PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF *Aquilaria subintegra* Ding Hou

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SULTAN IDRIS EDUCATION UNIVERSITY
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ABSTRACT

This study aims to investigate the phytochemicals and biological activities from the bark and leaf of *Aquilaria subintegra* Ding Hou. The samples were air-dried, ground, and gradient extracted by cold extraction using *n*-hexane, dichloromethane, and methanol successively. The bark and leaf crude extracts were purified *via* chromatographic techniques to produce pure compounds. The chemical structure of compounds were determined using spectroscopic methods such as nuclear magnetic resonance, ultraviolet, infrared, mass spectrometry and comparison from the literature review. The crude extracts and isolated compounds were tested for their anti-lipase, antioxidant and anticancer activities through colorimetric test, DPPH free radical scavenging and cytotoxicity assay, respectively. This study has successfully afforded 22 compounds, including three new compounds, namely aquilene A, aquilene B, and subintegranol. Other compounds were 5-hydroxy-7,4′-dimethoxyflavone, luteolin-7,3′,4′-trimethyl ether, 5,3′-dihydroxy-7,4′-dimethoxyflavone, 7,3′-dimethoxyluteolin, 5,7-dihydroxy-4′-methoxyflavone, *β*-sitosterol, *β*-sitostenone, stigmasterol, friedelin, epifriedelanol, phytol, and hentriacontane. Anti-lipase activity of *n*-hexane extracts of leaf and methanol extracts of bark showed the highest inhibition, 46% and 52% compared with the control, respectively. Meanwhile, through computational study of 5-hydroxy-7,4′-dimethoxyflavone exhibited the highest inhibition and predicted as a competitive lipase inhibitor. Besides, the leaf of methanolic extract showed the strongest DPPH radical scavenger with IC_{50} value of 29.56 ± 6.37 µg/mL. However, the tested isolated compounds showed less ability in antioxidant activity. In cytotoxic activity, the leaf and bark of methanolic extracts exhibited highest anticancer activity with IC_{50} values of 24.30 ± 0.07 and 17.46 ± 0.08 µg/mL, respectively. Besides, 5-hydroxy-7,4′-dimethoxyflavone showed the highest cytotoxicity activity with IC_{50} value of 38.48 ± 0.06 µg/mL. In conclusion, this study successfully revealed the presence of various phytochemicals in *A. subintegra* and their potential as anti-lipase, antioxidant and anticancer agents. In implication, this study enhances knowledge on the diversity of chemical compounds and their potential in modern medicine.

FITOKIMIA DAN AKTIVITI BIOLOGI DARIPADA *Aquilaria subintegra* Ding Hou

ABSTRAK

Kajian bertujuan untuk mengenal pasti fitokimia dan aktiviti biologi daripada kulit batang dan daun *Aquilaria subintegra* Ding Hou. Sampel dikering, dikisar dan diekstrak secara pengekstrakan dingin menggunakan pelarut *n*-heksana, diklorometana dan metanol secara berturutan. Ekstrak mentah kulit batang dan daun dipisahkan melalui teknik kromatografi untuk mendapatkan sebatian tulen. Struktur kimia sebatian tulen dikenalpasti menggunakan kaedah spektroskopi seperti resonans magnet nukleus, ultra lembayung, infra merah dan spektrometri jisim dan perbandingan daripada kajian lepas. Ekstrak mentah dan sebatian tulen diuji terhadap aktiviti anti-lipase, antioksidan dan antikanser masing-masingnya menerusi ujian warna, DPPH radikal bebas dan assai kesitotoksikan. Kajian ini berjaya mengasingkan 22 sebatian termasuk tiga sebatian baharu iaitu aquilen A, aquilen B dan subintegranol. Sebatian lain adalah 5-hidroksi-7,4′-dimetoksiflavon, luteolin-7,3′,4′-trimetil eter, 5,3′-dihidroksi-7,4′-dimetoksiflavon, 7,3′-dimetoksi luteolin, 5,7-dihidroksi-4′-metoksiflavon, *β*-sitosterol, *β*-sitostenon, stigmasterol, fridelin, epifridelanol, phytol dan hentriakontan. Aktiviti anti-lipase ke atas ekstrak *n*-heksana daun dan ekstrak metanol kulit batang masing-masingnya menunjukkan perencatan tertinggi, 46% dan 52% berbanding kawalan. Manakala, kajian simulasi berkomputer terhadap sebatian 5-hidroksi-7,4′-dimetoksiflavon mempamerkan perencatan tertnggi dan diramalkan sebagai perencat lipase yang kompetitif. Selain itu, ekstrak daun mentah metanol menunjukkan aktiviti perencatan radikal DPPH yang kuat dengan nilai IC_{50} 29.56 ± 6.37 μg/mL. Namun, sebatian tulen yang diuji mempamerkan kebolehan yang rendah bagi aktiviti antioksidan. Bagi aktiviti sitotoksik, ekstrak metanol daun dan kulit batang masing-masingnya menunjukkan aktiviti antikanser yang kuat dengan nilai IC_{50}, 24.30 ± 0.07 dan 17.46 ± 0.08 μg/mL. Selain itu, 5- hidroksi-7,4′-dimetoksiflavon menunjukkan kesan sitotoksik yang kuat dengan nilai IC_{50} 38.48 ± 0.06 μg/mL. Kesimpulannya, kajian ini berjaya mendedahkan kepelbagaian fitokimia daripada *A. subintegra* dan potensinya sebagai agen anti-lipase, antioksidan dan antikanser. Implikasinya, kajian dapat meningkatkan pengetahuan mengenai kepelbagaian sebatian kimia dalam tumbuhan serta potensinya dalam perubatan moden.

