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PHYTOCHEMISTRY OF Calophyllum inophyllum L. AND Calophyllum teysmannii Miq. AND THEIR BIOLOGICAL ACTIVITIES

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By

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PHYTOCHEMISTRY OF *Calophyllum inophyllum* L. AND *Calophyllum teysmannii* Miq. AND THEIR BIOLOGICAL ACTIVITIES

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Chairman : Professor Gwendoline Ee Cheng Lian, PhD
Faculty : Science

The genus *Calophyllum* is consisted of 180-200 species of evergreen trees which are widely distributed in Asia and Africa. This genus is popularly known for their bioactive compounds such as terpenoids, xanthones and coumarins. The need of new drugs are on the rise due to the discovery of various new diseases. Besides, the development of drug resistant diseases also makes drug discovery research significant. Hence, natural products research is important as they provide new templates in the field of drug discovery. Detailed studies were carried out chemically and biologically on the stem bark of two selected plants species, *Calophyllum inophyllum* and *Calophyllum teysmannii*. A total of two terpenoid compounds and five xanthones were isolated from the n-hexane and chloroform crude extracts of *Calophyllum inophyllum* respectively. The terpenoids are friedelin **(82)** and stigmasterol **(83)** whereas the xanthones are caloxanthone A **(23)**, caloxanthone B **(24)**, caloxanthone C **(43)**, pyranojacareubin **(6)** and macluraxanthone **(5)**. On the other hand, a total of two xanthones were isolated from the n-hexane and methanol crude extracts of *Calophyllum teysmannii* correspondingly namely ananixanthone **(29)** and *β*-mangostin **(84)**. The structures of the acquired compounds were elucidated by analyzing the spectroscopic data such as 1D NMR, 2D NMR, IR, and MS. Structural modifications on the parent compound ananixanthone **(29)** afforded four other new synthesized xanthone derivatives namely ananixanthone monoacetate **(85)**, ananixanthone diacetate **(86)**, 5-methoxyananixanthone **(87)**, and 5-O-benzylananixanthone **(88)**. Their structures were concluded through comparison with the parent compound's NMR spectra.

The crude extracts of both the plants were subjected to toxicity test against LPS stimulated RAW264.7 cells. The results indicated that only chloroform crude extract of *Calophyllum inophyllum* exhibited a promising result with an IC_{50} value of 14.81±0.0417 µg/mL. However, the rest of the crude extracts showed weak or no bioactivities.

Determination of the antioxidant activities through DPPH scavenging assay indicated that the methanol crude extract of *Calophyllum teysmannii* exhibited the most significant

antioxidant properties with the EC_{50} value of 33.06±0.36 µg/mL followed by moderate activities by the methanol crude extract of *Calophyllum inophyllum*. The remaining crude extracts showed weak or no activities.

Besides, antimicrobial assay via disc diffusion method was carried out against seven different microbe strains. The n-hexane and chloroform crude extract of *Calophyllum inophyllum* showed strong inhibition against *Staphylococcus epidermidis S273* and moderate inhibition against *Bacillus Subtillis B145*. The rest of the crude extracts showed weak or no inhibition against the microbes.

Total Phenolic Content (TPC) of the crude extracts via the Folin-Ciocalteu method showed that the most polar crude extracts, the methanol crude extracts of both plants had the highest TPC value with 138.56 and 204.93 µg of gallic acid/mg of crude extracts individually.

