ELEMENTAL AND PHYTOCHEMICAL STUDIES OF TWO *RHOICISSUS* SPECIES (*RHOICISSUS DIGITATA* AND *RHOICISSUS TOMENTOSA)* FOUND IN KWAZULU-NATAL, SOUTH AFRICA

BY

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Submitted in the fulfilment of the academic requirements for the degree

MASTER OF SCIENCE IN CHEMISTRY

In the School of Chemistry and Physics

College of Agriculture, Engineering and Science

University of KwaZulu-Natal, Durban

July 2019

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DECLARATION

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I Valentine Uwumubyeyi declare that

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CONFERENCES

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Poster: Chemical constituents and *in vitro* antioxidant activity of crude extracts and compounds from *Rhoicissus digitata*

Authors: Valentine Uwumubyeyi, Dr. Bongiwe Mshengu, and Dr. Roshila Moodley

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2. SACI/ChromSA postgraduate research colloquium, DUT (Ritson campus), 2019

Poster: Chemical constituents and *in vitro* antioxidant activity of crude extracts and compounds from *Rhoicissus tomentosa*

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In all the manuscripts prepared, I have performed all the experimental work and written the manuscripts. The co-authors were involved in discussion of the results and were responsible for verifying the scientific content and accuracy of the results as well as editing the manuscripts.

Signed 

ACKNOWLEDGEMENTS

Thanks to Almighty God, through his favour, this study is successfully completed.

I would like to express my sincere gratitude and appreciation to my supervisors: Dr. Bongiwe Mshengu, and Dr. Roshila Moodley for their tireless supervision, suggestions, advices, corrections, and their encouragement during the course of this work.

I would like to acknowledge the University of KwaZulu-Natal and my supervisors for the research facilities.

I would like to acknowledge my husband Wellars Kuradusenge for the financial support and words of encouragement.

ABSTRACT

Traditional medicine is used worldwide to prevent and treat various human diseases. It is estimated that 70-80% of Africans depend on medicinal plants to meet their primary health care needs. *Rhoicissus digitata* and *Rhoicissus tomentosa* (Vitaceae) are indigenous medicinal plant species that are commonly used by South Africans for the treatment of gynaecological and obstetric related conditions. The fruits of these plants are eaten by the local people and birds, and are used to make jelly, vinegar and wine.

In this study, a phytochemical analysis of the roots and fruit extracts of *R. digitata* was conducted, and this resulted in the isolation of two flavonoids ((+)-catechin and quercetin) and three triterpenes (12,13-dehydrolupeol, β-sitosterol, and oleanolic acid). Chromatographic purification of the roots, leaves and fruits of *R. tomentosa* that was also conducted, led to the isolation of four terpenoids (3β-taraxerol, stigmasterol, oleanolic acid and β-sitosterol), three flavonoids (quercetrin, (+)-catechin and aromadendrin7-O-β-glycopyranoside), and two pigment compounds (lutein and pheophytin a). The *in vitro* antioxidant activity of the crude methanol extracts and isolated flavonoids was comparable to that of known antioxidants (ascorbic acid, and butylated hydroxytoluene). The methanol extracts from the roots of both plants showed higher antioxidant activity compared to the other plant parts and quercetrin and quercetin showed the highest antioxidant potential of the tested flavonoids. These results suggest that *R. digitata* and *R. tomentosa* may be used as natural antioxidants to prevent or cure oxidative stress-related diseases.

This study also reports on the elemental composition of the roots, leaves and fruits of *R. digitata* and *R. tomentosa* in order to assess for nutritional value and potential metal toxicities. The

analytical results indicate *R. digitata* and *R. tomentosa* to be good sources of essential dietary nutrients including Mg, Mn, Cu, Ca, Fe, Zn and Se with low levels of the toxic metals, Pb and Cd. However, both plant species were found to take up As from the environment. Therefore, consumption by pregnant women, that use the plant medicinally to facilitate labour, should be controlled due to the plants potential to accumulate this toxic element.

The findings of this study validates the ethno-medicinal uses of *R. digitata* and *R. tomentosa* which is attributable to the presence of known bioactive molecules including triterpenes, sterols and flavonoids and provides evidence on the nutritional value of the edible fruits, which, if consumed by vulnerable communities, may contribute towards a diverse, more nutritious diet. The findings also suggest that moderate consumption by humans should be safe and is not likely to pose the risk of metal toxicity.

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ABBREVIATIONS

NMR	nuclear magnetic resonance
CC	column chromatography
COSY	correlated spectroscopy
CRM	certified reference material
d	doublet
dd	double of doublet
DEPT	distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRI	dietary reference intake
EtOAc	ethyl acetate
FDA	Food and Drug Administration
FRAP	ferric reducing antioxidant potential
GC-MS	gas chromatography-mass spectrometry
HMBC	heteronuclear multiple bond coherence
HSQC	heteronuclear single quantum coherence
ICP-OES	inductively coupled plasma-optical emission spectrometry
IR	infrared
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
m	multiplet
MeOH	methanol
ND	not determinable
ppm	part per million
RDA	recommended dietary allowance
s	singlet
t	triplet
TLC	thin layer chromatography
UL	tolerable upper intake level

CHAPTER 1: INTRODUCTION

1.1 Introduction to medicinal plants

The use of plants in improving the quality of human life is becoming increasingly popular (Sapam *et al*., 2018). It is estimated that 80% of the world's population depends on medicinal plants to meet their primary health care needs (Meena *et al*., 2009). People depend on medicinal plants as they are affordable, readily available, and are culturally acceptable. Knowledge on the medicinal uses of these plants is passed on from generation to generation or imparted by traditional healers (Olajuyigbe & Afolayan, 2012). The therapeutic effectiveness of medicinal plants is due to the presence of bioactive molecules (natural products) in them (Meena *et al.*, 2009). However, traditional healers are not aware of the identity of these biomolecules.

The isolation and identification of biologically active constituents from natural products such as crude plant extracts, to validate their traditional uses, started approximately 200 years ago (Brown *et al.*, 2014). Plant-derived drugs discovered in the early years include morphine (an analgesic and sleep-inducing agent obtained from the opium poppy), digoxin (a drug that was first isolated from *Digitalis lanata* that is used to treat various heart disorders), quinine and artemisinin (antimalarial drugs isolated from the cinchona tree and *Artemisia annua*, respectively), and aspirin (a drug used to treat pain, fever and inflammation which is derived from salicylic acid, an active component of the willow tree) (Adeleye *et al.*, 2019; Atanasov *et al.*, 2015; Klayman *et al.*, 1984). These drugs continue to play an important role in modern medicine for their therapeutic effects and as lead compounds for new drug development.

To date, approximately 120-126 of the commercially available drugs worldwide are derived from plants (Atanasov *et al.*, 2015; Dewick, 2002; Marles, 1996). Plant-derived drugs contribute to the treatment of numerous infectious, neurological, cardiovascular, metabolic, immunological, inflammatory and oncological diseases (Atanasov *et al.*, 2015). Recently, various plant-derived drugs have made an impact on modern medicine. These include reserpine, which was isolated from *Rauvolfia serpentina* (Apocynaceae) and is used as a tranquilizer and for the treatment of high blood pressure (Ascani & Smith, 2011). This drug was first approved by the Food and Drug Administration (FDA) in 1955 and is normally used together with a thiazide diuretic or vasodilator. Reserpine was the first drug involved in sympathetic neurotransmission in the human body (Berger *et al.*, 2005).

Homoharringtonine (HHT) was obtained from the *Cephalotaxus* species, *C. hainanensis* and *C. qinensis*. HHT was approved as an anticancer agent by the FDA in 2012 (Cragg & Newman, 2005; Newman & Cragg, 2016). HHT is an ester of cepholotaxine, which is isolated from the aqueous extract of the root, stem and seeds of *C. harringtonii* (Powell & Weisleder, 1970). Another recently approved anticancer agent is ingenol mebutate. This drug was approved by the FDA in 2012 for the treatment of actinic keratosis and was isolated from *Euphorbia pelpus*, a medicinal plant used to treat skin lesions (Lebwohl *et al.*, 2013).

Vernodalin and wighteone are plant-derived drugs used to treat infectious diseases. Vernodalin was isolated from *Vernonia colorata* (Asteraceae), a traditional medicinal plant used to treat fever, diarrhoea, abdominal pain, rheumatism and infertility in women (Reid *et al.*, 2001). Wighteone was obtained from the stem bark of *Erythrina variegata* in 1977 and later from *Erythrina lysistemon* (Fabaceae). *E. lysistemon* is commonly used by the Xhosa and Zulu

people to treat respiratory infections, earache, toothache, abscesses, sprains, and to disinfect wounds (Deshpande *et al*., 1977; Hutchings, 1996; Pillay *et al.*, 2001).

Hypericin and pseudohypericin are used as antibiotic and antiviral agents. These compounds inhibited the *in vitro* and *in vivo* propagation and radiation of Friend leukaemia virus (Kirakosyan *et al*., 2004. Hypericin and pseudohypericin were isolated from *Hypericum perforatum,* a medicinal plant used to treat wounds and inflammation. The crude extracts from *H. perforatum* were reported to show antiviral, anticancer, and antidepressant activities (Kirakosyan *et al.*, 2004).

1.2 Statement of the problem

While the search for drugs and lead compounds from plants have been successful, most of the plant biodiversity still remains unexplored. It is estimated that there are about 250 000 to 500 000 plant species on earth and only 1 to 10% of these species are used as food, while the rest are used for medicinal purposes (Cowan, 1999). Biological activities and phytochemistry have only been reported for about 6% and 15% of the medicinal plants, respectively (Atanasov *et al.*, 2015). Therefore, further research on the phytochemistry and pharmacology of medicinal plants is of great importance as this may contribute towards the identification of lead compounds and the discovery of new drugs.

Traditional medicinal plants are normally assumed to be safe for human consumption as plants have a long history of usage for the treatment of various diseases. In addition, the indigenous people from poor communities have relied on wild plants for their nutritional needs since time immemorial. However, plants are prone to heavy metal contamination which can cause adverse health effects if consumed, due to metal toxicities. A study on the elemental composition of

medicinal plants to monitor the trace metal levels is crucial to assess the suitability of these plants for human consumption (as food or for medicinal purposes).

1.3 Aim and research objectives

The aim of this study was to conduct a phytochemical and elemental investigation of two *Rhoicissus* plant species (*R. digitata* and *R. tomentosa*) that are indigenous to South Africa.

The objectives of this study were:

- to extract and isolate the secondary metabolites from the roots and fruits of *R. digitata* and the roots, leaves, and fruits of *R. tomentosa* using chromatographic techniques (thin layer chromatography (TLC) and column chromatography (CC)).
- to characterise and identify the isolated phytocompounds using different spectroscopic techniques (nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), infrared spectroscopy (IR) and ultraviolet-visible (UV/Vis) spectroscopy).
- to evaluate the antioxidant activity of the crude methanol extracts and pure compounds isolated from *R. digitata* and *R. tomentosa*.
- to determine the elemental composition of the roots, leaves, and fruits of *R. digitata* and *R. tomentosa* in order to assess their nutritional value and to evaluate for potential metal toxicities.

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CHAPTER 2

This chapter is a review of the literature on Vitaceae species with focus on *Rhoicissus* species, particularly, *Rhoicissus digitata* and *Rhoicissus tomentosa*. Plants as antioxidants, essential nutrients and heavy metal toxicity, as well as instruments used to fulfil the aims of this study are also discussed.

2.1 Introduction

The family Vitaceae (commonly known as the grape family) is mainly comprised of dicotyledonous flowering plants with 14 genera and about 900 species (Christenhusz & Byng, 2016; Ren *et al.*, 2011). The name Vitaceae is sometimes confused with Vitidaceae in old literature, but the accepted name is Vitaceae. Vitaceae plants are woody climbers, herbaceous vines and succulent shrubs, which are important sources of food and raw materials for the production of medicine, wine and perfume (Najmaddin *et al.*, 2011; Ren *et al.*, 2011).

Plant species of the Vitaceae family are characterised by leaves with tendrils on opposite sides which are used to support the plant as it climbs over trees and bushes (Zhang *et al.*, 2015). In addition, the leaves have small multicellular, spherical epidermal structures with a short stalk and opposite the leaves are small greenish, inconspicuous flower clusters (Gerrath *et al.*, 2015). Plants in this family are grouped into different classes based on the shoot architecture (Wilson *et al.*, 2006).

2.2 Distribution and traditional uses of Vitaceae plants

Vitaceae plants originated from the Laurasian region and are found in other regions, such as the Southern hemisphere (Wen *et al.*, 2013). Plants in this family are distributed in the tropical regions of Africa, Asia, Australia, the Neotropics, and in the Pacific Islands (Ren *et al.*, 2011; Soejima & Wen, 2006). *Parthenocissus* Planch and *Ampelopsis* Michx are the two genera in the Vitaceae family that are found in the northern temperate regions (Ren *et al.*, 2011). *Cissus* Linn. is the largest genus, with about 350 species that are grown in tropical and temperate zones (Gerrath & Posluszny, 1994). The smallest genera in this family (*Acareosperma* Gagnepain and *Clematicissus* Planch) only have one species each and are found in Laos and Western Australia, respectively (Soejima & Wen, 2006).

Vitaceae species are well known in traditional medicine for treating gynaecological and obstetric problems (Table 2.1) (Abdillahi & Van Staden, 2013). Examples of plants and plant parts used to treat these disorders are highlighted in Table 2.1. Other uses of plants in this family include treatment of backache, malaria, stomach-aches, kidney and bladder infections, and gastrointestinal problems. The plant parts used as medicine vary from one species to another, from practitioner to practitioner and also depends on the nature and state of the disease (Dewick, 2002).

Table 2.1: Traditional uses of some Vitaceae plants

Plants species	Part used	Therapeutic uses	References
Ampelopsis brevipedunculata and *A. sinica*	Root/stem Leaf/bulb	Used to treat liver and inflammation	(Parekh & Chanda, 2006)
Cissus populnea	Root/leaf	Used to treat sores on the breast of women after child birth and to increase milk production	(Steenkamp, 2003)
Cissus sicyoides	Leaf/root	Used to treat diabetes and inflammation	(Parekh & Chanda, 2006).
Cyphostema species	Roots/leaf/ stem	Used to ensure safe delivery during childbirth, used to treat cancer and toothache	(Hutchings, 1996)
Parimari curatellifolia	Bulb	Used to enhance fertility	(Katsoulis, 2014)
Homeria pallida	Bulb	Used to enhance fertility	(Katsoulis, 2014)
Tetrastigma leucostaphylum (Dennst) Alstone	Leaf	Used to treat headache and fever	(Steenkamp, 2003)
Tetrastigma hemsleyanumis	Roots/stem	Used to treat fractures, traumatic injury and swelling and pain	(Steenkamp, 2003)
Vitis venifera Linn	Fruits	Used to treat wounds, haemorrhages, anaemia, leprosy, skin diseases, syphilis, asthma, jaundice, diarrhoea and bronchitis	(Parekh & Chanda, 2006)
Wedilia calendula Linn	Root/leaf	Used to treat hepatic disorders, stomach and lung cancer	(Meena *et al.*, 2009)

2.3 Biological activity of Vitaceae plants

Several biological activities have been reported for the plants belonging to the Vitaceae family. These include anticancer, antioxidant, antiviral, anti-inflammatory and antimicrobial activities. The methanol (MeOH) extract of three *Cyphostemma* species (*C. flaviflorum*, *C. lonigerum* and *C. natalitium*) showed antimicrobial and anti-inflammatory activities against various microorganisms (Lin *et al.*, 1999). In another study, these *Cyphostemma* species also showed anti-proliferative effects against the HepG2 cell line (Opoku *et al.*, 2000). The ethanol extract

of the bark of *Cyphostema greveana* showed anti-proliferative activity against the A2780 human ovarian cancer cell line (Cao *et al.*, 2011).

Parekh and Chanda (2006) studied the antimicrobial activity of the aqueous and ethanol extracts of the leaves of *Vitis venifera* L. In this study, the leaves of *V. venifera* showed the highest inhibition of growth of microorganisms compared to the other plants tested. In particular, the ethanol extract of the leaves of *V. venifera* showed more than 85% inhibition, while the aqueous extract inhibited more than 65% of microbial growth (Parekh & Chanda, 2006). The acetone and water extracts from the rhizomes of *Ampelocissus grantii* exhibited antimicrobial and antioxidant activities. The acetone extract showed higher antioxidant and antimicrobial activities than the water extract (Zongo *et al.*, 2010).

Akram Ali Mohamed Shalabi and Abdel-Sattar (2016) studied the antidiabetic activity of the ethanol extract from the leaves and stem of *Cissus rotundifolia*. The ethanol extract and its fractions showed antidiabetic activity against streptozotocin (STZ) diabetic rats with the activity of the ethanol extract comparable to the diabetic control agent (gliclazide) (Akram Ali Mohamed Shalabi & Abdel-Sattar, 2016). In a study conducted by Omale and Okafor (2008), the extracts of the stems and leaves of *Cissus multistriata* demonstrated antioxidant capacity, membrane stabilisation and cytotoxic activity against brine shrimps (*Arthemia salina*). In this study, the stem showed higher antioxidant and membrane stabilising activity than the leaves with both extracts showing lower cytotoxicity than *Arthemia salina* (Omale & Okafor, 2008). The MeOH extract of the stem of *Cissus quandingularis* showed good *in vitro* antiviral activity against vero cells and HSV (type1 and type 2) (Balasubramanian *et al.*, 2010). The ethanol extract of the fruits of *Ampelocissus latifolia* showed dose-dependent anti-inflammatory and analgesic activities (Das *et al.*, 2014).

The diverse biological activities demonstrated by Vitaceae plants are attributed to the chemical constituents identified in this family and examples of these secondary metabolites are discussed in the following section (Lin *et al.*, 1999).

2.4 Phytochemistry of Vitaceae plants

The family Vitaceae is rich in stilbenoids and other bioactive molecules from different classes including terpenoids, flavonoids, coumarins and alkaloids (Das *et al.*, 2014). The following groups of compounds are often /commonly identified or isolated from the Vitaceae family.

2.4.1 Stilbenoids

Stilbenoids are a group of polyphenols commonly known for their complex structures and their numerous biological activities such as anti-oxidant, anti-inflammatory, anti-fungal, and anti-microbial activities (Rivière *et al.*, 2012). Complex oligomeric stilbenoids can be formed from the monomeric stilbene units, which are synthesised using stilbene synthase (STS) and chalcone synthase (CHS) (Fig. 2.1). The first reaction in the synthesis of stilbenes (Fig. 2.1) involves the deamination of phenylalanine (**A**) to trans-cinnamic acid (**B**) using phenylalanine ammonia-lyase (PAL) and cinnamic acid 4 hydroxylase (C4H). This is followed by the conversion of *p*-coumaric acid (**C**) to 4-coumaroyl: CoA (**D**) by 4-coumarate: CoA ligase (4CL). The addition of the acetate derived from malonyl CoA (**E**) results in a linear chain of tetraketide (**F**), which is further converted to a chalcone (**G**) or stilbene (**H**) depending on the activity of polyketide synthase (Fig. 2.1) (Deng *et al.*, 2017).

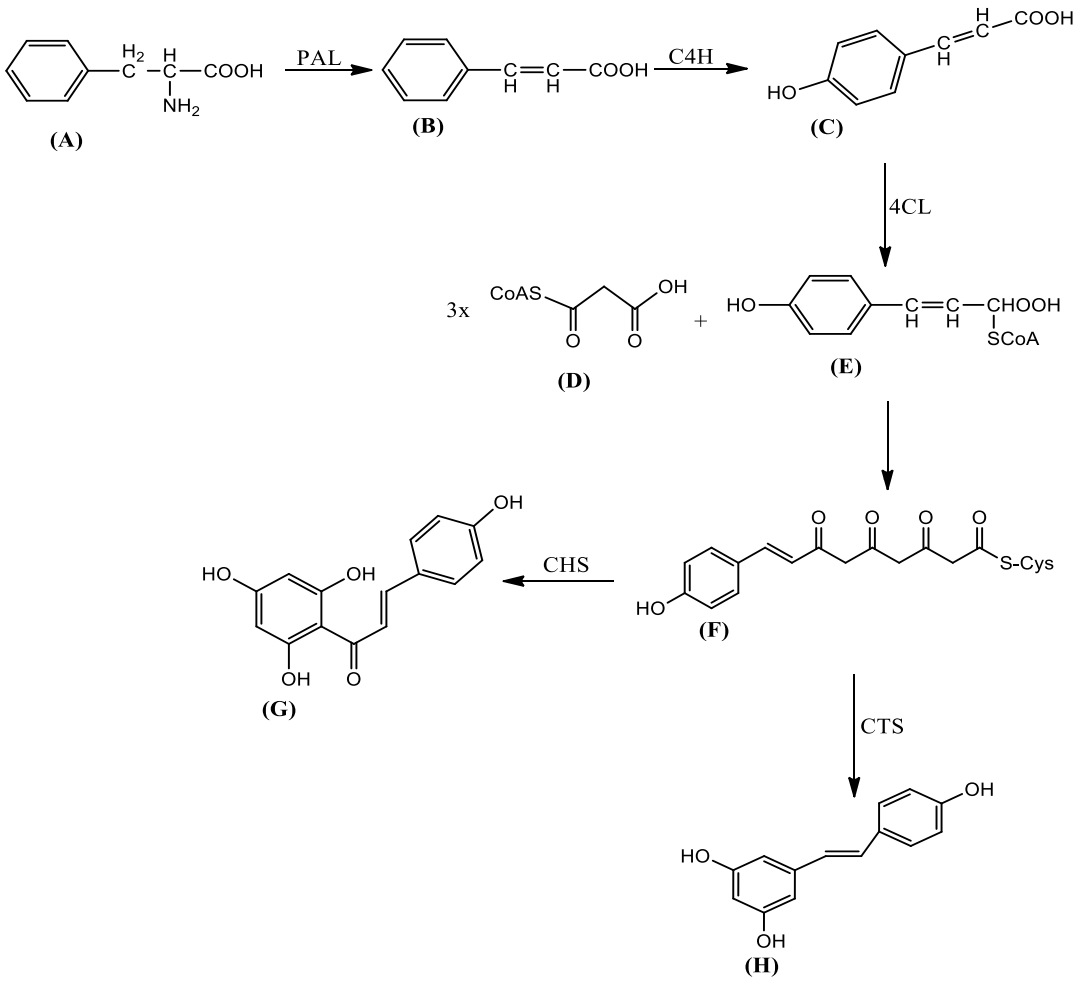


Figure 2.1: The biosynthetic pathway of stilbenes

The monomer resveratrol has been isolated from several Vitaceae genera such as *Ampelopsin*, *Cayiratia*, *Cissus*, *Cyphostemma*, *Muscadinia*, *Partenocissus*, *Rhoicissus*, and *Vitis*. Some stilbenes: longistylin A (**1**), longistylin C (**2**) and epiceid-(1→6)- β-*D*-glycopyranoside (**7**) were obtained from the MeOH extract of the leaves of *Parthenocissus tricuspidata* (Vitaceae) (Rivière *et al.*, 2012). Son *et al* (2007) reported that these compounds showed moderate *in vitro* antiplasmodium activity against the chloroquine sensitive strain of *Plasmodium falciparum*. Cissusin (**3**) and 2-(2',4'-dimethoxyphenyl)-5,6-methylenedioxybenzofuran (**4**) were isolated from the MeOH extract of the aerial parts of *Cissus sicyoides*, a medicinal plant used to treat

diabetes, inflammation and stomach-ache (Xu *et al.*, 2009). Six stilbenes, *E*-resveratrol-2-C-β-glucoside (**5**), *E*-3-O-methyl resveratrol-2-C-β-glucoside (**6**), cissuside A (**8**), cissuside B (**9**), *Z*-resveratrol-2-C-β-glucoside (**10**) and *Z*-3-O-methyl resveratrol-2-C-β-glucoside (**11**) (Fig. 2.2), were obtained from the roots and stems of *Cissus repens* which is used to treat malaria, burns, backache and gastrointestinal disorders (Wang *et al.*, 2007).

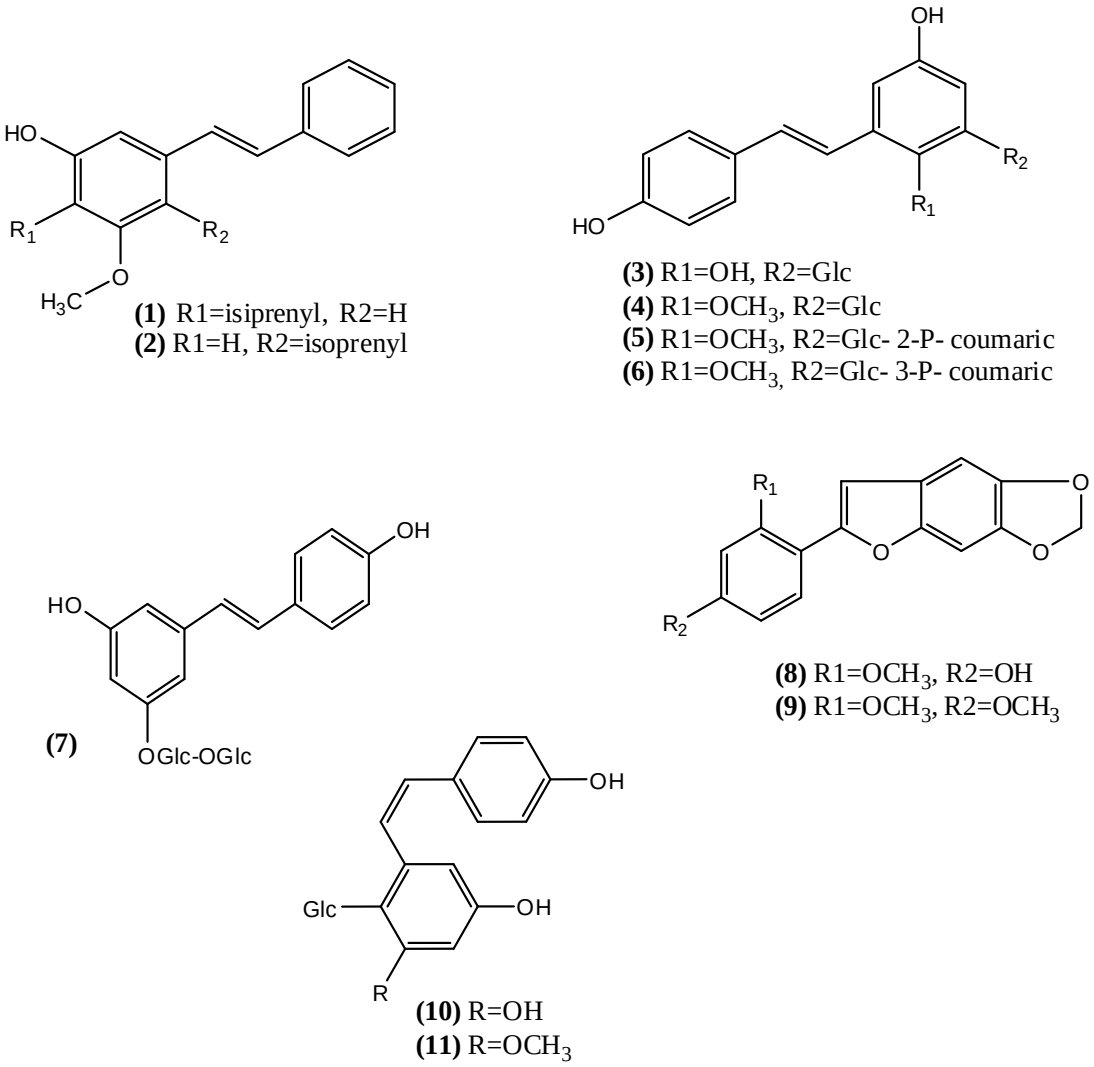
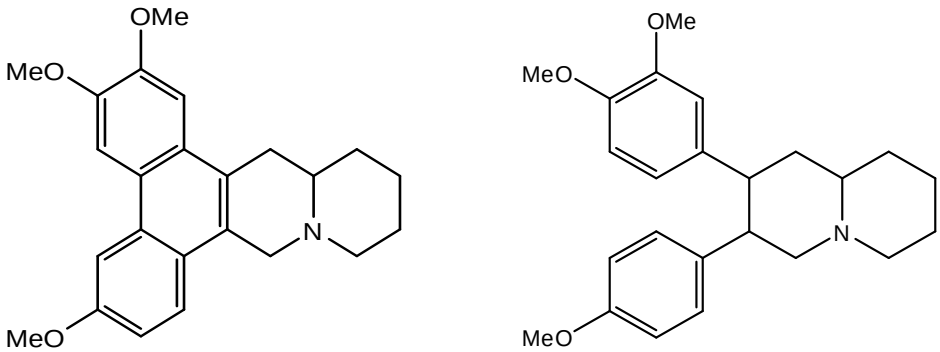


Figure 2.2: The stilbene monomers (1-11) identified from Vitaceae genera

2.4.2 Alkaloids

Alkaloids are secondary metabolites derived from amino acids and they are referred to as nitrogen containing compounds (Croteau *et al.,* 2000). Natural alkaloids and their derivatives are useful in the treatment of several neurodegenerative diseases such as Alzheimer disease, epilepsy, stroke, schizophrenia, Huntington's disease, and Parkinson's disease (Hussain *et al.*, 2018). This class of compounds was reported as a major component in some Vitaceae plants. In a study conducted by Soladoye and Chukwuma (2012), the stem of *Cissus populnea* showed high content of alkaloids (49.8%) compared to flavonoids (15.4%). A phytochemical investigation of the roots and leaves of *Cissus rheifolia* led to the isolation of two alkaloids, (-)-cryptopleurine (**12**) and kayawongine (**13**) (Fig. 2.3) (Saifah *et al*., 1983; Saifah *et al.*, 1987). *C. rheifolia* is known to possess various biological activities including anti-inflammation, antilipemic, and hypoglycemic activities (Garcıa *et al*., 2000; Viana *et al.*, 2004).



(**12**) (**13**)

Figure 2.3: Alkaloids (12 and 13) identified from Vitaceae plants

2.4.3 Terpenoids

Terpenoids are hydrocarbons based on an isoprene unit containing five carbon atoms (Devarenne, 2001). Based on the number of isoprene units, terpenoids are classified into various groups such as hemiterpenoid, monoterpenoid, sesquiterpenoid, diterpenoid, triterpenoid, tetraterpenoid and polyterpenoid. Some terpenoids (Fig. 2.4) were identified from Vitaceae plants. These include sitosterol-3-O-β-*D*-glycoside (**14**) sitosterol (**15**), which were isolated from the aerial part of *C. sicyoides*. Terpenoids from this plant showed antibacterial activity against *Bacillus subtilis* (Beltrame *et al.*, 2002). A phytochemical screening of the roots and stem of *C. quadrangularis* resulted in the identification of teraxerol (**16**) and β-amyrin (**17)** (Sudmoon *et al.*, 2016). *C. quadrangularis* is used in traditional medicine to treat gout, syphilis, piles, tumours, haemorrhoids peptic ulcers and venereal diseases (Nagani *et al*., 2011).

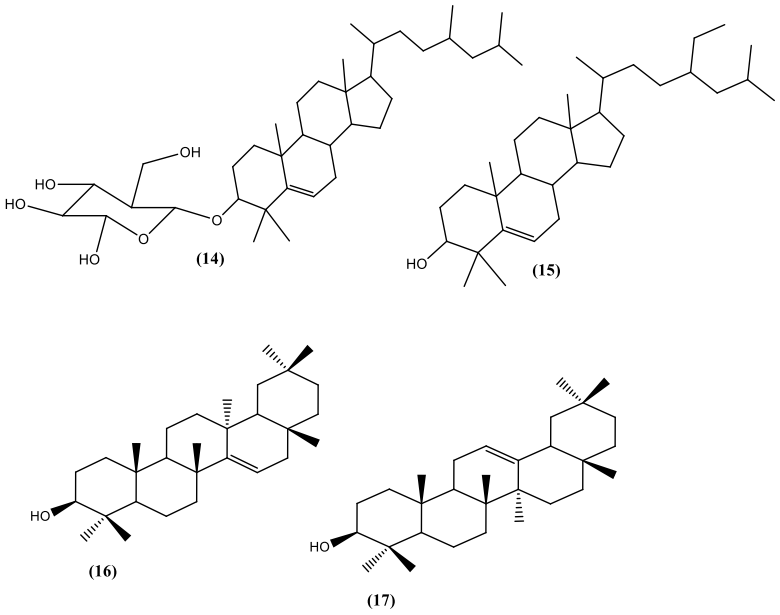


Figure 2.4: Terpenoids (14-17) identified from Vitaceae plants

2.4.4 Flavonoids

Flavonoids are the largest group of phenolic compounds that are found in plants in their free form or as glycosides (Kumar & Pandey, 2013). These compounds are formed by the condensation reaction of ring A, B and C. Flavonoids can be categorised as flavones (**A**), flavonols (**B**), flavonones (**C**), isoflavonones (**D**) and chalcones (**E**) (Fig. 2.5) (Mabry *et al*., 2012).

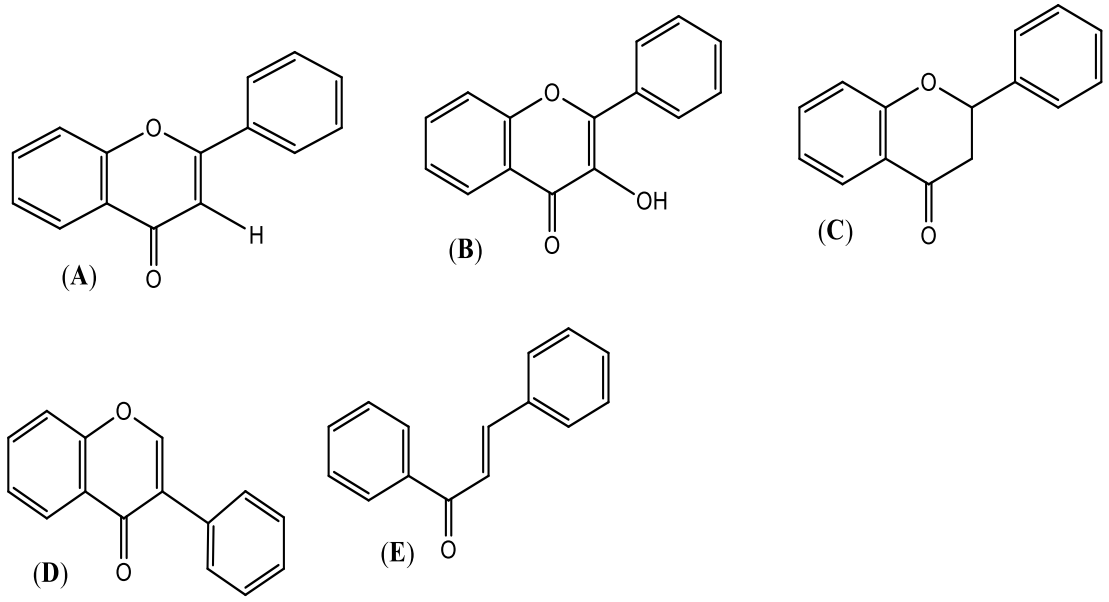


Figure 2.5: Chemical structures of the major classes of flavonoids in plants

Vitaceae plant species are known to possess high amounts of polyphenols. Figure 2.6 shows the structures of some flavonoids isolated from Vitaceae plants. Aromadendrine (**18**) was obtained from the leaves of *Ampelopsis contaniensis*, while vitexin (**19**) was isolated from the leaves of *Cissus rheifolia* (Saifah *et al.*, 1983; Van Thu *et al.*, 2015). Kaempferol-3-rhaminoside (**21**) and quercetin-3-rhamnoside (**20**) were isolated from the leaves of *C. sicyoides* (Almeida *et al*. 2009; Beltrame *et al.*, 2002).

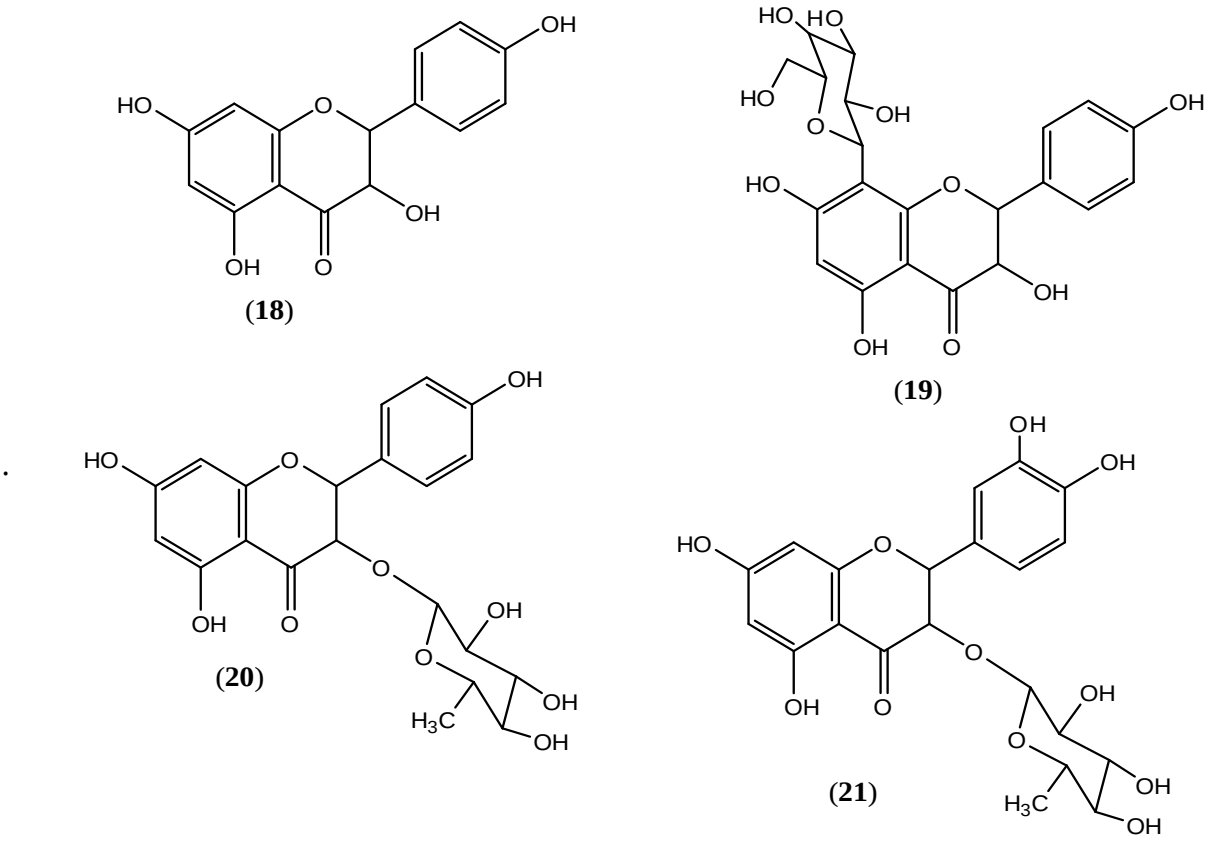
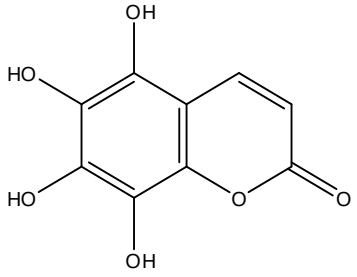


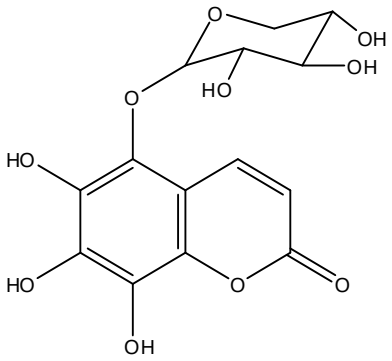
Figure 2.6: Flavonoids (18-21) identified from some Vitaceae plants

2.4.5 Coumarins

Coumarins are phenolic compounds with the basic skeleton of C6-C3. These compounds occur in plants in their free or glycosidic forms (Nqolo, 2008). Coumarins were reported to have antimicrobial, anti-inflammation, antioxidant, antitumor, and hypotensive activities (Nqolo, 2008). Sabandin (**22**) and 5,6,7,8-tetrahydroxycoumarin-5β-xylopiranoside (**23**) (Fig. 2.7) are examples of coumarins identified from the leaves of *Cissus sicyoides* (Beltrame *et al.*, 2002).



(**22**)



(**23**)

Figure 2.7**:** Coumarins (22 and 23) identified from some Vitaceae plants

2.5 The genus *Rhoicissus*

The genus *Rhoicissus* Planch (Vitaceae) derives its name from the Greek words *rhoia* (pomegranate) and *kissos* (ivy). This genus consists of about 12 species that are widely distributed in tropical regions and southern Africa. The species include *R. sessilifolia*, *R. revoilli*, *R. tomentosa*, *R. digitata*, *R. romboidea*, *R. tridentata subsp. tridentata*, *R. tridentata subsp. cuneifolia*, *R. laetans, R. microphylla*, *R. sekhukhuniensis, R. kougarbegensis,* and *R. schlechteri* (Kunene, 2015; Notten, 2004; Retief *et al*., 2001). *Rhoicissus* plants are climbing shrubs that are characterised by tendrils with no adhesive disc, uniform flowers at maturity, and short pedunculated chymes (Gerrath *et al*., 2004).

2.5.1 Traditional uses

Rhoicissus species, commonly known as *Isinwazi* (isiZulu), are usually prepared as liquid decoctions which are used to facilitate safe delivery during pregnancy and to improve fertility. These plants are also used to treat conditions related to the kidneys, stomach, liver and bladder and to treat wounds, malaria and cancer (Lin *et al.*, 1999; Opoku *et al.*, 2000; Opoku *et*

al.,2002). The fruits of *Rhoicissus* species are edible and are eaten by the local people and animals, and are also used in making jam, wine, vinegar, perfume and raisins (Kunene, 2015; Notten, 2004).

2.5.2 Biological activity and phytochemistry of *Rhoicissus* species

Rhoicissus plants have been reported to exhibit several biological activities such as anticancer, antioxidant, antiviral, anti-inflammatory, anti-hepatoprotective, antifungal and antimicrobial activities (Lin *et al.*, 1999; Opoku *et al.*, 2000; Opoku *et al.*, 2002).

Opoku *et al* (2002) evaluated the antioxidant activity of the MeOH extracts of four *Rhoicissus* plants (*R. tomentosa, R. digitata, R. tridentata, and R. rhomboidea)* using the 1-1-diphenyl-2-picryhydrazyl (DPPH) free radical, xanthine oxidase, DNA sugar damage, NDPPH free radical, trolox equivalent antioxidant capacity assay and chelating assays. Finding from this research showed that the antioxidant capacity is dependent on the sample concentrations with *R. tomentosa* and *R. digitata* showing pro-oxidative capacity at high concentrations. In addition, *R. tridentata* and *R. rhomboidea* exhibited more than 50% activity, which was comparable to the commercial antioxidants used in the study (vit E (63%), BHT (50.1%), and BHA (42.5%)) (Opoku *et al.*, 2002).

In another study, Opoku *et al* (2000) studied the anti-proliferative activity of the aqueous and MeOH extracts of *R. tomentosa, R. digitata, R. tridentata,* and *R. rhomboidea*. The root extracts from *R. tridentata* exhibited the highest anti-proliferative activity against HepG2 cell lines, with 96.27% and 87.01% inhibition for the aqueous and MeOH extracts, respectively. The other three *Rhoicissus* species studied showed potential antineoplastic activity, with more than 50%

inhibition of proliferation (Opoku *et al.*, 2000). Opoku *et al* (2007) also demonstrated the *in vivo* hepatoprotective activity of the roots aqueous extract from *R. tridentata* subsp. *cuneifolia*.

The anti-inflammatory and antimicrobial activity of *Rhoicissus* plants was reported by Lin *et al* (1999). The MeOH extracts of *R. tomentosa* leaf/stem, *R. digitata* leaf, *R. tridentata* root, and *R. rhomboidea* root extracts displayed good inhibitory activity against cyclooxygenase (COX-1) compared to the known anti-inflammatory agent (indomethacin). These plants also showed high antimicrobial activity against Gram-positive than Gram-negative microorganisms. In addition, the aqueous extracts of these plants exhibited low inhibition of prostanglandin synthesis (< 30%) (Lin *et al.*, 1999).

Rhoicissus revoilli exhibited good antimicrobial activity against microorganisms such as *Salmonella typhi*, *Streptococcus pyrogenes*, and *Aspergillus niger* (Arwa *et al.*, 2008). The phytochemical screening of the MeOH extracts from the roots and leaves of *R. revoilli* confirmed the presence of flavonoids, coumarins, alkaloids, steroids glycosides, anthraquinones, quinones and tannins (Arwa *et al.*, 2008; Katsoulis, 2014). In a study by Makhuvele *et al* (2018), the MeOH extract of the leaves of *R. sekhukhuniensis*, *R. laetans*, and *R. rhomboidea* showed good antigenotoxic activity against aflatoxin B1 induced mutagenicity.

While the biological activities of *Rhoicissus* species have been extensively studied, the phytochemistry of most plants in this genus has not received enough attention. The preliminary screening of chemical constituents in most species has been reported, but the isolation of pure compounds is only reported for *R. tridentata*. Qualitative screening of the leaves of *R. tridentata* led to the identification of flavonoids, coumarins, triterpenes, steroids, saponins, essential oils and anthocyanins (Katsoulis, 2014). Some terpenoids isolated from the roots of *R. tridentata*

include sitosterol-3-O-β-D-glycoside (**24**), sitosterol (**25**), oleanolic acid (**26**), imberbic acid (**27**), 20(29)-lupen-3-one (**28**), epi-psi-taraxastanonol (**29**), asiatic acid (**30**), and arjunolic acid (**31**) (Fig. 2.8) (Brookes & Katsoulis, 2006; Dube, 2014).

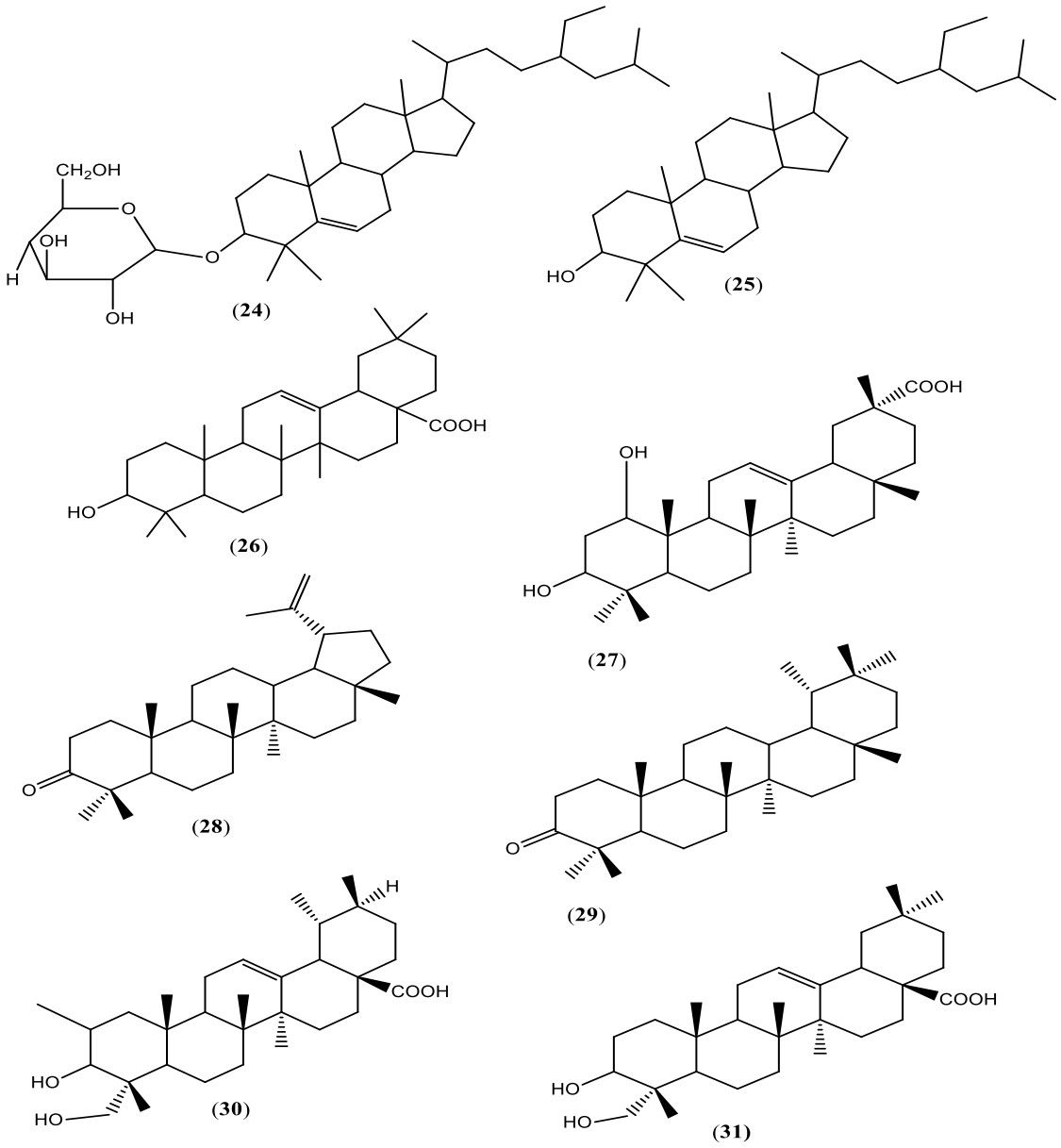


Figure 2.8**:** Chemical structures of terpenoids (24 to 31) identified from the roots of *R. tridentata*

The phytochemical investigation of the roots of *R. tridentata* also led to the isolation of polyphenols, such as, gallic acid (**32**) +(-) catechin (**33**), quercetrin (**34**), epigallocatechin gallate (**35**), (-)-epicatechin gallate (**36**) and epi-catechin (**37**) (Fig. 2.9). *R. tridentata* possesses considerable biological activities, for instance antimicrobial, antioxidant, antifungal, anticancer, and antimalarial activities (Brookes & Katsoulis, 2006; Dube, 2014)

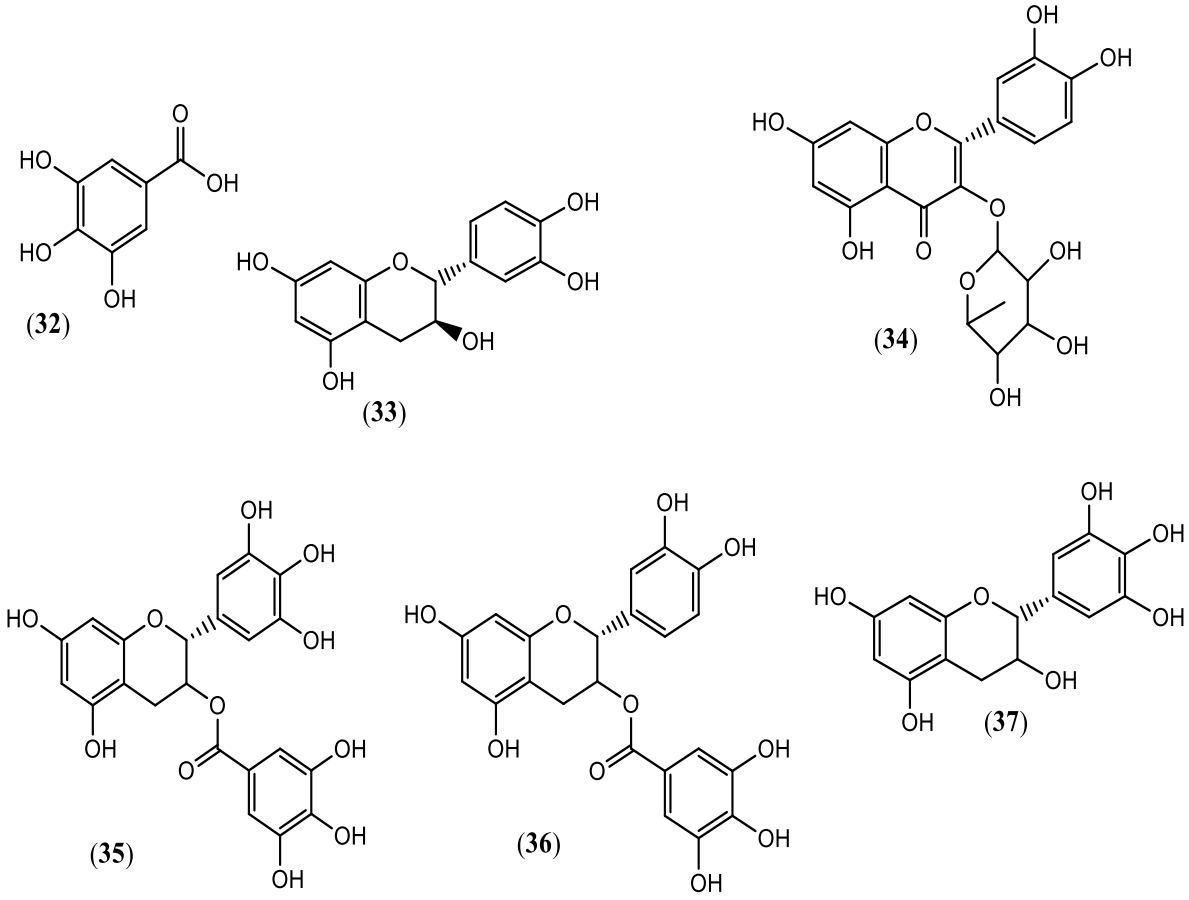


Figure 2.9**:** Flavonoids (32-37) identified from the roots of *R. tridentata*

The roots, stem, and leaves of *R. digitata, R. tomentosa,* and *R. romboidea* tested positive for the presence of flavonoids, coumarins, terpenoids, alkaloids, steroids, tannins and saponins. The chemical constituent in the leaves of *R. tomentosa* were identified using GC-MS (Table 2.2) (Nqolo, 2008; Uche-Okereafor *et al.,* 2016).

Table 2.2: Compounds identified from the crude extracts of the leaves of *R. tomentosa*

Hexane extract	**Ethyl acetate: hexane (1:4)**
carda-16,20(22)-dienolide	canthaxanthin
4,4-dimethylcholestan-3-one	carotene
11-hydroxygen-4-ene-3,20-dione	cedran,8s,14-diol
lycoxanthin	diepicedrene-1-oxide
methyltetraacethylmannopyranoside	2,3-dehydro-7,7dihydro-4-oxo-alpha-ionone
3,12-oleandione	4-ethyl-1-methylcyclohexanol
2-octadecyl-1,3,5-trimethylcyclohexane	eucalptol
6,10,14-trimethyl-2-pentadecanone	globulol
7,8,12-ti-o-acetylingol	4-hydroxymethyl-1,3,3trimethylcyclohexene
	isolongifolan-8-ol
	lycoxanthin
	resveratrol
	terpineol
	6,10,14-trimethyl-2-pentadecanone

It is clear that the genus *Rhoicissus* is popularly used in traditional medicine and plants in this genus exhibit interesting biological activities. The phytochemistry of *Rhoicissus* plants in not well known and further research is required to isolate bioactive secondary metabolites from these plants to validate the reported traditional uses, hence the phytochemistry of two *Rhoicissus* plants (*R. digitata* and *R. tomentosa*) was studied in this project. The antioxidant

activities of the crude extracts and secondary metabolites from these two plants were also studied as well as the heavy metals distribution in them.

2.6 *Rhoicissus digitata*

Rhoicissus digitata (Fig. 2.10), commonly known as baboon grape (English) and *Isinwazi* (isiZulu), is a robust woody climber with glossy ornamental leaves shaped like an open hand of three or five foliolates (Boon & Pooley, 2010). *R. digitata* is endemic to South Africa, but it is also found in other countries such as Mozambique, Swaziland, Zimbabwe and Zambia (Kunene, 2015; Pooley, 1993). The roots and leaves of *R. digitata* are used to treat stomach-aches, dysmenorrhea, amenorrhea, wounds, ringworms and intestinal worms, and are used to increase milk production in mothers and cows (Lin *et al.*, 1999; Opoku 2002). The fruits from *R. digitata* are consumed by the local people and used to make jam.

Figure 2.10*:* Aerial parts of *Rhoicissus digitata*

2.7 *Rhoicissus tomentosa*

Rhoicissus tomentosa (Fig. 2.11), commonly known as wild grape (English) and *isiNwazi* (isiZulu), is mostly grown in riverine fringes where it climbers over the trees and bushes. This plant has simple kidney shaped leaves with the lobes along the margin and three nerves at the base of leaves which are unique in the family Vitaceae (Notten, 2004). Different plant parts of *R. tomentosa* are used to treat intestinal worms, facilitate delivery during pregnancy and enhance fertility (Corrigan *et al.*, 2011). The fruits are eaten by the local people and birds, and are used to make jelly, vinegar and wine (Notten, 2004).

Figure 2.11*:* Aerial parts of *Rhoicissus tomentosa*

2.8 Plants as antioxidants

An antioxidant is any substance that delays or inhibits oxidation of the substrate at low concentration (Madhavi *et al.*, 1995). The term oxidisable substrate refers to almost everything in living cells, for example, DNA, lipids and carbohydrates. The human body is rich in the substances that can stop the formation of free radicals or limit the damage caused by these free radicals. Plants are a major source of antioxidants, but people can also get antioxidants from

other foods and food supplements. Plants contain various classes of compounds that exhibit free radical scavenging capacity, with polyphenols being the major class (Chanda & Dave, 2009). Phenolic compounds have received great attention because they interact with biological systems in such a way that result in disease prevention. Flavonoids are polyphenolic molecules, which are well known for their antioxidant properties. The activity of flavonoids is due to their hydroxyl groups that can act as free radical scavengers, reducing agents or as metal chelators (Rice-Evans *et al.*, 1997). The antioxidant activity of plant extracts and pure compounds may be evaluated using various assays including 1,1-diphenyl-2-picryhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and phosphomolybdate (Chanda & Dave, 2009).

2.8.1 The 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging activity

The free radical, DPPH, is widely used to test the ability of samples to act as free radical scavengers or hydrogen donors. A methanolic solution of DPPH is reduced to DPPH-H (Fig 2.12), which causes a change in colour from purple to yellow (Blois, 1958). The disappearance of the purple colour of DPPH is influenced by the number of electrons captured (if more electrons are captured, the DPPH colour is completely changed to yellow) and is monitored using a UV/Vis spectrophotometer at 517 nm. The positive controls for this assay are normally ascorbic acid, butylated hydroxyl toluene (BHT), gallic acid and butylated hydroxyanisole (BHA) (Chanda & Dave, 2009).

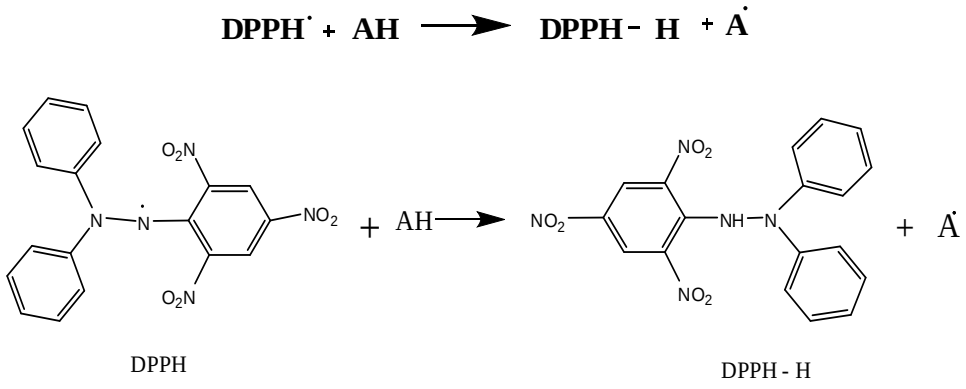


Figure 2.12**:** Reduction of DPPH to DPPH-H

2.8.2 Ferric reducing antioxidant power (FRAP) antioxidant

The FRAP assay is based on the capacity of antioxidants to reduce Fe^{3+} to Fe^{2+}. The reduction of an Fe^{3+} complex and 2,3,5-triphenyl-1,3,4-triaza-2-azonizcyclopenta-1,4-diene chloride (TPTZ) in acidic solution (pH 3.6) causes an appearance of a blue coloured ferrous tripyridyltriazine complex (Fe^{2+}TPTZ). The absorbance of the solution is spectrophotometrically measured at 700 nm (Alam *et al.*, 2013). The reduction potential of an antioxidant is calculated from the linear curve of absorbance versus concentration. The positive controls used in this method are similar to the DPPH assay (Chanda & Dave, 2009).

2.8.3 Phosphomolybdate reduction antioxidant

This assay is based on the reduction of Mo(VI) to Mo(V) by an antioxidant in acidic pH and high temperature, resulting in the appearance of a green coloured solution. The absorbance of the green Mo(V) complex is measured at 695 nm using a UV/Vis spectrophotometer. Ascorbic acid and BHT are used as positive controls in this assay (Chaouche *et al.*, 2014).

2.9 Essential nutrients to humans

Essential nutrients are chemical components necessary for the growth and development of humans (Stipanuk & Caudill, 2013). Essential nutrients are also important for normal metabolism in human and animals (Bohn & Science 2008). There are two groups of nutrients, the organic and inorganic nutrients. Organic nutrients (macronutrients) are synthesised by the living cell in small molecules and then converted to large molecules, for instance, proteins, carbohydrates and fats. The structure of carbohydrates and fats consists of carbon, hydrogen and oxygen while proteins have an additional nitrogen (Sarwar *et al.*, 2015). Inorganic nutrients (micronutrients) do no need to be produced by living organisms; they are present in the earth's crust, where they are taken up by plants or microorganisms. Micronutrients are usually required in small amounts to maintain the essential physicochemical processes in the human body.

The 21 elements (C, O, N, P, K, S, Ca, Mg, Fe, Cu, Co, Mn, Mo, B, Na, Cr, F, I, Ni, Se, and Zn) are regarded as inorganic nutrients or micronutrients. The micronutrients that are required by living organisms are referred to as minerals. Minerals are naturally found in foods or they can be artificially added to the diet as supplements. The dietary minerals are grouped into two categories (macro-minerals and trace-minerals). Macro-minerals are required in large amounts and their recommended dietary allowances (RDAs) are greater than 150 mg day^{-1}. Trace-minerals (Co, Cu, Cr, I, Fe, Mn, Zn, Ni, Se, and Mo) are needed in trace amounts and their RDAs are less than 200 mg day^{-1} (Sarwar *et al.*, 2015).

The amount and identity of micronutrients that people obtained from plants is influenced by variety of factors. Some of these factors are soil, agricultural produce post-harvest processing including cooking. For example, Zn and Fe in rice are found in the outer layer of the grains,

which is removed during processing (milling). On the hand, Zn in wheat is found in the inner part of the grain which is not removed during bread making (Soetan *et al*., 2010). Therefore, people consuming wheat obtain Zn, whereas rice has little or no Zn.

The availability of micronutrients in the soil is affected by pH, content of organic matter, soil aeration, moisture and interaction with other elements (De Valença & Bake, 2016). The organic matter in the soil serves various functions including storage of nutrients and soil aggregation. Environmental changes, such as soil erosion, causes loss of natural soil fertility and decreases the amount of micronutrients in the soil. This is due to the deterioration of physical, chemical and biological properties resulting from soil degradation (Mccauley *et al.*, 2009). The concentration of micronutrients is also affected by soil pH. Soil pH plays an important role in the dissolution of metals making them readily available to be taken up by plants. At low pH (pH < 4.5), the nutrients are very soluble while they are insoluble at high pH (pH > 8.5) and they cannot be absorbed by the plants. Excess soil moisture has been reported to inhibit the growth of plants, thus impacting on the uptake and concentration of nutrients in the plant (Mccauley *et al.*, 2009). Poor aeration was also reported to limit the soil biological processes and the quality of nutrients in the soil (Sajedi *et al.*, 2012).

Table 2.3: Recommended dietary allowances (RDAs) for individuals

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

The RDAs (Table 2.3) and tolerable upper intake levels (ULs) (Table 2.4) of nutrients for individuals were reported by NCBI, Food and Nutrition Board, Institute of Medicine in 2011. The RDA represents the average daily intake level of the nutrients sufficient to meet the requirements of healthy individuals and the ULs represents the highest allowable level of daily nutrient intake.

Table 2.4: Tolerable upper intake levels (ULs)

Life stages	**Ca (mg/d)**	**Cu (µg/d)**	**Fe (mg/d)**	**Mg (mg/d)**	**Mn (mg/d)**	**Ni (mg/d)**	**Se (µg/d)**	**Zn (mg/d)**
14-18 y	3 000	8 000	45	350	9	1	400	34
19-50 y	2 500	10000	45	350	11	1	400	40
>51 y	2 500	10000	45	350	11	1	400	40

ND- Not determined

Calcium (Ca) and magnesium (Mg) are required in large amounts compared to other elements (Table 2.3). Calcium together with the phosphorus (P) form a salt called hydroxyapatite, which is a major component of teeth and bones. An excess of Ca and phosphorus (P) in the diet has been reported to inhibit the absorption of Mn (Soetan *et al.*, 2010). Magnesium is also a constituent of bones and it is important in activating enzymes (Bohn & Science, 2008). Zinc (Zn) is an essential trace element that is a constituent of many enzymes such as RNA and DNA polymerase, carboxypeptidase, lactate dehydrogenase, alcohol dehydrogenase and superoxide dismutase (Soetan *et al.*, 2010). Zinc is useful in metabolism, cell replication, gene expression, catalytic activity, proper sense of taste and smell, immune function, wound healing, tissue repair, proteins synthesis and DNA synthesis (Hung *et al.*, 2016).

An excess Zn in the diet causes fever, vomiting, diarrhoea, nausea and anaemia. Zinc deficiency results in impairment of wound healing and sensory perception (Burch *et al.*,1975). A study on

the homeostatic regulation of Zn absorption in rats showed that the normal percentage of Zn in the blood is 75-85% (in erythrocytes), 12-22% (in plasma) and 3% (in leukocytes) (Evans *et al.*, 1973).

Copper (Cu) is useful in the production of melanin, myelin and connective tissues. This nutrient is associated with the functioning of enzymes. Copper deficiency causes anaemia, bone abnormalities and neutropenia diseases (Hawkesford *et al.*, 2012). Copper acts as a pro-antioxidant to promote the damage of free radicals and contribute to the development of Alzheimer's disease (Hung *et al.*, 2016).

Manganese (Mn) is an important element required for various physiological processes including bone mineralisation, protein and energy metabolisms (Arora *et al.*, 2011). Manganese is a cofactor of enzymes like hydrolase, decarboxylase and transferase (Harper *et al.*, 2000). Miller (1973) reported that there is a high concentration of Mn on the earth's crust and in most plants, however, animal bodies have a specific regulatory mechanism that controls the concentration of Mn in the body.

Iron (Fe) is an essential component of every living orgasm and is abundant on earth. In animal bodies, Fe can be recycled and conserved (Abbaspour *et al.*, 2014). Iron is important in energy production, DNA synthesis, oxygen transport and some enzymatic processes. The absorption and utilisation of Fe are enhanced by the presence of lead (Pb) (Soetan *et al.*, 2010).

Selenium (Se) is essential for the protection of the human body against free radicals and its activity is almost similar to that of anti-oxidative properties of α-tocopherol and coenzyme Q. It was reported that Se was used to prevent liver necrosis in rats, white muscle disease in lambs

and exudative diathesis in chicks (Soetan *et al.*, 2010). This element is a constituent of the amino acid, selenocysteine, which is used for protein synthesis in humans (Roman *et al.*, 2014).

