PHYTOCHEMICAL STUDIES AND CYTOTOXICITY ASSESSMENT OF STEM BARK EXTRACTS FROM *Calophyllum macrocarpum* HOOK. F. AND *Calophyllum recurvatum* P. F. STEVENS

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by

NOOR SYARAFANA BINTI FIROUZ

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LIST OF SYMBOLS & ABBREVIATIONS

^{o}C	Degree Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
1D	1-Dimension
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D	2-Dimension
A. aegypti	*Aedes aegypti*
A. fumigatus	*Aspergillus fumigatus*
A. salina	*Artemia salina*
A549	Lung Carcinoma Epithelial cell
AChE	Acetylcholinesterase
APR III	Angiosperm Phylogeny Group III
AR	Analytical Reagent
B. cereus	*Bacillus cereus*
B. malayi	*Brugia malayi*
B. megaterium	*Bacillus megaterium*
B. pumilus	*Bacillus pumilus*
B. subtilis	*Bacillus subtilis*
BChE	Butyrylcholinesterase
C. difficile	*Clostridioides difficile*
C. macrocarpum	*Calophyllum macrocarpum*
C. perfringens	*Clostridium perfringens*
C. recurvatum	*Calophyllum recurvatum*
C	Carbon
C_2	Carbon-2
C_3	Carbon-3
C_4	Carbon-4
C-C	Carbon-carbon
CA, USA	California, United States of America

Caco-2	Immortalized Human Colorectal Adenocarcinoma cell
CC	Column Chromatography
CC_{50}	Concentration of Test Compound required to reduce cell viability by 50%
$CHCl_3$	Chloroform
CM-C	*C. macrocarpum* Chloroform extract
CM-EA	*C. macrocarpum* Ethyl acetate extract
CM-H	*C. macrocarpum n*-Hexane extract
CM-M	*C. macrocarpum* Methanol extract
CNE1	Human nasopharyngeal Carcinoma cell
CO	Carbonyl
COSY	Correlated Spectroscopy
COX	Cyclooxygenase
CO_2	Carbon dioxide
CR-C	*C. recurvatum* Chloroform extract
CR-EA	*C. recurvatum* Ethyl acetate extract
CR-H	*C. recurvatum n*-Hexane extract
CR-M	*C. recurvatum* Methanol extract
CYP2E1	Cytochrome P450 2E1 - member of the cytochrome P450 mixed-function oxidase system
CyQuant	A highly sensitive fluorescence-based method for quantifying cells and assessing cell proliferation and cytotoxicity
d	Doublet
dd	Doublet of doublet
DENV-2	Dengue Virus 2
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E. coli	*Escherichia coli*

ED_{50}	Dose of Medication that produces desired pharmacologic effect in 50% of the studied population that takes the medication
EIMS	Electron Ionization Mass Spectrometry
EMEM	Eagle's Minimum Essential Medium
EtOAc	Ethyl acetate
FBS	Fetal Bovine Serum
FTIR	Fourier-Transform Infrared Spectroscopy
g	Gram
G. trabeum	*Gloeophyllum trabeum*
GCMS	Gas Chromatography – Mass Spectrometry
H. pylori	*Helicobacter pylori*
H	Hydrogen
H37Rv	Mycobacterium tuberculosis strain
HBOC	Hereditary Breast and Ovarian Cancer
HCT-116	Human Colon Cancer cell
HEK-293	Human Embryonic Kidney Cell
HeLa	Henrietta Lacks Cervical Cancer Cell
Hela S-3	Clonal derivative of HeLa cell
Hep-G2	Immortalized Liver Hepatocellular Carcinoma cell
HIV	Human Immunodeficiency Virus
HIV-1 RT	HIV-1 Reverse Transcription
HK1	Hexokinase-1 enzyme
HL-60	Promyelocytic Human Leukimia cell
HMBC	Heteronuclear Multiple Bond Coherence Spectroscopy
HPLC	High-Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
HT-29	Human Colon Cancer cell
HTS	High-Throughput Screening
IC_{50}	The Half Maximal Inhibitory Concentration
IMR-32	Human Caucasian Neuroblastoma cell
IUPAC	International Union of Pure and Applied Chemistry
J774G8	Mouse Macrophages cell

K562	Human Chronic Myeloid Leukimia cell
KB	Human Epithelial Carcinoma cell
L. amazonensis	*Leishmania amazonensis*
L. infantum	*Leishmania infantum*
L. monocytogenes	*Listeria monocytogenes*
L1210	Mouse Lymphotic Leukimia cell
LOX	Lipooxygenase
LPS	Lipopolysaccharides
LS147T	Colorectal Tumor cell
LS174T	Human Colon Carcinoma cell
m	Multiplet
MCF-7	Human Breast Cancer cell
MD, USA	Maryland, United States of America
MDA-MB-231	Triple-negative Breast Cancer cell
mg	Milligram
MHz	Mega-Hertz
MIC	Lowest Concentration of Antibiotic at which bacterial growth completely inhibited
mL	Millilitre
mM	Millimolar
Molt-4	Human T Lymphoblast cell
MRC-5	Human Fetal Lung Fibroblast cell
MS	Mass Spectrometry
MTT	(3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay
NCI-H23	Human non-small cell lung carcinoma
NCI-H460	Hypotriploid human cell
nm	Nanometre
nMol	Nanomoles
NMR	Nuclear Magnetic Resonance
O	Oxygen
OH	Hydroxyl
P. aeruginosa	*Pseudomonas aeruginosa*

