CHEMICAL CONSTITUENTS, ANTICARIES, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF THE ROOT OF *Mezoneuron benthamianum* BAILL.

BY

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ABSTRACT

Chewing sticks are the common means of maintaining oral hygiene in Africa. The roots of *Mezoneuron benthamianum*are commonly used as chewing stick in the southwestof Nigeria. However, their usage in preventing dental caries, a disease caused by bacteria especially *Streptococcus mutans*, has not been investigated.This study was therefore designed to identify the chemical constituents, the anticaries and other biological activities of *M. benthamianum.*

Roots of *M. benthamianum* were purchased in Ibadan and authenticated at the University of Ibadan herbarium (No.: UIH-22401).The pulverized root sample (2.7 kg) was extracted with methanol to obtain a crude extract which was successively partitioned with n-hexane, dichloromethane and ethyl acetate. The fractions were subjected to column and thin layer chromatography to obtain pure compounds. Thechemical structures of the isolated compounds were elucidated by the use of Infrared (IR), Nuclear Magnetic Resonance (NMR) and mass spectroscopy. The extracts and isolated compounds were evaluated for antibacterial activities against *Streptococcus mutans, Pseudomonas aeruginosa, Escherichia coli* and *Staphyloccus aureus* at concentrations ranging from 0.0125 to 20.0 mg/L.Their antioxidant and cytotoxic activities were determined using 2,2-diphenyl-1-picrylhydrazyl methodand brine shrimpbioassay. Data were analysed using descriptive statistics.

The successive partitioning of the crude methanol extract (200.0 g) of *M. benthamianum* yielded 5.0 g (n-hexane),14.0 g (dichloromethane) and 70 g (ethyl acetate) fractions. Column chromatographic purification of the n-hexane and ethyl acetate fractions gave six compounds which were elucidated to be taepeeninA, nortaepeeninA, stigmasterol, *trans*-resveratrol, piceatannol and gallic acid. The most abundant isolated compound, *trans*-resveratrol, showed the IR absorptions at

3421 (O-H) and 1633 cm^{-1} (aromatic C=C). The mass spectrum gave the $(MH)^{+}$ion at *m/z*229.0, suggesting the molecular formula $C_{14}H_{12}O_3$. The ^{1}HNMR spectrum displayed four sets of doublet signals indicating two ortho-coupled aromatic protons (δ 7.35, d,*J* = 8.6 Hz, H-2′, H-6′ and 6.78, d, *J* = 8.6 Hz, H-3′, H-5′) and two *trans*-olefinic protons(δ 6.97, d,*J* = 16.3 Hz, H-8 and 6.81, d,*J* = 16.4 Hz, H-7). The ^{13}C NMR spectrum showed the presence of seven aromatic methine carbons (δ 102.62, C-4; 105.80, C-2, C-6; 116.48, C-3′, C-5′ and 128.83, C-2′, C-6′), two olefinic methine carbons (δ 126.95, C-7 and 129.40, C-8) and five aromatic quaternary carbons (δ 130.39, C-1′;141.32, C-1; 158.27, C-4′ and 159.57, C-3, C-5),suggestive of fourteen carbon atoms. The *trans*-resveratrol and piceatannol exhibited strong antimicrobial activity against *S.mutans, P. aeruginosa, E. coli* and *S. aureus* with *trans*-resveratrol having the same minimum inhibitory concentration (MIC) of 0.025 mg/mL against all the organisms and piceatannol with MIC ranging from 0.025 – 0.30 mg/mL. They also showed potent antioxidant activity with IC_{50} of 35.81 and 30.35 μM,when compared with ascorbic acid (IC_{50} = 38.20 μM) and cytotoxic activity against brine shrimp with LC_{50} of 7.15 and 99.99 μg/ml.

Taepeenin A, nortaepeenin A and piceatannol were isolated from *Mezoneuron benthamianum*. The *trans*-resveratrol and piceatannol were identified as the major compoundswhich may be responsible for itsanticaries activity.

Keywords: *Mezoneuron benthamianum*, Dental caries, Chewing sticks, *trans*-Resveratrol, Piceatannol

Word count: 494

DEDICATION

To

God
My Creator, Helper and Pillar of support who has made it possible for me
to begin and to complete this programme
and
To My Family

ACKNOWLEDGEMENT

I give all glory and honour to God Almighty the creator of heaven and earth for the grace given me to commence and successfully complete this research work. It is not by might, nor by power, it is purely by the mercy and the grace of God.

I appreciate my supervisor and my mummy, Prof. (Mrs.) Olapeju O. Aiyelaagbe, for the encouragement, support, assistance, thoroughness and guidance given to me during the course of this project and for always being there for me. Your motherly supervision has created in me the inspiration and perseverance to carry on with this research work even when it seemed there was no way out. I say thank you ma.

I appreciate the Head of Department Professor T.I. Odiaka for setting a standard of excellence in the Department. I am also grateful to the head of the Organic Unit, Prof. O. Ekundayo for his innumerable assistance during this programme, the immediate past Head of Department, Prof. A. Adesomoju for being an inspiration and creating a model for me to emulate and all other lecturers in the Organic Unit of the Department- Dr I.A. Oladosu - for the support and assistance given to me during the course of this work - Prof. Sonibare, Dr Onocha, Dr Aboaba, Dr Oloyede, Dr Moronkola and Dr Olaoluwa, I say a very big thank you to you all.

I am grateful to the Director and scientists of the Indian Institute of Integrative Medicine (IIIM), of the Council of Scientific and Industrial Research (CSIR) of the government of India and The World Academy of Sciences (TWAS) for the award of the CSIR-TWAS Postgraduate Fellowship which facilitated my research visit to the Indian Institute of Integrative Medicine, Jammu where I did part of this research work.

My appreciation also goes to all other lecturers in the Department of Chemistry, University of Ibadan, who have helped shaped my life since my undergraduate days -

Prof. B.B. Adeleke, who magnanimously included my name in the HOD's list for me to get admission in 100 level to study chemistry during the 1998/99 session, Prof. Babalola, Dr L.M. Nwokocha, Dr Alabi, Dr Ajayi, and many technologists whose name I may not remember, God bless you all.

I won't forget to mention my colleagues who have assisted me in several ways during the course of this PhD research, Dr Hamid, Dr Akoro, Dr Atewolara-Odule, Dr Ajibade, Mr Sola Akande, Mr Ogunyemi Oderinlo, I say God will honour you all in Jesus name.

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Finally, I also sincerely appreciate the understanding, support and prayers of my wife, my jewel of inestimable value, Fisayo and our precious children – Tim, John and Glory. I am forever grateful to you all, God bless you all.

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Paul Matthew OSAMUDIAMEN
OCTOBER, 2018

CERTIFICATION PAGE

I certify that this work was carried out by Mr Paul Matthew OSAMUDIAMEN in the Department of Chemistry, University of Ibadan, Ibadan, Nigeria.

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ABBREVIATIONS

Techniques

HPLC	High-Performance Liquid Chromatography
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
ESI-MS	Electrospray Ionisation - Mass spectrometry
HRMS	High-Resolution mass spectrometry
IR	Infrared spectroscopy
Mp	melting point
UV	Ultra-Violet spectroscopy
ORTEP	Oak Ridge Thermal Ellipsoid Plot Program

NMR Related Terms

^{13}C	Carbon - 13 NMR
^{1}H	Proton NMR
COSY	^{1}H-^{1}H Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
DEPT	Distortionless Enhancement by Polarisation Transfer
NOESY	Nuclear Overhauser Effect Spectroscopy
s	singlet
d	doublet
dd	doublet of doublet
dt	doublet of triplet
m	multiplet

q	quartet
s	singlet
t	triplet
td	triplet of doublet
TMS	Tetramethylsilane

Miscellaneous

CAS	Ceric Ammonium Sulphate
MIC	Minimum Inhibition Concentration
IC_{50}	The concentration of substance that can inhibit 50 % of cell lines.

CHAPTERONE

INTRODUCTION

1.1 THE ROLE OF NATURAL PRODUCTS IN HEALTH CARE

The use of natural products, especially from plants, for healing is as ancient and universal as medicine itself. Plants have been used as a traditional method of treatment for many human diseases from the past. Written records about medicinal plants date back at least 5000 years to the Sumerians, and archeological records suggest even earlier use of medicinal plants (Palombo, 2009). Natural products played a significant role in several ancient traditional medicine systems, such as Chinese, Ayurveda and African traditional medicine which are still in common use today (Sarker *et al*, 2005).

Today plant-based drugs continue to play an essential role in health care and it has been estimated by the World Health Organization that 80% of the population of the world rely mainly on traditional medicines for their primary health care (Farnsworth *et al*, 1985). The remaining 20% of the world's population, who mainly reside in developed countries, are also recipients of the benefits of natural products (Arvigo and Balick, 1993), as about 121 drugs prescribed in the USA today come from natural sources and 90 of which come either directly or indirectly from plant sources (Benowitz, 1996). There are about 5,000,000 plant species in the world today, but the phytochemistry of only about 1% has been investigated (Raskin *et.al*., 2002). Hence, there is an enormous potential of new bioactive compounds that are yet undiscovered.

1.2 NATURAL PRODUCTS AND ORAL HEALTH

Oral health is a state of being free from chronic mouth and facial pain, oral and throat cancer, oral sores, periodontal disease, tooth decay and tooth loss, and other diseases and disorders that affect the oral cavity (Kapoor, 2015). Natural products have been used from the past for their antibacterial effect, but recently there have been investigations about promising agents for the prevention of oral diseases especially plaque-related diseases such as dental caries and periodontal disease (Borhan-mojabi and Azimi, 2013). Dental caries is a bacterial initiated demineralization of the hard tissue structures of the tooth. Periodontal disease is an inflammatory disease that affects the supporting structures of teeth including the gingiva, periodontal ligament and alveolar bone Ozden *et al*, 2015). It is characterized by inflammation of the supporting structures of teeth (Pihlstrom *et al*., 2005). If left untreated, it may cause the annihilation of these supporting structures and ultimately tooth loss. The global age-standardized prevalence of severe periodontitis was estimated at 10.5–12.0% as at the year 2010. (Kassebaum *et al*., 2014).Dental plaque is the primary etiologic factor in dental caries, gingivitis, and periodontal disease. The most common risk factors associated with Periodontal disease and tooth loss include poor oral hygiene maintenance (Javed *et al*., 2007), ageing (Fah and Schatzle, 2014; Schatzle *et al*., 2010), lifestyles such as smoking (Bergstrom, 2004; Fah and Schatzle, 2014) and chewing smokeless tobacco (Javed *et al*., 2013). It is noteworthy that some of the risk factors of periodontal disease, particularly tobacco have also been linked with the etiology of oral cancer (Warnakulasuriya,2009a; Johnson *et al*., 2011).Cancer is among the leading causes of global mortality and oral and pharyngeal cancers are its most common forms (Warnakulasuriya, 2009b).The annual global incidence of oral cancer has been reported to be approximately 500,000 (Siegel *et al*., 2013). Research

has shown the potential of natural products in remineralising tooth surfaces affected by dental caries (He *et al*, 2015). Plant based extracts and their derivatives have been explored for the anti-cariogenic and anticancer effects (Ferrazzano *et al.,* 2011; Hambire *et al*, 2015).

1.3 JUSTIFICATION OF THE PRESENT STUDY

The global need for alternative prevention and treatment options and products for oral disease that are safe, effective and economical comes from the rise in disease incidence (particularly in developing countries), increased resistance by pathogenic bacteria to currently used antibiotics and chemotherapeutics, opportunistic infections in immunocompromised individuals and financial considerations in developing countries (Badria and Zidan 2004; Tichy and Novak, 1998). Despite several chemical agents being commercially available, these can alter oral micro biota and have undesirable side-effects such as vomiting, diarrhoea and tooth staining (Chung *et al*, 2006; Park *et al*, 2003). Furthermore, western medicine has had only limited success in the prevention of periodontal disease and in the treatment of a variety of oral diseases. Hence, the search for alternative products continues and natural products isolated from plants used in traditional medicine are considered as good alternatives to synthetic compounds (Prabu *et.al*, 2006). As most of the oral diseases are due to bacterial infections and it has been well-documented that medicinal plants, (particularly chewing sticks) confer considerable antibacterial activity against various microorganisms including bacteria's responsible for dental caries (Kelmanson *et al.*, 2000).

Traditionally, *Mezoneuron benthamianum* is reportedly used in antiseptic cleansing and healing of ulcers and in the treating of enteralgia (Dickson *et al.*, 2011a) and as chewing sticks in southwestern states of Nigeria (Burkill, 1985). It is known locally

among the Yoruba's as "*meyinro*" meaning "*that whichmakes the teeth to be strong*". Despite, the importance of *M. benthamianum* in African traditional medicine, there are few reports of its phytochemical constituents and biological activities especially activities against oral microbes; even though it has the potential of being a source of bioactive constituents which could serve as new leads for the development of drugs against oral infections.

1.4 RESEARCH OBJECTIVES

1.4.1 GENERAL OBJECTIVE

The general objective of this study is to isolate and characterise bioactive compounds from *Mezoneuron benthamianum* and determine their antimicrobial activity against oral pathogens and other biological activities.

1.4.2 SPECIFIC OBJECTIVES

The specific objectives of this research are, therefore:

1. To prepare extracts of *Mezoneuron benthamianum* roots using standard methods.
2. To determine the anticaries activities of the extracts and other biological activities such as antioxidant, cytotoxic and anticancer activities.
3. To isolate pure compounds from the plant extracts using chromatographic techniques.
4. To characterise the pure isolates using spectroscopic techniques.
5. To determine the anticaries, antioxidant, cytotoxic and anticanceractivities of the pure isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 The genus *Mezoneuron*

Mezoneuron is a genus comprising 24 species. It has been considered by previous authors as either a subgenus of *Caesalpinia* Linnaeus (Hattink, 1974; Herendeen and Zarruchi 1990; Hou *et al*, 1996; Larsen *et al.* 1980; Larsen *et al*. 1984) or as a distinct genus (Prain, 1892; Lewis *et al.* 2005). The latest molecular evidence indicates that *Mezoneuron* should be considered as a genus distinct from *Caesalpinia sensu stricto*, together with about12 additional genera segregated from within *Caesalpinia sensu lato* (Lewis and Schrire 1995; Simpson *et al*. 2003; Gagnon *et al*. 2013; Gagnon, 2015; Gagnon *et al*. 2015).

Geographically, the genus *Mezoneuron* is most diverse in South East Asia, with about 12 species distributed in tropical or subtropical areas from China to Papua New Guinea and Australia. A further four species are endemic to Australia, and five endemic species occur in New Caledonia (Clark and Gagnon, 2015). Additionally, there are two species in continental Africa, one endemic in Madagascar, and one endemic in Hawaii (Herendeen and Dilcher, 1991; Herendeen and Crane, 1992).

2.1.1 The species of *Mezoneuron*

The species of *Mezoneuron* are scrambling shrubs or lianas with bipinnate leaves, and usually with recurved prickles on the stems and leaf rhachises. The flowers are usually yellow with red markings on the median petal, or occasionally entirely pink to or white varying in size from a few millimetres in length up to about 3 cm. The fruits range in length from 2 to 20 cm, the sutural wing measuring between 0.5 to 20 mm in width, and may be single or multiple seeded (Clark, 2016). The species include *M. kauaiense, M. hildebrandtii, M. angolense and M. benthamianum*

2.1.2 *Mezoneuron benthamianum* Baill.

*M. benthamianum*Baillion is a shrub in the secondary jungle and savanna forest from Senegal to Nigeria. It is a climbing or a straggling shrub about 10 m in length. The body surface of the stem is filled with recurved spines, to about 1.5 cm, corky spine tipped tubercles on older stems; glabrous or very sparsely orange tomentose. Its leaves are bipinnate showing alternate arrangement with 5 – 6 pairs of pinnae. The stipules are small and inconspicuous. The leaf petiole is usually between 5 to 10 cm long with a swollen base and has rachis that maybe 15 to 20 cm long bearing the recurved spines at the base of the pinnae. The leaflets are arranged alternately with 5 pairs per pinna, usually, they are elliptical, ranging from 3 to 4 cm by 1.5 to 2.5 cm. The base and apex are rounded and glabrous. The inflorescence (terminal raceme) is usually hairy and densely flowered, may or may not form branches, and can be up to 20cm in length. The flowers are bisexual, zygomorphic and pentamerous (Bosch, 2007).

Figure 2.1a: *Mezoneuron benthamianum* Baill.

Figure 2.1b: *Mezoneuron benthamianum* packed as local chewing sticks

2.1.3 Traditional uses of *Mezoneuron benthamianum*

The roots of *M. benthamianum* are considered to be an effective dysentery remedy in Ghana. The powdered roots are used mixed with shea butter or palm kernel oil to treat skin diseases and wounds in Ghana. A decoction of the root, bark, and leaves is used in Guinea for urethral discharges. An infusion of the dried root is drunk or used as a bath for general malaise in Senegal. The aqueous decoction of the roots is used in traditional medicine as aphrodisiacs and the vasorelaxant properties have been reported (Dickson *et al*., 2011a).

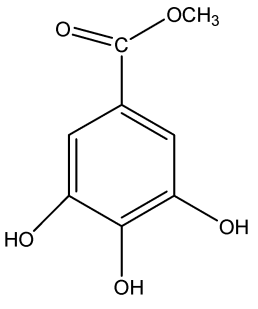
Young leaves of *M. benthamianum* are similarly used in Sierra Leone after mashing up on wounds and swollen parts of the body; young leaves are burnt for the same purpose. In Ivory Coast, the plant is used for troublesome cough and headache and as a plaster for a soothing suppository on piles or in mouthwash for toothache. A macerate of leafy twigs is prescribed in Senegal for male impotence when it is attributed to venereal disease. A decoction of leaves, bark and root is used in Guinea, Senegal and Nigeria for urethral discharge. The young bright red leaves are chewed in Guinea as depurative or simply as a masticatory. Fumigation with the leaf is said to be effective in oedemas. The leaf is administered as a mild laxative in Senegal in treating enteralgia. The root, scorched to remove the outermost skin, is put into palm wine in Sierra Leone to enhance the strength. The roots are considered aphrodisiac in Ivory Coast. In Nigeria, the root is reportedly used in Ibadan area as a chewing stick with a claim of effectiveness against dental caries (Burkill, 1985). It has been traditionally used in the management of several diseases including erectile dysfunction, dysentery, urethral discharges, skin diseases and wounds (Dickson *et al*, 2011a).

2.1.4 Chemical Constituents of *M. benthamianum*

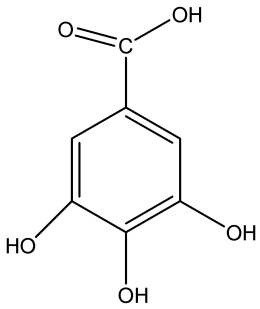
The aqueous extract of the whole plant has been analysed to contain flavonoids,

phenols, anthraquinones, reducing sugars, tannins and saponins (Mbagwu and Adeyemi, 2008). The leaves of *M. benthamianum* are rich in saponins and tannins (Fayemi and Osho, 2012). Sixteen compounds have been isolated from the chloroform, butanol and hydroethanolic extracts of the leaves namely: methyl gallate **(1)**, gallic acid **(2)**, shikimic acid-3-*O*-gallate **(3)**, 1-*O*-methyl-*D*-*chiro*-inositol **(4)**, (-)-epicatechin **(5)**, (-)-epicatechin-3-*O*–gallate **(6)**, Kaempferol-3-(6'-galloyl) glucoside **(7)**, Mezobenthamic acid A (**8**), Mezobenthamic acid B (**9**), neocaesalpin H (**10**), quercetin (**11**), kaempferol (**12**), resveratrol (**13**), ethyl gallate (**14**), β-sitosterol glucoside (**15**) and 13b-hydroxy-pheophorbide A (**16**), while three compounds have been isolated from the petroleum ether extract of the roots namely: deoxycaesaldekarin C **(17)**, Benthaminin 1 **(18)** and Benthaminin 2 **(19)** (Binutu and Cordell, 2000; Dickson *et al*, 2007, Jansen *et al*, 2017).

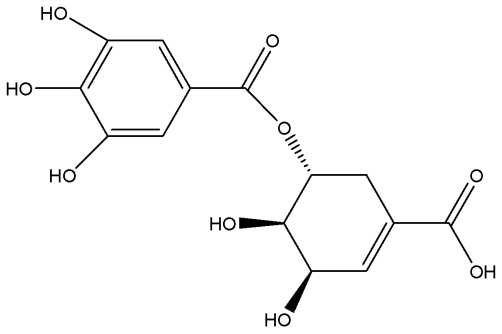
Fifteen compounds were also identified from the essential oil of the aerial parts of *M. benthamianum* constituting monoterpenes (36.5%), sesquiterpenes (20.4%), sesquiterpenoids (19.6%) and a non-ubiquitous apocarotenoid $C_{20}H_{30}O$ (16.7%) (Moronkola *et al*, 2009).



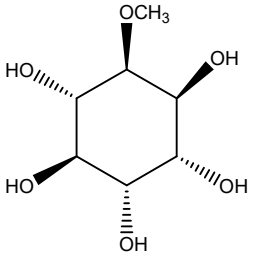
methyl gallate (**1**)



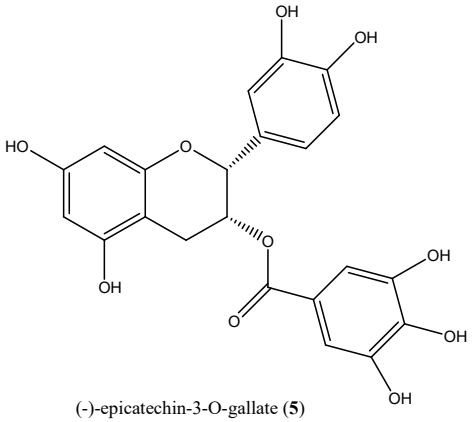
gallic acid (**2**)



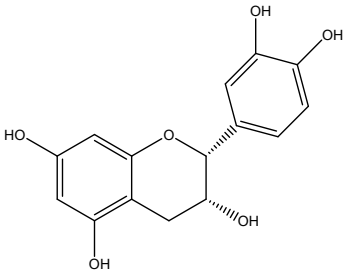
shikimic acid-3-O-gallate (**3**)



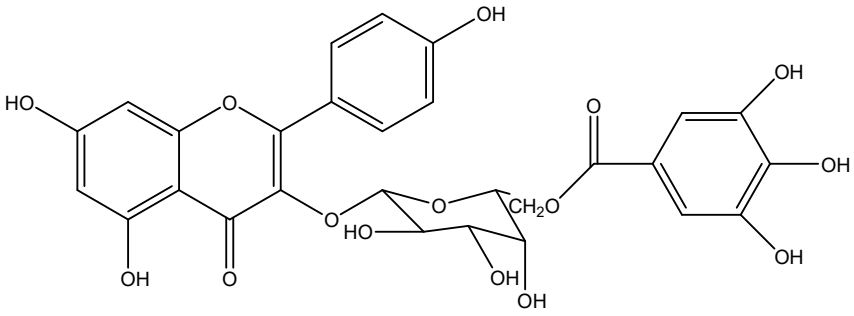
1-O-methyl-D-chiro-inositol (**4**)



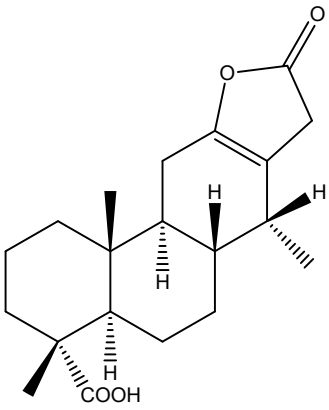
(-)-epicatechin-3-O-gallate (**5**)



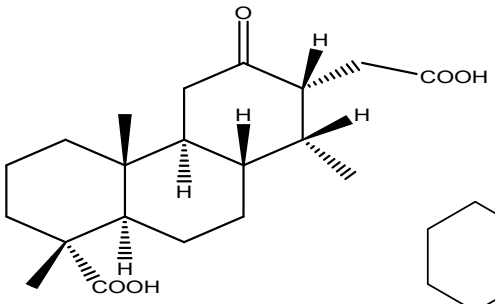
(-)-epicatechin (6)



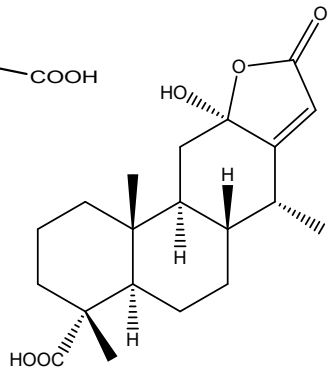
kaempferol-3-(6'-galloyl) glucoside (**7**)



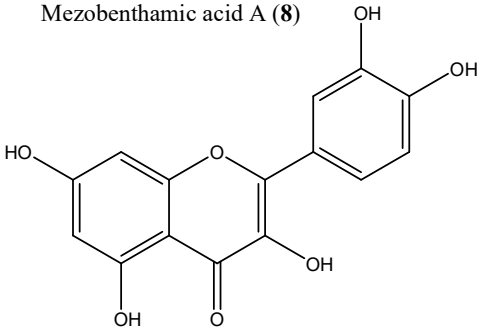
Mezobenthamic acid A (**8**)



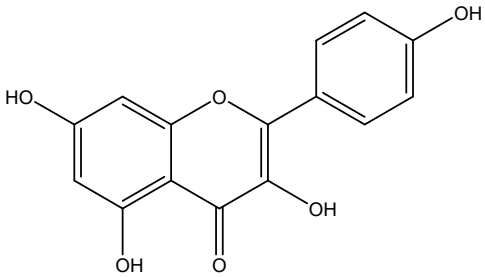
Mezobenthamic acid B (**9**)



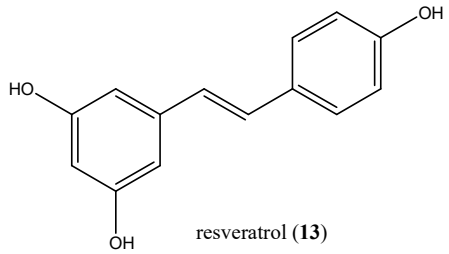
neocaesalpin H (**10**)



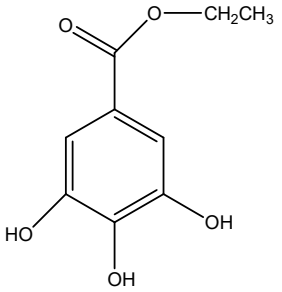
quercetin (**11**)



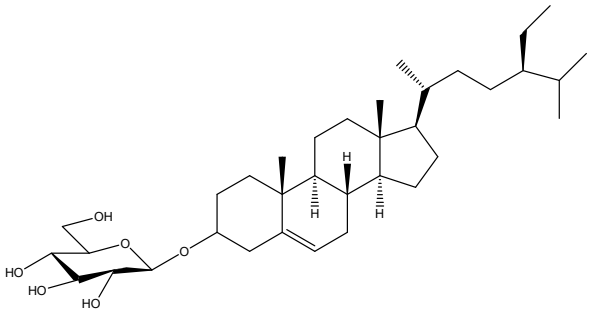
kaempferol (**12**)



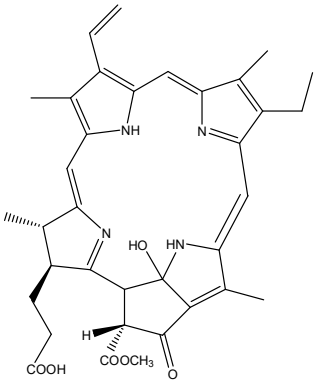
resveratrol (**13**)



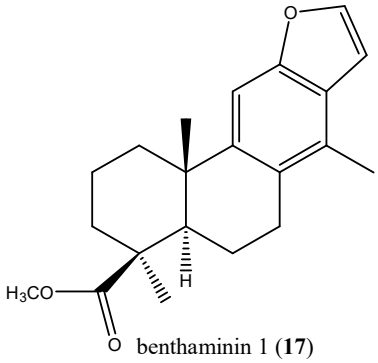
ethyl gallate (**14**)



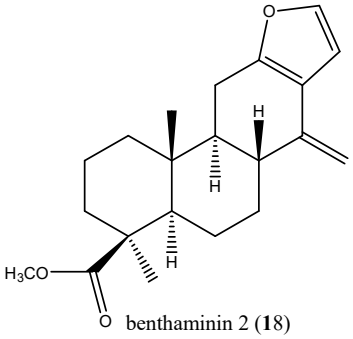
beta-sitosterol glucoside (**15**)



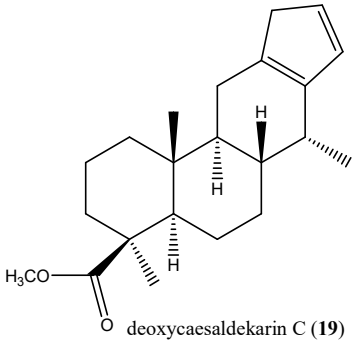
13b-hydroxy-pheophorbide A (**16**)



benthaminin 1 (**17**)



benthaminin 2 (**18**)



deoxycaesaldekarin C (**19**)

2.1.5 Anti-inflammatory Properties

The essential oil of the leaves of *M. benthamianum* inhibited the 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) ear induced oedema in mice as mice treated with 2.5 and 5.0 mg dose had a reduction of the ear oedema from 76.9 % and 92.3 % respectively. The oil significantly performed better against indomethacin, which is a standard anti-inflammatory drug (Moronkola *et al*, 2009).

2.1.6 Anti-diarrheal Properties

M. *benthamianum* showed a strong anti-diarrheal effect when tested on Wistar rat and Swiss mice weighing between 170 to 200 g and 25 to 30 g respectively. When the mice, which were induced with castor oil (0.2 ml/animal), were orally fed with aqueous extract (400 – 1600 mg/kg) 30 minutes before the administration of castor oil, a dose dependent and significant ($p<0.05$) delay in the onset of diarrhea was observed. There was also reduction in the frequency of the stooling and a general decrease in the weight of wet stools, hard, mild and copious diarrhea (Mbagwu and Adeyemi, 2008).

2.1.7 Vasorelaxation Property

The aqueous root bark extract of *M. benthamianum* also exhibited vasorelaxant properties. In an experiment involving the aorta ring of rat strip, the application of the aqueous extract (1 - 20 mg/L) on the aorta ring whose contraction was induced with phenylephrine, resulted in an immediate relaxation of the aorta which continued to reach a plateau after 15 minutes. Furthermore, the QPCR analysis revealed that the extract triggered eNOS mRNA expression ($p < 0.001$) of 2.4 ± 0.5, 4.3 ± 0.7 and 5.7 ± 0.7 at 0.1, 1 and 10 mg/L concentrations of root bark extract respectively, these are of great interest considering the fact that, scale stimulation only occurs when relative quantity is superior to 1.5 in eNOS QPCR evaluation (Zamble *et al*, 2008).

2.1.8 Antibacterial and Anti-Candida activities

Petroleum spirit, chloroform and ethanol extracts of the root bark of *M. benthamianum* showed recorded activity against gram-positive and gram-negative bacteria and some dermatophytes such as: *Micrococcus flavus* (NCTC 7743), *Bacillus subtillis* (NCTC 10073), *Staphylococcus aureus* (NCTC 4163) multidrug-resistant *S. aureus* (SA-1199B), tetracycline-resistant *S. aureus* (XU212) , erythromycin-resistant *S. aureus (*RN 4220), *Streptococcus faecalis* (NCTC 775), *Salmonella abony* (NCIMB 6017), *Pseudomonas aeruginosa* (NCIMB 10421), *Escherichia coli* (NCTC 9002), *Klebsiella aerogenes* (NCTC 5055), *Candida albicans* (NCPF 3179), *Saccharomyces cerevisiae* (NCTC 080178), *Trichophyton interdigitale* (NCPF 654) and *Microsporum gypseum* (NCPF261). The MIC values against these organisms ranged from 31.2 to 1000 µg/mL (Dickson, 2006). However, the gallic acid and its methyl ester isolated from its leaf were found to have weak activity against *Staphylococcus aureus (*ATCC 29213*)*, *Bacillus subtilis (*ATCC 21394*), Escherichia coli (*ATCC 25922*)* and *Pseudomonas aeruginosa* (ATCC 27853) (Binutu and Cordell, 2000)

The three compounds (benthaminin 1, benthaminin 2 and deoxycaesaldekarin C) which were isolated from the petroleum ether fraction of the root bark of the plant gave high antibacterial activities with benthaminin 2 having better performance than the others, it had a activity of 47.0 µM against *S. aureus* and *M. flavus* (Dickson, *et al*, 2007). Furthermore, when *M.benthamianum* was evaluated against *Candida* species gave Minimum inhibitory concentration (MIC) of 5 mg/ml against *C. glabrata,* 6 mg/ml against *C. torulopsis,* 8 mg/ml against *C. albicans and C. stellatoidea and* 15 mg/ml against *C. krusei* (Fayemi and Osho, 2012).

2.1.9 Antioxidant and Scavenging Properties of the Root Bark

M. benthamianum also shows a high level of antioxidant properties as the petroleum spirit and chloroform extracts of *M. benthamianum* recorded high antioxidant properties with IC_{50} values of 15.55 µg/mL and 19.72ug/mL for free radical scavenging activity and 23.15 and 30.36 µg/mL respectively for inhibition of lipid peroxidation of bovine brain liposomes (Dickson *et al*, 2006). Of the three compounds (benthaminin 1, benthaminin 2 and deoxycaesaldekarin C) isolated from the petroleum ether fraction of the root bark of the plant, deoxycaesaldekarin C and benthaminin 2 showed high antioxidant properties with benthaminin 2 recording better results having IC_{50} values of 42.7 and 74.2 µM for DPPH (2, 2-diphenyl-1-picrylhydrazyl) spectrophotometric and TBA (thiobarbituric acid) lipid peroxidation assays respectively. The performance of benthaminin 2 was attributed to the presence of an exocyclic methylene functional group in the compound. (Dickson, *et al*, 2007)

2.1.10 Aphrodisiac property

In a test carried out to determine the aphrodisiac property of the aqueous extract of the root, two groups of five sexually matured rats were given 1mL of tap water (control group) and 50 mg/kg body weight of the aqueous extract of *M. benthamianum* (test group). Results indicate that the mounting frequency (MF) ($p< 0.001$) increased significantly while the mounting latency (ML) decreased after 30 minutes, 1.15 hr and 3.15 hrs of observations when compared to that of untreated rats (Zamble, *et al*, 2008).

2.1.11 *In-vivo* Toxicity Properties

The aqueous extract of the whole plant has been found to be non-toxic to wistar rats and Swiss mice. The LD_{50} of the extract administered intraperitoneally was 1021.31 mg/kg. Oral administration up to 2 g/kg produced no deleterious effect 24 h after dosing and up to7 days afterwards (Mbagwu and Adeyemi, 2008).

2.2 PLANT PHYTOCHEMICALS

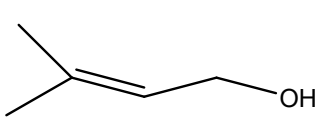
2.2.1 Primary and Secondary Metabolites

Natural products are classified into three: firstly, are the primary metabolites, which are the compounds that are in all cells and play a central role in the metabolism and reproduction of those cells, they include nucleic acids and the common amino acids and sugars. Secondly, there are those that have high-molecular-weight polymeric materials such as cellulose, lignins and proteins which form the cellular structures. Finally, there are the secondary metabolites which are compounds that are the characteristic of a limited range of species. Most primary metabolites exert their biological effect within the cell or organisms that are responsible for their production, while secondary metabolites, on the other hand, have often attracted interest because of their biological effect on other organisms (Hanson, 2003). The biosynthesis and breakdown of primary metabolites is known as primary metabolism (Dewick, 2002). The mechanism by which an organism biosynthesizes secondary metabolites is referred to as secondary metabolism and it is often found to be unique to an organism or is an expression of the individuality of a species (Dewick, 2002; Maplestone *et al*., 1992). Secondary metabolites are generally not necessary for the development, growth or reproduction of an organism and they are formed either as an outcome of the organism adapting to its environment or as a possible defense mechanism against predators to aid in the survival of the organism (Colegate and Molyneux, 1993; Dewick, 2002). Depending on their biosynthetic origin, secondary plant metabolites, could be classified as; terpenoids (monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids), phenolics (flavonoids, tannins, phenylpropanoids and related compounds) and alkaloids (a group of nitrogen containing compounds gotten from amino acids) (Edwards and Gatehouse, 1999).

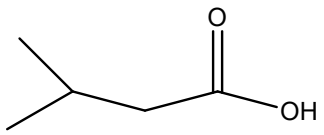
2.2.2 Terpenoids

Terpenoids are secondary metabolites consisting of one or more isoprene units linked together in a head to tail, or rarely tail to tail manner. Two different biosynthetic pathways produce the main terpene building block, isopentenyl pyrophosphate (IPP). The first is referred to as either the MEP (methylerythritolphosphate) or DOX (1-deoxy-D-xylulose) pathway. Here, IPP is formed in the chloroplast. The second biosynthetic route is known as the MVA (mevalonic acid) pathway (Figures 2.2 and 2.3). (Finar, 2000; Eisenreich *et al*, 2001; Rohdich *et al.,* 2001).They are classified by the number of isoprene units they contain into: Hemiterpenes e.g prenol (**20**) and isovaleric acid (**21**), Monoterpenes e.g geraniol (**22**), terpineol (**23**), myrcene (**24**) and iridoids (**25**) Sesquiterpenes e.g humulene (**26**), artemisinin (**27**) and farnesol (**28**), Diterpenes e.g Pleuremutillin (**29**), and taxadiene (**30**) Sesterterpenes e.g geranylfarnesol (**31)**, Triterpenes e.gUrsolic acid **(32)** and tetraterpenoids e.g Lycopene (**33**).

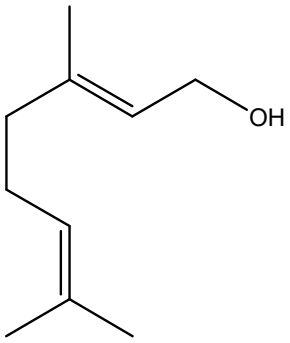
The function of terpenes in plants is generally considered to be both ecological and physiological. Many of them inhibit the growth of competing plants (allelopathy). Some are known to be insecticidal (Larcher, 1995; Becerra, 1997; Taiz and Zeiger, 2002) others are found to attract insect pollinators (Mauseth, 2003).



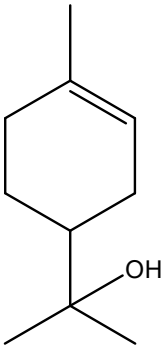
Prenol (**20**)



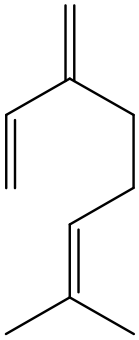
Isovaleric acid (**21**)



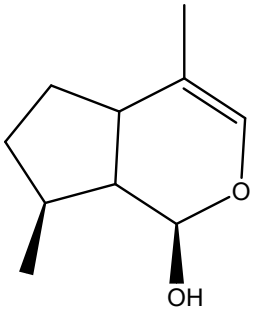
Geraniol (**22**)



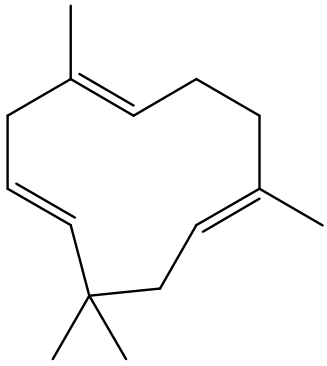
α-terpineol (**23**)



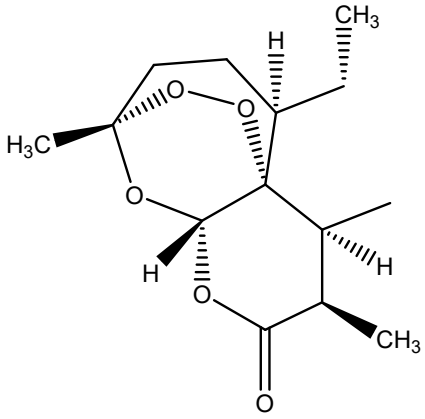
myrcene (**24**)



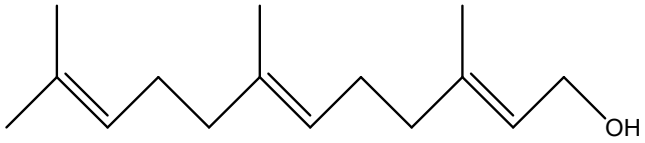
Iridoid (**25**)



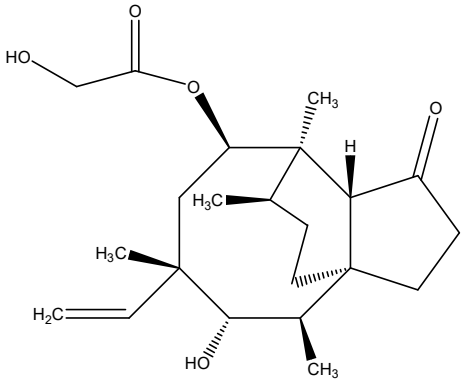
Humulene (**26**)



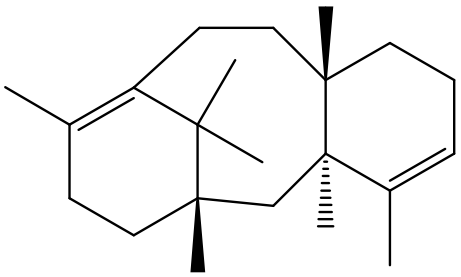
Artemisinin (**27**)



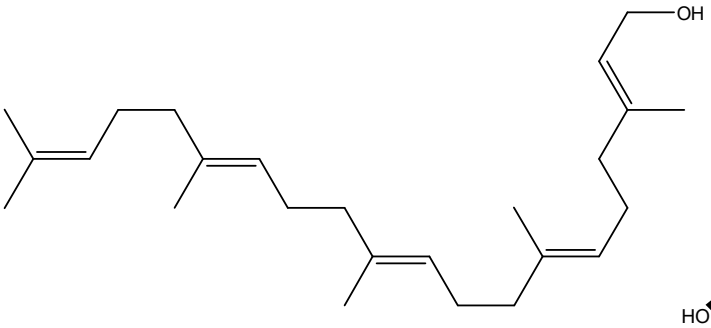
farnesol (**28**)



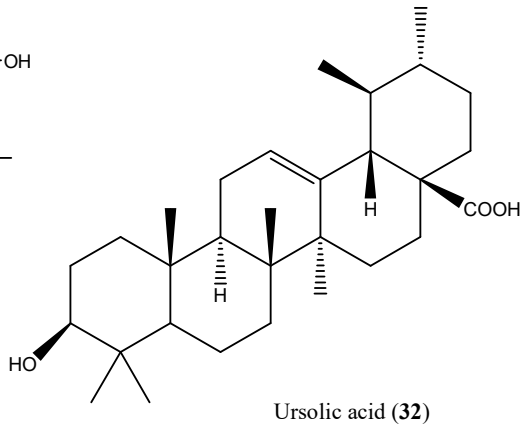
Pleuremutillin (**29**)



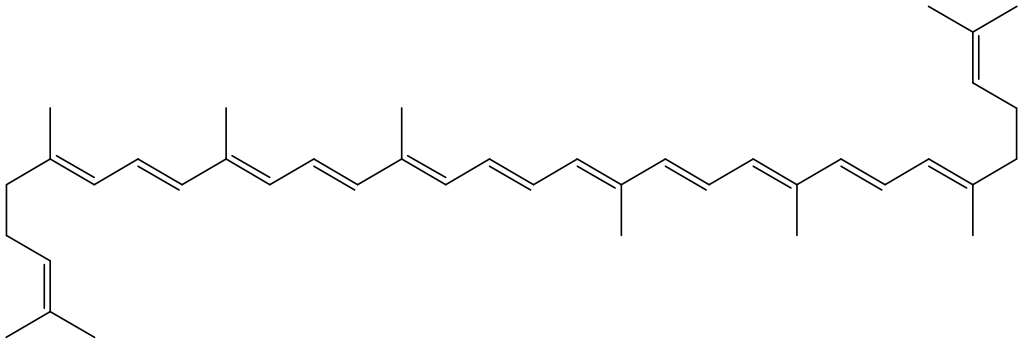
Taxadiene (**30**)



Geranylfarnesol (**31**)



Ursolic acid (**32**)



lycopene (**33**)

2.2.2.1 Biosynthesis of Terpenes

The biosynthesis of terpenes will be considered under two sub-sections: first is the production of isopentenyl pyrophosphate (IPP) and dimethy allyl pyrophosphate (DMAPP) (Fig. 2.2). Secondly is the condensation of IPP and DMAPP to form terpenes (Fig. 2.3).

2.2.2.2 Synthesis of IPP and DMAPP

The precursor of the isoprene unit is acetyl-CoA. The isoprene unit is formed via the mevalonic acid pathway (Fig. 2.2). when three molecules of acetyl-CoA combine in a step-by-step procedure to form the mevalonic acid, a six-carbon compound intermeiate. The Mevalonic acid then undergoes pyrophosphorylation by two enzymes, the mevalonate kinase and phosphomevalonate kinase using two molecules of ATP.

When MVA-PP is decarboxylated and dehydrated, it leads to the production of isopentenyl pyrophosphate (IPP), which can also be rearranged to dimethylallyl pyrophosphate (DMAPP) by isopentenyl isomerise.

2.2.2.3 Condensation of IPP and DMAPP to form terpenes

One molecule of IPP and DMAPP fuse together by the enzyme dimethylally transferase to produce geranyl pyrophosphate (GPP), which is the precursor of all monoterpenes. The successive combination of isoprene C_5 units obtained from IPP to the GPP unit obtained from DMAPP leads to the gradual buildup of the terpenes up to C_{25} compounds. Consequently, the sesquiterpened are produced from 2E, 6E-farnesyl pyrophosphate (FPP), and diterpenes from 2E, 6E, 10E-geranylgeranyl pyrophosphate (GGPP). The parents of triterpenes and tetraterpenes are formed by the reductive coupling of two FPPs or GGPPs, respectively. (Satyajit and Lutfun, 2007).

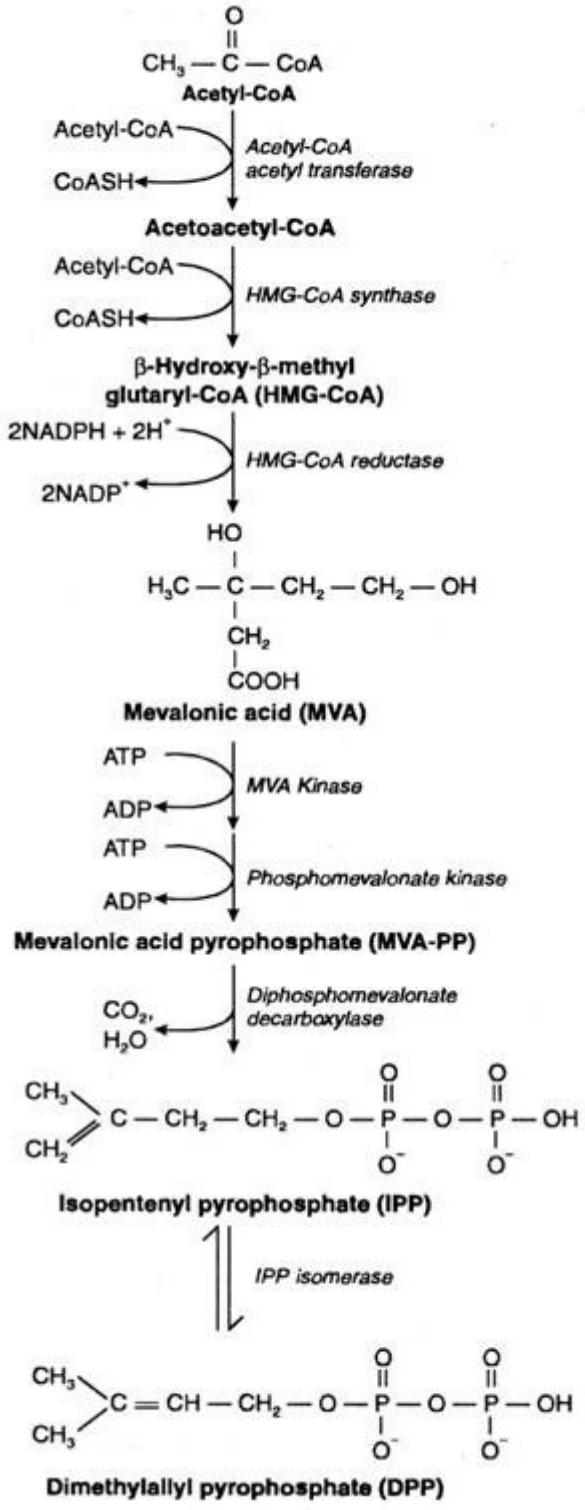


Figure 2.2: Biosynthesis of Isopentenyl Pyrophosphate (IPP) and Dimethylallyl pyrophosphate (DMAPP)(Khandelwal, 2016)

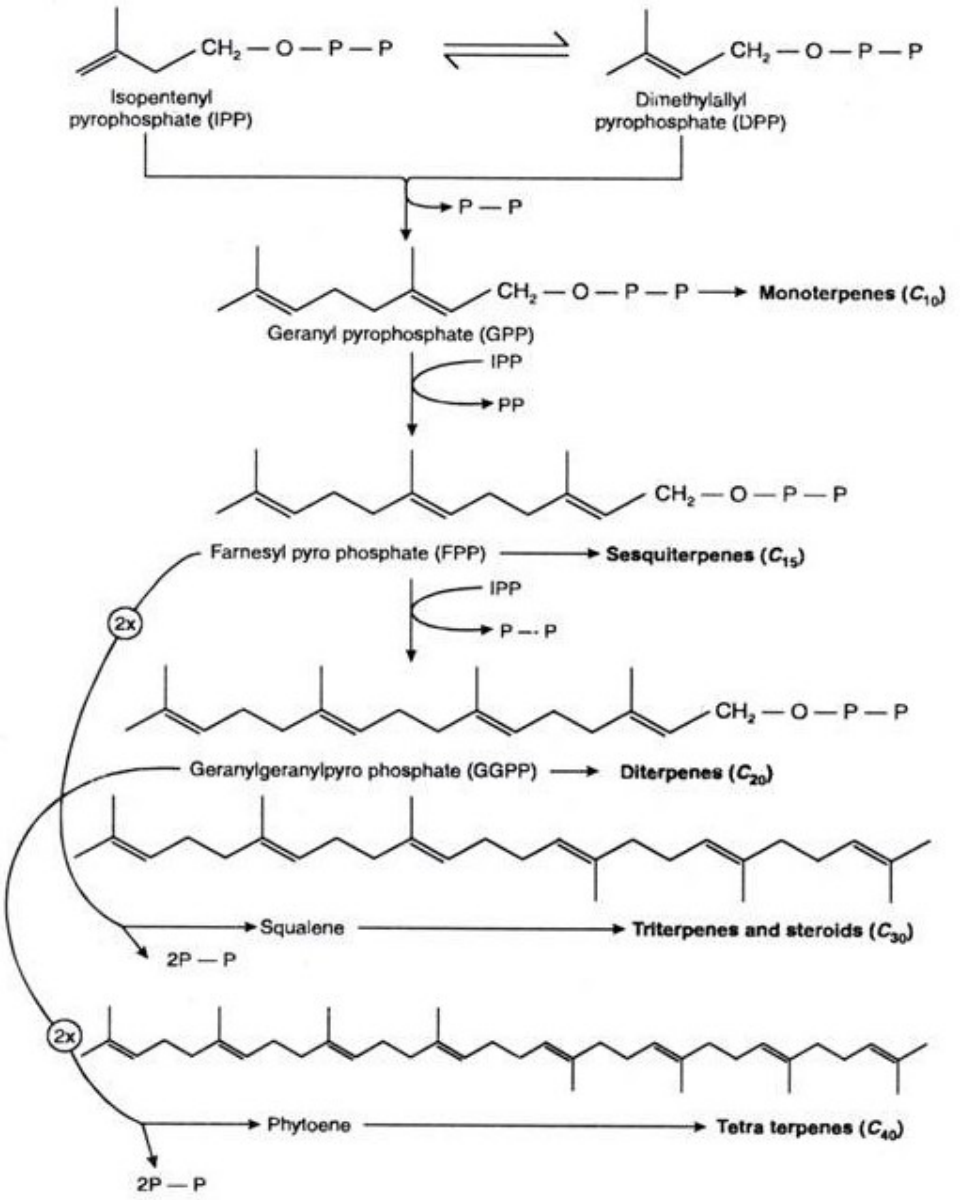


Figure 2.3: Biosynthesis of different classes of terpenes (Khandelwal, 2016)

2.2.2.4 CASSANE DITERPENOIDS

The name cassane is derived from the native name “Cassa” for *Erythrophleumguineense*, the first source of a crystalline diterpene alkaloid called Cassaine (Dalma,1939). The basic cassane skeleton is structurally characterized as a tricyclic diterpenewith a substitution of an ethyl group at C-13 and one methyl group at the C-14 positionwhile norcassanes have one carbon less from cassane either from C-17 or C-16positions (Fig. 2.4a). In literature, it was reported that cassane skeleton may bederived from pimarane by the methyl migration from C-13 to C-14. However, 17-norcassane diterpenes may be biosynthesized through decarboxylation of cassane-typediterpenes and 16- norcassane-type diterpenes, probably derived from oxidativecleavage of the C-15,16 double bond (Arjun *et al*, 2003; Overtone 1974) (Fig. 2.4b).Cassane diterpenes exhibited wide range of pharmacological activities such as antitumor,antimalarial, anti-inflammatory, anti-viral, anti-microbial and antitrypanosomalproperties.

