PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM GRACILENTUM*

By

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ABSTRACT

PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM GRACILENTUM*

Ham Yenn Pinn

Plants from the genus *Calophyllum* are locally known as "Bintagor" or "Penaga" trees. They have been used by the native peoples of Africa for treatment of various ailments such as rheumatism, lesions, diabetic sores, eye inflammation, blisters and others. In this project, phytochemical investigation on the stem bark of *Calophyllum gracilentum* resulted in the isolation of two new coumarins, namely 5,7-dihydroxy-4-(2-butyl)coumarin **[23]**, 5,7-dihydroxy-4-(2-butyl)-3,4dihydrocoumarin **[24]**, along with three known compounds, friedelin **[22],** 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid **[25]** and euxanthone **[26]** All these isolated compounds were characterized and elucidated for their structures via various spectroscopic analyses including ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, IR, UV-Vis and MS.

All the isolated compounds along with the crude extracts obtained were evaluated for their antioxidant activity through DPPH assay. In the assay, methanol crude extract was found to exhibit strong antioxidant activity with

IC_{50} value of 10.0 μg/mL which is comparable to the positive controls used, ascorbic acid (IC_{50} = 7.5 μg/mL) and kaempferol (IC_{50} = 11.5 μg/mL). Meanwhile, ethyl acetate crude extract and compound **24** displayed weak activities with IC_{50} values of 210 μg/mL and 178.5 μg/mL, respectively.

ABSTRAK

Tumbuhan daripada genus *Calophyllum* dikenali sebagai “Bintagor” atau “Penaga” pokok. Mereka telah digunakan oleh Africa orang asli Africa untuk rawatan pelbagai penyakit seperti rematik, luka, kencing manis, keradangan mata, lepuh dan lain-lain. Dalam projek ini, fitokimia mengkaji tentang batang *Calophyllum gracilentum* mangakibatkan pengasingan dua koumarin baru yang bernama 5,7-dihidroksi-4-(2-butil)koumarin **[23]**, 5,7-dihidroksi-4-(2-butil)-3,4-dihydrokkoumarin **[24]**, bersama dengan tiga sebatian yang diketahui, friedelin **[22],** 4-metil-3-[2,4,6-trihidroksi-3-(2-metilbutanoil)fenil]heksanoik asid **[25]** dan euxanthone **[26]**.Semua sebatian yang diasingkan telah dicirikan dan dijelaskan melalui pelbagai spektroskopi teknik seperti ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, IR, UV-Vis dan MS. Semua sebatian yang diasingkan bersama dengan ekstrak mentah diperolehi menjelaskan aktiviti antioksidan masing-masing menggunakan kaedah DPPH. Dalam asai, ekstrak mentah metanol didapati memberi aktiviti antioxidant yang kuat dengan nilai IC_{50} 10 μg/mL iaitu setanding dengan kawalan positif , acid askorbik (nilai IC_{50} = 7.5 μg/mL) dan kaempferol (nilai IC_{50} = 11.5 μg/mL). Sementara itu, ekstrak mentah etil asetat dan sebatian **24** mempaparkan aktiviit lemah dengan nilai IC_{50} 210 μg/mL dan 178.5 μg/mL masing-masing.

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ACKNOWLEDGEMENT

Hereby, I would like to thank my supervisor, Dr Lim Chan Kiang who gave me full support and supervised me through the whole course of my final year research project. Because of him, I managed to finish my project smoothly and his supervision is much appreciated.

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Last but not least, a million thanks to my beloved family members who supported me from time to time during the project. A special thanks also extended to my friends Boo Xin Lik and Kee Xin Lee for giving me good suggestion and taught me a lot of skills in this project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions

_____

(HAM YENN PINN)

APPROVAL SHEET

The project report entitled "**PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALLOPHYLLUM GRACILENTUM***" was prepared by HAM YENN PINN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____ Date: _____

(Dr Lim Chan Kiang)

Supervisor

Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahma

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **HAM YENN PINN** (ID No: **14ADB03218**) has completed this final year project entitled "**PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALLOPHYLLUM GRACILENTUM***" supervised by Dr. Lim Chan Kiang from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(HAM YENN PINN)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

δ	Chemical shift
δ_C	Chemical shift of carbon
δ_H	Chemical shift of proton
µg	Microgram
µL	Microliter
^{13}C	Carbon -13
1D-NMR	One Dimension Nuclear Magnetic Resonance
^{1}H	Proton
2D-NMR	Two Dimension Nuclear Magnetic Resonance
A_0	Absorbance of the blank (negative control)
A_1	Absorbance of the test sample
Acetone-d_6	Deuterated acetone
C=C	Carbon=Carbon
C=O	Carbon=Oxygen (or Carbonyl)
CC	Column Chromatography
$CDCl_3$	Deuterated chloroform
cm	Centimeter
C-O	Carbon-Oxygen (or Carbinol)
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
DPPH	1,1-diphenyl-2-picrylhydrazyl
EtOAc	Ethyl acetate
FTIR	Fourier-Transform Infrared Spectoscopy
g	Gram
GC-MS	Gas Chromatography –Mass Spectrometry

HMBC	Heteronuclear Multiplet Bond Coherance
HMQC	Heteronuclear Multiplet Quantum Coherance
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
IR	Infrared
J	Coupling constant in Hertz
KBr	Potassium bromide
kg	Kilogram
LC-MS	Liquid Chromatography –Mass Spectrometry
m	Multiplet
MeOH	Methanol
mg	Miligram
mL	Mililiter
mm	Milimeter
mol	Mole
nm	Nanometer
NMR	Nuclear Magnetic Resonance
ºC	Degree in Celsius
O-H	Oxygen-Hydrogen (or Hydroxyl)
ppm	Part per million
R_f	Retention factor
s	Singlet
t	Triplet
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV-Vis	Ultraviolet-Visible

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Natural products are referring the chemical composition those are produced by living organisms including plants, animals and microorganisms. In other words, they are referring to the chemical substances those are found in nature. These substances can be divided into two main classes, which are primary and secondary metabolites. Primary metabolites are found to be important and play a significant role in life processes. Primary metabolites include proteins, carbohydrates, nucleic acid and enzyme that can be found in all living organisms as these substances are substantial for healthy growth of living organisms. Meanwhile, secondary metabolites are not essential for development and growth of living organisms. They are synthesized for the homeostasis and functional statue of cells in organisms. The absence of these molecules will not give adverse effect to the plants. Usually, plants used their secondary metabolites as defensive weapons against predators. Besides, some secondary metabolites give colour and fragrance to assist in pollination. Owning to the plants' biological activities, for centuries traditional medicine has made use of their secondary metabolites extensively. They are still valued today as unique raw materials for agriculture, food and pharmaceuticals industries (Muranaka and Saito, 2010).

In ancient civilization, natural products were served as drugs to treat various illnesses. The knowledge of use of natural product for disease treatment was developed and resulted from mankind's continuous excavation and experiments to search for suitable foods to cure infection. Besides, the Greek physician and ancient Egyption also recorded the uses of natural product and medicinal herbs (Cragg and Newman, 2002). In folklore, lichens had been used to manufacture medicine in the early Chinese and Egyptian civilizations. It was recorded that *Usnea dillenius* ex Adanson was one of the ingredients in anti-dandruff shampoos (Esslinger, 2001).

Phytochemistry is the study of a huge variety of chemicals derived from plants. The common methodologies involved are extraction, isolation, purification and structure elucidation of phytochemicals. Innovation of technology and equipment produces drugs through the chromatographic isolation and purification of bioactive compounds from plants species. Natural products are used as the templates for chemical synthesis. Once the structure and biological function of a natural compound is identified, mankind will produce them synthetically. In 1870s, crude morphine was extracted from the plant *Papaver somniferum L (opium poppy)*. Morphine was boiled in acetic anhydride to yield diacetylmorphine (heroin) and was found to be readily converted to codeine (pain killer) (Dias, et al., 2012).

Secondary metabolites are classified based on their chemical structures such as alkaloids, coumarins, flavanoids, xanthones which are non-nutritive and not essential to life processes. However, according to Lee, et al. (1998), these chemical constituents have received considerable attention as cancer chemopreventive agents. Some phytochemicals showed antioxidant properties which are natural antioxidants which may benefit human health. Cellular metabolism produces reactive and unstable free radicals. These free radicals can lead to cell damage, causing oxidation stress resulted from unbalance between pro-oxidants and antioxidant mechanisms due to excessive oxidative metabolism. The oxidative metabolism can increase the risk of developing certain type of cancers and heart diseases such as mutagenesis and carcinogenesis. An antioxidant is a molecule that inhibits the oxidation of other molecules. It terminates the radical chain reaction by oxidizing itself and converts the free radical intermediates to a relatively stable compound. Therefore, antioxidants are termed as free radical scavengers. In 1997, resveratrol, a phytoalexin was found in grapes which exhibited cancer chemopreventive activity and acted as anti-initiation, anti-promotion and anti-progression of cancer in human body (Jang, et al., 1997).

According to National Cancer Institute (NCI), nearly half of the 200 most widely-prescribed drugs in the market are originated from natural product or its derivatives (Gupta, et al., 2005). From 2000 to 2003, 15 new natural product-derived drugs were launched and registered at the end of 2003 (Butler, 2004). In order to discover more and new biological active natural products, intensive researches focusing on a wide variety of plants species are carrying

out. This project has been focused on the isolation of bioactive secondary metabolites from *Calophyllum gracilentum* and evaluation for their antioxidant activity.

1.2 Botany of Plant Species Studied

Calophyllum is the greatest genus in the family of Calophyllaceae with about 190 species. It can be easily recognized by its opposite leaves with close parallel venation sequential with resin canals (Stevens, 1980). The *Calophyllum* tree normally can grow up to 30 m height. The tree and leaves of *Calophyllum* species is shown in Figure 1.1.

Figure 1.1: The tree and leaves of *Calophyllum* species

1.2.1 Taxonomy of Plant Studied

The taxonomy of *Calophyllum gracilentum* is shown in Table 1.1

Table 1.1: Taxonomy of *Calophyllum gracilentum*

Kingdom	Plantae
Division	Tracheophyta
Class	Spermatopsida
Order	Malpighiales
Family	Calophyllaceae
Genus	*Calophyllum*
Species	*Calophyllum gracilentum*

1.3 Geographical Distribution and Habitat

The *Calophyllaceae* family is a popular and significant group of trees in tropical Asia and Africa. It is locally known as *Bintagor* or *Penaga* tree. Some *Calophyllaceae* trees can be found in Malaysia, India, Sri Lanka and Australia. They grow in the lowland and hill forest, some of which on rocky and sandy sea shores. The distribution of *Calophullum* around the World is shown in Figure 1.2.

Figure 1.2: The distribution of *Calophyllum* around the World

1.4 Ethnomedicinal Uses and Pharmacological Studies

Calophyllum species have been used as traditional medicines by native peoples. The oil of *Calophyllum* is used for treatment of rheumatism and arthritis as well as lesions in southern Africa. Besides that, the oil can also be used to treat sun burn and diabetic sores. The leaves are used for healing eye inflammations and skin care. Its wood is strong and hard and hence, it is used for furniture, ship building and construction (Sunder, et al., 2014).

According to literature reports, *Calophyllum* species are rich in different classes of bioactive phytochemicals including coumarins, xanthones, chromanones, terpenes and steroids. Therefore, it has attracted the interest of

many researchers to carry out their investigation on the chemical and biological potentials of *Calophyllum* plants. Furthermore, Filho et al. (2009) reported that *Calophyllum* species showed cytotoxic activity against various cancer cell lines, inhibition of HIV-1 reverse transcriptase, anti-secretory and cytoprotective properties. Apart from that, they also displayed antinoceptive, molluscidal and antimicrobial effects.

1.5 Problem Statement

Antioxidant is a molecule that inhibits the adverse effects of the reactive oxygen species (ROS). ROS is known as unstable free radical that induces oxidation and causes cell damage. Natural antioxidant normally comes from plants such as vegetables and fruits. Some of them contain the phenolic compounds which can stabilize the free radical and minimize the risk of cancer.

There are a lot of synthetic antioxidants in the market including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tertiary butylhydroxyanisole (TBHQ). However, people still more relying to the natural antioxidant as synthetic antioxidant contains side effects which might cause pathological and carcinogenic effects. In addition, synthetic antioxidant is more toxic and not good for human health as compared with natural antioxidant.

In this study, *Calophyllum* gracilentum was investigated for its antioxidant activity via DPPH assay by using ascorbic acid and kaempferol as positive controls.

1.6 Objectives of Study

The purposes of conducting this study are:

- To extract and isolate chemical constituents from the stem bark of *Calophyllum gracilentum*.
- To identify and characterize the chemical structures of isolated compounds through the use of modern spectroscopic techniques.
- To study the antioxidant activity of the isolates and crude extracts of *Calophyllum gracilentum* through DPPH assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemical Studies

Phytochemistry is the study of chemical constituents that are naturally derived from plants. Those chemical constituents are also known as secondary metabolites or in their general term as "phytochemicals". The main concern of phytochemical study is to investigate the chemical composition that obtained from plants and to study the chemical phenomena that occur in various plant processes (Burrell, 1937). In comparison with primary metabolites, secondary metabolites are mostly composed of non-nutrient chemicals and most of them are commonly found in grains, vegetables and fruits (Tuorkey, 2015). Many biological active secondary metabolites found their uses in various industries such as pharmaceutical, nutraceutical and cosmeceutical industries.

Plants produce different classes of secondary metabolites including alkaloid, xanthone, coumarin, flavonoid and tannis that are highly valuable to humans. These bioactive compounds have been used for healing of patients and protection against various diseases. According to World Health Organization (WHO), a majority of people are still relying on the plant-based traditional medicines that are closely related to their original ethnopharmacological practice (McRae, et al., 2007). Undeniably that the bioactive natural compounds are of structural novelty and can be served as lead compounds for

chemical synthesis to give a variety of chemical derivatives with enhanced biological activities (Naithani, et al., 2010). Therefore, drugs industries are rapidly developing which aim to discover more useful natural products to serve the demand in the market. Some natural products were found to exhibit biological activities such as anti-malarial, antioxidant, antimicrobial, antiviral and antitumor. This discovery may help to reduce the risk and to prevent chronic and degenerative diseases (Bellik, et al., 2012).

2.1.1 Coumarins

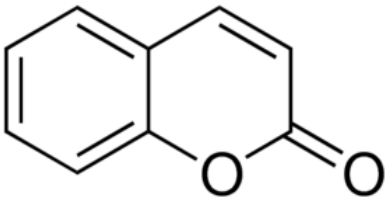


Figure 2.1: Basic building block of coumarins

Coumarins (1-benzopyran-2-one) are a subgroup of lactose. They are chemical compounds in the benzopyrone class of organic compounds, mostly found in plants. Structurally, they are oxygenated heterocyclic compounds which contain an oxygen atom double bonded to a carbon atom that attached to another oxygen atom in a closed ring with a benzene ring attached to it giving the molecular formula of $C_9H_6O_2$. There are more than 3400 coumarin derivatives that occur in nature (Boysen, et al., 2010).

Previous studies reported coumarins to exhibit a wide range of biological activities such as antioxidant, anti-inflammatory and anti-HIV. This may be due to their unique characteristic pharmacophore of planar aromatic nucleus connected with a hydrogen bond acceptor and the lactone group as a facilitator of protein-ligand binding (Torres, et al., 2014). Furthermore, different types of coumarins show particularity to different molecular targets, as antiviral agents.

2.1.2 Xanthones

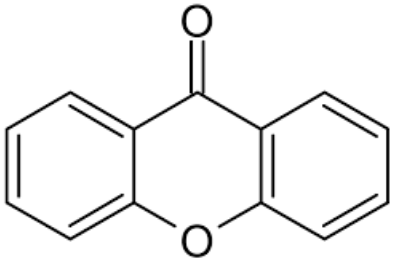


Figure 2.2: Structure of xanthone core

Xanthone (*9H*-xanthen-9-one) consists of two benzene rings linked by an oxygen bridge and a carbonyl group to give fused ring xanthone framework. It is commonly found in some higher plants, fungi and lichens. Xanthones can be divided into several classes based on the types of substituents present in the dibenzo-γ-pyrone scaffold which include simple oxygenated xanthones, prenylated xanthones and glycosylated xanthones (Sung, et al., 2012).

The biological activities elicited by xanthones are largely depending on the types and positions of the substituents in the nucleus. Previous study reported, γ-mangostin (γ-MG), a xanthone derivative isolated from *Garcinia mangostana* was used for treatment of chromic ulcers, diarrhoea, abdominal pain, dysentery and wound infections in folk medicine (Sung, et al., 2012). Furthermore, it also displayed cytotoxic effects on gastric, liver, breast and leukemia cancer cell lines (Sharma, 2013).

2.1.3 Terpenes

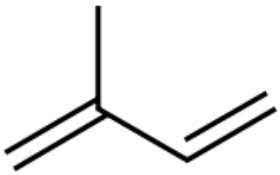


Figure 2.3: Molecular structure of isoprene

Terpenes are a large groups of compounds made up of one or more five-carbon isoprene units (CH_2=C(CH_3)-CH=CH_2) giving a general molecular formula of $(C_5H_8)_n$ whereby n indicated the number of isoprene unit. The isoprene units are linked one to another in head to tail manner. The difference in the number of isoprene units linked together gives different classes of terpenes such as monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes (Aldred, et al., 2009). Example of monoterpenes and sesquiterpenes are pinene and farnesol, respectively. Diterpenoids incur some

bitter flavour while triterpenes are found in small amount in edible oil. Carotenoids are the major constituent of tetraterpenoids (Gordon, 2013).

Terpenoids in the plant serve as an important antimicrobial agent against pathogens, enhance interactions with beneficial organisms, defend from herbivores, act as internal signalling and resistance to abiotic stresses (Jia, et al., 2018). Terpenoids show a diverse range of biological activities including antiviral, antibacterial, antifungal, anti-inflammatory, antiallergenic, antihyperglycemic, antiparasitic, antispasmodic, immunomodulatory and antitumoral activities *(*Soltani, 2016).

2.1.4 Flavonoids

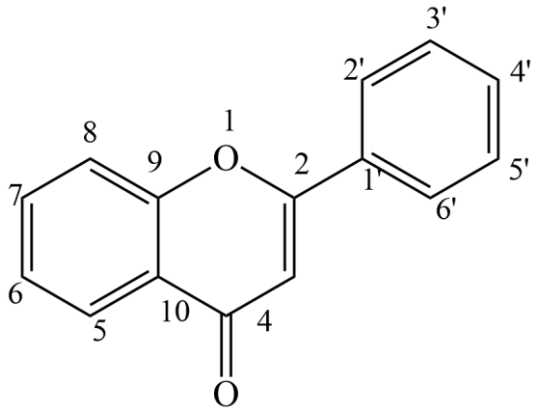


Figure 2.4: Molecular structure of flavonoids

Flavonoids mostly are phenolic compounds which consist of a benzene ring joined to a benzo-γ-pyrone moiety. They serve as plant's pigments that responsible for the colours of flowers and fruits. They also protect the plants

from ultraviolet radiation, pathogens and herbivores. Among the 2000 flavonoids isolated, 500 of them are in free (aglycone) state while the others are in O- or C-glycosides state. The three main types of flavonoids having their carbonyl carbon at position 4 are flavones, flavonols and flavanones which are water-soluble (Bone and Mills, 2013). The most common flavonoids found in nature are quercetin and kaempferol.

Flavonoids are indispensable for nutraceutical, pharmaceutical, medicinal and cosmetic applications (Panche, et al., 2016). Researchers had screened for their therapeutic potential for treatment of diabetes, cardiovascular diseases, cancers, neurodegenerative diseases, and osteoporosis (Ziberna, et al., 2014). This is due to that most flavonoids show good ability in scavenging free radicals such as hydroxyl, peroxyl, superoxide radicals, and singlet oxygen (Wiart, 2013).

2.2 Chemistry Biological Studies of *Calophyllum* Species

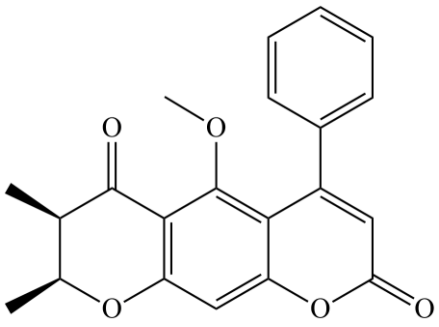
The genus *Calophyllum* consists of about 200 species but not even half number of them have been phytochemically studied. Among the *Calophyllum* species, *C. inophyllum*, *C. brasiliense*, *C. flavoramulum* and *C. membranaceum* are more well-studied than others. Previous studies on *Calophyllum* species have found them to be rich in chromanone acids, coumarins, xanthones, flavonoids and triterpenoids (Su, et al., 2008). Their

biological properties were also evaluated through modern *in vivo* and *in vitro* studies.

2.2.1 *Calophyllum incrassatum*

In year 2016, Aminudin et al., have successfully isolated four new coumarins, namely (7*R*,8*S*)-7,8-dihydro-5-methoxy-7,8-dimethyl-4-phenyl-2*H*,6*H*-benzo[1,2-*b*;5,4-*b*']dipyran-2,6-dione **[1]**, (7*S*,8*S*)-4-butyl-7,8-dihydro-5-hydroxy-7,8-dimethyl-*2H,6H*-benzo[1,2-*b*;5,4-*b*']dipyran-2,6-dione **[2]**, (7*S*,8*S*)-4-ethyl-7,8-dihydro-5-hydroxy-7,8-dimethyl-*2H,6H*-benzo[1,2-*b*;5,4-*b*']dipyran-2,6-dione **[3]**, (7*S*,8*S*)-7,8-dihydro-5-hydroxy-4,7,8-trimethyl-*2H,6H*-benzo[1,2-*b*;5,4-*b*']dipyran-2,6-dione **[4]**, along with 7,8-dihydro-5-hydroxy-7,8-dimethyl-4-propyl-*2H,6H*-benzo[1,2-*b*;5,4-*b*']dipyran-2,6-dione **[5]** from the plant *Calophyllum incrassatum* (Aminudin, et al., 2016).

All of the isolated compounds were studied for their α-glucosidase enzymatic inhibitory and cytotoxic activities. However, only compound **1** showed weak activities against α-glucosidase enzymatic activity and A-549 cancer cell line with high IC_{50} values of 93.25 and 87.71 μM, respectively. Other coumarin derivatives **2** to **5** were found inactive toward A-549 and MCF-7 cancer cell lines and α-glucosidase enzymatic inhibitory assay (Aminudin, et al., 2016).



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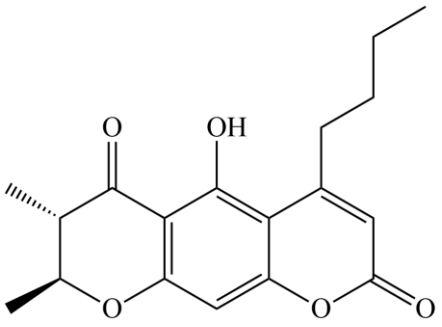

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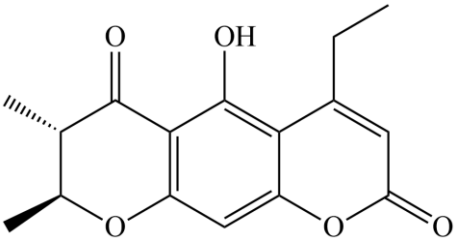

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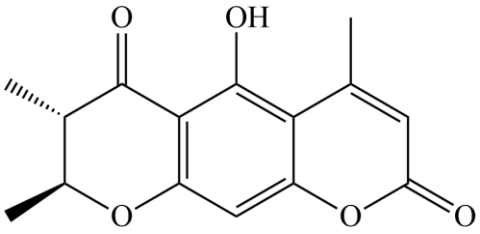

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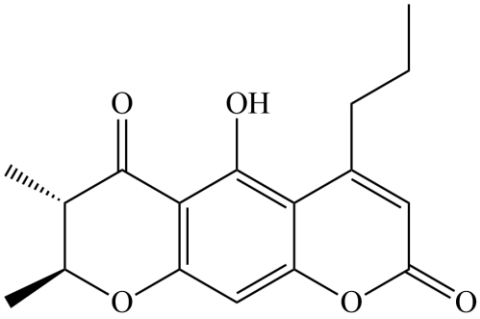


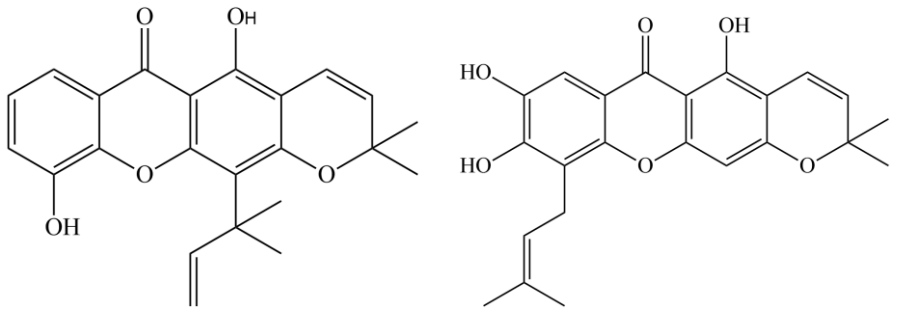
[5]

Figure 2.5: Molecular structures of chemical constituents isolated from *Calophyllum incrassatum*

2.2.2 *Calophyllum inophyllum*

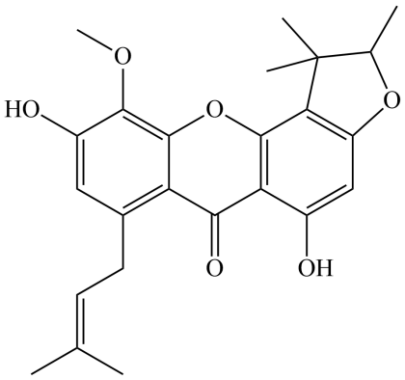
In year 2004, chemical study on the root bark and the nuts of *Calophyllum inophyllum* yielded a new xanthone derivative, namely inoxanthone **[6]** along with twelve known compounds which were caloxanthones A [**7]**, caloxanthones B [**8]**, macluraxanthone [**9]**, 1,5-dihydroxyxanthone [**10]**, calophynic acid [**11]**, brasiliensic acid [**12],** inophylloidic acid [**13]**, friedelan-3-one [**14]**, calaustralin [**15]**, calophyllolide [**16]**, inophyllums C [**17]** and inophyllums E [**18]** (Yimdjo, et al., 2004).

Compounds **7**, **11**, **12** and **13** and **16** displayed a strong cytotoxic activity with IC_{50} values of 7.4, 10.5, 11.0, 9.7 and 3.5 μg/ml, respectively, while the other compounds showed moderate to weak inhibitory activities toward KB cells (Yimdjo, et al., 2004).



[6] **[7]**

Figure 2.6: Molecular structures of chemical constituents isolated from *Calophyllum inophyllum*



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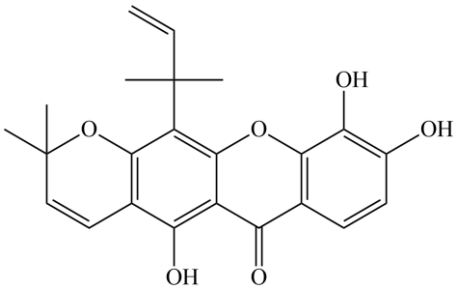

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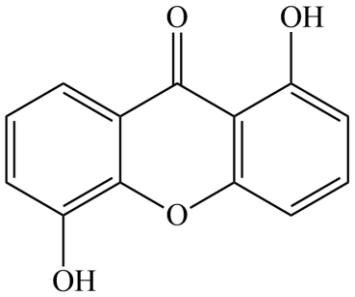

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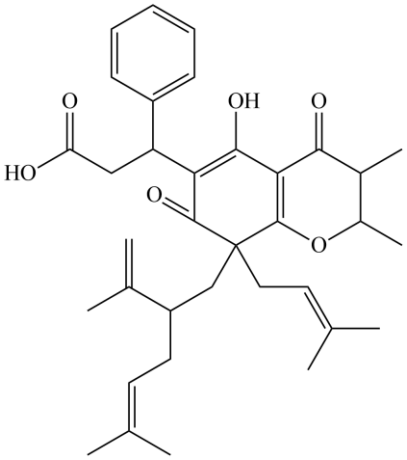

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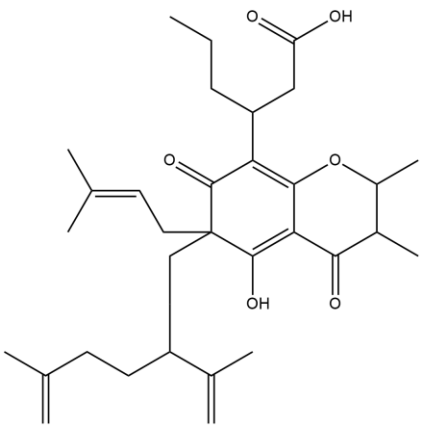

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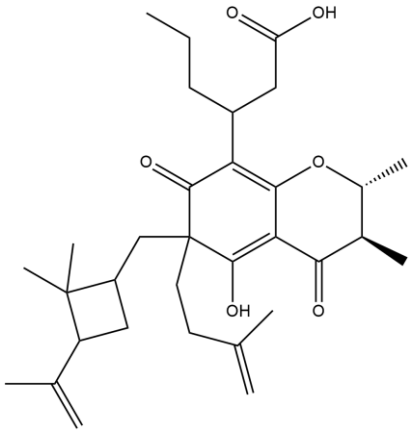
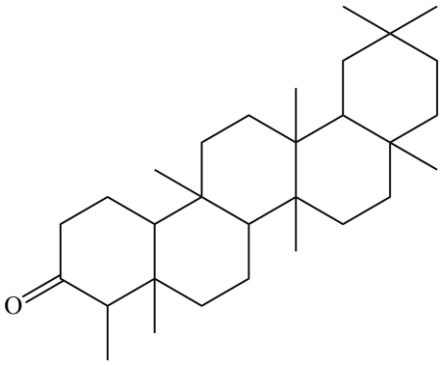


[13]

Figure 2.7: Molecular structures of chemical constituents isolated from *Calophyllum inophyllum* (continued)



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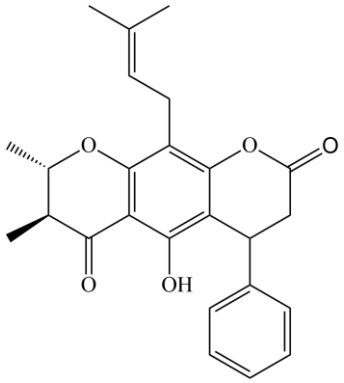

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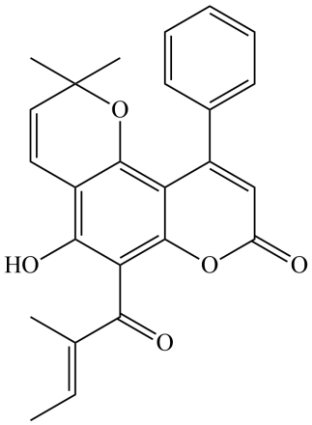

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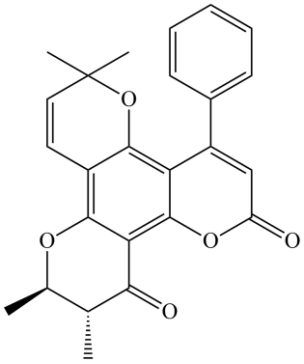

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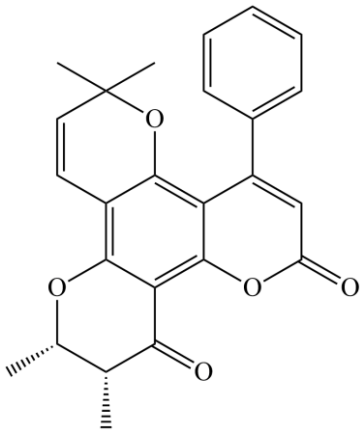
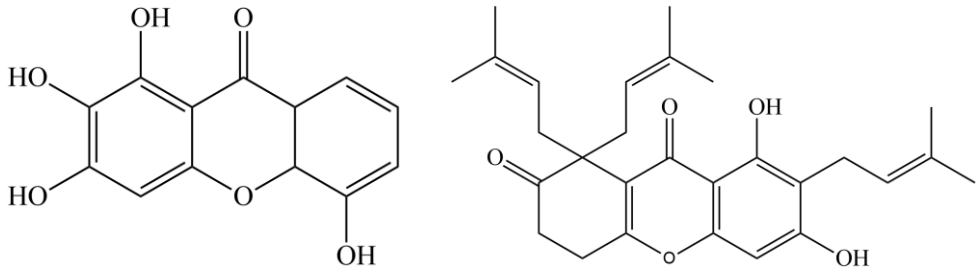


[18]

Figure 2.8: Molecular structures of chemical constituents isolated from *Calophyllum inophyllum* (continued)

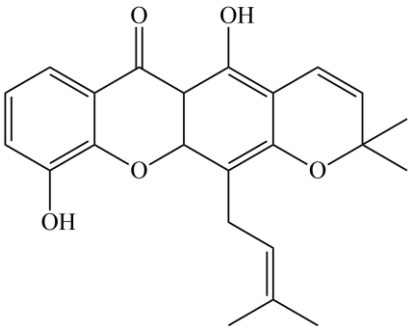
2.2.3 *Calophyllum gracilipes*

In year 2002, three xanthones, namely gracixanthone **[19]**, zeyloxanthone **[20]** and trapezifolixanthone **[21]** were isolated from the stem bark of *Calophyllum gracilipes* (Nasir, et al., 2013). These three compounds were studied for their cytotoxic activity by using colon carcinoma (HTC-116), prostrate carcinoma (PC3), mouse macrophages (RAW264.7), African Green Monkey kidney (VERO) and human breast adenocarcinoma (MCF-7) cell lines. From the assay, compound **20** displayed strong cytotoxicity with IC_{50} values of 9.56, 8.00, 8.22, 26.00 and 10.00 μg/mL toward HTC-116, PC3, RAW264.7, VERO and MCF-7 cell lines, respectively. The remaining compounds **19** and **21** showed no significant activity against all the cell lines (Nasir, et al., 2013).



[19] **[20]**

Figure 2.9: Molecular structures of chemical constituents isolated from *Calophyllum gracilipes*



[21]

Figure 2.10: Molecular structures of chemical constituents isolated from *Calophyllum gracilipes* (continued)

2.3 Summary of Literature Review on the Genus *Calophyllum*

Table 2.1: Summary of literature data on the Genus *Calophyllun*

Plant Species	Classes of Compound	Biological Activities	References
C. antilanum	• Chromanones	• Antimalarial • Cytotoxic	• Cuesta-Rubio, et al., 2015
C. apetalum	• Coumarins • Chromanone acids • Triterpenes • Xanthones	-	• Inuma, et al., 1997 • Nigam and Mitra, 1967 • Govindachari, Prakash, and Viswanathan, 1968
C. austroindicum	• Xanthones	-	• Iinuma, et al., 1996
C. blancoi	• Coumarins	• Antitumor	• Shen, et al.,

	• Chromanones • Xanthones	• Antiviral • Cytotoxic	2004; 2005 • Stout and Karl, 1968
C. bracteatum	• Xanthones	-	• Dharmaratne, 1986
C. brasiliense	• Chromanones • Xanthones • Coumarins • Flavonoids	• Antibacterial • Antiulcer • Antioxidants • Cytotoxic • Anticancer • Antifungal • Anti-HIV-1	• Cottiglia, et al., 2004 • Ito, et al., 2002; 2003 • Lemos, et al., 2012 • Kimura, et al., 2005 • Goncalves, et al., 2013 • Huerta-Reyes, et al., 2004 • Reyes-Chilpa, et al., 2004
C. calaba	• Chromanones • Terpenoid • Biflavonoid	-	• Gunatilaka, et al., 1984
C. caledonicum	• Xanthones	• Antifungal • Antimalarial	• Larcher, et al., 2004 • Morel, et al., 2002
C. cardio-oblongum	• Coumarins • Triterpenoids	-	• Ranjith, et al., 1985
C. dispar	• Coumarins	• Cytotoxic	• Guilet, et al., 2001a; 2001b
C. dryobalanoides	• Chromanones	-	• Ha, et al., 2012

C. enervasum	• Xanthones	• Antimicrobial	• Taher, et al., 2005
C. flavoramulum	• Flavonoids • Triterpenes • Xanthones	• Anti-AGEs	• Ferchichi, et al., 2012
C. gracilipes	• Triterpenes	-	• Cao, et al., 1997a; 1997c
C. hosei	• Coumarin • Xanthone	-	• Daud et al., 2014
C. inophyllum	• Coumarins • Tritepenes • Xanthones	• Antibacterial • Anti-HIV-1 • Cytotoxic	• Gomez-Verjan, et al., 2015 • Li, et al., 2010 • Laure, et al., 2008 • Patil, et al., 1993 • Pawar, et al., 2007 • Yimdjo, et al., 2004
C. lanigerum	• Coumarins	• Anti-HIV	• McKee, et al., 1996 • Kashman, et al., 1992
C. macrocarpum	• Flavonoids	-	• Ampofo and Waterman., 1986
C. membranaceum	• Chromanones • Xanthones	• Anti-inflammatory	• Ming, et al., 2016

			• Zou, et al.,2005
C. moonii	• Xanthones	-	• Dharmaratne and Wijesinghe, 1997
C. nadosum	• Xanthones	-	• Nasir, et al., 2011
C. papuanum	• Chromanone acids	-	• Stout, Hickernell and Sears, 1968
C. polyanthum	• Chromanone acids • Chromanones • Coumarins	-	• Ma, et al., 2004 • Wang, et al., 2010
C. ramiflorum	• Xanthones		• Bhanu, et al., 1975
C. sclerophyllum	• Flavonoids • Xanthones	• Antioxidant	• Rissyelly, et al., 2014
C. soulattri	• Coumarins • Terpenoids • Xanthones	• Antimicrobial • Cytotoxic	• Ee, et al., 2011 • Khan, Kihara and Omoloso, 2002 • Mah, et al., 2012 • Nigam, et al., 1988
C. symingtanianum	• Coumarins • Flavonoids	• Antioxidants • Anti-diabetic • Cholinesterase	• Aminudin, et al., 2015; 2016

		inhibitors • Cytotoxic • Antibacterial	
C. teysmannii	• Coumarins	• Anti-HIV-1	• Morel, et al., 2002 • Hay, et al., 2004
C. thwaitesii	• Phloroglucinols • Xanthones	• Cytotoxic	• Nguyen, et al., 2012
C. venulosum	• Flavonoids	-	• Cao, Sim and Goh, 1997b
C. verticilatum	• Flavonoids	-	• Ravelonjato, Knesch and Poisson, 1987
C. walkeri	• Flavonoids • Xanthones	-	• Ampofo and Waterman., 1986

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Plant Materials

The plant studied in this project was *Calophyllum gracilentum*. The stem bark of this plant was collected from the jungle in Landeh district, Sarawak and was identified by Mr. Tinjan Anak Kuda, a botanist from the Forest Department of Sarawak. A voucher specimen (UITM 3019) was deposited at the Herbarium of Universiti Teknologi MARA, Sarawak.

3.2 Chemical Reagents

The materials and solvents used in this project are listed in Tables 3.1 to 3.6.

Table 3.1: The materials and industrial grade solvents used for extraction, isolation and purification of chemical constituents

Solvents/ Materials	**Molecular formula**	**Density, ρ (g cm^{-3})**	**Source, Country**
Acetone	CH_3COCH_3	0.791	QReC, Malaysia
Dichloromethane	CH_2Cl_2	1.325	Fischer Scientific, UK
Ethyl acetate	$CH_3COOC_2H_5$	0.902	Lab Scan, Ireland
Methanol	CH_3OH	0.791	Mallinckrodit Chemicals, Phillisbury
n- Hexane	$CH_3(CH_2)_4CH_3$	0.659	Merck, Germany
Sephadex® LH-20	-	-	New Jersey, USA
Silica gel (60 Å)	SiO_2	-	Nacalai Tesque, Japan
Sodium sulphate anhydrous	Na_2SO_4	2.66	John Kollin Corporation, USA

Table 3.2: Material and analytical grade solvents used in TLC analysis

Solvents/ Materials	Molecular formula	Source, Country
Dichloromethane	CH_2Cl_2	QReC (Malaysia)
Ethyl acetate	$CH_3COOC_2H_5$	Fischer Scientific, UK
Iodine	I_2	Fischer Scientific, UK
n- Hexane	$CH_3(CH_2)_4CH_3$	R & M Chemicals, UK
TLC silica gel 60 F_{254}	-	Merck, Germany

Table 3.3: Deuterated solvents used in NMR analysis

Solvents/ Materials	Molecular formula	Source, Country
Acetone-d_6	CD_3COCD_3	Acros Organics, Belgium
Deuterated chloroform	$CDCl_3$	Acros Organics, Belgium
Methanol-d_4	CD_3OD	Acros Organics, Belgium

Table 3.4: Material and analytical grade solvent used in UV-Vis analysis

Solvents/ Materials	Molecular formula	Source, Country
Chloroform	$CHCl_3$	Fischer Scientific, UK
Cuvette (quartz)	-	Membrane solution, USA

Table 3.5: Material and HPLC grade solvents used in LC- and GC-MS analysis

Solvents/ Materials	**Molecular formula**	**Molecular weight (g mol^{-1})**	**Source, Country**
Acetonitrile	CH_3CN	41.05	Fischer Scientific, UK
Methanol	CH_3OH	32.04	Fischer Scientific, UK
Nylon syringe filter (0.5 μm)	-	-	Membrane solution, USA

Table 3.6: Material and chemical reagents used in antioxidant assay

Materials/ Chemical reagents	**Source, Country**
Ascorbic acid (Vitamin C)	Sigma-Aldrich, USA
Kaempferol	Sigma-Aldrich, USA
1,1-Diphenyl-2-picryhydrazyl (DPPH)	Sigma-Aldrich, USA
96-well plate	Techno Plastic, Switzerland

3.3 Methodology

3.3.1 Sequential Solvent Extraction, Isolation and Purification of Chemical Constituents from *Calophyllum gracilentum*

Approximately 2.0 kg of stem bark of *Calophyllum gracilentum* was collected, air-dried and finely ground into powder form. The powdered plant material was soaked with dichloromethane in a closed container for 48 hours at room temperature. The dichloromethane solvent extract was filtered and the solvent was removed using a rotary evaporator. These steps were repeated one more time and the crude extracts collected were combined.

The stem bark material was subsequently extracted twice with ethyl acetate and lastly twice with methanol. The dichloromethane, ethyl acetate and methanol crude extracts obtained were 199.04, 53.99 and 302.96 g, respectively.

Each of the crude extracts of *Calophyllum gracilentum* was subjected to gravity column chromatography via gradient elution to give a series of fractions which were then monitored for their chemical composition by using Thin Layer Chromatography (TLC). Fractions that showed similar composition on TLC plate were combined and subjected to further purification.

These processes were repeated until pure compounds were obtained. The fractions that showed single spot on the TLC plate were sent for spectroscopic analyses including NMR, IR, UV-Vis and LC-MS.

3.3.2 Chromatography

3.3.2.1 Column Chromatography

Column chromatography is a widely used method to separate and isolate pure compounds from a mixture of compounds. Gravity column chromatography was applied in this study to purify chemical constituents from the plant crude extracts. The stationary phase used was silica gel and it was packed in a glass column and eluted with a mixture of solvents in increasing polarity as mobile phase.

The size of the column used is dependent on the amount of sample being introduced. The sizes of columns available are 25, 30 and 80 mm in internal diameter. Dry packing method was used to prepare the sample. Firstly, the sample was dissolved with a suitable amount of solvent and was then added drop-wise into a small amount of silica gel and mixed them well. The sample mixture was left to dry overnight.

In the packing of column, silica gel was mixed with hexane to form a slurry which was then quickly poured into the glass column. The slurry was allowed to settle down in the column overnight before it was used for purification of sample.

The dry sample mixture was carefully introduced into the packed column to form a thin sample layer. A small amount of anhydrous sodium sulphate was added on top of the sample layer to protect the sample layer. The column was then eluted with a series of solvent mixtures (e.g. hexane/dichloromethane, dichloromethane/acetone and acetone/methanol) in increasing polarity. The eluents were collected as fractions according to the separated colour bands or volumes.

In order to get a good separation, the ideal height of the packed silica gel in the glass column is about 2/3 of the height of glass column. The flow rate of the mobile phase was monitored. Figure 3.1 shows the setup of the column chromatography.

