CHEMICAL CONSTITUENTS FROM THE ENDEMIC PLANT OF SARAWAK, CALOPHYLLUM CASTANEUM AND THEIR ANTIOXIDANT ACTIVITY

GAN SHU YING

BACHELOR OF SCIENCE (HONS) CHEMISTRY

FACULTY OF SCIENCE

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By

GAN SHU YING

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ABSTRACT

CHEMICAL CONSTITUENTS FROM THE ENDEMIC PLANT OF SARAWAK, *CALOPHYLLUM CASTANEUM* AND THEIR ANTIOXIDANT ACTIVITY

GAN SHU YING

In this project, the chemical constituents from the plant species, *Calophyllum castaneum* was studied. *Calophyllum castaneum* belongs to the genus of *Calophyllum*, is grouped into the family of Guttiferae. By far there has been no phytochemical and biological studies reported on this plat species.

Phytochemical study on the dichloromethane crude extract of the stem bark of *Calophyllum castaneum* yielded two triterpenoids, one xanthone and two chromanone acids. The compounds isolated were identified as friedelin, friedelinol, euxanthone, isoblancoic acid and blancoic acid. The structure of these isolated compounds were elucidated through the application of ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, GC-MS, LC-MS, IR and UV-Vis spectroscopic techniques.

Moreover, the methanol, ethyl acetate and dichloromethane crude extracts as well as all the five isolated compounds were screened in view for their antioxidant activity. However, only methanol and ethyl acetate crude extracts were found to show positive results in the assay with their IC_{50} values of 12 μg/mL and 84 μg/mL, respectively.

ABSTRAK

Dalam projek ini, sebatian-sebatian semula jadi yang terdapat dalam spesis flora, *Calophyllum castaneum* telah dikaji. *Calophyllum castaneum* adalah salah satu spesis yang terkandung dalam genus *Calophyllum* dan disenaraikan dalam keluarga Guttiferae. Setakat ini, tiada kajian fitokimia dan biologi telah dilaporkan ke atas *Calophyllum castaneum*.

Kajian fitokimia ke atas ekstrak diklorometana daripada kulit pokok *Calophyllum castaneum* telah menghasilkan dua triterpenoids, satu xanthone and dua asid chromanone. Sebatian- sebatian yang diperoleh telah dikenalpasti sebagai friedelin, friedelinol, euxanthone, isoblancoic asid and blancoic asid. Struktur-struktur bagi sebatian-sebatian ini telah dikajikan melalui pelbagai teknik spektroskopi sperti ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, GC-MS, LC-MS, IR and UV-Vis.

Selain itu, ekstrak methanol, etil asetat dan diklorometana serta semua sebatian-sebatian yang dipisahkan telah diselidik ke atas aktiviti atiosidan masing-masing. Hasil kajian menunjukkan bahawa hanya ekstrak methanol dan etil asetat memberi keputusan postitif dengan menunjukkan nilai IC_{50} 12 μg/mL dan 84 μg/mL masing-masing.

ACKNOWLEDGEMENT

First and foremost, I would like to take this opportunity to express my sincere appreciation to my supervisor, Dr. Lim Chan Kiang for his guidance, patience, support and advice throughout the whole project. I am thankful for his unselfishness in leading me and my laboratory mates in performing this project with success. I have gained a lot of extra knowledge from him during the research period. In addition, I am also grateful and touched for his concern and understanding about the hard times faced by my family during the research period.

Secondly, I would like to express my gratitude to the staffs of Chemistry Department of Universiti Tunku Abdul Rahman for their assistance and cooperation in making this project a success. Moreover, special thanks to my laboratory mates, Lai Jing Yi and Kisantini A/P Murugesu for their unconditional help and cooperation on problem solving and scientific discussion.

Lastly, my gratitude goes to my family members especially for my mother in giving me invaluable support and encouragement in order for me to finish this research successfully.

DECLARATION

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

_____

GAN SHU YING

APPROVAL SHEET

This project report entitled "**CHEMICAL CONSTITUENTS FROM THE ENDEMIC PLANT OF SARAWAK, *CALOPHYLLUM CASTANEUM* AND THEIR ANTIOXIDANT ACTIVITY**" was prepared by GAN SHU YING 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 Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **GAN SHU YING** (ID: 10ADB04803) had completed this report entitled "**CHEMICAL CONSTITUENTS FROM THE ENDEMIC PLANT OF SARAWAK, *CALOPHYLLUM CASTANEUM* AND THEIR ANTIOXIDANT ACTIVITY**" under the supervision of Assistant Professor DR. LIM CHAN KIANG from the Department 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,

_____

(GAN SHU YING)

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LIST OF ABBREVIATIONS

α	Alpha
δ	Chemical shift
δ_C	Chemical shift of carbon
δ_H	Chemical shift of proton
λ	Wavelength
λ_{max}	Maximum wavelength
μg	Microgram
ρ	Density
%	Percentage
^oC	Degree in Celsius
^1H	Proton
^{13}C	Carbon-13
g	Gram
d	Doublet
m	Multiplet
q	Quartet
s	Singlet
t	Triplet
J	Coupling constant in Hertz
dd	Doublet of doublets
dt	Doublet of triplets
cm	Centimetre
nm	Nanometer

kg	Kilogram
mL	Millilitre
Hz	Hertz
R_f	Retention factor
IR	Infrared
O-H	Hydroxyl
ppm	Parts per million
IC_{50}	Half Maximal Inhibitory Concentration
A_1	Absorbance of the test compound
A_o	Absorbance of the negative control (blank)
C=O	Carbon=Oxygen
C-H	Carbon-hydrogen
C-O	Carbon-Oxygen
$CDCl_3$	Deuterated chloroform
CD_3COCD_3	Deuterated acetone
DPPH	1,1-diphenyl-2-picryhydrazyl
FTIR	Fourier-Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
LC-MS	Liquid Chromatography- Mass Spectroscopy
HIV-1	Human Immunodeficiency Virus
NMR	Nuclear Magnetic Resonance
MIC	Minimal Inhibitory Concentration
MS	Mass Spectrometry
TLC	Thin Layer Chromatography
TMS	Trimethylsilane

KBr	Potassium Bromide
UV-Vis	Ultraviolet-Visible
1D-NMR	One Dimension Nuclear Magnetic Resonance
2D-NMR	Two Dimension Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Natural product chemistry is the branch of chemistry which deals with the isolation, identification, structure elucidation and chemical characteristic of natural products. Natural products are the chemical compounds or substances that produced by living organism, but the term is usually referred to secondary metabolites. As organisms need to adapt to their surrounding environment and protect themselves from predators, secondary metabolites are produced in order to help in the survival of the organisms. Secondary metabolites are generally not essential for the growth, development or reproduction of an organism but they play an important role in the pharmaceutical field (Cannell, 1998). Phytochemistry is a branch of natural product chemistry concerned with study of a huge variety of secondary metabolic compounds found in plants. The techniques commonly used in the field of phytochemistry including extraction, isolation, structural elucidation of natural products via various chromatographic and spectroscopic techniques (Phillipson, 2007).

The five senses of human being and curiosity that whether a compound can be used as a drug have aroused the interest of mankind in investigating the benefit and usefulness of herbs, and this has originated the natural product chemistry. Herbal drugs are use traditionally by people around the world. Undeniable, trial

and error is likely the method employed to develop the knowledge of traditional medicine regardless of any country. Historically, it was known that China acted as the pioneer of the world which can be dated back thousands of years ago in the use of natural product for healing, and is found to have the most historic medicine system. Sheng Nung Ts'ao Ching (Catalogue of Herbs by Shen Nung) and The Pen Ts'ao Kang Mu (General Catalogue of Herbs) are the two most representative collections of Chinese herbs, and these compilations are spread widely until today and contributed a lot to the pharmaceutical field. Other than China, there are still other countries which have a long history in medicine system, including India, Egypt and Greece, and the medicine system of India is said to be comparable with China for its long history (Nakanishi, 1998).

Pythagoras (580-500 BC), Aristotle (384-322 BC) and Galen (129-216) are the medical thinkers of Greece that are well known by everyone (Nakanishi, 1998). The born of phytochemistry is widely regarded as the result of isolation of tartaric acids from grapes in 1976 (Cordell, 2011). The era of natural product chemistry can be said that only emerged at the early 19th century and the identification of phytochemicals in plants has become important in modern drug discovery. Natural product chemistry is at the stage of stable development and it acts as the primary source of drugs, leading to the discovery of potential medicine. Today, modern and advanced techniques used in natural product chemistry have allowed isolation of various bioactive natural products from different sources to be chemically investigated and discovered. Currently, there are many phytochemical have been discovered and found to fight against various pharmacological targets such as HIV, cancers and Alzheimer's disease.

In future, natural product chemistry is believed to continue to grow and develop new and more advanced methodologies and techniques in order to intensify the discovery of more and more phytochemicals that have medicinal properties which may contribute to the modern drug development for treatment of diseases, especially those incurable diseases that become the main killers of human being.

Phytochemicals are nonessential nutrients, meaning that they are not essential to life processes. Although phytochemicals are not directly involved in the life processes, but they are proven to be benefit for human health therapeutic agents. Most of the phytochemicals show antioxidant activity, which are natural antioxidants that human valued. Oxidation is a chemical reaction that produces free radicals, which starts the chain reactions that damage cells and increase the risk of developing certain type of cancers and heart diseases. Antioxidants terminate these radical chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by themselves being oxidized. For other instances, paclitaxel which is isolated from the bark of *Taxus brevifolia* (Pacific Yew), is the drug that is most widely used in the treatment of breast cancer (Phillipson, 2007). In addition, many plants and their secondary metabolites were also found to exhibit various therapeutic properties which can be used for treatment of urinary tract infections, bronchitis, diarrhoea, cutaneous abscesses and parasitic diseases (Yasunaka, et al., 2005). In this project, the chemical constituents and antioxidant activity of *Calophyllum castaneum* were investigated.

1.2 Botany of Plant Studied

1.2.1 Taxonomy of Plant Studied

The taxonomy of *Calophyllum castaneum* is shown in the Table 1.1.

Table 1.1: Taxonomy of *Calophyllum castaneum*

Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Spermatopsida
Order	:	Malpighiales
Family	:	Guttiferae
Genus	:	*Calophyllum*
Species	:	*Calophyllum castaneum*

1.2.2 *Calophyllum castaneum*

1.2.2.1 Distribution and Habitat

Calophyllum castaneum is originated from the genus *Calophyllum*, which is one of the genera in the Guttiferae family, also known as Clusiaceae family (Crane, et al., 2005). Guttiferae family acts as the major canopy of the forest and there is no record to show any species from this family which has grown to become an emergent tree. The timber of the species from this family are valued by human because of their rich sources of secondary metabolites. *Bintangor* is the local name of the genus *Calophyllum* and it consists of around 180-200 different species distributed mostly in tropical areas of the world (Nasir, et al., 2011). For instances, the genus *Calophyllum* normally can be found in Sarawak (Malaysia),

Brunei, West Kalimantan and East Kalimantan. *Calophyllum* is normally found in swamp forests from sea level to the peak of hill.

1.2.2.2 Morphology of Plant Studied

Slander, small to medium trees are normally the appearance of the genus *Calophyllum* and they are occasionally shrubs (Nasir, et al., 2011). *Calophyllum castaneum* grows up to 85 ft. tall and 3 ft. girth with the bark surface shallowly fissured with greyish brown. The stalk of *Calophyllum castaneum* is covered with very fine yellowish latex while the green twig of the tree is covered with very fine brown hair. The leaves of the trees are opposite, petiolate, leathery, usually glabrous and have many secondary vein perpendicular to midvein but absence of tertiary vein. The flower of *Calophyllum castaneum* is usually displayed the properties of hermaphroditic, also known as bisexual and the ovary is a single, basal and anatropous ovule. The appearance of the fruit is just like the drupelike berry and is in green colour (Sungkaew, et al., 2009). The picture of *Calophyllum castaneum* is shown in Figure 1.1.

Figure 1.1: *Calophyllum castaneum* tree

1.2.2.3 Uses

According to previous phytochemical studies, *Calophyllum* is well known as a source of coumarins (Ito, et al., 2003), xanthones (Ito, et al., 2003), biflavonoids (Ito, et al., 1999), chalcone, benzofurans and triterpenoids (Ito, et al., 2002). Coumarins have been reported to exhibit anti-HIV and cancer chemo-preventive properties. Meanwhile, antifungal, antimicrobial and molluscicidal effects were reported from various xanthones derivatives (Gwendoline, et al., 2011). Apart from that, the acidic and sticky latex of *Calophyllum brasiliense* is also reported to be used in the treatment of pain, diabetes, diarrhoea, hypertension, herpes, rheumatism and ulcer (Lemos, et al., 2012). Besides medicinal uses, the hard timber of *Calophyllum* species can be used for building purposes in construction field.

1.3 Objectives of Study

The purposes of performing this study are:

I. To extract and isolate chemical constituents from the stem bark of *Calophyllum castaneum.*

II. To characterize and identify the structures of isolated compounds from the crude extracts of *Calophyllum castaneum* via modern spectroscopic techniques.

III. To investigate the antioxidant activity of the crude extracts as well as isolated compounds via DPPH assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemical Studies

Phytochemicals refer to the chemical constituents that are found naturally in plants. They are not nutrients that consist of the essential nutritive values for life but many of them are known to have potential bioactive properties. The term secondary metabolites is referring to the phytochemicals that produced naturally in plants which are not essential in the growth and development of living organisms but their protective or disease preventive properties are highly valued by human. Plants produce phytochemicals through secondary metabolisms which are the metabolisms that are not directly responsible for maintaining the survival of organisms. Phytochemicals which are produced through secondary metabolisms are found to be specific with reference to the genus of plants studied as the formation of phytochemicals only occurred in certain plants, or group of plants.

There are more than thousands of existing phytochemicals that have been identified from previous researches and studies. Many of them were reported to act as the protective agents against acute, chronic and degenerative diseases (Bellik, et al., 2012). Some of the phytochemicals were found to possess antioxidant property, protecting cells from cell damage due to oxidation, and hence reduce the developing of certain cancer diseases. In general, phytochemicals can be divided into several groups, which are carotenoids,

phenolics, alkaloids, nitrogen-containing compounds, and organosulphur compounds (Bellik, et al., 2012). Some of the well-known phytochemical compounds are alkaloids, flavonoids, xanthones, terpenes, and saponins. Phytochemical studies revealed that plants from the genus *Calophyllum* are to be rich in flavonoids, xanthones, triterpenes, coumarins and chromanones.

2.1.1 Flavonoids

Flavonoids are normally known as bioflavonoids, describing a group of water soluble polyphenolic compounds that have a C_6-C_3-C_6 carbon framework which specifically known as phenylbenzopyran functionality. Flavonoids are classified into different categories according to the difference in their molecular structures, namely chalcones, flavanols, catechin, flavones, flavanoes, isoflavone, dihydroflavonol and anthocyanidin (Sandhar, et al., 2011).

Flavonoids are found to have various biological functions, including the pharmacological uses and their functions in plants. For example, pigment present in the flowers gives colour that attracted insects for pollination. In addition, flavonoids contained in leaves also have the ability to prevent fungal infections and protect leaves from UV radiations (Sandhar, et al., 2011). In pharmacological aspect, flavonoids are found to interact with cytochrome P 450 and are used to treat heart diseases due to their mild vasodilators properties. Flavonoids are well known for their antioxidant activity and this free radical scavenging property is useful in the area of anticancer and anti-ageing. In additions, flavonoids also indicate antileukemic property, vitamin C sparing

activity, and the 5-lipoxygenase, cyclooxygenase, protein kinase C, tyrosine kinase activities and genetic toxicity (Sharma, D. K., 2006). Figure 2.1 shows the molecular structure of a flavone backbone.

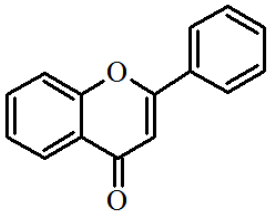


Figure 2.1: Molecular structure of flavone backbone

2.1.2 Xanthones

Xanthones are a class of polyphenolic compounds that occur naturally in nature with the molecular formula of $C_{13}H_8O_2$. Xanthones consist of a unique backbone with two benzene rings bridged across a carbonyl group and an oxygen. The conjugated ring system inhibiting the free rotation of the carbon-carbon bonds. The particular properties of xanthones are determined by the type and position of chemical substituents that attached to their unique backbone. Xanthones can be found in selected tropical plants, but they are even found more generally in the pericarp of the mangosteen fruits.

Xanthones are found to have various bioactive properties with the most famous one is their antioxidant ability. Mangostin which can be isolated from *Garcinia mangostana* was found to act as the free radical scavenger and hence prevented the oxidative damage of low density lipoprotein (Williams, et al., 1995). Besides that, xanthones isolated from *Garcinia mangostana* were also found to inhibit

the human leukemia cell line HL60 (Matsumoto, et al., 2003). α-mangostin extracted from *Garcinia mangostana L.* was reported to exhibit antibacterial activity against vancomycin resistant *Enterococci* (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA) (Sakagami, et al., 2005). Basic molecular structure of xanthone is shown in Figure 2.2.

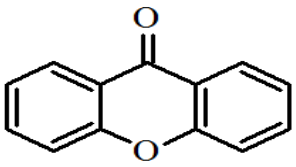


Figure 2.2: Basic molecular structure of xanthone

2.1.3 Terpenes

Terpenes are a group of naturally occurring compounds which are derived biosynthetically from the isoprene C-5 unit with the molecular formula of C_5H_8. Hence, terpenes are generally expressed in the formula of $(C_5H_8)_n$ with n representing the number of isoprene units that is present, giving the number of carbons in terpenes which is in the multiple of 5. Isoprene units are linked together in a head to tail fashion, which is known as the isoprene rule. Terpenes are classified into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes and polyterpenes. Terpenoids are compounds that with an isoprenoids structure which contain oxygen inside their structure and can be act as either ketones, aldehydes or alcohols.

Terpenes are widely used by human because of their potentials as medicine and flavour enhancers. The most commonly known and largely used terpenes is

rubbers. Low molecular weight terpenes such as essential oils are made from plants which are widely used as the natural food additives and aroma in perfumery. The most famous use of terpenes is the anticancer drug taxol, which is a diterpene. Taxol is used in the treatments against breast, ovarian and lung cancers. Apart from that, the diterpenes gingkgolides with their antagonistic property were found for their uses in treatment of inflammation, shock, ulceration, skin diseases and burn, platelet-activating factor increases (Ramawat and Merillon, 2013). Figure 2.3 shows the molecular structure of isoprene.

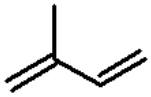


Figure 2.3: Molecular structure of isoprene

2.1.4 Coumarins

Coumarins or benzo-α-pyrones are one of the member of the benzopyrone family of compounds. In the structure of coumarins, there is a benzene ring linked to a pyrone ring (Bezwada, 2008). Coumarins can be divided into four main types which include simple coumarins, pyranocoumarins, furanocoumarins and pyrone-substituted coumarins. All of the reactions of coumarins are focused at the activated C-3,4- double bond of the α,β-unsaturated lactone and thus building the heterocyclic systems (Sethna and Shah, 1945).

There are different biological activities possessed by coumarins and they are broadly used in the agrochemical, perfume, medication and toiletries industries. Coumarins are compounds that are attractive to human for their physiological,

bacteriostatic and anti-tumour activity. The antitumor activity of coumarins such as 7-hydroxylcoumarin against several human tumour cell lines has been identified. In addition, coumarins and its derivatives are also acted as potential inhibitors against the cellular proliferation in various carcinoma cell lines (Lacy and O'Kennedy, 2004). Besides that, coumarins also found to exhibit other properties including anticoagulant, antioxidant, antimicrobial, antiviral, anti-inflammatory, antimalarial and analgesic (Sahoo, et al., 2012). Figure 2.4 shows the molecular structure of coumarins.

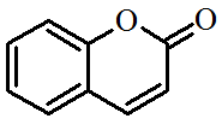


Figure 2.4: Molecular structure of coumarins

2.2 Chemistry of the Genus *Calophyllum*

There is around 200 *Calophyllum* species all around the world, but so far only about 30 species have been phytochemically studied and with a lesser number of them have been studied for their biological potentials. *Calophyllum* species act as a rich source of various secondary metabolites and most of the isolated secondary metabolites are bioactive compounds. These are the reasons why *Calophyllum* species are being phytochemically studied by many researchers. Among the species that have been extensively studied are *Calophyllum inophyllum*, *Calophyllum brasiliense*, *Calophyllum soulattri* and *Calophyllum lanigerum*. Previous researches have reported the isolation of xanthones, triterpenes, flavonoids, chromanones and coumarins from the *Calophyllum* species.

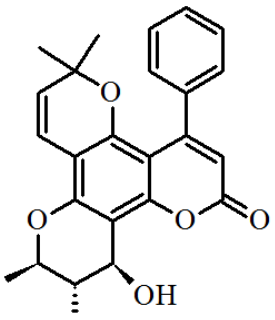
2.2.1 Chemistry of *Calophyllum castaneum*

The *Calophyllum* species that was studied in this project was *Calophyllum castaneum*. By far, there has been no phytochemical and biological studies reported on *Calophyllum castaneum*. Hence, review on the published works of other *Calophyllum* species that have been phytochemical and biological studied was carried out.

2.2.2 Chemistry of Other *Calophyllum* species

2.2.2.1 Chemistry of *Calophyllum inophyllum*

In year 2008, there were a total of 136 leaf extracts of *Calophyllum inophyllum* from French Polynesia Islands were being studied. By applying high pressure liquid chromatography-UV-diode array detection (HPLC-UV-DAD) technique, researchers have successfully determined various pyranocoumarins, including inophyllums B **[1]**, C **[2]**, G_1 **[3]**, P **[4]** and calophyllolide **[5]**. Compound **1** has a (10*R*, 11*S*, 12*S*)-10,11-dimethyl 12 chromanol ring which is important for the anti-HIV-1 activity. Compound **5** is also a HIV-1 active compound, but less active than compound **1**, having a *trans*-10,11-dimethylchromanol structure. Compound **5** is the 12-epimer of compound **1**. For compounds **3** and **4**, they showed less anti-HIV-1 activity. There is a 12-chromanone ring in compounds **2**, **3** and **4** (Laure, et al., 2008).



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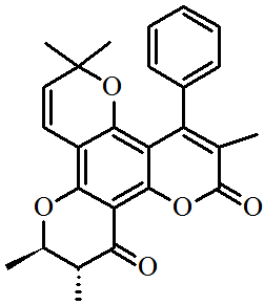

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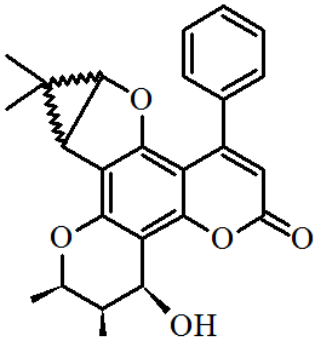

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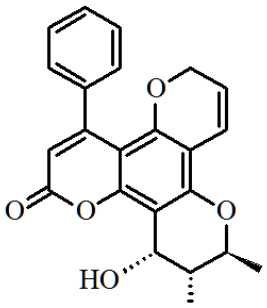

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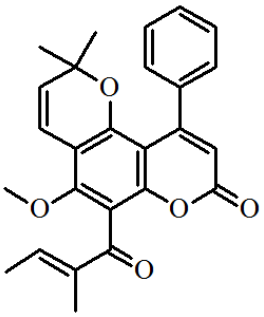
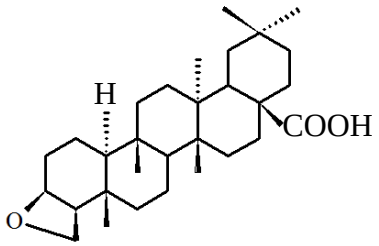


[5]

In year 2010, Li, et al. have isolated a new friedelane-type triterpene, namely 3β, 23-epoxy-fridelan-28-oic acid **[6]**. In the same study, a total of seven other known triterpenoids were also isolated which are friedelin **[7]**, epifriedelanol **[8]**, canophyllal **[9]**, canophyllol **[10]**, canophyllic acid **[11]**, 3-oxo-friedelan-28-oic acid **[12]** and oleanolic acid **[13]**. All these isolated compounds were being tested for their inhibitory ability on the growth of the human leukemia

HL-60 cells. The control human leukemia HL-60 cells was used to compare with the cells that were treated with different concentrations.The cell growth inhibitory effect and the concentration that suppressed half of the cell growth were determined. Compounds **6**, **11**, **12** and **13** were identified and found to possess antiproliferative effects. Compound **12** showed a stronger antiproliferative effect than that of other three compounds with the IC_{50} value of 2.67 μM.



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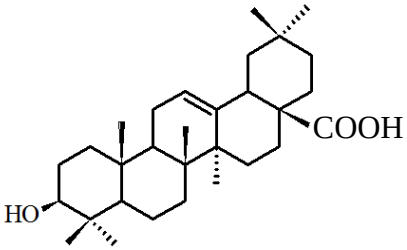
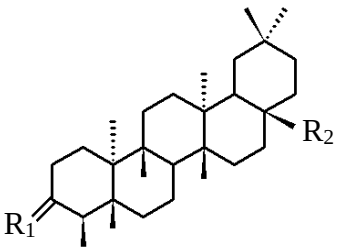


[13]

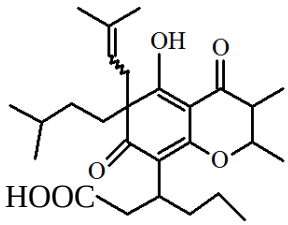


[7] R_1=O R_2=CH_3
[8] R_1=β-OH R_2=CH_3
[9] R_1=O R_2=CHO
[10] R_1=O R_2=CH_2OH
[11] R_1= β-OH R_2=COOH
[12] R_1=O R_2=COOH

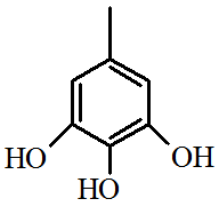
2.2.2.2 Chemistry of *Calophyllum brasiliense*

In year 2004, crude extracts, fractions and isolated compounds from different parts of *Calophyllum brasiliense* were examined for their antimalarial activity.

A total of six compounds have been successfully isolated in this study, which are brasiliensic acid **[14]**, gallic acid **[15]**, epicatechin **[16]**, protocatechuic acid **[17]**, friedelin [**7]** and 1,5-dihydroxyxanthone **[18]**. After 24 hours of incubation at 37 ℃, minimal inhibitory concentration (MIC) value which represents the lowest concentration that suppresses the growth of the organism was determined. The antimalarial activity of the crude extracts, fractions and isolated compounds were identified based on the calculated MIC values. Good antimalarial activity was shown in the extracts with the MIC values less than 100 µg/ml. All the growth of the tested microorganisms including Gram-positive, Gram-negative bacteria and yeasts have been suppressed by compound **17** to show antimalarial activity. Compound **18** also showed antimalarial activity, but only against Gram-positive bacteria. Others isolated compounds did not show any antimalarial activity against the tested microorganisms (Cottiqlia, et al., 2004).



[14]



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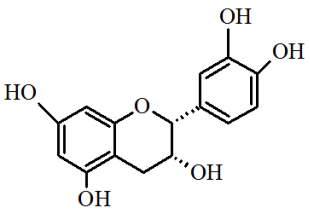
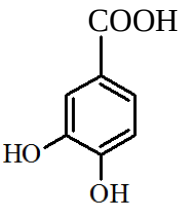
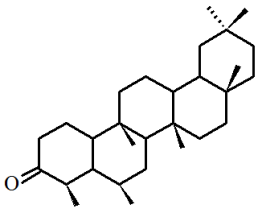


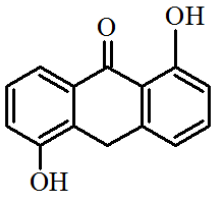
[16]



[17]

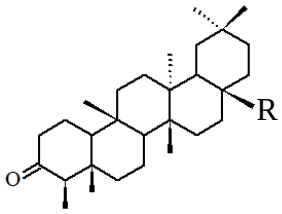


[7]



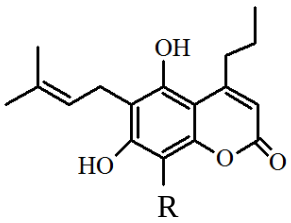
[18]

In the same year, another study on the leaves extract of *Calophyllum brasiliense* was carried out to examine the cytotoxic effects of the isolated compounds. From the hexane extract, two triterpenoids which are friedelin **[7]** and canophyllol **[10],** and eight other mammea type coumarins: mammea A/BA **[19]**, A/BB **[20]**, B/BA **[21]**, B/BB **[22]**, C/OA **[23]**, C/OB **[24]**, B/BA cyclo F **[25]** and B/BB cyclo F **[26]** were isolated. Meanwhile, the acetone extract afforded the biflavonoid amentoflavone **[27]**, isomammeigin **[28]**, protocatechuic acid **[29]** and compounds **18**, **20**, **21**, **22**, **27** and **28**. From the methanol crude extract, shikimic acid **[30]** and compound **7** were isolated. Cytotoxic activity against PC3, K562 and U251 was observed on all the isolated mammea type coumarins with the most active were the mixture of compounds **19** and **20** and mixture of compounds **23** and **24**. Compounds **28** and mixture of compounds **25** and **26** also exhibited the cytotoxic effects against all three cell lines, but with a lower inhibitory values of 38% to 69%. Compounds **7**, **10** and **30** only inhibited against the PC3 and U251. However, compound **29** did not show any cytotoxic effect against all the three cell lines. None of the isolated compounds have the potential to inhibit the activity of HIV-1 reverse transcriptase. Growths of *Staphylococcus aureus, S. epidermidis* and *Bacillus subtilis* were being suppressed by mixture of compounds **19** and **20,** and **23** and **24**, but most of the isolated compounds were unable to suppress the growth of fourteen bacterial strains (Reyes-Chilpa, et al., 2004).



