PHYTOCHEMISTRY OF *Calophyllum havilandii* P.F. Stevens AND *Calophyllum lowei* Planch. & Triana AND THEIR BIOLOGICAL ACTIVITIES

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UNIVERSITI SAINS MALAYSIA

2024

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

July 2024

ACKNOWLEDGEMENT

First of all, I would like to express my humblest gratitude and praise to Allah SWT for his generosity and blessings throughout the journey of my postgraduate studies and my overall life. I would also like to take this opportunity to express my utmost appreciation to my main supervisor, Dr. Thiruventhan Karunakaran, for his unwavering dedication in being the best mentor in teaching, advising, guiding, as well as providing me with helpful constructive criticism throughout my entire Master's journey. He has given me his entire support in making me understand each aspect of this research. Without his assistance and guidance, I could not have finished this thesis. My deepest gratitude also goes to both my co-supervisors, Assoc. Prof. Dr. Vivien Jong Yi Mian and Dr. Hafizi Abu Bakar, as well as Dr. Rameshkumar Santhanam and Dr. Amira Suriaty Yaakop for their steadfast support and guidance in terms of materials, advice, as well as continuous assistance in completing my research. Also not to forget, I would like to express my sincerest appreciation to former CDR Director, Prof. Dr. Vicknasingam Kasinather and current CDR director, Prof. Vikneswaran Murugaiyah for giving me the opportunity to successfully conduct my research at CDR. Also, I would like to express my appreciation to the science officers and laboratory officers here at CDR, Mr. Hilman and Mr. Razak for their help in assisting me with various laboratory matters. I would also like to thank Institute for Postgraduate Studies (IPS) for providing me with GRA-Assist funding during my first two semesters of my studies. Finally, a special gratitude and utmost appreciation to my mother, Dr. Siti Haliza Asat for being the source of my constant inspiration to persevere in the face of numerous obstacles in life. I promise to you and myself that I'll keep moving forward and make you proud. Thank you from the bottom of my heart.

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β	Beta
δ	Chemical shift in ppm
δ_H	Chemical shift of proton in ppm
δ_C	Chemical shift of carbon in ppm
λ	Wavelength
λ_{max}	Wavelength maxima in nm
ν_{max}	Wavenumber maxima in cm^{-1}
g	Gram
L	Litre
µg	Microgram
µL	Microlitre
µM	Micromolar
$[M]^+$	Molecular ion
$[M+H]^+$	Protonated molecular ion
m/z	Mass to charge ratio
°C	Degree Celsius
%	Percentage
^1H	Proton
^{13}C	Carbon-13
1D-NMR	One-Dimensional Nuclear Magnetic Resonance
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
d	Doublet
m	Multiplet
q	Quartet
s	Singlet
t	Triplet
dd	Doublet of doublet
J	Coupling constant in Hertz
cm	Centimetre
cm^{-1}	Reciprocal centimetre (wavenumber)
nm	Nanometre
kg	Kilogram
mg	Milligram
mL	Millilitre
ppm	Parts per million
Hz	Hertz
MHA	Mueller-Hinton Agar
MHz	Megahertz
Acetone-d_6	Deuterated acetone

AGE	Advanced Glycation End Products
APG	Angiosperm Phylogeny Group
ATCC	American Type Culture Collection
C. havilandii	*Calophyllum havilandii*
C. lowei	*Calophyllum lowei*
$CDCl_3$	Deuterated chloroform
CC	Column chromatography
COSY	Correlation Spectroscopy
COVID-19	Coronavirus Disease
DAD	Diode Array Detector
DEPT	Distortionless Enhancement by Polarization Transfer
DMAPP	Dimethylallyl Diphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EIMS	Electron Impact Mass Spectrometry
ESI	Electrospray Ionization
FTIR	Fourier-Transform Infrared Spectroscopy
FBS	Fetal bovine serum
GCC	Gravity column chromatography
GCMS	Gas Chromatography-Mass Spectrometry
GPS	Global Positioning System
HEP-G2	Cancer Human Liver Cell Line
HL-7702	Normal Human Liver Cell Line
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HTS	High-Throughput Screening
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Coherence
HRESIMS	High Resolution Electrospray Ionization Mass Spectrometry
IC_{50}	Half maximal inhibitory concentration
IPP	Isopentenyl Pyrophosphate
IR	Infrared
KBr	Potassium Bromide
Lit.	Literature
MIC	Minimum Inhibitory Concentration
MEM	Eagle's Minimum Essential Media
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear Magnetic Resonance
NCE	New Chemical Entities

PBS	Phosphate Buffer Saline
QTOF	Quadrupole Time-of-Flight
RPM	Revolutions per minute
Rel. int.	Relative intensity
SAR	Structure-Activity Relationship
SD	Standard Deviation
TCM	Traditional Chinese Medicine
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
WHO	World Health Organization

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FITOKIMIA DARI *Calophyllum havilandii* P.F. Stevens DAN *Calophyllum lowei* Planch. & Triana DAN AKTIVITI BIOLOGINYA

ABSTRAK

Tumbuhan dari genus *Calophyllum* (Calophyllaceae) adalah sumber khazanah yang kaya dengan sebatian hasil semulajadi yang menarik dan mempunyai kepelbagaian struktur yang mengagumkan. Sebatian fitokimia ini sering ditemui sebagai terbitan kelas fenolik, seperti xanthon, kumarin, kromanon, floroglucinol, serta flavonoid. Kebanyakan fitokimia ini telah terbukti berkesan dalam menunjukkan pelbagai aktiviti farmakologi dan biologi, seperti sitotoksik, anti-virus, anti-radang dan anti-mikrob. Dalam kajian ini, proses pengekstrakan, pemencilan dan pengenalpastian sebatian fitokimia telah dijalankan keatas kulit batang kayu dari dua spesies endemik yang tergolong dalam genus *Calophyllum* Malaysia dari Sarawak, iaitu *Calophyllum havilandii* dan *Calophyllum lowei*. Kesemua sebatian fitokimia ini telah dikenalpasti dan ditentukan menggunakan pelbagai teknik spektroskopi yang merangkumi MS, IR, UV, dan NMR. Ekstrak dan fitokimia terpilih dari kedua-dua spesies *Calophyllum* kemudian dinilai secara biologikal menggunakan ujian sitotoksik terhadap sel HL-7702 serta ujian anti-*bacillus* terhadap empat strain *bacillus* (*B. cereus, B. endophyticus*, *B. tropicus,* and *B. subtilis*). Proses pemencilan dan penulenan yang dilakukan keatas ekstrak *C. havilandii* berjaya menghasilkan satu terbitan 4-propildihidrokumarin baharu iaitu havilarin **(158),** bersama lima sebatian lain yang diketahui, iaitu asid kalolongik **(117)**, asid isokalolongik **(120)**, asid kaloteysmannik **(116)**, euxanthon **(17)**, dan β-sitosterol **(138)**, manakala kajian ke atas ekstrak *C. lowei* menghasilkan lima sebatian diketahui yang dikenalpasti sebagai trapezifolixanthon **(37)**, ananixanthon **(1)**, kaloxanthon C **(10)**, β-amirin **(159)**, dan stigmast-4-en-3-on