Lastly, cytotoxicity screening via MTT assay of ananixanthone **(29)** and four of its modified derivatives against three cancer cell lines, SNU-1, LS-174T and K562 resulted in ananixanthone **(29)** showing the greatest activities against SNU-1 and K562 cell lines with the IC_{50} values of 8.97±0.11 µg/mL and 2.96±0.06 µg/mL respectively. On the other hand, 5-methoxyananixanthone **(87)** indicated greater cytotoxic activity than its parent compound against LS-174T cell line with the IC_{50} value of 5.76±1.07 µg/mL.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

FITOKIMIA DARIPADA *Calophyllum inophyllum* L. DAN *Calophyllum teysmannii* Miq. DAN AKTIVITI-AKTIVTI BIOLOGI

Oleh

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April 2017

Pengerusi : Profesor Gwendoline Ee Cheng Lian, PhD
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Genus *Calophyllum* terdiri daripada 180-200 spesies pokok malar hijau yang dijumpai secara meluas di rantau Asia dan Afrika. Genus ini dikenali untuk sebatian bioaktif mereka seperti *terpenoid*, xanton dan *coumarin*. Keperluan ubat-ubatan baru adalah semakin meningkat kerana penemuan pelbagai jenis penyakit baru. Selain itu, perkembangan penyakit tahan dadah juga menjadikan penemuan ubat-ubatan baru semakin penting. Oleh sebab itu, penyelidikan produk semula jadi adalah penting kerana ia memberikan templat baru dalam bidang ubat-ubatan. Kajian terperinci telah dijalankan secara kimia dan biologi terhadap kulit batang dua spesies tumbuhan terpilih, iaitu *Calophyllum inophyllum* dan *Calophyllum teysmannii*. Sebanyak dua sebatian *terpenoid* berjaya diperolehi daripada ekstrak mentah n-heksana manakala lima xanton berjaya diperolehi daripada ekstrak mentah klorofom *Calophyllum inophyllum*. *Terpenoid* itu adalah *friedelin* **(82)** dan *stigmasterol* **(83)** manakala xanton-xanton adalah *caloxanthone A* **(23)**, *caloxanthone B* **(24)**, *caloxanthone C* **(43)**, *pyranojacareubin* **(6)** dan *macluraxanthone* **(5)**. Sebaliknya, sebanyak dua xanton telah diasingkan daripada ekstrak mentah n-heksana dan metanol *Calophyllum teysmannii* iaitu *ananixanthone* **(29)** dan *β-mangostin* **(84)**. Struktur sebatian yang diperolehi telah dijelaskan secara mendalam dengan menganalisakan data spektroskopi seperti 1D NMR, 2D NMR, IR, dan MS. Pengubahsuaian struktur *ananixanthone* **(29)** menghasilkan empat derivatif xanton lain iaitu *ananixanthone monoacetate* **(85)**, *ananixanthone diacetate* **(86)**, *5-methoxyananixanthone* **(87)**, dan *5-O-benzylananixanthone* **(88)**. Struktur-struktur komponen ini disimpulkan melalui perbandingan dengan struktur induk mereka iaitu *ananixanthone* **(29)**.

Ekstrak mentah kedua-dua tumbuh-tumbuhan telah tertakluk kepada ujian ketoksikan terhadap sel RAW264.7 yang dirangsangkan oleh *Lipopolysaccharide* (LPS). Keputusan menunjukkan bahawa hanya ekstrak mentah klorofom daripada *Calophyllum inophyllum* mempamerkan hasil yang memberangsangkan dengan nilai IC_{50} 14.81±0.0417 µg/mL. Namun demikian, ekstrak-ekstrak mentah yang lain menunjukkan bioaktiviti yang lemah atau sifar keaktifan bioaktivitinya.

Penentuan aktiviti antipengoksidaan melalui DPPH menunjukkan bahawa ekstrak mentah metanol *Calophyllum teysmannii* mempamerkan sifat-sifat antipengoksidaan yang paling memberangsangkan dengan nilai EC_{50} 33.06±0.36 µg/mL diikuti dengan aktiviti sederhana oleh ekstrak mentah metanol *Calophyllum inophyllum*. Ekstrak-ekstrak mentah baki menunjukkan lemah atau tiada aktiviti.

