (Khanduja and Bhardwaj, 2003) and are sometimes used as standards in antioxidant studies. These compounds are found in a wide variety of fruits and green leafy vegetables.

1.10.1 Antioxidant assays

The 2,2-diphenyl-β-picrylhydrazyl (DPPH) and the ferric reducing antioxidant potential (FRAP) assay are the two most commonly used methods of testing for antioxidant activity *in vitro* (Prior *et al*., 2005).

The DPPH assay is a simple, inexpensive assay based on the reduction of DPPH, a stable free radical by test compounds. Antioxidants react with DPPH which becomes paired off in the presence of a hydrogen donor producing DPPH-H. DPPH which is deep purple in colour gives a strong absorption maximum at 517 nm and changes to a yellow colour upon reduction by a strong antioxidant (Figure 1.21). Some of the standards usually used in this test include trolox, ascorbic acid, uric acid and α-tocopherol.



DPPH (purple) DPPH-H (yellow)

Figure 1.21: Reduction of DPPH (purple) by antioxidants to DPPH-H (yellow).

The level of decolourisation depends on the number of electrons donated (Rajesh and Natvar, 2011). The absorbances obtained are used to calculate the percentage scavenging ability of a compound using the following equation:

% scavenging = [Absorbance of control-Absorbance of sample/Absorbance of control] *100

1.11 Aim and objectives

The main aim of this study was to conduct a phytochemical investigation of two plant species, namely *Moringa oleifera* and *Prunus africana* that are commonly used to treat cancer in Kenya and to further determine the biological activity of the isolated compounds with focus on anti-cancer activity. The specific research objectives were:

1. To extract and purify compounds from different parts of the plants by means of column and thin layer chromatography (TLC).

2. To identify and characterize the compounds isolated using spectroscopic techniques viz. NMR, GC-MS, UV-Vis and IR.
3. To test the antioxidant activity of isolated compounds using the DPPH method.
4. To test the isolated compounds for cytotoxicity on selected mammalian cancer cell lines viz. HepG2, Caco-2 and non-cancer cell line HEK293.
5. To study the apoptosis induction of the compounds isolated on selected cell lines using the acridine orange-ethidium bromide dual staining method.

1.12 Outline of thesis

This thesis is written in the format of two papers and is divided into four chapters. Chapter 1 is the introduction and literature survey.

Chapter 2 is presented in the form of a research paper. The title of the paper is **'**Cytotoxicity, antioxidant and apoptosis studies of quercetin-3-O-glucoside and 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate from *Moringa oleifera'* and it addresses objectives met from the study of the first plant *Moringa oleifera.*

Chapter 3 is also presented in the form of a research paper. The title is **'**Phytochemistry profiling, cytotoxicity and apoptosis study of β-sitosterol-3-O-glucoside and β-amyrin from *Prunus africana'* and addresses the objectives met, based on results from the study of the second plant *Prunus africana*.

Lastly chapter 4 provides a summary of the discussion and concluding remarks based on the entire study. Recommendations for further studies are also outlined in this chapter.

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CHAPTER 2

CYTOTOXICITY, ANTIOXIDANT AND APOPTOSIS STUDIES OF QUERCETIN-3-O GLUCOSIDE AND 4-(β-D-GLUCOPYRANOSYL-1→4-α-L-RHAMNOPYRANOSYLOXY)-BENZYL ISOTHIOCYANATE FROM *MORINGA OLEIFERA*

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Abstract

Of all the potent anticancer drugs currently available in the market, over 60% of them are of natural origin. *Moringa oleifera,* from the family Moringaceae, is used as a source of vegetable and herbal medicine and in the treatment of various cancers in many African countries including Kenya. The present study involved the phytochemical analyses of the crude extracts of *M.oleifera* and biological activities (antioxidant, cytotoxicity and induction of apoptosis *in vitro*) of selected isolated compounds. The compounds isolated from the leaves and seeds of the plant were quercetin-3-O-glucoside, 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate, lutein and β-sitosterol. Antioxidant activity of quercetin -3-O-glucoside was significant compared to that of the control while 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate showed moderate activity. The cytotoxicity of 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate and quercetin-3-O-glucoside were tested in three cell lines, viz. hepatocellular carcinoma (HepG2), colon carcinoma (Caco-2) and a non-cancer cell line, Human Embryonic Kidney (HEK293) using the MTT cell viability assay. Apoptosis studies were carried out using the acridine orange/ethidium bromide dual staining method. The compounds isolated showed selective *in vitro* cytotoxic and apoptotic activity against human cancer and non-cancer cell lines, respectively. Quercetin-3-O-glucoside showed significant cytotoxicity against the Caco-2 cell line with an IC_{50} of 79 μg mL^{-1} and moderate cytotoxicity against the HepG2 cell line with an IC_{50} of 150 μg mL^{-1} while 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate showed significant cytotoxicity against the Caco-2 cell line and HepG2 cell line with an IC_{50} of 45 μg mL^{-1} and 60 μg mL^{-1}, respectively. Comparatively both compounds showed much lower cytotoxicity against the HEK293 cell line with IC_{50} values of 186 μg mL^{-1} and 224 μg mL^{-1}, respectively.

Keywords: anti-cancer activity, quercetin -3-O-glucoside, 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate

2.1. Introduction

Cancer is a leading cause of mortality worldwide with over 7.6 million deaths reported in 2008 alone (American Cancer Society, 2011) and 7 out of 10 deaths occurring in low and middle-income countries. Medicinal plants have been in use in many cultures around the world for thousands of years to cure and prevent several diseases, including cancer. In Kenya, the majority of the population in rural and urban areas use traditional medicine to treat illnesses ranging from stomach ache and HIV/AIDS to cancer (Wagate *et al*., 2008). Plants possess activity against several known diseases, mainly due to the presence of bioactive compounds. A large number of bioactive plant compounds have been isolated and are commercially available, with over 60% of them being employed as anti-cancer drugs. These include Vincristine and Vinblastine isolated from *Catharanthus roseus* and paclitaxel (Taxol) isolated from *Taxus brevifolia* (Cragg and Newman, 2005).

Moringa oleifera, from the family Moringaceace, is a fast growing tree indigenous to India, and is believed to have been introduced to Kenya in the early 1900s by the Asians (Odee *et al*., 2001). In Kenya, the *Moringa* tree is mainly used as a source of vegetable and herbal medicine leading to it often being referred to as the miracle tree because of its versatility. The plants nutritional and medicinal value is largely due to the presence of essential phytochemicals, minerals, proteins and vitamins. All parts of this tree are edible and are consumed in different ways. The plant is a rich source of nutrients, containing high concentrations of vitamins A and C, calcium and iron. The leaves are used as animal feed, in biogas production, as manure and also

as a biopesticide due to its antifungal activity (Wadhwa *et al*., 2013; Mishra *et al*., 2011). The seeds are used as fertilizer, and in water purification and treatment owing to the active coagulating agent found therein and due to its antimicrobial activity (Dalen *et al*., 2009; Gamila *et al*., 2007). The *Moringa* tree is the main source of medicine for some rural communities in Kenya. All parts of the plant are used in traditional medicine to treat a plethora of diseases including abdominal pains, eye, throat and skin infections, sexually transmitted diseases and cancer (Odee *et al*., 2001).

Crude extracts of the leaves and seeds have been shown to possess cytotoxic and antioxidant activity (Mishra *et al*., 2011; Wadhwa *et al*., 2013; Pandey *et al*., 2012). Compounds previously isolated from this plant include phenolic compounds, nitrile glycosides, sterols and alkaloids. Apoptosis is a term coined by Kerr *et al* (1972) referring to programmed cell death. This process is defective in most cancers and is a valuable drug target in cancer research. Sreelatha *et al* (2011) studied the anti-proliferative activity of the *Moringa* leaf extract on human nasopharyngeal carcinoma (KB) cells and reported apoptosis as a possible cause of cell death.

The antioxidant activity of the leaf extracts of this plant were reported to be comparable to those of reference antioxidants (Wadhwa *et al*., 2013). This is attributed to the presence of high quantities of flavonoids and other polyphenols in the leaves. Antioxidants are important scavengers of free-radicals that are the main cause of oxidative stress in the body. Oxidative stress is believed to contribute significantly to the causation of a number of diseases including cancer mainly through DNA damage (Chandra *et al*., 2000) and hence plays a major role in the pathophysiology of carcinomas (Fuchs-Tarlovsky, 2013). Flavonoids are polyphenolic compounds with potent free radical scavenging ability and their biological activity has been related to this activity (Kumbhare *et al*., 2012). Strong antioxidants have been reported to cause

cell death *in vitro* and enhance the activity of chemotherapy drugs (Boik, 2001), causing improved patient remissions by reducing oxidative stress caused by conventional chemotherapy drugs.

Novel approaches for cancer chemo-prevention and therapy are of great importance to overcome the challenges associated with modern chemotherapy. In this study, we isolated and identified compounds through chromatographic and spectroscopic techniques. Compounds of interest were tested for antioxidant, cytotoxic and apoptosis induction in cancer and non-cancer mammalian cell lines. Our research sought to identify compounds from *M. oleifera* with possible anticancer activity that would lead to the production of potential drug candidates.

2.2 Materials and methods

2.2.1 General experimental procedures

The solvents used for extraction and chromatography were of analytical reagent grade and were supplied by either Sigma or Merck chemical companies. Silica gel was used for column chromatography and pre-coated aluminium plates for thin layer chromatography (TLC). Both procedures were conducted at room temperature. Compounds were visualized on TLC under UV light using a H_2SO_4 in methanol (MeOH) spray reagent. 1D (^{1}H and ^{13}C) and 2D (HMBC, HSQC and COSY) NMR spectra were recorded at 400 MHz or 600 MHz on a Bruker AVANCE III spectrometer with tetramethylsilane (TMS) as the internal standard. The IR spectra for identification of functional groups in the compound were obtained on a Perkin Elmer Spectrum 100 FT-IR spectrometer with a universal ATR sampling accessory. UV spectra were obtained on a UV-Vis-NIR Shimadzu UV 3600 spectrophotometer.

The cell lines used were human cancer cell lines HepG2 (human hepatocellular carcinoma) and Caco-2 (human colon carcinoma) and HEK293 (human embryonic kidney) cells as the control. The HEK293 cells were obtained from the Anti-Viral Gene Therapy Unit, University of Witwatersrand, and the Caco-2 and HepG2 cells were purchased from Highveld Biological, Lyndhurst, South Africa.

2.2.2 Plant material

Fresh leaves, stems, bark and seeds were collected in Maseno, Kisumu County, Kenya. The plant was identified at the Kenya Forestry Research Institute (KEFRI). The plant material was air-dried in the shade for 6 weeks and ground into a fine powder.

2.2.3 Extraction and purification

Dried and ground samples of leaves (643.24 g), bark (580.60 g) and seeds (372.89 g) were sequentially extracted with hexane, dichloromethane (DCM) and MeOH for 48 hours on an orbital shaker at room temperature. The collected extracts were concentrated in a rotary evaporator to obtain the crude extracts which were then subjected to column chromatography using silica gel (Merck Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM) as the stationary phase and different solvent systems as the mobile phase. Collected fractions were monitored by TLC. Prior to column chromatography, the aqueous MeOH extract (139.64 g) was further partitioned in triplicate with equal volumes (200 mL) of DCM and then ethyl acetate to produce a DCM fraction (21.87 g) and ethyl acetate fraction (9.12 g).

Fractions 123-142 (50 mL each) were obtained from the ethyl acetate fraction of the MeOH extract (9.12 g) of leaves after using a 95% ethyl acetate: 5% MeOH mobile phase was dried and further purified using sephadex LH 20 in a 1 cm column. After collecting 2 mL fractions using 100% MeOH, compound **1a** (293.46 mg) was obtained in fractions 20-28.

Fractions 50-55 (50 mL) were obtained from the ethyl acetate fraction (1.86 g) of the MeOH extract (4.01 g) of seeds after using a 70% ethyl acetate: 30% methanol solvent system were combined and purified in sephadex LH 20 to yield compound **2a** (22.56 mg).

The DCM extract of leaves and bark showed similar TLC profiles. Therefore, they were combined (3.67 g), and subjected to column chromatography and eluted with a hexane: ethyl acetate (v/v) solvent system, starting with 100% hexane and stepped by 10% to100% ethyl acetate. Fractions (100 mL) were collected and fraction 75 collected at 30% hexane: 70% ethyl acetate was further purified in a 1 cm column resulting in the isolation of compound **3a** (73.45 mg).

Compound **4a** (29.14 mg) was obtained from the DCM fraction of the MeOH extract (21.87 g) of leaves eluted at 60% hexane: 40% ethyl acetate in fractions 55-63 after collecting 50 mL fractions. The combined fractions (1.80 g) were further purified to yield the pure compound.

2.2.4 Cell culture

The cells were grown to confluency in 25 cm^2 tissue culture flasks in Eagle's Minimum Essential Medium (EMEM) supplemented with glutamine, 10% fetal bovine serum and antibiotics (100 µg mL^{-1} penicillin, 100 µg mL^{-1} streptomycin). Cells at a seeding cell density of 1.8 x 10^5 cells per well were plated into a 48 well plate containing 200 µL of medium. The cells were then

incubated overnight at 37 °C in 5% CO_2, after which the medium was removed and 200 µL fresh medium was added. The compounds were first dissolved in 20 % dimethylsulfoxide (DMSO) and further diluted with phosphate buffered saline (PBS) for the cytotoxicity assay. Compounds **1a** and **2a** at concentrations of 20 µg mL^{-1}, 40 µg mL^{-1}, 60 µg mL^{-1} and 80 µg mL^{-1} were then added in triplicate to the cells and incubated for 48 hours at 37 °C.

2.2.5 Cytotoxicity assay

2.2.5.1 MTT assay

This assay measures the metabolic activity of cells and their ability to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan using the succinate-tetrazolium reductase system (Mosman, 1983). After 48 hours of incubation with the test compounds (as in **2.4**), the old medium was discarded and fresh medium added to the wells. Cells with no test compounds were used as positive controls (100% viability). Approximately, 200 µL of MTT at 5 mg mL^{-1} in PBS was added to each well. The cells were incubated with the MTT solution for 4 hours at 37 °C, after which the medium and MTT were removed from the wells,\ and 200 µL of DMSO was added to each well to dissolve the formazan salt. The presence of the formazan salt is indicative of cell viability and upon addition of DMSO produces a purple solution, the absorbance of which was read in a Mindray 96A microplate reader at 570 nm. The absorbance is proportional to the number of viable cells in each well. The tests were carried out in triplicate and graphs that were generated were used to calculate the concentration at which 50% cell death was achieved (IC_{50}) using Microsoft Excel 2010 ™.

2.2.6 Apoptosis assay

The Acridine Orange/Ethidium Bromide (AO/EB) dual staining method is a simple, inexpensive qualitative and quantitative method of studying cell apoptosis. Acridine orange is taken up by both viable and non-viable cells. It intercalates into the DNA making viable cells appear green under a fluorescent microscope. Conversely, ethidium bromide is only taken up by non-viable cells whose membrane integrity has been compromised making their nucleus to fluoresce bright orange (Bezabeh *et al*., 2001). Cells were seeded at a density of 1.2 x 10^5/well into a 24-well plate and incubated overnight to allow the cells to attach. Thereafter, compounds **1a** and **2a** were added to the cells at concentration of 20 μg mL^{-1} and incubated for 48 hours. The cells were then washed with PBS, and 10 μL of AO/EB dye (100 μg mL^{-1} acridine orange and 100 μg mL^{-1} Ethidium bromide in PBS was added and cells left at room temperature for 5 minutes. The cells were then viewed under a fluorescence microscope (Olympus inverted fluorescence microscope with a CC12 fluorescent camera (excitation filter of 450-490 nm and a barrier filter of 520 nm) (Wirsam Scientific & Precision Eq. LTD, Johannesburg, South Africa) at X200 magnification and examined for morphological changes due to apoptosis. The apoptotic index was calculated as shown below:

Apoptotic index = number of apoptotic cells/ number of total cells counted

2.2.7 Antioxidant activity

The antioxidant activity of compounds **1a** and **2a** was determined using the DPPH assay. This assay measures the reducing ability of a compound on the stable free radical DPPH. Antioxidants reduce DPPH to DPPH-H by donating hydrogen atoms. The antioxidant activities of the compounds were compared to that of ascorbic acid as the standard.

Compounds **1a** and **2a** and standard ascorbic acid were prepared at concentrations of 20 μg mL^{-1}, 50 μg mL^{-1}, 100 μg mL^{-1}, 250 μg mL^{-1} and 500 μg mL^{-1}. DPPH (0.01 M in MeOH) was added to the compounds in a 3:1 ratio. Methanol only was used as the blank. The solution was left in the dark for 30 minutes since DPPH decays in the presence of light. Absorbance was then read on a Biomate UV-Vis spectrometer at 517 nm. IC_{50} values were calculated on Microsoft Excel 2010™. IC_{50} value in this assay represented the concentration of the sample required to scavenge 50% of DPPH radical.

2.3 Results and Discussion

In the present study phytochemical analysis of the plant led to isolation and purification of four compounds, two of which were tested further for their antioxidant, cytotoxic and apoptotic activities in the HepG2, Caco-2 and HEK293 cell lines.

2.3.1 Phytochemistry

2.3.1.1 Compounds isolated

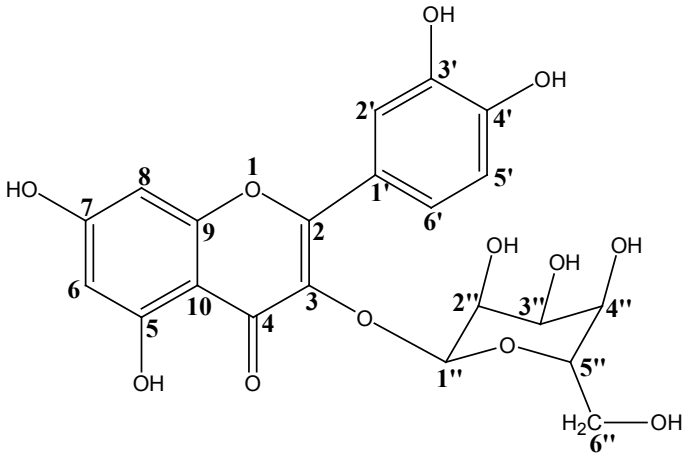
Quercetin-3-O-glucoside (**1a**): yellow powder; $C_{21}H_{20}O_{12}$; UV λ_{max} nm MeOH: 266, 361: ^{1}H-NMR (MeOH, 400 MHz): δ ppm 7.7 (1H, d, *J*=2.16 Hz H-2'), 7.59 (1H, dd, *J*=8.53 Hz *J*=2.26 Hz, H-6'), 6.86 (1H, d, *J*=8.40 Hz, H-5'), 6.38 (1H, d, H, *J*=2.14 Hz, H-8), 6.20 (1H, d, *J*=2.11 Hz, H-6), 5.24 (1H, d, *J*=7.44 Hz, H-1''), 3.34-3.95 (Glucoside). ^{13}C NMR (MeOH, 400 MHz): δ 159.0 (C-2), 135.6 (C-3), 179.4 (C-4), 163.0 (C-5), 99.9 (C-6), 166.0 (C-7), 94.7 (C-8), 158.4 (C-9), 123.0 (C-1'), 117.6 (C-2'), 145.9 (C-3'), 149.8 (C-4'), 116.0 (C-5'), 123.2 (C-6'), 104.3 (C-1''), 75.7 (C-2''), 78.3 (C-3''), 71.2 (C-4''), 78.1 (C-5''), 62.5 (C-6'').

4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2a**): light brown oily solid; $C_{20}H_{27}NO_{10}S$; UV λ_{max} nm MeOH: 222, 270: ^{1}H-NMR (MeOH), 600 MHz): 7.38

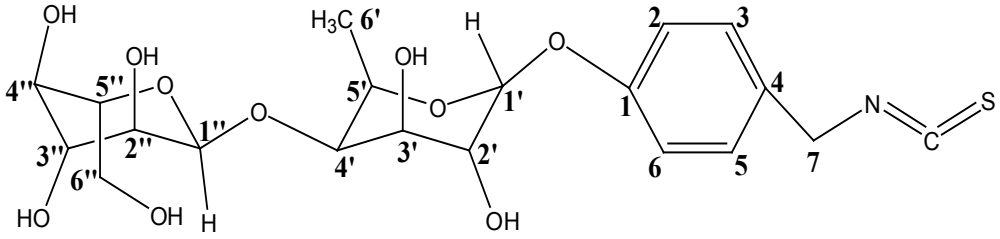
(2H, d, *J*=8.28 Hz), 7.08 (2H, d, *J*=8.57 Hz), α-rhamnose; 5.43 (1H, d, *J*=3.7 Hz, H-1'), 4.13(1H, m, H-2'), 4.05(1H, m, H-3'), 3.38(1H, m, H-4'), 3.66(1H, m, H-5'), 1.26(3H, d, *J*=6.0 Hz, H-6'), Glucose 5.42 (1H, d, *J*=2.3 Hz, H-1''), 3.75(1H, m, H-2''), 40.2-4.03(1H, m, H-3''), 3.46-3.48(1H, m, H-4''), 3.80-3.84(1H, m, H-5''), 3.68-3.72(1H, m, H-6''). ^{13}C-NMR (MeOH, 600 MHz): δ 155.7 (C-1), 116.5 (C-2), 129.0 (C-3), 129.9 (C-4), 129.0 (C-5), 116.5 (C-6), 60.8 (C-7), 92.2 (C-1'), 77.9 (C-2'), 72.4 (C-3'), 81.4 (C-4'), 69.2 (C-5'), 16.6 (C-6'), 98.5 (C-1''), 82.3 (C-2''), 72.9 (C-3''), 71.8 (C-4''), 70.8 (C-5''), 61.9 (C-6''), 131.1 (N=C=S).

β-Sitosterol (**3a**): white powder: $C_{35}H_{61}O_6$; UV λ_{max} nm MeOH: 234: ^{1}H-NMR ($CDCl_3$, 400 MHz): 5.33 (1H, d, *J*=5.06 Hz, H-6), 3.53 (1H, m, H-3), 0.90 (3H, d, *J*=6.5 Hz, H-21) 0.84 (3H, s, H-29), 0.82 (3H, s, H-26)0.80 (3H, d, *J*=1.5 Hz, H-27), 0.65 (3H, s, H-18, H-19). ^{13} C NMR ($CDCl_3$, 400 MHz): δ 37.1 (C-1), 31.5 (C-2), 71.7 (C-3), 42.2 (C-4), 140.6 (C-5), 121.6 (C-6), 31.5 (C-7), 31.8 (C-8), 50.6 (C-9), 36.4 (C-10), 20.9 (C-11), 39.6 (C-12), 42.2 (C-13), 56.6(C-14), 24.2 (C-15), 28.9 (C-16), 55.9(C-17), 11.7 (C-18), 19.2 (C-19), 36.0 (C-20), 18.6 (C-21), 33.8 (C-22), 25.9 (C-23), 45.7 (C-24), 29.0 (C-25), 18.9 (C-26), 19.7 (C-27), 22.9 (C-28), 11.8 (C-29).

Lutein (**4a**): reddish solid: $C_{40}H_{56}O_2$: UV λ_{max} nm MeOH: ^{1}H-NMR ($CDCl_3$ 400 MHz): 6.58-6.62 (m, H-11, H-11', H-15, H-15'), 6.35 (d, *J*=14.92 Hz, H-12, H-12'), 6.27 (m, H-14, H-14'), 6.17 (m, H-8, H-10, H-10'), 6.13 (s, H-7, H-8), 5.55 (1H, s, H-11), 5.43 (dd, *J*=25.59, *J*=10.04, H-7'), 4.22 (1H, s, H-3'), 4.02 (1H, m, H-3), 2.39 (1H, m, H-6'), 2.03 (1H, m, H-4), 1.97 (s, H-19, H-20, H-20'), 1.91 (s, H-19'), 1.63 (d, H-18, H-18'), 1.05 (s, H-16, H-17), 1.09 (s, H-16'), 0.82 (s, H-17'). ^{13}C NMR ($CDCl_3$, 400 MHz): 37.1 (C-1), 48.4 (C-2), 65.0 (C-3), 42.5 (C-4), 126.1 (C-5), 137.9 (C-6), 125.5 (C-7), 138.5 (C-8), 135.6 (C-9), 131.3 (C-10), 124.9 (C-11), 137.7 (C-12), 136.4 (C-13), 132.5 (C-14), 130.0 (C-15), 29.5 (C-16), 24.2 (C-17), 21.6 (C-18), 16.0 (C-19), 12.8 (C-20), 34.0 (C-1'), 44.6 (C-2'), 65.9 (C-3'), 124.8 (C-4'), 137.7 (C-5'), 54.9 (C-6'), 128.7 (C-7'), 137.7 (C-8'), 135.0 (C-9'), 130.8 (C-10'), 124.4 (C-11'), 137.5 (C-12'), 136.4 (C-13'), 132.5 (C-14'), 130.0 (C-15'), 28.7 (C-16'), 24.2 (C-17'), 22.8 (C-18'), 13.1 (C-19'), 12.7 (C-20').



Quercetin-3-O-glucoside (**1a**)



4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2a**)

Figure 2.1**:** Compounds **1a** and **2a** isolated from *Moringa oleifera* and tested for cytotoxic, antioxidant and apoptotic activity.

2.3.1.2 Isolation of compound 1a

Column chromatography of the methanol extract of the leaves yielded compound **1a** which was isolated as a yellow powder (293.46 mg). The ^{1}H-NMR and ^{13}C-NMR spectra showed characteristic resonances for flavonols namely quercetin. From the ^{1}H-NMR spectrum, resonances for the A-ring protons at δ_H 6.1 (H-6) and at δ_H 6.3 (H-8) that were meta-coupled were observed. The B-ring proton that resonated at δ_H 7.5 (1H, dd, *J*=8.53, 2.26 Hz, H-6') was

ortho coupled to the proton at δ_H 6.8 ppm (1H, d, *J*=8.40 Hz, H-5') and meta coupled to the proton at δ_H 7.7 (1H, d, *J*=2.14 Hz, H-2'). The presence of the sugar moiety was confirmed by the presence of the anomeric proton resonating at δ_H 5.2 (1H, d, *J*=7.44 Hz, H-1'') and resonances between δ_H 3.32-4.24. In the DEPT experiment, the resonance at δ_C 62.5 for C-6'' was shown to be a methylene indicating that the sugar is a glucose. The $[M]^+$ ion at *m/z* 465 is in agreement with the molecular formula $C_{21}H_{20}O_{12}$ for quercetin-3-O-glucoside. The IR spectrum showed characteristic absorption bands for the O-H group (3400–3100 cm^{-1}), C=C group (1615 cm^{-1}), and C-O group (1150–1010 cm^{-1}). The UV-Vis spectrum showed two absorption bands, a strong one at 238 nm and a weaker one at 281 nm originating from the A and B rings. The physical and spectroscopic data for compound **1a** matched those published in literature (Kazuma *et al*., 2003; Lam *et al*., 2008). Therefore, compound **1a** was identified as quercetin-3-O-glucoside.

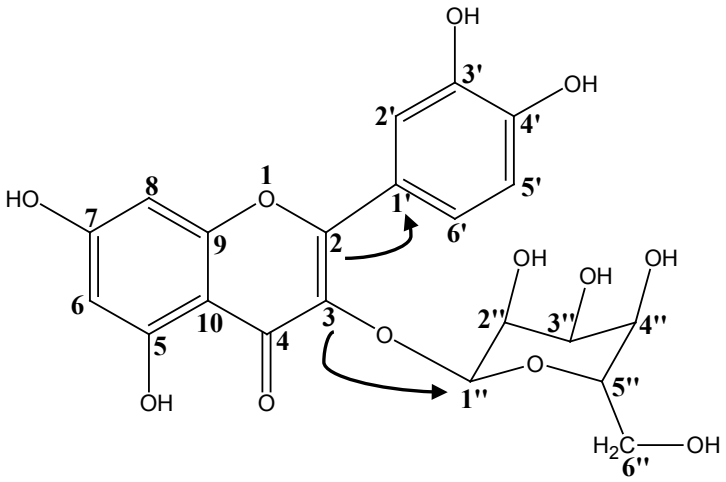


Figure 2.2: Important HMBC correlations of compound **1a**

2.3.1.3 Isolation of compound 2a

Compound **2a** was isolated as light brown and oily with a mass of 22.56 mg. The ^{1}H-NMR spectrum of compound **2a** showed resonances for two ortho coupled aromatic protons at δ_H 7.08 (2H, d, *J*=8.57 Hz, H-2,H-6) and δ_H 7.38 (2H, d, *J*=8.28 Hz, H-3,H-5) indicating a para-substituted benzene ring. This was confirmed by the ^{13}C-NMR data which showed resonances at δ_C 116.5, 129.0 for (C-2, C-6) and (C-3, C-5), respectively. Anomeric proton resonances at δ_H 5.43 (H, d, *J*=3.75 Hz, H-1') correlated with C-1' (δ_C 92.2) and δ_H 5.42 (H, d, *J*=2.3 Hz, H-1'') correlated with C-1'' in the HSQC spectrum indicating the presence of 2 sugars. Methyl doublets at δ_H 1.26 (3H, d, *J*=6.0 Hz, H-6') was assigned to the proton at C6' and confirmed one of the sugars to be a rhamnose. The DEPT experiments showed the presence of three quaternary carbons resonating at δ_C 155.7, 129.1, and 131.1 which were assigned to C-1, C-4 and C=N, respectively. Two methylene carbons at δ_C 61.99 and δ_C 60.80 were assigned to C-6'' and C-7, respectively. HMBC correlations of H-1' and C-1 showed that the rhamnose is attached at C-1 and further indicated that the glycosidic linkage between glucose and rhamnose is at 4' and 1''.

The IR spectrum showed characteristic bands at 1630-1510 cm^{-1}, indicating the presence of the C=S and C=N bonds of the thiocarbonate linkage attached to C7. A broad absorption band at 3307 cm^{-1} indicated the presence of hydroxyl groups (-OH) in the molecule. The $[M]^+$ ion at *m/z* 310 is in agreement with the molecular formula $C_{20}H_{27}NO_{10}S$ for 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate. This information was consistent with published data (Oluduro *et al*., 2012; Oluduro *et al*., 2010) and led to the identification of compound **2a** as 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate.

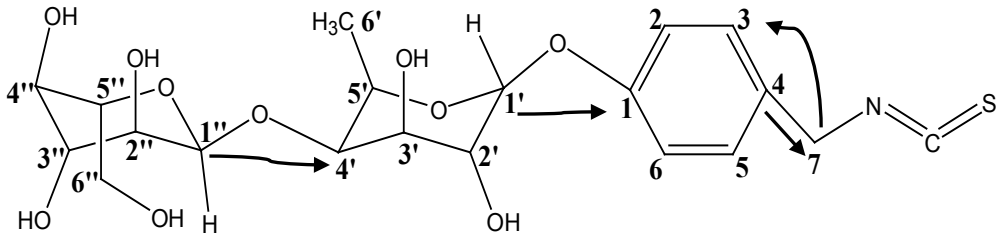


Figure 2.3: Important HMBC correlations of compound **2a**

The DCM extract of the leaves and bark yielded compound **3a** and its structure was further confirmed to be β-sitosterol, a ubiquitous plant sterol, by comparing spectroscopic data with that in literature (Ragasa *et al*., 2012). Compound **4a** was obtained from the leaves of the plant and the structure was confirmed to be lutein by comparing spectroscopic data with that in literature (Eldahshan *et al*., 2013; Ragasa *et al*., 2012; Shotipruk *et al*., 2012).

2.3.2 Antioxidant activity

The antioxidant test showed that the compound with the highest antioxidant activity was quercetin-3-O-glucoside (**1a**) whose activity with an IC_{50} of 26.73 µg mL^{-1} which was slightly higher than that of the control (31.68 µg mL^{-1}). Quercetin is a strong antioxidant and is commonly used as a standard in antioxidant assays. 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate (**2a**), showed moderate antioxidant activity with an IC_{50} value of 78.96 µg mL^{-1} as shown in Table 2.1.

Table 2.1: Radical scavenging activity and related IC_{50} values of compounds **1a** and **2a** against the standard, ascorbic acid. Data represented as mean ± SD (n=3).

Concentration in µg mL^{-1}	**% Scavenging activity**		
	Ascorbic acid	**1a**	**2a**
500	98.99±0.002	93.81±0.005	61.20±0.003
250	97.74±0.005	93.01±0.001	60.32±0.004
100	65.15±0.004	68.21±0.006	50.81±0.020
50	59.92±0.004	53.80±0.001	52.90±0.090
20	41.90±0.020	49.53±0.060	35.38±0.500
IC_{50} Values	31.68	26.73	78.96

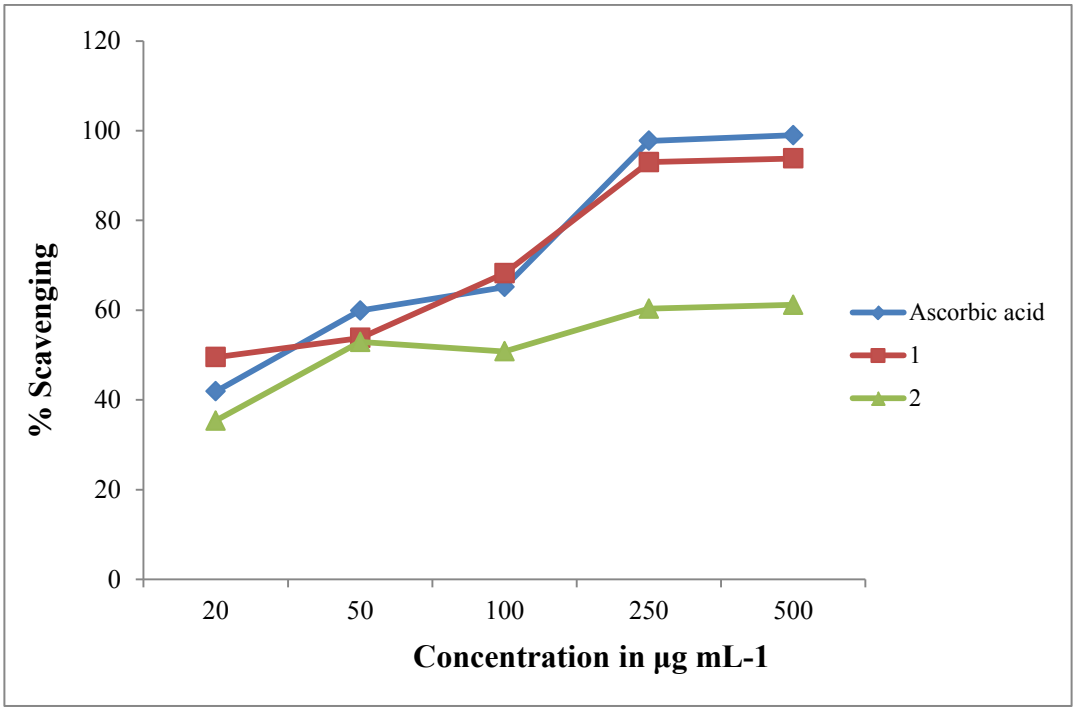
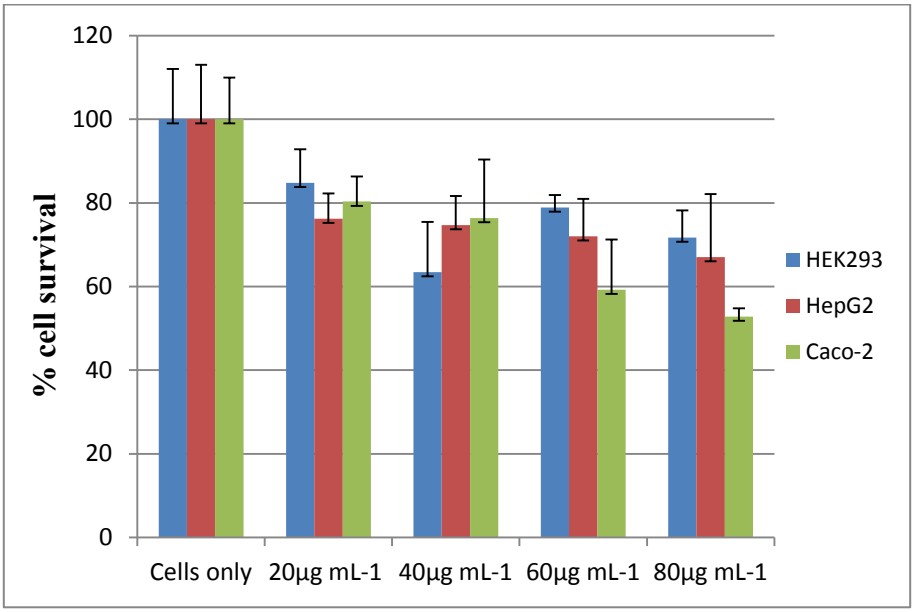


Figure 2.4**:** Antioxidant activity of compounds **1a** (quercetin-3-O-glucoside) and **2a (**4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate) and ascorbic acid as standard in the radical scavenging activity assay

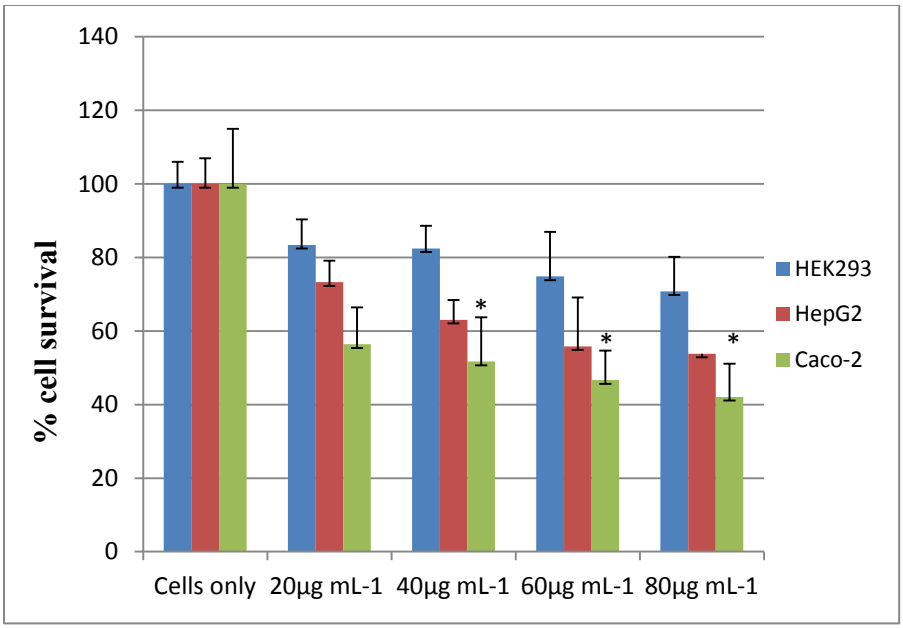
2.3.3 Cytotoxicity tests

Table 2.2: IC_{50} Values of compounds **1a** (quercetin-3-O-glucoside) and **2a (**4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate) on HEK293, HepG2 and Caco-2 cell lines.

CELL LINES	IC_{50} VALUES (µg mL^{-1})	
	1a	**2a**
HEK293	186	224
HepG2	150	60
Caco-2	79	45



COMPOUND 1a



COMPOUND 2a

Figure 2.5: MTT cell viability assay of compounds **1a** (quercetin-3-O-glucoside) and **2a (**4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate) in HEK293, HepG2 and Caco-2 cells. Data represented as mean ±SD (n=3). Compound **2a** showed significant cytotoxicity against cancer cell lines HepG2 and Caco-2 compared to compound **1a** ($p \leq 0.05$).

The cytotoxicity of compounds **1a** (quercetin-3-O-glucoside) and **2a (**4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate) on the three cell lines, Caco-2, HepG2 and HEK293 was carried out using the MTT assay. The results (Figure 2.5, Table 2.2) indicate that quercetin-3-O-glucoside showed selectivity but moderate cytotoxicity across all three cell lines. It was more cytotoxic to the Caco-2 cell line with an IC_{50} of 79 µg mL^{-1} when compared to the HepG2 cell line with an IC_{50} of 150 µg mL^{-1} but showed lower cytotoxicity in the control HEK293 with an IC_{50} of 186 µg mL^{-1}. 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate displayed the greatest cytotoxic activity in the Caco-2 cell line with an IC_{50} of 45 µg mL^{-1} but slightly lower in the HepG2 cell line with an IC_{50} of 60 µg mL^{-1}. From the cytotoxicity studies it can be deduced that 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate is more selective to cancer cells considering the

very low cytotoxicity seen in the HEK293 non-cancer control cells (IC_{50} of 224 μg mL^{-1}). Statistical analysis of the results using ANOVA with subsequent LSD post hoc test using SPSS ver. 20 showed 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate having significantly higher activity across all concentrations as compared to quercetin-3-O-glucoside. Moreover 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate had significant activity as compared to the control untreated cells on Caco-2 cell line. Both compounds displayed no significant activity against HEK293 cell lines at 95% confidence level, however 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate had significantly higher activity as compared to quercetin-3-O-glucoside on HepG2 cell line.

2.3.4 Apoptosis assay

Apoptosis studies were carried out to determine whether the isolated compounds induced apoptosis in the selected cell lines. We used the double staining method with acridine orange and ethidium bromide and detected the characteristic morphological changes in the cells due to apoptosis under a fluorescent microscope (Olympus). These changes include cell shrinkage, chromatin condensation, fragmentation of the nucleus and cell membrane blebbing. Viable cells fluoresce green under the microscope, early apoptotic cells a brighter green, late apoptotic cells orange with a condensed chromatin, and necrotic cells orange without condensed chromatin (Khasibhatla *et al*., 2006). Results of exposure of the cells lines to the compounds for 24 hours at a concentration of 10 μg mL^{-1} are shown in Figure 2.6. Compounds **1a** (quercetin-3-O-glucoside) and **2a (**4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate) were seen to induce apoptosis in all cell lines. Apoptotic features were clearly observed in these cells.

Apoptosis induction was selective as evidenced by the high apoptotic index in the cancer cells compared to the non-cancer cell line. The apoptotic index was much higher in all the control cells compared to cells treated with quercetin-3-O-glucoside and 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate as shown in Table 2.3 below.

Table 2.3: Apoptotic index values of compounds **1a** (quercetin-3-O-glucoside) and **2a (**4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate) on HEK293, HepG2 and Caco-2 cell lines.