P. falciparum	*Plasmodium falciparum*
P. sanguineus	*Pycnoporus sanguineus*
P-388	Lymphoma cell
P-450	Cytochromes P450
PBS	Phosphate Buffered Saline
PC3	Human prostate cancer cell
ppm	Part per million
Raji cell	Human B lymphoblastoid cell
RAW 264.7	Macrophage cell
Rf	Retention Factor
RPMI-8226	B lymphocytes
RXRα	Retinoid X Receptor Alpha
S. aureus	*Staphylococcus aureus*
s	Singlet
SC_{50}	Sample Concentration Reducing DPPH Concentration to its Initial Value
SGC-7901	Human gastric cancer cell
SK-MEL-28	Human melanoma cell
SNU-1	Near-diploid cell (modal chromosome no. 47)
T. brucei	*Trypanosoma brucei*
T. cruzi	*Trypanosoma cruzi*
T. rubrum	*Trichophyton rubrum*
t	Triplet
TLC	Thin Layer Chromatography
TNF-α-U1	Tumor necrosis factor alpha in U1 cell
tt	Triplet of triplet
TW01	Nasopharyngeal carcinoma cell
U251	Glioma stem cell
VERO	COVID-19 Vaccine
WHO	World Health Organization

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KAJIAN FITOKIMIA DAN PENILAIAN KESITOTOKSIAN DARIPADA KULIT BATANG KAYU *Calophyllum macrocarpum* HOOK. F. DAN *Calophyllum recurvatum* P. F. STEVENS

ABSTRAK

Spesies daripada genus *Calophyllum* telah digunakan sekian lamanya dalam bidang perubatan tradisional Asia, terutamanya di Malaysia. Penyelidikan hasilan semula jadi ke atas genus *Calophyllum* telah mengenalpasti pelbagai fungsi biologi yang dipamerkan oleh tumbuhan ini, contohnya, perencatan HIV, antioksidan dan juga aktiviti sitotoksik. Spesies daripada genus *Calophyllum* mempunyai kandungan sebatian fenolik yang tinggi terutamanya xanton dan kumarin. Namun, *Calophyllum macrocarpum* dan *Calophyllum recurvatum* daripada genus *Calophyllum* adalah antara spesies yang masih memerlukan pendokumentasian saintifik terutamanya tentang aspek fitokimia dan farmakologi. Dalam kajian ini, kulit batang kayu pokok *Calophyllum macrocarpum* dan *Calophyllum recurvatum* telah menjalani kajian fitokimia yang berfokus ke arah proses pemencilan sebatian kimia. Teknik kromatografi dalam pengasingan dan pemurnian terperinci ke atas kulit batang kayu *C. macrocarpum* (ekstrak *n*-hexana dan kloroform) telah mengasingkan xanton-xanton prenil; ananixanton (**1**), trapezifolixanton (**2**), 8-dioksigartanin (**3**) serta triterpenoid seperti stigmasterol (**4**) dan juga friedelin (**5**). Malah sebatian kimia seperti thwaitesixanton (**6**), teysmanon A (**7**), soulattrolid (**8**), kalanon (**9**), dan juga friedelin (**5**) telah berjaya dikenalpasti daripada ekstrak kulit batang kayu *C. recurvatum* (ekstrak *n*-hexana dan kloroform). Struktur-struktur sebatian kimia ini telah dicirikan dan ditentukan menggunakan teknik spektroskopi canggih. Aktiviti sitotoksik ekstrak, sebatian **1**-**2** dan **7**-**8** daripada kedua-dua pokok *C. macrocarpum* dan *C. recurvatum*

dinilai dan diuji terhadap sel kanser hati manusia (hati HeLa Chang) dan juga sel normal hati manusia (HL-7702). Antara kesemua ekstrak daripada *C. macrocarpum*, ekstrak *n*-heksana dan kloroform mempamerkan aktiviti sitotoksik yang bagus terhadap sel hati HeLa Chang. Sebatian ananixanton yang ditemui dalam kedua-dua ekstrak *C. macrocarpum* tersebut mempamerkan aktiviti sitotoksik yang tinggi terhadap sel hati HeLa Chang, berbanding dengan sebatian trapezifolixanton. Hal ini mungkin kerana kehadiran kumpulan prenil dan pirano yang melekat di posisi C-2, C-3 dan C-4 di ananixanton (**1**). Di samping itu, kesemua ekstrak dan kompaun-kompaun terpencil yang telah diuji terhadap sel normal hati manusia, HL-7702 tidak mempamerkan sebarang aktiviti sitotoksik. Daripada *C. recurvatum* pula, ekstrak *n*-heksana mempamerkan aktiviti sitotoksik yang baik terhadap sel hati HeLa Chang, manakala kedua-dua sebatian teysmanon A (**7**) dan soulattrolid (**8**) tidak mempamerkan sebarang aktiviti sitotoksik terhadap sel hati HeLa Chang. Kesemua ekstrak dan kompaun-kompaun terpencil daripada *C. recurvatum* juga tidak mempamerkan aktiviti sitotoksik terhadap sel normal hati manusia, HL-7702. Berdasarkan penemuan awal hasil aktiviti biologi yang dijalankan ke atas ekstrak-ekstrak dan sebatian-sebatian fitokimia yang dipencilkan, ekstrak *n*-heksana (daripada *C. macrocarpum* dan *C. recurvatum*), ekstrak kloroform (daripada *C. macrocarpum*), dan ananixanton (**1**) daripada *C. macrocarpum* perlu diuji dalam kajian praklinikal yang lebih lanjut untuk mengkaji potensi aplikasi terapeutik mereka terhadap aktiviti antikanser dan kesitotoksian yang bersasarkan mahupun tidak bersasarkan.