Selain itu, penentuan bioaktiviti antimikrob telah dijalankan terhadap tujuh jenis organisma mikrob yang berbeza. Ekstrak-ekstrak mentah n-heksana dan klorofom *Calophyllum inophyllum* menunjukkan aktiviti yang kuat terhadap *Staphylococcus epidermidis S273* dan aktiviti sederhana terhadap *Bacillus Subtillis B145*. Ekstrak-ekstrak mentah yang lain menunjukkan aktiviti yang lemah terhadap organisma mikrob selebihnya.

Jumlah Kandungan Fenolik (TPC) daripada ekstrak-ekstrak mentah melalui kaedah *Folin-Ciocalteu* menunjukkan ekstrak metanol daripada *Calophyllum inophyllum* dan *Calophyllum teysmannii* menunjukkan nilai TPC yang paling tinggi iaitu 138.56 dan 204.93 µg asid galik/mg ekstrak mentah.

Akhir sekali, saringan sitotoksiti melalui kajian MTT yang dilakukan ke atas *ananixanthone* **(29)** dan empat derivatifnya terhadap tiga jenis sel kanser iaitu SNU-1, LS-174T dan K562 menunjukkan *ananixanthone* **(29)** mempamerkan aktiviti-aktiviti yang baik terhadap sel kanser SNU-1 dan K562 dengan nilai IC_{50} 8.97±0.11 µg/mL dan 2.96±0.06 µg/mL. Sebaliknya, *5-methoxyananixanthone* **(87)** menunjukkan aktiviti sitotoksik yang lebih memberangsangkan daripada sebatian induknya terhadap sel kanser LS-174T dengan nilai IC_{50} 5.76±1.07 µg/mL.

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I certify that a Thesis Examination Committee has met on 11 January 2017 to conduct the final examination of Lee Geok Imm on her thesis entitled "Stance and Stance-Support Strategies in English Argumentative Writing by Malaysian Undergraduate Writers" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
δ	Chemical shift in ppm
%	percentage
λ_{max}	Wavelength maxima in nm
°C	Degree celcius
^{1}H	Proton
^{13}C	Carbon-13
EC_{50}	Half-maximal effective concentration
µg	Micro gram
br*s*	Broad singlet
Ac_2O	Acetic anhydride
Me_2CO	Acetone
CC	Column Chromatography
COSY	Correlation Spectroscopy
cm	Centimeter
cm^{-1}	Per centimeter
d	Doublet
dd	Doublet of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulphoxide
EIMS	Electron Ionization Mass Spectrometry
EtOAc	Ethyl Acetate
FeCl3	Ferric Chloride

FTIR	Fourier Transform Infrared
g	Gram
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GPC	Gel Permeation Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
Hz	Hertz
IC_{50}	Half Maximal Inhibitory Concentration
IR	Infrared
J	Coupling constant in Hz
kg	Kilogram
L	Liter
Lit.	Literature
m	Multiplet
M^+	Molecular ion
mg	Milligram
µg	Microgram
mL	Milliliter
mm	Millimeter
mM	Millimolar
MeOH	Methanol
MHz	MegaHertz
m.p.	Melting point
MS	Mass spectrum/spectra/spectrometer/spectrometry
m/z	Mass per charge
nm	Nanometer

NMR	Nuclear Magnetic Resonance
ppm	Parts per million
q	quartet
s	singlet
t	triplet
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
ν_{max}	Wavenumber maxima in cm^{-1}
UATR	Universal Attenuated Total Reflection
UV	Ultra Violet
UV-Vis	Ultra Violet-Visible

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Evolution plays an important role in shaping natural products derived from macro-organisms such as plants, animals, marine organism and micro-organisms as well. It is through evolution that enables the biosynthetic engines of nature to produce a wide diversity of complex natural products. Natural products are very distinctive in their very own way due to their intricate stereochemistry characteristics and diverse functional groups (Khazir *et al*. 2013). The discovery and development of natural products research flourished during the World War II era after large scale production of penicillin took place followed by the search for new antibiotics (Baker *et al*. 2007).