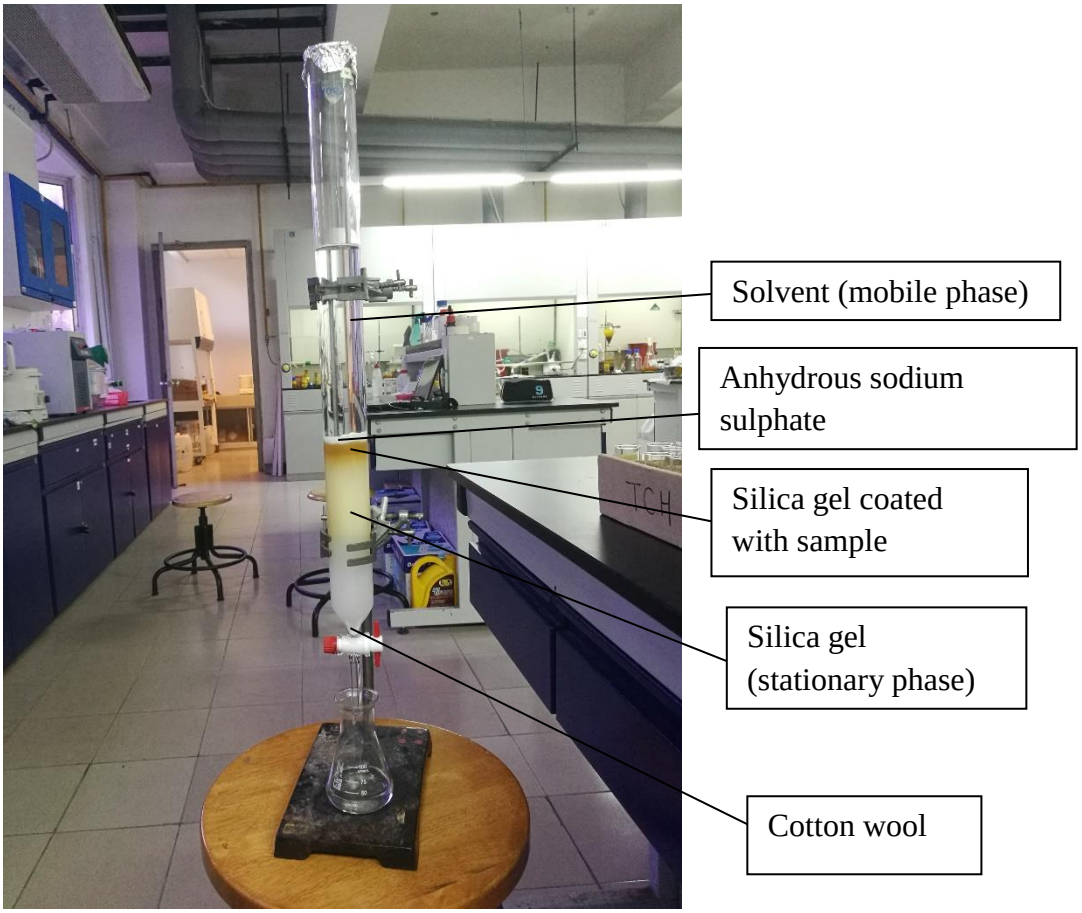


Figure 3.1: Setup of the column chromatography

3.3.2.2 Gel Permeation Chromatography (GPC)

Gel permeation chromatography is a type of size exclusion chromatography used to separate compounds based on the differences in their molecular weights and sizes. In this project, the stationary phase used was Sephadex® LH-20 whereas the mobile phase used was a polar mixture of solvent.

The sample was dissolved in methanol before being introduced into the gel permeation column. The sample solution was introduced onto the top of packed column to form a thin layer of sample solution. Isocratic elution via a solvent mixture of 90% methanol and 10% dichloromethane was used. During the elution, the larger size compounds were eluted out first while the smaller size molecules were eluted out later. The smaller size molecules stayed longer in the column as it was trapped inside the pores of packed materials and have to travel a longer distance along the column as compare to the larger size molecules.

3.3.2.3 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was used to check the purity of isolated compounds and to monitor the chemical composition of fractions collected from column chromatography. TLC plate was coated with silica gel 60 F_{254} and was prepared in 8 cm × 4 cm dimension. A baseline and a solvent front line were drawn 0.5 cm from the bottom and from the top of the TLC plate. Then a sufficient amount of sample solution was spotted on the baseline using a capillary tube. A solvent mixture as mobile phase was prepared and poured into the developing chamber. A piece of filter paper was placed inside the chamber. The wet filter paper helped to saturate the developing chamber with solvent vapour. After that, the TLC plate was placed into the developing chamber and the chamber was capped. Due to the capillary action of silica gel, the mobile phase migrated up the plate and the plate was removed from the

chamber when the solvent reached the solvent front line. The spots of compounds on the developed TLC plate were then visualized under the UV light and in iodine chamber. Different compounds travelled at different rates. The R_f value of each compound can be calculated by using the following formula:

$$R_f = \frac{\text{distance travelled by the compound (cm)}}{\text{distance travelled by solvent (cm)}}$$

3.3.3 TLC Detection Methods

3.3.3.1 UV Detection

After the TLC plate was completely developed, the plate was visualized under the UV lights of both short (254 nm) and long (365 nm) wavelengths. The spots visualized under short wavelength appeared as dark grey colour on a bright green background. At long wavelength, the spots appeared as fluorescence colour on a purple background. Compounds with aromatic ring and conjugated system can be detected effectively under the UV lights.

3.3.3.2 Iodine Vapour Detection

About 1 g of iodine crystals was placed in a 250 mL iodine chamber. The developed TLC plate was put into the chamber and the chamber was then capped and left aside for a few minutes. The compounds were stained with iodine vapour to form dark brown colour spots on the plate. The spots were quickly marked down as the staining effect of iodine was temporary.

3.4 Instruments

3.4.1 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is an analytical method used to determine the number and types of protons and carbons present in a molecule as well as to study the molecular structure of a compound. Basically, NMR is a non-destructive technique, it requires only a few milligrams of sample to obtain a good data.

In this project, JEOL JNM-ECX 400 MHz spectrometer was used to carry out 1D NMR and 2D NMR experiments on the samples. 1D NMR includes proton NMR (^{1}H), carbon NMR (^{13}C) and Distortionless Enhancement by Polarization Transfer (DEPT), while 2D NMR includes Heteronuclear

Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Coherence (HMBC). Tetramethylsilane (TMS) was used as the standard.

The sample was dissolved in a suitable amount of deuterated solvents used such as methanol-d_4, acetone-d_6 or deuterated chloroform. Then the sample solution was filled into an NMR tube up to 4 cm height. The NMR tube was then capped, labelled and ready for NMR experiments.

3.4.2 Infrared (IR) Spectroscopy

Infrared spectroscopy is used to determine the types of functional groups present in a molecule. It measures the bond vibrations of molecules. The sample was prepared in the form of potassium bromide (KBr) pellet. The sample was first ground with KBr in a ratio of 1:10 until it become a homogenous mixture. Then the sample mixture was pressed under a high pressure to obtain the KBr pellet. The KBr pellet was analyzed by the Perkin Elmer 2000-FTIR spectrophotometer to obtain the IR spectrum in the range of 4000 to 400 cm^{-1}.

3.4.3 Ultraviolet- Visible (UV-Vis) Spectroscopy

The UV-Vis spectroscopy is used to determine the presence of chromophores in a molecule by defining its conjugation system. The sample was dissolved in a suitable amount of solvent, and the solvent was used as the blank. Perkin Elmer Lambda 35, a double beam spectrophotometer was used in the analysis. The absorption was measured in the range of 200-400 nm in order to obtain the UV spectrum.

3.4.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

The coupling of liquid chromatography (LC) with mass spectrometry (MS) gives spectral information about the accurate mass and molecular formula of a non-volatile compound. Agilent Technologies 6520 LC/MS was used to analyze the compound by ionizing the sample solution into free cations. A minimum amount of sample was dissolved in HPLC grade solvent. The undissolved solid particles were filtered out before being introduced into the LC-MS. 5 µL of sample solution was injected into the column and eluted with a mixture of water and methanol in a ratio of 3:7 v/v at flow rate of 0.6 mL/min.

3.4.5 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is used to study the nominal mass, molecular formula and fragmentation pattern of a volatile compound. Mass spectra were obtained from Shimadzu GC-MS QP2010 equipped with a flame ionization detector (FID). HPLC grade solvent was used to prepare sample solution at concentration of 100 ppm. 5 µL of sample solution was injected into the non-polar BPX5 column and eluted with inert helium gas at flow rate of 0.6 mL/min.

3.4.6 Melting Point Apparatus

Stuart SMP 10 melting point apparatus was used to measure the melting point of isolated compounds. Sample was filled into a haematocrit capillary tube and heated until it melted.

3.5 Antioxidant Assay

The crude extracts, isolated compounds and standard compounds (ascorbic acid and kaempferol) were separately prepared in methanol at a concentration of 2 mg/mL. The DPPH solution was prepared by dissolving it in methanol at a concentration of 2 mg/mL. These solutions were sonicated for 5 minutes to

form homogeneous solution. The master stock solution of sample and DPPH solution were kept in dark condition to avoid decomposition due to exposure to light.

A serial dilution of master stock solution of test compounds gave concentrations of 240, 120, 60, 30, 15, 7.5 and 3.75 µg/mL in a 96-well plate. Each well was added with 10 µL of DPPH solution and 90 µL of methanol. The well with only methanolic DPPH solution served as the negative control, while the well with ascorbic acid or kaempferol served as the positive controls.

The plate was wrapped with aluminum foil and incubated for 30 minutes in the dark at room temperature. The absorbance of each well was measured at wavelength of 520 nm using a microplate reader. Each test sample was performed in triplicate and the absorbance was calculated in average. The percentage inhibition rate of test compound was calculated using the following formula:

$$\text{Inhibition rate (\%)} = \frac{A_{\circ} - A_1}{A_{\circ}} \times 100\%$$

Where A_o= absorbance of the negative control

A_1 = absorbance of the test compound

A graph of percentage inhibition rate against the test compound concentration was plotted. The IC_{50} value of the test compound was determined from the

graph. IC_{50} is the concentration of the test compound required to inhibit 50% of DPPH radical scavenging activity.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical Constituents Isolated from *Calophyllum gracilentum*

The stem bark of *Calophyllum gracilentum* was extracted successively with three different solvents – dichloromethane, ethyl acetate and methanol. From the extractions, 302.96 g of dichloromethane, 53.99 g of ethyl acetate and 199.04 g of methanol crude extracts were obtained.

About 131.36 g of dichloromethane crude extract was subjected to a 8.5 cm inner diameter silica gel column and eluted with solvent mixtures in increasing polarity (hexane/dichloromethane, dichloromethane/ethyl acetate, ethyl acetate/methanol). This afforded a total of 21 fractions (YZA1-21). All the fractions were monitored for their chemical compositions by using TLC plate. Fraction YZA4 was recrystallized from methanol to yield white needle-like crystals, friedelin **[22]**.

Fraction YZA9 was further purified via a 3.5 cm inner diameter column packed with silica gel and eluted with the solvent mixture similar to above to give 41 subfractions (YZB1-41). Subfractions YZB6-8 were combined and further purified via a 2.5 cm internal diameter column chromatographic

column packed with silica gel and eluted with a series of solvent mixtures (hexane/ethyl acetate, ethyl acetate/ acetone) in increasing polarity to give 35 subfractions (YZE1-35).

Subfraction YZE13 was subjected to a gel permeation column packed with Sephadex LH-20 and eluted with a solvent mixture of 90% methanol and 10% dichloromethane to give 32 subfractions (YZF1-32). Subfraction YZF9 yielded light-brownish solid, 5,7-dihydroxy-4-(2-butyl)coumarin [**23]**

Meanwhile, subfractions YZE9-11 were combined and subjected to the gel permeation column chromatography as above to give a total of 20 subfractions (YZG1-20). Further purification of the combined subfractions YZG10-11 via the same gel permeation column chromatography gave 34 subfractions (YZH1-34). Subfraction YZH29 yielded brown crystals, 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin **[24]**.

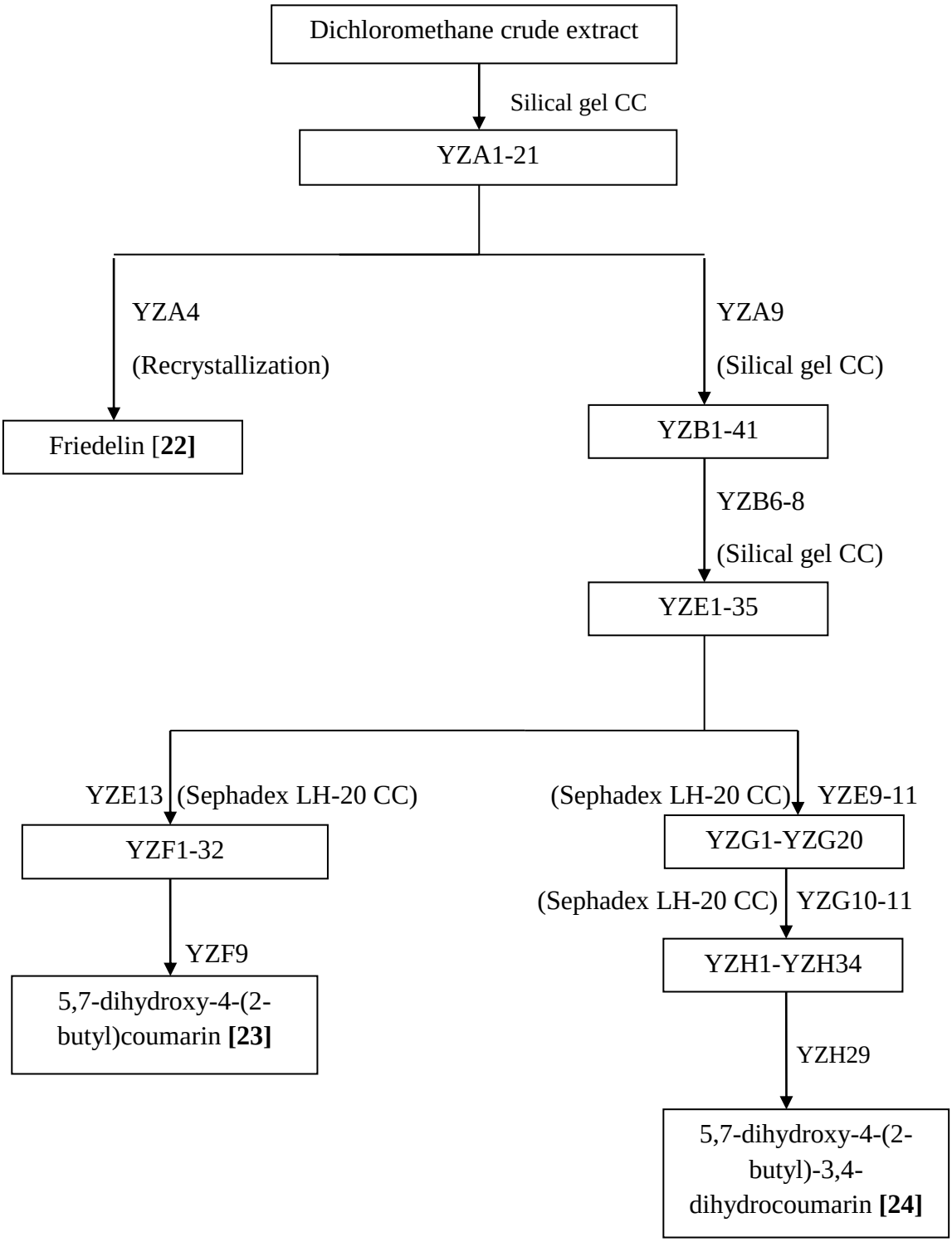


Figure 4.1: Pathway of isolation of pure compounds from dichloromethane crude extract

About 100.02 g of methanol crude extract was subjected to a 8.5 cm inner diameter silica gel packed column and eluted with solvent mixtures in increasing polarity (hexane/dichloromethane, dichloromethane/acetone, acetone/methanol). This afforded a total of 31 fractions (YPA1-31). All the fractions were monitored for their chemical compositions by using TLC plate.

Fractions YPA17-18 with their similar chemical composition were combined and subjected to a 3 cm inner diameter glass column packed with silica gel as stationary phase. Elution with mobile phase of increasing polarity (hexane/ethyl acetate, ethyl acetate/acetone) gave a total of 42 subfractions (YPB1-42)

Subfraction YPB40 was then further recrystalized from dichloromethane to yield white solid,4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid **[25]**.

Subfractions YPB58-61 were combined and subjected to a gel permeation column packed with Sephadex LH-20 and eluted with a solvent mixture of 90% methanol and 10% dichloromethane. A total of 31 subfractions (YPC1-31) were obtained and subfraction YPC9 gave yellow needle-like crystals, euxanthone **[26]**.

However, column chromatography carried out on the ethyl acetate crude extract of *Calophyllum gracilentum* failed to yield any pure compound.

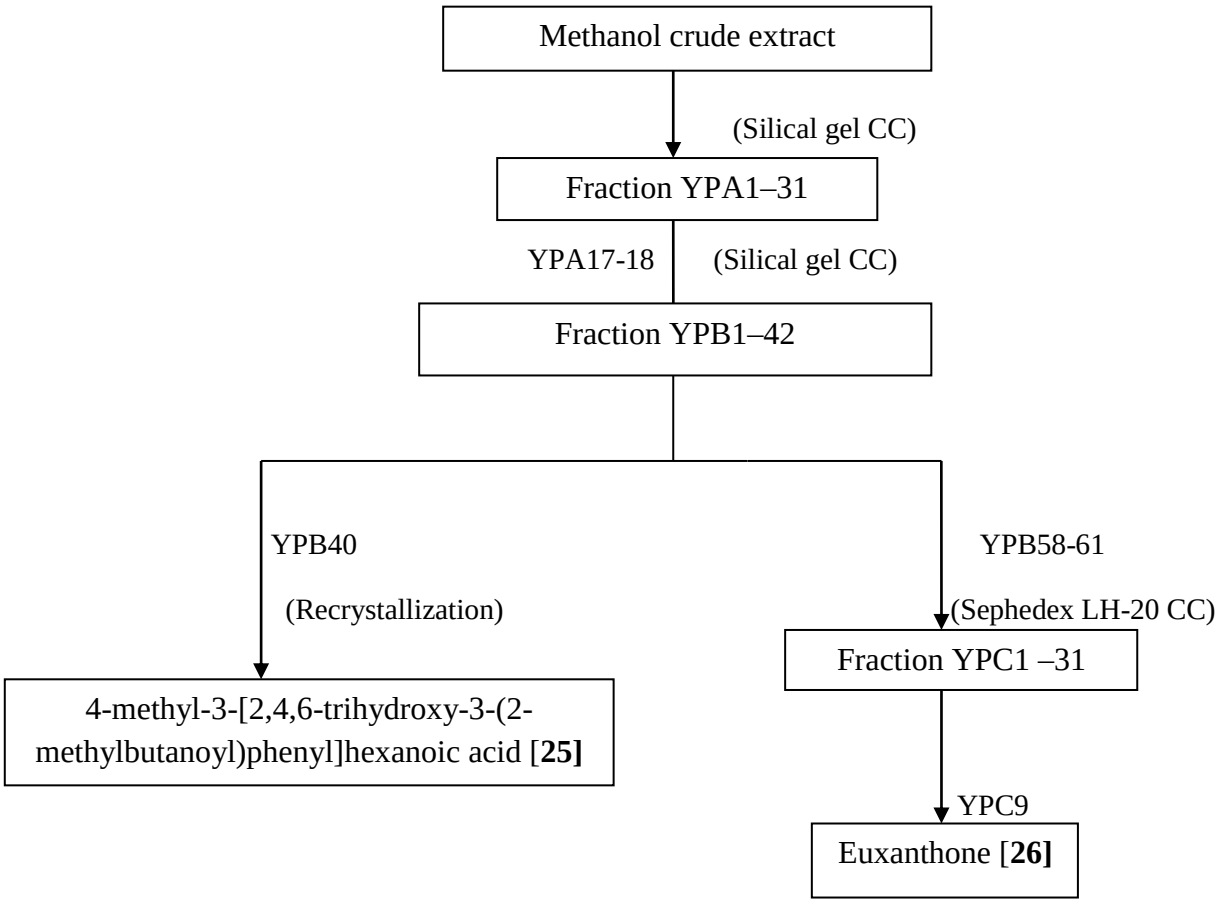
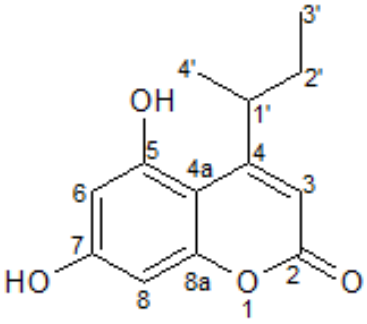


Figure 4.2: Pathway of isolation of pure compounds from methanol crude extract

4.2 Structural Characterization and Elucidation of 5,7-Dihydroxy-4-(2-butyl)coumarin [23]



[23]

About 88.2 mg of compound **23** was obtained as light-brownish solid. It showed a molecular weight of 234.23 g/mol corresponding to the molecular formula of $C_{13}H_{14}O_4$. This compound showed a melting point of 247 – 249 ºC. In the TLC analysis, it gave a single spot with a retention factor, R_f value of 0.72 by using a solvent mixture of 90% dichloromethane and 10% acetone.

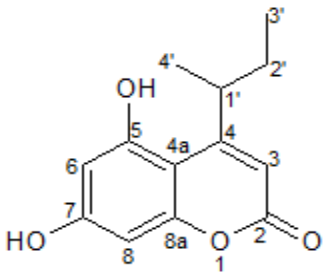
In the IR spectrum (Figure 4.3), a broad peak was observed at 3230 cm^{-1} which indicated the presence of hydroxyl group. Meanwhile, the presence of carbonyl group was indicated by absorption band at 1662 cm^{-1}. The absorption bands for aromatic C=C (1591 and 1458 cm^{-1}), C-H stretch (2876 – 2962 cm^{-1}) and C-O (1275 cm^{-1}) were also observed in the spectrum. In the UV-Vis spectrum (Figure 4.4), compound **23** showed the absorption maxima at 257.9 and 323.6 nm owning to the conjugated system as reflected by the coumarin nucleus in the assigned structure.

In the ^{1}H NMR spectra (Figures 4.5 and 4.6), two highly deshielded broad singlet signals at δ 9.65 and 9.28 were assigned to the two hydroxyl protons, 5-OH and 7-OH which might be interchangeable. Meanwhile, the methine protons H-6, H-8 and H-3 in the coumarin nucleus gave signals at δ 6.33 (d, *J* = 1.8 Hz), 6.26 (d, *J* = 2.4 Hz) and 5.86 (s), respectively. Proton signals below 4.0 ppm were all assigned to the protons in the 2-butyl side chain.

In the ^{13}C NMR spectra (Figures 4.7 to 4.9), a total of 13 carbons were observed in correspondence to the presence of 13 non-equivalent carbons in the proposed structure of compound **23**. The highly deshielded carbon signal at δ 160.8 was assigned to the lactone carbonyl group at carbon C-2. The oxygenated aromatic carbons C-7, C-8a and C-5 gave relatively deshielded signals at δ 160.6, 157.5 and 157.2, respectively. The other aromatic carbons in the coumarin nucleus which were C-3, C-4a, C-6 and C-8 gave signals at δ160.1, 102.1, 99.7 and 95.6, respectively. Apart from that, the 2-butyl side chain displayed characteristic carbon signals at δ 11.4 (C-3'), 18.9 (C-4'), 29.5 (C-2') and 36.7 (C-1').

The HMQC spectra (Figures 4.10 to 4.12) showed the direct ^{1}J coupling between protons and their respective carbons. Only protonated carbons showed their correlations in these spectra. For example, the methine proton signals at δ 3.88, 5.86, 6.26 and 6.33 were correlated to carbon signals at δ 36.7 (C-1'), 106.1 (C-3), 95.6 (C-8) and 99.7 (C-6), respectively.

The proposed structure of compound **23** was established based on the long range ^{2}J and ^{3}J couplings between protons and their neighbouring carbons. In the HMBC spectra (Figures 4.13 to 4.17), the methyl proton signal H-4' at δ1.17 showed correlations to carbon signals at δ36.7 (C-1'), 29.5 (C-2') and 163.9 (C-4) indicating that the 2-butyl group was attached to the coumarin nucleus at the carbon position C-4. The remaining of the HMBC correlations for the assignment of the coumarin nucleus were given in Table 4.1. Based on the spectral data, compound **23** was identified as 5,7-dihydroxy-4-(2-butyl)coumarin and it was reported for the first time as a new compound.



[23]

Table 4.1: Summary of NMR data for 5,7-dihydroxy-4-(2-butyl)coumarin [23]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			^2J	^3J
1	-	-	-	-
2	-	160.8	-	-
3	5.86 (1H,s)	106.1	C-2	C-1', C-4a
4	-	163.9	-	-
4a	-	102.1	-	-
5	-	157.2	-	-
6	6.33 (1H, d, J =1.8 Hz)	99.7	C-7	C-4a, C-8
7	-	160.6	-	-
8	6.26 (1H, d, J = 2.4 Hz)	95.6	C-7, C-8a	C-4a, C-6
8a	-	157.5	-	-
1'	3.88 (1H, m)	36.7	-	-
2'	1.39 (1H, m), 1.74 (1H, m)	29.5	C-1', C-3'	C-4'
3'	0.91 (3H, t, J = 7.3 Hz)	11.4	C-2'	-
4'	1.17 (3H, t, J = 6.7 Hz)	18.9	C-1'	C-2', C-4
5-OH	*9.28 (1H, brs)	-	-	-
7-OH	*9.65 (1H, brs)			

*Interchangeable

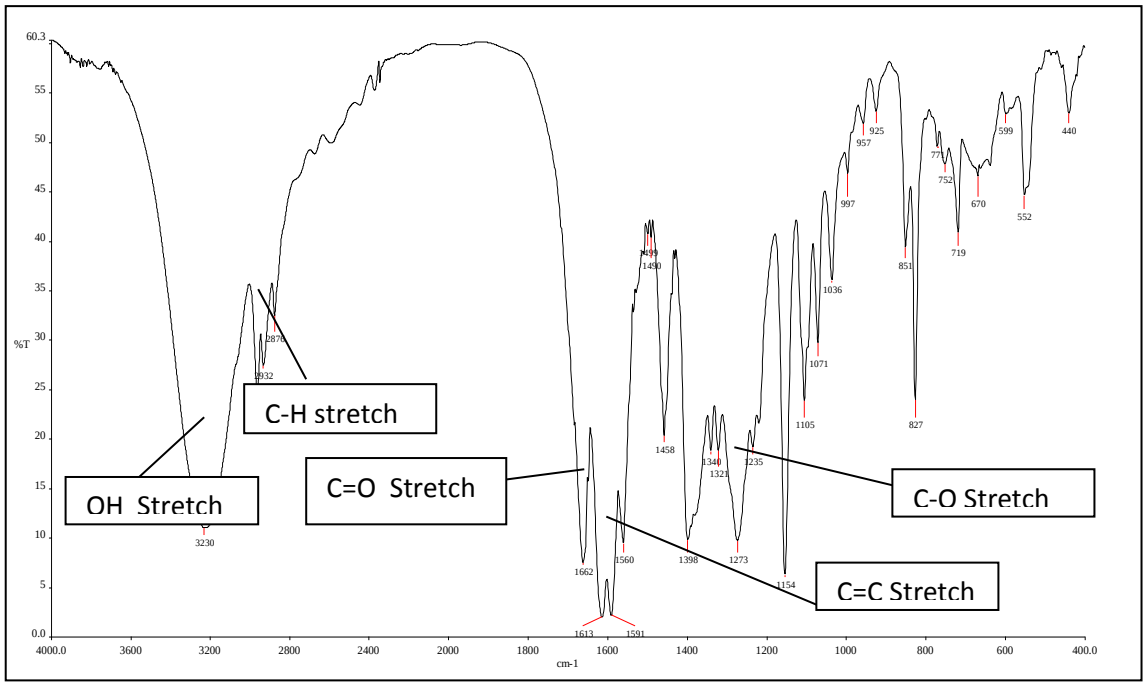


Figure 4.3: IR spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]

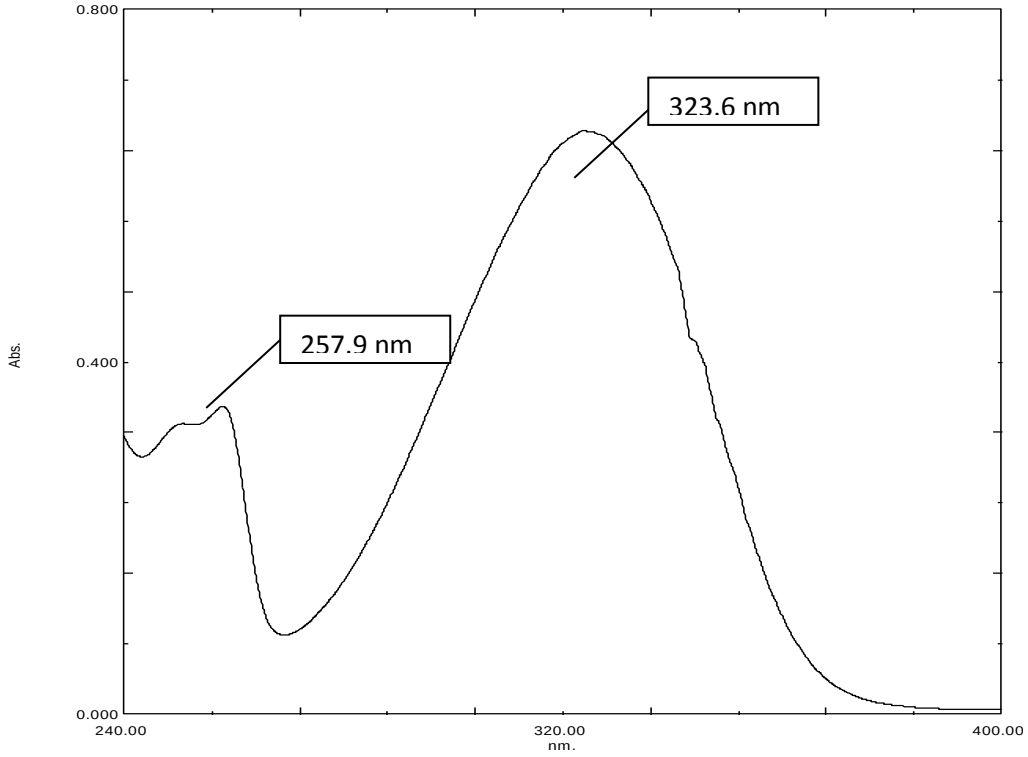
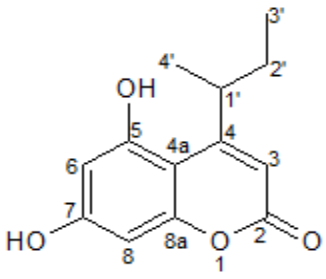


Figure 4.4: UV-Vis spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

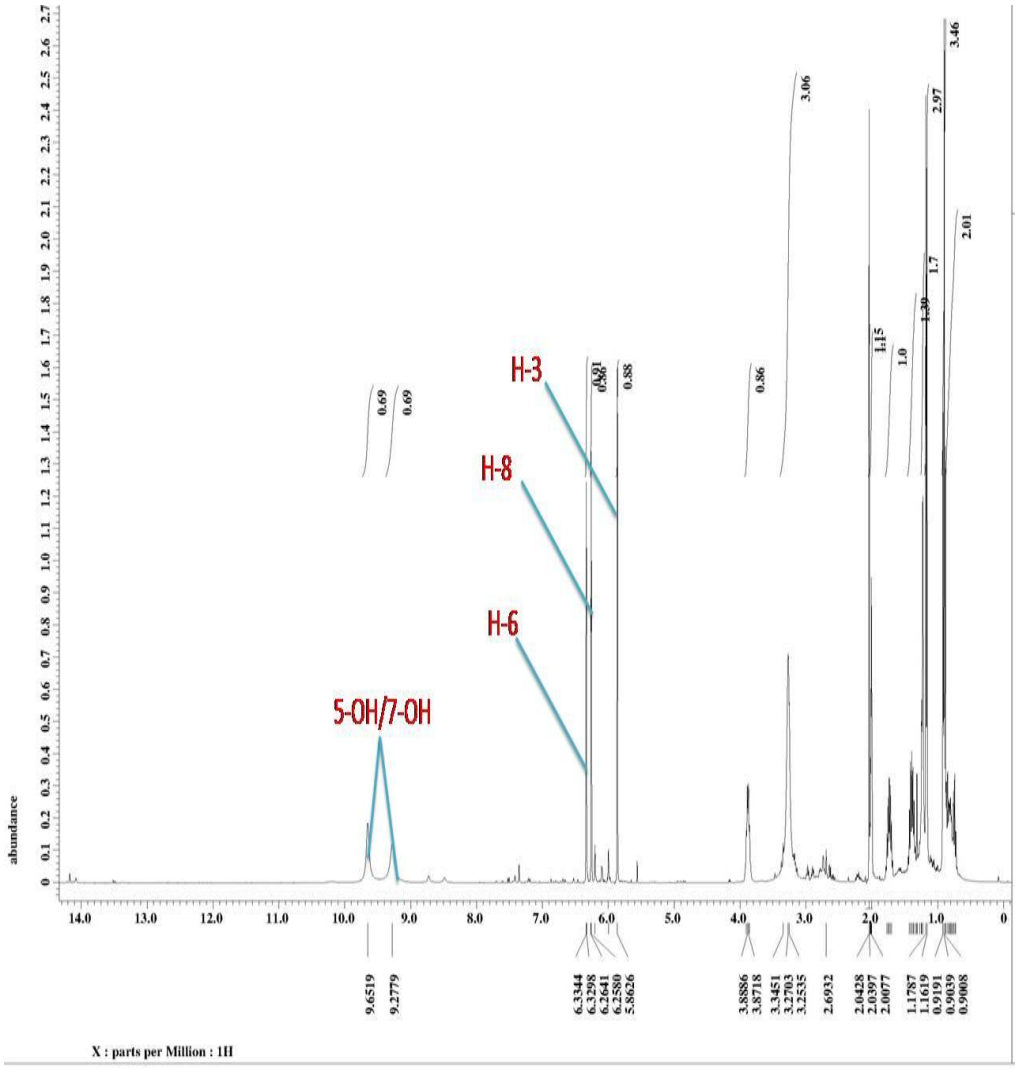
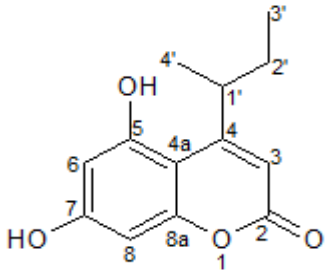


Figure 4.5: ^{1}H NMR spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23] (400 MHz, acetone-*d_6*)



[23]

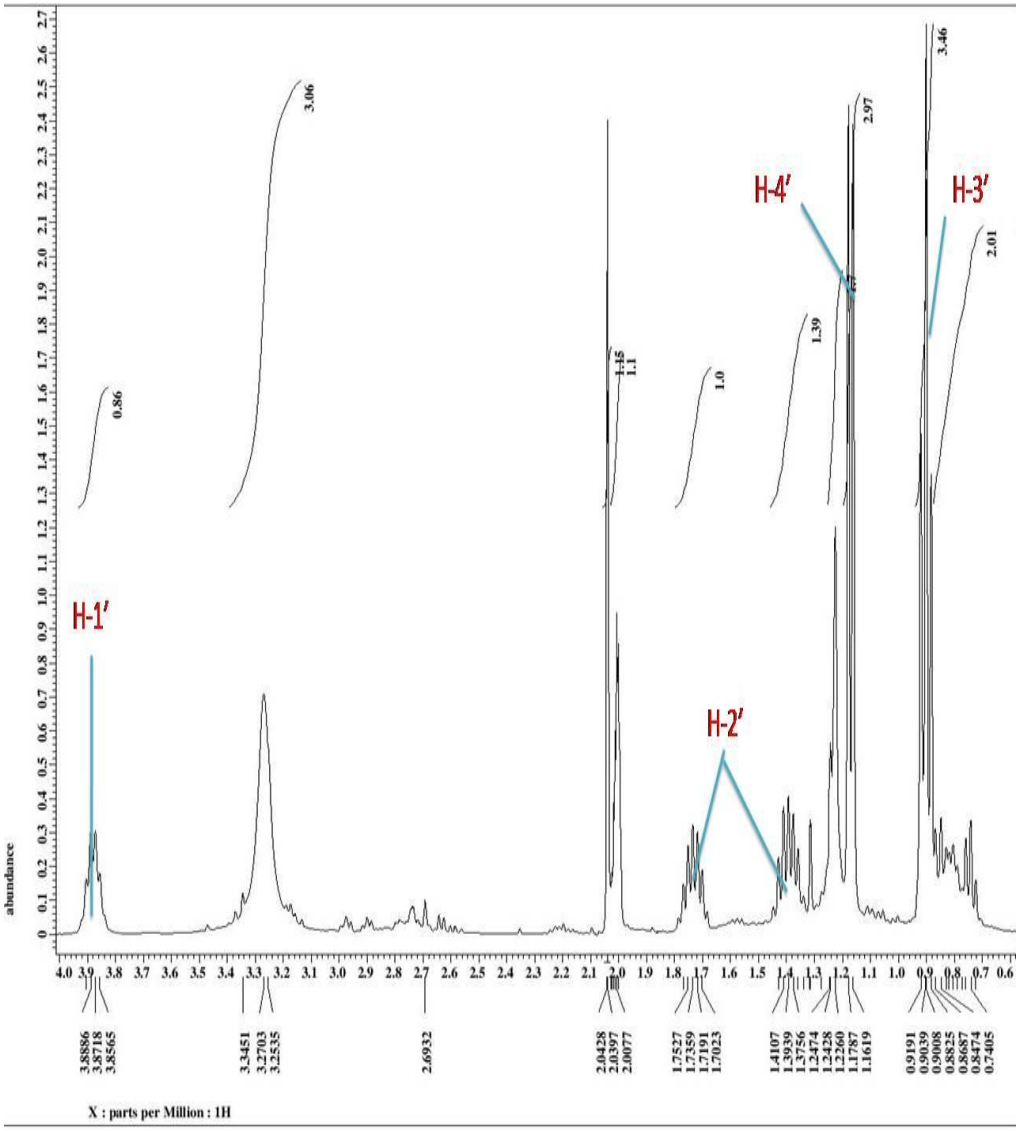
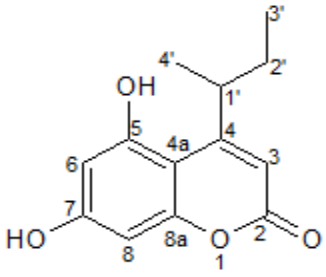


Figure 4.6: Expanded ^{1}H NMR spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23] (400 MHz, acetone-d_6)



[23]

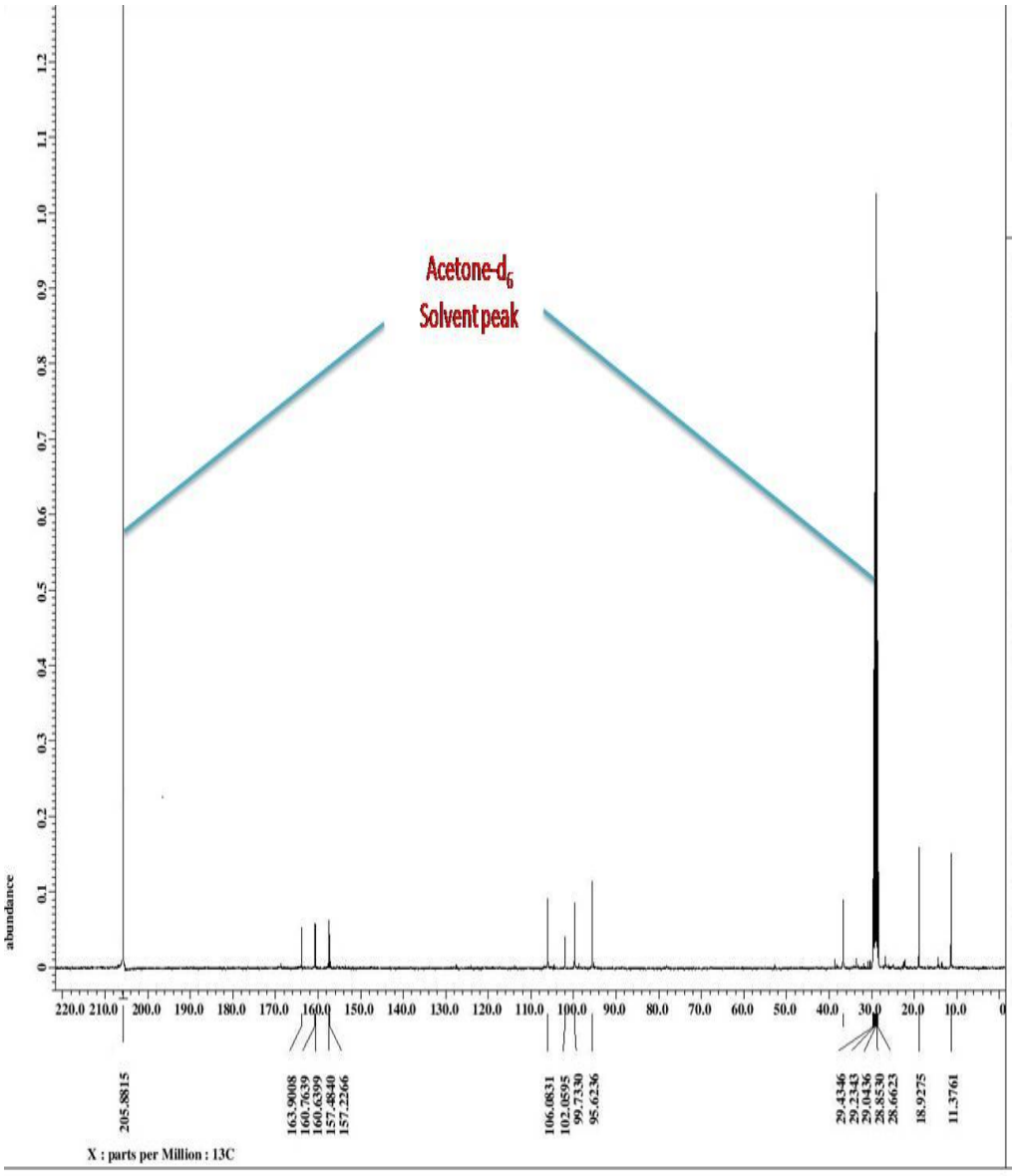
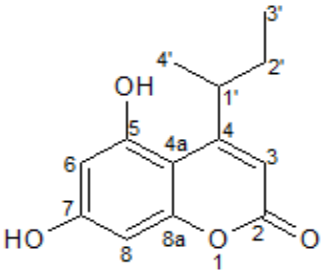


Figure 4.7: ^{13}C NMR spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23] (100 MHz, acetone-d_6)



[23]

