CHEMICAL COMPOUNDS FROM THE STEM BARK OF *CALOPHYLLUM GRACILENTUM* AND THEIR ANTIOXIDANT ACTIVITIES

By

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ABSTRACT

CHEMICAL COMPOUNDS FROM THE STEM BARK OF *CALOPHYLLUM GRACILENTUM* AND THEIR ANTIOXIDANT ACTIVITIES

Lim Li Qing

Chemical study on the medicinally important plant species has led to the isolation and identification of various useful compounds for further study into their pharmacological activities. In this project, sequential solvent extraction on the powdered stem bark material of *Calophylum gracilentum* gave dichloromethane, ethyl acetate and methanol crude extracts. Each of these crude extracts was subsequently purified using column chromatography. From the dichloromethane extract, a new coumarin namely, 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin **[31]** and a triterpenoid, friedelin **[30]** were successfully isolated. Meanwhile, purification on the methanol extract afforded euxanthone **[32]**. Structural characterization of these pure compounds was carried out via various spectroscopic analyses, including 1D- and 2D-NMR, UV-Vis, IR and MS.

All the crude extracts and isolated compounds obtained were investigated for their antioxidant potential via DPPH assay. Methanol crude extract showed the

most potent radical scavenging activity with IC_{50} value of 9.0 μg/mL. Meanwhile, dichloromethane and ethyl actate crude extracts showed weak antioxidant activities with IC_{50} value of 195.0 and 145.0 μg/mL, respectively. On the other hand, all the three isolated compounds **30**, **31** and **32** showed insignificant activity with IC_{50} values of above 240.0 μg/mL.

ABSTRAK

Kajian kimia tentang metabolit tumbuhan yang mempunyai nilai perubatan telah membawa pengenalan pelbagai kompaun berguna untuk kajian activiti farmakologi masa depan. Dalam projek ini, kulit kayu batang bubuk *Calophyllum gracilentum* telah diekstrak berturutan dengan menggunakan diklorometana, etil asetat dan metanol. Kompaun-kompaun dalam ekstrak mentah seterusnya diasingkan dengan menggunakan kromatografi. Daripada ekstrak dikloromentana satu koumarin baru, iaitu 5,7-dihydroksi-4-(butan-2-yl)-8-(3-metibutiril)-3,4-dihydrkoumarin **[31]** dan satu triterpenoid, friedelin **[30]** telah berjaya diassingkan. Sementara itu, pembersihan pada ekstrak metanol diberikan euxanthone **[32]**. Struktur-struktur kompaun telah diperolehi melalui pelbagai sepktroskopi analisis, termasuk 1D- dan 2D- NMR, UV-Vis, IR dan MS.

Semua ekstrak mentah dan kompau-kompaun tulen telah diujikan bagi potensi antioksiden dengan menggunakan kaedah DPPH. Ekstrak metanol bagi paling kuat aktiviti memerangkap radikal degan IC_{50} nilai 9.0 μg/mL. Sementara itu, ekstrak diklorometana dan etil asetat menunjukkan aktiviti antioksidan yang lemah degan IC_{50} nilai 195.0 and 145.0 μg/mL, masing-masing. Sebalikanya, semua kompau-kompaun tulen (**30**, **31**, **32**) menunjukkan aktiviti yang tidak ketara degan IC_{50} nilai di atas 240.0 μg/mL.

ACKNOWLEDGEMENT

It was never an individual accomplishment to complete this final year project and therefore, I would like to take this opportunity to express my heartiest thanks to my supervisor, Dr. Lim Chan Kiang for his patient guidance and advices throughout the course of this project. I am grateful and touched for having him to spend his precious time in teaching me patiently.

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A million thanks to my family members especially to my father in giving me invaluable support and encouragement, and for being understanding. Last but not least, I wish to express my gratitude to my friends, Emily Khong and Ong Wuey Chen 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 duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

Lim Li Qing

APPROVAL SHEET

This project report entitled "**CHEMICAL COMPOUNDS FROM THE STEM BARK OF *CALOPHYLLUM GRACILENTUM* AND THEIR ANTIOXIDANT ACTIVITIES"** was prepared by LIM LI QING 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 SCINCE

UNVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **LIM LI QING** (ID No: **15ADB01845**) has completed this final year project entitled "CHEMICAL COMPOUNDS FROM THE STEM BARK OF *CALOPHYLLUM GRACILENTUM* AND THEIR ANTIOXIDANT ACTIVITIES" under supervision of 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 UTAR community and public.

Yours truly,

_____

(LIM LI QING)

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LIST OF ABBREVIATIONS

°C	Degree in Celsius
^{13}C	Carbon-13
1D-NMR	One Dimension Nuclear Magnetic Resonance
^{1}H	Proton
2D-NMR	Two Dimension Nuclear Magnetic Resonance
A_1	Absorbance of test sample
Acetone-d_6	Deuterated acetone
A_o	Absorbance of blank (negative control)
C=O	Carbonyl
$CDCl_3$	Deuterated chloroform
cm	Centimeter
C-O	Carbinol
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EA	Ethyl acetate
FTIR	Fourier-Transform Infrared Spectroscopy
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
IR	Infrared
J	Coupling constant in Hertz
KBr	Potassium bromide
kg	Kilogram
LC-MS	Liquid Chromatography-Mass Spectrometry

m	Mutiplet
MeOH	Methanol
mg	Miligram
mL	Mililiter
mm	Milimeter
mol	Mole
nm	Nanometer
NMR	Nuclear Magnetic Resonance
O-H	Hydroxyl
ppm	Part per million
R_f	Retention factor
s	Singlet
t	Triplet
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet-Visible
δ	Chemical shift
δ_C	Chemical shift of carbon
δ_H	Chemical shift of proton
λ_{max}	Maximum wavelength
μg	Microgram
μL	Microliter

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Historically, herbal medicines were used by our ancestors as their traditional health care products. Due to its minimal side effect on well-being of patients, scientist and researchers have realized the importance of traditional herbal medicine and have shown their growing interest in studying herbal medicine worldwide. The term "natural products" used nowadays can be referred to the traditional Chinese medicine (Shayne, 2005). China is one of the pioneer countries in the world that implemented this traditional health care system for more than 3000 years, and the earliest use of herbal medicine was recorded circa 722 B.C. in the Yellow Emperor's Canon of Internal Medicine (Huang Di Nei Jing) (Wong and Dahlen, 1999). As time goes by, medicinal herbs are widely used nowadays based on the historical studies recorded by the physicians, pharmacist, philosophers and scientists from different countries such as Greek, Egypt, Arab, England and others (Dias, Urban and Roessner, 2012). The valuable knowledge about the medicinal plants recorded has been acquired from generation to generation which has contributed in the development of medical field today (Wong and Dahlen, 1999).

Natural products are chemical compounds that are derived from living organisms such as plants, animals, and microorganisms, and these compounds can be categorised into two fundamental classes which are primary and secondary metabolites. Primary metabolites are compounds that are required for basic development and wellbeing of living organisms, such as carbohydrates, lipids, amino acids and nucleic acids. In contrast, secondary metabolites are not essential for development and growth of body, however, they are vital for the survival of living organisms, e.g. secondary metabolites help in adaptation and protection against predators in the surrounding environment.

Secondary metabolites play a crucial role in the pharmaceutical industry as natural products have been known to provide more than one third of the therapeutic drugs in the world. This has evoked a great interest in phytochemical study of plants' secondary metabolites to be developed into drug candidates. The common techniques used in phytochemical study are extraction, isolation, structural elucidation of natural products via various chromatographic and spectroscopic techniques (Phillipson, 2007). Natural product chemistry started to flourish in early 19^{th} century when new and more advanced technology has been developed for extraction, isolation and purification of natural products for further drug discovery.

Throughout the history of mankind, many phytochemicals have been discovered to show distinctive pharmacology properties including anti-inflammatory, antibacterial, anticancer and other therapeutic effects. Natural products have

served as a crucial source of drug leads for drug findings and development. For example, salicin which was isolated from the bark of willow and *Populus* species was investigated to show anti-inflammatory and analgesic properties. This compound was later developed into aspirin which shows less side effect and pharmacologically similar to salicin (Butler, 2004). Meanwhile, the anti-malarial drug, quinine was isolated from the bark of *Cinchona succiruba* Pav.Ex Klotsch (Dias, Urban and Roessner, 2012). Nowadays, the development of natural drugs has become more advanced as the drugs can be prepared in capsule, ointments or liquid forms which are easier for people to use them.

Phenolic compounds present in plant kingdom are a valuable source of antioxidants. Free radicals generated during oxidation are unstable and able to initiate radical chain reaction in human body which may lead to oxidative stress and increase the risk of cancer development, ageing process, cell mutation, stroke and others. Antioxidant can be termed as "free radical scavengers" as it provides protection against free radicals. This can be done by removal of free radical intermediates to terminate the radical chain reaction. Flavonoids, coumarins and anthocynins are some examples of phytochemicals that show radical scavenging property (Khalaf, et al., 2008).

Nowadays, antioxidants have been broadly used in different industries such as food, cosmetic, polymer and other industries. For instance, they are used to prolong the shelf life and to maintain the quality of foods and beverages as well as used in the formulation of cosmetic product due to their potent antioxidant

activity (Angerhofer, Maes and Giacomoni, 2008). Antioxidants also prevent the formation of engine-fouling residue by avoiding polymerization occurs in gasoline and to avoid degradation occurs in polymer products (Meurant, 2012). Antioxidant can be categorised into two major classes: natural antioxidants and synthetic antioxidants. Natural antioxidants are phenolic compounds that can be found mostly in plant materials, e.g. flowers, vegetables and herbal plants, while synthetic antioxidant are artificial and obtained from chemical processes conducted in laboratory.

Synthetic antioxidants are widely used nowadays as compared to natural antioxidants in many industries for preservation of canned food, rubbers and others as they are relatively cheaper, more efficient and easily available. However, they show some limitations such as carcinogenic and relatively more toxic which are not safe enough for human consumption (Madhavi, Deshpande and Salunkhe, 1995). For instance, natural antioxidants such as thymol and 6-gingerol have been used to prevent lipid peroxidation, replacing the synthetic butylated hydroxynisole, BHA and butylated hydroxytoluene, BHT (Aeschbach, et al., 1994). Hence, the efforts in the search for new and safer natural antioxidants are necessary to overcome the limitation of use of synthetic antioxidants especially their applications in foods and beverages, and cosmetic industries. In line with this, this project was carrying out to look for new natural antioxidants from the stem bark extracts of *Calophyllum gracilentum*.

1.2 Background of Plant Species Studied

1.2.1 Taxonomy of Plant Studied

The plant kingdom represents an extraordinary reservoir of novel molecules. However, only a little has been phytochemically investigated out of 400 000 – 500 000 plant species around the globe (Corrado, 2001). *Calophyllum* is the largest genus in Calophyllaceae family that encompasses of about 190 plant species (Diaz, 2013). The taxonomy of *Calophyllum gracilentum* is shown in Table 1.1.

Table 1.1: Taxonomy of *Calophyllum gracilentum*

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

1.2.2 Morphology, Geographical Distribution and Habitat

The family of Calophyllaceae comprises of 14 genera and 475 species (Christenhusz and Byng, 2016). The sizes of the evergreen trees can be varied from large to medium and the trees can grow up to 30 m in height (Nasir, et al., 2013). The word "*Calophyllum*" was originated from Greek which means "beautiful leaf" (Ong, et al., 2011).

"Bintangor" is the local name for the genus *Calophyllum* and it comprises of around 180-200 distinct species and many are found to disperse in the tropical rainforest of Asia such as in Sarawak of Malaysia, Thailand, West and East Kalimantan and Brunei (Nasir, et al., 2011). It is was also found in swamp forests, some of which on sandy sea shores (Corner, 1978).

1.2.3 Ethno Medicinal Uses and Pharmacological Studies

Calophyllum is well known to be rich in bioactive phytochemicals such as coumarins, bioflavonoids, xanthones, terpenes and steroids (Su, et al., 2008). Due to its interesting chemical and biological profiles, *Calophyllum* plants have been intensively studied for their medicinal properties such as anticancer, antifungal, anti-inflammatory and others (Su, et al., 2008). Previous study on *Calophyllum* species has reported the isolation of bioactive coumarins which exhibited anti-HIV and anti-cancer properties (Gwendoline, et al., 2011). Apart

from that, seed oil *Calophyllum* species was also reported as a therapeutic remedy for treatment of ulcer and rheumatism in Malaysia (Dweck and Meadows, 2002).

1.3 Objectives of Study

The purposes of performing this study are:

1. To extract and isolate chemical compounds from the stem bark of *Calophyllum gracilentum*.
2. To identify and characterize the chemical structures of isolated compounds through various modern spectroscopic techniques.
3. To examine the DPPH radical scavenging activity of pure compounds and crude extracts of *Calophyllum gracilentum*.

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemical Studies

Secondary metabolites produced naturally in plants are chemical constituents those are not essential for primary metabolism of plants as well as for reproduction and photosynthesis. However, they are very valuable to humans as they show potential biological properties which can be used for protection and prevention against various diseases. Phytochemicals produced in plant may provide protection of plants against fungi, bacteria, competing plants and herbivores. They are also important for pollination attraction of animals and adaption of plant to the surrounding environment (Wink, 2010).

Phytochemicals isolated from the plants can be used as flavours, fragrances or drugs. These secondary metabolites are also a good source of nutrients to humans. For instance, tuberous root of *Ipomoea batatas* which is also known as sweet potato, has high content of β-carotene which is a useful source of vitamin A needed for growth of human body (Groppo, et al., 2011).

Nowadays due to the increasing risk of illnesses, people tend to use traditional medicine for the treatment of various diseases as it has a diverse health related benefits. According to Vasanthi, ShriShriMal and Das (2012), approximately 5000 plant species were recorded to show therapeutic qualities such as *Achillea millefolium* (yarrow), *Allium sativum* (garlic) and others which contain bioactive phytochemicals that are able to reduce the risk of cardiovascular diseases and artherosclerosis. Many phytochemicals were reported to exhibit various biological activities such as anticancer, antibacterial, antiviral, antitumor, antioxidative properties (Su, et al., 2008). The genus *Calophyllum* selected in this study is known to be rich in phytochemicals such as coumarins, xanthones, flavonoids, chromanones and triterpenes.

2.1.1 Xanthones

Xanthone is a natural heterocyclic compound that has a basic molecular formula of $C_{13}H_8O_2$. It is also identified as 9*H*-xanthen-9-one and usually appears in yellow colouration. Backbone skeleton of xanthones consists of two benzene ring which bridge across an oxygen atom and a carbonyl group. This structure is known as dibenzo-γ-pyrone (Khan and Ather, 2006). Figure 2.1 shows the core structure of xanthone.

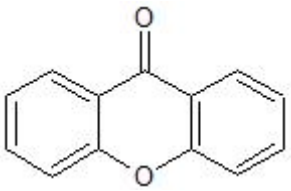


Figure 2.1: The core structure of xanthone

According to Khan and Ather (2006), xanthones were predominately isolated from *Guttiferae* and *Gentianaceae* families during the period from 1992 to mid-2003. It can also be obtained from fungi and lichens. Xanthones were reported to display various biological properties such as antioxidant, antimicrobial and antifungal. They also showed pharmacological properties such as antitumor, cytotoxic and hepatoprotective activities (Bräse, 2016).

Xanthones isolated from *Garcinia mangostana* were reported to exhibit potent cytotoxicity against human leukemia HL60 cell (Matsumoto, et al., 2005). Apart from that, xanthones extracted from *Garcinia subelliptica* were found to inhibit lipid peroxidation (Minami et al., 1994). Recently, xanthones were also reported to be used as antidepressive drugs. For instance, xanthones isolated from *Hypericum perforatum* were found to possess antidepressant action which could be developed into antidepressive drugs (Zhao, et al., 2014).

2.1.2 Flavonoids

Flavonoids are also known as bioflavonoids which can be found in the leaves, flowers, fruits of plants and also in beverages such as wine and tea. They are polyphenolic compounds which carry a basic structure of C_6-C_3-C_6 carbon

skeleton. They are diphenylpropane derivatives. Figure 2.2 shows the core structure of flavonoids.

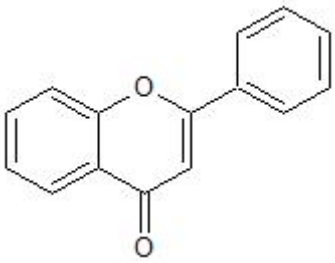


Figure 2.2: The core structure of flavonoids

Based on the differences in chemical structure of the heterocyclic C-ring, flavonoids can be classified into different categories such as flavones, flavanones, flavanols, isoflavones, dihydroflavanols, anthocyanins and catechin (Grotewold, 2006).

The epidemiological study found out that people who have dietary flavonoid intake (for example tea) show a lower risk of mortality in coronary heart disease. Flavonoids are free radical scavengers which cause macrophages *in vitro* to inhibit the oxidative modification of low density lipoproteins (LDL). This prevents the growth of artherosclerotic plaques and eventually decreases the risk of heart disease (Hertog, et al., 1993).

Flavonoids are not only useful to human being, they are also known as protective and pollinating agent to the plants. For example, flavonoids present in plants can act as antifeedants to protect the plants from insects. Flavonoids also provide

protection to the leaves from the UV radiation. In another way, flavonoids play an important role in pollination where the colour pigments present in the flowers attract pollinators (Treutter, 2006).

2.1.3 Coumarins

Coumarin was first isolated from the tonka tree (*Dipteryx odorata*) and its name was originated from the Caribbean word "coumarou" (Stewart, 2005). It is classified as benzopyrone family of compounds. Coumarins show unique ordor properties and they give pleasant smell with different odour characteristics. They have a basic molecular formula of $C_9H_6O_2$, and their core structure consists of benzene and α-pyrone ring (Hoult and Payá, 1996), as shown in Figure 2.3

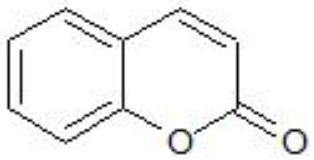


Figure 2.3: The core structure of coumarins

Coumarins can be classified into six main groups based on various substitutions on the coumarin core, such as simple coumarins, pyranocoumarins, furanocoumarins, dihydrofuranocoumarins, phenylcoumarin and bicoumarins. (Venugopala, Rashmi and Odhav, 2013).

Coumarins were found to show a diverse therapeutic application. Some coumarin dervatives were reported to exhibit anti-HIV, anti-microbial, anti-cancer, anti-inflammatory and antioxidant properties (Grazul and Budzisz, 2009). According to Belluti et al. (2010), coumarins inhibits the proliferation of the squamous cell carcinoma (A431) and melanoma (JRS) via apoptosis. Both of these cells have the potential of inducing cancer. In addition, the ability of coumarins to scavenge the peroxyl radicals inhibits the lipoxygenase (LOX) and cyclooxygenase (COX) pathway of arachidonic acid metabolism in growth control of cancer cells (Melagraki, et al., 2009).

2.1.4 Terpenes

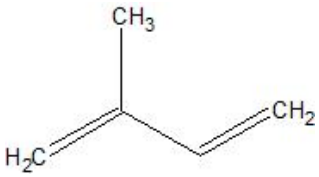


Figure 2.4: The core structure of isoprene

Figure 2.4 shows the core structure of isoprene from which terpenes are derived from. Isoprene has a molecular formula of C_5H_8, therefore terpenes are usually demonstrated as $(C_5H_8)_n$, where n is the number of isoprene units which are linked together in a head to tail manner. The origin of terpenes was derived from the extract of a pine tree, which is called turpentine (Hornback, 2006). Terpenes can be classified into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes,

sesterterpenes, triterpenes and so on based on a difference in the number of isoprene units having in the compounds.

Monoterpenes show good potential application in food and perfume industries as they are the major components for different aromas. They are also known as the essential oil which was reported to show antimicrobial activity by disrupting the microbial cytoplasmic membrane leading to the loss of impermeability to protons and ions that have bigger size (Cristani, et al., 2007).

2.2 Chemistry of the Genus *Calophyllum*

The genus *Calophyllum* consists of around 200 species all around the world. It is a well-known genus that is rich in secondary metabolites such as xanthones, flavonoids, coumarins, triterpenes and chromanones. However, not all the species from the genus have been phytochemically investigated. Among these, the more extensively studied species are *Calophyllum inophyllum*, *Calophyllum brasiliense*, *Calophyllum soulattri* and *Calophyllum lanigerum*.

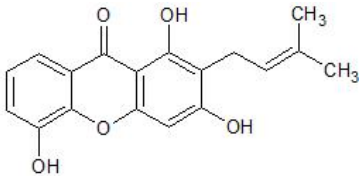
The plant selected in this study was *Calophyllum gracilentum*. However, there were no phytochemical and biological studies reported so far on this species. Therefore, the review was done based on other related *Calophyllum* species.

2.2.1 *Calophyllum symingtonianum*

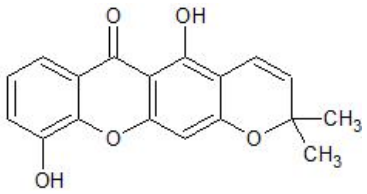
Calophyllum symingtonianum which was collected from Malaysia had been studied by Kawamura et al. in year 2012. Two major xanthones were successfully isolated from the heartwood of *Calophyllum symingtonianum*, named 1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone **[1]**, and 6-desoxyjacareubin **[2]**. Compound **2** exhibited trypanocidal activity against epimastigoes of *Trypanosoma cruzi*, while compound **1** was the putative biogenetic precursor of compound **2**. In this study, compound **2** showed a lesser anifungal activity towards brown-rot fungus, *Gloeophyllum trabeum*, than compound **1**. However, compound **2** showed a higher activity than compound **1**, against white-rot fungus, *Pycnoporus sanguineus* (Kawamura, et al., 2012).

In year 2015, Aminudin et al. isolated seven compounds from the stem bark of *Calophyllum symingtonianum*. These seven compounds were reported for the first time from this plant species and they were inophyllum D **[3]**, inophyllum H **[4]**, and calanone **[5]** which are coumarins, one terpene named lupenone **[6],** a chromanone carboxylic acid named isocordato-oblongic acid **[7]** and two flavonoids named amentoflavone **[8]** and carpachromene **[9]**. The assay results showed that both compounds **8** and **9** exhibited significant α-glucosidase inhibitory activity with IC_{50} values of 6.4 and 13.5 μM, respectively. Among the coumarin compounds, compound **3** (IC_{50} = 35.7 μM) showed the strongest

inhibiton, followed by compound **4** (IC_{50} = 62.3 μM) and lastly compound **5** (IC_{50} > 100 μM). Due to the presence of a greater number of hydroxyl group, compound **8** showed the greatest ability in inhibition of 15-LOX with the lowest IC_{50} value of 0.04 μM (Aminudin, et al., 2015).