Today, a huge array of compounds derived from natural products are extensively applied in the field of medicine, pharmacy and general biology. Due to their structural diversity, they inspire scientists and researchers to carry out screening tests with the hope of discovering new lead compounds to cure different diseases. Besides that, natural products derived drugs possess a high specificity with their biological targets which make them suitable candidates to act as curing agents. More interestingly, structural analogs by molecular modifications of the functional groups of the lead compounds are able to generate drugs with greater pharmacological activities and fewer side effects. It was reported that more than half of the currently available drugs are mainly from natural products and only 36% of 1073 small-molecule approved drugs for all diseases are synthetically made (Khazir *et al*. 2013). This shows how great the impact of natural products are on the world today.

Cancer, for example, is a silent killer which is responsible for many deaths of human beings. From a statistics by WHO, a total of 14 million cancer fatalities occurred in 2012 and it is expected to rise to 19 million by 2025. Natural products play a highly significant role in the discovery and development process of novel anti-cancer drugs. A survey by Newman and Cragg shows that 112 or 54% of the 206 anti-cancer drugs approved from 1940s to December 2010 are either natural products or their derivatives (Chen *et al*. 2015).

Hence, we can conclude that natural products have great potentials to serve mankind as primary options in human healthcare with the objective of creating a disease free world.

1.2 Botany of Plants Studied

1.2.1 The Family Clusiaceae

The family Clusiaceae or previously known as Guttiferae belongs to the order of Malpighiales. This family is widely comprised of 40 genera and 1000 species of shrubs and trees (Goh *et al*., 1992). Of the 40 genera, there are a few which are popular and commonly used in research due to their bioactivitiy potentials. They are namely *Calophyllum*, *Garcinia*, *Mesua* and *Mammea*.

Species from this family comprise of trees and shrubs with monopodially branched stems. They have stilt roots system. The stems are filled with white, yellow, or orange clear sap which flows from the resin canal. However, opaque latex are more commonly observed from this plant family. The leaves on the other hand are usually reddish in colour when young. The leaves do not have stipule-like scales but are frequently gland-dotted. Flowers from this family are usually bee-pollinated and display flowers with two to six imbricate sepals and petals.

The species from Clusiaceae family are very useful and commonly harvested as timber for the furniture industries. Some of the species also produce edible fruits and juices which contain high nutrition values and remedial properties such as *Garcinia mangostana* or commonly known as mangosteen.

1.2.2 The Genera *Calophyllum*

The genus *Calophyllum* belongs to the family of Clusiaceae. This genus is comprised of approximately 150 species worldwide and are mostly distributed in the tropical Asian region and the Pacific Islands. These plants flourish in the hill forests of the tropics at the altitude of 100-150m. Besides, these endemic trees are also known to grow in the wet lowlands. Due to their high tolerance against seawater, this genus can also be found growing along the seashores. In Malaysia, the biodiversity of our tropical jungles and humid weather give rise to the existence of several species from this genus such as *Calophyllum inophyllum*, *Calophyllum teysmannii*, *Calophyllum lowii*, *Calophyllum nodusum* and etc.

Calophyllum are locally known as bintangor. The trees can reach to a maximum height of 60m with a rough grey bark and a rounded head. Trees from this genus have evergreen broad leaves. Besides, the leaves are also shiny, leathery and oblong in shape. The trees also produce decorative figures on flat-sawn boards. The timbers' distinctive colour enhances their ability to serve as decorative objects such as furniture, parquet flooring, solid door construction, veneer and plywood.

Plants from this genus are widely known for their rich source of secondary metabolites such as xanthones, coumarins, and biflavonoids. These secondary metabolites also have remedial properties. Therefore, parts from the plants are commonly used by the locals as folk medicine. Decoctions of the bark and latex are used as medicine to heal diseases internally against diarrhea and after childbirth. The plant can also be utilized as an antiseptic and disinfectant to cure external illness such as skin and eye diseases.