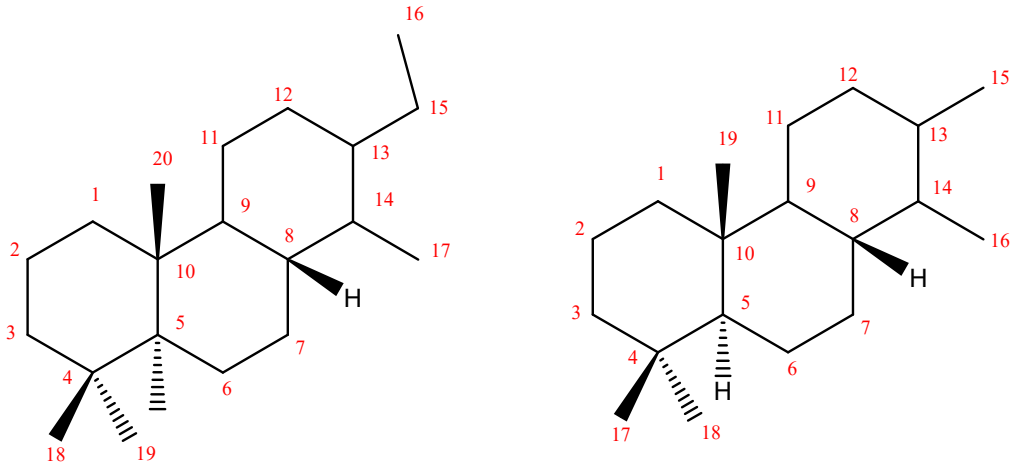


Fig. 2.4a: Cassane and norcassane diterpenes skeleton

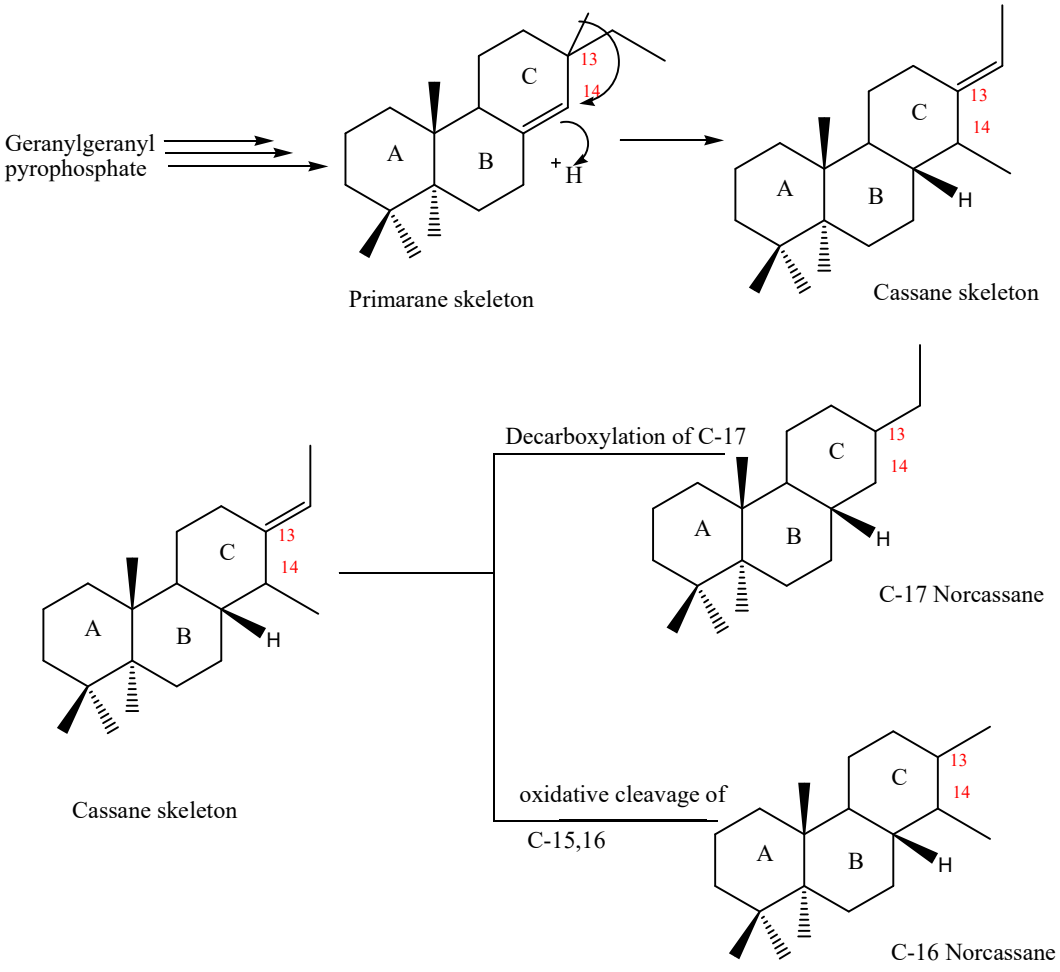


Fig.2.4b: Proposed Biosynthetic pathway for Cassane and Norcassane diterpene skeleton

2.2.3 Phenolic Compounds

Approximately 8,000 naturally occurring compounds belong to the category of"phenolics," all of which share a common structural feature: an aromatic ring bearing

at least one hydroxyl substituent, that is, a phenol (Croteau *et al*, 2000). The hydroxylgroup(s) can be free or engaged in another function as ethers, esters, or glycosides(Herrmann and Weaver, 1999). A straightforward classification attempts to divide the

broad category of phenolics into simple phenols and polyphenols, based exclusively on the number of phenol subunits present, but many plant phenolic compounds arepolymerized into larger molecules. Thus, the term "plant phenolics" are characterized

by the presence of several phenols and include simple phenols, free phenolic acids,phenylpropanoids, coumarins, flavonoids, stilbenes, tannins, and quinone pigments.Their biosynthetic origin is through the shikimate pathway (Fig. 2.5). (Clifford, 1999;

Finar, 2000; Petrussa *et al.,* 2013).

2.2.3.1. Phenolic acids

Phenolic acids (phenol carboxylic acids) are a subclass of the larger phenolic category (Fig. 2.6), that includes substances containing a phenolic ring and at least one organic carboxylic acid function. Depending on the carbon units of the lateral chain attached to the phenolic ring, the phenolic acids can be divided into C6-C3, C6-C2, and C6-C1 compounds, the most important being C6-C3 (derived from the hydroxycinnamic acid) and C6-C1 (compounds with a hydroxybenzoic structure). Examples of hydroxybenzoicacids include salicylic acid (**34**) and gallic acid (**35**) and examples of

compounds withhydrocinnamic acid structure include caffeic acid (**36**) and ferullic acid (**37**).

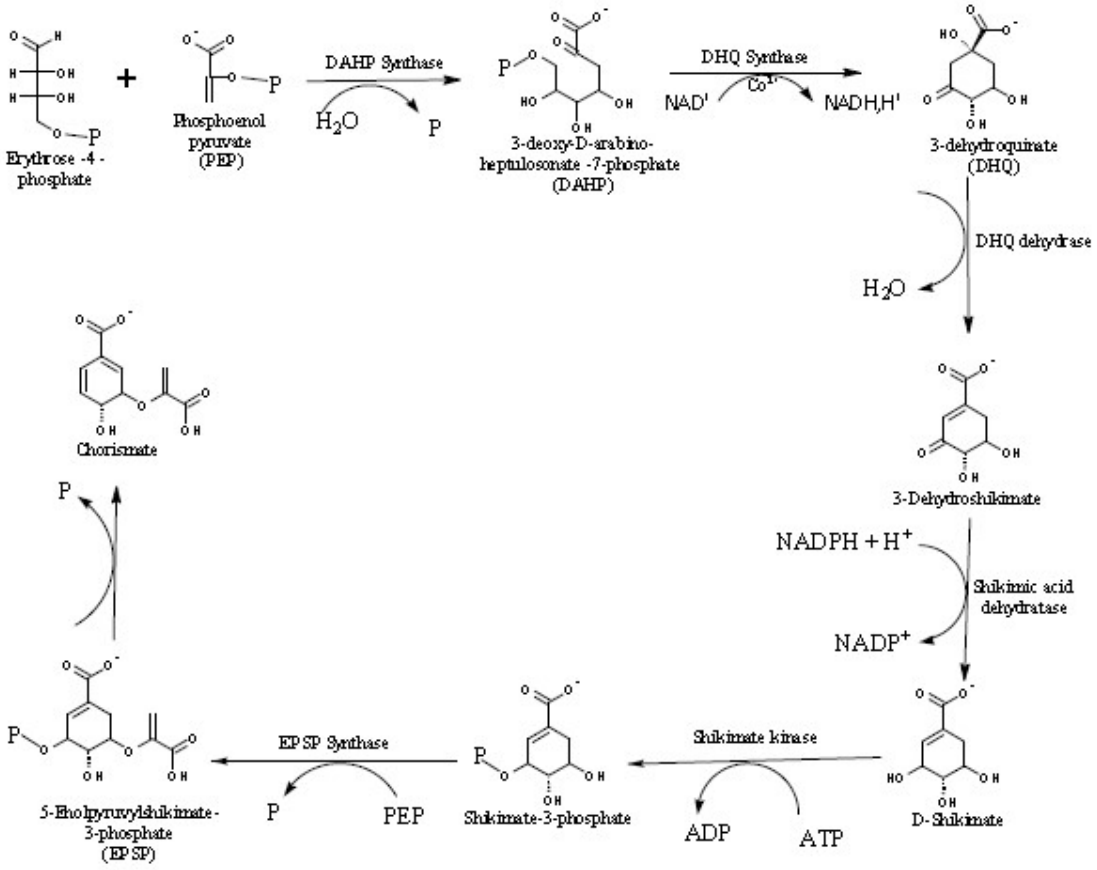


Figure 2.5: The Shikimate pathway (Petrussa *et al*, 2013)

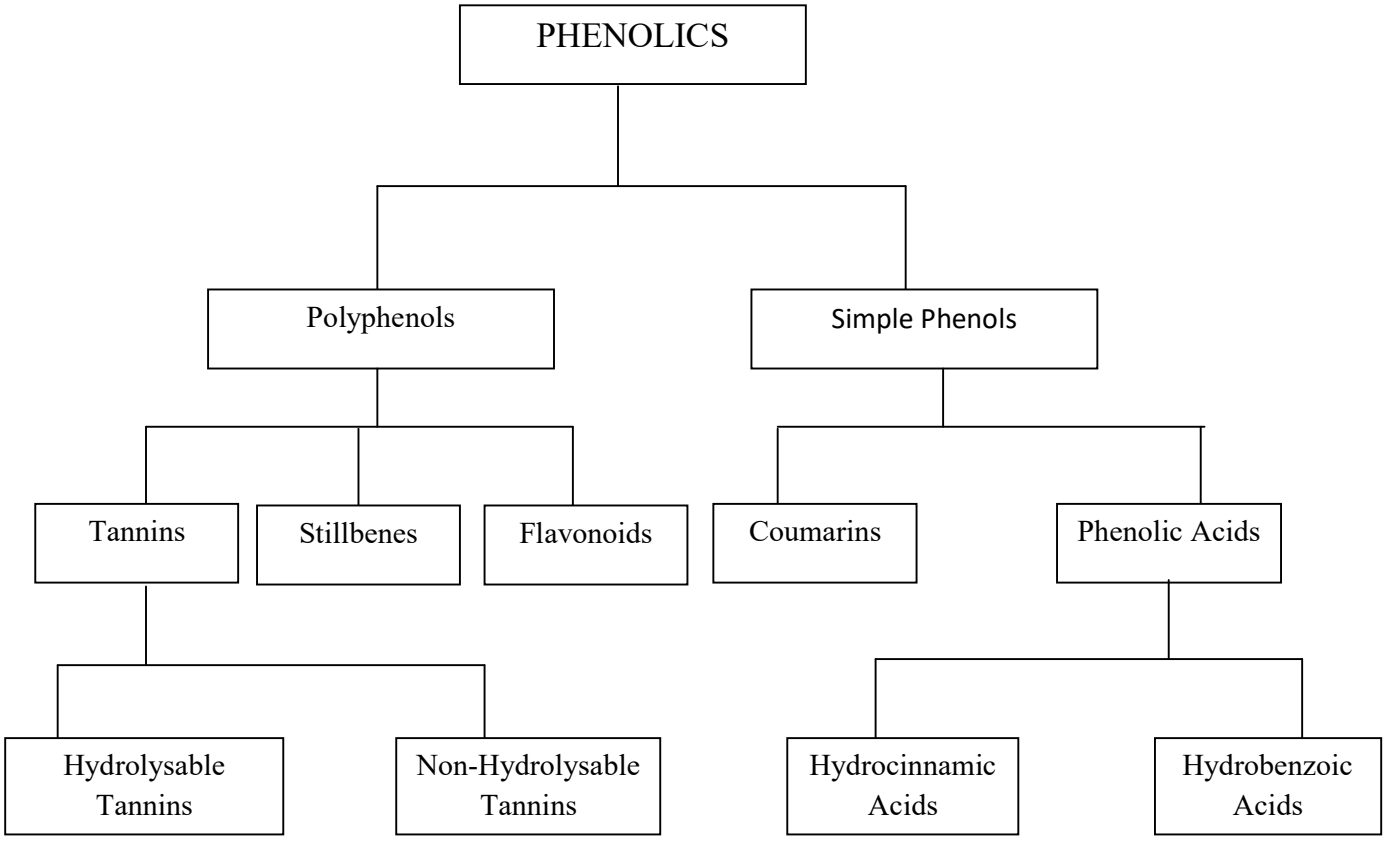
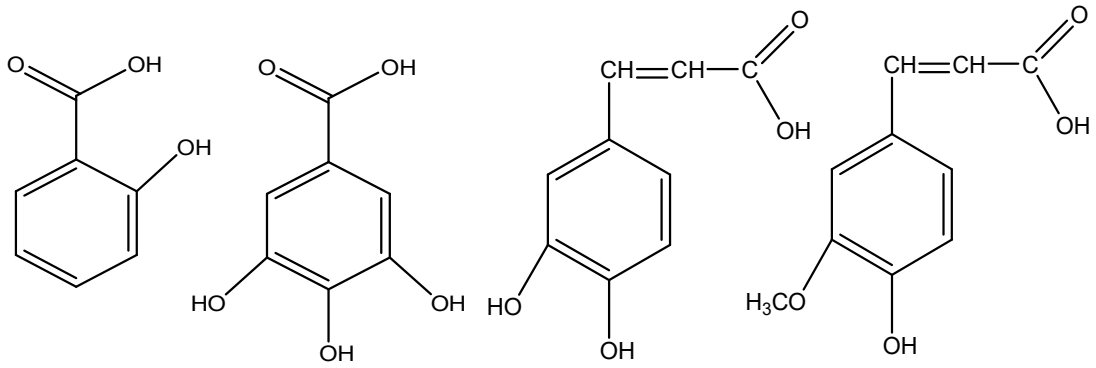


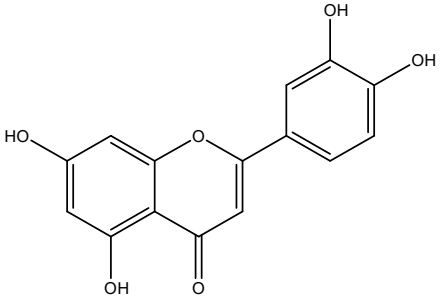
Figure 2.6: Schematic representation of the different groups of the Phenolic components in Plants (Goleniowski*et.al*., 2013)



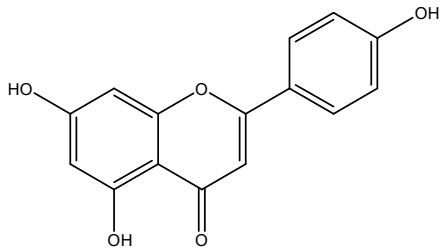
salicylic acid (**34**) gallic acid (**35**) caffeic acid (**36**) ferulic acid (**37**)

2.2.3.2 Flavonoids

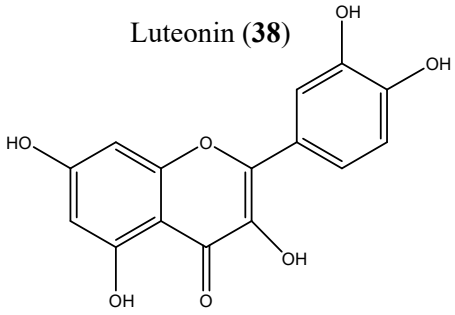
Flavonoids are secondary metabolites of plants that share the basic C6-C3-C6 structuralskeleton, consisting of two aromatic C6 rings (A and B) and a heterocyclic ring (C) thatcontains one oxygen atom (Fig. 2.7). They are synthesized by the polypropanoidpathway and the start-up component is phenylalanine molecule. The biological effectsof these compounds vary. They have been classified into six subgroups (Fig. 2.7)namely: Flavones e.g. luteonin (**38**) and apigenin (**39**), Flavonols e.g. quercetin (**40**)and kaempferol (**41**), Flavanones e.g. hesperetin (**42**) and eriodictyol (**43**), Flavan-3-ols: e.g. catechin (**44**) and epicatechin (4**5**), Isoflavones e.g. genistein (**46**) and daidzein(**47**), Anthocyanidins compounds e.g. cyanidin (**48**) and delphinidin (**49**)(Ghasemzadeh and Ghasemzadeh, 2011)



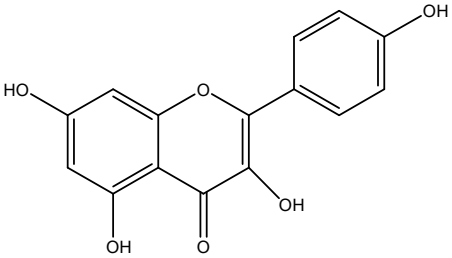
Luteonin (**38**)



apigenin (**39**)



quercertin (**40**)



kaempferol(**41**)

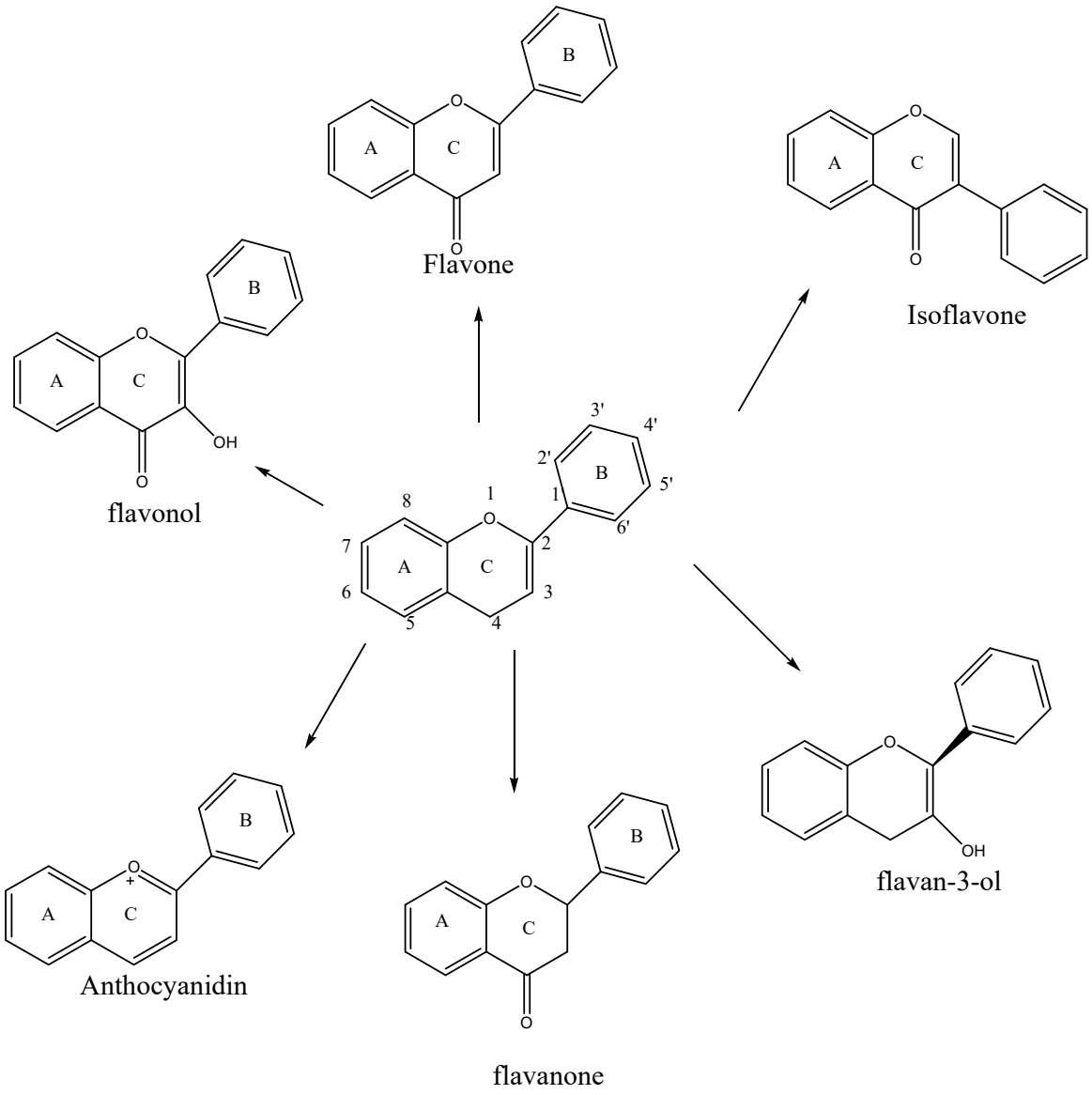
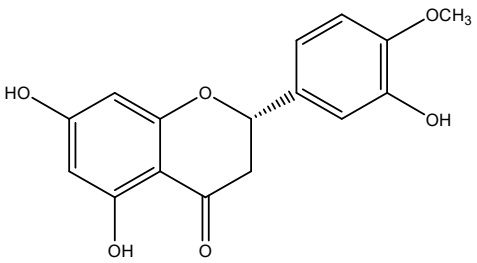
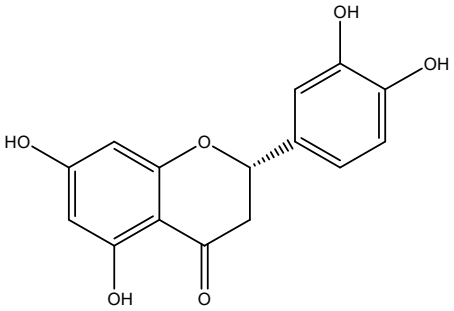


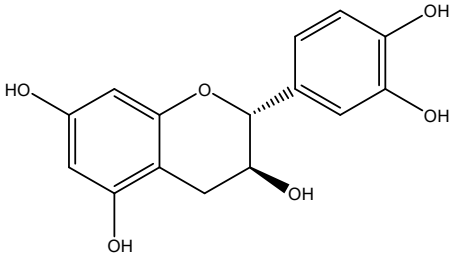
Figure 2.7: Structures of the six main classes of flavonoids (Crozier *et al*. 2006)



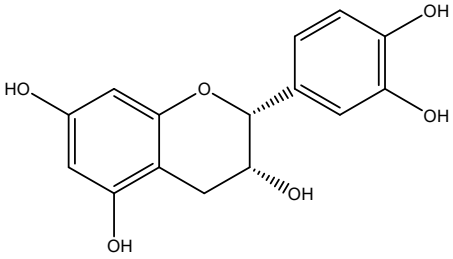
hesperetin(**42**)



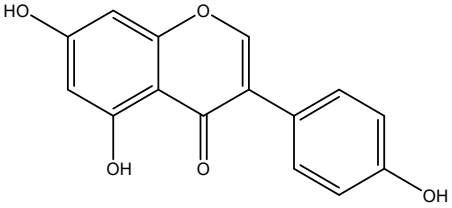
eriodictyol(**43**)



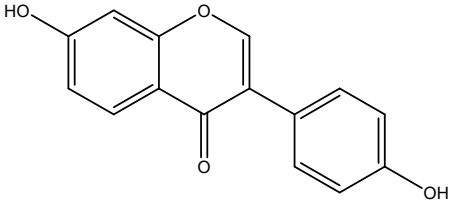
catechin(**44**)



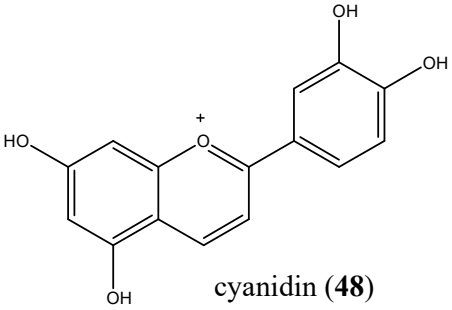
epicatechin(4**5**)



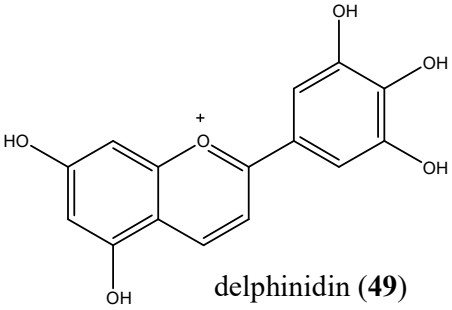
geinstein (**46**)



Daidzein (**47**)



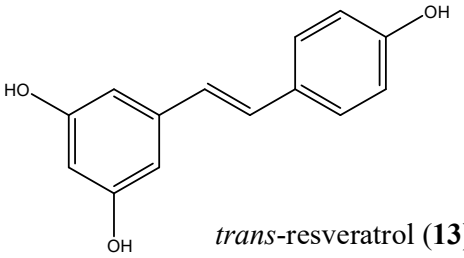
cyanidin (**48**)



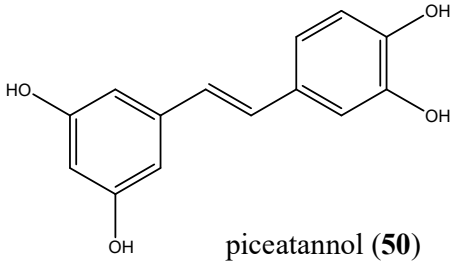
delphinidin (**49**)

2.2.3.3 Stilbenes

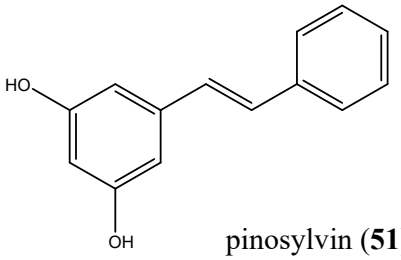
Stilbenes are from the class of plant polyphenols that share the 1,2-diphenylethene structural feature. The *trans*-resveratrol (3, 4',5 -trihydroxy-trans- stilbene) (**13**)is the basic unit of stilbenes. Other examples of stilbenes are piceatannol (**50**), pinosylvin (**51**) and *trans*-pterostilbene (**52**). (Chong *et al*, 2009; Shen *et al*, 2009).



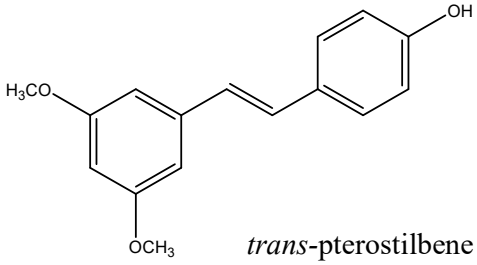
trans-resveratrol (**13**)



piceatannol (**50**)



pinosylvin (**51**)

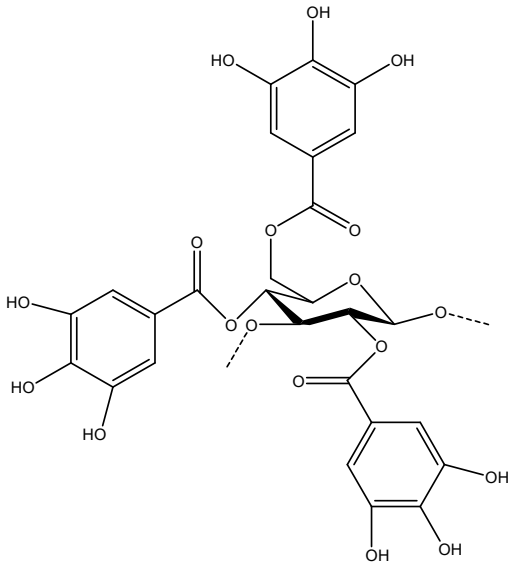


trans-pterostilbene (**52**)

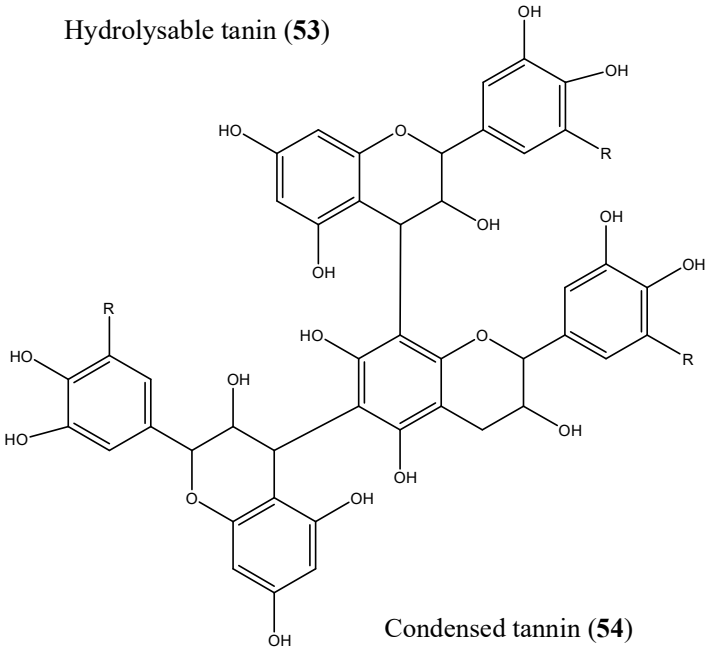
2.2.3.4 Tannins

The term "tannin" was first used by Seguin to explain the presence of some chemicals in extracts obtained from vegetables, which were involved in the conversion of animal skins to leather (Seguin, 1796). These chemical substances are present as polyphenols of different complexities and molecular mass. They are usually classified into two groups namely: the hydrolysable and non-hydrozable tannins (Freudenberg, 1920). Hydrolyzable tannins (**53**) are characterized by the presence of a hydroxyl groups which are combined partly or fully with gallic acids (gallotannis) or hexahydroxdiphenic acid (ellagitannins). Gallotannins produce glucose and gallic acids

after they have been hydrolysed by acids,bases or enzymes. On the other hand, the structures of condensed tannins (**54**) are yet to be determined because they are found to be more complicated than hydrolyzable tannins. They are primarily polymers made up of flavan-3-ols and flavan-3,4-diols monomer subunits or a combination of the two monomers. (Chung *et al*, 1998).



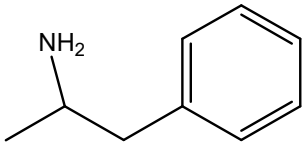
Hydrolysable tanin (**53**)



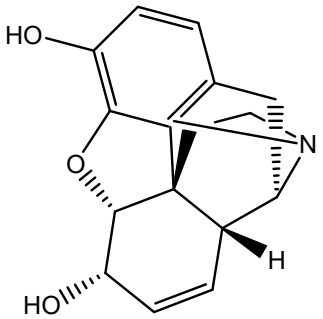
Condensed tannin (**54**)

2.2.4 Alkaloids

Alkaloids refer to a class of basic organic chemical compounds containing nitrogen atoms. They can either be naturally occurring or synthetic. They have been found to have significant physiological effects on humans as some can act as narcotics e.g. amphetamine **(55)** and morphine **(56)**.There are numerous biosynthetic pathways of alkaloids and the starting molecule of most of these alkaloids are amino acids such as ornithine, lysine and phenylalanine. Although alkaloids are basic in nature, some non-basic class such as N-oxides and quaternary nitrogen compounds also exist.. Alkaloids play several roles in biological systems such as gene coding in genotype, actively stimulating, inhibiting and terminating growth. They also show antimicrobial and anti-parasitic properties (Aniszewski, 2007).



Amphetamine (**55**)



Morphine (**56**)

2.3 EXTRACTION OF NATURAL PRODUCTS FROM PLANTS

Plants are complex matrices, producing varying secondary metabolites with different functional groups and polarities. The classes of commonly encountered natural products include terpenoids, , essential oils, alkaloids, waxes and fatty acids, steroids, phenolics, saponins and cardiac glycosides.

Several methods are employed to extract bioactive substances from plant material. Although water is employed as the major extractant in many traditional procedures, organic solvents of different polarities are usually selected in contemporary methods of extraction to take advantage of the various solubilities of plant chemical constituents. The different extraction procedures used to extract natural products from plant include: Maceration, Percolation, Soxhlet Extraction, Extraction under Reflux, and Steam Distillation.

2.3.1 Methods of Solvent Extraction

Methods of solvent extraction can be classified as continuous or discontinuous. In the continuous method (e.g., percolation and Soxhlet extraction), solvent flows through the plant material continuously. As constituents diffuse from the plant material into the surrounding solvent, the solvent becomes increasingly saturated, but because the solvent is continually flowing, the saturated solvent is replaced with less-saturated solvent.

In the case of discontinuous methods, the solvent is added and removed in batches. Hence, once equilibrium is reached between the concentration of solute inside the plant material and the concentration in the solvent, extraction essentially stops until the solvent is decanted and replaced with new solvent. Regardless of the extraction technique used, the resulting solution should be filtered to remove any remaining particulate matter. Extracts can be concentrated at reduced pressure on a rotary

evaporator or dried under a stream of nitrogen. If a rotary evaporator is used, it is advisable to keep the water bath temperature below 40^{o}C to prevent decomposition of thermolabile components. Especially when extracting large amounts of a single sample, the solvent collected from the rotary evaporator condenser during the concentration of one extraction batch may be recycled for further extraction of that same sample, but the use of recovered solvent in the extraction of other samples is not advised, because it may lead to cross-contamination of later extracts (Seidel, 2006).

2.3.2 CONTINUOUS METHOD OF EXTRACTION

2.3.2.1 Percolation

Percolation is an efficient method of extraction, suitable for medium to large sample sizes. A variety of different vessels can serve as percolators. The main requirements are that they have a wide opening at the top to accommodate addition and removal of plant material, and a valve at the base to regulate solvent flow. With the valve in the closed position, the plant material is added to the container, leaving room for expansion. Then, enough solvent is added to cover the sample. If the plant material is too loosely packed, it will not percolate efficiently, and it may need to be compressed to reduce the amount of solvent needed to cover the sample (glass or nonporous ceramic labware may be used as weights for compressing plant material). The plant material is allowed to soak for several hours or overnight, with adequate amounts of the solvent being added to keep the plant material covered. Next, the valve is opened slightly to allow the solvent to flow slowly into a container. The flow rate is regulated to ensure that the solvent exiting is nearly saturated with solute, and the solvent is added at the top of the percolator to replace that lost from the bottom. Although in principle more efficient and economical of solvent than maceration, percolation is

usually not practical for small amounts of plant material or for large numbers of samples (Seidel, 2006).

2.3.2.2 Soxhlet extraction

Soxhlet extraction is used widely in the extraction of plant metabolites because of its convenience. This method is adequate for both initial and bulk extraction. The plant powder is placed in an extraction chamber, which is placed on top of a collecting flask beneath a reflux condenser. A suitable solvent is added to the flask, and the set-up is heated under reflux. When a certain level of condensed solvent has accumulated in the thimble, it is siphoned into the flask beneath. The main advantage of Soxhlet extraction is that it is a continuous process. As the solvent (saturated in solubilized metabolites) empties into the flask, fresh solvent is recondensed and extracts the material in the thimble continuously. This makes Soxhlet extraction less time- and solvent-consuming than maceration or percolation. However, the main disadvantage of Soxhlet extraction is that the extract is constantly heated at the boiling point of the solvent used, and this can damage thermolabile compounds and initiate the formation of artefacts (Seidel, 2006).

2.3.3 DISCONTINUOUS METHOD OF EXTRACTION

2.3.3.1 Maceration

Maceration is a common method for extraction of small amounts of plant material in the laboratory because it can be carried out conveniently in Erlenmeyer flasks. As a rough guideline, after each addition of fresh solvent, the plant material should be left to macerate overnight. The solvent should be decanted through a screen or filter, and fresh solvent added to the flask. The sample is then mixed with the fresh solvent by stirring or swirling, and left to macerate again. Sonication of the macerating sample is sometimes used to reduce the time needed for thorough extraction. Experience indicates that after three solvent changes, the plant

material is almost completely exhausted. Large samples may also be macerated, usually in a large container with a tap at the base, as with large-scale percolation, except that the solvent is changed in batches. It has the advantage of moderate extraction conditions but suffers from high solvent consumption, long extraction times and low extraction yields (Jones and Kinghom, 2006).

2.3.4 CHOICE OF SOLVENT

Factors that should be considered when choosing a solvent or solvent system for extracting plant material include solubility of the target constituents, safety, ease of working with the solvent, potential for artefact formation, and the grade and purity of the solvent. Following the principle of "like extracts like", it is often possible to tailor the solvent choice to maximize the yields of the compounds of interest while minimizing the extraction of unwanted compounds. Solvents with low boiling points are generally easier to use from the standpoint that they are more easily concentrated. Acetone, chloroform ($CHCl_3$), dichloromethane (DCM), ethyl acetate (EtOAc), and n-hexane/petroleum ether evaporate relatively quickly, whereas water and butanol are more difficult to remove (Jones and Kinghom, 2006).

2.4 CHROMATOGRAPHIC METHODS USED IN THE ISOLATION OF COMPOUNDS

Chromatography is a separation process which depends on the differential distributions of the components of a mixture between a mobile bulk phase and an essentially thin film stationary phase. The stationary phase may either be in the form of a packed column (Column chromatography) through which a mobile phase is allowed to flow or in the form of a thin-layer adhering to a suitable form of backing material (thin layer chromatography) over which the mobile phase is allowed to ascend by capillary action.

The thin film stationary phase may be either a liquid or a solid, and the mobile phase may be a liquid or a gas. When the mobile phase is a gas, then the technique is *gas chromatography* and when it is a liquid, it is called *liquid chromatography*.

For a gas chromatography, if the stationary phase is a liquid, we have *gas-liquid chromatography* and if it is solid, the technique is *gas-solid chromatography*. For a liquid chromatography, we have *liquid-liquid chromatography* and *liquid-solid chromatography* (Furniss *et al*, 1980).

In liquid-liquid chromatography, the stationary phase is a liquid and the separation results from the differential solubility of the component in this liquid phase and the chromatographic technique is called partition *chromatography*. An example is paper *chromatography* and the stationary phase, in this case, is water held in the pores of filter paper where the cellulose network acts as solid support. In liquid-solid chromatography, the stationary phase is solid and the separation is due to the differential adsorption of the components on the solid support. In this case, the technique is called *adsorption chromatography* and an example is *thin layer chromatography*.

Chromatographic separation may also be due to differential retention of oppositely charged groups by an ion exchanger; this technique is known as *ion-exchange chromatography*. While the separation technique due to the difference in the time spent within the column by solute molecules having different sizes is known as *molecular exclusion chromatography*. When chromatography is used to separate components in order to identify them qualitatively and quantitatively, it is known as *analytical chromatography* and when it is carried out in order to isolate the separate components in a mixture, it is referred to as *Preparative Chromatography* (Furniss *et al*, 1980).

The chromatographic techniques usually employed in isolation of organic compounds are:

1. Thin-layer chromatography
2. Column chromatography.
3. High-performance liquid chromatography
4. Gas liquid chromatography

2.4.1 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is primarily for rapid qualitative analysis and is extremely effective. The chromatography is carried out on a small plate, such as a microscope slide, which is covered on one side with a thin coating of the adsorbent. Precoated TLC plates (glass, plastic or aluminium foil backing) for analytical and preparative work are also available commercially. Very small amount of samples are applied to the adsorbent surface as small spots in a row at one end of the plate with a fine capillary tube. The plate is then placed, sample end down, in a wide mouth jar containing a shallow pool of solvent. The solvent rises over the adsorbent layer by capillary action. The individual compound can be detected as separate spot on the plate by treating the developed TLC plate in iodine vapour, where all compounds absorb iodine or react with it, to form violet or brown spots on the slides. Other methods of visualizing spots are by illumination with an ultraviolet lamp and spraying the plate with special reagents. For an efficient separation, it is important that the plates are coated evenly, not overloaded with samples and the eluting solvent chosen properly (Touchstone and Dobbins, 1978).

2.4.2 COLUMN CHROMATOGRAPHY

Column chromatography is a chromatographic technique based on adsorption in which the stationary phase is a finely divided solid adsorbent packed in a glass column while the mobile phase is usually an organic solvent. The common adsorbents used as

stationary phase are silica gel, alumina, cellulose and bounded silica gel (RP-8 or RP-18) for very polar mixtures. The apparatus consists essentially of a long narrow glass tube (about 10-90 cm long and 1-5cm diameter) to which the adsorbent is loaded in the form of a slurry in a non-polar organic solvent. The adsorbent is allowed to settle evenly in the column and the mixture or extract to be separated is introduced onto the top of the column and the mixture or extract to be separated is introduced onto the top of the column. The extract could be introduced in form of a concentrated solution with a dropping pipette or in solid form pre-adsorbed on silica gel. The mobile phase is then allowed to pass through the column starting with nonpolar solvent used to pack the column. The solvent polarity is increased gradually until all the components of the mixture are eluted. The eluents are monitored by analytical TLC and subjected to further purification as necessary. (Snyder and Kinklamd, 1979).

2.4.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY(HPLC)

High-Performance Liquid Chromatography (HPLC) is an analytical process utilizing special instruments designed to separate, quantify and analyse components of a chemical mixture. Samples of interest are introduced to a solvent flow path, carried through a column packed with specialized materials for component separation, and component data is obtained through the combination of a detection mechanism coupled with a data recording system. All this occurs under pressures, which may reach or exceed 6,000 psi. The basic components of an HPLC system include a solvent reservoir, pump, injector, analytical or preparative column, detector, recorder and waste reservoir. Other important elements are an inlet solvent filter, post-pump inline filter, sample filter, pre-column filter, guard column, back pressure regulator and/or solvent sparging system. The function of each of these components is briefly described below.

An HPLC system begins with the solvent reservoir, which contains the solvent used to carry the sample through the system. The solvent should be filtered with an inlet solvent filter to remove any particles that could potentially damage the system's sensitive components. The solvent is propelled through the system by the pump. This often includes internal pump seals, which slowly break down over time. As these seals break down and release particles into the flow path, an inline solvent filter prevents any post-pump components damage.

The next component in the system is the sample injector, also known as the valve. This valve, equipped with a sample loop of the appropriate size for the analysis being performed, allows for the reproducible introduction of the sample into the flow path. Because the sample often contains particulate matter, it is important to utilize either a sample filter or a pre-column filter to prevent valve and column damage.

Following the injector, the column allows the primary sample separation to occur. This is based on the differential attraction of the sample components for the solvent and the packing material within the column. However, a sacrificial guard column is often included just prior to the analytical column to chemically remove components of the sample that would otherwise foul the main column. The separated components pass through a detector flow cell before they pass into the waste reservoir. The presence of the components of a sample in the flow cell prompts an electrical response from the detector, which is digitized and sent to a recorder. The recorder helps to analyse and interpret the data.

As a final system enhancement, a back pressure regulator is often installed immediately after the detector. This device prevents solvents bubble formation until the solvent is completely through the detector. This is important because bubbles in a flow cell can interfere with the detection of sample components. Alternatively, an

inert gas sparging system may be installed to force dissolve gasses out of the solvent being stored in the solvent reservoir (Fifield and Kaeley, 1995)

2.4.4 VACUUM LIQUID CHROMATOGRAPHY

Vacuum liquid chromatography (VLC) is a separation technique that uses reduced pressure to increase the flow rate of the mobile phase. The equipment is extremely simple and the procedure easy to apply. The column is a standard sintered glass filter funnel of various sizes, depending on the amount of extract. The column is often connected to the vacuum through a filter flask. Modern column consists of sintered glass funnel with ground glass joints that can be connected directly to vacuum and a filter funnel or flask. Different types of chromatographic adsorbents may be used for VLC ranging from alumina to silica gel and polyamide. TLC grade silica gel (e.g. Merck gel 60) and aluminium oxide are the most commonly used.

The chromatographic column is dry-packed with the TLC grade adsorbent under vacuum in order to achieve maximum packing density. The vacuum is released and low polarity solvent is poured onto the surface of the adsorbent and the vacuum is reapplied. The solvent is sucked dry and the column is ready for loading.

The sample may be dissolved in suitable solvent and applied into the column or pre-adsorbed on a small amount of silica gel, dried and applied evenly to the surface of the silica gel (support). Suction is then applied to the column to compress the sample to the silica gel. The choice of solvent is guided by the TLC characteristics of the mixture depending on the objective of the exercise. It is better to increase polarity slowly with earlier fractions and more rapidly with later fractions. The column could then be washed with a very polar solvent like methanol.

VLC is extremely very useful for bulk fractions of crude extracts and for separation of complex mixtures of terpenoids and alkaloids. VLC is simple, requires a small amount

of adsorbent and consumes the small volume of solvent, it also gives a pretreatment of samples prior to HPLC separation (Fifield and Kaeley, 1995).

2.5 STRUCTURAL DETERMINATION TECHNIQUES

In recent times, Natural Products chemistry has experienced great progress due to the introduction of modern physical methods of analysis, which has made structural elucidation of complex organic compounds, which would have been tedious to elucidate or determine, easy and accurate. Some of these methods rely on spectroscopic techniques which involve the selective absorption of electromagnetic radiation by the organic molecules. Some of these techniques are summarized below.

2.5.1 INFRARED SPECTROSCOPY

Infrared spectroscopy is used to detect and measure molecular vibrations in a molecule, resulting in the identification of various bond types and functional groups, since different functional groups have their corresponding vibrational frequencies. This makes infrared spectrum the most rapid and simplest means for assigning a compound to its class.

2.5.2 ULTRAVIOLET AND VISIBLE SPECTROSCOPY

UV-Vis spectra of the samples are recorded on a UV-Vis spectrophotometer. The samples are normally dissolved in methanol and are then scanned to know the λ_{max} and absorption coefficient in a particular concentration (Rahman, 2015).The chief value of ultraviolet spectrum is to provide information about the nature of a conjugated system. If the molecular formula shows some double-bond equivalents, comparison of the ultraviolet spectrum with reference data may identify the chromophore. The ultraviolet spectrum is often useful in differentiating between isomeric structures, e.g. between conjugated and non-conjugated systems, or between *cis*- and *trans*-stilbenes, or

between anthracene or phenanthrene structures. In general, Ultraviolet Spectroscopy is used to detect unsaturated and conjugated system in organic molecules (Scheinmann, 1973).

2.5.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear Magnetic Resonance spectroscopy is concerned with the magnetic properties of certain atomic nuclei, Common NMR active nuclei are ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, ^{29}Si etc. But of more importance to the Natural Product chemist is the nuclei of a hydrogen atom and that of carbon-13 isotopes. NMR uses a longer wavelength of the electromagnetic spectrum to detect changes in the alignment of nuclear magnets in strong magnetic fields. It enables us to record difference in the magnetic properties of the various magnetic nuclei present and to deduce the position of these nuclei are within the molecule, and also to know how many different kinds of environment are there in a molecule and measured how many atoms are present in each of these environments.

The utility of NMR stems from the fact that chemically distinct nuclei differ in resonance frequency in the same magnetic field. This phenomenon is known as the chemical shift. In addition, the resonance frequencies are perturbed by the existence of neighboring NMR active nuclei, in a manner dependent on the bonding electrons that connect the nuclei. This is known as spin-spin or *J* coupling. The spin-spin coupling allows one to identify connections between atoms in a molecule, through the bonds that connect them (Pavia *et al.,* 2001; Kalsi, 2004).

2.5.3.1 Two-dimensional Nuclear Magnetic Resonance (2D NMR) Spectroscopy

This is a set of nuclear magnetic resonance spectroscopy (NMR) methods which give data plotted in a space defined by two frequency axes rather than one. 2D NMR experiments include (i) Homonuclear through-bond correlation methods such as

COrrelation SpectroscopY (COSY),) (ii) Heteronuclear through-bond correlation methods, such as Heteronuclear Single-Quantum Correlation spectroscopy (HSQC) and Heteronuclear Multiple-Bond Correlation spectroscopy (HMBC). (iii) Through-space correlation methods, like Nuclear Overhauser Effect. SpectroscopY (NOESY) and Rotating frame nuclear Overhauser Effect SpectroscopY (ROESY). The most common types of 2D experiments are homonuclear correlation (COSY) and Heteronuclear CORrelation (HETCOR) spectroscopy (Schram and Bellama, 1988).

2.5.3.2 Homonuclear Correlation Spectroscopy (^{1}H-^{1}H COSY)

This is homonuclear correlation spectroscopy which shows a correlation between protons that are coupled to each other. There are many modified version of the basic COSY experiment: DQF-COSY (Double-Quantum Filtered), COSY45, Long Range Correlation SpectroscopY (LRCOSY) and Exclusive Correlation SpectroscopY (ECOSY) (Macomber, 1998).

2.5.3.3 Heteronuclear Correlation (HETCOR)

This 2D NMR indicates heteronuclear correlation, usually between ^{1}H and ^{13}C resonances mediated by $J_{C\text{-}H}$. The experiment can be run using either $^1J_{C\text{-}H}$ or longer range couplings. It has poor sensitivity because the observed nucleus is ^{13}C and has been largely replaced by the inverse detection experiments such as Heteronuclear Single Quantum Coherence **(**HSQC) and Heteronuclear Multi-Quantum Coherence (HMQC) (Lambert and Mazzola, 2002**)**.

2.5.3.4 Heteronuclear Single Quantum Coherence (HSQC)

This is a CH correlation experiment which uses proton detection of the ^{13}C signals using an Insensitive Nuclear Enhancement by Polarisation Transfer (INEPT) sequence. It shows higher resolution in the C-dimension than does the related HMQC experiment (Schram and Bellama, 1988).

2.5.3.5 Heteronuclear Multiple Bond Correlation (HMBC)

This experiment is similar to HSQC because it shows correlations between ^{1}H and ^{13}C through bonds in the molecule. However, it is different from HSQC in that it shows correlations that are separated by 2, 3 or more bonds.It is very useful for assigning molecules as it can be used to find near neighbours in a molecule (Woodman, 2013)

2.5.3.6 Nuclear Overhauser Spectroscopy (NOESY)

This experiment shows the correlation between protons that are close in space. The nuclear overhauser effect arises throughout radio frequency saturation of one spin, the effect causes the perturbation via dipolar interactions with further nucleus spins. This enhances the intensity of other spins. This method is a very useful tool to study the conformation of molecules. This is an NMR technique for determining the 3-dimensional structure of molecules (Lambert and Mazzola, 2002).

2.5.4 MASS SPECTROMETRY

Mass spectrometry does not depend on electromagnetic radiation but the bombardment of atoms and molecules with free electrons as a means of raising them to the state of positively charged ions by complete removal of outer electrons. This provides the molecular mass measurement and measures mass-to-charge ratio of the ions produces in the spectrometer. Structural information could be deduced from the fragmentation pattern of the compounds and the masses of the fragment ions can be related to likely structures. (William, 1996). A typical mass spectrometer consists of three operational components. These are the ionisation source, mass analysers and detector.

2.5.4.1 THE IONISATION SOURCE

Based on the ionization mode, mass spectroscopic techniques are classified to include the following:

2.5.4.1.1 Chemical Ionisation (CI)

In this method, a reagent gas is first ionized by electron impact and then subsequently reacts with analyte molecules to produce analyte ions. This method gives molecular weight information and reduced fragmentation in comparison to EI.

2.5.4.1.2 Electron Impact (EI)

A beam of electrons passes through a gas-phase sample and collides with neutral analyte molecules to produce a positively charged ion or a fragment ion. Generally, electrons with energies of 70 eV are used. This method is applicable to all volatile compounds (>10^3 Da) and gives reproducible mass spectra with fragmentation to provide structural information (Rose and Johnstone, 2001).

2.5.4.1.3 Fast Atom Bombardment (FAB)

Ions are produced by using a high current of bombarding particles. This is a soft ionisation technique and is suitable for analysis of low volatility species. It produces large peaks for the pseudo-molecular ion species $[M+H]^+$ and $[M-H]^-$ along with other fragment ions and some higher mass cluster ions and dimers (Kalsi, 2004).

2.5.4.1.4 Electro-Spray Ionization (ESI)

A solution is nebulized under atmospheric pressure and exposed to a high electrical field which creates a charge on the surface of the droplet. The production of multiple charged ions makes electrospray extremely useful for accurate mass measurement, particularly for thermally labile, high molecular mass substances (ie. proteins,

oligonucleotides, synthetic polymers, etc.) (Hoffman and Stroobank, 2002)

2.5.4.1.5 Matrix Assisted Laser Desorption Ionisation (MALDI)

This is a soft ionization technique suitable for the analysis of molecules which tend to be fragile and fragment when ionized by more conventional ionization methods such as biomolecules (biopolymers e.g DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules). It is similar in character to electrospray ionization both in relative softness and the ions produced (although it causes much fewer multiple charged ions). MALDI is also more tolerant of salts and complex mixture analysis than ESI (Rose and Johnstone, 2001).

2.5.4.1.6 Thermospray Ionisation (TI).

Thermospray ionisation, is a soft ionization technique producing predominantly MH^+ or $(M-H)^-$ ions, which may be accompanied by some degree of fragmentation under certain conditions. Thermospray is best suited to the analysis of organic compounds with molecular masses of less than 1000 da that exhibit some polarity. Into this wide category fall molecules such as drugs and drug metabolites (Beattie and Blake, 1989).

2.5.4.2 The Mass Analyser

This sorts the ions by their masses by applying electromagnetic fields. Examples include Time-of-Flight (TOF), magnetic sectors, Fourier transform and quadrupole ion traps.

The high-resolution mass spectrometry (HR-MS) is applied for the establishment of the exact molecular formula since it gives information about the elemental composition through exact mass measurements.

2.5.5 X-RAY CRYSTALLOGRAPHY

X-ray crystallography is a technique in which the pattern produced by the diffraction

of x-rays through the closely spaced lattice of atoms in a crystal is recorded and then analysed to reveal the nature of that lattice. X-ray crystallography allows precise determination of the atomic positions and consequently the bond lengths and angles of molecules within a single crystal. X-rays are electromagnetic radiation with wavelengths between about 0.02 Å and 100 Å (1Å = 10^{-10} meters).

When X-rays are beamed at the crystal, electrons diffract the X-rays, which cause a diffraction pattern. Through the use of the mathematical Fourier transform these patterns can be converted into electron density maps. These maps show contour lines of electron density. Since electrons more or less surround atoms uniformly, it is possible to determine where atoms are located; only hydrogen is difficult to map because it has one electron thus resulting to very low electron density around it. The crystal is rotated while a computerized detector produces two-dimensional electron density maps for each angle of rotation. The third dimension comes from comparing the rotation of the crystal with the series of images. Computer programs use this method to come up with three-dimensional spatial coordinates(Rhodes, 1993; Carter, 1997).

2.6 PERIODONTAL DISEASE

Periodontal disease (PD) is an inflammatory disease that affects the supporting structures of teeth including the gingiva, periodontal ligament and alveolar bone (Ozden *et al*., 2015, Pihlstrom *et al*., 2005). If left untreated, PD may cause the annihilation of these supporting structures and ultimately tooth loss.Plaque induced gingivitis is confined to the gingival tissues, whereas the various other forms of periodontitis affect all of the components of the periodontium i.e., gingival, periodontal ligament, cementum and alveolar bone. In general, both conditions demonstrate all of the classic signs and symptoms of chronic inflammation, including redness and

swelling of the tissues, loss of architectural form and reduced function. If the inflammatory response is not balanced by the host or is left untreated, inflammatory destruction can be so severe as to put the teeth at risk and tooth loss can be the ultimate outcome of the periodontal disease. Recently the global epidemiological data suggests periodontal disease to be a major burden on oral diseases (Fujita, 1990; Inoue *et al*, 1989; Petersen, 2003; WHO, 1997; WHO 2003). The most common risk factors associated with PD and tooth loss include poor oral hygiene maintenance (Javed *et al*., 2007), ageing (Fah and Schatzle, 2014; Schatzle *et al*., 2010) systemic disorders such as pre-diabetes and poorly-controlled diabetes mellitus (Javed *et al*., 2007; Taylor *et al*., 2013; Chapple and Genco, 2013), lifestyles such as smoking (Fah and Schatzle, 2014; Bergstrom, 2004) and chewing smokeless tobacco (Javed *et al*., 2013). Interestingly, some of the risk factors of PD, particularly tobacco and betel quid use, have also been linked with the etiology of oral cancer (Warnakulasuriya,2009a; Johnson *et al*., 2011).

