CHEMICAL CONSTITUENTS AND BIOACTIVITIES OF THE FRUITS AND LEAVES OF *Phaleria macrocarpa* (SCHEFF.) BOERL.

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Special dedication to

My beloved bonda, Pn. Hajjah Siti Zarah Mahpok.

My sisters and brothers,
Saiful Nizam
Fahrul Rozi
Siti 'Ainur Iza Hairani
Shahrul Bazli
Siti Nur 'Aqila

For all your love, prayers, support, and sacrifice.

Thank you so much.

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PREFACE

This thesis is the results of my own work carried out at the Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia and Department of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia between July 2011 and January 2014 under the supervision of Dr. Norazah Basar and Dr. Siti Pauliena Mohd Bohari. Part of my work describe in this thesis has been reported in the following publication:

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ABSTRACT

Phytochemicals and bioactivities of the fruits and the leaves of *Phaleria macrocarpa* have been studied. Successive extraction of the dried fruits using cold extraction followed by fractionation and purification afforded two benzophenones identified as 2,6,4'-trihydroxy-4-methoxybenzophenone and 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-*β*-D-glucopyranoside and two triterpenoids identified as 24-methylenecycloartan-3-one and 24-methyl-9,19-cyclolanost-25-en-3-ol. Meanwhile, cold extraction of the dried leaves followed by fractionation and purification yielded two sterols known as stigmasterol and β-sitosterol. Fractionation and purification process were done using vacuum liquid chromatography and column chromatography, respectively. The structures of isolated compounds were elucidated on the basis of their spectral data including 1D and 2D Nuclear Magnetic Resonance (^{1}H, ^{13}C, DEPT, COSY, HMQC, HMBC), Infrared, Ultraviolet spectroscopies and mass spectrometry. The antibacterial activities of all crude extracts and isolated compounds were performed using disc diffusion method followed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against two Gram-positive bacterial strains, *Bacillus subtilis* and *Staphylococcus aureus* together with two Gram-negative bacterial strains including *Escherichia coli* and *Pseudomonas putida*. The results have demonstrated that all extracts and isolated compounds exhibited weak activity against all tested bacteria with the MBC values exceeded 1000 μg/mL. Evaluation of antioxidant activity using 2,2-diphenyl-1-picrylhydydrazyl (DPPH) assay has revealed strong free radical scavenging properties of ethyl acetate and ethanol extracts from the fruits with SC_{50} of 14.00 and 19.97 μg/mL, respectively. The chloroform extract from the fruits and all extracts from the leaves exhibited moderate antioxidant activities with SC_{50} ranging between 58.07 – 94.10 μg/mL. Among the isolated compounds, 2,6,4'-trihydroxy-4-methoxy-benzophenone exhibited high free radical scavenging activity with SC_{50} of 29.73 μg/mL whereas the other isolated compounds were inactive against DPPH. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to evaluate the cytotoxic activity of crude extracts and isolated compounds. MTT assay of CHO, MCF-7, MDA-MB-231 and MDA-MB-468 cells were treated with all extracts and isolated compounds whereas the cell viability of 3T3, HeLa and HT-29 were treated by extracts of the fruits and two isolated benzophenones. The result has demonstrated good activity of dichloromethane extract from the leaves against MDA-MB-231 cell line (IC_{50} 70 μg/mL) and moderate cytotoxic effect of ethanol extract from the fruits towards MDA-MB-468 and HT-29 cell lines with the same IC_{50} of 100 μg/mL. Chloroform extract from the fruits was found to have moderate cytotoxic properties against HT-29 cell line (IC_{50} 100 μg/mL). All isolated compounds were found to have weak cytotoxic activity towards all tested cell lines.