CELL LINES	**APOPTOTIC INDEX**		
	Control	**Compound 1a**	**Compound 2a**
HEK293	0.000	0.057	0.03
HepG2	0.000	0.37	0.132
Caco-2	0.000	0.5	0.6

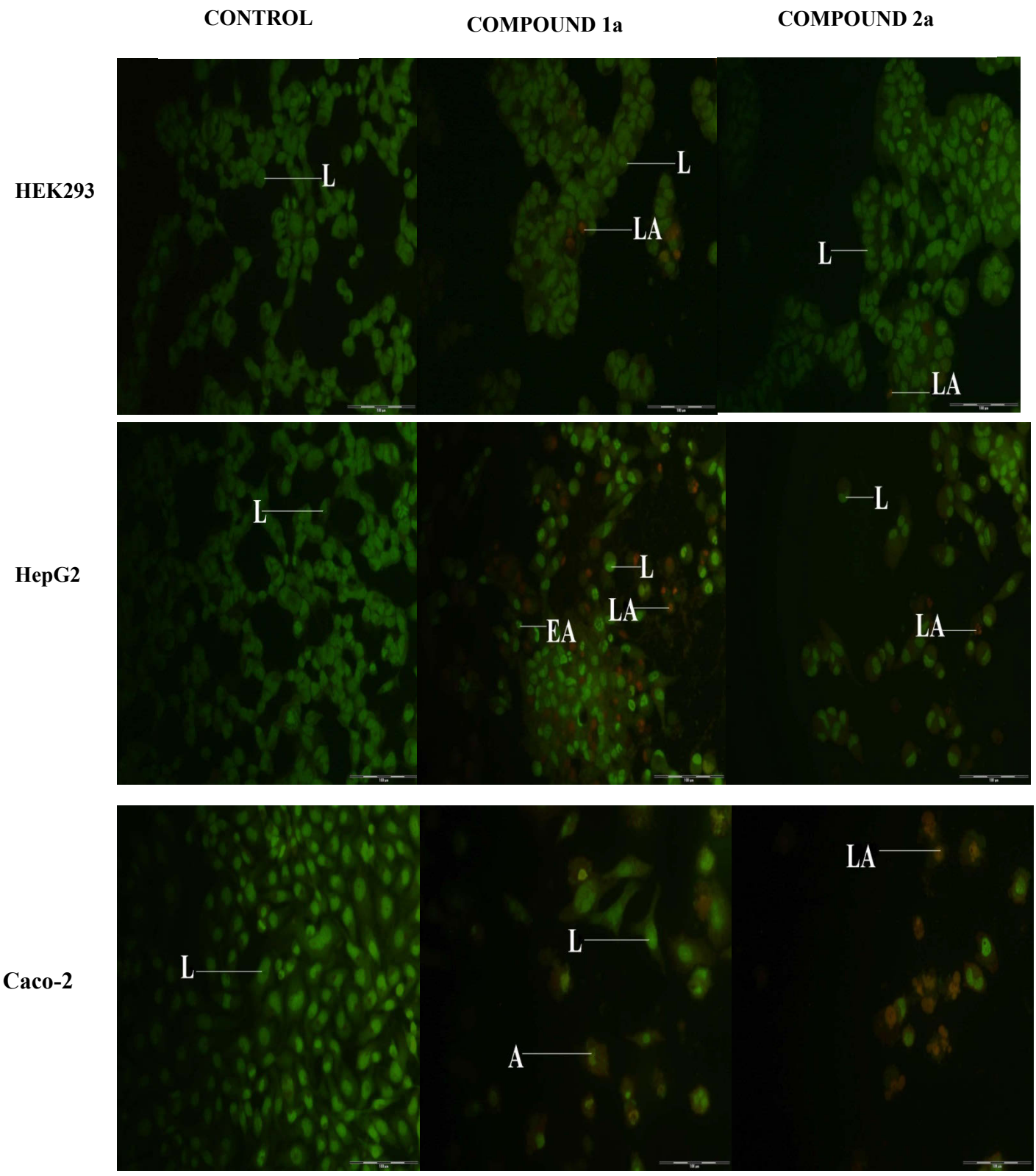


Figure 2.6: Induction of apoptosis by compounds **1a** and **2a** on HEK293, HepG2 and Caco-2 at a concentration of 10 µg mL^{-1}. L=Live A=Apoptotic LA= Late apoptotic EA= Early apoptotic. Scale is given as 100 µm.

Some synthetic drugs have their structure based on natural compounds or are synthetic derivatives of these compounds e.g. flavopiridol (Shoeb, 2006). *In vitro* cytotoxicity testing is a valuable tool for screening compounds with potential anti-cancer activity. In this study, we have correlated growth inhibition with induction of apoptosis. Crude extracts of the *Moringa* tree have been shown to possess cytotoxic and antioxidant activity (Charoensin, 2014; Singh *et al*., 2013). The high antioxidant activity of quercetin-3-O-glucoside is important in ridding the body of reactive oxygen species, thereby protecting DNA from damage and thus lowering mutation rates and occurrence of tumour cells. However when quercetin is used as an antioxidant it is then broken down and oxidised to quinone-type metabolites which have a pro-oxidant action that binds and fragments DNA (Russo *et al*., 2012) which would explain its apoptosis induction activity (Fig.2.6). Flavonoids have been reported to cause cell death and apoptosis in A549 (human lung adenocarcinoma) cells by inducing transcription of *p21,* a tumour suppressor gene that is only activated in the presence of *p53* which is inactivated in most tumours (Colic and Pavelic, 2000; Patil *et al*., 2010). Quercetin has been proposed to induce apoptosis via the intrinsic/mitochondrial pathway through the inhibition of protein kinase B phosphorylation and survivin which are responsible for the activation of caspases and down regulation of anti-apoptotic proteins Bcl-2 and Mcl-1 (Kuno *et al*., 2012; Russo *et al*., 2012).

4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate has been isolated once previously by Oluduro *et al* in 2012 and here we report the first evidence of its cytotoxic and apoptotic activity on mammalian cell lines to the best of our knowledge. This group of compounds is unique to the *Moringa* family and similar compounds are reported to possess cytotoxic activity *in vitro* and *in vivo,* and it was suggested that the isothiocyano group is an essential part of this activity (Guevara *et al*., 1999; Ragasa *et al*., 2012). Studies on

isothiocyanates report that these compounds inhibit cancer cell growth and induce apoptosis through different targets. Srivastava and Singh (2004) reported growth inhibition and apoptosis induction by benzyl isothiocyanate in animal models. They postulated that the apoptosis induction was due to down-regulation of Bcl-2 and up-regulation of Bax which are anti-apoptotic and pro-apoptotic proteins, respectively. Regulation of phase I and phase II enzymes which are implicated in cancer risk have also been proposed to be major mechanisms of action of isothiocyanates (Wu *et al*., 2009; Navarro *et al*., 2011). The aqueous extract of the *Moringa* leaf has been reported to induce apoptosis *in vitro* and this was attributed to the presence of a high concentration of the flavonoids quercetin and kaempferol in the leaves (Sreelatha *et al*., 2011).

The compounds in this study showed a concentration dependent cytotoxicity against both HepG2 and Caco-2 cell lines. They also displayed selective cytotoxicity on cancer cells versus normal cells with an IC_{50} of 186 µg mL^{-1} and 224 µg mL^{-1}. 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate was the more active of the two compounds with an IC_{50} value of 45 µg mL^{-1} in the Caco-2 cell line. Both compounds were not well tolerated by the Caco-2 compared to the HepG2 cell lines. Apoptosis induction is an important indicator of the ability of chemotherapy drugs to induce death in tumour cells. The results suggest that one of the causes of cell death upon exposure to these compounds is apoptosis. β-sitosterol and lutein have been isolated previously from this plant and occur in a wide variety of plants. They have been widely studied for their anticancer and chemopreventative potential (Ragasa *et al*., 2014; Patil *et al.*, 2013).

2.4 Conclusion

Four compounds were isolated from *M.oleifera* namely quercetin-3-O-glucoside, 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate, lutein and β-sitosterol. Two of these compounds were tested for antioxidant, cytotoxicity and apoptosis induction. 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate and quercetin-3-O-glucoside demonstrated varying degrees of cytotoxicity against two human cancer cell lines. Their cytotoxic selectivity to cancer cells *in vitro* warrants their future investigation as potential chemotherapeutic agents with fewer side effects than drugs currently in usage. Future studies need to be undertaken to understand the mechanism of action of these compounds and their specific target at the molecular level with regards to induction of apoptosis. *In vivo* studies also need to be conducted to investigate their effect on solid tumours and also to evaluate the pharmacological and pharmacokinetic activities of these compounds in an animal model.

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CHAPTER 3

PHYTOCHEMISTRY, CYTOTOXICITY AND APOPTOSIS STUDIES OF β-SITOSTEROL-3-O-GLUCOSIDE AND β-AMYRIN FROM *PRUNUS AFRICANA*

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Abstract

Prunus africana is used traditionally in Kenya in the treatment of cancer and worldwide in the treatment of benign prostate hyperplasia. In this study, compounds from the leaves and bark of this plant were isolated and tested for their cytotoxicity and apoptosis induction in two human cancer cell lines viz. hepatocellular carcinoma (HepG2), colorectal carcinoma (Caco-2) cells and a non-cancer cell line, viz. embryonic kidney (HEK293) cells. GC-MS profiling of the extract was also conducted. Three compounds, viz. β-sitosterol, β-amyrin and β-sitosterol-3-O-glucoside were isolated and the cytotoxic activity of β-amyrin and β-sitosterol-3-O-glucoside on the HepG2, Caco-2 and HEK293 was determined using the MTT cell viability assay. Both compounds had significant cytotoxic activity towards the Caco-2 cell line with IC_{50} values of 81 μg mL^{-1} and 54 μg mL^{-1} for β-amyrin and β-sitosterol-3-O-glucoside, respectively while low cytotoxicity was observed on HepG2 cell lines with IC_{50} values of 206 μg mL^{-1} and 251 μg mL^{-1} for β-amyrin and β-sitosterol-3-O-glucoside, respectively. Apoptosis induction in cells was studied using acridine orange/ethidium bromide dual staining. In both cases, the compounds tested demonstrated selective cytotoxicity towards cancer cells with high apoptosis indices in cells exposed to β-amyrin. Low IC_{50} values of 156 μg mL^{-1} and 937 μg mL^{-1} for β-amyrin and β-sitosterol-3-O-glucoside, respectively, were observed in the HEK293 cell line. GC-MS profiling of the extracts showed the plant to be rich in phenolics, pentacyclic triterpenoids and sterols.

Keywords: triterpenoids, phytosterols, MTT cell viability, apoptosis induction, GC-MS profiling.

3.1 Introduction

Cancer is a leading cause of death in Africa with 70% of the deaths occurring in low and middle income countries in the continent. However, it is given low public health priority due to the burden of infectious diseases (Jamison *et al*., 2006). For the majority of the population in Africa, healthcare is inaccessible. Therefore, many people rely on herbal medicine for treatment, particularly with regards to chronic diseases. Medicinal plants are a source of affordable medicine for the majority of low-income earners in most parts of Africa and, furthermore, most of the locals and rural poor continue to rely on herbal medicine even with access to modern medicine (Kigen *et al*., 2013).

Current cancer treatment proves to be challenging with chemotherapy drugs being non-specific and multi-drug resistance developing. This results in chemotherapy related toxicities leading to patients succumbing to the adverse effects of the drug rather than the disease (Zhang *et al*., 2011). Researchers are continually searching for alternative drugs that will overcome the challenges facing modern chemotherapy with bioprospecting being a viable route for the discovery of therapeutic agents. The success of natural products in cancer chemotherapy is well documented and has led to the development of drugs such as Taxol, from the *Taxus brevifolia*, and Maytansine from *Maytenus serrata* (developed using a monoclonal antibody as a targeting moiety which is currently in clinical trials) (Cragg and Newman, 2013).

Prunus africana is an evergreen tree from the family Rosaceae. It grows widely in Africa, stretching from western to southern Africa. In Kenya, it grows in the wild in the highland regions of the country, but has also been domesticated by several communities (Gachie *et al*., 2012). *P. africana* is used traditionally for the treatment of colorectal, breast and skin cancer (Ochwang'i

et al., 2014). The bark and leaf extracts are also used to treat fevers, gonorrhea and stomach pains (Stewart, 2003a).

Studies on *P. africana* have led to the isolation of sterols and pentacyclic triterpenes (Jimu, 2011). The use of this plant has been attributed to the anti-inflammatory activity of the isolated compounds (Otieno *et al*., 2013). Extracts from the bark of *P. africana* are used worldwide in the treatment of benign prostate hyperplasia, which is an inflammation of the prostate gland mostly found in men over 50 years of age. The tree is also reported to contain ferulic acids, n-docosanol and n-tetracosanol which lower blood cholesterol levels (Stewart, 2003b). *Prunus africana* is currently facing extinction (World Conservation Monitoring Centre, 1998) owing to massive overharvesting for export where it is sold in the form of herbal formulations.

Evidence and epidemiological studies suggest that phytosterols are protective against a wide range of diseases and possess anticancer activity on various cancers (Bradford and Awad, 2007). Few studies have documented the anticancer potential of this plant against different cancers. We herein report on the isolation and purification of three compounds, two of which were evaluated for cytotoxic activity on cancer cells, HepG2 and Caco-2 and for apoptosis induction as a possible form of death using immunofluorescent techniques. GC-MS profiling of the plant extracts was also conducted and the identified compounds were evaluated for their anticancer activity.

3.2 Materials and methods

3.2.1 Materials

Organic solvents (deuterated and undeuterated) and HPLC grade solvents were purchased from Sigma Co. (St. Louis, USA) and Merck (Darmstadt, Germany). Silica gel (Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM), thin layer chromatography (TLC) aluminium backed plates pre-coated with silica gel and MTT salt (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) were bought from Merck (Darmstadt, Germany). Human embryonic kidney cells (HEK293) was obtained from the Antiviral Gene Therapy Unit, University of the Witwatersrand, while the hepatocellular carcinoma (HepG2) cells and colorectal adenocarcinoma (Caco-2) cells were purchased from Highveld Biological (PTY) LTD (Lyndhurst, South Africa). Eagle's minimum essential medium (EMEM), penicillin/streptomycin and trypsin-EDTA, were purchased from Lonza BioWhittaker (Walkersville, USA). PBS was obtained from Calbiochem® (Canada). Foetal bovine serum was sourced from Hyclone GE Healthcare (Utah, USA). Acridine orange and ethidium bromide for apoptosis studies were obtained from Promega (Madison, USA). All other reagents were of analytical reagent grade.

3.2.2 General experimental procedures

Organic solvents hexane, dichloromethane (DCM) and methanol (MeOH) used for extraction and purification were distilled before use. Crude extracts were subjected to column chromatography, with silica gel as stationary phase, for purification. TLC was used to profile column fractions and spots were visualised under UV light and by use of an anisaldehyde spray reagent (20% H_2SO_4 and 80% MeOH). NMR spectra were obtained from a Bruker Avance 400

MHz or 600 MHz using TMS as an internal standard. The compounds were dissolved in deuterated solvents either chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO). Chemical shifts (δ) are expressed in ppm. UV spectra were obtained on a Perkin-Elmer Lambda 35 UV-Vis spectrophotometer using MeOH and hexane as solvents. Extract profiling was conducted on a GC-MS (Agilent GC-MSD equipped with a DB-5SIL MS fused silica capillary column) for identification of compounds. Extracts were dissolved using HPLC reagent grade solvents, MeOH and DCM. Phytocompounds were identified using the National Institute of Standards and Technology (NIST) database library.

3.2.3 Plant material

Samples of leaves and bark were collected from a traditional healer in Eldama Ravine Town (0º 03'N 35º 43'E/0.05º N 35.72º E), Koibatek District in Baringo County, Kenya. The identity of the tree was confirmed by the Kenya Forest Research Institute (KEFRI). The leaves and bark were air-dried in the shade for 2 weeks and cut into smaller pieces. The plant material was ground into a fine powder and weighed. This yielded 674.52 g of leaves and 423.45 g of bark.

3.2.4 Extraction and purification

Sequential extraction of the ground leaves and bark with hexane, DCM and MeOH was carried out for 48 hours on an orbital shaker. Extracts were filtered using Whatman No. 1 filter paper and concentrated under vacuum in a Buchii rotary evaporator to obtain a viscous concentrate. This was weighed and stored at 4 ºC in a refrigerator.

Purification of compounds was achieved using column chromatography. The combined hexane and DCM extract of the leaves (157.17 g) was separated using a hexane: ethyl acetate solvent system starting with 100% hexane and stepped by 10% to 100% ethyl acetate. Ten 100 mL fractions were collected for each solvent system. Fractions 37-40 was re-chromatographed to obtain compound **1b** (112 mg) in fractions 13-17 using a hexane (85%): ethyl acetate (15%) solvent system ($^{v}/_{v}$) after collecting 20 mL fractions. Fraction 51-55 from the crude extract yielded compound **2b** (23.9 mg).

The aqueous MeOH extract of the plant was partitioned in triplicate with 200 mL DCM and 200 mL ethyl acetate to obtain a DCM fraction (25 g) and ethyl acetate fraction (3.05 g). The DCM fraction obtained was subjected to column chromatography starting with 100% hexane and was stepped gradually with ethyl acetate. Fractions 92-103 (100 mL each), collected with hexane (10%): ethyl acetate (90%) ($^{v}/_{v}$) were pooled and further purified on sephadex LH20. MeOH (100%) was used to elute the compounds on a 1 cm column and 3 mL fractions were collected to obtain compound **3b** (19.5 mg) in fraction 3.

3.2.5 Extract profiling by GC-MS

An investigation of the hexane and DCM extract of the leaves and bark was done by GC-MS analysis. The injection volume was 1.0 μL with an injector temperature of 25 °C. Helium was used as the carrier gas with a column flow of 1.0 mL min^{-1}. The oven programme was 60 °C ramped to 260 °C at 4 °C min^{-1} for 10 minutes and held at 260 °C for 10 minutes. Total running time was 30 minutes. Hexane and DCM fractions collected using column chromatography were dissolved in HPLC grade DCM and then transferred to 1.5 mL clear autosampler vials for GC-MS analysis.

3.2.6 Cell culture

The cancer cell lines HepG-2, and Caco-2 and the non-cancer cell line HEK293 were grown in EMEM+Glutamine medium supplemented with 10% FBS and 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. They were cultured and maintained in 25 cm^2 culture flasks at 37 °C and 5% CO_2. At 90% confluency, the cells were trypsinised by overlaying the cells with 1mL trypsin-EDTA for 2 minutes. Once cells were trypsinised as evidenced by cells rounding off, fresh medium was added and the cells were split as desired or plated in 48 well plates for assay purposes.

3.2.7 MTT cell viability assay

This is one of the most widely used assays to measure cell viability. This assay measures the ability of viable cells to reduce MTT by mitochondrial dehydrogenases to the insoluble formazan salt. The salt once solubilised forms a pink/violet colour whose absorbance is measured spectrophotometrically (Mosman, 1983).

In this study, cells were plated on a 48 well plate with 200 μL media at a seeding density of 1.2×10^5 and incubated for 24 hours to allow attachment. Thereafter, the medium was changed and 10 μL of the compounds dissolved in DMSO and further diluted with PBS were added to the cells at concentrations of 20 μg mL^{-1}, 40 μg mL^{-1}, 60 μg mL^{-1} and 80 μg mL^{-1} in triplicate and incubated for 48 hours. Approximately 200 μL of MTT at a concentration of 5 mg mL^{-1} in PBS was added and incubated for 4 hours at 37 °C. After four hours, the medium was removed from the wells, leaving the formazan salt at the bottom. Subsequently, 200 μL of DMSO was added to solubilise the salt producing a pink colour whose absorbance was measured on a Mindray MR-96A

microplate reader (Vacutec, Hamburg, Germany) at 570 nm. Cytotoxicity was represented as percentage cell survival against compound concentration. IC_{50} values were calculated from graphs of percentage inhibition against concentration in Microsoft Excel™.

3.2.8 Apoptosis studies

Dual staining with acridine orange and ethidium bromide is a rapid technique for studying cell apoptosis *in vitro*. It enables the distinguishing of live, late and early apoptotic and necrotic cells. Acridine orange stains live cells by intercalating with the cell's DNA to emit green fluorescence, while the ethidium bromide stains dead cells emitting yellow to red fluorescence based on the stage of apoptosis. Approximately 100 mg mL^{-1} of both dyes was prepared in PBS. The dyes were mixed at a ratio of 1:1. Cells at a density of 1.2×10^5 were plated on 24 well plates and 20 µL of each compound at 10 µg mL^{-1} was added to the cells and incubated for 24 hours. Subsequently, old medium was removed from the plate and the cells were washed with 100 µL cold PBS. Thereafter, 15 µL of the dye solution was added to the cells which were then viewed under an Olympus fluorescent microscope at ×200 magnification. Apoptotic indices were calculated as shown:

Apoptotic index = number of apoptotic cells/ number of total cells counted

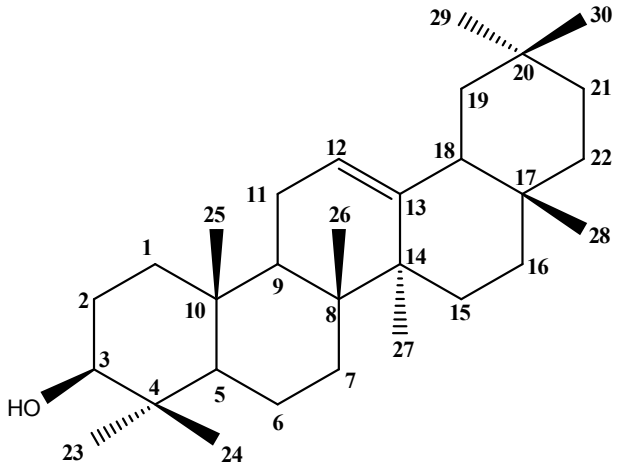
3.3 Results and Discussion

3.3.1 Phytochemistry

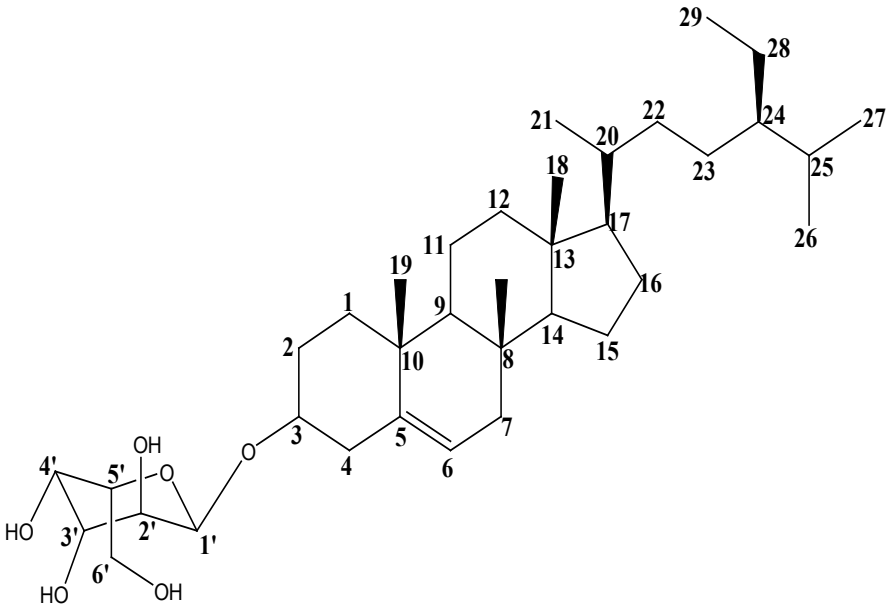
NMR studies led to the confirmation of 3 major compounds i.e. β-sitosterol (**1b**), β-amyrin (**2b**) and β-sitosterol-3-O glucoside (**3b**).

3.3.1.1 Isolated compounds

β-Sitosterol-3-O-glucoside (**3b**): white amorphous powder: $C_{35}H_{61}O_6$: UV λ_{max} nm MeOH: 204, 233. ^{1}H-NMR (DMSO, 400 MHz): δ 5.34 (1H, m, *J*=5.06 Hz, H-6), 4.86 (3H, dd, *J*=4.54 Hz, 13.61 Hz, OH-2', OH-3', OH-4'), 4.41 (1H, t, *J*=5.64 Hz, 11.49 Hz, OH-6'), 4.24 (1H, d, *J*=7.70 Hz, H-1'), 3.67 (1H, m, H-6'), 3.48 (1H, m, H-3), 3.14 (1H, dd, *J* = 3.72 Hz, *J*= 8.7 Hz, H-3'), 3.10 (1H, m, H-5'), 3.08 (1H, m, H-4'), 2.92 (1H, m, H-2'), 2.16 (2H, m, H-4), 0.97 (1H, s, H-19) 0.92 (1H, d, *J* = 6.74 Hz, H-21), 0.84 (1H, d, *J* = 6.83 Hz, H-27), 0.82 (1H, d, *J* = 7.10 Hz, H-26), 0.67 (1H, s, H-18). ^{13}C-NMR (DMSO, 400 MHz): δ 36.7 (C-1), 29.2 (C-2), 77.4 (C-3), 37.3 (C-4), 140.9 (C-5), 121.6 (C-6), 31.8 (C-7), 31.9 (C-8), 50.1 (C-9), (C-10) 20.1 (C-11), 38.8 (C-12), 42.3 (C-13), 56.6 (C-14) 24.3 (C-15), 28.2 (C-16) 55.9 (C-17) 12.1 (C-18), 19.5 (C-19), (C-20), 19.1 (C-21), 33.3 (C-22), 25.9 (C-23), 45.6 (C-24), 28.6 (C-25), 19.4 (C-26), 21.0 (C-27), 23.1 (C-28) 12.2 (C-29), 101.2 (C-1'), 73.9 (C-2'), 77.2 (C-3'), 70.6 (C-4'), 77.2 (C-5'), 61.6 (C-6')



(**2b**)



(**3b**)

Figure 3.1: Chemical structures of compounds **2b** (β-amyrin) and **3b (**β-Sitosterol-3-O-glucoside) isolated from *P. africana*.

3.3.1.2 Isolation of compound 2b

Compound **2b** was isolated as a white powder with a mass of 19.5 mg. The ^{1}H-NMR spectrum of compound **2b** showed characteristic resonances for a pentacyclic triterpene of the oleanane type. These were a downfield vinylic proton resonance at δ_H 5.25 (1H, t, *J*=6.6 Hz, 3.3 Hz, H-12), a carbinylic proton resonance at δ_H 3.20 (1H, dd, *J*=10.6 Hz, 4.1 Hz, H-3), a double doublet at δ_H 2.81 (1H, dd, *J*=14.3 Hz, 4.4 Hz, H-18) and seven methyl resonances between δ_H 0.77-1.19 (s, H-23-H-30). The ^{13}C-NMR spectrum showed resonances at δ_C 143.8 (C-13) and δ_C 122.2 (C-12) due to the vinylic carbons and a resonance at δ_C 78.8 (C-3) due to the carbinylic carbon. The DEPT 90 and 135 experiments resolved 8 methyl, 10 methylene, 5 methine and 7 quaternary carbon resonances.

The UV spectrum of compound **2b** gave maximum wavelength (λ_{max}) at 229 nm. The IR spectrum showed a broad absorption band at 3263 cm^{-1} (OH group) and a band at 1459 cm^{-1} (C=C). GC-MS data showed molecular ion peak [M^+] at m/z 426 which is in agreement with the molecular formula $C_{30}H_{50}O$. This data corresponded with that in literature (Mohato and Kundu, 1994; Vasquez *et al*., 2012) and confirmed compound **2b** to be β-amyrin.

3.3.1.3 Isolation of compounds 1b and 3b

Compound **1b** was isolated as an amorphous white solid with a mass of 112 mg and compound **3b** was isolated as an amorphous white solid with a mass of 23.9 mg. The ^{1}H-NMR spectra for both compounds showed characteristic resonances for β-sitosterol. These were resonances at δ_H 5.34 (1H, t, J=5.1 Hz, H-6) for the olefinic proton and a resonance at δ_H 3.53 (1-H, m, H-3) due to the carbinylic proton. However, for compound **3b**, the resonance at δ_H 4.24 (1H, d, *J*=7.7 Hz, H-1') due to the anomeric proton indicated the presence of a glycosidic linkage. This was confirmed by resonances between δ_H 2.92-4.86 which were assigned to H-2', H-3', H-4', H-5' and H-6' and the sugar identified as glucose.

The ^{13}C NMR spectrum for compound **1b** resolved 29 carbon resonances and for compound **3b** resolved 35 carbon resonances with characteristic olefinic resonances at δ_C 140.9 (C-5) and δ_C 121.6 (C-6) in sitosterol. The resonance at δ_C 101.2 (C-1') in compound **3b** was assigned to the anomeric carbon due to HSQC correlations. HMBC experiments confirmed the attachment of the sugar at C-3 (Figure 2) and the methylene at δ_C 61.6 was assigned to C-6' of the glucoside.

The UV spectra of compounds **1b** and **3b** showed two bands at 204 nm (0.3538 Å) and 231 nm (0.0984 Å). The IR spectra showed a broad absorption band at 3382 cm^{-1} (OH group), 2920 cm^{-1}

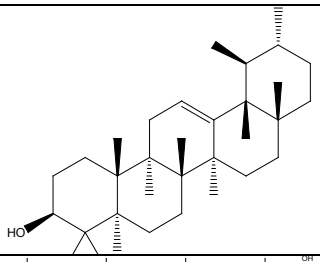
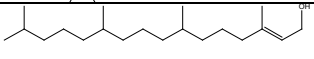
(CH groups) and 1461 cm^{-1} (C=C). GC-MS data for compound **1b** showed molecular ion peak [M^+] at m/z 414 which is in agreement with the molecular formula $C_{29}H_{50}O$. GC-MS data for compound **3b** showed molecular ion peak [M^+] at m/z 576, which is in agreement with the molecular formula $C_{35}H_{60}O_6$.

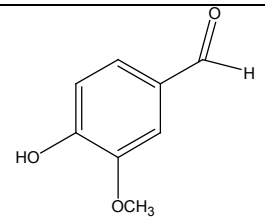
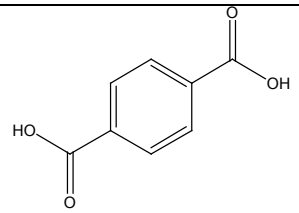
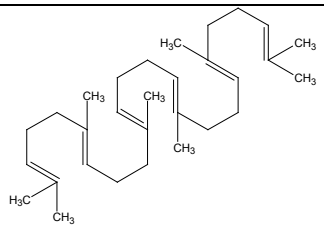
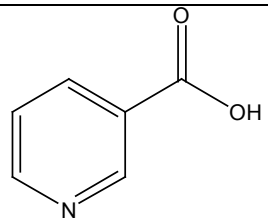
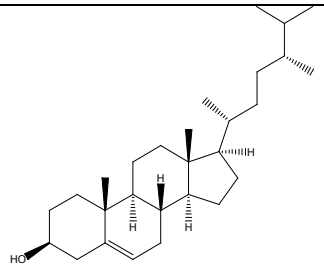
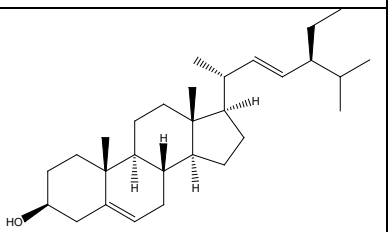
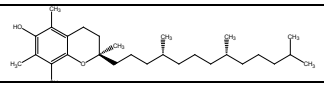
This data corresponded with that in literature (Arora and Kalia, 2013; Jayaprakasha et al., 2010) and confirmed compound **1b** to be the aglycone β-sitosterol and compound **3b** to be the glycoside, β-sitosterol-3-O-glucoside.

3.3.2 GC-MS profiling

GC-MS profiling led to the identification of 9 compounds using the NIST library. The fragmentation pattern, name, structure and retention time were compared to those of known compounds in the library. These are given in Table 3.1.

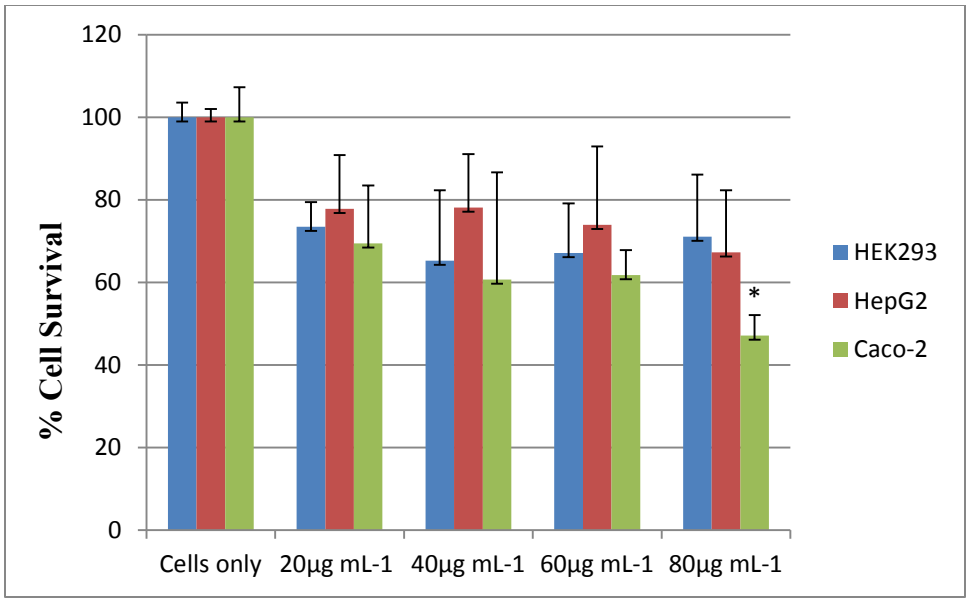
Table 3.1: Compounds of interest identified by GC-MS in the hexane and DCM extract of *P. africana* leaves.

Compound	Molecular ion/base ion peak	T_R (min)	Structure
α-amyrin	**426/218**	**10.7**	
Phytol	**123/71**	**11.3**	

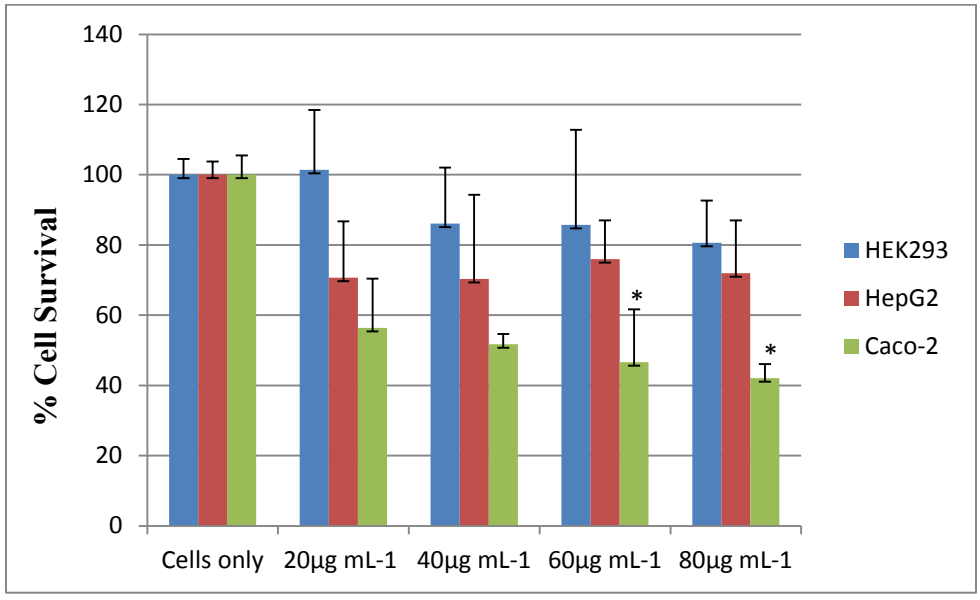
Vanillin	**152**	**7.3**	
Benzenedicarboxylic acid	**279/149**	**12.7**	
Squalene	**410/69**	**16.2**	
Vitamin B3/Nicotinic acid/Niacin	**122**	**8.3**	
Campesterol	**400**	**24.2**	
Stigmasterol	**412**	**12.6**	
α-tocopherol	**430/165**	**21.7**	

3.3.3 MTT cell viability studies

The MTT cell viability assay was used to study the cytotoxic effect of β-amyrin and β-Sitosterol-3-O-glucoside in the two cancer cell lines HepG2 and Caco-2 and non-cancer cell line, HEK293. Compounds tested exhibited a dose dependent cytotoxicity on the cancer cell lines. Both compounds showed selective cytotoxicity to cancer cells as evidenced by the high IC_{50} values 156 μg mL^{-1} and 937 μg mL^{-1} on the control non-cancer cell line. Significant cytotoxic activity was observed on Caco-2 cell lines on exposure to both compounds with IC_{50} values of 54 μg mL^{-1} and 81 μg mL^{-1} for β-amyrin and β-Sitosterol-3-O-glucoside, respectively. Our results showed that β-Sitosterol-3-O-glucoside was more potent against Caco-2 cells than β-amyrin which is in agreement with previously published studies (Jayaprakasha *et al*., 2010). Statistical analysis carried out using ANOVA with subsequent LSD post hoc test revealed that both compounds had significantly different activity across all the concentrations with sitosterol-3-O-glucoside showing higher activity. However sitosterol-3-O-glucoside and β-amyrin compounds had no significant cytotoxic effect on HepG2 cells with IC_{50} values of 206 μg mL^{-1} and 251 μg mL^{-1}, respectively at 95% confidence level. β-Sitosterol-3-O-glucoside exhibited significant cytotoxicity against Caco-2 cell line as compared to the control untreated cells. Statistical evaluation revealed low cytotoxicity of β-amyrin across all cell lines which is consistent with published reports on the weak anticancer activity of some pentacyclic triterpenes stating that derivatives of these compounds may improve activity (Barros *et al.,* 2011).



β-amyrin



β-Sitosterol-3-O-glucoside

Figure 3.2: MTT cell viability assay of β-amyrin and β-Sitosterol-3-O-glucoside on HEK293, HepG2, and Caco-2. Data is represented as mean ±SD (n=3). Greater cytotoxicity was observed in the Caco-2 cell line ($p \leq 0.05$).

Table 3.2: IC_{50} Values of β-amyrin and β-Sitosterol-3-O-glucoside on HEK293, HepG2 and Caco-2 cell lines.

CELL LINES	β-amyrin	β-Sitosterol-3-O-glucoside
	IC_{50} values in mg mL^{-1}	
HEK293	156	937
HepG2	206	251
Caco-2	81	54

3.3.4 Apoptosis studies

Apoptosis studies were conducted to investigate the ability of test compounds to induce apoptosis in the cancer cell lines. Dual staining with acridine orange and ethidium bromide is widely used in studying nuclear cell morphology in apoptotic cells. Examination of HepG2 and Caco-2 cell lines upon exposure of 10 μg mL^{-1} of each compound for 24 hours revealed that unlike the untreated controls, the treated cells showed features characteristic of apoptosis. Live cells appeared green, early apoptotic bright green or yellow and late apoptotic cells red/orange. Observed in treated cells was chromatin condensation, nuclear fragmentation and cell shrinkage, unlike the controls which had round homogenous nuclei. A significant reduction in viable cell numbers was also observed, suggesting that one of the causes of cell death was apoptosis. Apoptotic bodies were not observed in the non-cancer HEK293 cell line. Apoptosis indices were higher in cells treated with β-amyrin that those treated with β-Sitosterol-3-O-glucoside.

Table 3.3: Apoptotic index values of β-amyrin and β-Sitosterol-3-O-glucoside on HEK293, HepG2 and Caco-2 cell lines.

CELL LINE	Control	β-amyrin	β-Sitosterol-3-O-
	Apoptosis index		
HEK293	0.00	0.00	0.00
HepG2	0.00	0.35	0.27
Caco-2	0.00	0.33	0.13

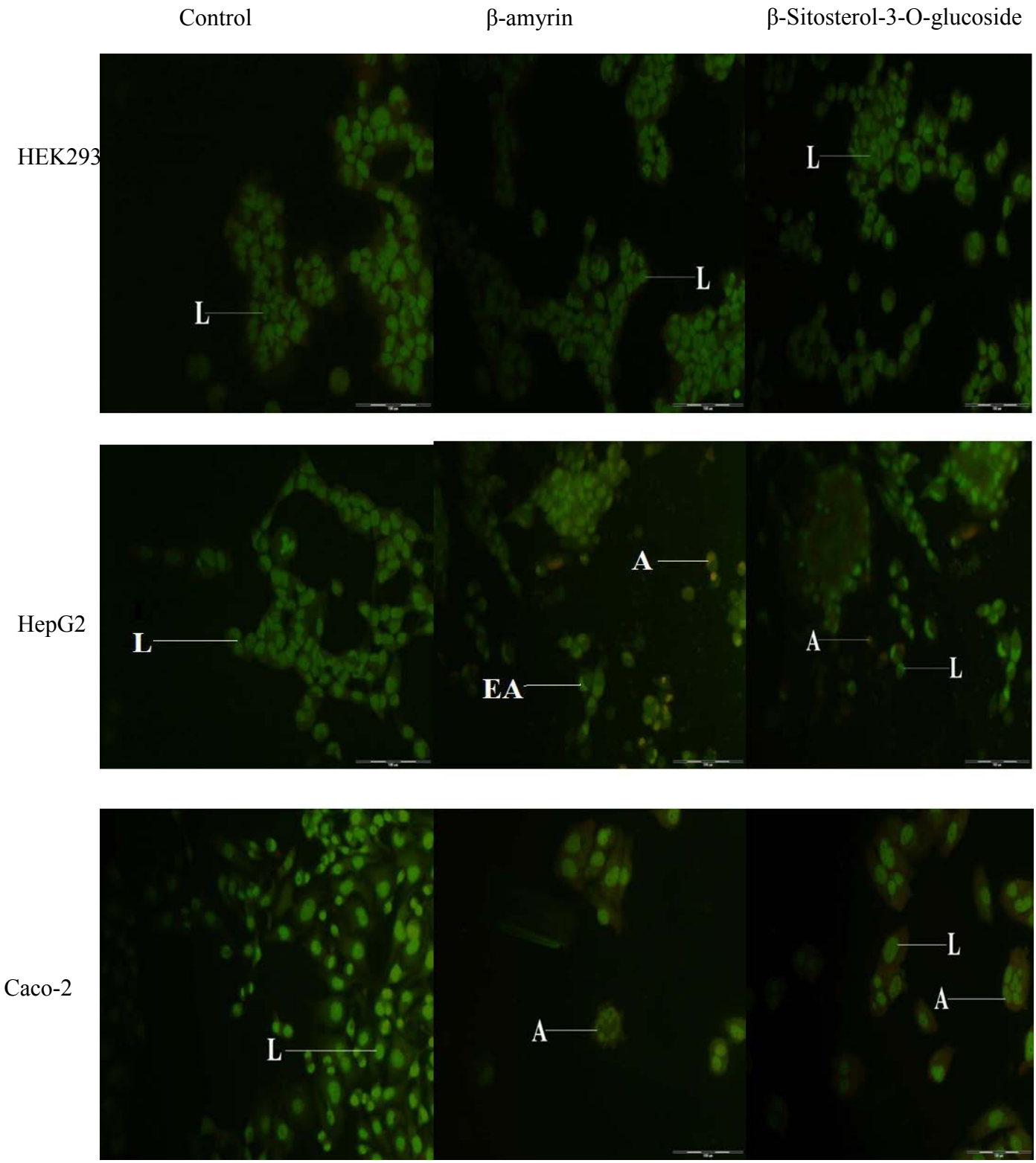


Figure 3.3: Fluorescence images of cell treated for 24 hours with β-amyrin and β-Sitosterol-3-O-glucoside at a concentration of 20 µg mL^{-1} showing induction of apoptosis on HEK293, HepG2 and Caco-2 L=Live A=Apoptotic EA= Early apoptotic. Scale is given as 100 µm.