(160). Ujian sitotoksisiti keatas kesemua ekstrak dan sebatian fitokimia dari *C. havilandii* telah menunjukkan aktiviti tiada kesan sitotoksik terhadap sel HL-7702 dalam julat kepekatan antara 3.125 – 200 μg/mL, manakala untuk *C. lowei*, hanya ekstrak *n*-heksana, sebatian trapezifolixanthon **(37)**, β-amirin **(159)**, dan stigmast-4-en-3-on **(160)** menunjukkan aktiviti sitotoksik yang lemah terhadap sel yang sama (< 70% daya maju sel berdasarkan ISO-190993-5). Tambahan pula, penilaian anti-*bacillus* oleh ekstrak dan sebatian fitokimia terpilih dari *C. havilandii* menunjukkan hanya ekstrak kloroform memberi aktiviti yang menjanjikan terhadap *B. endophyticus* dengan nilai MIC 0.5 μg/mL. Dalam pengetahuan kami, kajian ini adalah antara penyelidikan terawal berkaitan kajian fitokimia dan biologikal terhadap dua spesies *Calophyllum* dari Malaysia yang kurang diterokai, iaitu *C. havilandii* dan *C. lowei*, di mana kajian ini menandakan kepentingan laporan fitokimia yang pertama dari *C. havilandii*. Oleh itu, ini adalah penyelidikan terawal berkaitan kajian fitokimia dan biologikal yang melibatkan dua spesies *Calophyllum* dari Sarawak yang boleh memberikan gambaran bernilai tentang potensi hasil semulajadi tersebut dalam aplikasi terapeutik atau klinikal pada masa hadapan.

PHYTOCHEMISTRY OF *Calophyllum havilandii* P.F. Stevens AND *Calophyllum lowei* Planch. & Triana AND THEIR BIOLOGICAL ACTIVITIES

ABSTRACT

Plants from the genus *Calophyllum* (Calophyllaceae) is a treasure trove of various interesting phytochemicals with impressive structural diversity. These phytochemical constituents were often discovered as derivatives in the classes of phenolics, such as xanthones, coumarins, chromanones, phloroglucinols, and flavonoids. Many of them are proven to exhibit wide range of pharmacological as well as biological activities, such as cytotoxic, anti-viral, anti-inflammatory, and anti-microbial. In this study, the process of extraction, isolation, and characterization of phytochemicals were conducted on the stem bark of two endemic Malaysian *Calophyllum* species from Sarawak, namely *Calophyllum havilandii* and *Calophyllum lowei*. All of the isolated phytochemicals were characterized and elucidated using extensive spectroscopic techniques such as MS, IR, UV, and NMR. The selected extracts and phytochemicals from both plant species of *Calophyllum* were then subjected to biological evaluation using cytotoxicity assay against HL-7702 cell line as well as anti-*bacillus* assay against four strains of *bacillus* (*B. cereus, B. endophyticus*, *B. tropicus,* and *B. subtilis*). Isolation and purification process performed on the extracts of *C. havilandii* successfully yielded one new 4-propyldihydrocoumarin derivative, namely havilarin **(158)** together with five other known phytochemicals, calolongic acid **(117)**, isocalolongic acid **(120)**, caloteysmannic acid **(116)**, euxanthone **(17)**, and β-sitosterol **(138)**, whereas investigation on the extracts of *C. lowei* yielded five known phytochemicals which were identified as trapezifolixanthone **(37)**, ananixanthone **(1)**, caloxanthone C **(10)**,

β-amyrin **(159)**, and stigmast-4-en-3-one **(160)**. The cytotoxicity assay results indicated that all the tested extracts and phytochemicals from *C. havilandii* displayed non-cytotoxic activity against HL-7702 cell line in the concentration range between 3.125 – 200 μg/mL, whereas for *C. lowei*, only the *n*-hexane extract, trapezifolixanthone **(37)**, β-amyrin **(159)**, and stigmast-4-en-3-one **(160)** exhibited weak cytotoxicity against similar cell line (< 70% cell viability based on ISO-190993-5). Besides, anti-*bacillus* evaluation of selected extracts and phytochemicals from *C. havilandii* indicated that only the chloroform extract showed promising activities against *B. endophyticus* with MIC value of 0.5 μg/mL. To the best of our knowledge, this investigation represents one of the earliest studies pertaining to the phytochemical and biological works from the two underexplored Malaysian species of *Calophyllum*, *C. havilandii* and *C. lowei*, in which this study significantly denotes the first phytochemical report from *C. havilandii.* Hence, this study presents the preliminary research for phytochemical and biological studies involving both species of *Calophyllum* from Sarawak that can provide valuable insights on their potential therapeutic or clinical applications in near future.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Plants have shaped our planet Earth to an amazing extent. It is said that plants are one of the most successful organisms that thrive on Earth. This is based on the fact that plants (predominantly terrestrial) take up the majority of 80 percent of total Earth's biomass, which is then followed by bacteria in a distant second at 15 percent (Bar-On et al., 2018). Up until now, researchers have deduced that this unwavering success is due to the distinct abilities of many plant species to generate 'unique' chemicals that maximize their chances of survivability (Yang et al., 2018). These 'unique' chemicals differ from primary metabolites — molecules that are essential for every living organism, such as carbohydrates, proteins, nucleic acids, and lipids (Canarini et al., 2019; Zaynab et al., 2019). In contrast, these 'unique' chemicals, often regarded as secondary metabolites or phytochemicals, are exclusive to a particular species (Rosan, 2008). These specialized (secondary) metabolites are usually of limited occurrence in nature, in contrast to primary metabolites, which are generated by metabolic pathways that are almost common to every organism.

Interestingly, plants are the best chemical factories. Although being a sessile organism, plants were able to collectively generate wide range of structurally and functionally diverse secondary metabolites, which play an important role in plant resistance and adaptation to an ever-changing environment, as well as tolerance towards biotic and abiotic stresses (Dixon, 2003; Maeda, 2019; Wang et al., 2019a). It is reported that the plant kingdom was able to generate nearly one million metabolites, whose structure, function, and utility have only been partially explored (Afendi et al.,

2012). These specialized (secondary) metabolites were synthesised or derived from a few key intermediates or precursors in the primary metabolism. Although primary metabolites are essentially accumulated in plant cells, these secondary metabolites are only detected within particular tissues and organs, at certain developmental stages, and under specific environmental conditions (Sulpice & McKeown, 2015; Fang et al., 2019).