PHYTOCHEMICAL STUDIES AND CYTOTOXICITY ASSESSMENT OF STEM BARK EXTRACTS FROM *Calophyllum macrocarpum* HOOK. F. AND *Calophyllum recurvatum* P. F. STEVENS

ABSTRACT

Numerous *Calophyllum sp*. have long been valued widely in Asian traditional medicine especially in Malaysia. The modern natural product-based research on the genus *Calophyllum* has further revealed a variety of biological functions exhibited by these plants, such as inhibition of HIV, antioxidant, and cytotoxicity. Species from the genus *Calophyllum* are also abundant in phenolic compounds especially xanthones and coumarins. However, *Calophyllum macrocarpum* and *Calophyllum recurvatum* are among the species that still need more scientific documentation on the phytochemistry and pharmacological aspects. In this research project, the stem bark of *Calophyllum macrocarpum* and *Calophyllum recurvatum* were being subjected for phytochemical investigation, which led to the isolation of various phytochemicals such as xanthones and coumarins. Extensive chromatographic separations and purifications on the stem bark of *C. macrocarpum* (*n*-hexane and chloroform extracts) resulted in isolation of prenylated xanthones; ananixanthone (**1**), trapezifolixanthone (**2**), 8-deoxygartanin (**3**), as well as triterpenoid; stigmasterol (**4**) and friedelin (**5**). Furthermore, thwaitesixanthone (**6**), teysmanone A (**7**), soulattrolide (**8**), calanone (**9**), as well as friedelin (**5**) were successfully being isolated from the stem bark of *C. recurvatum* (*n*-hexane and chloroform extracts). The structures of the compounds were characterised and elucidated using advanced spectroscopic techniques. The cytotoxic activities of the extracts, compounds **1-2** and **7-8** from both *C. macrocarpum* and *C. recurvatum* were evaluated against cancerous human liver (HeLa Chang liver) and normal human

liver (HL-7702) cell lines. Among the extracts from *C. macrocarpum*, *n*-hexane and chloroform extracts offer significant cytotoxic effect against HeLa Chang liver cell line. Ananixanthone (**1**) presented in both extracts of *C. macrocarpum* exhibited high cytotoxicity towards HeLa Chang liver cell line, compared to trapezifolixanthone (**2**). This might be due to the presence of prenyl and pyrano group attached in the C-2, C-3 and C-4 positions of ananixanthone (**1**), respectively. Meanwhile, all of the tested extracts and isolated compounds from *C. macrocarpum* offer no cytotoxicity activities against normal human liver, HL-7702 cell line. From *C. recurvatum*, *n*-hexane extract portrayed good cytotoxicity activities against HeLa Chang liver cell line, while both of compounds, teysmanone A (**7**) and soulattrolide (**8**) did not portrayed any cytotoxicity activities against HeLa Chang liver cell line. All of the tested extracts and isolated compounds from *C. recurvatum* also offer no cytotoxicity activities against normal human liver, HL-7702 cell line. Taken into consideration from the outcome of these preliminary biological activities conducted on the extracts and isolated phytochemicals, *n*-hexane extract (from *C. macrocarpum* and *C. recurvatum*), chloroform extract (from *C. macrocarpum*), and ananixanthone (**1**) from *C. macrocarpum* need to be assigned to undergo more preclinical studies in studying their potential therapeutic applications towards targeted and non-targeted anticancer and cytotoxicity activities.

CHAPTER 1
INTRODUCTION

1. General Introduction

1.1 Evolutionary History of Phytochemistry

Phytochemicals are important because they play a crucial role in societal well-being and world health. Customarily, nearly all medicinal prescriptions have been derived from natural products, and phytochemicals have persisted to submit clinical studies; to serve as leading agents, especially as antibacterial and also anticancer agents (Harvey et. al. 2015). The pursuit of novel natural product-based drugs for pharmaceutical industry is indeed a continuous research that calls for constant optimisation in investigations and researches of the phytochemicals in plants which encompasses their metabolism (biosynthesis and biodegradation), phytochemical structures, natural variation in plants, biological significance, extraction, and both qualitative as well as quantitative assessment (Mendoza et. al. 2018). In phytochemistry, various methods and techniques have been evolved and employed including utilising advanced techniques for plant sample preparation, and also identifying structural elucidation of the phytochemical constituents. Phytochemical investigations of natural products include several features; extraction of the phytochemical constituents to be analysed from a plant, separation and isolation of the phytochemical constituents, identification and characterisation of the isolated phytochemical constituents, study of the biosynthesis pathway of selected phytochemical constituent, and also quantitative evaluation (Mendoza et. al. 2018). In fact, in the current millennium, the emerging discipline of genome sequencing and mining of microorganisms in this area of study is being driven by developments in computational biology, spectrometry, gene expression, metabolomics and a few others (Katz and Baltz. 2016).

What has intrigued phytochemists are the variety and complexity of phytochemical constituents themselves. In addition to the wide range of molecular weights of the phytochemicals in plants, a considerable number of phytochemical constituents contain significant stereo-specific carbon centres, which intrigued synthetic organic chemists in flourishing pathways as well as chemical reaction schemes. Cell biologists investigated for anticancer and other pharmacological properties while biochemists as well as molecular biologists established biosynthesis, genetics, and regulation of enzyme synthesis. Meanwhile, the industrial demand for large amount of natural product-based drugs has piqued interests of chemists, pharmacists and pharmacologist. The collaborative efforts from academic and industrial researchers had founded the field; natural products, specifically phytochemistry (Katz and Baltz. 2016).

1.2 Bridging Phytochemistry Knowledge for Human Health Benefits

Dating back to the dawn of civilisation, people have utilised different plants and their derivatives for medicinal treatment, for instance, the treatment of infectious diseases. There are many traditional healing practices that use different salves or concoctions that are extracted from plants to treat different diseases. Many of these conventional approaches are still in used until today. On top of that, some widely used medications in modern medicine had a rudimentary crude form taken from conventional medicine (Gorlenko et. al. 2020). This is because, plants provide not just vital nutrients required by us human, but also health-promoting bioactive phytochemicals that contribute to greater longevity, and disease treatment (Yoo et. al. 2018). In this recent age of pharmaceutical industry, plants are a valuable source to obtain various active chemical constituents as these active secondary metabolites exhibit a wide range of pharmaceutical effects to remedy bacterial and fungal related illnesses, and also some chronic degenerative illnesses namely diabetes and cancer (Mendoza et. al. 2018).