In vitro cytotoxicity screening is a valuable tool in drug discovery and is used widely by researchers especially when bio-prospecting for potentially active cancer drugs. Triterpenes are compounds of great interest in drug discovery owing to their diverse structures and wide pharmacological applications. Diet has been implicated in the development of many cancers, including colorectal cancer (Woyengo *et al*., 2009). Phytosterols are found in most plants and have shown evidence of anticancer activity in several studies (Pegel, 1997; Bradford and Awad, 2007; Jaiprakash *et al*., 2010).

Jaiprakash and co-workers in 2010 tested the inhibition of colon cancer cell line HT-29 with sitosterol glucoside isolated from *Citrus aurantium* which demonstrated potent activity with an IC_{50} value of 40 µM. β-amyrin esters, previously studied, exhibited potent cytotoxic activity against HL-60 leukemia cells with IC_{50} values of 1.8 to 5 µM. These compounds also induced apoptosis by activating caspase 3 (Barros *et al*., 2011). In this study, we isolated one pentacyclic triterpene and further identified its structural isomer using GC-MS analysis. We also isolated two plant sterols and identified another two by GC-MS analysis.

In our findings, β-Sitosterol-3-O-glucoside and β-amyrin demonstrated dose dependent cytotoxicity across all cell lines with low cytotoxicity observed on HEK293 and HepG2 cell lines. In both compounds, greater activity was observed on the colon cancer cell line Caco-2 rather than the hepatocellular carcinoma cell line HepG2, with β-Sitosterol-3-O-glucoside showing slightly higher activity (IC_{50} 54 mg mL^{-1}) than β-amyrin (IC_{50} 81 mg mL^{-1}).

Apoptosis is programmed cell death and is characterized by nuclear condensation and fragmentation, cell shrinkage, chromatin condensation and membrane blebbing (Hunter *et al.,* 2007). Apoptotic cells are eventually phagocytised *in vivo* by macrophages. Thus no

inflammation occurs. However, apoptotic cells cultured *in vitro* ultimately undergo secondary necrosis due to the absence of phagocytes. Impaired apoptosis through up or down regulation of apoptotic proteins is implicated in a number of diseases including autoimmune and neurodegenerative diseases and in this case cancer. Mutation and down regulation of *p53* are reported in most cancers, including colon and liver cancer (Jaiprakash *et al*., 2010). The molecular players in apoptosis are the caspases and Bcl-2 family proteins which upon activation initiate apoptosis through the intrinsic or extrinsic pathway. Bax/Bcl-2 proteins ratio is an important indicator of how a cell will respond to apoptosis induction.

To further investigate the influence of isolated compounds on apoptosis AO/EB, dual staining was conducted on the three cell lines upon exposure to test compounds. Both compounds tested induced apoptosis in Caco-2 cell line with high apoptosis indices of 0.33 and 0.13 compared to the control. Reduced cell numbers was also observed on this cell line on treatment with the two compounds. This was, however, not observed in HepG2 and HEK293 cell lines which both showed low apoptotic indices upon exposure to the compounds for 24 hours. This was in agreement with the studies done by Woyengo *et al* (2009) who reported apoptosis induction by β-sitosterol and Park *et al* (2007) who reported that β-sitosterol inhibited cellular proliferation and induced apoptosis in U937 leukemia cells by down regulation of the pro-apoptotic protein Bcl-2 and activation of caspase 3. This suggests that apoptosis induction by these compounds occurs via the intrinsic pathway. This has also been reported in stomach (Zhao *et al*., 2009) and colon cancer.

GC-MS studies of *P. africana* led to the identification of a number of compounds some of which have reported anti-cancer activity. Triterpenes, phytosterols and phenolic compounds were identified in the hexane and DCM extracts by GC-MS. Vanillin, a widely used flavouring agent

was identified and this compound is also reported to successfully inhibit proliferation of HT-29 colon cancer cells by inducing apoptosis (Ho *et al*., 2009). Studies on phytol, also identified in the extracts, report significant cytotoxicity on nasopharyngeal carcinoma (KB) cell line (23 µg mL^{-1}) (Malek *et al.,* 2009).

3.4 Conclusion

This study investigated the secondary metabolites of *P. africana* and their potential in cancer treatment by studying the *in vitro* cytotoxic and apoptotic activity on cancer cells. The pentacyclic triterpene, β-amyrin and a sterol, β-sitosterol with its glycoside, β-sitosterol-3-O-glucoside were isolated from the leaves and bark of the plant. The MTT cell viability study of β-amyrin and β-Sitosterol-3-O-glucoside showed β-sitosterol-3-O-glucoside to be the more active of the two compounds against the Caco-2 cell line. Both compounds were, however, inactive against the HepG2 cell line. Dual staining with ethidium bromide and acridine orange confirmed apoptosis induction as a mode of cell death with β-amyrin exhibiting higher apoptotic indices in both cancer cell lines than β-sitosterol-3-O-glucoside. The compounds tested showed selective cytotoxic and apoptotic activity as evidenced by the high IC_{50} values and low apoptotic indices on HEK293, the control non-cancer cell line. From the compounds isolated and those identified using GC-MS analysis, the plant appears to be rich in phenolics, pentacyclic triterpenes and sterols (classes of compounds that are known to be biologically active) which validates its ethno-medicinal use.

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CHAPTER FOUR

CONCLUSION

4.1 Overall summary

4.1.1 Findings from *Moringa oleifera*

Four pure compounds quercetin-3-O-glucoside (**1a**), 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2a**), β-sitosterol (**3a**) and lutein (**4a**) were isolated from *M. oleifera*. Quercetin-3-O-glucoside and β-sitosterol were isolated from the leaves, bark and seeds, lutein from the leaves and 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate from the seeds. β-sitosterol and lutein have been studied widely and their biological activities, particularly anti-cancer potential, is well documented. 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate and quercetin-3-O-glucoside were tested for their antioxidant activity using the DPPH assay and both compounds showed significant antioxidant activity with quercetin-3-O-glucoside showing higher activity than the standard ascorbic acid. This confirmed that the plant is a rich source of natural antioxidants. Cell viability studies of quercetin-3-O-glucoside and 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate showed both compounds to exhibit selective cytotoxicity on the non-cancer cell line HEK293 with low IC_{50} values. 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate showed greater cytotoxicity across both cancer cell lines compared to quercetin-3-O-glucoside. A study of apoptosis induction as a possible cause of cell death in the three cell lines revealed apoptosis to be one of the causes of cell death with selectivity for cancer cell lines. 4-(β-D-glucopyranosyl-1→4-α-L-

rhamnopyranosyloxy)-benzyl isothiocyanate has been isolated once before and, to the best of our knowledge, this is the first report of its cytotoxic and apoptosis activity on the three cell lines.

4.1.2 Findings from *Prunus africana*

Phytochemical studies on *P. africana* led to the isolation of three pentacyclic triterpenes: β-sitosterol (**1b**), β-amyrin (**2b**) and β-sitosterol-3-O-glucoside (**3b**) from the leaves and bark. GC-MS profiling of the hexane and DCM extracts of the leaves and bark led to the identification of several known pharmacologically active compounds. β-amyrin and sitosterol-3-O-glucoside were tested for their *in vitro* cytotoxic and apoptosis induction on two cancer cell lines. Both compounds showed moderate cytotoxic activity on the cancer cell lines. This plant species is facing extinction and has been declared endangered by the International Union for Conservation of Nature and Natural resources (IUCN) due to massive overharvesting of its bark for export. This study confirms that pharmacologically active compounds such as sterols and pentacyclic triterpenes in the bark are also present in the leaves. Therefore, the bark can be substituted with the leaves for treatment of BPH and cancer, thereby improving the conservation status of the plant.

4.2 General conclusions

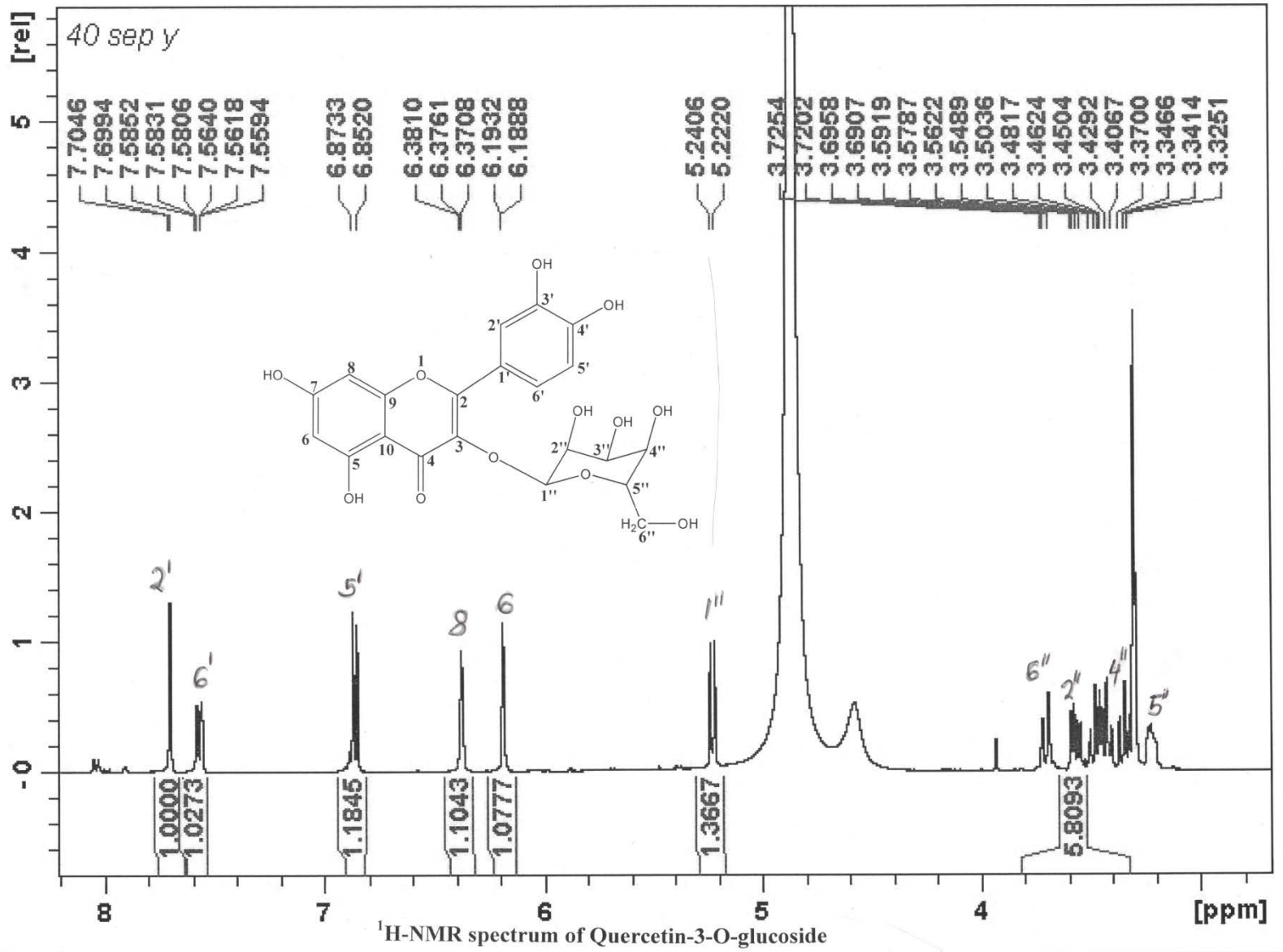
The focus of this study was to conduct a phytochemical analysis and to evaluate the anti-cancer potential of two medicinal plant species, *M. oleifera* and *P. africana,* that are commonly used for treatment of cancer in Kenya. Herbal formulations concocted from these trees are currently taken orally or used topically to treat various cancers. The highly acclaimed anti-cancer activity by traditional healers led us to this study in an effort to produce a scientific basis for its use in

traditional medicine and also to source compounds that could be important future drug leads. To a great extent, the objectives of this study have been met. Overall, the studied compounds showed varying degrees of anti-cancer activity to the cancer cell lines utilised. They have shown that they can be potential anti-cancer compounds and hence warrant further investigation.

4.3 Recommendations for further study

1. Further studies on the mechanism of action, especially with regards to apoptosis induction, should be carried out. Effects of isolated compounds on pro and anti-apoptotic proteins should be investigated.
2. Studies on the safety, dosage and efficacy of plant extracts used as well as compounds tested should be investigated.
3. Structural modification by addition of other functional groups to improve the activity of isolated compounds should be considered.
4. *In vivo* antitumour studies of the active compounds reported in this work should be considered.
5. Conservation measures of *P. africana* should be put in place by the Kenyan government by educating the public on the use of leaves instead of the bark in the preparation of herbal formulations.

APPENDIX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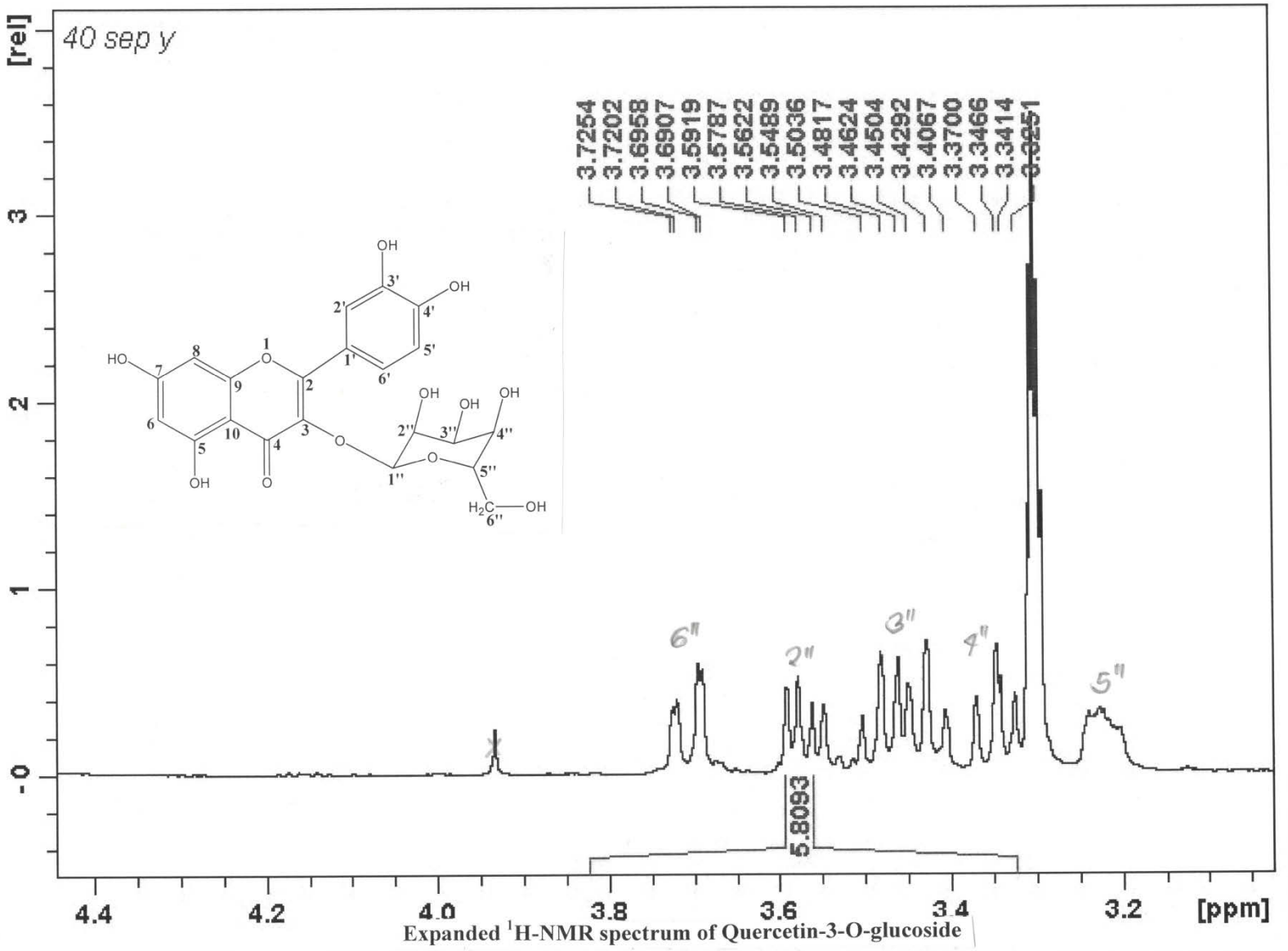

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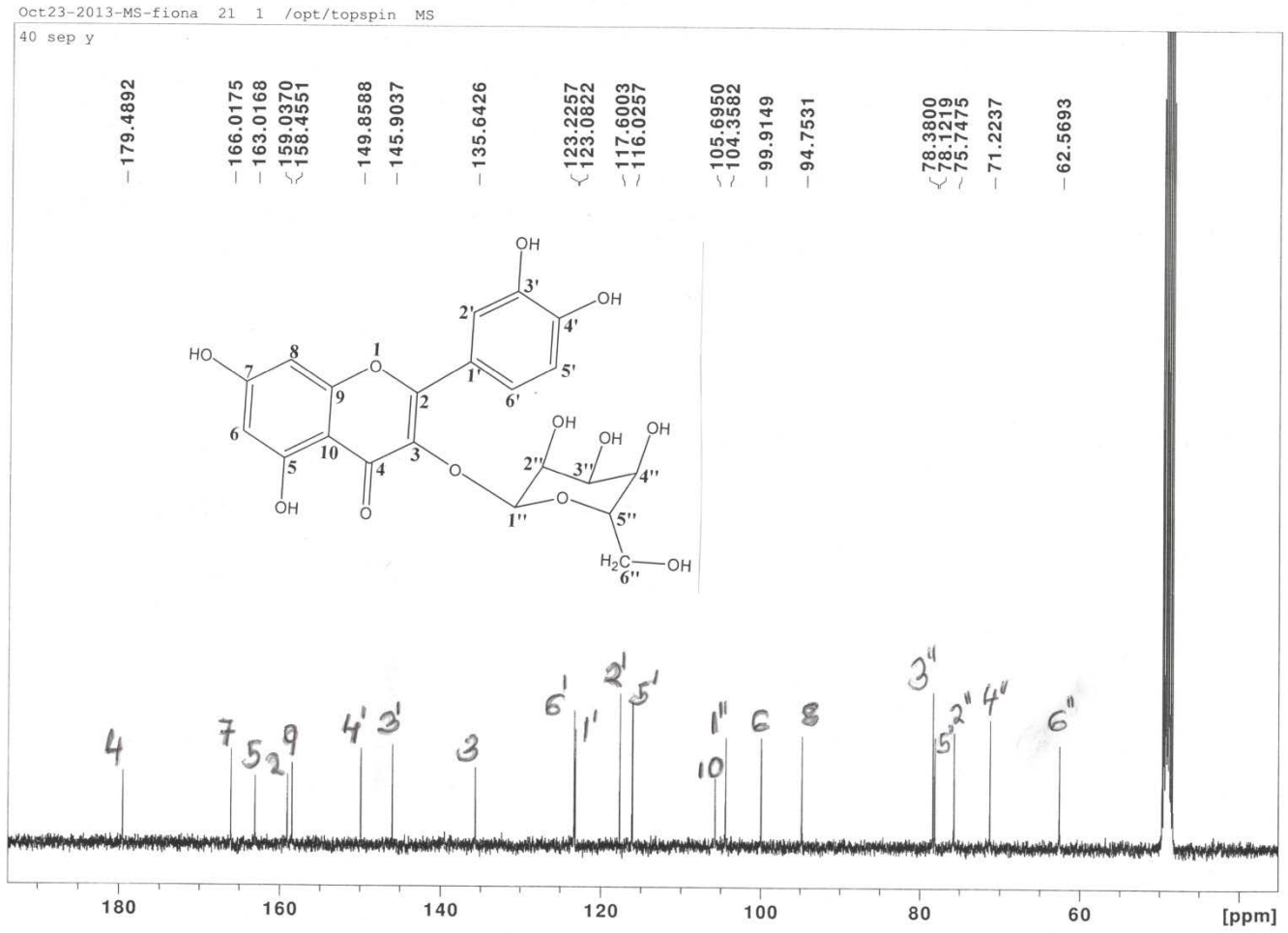

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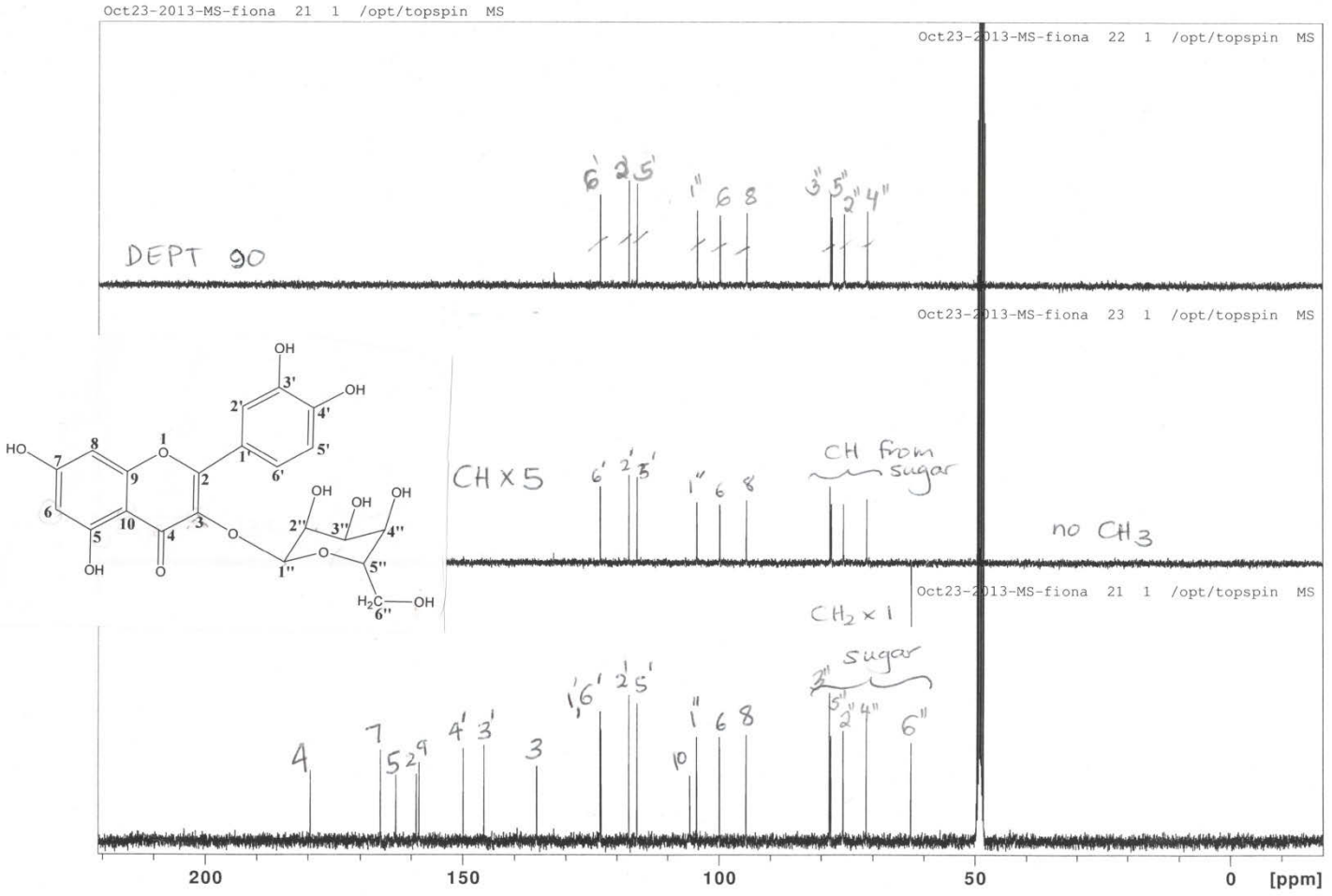

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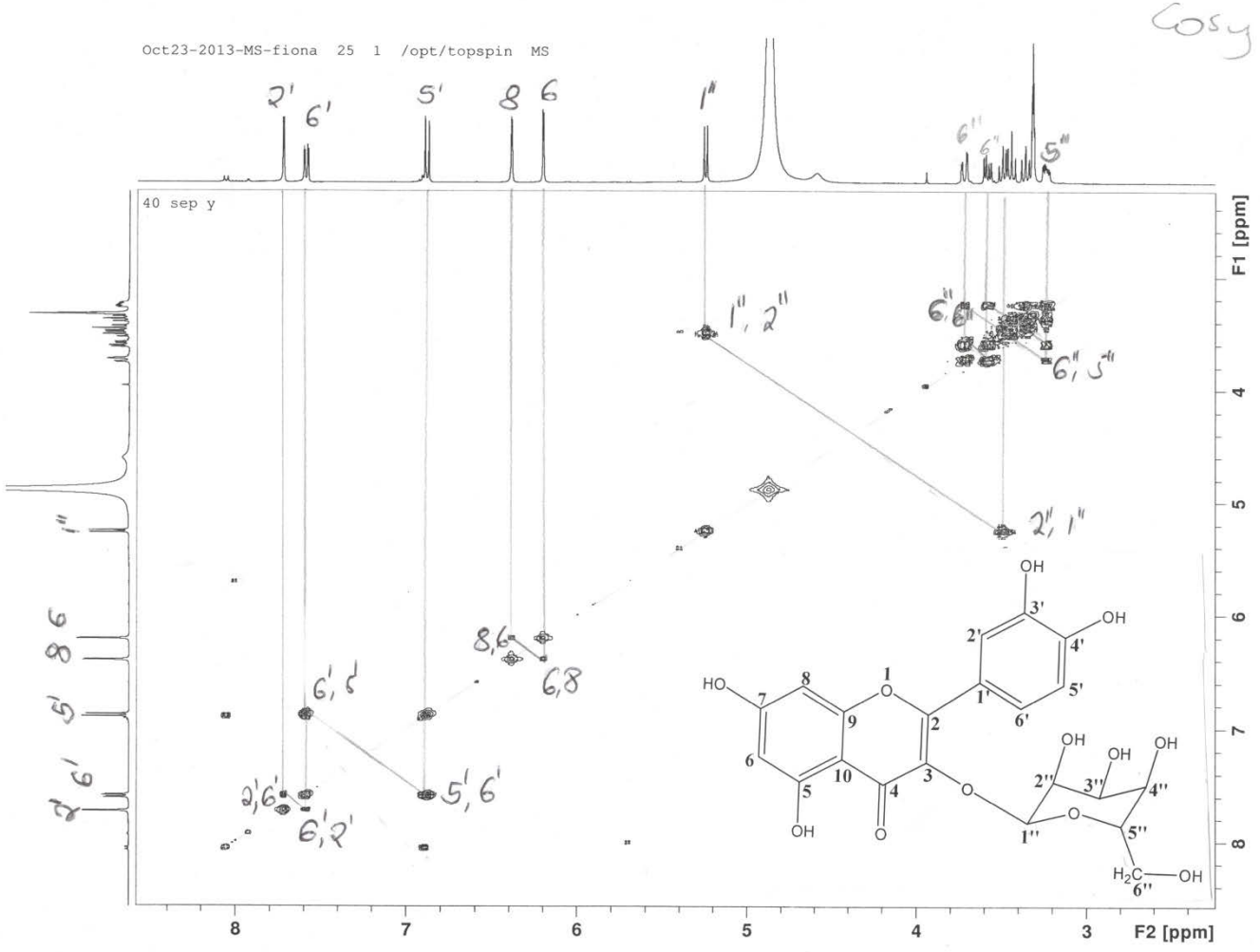

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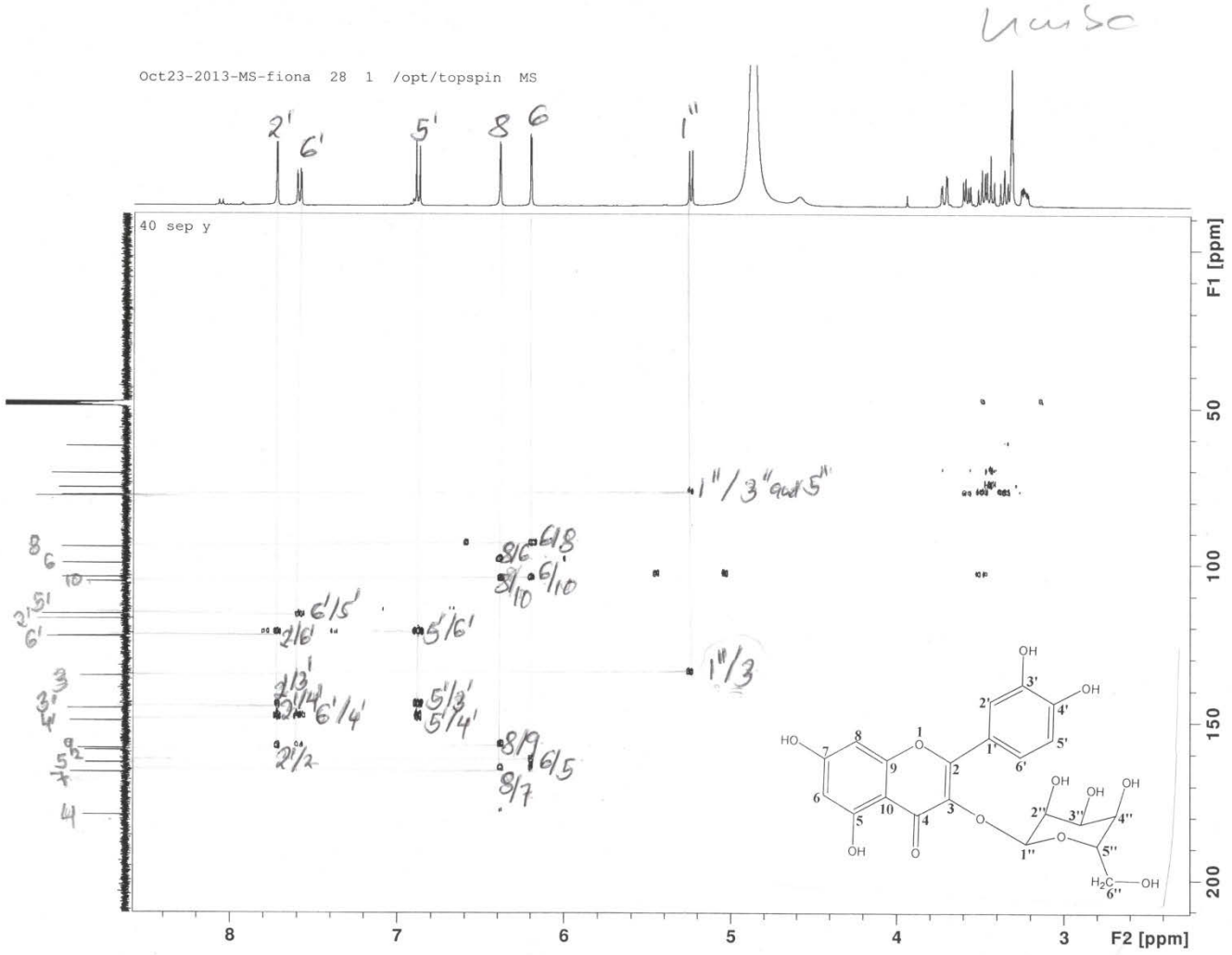

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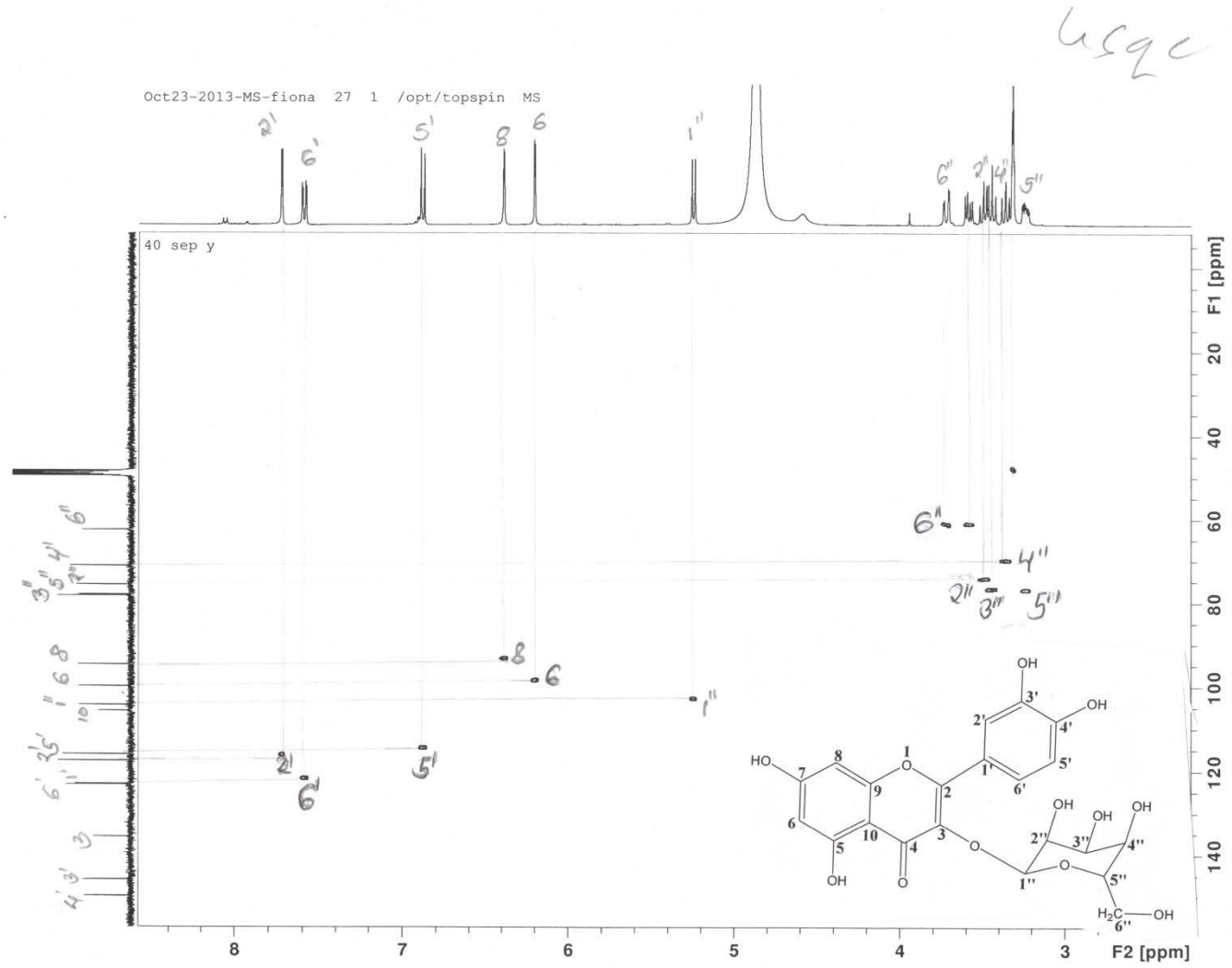

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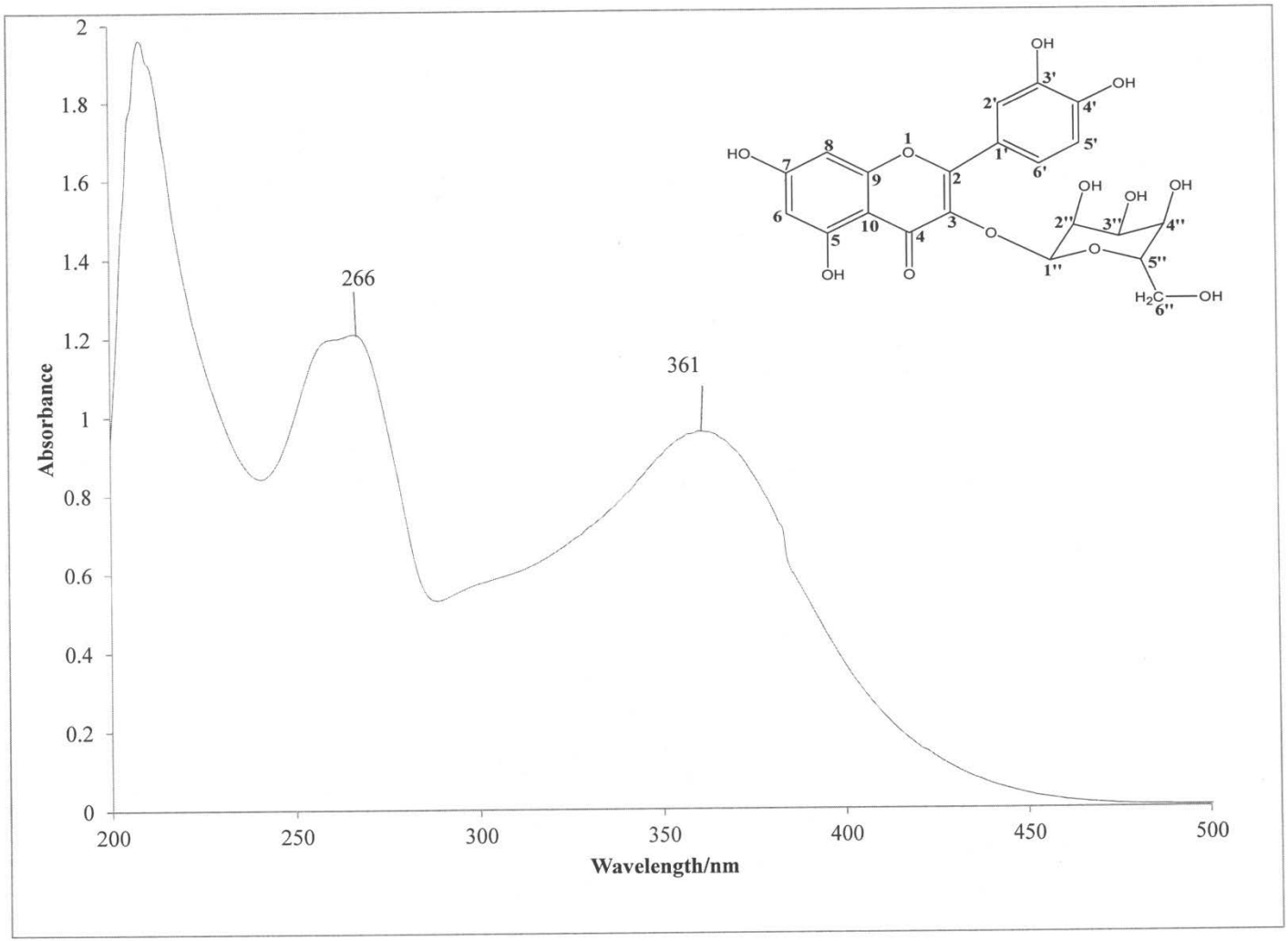

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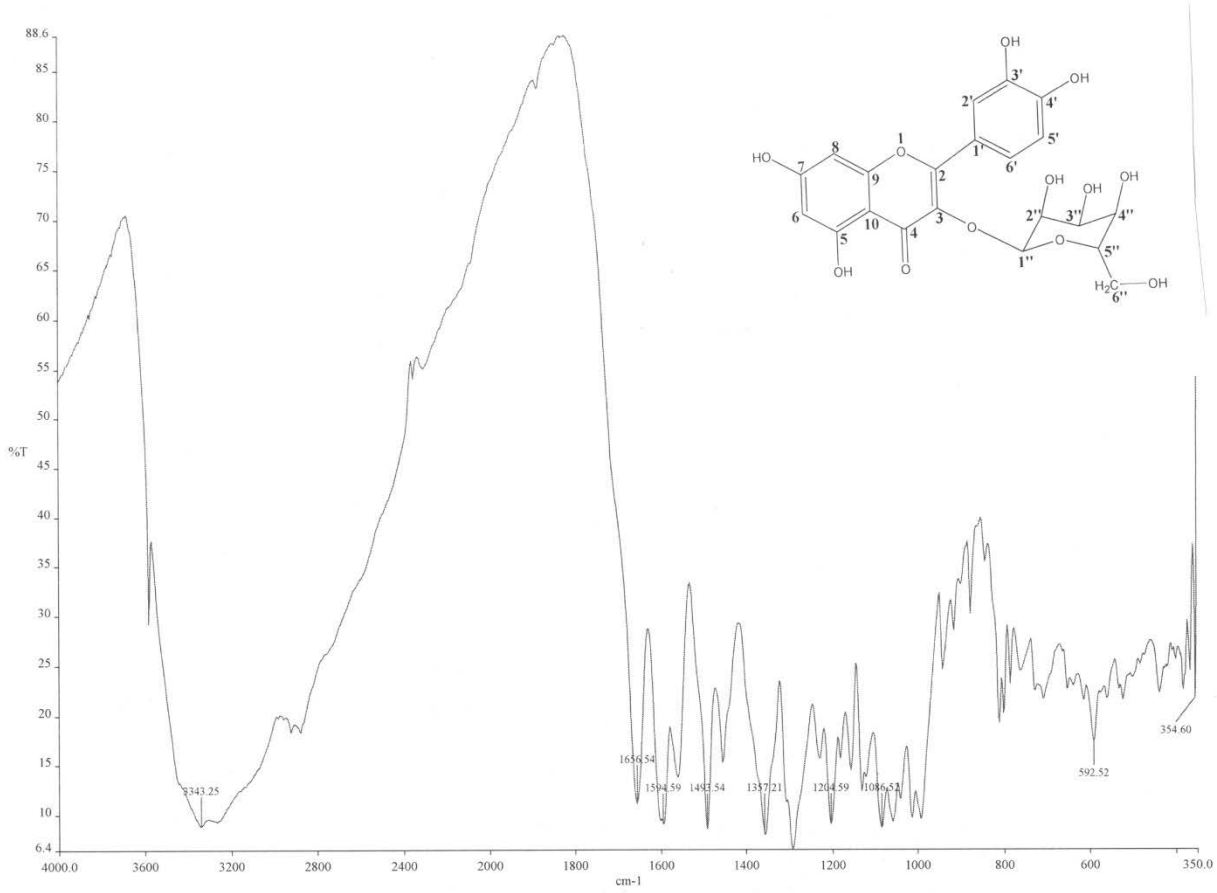