Secondary metabolites are produced from biosynthetic key intermediates (like acetyl coenzyme A, shikimic acid, mevalonic acid, and 1-deoxyxylulose-5-phosphate) of the most basic metabolic processes, such as photosynthesis, glycolysis, and Krebs cycle, after modification through numerous different mechanisms and reactions (e.g., alkylation, decarboxylation, aldol, Claisen, and Schiff base formation) that allow to produce an endless possibilities of unique secondary metabolites from a limited number of intermediates (Rosan, 2008; Bernardini et al., 2018). This metabolite diversity is, to a large extent, due to chemical modification of the basic skeletons of metabolites.

According to literature, there are numerous different classification methods to categorize the enormous chemodiversity of natural products. One of them is by following a rule: all secondary metabolites (not including alkaloids) are classified according to their biosynthetic origin (Heinrich et al., 2017). This rule is composed of grouping them according to the resulting final products of similar biosynthetic pathways. Some of the important classes of natural products were broadly classified as phenolics, alkaloids, polyketides, terpenoids, glycosides, and sulphur-containing compounds (Gershenzon et al., 2012; Heinrich et al., 2017; Guerriero et al., 2018).

While plants manufacture secondary metabolites to deter any interference to their growth and survivability, these metabolites are also known to be beneficial for human use (Facchini et al., 2012). Throughout history, there have been many utilizations of herbs with specialised metabolites, such as for fragrances, flavourings, pigments, insecticides, and therapeutics (Rai et al., 2017). Since then, plant natural products have found their way to assimilate into human's daily lives. For centuries, natural products have been used exclusively as therapeutic agents to treat various ailments and still today continue to be the most prominent source of new potential therapeutic preparations (Dias et al., 2012). In addition, a survey by Newman and Cragg (2020) pointed to the fact that many drugs on the market are either of natural origin or derived from them (Lautié et al., 2020). Famous instances of therapeutic use of natural products such as morphine (*Papaver somniferum*), Taxol (*Taxus brevifolia*), and quinine (*Cinchona spp.*) have found their way as an effective drug treatment to treat various debilitating diseases (Dzobo, 2022). In the year 2015, the scientific breakthrough for the therapeutic effects of natural products was reinvigorated globally. Prof. Youyou Tu was honoured with Nobel Prize in Physiology or Medicine for her discovery and development of artemisinin, one of the revolutionary antimalarial drugs, as well as to Prof. William C. Campbell and Prof. Satoshi Ōmura for the development of fungal metabolites avermectins isolated from *Streptomyces avermitilis* (Van Voorhis et al., 2015; Tu, 2016).

The remarkable complexity and molecular diversity of natural products have humbled to remind us that our knowledge on the untapped potential of natural products is still limited. This reflects the fact that the surface of most natural resources has barely been scratched. Despite the extensive investigation conducted on approximately 300,000 species of higher terrestrial flora globally, only about 15% have been

systematically examined phytochemically, and only 6% have been investigated pharmacologically (Newman & Cragg, 2020).

Given these findings in the context of Southeast Asian countries, Malaysia is among the world's 12 megadiverse countries with the oldest and richest floral diversities of all tropical Asia, with exceptional and endemic species that have been recently discovered (Abu Bakar et al., 2018; Hamidah et al., 2020). According to Saw et al. (2010), Malaysia has approximately 15,000 species of vascular plants, with 8,300 species in Peninsular Malaysia and about 12,000 species in Sabah and Sarawak. Among these figures, Malaysia was also endowed with more than 2,000 species of medicinal plants among its over 15,000 flowering plants (Abu Bakar et al., 2018; Idris Adewale, 2021). This huge biodiversity of Malaysian flora indicates that there is a varied complexity of interesting natural products awaiting to be discovered. But despite its long history of botanical explorations, the current state of Malaysia's flora has not yet been fully documented.

Therefore, this research aimed to investigate the phytochemistry from the stem bark of two indigenous plant species discovered in the tropical rainforest of Borneo, specifically in the Sarawak region of East Malaysia, namely *C. havilandii* and *C. lowei*. The selected extracts as well as the phytochemicals isolated from both plant species were subsequently subjected to biological evaluation to assess their possible cytotoxic and anti-*bacillus* activities.

1.2 Problem Statement

The genus *Calophyllum*, which belongs to the family Calophyllaceae is one of the largest genus of tropical evergreen trees that is endemic to Malaysia, however, some of them remained to be underexplored and unresolved (Gupta & Gupta, 2020). Despite that, the genus was of great interest to researchers due to previously discovered anti-HIV coumarin, calanolide A **(56)** from the stem bark of *Calophyllum lanigenum* in 1992 (Kashman et al., 1992). Since then, a great diversity of interesting bioactive secondary metabolites has been isolated from many species in the genus, which consists of mainly phenolic compounds in the classes of xanthones, coumarins, chromanones, and phloroglucinols (Taher et al., 2021; Lizazman et al., 2022a). Despite their extensive history of botanical and phytochemical studies, there is still insufficient amount of previous literature pertaining to phytochemical investigations conducted on two species of *Calophyllum* utilized in this study, *C. havilandii* and *C. lowei*, in which this will be the first report concerning on the phytochemical study from *C. havilandii*. Besides, both *Calophyllum* plants were regarded as an underexplored endemic plant species from Sarawak origin which potentially contains bioactive phytochemicals. Hence, there is a necessity to address the need in filling up the gap of knowledge on the phytochemical constituents that were present from these two species of *Calophyllum* to supply the demand of prospective phytochemical database for the development of new therapeutic agents against various infectious and non-infectious diseases.

1.3 Objectives

The aim of this research is to discover and identify the phytochemicals from the stem bark of two endemic Malaysian *Calophyllum* species, *Calophyllum havilandii* (*C.*

havilandii) and *Calophyllum lowei* (*C. lowei*) as well as to assess their possible biological activities. Hence, the specific objectives of this research are as follows:

(a) To extract, isolate, and purify the phytochemicals using various chromatographic techniques from the stem bark of two Malaysian *Calophyllum* species, namely *C. havilandii* and *C. lowei*.

(b) To elucidate and characterize the chemical structures of the isolated phytochemicals using various spectroscopic techniques such as MS, IR, UV, as well as 1D and 2D NMR.

