SECONDARY METABOLITES FROM *Calophyllum gracilentum*

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Final Year Project Report Submitted in
Partial Fulfillment of the Requirement for the
Degree of Bachelor of Science (Hons.) Chemistry with Management
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This Final Year Report Project entitled **"Secondary Metabolites from *Calophyllum gracilentum*"** was submitted by Nur Adibah Binti Zamri @ Mohd Zamri, in partial fulfilment of the requirement for Degree of Bachelor of Science (Hons.) Chemistry with Management, in the Faculty of Applied Sciences, and was approved by

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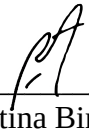

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Nur Adibah Binti Zamri

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LIST OF ABBREVIATIONS

^{13}C	:	Carbon-13
cm	:	Centimeter
CPC	:	Centrifugal Partition Chromatography
CC	:	Column Chromatography
^{o}C	:	Degree in Celsius
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
GAE	:	Gallic acid equivalent
g	:	Gram
IC_{50}	:	Half maximal inhibitory concentration
HIV	:	Human Immunodeficiency Virus
IR	:	Infrared
kg	:	Kilogram
LC-MS	:	Liquid Chromatography-Mass Spectrometry
µg	:	Microgram
mL	:	Milliliter
M	:	Molar concentration
nm	:	Nanometer
NMR	:	Nuclear Magnetic Resonance
K_D	:	Partitioning coefficient

KBr	:	Potassium bromide
^{1}H	:	Proton
QE	:	Quercetin equivalent
R_f	:	Retention factor
SAR	:	Structure-Activity Relationship
TLC	:	Thin-Layer Chromatography
TFC	:	Total Flavonoid Content
TPC	:	Total Phenolic Content
UV-Vis	:	Ultraviolet-Visible

ABSTRACT

SECONDARY METABOLITES FROM *Calophyllum gracilentum*

Chemical study of medicinally significant plant secondary metabolites resulted in the discovering of various beneficial compounds that will be further studied for their pharmacological activities. The process involves extraction, isolation, purification and structural elucidation of compounds isolated from plants. The *Calophyllum gracilentum* species is still not adequately documented on its chemical constituents and biological activities. In this study, the stem bark of *Calophyllum gracilentum* was subjected to solvent extraction using hexane. The hexane crude extract was subsequently isolated and purified using column chromatography and radial chromatography to obtain pure compounds. A xanthone, brasixanthone B **(15)** was successfully isolated from the hexane crude extract. The structure of the compound was characterized based on physical appearance, melting point and elucidation through spectroscopic methods (IR, ^{1}H NMR, ^{13}C NMR). The structural assignment was further confirmed by matching the data with the previous study. Next, the methanol, ethyl acetate, chloroform, and hexane crude extracts of *C. gracilentum* were studied for their antioxidant potential via Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical Scavenging assay. The methanolic and ethyl acetate extract exhibited high TPC at 93.89 and 162.93 mg GAE/g, respectively, and TFC at 74.55 and 158.47 mg QE/g, respectively. The IC_{50} value of methanol fraction (7.62 μg/mL) showed comparable, but less potent antioxidant activity with positive control ascorbic acid (4.97 μg/mL) followed by ethyl acetate extract (8.10 μg/mL). Therefore, the methanolic and ethyl acetate extract has a higher free radical scavenging ability than other extracts. The findings of the study support the traditional medicinal uses of these plant extracts. With the extracts having good antioxidant properties, they could be used for the potential development of antioxidant herbal formulations.

ABSTRAK

METABOLIT SEKUNDER DARI *Calophyllum gracilentum*

Kajian kimia mengenai metabolit tumbuhan penting yang mempunyai nilai perubatan telah membawa penemuan pelbagai kompaun berguna untuk kajian aktiviti farmakologi yang lebih mendalam pada masa hadapan. Proses ini melibatkan pengekstrakan, pengasingan, penulenan, dan analisis struktur kompaun. Spesis *Calophyllum gracilentum* masih tidak didokumentasikan dengan secukupnya berkenaan unsur kimia dan aktiviti biologinya. Dalam kajian ini, kulit batang kayu *Calophyllum gracilentum* telah diekstrak menggunakan heksana. Ekstrak mentah heksana telah diasingkan dan ditulen berturutan menggunakan kromatografi kolum dan kromatografi radial untuk mendapatkan kompaun tulen. Xanthone bernama brasixanthone B **(15)** telah berjaya diekstrak daripada ekstrak mentah heksana. Struktur kompaun tersebut telah dianalisis berdasarkan rupa fizikal, takat lebur dan analisis melalui metod spektroskopi (IR, ^{1}H NMR, ^{13}C NMR). Pembahagian struktur kompaun telah dikenalpasti dengan memadankan data dengan kajian lepas. Seterusnya, ekstrak mentah metanol, etil asetat, klorofom, dan heksana *Calophyllum gracilentum* telah dikaji potensi antioksidan melalui kaedah Jumlah Kandungan Fenol (TPC), Jumlah Kandungan Flavonoid (TFC), dan Ujian Aktiviti Penyingkiran Radikal Bebas (DPPH). Ekstrak metanol dan etil asetat menunjukkan nilai TPC 162.93 dan 93.89 mg GAE/g, masing-masing, dan TFC yang tinggi di 158.47 dan 74.55 mg GAE/g, masing-masing. Ekstrak metanol (7.62 μg/mL) menunjukkan nilai IC_{50} yang setanding, tetapi nilai IC_{50} yang setanding, tetapi kurang kuat dengan kawalan positif asid askorbik (4.97 μg/mL) diikuti dengan ekstrak etil asetat (8.10 μg/mL). Jadi, ekstrak metanol dan etil asetat mempunyai keupayaan pelupusan radikal bebas berbanding ekstrak yang lain. Hasil kajian ini menyokong penggunaan ekstrak tumbuhan ini dalam perubatan tradisional. Dengan ciri-ciri antioksidan baik dimiliki oleh ekstrak, ia berpotensi untuk digunakan dalam penghasilan rumusan herba antioksidan.

CHAPTER 1

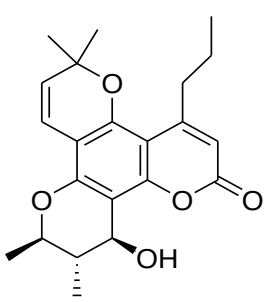
INTRODUCTION

1.1 Background of Study

Various plant species were found in the tropical rainforests in Malaysia. One of them is the *Calophyllum* genus, an endemic plant to Sarawak (Noh and Jong, 2020). They are also known as Bintangor locally. The *Calophyllum* genus falls under the Calophyllaceae family and is made up of around 180 – 200 different species (Lim *et al*., 2015). In addition, this genus has garnered many interests among phytologists to do extensive studies on their phytochemical and biological properties (Lim *et al.*, 2019). Several species under this genus were used traditionally as herbal medicine for diseases such as ulcers, inflammations, infections, and pain (Lemos *et al*., 2016).

Over the years, it was found that the plants from *Calophyllum* genus exhibit potential biological activities such as antibacterial, antioxidant, antimalarial, and cytotoxic (Lim *et al*., 2015; Karunakaran *et al*., 2020). This is due to the secondary metabolites such as coumarins, xanthones, triterpenoids, chromanones, and benzophenones found in different parts of the tree (Noh and Jong, 2020; Karunakaran *et al*., 2020). These metabolites also play an important role in protecting against pathogens in plants.

Kumar and Garg (2020) stated that *Calophyllum* species contain various dipyranocoumarins, chemical that shows strong anti-HIV-1Reverse transcriptase activity. Kashman and his co-researchers had isolated calanolide A **(1)** from the tree of *Calophyllum lanigerum* in 1992, and the compound was found to possess strong anti-HIV-1 activity. A year later, isolation of (+)-inophyllum B **(2),** and other new compounds from *Calophyllum inophyllum* was performed by Patil *et al*. (1993) also showed anti-HIV-1Reverse transcriptase activity.



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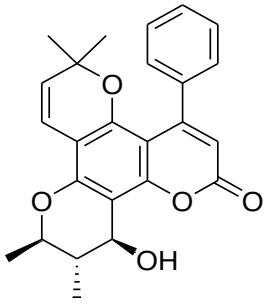

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1.2 Problem Statement

Previous studies on *Calophyllum* genus have revealed numerous phytoconstituents such as xanthone in its roots, stems, and leaves which possess potential biological properties such as antimalarial, antioxidant, antibacterial, and antitumor (Marliyana *et al*., 2021). In addition, the *Calophyllum gracilentum* species are also commonly found in tropical rainforests in Sarawak. Since this endemic *Calophyllum gracilentum* belongs to the same

family, its extracts may possess similar antioxidant activities. Besides, no study on *Calophyllum gracilentum* regarding its antioxidant properties is reported. Therefore, this study aims to identify the bioactive compounds present in the plant stem bark that correspond to the antioxidant activity. The findings are hoped to provide useful information for further development as potent antioxidant agents.

1.3 Significance of Study

According to the International Union of Conservation of Nature (IUCN), several species of the *Calophyllum* genus such as *C. rigidulum* and *C. afiricanum* have been listed as critically endangered. Therefore, owing to the importance of this genus, its ethnomedicinal uses, phytochemistry, and pharmacology should be reviewed. As the *C. gracilentum* plant itself is commonly used as traditional medicine by locals, an attempt was made to study its antioxidant properties. The outcome of this study can be very beneficial to scientists and chemists in developing antioxidant herbal formulations.

1.4 Objectives of Study

The general purpose of this research is to obtain the information on the secondary metabolites of C. *gracilentum* and the antioxidant properties of its extracts.

The specific objectives for this research are:

1. To extract and isolate compounds from the hexane extract of *C. gracilentum* stem bark.
2. To characterize the compounds isolated from the hexane extract of *C. gracilentum* stem bark.
3. To determine the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity assay of *C. gracilentum* extracts.

CHAPTER 2

LITERATURE REVIEW

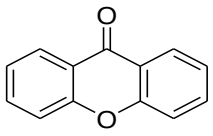
2.1 Phytochemical studies of *Calophyllum* genus

Previous phytochemical studies on the *Calophyllum* genus have shown that this genus contains potential lead compounds that can be used in drug discovery studies (Tee *et al*., 2018). In relation to that, bioactive secondary metabolites such as xanthones, coumarins, flavonoids, terpenoids, and chromanones are most reported in the chemical studies of the *Calophyllum* genus (Mah *et al*., 2012).

2.1.1 Xanthones

Xanthone **(4)** is a polyphenolic compound with a dibenzo-γ-pyrone group and has the molecular formula of $C_{13}H_8O_2$ (Zailan *et al*., 2021; Pinto *et al*., 2021). The name "xanthone" was originated from the word "xanthon" which is derived from the Greek word *xanthos*, which means yellow (Mazimba *et al*., 2013). The xanthone **(4)** structure is a planar, conjugated benzene ring system bridged across a carbonyl group and an oxygen atom (Mazimba *et al*., 2013). About 650 xanthones came from natural sources (Mazimba et al., 2013). Most xanthones occur in two plant families which are Guttiferae (*Calophyllaceae* and *Garciniaceae)* and Gentianaceae (*Gentiana* and *Gentianella*) (Tovilovic-

Kovacevic *et al*., 2020). Xanthones are well-known in phytochemical studies due to their biological and pharmacological properties (Tovilovic-Kovacevic *et al*., 2020). Previous studies on the *Calophyllum* genus also found that this genus is rich in xanthone and its derivatives. The number of xanthones isolated from *Calophyllum* species are summarized in Table 2.1.