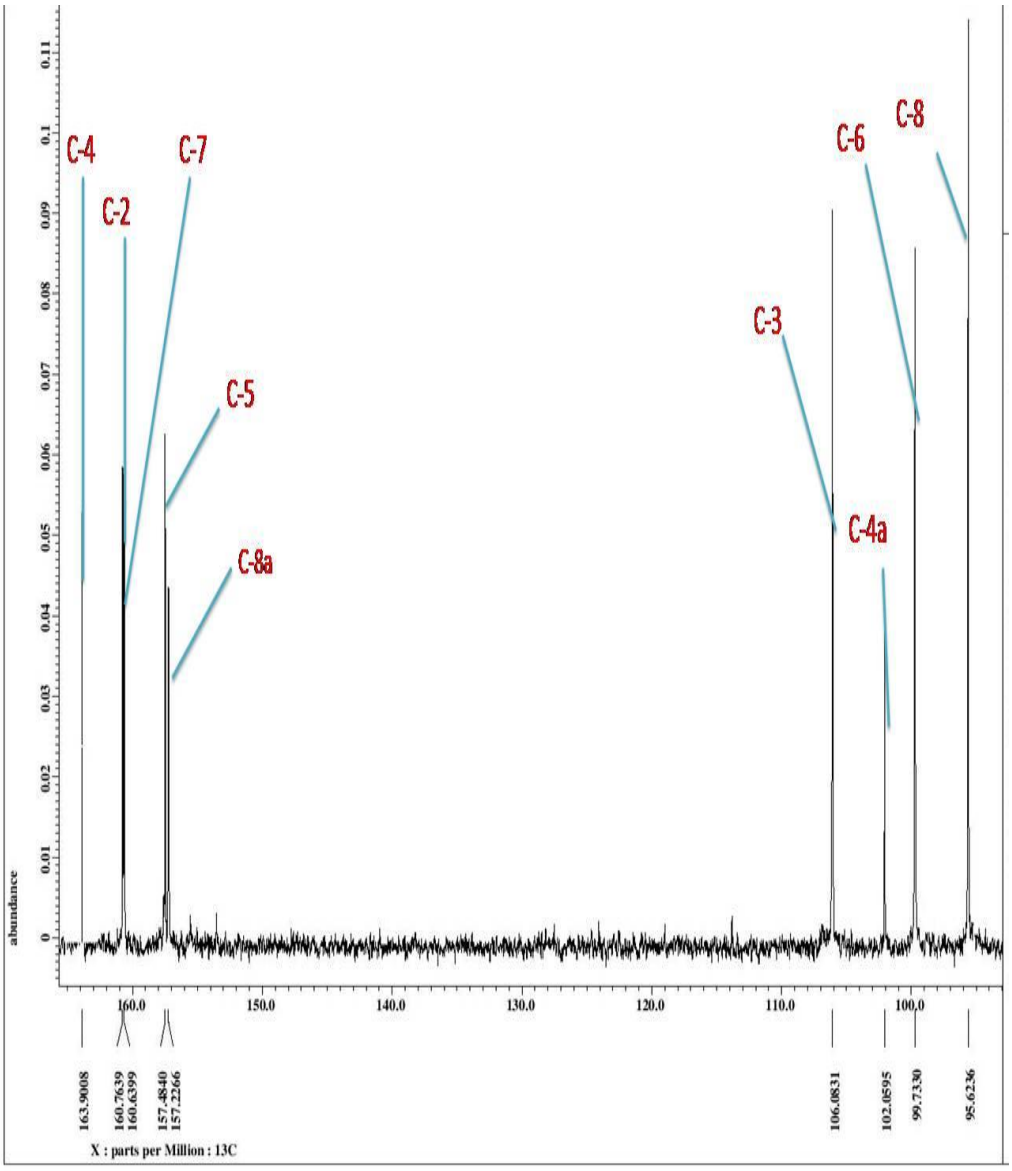
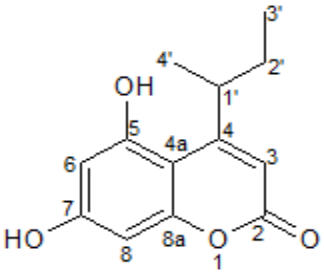


Figure 4.8: Expanded ^{13}C NMR spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23] (100 MHz, acetone-d_6)



[23]

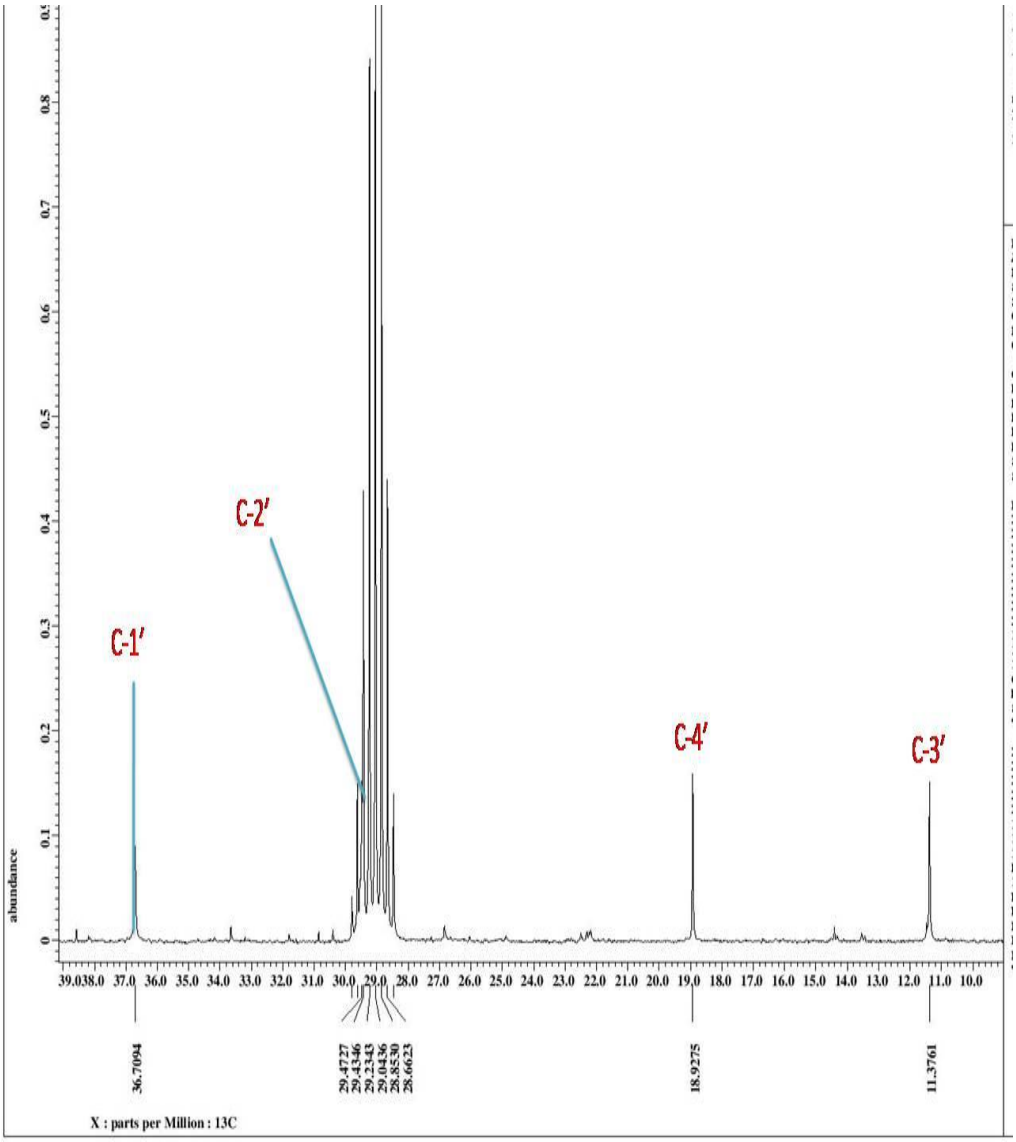
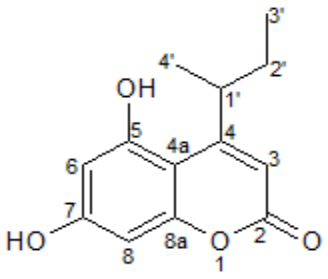


Figure 4.9: Expanded ^{13}C NMR spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23] (100 MHz, acetone-d_6)



[23]

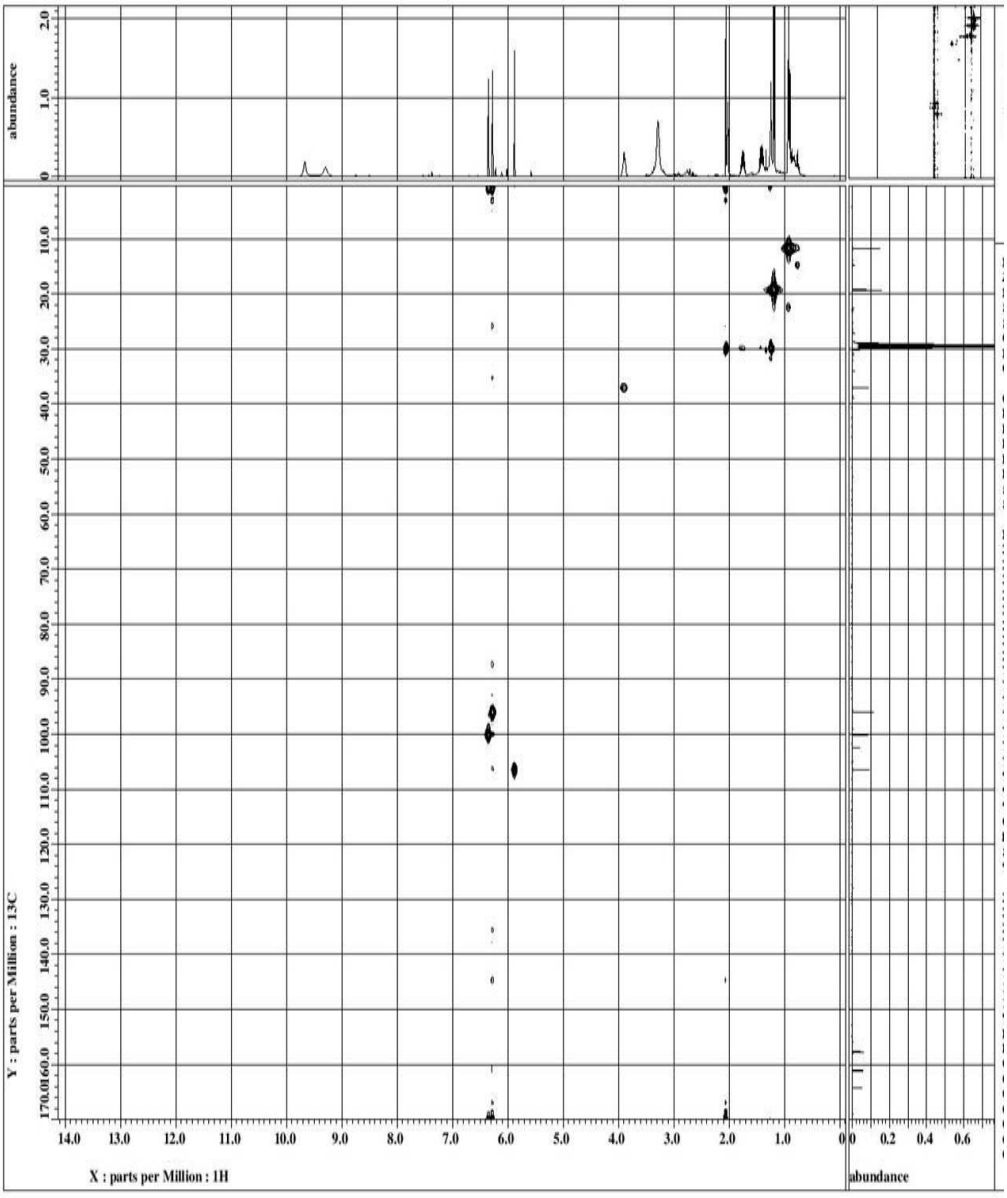
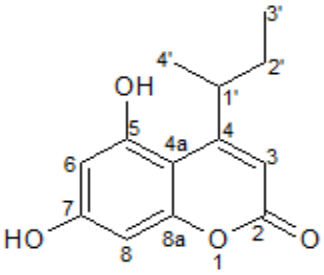


Figure 4.10: HMQC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

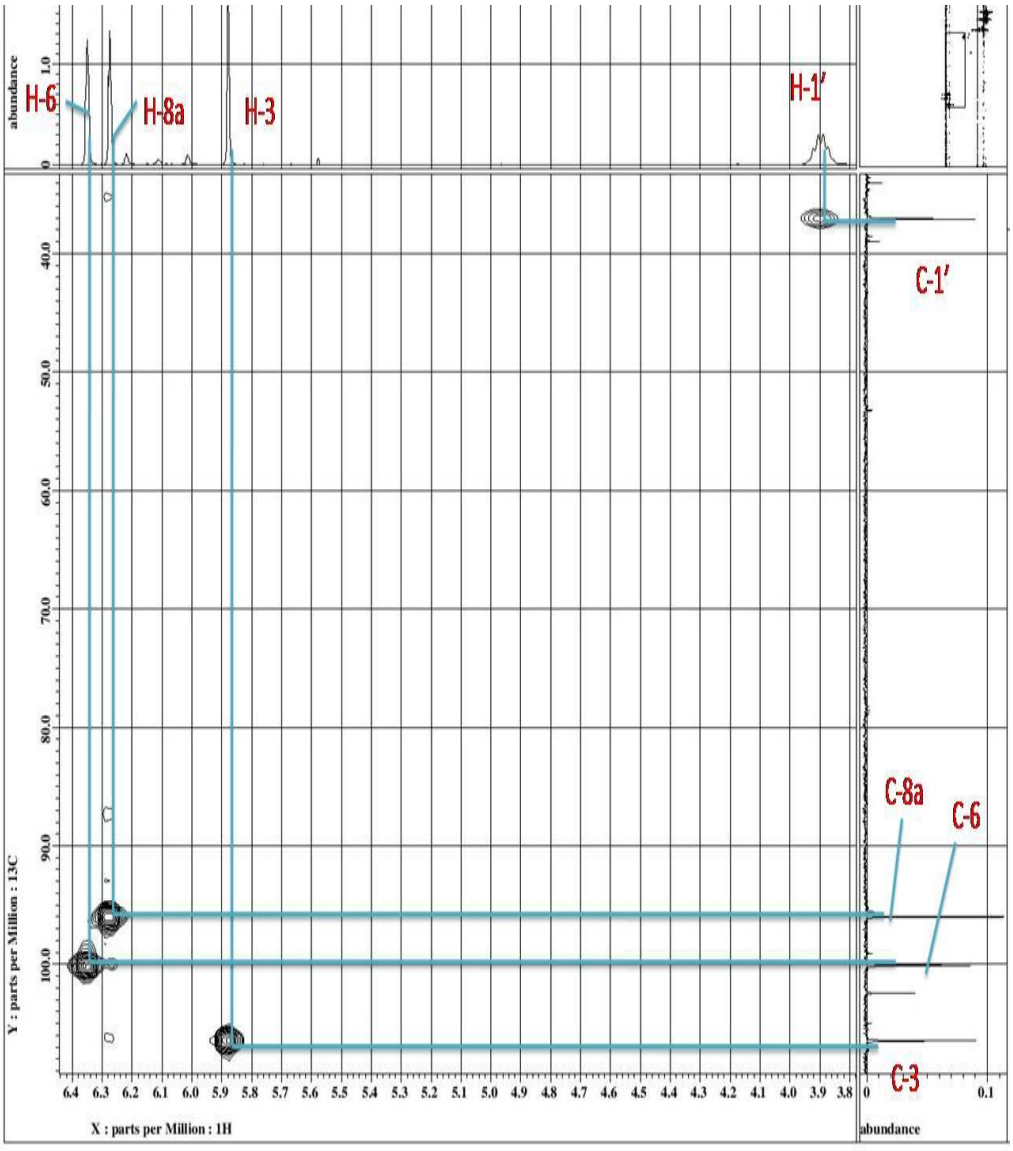
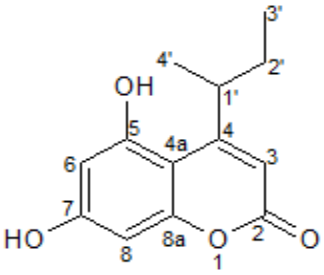


Figure 4.11: Expanded HMQC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

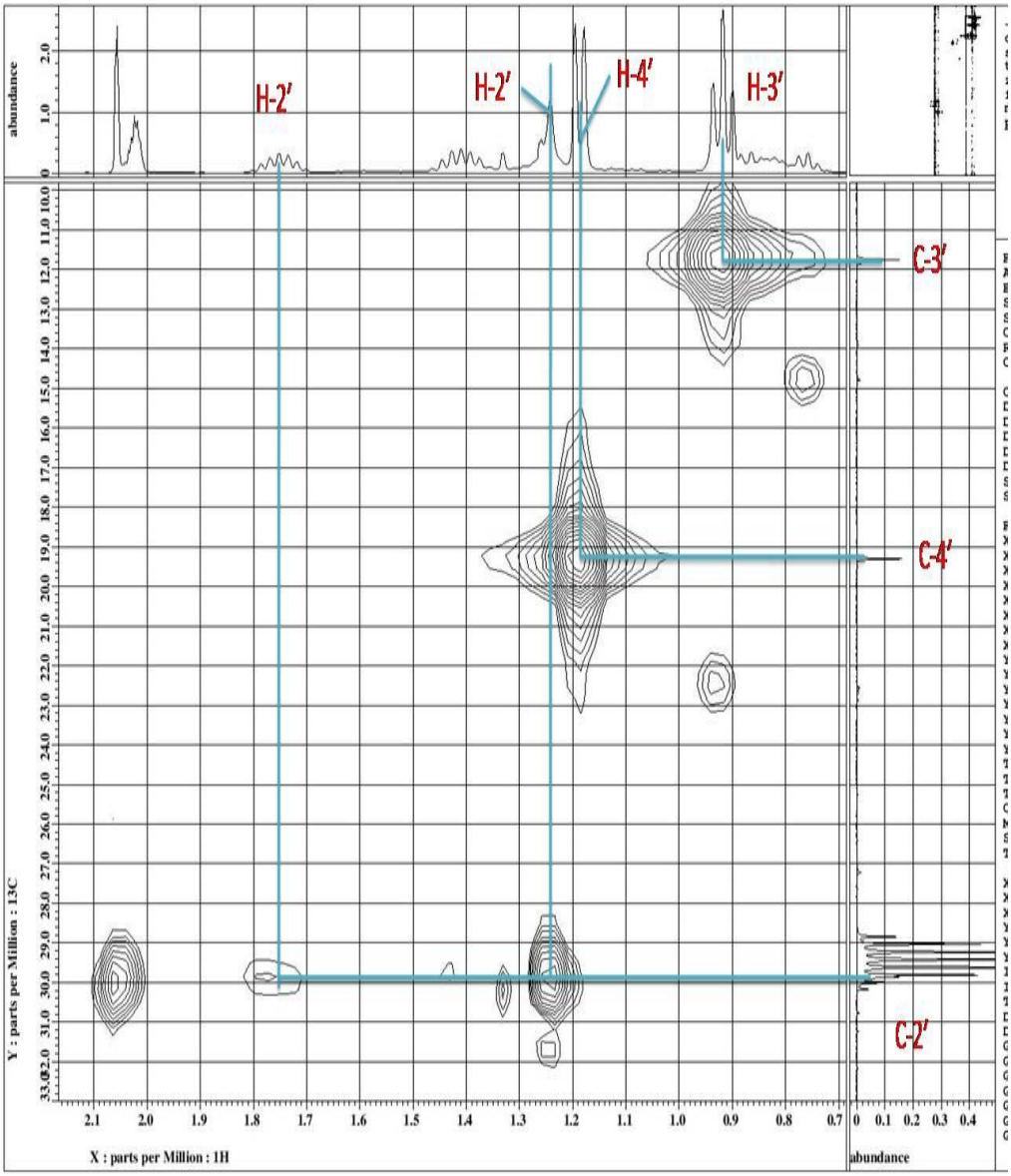
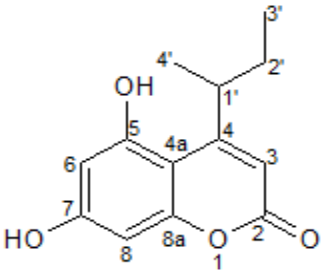


Figure 4.12: Expanded HMQC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

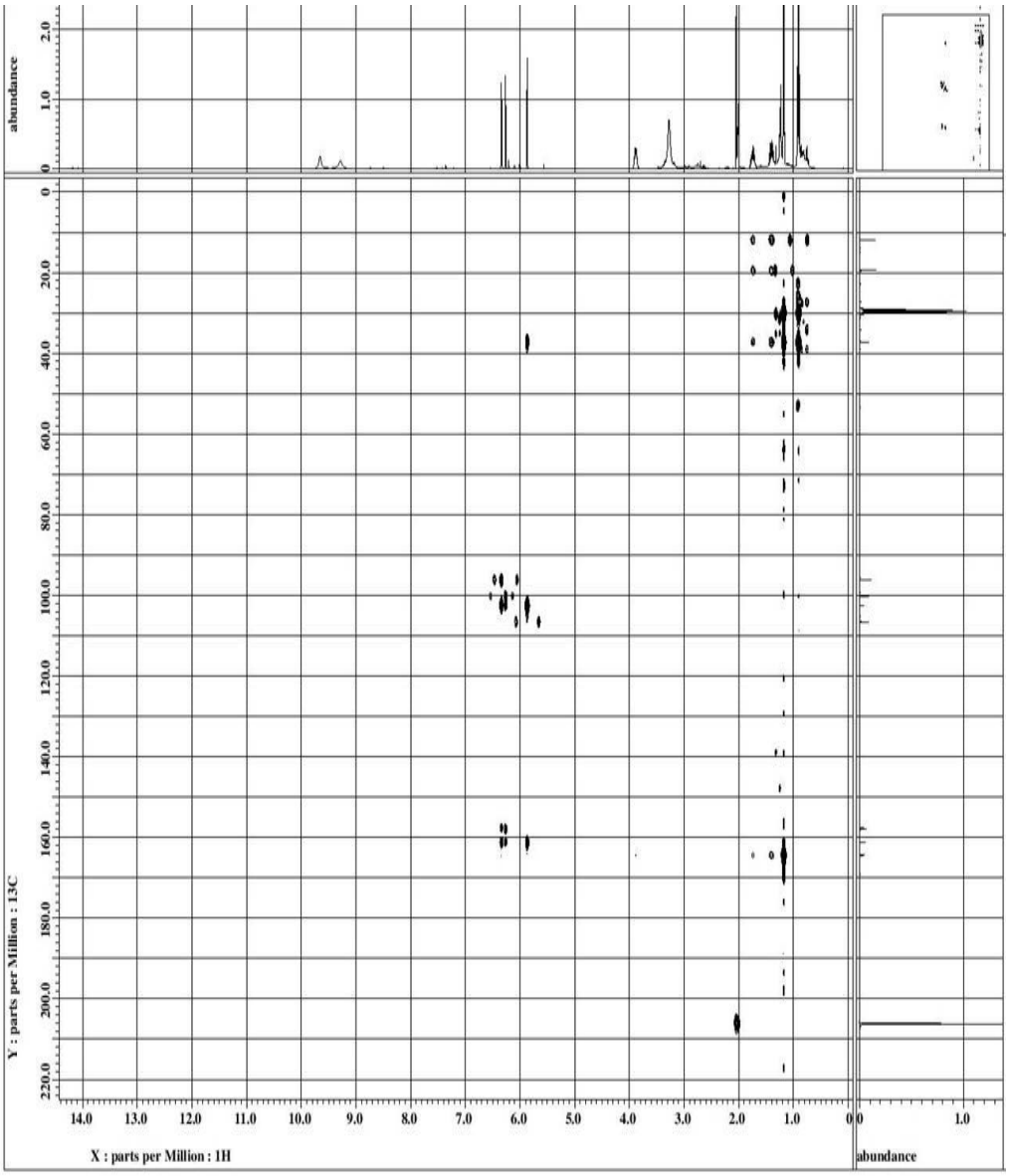
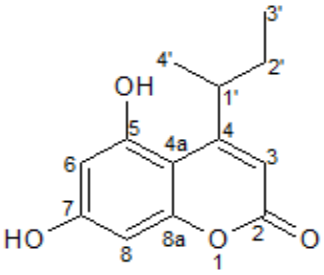


Figure 4.13: HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

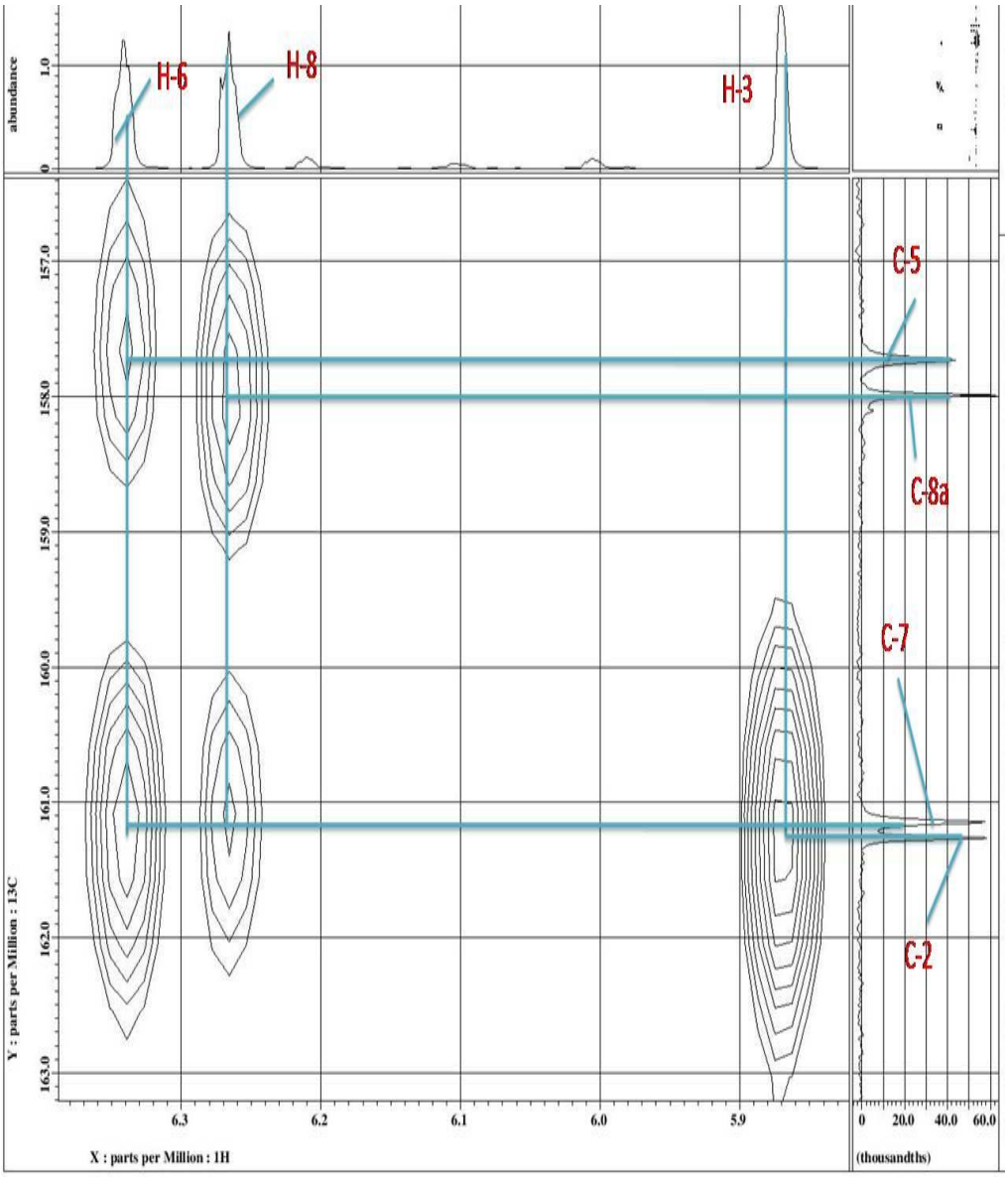
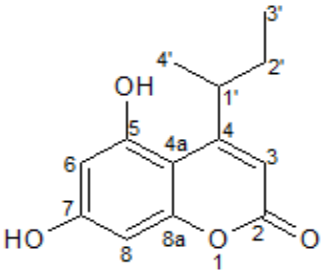


Figure 4.14: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

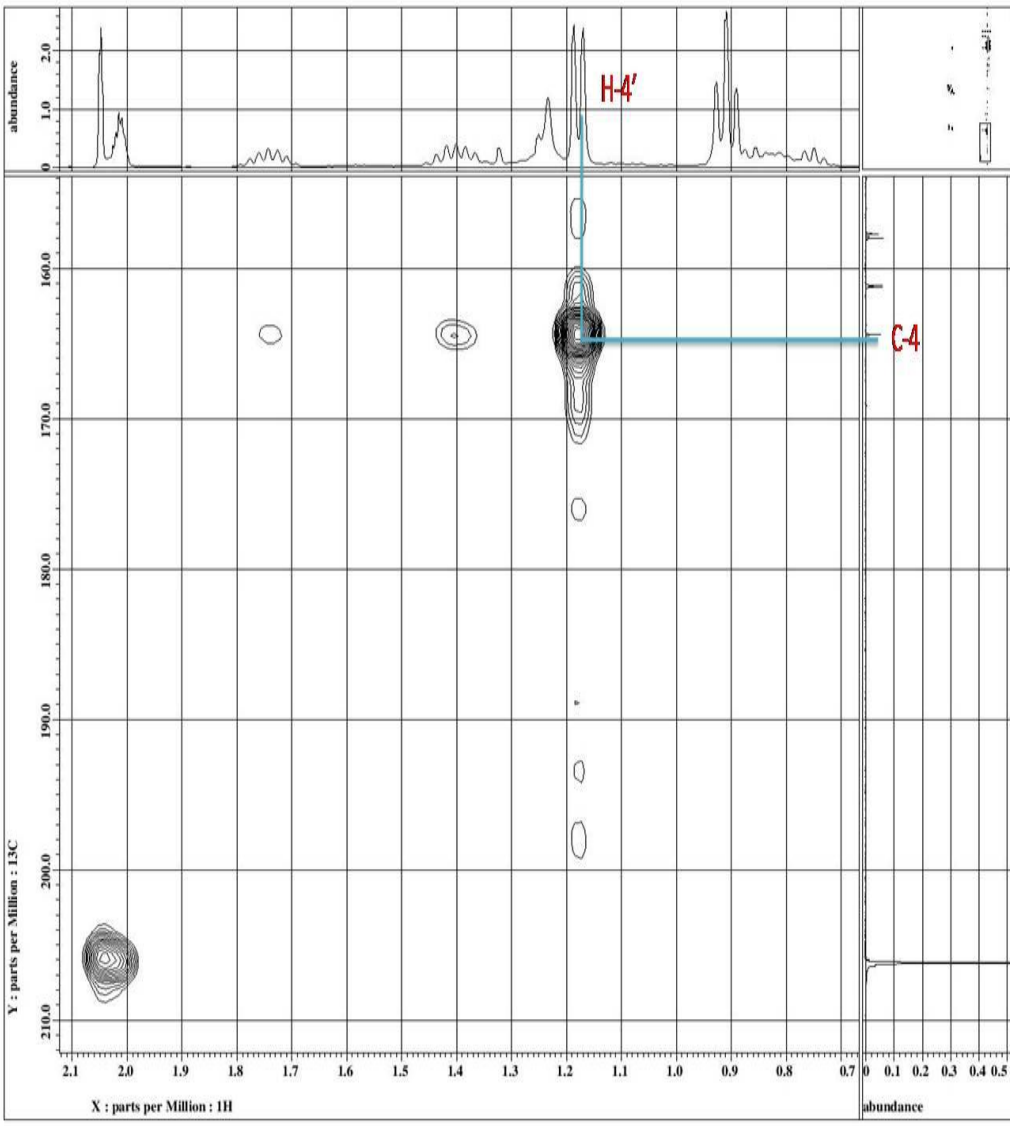
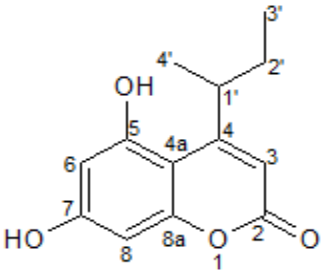


Figure 4.15: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

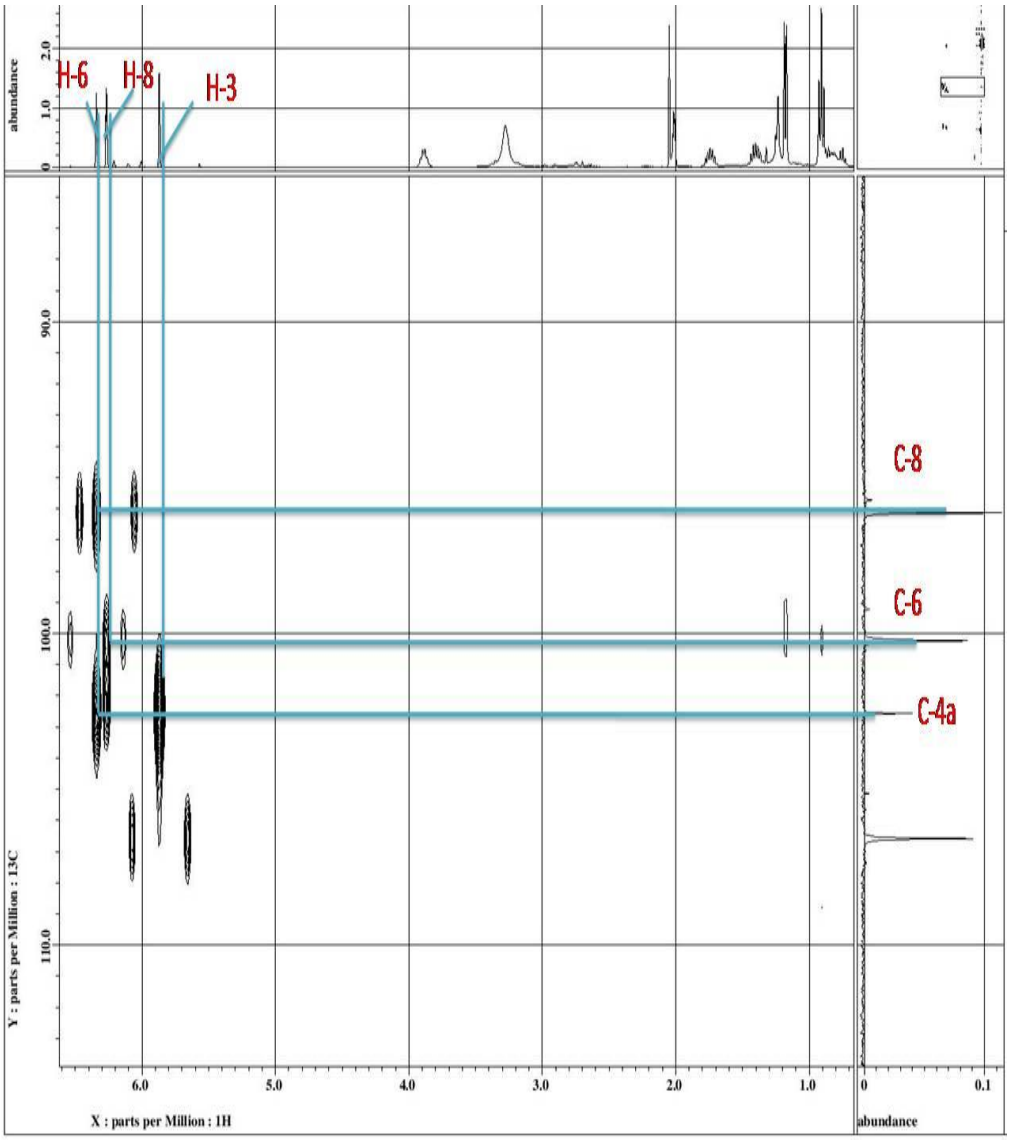
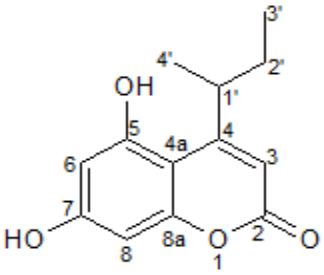


Figure 4.16: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]



[23]

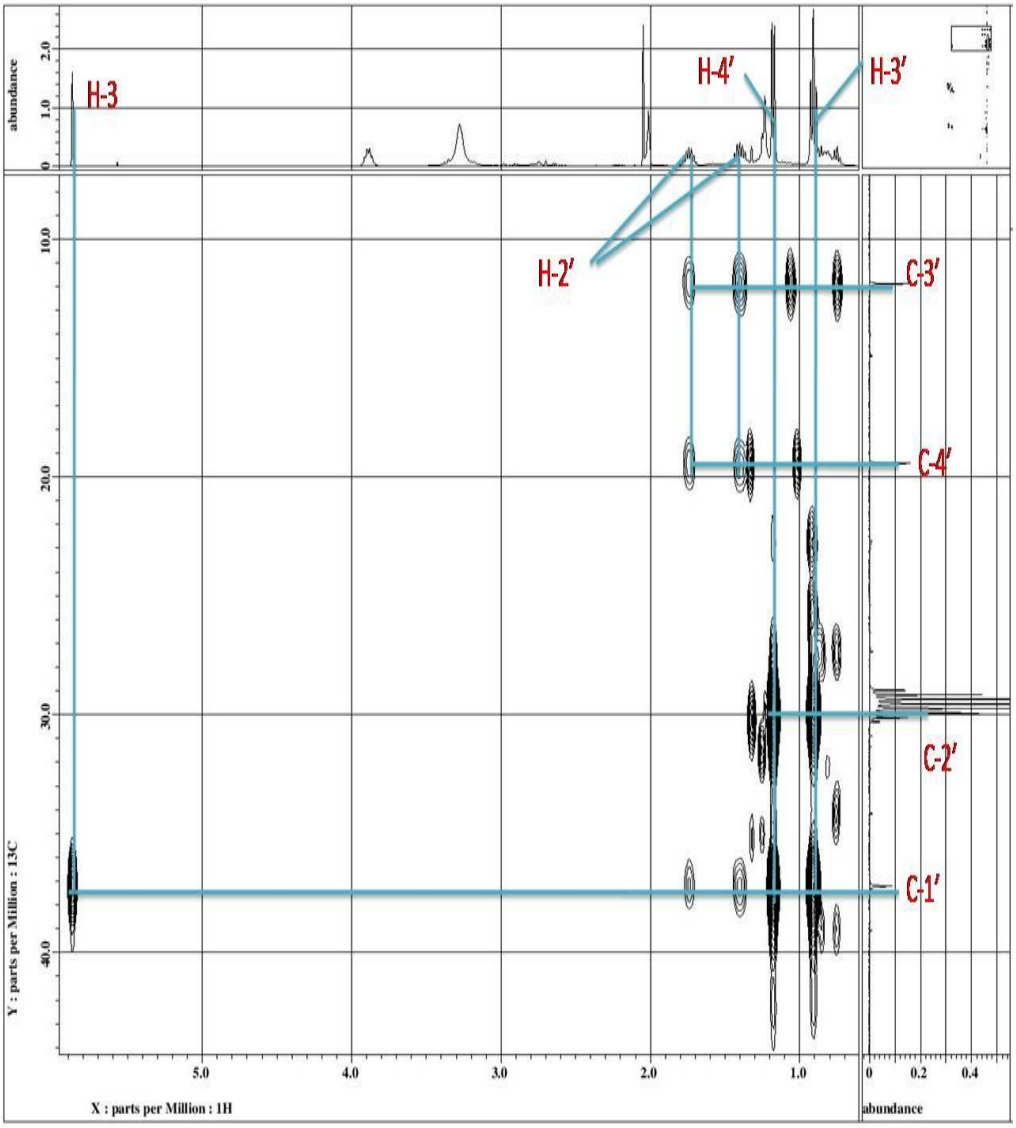
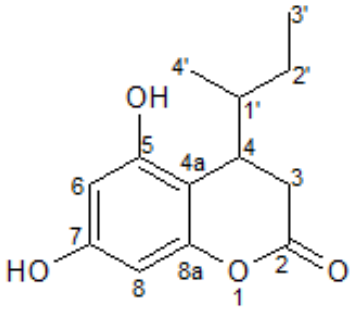


Figure 4.17: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)coumarin [23]

4.3 Structural Characterization and Elucidation of 5,7-Dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]



[24]

A total of 70.8 mg of compound **24** was obtained in the form of white solid. The molecular formula of this compound was $C_{13}H_{16}O_4$ giving a molecular weight of 236.24 mg/mol. Compound **24** gave a melting point of 256 – 257 ^oC. From the TLC analysis, a dark single spot was observed under UV light of wavelength 254 nm, and in the iodine chamber. The retention factor, R_f value obtained for the compound **24** was 0.57 by using a mixture of 90% dichloromethane and 10% acetone as mobile phase.

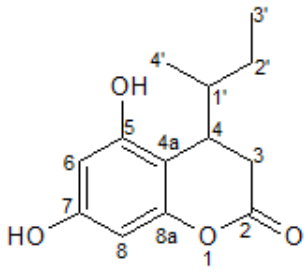
From the IR spectrum (Figure 4.18), a broad absorption band at 3345 cm^{-1} indicated the presence of hydroxyl group in the assigned structure. The carbonyl group (C=O) was observed at 1741 cm^{-1} as a sharp and intense band. Meanwhile, absorption bands observed at 2920, 1481 and 1250 cm^{-1} revealed the presence of C-H (stretch), C=C (stretch) and C-O (stretch) functionalities in compound **24**. In the UV-Vis spectrum (Figure 4.19), compound **24** showed absorption maxima at 281.8 nm which was higher than the

unsubstituted 3,4-dihydrocoumarin with its absorption maxima at 250 nm. This bathchromic effect was induced by the two hydroxyl groups in compound **24**.

In the ^{1}H NMR spectra (Figures 4.20 to 4.22), two broad deshielded singlet signals at δ 8.88 and 8.99 were assigned to the two hydroxyl protons, 5-OH and 7-OH which might be interchangeable. The two lone aromatic protons H-6 and H-8 in the coumarin nucleus gave a doublet and another doublet signals at δ 6.22 (1H, d, J = 2.4 Hz) and 6.03 (1H, d, J = 2.4 Hz), respectively. The two methylene protons H-3 (δ 2.57 and 2.72) and the methine proton H-4 (δ 3.15) in the lactone ring gave relatively more deshielded proton signals as compared to the proton signals exhibited by 2-butyl side chain in the region 1.58 to 0.69 ppm.

The ^{13}C NMR spectra (Figures 4.23 and 4.24) showed a total of 13 carbon signals which is in agreement with the total number of carbons present in the assigned structure. The most deshielded carbon signal at δ 170.3 was assigned to the lactone carbonyl carbon C-2. Meanwhile the deshielded carbon signals at δ 157.3, 155.5 and 153.2 were assigned to the oxygenated aromatic carbons C-7, C-5 and C-8a, respectively. The remaining non-oxygenated aromatic carbons C-4a, C-6 and C-8 gave signals at δ 104.7, 99.2 and 95.4, respectively. The six carbon signals observed below 40 ppm were assigned to the remaining sp^3 hybridized carbons C-3 (δ 30.1), C-4 (δ 33.6), C-1' (δ 38.5), C-2' (δ 26.9), C-3' (δ 11.7) and C-4' (δ 14.4).

The ^{1}J correlations exhibited in HMQC spectra (Figures 4.25 and 4.26) and the ^{2}J and ^{3}J correlations displayed in HMBC spectra (Figures 4.27 to 4.30) were summarized in Table 4.2. In the HMBC spectrum (Figure 4.30), the methyl proton H-4' at δ 0.70 showed correlations to carbon signals C-1' (δ 38.5), C-2' (δ 26.9) and C-4 (δ 33.6) which confirmed the 2-butyl side chain was linked to the coumarin core at carbon position C-4. Based on the spectral evidence, compound **24** was identified as 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin and this compound was reported for the first time as a new compound.



