CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *AQUILARIA MALACCENSIS* LAMK. (AGARWOOD) LEAVES

NIK NOOR ASMA BINTI NIK WIL

MASTER OF SCIENCE

(INDUSTRIAL CHEMISTRY)

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

DECLARATION OF THESIS AND COPYRIGHT

Author's full name : NIK NOOR ASMA BINTI NIK WIL

Date of birth : 23 MARCH 1989

Title : CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *AQUILARIA MALACCENSIS* LAMK. (AGARWOOD) LEAVES

Academic Session : SEM 2 2018/2019

I declare that this thesis is classified as:

☐	CONFIDENTIAL	(Contains confidential information under the Official Secret Act 1997)*
☐	RESTRICTED	(Contains restricted information as specified by the organization where research was done)*
☑	OPEN ACCESS	I agree that my thesis to be published as online open access (Full Text)

I acknowledge that Universiti Malaysia Pahang reserve the right as follows:

1. The Thesis is the Property of Universiti Malaysia Pahang
2. The Library of Universiti Malaysia Pahang has the right to make copies for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified by:

_____	_____
(Student's Signature)	(Supervisor's Signature)
890323-03-5652	_____
New IC/Passport Number	Name of Supervisor
Date:	Date:

NOTE : * If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter page 2 from the organization with the period and reasons for confidentiality or restriction.

SUPERVISOR'S DECLARATION

We hereby declare that We have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Master of Science (Industrial Chemistry).

_____

(Supervisor's Signature)

Full Name : DR. SAIFUL NIZAM BIN TAJUDDIN

Position : ASSOCIATE PROFFESOR

Date :

_____

(Co-supervisor's Signature)

Full Name : NOR ADILA BINTI MHD OMAR

Position : LECTURER

Date :

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Malaysia Pahang or any other institutions.

_____

(Student's Signature)

Full Name : NIK NOOR ASMA BINTI NIK WIL

ID Number : MKD13003

Date :

CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *AQUILARIA MALACCENSIS* LAMK. (AGARWOOD) LEAVES

NIK NOOR ASMA BINTI NIK WIL

Thesis submitted in fulfillment of the requirements
for the award of the degree of
Master of Science (Industrial Chemistry)

Faculty of Industrial Sciences & Technology

UNIVERSITI MALAYSIA PAHANG

AUGUST 2019

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratefulness to Allah S.W.T for giving me a strength, inspiration, and wisdom to finish this thesis without any obstacles. All the experience and knowledge along my studies hopefully will give me the opportunity to develop my future career. I also hope this thesis can serve as references to other people who want guidance from this research study.

First and foremost, millions of thanks and highest appreciation dedicated to my supervisor, Assoc. Prof. Dr. Saiful Nizam Tajuddin for all his support, guidance and patience throughout completing this project. He also helps me in giving some advices and motivates in everything about this research until achieve a main objective. I would like to extend my deepest gratitude to my Co-Supervisor Mrs Adila Mhd Omar who gave me a lot of knowledge in terms of the bioassay study. Not forgetten to Assoc. Prof. Dr. Nadeem and Dr. Seema who teaches me in all technique related to the spectroscopic study. All these people have graciously provided me with their expertise and time.

Also, a very special thank you to my research group mates and members of the staff faculty FIST and Bio Aromatic who have gladly given their time to help me throughout my research. Last but not least, my dearest appreciation and heartfelt thanks to my beloved parents and family for their everlasting prayer and support the strengthen me to complete this project. Lastly, I am deeply indebted to my wonderful husband and daughter for giving me a spirit to achieving my dream as a graduate student's master. Without their aid and support this project would not have made it far.

ABSTRAK

Spesies *Aquilaria* mengandungi pelbagai metabolit sekunder termasuk pelbagai sebatian fenolik yang dikatakan bertindak sebagai antioksidan yang sangat berkesan. Kajian terhadap sifat fitokimia dan antioksidan spesies *Aquilaria malaccensis* dari keluarga Thymelaeaceae telah dilakukan. Pengekstrakan fitokimia dari daun dilakukan dengan menggunakan kaedah pengekstrakan konvensional; pengekstrakan refluks menggunakan air dan penyerapan dengan metanol yang menghasilkan ekstrak air (WE) dan ekstrak metanol (ME). Pengekstrakan, pemeringkatan dan pemisahan campuran menggunakan beberapa teknik kromatografi (kromatografi kolum dan kromatografi lapisan nipis) telah digunakan dalam proses pemisahan sebatian tulen. Untuk mengenal pasti sebatian yang mudah meruap dalam *A. malaccensis* daun, dua teknik digunakan, iaitu, pengekstrakan mikro fasa pepejal (SPME) dan desorpsi haba langsung (DTD), kemudian dianalisis dengan menggunakan teknik gas kromatografi iaitu, GC-FID dan GC-MS. Sementara itu, kromatografi cecair ultra tinggi dengan kuadrupol spektrometri jisim masa penerbangan (UPLC-QToF / MS) digunakan untuk menentukan sebatian kimia yang tidak mudah meruap di dalam tumbuhan tersebut. Potensi antioksidan daripada ekstrak *A. malaccensis* dikawal oleh kadar perangkap radikal bebas (DPPH) dan pengurangan kapasiti antioksidan melalui tembaga (CUPRAC). Perbezaan signifikasi adalah berdasarkan nilai p di mana $p<0.05$ dianggap ada perbezaan dan sebaliknya. Beberapa komponen yang biasa ditemui adalah asid *n*-heksadekanoik, eudesmol dan oxo-agarospirol. Ekstrak air dan metanol, yang mana mengandungi jumlah kandungan fenolik yang tinggi (191.005 ± 0.002 dan 177.927 ± 0.001 mg dari GAEs/g ekstrak) menunjukkan kuasa penurunan dan menghapuskan aktiviti radikal bebas yang tinggi. Pemisahan ekstrak heksana membawa kepada pengasingan friedelanol dan pemisahan ekstrak diklorometana membawa kepada pengasingan friedelin. Struktur sebatian tulen tersebut dikenalpasti melalui kaedah seperti 1D (^{1}H, ^{13}C, DEPTQ), 2D (COSY, HSQC, HMBC) NMR, MS, UV, FTIR dan juga melalui perbandingan maklumat spektra daripada kajian lepas.

.