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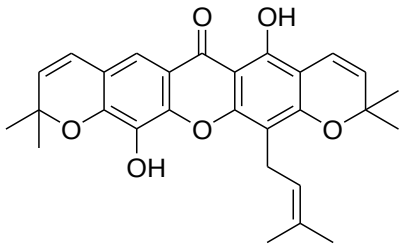

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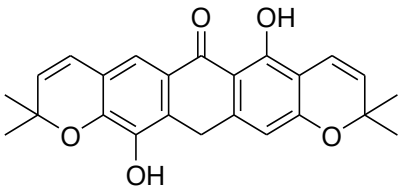

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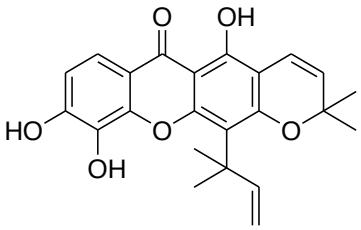

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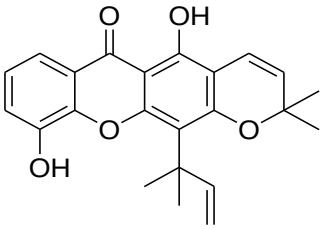

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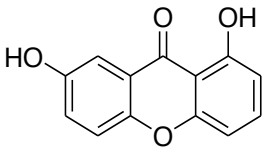

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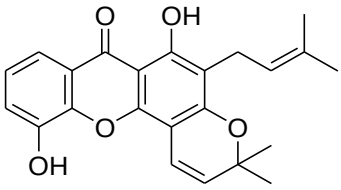

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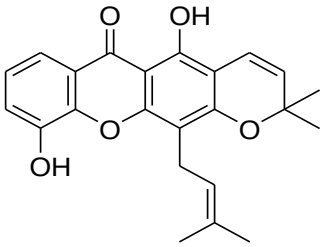

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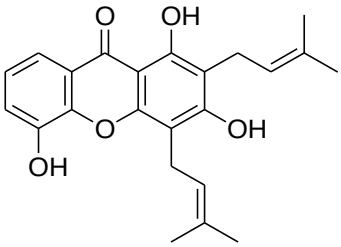

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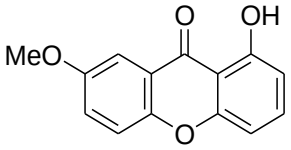

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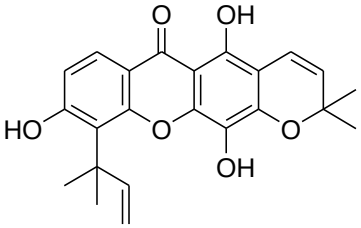

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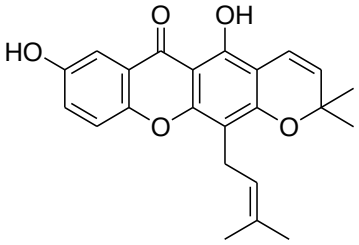

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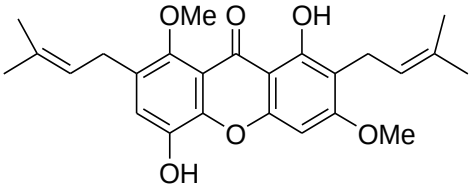

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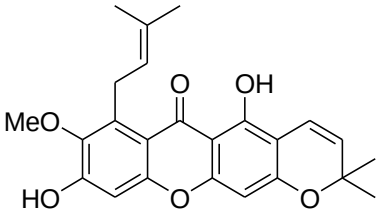

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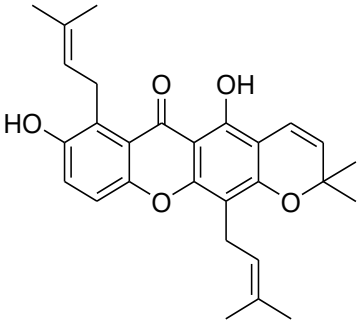

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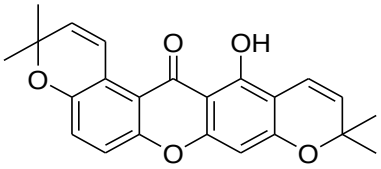

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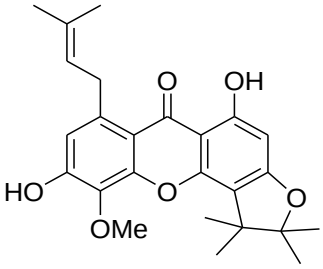

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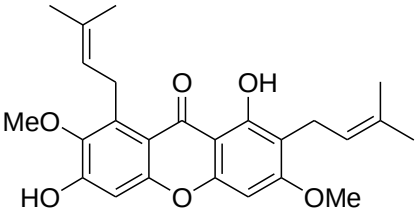

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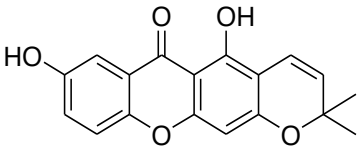

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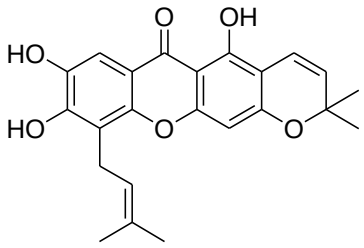

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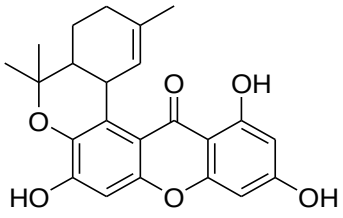

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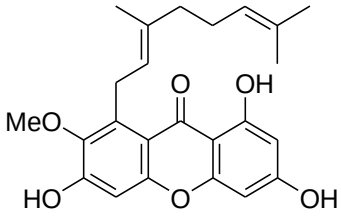

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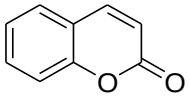
Table 2.1 Summary of xanthones isolated from *Calophyllum* species

Plant Species	Compound Name	References
C. andersonii	Caloxanthone I (**5**)	Tee *et al.*, 2018
	Pyranojacareubin **(6)**	
	Macluraxanthone **(7)**	
	Caloxanthone C **(8)**	
	Euxanthone **(9)**	
C. macrocarpum	Ananixanthone **(10)**	Karunakaran *et al.*, 2020
	Trapezifolixanthone **(11)**	
	8-deoxygartanin **(12)**	
C. ferrugineum	1-hydroxy-7-methoxy-9H-xanthen-9-one **(13)**	Noh and Jong, 2020
C. soulattri	Soulattrin **(14)**	Mah *et al.*,2012
	Brasixanthone B **(15)**	
	Phylattrin **(16)**	
C. hosei	9-hydroxycalabaxanthone **(17)**	Daud *et al.*, 2014
	Dombakinaxanthone **(18)**	
	Thwitesixanthone **(19**)	
	Caloxanthone B **(20)**	
	β-mangostin **(21)**	
	Osajaxanthone **(22)**	
	Caloxanthone A **(23)**	
	Calozeyloxanthone **(24)**	
	Rubraxanthone **(25)**	

2.1.2 Coumarins

Coumarin **(26)** (1,2-benzopyrone) is a naturally occurring compound comprising of fused benzene and pyrone rings and is the largest class of 1-benzopyran derivatives (Kostova *et al*., 2011; Sarker *et al*., 2017). More than 1300 coumarins have been identified from natural sources (Annunziata *et al*., 2020). Coumarin **(26)** is a fragrant colourless compound and was first isolated in 1820. In addition, the name coumarin was derived from the French word *"coumarou"* which means tonka bean (Sarker *et al*., 2017). Coumarin and its derivatives possess a broad spectrum of biological activities due to their ability to interact with various enzymes and receptors in living organisms (Al-Majedy

et al., 2017; Annunziata *et al*., 2020). Previous pharmacological studies on the *Calophyllum* genus reported many discoveries of coumarins. The coumarins isolated from *Calophyllum* species are summarized in Table 2.2.



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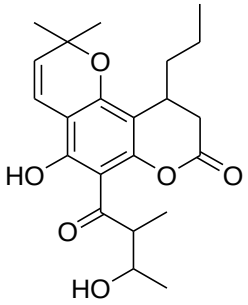

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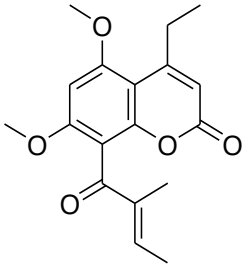

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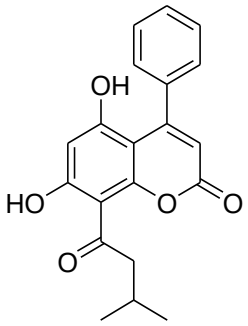

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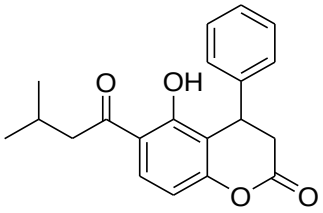

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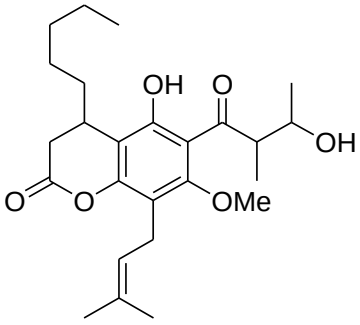

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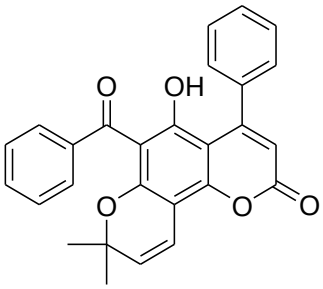

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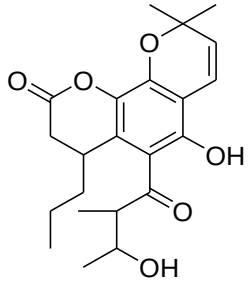

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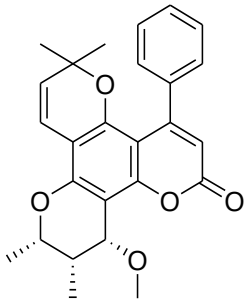

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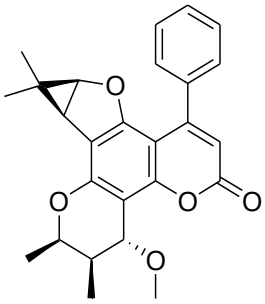

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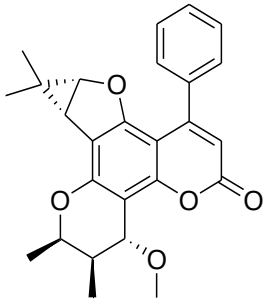

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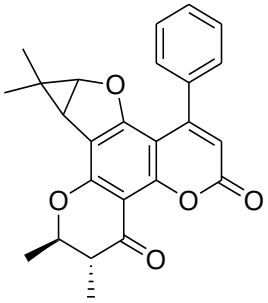

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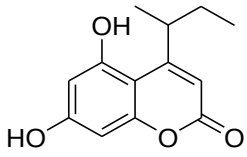

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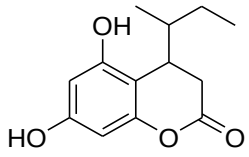

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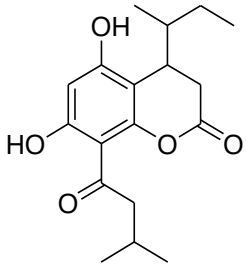


(40)

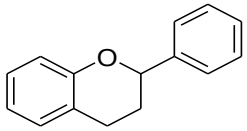
Table 2.2 Summary of coumarins isolated from *Calophyllum* species

Plant Species	Compound Name	References
C. wallichianum	Calanolide E **(27)**	Tee *et al.*, 2018
	Wallimarin T **(28)**	
C. sclerophyllum	Isodispar B **(29)**	Lim *et al.*, 2016
C. teysmannii	5,7-dihyroxy-6-(3-methylbutyryl)-4-	
C. castaneum	phenylcoumarin **(30)**	
C. canum		
C. hosei	Hoseimarin **(31)**	Daud *et al.*, 2014
C. ferrugineum	Isocalanone **(32)**	Noh and Jong, 2020
C. soulattri	Soulamrin **(33)**	Ee *et al.*, 2011
C. inophyllum	(-)-12-methoxyinophyllum A **(34)**	Li *et al.*, 2019
	(-)-12-methoxyinophyllum H-1 **(35)**	
	(-)-12-methoxyinophyllum H-2 **(36)**	
	Inophyllum J **(37)**	
C. gracilentum	Gracilenins A **(38)**	Lim *et al.*, 2019
	Gracilenins B **(39)**	
	Gracilenins C **(40)**	

2.1.3 Flavonoids

Flavonoids **(41)** are a large subgroup of small molecular secondary metabolites produced in different parts of plants and widely distributed throughout plants and prokaryotes (Samanta *et al*., 2011; Go´rniak *et al*., 2018). Flavonoid **(41)** has the main structure of a phenylpropanoid core (C_6-C_3-C_6), where it contains two aromatic rings (represented as rings A and B) linked by a heterocyclic ring (Zailan *et al*., 2021). There are more than 6,500 flavonoids have been identified

(Samanta *et al*., 2011). The four main groups of flavonoids are flavones, flavanones, catechins, and anthocyanins (Agrawal, 2011). Flavonoids are gaining much interest from the pharmaceutical industries due to their wide range of pharmacological activities (Kumar and Pandey, 2017; Górniak *et al*., 2018). Previous pharmacological studies also reported several discoveries of flavonoids in the *Calophyllum* genus. The flavonoids isolated from *Calophyllum* species are summarized in Table 2.3.