[24]

Table 4.2: Summary of NMR data for 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			^2J	^3J
1	-	-	-	-
2	-	170.3	-	-
3	2.57 (1H, dd, *J* = 16.5, 7.3 Hz) 2.72 (1H, d, *J* = 16.5 Hz)	30.1	C-2, C-4	C-4a , C-1’
4	3.15 (1H, t, *J* = 5.8 Hz)	33.6	-	C-4’
4a	-	104.7	-	-
5	-	155.5	-	-
6	6.22 (1H, d, *J* = 2.4 Hz)	99.2	C-7	C-4a, C-8
7	-	157.3	-	-
8	6.03 (1H, d, *J* = 2.4 Hz)	95.4	C-7, C-8a	C-4a, C-6
8a	-	153.2	-	-
1’	1.54 (1H, m)	38.5	-	-
2’	1.18 (1H, m) 1.33 (1H, m)	26.9	C-1’, C-3’	C-4’
3’	0.80 (3H, t, *J* = 7.3 Hz)	11.7	C-2’	C-1’
4’	0.70 (3H, t, *J* = 6.7 Hz)	14.4	C-1’	C-2’, C-4
5-OH	*8.88 (1H,brs)	-	-	-
7-OH	*8.99 (1H, brs)	-	-	-

* Interchangeable

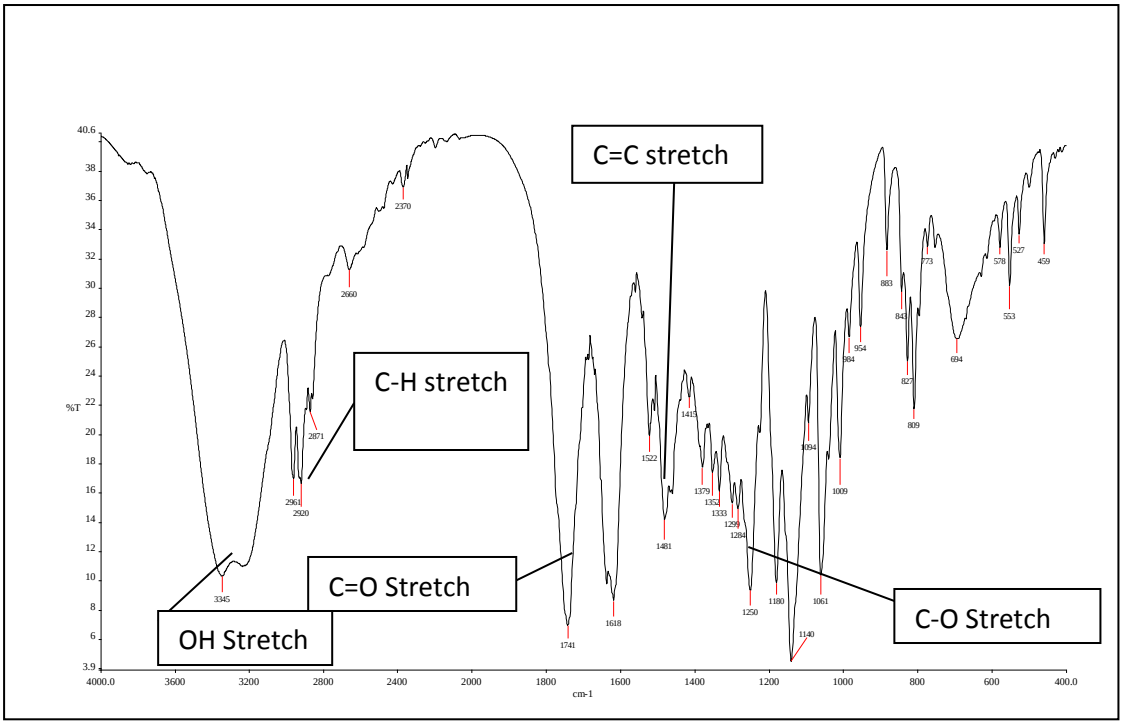


Figure 4.18: IR spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]

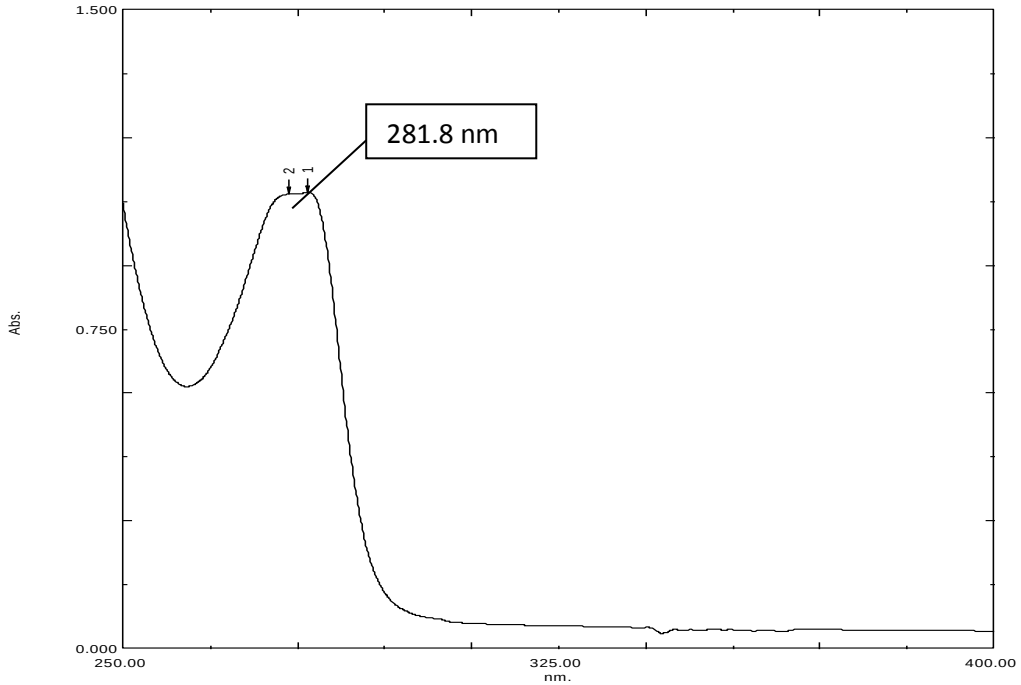
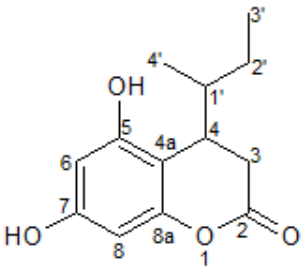


Figure 4.19: UV-Vis spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]



[24]

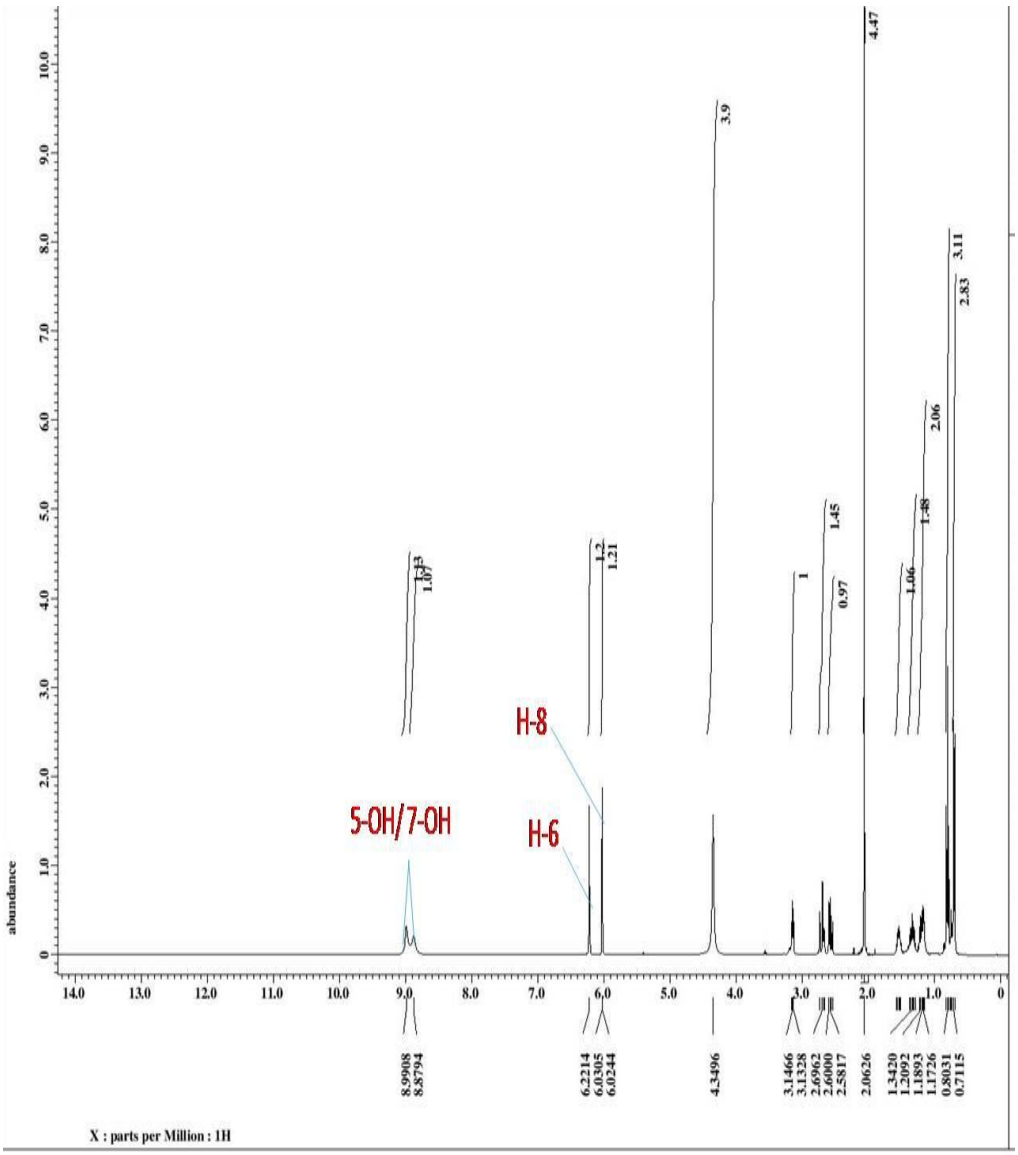
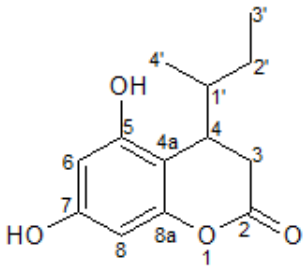


Figure 4.20: ^{1}H NMR spectrum of 5,7-dihydroxy-4-(2-butyl)3,4-dihydrocoumarin [24] (400 MHz, acetone-d_6)



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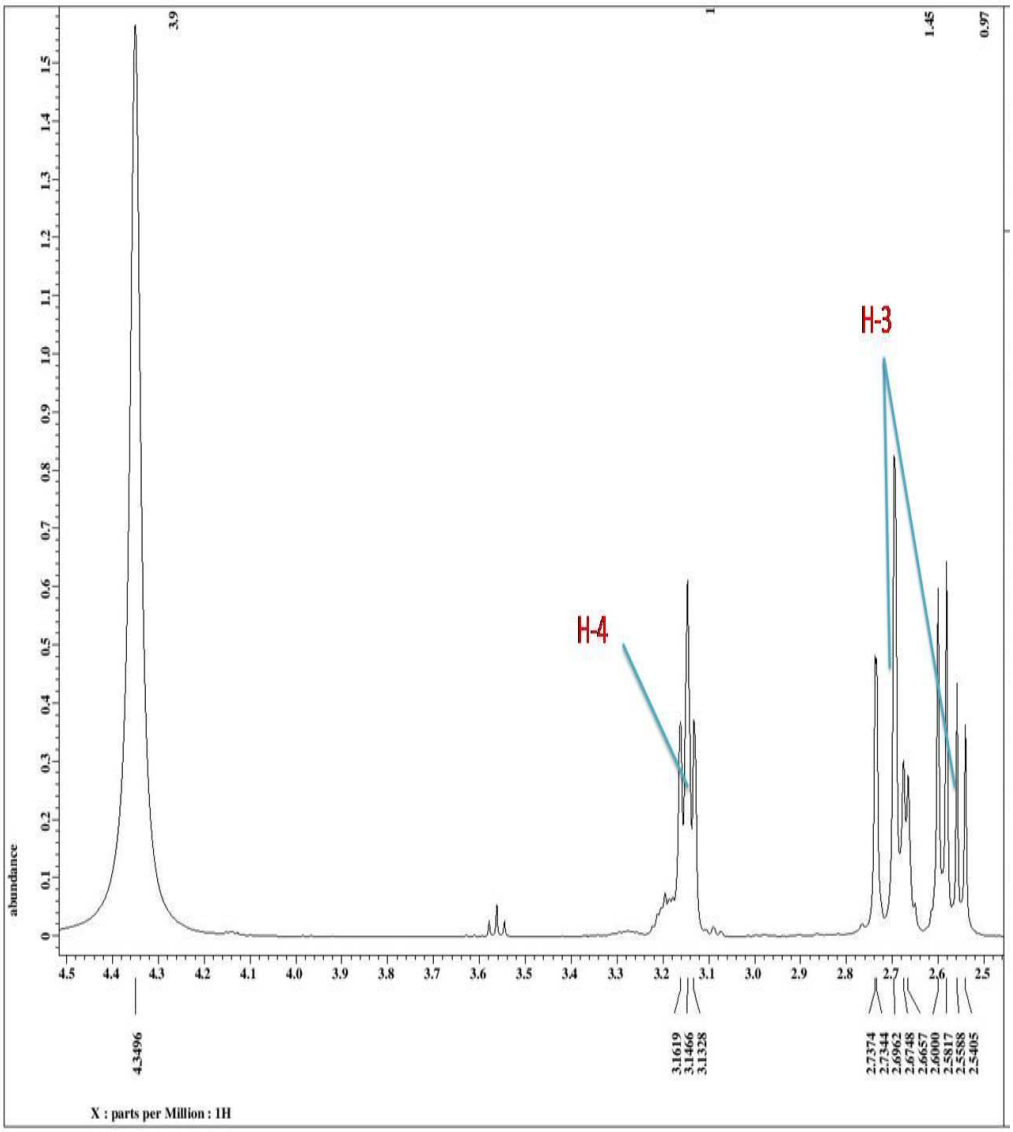
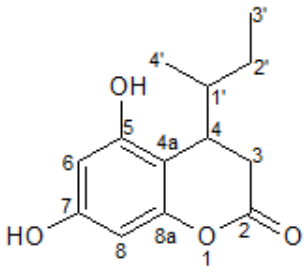


Figure 4.21: Expanded ^{1}H NMR spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24] (400 MHz, acetone-d_6)



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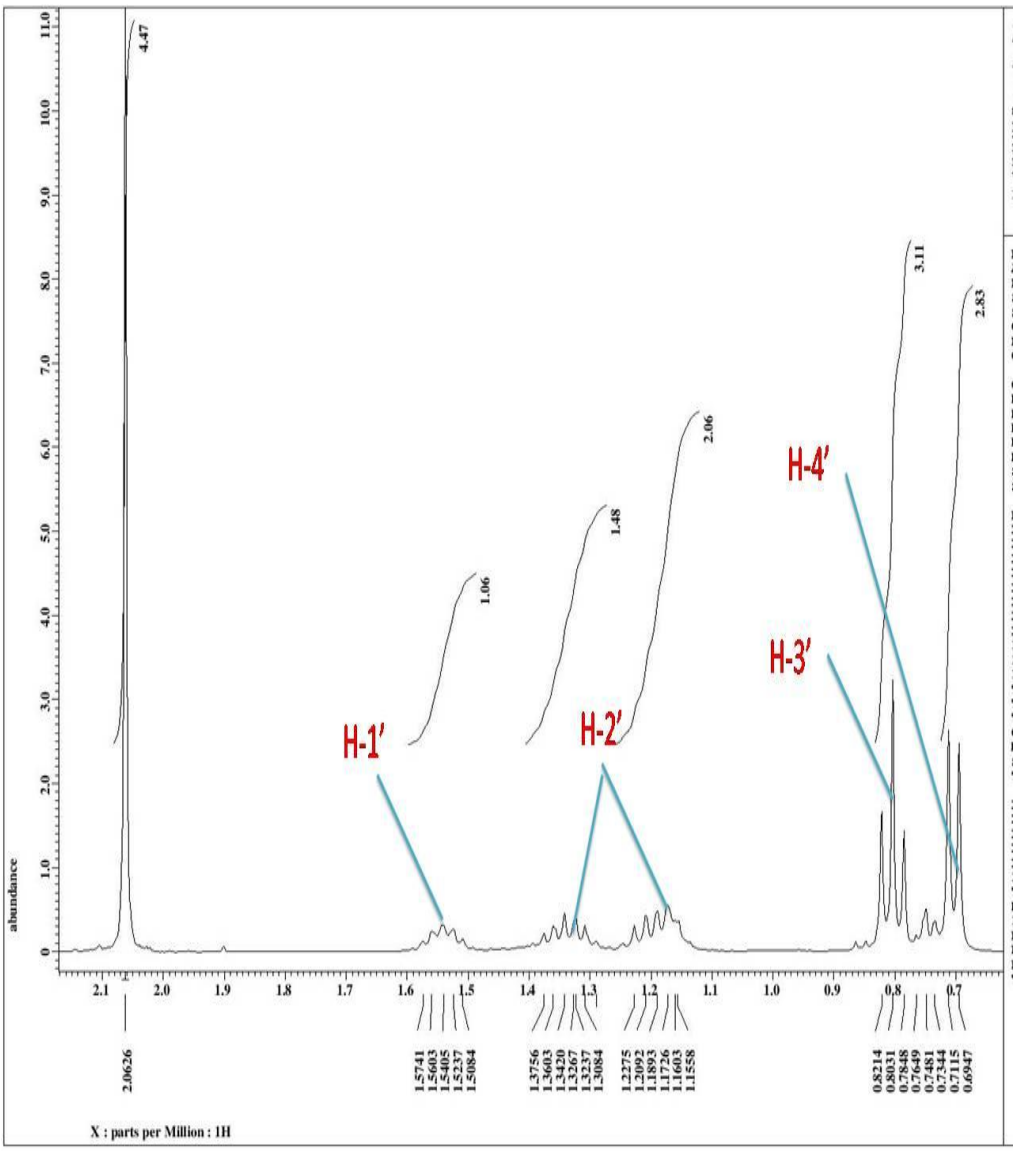
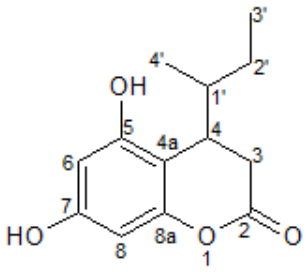


Figure 4.22: Expanded ^{1}H NMR spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24] (400 MHz, acetone-d_6)



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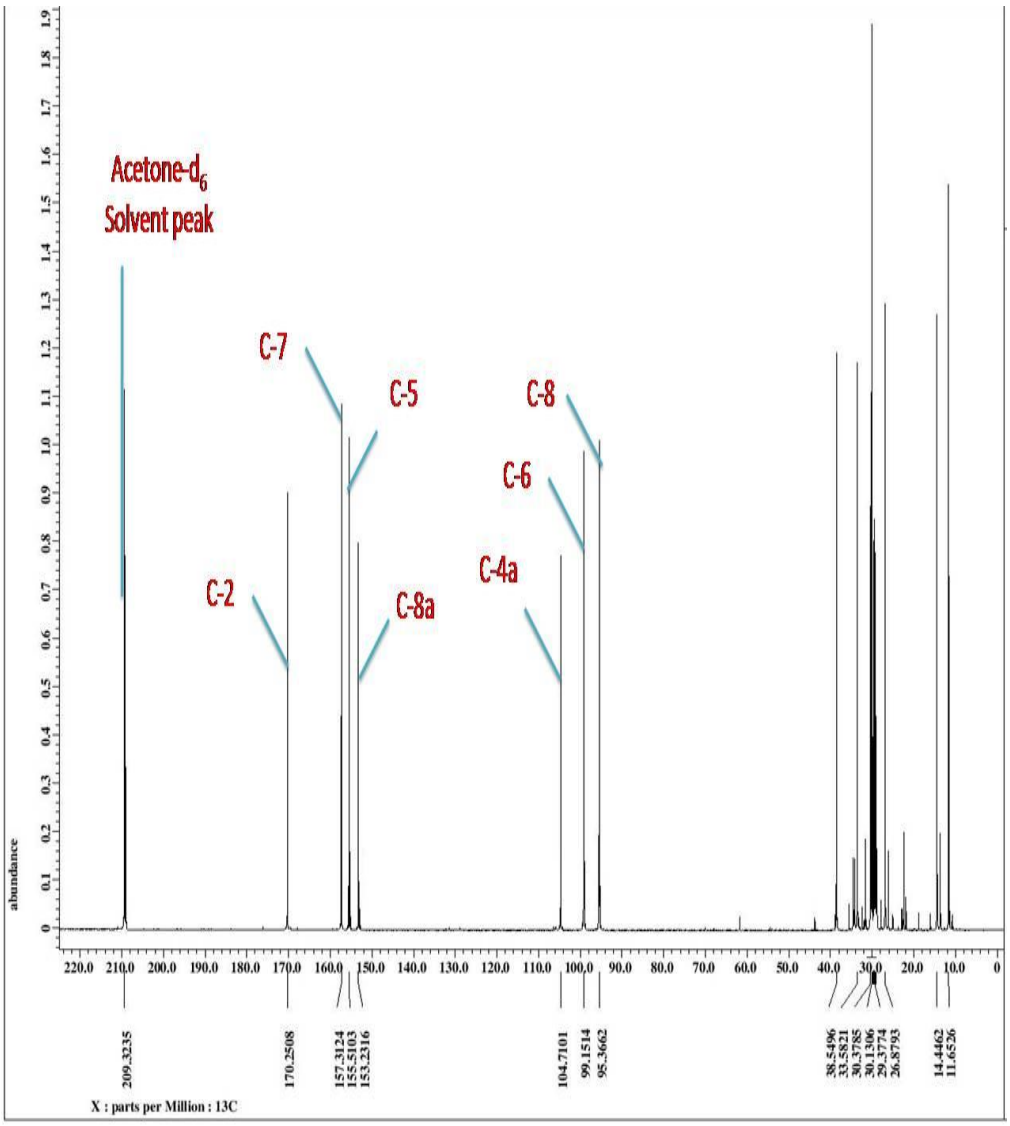
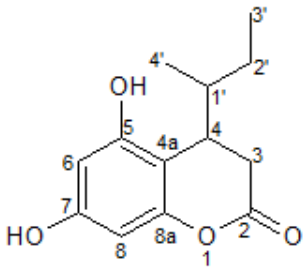


Figure 4.23: ^{13}C NMR spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24] (100 MHz, acetone-d_6)



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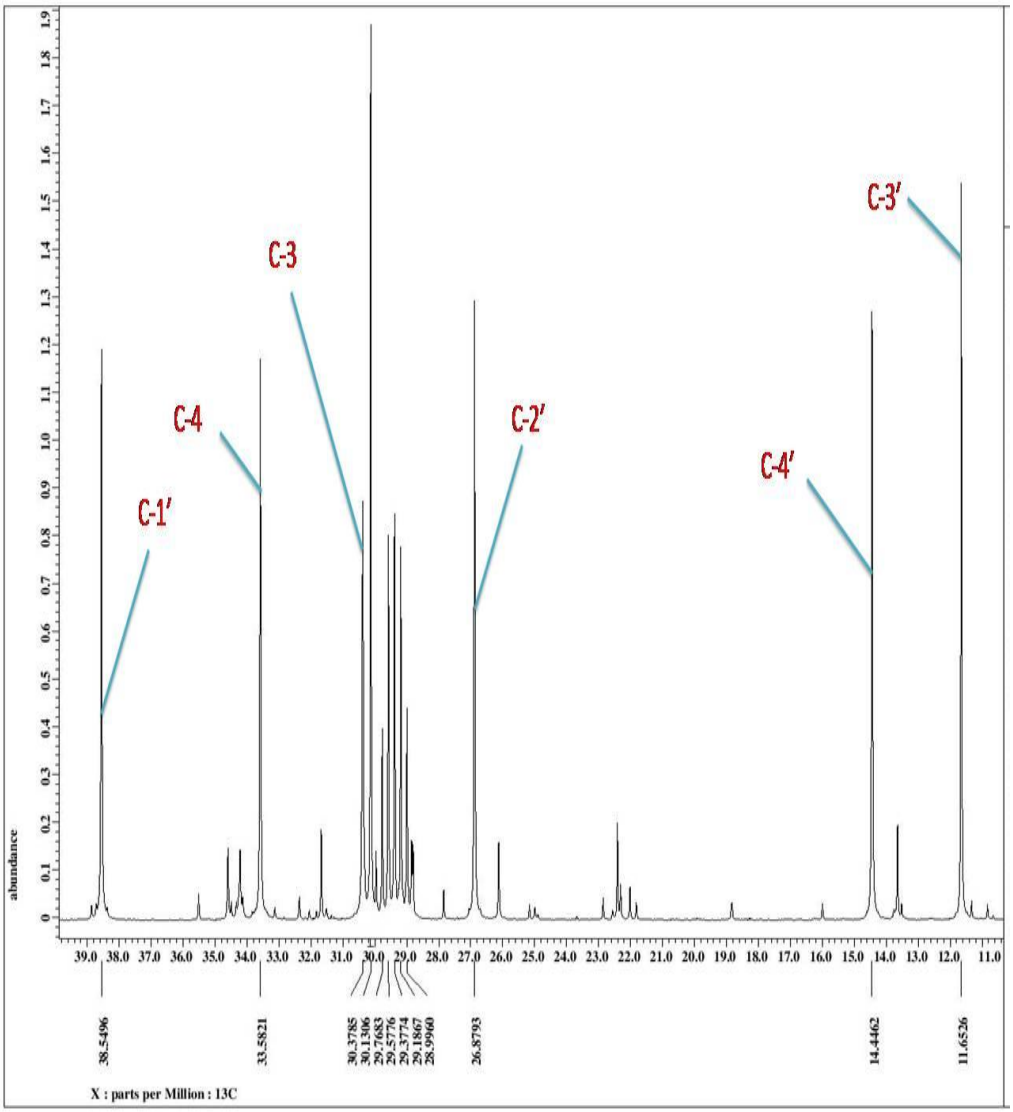
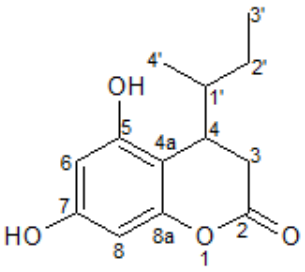


Figure 4.24: Expanded ^{13}C NMR spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24] (100 MHz, acetone-d_6)



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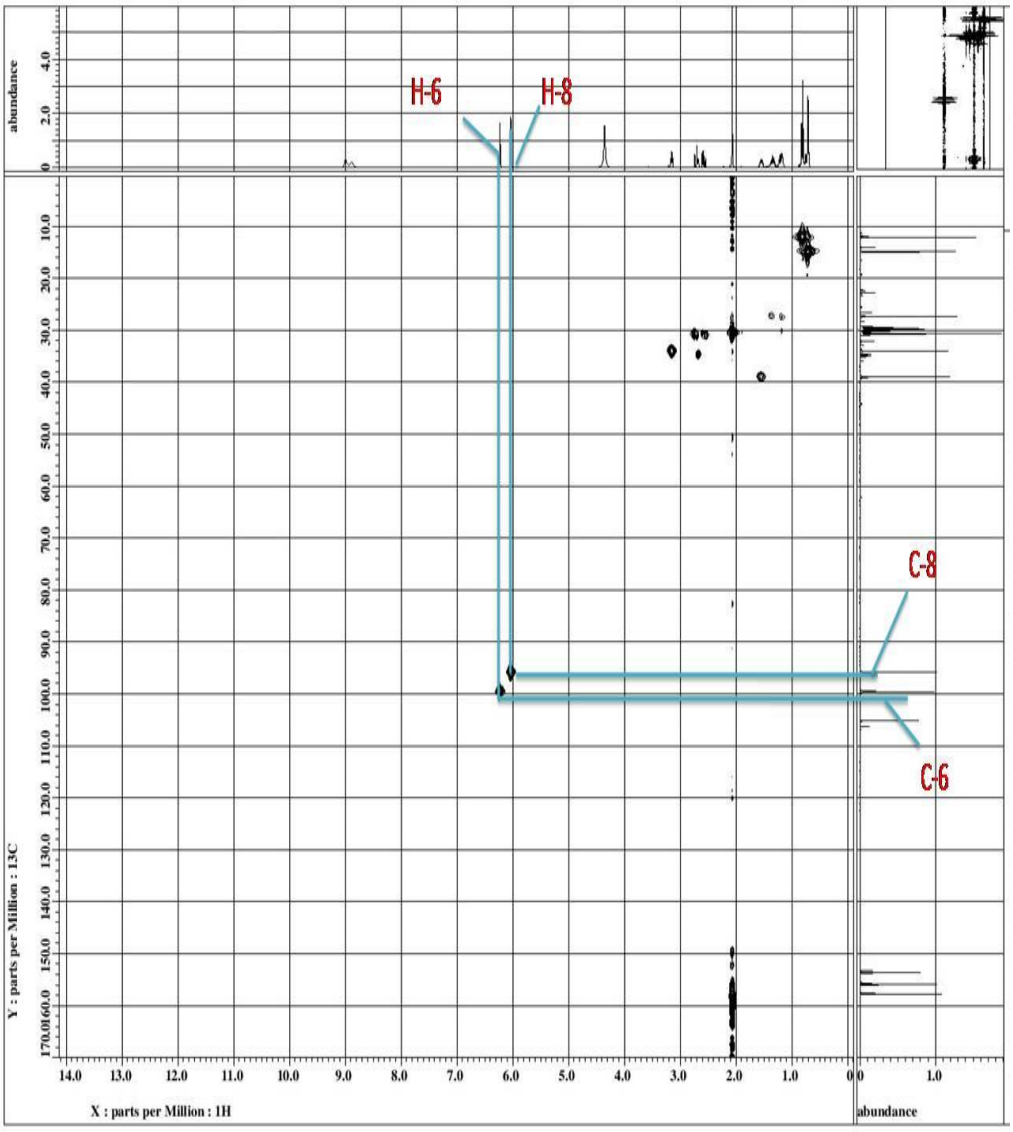
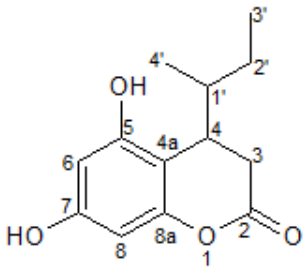


Figure 4.25: HMQC spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]



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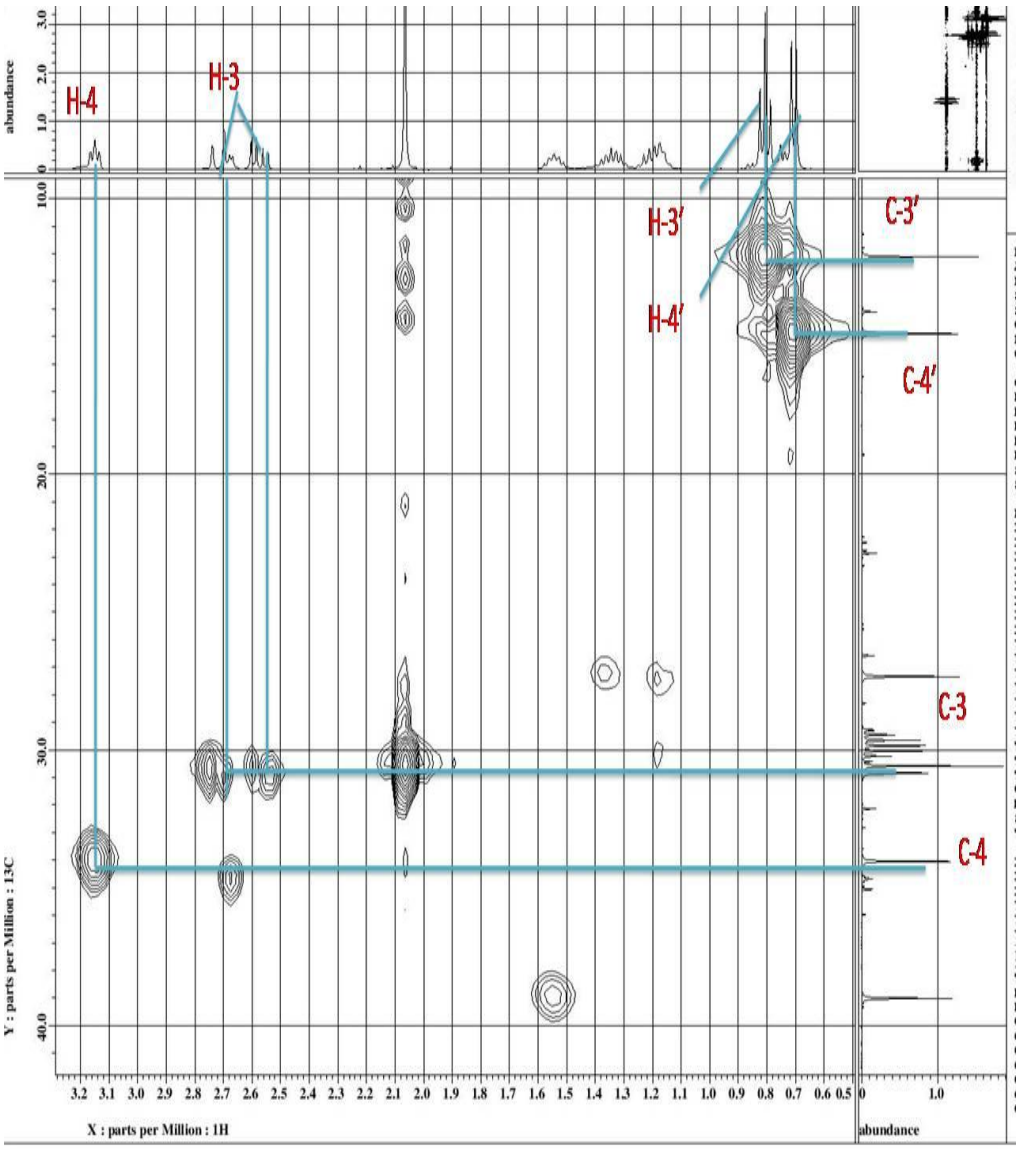
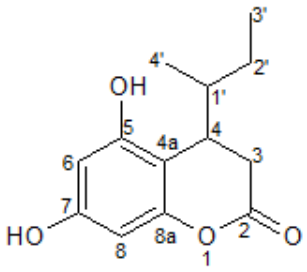


Figure 4.26: Expanded HMQC spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]



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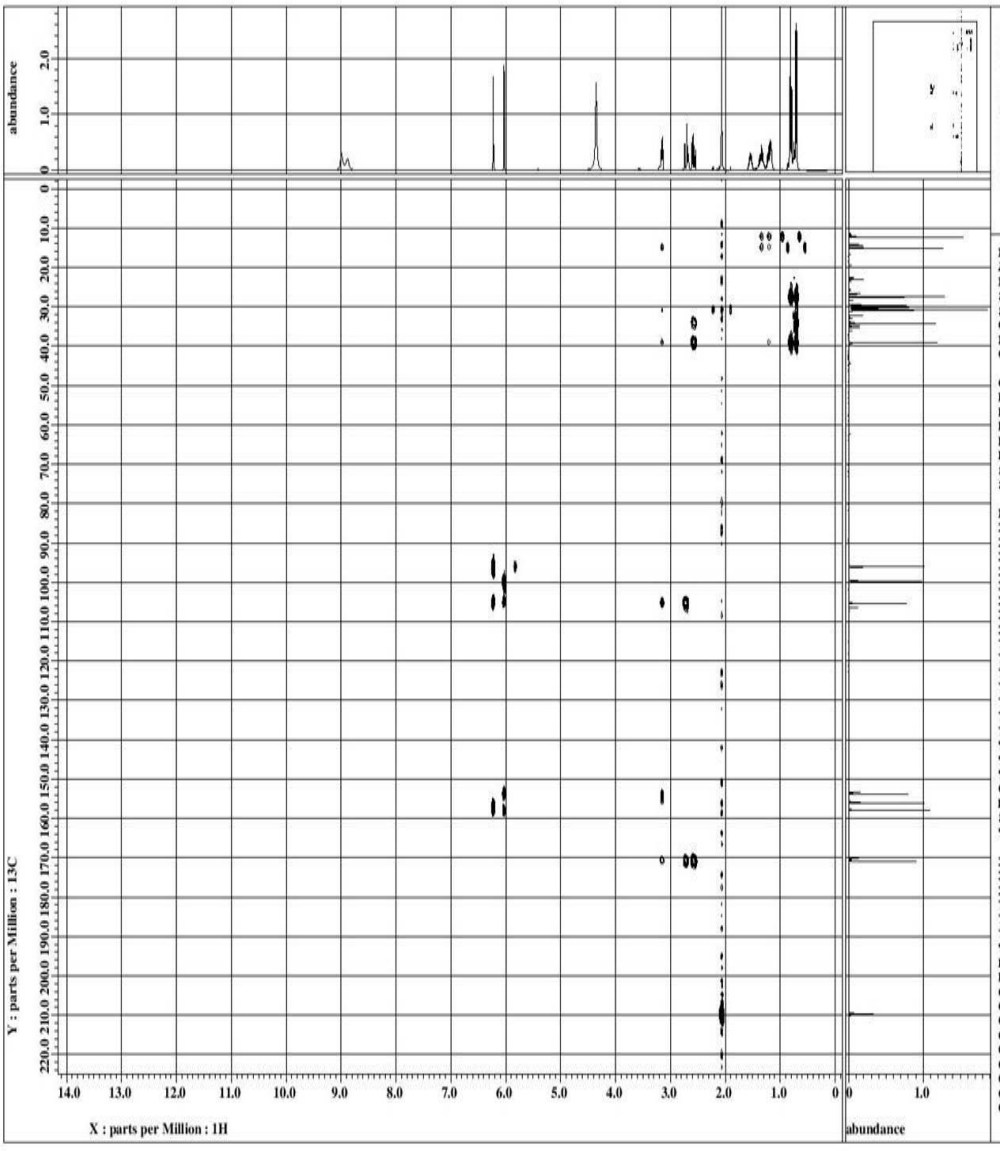
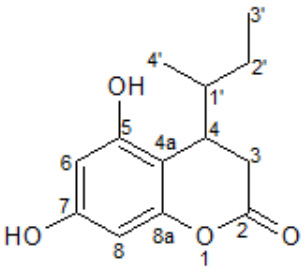


Figure 4.27: HMBC spectrum of 5,7-dihydroxy-4-(3-butyl)-3,4-dihydrocoumarin [24]



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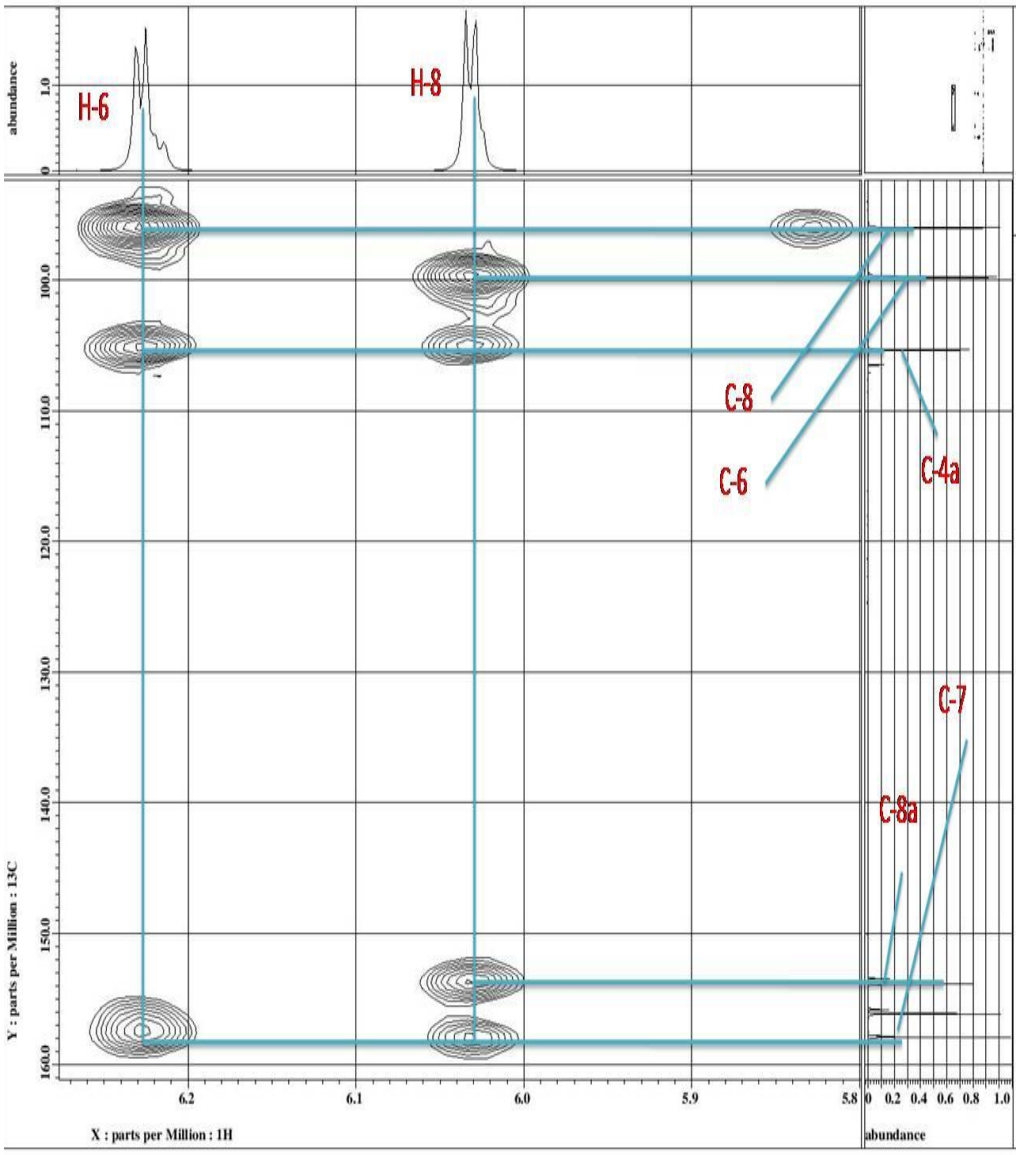
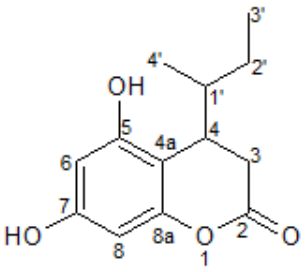


Figure 4.28: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyll)-3,4-dihydrocoumarin [24]



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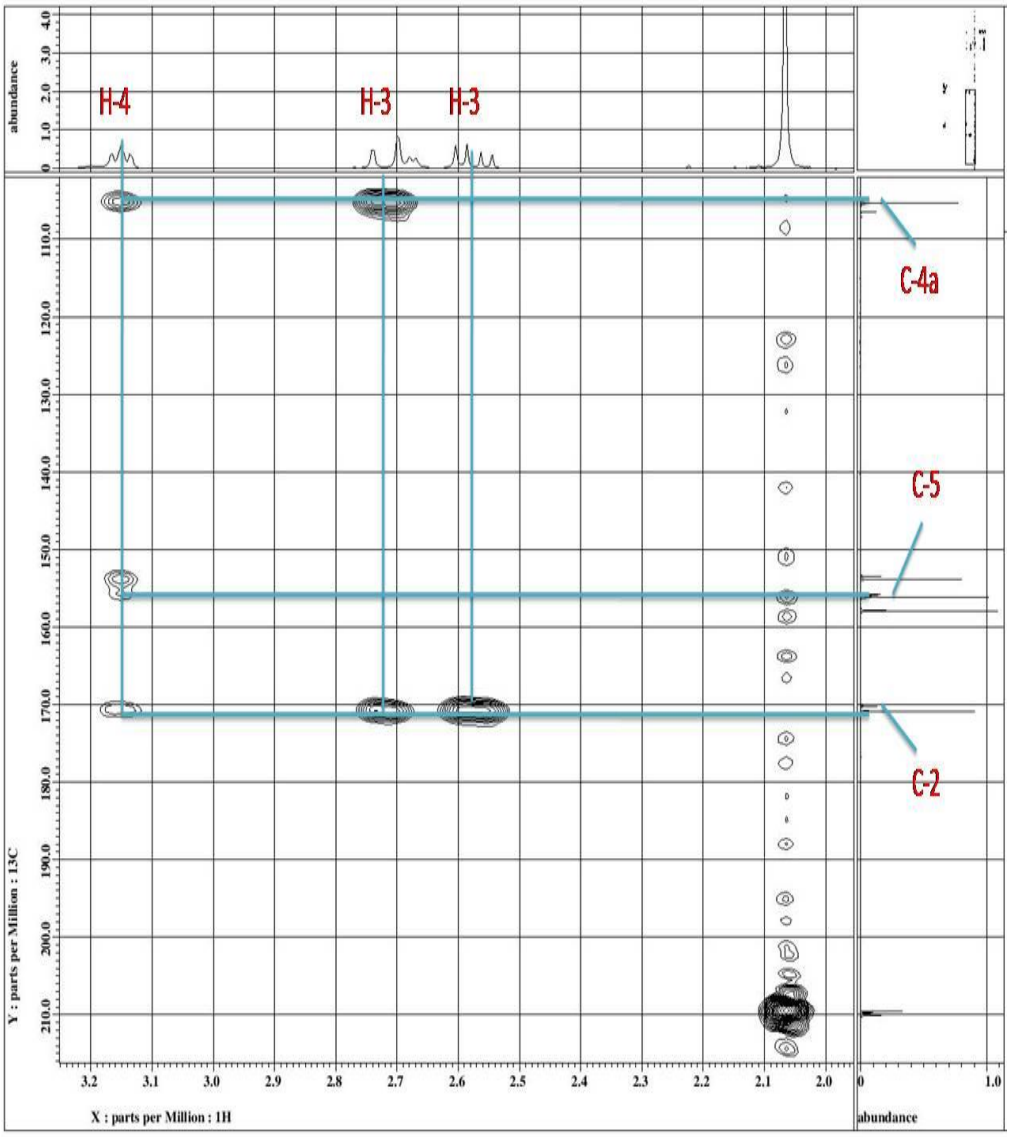
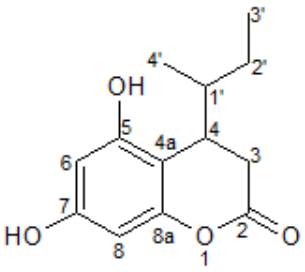


Figure 4.29: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]



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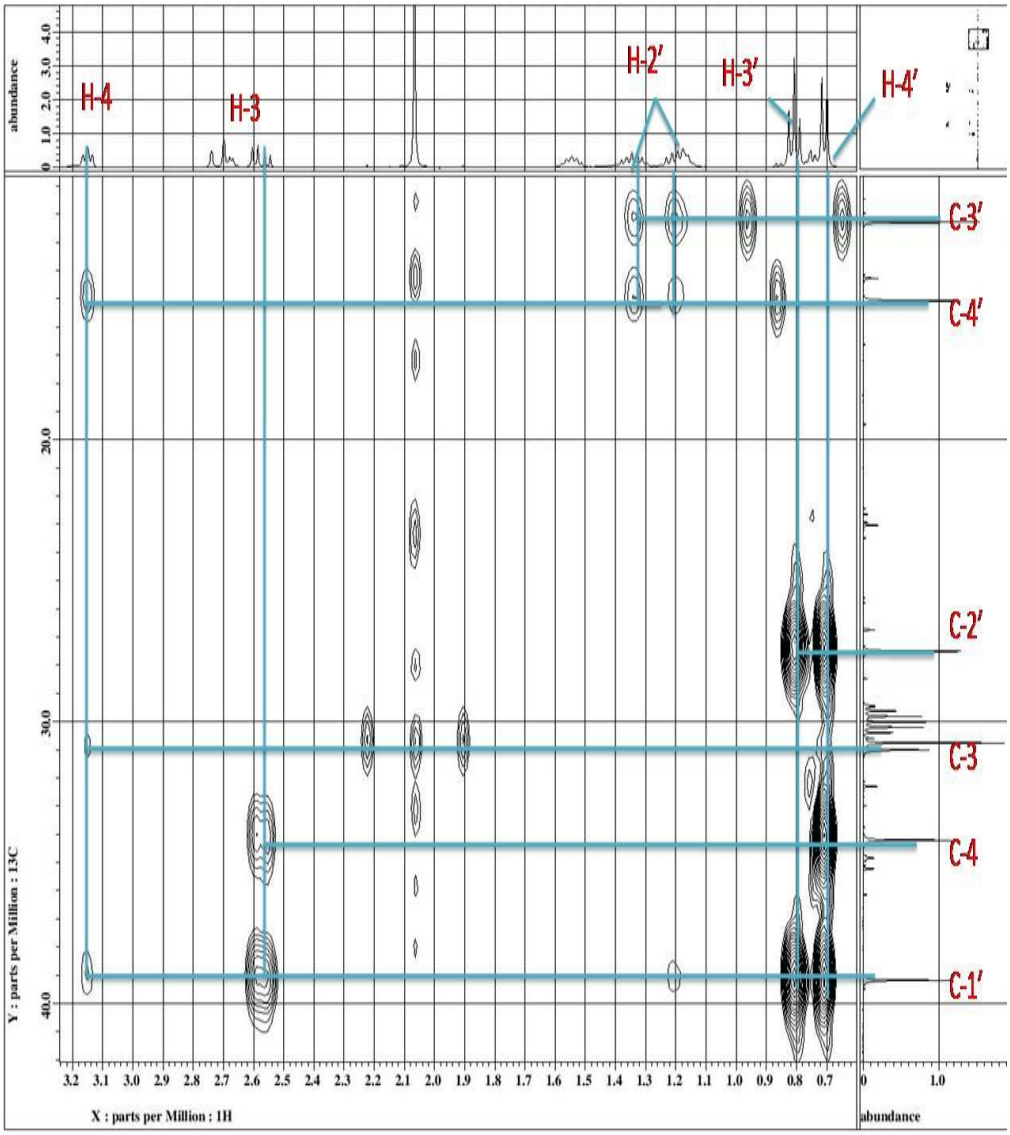
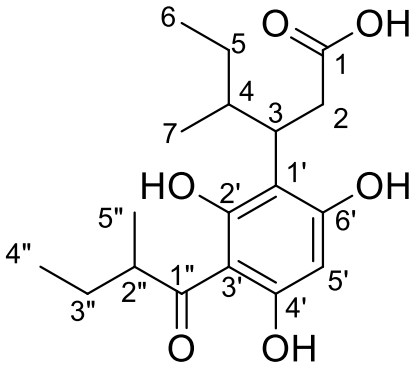


Figure 4.30: Expanded HMBC spectrum of 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin [24]

4.4 Structural Characterization and Elucidation of 4-Methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

About 9.5 mg of compound **25** was obtained as white solid giving a melting point of 169-171 ºC. This compound showed a molecular weight of 338.4 g/mol which was in agreement with the molecular formula of $C_{18}H_{26}O_6$ for the assigned structure. In the TLC analysis, compound **25** showed a single spot under both UV light of long wavelength (365 nm) and short wavelength (254 nm) and in iodine chamber. This compound gave a retention factor, R_f value of 0.32 using 100% dichloromethane as mobile phase.

