UNIVERSITI PUTRA MALAYSIA

CHEMICAL COMPOSITION, ANTIBACTERIAL AND TOXICITY ACTIVITIES OF Aquilaria LEAVES FROM THREE COMMONLY PLANTED SPECIES IN MALAYSIA

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FH 2017 6

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By

AIMI ZAFIRAH BINTI ADAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September 2017

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DEDICATION

In the name of Allah S.W.T., the most Benevolent and ever Merciful
All praise be to Allah S.W.T.

Specially dedicated to:

My Parents

ADAM TASEH & UMI KALSUM SHAARI

and

My Brothers

MUHAMMAD AZFAR ADAM

MUHAMMAD ANIQ ADAM

MUHAMMAD AFIF ADAM

MUHAMMAD ZAKWAN ADAM

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

CHEMICAL COMPOSITION, ANTIBACTERIAL AND TOXICITY ACTIVITIES OF *Aquilaria* LEAVES FROM THREE COMMONLY PLANTED SPECIES IN MALAYSIA

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September 2017

Chairman : Associate Professor Rozi Mohamed, PhD
Faculty : Forestry

The *Aquilaria* genus (Thymelaeaceae), is an eminent agarwood-producing tree, commonly found in the Indomalesia region. Illegal harvesting and random logging had caused *Aquilaria* trees to be listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In recent years, *Aquilaria* tree cultivation at large scale has been implemented to promote sustainable agarwood production. Besides utilizing agarwood induced in the tree trunk, the leaves can be processed into food products such as tea, which can give some economic return while farmers wait for their agarwood to mature. In this study, three *Aquilaria* species, *Aquilaria crassna*, *Aquilaria malaccensis* and *Aquilaria sinensis* were selected because they are widely planted in Peninsular Malaysia. The aim of this study was to validate the leaf specimen of the three *Aquilaria* species based on morphology and molecular identification, to identify the chemical compounds of the leaves using Gas Chromatography-Flamed Ionization Detector (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS) as well as to evaluate the antibacterial and toxicity activity of the leaves. A total of 58 compounds were obtained from the essential oils, and nine and 48 compounds from the hexane and methanol extracts, respectively. Among the major compounds identified were hexadecanoic acid and squalene, which are known to have antimicrobes and antioxidants properties, respectively. Methanol extracts of *A. malaccensis* exhibited maximum inhibition zones against *Staphylococcus aureus* and *Salmonella choleraesuis*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts (31.25 mg/ml) produced remarkable inhibitory effect on *S. aureus* compared to *S. choleraesuis* (250 mg/ml). The MTT test proved that both the essential oils and hexane extracts of the three leaves had no significant effects on the viability of the PBMCs. However, only the methanol extract of *A. malaccensis* had low levels of IC_{50} (24.5 mg/ml) and LD_{50} (4537 mg/kg) values, suggesting this species is slightly hazardous. For the comet test, the three *Aquilaria* species also did not show any DNA damages ($p>0.05$). Similar results were obtained when using the methanol extracts of *A. crassna* and *A. sinensis* at 2 mg/ml and 3 mg/ml,

respectively. However, for the methanol extracts of *A. malaccensis* leaves, DNA damage was observed ($p<0.05$). The tests conducted on PBMCs indicated that the essential oils and crude extracts of *Aquilari*a leaves from these three species are relatively safe to consume without major toxicity concern, but should be wisely taken in a dose-dependent manner. This study provides a benchmark in setting up indicators for measuring toxicity threats from the use of *Aquilaria* leaves, which is becoming a popular ingredient in food or drinks preparation.