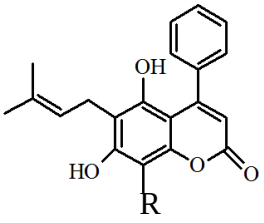
[7] R= Me

[10] R= CH_2OH



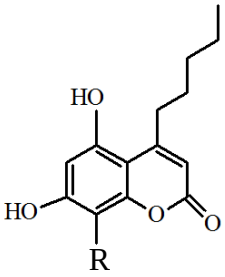
[19] R= 

[20] R= 



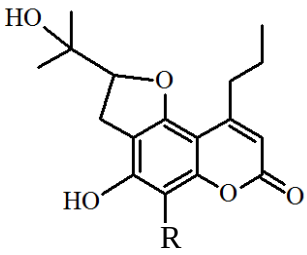
[21] R= 

[22] R= 



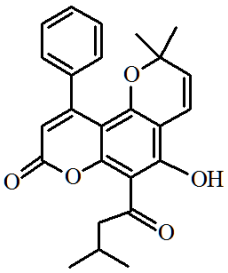
[23] R= 

[24] R= 

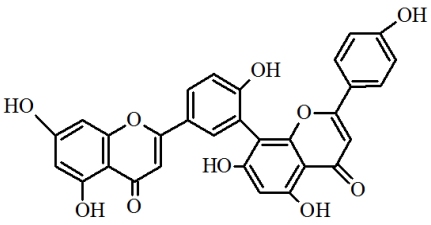


[25] R= 

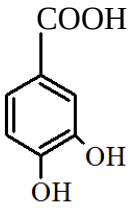
[26] R= 



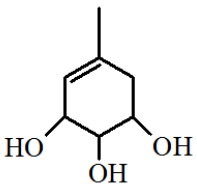
[28]



[27]



[29]



[30]

2.2.2.3 Summary of the Literature Investigation on *Calophyllum* Species

Table 2.1: Summary of the literature investigation on *Calophyllum* species

Plant Species	Types of Compound	Biological Activities	References
C. nodusum	Xanthones Triterpenes	-	• Nasir, et al., 2011
C. canum Hook f.	Xanthones	-	• Carpenter, et al., 1969
C. thorelii	Xanthones Polyisoprenylated acylphloroglu ci-nols	Cytotoxic agents Antioxidant	• Nguyen, et al., 2012 • Nguyen, et al., 2013
C. Caledonicum	Xanthones	Antimalarial Antifungal	• Hay, et al., 2004 • Morel, et al., 2002
C. cordato-oblongurn Thw	Xanthones Triterpenes Resin acids	-	• Gunasekera and Sultanbawa, 1975
C. inophyllum	Xanthones Coumarins Flavanoids Shikimic acids Tritepenes Chromanones	Antimicrobial Cytotoxic agents Anti-HIV-1 Antidyslipidemic Antioxidant Growth inhibitors on human HL- 60 cells Anti-inflammatory Antiarthritic Cancer chemoprevenative agents	• Yimdjo, et al., 2004 • Laure, et al., 2008 • Prasad, et al., 2012 • Li, et al., 2010 • Dweck and Meadows, 2002 • Prabakaran and Britto, 2012
C. flavoramulum	Flavonoids Xanthones Triterpenes	Anti-AGEs activity Radical scavenging potential	• Ferchichi, et al., 2012
C. polyanthum	Coumarins	Antiherpetic acitivity	• Ma, et al., 2004
C. gracilipes	Xanthones Triterpenes	Cytotoxic agents	• Nasir, et al., 2013

Plant Species	Types of Compound	Biological Activities	References
C. brasiliense	Chromanones Coumarins Triterpenes Shikimic acids Xanthones Flavonoids Steroids Tannins	Antiulcer Cytotoxic agents Anti-HIV-1 Antileishmanial Antinociceptive agents Antimicrobial Anti-Helicobacter pylori action Anti-Cancer Antisecretory Cytoprotective Antibacterial	• Lemos, et al., 2012 • Reyes-Chilpa, et al., 2004 • Isaias, et al., 2004 • Pretto, et al., 2004 • Souza, et al., 2009 • Kimura, et al., 2005 • Sartori, et al., 1999 • Ito, et al., 2003 • Cottiqlia, et al., 2004
C. enervosum	Polyisoprenylated ketone Xanthones	Antimaicrobial	• Taher, et al., 2005
C. Dryobalanoies	Chromanone Xanthones Flavonoids	-	• Ha, et al., 2012
C. symingtonianu-m	Xanthones	Antifungal Antioxidant Antimicrobial	• Kawamura, et al., 2012 • Attoumani, Susanti and Taher, 2013
C. cerasiferum	Coumarins	Potent HIV reverse transcriptase inhibitors	• Spino, Dodier and Sotheeswaran, 1998
C. decipiens	Triterpenes Xanthones Chromanones	Radical scavenging activity Antibacterial against *Mycobacterium tuberculosis* H37Rv	• Ajithabai, et al., 2012

Plant Species	Types of Compound	Biological Activities	References
C. membranace-um	Xanthones Triterpnes Flavonoids Chromanones	Cytotoxic agents Anti-inflammatory Anti-tumour	• Chen, et al., 2008 • Zou, et al., 2005 • Chen, et al., 2011
C. soulattri	Xanthones Triterpenes Coumarins	Antimicrobial Cytotoxic agents	• Mah, et al., 2011 • Gwendoline, et al., 2011 • Mah, et al., 2012 • Khan, Kihara and Omoloso, 2002
C. wighfianum	Xanthones Fatty acids	-	• Dean, et al., 1980
C. incrasaptum	Polycyclic aromatic natural products	Antimicrobial	• Abbas, et al., 2007 (a)
C. sundaicum	Polyprenylated Acylphloroglucinol	Activity against glucocorticoid receptor	• Cao, et al., 2006
C. rubiginosum	Flavanoids	Antioxidant Antimicrobial Anti-cancer Cytotoxic agents	• Taher, et al., 2010 • Alkhamaiseh, Taher and Ahmad, 2011 • Alkhamaiseh, et al., 2011
C. sclerophyllu-m	Xanthones	-	• Jackson, Locksley and Scheinmann, 1966
C. teysmannii	Coumarins	HIV inhibitors	• Vital, et al., 1998 • Fuller, et al., 1994
C. thwaitesii	Xanthones Triterpenes	Antibacterial Antifungal Antioxidant	• Napagoda, et al., 2009 • Dahanayake, et al., 1974
C. tetrapterum	Coumarins	Antimicrobial	• Abbas, et al., 2007 (b)

Plant Species	Types of Compound	Biological Activities	References
C. venulosum	Flavonoids	-	• Cao, Sim and Goh, 1997 • Cao, Sim and Goh, 2001
C. scriblitifoliu-m	Xanthones	-	• Jackson, Locksley and Scheinmann, 1967
C. walkeri Wight	Xanthones Triterpenes	-	• Dahanayake, et al., 1974
C. zeylanicum	Xanthones	-	• Gunasekera, Sotheeswaran and Sultanbawa, 1981 • Karunanayake, Sotheeswaran and Sultanbawa, 1979
C. austroindicu-m	Triterpenes Triterpene lactones Apetalic acids	-	• Govindachari, Prakash and Viswanathan, 1968 • Govindachari, Prakash and Viswanathan, 1967
C. trapezifolium Thw.	Xanthones Triterpenes	-	• Somanathan and Sultanbawa, 1974
C. calaba	Xanthones Triterpenes Flavonoids	-	• Kumar, et al., 1982
C. dispar	Coumarins	Cytotoxic agents	• Guilet, et al., 2001
C. panciflorum	Flavonoids	Antitumor promoting activity	• Ito, et al., 1999

Plant Species	Types of Compound	Biological Activities	References
C. lanigerum	Coumarins	Anti-HIV activity	• Mckee, et al., 1996
C. cordato-oblongum	Chromanone acid	-	• Dharmaratne, et al., 1999
C. papuanum	Chromanone acid	-	• Stout, Hickernell and Sears, 1968
C. macrocarpu-m	Neoflavonoids	-	• Ampofo and Waterman, 1985
C. chapelieri	Apetalic acids	-	• Guerreiro, Kunesch and Polonsky, 1971
C. austroindicu-m	Xanthones, coumarins, chromanone acids		• Iinuma, et al.,1996
C. blancoi	Chromanones acids, xanthones	Anticancer	• Stout and Sears, 1968 Shen, et al., 2005
C. bracteatum	Xanthones, triterpenoids, chromanone acids		• Somanathan and Sultanbawa, 1972
C. cuneifolium	Xanthones, chromanone acids, triterpenes		• Gunasekera, et al., 1977
C. fragrans	Xanthones		• Locksley and Murray, 1969
C. macrocarpu-m	Chromanone acids, triterpenoids		• Ampofo and Waterman, 1985
C. mucigerum	Coumarins, xanthones, triterpenes	Anti-luekemic, insecticide	• Ee, et al., 2004
C. scriblitifoliu-m	Xanthones		• Jackson, Locksley and Scheinmann, 1966

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

The plant studied in this project was *Calophyllum castaneum*. The stem bark of *Calophyllum castaneum* was collected from the jungle in Landeh, Sarawak. A voucher specimen of the plant was kept in herbarium at Universiti Teknologi MARA, Sarawak.

3.1.2 Chemicals

The solvents and materials used in the extraction, isolation and purification of chemical constituents from *Calophyllum castaneum* are listed in Table 3.1. The deuterated solvents used in NMR analyses are listed in Table 3.2. The HPLC grade solvents used in LC-MS and materials used in chemical analysis are listed in Table 3.3. The HPLC grade solvents used in GC-MS and materials used in chemical analysis are listed in Table 3.4. Chemical reagents and materials used in antioxidant assay are listed in Table 3.5.

Table 3.1: The solvents and materials used in the extraction, isolation and purification of chemical constituents from *Calophyllum castaneum*

Chemical reagents	Molecular formula	Density, ρ ($g.mL^{-1}$)	Source, Country
n-Hexane	$CH_3(CH_2)_4CH_3$	0.659	Merck, Germany
Dichloromethane	CH_2Cl_2	1.325	Fisher Scientific, UK
Ethyl acetate	$CH_3COOC_2H_5$	0.902	Lab-Scan, Ireland
Acetone	CH_3COCH_3	0.791	QREC, Malaysia
Methanol	CH_3OH	0.791	Mallinckrodit Chemicals, Phillipsburg
Silica gel (60Å)	-	-	a) Silicycle, Canada b) Merck, Germany
Sephadex®LH-20	-	-	New Jersey, United State

Table 3.2: Deuterated solvents used in NMR analyses

Deuterated solvents/ Materials	Source, Country
Acetone-d_6	Acros Organics, Belgium
Deuterated chloroform ($CDCl_3$)	Acros Organics, Belgium
Methanol-d_4	Acros Organics, Belgium

Table 3.3: Solvents and materials used in LC-MS analysis

Solvent/Materials	Molecular formula	Density, ρ ($g.mL^{-1}$)	Source, Country
Acetonitrile	C_2H_3N	41.05	Fisher Scientific, UK
Methanol	CH_3OH	32.04	Fisher Scientific, UK
Nylon syringe filter	-	-	Membrane-Solution, USA

Table 3.4: Solvents used in GC-MS analysis

Solvent/Materials	Molecular formula	Density, ρ ($g.mL^{-1}$)	Source, Country
Methanol	CH_3OH	32.04	Fisher Scientific, UK
Acetonitrile	C_2H_3N	41.05	Fisher Scientific, UK

Table 3.5: List of materials and reagents used in antioxidant assay

Reagents/Materials	Source, Country
96-well plate	Techno Plastic Products AG, Switzerland
Kaempferol	Sigma- Aldrich, USA
Vitamin C	Sigma- Aldrich, USA
1,1-diphenyl-2-picryhydrazyl (DPPH)	Sigma- Aldrich, USA

3.2 Methodology

3.2.1 Sequential Solvent Extraction of Chemical Constituents from *Calophyllum castaneum*

Approximately 2.0 kg of stem bark of *Calophyllum castaneum* was collected, air dried and ground into fine powder. The powdered materials of stem bark were then subjected to sequential solvent extraction by soaking in three different solvents, started with dichloromethane, followed by ethyl acetate and methanol, twice for each solvent, each time for two days at room temperature. For each solvent extraction, the extract was filtered and the filtrate was treated with anhydrous sodium sulphate in order to remove water residue. The filtrate was then concentrated by using rotary evaporator in a water bath at 40 ℃. The dried mass of the dichloromethane, ethyl acetate and methanol extracts were 40.6336 g, 27.8285 g and 41.9768 g, respectively.

3.2.2 Column Chromatography

Column chromatography is a common method used to isolate individual chemical compounds from a mixture of compounds. Column chromatography was performed in glass columns with the packing of solid particles coated with an adsorbed liquid that formed the stationary phase. A chemical solvent that either polar or non-polar that acts as the mobile phase was added through the top of the column and run down the column via gravity or external pressure.

Before stationary phase was introduced into the column, a thin layer of drying agent or sea sand was introduced first to form a layer of column bed to prevent the leakage of the stationary phase. Silicycle or Merck silica gel (40-63 μm) was mixed with hexane to form slurry and then it was poured into a glass chromatographic column. A compact packing of stationary phase in the column is necessary by allowing the slurry to settle down well by tapping the column. The sample was prepared via dry packing method in which the sample was dissolved in a minimum amount of suitable solvent, and was then mixed dropwisely with dry silica gel. The prepared sample was finely-ground and was left to dry at room temperature overnight. The prepared sample was subjected to the column followed by gradient elution in increasing polarity in order to separate the compounds with different polarity. After the sample was introduced, a thin layer of drying agent or sea sand was introduced on the top of packing column as a protection layer. The fractions collected were then concentrated by using rotary evaporator and analysed by using Thin Layer Chromatography (TLC).

Figure 3.1: Column chromatography apparatus

3.2.3 Thin Layer Chromatography (TLC)

Thin layer chromatography is a simple chromatographic technique that can be used to separate a mixture of compounds based on difference in their polarity. This was performed using the Merck brand TLC plate with silica gel coated on aluminium plate in the size of 4 cm x 8 cm. The coated silica gel was the stationary phase while a suitable solvent system was used as the mobile phase. Firstly, the sample was dotted on the marked baseline of the plate by using a thin capillary tube. Next, a developing chamber was filled with a known composition of solvent system. A piece of filter paper was placed in the developing chamber, and then the chamber was covered and left aside for a while in order to ensure the chamber to be saturated with the solvent vapour. The spotted TLC plate was then placed into the chamber to allow the mobile phase to move up on the plate via capillary action. The strength of adsorptivity of the compounds with the stationary phase and their degree of solubility in the mobile phase would influence their rate of migration on the TLC plate. The solvent molecules in the mobile phase will compete with solute molecules for the sites on the adsorbent. Hence, an appropriate solvent system should be used to develop the TLC. The TLC plate was then taken out from the developing chamber when the solvent reached the solvent front line and the plate was visualized under UV light and in an iodine vapour chamber. The R_f value of each separated compound was calculated by using the following equation:

$$R_f = \frac{\text{Distance traveled by the compound (cm)}}{\text{Distance of the solvent front (cm)}}$$

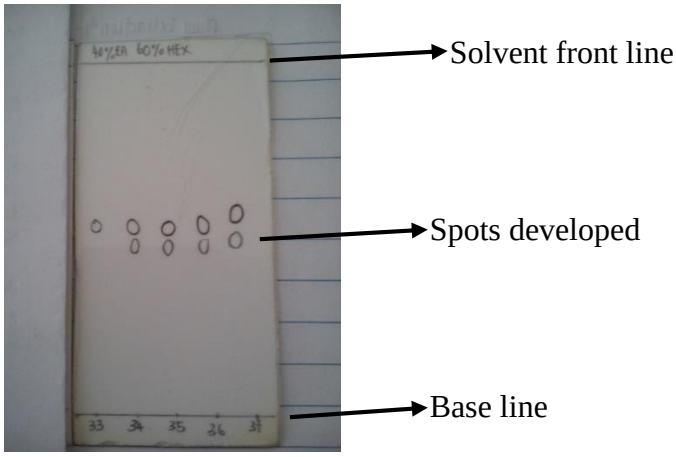


Figure 3.2: The developed TLC plate

3.2.4 Detection Method for TLC

3.2.4.1 UV Detection Method

The developed TLC plates were visualised under UV light with both the short wavelength (254 nm) and long wavelength (365 nm). The dark spots and colour fluorescence spots that were detected under the UV long and short wavelengths, respectively were circled and labelled for their physical appearances.

3.2.4.2 Iodine Vapour Detection Method

An iodine vapour chamber was prepared by weighing 1.5 g of iodine crystals into a covered glass container and the chamber was allowed to be saturated with iodine vapour. In order to detect non-conjugated compounds or terpenoids that cannot be detected under UV light, the developed TLC plates were placed into the iodine vapour chamber for few minutes. Distinct dark yellow spots that appeared on the plate indicated the presence of non-conjugated compounds or

terpenoids, and the spots were circled and labelled. The marking action was done immediately as the iodine spots might dissipate over time after took out from the chamber.

3.2.4.3 Iron (III) Chloride Solution

The iron (III) chloride solution was prepared by dissolving 1.5 g of iron (III) chloride in 100 mL of methanol. The developed TLC plate was sprayed with the iron (II) chloride solution to give coloured complexes. Compounds like phenols gave a dark blue or greenish spot while the hydroxamic acids gave a red spot on the TLC plate.

3.2.5 Gel Permeation Chromatography

Gel permeation chromatography is a type of size exclusion chromatography with separation of compounds based on their sizes when passing through the packed column. This type of chromatographic method employs beads of porous polymeric material as the stationary phase and organic solvent as the mobile phase. Different sizes of molecules are flown through the column at different rates. The porous polymeric materials which acts as stationary phase are designed to have pores of different sizes. Smaller molecules tend to elute slower than larger molecules because they enter into the pores which larger molecules cannot. Larger molecules thus have less overall volume to traverse along the column and they are eluted first compared to smaller molecules. By applying this chromatographic method, individual compounds of different sizes in a mixtures can be separated easily. The packing material that is used in the gel

permeation chromatography is Sephadex. The name Sephadex is came from the sentence of 'SEparation PHArmacia DEXtran'. It is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. There are four grades for Sephadex, which includes superfine grade, fine grade, coarse and medium grade and coarse grade. Different grades of Sephadex are suitable for different purposes. Irreversibly damaged of the bed structure of Sephadex will occur upon freezing and melting of Sephadex when heating above 40 ℃. The important characteristics of Sephadex are wide selection of exclusion limits, high-recovery desalting and buffer exchange, prepacked in a range of formats and well-proven. In this study, the mobile phase used was a solvent mixture of 10% dichloromethane and 90% methanol with Sephadex®LH-20 employed as stationary phase.

3.3 Instruments

3.3.1 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) is a spectroscopic method that studies the magnetic properties of nucleus of an atom. As each nucleus of an atom has different surrounding environment, they are going to experience different magnetic field from their neighbours and produce distinctive signals that help in the elucidation of chemical structure of molecule. In this project, all of the samples were dissolved in appropriate deuterated-solvents, such as deuterated-chloroform, acetone and methanol according to the extent of solubility of the samples in the solvent. Dissolved samples were filled into the NMR tubes up to

a height of approximately 4 cm and were then labelled, capped and wrapped with parafilm in order to prevent solvent evaporation. All of the prepared samples were run by JEOL JNM-ECX 400MHz NMR spectrometer with the use of trimethylsilane (TMS) as internal standard and reference to obtain ^{1}H-NMR, ^{13}C-NMR, HMQC (Heteronuclear Multiple Quantum Coherence), and HMBC (Heteronuclear Multiple Bond Coherence) spectra for structural elucidation of compounds.

3.3.2 Infrared Spectrophotometer (IR)

Bonds of all atoms in molecules are in vibrational mode, Infrared spectroscopy is used to study different vibrational mode of bonds in the molecule. Sample tend to absorb infrared radiation when the frequency of bond vibration of sample matches the frequency of infrared radiation directed on it. IR spectrum provides valuable information about specific functional group that are present in the analysed sample which may help in the elucidation and identification of molecular structure. In this project, sample was prepared in sample pellet by grinding a small amount of sample with potassium bromide, KBr powder in a ratio of 1: 10 followed by compression under high pressure. Perkin Elmer 2000-Fourier transform infrared (FTIR) spectrometer was used in this study to obtain IR spectra in the range of 4000 to 400 cm^{-1}.

3.3.3 Ultraviolet-Visible Spectrophotometer (UV-Vis)

Ultraviolet-Visible spectrophotometer is the absorption spectroscopy that uses visible and/or ultraviolet light source to provide qualitative information about

highly conjugated organic compounds. Highly conjugated organic compounds absorb light in the ultraviolet or visible region which induce electronic transition of electron from a lower energy state to a higher energy state. As different compounds show different characteristic value in absorbance, absorption maxima and wavelength of maximum absorbance, informations on the structure of a compound can be obtained. All samples were prepared by dissolving a small amount of sample in ethanol. In this project, Perkin-Elmer Lambda (25/35/45) UV-Vis spectrophotometer was used for sample analysis with a solvent blank prepared in 98% absolute ethanol.

3.3.4 Mass Spectrometry

Mass spectrometry is a powerful technique used for identification of unknown compounds and studying of their molecular structures. Mass spectrometry converts the sample molecules into charge particles and measures the molecular masses of individual compounds and atoms precisely. Generally, there are three steps in mass spectrometry technique, which are ionization of sample molecules into charged ions, separation and mass analysis of the molecular ions and their charged fragments and measurement of intensity of ion current formed from the mass-separated ions which is display in the mass spectrum.

3.3.4.1 Gas Chromatography-Mass Spectrometry (GC-MS)

GC technique allows the separation of individual compounds from a complex mixture. When gas chromatography is coupled with mass spectrometry, sample consisting of a mixture of compounds is first running through the GC column

to effect separation of compound before they are directed to the mass spectrometer, where ionisation, fragmentation and mass detection take place. In this project, Shimadzu GC-MS-QP2010 Plus model was used for providing mass data for sample analyses.

3.3.4.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

This is a coupled technique in which the liquid chromatography is paired with mass spectrometry for the structure elucidation of unknown compounds. In this coupled technique, sample containing a mixture of compounds is first running through the HPLC column for separation and then directed into the mass spectrometer for structural analysis. Based on the solubility of the samples, the samples were prepared by dissolving them either in HPLC grade methanol or acetonitrile to a concentration of below 100 ppm. Before injected into the LCMS column, the prepared samples were filtered to remove any undissolved solids or impurities by using the nylon syringe filter (pore size = 0.22 μm). A soft ionization method which is electrospray ionization (ESI) was used to determine the molecular weight of the separated compounds with less fragmentation occurred to the molecular ion formed. In this study, high resolution mass spectra were obtained by using G6520B Q-TOF LC/MS spectrometer.

3.3.5 Melting Point Apparatus

Melting point is a narrow range of temperature measured when compound changes from solid state into liquid state. Pure crystalline compounds have clear and sharp defined melting point, so the enlargement of the melting point value

indicates the presence of impurities inside the compounds. Hence, the purity of a compound can be determined by measuring its melting point value and compare with the value of pure compound. The solid form sample was introduced into haematocrit capillary and the filled capillary was inserted into the melting point apparatus. The temperature range in which the solid start to melt and completely melted was recorded. In this study, the melting point measurement was carried out by using Barnstead Electrothermal 9100 melting point apparatus.

3.3.6 Polarimeter

Optical activity is a physical property, and a compound is considered to be optically active if linearly polarized light is rotated clockwise (+) or counterclockwise (-) when passing through it. Molecular structure and concentration of chiral molecules in the substance determine the amount of optical rotation. In this study, the polarization measurement was carried out by using Jasco Europe P-2000 digital polarimeter.

3.3.7 Antioxidant Assay

In this study, antioxidant assay was carried out to investigate the antioxidant activities of the crude extracts and isolated compounds. Firstly, crude extracts, isolated compounds and standard compounds (Vitamin C and Kaempferol) were dissolved in methanol for preparation of master stock at concentration of 1 mg/mL. The master stocks were sonicated for 5 minutes for the samples to be fully dissolved. The DPPH solution was prepared by dissolving DPPH powder

in methanol to obtain concentration of 2 mg/mL. The DPPH solution was sonicated for 5 minutes for the powder to be fully dissolved. All the prepared solutions were then stored in a 4 ℃ chiller in a dark condition.

Crude extracts, isolated compounds and standard compounds for the assay were then prepared at different concentration of 240, 120, 60, 30, 15, 7.5 and 3.75 μg/mL in methanol through serial dilution of their master stocks. All the prepared solutions were then transferred into 96-well plate and 5μL of DPPH solution was added. Negative control was prepared to contain only the DPPH and methanol. All samples were run in triplicate and averaged for their results.

Immediately after the addition of reagents, the plate was wrapped with aluminium foil to avoid evaporation of solvent and was stored in dark at room temperature for 30 minutes. In this study, microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the absorbance of the mixtures in each well at 520 nm. The results were then interpreted by using the Microplate Manager®, Version 5.2.1 software. Inhibition rates of the compounds were calculated and a graph of inhibition rate against concentration of the samples was plotted to determine the IC_{50} value of the test compounds.

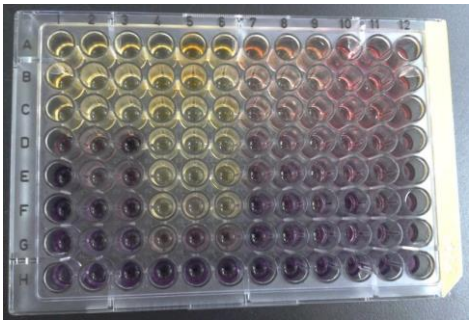


Figure 3.3: DPPH antioxidant assay using 96-well plate

3.4 Calculation

3.4.1 Inhibition Rate

Inhibition rate of the test compounds was calculated by using the formula below:

$$\text{Inhibition rate (\%)} = \frac{A_o - A_1}{A_o} \; x \; 100\%$$

Where A_o = Absorbance of the negative control (blank)

A_1 = Absorbance of the test compound

In order to obtain IC_{50}, a graph of inhibition rate against the sample concentration was plotted. IC_{50} is the concentration of sample necessary to cause 50% inhibition to the DPPH radical scavenging activity.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Compounds Isolated from *Calophyllum castaneum*

The stem bark of *Calophyllum castaneum* was extracted via sequential extraction using dichloromethane, ethyl acetate and methanol. A dichloromethane crude extract of 40.6336 g was obtained and about 35.0000 g of it was subjected to gravity column chromatography packed with silica gel as the stationary phase, eluted with a series of solvent mixtures of increasing polarity (hexane/dichloromethane, dichloromethane/ethyl acetate, ethyl acetate/acetone and acetone/methanol) as the mobile phase. This column afforded a total of 37 fractions, CCF (F1-F37).

Fractions 15 and 16 (CCF) were combined and subjected to a 4.0 cm diameter chromatographic column packed with silica gel and eluted with two different solvent mixtures of increasing polarity (hexane/ethyl acetate and dichloromethane and acetone) as the mobile phase. This yielded a total of 48 fractions, CCG (F1-F48). Subfractions 4 and 5 were combined and washed with analytical grade methanol to give white needle-like crystals, which is friedelin **[18]**. Same method as mentioned above was applied on the subfraction 11 which afforded another white needle-like crystals, friedelinol **[33]**.

Besides that, fraction 21 (CCF) was further purified by subjecting to a 4.0 cm diameter chromatography column packed with silica gel and eluted with a series

of solvent mixtures of increasing polarity (hexane/ethyl acetate, dichloromethane/acetone and acetone/methanol) as the mobile phase. This column afforded a total of 41 subfractions, CCH (F1-F41). Subfractions 16 and 17 were combined and subjected to a 2.5 cm diameter column chromatography eluted with similar solvent mixtures as above to give total of 32 subfractions, CCI (F1-F32). Subfractions 10-13 were further purified by gel permeation chromatography packed with sephadex®LH-20 and eluted with a solvent mixture of 10% dichloromethane and 90% methanol to give 52 subfractions, CCM (F1-F52). Subfractions 23-32 gave yellow-needle like crystals and was identified as euxanthone **[34]**.

In addition, subfractions CCH (F18-20) were further subjected to a 2.5 cm diameter chromatography column packed with silica gel and eluted with solvent mixtures of hexane/ethyl acetate and ethyl acetate/acetone to afford 33 subfractions, CCK (F1-F33). Subfractions 18 and 19 gave yellow gummy resins which is blancoic acid **[36]**. Apart from that, subfractions CCH (F25-F33) were further purified by using gel permeation chromatography with sephadex®LH-20 as the packing materials and eluted with a solvent mixture of 10% dichloromethane and 90% methanol to afford 63 subfractions, CCL (F1-F63). Subfractions 22-51 which are yellow gummy resins afforded isoblancoic acid **[35]**. All the structures of the isolated compounds were elucidated through GCMS and LCMS, 1D-NMR, 2D-NMR, IR and UV-Vis analyses.

Figure 4.1 shows the pathway of isolation of friedelin **[18]**, friedelinol **[33]**, euxanthone **[34]**, isoblancoic acid **[35]** and blancoic acid **[36]**.