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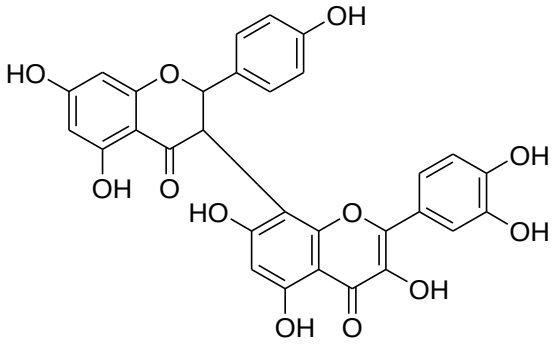

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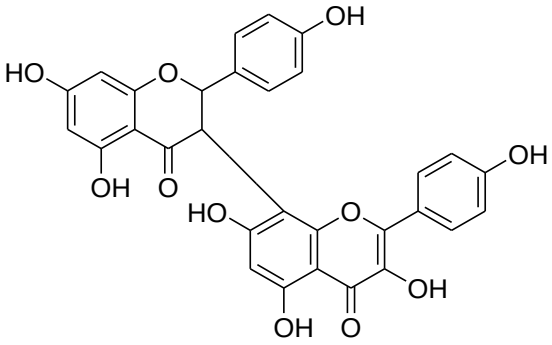

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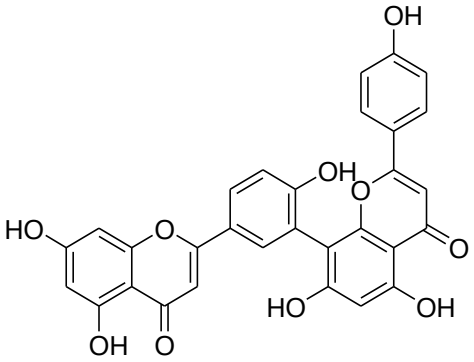

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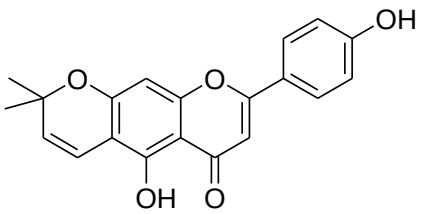


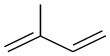
(45)

Table 2.3 Summary of flavonoids isolated from *Calophyllum* species

Plant Species	Compound Name	References
C. panciflorum	Pancibiflavonone **(42)**	Ito *et al.*, 1999
	Garcinianin **(43)**	
C. symingtoniamum	Amentoflavone **(44)**	Aminuddin *et al.*, 2015
	Carphachromene **(45)**	

2.1.4 Terpenoids

Terpenoids are the largest group of secondary metabolites found in plants, with over 40,000 different molecules (Liu *et al.*, 2021). They are biosynthetically derived and originate from isoprene **(46)**, which is commonly found in the *Calophyllum* genus in Malaysia. Terpenoids are usually expressed in the formula $(C_5H_8)_n$ where n refers to the amount of isoprene **(46)** in the carbon structure. In addition, terpenoids are classified under seven classes: hemiterpenes, monoterpenes, sesquiterpenes, sesterterpenes, triterpenes, polyterpenes, and tetraterpenes (Zailan *et al*., 2021). Terpenoids are diverse due to their wide range of biological activities (Ludwiczuk *et al.*, 2017). Several studies on terpenoids in the *Calophyllum* genus were reported with friedelin and stigmasterol being the two most common triterpenoids. The number of terpenoids isolated from *Calophyllum* species are summarized in Table 2.4.



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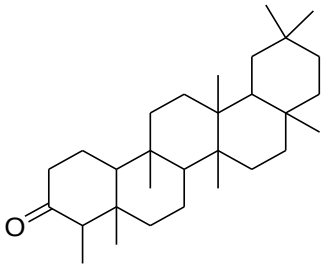

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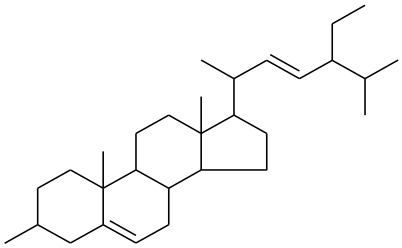

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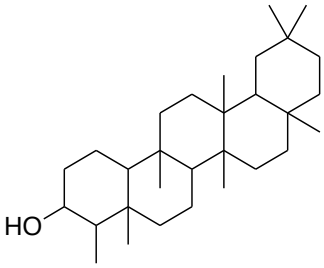

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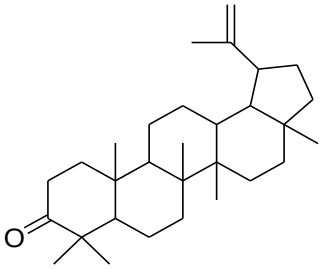

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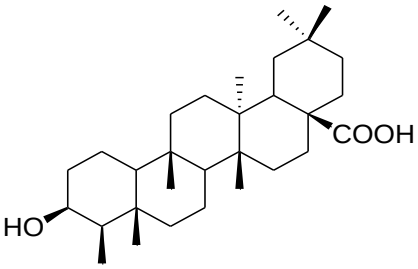

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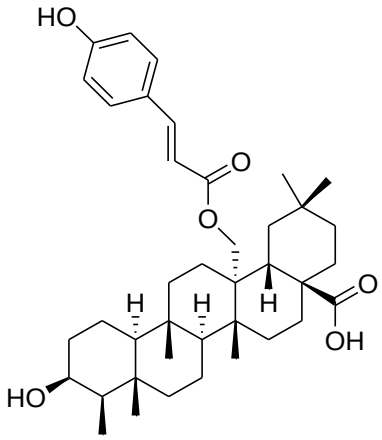

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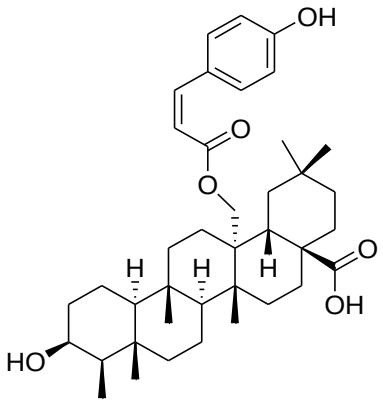


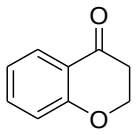
(53)

Table 2.4 Summary of terpenoids isolated from *Calophyllum* species

Plant Species	Compound Name	References
C. sclerophyllum	Friedelin **(47)**	Lim *et al.,* 2016
C. castaneum	Stigmasterol **(48)**	Karunakaran *et al.,* 2020
C. canum	Friedelinol **(49)**	Tee *et al.,* 2018
C. macrocarpum		
C. wallichianum		
C. ferrugineum	Friedelin **(47)**	Noh and Jong, 2020
	Stigmasterol **(48)**	
C. symingtonium	Lupenone **(50)**	Aminudin *et al.,* 2015
C. inophyllum	Canophyllic acid **(51)**	Prasad *et al.,* 2012
	27-[(E)-p-coumaroyloxy]canophyllic acid **(52)**	Van Thanh *et al.,* 2019
	27-[(Z)-p-coumaroyloxy]canophyllic acid **(53)**	

2.1.5 Chromanones

Chromanones **(54)** are a group of heterolytic secondary metabolites that contains a benzene ring fused to a 2,3-dihydro-γ-pyranone forming a chromanone nucleus (Zailan *et al*., 2021). The nucleus consists of two stereocenters at carbons C-2 and C-3 in addition to the stereocenter located in the attached alkyl side chain containing 5-8 carbons in length (Lim *et al*., 2015). Previous studies reported that chromanone derivatives have unique structures. Hence, they possess interesting stereochemical diversity (Lim *et al*., 2016). The chromanones isolated from *Calophyllum* species are summarized in Table 2.5**.**



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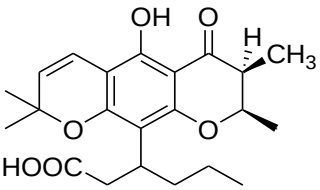

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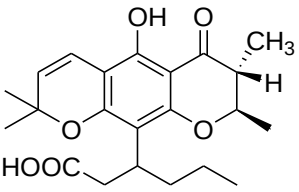

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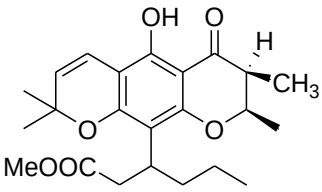

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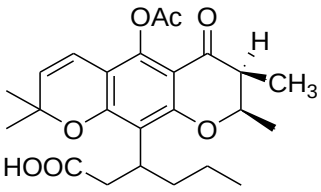

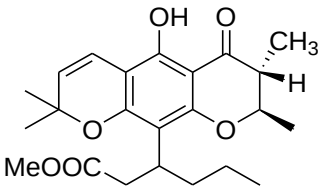

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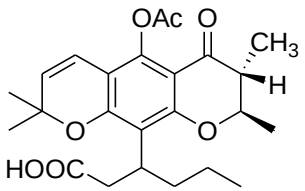

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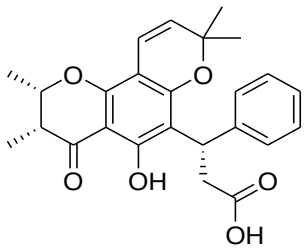

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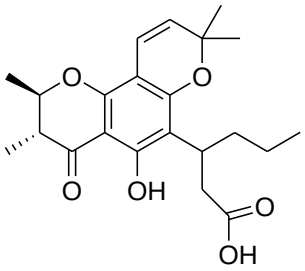

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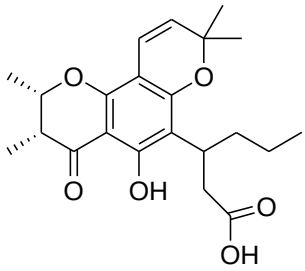

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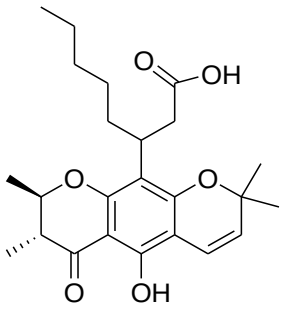

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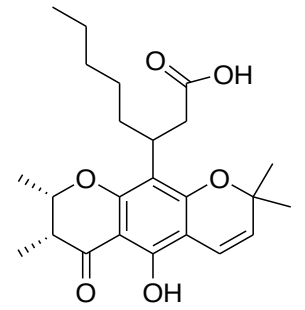

(64)

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Table 2.5 Summary of chromanones isolated from *Calophyllum* species.

Plant Species	Compound Name	References
C. blancoi	Apetalic acid **(55)**	Shen *et al*., 2004
	Isoapetalic acid **(56)**	
	Apetalic acid methyl ester **(57)**	
	Apetalic acid 5-*O*-acetate **(58)**	
	Isoapetalic methyl ester **(59)**	
	Isoapetalic acid 5-*O*-acetate **(60)**	
C. teysmannii	Caloteysmannic acid **(61)**	Lim *et al.*, 2016
C. castaneum	Calolongic acid **(62)**	Lim *et al.*, 2016
	Isocalolongic acid **(63)**	
	Blancoic acid **(64)**	
	Isoblancoic acid **(65)**	

2.2 Pharmacological activities of *Calophyllum* genus

Malaysian *Calophyllum* genus is starting to appear as a potential source for lead compounds against targeted diseases in the development of drugs. Previous scientific studies on the genus revealed that the unique phytoconstituents from the genus have led to promising pharmacological activities such as antioxidant, antibacterial, anti-inflammatory, anticancer, and anti-HIV.

2.2.1 Antioxidant activity

Antioxidant refers to a substance's ability to delay, prevent, or remove oxidative damage to a target molecule (Pintea and Rugină, 2014). It is also a defense mechanism to neutralize the effects of reactive oxygen species (ROS) (Mulgund *et al*., 2015). ROS consist of oxygen-containing molecules that are highly reactive, resulting from the incomplete reduction of molecular oxygen in cells (Shields *et al*., 2021). A high level of ROS can lead to oxidative damage to the body's lipids, proteins, and nucleic acids (Pintea and Rugină, 2014).

Secondary metabolites such as phenolic groups: coumarins, and flavonoids possess antioxidant properties. These compounds can scavenge free electrons and chelate metal ions that generate free radicals due to their structure consisting of an aromatic ring and hydroxyl groups (Tovilovic-Kovacevic *et al*., 2020). The antioxidant properties of compounds in *C. ferrugineum* were studied and found compound **32** shows good antioxidant properties with a mechanism by reduction of DPPH radical (Noh and Jong, 2020). Apart from that, Prasad *et al*. (2012) reported that compound **51** is active in the hyperlipidemia model and exhibits good antioxidant activity. However, compounds **61**, **62**, and **63** showed no significant antioxidant activity in the DPPH radical scavenging capacity assay of *C. teysmannii* (Lim *et al*., 2015).

2.2.2 Antibacterial activity

Compounds with antibacterial properties can kill or inhibit the growth of microorganisms such as bacteria, fungi, and algae (Burnett-Boothroyd and McCarthy, 2011). These antibacterial agents play a crucial role in discovering and developing new drugs (Kumar and Garg, 2020). The lead molecule for further development as a potent antimicrobial agent can be identified by screening their extracts for antimicrobial activity against various organisms. Ludwiczuk *et al*. (2017) reported that terpenoids display a broad spectrum against various Gram-positive and Gram-negative pathogenic bacteria. This is due to the structure being lipophilic, hence they can permeate easily through

the cell wall and cell membrane. As a result, the bacterial cell will die due to the disruption of microbial membranes (Kumar and Pandey, 2013).