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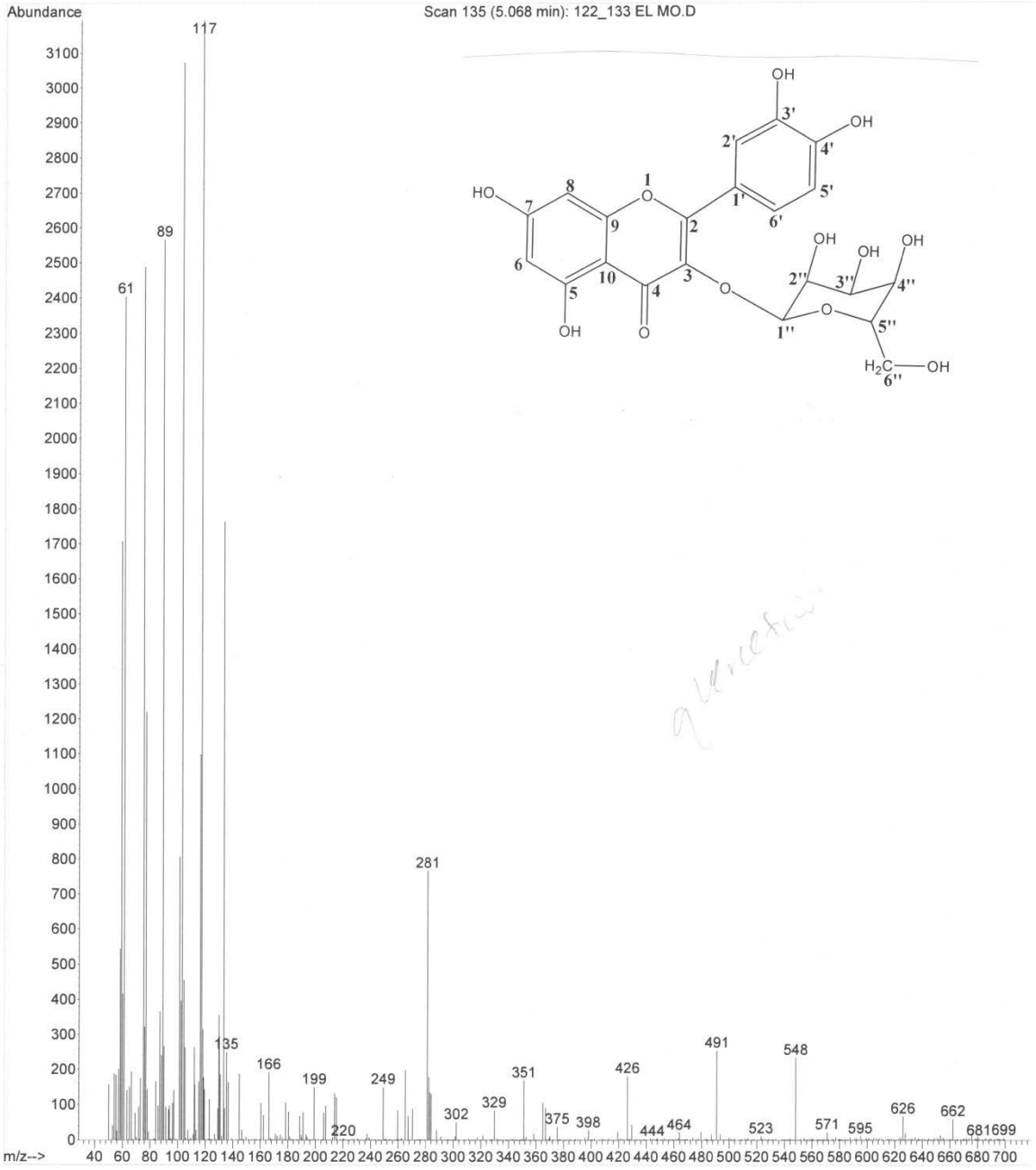

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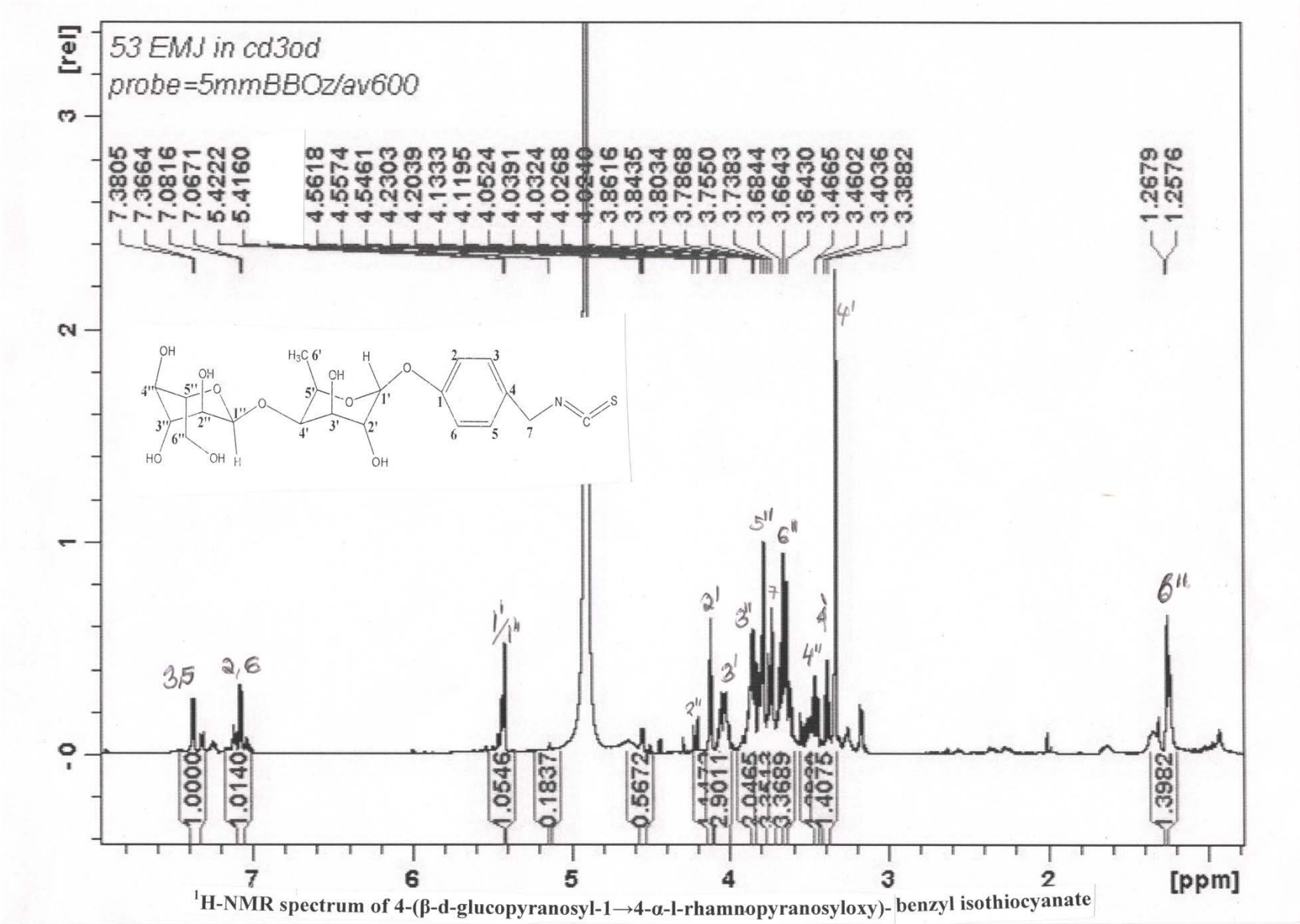

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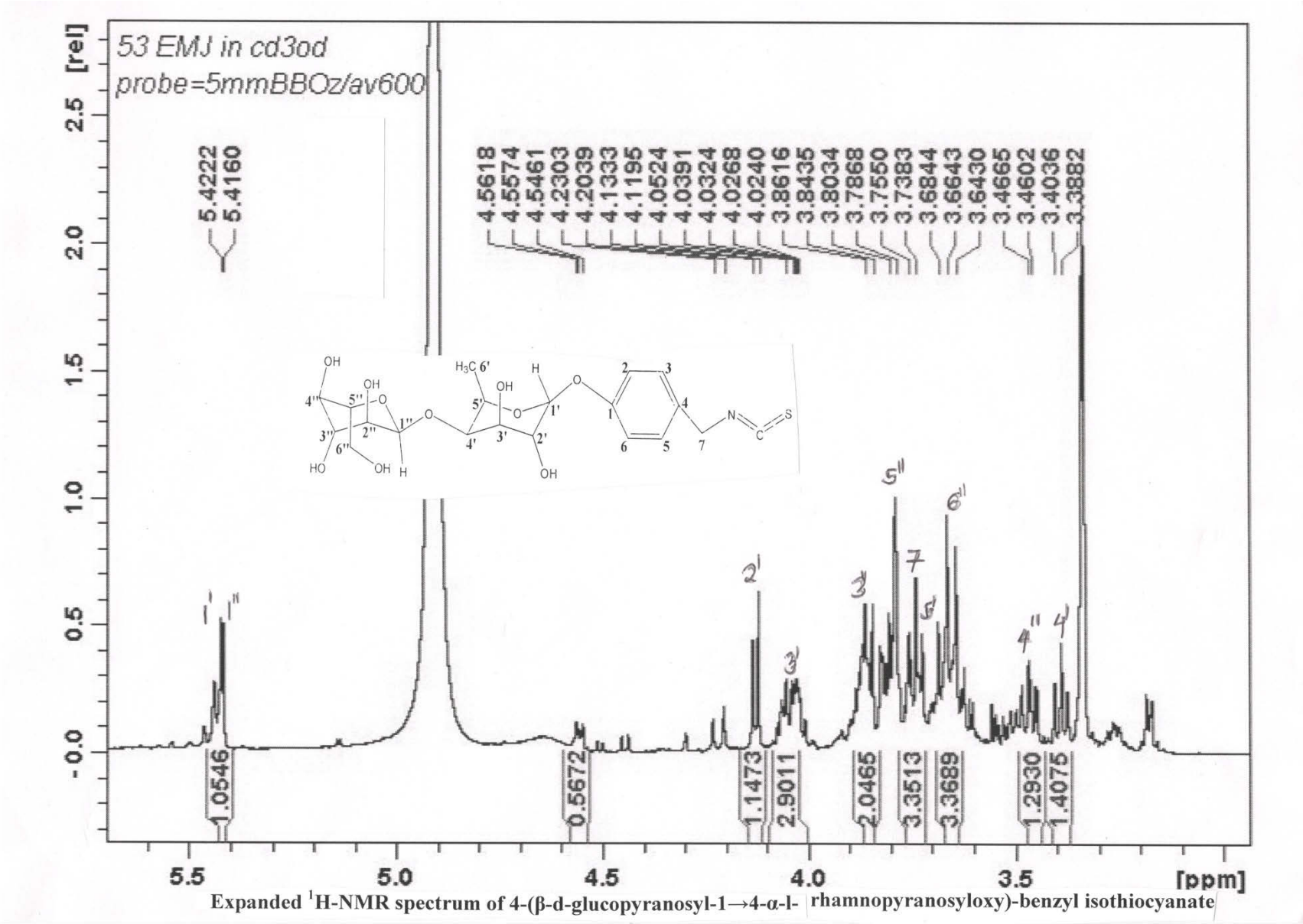

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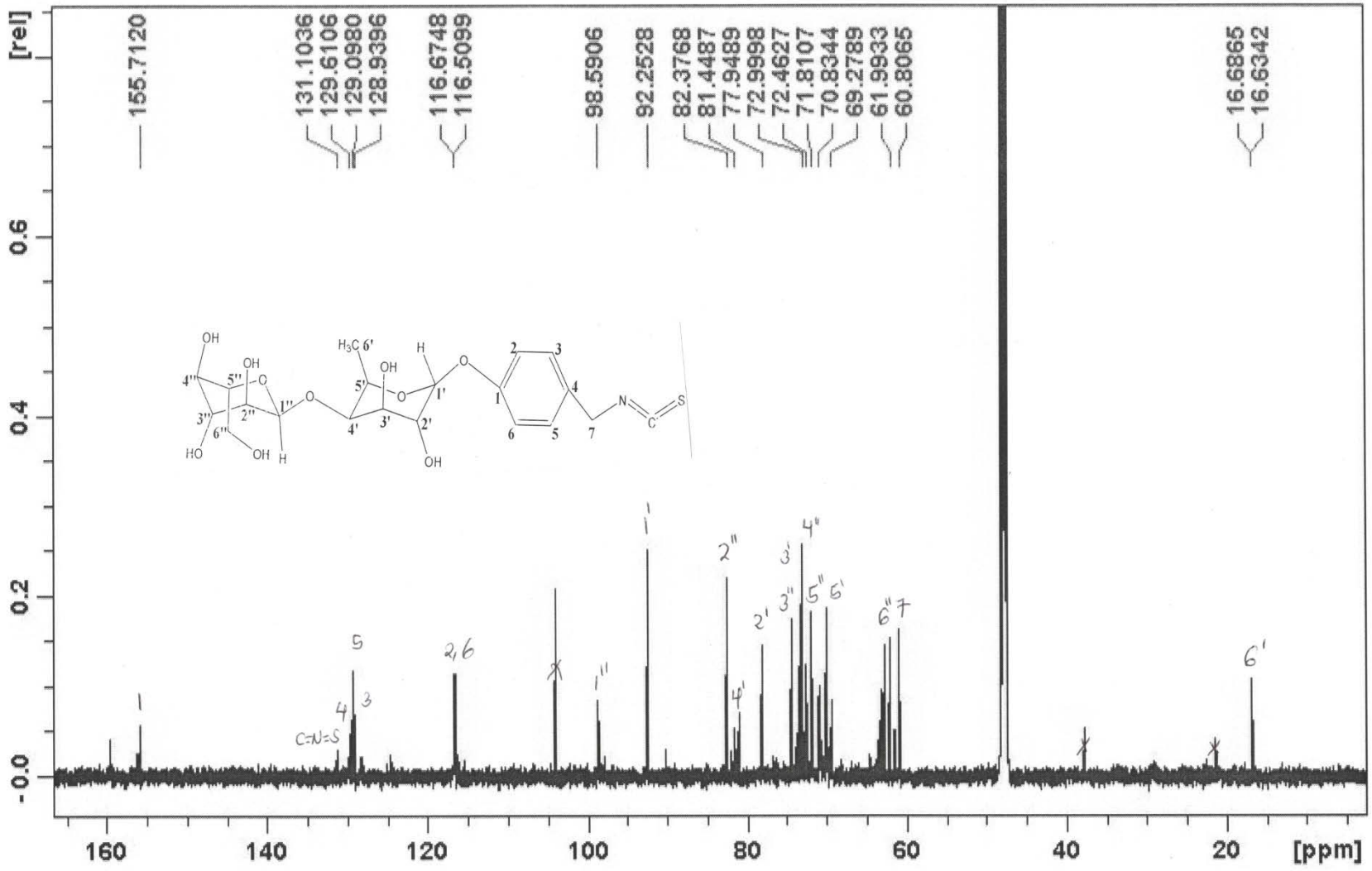

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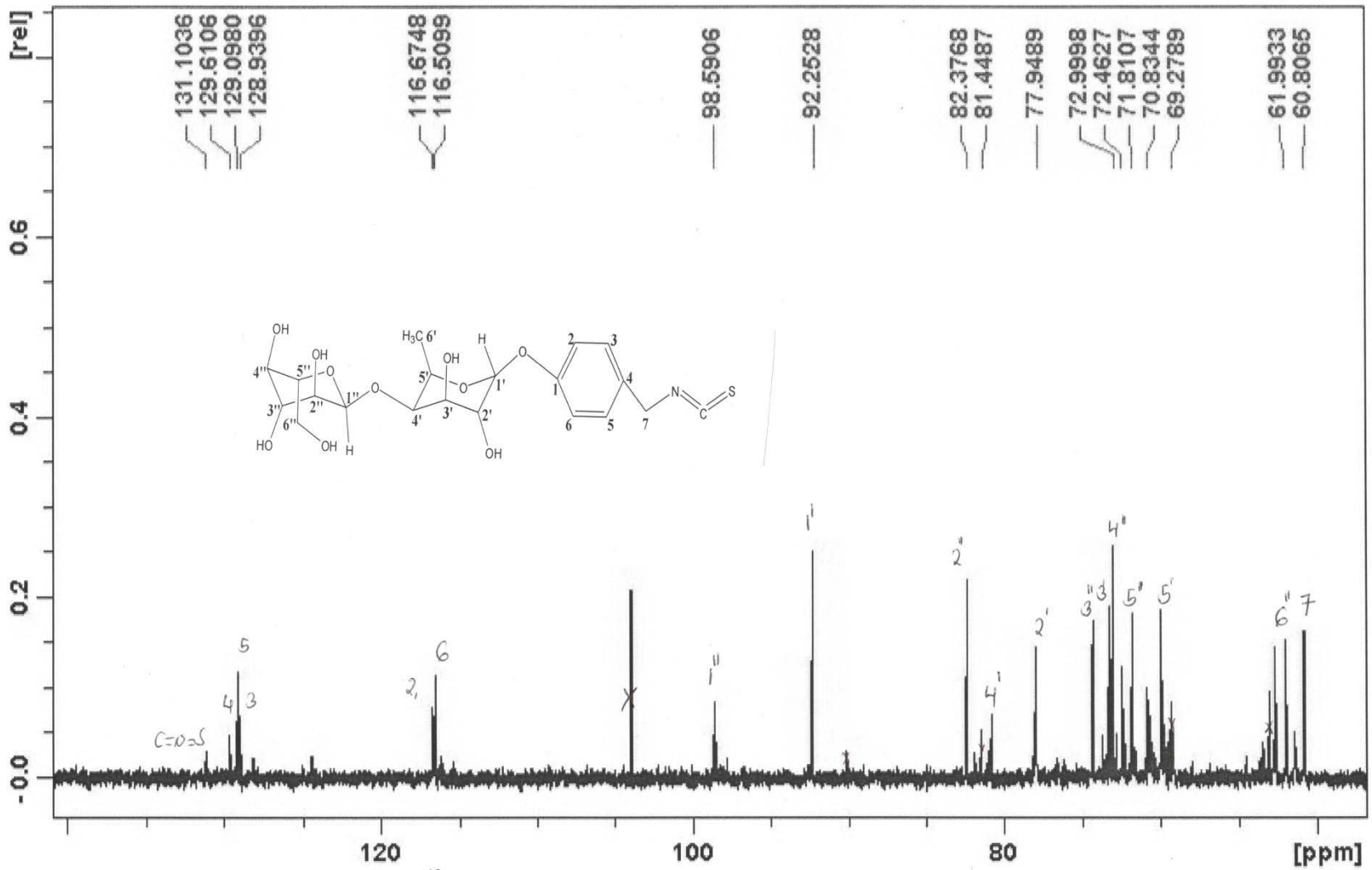

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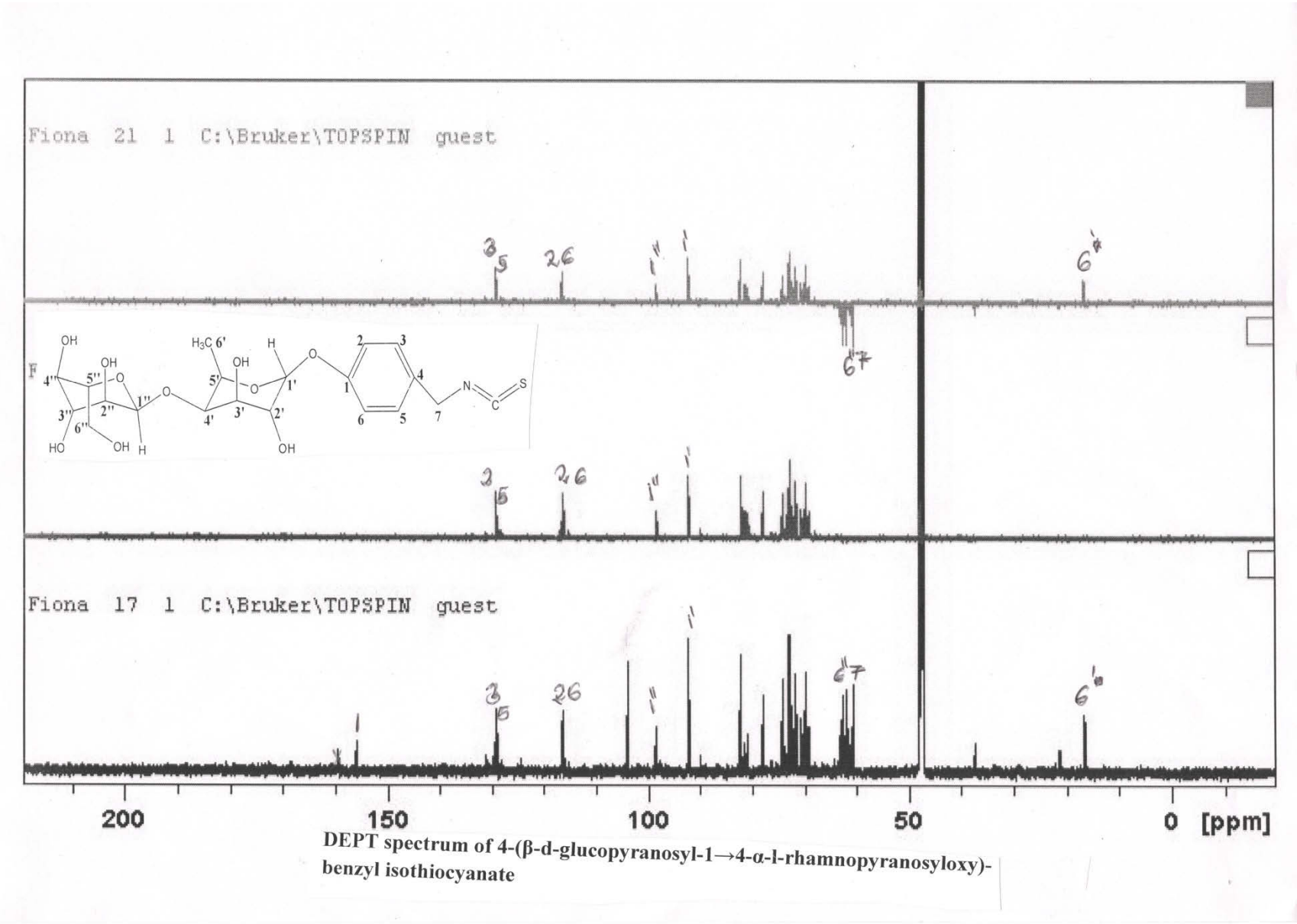

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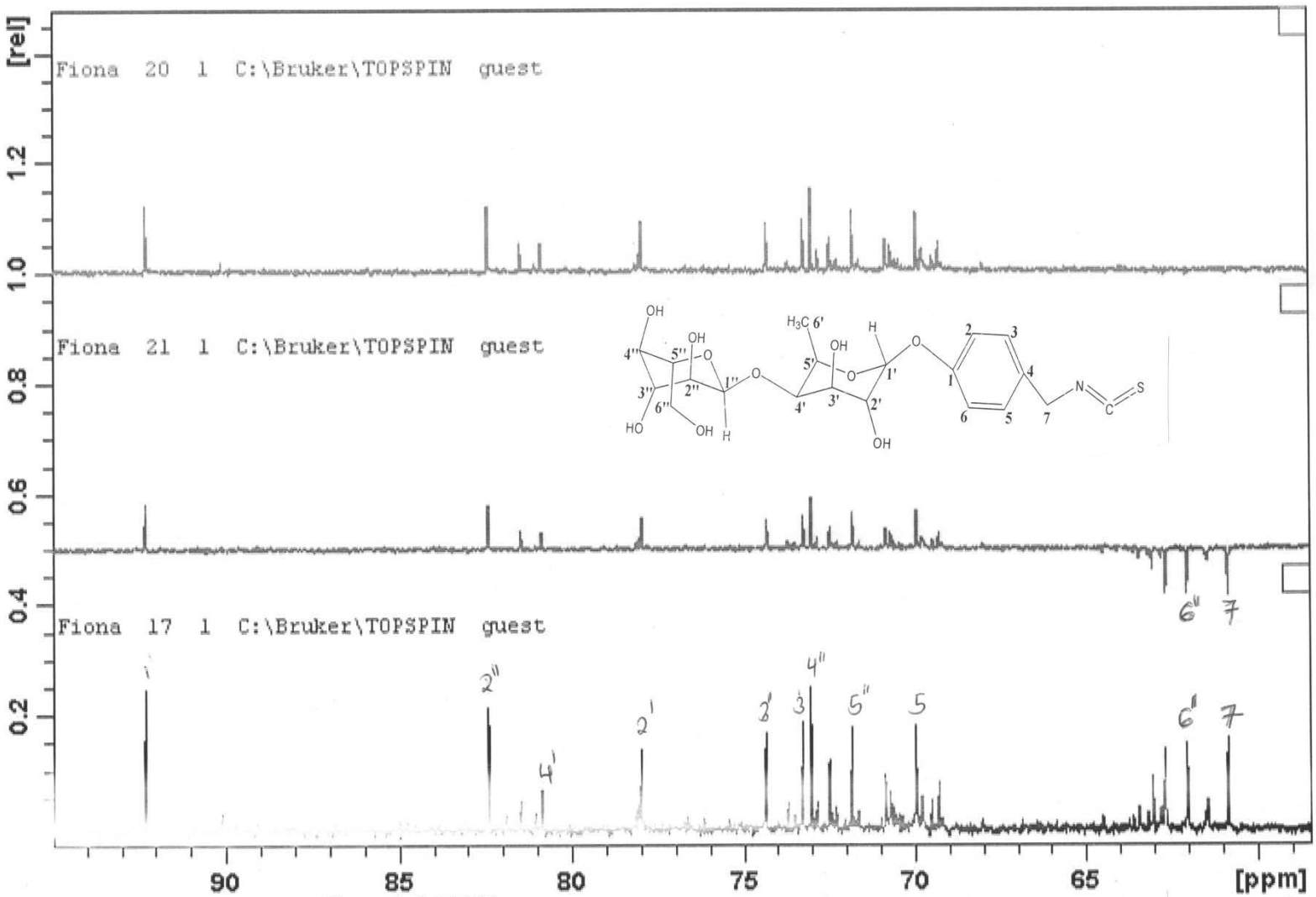

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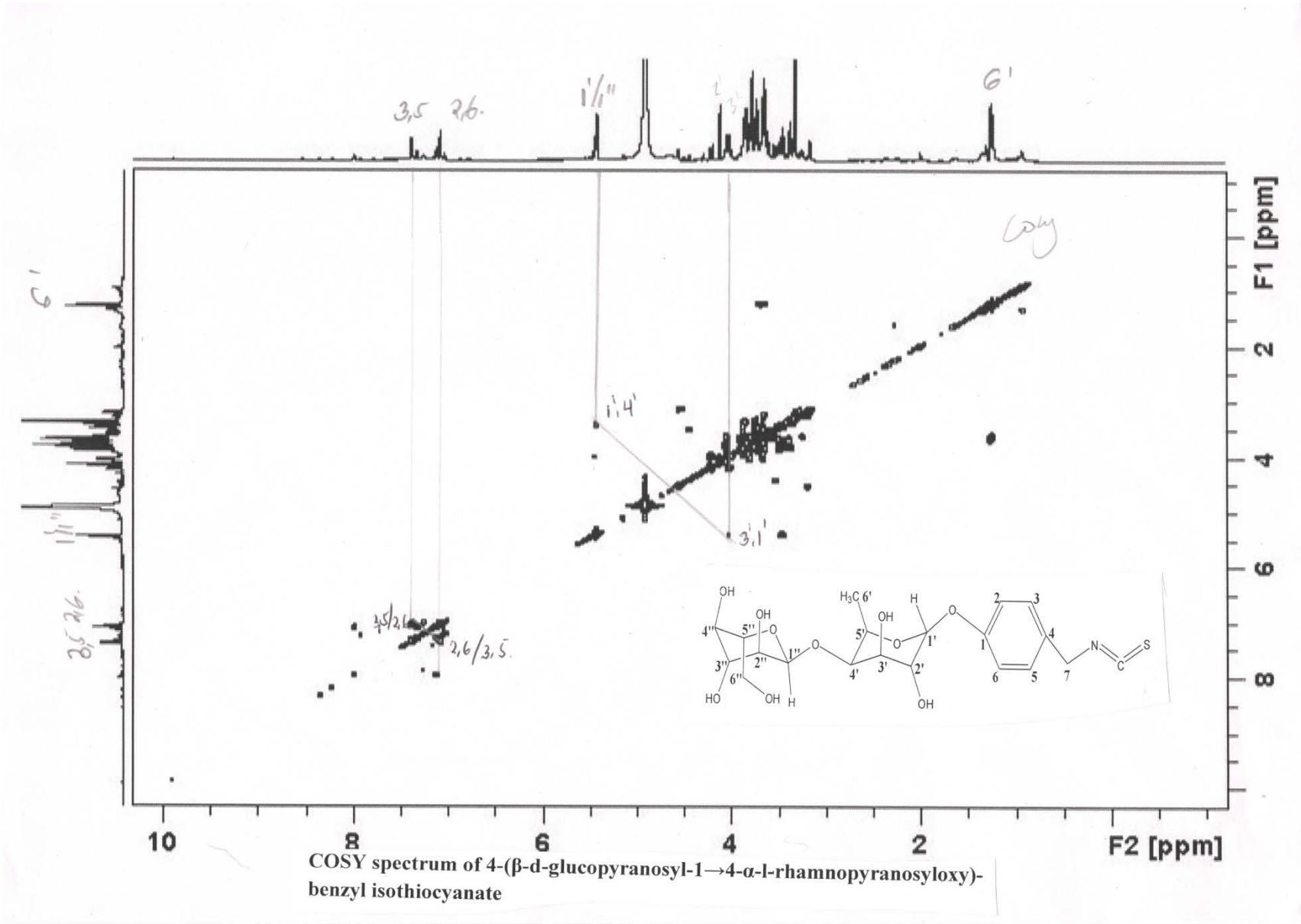

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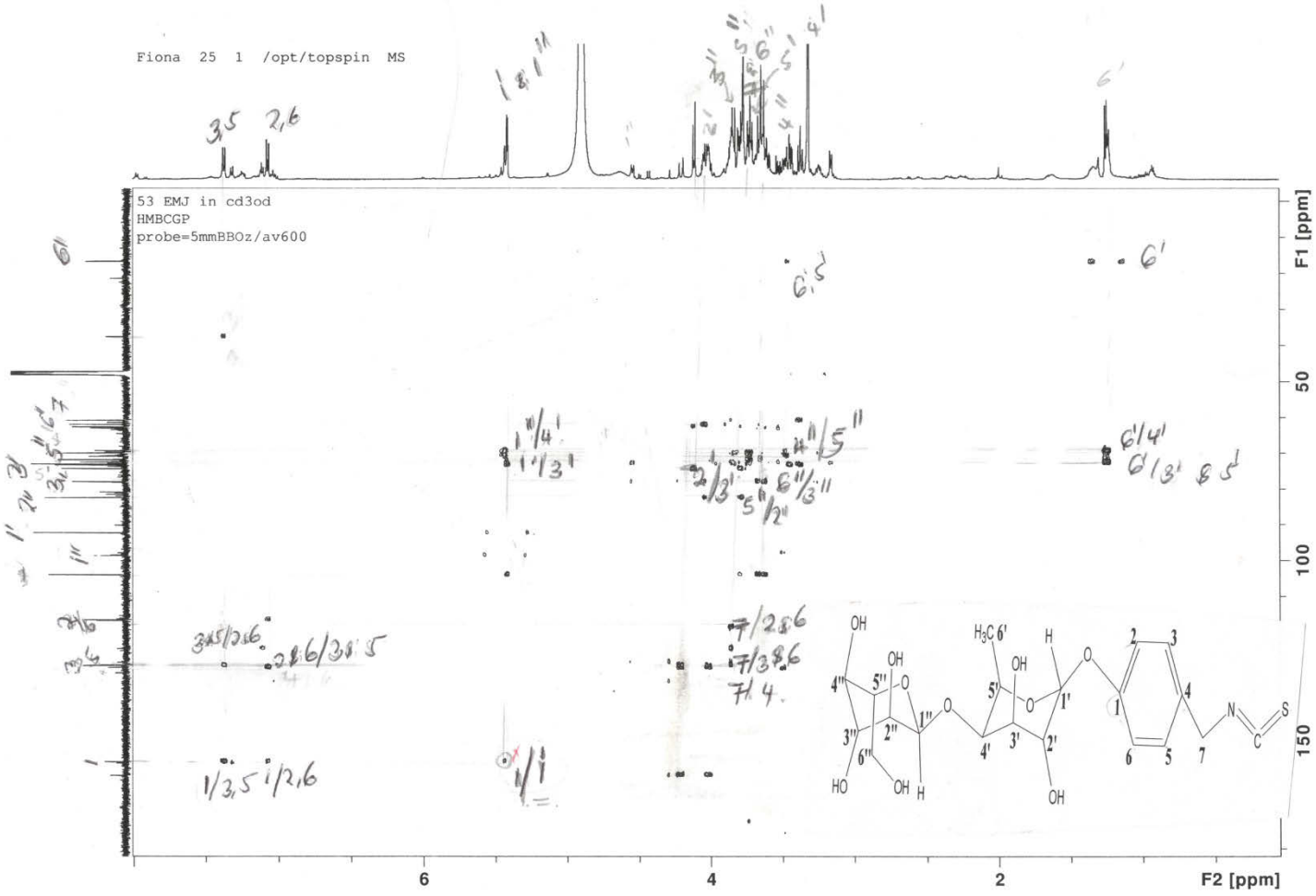

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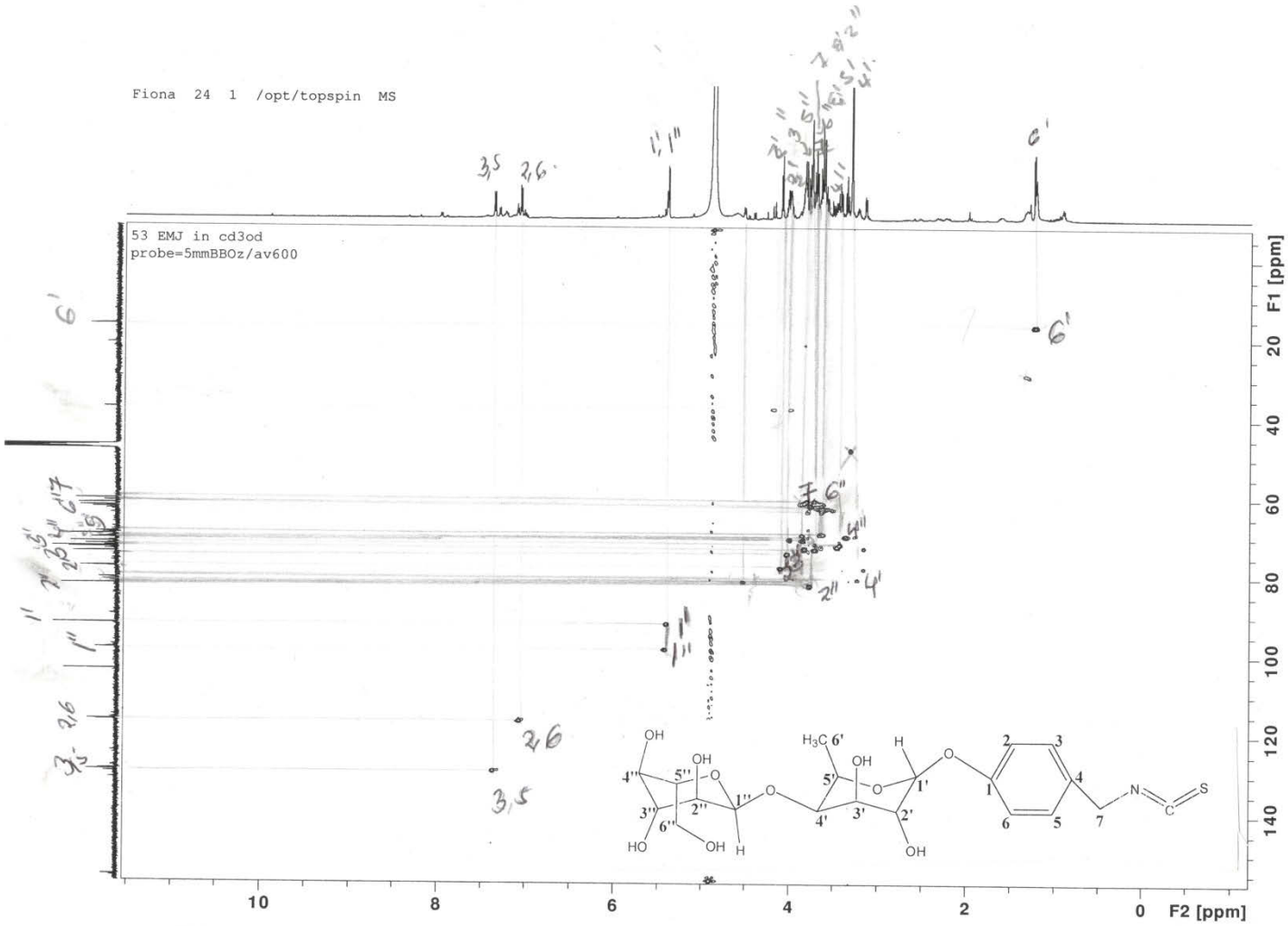

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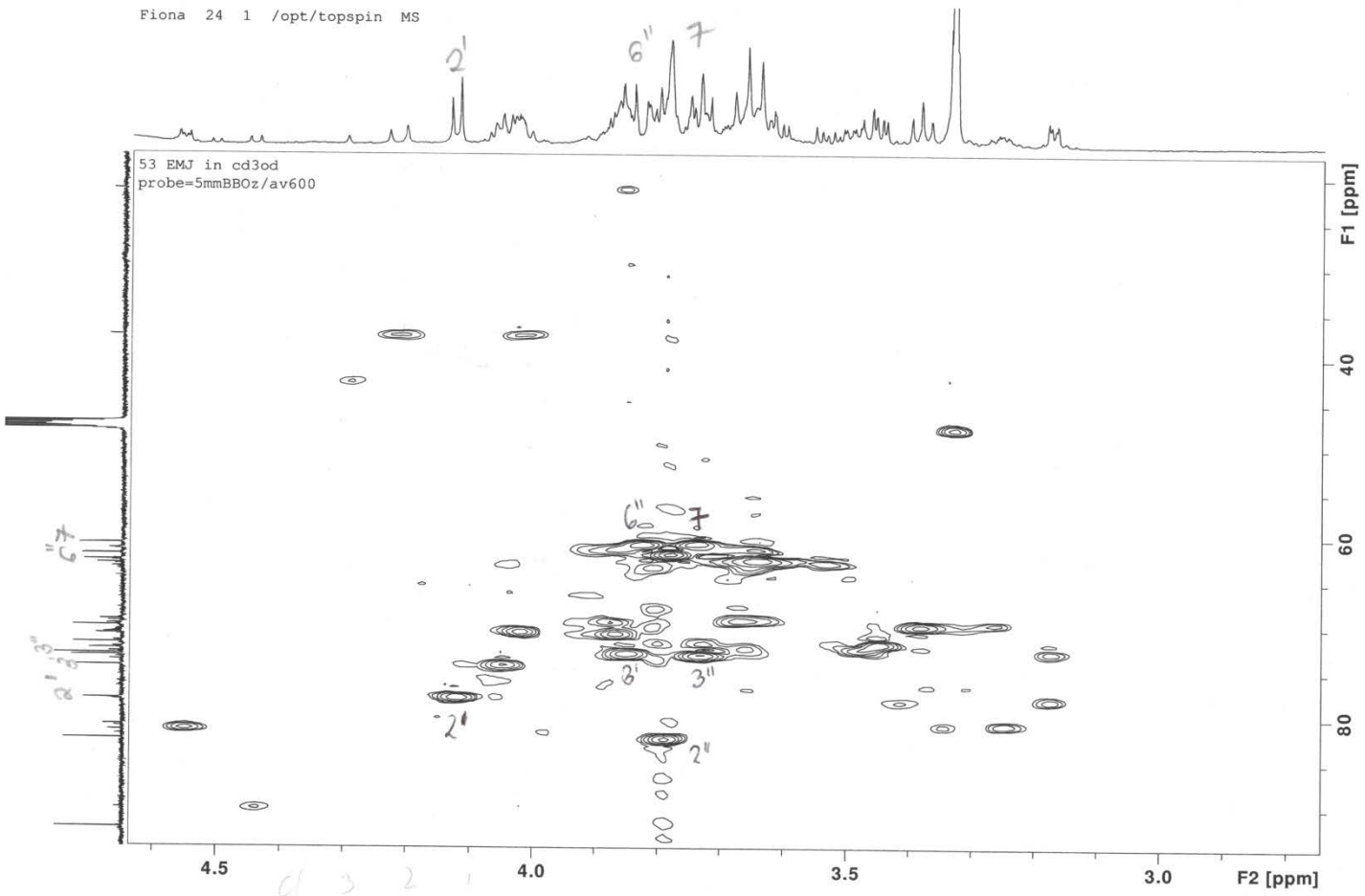