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

KOMPOSISI KIMIA, AKTIVITI ANTIBAKTERIA DAN TOKSISITI BAGI DAUN *Aquilaria* DARIPADA TIGA SPESIES TANAMAN DI MALAYSIA

Oleh

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Genus *Aquilaria* (Thymelaeaceae), adalah sebuah pokok yang menghasilkan gaharu yang terkenal, yang biasanya ditemui di rantau Indomalesia. Penuaian haram dan pembalakan rawak telah menyebabkan pokok *Aquilaria* disenaraikan dalam Konvensyen Perdagangan Antarabangsa Spesies Terancam Flora dan Fauna (CITES). Dalam tahun-tahun kebelakangan ini, penanaman pokok *Aquilaria* pada skala besar telah dilaksanakan untuk menggalakkan pengeluaran gaharu mampan. Selain menggunakan gaharu teraruh di dalam batang pokok, daun boleh diproses menjadi produk makanan seperti teh, yang boleh memberikan beberapa pulangan ekonomi sementara peladang menunggu gaharu untuk matang. Dalam kajian ini, tiga spesies *Aquilaria, Aquilaria crassna, Aquilaria malaccensis* dan *Aquilaria sinensis* telah dipilih kerana mereka ditanam secara meluas di Semenanjung Malaysia. Tujuan kajian ini adalah untuk mengesahkan spesimen daun tiga spesies *Aquilaria* berdasarkan morfologi dan pengenalpastian molekul, untuk mengenal pasti sebatian kimia daun menggunakan kromatografi gas-pengesan pengionan nyalaan (GC-FID) dan kromatografi gas-spektrometri jisim (GC-MS) serta menilai aktiviti antibakteria dan ketoksikan daun. Sebanyak 58 kompaun telah diperolehi daripada minyak pati dan masing-masing sembilan dan 48 sebatian daripada ekstrak heksana dan metanol. Antara sebatian utama yang dikenalpasti adalah asid hexadecanoic dan squalene, yang masing-masing mempunyai ciri-ciri antimikrob dan antioksidan. Ujian antimikrobial oleh kaedah penyerapan agar dan penyerapan mikro mendapati ekstrak metanol *A. malaccensis* menunjukkan zon pencegahan maksimum terhadap *Staphylococcus aureus* dan *Salmonella choleraesuis*. Kepekatan perencatan minimum (MIC) dan kepekatan bakterisida minimum (MBC) ekstrak (31.25 mg/ml) menghasilkan kesan perencatan yang lebih pada *S. aureus* berbanding *S. choleraesuis* (250 mg/ml). Ujian sitotoksik oleh 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium pengurangan bromida tetrazolium (MTT) dan ujian genotoksik oleh analisis komet telah dijalankan terhadap sel-sel mononuklear darah periferi manusia (PBMCs) untuk menentukan ketoksikan tumbuhan. Ujian MTT membuktikan bahawa kedua-dua minyak pati dan ekstrak heksana ketiga-tiga spesies daun *Aquilaria* tidak mempunyai kesan yang besar ke atas daya maju

PBMCs. Walau bagaimanapun, hanya ekstrak metanol daun *A. malaccensis* sahaja yang mempunyai nilai IC_{50} pada tahap rendah (24.5 mg/ml) dan LD_{50} (4537 mg/kg), yang menunjukkan spesies ini sebagai sedikit berbahaya. Dalam analisis komet, ketiga-tiga spesies daun *Aquilaria* tidak menunjukkan sebarang kerosakan DNA ($p>0.05$). Keputusan yang sama telah diperolehi dengan menggunakan ekstrak metanol *A. crassna* dan *A. sinensis*, masing-masing pada kepekatan 2 mg/ml dan 3 mg/ml. Walau bagaimanapun, terdapat kerosakan DNA ($p<0.05$) bagi ekstrak metanol *A. malaccensis*. Ujian yang dijalankan ke atas PBMCs menunjukkan bahawa minyak pati dan ekstrak mentah daun *Aquilaria* daripada ketiga-tiga spesies agak selamat digunakan tanpa kebimbangan ketoksikan utama, tetapi harus diambil berpada-pada, bergantung kepada dos yang diambil. Kajian ini merupakan penanda aras dalam menjadi petunjuk untuk mengukur ancaman ketoksikan daripada penggunaan daun *Aquilaria*, yang menjadi bahan popular dalam penyediaan makanan atau minuman.

ACKNOWLEDGEMENTS

In the name of Allah, the most Benevolent and Merciful.

Primarily, all praise to the almighty Allah S.W.T. for His blessing, endless love, and gracious upon me for the strength and patience given throughout the whole study period. I would like to express my deepest appreciation to my supervisor, Assoc. Prof. Dr. Rozi Mohamed for her unwavering support and mentorship in guiding me from the initial to the final stage of my study. My sincere thanks to my co-supervisor, Dr. Ummi Hani Abdullah for her thoughts and advices during the completion of this study.

