PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM LANIGERUM*

BY

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ABSTRACT

PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF

Calophyllum lanigerum

Mong Lai Wan

Chemical study on medicinally important plant secondary metabolites had led to the identification of various useful compounds for further study into their pharmacological activities. It involves extraction, isolation, purification and structural characterization of compounds isolated from plants. In this project, the stem bark of *Calophyllum lanigerum* was subjected to sequential solvent extraction by using dichloromethane and methanol. The chemical compounds present in the crude extracts were subsequently separated using column chromatography. From the dichloromethane crude extract, a xanthone and a triterpenoid were successfully isolated, namely euxanthone **[18]** and friedelin **[19]**. Characterization and identification of these pure compounds were carried out through various spectroscopic analyses, including 1D- and 2D-NMR, UV-Vis and IR.

Besides, all the crude extracts of *Calophyllum lanigerum* and isolated compounds were investigated for their antioxidant potential via DPPH assay. In the assay, methanol crude extract was found to exhibit strong antioxidant

activity with IC_{50} value of 3.0 μg/mL, which is comparable to the positive control used, ascorbic acid (IC_{50} = 4.0 μg/mL). Meanwhile, dichloromethane crude extract, compounds **18** and **19** displayed inactive antioxidant activities with IC_{50} values of more than 240.0 μg/mL.

ABSTRAK

Kajian kimia tentang metabolit tumbuhan yang mempunyai nilai perubatan telah membawa kepada pengenalan pelbagai kompaun berguna untuk kajian aktiviti farmakologi masa depan. Ia melibatkan pengekstrakan, pengasingan, pembersihan dan analisis struktur kompaun. Dalam projek ini, kulit batang pokok *Calophyllum lanigerum* telah diekstrak berturutan dengan menggunakan diklorometana dan metanol. Kompaun-kompaun dalam ekstrak mentah seterusnya diasingkan dengan menggunakan kaedah kromatografi. Daripada ekstrak diklorometana, xanthone dan triterpenoid telah diasingkan, iaitu euxanthone **[18]** dan friedelin **[19]**. Struktur-struktur kompaun ini telah diperolehi melalui pelbagai spektroskopi analisis, termasuk 1D- dan 2D-NMR, UV-Vis dan IR.

Selain itu, ekstrak mentah *Calophyllum lanigerum* dan kompaun-kompaun tulen telah diuji mengenai potensi antioksiden dengan menggunakan kaedah DPPH. Dalam ujian ini, ekstrak mentah metanol didapati menunjukkan aktiviti antioksidan yang kuat dengan nilai IC_{50} 3.0 µg/mL, iaitu sebanding dengan kawalan positif, asid askorbik (nilai IC_{50} = 4.0 µg/mL). Sementara itu, ekstrak mentah dichloromethane, kompaun [18] dan kompaun [19] menunjukkan aktiviti antioksidan tidak aktif dengan nilai IC_{50} lebih daripada 240.0 µg/mL.

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A million thanks to my family members for their motivation, enduring love and financial support. Thank you for being understanding. Last but not least, I wish to express my special gratitude to my friends, Ang Yen Yuan and Chelsea Quek Mei Ing for their sincere advice and emotional support during my difficult times.

DECLARATION

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

(MONG LAI WAN)

APPROVAL SHEET

The project report entitled "**PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM LANIGERUM***" was prepared by MONG LAI WAN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **MONG LAI WAN** (ID No: **15ADB07643**) has completed this final year project entitled "**PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM LANIGERUM***" supervised by Dr. Lim Chan Kiang from the Department of Chemical Science, Faculty of Science.

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

Yours truly,

____

(MONG LAI WAN)

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation end-product
B.C.	Before Christ (used in timeline)
β	Beta
^{13}C	Carbon-13
C=C	Carbon=Carbon
C-H	Carbon-Hydrogen
C-O	Carbon-Oxygen (or Carbinol)
C=O	Carbon=Oxygen (or Carbonyl)
cm	Centimeter
δ	Chemical shift
c	Concentration of sample in g/mL
J	Coupling constant in Hertz
^{o}C	Degree in Celsius
Acetone-d_6	Deuterated acetone
$CDCl_3$	Deuterated chloroform
d	Doublet
dd	Doublet of doublet
DCM	Dichloromethane
EtOAc	Ethyl acetate
FTIR	Fourier-Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
g	Gram

IC_{50}	Half maximal inhibitory concentration
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
Hz	Hertz
HRESIMS	High Resolution Electrospray Ionization Mass
HPLC	High Performance Liquid Chromatography
IR	Infrared
kg	Kilogram
LC-MS	Liquid Chromatography-Mass Spectrometry
m/z	Mass-to-charge ratio
λ_{max}	Maximum wavelength
MeOH	Methanol
μg	Microgram
μL	Microliter
μmol	Micromole
mg	Miligram
mL	Mililiter
mm	Milimeter
mM	Milimoles
mol	Mole
m	Multiplet
nm	Nanometer
NMR	Nuclear Magnetic Resonance
1D-NMR	One Dimension Nuclear Magnetic Resonance

O-H	Oxygen-Hydrogen (or Hydroxyl)
ppm	Part per million
KBr	Potassium bromide
^{1}H	Proton
R_f	Retention factor
s	Singlet
TMS	Tetramethyl silane
TLC	Thin Layer Chromatograph
t	Triplet
TE	Trolox Equivalent
2D-NMR	Two Dimension Nuclear Magnetic Resonance
UV-Vis	Ultraviolet-Visible
DPPH	2,2-Diphenyl-1-picrylhydrazyl

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Natural products have been a source for remedies since ancient time. In the present day, the use of medicines that are derived from natural product is growing worldwide due to their incomparable chemical diversity and novel mechanism of action (Yuan et al., 2016). Generally, natural products are referring to the chemical compounds produced by living organisms such as plants, animals, microorganisms. These chemical compounds can be categorized into two classes, which are primary and secondary metabolites. Primary metabolites such as proteins, vitamins and lipids play an integral role in life processes such as growth development, reproduction and metabolism. In contrast, secondary metabolites are not directly involved in those processes, but they are produced for the homeostasis and maintenance of the living organisms. As an illustration, pigments such as terpenoid, carotenes and flavonoids give color and phenolic odors to attract pollinators. Apart from that, secondary metabolites serve as competitive or defensive weapons against competitors. It is the area of secondary metabolites that possess pharmacologically active products that can be used for treatment of diseases (Cragg, Newman and Snader, 1997).

In the early drug discovery, primary sources of various medicines are plants with ethnopharmacological uses. The significance of natural products in drug discovery has been reviewed and targeted in the investigation of natural products as the source for treatment of cancer. An analysis of the number and sources of chemotherapeutic agents indicates that over 60% of the approved drugs are derived from plants (Cragg, Newman and Snader, 1997). According to Butler, from 2005 to 2007, a total of 13 natural products related drugs were approved for marketing worldwide, and, five of them are new human drug classes which are ziconotide, exenatide, retapamulin, trabectedin and ixabepilone (Butler, 2008).

Phytochemistry is the study of chemistry of natural products, chemicals derived from plant species either qualitatively or quantitatively. The methodologies used are extraction, isolation, purification and structure elucidation of isolated compounds. Drugs are produced with the aid of advanced extraction technology, in which the bioactive compounds from plants can be chromatographically isolated, purified and used to produce drugs. Furthermore, the identified natural products will be used as templates to synthesize new drugs (Lahlou, 2013).

Secondary metabolites produced by plants consist of a variety of classes including alkaloids, xanthones, terpene, coumarins are categorized based on their chemical structures. Although they are not essential to life processes, but their antioxidant properties are benefit to human health. Reactive and unstable free radicals are dangerous products of cellular metabolism and it can directly

harm the cell growth and development. These free radicals can damage cells by attacking important macromolecules including lipids, nucleic acids and proteins which cause oxidative stress leading to the unbalance system between pro-oxidants and antioxidants. Oxidative stress has significantly contributed to all inflammatory diseases and to increase the risk of developing certain type of cancer and cardiovascular diseases. Antioxidants can decrease oxidative stress by inhibiting the oxidation of substrate. They are stable molecules which are able to neutralize the rampaging free radicals by donating an electron to them to form a relatively stable compounds, thus reducing their capacity to damage the cells (Lobo et al., 2010).

In 1970, combretastatin was found from the stembark of South African "bush willow", *Combretum caffrum* which exhibited cancer chemo preventive activity and acted as anti-angiogenic agent. It was able to inhibit the growth of tumor blood vessel, and tumor cell death and necrosis occurs (Cragg and Newman, 2005). Malaysia is ranked as the 12th megadiverse country, which 60% of the country's land is still forested. It is estimated that there are around 15,000 species of vascular plants and around 10% of them have medicinal potential (Jamal, Barkat and Amid, 2010). In this project, works have been focused on the isolation of bioactive secondary metabolites from *Calophyllym lanigerum* and to investigate for their antioxidant activity.

1.2 Botany of Plant Species Studied

1.2.1 Taxonomy

The taxonomy of *Calophyllum lanigerum* is shown in Table 1.1.

Table 1.1: Taxonomy of *Calophyllum lanigerum*

Species	*Calophyllum lanigerum*
Genus	*Calophyllum*
Family	Calophyllacae
Order	Malpighiales
Class	Spermatospida
Division	Tracheophyta
Kingdom	Plantae

1.2.2 Morphology

The Calophyllaceae family consists of about 190 species of evergreen trees which can grow up to 21 m in height and up to 81 cm in diameter. The trees have long-stalked leaves with thickly and leathery leaf blades and have shape in narrowly egg to oblong. The flowers are white and about 3-7 cm in length.

Calophyllum lanigerum produces egg-shaped fruits which is in green to yellow color (Stevens, 2018). The picture of *Calophyllum lanigerum* is shown in Figure 1.1.

Figure 1.1: *Calophyllum lanigerum*'s fruits, leaves and flowers

1.2.3 Geographical Distribution and Habitat

Calophyllum lanigerum can be found in the tropical rainforest of Asia such as Singapore, India and Malaysia (Dweck and Meadows, 2002). *Calophyllum* is locally known as *bintangor*. They usually grow in lowland primary rainforest, forests near the sea and some in the swamp-forest.

1.2.4 Ethnomedical Uses and Pharmacological Studies

The *Calophyllum* species have been used in traditional medicines and the oil extracted from the fruit is used for treatment for rheumatism, ulcers and skin diseases. The leaves and stem barks of these plants are used to treat different diseases including pain, infections, inflammation, and against diarrhea. According to phytochemical studies, *Calophyllum* species contain mainly phenolic compounds like xanthones, coumarins and flavonoids. Many studies stated that some of the compounds extracted such as calanolides and inophyllums, were shown the ability to inhibit HIV-1 replication (Shen et al., 2004).

1.3 Problem Statement

Antioxidants are molecules that inhibit oxidation of other molecules by quenching the free radicals. It can neutralize free radicals by accepting or donating electrons to eliminate the unstable free radical and to form a relatively stable molecule. Free radicals can induce oxidative stress and cause a significant cellular damage in the body.

Antioxidant is classified into natural antioxidant and synthetic antioxidant. Natural antioxidants usually can be obtained from plants like fruits and vegetable. Nowadays, synthetic antioxidants such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) are widely used in market. Despite synthetic antioxidants show the advantage that they can be produced in large amount, they still have some limitations. Synthetic antioxidants may cause adverse effects in human health which may lead to cancer whereas natural antioxidants are more readily accepted by our body (Thorat, 2013).

Study in natural antioxidants are needed to complement the synthetic antioxidants. In this study, we are going to study the antioxidant activity of *Calophyllum lanigerum* via DPPH assay by using kaempferol and ascorbic acid as positive controls.

1.4 Objectives of Study

There are three main purposes of this study are:

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

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemical Studies

Phytochemistry is generally known as the study of chemicals constituents that are derived from plants. Phytochemicals are also known as secondary metabolites that are produced via secondary metabolism pathway. They are not important in growth and development of plants, but plants synthesize them to protect themselves against diseases or insect attacks. For instance, plants produce benzopyranones to kill pathogenic microbes and show germination inhibitory properties (Tiwari, 2015). The main concerns of phytochemical study are to isolate chemical constituents from plants and to study for their biological activity.

Secondary metabolites produced by plants are classified into different chemical classes including alkaloids, xanthones, terpene, coumarins which are biologically active and highly valuable to humans. Due to their biological activities, these bioactive compounds have been used as remedy to cure various diseases such as antitumor, anti-malarial and antiviral (Kathirvel and Sujatha, 2016). Phytochemicals are a unique resource for fine chemicals, pharmaceuticals and food additives. Therefore, phytochemicals play a significant role in health care system, they are served as lead compound for

chemical synthesis giving a wide range of compounds with diverse health-related benefits (Naithani et al., 2010).

2.1.1 Coumarins

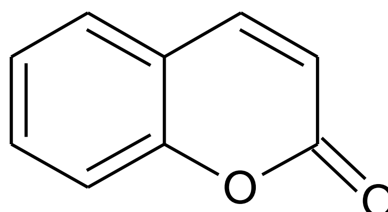


Figure 2.1: Basic building block of coumarins

Coumarins (1-benzopyran-2-one) are colorless and crystalline compounds with a vanilla like flavor. They are compounds from benzopyrones family, which consist of a benzene ring joined with a pyrone ring as shown in Figure 2.1, giving the molecular formula of $C_9H_6O_2$ (Rohini, 2014). Coumarins can be categorized into four main types based on their chemical structure which are simple coumarins, pyranocoumarins, pyrone-substituted coumarins and furanocoumarins. It can be found in several plants such as Tonka beans, lavender, cinnamon, and strawberries.

Numerous reports have revealed that coumarins possessed different biological activities such as anticoagulant, anti-tumor and immunostimulatory activities. Thus, they are broadly used in clinical medicine (Jain and Joshi, 2012). For instance, scoparone isolated from the Chinese herb *Artemisia scoparia*

(Yinchen) is used to treat jaundice (Hoult and Payá, 1996). In 1994, a pyranocoumarin with anti-HIV activity was isolated from the latex of the *Calophyllum teysmannii* (Spino, Dodier and Sotheeswaran, 1998).

2.1.2 Xanthones

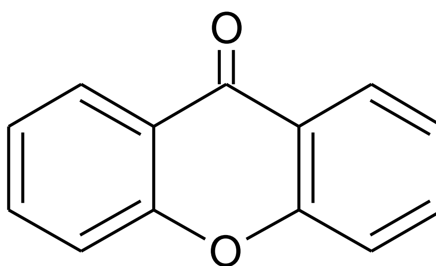


Figure 2.2: Basic building block of xanthones

Xanthones (xanthen-9-one) are crystalline compounds with yellow in color. They are polyphenolic compounds which compose of two benzene rings linked by a carbonyl group and an oxygen bridge as shown in Figure 2.2 and having a core molecular formula of $C_{13}H_8O_2$. Xanthones are commonly found in higher plant families like Gentianaceae, Clusiaceae, Moraceae, Guttiferae, and Polygalaceae. Xanthones can be categorized into five main groups which are simple xanthone, prenylated xanthones, xanthone glycoside, bisxanthone and xanthonolignoids. (Negi et al., 2013).

Xanthones show diverse biological activities including, antioxidative, anticancer, antihypertensive and antithrombotic. The biological activities of xanthones are influenced by the substituents present in the ring system (Na,

2009). According to Akao, Nakagawa and Nozawa, they isolated α-mangostin, β-mangostin, γ-mangostin from *Garcinia mangostana* and found that they strongly inhibited cell growth (Akao, Nakagawa and Nozawa, 2008).

2.1.3 Terpenes

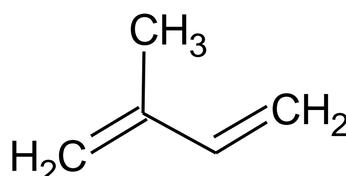


Figure 2.3: Basic building block of terpenes (isoprene)

Terpenes are a large class of phytochemical that are built up from isoprene with a molecular formula of $(C_5H_8)_n$ as shown in Figure 2.3. Terpene hydrocarbons can be categorized according to their number of isoprene units giving rise to monoterpenes, diterpenes, sesquiterpenes, triterpenes, tetraterpenes and polyterpenes. They usually have a strong smell that used to protect the plant by deterring herbivores or attracting predators and parasites of herbivores (Gershenzon and Dudareva, 2007).

Due to the high lipophilic nature of these compounds, the loss of chemiosmotic control is due to their toxicity and their principal target is on the cell membrane. For instance, one of the common antimalarial drugs, the sesquiterpene lactone artemisinin from *Artemisia annua.* Moreover, diterpene paclitaxel (Taxol) exhibits anticancer activity, which is able to kill tumor cells by interfering the

microtubule dynamics and arrests mitosis by binding to the tubulin (Gershenzon and Dudareva, 2007).