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LIST OF ABBREVIATIONS

α	-	Alpha
AA	-	Ascorbic acid
Abs	-	Absorbance
ASBH	-	*n*-hexane crude of bark *A. subintegra*
ASBD	-	Dichloromethane crude of bark *A. subintegra*
ASBM	-	Methanol crude of bark *A. subintegra*
ASLH	-	*n*-hexane crude of leaf *A. subintegra*
ASLD	-	Dichloromethane crude of leaf *A. subintegra*
ASLM	-	Methanol crude of leaf *A. subintegra*
β	-	Beta
br	-	broad
δ	-	Chemical Shift
CC	-	Column Chromatography
^{13}C-NMR	-	Carbon NMR
$CDCl_3$	-	Chloroform-D
CD_3OD	-	Methanol-D
CH_2Cl_2	-	Dichloromethane
COSY	-	Correlation spectroscopy
1D	-	1-dimensional
2D	-	2-dimensional
d	-	Doublet
dd	-	Doublet of doublet
DEPT	-	Distortionless Enhancement by Polarization Transfer
DMSO	-	Dimethyl Sulfoxide
DL	-	Dried Leaves
DPPH	-	2,2-diphenyl-1-picrylhy-drazyl
EIMS	-	Electron Ionization Mass Spectrometry

ESI-MS	-	Electron Spray Ionization Mass Spectrometry
EtOAc	-	Ethyl acetate
n-Hex	-	Hexane
HCl	-	Hydrochloric acid
H_2SO_4	-	Sulfuric acid
Hz	-	Hertz
^{1}H-NMR	-	Proton NMR
HMBC	-	Heteronuclear Multiple Bond Correlation
HMQC	-	Heteronuclear Multiple Quantum Coherence
IR	-	Infrared
J	-	Coupling constant
M	-	Multiplet
MeOH	-	Methanol
m/z	-	Mass to charge ion
MS	-	Mass Spectrometry
mg	-	miligram
mL	-	mililiter
min	-	minute
NMR	-	Nuclear Magnetic Resonance
nm	-	nanometer
PPL	-	Porcine Pancreatic Lipase
s	-	Singlet
sec	-	second
SD	-	Standard deviation
SiO_2	-	Silica gel
t	-	triplet
TLC	-	Thin Layer Chromatography
UV	-	Ultra Violet
µL	-	microliter
µmole	-	micromole
PHE	-	Phenylalanine
ILE	-	Isoleucine

ASP	-	Aspartate
TYR	-	Tyrosine
HIS	-	Histidine
SER	-	Serine
LEU	-	Leucine
ALA	-	Alanine
PRO	-	Proline
ILE	-	Isoleucine
PHE	-	Phenylalanine
TRP	-	Tryptophan
ARG	-	Arginine
VAL	-	Valine
GLU	-	Glutamate
THR	-	Threonine

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Natural products (NPs) give a massive impact on science because of the discovery of plentiful medicinal drugs precursor (Crane & Gademann, 2016). NPs universally refer to substances which are isolated from living organism, and it has usually formed in secondary metabolites. NPs have always been a keystone in finding new lead molecules for drug candidates (Orhan, 2016). They are representing a vast family of diverse chemical constituents with their valuable biological activities, particularly in human, veterinary medicine, and agriculture, as well as significant agents in pharmaceuticals, herbicides, and insecticides (Katz & Baltz, 2016). NPs have brought significant effects of economic due to their role in pharmaceuticals, and fragrances (Anulika, Ignatius, Raymond, Osasere, & Abiola, 2016).