As phytochemists, one of the top questions that we often ask ourselves is whether the secondary metabolites derived from plants are good or bad. Do they have the chance on becoming a panacea against disease, or do they do harm to us instead? Chemical substances known as natural products typically have applications that benefit human health. Yet, the same chemical substances may also generate poisonous properties. Secondary metabolites have been suggested to be biosynthesized as waste or even detoxification products (Madariaga-Mazón et. al. 2019). Additionally, it has been proposed that secondary metabolites play important metabolic roles. It is interesting to note who these substances are hazardous to (Madariaga-Mazón et. al. 2019), as the chemical composition and characteristics of plant secondary metabolites determine how they work (Gorlenko et. al. 2020).

With recent studies have shown the effects of phytochemical-abundant supplements on health, it is firmly emphasized that ingesting these phytochemicals can enhance health (Yoo et. al. 2018). Hence, many researchers conducted investigations to study the benefits of phytochemicals for health. Yet, studies on specific uses of phytochemicals encountered a number of complications.

Despite playing a significant part in the drug development process, certain phytochemicals are extremely hazardous. Toxicity in this context refers to the negative impact on an organism as a whole, as well as the impact on an organism's substructure, such as a cell or an organ. Toxic phytochemicals can, however, also be utilised to treat some diseases; for instance, cytotoxic phytochemicals are employed in the therapy of cancer. Thus, secondary metabolites may so have two distinct roles (Madariaga-Mazón et. al. 2019).

Of course, not every plant-derived phytochemical is immediately ready for use in standard clinical practise. Nevertheless, thanks to recent developments in bio-screening research, we will be able to both detect and identify even extremely limited amount of active phytochemicals and elucidate the precise molecular mechanisms underlying their effect(s) on targets. The chemical alteration of potentially beneficial phytochemicals in order to enhance their bioactive capabilities and lessen their harmful properties and side effects is another important and promising research.

Recognising the health perks of phytochemical constituents is indeed a crucial action in medicinal drugs and food development. Knowledge on the structural variety, sources, synthetic practicability, as well as its inherent toxicities of these plant phytochemicals will help expand their usages, for the benefit of mankind as well as the environment.

1.3 Problem Statement

The plants in genus *Calophyllum* are well known among locals for their usefulness and benefits in traditional medicines. This genus is also renowned for its vast structural diversity and prosperous foundation of pharmaceutical active phytochemicals. Yet nevertheless, until today, there are still many *Calophyllum* species that remain unexplored, especially in phytochemical aspects. Among them, *Calophyllum macrocarpum* and *Calophyllum recurvatum* still need more proper scientific documentation on the phytochemical and pharmacological investigations, as existed data do not provide enough knowledge on the phytochemical contents of both species. Thus, there is a necessity for us scientific community members to conduct phytochemical investigations on the species *C. macrocarpum* and *C. recurvatum*, at the same time, to study the structural diversity of the phenolic compounds present in it as well as its potency towards biological activities and pharmacological activities of both species.

Hence, phytochemical investigations were conducted to understand its chemistry, especially on identifying new and existing chemical entities with diverse chemical structures and potential bioactivities from *C. macrocarpum* and *C. recurvatum*. The discovery of potential bioactive constituents towards selected bioactivities apparently brings great importance to the medicinal field, as the helpful information would be essential for future references by natural product chemists. Also, the establishment and development of these prospective as drug towards selectivity can serve as alternatives or supplementary to the present existing drugs on demand.

1.4 Objective of Study

This project is conducted to isolate, purify, and characterise the structures of phytochemical constituents from *Calophyllum macrocarpum* and *Calophyllum recurvatum*. Structural identification and elucidation will be conducted using detailed spectroscopic techniques on the isolated phytochemicals. The key resolution of this research project is to understand the phytochemistry of the genus *Calophyllum* especially on these species, *C. macrocarpum* and *C. recurvatum*, along with their potential biological properties. Thus, the specific objectives of the research are:

i. To extract and isolate chemical constituents from the stem barks of *Calophyllum macrocarpum* and *Calophyllum recurvatum*.
ii. To perform structural characterization and elucidation of isolated compounds using advance spectroscopic techniques.
iii. To investigate the cytotoxic properties of the isolated phytochemicals and the extracts against HeLa Chang liver and HL-7702 cell lines by using MTT assay technique.

CHAPTER 2
LITERATURE REVIEW

2.1 Taxonomy of Calophyllaceae

Calophyllaceae (Order: Malpighiales) is one of flowering-plants families which is acknowledged by APG III classification system (Gupta & Gupta. 2020). The majority of 475 species and 14 genera that comprise this family were previously known to belong to the *Calophylleae* of the family *Clusiaceae* (Christenhusz et. al. 2016) but Angiosperm Phylogeny Group has concluded that it was essential to separate this branch of genera into its own family (Bremer et. al. 2009).

With the exception of *Calophyllum* L. and *Mammea* L., which are found in tropical rainforest of both Old and New World, most genera of *Calophyllaceae* are restricted to either the Old or New World tropics (Díaz. 2012). Furthermore, APG III classification system has reclassified that genus *Calophyllum*, which was formerly classified as part of the *Guttiferae* family into a part of *Calophyllaceae* family (Gupta & Gupta. 2020). Figure 2.1 shows some parts of *Calophyllum* plants.


Figure 2.1: Parts of *Calophyllum* plants.