2.1.4 Flavonoids

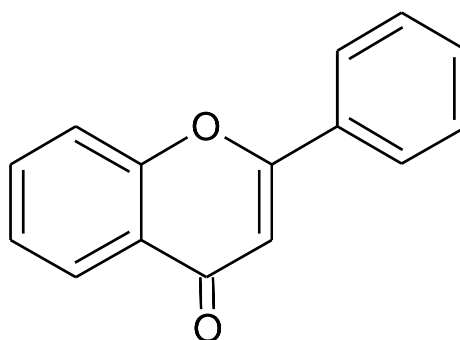


Figure 2.4: Basic building block of flavonoids

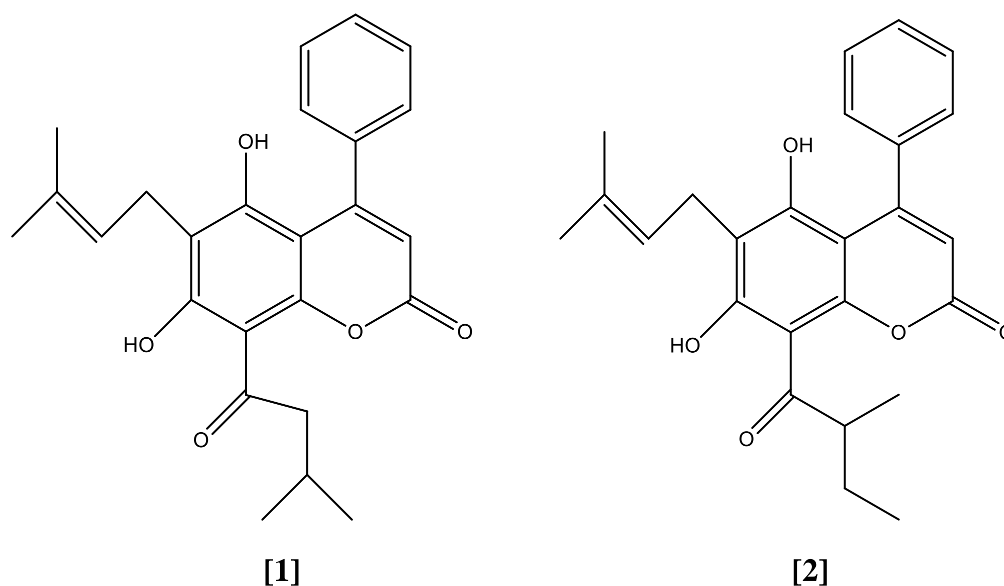
Flavonoids have a general structure which consists of a heterocyclic ring and two phenyl rings as shown in Figure 2.4. The name of flavonoid was arised from Latin word flavus meaning yellow, because most of the flavonoids are yellow in color. They serve as plant pigments or co-pigments which responsible for various color exhibited by seeds of plants, barks, flowers, barks and leaves (Bhat, Nagasampagi and Meenakshi, 2009). In pharmacological aspect, flavonoids show oxidation-reduction potentials since flavonoids are phenolic compounds. It is capable to protect unsaturated fatty acids in membrane as ascorbate against oxidation (Havsteen, 2002). In addition, it also can act as inhibitors of cyclin-dependent kinases for treatment of breast carcinoma cells (Rice-Evans, Miller and Peganga, 1996).

2.2 Chemistry of *Calophyllum* Species

The genus *Calophyllum* is a valuable source of biologically active compounds which is highly demanded in pharmaceutical industry. There is around 200 *Calophyllum* species but only around 15 % of them have been phytochemically studied. Among all these species, only *Calophyllum inophyllum, Calophyllum teysmannii*, *Calophyllum soulattri* and *Calophyllum symingtonianum* have been extensively studied. Based on the previous studies, *Calophyllum* species have shown to be rich in xanthones, coumarins, triterpenoids, and flavonoids (Su et al., 2008).

2.2.1 *Calophyllum brasiliense*

In year 2007, Ruiz-Marcial et al. have collected the leaves of *Calophyllum Brasiliense* from Brazil and successfully isolated two mammea type of coumarins, namely mammea A/BA **[1]** and mammea A/BB **[2]**. Cytotoxic activity of isolated compounds was studied via baby mouse kidney (BMK) cells assay. From the assay, the survival of BMK cells was obviously decreased by compound **1** and **2** and showed a concentration dependent inhibitory effect on the growth of BMK cells. When coumarins were at lowest concentration (3 µg/mL), the surviving BMK cells were reduced by 20 % whereas at highest concentration (20 µg/mL), the declined in cell survival was greater than 50 % (Ruiz-Marcial et al., 2007).

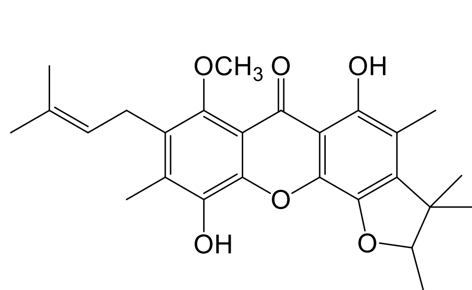


[1] **[2]**

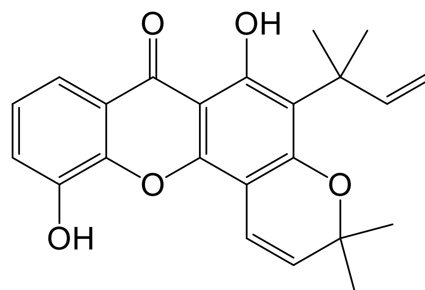
Figure 2.5: Molecular structure of chemical constituents isolated from *Calophyllum brasiliense*

2.2.2 *Calophyllum inophyllum*

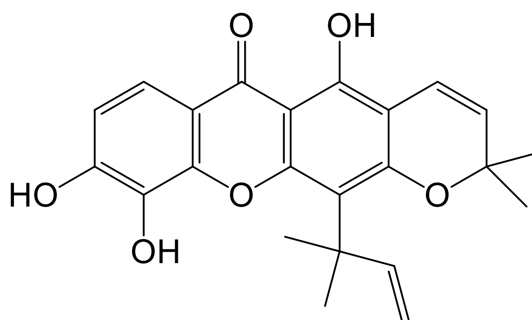
In year 2011, chemical study on the stem bark of *Calophyllum inophyllum* yielded one new furanoxanthone, namely inophinnin **[3]**, with 8 known compounds which were inophyllin A **[4]**, macluraxanthone **[5]**, pyranojacareubin **[6]**, 4-hydroxyxanthone **[7]**, friedelin **[8]**, stigmasterol **[9]** and betulinic acid **[10]**. Their anti-inflammatory activity was tested by using nitric oxide assay. Compound **3** was found to be potent anti-inflammatory activity showing IC_{50} value of 25 µg/mL (Ee et al., 2011).



[3]



[4]



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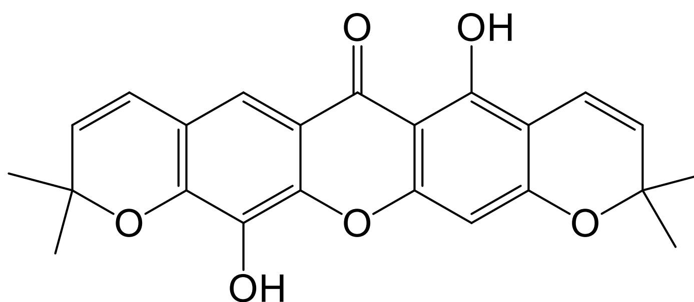


[6]

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

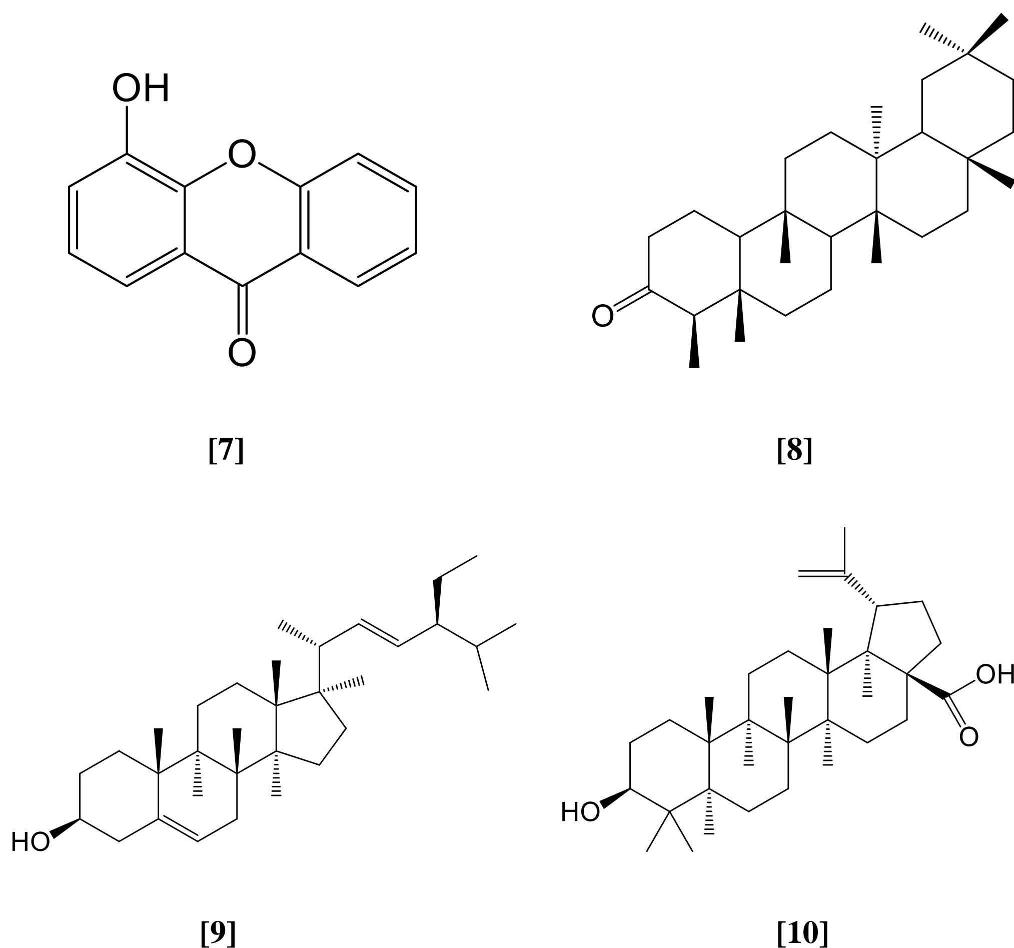


Figure 2.7: Molecular structure of chemical constituents isolated from *Calophyllum inophyllum* (continued)

2.2.3 *Calophyllum symingtonianum*

In later year 2015, Aminudin et al. have isolated 7 compounds which were identified as inophyllum D **[11]**, inophyllum H **[12]**, calanone **[13]**, isocordato-oblongic acid **[14]**, amentoflavone **[15]**, carpachromene **[16]** and lupenone **[17]**. All these compounds displayed a significant α-glucosidase inhibitory activity while only compound **15** showed a positive result against 15-lipoxygenase (15-LOX) inhibition. Both flavonoids, **15** and **16** displayed a strong α-glucosidase

inhibitory activity with IC_{50} values of 6.4 and 13.5 µg/mL respectively which were much lower than the standard used, acarbose with IC_{50} value of 456.4 µg/mL. The IC_{50} values of compounds **11**, **12** and **13** were 35.7, 62.3 and more than 100 µg/mL, respectively. From the IC_{50} inhibition trends, it was proven that the 2,3-dimethylchromanol ring in the coumarin structure played a significant role for the inhibition. Moreover, compound **15** was the most potent inhibitor for 15-LOX with the lowest IC_{50} value of 0.04 µg/mL, much lower than the standard used, quercetin (IC_{50} = 3.6 µg/mL). This might be due to the high number of hydroxyl groups present in compound **15** (Aminudin et al., 2015).

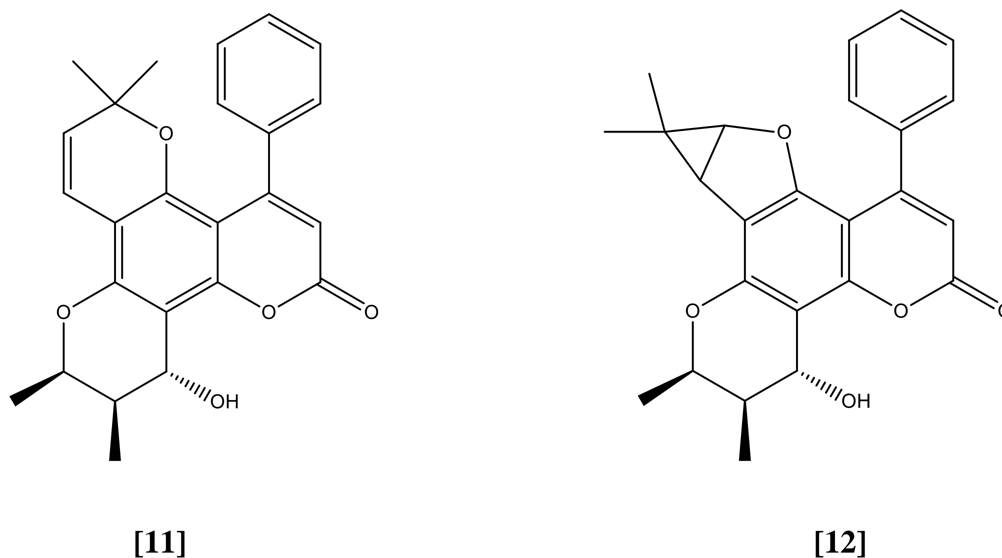
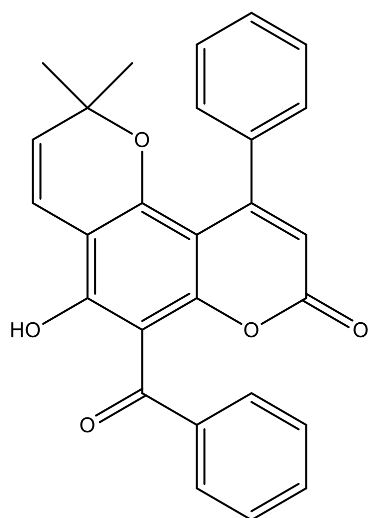
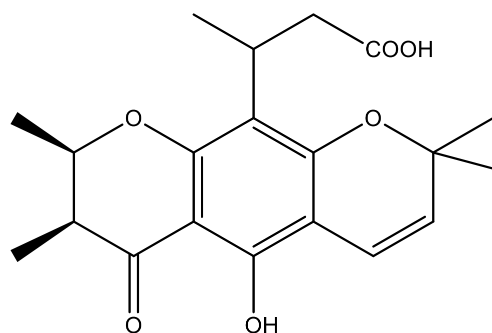
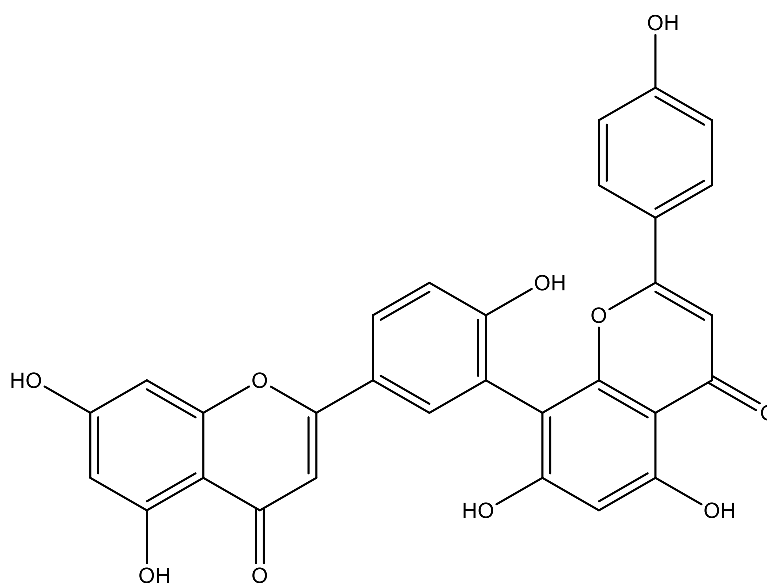


Figure 2.8: Molecular structure of chemical constituents isolated from *Calophyllum symingtonianum*





[13] **[14]**



[15]

Figure 2.9: Molecular structure of chemical constituents isolated from *Calophyllum symingtonianum* (continued)

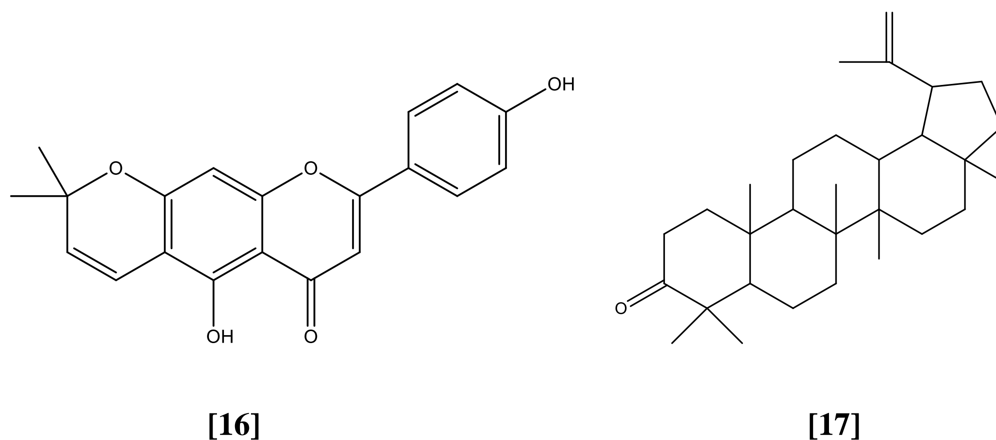


Figure 2.10: Molecular structure of chemical constituents isolated from *Calophyllum symingtonianum* (continued)

2.3 Summary of Literature Review on the Genus *Calophyllum*

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

Plant Species	Classes of Compounds	Biological Activities	References
C. antilanum	• Chromanones	• Antimalarial • Cytotoxic	• Cuesta-Rubio et al., 2015
C. brasiliense	• Chromanones • Coumarins • Flavonoids • Terpenes • Triterpenes • Xanthones	• Anti-cancer • Anti-HIV • Antioxidant	• Kudo et al., 2013 • Lemos et al., 2012 • Pires et al., 2014

C. caledonicum	• Xanthones	• Antimalarial	• Morel et al., 2002
C. dispar	• Coumarins	• Cytotoxic	• Guilet et al., 2001
C. enervosum	• Flavonoids • Xanthones	• Antioxidant • Cytotoxic	• Taher et al., 2005
C. flavoramulum	• Flavonoids • Terpenes • Xanthones	• Anti-diabetic • Inhibit AGEs formation	• Kulkarni et al., 2016 • Ferchichi et al., 2012
C. inophyllum	• Triterpenes • Flavonoids • Coumarins	• Anti-microbial • Anti-tumor • Anti-HIV	• Gómez-Verjan et al., 2015 • Li et al., 2010 • Laure et al., 2008
C. membranaceum	• Xanthones	• Anti-inflammatory • Cytotoxic	• Chen, et al., 2008 • Zou, et al., 2005

C. moonii	• Triterpenes • Xanthones	• Antioxidant	• Dharmaratne, Wijesinghe and Thevanasem, 1999
C. panciflorum	• Flavonoids	• Anti-tumour	• Ito et al., 1999
C. polyanthum	• Coumarins	• Anti-herpetic activity	• Ma et al., 2004
C. soulattri	• Coumarins • Terpenoids • Xanthones	• Cytotoxic	• Nigam et al., 1988 • Mah et al., 2015
C. teysmannii	• Chromanone acids • Coumarins	• Anti-HIV • Cytotoxic	• Lim et al., 2015 • McKee et al., 1996
C. thorelii	• Benzophenon-es • Triterpenes • Xanthones	• Cytotoxic	• Nguyen et al., 2012
C. thwaitesii	• Xanthones	• Anti-fungal • Anti-oxidant	• Dharmaratne et al., 2009

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Plant Materials

The plant studied in this project was *Calophyllum lanigerum* which the stem bark was collected from the jungle in Landeh, Sarawak and was identified by Mr. Tinjan Anak Kuda, a botanist from the Forest Department of Sarawak. A voucher specimen (UITM 3008) was deposited at the herbarium of Universiti Teknologi MARA, Sarawak.

