CHEMICAL CONSTITUENTS AND BIOACTIVITIES OF *Garcinia griffithii* T. ANDERSON

NIK SHAZWANI AFIFAH BINTI NIK SAZALI

UNIVERSITI TEKNOLOGI MALAYSIA

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NIK SHAZWANI AFIFAH BINTI NIK SAZALI

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Dedicated to

My most beloved mother and father
My dearest sisters and brothers
My friends

Who always supportand inspire me all the time.

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PREFACE

This thesis is the result of my work carried out in the Department of chemistry, Universiti Teknologi Malaysia under the supervision of Assoc. Prof. Dr. Farediah Ahmad. Parts of my work described in this thesis have been reported in the following publications:

1. Nik Shazwani Afifah Nik Sazali and Farediah Ahmad (2011). Chemical Constituents and Biological Activities of The Leaves of *Garcinia Griffithii* (Guttiferae). Poster presented at International Conference on Natural Products (ICNP) 2011, Palm Garden Hotel, IOI Resort, Putrajaya. 13 – 16 November 2011.

ABSTRACT

Chemical and bioactivity investigations were carried out on the leaves and stem barks of *Garcinia griffithii* T. Anderson from Guttiferae family. Sample for each part of the plant was extracted consecutively with increasing polarity of solvents by Soxhlet method. Vacuum liquid chromatography and column chromatography were used to purify the crude extracts. The pure compounds were elucidated by using combined spectroscopic techniques which include UV, IR, NMR (1D and 2D) and MS. Chromatographic purification of the leaves extracts have afforded nine pure compounds identified as squalene, 28-hydroxyfriedelan-3-one, friedooleanan-3-one, olean-12-en-3-ol, amento-4'-methyl ether, 3,8''-binaringenin, 3,8''-binaringenin-7''-*O*-glucoside, morelloflavone and morelloflavone-7''-*O*-glucoside. Chromatographic purification of the ethyl acetate and methanol extracts of the stem barks yielded two compounds identified as amento-4'-methyl ether and morelloflavone. The crude extracts and pure compounds isolated from methanol crude extract of the leaves were screened for various types of antioxidant assay and tyrosinase inhibition activities. The antioxidant assay on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical showed that the crude *n*-hexane extract of the stem barks had the highest radical scavenging activity with IC_{50} = 96.43 ± 2.69 μg/mL, while morelloflavone was found to be the strongest antioxidant compound with IC_{50} = 57.57 ± 0.53 μg/mL compared to other compounds. The crude methanol extract of the stem barks showed the highest total antioxidant with 260.81 ± 2.21 mg/g of ascorbic acid equivalent (AAE/L) and 871.43 ± 6.62 mg/g of butylated hydroxyl toluene equivalent (BHTE/L) while the crude methanol extract of the leaves showed the highest total phenolic content with 444.10 ± 6.67 mg/g of gallic acid equivalent (GAE/L) and 423.10 ± 6.67 mg/g of (±)-cathechin equivalent (CE/L). Morelloflavone showed the highest value for both assays with values 58.50 ± 3.15 mg/g of AAE/L and 264.50 ± 9.45 mg/g of BHTE/L; and 841.33 ± 38.28 mg/g of GAE/L and 822.97 ± 33.93 mg/g of CE/L, respectively. The crude extracts and all compounds were found to have weak anti-tyrosinase activity. The antimicrobial assay of all the crude extracts were carried out by using disc diffusion method, followed by minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). The methanol crude extract of the leaves showed the most significant antimicrobial activity towards *E. faecalis* and *K. pneumoniae* with MIC and MBC value ranged between 225 – 450 μg/mL compared to the other crude extracts.