(Left) Taken by Chitranshi Tewari, iNaturalist, https://www.inaturalist.org/photos/34 5665984

(Right) Taken by Jmole, iNaturalist, https://www.inaturalist.org/photos/254222939

2.1.1 Taxonomy of Genus *Calophyllum* and its ethnobotanical and ethnomedicinal uses.

Calophyllum is a large genus that belongs to the *Calophyllaceae* family and has approximately 200 species (Nasir et al. 2013), with pantropical distribution. *Calophyllum* gets its name from Greek words; "*kalos*" that denotes "good" while "*phullon*" signifies "leaf" (Ismail et al. 2016).

Table 2.1: The Taxonomy of genus *Calophyllum.*

Domain	Eukaryota
Kingdom	Plantae
Phylum	Tracheophyta
Subphylum	Angiospermae
Class	Magnoliopsida
Order	Malpighiales
Family	*Calophyllaceae*
Genus	*Calophyllum*

Calophyllum species are quite tricky to categorise because of the obstacles in creating certain parameters (Gupta & Gupta. 2020). *Calophyllum* species range in height from very tall trees to small shrubs. The majority of the plants, however, are medium-sized trees. The species' habitation spans from wet tropical rainforest in the lowlands to drier environments at higher altitudes. Some of the species can also be found in zones that have been waterlogged (Gupta & Gupta. 2020).

Nevertheless, the species of this genus can be still distinguished through taxonomical features like the bark, wood, twigs, bud, leaves, inflorescence, flowers, fruit, germination, seedlings, and leaf anatomy (Stevens. 1980). Certain species can be identified by the appearance of the outer bark's inner surface, but most species cannot be identified in this way. All species' trunks have an inner bark that secretes latex, which is often a clear yellowish colour. For some species, the colour of the latex that leaks from the trunk may differ. In a few larger *Calophyllum* species, the sapwood has a rippling surface at the cambium, however it is uncertain how many species possess this characteristic. Each species has a unique outer bark colour as well as surface and latex colours (Stevens. 1980).

Nearly every species of *Calophyllum* can be recognised merely by its leaves, both in its natural habitat and in the herbarium. The characteristics that give the leaf its particular appearance include the midrib and venation, along with size, prominence of the edge, toughness, form, and, to some extent, the colour of the dried leaf. Venation prominence is described subjectively and varies depending on the species (Stevens. 1980).

The inflorescence's position, size, and kind can all vary infra-specifically. All inflorescences have flowers at the ends of their main and lateral branches, and they are all cymose in character. The investigation of floral characteristics in *Calophyllum* is complicated by our lack of knowledge on its sex-forms. Some of the collections are hermaphrodite or perhaps polygamous, while others are dioecious. Some species' flowers are unknown, while the number of flowering specimens from other species is insufficient to say whether or not those species have sex-forms. Resurrected herbarium material is used to calculate the size of the tepals. The number of tepals varies between species. The optimal time to count stamens is just before a flower blooms, however in most cases, only a few blossoms were examined, thus the results are estimations (Stevens. 1980).

Despite the lack of ripe fruit material, the drupe-shaped fruit of *Calophyllum* frequently exhibits recognisable diagnostic traits. Both the mesocarp's thickness and the dried fruit's surface's properties can be used to distinguish one dried fruit from another. Despite the fact that the majority of stones have an ovoid or subspherical shape with a rounded apex, some of them have angled or sharply pointed apexes. Trees can produce what appear to be regular fruits even when flowers are not pollinated; the corky layer fills the interior of the stone in these fruits (Stevens. 1980).

Even though only a small number of germination of *Calophyllum* species has been seen, their differences have a significant taxonomic impact. It takes 1-2 months for seeds to germinate (occasionally up to 4 months). The first leaves of seedlings are pseudo-verticillate or have well-developed internodes; intermediates have also been found. The rate of growth of the seedlings and the geotropism performance of each species' shoots seems to be identical. Seedling and sapling leaves are totally distinctive from those of mature trees in terms of size, form, and anatomy. They frequently have a longer, pointed, and bigger apex (Stevens. 1980).

Members of this genus are found in Eastern Africa, tropical America, Australia, Madagascar, the Pacific Islands, the Atlantic Forest and Brazil, in addition to South and Southeast Asia (Abbas et al. 2019), as the genus is restricted to the hot and humid tropics climate (Ismail et al. 2016). *Calophyllum* species are large hardwoods that grow in a variety of habitation from mountainous forests to coastal wetlands, but are only found in the hot and humid tropics (Noldin. 2006).

Furthermore, stem bark usually protects trees from weather conditions, insect pests, and browsing (Pásztory et. al. 2016). The stem barks are also the rich sources of tannins and other phenolic compounds (Singh et. al. 2010). In fact, the first ever chemical investigation being conducted on the genus was recorded back in 1950s by Polonsky and Ormancey-Potier (Polonsky. 1957). Coumarins and xanthones are quite common in this genus and have been isolated from the wood and bark of several species of this genus (Gómez-Verjan et. al. 2017).

Moreover, parts of *Calophyllum* species have long been used for their numerous health benefits. Locals have long employed various parts of the genus *Calophyllum* plant; such as tree barks and seed oil, to aid more minor and persistent ailments. Despite the availability of ethnobotanical and ethnomedicinal knowledge for some *Calophyllum* species, some traditional uses for some of the species listed have yet to be documented or published. Table 2.2 summarises the ethnobotanical and ethnomedicinal uses for available species of the genus *Calophyllum*.

Table 2.2: Ethnobotanical and ethnomedicinal uses of selected *Calophyllum* species.