2.6.1 RELATIONSHIP BETWEEN PERIODONTAL DISEASE AND ORAL CANCER

Oral cancer originating in any of the tissues in the mouths or any cancerous tissue growth located in the oral cavity is a type of head and neck cancer (Santosh *et al*, 2016). It is reported that about 75% oral cancers were related to bad living habits such as tobacco use and excessive alcohol consumption (Kumar *et al*, 2016). Moreover, other risk factors for oral cancer are poor oral hygiene, irritation caused by ill‑fitting dentures and other rough surfaces on the teeth, poor nutrition, and some chronic infections caused by fungi, bacteria, or viruses (Patel *et al*, 2016). Oral cancer is the 15th most diagnosed malignant carcinoma with the incidence rate of 3.9/100,000 (Garrote *et al*, 2001). Oral cancer has become a major public health problem

worldwide for its increasing incidence and mortality (Buglione *et al*, 2016; Sultana *et al*, 2014). And recently, for the progress of chemo-radiation therapy, the 5‑year survival rate was on the rise. The early diagnosis and treatment for oral cancer are essential for prognosis. Thereafter, screening the risk factors for oral cancer is important for its prevention. It is reported that about 75% oral cancers were associated with bad living habits such as smoking and excessive alcohol consumption (Rosenquist *et al*, 2005). Moreover, other risk factors for oral cancer are poor oral hygiene, irritation caused by ill‑fitting dentures and other rough surfaces on the teeth, poor nutrition, and some chronic infections caused by fungi, bacteria, or viruses. Several studies have also found that periodontal disease may increase the risk of developing oral cancer. However, each related studies have small number of patients with limited statistical power. The results indicated that periodontal disease can increase oral cancer risk by nearly 2‑fold (Ye *et al*, 2016).

2.7 BIOASSAYS

The methods for the detection of biological activity of e.g. plant extracts can best be divided into two groups for screening purposes: Primary screening bioassays and specialized screening bioassays.

2.7.1 GENERAL BIOASSAY

A broad screening bioassay is probably most useful if one is randomly screening chosen organisms for any kind of pharmacological activity. Most phytochemical laboratories which engage in the bioassay-guided isolation of actives from plant extracts hardly use complex bioassays, as efforts have been made to introduce simple, inexpensive 'frontline" or 'bench-top" bioassays for the rapid screening of plant extracts and fractions.(Ghisalberti, 1993; Vlietinck , 1999). An example of the general

bioassay is the brine shrimp lethality assay.

2.7.1.1 Brine shrimp lethality assay

Brine Shrimp lethality bioassay (McLaughlin *et al*., 1998; Meyer *et al*., 1982) is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. By this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity. The method is a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. Brine toxicity is closely correlated with 9KB (human nasopharyngeal carcinoma) cytotoxicity (p=0.036 and kappa = 0.56). ED_{50} values for cytotoxicities are generally about one-tenth the LC_{50} values found in the Brine Shrimp test. Thus, it is possible to detect and then monitor the fractionation of cytotoxic extracts using the Brine lethality bioassay (Alkofahi *et al*., 1988; Mclaughlin *et al*., 1998; Meyer *et al*., 1982). The Brine Shrimp assay has advantages of being rapid (24 hours), inexpensive, and simple (e.g., no aseptic techniques are required). It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less). Furthermore, it does not require animal serum as is needed for cytotoxicities (Mclaughlin *et al*., 1998).

2.7.2 SPECIALIZED BIOASSAYS

A specialized assay is directed at finding some effect against a specific disease or activity. The specialized assays employed in this study include: Antimicrobial, anticancer and antioxidant assays.

2.7.2.1 Anti-microbial assay

Antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome (Balouiri *et al*, 2016). Plants and other natural

sources can provide a huge range of complex and structurally diverse compounds as potential antimicrobial agents (Runyoro *et al*, 2006; Mabona *et al*, 2013). A variety of laboratory methods can be used to evaluate or screen the *in vitro* antimicrobial activity of an extract or a pure compound. The most known and basic methods are the disk-diffusion and broth or agar dilution methods. To further study the antimicrobial effect of an agent in depth, time-kill test and flow cytofluorometric methods are recommended, which provide information on the nature of the inhibitory effect (bactericidal or bacteriostatic) (time-dependent or concentration-dependent) (Balouiri *et al*, 2016) . The two methods employed for detection of antimicrobial activity in this study are disc diffusion and dilution methods.

2.7.2.1.1 Agar Disc Diffusion Method

In this procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured. The advantages of disc-diffusion assay includes: simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and the ease to interpret results provided.Moreover, the agar disc-diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC), as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium (Balouiri *et al*, 2016).

2.7.2.1.2 Agar Well Diffusion Method

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Valgas *et al*, 2007). Similarly to the procedure used in

disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer, and a volume (20–100 μL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested (Balouiri *et al*, 2016).

2.7.2.1.3 The Dilution Methods

Dilution methods are the most appropriate ones for the determination of MIC values, since they offer the possibility to estimate the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macrodilution or microdilution). Either broth or agar dilution method may be used to quantitatively measure the in vitro antimicrobial activity against bacteria and fungi. MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested, and it is usually expressed in mg/mL or mg/L (Balouiri *et al*, 2016).

2.7.2.1.3.1 Agar dilution method

The agar dilution method involves the incorporation of varying desired concentrations of the antimicrobial agent into an agar medium (molten agar medium), habitually using serial two-fold dilutions, followed by the inoculation of a defined microbial inoculum onto the agar plate surface. The MIC endpoint is recorded as the lowest concentration of antimicrobial agent that completely inhibits growth under suitable incubation conditions. This technique is suitable for both antibacterial and antifungal susceptibility testing. If multiple isolates are being tested against a single compound, or if the compound (or extract) tested masks the detection of microbial growth in the liquid medium with its colouring, agar dilution method is often preferred to broth

dilution for the MIC determination (Balouiri *et al*, 2016).

2.7.2.1.3.2 Broth Dilution Method

Broth micro- or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. The procedure involves preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in tubes containing a minimum volume of 2 mL (macrodilution) or with smaller volumes using 96-well microtitration plate (microdilution). Then, each tube or well is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. After well-mixing, the inoculated tubes or the 96-well microtitration plate are incubated (mostly without agitation) under suitable conditions depending upon the test microorganism. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in tubes or microdilution wells as detected by the unaided eye (CLSI, 2012). Unlike microdilution method, the main disadvantages of the macrodilution method are the tedious, manual undertaking, risk of errors in the preparation of antimicrobial solutions for each test, and the comparatively large amount of reagents and space required (Jorgensen, 2009).

2.7.2.2 Anti – Cancer assay

It is important to distinguish between the terms “anticancer”, “cytotoxic” and “antitumor”. “Anticancer” compounds are those which are effective in cancers in humans. “Cytotoxic” compounds are toxic to cells in culture but they may not show any selective toxicity to cancer cells as against normal cells. Cytotoxic compounds may be cytostatic (*i.e.* stop cell growth, reversibly or irreversibly) or cytocidal (kill cells). While, “Antitumor” compounds are those which are active in an *in-vivo* tumour system. Such compounds would therefore show selectivity against tumour cells. *In*

vitro anticancer assays are primarily of two types: molecular assays or cellular assays. Molecular assays are directed at a single subcellular target and they are therefore highly particular, while cellular assays are based either on cytotoxicity or morphological assays. A simple example of a cytotoxicity assay may be to measure the 50% growth inhibitory concentration against a single cell line (i.e. IC_{50}) (Atta-ur-Rahman *et al*, 2005). Cellular assay method was employed in this study. The IC_{50} value was determined by plotting the data on a dose response curve of percent inhibition versus concentration. The smaller the IC_{50} value the more active is the substance.

2.7.2.2.1 Sulforhodamine B (SRB) Cytotoxicity assay

The sulforhodamine B (SRB) assay is used for in vitro cytotoxicity screening through cell density determination, which is based on the measurement of cellular protein content. The assay relies on the ability of the SRB to bind with basic amino acid residues of trichloroacetic acid (TCA) fixed protein contents of the cells. SRB is a bright-pink amino xanthene dye with two sulfonic groups that bind to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass (Lillie, 1977; Vicha and Kirtikara, 2006). The effectiveness of the SRB assay is frequently compared to that of another method using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT assay requires cellular metabolic activity to convert the colourless tetrazolium to the purple-coloured formazan dye. The SRB assay has some advantages over the MTT assay, for example, some compounds can directly interfere with MTT reduction without having any effects on cell viability (Plumb *et al.,* 1989), while SRB staining is rarely affected by this type of interference. In addition, SRB staining is independent of cell metabolic activity; therefore, fewer steps are required to optimize

assay conditions for specific cell lines than in the MTT assay (Keepers, 1991).

2.7.2.3. Antioxidant assay

Oxidative stress has been identified as the root cause of the development and progression of several diseases. Plants have long been source of dietary antioxidants. It is believed that two-thirds of the world's plant species have medicinal importance, and almost all of these have excellent antioxidant potential (Krishnaiah *et.al*, 2011). The interest in the exogenous plant antioxidants was first evoked by the discovery and subsequent isolation of ascorbic acid from plants (Szent-Giörgyi, 1963). Since then, the antioxidant potential of plants has received a great deal of attention because increased oxidative stress has been identified as a major causative factor in the development and progression of several life threatening diseases. In addition, supplementation with exogenous antioxidants or boosting of endogenous antioxidant defences of the body has been found to be a promising method of countering the undesirable effects of oxidative stress (Kasote *et al*, 2013). *In vitro* antioxidant activity assessment methods are often used to screen and confer antioxidant potential to plants or their phytochemicals. There are several in vitro assays used to measure and confer antioxidant activity to plants; however, each of these has its own limitations regarding applicability. Therefore, multiple assay strategies have frequently been adapted to confer antioxidant potential. In these assays, plants are generally assessed for their function as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators, after which they are classified as primary (chain-breaking) and secondary (preventive) antioxidants. Primary antioxidants act by donating a hydrogen atom, while secondary antioxidants function via binding of metal ions capable of catalysing oxidative processes and scavenging oxygen, absorbing UV radiation, inhibiting enzymes or decomposing hydroperoxides (Kasote, 2013). Based on the inactivation

mechanism involved, antioxidant activity assessment methods are classified into hydrogen atom transfer (HAT) and electron transfer (ET) reaction-based methods. Bond dissociation energy and ionization potential are two major factors that determine the mechanism and efficiency of antioxidants (Karadag *et al*, 2009). HAT-based methods measure the ability of an antioxidant to scavenge free radicals via hydrogen donation to form stable compounds. While these methods are more relevant to the radical chain-breaking antioxidant capacity, SET-based methods measure the ability of an antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and free radicals ((Karadag *et al*, 2009; Prior*, et al*, 2005). Total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC), lipid peroxidation inhibition capacity (LPIC) and carotene or crocin-bleaching assays are HAT-based methods. Other commonly used antioxidant activity assessing methods such as ferric reducing antioxidant power (FRAP) and copper reduction assay involve SET mechanisms (Ou *et al*, 2001). However, some methods, such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and {2,2'–azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)} (ABTS), involve both HAT and SET mechanisms (Prior *et al*. 2005).

2.7.2.3.1 DPPH *In-Vitro* Antioxidant Assay

This method was developed in 1958 (Blois, 1958) with the viewpoint to determine the antioxidant activity in a like manner by using a stable free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, $M = 394.33$). The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine (Contreras-Guzman, 1982). DPPH is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerize, like most

other free radicals. The delocalisation also gives rise to the deep violet colour, with an absorption in ethanol solution at around 520 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet colour. While DPPH can accept an electron or hydrogen radical to become a stable, diamagnetic molecule, it can be oxidized only with difficulty, and then irreversibly. DPPH shows a strong absorption band at 517 nm due to its odd electron and solution appears a deep violet colour, the absorption vanishes as the electron pairs off. The resulting decolorization is stoichiometric with respect to the number of electrons taken up. The alcoholic solutions of 0.5 mM are densely coloured and at this concentration, the Lambert-Beer law is obeyed over the useful range of absorption (Blois, 1958).

It is a rapid, simple, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity of foods. It can also be used to quantify antioxidants in complex biological systems, for solid or liquid samples. This method is easy and applies to measure the overall antioxidant capacity (Prakash 2001) and the free radical scavenging activity of fruit and vegetable juices (Sendra *et al*. 2006). This assay has been successfully utilized for investigating antioxidant properties of wheat grain and bran, vegetables, conjugated linoleic acids, herbs, edible seed oils, and flours in several different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol and benzene (Yu, 2001; Parry *et al*. 2005). It is a convenient method for the antioxidant assay of cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds (Masahiro *et al*. 2005), for olive oil, fruits, juices and wines (Sanchez-Moreno, 2002).

The advantage of this method is that DPPH is allowed to react with the whole sample and sufficient time given in the method allows DPPH to react slowly even with weak antioxidants (Prakash 2001). DPPH method may be utilized in aqueous and nonpolar

organic solvents and can be used to examine both hydrophilic and lipophilic antioxidants (Prior *et al*. 2005).

DPPH assay is considered a valid accurate, easy and economical method to evaluate radical scavenging activity of antioxidants since the radical compound is stable and need not be generated.

CHAPTER THREE

MATERIALS AND METHODS

3.1 GENERAL EXPERIMENTAL PROCEDURE

3.1.1 Materials

The solvents used for extraction were laboratory grade chemicals and were purified prior to use. Silica gel 60–120, 100–200 and 200 – 400 mesh sizes were used for column chromatography. Thin layer chromatography (TLC) was used for monitoring the fractions using aluminum sheets pre-coated with silica gel 60 F_{254} (Merck, Germany). Isolated compounds were detected under UV light at 254nm and 365nm wavelength respectively and also either sprayed with ceric ammonium sulphate solution followed by heating on a TLC heater at about 150^{O}C for few minutes or the developed TLC plate was treated in iodine vapour. Materials used for Brine shrimp cytotoxicity assay: Brine shrimp (*Artemia salina),* eggs and salt, were purchased from Essex Marine Aquatics, UK. The microbial media used for antimicrobial assay - Mueller-Hilton agar, MacConkey agar and nutrient broth, were all obtained from LAB M, Bury, UK while sterile normal saline was supplied by Dana Ashmina Ltd, Ibadan, Nigeria. Reagents used for Anti-cancer assay: RPMI-1640, fetal calf serum, trypsin, PBS, trypan blue, penicillin, streptomycin, DMSO, sulphorhodamine, paclitaxel (taxol), 5-fluorouracil, were obtained from Sigma Chemical Co. USA.

3.1.2 Instrumentation

^{1}H and ^{13}C NMR were run on Bruker Avance III 400 MHz and Bruker Avance 500 MHz using $CDCl_3$, MeOD or DMSO-*d* as solvent. The chemical shifts are reported

downfield from TMS used as internal standard. Values of coupling constant *J* are reported in Hz. Optical rotation was recorded on Perkin Elmer Polarimeter model 241. Infra-Red spectrometer was done using Perkin Elmer Fourier Transform Infra-red spectrometer Model Spectrum 2. The mass of the compounds were determined using Agilent 1260 liquid chromatography (Agilent, USA) equipped with a quaternary solvent delivery system, an auto sampler, a column compartment and Triple quad 6410 MS system and Agilent technologies 6540 UHD Accurate mass Q-TOF Liquid chromatography-mass spectrometer (Agilent, USA). Melting points were determined in open capillaries using automated melting point apparatus. Ultraviolet spectroscopy was done using Labdomed Inc. Spectrum UVVIS Double PC 8 Auto Cell Scanning Spectrophotometer UV-VIS Double Beam Model UVD- 3200. Crystal X-ray diffraction was determined using X'calipur single crystal X-ray diffractometer with CCD camera. Absorbance measurements for DPPH antioxidant assay was determined using Uniscope UV Spectrophotometer Model SM 7504.

3.2 PLANT COLLECTION

*Mezoneuron benthamianum*root was collected in Ibadan in the month of October 2012 and was identified and authenticated by the Plant Taxonomist at the Department of Botany of the University of Ibadan where a voucher specimen (No: UIH-22401) was deposited in the herbarium.

3.3 PLANT PREPARATION AND EXTRACTION

The dried root of *M. benthamianum* (2.7kg) was pulverised with the milling machine at the Department of Chemistry, University of Ibadan, Nigeria and subsequently, extracted by maceration with methanol (10L x 2, for 72 hours each). The extracts were concentrated under reduced pressure with rotary evaporator to give the crude methanol extract (200 g). This was subsequently dissolved in aqueous methanol and

further partitioned into hexane, dichloromethane, ethyl acetate and aqueous methanol fractions to give 5 g, 14 g, 70 g and 100 g of hexane, dichloromethane, ethyl acetate and aqueous methanol fractions respectively. The flowchart for the fractionation of the extracts and isolation of the compounds is shown in Fig. 3.1.

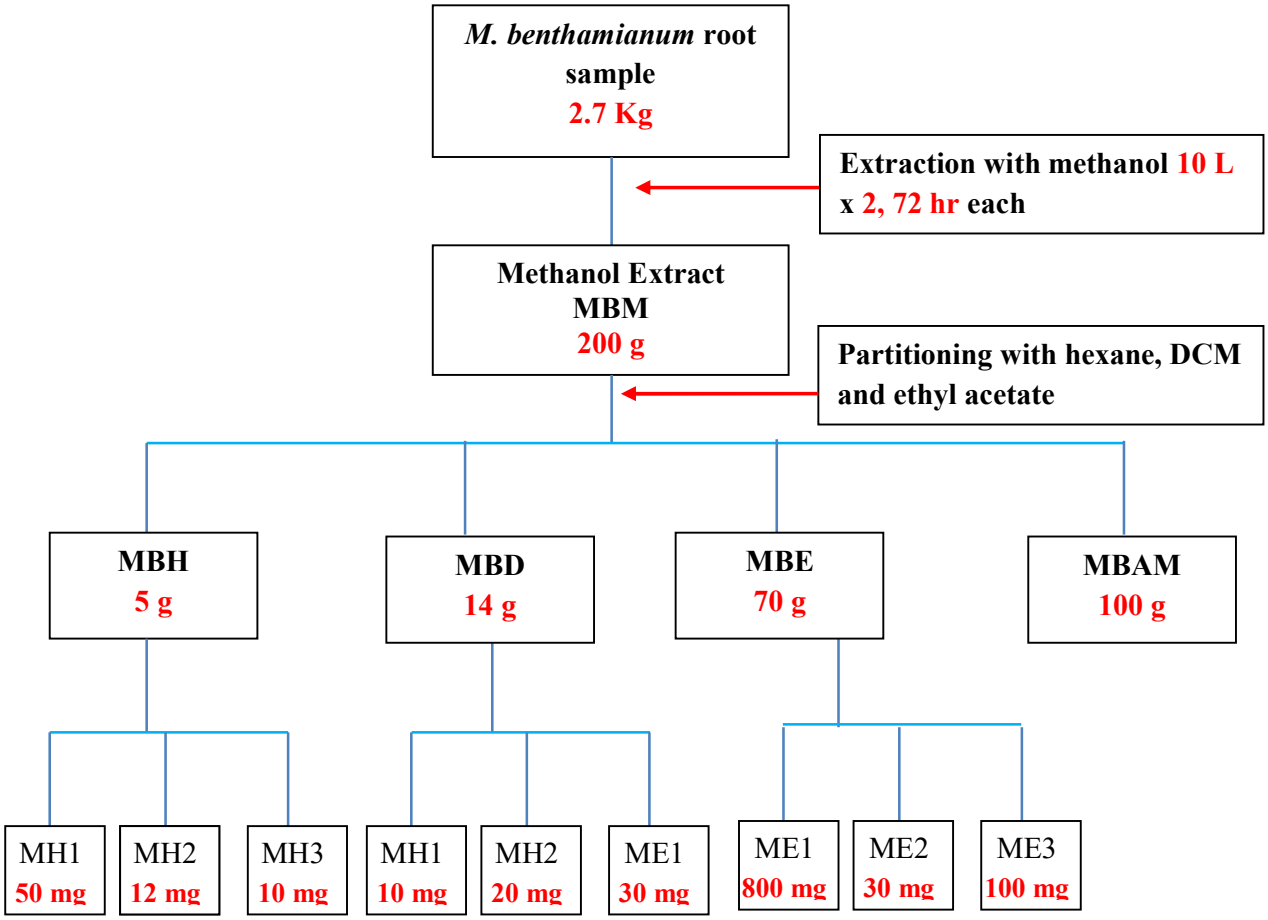


Figure 3.1: Scheme for the isolation of compounds from *M. benthamianum*

Key: MBM - *M.benthamianum* methanol extract
MBH - *M.benthamianum* Hexane fraction
MBD - *M.benthamianum* Dichloromethane fraction
MBE - *M.benthamianum* Ethyl acetate fraction
MBAM - *M.benthamianum* Aqueous methanol fraction

3.4 FRACTIONATION OF HEXANE EXTRACT

The hexane extract (4 g) was fractionated using column chromatography (CC) over silica gel (60-120 mesh size), and eluted with hexane in increasing proportions of ethyl acetate and methanol to give three hundred and eighty-eight fractions. The eluted fractions were combined on the basis of TLC analysis to give twelve sub-fractions Fractions F_1 to F_{12}. Fractions F_2 to F_4 were further purified to afford three compounds, while further purification on fractions F_5 to F_{12} could not afford any pure compound.

3.4.1 ISOLATION OF MH1

Sub-fraction F_2 (100 mg) eluted with ethyl acetate: hexane (5:95)gave crystalsupon concentration using rotary evaporator. This was subsequently purified by recrystallization from hexane to afford compound **1** and labeled MH1 (60 mg).

3.4.2 ISOLATION OF MH2

Sub-fraction F_3 (50 mg) eluted with ethyl acetate: hexane (5:95) gave a mixture of two compounds, this was further purified by thin layer chromatography using with ethyl acetate: hexane (2:98), followed by preparative column chromatography to afford compound **2** (20 mg) and labeled MH2.

3.4.3 ISOLATION OF MH3

Sub-fraction F_4 (40 mg) eluted with ethyl acetate: hexane (5:95) gave a white crystalline compound which was purified by recrystallization from hexane to afford compounds 3 (30 mg) and labeled MH3.

3.5 FRACTIONATION OF DICHLOROMETHANE EXTRACT

The Dichloromethane extract (12 g) was subjected to column chromatography using

silica gel (100 – 200 mesh size) and eluted with hexane in increasing proportion of ethyl acetate and subsequently, ethyl acetate in increasing proportion of methanol to give thirty fractions with where pooled on the basis of TLC analysis to give four sub-fractions. Purification of these four sub-factions also led to the isolation of MD1, MD2 and MD3. Upon analysis, MD1 and MD2 were found to be the same as MH1 and MH2. While MD3 was found to be the same as another compound also isolated from the ethyl acetate extract, ME1.

3.6 FRACTIONATION OF ETHYL ACETATE EXTRACT

The ethyl acetate extract (40 g) was subjected to column chromatography using silica gel (200 – 400 mesh size) and eluted with hexane in increasing proportions of ethyl acetate and subsequently, ethyl acetate in increasing proportion of methanol to give fifty fractions. Similar fractions were combined on the basis of TLC analysis to give seven sub-fractions. Purification of sub-fractions F_3, F_5 and F_6 gave three pure compounds ME1, ME2 and ME3. These three compounds were subjected to different spectroscopic techniques to fully characterize them.

3.6.1 ISOLATION OF ME1

Fractions 17 – 22 eluted with ethyl acetate: hexane (40:60) were pooled together on the basis of TLC analysis to give sub-fractions F_3 (1.0 g). This was purified by column chromatography using ethyl acetate: hexane (40:60) to afford compound **5** (1.2 g) and labeled **ME1.**

3.6.2 ISOLATION OF ME2

Fractions 24 – 29 eluted with ethyl acetate: hexane (50:50) were pooled together on the basis of TLC analysis to give sub-fractions F_5 (200 mg). This was purified by column chromatography with ethyl acetate: hexane (40:60) to afford compound **6** (80

mg) and labeled **ME2.**

3.6.3 ISOLATION OF ME3

Fractions 31 – 35 eluted with ethyl acetate: hexane (50:50) were pooled together on the basis of TLC analysis to give sub-fractions F_5 (80 mg). This was purified by column chromatography using with ethyl acetate: hexane (40:60) to afford compound **7** (40 mg) and labeled **ME3.**

3.7 FRACTIONATION OF METHANOL EXTRACT

The methanol extract was fractionated using column chromatography with silica gel (100 – 200 mesh size) and eluted with different solvent systems such as hexane in increasing proportion of ethyl acetate and dichloromethane in increasing proportion of methanol, however, no pure compound could be isolated.

3.8 BRINE SHRIMP *IN-VITRO*CYTOTOXIC ASSAY

The brine shrimp lethality test (Meyer *et al*., 1982) was used to predict the bioactivity of the extracts and isolated compounds.

3.8.1 Hatching Shrimp

Brine shrimp eggs, *Artemia salina* were hatched in a vessel containing distilled water to which the salt and eggs were added. The vessel was kept under an inflorescent bulb and facilitated with good aeration for 48 hr at room temperature. After hatching, nauplii released from the egg shells were collected at the bright side of the vessel (near the light source) by using micropipette.

3.8.2 Preparing the Serial Dilutions.

The stock solution of 1000 ppm was prepared by weighing 20 mg of sample into 2 ml of 1% DMSO in sea water. Serial dilutions of 100 ppm and 10 ppm were prepared by transferring 0.2 ml of the stock solution to 1.8 ml of 1 % DMSO in sea water in another test-tube to give 100 ppm which was also further diluted accordingly to give 10 ppm. This was done in triplicate.

3.8.3 Brine Shrimp Test

After two days (when the shrimp larvae are ready), 4ml of sea water is added to each test tube and 10 shrimps are introduced into the test tube after which the volume is adjusted with sea water to 5 ml /test tube. The test-tubes are placed uncovered, under the lamp and the setup was allowed to stand for 24 hr after which the number of survivors are counted and recorded.

3.8.4 Statistical Analysis

The results are analysed with Finney computer program for probit analysis to determine the LC_{50} values at 95% confidence intervals.

3.9 *IN-VITRO* ANTICARIES ASSAY OF EXTRACTS AND ISOLATED COMPOUNDS

The agar well-dilution method was used to determine the inhibition zones and minimum inhibition concentration (MIC) of the extracts and isolated compounds.

3.9.1 Determination of Zones of Inhibition

The extracts and isolated compounds were tested against four microorganisms [*Staphylococcus aureus* (gram positive bacteria), *Escherichia coli* (gram negative bacteria), *Pseudomonas aeruginosa* (gram negative bacteria) and *Streptococcus mutans* (gram positive bacteria)] that were clinically isolated at the Department of Pharmaceutical Microbiology from the swabs taken from the oral cavity of patients at the Dental clinic of the University of Ibadan and three typed microorganism strains [*S. aureus* (ATCC 29213), *E. coli* (ATCC 35218), *P. Aeruginosa* (ATCC 27855)]. Each plant extract was prepared into a concentration of 200 mg/mL from the dried extract by dissolving 2 g of the individual plant extract in 10 ml of methanol, then 2 ml of the mixture was diluted serially four times with 2 ml of the methanol to give 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml. a volume of 0.1 ml of a particular concentration was introduced into the well of the agar medium (6 mm in diameter) using calibrated Pasteur pipette. Gentamicin at 10 µg/ml concentration was introduced into one of the wells to serve as the positive control while methanol was used as negative control. The plates were left for 45 minutes to allow the diffusion of the extracts and controls in the wells through the agar medium after which they were incubated at 37^{o}C for 24 hours. This was done in duplicates. Zones of growth inhibition of the various concentrations of the extracts and the controls were measured with ruler and recorded. The mean zone of inhibition less than 8 mm is considered as no activity.

3.9.2 MINIMUM INHIBITORY CONCENTRATION DETERMINATION

3.9.2.1 INOCULUM PREPARATION OF THE TEST ORGANISMS

The inoculum was prepared from overnight grown culture on Mueller-Hilton agar plates by suspending well-isolated colonies of culture in 2 ml sterile saline and the turbidity of the suspension was adjusted to 0.5 McFarland (1.5 x 10^8 CFU/ml). The cultures were further diluted in medium to get 2 times the final inoculum (5 x 10^5CFU/ml), blank media used as negative control.

3.9.2.2 PREPARATION OF STOCK SOLUTION

1 g or 1000 mg of extract was dissolved in 5 ml of methanol to give 200 mg/ml after which two fold serial dilutions were prepared from the stock solution to give 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78, 0.39 and 0.2 mg/ml. Furthermore, 2 ml of these solutions were added to 18ml of Nutrient Agar solution to give 20, 10, 5, 2.5 and 1.25, 0.625, 0.3125, 0.1563, 0.078, 0.039 and 0.020 mg/ml.

10 mg of the compounds was dissolved in 5 ml of methanol to give 2 mg/ml after which serial dilutions were prepared from the stock solution by adding 2 ml of this solution to 18 ml of Nutrient Agar solution to give 0.20 mg/mL further dilutions were carried out to give 0.10, 0.05, 0.025 and 0.0125 mg/mL.

3.9.2.3 PREPARATION OF TEST PLATE

One milliliter of each dilution of the extract was mixed with 9 mL of Mueller Hinton agar, poured into 10 cm diameter petri dishes and allowed to set. After allowing the agar to dry for about 30 mins, each plate was inoculated with 1:100 dilution of overnight broth cultures of each test organisms containing 1.0 x 10^8 CFU/mL (according to 0.5 MacFarland standard). Nutrient agar plates with extract but without an organism and one containing only the organism but without extract served as positive and negative control respectively.

3.9.2.4 INCUBATION OF PLATES

All the plates including *Pseudomonas aregonosa*, *Staphylococos aureus, Escherichia coli* and *Streptococcus mutans* were incubated at 37^{o}C overnight. The lowest concentration of samples in the well showing no growth is considered as MIC.

3.10 *IN-VITRO* ANTIOXIDANT ASSAY

The free radical scavenging activity of the compounds was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by, Brand-Williams *et al*., (1995) with slight modifications. Briefly, a 0.1 mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 0.5 ml of the samples dissolved in methanol and using a range of 12.5-200 µg/ml. After 20 min, the absorbance was measured at 517 nm. The DPPH radical scavenging activity was calculated according to the following equation:

DPPH scavenging activity (%) = $[(A_o - A_I)/A_o]$ x 100

Where $A_o$is the absorbance of the control and A_I is the absorbance in the presence of the test substance. DPPH solution alone served as control (A_o). A graph of % inhibition against concentration was plotted and IC_{50} determined using the GraphPad Prism 7.0 programme.

3.11 *IN-VITRO* ANTICANCER ASSAY

The crude extracts and the isolated compounds from *M. benthamianum* were also tested against different cell lines such as Lung (A549), Prostate (PC-3), Lung (NCI-H322) and Breast (T47D).

The human cancer cell lines were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2 mM glutamine, pH 7.4, supplemented with 10 % fetal calf serum, 100 µg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37 °C, 5 % CO2, 90 % RH). The cells at sub confluent stage were

harvested from the flask by treatment with trypsin [0.05 % in PBS (pH 7.4) containing 0.02 % EDTA]. Cells with the viability of more than 98 % as determined by trypan blue exclusion, were used for determination of cytotoxicity The cell suspension of 1 x 10^5 cells/ml was prepared in complete growth medium.Stock solutions (20 mM/ml) of compounds and (20 mg/ml) extracts were prepared in DMSO. The stock solutions were serially diluted with complete growth medium to obtain working test solutions of required concentrations.

In vitro cytotoxicity against human cancer cell lines was determined (Monks *et al*., 1991) using 96-well tissue culture plates. The 100 µl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were allowed to grow in carbon dioxide incubator (37 °C, 5 % CO_2, 90 % RH) for 24 hours. Test materials in complete growth medium (100 µl) were added after 24 hours of incubation to the wells containing cell suspension. The plates were further incubated for 48 hours in a carbon dioxide incubator. The cell growth was stopped by gently layering trichloroacetic acid (50 %, 50 µl) on top of the medium in all the wells. The plates were incubated at 4 ^oC for one hour to fix the cells attached to the bottom of the wells. The liquid of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove trichloroacetic acid, growth medium low molecular weight metabolites, serum proteins etc. and air-dried. The plates were stained with Sulforhodamine B dye (0.4 % in 1 % acetic acid, 100 µl) for 30 minutes. The plates were washed five times with 1 % acetic acid and then air-dried. (Skehan *et al*., 1990). The adsorbed dye was dissolved in Tris-HCl Buffer (100 µl, 0.01 M, pH 10.4) and plates were gently stirred for 10 minutes on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of

theexperimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 EXTRACTION OF PLANT SAMPLES

The ground root of *Mezoneuron benthamianum* (2.7kg) were extracted with methanol and concentrated under reduced pressure to give dark brownish extract (200 g). This was dissolved in aqueous methanol and further partitioned with hexane, dichloromethane, ethyl acetate and methanol to give their respective fractions which were concentrated under reduced pressure.The yields of the extract and fractions are presented in Table 4.1 with their physical appearance.

Table 4.1:The Percentage yield of extracts of *Mezoneuron benthamianum* and their physical appearance

Plant extracts	Weight of plant (g)	Weight of extract (g)	% yield	Physical appearance of extract
MB	2375	200	8.42	Dark brown
MBH	2375	5	0.21	light brown
MBD	2375	14	0.59	Dark brown
MBE	2375	70	2.95	Dark brown
MBM	2375	100	4.21	Dark brown

Key: MBM - *M.benthamianum* methanol extract
MBH - *M.benthamianum* Hexane fraction
MBD - *M.benthamianum* Dichloromethane fraction
MBE - *M.benthamianum* Ethyl acetate fraction
MBAM - *M.benthamianum* Aqueous methanol fraction

4.2 FRACTIONATION AND PURIFICATION OF THE HEXANE EXTRACT

The hexane extract was subjected to open column chromatography using silica gel (60-120 mesh) and eluted with hexane in increasing proportions of ethyl acetate and methanol to yield three hundred and eighty-eight fractions which were combined based on TLC analysis to give twelve sub-fractions. Purification of these fractions gave three pure compounds MH1, MH2 and MH3. These compounds were subjected to different spectroscopic techniques in order to characterize them.

4.2.1 ISOLATION OF MH1

Fractions 21 – 36 eluted with 5% ethyl acetate in hexane were combined based on TLC analysis to give sub-fraction F_2 whichgave crystals upon concentration under reduced pressure. This was subsequently purified by recrystallization from hexane to give white crystals designated as MH1 (60 mg).

4.2.2 CHARACTERIZATION OF MH1

Appearance: White crystals

Melting point: 158-159^{o}C

Optical rotation: $[\alpha]_D$ = +54.7 (*c*= 0.0015, $CHCl_3$)

Infra-Red (KBr, $V_{max,}$ cm^{-1}):, 1723 (RCO_2R), 771 (furan moeity)

Ultra Violet (CH_2Cl_2, nm): λ_{max} 253, 282 and 292

Molecular formula (HRESIMS): *m/z* $(MH)^+$ 327.1952, (calculated for $C_{21}H_{27}O_3$, 327.1960)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 38.7 (C_1), 18.7 (C_2), 36.6 (C_3), 47.6 (C_4), 44.4 (C_5), 21.8 (C_6), 27.6 (C_7), 127.4 (C_8), 147.2 (C_9), 37.9 (C_{10}), 104.3 (C_{11}), 153.6 (C_{12}), 125.3 (C_{13}), 128.3 (C_{14}), 106.2 (C_{15}), 144.2 (C_{16}), 16.1 (C_{17}),`179.2 (C_{18}), 16.7 (C_{19}), 25.8 (C_{20}), 52.3 (C_{21})

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.39, d, *J* = 9.7 Hz / 1.60, d, *J* = 3.62 (2H, $H_{1\alpha/\beta}$), 1.73, m / 1.83, dd, *J* = 11.03, 3.86 Hz (2H, $H_{2\alpha/\beta}$), 1.82, m / 1.66, m (2H, $H_{3\alpha/\beta}$), 2.28, dd, *J* = 12.69, 2.12 Hz (1H, H_5), 1.88, m / 1.53 dd, *J* = 13.20, 7.85 Hz (2H, $H_{6\alpha/\beta}$), 2.89, dd, *J* = 16.94, 6.57 Hz/ 2.79, m (2H, $H_{7\alpha/\beta}$), 7.33, s (1H, H_{11}), 6.73, d, *J* = 2.08 Hz (1H, H_{15}), 7.53, d, *J* = 2.18 Hz (1H, H_{16}), 3.70, s (3H, H_{21}), 2.34, s (3H, H_{17}), 1.31, s (1H, H_{19}), 1.26, s (1H, H_{20}).

4.2.3 STRUCTURAL ELUCIDAITON OF MH1

The structure of MH1 was determined through analysis of its EIMS, IR, UV, ^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC spectra. The molecular formula of MH1 was deduced to be $C_{21}H_{26}O_3$ based on the HRESIMS. HRESIMS, *m/z* 327.1952 $[M+H]^+$, (Fig. 4.2 and 4.3). The IR spectrum (Fig. 4.4) gave signals typical of ester and furan functional groups at 1723 and 771 cm^{-1} respectively. The UV spectrum (Fig. 4.5) had absorption maxima, λ_{max} at 253, 282 and 292 nm which suggested the presence of a benzofuran chromophore (Yodsaoue, 2010). The ^{1}H NMR spectrum (Fig. 4.6) displayed the resonances indicative of a 1,2-disubstituted furan in the downfield regions at δ 7.53 (d, *J* = 2.2Hz) and 6.73 (d, *J* = 2.1 Hz). The presence of another aromatic proton at δ 7.33 (s), along with an aromatic methyl group at δ 2.34, confirmed the presence of a trisubstituted benzofuran moiety. This is in addition to a singlet at δ 3.70 (3H) which is due to the presence of a methoxy group. The proton at δ 7.33 attributed to the carbon at δ 104.2 (C-11) on the basis of HSQC showed correlations to the aromatic carbons at δ 153.4 (C-12), 147.2 (C-9), and 127.4 (C-8), and a quaternary carbon at δ 37.9 (C-10), thus confirming its position as proton 11 on the cassane skeleton (Fig. 4.1a). The HMBC spectrum also revealed that the aromatic methyl protons at δ 2.34 (H-17) have long-range correlations to the aromatic carbons at δ 128.3 (C-14), 127.4 (C-8), and 125.3 (C-13). Similarly, the methyl protons at δ

1.31 (H-19) displayed HMBC correlations to the ester carbonyl at δ 179.2 (C-18), the methine carbon at δ 44.4 (C-5), and a methylene carbon at δ 36.6 (C-3), thus establishing that it was attached to C-4. The stereochemistry at C-4 was assigned on the basis of NOESY data (Fig. 4.12) and X-ray analysis (Fig. 4.13). The two tertiary methyl groups on C-4 and C-10 were shown to be of β-axial orientation, whereas the methine proton H-5 should be α-axial oriented. Based on these data, the orientation at C-4 was assigned. These spectra data thus fitted that of methyl-1,2,3,4,4a,5,6,11b-octahydro-4,7,11b-trimethylphenanthro[3,2-b]-furan-4-carboxylate which was previously reported as Taepeenin A (Fig. 4.1b), isolated from *Caesalpinia crista* (Cheenpracha, 2005). Taepeenin A is a enantiomer of benthaminin 1 which was previously isolated from *M. benthamianum* (Dickson, 2007).

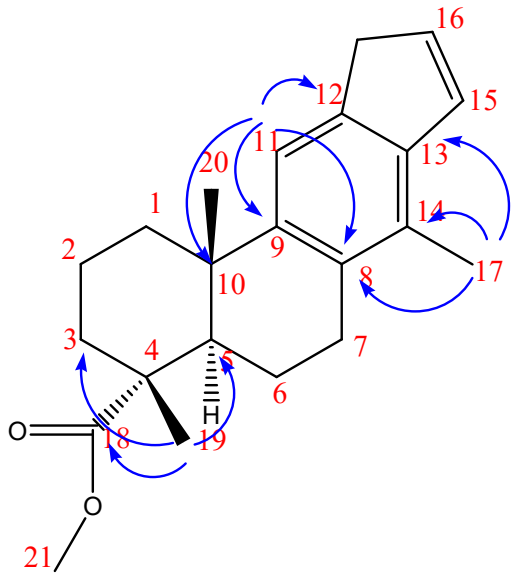


Figure 4.1a: HMBC correlations of compound MH1

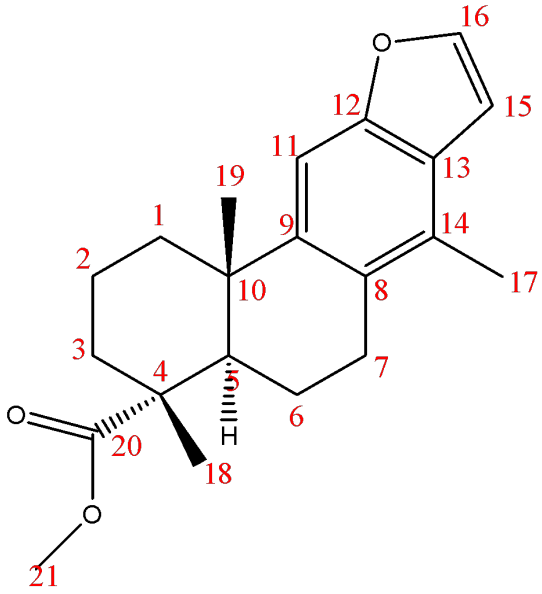


Figure 4.1b: Compound MH1 – Taepeenin A (methyl 1,2,3,4,4a,5,6,11b-octahydro-4,7,11b-trimethylphenanthro[3,2-b]furan-4-carboxylate)

Table 4.2: ^{13}C (100 MHz, $CDCl_3$) and ^{1}H NMR (400 MHz, $CDCl_3$) data of compound MH1 compared with literature

Carbon No	δ ^{13}C ppm	δ ^{13}C ppm (Cheenpracha *et al*., 2005)	DEPT	δ ^{1}Hppm, multiplicity (J_{Hz})	δ ^{1}Hppm, multiplicity (J_{Hz}) (Cheenpracha *et al*., 2005)	COSY	HMBC
1	38.7	38.9	CH_2	2.39 *d*, (16.44); 1.60 *d*, (3.62)	2.38 m, 1.55 *m*		C_2, C_3, C_5, C_{20}
2	18.8	18.7	CH_2	1.83 *dd*, (3.86, 11.03); 1.73 *m*	1.81 *m*, 1.74 *m*		C_4
3	36.7	36.6	CH_2	1.82 *m,* 1.66 *m*	1.80 *m*, 1.68 *m*		C_{18}, C_4
4	47.7	47.7	C	-		-	-
5	44.4	44.4	CH	2.28 *dd*, (12.69, 2.12)	2.27 *dd* (12.9, 2.4)	H_6	C_4, C_9, C_{10}, C_{18}, C_{20}
6	21.8	21.8	CH_2	1.88 *m;* 1.53 *dd* (7.85, 13.20)	1.92 *m*, 1.55 *m*	H_5, H_7	C_5, C_7, C_{10}
7	27.6	27.6	CH_2	2.89 *dd* , (6.57, 16.94);2.79 *m*	2.83 *m*	H_6	C_5, C_6, C_8, C_9
8	127.5	127.5	C	-		-	-
9	147.3	147.2	C	-		-	-
10	37.9	37.8	C	-		-	-
11	104.3	104.3	CH	7.33 *s*	7.32 br *s*	-	C_8, C_9, C_{10}, C_{12}
12	153.6	153.6	C	-		-	-
13	125.3	125.4	C	-		-	-
14	128.3	128.3	C	-		-	-
15	105.0	105.0	CH	6.73 *d* (2.08)	6.72 *dd* (2.1, 0.9)	H_{16}	C_{12}, C_{13}, C_{16}
16	144.2	144.2	CH	7.53 *d* (2.18)	7.53 d (2.1)	H_{15}	C_{12}, C_{13}, C_{15}
17	15.9	15.9	CH_3	2.34 *s*	2.35 *s*	-	C_8, C_9, C_{12}, C_{13}, C_{14}
18	179.2	179.2	C	-		-	-
19	16.6	16.6	CH_3	1.31 *s*	1.31 *s*	-	C_3, C_4, C_5, C_{18}
20	25.6	25.6	CH_3	1.26 *s*	1.27 *s*	-	C_1, C_5
21	52.0	52.0	CH_3	3.70 *s*	3.70 *s*	-	C_{18}

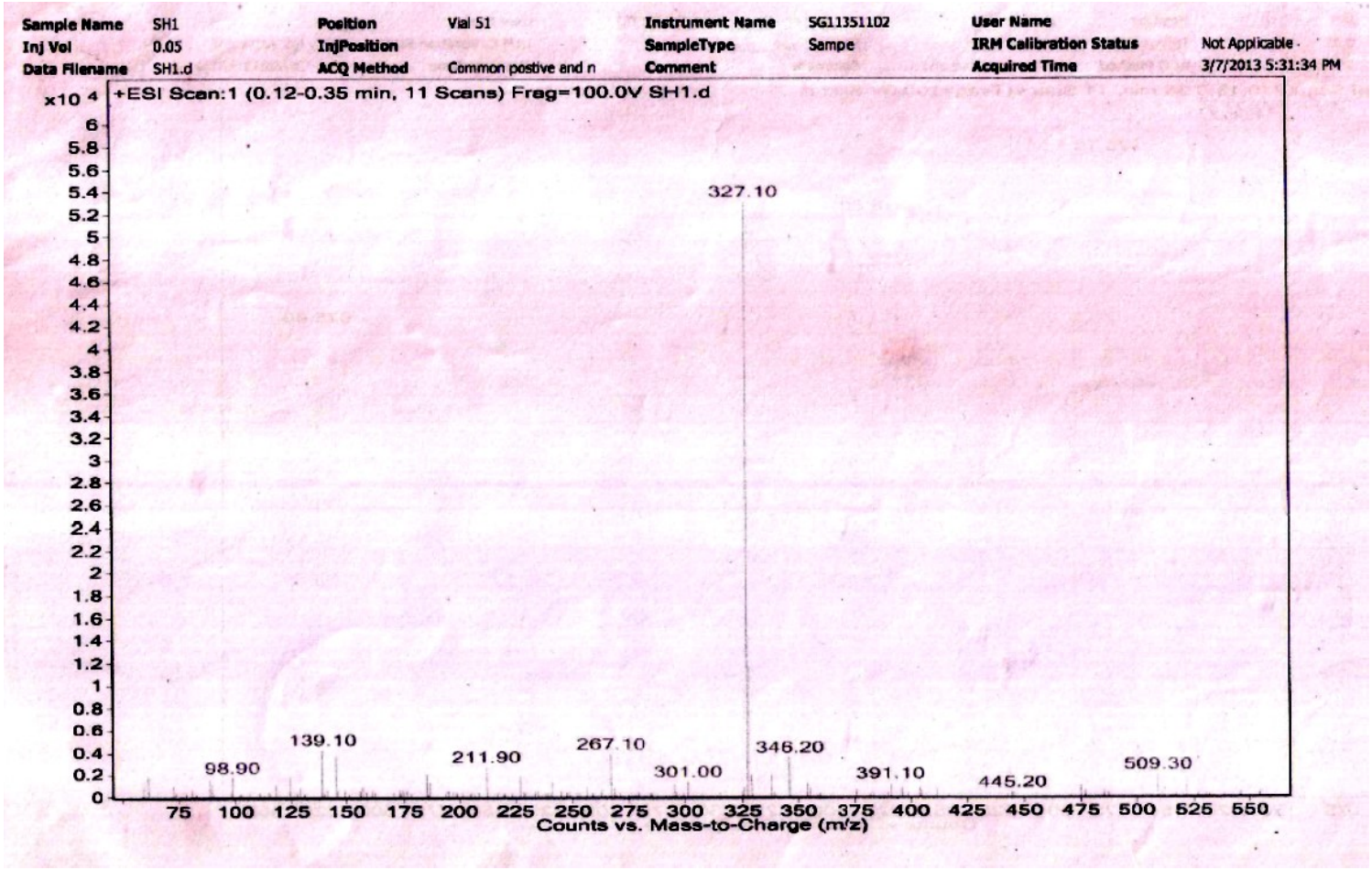


Figure 4.2: ESI-Mass spectrum of Compound MH1

Qualitative Compound Report

Data File	SH1.d	**Sample Name**	SH1
Sample Type	Sample	**Position**	Vial 8
Instrument Name	Instrument 1	**User Name**	vishal
Acq Method	vishal_12-01-13.m	**Acquired Time**	15-04-2013 PM 12:56:35
IRM Calibration Status	Success	**DA Method**	daily_report.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 14: C21 H26 O3	0.194	326.1881	C21 H26 O3	C21 H26 O3	0.24	C21 H26 O3

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 14: C21 H26 O3	327.1952	0.194	Find by Molecular Feature	326.1881