ABSTRAK

Fitokimia dan bioaktiviti terhadap buah dan daun *Phaleria macrocarpa* telah dikaji. Pengekstrakan ke atas buah kering menggunakan pengekstrakan sejuk diikuti dengan fraksinasi dan penulenan telah berjaya menghasilkan dua benzofenon yang dikenal pasti sebagai 2,6,4'-trihidroksi-4-metoksibenzofenon dan 6,4'-dihidroksi-4-metoksibenzofenon-2-*O*-*β*-D-glukopiranosida dan dua triterpenoid yang dikenal pasti sebagai 24-metilensikloartanon-3-on dan 24-metil-9,19-siklolanost-25-en-3-ol. Manakala, pengekstrakan sejuk ke atas sampel daun kering diikuti dengan fraksinasi dan penulenan telah menghasilkan dua sebatian sterol iaitu stigmasterol dan β-sitosterol. Proses fraksinasi dan penulenan telah dilakukan menggunakan kromatografi cecair vakum dan kromatografi turus. Struktur bagi semua sebatian tulen telah dikenal pasti berdasarkan data spektoskopi termasuk 1D dan 2D Resonan Magnetik Nuklear (^{1}H, ^{13}C, DEPT, COSY, HMQC, HMBC), Inframerah, Ultralembayung dan spektrometri jisim. Aktiviti antibakterial semua ekstrak mentah dan sebatian tulen telah dijalankan menggunakan kaedah pembauran cakera diikuti dengan penentuan kepekatan perencatan minimum (MIC) dan kepekatan bakterisida minimum (MBC) terhadap dua bakteria Gram-positif, *Bacillus subtilis* and *Staphylococcus aureus* bersama-sama dengan dua bakteria Gram-negatif termasuk *Escherichia coli* and *Pseudomonas putida*. Keputusan menunjukkan bahawa semua ekstrak mentah dan sebatian tulen menunjukkan aktiviti lemah terhadap semua bakteria yang diuji dengan nilai MBC yang melebihi 1000 μg/mL. Penilaian aktiviti antioksidan menggunakan asai 2,2-difenil-1-pikrilhidrazil (DPPH) telah mendedahkan sifat penangkapan radikal bebas yang kuat oleh ekstrak etil asetat dan etanol dari buah dengan SC_{50} masing-masing ialah 14.00 dan 19.97 μg/mL. Ekstrak kloroform daripada buah dan semua ekstrak dari daun menunjukkan aktiviti antioksidan yang sederhana dengan SC_{50} antara 58.07–94.10 μg/mL. Antara semua sebatian tulen, 2,6,4'-trihidroksi-4-metoksibenzofenon menunjukkan aktiviti penangkapan radikal bebas yang tinggi dengan SC_{50} 29.73 μg/mL manakala sebatian tulen yang lain tidak aktif terhadap DPPH. Asai MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida) telah dijalankan untuk menilai aktiviti sitotoksik ekstrak mentah dan sebatian tulen. Asai MTT bagi jujukan sel CHO, MCF-7, MDA-MB-231 dan MDA-MB-468 telah diuji ke atas semua ekstrak mentah dah sebatian tulen manakala daya maju sel bagi 3T3, HT-29 dan HeLa telah diuji ke atas ekstrak mentah dari buah dan dua benzofenon tulen. Keputusan menunjukkan aktiviti yang baik bagi ekstrak diklorometana dari daun terhadap jujukan sel MDA-MB-231 (IC_{50} 70 μg/mL) dan kesan sitotoksik yang sederhana bagi ekstrak etanol dari buah terhadap jujukan sel MDA-MB-468 dan HT-29 dengan nilai IC_{50} yang sama iaitu 100 μg/mL. Ekstrak klorofom telah dijumpai untuk memiliki sifat sitotoksik yang sederhana terhadap jujukan sel HT-29 (IC_{50} 100 μg/mL). Semua sebatian tulen telah dijumpai untuk memiliki aktiviti sitotoksik yang lemah terhadap semua jujukan sel yang diuji..