2.10 Arsenic toxicity

Arsenic (As) is a widespread element in foods and the environment (water, soil and air). The greatest route of human exposure to As is through consumption of contaminated groundwater. It is reported that 200 millions of people in 70 countries around the world are exposed to As through drinking water (Minatel *et al.*, 2018). According to WHO guidelines, the maximum contaminant levels of As in drinking water should be 0.01 mg/L (10 parts per billion) (WHO, 2011). Groundwater and soil contamination by As is as a result of anthropogenic activities such as mining, irrigation, and the use of arsenical herbicides, insecticides and wood preservatives. This contamination of soil and groundwater then leads to crop plants being contaminated with As. Excessive uptake of As by crop plants causes yield loss and may pose a health risk to humans (FAO, 2011; Kabata-Pendias & Mukherjee, 2007; Saha *et al.*, 1999). Other food products such as rice, wheat, maize and tomato (except plants) have also been reported as sources of exposure to As (Huang *et al.*, 2015).

Arsenic mainly exists in two forms (organic and inorganic). In the inorganic compounds, arsenic exists as arsenite (As(III)) and arsenate (As(V)) with As(III) being 60 times more toxic than As(V). In addition, inorganic As is highly toxic and more abundant in water and soil in comparison to the organic forms. The inorganic form(As(V)) dominates in aerobic soils, while As(III) is a major form in anaerobic conditions like paddy soils. Organic As compounds are normally found in food as monomethylarsonic acid, dimethylarsinic acid, arsenosugars, arsenocholine, arsenobetain and arsenolipids (Sarkar & Paul, 2016). A common source of the

less toxic organic arsenic (arsenobetaine) is seafood. Other foodstuffs that are a source of As exposure to humans are cereals such as rice, wheat, corn, oats, buckwheat, vegetables and meat products. Various foods have different amounts of As and these are shown in Table 2.5. Arsenic toxicity in humans causes serious health conditions, such as, liver, bladder, lung and skin cancer (WHO, 2011).

Table 2.5: Maximum level of Arsenic in foodstuffs

Foodstuff	**mg kg^{-1}**
Edible fats and oils	0.10
Fat spreads and blended spreads	0.10
Natural mineral water	0.01
Salt, food grade	0.50
Rice, husked	0.35
Rice, polished shed	0.20

2.11 Phytochemical and analytical techniques

The following instrumental techniques have been employed in this study.

2.11.1 Column chromatography

Column chromatography packed with silica gel F_{254} or sephadex LH-20 was used to separate the secondary metabolites in the plant extracts. Based on the polarity, the extracts and compounds were fractionated and purified using solvent systems of different polarities. Figure 2.13 shows a packed column used in the current study.

The eluted fractions were spotted on thin layer chromatography (TLC) to determine their purity or chemical profiles. TLC is made up of an adsorbent material, commonly known as stationary

phase. A TLC plate is spotted with a sample and placed in a chamber containing a solvent system (also known as mobile phase). The mobile phase consists of one or a mixture of solvents of different polarities. In a chamber, the mobile phase moves up and the compounds in a sample also moves up the TLC plate at different rates depending on their polarities. The ratio of distance travelled by compounds over distance travelled by the solvent (commonly known as the retention factor, R_f) is calculated. Generally, different compounds have different R_f values based on their polarities.

Figure 2.13**:** A silica-packed column used in this study

2.11.2 Nuclear magnetic resonance (NMR) spectroscopy

NMR is used to characterise the structure of a compound by determining the number and identity of protons and carbons in the molecule (1D NMR), as well as, determining their connectivity (2D NMR). The ^{1}H-NMR (1D) shows coupled peaks in the range of 0-13 ppm, while signals appear as singlet peaks in the ^{13}C NMR from 0-230 ppm. Distortionless enhancement by polarization transfer (DEPT) NMR technique is used to classify carbon atoms

as methyl (CH_3), methylene (CH_2), methane (CH) and quaternary carbons. In the DEPT 90 experiment, only CH group resonances appear while DEPT 135 shows CH_3, CH and CH_2 groups. CH_2 signals appear in an opposite phase to the other groups in DEPT 135. Structural elucidation of the compounds is also supported by 2D NMR experiments. The ^1H-^1H homonuclear correlation spectroscopy (COSY) shows proton-proton correlations and nuclear overhauser effect spectroscopy (NOESY) shows correlations between protons which are close in space. Heteronuclear single quantum coherence (HSQC) shows single bond proton to carbon correlations and heteronuclear multiple bond correlation (HMBC) shows correlations between proton and carbon that are separated by two or three bonds.

2.11.3 Fourier transform infrared (FT-IR) spectroscopy

FT-IR provides information about the functional groups in a solid, liquid or a gas sample (Kim *et al*., 2005). In this technique, the IR radiation is passed through a sample and it initiates the excitation of bending or stretching vibrations of the covalent bonds in a molecule. As a molecule contains different functional groups, different IR frequencies are absorbed. The FT-IR instrument (Fig 2.14) measures the energy absorbed at different wavelengths and an absorbance or transmittance spectrum is created (Belin & Epron, 2005). The IR spectrum is a plot of absorbance/transmittance versus frequencies or wavelength. The advantage of FT-IR is that it provides precise measurements with no external calibration required (Belin & Epron, 2005).

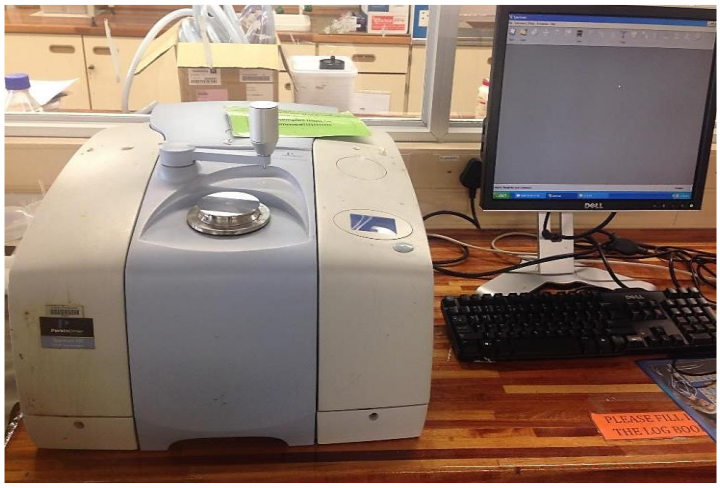


Figure 2.14: The FT-IR spectrometer at UKZN

2.11.4 Gas chromatography-mass spectrometry (GC-MS)

The molecular mass and identity of various substances within a tested sample was conducted with a combined GC-MS technique. In mass spectrometer, the molecules are bombarded and fragmented into positive, negative, and neutral species and these ions are separated based on the adsorption between the stationary and mobile phases. The mass spectrometer determines the mass-to-charge ratio (*m/z*) of the ions, hence the molecular weight of compounds (Krone *et al.*, 2010).

2.11.5 Ultraviolet-visible (UV/Vis) spectroscopy

In UV/Vis spectroscopy, molecules or atoms absorb UV and visible radiation and this enables excitation of electrons from the lower energy level (ground state) to a high energy level (excited state). A diffraction grating splits the light into the component colors of different wavelengths. The light source used in the UV/Vis spectrophotometer is a combination of tungsten/halogen and deuterium lamps, and it provides radiation covering a range of 200 to 800 nm. Conjugated

organic compounds absorb light in the UV or visible regions, and the wavelength and intensity of absorbance is influenced by functional groups (chromophores) in the molecule (Workman & Springsteen, 1998). In this study, the UV/Vis spectrum was used to predict the conjugation in various organic compounds by comparing the absorption maxima to reported values for different groups of compounds.

2.11.6 Microwave digestion

This technique is used to digest the sample and release the heavy metals found in organic substances before elemental analysis. The solubility of the heavy metal is achieved by exposing the sample to a strong acid (HNO_3) in a closed vessel under high pressure and temperature. In this technique, the samples are placed in a bomb (vial) made of polytetrafluoroethylene (PTFE) or perfluoroalkoxy (PFA) before irradiation in the microwave (Fig. 2.15) (Lamble & Hill, 1998).

Figure 2.15**:** The microwave used in this study

2.11.7 Inductively coupled plasma-optical emission spectrometry (ICP-OES)

The inductively coupled plasma-optical emission spectrometer (Fig. 2.16) is an analytical instrument that is used to determine the identity and concentration of elements in a sample. The concentration is measured by the intensity of the emitted radiation. In ICP-OES, the spontaneous emission of photons from atoms or ions is excited in the radio frequency field. The excited atoms or ions relaxes back to the ground state through the emission of photons. These photons have characteristic energies or wavelengths, which is used determine the structure of the atoms or ions, and subsequently, the identity of elements in the sample.

The concentration of elements in the sample is directly proportional to the total number of photons (Hou & Jones, 2000). Liquid samples are aspirated directly into the instrument, but solids need extraction or acid digestion before being injected. The sample of known analytical composition is required in this technique to calibrate and assess the accuracy of analytical instrumentation. This instrument has several advantages such as high stability leading to high accuracy and precision, limited spectral interferences, fairly simple to run, simultaneous multi-elements capability, and excellent detection limits for most elements.

Figure 2.16**:** Inductively coupled plasma-optical emission spectrometer

2.12 References

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CHAPTER 3

CHEMICAL CONSTITUENTS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF CRUDE EXTRACTS AND COMPOUNDS FROM *RHOICISSUS DIGITATA* AND *RHOICISSUS TOMENTOSA*

3.1 Abstract

The indigenous medicinal plant species, *Rhoicissus digitata* and *Rhoicissus tomentosa* (Vitaceae), are popular amongst South Africans for the treatment of various diseases. In this study, the phytochemical analysis of the roots and fruits extracts of *R. digitata* resulted in the isolation of two flavonoids ((+)-catechin and quercetin) and three triterpenes (12,13-dehydrolupeol, β-sitosterol, and oleanolic acid); the phytochemical analysis of the roots, leaves and fruits of *R. tomentosa* led to the isolation of four terpenoids (3β-taraxerol, stigmasterol, oleanolic acid and β-sitosterol), three flavonoids (quercetrin, (+)-catechin and aromadendrin7-O-β-glycopyranoside), and two pigment compounds (lutein and pheophytin a). The *in vitro* antioxidant activity of the crude methanol extracts and isolated flavonoids was comparable to that of known antioxidants, ascorbic acid and butylated hydroxytoluene. The methanol extract of the roots of both plants showed higher antioxidant activity compared to the other plant parts. These results suggest that *R. digitata* and *R. tomentosa* can be used as natural antioxidants to prevent or cure oxidative stress-related diseases.

Keywords: *Rhoicissus*, flavonoids, terpenoids, antioxidant, pheophytin a, lutein

3.2 Introduction

The genus *Rhoicissus* Planch (Vitaceae) comprises of 12 species which are native to South Africa. *Rhoicissus* plants are climbing shrubs with tendrils that have no adhesive discs and are mostly distributed in coastal dune forests (Gerrath *et al*., 2004). Plants from this genus are widely used in South Africa to ensure safe delivery during pregnancy. *Rhoicissus digitata*, commonly known as baboon grape (English) and *Isinwazi* (isiZulu), is endemic to South Africa, but it is also found in other countries such as, Mozambique, Swaziland, Zimbabwe and Zambia (Kunene, 2015; Pooley, 1993). The roots and leaves of *R. digitata* are used to treat stomach-ache, dysmenorrhea, amenorrhea, wounds, ringworms and intestinal worms, and are used to increase milk production in mothers and cows (Lin *et al.*, 1999; Opoku *et al.*, 2002). The fruits from *R. digitata* are consumed by the local people and used to make jam.

Rhoicissus tomentosa, commonly known as wild grape (English) and *isiNwazi* (isiZulu), is used in traditional medicine to treat intestinal worms, facilitate delivery during pregnancy and enhance fertility (Corrigan *et al.*, 2011). The fruits are eaten by local people and birds, and are used to make jelly, vinegar and wine (Notten, 2004). The crude methanol extract from *R. tomentosa* and *R. digitata* have been investigated for different biological activities. The extracts demonstrated good antibacterial, anti-inflammatory, anticancer and antioxidant activity (Lin *et al.*, 1999; Opoku *et al.*, 2000; Opoku *et al.*, 2002; Uche-Okereafor *et al.*, 2016).

The biological activities of these plants have been extensively studied, however, their phytochemistry have not been reported. Preliminary screening of the different parts of *R. digitata* indicated polyphenols to be the major constituent in the plant (Opoku *et al.*, 2002). Screening of the crude extracts of *R. tomentosa* indicated the presence of flavonoids, coumarins,

sterols, alkaloids essential oils, reducing sugars, saponins and terpenoids (Nqolo, 2008; Uche-Okereafor *et al.,* 2016). In this study, we report on the isolation and characterisation of the secondary metabolites from *R. digitata* (roots and fruits) and *R. tomentosa* (roots, leaves and fruits). In addition, we report on the *in vitro* antioxidant activity of the crude methanol extracts of the roots and fruits and selected isolated compounds using a multi-method approach.

3.3 Experimental

3.3.1 General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance III spectrometers (400 MHz or 600 MHz). Isolated compounds were dissolved in deuterated chloroform ($CDCl_3$) or deuterated methanol (MeOD) for NMR analysis. Tetramethylsilane (TMS) was used as an internal standard. Chemical shifts (δ) were reported in ppm and coupling constant (*J*) in Hz. Column chromatography (CC) was performed with Merck silica gel 75-230 mesh or sephadex LH-20. Thin layer chromatography (TLC) was performed on pre-coated TLC plates (Merck, kieselgel 60 F_{254}). Compounds were visualised as spots on TLC plates under UV light (254 nm) or by spraying the plate with 5% concentrated H_2SO_4 in methanol (MeOH) followed by heating. The retention factor (R_f) was used to profile different fractions. Mass spectra were recorded on an Agilent GC-MSD apparatus or Agilent LC/MSD Trap 1100 Series. Infrared (IR) spectra were recorded using a Perkin- Elmer Universal ATR spectrometer. UV/Vis spectra were obtained from Hewlett Packard UV-3600 spectrophotometer.

3.3.2 Plant material

R. digitata (roots and fruits) and *R. tomentosa* (roots, leaves and fruits) (Fig. 3.1) were collected from various sites in Durban, KwaZulu-Natal, South Africa from August-December 2017. The plants were identified by Dr Syd Ramdhani from the School of Life Science, University of KwaZulu-Natal (UKZN), and the voucher specimens (V. Uwumubyeyi 01 and V. Uwumubyeyi 02) were deposited in the ward herbarium. Plant materials were dried in an oven at 40 ^{0}C for two weeks and pulverized into a powder using a pestle and mortar.

(A) **(B)**

The roots (**A**) and fruits (**B**) of *R. digitata*

Roots **Leaves** **Fruits**

Plant parts of *R. tomentosa*

Figure 3.1: Plant parts used in this study

3.3.3 Extraction

The dried powdered roots (1524 g and 1694 g) and fruits (562 g and 387 g) of *R. digitata* and *R. tomentosa*, respectively, were extracted with dichloromethane (DCM) and methanol

(MeOH), consecutively for 48 hours using an orbital shaker. The same extraction procedure was followed for the leaves of *R. tomentosa* (843 g). The extracts were filtered and concentrated by evaporation under reduced pressure using a rotary evaporator at 45 ^{0}C to yield the crude extracts. The crude extracts were kept in the fridge until further used.

3.3.4 Preliminary phytochemical analysis

3.3.4.1 Test for alkaloids (Wagner test)

The Wagner's reagent was prepared by dissolving a mixture of potassium iodide (2 g) and iodine (1.27 g) in 2 mL of distilled water and diluting this solution to 100 mL in a volumetric flask. 15 mg of the crude plant extracts were mixed with 6 mL of 1% HCl and heated in a water bath for 5 minutes at 60 ^{0}C followed by filtration. The formation of reddish brown coloured precipitate on the addition of few drops of Wagner reagent to 2 mL of extract solution indicated the presence of alkaloids (Abdullahi *et al*., 2013).

Test for terpenoids and steroids

The formation of a reddish brown coloration at the interface on addition of 2 mL of chloroform and concentrated H_2SO_4 (2 mL) to 100 mg of extract indicated the presence of terpenoids (Ayoola *et al.,* 2008). The appearance of blue or green color when 2 mL of acetic anhydride and 2 mL H_2SO_4 were added to 100 mg of extract indicated the presence of steroids (Soni & Sosa, 2013).

Test for tannins and saponins

The formation of black precipitate on addition of a few drops of 5% ferric chloride to a solution of 0.5 g of extract in distilled water (10 mL) indicated the presence of tannins (Banso & Adeyemo, 2006). The formation of frothing on the addition of distilled water (10 mL) to the crude extract (0.5 g) indicated the presence of saponins (Banso & Adeyemo, 2006).

Test for anthraquinone glucosides and cardiac glucosides

The mixture of extract (1 g) and 5% H_2SO_4 (1 mL) was boiled in water bath and then filtered. The mixture of filtrate (1 mL) and chloroform (1 mL) were allowed to stand for 5 minutes. The formation of a rose pink color when the lower layer of chloroform (1 mL) was mixed with diluted ammonia (0.5 mL) indicated the presence anthraquinone glucosides (Joshi *et al*., 2013). The formation of brown ring at the interface when 0.5 g of extract was shaken with 5 mL of distilled water, 2 mL of glacial acetic acid, few drops of ferric chloride, and 1 mL H_2SO_4 indicated the presence of cardiac glucosides (Ayoola *et al.*, 2008).

Test for flavonoids and phenols

The appearance of red coloration after heating the mixture of extract (1 g) and 50 % methanol (1 mL) followed by the addition of metal magnesium (0.5 g) and few drops of concentrated HCl (1 mL) indicated the presence of flavonoids (Auwal *et al.*, 2014). The formation of bluish green color when 1 g of extract was mixed with 1% $FeCl_3$ (2 mL) indicated the presence of phenols (Santhi & Sengottuvel, 2016).

3.3.5 Isolation and purification of *R. digitata* and *R. tomentosa*

The DCM crude extract of the roots (5.6 g and 3.82 g) and fruits (11.84 g and 8.42 g) of *R. digitata* and *R. tomentosa*, respectively, as well as the leaves extract of *R. tomentosa* (13.12 g) were subjected to the silica gel column chromatography (CC) using a gradient elution system of n-hexane: ethyl acetate (EtOAc) (starting from 100% n-hexane that was stepped by 10% to 100% EtOAc), then finally washing the column with 100% MeOH. Fractions (40 mL) were collected for each eluent step and analysed on TLC. Seven and six main fractions were obtained from the *R. digitata* and *R. tomentosa* roots extracts, respectively. The fruits extracts of both plants yielded four main fractions, while five fractions were obtained from the leaves extract of *R. tomentosa*. Compounds **A1** (235 mg) and **A2** (320 mg) were obtained from fraction 3 and 4, respectively, from the roots of *R. digitata*. Fractions 2 and 4 from the roots of *R. tomentosa* yielded compounds **B1** (120 mg) and **B2** (220 mg), respectively. Compound **A4** (80 mg) was purified from fraction 3 from the fruits of *R. digitata*, while compounds **B7** (9 mg) and **B8** (560 mg) were obtained from fraction 2 and 4, respectively, from the fruits of *R. tomentosa*. Compounds **B4** (145 mg) and **B5** (110 mg) were isolated from fractions 2 and 3, respectively, from the leaves of *R. tomentosa*.

The MeOH extracts from the roots (10.02 g and 8.35 g), fruits (13.82 g and 13.48 g) of *R. digitata* and *R. tomentosa*, respectively, as well as the leaves extract from *R. tomentosa* (12.13 g) were fractionated by CC using a gradient elution starting from 100% n-hexane that was stepped by10% to 100% EtOAc. This was followed by a gradient elution system of DCM-MeOH (starting from 100% DCM that was stepped by10% to 100% MeOH). Fractions (50 mL) were collected for each eluent step and analysed on TLC to obtain four main fractions from the

roots and fruits of both plants, and three fractions from the leaves of *R. tomentosa*. Compound **A3** (90 mg) and **A5** (35 mg) were obtained from fraction 2 from the MeOH roots and fruits extracts of *R. digitata*, respectively. Purification of fraction 2 from the MeOH roots and leaves extracts of *R. tomentosa* led to the isolation of compounds **B3** (60 mg) and **B6** (50 mg), respectively. Compound **B9** (220 mg) was obtained from fraction 3 from the MeOH fruits extract of *R. tomentosa*.

3.3.6 Spectroscopic data of compounds isolated from *R. digitata*

Compound A1: white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.11 (1H, tt, *J* = 6.92, 2.56 Hz, H-12), 4.72 (1H, d, *J* = 12.69 Hz, H-29a), 4.68 (1H, d, *J* = 12.69 Hz, H-29b), 3.18 (1H, q, *J* = 5.43 Hz, H-3), 2.20 (1H, m, H-19), 2.18 (2H, m, H-7), 2.10 (2H, q, *J* = 7.52 Hz, H-11), 1.95 (2H, m, H-21), 1.86 (2H, m, *J* = 8.80 Hz, H-16), 1.70 (3H, t, *J* = 7.00 Hz, H-30), 1.67 (3H, m, H-28), 1.63 (3H, m, H-24), 1.60 (1H, m, H-9), 1.56 (2H, m, H-1), 1.53 (2H, m, H-2), 1.51 (3H, m, H-26), 1.50 (1H, m, H-18), 1.23 (3H, m, H-27), 1.05 (2H, m, H-15), 0.96 (2H, m, H-22), 0.85 (3H, m, H-23), 0.83 (3H, m, H-25), 0.75 (1H, m, H-5). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C (ppm): 152.89 (C-20, C), 131.59 (C-13, C), 124.62 (C-12, CH), 107,63 (C-29, CH2), 79.12 (C-3, CH), 56.03 (C-5, CH), 51.10 (C-18, CH), 49.59 (C-17, C), 48.00 (C-19, CH), 45.42 (C-9, CH),40.62 (C-14, C), 39.26 (C-8, C), 39.13 (C-22, CH_2), 37.38 (C-10, C), 35.58 (C-1, CH_2), 34.26 (C-16, CH_2), 31.54 (C-7, CH_2), 29.85 (C-4, C), 29.06 (C-21, CH_2), 28.18 (C-23, CH_3), 27.5 (C-15, CH_2), 27.2 (C-11, CH_2), 25.87 (C-30, CH_3), 21.53 (C-2, CH_2), 18.44 (C-6, CH_2), 17.87 (C-28, CH_3), 16.37 (C-25, CH_3), 16.08 (C-26, CH_3), 15.80 (C-24, CH_3), 15.53 (C-27, CH_3) (Prakash & Prakash, 2012).

Compound A2: white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.32 (1H, d, *J* = 5.16 Hz, H-6), 3.49 (1H, m, H-3), 2.26 (2H, m, H-4), 2.17 (2H, q, *J* = 8.63 Hz, H-12), 1.84 (2H, m, H-1), 1.79 (1H, q, *J* = 4.27 Hz, H-8), 1.64 (2H, m, H-23), 1.56 (1H, m, H-25), 1.48 (2H, m, H-16), 1.41 (2H, d, m, H-28), 0.98 (2H, m, H-11), 0.89 (2H, d, *J* = 6.56 Hz, H-15), 0.83 (3H, m, H-27), 0.81 (3H, m, H-26), 0.79 (3H, m, H-21), 0.65 (3H, m, H- 29), 0.62 (3H, m, H- 18). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C (ppm): 140.86 (C-5, C), 121.74 (C-6, CH), 71.83 (C-3, CH), 56.87 (C-14, CH), 56.15 (C-17, CH), 50.24 (C-9, CH), 45.94 (C-24, CH), 42.43 (C-4, CH_2), 42.41 (C-13, C), 39.88 (C-12, CH_2), 37.76 (C-1, CH_2), 36.61 (C-10, C), 36.25 (C-20, CH), 34.05 (C-22, CH_2), 32.01 (C-8, CH), 31.17 (C-2, CH_2), 29.81 (C-7, CH_2), 29.26 (C-25, CH), 28.35 (C-16, CH_2), 26.18 (C-23, CH_2), 24.41 (C-15, CH_2), 23.17 (C-28, CH_2), 21.19 (C-11, CH_2), 19.92. (C-27, CH_3), 19.50 (C-19, CH_3), 19.14 (C-26, CH_3), 18.88 (C-21, CH_3), 12.08 (C-29, CH_3), 11.96 (C-18, CH_3) (Kovganko *et al*., 1999; Mbambo *et al*., 2012).

Compound A3: light brown solid, ^{1}H NMR (400 MHz, MeOD) δ_H (ppm): 6.85 (1H, d, *J* = 1.96 Hz, H-2′), 6.77 (1H, d, *J* = 8.16 Hz, H-5′), 6.73 (1H, dd, *J* =1.96 Hz, H- 6′), 5.94 (1H, d, *J* = 2.28 Hz, H-8), 5.87 (1H, d, *J* =2.32 Hz, H-6), 4.57 (1H, d, *J* =7.56 Hz, H-2), 3.98 (1H, m, H-3), 2.86 (1H, dd, *J* = 16.0, 5.2 Hz, H- 4a), 2.51 (1H, dd, *J* = 16.4, 7.6 Hz, H-4b). ^{13}C NMR (400 MHz, MeOD) δ_C (ppm): 157.84 (C-7, C), 157.59 (C-5, C), 156.93 (C-9, C), 146.27 (C-3′, C), 146.24 (C4′, C), 132.24 (C-1′, C), 120.07 (C-6′, CH), 116.12(C-5′, CH), 115.29 (C-2′, CH), 100.86 (C-10, C), 96.33 (C-8, CH), 95.54 (C-6, CH), 82.87 (C-2, CH), 68.83 (C-3, CH), 28.53 (C-4, CH_2) (Abdulah *et al.*, 2017).

Compound A4: white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.27 (1H, t, *J* = 3.40 Hz, H-12), 3.21 (1H, q, *J* = 5.23 Hz, H-3), 2.81 (1H, q, *J* = 5.94 Hz, H-18), 2.04 (3H, m, H-30),

1.96 (2H, q, *J* = 5.75 Hz, H-2), 1.59 (3H, m, H-27), 1.53 (1H, m, H-9), 1.25 (2H, m, H-21), 1.13 (3H, m, H-23), 0.98 (2H, m, H-15), 0.92 (2H, m, H-11), 0.91 (2H, m, H-2), 0.90 (3H, m, H-29), 0.77 (3H, m, H-27), 0.75 (3H, m, H-24), 0.74 (3H, m, H-25), 0.71 (3H, m, H-26). ^{13}C NMR (400 MHz, $CDCl_3$) δ_H (ppm): 182.99 (C-28, C), 143.59 (C-13, C), 122.63 (C-12, CH), 79.03 (C-3, CH), 55.21 (C-5, CH), 47.62 (C-9, CH), 46.51 (C-17, C), 45.87 (C-19, CH_2), 41.60 (C-14, C), 40.99 (C-18, CH), 39.27 (C-8, C), 38.75 (C-4, C), 38.39 (C-1, CH_2), 37.08 (C-10, C), 33.79 (C-21, CH_2), 33.06 (C-29, CH_3), 32.61 (C-7, CH_2), 32.43 (C-22, CH_2), 30.67 (C-20, C), 28.09 (C-27, CH_3), 27.68 (C-15, CH_2), 27.18 (C-2, CH_2), 25.92 (C-23, CH_3), 23.57 (C-30, CH_3), 23.39 (C-11, CH_2), 22.93 (C-16, CH_2), 18.29 (C-6, CH_2), 17.12 (C-26, CH_3), 15.53 (C-24, CH_3), 15.31 (C-25, CH_3) (Baek *et al.*, 2010).

Compound A5: yellow powder**,** ^{1}H NMR (400 MHz, MeOD) δ_H (ppm) 7.72 (1H, d, *J* = 2.00 Hz, H-2′), 7.62 (1H, dd, *J* = 2.04, 8.48 Hz, H-6′), 6.88 (1H, d, *J* = 8.48 Hz, H-5′), 6.38 (1H, d, *J* =1.80 Hz, H-8), 6.18 (1H, d, *J* = 1.88 Hz, H-6). ^{13}C NMR (400 MHZ, MeOD) δ_C (ppm): 177.35 (C-4, C), 165.58 (C-7, C), 162.43 (C-5, C), 158.22 (C-9, C), 148.77 (C-4′, C), 148.09 (C-2, C), 146.21 (C-3′, C), 137.22 (C-3, C), 124.15 (C-1′, C), 121.74 (C-6′, CH), 116.29 (C-5′, CH), 116.04 (C-2′, CH), 104.54 (C-10, C), 99.31 (C-6, CH), 94.50 (C-8, CH) (Kyriakou *et al.*, 2012).

3.3.7 Spectroscopic data of compounds isolated from *R. tomentosa*

Compound B1: white powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.55 (1H, dd, *J* = 4.96, 11.33 Hz, H-15), 3.21 (1H, dd, *J* = 4.68, 11.01 Hz, H-3), 2.05 (2H, tt, *J* =3.04, 12.56 Hz, H-19), 1.93 (2H, dd, *J* = 2.93, 14.72 Hz, H-16), 1.64 (2H, m, H-2), 1.49 (1H, m, H-18), 0.96 (3H, m, H-29), 0.83 (3H, m, H-28), 0.79 (1H, m, H-5). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C (ppm):

158.19 (C-14, C), 116.98 (C-15, CH), 79.18 (C-3, CH), 55.63 (C-5, CH), 49.38 (C-18, CH), 48.85 (C-9, CH), 41.42 (C-19, CH_2), 39.08 (C-8, C), 38.86 (C-4, C), 38.10 (C-17, C), 37.84 (C-1, CH_2), 37.67 (C-13, C), 36.77 (C-16, CH_2), 35.89 (C-10, C), 35.22 (C-12: CH_2), 33.80 (C-21: CH_2), 33.45 (C-29: CH_3), 33.19 (C-22: CH_2), 30.02 (C-28: CH_3), 29.93 (C-26, CH_3) ,29.81 (C-22, CH_2), 28.90 (C-22, CH_2), 28.10 (C-23, CH_3), 27.24 (C-2, CH_2), 26.01 (C-27, CH_3), 21.42 (C-30, CH_3), 18.90 (C-6, CH_2), 17.60 (C-11, CH_2), 15.55 (C-24, CH_3), 15.53 (C-25, CH_3) (Koay *et al*., 2013; Oladoye *et al*., 2015).

Compound B2: white powder**,** ^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.37 (1H, d, *J* = 5.08 Hz, H-6), 5.16 (1H, dd, *J* = 8.52, 8.56 Hz, H-22), 5.04 (1H, dd, *J* = 8.60, 8.68 Hz, H-23), 3.54 (1H, dt, *J* = 9.61, 11.11 Hz, H-3), 2.29 (2H, m, H-4), 2.01 (2H, m, H-12), 1.86 (1H, m, H-25), 1.63 (2H, m, H-15), 1.27 (2H, m, H-16), 1.13 (1H, m, H-17), 1.03 (3H, m, H-19), 0.95 (3H, m, H-27), 0.84 (3H, m, H-26), 0.71 (3H, m, H-29). ^{13}C NMR (400 MHz, $CDCl_3$) δ_C (ppm): 141.07 (C-5, C), 138.62 (C-22, CH), 129.59 (C-23, CH), 122.03 (C-6, CH), 72.13 (C-3, CH), 57.08 (C-14, CH), 56.37 (C-17, CH), 51.55 (C-24, CH), 50.45 (C-9, CH), 42.61 (C-13, C), 42.53 (C-13, CH_2), 40.80 (C-20, CH), 40.09 (C-12, CH_2), 37.51 (C-1: CH_2), 36.82 (C-10: C), 36.46 (C-8: CH),34.26 (C-2: CH_2), 32.22 (C-25: CH), 31.97 (C-7, CH_2), 28.56 (C-16, CH_2), 26.39 (C-28, CH_2), 24.61 (C-15, CH_2), 21.39 (C-11, CH_2), 20.13 (C-21, CH_3), 19.71 (C-26, CH_3), 19.34 (C-19, CH_3), 19.09 (C-27, CH_3), 12.29 (C-29, CH_3), 12.17 (C-18, CH_3) (Koay *et al.*, 2013).

Compound B4: dark green solid, ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 9.49 (1H, d, *J* = 22.56 Hz, H-10), 9.33 (1H, d, *J* = 24.42 Hz, H-5), 8.55 (1H, d, *J* = 37.81 Hz, H-20), 6.29 (1H, d, *J* = 16.50 Hz, H-3″ a), 6.19 (1H, t, *J* = 5.73 Hz, H-3″ b), 4.25 (1H, t, *J* = 7.44 Hz, H-17), 3.93 (3H, m: H-13^{IV}), 3.86 (3H, m, H-12′), 3.41 (2H, m, H-2′), 3.20 (3H, m, H-7′), 1.71 (1H, t, *J* = 7.62 Hz, H-18), 1.68 (3H, m, *J* = 9.51 Hz, H-8″). ^{13}C NMR (400 MHz, $CDCl_3$) δ_H (ppm): 189.63 (C-

13, C′), 172.94 (C-17‴, C), 172.22 (C-13″, C′), 169.62 (C-19, C), 161.29 (C-16, C), 155.58 (C-6, C), 150.93 (C-9, C), 149.70 (C-14, C), 145.17 (C-8, C), 142.79 (C-1, C), 137.96 (C-11: C), 136.51 (C-7, C), 136.20 (C-4, C), 136.17 (C-3, C), 131.82 (C-2, C),129.09 (C-3′, CH), 129.03 (C-12, C), 129.00 (C-13, C), 122.70 (C-3″, CH), 105.30 (C-15, C), 104.37 (C-10, CH), 97.51 (C-5, CH), 93 (C-20, CH), 64.73 (C-13″, C), 52.80 (C-13^{IV}, CH_3), 51.21 (C-17, C), 50.16 (C-18, C), 31.25 (C-17″, CH_2), 29.70 (C-17′, CH_2), 23.06 (C-18′, CH_3), 19.65 (C-8′, CH_3), 17.54 (C-8″, CH_3), 12.07 (C-12′, CH_3), 12.04 (C-2′, C), 11.16 (C-7′, CH_3) (Chaves *et al.*, 2013; Kapewangolo *et al*., 2017).

Compound B5: yellow coloured solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm): 6.62 (1H, m, *J* = 5.75 Hz, H-4), 6.36 (1H, t, *J* = 7.40 Hz, H-10), 6.28 (1H, t, *J* = 14.23 Hz, H-14), 6.16 (1H, q, *J* = 3.58 Hz, H-7), 6.12 (1H, s, H-8), 3.94 (1H, m, *J* = 6.81 Hz, H-3), 2.36 (1H, m, *J* = 9.39 Hz, H-6), 1.97 (3H, m, H-19), 1.91 (3H, m, H-20), 1.73 (3H, m, H-20′), 1.68 (3H, m, H-19′), 1.61 (3H, m, H-18,18′), 1.07 (3H, m, H-17), 1.00 (3H, m, H-16′), 0.85 (1H, m, H-17′). ^{13}C-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 138.50 (C-8, CH), 137.99 (C-12, CH), 137.77 (C-4′, CH), 137.57 (C-6, CH), 136.49 (C-13, C), 136.41 (C-9, C), 135.69 (C-9′, C), 132.57 (C-14, CH), 131.30 (C-10, CH), 130.81 (C-10′, CH), 130.09 (C-15, CH), 128.73 (C-15′, CH), 126.17 (C-5, C), 125.59 (C-7, CH), 124.94 (C-11, CH), 124.81 (C-11′, CH), 124.49 (C-4, CH), 65.93 (C-3′, CH), 65.09 (C-3, CH), 54.97 (C-6′, CH), 48.44 (C-2, CH_2), 44.63 (C-2′, CH_2), 42.56 (C-4, CH), 37.12 (C-1, C), 34.03 (C-1′, C), 30.26 (C-16, CH_3), 29.50 (C-16′, CH_3), 28.73 (C-17, CH_3), 24.28 (C-17′, CH_3), 22.86 (C-18′, CH_3), 21.62 (C-18, CH_3), 13.11 (C-19′, CH_3), 12.87 (C-19, CH_3), 12.75 (C-20, CH_3) (El-Raey *et al*., 2013; Otaka *et al*., 2016).

Compound B6: bright yellow solid, ^{1}H NMR (400 MHz, MeOD) δ_H (ppm): 7.37 (1H, s, H-2′), 7.33 (1H, d, J=8.30 Hz, H-6′), 6.94 (1H, d, J=8.29 Hz, H-5′), 6.39 (1H, s, H-6), 6.23 (1H, s, H-8), 5.38 (1H, s, H-1″), 4.26 (1H, s, H-2″), 3.79 (1H, q, *J* = 4.12 Hz, H-3″), 3.45 (1H, m, H-5″), 3.38 (1H, d, *J* = 9.47 Hz, H-4″), 0.98 (3H, m, H-6″). ^{13}C NMR (400 MHz, MeOD) δ_C (ppm): 179.74 (C-4, C), 165.96 (C-7, C), 163.16 (C-5, C), 159.51 (C-9, C), 158.61 (C-2, C),149.88 (C-4′: C), 146.46 (C-3′, C), 136.36 (C-3, C), 123.12 (C-6′, CH), 117.22 (C-1′, CH), 116.65 (C-5′, CH), 110.61 (C-2′, CH),103.65 (C-1″, CH), 100.06 (C-6, CH), 95.01 (C-8, CH), 73.41 (C-4″, CH), 72.28 (C-3″, CH), 72.18 (C-5″, CH), 72.03 (C-2″, CH), 17.08 (C-6″, CH3) (Guimarães *et al.*, 2015).

Compound B9: brown yellow solid, ^{1}H NMR (400 MHz , MeOD) δ_H (ppm): 7.39 (1H, d, *J* = 8.52 Hz, H-2′), 7.36 (1H, d, *J* = 8.58 Hz, H-6′), 6.87 (1H, s, H-3′), 6.85 (1H, s, H-5′), 5.96 (1H, d, *J* = 1.98 Hz, H-6), 5.93 (1H, d, *J* = 1.86 Hz, H-8), 5.30 (1H, d, *J* = 10.02 Hz, H-2), 4.97 (1H, d, *J* = 10.02 Hz, H-3″), 4.23 (1H, m, *J* = 4.22 Hz, H-2″), 3.86 (1H, d, *J* = 7.80 Hz, H-1″), 3.64 (2H, m, *J* = 5.49 Hz, H-6″), 3.33 (1H, t, *J* = 2.31 Hz, H-3), 3.28 (1H, m, *J* = 9.32 Hz, H-4″), 3.03 (1H, m, *J* = 17.93 Hz, H-5″). ^{13}C NMR (400 MHz, MeOD) δ_C (ppm): 196.14 (C-4, C), 168.99 (C-7, C), 165.40 (C-9, C), 164.21 (C-5, C), 159.21 (C-4′, C), 130.59 (C-2′, CH), 130.48 (C-6′, CH), 128.58 (C-1′, C), 116.44 (C-3′, CH), 115.91 (C-5′, CH), 103.21 (C-10, C), 102.70 (C-1″, CH), 97.57 (C-6, CH), 96.55 (C-8, CH), 83.55 (C-2, CH), 78.55 (C-5″, CH), 77.56 (C-3″, CH), 74.58 (C-2″, CH), 73.90 (C-3, CH), 71.22 (C-4″, CH), 62.58 (C-6″, CH_2) (Markham *et al.*, 1985; Slimestad *et al.*, 1994).

3.3.8 Antioxidant activity

3.3.8.1 DPPH free radical-scavenging activity assay

The free radical scavenging activity of the crude MeOH extracts and isolated compounds from *R. digitata* and *R. tomentosa* was measured using the DPPH (1,1-diphenyl-2-picrylhydrazine) stable free radical method reported by Soni and Sosa (2013) with some modifications. The radical scavenging activity of the test sample is detected by the colour change of the DPPH solution from purple to yellow depending on the concentration of the sample. A volume (150 µL) of the crude MeOH extracts at different concentrations (10-1000 µg mL^{-1}) was mixed with 2850 µL of a methanolic DPPH solution (0.1 Mm). The mixture was shaken and kept at room temperature (in the dark) for 30 minutes. The absorbance of this mixture was measured at 517 nm using a UV/Vis spectrophotometer. Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as positive standards and the MeOH was used as control. The % scavenging activity was calculated using the following formula:

$$\% \text{ Scavenging} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

$A_{control}$ = Absorbance of DPPH + MeOH

The IC_{50} value which is the amount of an antioxidant required to decrease the DPPH absorbance by 50% compared to the negative control was determined by plotting the absorbance of DPPH against sample concentration in µg/mL for standards and test samples (Soni & Sosa, 2013).

3.3.8.2 Ferric reducing antioxidant power (FRAP) assay

The reducing power of extracts and compounds was determined using the procedure outlined by Iqbal *et al* (2015) with a few modifications. The presence of an antioxidant agent causes the reduction of Fe^{3+} to Fe^{2+} in the FRAP assay, which creates an intense navy blue colour. The absorbance (measured at 700 nm) tests the amount of iron reduced, which is related to the amount of the antioxidant. The mixture of a test sample (1 mL) at different concentrations (10-1000 μg mL^{-1}), 1 mL of phosphate buffer (K_2HPO_4 and KH_2PO_4), and 1 mL of 1% potassium ferric cyanide ($K_2Fe(CN)_6$) was incubated in a water bath at 50 ^{0}C for 20 minutes followed by the addition of 1 mL of 10% trichloroacetic acid (TCA). This mixture was allowed to stand at room temperature for 10 minutes and the top layer (2 mL) was mixed with 7 mL of distilled water and 1 mL of 0.1% ferric chloride ($FeCl_3$). The absorbance was measured at 700 nm with MeOH as a negative control and ascorbic acid and BHT as positive controls (Iqbal *et al.*, 2015).

3.3.8.3 Phosphomolybdate assay

The crude MeOH extracts and isolated compounds were evaluated for their reducing potential according to the method described by Picot and Mahommodally (2017). In this assay, reduction of Mo(VI) to Mo(V) indicates the antioxidant activity of the sample. The reduction is confirmed by the appearance of a green colour due to the formation of a phosphate/Mo(V) complex in acidic pH. Samples (0.3 mL) of various concentrations (10-1000 μg mL^{-1}) and 3 mL of phosphomolybdate reagent (0.6 M H_2SO_4, 287 MM sodium phosphate, and 4 MM ammonium molybdate) were incubated at 95 ^{0}C for 90 minutes. The mixture was cooled to room temperature and absorbance measured at 695 nm using a UV/Vis spectrophotometer (Picot &

Mahomoodally, 2017). MeOH was used as a negative control, while ascorbic acid and BHT were the positive standards.

3.3.9 Statistical analysis

The statistical analysis was done using the Statistical Package for Social Sciences (PASW Statistics 22, IBM Corporation, Cornell, New York). Linear regression was used to calculate the IC_{50} value. One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was used to determine significant differences between the means ($p < 0.05$). The results were expressed as mean ± standard deviation (SD) of the three replicates.

3.4 Results and Discussion

3.4.1 Qualitative analysis of compounds

The phytochemical screenings showed the presence of various classes of compounds (Table 3.1) in DCM and MeOH crude extracts of *R. digitata*. The results indicated the presence of steroids, terpenoids, saponins, and cardiac glycoside in all samples. Flavonoids, phenols, and tannins were detected in methanol extracts, while alkaloids were only detected in DCM extracts. Antroquinone glucoside was not detected in any sample.

Table 3.1**:** Phytochemical screening of crude extracts of *Rhoicissus digitata*

Test	Roots		Fruits	
	DCM extract	**Methanol extract**	**DCM extract**	**Methanol extract**
Alkaloids	+	-	+	-
Steroids	+	+	+	+
Terpenoids	+	+	+	+
Tannins	-	+	-	+
Saponins	+	+	+	+
Antroquinone glucoside	-	-	-	-
Cardiac glucoside	+	+	+	+
Flavonoids	-	+	-	+
Phenols	-	+	-	+

(+) for the presence and (-) for the absence

3.4.2 Structure elucidation of compounds from *R. digitata*

Five compounds were isolated from the roots and fruits of *R. digitata.* The chemical structures of these compounds (Fig. 3.2) were identified using spectroscopic data (1D and 2D NMR, mass, IR, and UV/Vis) and by comparison of experimental data with literature.

Compound **A1** (230 mg) was obtained as a white powder from the roots of *R. digitata*. The GC-MS spectrum showed a molecular ion peak at *m/z* 426 (M+H), which corresponds to the molecular formula of $C_{30}H_{50}O$. The IR spectrum of compound **A1** showed broad absorption peaks at 3311 cm^{-1} for the O-H group, 1032 cm^{-1} for the C-O group, and 1643 cm^{-1} for the C=C group. The ^{13}C and DEPT NMR spectra resolved thirty carbon resonances corresponding to six quaternary, seven methyl, twelve methylene, and five methine carbons. The ^{1}H NMR spectrum showed a multiplet at δ_H 3.18 (H-3) which is characteristic of a terpenoid nucleus (Bin Sayeed

et al., 2016), two olefinic protons at δ_H 4.68 (H-29a) and 4.72 (H-29b) that showed cross peaks to the same carbon in HSQC NMR experiment, and an olefinic proton at δ_H 4.72 (H-12). The ^{1}H NMR spectrum showed seven signals at δ_H 1.70, 1.67, 1.63, 1.51, 1.23, 0.85, and 0.83, attributed to the seven methyl protons (H-30, H-28, H-24, H-26, H-27, H-23, and H-25). The UV/Vis absorption spectrum of this compound showed an absorbance band at 239 nm, which is consistent with that of terpenoids.

Compound **A1** was identified as 12,13-dehydrolupeol as the experimental data closely matched that reported in literature for lupeol (Prakash & Prakash, 2012). 12,13-Dehydrolupeol is isolated for the first time from the family Vitaceae, however lupeol was isolated from the leaves of some Vitaceae plants including *Cyphostemma digitatum* and *Cissus quandranglaris* Linn (Al-Mahweety, 2016; Rao *et al*., 2011). Lupeol has been reported to possess several biological activities including reducing exudate volume and the total count of leukocytes, inhibiting trypsine and chemotrypsin, as well as anti-inflammatory activity (Ahmad *et al*., 2010; Longhi-Balbinot *et al.*, 2012).

Compound **A2** (320 mg) was obtained as a white powder from the roots of *R. digitata* with a molecular ion peak at *m/z* 414 corresponding with the molecular of $C_{29}H_{50}O$ (Mbambo *et al.*, 2012). The ^{1}H NMR spectrum showed a multiplet at δ_H 3.49 (H-3), an olefinic proton at δ_H 5.32 (H-6) and six methyl protons at δ_H 1.00 (H-27), 0.94 (H-19), 0.84 (H-26), 0.80 (H-21), 0.69 (H-29), and 0.68 (H-18). The ^{13}C and DEPT (90 and 135) NMR spectra resolved twenty-nine carbon signals corresponding to six methyl, eight methine, four quaternary, and eleven methylene carbons (Kovganko *et al.*, 1999). The IR spectrum showed absorption frequencies of O-H at 3313 cm^{-1}, C-O at 1046 cm^{-1}, and C=C at 1650 cm^{-1}. The UV/Vis absorption spectrum of compound **A2** showed a band peak at 279 nm. The experimental corresponded with literature

data and confirmed compound **A2** to be β-sitosterol (Kovganko *et al.*, 1999). β-sitosterol was previously isolated from the genus *Rhoicissus tridentata* (Brookes & Katsoulis, 2006; Dube, 2014). This compound is used in the cosmetics, pharmaceutical and food industries and has been shown to possess anti-cancer activity (Longhi-Balbinot *et al.*, 2012).

Compound **A3** (90 mg) was obtained as a light brown solid from the roots of *R. digitata*. The LC-ESI-MS spectrum showed a molecular ion peak at *m/z* 291, which is in agreement with the molecular formula of $C_{15}H_{14}O_6$ (Abdulah et al., 2017). The ^{1}H NMR spectrum showed three signals in the aromatic region at δ_H 6.85 (d, J = 1.96 H_Z, H-2′), 6.77 (d, J = 8.16, H-5′), and 6.37 (dd, J = 1.96, 8.16 H_Z, H-6′), which is characteristic of a B ring of flavonoids (Mshengu, 2015). Two meta-coupled aromatic signals were observed at δ_H 5.83 (H-8), 5.76 (H-6), confirming the presence of an A ring (Kyriakou *et al.*, 2012). The proton resonances at δ_H 2.86 (dd, J = 16.0, 5.2 Hz) and 2.51 (dd, J = 16.4, 7.6 Hz) correlated to the same carbon peak (δ_C 28.5) in the HSQC NMR spectrum (Abdulah et al., 2017). These signals were assigned as H-4a and H-4b. The ^{13}C and DEPT NMR spectra resolved fifteen carbon signals, including seven quaternary, seven methine and a methylene signal. The absorption frequencies at 3198 cm^{-1}, 1605 cm^{-1}, and 1141-1019 cm^{-1} are due to the O-H, C=C and C-O groups, respectively. The UV spectrum of compound **A3** showed absorption bands at 242 nm and 279 nm, which is consistent with the reported maximum wavelength of flavonoids (Kyriakou *et al.*, 2012). Based on experimental and literature data, compound **A3** was identified as (+)-catechin (Abdulah *et al.*, 2017). This compound was previously isolated from *R. tridentata* (Mshengu, 2015). Some biological activities of (+)-catechin include anti-inflammatory, anti-allergic, anti-mutation, antioxidant, and anti-aging (Bagchi *et al.*, 2000).

Compound **A4** (80 mg) was isolated as a white powder from the fruits of *R. digitata*. A molecular ion peak at *m/z* 456, consistent with the molecular formula $C_{30}H_{48}O_3$ (Baek et al., 2010), was observed from the GC-MS spectrum. The olefinic methine proton at δ_H 5.27 (H-12) and an oxygenated methine proton at δ_H 3.22 (H-3) were observed on the ^{1}H NMR spectrum. The ^{13}C and DEPT NMR spectra resolved thirty carbons resonances including eight quaternary, seven methyl, ten methylene, and five methine carbons. A carboxylic carbon resonance was observed at δ_C 182.99 (C-28) and a quaternary olefinic carbon resonated at δ_C 143.59 (C-13) in ^{13}C NMR spectrum, indicative of an oleanane triterpenoid (Baek *et al.*, 2010). The IR spectrum showed characteristic absorption bands for O-H at 3454 cm^{-1} and C=O group at 1685 cm^{-1}.

Compound **A4** showed an absorption peak at 243 nm in the UV spectrum, which is in agreement with the reported maximum wavelength of terpenoids (Baek et al., 2010). Compound **A4** was identified as oleanolic acid as the experimental data was the same as that reported in the literature. (Baek *et al.*, 2010). Oleanolic acid was previously isolated from *R. tridentata* (Brookes & Katsoulis, 2006). This compound was reported to be an anti-inflammatory, antioxidant, hepatoprotective, and anticancer agent (Jesus *et al*., 2015).

Compound **A5** (35 mg) was obtained as a yellow powder from the fruits of *R. digitata*, with a molecular ion peak at *m/z* 301, which is in agreement with the molecular formula of $C_{15}H_{10}O_7$ in the LC-ESI-MS spectrum (Kyriakou *et al.*, 2012). The ^{1}H and COSY NMR spectra showed the presence of an ABX aromatic spin system at δ_H 7.72 (1H, d, J = 2.00 Hz, H-2′), 7.62 (1H, d, J = 8.48 Hz, H-5′), and 6.88 (1H, dd, J = 8.48 and 2.00 Hz, H-6′). The meta-coupled proton signals observed at 6.38 (1H, d, J =1.80 Hz) and 6.18 (1H, d, J = 1.80 Hz) in the ^{1}H NMR spectrum were assigned to the A ring protons (Abdulah et al., 2017). Fifteen signals, consisting of ten quaternary and five methine carbons were observed in the ^{13}C and DEPT NMR spectra.

The UV/Vis spectrum of compound **A5** showed two absorption bands with maximum peak at 370 nm which is characteristic of quercetin (Kyriakou *et al.*, 2012). The IR spectrum showed absorption bands of O-H, C=C, and C=O groups at 3244 cm^{-1}, 1602 cm^{-1}, and 1660 cm^{-1}, respectively. Compound **A5** was identified as quercetin as the experimental data was in agreement with that in literature (Kyriakou *et al.*, 2012). This is a first report on the isolation of quercetin from the genus *Rhoicissus*, however it was isolated from *Cissus rotundifolia* (Vitaceae) (Akram Ali Mohamed Shalabi, 2016). Quercetin is used for the treatment of stage I hypertension and has anti-influenza and anti-diarrhoeal properties (Perez-Vizcaino *et al*., 2009).

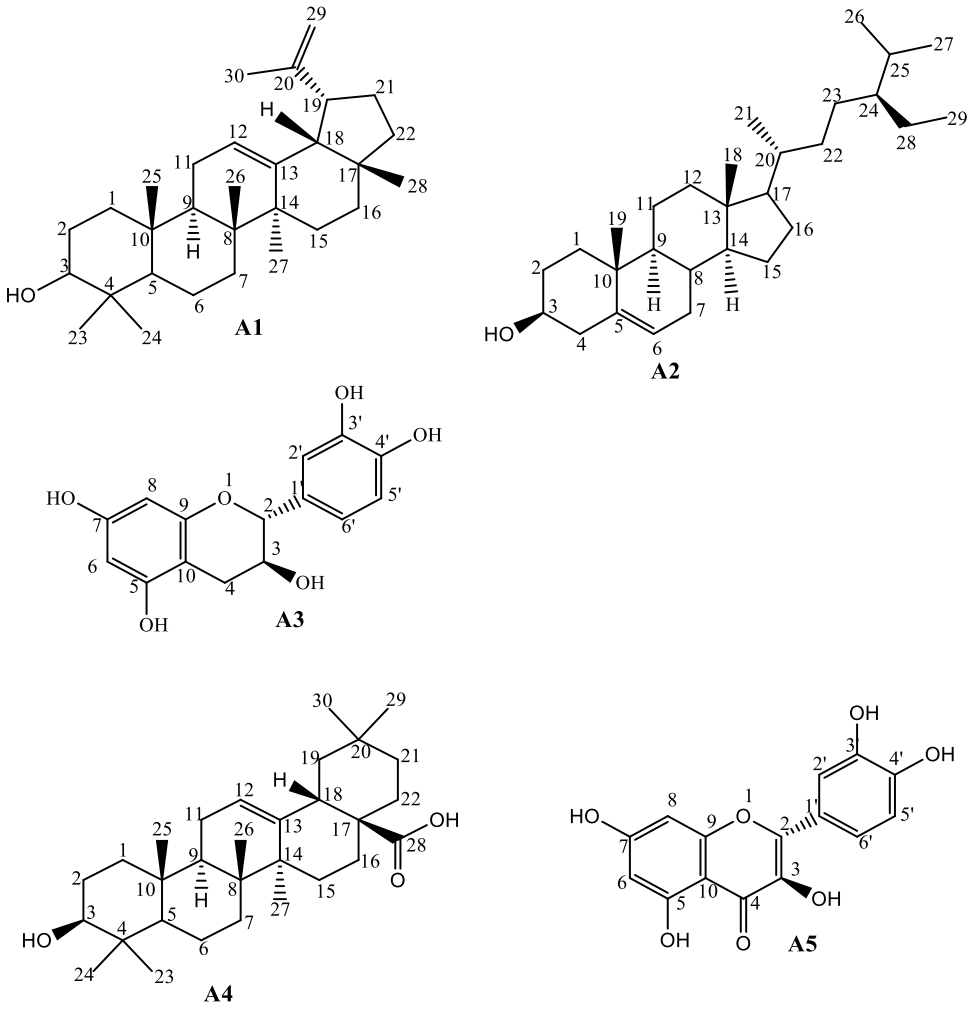


Figure 3.2: The chemical structures of the compounds (**A1**-**A5**) isolated from *R. digitata*

3.4.3 Structural elucidation of compounds from *R. tomentosa*

The chemical structures of the nine compounds isolated from *R. tomentosa* are presented in Figure 3.3. Compound **B1** (120 mg) was obtained as a white powder from the roots of *R. tomentosa*. The GC-MS spectrum showed molecular ion peak at *m/z* 426, corresponding to the molecular formula of $C_{30}H_{50}O$ (Koay *et al*., 2013). The UV/Vis absorption spectrum showed a band peak at 218 nm, this is in agreement with the reported UV_{max} of triterpenoid (Belsner *et al*., 2003). The ^{1}H NMR spectrum showed a doublet of doublet at δ_H 3.21 (H-3) and an olefinic proton signal at δ_H 5.55 (H-15) that correlated to the carbon signals at δ_C 79.18 and δ_C 116.98 in the HSQC experiment. Thirty carbon signals (seven quaternary, eight methyl, five methine, and ten methylene signals) were observed in the ^{13}C and DEPT NMR spectra. The IR spectrum exhibited absorption peaks at 3396 cm^{-1} for O-H, 1641 cm^{-1} for C=C, and at 1104 cm^{-1} for C-O. Compound **B1** was identified as 3β-taraxerol as the experimental data was in agreement with that reported data in literature (Oladoye *et al.*, 2015). To the best of our knowledge, this is the first report on the isolation of 3β-taraxerol from the genus *Rhoicissus*, however it was identified from the roots and stem of *Cissus quadriangularis* of the family Vitaceae (Sudmoon *et al*., 2016). 3β-Taraxerol possesses various biological activities such as anti-inflammatory and anticancer activity (Nithyamol Kalappurakkal *et al*., 2018).

A white powder (compound **B2**) (220 mg) was obtained from the roots of *R. tomentosa*. This compound showed a molecular ion peak at *m/z* 412, corresponding to $C_{29}H_{48}O$ in the GC-MS spectrum (Huang *et al.*, 2011). The ^{1}H NMR spectrum indicated a proton resonance at δ_H 3.54 (H-3) and three olefinic proton resonances at δ_H 5.37 (H-6), 5.16 (H-22), and 5.04 (H-23). The ^{13}C NMR spectrum indicated a signal at δ_C 72.13 (C-3). The carbon signals resonating at δ_C

19.34, 12.17 were due to angular methyl carbons (C-19 and C-18, respectively). This is consistent with the reported data for stigmasterol (Huang *et al*., 2011). The broad absorption band for O-H group was observed at 3423 cm^{-1}in the IR spectrum. The IR spectrum also showed absorption peaks for cyclic olefinic group at 3290 cm^{-1}, C-O at 1192 cm^{-1}, and aliphatic group vibrations at 2933 cm^{-1} and 2865 cm^{-1}. The UV/Vis absorption spectrum of this compound showed a peak at 236 nm, which is in agreement with the reported maximum wavelength of stigmasterol (Koay *et al.*, 2013). The structure of compound **B2** was identified as stigmasterol (Huang *et al.*, 2011). This is the first isolation of stigmasterol from the genus *Rhoicissus* however, this compound was isolated from rhizomes of *Cissus poulnea* in the Vitaceae family (Nyemb *et al.*, 2018). Stigmasterol has been reported to cure osteoarthritis, paw oedema, and neutrophil infiltration (Gabay *et al.*, 2010).

Compound **B3** was isolated as a light brown solid (60 mg) from the roots of *R. tomentosa*. The LC-ESI-MS spectrum of this solid showed a molecular ion peak at *m/z* 291, indicating a molecular formula of $C_{15}H_{14}O_6$ (Abdulah et al., 2017). The 1D and 2D NMR data of this solid was similar to that of (+)-catechin (compound **A3**) isolated from roots of *R. digitata* in the present study.

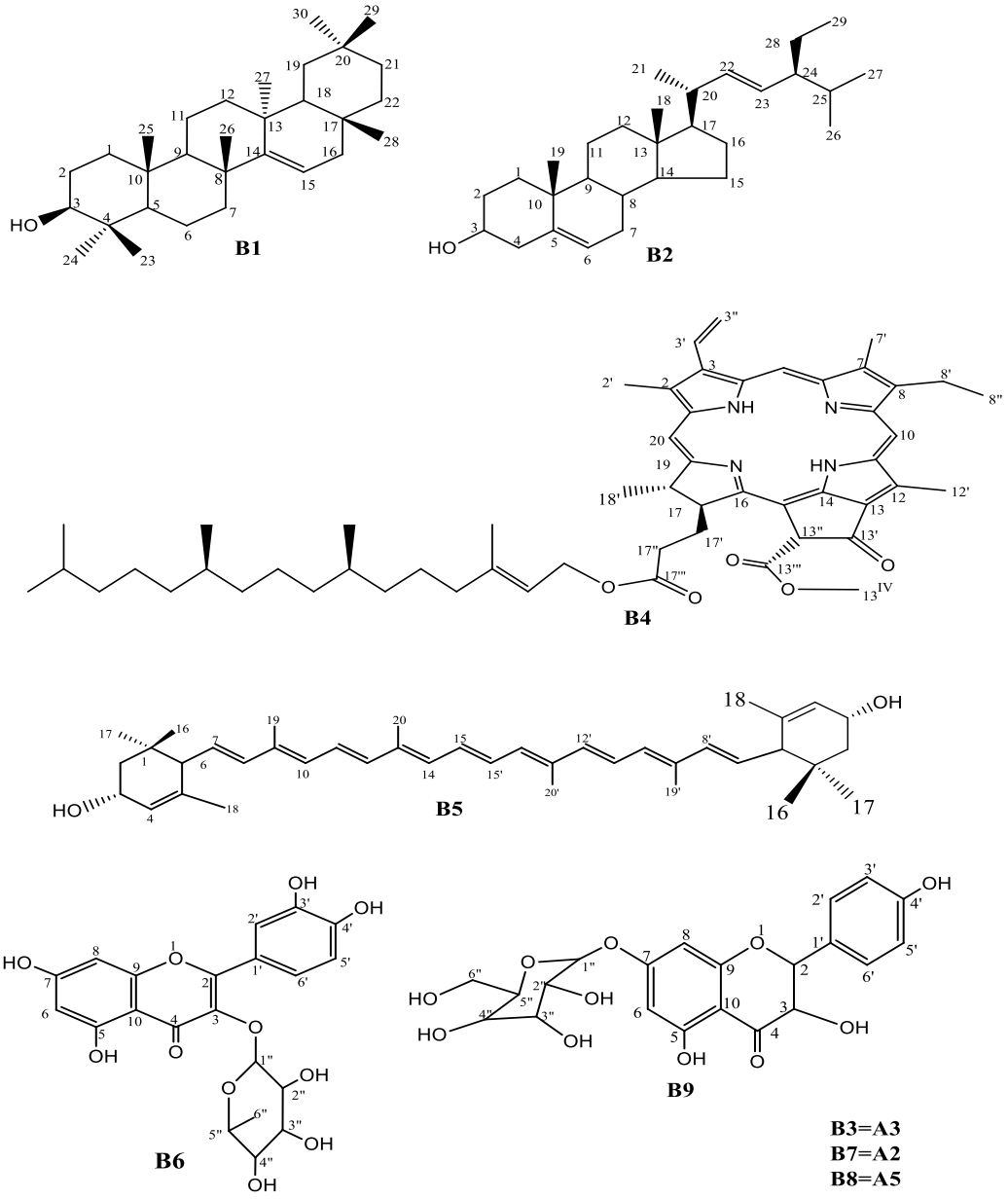
Compound **B4** was isolated as dark green solid (145 mg) from the leaves of *R. tomentosa*. A molecular ion peak at *m/z* 871, which is in agreement with the molecular formula of $C_{55}H_{74}N_4O_5$ was obtained from the GC-MS spectrum (Chaves et al., 2013). The ^{1}H NMR spectrum showed an upfield signal at δ_H -1.60, corresponding to the NH proton from the pyrrole ring and four olefinic proton resonances at δ_H 6.30 (H-3′), 6.19 (H-3″), 8.58 (H-20), and 9.50 (H-10). The ^{13}C and DEPT NMR spectra resolved fifty-five carbon peaks (eleven methyl, fourteen methylene, eleven methine and nineteen quaternary signals). The IR spectrum showed broad absorption

bands at 3387 cm^{-1} for N-H, 1695 cm^{-1} for C=O, 1454 cm^{-1} for C≡N, and 1159 cm^{-1} for C-O. The UV/Vis spectrum showed an absorption band at 411 nm, which is in agreement with the reported maximum wavelength of pheophytins (Ahn *et al.*, 2002). Compound **B4** was identified as pheophytin a as confirmed by literature data (Ogunlaja *et al*. 2016). This is the first report on the existence of pheophytin a in *Rhoicissus* species, but the occurrence of this compound was previously reported from some plants in Vitaceae family such as *Cissus trifoliate* (Reddy *et al*., 2003).

Compound **B5** (110 mg) was isolated as yellow solid from the leaves and its GC-MS spectrum showed a molecular ion peak at *m/z* 568, corresponding to the molecular formula of $C_{40}H_{56}O_2$ (Al-Duais *et al*., 2009). The ^1H NMR spectrum showed an olefinic proton signals between δ_H 6.06 and 6.67, and OH peak at δ_H 4.23, characteristic of lutein) (Otaka *et al*., 2016) . The ^{13}C NMR spectrum indicated forty carbon peaks assigned as ten methyl, two methylene, twenty methine, and eight quaternary carbons. The IR spectrum showed peaks at 3386 cm^{-1} (O-H), 1707 cm^{-1} (C=C), and 1251 cm^{-1} (C-O). The UV/Vis spectrum showed an absorption band at 451 nm, which is consistent with the maximum wavelength of lutein (Ogunlaja *et al.*, 2016). Compound **B5** was identified as lutein. This compound was previously isolated from *Cyphostemma digitatum* (Vitaceae), a medicinal plant used as a culinary herb and as source of food flavouring (Al-Duais *et al*., 2009).

Compound **B6** (50 mg) was obtained as bright yellow solid from the leaves. The GC-MS spectrum showed molecular ion peak at *m/z* 430, which is in agreement with the molecular formula of $C_{21}H_{20}O_{11}$ (Guimarães et al., 2015). The ^1H and ^{13}C NMR spectra were similar to compound **A5** (quercetin) except for the anomeric proton signal at δ_H 5.38 (H-1″) that suggested the presence of a deoxy sugar moiety in this compound. Compound **B6** was therefore identified

as quercetrin after comparison of experimental data with that in literature (Devi & Kumar, 2018). Quercetrin was previously obtained from some Vitaceae plants, such as *R. tridentata* and *Cissus rotindifolia* (Akram Ali Mohamed Shalabi, 2016; Mshengu, 2015). Some biological activities of quercetrin include anticancer and antioxidant activities (Choiprasert *et al*., 2010; Mshengu, 2015).



SFigure 3.3: The chemical structures of the compounds (**B1** to **B9**) isolated from *R. tomentosa*

Compound **B7** was obtained as a white powder (9 mg) from the fruits. The spectroscopic data of this compound was similar to that of β-sitosterol (compound **A2**), which was obtained from the roots of *R. digitata*. This is the first report on the isolation of β-sitosterol from *R. tomentosa*.

Compound **B8** was isolated as a white powder (560 mg) from the fruits of *R. tomentosa*. This powder was identified as oleanolic acid as the experimental data was similar to that reported in literature (Baek *et al.*, 2010; Yang, *et al*., 2012). This is the first report on the isolation of oleanolic acid from *R. tomentosa*. This compound was isolated from the fruits of *R. digitata* (compound **A4**) in the present study, and was previously obtained from the roots of *R. tridentata* (Brookes & Katsoulis, 2006).