ABSTRACT

Aquilaria species contains variety of secondary metabolites including various phenolic compounds which have been reported as excellent antioxidants. A study on phytochemical and antioxidant properties of *Aquilaria malaccensis* from Thymelaeaceae family was performed. The extraction of phytochemicals from the leaves were performed using conventional extraction methods: reflux extraction using water and maceration using methanol to obtain water extract (WE) and methanol extract (ME), respectively. Solvent–solvent extraction, fractionation and separation using different chromatographic techniques (column chromatography and thin layer chromatography) were used for isolation of pure compounds. In order to identify the volatile aroma compounds in the leaves of *A. malaccensis*, two methods were used namely solid-phase microextraction (SPME) and direct thermal desorption (DTD), in combination with gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis. The ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QToF/MS) was used for determination of non-volatile chemical compounds present in the leaves. The potential antioxidative activity of the leaves extracts of *A. malaccensis* was evaluated via radical scavenging assay (DPPH) and copper reducing antioxidant capacity (CUPRAC) assays. Significant differences were based on p values where $p<0.05$ were considered significantly different and vice-versa. Some of the commonly identified chemical components were *n*-hexadecanoic acid, eudesmol and oxo-agarospirol. WE and ME extracts with the highest total phenolic contents (191.005 ± 0.002 and 177.927 ± 0.001 mg of GAEs/g extract) showed strong reducing power and scavenging radical activity. Friedelanol and friedelin were isolated from the fractionation of hexane and dichloromethane extracts, respectively. The structure of the isolated compounds was elucidated by using spectroscopic methods namely 1D (^{1}H, ^{13}C, DEPTQ), 2D (COSY, HSQC, HMBC) NMR, MS, UV, FTIR and by comparison with literature values of published data spectra.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

α	alpha
β	beta
°C	Celsius
cm	centimetre
cm^{-1}	per centimetre
$CuCl_2$	copper (II) chloride
δ	chemical shift in ppm
eV	electron volt
γ	gamma
g	gram
hr	hour
KBr	potassium bromide
kg	kilogram
Kv	kilo volt
λ_{max}	maximum wavelength
μM	micromolar
μ	micro
μL	microlitre
m	metre
mL	millilitre
mm	millimetre
mM	millimolar
μM	micromolar
mg/mL	milligram per millilitre
$MgCl_2$	magnesium chloride
MHz	mega Hertz
m/z	mass to charge ratio
Na	sodium
NH_4OH	ammonium hydroxide
nm	nanometre
%	percentage

LIST OF ABBREVIATION

AA	ascorbic acid
BPI	based peak ion
^{13}C NMR	carbon nuclear magnetic resonance
$CDCl_3$	deuterated chloroform
COSY	correlation spectroscopy
CUPRAC	cupric reducing capacity
dbh	diameter at breast height
DCM	dichloromethane
DEPTQ	distortionless enhancement by polarization transfer with retention of quaternaries
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTD	direct thermal desorption
EIMS	electron impact mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
Hx	hexane
HPLC	high performance liquid chromatography
^{1}H NMR	proton nuclear magnetic resonance
HMBC	heteronuclear multiple bond coherence
HSQC	heteronuclear single quantum coherence
EC_{50}	concentration required to obtain a 50 % antioxidant effect
GAE	mg/g gallic acid equivalents
IC_{50}	concentration of drug required to inhibit cell growth by 50 %
IR	infrared
J	coupling constant
M^+	molecular ion
ME	methanol extract
MS	mass spectrum
NIST	National Institute of Standards and Technology

NMR	nuclear magnetic resonance
pH	power of hydrogen
RI	retention index
RP-18	reverse phase silica gel
SPME	solid phase microextraction
t_R	retention time
TIC	total ion current chromatogram
TPC	total phenolic content
TLC	thin layer chromatography
UPLC-QToF/MS	ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
UV-Vis	ultraviolet-visible spectroscopy
WE	water extract

CHAPTER 1

INTRODUCTION

1.1 Introduction

The uses of medicinal plants as raw materials in traditional medicines has led to the discovery of plant extracts, which have been commonly used in formulation of pharmaceutical products for treatment of various diseases and infections. The application of traditional and modern experimental techniques to separate and purify chemical compounds from plant, either through chemical or biological methods, has enabled the examination of their structure and the respective chemical profile in detail. Isolation of pure compound from plant is important to establish a reference that can be used to design synthetic compounds and to allow for structural determination of bioactive compounds.

Malaysia is one of the countries that are rich in biodiversity, which has about 1300 medicinal plant species growing in Peninsular Malaysia and Sabah, respectively (Kulip *et al*., 2010). *Aquilaria* is one of the plant genera that are widely distributed throughout Peninsular Malaysia. *Aquilaria* is a genus to a number of species of aromatic non-timber forest tree, which belongs to Thymelaeaceae family. The tree is one of the world's most valuable aromatic forest products. *Aquilaria* is also known by various names such as agarwood, aloeswood, eaglewood, gaharu, kalamabak or oudh depending on the region. It can be mostly discovered in the Southern and Southeastern parts of Asia, which comprise Cambodia, Vietnam, Laos, Thailand, Myanmar, Malaysia, Indonesia, and Papua New Guinea (Zich & Compton, 2002).

Several *Aquilaria* species have been found growing in Malaysia including *A. malaccensis, A beccariana, A. microcarpa, A. hirta, and A. rostrata*. Among these species, *A. malaccensis* is the most common species found in Malaysia, particularly in

Peninsular Malaysia, Sabah and Sarawak (Chua, 2018). In Malaysia and Indonesia, this resin is mainly produced by the tree of the genus *Aquilaria*, of which *A. malaccensis* is known as the main species to produce agarwood or gaharu (Barden, 2000; Mohamed, 2010). *A. malaccensis* wood has been used as tonic, stimulant, diuretic and flatulence relief while the grated wood has been used in preparation of traditional medicine to treat smallpox and illness during and after childbirth. Meanwhile, the leaves are applied in traditional medicine and herbal drink tea.

Aquilaria or agarwood have been used in various commercial and medicinal concoctions, and not restricted to application as incense and perfumery products. Historically, agarwood has been used extensively by Buddhist, Hindu, and Muslim for cultural, religious and medicinal purposes as well as in special ceremonies (Barden *et al.*, 2000). This plant contains important bioactive constituents, which are usually the secondary metabolites such as flavonoids, tannins and saponins (Dahham *et al*., 1997; Dash *et al*., 2008; Huda *et al*., 2009; Khalil *et al*., 2013; Wil *et al*., 2014), as well as alkaloid and terpenoids (Bahrani *et al*., 2014; Dahham *et al*., 1997; Dash *et al*., 2008; Huda *et al*., 2009; Khalil *et al*., 2013). Phytochemical screening is a technique that has been commonly used to evaluate the pharmacological actions of agarwood, including antioxidant, antidiabetic, sedative, analgesic, antipyretic, anti-inflammatory, hepatoprotective, anticancer and antimicrobial properties (Table 1.1). Based on these ethnopharmacological evidence, the present study is designed to explore the chemical constituents extracted from agarwood plant.

Table 1.1 Pharmacological activities of agarwood plant

Pharmacological activities	**References**
Antioxidant	Adam *et al.* (2018); Dahham *et al*. (2014); Duan *et al*. (2015); Hendra *et al.* (2016); Huda *et al*. (2009); Kamonwannasit *et al*. (2013); Miniyar *et al.* (2008); Moosa (2010); Sattayasai *et al*. (2012); Tay *et al.* (2014); Wil *et al.* (2014)
Antidiabetic	Jiang *et al.* (2011); Pranakhon *et al.* (2011); Zulkifle *et al.* (2013)
Sedative	Gao *et al.* (2012)
Analgesic, Antipyretic	Sattayasai *et al.* (2012)
Anti-inflammatory	Rahman *et al*. (2012); Zhou *et al.* (2008)
Hepatoprotective	Alam *et al.* (2017)
Anticancer	Dahham *et al.* (2015)
Antimicrobial	Cui *et al.* (2011); Wetwitayaklung *et al.* (2009)

Agarwood is composed of a complex mixture of organic compounds, predominantly the sesquiterpenes and chromones (Ishihara *et al.*, 1993). These two groups of compounds are responsible for its unique aroma characteristics. More than 70 sesquiterpenes have been identified from various *Aquilaria* species (Naef, 2011). Sesquiterpene hydrocarbons and oxygenated sesquiterpenes are the main types of sesquiterpene in agarwood oil. In a review on chemical constituents of agarwood leaves, the phytochemicals present are from broad range of chemical classes namely phenolic acids, benzophenones, xanthonoids, flavonoids, terpenoids, phytosterols and fatty acids (Hashim *et al.*, 2016). Some of these phytochemicals have shown pharmacological effects which are probably useful for future drug discovery.