(c) To evaluate the cytotoxic and anti-*bacillus* activities of selected extracts and isolated phytochemicals from both *Calophyllum* species against normal human hepatic cell line (HL-7702) as well as four strains of *bacillus* (*B. cereus, B. endophyticus, B. tropicus,* and *B. subtilis*).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Genus *Calophyllum*

Calophyllum, as uniquely named by the botanist Carl Linnaeus has its etymology originated from the Greek word καλός (kalos, "beautiful") and φύλλον (phyllon, "leaf"), which collectively mean 'beautiful leaf' (Damon, 2016; Susanto et al., 2019). Genus *Calophyllum* is generally a large genus of evergreen trees that extends across all pantropical continents, in which most of the discovered species were found predominantly in the warm, tropical region of Southeast Asia (Stevens, 1980; Karunakaran et al., 2022). The genus was considered the largest genus in Calophyllaceae family as it comprises about 180 to 200 species of flowering trees being found worldwide, whereas in Malaysia alone, about 45 different species of *Calophyllum* have been successfully identified (Wang et al., 2018). The genus has many vernacular names according to its origins. To name a few, in Malaysia it is commonly called as "*bintangor*" or "*penaga*", while in some parts of Madagascar it seemed to be known as "*vintanina*" (Damon, 2016). However, there seem to exist many other vernacular names to indicate distinct species of *Calophyllum* based on their regional variation and differences in appearance (Damon, 2016).

Up until now, the genus *Calophyllum* has so far received enormous attention from the scientific community due to the astounding therapeutic potential it possesses (Gupta & Gupta, 2020). The genus is well known for its medicinal uses and has been traditionally used for the treatment of various potentially chronic diseases such as

ulcers, eye infections, haemorrhoids, inflammation, malaria, and certain venereal diseases (Gupta & Gupta, 2020).

2.2 Taxonomic Description of Genus *Calophyllum*

Genus *Calophyllum* which was classified in the Calophyllaceae family, was included in the order Malpighiales and phylum Magnoliophyta. Plants from the order Malpighiales are highly diverse, comprising nearly 16,000 species, corresponding to about 7.8% of eudicot diversity and they have been one of the most important components of lowland tropical rainforests since ancient times (Davis et al., 2005; Díaz, 2013; Khan et al., 2017; Trad et al., 2021). The order encompasses approximately 36 families of plants, including Calophyllaceae, with about 12 genera and approximately 437 species occurring in both the Old and New World (The Angiosperm Phylogeny, 2009; Trad et al., 2021). Meanwhile in the family Calophyllaceae, the genus *Calophyllum* was regarded as the largest genus amongst all species in the family, with approximately 200 species being found worldwide (Khan et al., 2017).

Previously, this genus had been categorized under the Clusiaceae (Guttiferae) family, until recent phylogenetic studies based on 13 genes indicated that Clusiaceae is paraphyletic and Calophyllaceae (Clusiaceae subfamily Kielmeyeroideae) should be separated. The study conducted by the Angiosperm Phylogeny Group (APG) then decided that the genus *Calophyllum* was necessary to be included along with several other genera (E.g: Kayea Wall., Mammea L. and Mesua L.) from the Clusiaceae subfamily Kielmeyeroideae to the reinstated pantropical family Calophyllaceae J. Agardh (The Angiosperm Phylogeny Group, 2009; Wurdack et al., 2009; Gómez-Verjan et al., 2017). The genus was last known to be revised by Stevens in 1980 for

the Old World *Calophyllum*, which focuses more on the Indo-Malayan tropical region where the species were mainly distributed (Seah et al., 2019). **Figure 2.1** below shows the general taxonomic hierarchy for the genus *Calophyllum*. It is to be noted that some of the Malaysian *Calophyllum* species taxonomy remained disputed and unresolved. Further taxonomical investigation needed to be carried out to detail the classification of the respective species in this genus.

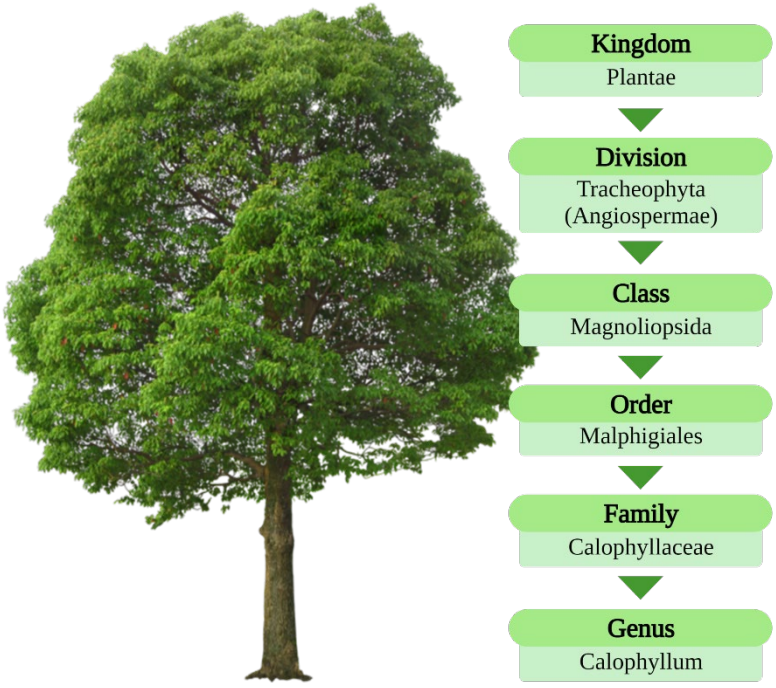

Figure 2.1 Taxonomic hierarchy of genus *Calophyllum*.

2.3 Botanical Description of Genus *Calophyllum*

Calophyllum is an easily identified genus with flowering evergreen trees or shrubs that exhibit characteristics such as low-branching, slow-growing, as well as a broad, irregular crown (Stevens, 1980; Stevens et al., 2007). The trees can grow up to 30 m without buttresses or root modifications (Tomlinson, 2016). However, knee roots, loop

roots, or pneumatophores are uncommon, which occurred only in swamp-dwelling species (Stevens, 1980). According to botanist Peter Stevens (1980), the thick, round to elliptical-shaped leaves with green semigloss coloration make the genus instantly recognizable even in the absence of fruits and flowers. Besides, the genus can be generally distinguished by the characteristics of the leaves which are decussate, oppositely arranged leathery blades borne on short petioles and narrow parallel veins that alternate with the resin canals (Díaz, 2013; Tomlinson, 2016). Trees from many species of *Calophyllum* possessed distinct, diamond to boat-shaped fissures on its bark, ranging from yellow to reddish hues depending on its age (Stevens, 1980). In addition, most of the species from the genus sometimes produce profuse and sticky sap from its bark, often have a clear to milky white or faint yellowish green coloration (Damon, 2016). The inflorescence was either terminal or axillary and could be up to 15 cm long with usually a raceme with five to 15 flowers, while the flowers are hermaphroditic and commonly whitish and sweet-scented, with numerous stamens as well as tetramerous with eight (to 13) tepals; two outer pairs were decussately arranged, with four inners in an imbricate whorl, little differentiated from the outer (Stevens, 1980; Tomlinson, 2016). Furthermore, the fruits are usually drupe when ripe, with thin flesh and large seed enclosed by a spongy layer as well as thin stony layer derived from the testa (Stevens, 1980).