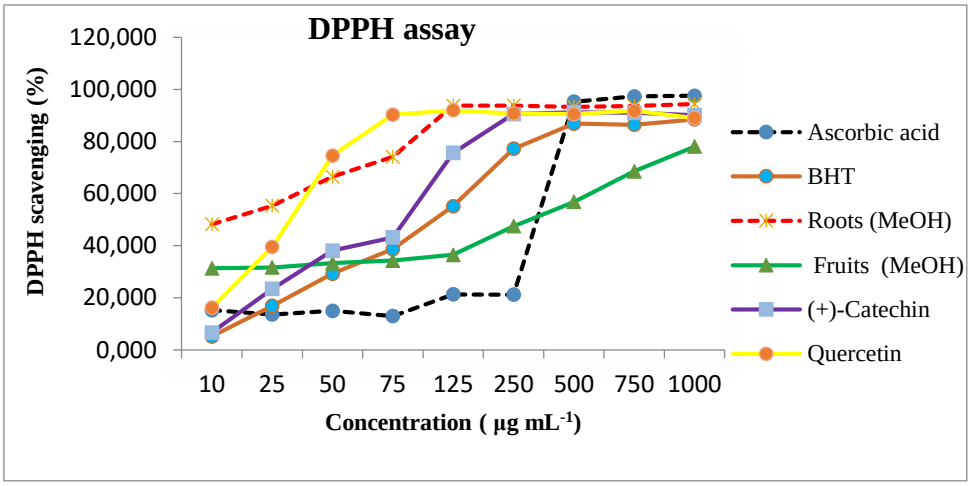
Compound **B9** (220 mg) was obtained as brown yellow solids from the fruits. The GC-MS spectrum showed molecular ion peak at *m/z* 455, in agreement with the molecular formula of $C_{21}H_{22}O_{11}$ (Slimestad *et al.*, 1994). The ^{1}H NMR spectrum showed sugar proton signals at δ_H 5.38, 4.26, 3.79, 3.45, 3.38, and 0.98. These protons correlated to the carbon signals between δ_C 78.55 and 62.58 in the HSQC spectrum. The ^{1}H NMR spectrum showed four downfield signals at δ_H 7.39 (H-2′), 7.36 (H-6′), 6.87 (H-3′), 6.85 (H-5′), consistent with the aromatic protons of the B ring. The presence of an A ring was confirmed by the presence of meta-coupled doublets at δ_H 5.96 (H-6) and 5.93 (H-8). Two coupled signals at δ_H 5.30 and 3.33 were due to the C-ring protons (H-2 and H-3, respectively). The ^{13}C NMR spectrum showed twenty-one signals assigned as seven quaternary, thirteen methine and a methylene carbon. The IR spectrum showed absorption peaks at 3324 cm^{-1} for O-H, 1723 cm^{-1} for C=O, and 1255 cm^{-1} for C-O. The UV/Vis absorption spectrum showed two peaks at 293 nm and 240 nm, this is in agreement with maximum wavelength of flavonoids (Slimestad *et al.*, 1994) . The experimental

and literature data led to the identification of compound **B9** as aromadendrin7-O-β-glucopyranoside (Markham *et al.*, 1985; Slimestad *et al.*, 1994). This is the first report on the isolation of this compound from the genus *Rhoicissus*, however the compounds, aromadendrin, has been obtained from the leaves of *Ampelopsis contaniensis* of Vitaceae family (Van Thu *et al.*, 2015).

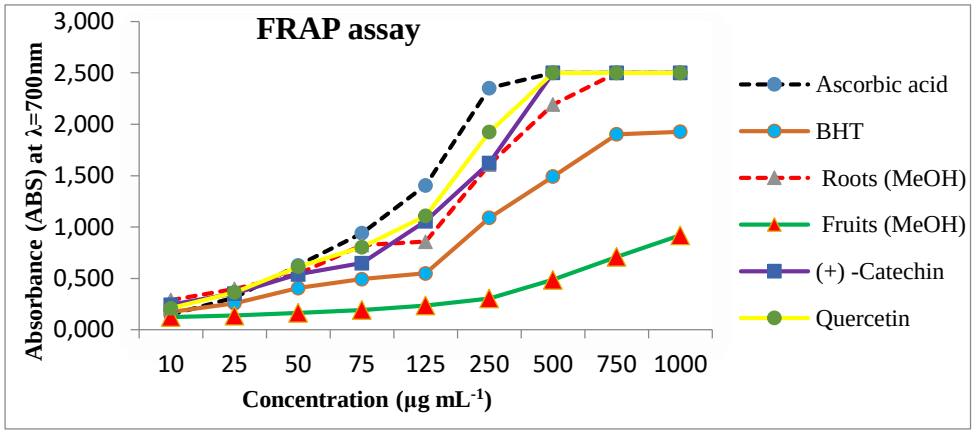
3.4.4 Antioxidant activity

The antioxidant activity of the crude MeOH extracts and compounds was evaluated using DPPH, FRAP and phosphomolybdate assays. For both plants, the scavenging effect and the reducing power of the crude MeOH extracts and isolated compounds increased with the increasing sample concentrations (Fig. 3.4 and 3.5).

A



B



C

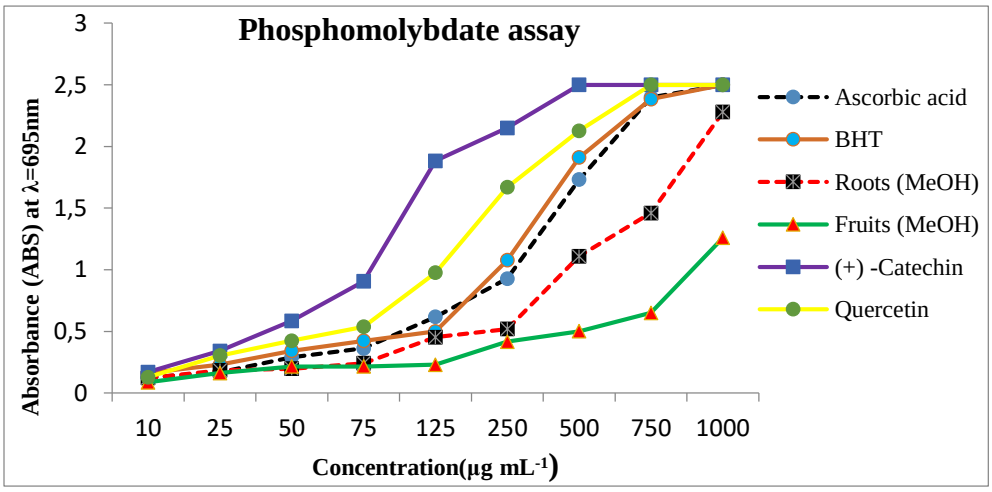
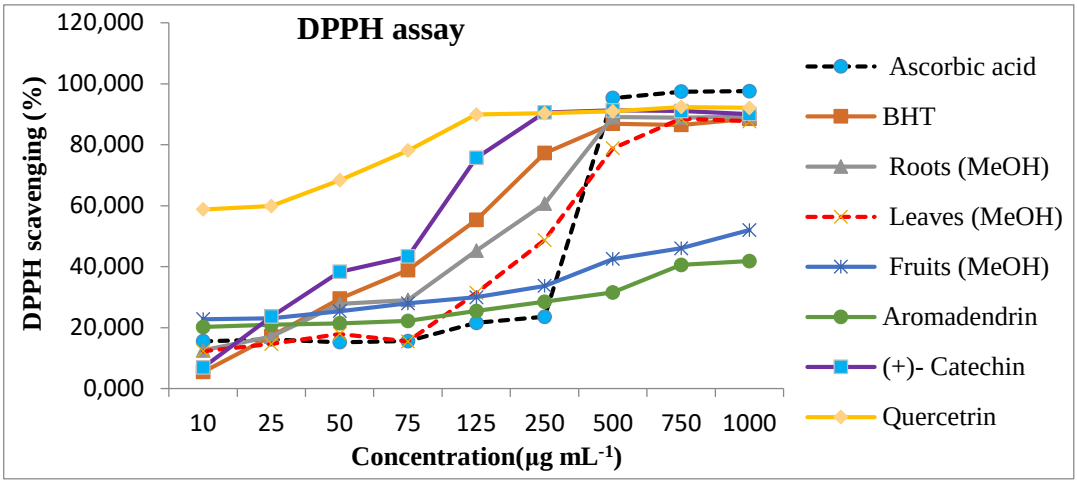
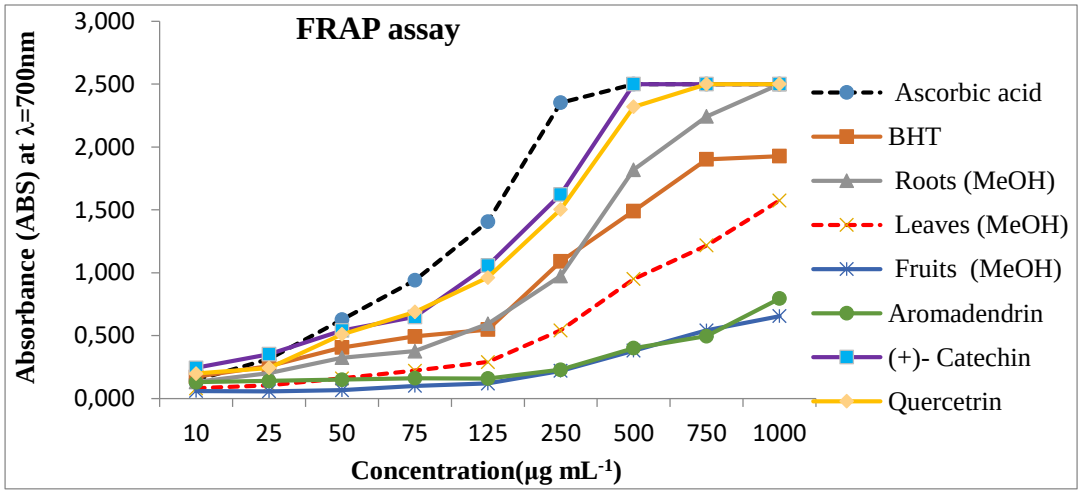


Figure 3.4: Antioxidant activity of the crude extracts and compounds from *R. digitata*.

A



B



C

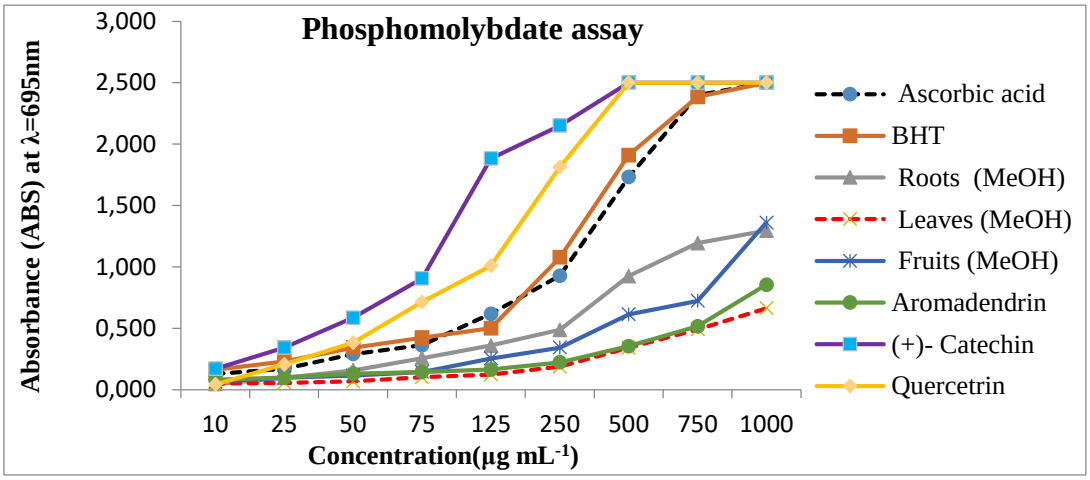


Figure 3.5: Antioxidant activity of the crude extracts and compounds from *R. tomentosa*.

The MeOH extract of roots of *R. digitata* (IC_{50} = 0.296 ± 0.20) and *R. tomentosa* (IC_{50} =4.907 ± 0.34) showed higher DPPH radical scavenging activity compared to the leaves and fruit extracts. Similar to the results obtained from the DPPH assay, the results from FRAP and phosphomolybdenum assays showed the reducing power of the MeOH root extracts to be higher than the MeOH leaves and fruits extracts.

At higher concentrations (≥ 500 μg mL^{-1}), the activity of the MeOH root extract showed no major difference in comparison to the positive controls but it was higher than the other tested samples. The isolated compounds (quercetin and quercetrin) showed significantly higher activity (in all three antioxidant assays) compared to their parent extracts. With exception of the DPPH assay for the methanol root extract of *R. digitata*, (+)-catechin also showed higher activity than its parent extracts. As discussed earlier, (+)-catechin was obtained from the MeOH roots extracts of both plants, quercetin from the fruits of *R. digitata*, while quercetrin was isolated from the leaves of *R. tomentosa*. The poor antioxidant activity of the extracts compared to the individual compounds may be attributed to the presence of other non- active or compound with antagonistic effects in the extract.

Of all the tested flavonoids, quercetin and quercetrin showed the highest antioxidant activity. The scavenging potential was found to be in the order of quercetrin > quercetin > (+)-catechin > aromadendrin7-O-β-glucopyranoside (Table 3.2). Previous studies on the structure activity relationship for antioxidant activity of flavonoids reported that good antioxidant requires the presence of a C_2=C_3 double bonds, the 3-hydroxyl group and the 4-carbonyl group in the C ring, as well as the catechol group (3′, 4′-OH) on ring B. Quercetin and quercetrin fulfilled all these requirements, hence they showed better activity than the other compounds and positive controls (Wang *et al*., 2018).

(+)-Catechin showed less activity due to the lack of double bond (C_2=C_3) and the ketogroup in the C-ring (Pekkarinen *et al*., 1999; Zhang *et al*., 2014). Aromadendrin7-O-β-glucopyranoside showed the least activity in all three assays relative to the other flavonoids (Table 3.2). This is due to the O-glycosylation in position 7 of the A ring, which interferes with the co-planarity of the molecule and delocalisation of electrons, thus decreasing activity (Wang *et al.*, 2018). This compound also lacks the C_2=C_3 double bond in the C-ring, which is required to improve the antioxidant potential (During & Larondelle, 2013; Wang *et al.*, 2018).

Table 3.2: IC_{50} values of methanol extracts and compounds from *R. digitata* (RD) and *R. tomentosa* (RT)

Extracts/compounds	**DPPH (µg mL^{-1})**	**FRAP (µg mL^{-1})**	**Phosphomolybdate (µg mL^{-1})**
AA	5.539 ± 0.43	142.85 ± 1.00	154.75 ± 0.95
BHT	4.672 ± 0.35	205.16 ± 0.70	154.804 ± 0.95
Roots (RD)	0.296 ± 0.20	156.991 ± 0.91	204.195 ± 0.74
Fruits (RD)	5.597 ± 0.19	530.797 ± 0.28	435.198 ± 0-.36
Roots **(**RT**)**	4.907 ± 0.34	158.209 ± 0.92	300.315 ± 0.48
Leaves **(**RT**)**	5.535 ± 0.35	269.6065 ± 0.54	365.575 ± 0.22
Fruits **(**RT**)**	9.327 ± 0.11	656.242 ± 0.22	684.903 ± 0.42
(+)-Catechin	4.037 ± 0.36	148.48 ± 0.97	143.828 ± 0.99
Quercetin	1.88 ± 0.30	147.545 ± 0.97	146.947 ± 0.96
Quercetrin	1.261 ± 0.15	147.97 ± 0.96	137.52 ± 1.03
Aromadendrin	12.661 ± 0.09	698.22 ± 0.22	698.286 ± 0.27

3.4 Conclusion

To the best of our knowledge, this is the first report on the isolation of chemical constituents from *R. digitata* and *R. tomentosa*. Findings from this study confirmed *R. digitata* and *R. tomentosa* to be rich in flavonoids and terpenoids that could be responsible for the antioxidant potential of these plants, which may be used in the prevention or treatment of degenerative diseases resulting from oxidative stress.

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CHAPTER 4

ELEMENTAL COMPOSITION AND NUTRITIONAL VALUE OF *R. DIGITATA* AND *R. TOMENTOSA*

4.1 Abstract

Rhoicissus digitata and *Rhoicissus tomentosa* (Vitaceae) are indigenous medicinal plant species found in South Africa and their fruits are consumed by the local people. In this study, the elemental distribution and nutritional value of the roots, leaves, and fruits of *R. digitata* and *R. tomentosa* were investigated. These *Rhoicissus* species were found to be rich sources of essential nutrients, with concentrations of elements in decreasing order of Ca > Mg > Fe > Mn > Cu >Zn > As > Se. *R. digitata* and *R. tomentosa* were found to be rich in Fe, with concentrations in the leaves being 904 mg kg^{-1} and 2445 mg kg^{-1}, respectively. The study confirms that these plants are suitable for human consumption with low levels of toxic elements.

Keywords: Elemental distribution, nutritional value, toxicity, medicinal plant

4.2 Introduction

Plants have been used as source of food and medicine since time immemorial. The medicinal properties of plants are mainly due to the presence of bioactive secondary metabolites and organic nutrients (Sapam *et al.*, 2018; Yadav & Agarwala, 2011). The nutritional properties of plants are mainly due to inorganic nutrients such as essential elements (Asfaw & Tadesse, 2001). Bosman *et al* (2011) reported that frequent consumption of plants decreased the risk of nutrition-related diseases. Food and medicinal plants are normally assumed to be safe for human consumption as plants have a long history of usage as food or medicine.

However, plants accumulate heavy metals from the environment, and poses a risk to human health if consumed for either nutritional or medicinal purposes. At low levels, some toxic metals can be carcinogenic or mutagenic to the human body (Moodley *et al*., 2012). At high levels, essential metals can also be toxic to the human body. Environmental and soil contamination by heavy metals is as a result of human activities (anthropogenic sources) such as agricultural, industrial, improper waste disposal, automobile emissions and mining (Bidar *et al.*, 2009). Pollution by metals is a major concern due to their toxicity, non-biodegradability, and negative impact on the environment, which is a risk to human health (Bidar *et al.*, 2007).

The family Vitaceae (commonly known as grape family) comprises plants which are useful in the production of wine, medicine and perfumery (Najmaddin *et al.*, 2011). Vitaceae plants, including *Rhoicissus* species, are popular in traditional medicine for their use in the preparation of herbal tonics such as *isihlambezo* (in isiZulu), which is taken by pregnant women in their last trimester of pregnancy to facilitate labour (Brookes & Katsoulis, 2006). *Rhoicissus digitata* and *Rhoicissus tomentosa* (Vitaceae), commonly known as *isiNwazi* (in isiZulu), are climbing

shrubs native to South Africa. The fruits of these plants are eaten by the local people and are used to make jam, raisins, perfume, vinegar and wine (Notten, 2004). The nutritional value of the fruits of *R. digitata* have previously been studied (Mlambo *et al*., 2016). This study showed good essential elements (such as Se, Cu and Mn) from the fruits of *R. digitata*. However, the nutritional value of the roots and leaves (that are ingested for medicinal purposes) have not been reported.

The elemental composition of the fruits of *R. tomentosa* (that are eaten), and roots and leaves (that are used medicinally), have not been studied. Therefore, the aim of this study was to determine the concentration of essential elements in the fruits, leaves and roots of *R. digitata* and *R. tomentosa* found in KwaZulu-Natal, South Africa, in order to assess their nutritional value and potential toxicities, if consumed.

4.3 Experimental

4.3.1 Sample collection

The roots, leaves and fruits of *Rhoicissus digitata* were collected from Suncoast, Durban beach, Durban (29.794^{o} S, 31.563^{0} E) in August 2017 and the samples of *Rhoicissus tomentosa* were collected in December 2017 from Varsity Drive, Durban (31.254^{0} S, 31.465^{0} E).

4.3.2 Sample preparation

The plant material was washed and rinsed with double distilled water, and then dried in the oven at 40 ^{0}C, for five days. Dried samples were powdered using a mortar and pestle, transferred into polyethylene bags, and placed in refrigerator until analysed. About 100 g of unwashed

roots, 10 g of leaves and 20 g of unwashed fruits were separately weighed and dried to constant weight in order to determine their moisture content.

4.3.3 Reagents and standards

The solvents used in the experiments were of analytical reagent grade and were obtained from Merck (Kenilworth, USA) and Sigma Aldrich (St. Louis, USA). All glassware and plastic bottles were washed with 3 M HNO_3, rinsed with double distilled water, and air dried to prevent contamination.

4.3.4 Sample digestion and elemental analysis

Sample digestion was achieved with the aid of the CEM microwave Accelerated Reaction System (MARS 6, CEM Corporation, Matthews, NC, USA) with MARSXpress™ vessels and infrared temperature sensor. The MARSXpress™ vessels contain liners, caps, and sleeves with a self-regulating pressure control. The powdered samples (0.25 g) and certified reference material (CRM0 (0.25 g) were mixed with 10 mL of 70% HNO_3 in the microwave vessel. Each sample was pre-digested for 30 minutes before digestion by microwave. During digestion, the power was set to 100% at 1600 W and the temperature was set to 180 ^{0}C in 20 minutes. The holding and cooling time was set for 15 minutes each. The digested samples were filtered with 0.42 µL, 25 mm nylon syringe filters, transferred into 50 mL volumetric flasks, and made up to the mark using double distilled water. The solutions were stored in 50 mL polyethylene plastic bottles and kept in a refrigerator at 4 ^{0}C until elemental analysis.

The digested samples were analysed for As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn using inductively coupled plasma-optical emission spectrometer (Perkin Elmer® Optima™

5300 with Dual View, Billerica, Massachusetts, USA). Wavelengths were selected based on maximum analytical performance and minimum spectral interferences. Analytical wavelengths that produced accurate readings for the CRM were also selected for the analysis of samples.

4.3.5 Quality assurance

Stock solutions (1 000 mg L^{-1}) (Fluka Analytical, Sigma, Switzerland) were used to prepare working standards in double distilled water and these were used to prepare the calibration curves. Validation of the analytical method was performed using the CRM, strawberry leaves (LGC-7162), from the Community Bureau of Reference of the Commission of the European Communities, Brussels, Belgium. The experimental data obtained from the CRM was compared to corresponding certified values for each element (Table 4.1). Measured values were comparable to those certified therefore the analytical method was accepted.

Table 4.1: Comparison of experimental values obtained for the certified reference material (strawberry leaves – LGC 7162) (mean ± SD, n=3) to certified values

Element	Wavelength (nm)	IDL (mg kg^{-1})	Measured Concentration (mg kg^{-1})	Certified Value (mg kg^{-1})
As	193.69	0.083	0.19 (0.05)	0.28 (0.07)
Ca	317.93	0.010	14098 (556)	15300 (700)
Cr	267.72	0.0071	1.56 (0.38)	2.15 (0.34)
Cu	324.75	0.0054	9.76 (0.28)	10
Fe	259.93	0.0062	759 (12)	818 (48)
Pb	220.35	0.042	1.1 (0.3)	1.8 (0.4)
Mg	279.07	0.0016	3902 (46)	3770 (170)
Mn	257.61	0.0014	197 (14)	171 (10)
Ni	231.60	0.048	1.8 (0.4)	2.6 (0.7)
Zn	206.20	0.0018	28 (1)	24 (5)

4.3.6 Statistical analysis

Statistical analysis was performed using one-way ANOVA and the Statistical Package for the Social Science (PASW Statistical 24, IBM Corporation, Cornell, New York).

4.4 Results and Discussion

4.4.1 Moisture content

The moisture content was found to be 73% in the roots, 55.1% in the leaves and 78% in the fruits of *R. digitata* and 74% in roots, 50% in the leaves and 61.5% in the fruits of *R. tomentosa*.

4.4.2 Elemental analysis

Table 4.2 shows the metal concentrations determined in the roots, leaves and fruits of *R. digitata* and *R. tomentosa*. Arsenic, Ca, Cu, Mg, and Fe were detected in all samples, while Zn was only detected in the leaves of both plants. Low Zn concentrations in plants could be as a result of low concentration of Zn in the soil. Neutral and alkaline soil that is exposed to phosphorus fertilizers have been reported to have low concentrations of Zn (Marschner, 1993). Previous analyses on the fruits of *R. digitata* indicated the presence of Cr, Mn and Zn (Mlambo *et al.*, 2016). These elements were not detected in the fruits in the current study; however, Mn was detected in the leaves and roots of *R. digitata*. The presence of higher concentrations of heavy metals in the leaves was not surprising as several studies have reported plants to have a tendency for accumulating higher amounts of heavy metals in the shoots (Soetan *et al*., 2010). This is because some heavy metals are easily stored in the shoots compared to the roots whilst some elements are required for certain metabolic processes in the shoots (Rascio & Navari-Izzo, 2011).

Table 4.2: Elemental concentrations (mg kg^{-1}) in different plant parts of *R. digitata* and *R. tomentosa*

Element	*R. digitata* (mg kg^{-1})			*R. tomentosa* (mg kg^{-1})		
	Roots	**Leaves**	**Fruits**	**Roots**	**Leaves**	**Fruits**
As	12.3 (0.06)	14.3 (0.06)	12.5 (0.06)	16.3 (0.01)	7.3 (0.01)	7.1 (0.01)
Ca	22893 (1.6)	43000 (0.9)	17334 (0.7)	24193 (2.4)	75246 (3.7)	8286 (0.3)
Cu	59 (0.001)	59 (0.002)	65 (0.006)	61 (0.158)	64 (0.002)	61(0.001)
Mg	4351 (0.2)	10917 (0.3)	3395 (0.3)	3865 (0.2)	6837 (0.1)	2188 (0.1)
Mn	5 (0.01)	151 (0.01)	ND	ND	245 (0.01)	ND
Fe	686 (0.06)	904 (0.04)	3.5 (0.01)	566 (0.05)	2445 (0.03)	80 (0.01)
Se	ND	2.7 (0.01)	2	2	9.2 (0.04)	ND
Zn	ND	43 (0.007)	ND	ND	30 (0.002)	ND

ND: not determined

The concentrations of elements were found to be in a decreasing order of Ca > Mg > Cu > Fe > As > Se in the fruits; Ca > Mg > Cu > Fe > As > Mn > Se in the roots, and Ca > Mg > Fe > Cu > Zn > Mn > As > Se in the leaves.

Calcium is an essential macro-nutrient required for the formation and maintenance of bones and for normal functioning of nerves and muscles (Soetan *et al.*, 2010). Magnesium is a constituent of bones and teeth, and is important in activating enzymes. Its deficiency causes various diseases, such as cardiovascular diseases, metabolic syndrome and diabetes mellitus type II (Bohn & Science, 2008; Soetan *et al.*, 2010). Calcium concentrations were found to be highest in *R. digitata* and *R. tomentosa* samples (8286 to 75246 mg kg^{-1}) with the leaves of *R. tomentosa* being richer in Ca than those of *R. digitata*. All samples of *R. digitata* and *R. tomentosa* were found to be rich in Mg with leaves of both plants having higher Mg content

than the roots and fruits. The high concentrations of Ca and Mg found in these plant species are in agreement with previous studies that show plants to accumulate high concentrations of these metals to meet their physiological requirement levels compared to other metals (Bohn & Science, 2008).

Iron is a micronutrient essential for the formation of red blood cells and transportation of oxygen to body cells (Soetan *et al.*, 2010; Srai & Sharp, 2012). The leaves of both plant species were found to be rich in Fe however, the fruits contained relatively low concentrations (3.5 to 80 mg kg^{-1}). Although Fe is an essential element required for the prevention of diseases such as anaemia, if in excess, it is highly toxic and can lead to cell or organ damage. It has been reported that a third of the world's population suffers from Fe deficiency disorders (Srai & Sharp, 2012). The results from this study suggest that the leaves of *Rhoicissus* species may be used for Fe supplementation.

Toxic elements, Pb and Cd, did not occur in tested plant parts of *R. digitata* and *R. tomentosa*. This may be because they occur at concentration below instrument detection limits (IDL) of 0.042 mg L^{-1} for Pb and 0.0027 mg L^{-1} for Cd. On the other hand, As was detected in all samples studied. The highest concentration of As (16.3 mg kg^{-1}) was found in the roots of *R. tomentosa*, with its fruits having the lowest concentration (7.1 mg kg^{-1}). Arsenic concentrations in terrestrial plants, under normal conditions, are usually less than 10 mg kg^{-1}, although an average of 40 mg kg^{-1} was reported in crop plants such as rice, wheat, maize, tomato and barley consumed by humans (Matschullat, 2000; Panda *et al*., 2010). Therefore, As concentrations obtained in this study fall within the reported concentration levels for similar terrestrial plants. Furthermore, terrestrial plants are reported to accumulate lower levels of the toxic inorganic form of As compared to marine organisms (Authority, 2014; Magura, 2015).

Crop plants, such as rice are grown in paddy soil and they accumulate high levels of As due to exposure to As-contaminated groundwater, which is used for irrigation. The use of As-contaminated groundwater in Bangladesh increased the concentration of As in paddy soil from 57 µg kg^{-1} to 83 µg kg^{-1}, which indicates that groundwater is one of the sources of As contamination in the soil (Alam & Sattar, 2000; Ullah, 1998). It is also reported that the more toxic inorganic form (As(III)) may predominate in paddy soil under anaerobic conditions, while under aerobic conditions, As(V) dominates. Both plants in the present study grow in the wild under aerobic conditions, therefore, the major form of As in this soil can be assumed to be As(V).

Most of the samples in this study (except the leaves and fruits of *R. tomentosa*) showed As concentration of more than 10 mg kg^{-1} but less than 20 mg kg^{-1}. It can be assumed that these plants are safe for human consumption as the levels of As are lower than those reported for some well-known edible plants. Since high concentrations of As were found in the roots of both plants, it is possible that plant uptake from soil is the main source of As contamination. Atmospheric deposition on aerial parts of plants could also result in As contamination of the leaves and fruits. Excessive consumption of As contaminated plants may pose a risk to human health which include liver and kidney malfunction, skin cancer, lung cancer and bladder infections (FAO, 2011; Kabata-Pendias & Mukherjee, 2007; Saha *et al.*, 1999).

Copper concentrations in all plant parts were within a small range of variation (59 mg kg^{-1} to 65 mg kg^{-1}). Copper is useful in maintaining the strength of blood vessels, skin, connective tissue and epithelial cells in the body and for normal functioning of the thyroid gland (Hung *et al.*, 2016; Uauy *et al*., 1998). Excess Cu intake can cause oxidative stress (Gaetke & Chow, 2003).

Manganese is an essential element involved in the formation of bones (Murray, 2000). Manganese was only detected in the roots (5 mg kg^{-1}) and leaves (151 mg kg^{-1}) of *R. digitata* and leaves (245 mg kg^{-1}) of *R. tomentosa*. An excess of Mn in the diet may cause liver, lung, and cardiovascular diseases while its deficiency may result in skin lesions and bone malformation (Crossgrove & Zheng, 2004).

Selenium protects living organisms against the harmful action of free radicals (Soetan *et al.*, 2010). Selenium was found in the leaves and fruits of *R. digitata*, and in the roots and leaves of *R. tomentosa*. A study on the relationship between soil and plant Se showed that the concentration of Se in the plant is dependent on the plant species and content of Se in the soil. A excess of Se in the diet causes hair loss, skin discoloration, nail abnormities and aesthesia (Zhao *et al.*, 2005). Its deficiency results in malfunction of reproductive organs in human (Mehdi *et al.*, 2013).

4.4.3 Elemental contribution of *R. digitata* and *R. tomentosa* to the human diet

The ripe fruits of *R. digitata* and *R. tomentosa* are eaten by the local people or used to make jam in South Africa. The elemental concentrations of 10 g (dry mass, average serving size) of the fruits were compared to dietary reference intakes (DRIs) for the essential elements (Table 4.3). The elemental concentrations of 10 g (dry mass, average amount ingested from herbal decoctions or infusions) of the roots and leaves of both plants were also compared to DRIs due to their use in traditional medicine. Elemental concentrations above tolerable upper intake levels (ULs) can be considered toxic in terms of contribution to the diet.

Table 4.3: Dietary reference intake (recommended dietary allowances (RDAs) and tolerable upper intake levels (ULs)) of elements for most individuals compared to average concentration of *R. digitata* and *R. tomentosa* elements (n=3).

	DRI (mg day^{-1})		**Average concentration: mg day^{-1} (%)**					
			R. digitata			***R. tomentosa***		
Element	RDA	UL	Roots	Leaves	Fruits	Roots	Leaves	Fruits
Ca	1000	2500	229 (23)	430 (43)	173 (17)	242(24)	752(75)	83 (8.3)
Cu	0.9	8	0.58 (64)	0.58 (64)	0.65 (72)	0.61 (67)	0.63 (70)	0.61 (68)
Mg	310	350	43 (14)	109 (35)	34 (11)	39 (12)	68 (22)	22 (7)
Mn	1.6	9	0.05 (3.1)	1.51(94)	ND	ND	2.46 (154)	ND
Fe	8	45	6.85 (86)	9.03 (113)	0.03 (0.3)	5.66 (71)	24.45 (306)	0.80 (10)
Se	0.055	0.4	ND	0.027(49)	0.02 (36)	0.02 (36)	0.09 (164)	ND
Zn	8	34	ND	0.43 (5.3)	ND	ND	0.30 (37)	ND

- ND: Not determined, DRI: Dietary Reference Intake

The results show that consumption of 10 g (dry mass) of roots, leaves, and fruits of *R. digitata* may contribute 86%, 113%, and 0.3%, respectively, toward the RDA for Fe. Consumption of 10 g (dry mass) of roots, leaves, and fruits of *R. tomentosa* may contribute 71%, 306%, and 10%, toward the RDA for Fe, respectively. The results showed roots and leaves of the two species to be rich in Fe. However, the Fe content is found in plant parts that are not consumed as food. The roots and leaves of the plant species can therefore be used as supplementary medications for individuals suffering from anaemia (Emery, 1982) . Although Fe was found to be above the RDA for the element in the leaves of both species, the concentrations did not

exceed the ULs therefore, the species can be considered safe for human consumption for nutritional and medicinal purposes.

All parts of both plant species studied were found to be rich in Cu (contribution greater than 64% towards the RDA for the element). The leaves of both plant species were found to be rich in Mn (contributing 94 – 154% towards its RDA). Consumption of 10 g per day of the leaves and fruits of *R. digitata* may contribute 49% and 36%, toward the RDA for Se, while the roots and leaves of *R. tomentosa* may contribute 36% and 164%, respectively. Additionally, Se in the leaves of *R. tomentosa* do not exceed its tolerable upper intake level (UL). The plant parts rich in Se can be used medicinally to reduce prostate cancer in men as Se is used to treat this disorder (Finley, 2007). *Bertholletia excelsa* (Brazil nuts) was found to have an average concentration between 8 and 83 µg $g^{-1}$and this plants is used in tumour prevention due to this high Se content (Thomson *et al*., 2008).

The findings from this study show that consumption of 10 g (dry mass, serving size per person/ day) of the roots, leaves, and fruits of *R. digitata* result in As intake of 0.12 mg, 0.14 mg, and 0.12 mg per day. Similarly, consumption of *R. tomentosa* may result in As intake of 0.16 mg, 0.07 mg, 0.07 mg per day from the roots, leaves and fruits, respectively. Sarkar and Paul (2016) reported that an average dietary intake of As of 0.02-0.3 mg day^{-1} may be safe for the general population. Meacher et al (2002) estimated that the safe dietary intake of As for males should be 0.0018 – 0.0114 mg day^{-1} and 0.0013 – 0.0094 mg day^{-1} for females (Meacher *et al.*, 2002; Panda *et al*., 2010). The dietary intake of As in the present study is higher than these reported in literature to be safe, therefore, people consuming these plants should exercise control and limit their intake.

4.5 Conclusion

The roots, leaves, and fruits of R. *digitata* and *R. tomentosa* were analysed for 13 elements and the concentrations were generally found to be in the decreasing order of Ca > Mg >Fe > M n > Cu >Zn > As > Se. The leaves of both plants were found to be rich in macro and micro-nutrients especially Fe, Mn and Se. The plant species were also found to accumulate As in the roots, shoots and fruits therefore, consumption for medicinal or nutritional purposes should be limited especially if taken by pregnant women to facilitate labour.

4.6 References

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CHAPTER 5: GENERAL CONCLUSIONS

5.1 Overall summary and conclusions

The study focused on two *Rhoicissus* species (*Rhoicissus digitata* and *Rhoicissus tomentosa*) found in Kwazulu-Natal, South Africa that were investigated both phytochemically and analytically. The investigation was impelled by the use of these plant species in traditional medicine to treat various diseases and the consumption of the edible fruits by the local people to meet their nutritional needs. Previously, studies on the biological activities of the crude extracts from both plant species have been conducted, however, the isolation and identification of the secondary metabolites or active biomolecules have not been reported. In this study, the phytochemistry and *in vitro* antioxidant potential of the crude extracts and isolated compounds from *R. digitata* and *R. tomentosa* was investigated. The elemental composition of the *Rhoicissus* species was also determined to assess for nutritional value and potential toxic effects.

Five compounds ((+)-catechin, quercetin, 12,13-dehydrolupeol, β-sitosterol and oleanolic acid) were isolated and identified from *R. digitata*, and nine compounds (3β-taraxerol, stigmasterol, oleanolic acid, β-sitosterol, quercetrin, (+)-catechin, aromadendrin7-O-β-glycopyranoside, lutein and pheophytin a) were isolated and identified from *R. tomentosa*. The identity of these compounds were confirmed using spectroscopic techniques and by comparison with literature values. The results indicate *Rhoicissus* species to be rich in triterpenes, sterols and flavonoids.

The *in vitro* antioxidant activity of the methanol extracts and the isolated flavonoids indicated that *R. digitata* and *R. tomentosa* may be used as alternative treatments of degenerative diseases related to oxidative stress. The elemental analysis showed *R. digitata* and *R. tomentosa* to be

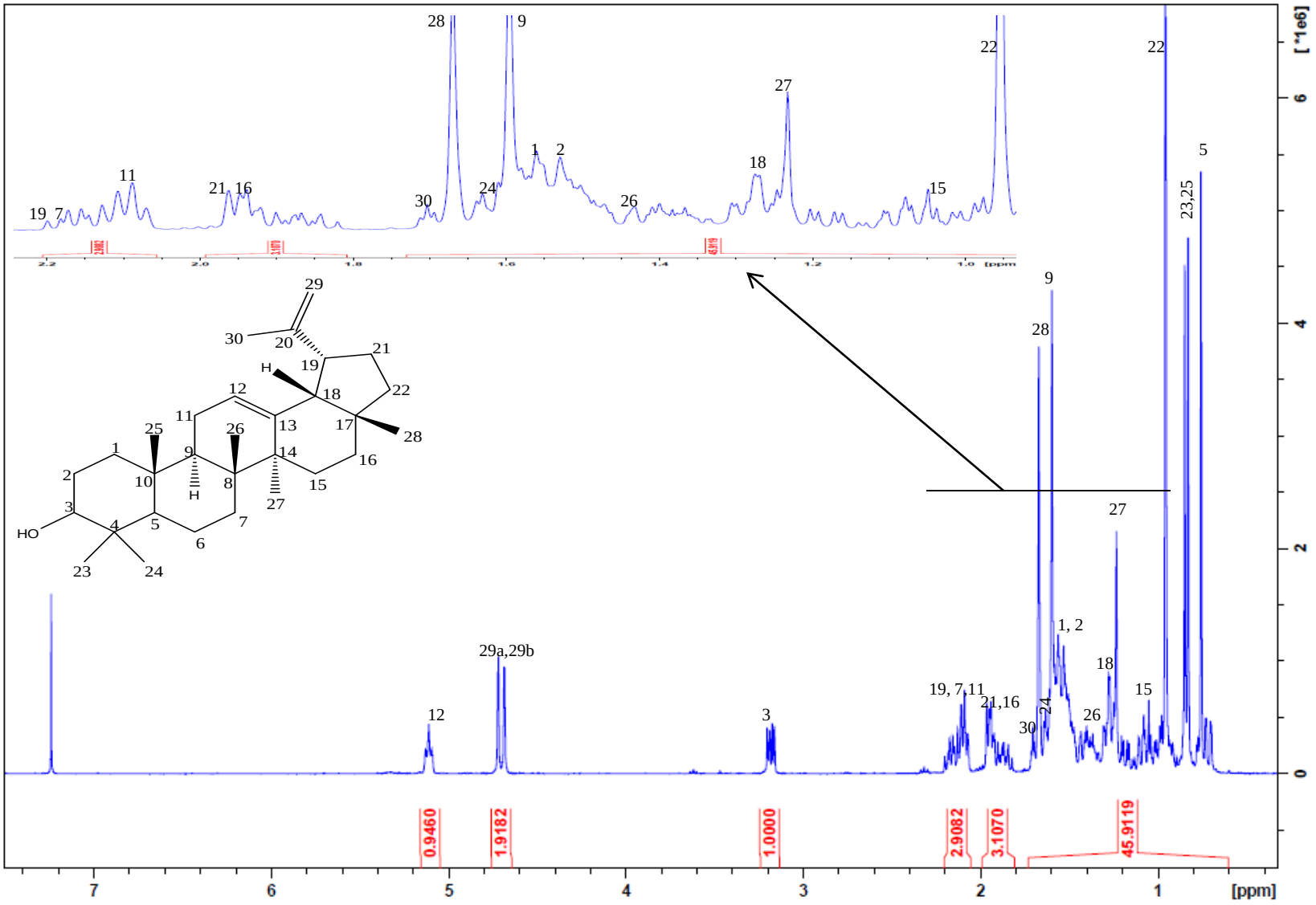
good dietary sources of essential nutrients with low concentrations of the toxic elements, Pb and Cd. However, consumption by pregnant women that use the plant medicinally to facilitate labour should be limited due to the plants potential to accumulate As. This study validates the ethno-medicinal uses of these plant species due to the presence of known bioactive molecules including triterpenes, sterols and flavonoids and provides evidence on the nutritional value of the edible fruits, which, if consumed by vulnerable communities, may contribute towards a diverse, more nutritious diet.

5.2 Recommendations for future research

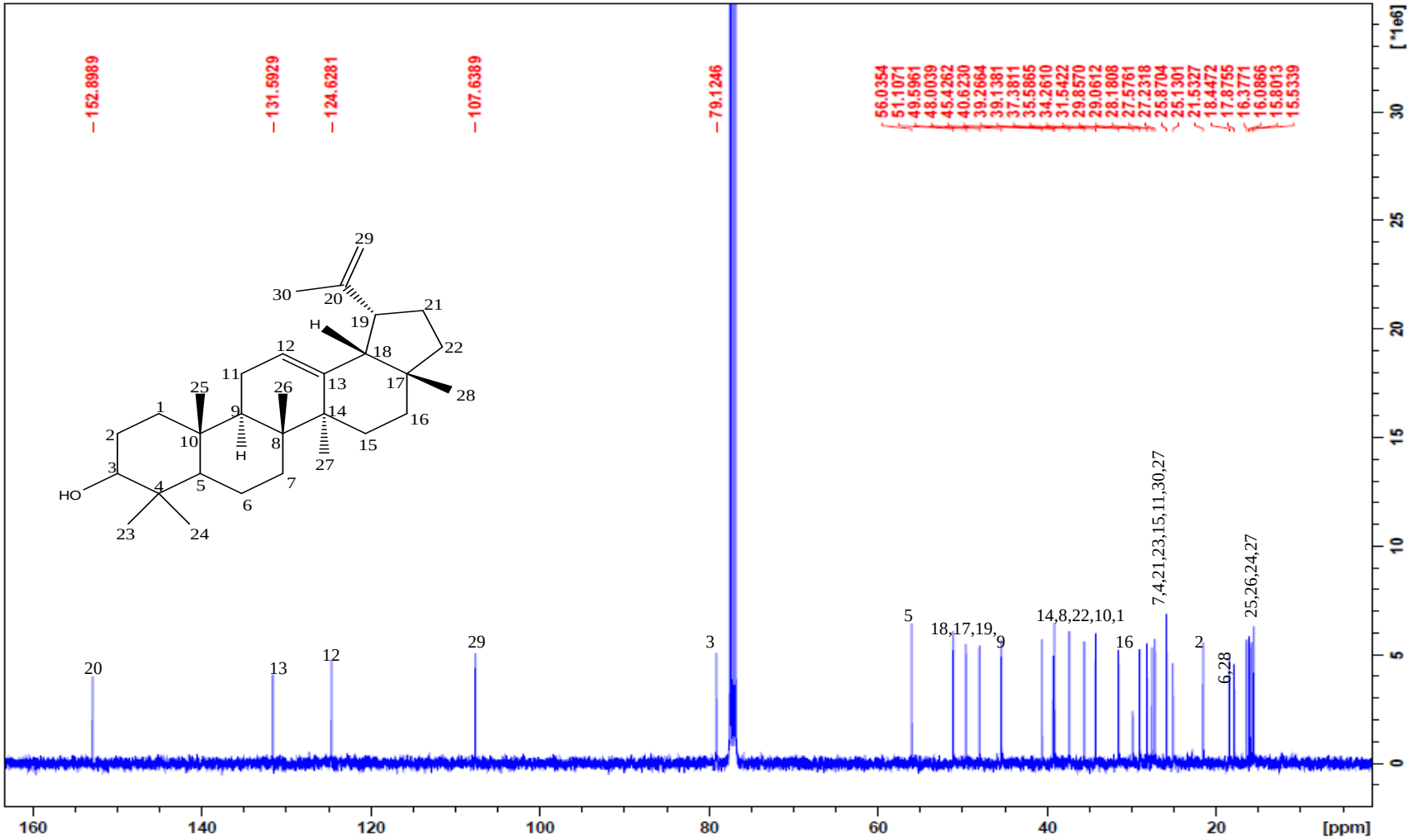
- Evaluation of other biological activities shown by the compounds isolated from *R. digitata* and *R. tomentosa*.
- Isolation, structural characterisation, and analysis of biological activities of compounds from other *Rhoicissus* species.
- Comparison of the nutritional value and toxicity of *R. digitata* and *R. tomentosa* collected from different locations in Kwazulu-Natal, as well as evaluating the impact of seasonal change in the elemental composition of these plants.
- Assessment of the nutritional value and toxicity of the crude extracts from other *Rhoicissus* plants.

APPENDIX

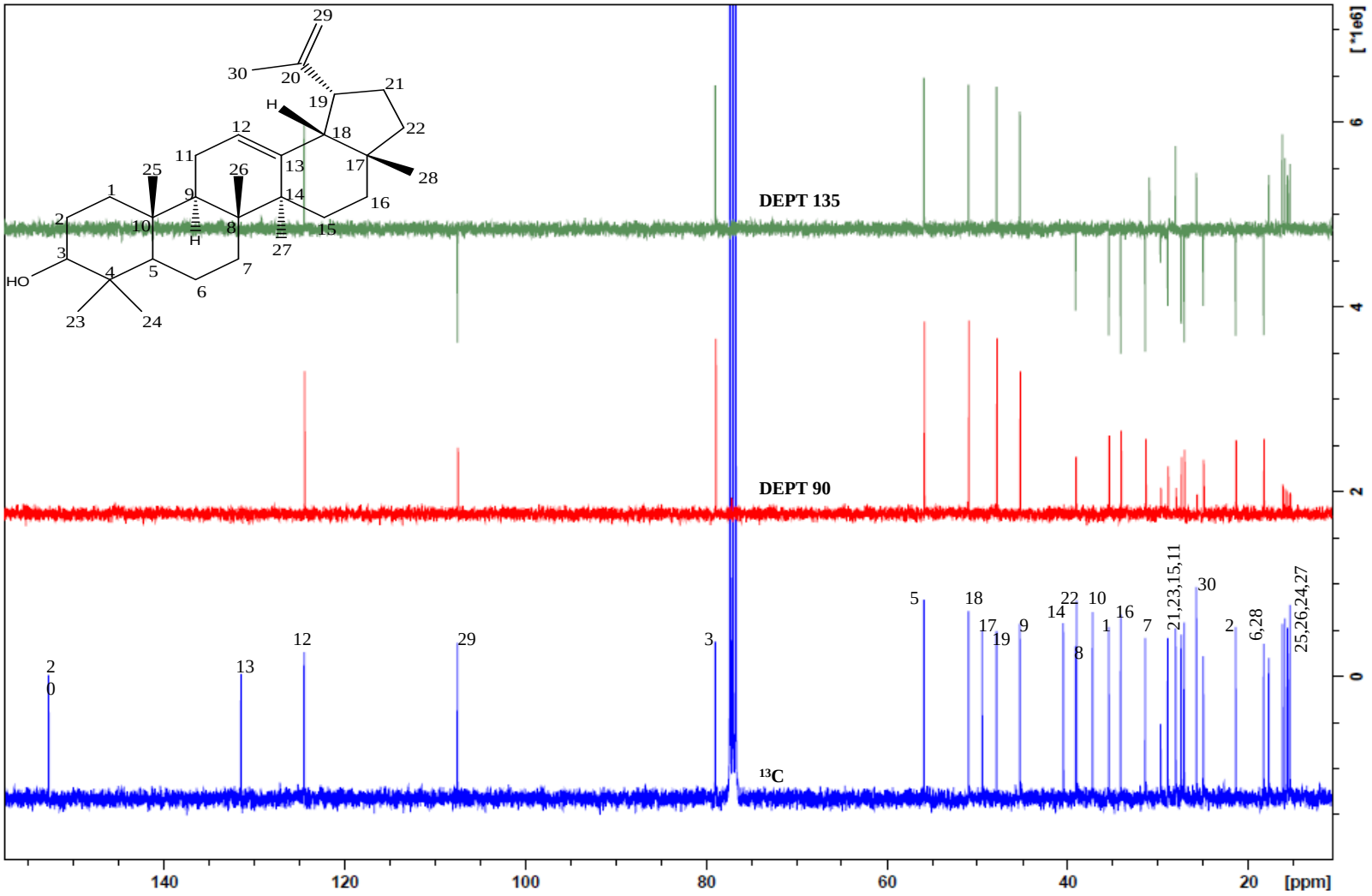
Supporting information consisting the NMR, MS, IR and UV spectra



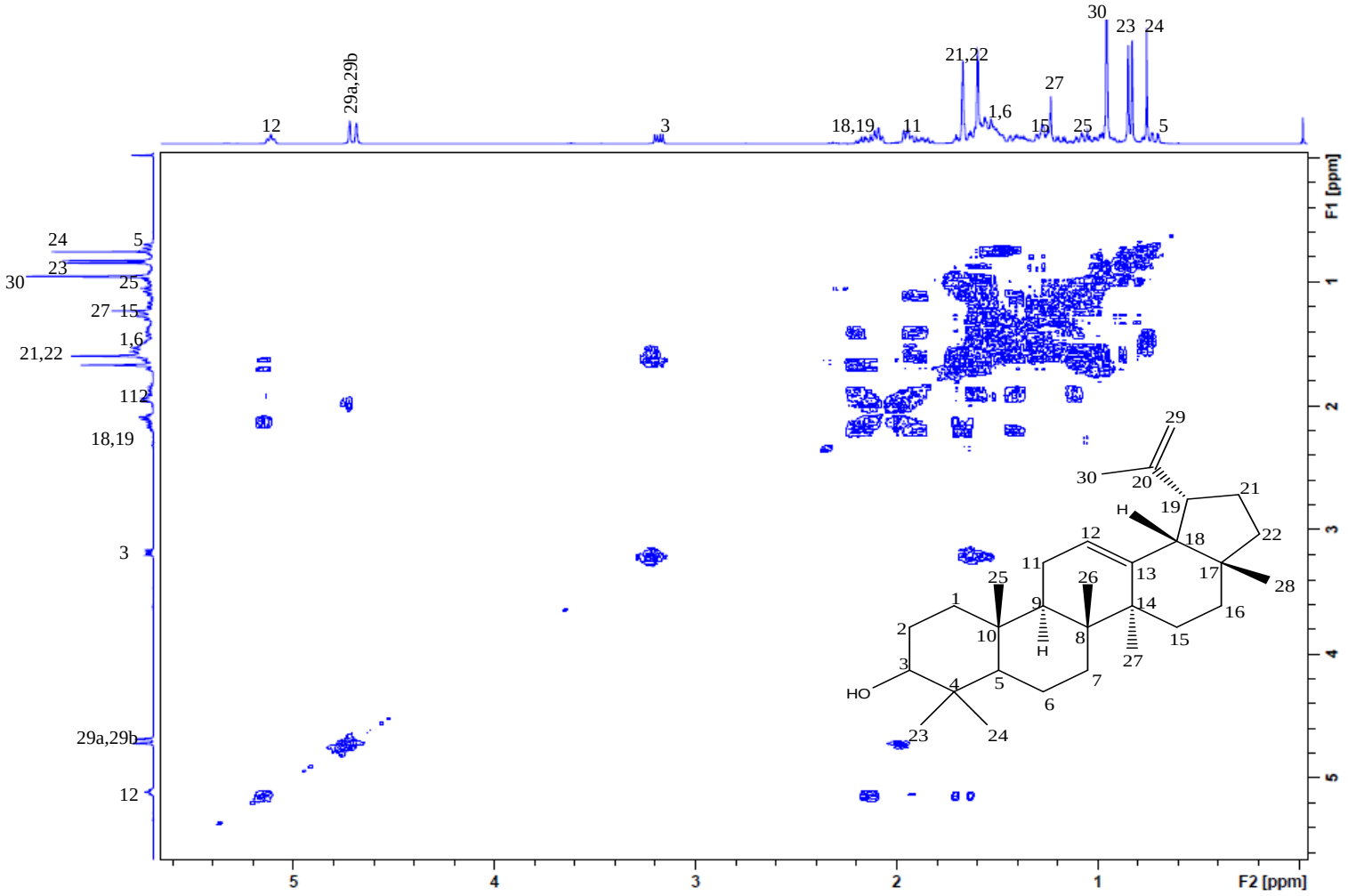
^{1}H NMR spectrum of 12,13-dehydrolupeol in $CDCL_3$



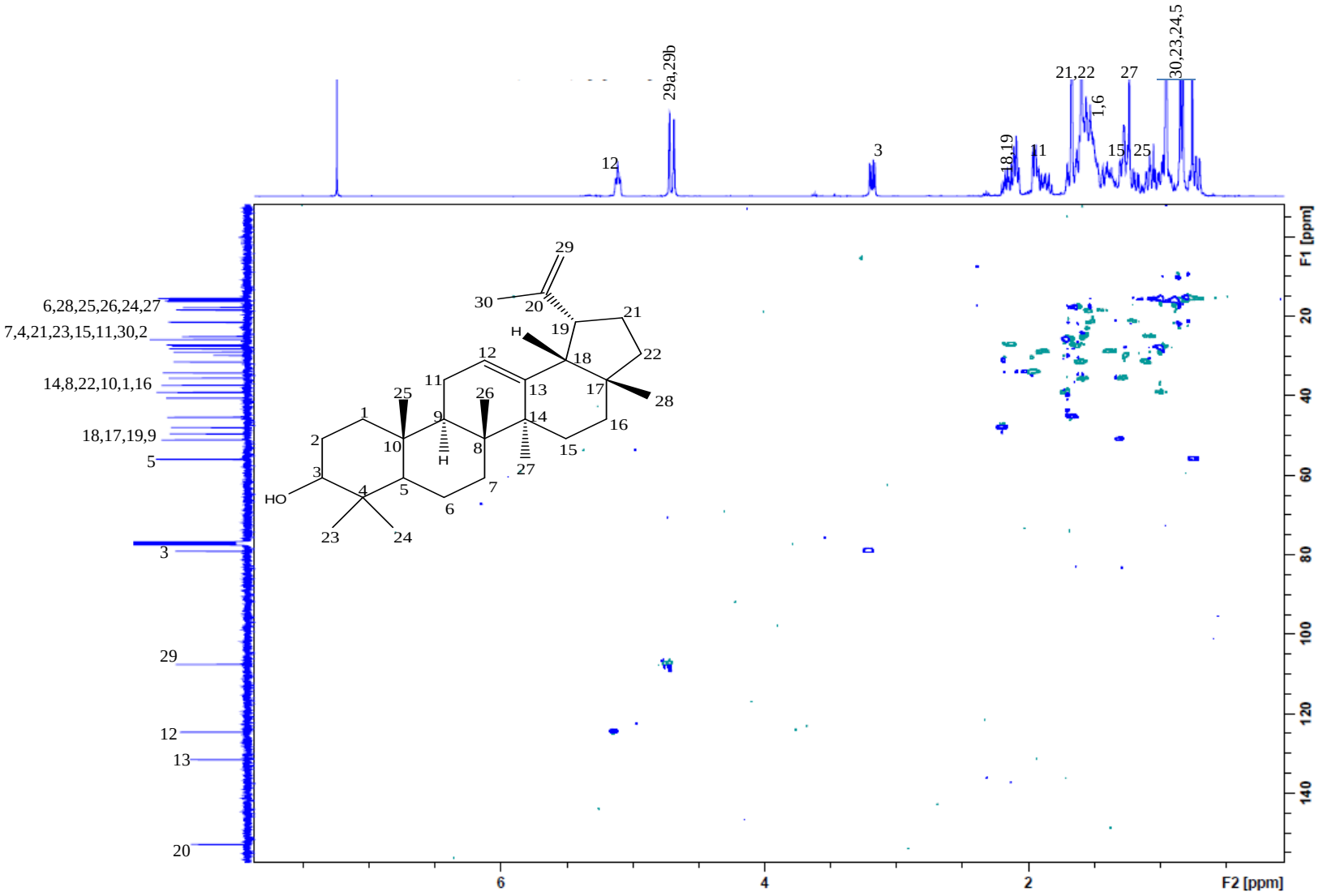
^{13}C NMR spectrum of 12,13-dehydrolupeol in $CDCL_3$



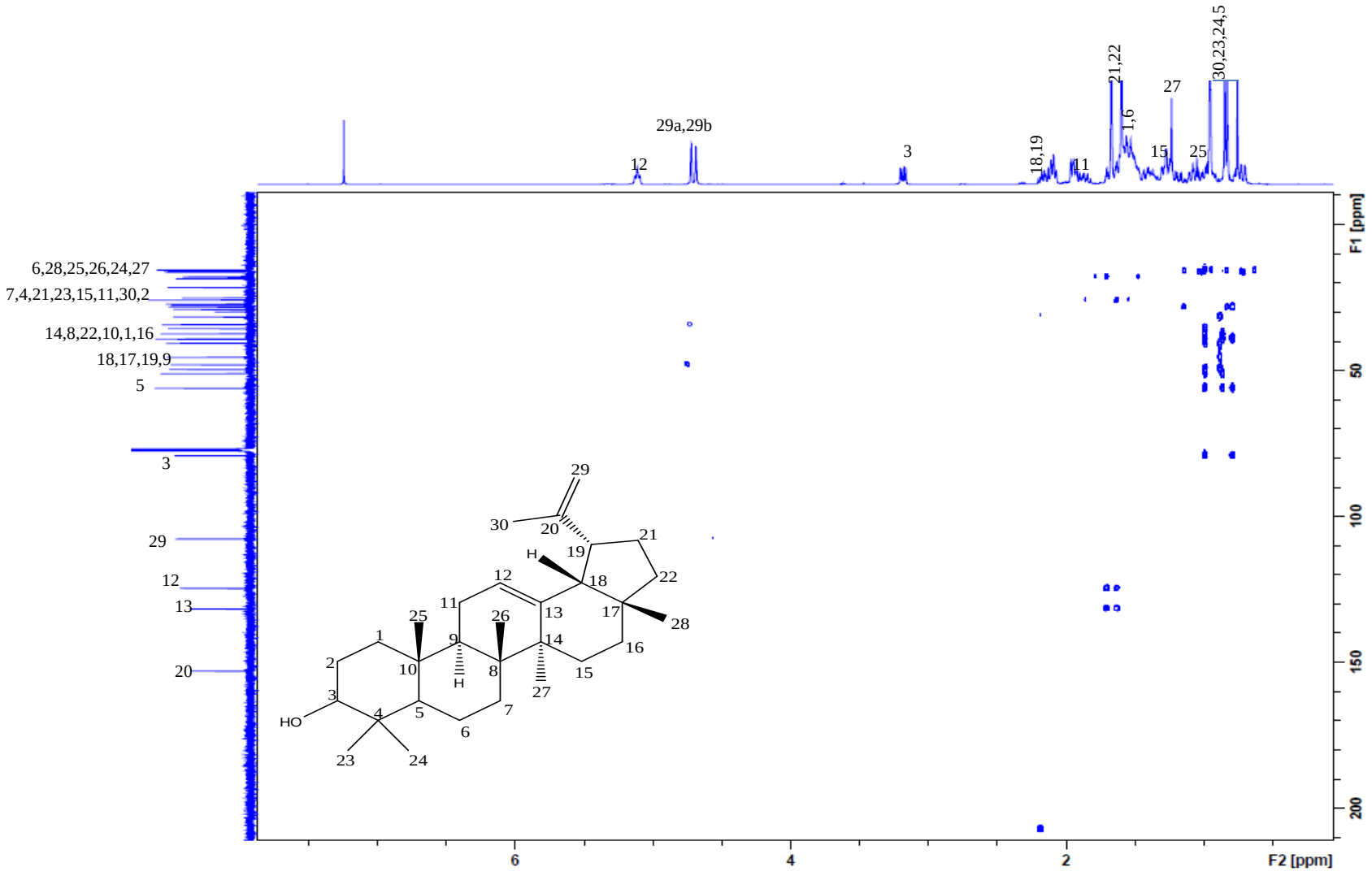
DEPT NMR spectrum of 12,13-dehydrolupeol in $CDCL_3$



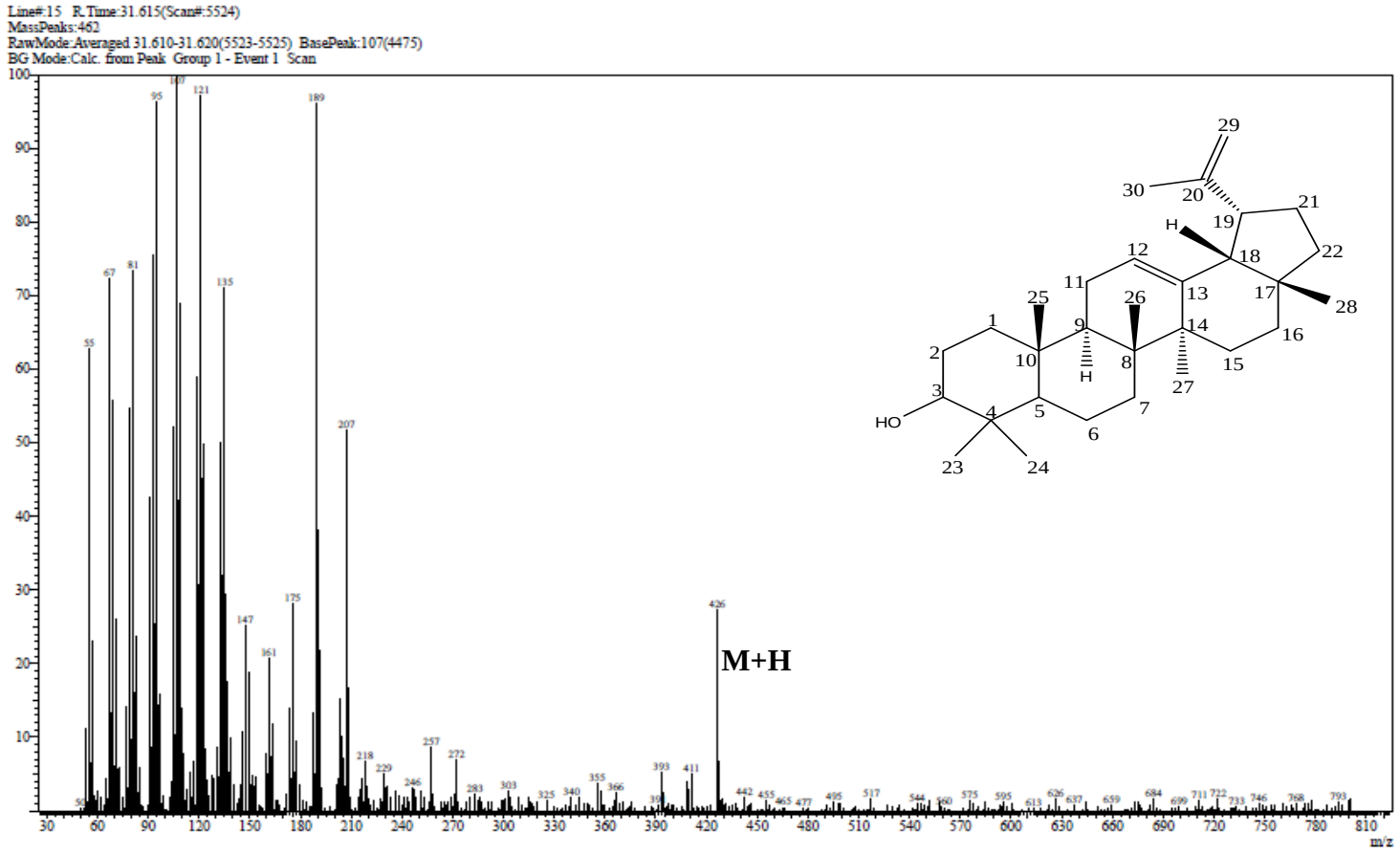
COSY NMR spectrum of 12,13-dehydrolupeol in $CDCL_3$



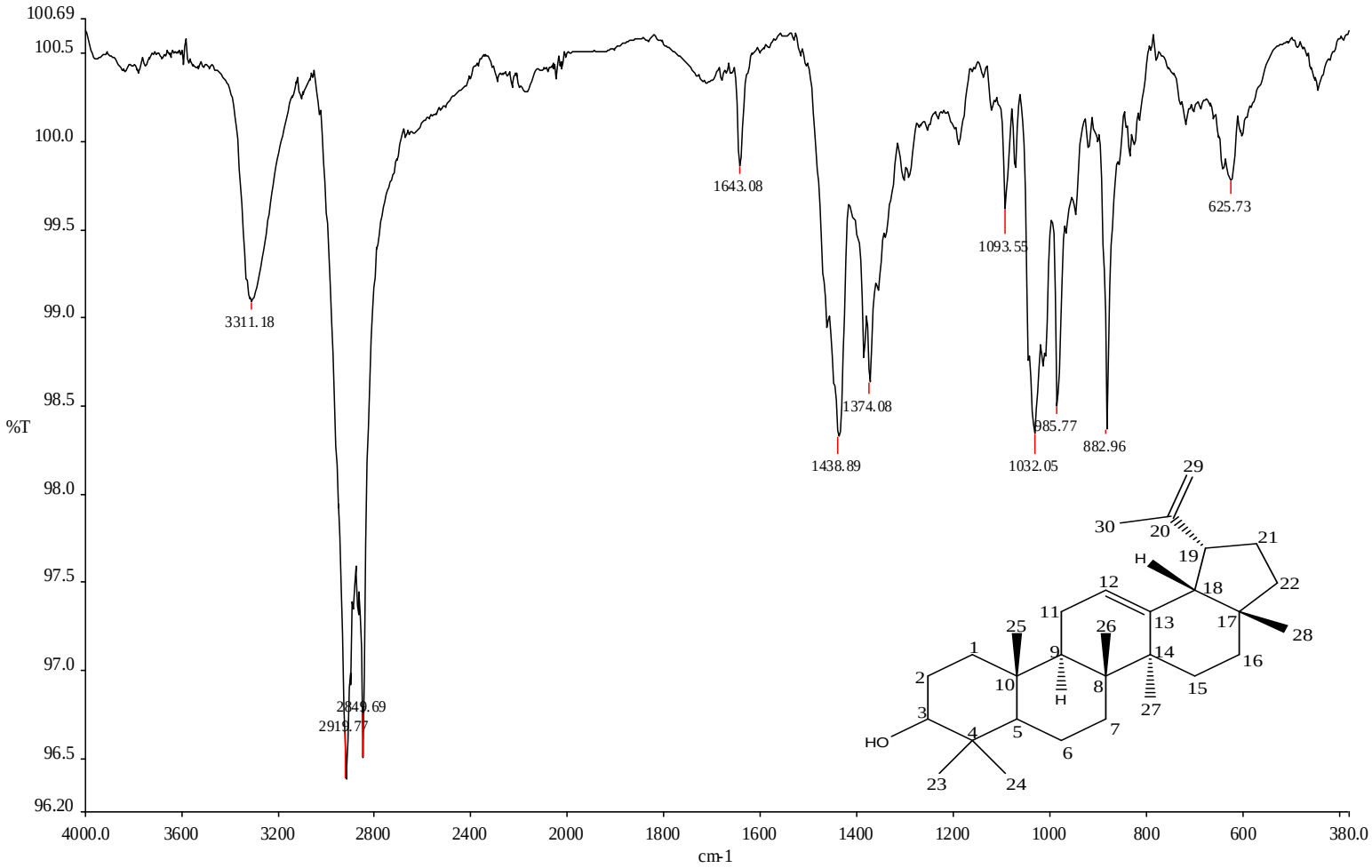
HSQC NMR spectrum of 12,13-dehydrolupeol in $CDCL_3$