Historically, since 60,000 years ago, NPs such as plants, animals, microorganisms and marine organisms have been used as a medicinal treatment. However it faced a massive challenge in the early stage to the humans because of the consumption risks such as vomiting, diarrhoea, coma or other toxic reactions and perhaps led to the death; still, early humans knowledgeable about edible materials and natural medicines (Yuan, Ma, Ye, & Piao, 2016).

The NPs such as bacterial, fungal, and the plant has served as front line therapeutics to treat diseases. For example, antibiotics, chemotherapeutics, immunosuppressants, cholesterol-lowering agents and anaesthetics. These molecules derive from large groups of chemicals including polyketides, nonribosomal peptides, saccharides, alkaloids, terpenoids, and comprise a staggering diversity of chemical scaffolds (Medema & Fischbach, 2015; Tang, Zou, Watanabe, Walsh, & Tang, 2017).

Today, natural medicines have been used principally in medical treatment, especially in developing countries (Greenwell & Rahman, 2015). Herbal plants, either one part (flowers, leaves, branches or roots) or entire parts of it might use in the treatment of acute and chronic diseases. Also, in the development of dietary products due to continuous advantages, showed minimal side effect contrasting to synthetic medicine which contributes a severe impact for the long term uses (Farzaneh & Carvalho, 2015). Therefore, in the 21st century, medicinal herbs gained attention as an alternative way to replace the usage of synthetic drugs.

The statistical data from the World Health Organization (WHO), showed about 80% of people in the world depend on traditional herbal medicine for their health care needs. Traditional Chinese Medicine, Indian Ayurvedic Medicine, and Unani Medicine of Arab cultures were globally used as references for herbal medicine (Rehman, Choe, & Yoo, 2016).

Most researchers attract Malaysian herbs due to the massive areas of rainforest and tropical weather. In Malaysia, approximated 15,000 known plant species, in which 2,000 species have medicinal values, and the rest species are not exploited and cultivated (Saad et al., 2015). Some examples of medicinal plants reported in Malaysia shown in Table 1.1 (Madaleno, 2015).

Table 1.1

Medicinal plants reported in Malaysia

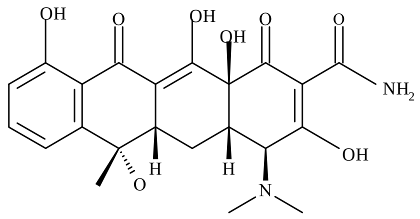
Plant Species	**Part**	**Medicinal action**
Strobilanthes crispus (L.) Bremek (Pecah Kaca)	Leaf	Anticancer, antioxidant, antidiabetic
Cananga odorata (Lam.) Hook. F. & Thomson (Ylang-ylang)	Flower	As a relaxing body oil
Cuminum cyminum L. (Cumin)	Seed	Herbal supplement for fatigue
Cocos nucifera L. (Chamomile)	Fruit	As a healing oil
Plectranthus barbatus Andrews (Long Pepper)	Whole plant	Healing body oils

(*continued*)

Table 1.1 *(continued)*

Plant Species	**Part**	**Medicinal action**
Pinellia nti-in (Thunb.) Ten. Ex Breitenb. (Musli)	Rhizome	Chinese remedy for cough and cold
Mentha arvensis L. (Jamun)	Leaf	Chinese remedy for pimples, skin rashes and bruises
Cinnamomum camphora (L.) J. Presl (PolygalaRhubarb)	Bark, leaf	As nti-inflammatory cream for itching and burns

The US Food and Drug Administration approved 1453 new NPs in the year 2013, and prominently used in medicinal treatment. For example, tetracycline (**1**) and chloramphenicol (**2**) as antibacterial, amphotericin B (**3**) (antifungal and antiparasitic), mitomycin C (**4**) and daunorubicin (**5**) as antitumor agents (Katz & Baltz, 2016). Thus, the significance of NPs are undoubtedly enormous and contribute massive benefits. Therefore, the scientist worldwide has a vital responsibility to identify medicinal plants which valuable to commercialize as herbal medicines.