Agarwood industry has become a huge business as it promises lucrative earnings. Demand for agarwood has increased and exceeded the supply to cater the markets in the Middle East, Europe and Asia for diverse applications in traditional and modern fragrance industry, as well as for religious and medicinal uses (Barden *et al.*, 2000). Overexploitation of agarwood from its natural habitat has driven the species to near extinction (Zhang *et al.*, 2008). As a result, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has listed *Aquilaria* as endangered species, putting its trade under tight control. As the source of natural agarwood diminishes, agarwood and its derivatives become more expensive (López-Sampson & Page, 2018).

The dire state of agarwood supply and insatiable demand have encouraged the cultivation of agarwood-producing species as an alternative source of agarwood. However, as the tree takes a long time to grow prior to harvest and inoculation process is required to trigger the production of resin in the tree, gaining investment becomes difficult especially for small-scale agarwood farmers, as it is considered high-risk with indefinite return on investments. Thus, exploring the commercial potential of the leaves such as producing tea derived from the abundant of *Aquilaria* leaves will help the planters to create additional revenue streams while waiting for the tree to mature and produce high-value agarwood (Adam *et al.*, 2018).

1.2 Problem Statement

Due to the variety of agarwood products in the market such as tea, a comprehensive study is needed for a simple sampling procedure in qualitative assessment to provide at least, the characteristic profiles of the volatile components present in these products. These flavour-contributing constituents signify the quality of the products manufactured for consumers use. Although previous studies on identification of chemical constituents of agarwood have mostly concentrated on the woods and essential oils, the information on different other parts is very limited, with existing publications focused on specific species, namely *A. agollocha* (Alam *et al.*, 2015) and *A. sinensis* (Li *et al.*, 2014). Unfortunately, there has not been any research conducted on identification flavour compounds in *A. malaccensis* leaves. Agarwood leaves have been proclaimed to exert various bioactivities including antioxidant, antidiabetic, anti-inflammatory, antimicrobial and anticancer. Antioxidants are generally the compounds that are able to prevent or delay the effects of highly reactive radicals by donating electron to convert these radicals into a relatively stable form. Some biochemical reactions caused by the presence of free radicals can damage crucial biomolecules in human body. While the leaves have been used as natural flavouring agent and ingredient in traditional medicine, research regarding their antioxidant activity and analysis of chemical constituents are important in order to evaluate its potential commercial use in future.

1.3 Research Objectives

There are some important tasks to be carried out in order to achieve the objectives, which are outlined as follows;

1. To extract and identify the chemical constituents of the leaves extracts of *A. malaccensis* of water and methanol extract.
2. To evaluate total phenolic content and antioxidant activity in different solvents extracts (water, methanol, hexane, dichloromethane and ethyl acetate) from leaves of *A. malaccensis*.
3. To isolate and characterize the compounds from *A. malaccensis* leaves.

1.4 Scope of Study

To achieve the objectives, the scope of the research has been identified, which are listed as follows;

1. Extraction of *A. malaccensis* leaves extract via water extraction and methanol extraction. The identification of chemical constituents of *A. malaccensis* via GC and UPLC-QToF/MS.
2. Evaluation of total phenolic content and antioxidant activity via analysis of radical scavenging activity and cupric reducing capacity.
3. Elucidation of isolated compounds was carried out by spectroscopic techniques such as NMR (^{1}H, ^{13}C, DEPTQ, COSY, HSQC, HMBC), UV-Vis, FT-IR and GC-MS.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The aim of this review is to provide an overview of phytochemical, ethnomedicinal and pharmacological aspects of *Aquilaria* species. As the current research explores the leaves of *Aquilaria*, this review will provide a platform to appraise the potential value of *Aquilaria* leaves as active ingredient in value-added health products from scientific perspectives.

2.2 Botanical Overview

Aquilaria malaccensis is classified into the family of Thymelaeaceae in the order of Malvales (Table 2.1).

Table 2.1 Taxonomic classification of *Aquilaria malaccensis*

Taxonomic Classifications	
Kingdom	Plantae
Division	Tracheophyta
Class	Magnoliopsida
Order	Malvales
Family	Thymelaeaceae
Genus	*Aquilaria*

Source: IUCN (2019)

2.2.1 Thymelaeaceae

Thymelaeaceae consists of 54 genera and about 938 species including the species of *Aquilaria*, *Daphne*, *Gonystulus* and *Gyrinops* (The Plant List, 2013). The plants from this family grow worldwide, well distributed particularly in tropical Africa and Australia.

The Thymelaeaceae includes shrubs, evergreens and herbaceous plants. The wood is usually fibrous and can be peeled off into long strips. The leaves are simple, exstipulate, arranged in alternate or opposite pattern. The flowers are dioecious and have a hollowed-out receptacle, forming a deep tube from the rim of which the floral parts are borne. The fruits mostly vary by category, which includes drupe, achene, berry and occasionally capsule (*Aquilaria*). The seeds have little or no endosperm, and the embryo is straight (Vernon *et al*., 2007).

2.2.2 Genus *Aquilaria*

The genus *Aquilaria* comprises about 21 accepted species out of the 57 scientific plant names submitted (The Plant List, 2013). *Aquilaria* spp. are widely distributed in southern China and several countries in Southeast Asia. The species grow in Bangladesh, Bhutan, India (Assam, Manipur, Meghalaya, Tripura), Indonesia (Kalimantan, Sumatera), Iran, Malaysia (Peninsular Malaysia), Myanmar, Philippines, Singapore, Laos, Cambodia, Vietnam and Thailand. *Aquilaria* is an evergreen tree mainly found in natural forests, growing to a height of 15 to 40 meter, with diameter at breast height (dbh) between 0.6 and 2.5 meter (Chakrabarty *et al*., 1994). *Aquilaria* spp. are among the most valuable tree species in the world, which produce aromatic resin, commonly called as gaharu, agarwood, or oud.

There are nine species of *Aquilaria* known to produce agarwood namely *A. rostrata*, *A. khasiana*, *A. crassna*, *A. filaria*, *A. sinensis*, *A. malaccensis*, *A. hirta*, *A. beccariana* and *A. microcarpa* (Ng *et al*., 1997). *A. khasiana* has been found growing in South Asia, particularly in India. *A. malaccensis* is mostly known to originate from Malaysia, Indonesia and India; *A. crassna* grows in Cambodia, Malaysia, Thailand and Vietnam; *A. beccariana* in Indonesia; *A. filaria* in New Guinea and Philippines; *A. hirta* in Thailand, Indonesia and Malaysia; *A. microcarpa* in Indonesia and Malaysia; *A. rostrata* in Malaysia; and *A. sinensis* in China (Akter *et al*., 2013).