MFE MS Spectrum

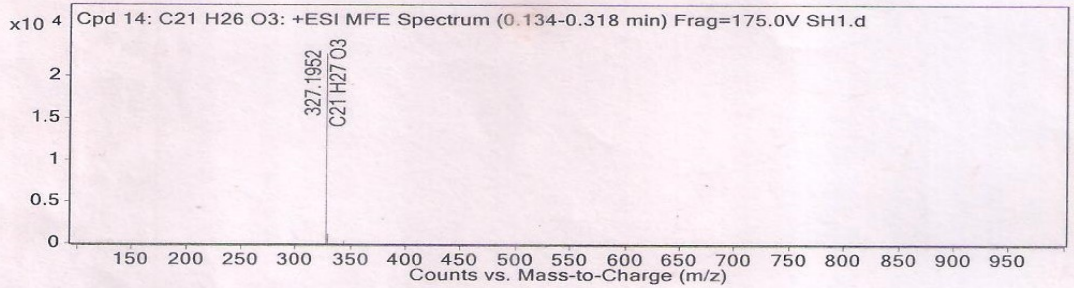


Figure 4.3: HRESI Mass Spectrum of Compound MH1

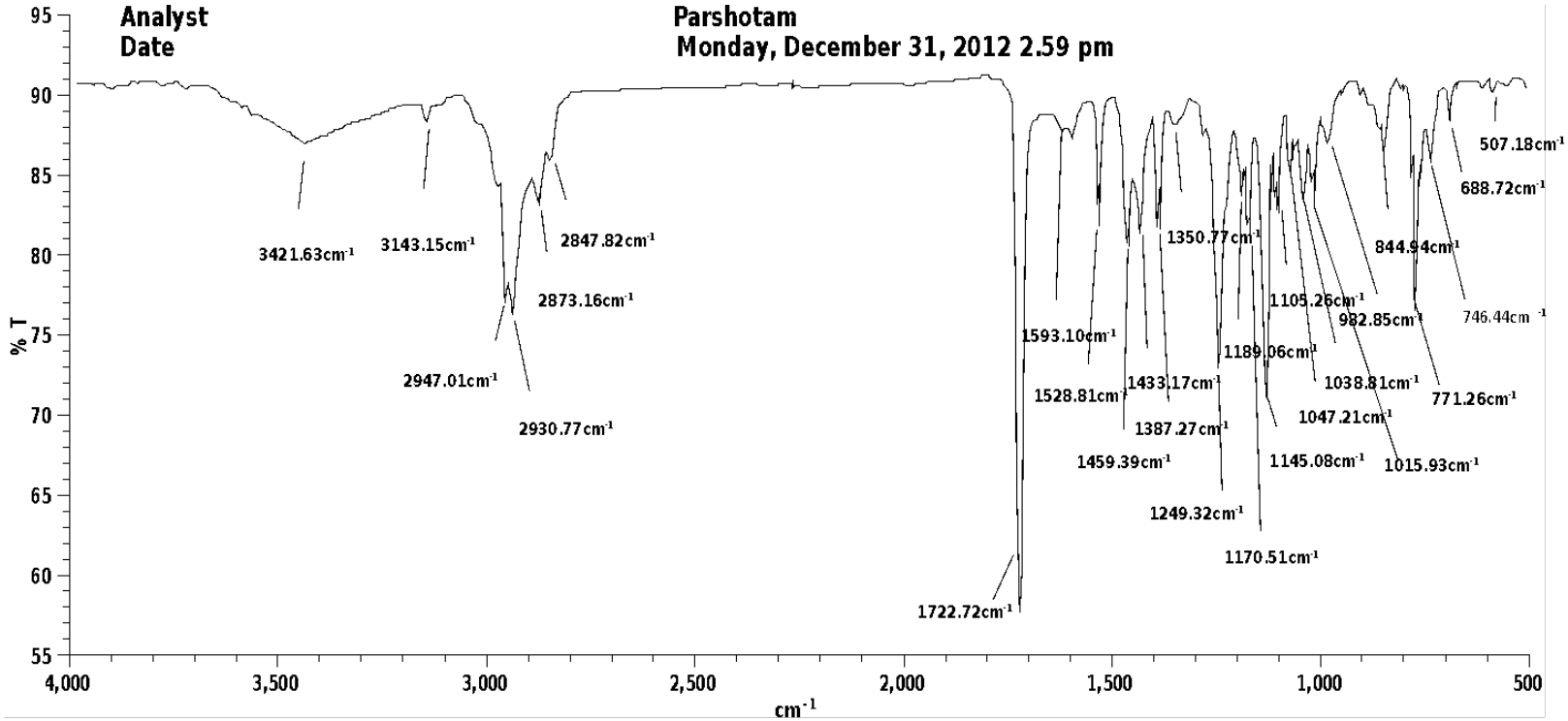


Figure 4.4: Infra-red spectrum of Compound MH1

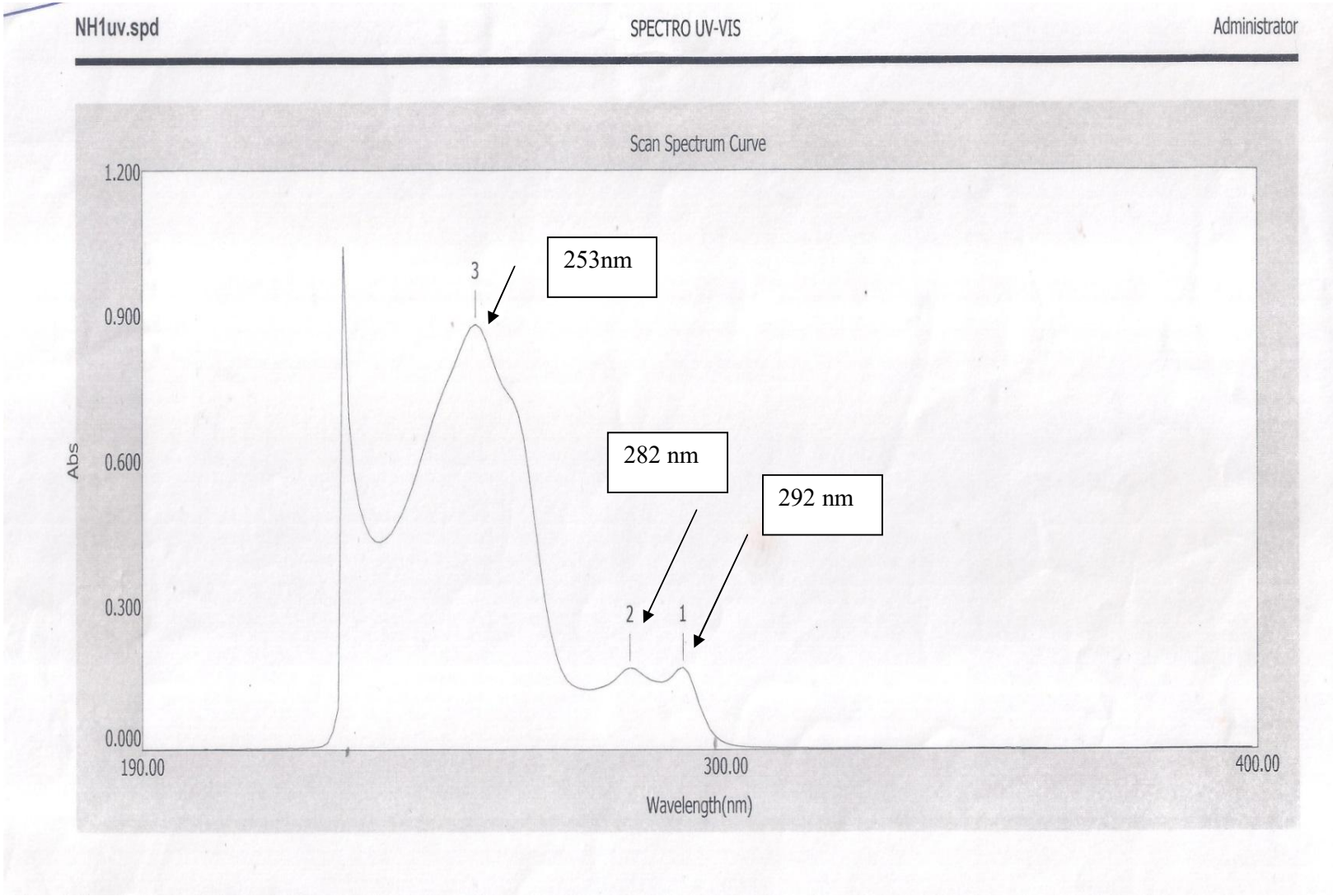


Figure 4.5: Ultra-Violet Spectrum of Compound MH1

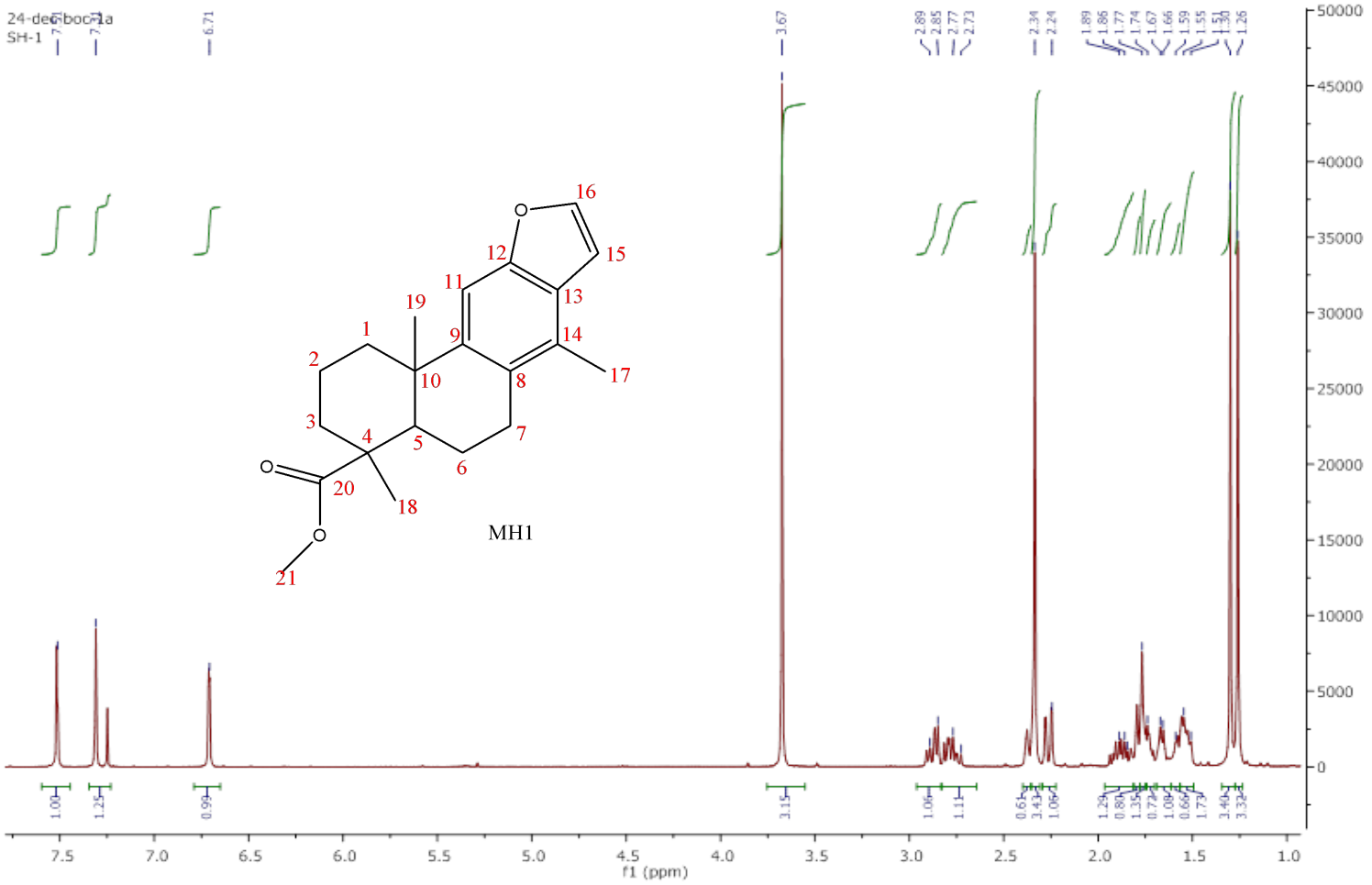


Figure 4.6: ^{1}H NMR (400 MHz, $CDCl_3$) Spectrum of Compound MH1

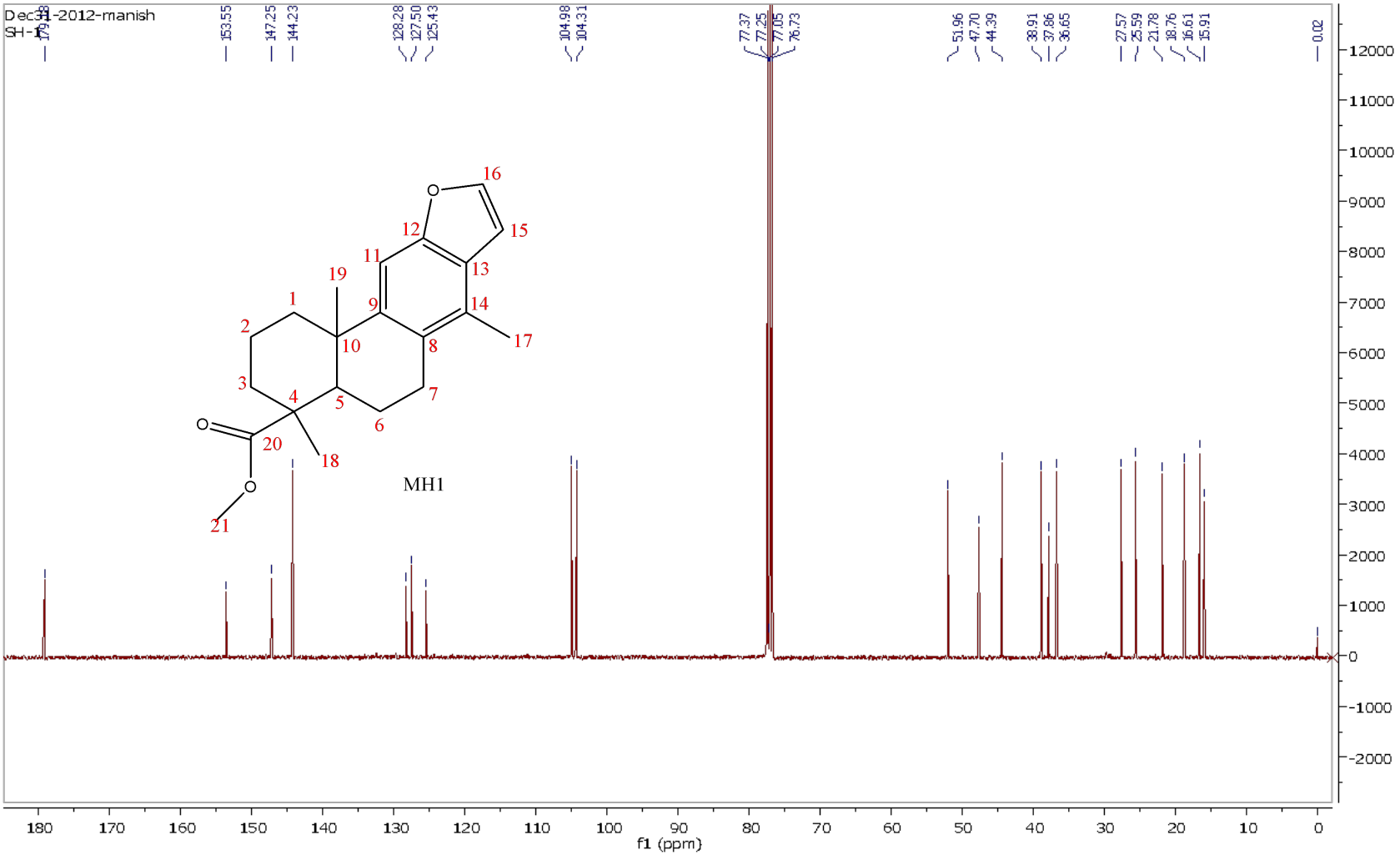


Figure 4.7: ^{13}C NMR (100 MHz, $CDCl_3$) Spectrum of Compound MH1

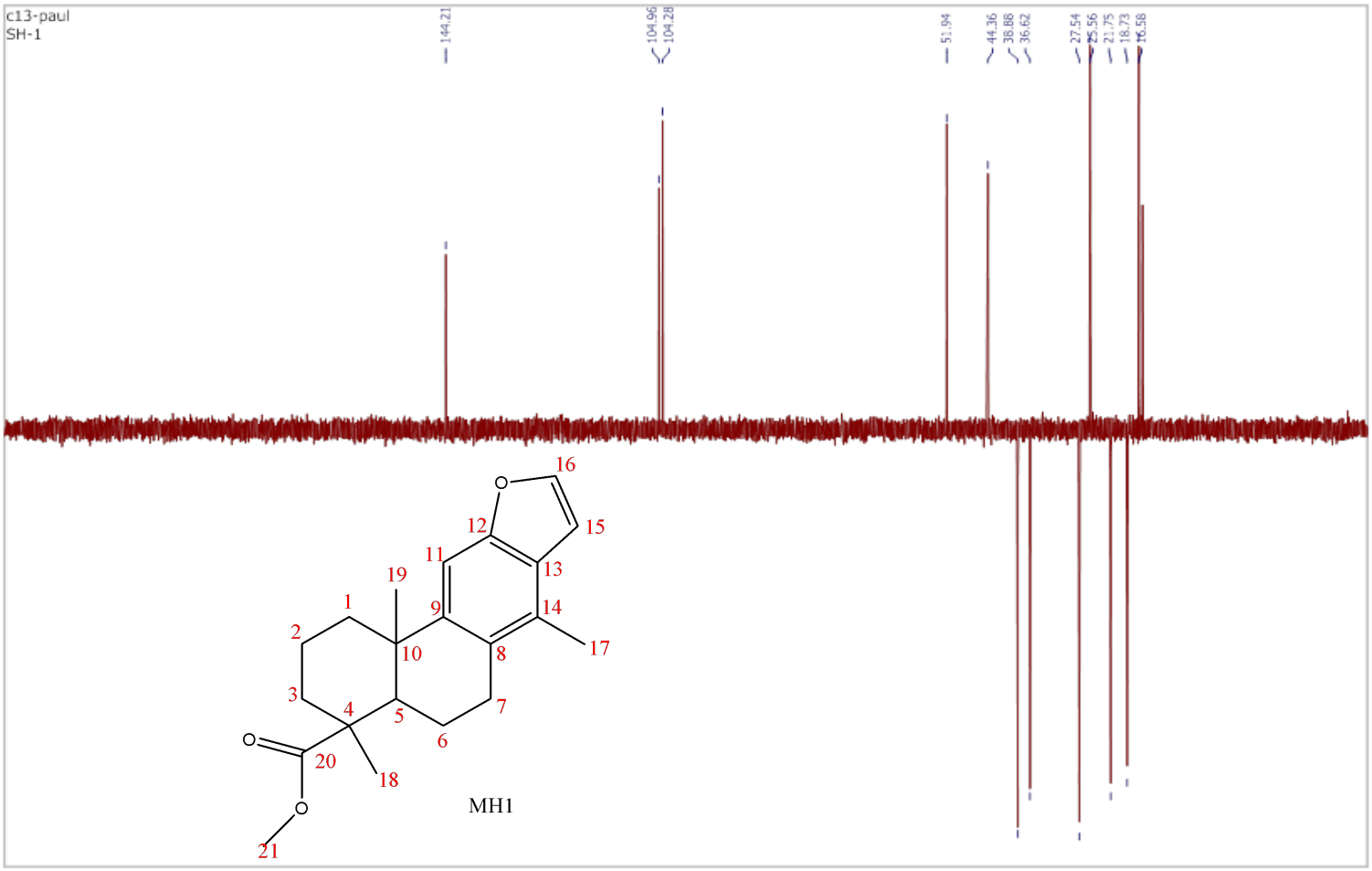


Figure 4.8: DEPT Spectrum of Compound MH1

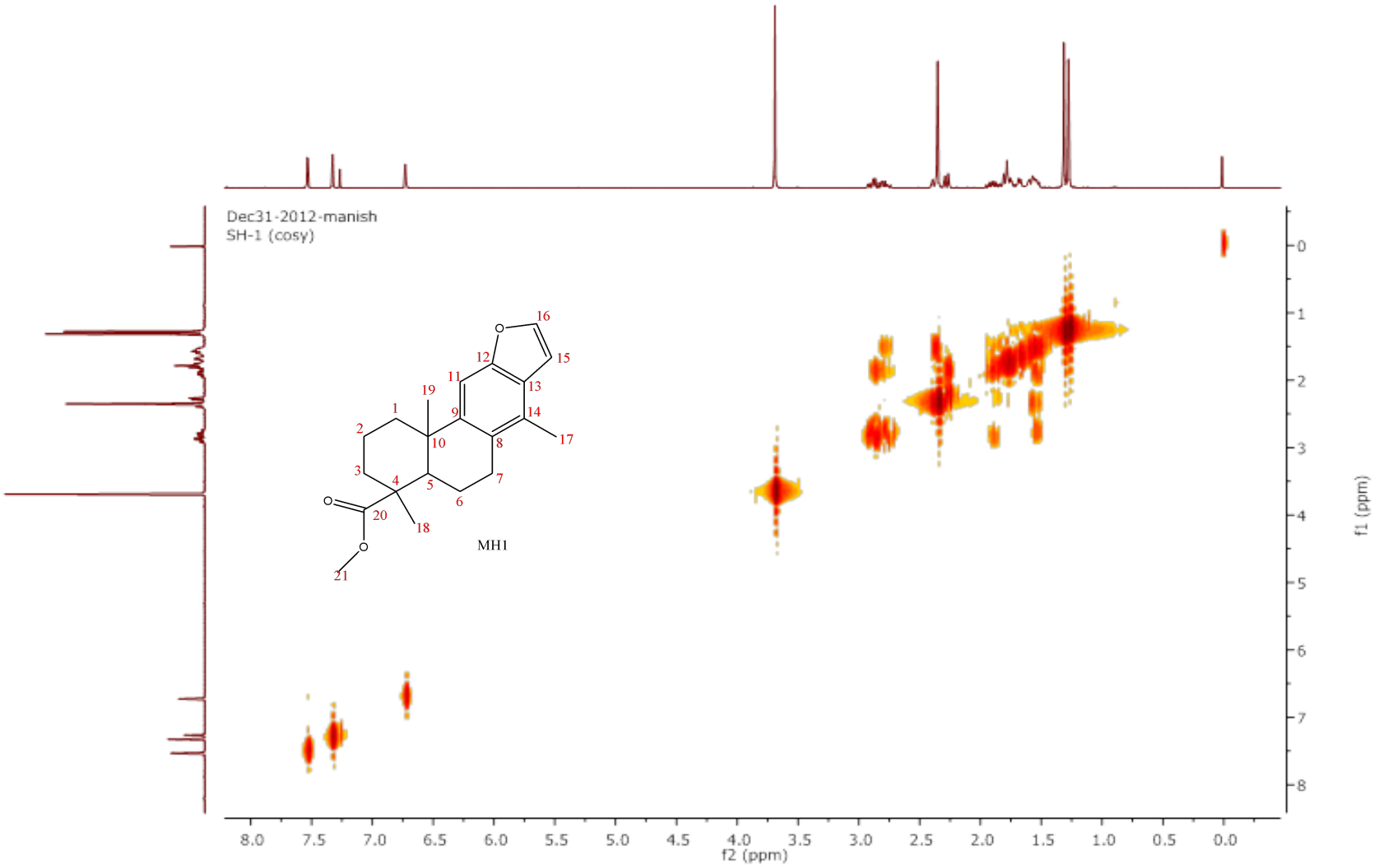


Figure 4.9: COSY spectrum of Compound MH1

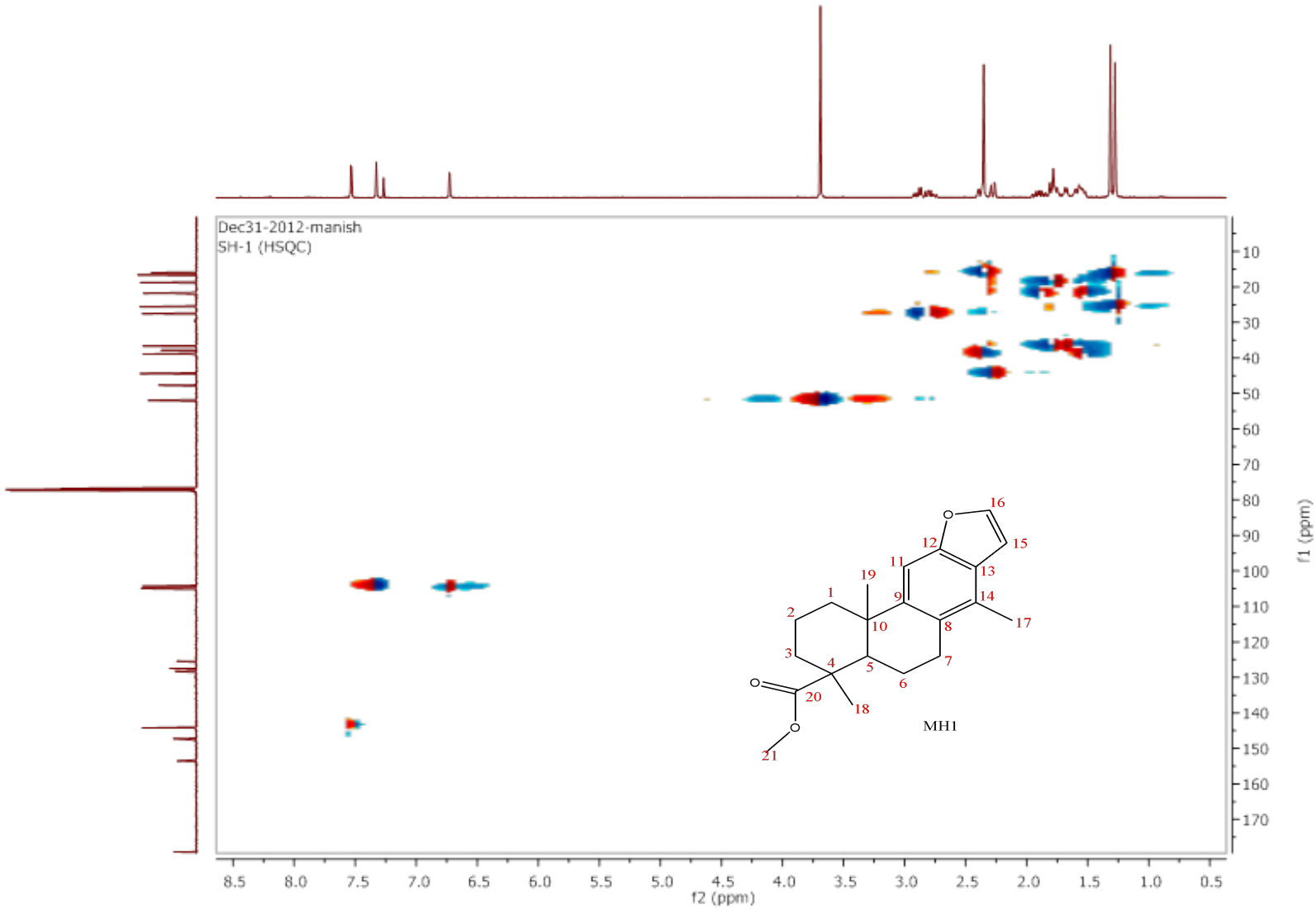


Figure 4.10: HSQC spectrum of Compound MH1

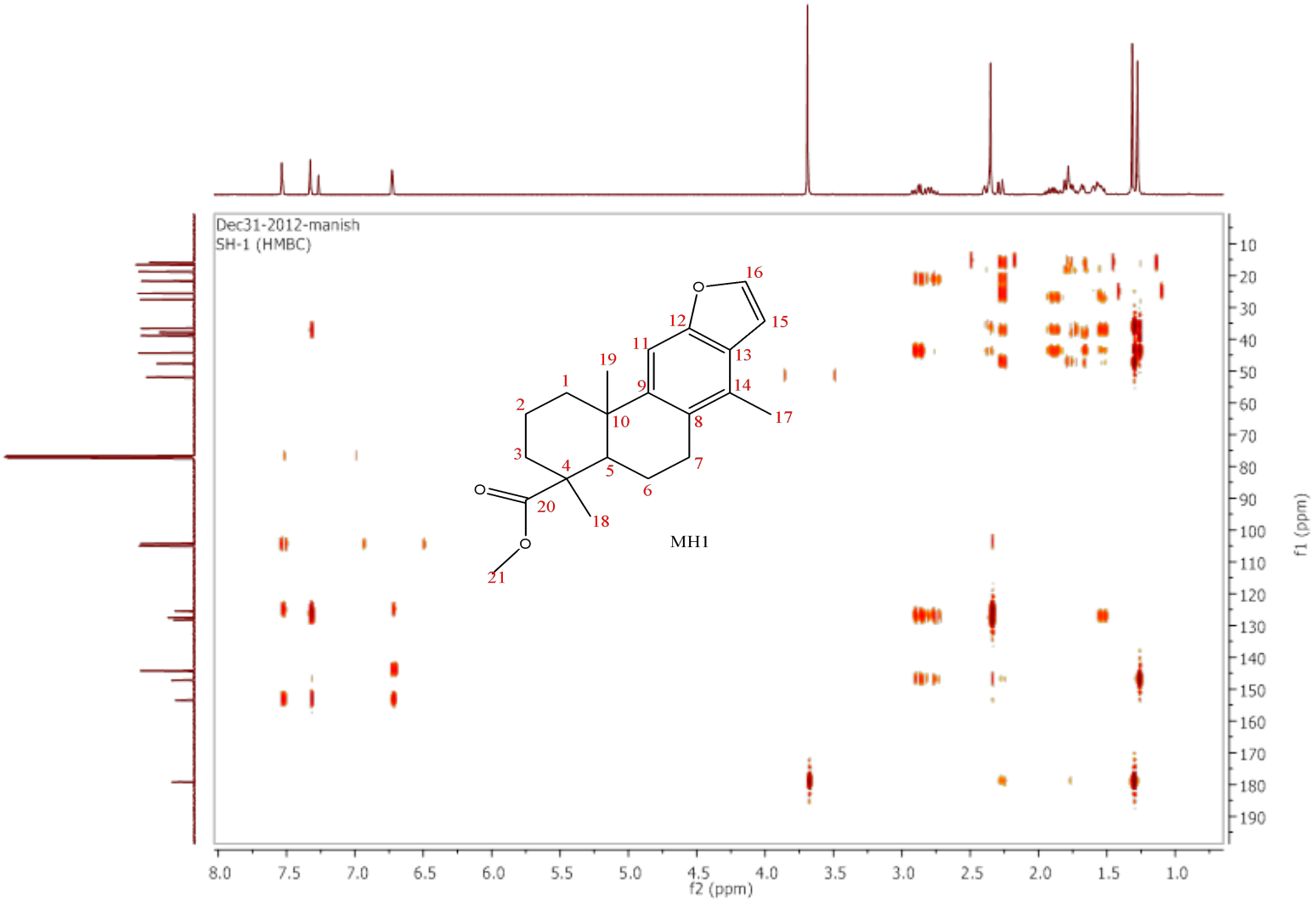


Figure 4.11:HMBC spectrum of compound MH1

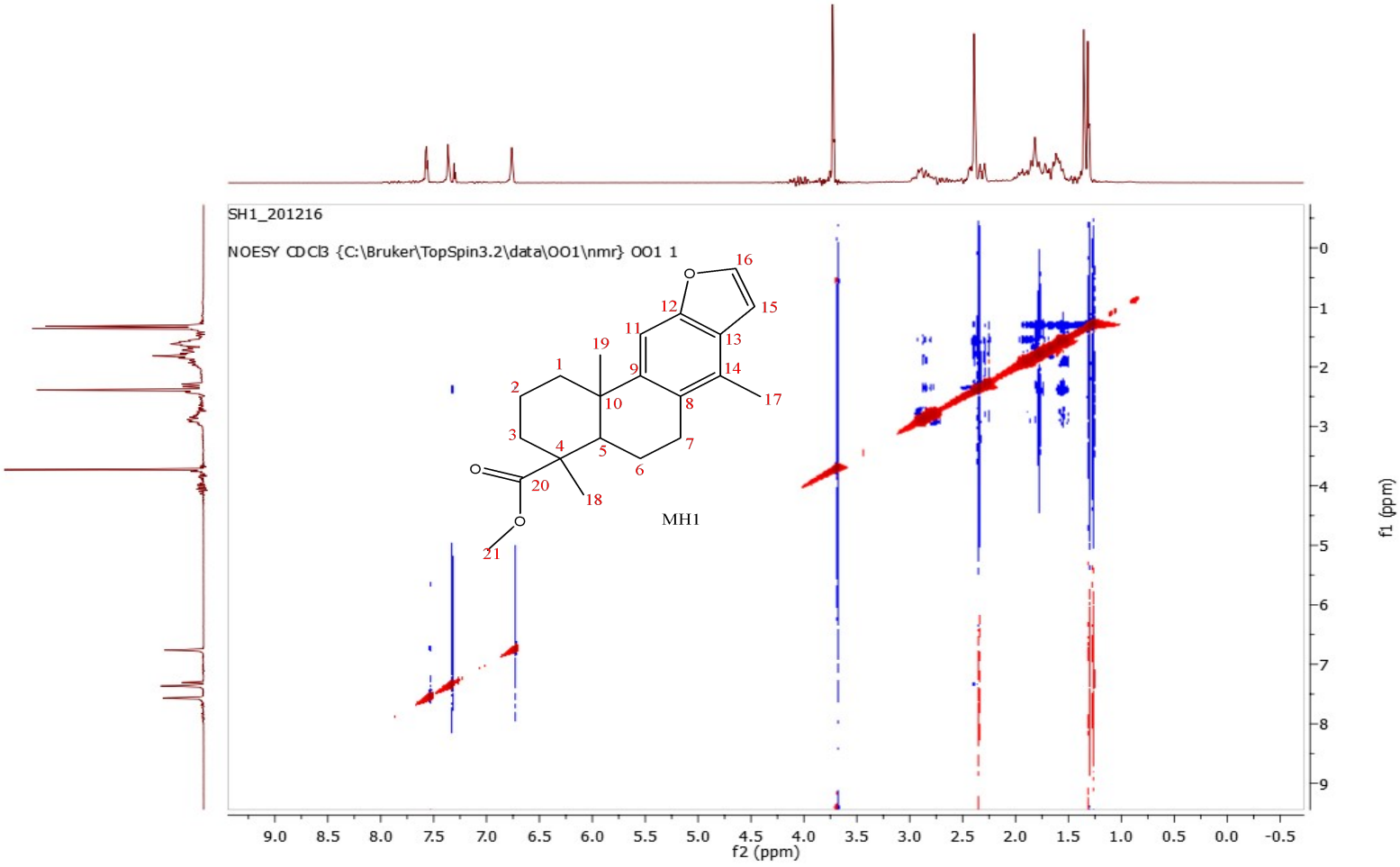


Figure 4.12: **NOESY spectrum of compound MH1**

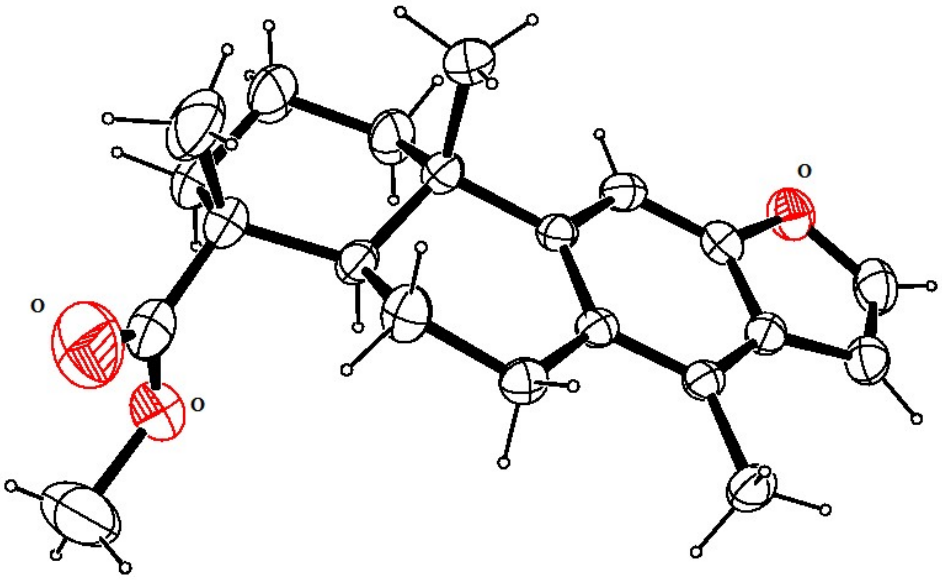


Figure 4.13: ORTEP (XRD) diagram of compound MH1

4.2.4 ISOLATION OF MH2

Sub-fraction F_3 (50 mg) eluted with ethyl acetate: hexane (5:95) gave a mixture of two compounds, this was further purified by column chromatography using with ethyl acetate: hexane (2:98), followed by preparative thin layer chromatography to afford compound **2** (20 mg) and labeled MH2.

4.2.5 CHARACTERISTICS OF COMPOUND 2 (MH2)

Appearance: White solid

Melting point: 155-156^{o}C

Optical rotation: $[\alpha]_D$ = -8.9 (c =0.00146, $CHCl_3$)

Infra-red (KBr, $V_{max,}$ cm^{-1}): 1726 (RCO_2R), 1712 (R_2CO), 1666 (C=C), 762 (furan)

Ultra-Violet (CH_2Cl_2): λ_{max} 257 nm

Molecular formula: $C_{20}H_{26}O_4$

Molecular Mass (HRESI-MS): 330.1834 g/mol, (calculated for, 330.1831 g/mol)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 37.9 (C-$_1$), 17.9 (C-$_2$), 36.7 (C-$_3$), 47.3 (C-$_4$), 49.0 (C-$_5$), 23.5 (C-$_6$), 26.8 (C-$_7$), 45.0 (C-$_8$), 52.9 (C-$_9$), 36.9 (C-$_{10}$), 22.8 (C-$_{11}$), 166.4(C-$_{12}$), 119.8 (C-$_{13}$), 195.9 (C-$_{14}$), 106.6 (C-$_{15}$), 142.8 (C-$_{16}$), 179.0 (C-$_{17}$), 16.8 (C-$_{18}$), 14.9 (C-$_{19}$), 52.1 (C-$_{20}$)

^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.13, m / 1.73, m (2H, H-$_{1\alpha/\beta}$), 1.61, m (2H, H-$_2$), 1.58, m (2H, H-$_3$), 1.78, d, *J* = 7.16 (1H, H-$_5$), 1.47, m / 1.25, m (2H, H-$_{6\alpha/\beta}$), 2.45, m /1.29, s (2H, H-$_{7\alpha/\beta}$), 2.31 (td, *J* = 12.07, 4.32 Hz, 1H, H-$_8$), 1.88, td, 12.0, 5.3 (1H, H-$_9$), 2.88, dd, *J* = 17.13, 5.26 Hz / 2.64, dd, *J* = 17.13, 11.93 Hz (2H, H-$_{11\alpha/\beta}$), 6.63, s (1H, H-$_{15}$), 7.30, s (1H, H-$_{16}$), 1.21, s (3H, H-$_{18}$), 1.00, s (3H, H-$_{19}$), 3.66, s (3H, H-$_{20}$)

4.2.6 STRUCTURAL ELUCIDAITON OF MH2

The structure of MH2 was determined through the analysis of its MS, IR and NMR data and by comparison with literature. The positive ESIMS and HRESIMS spectra of MH2 (Fig. 4.15 and 4.16) gave the $(M+H)^+$ ion at 331.10 and 331.1902 respectively, corresponding to molecular formula of $C_{20}H_{26}O_4$ (DBE=8).

The IR spectrum (Fig. 4.17) gave signals typical of ester, ketone and furan

functionalgroups at 1726, 1713 and 761 cm^{-1} respectively. The ^{1}H NMR spectrum (Fig. 4.18) of MH2displayed the resonances indicative of a 1, 2-disubstituted furan in the downfield positions at δ 7.30 (s) and 6.63 (s) which were similar to those of compound **MH1**. The ^{1}H and ^{13}C NMR spectra (Fig. 4.18 and 4.19) revealed that compound MH2 had the same A and B rings as compound MH**1** with the exception of ring C, which was non-aromatic in compound MH**2**. This was supported by the absence of the signal due to the aromatic proton at δ 7.33 (H-11), and the presence of methine signals at δ 1.88 (td, *J* = 5.3, 12.0, 12.03, H-9) and 2.31 (td, *J* = 4.32, 12.07, 12.02, H-8) and methylene signals at δ 2.64 (dd, *J*= 5.26, 17.13 Hz,H-11β) and 2.88 (dd, *J* =5.26, 17.13 Hz, H-11α). In addition, the ^{13}C NMR spectrum (Fig. 4.19) of Compound **MH2** showed a carbonyl signal at δ 195.9 ppm. The HSQC spectrum (Fig. 4.22) indicated that the methine protons at δ 1.88 and 2.31 and the methylene protons at (δ 2.64 and 2.88) were directly attached to carbons at δ 52.9 (C-9), 45.0 (C-8) and δ 22.8 (C-11) respectively. The HMBC (Fig. 4.23) also revealed that the protons at δ 2.31 (H-8), 1.88 (H-9) and 6.63 (H-15) showed long-range correlations with the carbonyl carbon at δ 195.9, thereby confirming the location of the carbonyl carbon at C-14. Similarly, themethyl protons at δ 1.21 (H_3-18) showed HMBC correlations to the ester carbonyl carbon at δ 179.0, the quaternary carbon at δ 47.3 (C-4), and a methylene carbon at δ 36.6 (C-3), thus establishing that it was attached to C-4. Furthermore, apart from the signal of the methoxy group, compound **MH2** only contained 19 carbons in the main carbon framework, suggesting it to be a norditerpene. The spectra data for compound MH**2** thus fitted that of methyl1,2,3,4,4a,5,6,6a,7,11,11a,11b-dodecahydro-4,11b-dimethyl-7-oxophenanthro-[3,2-b]-furan-4-carboxylate, which was previously reported as Nortaepeenin A (Fig. 4.14), isolated from *Caesalpinia crista* (Cheenpracha, 2005).

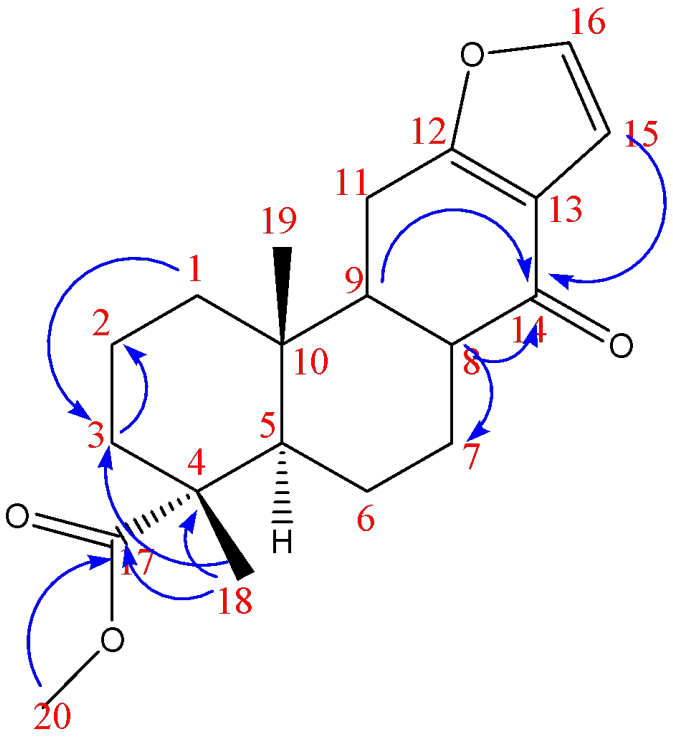


Figure 4.14a: HMBC correlation of compound MH2

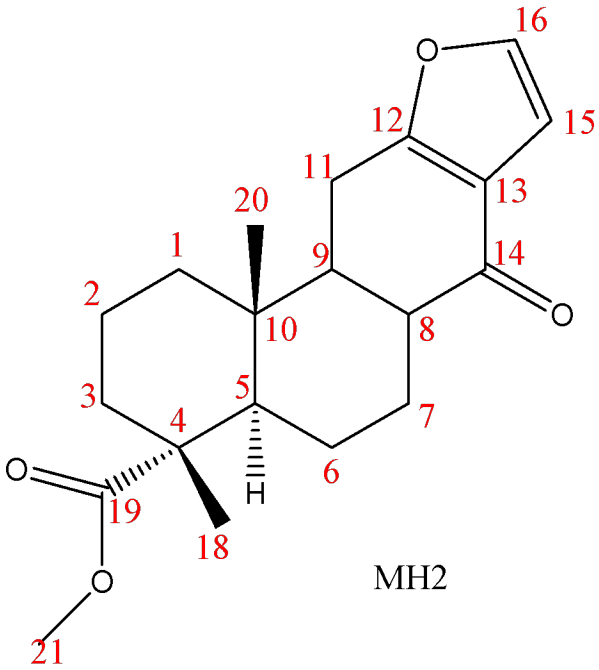


Figure 4.14b: Compound MH2 – Nortaepeenin A (Methyl-1,2,3,4,4a,5,6,6a,7,11, 11a,11b-dodecahydro-4,11b-dimethyl-7-oxophenanthro[3,2-b]furan-4-carboxylate)

Table 4.3: ^{13}C (100 MHz, $CDCl_3$) and ^{1}H NMR (400 MHz, $CDCl_3$) data of compound MH2 compared with literature

Assignment	δ ^{13}C ppm	δ ^{13}C ppm (Cheenpracha *et al*., 2005)	DEPT	δ ^{1}Hppm, multiplicity (J_{Hz})*	δ ^{1}Hppm, multiplicity (J_{Hz}) (Cheenpracha *et al*., 2005)	COSY	HMBC
1	37.9	37.9	CH_2	1.13 *m;*1.73 *m*	1.74 *m*, 1.14 *m*	H_2	C_3, C_4
2	17.9	17.9	CH_2	1.61 *m*	1.69 *m*	H_1	C_4, C_5
3	36.7	36.7	CH_2	1.58 *m*	1.62 *m*	-	C_2
4	47.3	47.3	C	-	-	-	-
5	49.0	49.0	CH	1.78 *d* (7.16)	1.78 *dd* (12.3, 2.4)	H_6	C_{17}
6	23.5	23.5	CH_2	1.47 *m*, 1.25	1.49 *m*, 1.29 *m*	H_5	C_7, C_8
7	26.8	26.8	CH_2	2.45 *m*, 1.29 *s*	2.46 *m*, 1.31 *m*		C_5, C_9, C_{14}
8	45.0	45.0	CH	2.31 *td;*(4.32, 12.07, 12.02)	2.31 *td* (12.0, 4.2)	H_7	C_7, C_9, C_{14}
9	52.9	52.9	CH	1.88 *td* (5.3, 12.0, 12.03)	1.88 *td* (12.0, 5.1)	H_8,H_{11}	C_{12}, C_{14}
10	36.9	36.9	C	-	-	-	-
11	22.8	22.8	CH_2	2.88 *dd* (5.26, 17.13) 2.64 *dd* (11.93, 17.13)	2.90 *dd* (17.1, 5.1), 2.66 *dd* (17.1, 12.0)	H_7, H_9	C_8, C_9, C_{12}
12	166.4	166.4	C	-		-	-
13	119.8	119.8	C	-		-	-
14	195.9	195.7	C	-		-	-
15	106.6	106.5	CH	6.63 *s*	6.63 *d* (1.8)	-	C_{12}, C_{14}, C_{16}
16	142.8	142.8	CH	7.30 *s*	7.30 *d* (1.8)	-	C_{12}, C_{13}, C_{15}
17	179.1	178.9	C	-		-	-
18	16.8	16.8	CH_3	1.21 *s*	1.21 *s*	-	C_3, C_5, C_{17}
19	14.9	14.8	CH_3	1.00 *s*	1.01 *s*	-	C_5, C_9, C_{10}
20	52.1	52.0	CH_3	3.66 *s*	3.65 *s*	-	C_{17}

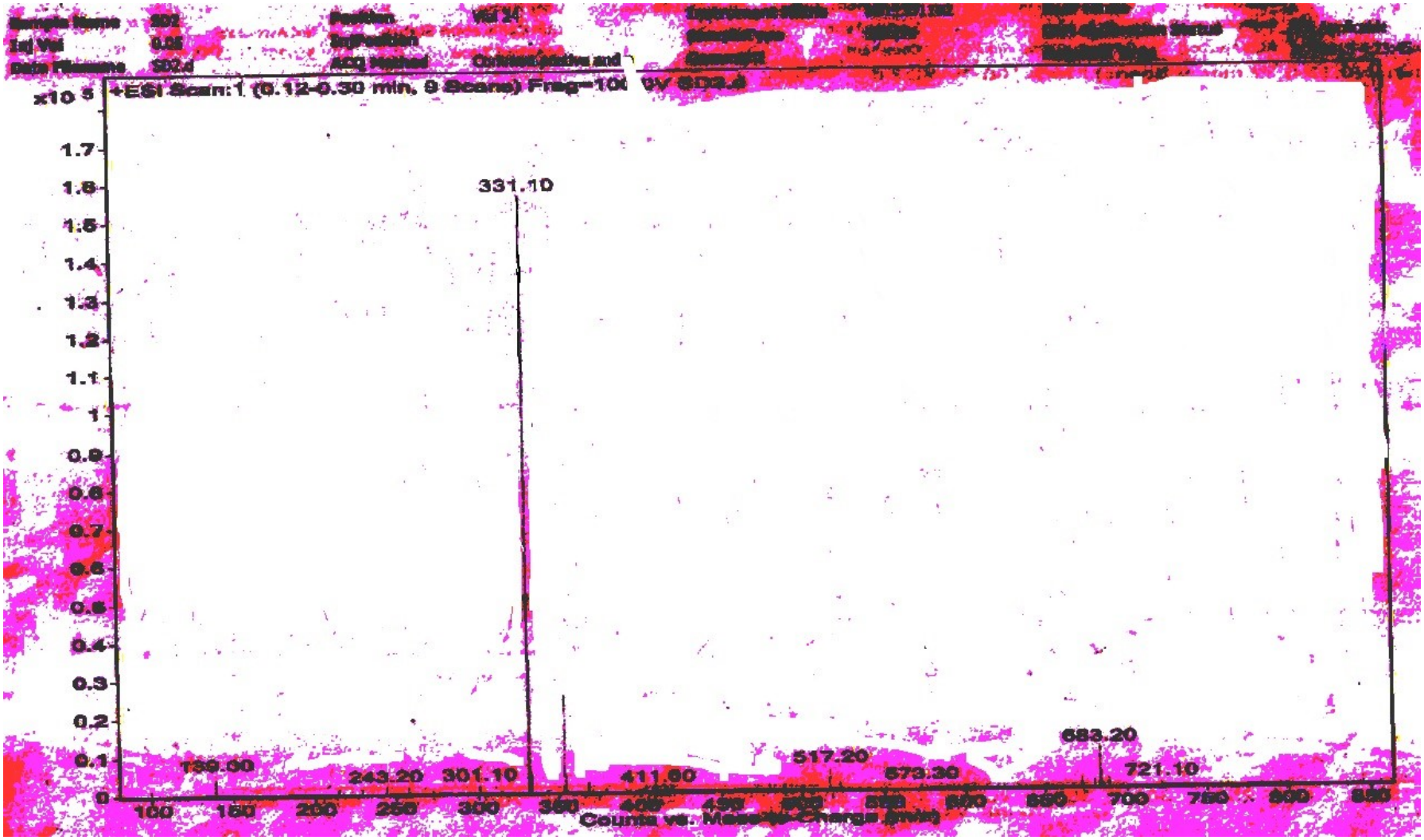
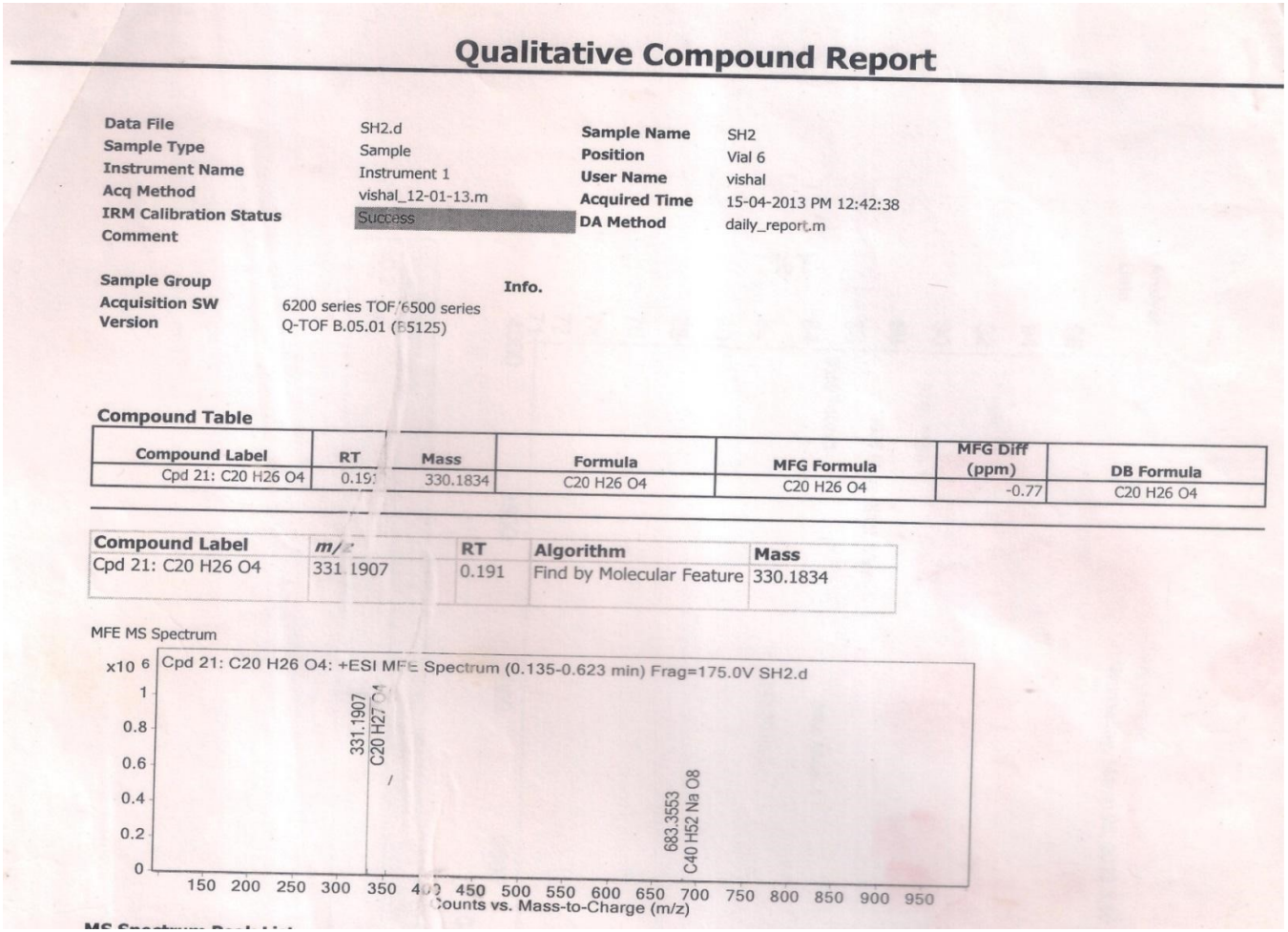


Figure 4.15: ESI Mass spectrum of compound MH2



Qualitative Compound Report

Data File	SH2.d	**Sample Name**	SH2
Sample Type	Sample	**Position**	Vial 6
Instrument Name	Instrument 1	**User Name**	vishal
Acq Method	vishal_12-01-13.m	**Acquired Time**	15-04-2013 PM 12:42:38
IRM Calibration Status	Success	**DA Method**	daily_report.m
Comment			

Sample Group Info.	
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 21: C20 H26 O4	0.191	330.1834	C20 H26 O4	C20 H26 O4	-0.77	C20 H26 O4

Compound Label	m/z	RT	Algorithm	Mass
Cpd 21: C20 H26 O4	331.1907	0.191	Find by Molecular Feature	330.1834