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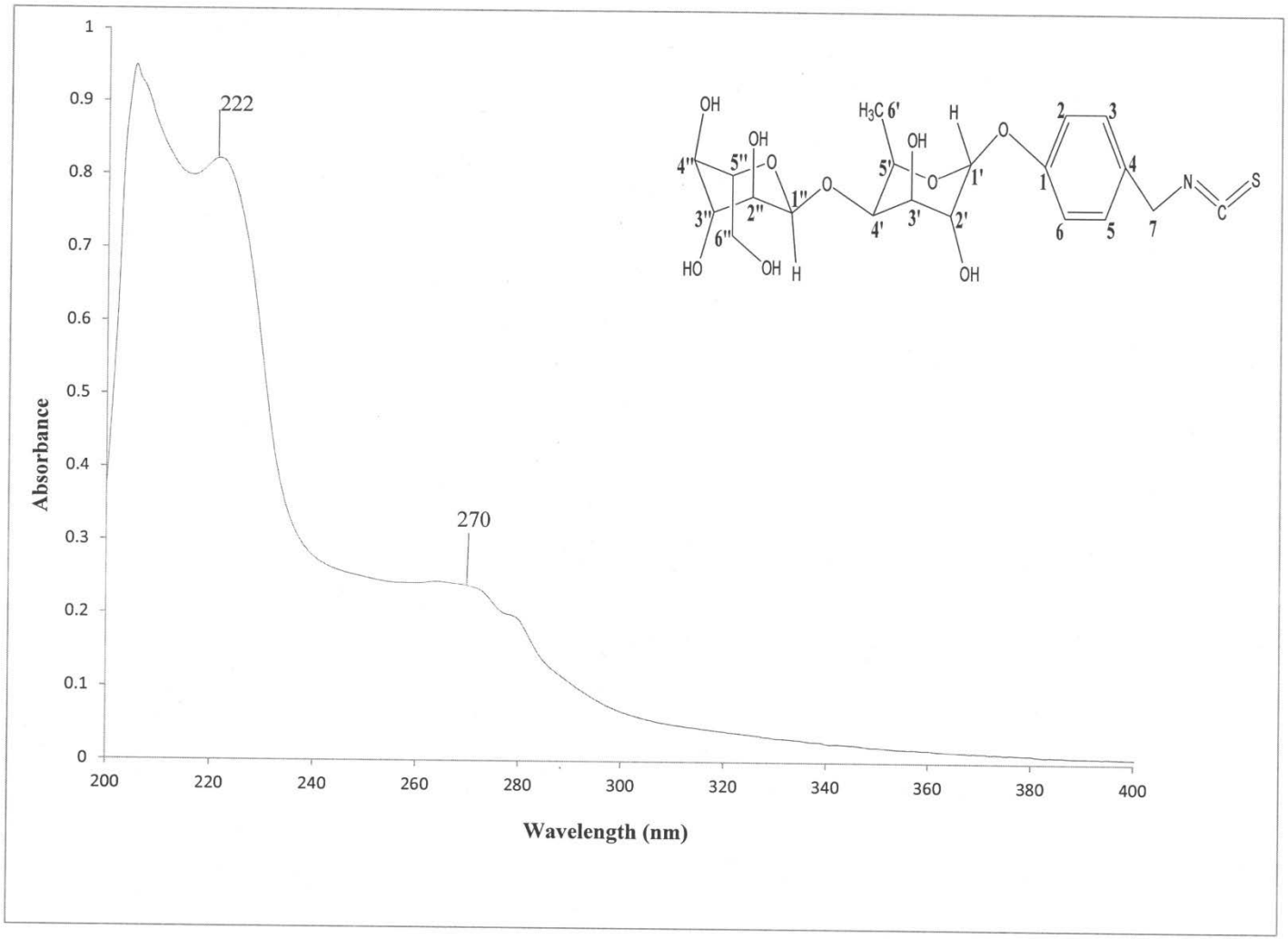

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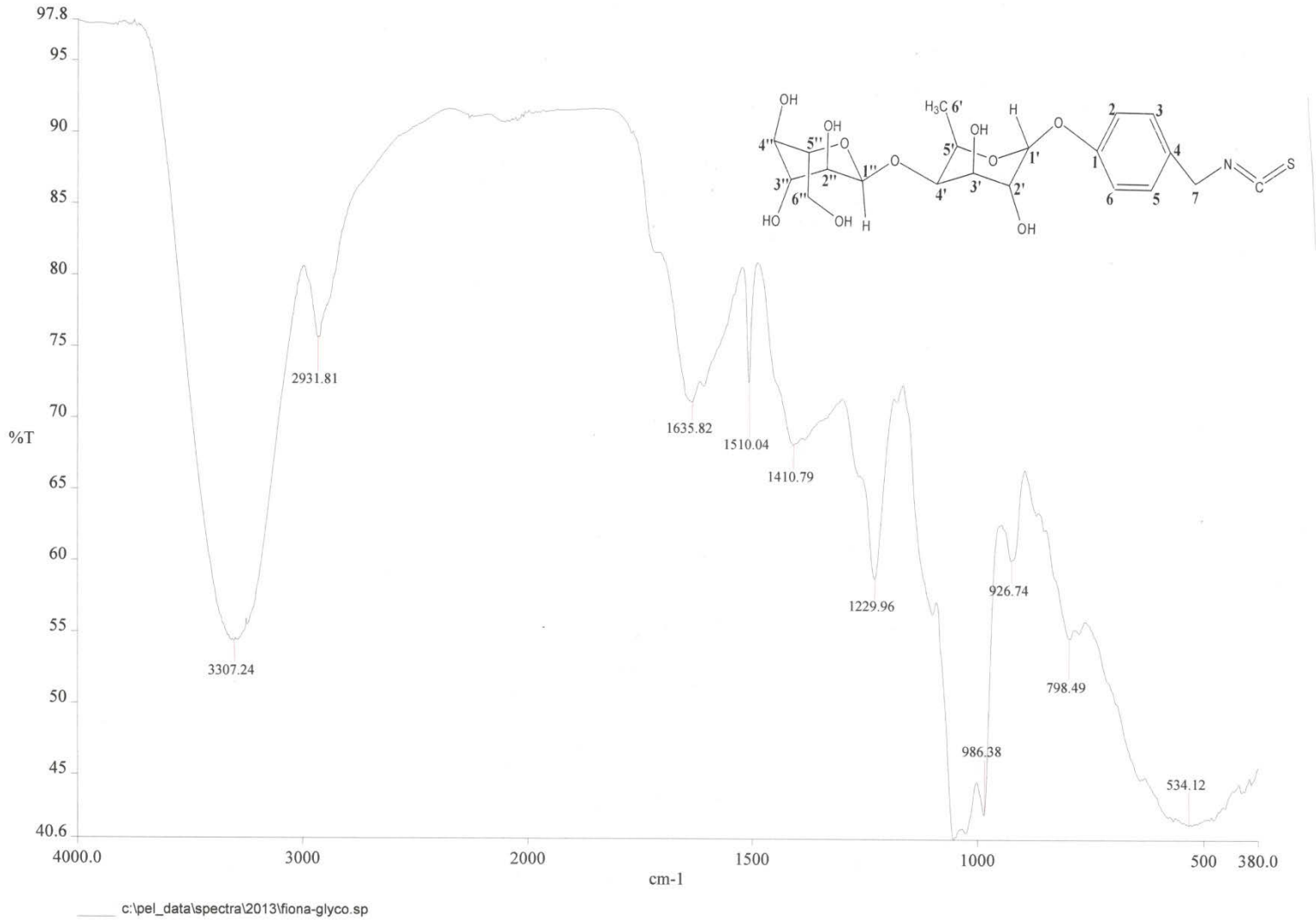

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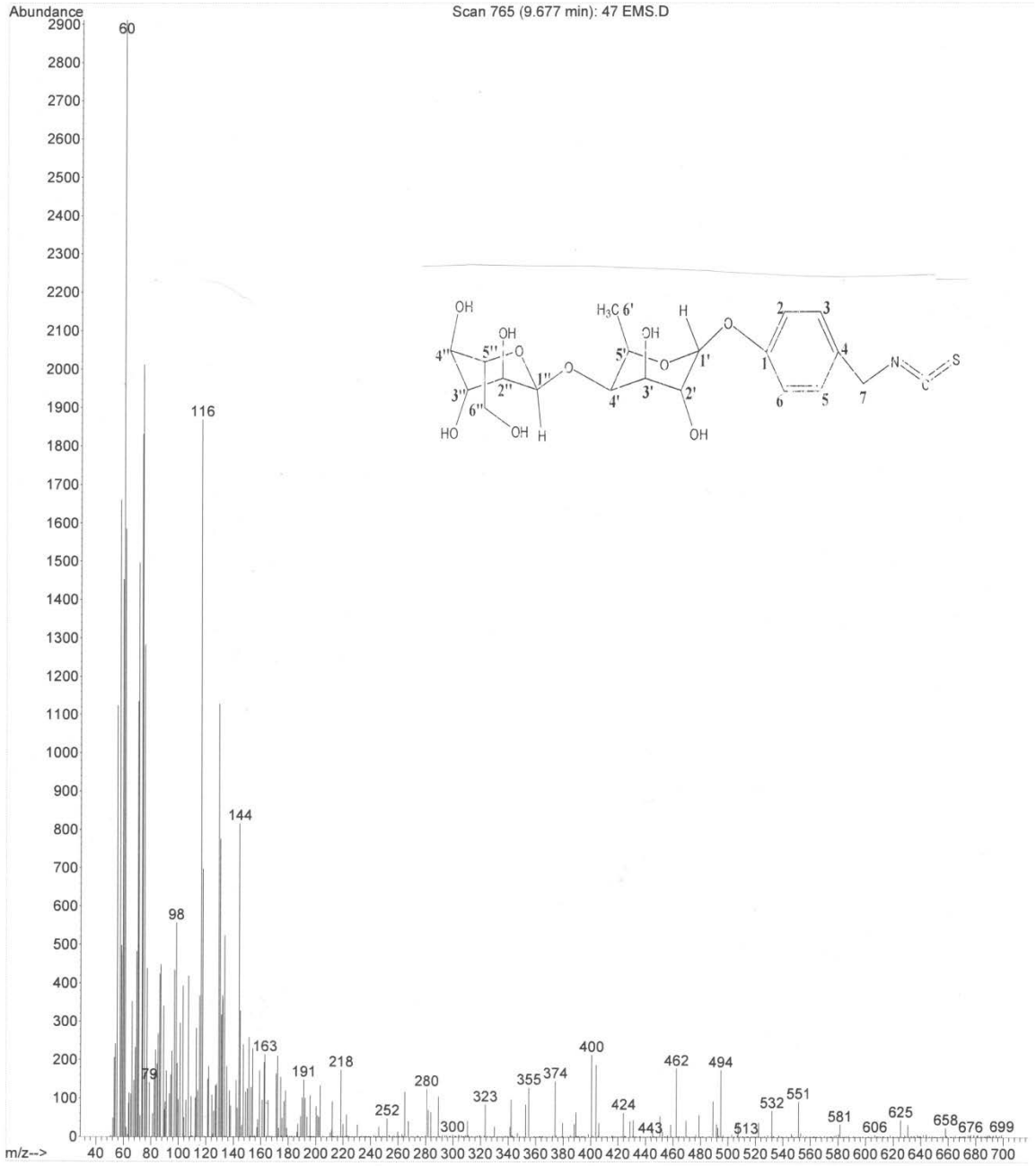

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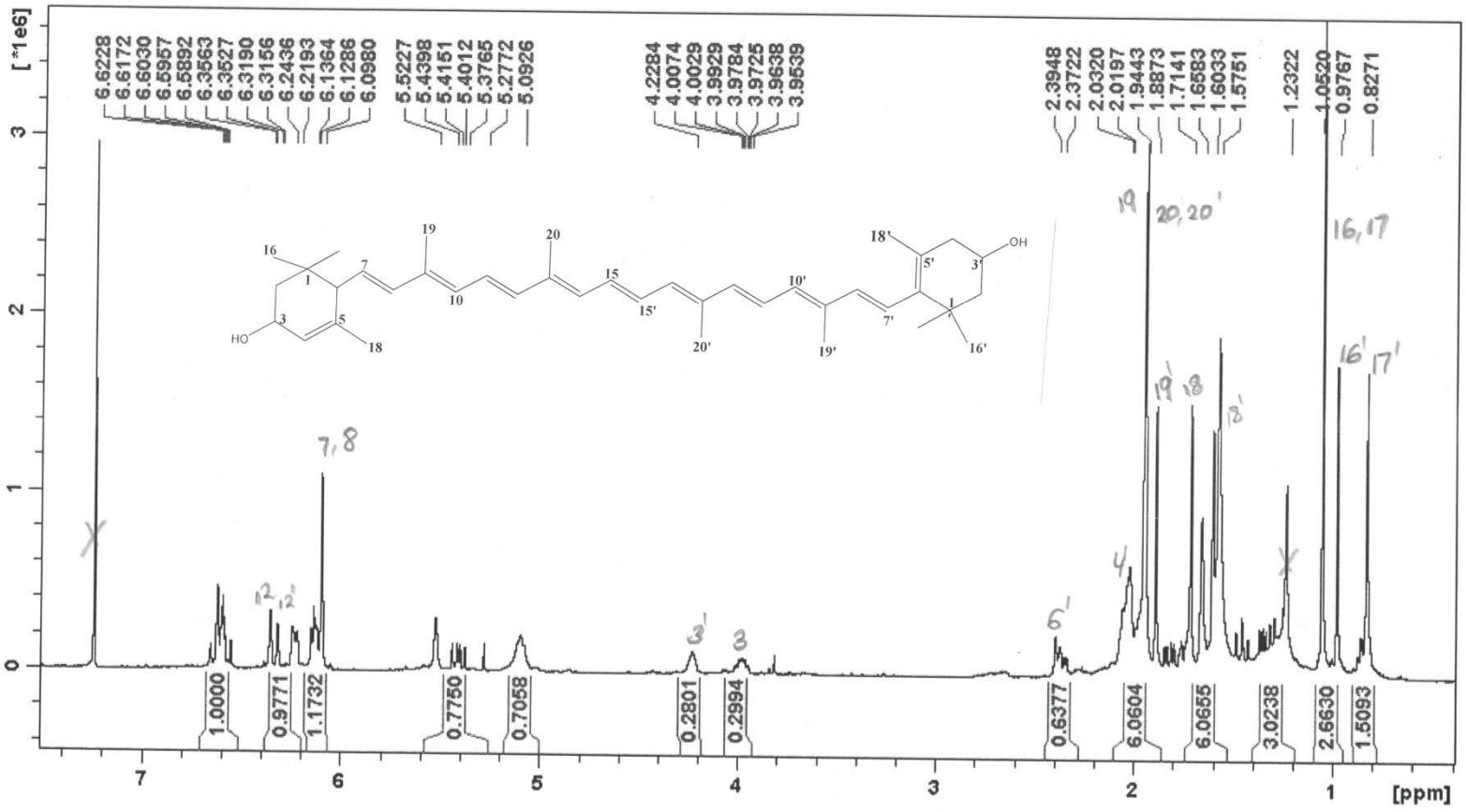

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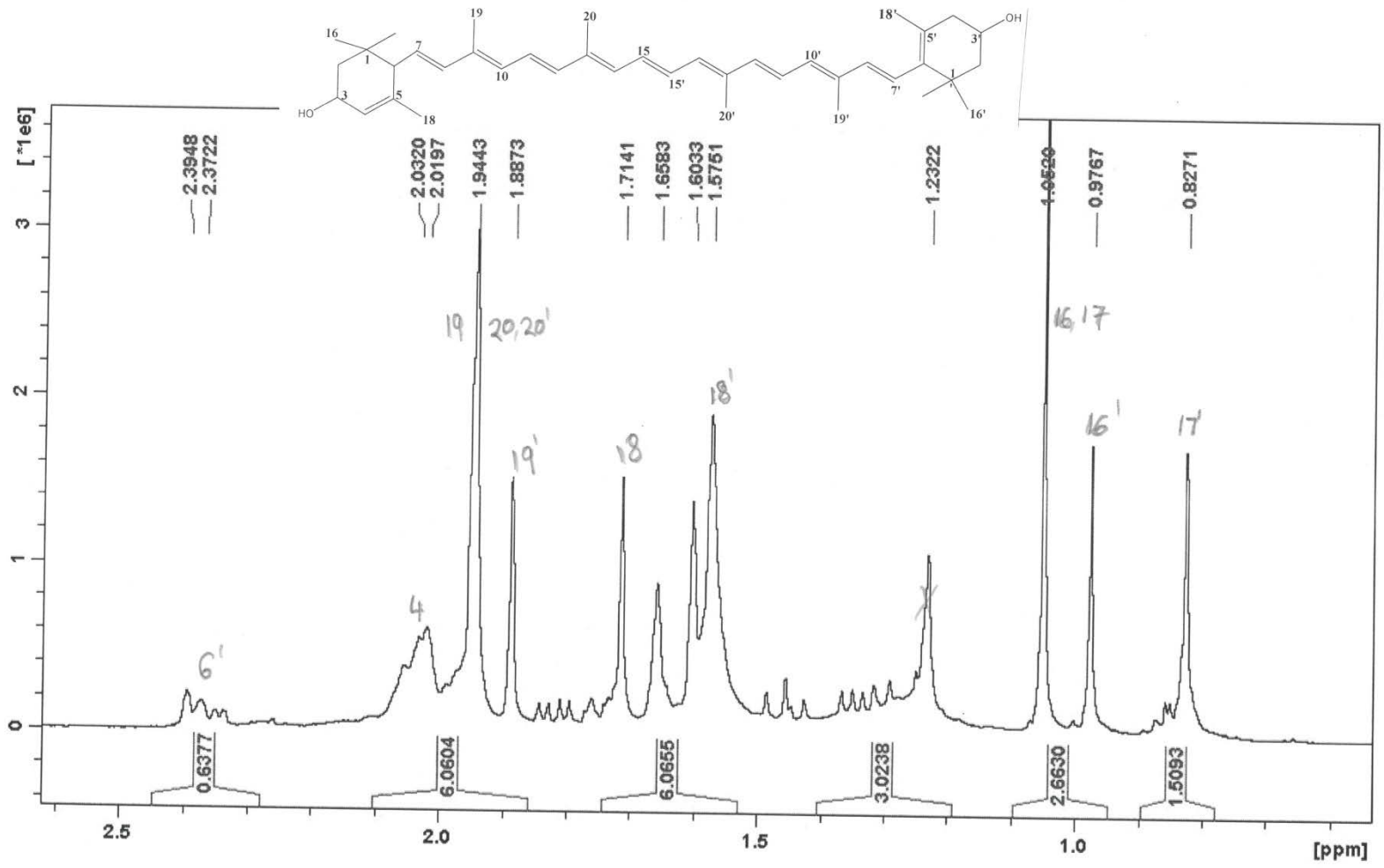

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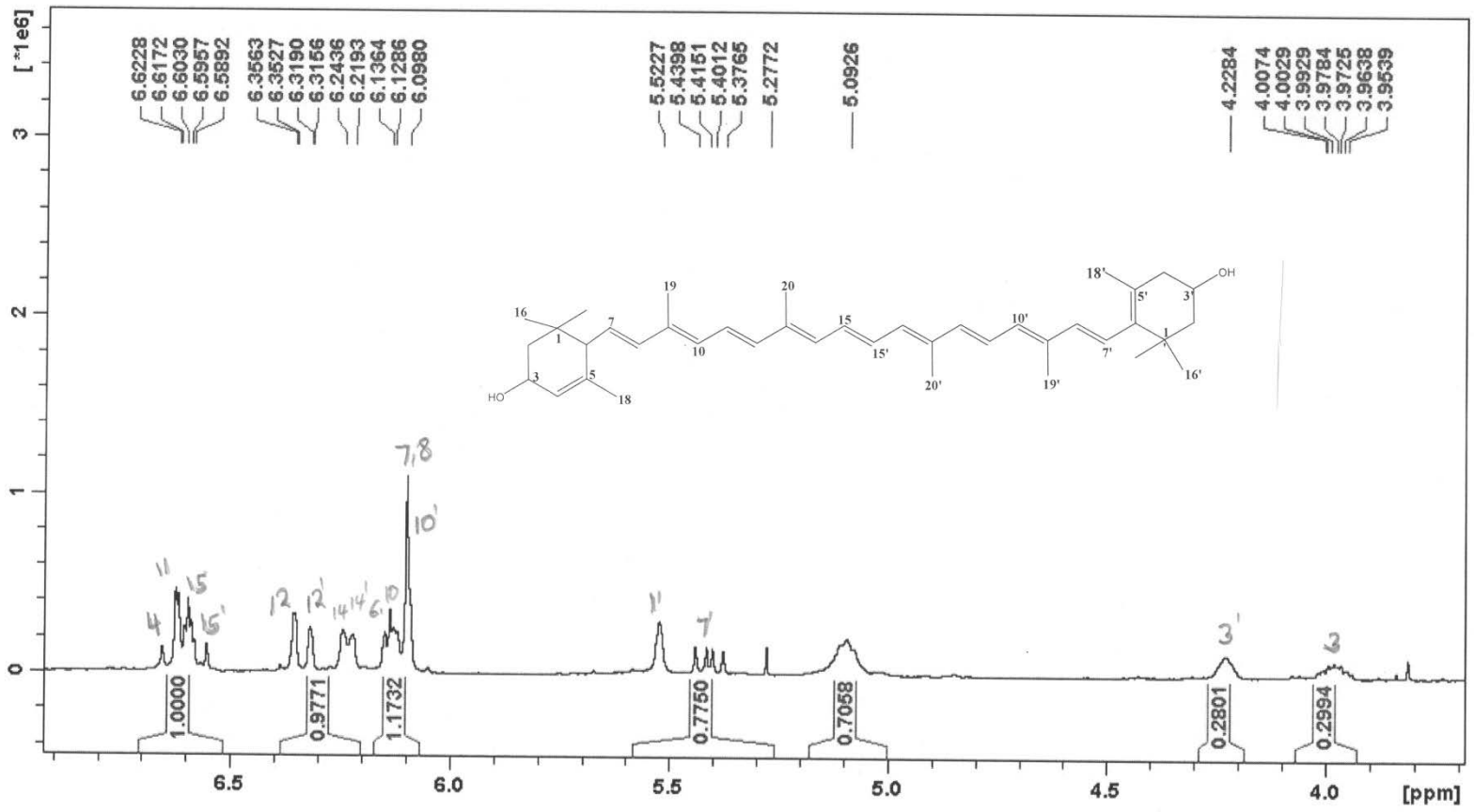

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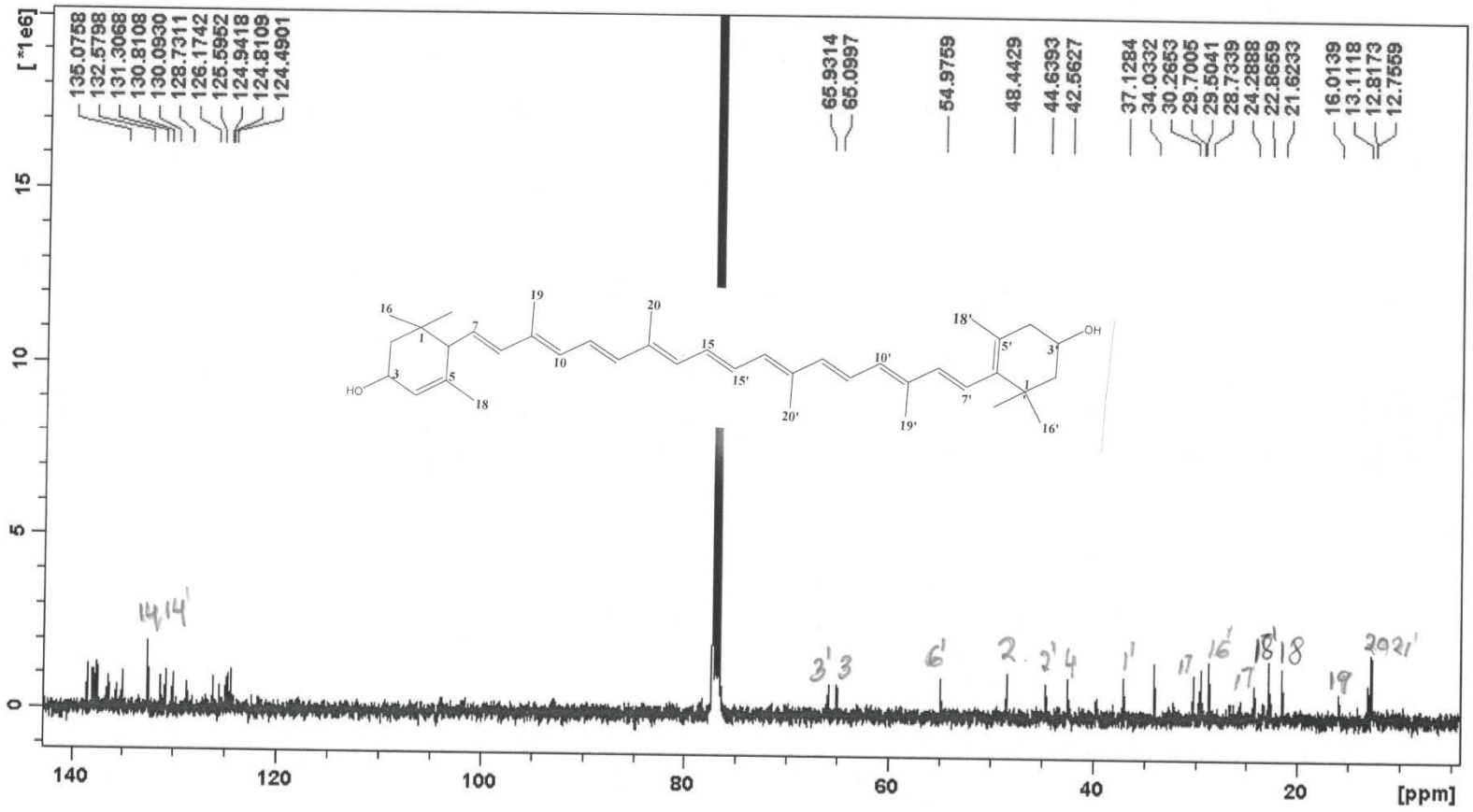

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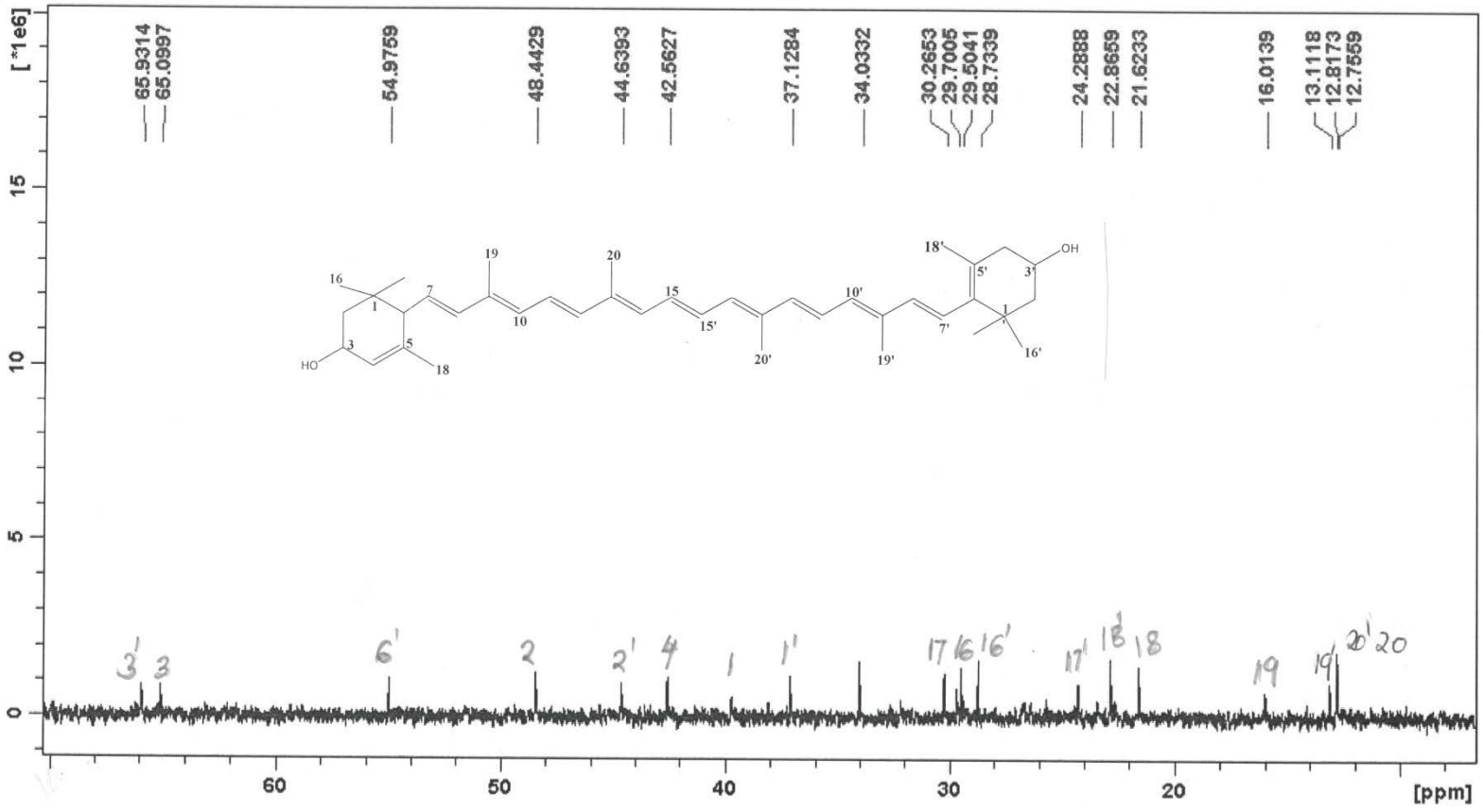

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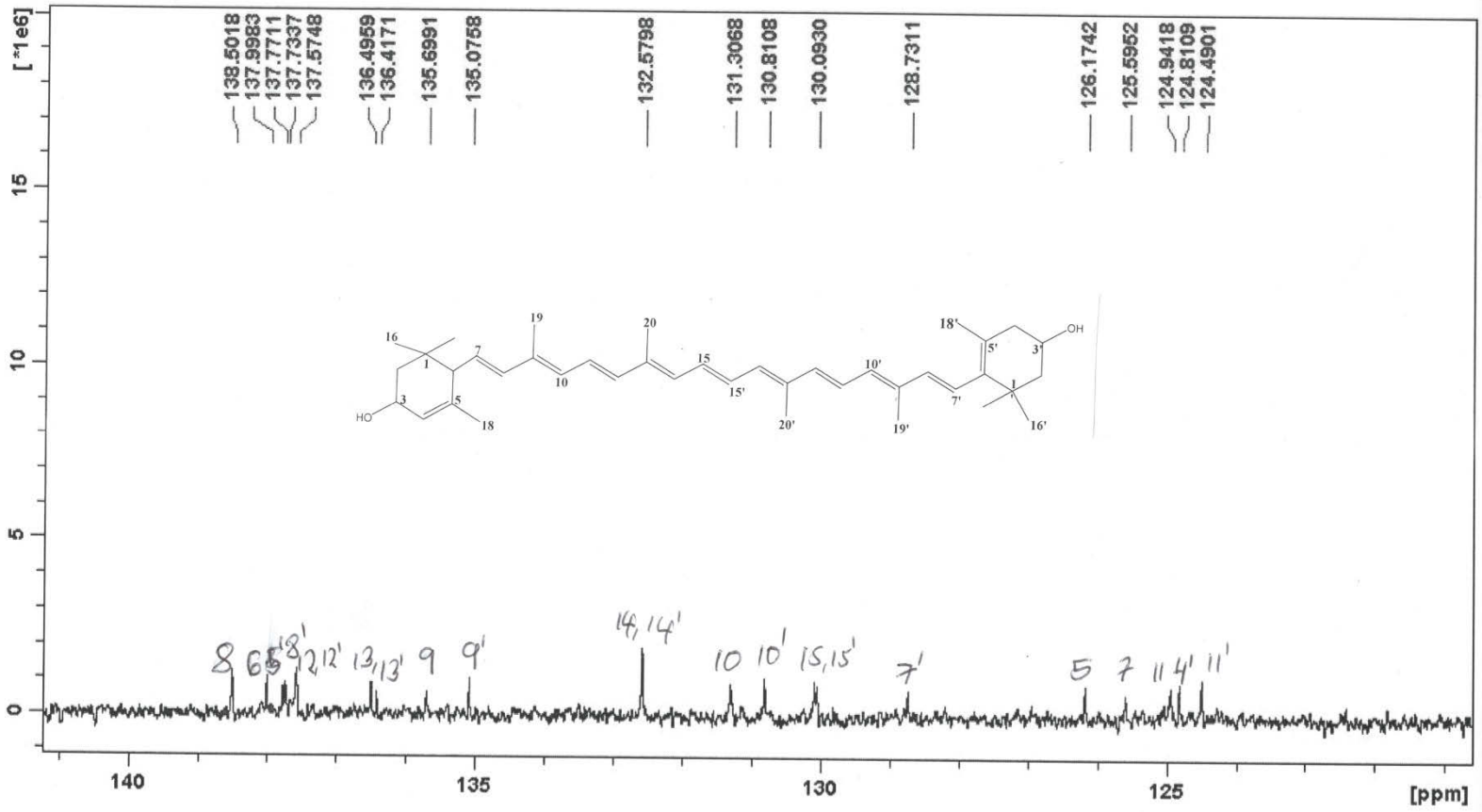

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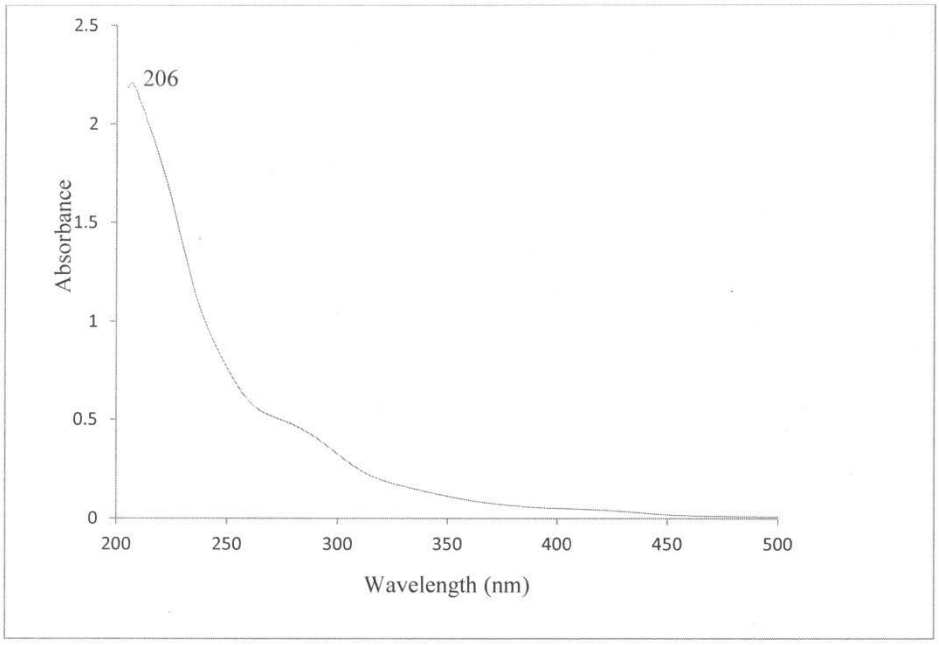
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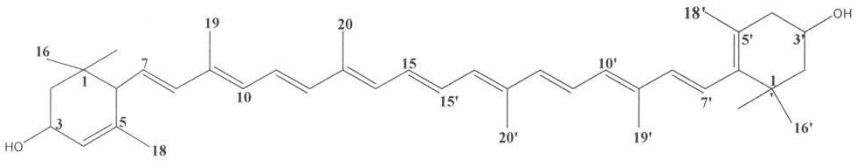
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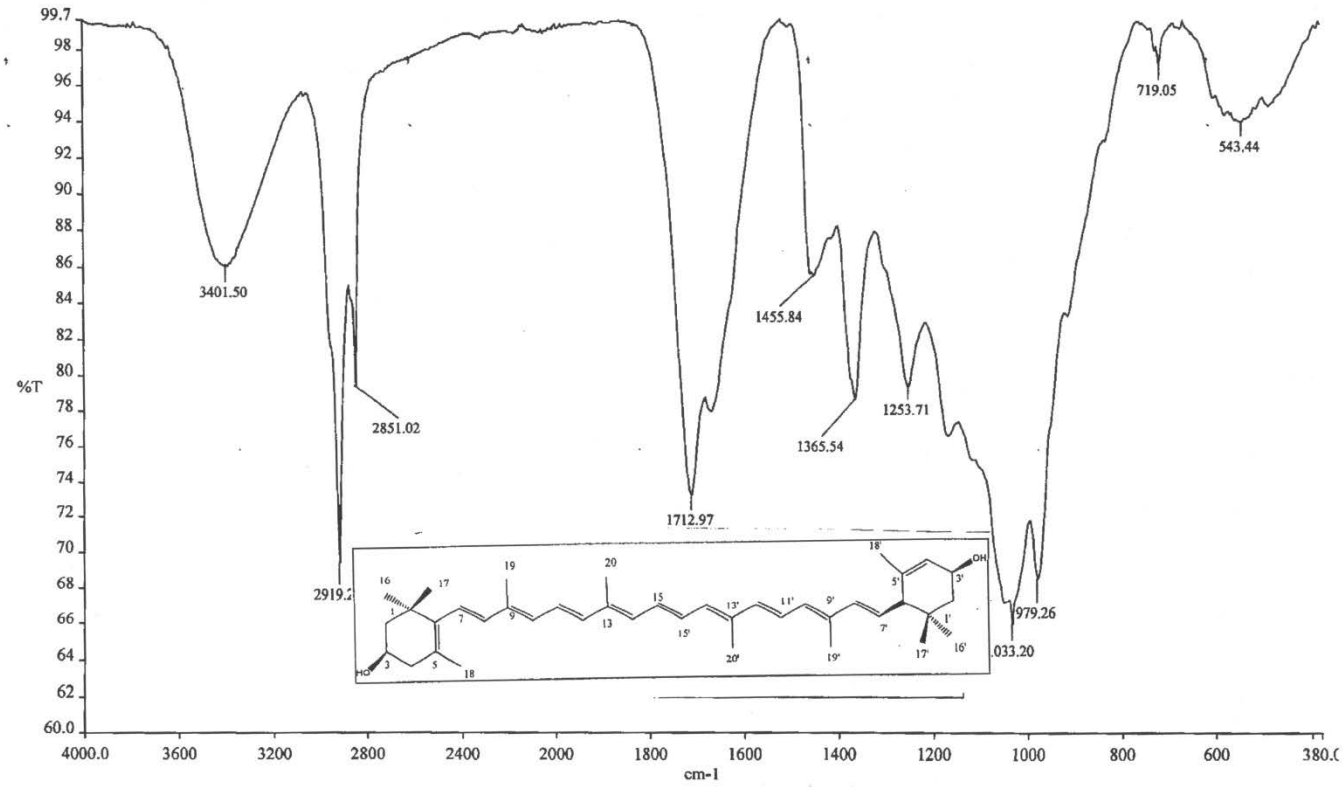
UV of Lutein



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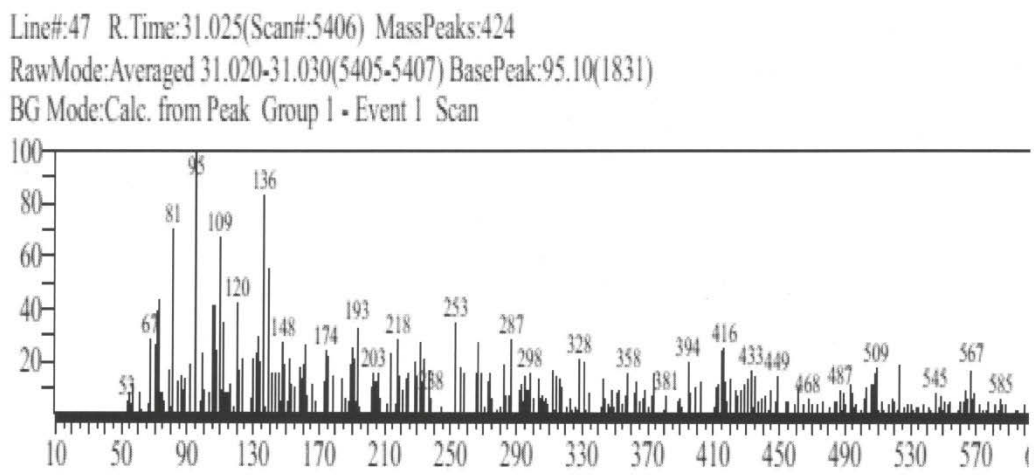

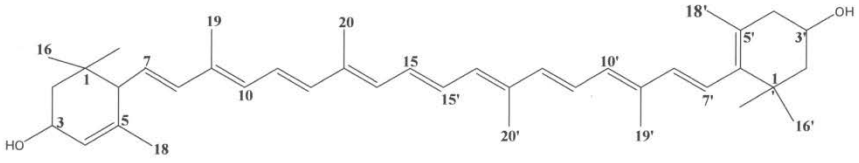

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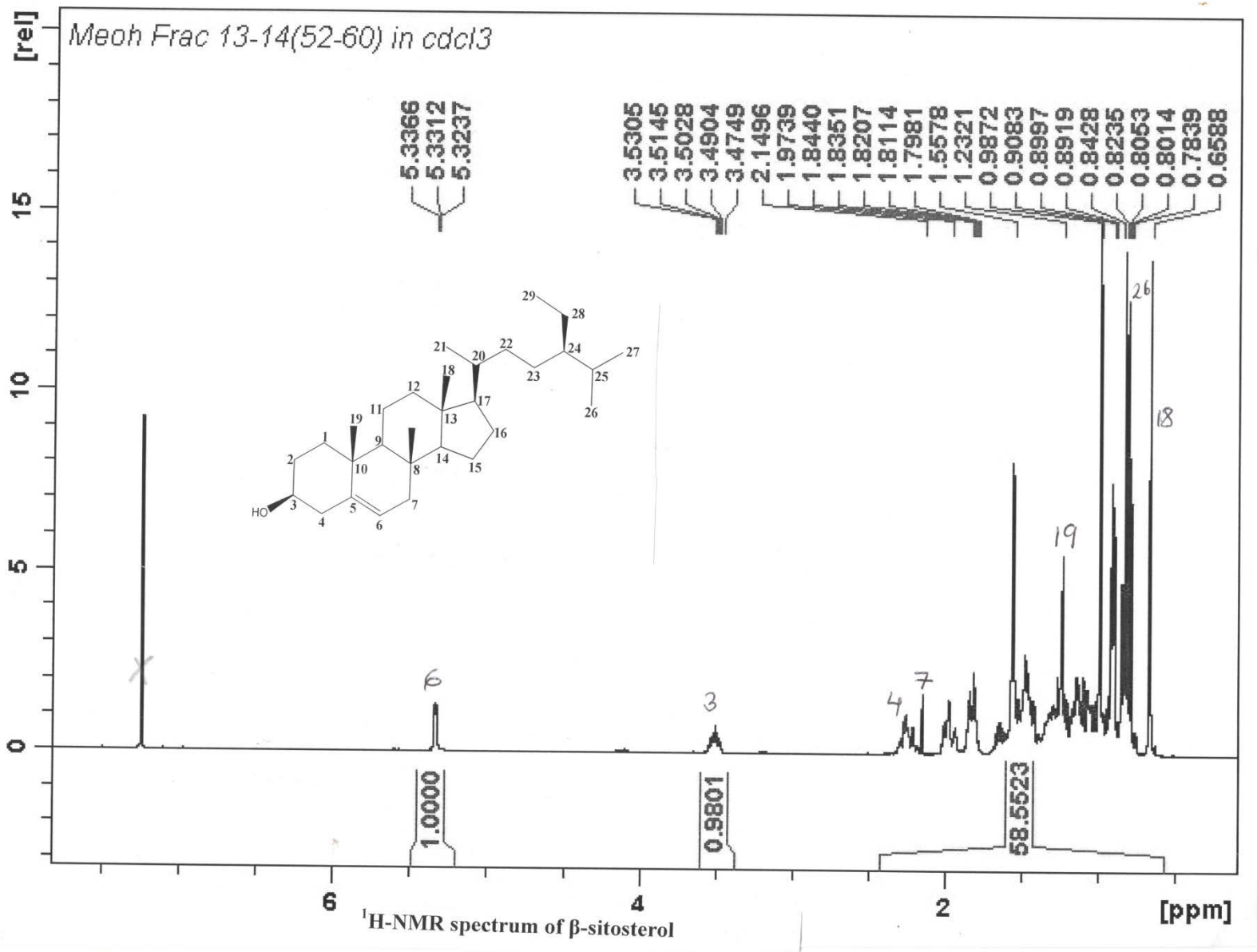