Species	Botanist	Ethnobotanical and ethnomedicinal uses (parts used)	References
C. apetalum	Willd.	Traditionally used as an eye remedy in Asian medicine (leaf decoction); Used in the treatment of rheumatism, leprosy, scabies, as septic poison, and other cutaneous afflictions (seed oil) as well as gastric troubles (leaf juice); Good for construction of boats, furniture and suitable for plywood (timber)	Ruma et al. 2014; Joshi et al. 2013; Prakash et al. 1984; Nair & Seeni 2003
C. austroindicum	Kosterm. ex P.F.Stevens	The tree yields good timber	Stevens. 1980
C. biflorum	M.R.Hend. & Wyatt-Sm.	Used to make planks in Kuching (wood), the latex of S3802 was noted as being poisonous, but there may have been confusion between this plant and a member of the Anarcardiaceae	Stevens. 1980
C. blancoi	Planch. & Triana	Used as a source of dye and in the treatment of wounds (no mention of plant part used); Used to treat wounds, boils, tumours, swellings and also to alleviate asthma (latex)	Stout & Sears. 1968; Stevens. 1980
C. bracteatum	Thwaites	Used for buildings, masts, and plywood (wood)	Stevens. 1980
C. brasiliense	Cambess.	Used for construction, flooring, and furniture (timber); Used to treat cutaneous infections (seed oil), tea for pregnant women to clean the womb (bark), toothache or to prevent infections (latex); Used to treat rheumatism, varicose veins, haemorrhoids and ulcers (stem bark), whereas the leaves have anti-inflammatory properties; Used in the treatment of pain, diabetes, diarrhoea, hypertension, herpes, rheumatism and ulcer (latex exudated from inner bark)	Reyes-Chilpa et al. 1997; Silva-Castro 2020; Rea et al. 2013; Lemos et al. 2012

Table 2.2: Ethnobotanical and ethnomedicinal uses of *Calophyllum* species. (continued)

Species	Botanist	Ethnobotanical and ethnomedicinal uses (parts used)	References
C. calaba	L.	Has been employed medicinally (latex of the trunk), and lamp oil is extracted (kernels that contained high lipid content)	Crane et al. 2015
C. caledonicum	Vieill. ex Planch. & Triana	Highly resistant towards fungi and termites (heartwood); Used as a diuretic (latex)	Morel et al. 2002; Hay et al. 2003; Hay et al. 2004
C. canum	Hook.f. ex T.Anderson	Used as antibacterial, anticancer and antioxidant (no mention of plant part used); Used to stupefy fish (latex); Used for construction, masts, and spars (wood)	Taher et al. 2020; Arifullah et al. 2014; Kawamura et al. 2012; Stevens. 1980
C. cerasiferum	Vesque	Used in construction (wood)	Stevens. 1980
C. chapelieri	Drake	Used in construction (wood), and oil is sometimes extracted from the seed	Stevens. 1980
C. depressinervosum	M.R.Hend. & Wyatt-Sm.	Used as antimicrobial (latex)	Arifullah et al. 2014
C. dioscurii	P.F.Stevens	Used to treat fever and skin disease (decoction of leaves and stem bark)	Tjahjandarie et al. 2019
C. dryobalanoides	Pierre	Used as a purgative in traditional medicine (root) but chemical constituents have not been previously investigated	Ha et al. 2012
C. ferrugineum	Ridl.	Used as decoction for women who has just given birth (wood; stem bark); Used for masts and house building (wood); Used in making walls for native house (bark)	Stevens. 1980
C. incrassatum	M.R.Hend. & Wyatt-Sm.	Used as poultice to treat itching and other skin diseases (no mention of plant part used)	Eswani et al. 2010

Table 2.2: Ethnobotanical and ethnomedicinal uses of *Calophyllum* species. (continued)

Species	Botanist	Ethnobotanical and ethnomedicinal uses (parts used)	References
C. inophyllum	L.	Used as poultice to treat skin diseases, wounds, eye ailments, rheumatism, inflammations, and haemorrhoids (seed oil; leaves; stem resin); Used as dye for fish net (stem bark); Used as fuels for lighting purpose, vehicles and generate electricity (seeds oil); Used for building ship (wood)	Kainuma et al. 2016; Stevens. 1980
C. lanigerum	Miq.	Used for building house and ship making (wood)	Stevens. 1980
C. macrocarpum	Hook.f.	Used as antimicrobial (no mention of plant part used); Used for building houses and make furniture (wood)	Arifullah et al. 2014; Stevens. 1980
C. macrophyllum	Scheff.	Used for construction purposes (wood) and the fruit is edible	Frengki et al. 2013
C. membranaceum	Gardner & Champ.	Used in Chinese folk medicine for the treatment of rheumatism, arthritis, lumbago and wounds in Hainan Island, PR China (stem and bark); Used to reduce inflammation around bruises and to kill pain, relieve rheumatic joint pain, lumbago, and wound pain (no mention of plant part used)	Chen et al. 2011; Zou et al. 2005; Stevens. 1980
C. pinetorum	Bisse	Used by local populations as a cicatrisant agent at Pinar del Rio Province of Cuba (no mention of plant part used)	Alarcon et al. 2008
C. polyanthum	Wall. ex Choisy	Used to treat traumatic bleeding and to relieve pain in Chinese folk medicine (no mention of plant part used)	Zhong et al. 2010
C. recedens	Jum. & H.Perrier	Used in the manufacture of furniture and canoes (wood), and has been used in the local pharmacopeia (latex)	Stevens. 1980

Table 2.2: Ethnobotanical and ethnomedicinal uses of *Calophyllum* species. (continued)