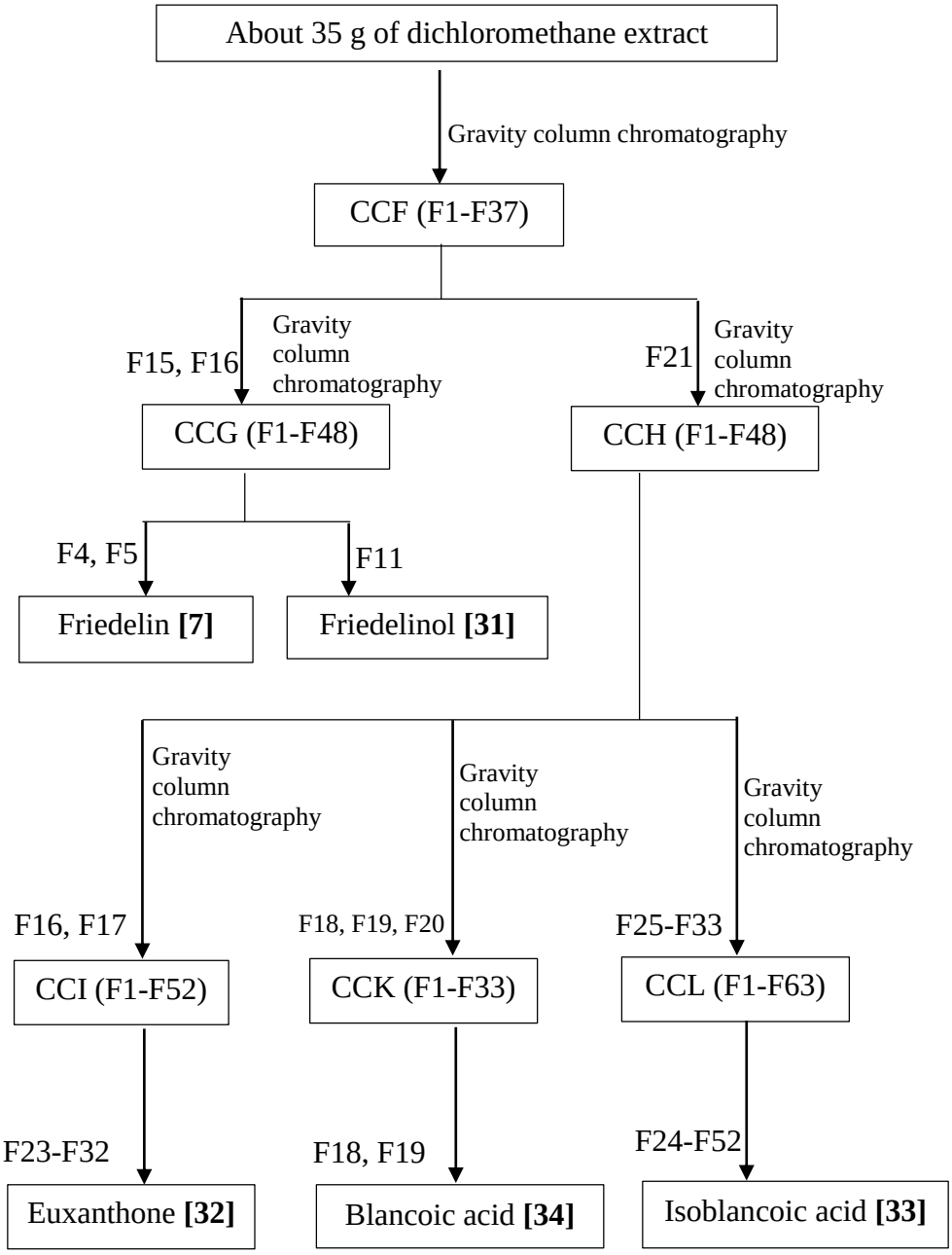
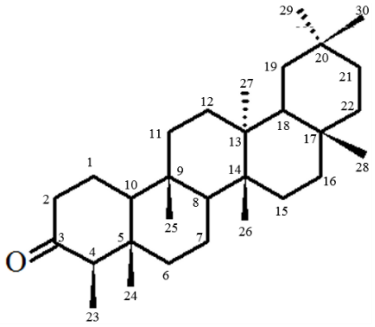


Figure 4.1: The pathway of isolation of friedelin [7], friedelinol [31], euxanthone [32], isoblancoic acid [33] and blancoic acid [34]

4.1.1 Characterisation and Structural Elucidation of Friedelin [7]



[7]

Figure 4.2: Structure of friedelin [7]

Compound **7** is a common triterpenoid. It was isolated from dichloromethane extract of *Calophyllum castaneum* as white-needle-like crystals with a melting point of 258-259 ^{o}C (Lit. 261-262 ^{o}C, Subhadhirasakul and Pechpongs, 2005). Compound **7** has a molecular formula of $C_{30}H_{50}O$ in corresponding to the molecular weight of 426.17 g/mol. Compound **7** was developed on TLC plate by using a solvent system of 10% ethyl acetate and 90% hexane. Figure 4.2 below shows the developed TLC plate for compound **7**.

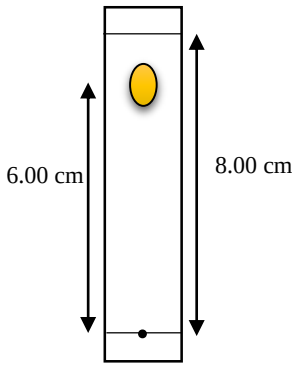


Figure 4.3: The developed TLC plate for friedelin [7]

Compound **7** gave a dark spot on TLC when exposed under UV light and iodine vapour chamber. Compound **7** gave R_f value of 0.75 with the use of a relatively non-polar solvent system. This means that this compound is a low polarity compound.

There were a total of seven methyl singlets observed at δ 0.70 (H-24), 0.84 (H-25), 0.93 (H-26), 1.03 (H-27), 1.16 (H-28) and 0.98 (H-29 & H-30) in the ^{1}H NMR spectrum (Figure 4.4 and 4.5). Besides, a doublet signal of methyl was found at δ 0.86 (*J*=6.1 Hz, H-23), a mutiplet signal of methine proton was found at δ 2.26 (H-4) and two groups of methylene protons gave signals at δ 1.95 &1.72 (m, H-1_a &H-1_b) and 2.37 (dd, *J*=11.3Hz & 5.4 Hz, H-2_a) & 2.26 (m, H-2_b). There was no vinylic proton signal observed and the remaining 17 protons signals were observed in the range of δ 1.2-2.00, which in a highly shielded region.

In the ^{13}C-NMR spectrum (Figure 4.6 and 4.7), a total of 30 carbons were observed. Among the 30 carbons, a keto carbon signal was observed at δ 213.4 (C-3) which was highly deshielded. There were a total of eight methyl groups as revealed by carbon signals at δ 6.9 (C-23), 14.7 (C-24), 18.0 (C-25), 20.3 (C-26), 18.8 (C-27), 32.1 (C-28), and 35.1 (C-29) and 31.9 (C30). Meanwhile, six quaternary carbon signals were observed at δ 42.2 (C-5), 37.5 (C-9), 28.3 (C-20), 20.3 (C-26), 18.8 (C-27) and 32.1 (C-28). The rest of the carbon signals were found at δ 22.3 (C-1), 41.6 (C-2), 58.3 (C-4), 41.4 (C-6), 18.3 (C-7), 53.2 (C-8), 59.5 (C-10), 35.7 (C-11), 30.6 (C-12), 39.8 (C-13), 38.4 (C-14), 32.8 (C-15), 36.1 (C-16), 30.1 (C-17), 42.8 (C-18), 28.3 (C-19), 32.5 (C-21) and 39.3

(C-22). Both ^{1}H-NMR and ^{13}C-NMR data of compound **7** were found to match closely with those of literature values reported for friedelin **[7]** (Abbas, et al., 2007). Table 4.1 shows the summary of NMR data for compound **7** in comparison with the literature values of friedelin.

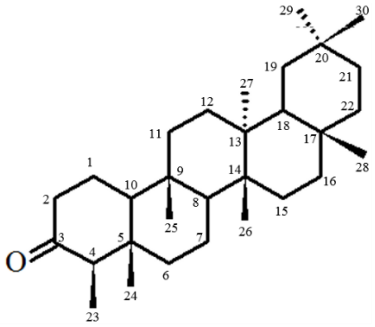
IR and UV-Vis spectroscopies were also used to confirm the structure of compound **7**. From the IR spectrum (Figure 4.8), an intense peak was observed at 1715 cm^{-1} indicating the presence of a carbonyl group. In addition, the presence of sp^3 C-H stretching and C-H bending were revealed by absorption band at 2925 and 1457 cm^{-1}, respectively. Apart from that, the UV-Vis spectrum (Figure 4.9) indicated a maximum absorbance at 206.05 nm. Hence, it can be further confirmed that compound **7** does not have a conjugated double bond structure.

Table 4.1: Summary of NMR data for compound 7 in comparison with literature values of friedelin

Position	δ_H (ppm)	δ_C (ppm)	*δ_H (ppm)	*δ_C (ppm)
1	1.95 ($1H_a$, m) 1.72 ($1H_b$, m)	22.3	1.71 (m) 1.97 (m)	22.3
2	2.37 ($2H_a$, dd, *J*= 11.3Hz, 5.4Hz) 2.26 ($2H_b$, m)	41.6	2.41 (dd, *J*= 13Hz, 3.5Hz) 2.31 (m)	41.5
3	-	213.4	-	213.2
4	2.26 (1H, m)	58.3	2.28 (m)	58.2
5	-	42.2	-	42.2
6	-	41.4	-	41.3
7	-	18.3	-	18.2
8	-	53.2	-	53.1

Position	δ_H (ppm)	δ_C (ppm)	*δ_H (ppm)	*δ_C (ppm)
9	-	37.5	-	37.4
10	-	59.5	-	59.5
11	-	35.7	-	35.6
12	-	30.6	-	30.5
13	-	39.8	-	39.7
14	-	38.4	-	38.3
15	-	32.8	-	32.4
16	-	36.1	-	36.0
17	-	30.1	-	30.0
18	-	42.8	-	42.8
19	-	35.4	-	35.3
20	-	28.3	-	28.2
21	-	32.5	-	32.8
22	-	39.3	-	39.3
23	0.86 （3H, d, *J*=6.1Hz)	6.9	0.92 (d, *J*=7.0 Hz)	6.8
24	0.70 (3H,s)	14.7	0.75 (s)	14.7
25	0.84 (3H,s)	18.0	0.90 (s)	18.0
26	0.93 (3H,s)	20.3	1.03 (s)	20.3
27	1.03 (3H,s)	18.8	1.07 (s)	18.7
28	1.16 (3H,s)	32.1	1.20 (s)	32.1
29	0.98 (3H,s)	35.1	0.98 (s)	35.0
30	0.98 (3H,s)	31.9	1.02 (s)	31.8

*Abbas, et al., 2007



[7]

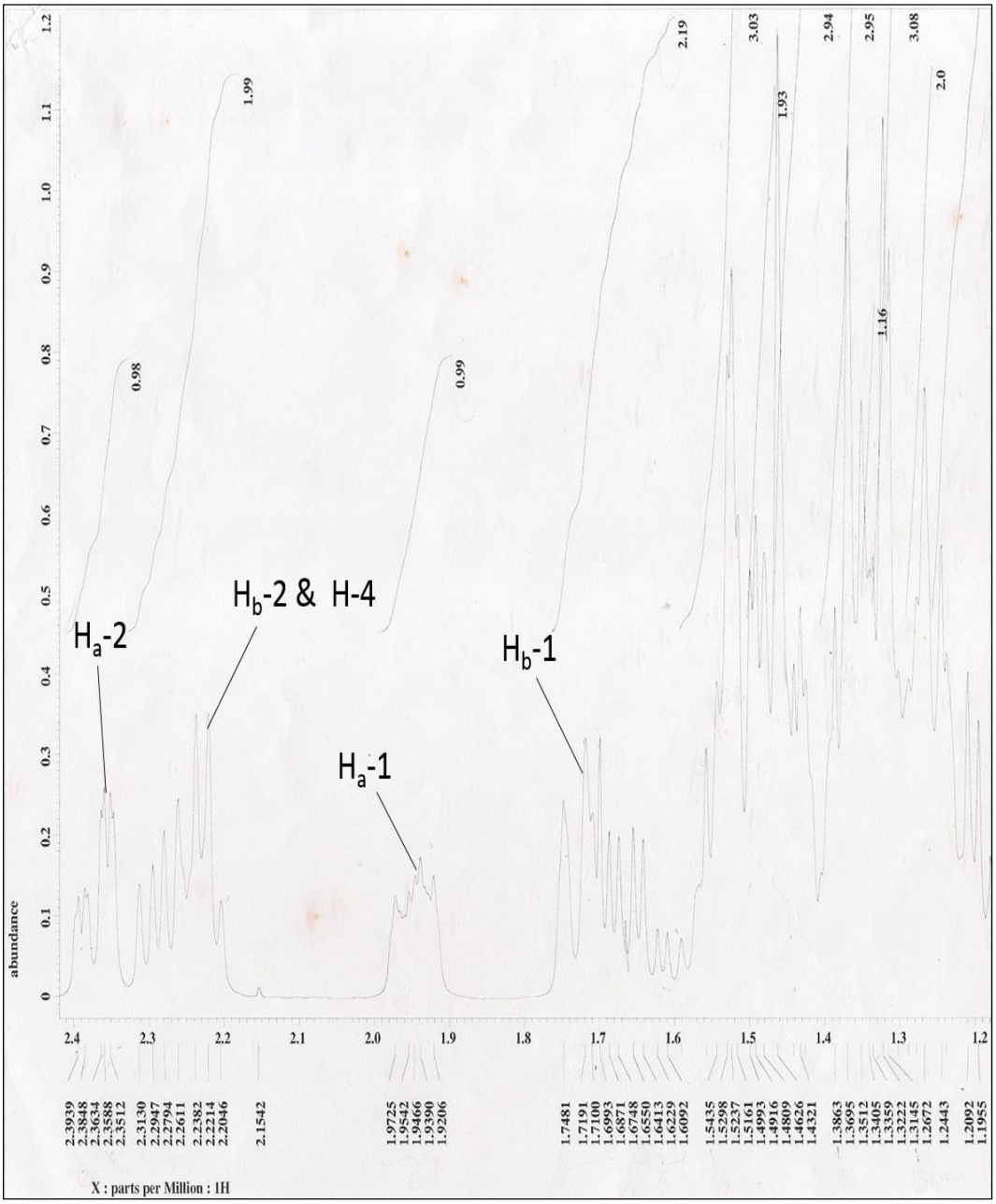
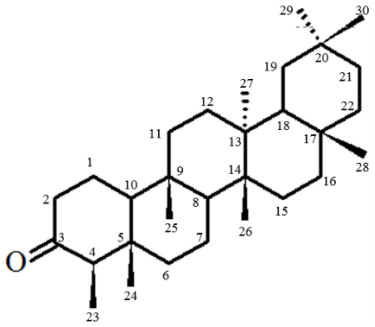


Figure 4.4: ^{1}H-NMR spectrum of friedelin [7] (400 MHz, $CDCl_3$) (expanded)



[7]

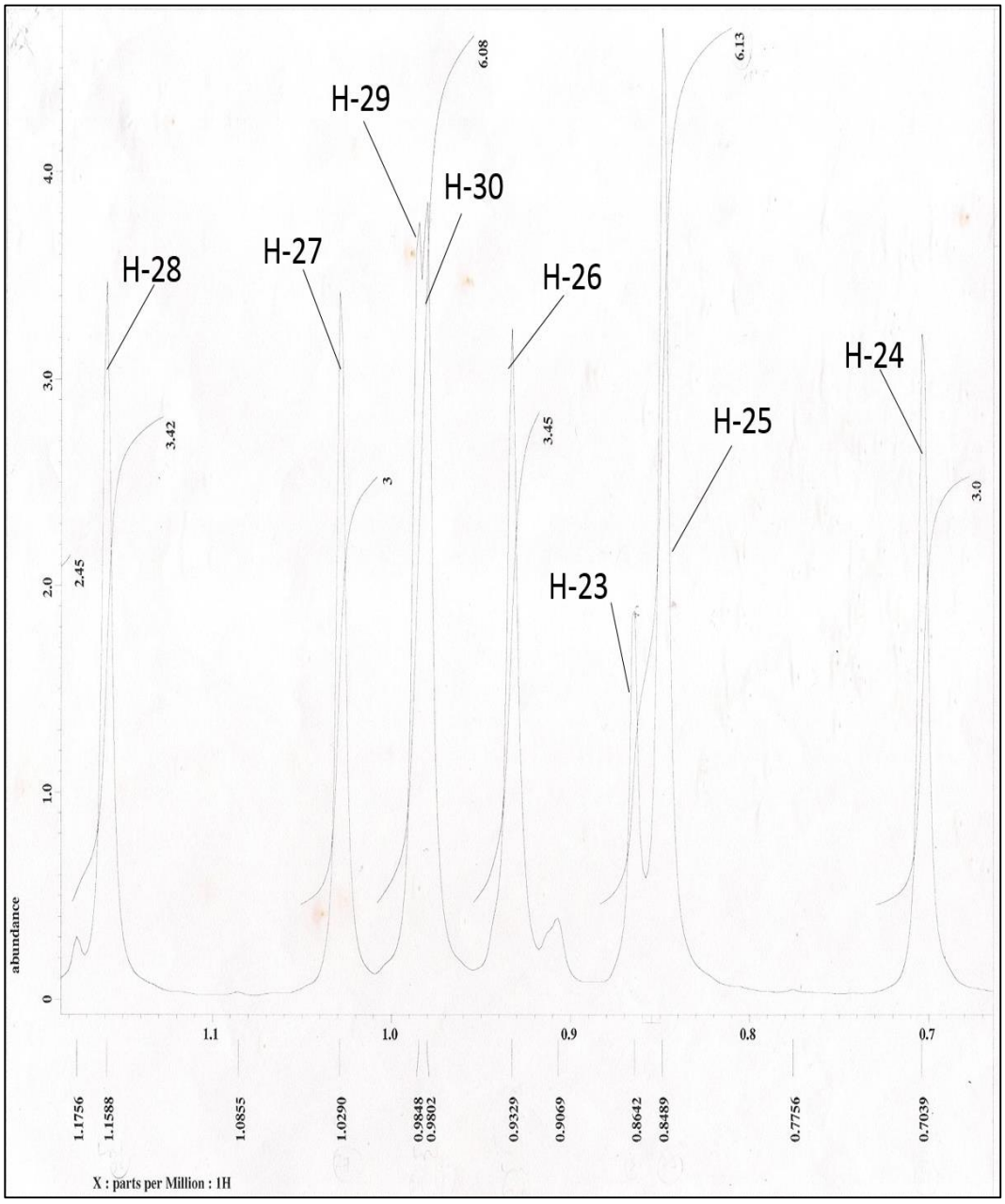
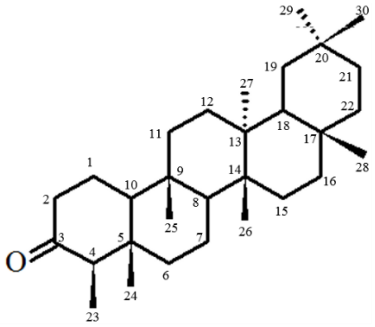


Figure 4.5: ^{1}H-NMR spectrum of friedelin [7] (400 MHz, $CDCl_3$) (expanded)



[7]

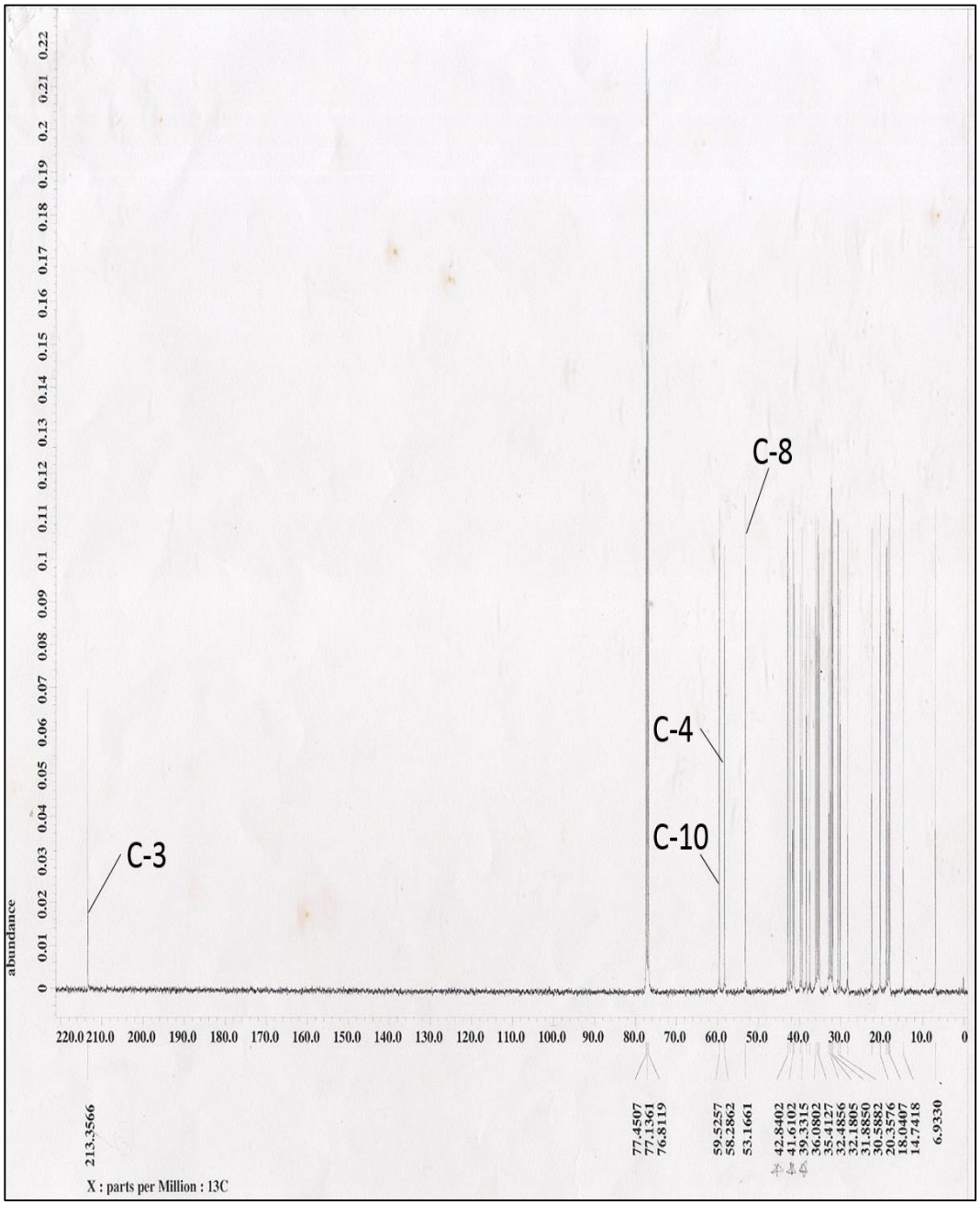
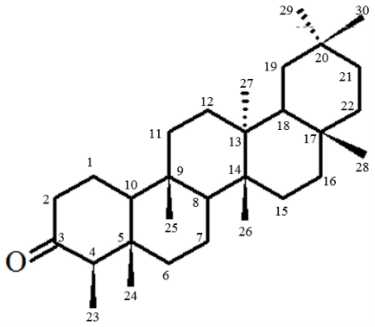


Figure 4.6: ^{13}C-NMR spectrum of friedelin [7] (400 MHz, $CDCl_3$)



[7]

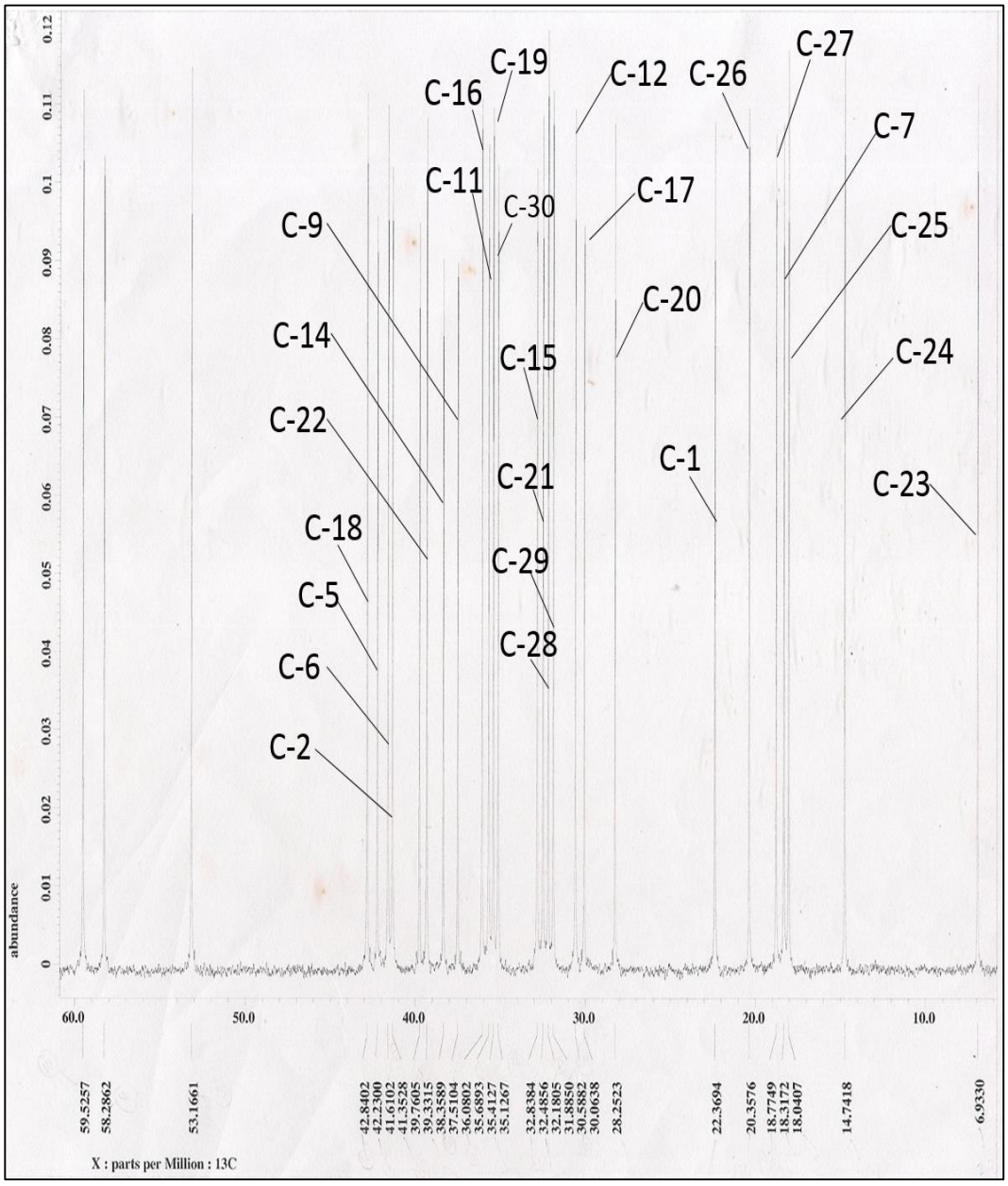
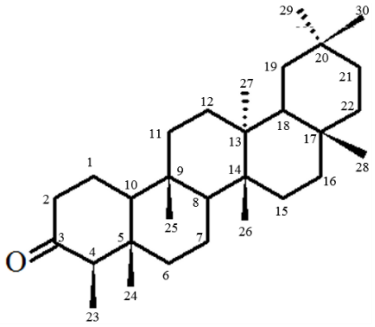


Figure 4.7: ^{13}C-NMR spectrum of friedelin [7] (100 MHz, $CDCl_3$) (expanded)



[7]

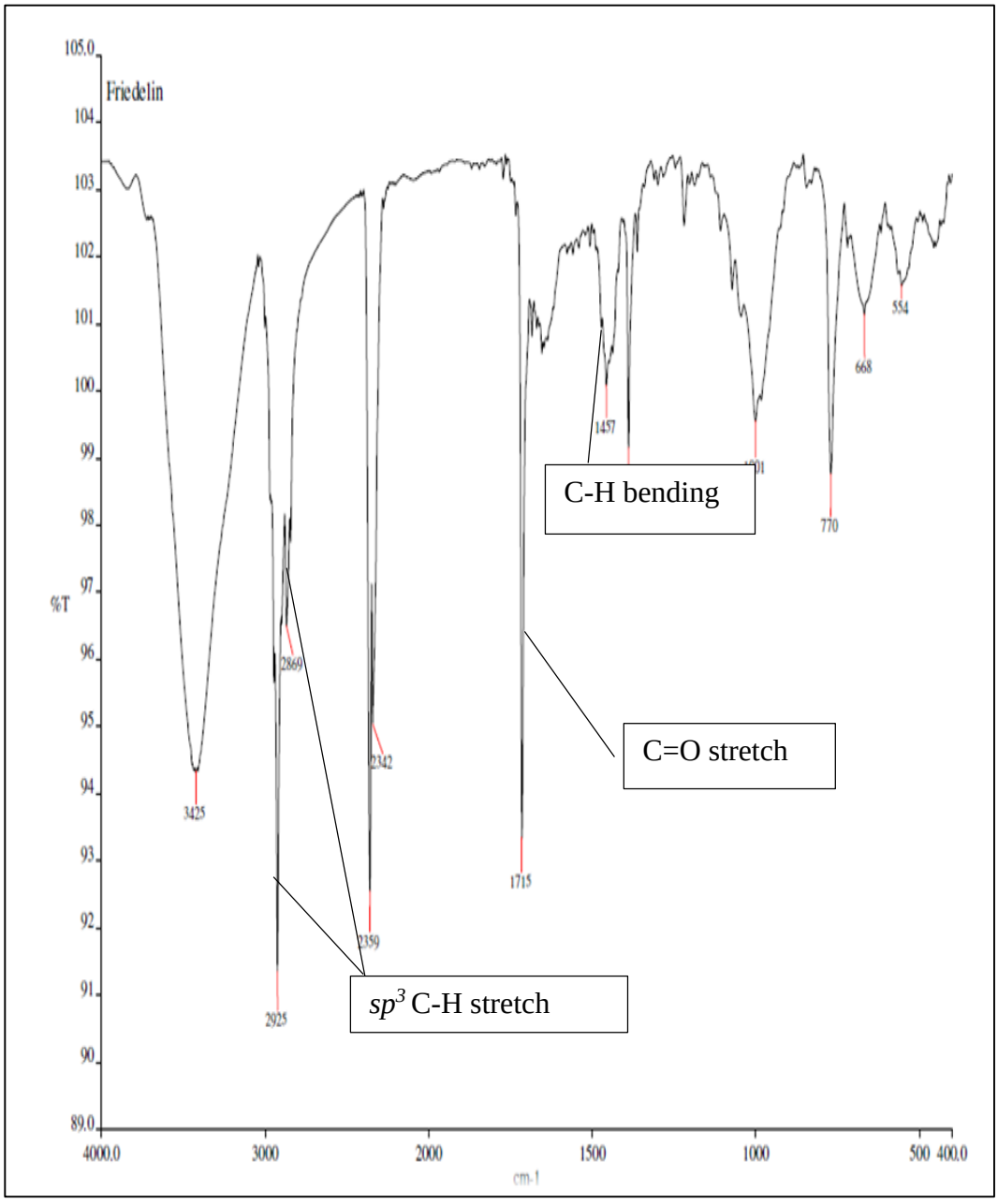
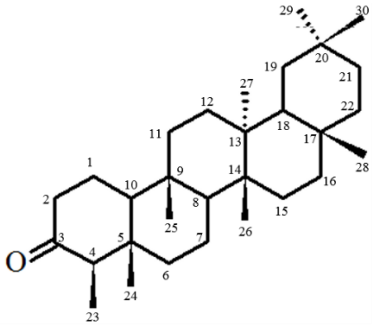


Figure 4.8: IR spectrum of friedelin [7]