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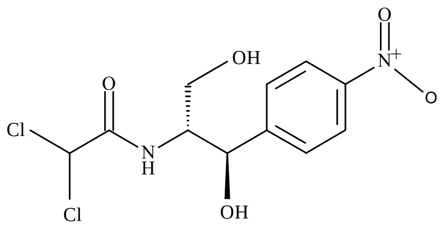

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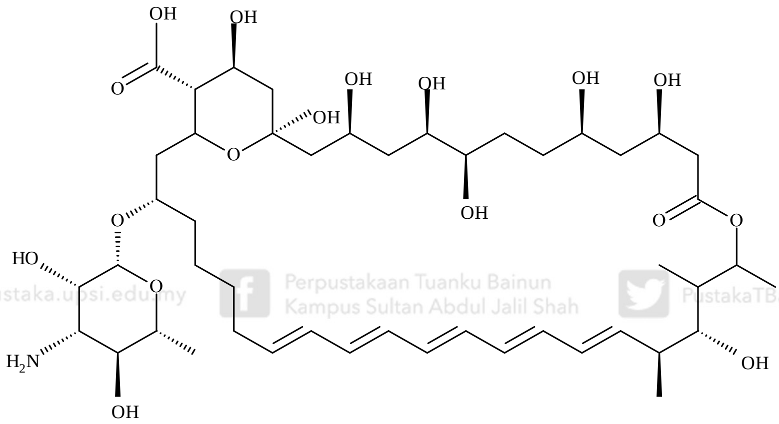

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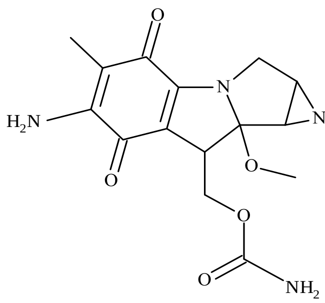

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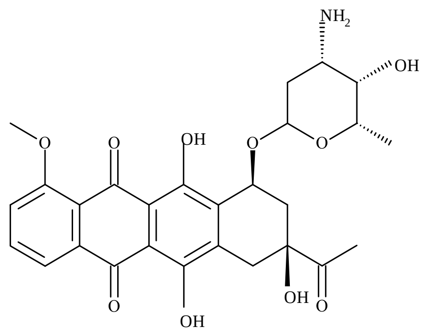

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1.2 Problem Statement

Aquilaria sp. is a fragrant tree which widely distributed in Asia including, Malaysia, Thailand, and Indonesia. This species was valuable due to the production of scented and aromatic wood which known as gaharu or agarwood. Besides that, agarwood tea was produced from *Aquilaria* leaves which mainly manufactured in China, Malaysia, and Indonesia. Yet, there is no official report commenced date of agarwood tea became as a commercial product, however, in 2007, there was first reported (Wu et al., 2007) toward toxicological safety of agarwood tea in the local market Hainan, China (Adam, Lee, & Mohamed, 2017).

However, there is a deficiency information specific population of *A. subintegra*. *A. subintegra* has found in Thailand, Narathiwat province, with an altitude range between 300 -500 m asl (Lee & Mohamed, 2016).

Furthermore, there is a limited number of phytochemical and pharmacological studies of *A. subintegra*. To the best of our knowledge, there is a tiny reference for isolation of *A. subintegra* in the phytochemical study. Besides, an inadequate study on the pharmacological study of the isolated compound from *A. subintegra* triggered us to reveal the chemical constituents and expected to obtained new bioactive potential compounds from this plant.

1.3 Significance of Study

This study revealed the chemical constituents obtained for the first time and examined their biological activities. Current results would be able to enhance knowledge, especially on species of study, the diversity of its chemical compounds derived from this plant, and its potential as a precursor to modern medicine.

1.4 Objectives of Study

The main objectives of the current study are:

i- To extract, isolate, and purify chemical constituents from the crude extracts of *A. subintegra* bark and leaf using chromatographic techniques.

ii- To identify and elucidate the chemical structures of the isolated compounds using several spectroscopic analyses such as nuclear magnetic resonances (NMR), ultraviolet-visible spectroscopy (UV), Fourier transform infrared spectroscopy (FTIR) and mass spectrometry (MS).

iii- To investigate biological properties including antioxidant, anti-lipase, and anticancer toward crude extracts and isolated compounds. Antioxidant activity was determined using DPPH free radical scavenging assay. The anti-lipase activity was measured by colorimetric assay; meanwhile, anticancer activity was examined using Cell Counting Kit-8 (CCK-8)/ WST-8 toward breast cancer cell, MCF-7.

iv- To determine the interaction of isolated compounds with receptor using computational study.