It is worthy to note that some characteristics that make this genus taxonomically difficult and biologically unique are due to the infraspecific variation in tepal number and breeding systems it had. The tepal number not only varies between individuals of the same species, but also among flowers from the same inflorescence (Stevens, 1980). Additionally, several genus of *Calophyllum* in the Indo-Malayan region often, but not always, occupy a wide geographic range, but the amount of

morphological variation within the genus somewhat differs (Stevens, 1980). Some of them exhibit considerable morphological variation, which is partly correlated with their geographical ecology. **Figure 2.2** below depicts the general botanical morphology associated with the genus *Calophyllum*.

Figure 2.2 General botanical morphology of genus *Calophyllum*.

2.4 Geographical Distribution and Habitat of Genus *Calophyllum*

Out of approximately 187 species in the genus *Calophyllum* so far documented, 179 species are present in the Old World (predominantly in the Indo-Malaysian region), and the remaining are from the New World (from Mexico, to the Caribbean and Argentina) (Ramesh et al., 2012). The New World species may be derived from a single ancestor originally from the Old World region (Stevens, 1980). Fossil and pollen records from Java, Sumatera, and Assam suggest that the genus has been around since the Miocene period, which is approximately 23 million years ago (Damon, 2016). The distribution of the species and distinctive characters within the genus *Calophyllum*

follows the east-west division of Malesia, which spans from the Indomalayan to Australasia regions. As indicated in the report by P.F. Stevens (1980), species from genus *Calophyllum* were proposed to move from West to East Malesia as continental drift brought the Australian plate near Southeast Asia approximately 15 to 20 million years ago, which suggests that there may have been earlier floristic contact. Diversification of the genus in East Malesia has been a subsequent result of this movement, which specifically reflects the vast difference in morphology of some species.

In fact, the relationship between Malaysian *Calophyllum* flora with that of relatively distant Borneo, is not an uncommon pattern. It is reported that endemicity of the genus was relatively high in Borneo, less high within Malaysia (mainly in montane species), and low in Sumatra (Stevens, 1980). Most of the species that were found within Malaysia thrive from wet tropical rainforests to dry areas at higher altitudes (Stevens, 1980; Stevens et al., 2007). The species from this genus were basically restricted to humid, closed, lowland to colline, or sometimes montane rainforest, although a few species grow in drier or more open habitats. Besides, some of the species were also found in marshy areas (Stevens, 1980). A few Bornean species (e.g., *C. hosei*, *C. scriblitifolium*, and *C. sclerophyllum*) grow both in peat swamps and in leached, arid sands. Stevens (1980) reported that such distributions are rather common and reflect certain essential ecological similarities, such as extreme oligotrophy, in rather disparate environments. Additionally, several species thrive predominantly above 1300 meters. As might be expected, the species that grow at high altitudes tend to have rather short, broad, thick leaves that are not much pointed at the apex. Since most of these genus in the Malesia region seem to be independently derived from lowland species, they have little else in common; in which the species

growing at high altitudes are closely related to each other. Also, some species were found to thrive at specific habitats, such as near stream sides, in forests with acid soils, or sometimes over limestone or ultramafic rock (Stevens, 1980). The summary of several Malaysian *Calophyllum* species that were reported by the corresponding botanist and their distribution in the Malaysian region are tabulated in **Table 2.1**.

Table 2.1 Malaysian *Calophyllum* sp. and its distribution in Malaysia.

Species	Botanist*	Distribution in Malaysia
C. andersonii	Peter Francis Stevens (P. F. Stevens)	Sarawak
C. benjaminum	Unresolved	-
C. buxifolium	Julien Joseph Vesque (Vesque)	Sarawak
C. canum	Joseph Dalton Hooker ex Thomas Anderson (Hook. f. ex T. Anderson)	Kedah, Pulau Pinang, Perak, Selangor, Negeri Sembilan, Melaka, Kelantan, Terengganu, Pahang, Sabah, and Sarawak
C. castaneum	Peter Francis Stevens (P.F. Stevens)	Sarawak
C. depressinervosum	Murray Ross Henderson & John Wyatt-Smith (M. R. Hend. & Wyatt-Sm.)	Kedah, Pulau Pinang, Selangor, Negeri Sembilan, Kelantan, Terengganu, Pahang, Johor, Sabah, and Sarawak
C. dispar	Peter Francis Stevens (P. F. Stevens)	Terengganu, Pahang, and Sabah
C. ferrugineum	Henry Nicholas Ridley (Ridl.)	Perak, Selangor, Pahang, Negeri Sembilan, Melaka, Kelantan, Terengganu, Johor, Sabah, and Sarawak
C. flavoramulum	Murray Ross Henderson & John Wyatt-Smith (M. R. Hend. & Wyatt-Sm.)	Terengganu
C. gracilentum	Unresolved	-
C. gracilipes	Elmer Drew Merrill (Merr.)	Sabah and Sarawak
C. hosei	Henry Nicholas Ridley (Ridl.)	Sarawak
C. incrassatum	Murray Ross Henderson & John Wyatt-Smith (M. R. Hend. & Wyatt-Sm.)	Selangor, Melaka, Kelantan, Terengganu, Pahang, Johor, Sabah, and Sarawak
C. inophylloide	George King (King)	Kelantan, Terengganu, Johor, Sabah, and Sarawak
C. inophyllum	Carl Linnaeus (Linn.)	Kedah, Pulau Pinang, Perak, Selangor, Negeri Sembilan, Melaka, Kelantan, Pahang, Johor, Sabah, and Sarawak
C. javanicum	Friedrich Anton Wilhelm Miquel (Miq.)	Sabah
C. lanigerum	Friedrich Anton Wilhelm Miquel (Miq.)	Johor and Sarawak
C. lowii	Joseph Dalton Hooker (Hook. f.)	Sarawak

* The respective name of the botanists that identified the plant species were reported in 1980 by Stevens.