[7]

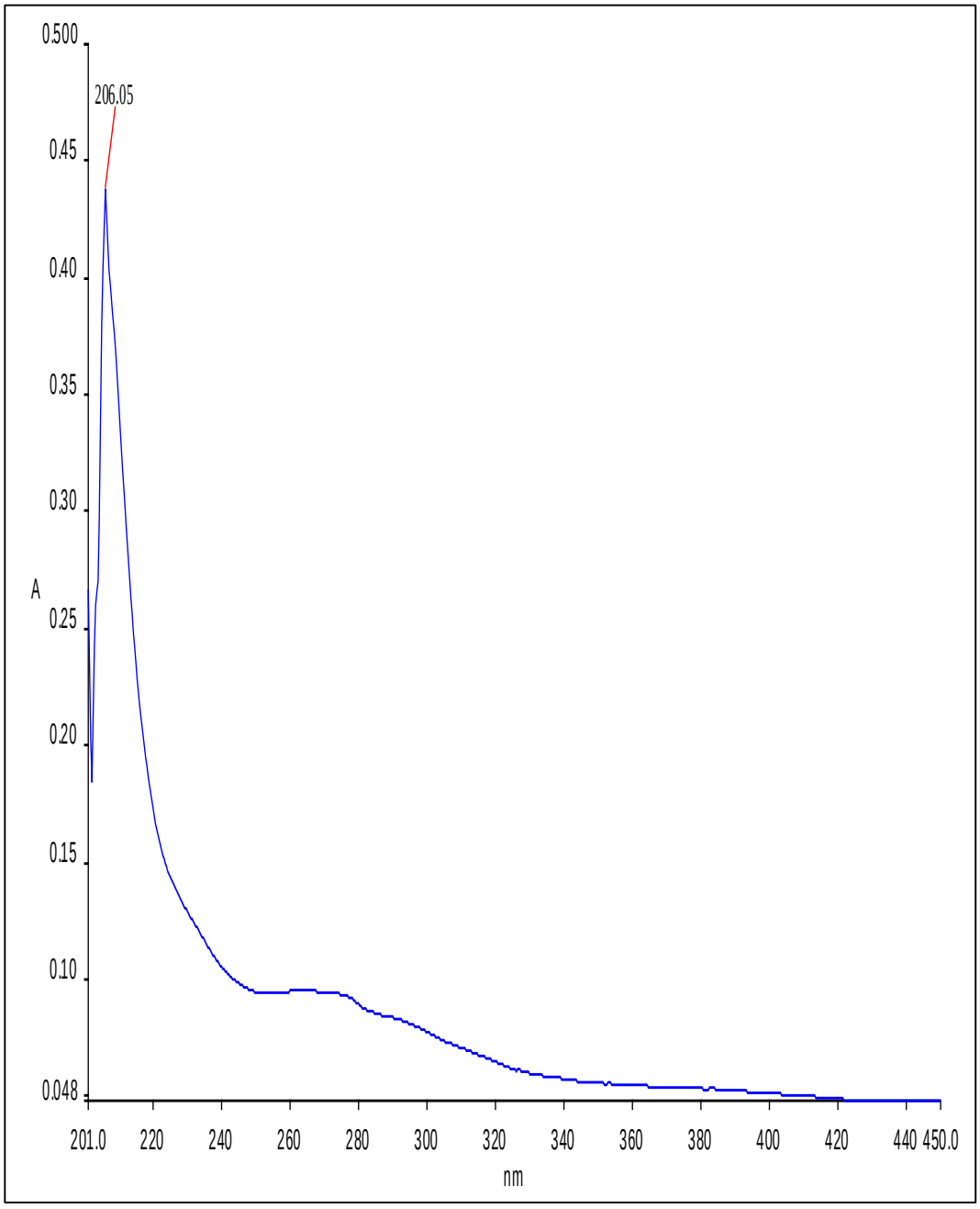
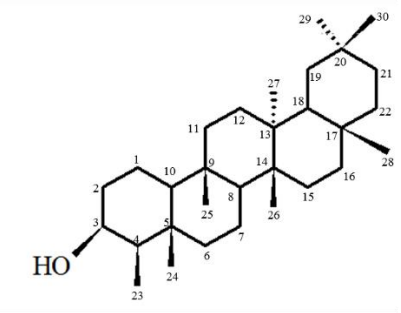


Figure 4.9: UV-Vis spectrum of friedelin [7]

4.1.2 Characterisation and Structural Elucidation of Friedelinol [31]



[31]

Figure 4.10: Structure of friedelinol [31]

Compound **31** was isolated as white needle-like crystals and identified as friedelinol, a triterpenoid. It has a melting point of 278- 279 ^{o}C (Lit. 280-282 ^{o}C, Queiroga, et al., 2000) and the mass spectrum (Figure 4.11) showed a molecular ion, M^{+} at m/z 428.0000 g/mol corresponding to the molecular formula of $C_{30}H_{50}O$. The developed TLC plate for compound **31** is shown in Figure 4.12 below.

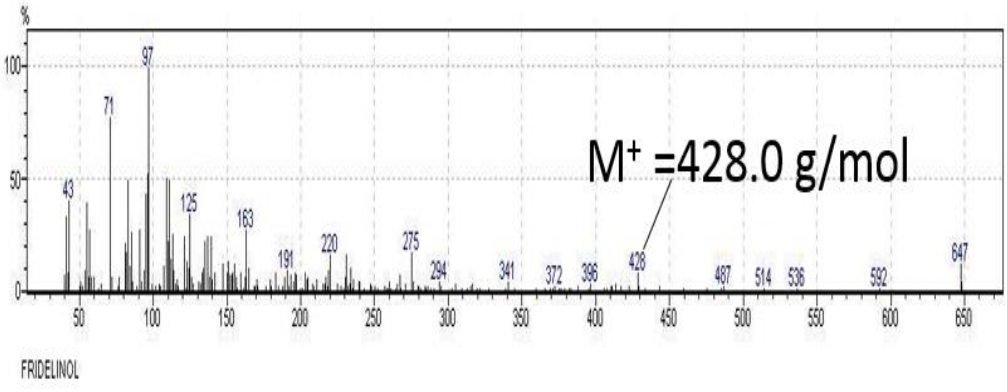


Figure 4.11: Mass spectrum of friedelinol [31]

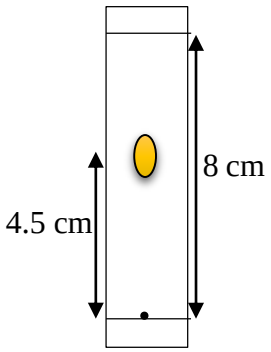


Figure 4.12: The developed TLC plate for friedelinol [31]

In TLC analysis, compound **31** gave a R_f value of 0.56 when developed using a solvent system of 10 % ethyl acetate and 90 % hexane. Developed TLC was later exposed under UV light as well as in iodine vapour to give a dark spot.

The ^{1}H-NMR spectrum of friedelinol (Figure 4.13 and 4.14) exhibited a total of eight methyl protons signals at δ 0.94 (H-23), 0.94 (H-24), 0.85 (H-25), 0.99 (H-26), 0.99 (H-27) and 1.16 (H-28), 1.00 (H-29) and 0.95 (H-30). The deshielded proton signal at 3.73 ppm was assigned to oxymethine proton, H-3 which was directly attached to a hydroxyl group. There were a doublet of triple signals observed at δ 1.90 (dt, *J*=9.2 Hz, 2.1 Hz) assigned to proton H-2 and a multiplet signal at 1.73 ppm assigned to proton H-6. The remaining proton signals were assigned to methylene protons, found in the range of δ 0.88-1.55.

^{13}C-NMR spectrum (Figure 4.15) indicated a total of 30 carbon signals revealing the presence of 30 carbons in the assigned compound. From these 30 carbons, there were eight methyl carbons which gave signals at δ 11.7 (C-23), 16.5 (C-24), 18.3 (C-25), 18.7 (C-26), 20.3 (C-27), 32.4 (C-28), 35.1 (C-29) and 31.9 (C-30). In addition, oxymethine carbon, C-3 gave a relatively deshielded signal at 72.8 ppm. The rest of the carbon signals were observed at δ 15.8 (C-

1), 35.4 (C-2), 49.2 (C-4), 37.2 (C-5), 41.8 (C-6), 17.6 (C-7), 53.3 (C-8), 37.9 (C-9), 61.4 (C-10), 35.3 (C-11), 30.7 (C-12), 38.4 (C-13), 39.4 (C-14), 32.9 (C-15), 36.2 (C-16), 30.1 (C-17), 42.8 (C-18), 35.6 (C-19), 28.3 (C-20), 32.2 (C-21) and 39.8 (C-22). Both the ^{1}H- and ^{13}C- NMR data of compound **31** were resembled to the literature values reported for friedelinol (Utami, et al., 2013). Table 4.2 shows the summary of NMR data for compound **31** in comparison with the literature values of friedelinol.

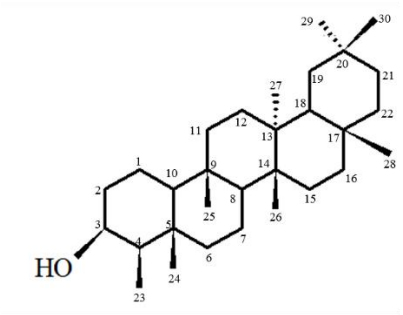
Apart from NMR data, verification of the structure of compound **31** was further supported by IR and UV-Vis analyses. The IR spectrum (Figure 4.16) showed an intense and broad peak of O-H stretch at 3429 $cm^{-1}$and a C-O stretch at 1009 cm^{-1}. These two bands further confirmed the presence of the O-H functional group in friedelinol. sp^3 C-H stretches at 2924 and 2869 cm^{-1} and a CH_3 bending at 1446 cm^{-1} were also observed in the spectrum. The UV-Vis spectrum (Figure 4.17) exhibited a maximum absorbance at 206.18 nm, supported that compound **31** was a non-conjugated compound.

Table 4.2: Summary of NMR data for compound 31 in comparison with literature values of friedelinol

Position	δ_H (ppm)	δ_C (ppm)	*δ_H (ppm)	*δ_C (ppm)
1	-	15.8	-	15.8
2	1.9 (1H_a, dt, *J*=9.2, 2.1 Hz) 1.59 (1H_b, m)	35.4	1.90 (dt, *J*= 10.1, 3.0 Hz) 1.57 (m)	35.3
3	3.73 (1H, s)	72.8	-	72.8
4	-	49.2	-	49.2
5	-	37.2	-	37.1
6	1.73 (1H, m)	41.8	1.73 (dt, *J*= 12.8, 3.0 Hz)	41.7
7	-	17.6	-	17.5

Position	δ_H (ppm)	δ_C (ppm)	*δ_H (ppm)	*δ_C (ppm)
8	-	53.3	-	53..2
9	-	37.9	-	37.8
10	-	61.4	-	61.3
11	-	35.3	-	35.2
12	-	30.7	-	30.6
13	-	38.4	-	38.4
14	-	39.4	-	39.3
15	-	32.9	-	32.8
16	-	36.2	-	36.1
17	-	30.1	-	30.0
18	-	42.8	-	42.1
19	-	35.6	-	35.6
20	-	28.3	-	28.2
21	-	32.2	-	32.1
22	-	39.8	-	39.3
23	0.94 (3H, d, *J*= 6.72)	11.7	0.94 (*d*, *J*= 7.0 Hz)	11.6
24	0.94 (3H, s)	16.5	0.93 (3H, s)	16.4
25	0.85 (3H, s)	18.3	0.84 (3H, s)	18.6
26	0.99 (3H, s)	18.7	0.98 (3H, s)	18.6
27	0.99 (3H, s)	20.3	0.98 (3H, s)	20.1
28	1.16 (3H, s)	32.4	1.16 (3H, s)	32.3
29	1.00 (3H, s)	35.1	0.99 (3H, s)	35.0
30	0.95 (3H, s)	31.9	0.95 (3H, s)	31.8

*Utami, et al., 2013



[31]

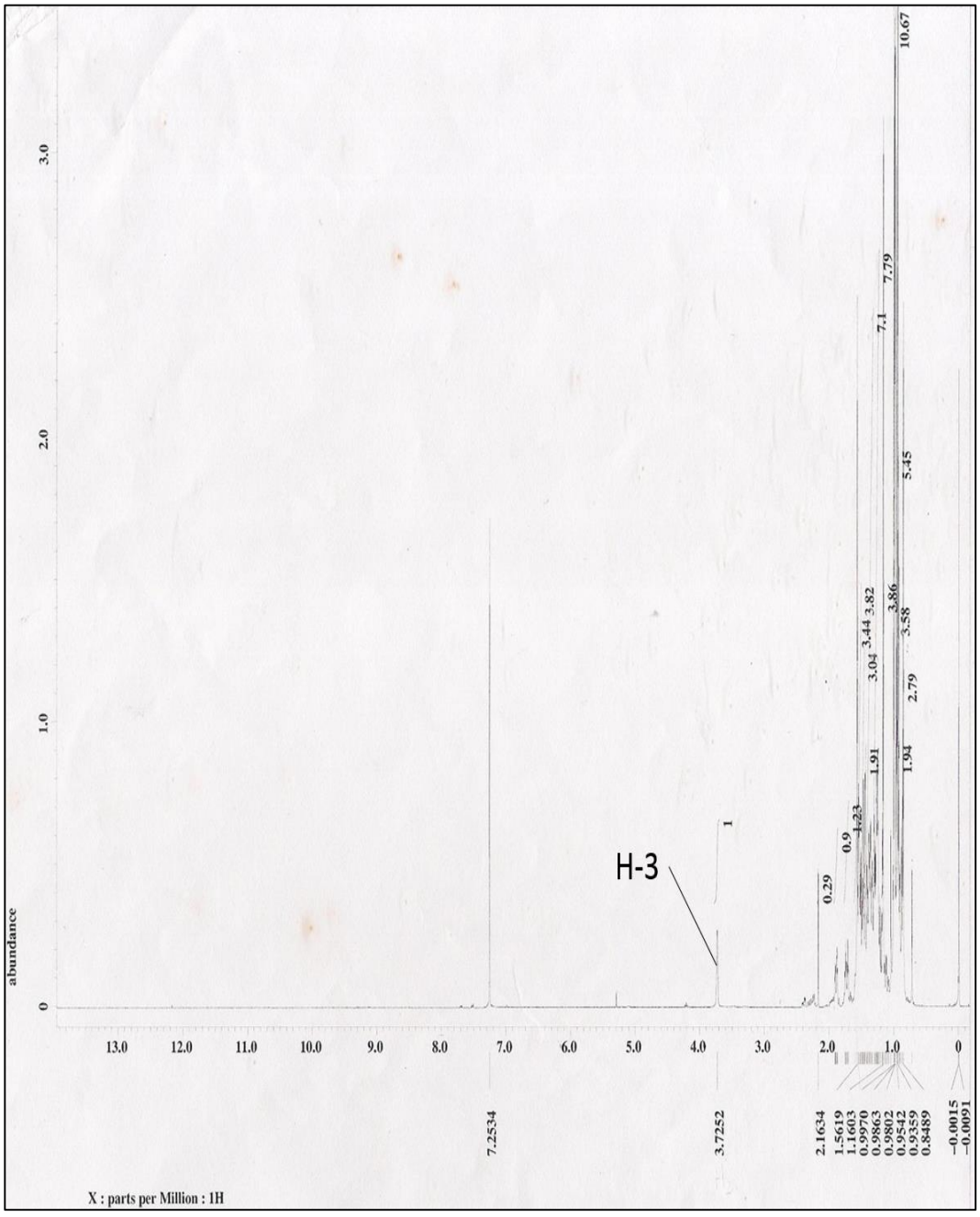
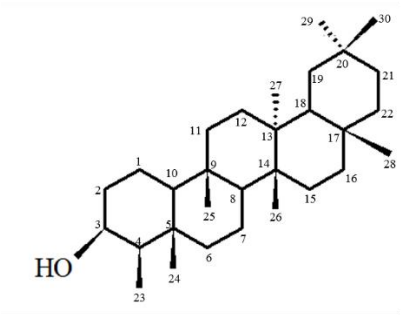


Figure 4.13: ^{1}H-NMR spectrum of friedelinol [31] (400 MHz, $CDCl_3$)



[31]

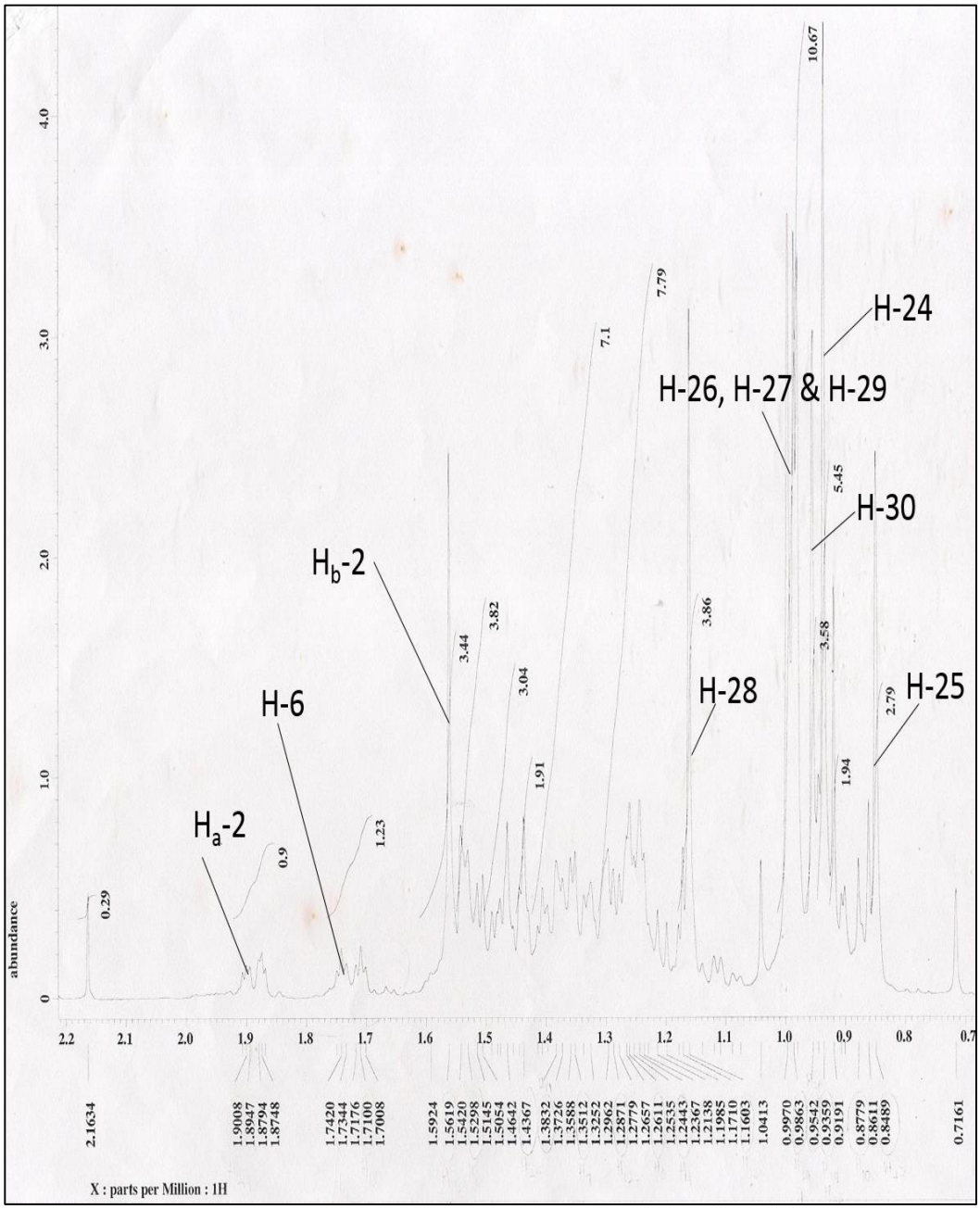
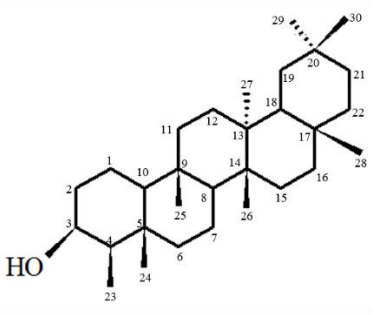


Figure 4.14: ^{1}H-NMR spectrum of friedelinol [31] (400 MHz,$CDCl_3$) (expanded)



[31]

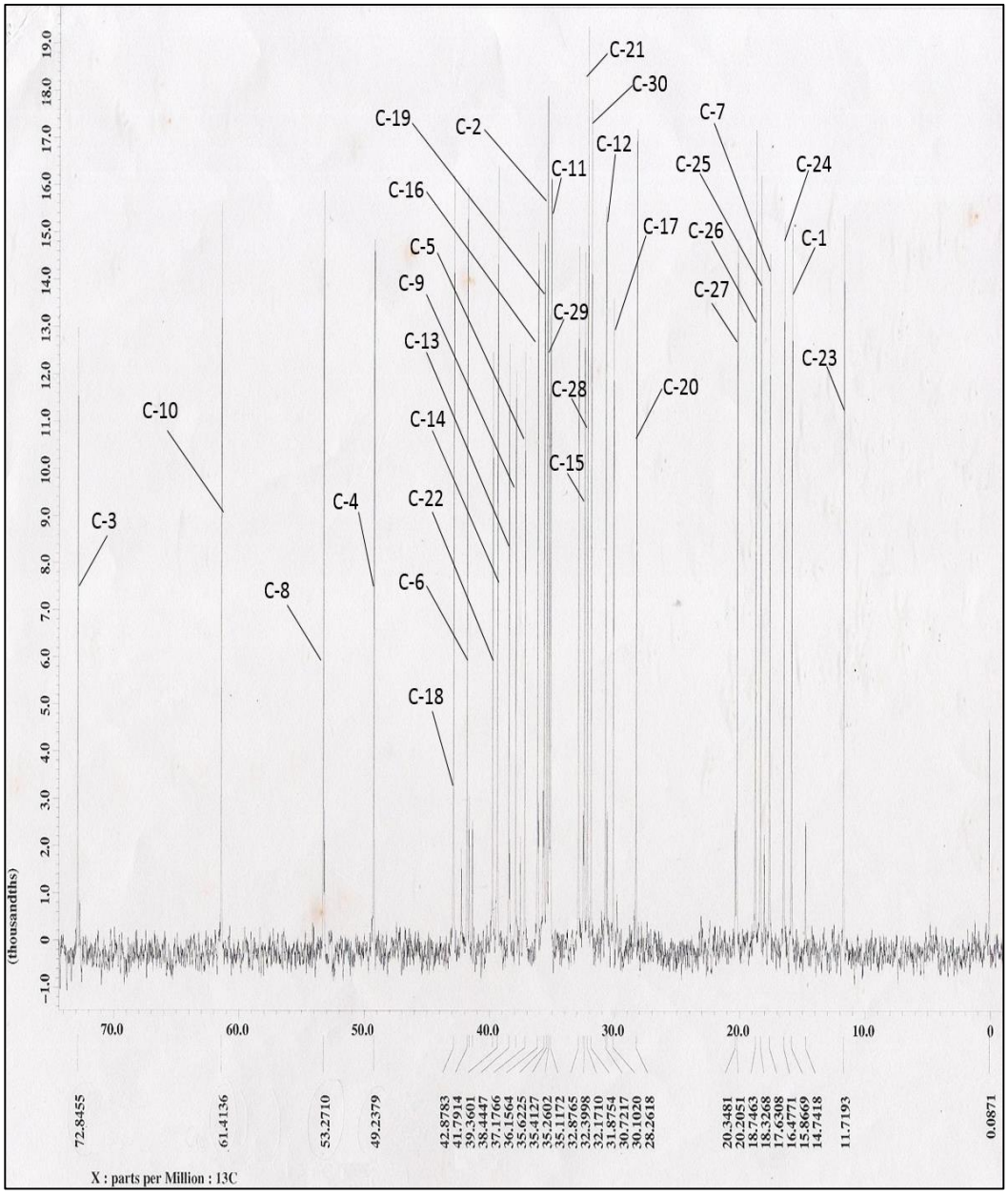
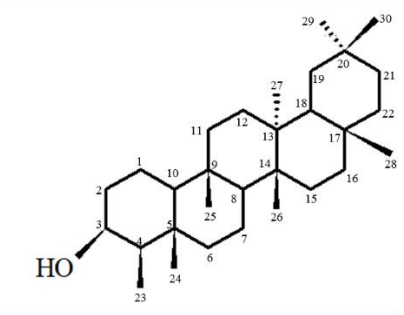


Figure 4.15: ^{13}C-NMR spectrum of friedelinol [31] (100 MHz, $CDCl_3$) (expanded)



[31]

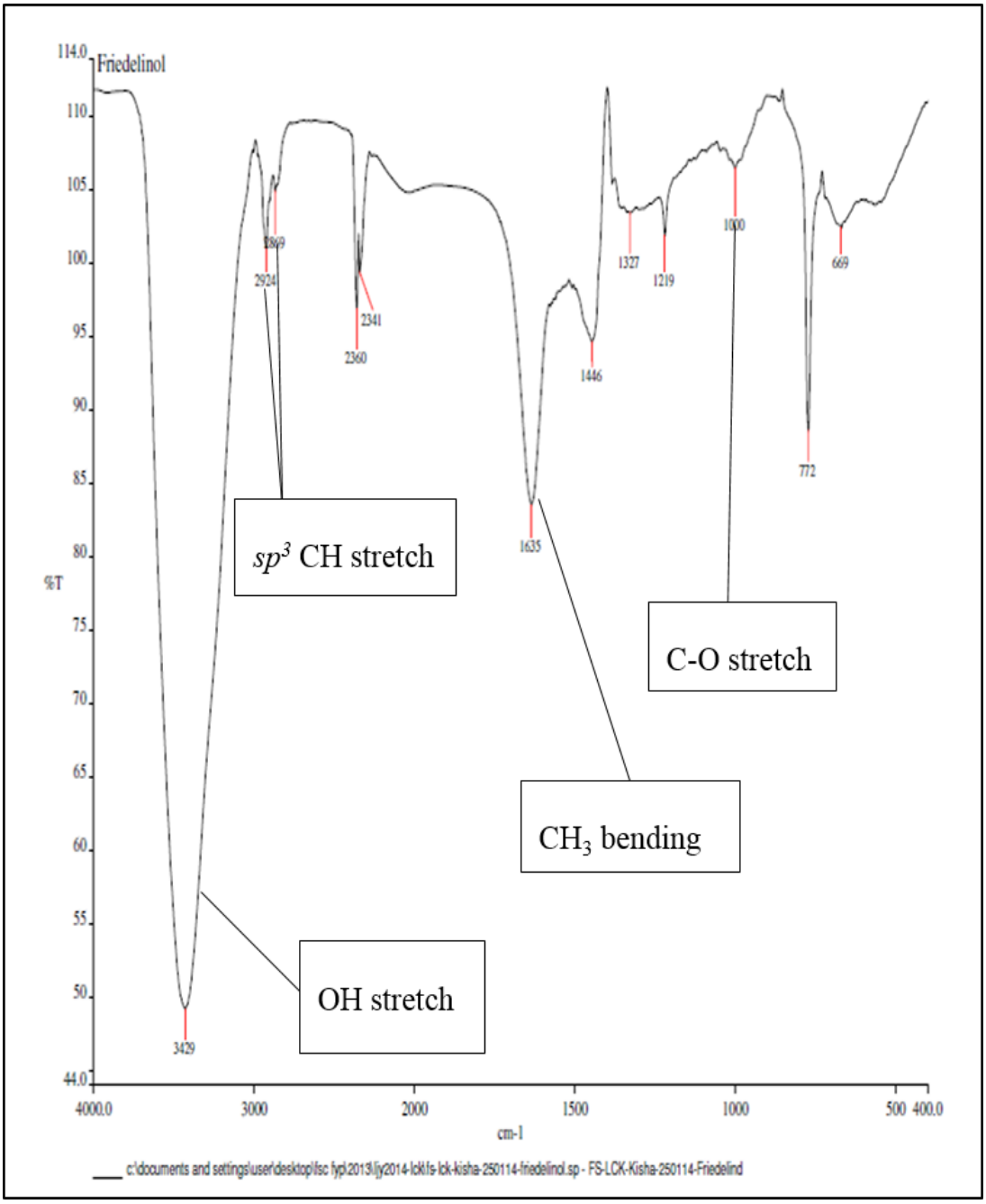
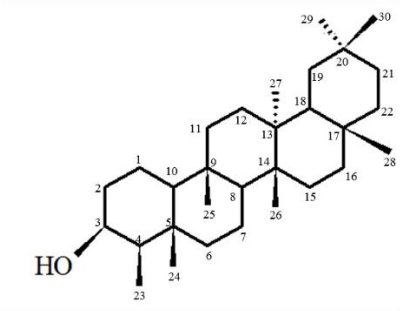


Figure 4.16: IR spectrum of friedelinol [31]



[31]

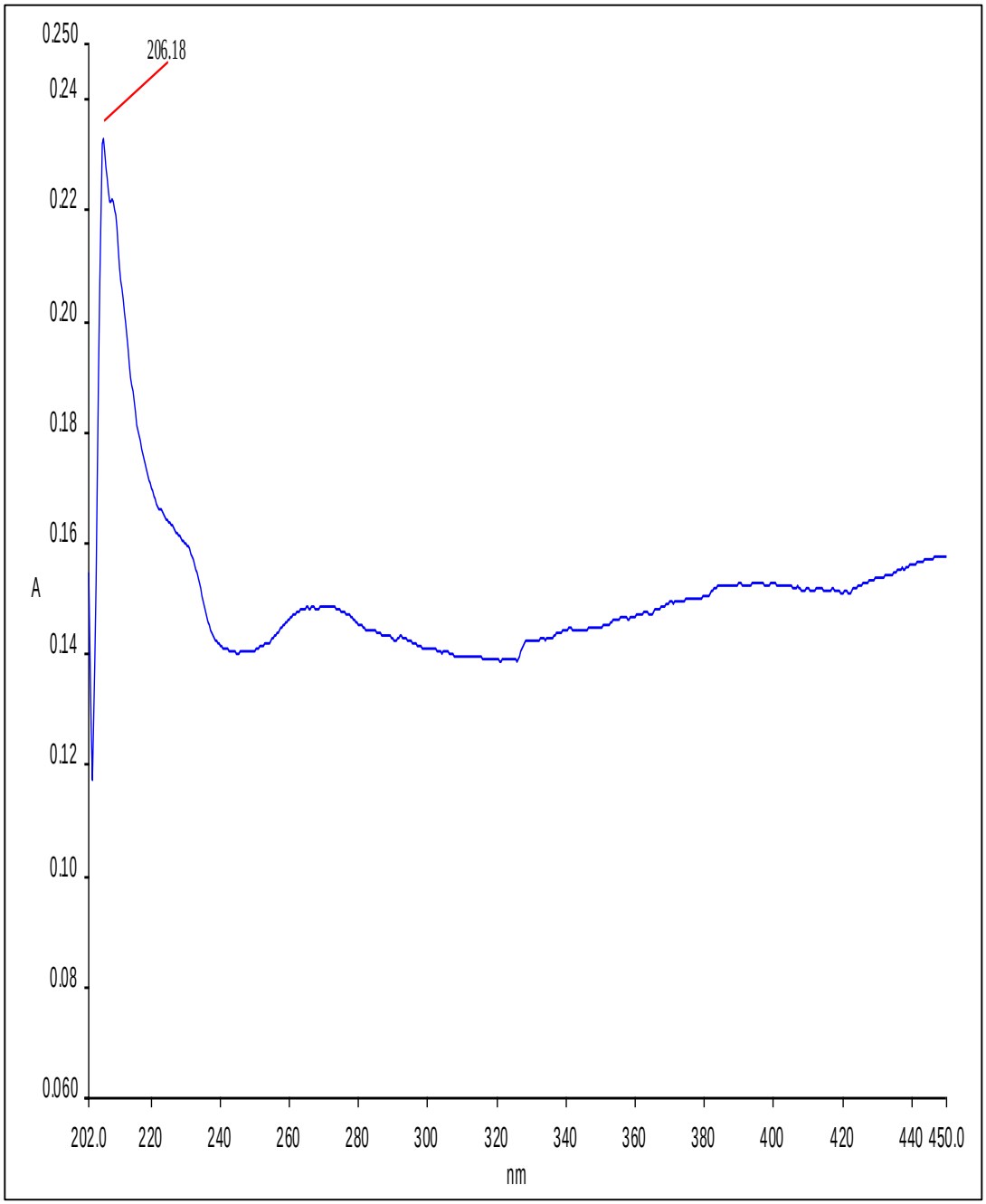
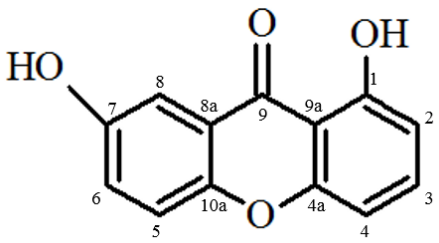


Figure 4.17: UV-Vis spectrum of friedelinol [31]

4.1.3 Characterisation and Structural Elucidation of Euxanthone [32]



[32]

Figure 4.18: Structure of euxanthone [32]

Compound **32** was obtained as yellow needle-like crystals which was established to have a molecular formula of $C_{13}H_8O_4$ corresponding to molecular weight of 228.20 g/mol. This compound showed a melting point of 234-236 ^oC with reference to the literature value of 239-241 ^oC (Kato, et al., 2004). Compound **32** was analysed by using TLC plate. Figure 4.19 below shows the developed TLC plate for compound **32**.