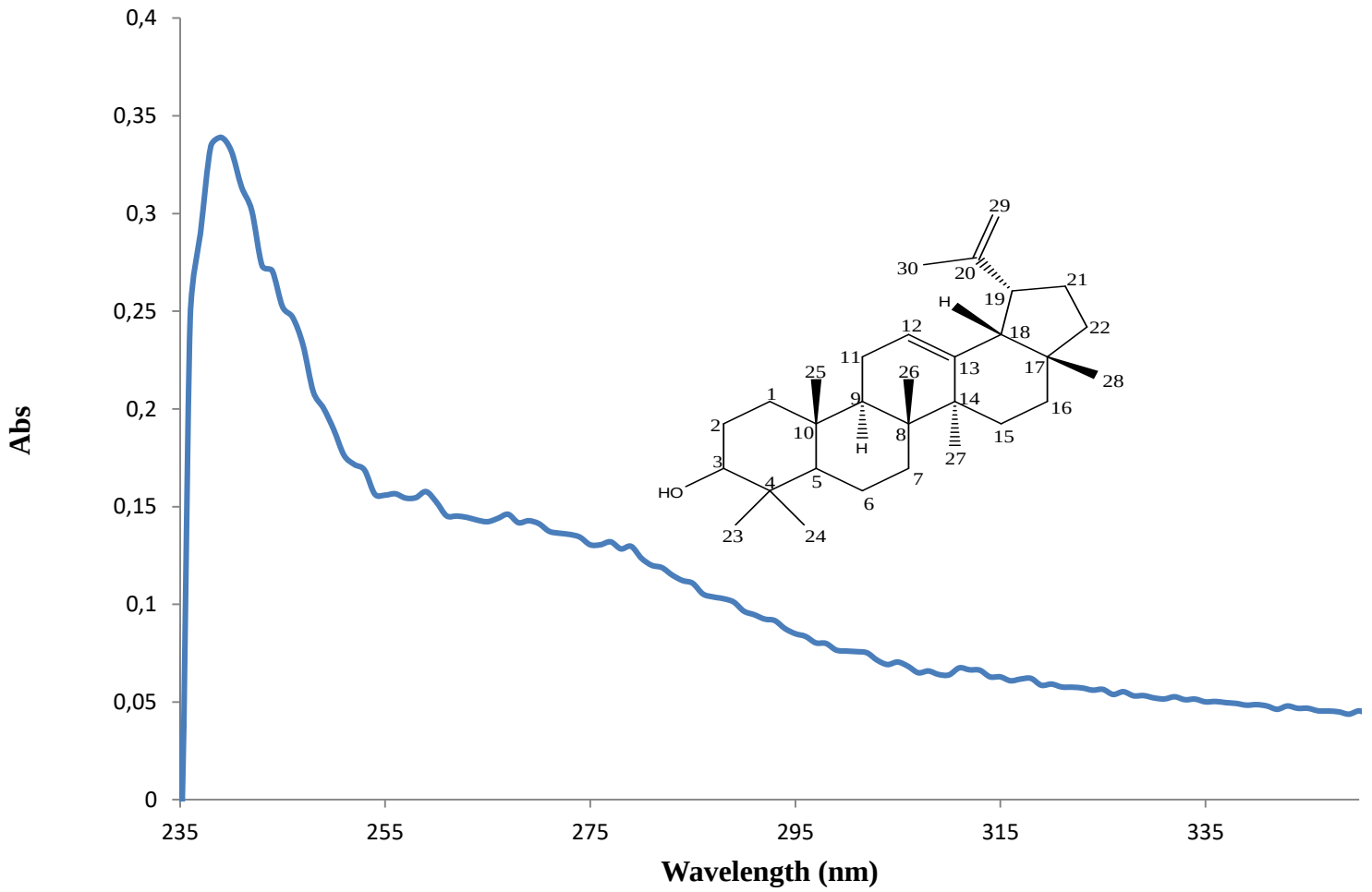
HMBC NMR spectrum of 12,13-dehydrolupeol in $CDCL_3$



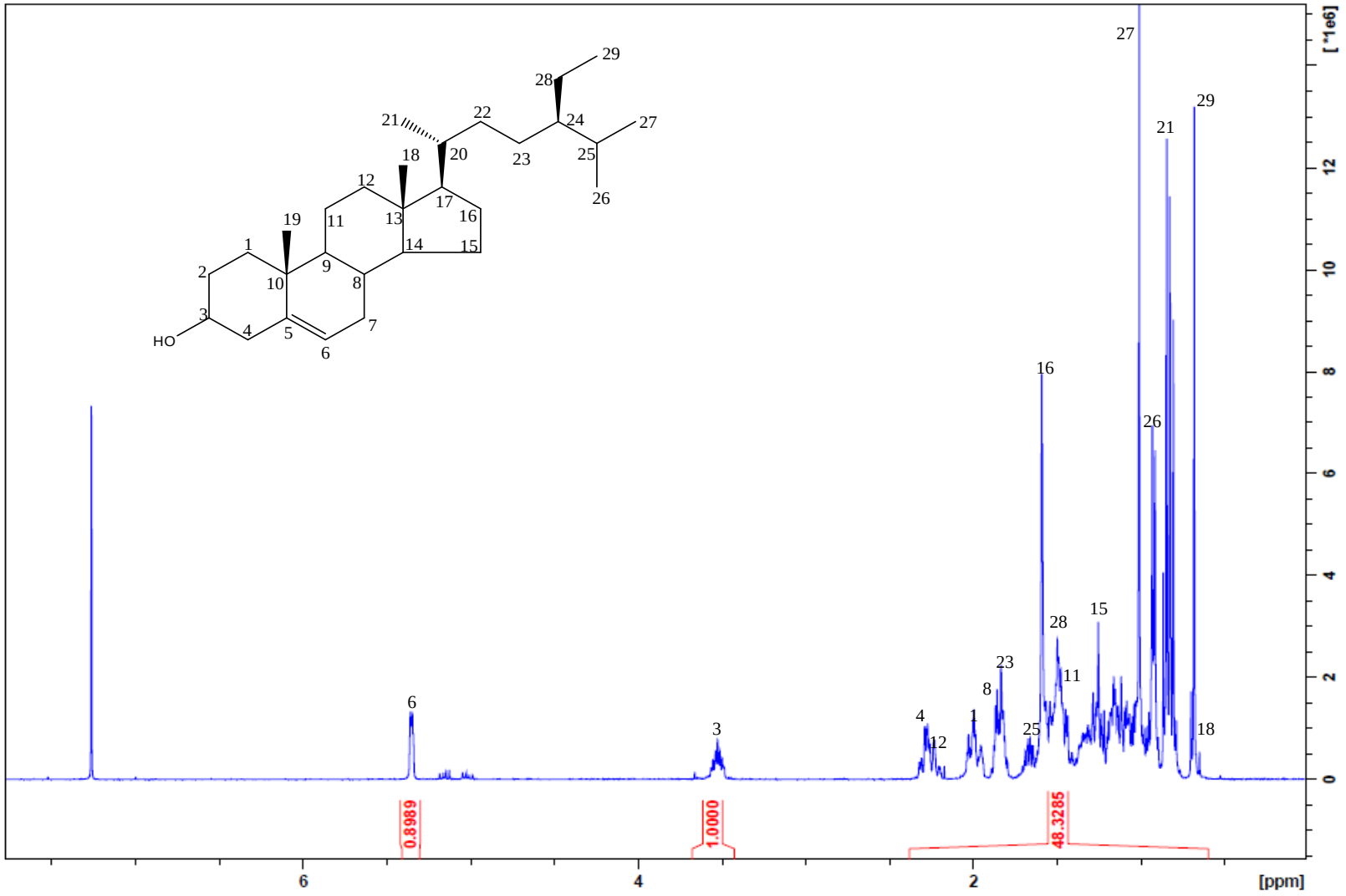
Mass spectrum of 12,13-dehydrolupeol



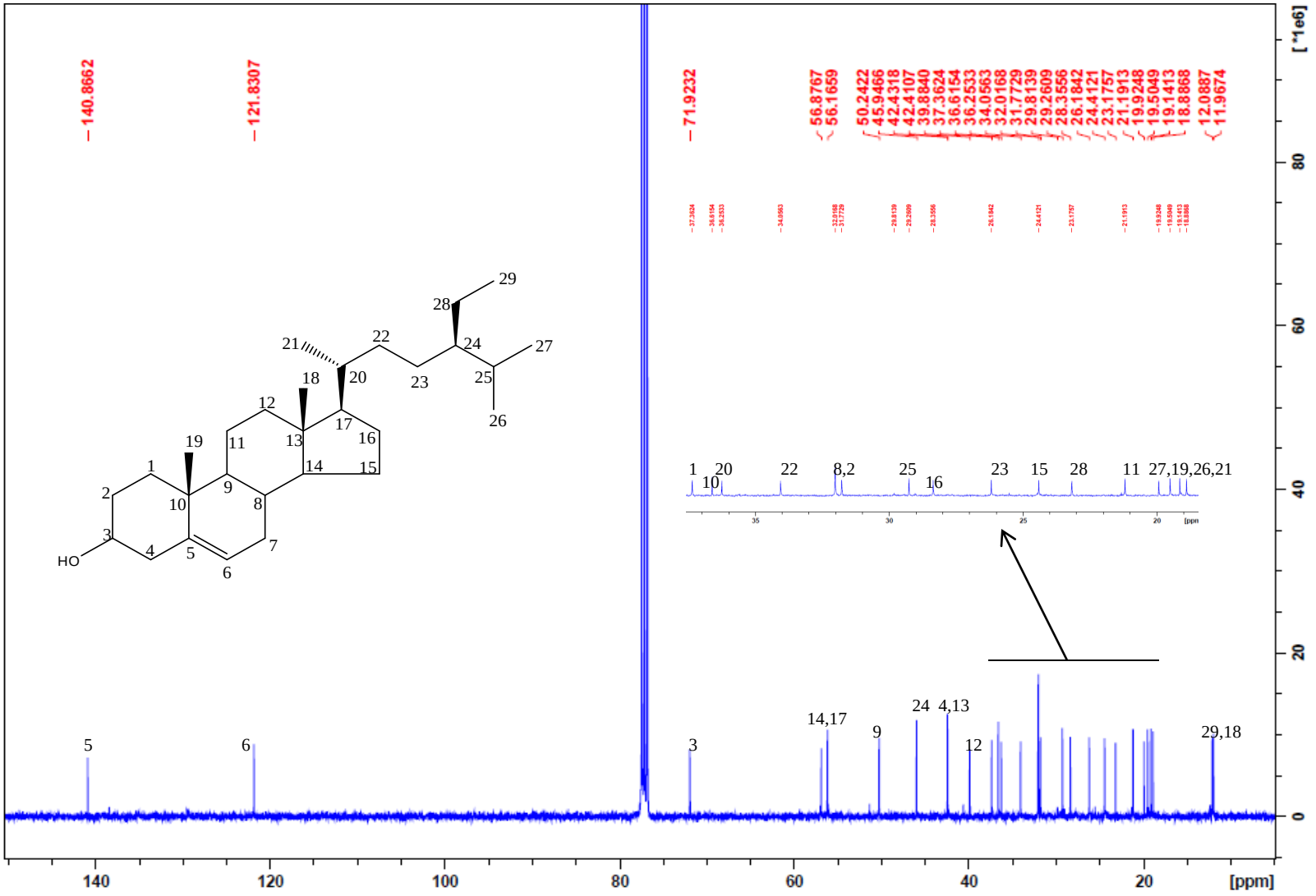
IR spectrum of 12,13-dehydrolupeol



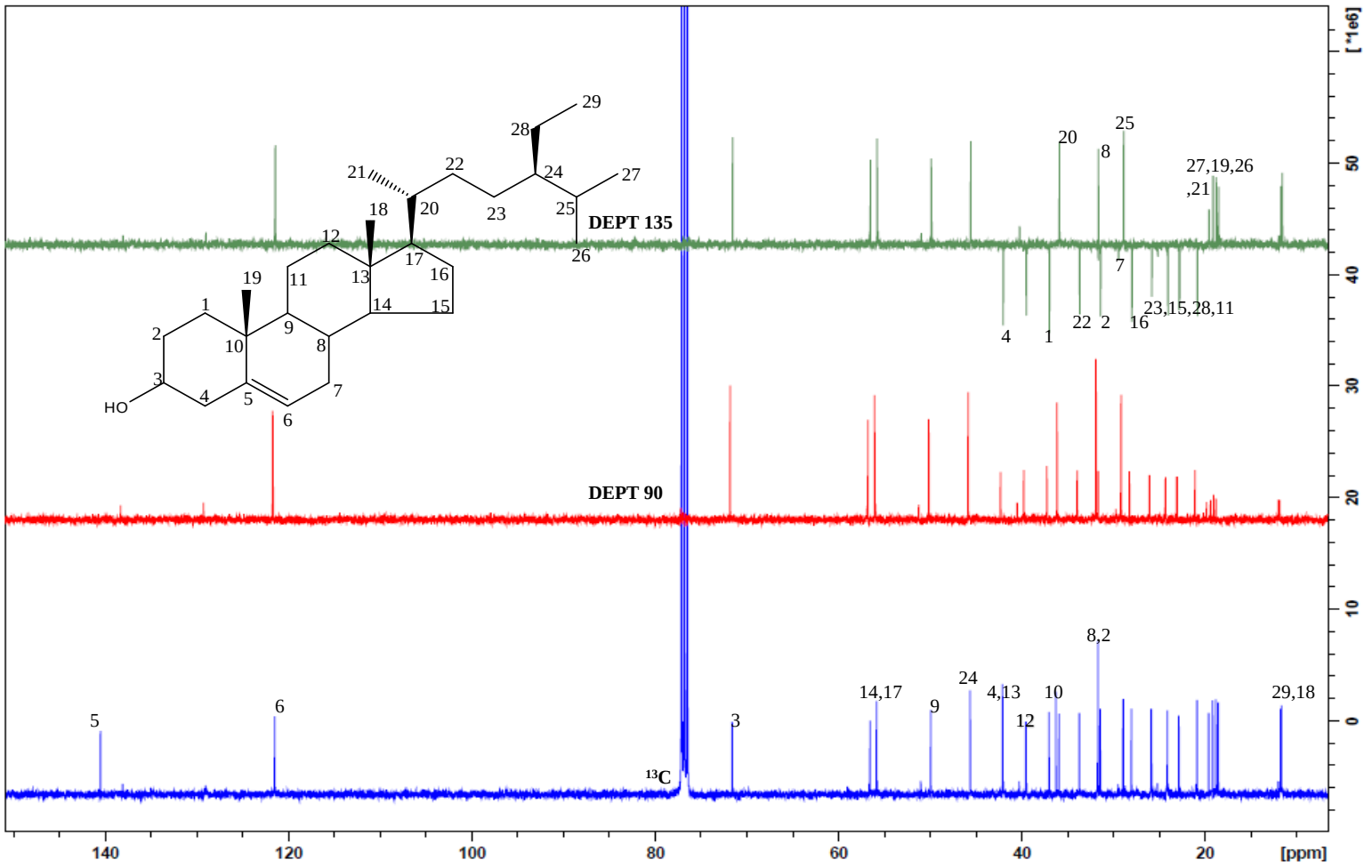
UV spectrum of 12,13-dehydrolupeol



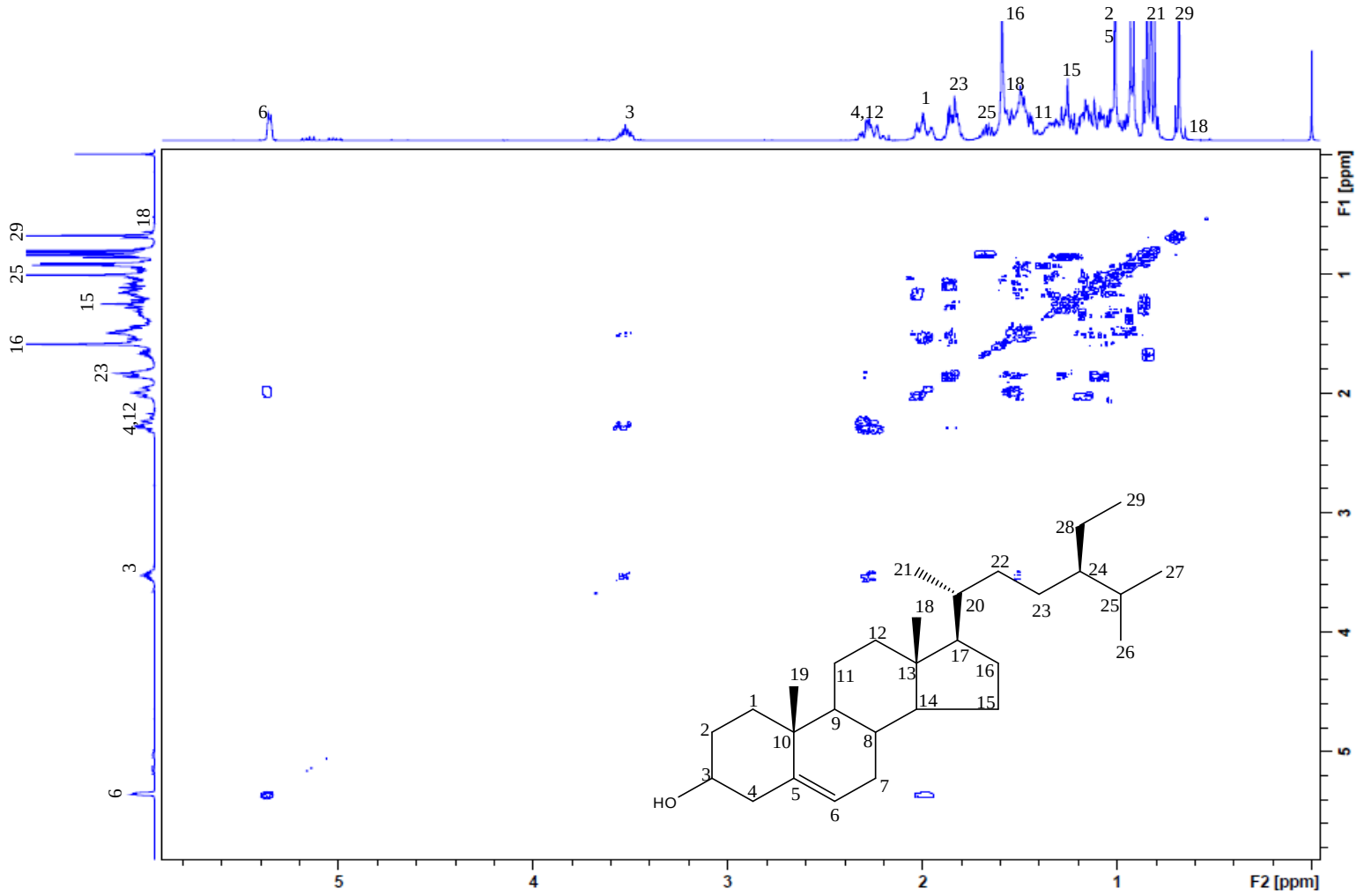
^{1}H NMR spectrum of β-sitosterol in $CDCL_3$



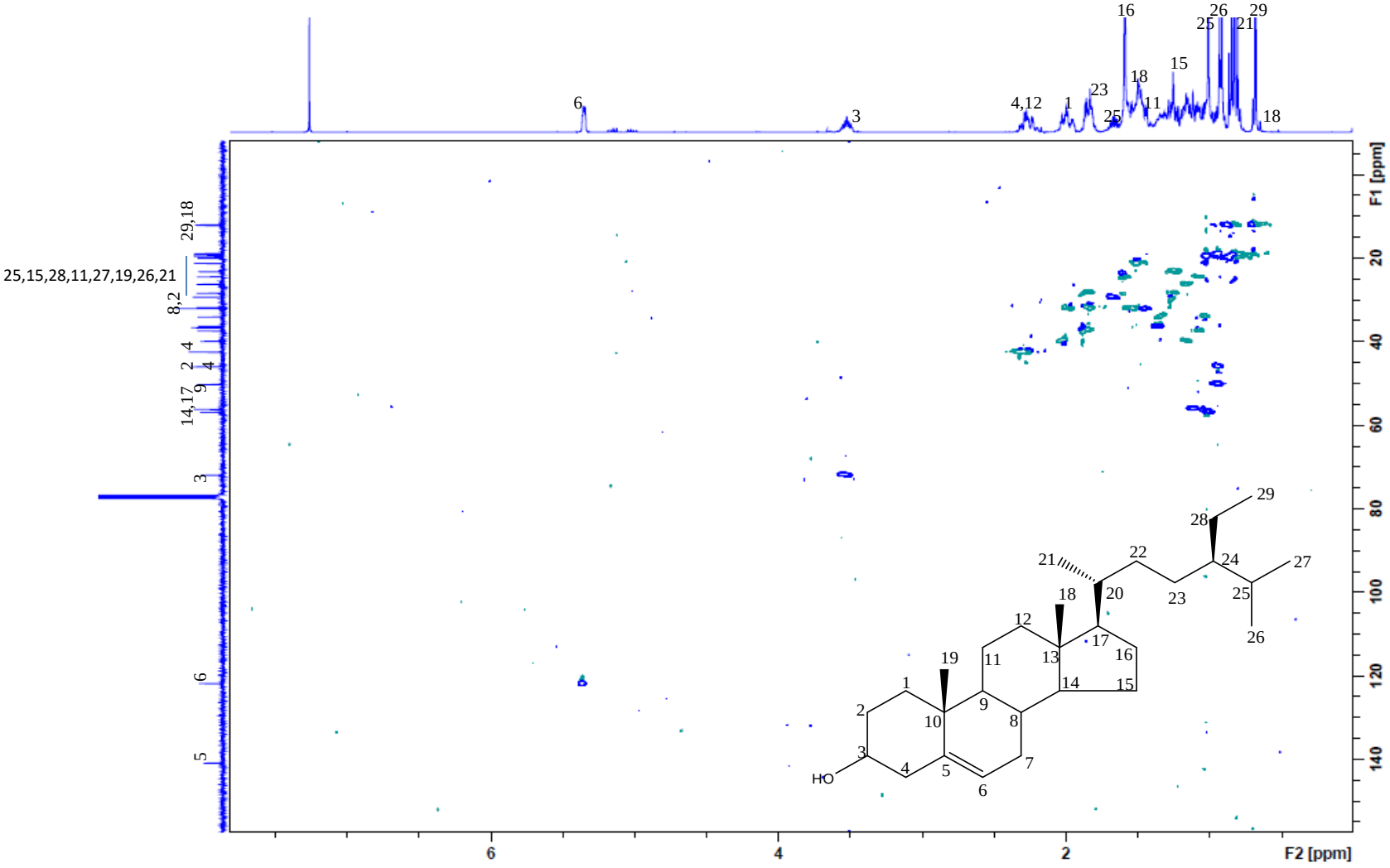
^{13}C NMR spectrum of β-sitosterol in $CDCL_3$



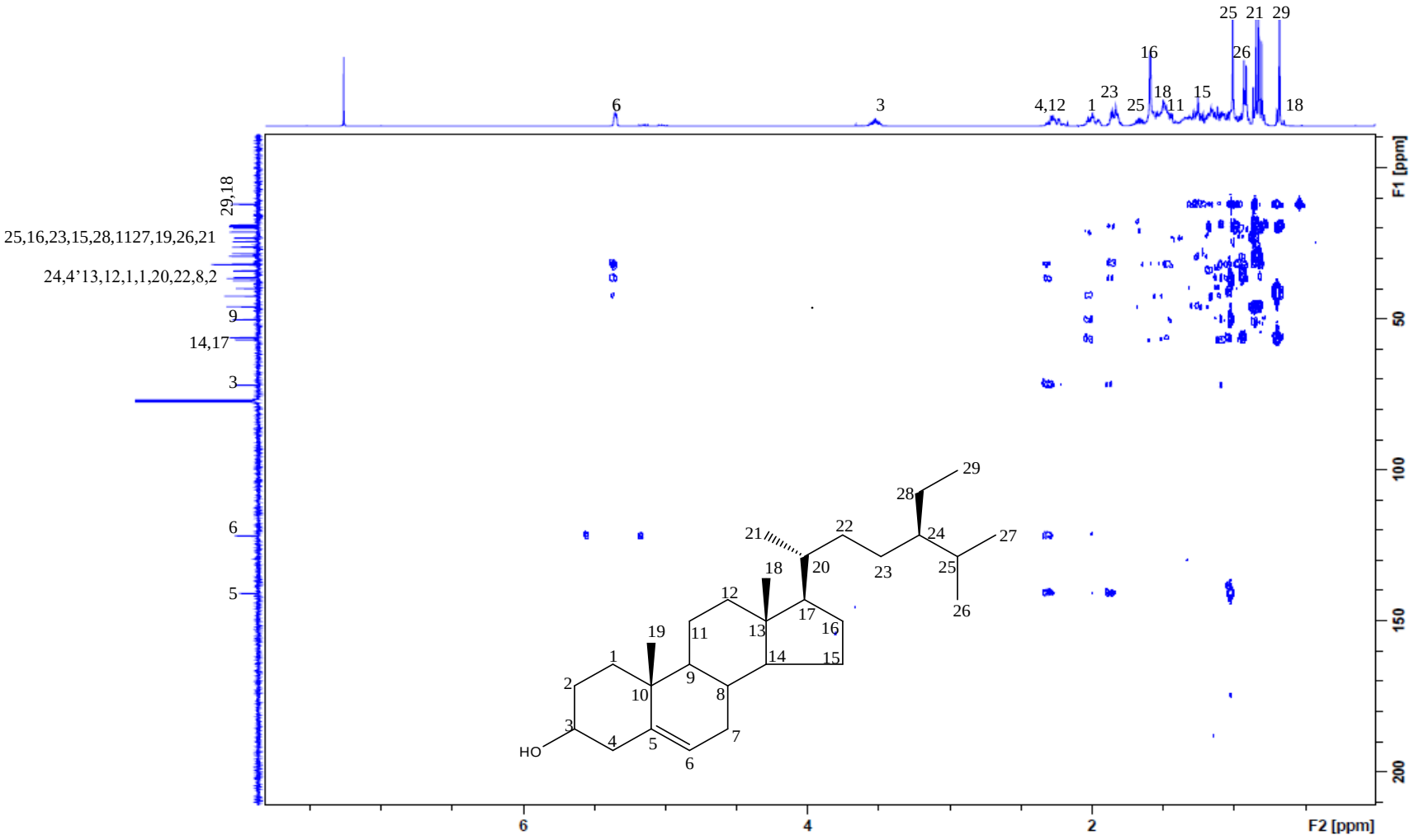
DEPT NMR spectrum of β-sitosterol in $CDCL_3$



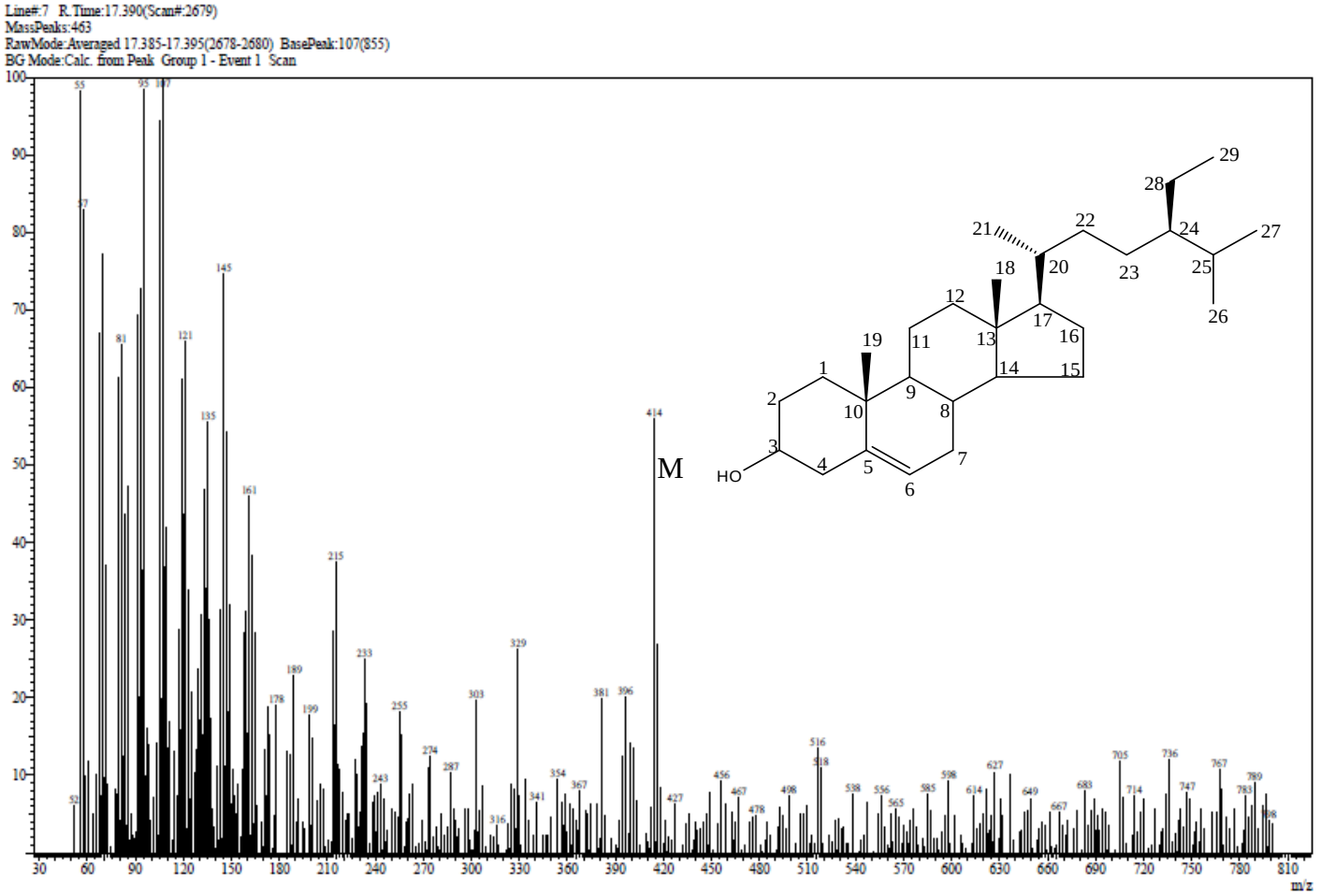
COSY NMR spectrum of β-sitosterol in $CDCL_3$



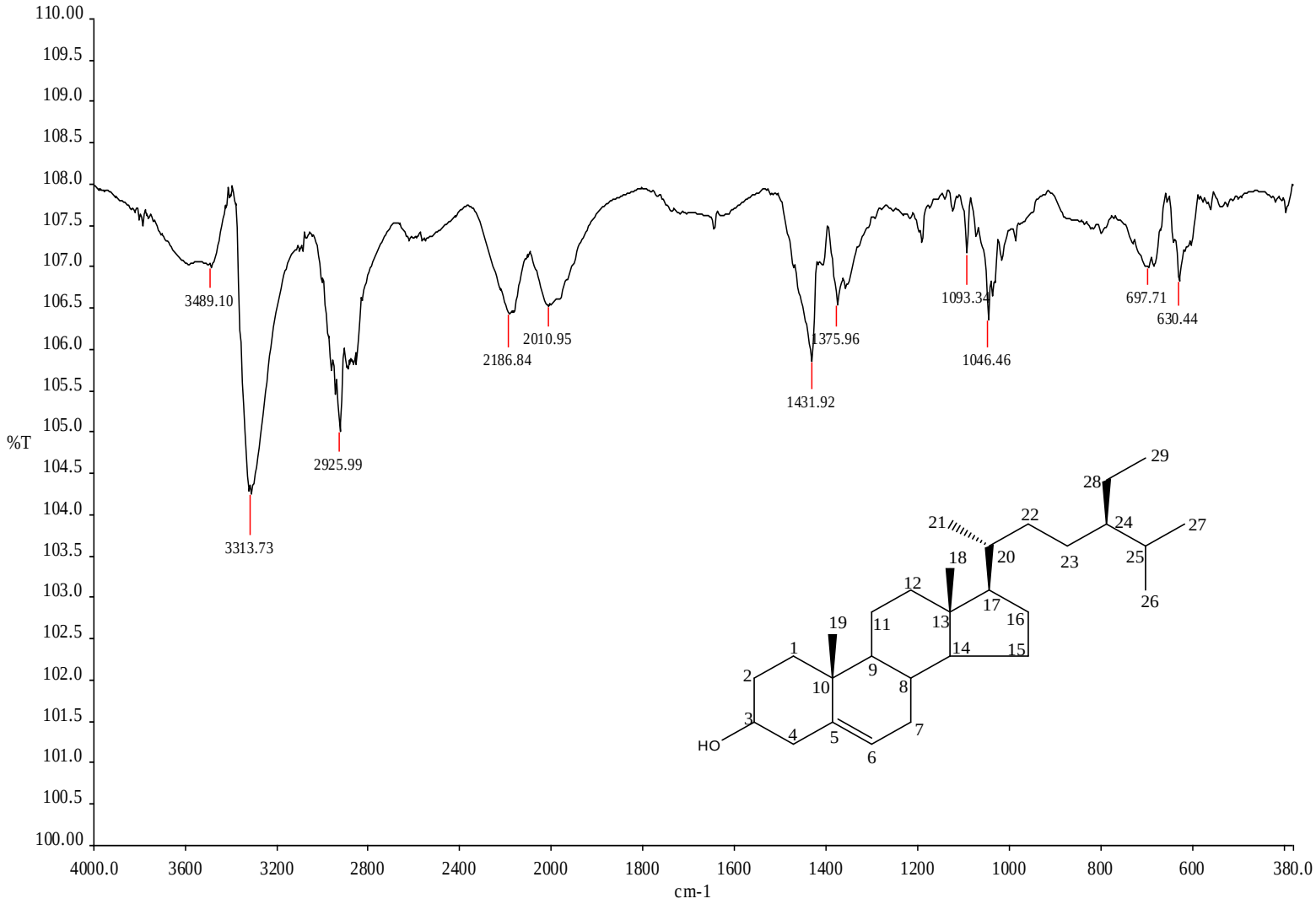
HSQC NMR spectrum of β-sitosterol in $CDCL_3$



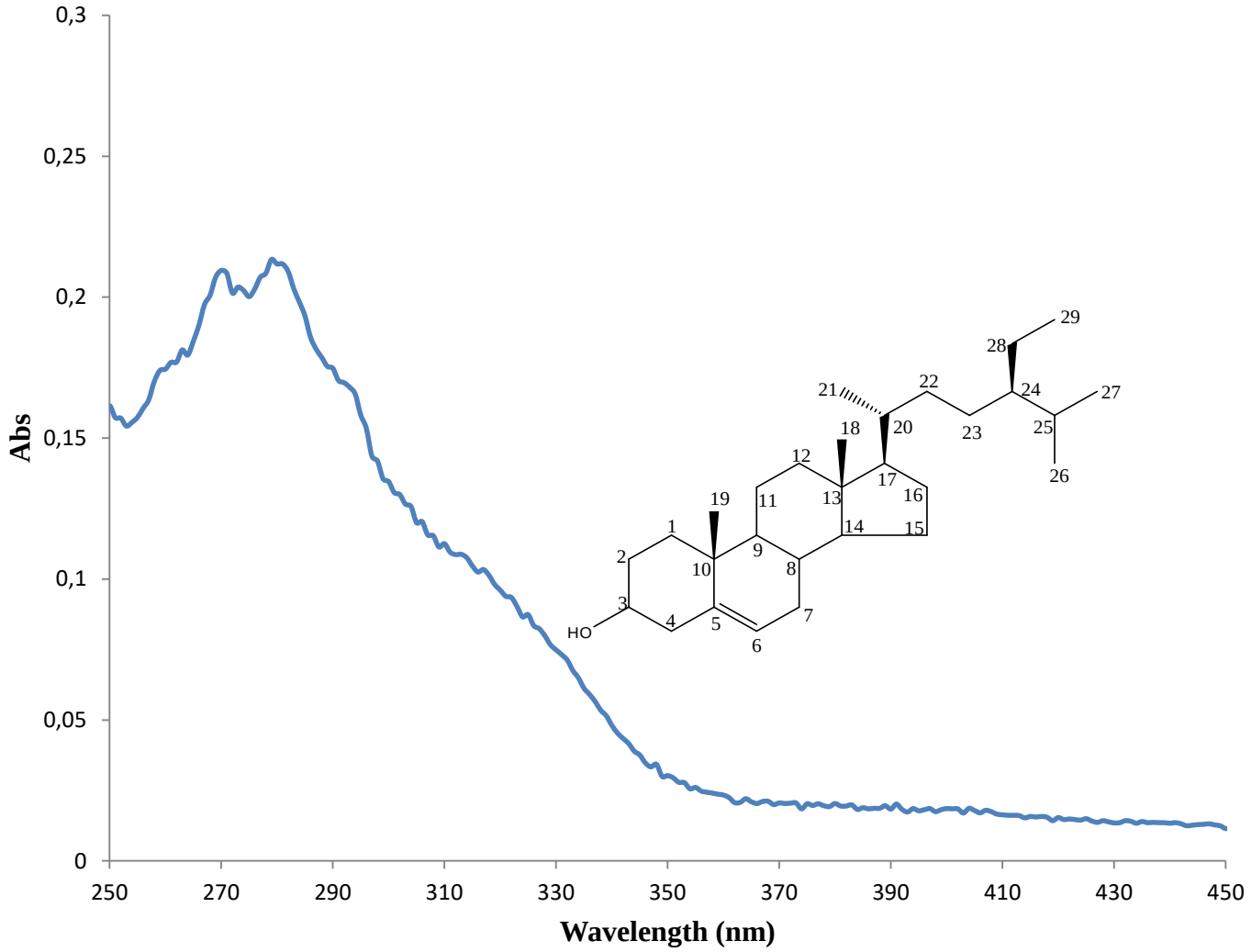
HMBC NMR spectrum of β-sitosterol in $CDCL_3$



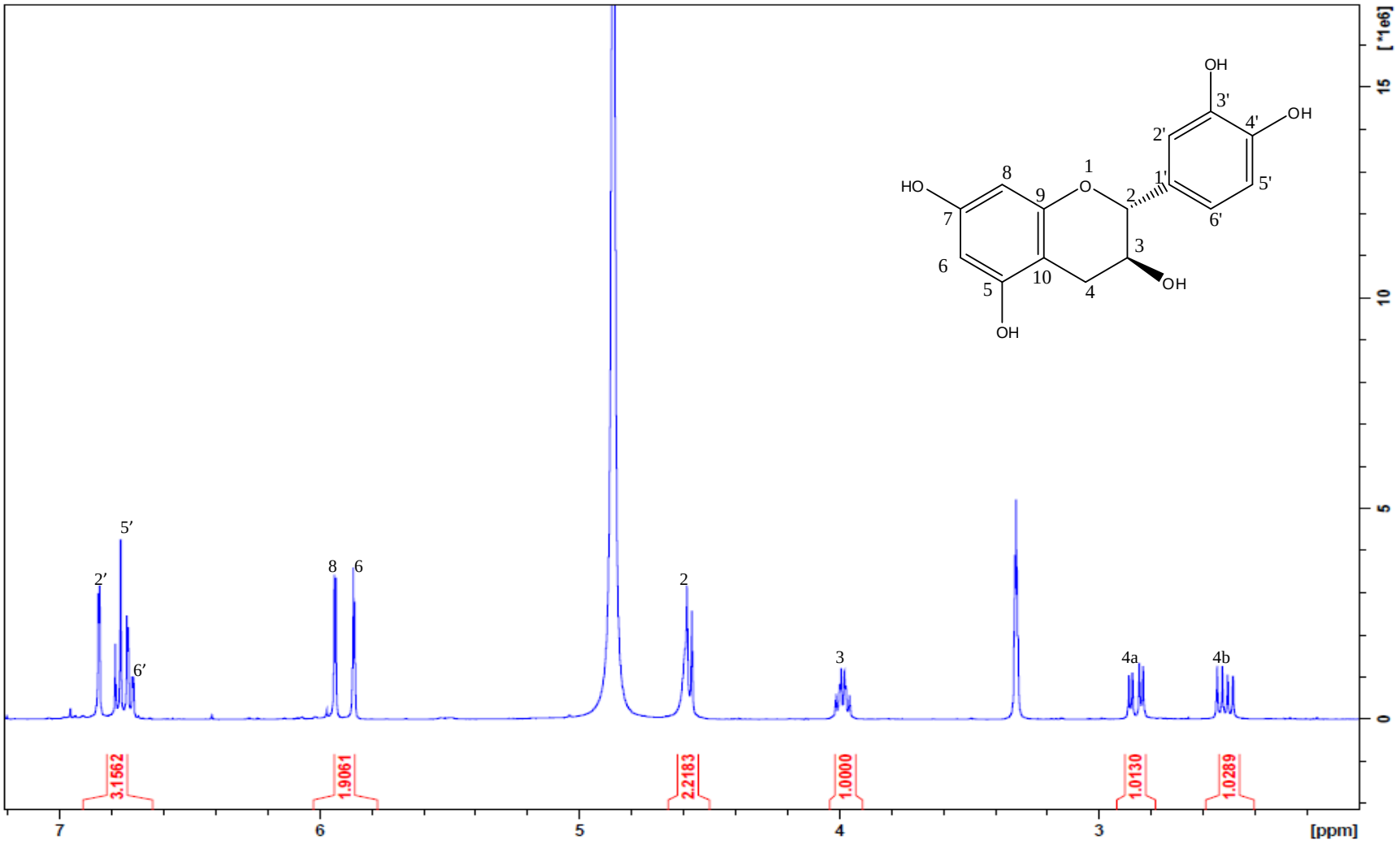
Mass spectrum of β-sitosterol



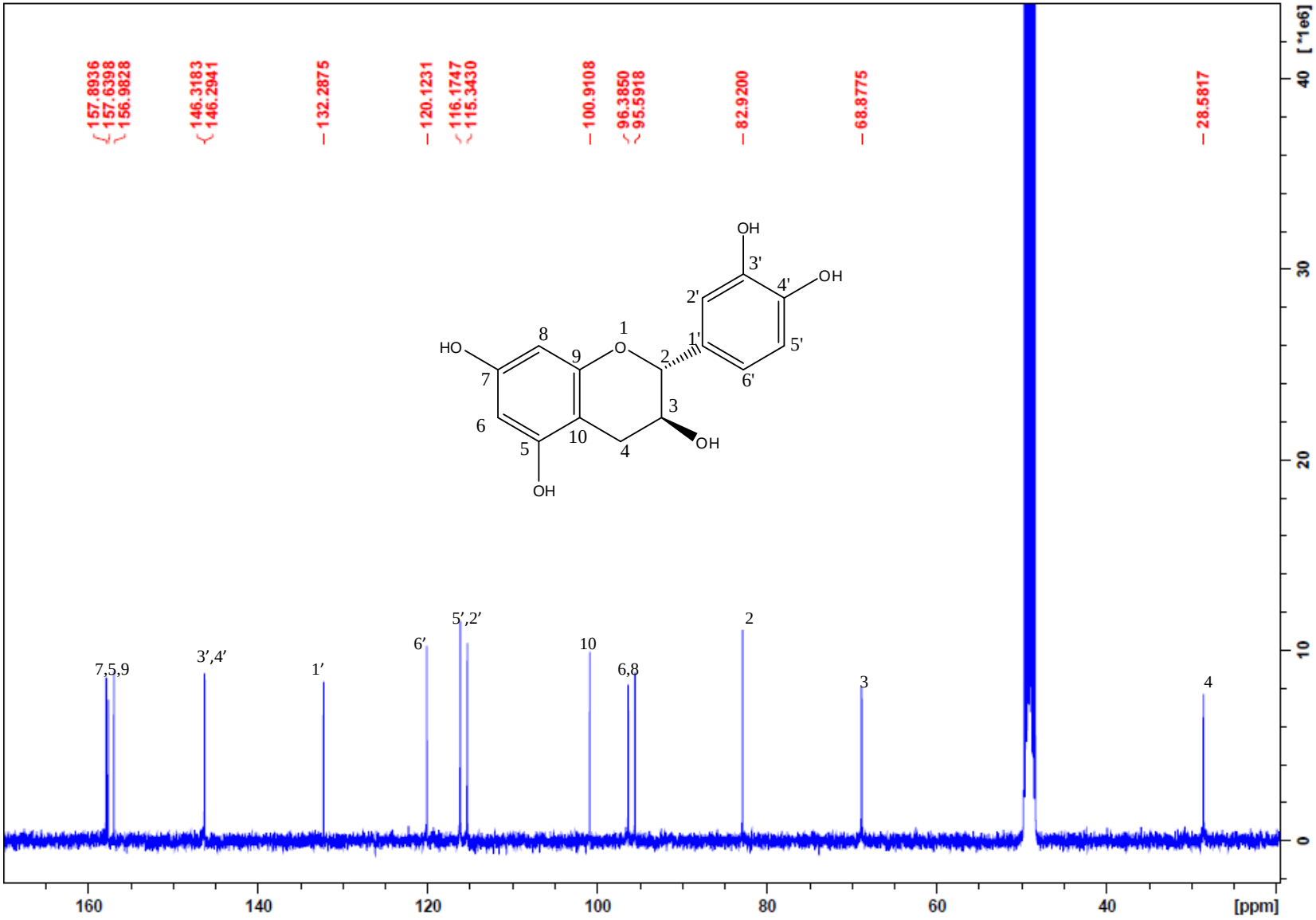
IR spectrum of β-sitosterol



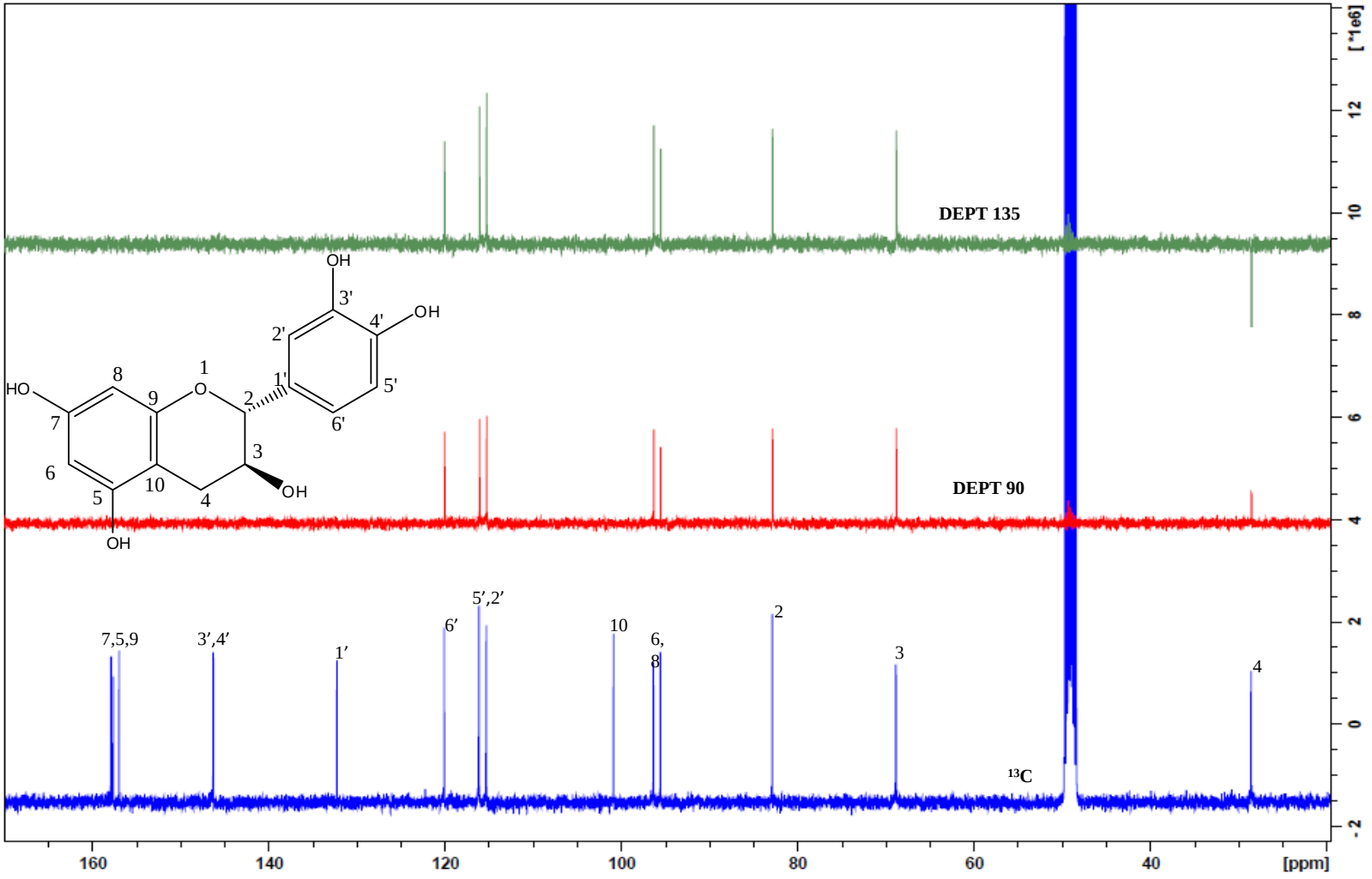
UV spectrum of β-sitosterol



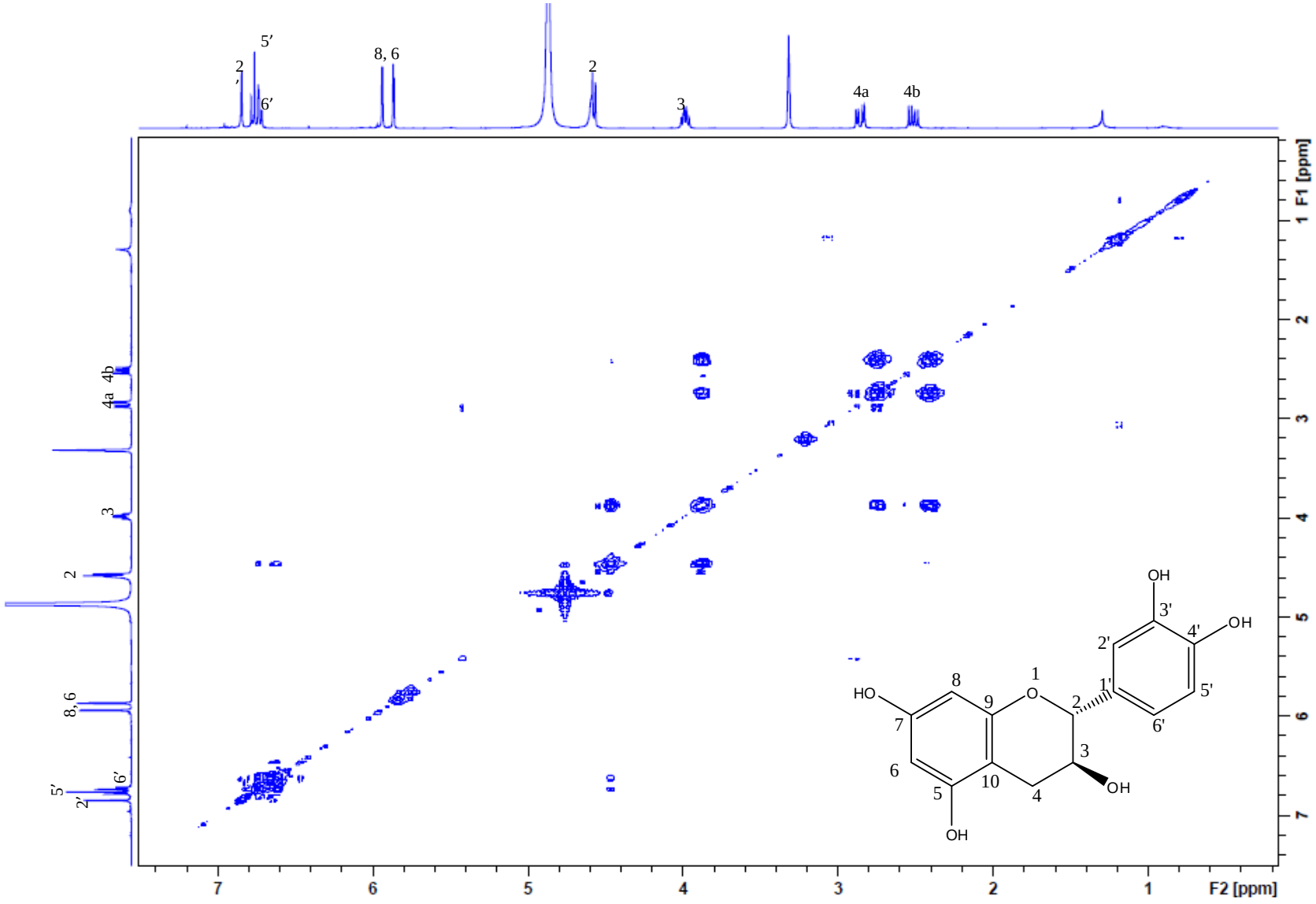
1H NMR spectrum of (+)-catechin in MeOD



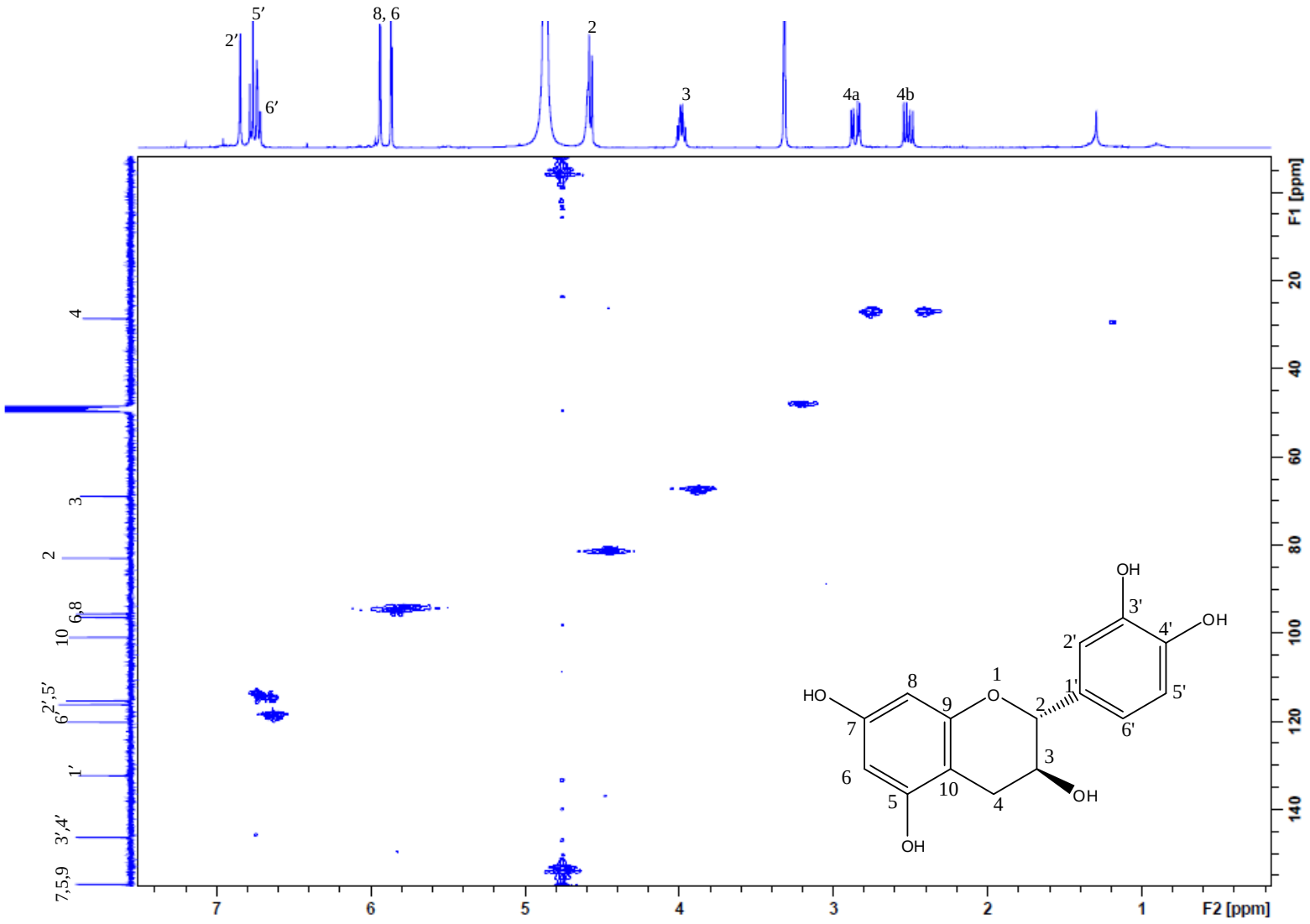
^{13}C NMR spectrum of (+)-catechin in MeOD



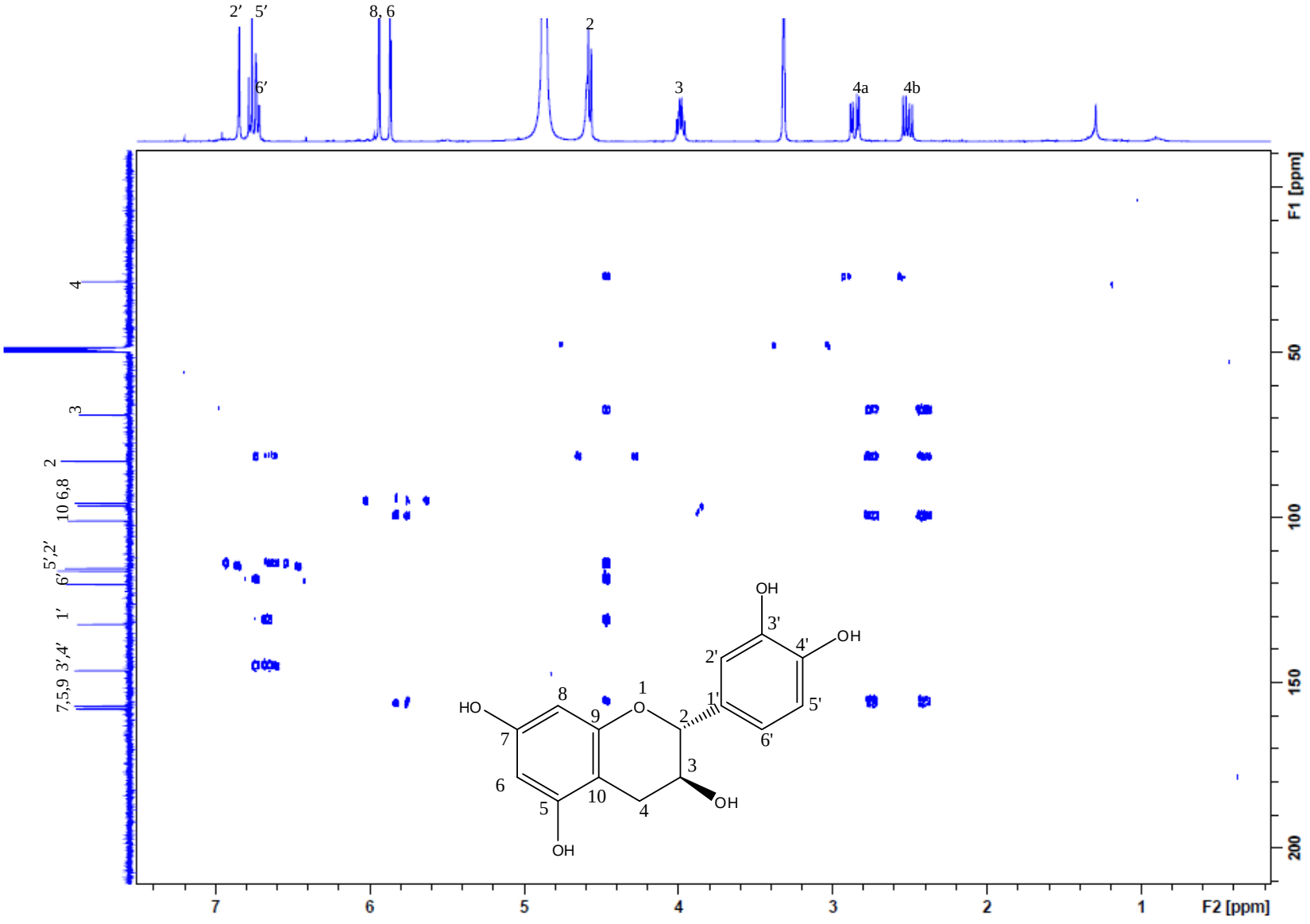
DEPT NMR spectrum of (+)-catechin in MeOD



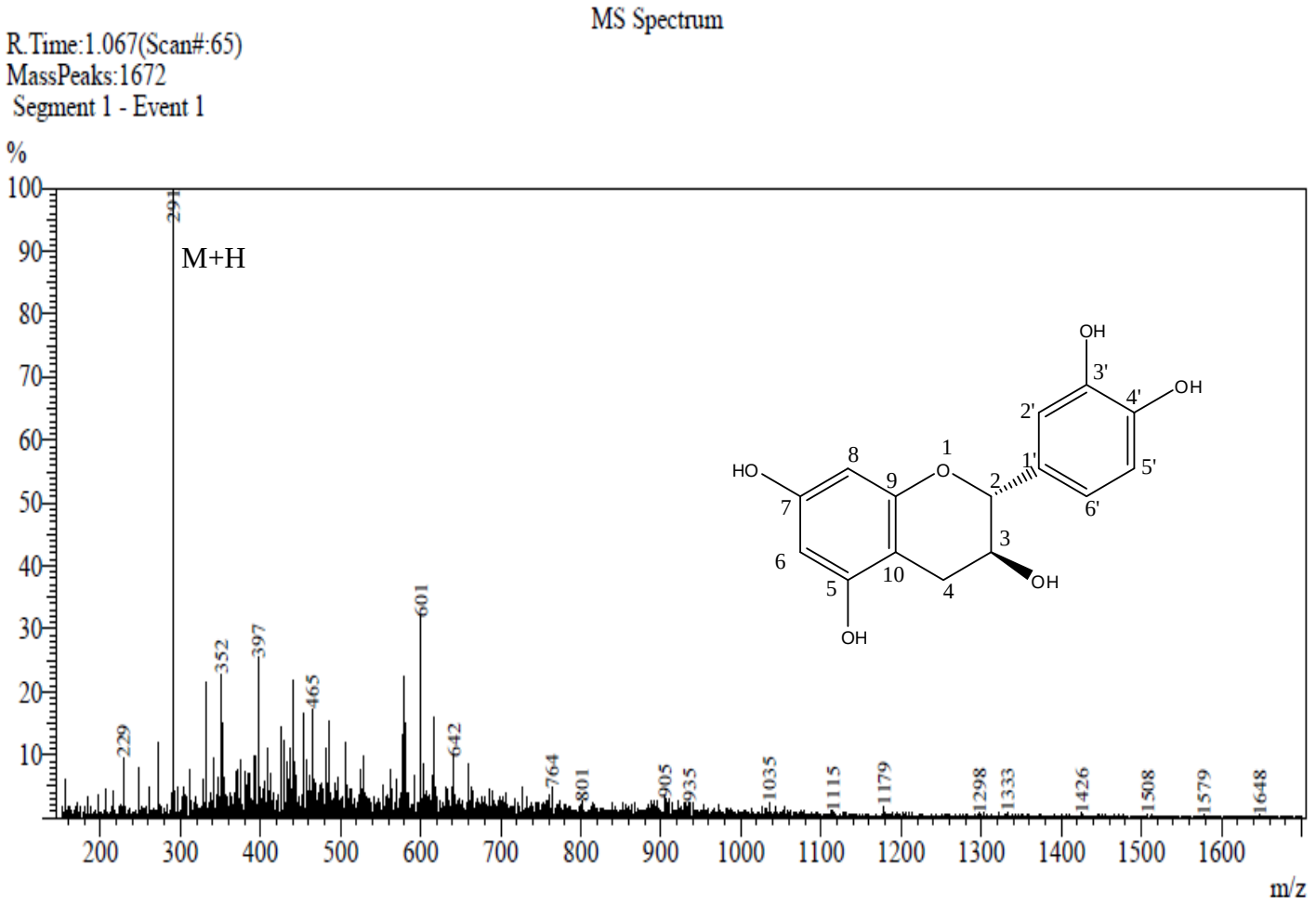
COESY NMR spectrum of (+)-catechin in MeOD



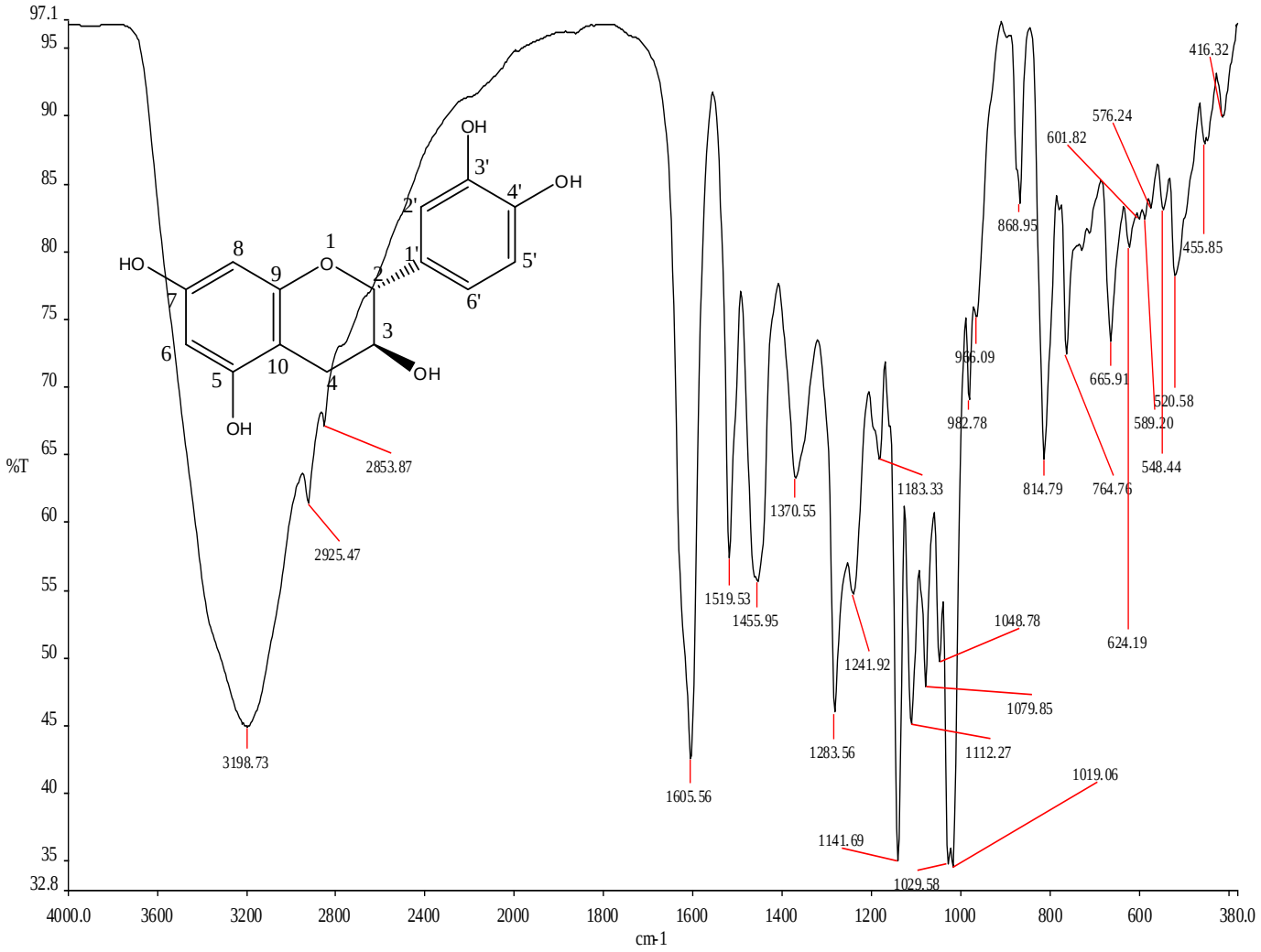
HSQC NMR spectrum of (+)-catechin in MeOD