ABSTRAK

Kajian kimia dan bioaktiviti telah dijalankan ke atas daun dan batang pokok *Garcinia griffithii* T. Anderson daripada keluarga Guttiferae. Sampel untuk setiap bahagian pokok telah diekstrak secara berturutan dengan menggunakan pelarut mengikut kekutuban menaik melalui kaedah Soxhlet. Kromatografi turus vakum dan kromatografi turus gravitI telah digunakan untuk penulenan ke atas ekstrak mentah. Struktur sebatian tulen dikenalpasti dengan menggunakan kaedah spektroskopi UV, IR, NMR (1D dan 2D) dan MS. Penulenan dan pencirian ke atas ekstrak mentah *n*-heksana daripada daun telah Berjaya menemukan Sembilan sebatian tulen yang dikenalpasti sebagai skualena, 28-hidroksifriedelan-3-on, friedooleanan-3-on, olean-12-en-3-ol, amento-4'-metil eter, 3,8"-binaringenin, 3,8"-binaringenin-7"-*O*-glukosida, morelloflavon dan morelloflavon-7"-*O*-glukosida. Penulenan secara kromatografi ke atas ekstrak etil asetat dan methanol batang pokok menghasilkan dua sebatian yang dikenalpasti sebagai amento-4'-metil eter dan morelloflavon. Ekstrak mentah dan sebatian tulen yang telah diasingkan daripada ekstrak mentah methanol daun disaring untuk beberapa ujian antioksidan dan aktiviti rencatan tirosinase. Ujian antioksidan ke atas radikal bebas 2,2-difenil-1-pikrilhidrazil (DPPH) menunjukkan bahawa ekstrak mentah *n*-heksana daripada batang pokok mempunyai aktiviti radikal tertinggi dengan nilai IC_{50} = 96.43 ± 2.69 μg/mL, manakala sebatian morelloflavon ditemui sebagai sebatian antioksidan terkuat dengan nilai IC_{50} = 57.57 ± 0.53 μg/mL berbanding dengan sebatian lain. Ekstrak mentah methanol daripada batang pokok menunjukkan nilai jumlah antioksidan yang paling tinggi dengan nilai 260.81 ± 2.21 mg/g setara dengan asid askorbik (AAE/L) dan 871.43 ± 6.62 mg/g setara dengan butil hidroksitoluena (BHTE/L) manakala ekstrak mentah methanol daripada daun mempunyai jumlah kandungan fenol yang paling tinggi, iaitu 444.10 ± 6.67 mg/g setara dengan asid galik (GAE/L) and 423.10 ± 6.67 mg/g setara dengan (±)-katekin (CE/L). Sebatian morelloflavon menunjukkan nilai tertinggi bagi kedua-dua saringan masing-masing dengan nilai 58.50 ± 3.15 mg/g AAE/L dan 264.50 ± 9.45 mg/g BHTE/L; dan 841.33 ± 38.28 mg/g GAE/L dan 822.97 ± 33.93 mg/g CE/L. Ekstrak mentah dan kesemua sebatian tulen didapati mempunyai aktiviti anti-tirosinase yang lemah. Saringan antimikrob telah dijalankan ke atas ekstrak mentah dengan menggunakan kaedah pembauran cakera, diikuti dengan penentuan nilai rencatan minimum (MIC) dan kepekatan bakterisida minimum (MBC). Ekstrak mentah methanol daripada daun menunjukkan aktiviti antimikrob yang paling signifikan terhadap *E. faecalis* and *K. pneumonia* dengan nilai MIC dan MBC antara 225 – 450 μg/mL berbanding dengan ekstrak mentah yang lain.

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LIST OF ABBREVIATIONS

AAE/L	-	Ascorbic acid equivalent
BHTE/L	-	Butylated hydroxytoluene equivalent
br	-	Broad
CE/L	-	(±)-Catechin equivalent
°C	-	Degree celcius
^{13}C	-	Carbon 13
CC	-	Column Chromatography
$CDCl_3$	-	Deuterated Chloroform
$CHCl_3$	-	Chloroform
CH_2Cl_2	-	Dichloromethane
CIMS	-	Chemical Ionization Mass Spectrometry
COSY	-	Correlation spectroscopy
δ	-	Chemical shift
cm^{-1}	-	Per centimeter
d	-	Doublet
dd	-	Doublet of doublet
DEPT	-	Distortionless Enhancement by Polarization Transfer
DMSO	-	Dimethyl sulfoxide
DPPH	-	2,2-Diphenyl-1-picryhydrazyl
EIMS	-	Electron Impact Mass Spectrometry
Et_2O	-	Diethyl ether
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol
FTIR	-	Fourier Transform Infrared
GAE/L	-	Gallic acid equivalent
^{1}H	-	Proton

H_2O	-	Water
H_3BO_3	-	Boric acid
HMBC	-	Heteronuclear Multiple Bond Correlation
HMQC	-	Heteronuclear Multiple Quantum Coherence
Hz	-	Hertz
IC_{50}	-	Inhibition concentration for 50%
IR	-	Infrared
J	-	Coupling constant
KBr	-	Potassium bromide
L	-	Liter
λ	-	Lambda
m/z	-	Mass to charge ratio
m	-	Multiplet
m.p.	-	Melting point
µg	-	Microgram
mg	-	Miligram
mL	-	Mililiter
MBC	-	Minimum bactericidal concentration
MeOH	-	Methanol
MIC	-	Minimum inhibitory concentration
nm	-	Nanometer
NA	-	Nutrient agar
NB	-	Nutrient broth
NMR	-	Nuclear Magnetic Resonance
ppm	-	Parts per million
quint	-	Quintet
R_f	-	Retention factor
s	-	Singlet
SiO_2	-	Silica gel
t	-	Triplet
TLC	-	Thin Layer Chromatography
UV	-	Ultraviolet
VLC	-	Vacuum Liquid Chromatography