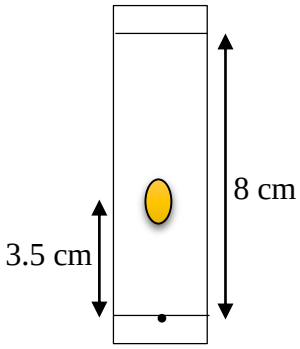


Figure 4.19: TLC plate developed for euxanthone [32]

In the TLC analysis, compound **32** gave R_f value of 0.44 when eluted with a solvent mixture of 15% acetone, 20% dichloromethane and 65% hexane,

showing that this compound is a relatively polar compound. The developed TLC plate gave a dark spot under the UV light and treatment with iodine vapour.

The compound was elucidated by applying NMR spectroscopy. From the ^{1}H-NMR spectrum (Figure 4.20), a total of eight proton signals were observed including two highly deshielded hydroxyl proton signals at δ12.70 assigned to proton 1-OH and 7-OH, respectively. Aromatic protons signals were indicated at δ 6.75 (d, *J*= 8.6 Hz, H-2), 7.68 (t, *J*= 8.2 Hz, H-3) and 6.98 (d, *J*=8.6 Hz, H-4) for xanthone ring A. Furthermore, ortho-coupled and meta-coupled aromatic protons were also found in the ring B of this compound with the signals found at δ 7.50 (d, *J*= 9.2 Hz, H-5), 7.41 (dd, *J*=9.2 Hz & 3.0 Hz, H-6) and 7.58 (d, *J*= 3.0 Hz, H-8).

A total of 13 carbon signals were revealed by the ^{13}C-NMR spectrum (Figure 4.21), including a highly deshielded carbonyl carbon found in the downfield region at δ 182.2 (C-9). Besides, four oxygenated aromatic carbons in the structure gave signals in the downfield region at δ 161.9 (C-1), 156.5 (C-4a), 154.2 (C-7) and 150.2 (C-10a). The remaining carbon signals were observed at δ 109.9 (C-2), 137.0 (C-3), 107.0 (C-4), 119.4 (C-5), 125.4 (C-6), 108.3 (C-8), 121.0 (C-8a) and 109.7 (C-9a).

The structure of compound **32** was further elucidated via 2D-NMR analysis which revealed the correlations between protons and their immediate and neighbouring carbons. The HMQC spectrum (Figure 4.22) provides information about direct ^{1}J coupling between the proton and carbon, and the assignment of protons to their immediate carbons were established according to these

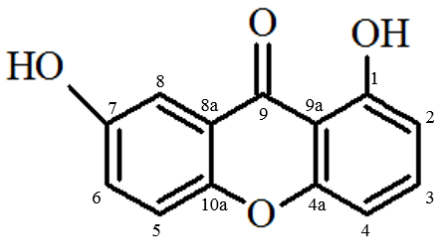
correlations. From the HMBC spectrum (Figure 4.23 and 4.24), long range correlations were observed between H-6 and C-8, H-2 and C-4, H-8 and C-6, H-6 and C-10a, H-5 and C-7 and H-3 and C-4a & C-1. The structure of compound **32** was assigned according to these correlation data. Table 4.3 shows the summary of NMR data for compound **32**.

Further confirmation of the structure was accomplished by IR and UV-Vis analyses. The IR spectrum (Figure 4.25) indicated broad band at 3133 cm^{-1}corresponding to the O-H stretch. In additions, C-O stretch at 1233 cm^{-1} and conjugated aromatic C=C stretch at 1479, 1458, 1437, 1420 cm^{-1} were also observed. Meanwhile, UV-Vis spectrum (Figure 4.26) showed absorption maxima at 206.14, 235.35, 260.02 and 387.33 nm which indicated the compound to be highly conjugated. Compound **32** was unambiguously assigned as euxanthone based on all the spectral NMR evidence given above.

Table 4.3: Summary of NMR spectral data for euxanthone [32]

Position	**δ_H (ppm)**	**δ_C (ppm)**	**HMBC**	
			***J*²**	***J*³**
1	-	161.9	-	-
2	6.75 (1H, d, *J*=8.6 Hz)	109.7	-	C-4
3	7.68 (1H, t, *J*= 8.2 Hz)	137.0	-	C-1, C-4a
4	6.98 (1H, d, *J*=8.6 Hz)	107.0	C-4a	C-2
4a	-	156.5	-	-
5	7.50 (1H, d, *J*=9.2Hz)	119.4	C-6	C-7
6	7.41 (1H, dd, *J*= 9.2 Hz, 3.0 Hz)	125.4	-	C-8, C-10a
7	-	154.2	-	-
8	7.58 (1H, d, *J*= 3.0 Hz)	108.3	-	C-6
8a	-	121.0	-	-
9	-	182.2	-	-
9a	-	109.7	-	-

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			J^2	J^3
10a	-	150.2	-	-
1-OH	12.70(OH, s)	-	-	C-2
3-OH	9.05 (OH, s)	-	-	-



[32]

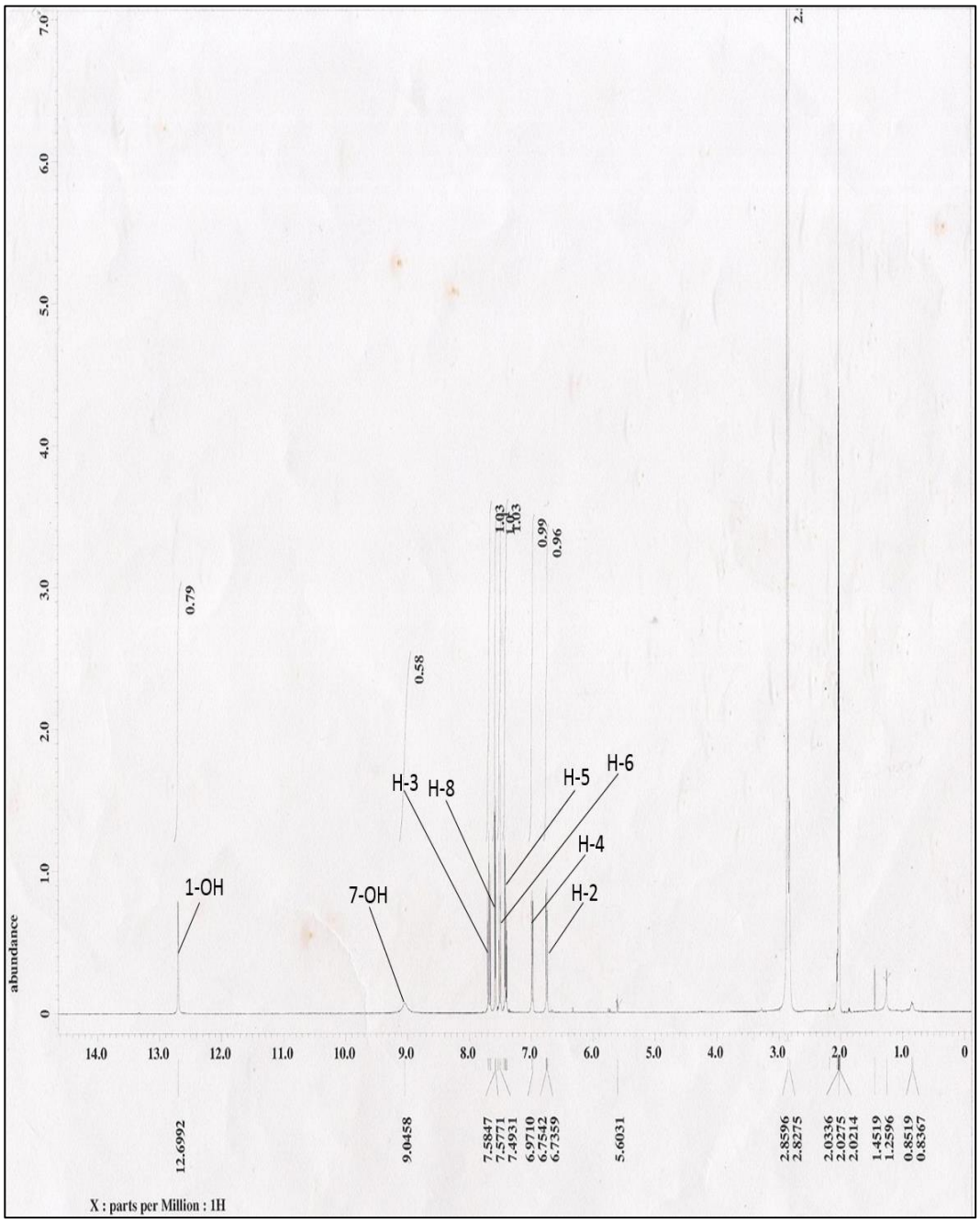
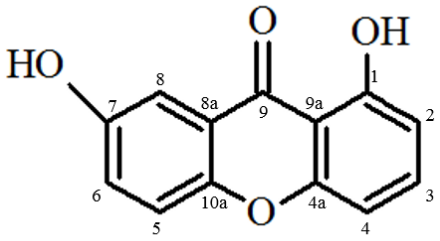


Figure 4.20: ^{1}H-NMR spectrum of euxanthone [32] (400 MHz, acetone-d_6)



[32]

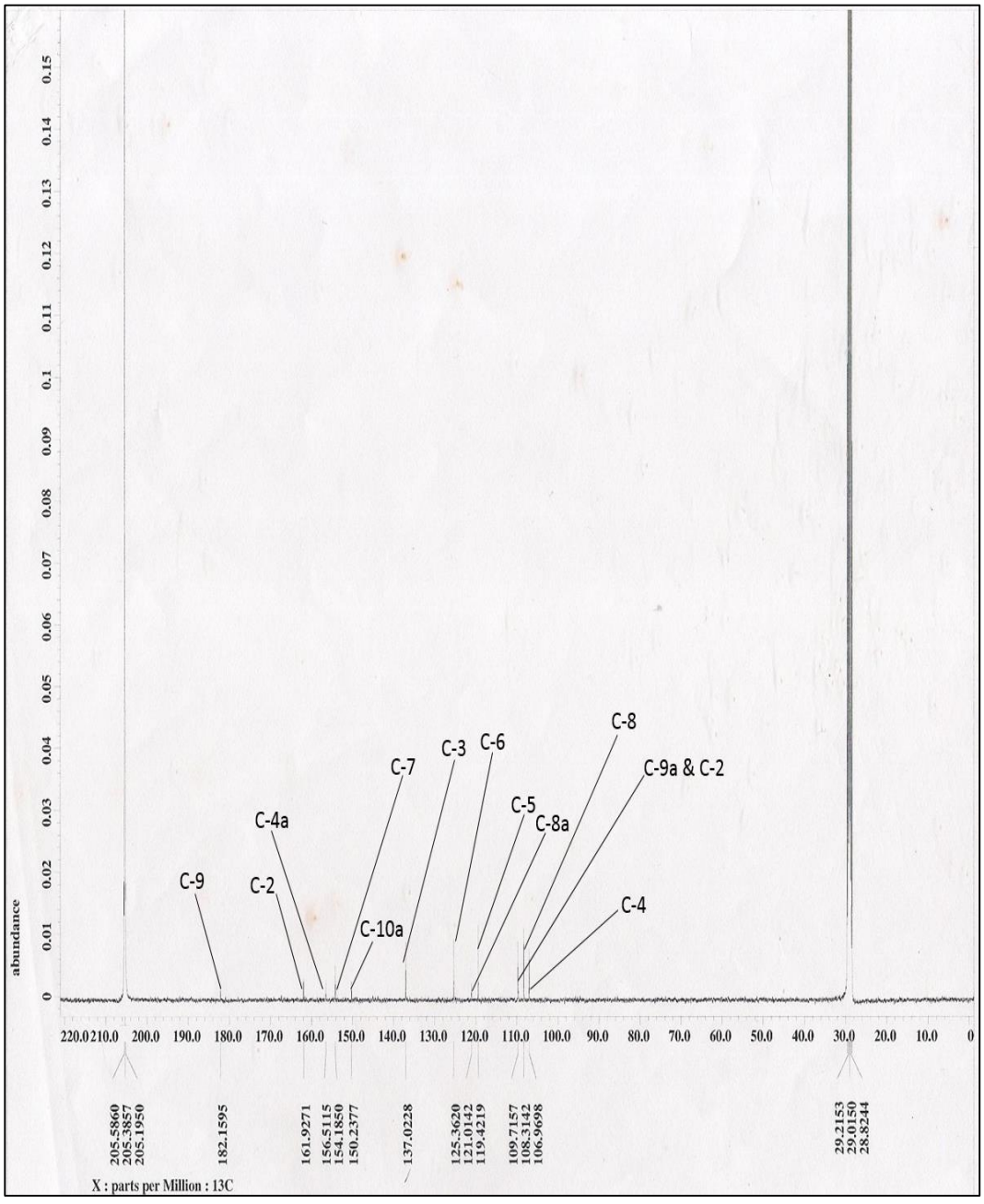
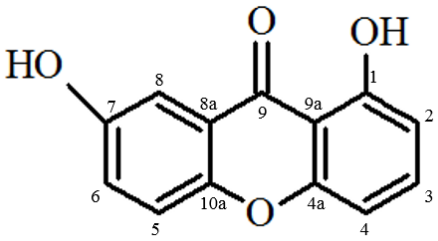


Figure 4.21: ^{13}C-NMR spectrum of euxanthone [32] (400 MHz, acetone-d_6



[32]

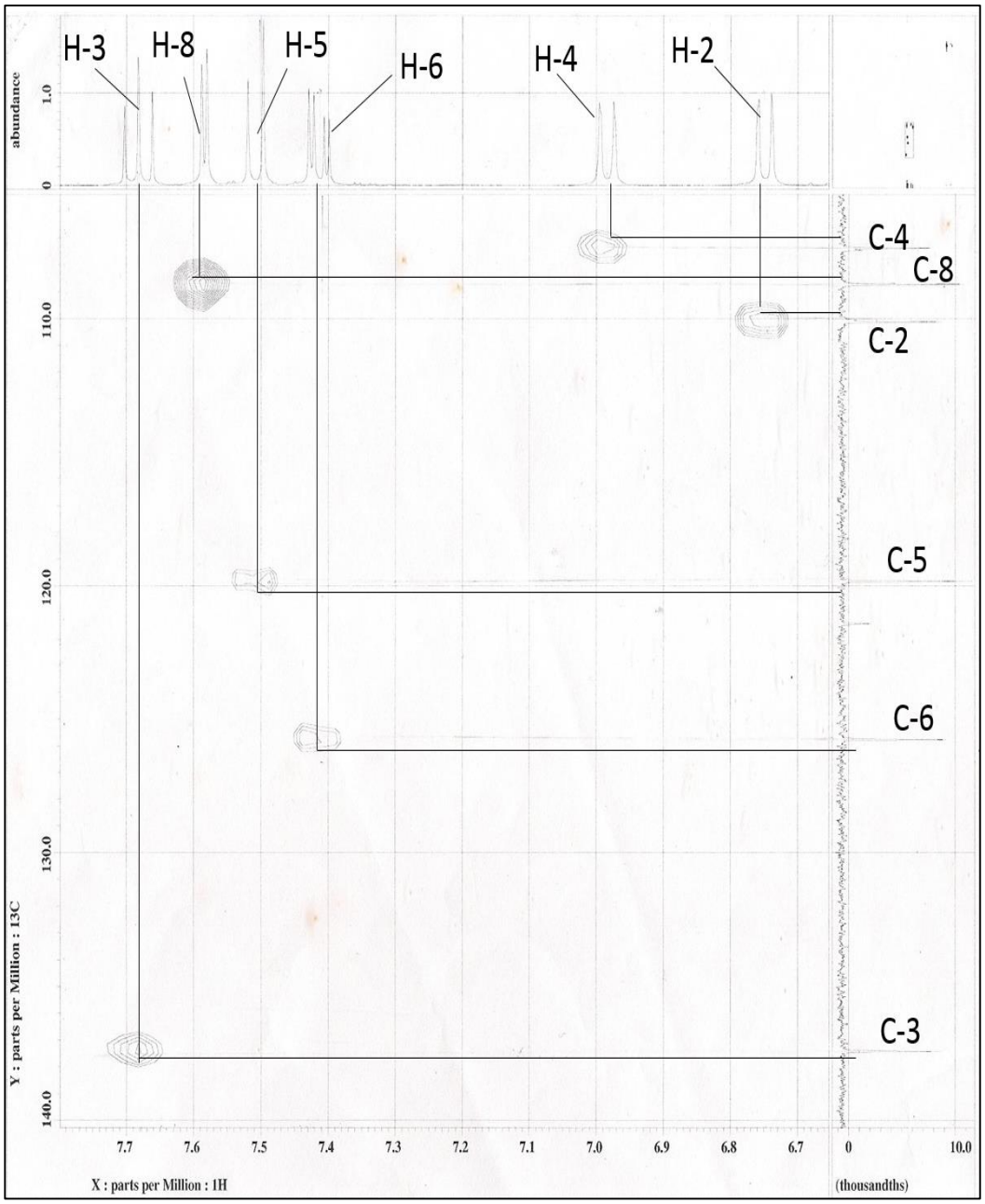
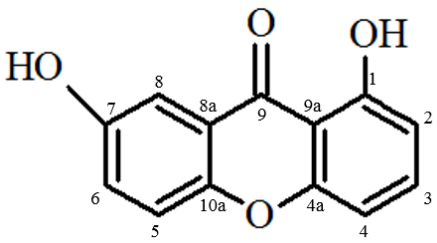


Figure 4.22: HMQC spectrum of euxanthone [32] (expanded)



[32]

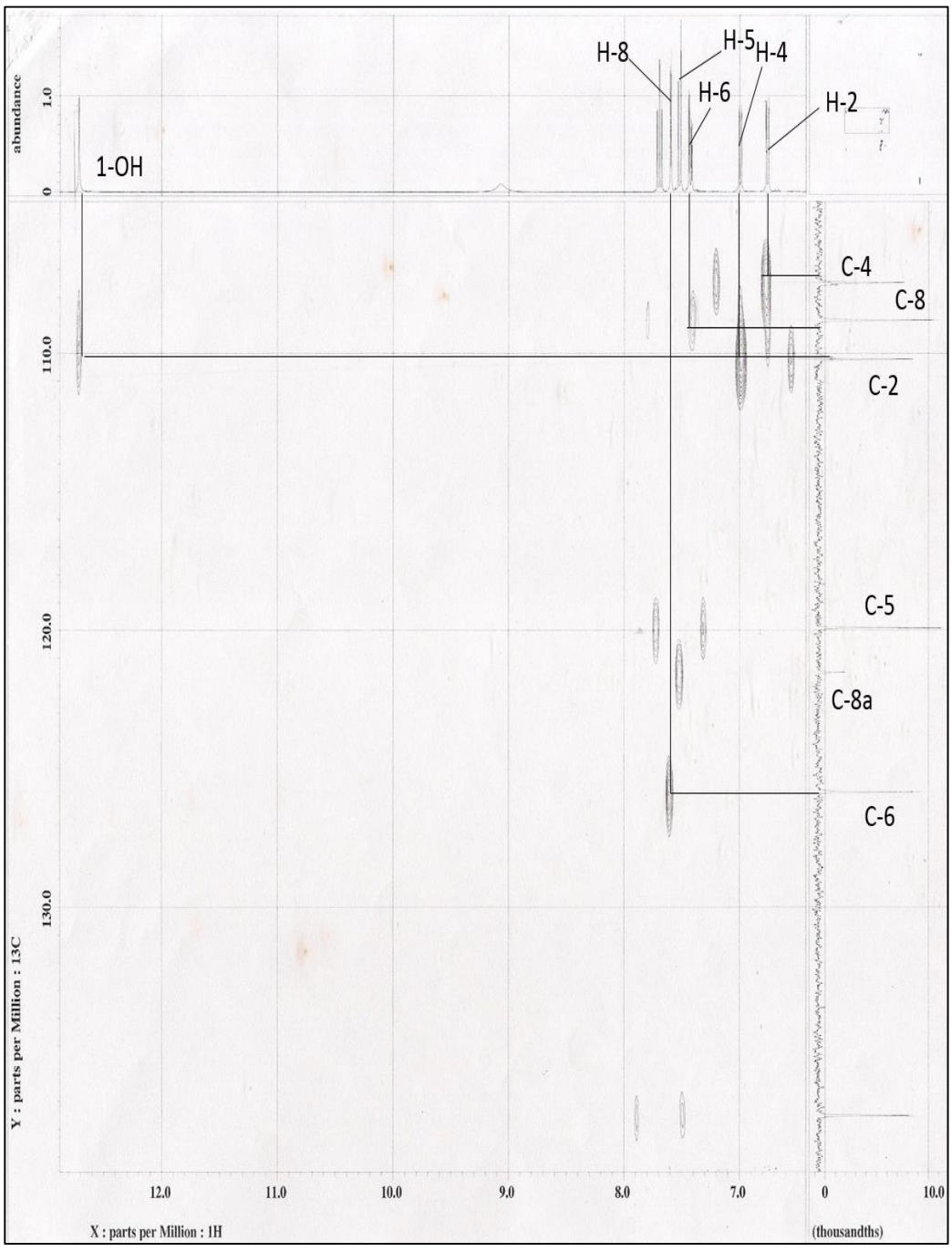
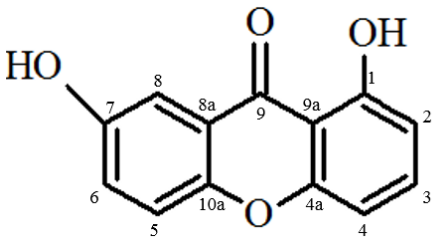


Figure 4.23: HMBC spectrum of euxanthone [32] (expanded)



[32]

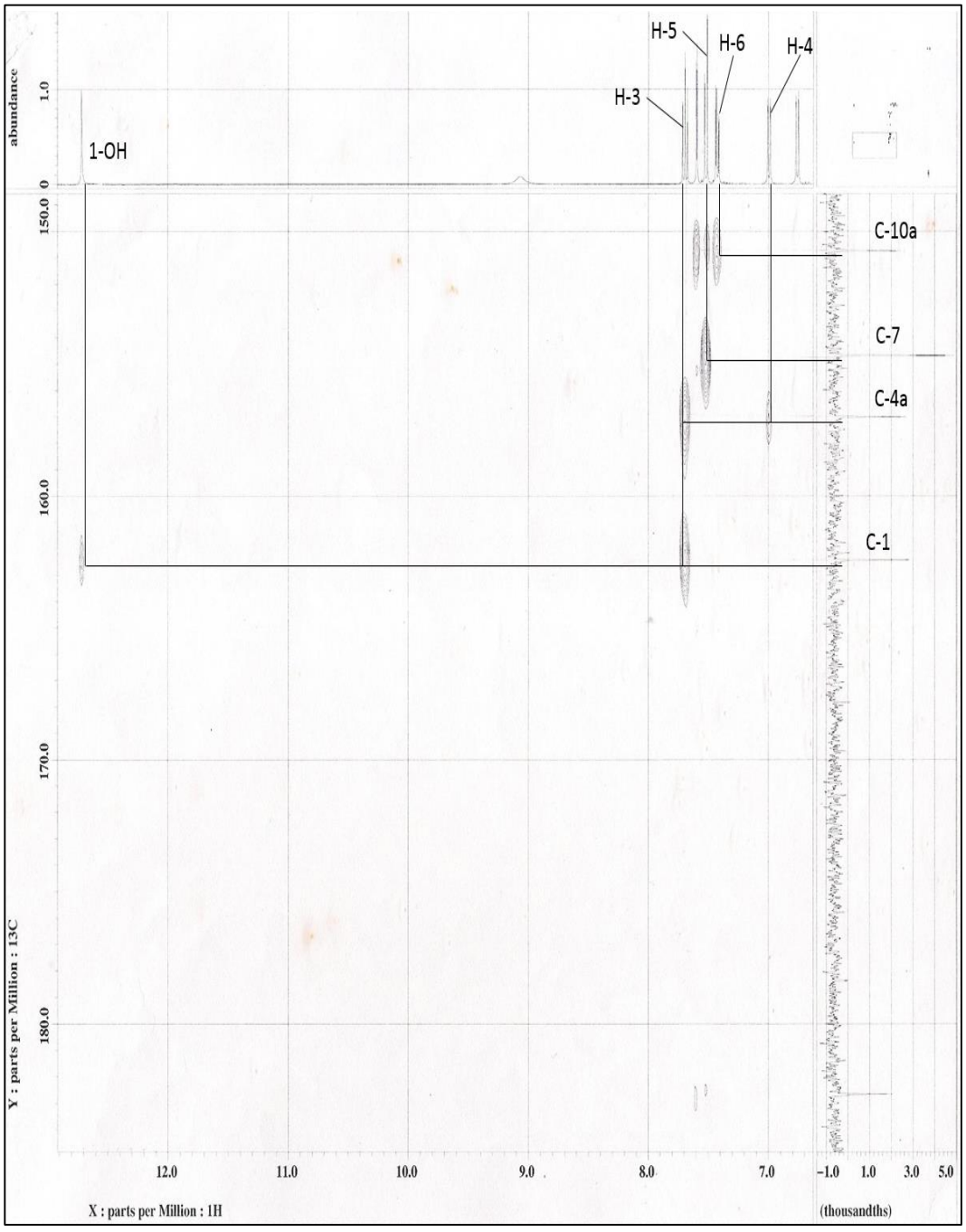
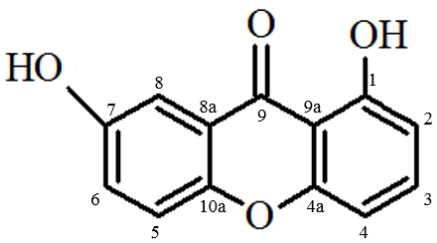


Figure 4.24: Expanded HMBC spectrum of euxanthone [32] (expanded)



[32]

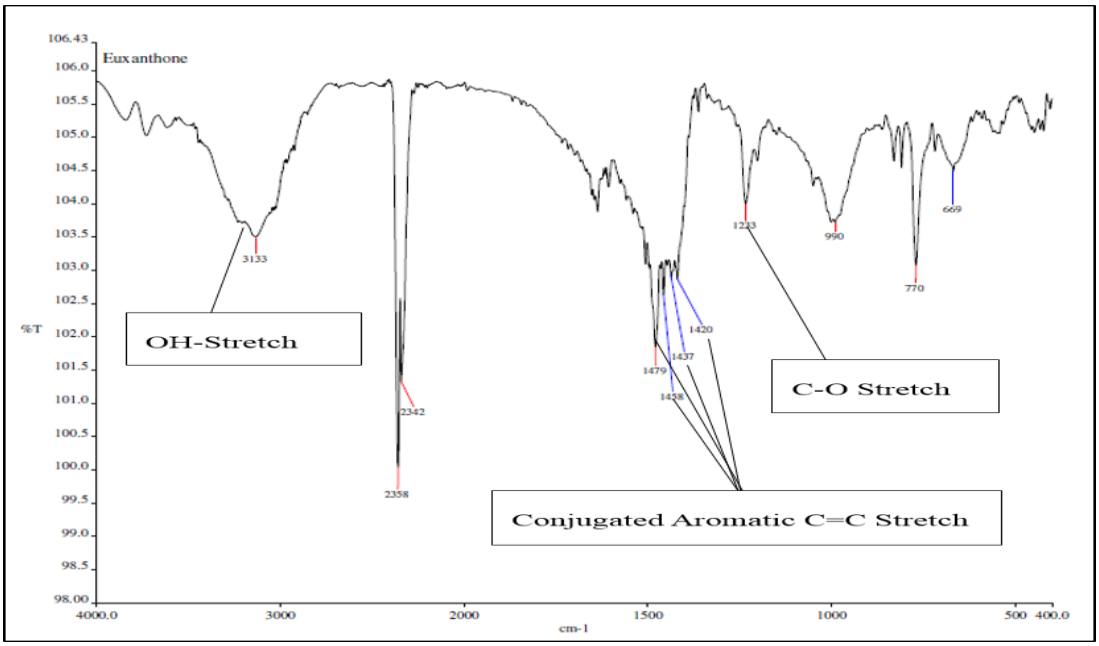


Figure 4.25: IR spectrum of euxanthone [32]

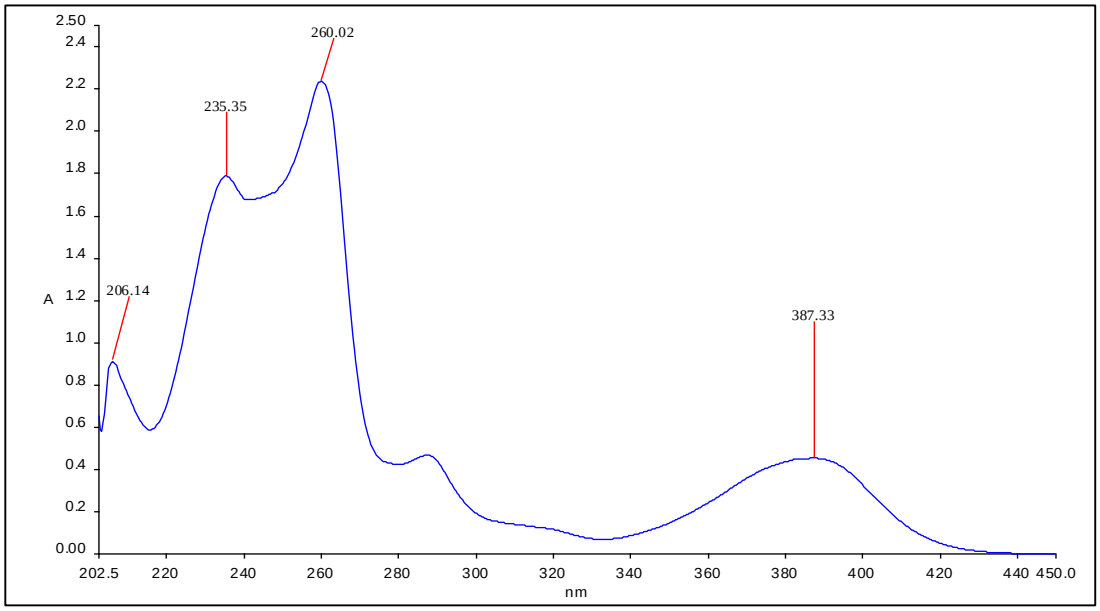
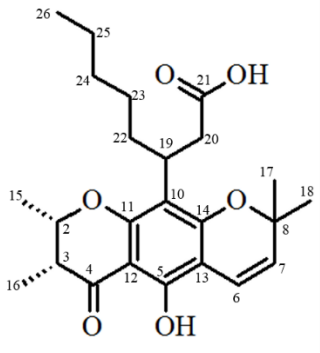


Figure 4.26: UV-Vis spectrum of euxanthone [32]

4.1.4 Characterisation and Structural Elucidation of Isoblancoic Acid [33]



[33]

Figure 4.27: Structure of isoblancoic acid [33]

Compound **33** is a chromanone acid. This monocarboxylic acid compound appeared as yellow gummy resin giving a molecular weight of 416.2202 g/mol as shown in HRESI mass spectrum (Figure 4.28) in corresponding to the molecular formula of $C_{24}H_{32}O_6$. Compound **33** was subjected to optical activity test and obtained a specific rotation, $[\alpha]_{\lambda}^{T}$ of -41.7°. The TLC plate developed for isoblancoic acid **[33]** is shown in Figure 4.29.