[1]



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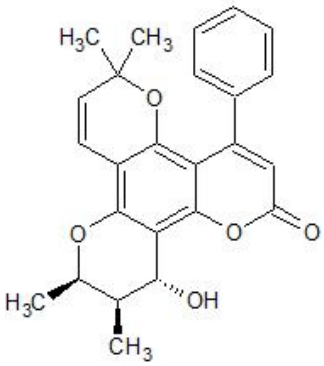

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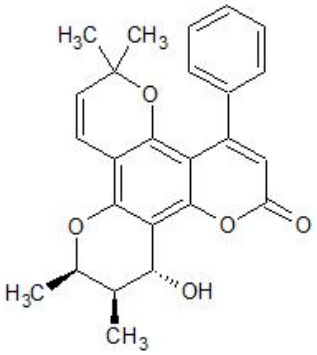

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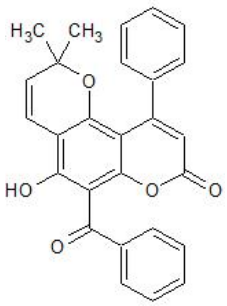

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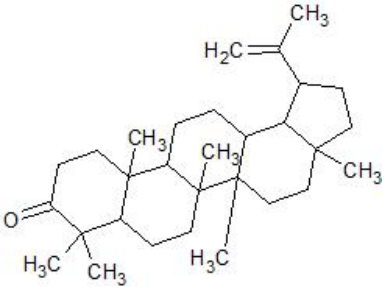
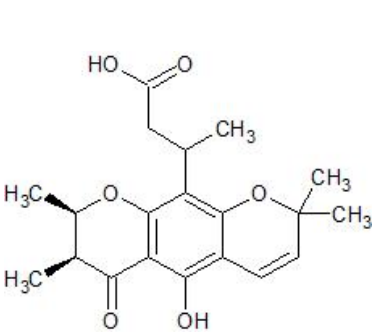
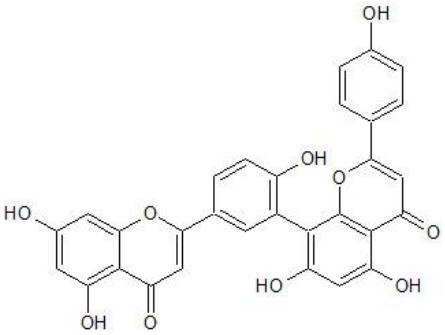


[6]

Figure 2.5: Molecular structures of isolated compounds from *Calophyllum symingtonianum*



[7]



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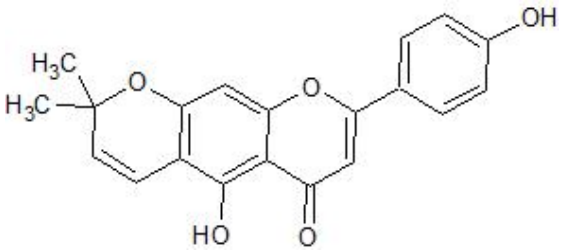


[9]

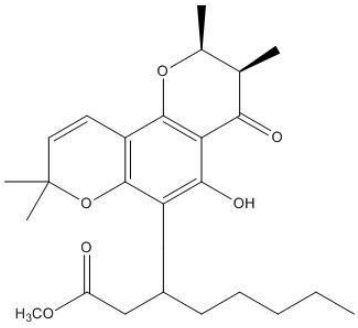
Figure 2.6: Molecular structures of isolated compounds from *Calophyllum symingtonianum* (continued)

2.2.2 *Calophyllum membranaceum*

Malfunction of RXRα causes various diseases such as cancer, diabetes, obesity and osteoporosis. *Calophyllum membranaceum* used in Traditional Chinese Medicine (TCM) has been studied by Ming et al. to show potential in treating the inhibitory of RXRα. In this study, two new chromanones, namely

calopolyanic acid methyl ester **[10]** and isopinetoric acid methyl ester **[11]**, two new xanthones, namely calophylixanthones A **[12]** and calophylixanthones **[13]** and a C-glycoside, namely calophymembranside C **[14]** were isolated from *Calophyllum membranaceum*. It was reported that compound **14** showed the transcriptional inhibitory activity of RXRα with IC_{50} value of 29.95 ± 1.08 μM (Ming et al., 2016).

Apart from that, Zou et al. (2005) revealed the isolation of five chemical compounds from *Calophyllum membranaceum*, which includes three xanthones, named calophymembranol A [**15]**, B **[16]**, C **[17]**, a biphenyl *C*-glycoside called calophymembranside A **[18]** and a phenylethanoid glycoside, named calophymembranside B **[19]**. All these isolated compounds were tested for their inhibitory activity towards cyclooxygenase-2 which is responsible for the inflammatory events. As a results, compound **15** exhibited strong inhibitory activity on cyclooxygenase-2 with IC_{50} value of 2.99 μM.



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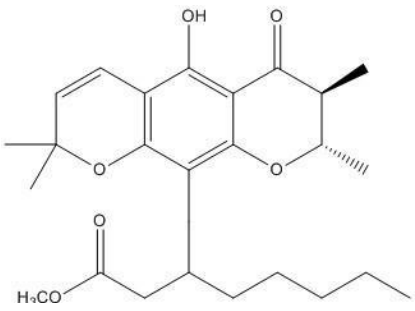
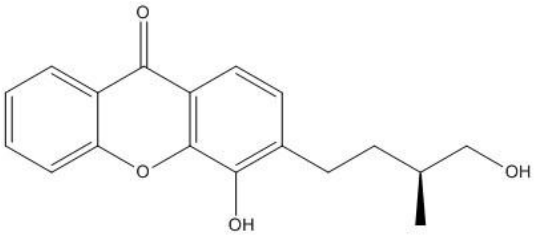
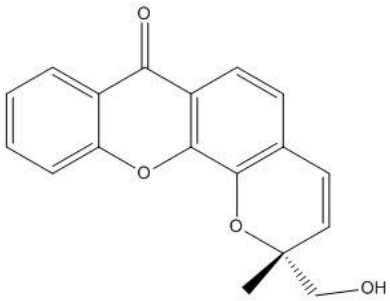


[11]

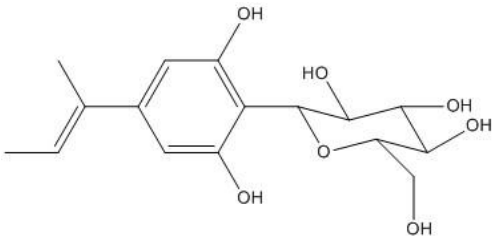
Figure 2.7: Molecular structures of isolated compounds from *Calophyllum membranaceum*



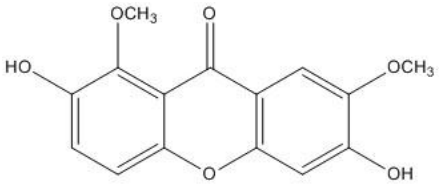
[12]



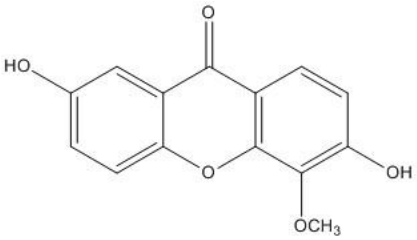
[13]



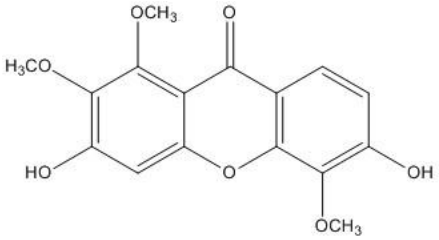
[14]



[15]

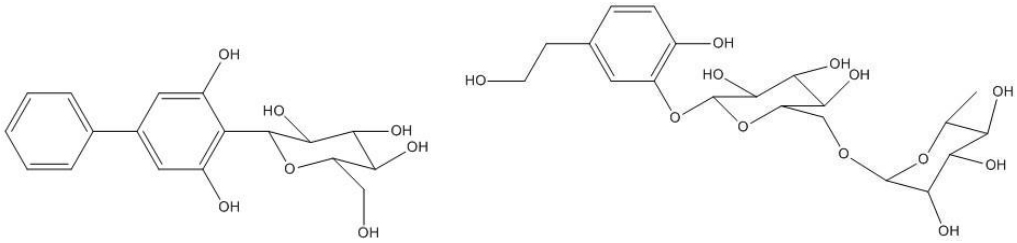


[16]



[17]

Figure 2.8: Molecular structures of isolated compounds from *Calophyllum membranaceum* (continued)



[18] **[19]**

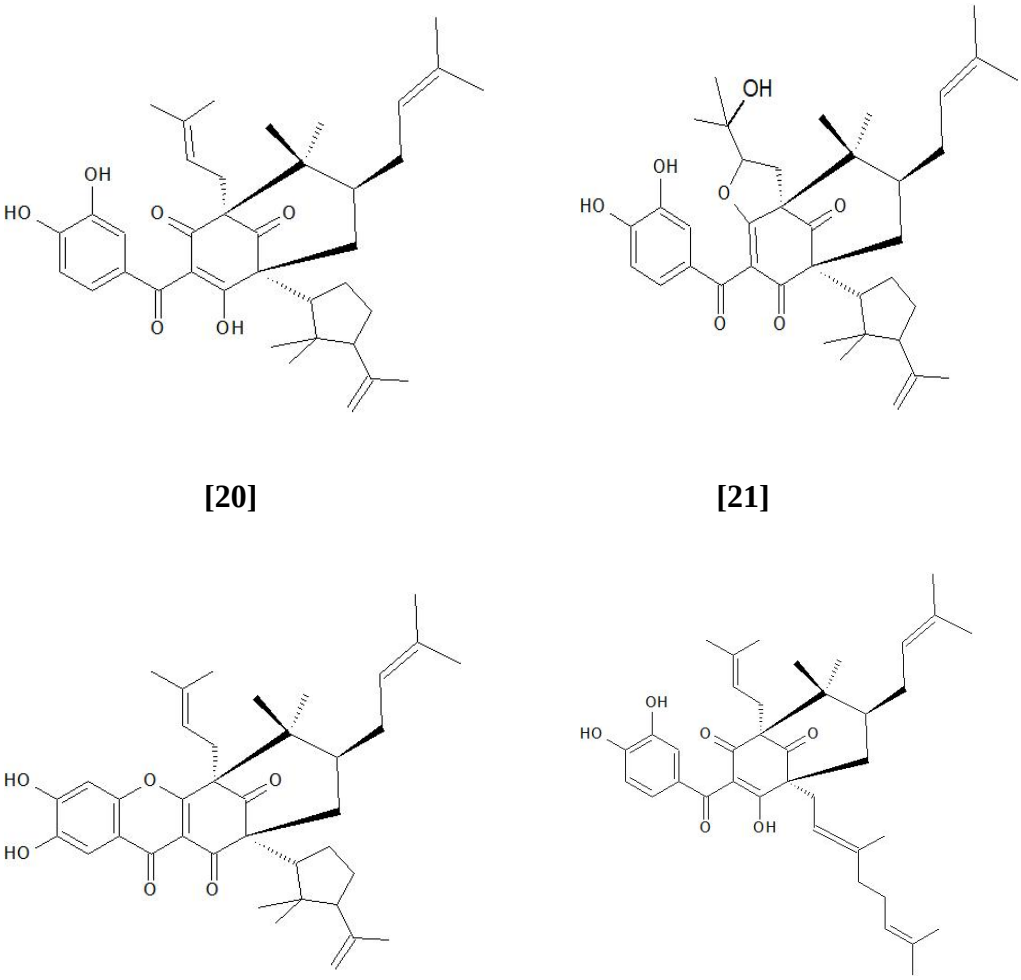
Figure 2.9: Molecular structures of isolated compounds from *Calophyllum membranaceum* (continued)

2.2.3 *Calophyllum thorelii*

According to Nguyen et al. (2012), chemical investigation on the stem bark of *Calophyllum thorelii* collected from Vietnam, yielded three new compounds including two polyisoprenylated acylphloroglucinols called thoreliones A **[20]** and B **[21]**, a polyisoprenylated tetracyclic xanthones, namely oxy-thorelione A **[22]**, together with three known compounds, namely guttiferone I **[23]**, F **[24]** and 30-epicambogin **[25].** All the isolated compounds were assayed on the three human cancer cell lines, MCF-7, HeLa and NCI-H460. Compound **20** showed moderate cytotoxic activity against three tumour cell lines with IC_{50} values of 7.4 ± 0.1, 9.3 ± 0.2 and 10.6 ± 0.5 μg/mL, respectively.

In year 2013, a new xanthone, calothorexanthone **[26]** was isolated from the stem bark of *Calophyllum thorelii* together with three known compounds, namely garbogiol **[27]**, δ-tocotrienol **[28]**, and globuxanthone **[29]**. All the

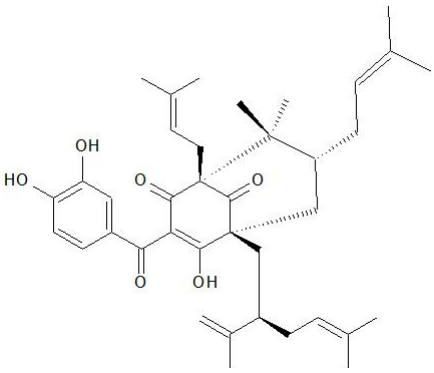
isolated compounds were screened for their DPPH free radical scavenging activites and the results showed that compound **27** displayed the strongest activity (IC_{50} = 15.07 ± 0.63 µg/mL), followed by compound **26** (IC_{50} = 17.46 ± 0.58 µg/mL), compound **28** (IC_{50} = 23.83 ± 0.31 µg/mL), and lastly compound **29** (IC_{50} = 38.19 ± 0.52 µg/mL) (Nguyen, et al., 2013).



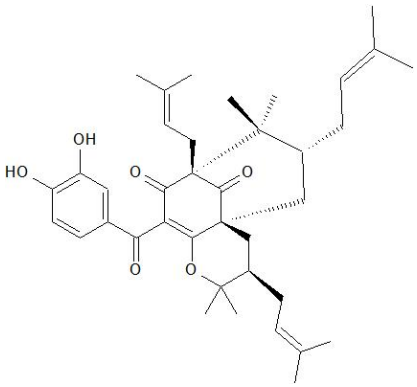
[20] **[21]**

[22] **[23]**

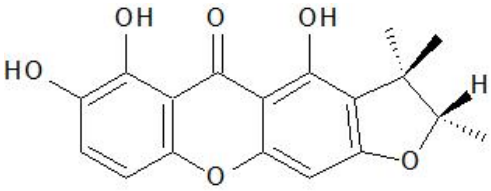
Figure 2.10: Molecular structures of isolated compounds from *Calophyllum thorelii*



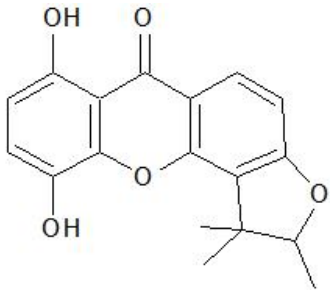
[24]



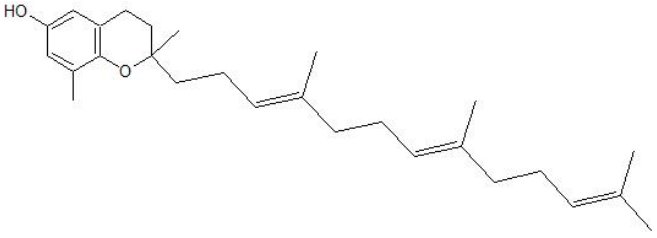
[25]



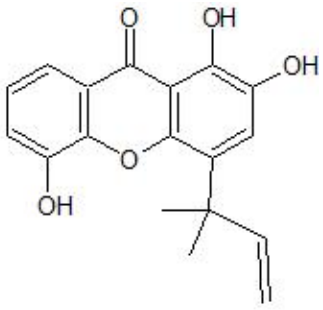
[26]



[27]



[28]



[29]

Figure 2.11: Molecular structures of isolated compounds from *Calophyllum thorelii* (continued)

2.2.4 Summary of Literature Review on *Calophyllum* Species

Table 2.1 Summary of the literature review on *Calophyllum* species

Name of the Plant Species	Type of Compound	Biological Activity	References
C. antillanum	• Chromanones	• Antimalarial • Cytotoxic	• Cuesta-Rubio, et al., 2015
C. apetalum	• Chromanone acids • Coumarins • Triterpenes • Xanthones	• Antitumor	• Inuma, et al., 1997 • Nigam and Mitra, 1967 • Govindachari, Prakash and Viswanathan, 1968a; 1968b
C. austroindicum	• Xanthones • Coumarins	-	• Iinuma, et al., 1996
C. benjaminum	• Coumarins • Xanthones • Triterpenes	-	• Sahimi, et al., 2015
C. blancoi	• Chromanones • Xanthones • Coumarins	• Cytotoxic • Antiviral • Antitumor	• Shen, et al., 2004; 2005 • Stout and Karl, 1968

C. brasiliense	• Xanthones • Flavonoids • Triperpenes • Chromanones • Coumarins	• Anticancer • Antibacterial • Anti-HIV-1 • Antiulcer • Cytotoxic • Antileishmanial	• Kimura, et al., 2005 • Lemos, et al., 2012 • Cottiqlia, et al., 2004 • Pires, et al., 2014 • Reyes-Chilpa, et al., 2004 • Ito, et al., 2003
C. calaba	• Xanthones • Terpenoids • Biflavonoids	-	• Kumar, et al., 1982 • Gunatilaka, et al., 1984
C. caledonicum	• Xanthones	• Antifungal • Antimalarial	• Morel, et al., 2002 • Hay, et al., 2004
C. chapelieri	• Chromanone acids	-	• Guerreiro, Kunesch and Polonsky, 1971
C. cordato-oblongurn	• Coumarins • Terpenoids	-	• Gunasekera and Sultanbawa, 1975 • Dharmaratne, et al., 1985
C. cuneifolium	• Xanthones • Chromanone acids • Triterpenes	-	• Gunasekera, et al., 1977

C. decipiens	• Triterpenes • Xanthones • Chromanones	• Antibacterial • Antioxidant	• Ajithabai, et al., 2012
C. dispar	• Coumarins	• Cytotoxic	Guilet, et al., 2001
C. dryobalanoies	• Chromanones • Xanthones	-	• Ha, et al., 2012
C. enervosum	• Xanthones	• Antimicrobial	• Taher, et al., 2005
C. flavoramulum	• Xanthones • Triterpenes • Flavonoids	• Antioxidant • Anti-AGEs	• Ferchichi, et al., 2012
C. fragrans	• Xanthones	-	• Locksley and Murray, 1969
C. gracilipes	• Xanthones • Triterpenes	• Cytotoxic	• Nasir, et al., 2013
C.incrasaptum	• Triterpenes	• Antimicrobial	• Abbas, et al., 2007
C. inophyllum	• Xanthones • Coumarins • Flavonoids • Triterpenes	• Anti-HIV-1 • Antimicrobial • Antioxidant • Cytotoxic • Antidyslipide-mic	• Yimdjo, et al., 2004 • Laure, et al., 2008 • Prasad, et al., 2012 • Li, et al., 2010
C. lanigerum	• Coumarins	• Anti-HIV	• Mckee, et al., 1996
C. macrocarpum	• Neoflavonoids • Triterpenoids	-	• Ampofo and Waterman, 1986

C. membranace-um	• Xanthones • Chromanones	• Cytotoxic • Anti-inflammatory	• Chen, et al., 2008 • Zou, et al., 2005
C. nodusum	• Xanthones • Triterpenes	-	• Nasir, et al., 2011
C. panciflorum	• Biflavonoids	• Antitumour	• Ito, et al., 1999
C. polyanthum	• Coumarins	• Antiherpetic	• Ma, et al., 2004
C. ramiflorum	• Xanthones	-	• Bhanu, et al., 1975
C. rubiginosum	• Flavonoids	• Anticancer • Antioxidant • Antimicrobial	• Bakhtiar, et al., 2010 • Alkhamaiseh, et al., 2011 • Alkhamaiseh, Taher and Ahmad, 2011
C. sclerophyllum	• Flavonoids • Xanthones • Chromanone acids	• Antioxidant	• Jackson, Locksley and Scheinnman, 1966 • Rissyelly, et al., 2014
C. soulattri	• Xanthones • Triterpenes • Coumarins	• Antimicrobial • Cytotoxic	• Mah, et al., 2011;2012 • Gwendoline, et al., 2011 • Khan, Kihara and Omoloso, 2002

C. symingtonianum	• Xanthones • Flavonoids • Coumarins	• Antifungal • Antioxidant • Antimicrobial • Cytotoxic • Anti-diabetic	• Kawamura, et al., 2012 • Attoumani, Susanti and Taher, 2013 • Aminudin, et al., 2015; 2016
C. teysmannii	• Coumarins	• Anti-HIV	• Fuller, et al., 1994
C. thorelii	• Xanthones • Benzophenones • Triterpenes	• Cytotoxic • Antioxidant	• Nguyen, et al., 2012 • Nguyen, et al., 2013
C. thwaitesii	• Xanthones • Triterpenes	• Antioxidant • Antibacterial • Antifungal	• Napagoda, et al., 2009 • Dahanayake, et al., 1974
C. venulosum	• Flavonoids	-	• Cao, Sim and Goh, 1997 • Cao, Sim and Goh, 2001
C. verticillatum	• Neoflavonoids • Triterpenes	• Mulloscicidal	• Ravelonjato, Kunesch and Poisson, 1987
C. walker	• Xanthones • Triterpenes	-	• Dahanayake, et al., 1974

C. zeylanicum	• Xanthones	-	• Gunasekera, Sotheeswaran and Sultanbawa, 1981

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Plant Materials

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

3.1.2 Chemical Reagents

The solvents and materials used for the extraction, isolation and purification of chemical constituents from *Calophyllum gracilentum* are summarized in Table 3.1. Table 3.2 shows the analytical grade solvents and materials used for TLC analysis. The deuterated solvents used for NMR analysis are listed in Table 3.3. Table 3.4 shows the list of the HPLC grade solvents and material used for LC- and GC-MS analyses. Next, analytical grade solvents and materials used for UV-

Vis analysis are listed in Table 3.5. Lastly, the chemical reagents and materials used for antioxidant assay are summarized in Table 3.6.

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

Solvents/Materials	Molecular formula	Density, ρ (g cm^{-3})	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 (60 Å)	SiO_2	-	Nacalai Tesque, Japan
Sephadex®LH-20	-	-	GE Healthcare, United State
Sodium sulphate anhydrous	Na_2SO_4	2.66	John Kollin Corporation, USA.

Table 3.2: Analytical grade solvents and materials used for TLC analysis

Solvents/Materials	Molecular formula	Source, Country
TLC silica gel 60 F_{254}	-	Merck, Germany
Iodine	I_2	Fisher Scientific, UK
n-Hexane	$CH_3(CH_2)_4CH_3$	R & M Chemicals, UK
Dichloromethane	CH_2Cl_2	QReC, Malaysia
Acetone	CH_3COCH_3	QReC, Malaysia
Ethyl acetate	$CH_3COOC_2H_5$	Fisher Scientific, UK

Table 3.3: Deuterated solvents used in NMR analysis

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

Table 3.4: HPLC grade solvents and materials used in LC- and GC-MS analysis

Solvents/Material	Molecular formula	Density, ρ (g cm^{-3})	Source, Country
Acetonitrile	CH_3CN	0.786	Fischer Scientific, UK
Methanol	CH_3OH	0.791	Fischer Scientific, UK
Nylon syringe filter (0.5 μm)	-	-	Titan2, USA

Table 3.5: Analytical grade solvents and materials used for UV-Vis analysis

Solvents/Materials	Molecular formula	Source, Country
Chloroform	$CDCl_3$	Fischer Scientific, UK
Cuvette (quartz)	-	Membrane solution, USA

Table 3.6: Chemical reagents and materials used for antioxidant assay

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

3.2 Methodology

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

Approximately 2.0 kg of stem bark of *Calophyllum gracilentum* was collected, air dried and ground into fine powder. The powdered material of the stem bark was then put into a closed container and soaked with dichloromethane at room temperature. After 48 hours, the dichloromethane extract was filtered and

anhydrous sodium sulphate was added to the filtrate in order to remove water residue. The solvent was then evaporated under reduced pressure via a rotary evaporator to give dry dichloromethane crude extract. These steps were repeated twice and the two dry dichloromethane crude extracts obtained were combined.