A. malaccensis, *A. hirta*, *A. beccariana*, *A. rostrata* and *A. microcarpa* are widely distributed in several states of Malaysia (Barden *et al*., 2000; Hashim *et al*., 2016). Distribution of *Aquilaria* species with different local names in Malaysia is shown in Table 2.2. In the State of Pahang, this plant provides income for the Orang Asli communities. They use agarwood from *Aquilaria* as fragrance ingredient for spiritual purposes

(Antonopoulou *et al.*, 2010). Penan community in Sarawak use agarwood in the traditional remedy to treat stomach ache, fever, and as an insect repellent (Donovan & Puri, 2004).

Table 2.2 Distribution of *Aquilaria* species in Malaysia

Scientific Name	**Local name**	**Distribution**
A, malaccensis	Karas, kekaras, rostrata, engkaras	Peninsular Malaysia, Sabah, and Sarawak
A. hirta	Chandan buluh	Peninsular Malaysia
A. rostrata	-	Peninsular Malaysia
A. beccariana	Gaharu tanduk	Peninsular Malaysia, Sabah, and Sarawak
A. microcarpa	Engkaras	Sabah, and Sarawak

Source: Ali *et al.* (2015)

Previous chemical studies on *Aquilaria* spp. have been concentrated on essential oils of the resinous wood, with limited work have been done to study the chemical compounds from other parts of the plant. Agarwood is rich in secondary metabolites namely sesquiterpenes and chromones (Ishihara *et al.*, 1993; Ueda *et al.*, 2006). These two groups have been reported as the major group of chemical compounds identified in agarwood, which are responsible for the warm, sweet, balsamic and long-lasting odors released when agarwood is burnt or heated (Naef, 2011). Agarwood has often been used in traditional medicine, perfumes, incense and religious ceremony across Asia, Middle East, and Europe (Persoon & van Beek, 2008). High demand for agarwood in the market has led to rapid depletion of *Aquilaria* trees in natural forests. As a result, several *Aquilaria* species have been classified as threatened according to the International Reunion for Conservation of Nature and Natural Resources (IUCN) Red List (IUCN, 2019).

In response to this situation, efforts have been undertaken toward the development of sustainable agarwood plantation and management, which uses artificial induction to induce agarwood formation in cultivated *Aquilaria* (Akter *et al.*, 2013; Mohamed *et al.*, 2014). However, there have been varying degrees of success rendered by the numerous induction techniques developed to improve agarwood production in term of yield and quality. In natural environment, agarwood is formed only when the tree is wounded as a

result of certain external factors such as lightning strike, animal attack, infestation, and microbial infection (Blanchette & Van Beek, 2009). Traditionally, agarwood farmers in rural area wound the tree by cutting the trunk using blade or hammering the nails into the trunk to trigger agarwood formation (Barden *et al*., 2000). The most recent approach in modern artificial induction of agarwood uses scientific technique, which involves the drilling of holes in the trunk and injection of inoculants composed of specific agents or chemicals into these holes (Blanchette & Van Beek, 2009). Unfortunately, according to Liu *et al*. (2013) and Mohamed *et al.* (2014), this method renders low yield of agarwood despite the long time taken for agarwood to form. Due to this reason, many farmers have started to consider other alternatives to generate income from agarwood farming; this include exploring the potential commercialization of other parts of the plant particularly the leaves, which are abundant in the plantation, to produce processed products such as tea (Pranakhon *et al.* 2011; Zhou *et al.* 2008).

2.2.3 *Aquilaria malaccensis*

Aquilaria malaccensis (Figure 2.1) in the genus *Aquilaria* has been found growing in different countries of South Asia and South East Asia namely Bangladesh, Bhutan, India, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore and Thailand (Oldfield *et al*., 1998). Chakrabarty *et al.*, (1994) documented the India's trade of *A. malaccensis* in northeast India, which described the species as critically endangered but highly demanded with great economic value.

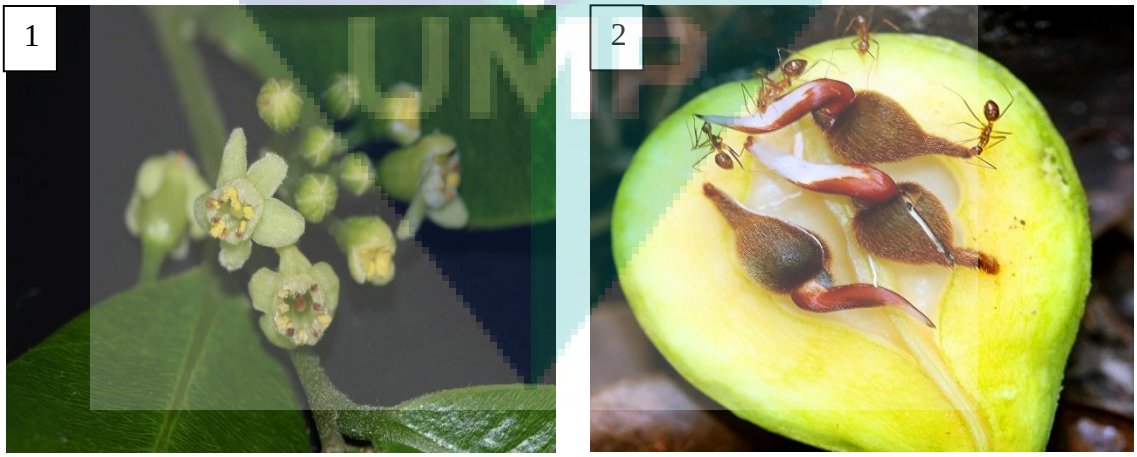


Figure 2.1 Part of *A. malaccensis*; (1) Flower (2) Fruit
Source: Lee & Mohamed (2016)

A. malaccensis is the major source of agarwood which has been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2003) due to overexploitation of its population in natural habitats for international trades. Appendix II aims to protect the endangered species from unsustainable use or illegal exploitation by using the permit system. In Malaysia, this species is commonly found throughout the Peninsular Malaysia, Sabah and Sarawak (Barden *et al*., 2000). *A. malaccensis* is a tree with sizes ranging from medium to large evergreen tree of about 20 - 40 m height, with 150 - 250 cm dbh.

The leaves of *A. malaccensis* are shiny, spirally arranged and elliptic, about 3 - 5 cm wide and 6-10 cm long with 12-16 pairs of veins. The diameter growth rate of *A. malaccensis* distributed in Peninsular Malaysia is relatively high for forest grown tree, with a mean of 0.33 cm per year (Lafrankie, 1994). The seed is small and round, and has a reddish short appendage. The shape of the capsule (fruit) is round at the apex while slender at the base, with tiny calyx lobes recurving outward. The flower is bell-shaped and yellowish according to Lee & Mohamed (2016). Figure 2.2 shows the diagram of botanical features of *A. malaccensis*.