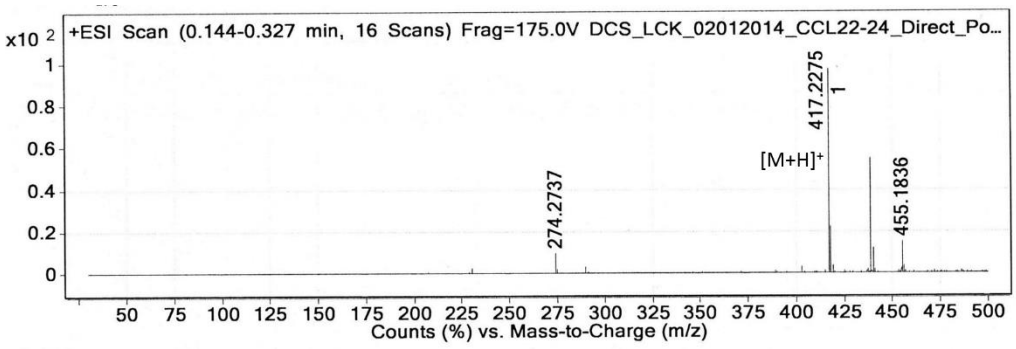


Figure 4.28: HRESI mass spectrum of isoblancoic acid [33]

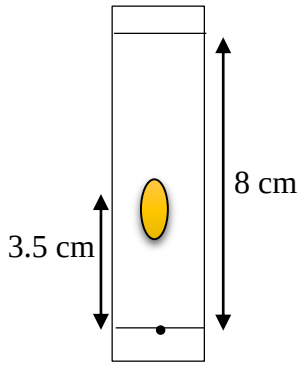


Figure 4.29: The developed TLC plate for isoblancoic acid [33]

The R_f value obtained was 0.44 eluted with a solvent system of 15% acetone, 25% dichloromethane and 65% hexane, showing that this compound is a relatively polar compound. Compound **33** gave a dark spot on the TLC plate when examined under UV light and treated with iodine vapour in the chamber.

In the ^{1}H-NMR spectrum (Figure 4.30 and 4.31), the highly deshielded singlet signal at δ12.38 was assigned to the chelated hydroxyl proton, 5-OH. The five methyl protons signals present in the compound gave intense signals at δ 1.33 (d, *J*= 6.7 Hz, H-15), 1.13 (d, *J*= 6.1 Hz, H-16), 1.35 (s, H-17), 1.42 (s, H-18) and 0.81 (H-26). Five groups of methylene protons gave signals at *δ* 2.81 (dd, *J*= 14.7, 6.7 Hz, H-20), 2.64 (m, H-20), 1.17 (m, H-24), 1.53 (m, H-22) and 1.21 (m,H-25) while five methine proton signals were observed at δ 4.42 (brs, H-2), 2.46 (m, H-3), 6.58 (d, *J*= 9.8 Hz) , 5.44 (d, *J*= 9.8 Hz) and 3.66 (m,H-19).

The ^{13}C- NMR spectrum (Figure 4.32) showed five methyl carbon signals at δ 16.4 (C-15), 9.4 (C-16), 28.5 (C-17), 28.2 (C-18) and 14.2 (C-26). On the other hands, the methylene carbons exhibited signals at 38.7 (C-20), 33.2 (C-22), 27.5 (C-23), 31.8 (C-24) and 22.7 (C-25) while the methine carbons gave signals at 76.1 (C-2), 44.3 (C-3), 115.7 (C-6), 125.8 (C-7) and 30.8 (C-19). Apart from that, nine quaternary carbon signals were observed in the ^{13}C-NMR spectrum,

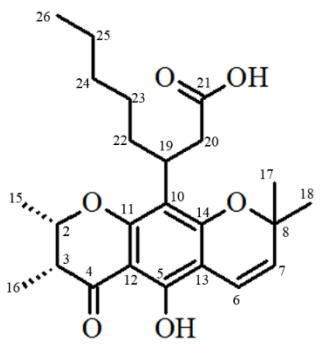
at δ 201.4 (C-4), 160.1 (C-14), 78.3 (C-8), 108.8 (C-10), 160.0 (C-11), 101.3 (C-12), 102.6 (C-13), 157.4 (C-15) and 179.6 (C-21).

2D NMR experiments, including HMQC and HMBC analyses were also used to establish the structure of compound **33**. The HMBC spectrum (Figure 4.35) displayed linkages between H-16 and C-2, C-4, H-20 and C-10, C-19, C-21, H-26 and C-24, C-25 and H-24 and C-3, C-25, and the 2D-NMR correlation data were summarised in Table 4.4. Based on all the above NMR data, compound **33** was identified as isoblancoic acid.

The assigned structure was further supported by IR and UV-Vis analyses. The IR spectrum (Figure 4.36) indicated the presence of the O-H stretch at 3419 cm^{-1} and C-O stretch at 1132 cm^{-1}. The C=O stretch at 1626 cm^{-1} has proven the presence of a carbonyl group in the assigned structure. Others absorption bands due to the sp^3 C-H stretch (2925 cm^{-1}), aromatic C=C stretch (1442 cm^{-1}), aromatic C-H bending (993 cm^{-1}) and CH_2 bending (771 cm^{-1}) were also observed. Moreover, the UV-Vis spectrum (Figure 4.37) gave absorption maxima at 209.05, 274.41, and 311.31 nm indicated compound **33** to have a conjugated structure which was in agreement with the structure of isoblancoic acid **[33]**.

Table 4.4: Summary of NMR spectral data for isoblancoic acid [33]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			J^2	J^3
2	4.42 (1H, brs)	76.1	-	-
3	2.46 (1H, m)	44.3	-	-
4	-	201.4	-	-
5	-	157.4	-	-
6	6.58 (1H, d, *J*=9,8Hz)	115.7	-	C-5, C-8
7	5.44 (1H, d, *J*=9.8 Hz)	125.8	C-8	C-13, C-17
8	-	78.3	-	-
9	-	-	-	-
10	-	108.8	-	-
11	-	160.0	-	-
12	-	101.3	-	-
13	-	102.6	-	-
14	-	160.1	-	-
15	1.33 (3H, *d*, *J*= 6.7 Hz)	16.4	C-2	C-3
16	1.13 (3H, *d*, *J*= 6.1 Hz)	9.4	-	C-2, C-4
17	1.35 (3H, s)	28.5	-	-
18	1.42 (3H, s)	28.2	C-8	C-17
19	3.66 (1H, m)	30.8	-	-
20	2.81 (1H, dd, *J*= 14.7, 8.5 Hz) 2.64 (1H, m)	38.7	C-19, C-21	C-10
21	-	179.6	-	-
22	1.53 (1H, m) 1.81 (1H, m)	33.2	-	-
23	1.19 (2H, m)	27.5	-	-
24	1.17 (2H, m)	31.8	C-25	-
25	1.21 (2H, m)	22.7	-	-
26	0.81 (3H, t, *J*=6.7 Hz)	14.2	C-25	C-24
5-OH	12.38 (1H, s)	-	-	-



[33]

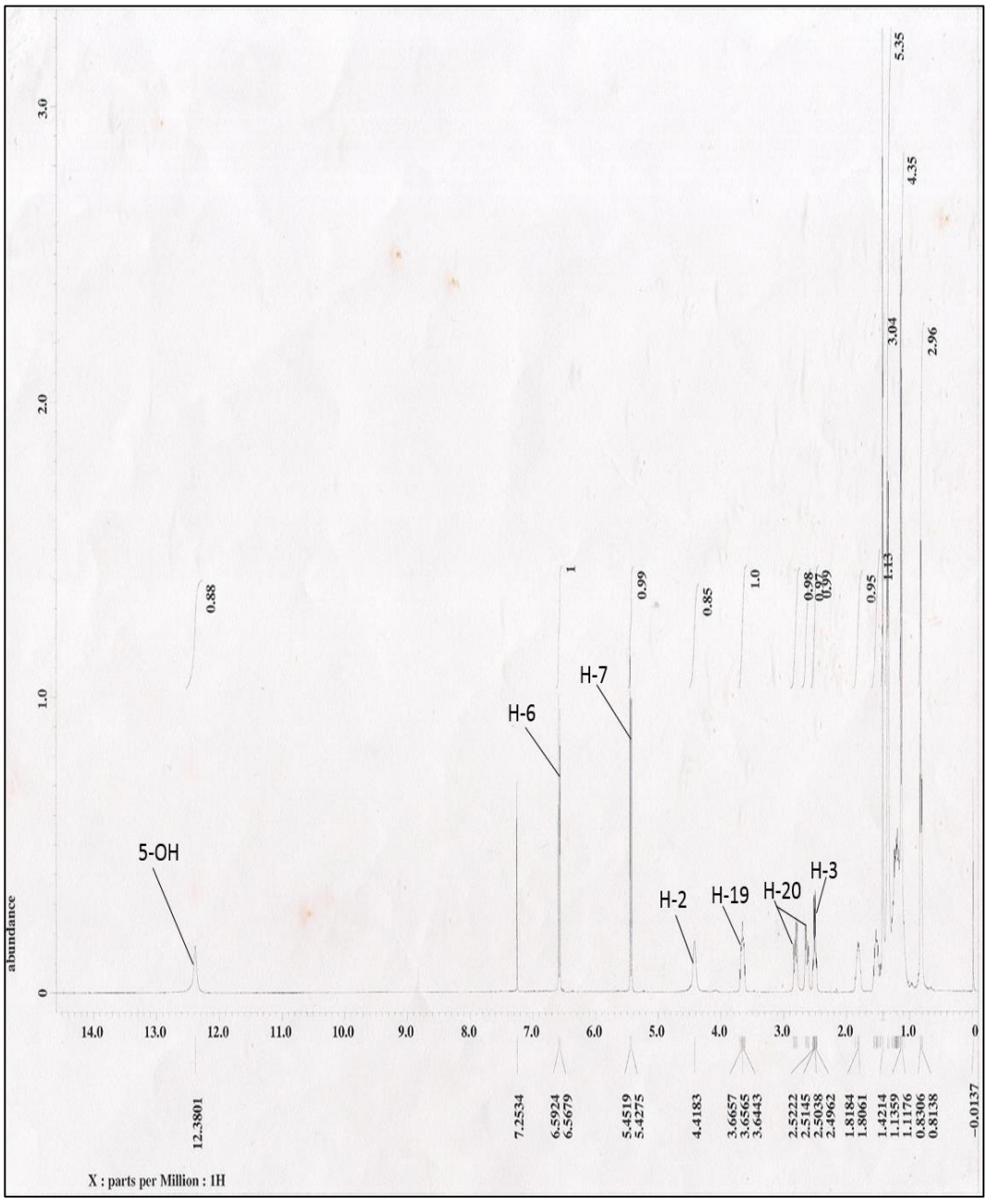
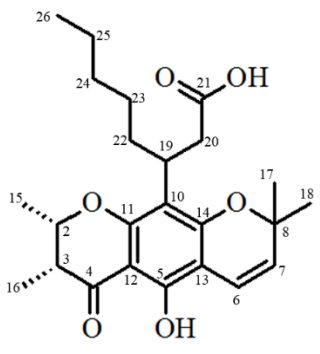
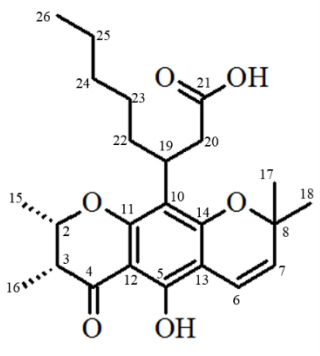


Figure 4.30: ^{1}H-NMR spectrum of isoblancoic acid [33] (400 MHz, $CDCl_3$)



[33]

Figure 4.31: ^{1}H-NMR spectrum of isoblancoic acid [33] (400 MHz, $CDCl_3$) (expanded)



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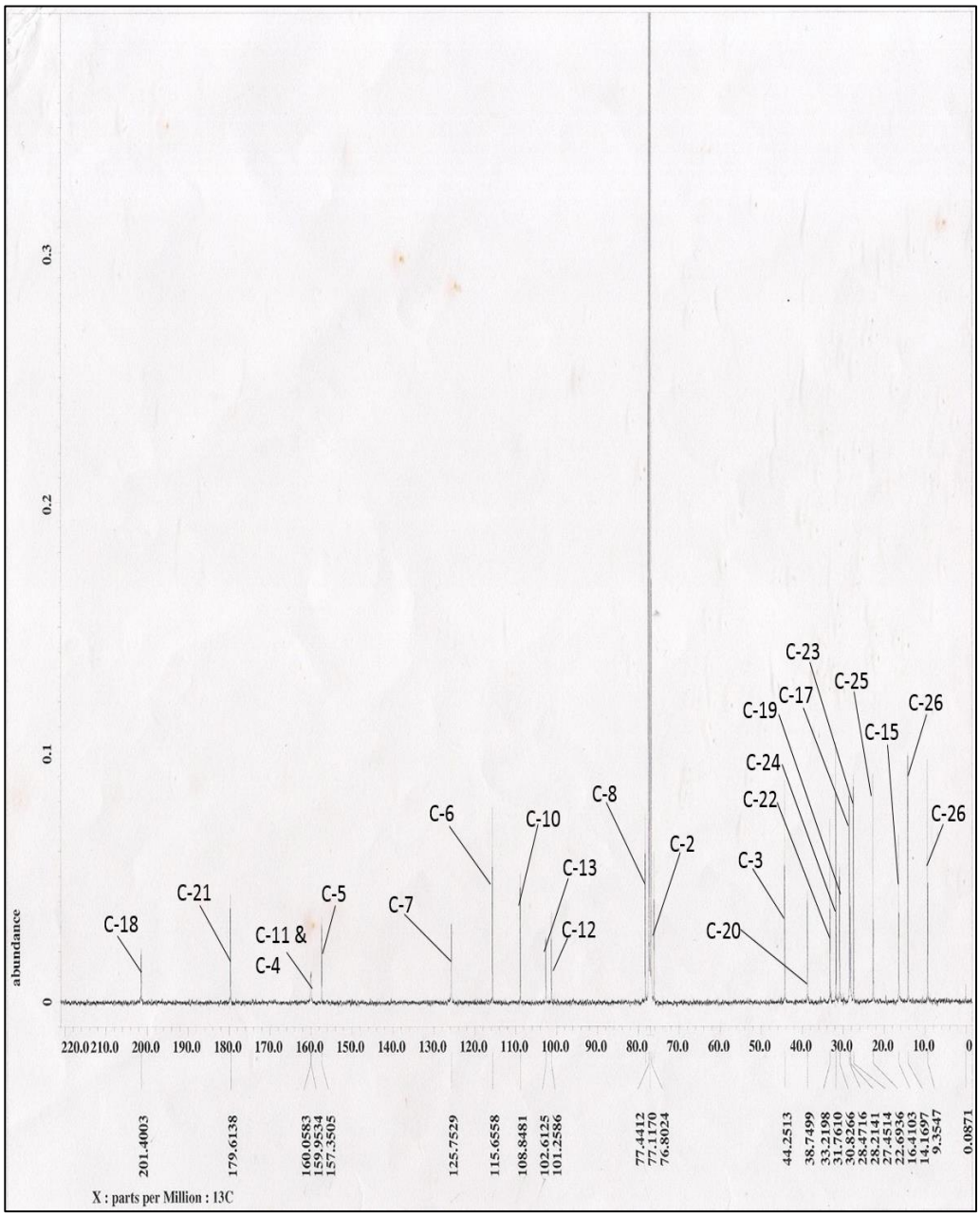
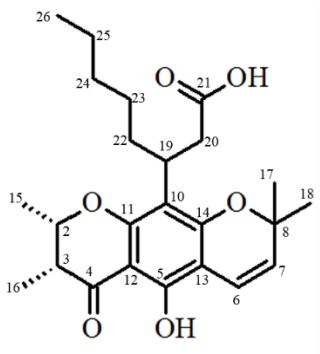


Figure 4.32: ^{13}C-NMR spectrum of isoblancoic acid [33] (100 MHz, $CDCl_3$)



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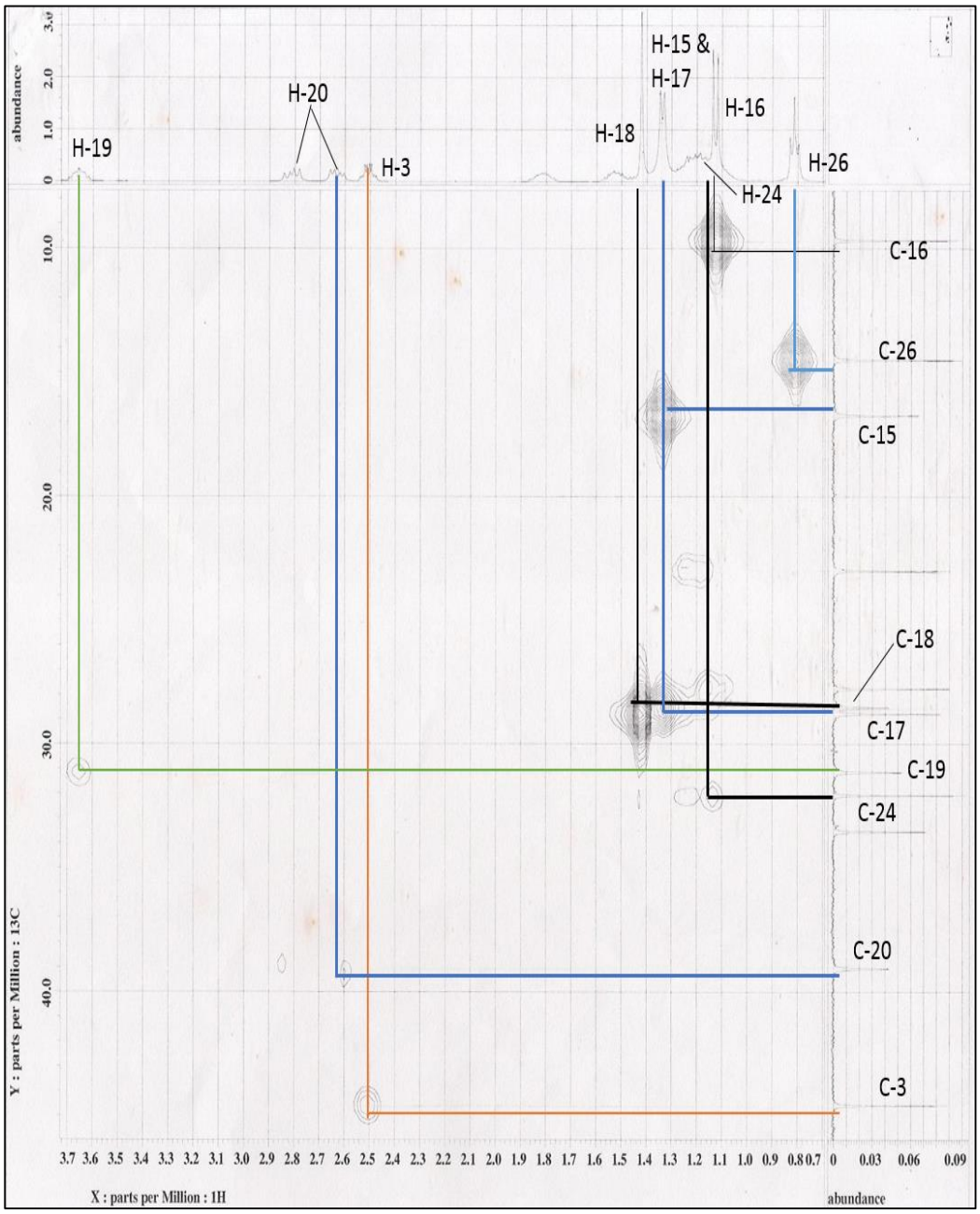
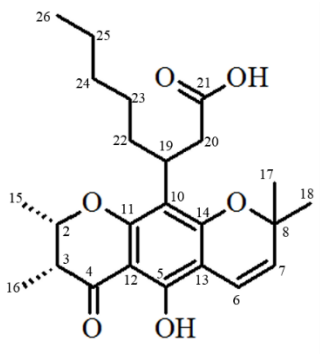


Figure 4.33: HMQC spectrum of isoblancoic acid [33] (expanded)



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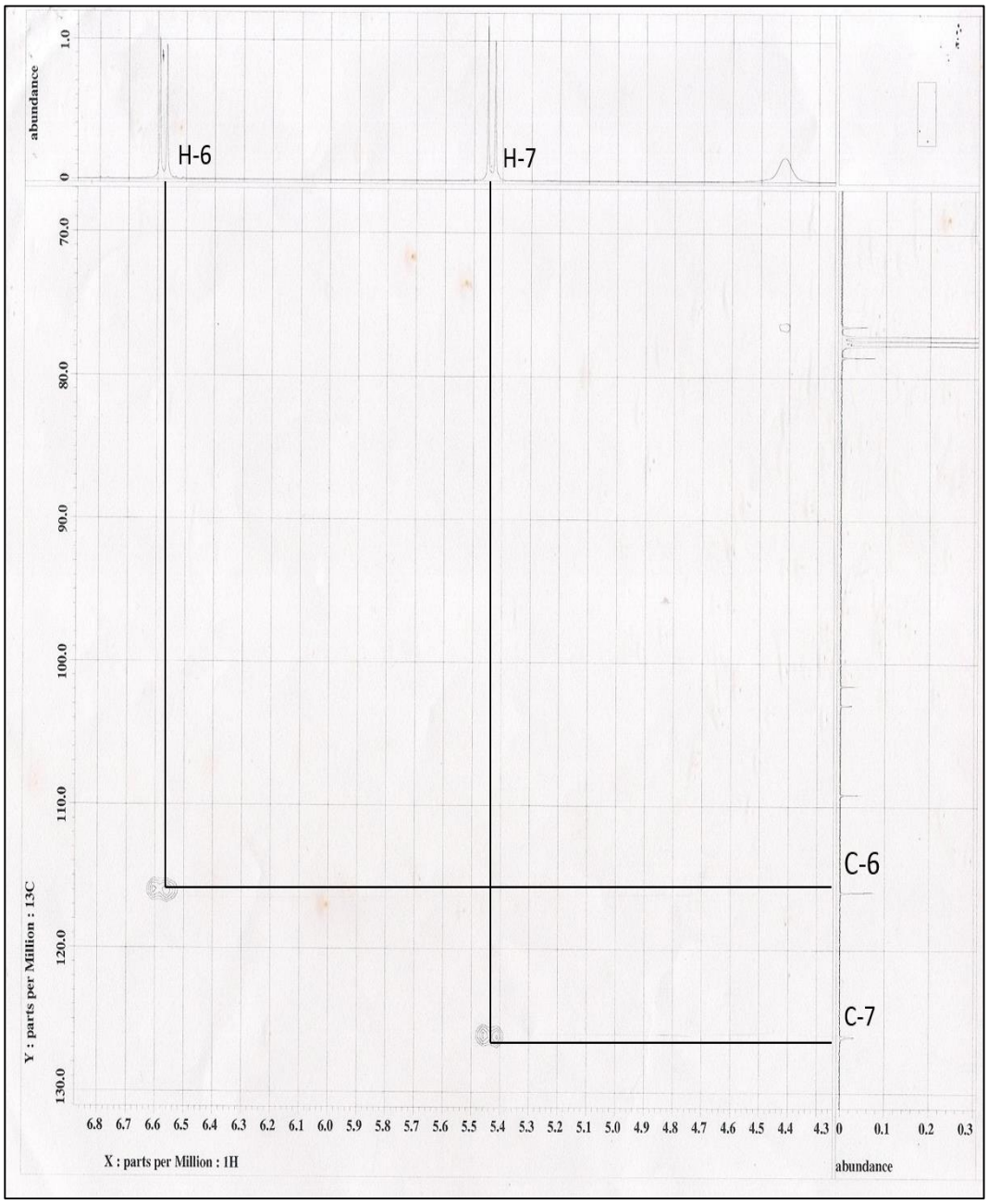
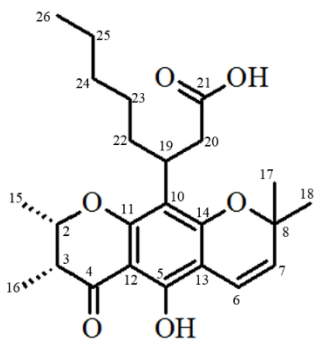


Figure 4.34: HMQC spectrum of isoblancoic acid [33] (expanded)