The plant material was then extracted twice with ethyl acetate followed by methanol in the order of increasing polarity. The weights of dichloromethane, ethyl acetate and methanol crude extracts obtained were 302.96, 53.99 and 199.04 g, respectively.

The crude extracts obtained were separately subjected to gravity column chromatography to give a series of fractions. The fractions collected were monitored for their chemical compositions via TLC analysis. Fractions found to have similar chemical composition were combined and subjected to further column chromatography until pure compounds were obtained. Pure compounds that gave a single spot on the TLC plate were characterized and identified for their structures using modern spectroscopic methods such as NMR, IR, UV-Vis and MS.

3.2.2 Column Chromatography

Column chromatography is a common method used to isolate chemical compounds from a crude extract. Silica gel was used as stationary phase while solvent mixtures in increasing polarity were used as the mobile phase. In gravity column chromatography, the mobile phase was driven down the silica gel packed column by gravitational force, and the separation was effected based on different affinity of the compounds for silica gel which resulted a difference in migration rates of the compounds. Eluents were collected according to the separated colour bands or volume.

Glass column of different sizes in internal diameter were used, depending on the amount of sample used. In this study, the sample was prepared via the dry packing method. Firstly, the crude extract was dissolved in a suitable amount of solvent and then was added dropwise into a small amount of silica gel in a beaker. The mixture was ground and left to dry overnight to form fine powdered sample.

A suitable amount of silica gel was mixed with *n*-hexane in a separate beaker to form a slurry and the slurry was then introduced into the glass column. After the silica gel was left to settle down in the column, the packed column was tapped with a rubber tube to facilitate even surface formation to the silica gel packing. The powdered sample was then subjected into the glass column to form a thin sample layer. A small amount of anhydrous sodium sulphate was added on top

of the sample layer to act as the protective layer and also in helping to absorb the water residue present in organic solvents used as mobile phase.

A stepwise gradient elution via solvent mixtures in increasing polarity (*n*-hexane/dichloromethane, dichloromethane/ethyl acetate, ethyl acetate/methanol) was used as mobile phase during elution of the column. Fractions collected were concentrated using a rotary evaporator and were then monitored for their chemical content via thin layer chromatography. Figure 3.1 shows the apparatus set up for column chromatography.

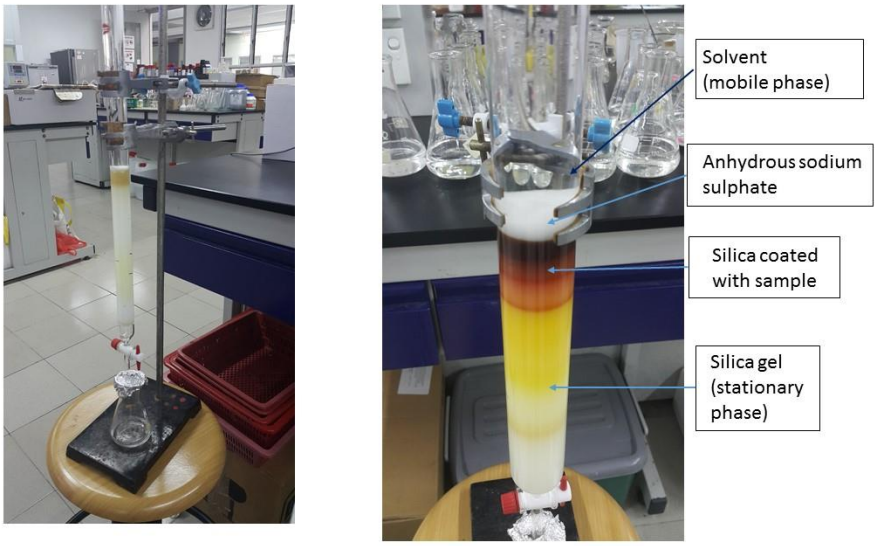


Figure 3.1 Column chromatography apparatus set up

3.2.3 Gel Permeation Chromatography

Gel permeation chromatography is a size exclusion chromatography in which compounds in solution are separated by differences in their sizes or molecular weights. The stationary phase and mobile phase used in this type of chromatography are different from the normal phase column chromatography mentioned in Section 3.2.2. It employs beads of porous polymeric material as the stationary phase such as Sephadex LH-20 which is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin and only polar solvents are usually used as the mobile phase. Smaller sized compound tends to enter into the pores of packing material which larger compound cannot. Therefore, larger compound travels along the column and eluted out first compared to the smaller compound. This chromatography method was used to separate individual compounds of different sizes in a mixture.

In this study, the mobile phase used was a solvent mixture of 90 % methanol and 10 % dichloromethane together with the Sephadex®LH-20 as the stationary phase.

3.2.4 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out to examine the purity of the isolated compounds and chemical composition of the fractions collected from column

chromatography. This was done by using 4 cm × 8 cm size of TLC plate with silica gel coated on the aluminium plate.

First, the sample was dissolved in a suitable amount of analytical grade solvents and it was then dotted on the marked baseline of the TLC plate by using a micro capillary tube. Next, the TLC plate with loaded samples was placed into the developing chamber that was filled with a suitable solvent mixture as the mobile phase. The chamber was closed with a lid or cap to allow the chamber to be saturated with the solvent vapour. The mobile phase started to migrate up the plate via capillary action and the rate of migration of compounds is depending on the degree of solubility of the compounds in the mobile phase and their interaction with stationary phase.

The TLC plate was taken out from the developing chamber when the mobile phase reached the solvent front line, and the plate was visualized under UV lights and later in an iodine vapour chamber.

Polar compounds moved up slower than non-polar compounds due to the former adsorbed more strongly to the stationary phase. Therefore, it has a smaller retention factor, R_f. The R_f value of each separated compound was calculated by using the following equation:

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

3.2.5 TLC Detection Method

3.2.5.1 UV Detection

UV detection technique was used to detect UV active compounds which have aromatic rings or conjugated system. The developed TLC plates were visualized under UV lights with both short (254 nm) and long (365 nm) wavelengths. As the TLC plate was coated with manganese-activated zinc silicate, it showed a bright green background and pale purple blue background when irradiated with UV lights of short and long wavelengths, respectively. Dark grey spot was observed at bright green background for the compound that absorbed the UV light at 254 nm, while fluorescence colour spot was observed on the pale purple background when the compound absorbed UV light at 365 nm.

3.2.5.2 Iodine Vapour Detection

Iodine vapour chamber was prepared by introducing a suitable amount of iodine crystals into a covered glass container. After the chamber was saturated with iodine vapour, the developed TLC plate was placed into the chamber for a few minutes to detect the presence of colourless terpenoids or non-conjugated compounds which were found to be invisible under UV light. When dark yellow-brown spots appeared, the TLC plate was taken out from the chamber and the

spots were circled before the iodine spots dissipated over time. These spots indicated the presence of terpenoids or non-conjugated compounds.

3.3 Instruments

3.3.1 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) is a spectroscopic method that provides information about the number and types of protons and carbons present in a molecule, and also the structural correlations among the atoms in the molecules. Each atom generates a magnetic field different from their neighbouring atoms as each of them experiences a difference in surrounding chemical and magnetic environment. Therefore, each of them produces distinctive signal that would help in the elucidation of chemical structure of the molecule.

In this study, the samples were separately dissolved in an appropriate amount of deuterated solvent such as deuterated chloroform, acetone or methanol according to the solubility of the samples in the solvents. Then, they were separately transferred into the NMR tubes up to 4 cm in height, and the tubes were capped, and labelled. The samples were run using JOEL JNM-ECX 400 MHz NMR spectrometer with the use of trimethylsilane (TMS) as internal standard and reference to obtain ^{1}H NMR, ^{13}C NMR, DEPT (Distortionless Enhancement by Polarization Transfer), HMQC (Heteronuclear Multiple

Quantum Coherence), and HMBC (Heteronuclear Multiple Bond Coherence) spectra for structural elucidation of the samples

3.3.2 Infrared Spectrophotometer (IR)

Infrared (IR) spectroscopy can be used to study different vibrational mode of bonds in a molecule. Molecule absorbs infrared radiation when the frequency of the bond vibration of the molecule matches the frequency of the infrared radiation directed on it. IR provides information about the functional groups present in a sample, and the specific fingerprint region in the spectrum is used for identification of molecular structure of the sample.

Preparation of sample was done by mixing homogenously a small amount of sample with potassium bromide, KBr powder in a ratio of 1:10 followed by compression under high pressure to give KBr pellet which was then inserted into a sample holder of IR instrument. 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 (UV-Vis) spectroscopy uses visible and/or ultraviolet light source to provide information about the presence of chromophores in relation to the conjugated system in a molecule. Highly conjugated compounds absorb light in the UV region to induce the electronic transition of electron from a lower energy state to a higher energy state.

In this project, Shimadzu double beam UV-Vis spectrophotometer was used for the sample analysis, where the radiation was split into two compartments: the blank and sample solution. An appropriate amount of analytical grade solvent such as methanol or dichloromethane was used to dissolve the sample and the absorption spectrum was obtained in the range of 200-400 nm.

3.3.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is a coupled technique that is used to obtain accurate molecular mass, molecular formula and fragmentation pattern of a non-volatile compound. Sample was prepared in HPLC grade solvent and was filtered to remove any undissolved solid particles before it was introduced to LC-MS analysis. Agilent Technologies 6520 LC/MS was used in this project to ionize the sample solution into free ions via electrospray ionization method. 5 μL of sample solution was

auto-injected into the column, followed by elution with a mixture of 30 % water and 70 % of methanol at a flow rate of 0.6 mL/min.

3.3.5 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS instrument is used to study the nominal molecular weight, molecular formula and mass fragmentation of a test compound. Shimadzu GC-MS QP2010 equipped with a flame ionization detector (FID) was used in this project to ionize the sample solution into free ions for spectral analysis. Sample was prepared by dissolving it in a suitable amount of AR grade solvent. 5 μL of sample solution was auto-injected into the non-polar BPX5 column and eluted with helium gas at a flow rate of 0.6 mL/min.

3.3.6 Melting Point Apparatus

Melting point measurement is carried out to determine the melting point and purity of a test compound. Pure compound normally has a narrow and sharp range of melting point. The melting point of the test compound was compared with the literature value of the pure compound to confirm the identity of the compound. Stuart SMP 10 melting point apparatus was used to determine the melting point of the test compound by heating the haematocrit capillary tube which was partially filled up with the sample. The temperature range at which the compound started to melt and entirely melted was recorded.

3.4 Antioxidant Assay

In this study, antioxidant activity of the crude extracts and isolated compounds was investigated via DPPH assay. The master stock at concentration of 1 mg/mL was prepared by dissolving the standard (ascorbic acid and kaempferol) and samples separately in methanol, and sonicated for 5 minutes to form homogenous solutions. DPPH powder was dissolved in methanol and sonicated to give DPPH solution at concentration of 2 mg/mL. All the prepared solutions were stored in a 4 ℃ chiller in dark condition to avoid exposure to light.

Test solutions were prepared through serial dilution, at various concentrations of 240, 120, 60, 30, 15, 7.5, 3.75 μg/mL in a 96-well plate. 5 μL of DPPH solution and 10 μL of methanol were added to each well and the wells with only DPPH solution and methanol were served as the blank, while ascorbic acid and kaempferol served as the positive controls in this assay.

After the addition of the reagents, the plate was immediately covered and wrapped with aluminium foil to prevent evaporation of the solvent and exposure to light. The plate was incubated in dark at room temperature for 30 minutes. The absorbance of the content in each well was measured at 517 nm using a microplate reader and each sample was performed in triplicate. The average absorbance for each concentration was recorded.

Finally, the percentage of the inhibition rate of the samples was calculated using the following equation:

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

where A_0 = absorbance of the negative control

A_1 = absorbance of sample

The data obtained were used for the plotting of graph of inhibition rate versus sample concentrations for the sample to determine the IC_{50} value. IC_{50} is defined as the concentration of sample which is required to inhibit 50 % DPPH radical scavenging activity.

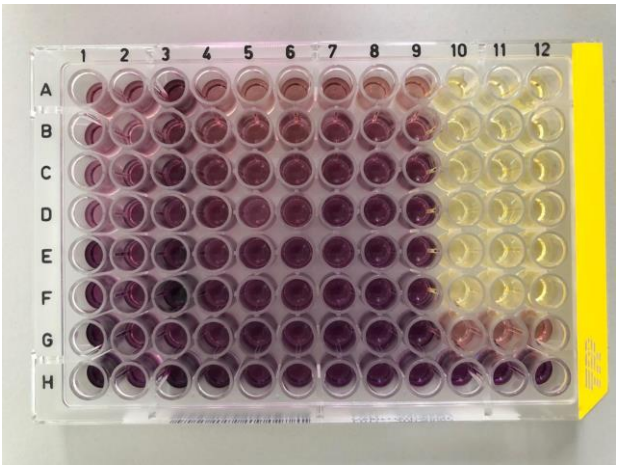


Figure 3.2: DPPH antioxidant assay using 96-well plate

Chapter 4

RESULTS AND DISCUSSION

4.1 Chemical Constituents Isolated from *Calophyllum gracilentum*

About 131.4 g of dichloromethane crude extract of *Calophyllum gracilentum* was subjected to silica gel gravity column chromatography, 8.5 cm in diameter packed in *n*-hexane. The column was eluted with a series of solvent mixture of increasing polarity (hexane/dichloromethane, dichloromethane/ethyl acetate, ethyl acetate/methanol) to give 30 fractions which were labelled as YZA1-30. Fractions YZA5-6 were combined and recrystallized from methanol to give white needle-like crystals, friedelin **[30]** (137.5mg). Fraction YZA10 was further purified by silica gel column chromatography, 4.0 cm in diameter eluted with a gradient of hexane/acetone in increasing polarity to give 20 subfractions (YZC1-20). Subfractions YZC15-19 were combined and subjected to gel permeation column packed with Sephadex LH-20 and eluted with a solvent mixture of 90 % methanol and 10 % dichloromethane to give subfractions YZI1-10. Further purification of the combined subfractions of YZI3-7 via the gel permeation column gave a total of 20 subfractions (YZM1-20). Subfractions YZM8-9 yielded brownish gum, 5,7-hydroxy-4-(butan-2-yl)-6-(3-methylbutyryl)-3,4-dihydrocoumarin **[31]** (34.6 mg). However, purification of ethyl acetate crude extract failed to yield any pure compounds.

On the other hand, fractionation of about 100.0 g of methanol crude extract by silica gel column chromatography, 8.5 cm in diameter packed in *n*-hexane and eluted with solvent mixtures of increasing polarity (hexane/dichloromethane, dichloromethane/acetone, acetone/methanol) afforded 35 fractions (LQA1-35). Fractions LQA19–20 were combined and subjected to a 4.0 diameter silica gel packed column eluted with solvent mixtures of increasing polarity (hexane/ethyl acetate and ethyl acetate/acetone) to yield subfractions LQC1–80. Subfractions LQC33–36 were combined and subjected to gel permeation column packed with Sephadex LH-20 and eluted with a solvent mixture of 90 % methanol and 10 % dichloromethane to yield subfractions LQS1–30. Subfractions of LQS26–28 gave yellow-needle like crystals, euxanthone **[32]** (47.8 mg).

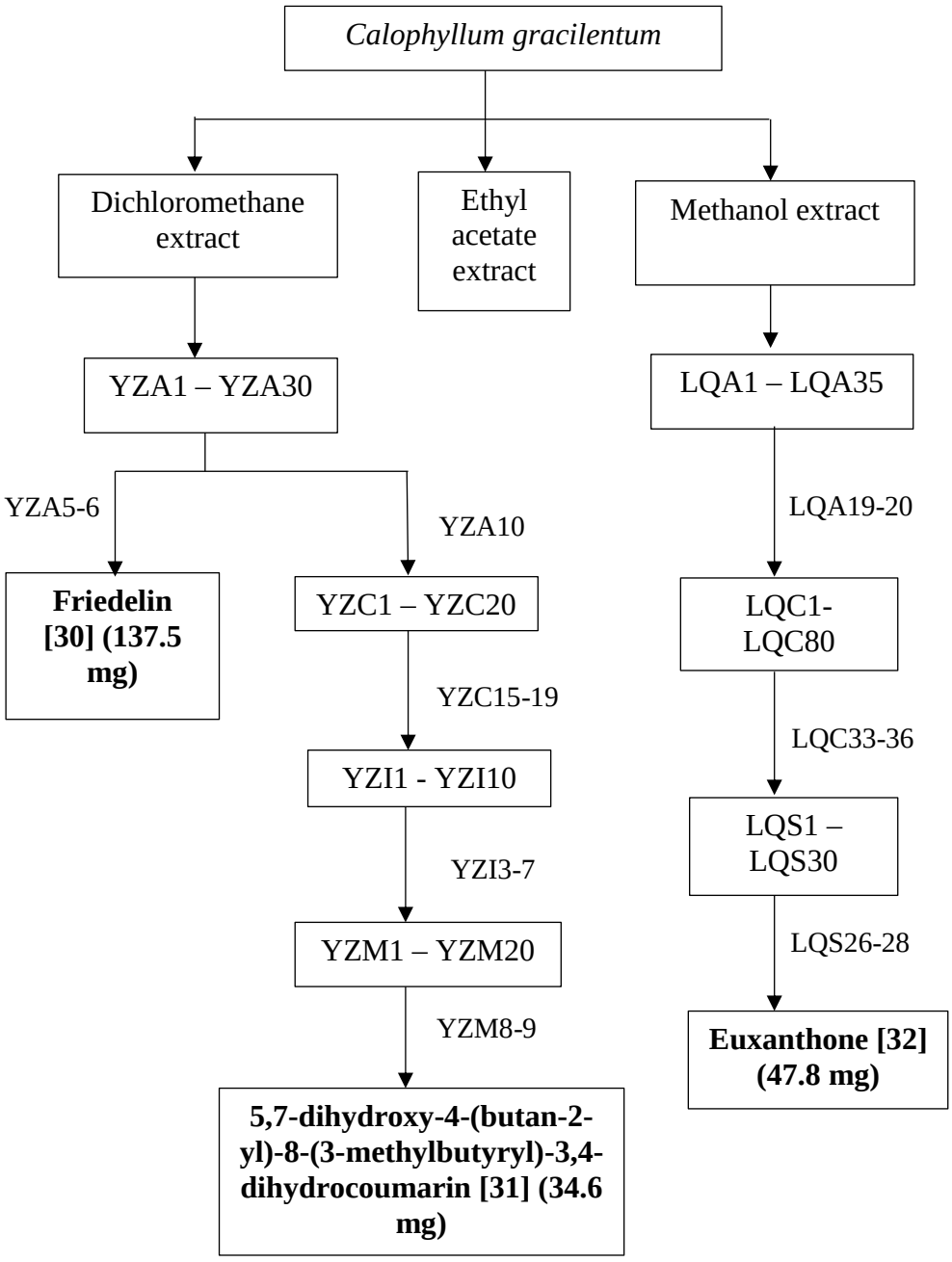


Figure 4.1: Pathway of isolation of pure compounds

4.2 Structural Characterization and Elucidation of Friedelin [30]

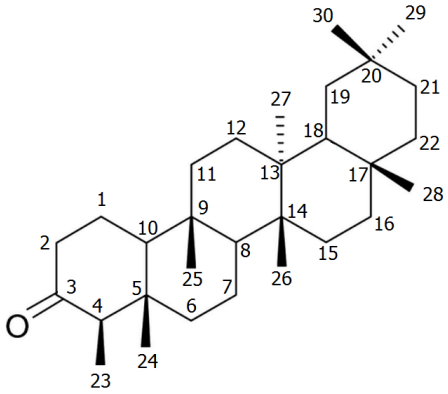


Figure 4.2: Molecular structure of friedelin [30]

A total of 137.5 mg of compound **30** was isolated from dichloromethane extract of *Calophyllum gracilentum* as white needle like-crystals with a melting point of 259-260°C (Lit. 261-262°C, Subhadhirasakul and Pechpongs, 2005). This compound appeared as a single dark spot under short wavelength of UV light at 254 nm during the TLC analysis. It gave a retention factor, R_f value of 0.59 using a mixture of 70 % dichloromethane and 30 % hexane as mobile phase. Compound **30** appeared as a dark brown spot when treated with iodine vapour.

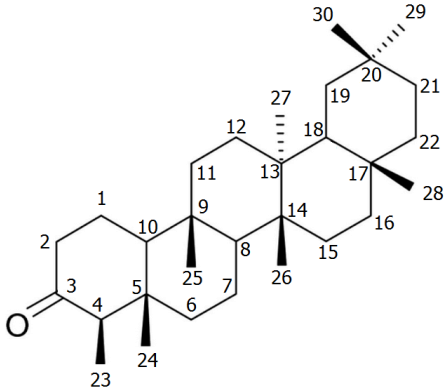
From the IR spectrum (Figure 4.3), an intense peak at 1715 cm^{-1} indicated the presence of a carbonyl group. In addition, the presence of sp^3 C-H stretching was revealed by the absorption peak at 2927 cm^{-1}. Apart from that, the UV-Vis spectrum (Figure 4.4) gave an absorption peak at 218 nm which is due to the n

$\rightarrow \sigma^*$ transition. Therefore, compound **30** does not have a conjugated double bond structure.

In the ^{1}H NMR spectrum (Figures 4.6 and 4.7), a total six singlet signals at δ 0.70 (H-24), 0.85 (H-25), 0.93 (H-26), 1.03 (H-27), 1.16 (H-28) and 0.98 (H-29 and H-30) and a doublet signal at δ 0.86 (*J* = 6.1 Hz, H-23) were the characteristic proton signals for the presence of eight methyl groups in compound **[30]** which resembles friedelin. Meanwhile, a multiplet signal of methine proton was observed at δ 2.23 (H-4). In addition, two groups of methylene protons gave signals at δ 1.94 (m, H-1_a) and 1.73 (m, H-1_b), and δ 2.36 (dd, *J* = 13.4 Hz and 5.4 Hz, H-2_a) and 2.28 (m, H-2_b). There was no vinylic proton signals observed in the region above δ 5.00 and the remaining protons signals of compound **30** appeared as multiplets in the upfield region of δ 1.90-1.20.