The antibacterial potency of about 50 coumarin derivatives, natural and synthetic, was evaluated and correlated by a Structure-Activity Relationship (SAR) study. The bacterial susceptibility to coumarins was evaluated by identifying the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC of ≤ 500 µg/mL indicates that the compound has a strong MIC value and potential as an effective antimicrobial agent (Noh and Jong, 2020). The extracts contained compounds **5 – 9** were moderately active against *B. subtilis.* It was believed that the rich source of xanthones in the compounds contributed to the activities of plant extracts against *B. subtilis* (Tee *et al*., 2018). Apart from that, Noh and Jong (2020) reported that the chloroform extract of *C. ferrugineum* displayed strong MIC values ranging from 112.5 µg/mL to 225 µg/mL. The extract has potential as a bactericidal agent against *P. aeruginosa* and *E. coli.*

2.2.3 Anti-inflammatory activity

Inflammation is a normal biological process to protect the body from infection against harmful agents such as bacteria and viruses. Inflammation is normally rapid and self-limiting. However, abnormal resolution and prolonged inflammation lead to multiple chronic disorders (Kumar and Pandey, 2013).

Anti-inflammatory agents are able to treat inflammation by blocking certain substances that cause inflammation in the body.

Zakaria *et al*. (2014) reported that the extract of *C. inophyllum* inhibited 77% and 88% of cyclooxygenase and lipoxygenase activities, respectively. This indicates that the compounds in the plant have potent anti-inflammatory agents. Besides, Nguyen *et al*. (2017) reported that calophyllide, a compound isolated from *C. inophyllum* displayed numerous positive effects on cutaneous wound healing as it reduced the formation of fibrosis and accelerated the wound area closure with epidermis and dermal layers being formed completely by day 14 post-treatment. Compounds **52** and **53** were found to exhibit potent inhibitory activity by inhibiting pro-inflammatory mediators such as NO, IL-1β, and TNF-α (Van Thanh *et al*., 2019).

2.2.4 Anticancer, cytotoxicity activity

Cytotoxicity studies are useful in identifying the potential toxicity of a substance. According to the guidelines from American National Cancer Institute (NCI), to consider a compound to have *in vitro* cytotoxicity activity, the IC_{50} value of the extract is ≤ 20 g/mL, while for pure compound, the IC_{50} value is < 4 µg/mL, following incubation between 48 and 72 hours (Alabsi *et al*., 2016).

Cytotoxicity activity of compounds **61**, **62**, **63**, and **48** were evaluated by Lim *et al*. (2015) against the HeLa cancer cell line. The compounds were found to display potent inhibitory activity comparable to cisplatin, which was used as the positive control. Next, compounds **10** and **11** were tested for their cytotoxicity against two cell lines such as human hepatocytes (HeLa Chang Liver) and human embryonic kidney cells (HEK-293) at a concentration range of 500 µg/mL to 7.8125 µg/mL after 20 hours of treatment. Compound **10** exhibited high cytotoxicity against the HeLa Chang liver cell line at the IC_{50} value of 11.08 ± 3.09 µM. However, it displayed limited cytotoxicity against the HEK-293 cell line. On the other hand, compound **11** exhibited lower cytotoxic activities against both cell lines with the IC_{50} value > 50 µM. This may be due to the substitution pattern of the pyrano (C-3 & C-4) and prenyl (C-2) group in compound **10** that leads to the enhancement of compound cytotoxicity towards HeLa Chang liver cell compared to compound **11** (Karunakaran *et al*., 2020). Next, Mah *et al*. (2015) reported that compound **11** exhibited potent cytotoxic activities against B-lymphocyte cells (Raji), colon carcinoma cells (LS174T), and skin carcinoma cells (SK-MEL-28). Compound **29** displayed potent cytotoxicity activity against nasopharyngeal cancer cell lines (SUNE1, TW01, CNE1, HK1) with an IC_{50} value range of 3.8 to 11.5 µM (Lim *et al*., 2016).

2.2.5 Anti-HIV activity

Compound **1** was first isolated from the tropical rainforest tree *C. lanigerum var. austrocoriaceum* in 1992 and has been widely studied by pharmacologists due to the compound possessing potent activity against Human Immunodeficiency Virus (HIV) type 1 reverse transcriptase (HIV-1 RT) (Huerta-Reyes *et al*., 2004). In Malaysia, compound **1** is the first member of coumarins possessing anti-HIV-1 activity with reverse transcriptase inhibition as a mediator, compound **1** is currently reaching clinical development to become the novel therapeutic option in treating HIV infection (Zailan *et al*., 2021).

Sundur *et al*. (2014) had evaluated the inhibitory activities of ethanolic and water extracts of *C. inophyllum* stem bark against two prime enzymes of HIV: HIV-1 protease (HIV-PR) and HIV-1 integrase (HIV-IN). It was found that both extracts showed potent anti-HIV-IN activity with IC_{50} values of 9.8 and 5.6 µg/mL, respectively. On the other hand, the IC_{50} values for anti-HIV-1 PR activity were found to be higher at 63.8 and 16.3 µg/mL, respectively. Kashman *et al*. (1992) reported that the anti-HIV activity from *C. lanigerum* was detected in the presence of calanolides. The isoprenylcoumarins isolated from *C. lanigerum* leaves were identified as active substances inhibiting the HIV-1 reverse transcriptase activity.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Plant Materials

1.2 kg of leaves and twig samples of *C. gracilentum* species was prepared and the herbarium voucher specimens were deposited at Universiti Teknologi MARA Sarawak.

3.2 Methods

In general, the process began with collecting herbarium voucher specimens of *C. gracilentum* deposited at UiTM Sarawak. Next, the powdered sample underwent an extraction process using the cold maceration method followed by purification processes using column chromatography, Thin-Layer Chromatography (TLC), and Centrifugal Partition Chromatography (CPC). Then, the purified compound underwent structural elucidation using Nuclear Magnetic Resonance (NMR) and Infrared (IR) spectroscopy. The extracts were studied for their Total Phenolics Content (TPC), Total Flavonoids Content (TFC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity Assay.

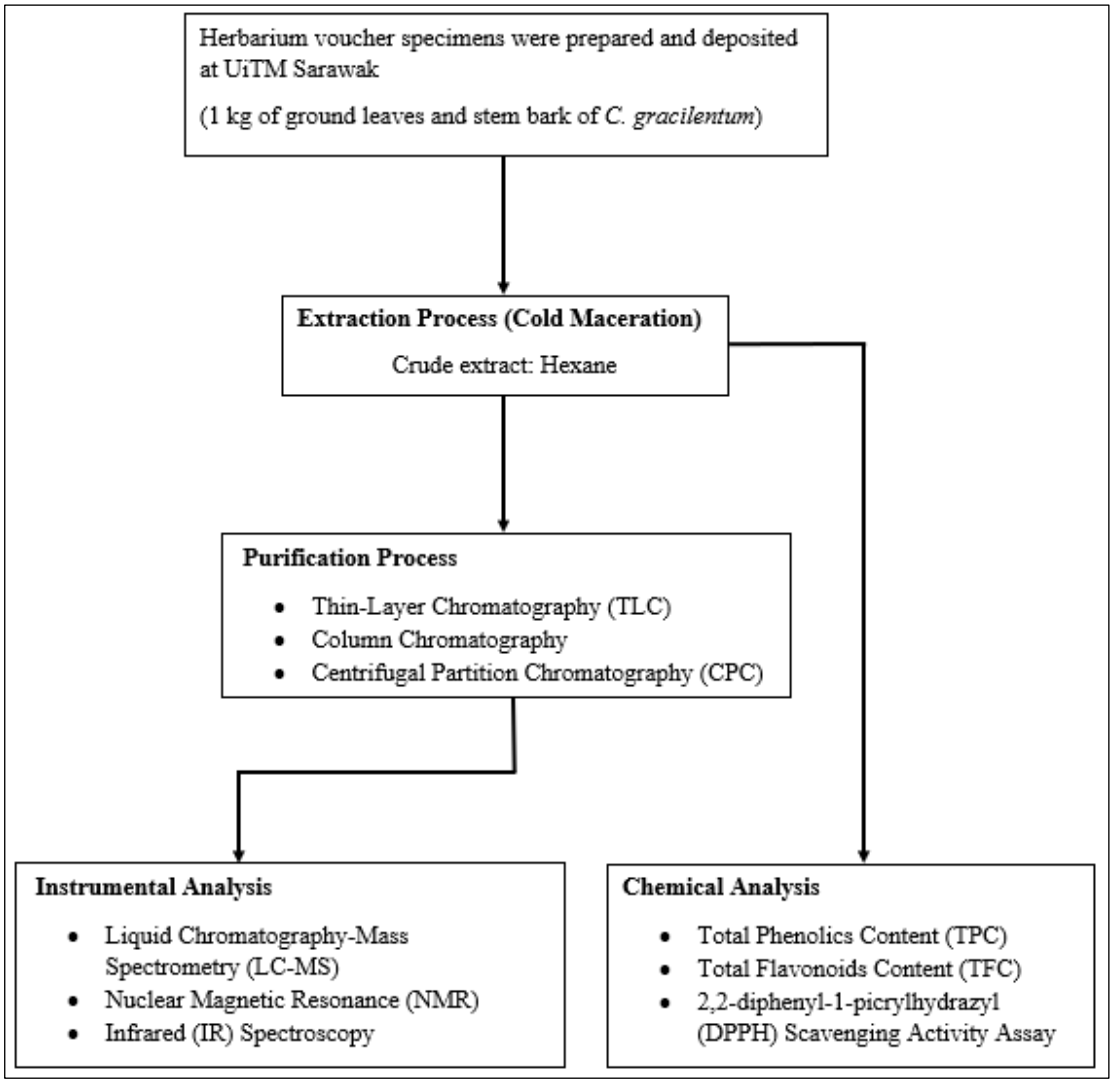


Figure 3.1 Flowchart of overall methodology of *C. gracilentum*

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

The first step to the isolation and purification work of plant samples would be the extraction of the sample. Thus, the cold maceration method was used to extract *C. gracilentum*. The plant material was transferred into a closed container, and solvent (hexane) was added until it covered the top of the sample. Approximately 1.2 kg of stem bark of *C. gracilentum* was collected, air-dried, and finely ground into powder form. The powdered form sample was used to ensures maximum surface area contact with the solvent, hence leading to an efficient extraction. After that, the powdered plant sample was soaked in hexane for about 48 hours at room temperature. The plant extracts should not be stored in the solvent for too long or exposed to sunlight as this can increase the risk of artifact formation and decomposition of extract constituents (Maltese *et al*., 2009). Then, the hexane crude solution was filtered, and the solvents were evaporated under reduced pressure via a rotary evaporator. About 2 g of each crude extract was kept for the antioxidant assay. The hexane extract was then purified using column chromatography, thin-layer chromatography, and centrifugal partition chromatography.

3.2.2 Column Chromatography

Column chromatography is a popular method for isolating and purifying chemical constituents from plant crude extracts. The column is made up of a long glass tube (5 cm – 1 m long) with a tap and glass wool filter at the bottom. The stationary phase (silica gel) was held in a glass column, which adsorbed and separated the compounds passing through it with the help of the mobile phase (solvent). The sample was prepared by dry packing method in which the sample was dissolved in suitable amount of solvent, followed by adding dropwise into silica gel and mixed homogenously. The silica gel was mixed with hexane in a separate beaker which formed a slurry. Then, the slurry was poured into a glass column. The prepared sample was then subjected to column chromatography using gradient elution in increasing polarity to separate compounds of different polarities. The fractions of hexane were collected at an interval. Separation is based on the component affinity for both phases, resulting in different migration rates (Srivastava *et al*., 2021). The higher the adsorption to the stationary phase, the slower the molecule traveled through the column. The solvent was removed from the collected fractions using a rotary evaporator after the purification process was completed, yielding the isolated material.

3.2.3 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was carried out to analyze the purity or chemical composition of the fractions using silica gel coated with aluminum sheets. Firstly, a baseline was drawn on the TLC plate, followed by spotting a suitable amount of sample solution onto the baseline using a microcapillary tube. Next, the plate was immersed in the TLC chamber to allow the development of spots. The TLC chamber also prevents solvent evaporation and keeps the developing process dust-free. When immersing the TLC plate, the spots were kept above the level of the mobile phase and not fully immersed so that the spots did not dissolve in the solvent. The solvent (mobile phase) migrated up the plate through the silica sorbent (stationary phase) due to capillary action (Gocan, 2002). The plate was taken out to dry once the spots were already developed. The sample spots were then observed under an ultraviolet (UV) lamp. Each spot has a retention factor (R_f) value expressed as:

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

Polar compounds were adsorbed strongly on the stationary phase, hence they moved slowly up the plate. As a result, these compounds produced a small R_f value. On the other hand, non-polar compounds have larger R_f since the compounds showed less affinity towards the stationary phase. Therefore, the components of the fraction were separated according to their relative polarities.

The most common non-destructive visualization method for TLC plates is UV light. UV corresponding compounds containing aromatic rings and conjugated systems can be detected with effective visualization. Compounds must have strong UV absorption in the range of 220 – 280 nm to be detectable under the UV lamp. At a short wavelength (254 nm), compounds appear as dark grey spots on a bright green background. At a long wavelength (365 nm), compounds will appear as fluorescence colour spots on a pale purple background under UV light.