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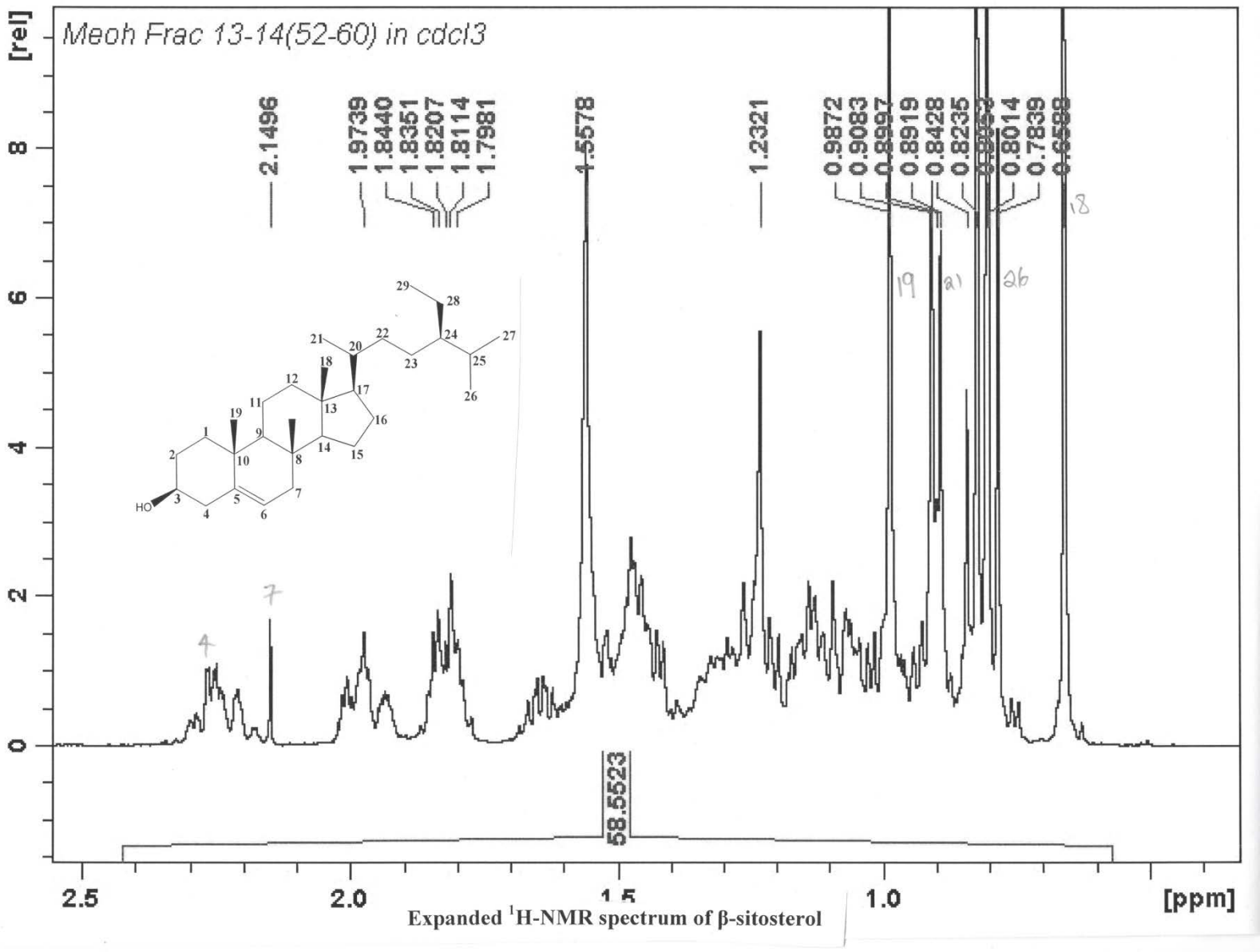

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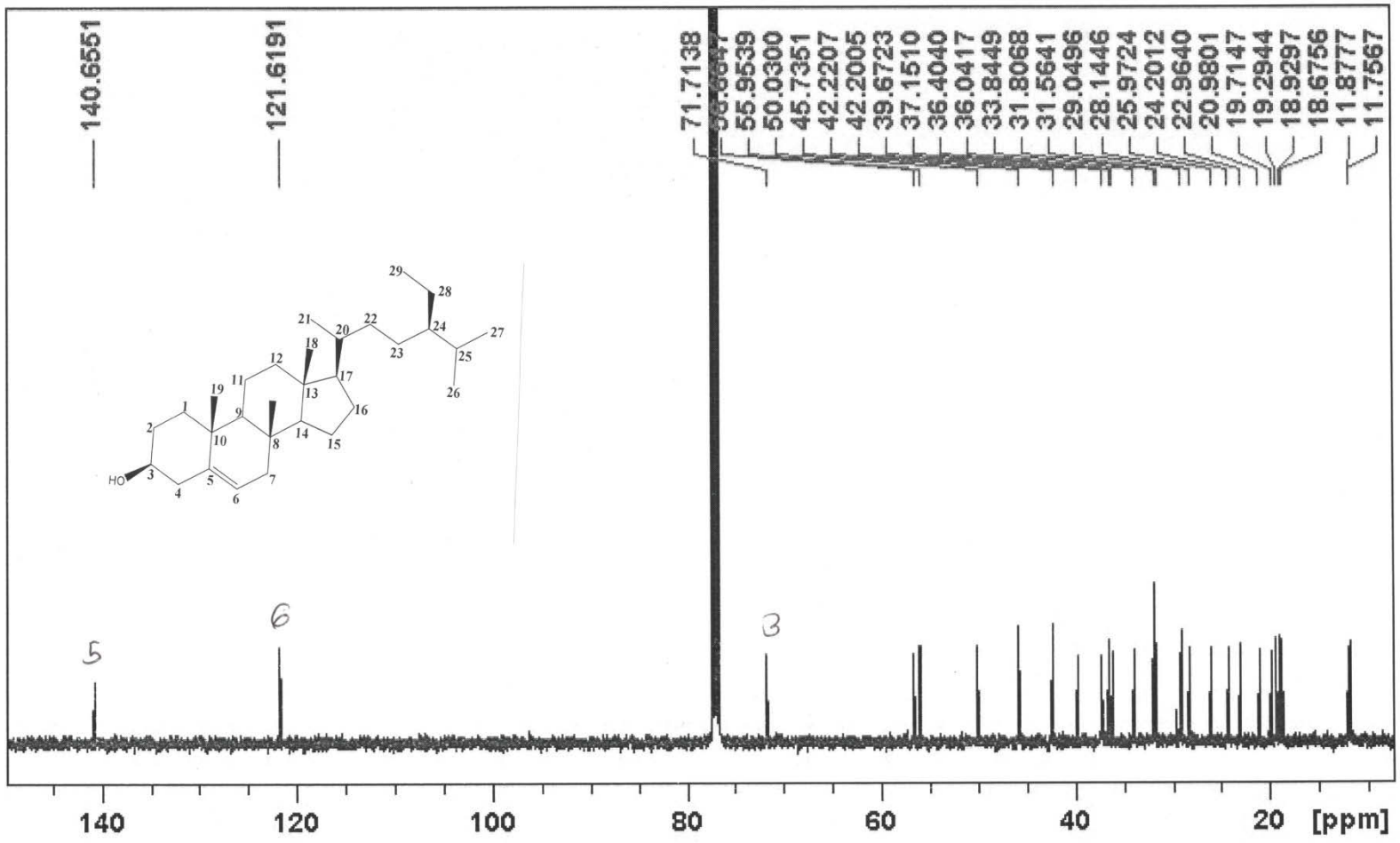

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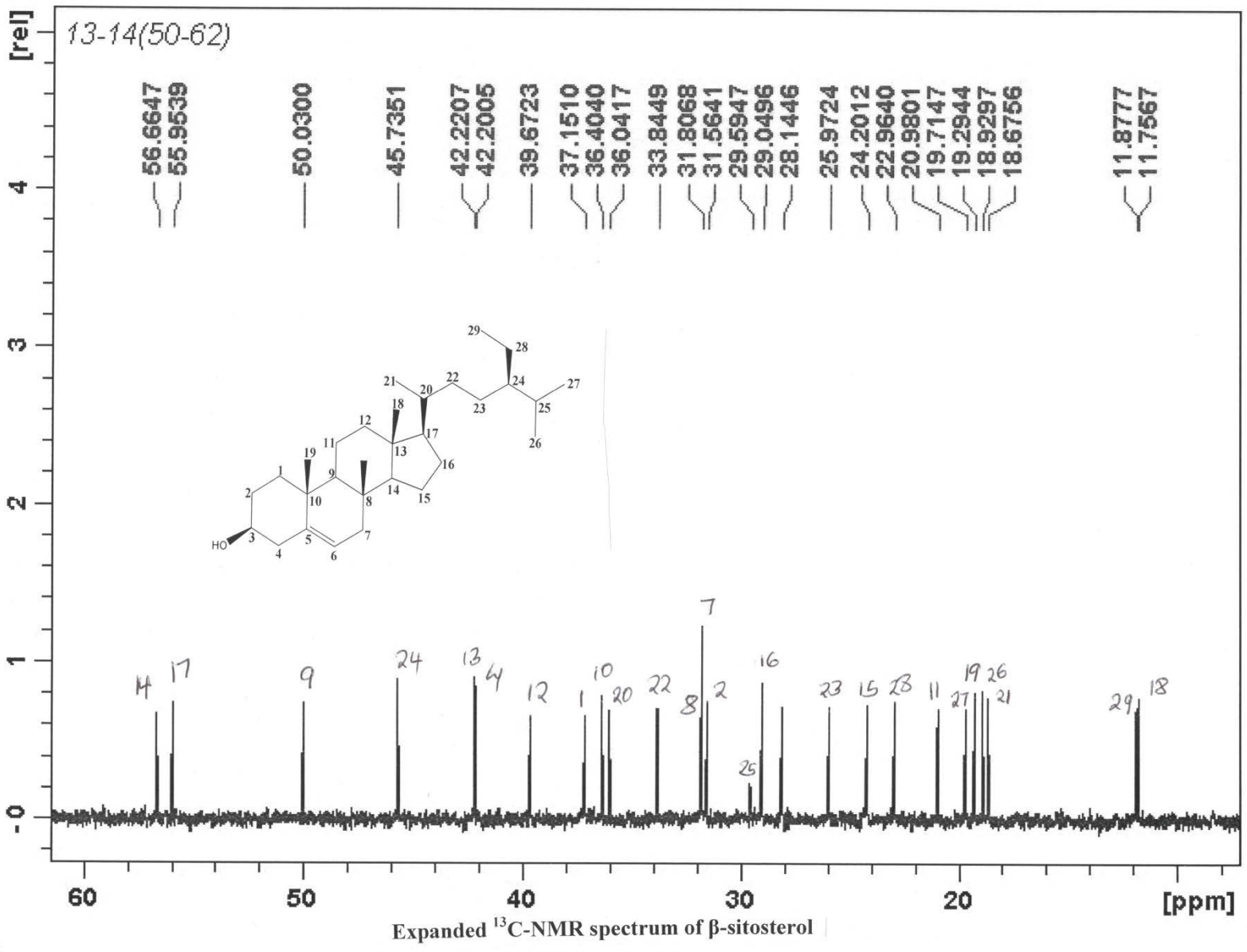

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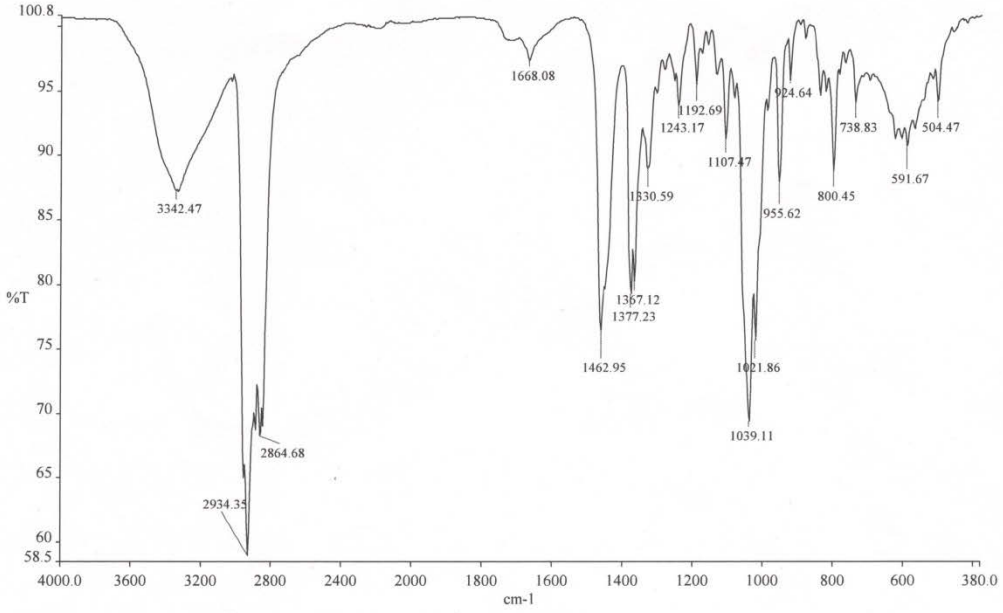

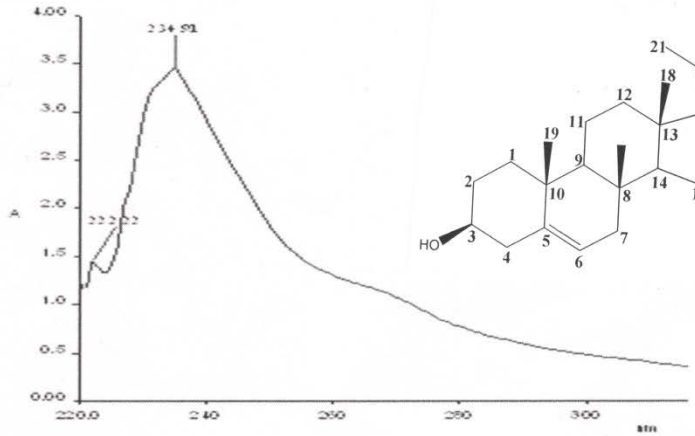

UV and IR spectrum of β-sitosterol

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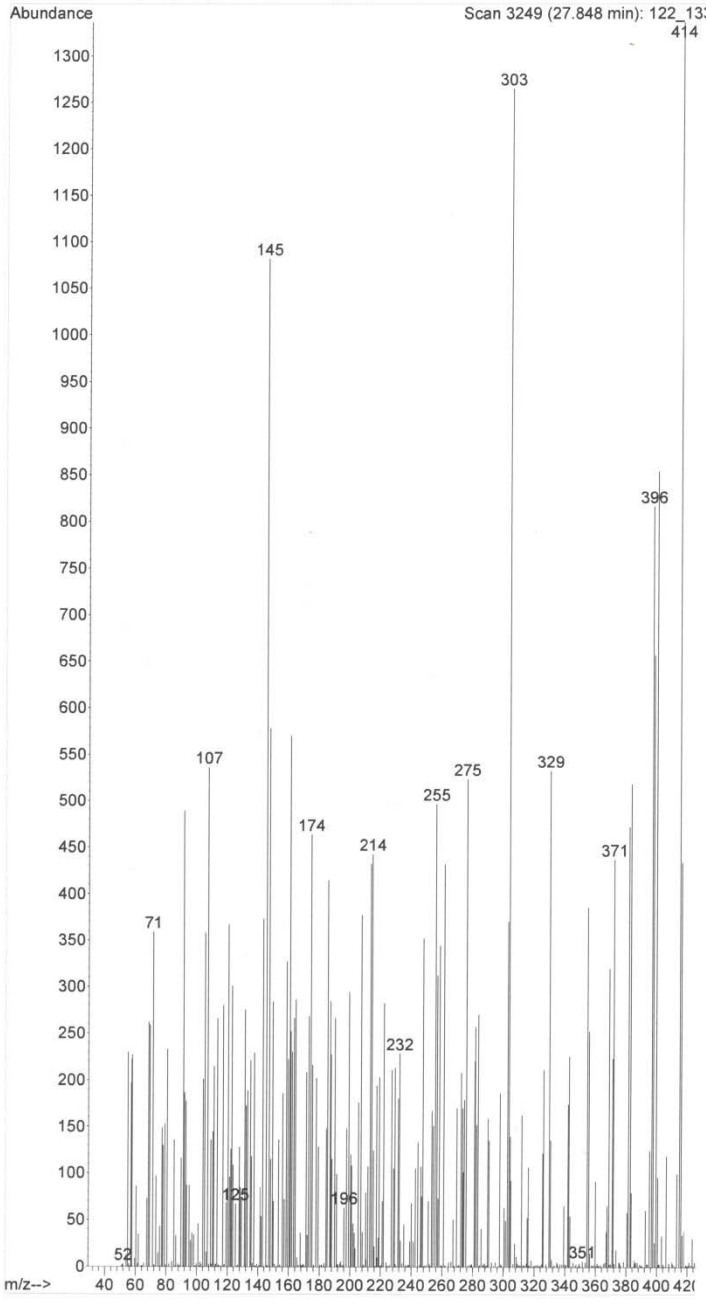
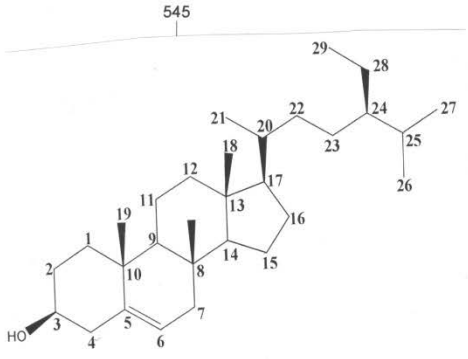
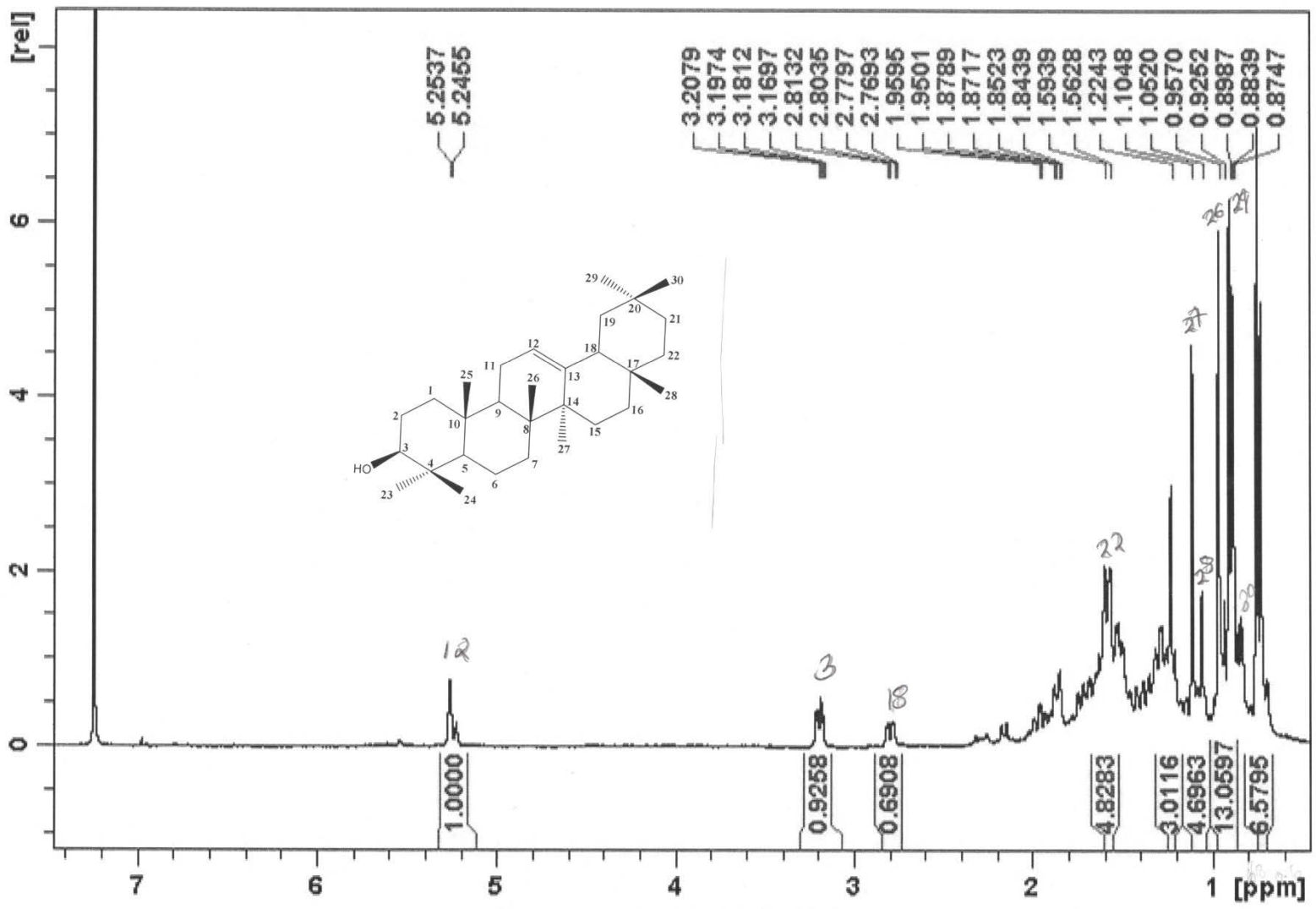
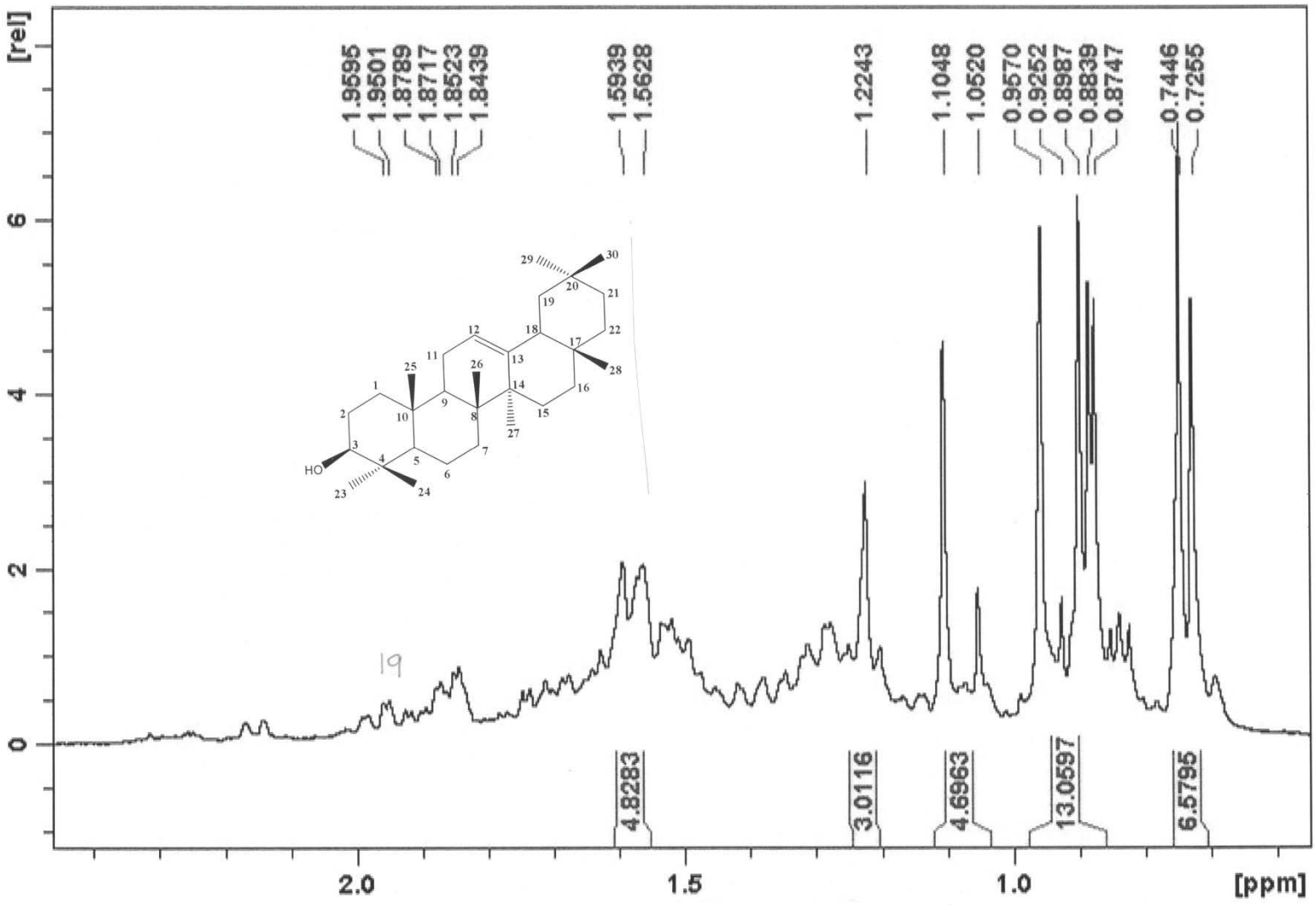