From the ^{13}C NMR spectrum (Figures 4.8 and 4.9), a total 30 carbon signals were observed corresponding to the presence of 30 carbons in the compound **30**. The most deshielded signal at δ 213.4 was assigned to the keto carbon (C-3). The eight methyl carbons showed signals at δ 6.9 (C-23), 14.7 (C-24), 18.0 (C-25), 20.3 (C-26), 18.8 (C-27), 32.2 (C-28), 35.1 (C-29) and 31.9 (C-30). Meanwhile, six quaternary carbon signals were observed at δ 42.2 (C-5), 37.5 (C-9), 39.8 (C-13), 38.4 (C-14), 30.1 (C-17) and 28.3 (C-20), and the remaining carbon signals were assignable to the methylene carbons. Both ^{1}H NMR and ^{13}C NMR data of compound **30** were found to be in agreement with the literature

values reported for friedelin (Abbas, et al., 2007). Table 4.1 shows the summary of NMR data for compound **30** in comparison with the literature values of friedelin.



[30]

Table 4.1: Summary of NMR data for compound 30 in comparison with literature values of friedelin

Position	δ_H (ppm)	*δ_H (ppm)	δ_C (ppm)	*δ_C (ppm)
1	1.94 ($1H_a$, m) 1.73 ($1H_b$, m)	1.97 (m) 1.71 (m)	22.5	22.3
2	2.36 ($2H_a$, dd, *J* = 13.4, 3.0 Hz) 2.28 ($2H_b$, m)	2.41 (dd, *J* = 13.0, 3.5 Hz) 2.31 (m)	41.6	41.5
3	-	-	213.4	213.2
4	2.23 (1H, m)	2.28 (m)	58.3	58.2
5	-	-	42.2	42.2
6	1.75 (1H, m) 1.29 (1H, m)	1.78 (m) 1.31 (m)	41.4	41.3
7	1.49 (1H, m) 1.40 (1H, m)	1.51 (m) 1.41 (m)	18.3	18.2
8	1.40 (1H, m)	1.41 (m)	53.2	53.1
9	-	-	37.5	37.4
10	1.52 (1H, m)	1.55 (m)	59.5	59.5
11	1.39 (1H, m)	1.40 (m)	35.7	35.6
12	1.29 (1H, m) 1.26 (1H, m)	1.30 (m) 1.28 (m)	30.6	30.5
13	-	-	39.8	39.7
14	-	-	38.4	38.3
15	1.48 (1H, m) 1.28 (1H, m)	1.50 (m) 1.30 (m)	32.8	32.8

16	1.53 (1H, m)	1.40 – 1.60 (m)	36.1	36.0
17	-	-	30.1	30.0
18	1.59 (1H, m) 1.37 (1H, m)	1.60 (m) 1.40 (m)	42.8	42.8
19	1.58 (1H, m)	1.60 (m)	35.4	35.3
20	-	-	28.3	28.2
21	1.50 (1H, m) 1.30 (1H, m)	1.51 (m) 1.31 (m)	32.5	32.5
22	1.50 (1H, m) 1.30 (1H, m)	1.51 (m) 1.31 (m)	39.3	39.3
23	0.86 (3H, d, *J* = 6.1 Hz)	0.92 (d, J = 7.0 Hz)	6.9	6.8
24	0.70 (3H, s)	0.75 (s)	14.7	14.7
25	0.85 (3H, s)	0.90 (s)	18.0	18.0
26	0.93 (3H, s)	1.03 (s)	20.3	20.3
27	1.03 (3H, s)	1.07 (s)	18.8	18.7
28	1.16 (3H, s)	1.20 (s)	32.2	32.1
29	0.98 (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.

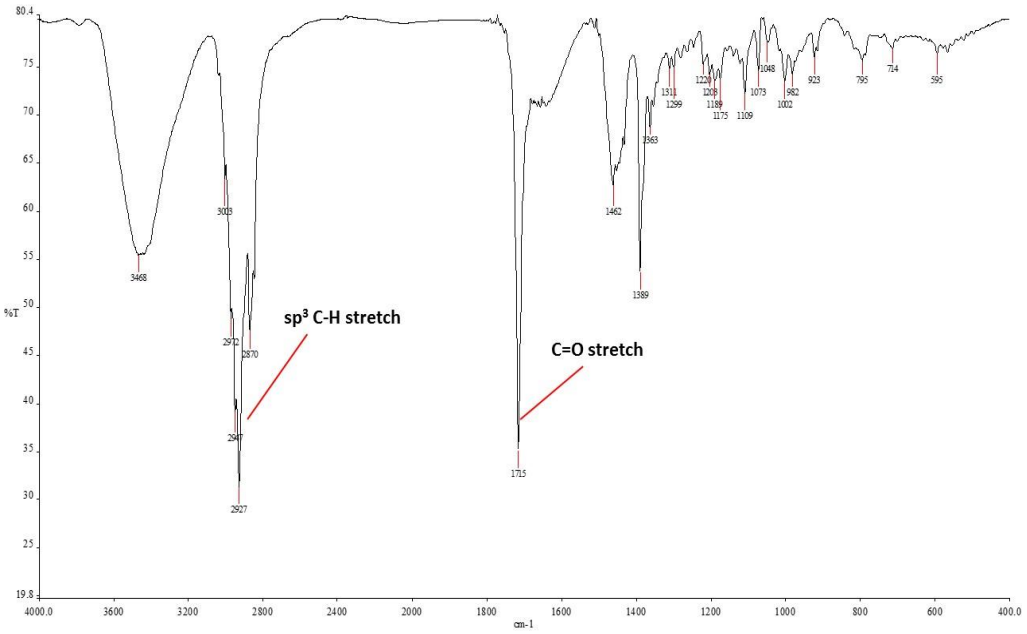


Figure 4.3: IR spectrum of friedelin [30]

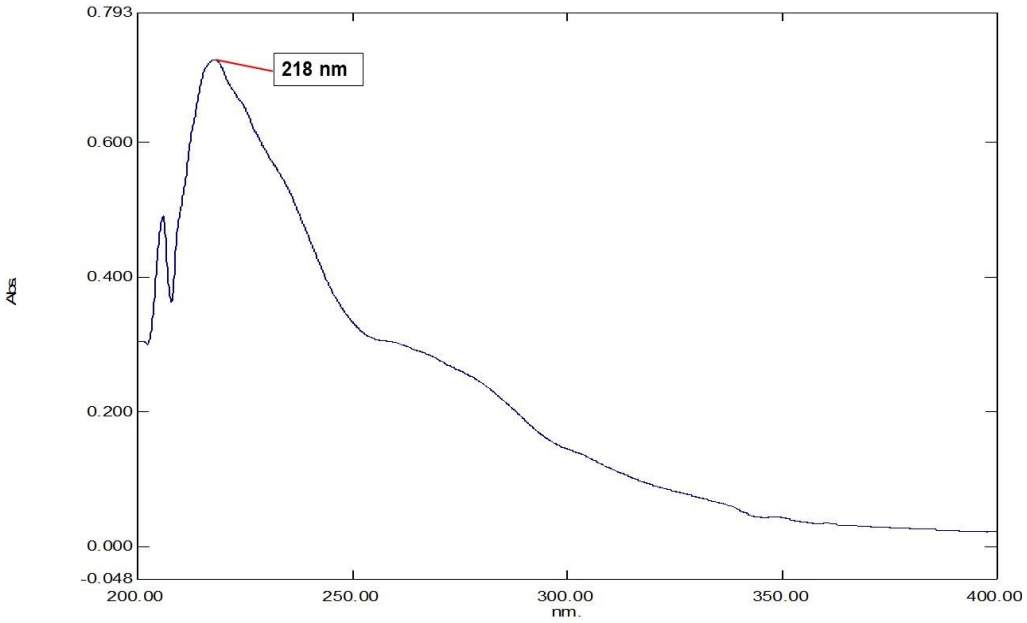
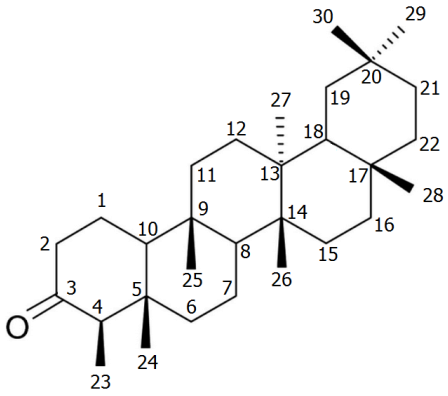


Figure 4.4: UV-Vis spectrum of friedelin [30]



[30]

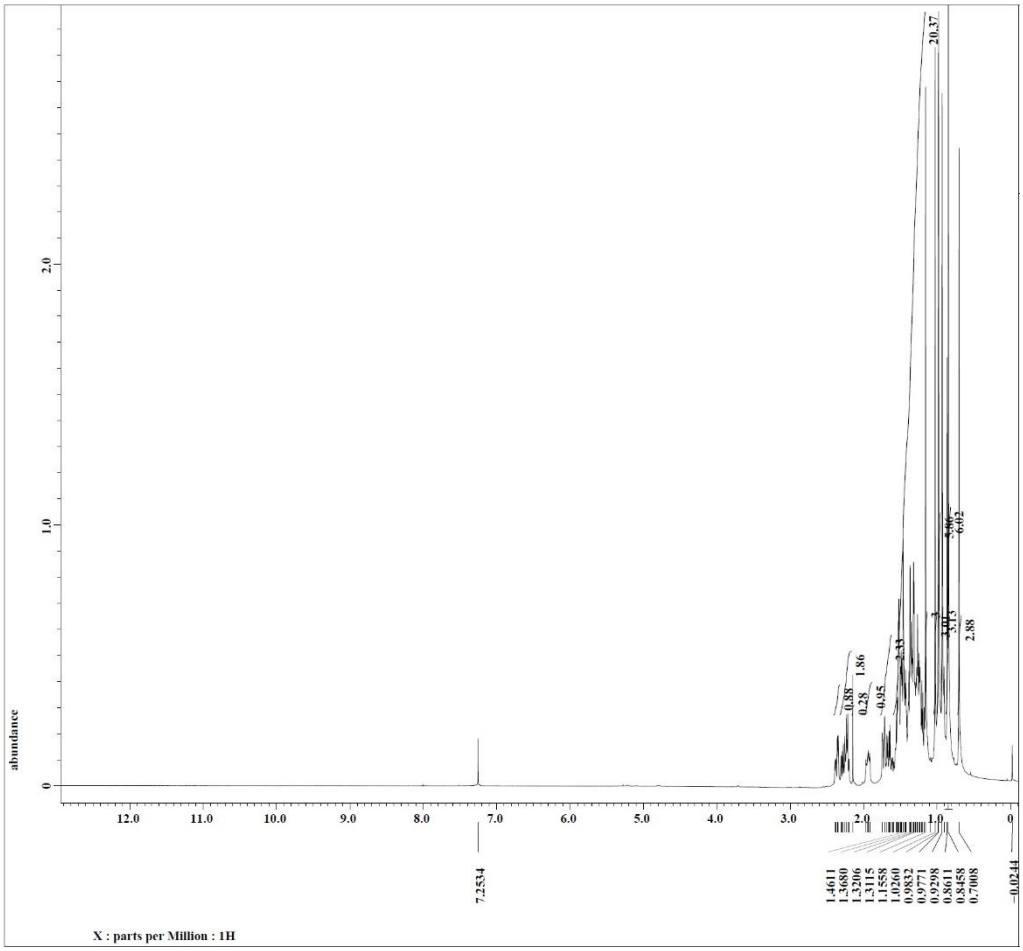
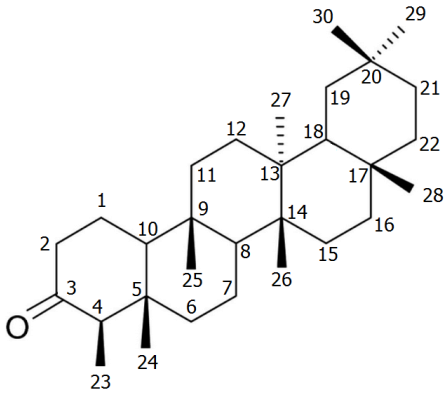


Figure 4.5: ^{1}H NMR spectrum of friedelin [30] (400 MHz, $CDCl_3$)



[30]

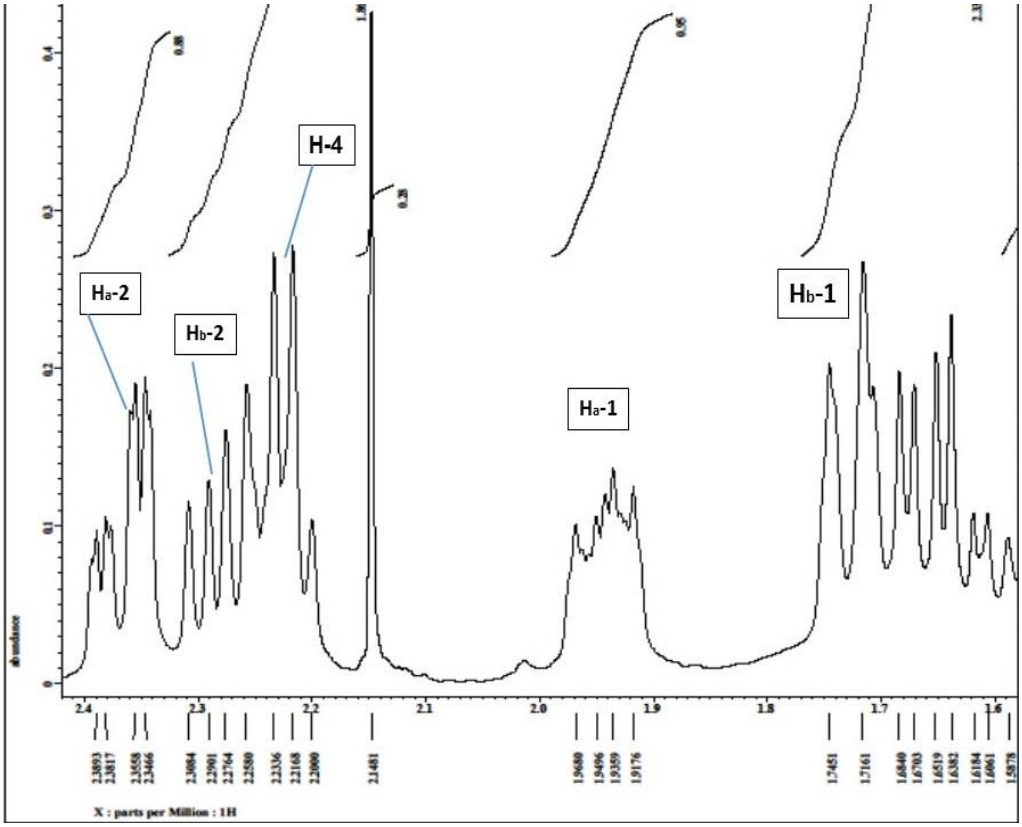
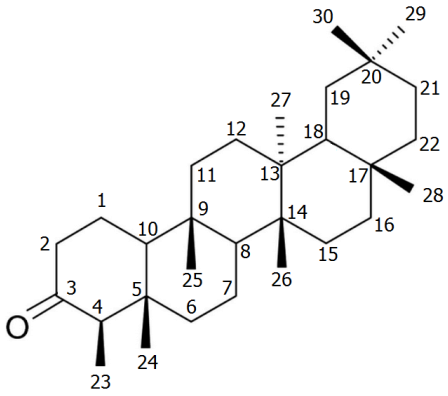


Figure 4.6: ^{1}H NMR spectrum of friedelin [30] (400 MHz, $CDCl_3$) (expanded)



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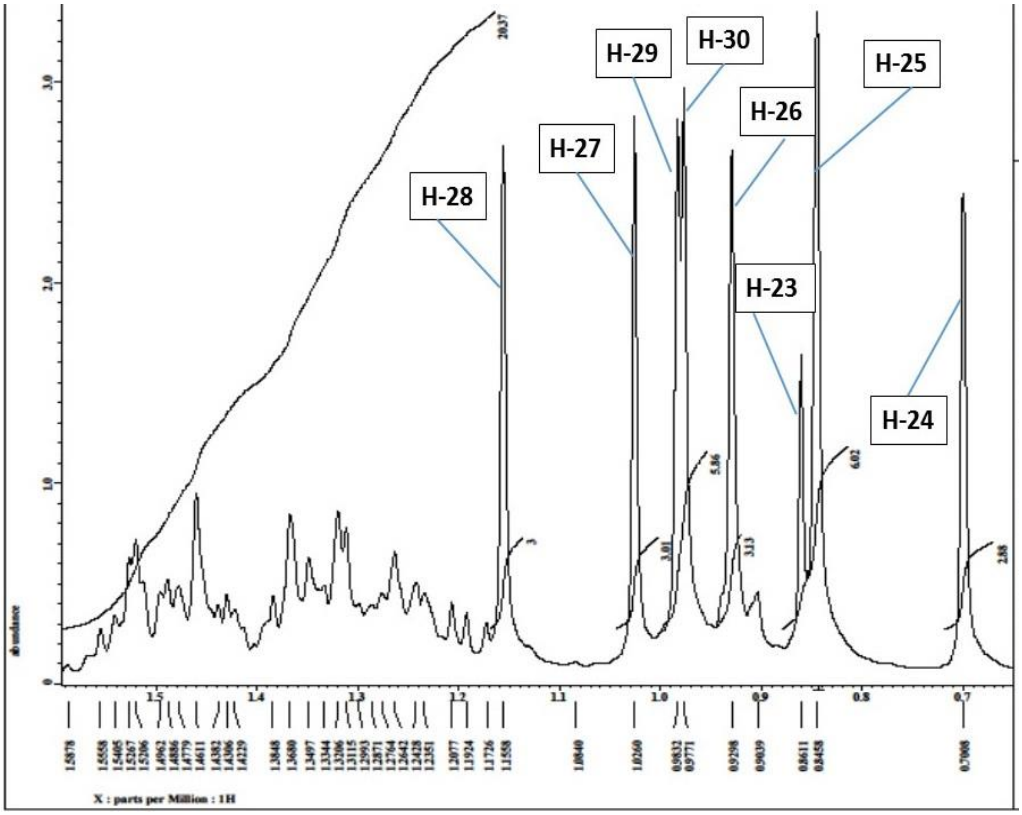
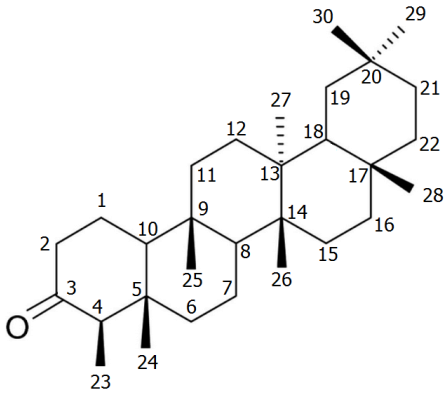


Figure 4.7: ^{1}H NMR spectrum of friedelin [30] (400 MHz, $CDCl_3$) (expanded)



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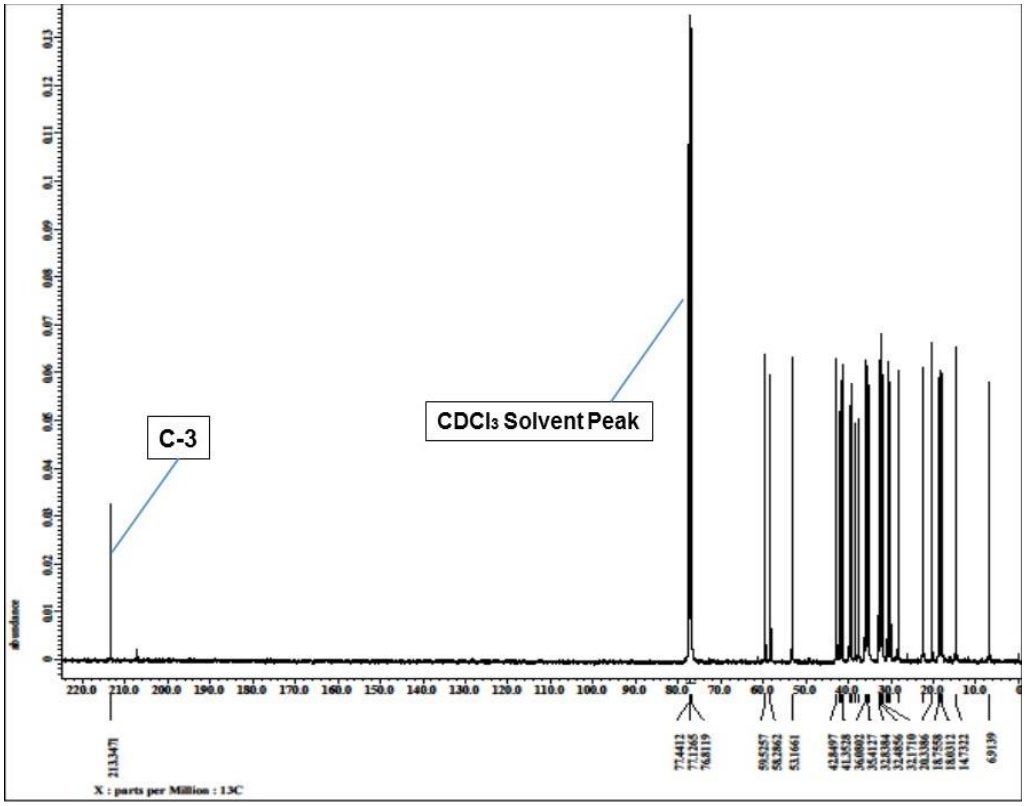
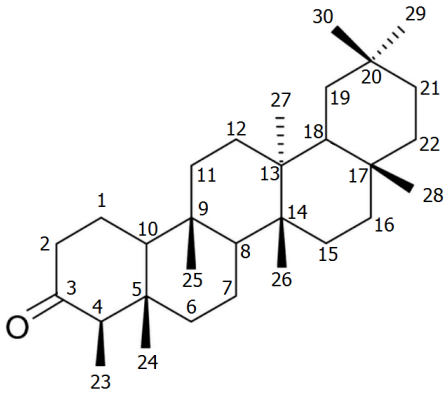


Figure 4.8: ^{13}C NMR spectrum of friedelin [30] (100 MHz, $CDCl_3$)



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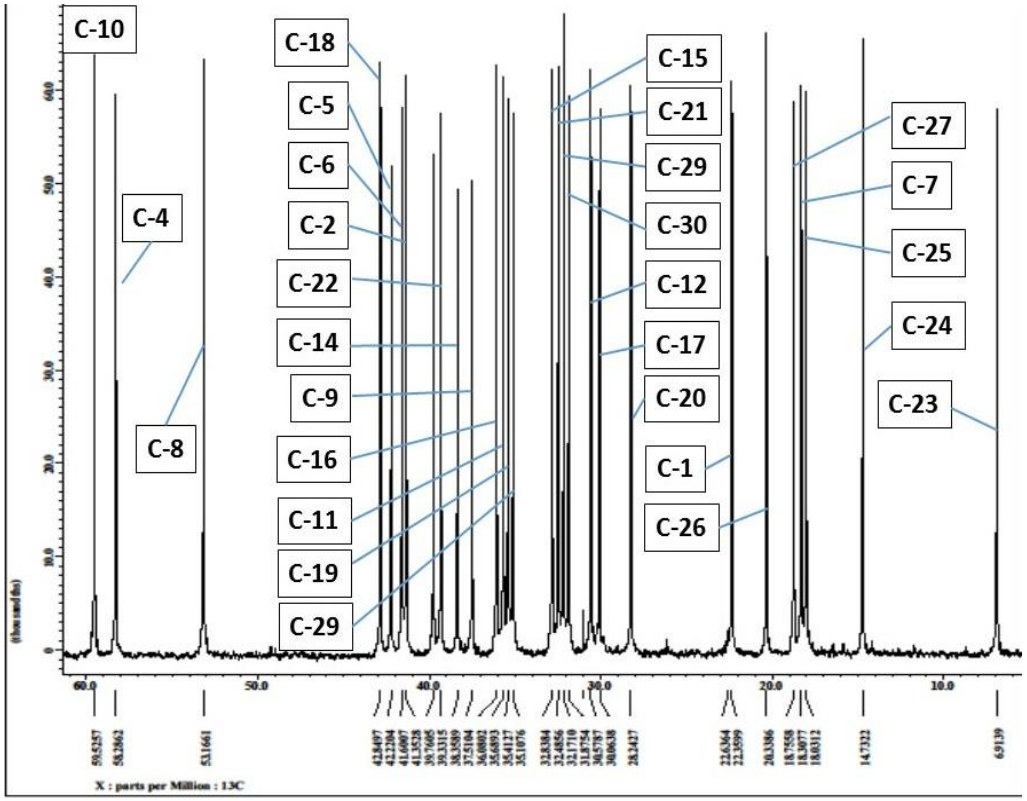


Figure 4.9: ^{13}C NMR spectrum of friedelin [30] (100 MHz, $CDCl_3$) (expanded)

4.3 Structural Characterization and Elucidation of 5,7-Dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31]

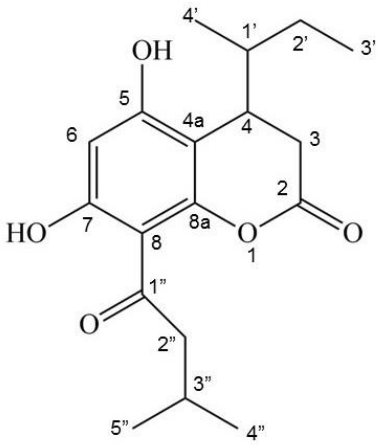


Figure 4.10: Molecular structure of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31]

About 34.6 mg of compound **31** was isolated as a brownish gum. This compound showed a molecular formula of $C_{18}H_{24}O_5$ which was in agreement with the molecular weight of 320.4 g/mol. In the TLC analysis, compound **31** appeared as single dark spot under short wavelength of UV light at 254 nm, and a brown spot in iodine chamber. The retention factor, R_f value obtained was 0.68 using a mixture of 70 % hexane and 30 % dichloromethane as mobile phase.