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acetone- d_6	-	Deuterated acetone
br	-	Broad
°C	-	Degree celcius
^{13}C NMR	-	Carbon Nuclear Magnetic Resonance
CC	-	Column Chromatography
$CDCl_3$	-	Deuterated chloroform
cm^{-1}	-	Per centimeter
CO_2	-	Carbon dioxide
COSY	-	Correlation Spectroscopy
δ	-	Chemical shift
d	-	Doublet
dd	-	Doublet of doublet
DEPT	-	Distortionless Enhancement by Polarization Transfer
DMEM	-	Dulbeccos Modified Eagles Medium
DMSO	-	Dimethyl sulfoxide
DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
EIMS	-	Electron Impact Mass Spectrometry
FBS	-	Fetal bovine serum
FTIR	-	Fourier Transform Infrared
GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography-Mass Spectrometry
^{1}H NMR	-	Proton Nuclear Magnetic Resonance
HMBC	-	Heteronuclear Multiple Bond Correlation
HMQC	-	Heteronuclear Multiple Quantum Coherence
Hz	-	Hertz
IC_{50}	-	Concentration of substrate that causes 50% growth inhibition of cell
IR	-	Infrared
J	-	Coupling constant

KBr	-	Potassium bromide
L	-	Liter
lit.	-	Literature
λ	-	Lambda
M^+	-	Molecular ion
m	-	Multiplet
m/z	-	Mass to charge ion
mg	-	Milligram
mL	-	Milliliter
m.p.	-	Melting point
μg	-	Microgram
MBC	-	Minimum Bactericidal Concentration
MIC	-	Minimum Inhibitory Concentration
MTT	-	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	-	Nutrient agar
NB	-	Nutrient broth
NMR	-	Nuclear Magnetic Resonance
nm	-	Nanometer
ppm	-	Part per million
R_f	-	Retention factor
SiO_2	-	Silica gel
S	-	Singlet
SC_{50}	-	Radical scavenging activity at concentration of 50%
RPMI 1640	-	Roswell Park Memorial Institute 1640
t	-	Triplet
t_R	-	Retention time
TLC	-	Thin Layer Chromatography
UV	-	Ultraviolet
VLC	-	Vacuum Liquid Chromatography

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Nature play an important role in providing the basic needs of human in the production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavors, fragrances, and not the least, medicines for the treatment of various disease [1-3]. Mineral, animal and plant products were utilized as the main sources of drugs and the use of natural products with therapeutic properties is as ancient as human civilization [4- 6].

Throughout history, natural products have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy and biology [2, 6-8]. Plants, in particular have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of year and continue to provide humankind with new remedies [3]. In addition, plant derived natural products have been historically proven to be very useful in the pharmaceutical industry and will continue to be important sources of leading compounds for the design and synthesis of other novel substances [9, 10].

Most of the plant compounds that have been found to be medicinally useful and interesting tend to be secondary metabolites including alkaloids, phenolics, flavanoids and terpenoids. Secondary metabolites represent features that can be expressed in terms of ecological, taxonomic and biochemical differentiation and diversity. The wide molecular diversity of secondary metabolites throughout the

plant kingdom represents an extremely rich biogenic resource for the discovery of novel drugs and for developing innovative drugs [11]. To date, natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. Higher plants contribute no less than 25% of the total. During the last 40 years, at least a dozen potent drugs have derived from flowering plants [11].

Being one of the 12 mega biodiversity countries in the world, Malaysia is home to a wealth unique plant with the presence of 15,000 species flowering plants and out of these approximately 5,000 have been reported to possess medicinal value [12]. However, increasing of degradation and cultivation of forest coupled with deforestation is contributing factor for the reduction of abundance of local prospective medicinal plants. Furthermore, the ethnobotanical information are not always transcending beyond generations. Therefore, the importance of the botanical and chemical studies of various local plants remains and need to be investigated extensively [9]. Among the flowering plant of interest is *Phaleria macrocarpa* or known as mahkota dewa which is belongs to Thymelaeaceae family.

1.2 Family of Thymelaeaceae

Thymelaeaceae family is a cosmopolitan family of flowering plants, which is established by Antoine Laurent de Jussieu in 1789 [13]. This family consists of 45 genera and 700 - 800 species and widely distributed in both hemispheres [14, 15]. Nine genera and 115 species of Thymelaeaceae plants which are 89 endemic were found in China [16]. There are some large genera in Thymelaeaceae family which are *Gnidia* with approximate number of species 160, *Pimelea* (110), *Daphne* (95), *Wikstroemia* (70), *Daphnopsis* (65), *Struthiola* (35), *Lachnaea* (30), *Thymelaea* (30), *Phaleria* (30) and *Gonystylus* (25) [17].