Figure 2.2 *A. malaccensis* (1) flowering branch, (2) flower, (3) longitudinal section of flower, (4) fruit and (5) longitudinal section of fruit
Source: Oyen & Dung (1999)

2.3 Medicinal Uses

The bark, root and leaves of *Aquilaria* spp. have been utilized for thousands of years for various purposes, as in the traditional medicinal practices of Ayurvedic, Tibetan and East Asian (Barden *et al.*, 2000; Persoon & van Beek, 2008). They believe that agarwood can reduce body pain, asthma and gout. Chakrabarty *et al*. (1994) reported that agarwood has also been prescribed as a tonic for pregnant women, to cure the diseases related to female genital organs, jaundice and body pains.

The Chinese have been using the leaves of *A. sinensis* to treat trauma-related injuries such as fractures and bruises (Zhou *et al*., 2008). A study by Hara *et al*. (2008) on oral administration of acetone extract of *A. sinensis* leaves demonstrated laxative effects in the treated mice. In Thailand, the leaves of *A. crassna* have been used as traditional medicine in the treatment of various disorders, and most recently used as an additive in health food and beverages (Sattayasai *et al.*, 2012). Another study on *A. crassna* demonstrated the hypoglycemic effects in diabetic patient, who consumed the extract orally (Pranakhon *et al*., 2011). *A. malaccensis* have been used traditionally in the treatments of various disorders, and most recently have been used as additive in health food and beverages, such as tea. Recent studies in Malaysia have demonstrated the potential use of crude leaves extracts to treat diabetes mellitus (Zulkifle et al., 2013) with antioxidant activities (Hendra *et al*., 2016; Huda *et al.*, 2009; Moosa, 2010; Wil *et al.*, 2014). The stembark of the plant may be potentially used as anticancer agent (Gunasekera *et al*., 1981). This could be interesting for further phytopharmacological investigations.

2.4 Phytochemical Constituents of Genus *Aquilaria*

Researchers have made great efforts to isolate and identify biologically active compounds and other major constituents from various species of *Aquilaria* using various extraction techniques on different plant parts including the leaves, stem and fruits. One of the most studied species is *A. malaccensis*, which several parts of this plant, particularly the wood and leaves, have been reportedly used in various traditional medicines to treat a wide range of disorders. Nowadays, the study on the characteristics of purified or synthesized constituents from essential oils or crude extracts has become important. Agarwood can be used by the food and cosmetic industries due to its unique flavor, fragrance and scent.

Table 2.3 Chemical constituents in different extracts of *Aquilaria* leaves

Species	Extraction method	Chemical constituents	References
A. malaccensis	hydrodistillation, solvent extraction	(i) hexadecanoic acid (ii) squalene	Adam *et al.*, (2018)
	hydrodistillation	(i) pentadecanal (ii) 9-octadecenal (ii) tetradecanal	Samadi *et al.* (2017)
	solvent extraction (hexane)	(i) stigmasterol (ii) β-sitosterol (iii) 3-friedelanol	Moosa (2010)
	solvent extraction (methanol)	(i) hexadecanoic acid (ii) phytol (iii) squalene	Khalil *et al.* (2013)
A. crassna	solvent extraction (hexane) solvent extraction (dichloromethane)	(i) 5-hydroxy-7,4-dimethoxyflavone (ii) epifriedelanol (iii) squalene (iv) phytol	Azziz *et al.* (2013)
	soxhlet extraction (water, hexane, isopropanol and ethanol)	(i) squalene (ii) *n*-hexadecanoic acid (iii) octadecatrienoic acid	Lee *et al.* (2016)
	soxhlet extraction (dichloromethane, petroleum ether, ethanol)	(i) mangiferin (ii) genkwanin	Ray *et al.* (2014)
	solvent extraction (ethanol)	(i) epigallocatechin gallate (ii) epicatechin gallate (iii) iriflophenone 3-C-β-glucoside	Tay *et al.* (2014)
A. sinensis	steam distillation	(i) 9-hexacosene (ii) pyridine-3-carboxamide (iii) 1-bromodocosane (iv) octacosane (v) docosane (vi) hexadecane,1-iodo- (vii) dodecane	Zhang *et al.* (2011)
	steam distillation	(i) hexadecanoic acid (ii) 6, 10, 14-trimethy l-2-pentadecanone (iii) tetradecanoic acid (iv) (E)-9-octadecenoic acid (v) pentadecanoic acid	Liu *et al.* (2007)

Table 2.3 Continued

Species	Extraction method	Chemical constituents	References
A. sinensis	steam distillation	(vi) 4, 8, 12, 16-tetramethylheptadecan-4-olide (vii) phytol (ix) nonanoic acid (x) isophytol (xi) octadecanoic acid	Liu *et al.* (2007)
	solvent extraction (hexane, ethyl acetate and methanol)	(i) 5-hydroxy-7, 4'-dimethoxyflavone (ii) genkwanin (iii) protocatechuic acid (iv) iriflophenone 3-C-β-glucoside (v) mangiferin	Pranakhon *et al*. (2015)
	solvent extraction (butanol)	(i) aquisiflavoside	Yang *et al.* (2012)
	solvent extraction (60 % ethanol)	(i) mangiferin (ii) genkwanin-5-O-β-primeveloside (iii) iriflophenone-2-O-α-rhamno-side	Kakino *et al.* (2010)
	solvent extraction	(i) 5-hydroxyl-7,4'-dimethoxyflavone (ii) luteolin (iii) genkwanin (iv) yuankanin (genkwanin-5-O-beta-D-primeveroside (v) adenosine (vi) genkwanin-5-O-beta-D-glucopyranoside (vi) hypoxanthine (vii) hypolaetin-7-O-beta-D-glucopyranoside (viii) 8-C-beta-D-galactopyranosylisovitexin	Feng *et al.*, (2012)
	solvent extraction (acetone and methanol)	(i) iriflophenone 2-O-α-rhamnoside (ii) iriflophenone 3, 5-C-β-diglucoside (iii) genkwanin 5-O-β-primeveroside (iv) mangiferin	Hara *et al.* 2008)
	solvent extraction	(i) β-sitosterol (ii) hexacosanic acid	Feng *et al.* (2011)

Table 2.3 Continued

Species	Extraction method	Chemical constituents	References
A. sinensis	solvent extraction	(iii) 2α-hydroxyursane dihydrotanshinone (iv) tanshinone I (v) tanshinone II (vi) 2α-hydroxyursolic acid (vii) p-hydroxybenzoic acid (viii) hydroquinone (ix)daucosterol	Feng *et al.* (2011)
	solvent extraction	(i) 7-hyroxy-5, 4'-dimethoxy flovone (ii) 5-hydroxy-7, 4'-dimethoxy flavone (iii) luteolin-7-3',4'-trimethyl isocorydine (iv) 4-hydroxybenzoic acid (v) triacontenoic (vi) hentriacontane (vi) α-stigmasterol (vii) epifriedelanol (viii) friedelan (ix) friedelin (x) genkwanin (xi) 5, 4'-dihyroxy-7, 3'-dimethoxy flovone	Nie *et al.* (2009)
	solvent extraction (methanol)	(i) 5-hydroxy-4',7-dimethoxyflavonoid (ii) luteolin-7,3',4'-trimethyl ether (iii) 5,3'-dihydroxy-7,4'-dimethoxyflavone (iv) vanillic acid (v) *p*-hydroxybenzoic acid (vi) methylparaben (vii) syringic acid (viii) isovanillic acid (ix) β-sitosterol (x) stigmasterol (xi) β-sitostenone (xii) stigmasta-4,22-dien-3-one	Kang *et al.* (2014)
	solvent extraction (70 % aqueous ethanol)	(i) aquilarisinin (ii) aquilarinoside (iii) hypolaetin 5-o-β-d-glucuronopyranoside	Kim *et al.* (2015)