The IR spectrum (Figure 4.11) indicated compound **30** to have functional groups such as hydroxyl O-H (3380 cm^{-1}), carbonyl C=O (1730 cm^{-1}), carbinol C-O (1210 cm^{-1}) and aromatic C=C (1615 and 1420 cm^{-1}). Apart from that, the presence of sp^3 C-H stretching was revealed by the absorption peak at 2920

cm^{-1}. The UV-Vis spectrum (Figure 4.12) showed the characteristic absorption maxima for compound **31** at 235 and 285 nm. The absorption peak in the range of 270-350 nm was due to the electronic transition of $\pi \rightarrow \pi^*$, while the absorption peak in the range of 150-250 nm was due to the electronic transition of $n \rightarrow \sigma^*$, in relation to the presence of oxygen atoms with non-bonding electrons and conjugated benzene ring in compound **31**.

The chemical structure of compound **31** was elucidated based on the 1D- and 2D-NMR. In the ^{1}H-NMR spectrum (Figure 4.13), a strong and sharp singlet observed at δ 13.75 was assigned to the chelated hydroxyl proton 7-OH. The hydroxyl proton 7-OH forms intramolecular hydrogen bonding with the keto group, C-1" in the acyl side chain. Meanwhile, the free hydroxyl proton (5-OH) gave a broad singlet at δ 8.32. The lone aromatic protons, H-6 display a singlet at δ 6.25. In addition, the methylene proton H-3 in the fused pyrano ring gave two signals, a doublet (J = 16.5 Hz) and a doublet of doublets (J = 16.5, 7.3 Hz) at δ 2.90 and 2.59 respectively. The slight difference in their chemical and magnetic environments due to the restriction of rotation about C-C bond in the ring causes one of the proton experiences a relatively higher deshieldng effect than the other one. The remaining methine proton H-4 in the pyrano ring exhibited a triplet (J = 6.1 Hz) at δ 3.23.

Apart from that, the presence of a 3-methylbutyryl group in the compound was evidenced from the characteristic proton signals: a doublet (J = 6.7 Hz) for methylene proton H-2" at δ 2.96, a multiplet for methine proton H-3" at δ 2.15

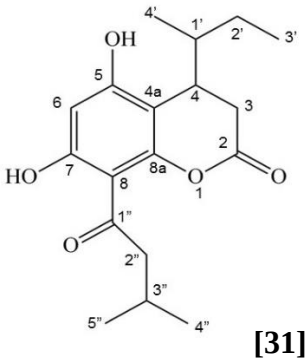
and a pair of doublets (J = 1.1 Hz) for methyl protons H-4" and H-5" at δ 0.97 and 0.95, respectively. Meanwhile, the presence of a butan-2-yl moiety was revealed by the three multiplets at δ 1.63, 1.39, 1.31 (H-1', Ha-2' and Hb-2'), a triplet (J = 7.3 Hz) at δ 0.88 (H-3') and a doublet (J = 4.3 Hz) at δ 0.83 (H-4').

A total of 18 carbon signals in the ^{13}C-NMR spectra (Figures 4.16 and 4.17) indicated compound **31** to have 18 carbons. The two downfield signals at δ 205.8 and 168.6 were assigned to the carbonyl carbons C-1" and C-2, respectively. Besides that, the oxygenated aromatic carbons C-5, C-7 and C-8a exhibited relatively deshielded signals at δ 160.2, 164.9 and 153.4, respectively. Meanwhile, the quaternary carbons C-8 and C-4a gave signals at δ 104.8 and 105.8, respectively. The remaining carbon signals below δ 60.0 were assigned to the sp^3 hybridized carbons in the pyrano ring and the side chain moieties: δ 30.2 and 33.5 for C-3 and C-4 in the pyrano ring; δ 53.2, 25.9, 22.7 (×2) for C-2", C-3" and C-4" & C-5" in the acyl group; δ 38.1, 27.0, 12.0, 15.0 for C-1', C-2', C-3' and C-4' in the butan-2-yl moiety.

2D-NMR analysis further confirmed the positions of protons and carbons in the structure. In HMQC spectra (Figures 4.18 and 4.19), all the protonated carbons including methylene, methine and methyl carbons, showed ^1J correlations with their respective protons in the spectrum. For instance, the singlet integrated for one proton at δ 6.25 was correlated to carbon signal C-6 signal at δ 99.8 indicating carbon C-6 is a methine carbon. On the other hand, the HMBC spectra (Figures 4.20 – 4.23) showed the long range coupling between protons and their

neighbouring carbons through ^{2}J and ^{3}J correlations. For instance, the hydroxyl proton 7-OH at δ 13.73 showed ^{2}J correlation to the oxygenated quaternary carbon, C-7 at δ 164.9 and ^{3}J correlations to neighbouring carbons C-6 at δ 99.8 and C-8 at δ 104.8. The complete assignment of ^{2}J and ^{3}J correlations of compound **31** was given in Table 4.2.

Based on the spectral evidence, compound **31** was identified as 5,7-hydroxy-4-(butan-2-yl)-6-(3-methylbutyryl)-3,4-dihydrocoumarin and it was reported for the first time as a new compound.



[31]

Table 4.2: Summary of NMR data and structural assignment for 5,7-hydroxy-4-(butan-2-yl)-6-(3-methylbutyryl)-3,4-dihydrocoumarin [31]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			^2J	^3J
1	-	-	-	-
2	-	168.6	-	-
3	2.90 (1H, d, *J* = 16.5 Hz) 2.59 (1H, dd, *J* = 16.5, 7.3 Hz)	30.2	C-2, C-4	C-1’
4	3.23 (1H, t, *J* = 6.1 Hz)	33.5	C-1’, C-3	C-5, C-2, C-8a, C-2’, C-4’
4a	-	105.8	-	-
5	-	160.2	-	-
6	6.25 (1H, s)	99.8	C-7, C-5	C-4a, C-8
7	-	164.9	-	-
8	-	104.8	-	-
8a	-	153.4	-	-
1’	1.63 (1H, m)	38.1	C-4, C-2’, C-4’	C-3
2’	1.39 (1H, m) 1.31 (1H, m)	27.0	C-1’, C-3’	C-4’
3’	0.88 (3H, t, *J* = 7.3 Hz)	12.0	C-2’	C-1’
4’	0.83 (3H, d, *J* = 4.3 Hz)	15.0	C-1’	C-4, C-2’
1”	-	205.8	-	-
2”	2.96 (2H, d, *J* = 6.7 Hz)	53.2	C-1”, C-3”	C-4”, C-5”
3”	2.15 (1H, m)	25.9	C-2”, C-4”, C-5”	-
4”	0.97 (3H, d, *J* = 1.1 Hz)	22.7	C-3”	C-2”, C-5”
5”	0.95 (3H, d, *J* = 1.1 Hz)	22.7	C-3”	C-2”, C-4”
5-OH	8.32 (OH, s)	-	-	-
7-OH	13.73 (OH, s)	-	C-7	C-6, C-8

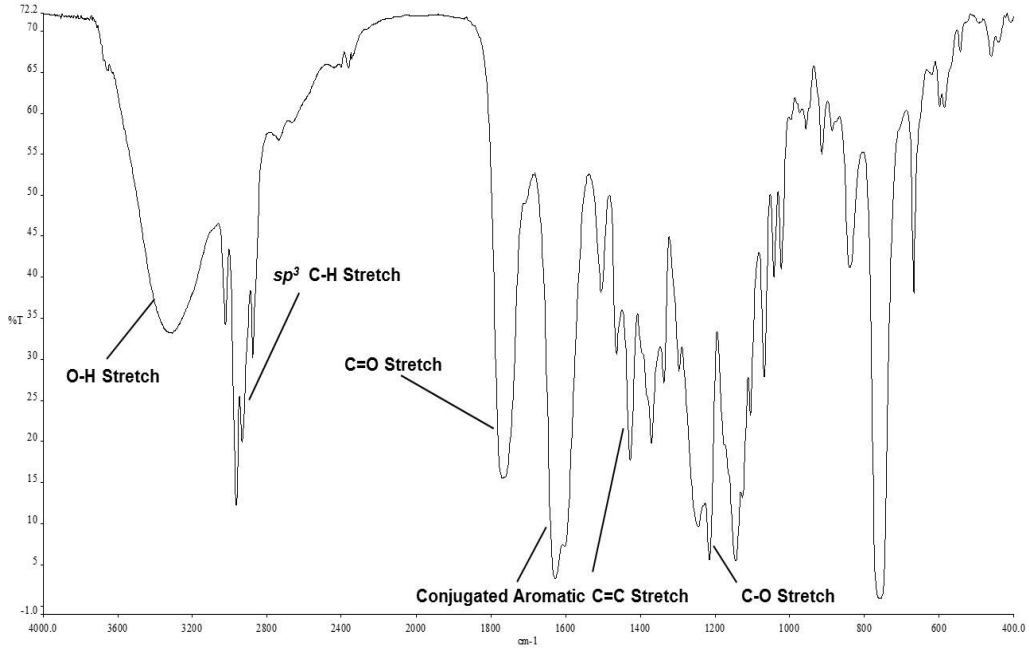


Figure 4.11: IR spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31]

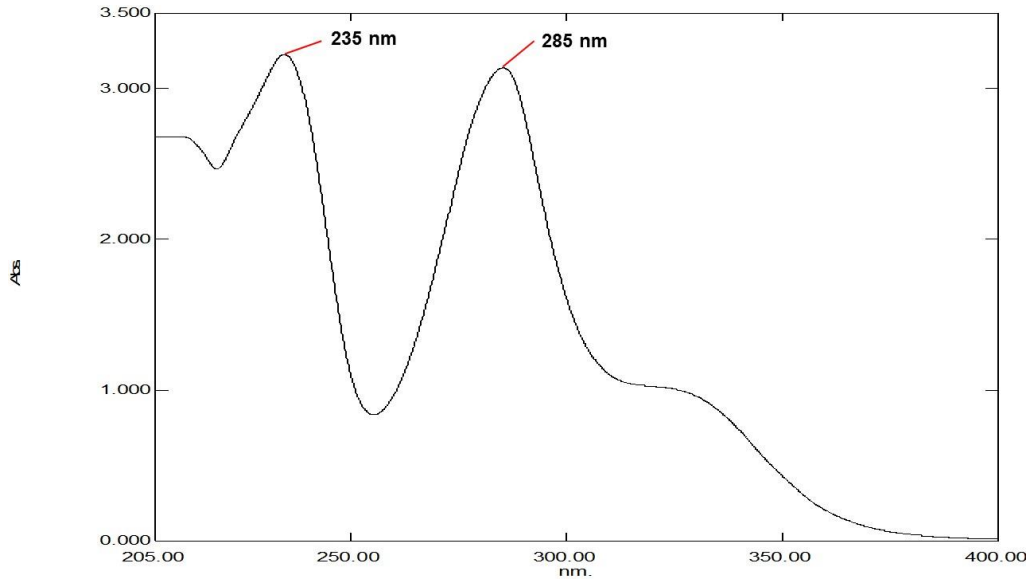
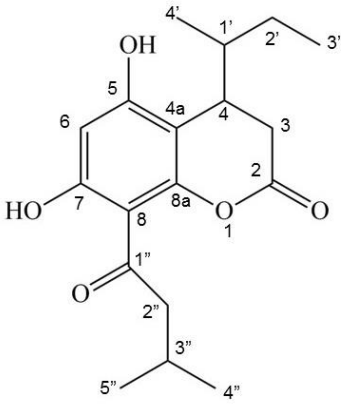


Figure 4.12: UV-Vis spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31]



[31]

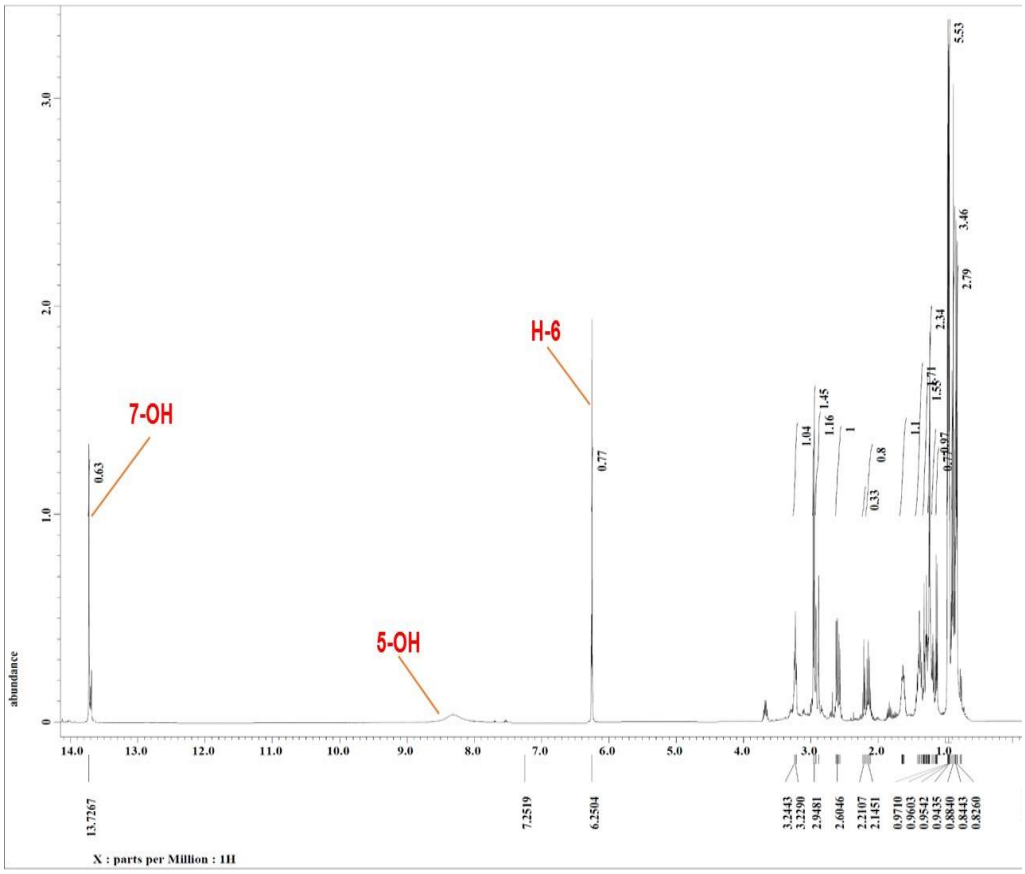
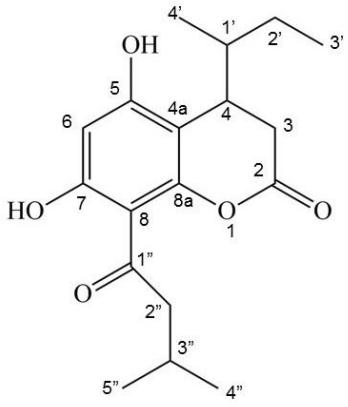


Figure 4.13: ^{1}H NMR spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (400 MHz, $CDCl_3$)



[31]

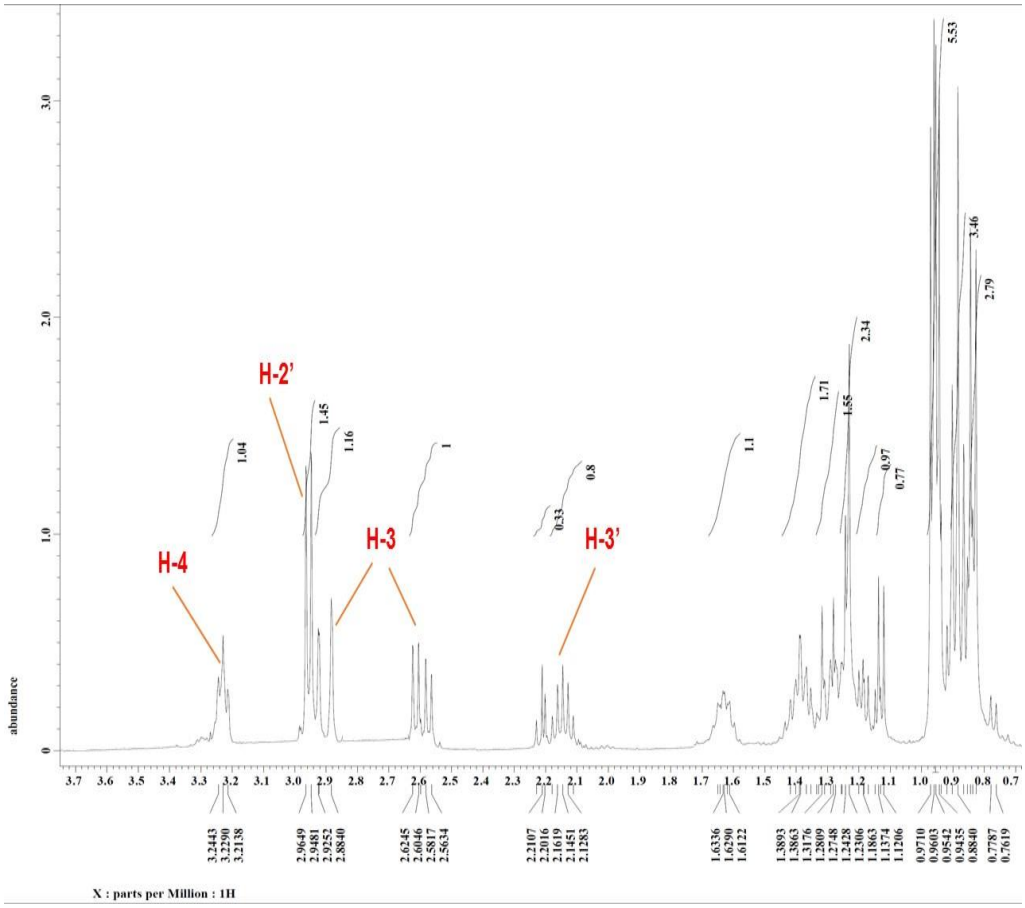
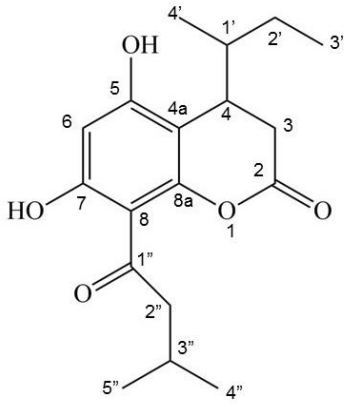


Figure 4.14: ^{1}H NMR spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (400 MHz, $CDCl_3$) (expanded)



[31]

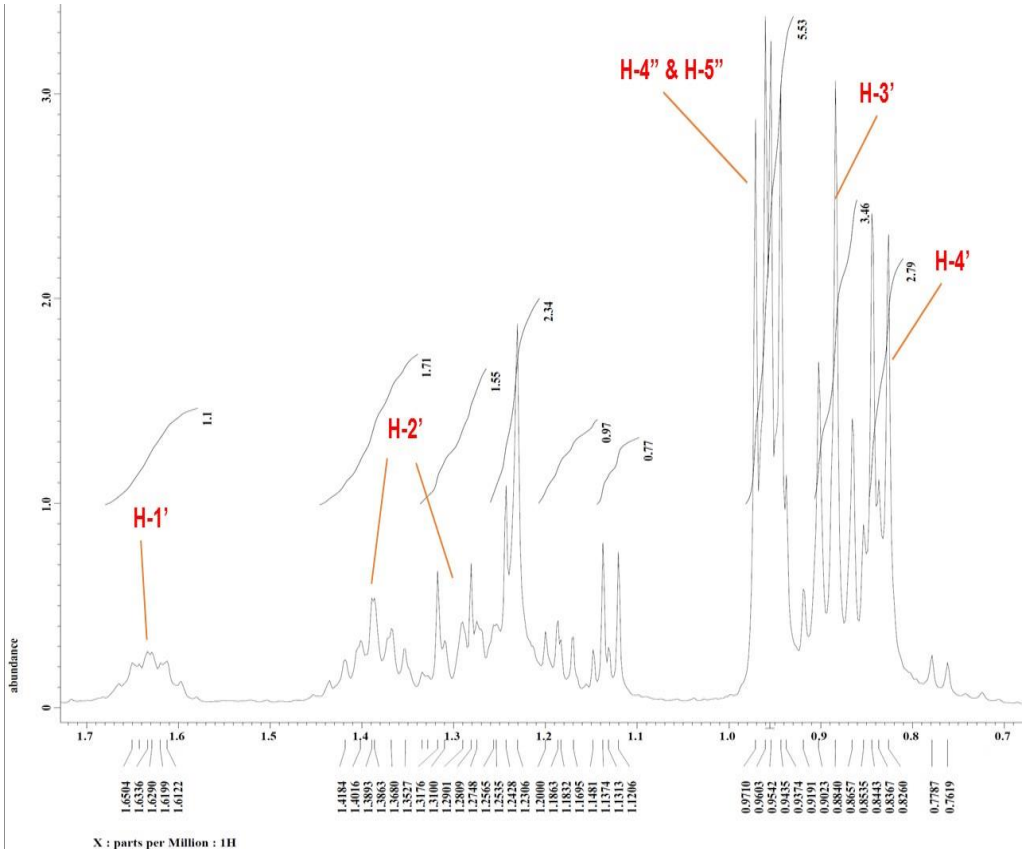
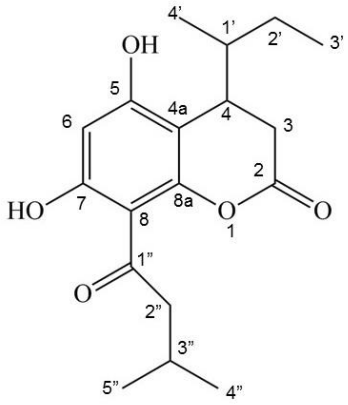