MFE MS Spectrum

Figure 4.16: HRESI Mass Spectrum of compound MH2

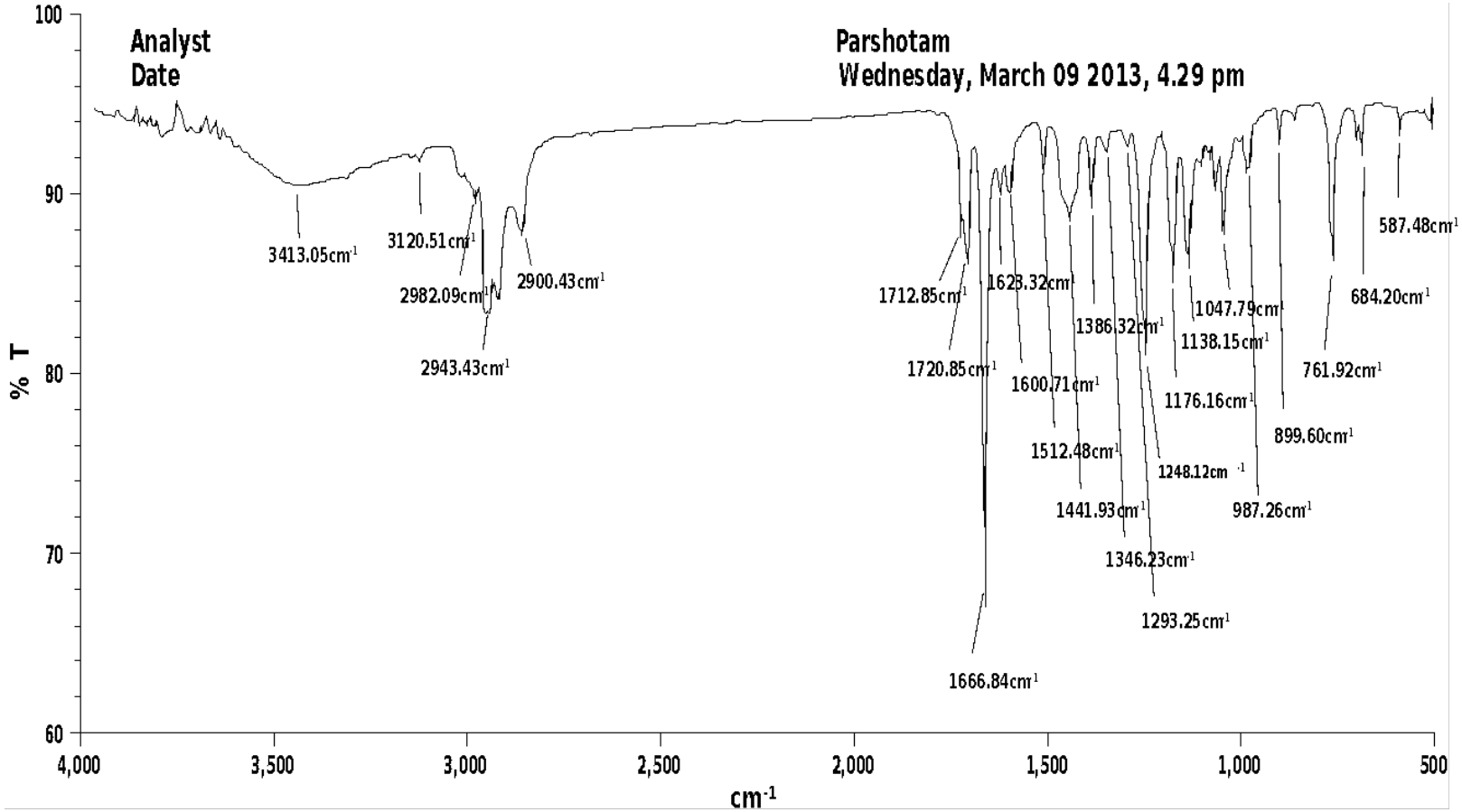


Figure 4:17: Infra-red spectrum of Compound MH2

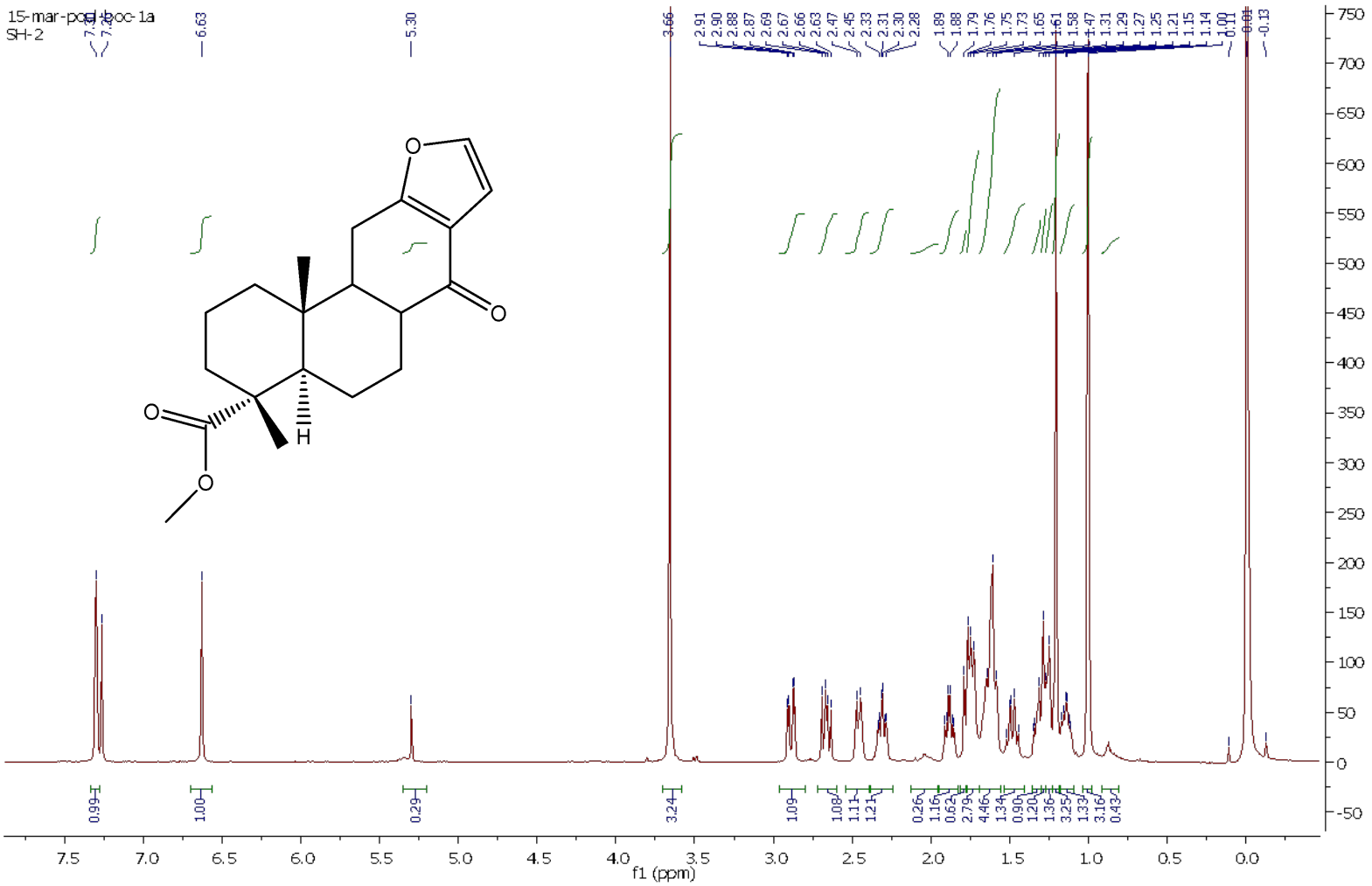


Figure 4.18: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound MH2

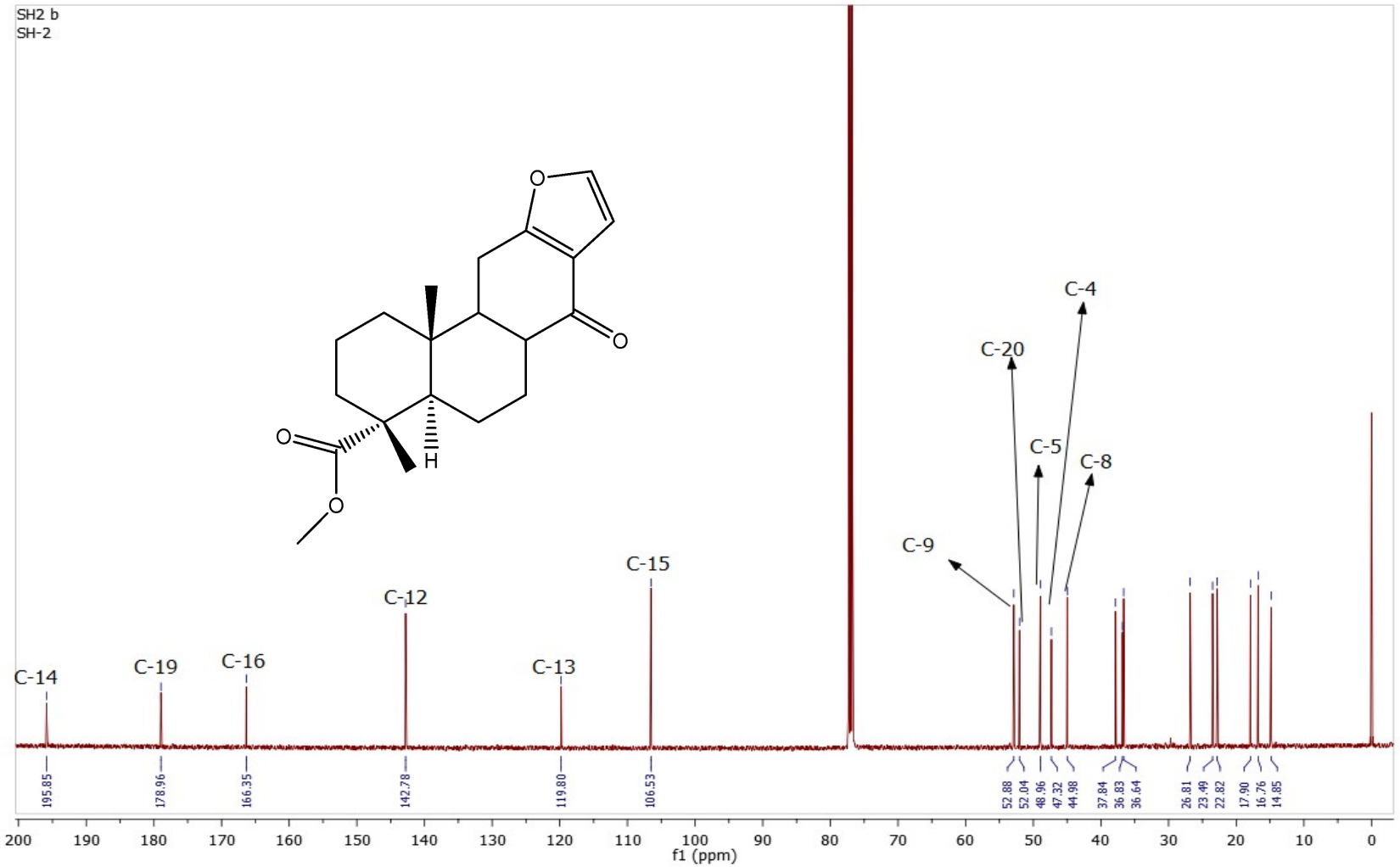


Figure 4.19: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound MH2

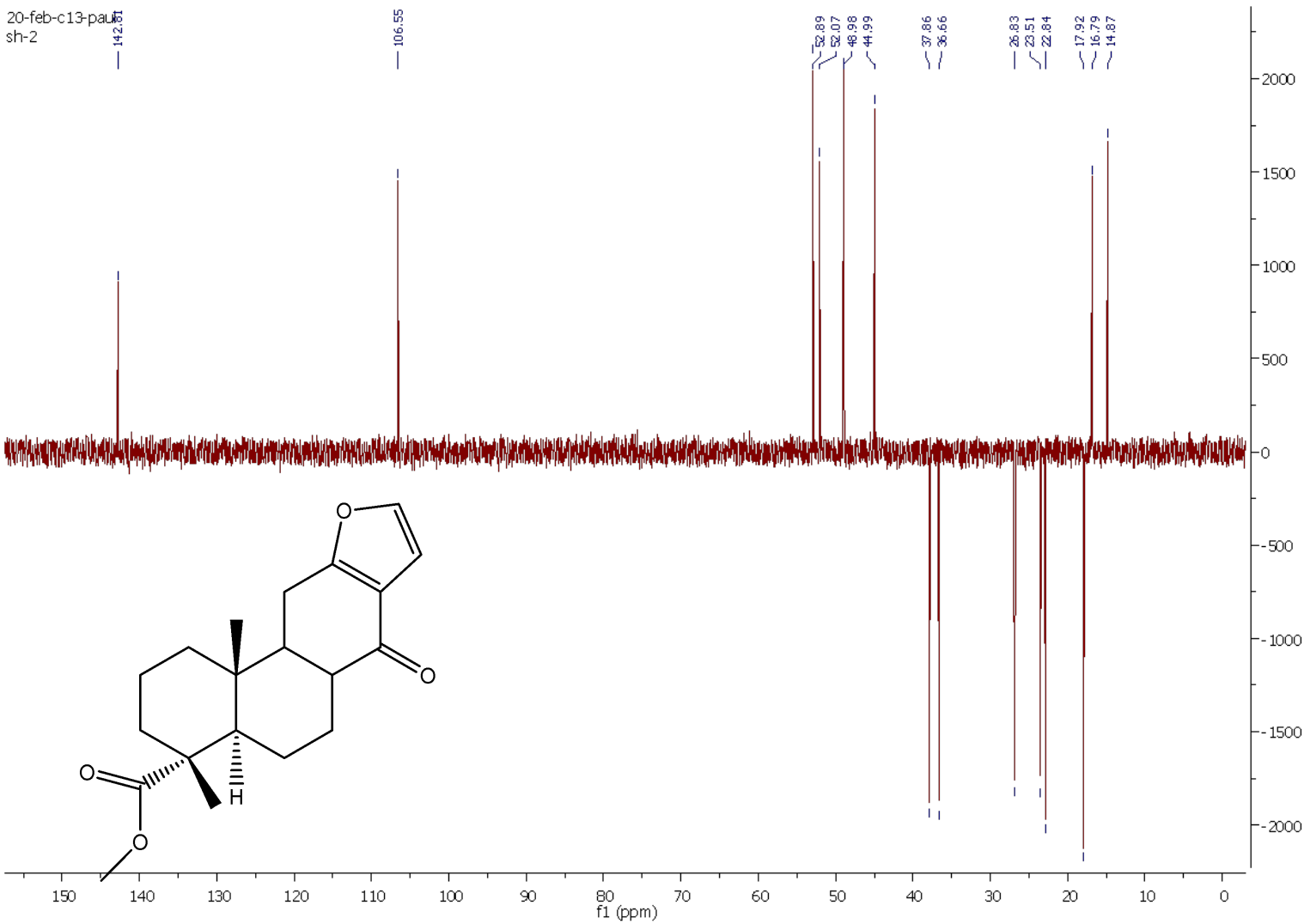


Figure 4.20: DEPT spectrum of compound MH2

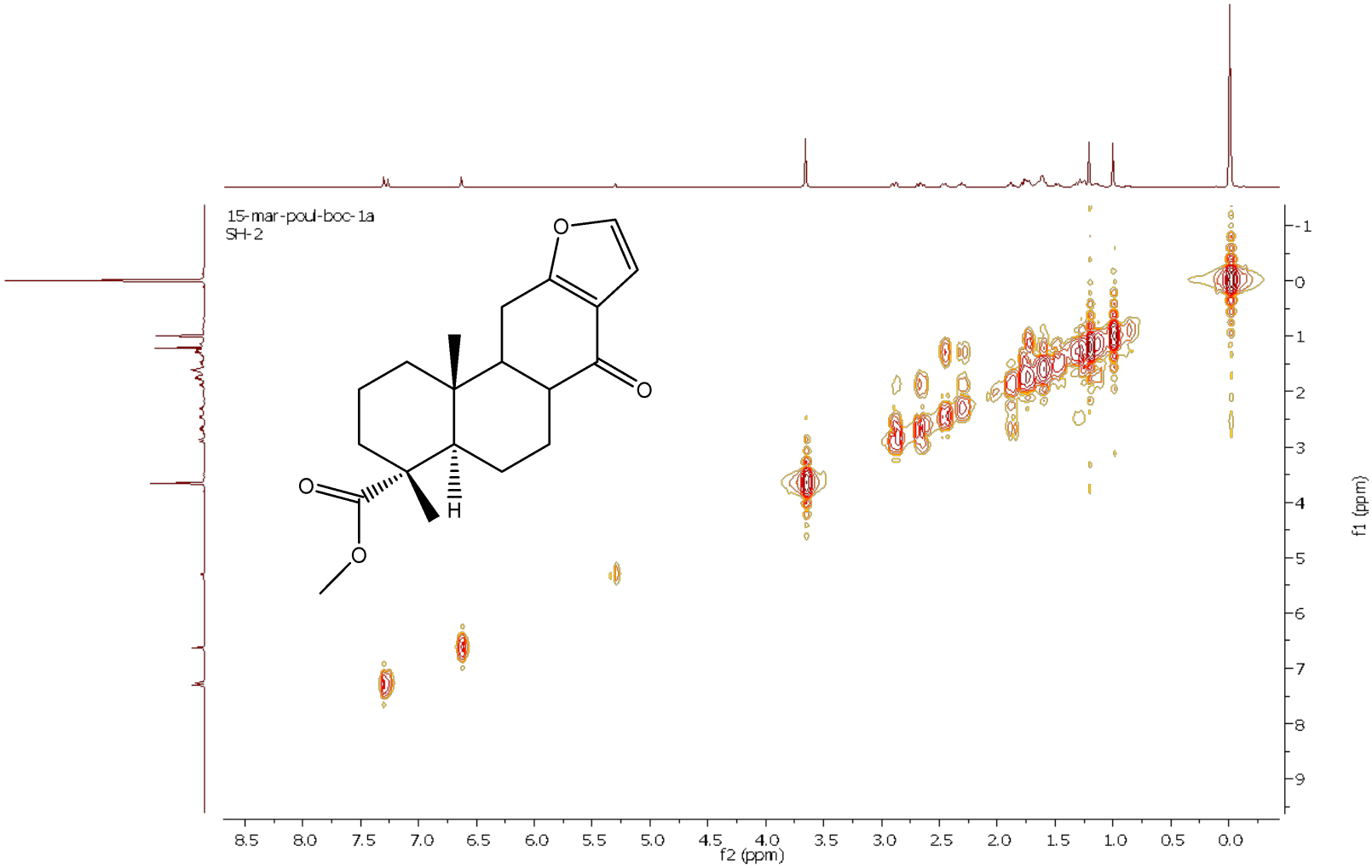


Figure 4.21: COSY spectrum of compound MH1

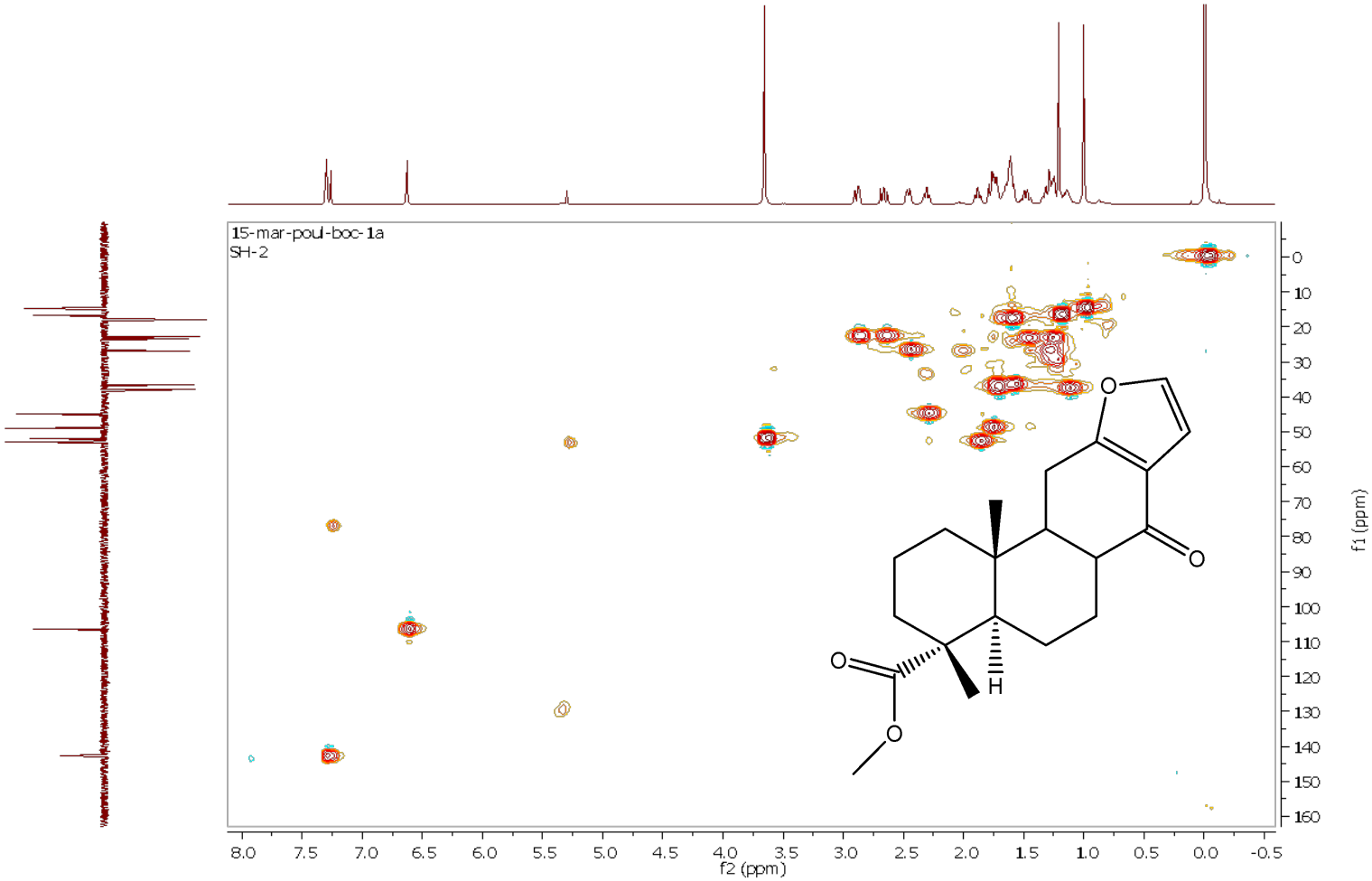


Figure 4.22: HSQC spectrum of compound MH2

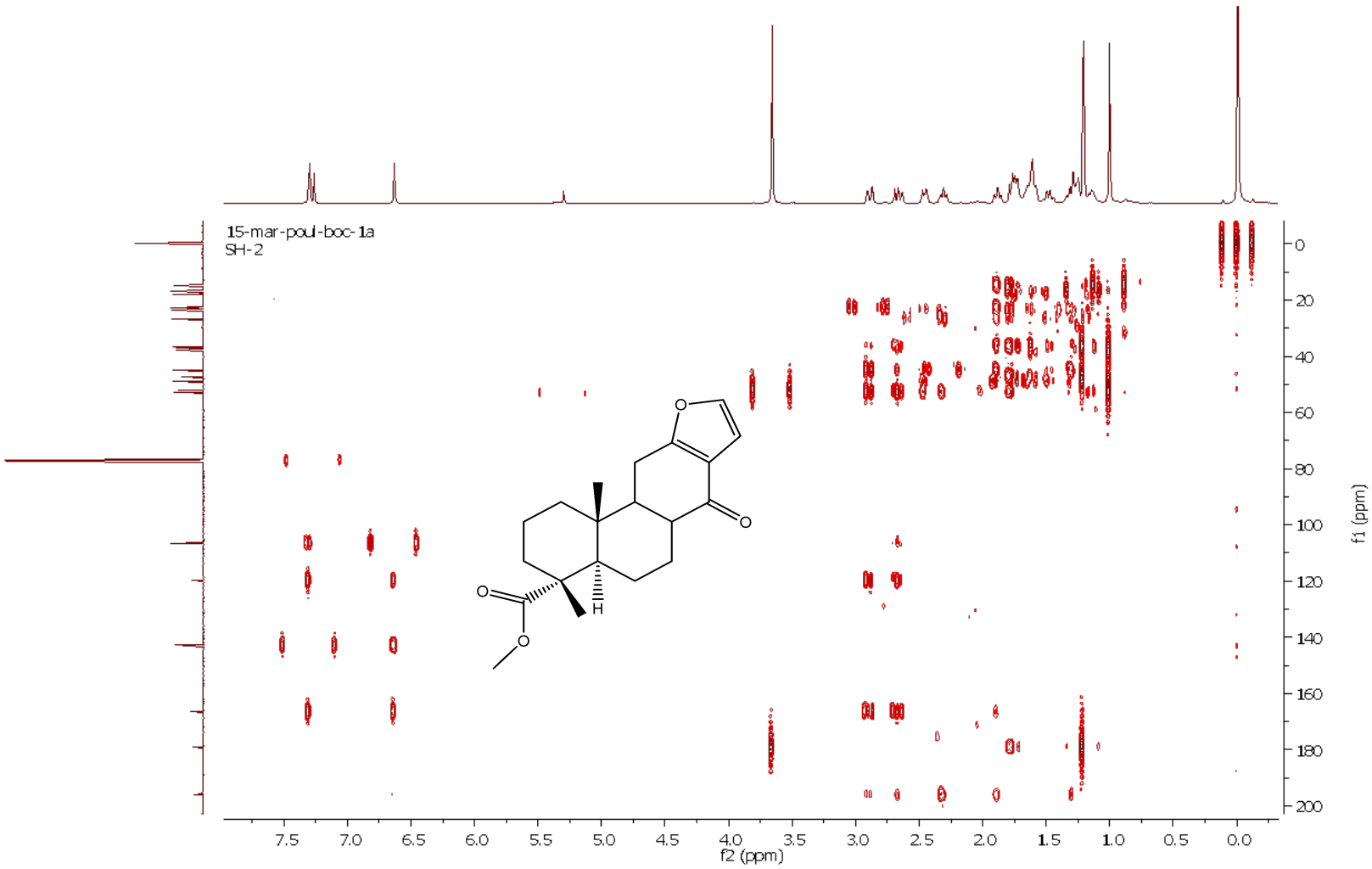


Figure 4.23: HMBC spectrum of compound MH2

4.2.7 ISOLATION OF MH3

Sub-fraction F_4 (40 mg) eluted with ethyl acetate: hexane (5:95) gave a white crystalline compound which was purified by recrystallization from hexane to afford compound**3** (30 mg) and labelled **MH3.**

4.2.8 CHARACTERISATION OF MH3

Appearance: White crystal

Infra-red (KBr, Vmax, cm^{-1}): 3435.71 (O-H), 2935.00, 2851.21 and 1619.75

Molecular formula: $C_{29}H_{48}O$

Molecular Mass: (HRESI-MS): 412.6908 g/mol (calculated for 412.3705 g/mol)

HREIMS Major peaks (% relative abundance): m/z 397.3826 (100 %) $[M\text{-}CH_3]^+$, 315.1605 (11.6) $[M\text{-}C_7H_{14}+H]^+$, 255.2101 (7.1) $[M\text{-side chain-}H_20]^+$

^{13}C NMR of Compound 3 ($CDCl_3$, 100 MHz): δ 37.26 C-1, 31.66 C-2, 71.82 C-3, 42.30 C-4, 140.76 C5, 121.75 C-6, 31.90 C-7, 33.94 C-8, 50.15 C-9, 36.51 C-10, 26.03 C-11, 39.77 C-12, 42.33 C-13, 56.87 C-14, 24.32 C-15, 29.13 C-16, 56.04 C-17, 12.00 C-18, 19.04 C-19, 40.54 C-20, 21.12 C-21, 138.33 C-22, 129.26 C-23, 51.25 C-24, 45.91C-25, 19.42 C-26, 19.85 C-27, 24.32 C-28, 12.28 C-29.

Selected ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.35 (1H, d, *J* = 5.0 Hz, H-6), 5.15 (1H,dd, *J* = 15.2, 8.6 Hz, H-22), 5.01 (1H, dd, *J* = 15.3, 8.7 Hz, H-23), 3.51 (1H, dd, *J* = 13.3, 8.4 Hz, H-3, 0.67 (1H, s, H-18), 1.01 (1H, s, H-19), 0.92 (3H, d, 6.5 Hz, H-21), 0.82 (3H, d, 1.68 Hz, H-26), 0.80 (3H, d, 7.0 Hz, H-27), 0.85 (3H, m, H-29).

4.2.9 STRUCTURAL ELUCIDAITON OF MH3

The structure of MH3 was determined through the use of different spectroscopic methods including Infra-red, HRESI-MS, ^{1}H NMR and ^{13}C NMR data and by comparison with literature. The IR spectrum of MH3 exhibit characteristic

absorptionbands at 3435.72 cm^{-1} indicating O-H stretching, 2935.00 $cm^{-1}$and 2851 cm^{-1} due to aliphatic C-H stretching. In addition to another band at 1691 cm^{-1} which is due to C=C stretching. The positive HR-ESI Mass spectrum of MH3 gave the characteristic peak at 397.3826 indicating [M-H_2O + H] ion and suggested the molecular formula as $C_{29}H_{48}O$. The ^{1}H-NMR spectrum of MH3 revealed two one proton signals at δ 3.51 and δ 5.36 typical for H3 and H6 of a steroidal nucleus. It also gave two characteristic downfield olefinic protons with signals at δ 5.15 (1H, dd, *J* = 15.2, 8.6 Hz) and 5.01 (1H, dd, *J* = 15.3, 8.7 Hz) which were identical with the chemical shift of H22 and H23 respectively of stigmasterol (Ahmed *et al.,* 2013). The spectrum further revealed two singlets signals at δ 0.67 and δ 1.01 which are assignable to two tertiary methyl groups at C-13 and C-10 respectively. This is in addition to two doublets at δ 0.82 (3H, d, 1.68 Hz) and 0.80 (3H, d, 7.0 Hz), which could be ascribed to the two methyl groups at H26 and H27 and another three-proton doublet at δ 0.92 (3H, d, 6.5 Hz) for H21, including one three-proton triplet at δ 0.85 (3H, m) which could be assigned to the primary methyl group of H29 (Ahmed *et al.,* 2013). The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon at δ 71·85, and two olefinic carbons at δ 138.33 and 129.0. Comparing the ^{13}C and the DEPT spectrum further reveals the presence of six methyl (CH_3) groups, nine methylene (CH_2), eleven methine (CH) and three quaternary carbons atoms. With the aid of these data and by comparing with data obtained in literature, the compound was therefore identified as stigmasterol. (Ahmed *et al.,* 2013; Jain and Bari, 2010; Kamboj and Saluja, 2011).

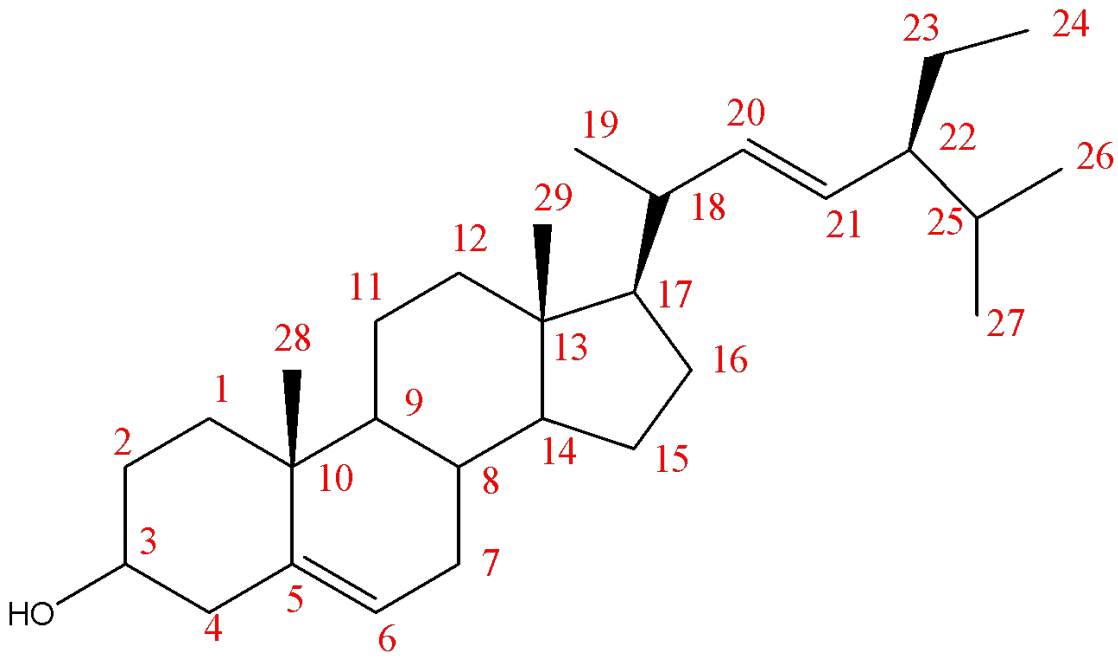


Figure 4.24 Compound MH3 – Stigmasterol

Table 4.4: ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) data of compound MH3

Carbon No	DEPT	δ ^{13}C NMR ppm		δ ^{1}H NMR ppm, multiplicity (J_{Hz})	
		MH3	Stigmasterol (Ahmed *et al*, 2013)	MH3	Stigmasterol (Ahmed *et al*, 2013)
1	CH_2	37.26	37.29		
2	CH_2	31.66	28.28		
3	CH	71.82	71.85	3.51 (1H, dd, *J* = 13.3, 8.4 Hz)	3.52 (1H, dd, *J* = 9.6, 4.8 Hz,),
4	CH_2	42.30	42.36		
5	C	140.76	140.81		
6	CH	121.75	121.74	5.35 (1H, d, *J* = 5.0 Hz)	5.34 (1H, d, J = 5.2 Hz)
7	CH_2	31.90	31.72		
8	CH	33.94	34.01		
9	CH	50.15	50.2		
10	C	36.51	36.54		
11	CH_2	26.03	26.17		
12	CH_2	39.77	39.83		
13	C	43.33	42.37		
14	C	56.87	56.82		
15	CH_2	24.32	24.33		
16	CH_2	29.13	29.23		
17	CH	56.04	56.12		
18	CH_3	12.00	12.02	0.67 (1H, s)	0.67 (1H, s)
19	CH_3	19.04	19.41	1.01 (1H, s)	1.00 (1H, s)
20	CH	40.54	40.50		
21	CH_3	21.12	21.13	0.92 (3H, d, 6.5 Hz)	0.92 (3H, d, *J* = 6.0 Hz)
22	CH	138.35	138.33	5.15 (1H , dd, *J* = 15.2, 8.6 Hz)	5.16 (1H, dd, *J* = 15.0, 8.4 Hz
23	CH	129.26	129.0	5.01 (1H, dd, *J* = 15.3, 8.7 Hz)	5.03 (1H, dd, J 15.0, 8.4 Hz)
24	CH	51.25	51.28		
25	CH	45.82	45.91		
26	CH_3	19.42	19.42	0.82 (3H, d, 1.68 Hz)	0.81 (3H, d, *J* = 7.2 Hz)
27	CH_3	19.85	19.84	0.80 (3H, d, 7.0 Hz)	0.79 (3H, d, *J* = 7.2 Hz)
28	CH_2	24.32	24.34		
29	CH_3	12.28	12.26	0.85 (3H, m)	0.85 (3H, d, *J* = 8.0 Hz)

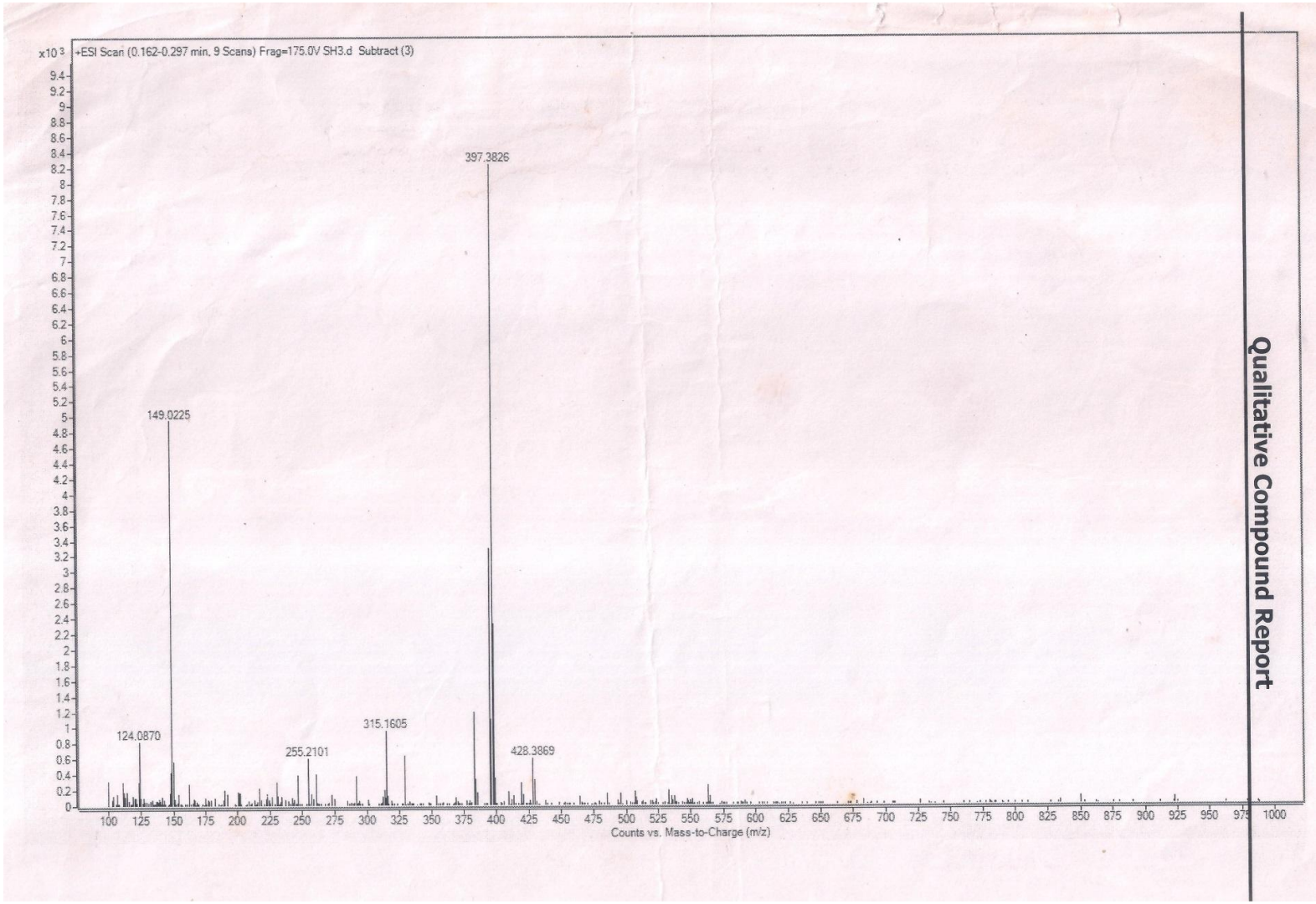


Figure 4.25: HRESI Mass Spectrum of compound MH3

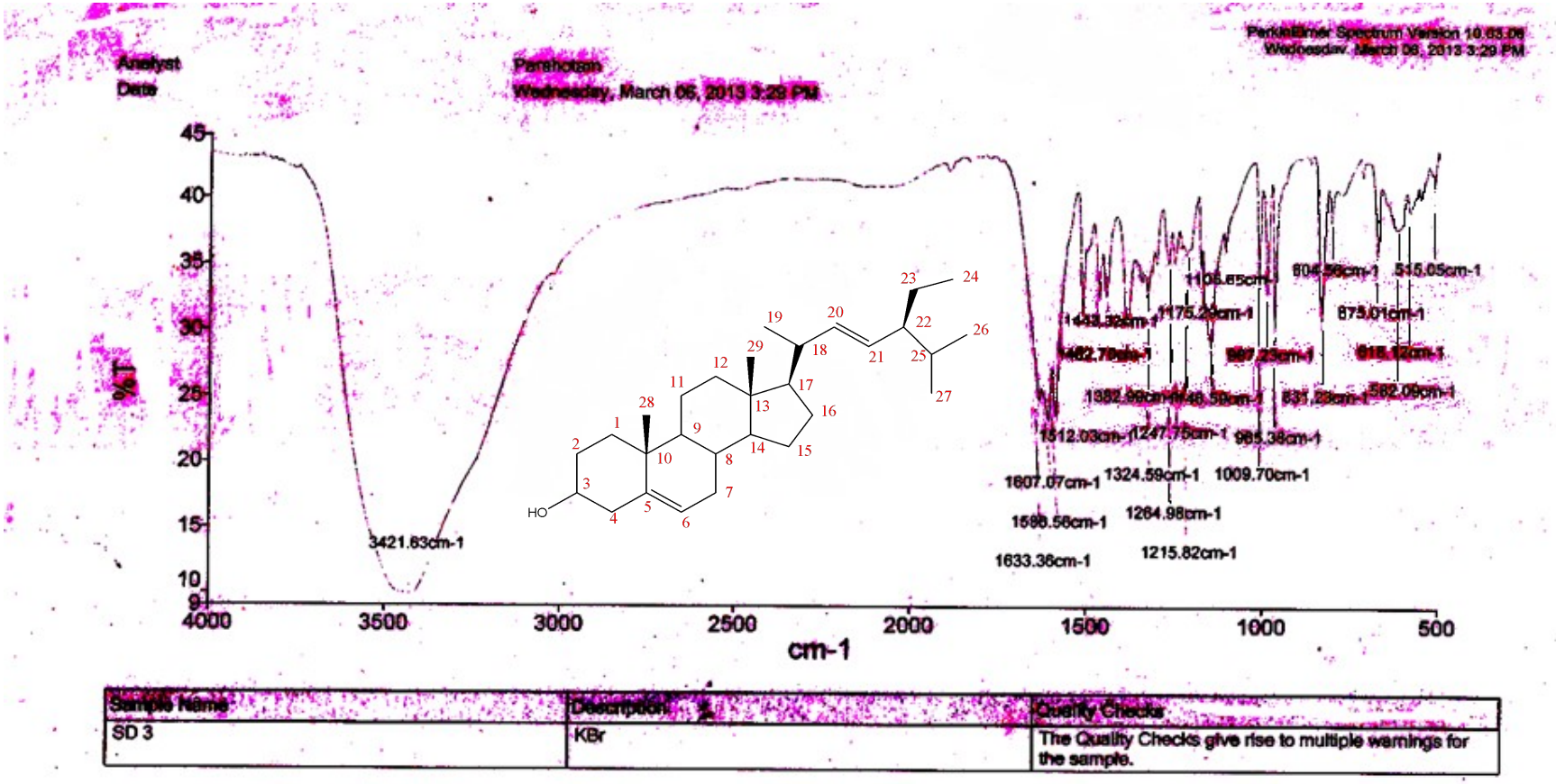


Figure 4.26: Infra-red spectrum of compound MH3

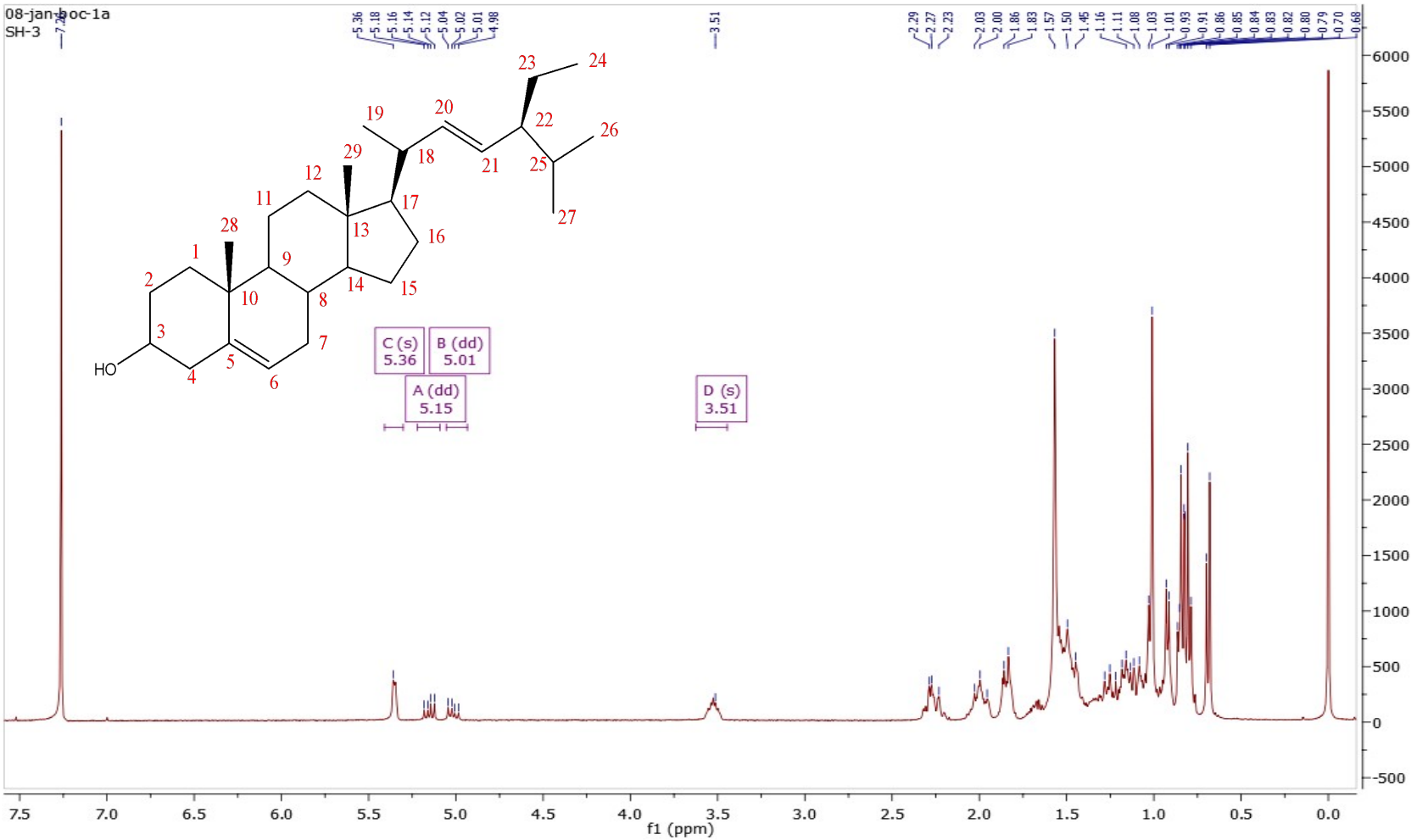


Figure 4.27: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound MH3

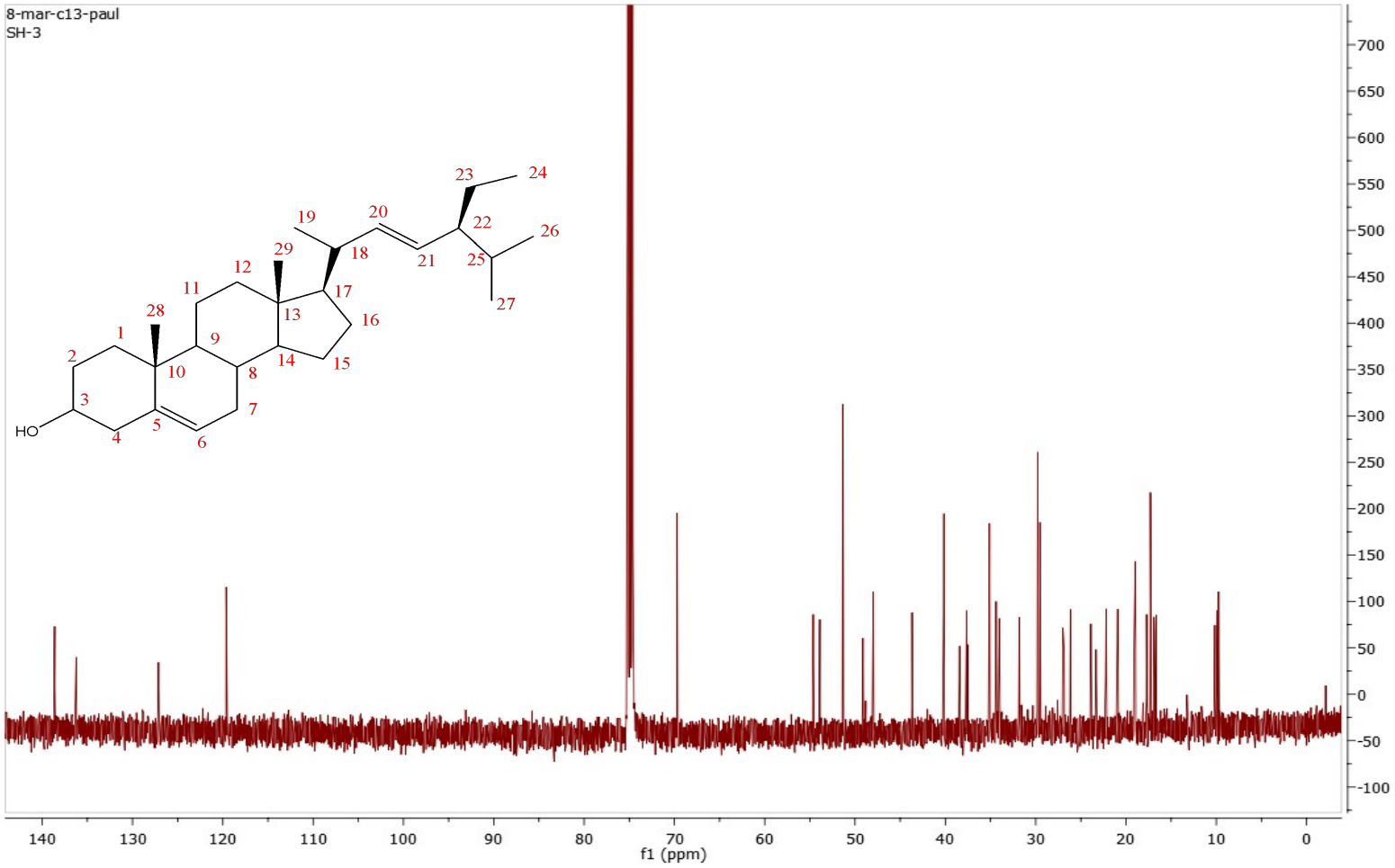


Figure 4.28: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound MH3

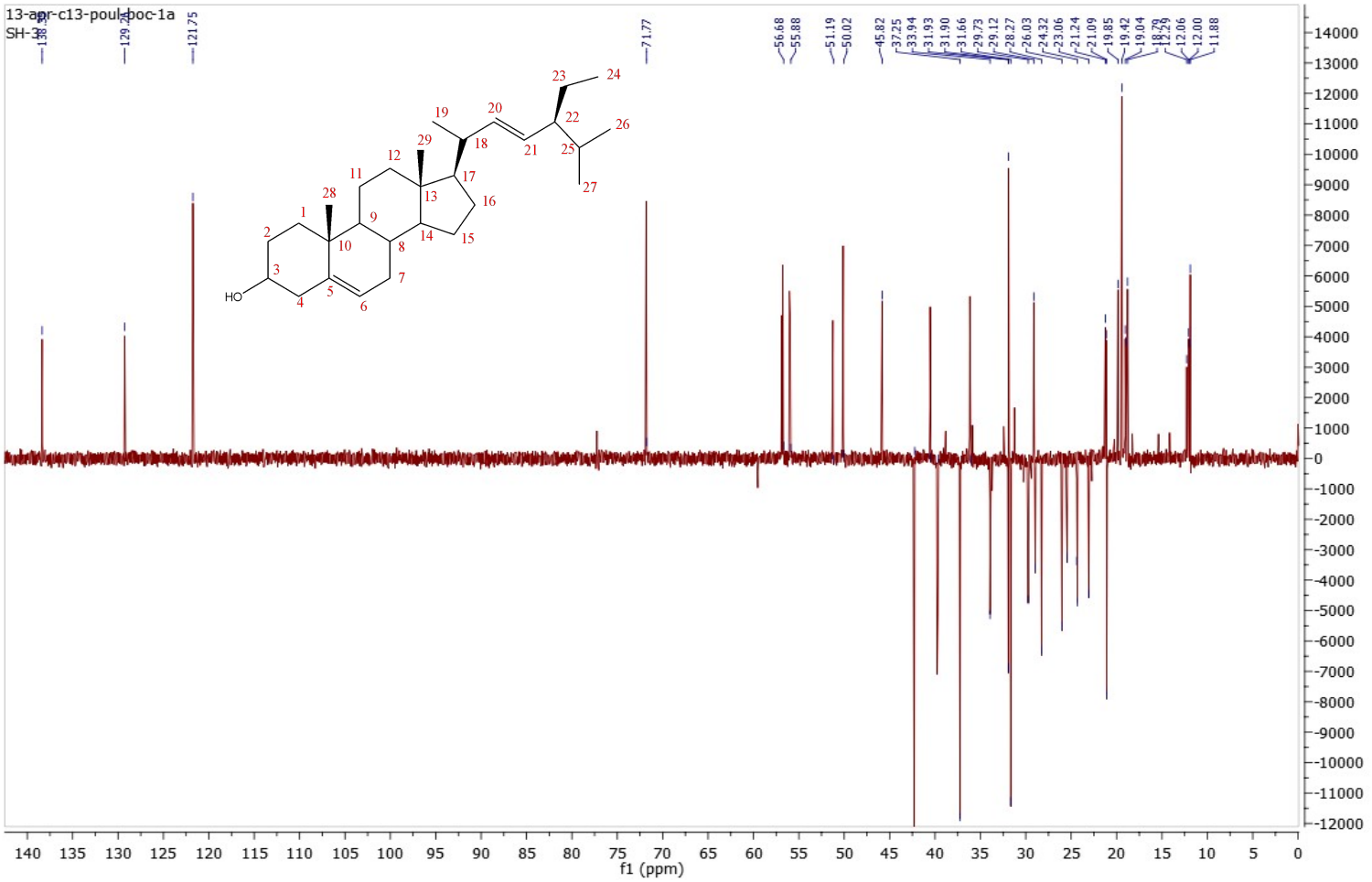


Figure 4.29: DEPT spectrum of compound MH3

4.3 FRACTIONATION OF ETHYL ACETATE EXTRACT OF *M. benthamianum*

The ethyl acetate extract (40 g) was subjected to column chromatography using silica gel (200 – 400 mesh size) and eluted with hexane in increasing proportions of ethyl acetate and subsequently, ethyl acetate in increasing proportion of methanol to give fifty fractions. Similar fractions were combined on the basis of TLC analysis to give six sub-fractions (Fig. 4.30). Purification of sub-fractions F_1, F_3 and F_4 gave three pure compounds ME1, ME2 and ME3. These three compounds were subjected to different spectroscopic techniques to fully characterize them.

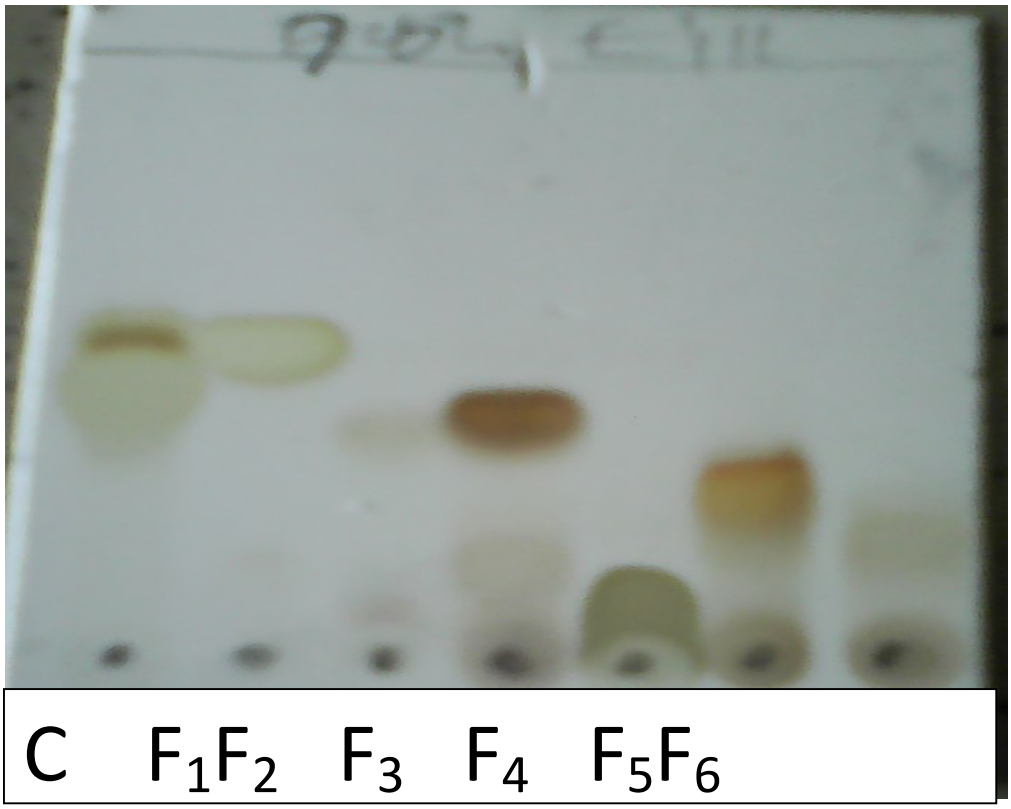


Figure 4.30: TLC Profile of the Sub-fractions F_1 to F_6 in 70 % EtOAc/Hex

4.3.1 ISOLATION OF ME1

Fractions 17 – 22 eluted with ethyl acetate: hexane (40:60) were pooled together on the basis of TLC analysis to give sub-fractions F_2 (1.5 g). This was purified by column chromatography using with ethyl acetate: hexane (40:60) to afford compound **5** (1.0 g) and labeled ME1.

4.3.2 CHARACTERISATION OF ME1

Appearance: creamy powder

Melting point: 260 – 262 ^{o}C

Infra-red (KBr, Vmax, cm^{-1}): 3421(OH stretch), 1633 (aromatic C=C)

UV-Visible spectra (nm): 248, 257, 322, 330

Molecular formula: $C_{14}H_{12}O_3$

Molecular Mass: (HRESI-MS): 228.2433 (calculated for 228.0786 g/mol)

EIMS Major peaks (% relative abundance): m/z 229.00 **(**100)$[MH]^+$

^{13}C NMR (MeOD, 125 MHz): δ 159.57 C-3, 5, 158.27 C-4', 141.32 C-1, 130.39 C-1', 129.40 C-8, 128.83 C-2', 6', 126.95 C-7, 116.48 C-3', 5', 105.80 C-2, 6, 102.62 C-4.

^{1}H NMR (MeOD, 500 MHz): δ 7.35 (d, J = 8.6 Hz, 2H, H-3', 5'), 6.97 (d, J = 16.3 Hz, 1H, H-8), 6.81 (d, J = 16.4 Hz, 1H, H-7), 6.78 (d, J = 8.6 Hz, 2H, 2', 6'), 6.48 (d, J = 2.1 Hz, 2H, H-2, 6), 6.19 (t, J = 2.1 Hz, 1H, H-4).