Table 2.3 Continued

Species	Extraction method	Chemical constituents	References
A. sinensis	solvent extraction (70 % aqueous ethanol)	(iv) aquilariaxanthone (v) iriflophenone 2-o-α-l-rhamnopyranoside (vi) iriflophenone 3-c-β-d-glucoside (vii) iriflophenone 3,5-c-β-d-diglucopyranoside	Kim *et al.* (2015)
	reflux extraction (ethanol-water)	(i) aquilarinoside A (ii) 7-β -D-glucoside of 5-O-methylapigenin (iii) iriflophenone (iv) mangiferin (v) 5-O-xylosylglycoside of 7-O-methylapigenin (vi) 5-O-xylosylglucoside of 7,4'-di-O-methylapigenin (vii) 5-β-d-glucoside of 7,3'-di-omethylluteolin (viii) luteolin (ix) genkwanin (x) hydroxy genkwanin	Qi *et al.* (2009)

The phytochemicals present in *Aquilaria* leaves are from diverse chemical classes including 2-(2-phenylethyl) chromones, phenolic acids, benzophenones, xanthonoids, flavonoids, terpenoids, phytosterols and fatty acids. Some of the phytochemicals have exhibited pharmacological effects, which might be the candidates for future drug discovery. Table 2.3 summarizes the literature review on the chemical constituents extracted from the leaves of *Aquilaria* by using different extraction techniques.

Samadi *et al*. (2017) reported that essential oil from the leaves of *A. malaccensis* consists of pentadecanal, 9-octadecanal, tetradecanal, hexadecanoic acid, δ-elemene, α-copaene, α-eudesmol, γ-eudesmol, caryophyllene. In addition, a study on the essential oil of *A. sinensis* extracted by using steam distillation and separated by capillary column chromatography identified the presence of nonanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, octadecanoic acid, (E)-9-octadecenoic acid, 6,10,14-trimethyl-2-pentadecanone and phytol as the major constituents (Liu *et al*., 2007). Meanwhile, Khalil *et al*., (2013) identified hexadecenoic acid, phytol and squalene as the major composition in the methanolic extract of *A. malaccensis*. Another study on

the extract of *Aquilaria* species obtained via maceration and soxhlet extraction techniques, revealed the presence of several major constituents like phytol, squalene, hexadecanoic acid and octadecatrienoic acid (Lee *et al.*, 2016). These chemical compounds were quantitatively analyzed by using gas chromatography-mass spectrometry (GC-MS) method. Based on review on identification of chemical constituent of *A. malaccensis* leaves, most identified compounds are from a group of terpenoids and fatty acids. Indeed, there is no scientific review on other classes like phenolic acids and flavonoids from this plant species.

Kang *et al*. (2014) isolated twelve pure chemical compounds from the methanolic extract of *A. sinensis*, which included groups of flavonoids, benzenoids and steroids. Other chemical compounds from flavonoids group were previously isolated from this species namely benzophenone, glucoside iriflophenone, mangiferin, 5-O xylosylglucoside of 7-O-methylapigenin, 5-O-xylosylglucoside 7,4-di-O-methylapigenin, 5-β-D-glucoside,7,3-di-O-methylluteolin, luteolin, genkwanin and hydroxy genkwanin (Qi *et al*., 2009).

Determination of the presence of bioactive compounds in the leaves of *Aquilaria* is important to officially register agarwood tea made of *Aquilaria* leaves as health beverage, which can be traditionally used in conjunction with the modern treatments to alleviate various health problems. The discovery is also important for the development of useful drugs from natural sources. The flavor of the tea which gives off unique taste and odor, is an important criterion to attract consumers buy-in.

Further analysis on the flavoring compounds present in *Aquilaria* leaves may be required using improved methods. It is important to conduct a study using simple sampling procedures to provide at least, the qualitative profiles of the volatile components present in *Aquilaria* leaves. Therefore, further research is necessary to examine the volatile constituents that may contribute to the unique flavor of the *Aquilaria* leaves extract. Determining the presence of these flavoring compounds in final products may require new or improved methods of analysis.

2.5 Method of Extraction

2.5.1 Solid-phase Microextraction (SPME)

Solid-phase microextraction (SPME) is a technique used to extract both volatile and non-volatile components in either liquid or gaseous states by establishing the equilibrium state between the sample matrix and the fiber coating. SPME is a simple and fast extraction technique that uses a fiber coated with sorbent without the need for solvent (Prosen & Zupancic-Kralj, 1999). SPME involves the transport of the analytes from the sample matrix to the coated fiber and analysis using analytical instrument of various types (Abkenar *et al.*, 2006; Richter & Schellenberg, 2007).

There are several factors influencing the effectiveness of SPME such as polarity and thickness of the fiber coating, and temperature of the sample. Previously, few researchers have been studying the effects of the polarity of fiber coatings used in SPME and the respective mechanisms of extraction on identification of volatile compounds in different kinds of matrices (Wardencki *et al*., 2004). It is important to select an appropriate fiber coating based on analyte size and polarity since different types of sorbents may bind to different groups of analytes. Pawliszyn, (2007) reported the amount of analyte adsorbed onto the fiber would increase by raising the thickness of fiber coating. However, increasing the temperature requires reduction in the extraction time, resulting in the decrease in the amount of analyte adsorbed onto the fiber. Another study did a comparison between the various types of fiber coating: polydimethylsiloxane (PDMS), polyacrylate (PA), PDMS/divinylbenzene (DVB), PDMS/carboxen, DVB/carboxen/PDMS (Shirey, 2000). The study indicated that PDMS has good affinity towards non-polar compounds, while PDMS/DVB binds better to polar compounds of low volatility.

2.5.2 Direct Thermal Desorption (DTD)

Direct thermal desorption (DTD) is the recently developed cost-effective alternative method, which enhance the analysis of volatile and semi-volatile organic compounds from solid, liquid and slurry samples in a very small quantity, by using GC/MS for aroma profiling. This technique is easy and fast, which requires little, or no sample preparation. It is a solvent-free method that involves the transport of the analytes

from a sorbent or sample matrix using heat and flow of inert gas. In addition, the use of this technique reduces the risk of sample contamination during sample preparation.

Direct sample probe captures and transport the vaporized sample in the GC-MS using carrier gas, while allowing temperature to be controlled. A spectrum match would be identified via GC-MS spectral library matching. Perez *et al.*, (1997) tested this technique using time as variable (10, 20, 30, 40, 50 and 60 min) at different temperatures (at 180°C, 200°C, 220°C and 240°C) to observe its effectiveness. Later, Falkovich & Rudich (2001) demonstrated that the DTD allows for temperature control and it is a time-dependent process. In addition, splitting samples would give advantages in DTD process by allowing the tube or trap desorption flows to be set higher than GC column flow; split flow can be adjusted to reduce the contamination and overloading onto a column and detector. The limit of ratio between estimates is approximately zero to 200:1. This tool would benefit the analysis of chemical compounds in the mixtures as the sorbent or matrix containing the analytes can be subjected to the high temperatures as required.