Figure 4.15: ^{1}H NMR spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (400 MHz, $CDCl_3$) (expanded)



[31]

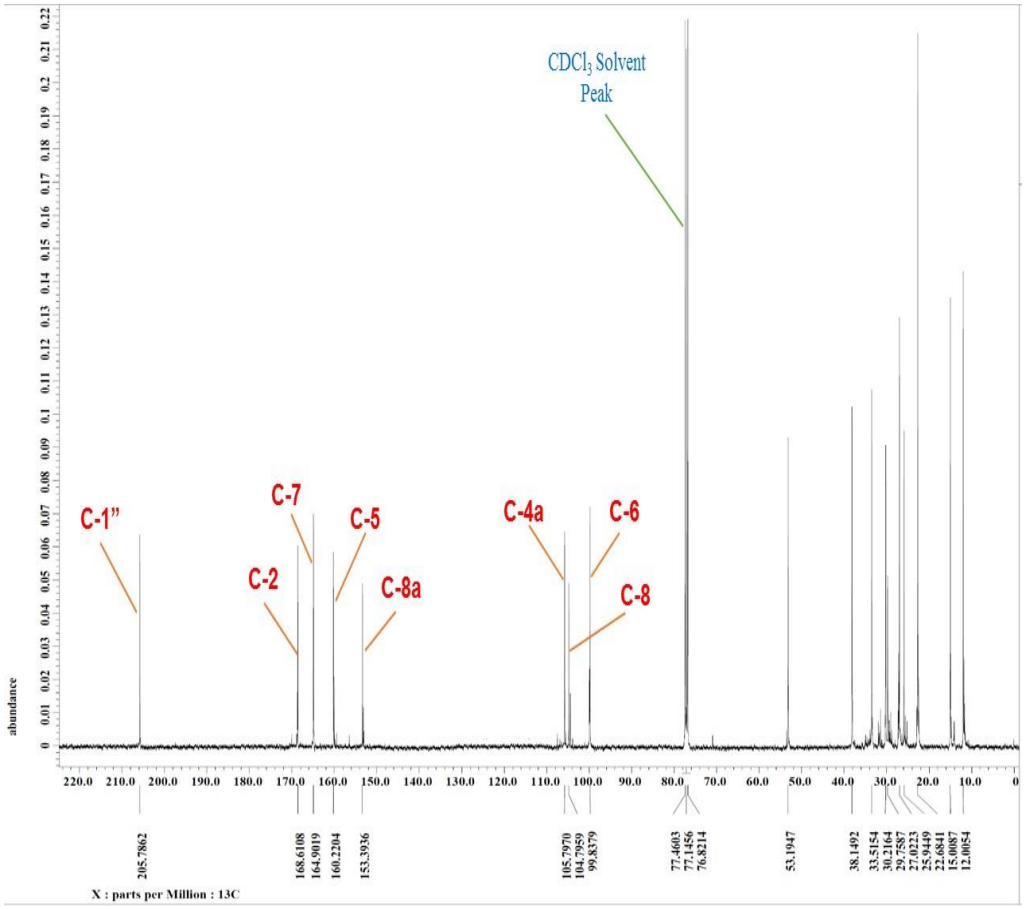
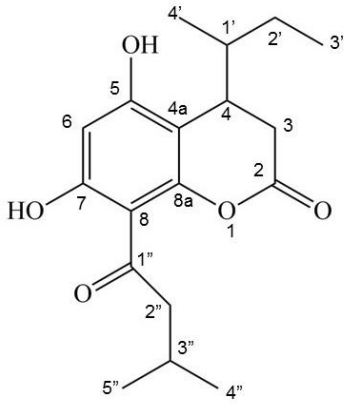


Figure 4.16: ^{13}C NMR spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (100 MHz, $CDCl_3$)



[31]

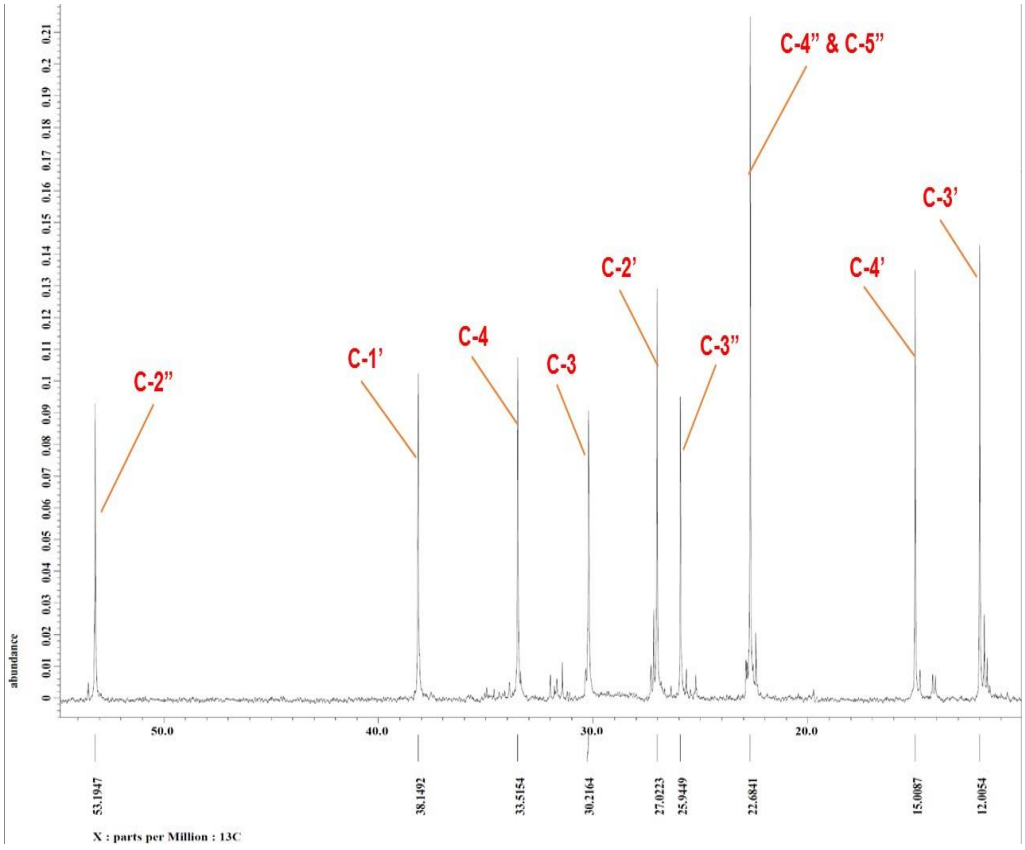
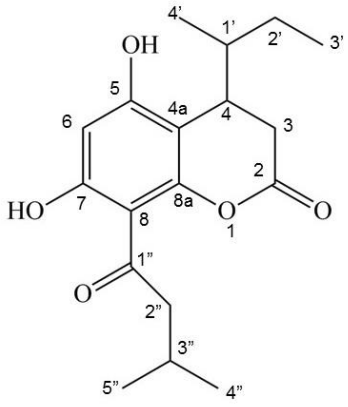


Figure 4.17: ^{13}C NMR spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (100 MHz, $CDCl_3$) (expanded)



[31]

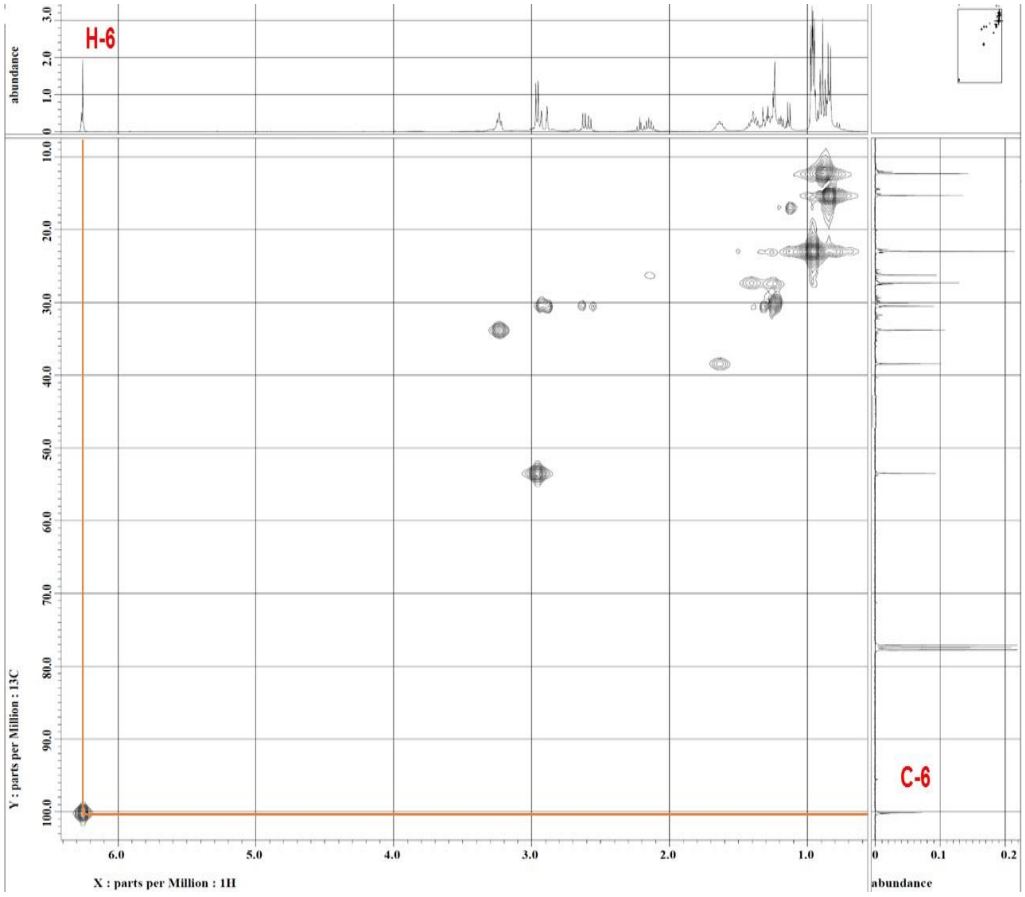
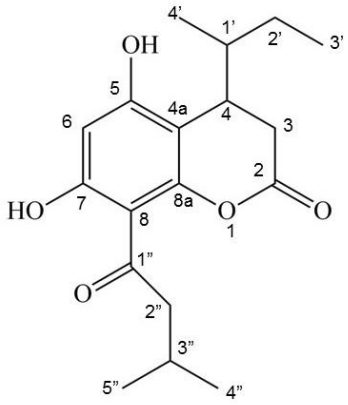


Figure 4.18: HMQC spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (expanded)



[31]

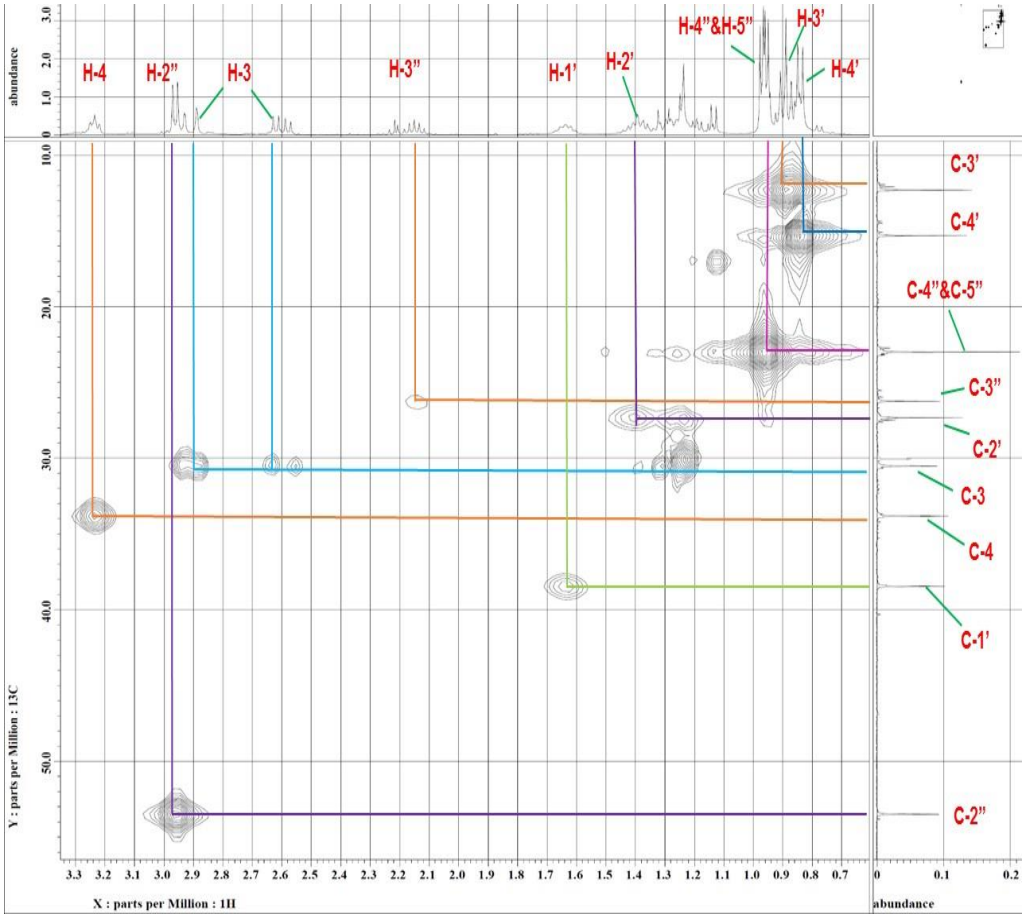
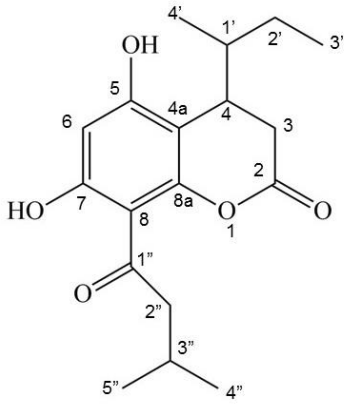


Figure 4.19: HMQC spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (expanded)



[31]

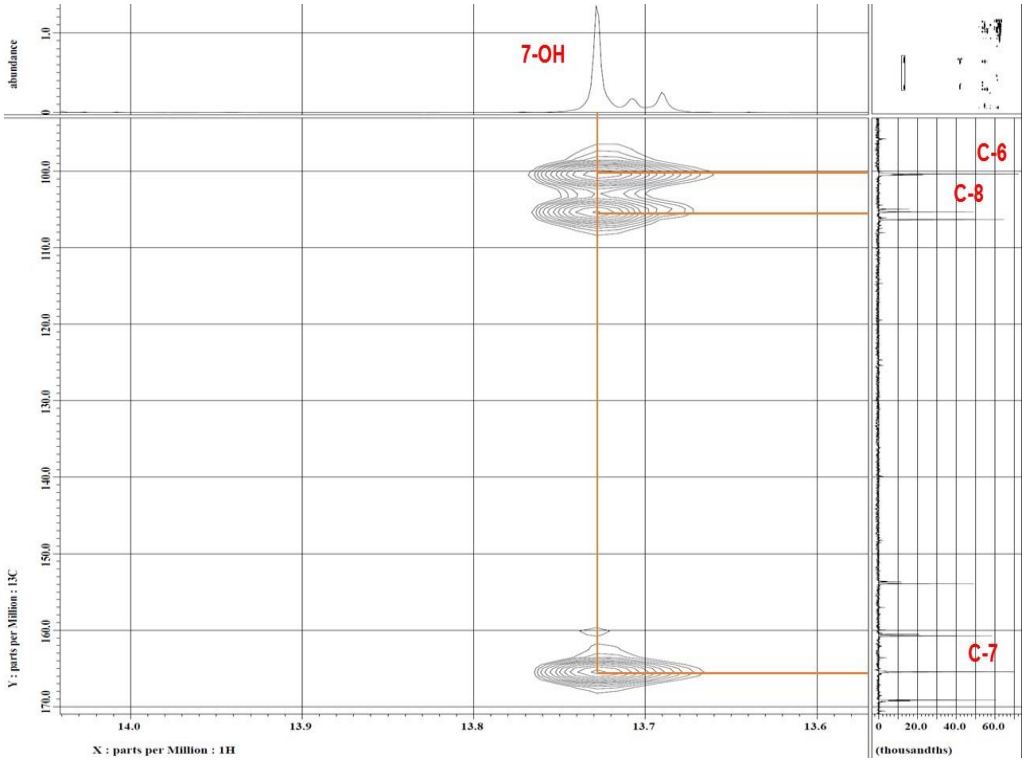
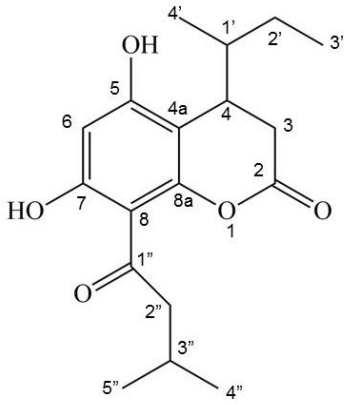


Figure 4.20: HMBC spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (expanded)



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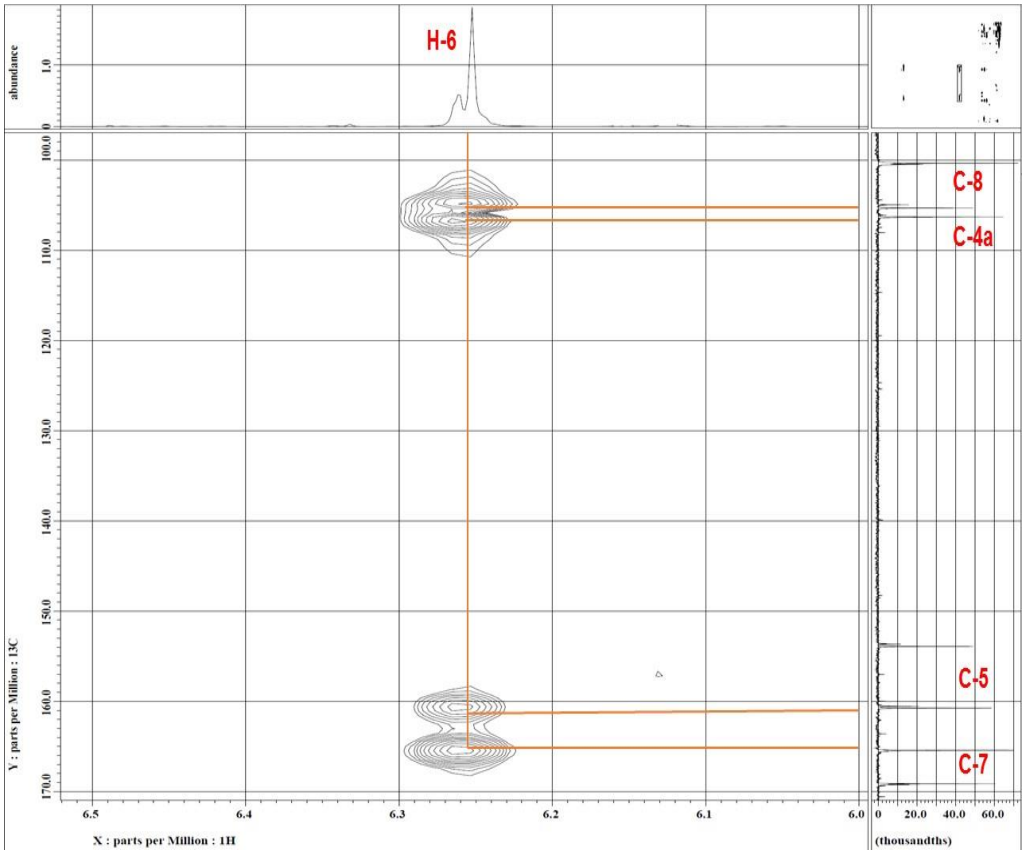
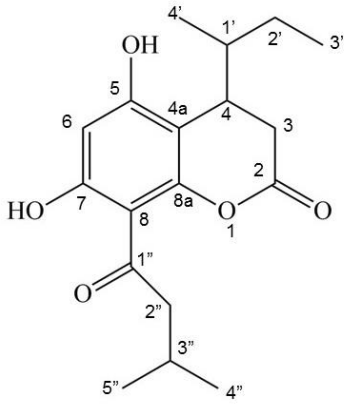


Figure 4.21: HMBC spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (expanded)



[31]