4.3.3 STRUCTURAL ELUCIDATION OF ME1

The structure of ME1 was determined through the analysis of its Infra-red, HREIMS and NMR data and by comparison with literature. The Infrared spectrum (fig. 4.32) displayed a broad spectrum at 3421.63, 1607 and 1633.36 cm^{-1} indicating vibrational frequencies due to hydrogen bonded O-H and C=C bonds respectively. The HR-EIMS spectra of ME1 (fig. 4.33) gave the MH^+ ion at *m/z* of 229.0859, suggesting the

formula of $C_{14}H_{12}O_3$ (DBE=9). The number of unsaturation could be accounted for as two benzene rings and one alkene group. The ^{1}H NMR spectrum (Fig. 4.34) displayed two doublet signals (δ 7.35, d, *J* = 8.56 Hz, H-2', H-6' and 6.78, d, *J* = 8.6 Hz, H-3', H-5') which are indicative of two aromatic ortho-coupled protons. It also showed another set of two doublets peaks (δ 6.97, d, *J* = 16.3 Hz, H-8 and 6.81, d, *J* = 16.44 Hz, H-7) which are indicative of coupled trans-olefinic protons. Furthermore, the presence of doublet (δ 6.48, d, *J* = 2.1 Hz, H-2) and triplet peaks (δ 6.19, t, *J* = 2.1 Hz, H-4) suggests meta-coupled aromatic protons. The ^{13}C NMR spectrum (Fig. 4.35) showed the presence of seven aromatic methine carbons (δ 102.62 C-4, 105.80 C-2 and C-6, 116.48 C-3' and C-5', 128.83 C-2' and C-6'), two olefinic methine carbons (δ 126.95 and 129.40) and five aromatic quaternary carbons (δ 130.39 C-1', 141.32 C-1, 158.27 C-4', 159.57 , C-3 and C-5). This accounted for the fourteen carbons atoms. The one-bond proton-carbon correlation observed in HSQC spectrum (Fig. 4.39) facilitated the assigning of the protons to their carbons with the assistance of the long range correlations observed in HMBC (fig. 4.40) and also compared with data obtained from literature (Mattivi *et al*, 1995). Thus ME1 is assigned as *trans*-resveratrol (3,4',5-trihydroxlstillbene). Resveratrol exists in two isomeric forms: *cis*-resveratrol and *trans*-resveratrol. The *trans* form is more stable and potent than the *cis* form (Aluyen *et al*., 2012)

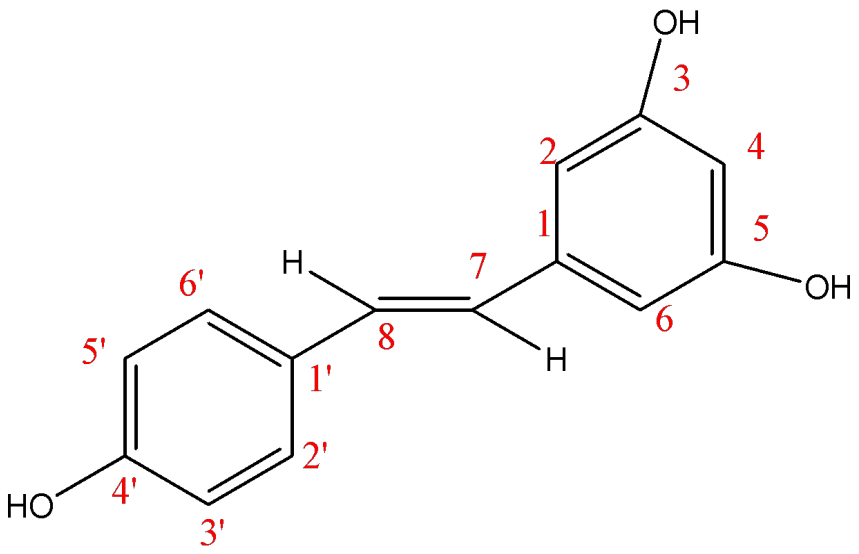


Figure 4.31: Compound ME1 – trans-resveratrol (3,4',5 – trihydroxylstillbene)

Table 4.5: ^{13}C (100 MHz, MeOD) and ^{1}H NMR (400 MHz, MeOD) data of compound ME1

	^{13}C NMR spectra		^{1}H NMR spectra	
Carbon No	ME1	Resveratrol Mattivi *et al*, 1995	ME1	Resveratrol Mattivi *et al*, 1995
1	141.3	140.9	-	-
2	105.8	105.7	6.48 d (J = 2.09 Hz, 2H)	6.54 d (J =2.1, 2H)
3	159.6	159.6	-	-
4	102.6	102.7	6.19 t (J = 2.09, 2.09 Hz, 1H)	6.28 t (J =2.1, 2.1, 1H)
5	159.6	159.6	-	-
6	105.8	105.7	6.48 d (J = 2.09 Hz, 2H)	6.54 d (J =2.1, 2H)
7	127.0	126.9	6.81 d (J = 16.44, 1H)	6.89 d (J =16.30, 1H)
8	129.4	129.1	6.97 d (J =16.28, 1H)	7.02 d (J =16.30, 1H)
1’	130.4	130.0	-	-
2’	128.8	128.8	7.35 d (J =8.56, 2H)	7.41 d (J =8.7, 2H)
3’	116.5	116.4	6.78 d (J =8.59, 2H)	6.83 d (J =8.7, 2H)
4’	158.3	158.2	-	-
5’	116.5	116.4	6.78 d (J = 8.59, 2H)	6.83 (J =8.7, 2H)
6’	128.8	128.8	7.35 d (J = 8.56, 2H)	7.41 d (J =8.7, 2H)

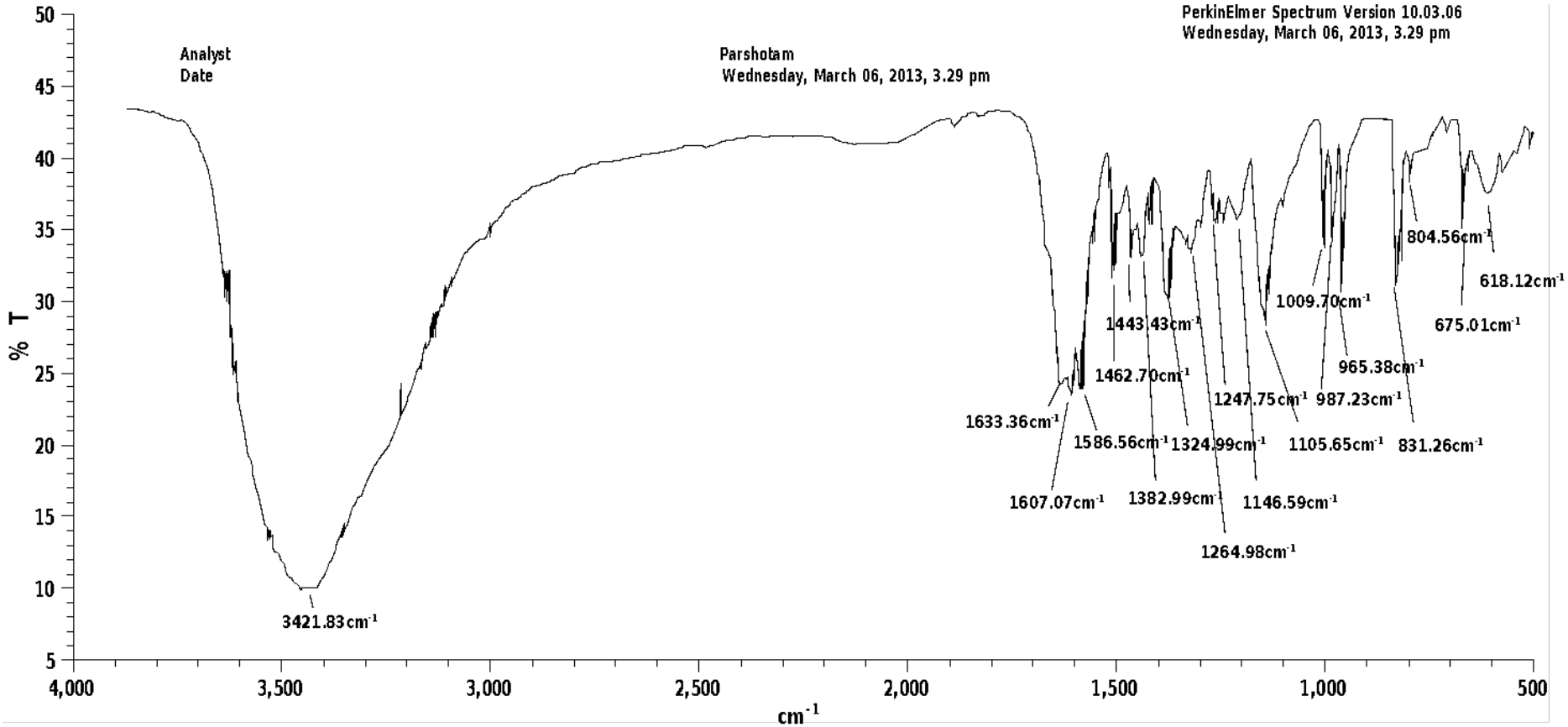


Figure 4.32: Infra-red spectrum of compound ME1

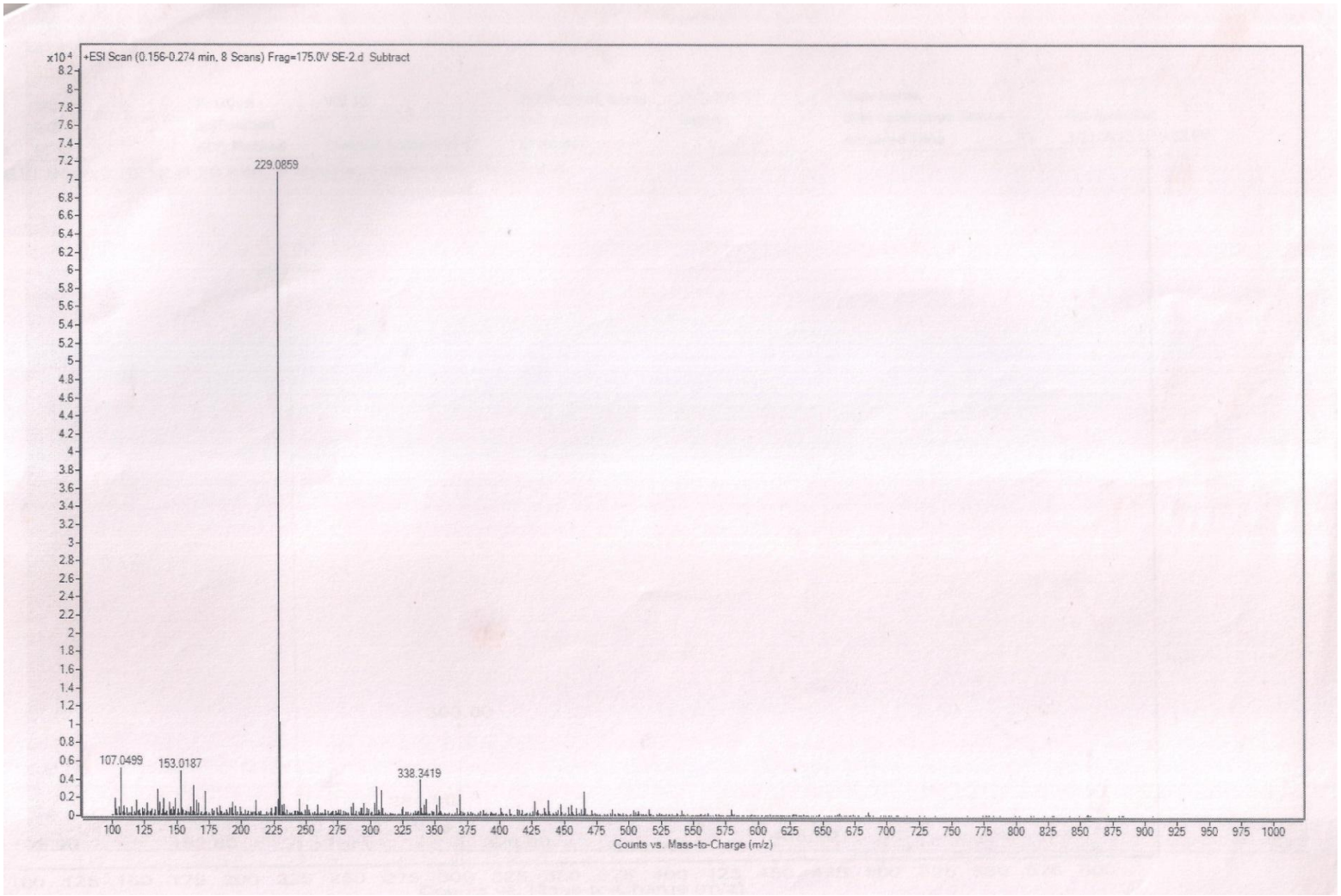


Figure 4.33: HRESI Mass spectrum of compound ME1

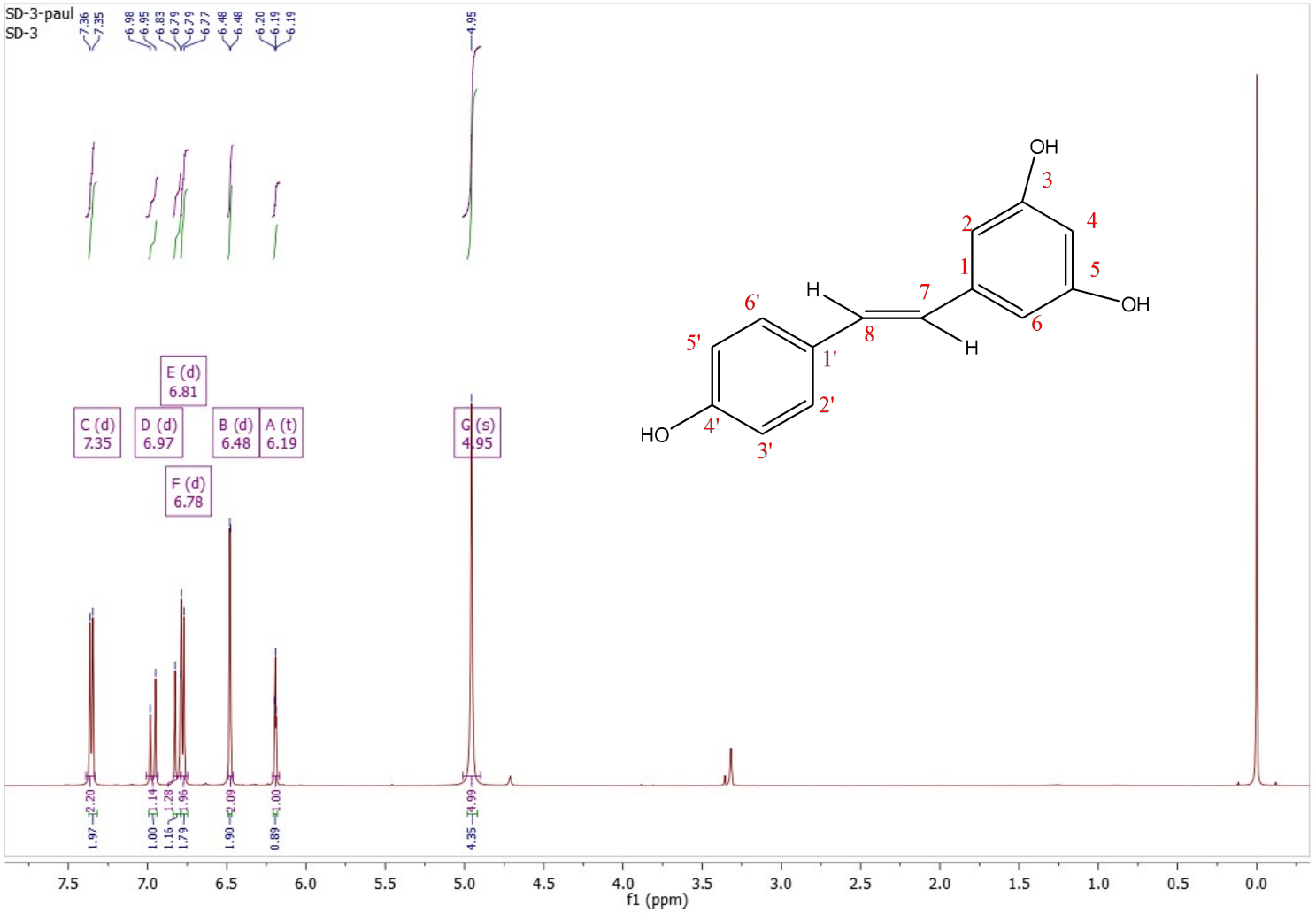


Figure 4.34: ^{1}H NMR (400 MHz, MeOD) of compound ME1

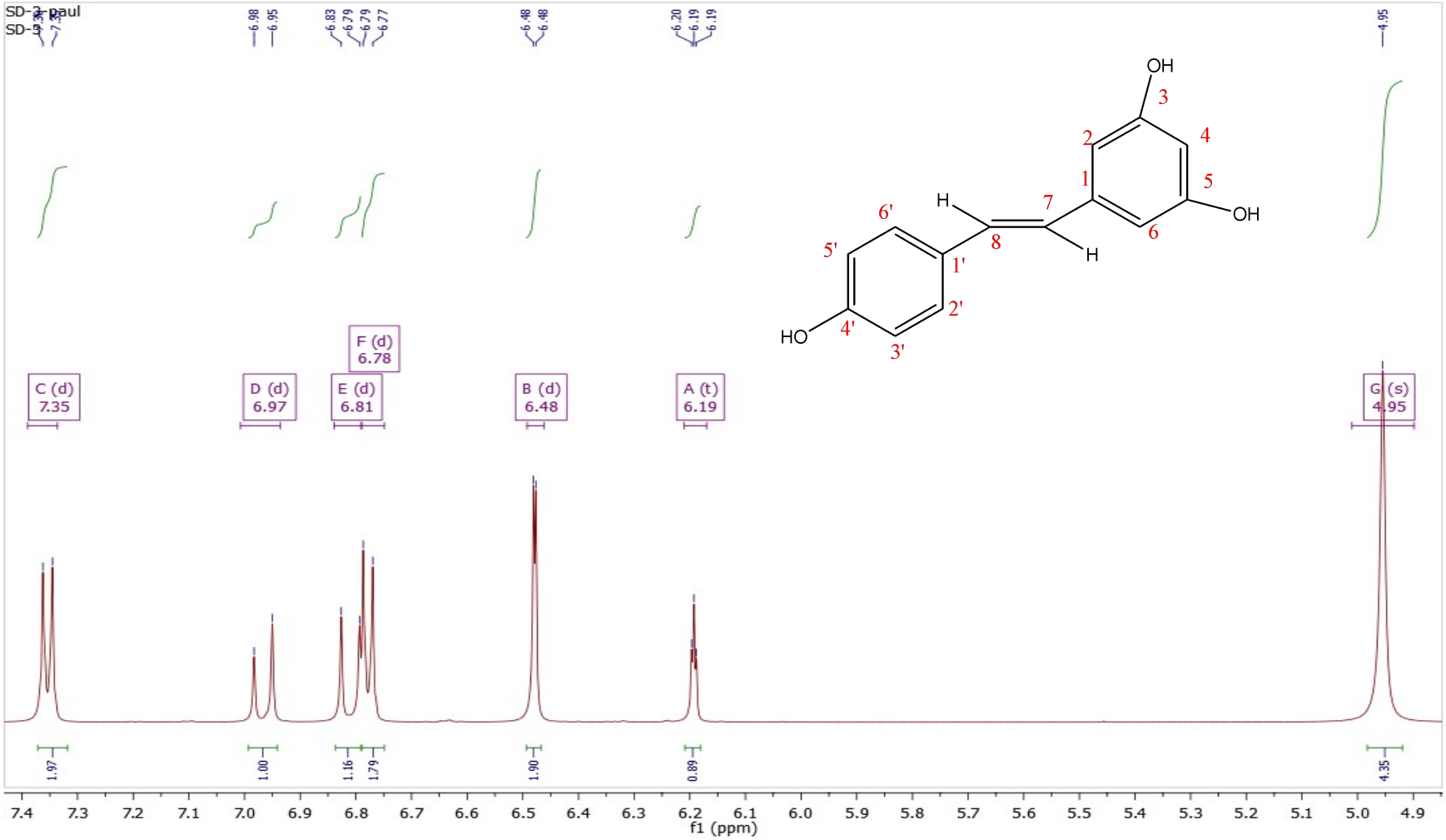


Figure 4.35: Expanded ^{1}H NMR (400 MHz, MeOD) of compound ME1

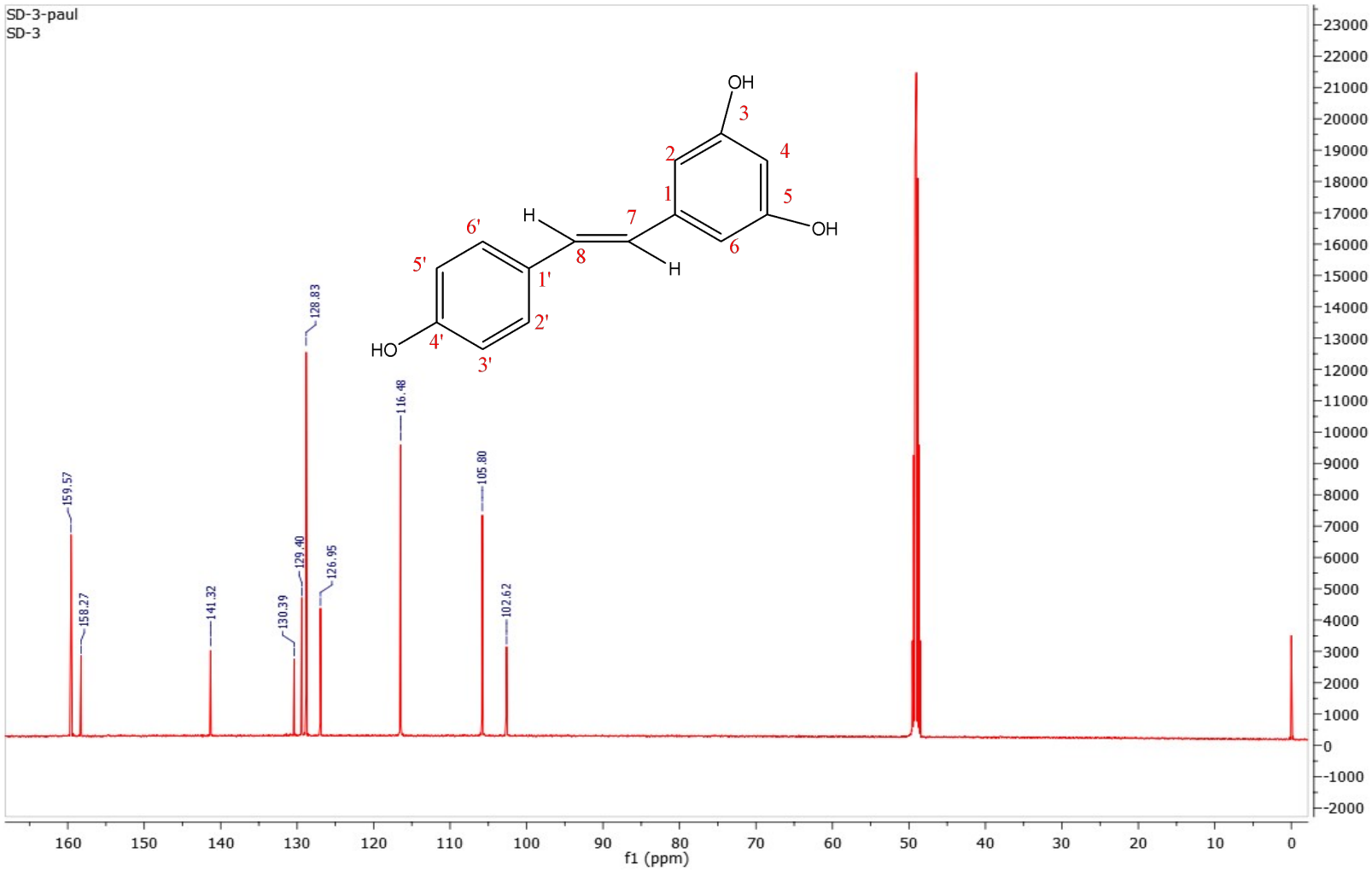


Figure 4.36: ^{13}C NMR (100 MHz, MeOD) of compound ME1

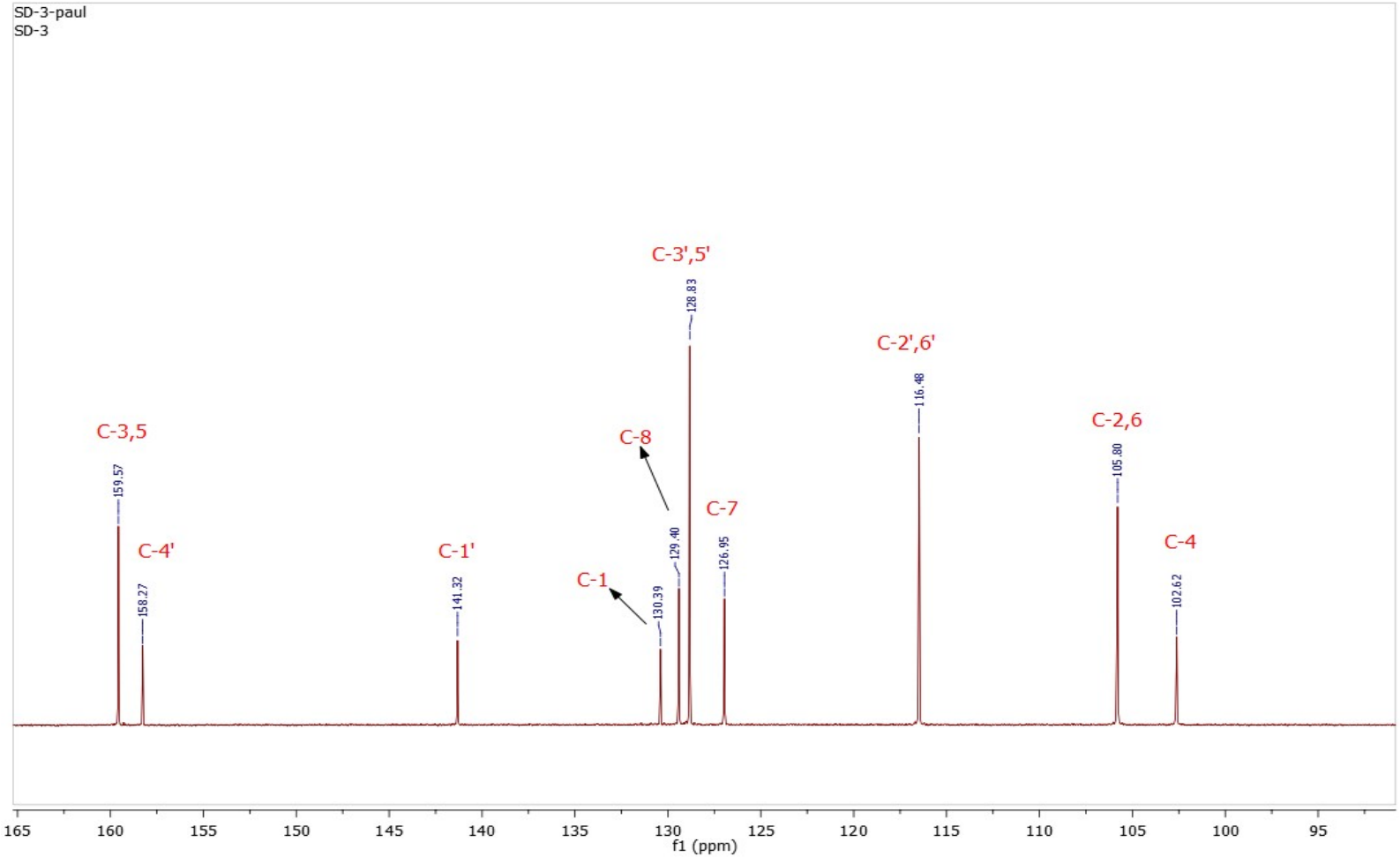


Figure 4.37: Expanded ^{13}C NMR (100 MHz, MeOD) spectrum of compound ME1

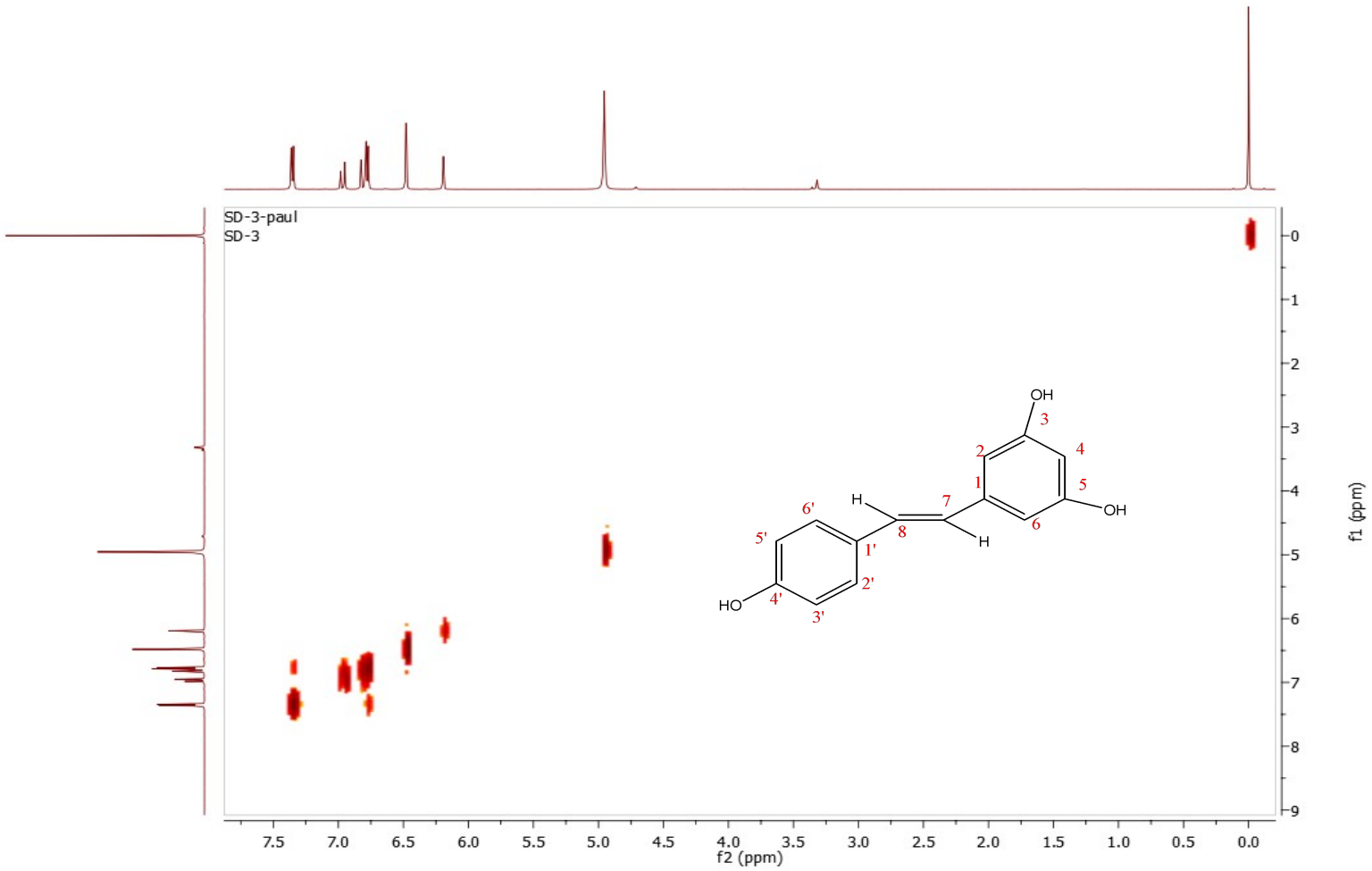


Figure 4.38: COSY spectrum of compound ME1

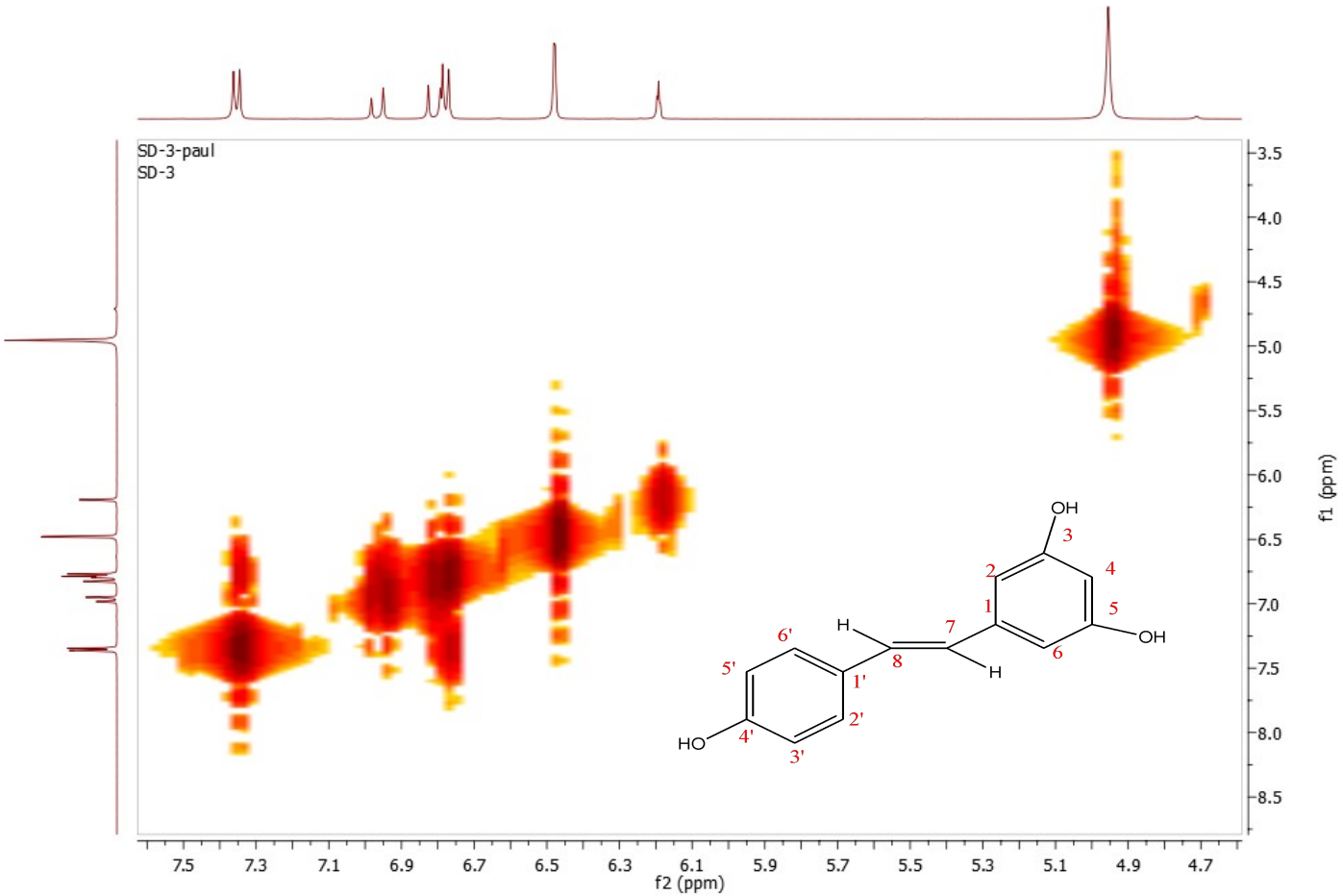


Figure 4.39: Expanded COSY spectrumof compound ME1

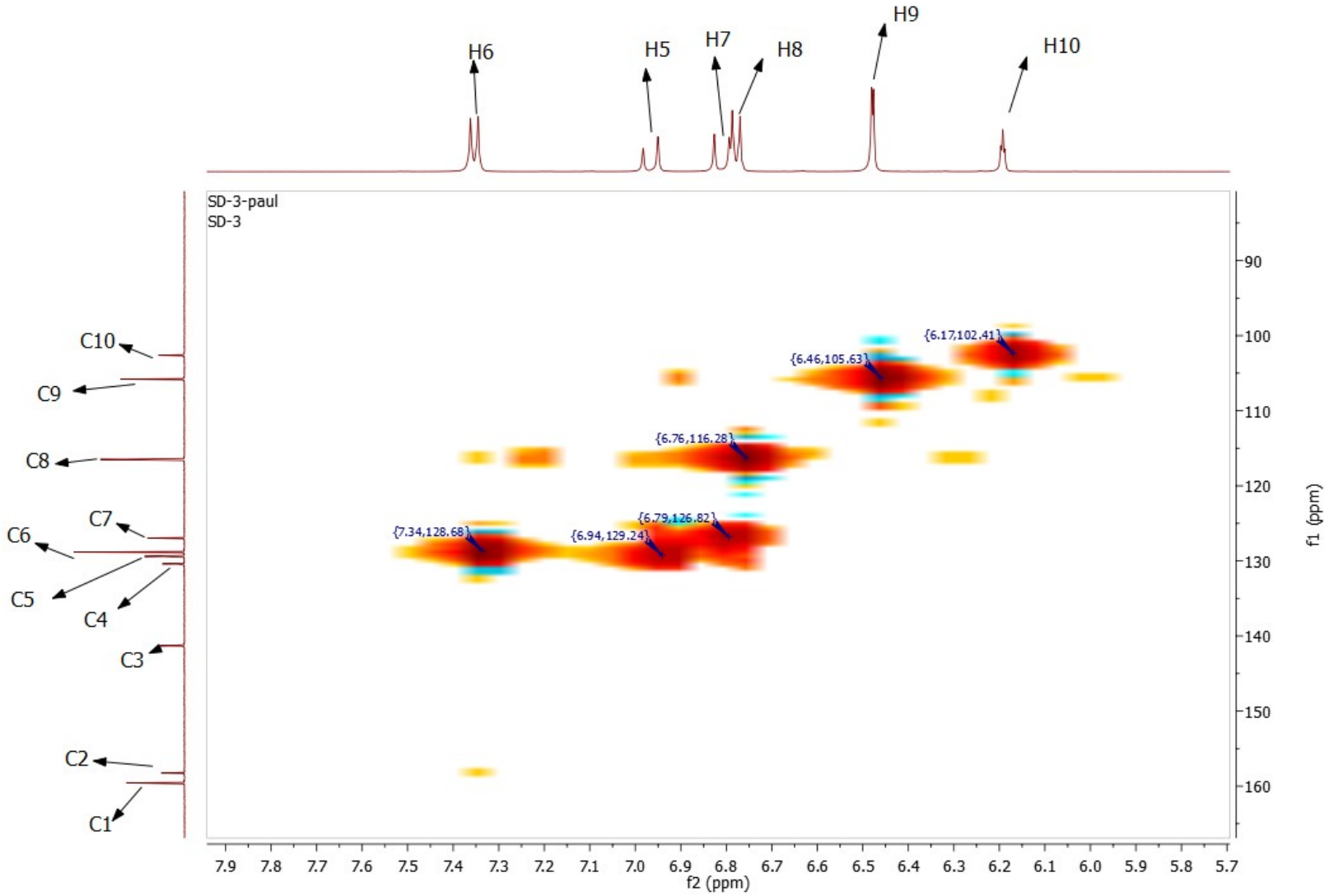


Figure 4.40: HSQC spectrum of compound ME1

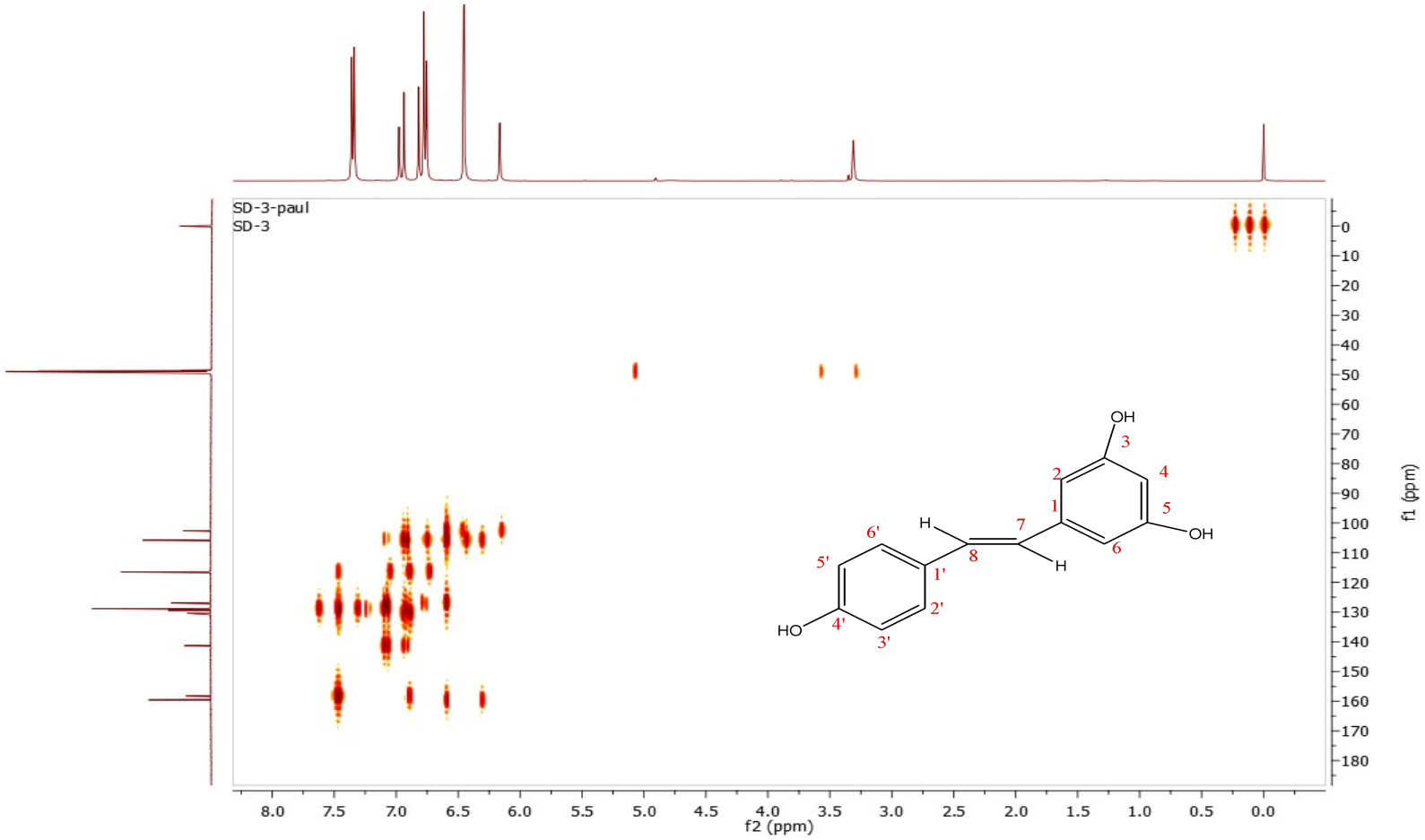


Figure 4.41: HMBC spectrum of compound ME1

4.3.4 ISOLATION OF ME2

Fractions 24 – 29 eluted with ethyl acetate: hexane (50:50) were pooled together on the basis of TLC analysis to give sub-fraction F_4 (200 mg). This was purified by column chromatography using ethyl acetate: hexane (40:60) to afford the compound labeled ME2 (80 mg).

4.3.5 CHARACTERISATION OF ME2

Appearance: dark orange solid

Melting point: 226-228 ^{o}C

Infra-red (KBr, Vmax, cm^{-1}): 3511, 3346, 2925, 2849, 1633, 1600

UV-Visible (methanol, nm): 250, 257, 331

Molecular formula: $C_{14}H_{12}O_4$

Molecular Mass: (HRESI-MS): 244.2472 g/mol (calculated for 244.0736 g/mol)

ESIMS peaks (% relative abundance): m/z 245 (100) $[MH]^+$, 227 (11.6) $[M - OH]^+$

^{13}C NMR ($CDCl_3$, 75 MHz): δ 139.69 (C-1), 104.64 (C-2,6), 158.93 (C3,5), 102.07 (C-4), 125.91 (C-7), 118.94 (C-8), 129.07 (C-1'), 113.65 (C-2'), 145.85 (C-3'), 145.97 (C-4'), 116.02 (C-5'), 128.67 (C-6').

^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.36 (d, *J* = 1.6 Hz, 1H, H-2,6), 6.10 (s, 1H, H-4), 6.72 (d, *J* = 3.2 Hz, 1H, H-7), 6.86 (d, *J* = 8.7 Hz, 1H, H-8), 6.95 (d, *J* = 1.4 Hz, 1H, H-2'), 6.68 (d, *J* = 4.7 Hz, 1H, H-5'), 6.82 (s, 1H, H-6'),

4.3.6 STRUCTURAL ELUCIDATION OF ME2

The structure of ME2 was established with the aid of the analysis of its ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HSQC, Infra-red, ESIMS data and by comparison with literature (Ferrigni and McLaughlin, 1984, Cardona *et al*, 1986). The Infrared spectrum (Fig. 4.43) showed a sharp peak at 3511.7 and a broad band at 3346.00 cm^{-1}representing a free O-H and an hydrogen bonded O-H vibrations respectively. It also

showed strong absorption at 1633 and 1600 cm^{-1} representing olefinic and aromatic C=C vibrations respectively. The positive ESI-MS spectrum of compound ME2 (fig. 4.44) gave the MH^+ ion at *m/z* 245.1, indicating the molecular formula of $C_{14}H_{12}O_4$ (DBE = 9). The number of unsaturation is accounted for as two benzene ring and one alkene group as in compound ME1. The ^1H NMR spectrum (Fig. 4.45) revealed a doublet (δ 6.48, d, *J* = 2.1 Hz, H-2) and triplet peaks (δ 6.19, t, *J* = 2.1 Hz, H-4) suggesting meta-coupled aromatic protons and two doublet peaks (δ 6.73, d, *J* = 15.57 Hz, H-7), and (δ 6.88, d, *J* = 16.21 Hz, H-8) indicating olefinic protons as in compound ME1. However, unlike compound ME1, it also showed two doublet signals (δ 6.96, *d*, J = 2.01 Hz, H-2') and (δ 6.73, *d*, J = 8.33 Hz, H-5') and a doublet of doublet (δ 6.82, *dd*, J = 2.06, 8.21 Hz, H-6') suggesting three protons coupled together in a 1,2,4-tri-substituted benzene ring. The ^{13}C NMR and the DEPT spectra (Fig. 4.46 and 4.47) showed fourteen carbon atoms being accounted for as six aromatic methine carbons (δ 104.7, C-2,6; 102.2, C-4; 116.2, C-2', 113.8, C-5' and 119.0, C-6') two olefinic carbon (δ, 126.0, C-7 and 128.7, C-8); and six aromatic quaternary carbons atoms (δ, 139.7, C-1; 158.9, C-3, C-5; 129.1, C-1'; 145.9, C-3' and 146.0, C-4') respectively. The one-bond proton-carbon correlation observed in HSQC spectrum (Fig. 4.50) and the long range correlations observed in HMBC (fig. 4.51) facilitated the assigning of the protons to their carbons and with other neighboring carbon atoms. These structural data were also compared with that obtained in literature. (Cardona *et.al.,* 1986 and Brinker and Seigler, 1991)

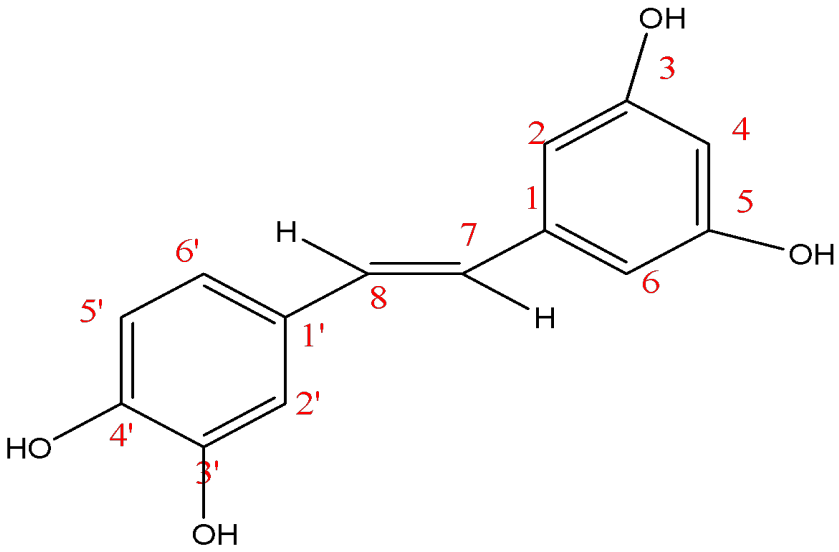


Figure 4.42: Compound ME2 – Piceatannol (3,3’ 4’,5-tetrahydroxyl stilbene)

Table 4.6: ^{13}C (100 MHz) and ^{1}H NMR (600 MHz) data of compound ME2

Carbon No	^{13}C NMR	Piceatannol Cardona *et al*, 1986	^{1}H NMR	Piceatannol Ferrigni and McLaughlin, 1984	HMBC
C1	139.69	140.7	-	-	-
C2	104.74	105.5	6.43 (2H, d, *J* =2.13 Hz)	6.37 (2H, d, *J* = 2.03 Hz)	C_3, C_4
C3	158.93	159.5	-	-	-
C4	102.13	102.5	6.15 (1H, t, *J* = 2.11 Hz)	6.12 (1H, t, *J* =2.01 Hz)	C_3, C_6
C5	158.93	159.5	-	-	-
C6	104.64	105.5	6.43 (2H, d, *J* =2.13 Hz)	6.37 (2H, d, *J* = 2.03 Hz)	C_4, C_5
C7	125.97	126.8	6.73 (1H, d, *J* =15.57 Hz)	6.7 (1H, d, *J* = 16 Hz)	C_6
C8	128.67	129.6	6.82 (1H, d, *J* = 8.21, 2.06)	6.83 (1H, dd, *J* = 8.5, 2 Hz)	$C_{3'}$, C_{-7}, $C_{6'}$
C1’	129.07	132.9	-	-	-
C2’	113.65	116.1	6.96 (1H, d, *J* =1.69 Hz)	6.96 (1H, d, *J*=1.96 Hz)	$C_{6'}$, $C_{3'}$
C3’	145.85	146.0	-	-	-
C4’	145.97	146.2	-	-	-
C5’	116.02	113.7	6.74 (1H, d, *J* = 7.25Hz)	6.77 (1H, d, *J* =8.5 Hz)	$C_{3'}$, C_8
C6’	118.94	119.8	6.88 (1H, d, *J* =16.21 Hz)	6.85 (1H, d, *J* = 16 Hz)	$C_{2'}$, C-3’, C-7, C-1