The species of Thymelaeaceae are mostly shrubs or small trees, rarely herbs, evergreen or deciduous. Most species are toxic but some have medicinal properties. The phloem contains very strong fibers, which make the bark of many species from

this family very beneficial in manufacturing of high quality paper especially bank notes. The stems have characteristics of very supple and difficult to break which used as a substitute for string [16].

1.3 Genus *Phaleria*

Phaleria is a genus of about 30 species of evergreen and deciduous trees that belong to the mulberry family of Thymelaeaceae. There are some selected species of *Phaleria* that have been recognized by Hou which are *Phaleria coccinea* (Gaud.) F. Muell., *Phaleria macrocarpa* (Scheff.) Boerl., *Phaleria nisidai* Kan., and *Phaleria perrottetiana* (Decne.) F-Vill. [18]. Three new species of *Phaleria* identified as *Phaleria longituba, Phaleria okapensis* and *Phaleria pilistyla* were found in the highlands of Papua New Guinea [19]. The determination of new species of *Phaleria* can easily be made by following the little disruption of the key that described by Hou [18].

1.4 Statement of Problem

In Indonesia, there are numerous bioactivities have been conducted on crude extracts of *Phaleria macrocarpa* such as antioxidant, anti-inflammatory, antibacterial, and antiproliferation activities. Despite of these well-known bioactive effects and qualitative composition of *P. macrocarpa*, there is still limited information available regarding the isolation and scientific evaluation of bioactivities of chemical compounds from this plant. Furthermore, no study has been reported on the phytochemicals and bioactivities of compounds isolated from *P. macrocarpa* species from Malaysia. Thus, this research is performed to determine the chemical composition and bioactivities of isolated compounds of local *P. macrocarpa*.

1.5 Research Objectives

The objective of this research is to determine the chemical constituents from the fruits, and leaves of *P. macrocarpa*. This study involved extraction of the samples, isolation and purification of the crude extracts to obtain pure compounds. The structures of isolated compound were characterized using spectroscopic methods. The crude extracts and isolated compounds were evaluated for antibacterial, antioxidant and cytotoxic activities.

1.6 Scope of the Study

This research is focused on the investigation of chemical constituents and bioactivities of *P. macrocarpa.* The fruits sample was extracted using cold extraction followed by back extraction while the leaves sample was extracted using sequential cold extraction method. The crude extracts were subjected to chromatographic fractionation using vacuum liquid chromatography (VLC) followed by purification of the fractions using column chromatography (CC) and recrystallization to obtain pure compounds. Structural elucidation of the isolated compounds was performed using spectroscopic methods including 1D and 2D NMR, UV, MS, GC-MS and IR.

The crude extracts and pure compounds were screened for antibacterial, antioxidant and cytotoxic activities. The antibacterial activities were performed using disc diffusion method, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas putida.* The *in vitro* free radical scavenging activities of the extracts and isolated compounds were evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was carried out to evaluate the cytotoxic activity.

1.7 Significance of Study

The medicinal values of *P. macrocarpa* in treating various types of diseases led to the verification of the claims made by the traditional medicine researcher. The biological activities of the crude extracts of this plant have been extensively reported to possess a wide range of biological activities including cytotoxic, antioxidant, antidiabetic, antibacterial and anti-inflammatory [20 - 22]. However, the results obtained are insufficient to prove these claims since the information regarding the specific compound that contributes to those activities is uncertain. Thus, this research was performed to isolate and elucidate the structure of pure compounds from *P. macrocarpa*. In addition, the association of the isolated compounds to the antibacterial, antioxidant and cytotoxic activities of this plant were also investigated. These findings may shed light on the actual properties of this plant in relation with the bioactivities.

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