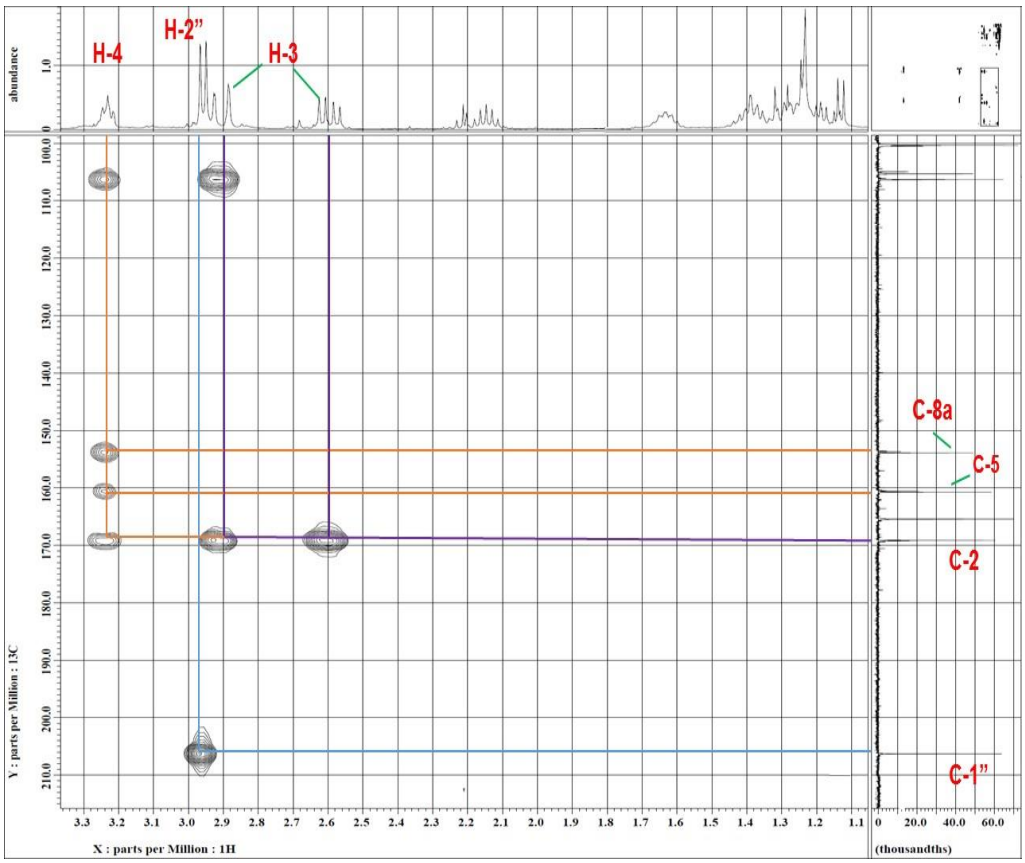
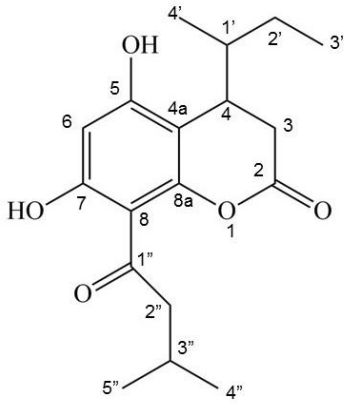


Figure 4.22: HMBC spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (expanded)



[31]

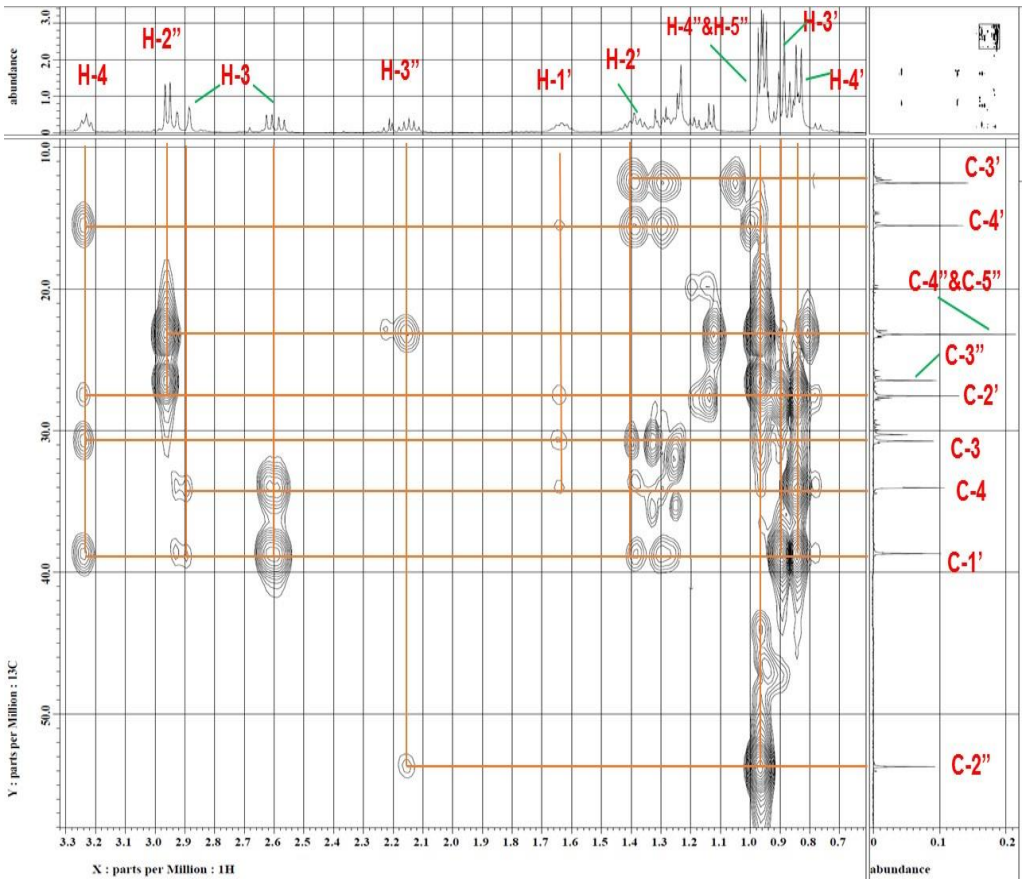


Figure 4.23: HMBC spectrum of 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin [31] (expanded)

4.4 Structural Characterization and Elucidation of Euxanthone [32]

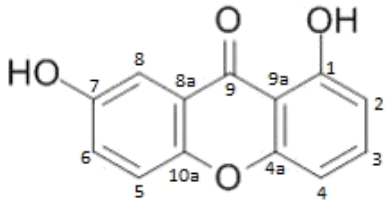


Figure 4.24: Molecular structure of euxanthone [32]

About 47.8 mg of compound **32** was isolated as yellow needle-like crystals which has a molecular formula of $C_{13}H_8O_4$ corresponding to the molecular weight of 228.2 g/mol. A protonated molecular ion, $[M+H]^+$ was observed in the HRESI mass spectrum (Figure 4.25) showed compound **32** have an accurate mass of 229.1375 g/mol which was 99.96 % agreement with the calculated value of 229.2110 g/mol. Compound **32** showed a melting point of 236 – 238 °C (Lit. 239 - 241°C, Kato, et al., 2004). In the TLC analysis, it appeared as a single dark spot under short wavelength of UV light at 254 nm, and a brown spot in iodine chamber. It gave a retention factor, R_f value of 0.85 using a mixture of 90 % dichloromethane and 10 % acetone as mobile phase.

The IR spectrum (Figure 4.26) showed a broad absorption band at 3399 cm^{-1} indicating the presence of O-H stretch in compound **32**. In addition, an intense peak observed at 1607 cm^{-1} and a sharp peak at 1234 cm^{-1} revealed the presence

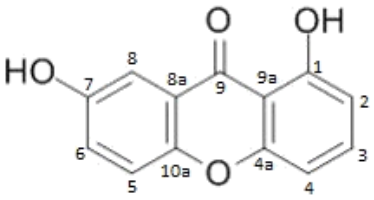
of C=O stretch and C-O stretch, respectively. Meanwhile, conjugated aromatic C=C stretch gave absorption peak at 1483 cm^{-1}. In the UV-Vis spectrum (Figure 4.27), absorption maxima at 208, 233, 259 and 382 nm were observed, indicating compound **32** to have a highly conjugated structure.

Compound **32** was elucidated by using 1D- and 2D-NMR spectroscopy. In the ^{1}H NMR spectrum (Figure 4.28), two highly deshielded proton signals observed at δ 12.70 and δ 9.0 were assigned to hydroxyl protons, 1-OH and 7-OH, respectively. The proton 1-OH experienced a greater deshielding effect than 7-OH due to the hydrogen in 1-OH forms hydrogen bond with the carbonyl group (C-9), while 7-OH remained as a free hydroxyl proton. Meanwhile, the remaining six methine proton signals observed at δ 7.68 (t, *J* = 8.5 Hz), 7.58 (d, *J* = 3.04 Hz), 7.50 (d, *J* = 9.2 Hz), 7.41 (dd, *J* = 9.2, 3.1 Hz), 6.98 (d, *J* = 8.5 Hz) and 6.74 (d, *J* = 9.2 Hz) were assigned to the aromatic ring protons H-3, H-8, H-5, H-6, H-4 nd H-2, respectively.

In the ^{13}C NMR spectrum (Figure 4.30), a total of 13 carbon signals were observed assignable to the 13 carbons in compound **32**. The keto carbon C-9 gave the most deshielded carbon signal in the highly downfield region in the spectrum at δ 182.2. In addition, the four oxygenated aromatic carbons C-1, C-4a, C-7 and C-10a gave carbon signals in the downfield region at δ 161.9, 156.5, 154.2 and 150.2, respectively. The remaining carbon signals below δ 140.0 were assigned to the non-oxygenated aromatic carbons which are observed at δ 109.7

(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 107.0 (C-9a).

Compound **32** was further subjected to 2D-NMR analysis to study the structural correlations of protons in the compound **32**, to their immediate and neighbouring carbons. HMQC spectrum (Figure 4.31) showed the information about the ^{1}J direct bonding of protons to their immediate carbons. Meanwhile, the HMBC spectra (Figures 4.32 and 4.33) showed the long range (^{2}J and ^{3}J) correlations between protons and their neighbouring carbons. The HMBC assignment of compound **32** was given in Table 4.3. Table 4.3 shows the summary of the NMR data and structural assignment for compound **32** which was identified as euxanthone.



[32]

Table 4.3: Summary of NMR data and structural assignment for euxanthone [32]

Position	δ_H (ppm)	δ_C (ppm)	HMBC	
			^2J	^3J
1	-	161.9	-	-
2	6.74 (1H, d, *J* = 9.2 Hz)	109.7	-	C-4
3	7.68 (1H, t, *J* = 8.5 Hz)	137.0	-	C-1, C-4a
4	6.98 (1H, d, *J* = 8.5 Hz)	107.0	-	C-2
4a	-	156.5	-	-
5	7.50 (1H, d, *J* = 9.2 Hz)	119.4	-	C-7
6	7.41 (1H, dd, *J* = 9.2, 3.1 Hz)	125.4	-	C-8, C-10a
7	-	154.2	-	-
8	7.58 (1H, d, *J* = 3.04 Hz)	108.3	-	C-6, C-10a
8a	-	121.0	-	-
9	-	182.2	-	-
9a	-	107.0	-	-
10a	-	150.2	-	-
1-OH	12.70 (OH, s)	-	C-1	C-2
3-OH	9.06 (OH, s)	-	-	-

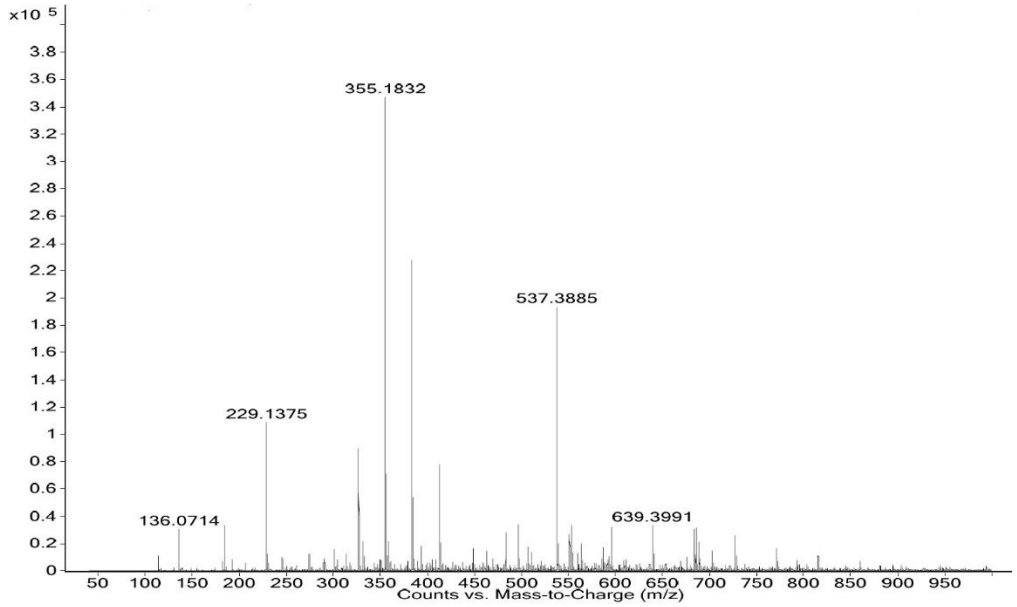


Figure 4.25: HRESIMS spectrum of euxanthone [32]

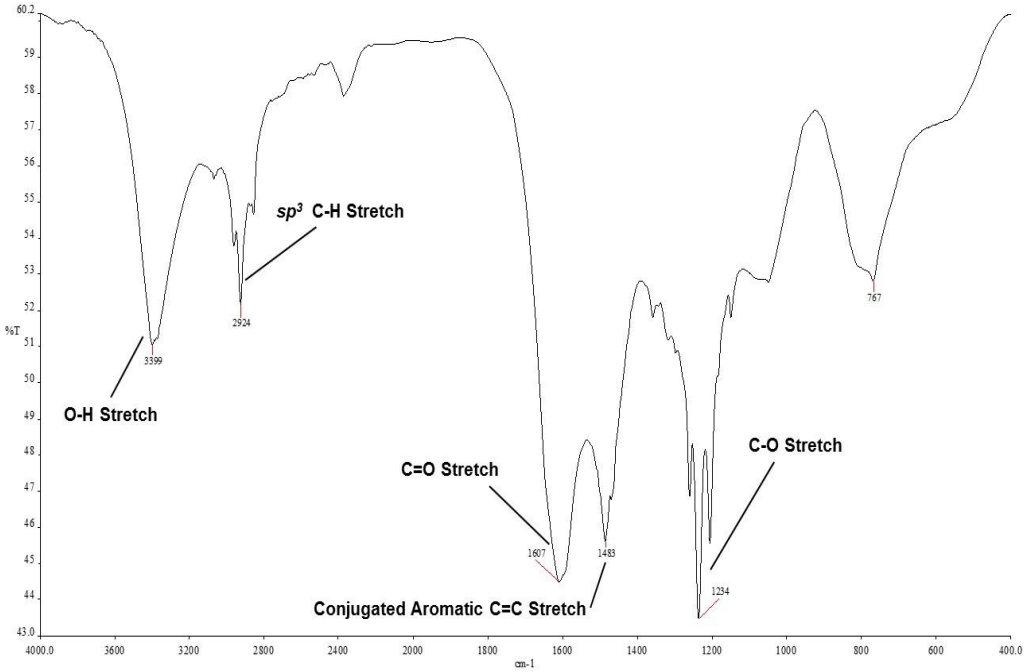


Figure 4.26: IR spectrum of euxanthone [32]

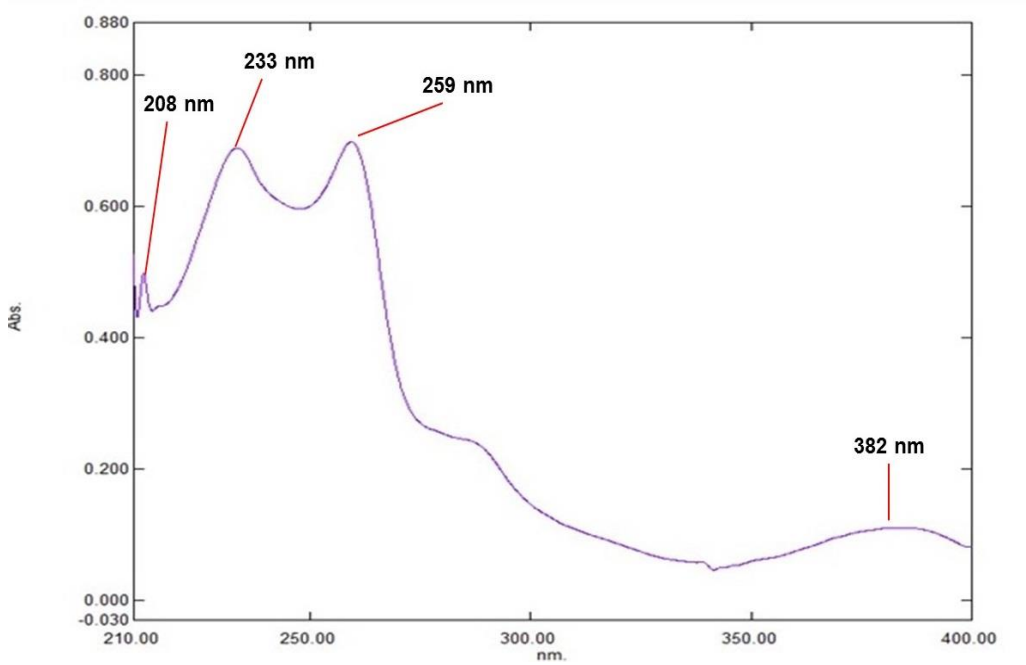
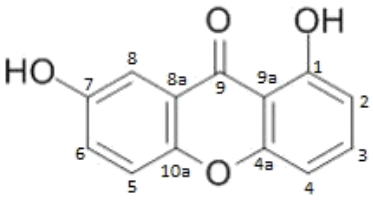


Figure 4.27: UV-Vis spectrum of euxanthone [32]



[32]

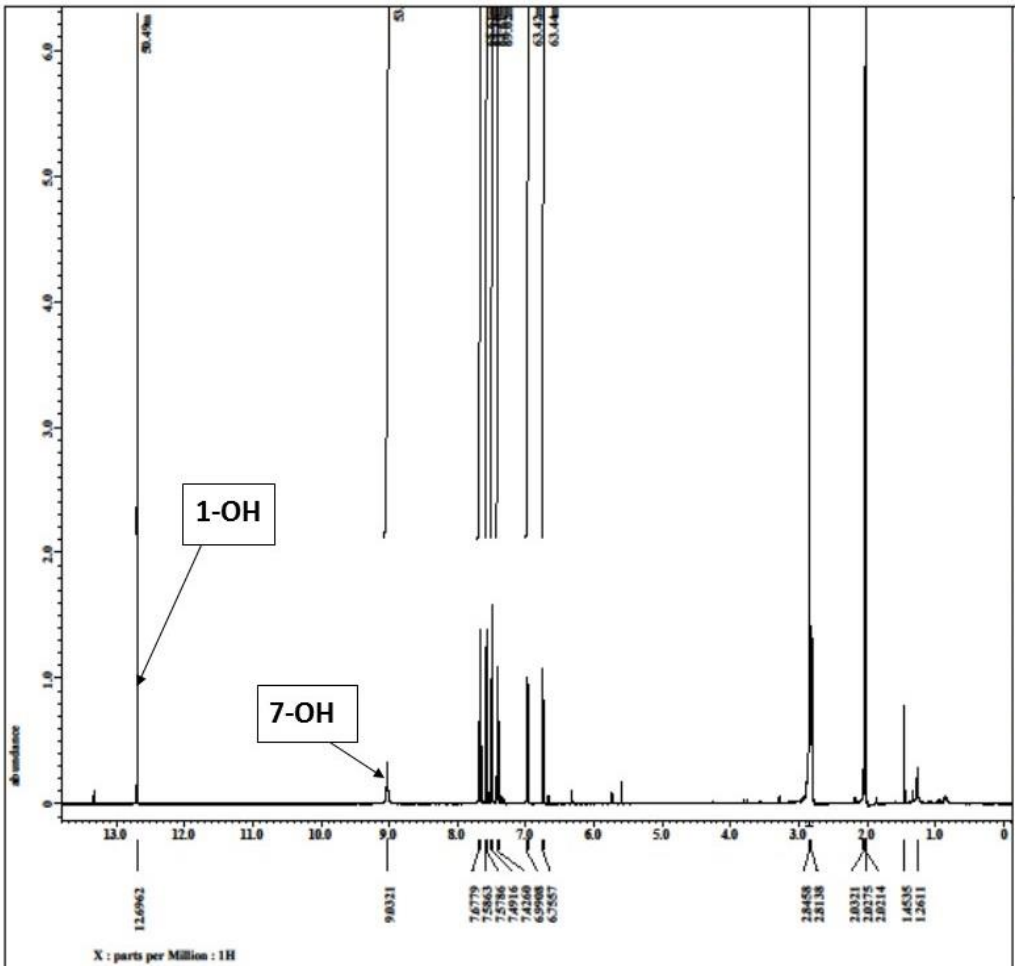
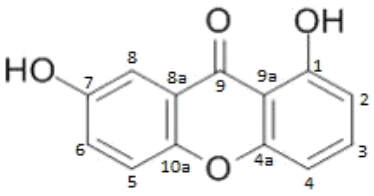


Figure 4.28: ^{1}H-NMR spectrum of euxanthone [32] (400 MHz, Acetone-d_6)



[32]

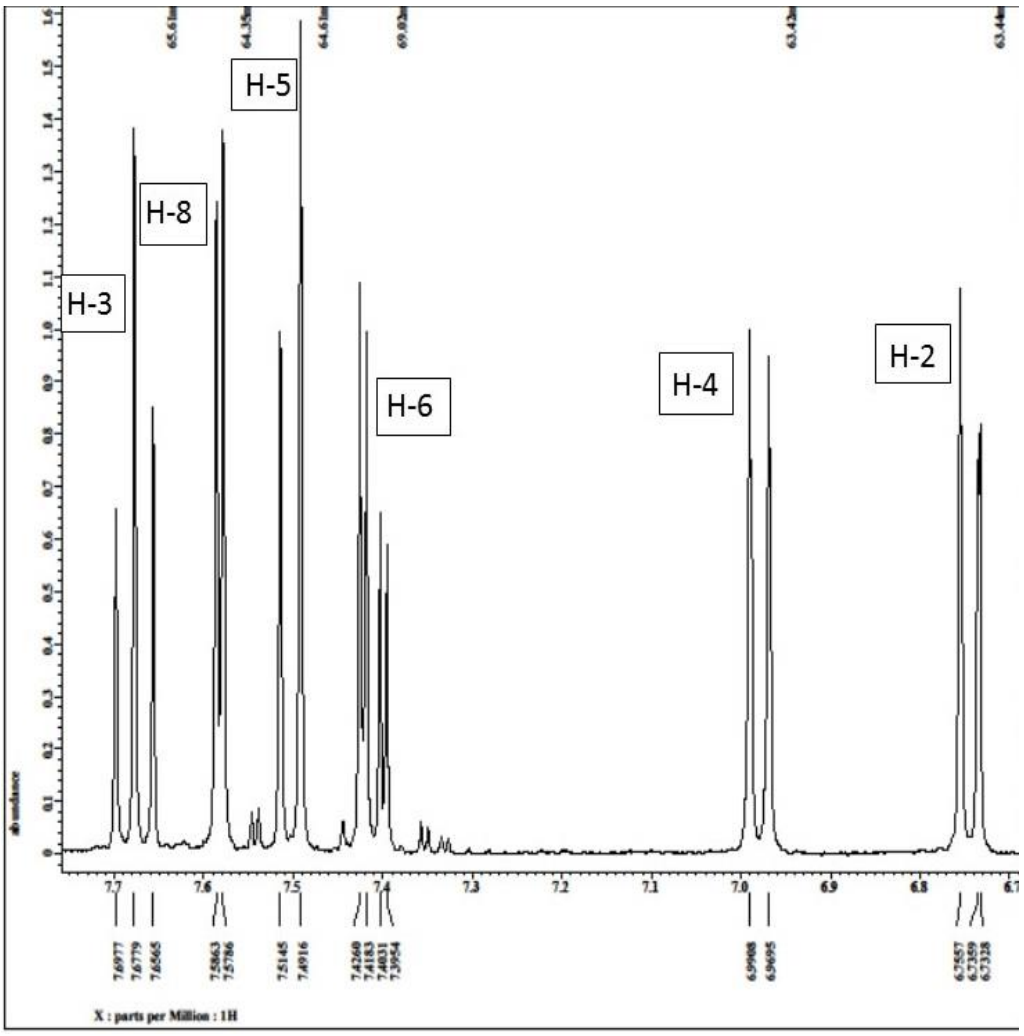
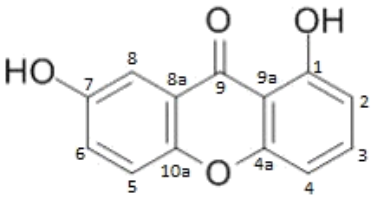