Table 2.1 Malaysian *Calophyllum* sp. and its distribution in Malaysia. (continued)

Species	Botanist*	Distribution in Malaysia
C. macrocarpum	Joseph Dalton Hooker (Hook. f.)	Kedah, Perak, Selangor, Negeri Sembilan, Kelantan, Terengganu, Pahang, Johor, Sabah, and Sarawak
C. mucigerum	Unresolved	-
C. nodusum	Julien Joseph Vesque (Vesque)	Johor, Sabah, and Sarawak
C. rubiginosum	Murray Ross Henderson & John Wyatt-Smith (M. R.Hend. & Wyatt-Sm.)	Selangor, Negeri Sembilan, Pahang, Johor, Sabah, and Sarawak
C. sclerophyllum	Julien Joseph Vesque (Vesque)	Kedah, Kelantan, Terengganu, Pahang, Sabah, and Sarawak.
C. scriblitifolium	Murray Ross Henderson & John Wyatt-Smith (M. R.Hend. & Wyatt-Sm.)	Perak, Selangor, Johor, and Sarawak
C. soulattri	Nicolaas Laurens Burman (Burm. f.)	Perlis, Kedah, Pulau Pinang, Negeri Sembilan, Melaka, Kelantan, Pahang, Johor, Sabah, and Sarawak
C. symingtonianum	Murray Ross Henderson & John Wyatt-Smith (M. R.Hend. & Wyatt-Sm.)	Kedah, Kelantan, and Pahang
C. teysmannii	Friedrich Anton Wilhelm Miquel (Miq.)	Kelantan, Terengganu, Pahang, Johor, Sabah, and Sarawak.
C. venulosum	Heinrich Zollinger (Zoll.)	Perak, Pahang, Johor, Sabah, and Sarawak
C. wallichianum	Jules Émile Planchon & José Jerónimo Triana (Planch. & Triana)	Kedah, Pulau Pinang, Perak, Terengganu, Negeri Sembilan, and Kelantan.

* The respective name of botanists that identified the plant species were reported in 1980 by Stevens.

2.5 Ethnobotany and Ethnomedicinal Uses of Genus *Calophyllum*

Historically, trees from genus *Calophyllum* used to be an important timber trees before the recent emergence of industrial-scale logging in Southeast Asia (Damon, 2016). It was then economically exported for its utilization as decorative figures on flat-sawn boards and also for decorative purposes, such as furniture, flooring, door construction, veneer, and plywood (Burgess et al., 1966). Apart from that, there was also important evidence of local ethnobotanical and ethnomedicinal usage of *Calophyllum* parts, which was documented by various reports dated from the 1980s (Damon, 2016; Stevens, 1980).

A prominent example on ethnobotanical usage of *Calophyllum* parts was reported from the indigenous people of Kula Ring, the Iban people, which mainly use the *Calophyllum* branches as firewood, while the trunks were often used for construction, including masts for boats and ships, as well as houses (Stevens, 1980; Damon, 2016). Some species of *Calophyllum* (e.g.; *C. dryobalanoides*) yielded fragrant oils that were used by the locals as hair dressing, while the latex was commonly used for shampoo, and the tree bark was utilized to make small articles, such as pails, baskets, and partitions. Besides, the hard wood was used to make bows and implement handles, and the bast is given to horses to keep them in good condition (Stevens, 1980). Another notable ethnobotanical evidence can be observed by the utilization of various parts of *C. inophyllum* (commonly known as tamanu or bintangor laut) by the local aborigines, with the use of the coloured stem bark as dye for fish nets, whereas the oil obtained from the seeds was used as fuel for lightning purposes as well as to generate electricity, and the wood was used to construct ships (Stevens, 1980; Kainuma et al., 2016). Additionally, the latex of many *Calophyllum* species was

considered poisonous, which often mixed together with other ingredients to numb fish or exterminate rats (Kawamura et al., 2012).

Although *Calophyllum* was deemed to be significant for timber and other ethnobotanical uses, it also belongs in the plant order of long-recognized medicinal usage (Stevens, 1980). *Calophyllum* was known to be included in the order Malpighiales, the same order as *Salix alba*, *Hypericum perforatum*, and *Erythroxylum coca,* which were all famous for their therapeutic properties. Some previous findings reported various parts of the *Calophyllum* tree that describe its medicinal values, such as the stem bark, seeds, resin, and roots, which were traditionally used to treat various kinds of ailments, from mild to chronic diseases such as gastric ulcers, pain, tumours, infections, and inflammations (Gupta & Gupta, 2020). Some important examples of ethnomedicinal usage include the usage of stem bark from *C. ferrugineum,* which was used as a decoction for women who had just given birth, and various parts of *C. inophyllum*, such as the barks, fruits, and latex, which were utilized to treat various diseases, from skin diseases, wounds, eye ailments, rheumatism, inflammations, to haemorrhoids (Stevens, 1980; Kainuma et al., 2016). A detailed list of ethnomedicinal usage of several other Malaysian species of genus *Calophyllum* can also be referred to in **Appendix A** for further review.

2.6 Descriptions of *Calophyllum havilandii*

Calophyllum havilandii P.F. Stevens (*C. havilandii*) is a widely distributed species in Borneo, specifically in Northwest Borneo (Sabah, Sarawak, and Brunei) (**Figure 2.3**). Additionally, it is a species with characteristics of 18-27 meters tall tree, trunk without buttresses, possessing yellowish brown to black fissured outer bark, as well as yellow to colourless latex. Besides, it thrives mostly in peat swamps and at lower altitudes,

and was seen flowering in November, while the submature fruit was found to develop in April (Stevens, 1980). *C. havilandii* superficially resembles *C. sclerophyllum*, in which both have been confused due to near similarities in characteristics. Some of the differences that can be distinguished were in the dried specimens, in which *C. havilandii* leaf specimens were darker in colour than *C. sclerophyllum*. Another notable difference can be seen in the fruit of *C. havilandii*, which lacked the strong outer layer being compared to *C. sclerophyllum*, and air spaces developed under the skin of the fruit, making it deeply wrinkled. This contrasts with the smooth, darker coloured fruit of *C. sclerophyllum*. The wood of *C. havilandii* was often used by the locals as planks for construction.

2.7 Descriptions of *Calophyllum lowei*

Calophyllum lowei Planch. & Triana (*C. lowei*) (previously known as *Calophyllum lowii*) have its distribution spanning from Sumatera and its adjacent islands (specifically in Riau, Jambi and Bangka) to the islands of Borneo (specifically in Sarawak and Kalimantan) (**Figure 2.3**). It possessed common characteristics such as 20 meters tall tree, shiny oblongated leaves, with yellow transparent latex, which make it closely resembling to *C. canum*. Generally, *C. lowei* is larger in all botanical segments (except stamen) compared to *C. canum*. Some of the distinct values between the two species can be observed from the appearance of the inflorescence, in which *C. lowei* has rarely branched, congested flowers, which is in contrast with *C. canum*, which usually develops branching and non-congested flowers. Another feature to note between them is that the fruit of *C. lowei* has sharply pointed ends with a dense and wrinkled surface, while the fruit of *C. canum* is rounded with shallowly striated surface. *C. lowei* have its ecology that flourishes mostly in lowland rainforests, some

of which are frequently inundated periodically, as well as at an altitude below 60 meters. The locals use the wood for house building, while the latex is often mixed with coconut milk to be used medicinally as treatment for itching and skin infection (Stevens, 1980).