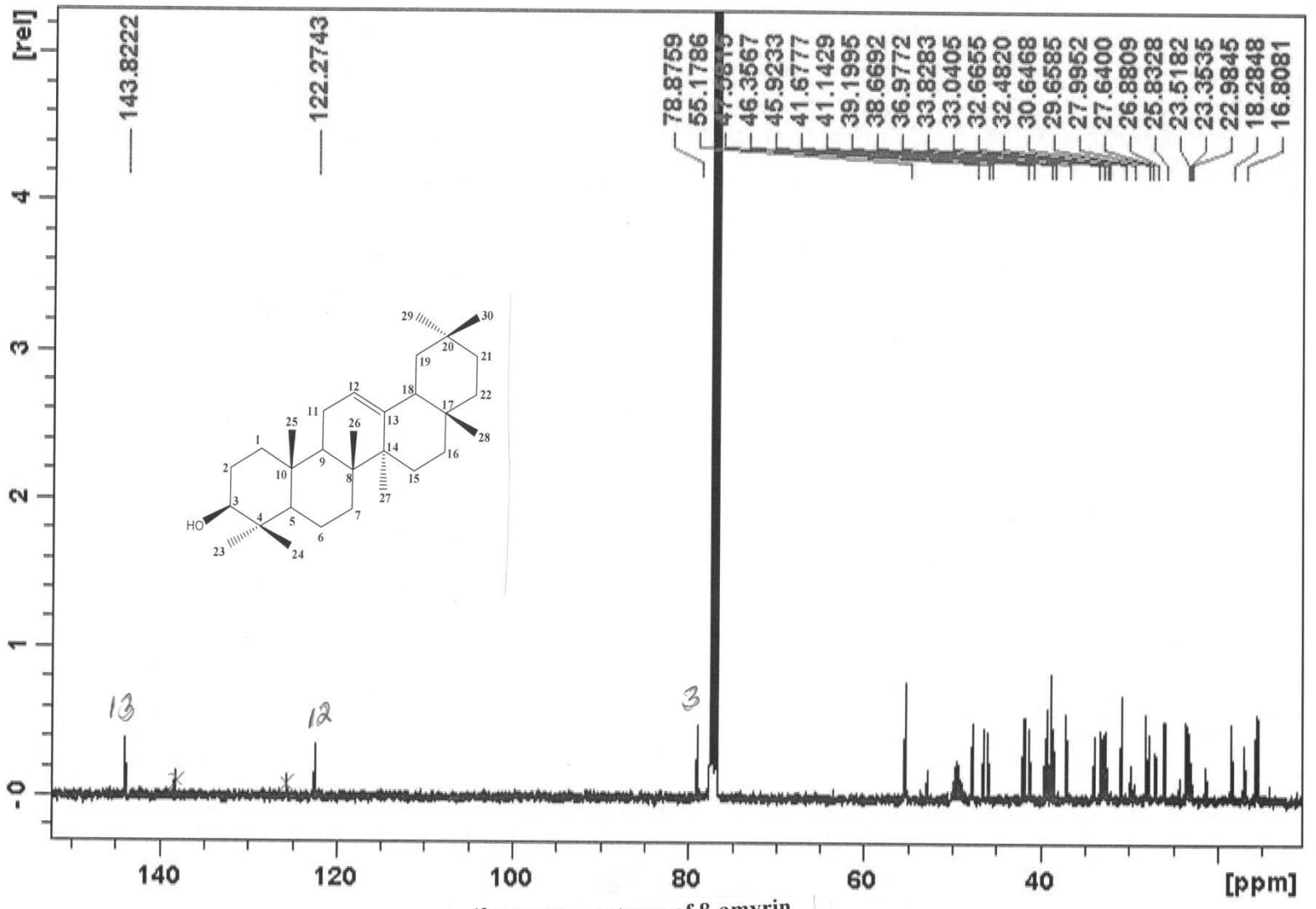
MS spectrum of β-sitosterol



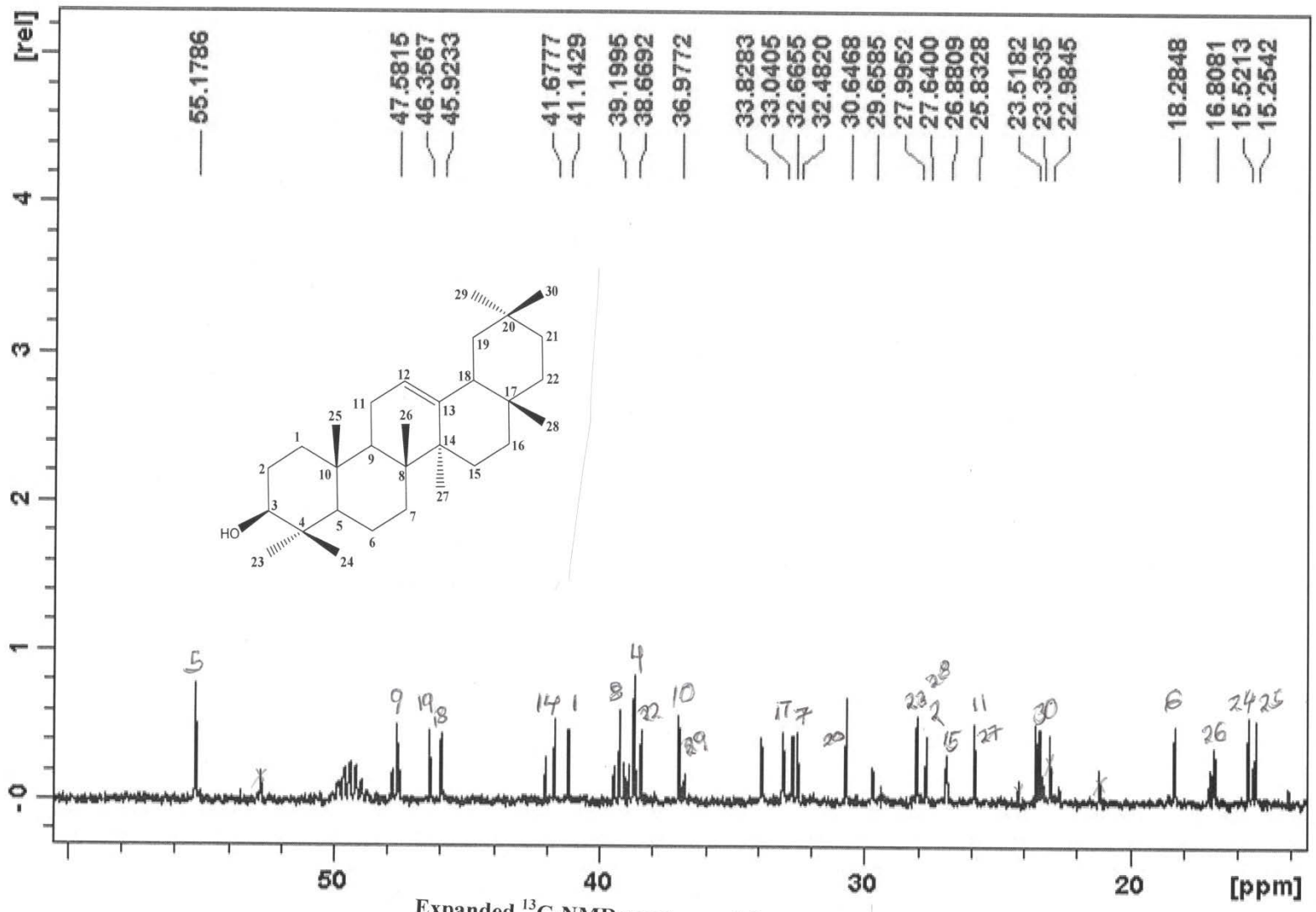
^{1}H-NMR spectrum of β-amyrin



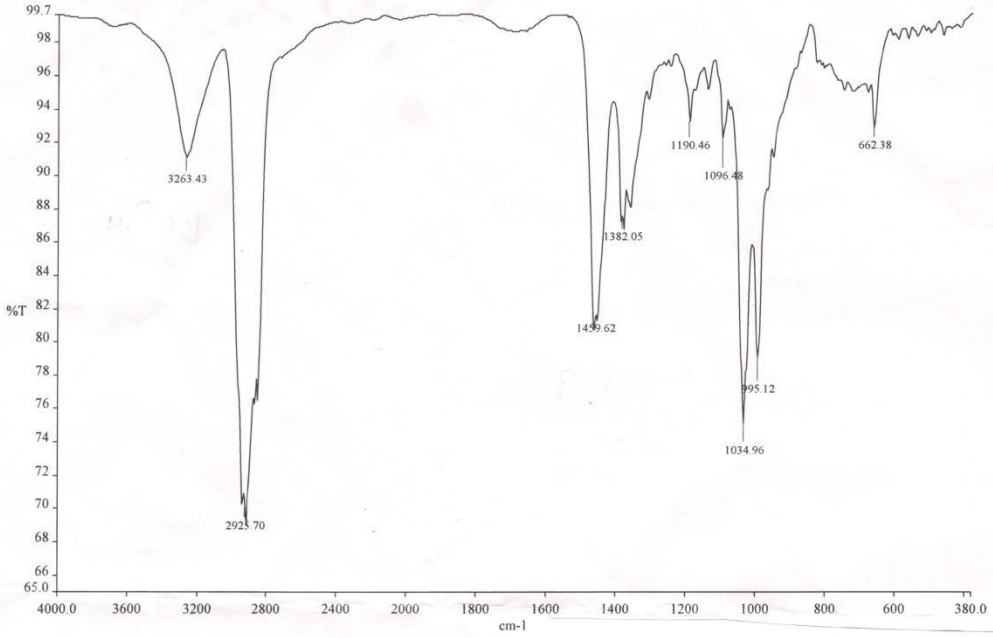
Expanded ^{1}H-NMR spectrum of β-amyrin

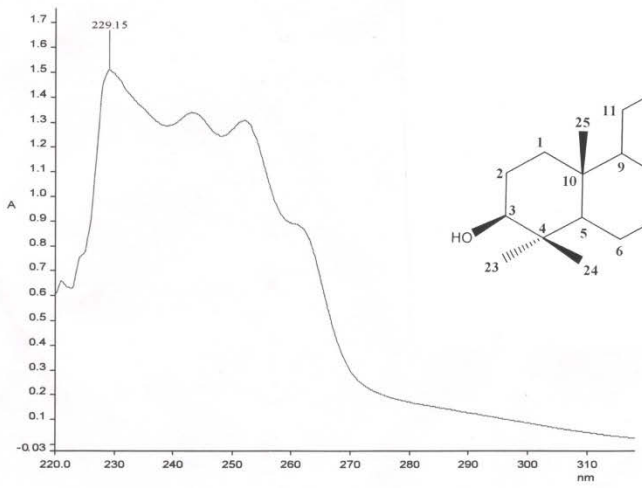


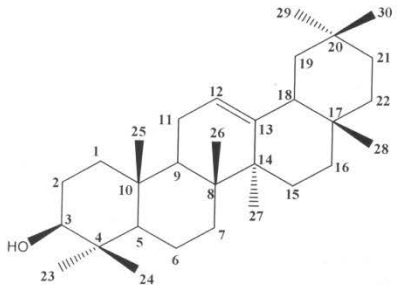
^{13}C-NMR spectrum of β-amyrin



Expanded ^{13}C-NMR spectrum of β-amyrin





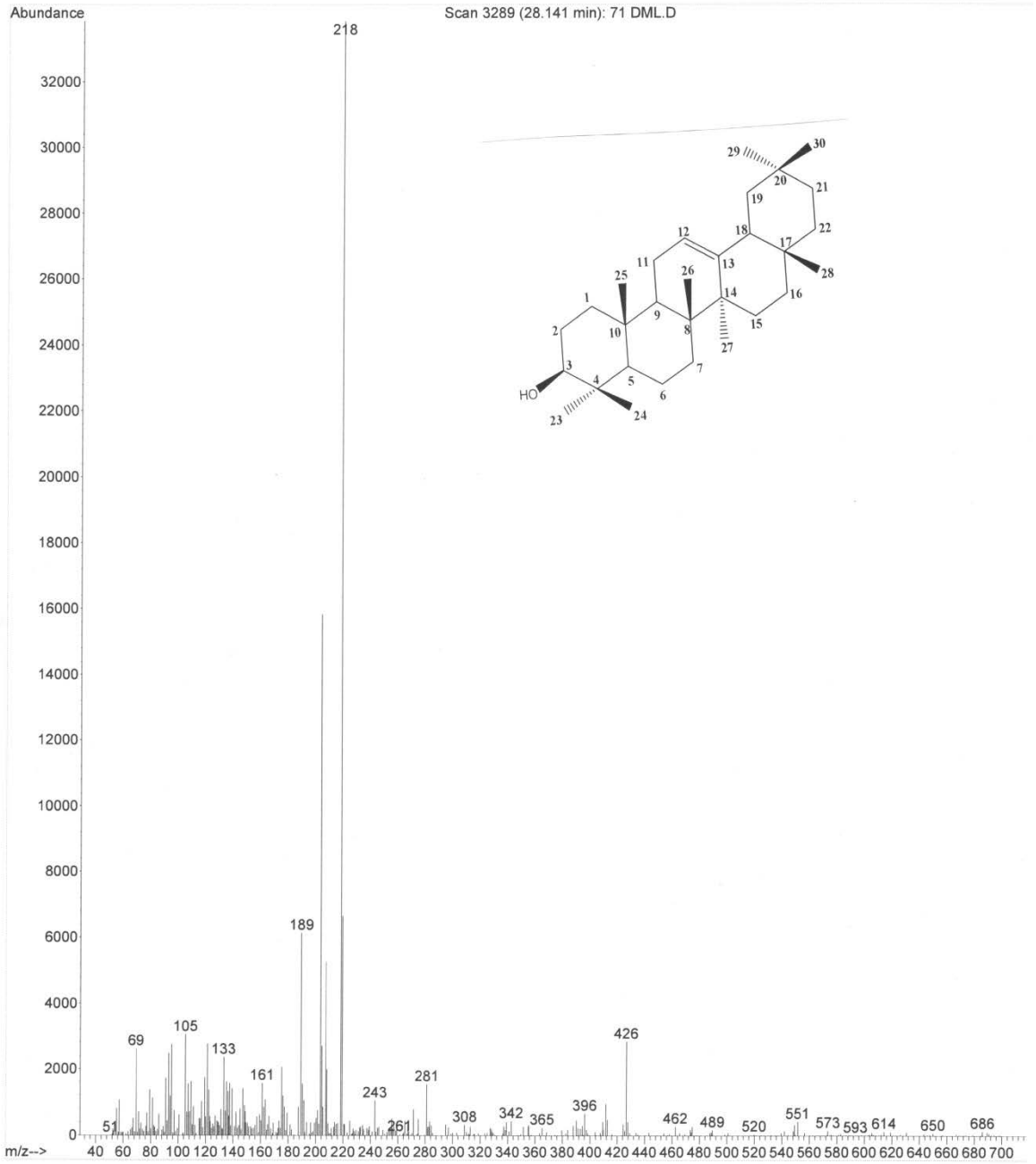


138

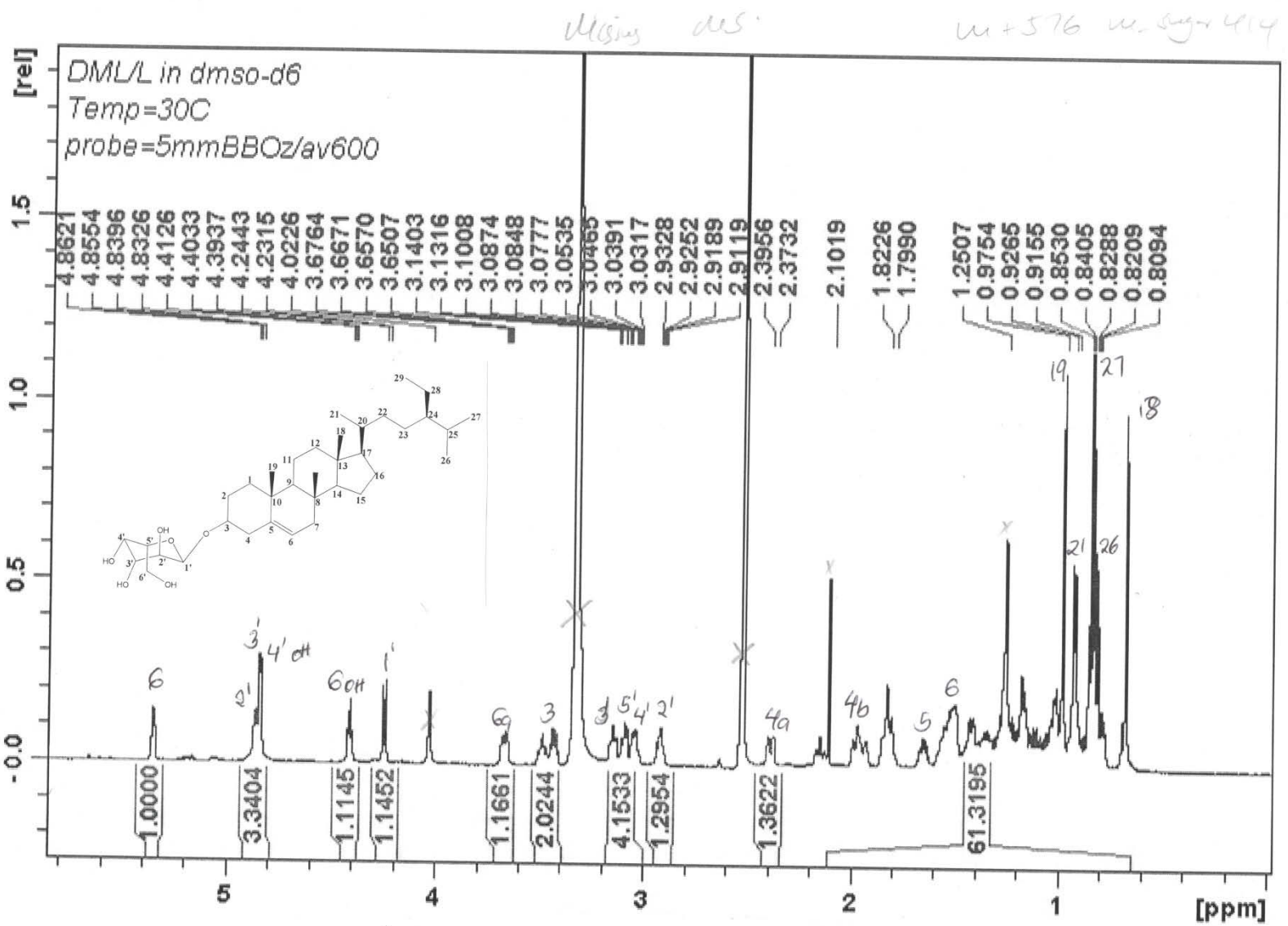
UV and IR spectrum of β-amyrin

158

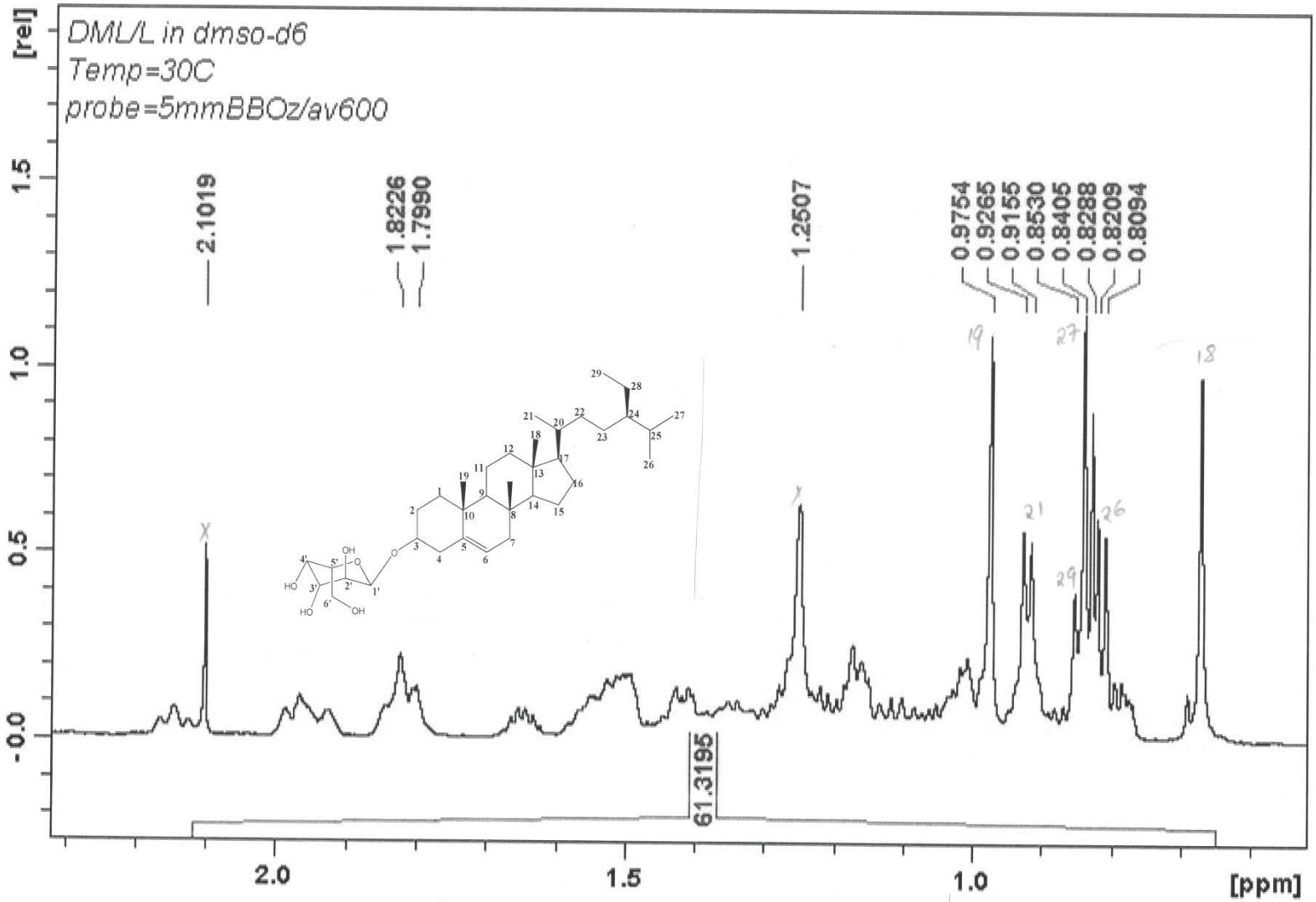
File : C:\MSDCHEM\1\DATA\FIONA\71 DML.D
Operator : fiona
Acquired : 5 Jun 2013 23:13 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973n
Sample Name: 71 DML
Misc Info :
Vial Number: 16



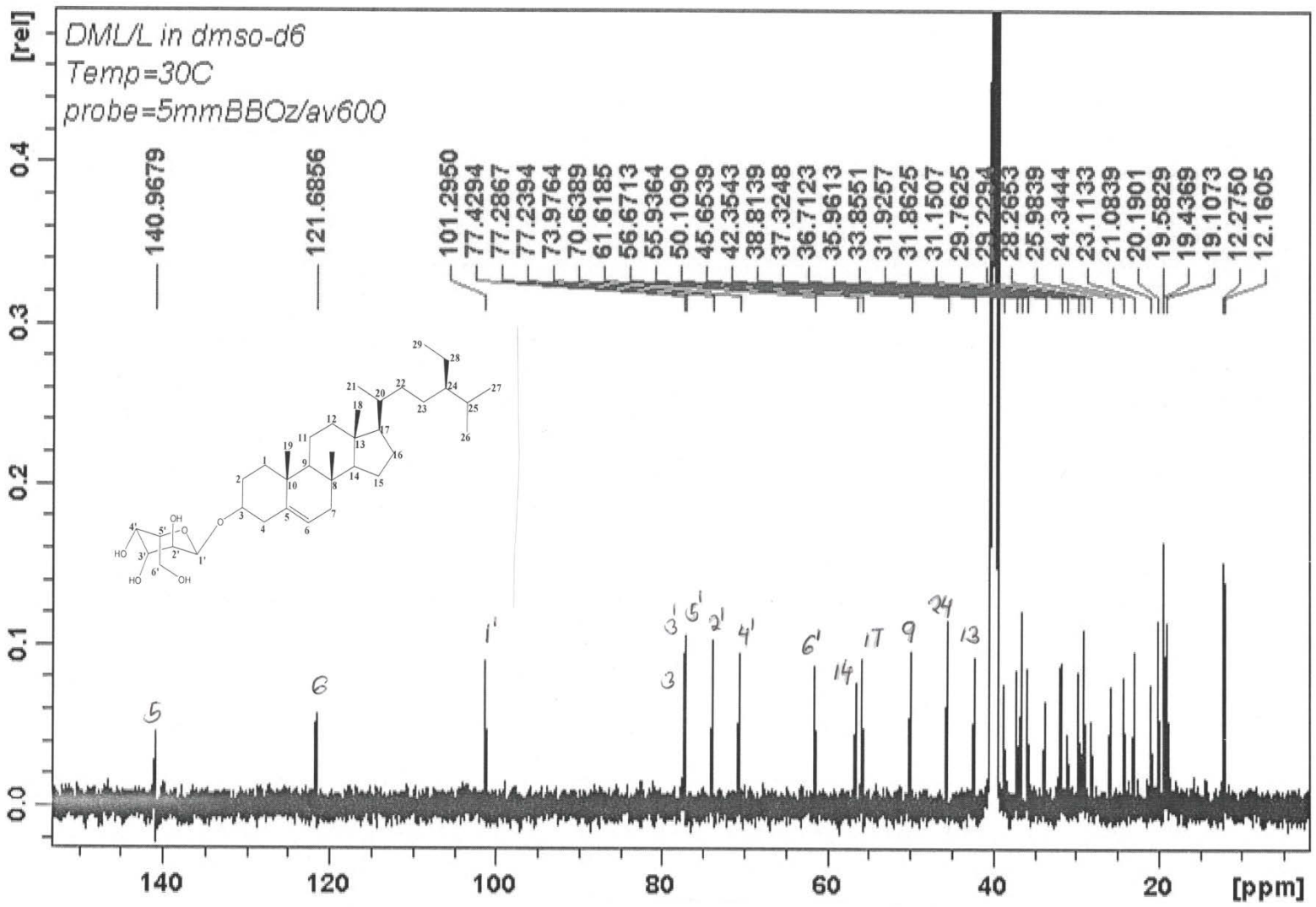
MS spectrum of β-amyrin



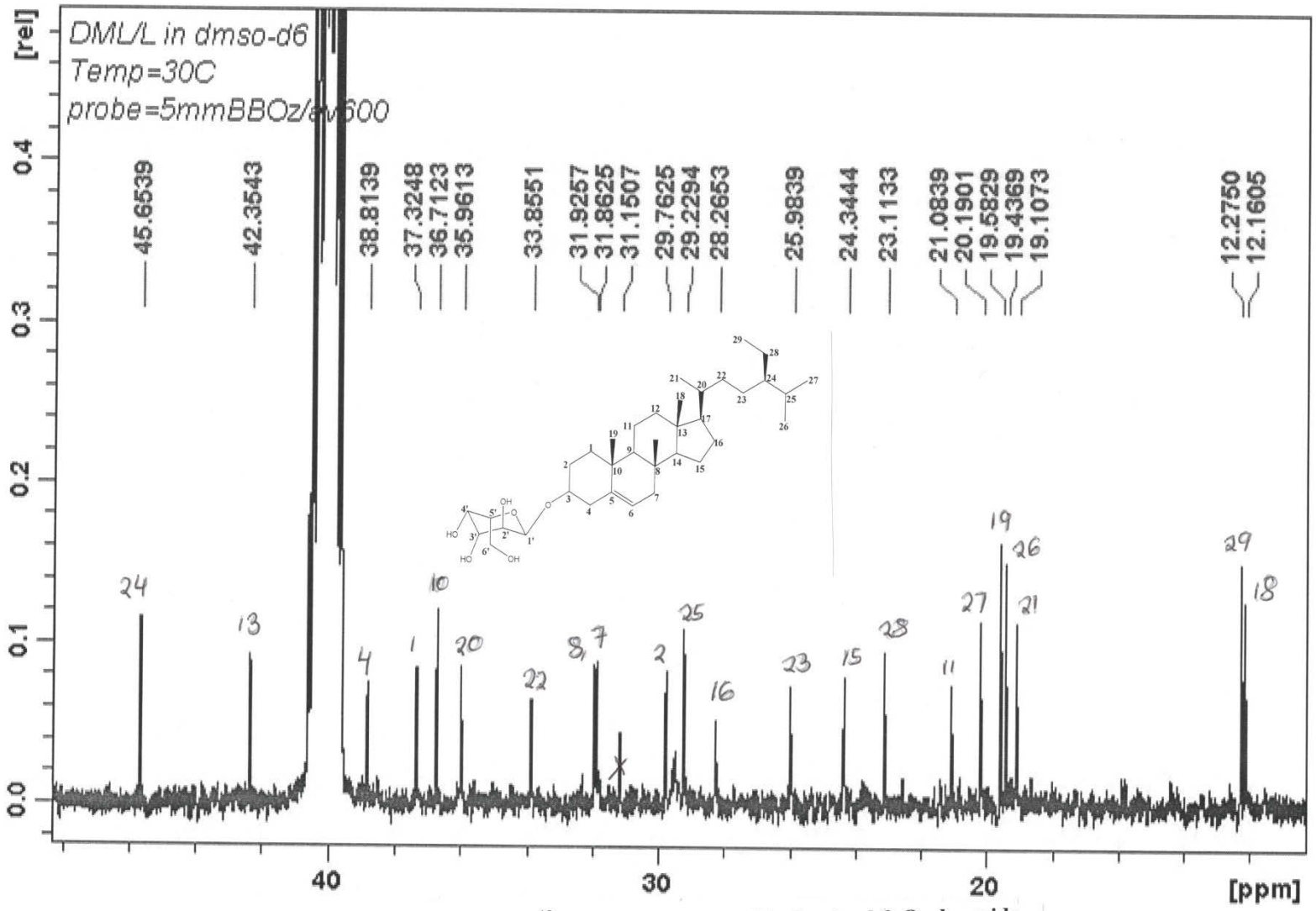
^{1}H-NMR spectrum of β-sitosterol-3-O-glucoside



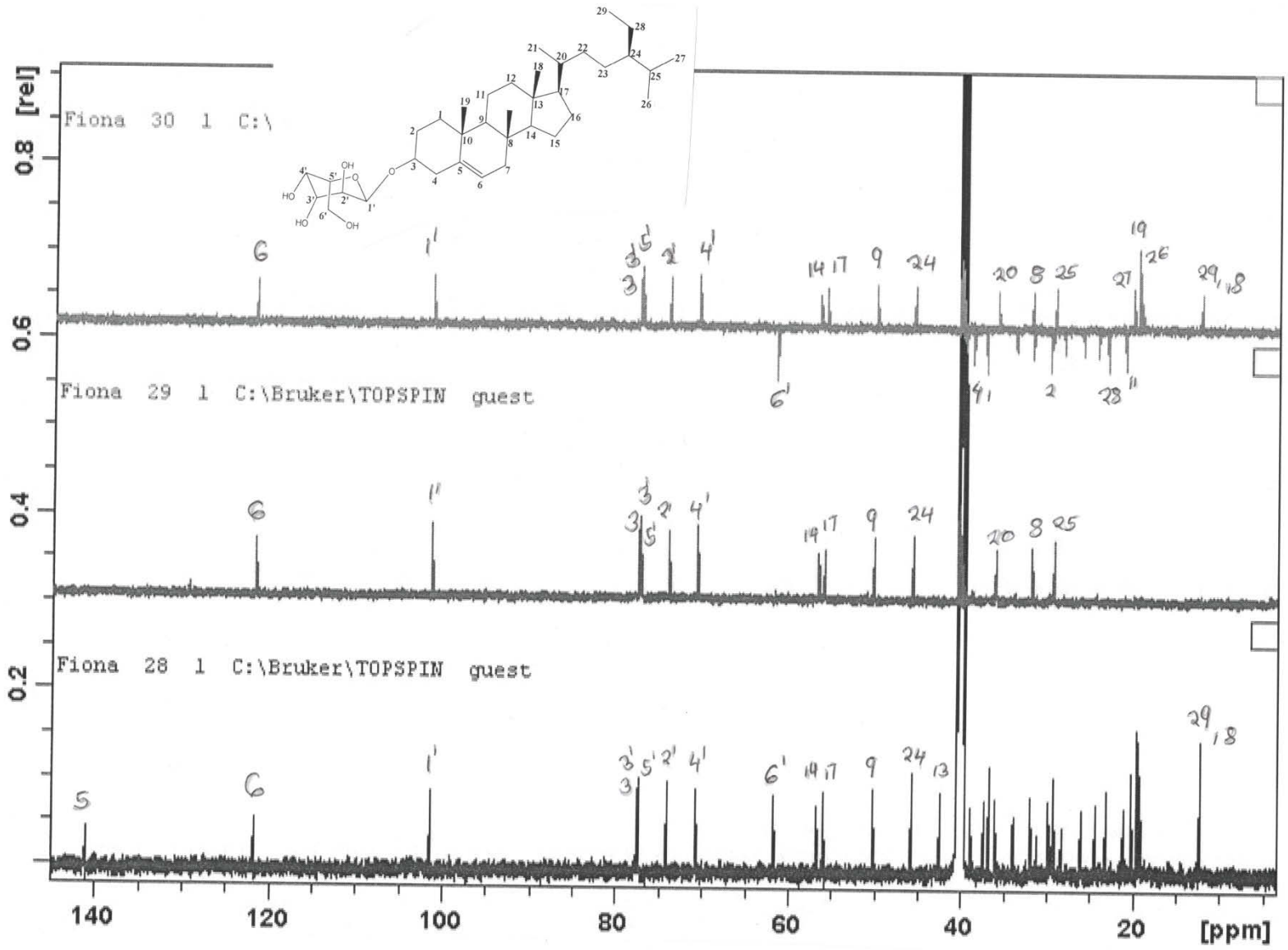
Expanded ^{1}H-NMR spectrum of β-sitosterol-3-O-glucoside



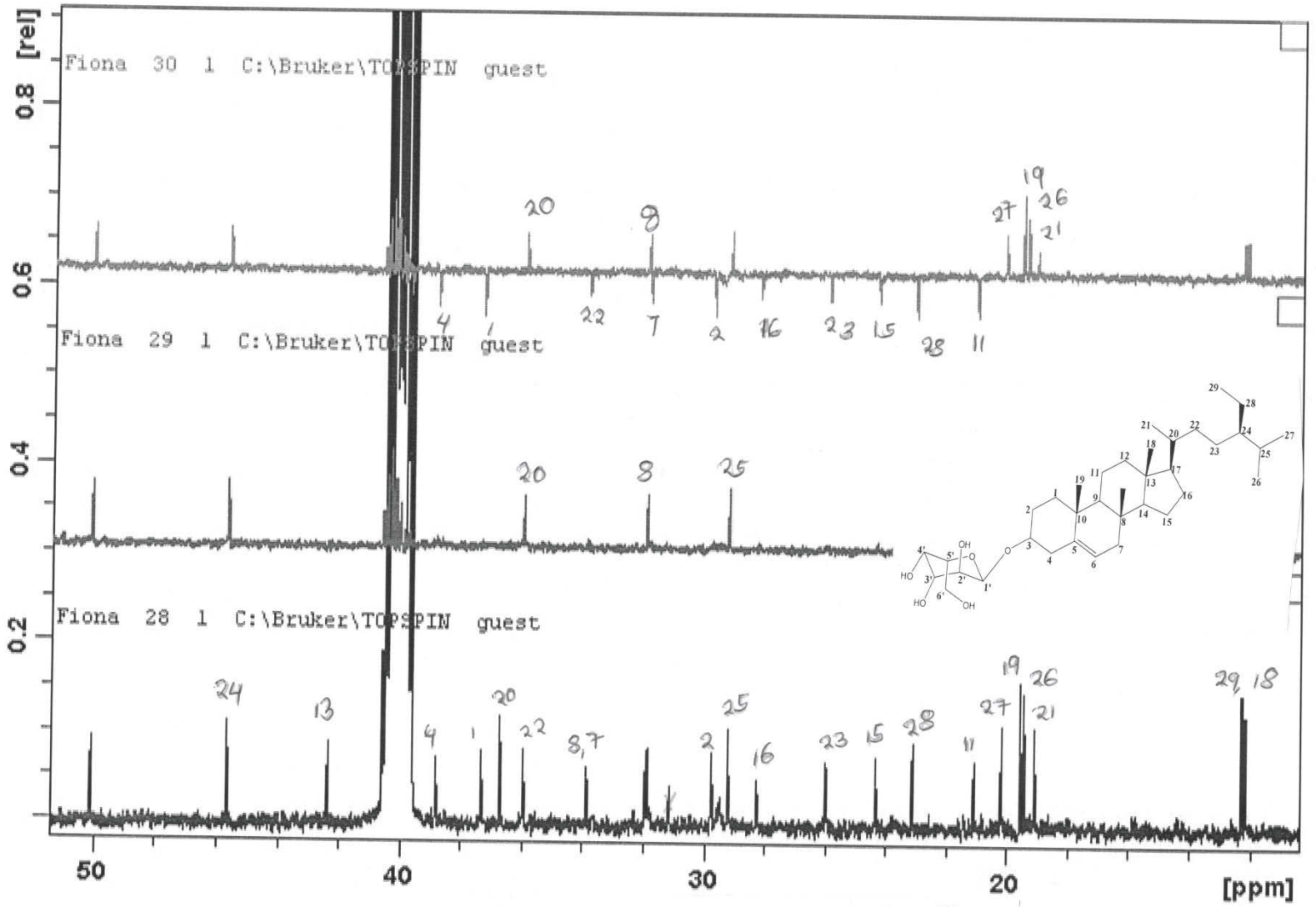
^{13}C-NMR spectrum of β-sitosterol-3-O-glucoside



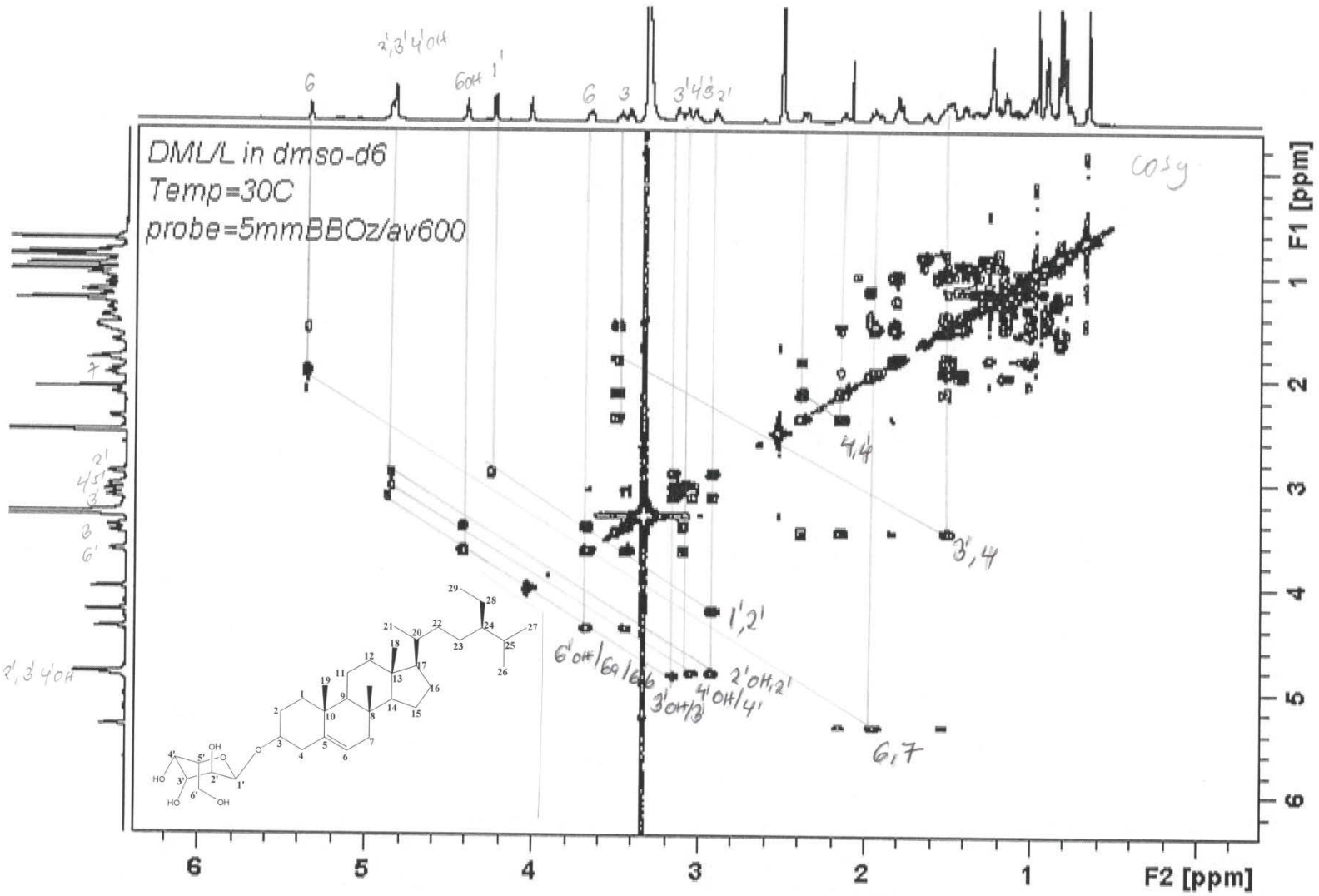
Expanded ^{13}C-NMR spectrum of β-sitosterol-3-O-glucoside



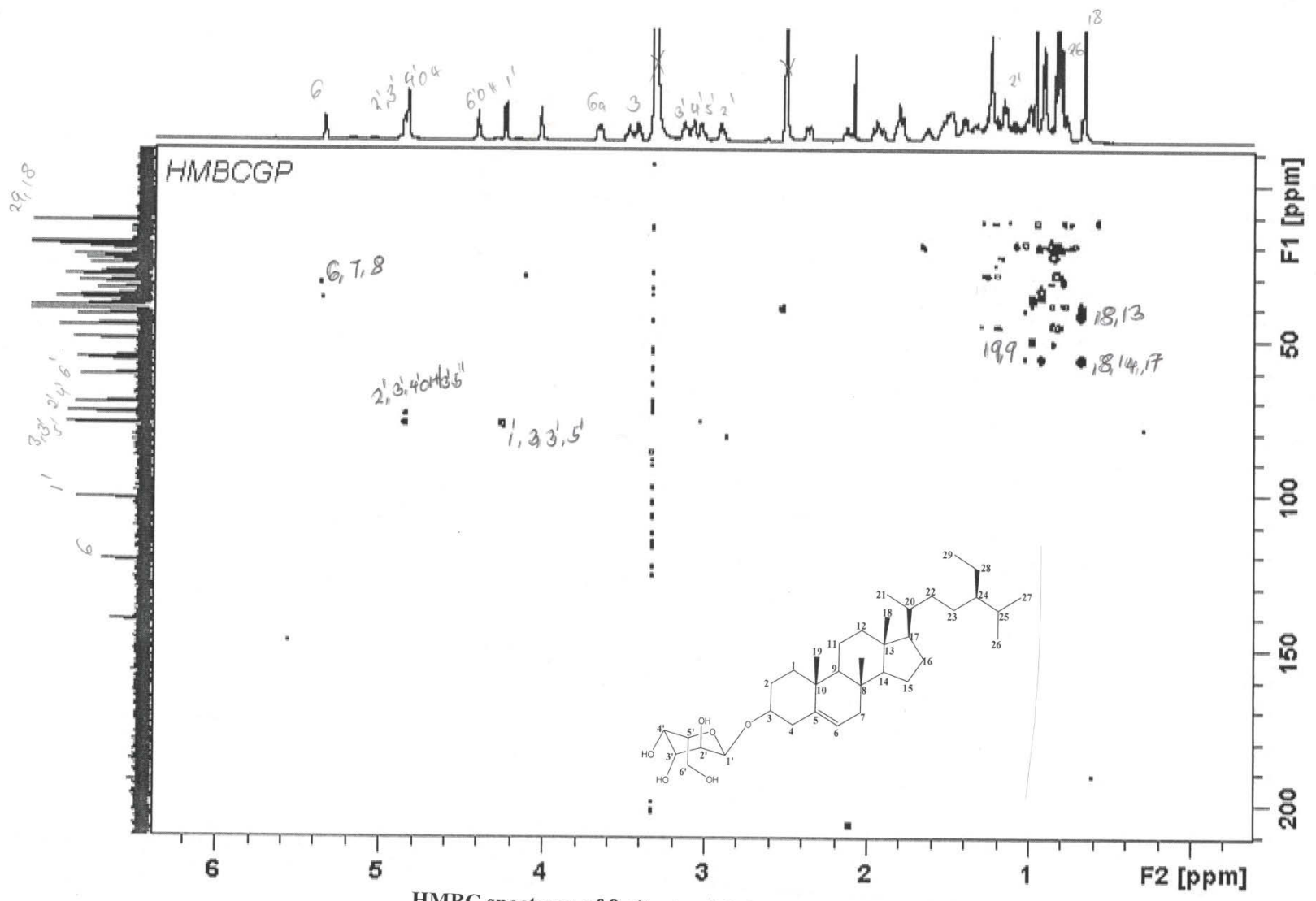
DEPT spectrum of β-sitosterol-3-O-glucoside



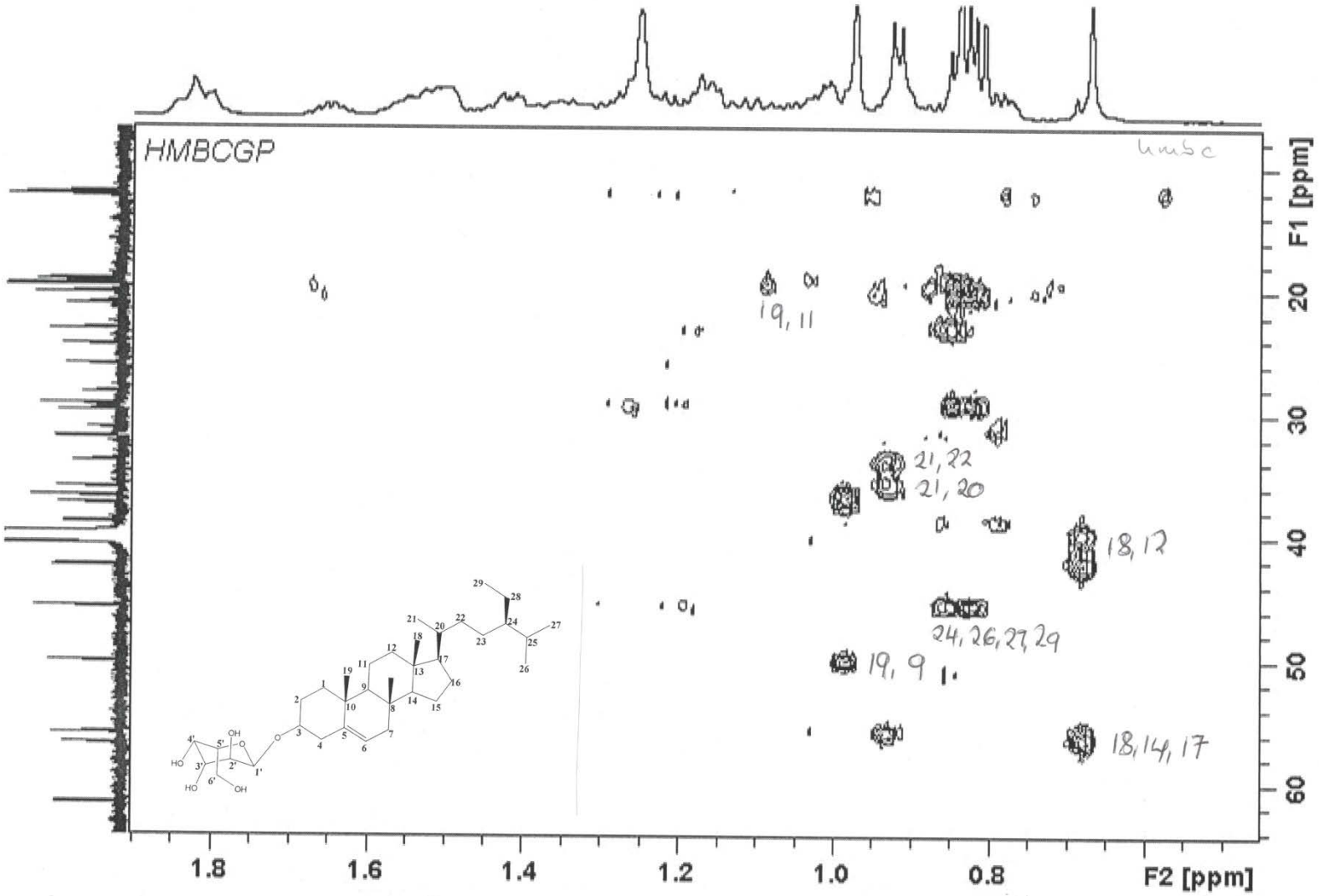
Expanded DEPT spectrum of β-sitosterol-3-O-glucoside



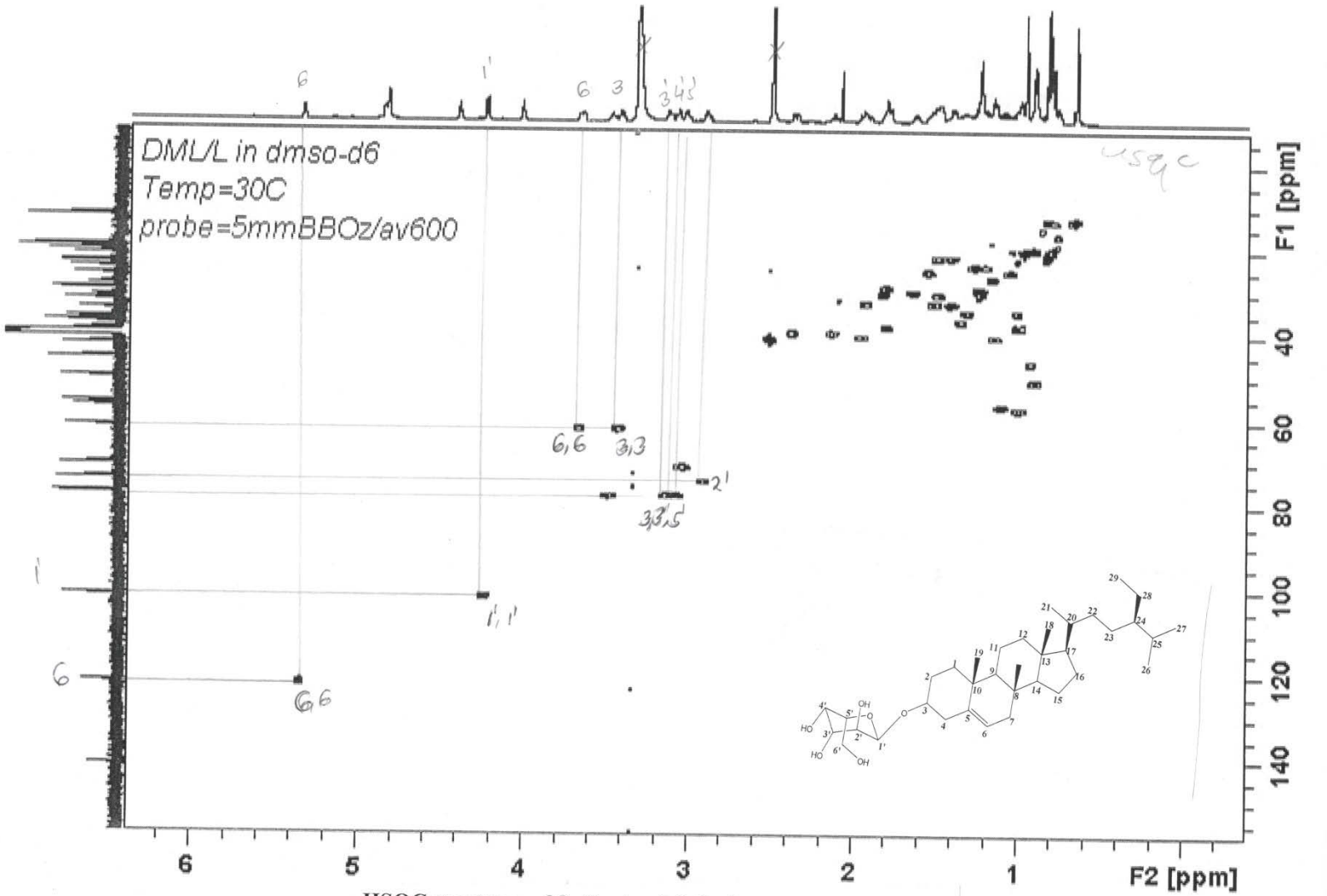
COSY spectrum of β-sitosterol-3-O-glucoside



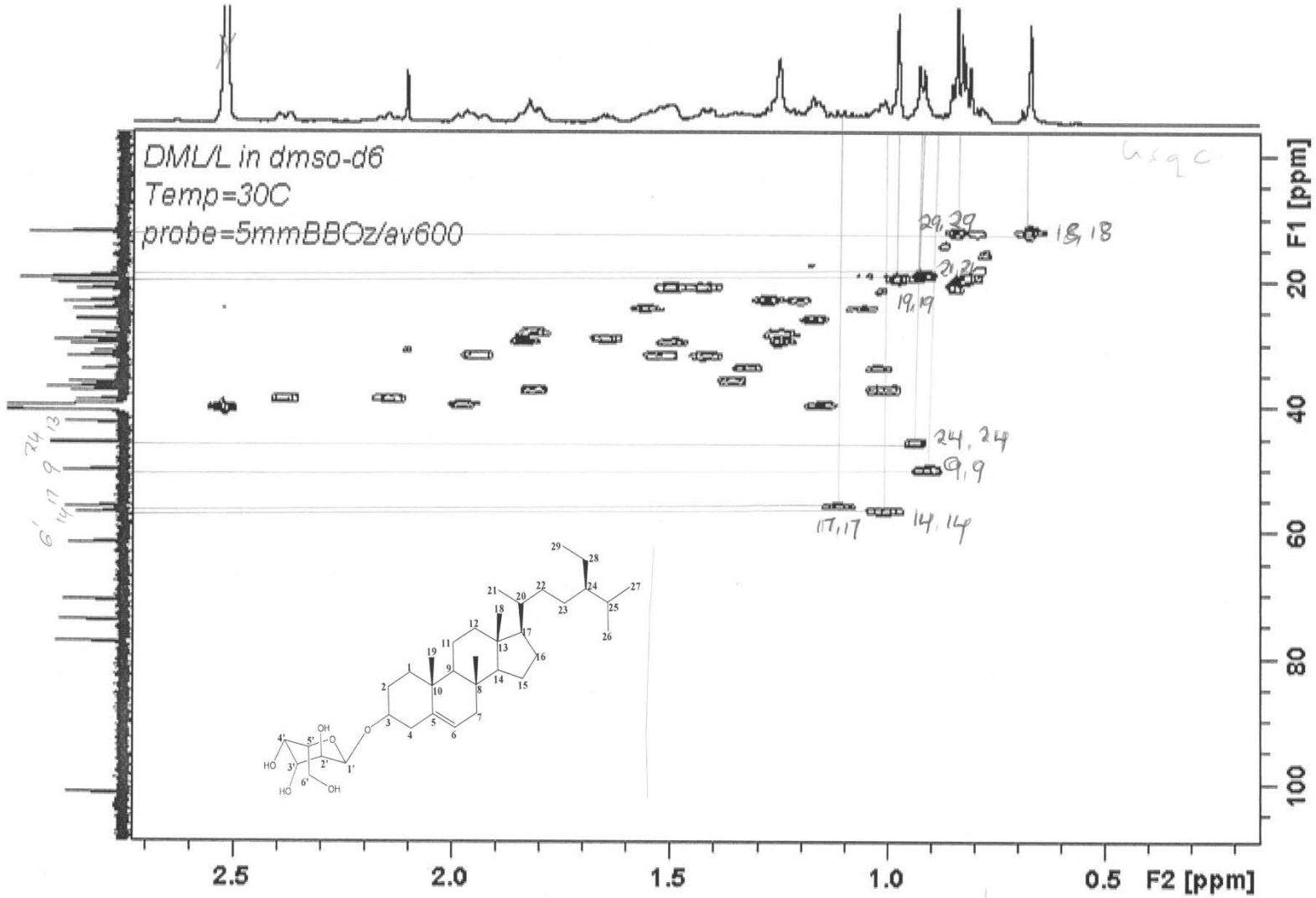
HMBC spectrum of β-sitosterol-3-O-glucoside