My earnest thanks to the management team of Forest Research Institute of Malaysia (FRIM), Kepong, Selangor, especially to Dr. Mohd Noor Mahat in providing the samples required for this research. My warmest gratitude towards Assoc. Prof. Dr. Saiful Nizam Tajuddin and Mr. Che Mohd Aizal Che Mohd for their guidance and practical support in conducting my research in Universiti Malaysia Pahang. Nevertheless, Prof. Dr. Arunrat Chaveerach, Dr. Runglawan Sudmoon, Miss Tikumporn Thooptianrat, and other lab members from Department of Biology, whom I had the privilege to work with during my research attachment in Khon Kaen University, Thailand for four months.

My heartiest appreciation to my beloved family especially my father, Adam Taseh, my mother, Umi Kalsum Shaari, my brothers, Muhammad Azfar, Muhammad Aniq, Muhammad Afif and Muhammad Zakwan for their concerns, loves, and constant moral supports. Their encouragements helped me to encounter the struggles, pains, and hardships through every step of this incredible journey.

Besides that, my grateful thanks to the labmates, Dr. Lee Shiou Yih, Siti Rahimah Jumaat, Tuan Noraida Tuan Hamzah, Farah Hanani Azman, Muhammad Syahmi Hishamuddin, and Mohamad Azren Putra Mat Desa for helping me out in numerous ways during various stages of my study. I would like to extend my thanks to all my friends for their help and support during the entire study duration. To those who have indirectly contributed in this research, your kindness means a lot to me. Thank you very much.

Finally, I convey my sincere gratitude towards the Ministry of Higher Education, Malaysia and School of Graduate Studies, UPM for the financial support and cooperation given during the study period.

I certify that a Thesis Examination Committee has met on 20 September 2017 to conduct the final examination of Aimi Zafirah binti Adam on her thesis entitled "Chemical Composition, Antibacterial and Toxicity Activities of *Aquilaria* Leaves from Three Commonly Planted Species in Malaysia" 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 Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microlitre
µm	Micrometer
µM	Micromol
AChE	Acetylcholinesterase
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
BSA	Bovine serum albumin
CASP	Comet Assay Software Project
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
eV	Electron-volt
FBS	Fetal bovine serum
FID	Flame ionization detection
FRIM	Forest Research Institute of Malaysia
g	Gram
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization
GC-MS	Gas Chromatography-Mass Spectrometry
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC	Inhibition concentration
kg	Kilogram
LD	Lethal dose
LMA	Low melting agarose
M	Molar
m	Meter
mA	Milliamps
MBC	Minimum bactericidal effect
mg	Miligram
MIC	Minimum inhibitory effect
min	Minute
mL	Mililitre
mM	Metermol
mm	Milimeter
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazl-2-yl)-2,5 diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards Technology
ng	Nanogram
nm	Nanometer
OTM	Olive tail moment
PBMC	Peripheral blood mononuclear cells

PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
PHA	Phytohemagglutinin
RI	Retention index
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
UV	Ultra violet
v	Volt
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 General

The World Health Organization (WHO) defined a medicinal plant as any plant, in which one or more of its organs contain substances that can be utilized for the remedial purpose or as pioneer for the production of useful drugs. Most of the species that are used in folk medicines are found in the Asia-Pacific region with an estimation of more than 70,000 plant species (WHO, 1977). There is a great native knowledge on the use of medicinal plants in almost every Asian country where traditional identification system has been existed for long. However, formal scientific identification of these plants only started in the 1900's (Rao and Arora, 2004). The medicinal value of these plants depends on the chemical compounds that generate a specific physiological action on the human body. For instance, alkaloids, flavonoid, tannin and phenolic compounds are one of the important chemical compounds in plants. Most native medicinal plants are also used for medicinal purposes (Edeoga, 2005).