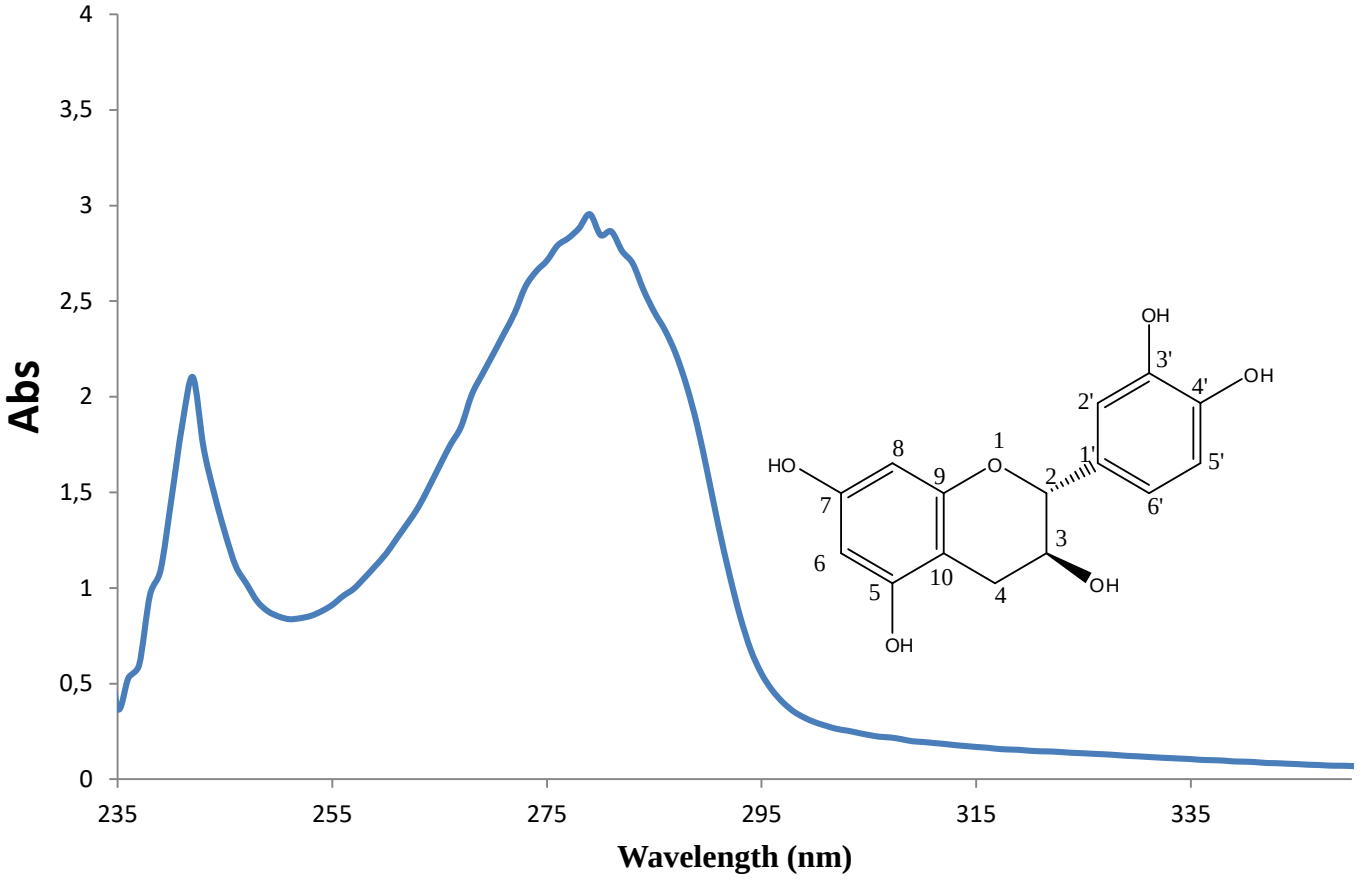
HMBC NMR spectrum of (+)-catechin in MeOD



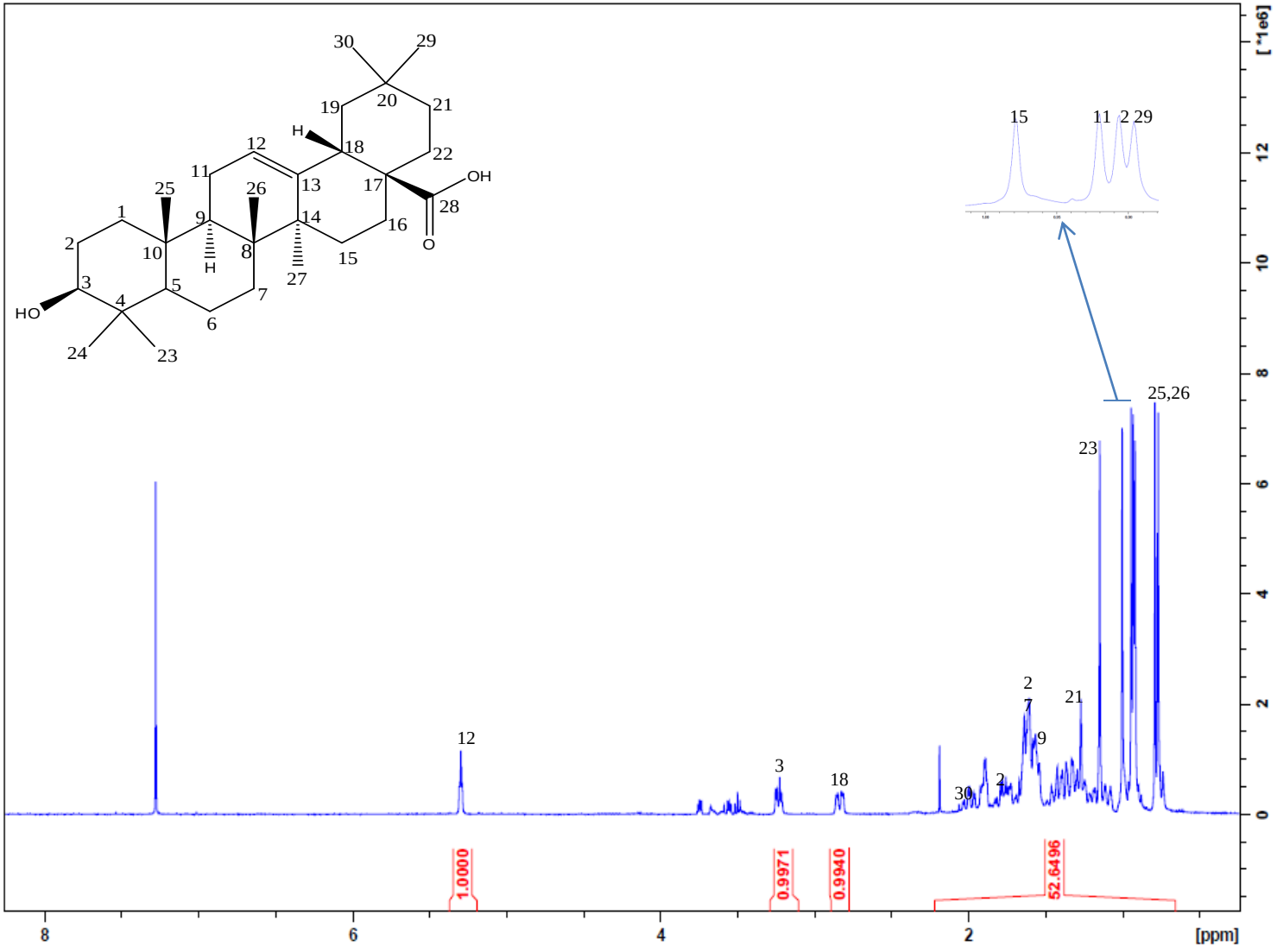
Mass spectrum of (+)-catechin in MeOH



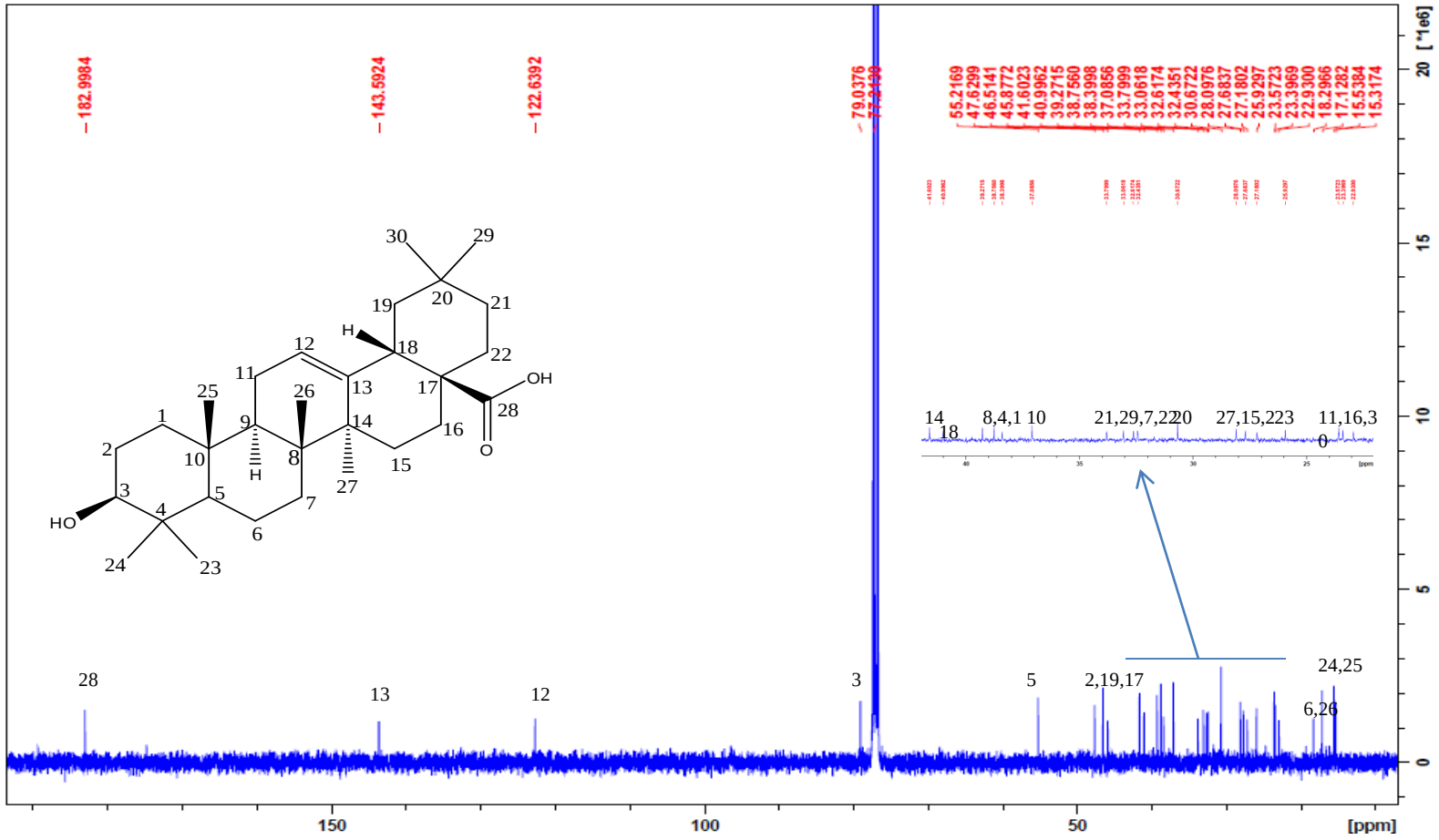
IR spectrum of (+)-catechin



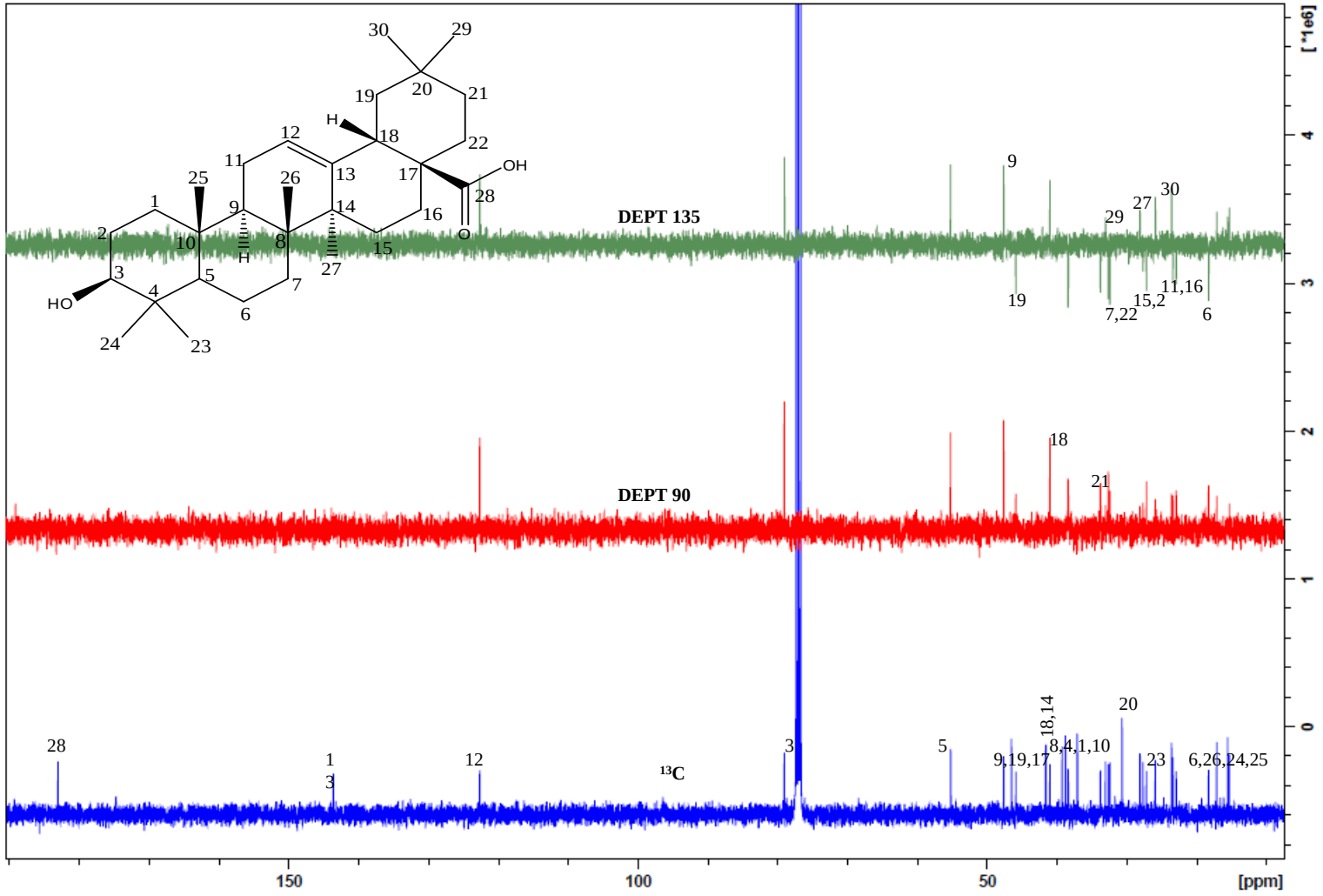
UV spectrum of (+)-catechin



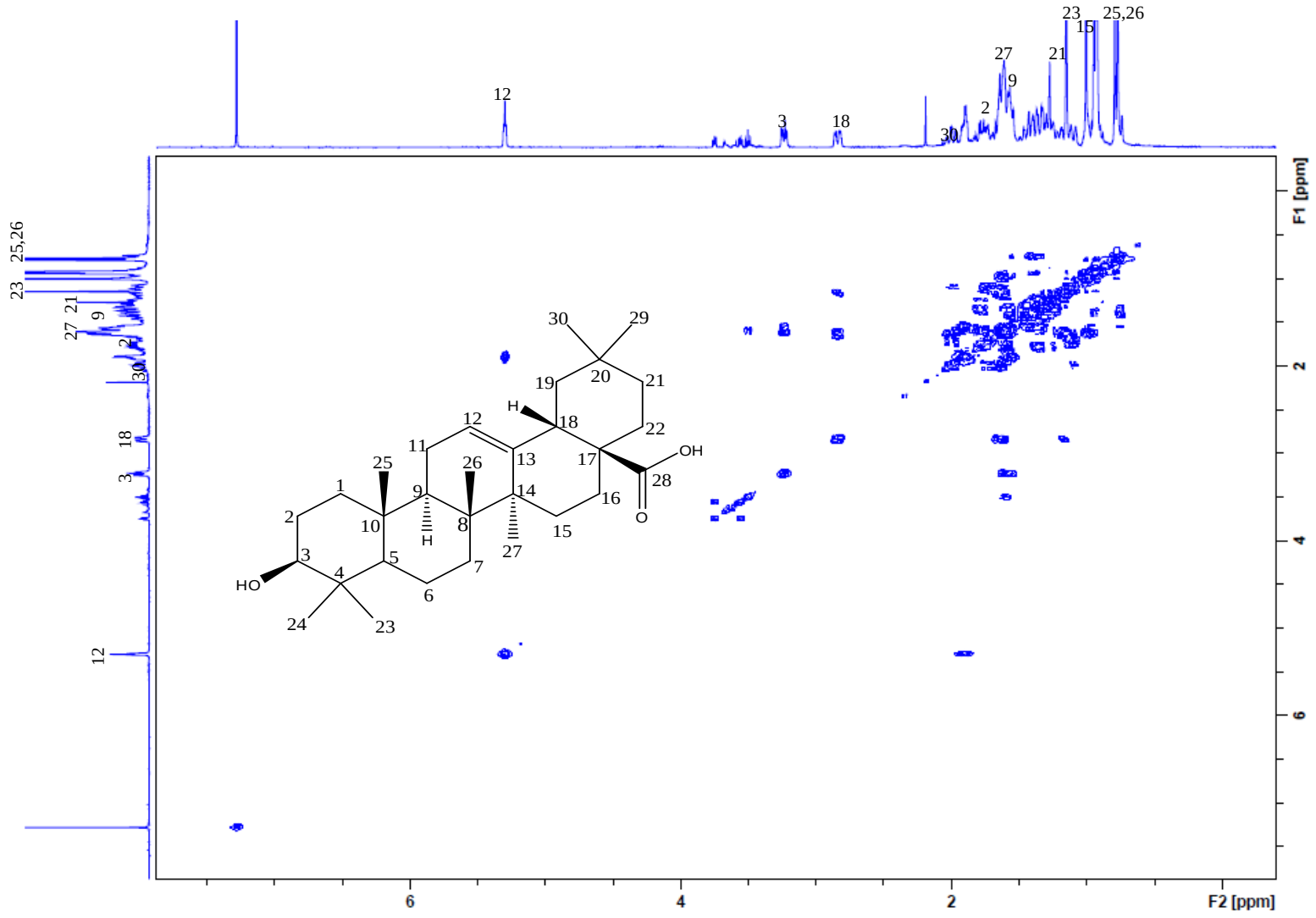
^{1}H NMR spectrum of oleanolic acid in $CDCL_3$



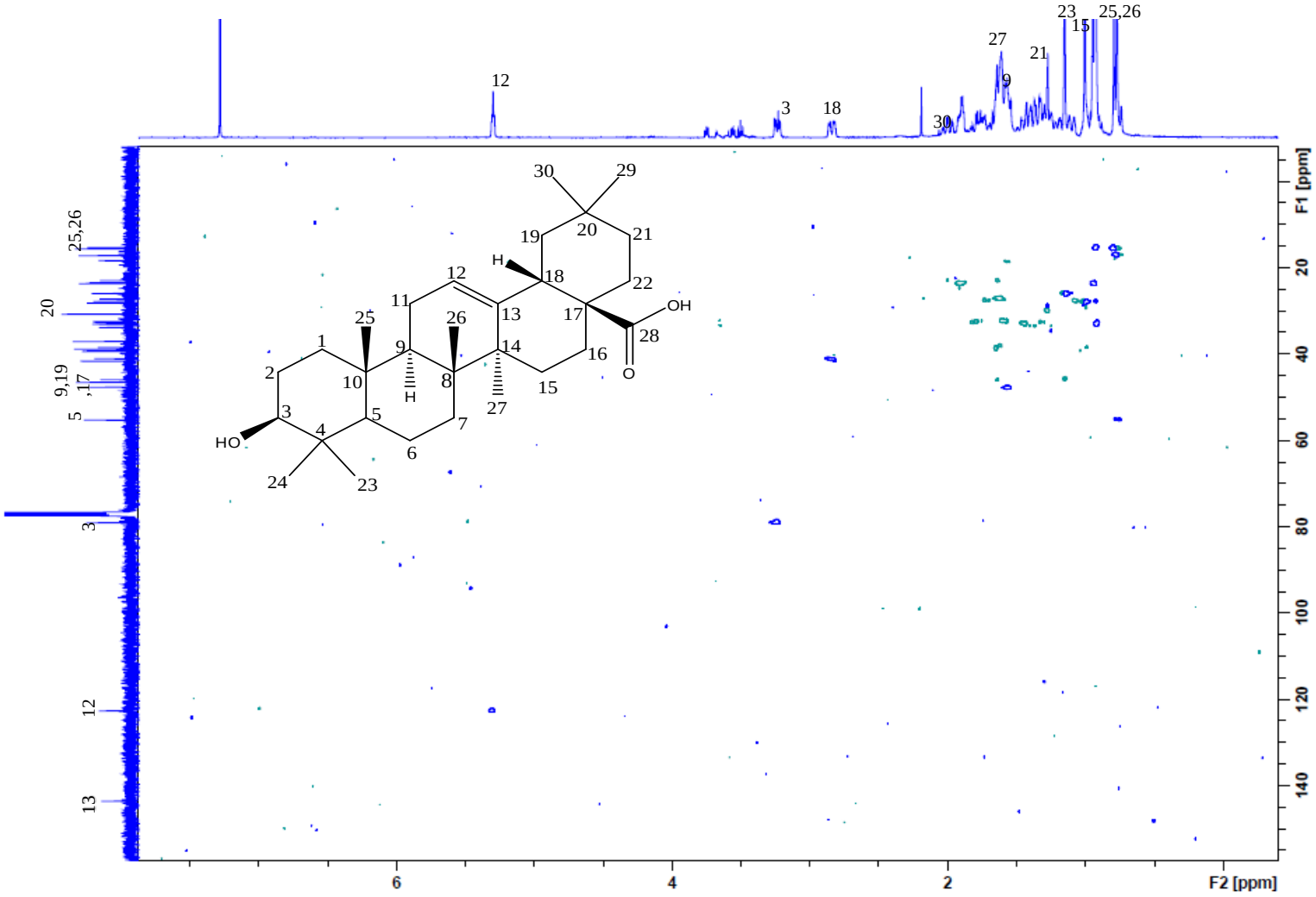
^{13}C NMR spectrum of oleanolic acid in $CDCL_3$



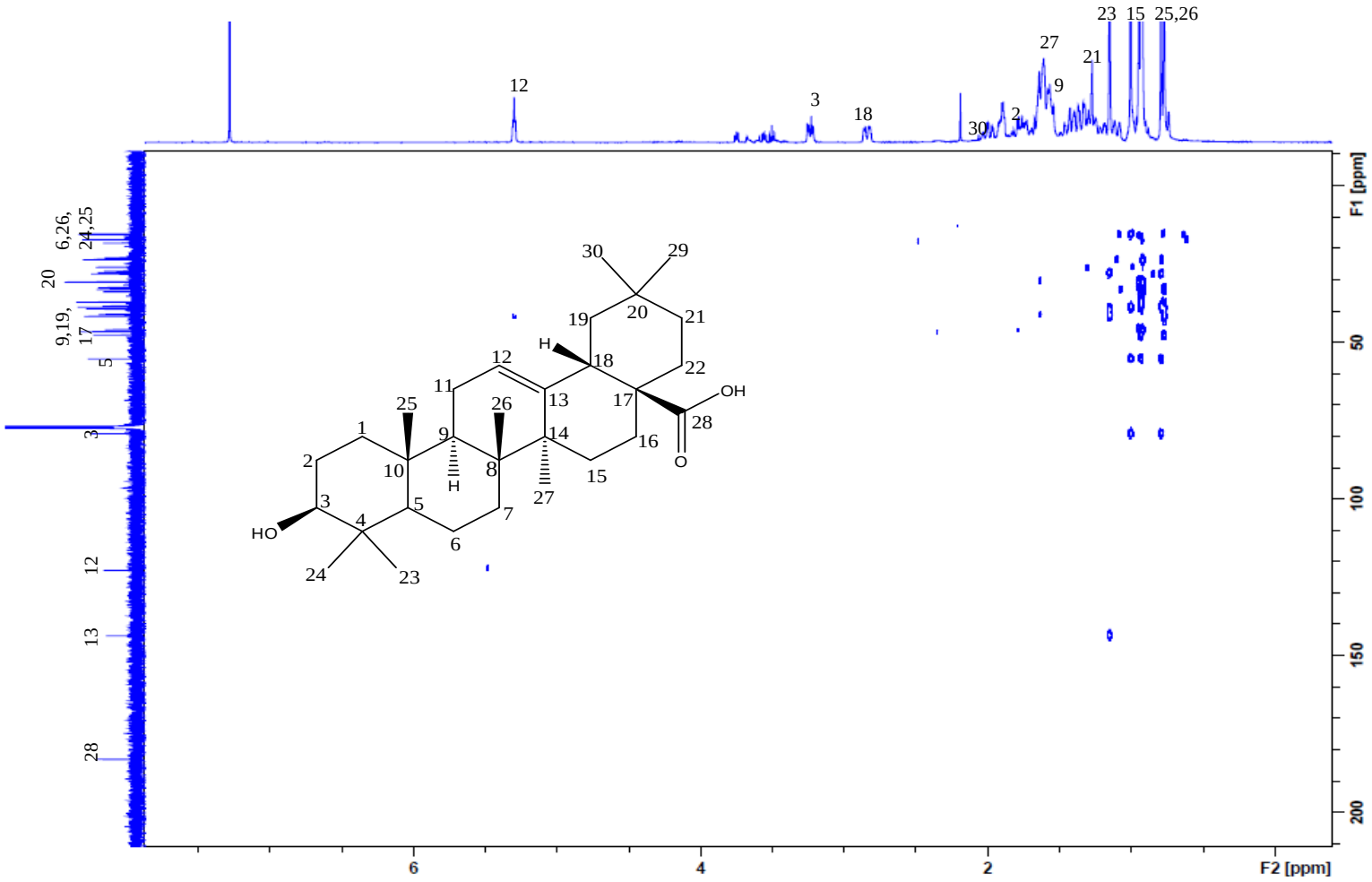
DEPT NMR spectrum of oleanolic acid in $CDCL_3$



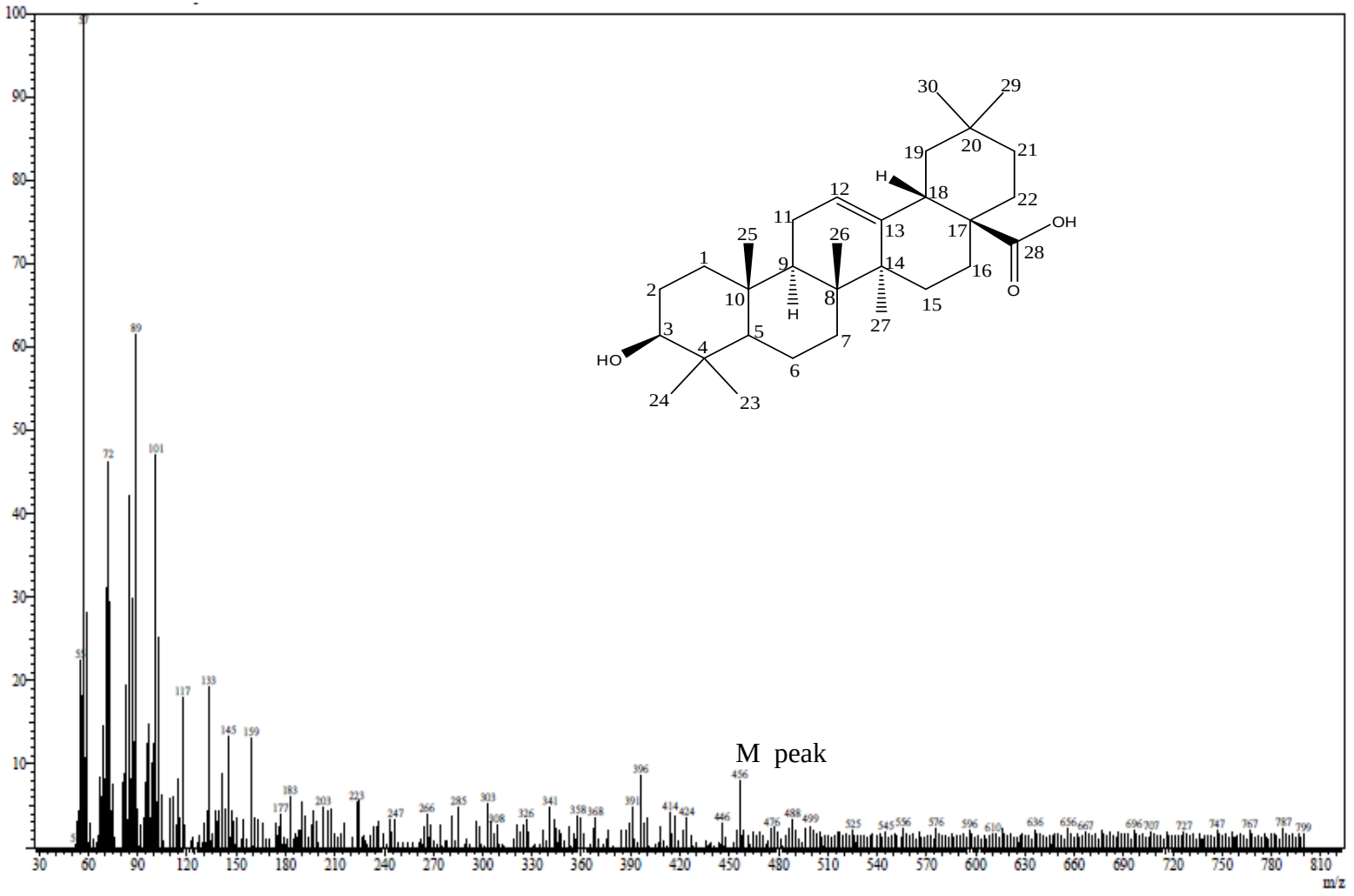
COSY NMR spectrum of oleanolic acid in $CDCL_3$



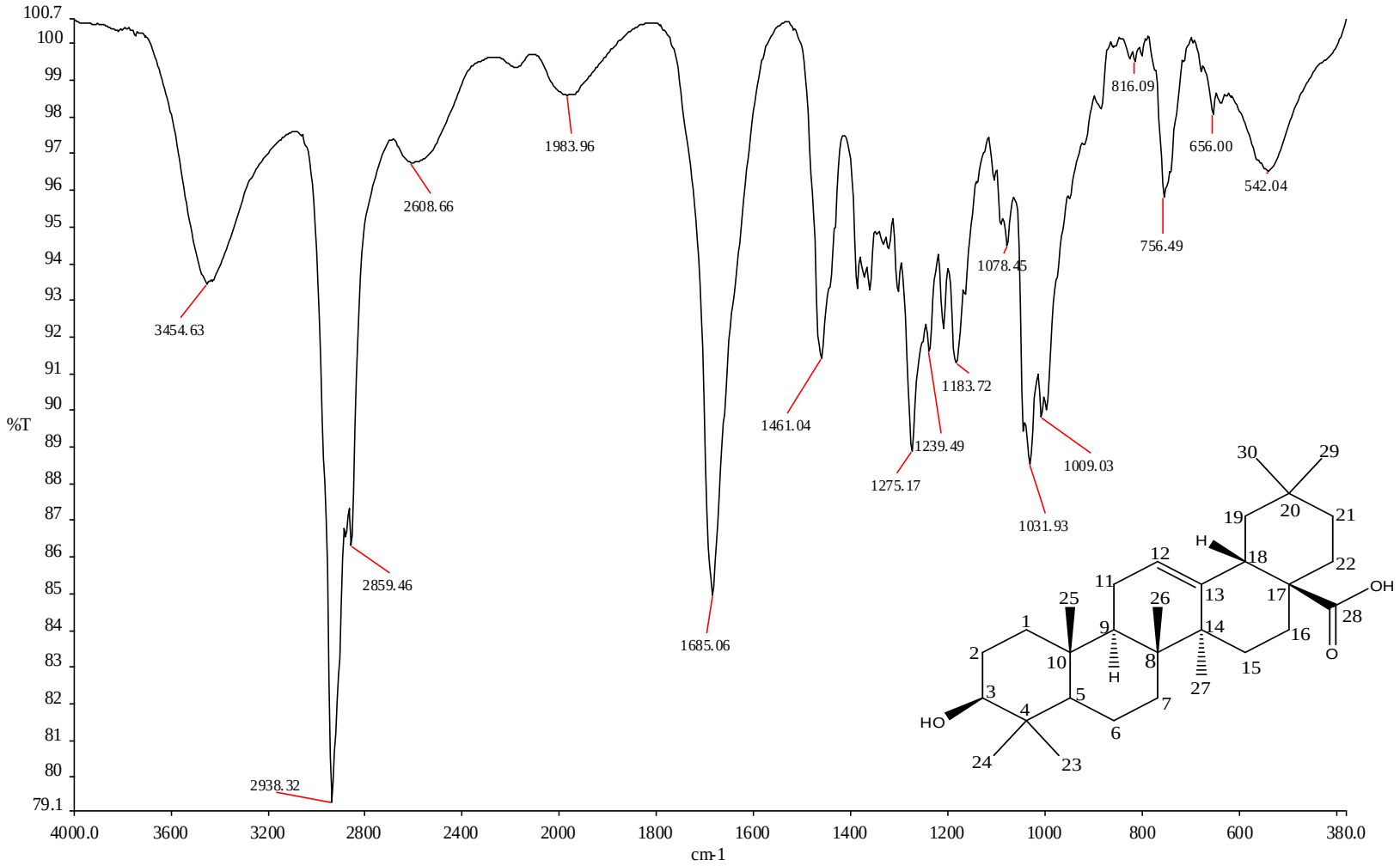
HSQC NMR spectrum of oleanolic acid in $CDCL_3$



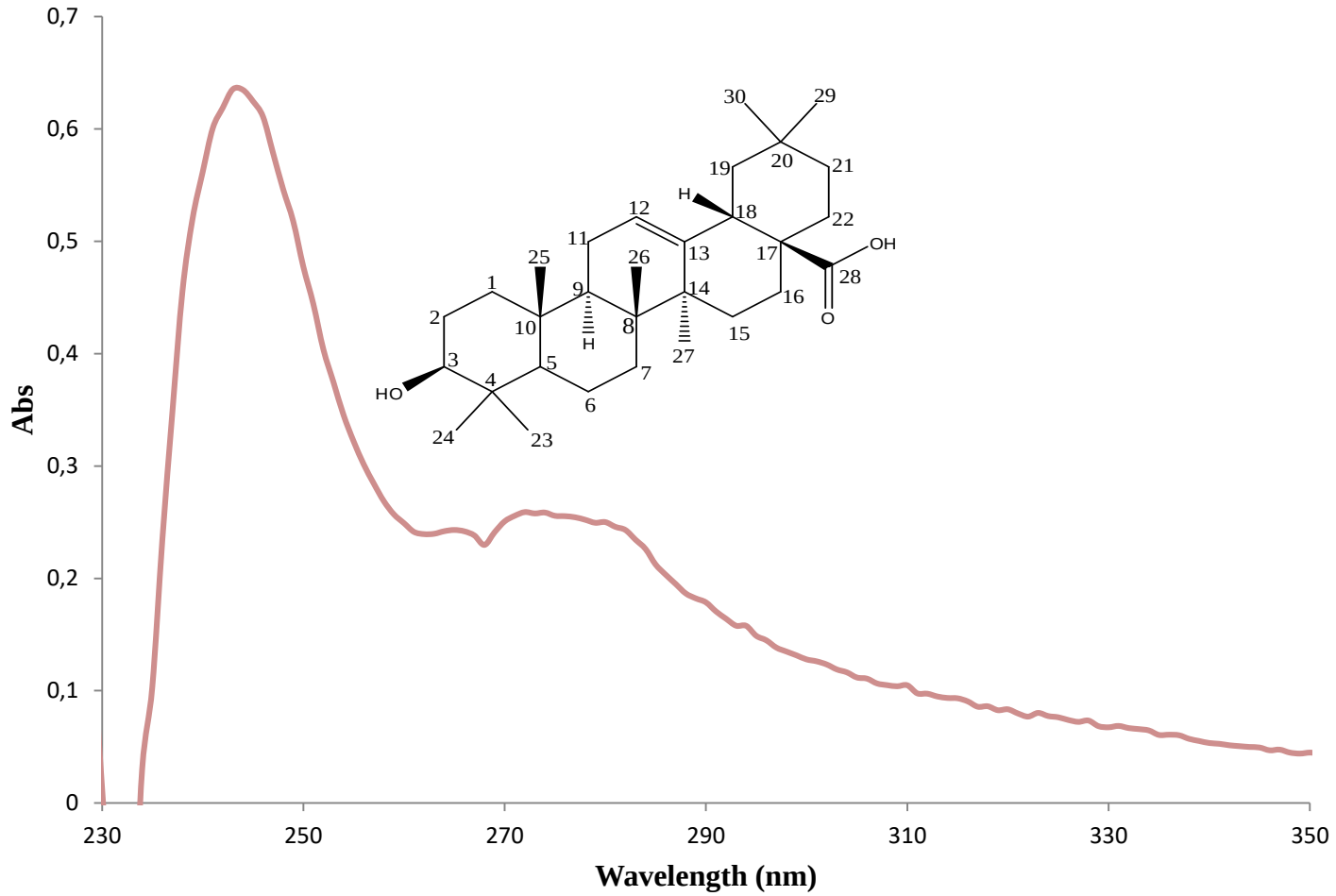
HMBC NMR spectrum of oleanolic acid in $CDCL_3$