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

A natural product is a chemical compound or a substance produced by living organisms, plants, animals, insects and microbes. These chemical constituents usually have pharmacological or bioactivities that can be applied in pharmaceutical and drug design [1]. They have, until recently, been the primary source for the commercial medicines and drug research development [2]. Natural products chemistry has always been concerned with the discovery of bioactive constituents and it remains one of the main keys that play an important role in the continuous research for new drugs in the industrial drug discovery process [3]. Besides, many natural products have reached the market without chemical modification, thus the potential to commercialize these small, drug-like molecules are very economical [4]. The abundance of natural renewable supply of plants and herbs indeed is a great source of affordable drugs, which is complimentary to the modern medicine [5].

The practices of modern medicine have yielded numerous purified compounds with medicinal properties as the result of the chemical investigations and purification of extracts of plants. These compounds have been developed into pharmaceutical agents [6]. To date about 25% of all available modern drugs such as morphine and salicylates are derived directly or indirectly from higher plants. These interesting compounds developed major classes of the analgesic drugs, namely, opioids which are classified as central nervous system depressants and non-steroidal anti-inflammatory drugs. Over the years, natural products and their derivatives are

not only used clinically, but also play an important role in discovery of new targets such as receptors, enzymes, transporters or ion channels involved in relevant physiological and pathological processes [7].

The analysis of plant components should thus begin with bioactivity-directed screening and bioactivity-directed fractionation leading to the isolation and characterization of pure biologically active compounds [8]. With the advancement of spectroscopic methods, numerous types of active compounds can be isolated from plants and are structurally characterized. In due course, many of these compounds are synthesized in the laboratory. Sometimes, better-tolerated drugs are produced by chemical modifications or by total synthesis of analogues of the active principles [9]. As a result, the findings from this area of research will assist in contributing to the evolution in practice of traditional and modern medicineby utilizing them on a larger commercial basis to eliminate health problems. Therefore, today, an increase of global interest for industrial production concurrently meet the demand for conserving biodiversity to enhance the development of renewable natural products for medicine [10].

1.2 Guttiferae Family

Guttiferae family is one of the families in order Guttiferals. The family contains about 48 genera and over 1000 species of perennial herbs, shrubs and trees, widely distributed by the tropical and temperate regions of the world [11]. Most members of the family are in view of their economical and medicinal purposes in many parts of the world for treatment of different illnesses [12]. In Malaysia, Guttiferae is an important component of the Malaysian Rain Forest as the second-storey forest trees which include some well-known and important trees such as ironwood tree, mangosteen and penaga laut. Whitemore [11] identified and classified four genera and 121 species that can be found in Peninsular Malaysia which are *Calophyllum* (45 species), *Garcinia* (49 species), *Mammea* (23 species) and *Mesua* (4 species).

The trees or shrubs of this family have inner barks with yellow or white latex in droplets. The bark is smooth, fissured or scaly patter and the bole rarely with stilt roots or buttresses [13]. The leaves are mostly opposite without true stipule and the secondary nerves often numerous, close and parallel. Flowers are bisexual or sometimes unisexual, have scented and can be on the twigs behind the leaves. The sepals and petals are four to five each and overlapping whereas, the stamens usually connate in bundles. The fruits of this family can be a drupe for *Calophyllum* and *Mammea*, a nut for *Mesua* or a berry with the seeds embedded in pulp, not splitting with wall leathery of fleshy for *Garcinia* [11, 14].

Primarily, the genus *Garcinia* is the biggest genus with the common village fruit-trees such as *G. atroviridis* (*asam gelugor*), *G. cowa* (village *kandis*) and *G. prainiana* (*kechupu*). The trees are small to medium, rarely taller than 30 m; therefore the trees are almost completely confined to the interior of the forest, in shade. Many species of *Garcinia* have very similar leaves but differ in flower and fruit characters [11]. The fruit hull of *G. mangostana* has found many uses in traditional medicine such as for healing skin infections and wounds in Thai folk medicine [15].