Aquilaria is known as one of the medicinal plants and widely used in a traditional Chinese medicine since 15th century (Gunasekera *et al.,* 1980). The genus *Aquilaria*, which is commonly known as 'Karas' in Malay, belongs to the family Thymelaeaceae, is an evergreen tropical woody tree that is well-known for its fragrant resin called agarwood or 'gaharu'. Agarwood is formed when a fungus or certain disease infects a wounded tree, or simply when a wound is inflicted on the tree. Agarwood is often used in several applications, such as incense, perfumery, medicine, religious ceremony and ornamental (Ng *et al.,* 1997; Compton and Zich, 2002; Lee and Mohamed, 2016). Incenses made from agarwood produce a pleasant aroma when burnt and are used in rituals in many beliefs, while the wood is carved into religious objects such as idols and praying beads (Lee and Mohamed, 2016). Agarwood is distilled into essential oil and is highly prized in Middle-Eastern countries. The oil is generally used in perfumery and cosmetic products (Barden *et al.,* 2000). All this while, farmers only utilized agarwood induced in the tree trunk before they came to know about agarwood leaves that can be processed into food products such as tea, which can give some economic return while waiting for their agarwood to mature. Such trend has been circulating in several countries such as China, Indonesia, Malaysia, Thailand and Vietnam (Zhou *et al.,* 2008).

The identification of bioactive compounds in the leaves are useful in the finding of new compounds that have potential as remedial agents. In recent years, *A. sinensis* leaves are applied in traditional medicine for treatments of trauma-related illnesses such as bruises and fractures (Zhou *et al.,* 2008). Meanwhile, *A. crassna* leaves are useful as a supplement to combat various health conditions such as high blood pressure, constipation, headache and diabetes (Pranakhon *et al.,* 2011), and in treating digestive ailment and as a mild sedative (Kakino *et al.,* 2010). Nowadays, the use of *Aquilaria* leaves in food products have been diversified and sold in the forms of tea in sachets,

mixed with coffee, biscuits and ice creams. It has also been used as essence in ointments production (Chakrabarty *et al.*, 1994).

The increasing demand to natural products originating from traditional medicines has led to a renaissance of the scientific interest in their biological effects. However, they need to go through scientific research and stringent regulation in checking their toxicity effects and safety for mass consumption. Some of the active ingredients in the extracts probably have incredibly high toxic dose which could be harmful to humans (Jain *et al.*, 2013).

1.2 Problem Statements

Many have studied the chemical compounds and biological activities in the agarwood resin from *Aquilaria* species due to its well-known values. However, the leaves also have potential economic value, specifically on health-related products such as tea. To date, studies on the chemical compounds and toxicity of *Aquilaria* leaves are still limited. There are very few reports on the chemical compounds of *Aquilaria* leaves, and none in its toxicity to human.

Many people choose herbal products because they mistakenly believe herbal products are superior to manufactured products. They opt for herbal remedies as alternative by assuming that these products are safe for consumption without thinking of the long term effect to their health. Some of the chemical compounds in the plants may be harmful to the body.

1.3 Justification

This study was conducted to evaluate the chemical compounds of the essential oils and extracts of *Aquilari*a leaves. It is important to determine the chemical compounds and toxicity of the *Aquilaria* leaves.

Not all natural products are safe, pure or effective, so people need to know the effects of the contents that may have on health. It is important to ensure that the use of natural products is based on scientific origin so that the plants are safe to consume by people. Hence, toxicity test on the plants such as cytotoxicity and genotoxicity need to be conducted before consuming.

1.4 Objectives

The general objective of this study was to identify the chemical compounds and evaluate the toxicity level of *Aquilaria* leaves from commercial species that are widely planted in Malaysia: *Aquilaria crassna*, *Aquilaria malaccensis* and *Aquilaria sinensis.*

The specific objectives of this study include:

1. To validate the leaf specimen of *Aquilaria* species based on morphology characteristics and molecular identification.
2. To identify the chemical compounds in the essential oils and crude extracts of the leaves using Gas Chromatography-Flame Ionization Detector (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS) techniques.
3. To evaluate the antibacterial activity of crude extracts of the leaves using agar well diffusion and micro dilution.
4. To evaluate the toxicity level of the essential oils and crude extracts of the leaves using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) assay and comet assay.

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