3.2.4 Centrifugal Partition Chromatography (CPC)

Centrifugal Partition Chromatography (CPC) is a method in which both the stationary and mobile phases are liquids, and separation is based on solute partitioning between the two immiscible liquid phases (Lorántfy *et al*., 2020). Firstly, the stationary phase was added into the column (rotor) and retained inside the rotor while spinning at a moderate rotational speed. Next, the mobile phase containing the sample extracts was fed into the rotor onto the chromatotron plate and was pumped through the stationary phase. This is where the exchange of molecules between the two phases takes place. UV light was used to continuously monitor the separation of the compounds on the plate. Then, the eluted fractions containing the purified solutes were collected into different vials.

3.3 Structural elucidation

3.3.1 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) is used to determine the molecular mass, molecular formula, and fragmentation pattern of a non-volatile compound. About 2 mg of sample was dissolved n 2 mL HPLC grade solvent and filtered to remove undissolved solid particles before it was introduced into the LC-MS. 5 µL of sample solution was auto-injected into the column, followed by flushing with 30% water and 70% methanol in a flow rate of 0.6 mL/min. Agilent Technologies 6520 LC/MS equipped with an electrospray source was used in this analysis.

3.3.2 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical method used to identify the sample content, purity, and molecular structure (Mohamed *et al*., 2020). The ^{13}C-NMR is used to determine the number of carbons present in the compound, while the ^{1}H NMR is used to identify the number of hydrogens present and how the hydrogen atoms are connected in the compound (Ingle *et al*., 2017). This technique depends on the interaction between the material and electromagnetic radiation. When sensitive radio receivers detect the excitation of material nuclei with radio waves into nuclear magnetic resonance, the NMR signal will be produced. These signals can provide information on the molecule's electronic structure and functional groups (Mohamed *et al*., 2020).

The samples were prepared by dissolving them in deuterated acetone. Next, the samples were transferred into NMR tubes up to about 4 cm in height. The solvents were selected based on the degree of dissolution. The NMR tubes were then capped and wrapped with parafilm to avoid solvent evaporation. In this analysis, JEOL JNM-ECX 400 MHz spectrometer was used to obtain ^{1}H-NMR and ^{13}C-NMR spectra for each pure compound. Tetramethyl silane (TMS) was used as an internal standard during analysis.

3.3.3 Infrared (IR) Spectroscopy

The Infrared (IR) spectroscopy is used to identify the functional groups present in a molecule of the plant extract (Ingle *et al*., 2017). The molecular structure of the compounds can also be identified by observing their fingerprint regions. The preparation of the sample for IR spectroscopy was done by mixing the powder sample with potassium bromide (KBr) in a ratio of 1:10, and the mixture was pressed under high pressure using a die-set for about 2 minutes. The pressed powder formed a KBr pellet. The pellet should be thin and almost transparent. An opaque KBr pellet will produce poor spectra because only little infrared can pass through them. White spots in the KBr pellet indicate that the powder has not been properly ground or compacted into a pellet. Then, the sample in the pellet was measured for IR using the Perkin Elmer 2000-FTIR spectrophotometer at the range of 4000 to 400 cm^{-1}.

3.4 Antioxidant assay

3.4.1 Total Phenolic Content (TPC)

The TPC was determined by using Folin-Ciocalteu method described by Wani, Prasad and Prakash (2019) with slight modification. Firstly, 10 mg of the sample was dissolved in 10 mL of ethanol. 300 µL of the sample was taken out and transferred into another vial. Then, 2250 µL of Folin-Ciocalteu reagent was added to it. The solution mixture stood for 5 minutes. 2250 µL of 6% of sodium carbonate was gently mixed into the vial. After 40 minutes, the absorbance values were measured by UV-Vis spectrophotometer (Lambda 25, Perkin Elmer, USA) with detection of 765 nm by using gallic acid (40, 80, 120, 160, and 200 µg/mL) as the reference standard. The results were expressed as mg GAE/g extract.

3.4.2 Total Flavonoids Content (TFC)

The TFC of the crude extract was determined by using aluminium chloride colorimetric method as described by Chukwumah, Walker and Verghese (2009) with a slight modification. Firstly, 5 mL of sample were mixed with 5 mL of 2% aluminium chloride in a vial. Next, the vial was shaken and left for 10 minutes. The analysis was carried out using UV-Vis spectrophotometer with the detection of 415 nm by using quercetin (40, 80, 120, 160, and 200 µg/mL) as the reference standard. The results were expressed as mg QE/g extract.

3.4.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity Assay

The DPPH scavenging activity assay was done using a method adapted from Mensor *et al.* (2001) with slight modifications. Firstly, 6 mg of sample was dissolved in ethanol as a stock solution. It was diluted to concentrations of 15, 30, 60, 120, and 240 µg/mL. Next, 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was added into a vial containing a mixture 1 mL of sample solutions at different concentrations and 3 mL of methanol. The solution mixture was allowed to react for 30 minutes. The analysis was carried out using UV-Vis spectrophotometer with detection of 517 nm with ethanol as blank, ascorbic acid as positive control and 1 ml methanol plus 3 ml DPPH as the negative control. The percentage of inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = [(Abc - Abs)/Abc] \times 100\%$$

Abc is the absorbance of negative control and **Abs** is absorbance of samples.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical constituent isolated from *C. gracilentum*

About 1.2 kg of stem barks of *C. gracilentum* was subjected to hexane extraction and yielded 20.88 g golden brown crude extract. Then, 10 g of *C. gracilentum* hexane (CGH) crude was isolated using column chromatography and afforded nine fractions of column crude. Fraction **F5** was chosen for this study to determine the secondary metabolites present in the plant extract. Fraction number 5 underwent further separation using column chromatography, which afforded 42 subfractions. The subfractions were labeled as 1 to 42 and TLC consisting of different solvent systems; hexane-ethyl acetate, hexane-chloroform, and hexane-acetone were done to observe any separations. Based on the TLC profiles, fractions **F12** to **F33** were combined to undergo radial chromatography using chromatotron with the solvent system hexane-ethyl acetate at the ratio of 8:2, gradually increasing the polarity to 6:4. Then, the front fraction collected was subjected to gel-filtration chromatography (Sephadex) which afforded 14 subfractions.

After further series of fractionation and TLC profiling, CGH F5 (12-33) (1-5) (1-4) showed promising results as the fraction displayed a single spot during TLC analysis. In order to confirm the purity of this single spot isolate, an adequate amount of the sample was taken out from the fraction and prepared for NMR analysis. According to the spectroscopic data obtained and comparison with literature data, the pure compound was identified as compound **15**. Another spectroscopic technique, such as FTIR, was also used in structural elucidation for the pure compound. Figure 4.1 shows the isolation pathways of brasixanthone B **(15)** from *C. gracilentum.*

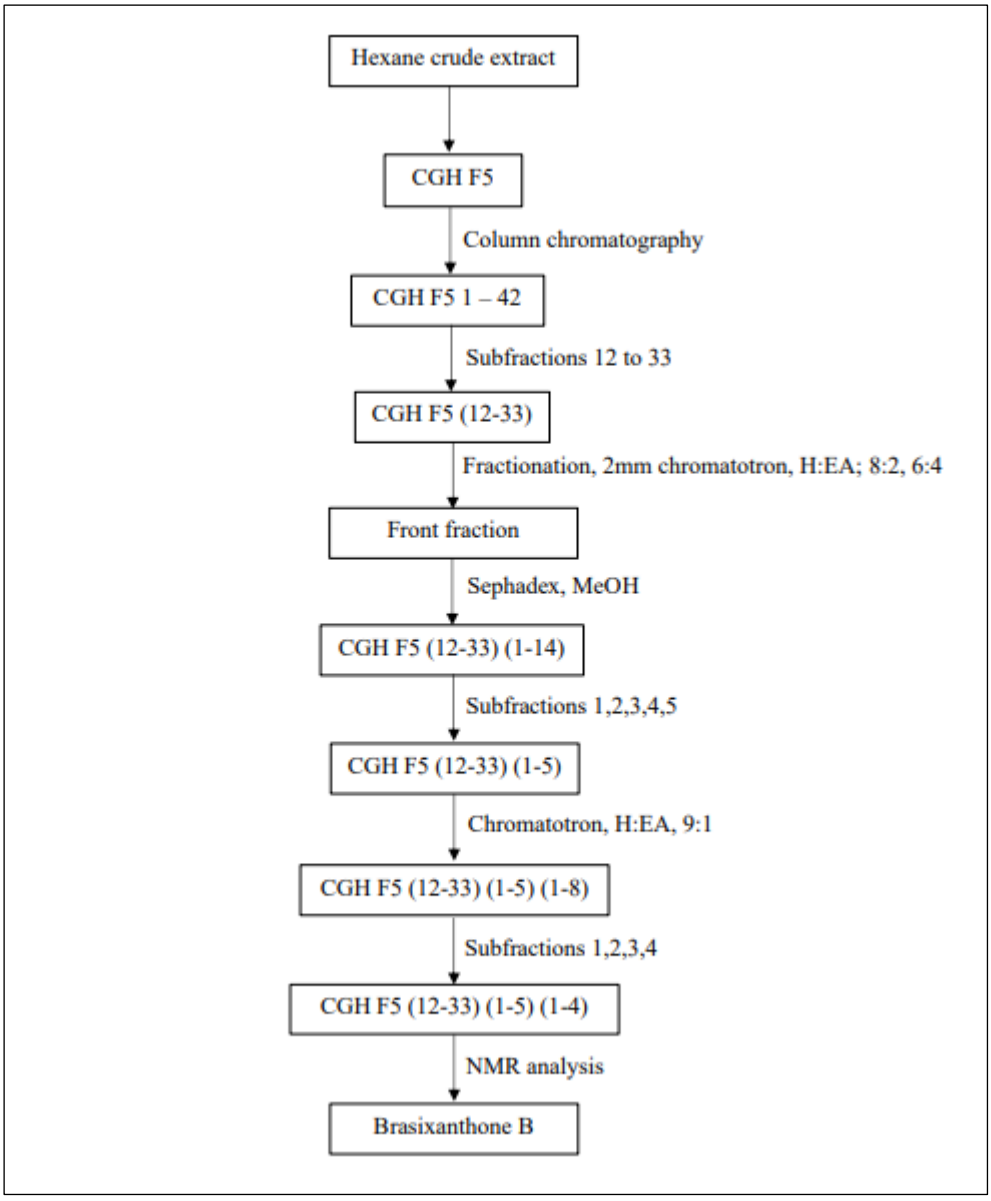


Figure 4.1 Isolation pathways of brasixanthone B **(15)** from *C. gracilentum*.

4.2 Chemical identification and structural elucidation of brasixanthone B (15)

A total of 1.90 mg of yellow crystals was isolated from the hexane extract. The LC-MS spectrum (Figure 4.2) showed a $[M+H]^+$ ion peak at m/z 379.1557. According to the LC-MS-MS data, the molecular ion peak at m/z 379.1557 corresponds to the formula of $C_{23}H_{22}O_5$. This compound has a melting point of 227 – 229 ^oC (Ito *et al*, 2002). It was developed on the TLC plate using the solvent system of 90% hexane and 10% ethyl acetate.

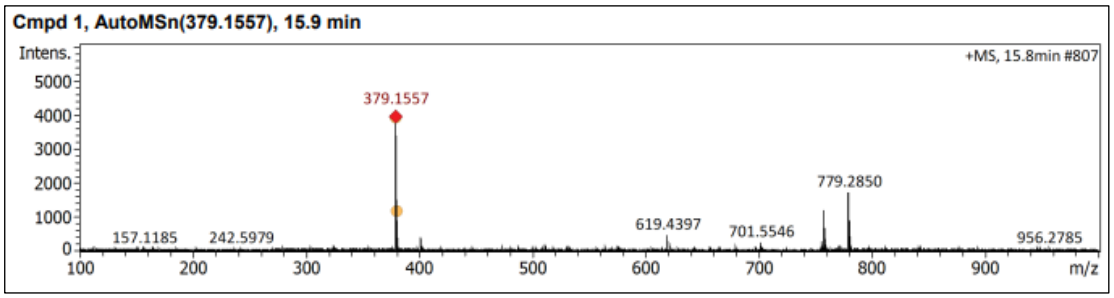


Figure 4.2 LC-MS spectrum of brasixanthone B **(15)**

Assignment of the chemical structure of compound **15** was supported by FTIR analysis. From the IR spectrum (Figure 4.3), the O-H stretch appeared as a broad peak at 3389.49 cm^{-1}. The absorption peaks at >3000 cm^{-1} (sp^2) and <3000 cm^{-1} (sp^3) were consistent with the C-H stretching band from methylene and methyl groups, whereas absorption peaks at 1606.67 cm^{-1} and 1483.32 cm^1 indicated C=C stretch from a cyclic alkene. A sharp band at 1647.62 cm^{-1} indicated ketone (C=O) stretching while 1210.39 cm^{-1} indicated C-O stretch. Finally, both peaks at 838.56 cm^{-1} and 805.91 cm^{-1} indicated the C-H bend.