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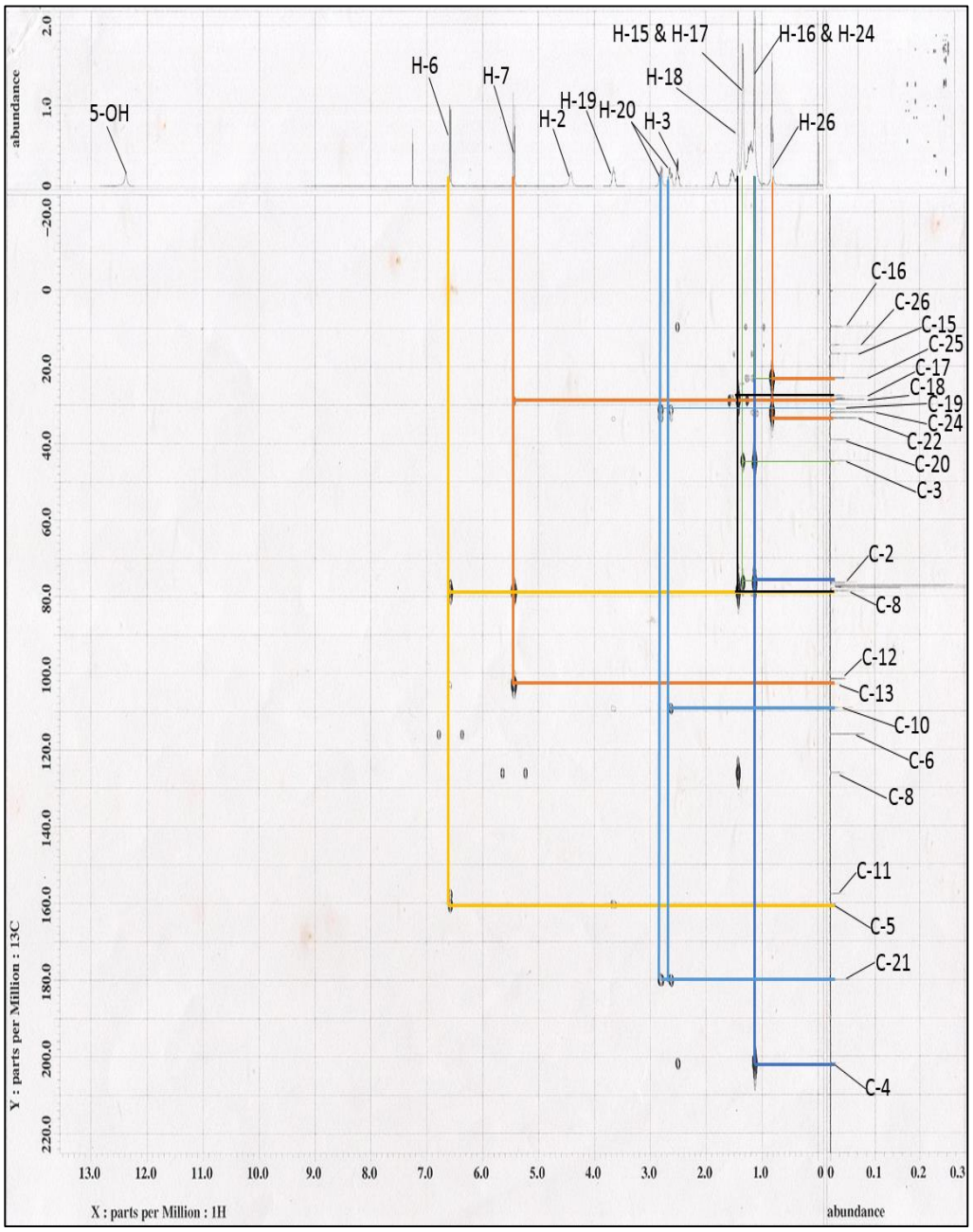
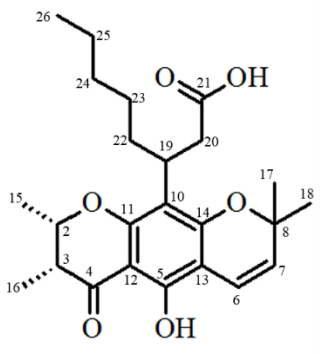


Figure 4.35: HMBC spectrum of isoblancoic acid [33]



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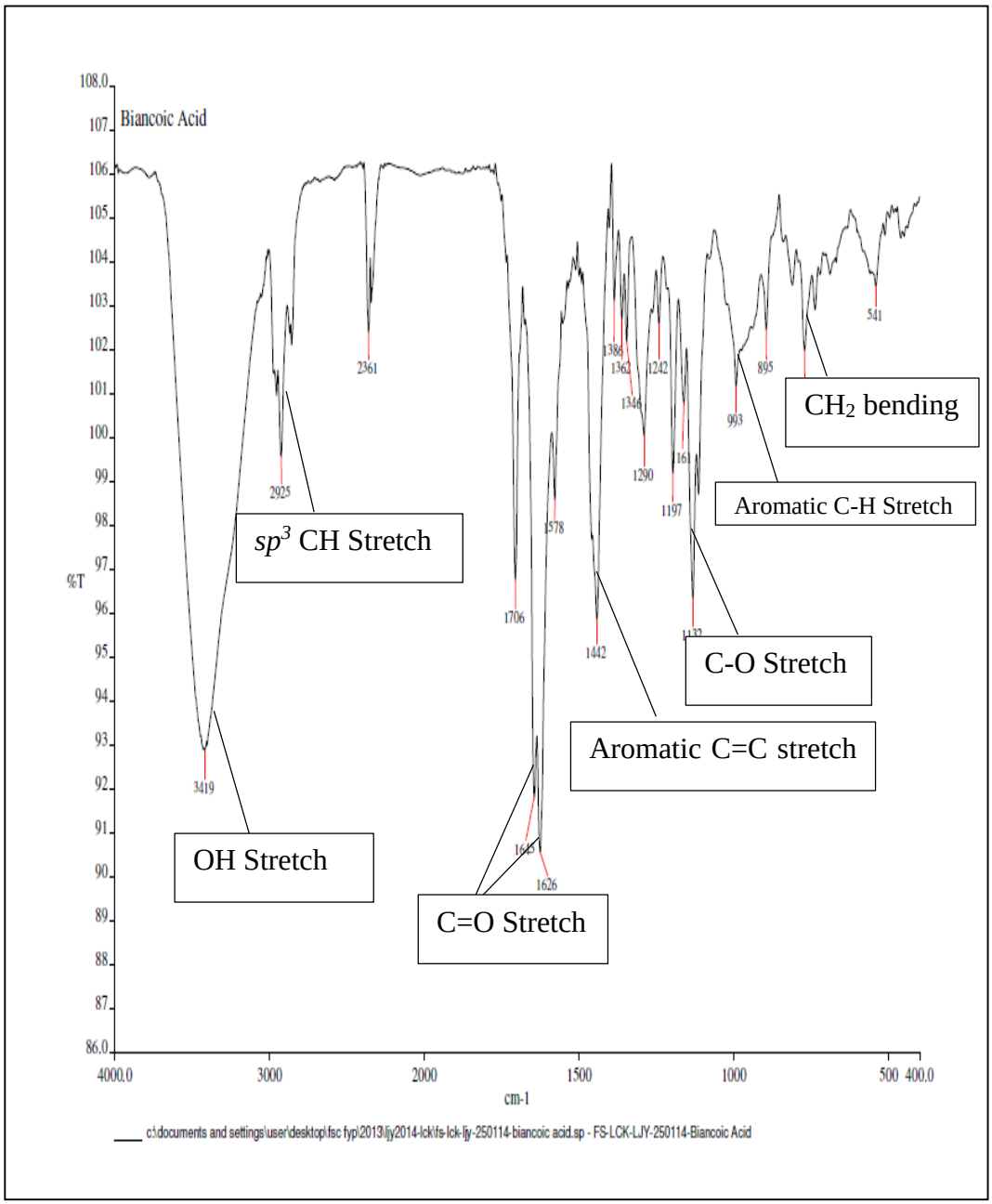
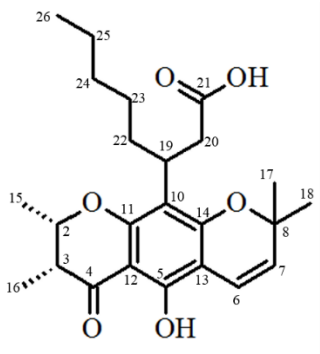


Figure 4.36: IR spectrum of isoblancoic acid [33]



[33]

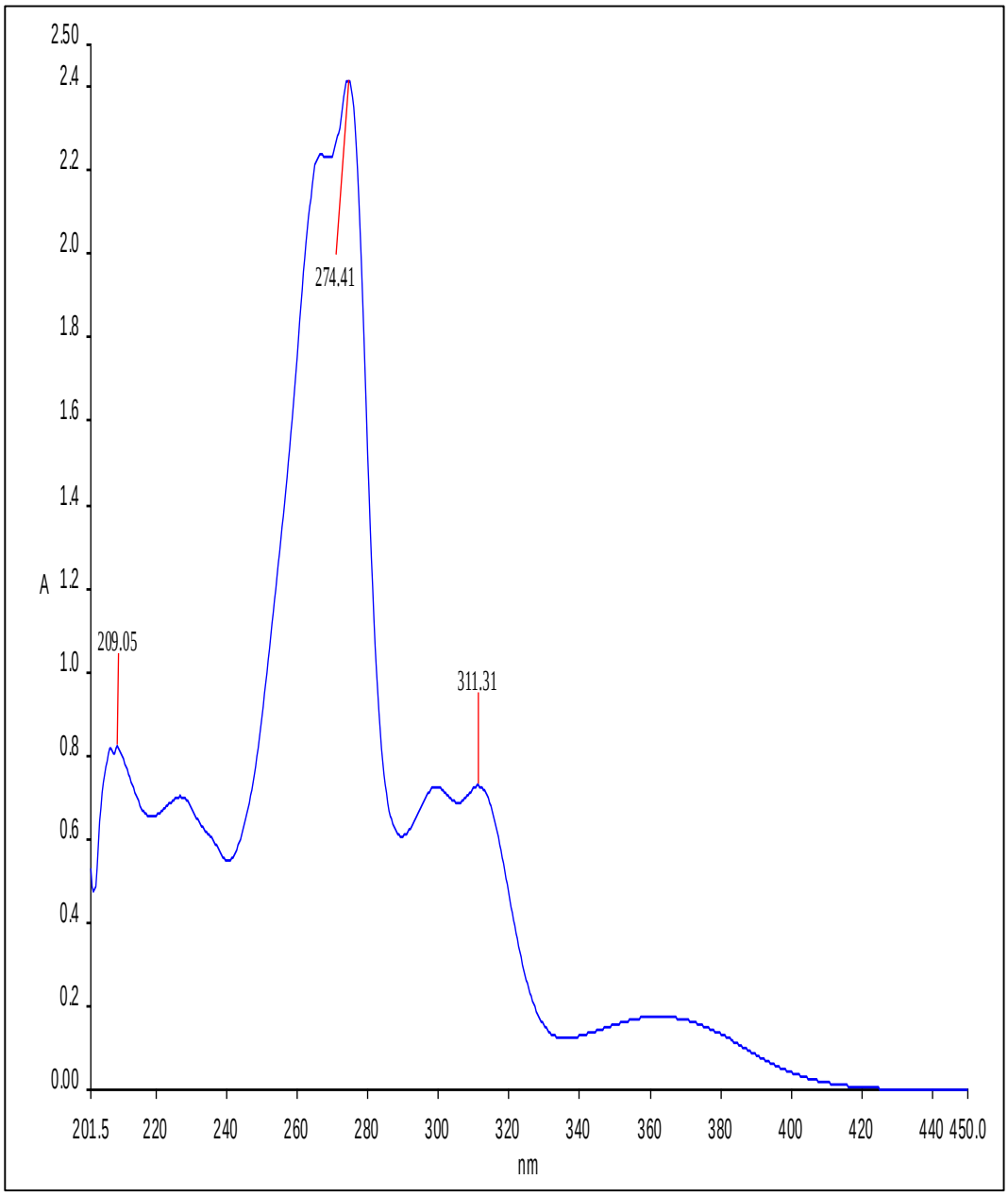
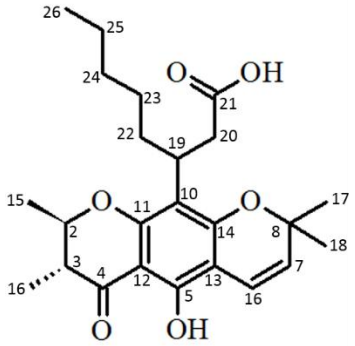


Figure 4.37: UV-Vis spectrum of isoblancoic acid [33]

4.1.5 Characterisation and Structural Elucidation of Blancoic Acid [34]



[34]

Figure 4.38: Structure of blancoic acid [34]

Compound **34** was identified as blancoic acid which is a stereoisomer of isoblancoic acid **[33]**, showing molecular formula of $C_{24}H_{32}O_6$. This compound was isolated as green gummy resin and was found to have a molecular weight of 416.2200 g/mol as shown in HRESI mass spectrum (Figure 4.39). Compound **34** was subjected to optical activity test and obtained a specific rotation, $[\alpha]_{\lambda}^{T}$ of -66.9^{o}, being different from compound **33**. In TLC analysis, this compound gave R_f value of 0.44, when eluted with a solution mixture of 30% ethyl acetate and 70 % hexane. The developed TLC plate developed for this compound is shown in Figure 4.40 below.

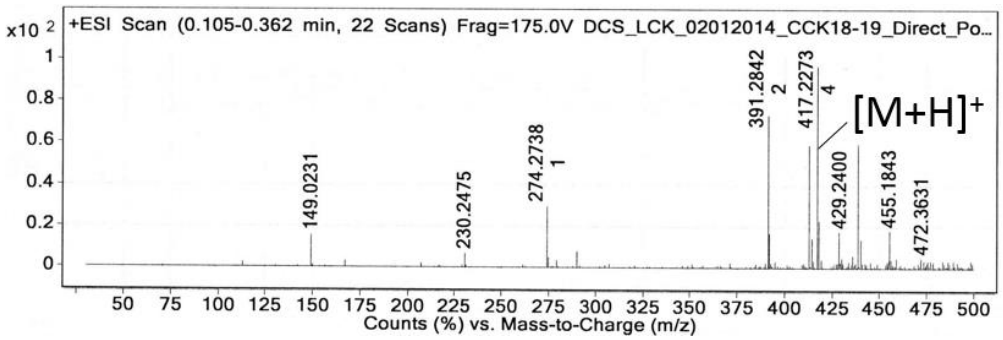


Figure 4.39: HRESI mass spectrum of blancoic acid [34]

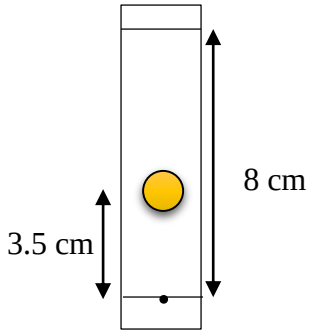


Figure 4.40: The developed TLC plate for blancoic acid [34]

Compound **34** showed a close match of NMR spectral data with isoblancoic acid. In the ^{1}H-NMR spectrum (Figures 4.41 and 4.42), the highly deshielded singlet signal at δ12.48 was assigned to the chelated hydroxyl protons, 5-OH. Signals at δ 1.48 (d, *J*= 6.1 Hz, H-15), 1.18 (d, *J*= 6.7 Hz, H-16), 1.42 (s, H-17), 1.38 (s, H-18) and 0.81 (t, *J*= 6.1 Hz, H-26) were corresponding to the five methyl protons present in the structure.

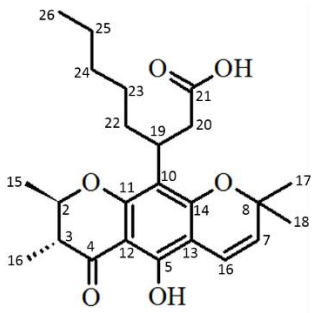
The ^{13}C-NMR spectrum (Figure 4.43) revealed five methyl carbon signals at δ 19.6 (C-15), 10.5 (C-16), 28.4 (C-17), 28.3 (C-18) and 14.2 (C-26). On the other hands, the signals observed at 38.8 (C-20), 33.2 (C-22), 27.5 (C-23), 31.8 (C-24) and 22.6 (C-25) were assigned to methylene carbons. Moreover, the methine

carbons gave signals at δ 79.0 (C-2), 45.8 (C-3), 115.7 (C-6), 125.7 (C-7) and 30.7 (C-19). Meanwhile, signals at δ 199.5, 157.1, 78.2, 109.1, 157.1, 101.9, 102.6, 160.0 and 179.7 were assigned to the nine quarternary carbons C-4, C-5, C-8, C-10, C-11, C-12, C-13, C-14 and C-21, respectively. The structure of compound was further deduced from the 2D-NMR analysis. According to HMBC spectrum (Figure 4.46), the methylene protons at δH 2.78 (H-20a) and 2.70 (H-20b) were ^{2}J coupled to the carbonyl carbon of COOH group (δ 179.7, C-21), and also ^{2}J coupled to a methine carbon (δ 30.7, C-19).All the 2D-NMR correlations between carbons and protons were summarised in Table 4.5. Based on 1D- and 2D-NMR data, compound **34** was identified as blancoic acid.

The assigned stricture was further supported by IR and UV-Vis spectroscopic analyses. In the IR spectrum (Figure 4.47), the presence of carbonyl functional group was revealed by C=O stretch appeared at 1628 cm^{-1}. Besides that, the intense and broad band observed at 3430 cm^{-1} was corresponding to the O-H stretch. The C-O stretch gave absorption band at 1189 cm^{-1}. Meanwhile, absorption band for aromatic C=C stretch, aromatic C-H stretch and CH_2 bending were also found at 1445 cm^{-1}, 994 cm^{-1} and 771 cm^{-1}, respectively. The UV-Vis spectrum (Figure 4.48) gave the characteristic absorption maxima of the compound at 209.05, 274.41 and 311.31 nm, revealing the compound to have a highly conjugated structure which is in agreement with the structure assigned, blancoic acid **[34]**.

Table 4.5: Summary of NMR spectral data of blancoic acid [34]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			J^2	J^3
2	4.09 (1H, m)	79.0	-	-
3	2.51 (1H, m)	45.8	C-2, C-4& C-16	C-15
4	-	199.5	-	-
5	-	157.1	-	-
6	6.58 (1H, d, *J*= 10.0 Hz)	115.7	C-13	C-5, C-8 & C-14
7	5.46 (1H,d, *J*= 10.0 Hz)	125.7	C-8	C-13 & C-17
8	-	78.2	-	-
10	-	109.1	-	-
11	-	157.1	-	-
12	-	101.9	-	-
13	-	102.6	-	-
14	-	160.0	-	-
15	1.48 (3H, d, *J*=6.1 Hz)	19.6	C-2	C-3
16	1.18 (3H, d, *J*= 6.7 Hz)	10.5	C-3	C-2 & C-4
17	1.42 (3H, s)	28.4	C-8	C-7 & C-18
18	1.38 (3H, s)	28.3	C-8	C-7 & C-17
19	3.65 (3H, m)	30.7	C-10, C-20 & C-22	C-14
20	2.78 (1H, dd, *J*= 15.3, 8.5 Hz) 2.70 (1H, dd, *J*= 15.3, 7.0 Hz)	38.8	C-19 & C-21	C-10 & C-22
21	-	179.7	-	-
22	1.82 (1H, m) 1.64 (1H, m)	33.2	C-23	C-10
23	1.22 (2H, m)	27.5	-	-
24	1.20 (2H, m)	31.8	-	-
25	1.24 (2H, m)	22.6	-	
26	0.81 (3H, t, *J*=6.12Hz)	14.2	C-25	C-26
5-OH	12.48 (1H,s)	-	C-12 & C-13	-



[34]

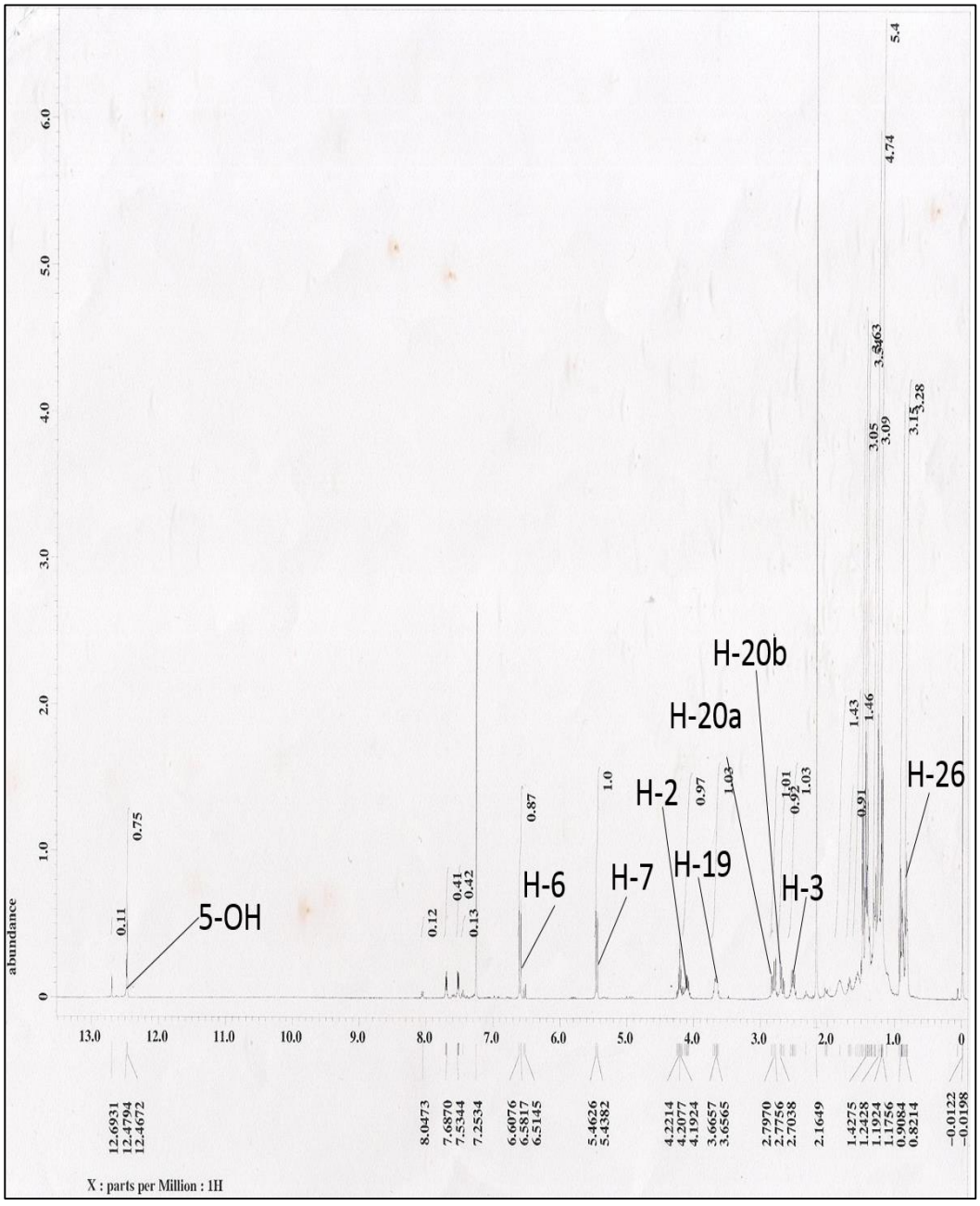
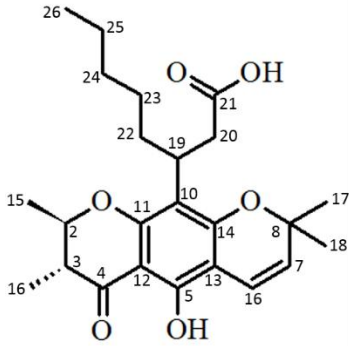


Figure 4.41: ^{1}H-NMR spectrum of blancoic acid [34] (400 MHz, $CDCl_3$)



[34]

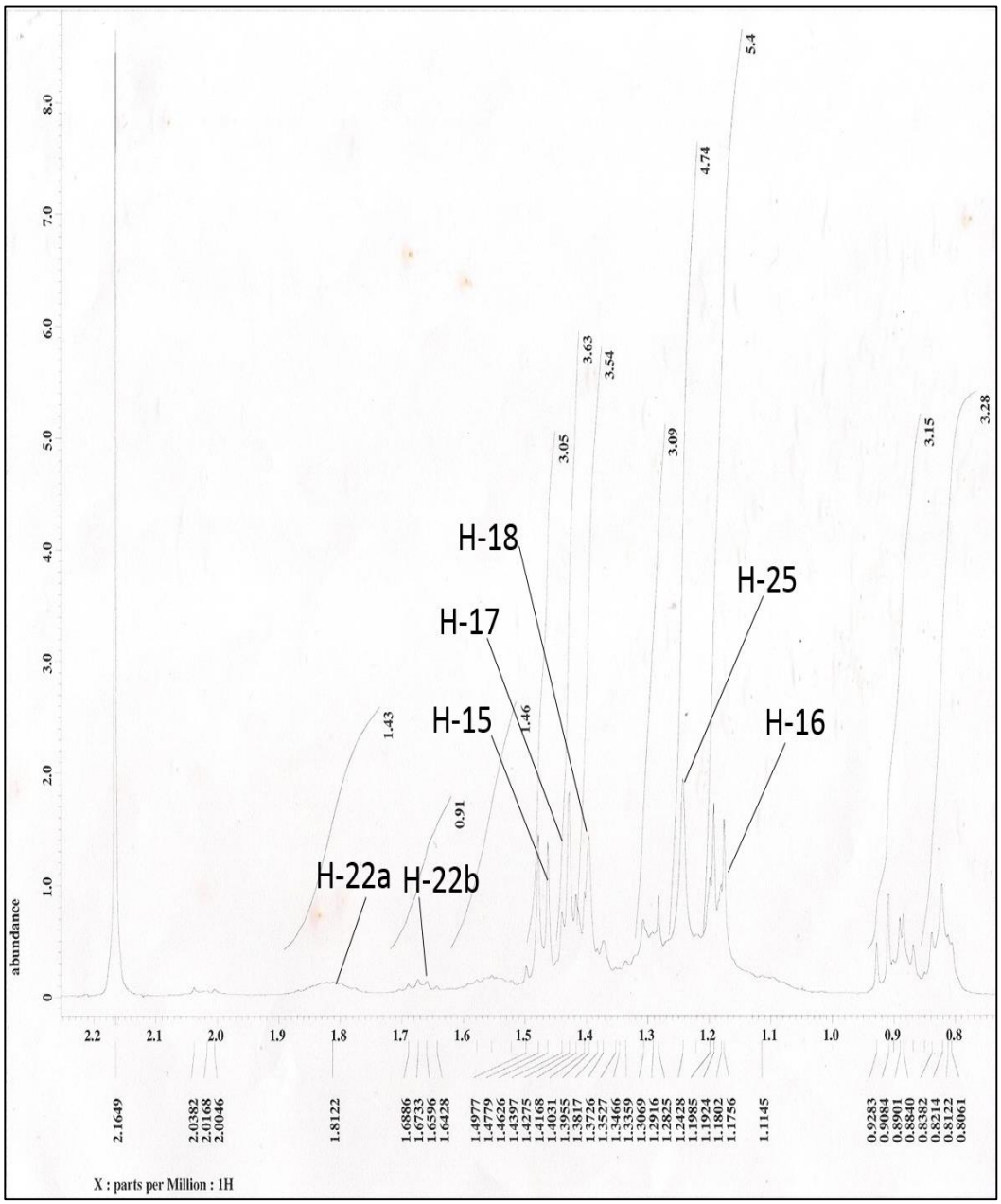
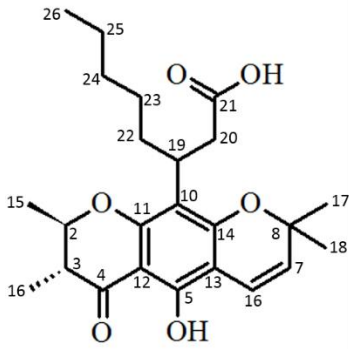


Figure 4.42: ^{1}H-NMR spectrum of blancoic acid [34] (400 MHz, $CDCl_3$) (expanded)



[34]

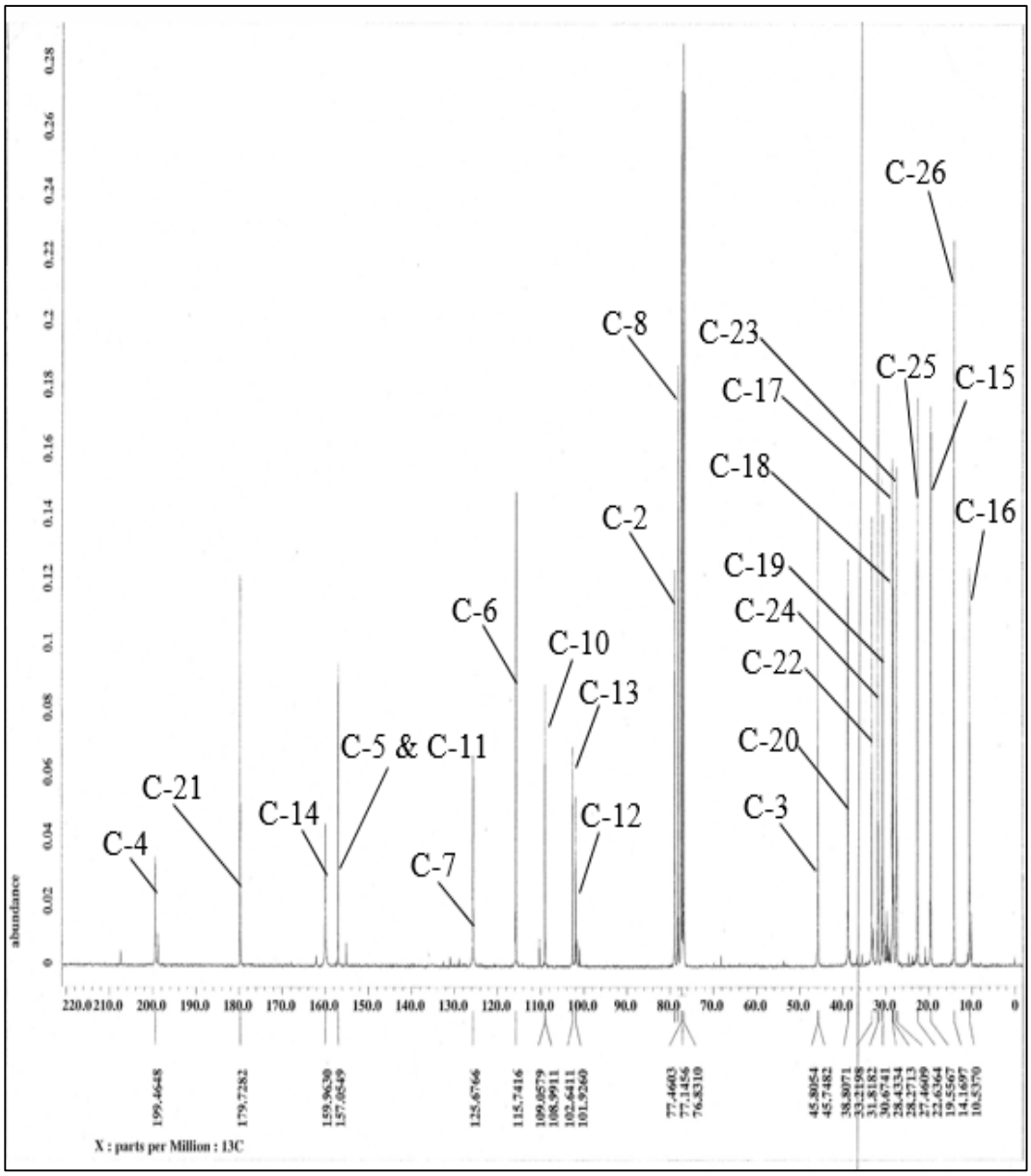
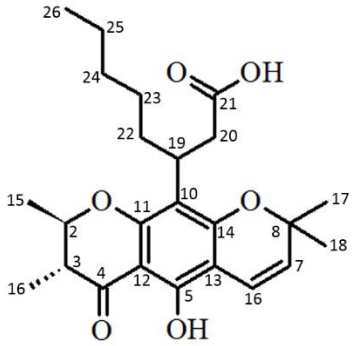