Species	Botanist	Ethnobotanical and ethnomedicinal uses (parts used)	References
C. rubiginosum	M.R.Hend. & Wyatt-Sm.	Used to treat itching, other skin diseases, as antimicrobial, and antioxidant (wood; latex); Used as poison bait to kill rats (latex)	Eswani et al. 2010; Arifullah et al. 2014; Stevens. 1980
C. sclerophyllum	Vesque	Used for construction (wood)	Stevens. 1980
C. soulattri	Burm.f.	Used as infusion for women who has just given birth, for treating rheumatic pain, and relieves pain in leprosy (root; stem bark; seed); Used for masts, spars and building house (wood)	Gupta and Gupta 2020; Stevens. 1980
C. symingtonianum	M.R.Hend. & Wyatt-Sm.	Used as antifungal and antioxidant (no mention of plant part used); Used for building house (wood)	Arifullah et al. 2014; Stevens. 1980
C. tacamahaca	Willd.	Treatment for eye diseases, rheumatism, headache, gout, arthritis, dermic problems, skin disorders, memory troubles, blood circulation (no mention of plant part used)	Lavergne. 2001
C. teysmannii	Miq.	Used as antiviral (no mention of plant part used); Used for construction (wood)	Arifullah et al. 2014; Stevens. 1980
C. thorelii	Pierre	Useful in construction, including that of boats and masts, apparently being resistant to the attacks of borers (wood), while, the flowers are very fragrant & used by the ladies	Stevens. 1980
C. tomentosum	Wight	Being practiced in Ayurveda to treat ulcers, snake bites and eye diseases (extracts); Used in the treatment of skin diseases (seed oil)	Govindappa et al. 2018; Stevens. 1980
C. venulosum	Zoll.	Used as oars (wood)	Stevens. 1980
C. verticillatum	P.F.Stevens	Used in construction (wood)	Stevens. 1980

Table 2.2: Ethnobotanical and ethnomedicinal uses of *Calophyllum* species. (continued)

Species	Botanist	Ethnobotanical and ethnomedicinal uses (parts used)	References
C. walkeri	Wight	Used in construction and joinery (wood), as well as lighting (seed oil)	Stevens. 1980
C. wallichianum	Planch. & Triana	Used as poultice to treat itching and other skin diseases (latex)	Eswani et al. 2010

2.1.1(a) Taxonomy of *Calophyllum macrocarpum*

C. macrocarpum could grow on seasonal freshwater swamp forest, riverine, hillsides, ridges, and low undulating land in pantropical country. The locals generally called *C. macrocarpum* by its local name; Bintagor Bunut or Bintagor Batu (Source: Malaysia Biodiversity Information System (MyBIS)). Figure 2.2 shows the herbarium samples of *C. macrocarpum*. The taxonomy of *C. macrocarpum* are shown below:

Table 2.3: The Taxonomy of *Calophyllum macrocarpum* (Source: Malaysia Biodiversity Information System (MyBIS)).

Domain	Eukaryota
Kingdom	Plantae
Phylum	Tracheophyta
Subphylum	Angiospermae
Class	Magnoliopsida
Order	Malpighiales
Family	*Calophyllaceae*
Genus	*Calophyllum*
Species	*C. macrocarpum*
Common name	Bintagor Bunut

Figure 2.2: Herbarium sample of *C. macrocarpum*.

Taken from Herbarium Catalogue Specimens; Digital Image © Board of Trustees, RBG Kew, http://creativecommons.org/licenses/by/3.0/

2.1.1(b) Taxonomy of *Calophyllum recurvatum*

The local people are more familiar with *C. recurvatum* as Bintagor. However, this species is considered relatively new and an unexplored species, since there are no literature studies or any present data on this plant. Figure 2.3 shows the herbarium samples of *C. recurvatum*. The taxonomy of *C. recurvatum* are shown below:

Table 2.4: The Taxonomy of *Calophyllum recurvatum* (Source: Global Biodiversity Information Facility (GBIF)).

Domain	Eukaryota
Kingdom	Plantae
Phylum	Tracheophyta
Subphylum	Angiospermae
Class	Magnoliopsida
Order	Malpighiales
Family	*Calophyllaceae*
Genus	*Calophyllum*
Species	*C. recurvatum*
Common name	Bintagor

Figure 2.3: Herbarium sample of *C. recurvatum*.

Taken from Naturalis Biodiversity Center; http://creativecommons.org/publicdomain/zero/1.0/

2.2 Chemistry of xanthones

Greek word "*xanthos,*" which means "yellow," is where the word "xanthone" originates (Diderot et. al. 2006). Xanthones are yellow-coloured natural polyphenolic compounds with a basic structural formula of C_6-C_1-C_6 and molecular formula of $C_{13}H_8O_2$. Xanthones are a subgroup of phenolic constituents is categorized under benzopyrone. Xanthones have dibenzo-γ-pyrone as core in the skeletal structure, in which the two benzene rings bridged across a carbonyl group and an oxygen. These system of conjugated rings hinder the carbon-carbon's free rotation. The properties of xanthones depend on the types and position of chemical substituents that attached to the core structure. Xanthones are divided into six main classes which are simple oxygenated xanthones, prenylated xanthones, xanthone glycosides, bisxanthones, xanthonolignoids and miscellaneous xanthones (Negi et al, 2013). Figure 2.4 shows the basic skeleton of a xanthone.

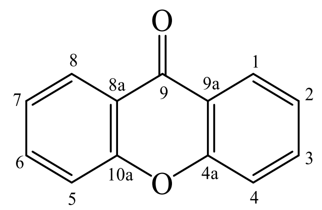

Figure 2.4: Basic skeleton of xanthone.

Simple oxygenated xanthones are further subclassed accordingly, in regards to the degree of oxygenation into non-, mono-, di-, tri-, tetra-, penta-, and hexa-oxygenated substances (Negi et al, 2013). In this type of xanthones, the substituent groups are rather just simple hydroxy, methoxy or methyl moieties. Mono-oxygenated xanthones are rare as there are only three compounds from the *Hypericaceae* and *Guttiferae* families were discovered (Diderot et. al. 2006).

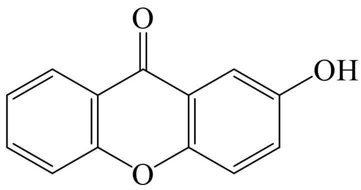

Figure 2.5: Example of mono-oxygenated xanthone.