3.2 Chemical Reagents

The solvents and materials used in this project are summarized in Tables 3.1 to 3.5.

Table 3.1: Industrial grade solvents and materials used in the extraction and isolation of chemical constituents

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

Table 3.2: Deuterated solvents used in NMR analysis

Solvents	Molecular formula	Source, Country
Acetone-*d6*	CD_3COCD_3	Acros Organics, Belgium
Methanold-*d4*	CD_3OD	Acros Organics, Belgium
Deuterated chloroform	$CDCl_3$	Acros Organics, Belgium

Table 3.3: Analytical grade solvents and materials used in TLC analysis

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

Table 3.4: Analytical grade solvent and materials used in UV-Vis analysis

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

Table 3.5: Chemical reagents and materials used in antioxidant assay

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

3.3 Methodology

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

A simple extraction method known as maceration was used in this study. Approximately 2.0 kg of stem bark of plant studied was collected then air dried and ground into fine powder. Dichloromethane was used to soak the powdered stem bark in a closed container at room temperature for two days. Then, dichloromethane crude extracts were filtered and water residue in the filtrate was removed by treating with anhydrous sodium sulphate. The solvent in the filtrate was then removed by using a rotary evaporator. These steps were repeated twice, and the dried dichloromethane crude extracts were combined.

The stem bark material was then extracted with methanol, twice following the procedures above to give dried methanol crude extracts. The dried mass of dichloromethane and methanol crude extracts obtained were 67.2819 and 22.8121 g, respectively. About 2 g of each crude extract were kept for antioxidant assay.

The two crude extracts of *Calophyllum lanigerum* were separately subjected to gravity column chromatography and gradient elution was used to separate the chemical constituents into a series of fractions. Chemical composition of fractions collected was determined by using thin layer chromatography (TLC).

Potential fractions that showed more than one spot on TLC were combined and subjected to another column chromatography for further purification. If there is a single spot showed on TLC plate, the compound was then subjected to spectroscopic analyses for structural identification. Repeat these steps until pure compounds were obtained.

3.4 Chromatography

3.4.1 Column Chromatography

Separation and isolation of pure chemical compounds from a mixture of compounds is commonly done by column chromatography technique. Silica gel acts as stationary phase and the solvent (mobile phase) were added into the glass column and the glass column was set vertically. The solvent eluted from column via gravitational force.

The size of column used were 25, 30 and 85 mm in internal diameter, depending on the amount of introduced sample. A slurry was formed by mixing silica gel and hexane homogenously and poured into the glass column. The column was tapped gently. This will allow silica gel settled down gradually and remove air bubbles to obtain a compact packing of stationary phase. In this study, dry packing method was used to prepare the sample. The sample was dissolved in a

minimum amount of suitable solvent. The dissolved sample was then added dropwise into silica gel and mixed homogenously. The prepared sample was introduced into the packed glass column. After that, a thin layer of anhydrous sodium sulphate that acts as drying agent and protective layer was added on top of the sample layer. Gradient elution in increasing polarity was served as mobile phase to separate compounds of different polarities. A series of fractions were collected and then concentrated by using a rotary evaporator. The collected fractions were analyzed for their chemical contents by using Thin Layer Chromatography (TLC).

3.4.2 Gel Permeation Chromatography

Size exclusion chromatography was used to separate compounds according to their molecular weights and sizes. Type of size exclusion chromatography used in this study was known as gel permeation chromatography, used Sephadex® LH-20 as stationary phase and a polar mixture of solvents as mobile phase.

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

materials and travelled a greater distance along the column as compare to the larger size molecules.

3.4.3 Thin Layer Chromatography

The purity of fractions collected from column chromatography was examined by TLC analysis. The TLC plate used was an aluminium sheets which coated a layer of silica gel 60 F_{254} Merck on top of it and cut in size of 4 cm x 8 cm. Firstly, the sample was diluted and then spotted on the marked baseline of the TLC plate by using a micro capillary tube. Next, the plate was placed in a developing chamber, which contains a suitable amount of solvent mixture. The solvent moved up to the solvent front line of the plate through capillary action. The spots were visualized under UV light and iodine vapor. Retention factor, R_f value of each compound was calculated by using the equation below:

$$R_f = \frac{\text{Distance travelled by the compund (cm)}}{\text{Distance travelled by solvent front (cm)}}$$

3.5 TLC Detection Methods

3.5.1 UV Detection

The completely developed TLC plate was then visualized under UV lights of both short (254 nm) and long (365 nm) wavelengths. Aromatic and conjugated

compounds can be detected under UV lights. At short wavelength, the spots appeared as dark grey spots on a bright green background while at long wavelength, the spots appeared as fluorescence color spots on a pale purple background.

3.5.2 Iodine Vapor Detection

Approximately 1 g of iodine crystals was added into a closed chamber to prepare an iodine vapor chamber. Then, the developed TLC plate was put into the chamber for few minutes. The brown spots that appeared on the TLC plate were marked down quickly as it will disappeared after take out from the iodine chamber.

3.6 Instruments

3.6.1 Nuclear Magnetic Resonance (NMR)

The information about the number and types of proton and carbon present in a molecule and their correlations for structural study were obtained by using the popular spectroscopic technique, Nuclear Magnetic Resonance (NMR). In this study, JEOL JNM-ECX 400 MHz spectrometer was used to carry out 1D and 2D NMR with the use of tetramethlysilane (TMS) as internal standard. 1D NMR

includes proton NMR (^{1}H), carbon NMR (^{13}C) and Distortionless Enhancement by Polarization Transfer (DEPT), while 2D NMR includes Heteronuclear Multiple Bond Coherence (HMBC) and Heteronuclear Multiple Quantum Coherence (HMQC).

A suitable amount of deuterated solvent such as methanol-d_4, acetone-d_6 or deuterated chloroform was used to dissolve the sample. The dissolved sample was then transferred into an NMR tube until 4 cm height. The NMR tube was capped, labelled and run for NMR experiments.

3.6.2 Infrared (IR) Spectroscopy

The functional groups present in a molecule was determined by using infrared (IR) spectroscopy. It measures the vibrations of atoms. The sample was first ground with potassium bromide, KBr in a ratio of 1:10 to form a homogenous mixture followed by compression under high pressure to form a pellet. The KBr pellet was analyzed by Perkin Elmer 2000-Fourier Transform Infrared (FTIR) spectrophotometer and the IR spectrum was obtained in the range of 4000 to 400 cm^{-1}.

3.6.3 Ultraviolet-Visible (UV-Vis) Spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy was used to analyze the presence of conjugated system in a compound. A suitable amount of analytical grade solvent was used to dissolve sample, then transferred into a quartz cuvette for analysis via a double beam spectrophotometer, Perkin Elmer Lambda 35. The solvent that used to dissolve sample was used as blank. The absorption spectrum was measured in the range of 200 – 400 nm.

3.6.4 Melting Point Apparatus

One of the techniques that used to determine the purity of a compound is determined its melting point. A pure compound should have a narrow melting range as compare to compound that is not pure. The purity of a compound can be determined by comparing the experimental melting point with the literature value of the pure compound. Sample was filled into a hematocrit capillary tube and heated until it melted by using the Stuart SMP 10 melting point apparatus.

3.7 Antioxidant Assay

To prepare 1 mg/mL of stock solutions, crude extracts, isolated and standard compounds (ascorbic acid and kaempferol) were dissolved separately in

methanol. After that, to ensure that all the sample were fully dissolved and mixed homogenously, the stocks solutions were sonicated for 5 minutes. Similarly, the DPPH powder was dissolved in methanol and followed by sonication for 5 minutes to prepare the 2 mg/mL of DPPH solution. All the prepared stock solutions and DPPH solution were kept in a 4°C chiller in dark condition.

A serial dilution of stock solutions was carried out in a 96-well plate to prepare the test solutions at various concentrations of 240, 120, 60, 30, 15, 7.5 and 3.75 µg/mL. About 10 µL DPPH solution and were then added to each well. The positive control used for this assay were kaempferol and ascorbic acid while the wells with DPPH solution and methanol without the test sample were served as blank.

After the addition of reagents, the plate was immediately capped and wrapped with aluminum foil to avoid evaporation of solvent. The plate was then incubated for 30 minutes. The absorbance of the content in each well was measured with a microplate reader at 520 nm. Each test sample was performed in triplicate and the average absorbance for each concentration was calculated and recorded. The following equation was used to calculate the percentage inhibition rates of the test compounds.

$$\text{Inhibition rate (\%)} = \frac{A_0 - A_1}{A_0} \text{ x } 100\%$$

Where A_0 = absorbance of the blank (negative control)

A_1 = absorbance of the test sample

A graph of inhibition rate against concentration of test sample was plotted to determine the IC_{50} value of test samples. IC_{50} is defined as the concentration of sample that required to inhibit 50 % of DPPH radicals presents.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical Constituents Isolated from *Calophyllum lanigerum*

The stembark of *Calophyllum lanigerum* was extracted with two different solvents which were dichloromethane and methanol. From the extractions, 67.2819 g of dichloromethane and 22.8121 g of methanol crude extracts were obtained. About 66.4566 g of dichloromethane was subjected to column chromatography using a 85 mm internal diameter glass column. Silica gel was used as stationary phase and eluted with solvent mixtures in rising polarity (hexane-dichloromethane, dichloromethane-ethyl acetate, ethyl acetate-methanol), in stepwise gradient elution to give 53 fractions (MC 1-53). All the fractions were monitored for their chemical compositions through TLC analysis. Fraction MC 10 was recrystallized from methanol to yield white needle-like crystals, friedelin **[19]**.

Fractions MC 24 - 28 were combined and subjected to a 30 mm inner diameter glass column packed with silica gel and eluted with mobile phase of increasing polarity (hexane-ethyl acetate, ethyl acetate-acetone) to give 25 sub fractions MLWA 1 - 25. Further purification of the combined sub fractions MLWA 4 and MLWA 5 gave 21 subfractions (MLWB 1 – MLWB 21). Subfractions MLWB 4 - 5 were combined and subjected to a 25 mm inner diameter silica gel packed column eluted with hexane-ethyl acetate to give 18 subfractions MLWE 1 – 18.

Subfractions MLWE 4 - 7 were further purified via a gel permeation chromatographic column that used Sephadex®LH-20 as stationary phase and eluted with solvent mixture of 90 % methanol and 10 % dichloromethane to give 40 subfractions MLWJ 1 - 40. Subfractions MLWJ 4 – 6 yielded yellow needle-like crystals, euxanthone **[18]**.

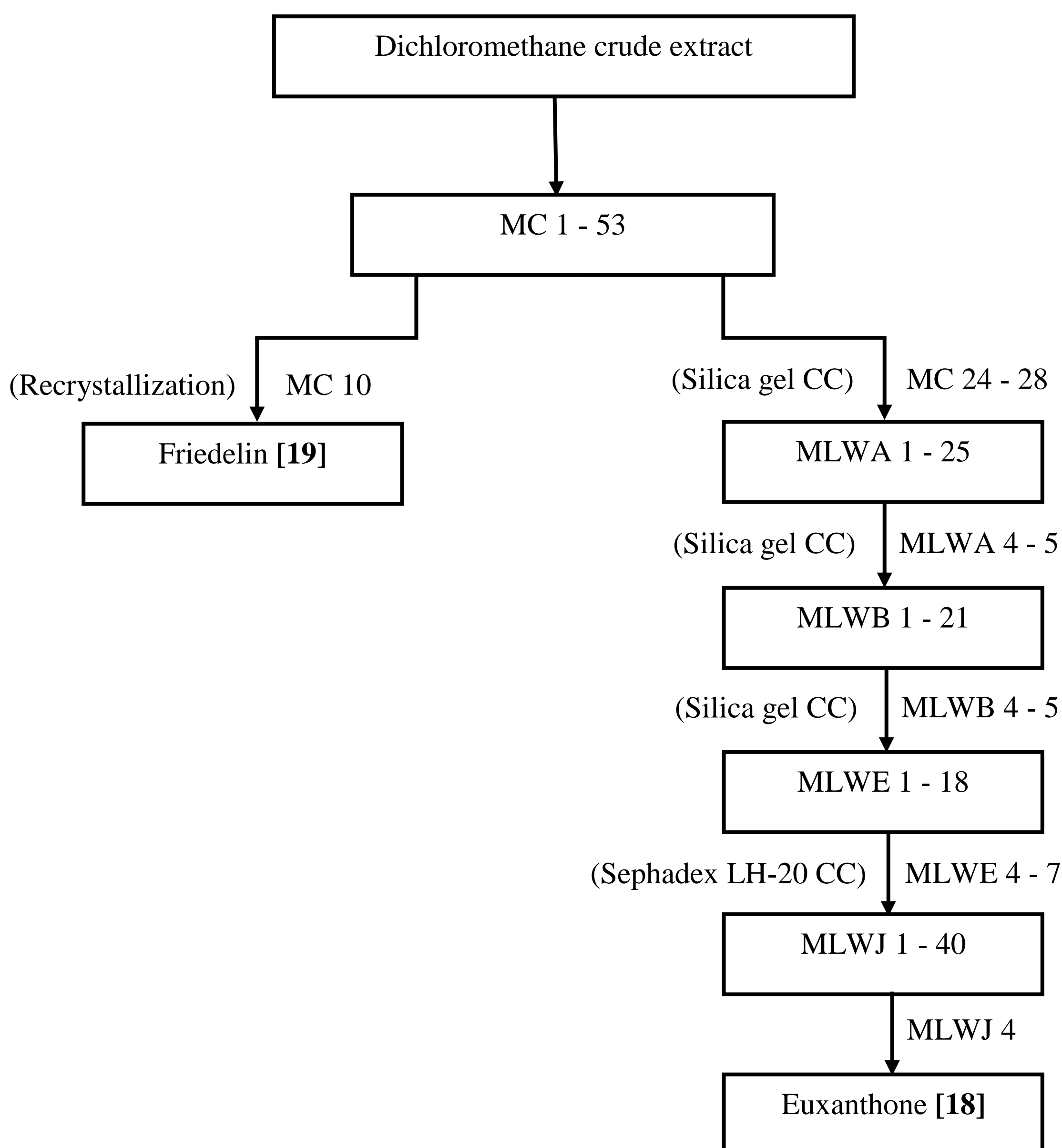
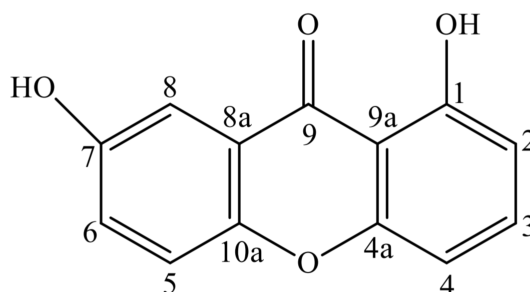


Figure 4.1: The pathway of isolation of pure compounds

4.2 Structural Characterization and Elucidation of Euxanthone [18]



[18]

A total of 228.2 mg of compound **18** was isolated as yellow-needle like crystals. It was isolated from dichloromethane extract of *Calophyllum lanigerum* with a melting point of 236 °C -238 °C (Lit. 239 °C - 241 °C, Locksley, Moore and Scheinmann, 1966). Compound **18** has a molecular formula of $C_{13}H_8O_4$ giving the molecular weight of 228.2 g/mol. In TLC analysis, this compound was developed using a solvent system of 50 % hexane and 50 % acetone to give a retention factor, R_f value of 0.75. It gave a single dark spot when visualized under UV light at wavelength 254 nm.