Figure 4.43: ^{13}C-NMR spectrum of blancoic acid [34] (100 MHz, $CDCl_3$)



[34]

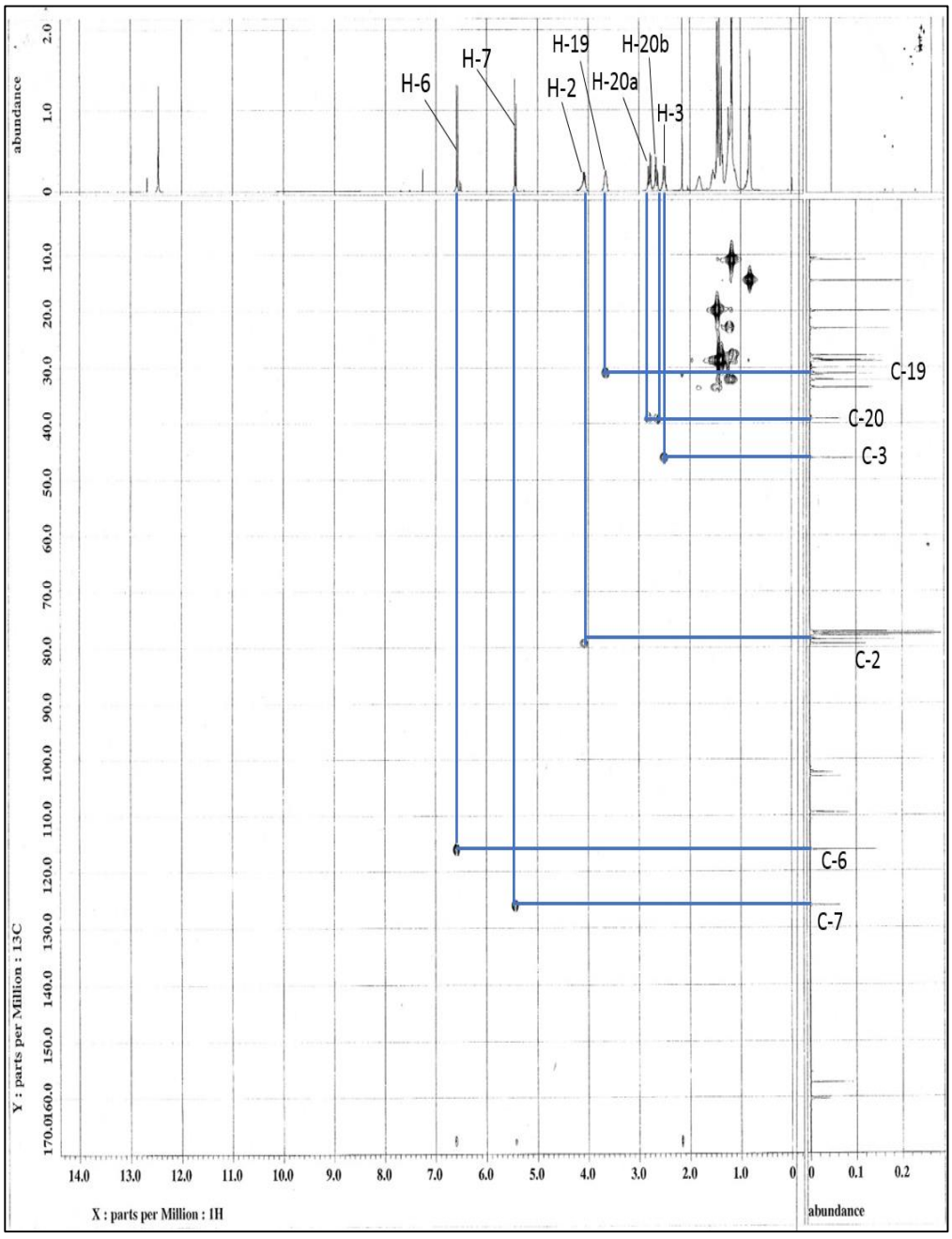
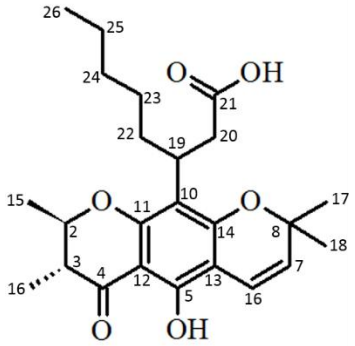


Figure 4.44: HMQC spectrum of blancoic acid [34]



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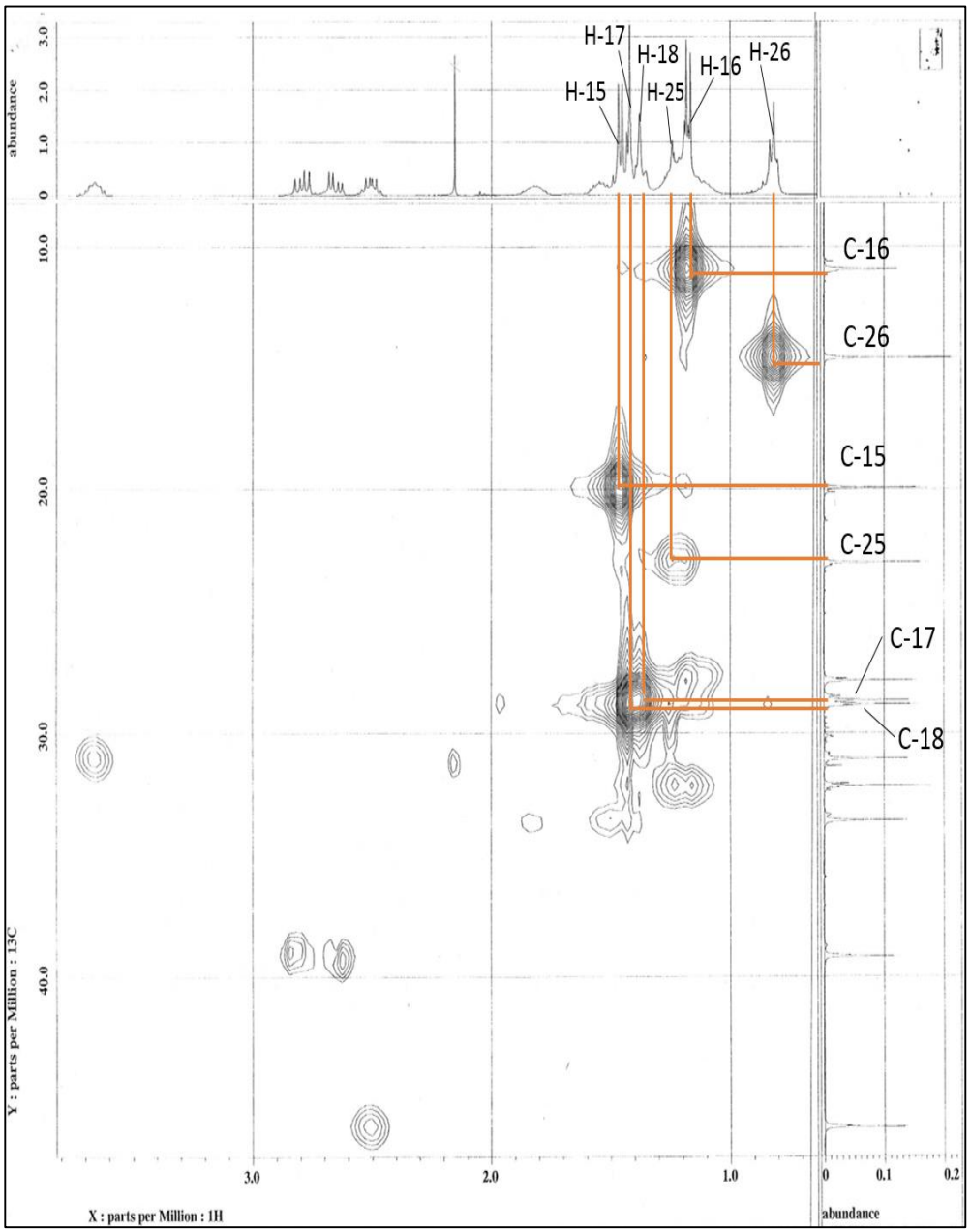
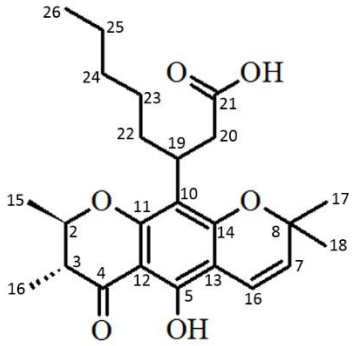


Figure 4.45: HMQC spectrum of blancoic acid [34] (expanded)



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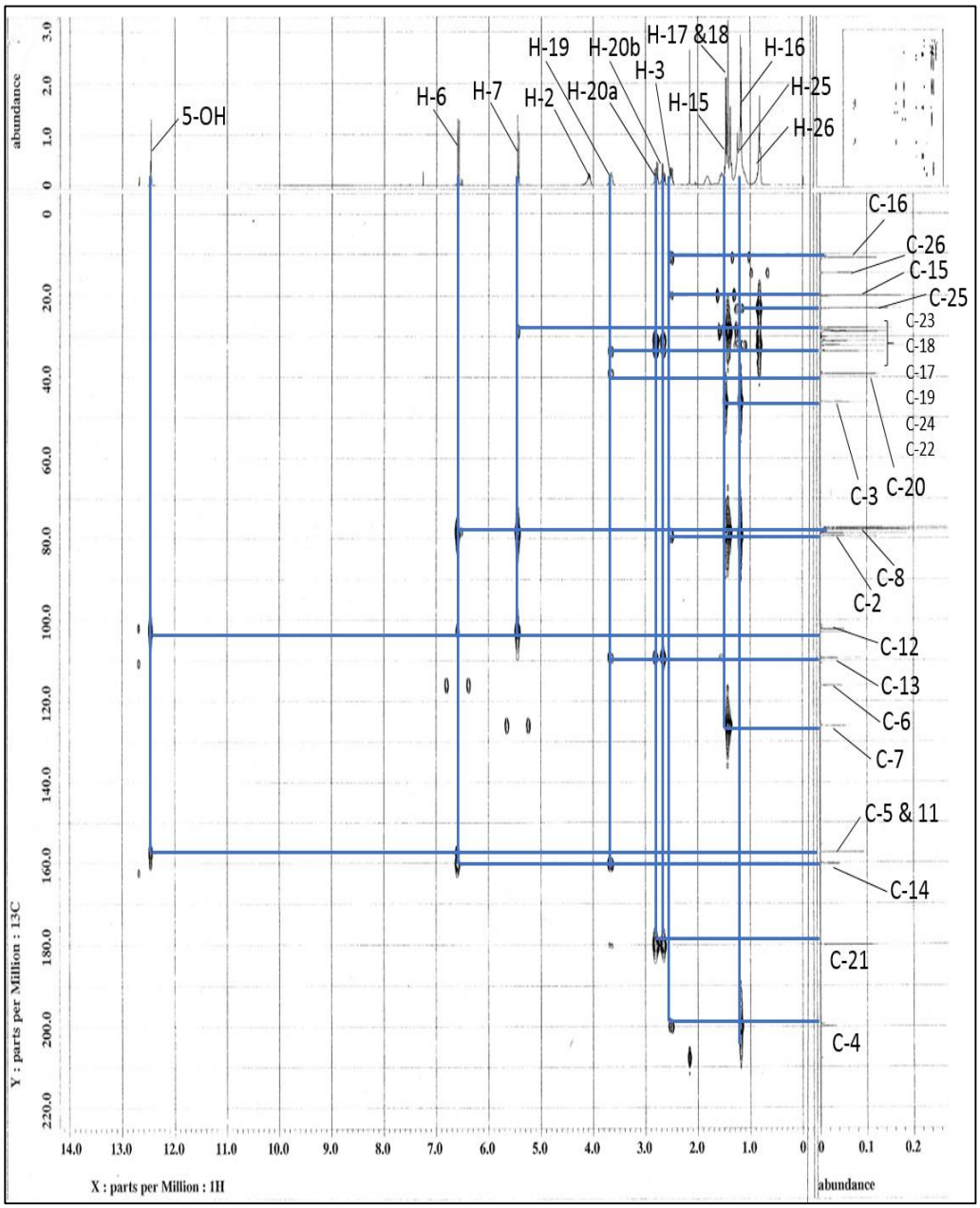
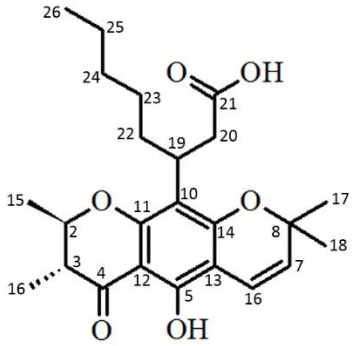


Figure 4.46: HMBC spectrum of blancoic acid [34]



[34]

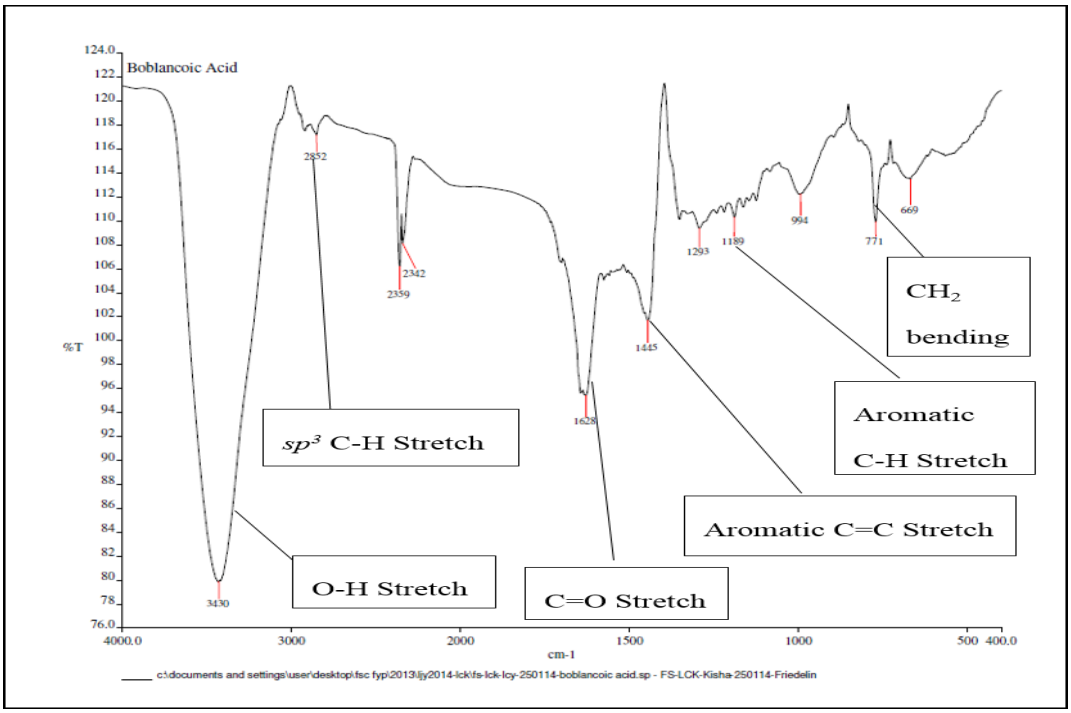


Figure 4.47: IR spectrum of blancoic acid [34]

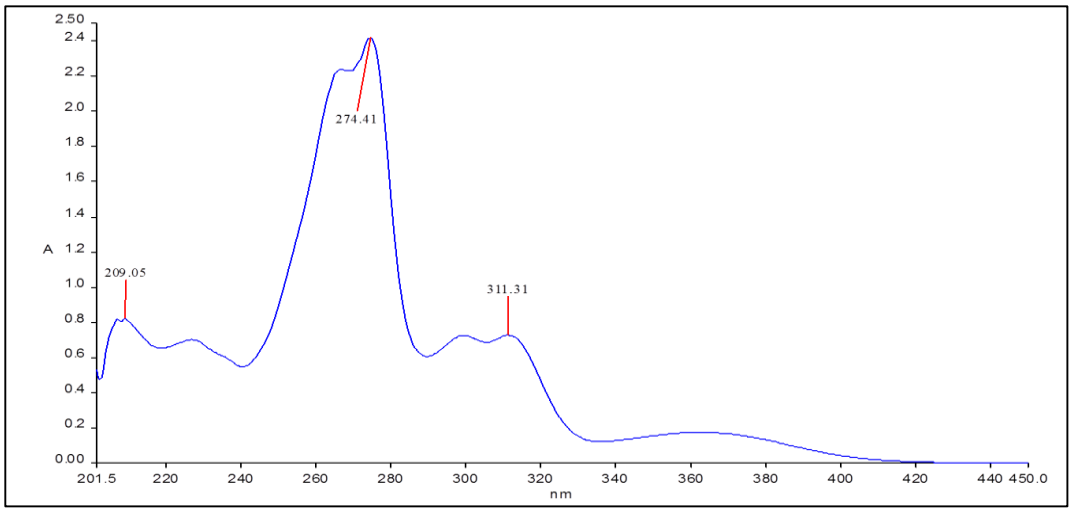


Figure 4.48: UV- Vis spectrum of blancoic acid [34]

4.2 Antioxidant Assay

All the stem bark crude extracts and the isolated compounds were evaluated for their antioxidant activities via DDPH methods. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical that found in the dark-coloured powder form. DPPH is able to resist dimerization due to delocalisation of the spare electron around the molecule (Alam, Bristi and Rafiquzzaman, 2013). The delocalisation of the electron contributed to the deep violet colour which shows a characteristic absorption wavelength at 520 nm. However, the violet colour is reduced to yellow colour when DPPH accepted proton radical from a substrate. IC_{50} value is defined as the effective concentration of a compound to inhibit the radical activity of DPPH by 50%. Linear regression analysis of the dose response curve was used to obtain the IC_{50} value by plotting graph of percentage of inhibition rate versus concentration of test sample as depicted in Figures 4.49, 4.50, 4.51 and 4.52.

From the assay on three crude extracts of *Calophyllum castaneum* and all t isolated compounds, only methanol and ethyl acetate crude extracts exerted inhibitory effect on the radical activity of DPPH although the isolated euxanthone, isoblancoic acid and blancoic acid possessed hydroxyl group in their structures, but negative results were obtained. The results of DPPH assay were summarised in Table 4.6 below.

Table 4.6: Results of DPPH assay for crude extracts and isolated compounds

Sample Tested	Antioxidant Activity, IC_{50} (µg/mL)
Methanol crude extract	12
Ethyl acetate crude extract	84
Dichloromethane crude extract	>240
Friedelin **[7]**	>240
Friedelinol **[31]**	>240
Euxanthone **[32]**	>240
Isoblancoic acid **[33]**	>240
Blancoic acid **[34]**	>240
Kaempferol	16
Ascorbic acid	10

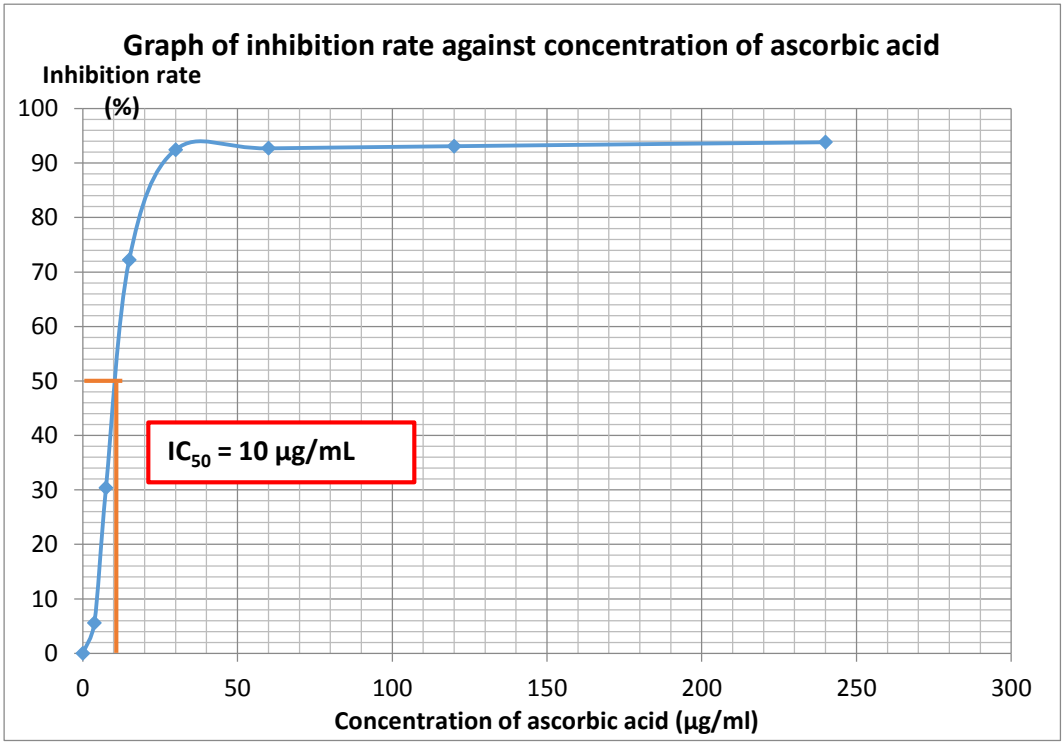


Figure 4.49: Graph of inhibition rate against concentration of ascorbic acid

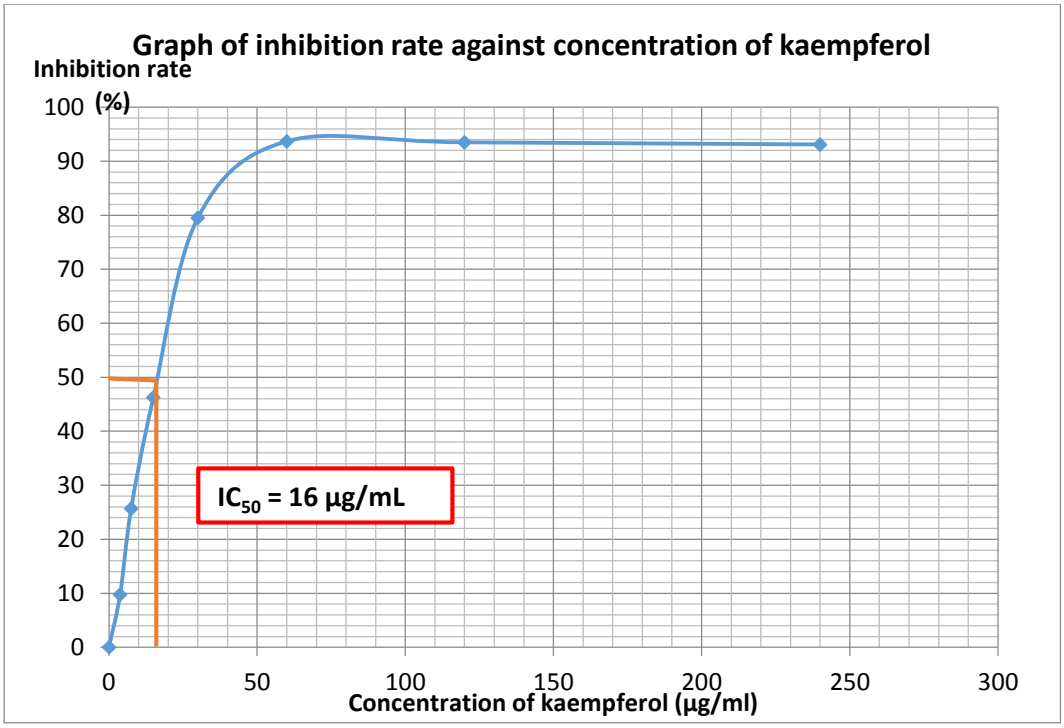


Figure 4.50: Graph of inhibition rate against concentration of kaempferol

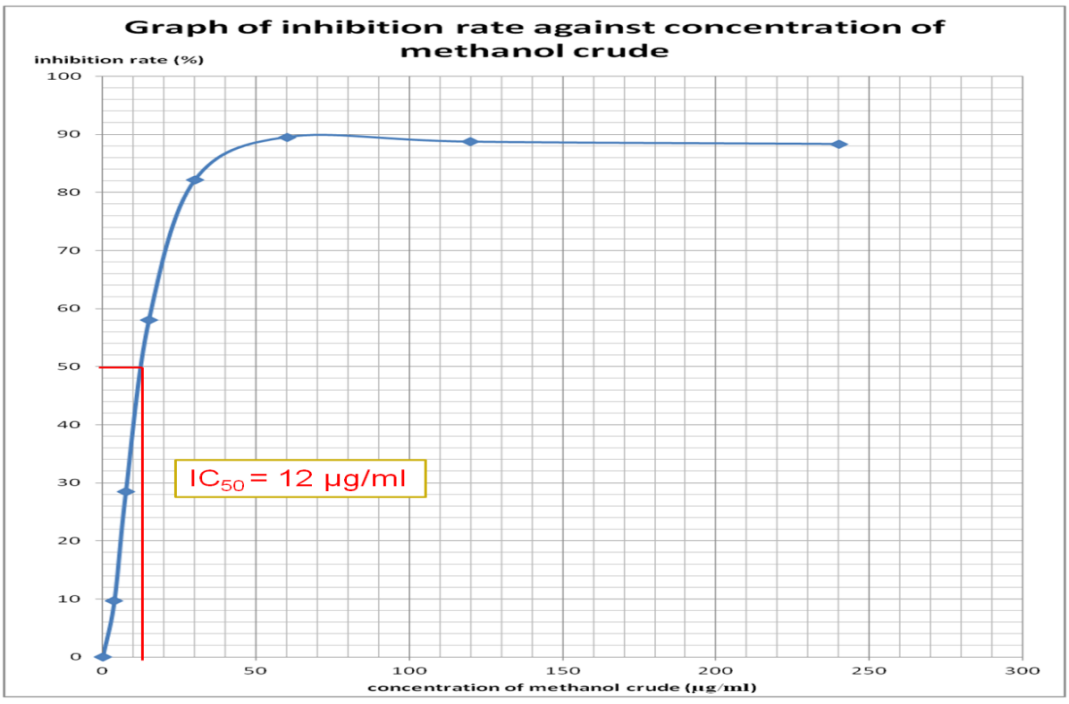


Figure 4.51: Graph of inhibition rate against concentration of methanol crude extract

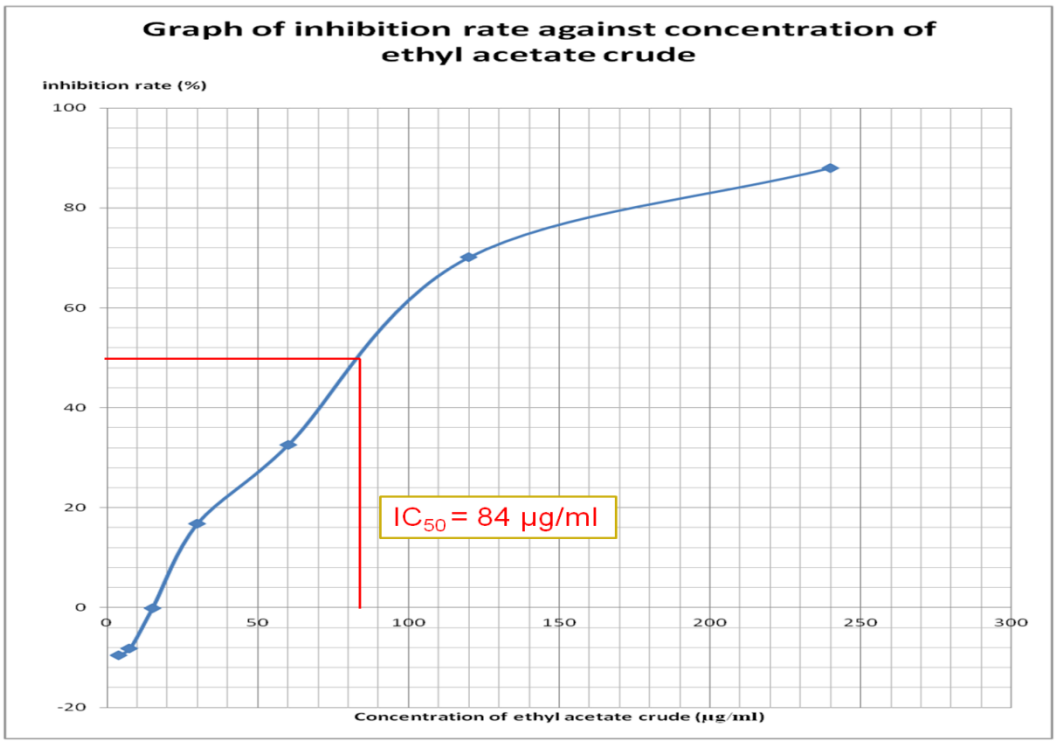


Figure 4.52: Graph of inhibition rate against concentration of ethyl acetate crude extract

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

In this study, a total of five compounds have been isolated from the dichloromethane crude extract of the *Calophyllum castaneum*. They have been identified for their structures as friedelin **[7]**, friedelinol **[31]**, euxanthone **[32]**, isoblancoic acid **[33]** and blancoic acid **[34]** through the application spectroscopic methods, as well as comparison with the literature values of published compounds. In the antioxidant assay, only the methanol and ethyl acetate crude extracts showed the positive results giving their IC_{50} values of 84 μg/mL and 12 μg/mL, respectively. However, dichloromethane crude extract and other isolated pure compounds were reported to be inactive.

5.2 Future Studies and Recommendations

Advanced chromatographic methods such as HPLC, centrifugal chromatography and flash column chromatography are suggested to be applied to improve the separation efficiency, including isolation of minor compounds as well as to fasten up the purification process. In this study, all the isolated compounds and DCM crude extract were found to be inactive in the antioxidant activity, it is recommended that a further investigation on other biological activities such as antimalarial, antimicrobial and anticancer to be carried out to the isolated compounds.

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