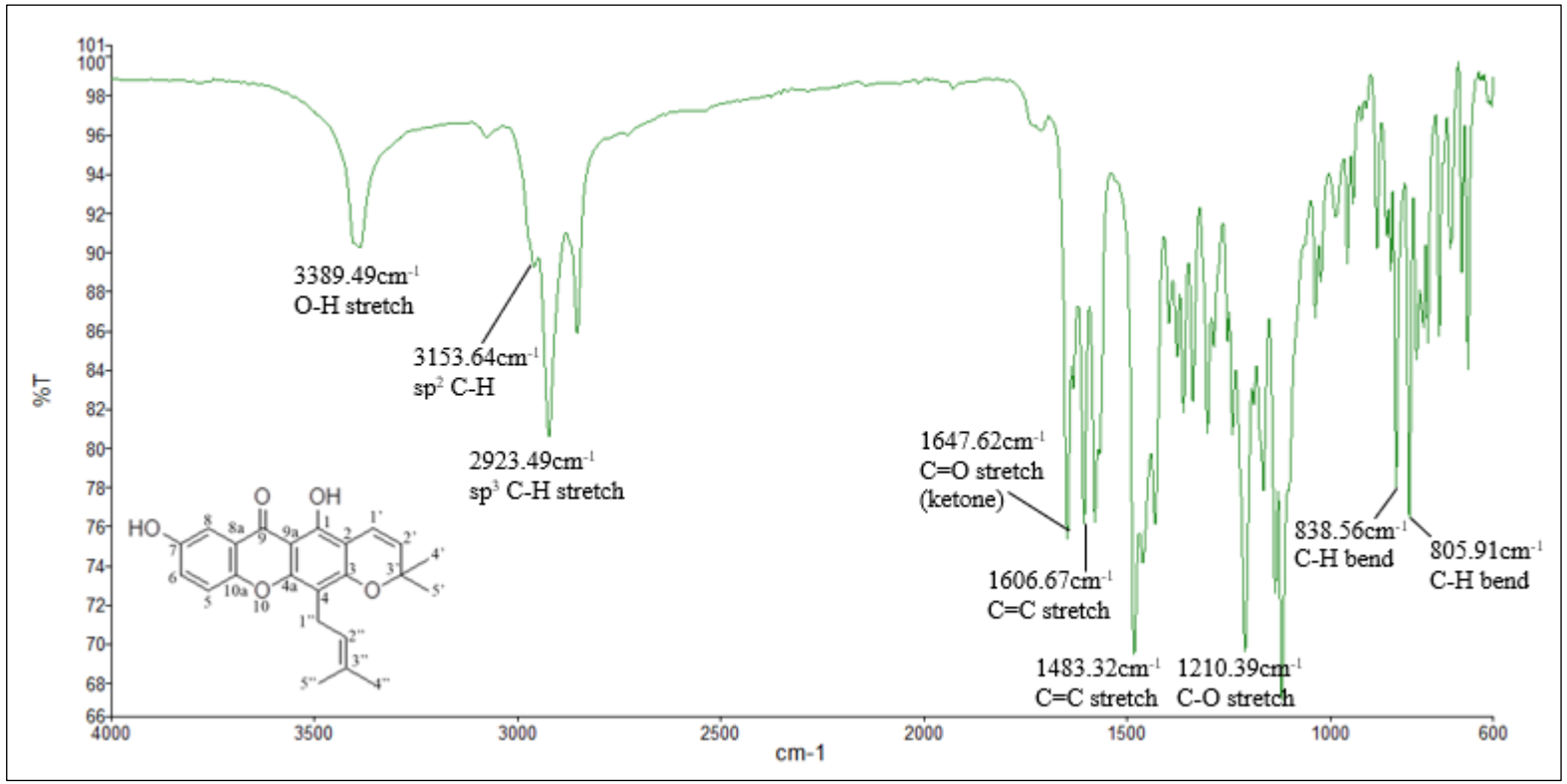
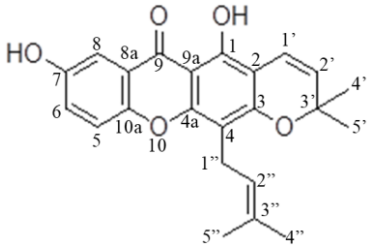


Figure 4.3 FTIR spectrum for brasixanthone B (**15)**.

The chemical structure of compound **15** was elucidated based on the 1D- and 2D-NMR spectral data. The ^{1}H-NMR spectrum (Figure 4.4) showed that aromatic protons H-5, H-6, and H-8 ranged between 6.0 ppm to 8.0 ppm indicating xanthone skeleton. In the COSY spectrum (Figure 4.6), the three proton signals H-6 (δH 7.38) were observed to be *ortho*- and *meta*-coupled with respective H-5 (δH 7.39) and H-8 (δH 7.55) which further supports the result. The two most shifted resonances in the low field region at δH 13.31 and 8.97 indicated chelated hydroxyl groups. The typical proton signals indicated the presence of a prenyl group at δH 3.50 (2H, *d*, *J* = 7.4 Hz) and 5.26 (*t*, *J* = 7.4 Hz), along with two singlet resonances δH 1.90 and 1.67. Besides, the ^{1}H-NMR spectrum exhibited a pair of doublets at δH 6.73 and δH 5.76 for H-1' and H-2', respectively, confirming presence of a pyrano ring. A sharp singlet signal integrated for six protons was observed at δH 1.51. The *ortho*- coupling of H-1' and H-2' were observed in the COSY spectrum.

The ^{13}C NMR spectrum (Figure 4.5) exhibited 23 peaks which is in accordance with the molecular formula $C_{23}H_{22}O_5$. The HSQC spectrum indicated the direct attachments of protons to carbons as shown in Figure 4.7 and Figure 4.8. In the HMBC spectrum (Figure 4.9), the chelated OH signal (δH 13.31) was correlated with δC 103.8 (C-9a), 104.8 (C-2), and 156.5 (C-1), suggesting its placement at C-1. The hydroxyl proton (7-OH) gave cross-peaks with C-8 (δC 107.9) and C-6 (δC 123.1), thus confirming the placement of the hydroxyl group at C-7 (Figure 4.10). The doublet resonance at H-1" (δH 3.49) gave cross-peaks with

C-4, C-3, and C-4a indicating the placement of the prenyl group at C-4 (Figure 4.12). The doublet signal at δH 6.73 (H-1') showed a correlation with carbon signals at δC 104.8 and 158.8, affirming the fusion of the pyrano ring to C-2 and C-3 (Figure 4.11). Based on the LC-MS, ^{1}H NMR, ^{13}C NMR, and IR spectral information, the comparison made with literature has confirmed compound **15**, identified as brasixanthone B, or 5,8- dihydroxy-2,2-dimethyl-12-(3-methylbut-2-en-1-yl)-*2H,6H*-pyrano[3,2b]xanthen-6-one.



(15)

Table 4.2 Summary of NMR data for compound in comparison with literature values of brasixanthone B (**15)**.

No.	δ ^{13}C (ppm)	δ ^{13}C (ppm) *	δ ^{1}H (Mult, *J* Hz, int)	δ ^{1}H (Mult, *J* Hz, int) *	^{1}H-^{13}C HMBC	^{1}H-^{1}H COSY
1	156.5	155.6	13.31 (*s*, OH)	13.05	C-9a, C-2, C-1	
2	104.8	104.2				
3	158.8	158.3				
4	107.9	107.3				
5	119.9	119.1	7.39 (*d*, 9.0, 1H)	7.36 (*d*, 8.8)	C-8, C-7, C-10a	H-6, H-8
6	123.1	123.7	7.38 (*dd*, 9.0, 3.0)	7.24 (*dd*, 8.9,2.2)	C-8, C-7 C-10a	H-8, H-5
7	152.8	152.1	8.97 (*s*, OH)	9.04 (*s*, OH)	C-8, C-6	
8	109.2	109.1	7.55 (*d*, 3.0)	7.55 (*d*, 2.2)	C-6, C-10a, C-7	H-5, H-6

9	181.7	180.8				
10						
4a	154.9	154.4				
8a	121.6	120.8				
9a	103.8	103.3				
10a	150.8	150.6				
1’	116.1	115.8	6.73 (*d*, 10.0)	6.74 (*d*, 10.1)	C-2, C-3’, C-1, C-3	H-2’
2’	128.6	127.3	5.76 (*d*, 10)	5.61 (*d*, 10.1)	C-4’, C-3’, C-2	H-1’
3’	79.0	78.1				
4’	28.4	28.4	1.51 (3H, *s*)	1.48 (3H, *s*)	C-5’, C-3’, C-1’, C-2’	
5’	28.4	28.4	1.51 (3H, *s*)	1.48 (3H, *s*)	C-4’, C-3’, C-1’, C-2’	
1”	21.9	21.4	3.50 (2H, *d*, 7.4)	3.46 (2H, *d*, 7.3)	C-4, C-2”, C-3”, C-4a	H-2”
2”	123.1	122.2	5.26 (*t*, 7.4)	5.22 (*m*)		H-1”
3”	131.8	131.4				
4”	18.1	17.9	1.90 (3H, s)	1.88 (3H, *s*)	C-5”, C-2”, C-3”	
5”	25.9	25.8	1.67 (3H, s)	1.68 (3H, *s*)	C-4”, C-2”, C-3”	

* Source: Ito *et al.* (2002).

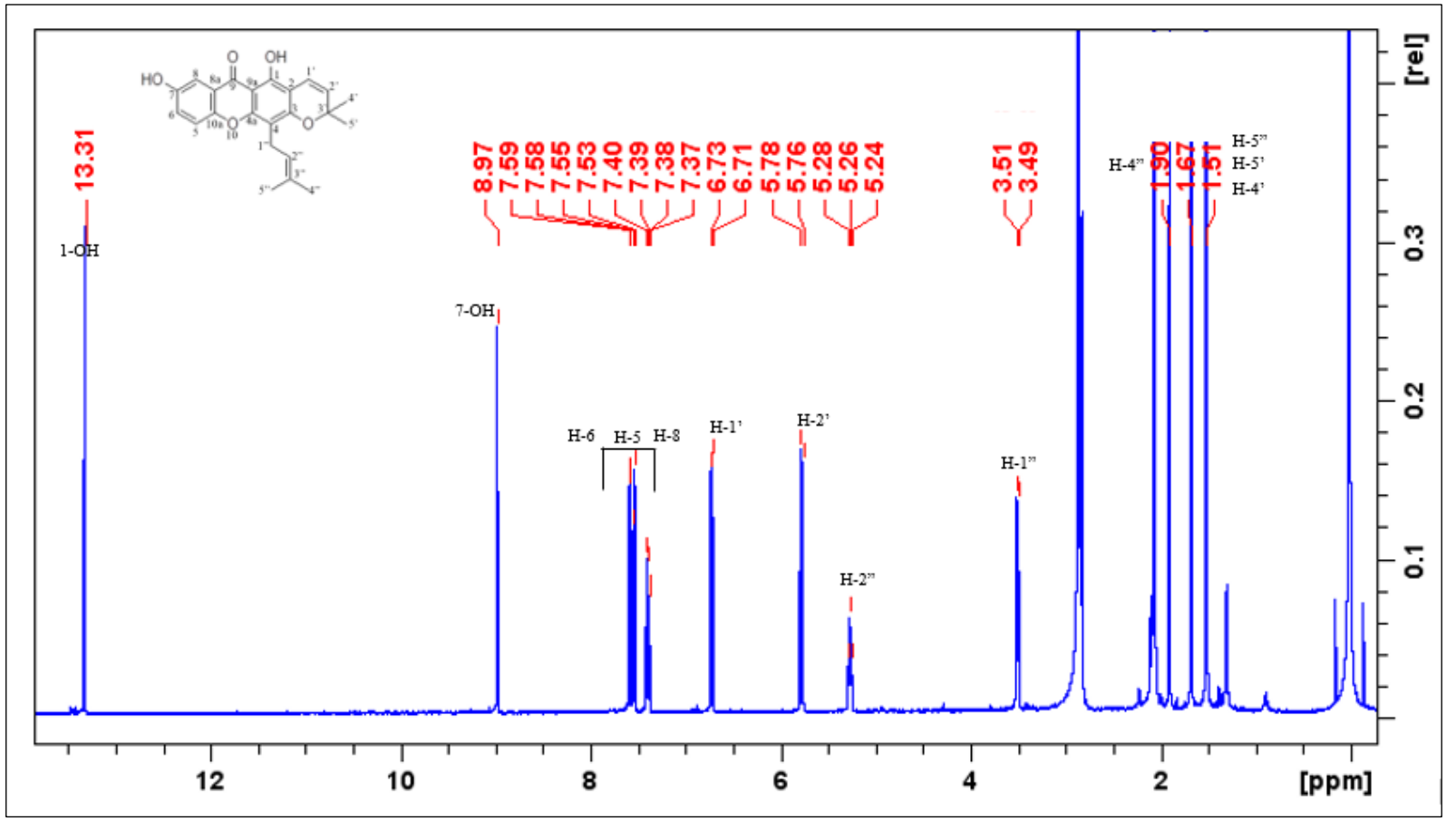


Figure 4.4 ^{1}H NMR spectrum of brasixanthone B (**15**) (400 MHz, acetone-d_6).