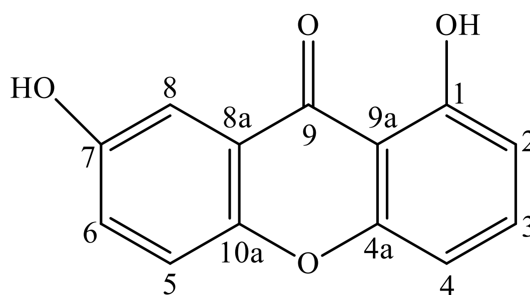
In the ^{1}H NMR spectra (Figures 4.2 and 4.3), a total of eight proton signals were observed including two highly deshielded proton signals at δ 12.69 and 9.06 which were assigned to the chelated hydroxyl proton, 1-OH and a free hydroxyl proton, 7-OH, respectively. Meanwhile, the remaining six aromatic protons H-2, H-3, H-4, H-5, H-6 and H-8 gave six signals with different multiplicities at δ 6.74 (d, J = 8.6 Hz), 7.67 (t, J = 8.6 Hz), 6.97 (d, J = 8.6 Hz), 7.50 (d, J = 9.2 Hz), 7.41 (dd, J = 9.2 Hz & 3.0 Hz) and 7.57 (d, J = 3.0 Hz), respectively.

A total of 13 carbon signals were shown in the ^{13}C NMR spectrum (Figures 4.4) which were in agreement with the number of carbons in the proposed structure. The most deshielded signal at δ 182.2 was assigned to the carbon C-9 which was a keto group (C=O). Besides, the four oxygenated aromatic carbons gave relatively more deshielded carbon signals at δ 161.9 (C-1), 156.5 (C-4a), 154.2 (C-7) and 150.2 (C-10a). Meanwhile, the remaining carbon signals appeared in the region below δ 140.0 were attributed to the eight non-oxygenated aromatic carbons, C-2, C-3, C-4, C-5, C-6, C-8, C-8a and C-9a.

The 2D NMR analysis indicated the structural correlations between protons and their surrounding carbons. The HMQC spectrum (Figures 4.6 and 4.7) showed the heteronuclear ^{1}J couplings between protons and their respective carbons whereas in the HMBC spectra (Figures 4.8, 4.9, 4.10 and 4.11), it indicated the long range heteronuclear ^{2}J or ^{3}J coupling between protons and their neighboring carbons. The spectral assignment was summarized in Table 4.1.

The assigned structure of compound **18** was further confirmed by IR and UV-Vis analyses. A broad band appeared at 3303 cm^{-1} on the IR spectrum (Figure 4.12) was corresponded to the O-H stretch in the compound. Moreover, the presence of conjugated aromatic C=C stretch was indicated by the peak at 1478 cm^{-1} and C=O stretch at 1607 cm^{-1}. In addition, absorption bands observed at 1235 cm^{-1} showed the presence of C-O stretch. Meanwhile, UV-Vis spectrum (Figure 4.13), displayed absorption maxima at 208.0, 212.9, 233.1 and 256.9

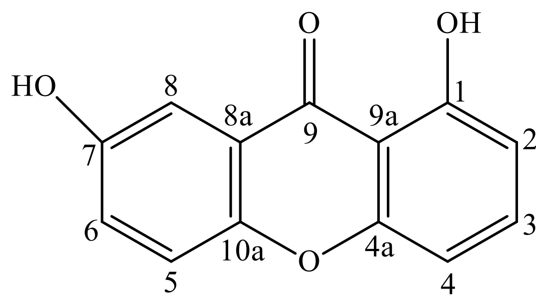
cm^{-1}, this due to compound **18** have a highly conjugated system. Based on the spectral evidence, compound **18** was unambiguously identified as euxanthone.



[18]

Table 4.1: Summary of NMR assignment for euxanthone [18]

Position	δH (ppm)	δC (ppm)	HMBC	
			²J	***³J***
1	-	161.9	-	-
2	6.74 (1H, d, *J* = 8.6 Hz)	109.7	C-1	C-4
3	7.67 (1H, t, *J* = 8.6 Hz)	137.0	-	C-1, C-4a
4	6.97 (1H, d, *J* = 8.6 Hz)	107.0	C-4a	C-2
4a	-	156.5	-	-
5	7.50 (1H, d, *J* = 9.2 Hz)	119.4	C-10a	C-7, C-8a
6	7.41 (1H, dd, *J* = 9.2 Hz, 3.0 Hz)	125.4	-	C-8, C-10a
7	-	154.2	-	-
8	7.57 (1H, d, *J* = 3.0 Hz)	108.3	C-7	C-6, C-9, C-10a
8a	-	121.0	-	-
9	-	182.2	-	-
9a	-	107.0	-	-
10a	-	150.2	-	-
7-OH	9.06 (OH, s)	-	C-6, C-8	-
1-OH	12.69 (OH, s)	-	C-2	-



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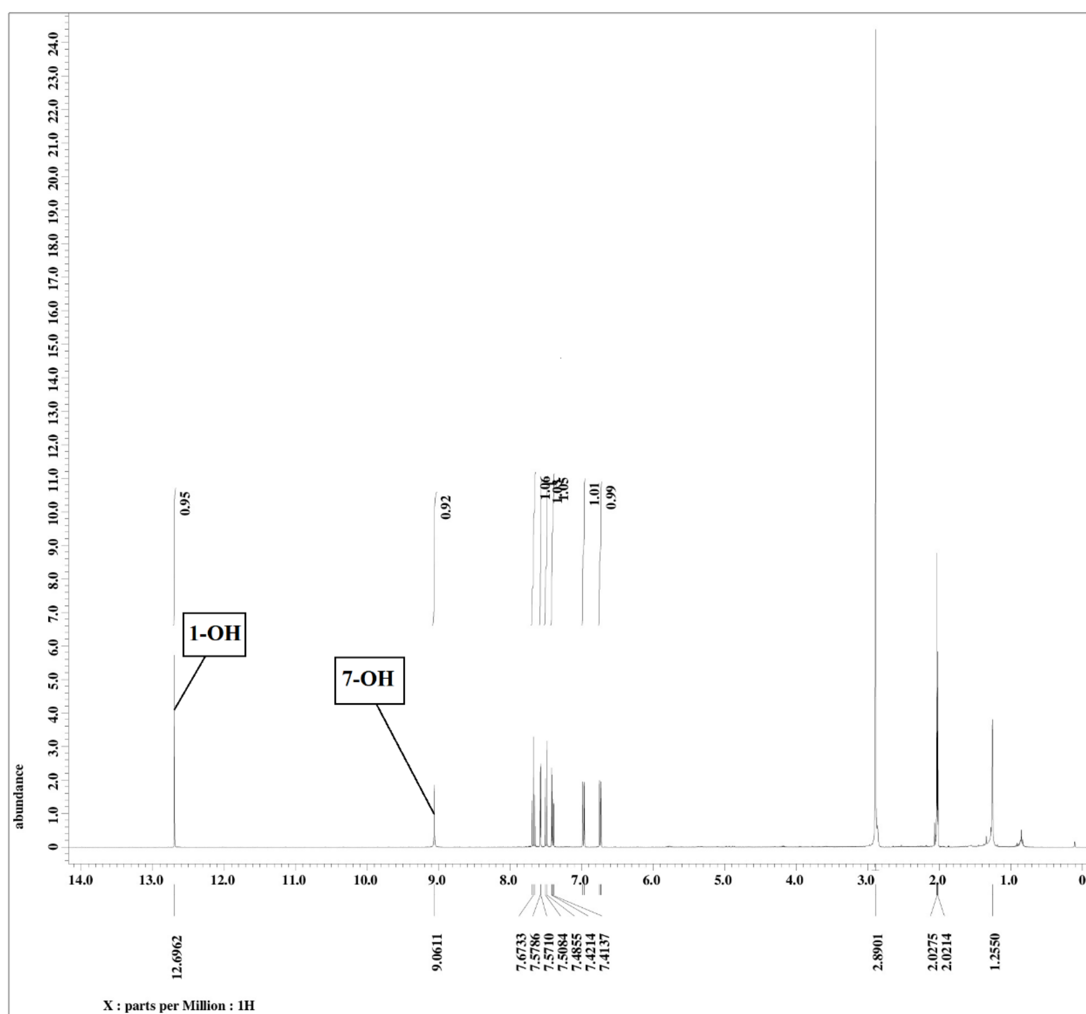
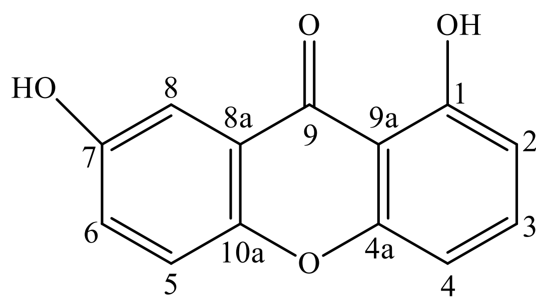


Figure 4.2: ^{1}H NMR spectrum of euxanthone [18] (400 MHz, acetone-d_6)



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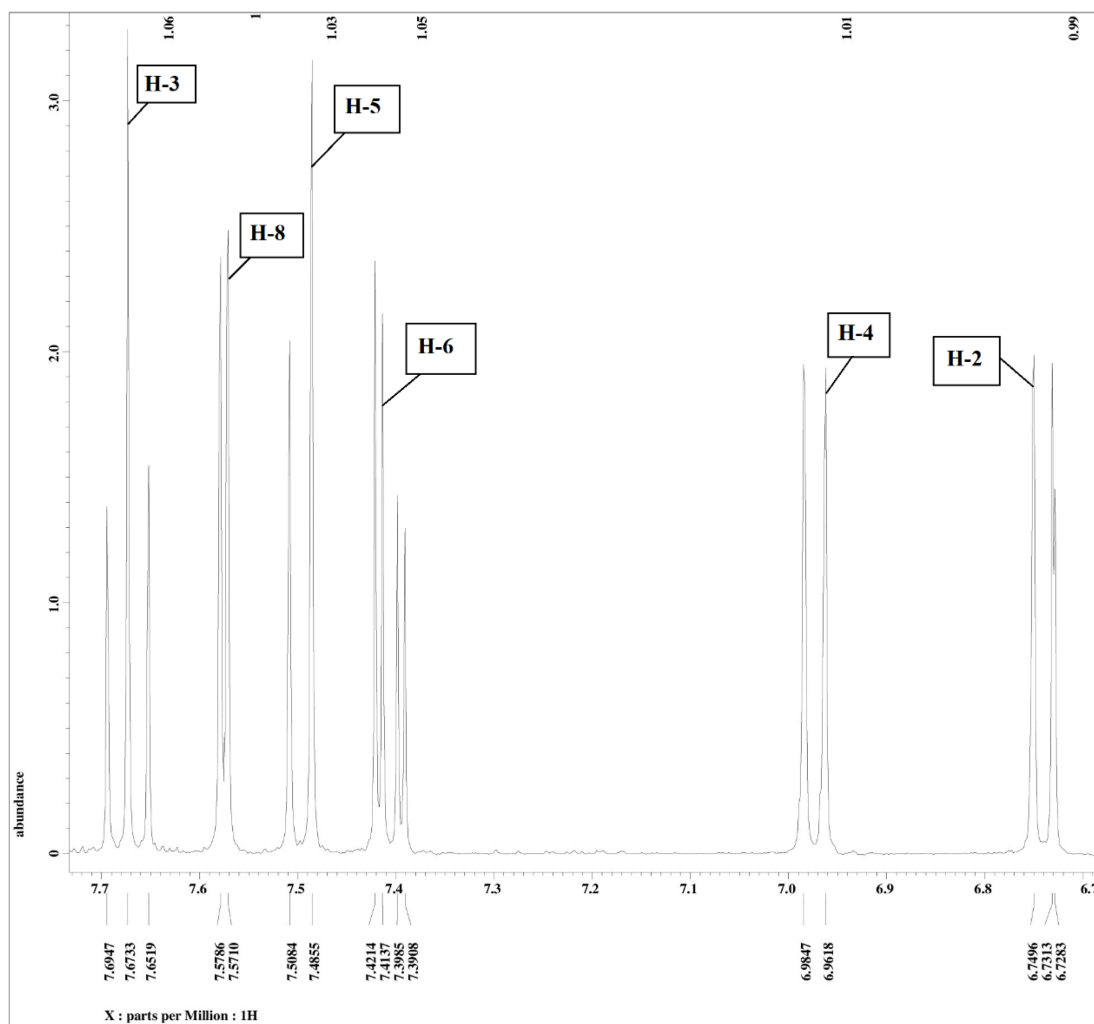
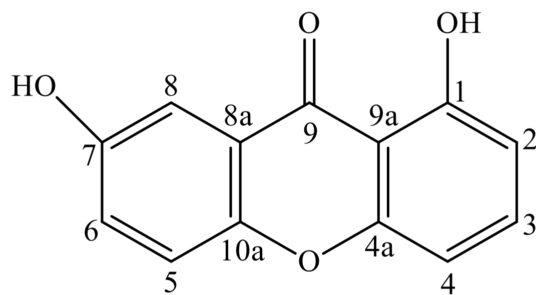


Figure 4.3: Expanded ^{1}H NMR spectrum of euxanthone [18] (400 MHz, acetone-d_6)



[18]

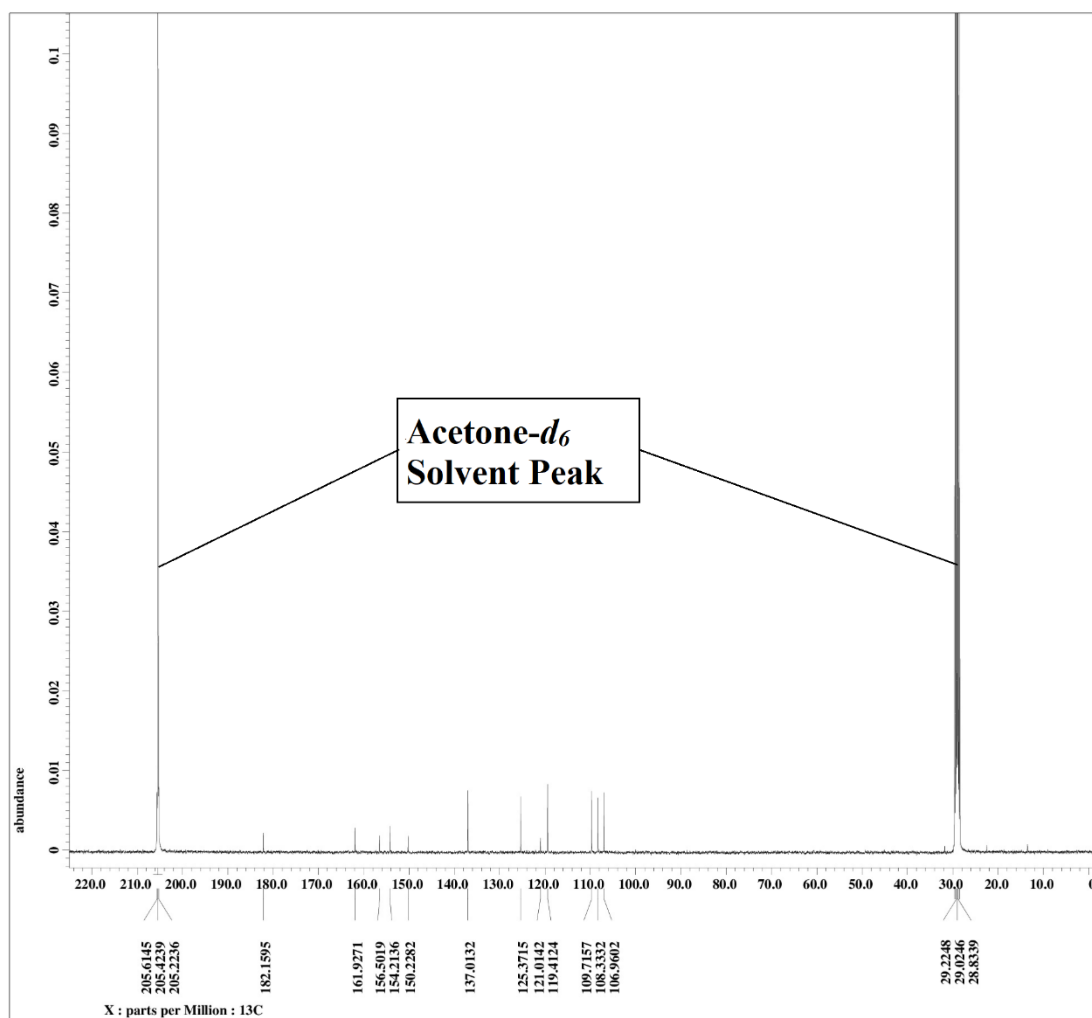
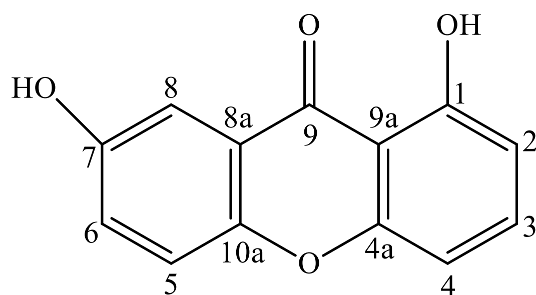