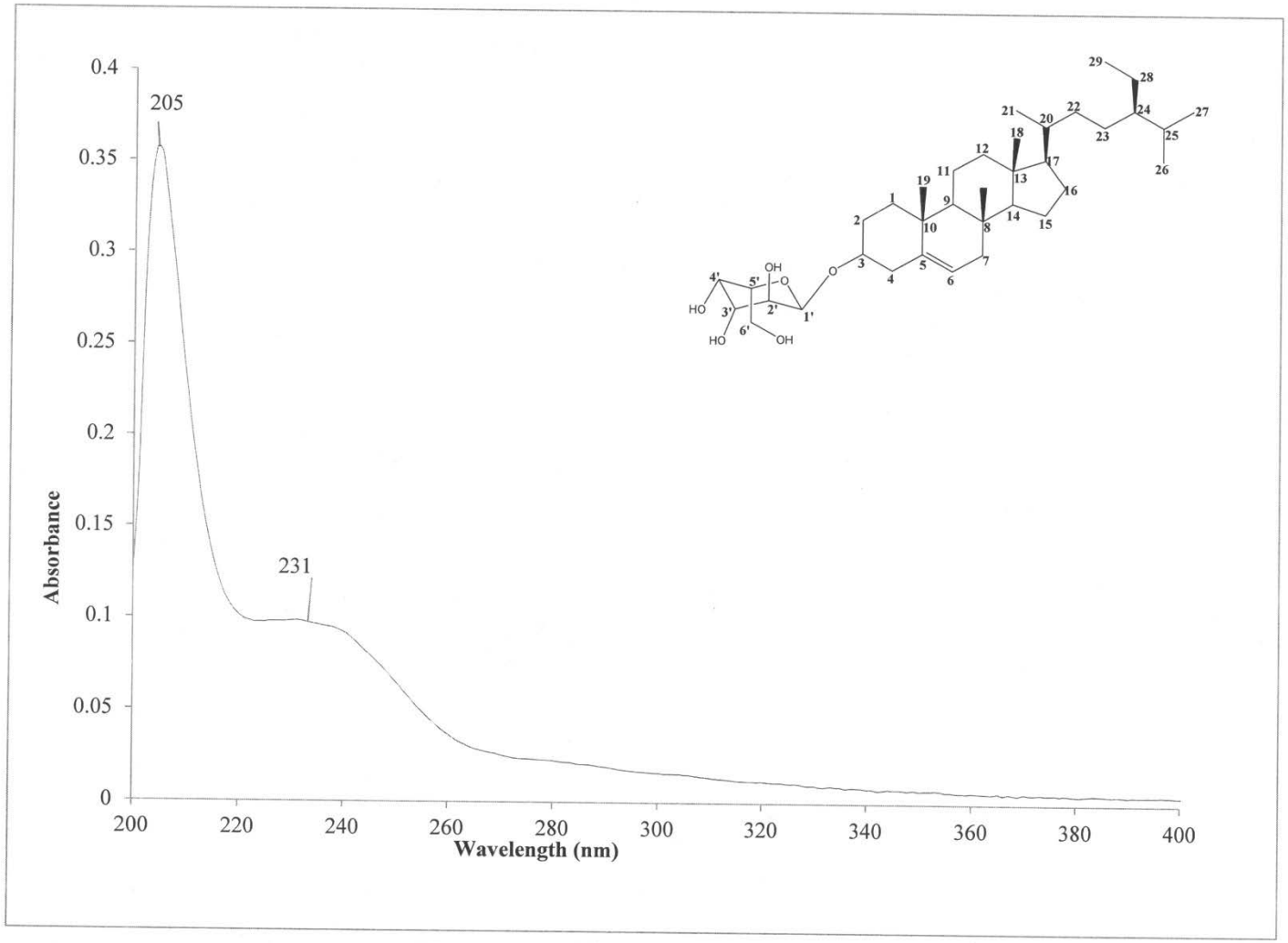
Expanded HMBC spectrum of β-sitosterol-3-O-glucoside



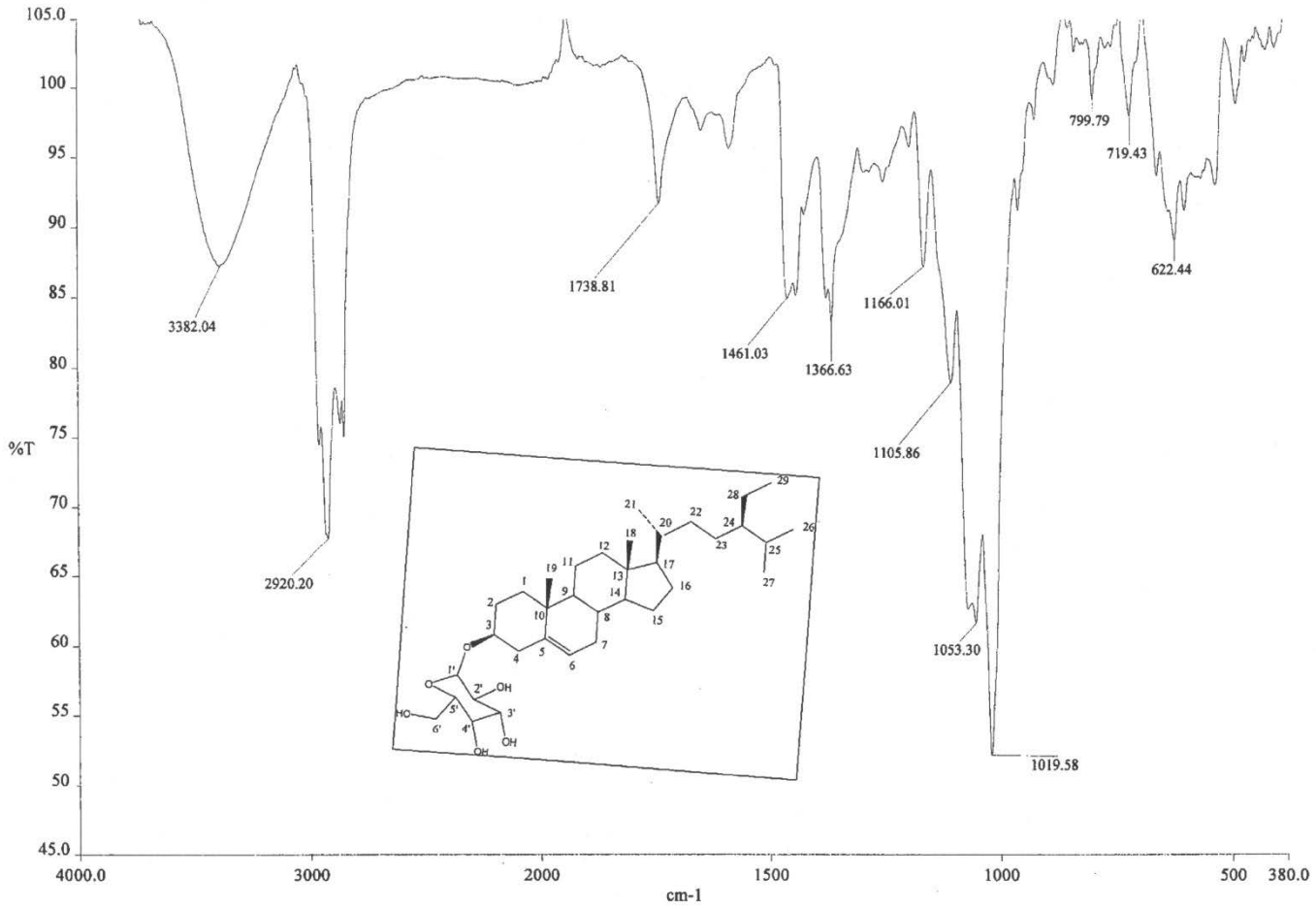
HSQC spectrum of β-sitosterol-3-O-glucoside



Expanded HSQC spectrum of β-sitosterol-3-O-glucoside

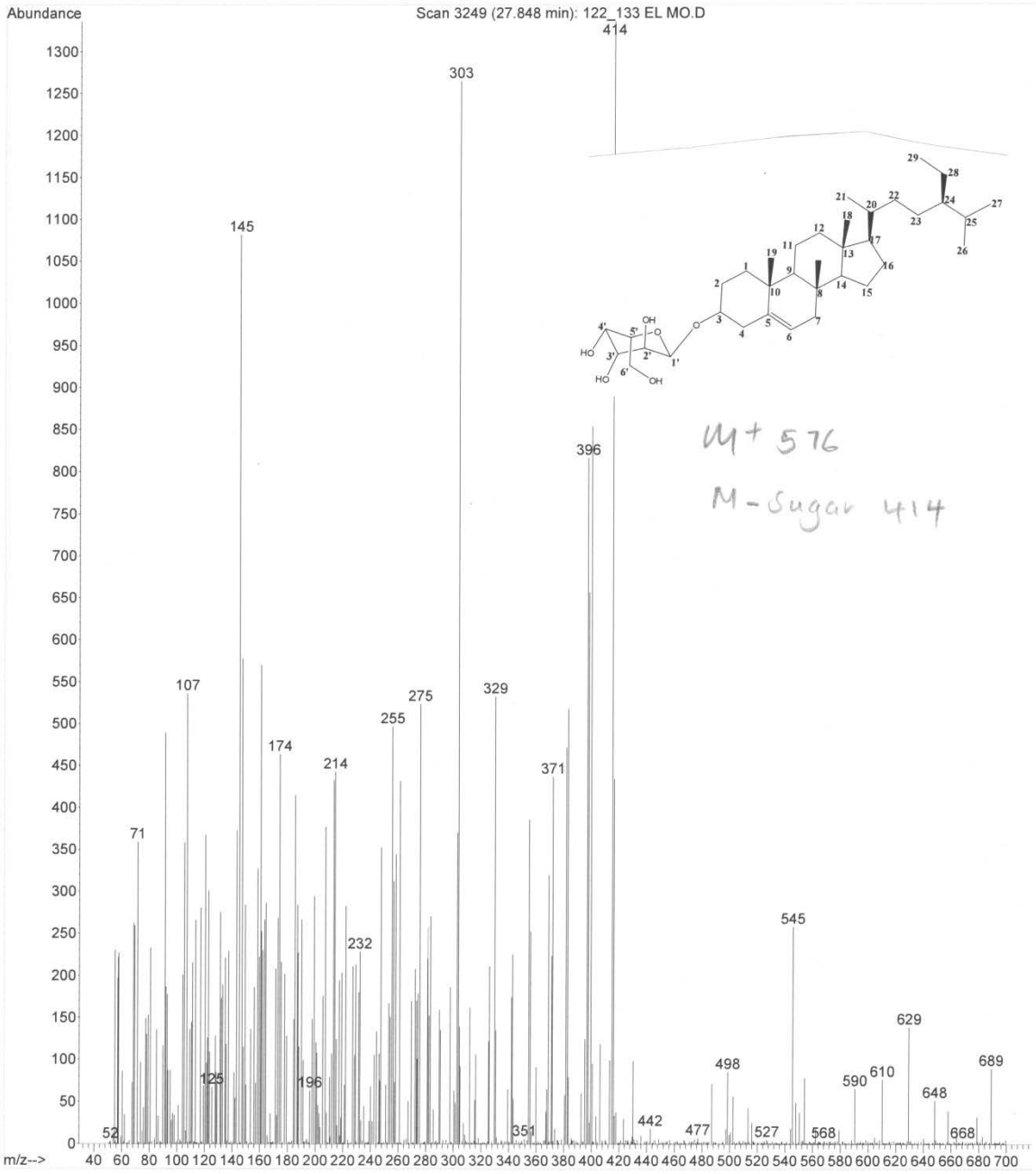


UV spectrum of β-sitosterol-3-O-glucoside



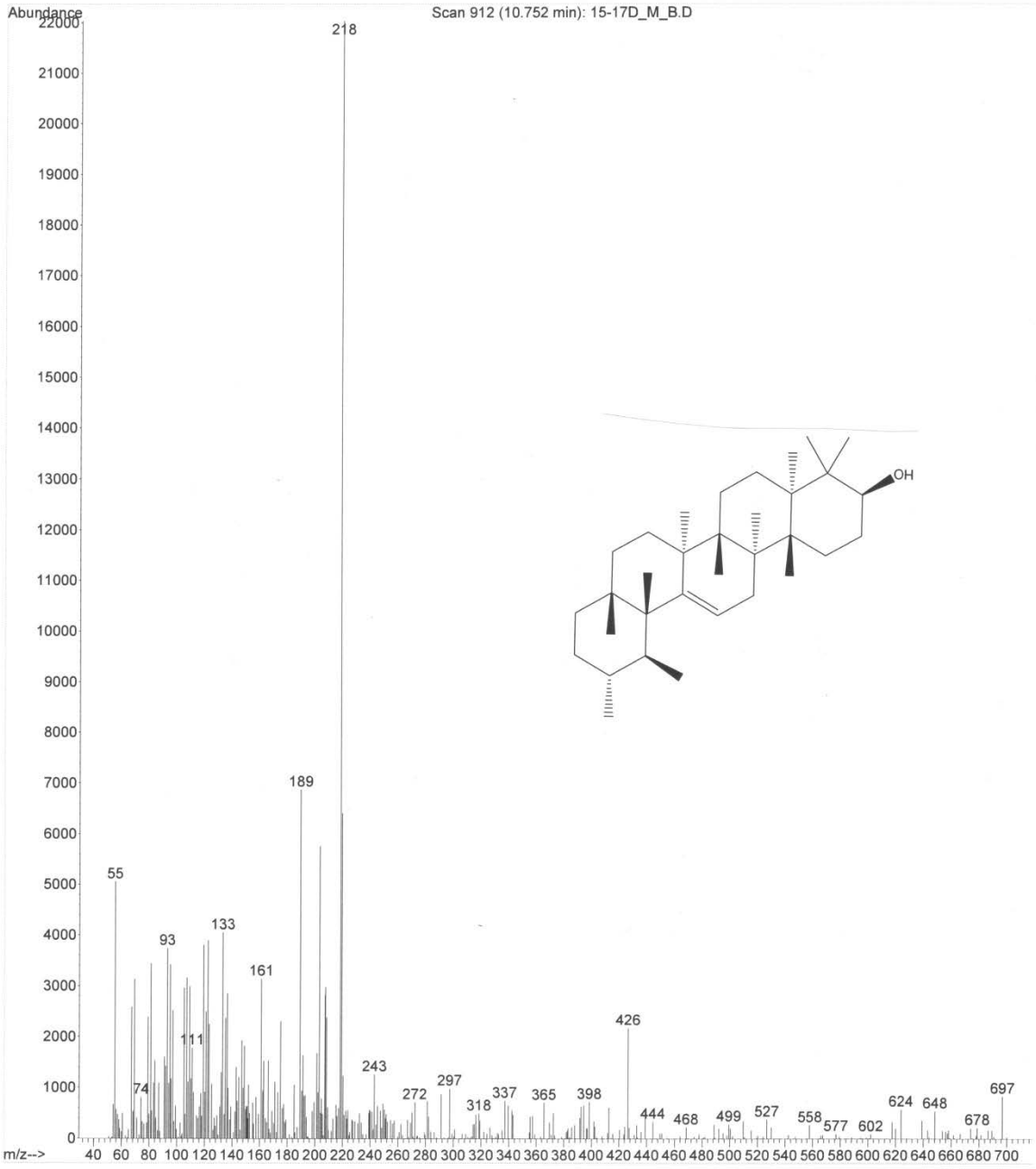
IR spectrum of β-sitosterol-3-O-glucoside

File : C:\MSDCHEM\1\DATA\FIONA\122_133 EL MO.D
Operator : fiona
Acquired : 6 Aug 2013 5:39 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973n
Sample Name: 122 133 EL MO
Misc Info :
Vial Number: 16



MS spectrum of β-sitosterol-3-O-glucoside

File : C:\MSDCHEM\1\DATA\FIONA\15-17D_M_B.D
Operator : fiona
Acquired : 5 Jun 2013 1:26 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973N
Sample Name: 15-17D/M/B
Misc Info :
Vial Number: 10



MS spectrum of α-amyrin

```
File       : C:\MSDCHEM\1\DATA\FIONA\17-26 D_M_L.D
Operator   : fiona
Acquired   :  5 Jun 2013    5:38      using AcqMethod NATPRODUCTS AUTOMAT
Instrument :   5973N
Sample Name: 17-26 D M L
Misc Info  :
Vial Number: 6
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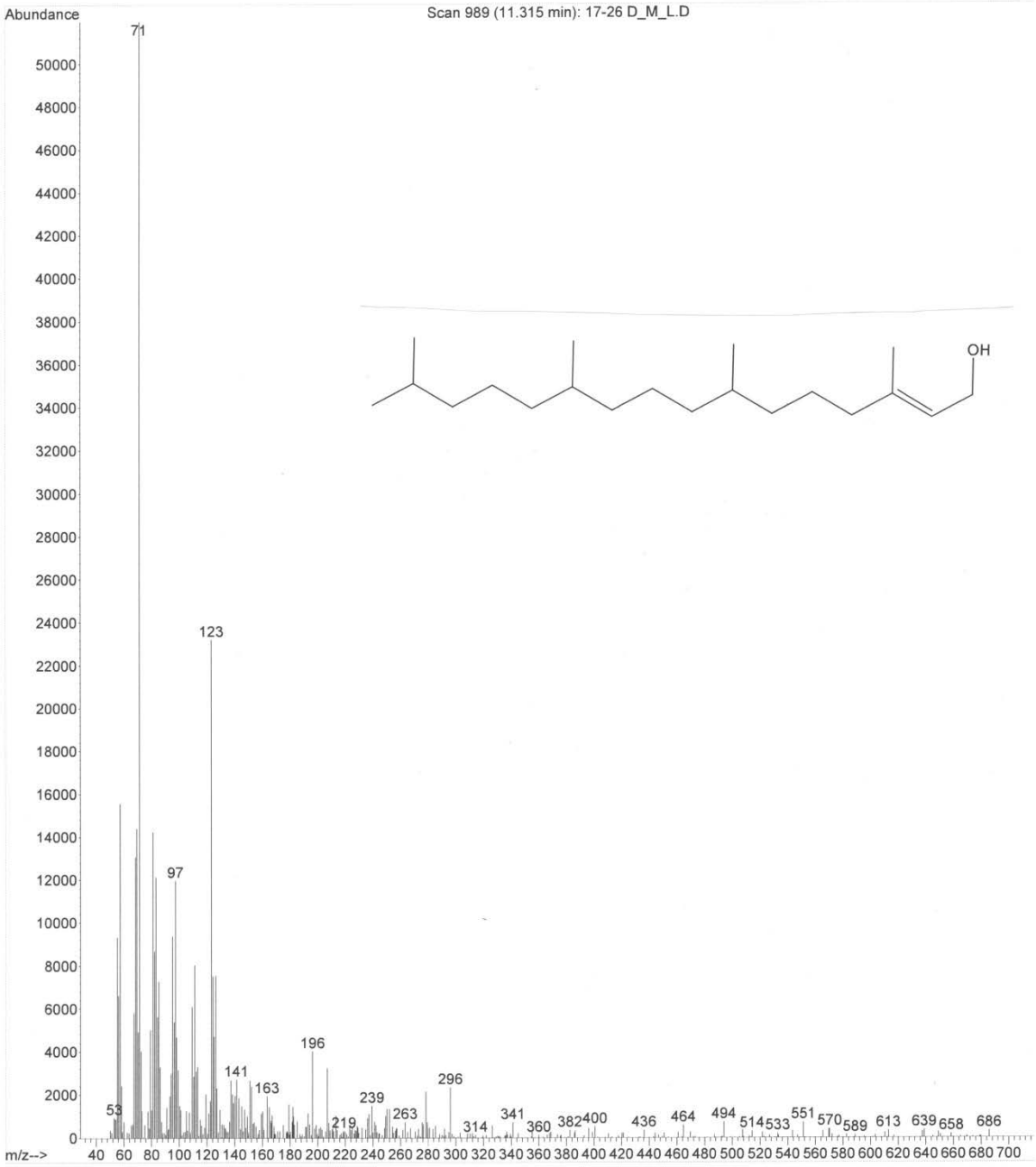
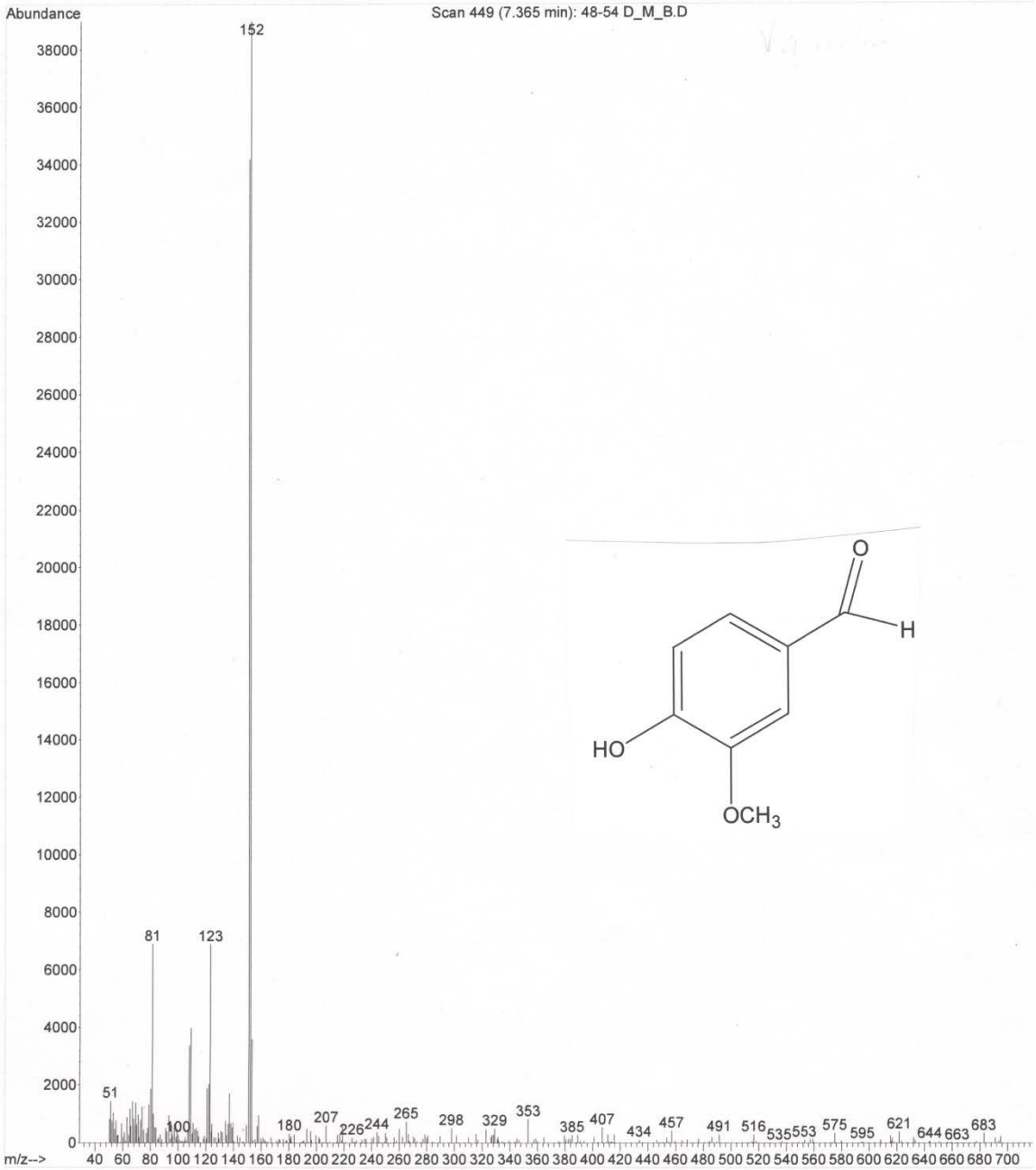


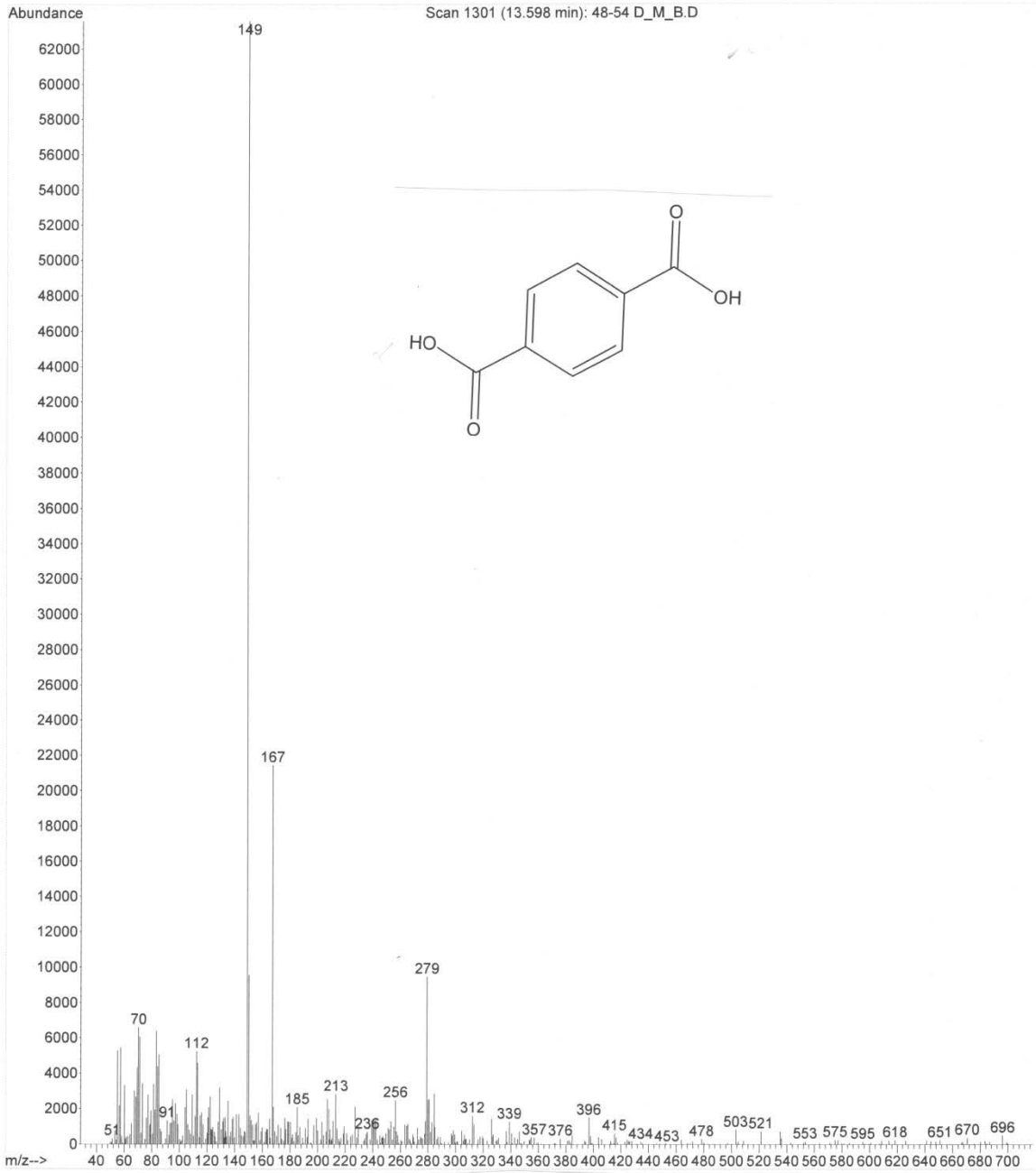
MS spectrum of phytol

File : C:\MSDCHEM\1\DATA\FIONA\48-54 D_M_B.D
Operator : fiona
Acquired : 5 Jun 2013 3:18 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973N
Sample Name: 48-54 D M B
Misc Info :
Vial Number: 2



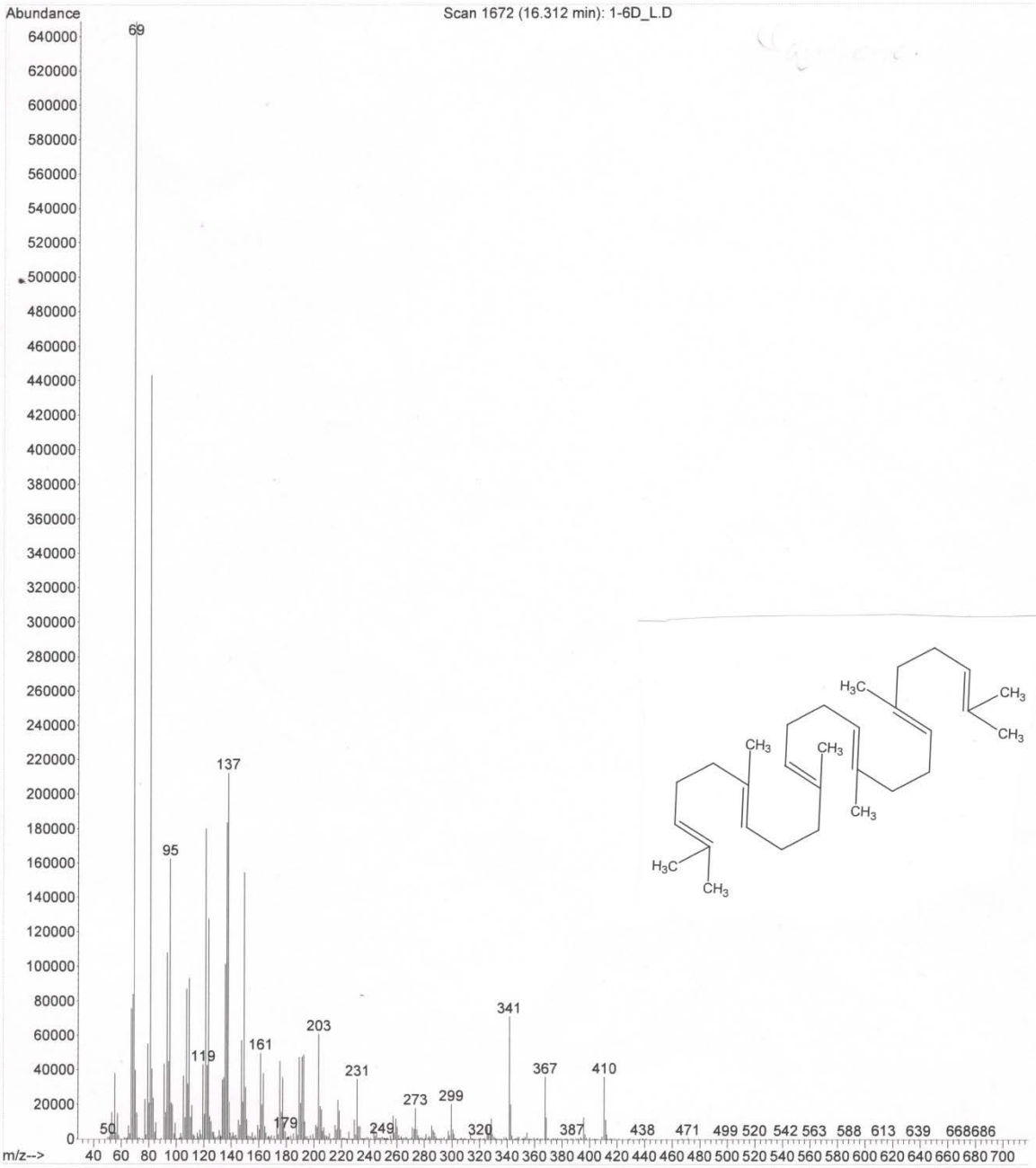
MS spectrum of Vanillin

File : C:\MSDCHEM\1\DATA\FIONA\48-54 D_M_B.D
Operator : fiona
Acquired : 5 Jun 2013 3:18 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973N
Sample Name: 48-54 D M B
Misc Info :
Vial Number: 2



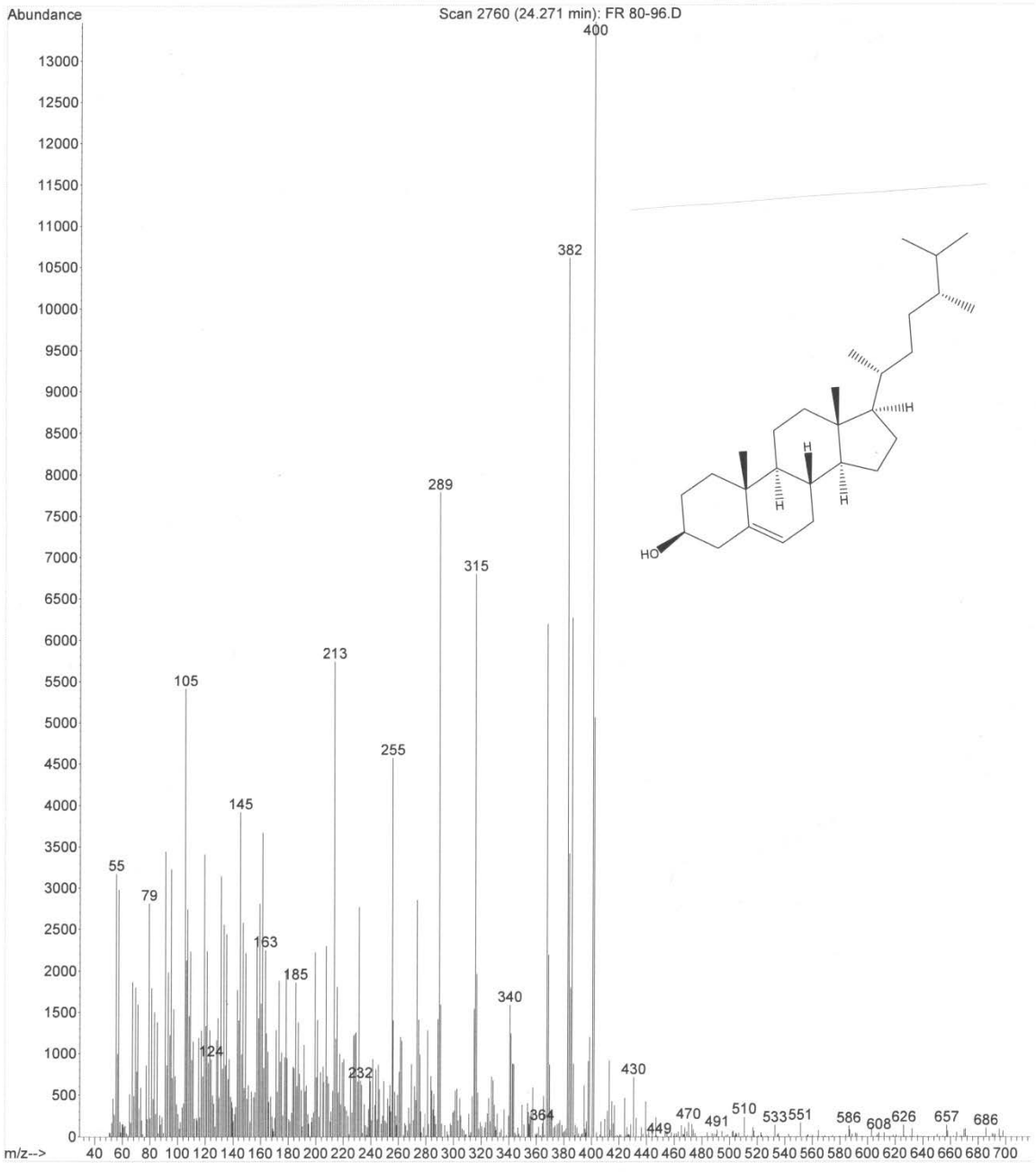
MS spectrum of Benzenedicarboxylic acid

File : C:\MSDCHEM\1\DATA\FIONA\1-6D_L.D
Operator : fiona
Acquired : 4 Jun 2013 20:10 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973N
Sample Name: 1-6D/L
Misc Info :
Vial Number: 1



MS spectrum of Squalene

File : C:\MSDCHEM\1\DATA\FIONA\FR 80-96.D
Operator : FIONA
Acquired : 10 Apr 2013 15:28 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973N
Sample Name: FR 80-96
Misc Info :
Vial Number: 2



MS of Campesterol

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File       : C:\MSDCHEM\1\DATA\FIONA\39-44 D_M_L.D
Operator   : fiona
Acquired   :  5 Jun 2013    6:13      using AcqMethod NATPRODUCTS AUTOMAT
Instrument :   5973N
Sample Name: 39-44 D M L
Misc Info  :
Vial Number: 7
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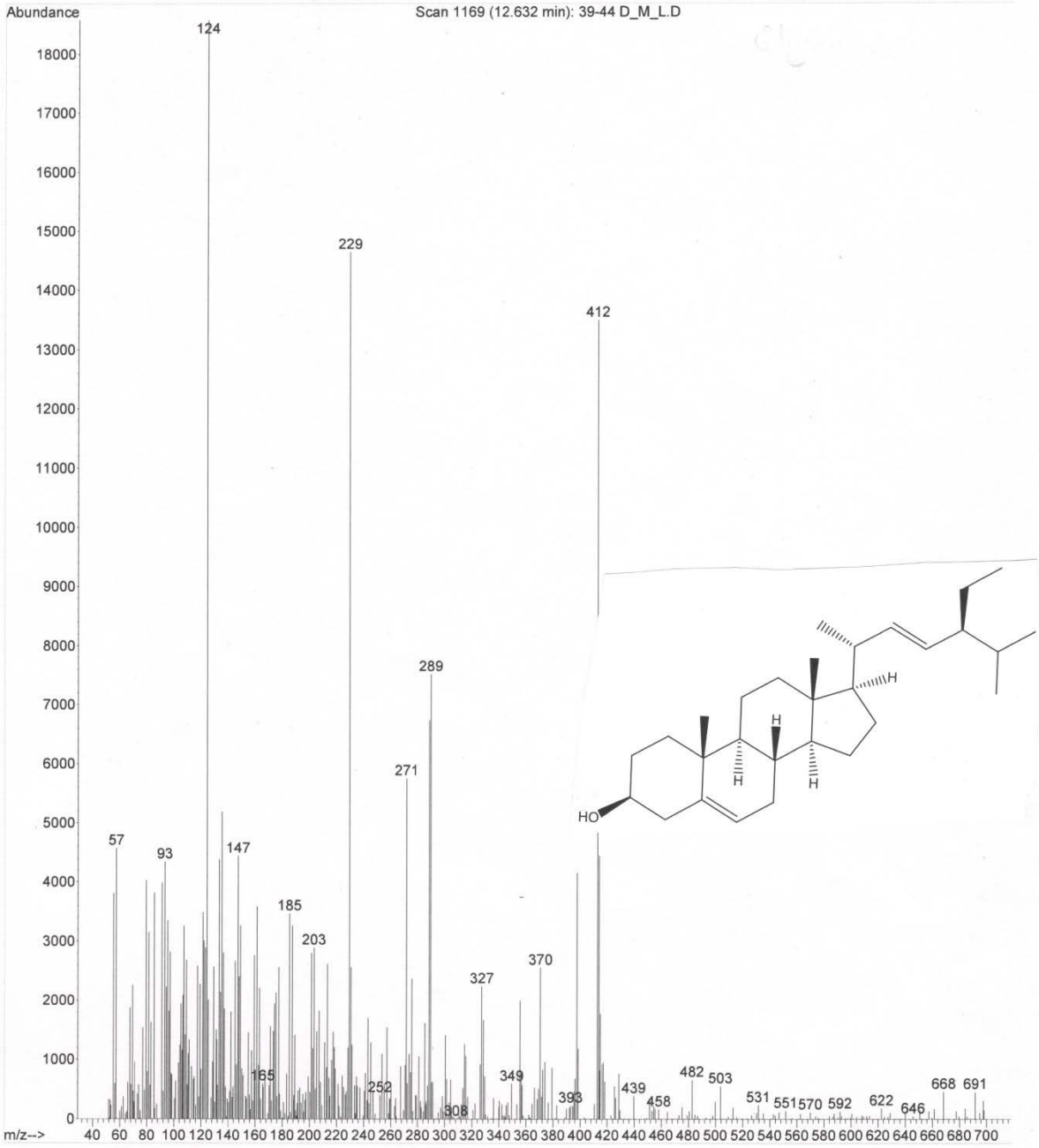
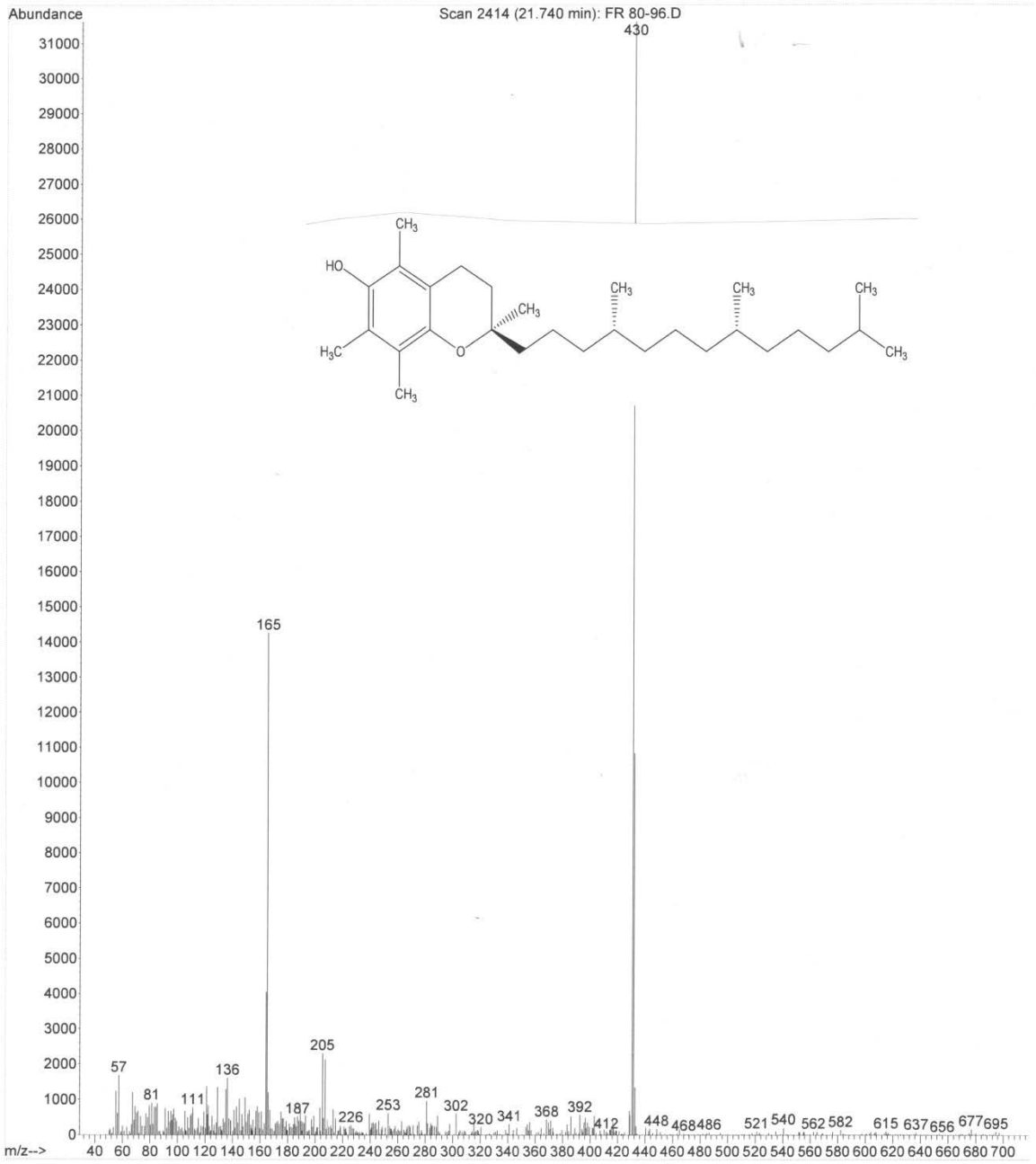


MS spectrum of Stigmasterol

File : C:\MSDCHEM\1\DATA\FIONA\FR 80-96.D
Operator : FIONA
Acquired : 10 Apr 2013 15:28 using AcqMethod NATPRODUCTS AUTOMAT
Instrument : 5973N
Sample Name: FR 80-96
Misc Info :
Vial Number: 2



MS spectrum of α-tocopherol