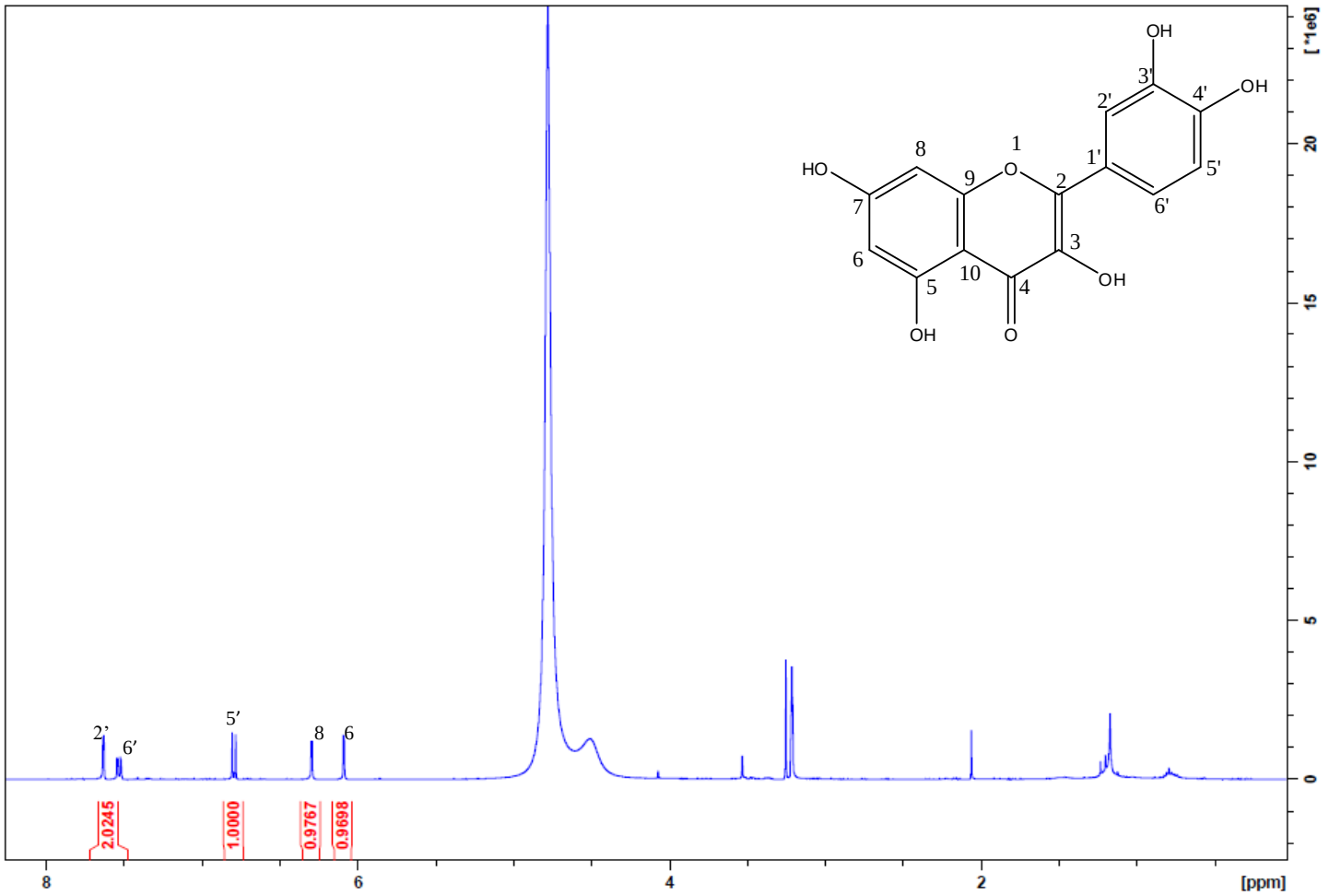
Mass spectrum of oleanolic acid



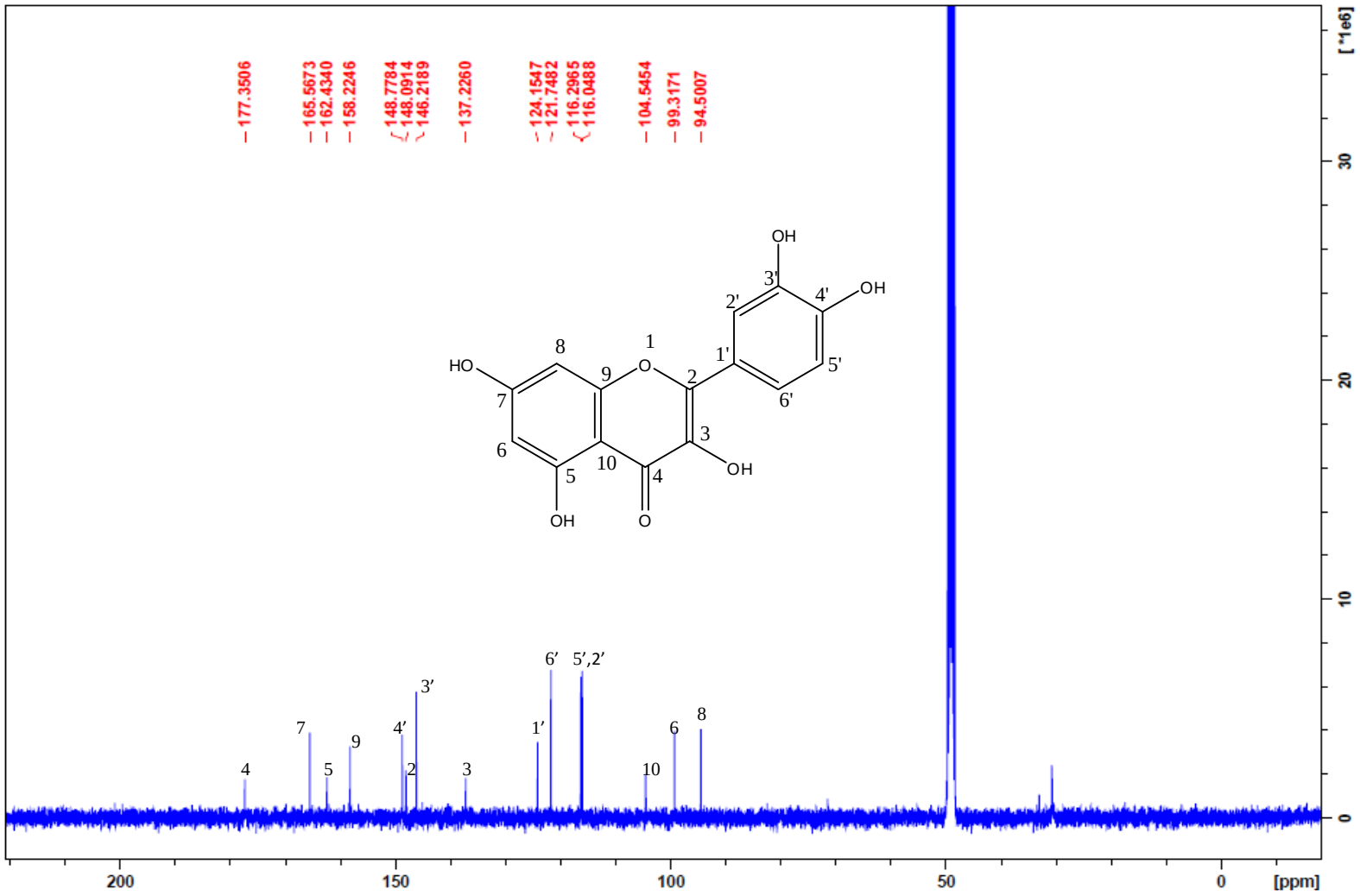
IR spectrum of oleanolic acid



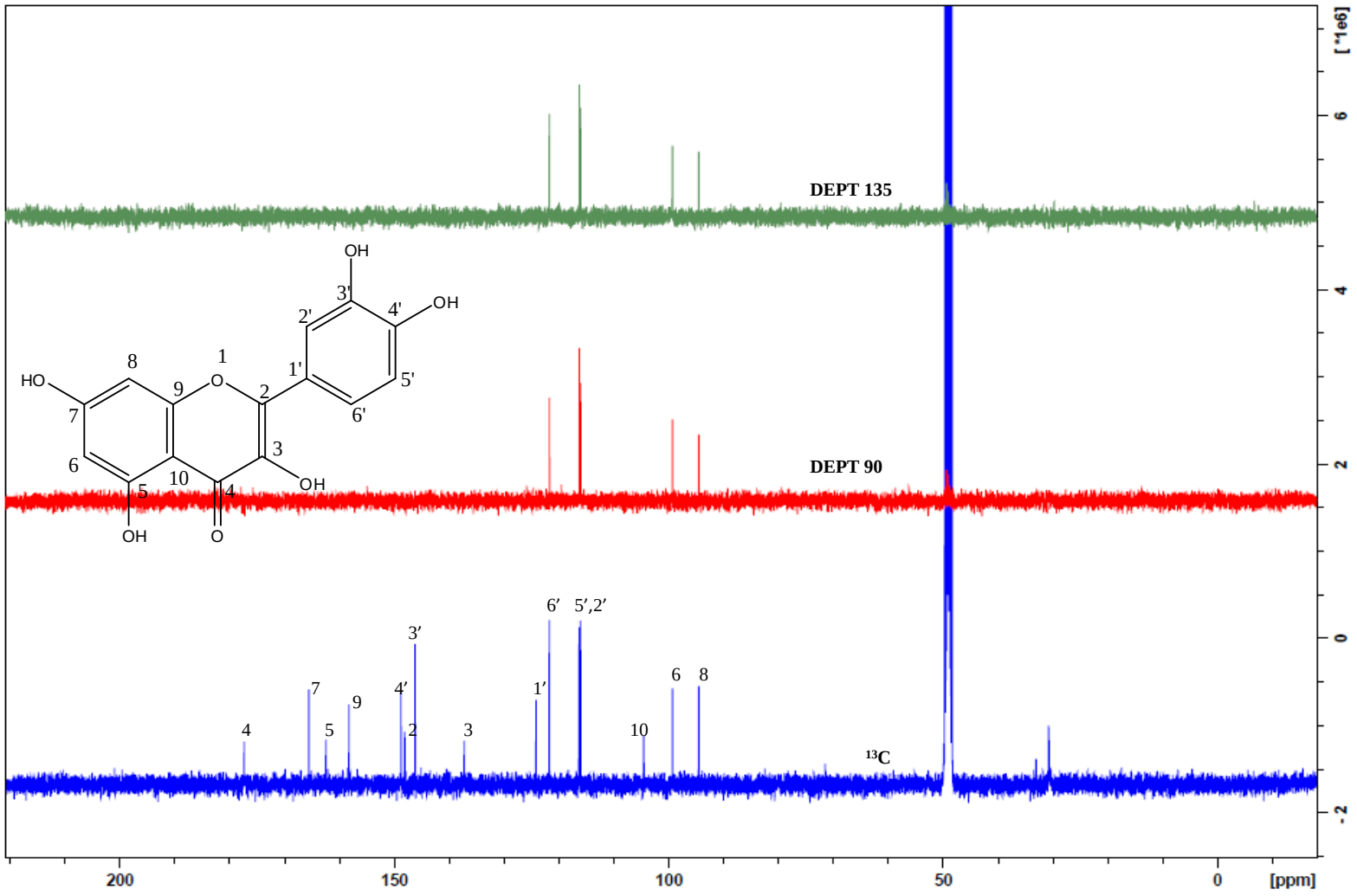
UV spectrum of oleanolic acid



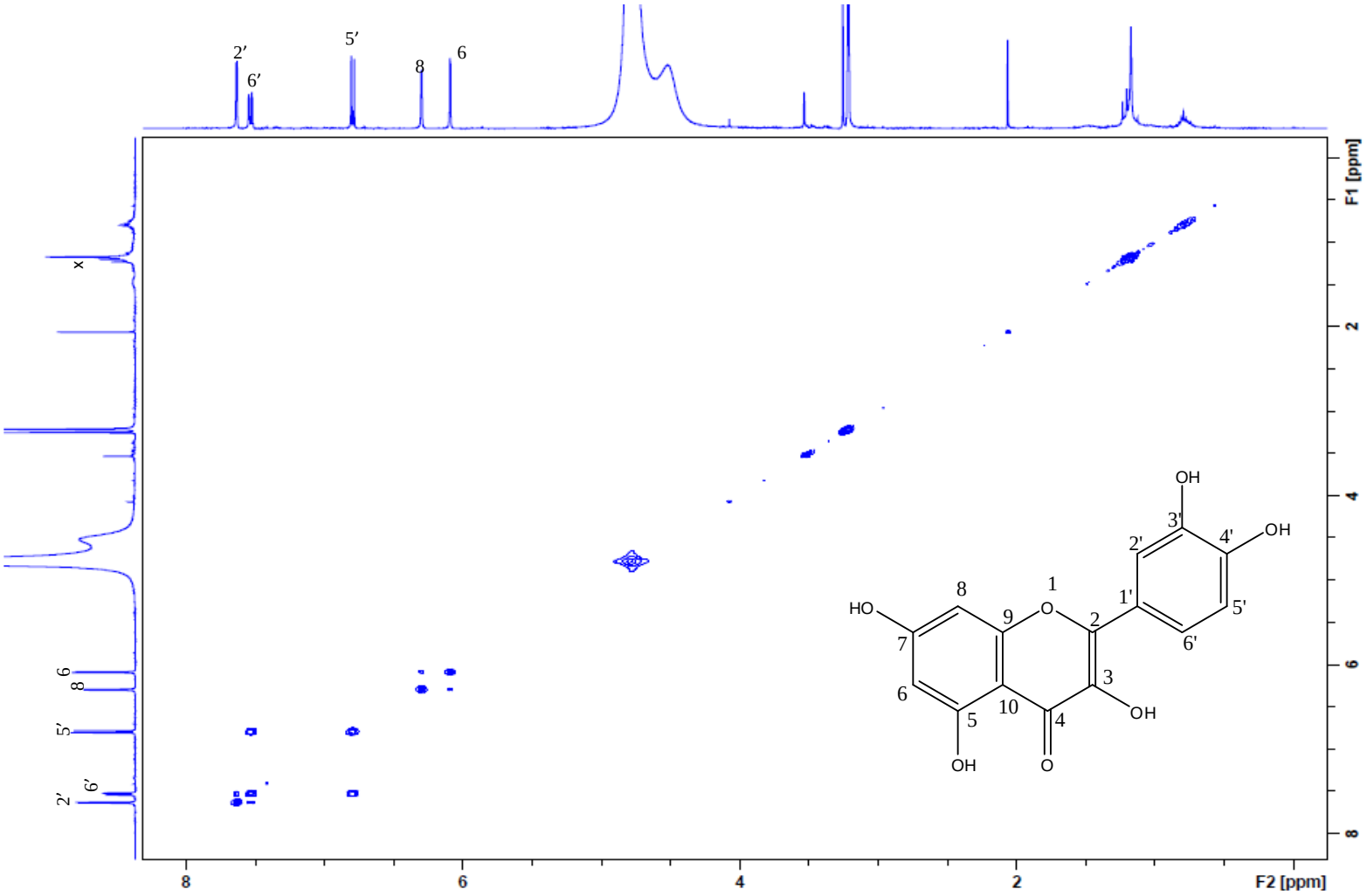
^{1}H NMR spectrum of quercetin in MeOD



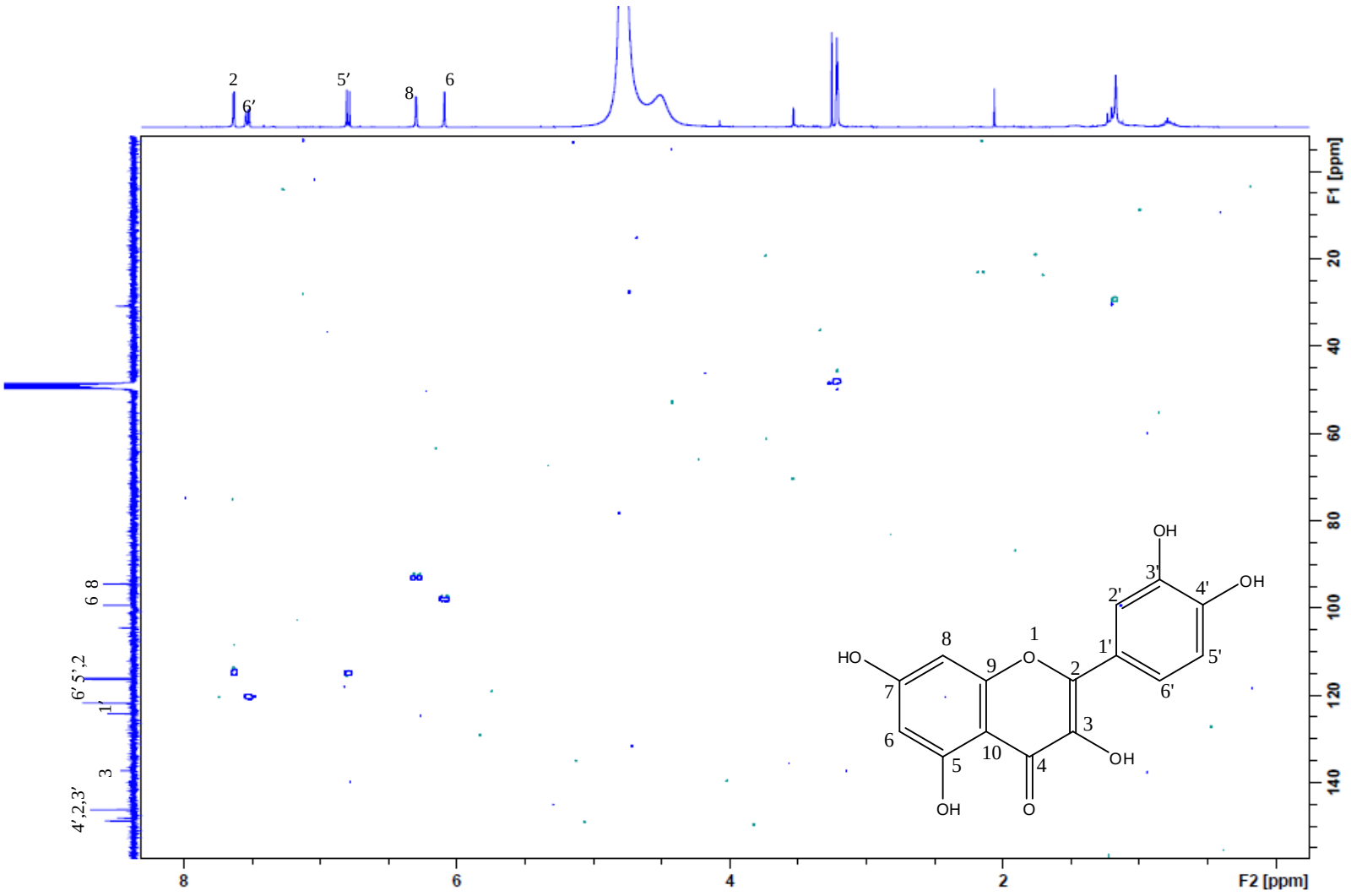
^{13}CNMR spectrum of quercetin in MeOD



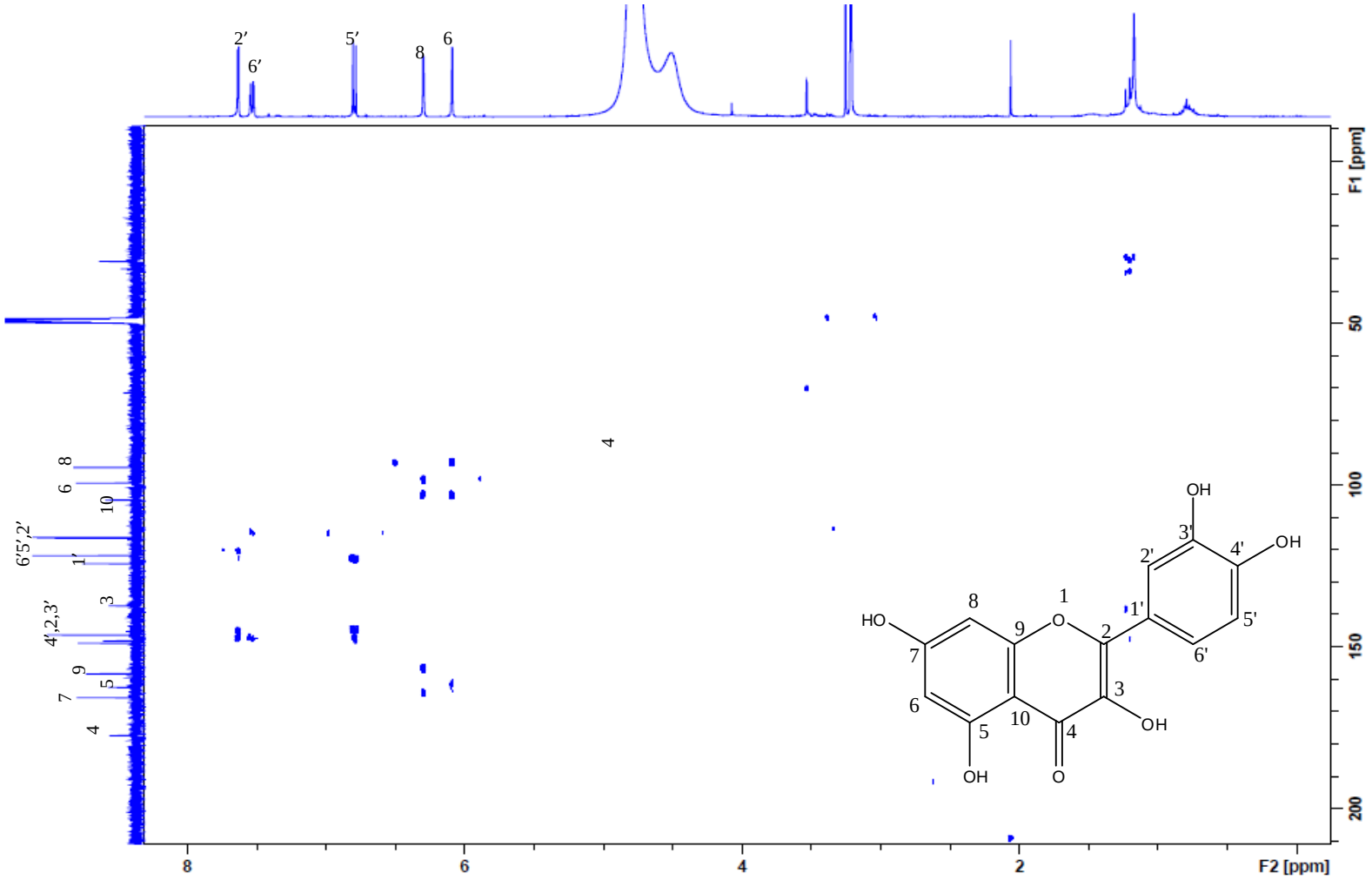
DEPT NMR spectrum of quercetin in MeOD



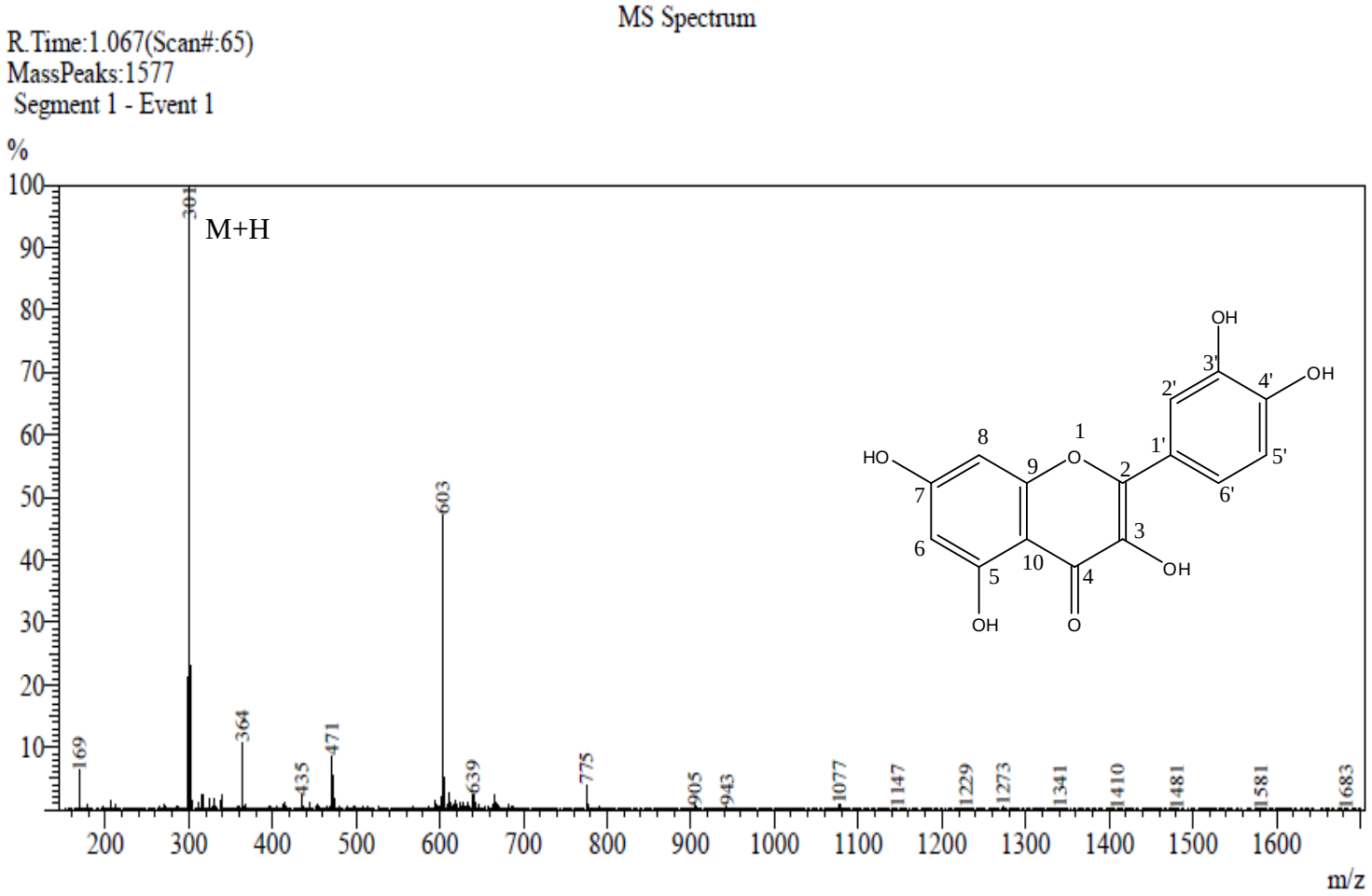
COSY NMR spectrum of quercetin in MeOD



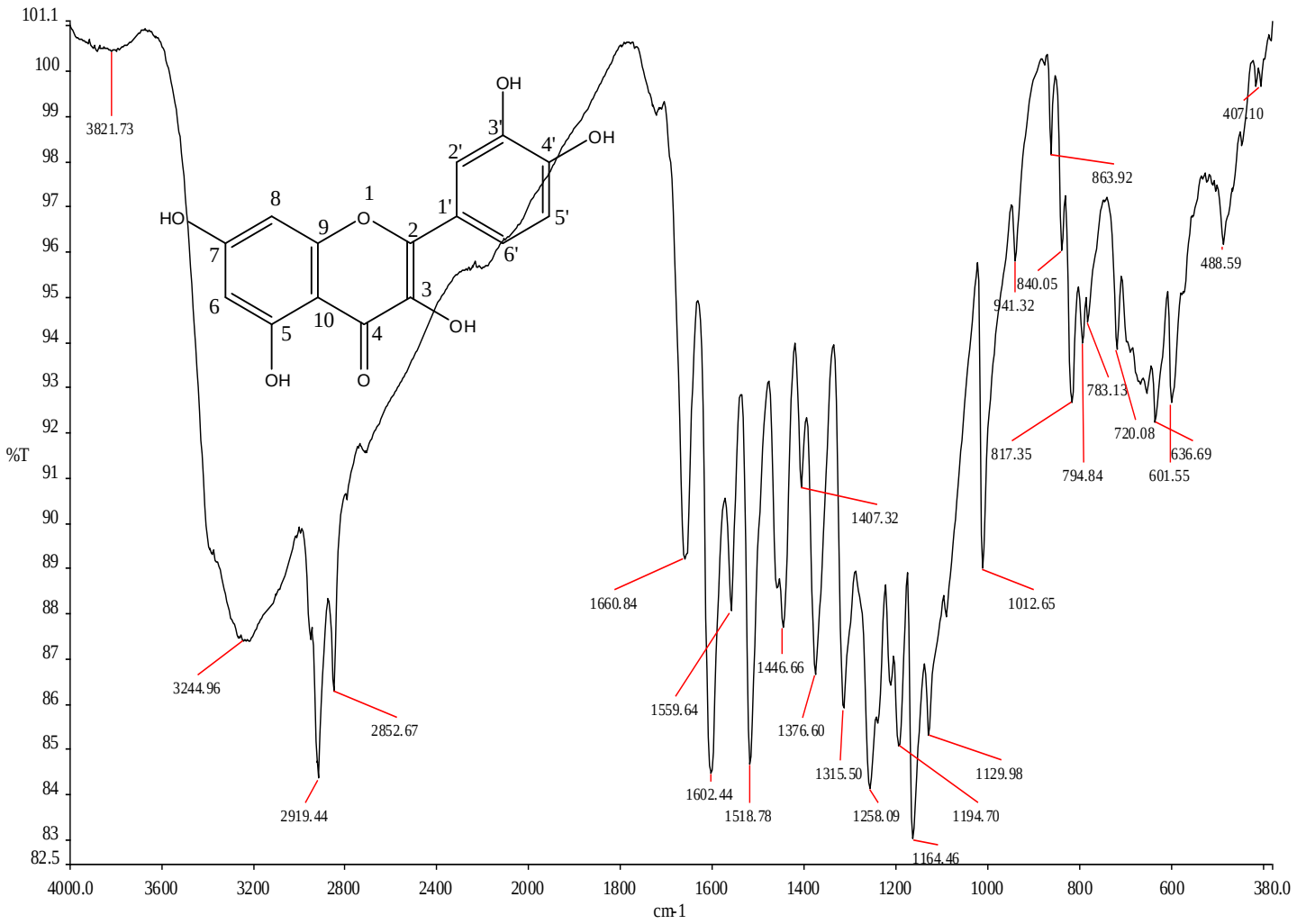
HSQC NMR spectrum of quercetin in MeOD



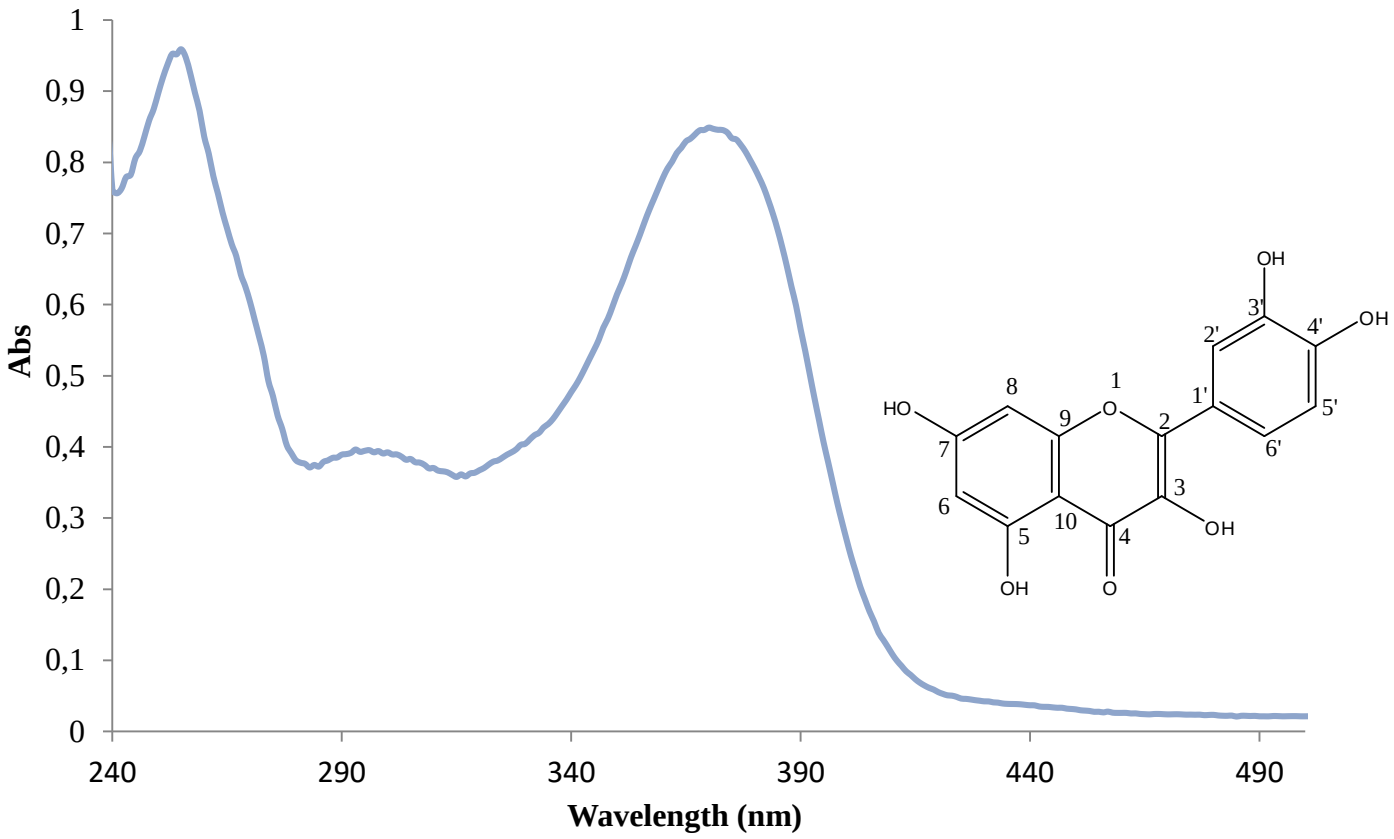
HMBC NMR spectrum of quercetin in MeOD



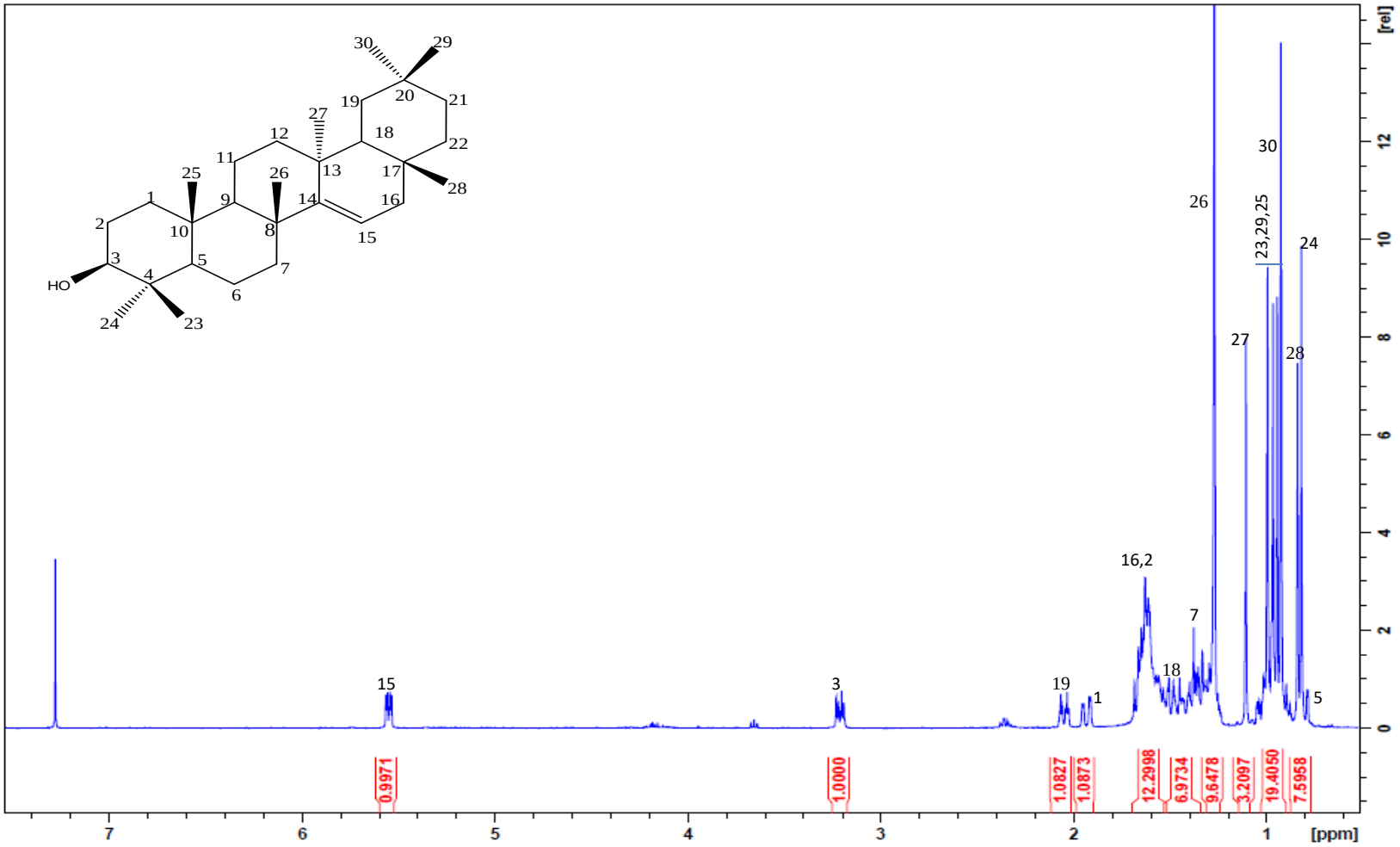
Mass spectrum of quercetin



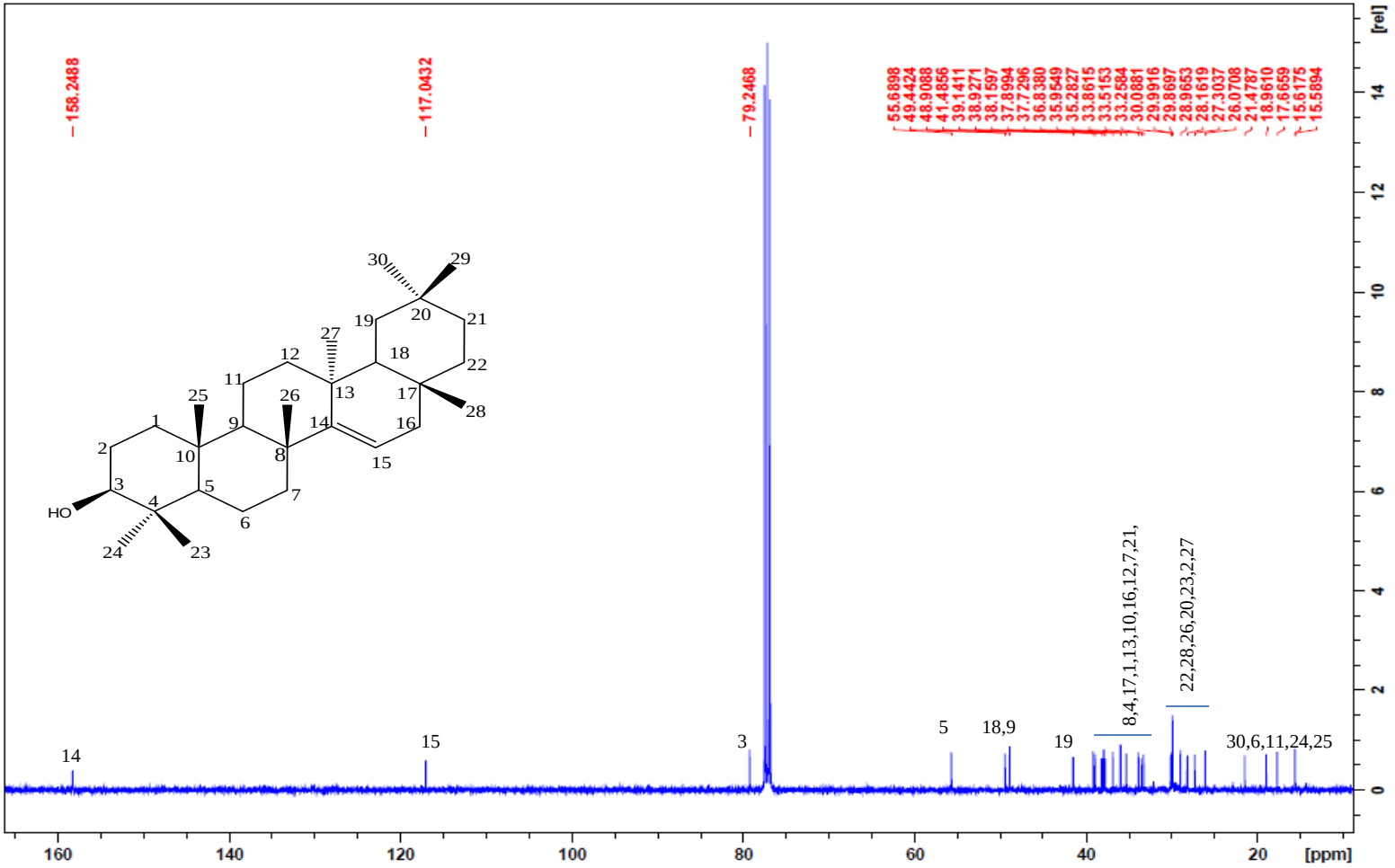
IR spectrum of quercetin



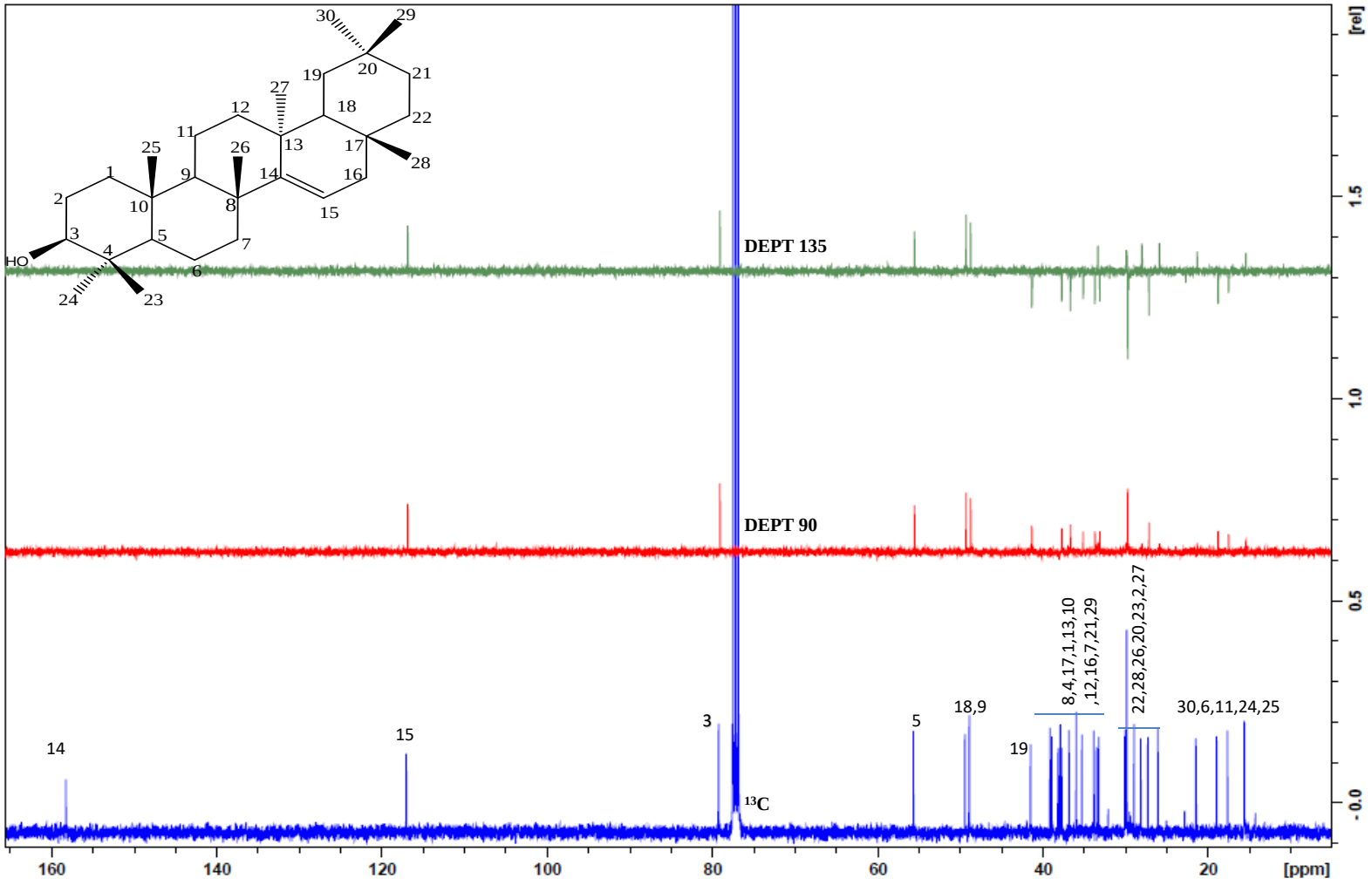
UV spectrum of quercetin



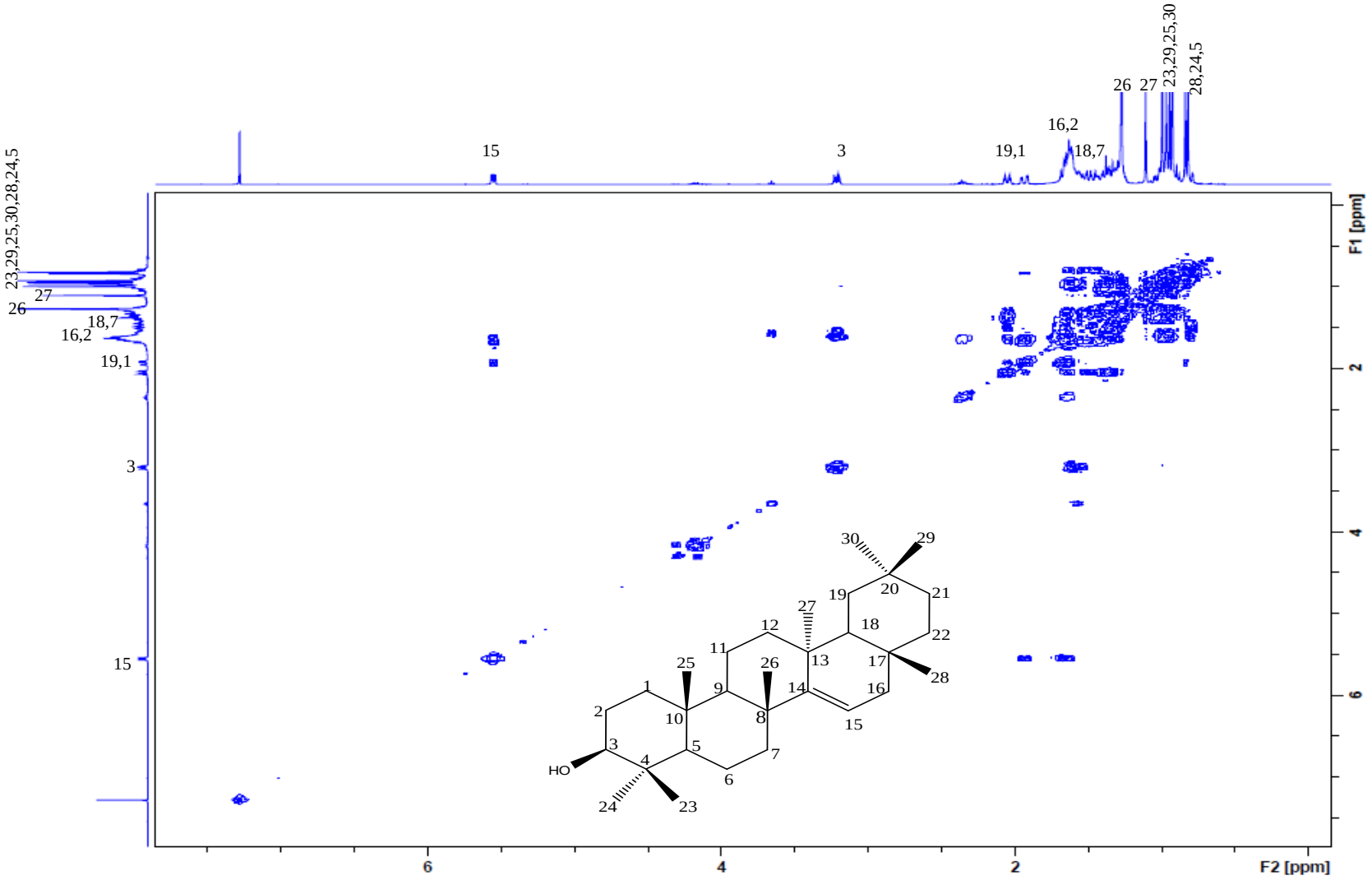
^{1}H NMR spectrum of 3β-Taraxerol in $CDCL_3$



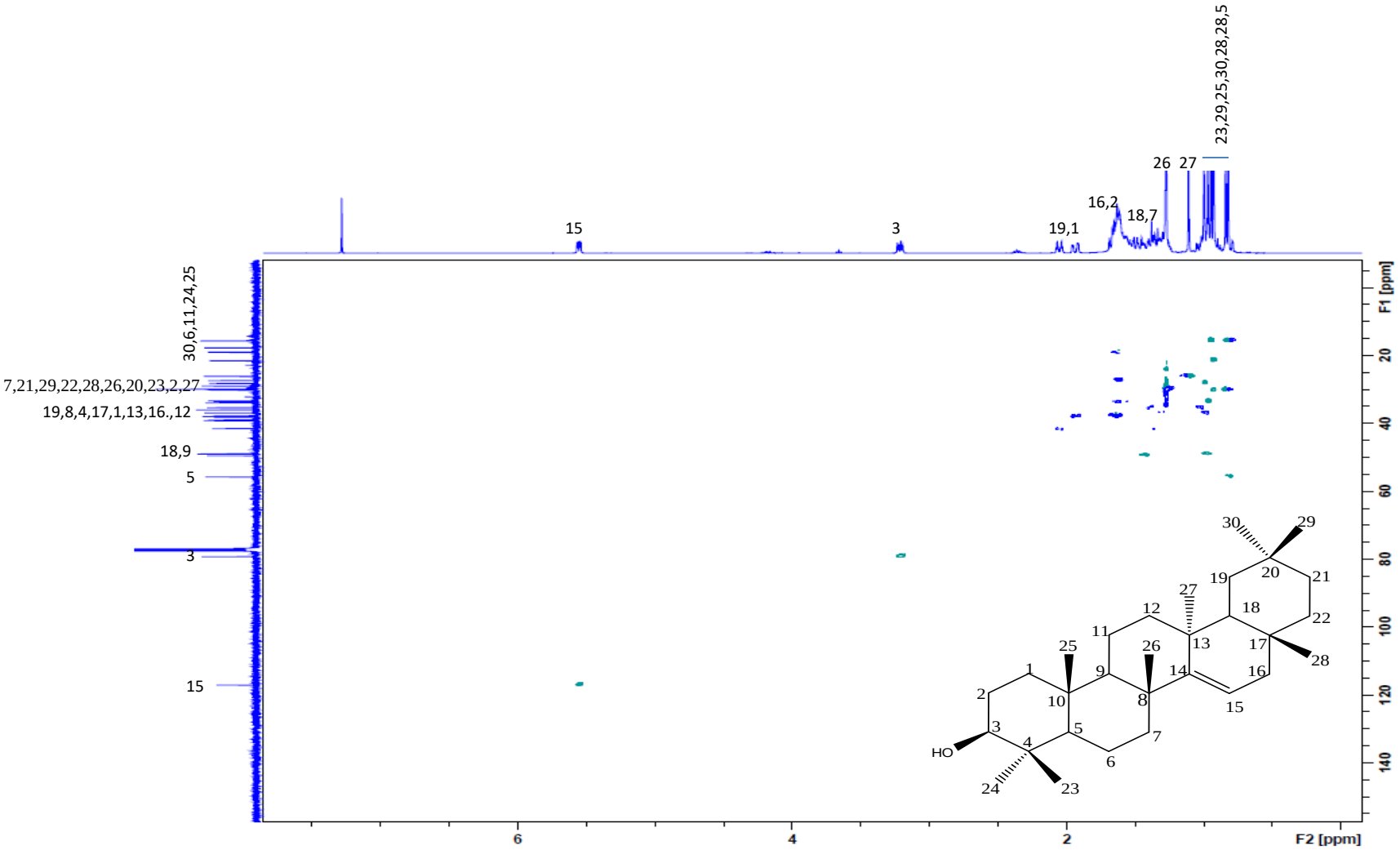
^{13}C NMR spectrum of 3β-Taraxerol in $CDCL_3$



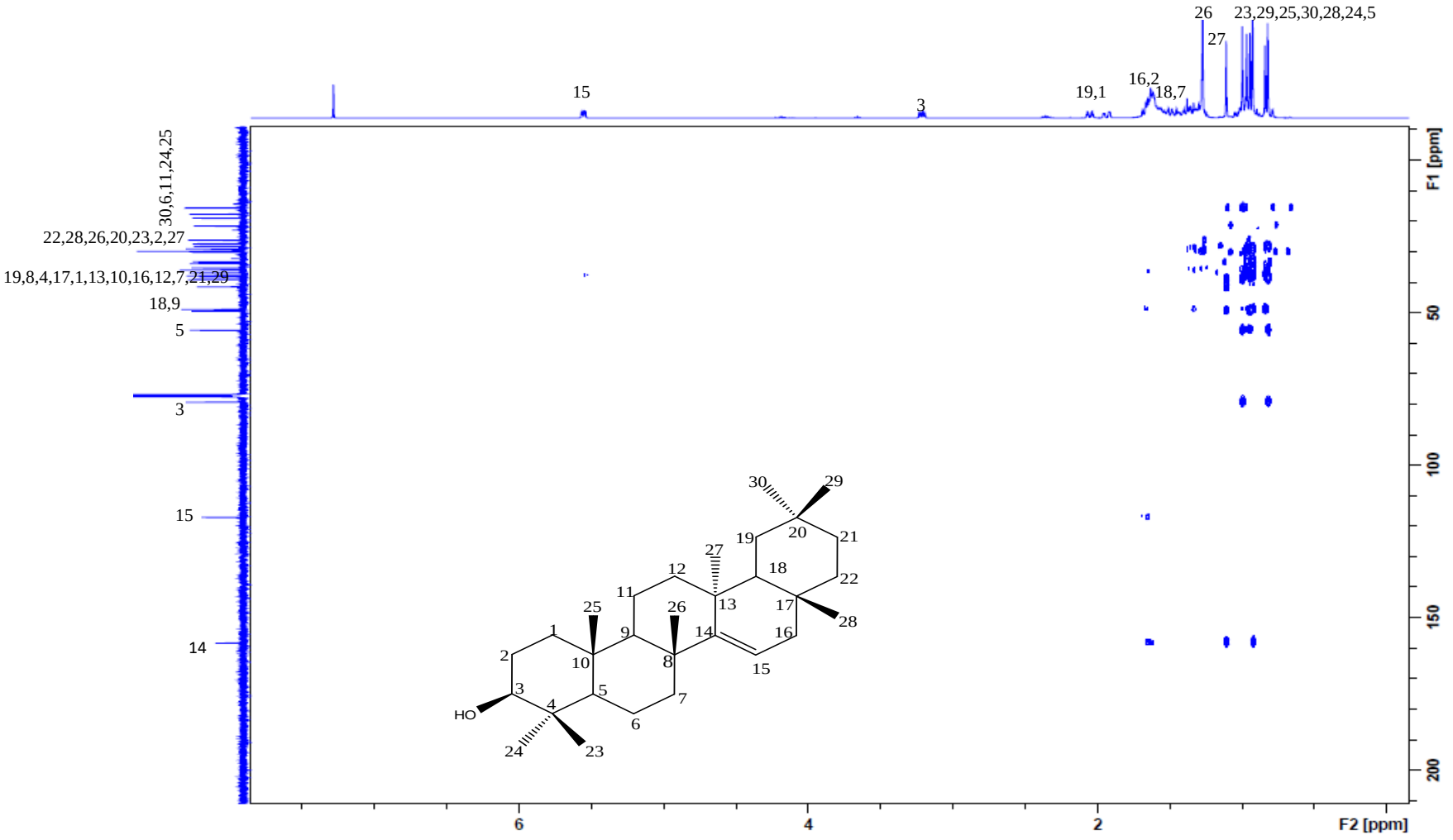
DEPT NMR spectrum of 3β-Taraxerol in $CDCL_3$



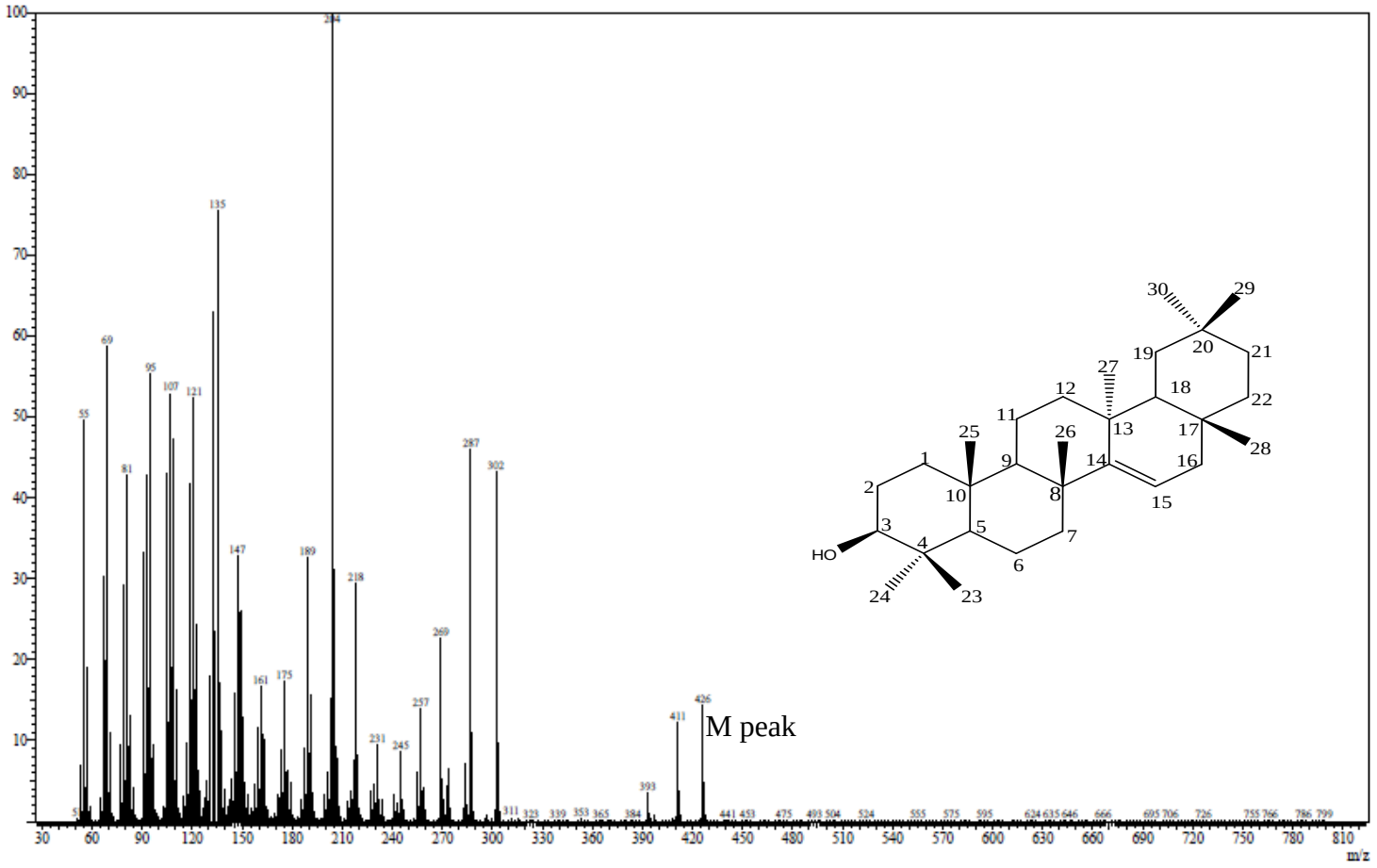
COSY NMR spectrum of 3β-Taraxerol in $CDCL_3$



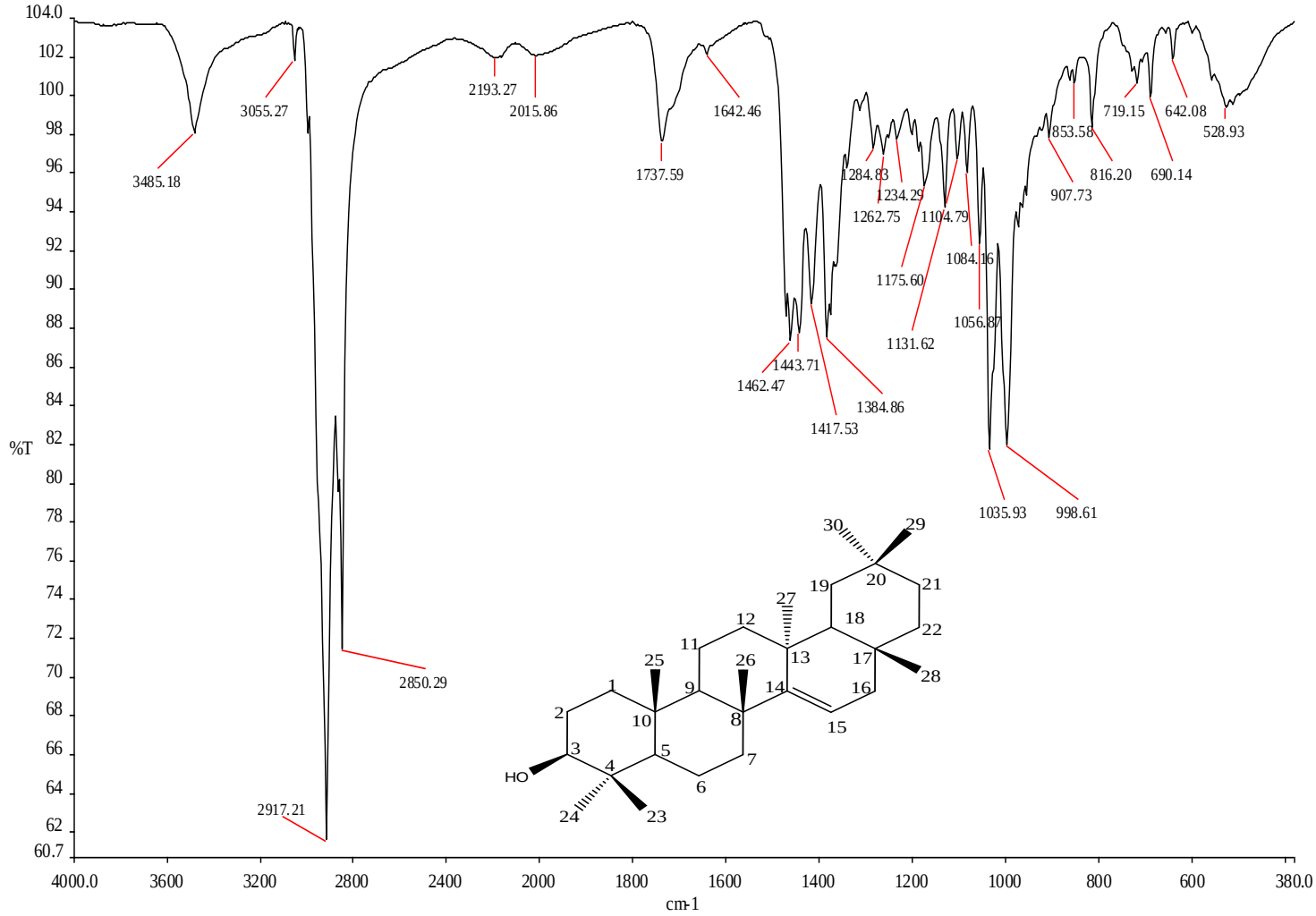
HSQC NMR spectrum of 3β-Taraxerol in $CDCL_3$



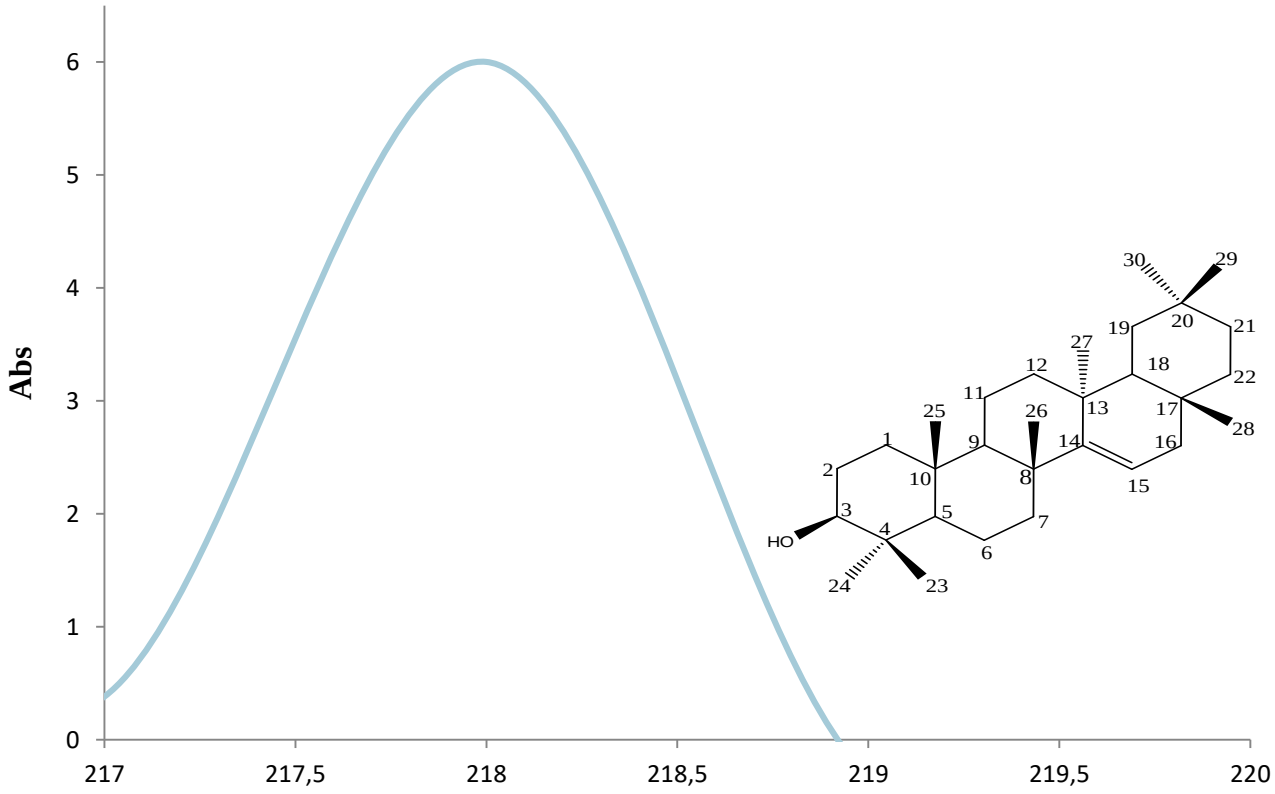
HMBC NMR spectrum of 3β-Taraxerol in $CDCL_3$



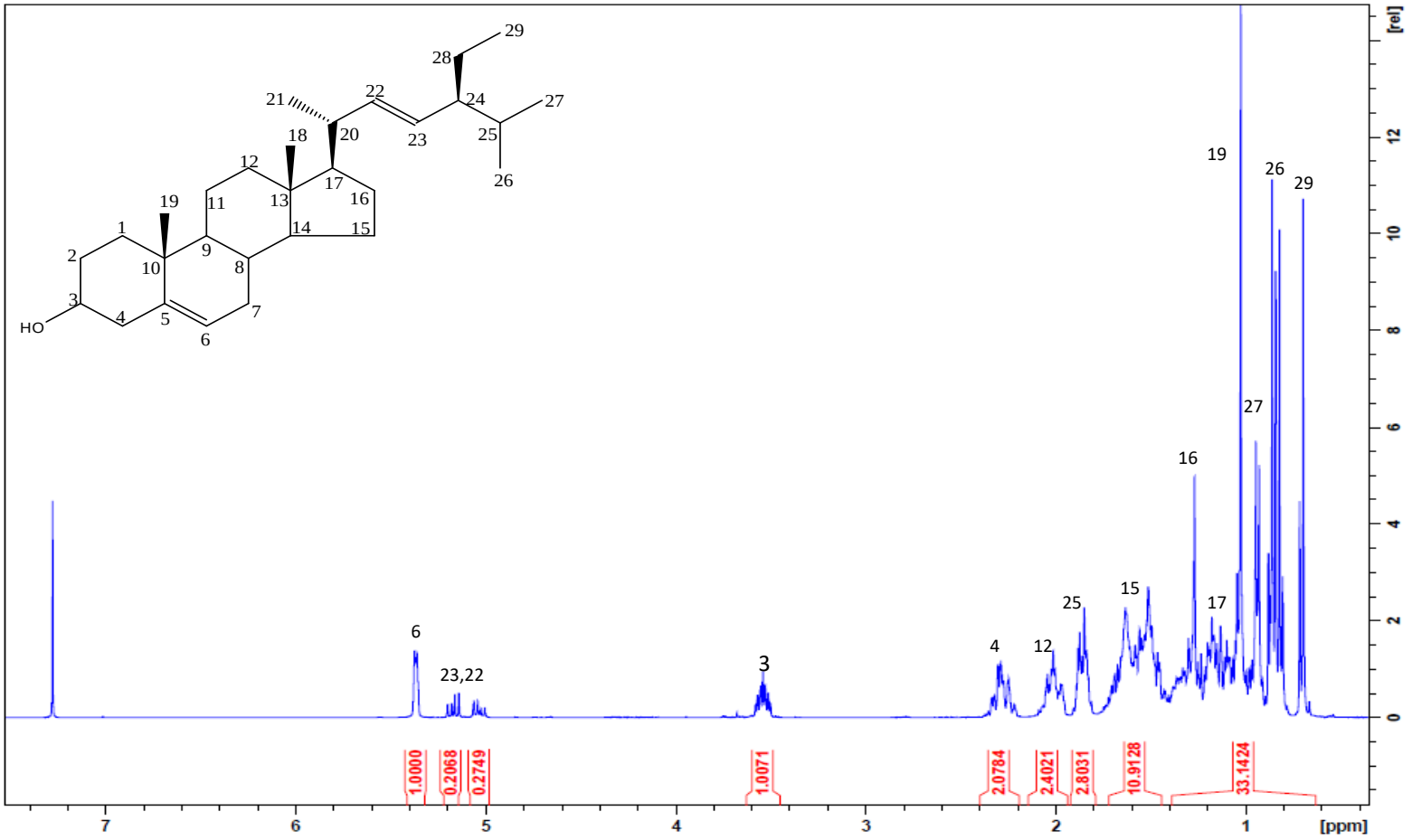
Mass spectrum of 3β -Taraxerol



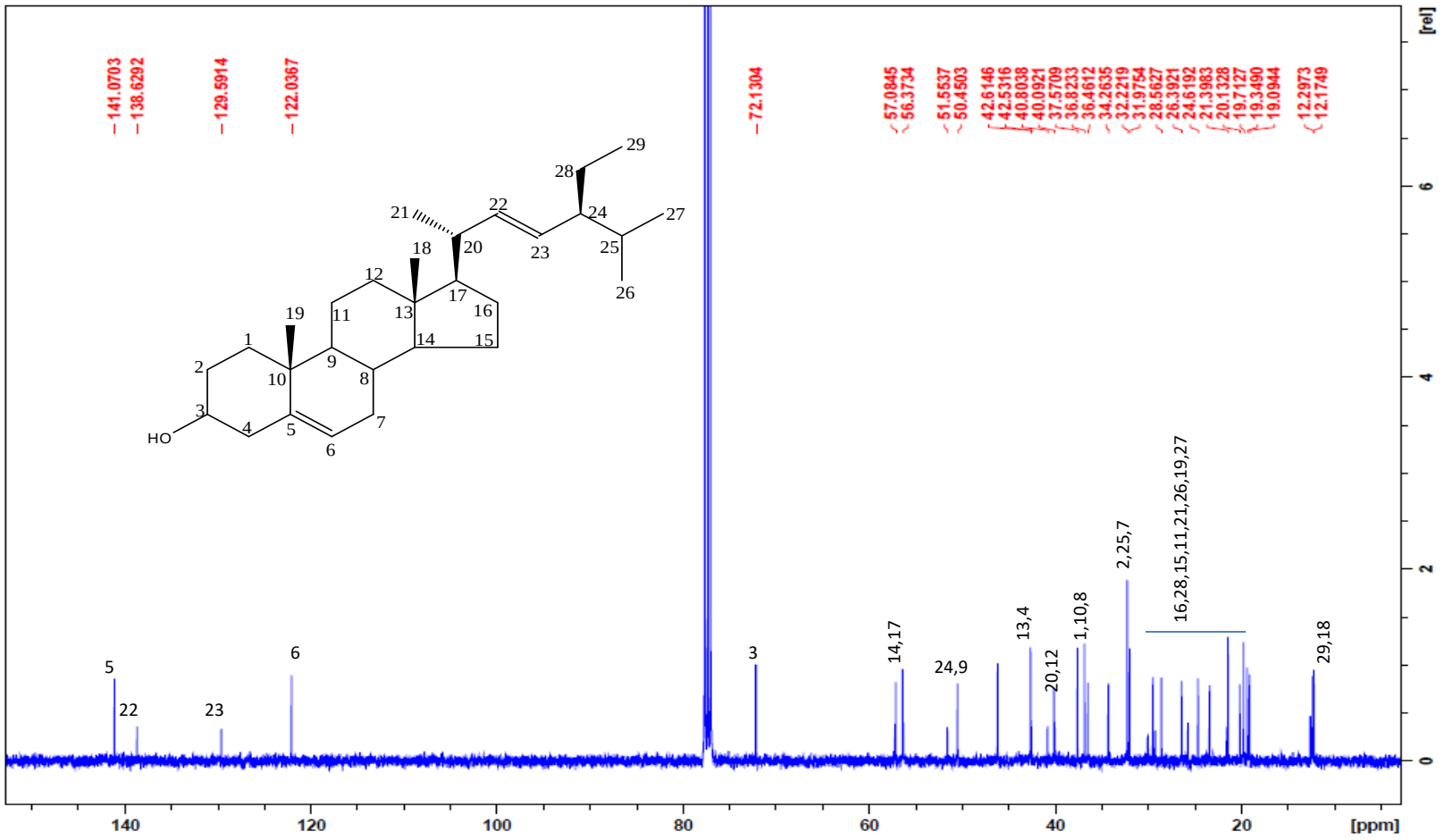
IR spectrum of 3β-Taraxerol



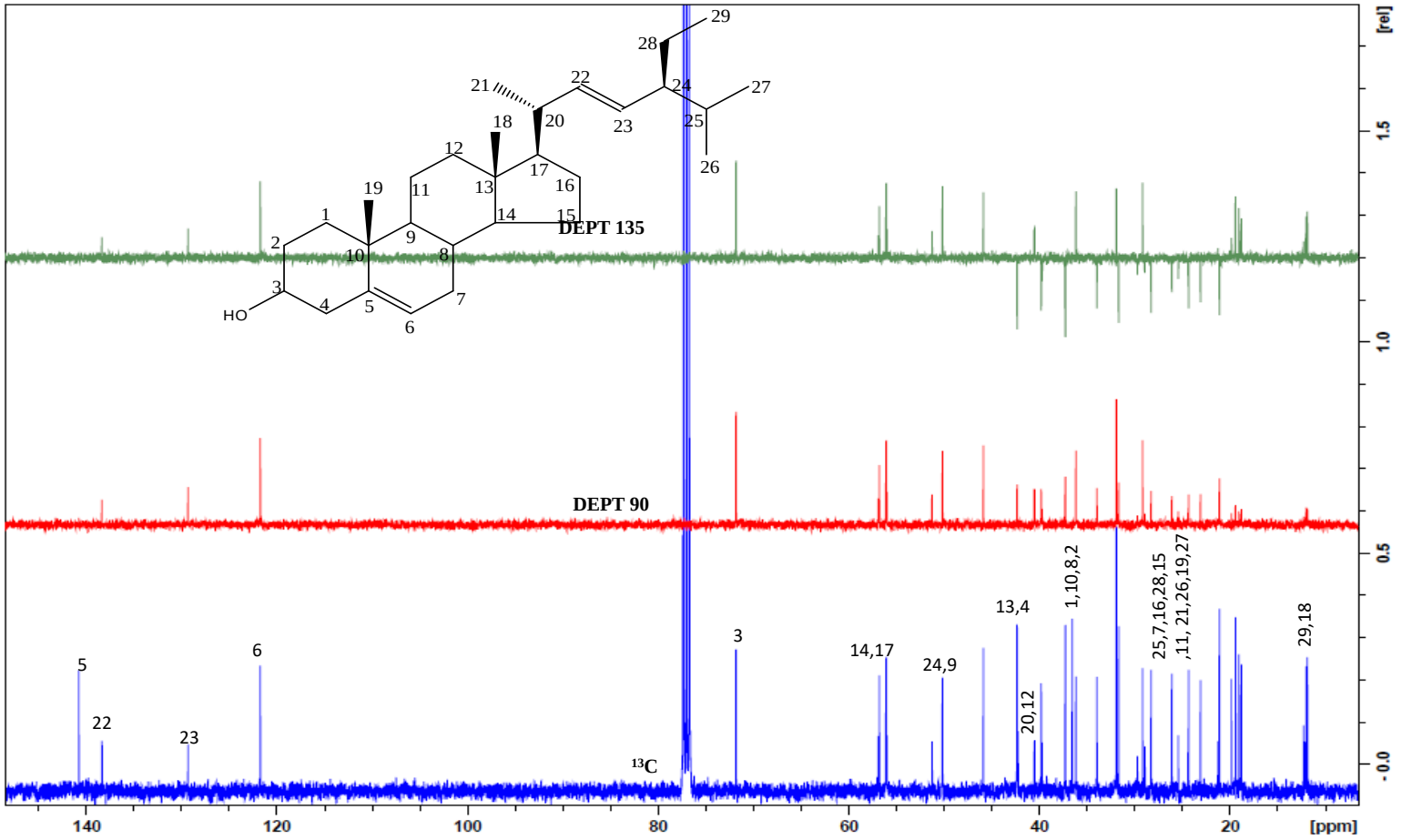
UV spectrum of 3β-Taraxerol



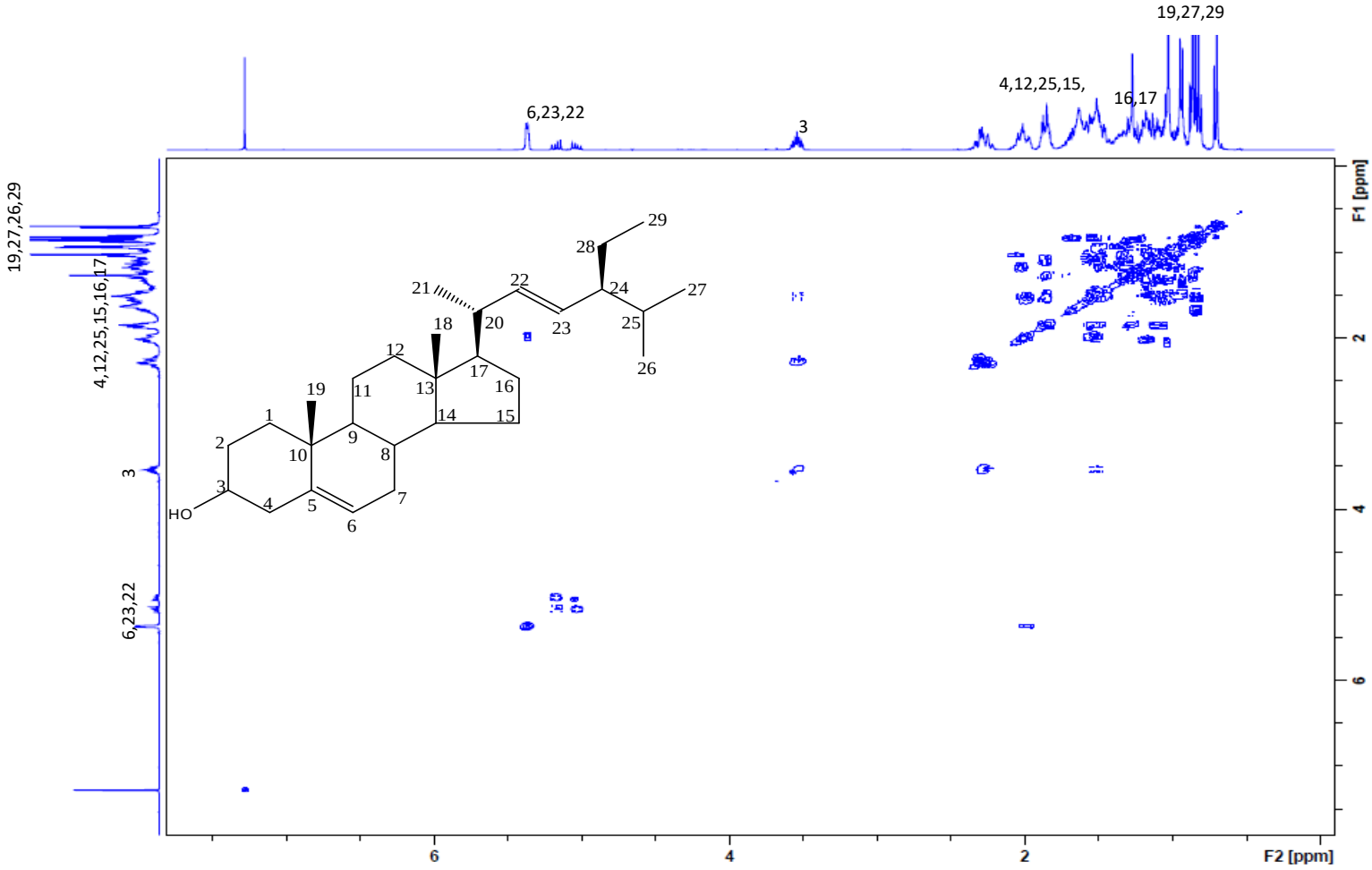
^{1}H NMR spectrum of stigmasterol in $CDCL_3$



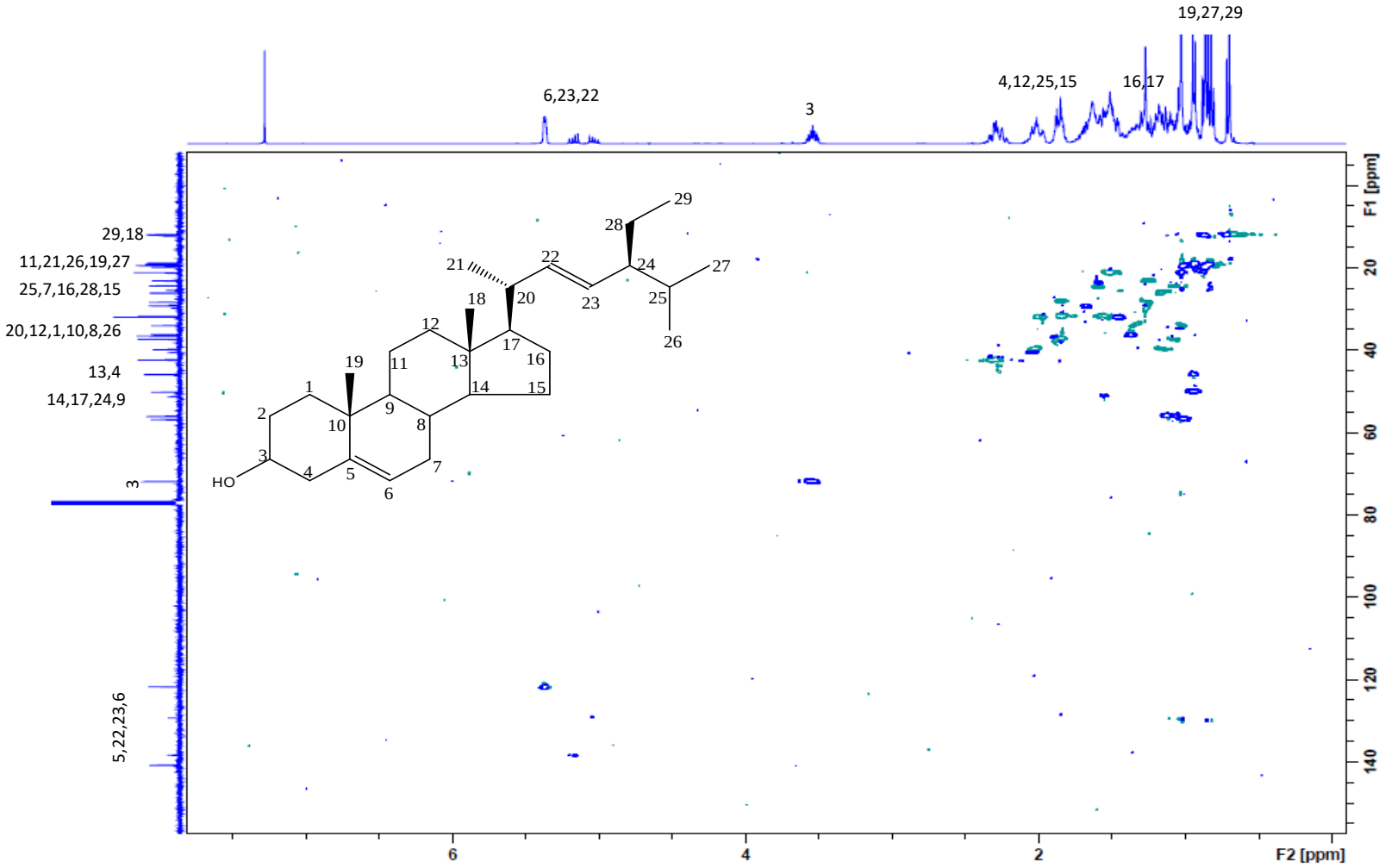
^{13}C NMR spectrum of stigmasterol in $CDCL_3$



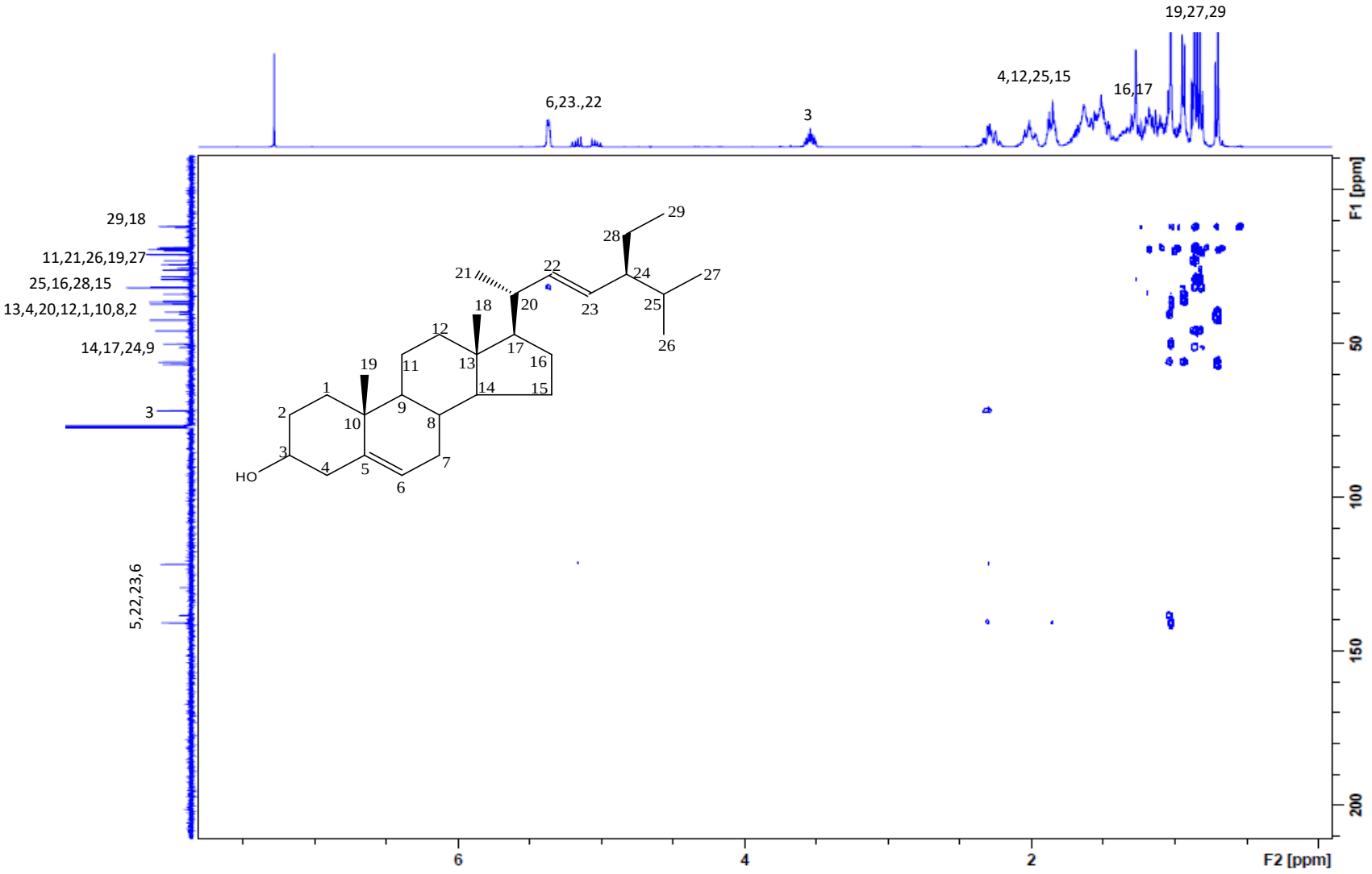
DEPT NMR spectrum of stigmasterol in $CDCL_3$



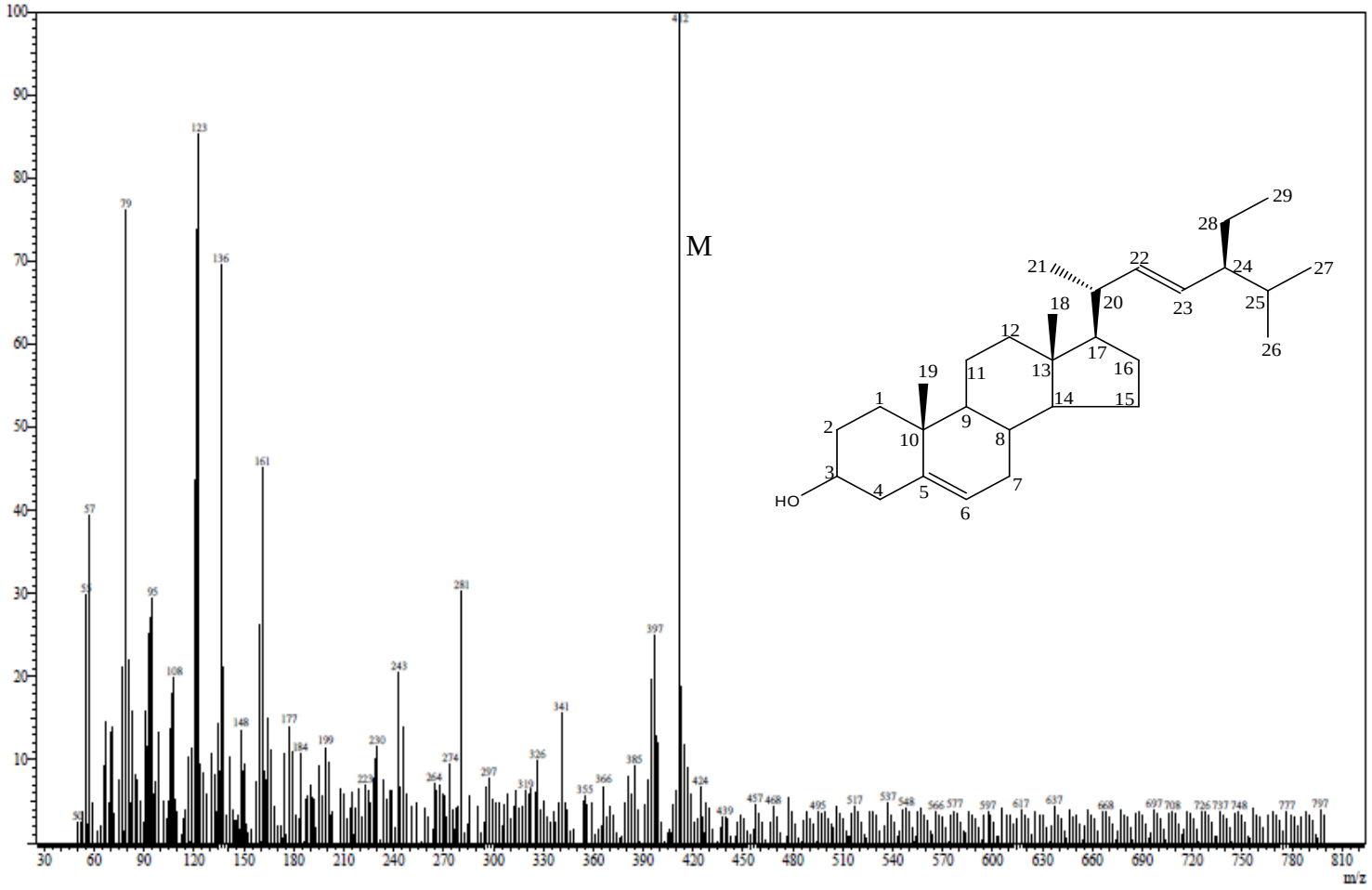
COSY NMR spectrum of stigmasterol in $CDCL_3$



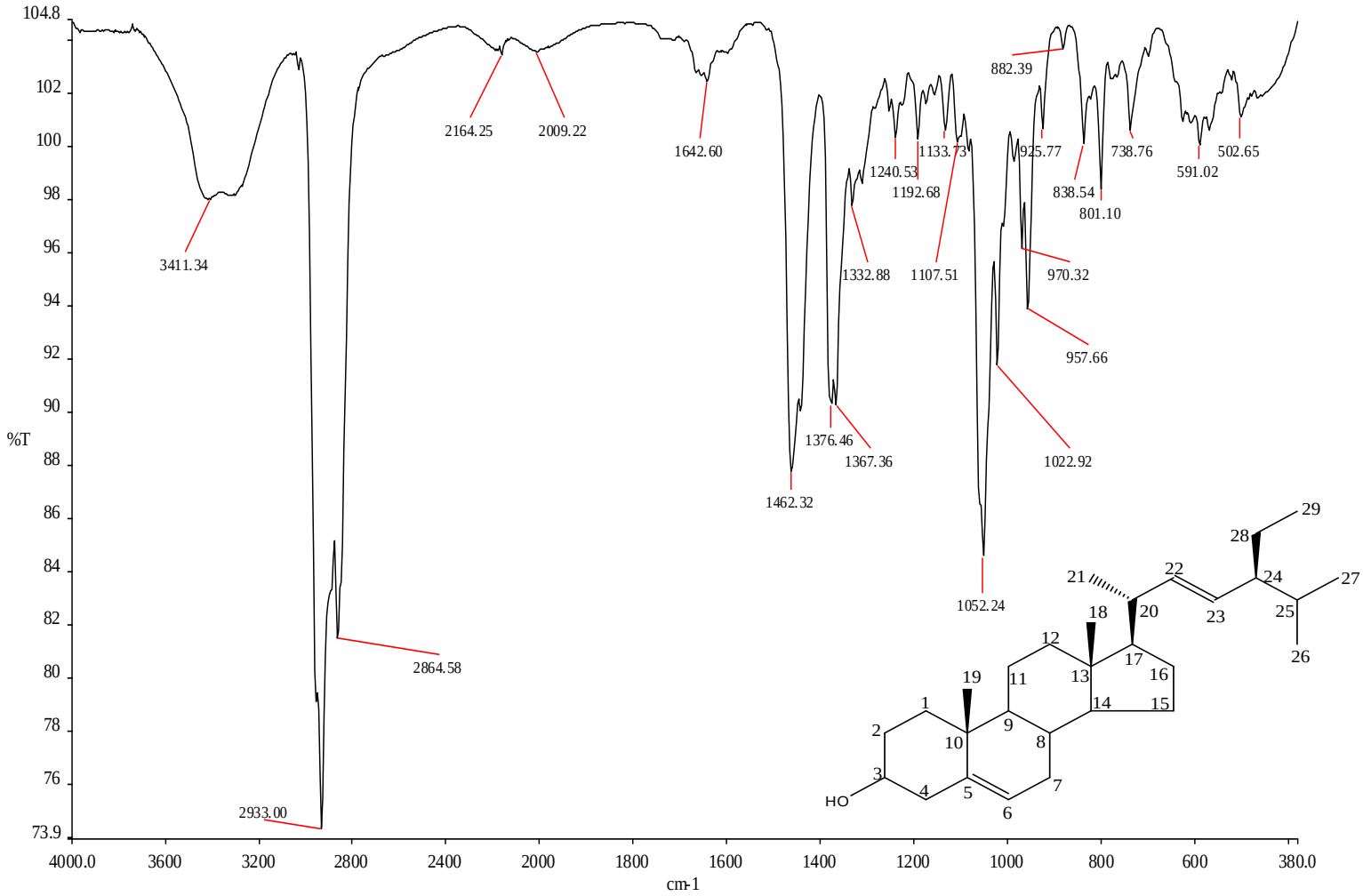
HSQC NMR spectrum of stigmasterol in $CDCL_3$