For di-oxygenated xanthones, twelve derivatives of this class were found (Diderot et. al. 2006).

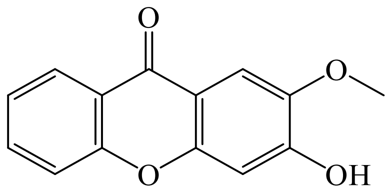

Figure 2.6: Example of di-oxygenated xanthone.

Gentisine, the first isolated xanthone, is an example of tri-oxygenated xanthones (Diderot et. al. 2006).

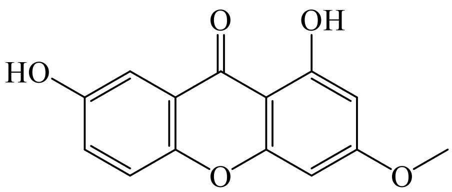

Figure 2.7: Example of tri-oxygenated xanthone.

Tetra-oxygenated: many of them were isolated from the *Gentianaceae* family and they were more abundant (Diderot et. al. 2006).

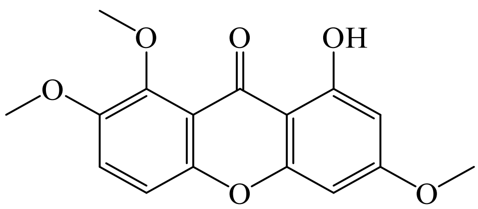

Figure 2.8: Example of tetra-oxygenated xanthone.

Only a few penta-oxygenated xanthones were found in nature (Diderot et. al. 2006).

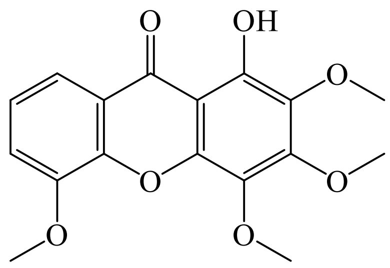

Figure 2.9: Example of penta-oxygenated xanthone.

Hexa-oxygenated xanthones are the highest level of oxygenation ever recorded for xanthones (Diderot et. al. 2006).

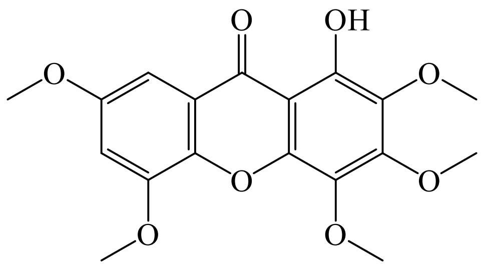

Figure 2.10: Example of hexa-oxygenated xanthone.

Naturally, occurring glycosylated xanthones are reported as *C*- or *O*-glycosides. *O*-glycosides have typical glycosidic linkage. In *O*-glycoside xanthones, the sugar moiety is typically presents at carbon-1 of xanthone nucleus, which is quite challenging to enlighten its presence over there given its close proximity of the carbonyl group and the potential for strain (Diderot et. al. 2006).

When compared to *O*-glycoside xanthones, *C*-glycoside xanthones are actually better hydrolysis-resistant xanthones, although their occurrence is exceedingly rare (Diderot et. al. 2006). In *C*-glycosides, the sugar moiety and the xanthone nucleus is linked by the C-C bond, which made them quite resistant to enzymatic hydrolysis (Negi et al. 2013).

Besides that, the existence of the prenylated xanthones is constrained to the family *Guttiferae* plants (Negi et al. 2013). It seems that the family *Guttiferae* produces a lot of xanthones with geranyl and isopentenyl substituents (Diderot et. al. 2006).

Naturally occurring xanthonolignoids are scarce; in previous studies, only five compounds were reported. Xanthonolignoids are a rather uncommon class of natural products that are mostly found in the genera *Kielmeyera*, *Caraipa*, and *Psorospermum* of the *Guttiferae* family. The skeletal patterns that are generated when the lignoid pattern and the xanthone nucleus combine to make these compounds are remarkably similar (coniferyl alcohol or syringenin). Candesin D is the most representative xanthonolignoid known so far. (Diderot et. al. 2006).

A number of 12 bisxanthones; five from the higher plants, six from fungi and one from lichen that have been reported to date. Last but not least, xanthones with substituents other than all that have been discussed before are all included in miscellaneous xanthones and they could not be categorised in the conventional way (Negi et al. 2013).

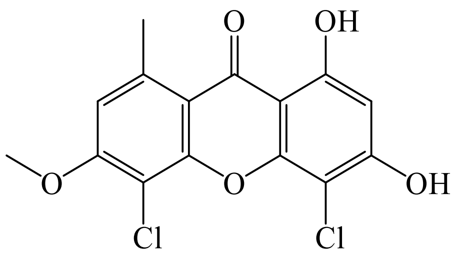

Figure 2.11: Example of miscellaneous xanthone (4,5-dichloro-6-*O*-methylnorlichexanthone).

Numerous researchers have thoroughly investigated the biosynthetic process that results in xanthones in plants both *in vivo* and *in vitro* utilizing markers. They made an effort to compare and contrast natural xanthones with established oxygenation patterns. They demonstrated that the synthesis is the result of two processes; acetate polymalonic pathway (lower plants - microorganisms and lichens) and mixed shikimate acetate pathway (higher plants) (Pogam & Boustie 2016).

It has been established that the biosynthesis of several xanthones in lower plants (microorganisms and lichens) was entirely obtained from seven acetate units, through acetate polymalonic route. An example of the acetate polymalonic pathway is provided by the biosynthetic mechanism of ravelenin from *Helminthosporium raverelii* proposed by Birch et al. back in 1976. The pathway involved is as shown in Figure 2.12 (Pogam & Boustie 2016).