UNIVERSITI PUTRA MALAYSIA

PHYTOCHEMICAL CONSTITUENTS FROM Calophyllum buxifolium Vesque AND Calophyllum hosei Ridl AND THEIR BIOLOGICAL ACTIVITIES

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By

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PHYTOCHEMICAL CONSTITUENTS FROM *Calophyllum buxifolium* Vesque AND *Calophyllum hosei* Ridl AND THEIR BIOLOGICAL ACTIVITIES

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

Extensive phytochemical and biological studies to search for bioactive secondary metabolites from our Malaysian flora stem bark of two *Calophyllum* spp., *C. buxifolium* and *C. hosei* were carried out. These two plants were subjected to detail isolation work which involved extraction and purification of chemical components using various chromatographic techniques. All the pure compounds were identified using numerous spectroscopic analysis, such as 1D and 2D NMR, MS, IR, UV and also by comparison with literature data. These techniques have led to the isolation and elucidation of several chemical constituents of different type of classes which are the xanthones, coumarins, flavonoid, terpenoid and sterols. The crude extracts and several pure compounds from both plants species were also screened for their cytotoxic, anti-inflammatory and antimicrobial activities using MTT (Microculture Tetrazolium Salt), nitric oxide (NO) and disc diffusion assay. Human promyelocytic leukemia (HL60) and human breast adenocarcinoma (MCF7) cell lines were used in the cytotoxic assay. The anti-inflammatory activity was tested against RAW 264.7 murine macrophage cells in vitro that were treated with lipopolysaccharide (LPS) which induce inflammatory response. The antimicrobial activity was tested against three Gram positive bacteria, *Bacillus subtilis* B145, *Staphylococcus aureus* S276 and *Staphylococcus epidermis S273* and two Gram negative bacteria, *Escherichia coli* E266 and *Serratia marcencens* S381.

Detail work on the stem bark of *C. buxifolium* a plant never reported before for its phytochemistry has led to the isolation of two new chemical components, buxixanthone **(215)** and benjaminin **(223)** together with another 14 known compounds that consists of xanthones, flavonoids and terpenoids. Meanwhile from the study on three different extracts from the stem bark of *C. hosei* also an unreported plant, has successfully result in one new coumarin, hoseimarin **(222)** along with other 12 known xanthones and one common triterpenoid. There has been no previous report on the phytochemical work on the *C. hosei*.

The crude extracts for both plants gave a weak cytotoxic activity through MTT assay against two human cancer cell lines, HL60 (human promyelocytic leukemia) and MCF7 (human breast adenocarcinoma) cell line. Only four compounds, benjaminin **(223)**, mangostingone **(220)**, rubraxanthone **(221)** and β-mangostin **(178)** gave some activities towards the HL60 cell line with IC_{50} values of 96.13, 46.92, 10.53 and 7.16 μg / mL, respectively.

Anti-inflammatory assay was carried out for the crude extracts and 14 pure compounds using the nitric oxide (NO) assay. Most of the crude extracts for both plants gave a weak inhibition of NO production in RAW 264.7 cells. Eight xanthones, rubraxanthone **(221)**, buxixanthone **(215)**, ananixanthone **(183)**, dombakinaxanthone **(95)**, macluraxanthone **(87),** β-mangostin **(178)**, 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)-xanthone **(218)** and mangostingone **(220)** gave IC_{50} values of 6.45, 6.84, 7.14, 7.57, 9.07, 11.68, 12.05 and 12.34 µg / ml indicating their strong efficacy in reducing the NO production in RAW 264.7 cells. On the other hand, six more pure compounds gave mild inhibition on NO production in RAW 264.7 cells.

All the crude extracts for *C. buxifolium* and *C. hosei* gave no inhibition towards three Gram positive bacteria, *Bacillus subtilis* B145, *Staphylococcus aureus* S276 and *Staphylococcus epidermis S273* and two Gram negative bacteria, *Escherichia coli* E266 and *Serratia marcencens* S381.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SEBATIAN FITOKIMIA DARI *Calophyllum buxifolium* Vesque DAN *Calophyllum hosei* Ridl DAN AKTIVITI BIOLOGIKALNYA

Oleh

SHAARI BIN DAUD

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Pengerusi: Profesor Gwendoline Ee Cheng Lian, PhD
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Kajian intensif fitokimia dan biologikal dalam pencarian metabolit sekunder dari flora Malaysia telah dijalankan terhadap dua spesies *Calophyllum*, iaitu *C. buxifolium* dan *C. hosei*. Dua pokok ini akan dikaji secara terperinci di mana ia melibatkan pengekstrakkan dan penulenan komponen kimia dengan menggunakan pelbagai kaedah kromatografi. Semua sebatian tulen dikenalpasti menggunakan pelbagai analisis spektroskopi seperti 1D dan 2D Resonan Magnetik Nuklear (RMN), spetroskopi jisim (MS), Infra merah (IR), ultra violet (UV) dan juga perbandingan dengan data terdahulu.