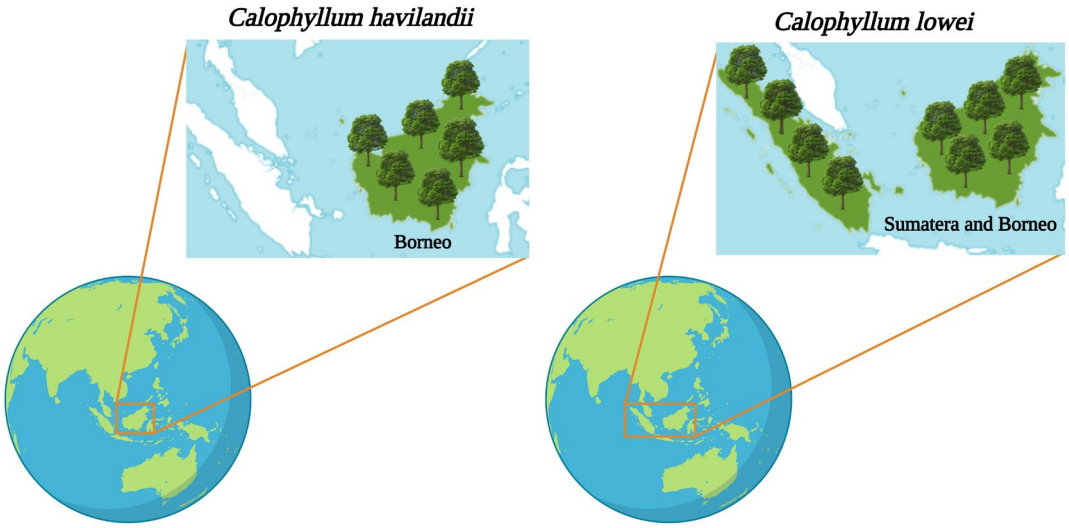

Figure 2.3 Geographical distribution of *C. havilandii* and *C. lowei*.

2.8 Phytochemistry of Genus *Calophyllum*

Genus *Calophyllum* is a treasure trove of interesting phytochemicals with diverse structural scaffold. The earliest phytochemical analyses of the genus *Calophyllum* were pioneered by Ormancey-Potier and Polonsky, in which they discovered and documented several phytochemicals from different parts of *Calophyllum* species (Ormancey-Potier et al., 1951; Polonsky, 1957). In context, these authors isolated and elucidated the chemical structures of calophyllolide **(62)**, inophylollide, and calophyllic acid from the leaves and fruits of *Calophyllum inophyllum,* in which up to later years until now, various other *Calophyllum* species along with other botanical parts (stem bark, roots, and resin) have been studied widely for their phytochemical content (Gómez-Verjan et al., 2017). These extensive phytochemical studies have led

to the identification of diverse important bioactive phytochemicals from the genus, revealing them to be a rich source of secondary metabolites prominently in the class of phenolics (specifically xanthones, coumarins, chromanones, phloroglucinols, and flavonoids) as well as terpenoids (Gupta & Gupta, 2020) (**Figure 2.4**). This phytochemical study mostly investigated and focused on the extraction, isolation, purification, and structural elucidation of the secondary metabolites isolated from various parts of *Calophyllum* species such as the stem bark, leaves, roots, fruits, latex, as well as the heartwood.

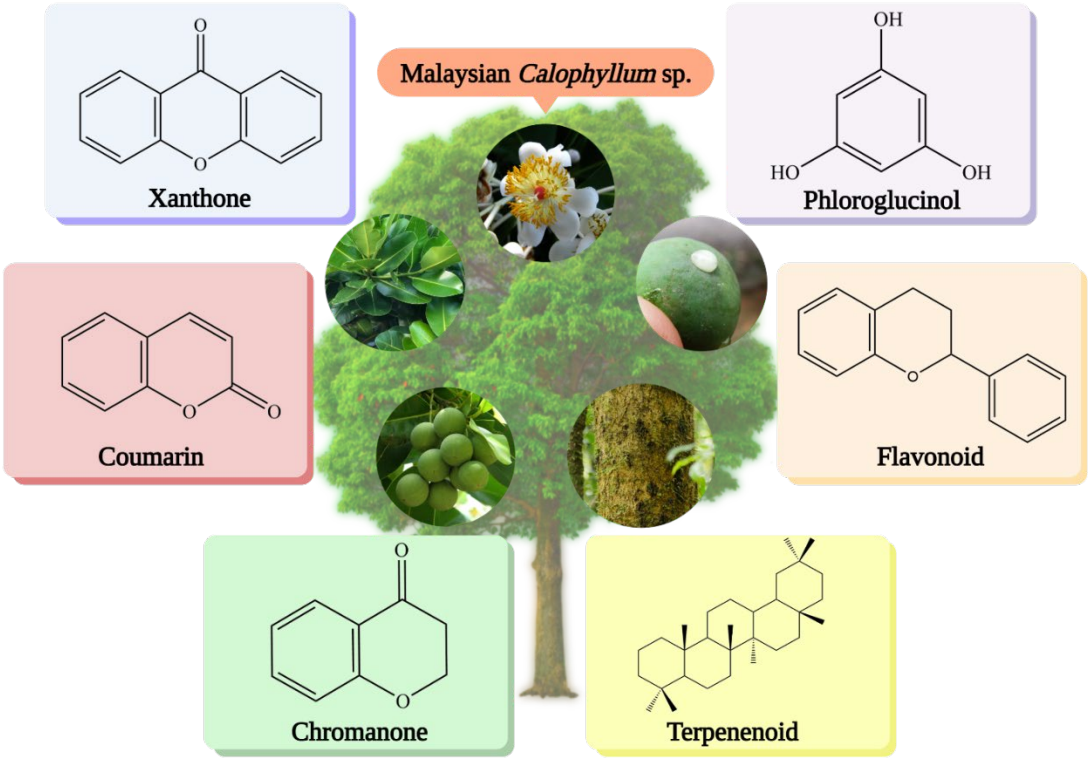

Figure 2.4 Summary of phytochemicals from Malaysian *Calophyllum* species.

2.8.1 Xanthones

Xanthones, or also known based on IUPAC nomenclature as 9*H*-Xanthen-9-one, are one of the important classes of phenolic compounds found in several restricted plant families such as Calophyllaceae, Clusiaceae, Gentianaceae, and Hypericaceae

(Tovilovic-Kovacevic et al., 2020). Xanthone has a basic molecular framework of 13 carbon atoms (C_6-C_1-C_6) in which it consists of two aromatic rings that are fused together by an oxygen atom and a carbonyl group. The two aromatic rings in the xanthone core were designated and numbered based on their biosynthetic foundation in higher plants. In the structure, the A-ring is acetate-derived (numbered 1–4), whereas the B-ring (numbered 5–8) is derived from the shikimate pathway, and both aromatic rings are connected by γ-pyrone to form the basic skeleton of xanthone (**Figure 2.5**). All the other carbon atoms are numbered according to IUPAC 2004 recommendations for structure elucidation purposes (Pinto et al., 2021). A recent review article by Remali et al. (2022) has comprehensively detailed all the intricacies of the biosynthetic mechanism of xanthones as well as its differences between plant species.