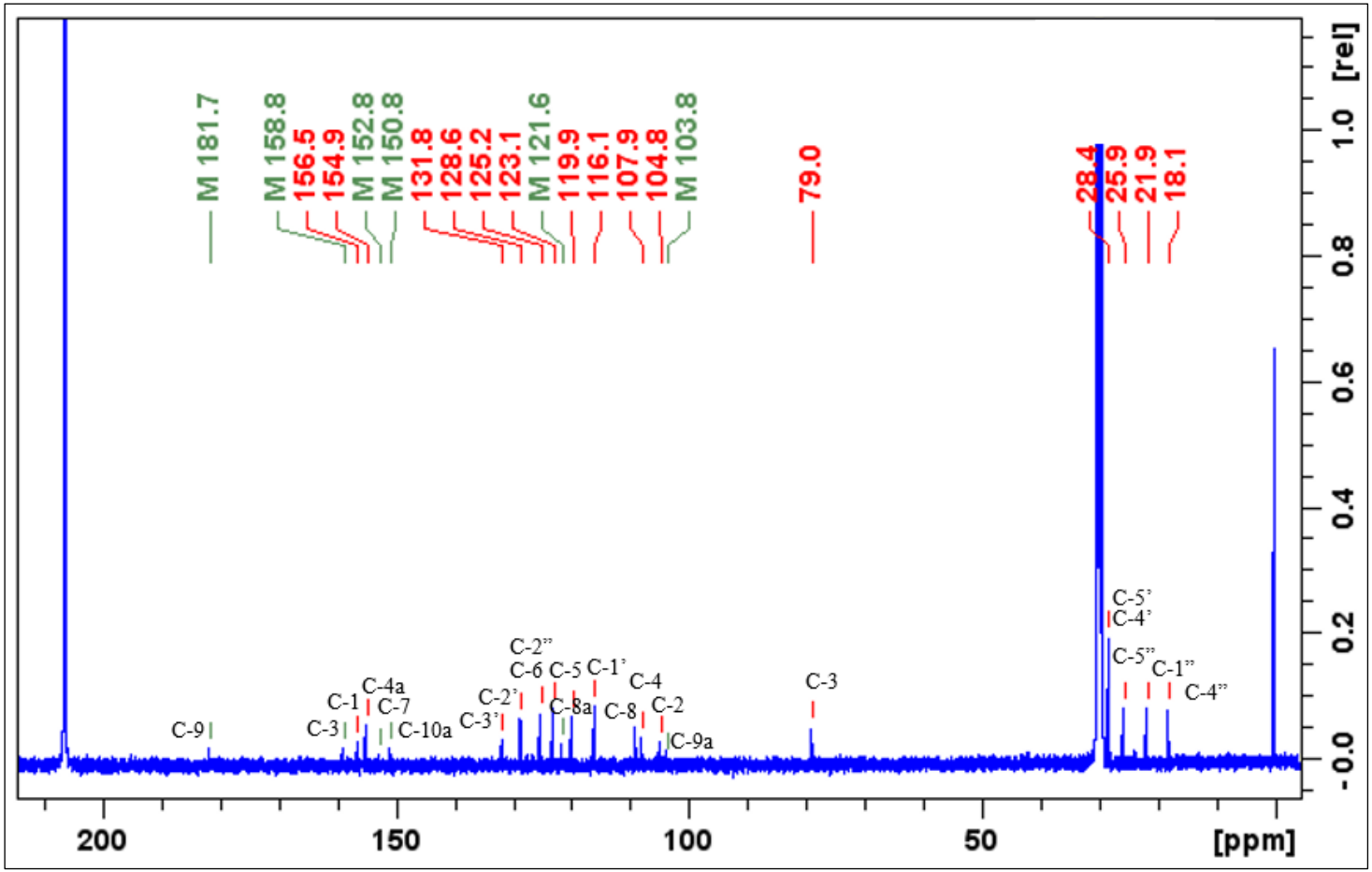


Figure 4.5 ^{13}C NMR spectrum of brasixanthone B (**15)** (400 MHz, acetone-d_6).

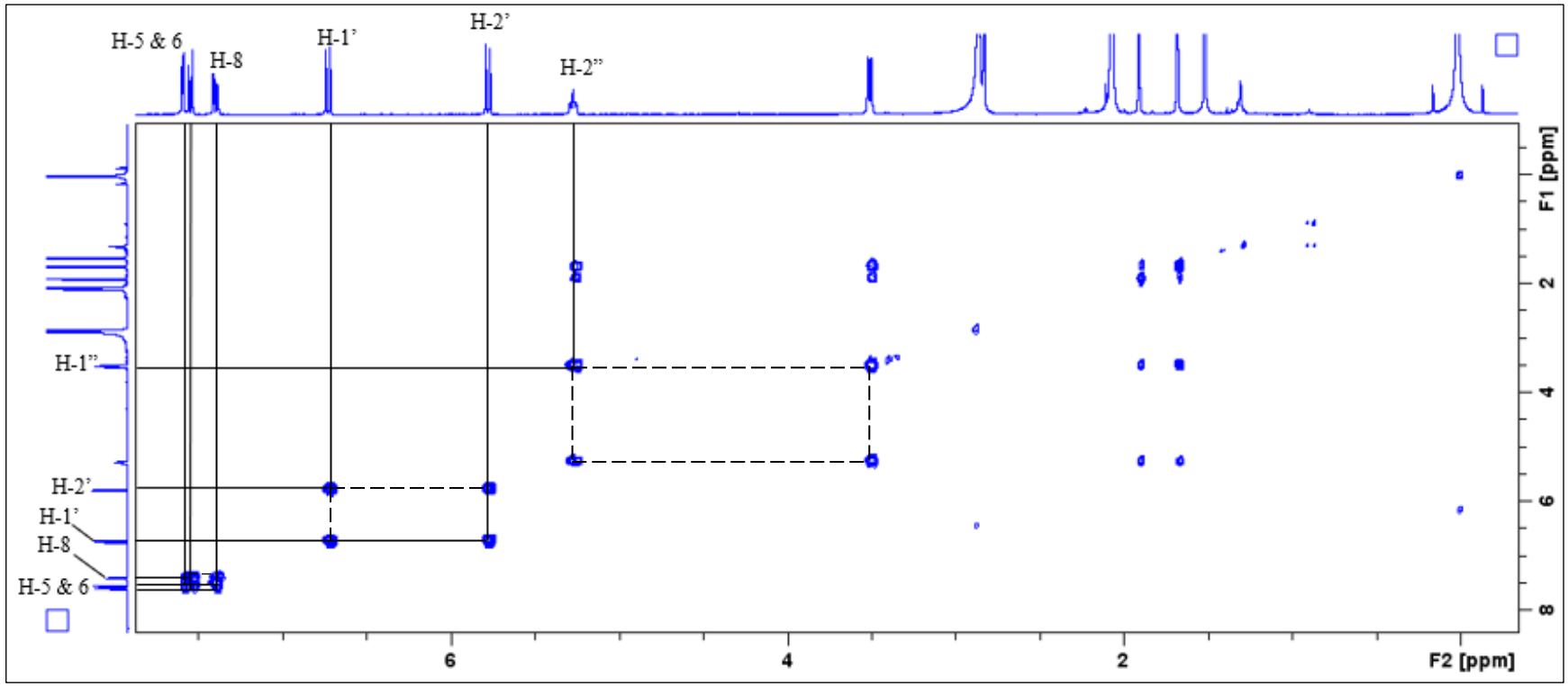


Figure 4.6 ^{1}H COSY spectrum of brasixanthone B (**15)** (400 MHz, acetone-d_6).

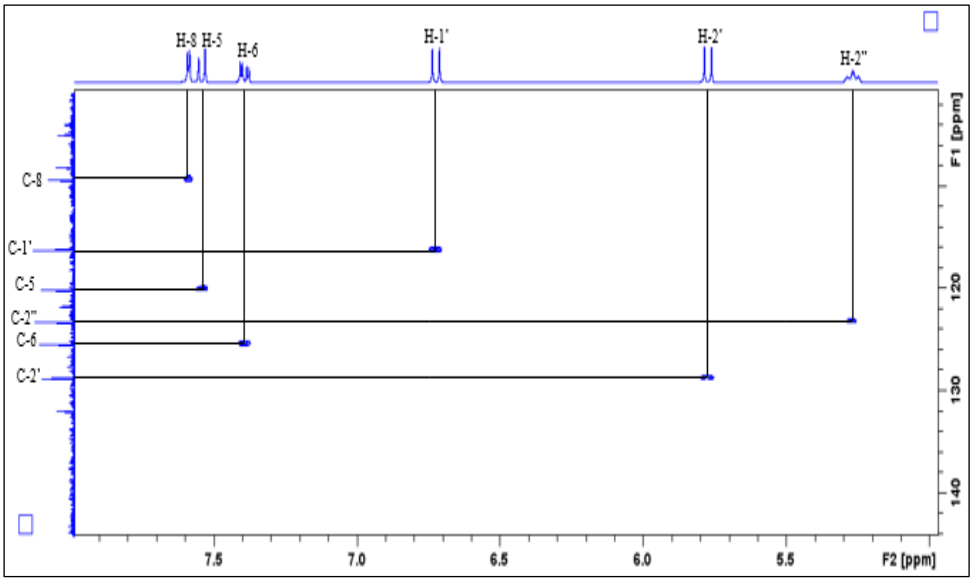


Figure 4.7 Expanded HSQC spectrum (5.0 – 8.0 ppm) of brasixanthone B (**15**) (400MHz, acetone-d_6).

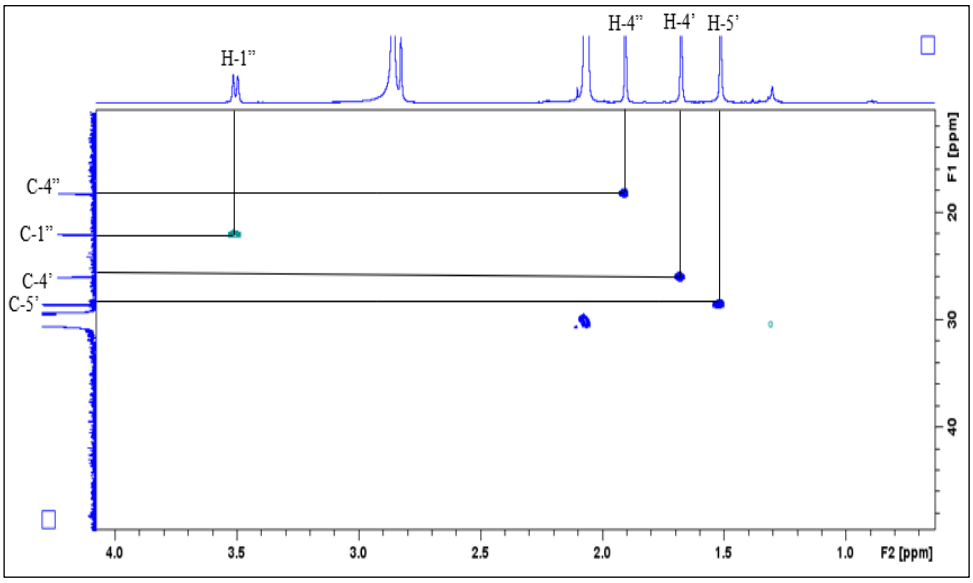


Figure 4.8 Expanded HSQC spectrum (1.5 – 3.5 ppm) of brasixanthone B (**15**) (400 MHz, acetone-d_6).

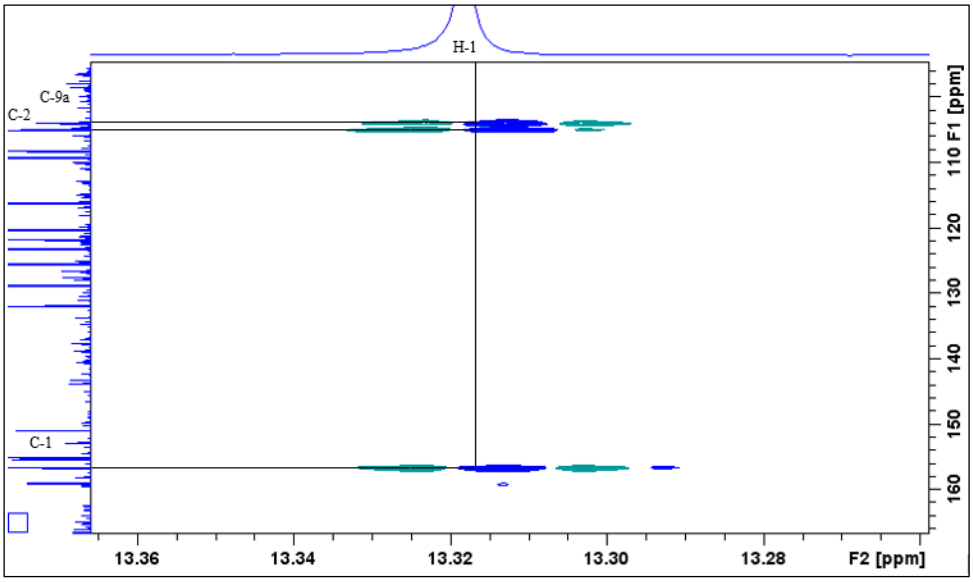


Figure 4.9 Expanded HMBC spectrum (13.28 – 13.36 ppm) of brasixanthone B (**15)** (400 MHz, acetone-d_6).