2.6 Methods of Analysis

Gas Chromatography (GC) is an analytical tool separation in research and commercial analytical laboratory. It widely used to identify (qualitative) and measure the amount (quantitative) to separating the compounds in a mixture into separate components. In GC analysis, compounds of the mixture travel through the GC column as gases or vapor phase. The compounds probably can be investigated by GC if compounds molecules have sufficient volatility and do not decompose at 400-450°C or below. The chromatographic separation involves the interaction of the sample between a stationary phase (solid or liquid) and a mobile phase (gas).

Previous research has shown that agarwood chemical constituents in agarwood chips were identified by GC-MS (Tajuddin & Yusoff, 2010). Richter & Schellenberg, (2007) were applied GC-MS coupled with SPME to analyze compound extracted from aromatic plant, namely, marjoram (*Origanum majorana L.*), caraway (*Carum carvi L.*), sage (*Salvia officinalis L.*) and thyme (*Thymus vulgaris L.).* Meanwhile, the chemical compositions for agarwood leaves by using GC-MS also have also been studied by Khalil *et al., (2013)* was found to be one of the major compounds.

2.7 Biological Activities of *Aquilaria* Leaves

Aquilaria leaves have been reported to exhibit various biological activities including antidiabetic, antibacterial, antimicrobial, antioxidant and anti-inflammatory. Some of these biological activities of *Aquilaria* spp. are relatively newly discovered, whereas some have been applied in traditional medicine which are now scientifically verified.

Table 2.4 Reported biological activities of crude leaves extracts from various species of *Aquilaria*

No.	Compounds	Species	Pharmacological activities	Reference
i.	Aglycone of aquilarisinin	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
ii.	Aquilarinoside A	*A. sinensis*	Anti-inflammatory	Qi *et al.* (2009)
iii.	Aquilarisinin (iriflophenone 2-O-β-D-glucopyranosyl-(1-4)-O-α-L-rhamnopyranoside)	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
iv.	Aquilarixanthone (2-C-β-D-xylopyranosyl-1,3,4,6,7-pentahydroxyxanthone)	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
v.	Aquisiflavoside	*A. sinensis*	Anti-inflammatory	Yang *et al.* (2012)
vi.	Genkwanin	*A. crassna*	Antioxidant	Ray *et al.* (2014)
vii.	Genkwanin 5-O-β-primeveroside	*A. crassna*	Laxative	Kakino *et al.* (2010)
		A. sinensis	Laxative	Kakino *et al.* (2010)
viii.	Hypolaetin 5-O-β-D glucorunopyranoside	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
ix.	Iriflophenone 2-O-α-L-rhamnopyranoside	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
x.	Iriflophenone 3-5-C-β-D-diglucopyranoside	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
xi.	Iriflophenone 3-c-β-D-glucoside	*A. sinensis*	α-glucosidase inhibition	Feng *et al.* (2011)
		A. sinensis	α-glucosidase inhibition	Pranakhon *et al.* (2015)
xii.	Mangiferin	*A. crassna*	Antioxidant	Ray *et al.* (2014)
		A. sinensis	α-glucosidase inhibition	Feng *et al.* (2011)
		A. crassna	Laxative	Kakino *et al.* (2010)
		A. sinensis	Laxative	Kakino *et al.* (2010)

Flavonoids and phenolics acids are the most important groups of secondary metabolites and bioactive compounds in plants, which can be potentially used in the treatment of cancer and other human diseases (Ghasemzadeh & Ghasemzadeh, 2011). According to Kumar & Pandey (2013), a number of flavonoids have shown significant antioxidative, anti-inflammatory, anti-cancer and antiviral activities, and free radical scavenging capacity, which may render hepatoprotective properties to prevent coronary heart disease. Previously, the anti-inflammatory activity *A. subintegra* leaves have been reported (Hashim *et al*,. 2012; Zhou *et al.*, 2008). This could be due to the presence of flavonoids in the leaves (Feng *et al*., 2011; Ito *et al*., 2012; Qi *et al.* 2009). Meanwhile, several study on *A. crassna* leaf extract by EtOH has been shown significant antioxidant (Dahham *et al.*, 2014; Ray *et al*., 2014; Tay *et al.*, 2014). On the other hand, a study on methanolic leaves extract of *A. malaccensis* identified the presence of alkaloids, steroids, triterpenoids, saponin and flavonoids, which may contribute to antioxidative activity (Huda *et al.*, 2009; Khalil *et al.*, 2013).

Several studies on the constituents of *Aquilaria* have been conducted, which led to isolation of various compounds from the plant. Hara *et al.*, (2008) and Kakino *et al.*, (2010) have identified the main compounds present in the leaves of *A. crassna* and *A. sinensis* namely mangiferin and genkwanin 5-O-β-primeveroside, which might contribute to laxative effects observed in the study. In 2011, Feng *et al.* reported the isolation of mangiferin, and iriflophenone 2-O kaempferol 3, 4, 7-trimethyl ether from the leaves of *A. sinensis*, which may act as antidiabetic agent that allows for lowering of blood glucose level by controlling carbohydrate absorption at the intestine. The biological activity of the chemical compounds isolated from *Aquilaria* leaves are summarized in the Table 2.4. Notably, most of the studies on *Aquilaria* leaves have been performed on *A. sinensis*, which may be due to the established use of the species in the traditional Chinese medicine.

2.7.1 Antioxidant Activity

Discoveries of medicinal properties of plants encourage the sourcing of antioxidants from nature, which have been extensively applied in the pharmaceutical and food industries (Sati *et al.*, 2010). Plant extract has also been very popular as health-promoting ingredient in herbal products. Antioxidants are capable of neutralizing the effect of unstable molecules or substances known as free radicals that damage the cells

in the body, and providing antioxidative defense (Reiter, 1995). Polyphenols, carotenoids and vitamins represent some of the major classes of natural antioxidants. In food, polyphenols may contribute to the bitter taste, astringency, color, flavor, odor and oxidative stability of the product (Pandey & Rizvi, 2009).

Considering the potential pharmaceutical effects of plant extract on human health, the use of efficient extraction technique is extremely important. The assessment of antioxidative activity in functional food and medicinal plants as well as the contributing chemical compositions have drawn remarkable attention in the study of food science and nutrition. The heated reflux extraction and maceration are conventional procedures frequently used to recover phenolics from solid samples (Khoddami *et al.*, 2013). The heated reflux methods are normally performed at 90 °C for several hours while maceration is performed over days at ambient temperature. According to Biesaga (2011) and Kalia *et al.* (2008), the advantages of these methods are simple, require relatively cheap apparatus and result in adequately high phenolic extraction rates.