Xanthones isolated from natural sources (e.g.; plants or fungi) have various substituents attached to the ring, thus leading to structural diversity with a wide spectrum of biological as well as pharmacological activities (Sousa et al., 2005; Tovilovic-Kovacevic et al., 2020). Xanthones are mainly categorized into six subclasses, which is simple xanthones, prenylated xanthones, glycosylated xanthones, bisxanthones, xanthonolignoids, as well as miscellaneous xanthones (Remali et al., 2022). However, plants mostly generate xanthones in the first three subclasses, in which they are formed from two main core xanthone structures, 1,3,5-tetrahydroxyxanthone or 1,3,7-tetrahydroxyxanthone (El-Seedi et al., 2010; Remali et al., 2022). In genus *Calophyllum*, xanthone derivatives were found in many parts of the plant, especially in the stem bark, heartwood, leaves, as well as the roots.

Some of the prominent examples of xanthone derivatives that can be discovered from the Malaysian genus *Calophyllum* are prenylpyranoxanthone

derivatives, such as the constitutional isomers ananixanthone **(1)** and trapezifolixanthone **(37)**, which were previously isolated from many species of *Calophyllum,* especially *C. canum*, *C. macrocarpum*, and *C. hosei* (Lizazman et al., 2022a; Lim et al., 2016, Karunakaran et al., 2020). Besides, several other groups of xanthone derivatives, such as the commonly found simple 1,7-dihydroxyxanthone, euxanthone **(17)**, and a rare bisxanthone, biscaloxanthone **(3)**, were also previously isolated from *C. gracilentum* and *C. canum*, respectively (Lizazman et al., 2022a; Lim et al., 2019a). Thus, the list and chemical structures of various xanthone derivatives that were isolated from the several Malaysian *Calophyllum* species were tabulated and portrayed in **Table 2.2** and **Figure 2.6**, respectively.

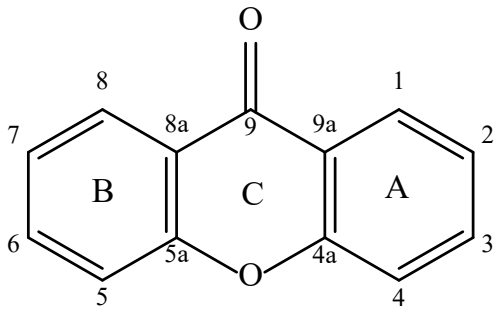

Figure 2.5 Structural framework of xanthone skeleton.

Table 2.2 Xanthones isolated from Malaysian *Calophyllum* sp.

Compound name	Species	Parts used	Reference
Ananixanthone **(1)**	*C. canum*	Stem bark	(Lizazman et al., 2022a; Lim et al., 2016)
	C. macrocarpum		(Karunakaran et al., 2020)
	C. hosei		(Daud et al., 2020)
	C. teysmannii		(Lee et al., 2018; Jamaluddin et al., 2013)
	C. buxifolium		(Daud et al., 2016)
	C. venulosum		(Ismail et al., 2015)
	C. lowii		(Jamaluddin et al., 2013)
Beta-mangostin **(2)**	*C. hosei*	Stem bark	(Daud et al., 2020)
	C. teysmannii		(Lee et al., 2018)
	C. benjaminum		(Sahimi et al., 2015)
	C. hosei		(Daud et al., 2014)
	C. lowii		(Jamaluddin et al., 2013)
	C. javanicum		(Sahimi et al., 2013)
Biscaloxanthone **(3)**	*C. canum*	Stem bark	(Taher et al., 2020)
Brasixanthone B **(4)**	*C. inophyllum*	Stem bark	(Mah et al., 2015)
	C. soulattri		
Brasilixanthone **(5)**	*C. inophyllum*	Stem bark	(Ee et al., 2009)
Brasilixanthone B **(6)**	*C. inophyllum*	Stem bark	(Ee et al., 2004a)
Buxixanthone **(7)**	*C. buxifolium*	Stem bark	(Daud et al., 2016)
Caloxanthone A **(8)**	*C. inophyllum*	Stem bark	(Lee et al., 2017)
	C. benjaminum		(Sahimi et al., 2015)
	C. inophyllum		(Lee et al., 2017; Ee et al., 2009)
Caloxanthone B **(9)**	*C. hosei*	Stem bark	(Daud et al., 2020)
	C. depressinervosum		(Zamakshshari et al., 2019)
	C. inophyllum		(Lee et al., 2017; Mah et al., 2011b; Ee et al., 2009)
	C. soulattri		(Mah et al., 2011a)

Table 2.2 Xanthones isolated from Malaysian *Calophyllum* sp. (continued)

Compound name	Species	Parts used	Reference
Caloxanthone C **(10)**	*C. canum*		(Lizazman et al., 2022a)
	C. andersonii		(Tee et al., 2018b)
	C. depressinervosum		(Zamakshshari et al., 2016)
	C. inophyllum	Stem bark	(Lee et al., 2017; Mah et al., 2015)
	C. soulattri		(Mah et al., 2011a)
	C. gracilipes		(Cao et al., 1997b)
Caloxanthone I **(11)**	*C. andersonii*	Stem bark	(Tee et al., 2018b)
	C. depressinervosum		(Zamakshshari et al., 2016)
	C. venulosum		(Ismail et al., 2015)
Caloxanthone J **(12)**	*C. depressinervosum*	Stem bark	(Zamakshshari et al., 2016)
Caloxanthone L **(13)**	*C. teysmannii*	Stem bark	(Jamaluddin et al., 2013)
	C. lowii		
Calozeyloxanthone **(14)**	*C. hosei*	Stem bark	(Daud et al., 2020)
Cudraxanthone C **(15)**	*C. mucigerum*	Stem bark	(Ee et al., 2004b)
Dombakinaxanthone **(16)**	*C. hosei*	Stem bark	(Daud et al., 2020)
	C. buxifolium		(Zamakshshari et al., 2019)
	C. benjaminum		(Sahimi et al., 2015)
	C. sclerophyllum		(Daud et al., 2013)
	C. javanicum		(Sahimi et al., 2013)
Euxanthone **(17)**	*C. canum*	Stem bark	(Lizazman et. al., 2022a; Lim et al., 2016)
	C. gracilentum		(Lim et al., 2019a)
	C. castaneum		(Lim et al., 2019b)
	C. andersonii		(Tee et al., 2018b)
	C. castaneum		(Lim et al., 2016)
Flavoramulone **(18)**	*C. flavoramulum*	Leaves	(Ferchichi et al., 2012)
Fuscaxanthone C **(19)**	*C. benjaminum*	Stem bark	(Sahimi et al., 2015)