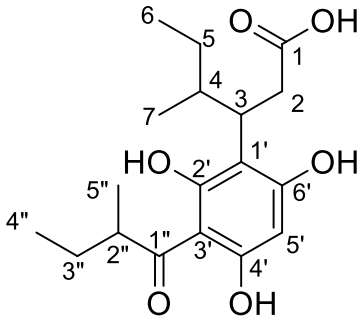
In the IR spectrum (Figure 4.31), a sharp and intense band observed at 3402 cm^{-1} indicated the presence OH group. The C-O stretch and C-H stretch were indicated by the absorption maxima at 1150 and 2935 cm^{-1}, respectively. Meanwhile, the C=O stretch for carboxyl and keto groups were observed at 1621 and 1753 cm^{-1}, respectively. The UV-Vis spectrum (Figure 4.32) showed the absorption maxima at 234.3, 285.0 and 321.6 nm indicating the compound to have a conjugated system.

In the 1H NMR spectra (Figures 4.33 to 4.35), the lone aromatic proton H-5' in the benzene ring gave a singlet signal at δ 6.28. The most deshielded singlet at δ 13.60 was assigned to the chelated hydroxyl proton 2'-OH or 4'-OH which can be interchangeable. The presence of 2-methylbutanoyl moiety in the structure displayed characteristic proton signals at δ 3.69 (1H, sextet), 1.41 and 1.27 (2H, m), 0.96 (3H, t) and 1.15 (3H, d) for protons H-2'', H-3'', H-4'' and H-5'', respectively. Meanwhile, the existence of hexanoic acid side chain was revealed by the characteristic proton signals at δ 2.59 and 2.91 (2H, dd), 3.22 (1H, t), 1.64 (1H, m), 1.85 (1H, m), 0.90 (3H, t) and 0.86 (3H, d) which were assigned to protons H-2, H-3, H-4, H-5, H-6 and H-7, respectively.

The ^{13}C NMR spectra (Figures 4.36 and 4.37) showed a total of 18 carbon signals wherein seven of them were assigned to quaternary sp^2 hybridized carbons C-1 (168.0 ppm), C-1' (105.5 ppm), C-2' (153.3 ppm), C-3' (104.8 ppm), C-4' (165.1 ppm), C-6' (159.2 ppm) and C-1'' (210.0 ppm). The remaining carbon signals appeared below δ 90.0 were assigned to the sp^3 hybridized carbons in the 4-methylbutanoyl and hexanoic acid moieties.

Compound **25** was a phloroglucinol derivative. The attachment of hexanoic acid side chain to the phloroglucinol core in the structure was confirmed by HMBC analysis. In the HMBC spectrum (Figure 4.43), proton signal, H-3 at δ 3.22 (1H, t) was correlated to carbon signals at δ 105.5 (C-1'), 153.3 (C-2') and 159.2 (C-6') which established the hexanoic acid moiety to the attached to the phloroglucinol core at carbon position C-1'. The second side chain was

found to be attached to either carbon position C-3’ or C-5’ which gave identical compound in the substitution. The remaining unsubstituted aromatic carbon in the phloroglucinol core was confirmed by the lone aromatic proton singlet signal for H-5’ at δ 6.21 (1H, s) in the ^{1}H NMR spectrum (Figure 4.33). The assignment of HMQC and the remaining of HMBC correlations for compound **25** was summarized in Table 4.3. Based on the spectral evidence, compound **25** was identified as 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid.



[25]

Table 4.3: Summary of NMR data for 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			^2J	^3J
1	-	168.0	-	-
2	2.59 (1H, dd, *J* = 16.5, 7.3 Hz 2.91 (1H, dd, *J*= 16.5, 1.2 Hz	30.3	C-1	C-1’
3	3.22 (1H, t, *J* = 5.5 Hz)	33.6	C-1’	C-2’, C-6’, C-1
4	1.64 (1H, m)	38.2	-	-
5	1.85 (2H, m)	27.2	C-4, C-6	C-7
6	0.90 (3H, t, *J* = 8.8 Hz)	12.0	C-5	C-4
7	0.86 (3H, d, *J* = 6.7 Hz)	15.1	C-4	C-5
1’	-	105.5	-	-
2’	-	153.3	-	-
3’	-	104.8	-	-
4’	-	165.1	-	-
5’	6.21 (1H, s)	99.9	C-4’, C-6’	C-3’
6’	-	159.2	-	-
1’’	-	210.0	-	-
2’’	3.69 (1H, sextet), *J* =	46.6	C-1’’	-
3’’	1.27 (1H, m) 1.41 (1H, m)	27.0	C-2’’, C-4’’	C-5’’
4’’	0.96 (3H, t, *J* = 15.3 Hz)	11.8	C-3’’	-
5’’	1.15 (3H, d, *J* = 6.7 Hz)	16.7	C-2’’	C-3’’
2’-OH/ 4’-OH	13.60 (1H, s)	-	C-3’ C-5’	C-4’
6’-OH	7.06 (1H, brs)	-	-	-

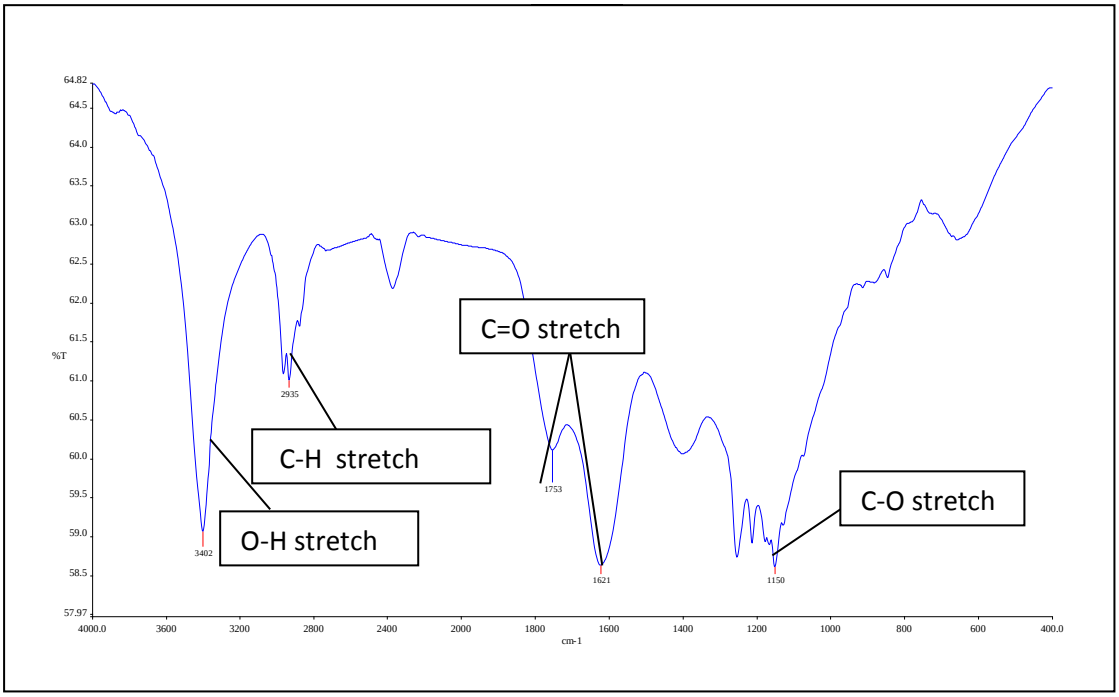


Figure 4.31: IR spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]

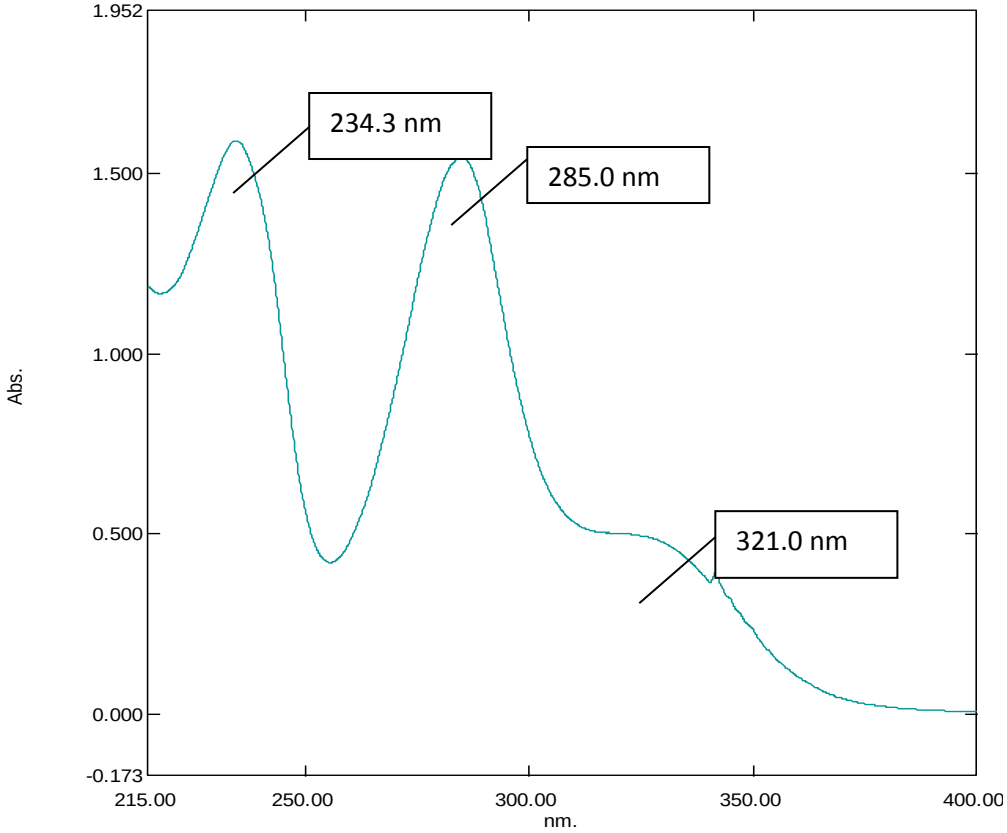
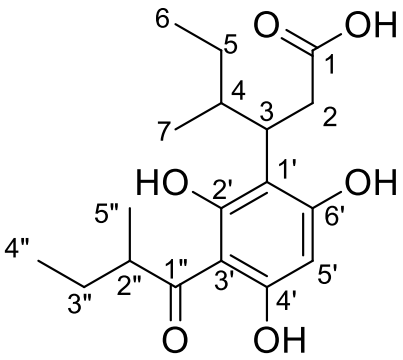


Figure 4.32: UV-Vis spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

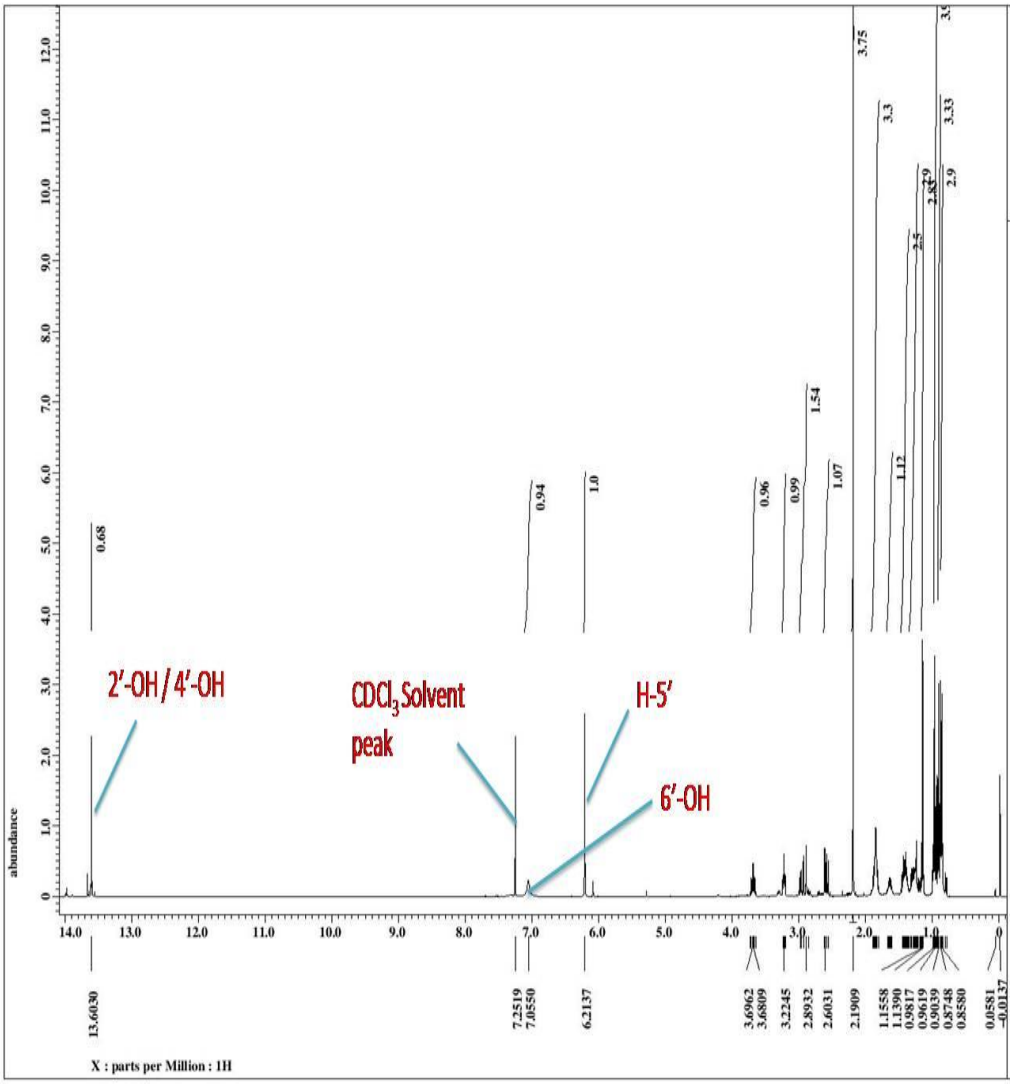
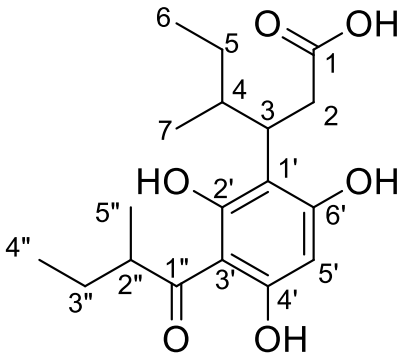


Figure 4.33: ^{1}H NMR spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25] (400 MHz, $CDCl_3$)



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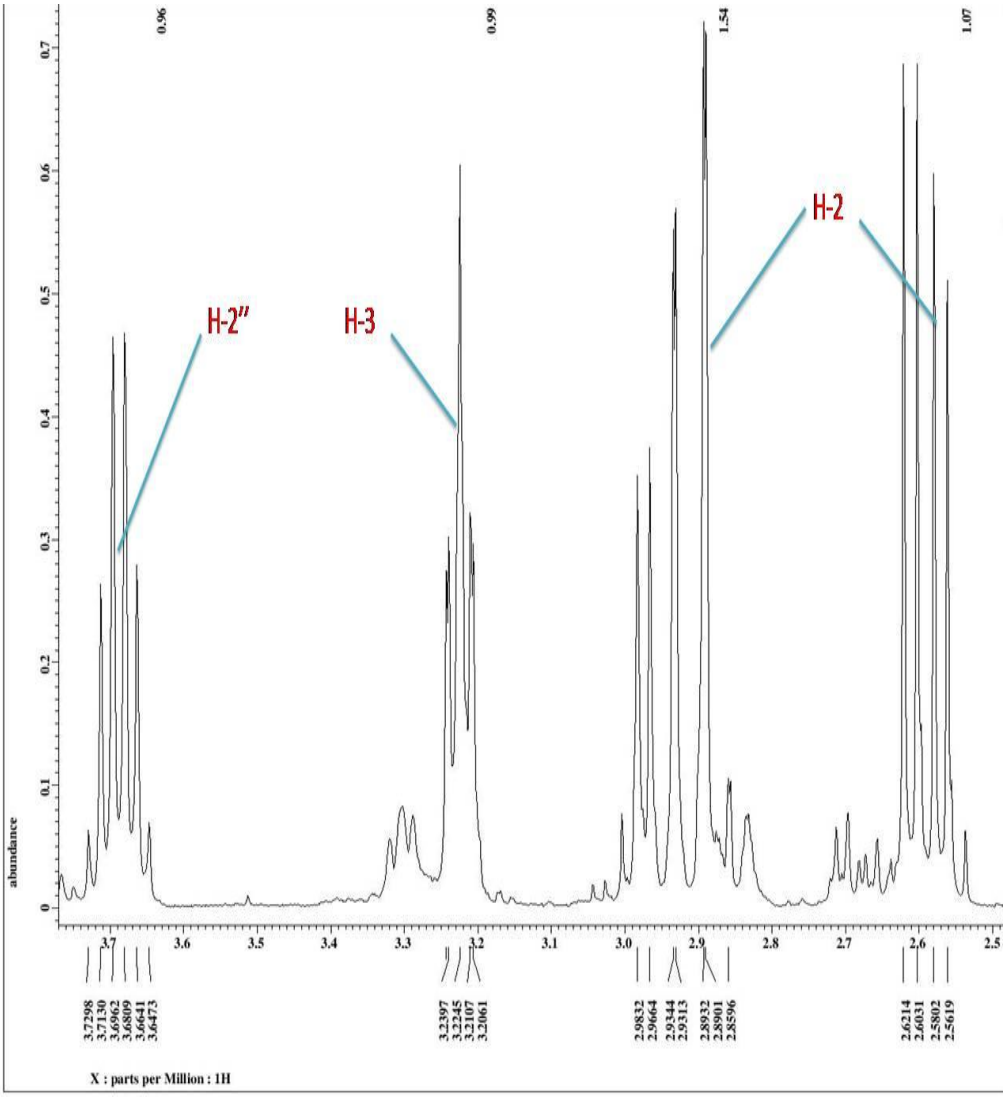
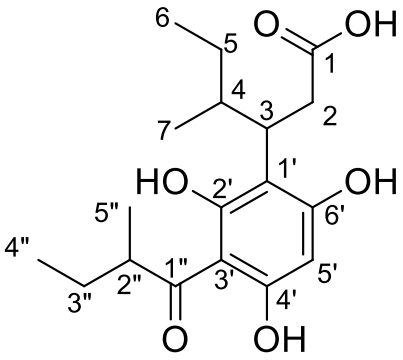


Figure 4.34: Expanded ^{1}H NMR spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25] (400 MHz, $CDCl_3$)



[25]

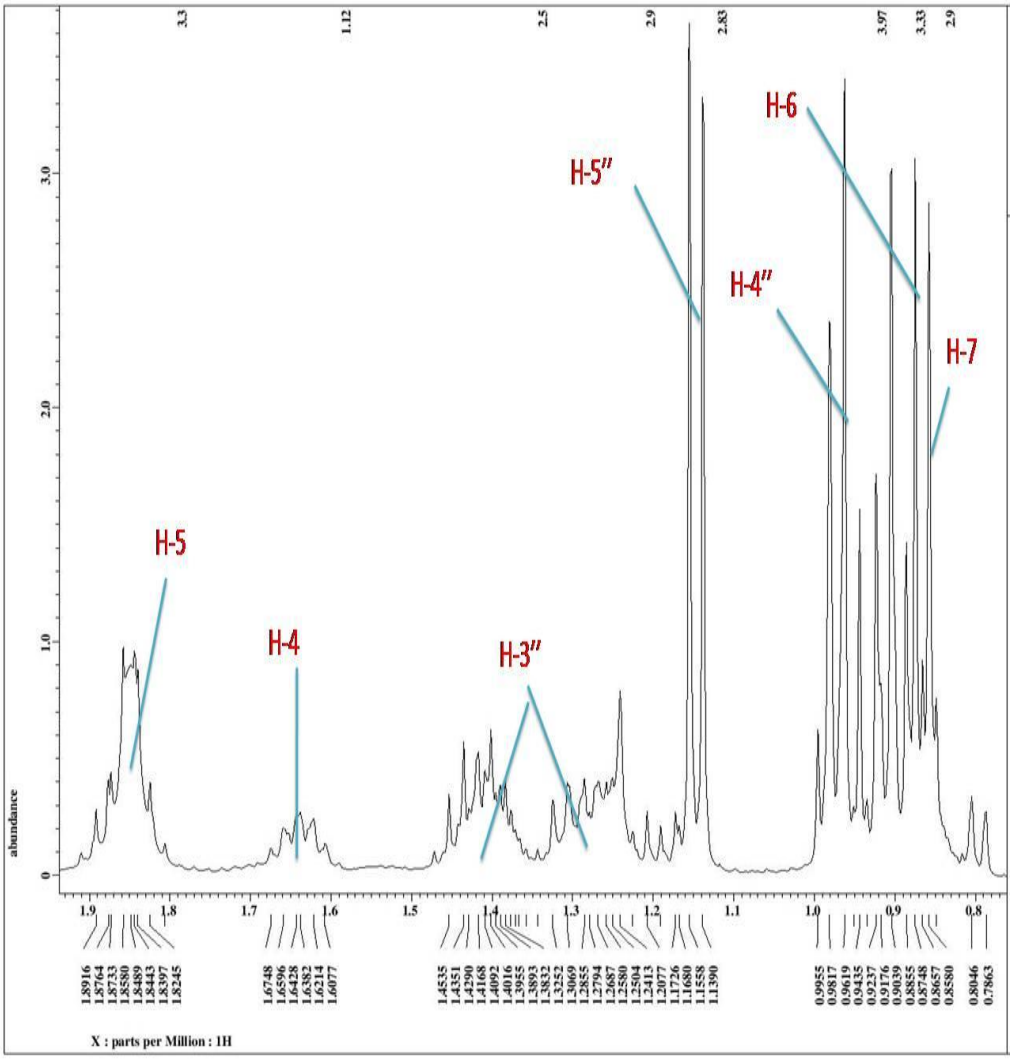
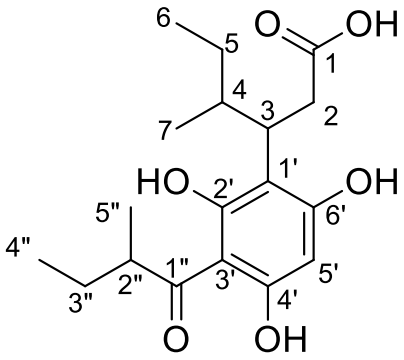


Figure 4.35: Expanded ^{1}H NMR spectrum of 4-methyl-3-[2,4,6 trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25] (400 MHz, $CDCl_3$)



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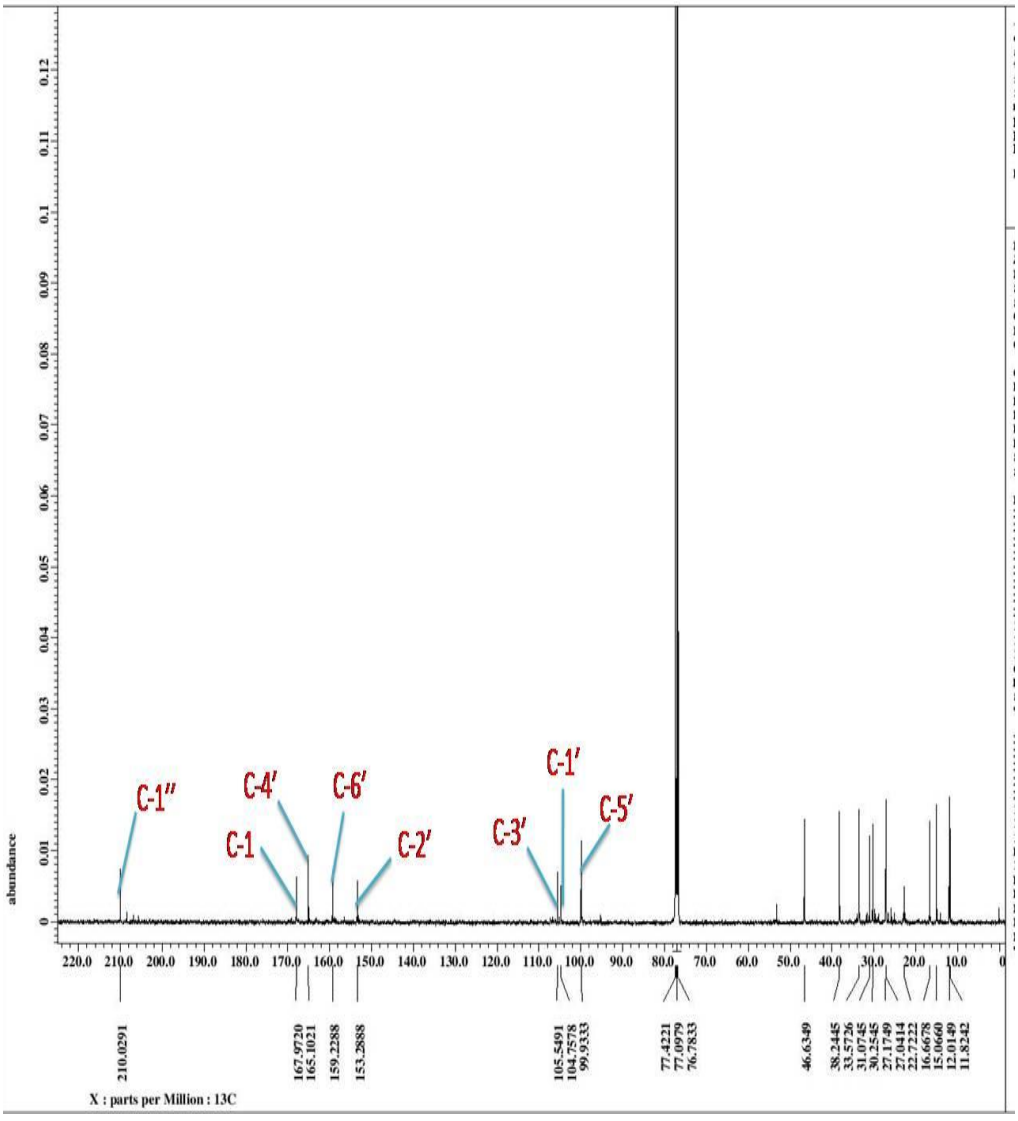
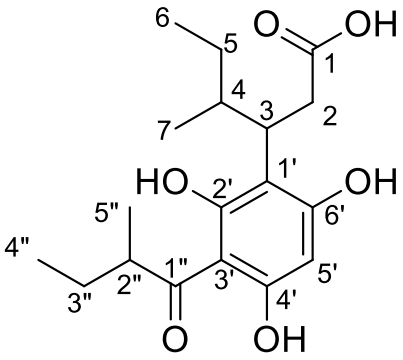


Figure 4.36: ^{13}C NMR spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25] (100 MHz, $CDCl_3$)



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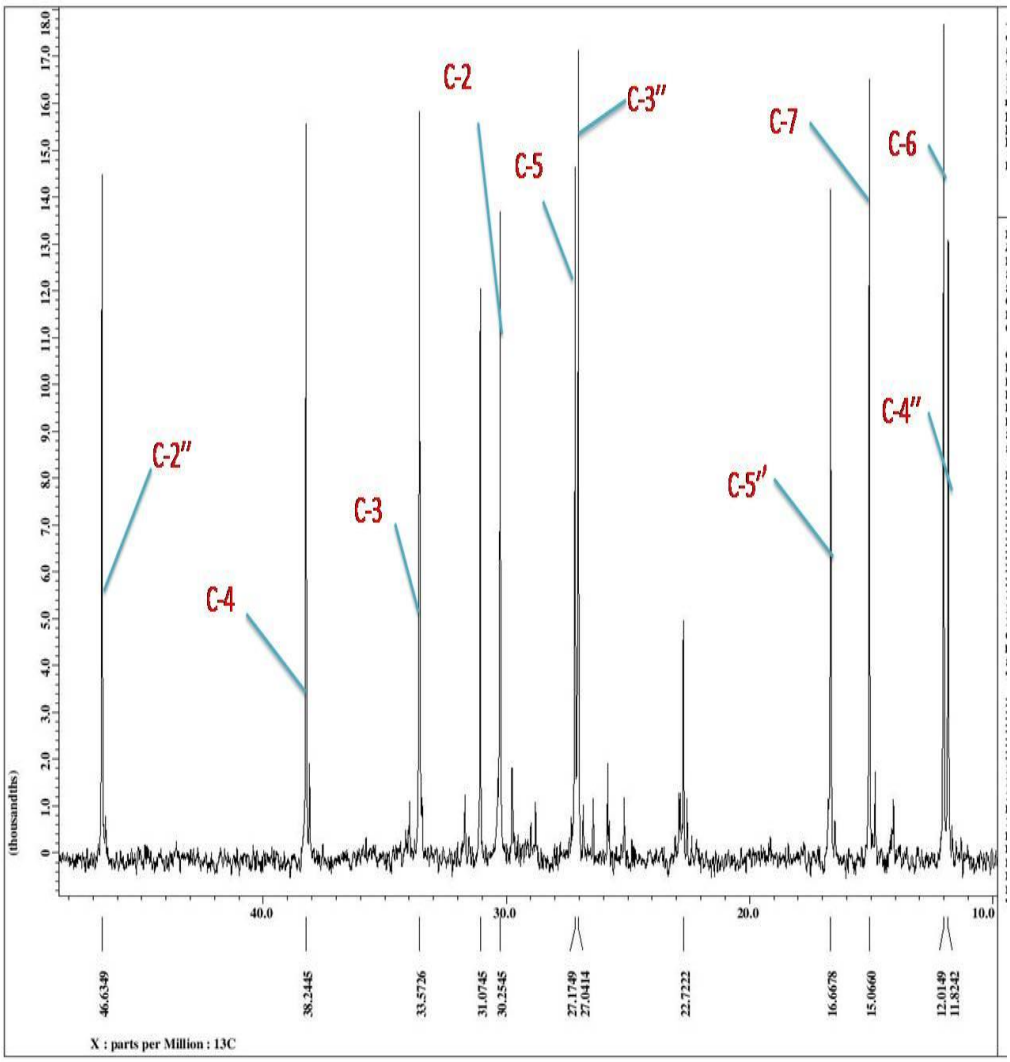
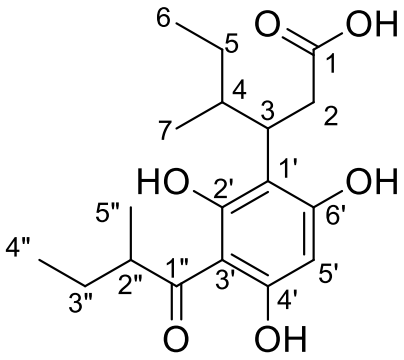


Figure 4.37: Expanded ^{13}C NMR spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25] ` (100 MHz, $CDCl_3$)



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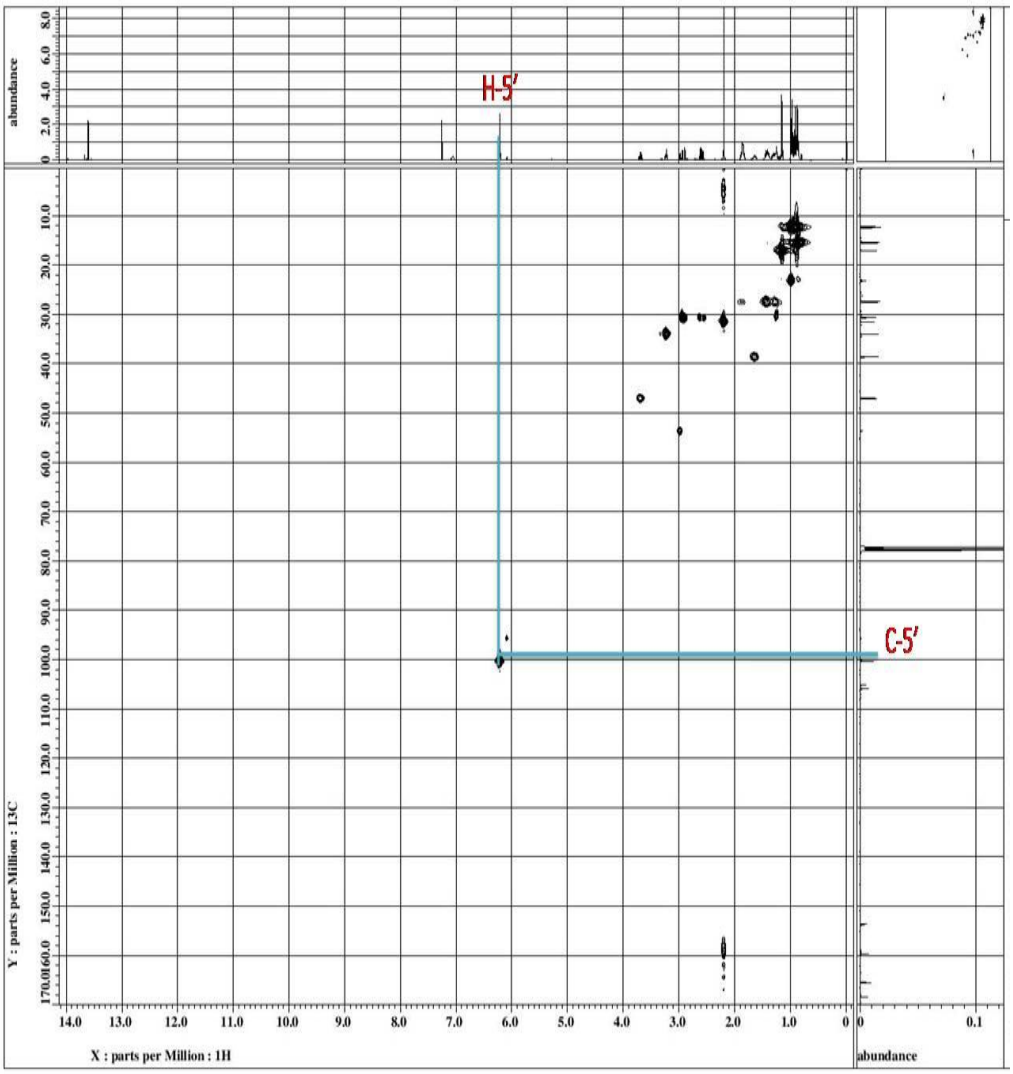
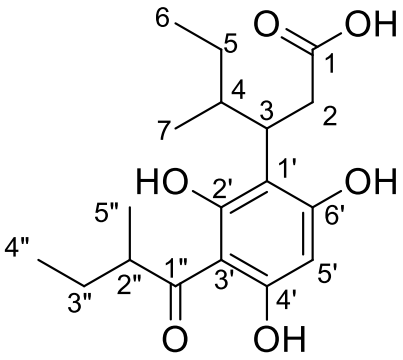


Figure 4.38: HMQC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



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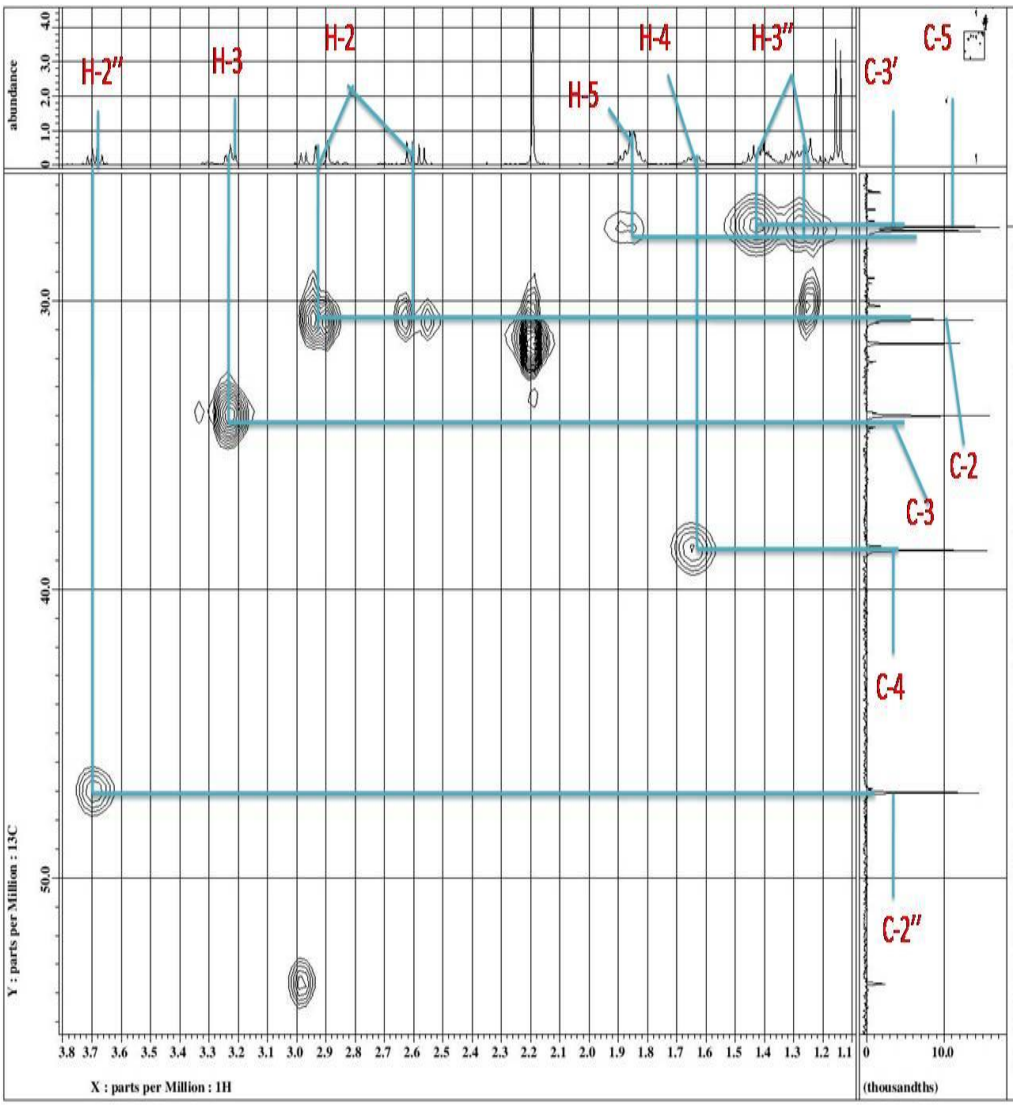
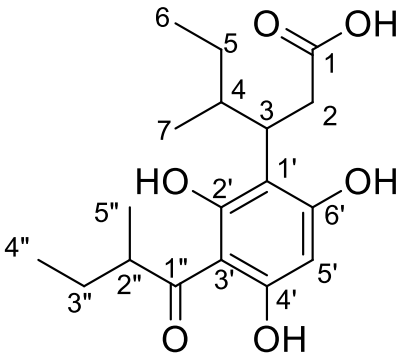