Figure 4.4: ^{13}C NMR spectrum of euxanthone [18] (100 MHz, acetone-d_6)



[18]

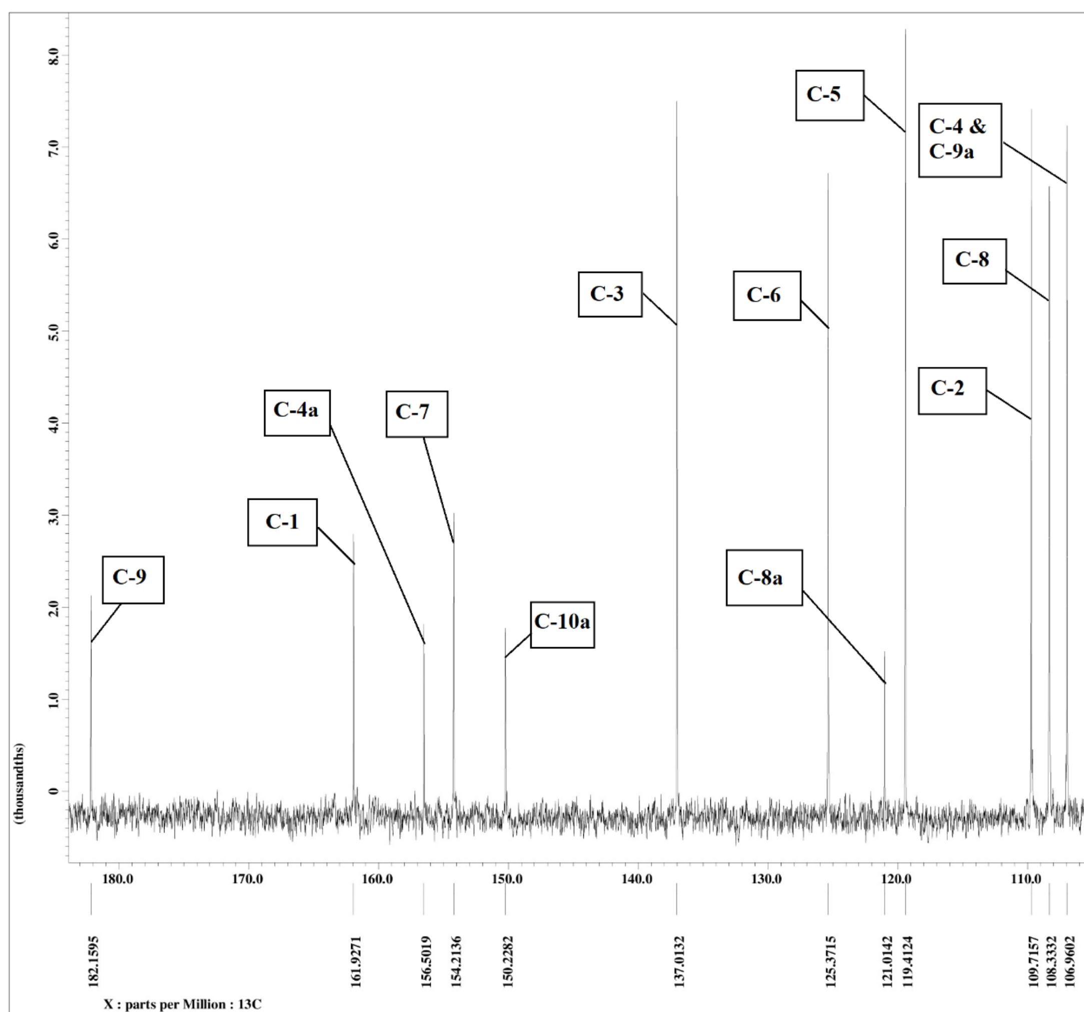
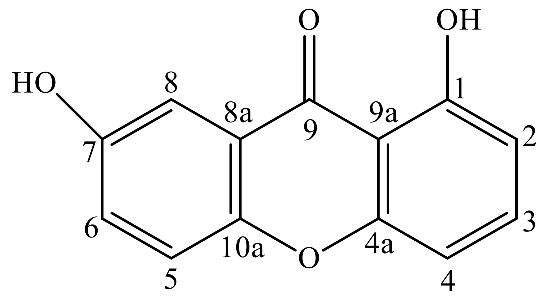


Figure 4.5: Expanded ^{13}C NMR spectrum of euxanthone [18] (100 MHz, acetone-d_6)



[18]

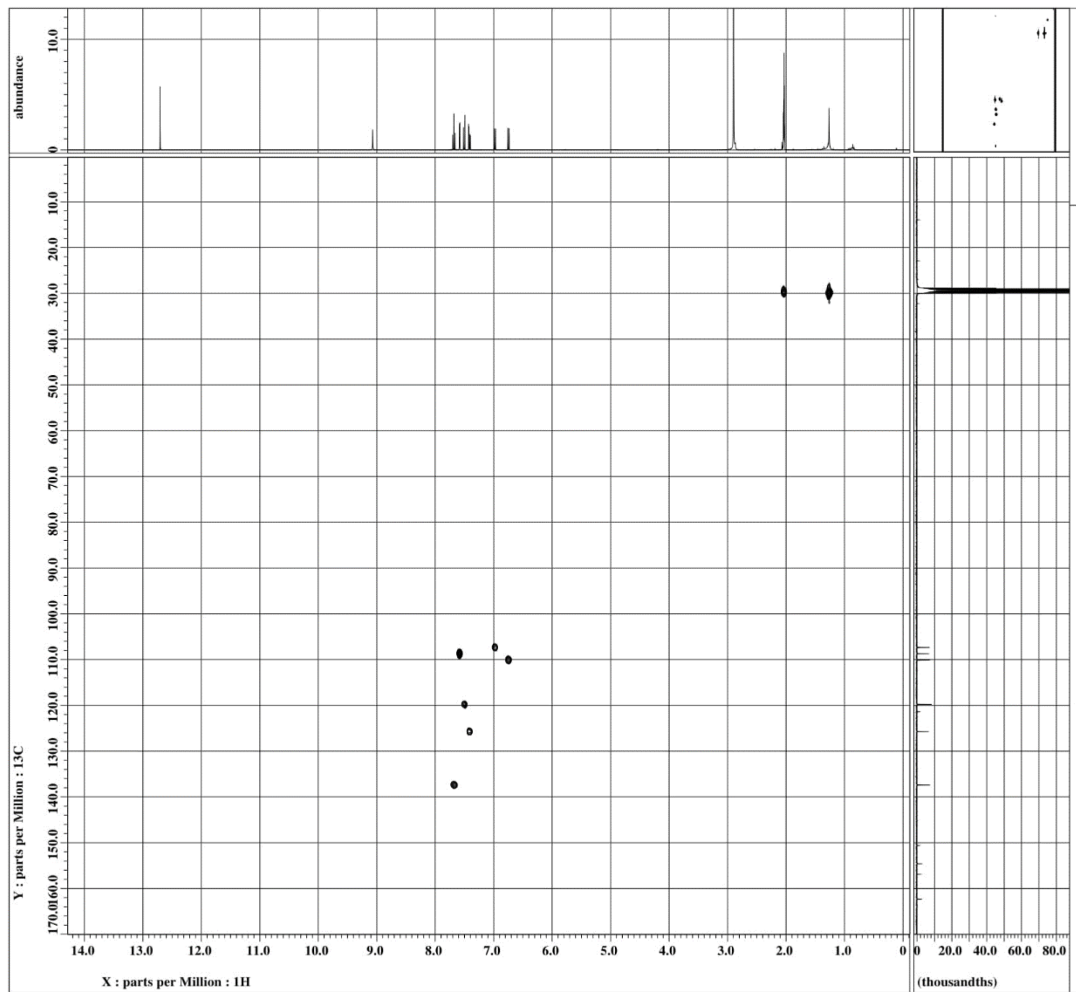
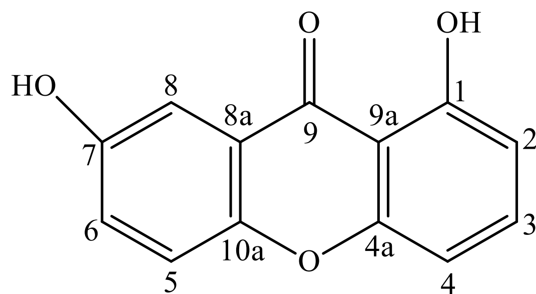


Figure 4.6: HMQC spectrum of euxanthone [18]



[18]

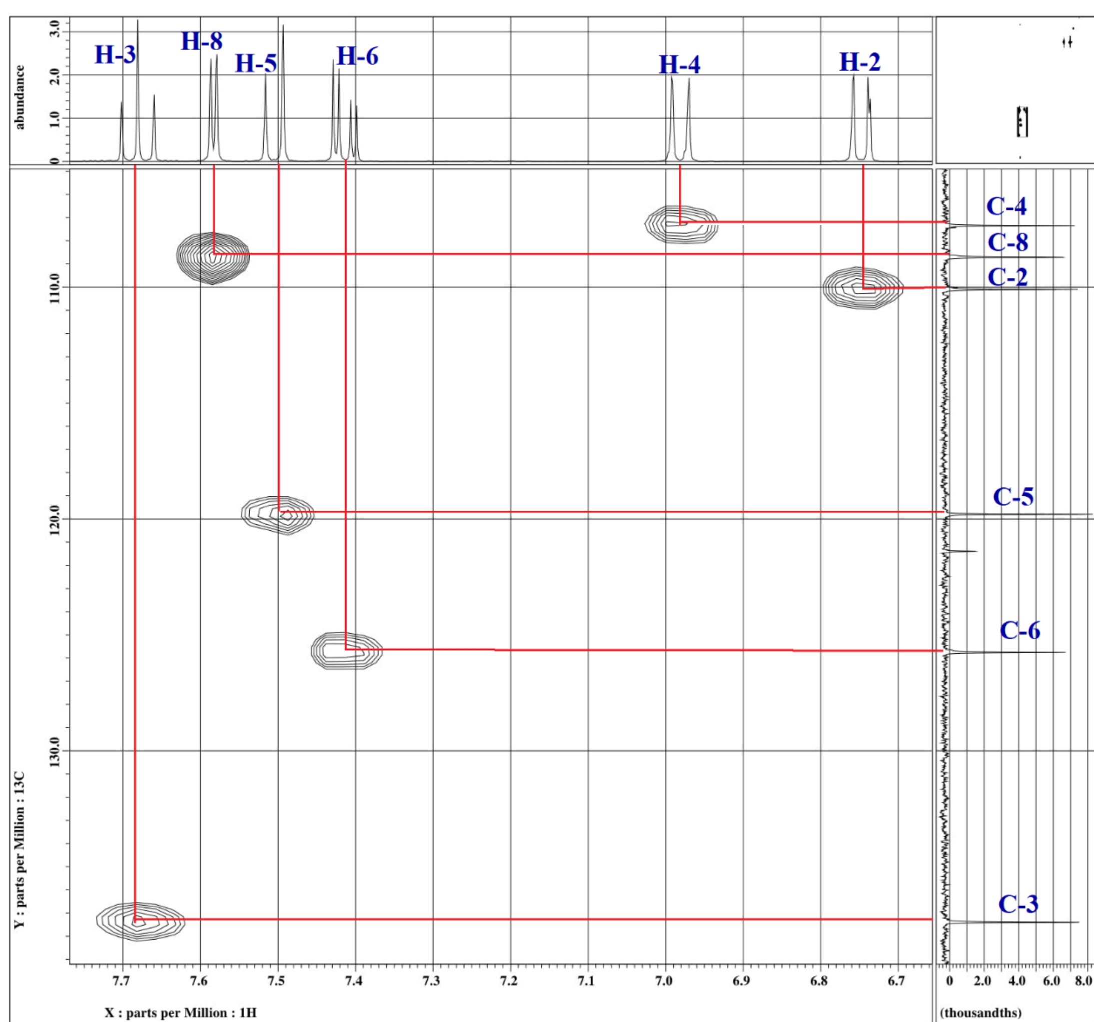
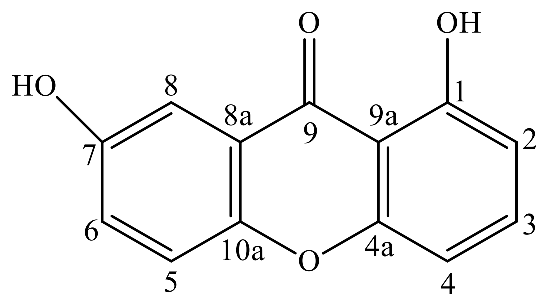


Figure 4.7: Expanded HMQC spectrum of euxanthone [18]



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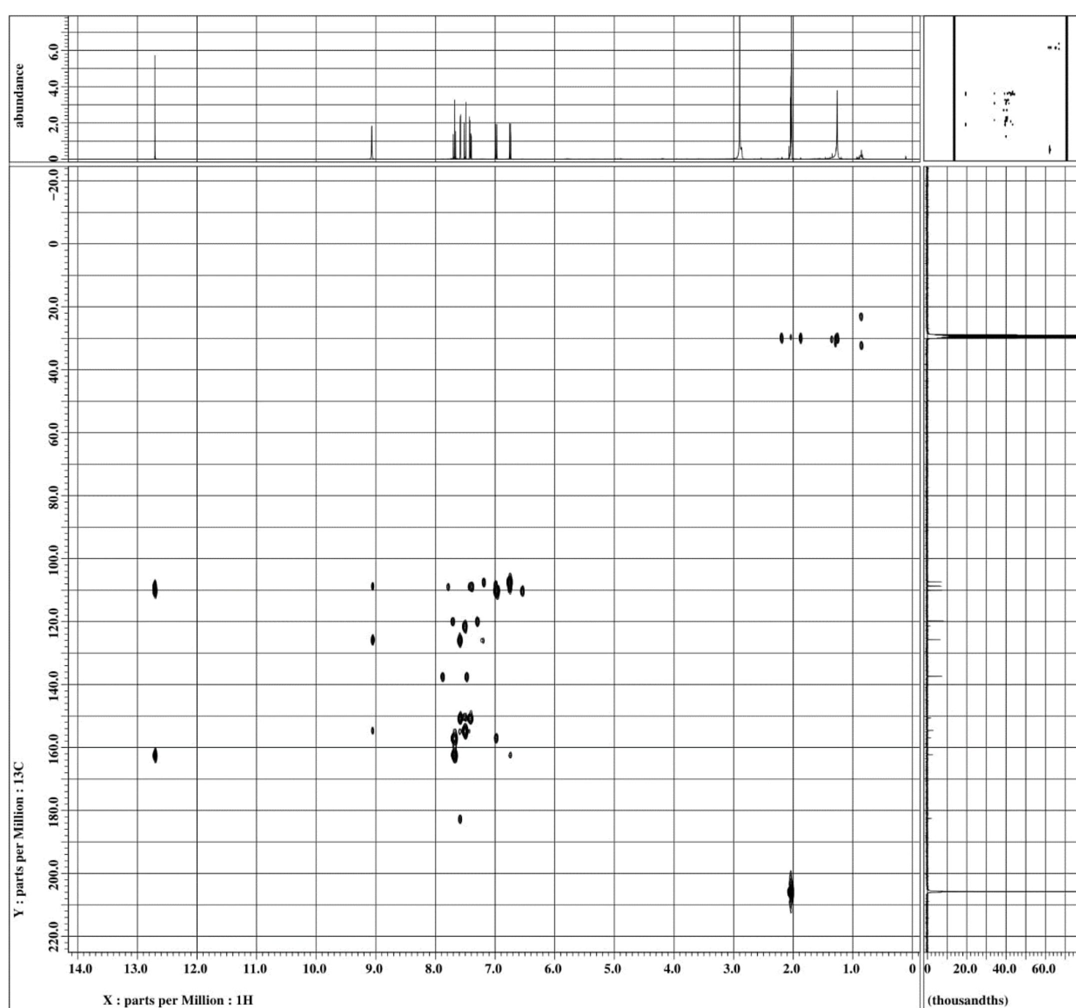
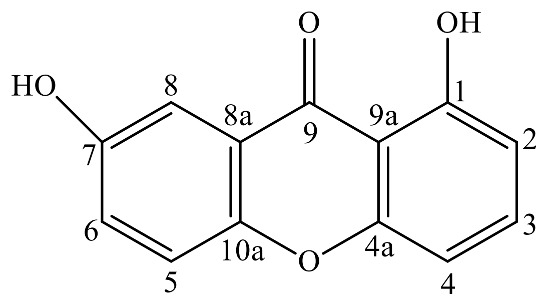


Figure 4.8: HMBC spectrum of euxanthone [18]



[18]