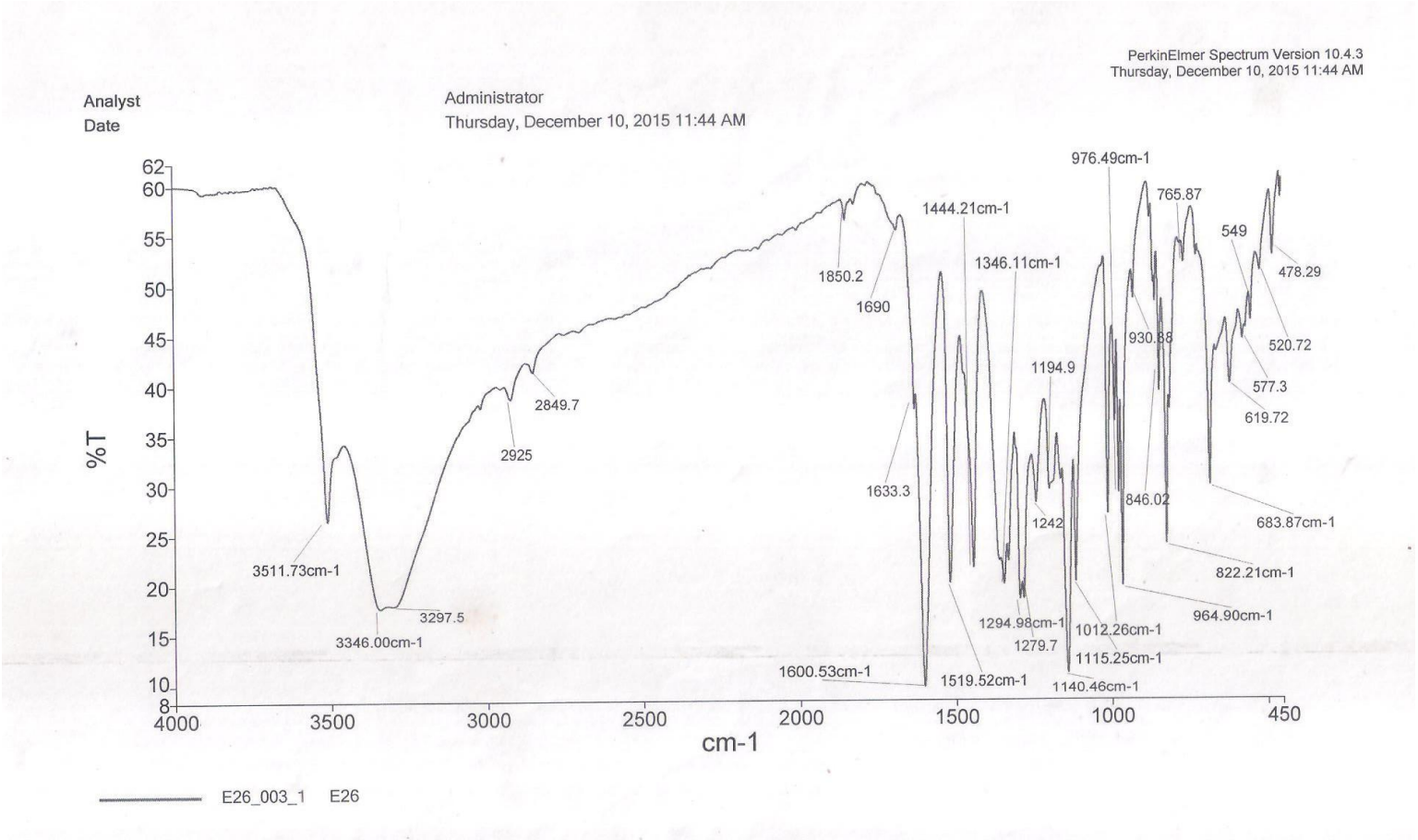


Figure 4.43: Infra-red spectrum of compound ME2

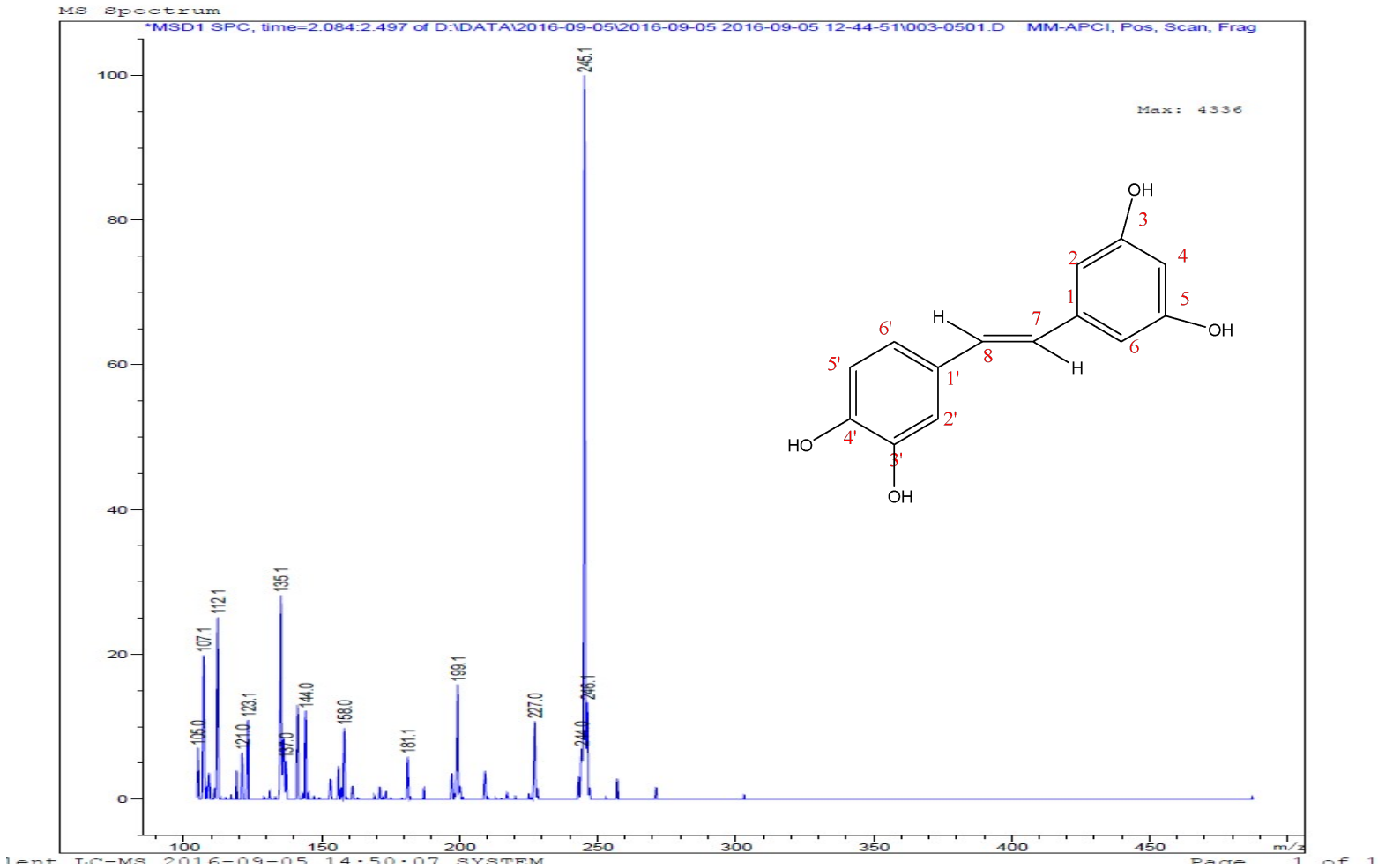


Figure 4.44: ESI-Mass spectrum of Compound ME2

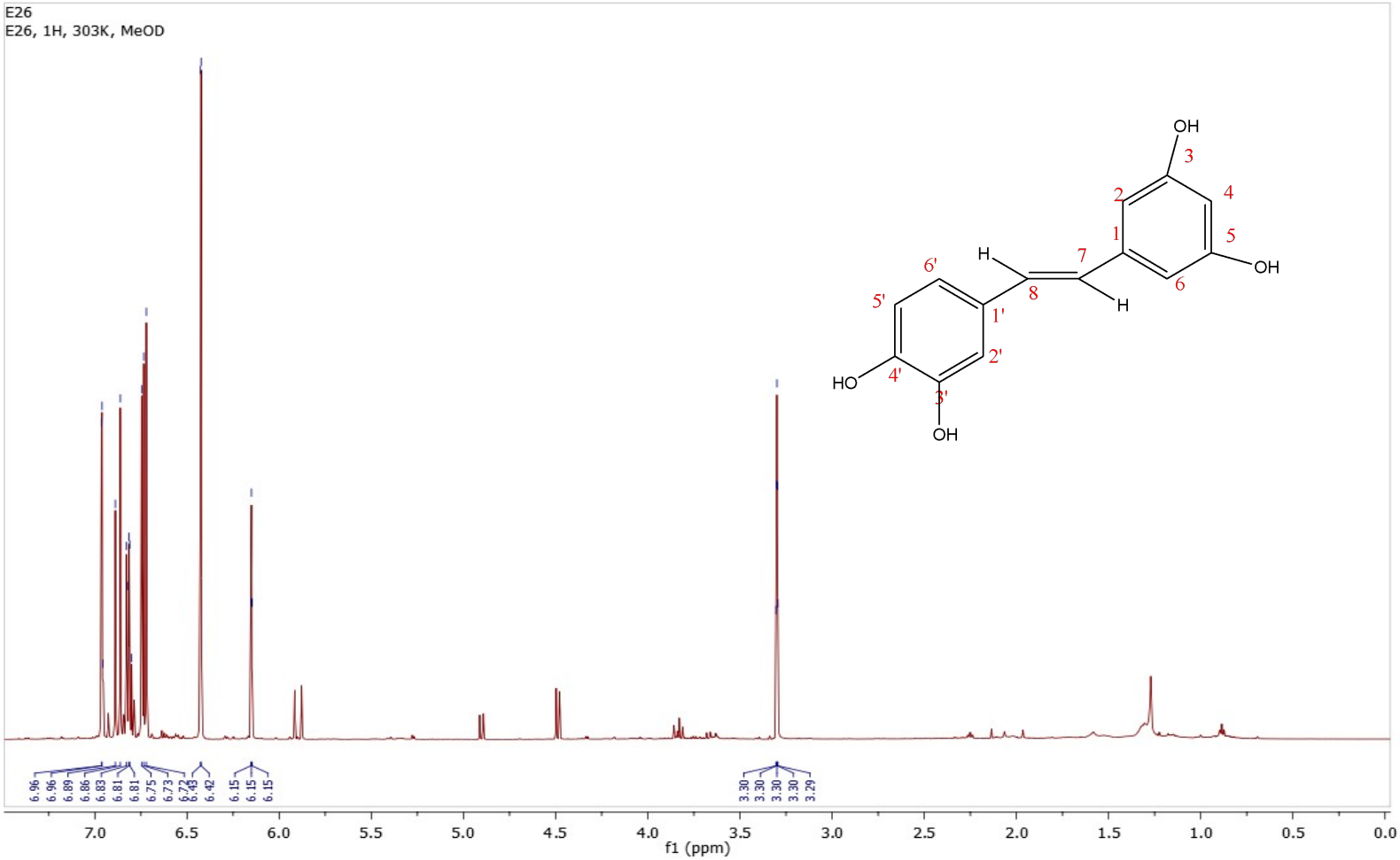


Figure 4.45: ^{1}H NMR (600 MHz, MeOD) Spectrum of compound ME2

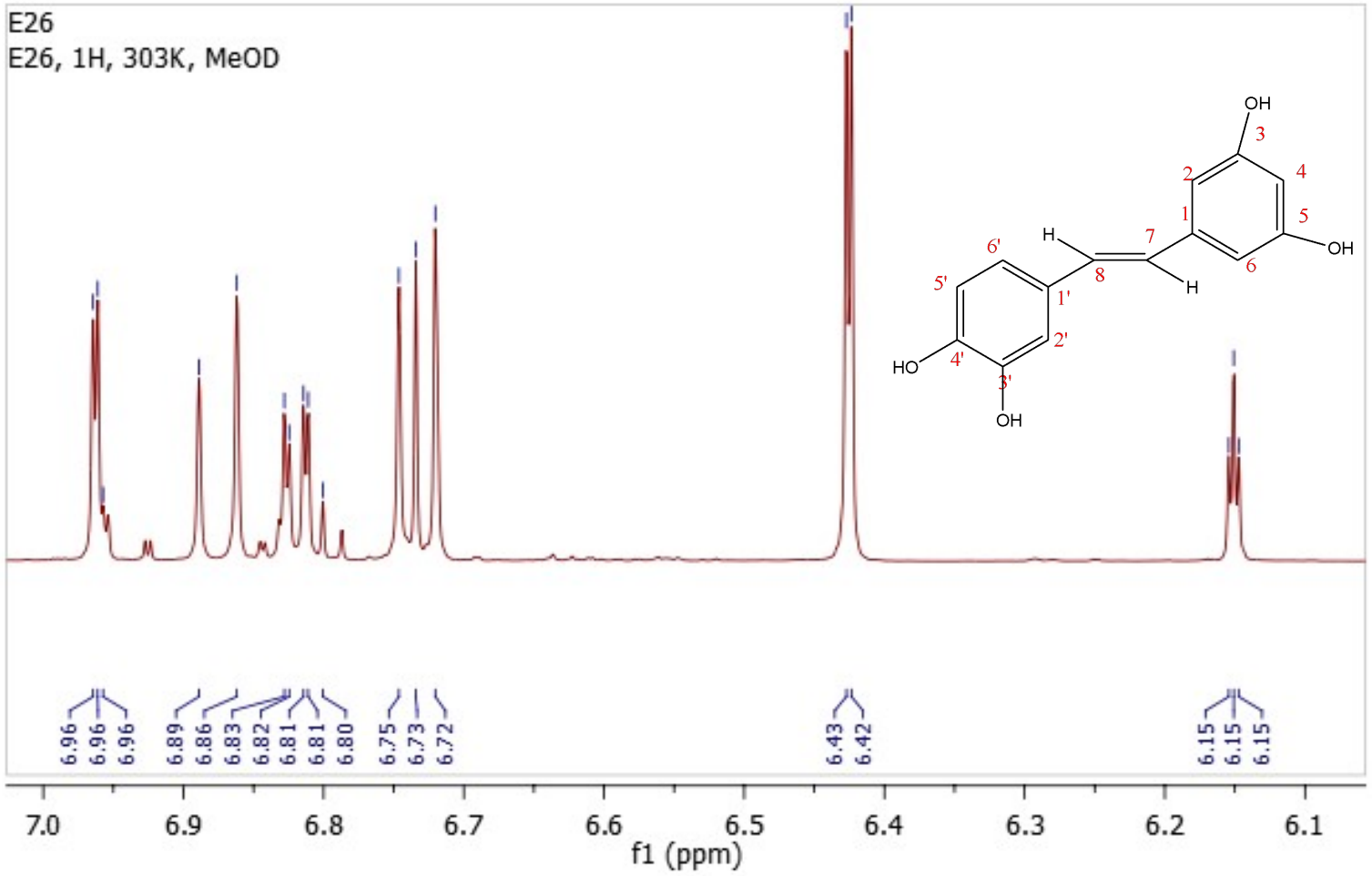


Figure 4.46: Expanded ^{1}H NMR (600 MHz, MeOD) Spectrum of compound ME2

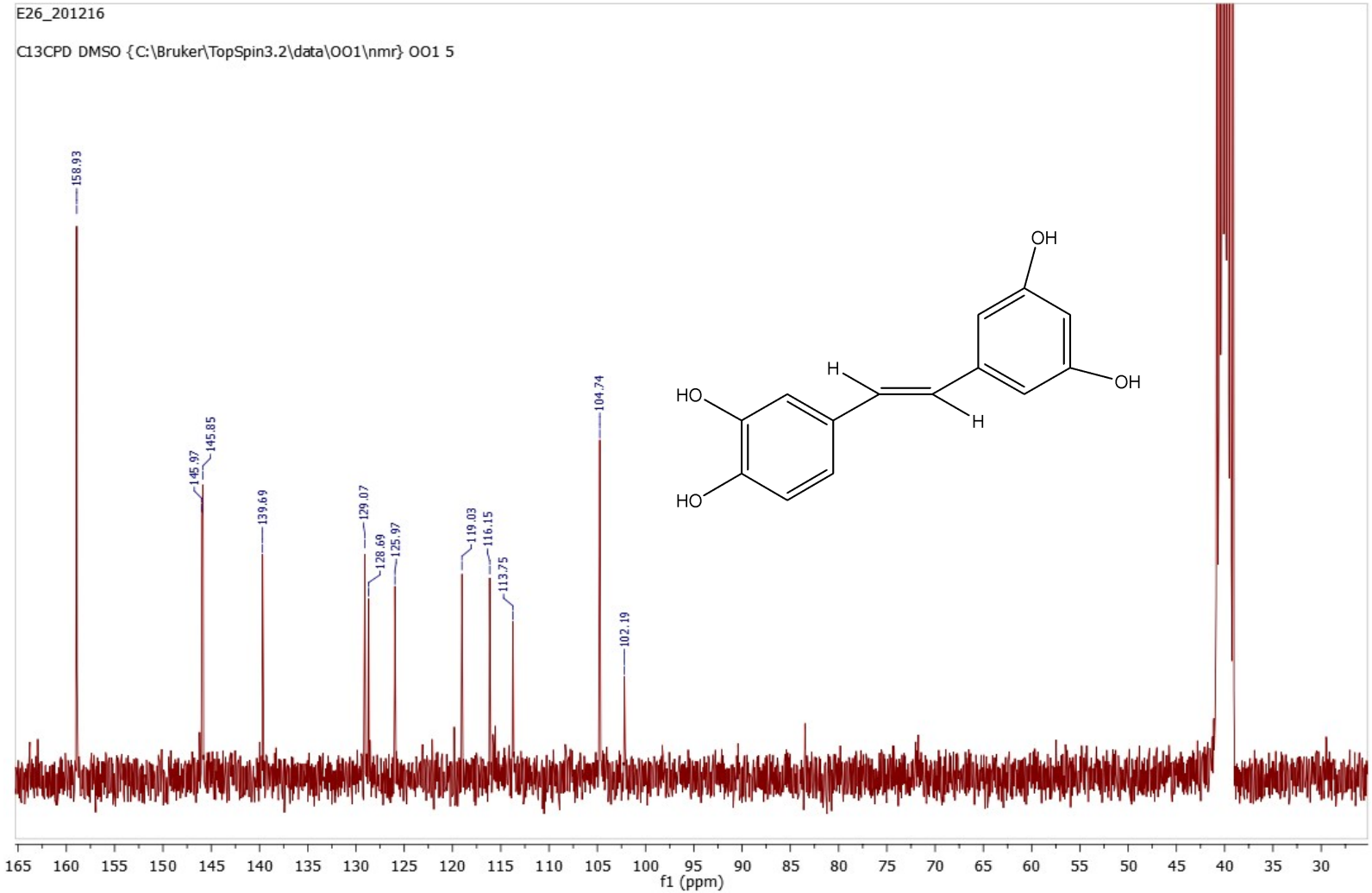


Figure 4.47: ^{13}C NMR (75 MHz, DMSO) Spectrum of compound ME2

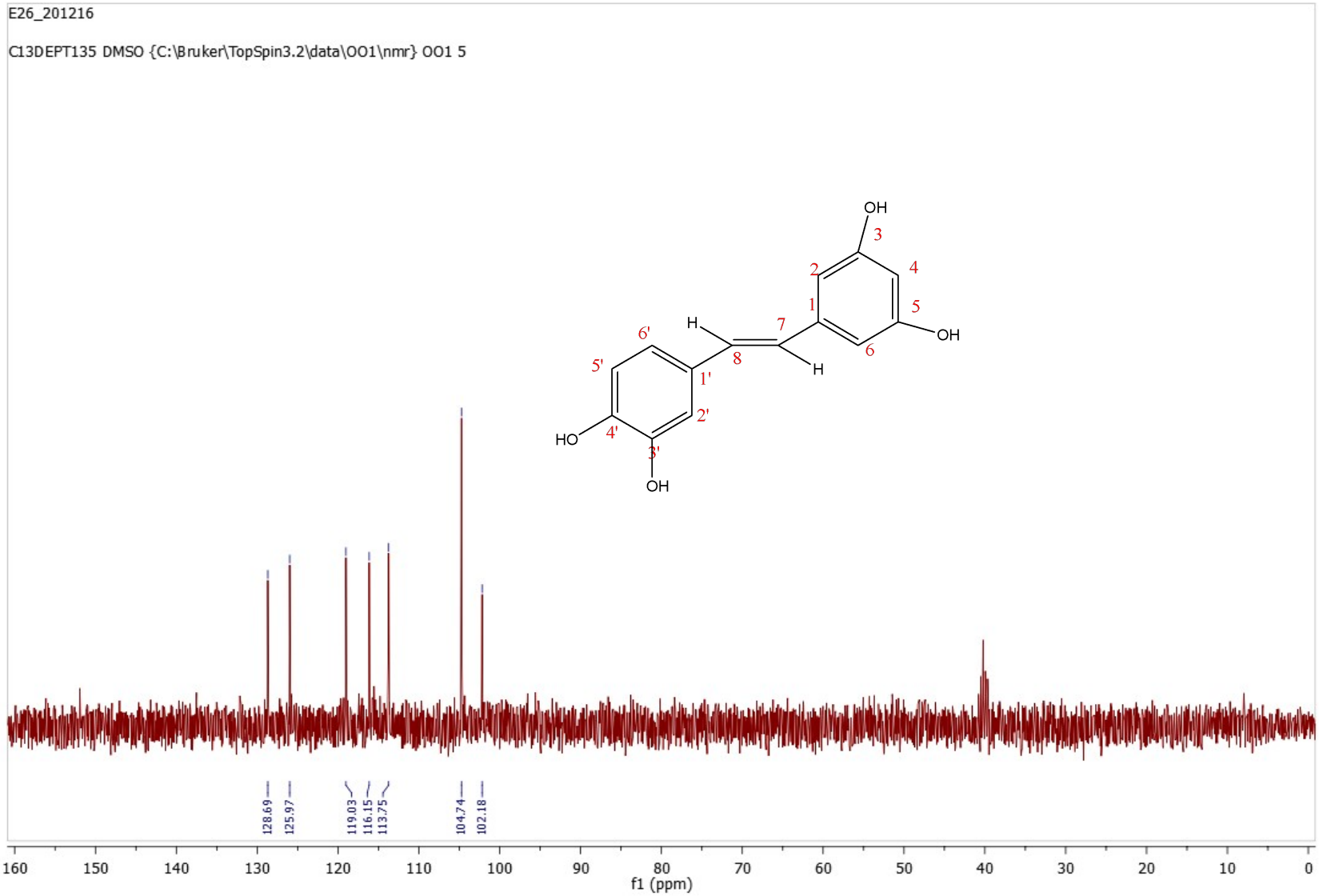


Figure 4.48: DEPT Spectrum of compound ME2

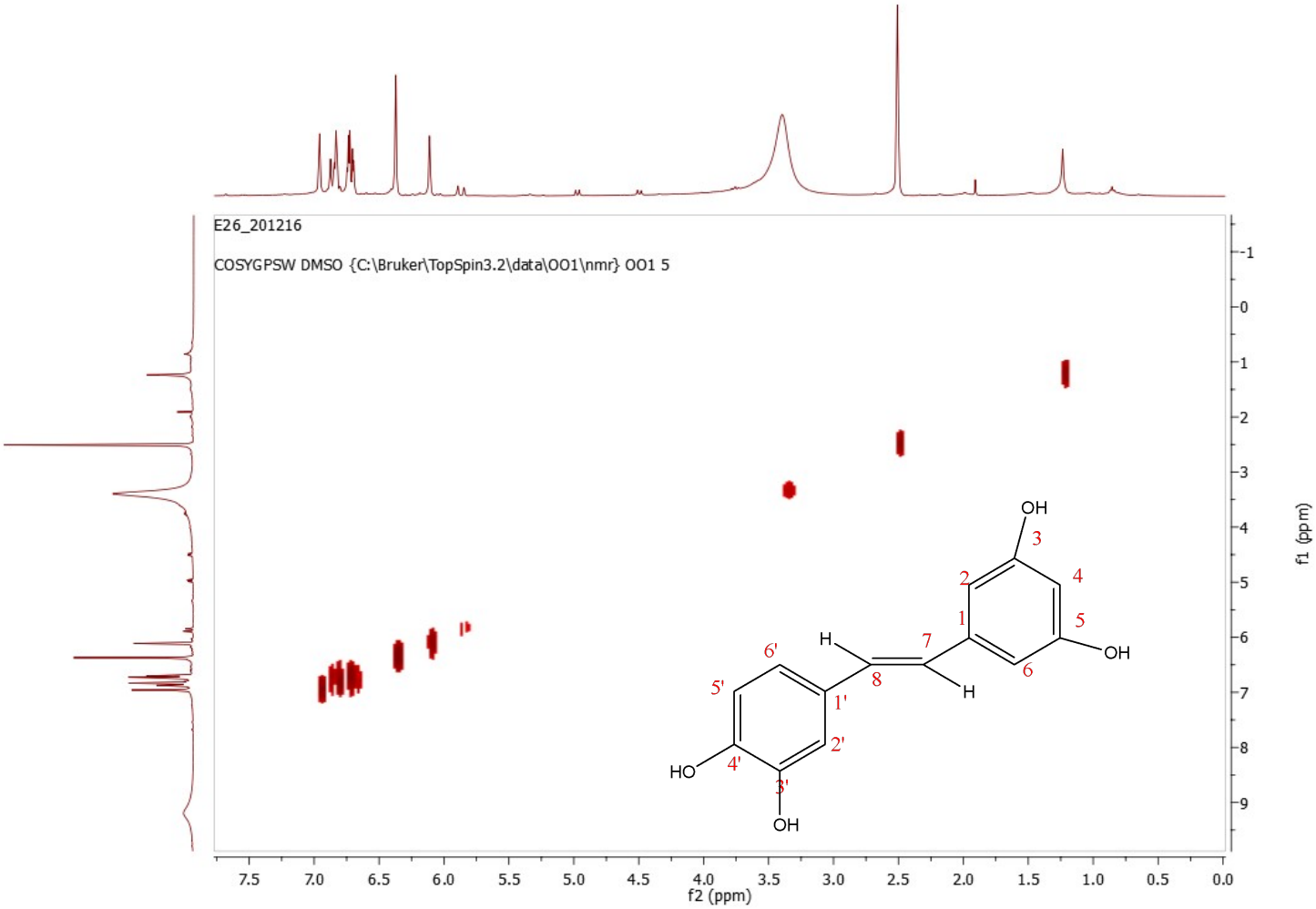


Figure 4.49: COSY Spectrum of compound ME2

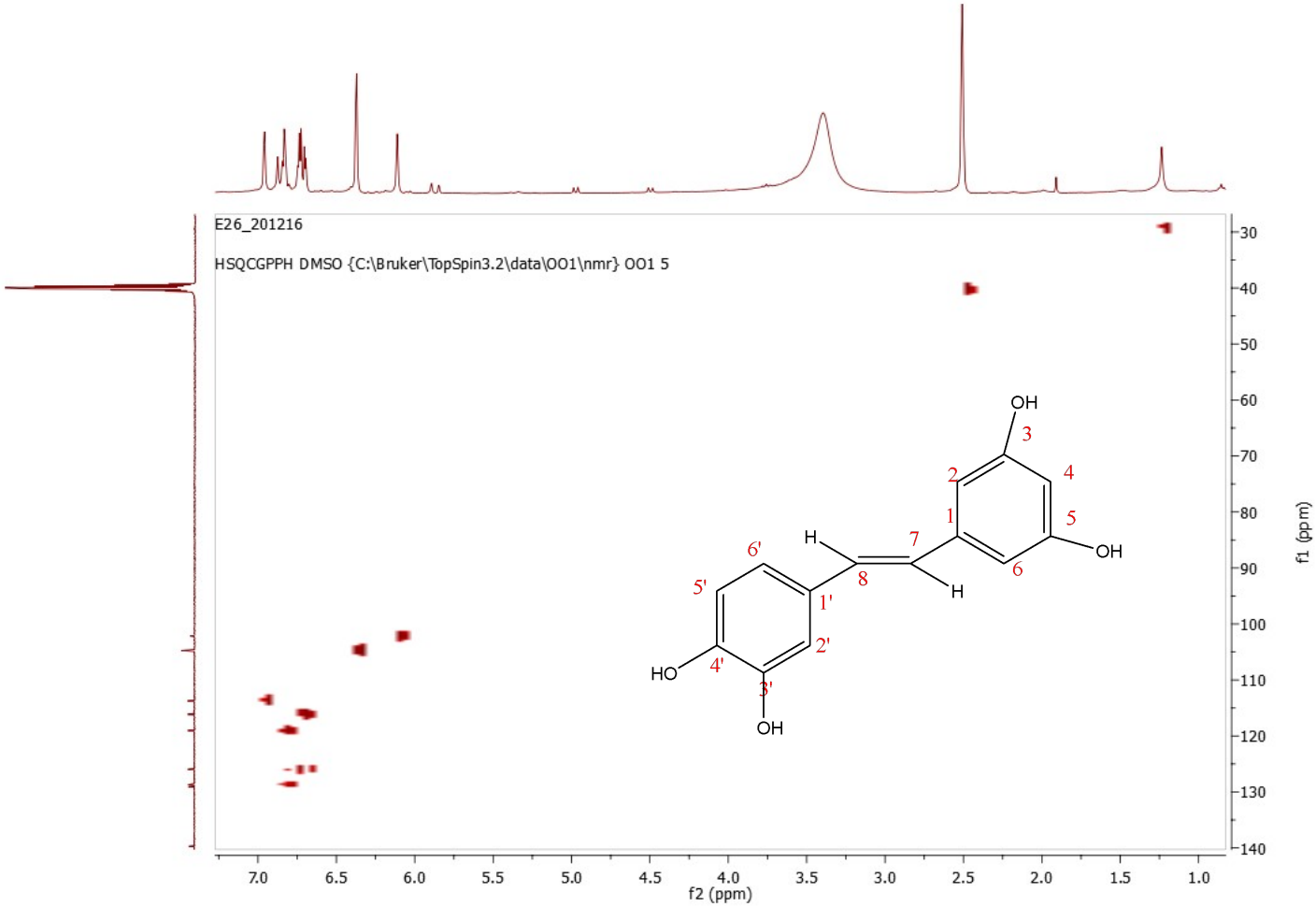


Figure 4.50: HSQC Spectrum of compound ME2

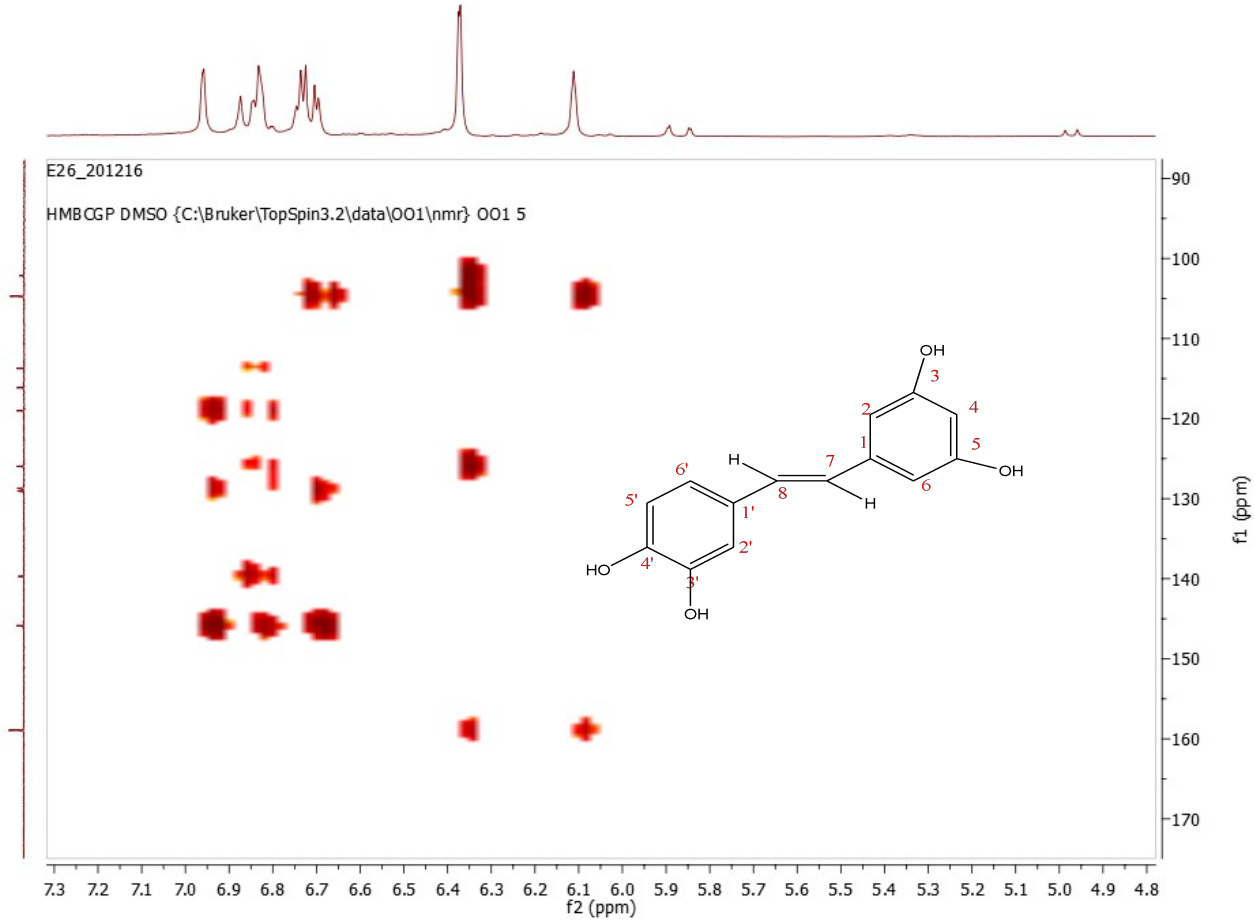


Figure 4.51: HMBC spectrum of compound ME2

4.3.7 ISOLATION OF ME3

Fractions 31 – 35 eluted with ethyl acetate: hexane (50:50) were pooled together on the basis of TLC analysis to give sub-fractions F_4 (80 mg). This was purified by column chromatography using ethyl acetate: hexane (40:60) to afford the compound labeled **ME3** (40 mg).

4.3.8 CHARACTERISATION OF ME3

Appearance: grey

Melting point: 257-259 ^{o}C

Infra-red (KBr, ν_{max}, cm^{-1}): 3368, 3288, 3066, 1701, 1617

Ultra-Visible (nm): 277, 330

Molecular formula: $C_7H_6O_5$

Molecular Mass: 170.1195 g/mol

^{13}C NMR ($CDCl_3$, 75 MHz): δ. 138.44 (C-4), 167.93 (COOH), 120.91 (C-1), 145.85 (C-3,5), 109.18 (C-2,6)

^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.08 (s, 1H, H-COOH), 6.91 (s, 2H, H-2,6), 3.40 (s, 3H, OH).

4.3.9 STRUCTURAL ELUCIDATION OF ME3

The structural elucidation of ME3 was based on its IR and ^{1}H and ^{13}C NMR spectral data and by comparison with data available in literature. The Infra-red spectrum (Fig. 4.53) gave a broad absorption at 3368 and 3288 cm^{-1} indicating hydrogen bonded O-H stretch, 3066 representing an unsaturated C-H stretching vibration, 1701 indicates a carboxylic carbonyl group and an absorption peak at 1617 indicates an aromatic C=C stretch. The ^{1}H NMR (DMSO-d_6) spectrum (Fig. 4.54) revealed the presence of a singlet at δ 6.91 (2H, s, H-2 and H-6) indicating aromatic protons. The ^{13}C spectrum (75 MHz, acetone-d_6) for compound ME3 revealed five peaks at δ 167.9 (C-7), 145.9 (C-3 and C-5), 138.4 (C-4), 120.9 (C-1) and 109.2 (C-2 and C-6). HSQC and HMBC

assisted in the assigning of the protons to their respective carbons. The structure of ME3 was thereby confirmed to be gallic acid by comparing with the values obtained in literature (Chanwitheesuk *et al*, 2007). The purity of ME3 was also confirmed by its melting point and co-spotting it with a standard sample of gallic acid compound.

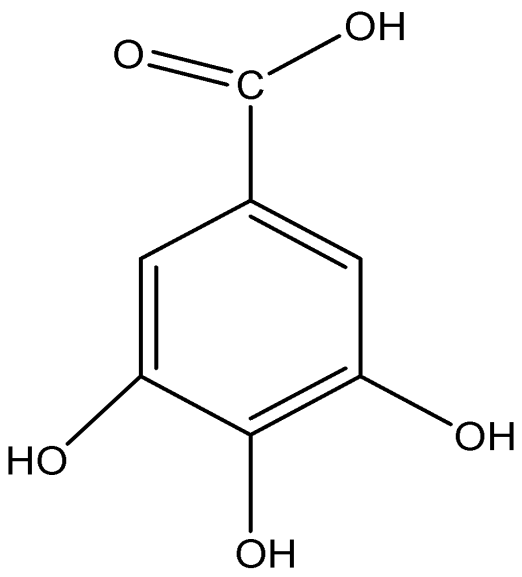


Figure 4.52: Compound ME3 – Gallic acid (3,4,5-trihydroxyl benzoic acid)

Table 4.7: ^{1}H NMR (300 MHz, DMSO) and ^{13}C (75 MHz, DMSO) data of compound ME3

Carbon No	^{13}C NMR spectra		^{1}H NMR spectra	
	ME1	Gallic acid Chanwitheesuk *et al*, 2007	ME1	Gallic acid Chanwitheesuk *et al*, 2007
1	120.9	120.8	-	-
2	109.2	109.1	6.91	7.15
3	145.9	144.9	-	-
4	138.4	137.8	-	-
5	145.9	144.9	-	-
6	109.2	109.1	6.91	7.15
7	167.9	167.4	-	-

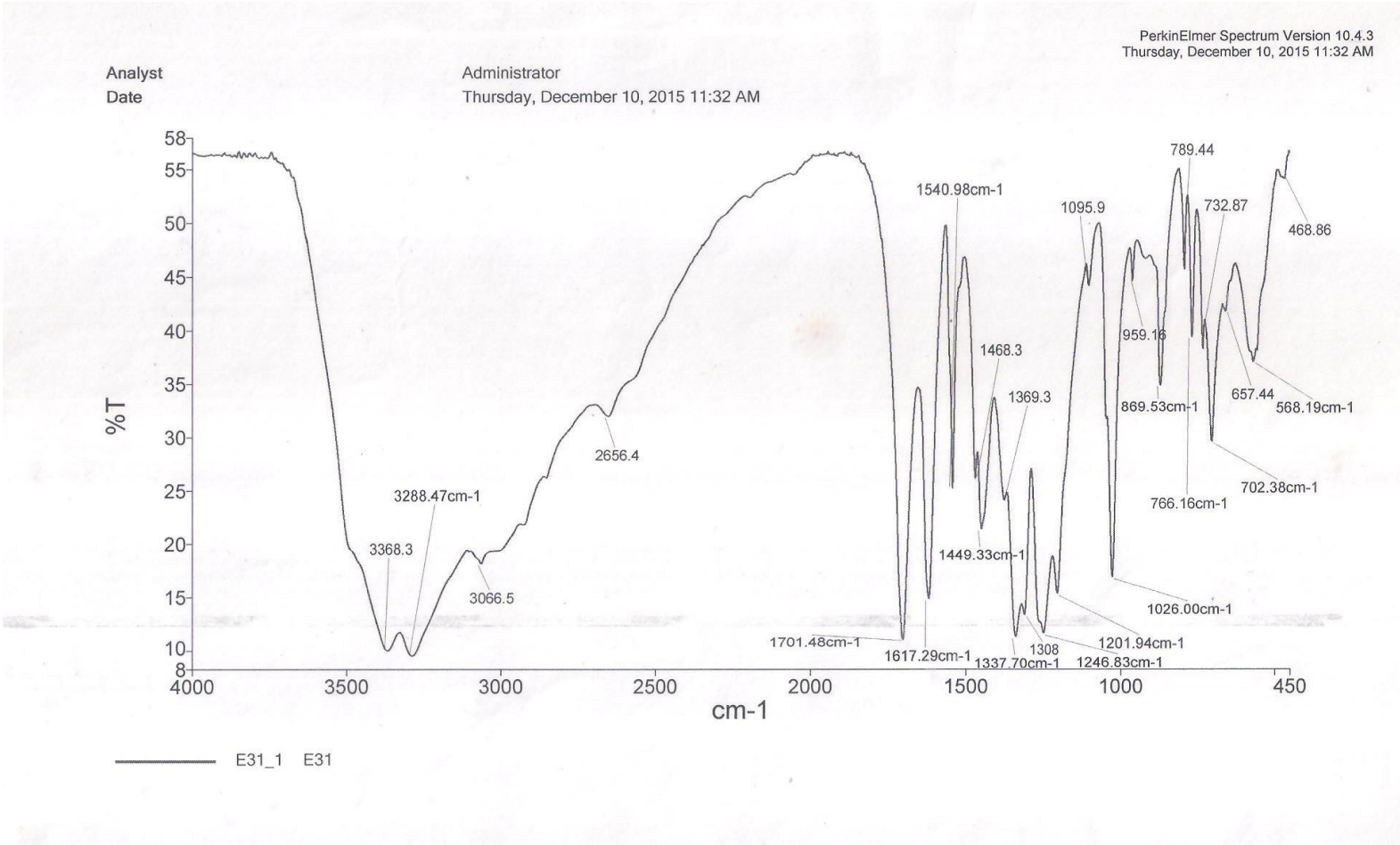


Figure 4.53: Infra-red spectrum of compound ME3

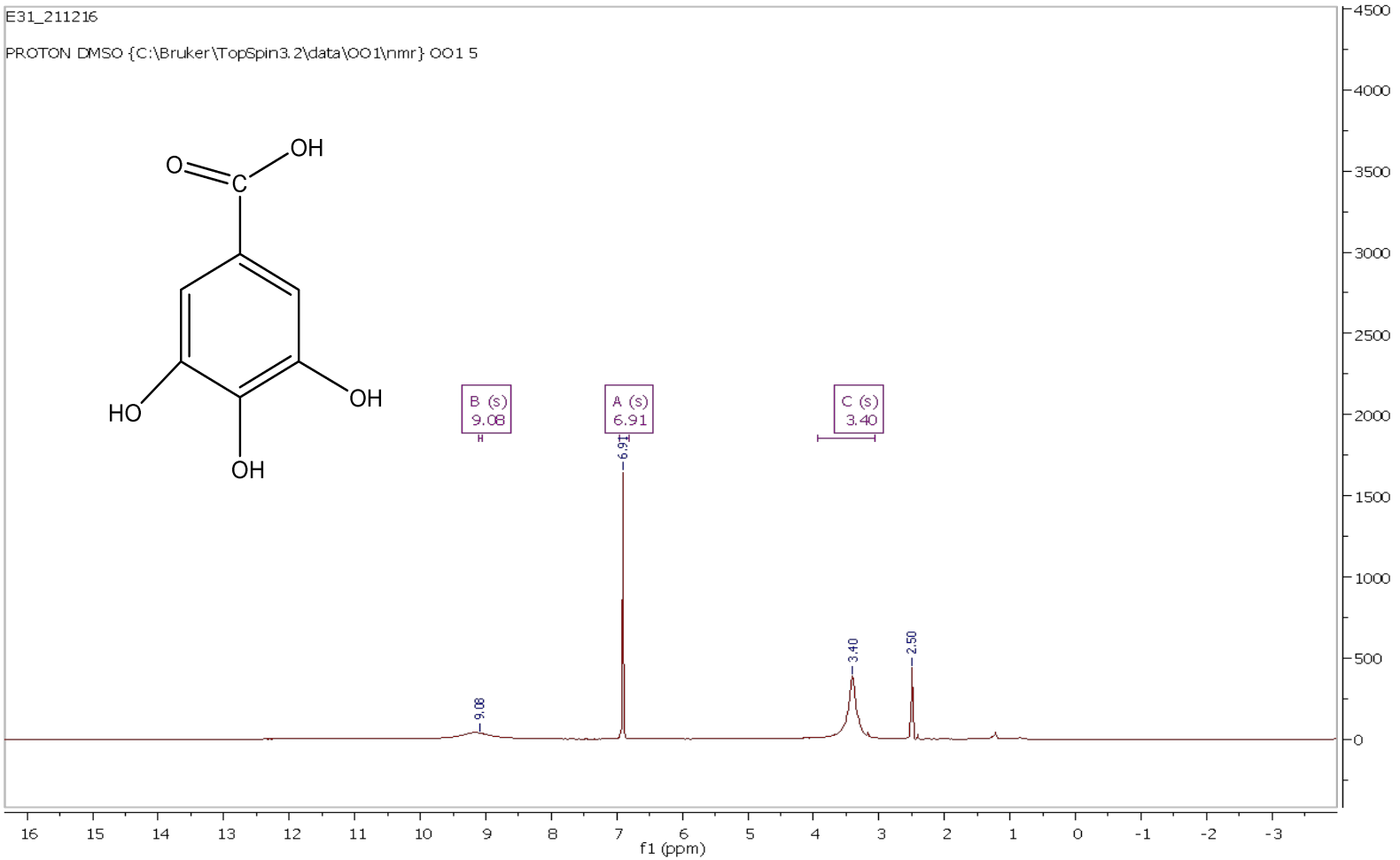


Figure 4.54: ^{1}H NMR (300 MHz, DMSO) spectrum of compound ME3

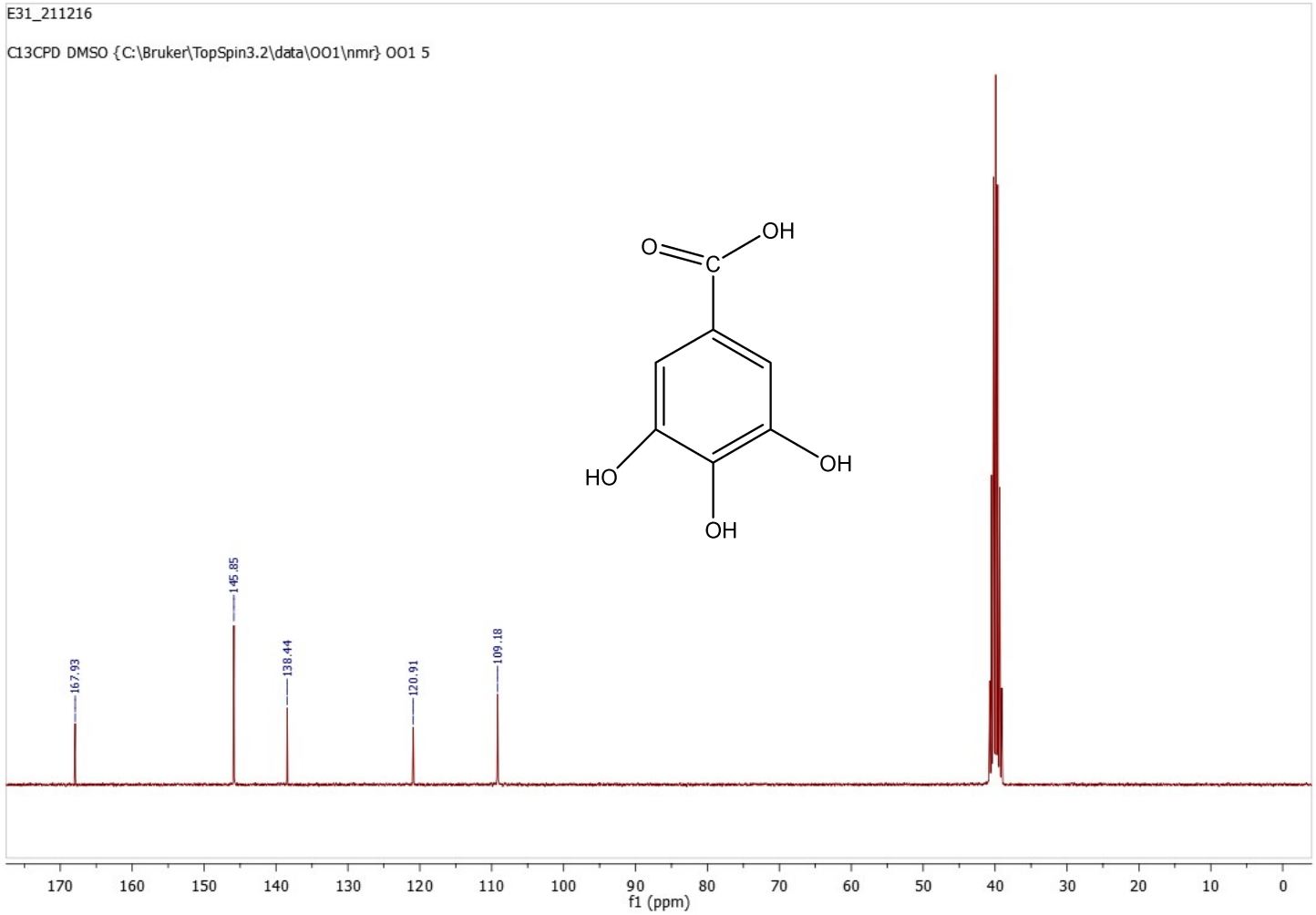


Figure 4.55: ^{13}C NMR (75 MHz, DMSO) spectrum of compound ME3

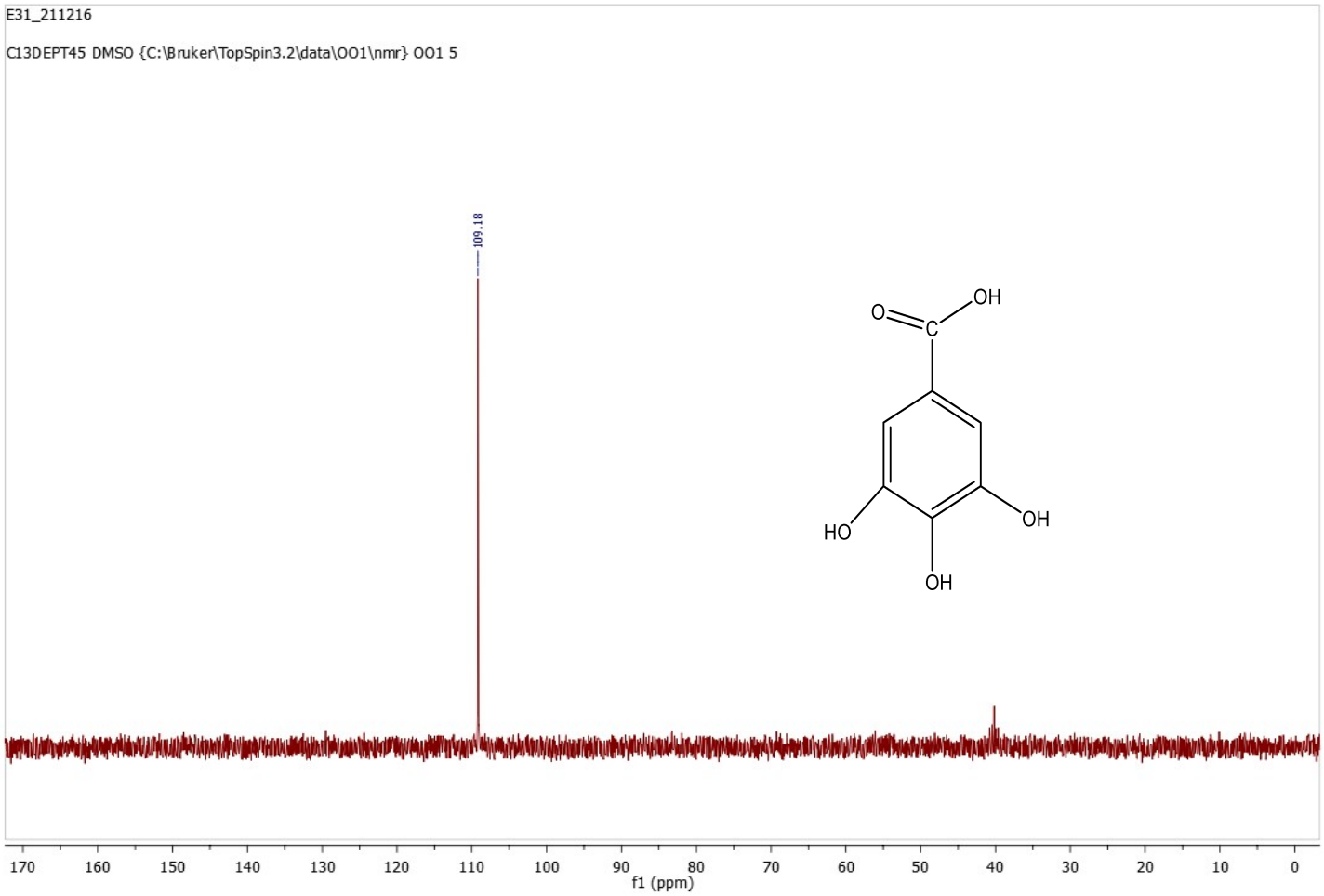


Figure 4.56: DEPT spectrum of compound ME3

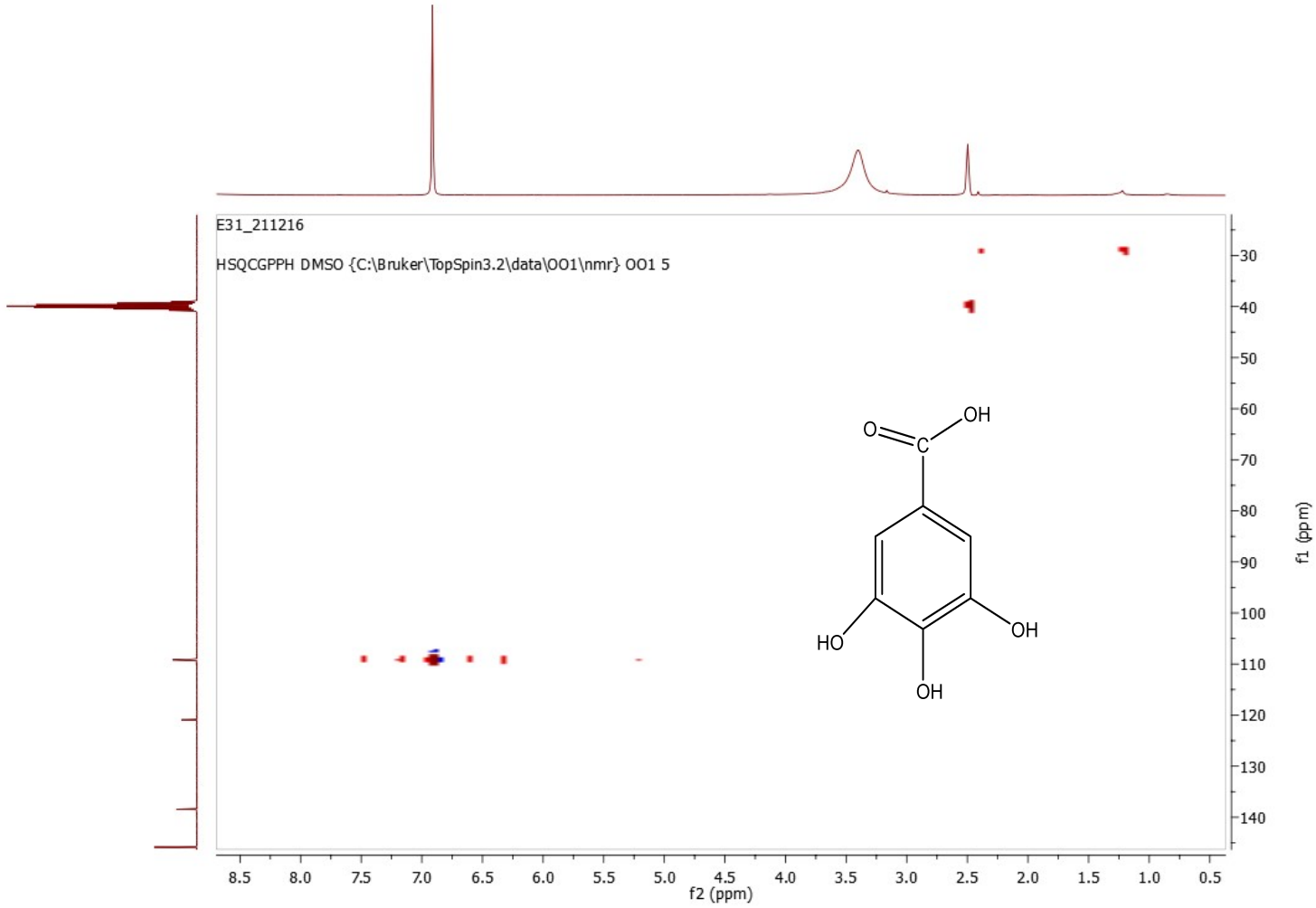


Figure 4.57: HSQC spectrum of compound ME3

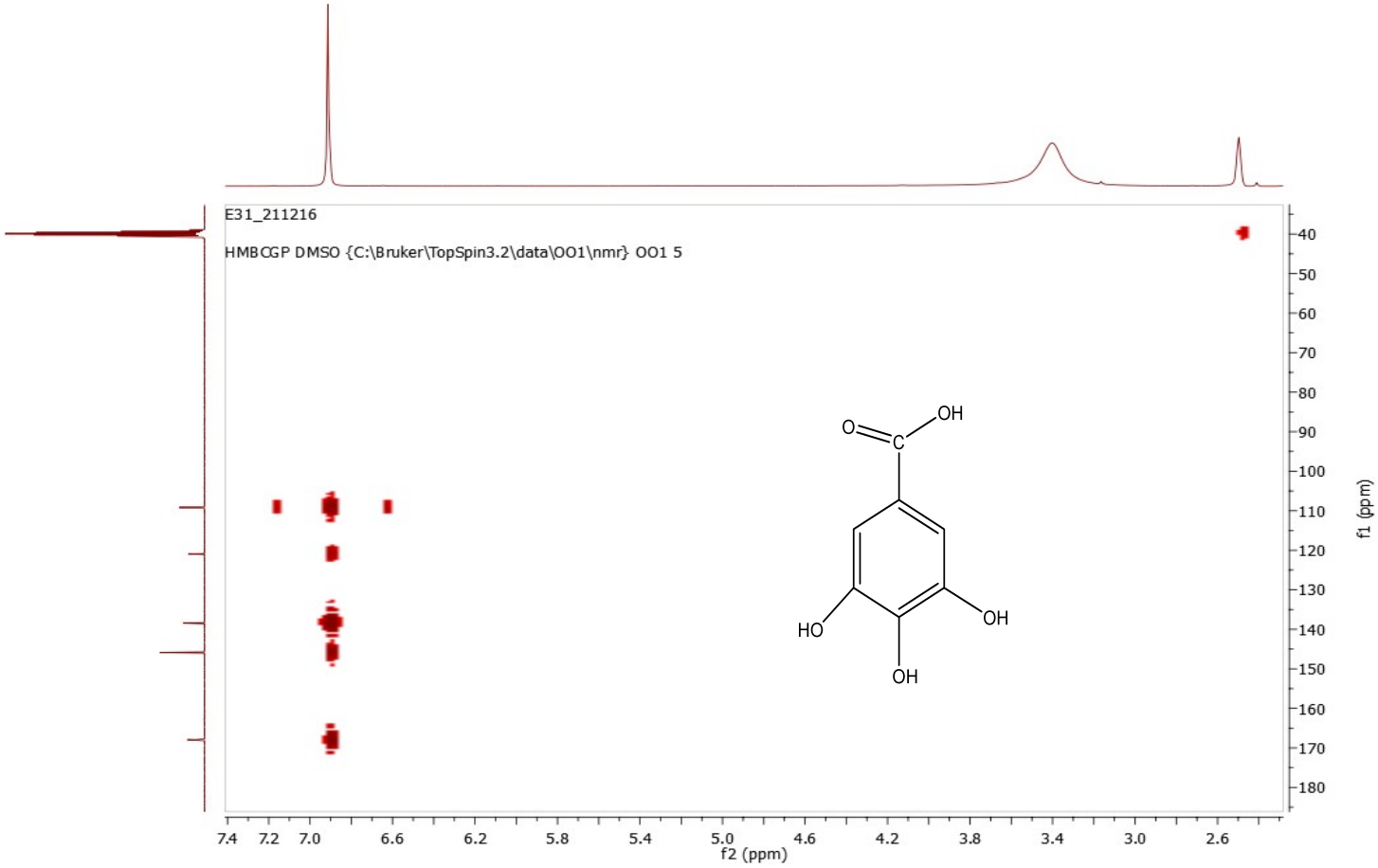


Figure 4.58: HMBC spectrum of compound ME3

4.4 BRINE SHRIMP *IN-VITRO*CYTOTOXICITY ASSAY

The Brine shrimp cytotoxity assay was proposed by Michael *et al*, 1956and further developed by several groups (Vanhaecke *et al*,1981; Sleet and Brendel, 1983). It is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. By this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity (Mclaughlin *et al*., 1998; Meyer *et al.,* 1982).It is used as a preliminary toxicity screening assay of plant extracts and isolated pure compounds or synthetic compounds (Quazi *et al*, 2017).

4.4.1 BRINE SHRIMP *IN-VITRO*CYTOTOXIC ACTIVITIES OF EXTRACTS

The results of the brine shrimp cytotoxicity assay on the extracts of *M. benthamianum* are shown in Figure 4.59. The result showed that all the extract exhibit strong cytotoxic effect with IC_{50} < 1000 (Meyer *et al.,* 1982). It also showed the dichloromethane extract to be the most active of the extracts with LC_{50} value of 29.29 µg/ml followed by the hexane extract with LC_{50} values of 99.96 µg/ml. This result further showed that there is a correlation between the brine shrimp cytotoxic assay and the *in vitro* anticancer assay in Fig. 4.64 which showed the non-polar extracts (MBH and MBD) as having a greater percentage growth inhibition against the cancer cell lines than the polar extracts (MBE and MBAM).