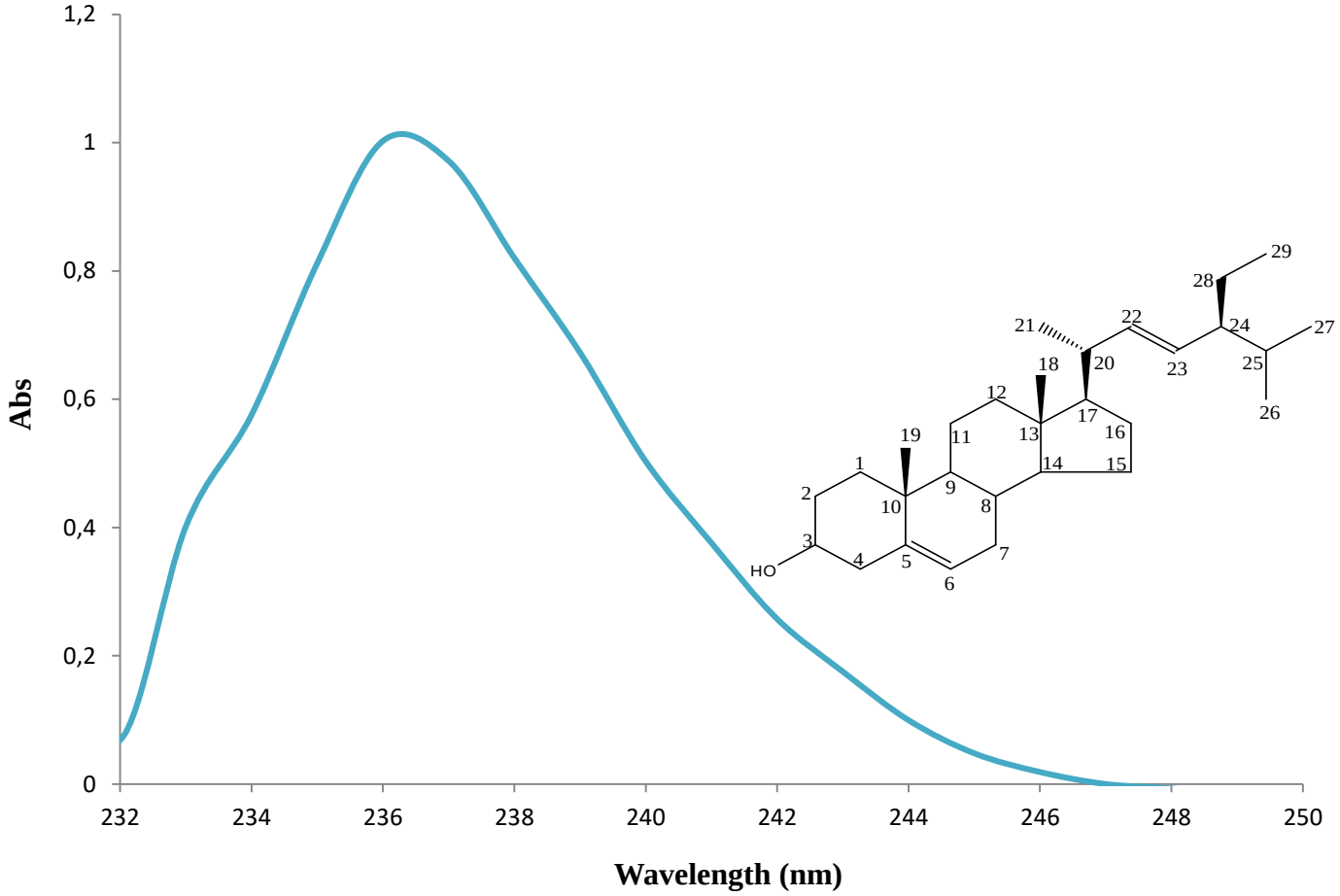
HMBC NMR spectrum of stigmasterol in $CDCL_3$



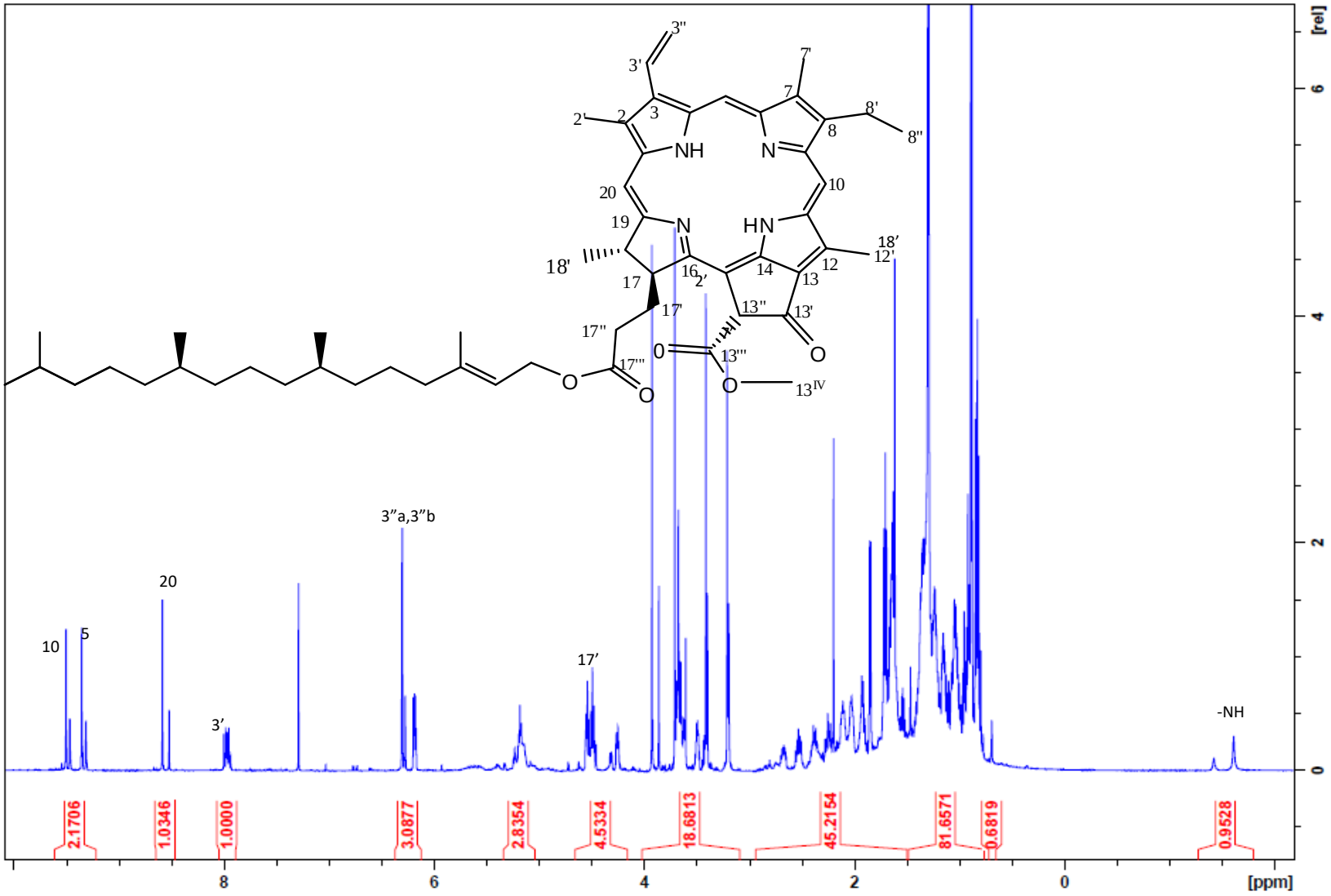
Mass spectrum of stigmasterol



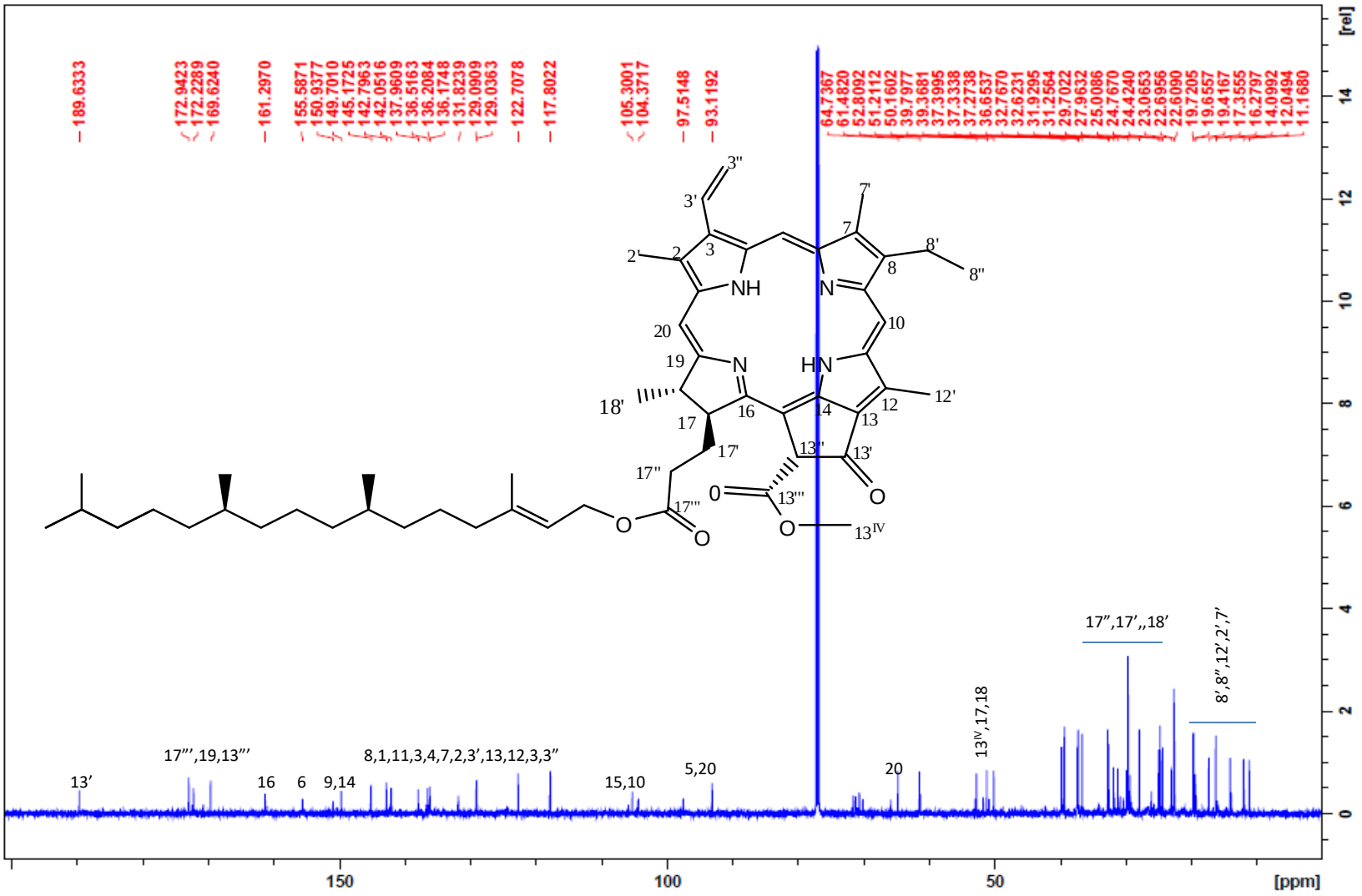
IR spectrum of stigmasterol



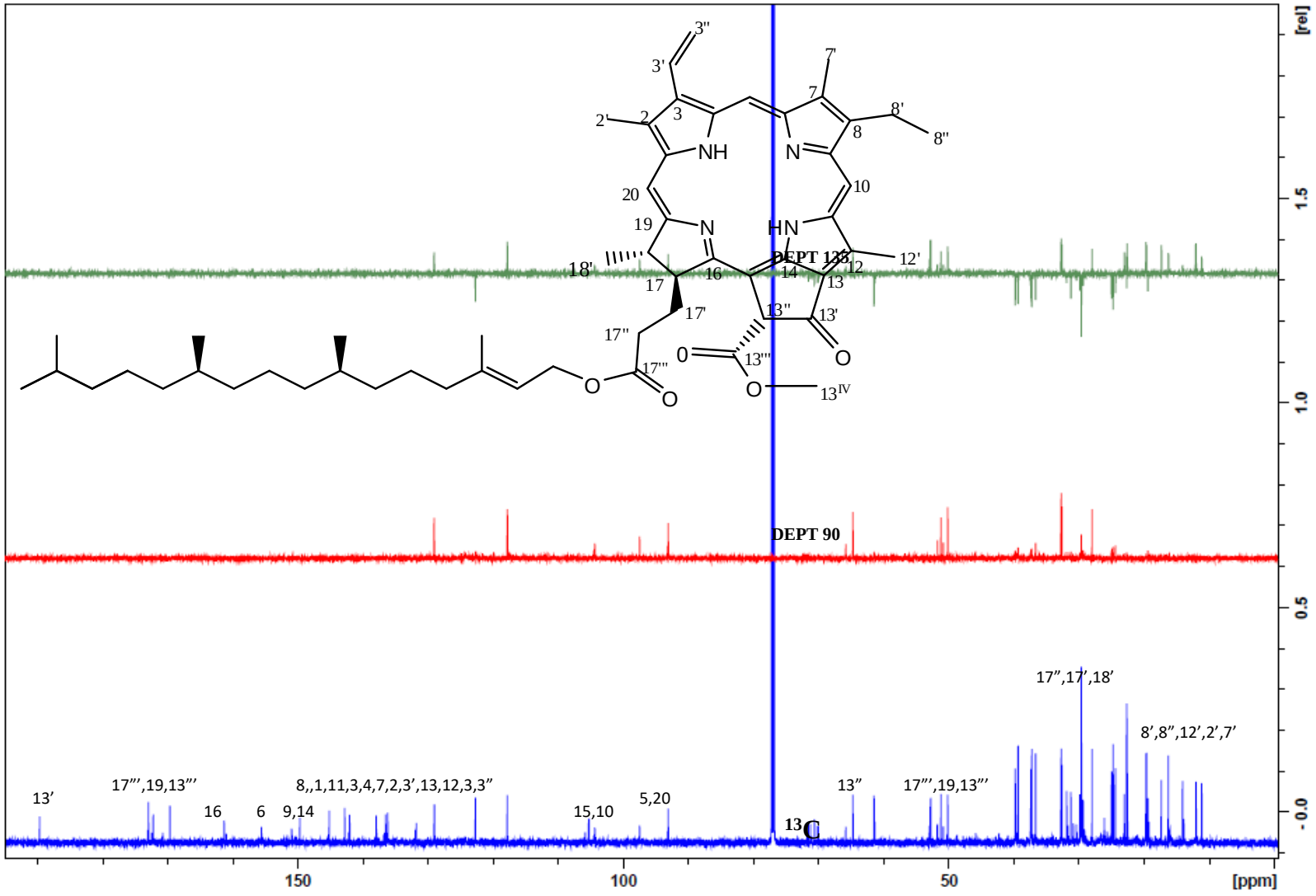
UV spectrum of stigmasterol in DCM



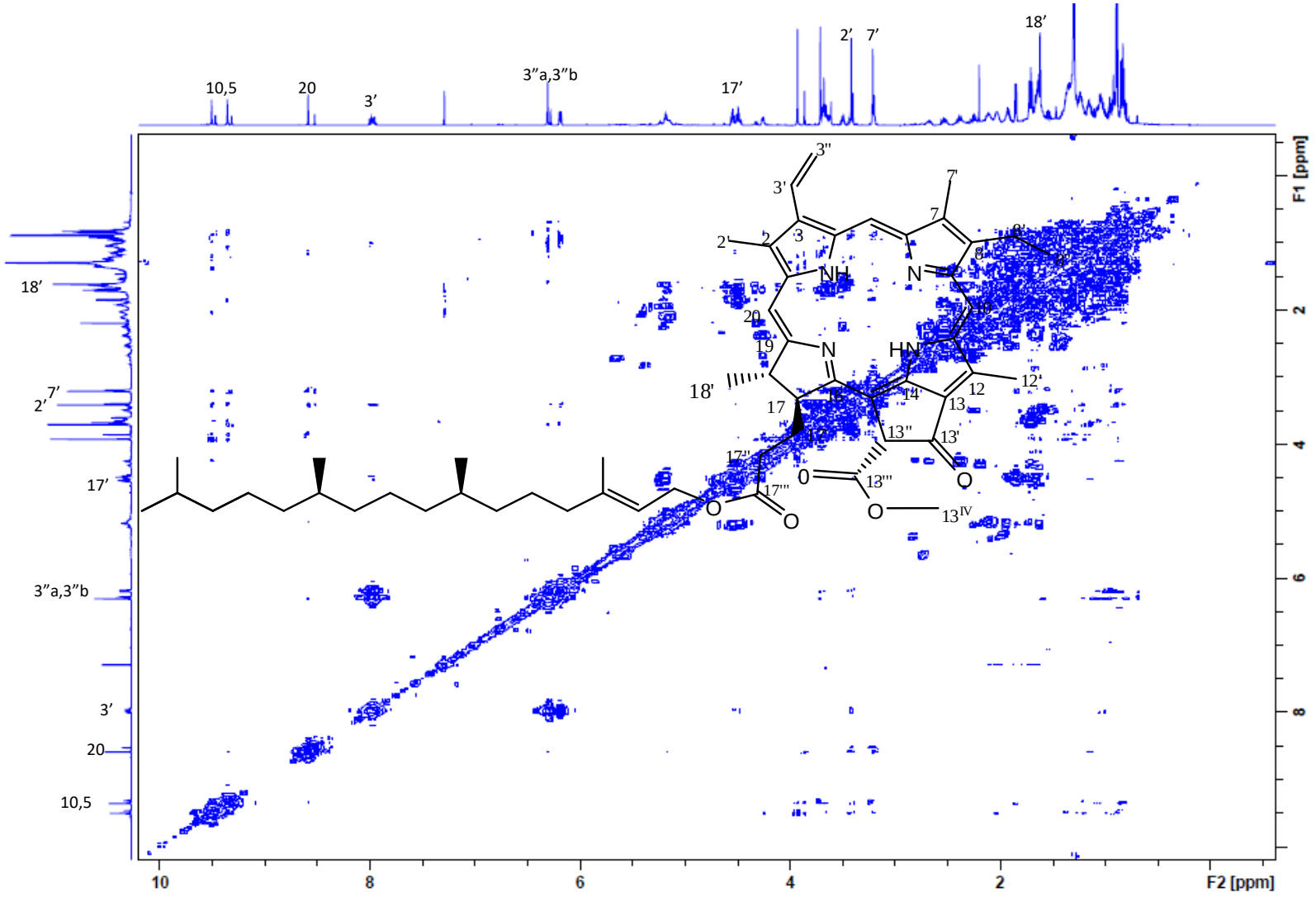
^{1}H NMR spectrum of pheophytin a in $CDCL_3$



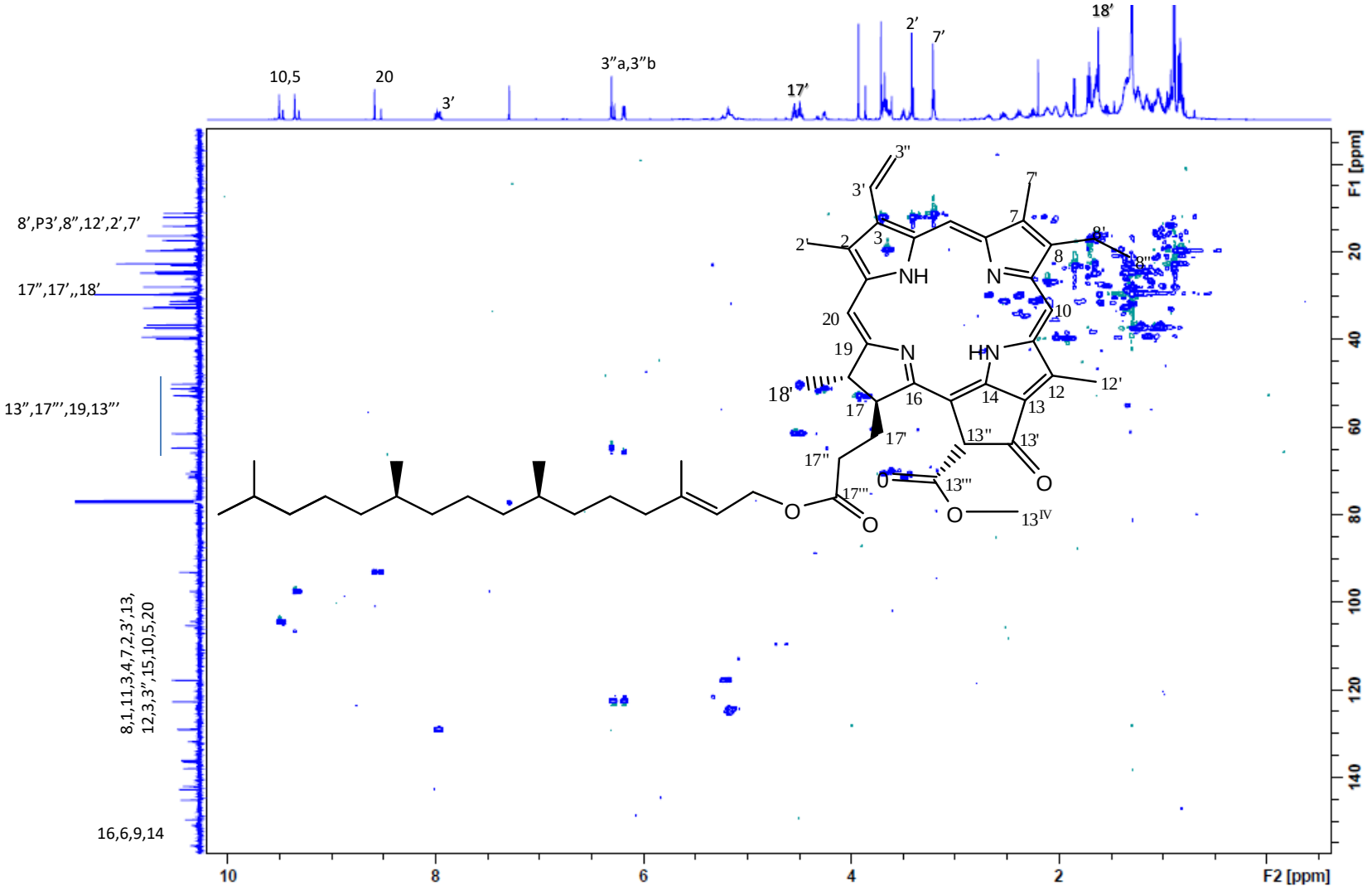
^{13}C NMR spectrum of pheophytin a in $CDCL_3$



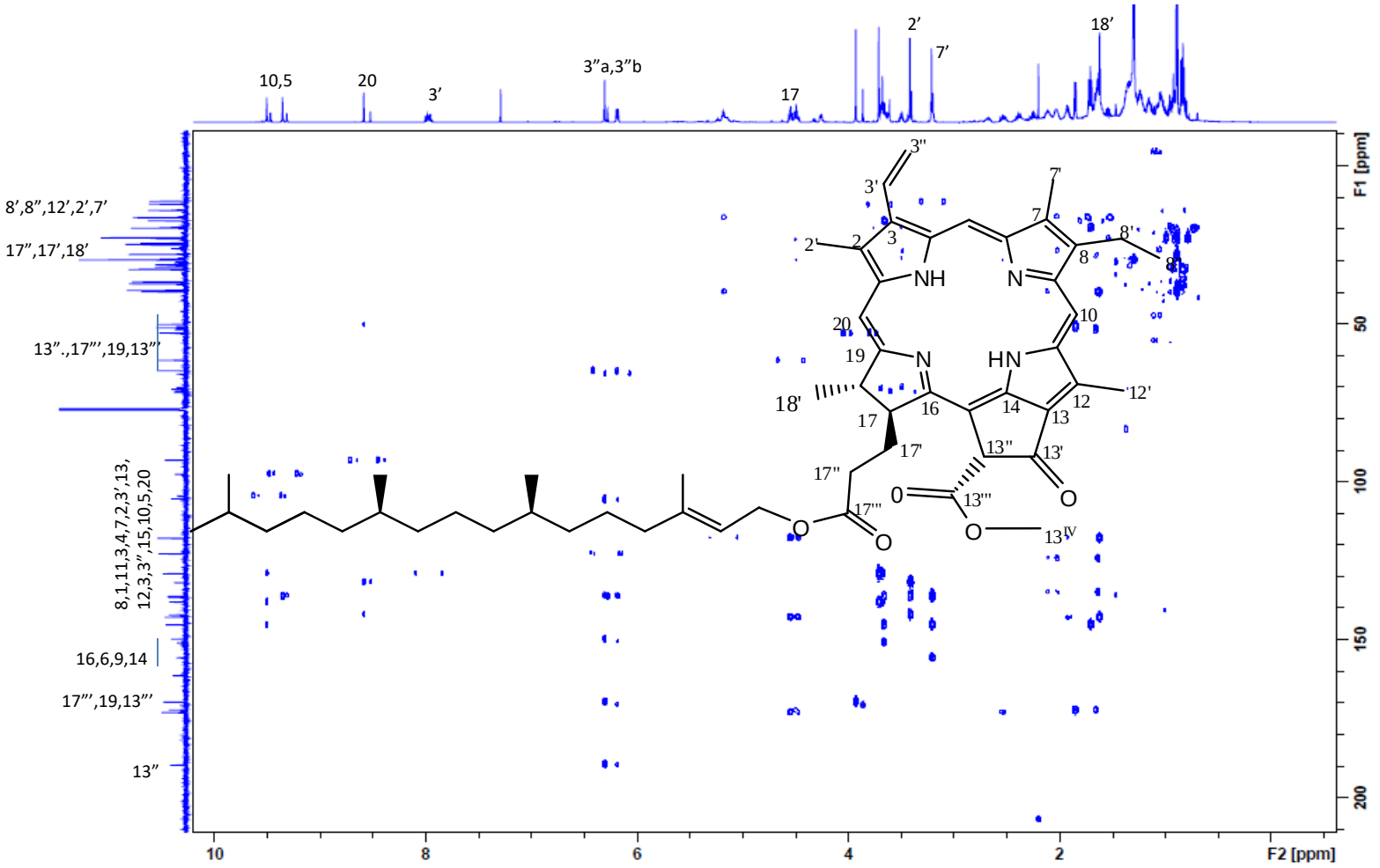
^{13}C NMR spectrum of pheophytin a in $CDCL_3$



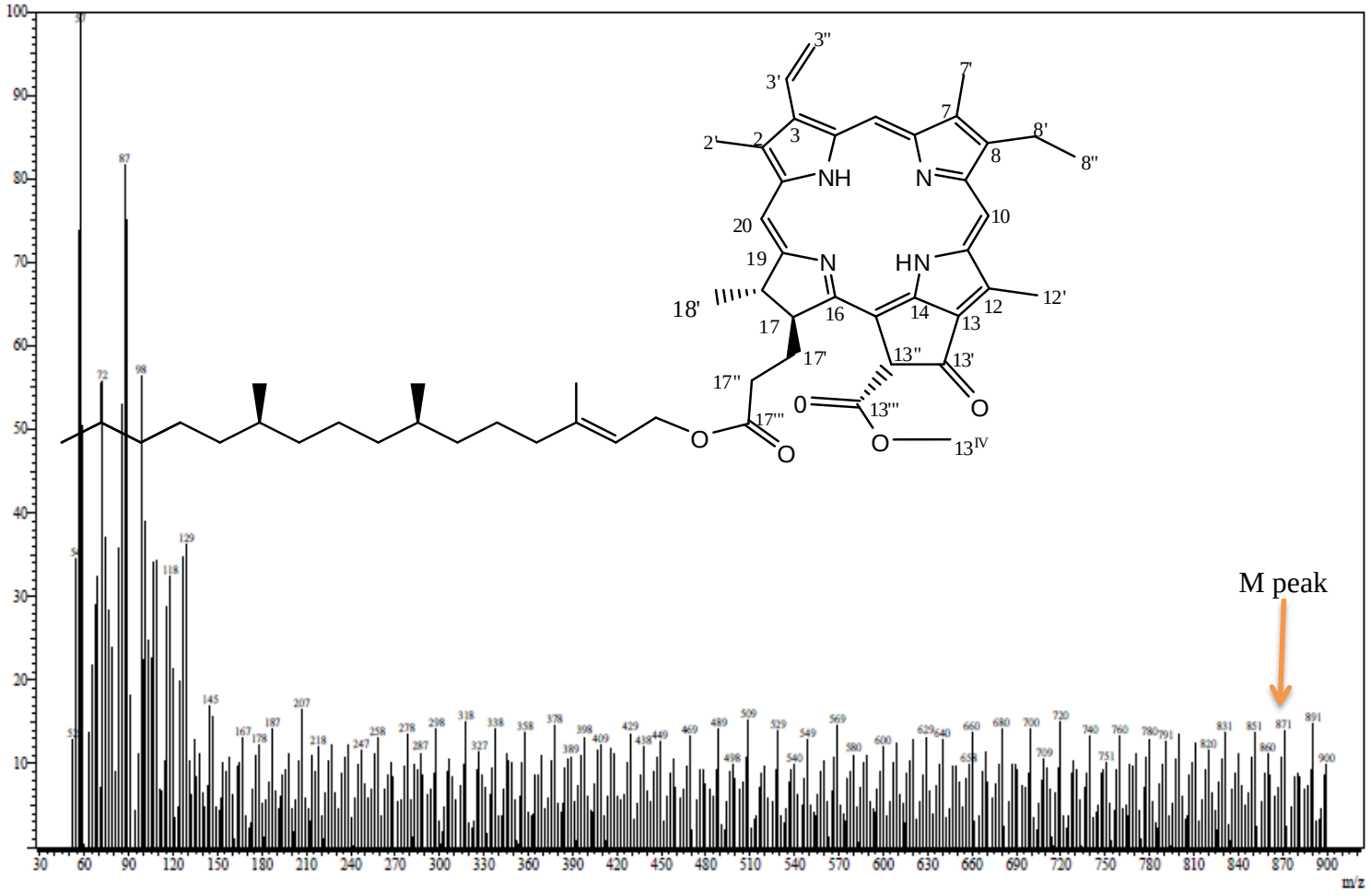
COSY NMR spectrum of pheophytin a in $CDCL_3$



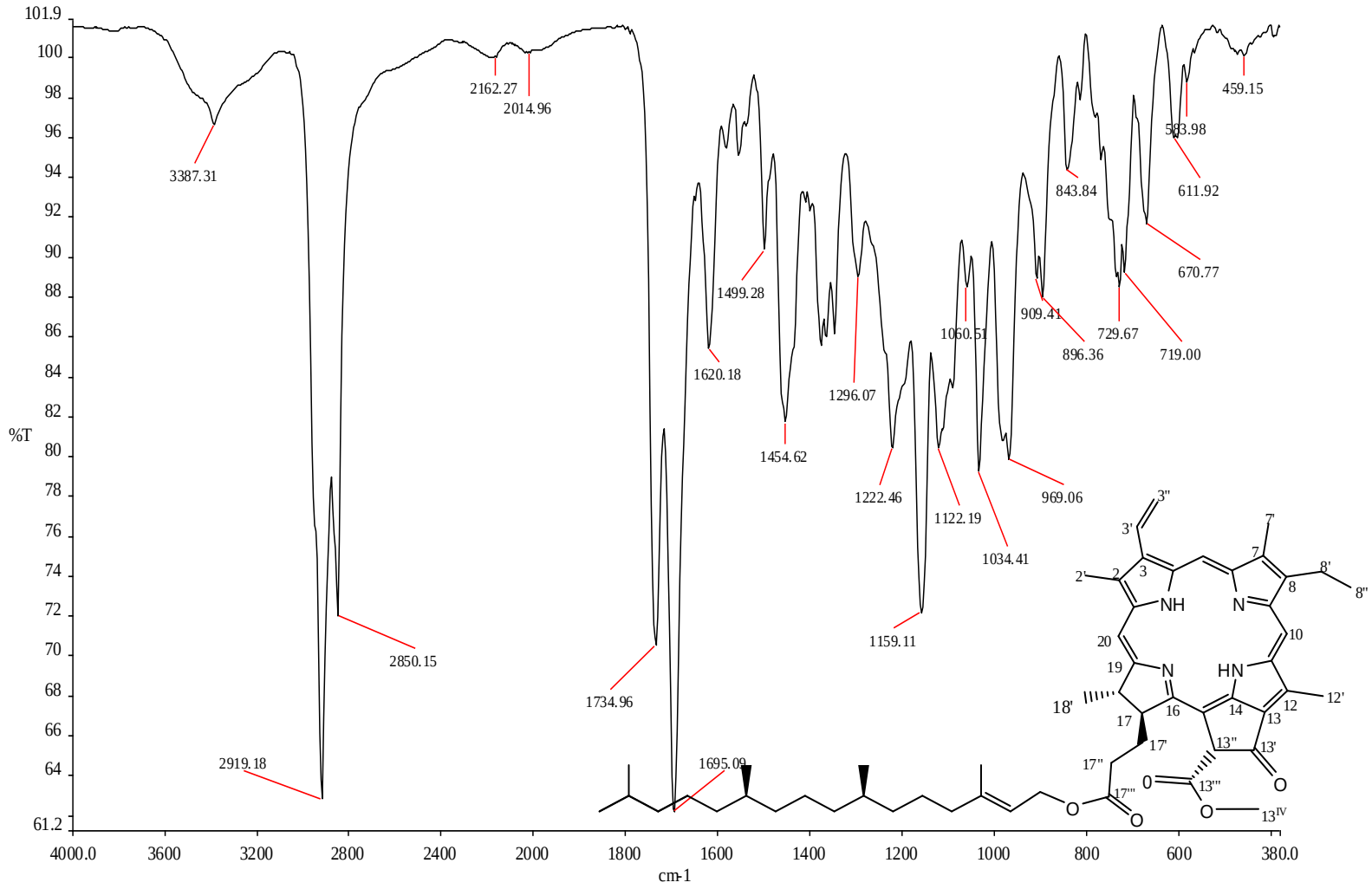
HSQC NMR spectrum of pheophytin a in $CDCL_3$



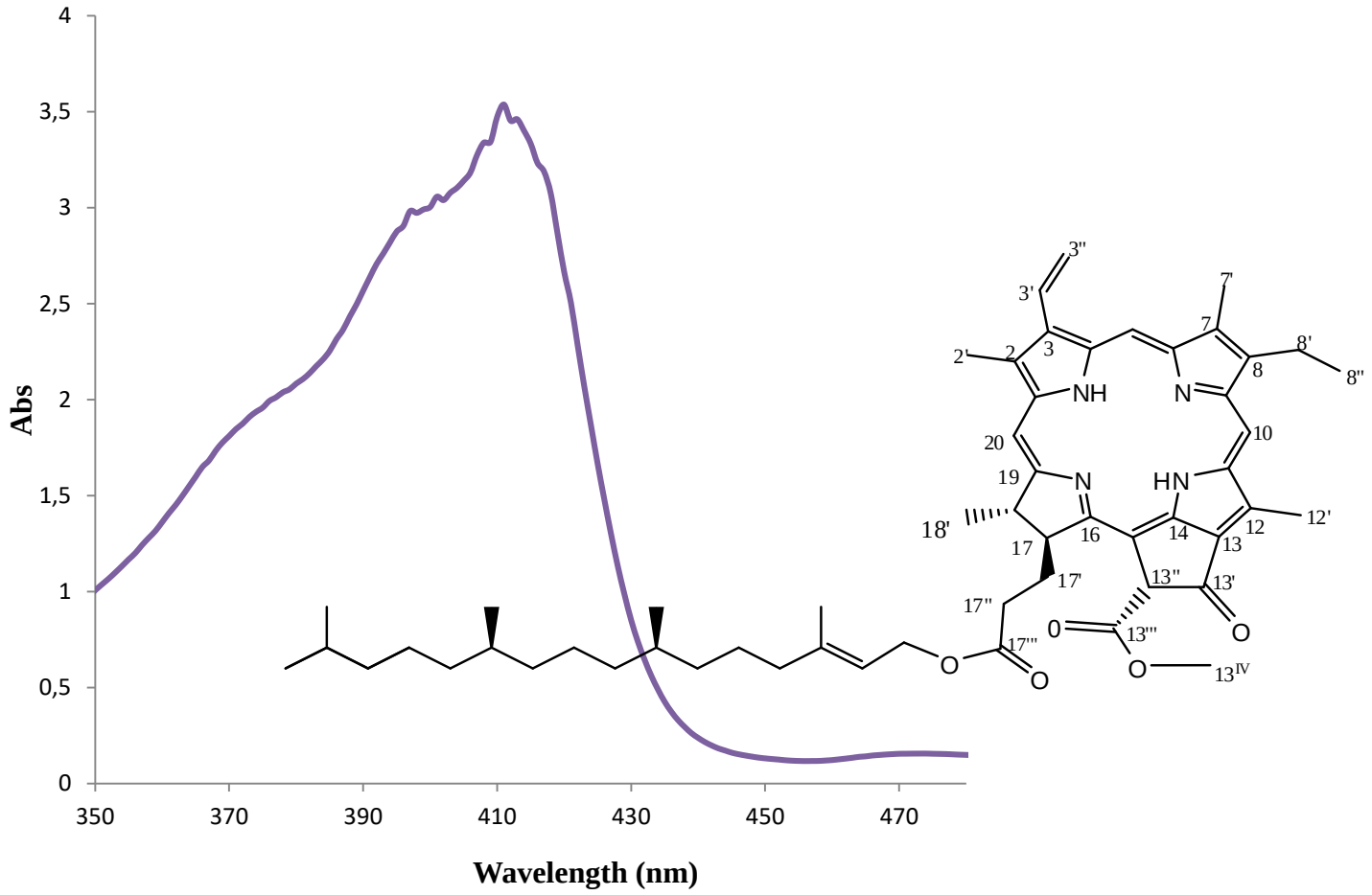
HMBC NMR spectrum of pheophytin a in $CDCL_3$



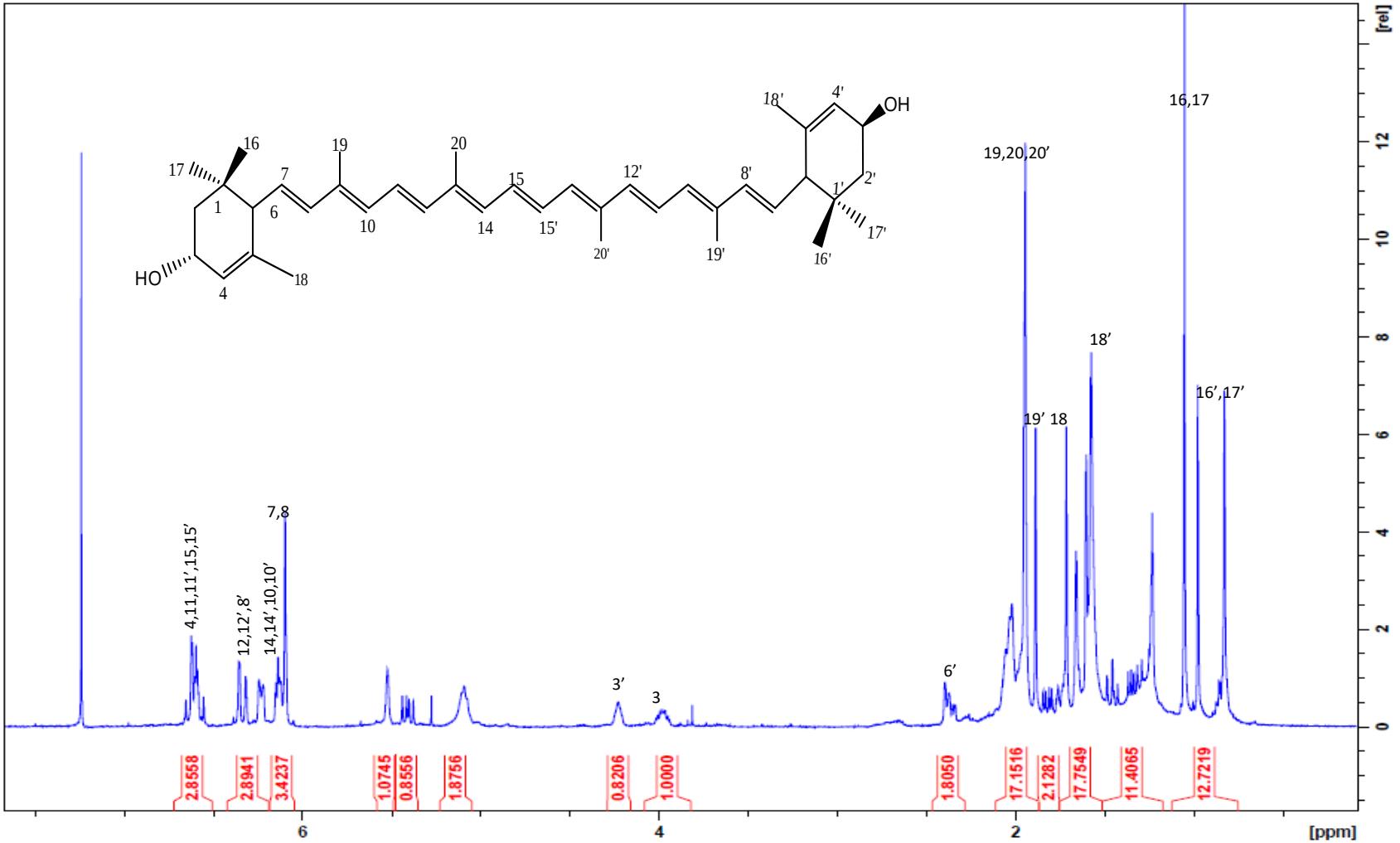
Mass spectrum of pheophytin a



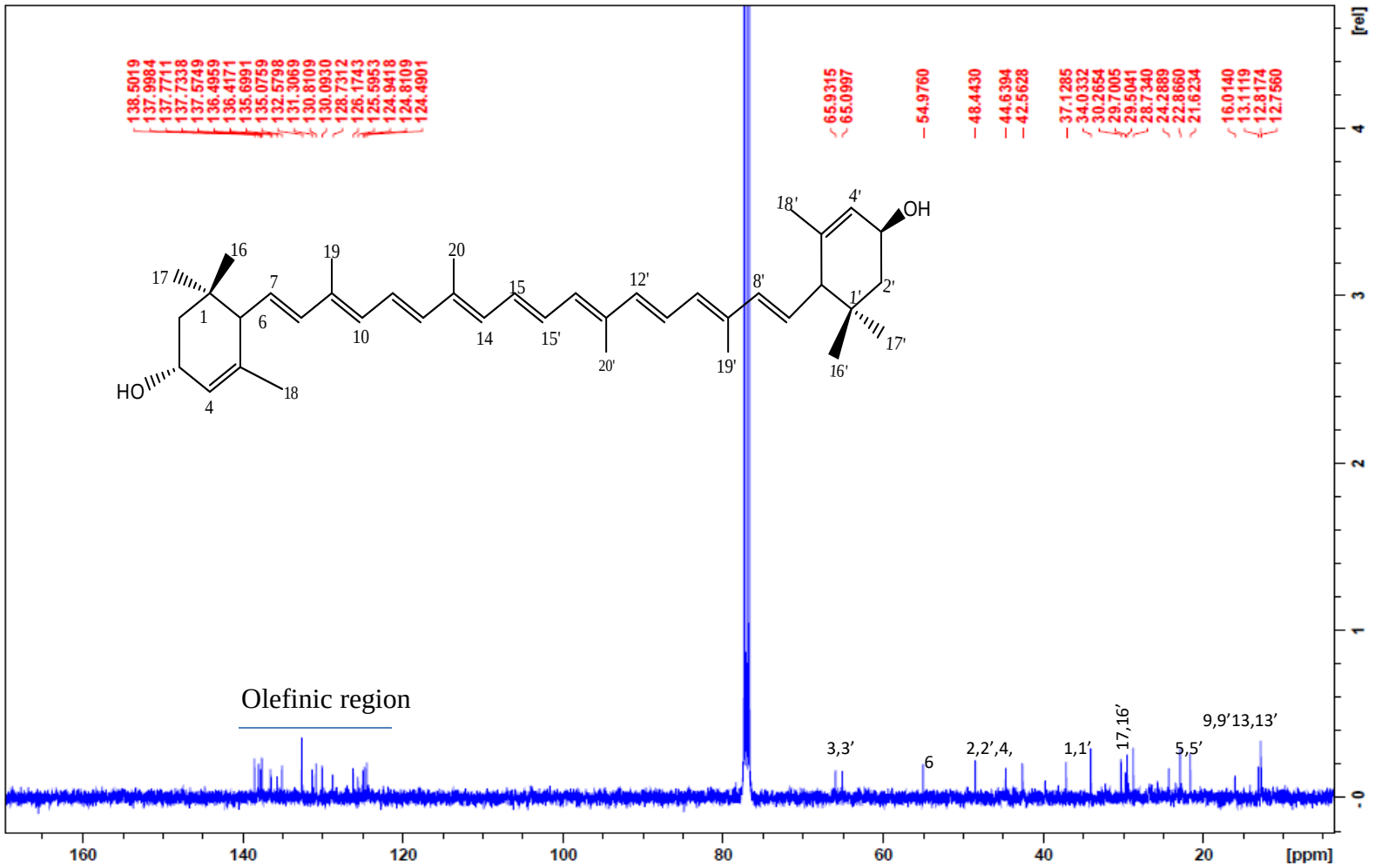
IR spectrum of pheophytin a



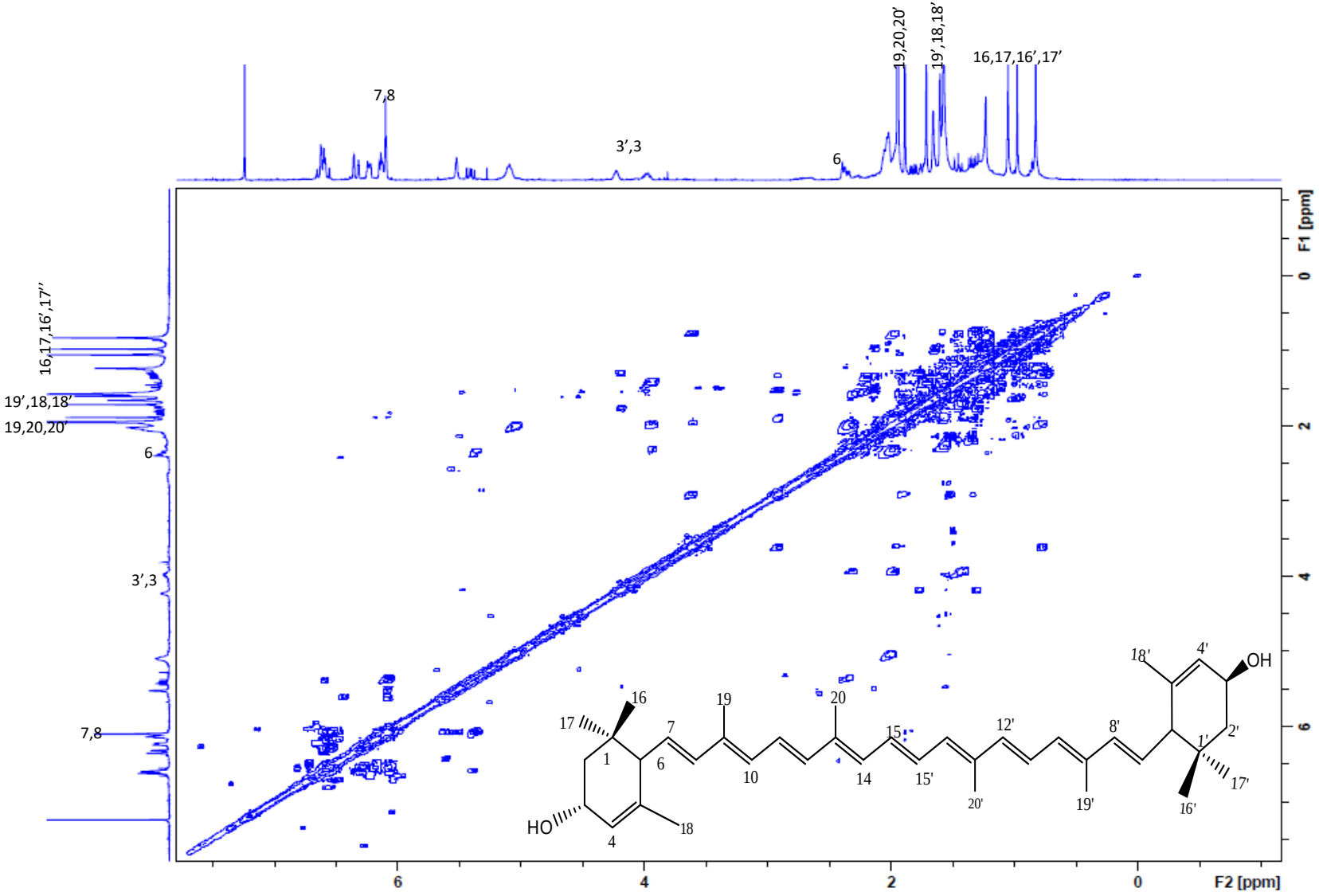
IR spectrum of pheophytin a



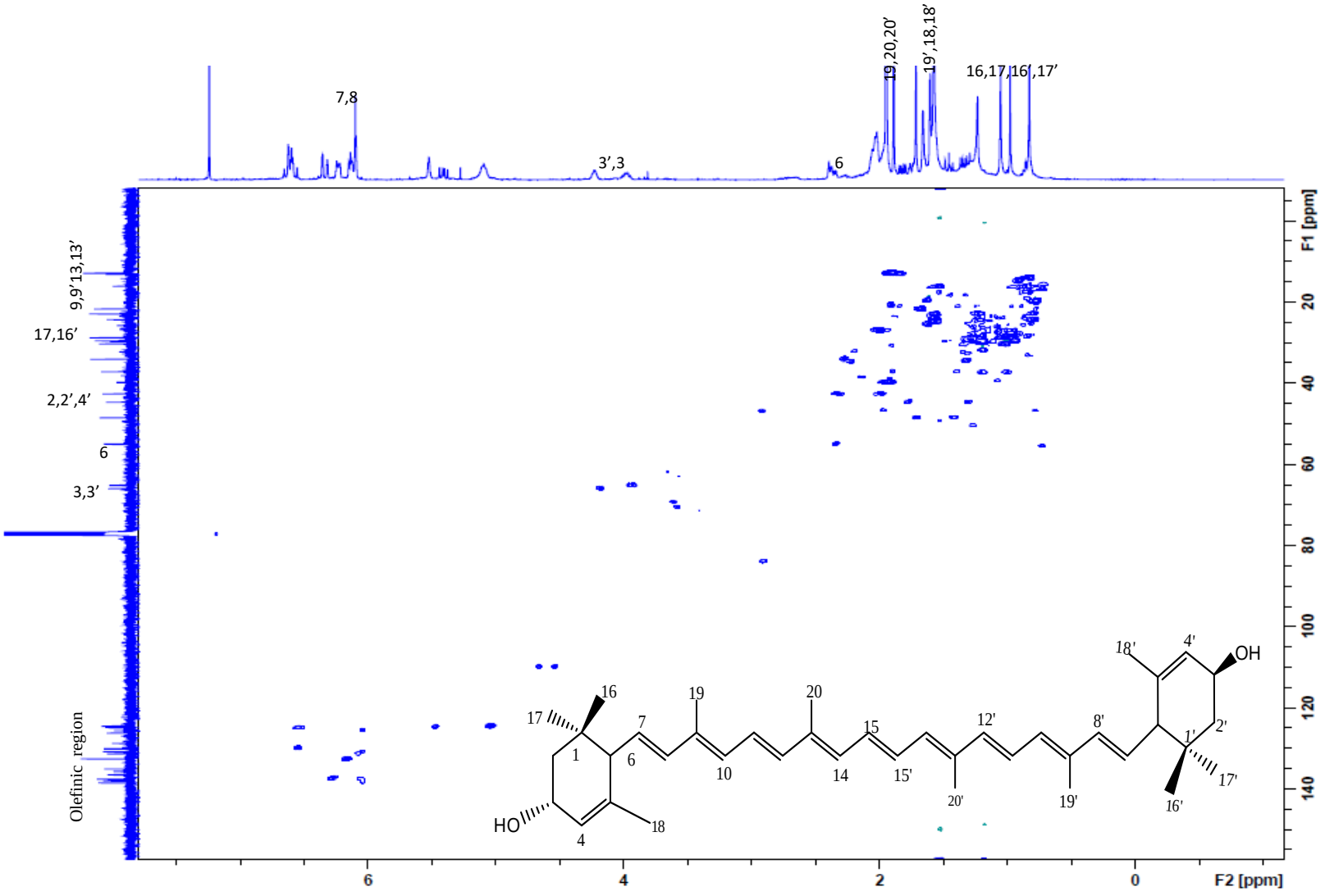
^{1}H NMR spectrum of lutein in $CDCL_3$



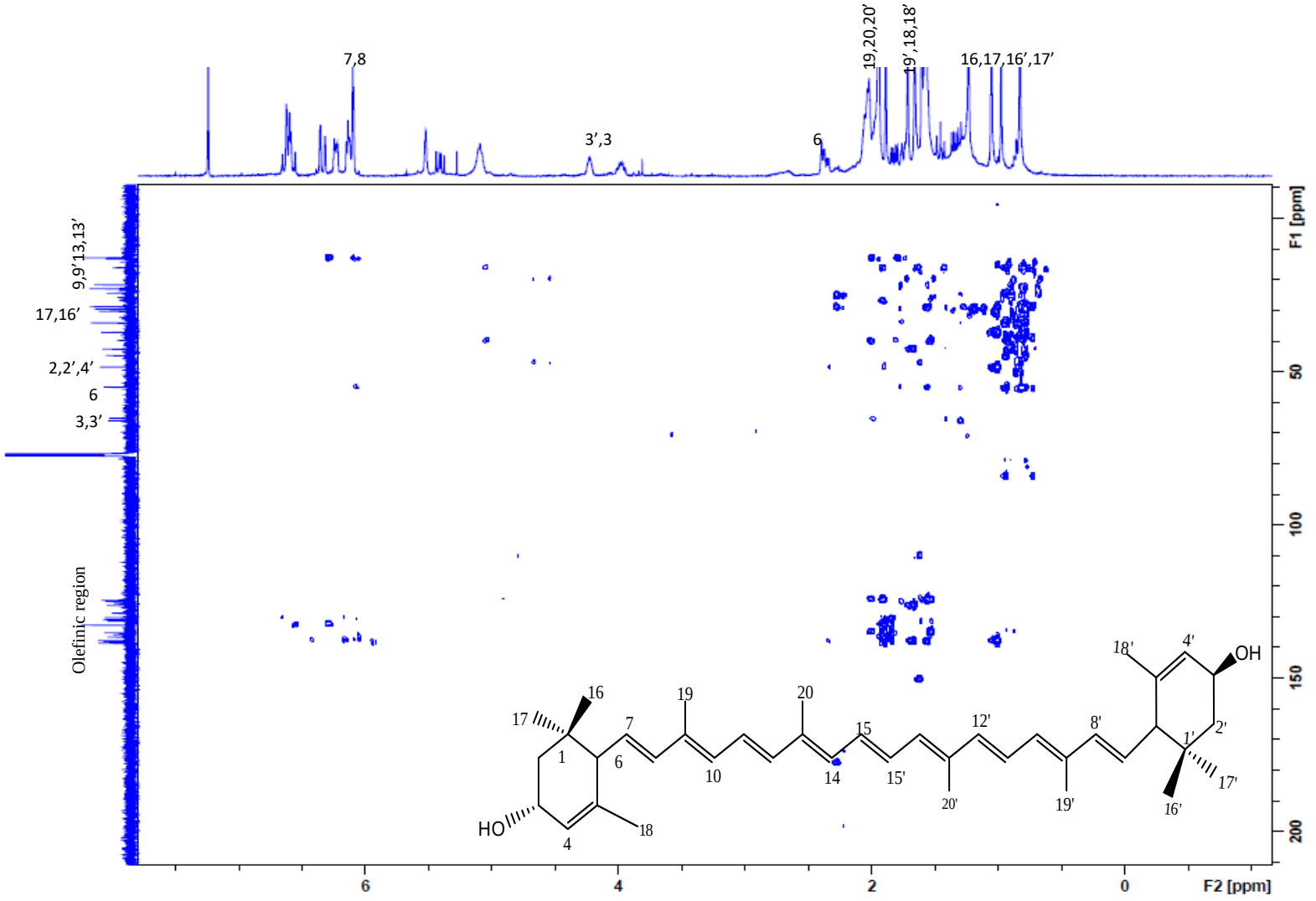
^{13}C NMR spectrum of lutein in $CDCL_3$