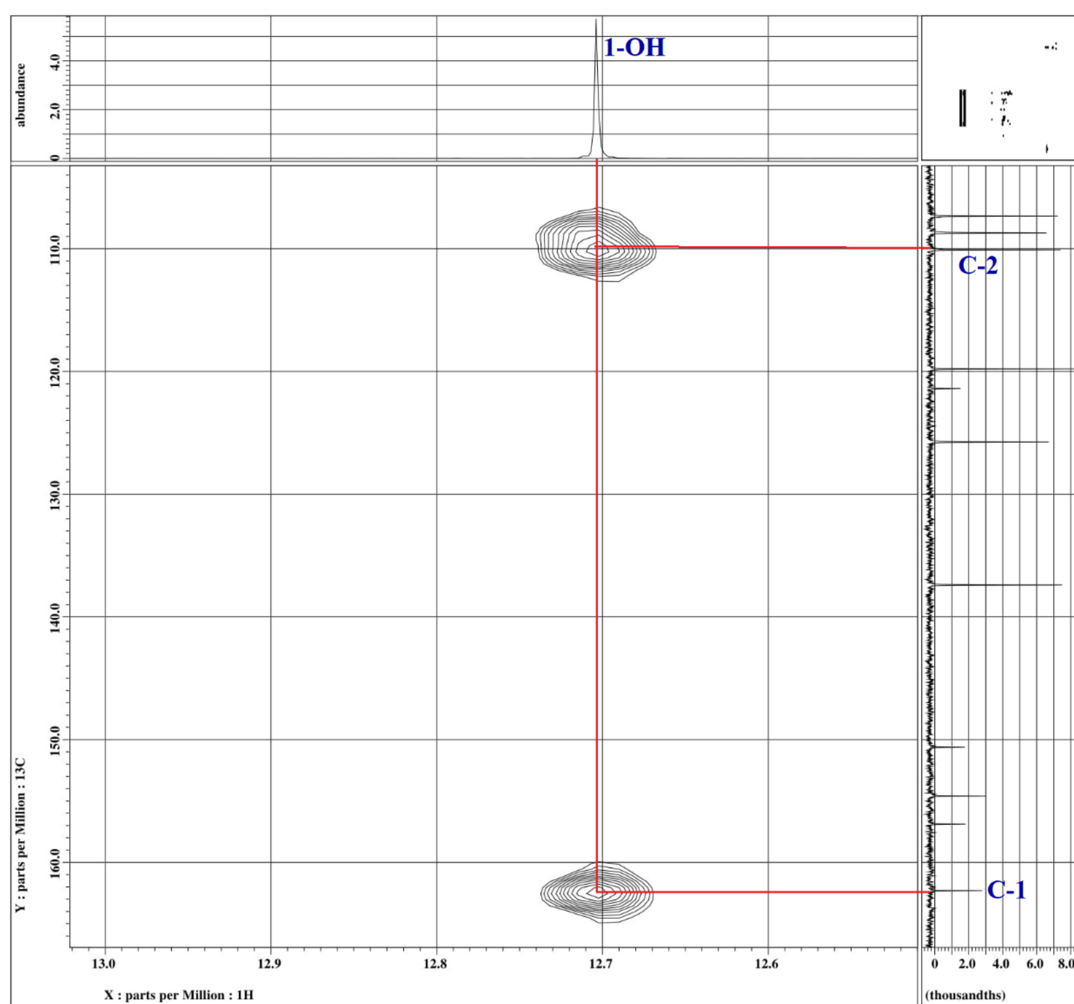
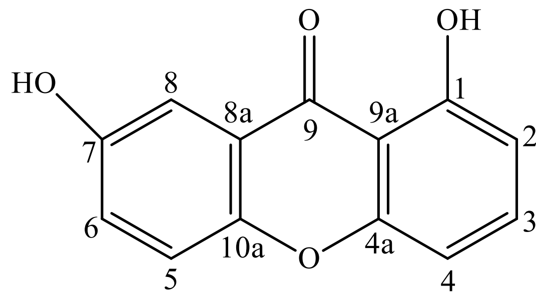


Figure 4.9: Expanded HMBC spectrum of euxanthone [18]



[18]

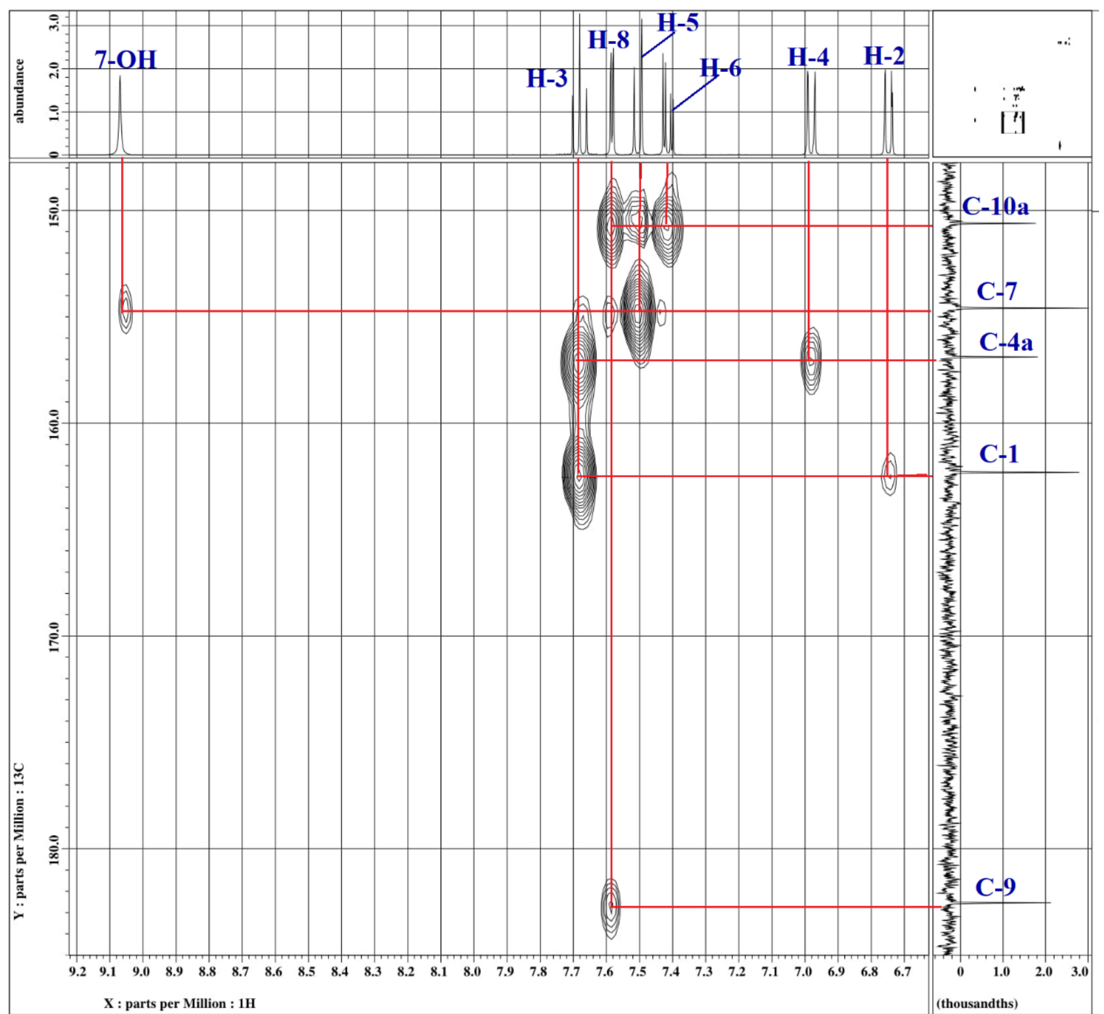
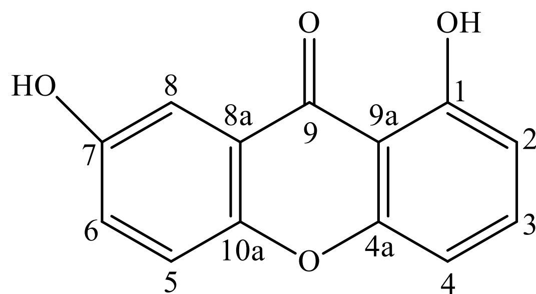


Figure 4.10: Expanded HMBC spectrum of euxanthone [18]



[18]

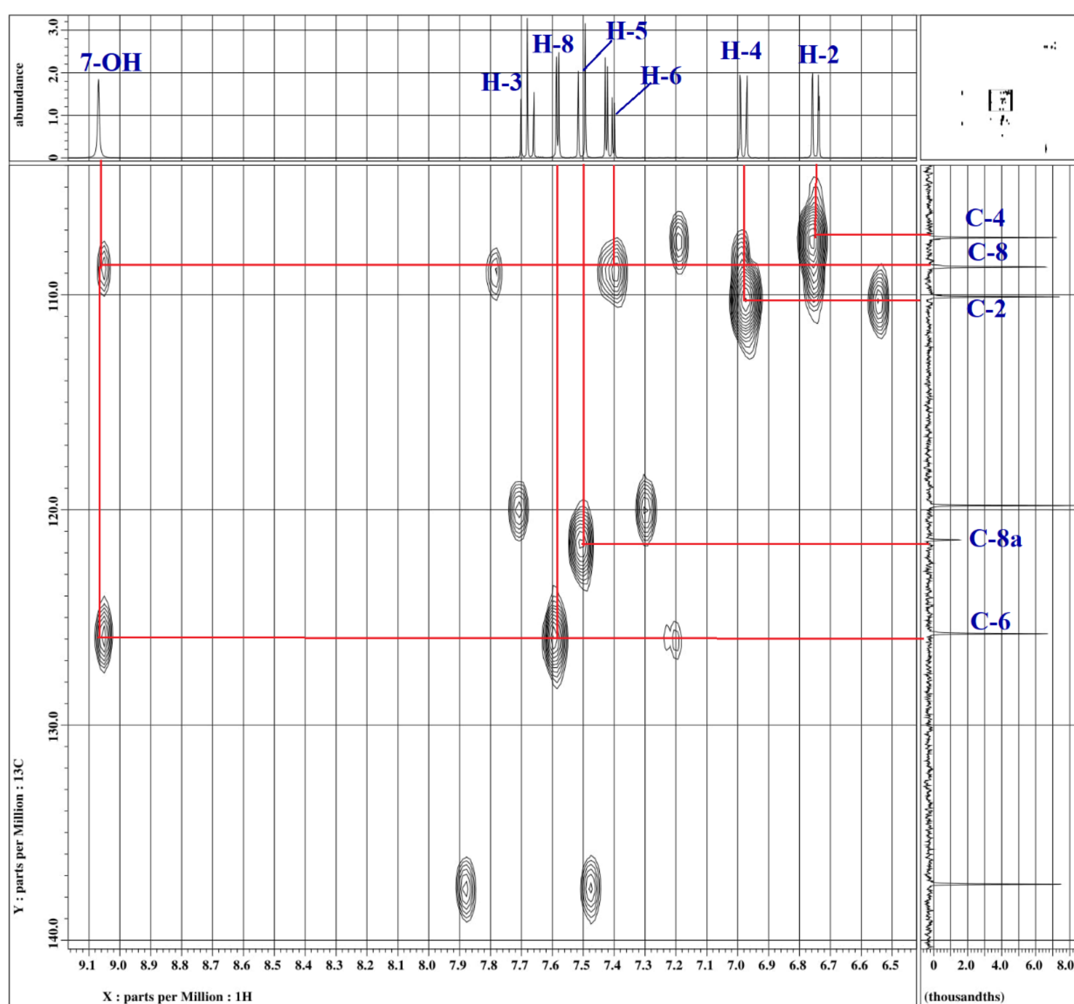


Figure 4.11: Expanded HMBC spectrum of euxanthone [18]

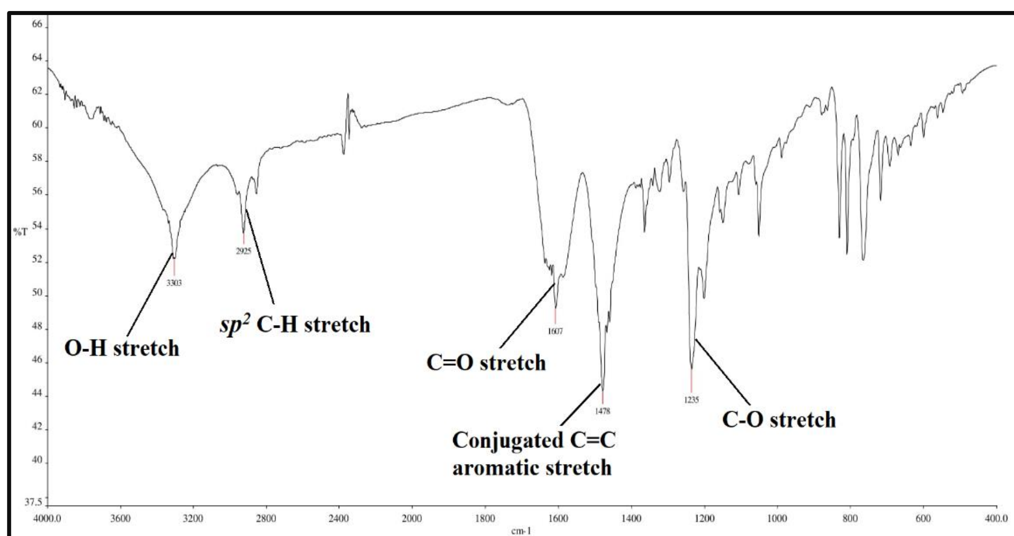


Figure 4.12: IR spectrum of euxanthone [18]

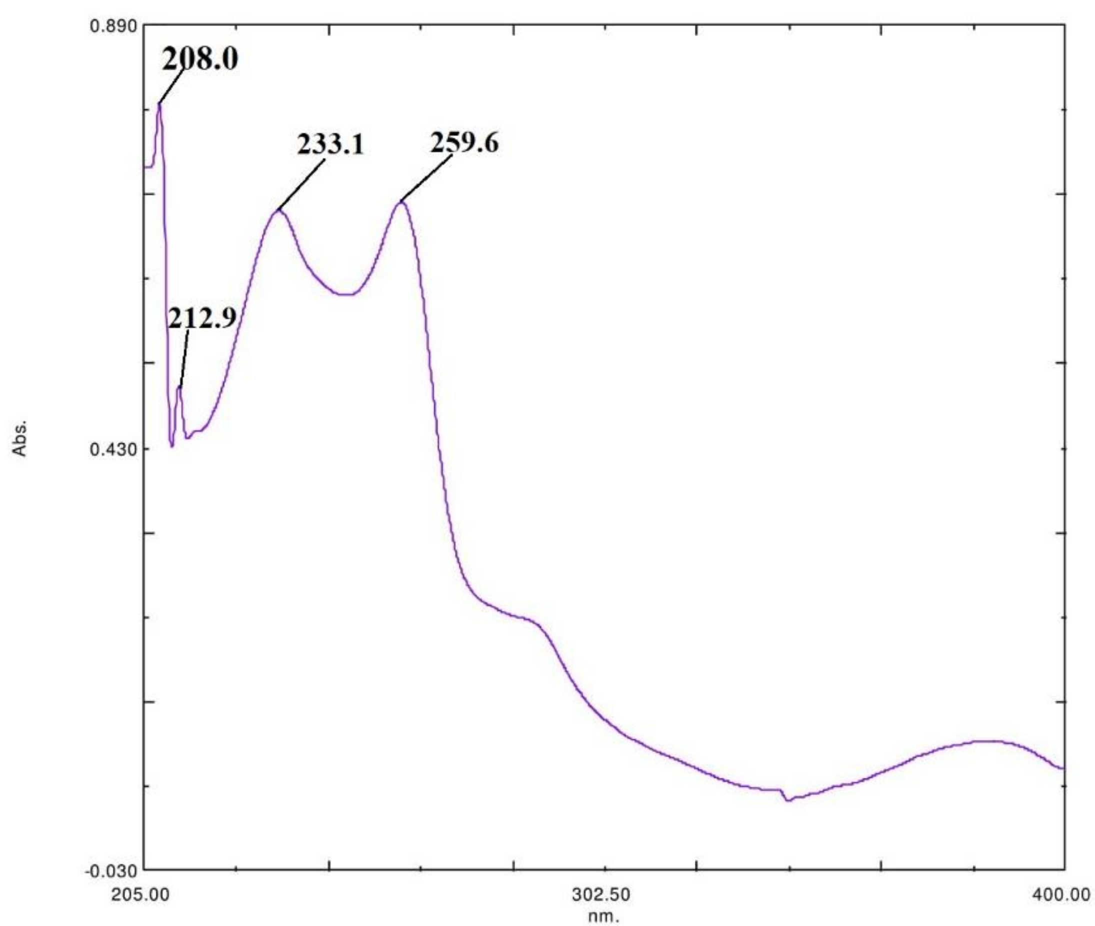
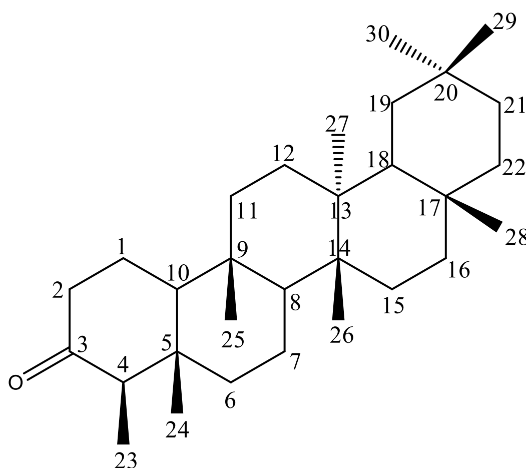


Figure 4.13: UV-Vis spectrum of euxanthone [18]

4.3 Structural Characterization and Elucidation of Friedelin [19]



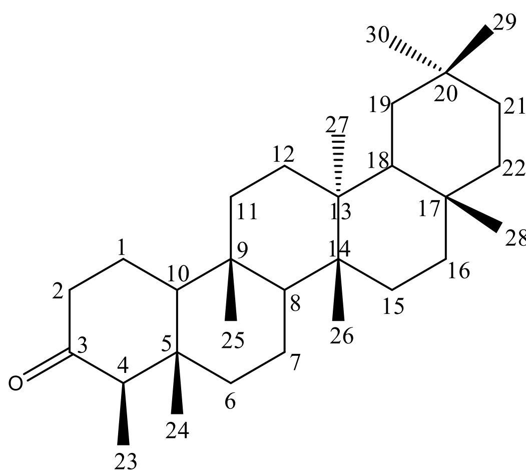
[19]

Compound **19** having a molecular formula of $C_{30}H_{50}O$, was obtained as white needle-like crystals. The melting point of compound **19** was 259 °C – 260 °C which is quite close to the literature value (261 °C - 262 °C) (Subhadhirasakul and Pechpongs, 2005). During TLC analysis, compound **19** looked invisible under the short (254 nm) and long wavelengths (365 nm) of UV light whereas a single yellow spot was observed after treated with iodine vapor in a chamber. It gave a retention factor, R_f value of 0.65 using a solvent mixture of 50 % hexane and 50 % dichloromethane as mobile phase.