Figure 4.39: Expanded HMQC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

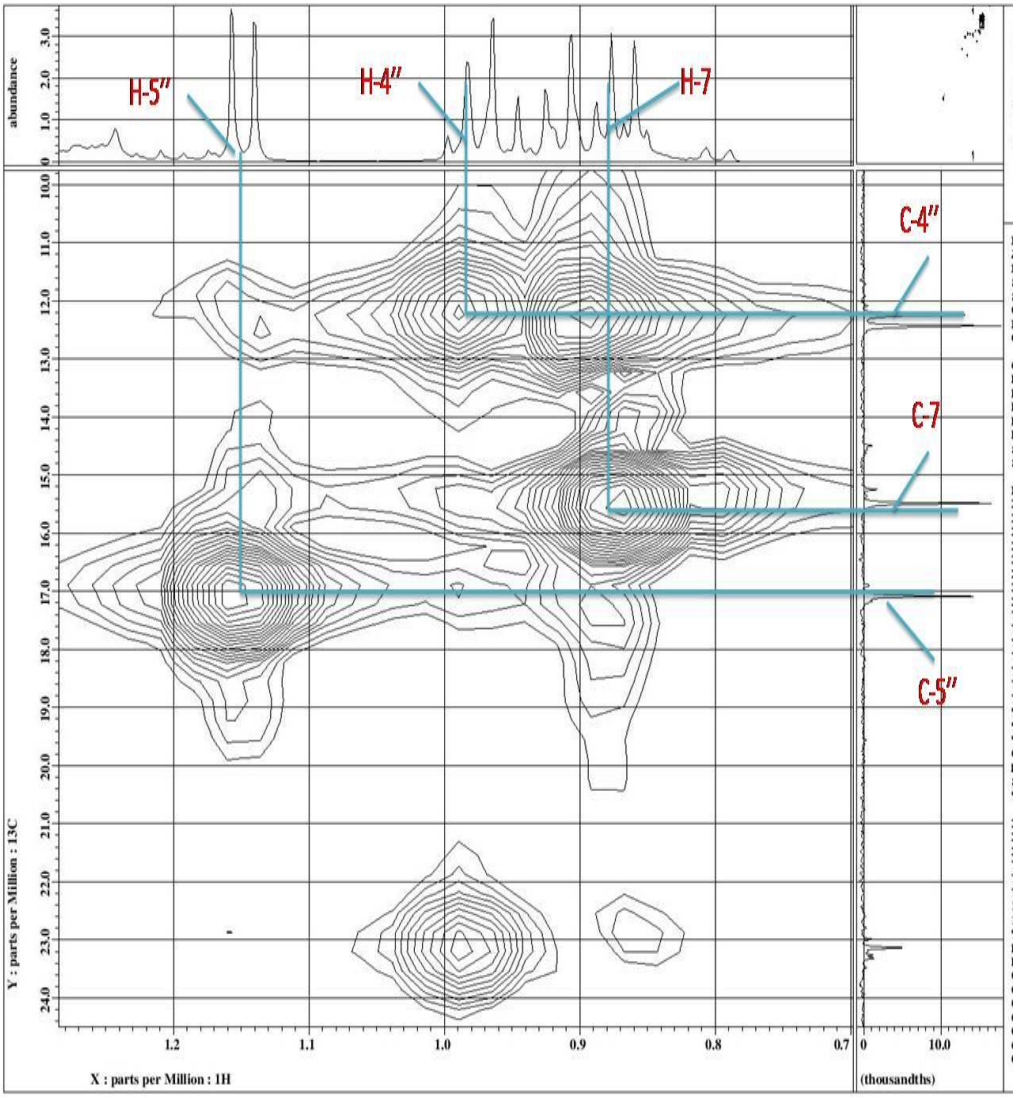
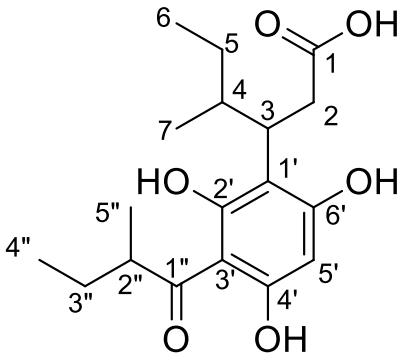


Figure 4.40: Expanded HMQC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

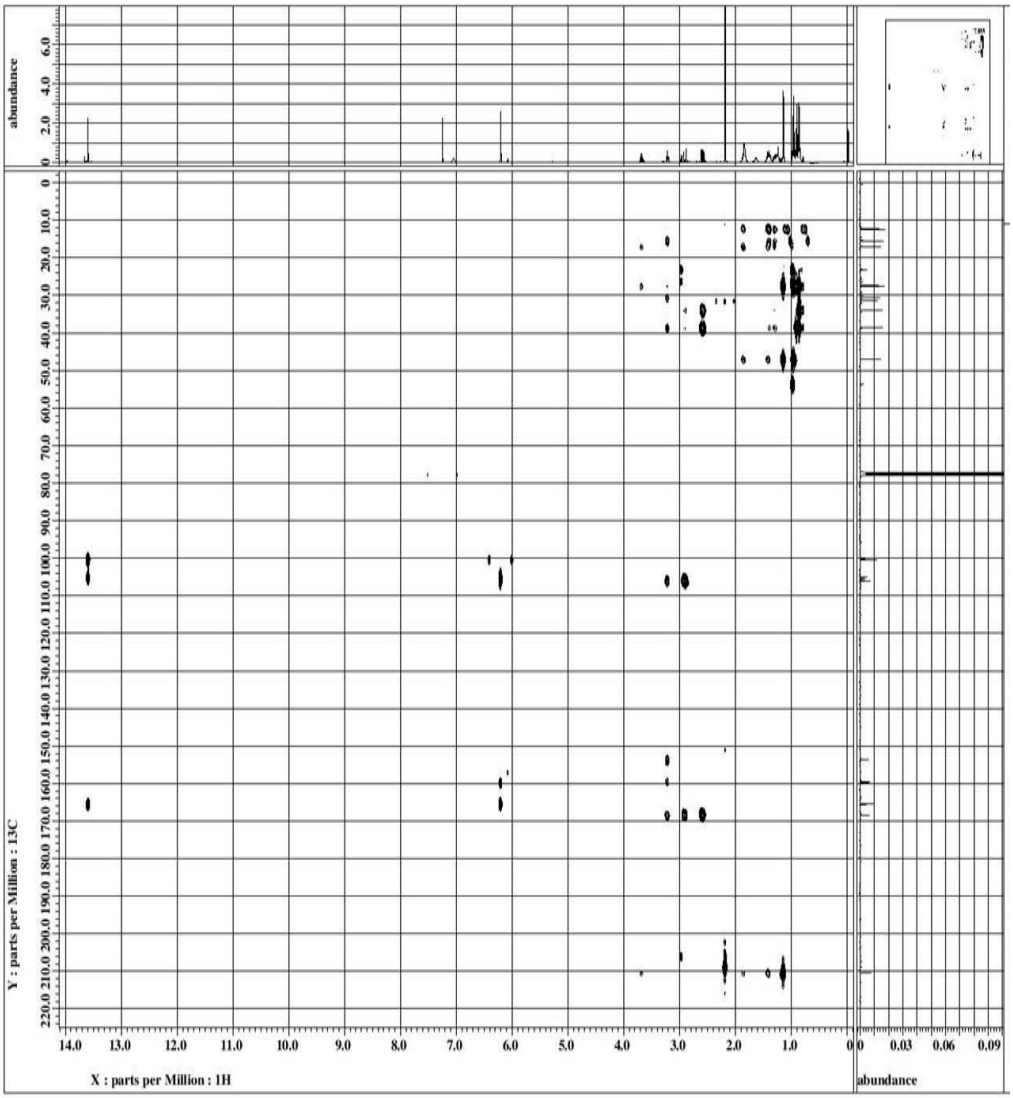
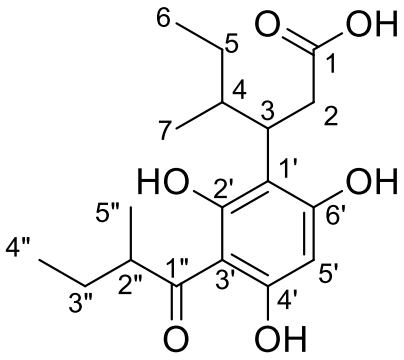


Figure 4.41: HMBC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

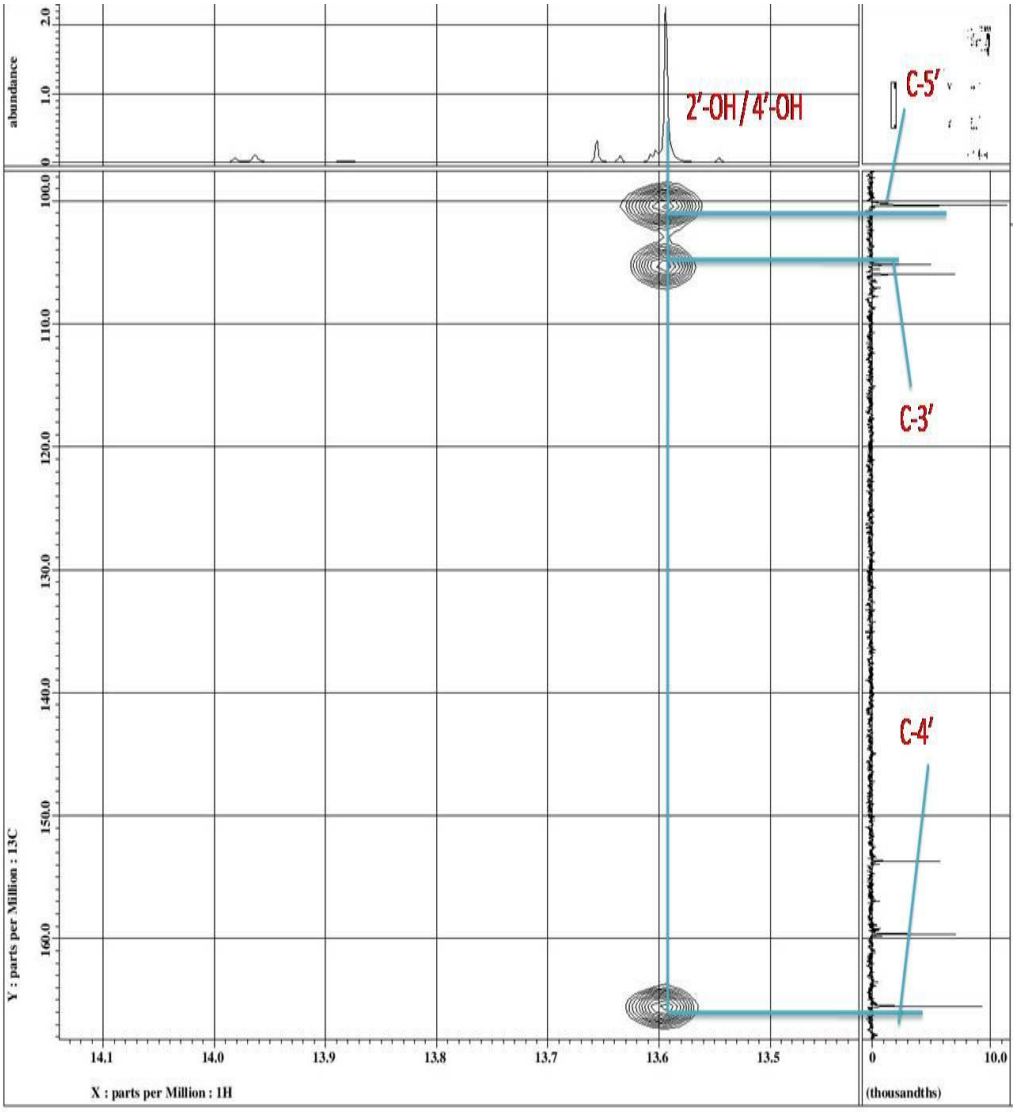
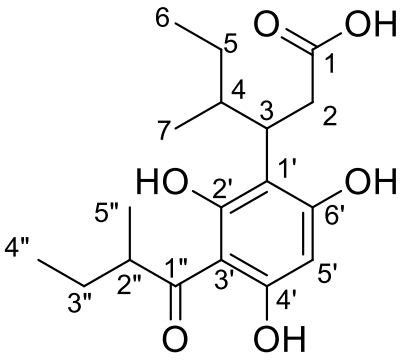


Figure 4.42: Expanded HMBC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

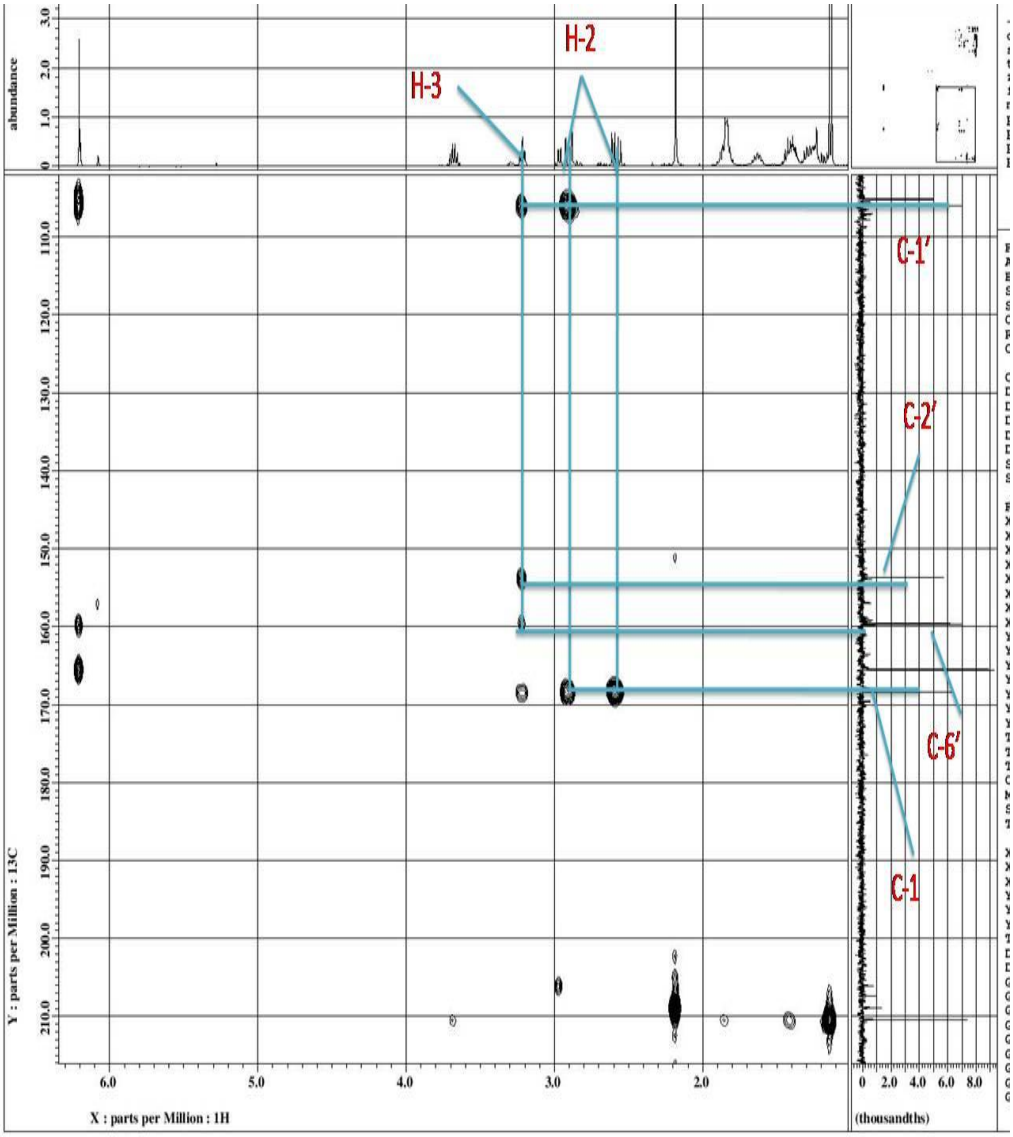
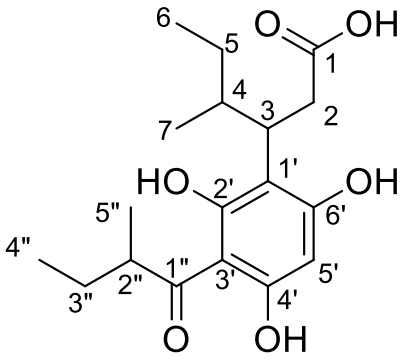


Figure 4.43: Expanded HMBC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

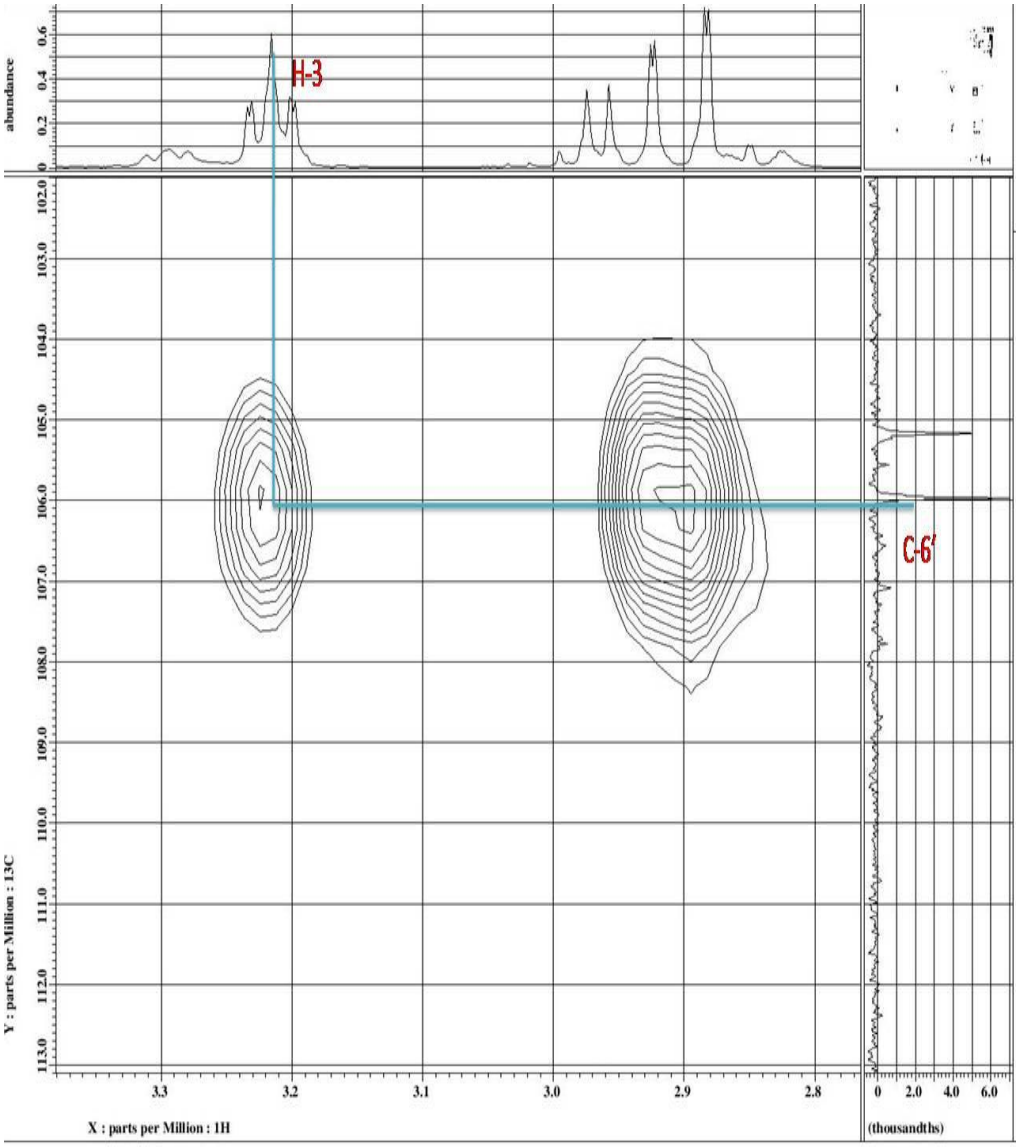
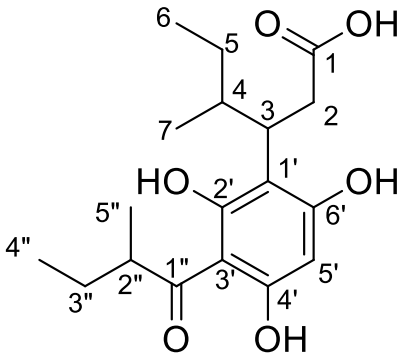


Figure 4.44: Expanded HMBC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

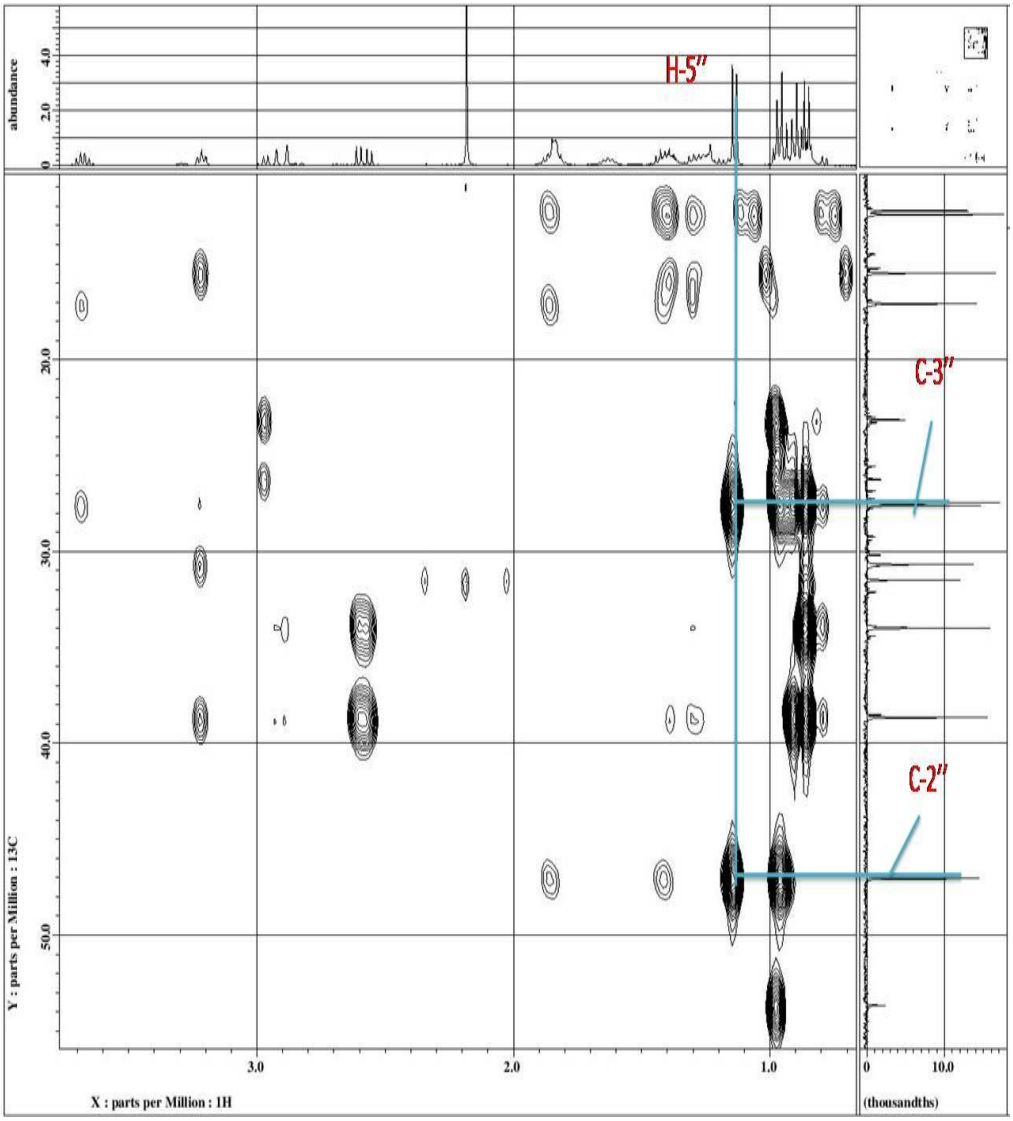
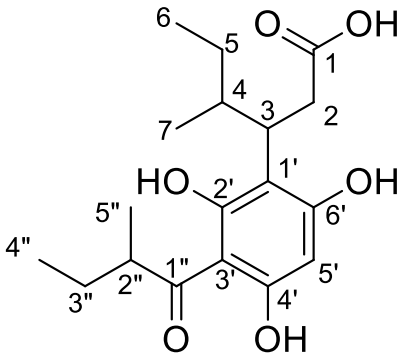


Figure 4.45: Expanded HMBC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]



[25]

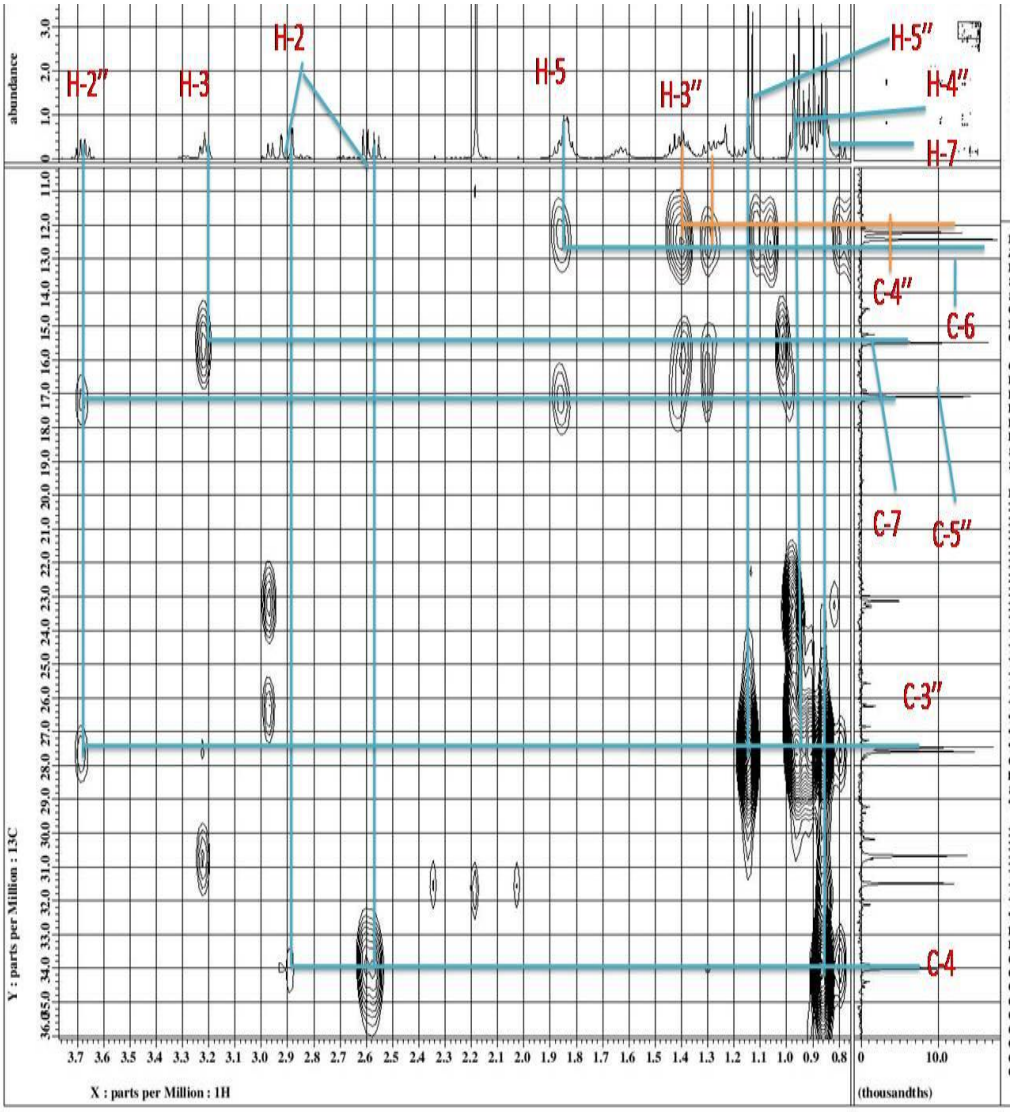
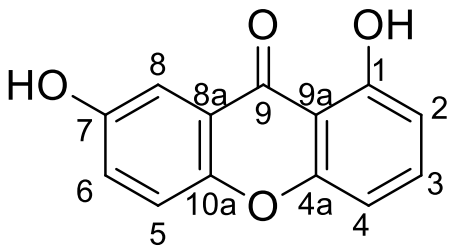


Figure 4.46: Expanded HMBC spectrum of 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid [25]

4.5 Structural Characterization and Elucidation of Euxanthone [26]



[26]

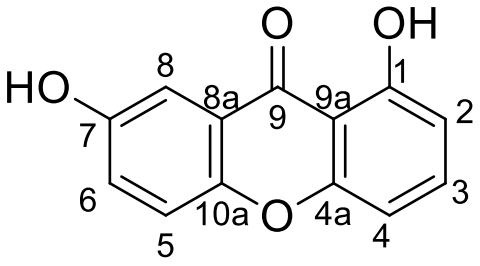
About 5.0 mg of compound **26** was isolated as yellow needle-like crystals. The compound has a molecular formula of $C_{13}H_8O_4$ giving the molecular weight of 228.2 g/mol. In the TLC analysis, it gave a single dark spot when visualized under UV light (254 nm) with a retention factor, R_f value of 0.81 using a solvent mixture of 90% methanol and 10% dichloromethane. This compound showed a melting point of 238 - 239 ºC (Lit. 239 – 241 ºC, Locksley, Moore and Scheinmann, 1966).

A broad band appeared at 3399 cm^{-1} in the IR spectrum (Figure 4.47) indicated the presence of O-H stretch in the compound. Furthermore, absorption bands observed at 1607, 1483 and 1234 cm^{-1} revealed the presence of C=O, aromatic C=C and C-O functionalities in the assigned structure. In the UV-Vis spectrum (Figure 4.48), the absorptions maxima observed at 234.0, 259.5, 286.7 and 385.7 nm were attributed to the highly conjugated system having in polyhydroxyxanthone.

In the ^{1}H NMR spectra (Figures 4.49 and 4.50), the highly deshielded sharp singlet at δ 12.70 and a broad singlet at δ 9.02 were assigned to the chelated hydroxyl proton, 1-OH and the free hydroxyl proton, 7-OH, respectively. The remaining six proton signals in the aromatic region δ 6.0 to 8.0 were attributed to the aromatic protons H-2 (δ 6.74, d), H-3 (δ 7.68, t), H-4 (δ 6.98, d), H-5 (δ 7.50, d), H-6 (δ 7.41, dd) and H-8 (δ 7.58, d).

A total of 13 carbon signals were observed in the ^{13}C NMR spectra (Figures 4.51 and 4.52) in correspondence to the 13 carbons present in the assigned structure. The most deshielded carbon signal at δ 182.2 was assigned to the carbon C-9 which was a keto group (C=O). In addition, the oxygenated aromatic carbons C-4a, C-1, C-7 and C-10a gave relatively more deshielded signals at δ 156.5, 161.9, 154.1 and 150.2, respectively. The remaining eight non-oxygenated aromatic carbons exhibited signal in the region δ 107.0 to 137.0.

The structure of compound **26** was further confirmed by 2D NMR experiments including HMBC and HMQC, and the spectral assignment was summarized in Table 4.4. Based on the spectral evidence, compound **26** was identified as euxanthone.



[26]

Table 4.4: Summary of NMR data for euxanthone [26]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			^2J	^3J
1	-	161.9	-	-
2	6.74 (1H, d, *J* = 8.6 Hz)	109.7	-	C-4
3	7.68 (1H, t, *J* = 8.6 Hz)	137.0	-	C-1, C-4a
4	6.98 (1H, d, *J* = 8.6 Hz)	107.0	-	C-2
4a	-	156.5	-	-
5	7.50 (1H, d, *J* = 9.2 Hz)	119.4	-	C-7
6	7.41 (IH, dd, *J* = 9.2, 3.1 Hz)	125.4	-	C-8, C-10a
7	-	154.1	-	-
8	7.58 (1H, d, *J* = 3.0 Hz)	108.3	-	C-6, C-9, C-10a
8a	-	121.0	-	-
9	-	182.2	-	-
9a	-	107.0	-	-
10a	-	150.2	-	-
1-OH	12.70 (OH, s)	-	C-1	C-2
7-OH	9.02 (OH, brs)	-	-	-

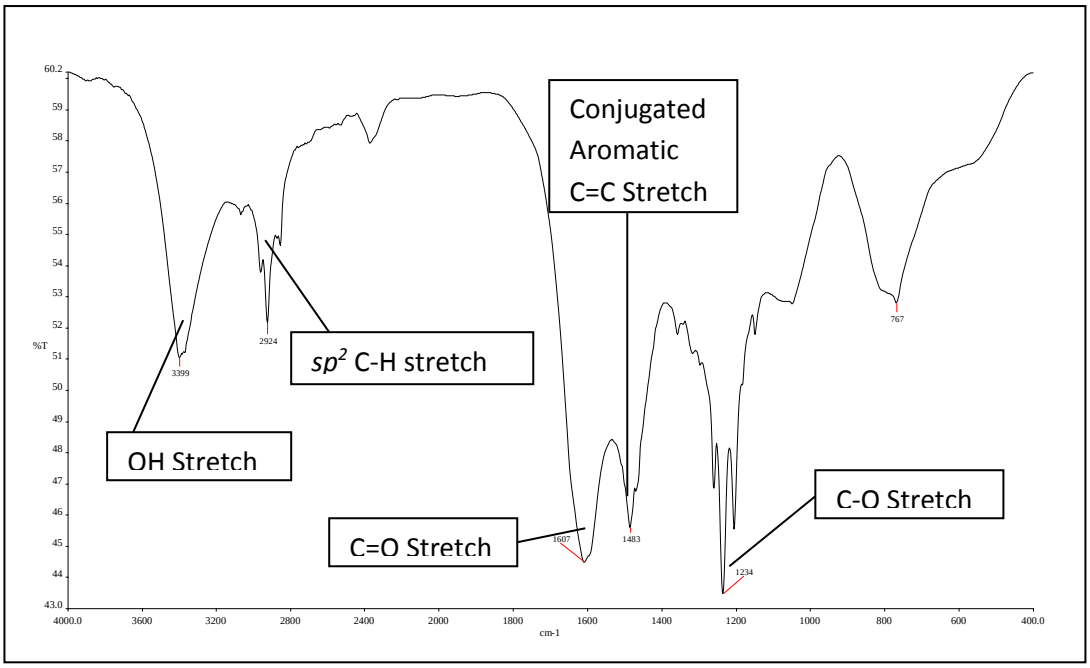


Figure 4.47: IR spectrum of euxanthone [26]

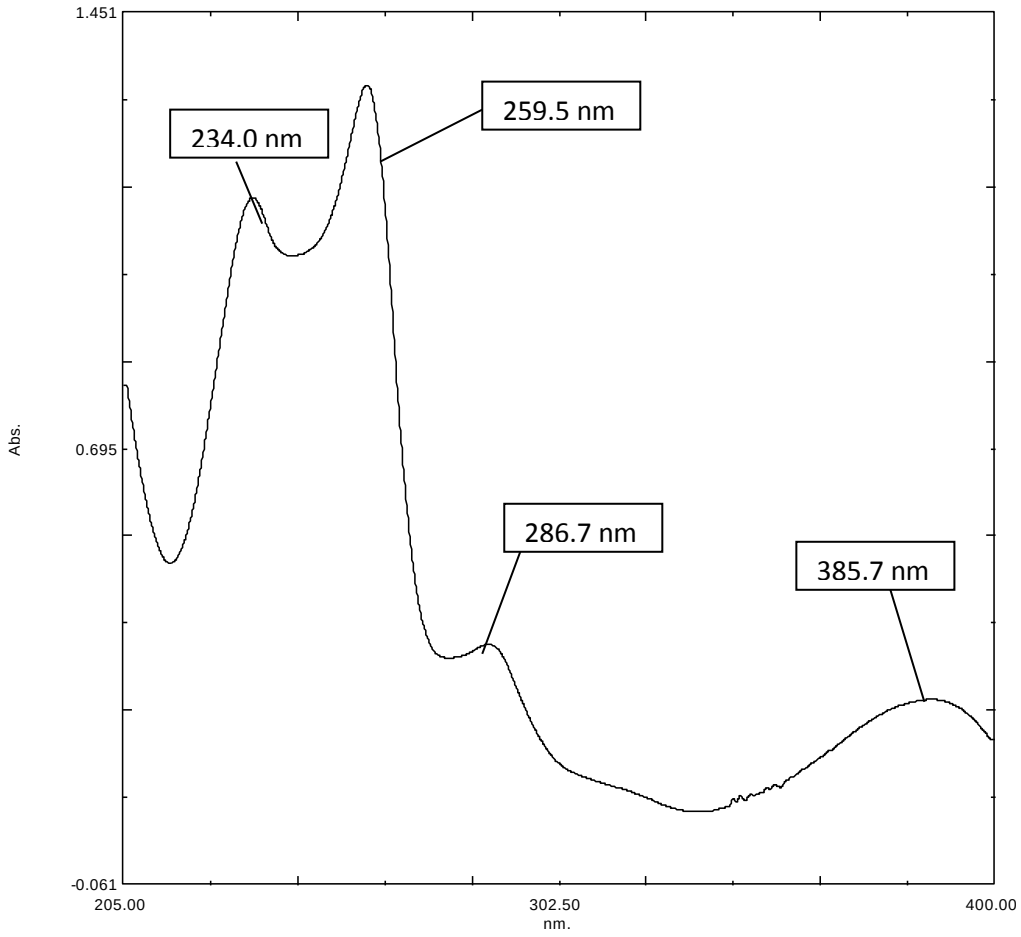
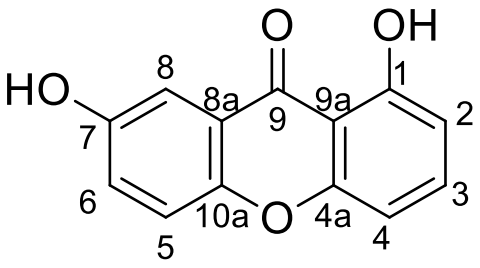


Figure 4.48: UV-Vis spectrum of euxanthone [26]



[26]

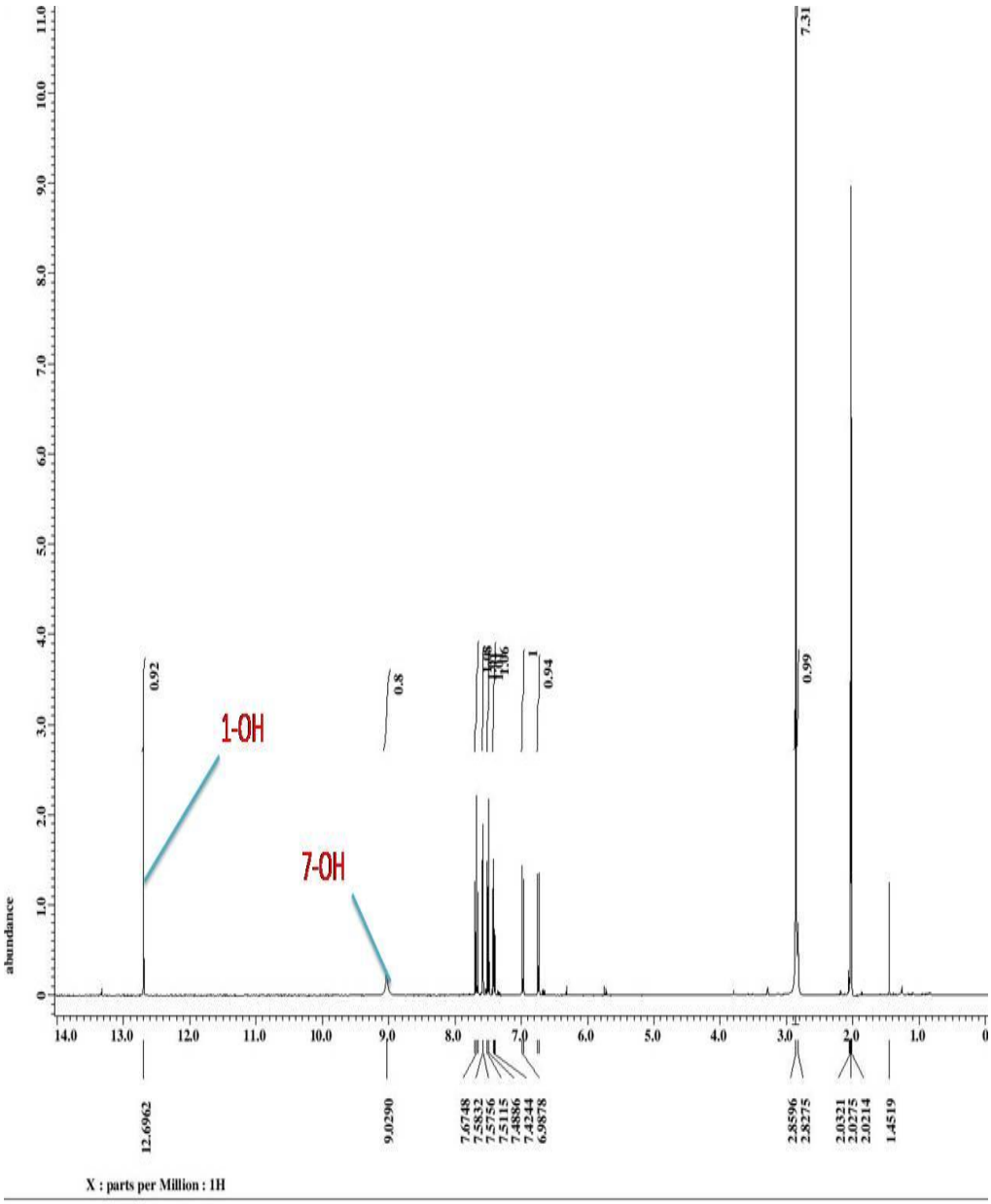
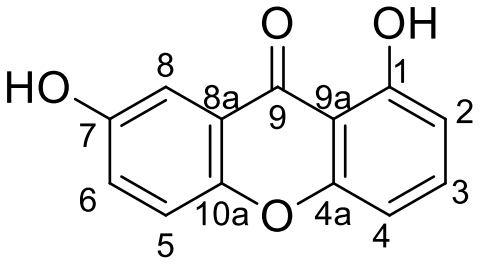


Figure 4.49: ^{1}H NMR spectrum of euxanthone [26] (400 MHz, acetone-d_6)



[26]