1.2.3 Species *inophyllum*

This species is a large evergreen plant which originates from east Africa, Australia, and southern coastal area of India and Melesia. Due to its wide distribution across the globe, many names are associated with this species. In Malaysia, this plant is known as bintangor or penaga, beauty leaf in Australia, *tamanu* in Tahiti, and *undi* in India. *Inophyllum* species is a slow growing and low branching plant which is able to grow to a height of 8 to 20 m. Flowering occurs all year round with the width of the flower reaching 25 mm in width. The fruit is round in shape and normally observed as a green drupe with a diameter of 2 to 4 cm. The interior of the fruit contains a single large seed and the fruit changes colour from green to yellow or brownish red when ripens. The wood of this plant is traditionally used in construction due to the hardness and strength of the wood. *Tamanu* oil, which is extracted from the seeds are used in traditional medicine to cure illness. In a region in India, the bark is ground into powder and mixed with water before being applied to plants affected by a plant disease called *neeru vembu*.

1.2.3 Species *teysmannii*

This plant species varies from small to big trees which are capable of growing up to a height of 33 m. In the aspect of physical appearance, this species has brown to grey-brown stem bark with narrow, rough and shallow fissures. The leaves are leathery and oblong in shape and the flowers have four petals. The fruits are round in shape. This species are widely distributed around southeast asia countries such as Malaysia and Indonesia. The habitats of this species comprise peat swamps and tropical rainforest at elevations up to 1220 m. This species is utilized in constructions due to its hard wood properties and applied as traditional medicine among the locals.

Figure 1.1: *Calophyllum* tree

Figure 1.2: *Calophyllum* stem bark

Figure 1.3: Leaves of *Calophyllum*

Figure 1.4: Fruits of *Calophyllum*

1.3 Plants Selection

It has been proven from previous research that plants from the genus *Calophyllum* contain various types of secondary metabolites namely xanthones, coumarins, terpenenoids and flavonoids. Therefore this research project focused on the isolation of secondary metabolites from the selected plant species, *Calophyllum inophyllum* and *Calophyllum teysmannii*. The factor behind the specified selection was due to their great application values which were documented globally. Therefore, this sparked our interests to further the process of searching for new compounds from both of them. This study on *Calophyllum inophyllum* and *Calophyllum teysmannii* comprised the isolation of various types of compounds and the determination of their bioactivities.

1.4 Problem Statements

The needs and demand for new drugs to cure various new tropical diseases are on the rise due to the development of drug resistant diseases (Cordell., 2002). Therefore, natural products are significant for the purpose of serving as templates for future drug design and development candidates. Besides, the development of drugs nowadays are not sustainable which depletes the non-renewable resources and only 11 % of the 252 essential and basic drugs on the list are derived from plants (natural products) (Cordell., 2002).

1.5 Objectives of Study

The ultimate goal of this research is to isolate, analyze and elucidate the structures of the phytochemical compounds obtained from *Calophyllum inophyllum* and *Calophyllum teysmannii*. Besides, the crude extracts and the compounds isolated will be subjected to biological assays for bioactivity determination.
As such, the specific objectives stated below are to be met accordingly in order to complete and fulfill the requirement of this research.

a. To extract and isolate pure compounds from crude extracts of *Calophyllum inophyllum* and *Calophyllum teysmannii* through column chromatographic methods.

b. To elucidate and determine the molecular structures of the compounds from their spectra and data obtained via different modern spectroscopic techniques.
c. To determine the degree of bioactivities of the crude extracts by subjecting them to various bioassay screenings such as toxicity, total phenolic content, antioxidant, antimicrobial activities.
d. To structurally modify selected compound(s) and subject them for cytotoxicity screening via MTT assay against three cancer cell lines to determine their cytotoxic activities.

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