Melalui teknik-teknik ini ia telah membolehkan pengasingan dan pengenalpastian untuk beberapa jenis kelas sebatian kimia iaitu xanthon, kumarin, flavonoid, terpenoid dan sterol. Ekstrak mentah dan beberapa sebatian tulen daripada kedua-dua spesis pokok ini juga diuji untuk aktiviti sitotoksik, anti keradangan dan aktiviti mikrobial dengan menggunakan asai MTT (Garam tetrazolium mikrokultur), nitrik oksida (NO) dan cerakin penyebaran cakera. Dua talian sel karsinoma manusia iaitu sel karsinoma promyelocytic leukemia (HL60) dan sel karsinoma payu dara (MCF7) telah digunakan di dalam ujian sitotoksik ini. Anti keradangan pula telah diuji secara *in vitro* dengan menggunakan sel RAW 264.7 murin makrofaj yang telah dirawat dengan lipopolisakarida (LPS) di mana ia bertindak sebagai agen keradangan. Tiga bakteria gram positif iaitu *Bacillus subtilis* B145, *Staphylococcous aureus* S276 dan *Staphylococcus epidermis* S273 dan dua bakteria gram negatif, *Escherichia coli* E266 dan *Serratia marcencens* S381 telah digunakan dalam menguji aktiviti antimikrobial.
Kajian fitokimia yang terperinci terhadap kulit batang pokok *C. buxifolium* telah membolehkan pengasingan dua sebatian kimia baru iaitu buksixanthon **(215)** dan benjaminin **(223)** bersama dengan 13 lagi xanthon sebatian biasa yang terdiri daripada xanthan, flavonoid, triterpenoid dan sterol. Sementara itu kajian terhadap tiga ekstrak mentah daripada satu lagi pokok yang belum pernah dikaji iaitu *C. hosei* telah berjaya mengasingkan satu kumarin yang baru iaitu hoseimarin **(222)** bersama dengan 12 lagi xanthon dan satu triterpenoid biasa. Tiada laporan tentang kajian fitokimia terhadap *C. hosei* dilaporkan sebelum ini.

Ekstrak mentah dari kedua-dua pokok memberi aktiviti sitotoksik yang lemah terhadap dua talian sel barah iaitu HL60 (human promyelocytic leukemia) dan MCF7 (human breast adenocarcinoma). Hanya empat sebatian kimia iaitu, benjaminin **(223)**, mangostingon **(220)**, rubraxanthon **(221)** and β-mangostin **(178)** memberi aktiviti terhadap sel HL60 masing-masing dengan nilai IC_{50} 96.13, 46.92, 10.53 dan 7.16 μg / mL.

Kebanyakkan ekstrak mentah daripada kedua-dua tumbuhan ini memberi nilai perencatan penghasilan NO pada sel RAW 264.7. Lapan xanthon, rubraxanthon **(221)**, buksixanthon **(215)**, ananixanthon **(183)**, dombakinaxanthon **(95)**, makluraxanthon **(87),** β-mangostin **(178)**, 1,3,7-trihidroksi-2-(3-metilbut-2-enil)-xanthon **(218)** dan mangostingon **(220)** memberi nilai IC_{50} 6.45, 6.84, 7.14, 7.57, 9.07, 11.68, 12.05 dan 12.34 µg / ml menunjukkan keberkesanan yang kuat dalam menurunkan penghasilan NO di dalam sel RAW 264.7. Manakala, enam lagi sebatian kimia tulen memberi perencatan sederhana pada penghasilan NO dalam sel RAW 264.7.

Semua ekstrak mentah untuk *C. buxifolium* dan *C. hosei* tidak memberikan sebarang perencatan terhadap tiga bakteria Gram positif, *Bacillus subtilis* B145, *Staphylococcus aureus* S276 dan *Staphylococcus epidermis S273* dan dua bakteria Gram negatif, *Escherichia coli* E266 dan *Serratia marcencens* S381.