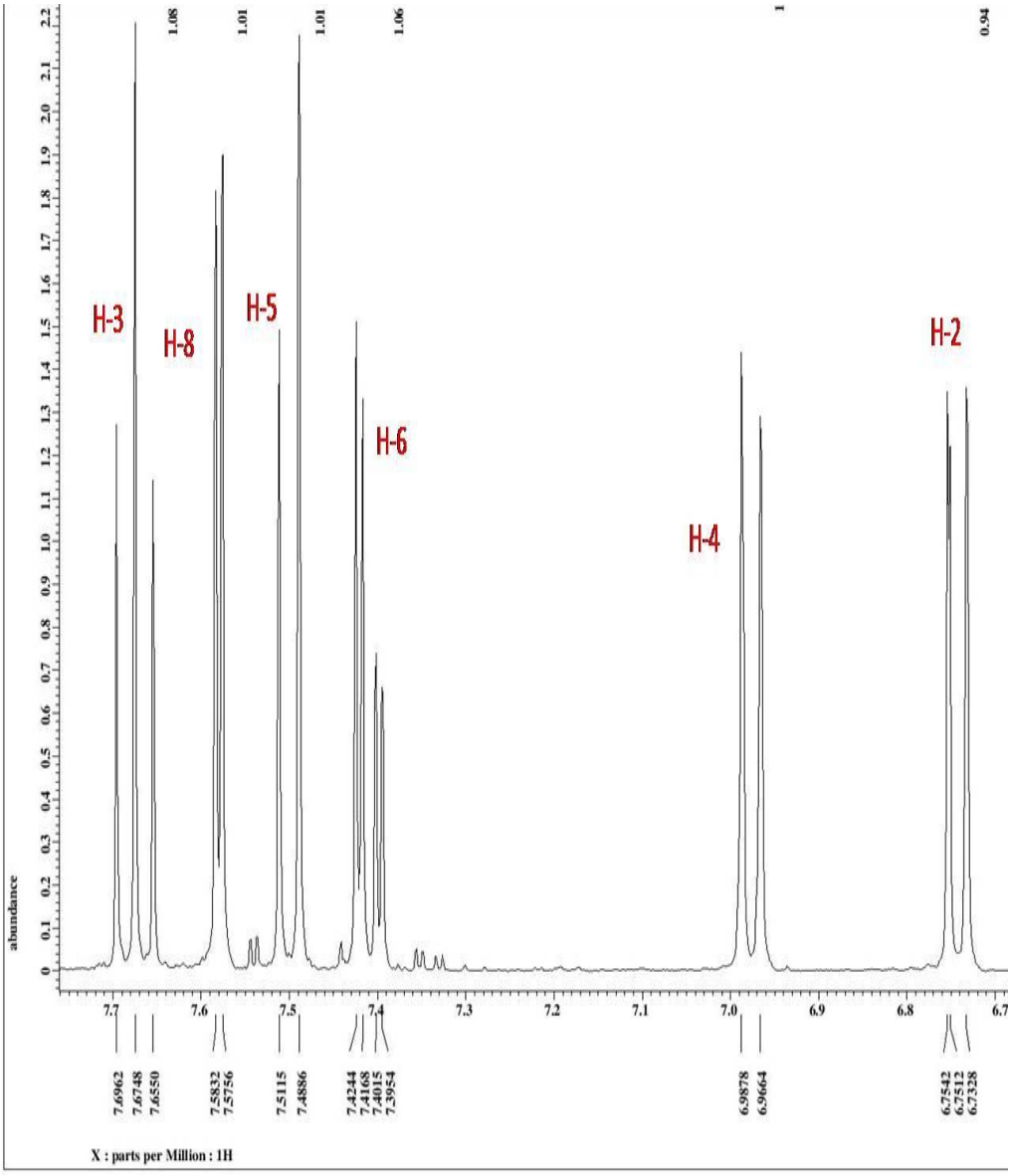
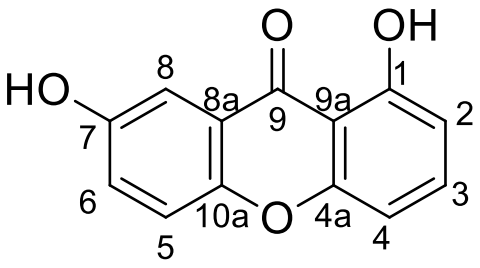


Figure 4.50: Expanded ^{1}H NMR spectrum of euxanthone [26] (400 MHz, acetone-d_6)



[26]

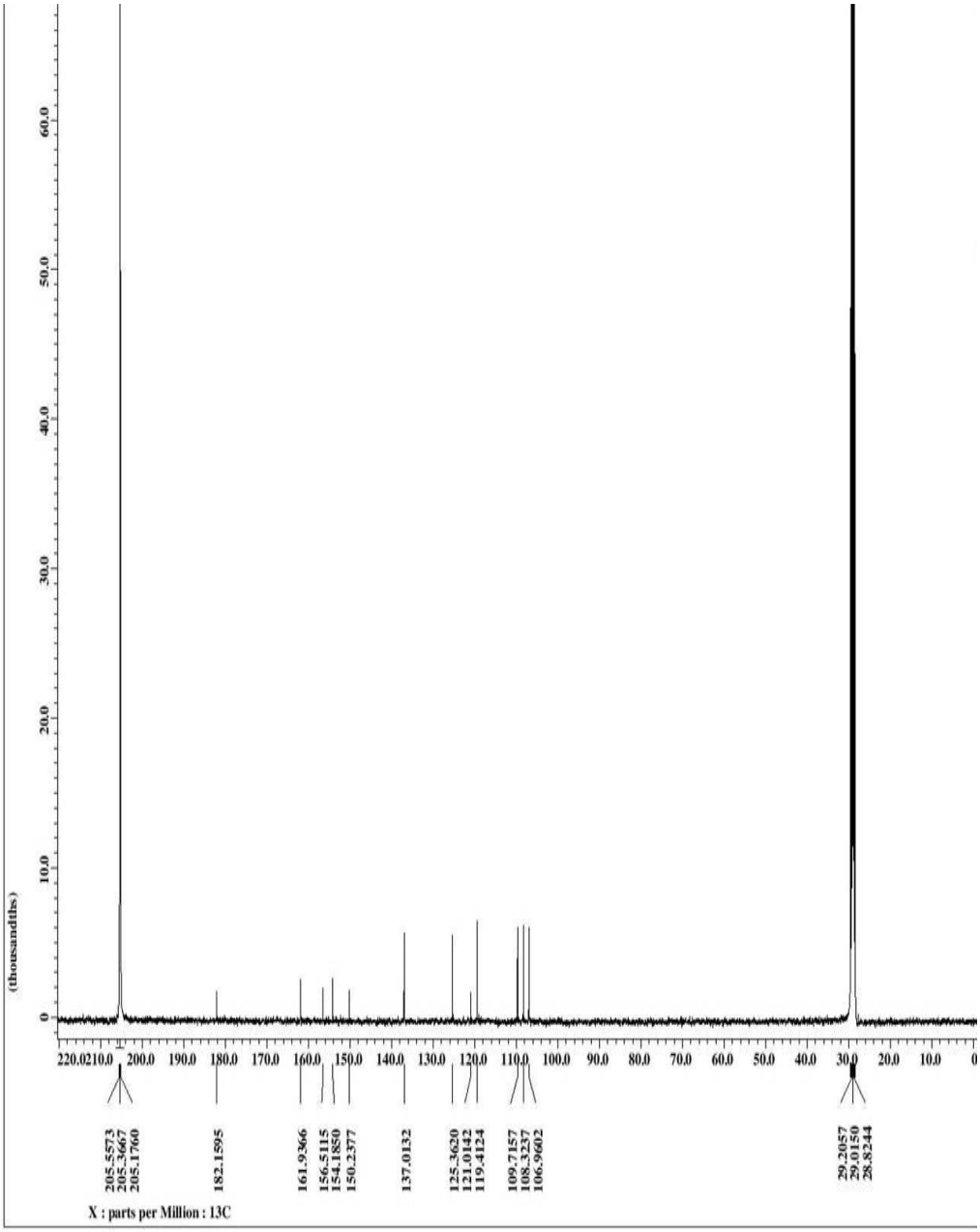
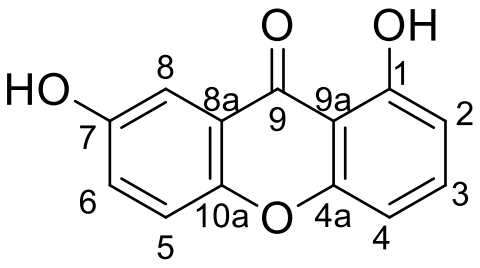


Figure 4.51: ^{13}C NMR spectrum of euxanthone [26] (100 MHz, acetone-d_6)



[26]

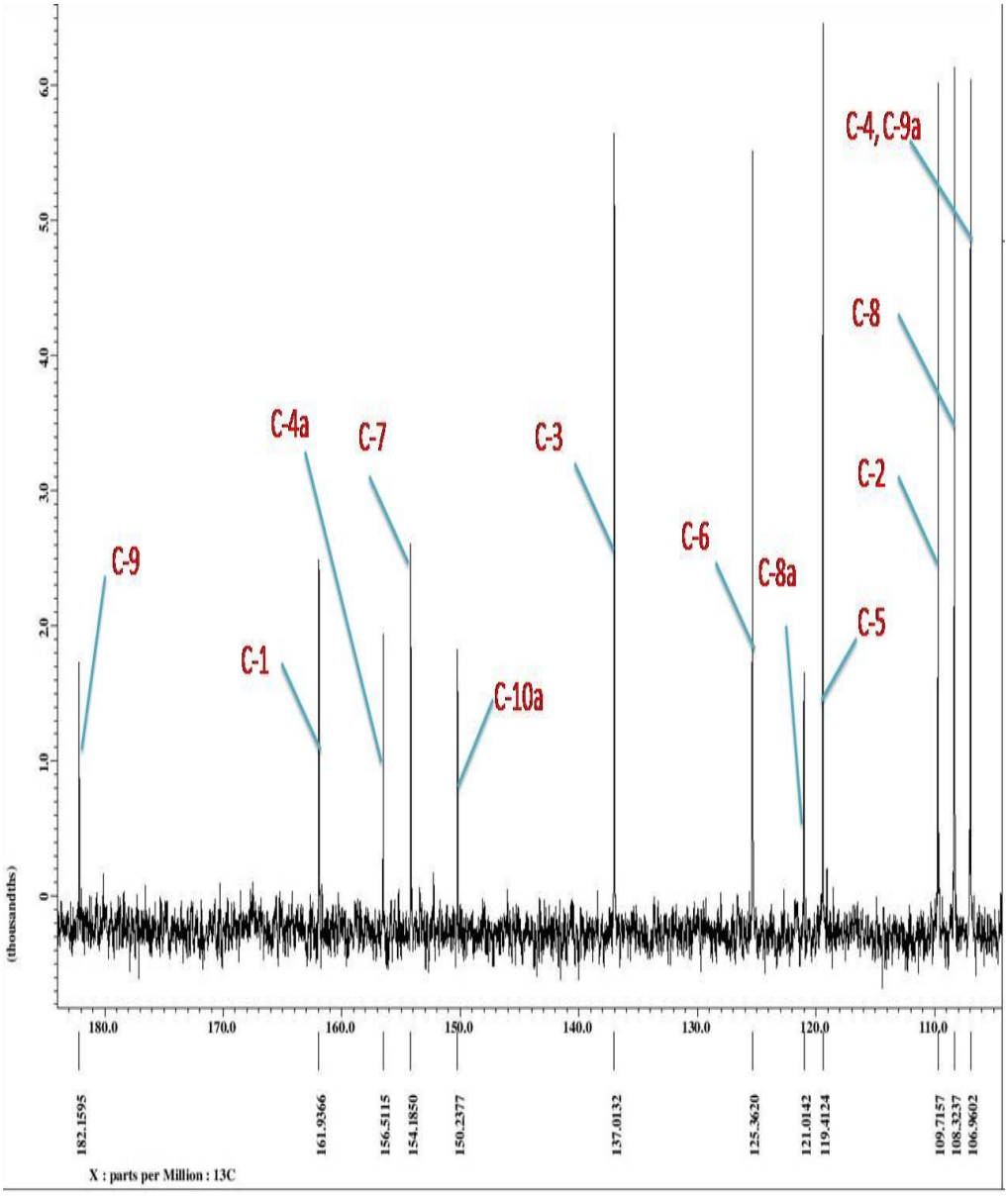
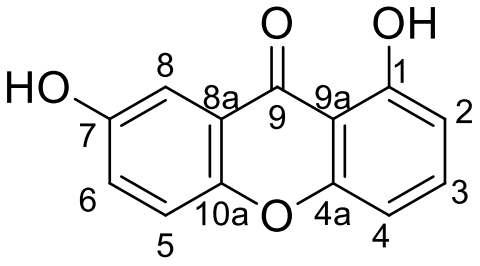


Figure 4.52: Expanded ^{13}C NMR spectrum of euxanthone [26] (100 MHz, acetone-d_6)



[26]

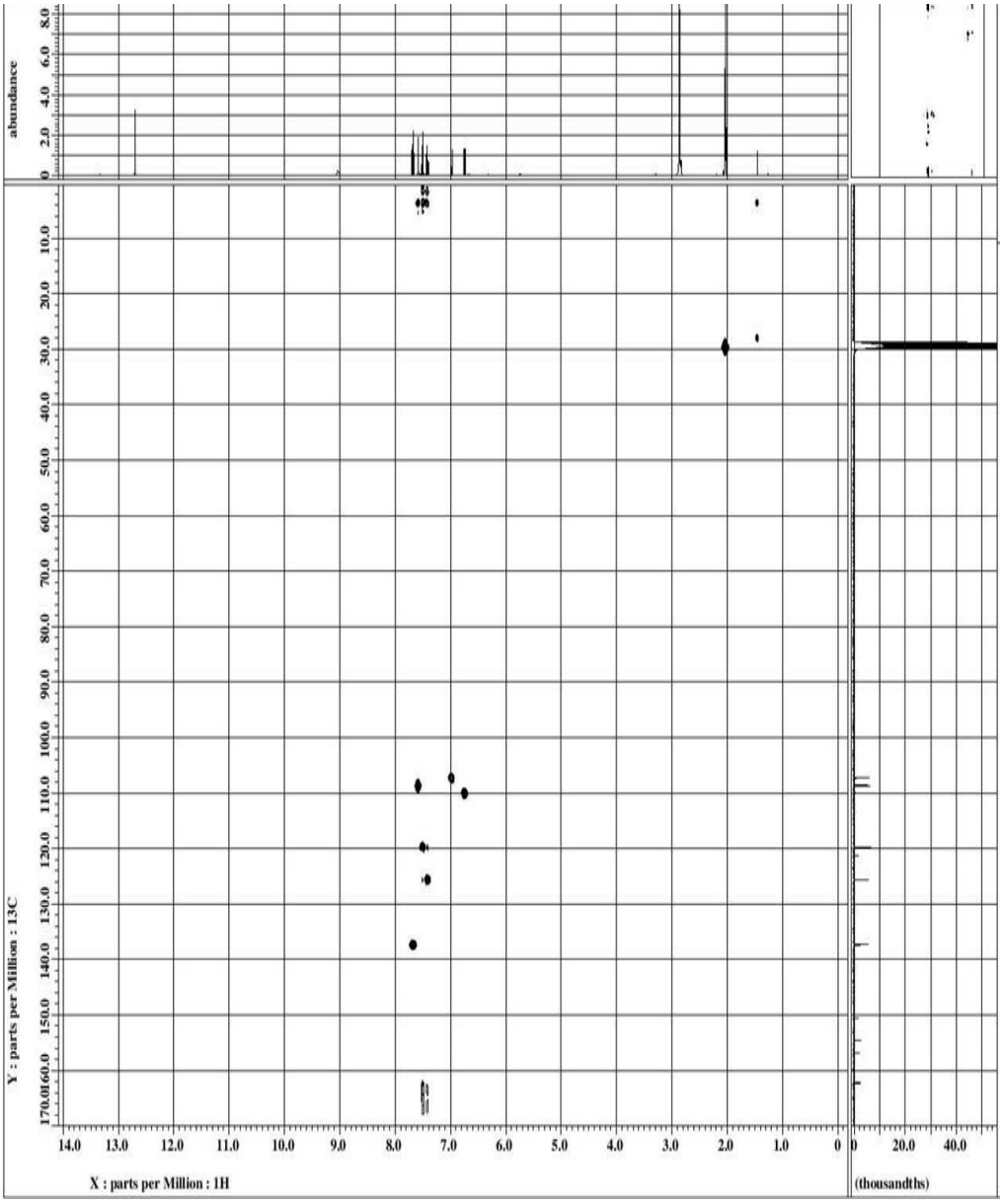
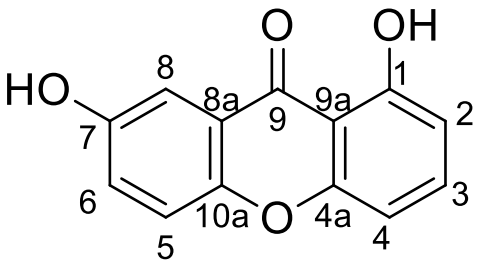


Figure 4.53: HMQC spectrum of euxanthone [26]



[26]

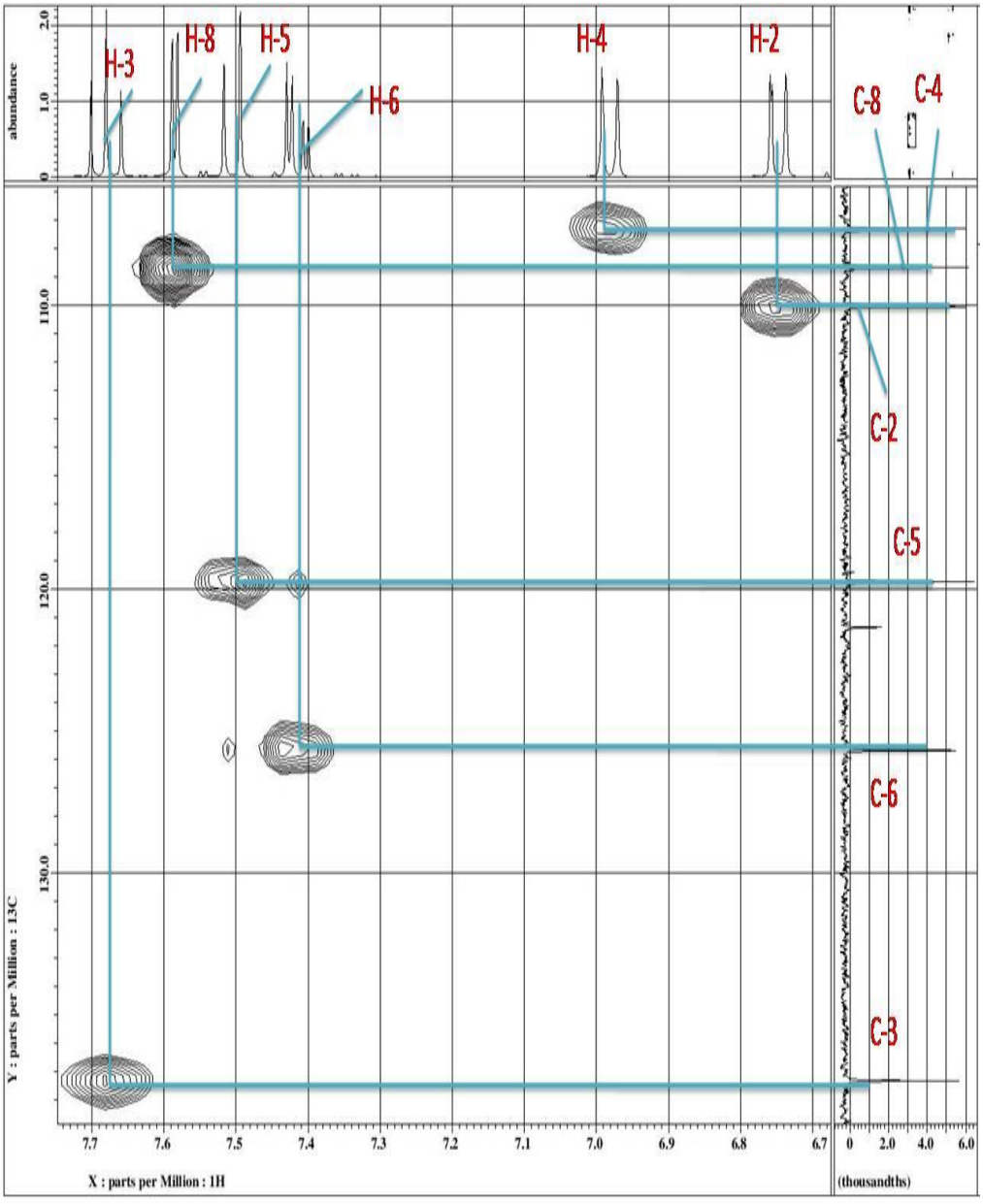
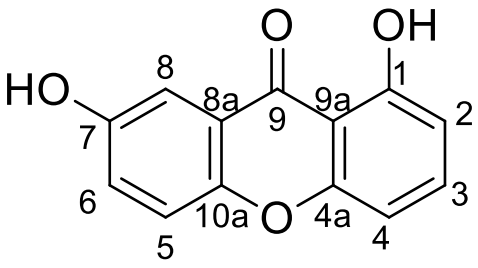


Figure 4.54: Expanded HMQC spectrum of euxanthone [26]



[26]

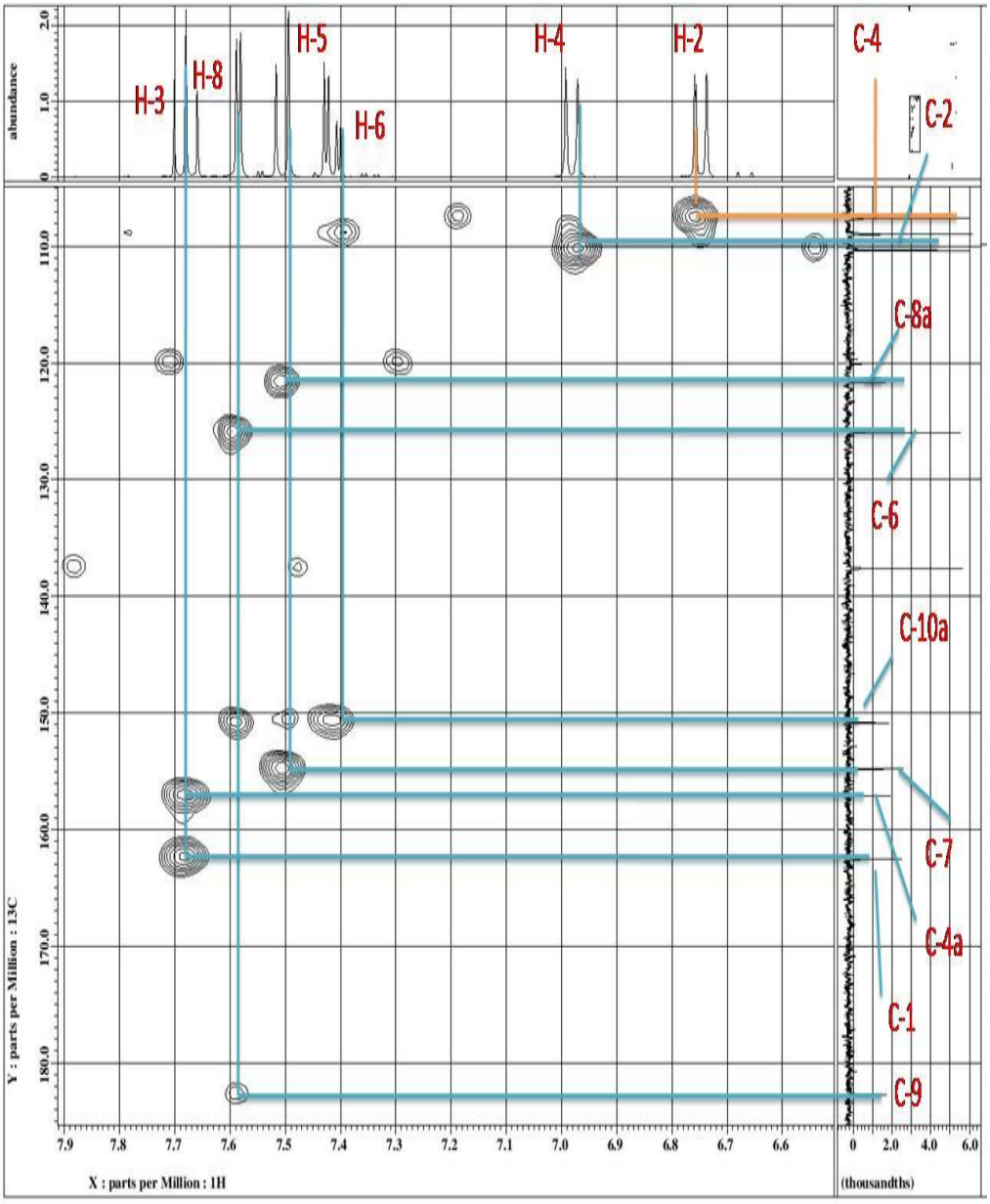
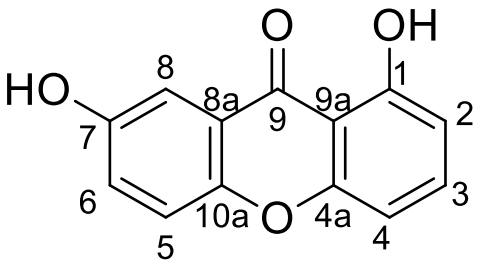


Figure 4.55: Expanded HMBC spectrum of euxanthone [26]



[26]

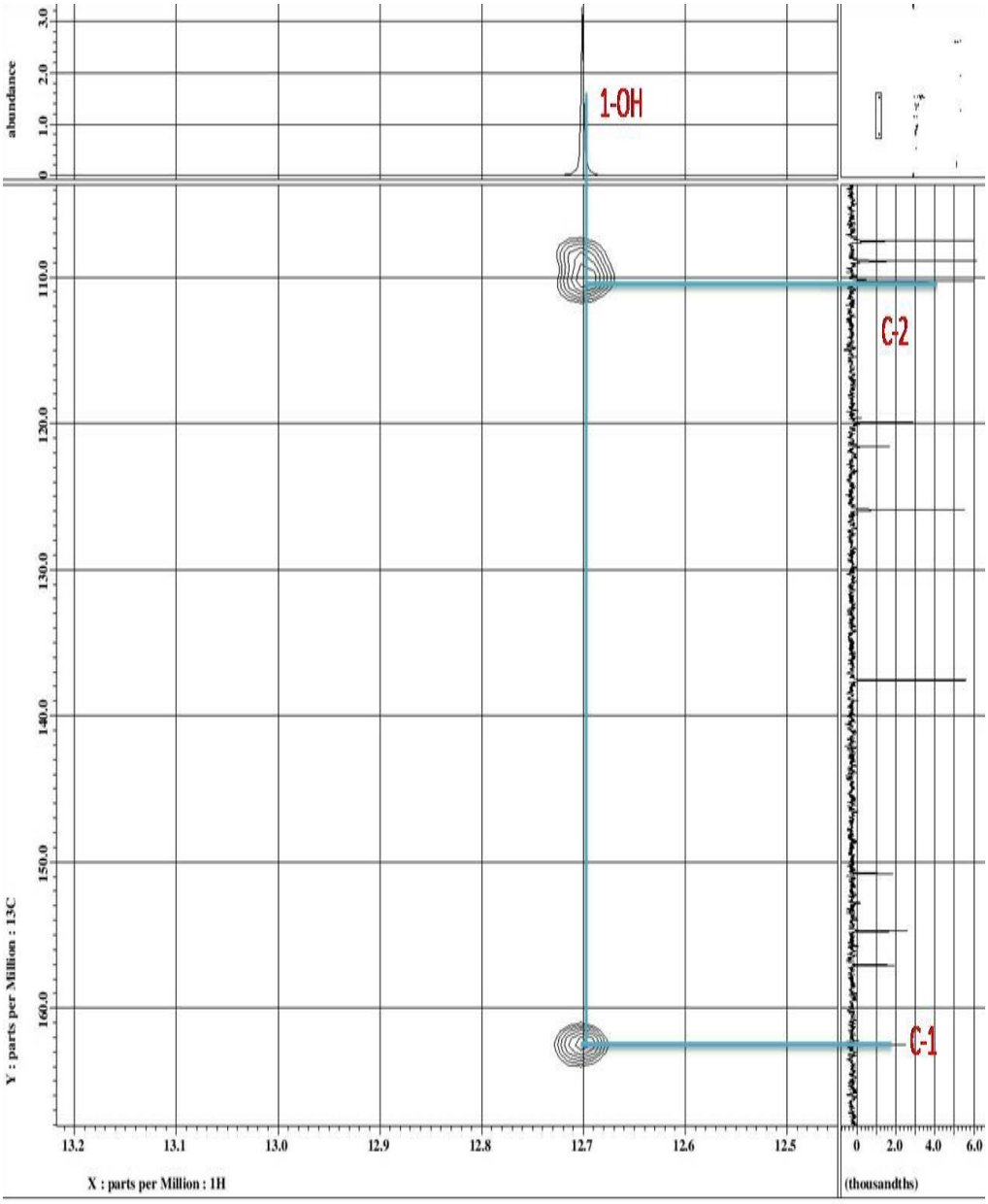
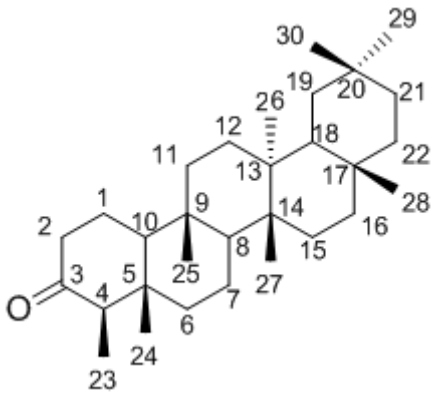


Figure 4.56: Expanded HMBC spectrum of euxanthone [26]

4.6 Structural Characterization and Elucidation of Friedelin [22]



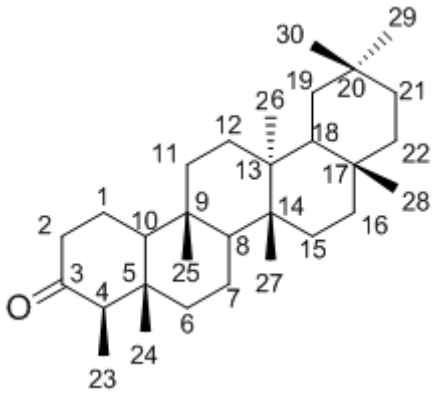
[22]

About 34.8 mg of compound **22** was isolated as white needle-like crystals, giving a molecular formula of $C_{30}H_{50}O$ which was in agreement with the molecular weight of 426.72 g/mol. This compound showed a melting point of 258-259 ºC (Lit. 259-260 ºC, Abbas, et al., 2007). In the TLC analysis using a mixture of 80% hexane and 20% of dichloromethane as mobile phase, it displayed a single spot with a retention factor, R_f value of 0.70 when visualized under short wavelength of UV light at 254 nm.

The compound was further subjected to FTIR analysis. From the IR spectrum (Figure 4.57), the absorption bands observed at 2927 and 1740 cm^{-1} revealed the presence of sp^3 C-H (stretch) and C=O (stretch) functionalities. Apart from that, this compound gave an absorption maximum at 218.0 nm in the UV-Vis spectrum (Figure 4.58) which was resulted from n → n* transition.

In the ^{1}H-NMR spectra (Figures 4.59 and 4.60), all the proton signals were observed in the upfield region below δ 3.0 mainly due to all the protons were covalently bonded to sp^3 hybridized carbons in the five-membered ring skeleton. The eight methyl groups H-23, H-24, H-25, H-26, H-27, H-28, H-29 and H-30 in the structure displayed characteristic proton signals at δ 0.86 (3H, d), 0.70 (3H, s), 0.85 (3H, s), 0.95 (3H, s), 1.03 (3H, s), 1.16 (3H, s), 0.99 (3H, s) and 0.98 (3H, s), respectively. The remaining of the proton signals were assigned by comparison with the literature values (Abbas, et al., 2007) and were summarized in Table 4.5.

A total of 30 carbon signals were observed in the ^{13}C NMR spectra (Figures 4.61 and 4.62) indicating the assigned structure to have a total of 30 carbons. The keto carbon C-3 gave the most deshielded signal at δ 213.0. The eight methyl carbon signals were observed at δ 6.9 (C-23), 14.7 (C-24), 18.0 (C-25), 18.8 (C-27), 20.3 (C-26), 31.9 (C-30), 32.2 (C-28) and 35.1 (C-29). The remaining of carbon signals were assigned based on comparison with the literature values (Abbas, et al., 2007) and were summarized in Table 4.5.



[22]

Table 4.5: Summary of NMR data for compound 22 in comparison with literature values of friedelin

Position	δ_H (ppm)	* δ_H (ppm)	δ_C (ppm)	*δ_C (ppm)
1	1.95 (1H$_a$, m) 1.72 (1H$_b$, m)	1.97 (m) 1.71 (m)	22.4	22.3
2	2.36 (1H$_a$, dd, *J* = 13.4 Hz, 3.0 Hz,) 2.29 (1H$_b$, m)	2.41 (dd, *J*= 13.0, 3.5Hz) 2.31 (m)	41.6	41.5
3	-	-	213.4	213.2
4	2.24 (1H, m)	2.28 (m)	58.3	58.2
5	-	-	42.2	42.2
6	1.78 (m) 1.31 (m)	1.78 (m) 1.31 (m)	41.3	41.3
7	1.51 (m) 1.41 (m)	1.51 (m) 1.41 (m)	18.3	18.2
8	1.41 (m)	1.41 (m)	53.2	53.1
9	-	-	37.5	37.4
10	1.55 (m)	1.55 (m)	59.5	59.5
11	1.40 (m)	1.40 (m)	35.7	35.6
12	1.30 (m) 1.28 (m)	1.30 (m) 1.28 (m)	30.6	30.5
13	-	-	39.8	39.7
14	-	-	38.4	38.3

15	1.50 (m) 1.30 (m)	1.50 (m) 1.30 (m)	32.8	32.8
16	1.40-1.60 (m)	1.40-1.60 (m)	36.1	36.0
17	-	-	30.1	30.0
18	1.60 (m) 1.40 (m)	1.60 (m) 1.40 (m)	42.8	42.8
19	1.60 (m)	1.60 (m)	35.4	35.3
20	-	-	28.3	28.2
21	1.51 (m) 1.31 (m)	1.51 (m) 1.31 (m)	32.5	32.5
22	1.51 (m) 0.97 (m)	1.51 (m) 0.97 (m)	39.3	39.3
23	0.86 (3H, d, *J* = 6.7 Hz)	0.92 (d, *J*= 7.0 Hz)	6.9	6.8
24	0.70 (3H, s)	0.75 (s)	14.7	14.7
25	0.85 (3H, s)	0.90 (s)	18.0	18.0
26	0.95 (3H, s)	1.03 (s)	20.3	20.3
27	1.03 (3H, s)	1.07 (s)	18.8	18.7
28	1.16 (3H, s)	1.20 (s)	32.2	32.1
29	0.99 (3H, s)	1.02 (s)	35.1	35.0
30	0.98 (3H, s)	0.98 (s)	31.9	31.8

* Literature source: Abbas, et al., 2007

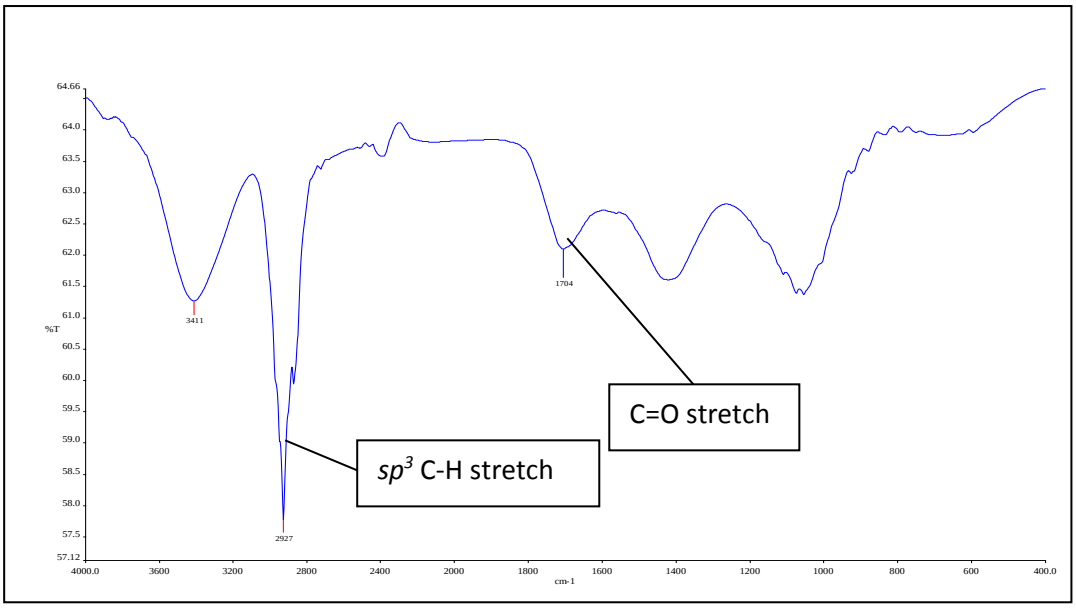


Figure 4.57: IR spectrum of friedelin [22]

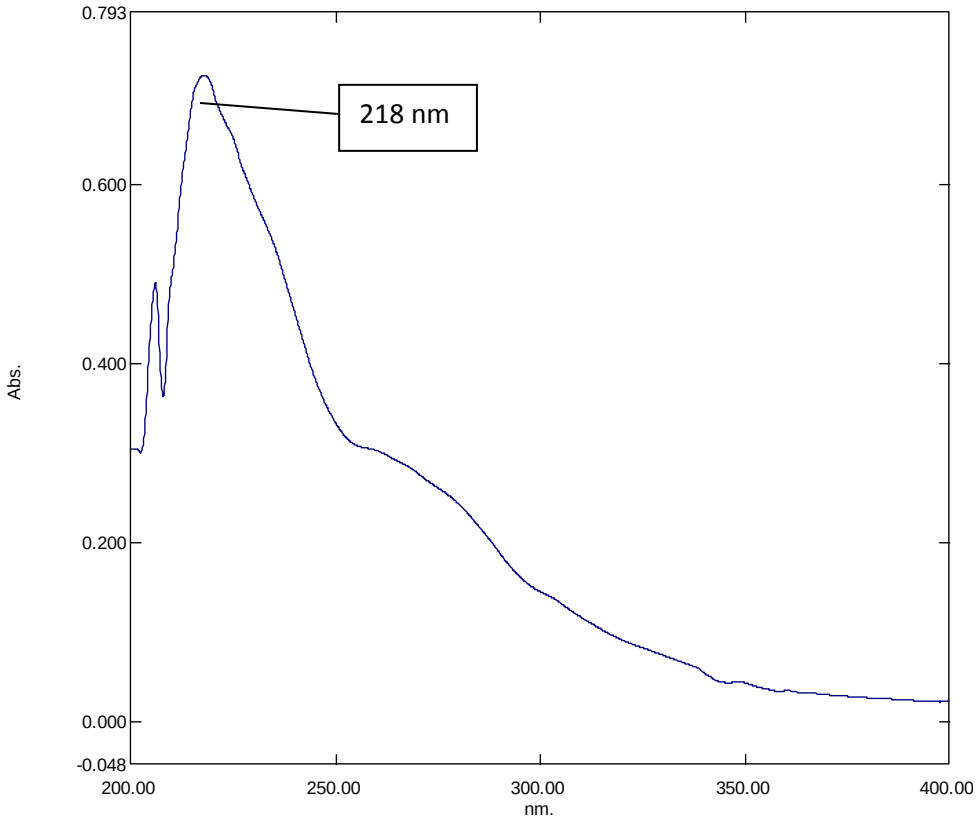
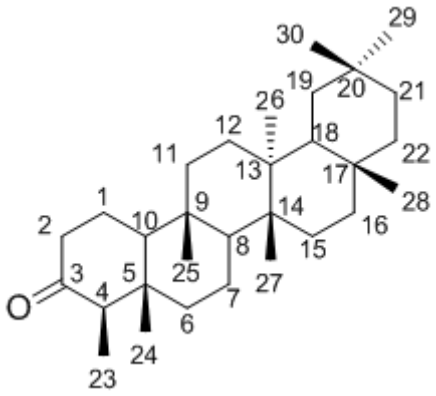


Figure 4.58: UV-Vis spectrum of friedelin [22]



[22]

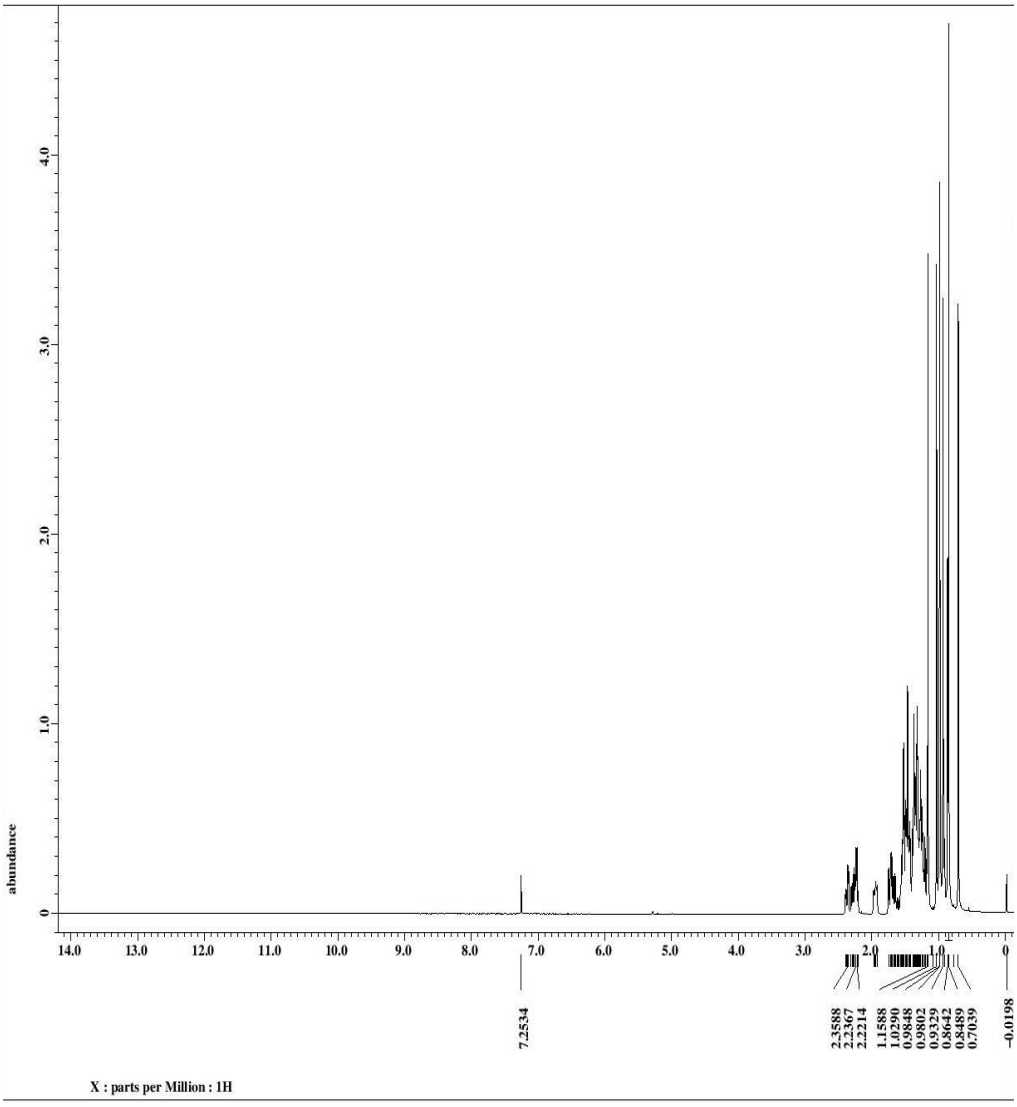
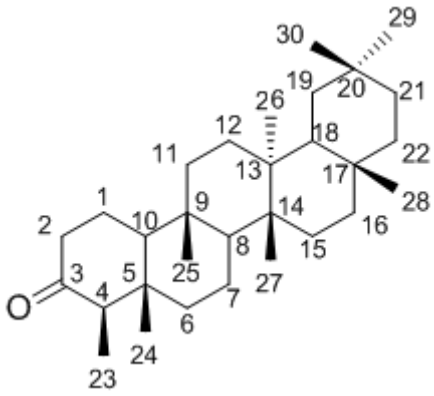


Figure 4.59: ^{1}H NMR spectrum of friedelin [22] (400 MHz, $CDCl_3$)



[22]