Figure 4.29: ^{1}H NMR spectrum of euxanthone [32] (400 MHz, Acetone-d_6) (expanded)



[32]

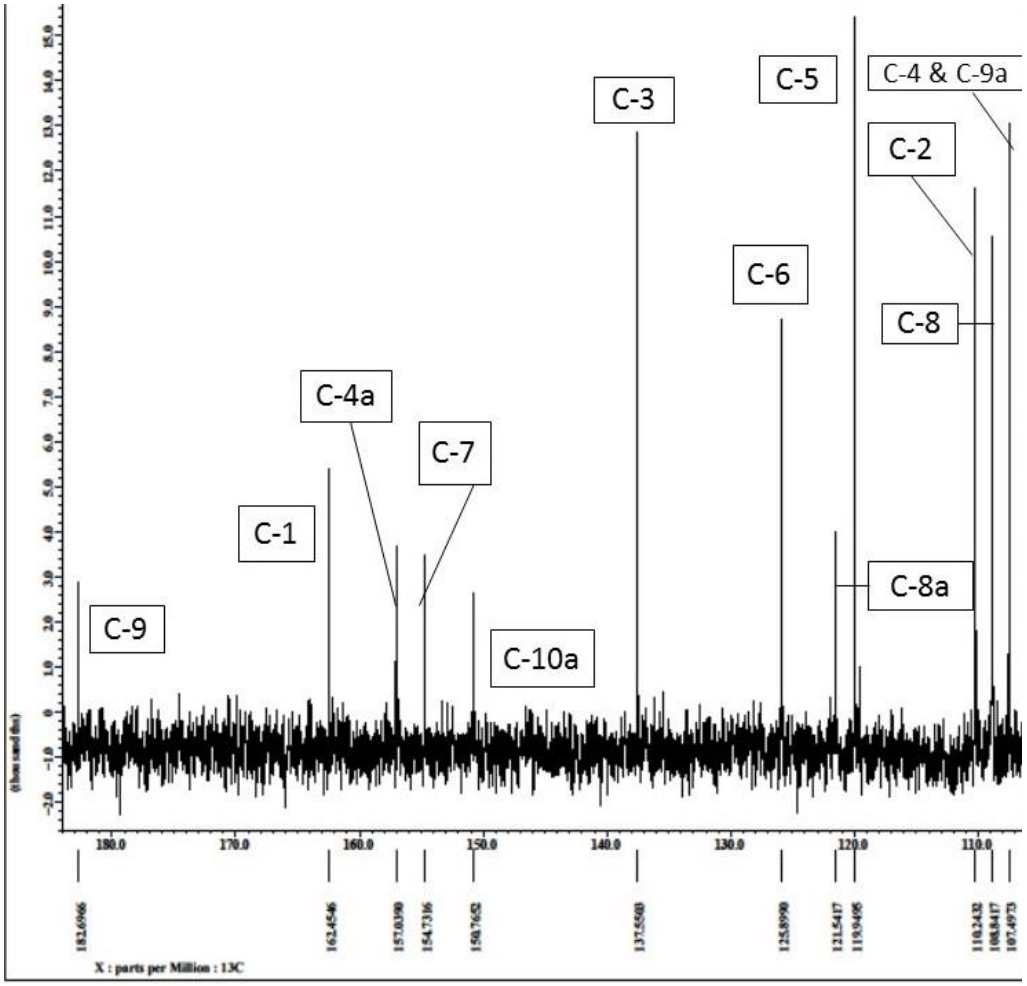
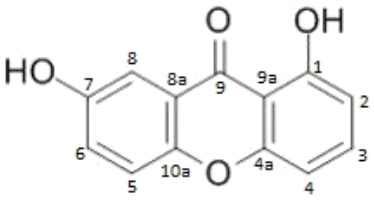


Figure 4.30: ^{13}C NMR spectrum of euxanthone [32] (100 MHz, Acetone-d_6) (expanded)



[32]

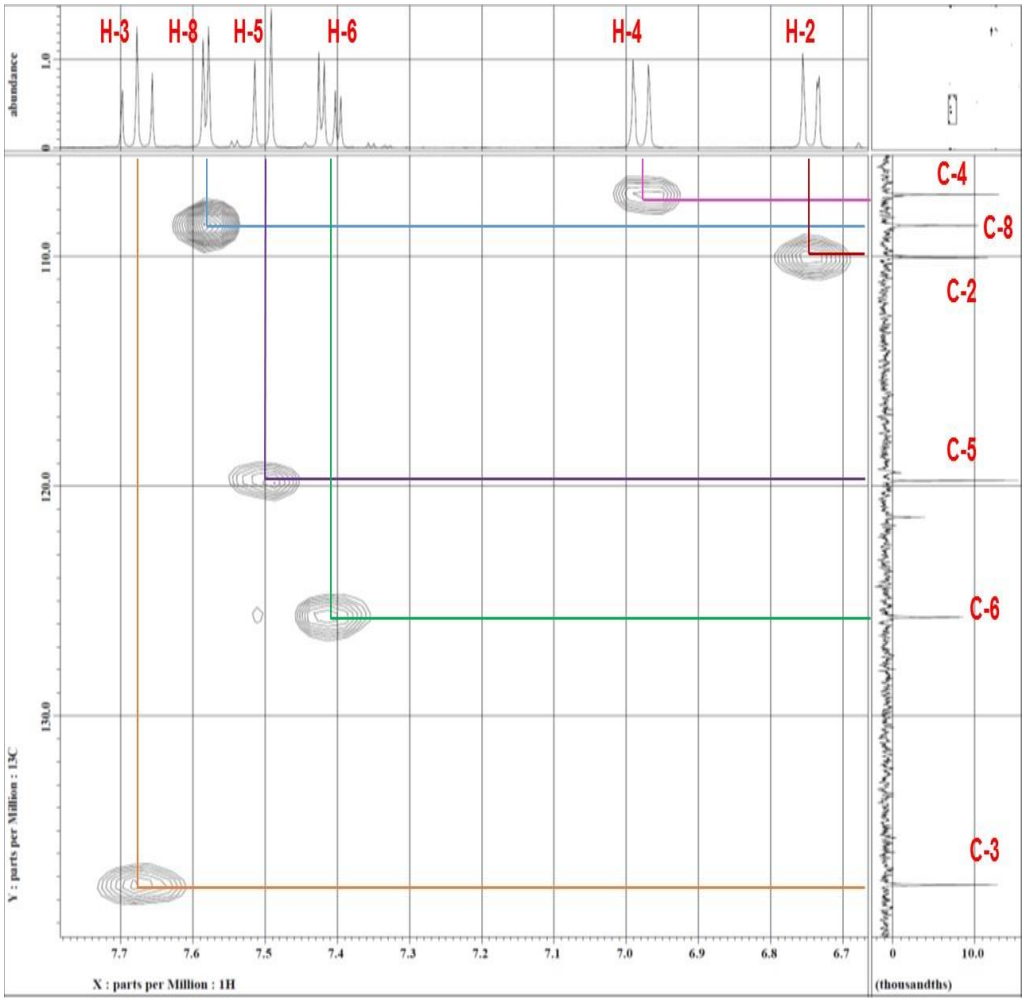
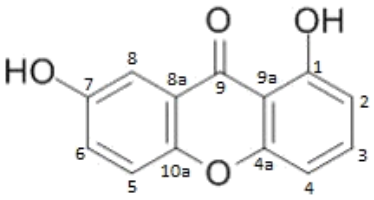


Figure 4.31: HMQC spectrum of euxanthone [32] (expanded)



[32]

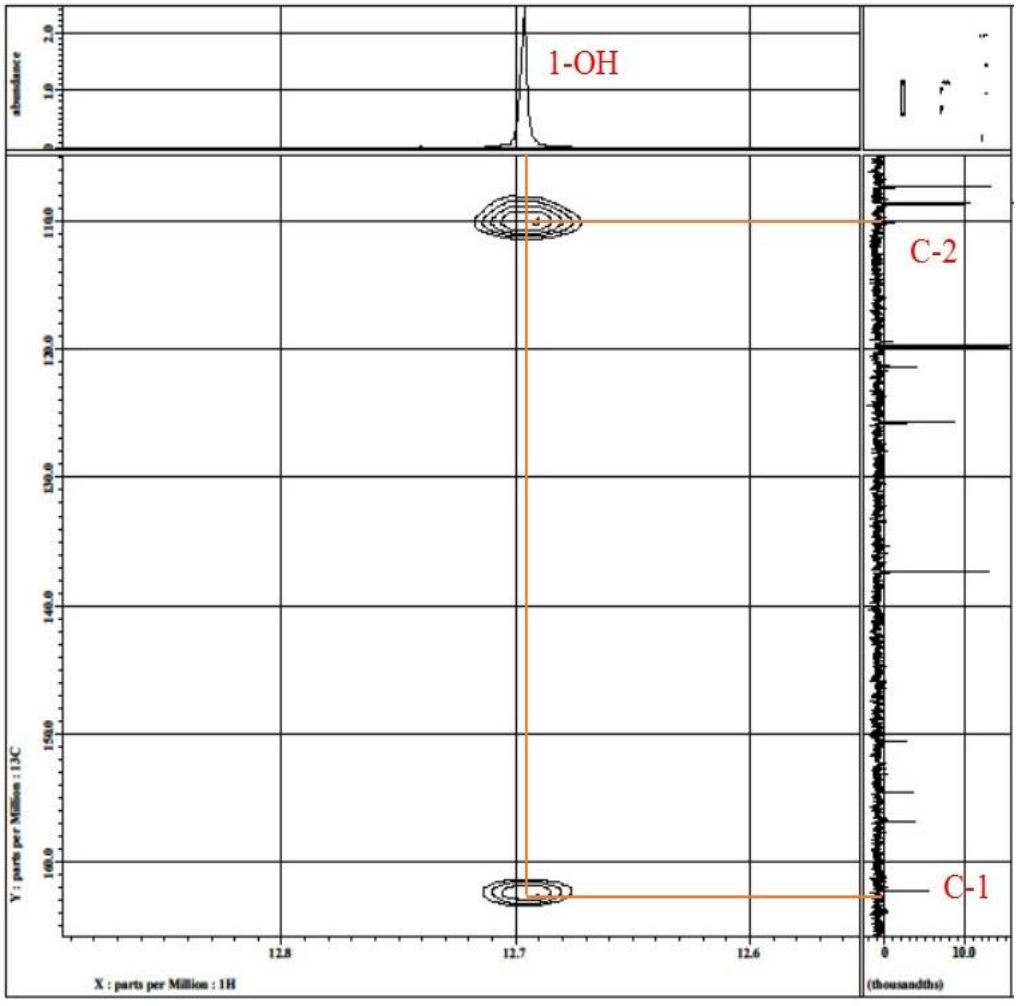
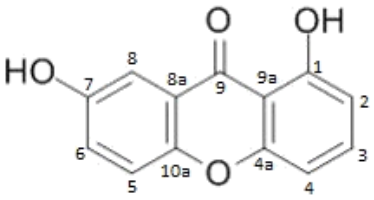


Figure 4.32: HMBC spectrum of euxanthone [32] (expanded)



[32]

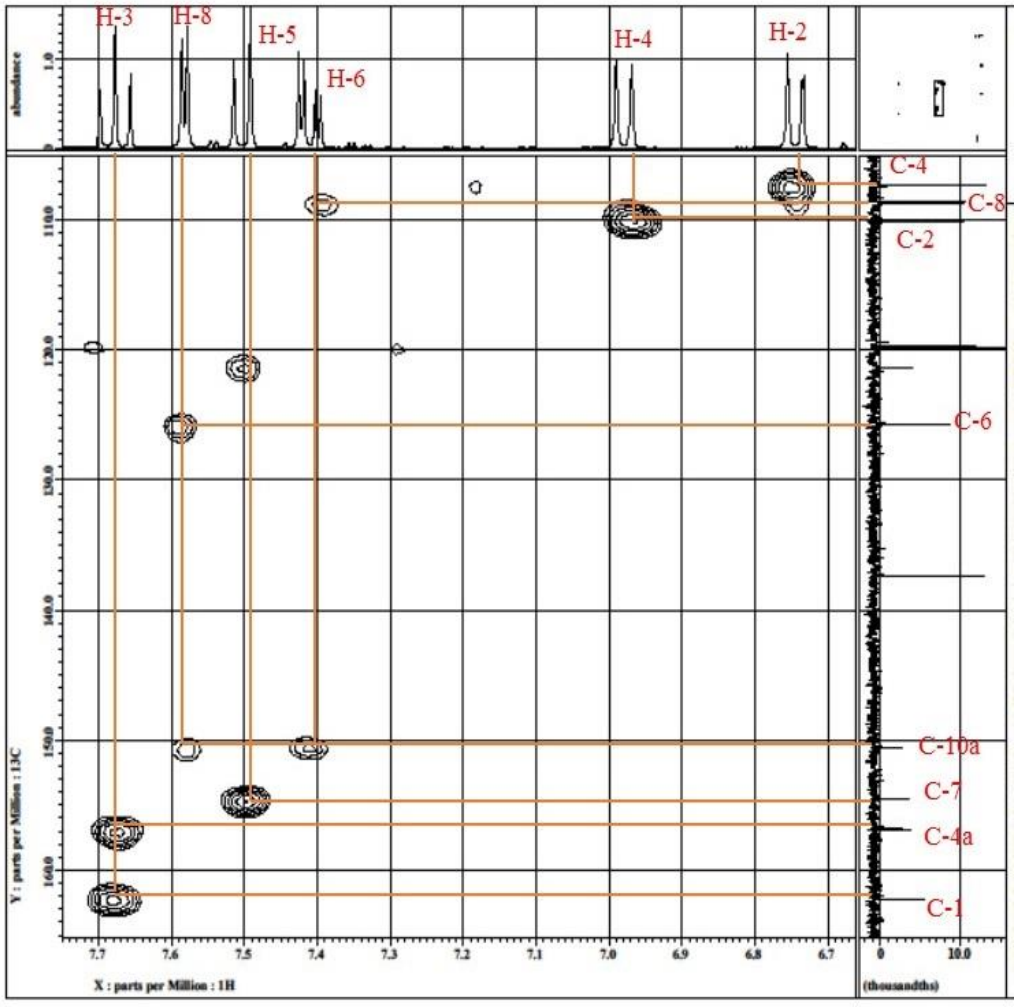


Figure 4.33: HMBC spectrum of euxanthone [32] (expanded)

4.5 Antioxidant Assay

All the crude extracts of *Calophyllum gracilentum* and the isolated compounds were evaluated for their antioxidant activities via 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH appeared in violet colour and acts as free radicals. When the DPPH radicals are scavenged and reduced, they change from violet colour to yellow colour. This leads to a decrease in absorbance reading at 520 nm for violet colour. IC_{50} value of the samples can be determined by the plotting of graph of inhibition rate (%) versus concentration of samples as shown in the Figure 4.34 and 4.35. The lower the IC_{50} value obtained for a sample, the stronger the antioxidant activity.

Table 4.4: Antioxidant result of test samples in DPPH assay

Test Samples	**IC_{50} (µg/mL)**
Positive Controls:	
1. Ascorbic acid	2.0
2. Kaempferol	15.0
Crude extracts:	
1. Dichloromethane (DCM)	195.0
2. Ethyl Acetate (EA)	145.0
3. Methanol (MeOH)	9.0
Isolated compounds:	
1. Friedelin **[30]**	> 240.0
2. 5,7-Dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin **[31]**	> 240.0
3. Euxanthone **[32]**	> 240.0

From the assay results, the dichloromethane, ethyl acetate and methanol crude extracts of *Calophyllum gracilentum* showed strong to weak antioxidant activities. The methanol crude extract gave the most potent radical scavenging

activity with IC_{50} value of 9.0 µg/mL comparable to that of positive control used, kaempferol (IC_{50} = 15.0 µg/mL). In contrast, dichloromethane and ethyl acetate crude extracts showed weak DPPH radical scavenging activities with IC_{50} values of 195.0 and 145.0 µg/mL, respectively. It was found that the more polar the crude extract, the higher the antioxidant potency exhibited. This may be due to the presence of a much larger amount of polyphenolic compounds in the methanol crude extract as polyphenolic compounds are known to be good antioxidants and showing a much solubility in polar protic solvent like methanol than the other two crude extracts. Meanwhile, the three isolated compounds **30**, **31** and **32** showed insignificant activity with IC_{50} value above 240.0 µg/mL.

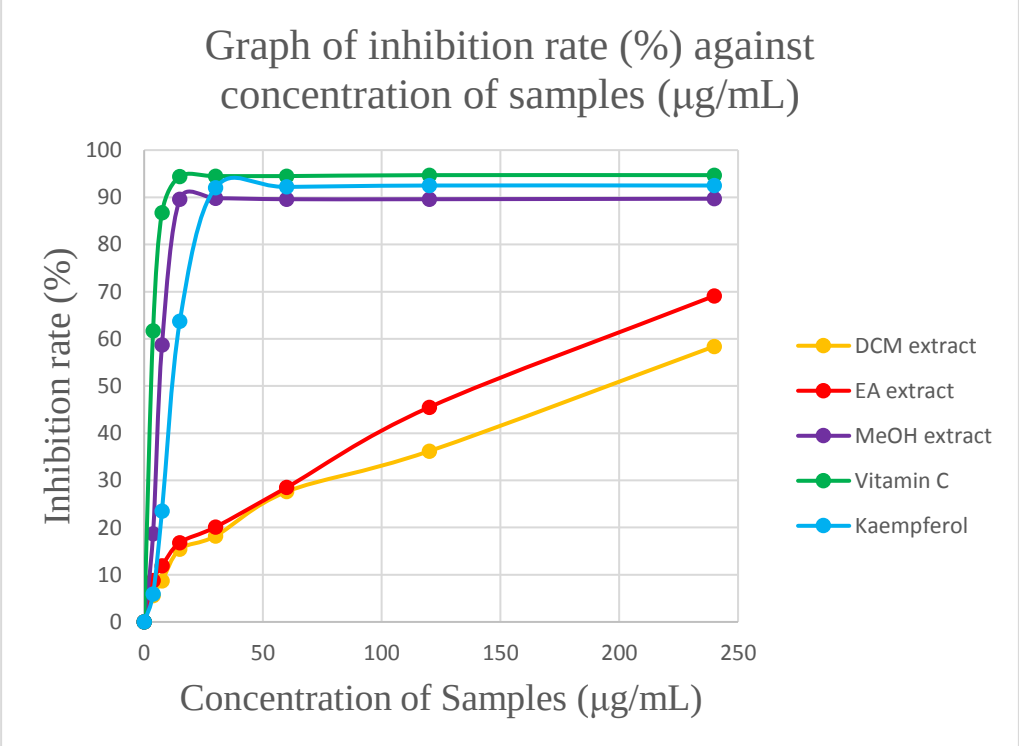


Figure 4.34: Graph of inhibition rate (%) against concentration of standard and crude extract samples

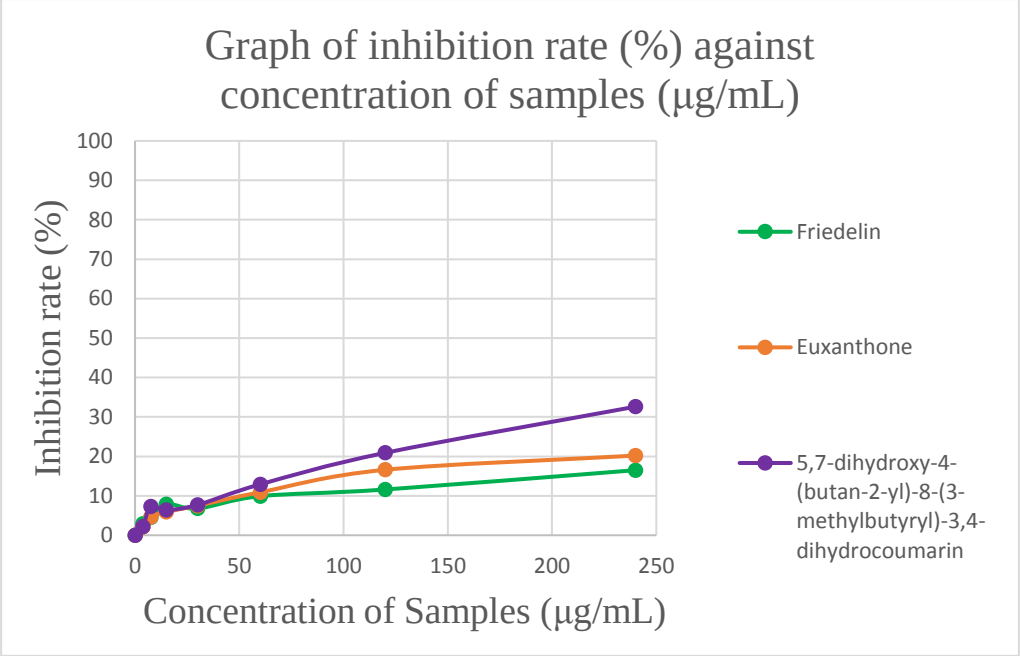


Figure 4.35: Graph of inhibition rate (%) against concentration of isolated samples

CHAPTER 5

CONCLUSIONS

5.1 Conclusion

In this study, a new compound, namely 5,7-dihydroxy-4-(butan-2-yl)-8-(3-methylbutyryl)-3,4-dihydrocoumarin **[31]** along with friedelin **[30]** and euxanthone **[32]** were successfully isolated from the stem bark extracts of *Calophyllum gracilentum*. The structure of isolated compounds **30**-**32** were established based on various modern spectroscopic techniques including 1D- and 2D-NMR, UV-Vis, IR and MS.

The crude extracts and isolated compounds were investigated for their antioxidant potency via the DPPH assay. Among all the crude extracts, methanol crude extracts showed the most potent radical scavenging activity with IC_{50} value of 9.0 μg/mL comparable to that of positive control used, kaempferol (IC_{50} = 15.0 μg/mL). Meanwhile, dichloromethane and ethyl actate crude extracts showed weak antioxidant activities with IC_{50} value of 195.0 and 145.0 μg/mL, respectively. On the other hand, all the three isolated compound **30**, **31** and **32** showed insignificant activity with IC_{50} values above 240.0 μg/mL.

5.2 Future Perspective

In order to improve the separation efficiency such as isolation of the minor compounds, more advanced chromatographic methods such as HPLC, centrifungal chromatography and flash chromatography are recommended to be used. Since all isolated compounds were found to be inactive in the antioxidant activity, further investigation on their biological activities such as antimicrobial, anticancer and antifungal are suggested to be carried out. Besides, the isolated pure compounds can also be studied for their chemical derivatives via organic synthesis in future for exploration of their biological potential.

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