The second largest genus is *Calophyllum* called '*bintangor*' by Malays. It provides timber such as *C. coriaceum* (*bintangor gunung daun besar*), *C. cuneatum* (*bintangor gunung daun kecil*) and *C. inophylloide* (*bintangor batu*). Trees of this genus have the largest size among the other three genera with heights of up to 30 – 36m and are common of the lowland and mountain forest. *C. inophyllum* has wide variety of cures found from its oil to its roots. Its gum, bark, leave, and all other parts of it are found to be curative in medicine hence known as the 'All Heal' plant [16]. The seed oil of *C. inophyllum*is reported to cure rheumatism and skin affections in Indian traditional medicine [17].

The Malay name for genus *Mesua* is '*penaga*' and also consists of valuable timber with very hard and heavy wood such as *M. grandis* (*penaga sabut*), *M. lepidota* (*penaga tikus*) and *M. nuda* (*penaga lilin*). The trees comprise small to medium trees with height up to about 23 m [11]. *M. ferrea*is a very well-known

species; the word '*ferrea*' is from the Latin word, '*ferrum*' which means 'iron' referring to its extremely hard wood [18]. The heartwood is very hard, dense, strong, heavy and durable like ebony and used extensively for heavy construction, in machinery work, vehicles and agricultural implements [19]. In Malaysia, the kernels of *M. ferrea* are pounded with the seed oil and applied to wounds as poultice [18].

The last genus identified in Malaysia is *Mammea* consists of *M. brevipes*, *M. malayana*, *M. siamensis* and *M. odorata*. The Malaysian wild *Mammea* species are rare trees and restricted to the lowland. The genus has small trees with about 15 m height and has little important uses to human. *M. siamensis* has a big flower which smell of violets and planted in temple. Its pollen is reputed to be used as a cosmetic. The well-known species in this genus is *M. americana*, the sole American species which produces large edible apricot-like fruits called as the Mammea apple [11].

1.3 *Garcinia griffithii*

Species of *Garcinia griffithii* (apple-*kandis* or *kandis gajah*) is a small to medium tree that may reach 23 m tall and conspicuous from the large leaves and fruits. Its latex is yellowish white and the inner bark with opaque yellow exudate. The sapwood and heartwood cannot be distinct and in dark red-brown colour. The leaves have very large blade with size from 15×7 to 28×16 cm, broady elliptic, strongly ribbed and pointed. The edges incurved and has base rounded. The young leaves are pink and the drying leaves are blackish green with thin-texture. The flowers mainly in short woody on the twigs behind the leaves and have four sepals and petals with yellow flushed red colours at the base. The fruits are characteristically globose, faintly ribbed, subsessile, fattened at the top, clustered on the branched, edible and turning brownish yellow with watery acid flesh like green apple. The stigma is usually sunken entirely, flat or slightly convex. The species is common in Peninsular Malaysia at the low land forest [11, 16, 20].

1.4 Problem Statement

To date, there are two reports on the phytochemicals and antiplasmodial activity of *Garcinia griffithii* collected from Indonesia [21] and Singapore [22]. However, the bioactivities of the plants were not studied thoroughly. Although the climate and ecology of Singapore and Indonesia are similar with Malaysia, this study is still being continued hoping to get variations of chemical constituents with interesting bioactivities. The data obtained will be utilized for future research on the chemical markers of *Garcinia* species from Malaysia.

1.5 Objectives of Research

The objectives of this research are:

- To isolate the chemical compounds from the leaves and stem barks of *G. griffithii*.
- To elucidate the structures of the chemical compounds by combined spectroscopic methods.
- To carry out the antioxidant, antimicrobial and tyrosinase inhibition activities on the crude extracts and pure compounds of *G. griffithii*.

1.6 Scope of Research

This research will focus on the leaves and stem barks of *G. griffithii*. The sample will be extracted by Soxhlet apparatus using *n*-hexane, dichloromethane, ethyl acetate and methanol as the solvent to afford the crude extracts. Separation and purification of the natural compounds from the crude extracts will be carried out with chromatographic techniques such as vacuum liquid chromatography and

column chromatography. The structure of the pure compounds will be elucidated with various spectroscopic techniques such as UV, IR, NMR (1D and 2D) and MS.

The crude extracts will be screened for bioactivity focusing on antioxidant, tyrosinase inhibition and antimicrobial activities. The antioxidant assay by DPPH method, the total antioxidant assay by formation of the green phosphomolybdenum complex and the total phenolic content assay using Folin-Ciocalteaumicro method will be used to screen the antioxidant activities of the samples. The antimicrobial assay will be carried out by disc diffusion method against two Gram-positive bacteria: *Enterococcus faecalis* and *Bacillus subtilis* and two Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. Further evaluation of the antimicrobial activity will be carried out by determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). The isolated compounds will be screened for antioxidant as above and tyrosinase inhibition activities only.

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