The IR spectrum (Figure 4.14) displayed absorption bands at 1715 and 2927 cm^{-1} indicating the presence of carbonyl group and sp^3 C-H stretching. Compound **19** showed an adsorption peak at 217 nm in the UV-Vis spectrum (Figure 4.15). This was due to the $n \longrightarrow \pi^*$ transition which was corresponded to the excitation of an electron from the lone pair electron to the π^* orbital.

From the ^{1}H NMR spectra (Figures 4.15 and 4.16), all the proton signals were found to appear in the upfield region below δ 2.5. This is due to all the protons in compound **19** were covalently bonded to sp^3 hybridized carbons. The eight methyl groups gave eight characteristic proton signals consisting of seven singlets and one doublet at δ 0.69, 0.84, 0.92, 1.02, 1.15, 0.98, 0.97 and 0.85 assignable to protons H-24, H-25, H-26, H-27, H-28, H-29 and H-23, respectively. Moreover, the assignment of these proton signals were done by comparing with the literature values reported by Abbas et al. in year 2007, and the spectral assignment was summarized in Table 4.2.

The ^{13}C NMR spectra (Figures 4.17 and 4.18) showed a total 30 carbon signals which are in agreement with the number of carbons in the proposed structure. Among the carbons, the keto carbon C-3 gave the most deshielded signal at δ 213.3 and the remaining of 29 carbon signals were found to appeared in the region below δ 70.0 which were assigned to the sp^3 hybridized carbons. Furthermore, the eight methyl carbons which appeared in the upfield region at δ 6.9, 14.7, 18.0, 20.3, 18.8, 32.2, 35.1 and 31.9 were attributed to carbons C-23, C-24, C-25, C-26, C-27, C-28, C-29 and C-30 respectively. The remaining assignment of carbon signals were completed by comparing with the literature values reported by Abbas et al., in year 2007.



[19]

Table 4.2: Summary of NMR assignment for friedelin [19] in comparison with literature values

Position	δ_H (ppm)	*δ_H (ppm)	δ_C (ppm)	*δ_C (ppm)
1	1.93 (1H_a, m)	1.97 (m)	22.4	22.3
	1.71 (1H_b, m)	1.71 (m)		
2	2.35 (1H_a, dd, J = 13.4 Hz, 3.0 Hz)	2.41 (dd, J = 13.0 Hz, 3.5 Hz)	41.6	41.5
	2.29 (1H_b, m)	2.31 (m)		
3	-	-	213.3	213.2
4	2.25 (1H, m)	2.28 (m)	58.3	58.2
5	-	-	42.2	42.2
6	1.78 (m)	1.78 (m)	41.3	41.3
	1.31 (m)	1.31 (m)		
7	1.51 (m)	1.51 (m)	18.3	18.2
	1.41 (m)	1.41 (m)		
8	1.41 (m)	1.41 (m)	53.2	53.1
9	-	-	37.5	37.4
10	1.55 (m)	1.55 (m)	59.5	59.5
11	1.40 (m)	1.40 (m)	35.7	35.6
12	1.30 (m)	1.30 (m)	30.6	30.5

	1.28 (m)	1.28 (m)		
13	-	-	39.8	39.7
14	-	-	38.4	38.3
15	1.50 (m) 1.30 (m)	1.50 (m) 1.30 (m)	32.8	32.8
16	1.40 – 1.60 (m)	1.40 – 1.60 (m)	36.1	36.0
17	-	-	30.0	30.0
18	1.60 (m) 1.40 (m)	1.60 (m) 1.40 (m)	42.8	42.8
19	1.60 (m)	1.60 (m)	35.4	35.3
20	-	-	28.2	28.2
21	1.51 (m) 1.31 (m)	1.51 (m) 1.31 (m)	32.5	32.5
22	1.51 (m) 0.97 (m)	1.51 (m) 0.97 (m)	39.3	39.3
23	0.85 (3H, d, *J* = 6.7 Hz)	0.92 (d, *J* = 7.0 Hz)	6.9	6.8
24	0.69 (3H, s)	0.75 (s)	14.7	14.7
25	0.84 (3H, s)	0.90 (s)	18.0	18.0
26	0.92 (3H, s)	1.03 (s)	20.3	20.3
27	1.02 (3H, s)	1.07 (s)	18.8	18.7
28	1.15 (3H, s)	1.20 (s)	32.2	32.1
29	0.98 (3H, s)	1.02 (s)	35.1	35.0
30	0.98 (3H, s)	0.98 (s)	31.9	31.8

* Literature source: Abbas et al., 2007

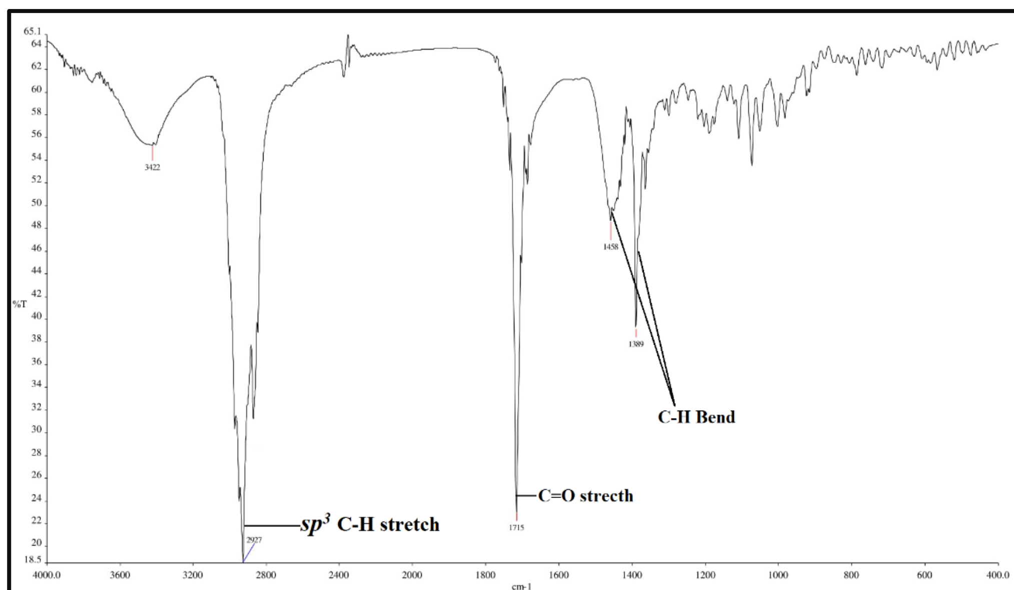


Figure 4.14: IR spectrum of friedelin [19]

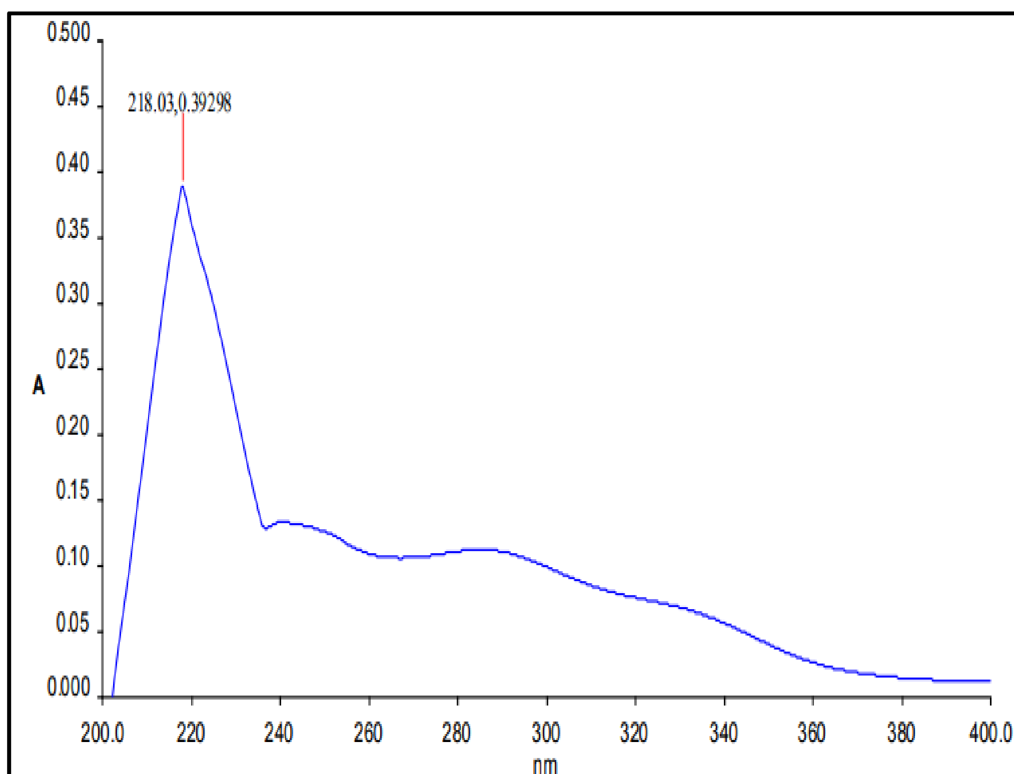
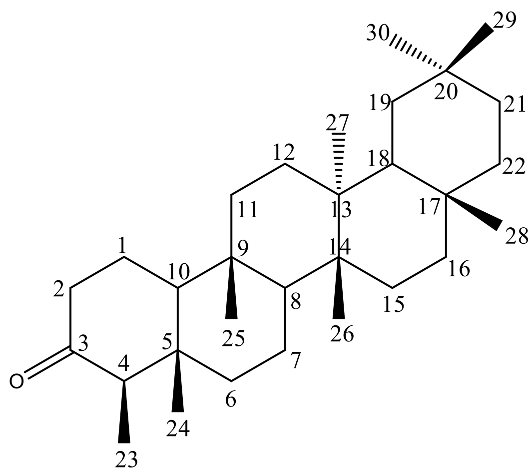


Figure 4.15: UV-Vis spectrum of friedelin [19]



[19]

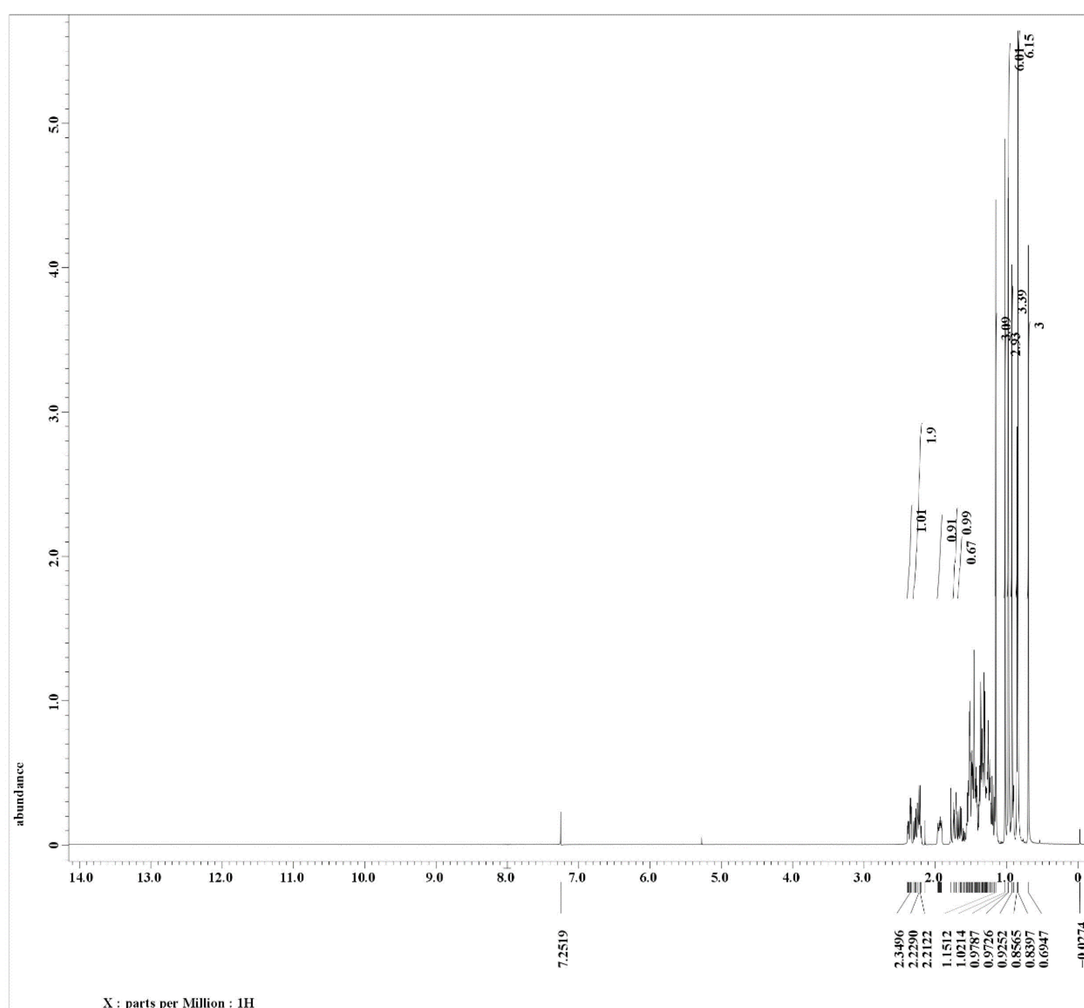
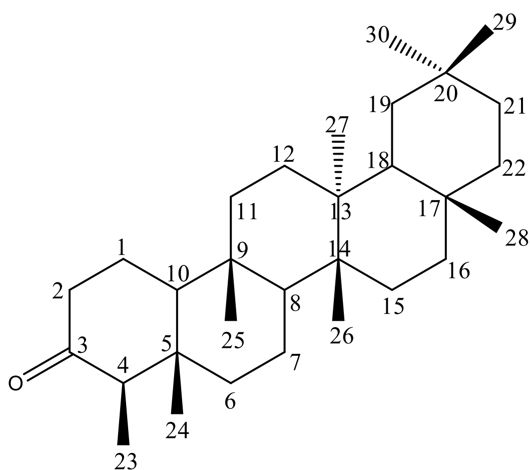


Figure 4.16: ^{1}H NMR spectrum of friedelin [19] (400 MHz, $CDCl_3$)



[19]