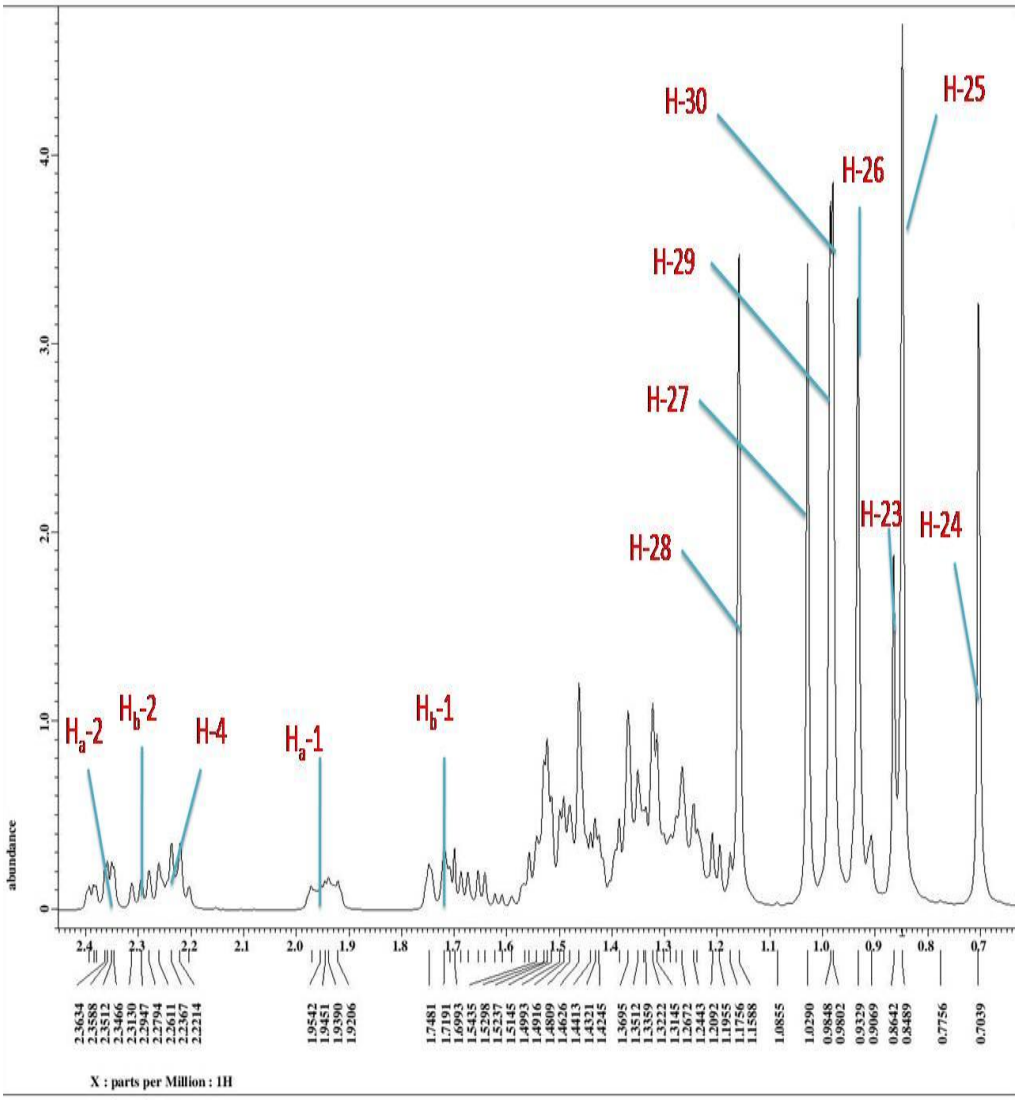
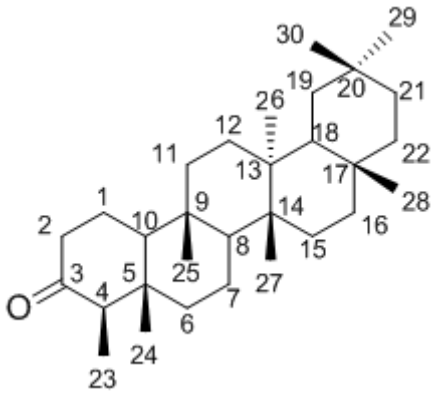


Figure 4.60: Expanded ^{1}H NMR spectrum of friedelin [22] (400 MHz, $CDCl_3$)



[22]

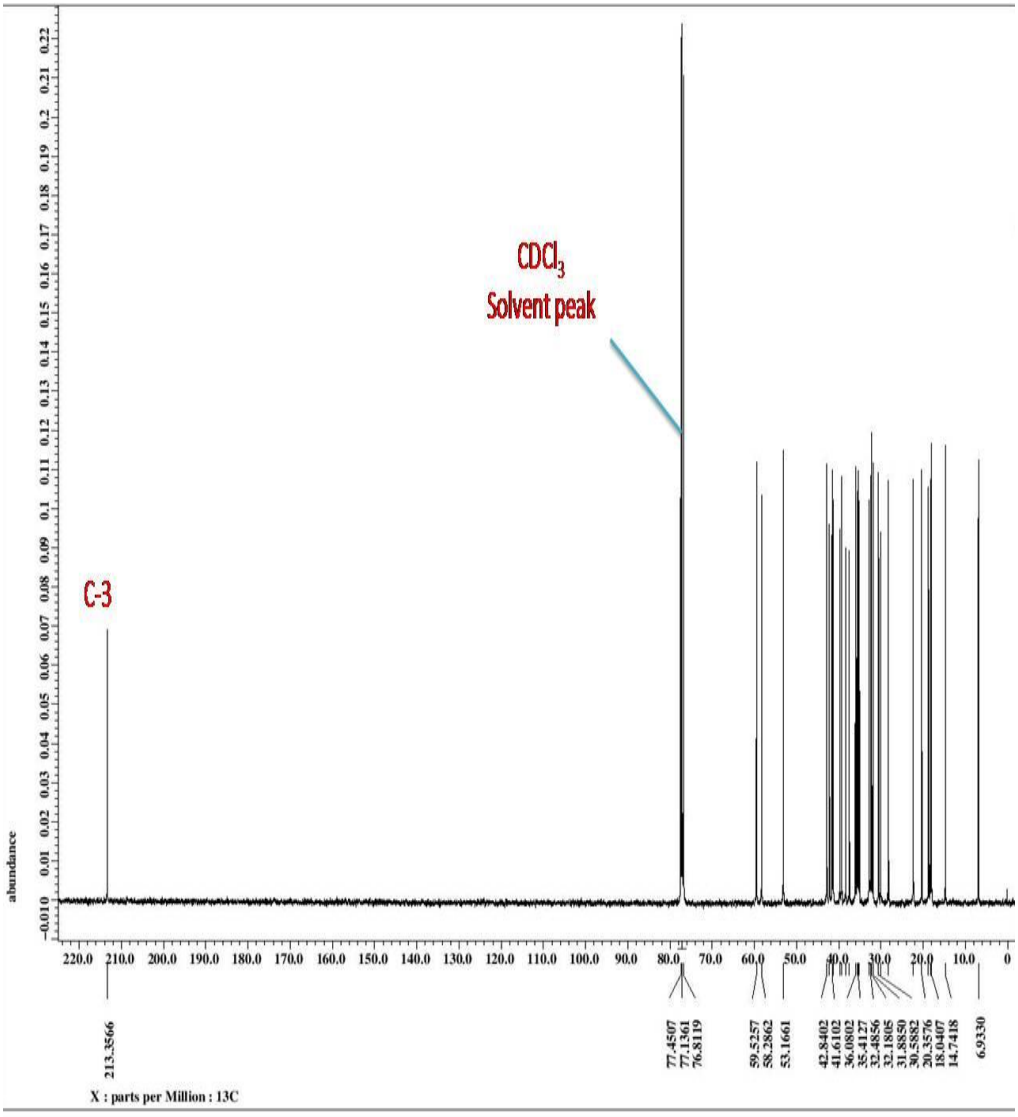
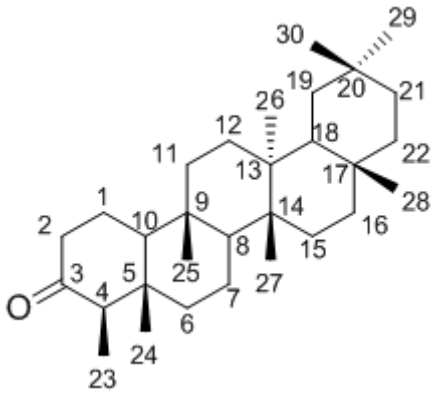


Figure 4.61: ^{13}C NMR spectrum of friedelin [22] (100 MHz, $CDCl_3$)



[22]

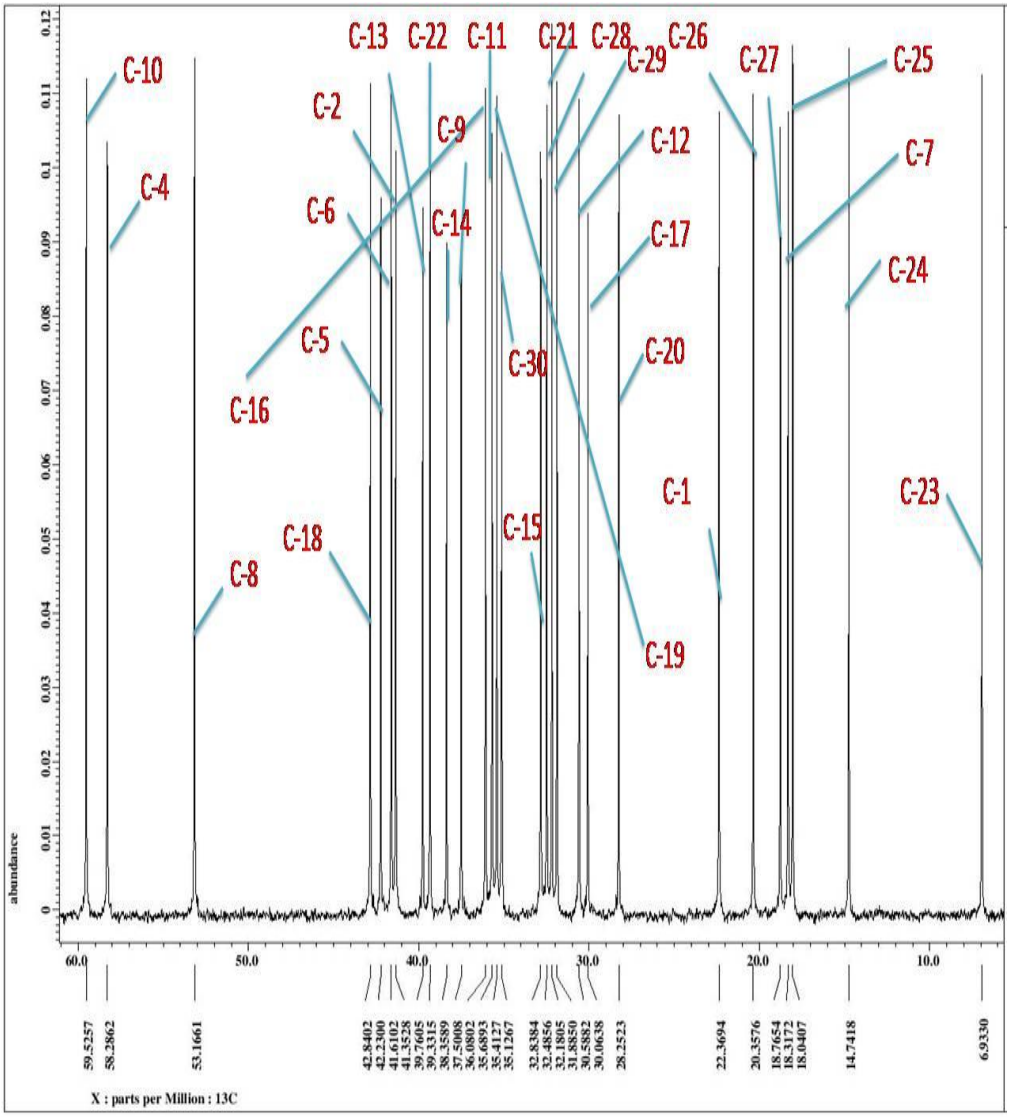


Figure 4.62: Expanded ^{13}C NMR spectrum of friedelin [22] (100 MHz, $CDCl_3$)

4.7 Antioxidant Test

The reactive and unstable free radicals are known as reactive oxygen species (ROS). ROS can cause cell damage due to the build up of oxidative stress in cells, resulted from unbalance mechanisms between pro-oxidants and antioxidant. A phenolic compound can act as the neutralizer that terminates the radical chain reaction by oxidizing itself and converts the free radical intermediates to a relatively stable compound.

In this study, 2-2-diphenyl-1-picrylhydrazyl (DPPH) assay was conducted to determine the radical scavenging activity of the crude extracts and isolated compounds from the stem bark of *Calophyllum gracilentum*. DPPH is a violet colour of free radical, however, it undergoes reduction and changes colour to yellow when it is scavenged. Hence, when the DPPH radicals were reduced, they resulted in the decrease of absorbance at wavelength of 520 nm. In this assay, DPPH was used as the colour indicator for the measurement of antioxidant activity of samples.

IC_{50} value is defined as the concentration of sample that required to inhibit 50% of DPPH radical activity. The lower the IC_{50} value obtained for a sample, the stronger the antioxidant activity. Graphs of percent inhibition rate against concentration of sample were plotted to obtain the IC_{50} values of the crude extracts and isolated compounds **22** to **26** as shown in Figures 4.63 and 4.64.

Table 4.6: Antioxidant results of test samples from the DPPH assay.

Test sample	IC_{50} (μg/mL)
Positive controls:	
Ascorbic acid	7.5
Kaempferol	11.5
Crude extracts:	
Ethyl acetate	210.0
Dichloromethane	>240.0
Methanol	10.0
Isolated compound:	
Friedelin **[22]**	>240.0
5,7-dihydroxy-4-(2-butyl)coumarin **[23]**	>240.0
5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin **[24]**	178.5
4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)hexanoic acid **[25]**	>240.0
Euxanthone **[26]**	>240.0

Table 4.6 showed the antioxidant activities of the positive controls, crude extracts and isolated compounds **22** to **26** in the DPPH assay. Among the crude extracts, methanol extract gave the lowest IC_{50} value of 10.0 μg/mL showing a strong antioxidant activity comparable to the positive controls, ascorbic acid and kaempferol with IC_{50} values of 7.5 and 11.5 μg/mL, respectively. The increasing order of antioxidant activity from strongest to weakest was arranged as methanol, ethyl acetate and dichloromethane extracts. Dichloromethane extract showed the weakest radical scavenging activity because it contained mostly low polarity compounds such as hydrocarbons and terpenoids which may not be good antioxidants because they were not phenolic compounds. Phenolic compounds are good antioxidants and were

found to be abundant in methanol extract as they can form hydrogen bonding with methanol to increase their solubility in methanol. Hence, methanol extract showed the highest activity among the crude extracts due to its high phenolic content.

On the other hand, all the isolated compounds **23**, **25**, **26** and **22** showed insignificant radical scavenging activity with their IC_{50} values of above 240 µg/mL except for compound **24** which showed a weak antioxidant activity with its IC_{50} value of 178.5 µg/mL.

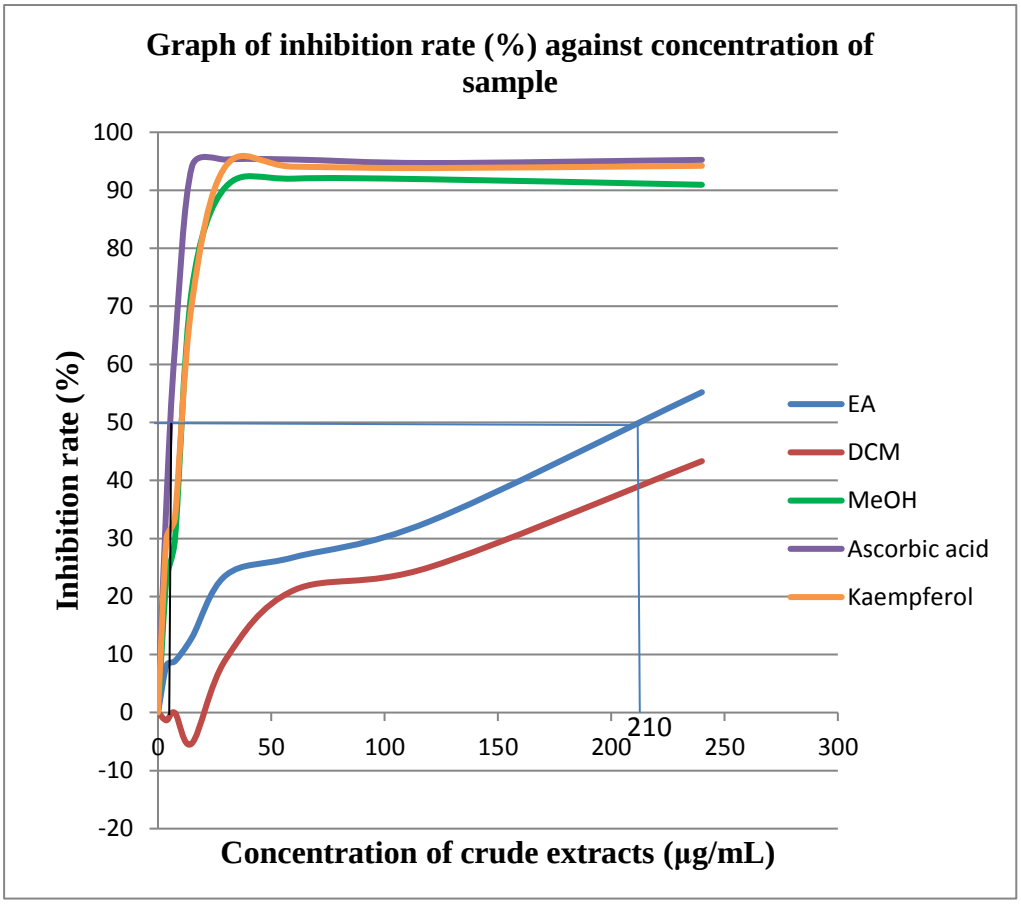


Figure 4.63: Graph of inhibition rate (%) against concentration for crude extracts

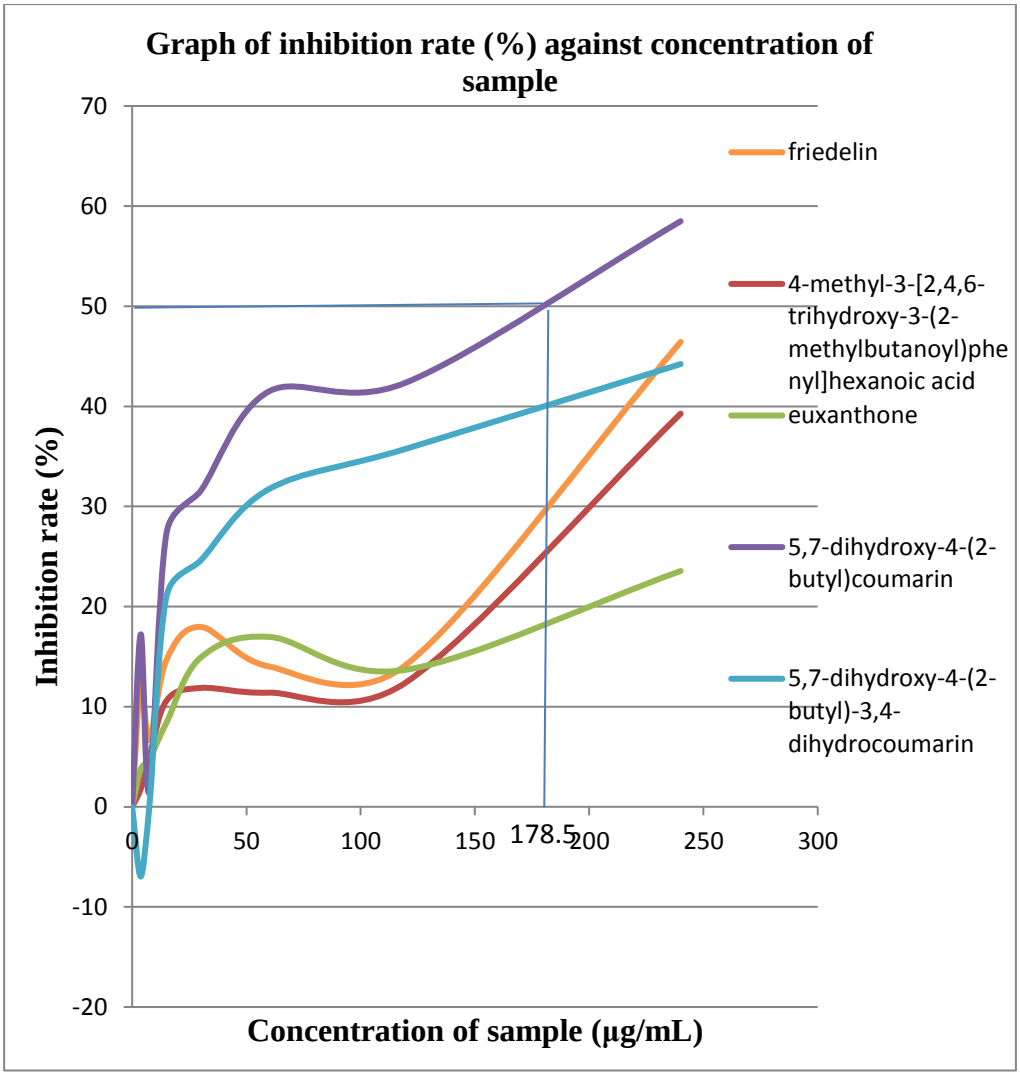


Figure 4.64: Graph of inhibition rate (%) against concentration for isolated compounds 22 to 26

CHAPTER 5

CONCLUSIONS

5.1 Conclusion

In this study, two new coumarins, namely 5,7-dihydroxy-4-(2-butyl)coumarin **[23]** and 5,7-dihydroxy-4-(2-butyl)-3,4-dihydrocoumarin **[24]** were successfully isolated along with 4-methyl-3-[2,4,6-trihydroxy-3-(2-methylbutanoyl)phenyl]hexanoic acid **[25]**, euxanthone **[26]** and friedelin **[22]**, from the stem bark extract of *Calophyllum gracilentum*. Their chemical structures were identified via various modern spectroscopic techniques including 1D- and 2D-NMR, IR and UV-Vis spectroscopies.

Three crude extracts and five isolated compounds were investigated for their antioxidant potency via DPPH assay. In the assay, methanol crude extract was found to exhibit strong antioxidant activity with IC_{50} value of 10 μg/mL which is comparable to the positive controls used, ascorbic acid (IC_{50} = 7.5 μg/mL) and kaempferol (IC_{50} = 11.5 μg/mL). Meanwhile, ethyl acetate crude extract and compound **24** displayed weak radical scavenging activities with IC_{50} values of 210 and 178.5 μg/mL, respectively. However, dichloromethane crude extract, compounds **22**, **23**, **25** and **26** were found to be inactive showing IC_{50} values of more than 240 μg/mL.

5.2 Future Perspectives

Extensive study should be carried out to the other parts of the plant e.g. root barks, leaves and heartwood in the search for new and biological active compounds from *Calophyllum gracilentum*. In addition, the isolated compounds obtained in this study can be subjected to other biological studies such as antimicrobial, anti-cancer and anti-fungi in future. Besides that, more advance chromatographic technique such as high performance liquid chromatography (HPLC) and centrifugal chromatography should be used to increase the efficiency for isolation of minor compounds from the plant extracts. Lastly, the pure compounds showing good biological potency should be studied for their potential derivatives via organic synthesis.

REFERENCES

Abbas, F.A., Al-Massarany, S.M., Khan, S., Al-Howiriny, T.A., Mossa, J.S. and Abourashed, E.A., 2007. Phytochemical and biological studies on Saudi *Commiphora opobalsamum* L. *Natural Product Research*, *21*(5), pp.383-391.

Aldred, E.M., Buck, C. and Vall, K., 2009. Chapter 22-Terpenes. *EMA b. B. Vall (Ed.), Pharmacology*, pp.167-174.

Aminudin, N.I., Ahmad, F., Taher, M. and Zulkifli, R.M., 2015. α-Glucosidase and 15-Lipoxygenase Inhibitory Activities of Phytochemicals from Calophyllum symingtonianum. *Natural product communications*, *10*(9), pp.1585-1587.

Aminudin, N.I., Ahmad, F., Taher, M. and Zulkifli, R.M., 2016. Incrassamarin A–D: Four new 4-substituted coumarins from Calophyllum incrassatum and their biological activities. *Phytochemistry Letters*, *16*, pp.287-293.

Ampofo, S.A. and Waterman, P.G., 1986. Xanthones and neoflavonoids from two Asian species of Calophyllum. *Phytochemistry*, *25*(11), pp.2617-2620.

Bellik, Y., Boukraâ, L., Alzahrani, H.A., Bakhotmah, B.A., Abdellah, F., Hammoudi, S.M. and Iguer-Ouada, M., 2012. Molecular mechanism underlying anti-inflammatory and anti-allergic activities of phytochemicals: an update. *Molecules*, *18*(1), pp.322-353.

Bhanu, S., Scheinmann, F. and Jefferson, A., 1975. Xanthones from the heartwood of *Calophyllum ramiflorum*. *Phytochemistry*, *14*(1), pp.298-299.

Bone, K. and Mills, S., 2013. *Principles and Practice of Phytotherapy-E-Book: Modern Herbal Medicine*. Elsevier Health Science

Boysen, R.I. and Hearn, M.T., 2010. High performance liquid chromatographic separation methods. *Comprehensive Natural Products II*, 9, pp. 5-49.

Burrell, R.C., 1937. Phytochemistry. What it is and how it has developed. *Journal of Chemical Education*, *14*(11), p.520.

Butler, M.S., 2004. The role of natural product chemistry in drug discovery. *Journal of natural products*, *67*(12), pp.2141-2153.

Cao, S.G., Lim, T.B., Sim, K.Y. and Goh, S.H., 1997a. A highly prenylated xanthone from the bark of *Calophyllum gracilipes* (Guttiferae). *Natural Product Letters*, *10*(1), pp.55-58.

Cao, S.G., Sim, K.Y. and Goh, S.H., 1997b. Biflavonoids of *Calophyllum venulosum*. *Journal of natural products*, *60*(12), pp.1245-1250.

Cao, S.G., Sim, K.Y., Goh, S.H., Xue, F. and Mak, T.C., 1997c. Gracilipene: a heterocyclic seco-trisnor-oleanane from *Calophyllum gracilipes* (Guttiferae). *Tetrahedron letters*, *38*(27), pp.4783-4786.

Cottiglia, F., Dhanapal, B., Sticher, O. and Heilmann, J., 2004. New Chromanone Acids with Antibacterial Activity from *Calophyllum brasiliense*. *Journal of natural products*, *67*(4), pp.537-541.

Cragg, G.M. and Newman, D.J., 2002. Drugs from nature: past achievements, future prospects. In *Advances in Phytomedicine* (Vol. 1, pp. 23-37). Elsevier.

Cuesta-Rubio, O., Oubada, A., Bello, A., Maes, L., Cos, P. and Monzote, L., 2015. Antimicrobial assessment of resins from *Calophyllum antillanum* and *Calophyllum inophyllum*. *Phytotherapy research*, *29*(12), pp.1991-1994.

Daud, S.B., Ee, G.C.L., Malek, E.A., Teh, S.S. and See, I., 2014. A new coumarin from *Calophyllum hosei*. *Natural product research*, *28*(19), pp.1534-1538.

Dharmaratne, H.R.W. and Wijesinghe, W.N.M., 1997. A trioxygenated diprenylated chromenxanthone from *Calophyllum moonii*. *Phytochemistry*, *46*(7), pp.1293-1295.

Dharmaratne, H.R.W., Sotheeswaran, S., Balasubramaniam, S. and Reisch, J., 1986. Xanthones from roots of three Calophyllum species. *Phytochemistry*, *25*(8), pp.1957-1959.

Dias, D.A., Urban, S. and Roessner, U., 2012. A historical overview of natural products in drug discovery. *Metabolites*, *2*(2), pp.303-336.

Ee, G.C.L., Mah, S.H., Teh, S.S., Rahmani, M., Go, R. and Taufiq-Yap, Y.H., 2011. Soulamarin, a new coumarin from stem bark of *Calophyllum soulattri*. *Molecules*, *16*(11), pp.9721-9727.

Esslinger, T.L., 2012. Recent Literature on Lichens, *The Brylogist*, 104(3), pp.473-486.

Ferchichi, L., Derbré, S., Mahmood, K., Touré, K., Guilet, D., Litaudon, M., Awang, K., Hadi, A.H.A., Le Ray, A.M. and Richomme, P., 2012. Bioguided fractionation and isolation of natural inhibitors of advanced glycation end-products (AGEs) from *Calophyllum flavoramulum*. *Phytochemistry*, *78*, pp.98-106.

Filho, C., V., Meyre-Silva, C. and Niero, R., 2009. Chemical and pharmacological aspects of the genus Calophyllum. *Chemistry & biodiversity*, *6*(3), pp.313-327.

Gomez-Verjan, J., Gonzalez-Sanchez, I., Estrella-Parra, E. and Reyes-Chilpa, R., 2015. Trends in the chemical and pharmacological research on the tropical trees *Calophyllum brasiliense* and *Calophyllum inophyllum*, a global context. *Scientometrics*, *105*(2), pp.1019-1030.

Gonçalves, R.M., Lemos, C.O.T., Leal, I.C.R., Nakamura, C.V., Cortez, D.A.G., da Silva, E.A., Cabral, V.F. and Cardozo-Filho, L., 2013. Comparing conventional and supercritical extraction of (−)-Mammea A/BB and the antioxidant activity of Calophyllum brasiliense extracts. *Molecules*, *18*(6), pp.6215-6229.

Gordon, M.H., 2003. FATS| Classification. *Encyclopedia of Food Sciences and Nutrition (Second Edition)*, pp. 2287-2292

Govindachari, T.R., Prakash, D. and Viswanathan, N., 1968. Structure of apetalic acid. *Tetrahedron*, *24*(21), pp.6411-6415.

Guilet, D., Hélesbeux, J.J., Séraphin, D., Sévenet, T., Richomme, P. and Bruneton, J., 2001a. Novel Cytotoxic 4-Phenylfuranocoumarins from *Calophyllum d ispar*. *Journal of natural products*, *64*(5), pp.563-568.

Guilet, D., Séraphin, D., Rondeau, D., Richomme, P. and Bruneton, J., 2001b. Cytotoxic coumarins from *Calophyllum dispar*. *Phytochemistry*, *58*(4), pp.571-575.

Gunatilaka, A.L., De Silva, A.J., Sotheeswaran, S., Balasubramaniam, S. and Wazeer, M.I., 1984. Terpenoid and biflavonoid constituents of *Calophyllum calaba* and *Garcinia spicata* from Sri Lanka. *Phytochemistry*, *23*(2), pp.323-328.

Gupta, R., Gabrielsen, B. and Ferguson, S.M., 2005. Nature's medicines: traditional knowledge and intellectual property management. Case studies from the National Institutes of Health (NIH), USA. *Current drug discovery technologies*, *2*(4), pp.203-219.

Ha, L.D., Hansen, P.E., Duus, F., Pham, H.D. and Nguyen, L.H.D., 2012. A new chromanone acid from the bark of *Calophyllum dryobalanoides*. *Phytochemistry Letters*, *5*(2), pp.287-291.

Hassan, M.Z., Osman, H., Ali, M.A. and Ahsan, M.J., 2016. Therapeutic potential of coumarins as antiviral agents. *European journal of medicinal chemistry*, *123*, pp.236-255.

Hay, A.E., Hélesbeux, J.J., Duval, O., Labaïed, M., Grellier, P. and Richomme, P., 2004. Antimalarial xanthones from *Calophyllum caledonicum* and *Garcinia vieillardii*. *Life sciences*, *75*(25), pp.3077-3085.

Huerta-Reyes, M., del Carmen Basualdo, M., Abe, F., Jimenez-Estrada, M., Soler, C. and Reyes-Chilpa, R., 2004. HIV-1 inhibitory compounds from *Calophyllum brasiliense* leaves. *Biological and Pharmaceutical Bulletin*, *27*(9), pp.1471-1475.

Iinuma, M., Ito, T., Tosa, H., Tanaka, T., Miyake, R. and Chelladurai, V., 1997. Prenylated xanthonoids from Calophyllum apetalum. *Phytochemistry*, *46*(8), pp.1423-1429.

Iinuma, M., Tosa, H., Toriyama, N., Tanaka, T., Ito, T. and Chelladurai, V., 1996. Six xanthones from Calophyllum austroindicum. *Phytochemistry*, *43*(3), pp.681-685.

Ito, C., Itoigawa, M., Mishina, Y., Filho, V.C., Enjo, F., Tokuda, H., Nishino, H. and Furukawa, H., 2003. Chemical constituents of Calophyllum brasiliense. 2. Structure of three new coumarins and cancer chemopreventive activity of 4-substituted coumarins. *Journal of natural products*, *66*(3), pp.368-371.

Ito, C., Itoigawa, M., Mishina, Y., Filho, V.C.,Mukainaka, T., Tokuda, H., Nishino, H. and Furukawa, H., 2002. Chemical Constituent of *Calophyllum brasiliensis*: Structure eludication of seven new xanthones and their cancer chemopreventive activity 1. *Journal of natural product*, 65(3), pp.267-272

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G. and Moon, R.C., 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, *275*(5297), pp.218-220.

Jia, Q., Köllner, T.G., Gershenzon, J. and Chen, F., 2018. MTPSLs: New Terpene Synthases in Nonseed Plants. *Trends in plant science*.

Kashman, Y., Gustafson, K.R., Fuller, R.W., Cardellina, J.H. and RW Jr, H., GM and Boyd, MR (1992) The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree, Calophyllum lanigerum. *J. Med. Chem*, *35*, pp.2735-2743.

Khan, M.R., Kihara, M. and Omoloso, A.D., 2002. Antimicrobial activity of *Calophyllum soulattri*. *Fitoterapia*, *73*(7-8), pp.741-743.

Kimura, S., Ito, C., Jyoko, N., Segawa, H., Kuroda, J., Okada, M., Adachi, S., Nakahata, T., Yuasa, T., Furukawa, H. and Maekawa, T., 2005. Inhibition of leukemic cell growth by a novel anti-cancer drug (GUT-70) from *Calophyllum brasiliense* that acts by induction of apoptosis. *International journal of cancer*, *113*(1), pp.158-165.

Larcher, G., Morel, C., Tronchin, G., Landreau, A., Séraphin, D., Richomme, P. and Bouchara, J.P., 2004. Investigation of the antifungal activity of caledonixanthone E and other xanthones against *Aspergillus fumigatus*. *Planta medica*, *70*(06), pp.569-571.

Laure, F., Raharivelomanana, P., Butaud, J.F., Bianchini, J.P. and Gaydou, E.M., 2008. Screening of anti-HIV-1 inophyllums by HPLC–DAD of *Calophyllum inophyllum* leaf extracts from French Polynesia Islands. *Analytica chimica acta*, *624*(1), pp.147-153.

Lee, S.K., Mbwambo, Z.H., Chung, H., Luyengi, L., Gamez, E.J., Mehta, R.G., Kinghorn, A.D. and Pezzuto, J.M., 1998. Evaluation of the antioxidant potential of natural products. *Combinatorial chemistry & high throughput screening*, *1*(1), pp.35-46.

Lemos, L.M.S., Martins, T.B., Tanajura, G.H., Gazoni, V.F., Bonaldo, J., Strada, C.L., da Silva, M.G., Dall'Oglio, E.L., de Sousa Júnior, P.T. and de Oliveira Martins, D.T., 2012. Evaluation of antiulcer activity of chromanone fraction from Calophyllum brasiliesnse Camb. *Journal of ethnopharmacology*, *141*(1), pp.432-439.

Li, Y.Z., Li, Z.L., Yin, S.L., Shi, G., Liu, M.S., Jing, Y.K. and Hua, H.M., 2010. Triterpenoids from *Calophyllum inophy*llum and their growth inhibitory effects on human leukemia HL-60 cells. *Fitoterapia*, *81*(6), pp.586-589.

Locksley, H.D., Moore, I. and Scheinmann, F., 1966. Extractives from guttiferae. Part IV. Isolation and structure of *ugaxanthone* and *mbarraxanthone* from *Symphonia globulifera* L. *Journal of the Chemical Society C: Organic*, pp.2265-2269.

Ma, C.H., Chen, B., Qi, H.Y., Li, B.G. and Zhang, G.L., 2004. Two pyranocoumarins from the seeds of *Calophyllum polyanthum*. *Journal of natural products*, *67*(9), pp.1598-1600.

Mah, S.H., Ee, G.C.L., Teh, S.S., Rahmani, M., Lim, Y.M. and Go, R., 2012. Phylattrin, a new cytotoxic xanthone from *Calophyllum soulattri*. *Molecules*, *17*(7), pp.8303-8311.

McKee, T.C., Fuller, R.W., Covington, C.D., Cardellina, J.H., Gulakowski, R.J., Krepps, B.L., McMahon, J.B. and Boyd, M.R., 1996. New pyranocoumarins isolated from *Calophyllum lanigerum* and *Calophyllum teysmannii*. *Journal of natural products*, *59*(8), pp.754-758.

McRae, J., Yang, Q., Crawford, R. and Palombo, E., 2007. Review of the methods used for isolating pharmaceutical lead compounds from traditional medicinal plants. *The Environmentalist*, *27*(1), pp.165-174.

Ming, M., Zhang, X., Chen, H.F., Zhu, L.J., Zeng, D.Q., Yang, J., Wu, G.X., Wu, Y.Z. and Yao, X.S., 2016. RXRα transcriptional inhibitors from the stems of *Calophyllum membranaceum*. *Fitoterapia*, *108*, pp.66-72.

Morel, C., Hay, A.E., Litaudon, M., Sévenet, T., Séraphin, D., Bruneton, J. and Richomme, P., 2002. Thirteen new xanthone derivatives from *Calophyllum caledonicum* (Clusiaceae). *Molecules*, *7*(1), pp.38-50.

Muranaka, T. and Saito, K., 2010. Production of pharmaceuticals by plant tissue cultures.

Naithani, R., Mehta, R.G., Shukla, D., Chandersekera, S.N. and Moriarty, R.M., 2010. Antiviral Activity of Phytochemicals: A Current Perspective. In *Dietary Components and Immune Function* (pp. 421-468). Humana Press, Totowa, NJ.

Nasir, N.M., Rahmani, M., Shaari, K., Ee, G.C.L., Go, R., Kassim, N.K., Muhamad, S.N.K. and Iskandar, M.J., 2011. Two new xanthones from *Calophyllum nodusum* (Guttiferae). *Molecules*, *16*(11), pp.8973-8980.

Nasir, N.M., Rahmani, M., Shaari, K., Kassim, N.K., Go, R., Stanslas, J. and Jeyaraj, E.J., 2013. Xanthones from *Calophyllum gracilipes* and their cytotoxic activity. *Sains Malaysiana*, *42*(9), pp.1261-1266.

Nguyen, L.T.T., Nguyen, H.T., Barbič, M., Brunner, G., Heilmann, J., Pham, H.D., Nguyen, D.M. and Nguyen, L.H.D., 2012. Polyisoprenylated acylphloroglucinols and a polyisoprenylated tetracyclic xanthone from the bark of *Calophyllum thorelii*. *Tetrahedron Letters*, *53*(34), pp.4487-4493.

Nigam, S.K., Banerji, R., Rebuffat, S., Pascard, C. and Bodo, B., 1988. Soulattrone A, A C24 terpenoid from Calophyllum soulattri. *Phytochemistry*, *27*(2), pp.527-530.

Nigam, S.K., Mitra, C.R., Kunesch, G., Das, B.C. and Polonsky, J., 1967. Constituents of *Calophyllum tomentosum* and *Calophyllum apetalum* nuts: Structure of a new 4- alkyl-and of two new 4-phenyl coumarins. *Tetrahedron Letters*, *8*(28), pp.2633-2636.

Panche, A.N., Diwan, A.D. and Chandra, S.R., 2016. Flavonoids: an overview. *Journal of nutritional science*, *5*.

Patil, A.D., Freyer, A.J., Eggleston, D.S., Haltiwanger, R.C., Bean, M.F., Taylor, P.B., Caranfa, M.J., Breen, A.L. and Bartus, H.R., 1993. The inophyllums, novel inhibitors of HIV-1 reverse transcriptase isolated from the Malaysian tree, *Calophyllum inophyllum* Linn. *Journal of Medicinal Chemistry*, *36*(26), pp.4131-4138.

Pawar, K.D., Joshi, S.P., Bhide, S.R. and Thengane, S.R., 2007. Pattern of anti-HIV dipyranocoumarin expression in callus cultures of *Calophyllum inophyllum* Linn. *Journal of biotechnology*, *130*(4), pp.346-353.

Ranjith, H., Dharmaratne, W., Sotheeswaran, S., Balasubramaniam, S. and Waight, E.S., 1985. Triterpenoids and coumarins from the leaves of *Calophyllum Cordato-Oblongum*. *Phytochemistry*, *24*(7), pp.1553-1556.

Ravelonjato, B., Kunesch, N. and Poisson, J.E., 1987. Neoflavonoids from the stem bark of *Calophyllum verticillatum*. *Phytochemistry*, *26*(11), pp.2973-2976.

Reyes-Chilpa, R., Estrada-Muñiz, E., Apan, T.R., Amekraz, B., Aumelas, A., Jankowski, C.K. and Vázquez-Torres, M., 2004. Cytotoxic effects of mammea type coumarins from *Calophyllum brasiliense*. *Life Sciences*, *75*(13), pp.1635-1647.

Rissyelly, Katrin, Berna, E. and Angger, M., 2014. Radical Scavenging Activity of Extract, Fraction and Chemical Compound from *Calophyllum sclerophyllum* vesq. Stembark by Using 1, 1-Diphenyl-2-Picryl Hydrazil (DPPH). *Int. J. PharmTech Res*, 6(1), pp.396-402

Sharma, R., 2013. Herbal Supplements or Herbs in Heart Disease: History, Herbal Foods, Coronary Heart Disease. In *Bioactive Food as Dietary Interventions for Cardiovascular Disease* (pp. 29-61).

Shen, Y.C., Wang, L.T., Khalil, A.T. and Kuo, Y.H., 2004. Chromanones and dihydrocoumarins from *Calophyllum blancoi*. *Chemical and pharmaceutical bulletin*, *52*(4), pp.402-405.

Shen, Y.C., Wang, L.T., Khalil, A.T., Chiang, L.C. and Cheng, P.W., 2005. Bioactive pyranoxanthones from the roots of *Calophyllum blancoi*. *Chemical and pharmaceutical bulletin*, *53*(2), pp.244-247.

Soltani, J., 2016. Secondary Metabolite Diversity of the Genus Aspergillus: Recent Advances. In *New and Future Developments in Microbial Biotechnology and Bioengineering*, pp. 275-292.

Stevens, P.F., 1980. A revision of the Old World species of Calophyllum (Guttiferae). *Journal of the Arnold arboretum*, *61*(2), pp.117-424.

Stout, G.H. and Sears, K.D., 1968. Calophyllum products. III. Structure of blancoic acids. *The Journal of Organic Chemistry*, *33*(11), pp.4185-4190.

Su, X.H., Zhang, M.L., Li, L.G., Huo, C.H., Gu, Y.C. and Shi, Q.W., 2008. Chemical constituents of the plants of the genus Calophyllum. *Chemistry & biodiversity*, *5*(12), pp.2579-2608.

Sundur, S, V.L.Jayasekhar, Shrivastava, D.B., Sharma, D.P. and Sunder,S., 2014. A Review Article of Pharmacological Activities And Biological, *International Journal of Advanced Research*, 2(12), pp.599-603.

Sung, B., Prasad, S., Gupta, S.C., Patchva, S. and Aggarwal, B.B., 2012. Regulation of inflammation-mediated chronic diseases by botanicals. In *Advances in Botanical Research*(Vol. 62, pp. 57-132). Academic Press.

Taher, M., Idris, M.S., Ahmad, F. and Arbain, D., 2005. A polyisoprenylated ketone from *Calophyllum enervosum*. *Phytochemistry*, *66*(6), pp.723-726

Torres, F.C., Brucker, N., Fernandes Andrade, S., Fabio Kawano, D., Cristina Garcia, S., Lino von Poser, G. and Lucia Eifler-Lima, V., 2014. New insights into the chemistry and antioxidant activity of coumarins. *Current topics in medicinal chemistry*, *14*(22), pp.2600-2623.

Tuorkey, M.J., 2015. Cancer therapy with phytochemicals: present and future perspectives. *Biomedical and Environmental Sciences*, *28*(11), pp.808-819.

Wang, H., Sun, Q.Y., Yang, F.M., Long, C.L., Wang, Y.H., Tang, G.H., Zhao, F.W., Niu, H.M., Huang, Q.Q., Xu, J.J. and Wataya, Y., 2010. Chromanone Derivatives from the Pericarps of *Calophyllum polyanthum*. *Helvetica Chimica Acta*, *93*(11), pp.2183-2188.

Wiart, C., 2013. *Lead compounds from medicinal plants for the treatment of cancer* (Vol. 1). Academic Press.

Yimdjo, M.C., Azebaze, A.G., Nkengfack, A.E., Meyer, A.M., Bodo, B. and Fomum, Z.T., 2004. Antimicrobial and cytotoxic agents from *Calophyllum inophyllum*. *Phytochemistry*, *65*(20), pp.2789-2795.

Ziberna, L., Fornasaro, S., Čvorović, J., Tramer, F. and Passamonti, S., 2014. Bioavailability of flavonoids: the role of cell membrane transporters. In *Polyphenols in human health and disease*, pp. 489-511.

Zou, J., Jin, D., Chen, W., Wang, J., Liu, Q., Zhu, X. and Zhao, W., 2005. Selective Cyclooxygenase-2 Inhibitors from *Calophyllum membranaceum*. *Journal of natural products*, *68*(10), pp.1514-1518.