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I certify that a Thesis Examination Committee has met on 19 May 2017 to conduct the final examination of Shaari bin Daud on his thesis entitled "Phytochemical Constituents from *Calophyllum buxifolium* Vesque and *Calophyllum hosei* Ridl and their Biological Activities" 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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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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δ	Chemical shift in ppm
λ_{max}	Wavelength maxima in nm
˚C	Degree celcius
^{1}H	Proton
^{13}C	Carbon-13
IC_{50}	Initial Concentration to kill 50% of cells
cm	centimeter
d	Doublet
D	Deuterated proton
DEPT	Distortionless Enhancement by Polarization Transfer
EIMS	Electron ionization mass spectrometry
EtOH	Ethanol
FTIR	Fourier Transform Infra-Red
g	Gram
GC-MS	Gas chromatography-mass spectroscopy
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
Hz	Hertz
IR	Infra-Red
J	Coupling constant
Lit.	Literature review
m	Multiplet
m	meter
[M]+	Molecular ion

mg	Milligram
MHz	Megahertz
m.p.	Melting point
MS	Mass Spectrometry
m/z	Mass per charge
NMR	Nuclear Magnetic Resonance
nm	nanometer
Rf	Retention Factor
s	Singlet
t	Triplet
TLC	Thin layer chromatography
TMS	Tetramethylsilane
µg	microgram
µM	Micro molar
UATR	Universal Attenuated Total Reflection
UV	Ultraviolet
ν_{max}	Wavenumber maxima in cm-1

CHAPTER 1

INTRODUCTION

1.1 General introduction

Phytochemistry are usually referred or deals to the analysis of plant chemical that found in plants, which gave health benefits for humans further than those attributed to macronutrients and micronutrients (Hasler *et al.*, 1999). Meanwhile, phytochemicals can be described as plant chemicals that protect plant cells from environmental hazards such as pollution, stress, UV exposure, drought and pathogenic attack. Phytochemicals can be classified to primary or secondary compounds. The common sugars, amino acids, proteins, purines and chlorophyll's are consider as primary constituents. On the other hand, alkaloids, flavonoids, saponins, terpenes, lignans, phenolics and glucosides are the remaining plant chemicals whereas classified as secondary constituents (Hana Ni, 1998). Phenolics are the most numerous and structurally diverse plant phytocontituents based on literature survey. Phytochemical constituents also can be referred as natural products since it source come from plants.

Natural products can be defined as secondary metabolites produced by organisms in response to external stimuli such as nutritional changes, infections and competition. Natural products are usually isolated as biologically active pharmacophores from plants, fungi, bacteria, protozoans, insects and animal. These valuable natural products can be highlighted by their usage today in medical and animal health industries such as lovastatin (anticholesterolemic agent), paclitaxel and doxorubicin (antitumor agents), erythromycin (antibiotic), and amphotericin B (fungicidal agent).

Natural products have played an important role in the drug discovery process. Most of the old society and agrarian societies use plant-derived natural products as therapies for quite a number of diseases ranging from infections to emphysema. About one third of the top selling drugs in the world are natural products or their derivatives. Natural products in extract form consist of various secondary metabolites. Some of these metabolites can react as active components and give positive effects to some diseases. In order to obtain these active pure compounds, extraction and isolation processes need to be carried out. However these processes are tedious and time consuming. The inavailability of sufficient plant materials, selection and implementation of appropriate screening bioassay and to obtain these bioactive compounds in large amounts are also the constraints for the search for new lead compounds in drug discovery.

Even though the process of finding new active compounds from natural product research is difficult compared to the synthetic conventional drug research most users still prefer drugs that are derived from natural products. This is probably due to the fact that the natural product secondary metabolites have fewer side effects as compared to synthetic drugs and they are also safe therapeutic agents (Deltito *et al.*, 1998).

Moreover, the increasing public concern for a healthy lifestyle has made the natural extracts to be more reliable as nutraceuticals and agrochemicals for them.

Since secondary metabolites are structurally complex molecules, characterization need to be carried out using modern spectroscopic instruments such as gas chromatography-mass spectrometry (GCMS), nuclear magnetic resonance (NMR), infrared (IR) and ultra-violet spectroscopy (UV). Identification of naturally occurring products can be solved by natural product chemists using those instruments. Alkaloids, steroids, coumarins, xanthones, acids and flavonoids are the commonly isolated secondary metabolites that have been reported to possess medicinal properties.

1.2 Botany of Plants Studied

1.2.1 The Family Clusiaceae

The Clusiaceae family, formerly known as the Guttiferae, belongs to the order of Malpighiales. They consists of about 40 genera and over 1600 species of trees and shrubs (Goh *et al.*, 1992). Basically there are 4 important genera of the Clusiaceae of importance namely *Garcinia, Calophyllum, Mesua* and *Mammea*. *Calophyllum* species are important for their timbers, while some species of *Garcinia* produce edible fruits and important resin in pigment manufacturing.