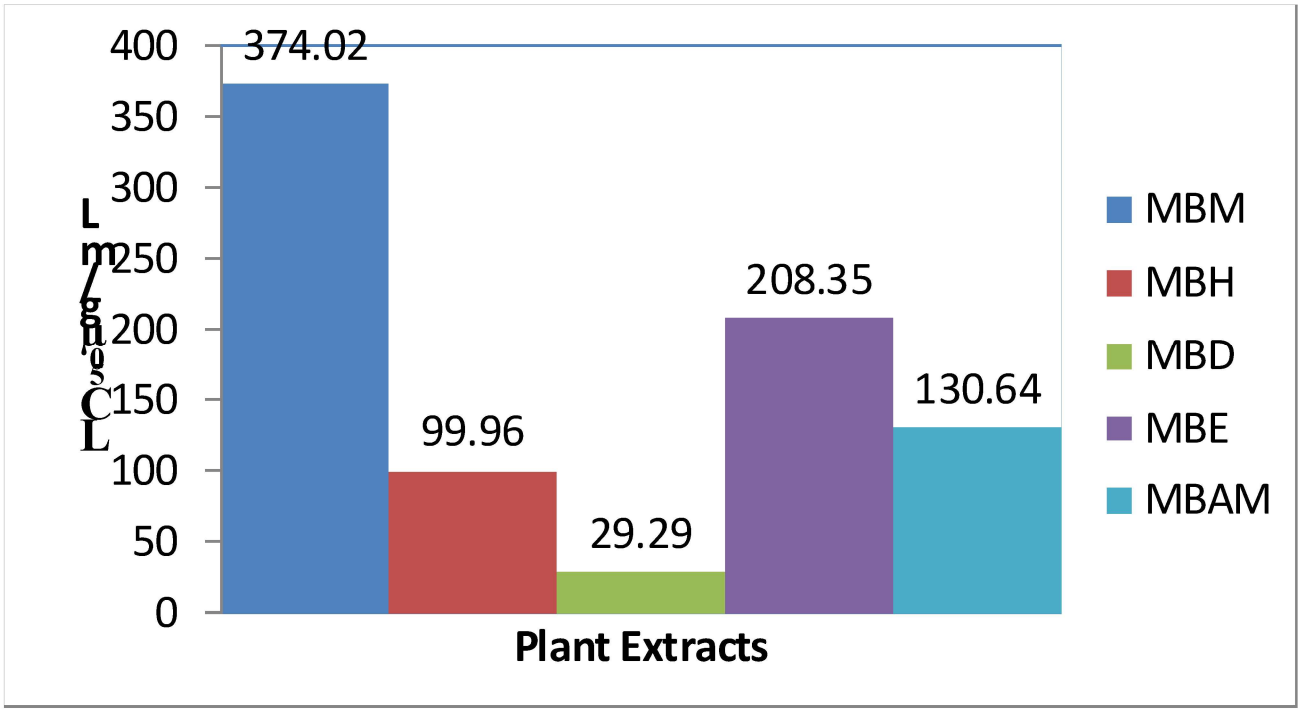


Fig. 4.59: Brine shrimp *in-vitro*cytotoxic activity of *M. benthamianum* extracts

Key:

MBM = *M. benthamianum* crude methanol extract
MBH = *M. benthamianum* hexane fraction
MBD = *M. benthamianum* dichloromethane fraction
MBE = *M.benthamianum* ethyl acetate fraction
MBAM = *M.benthamianum* methanol fraction

4.4.2 BRINE SHRIMP *IN-VITRO*CYTOTOXIC ACTIVITIES OF ISOLATED COMPOUNDS

The result of the brine shrimp cytotoxic activity of the isolated compounds (Fig. 4.0) revealed that *trans*-resveratrol has the highest activity with LC_{50} value of 7.15 while taepeenin A had the least activity with IC_{50} value of 179.18 µg/ml as is shown in Fig. 4.60. Piceatannol and gallic acid had similar LC_{50} values of 99.99 and 98.71 µg/ml respectively. This is the first report of the brine shrimp cytotoxic activity of taepeenin A. However, the brine shrimp cytotoxic activities of the polyphenols are comparable to those previously reported in literature. For instance, *trans*-resveratrol, Piceatannol and gallic acid were reported to have LC_{50} values of 56.8, 279 and 54.3 µg/ml, respectively (Ferrigni and McLaughlin, 1984and Wanjala and Majinda, 2001 results further justify the cytotoxic activity of the dichloromethane extract as resveratrol, which had the highest activity was also isolated from dichloromethane extract.

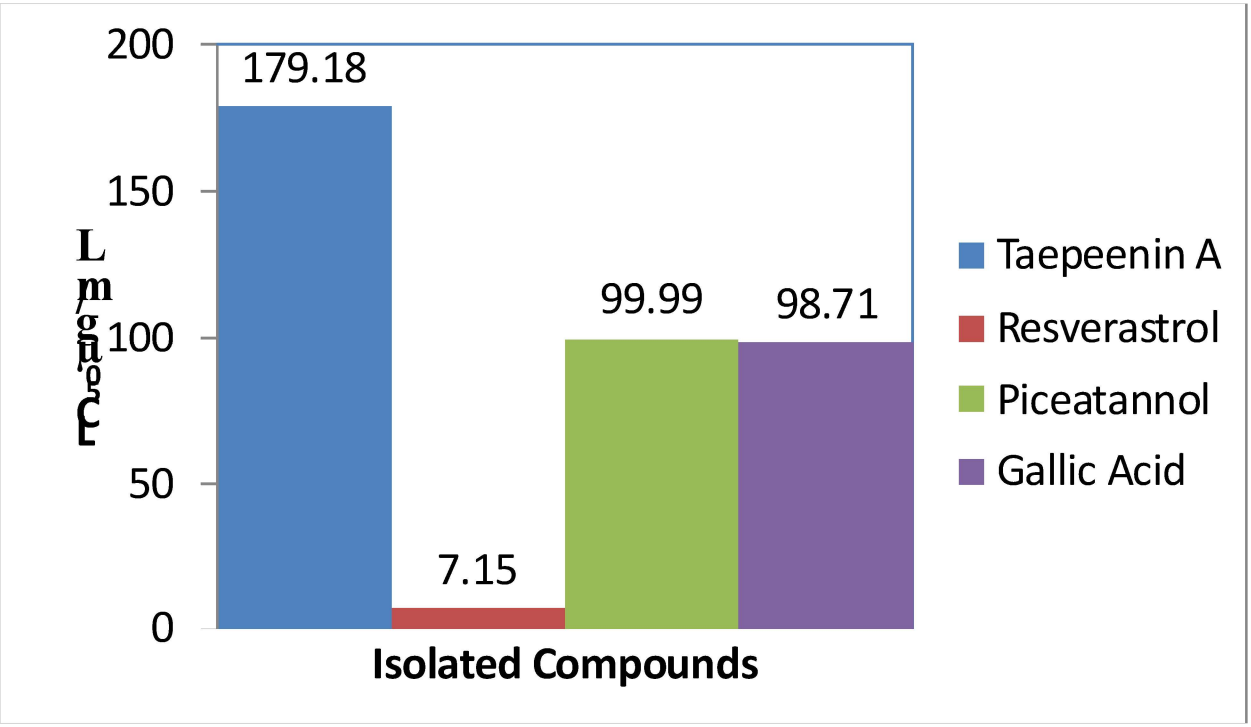


Fig. 4.60: Brine shrimp *in-vitro*cytotoxic activities of isolated compounds from *M. benthamianum*

4.5 *IN-VITRO* ANTICARIES ACTIVITIES

4.5.1 *IN-VITRO* ANTICARIES ACTIVITIES OF *M. benthamianum* EXTRACTS

The results of the antimicrobial assay of*Mezoneuron benthamianum* extracts (Tables 4.8 and 4.9; Figures 4.61 and 4.62) showed that the crude methanol extract, the ethyl acetate as well as the aqueous methanol extract of *M. benthamianum* had high antimicrobial activity against the eight strains of microorganisms used with activity indices (ratio of the zone of inhibition of the extracts to the zone of inhibition of the positive control – gentamicin) in the range of 3.2 – 5.25, 3.1 – 6.0 and 2.7 – 5.6 respectively, while the hexane extract had the least antimicrobial activity with index in the range of 0.0 – 4.38. This result further showed that the polar extract exhibited stronger antimicrobial activity against the microorganisms used in this study. This is further confirmed from the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values as shown in Fig. 4.62 and Table 4.9 respectively. The polar extracts of *M. benthamianum* are rich in polyphenols, which have been established to have antimicrobial and anti-cariogenic properties (Ferrazzano *et al*, 2011). For instance, the antimicrobial activity of the petroleum spirit, chloroform and ethanol extracts of *M. benthamianum* produced good MIC values ranging from 31.2 to 1000 µg/ mL against both gram-positive and gram-negative bacteria such as *Staphylococcus aureus, Bacillus subtillus,Micrococcus flavus,* and *Pseudomonas aeruginosa* (Dickson *et al.,* 2006),

Table 4.8: *In-vitro* Antimicaries activities of *M. benthamianum* extracts

Plant Extracts/ Control	Concentration (mg/ml)	Organisms / Zone of inhibition (mm)						
		*P. aeruginosa** (ATCC 27855)	*P. aeruginosa*	*S. aureus** (ATCC 29213)	*S. aureus*	*E. Coli** (ATCC 35218)	*E. Coli*	*S. mutans*
MBM	100	24	24	26	22	22	24	43
	50	18	18	22	18	16	18	19
	25	16	16	18	16	14	16	17
	12.5	14	14	16	14	12	14	14
	6.25	12	12	14	12	-	12	12
MBH	100	18	18	10	10	-	10	14
	50	16	16	-	-	-	-	12
	25	14	14	-	-	-	-	11
	12.5	-	12	-	-	-	-	10
	6.25	-	10	-	-	-	-	10
MBE	100	22	24	26	24	16	24	23
	50	16	22	24	22	14	22	20
	25	14	20	20	20	12	18	17
	12.5	-	14	-	12	-	-	14
	6.25	18	16	24	22	20	20	12
MBAM	100	18	20	20	18	18	28	19
	50	16	18	18	16	16	26	16
	25	14	16	16	14	14	24	14
	12.5	12	-	14	12	12	18	13
	6.25	12	-	10	-	-	16	11
Gentamicin	(10 µg/ml)	18	16	24	22	20	20	22

KEY: MBM = *M. benthamianum* methanol extract, MBH = *M. benthamianum* hexane extract, MBE = *M.benthamianum* ethyl acetate extract, MBAM = *M.benthamianum* methanol extract. "–" = No inhibition

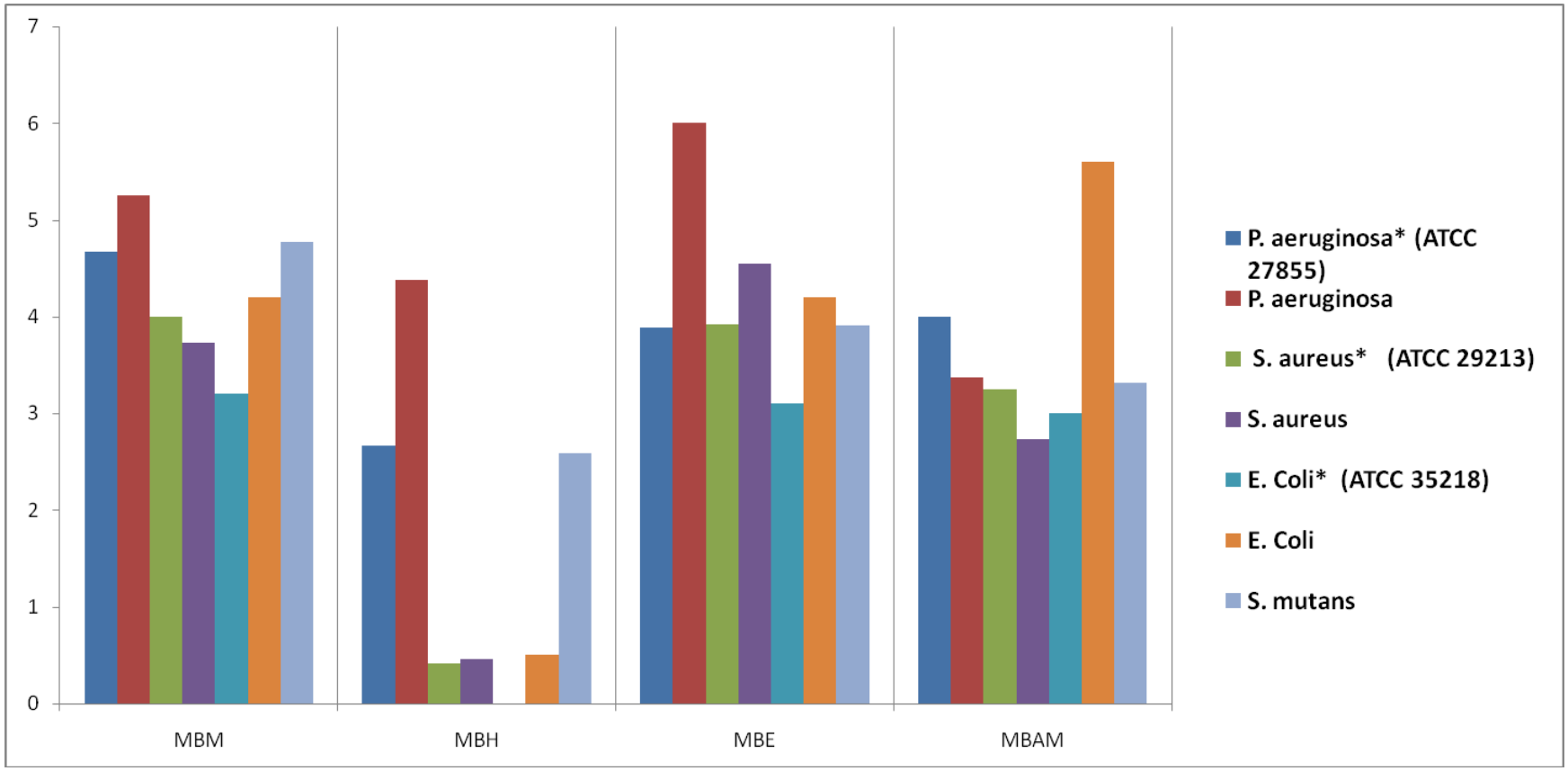


Figure 4.61: Antiicaries Activity indices of *M. benthamianum* extracts

Key:

MBM = *M. benthamianum* unpartitioned extract, MBH = *M. benthamianum* hexane extract, MBE = *M. benthamianum* ethyl acetate extract, MBAM = *M. benthamianum* methanol extract

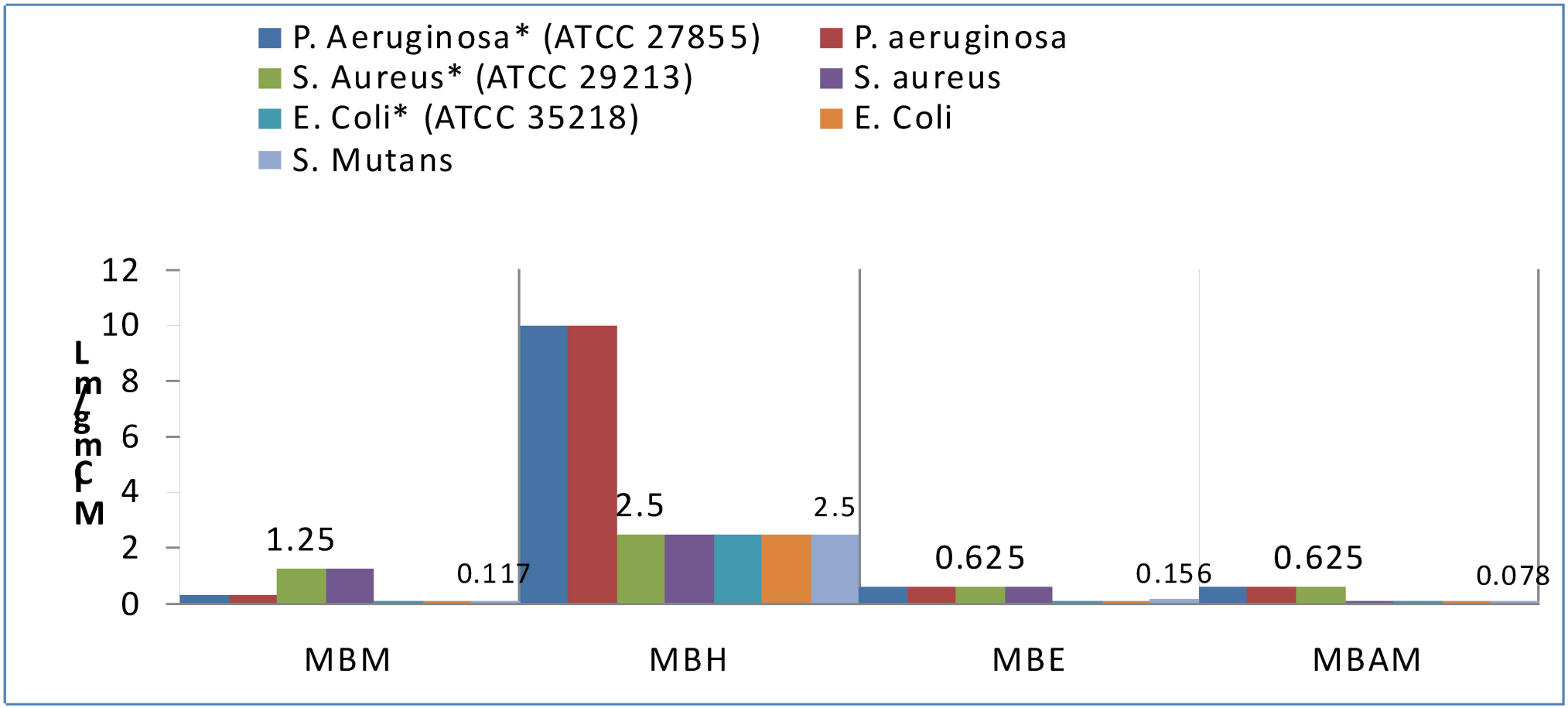


Figure 4.62: Minimum Inhibitory Concentration (MIC) of *M. benthamianum* Extracts

Key:

MBM = *M. benthamianum*methanol crude extract, MBH = *M. benthamianum* hexane fraction, MBE = *M. benthamianum* ethyl acetate fraction, MBAM = *M. benthamianum* methanol fraction

Table 4.9: Minimum Bactericidal Concentration of Plant Extracts

Plant Extracts	*P. aeruginosa** (ATCC 27855)	*P. aeruginosa*	*S. aureus** (ATCC 29213)	*S. aureus*	*E. Coli** (ATCC 35218)	*E. Coli*	*S. mutans*
MBM	> 0.625	> 0.625	> 0.625	> 0.625	>0.625	0.078	>0.625
MBH	>20.0	20.0	>20.0	2.5	20.0	>20.0	15.0
MBE	> 0.625	> 0.625	> 0.625	0.625	0.156	0.625	0.4688
MBAM	> 0.625	> 0.625	> 0.625	> 0.625	>0.625	0.078	>0.625

Key:

MBM = *M. benthamianum* methanol crude extract

MBH = *M. benthamianum* hexane fraction

MBE = *M. benthamianum* ethyl acetate fraction

MBAM = *M. benthamianum* aqueous methanol fraction

4.5.2 *IN-VITRO* ANTICARIES ACTIVITIES OF ISOLATED COMPOUNDS

The MIC values of four of the isolated compounds (Fig. 4.63) showed that they were active against all the strains of microorganisms tested with resveratrol having the highest activity with MIC value of 0.025 µg/ml against seven pathogens and 0.2 against *P. aeruginosa* (ATCC 27855), followed by piceatannol which had an MIC values of 0.025 µg/ml against four pathogens, 0.038 µg/ml against *S. mutans* and > 0.2 µg/ml against *P. aeruginosa.* Gallic acid was also effective against the oral pathogens with MIC values of 0.025 µg/ml against *E. coli*, 0.0625 against *S.mutans* and 0.1 µg/ml against *P.aeruginosa* and *S. aureus*. Taepeenin A had the least antimicrobial activity with MIC values in the range of 0.025 – 0.1 µg/ml. While this is the first report of the antimicrobial activity of Taepeenin A, the antimicrobial activity of resveratrol, piceatannol and gallic acid are well reported in previous literature. Both resveratrol and piceatannol are very important naturally occurring polyphenolic stilbenes, produced by plants in response to fungal infection, mechanical damage, or ultra-violet irradiation. Paulo et.al., (2010) reported the antimicrobial activity of resveratrol against *Bacillus cereus* ATCC 11778, *Staphyloccus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 with MIC values ranging from 50 – 200 µg/ml.

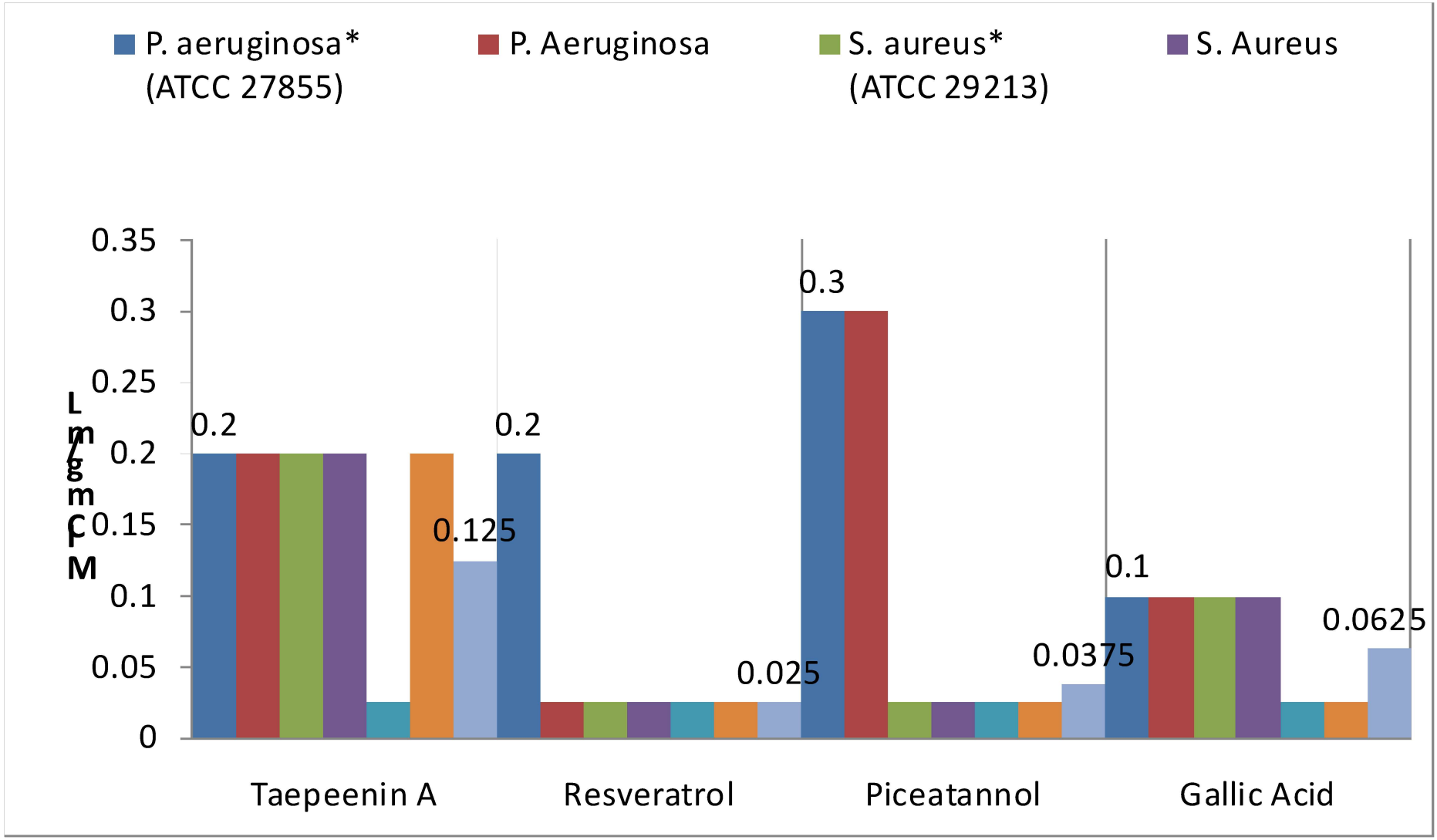


Figure 4.63: Minimum Inhibitory Concentration (MIC) of Isolated Compoundsfrom *M. benthamianum*

4.6 *IN-VITRO* ANTICANCER ACTIVITIES

4.6.1 *IN-VITRO* ANTICANCER ACTIVITIES OF PLANT EXTRACTS

The result of the *in-vitro* cytotoxic activity of the crude extracts against the four different cancer cell lines [Lung (A549), Prostate (PC-3), Lung (NCI-H322) and Breast (T47D)] is shown in Fig. 4.64. The result shows that the unpartitioned methanol, hexane and dichloromethane extracts exhibit selective inhibitory activity against Lung (A549), Lung (NCI-H322) and Breast (T47D) cell lines with % growth inhibition of 63, 50, 38; 59, 44, 41 and 52, 44, 36 % respectively. The result shows that the non-polar extract exhibited a greater cytotoxic activity against the polar extract.

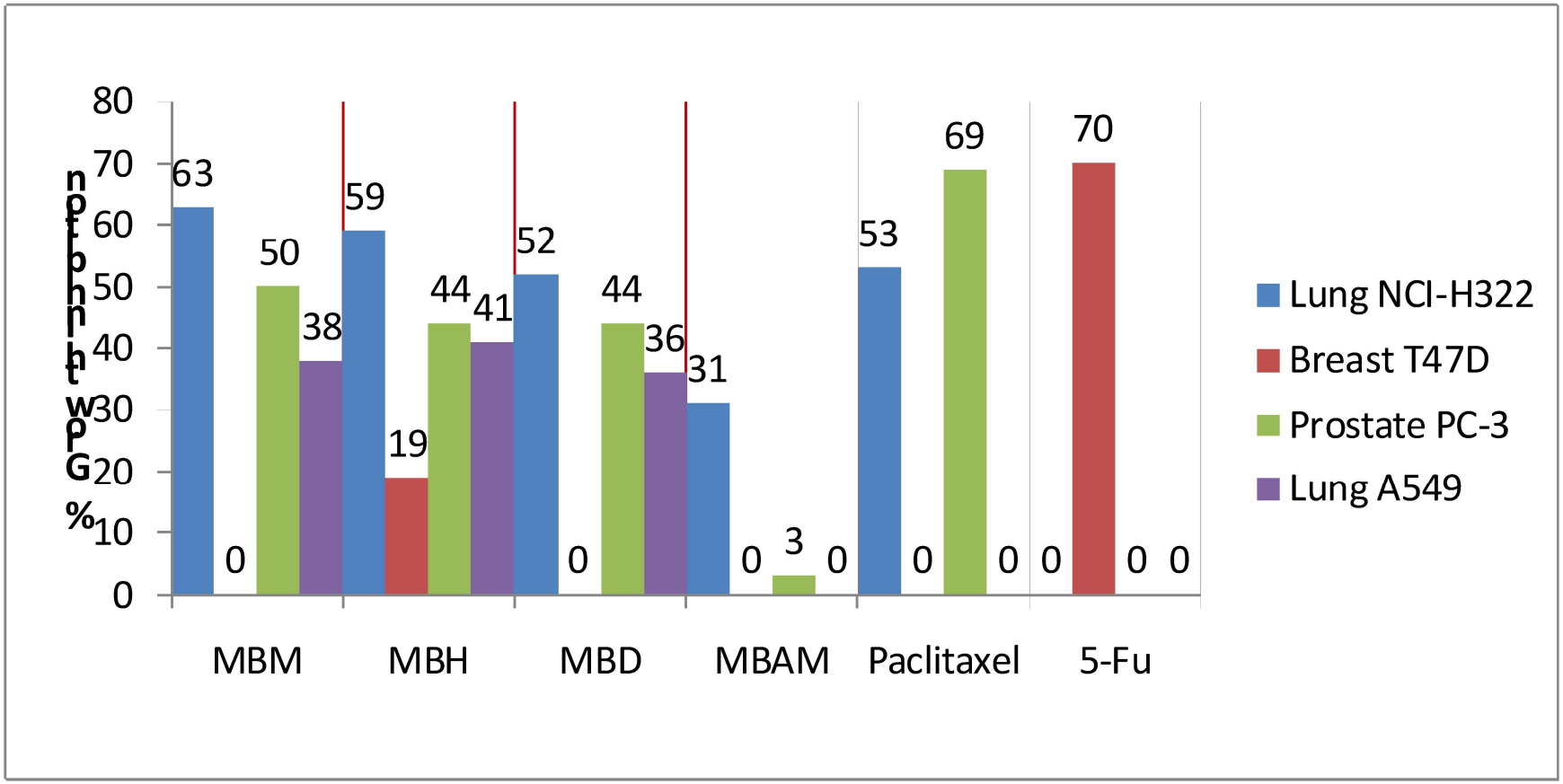


Figure 4.64: Percentage Growth Inhibition of cancer cell lines by *M. benthamianum*extracts

4.6.2 *IN-VITRO* ANTICANCER ACTIVITIES OF ISOLATED COMPOUNDS

Four of the isolated compounds (Taepeenin A, Nortaepeenin A, Stigmasterol and Resveratrol) were tested against the different cell lines - Lung (A549), Prostate (PC-3), Lung (NCI-H322) and Breast (T47D). The results of the *in-vitro* cytotoxic activity of four of the isolated compounds are shown in Table 4.10 and Fig. 4.65 respectively. The results showed that nortaepeenin A had broad spectrum activity against four of the cancer cell lines tested against with IC_{50} values of 49.4, 42.8, 38.3 and 76.2 µM against Lung (NCI-H322), Breast (T47D), Prostate (PC-3) and Lung (A549) cancer cell lines respectively. Taepeenin A showed a moderate selective activity against Lung (NCI-H322) with IC50 of 71.8 µM. It also showed that stigmasterol had a highly significant and selective activity against Breast (T47D) cancer cell line with IC_{50} value of 5 µM while resveratrol showed weak activity against Lung (NCI-H322), Breast (T47D), Prostate (PC-3), and Lung (A549) cancer cells with IC50 values of >100, 96.1, 96.6 and 75.2 µM respectively

Table 4.10: *In-vitro*anticancer activities of isolated compounds

(% Growth inhibition)

Samples	Conc (μM)	Tissue/Cell line type			
		Lung	Breast	Prostate	Lung
		NCI-H322	T47D	PC-3	A549
		% Growth Inhibition			
Taepeenin A MH1	1	26	0	0	0
	10	26	25	0	2
	30	31	29	0	5
	50	47	34	0	15
	100	**58**	37	11	23
Nortaepeenin A MH2	1	12	29	0	5
	10	15	31	1	7
	30	36	43	54	15
	50	**60**	**58**	**63**	48
	100	**61**	**59**	**65**	**57**
Stigmasterol	1	8	32	0	0
	10	9	58	0	0
	30	11	**61**	0	0
	50	15	**66**	0	0
	100	22	**68**	0	34
Resveratrol	1	0	0	0	2
	10	8	17	0	11
	30	14	22	0	19
	50	26	31	15	30
	100	44	52	**58**	**68**
Paclitaxel	1	53	-	69	-
5-Fu	20	-	70	-	-

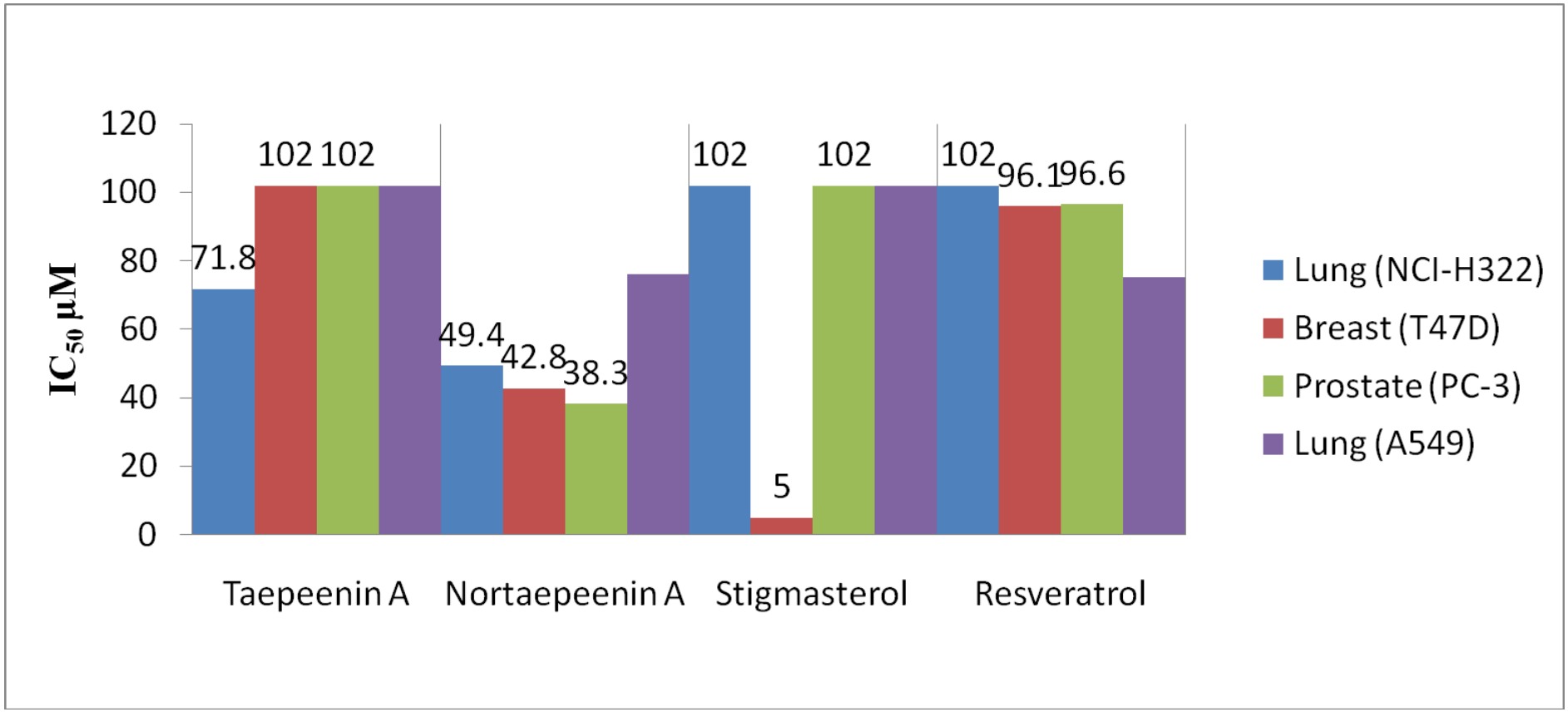
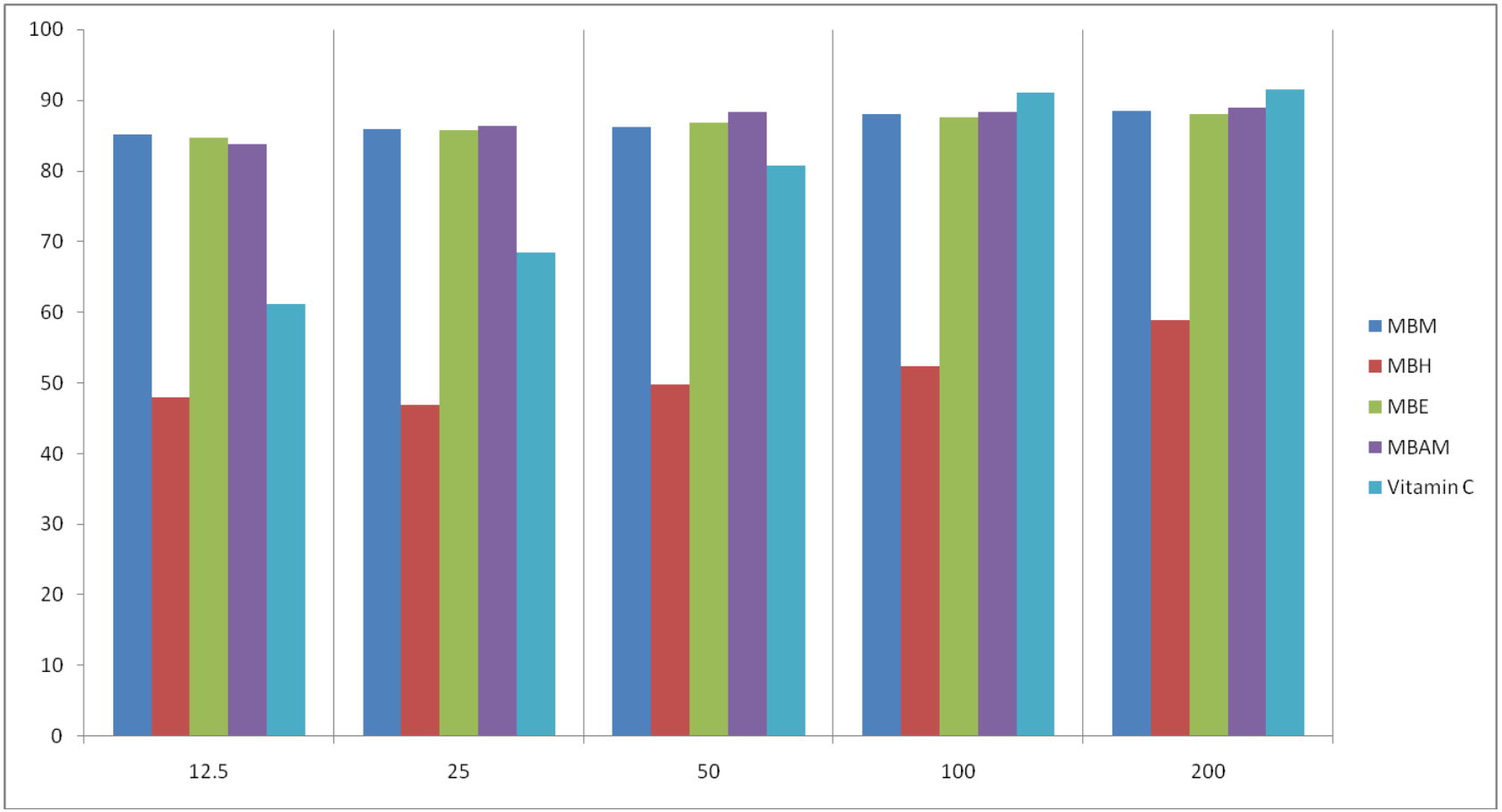


Fig. 4.65: *In vitro*Anticancer activities (IC_{50}) of isolated compounds from *M. benthamianum*

4.7 *IN-VITRO* ANTIOXIDANT ACTIVITIES

4.7.1 *IN-VITRO* ANTIOXIDANT ACTIVITIES OF PLANT EXTRACTS

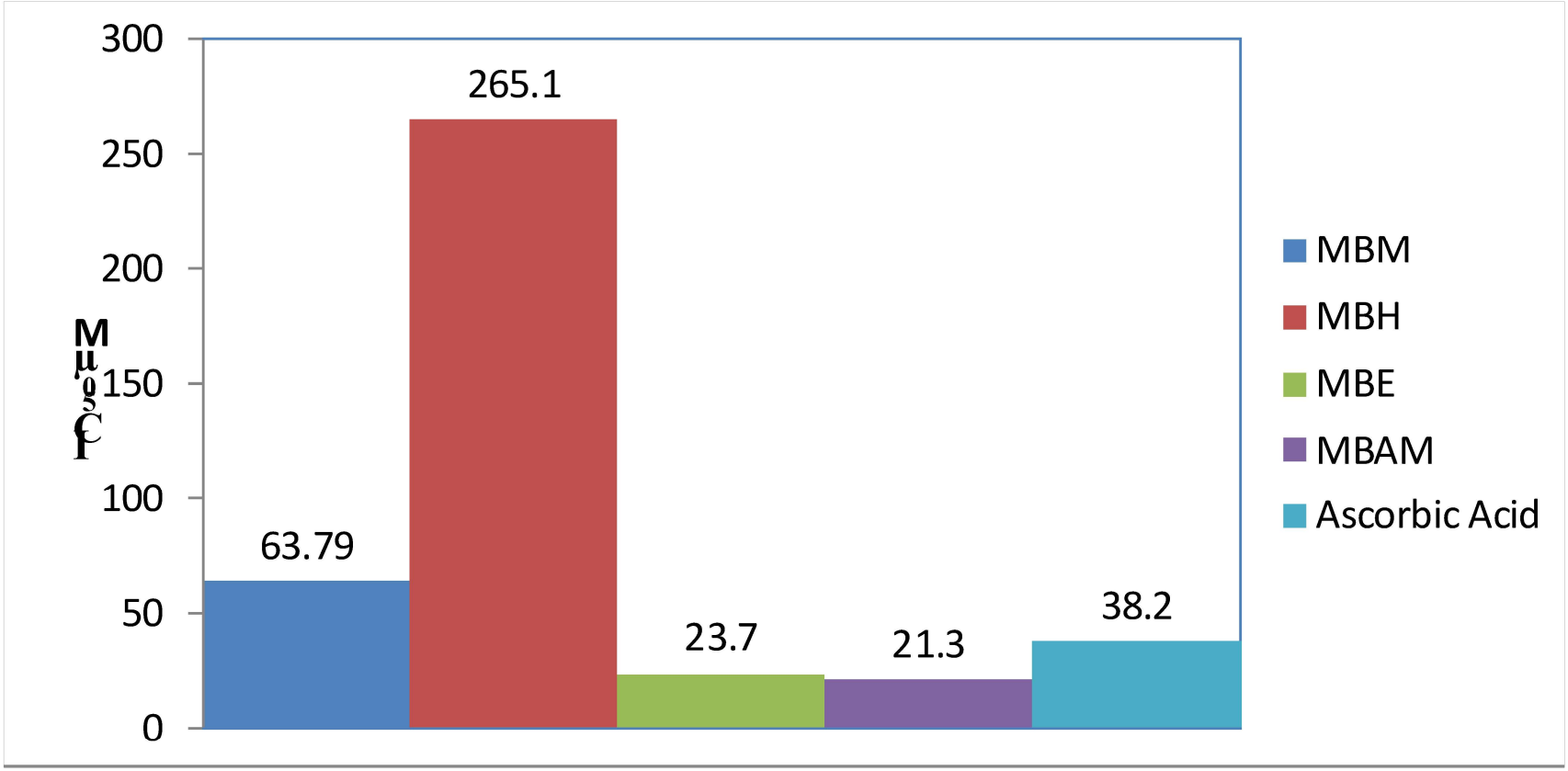
The result of the antioxidant activities of the crude extracts are shown in Fig. 4.66 and 4.67. The result showed that the ethyl acetate and methanol extracts exhibited strong antioxidant activity with IC_{50} of 23.7 and 21.3 μM respectively; while the crude methanol and hexane extracts had moderate antioxidant activity with IC_{50} of 63.79 and 265 μM respectively. The ethyl acetate and methanol extracts have higher antioxidant activity than the standard drug ascorbic acid, which showed that *M. benthamianum* is a good source of antioxidant compounds.



Key:

MBM = *M. benthamianum* crude methanol extract, MBH = *M. benthamianum* hexane extract, MBE = *M. benthamianum* ethyl acetate extract, MBAM = *M. benthamianum* aqueousmethanol extract

Fig. 4.66: Percentage Scavenging activity of *Mezoneuron benthamianum* extracts



Key:

MBM = M. benthamianum methanol crude extract, MBH = M. benthamianum hexane fraction, MBE = M. benthamianum ethyl acetate fraction, MBM = M. benthamianum methanol fraction

Figure 4.67: *In-vitro*Antioxidant Activity (IC_{50}) *M. benthamianum* extracts

4.7.2 *IN-VITRO* ANTIOXIDANT ACTIVITIES OF ISOLATED COMPOUNDS

The results of the antioxidant activity of the isolated compounds are shown in Fig. 4.68 and 4.69 respectively. The result showed that gallic acid had a very strong antioxidant activity with IC_{50} of 11.73 µM, resveratrol and piceatannol equally had strong antioxidant activity with IC_{50} of 35.81 and 30.35 µM respectively, while taepeenin A had a weak antioxidant activity with IC_{50} of 127.1 µM. Gallic acid has been established to possess the highest antioxidant potential having reduced 6 DPPH molecules per radical (Brand-Williams *et, al*., 1995). Apart from DPPH assay, gallic acid emerged as the strongest antioxidant in other well-known assays as well, such as Trolox equivalent antioxidant capacity (TEAC I-III) assay, Total radical-trapping antioxidant parameter (TRAP) assay, Photochemiluminescence (PCL) assay and Ferric reducing ability of plasma (FRAP) assay. It is found to be a better antioxidant than popular antioxidants like ascorbic acid, Trolox and uric acid (Schlesier *et al*, 2002). It has been demonstrated as the chief antioxidant component responsible for the antioxidant properties of a number of plant extracts (Veluri *et al*, 2006; Chia,*et al*, 2010; Bhadoriya *et al*, 2012 and Palafox-Carlos *et al*, 2012 and Gonzalez-Abuín *et.al*, 2014). Similarly, resveratrol and piceatannol have been shown to possess strong antioxidant activities in a number of studies (He and Yan, 2013). Piceatannol has also been shown to possess a stronger antioxidant activity than resveratrol. (He and Yan, 2013). The presence of gallic acid and piceatannol, resveratrol in *M. benthamianum* is a very good indication as one of the factors responsible for its effectiveness as a strong tool in promoting oral hygiene.

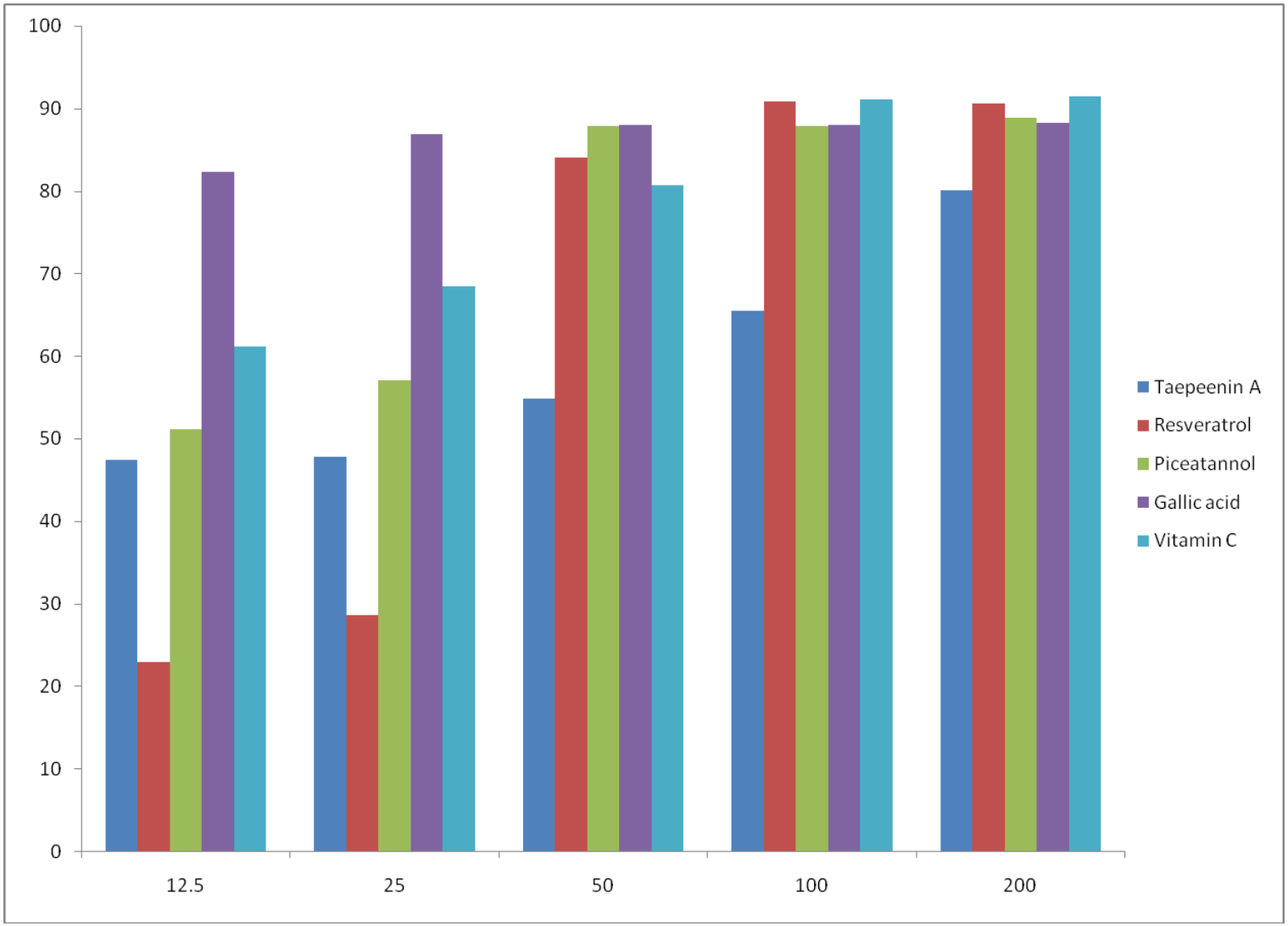


Fig. 4.68:Percentage Scavenging Activity of isolatedcompounds from *M. benthamianum*

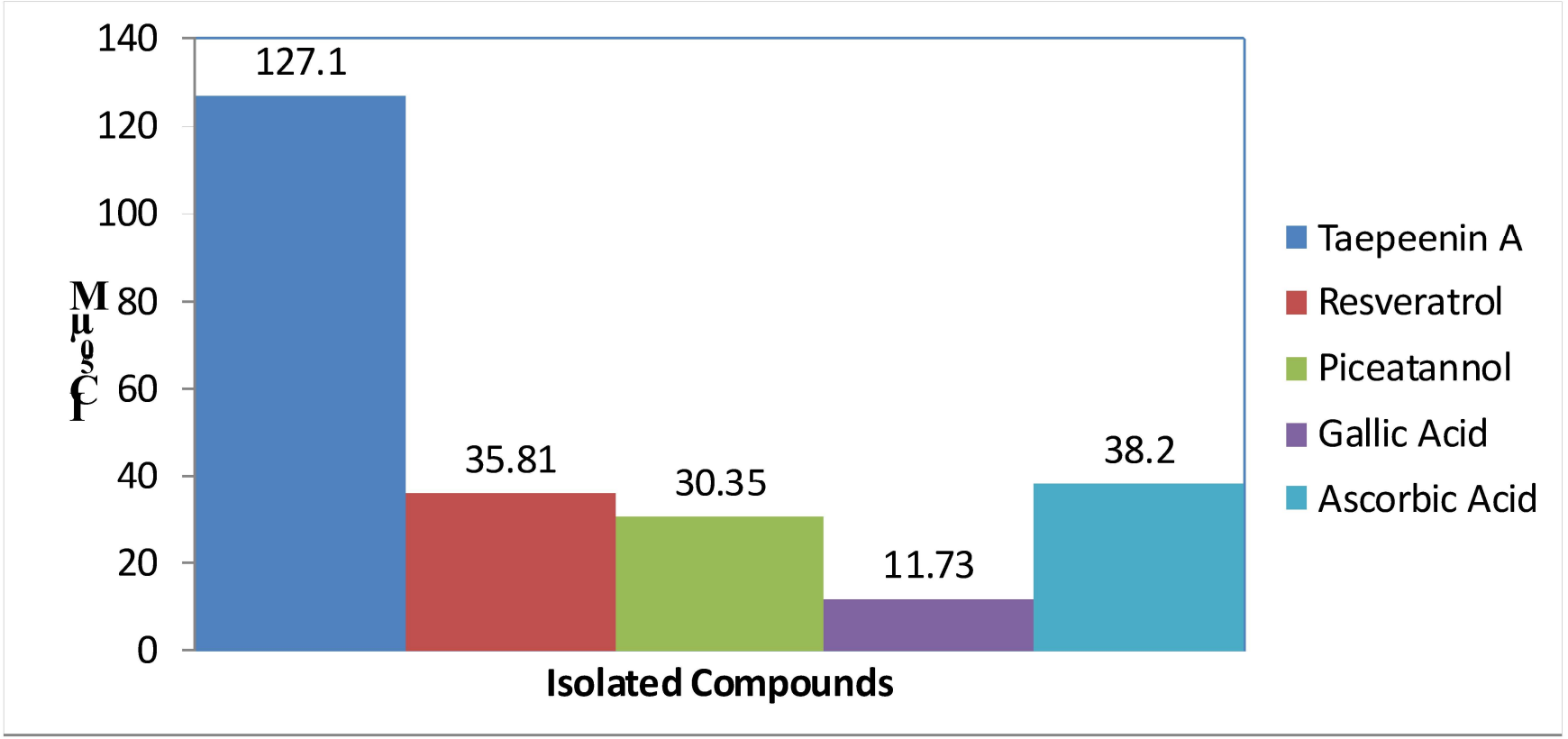


Figure 4.69: *In-vitro*Antioxidant Activity (IC_{50}) of Isolated Compounds from *M. benthamianum*

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

Medicinal properties associated with gum healing, analgesia, antisickling, haemostasis and astringence have been attributed to chewing sticks, as well as the possession of antimicrobial and plaque inhibiting effects (Wolinsky and Sote, 1983; 1984). *M. benthamianum* is a shrub that is used as chewing stick in Nigeria. Phytochemical investigation of the hexane, dichloromethane and ethyl acetate extracts of the roots led to the isolation of six compounds namely: Taepeenin A (methyl 1,2,3,4,4a,5,6,11b-octahydro-4,7,11b-trimethylphenanthro[3,2-b]furan-4-carboxylate), nortaepeenin A (methyl-1,2,3,4,4a,5,6,6a,7,11,11a,11b-dodecahydro-4,11b-dimethyl-7-oxophenanthro[3, 2-b]-furan-4-carboxylate), stigmasterol, resveratrol (3,4',5 – trihydroxylstillbene), piceatannol (3, 3'4',5-tetrahydroxyl stilbene) and gallic acid (3,4,5 – trihydroxyl benzoic acid). Taepeenin A, nortaepeenin A and piceatannol are isolated for the first time from *M. benthamianum*.

The antimicrobial activities of the extracts and isolated compounds showed that the ethyl acetate and methanol extracts had high antimicrobial activities against the eight strains of microorganism used with activities indices ranging from 3.1 – 6.0 and 2.7 – 5.6 respectively. The result showed that the crude methanol, hexane and dichloromethane extracts exhibited selective inhibitory activity against Lung (A549), Lung (NCI-H322) and Breast (T47D) cell lines with percentage growth inhibition of 63, 50, 38; 59, 44, 41 and 52, 44, 36% respectively. The result also showed that

the ethylacetate and methanol extracts exhibited strong antioxidant activity with IC_{50} values of 23.70 and 21.30 µM respectively. The crude methanol extract had a moderate activity of 63.79 µM while the hexane extract had poor antioxidant activity with IC_{50} value of 265. The result also showed that gallic acid had the highest antioxidant activity with IC_{50} of 11.73 µM and resveratrol and piceatannol had strong antioxidant activity with IC_{50} of 35.81 and 30.35 µM respectively.

Both resveratrol and piceatannol are very important naturally occurring polyphenolic stilbenes. Compared with resveratrol, piceatannol is a more potent anticancer agent and preferable agent with other biological activities like antioxidant activity (Kukreja *et. al.,* 2014).

This study justified the use of *Mezoneuronbenthamianum* as an effective tool for maintaining oral hygiene as it protects the mouth against oral microorganisms as well as scavenge free radicals in the mouth thereby preventing infections which may affect the body.

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