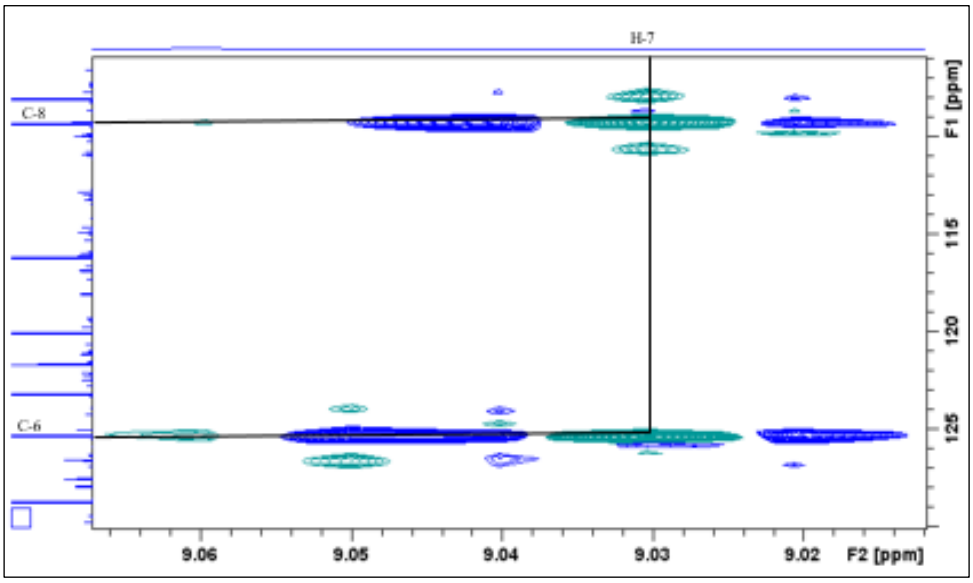


Figure 4.10 Expanded HMBC spectrum (9.0 – 9.07 ppm) of brasixanthone B **(15)** (400 MHz, acetone-d_6).

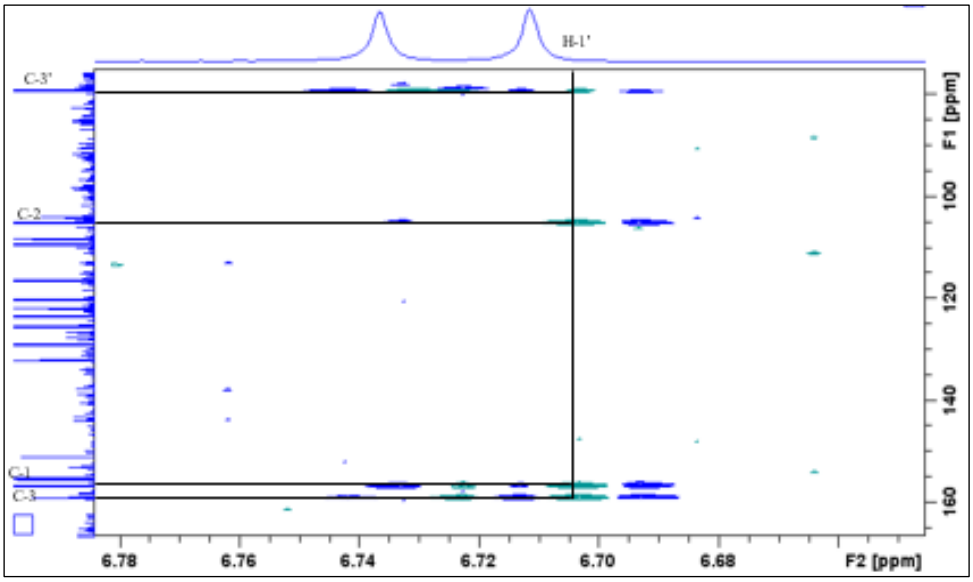


Figure 4.11 Expanded HMBC spectrum (6.67 – 6.78 ppm) of brasixanthone B (**15**) (400 MHz, acetone-d_6).

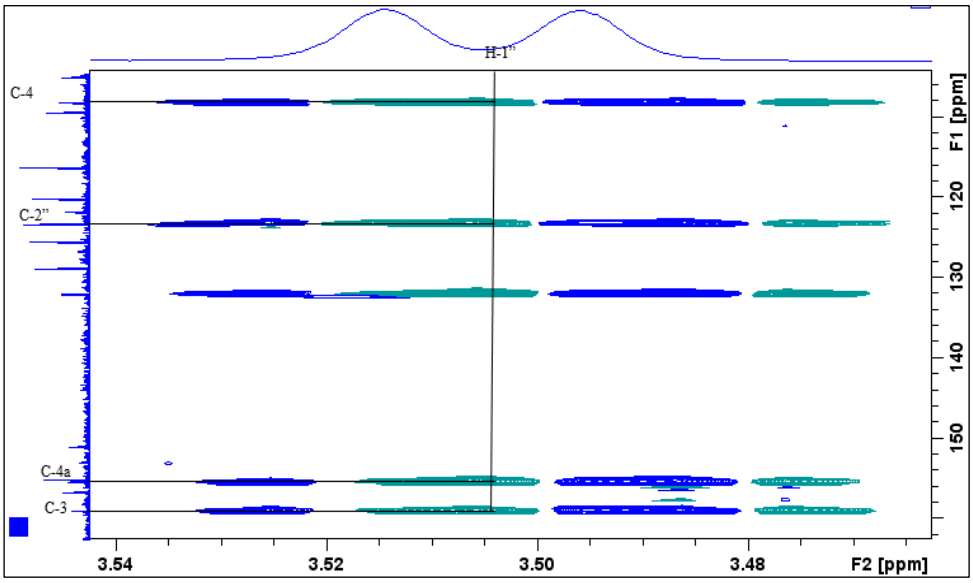


Figure 4.12 Expanded HMBC spectrum (3.46 – 3.54 ppm) of brasixanthone B (**15**) (400 MHz, acetone-d_6).

4.3 Antioxidant Test

4.3.1 Total Phenolics Content (TPC) and Total Flavonoid Content (TFC)

TPC and TFC activity are the methods to determine the amount of phenolic and flavonoid content in samples, respectively. Phenolic compounds are secondary metabolites found abundant in plants and food, while flavonoids are a part of phenolic compounds (Alkhamaiseh *et al*, 2012). Phenolic compounds have redox properties, allowing them to act as antioxidants (Johari and Khong, 2018). Table 4.3 shows the TPC and TFC of *C. gracilentum* in four different extracts; hexane, ethyl acetate, chloroform, and methanol. The TPC of the *C. gracilentum* extracts were 35.65, 48.67, 93.89, and 162.93 mg GAE/g for hexane, chloroform, ethyl acetate, and methanol, respectively. Meanwhile, the TFC results were 17.74, 20.81, 74.55, and 158.47 mg QE/g for hexane, chloroform, ethyl acetate, and methanolic extract, respectively. Based on the result, it is found that the methanolic extract of *C. gracilentum* exhibited the highest TPC and TFC. This may be because higher amounts of phenolics are often extracted in a more polar solvent like methanol than acetate, chloroform, and hexane.

Table 4.3 Total phenolic and flavonoid content of *C. gracilentum* hexane, ethyl acetate, chloroform, and methanolic extracts.

Fraction	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
Hexane	35.65±0.004	17.74±0.0057
Chloroform	48.67±0.0025	20.81±0.0015
Ethyl acetate	93.89±0.0033	74.55±0.0042
Methanol	162.93±0.042	158.47±0.014

4.3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The radical scavenging activity of crude extracts was evaluated by means of DPPH assay. DPPH is used due to its stability as a radical, giving purple colour and strong absorbance at 517 nm. DPPH is reduced upon reaction with antioxidant forming yellow-coloured anti-radical diphenylpicryl hydrazine (DPPH-H) which leads to a decrease in absorbance at 517 nm. The conversion of colour from purple to yellow indicated the scavenging potential of the antioxidants in the extracts in terms of hydrogen donating ability (Perera *et al*, 2016). IC_{50} refers to the concentration of sample required to inhibit 50% of DPPH radical activity. DPPH is reduced upon reaction with antioxidants leading to a decrease in absorbance at 517 nm. The radical scavenging activity was examined and compared with ascorbic acid as references. The DPPH assay was assessed by IC_{50} value where a lower IC_{50} value, indicates higher antioxidant activity. Figure 4.13 depicts the % inhibition rate versus concentration of samples, and Table 4.4 shows the IC_{50} values of the crude extracts obtained from the graph.

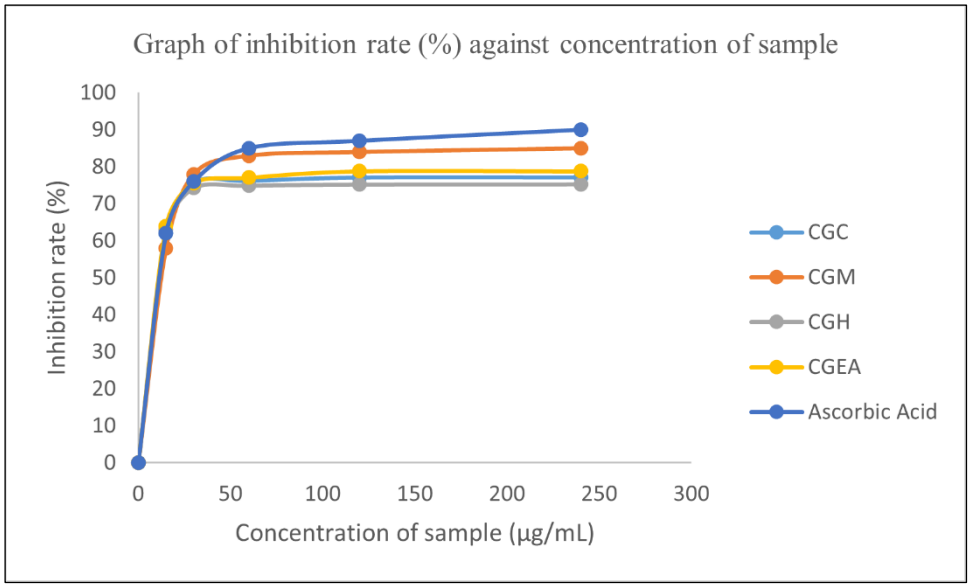


Figure 4.13 Inhibition rate (%) of free DPPH radicals against concentration of samples.

Table 4.4 Antioxidant result of test samples in DPPH assay.

Test sample	IC_{50} (µg/mL)
Positive control:	
Ascorbic acid	4.97±0.02
Crude extracts:	
Hexane	13.54±0.043
Chloroform	11.39±0.031
Ethyl acetate	8.10±0.027
Methanol	7.62±0.052

Based on the results, the hexane, chloroform, ethyl acetate, and methanol crude extracts of *C. gracilentum* gave moderate to strong antioxidant activities in DPPH radical scavenging test. In comparison with the positive control ascorbic acid (4.97 µg/mL), the methanol fraction showed comparable but less potent antioxidant activity, followed by ethyl acetate extract with an IC_{50} value of 7.62 µg/mL and 8.10 µg/mL accordingly. Meanwhile, the hexane and chloroform

fractions showed moderate antioxidant activity with IC_{50} values of 13.54 μg/mL and 11.39 μg/mL, respectively.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

In this study, a pure compound has been isolated and purified from the hexane crude extract of *Calophyllum gracilentum*. The chemical structure of the pure compound has been elucidated via spectroscopic methods, including LC-MS, ^{1}H NMR, ^{13}C NMR, and IR. The comparison made with literature has confirmed the isolated pure compound identified as brasixanthone B **(15)**. All the crude extracts (methanol, ethyl acetate, chloroform, and hexane) were studied for their antioxidant potential via TPC, TFC, and DPPH assay. To the best of our knowledge, there was no report available on the antioxidant activity of *C. gracilentum.* The IC_{50} value from the crude extracts showed moderate to strong antioxidant activity. The methanolic and ethyl acetate extract exhibited high TPC at 93.89 and 162.93 mg GAE/g, respectively, and TFC at 74.55 and 158.47 mg QE/g, respectively. The IC_{50} value of methanol fraction (7.62 μg/mL) showed comparable but less potent antioxidant activity with positive control ascorbic acid (4.97 μg/mL), followed by ethyl acetate extract (8.10 μg/mL). Therefore, the methanolic and ethyl acetate extracts have a higher free radical scavenging ability than other extracts. With the extracts having good antioxidant properties, they could be used for the potential development of antioxidant herbal formulations.

The amount of pure compound obtained was too little making the full run NMR spectrum and the antioxidant assay of the pure compound difficult to perform.

Therefore, a recommendation, more *C. gracilentum* hexane extract (>10g) should be isolated to yield a higher amount of compounds. Next, isomeric compounds can be difficult to isolate using only column chromatography and radial chromatography. The similar retention time produced by the isomeric compounds causes overlapping spots on TLC. Thus, it is suggested to use more advanced chromatographic techniques such as High-Performance Liquid Chromatography (HPLC) to improve the separation efficiency due to its rapid separation and high-resolution feature. Finally, it is recommended for the crude extracts in this study to be subjected to further evaluation for other biological activities such as cytotoxic and antimicrobial activities.

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