Members of the Clusiaceae are usually found as trees or shrubs and can be recognized by the latex in their stem, leaves, fruit and bark and fruits of capsules for seeds. The flowers are bee-pollinated, actinomorphic with numerous stamens and separate petals. The leaves are variable in size with opposite leaves often with fine parallel nerves and without true stipulates.

1.2.2 The Genus *Calophyllum*

The *Calophyllum* plants are usually referred to as "beautiful leaf". This is because the name "*Calophyllum*" comes from the Greek language, in which "kalos" means beautiful, while "phullon" means leaf. This genus has around 180-200 species of tropical evergreen trees in the family Clusiaceae. It is widely distributed in Australasia, Madagascar, Eastern Africa, South and Southeast Asia, the Pacific islands, the West Indies and Latin America (Morel *et al.*, 2000). There are a few common names for *Calophyllum*, whereas based on the geographical areas such as Malaysia it is known as Bintagor tree, India as Poon tree, and Latin America as Guanandi, Jacareuba or Santa Maria.

Calophyllum plants are mainly widespread from the sea-shore to the highest mountain tops, mixed peat swamps, freshwater swamp and mixed dipterocarp forests. Trees from

this genus are usually found as small to medium-sized, without buttresses. Meanwhile, the inner barks of this genus are pink to red, laminated, exuding sticky and yellow or white varnish. On the other hand, leaves of this genus are generally narrow and less channeled above while the flowers and fruits are favoured by bees, birds and small mammals. Wood from this genus are usually used for local purposes while the large round nuts have contributed to the main source for seed oil production (Dweck *et al.*, 2002).

1.2.3 The Species *Calophyllum buxifolium*

Calophyllum buxifolium is a small gnarled tree with low buttresses. This tree can reach 80 ft. in height. This tree has ruggedly thick flaky bark surface and is chocolate-brown colour. Furthermore, the inner bark is orange-brown and exudate is yellow in colour while the leaves are broadly elliptic and variable in shape but always thickly coriaceous. The flower is 7 mm wide and the fruit 2.5 cm long, 1.8 cm wide, ellipsoid and apiculate. This fruit will be chocolate-brown in colour when it dries.

This species is usually distributed at Philippines, Sabah, Sarawak and Brunei. Their habitat is common on skeletal soils or on sandy plateaux. There are no phytochemical works yet for this species.

Figure 1.1: The Leaves and Twigs of *Calophyllum buxifolium*

1.2.4 The Species *Calophyllum hosei*

Calophyllum hosei is widely distributed in Borneo, Sarawak and Brunei. *Calophyllum hosei* is a small to medium sized tree which occasionally can grow up to 80 ft tall and 5 ft wide. This species also has a glabrous pubescent bud and shiny leaf blade which then changes to copper-brown when the leaf dries up. *Calopyllum hosei* commonly grows in ground-water podsols and shallow peat.

There are no reports on their uses and phytochemistry.

Figure 1.2: The Leaves and Twigs of *Calophyllum hosei*

1.3 Problem Statement

Cancer disease is one of the major threats to human beings in this world. The number of people suffering from this disease keeps on increasing year by year. As we know inflammation is one of the caused that contribute to cancer disease. Inflammation is a normal and main process created by naturally by our body. The process of inflammation helps to get rid of unwanted bacteria, and other invaders. Inflammation also helps our body to cleaning up dead cells from infections. Chronic inflammation can drive to cancer disease. Thus, research on the finding of active crude extracts or pure compounds from natural resources for future anticancer and anti-inflammatory drugs need to be carried out.

The use of natural products as therapeutics and their medicinal properties have been known since a long time ago. This can be clearly seen by the ancient society using plant crude extracts in their routine life in therapies for treating diseases. Synthetic drugs that we have been using to threat disease are now believed to give long term side effects. Hence, work on the discovery of drugs from natural active compounds that have less side effects from plants is still in high demand.

The discovery of new chemical constituents that have bioactive properties through screening of natural products from *Calophyllum* which are not accessible by other technologies will be of great importance to the pharmaceutical industry and public health. The isolation techniques and structural elucidation of new compounds as well as bioassay results will also be useful for other natural product chemists for future research work. There are no reports on the phytochemistry of *Calophyllum buxifolium* and *Calophyllum hosei*. The significant discovery of lead compounds from this research

can contribute to the search for alternative drugs to the presently available drugs in the market.

1.4 Objectives of Study

The main objectives of this study are:

1. To extract and isolate the chemical components from the stem bark of *Calophyllum buxifolium* and *Calophyllum hosei*

2. To identify and elucidate the structures of the pure components using varies spectroscopic methods.

3. To screen and evaluate the anti-inflammatory, anti-cancer and anti-microbial biological activities of each crude extract and pure isolated constituents.

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