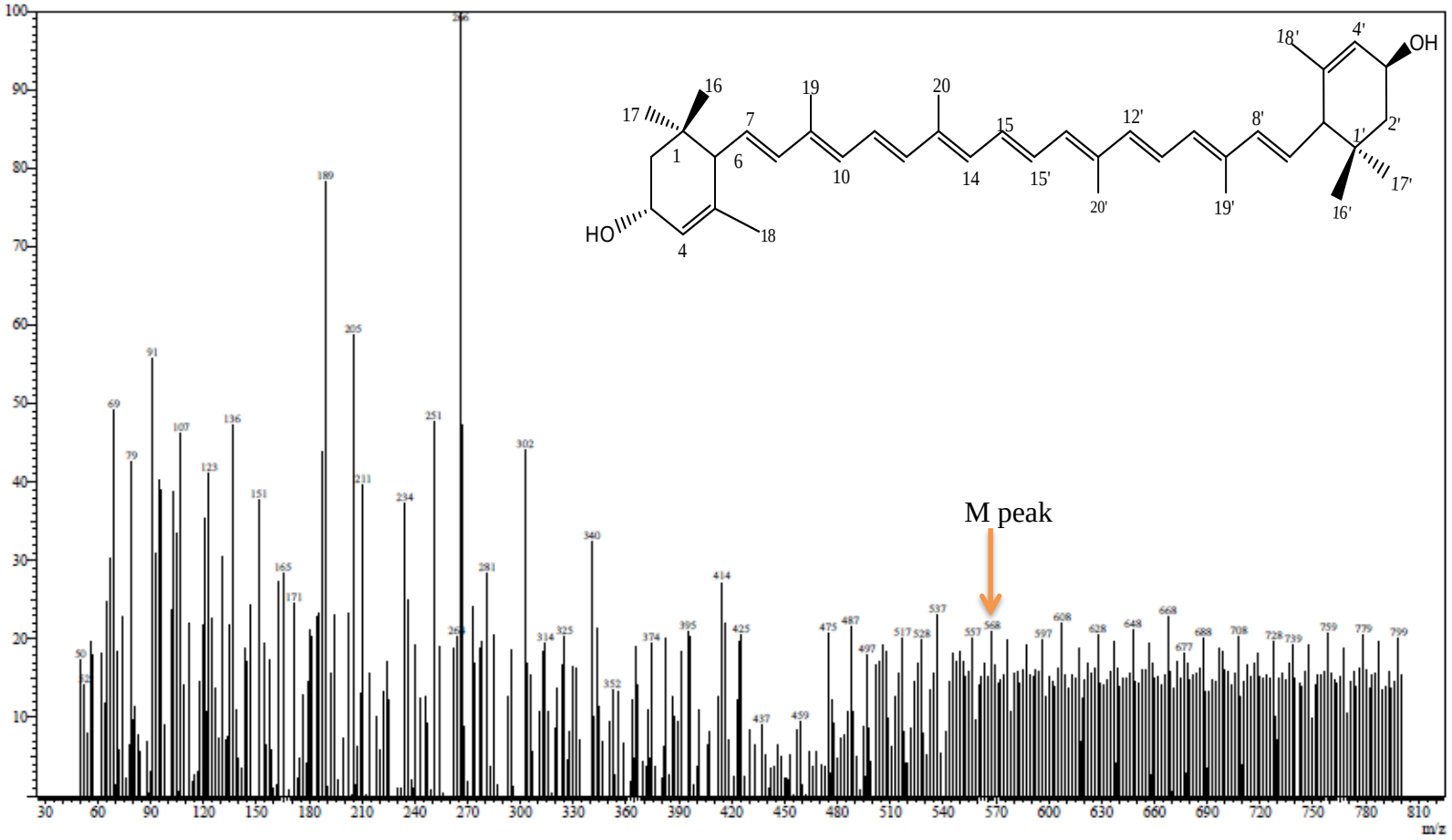
COSY NMR spectrum of lutein in $CDCL_3$



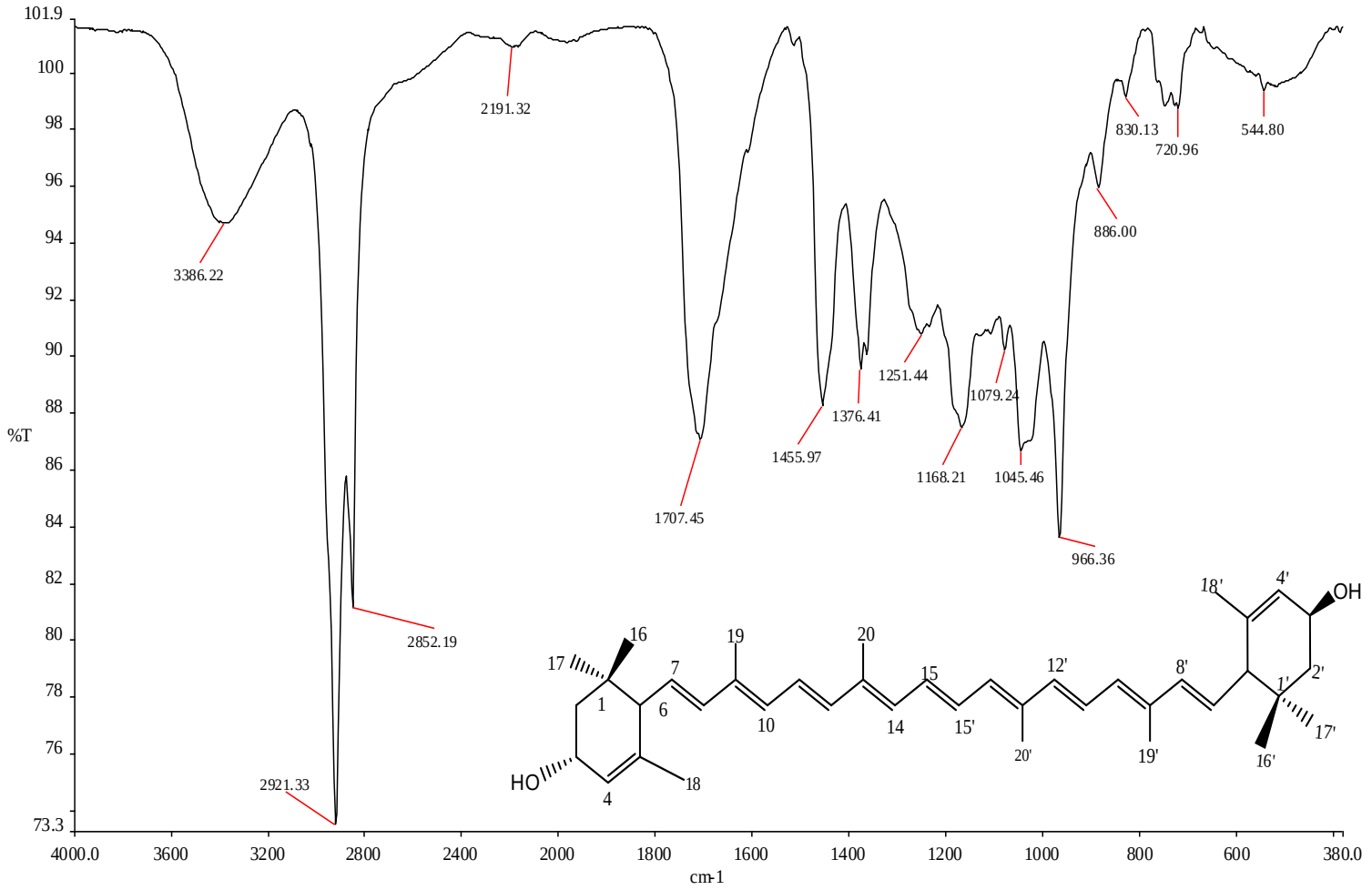
HSQC NMR spectrum of lutein in $CDCL_3$



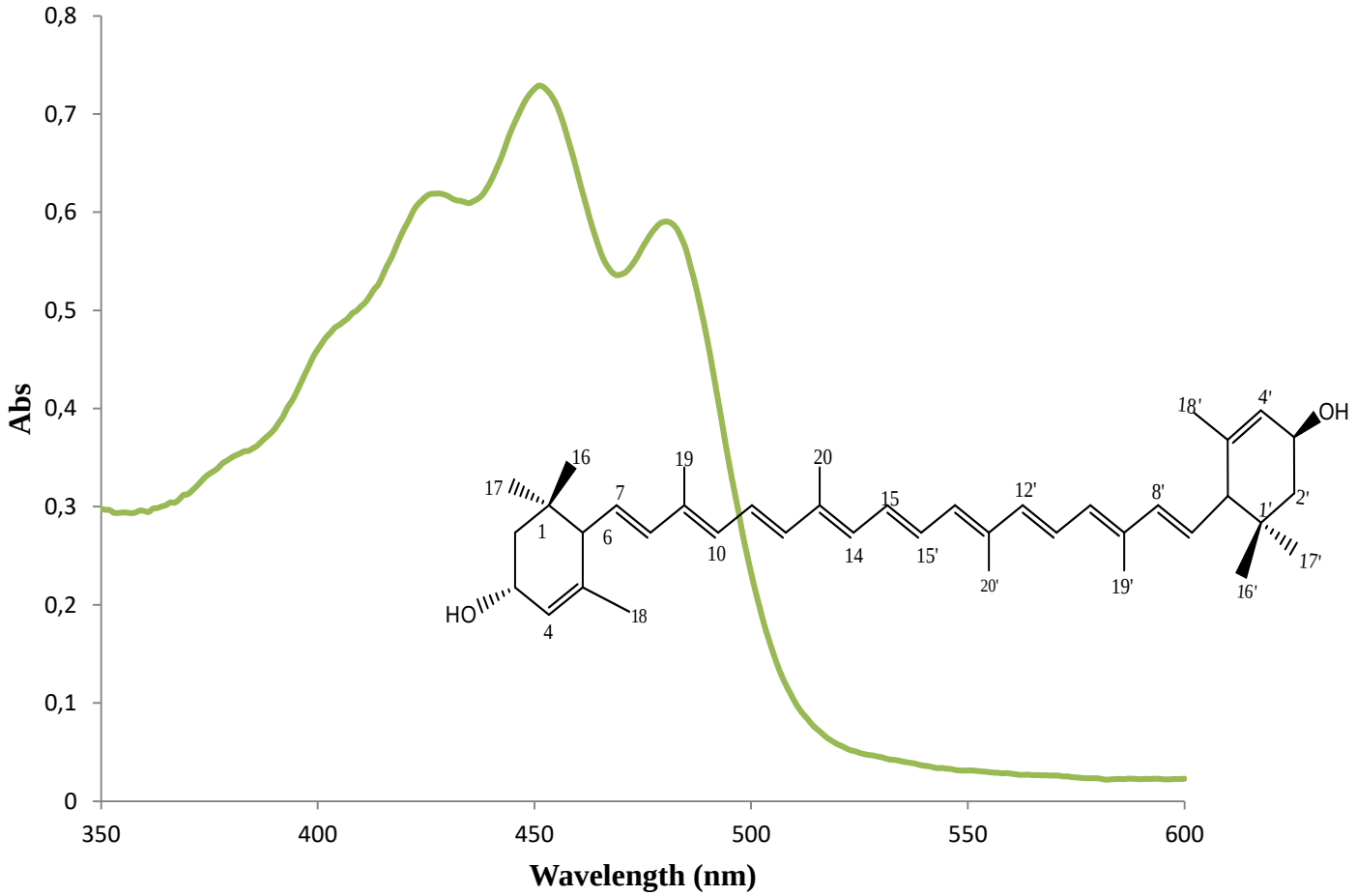
HMBC NMR spectrum of lutein in $CDCL_3$



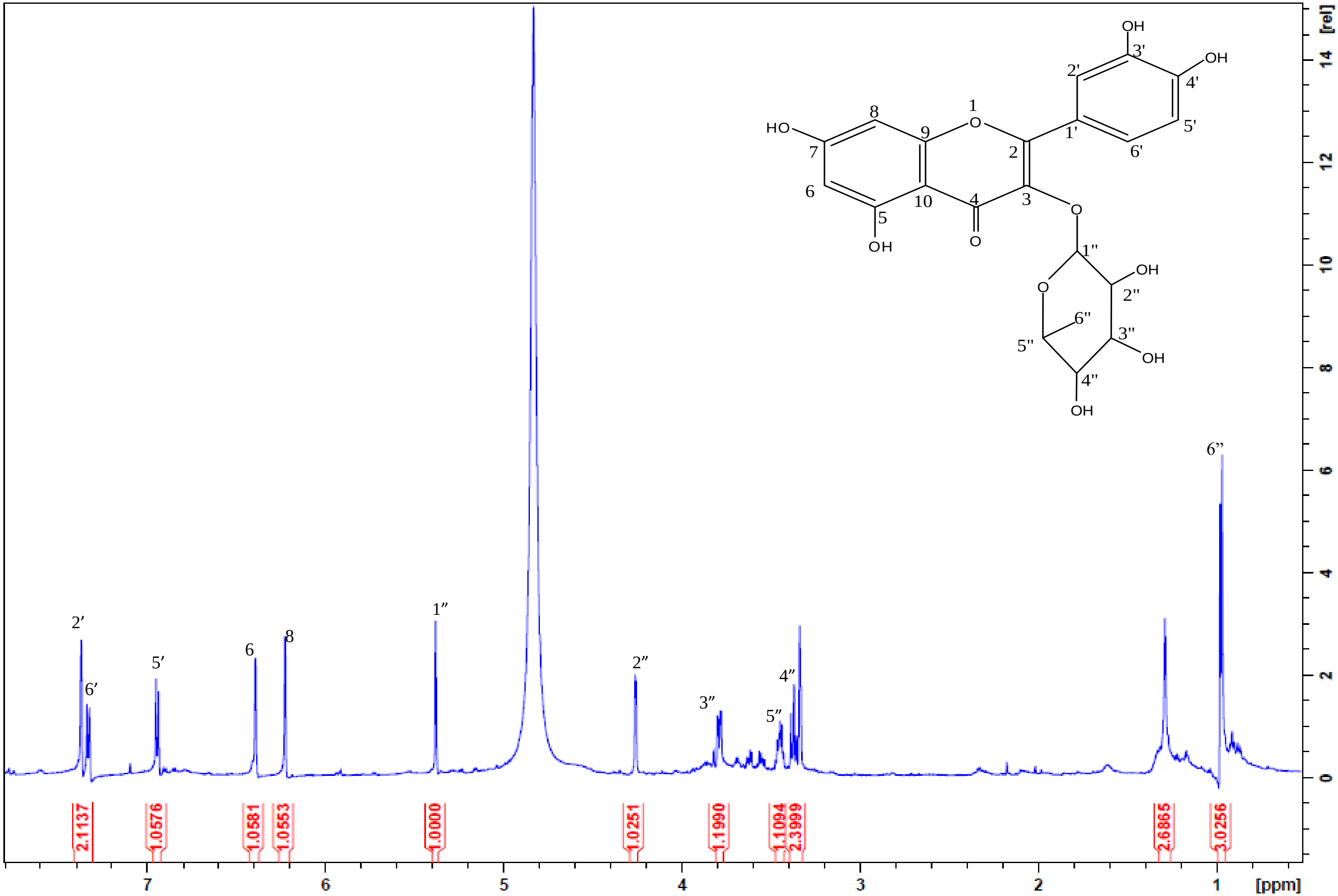
Mass spectrum of lutein



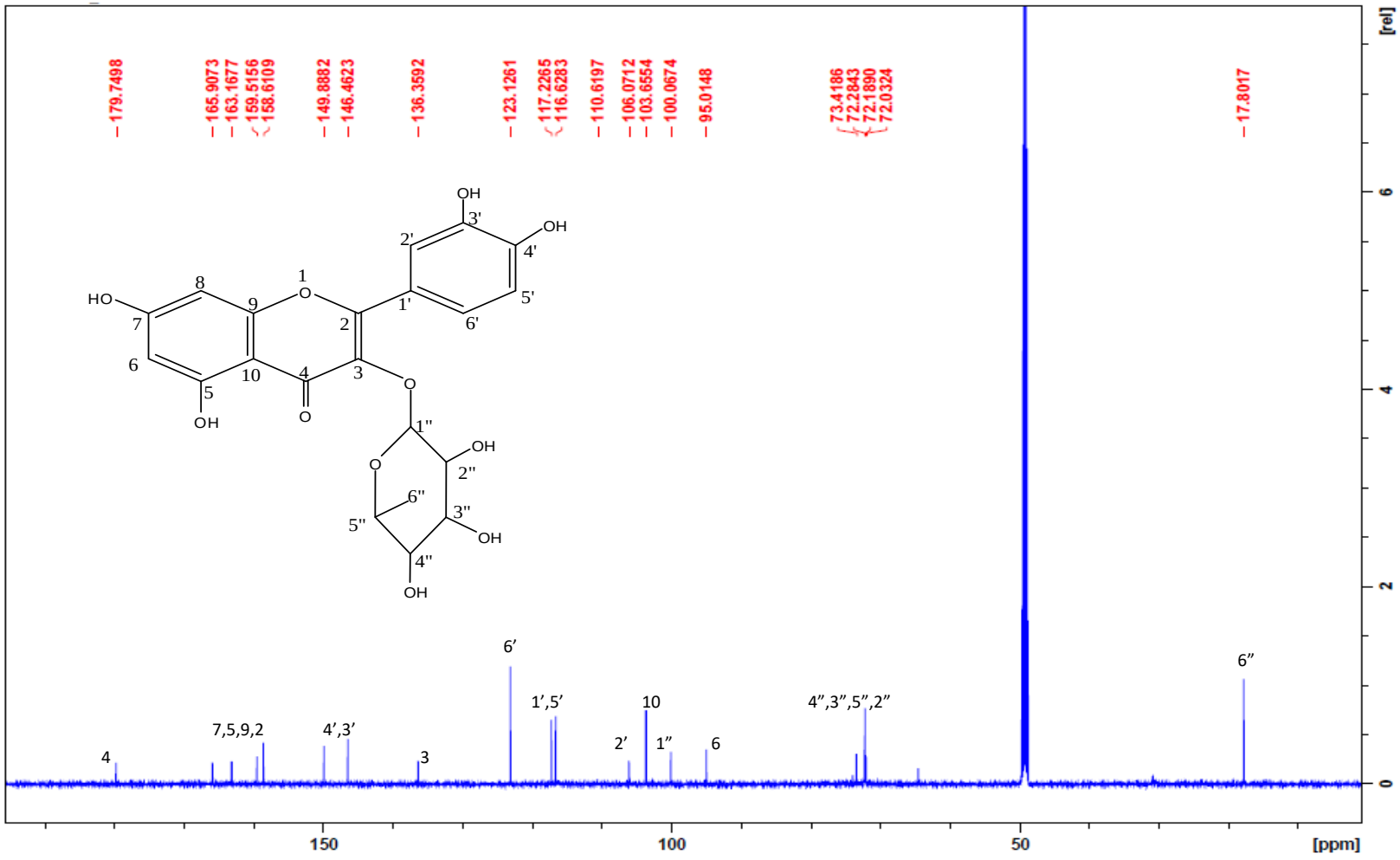
IR spectrum of lutein



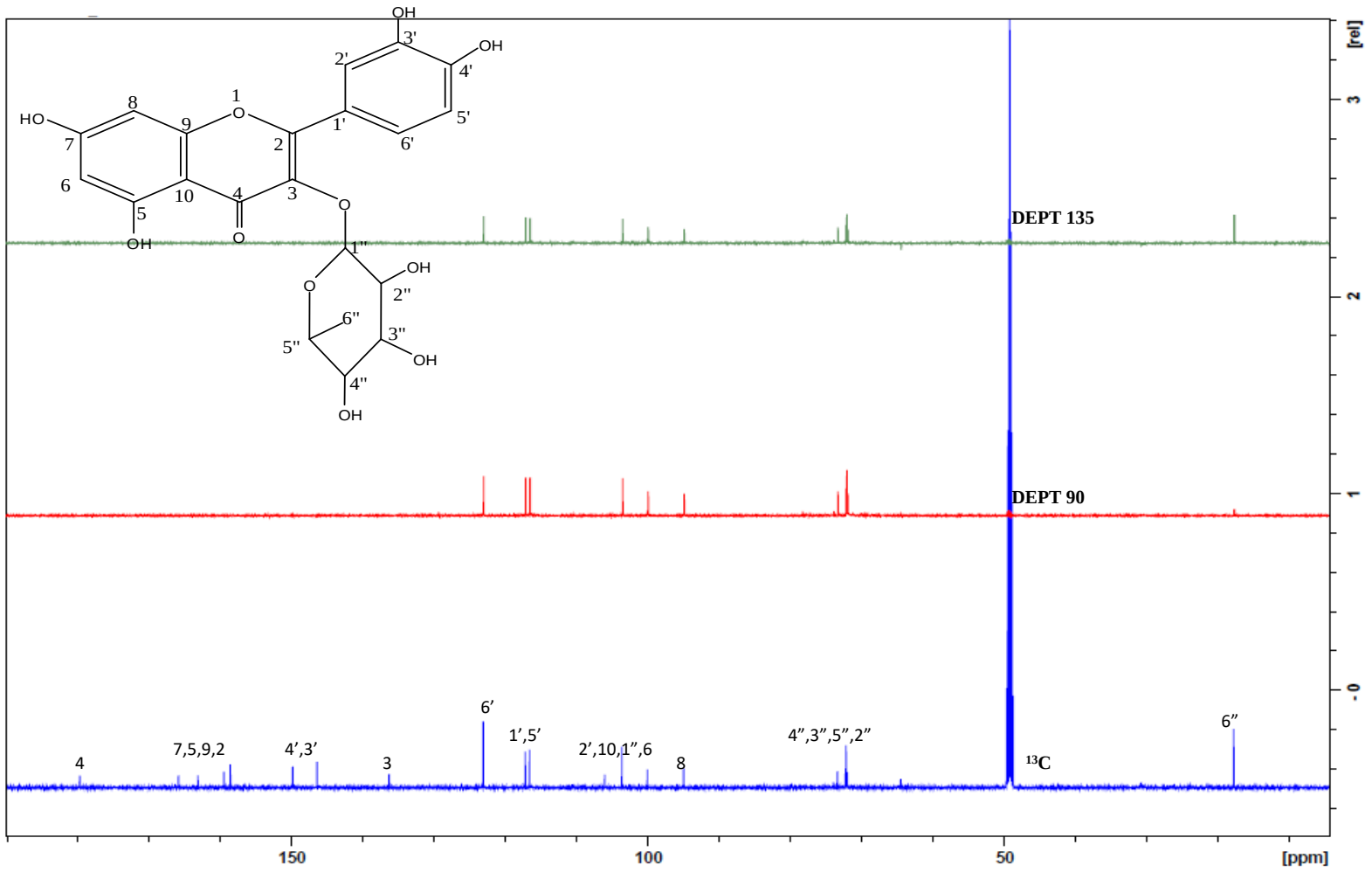
UV spectrum of lutein



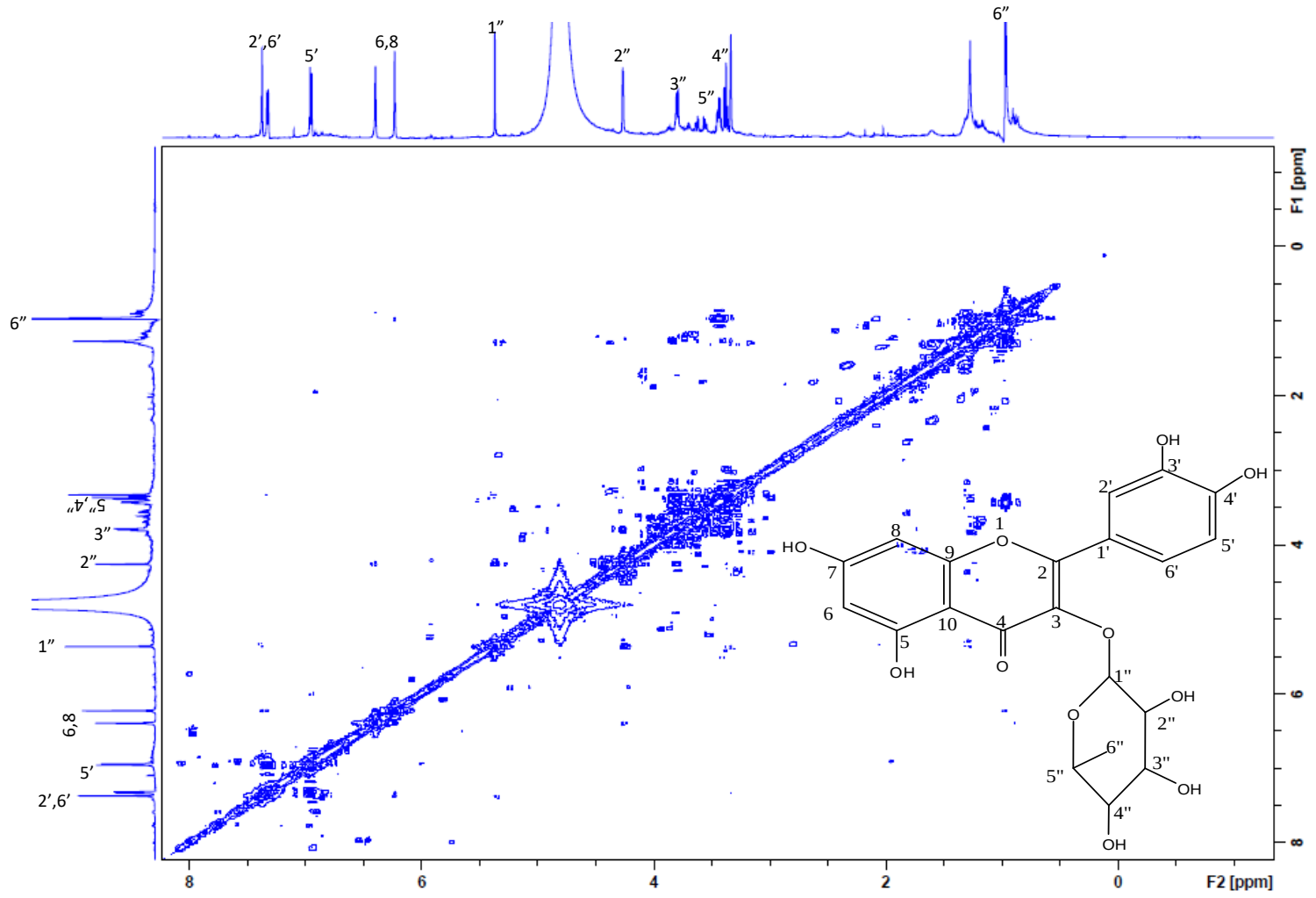
[1]H NMR spectrum of quercetrin in MeOD



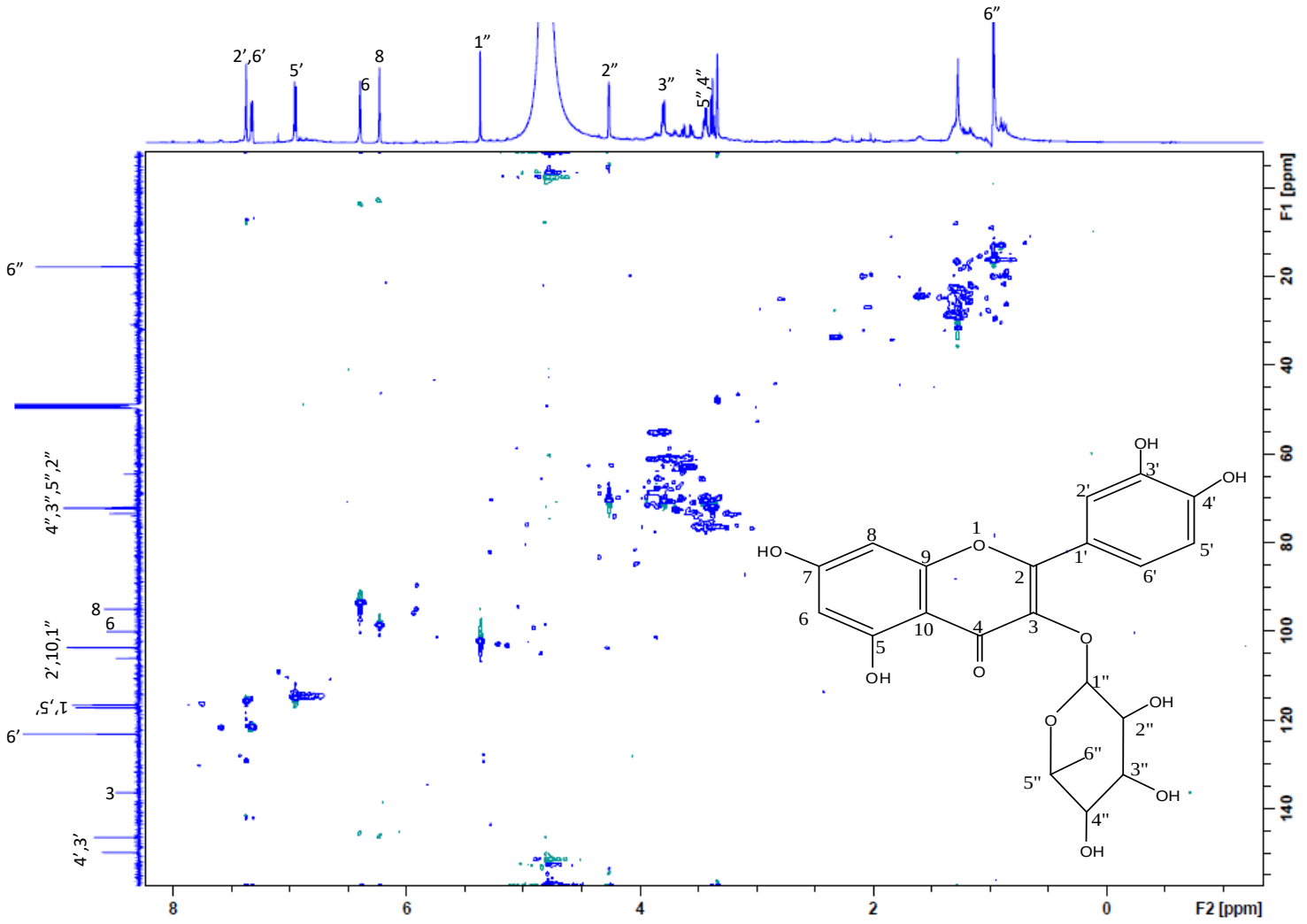
^{13}CNMR spectrum of quercetrin in MeOD



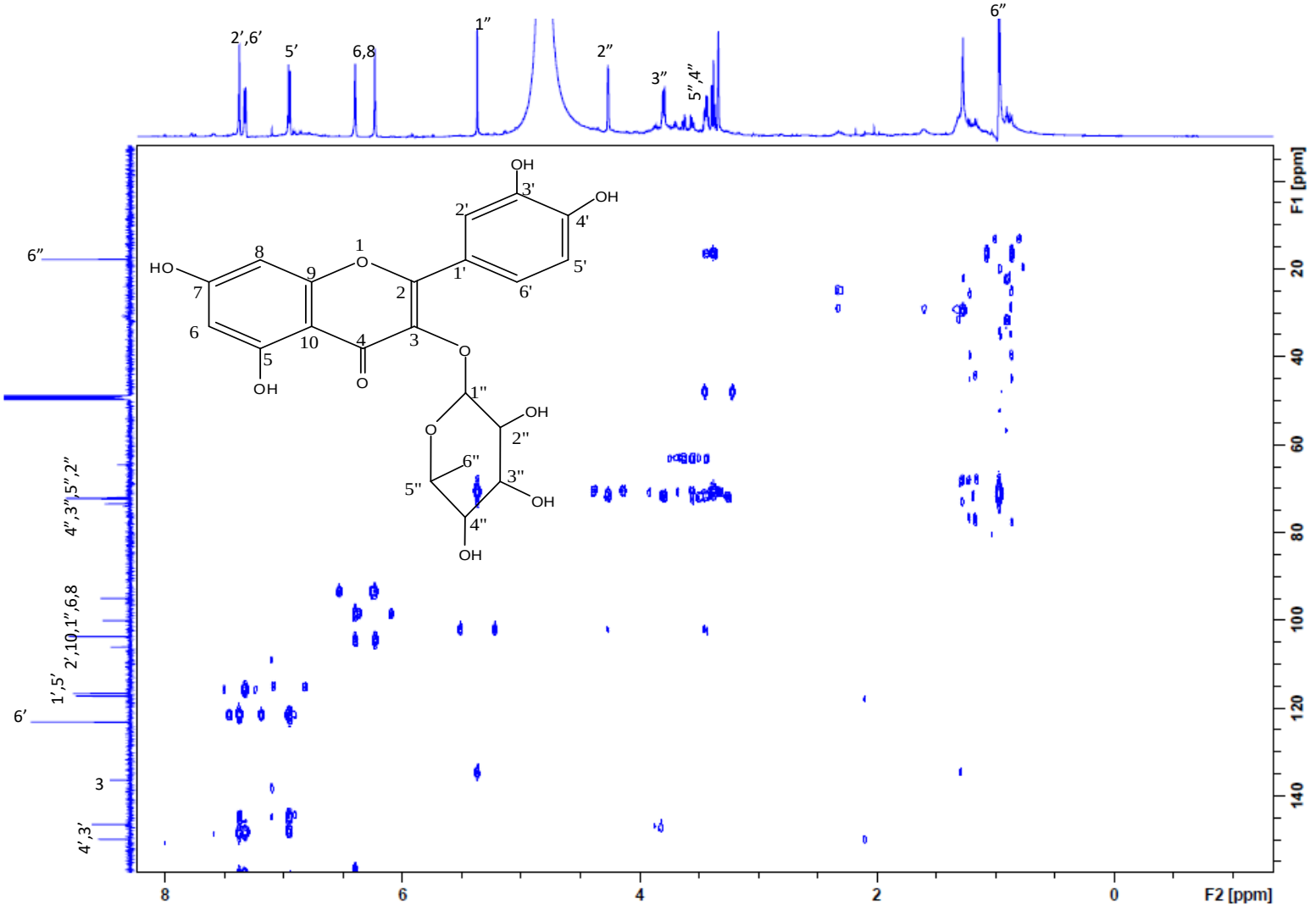
DEPT NMR spectrum of quercetrin in MeOD



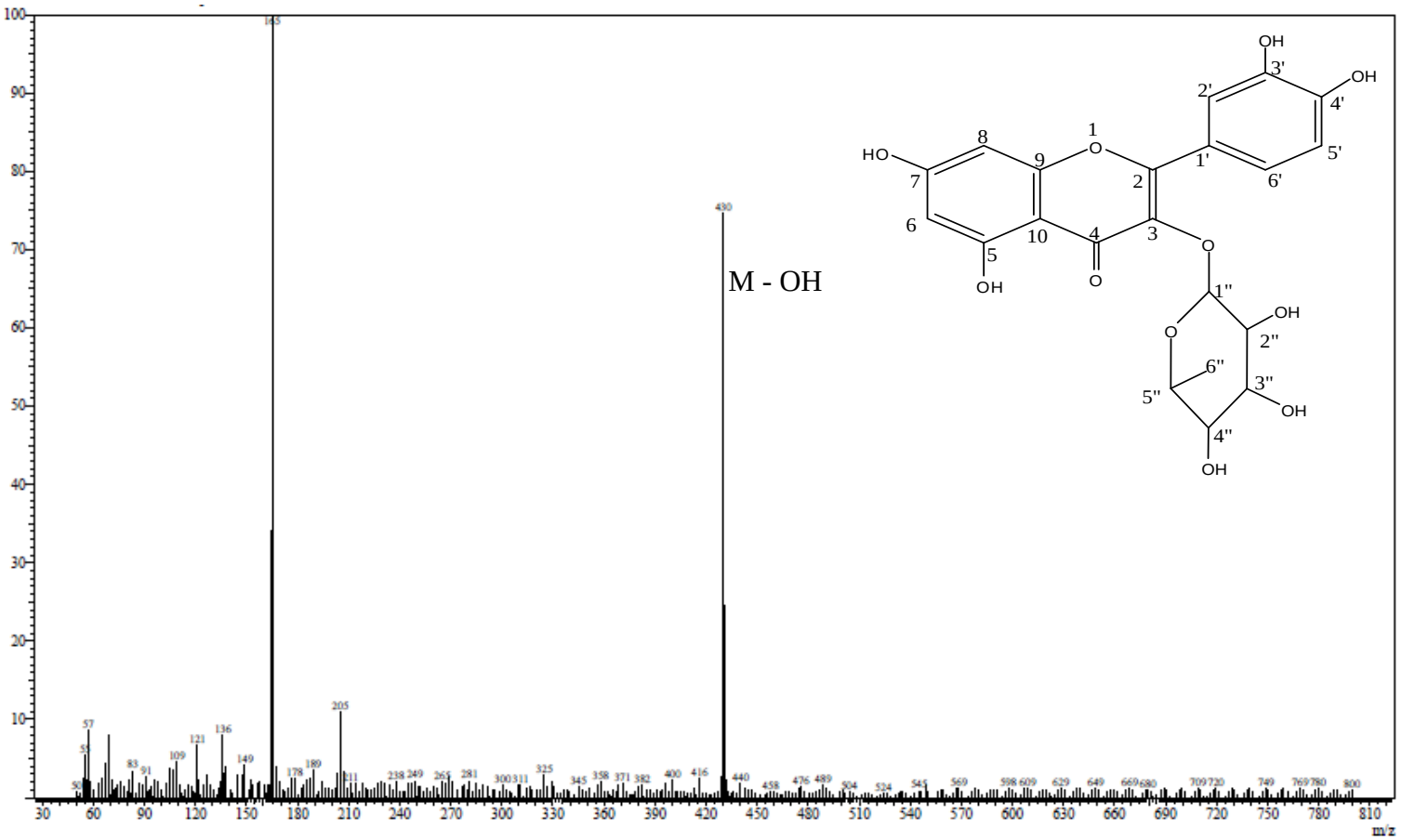
COSY NMR spectrum of quercetrin in MeOD



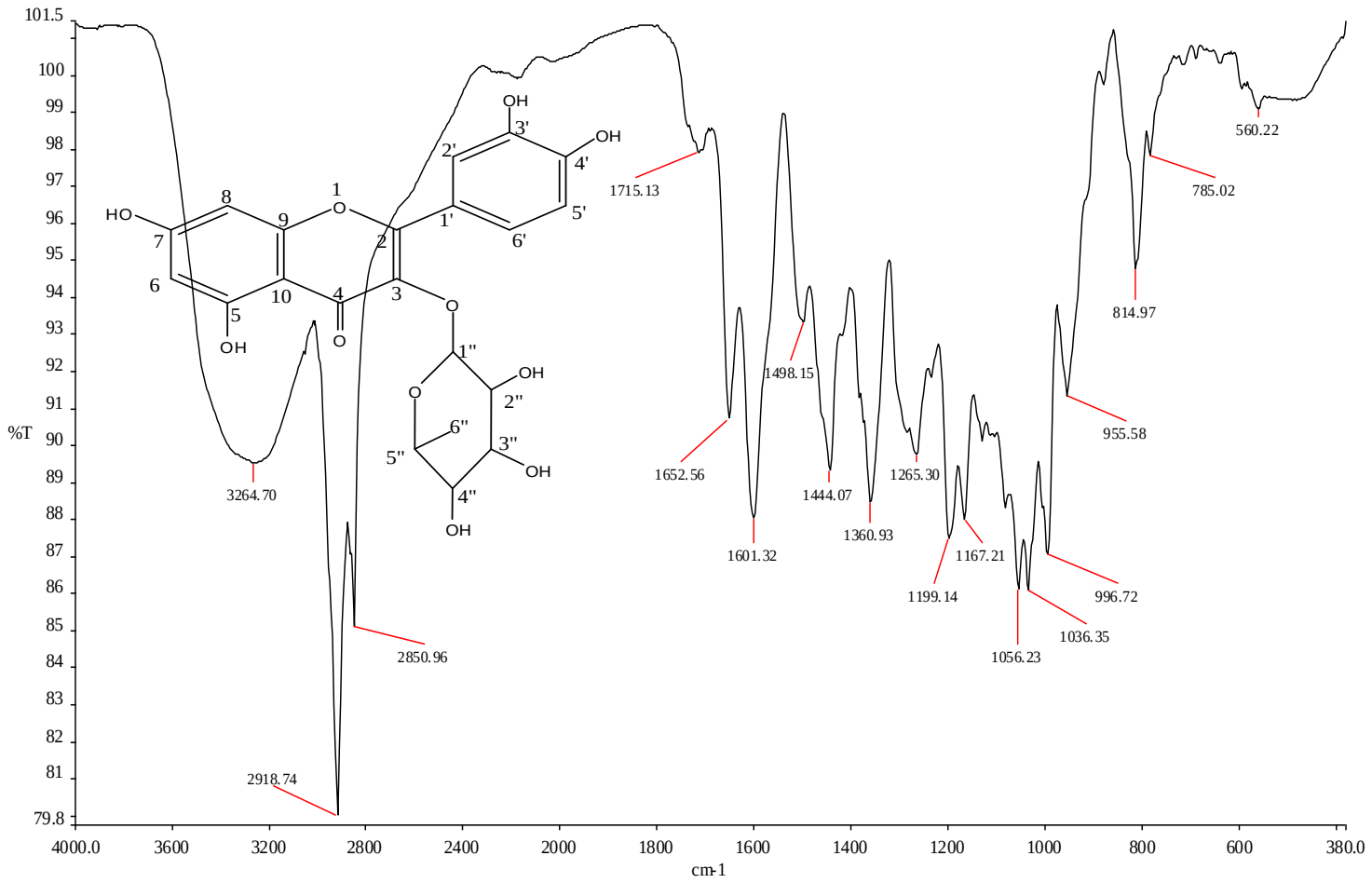
HSQC NMR spectrum of quercetrin in MeOD



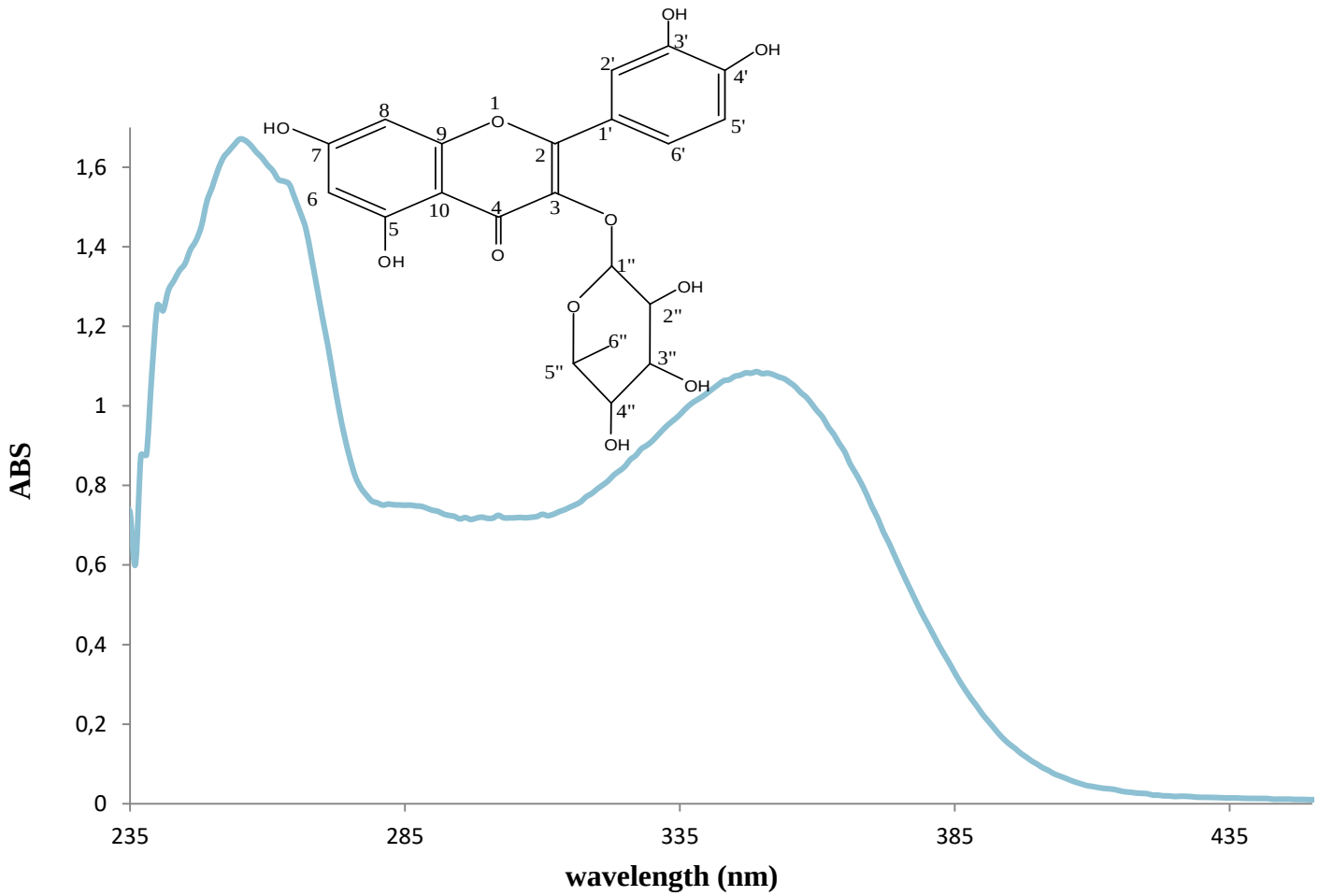
HMBC NMR spectrum of quercetrin in MeOD



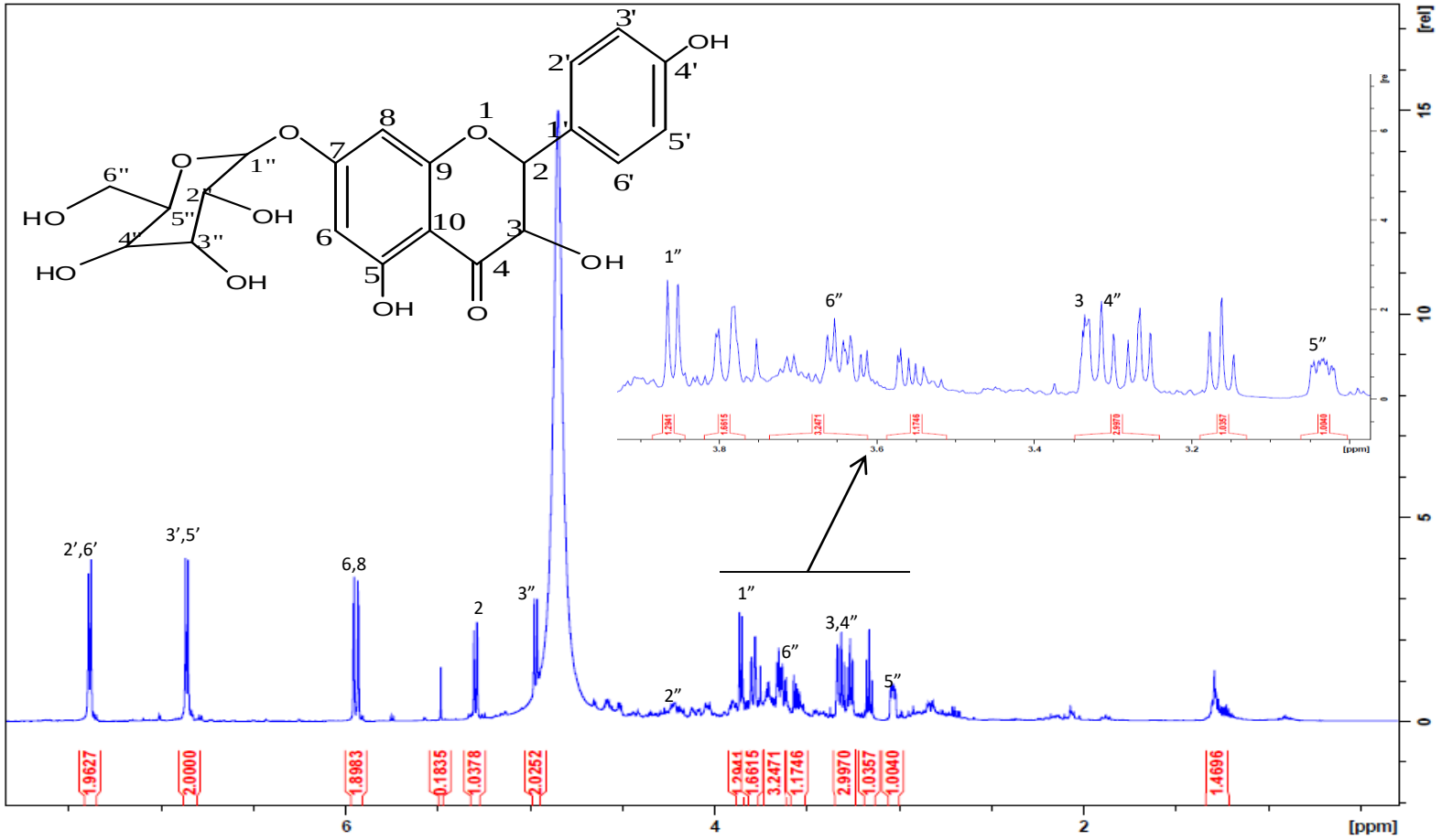
Mass spectrum of quercetrin



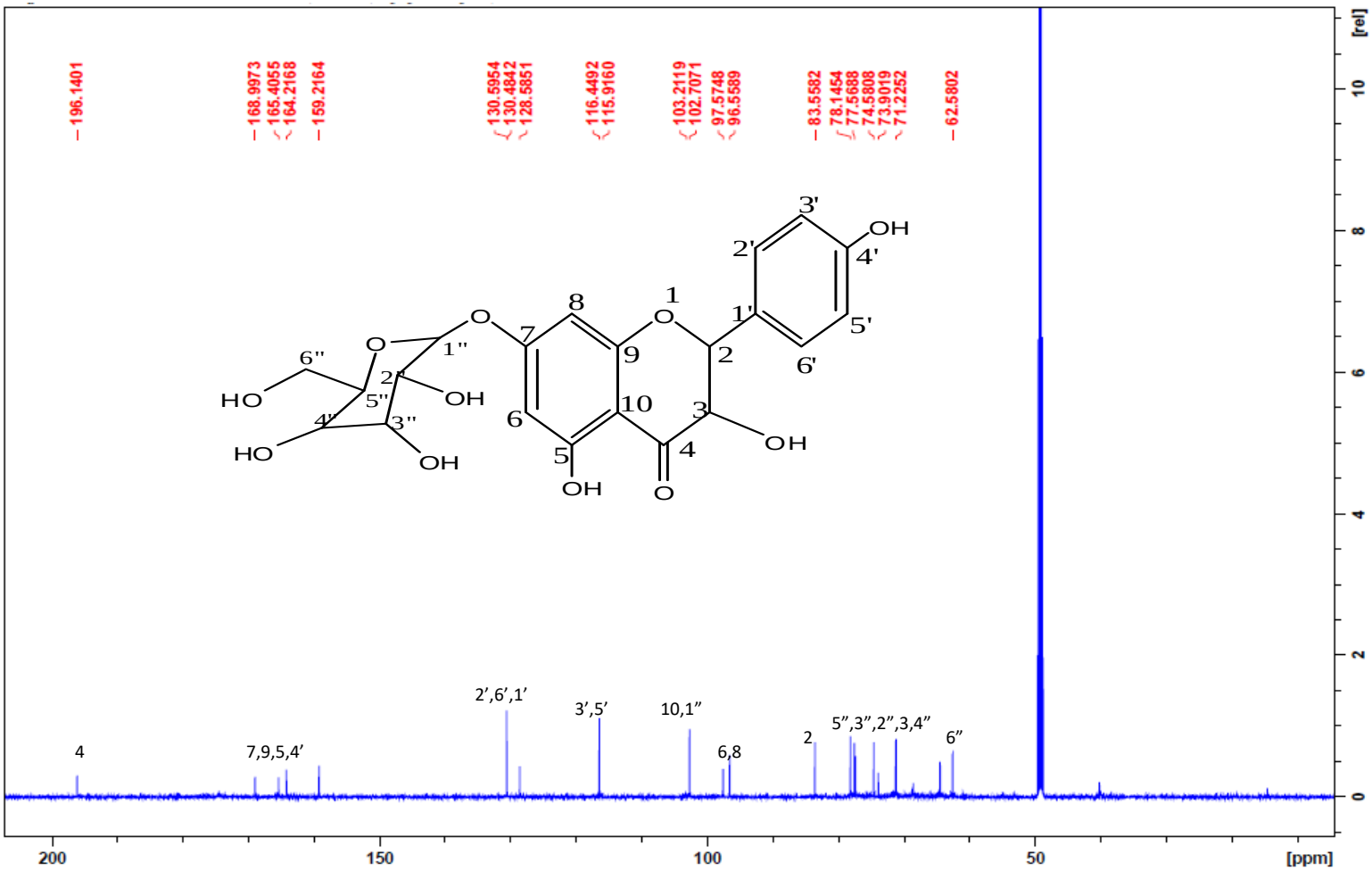
IR spectrum of quercetrin



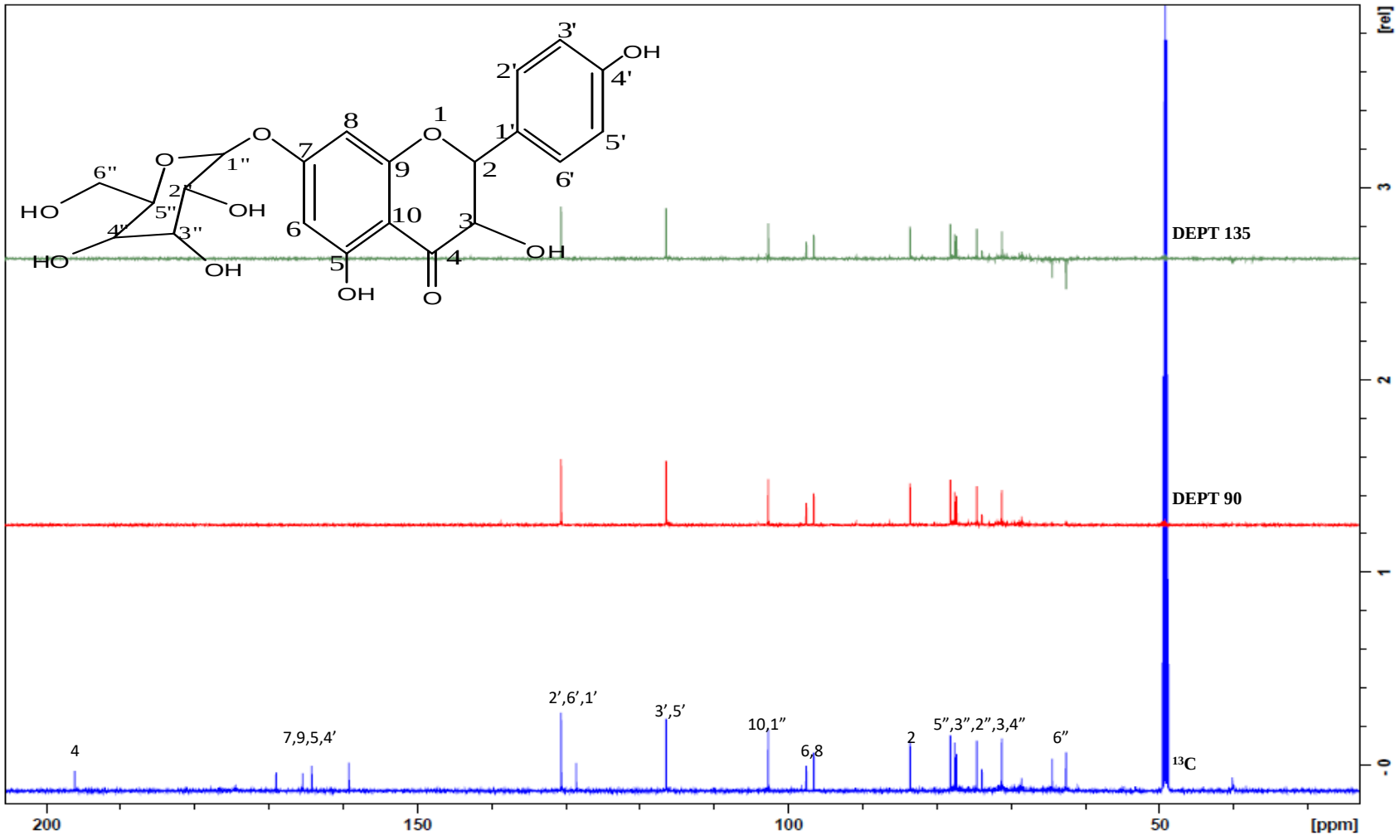
UV spectrum of quercetrin



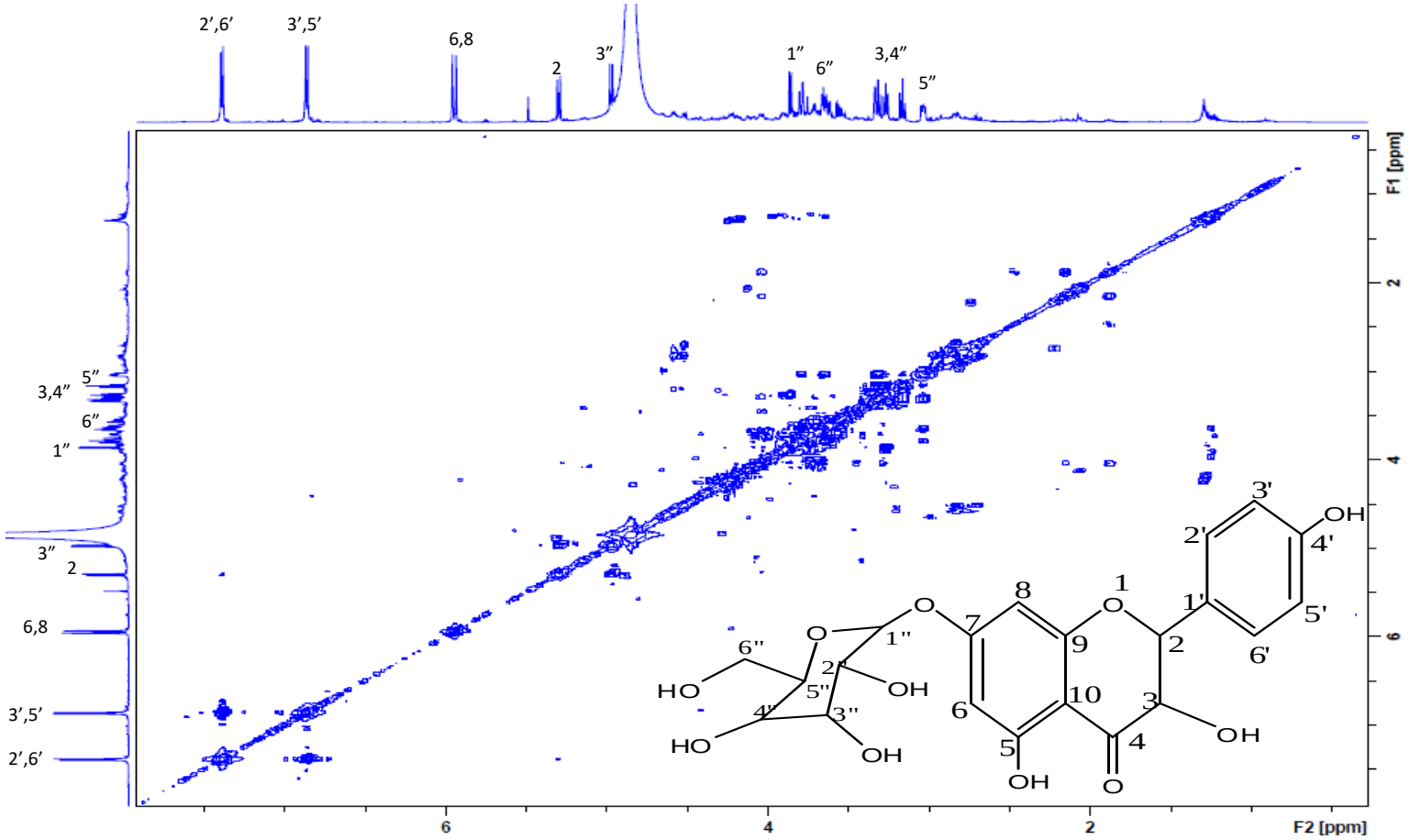
^{1}H NMR spectrum of aromedendrin7-O-β-glycopyranoside in MeOD



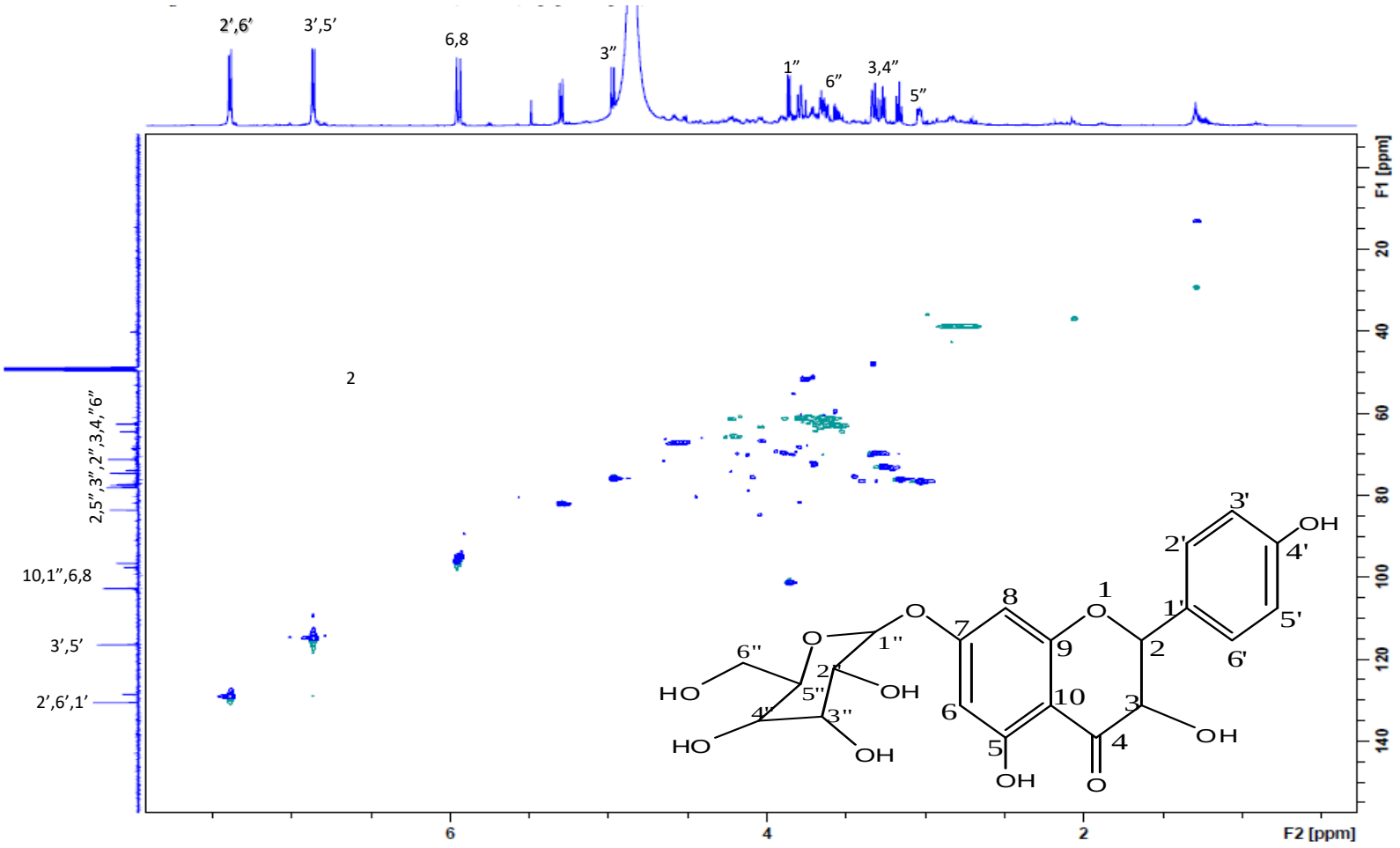
^{13}C NMR spectrum of aromedendrin7-O-β-glycopyranoside in MeOD



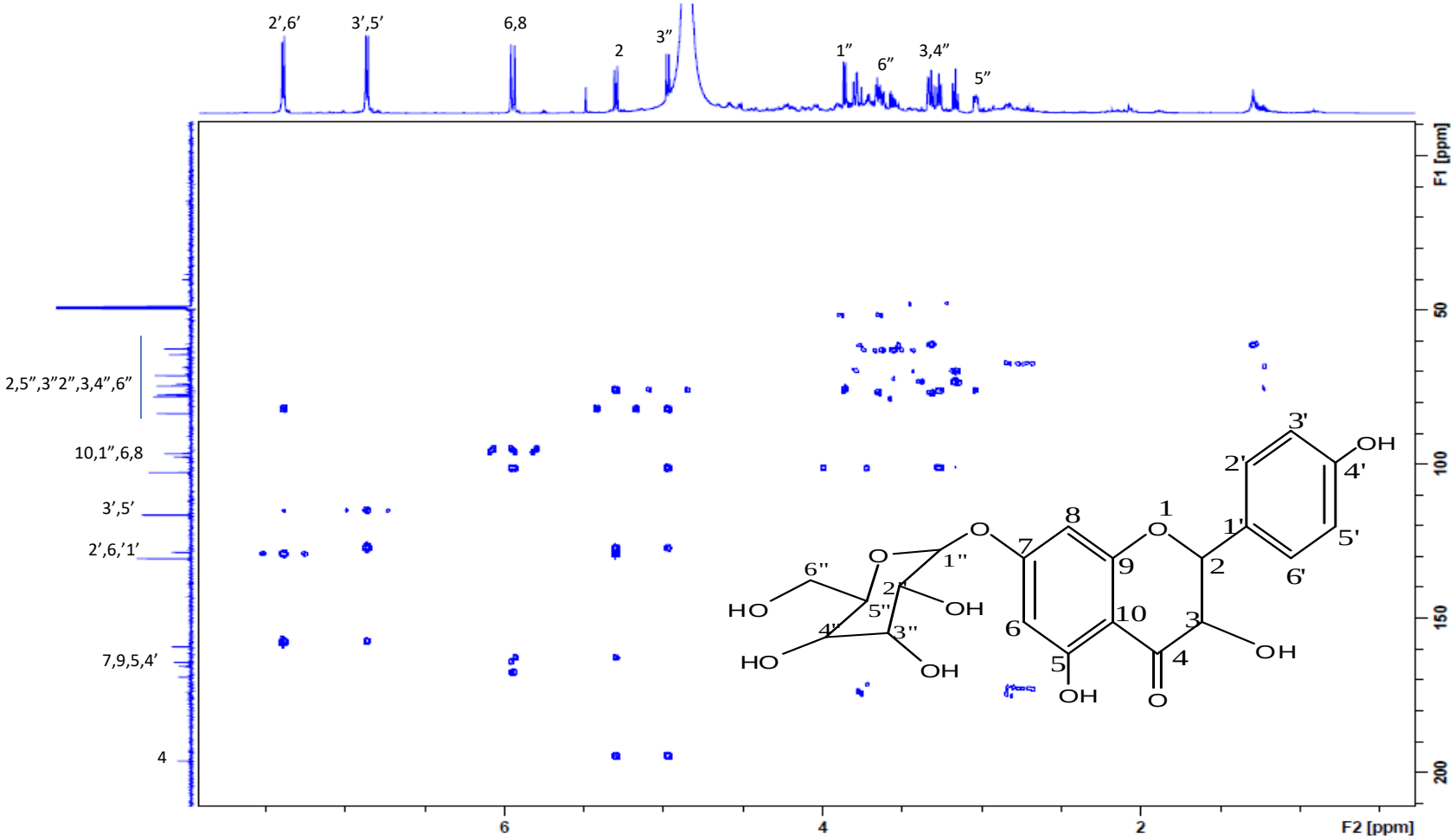
DEPT NMR spectrum of aromedendrin7-O-β-glycopyranoside in MeOD



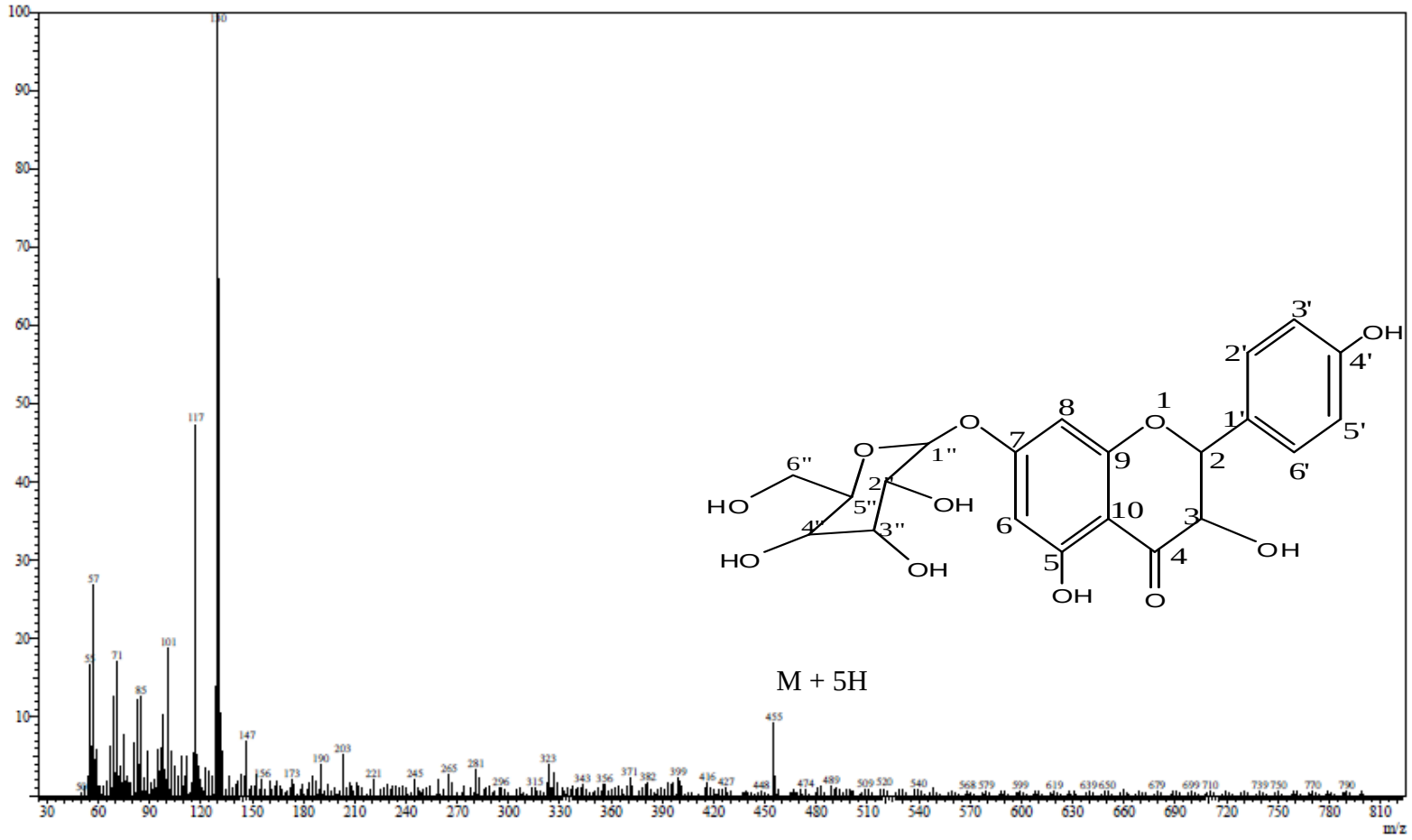
COSY NMR spectrum of aromedendrin7-O-β-glycopyranoside in MeOD



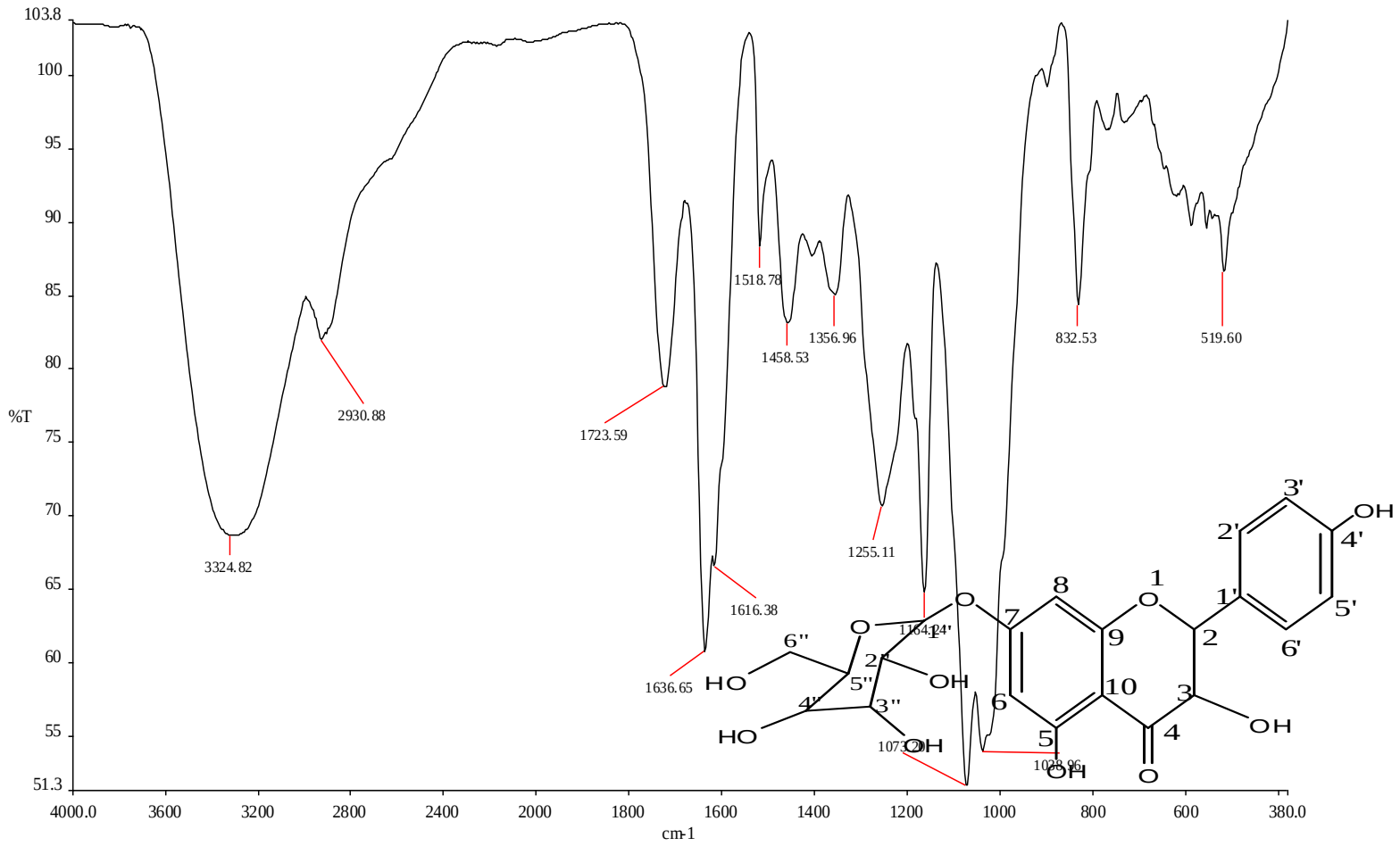
HSQC NMR spectrum of aromedendrin7-o-β-glycopyranoside in MeOD



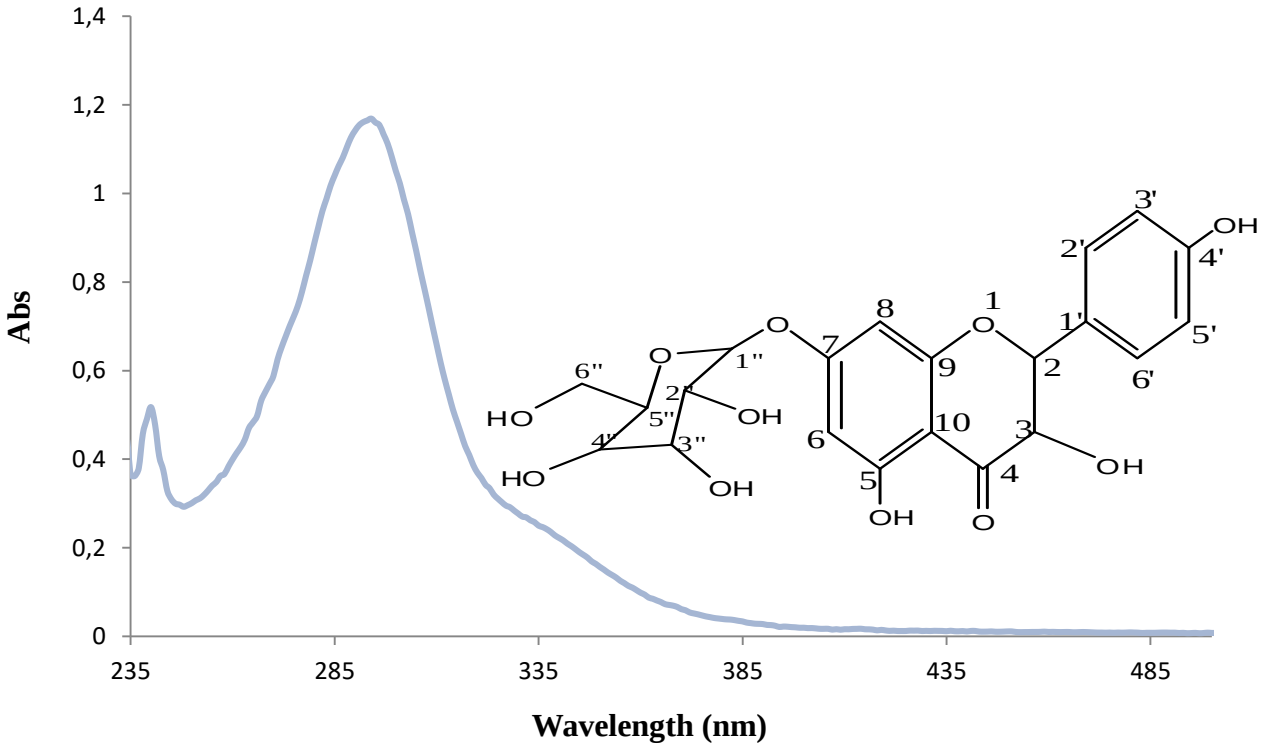
HMBC spectrum of aromedendrin7-O-β-glycopyranoside in MeOD



Mass spectrum of aromedendrin7-O-β-glycopyranoside



IR spectrum of aromedendrin7-O-β-glycopyranoside



UV spectrum of aromadendrin7-O-β-glycopyranoside