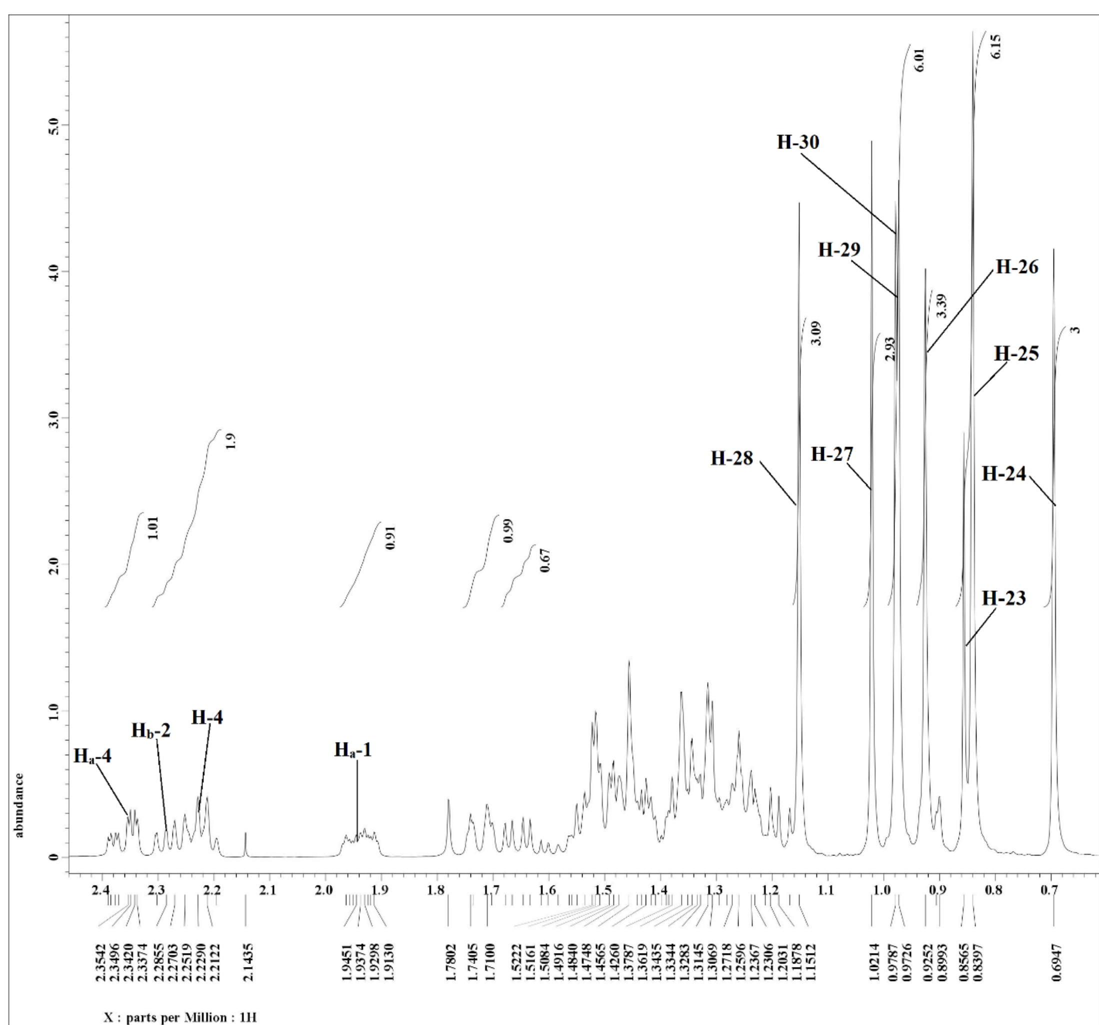
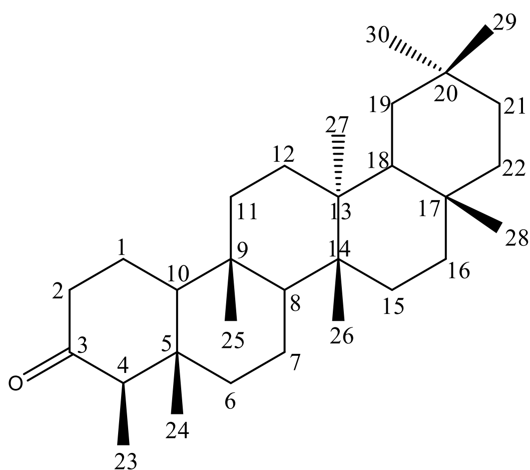


Figure 4.17: Expanded ^{1}H NMR spectrum of friedelin [19] (400 MHz, $CDCl_3$)



[19]

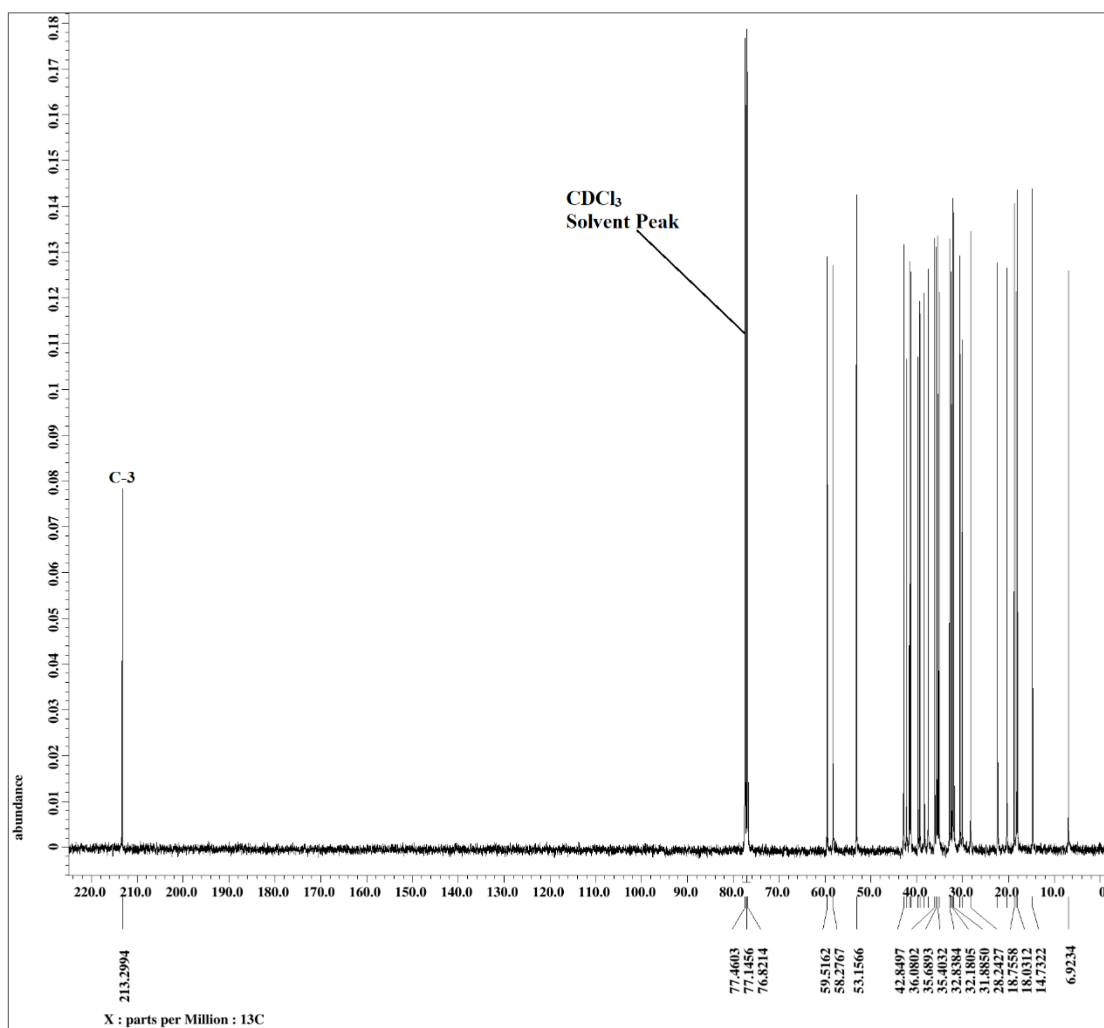
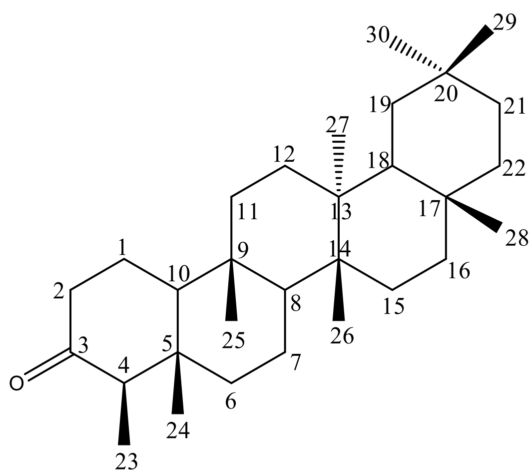


Figure 4.18: ^{13}C NMR spectrum of friedelin [19] (100 MHz, $CDCl_3$)



[19]

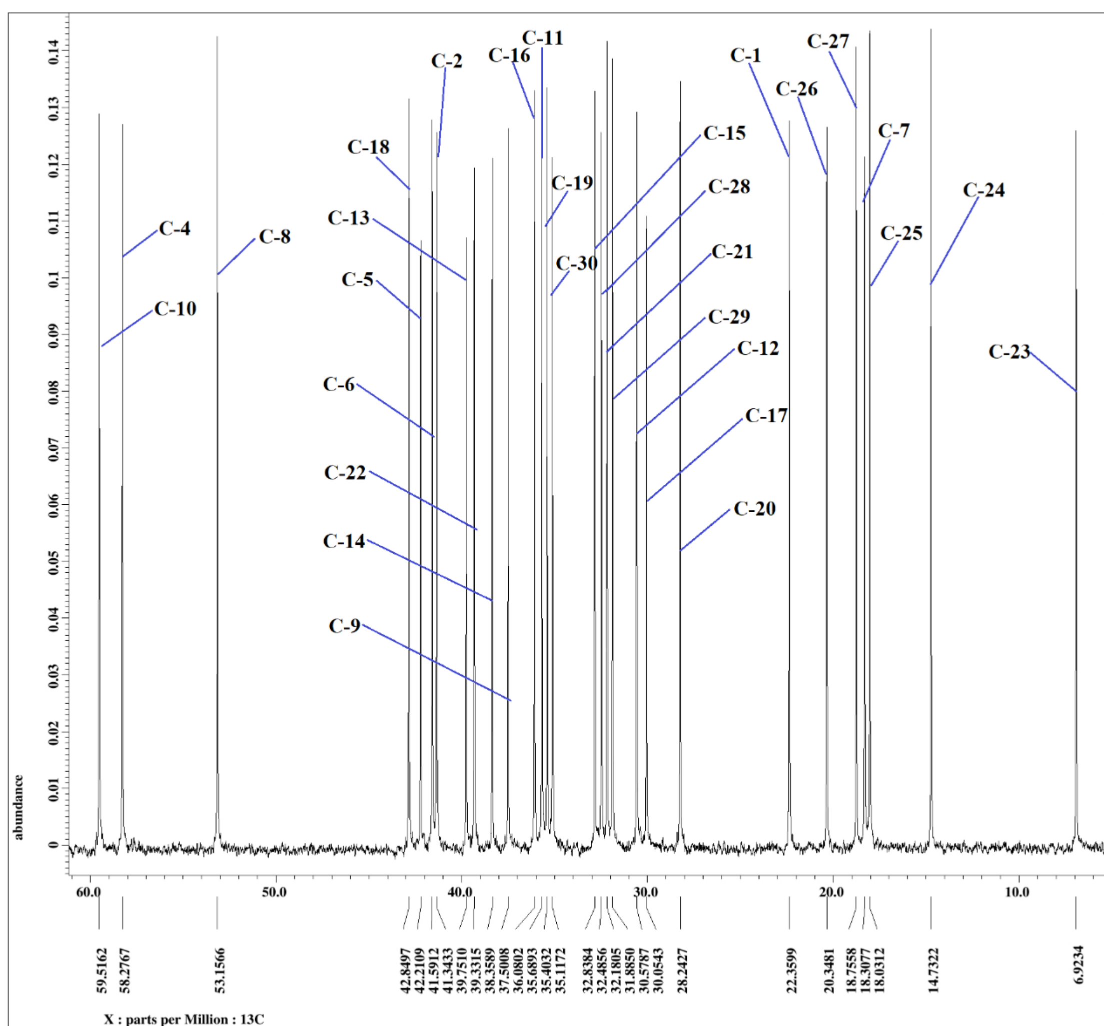


Figure 4.19: Expanded ^{13}C NMR spectrum of friedelin [19] (100 MHz, $CDCl_3$)

4.4 Antioxidant Assay

In this study, antioxidant activity of all the crude extracts and isolated compounds from *Calophyllum lanigerum* were tested via 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH is a crystalline powder with dark purple color which composed of stable free radical molecules and it changes to yellow color when it is scavenged. When DPPH solution was introduced with the antioxidant, it turned from dark purple to yellow color because it is reduced by accepting a hydrogen atom from the antioxidant, and this resulted a decrease in absorbance detected at 520 nm. The antioxidant activity of test compound was expressed as IC_{50} (concentration of sample that needed to inhibit 50 % of DPPH radical activity). The IC_{50} value of test samples were determined from the plotting of the graph of inhibition rate against concentration of sample as shown in Figure 4.20.

Table 4.3: Antioxidant results of test samples in DPPH assay

Test Samples	Antioxidant Activity, IC_{50} (μg/mL)
Positive controls:	
1. Ascorbic Acid	4.0
2. Kaempferol	9.0
Crude Extracts:	
1. Dichloromethane (DCM)	>240
2. Methanol (MeOH)	3.0
Isolated compounds:	
1. Euxanthone **[18]**	>240
2. Friedelin **[19]**	>240

From the assay, methanol crude extract of *Calophyllum lanigerum* was found to show the most potent activity with the lowest IC_{50} value of 3.0 μg/mL which was comparable with the positive control used, ascorbic acid (IC_{50} = 4 μg/mL). Moreover, dichloromethane crude extract and both isolated compounds were found to be inactive showing IC_{50} values of more than 240 μg/mL. Friedelin **[19]** is not a good antioxidant because it is not a phenolic compound. Meanwhile, euxanthone **[18]** is a phenolic compound but the hydroxyl group in the molecule is chelated to the keto group, thus, it is unable to donate hydrogen.

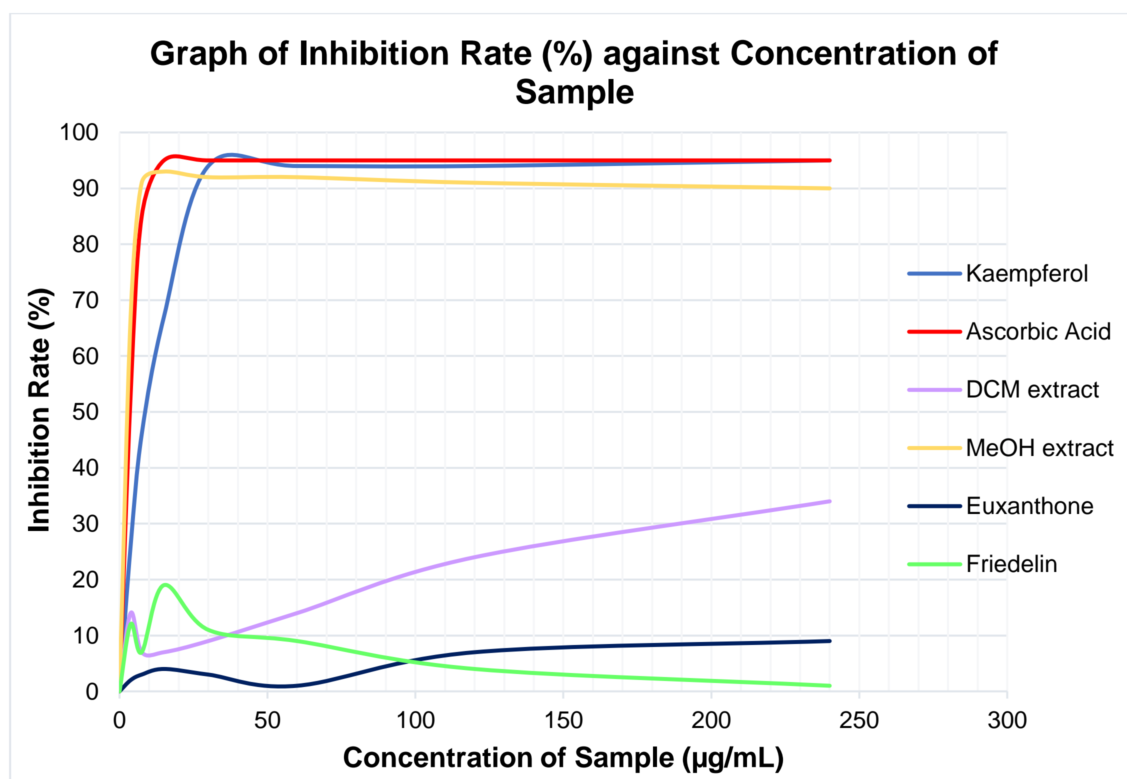


Figure 4.20: Graph of inhibition rate (%) against concentration of sample (μg/mL)

CHAPTER 5

CONCLUSION

5.1 Conclusion

In conclusion, two phytochemicals, namely euxanthone **[18]** and friedelin **[19]** were isolated from the dichloromethane crude extract of *Calophyllum lanigerum*. The structures of these isolated compounds were identified through modern spectroscopic analyses including 1D and 2D-NMR, UV-Vis and IR.

The dichloromethane, methanol crude extracts and both isolated compounds **18** and **19** were screened for their antioxidant activity via DPPH assay. Among all the test samples, only methanol crude extract showed the most potent antioxidant activity with the lowest IC_{50} value of 3.0 μg/mL which was comparable with the positive control used, ascorbic acid (IC_{50} = 4.0 μg/mL). Meanwhile, dichloromethane crude extract, compounds **18** and **19** were reported to show insignificant antioxidant activity with IC_{50} values of above 240 μg/mL.

5.2 Future Perspectives

In this project, one xanthone and one triterpenoid had been successfully isolated. In future studies, it is highly recommended the use of more advanced separation techniques such as high-performance liquid chromatography (HPLC) to isolate minor compounds from the stem bark of *Calophyllum lanigerum*. Secondly, the other biological activities such as anti-cancer, anti-microbial and anti-malarial of crude extracts and isolated compounds should be further investigate. Last but not least, actuve compounds should be studied for their potential chemical derivatives via organic synthesis.

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