Spigno *et al.* (2007) was report the effect of both temperature and time are strongly influence the antioxidant in grapes extract. This study indicated a reduction of phenolic content at 333K (60° C) for 20 hours, even the highest percentage yield (about 2.5 %) produced. The authors also observe the influence of lower temperature at 317K (44° C) at longer time (24 hour) obtained higher extraction yield about 3.0 %. Generally, sensitive compound is preferable to use a longer time extraction with lower temperature. Increase the solvent to solid ratio in extraction of phenolic compounds is consistent with mass transfer principle and will increase the total amount of extraction of solids by solvent. These effects have been recently approached by a work developed by Wong *et al.* (2013). The evaluation of their studies reported the solid-to-solvent ratio (1:5. 1:10, 1:15 and 1:20) on *Phyllanthus niruri* (Dukung Anak) reported solid-to-solvent ratio at 1:20 exhibited high antioxidant capacities. It has been shown that different extraction methods allow for extraction of different classes of chemical compounds in the plant with different biological activities. The findings signify the importance of selecting the most suitable extraction method based on the nature of the sample used.

Additionally, several evaluation assays such as Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl- 1-picrylhydrazyl (DPPH), radical absorbance capacity (ORAC), cupric ion reducing

antioxidant capacity (CUPRAC) have been developed to screen the antioxidative activity of the extracts from natural products, particularly those that are frequently consumed by people.

Reducing power can be measured as a significant reflection of the antioxidative activity (Oktay et al., 2003). CUPRAC test is designed to measure the capacity of antioxidants to bind with the metal ion or interact with free radical. The bis (neocuproine) copper (II) chelate (Cu (II)-Nc) is used as the chromogenic redox reagent in this assay. The color of the test solution would change due to the Cu(I)-Nc chelate formed as a result of redox reaction with reducing polyphenols, which can be measured spectrophotometrically at 450 nm (Apak *et al.*, 2004). Ascorbic acid, BHA, trolox, α-tocopherol and BHT are used as positive controls.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable organic radical widely used to assess the radical scavenging activity of antioxidants. The absorbance of the radical is measured in the range of 515–520 nm. Due to limited solubility of DPPH in aqueous solution, strong basic solution like methanol and ethanol are usually used. Sharma & Bhat (2009) reported the use methanol or buffered methanol as appropriate solvents for the assessment of antioxidative activity of non-polar, less polar and polar compounds against DPPH free radical. In this assay, ascorbic acid, BHA, vitamin E, gallic acid, catechin, quercetin, BHT and glutathione can be used as positive controls (Chanda & Dave, 2009). The deep purple solution of DPPH changes to violet when there is an exchange (donation and receiving) of electrons (Molyneux, 2004). The quality of the antioxidants in the extracts can be determined by calculating the half maximal inhibitory concentration (IC_{50} value as µg/mL), which represent the concentration of antioxidant required to decrease the initial free radical concentration by 50%.

These scientific evidences discussed in previous studies indicate that agarwood contains a lot of biologically active components, which may offer protection against degenerative diseases. Since the role of free radicals has been implicated in various diseases, the study on antioxidative activity of agarwood is important to exploit their therapeutic potentials. Previous study showed that the aqueous extracts of dried leaves of *A. crassna* exhibited radical scavenging capacities, which were measured using ABTS, FRAP and DPPH assays, giving the IC_{50} values of 7.25 ± 2.05 µg/m, 218.93 ± 29.77 µg/mL and 1.18 ± 0.07 µmol, respectively (Kamonwannasit *et al.*, 2013). Another study

also reported the antioxidative activity of the methanolic leaves extract of *A. crassna* (Sattayasai *et al.* 2012). Huda *et al.* (2009) investigated the antioxidative activity of the leaves extracts of *A. malaccensis* extracted using ethyl acetate, dichloromethane, methanol and hexane; the highest antioxidative properties was observed in the methanolic leaves extract.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter describes materials and methods used in this study. The research started with the leaves of *A. malaccensis*, which were subjected to extraction using water and methanol as solvents. The extracts obtained from the water and methanol extraction were then subjected to analysis using GC and UPLC-QToF/MS for identification of volatile and non-volatile chemical constituents. Both water and methanol extracts were then partitioned via liquid-liquid extraction method. The different partitions of crude extracts of *A. malaccensis* were further screened for antioxidative activities of bioactive compounds present in the samples. Then, extracts were chromatographed and fractionated to isolate the chemical compounds from this plant for further identity determination. The isolated compounds were characterized by spectroscopy techniques in comparison to the previously reported spectral data.

3.2 Materials

3.2.1 Plant Sample

The leaves of *A. malaccensis* were collected using bulk sampling from Ladang Karas, Merchang in Terengganu. The species of the plant was identified and verified at Institute of Bioscience, University Putra Malaysia, Malaysia. A voucher specimen (herbarium no. SK 2422/14) was collected and deposited at Herbarium of Laboratory of Biodiversity, Institute of Bioscience, Universiti Putra Malaysia, Malaysia.

3.2.2 Materials for Extraction and Isolation

Solvents namely hexane (Hx), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (ME) of industrial grade were used for extraction and isolation purposes.

Solvents of analytical grade were used for purification and analysis using chromatography. The spraying reagent used for TLC was diluted in sulphuric acid. Silica gel 60 GF_{254} (0.063 - 0.200 mm) (70 - 230 mesh ASTM) was used as a stationary phase in column chromatography, while aluminum and glass-supported silica gel 60 GF_{254} plates were used in TLC analysis. The silica gel and TLC sheets were purchased from Merck Millipore, Malaysia.

3.2.3 Plant Sample Preparation

After collection, plant materials were transferred to the laboratory. The leaves were separated from the twigs and cleaned under the running tap water to remove dust and foreign materials. The clean leaves were air dried in a hot oven at 40 °C (Prieto *et al*., 1999) until a constant weight was reached, which will be hereafter referred to as dried form. The dried material was ground using laboratory grinder to obtain fine powder with particle sizes < 0.5 mm. Samples of fine powder were sealed in plastic bag and stored at 4 °C prior to extraction.

3.3 Extraction Methods

3.3.1 Reflux Extraction

Sample of plant materials in fine powder form was weighed and transferred into a 2 L distillation flask. Distilled water was used as a solvent. The ratio between the weight of the samples and the volume of solvent used was 1:20. During extraction, the mixture was boiled at 90°C for 120 min. Afterwards, extracts were filtered through Whatman No. 1 filter paper. The extraction was conducted in triplicates using different set of samples. The samples were freezed at -80 °C before subjected to vacuum freeze drying for 72 hr. The extracts were placed in a glass bottle and stored at −4 °C until further use.

3.3.2 Maceration

150 g of plant sample in fine powder form were transferred into 5 L beaker, and added with methanol as a solvent at the ratio of 1:20 between sample and solvent, and left for 3 days at room temperature. The extract was filtered using Whatman No.1 filter paper. The filtrate was collected and the residue was re-extracted twice under the same conditions described previously. The solvent were removed under the reduced pressure at 60 ^{o}C using rotary evaporator (BÜCHI Rotavapor 114 equipped with a BÜCHI Water

bath B-480, Flawil, Switzerland and OAKTON Aspirator pump Model WP-15, Metex Corporation Limited, Toronto, ON) to produce crude methanol extract (ME).

3.3.3 Liquid-Liquid Extraction

The concentrated water and methanol extracts were suspended in water and fractionated with hexane, dichloromethane and ethyl acetate, consecutively, followed by evaporation in vacuo to obtain hexane (Hx), dichloromethane (DCM) and ethyl acetate (EtOAc) fractions. Figure 3.1 and 3.2 shows the procedure for extraction and fractionation of *A. malaccensis* leaves from both reflux and methanol extraction.

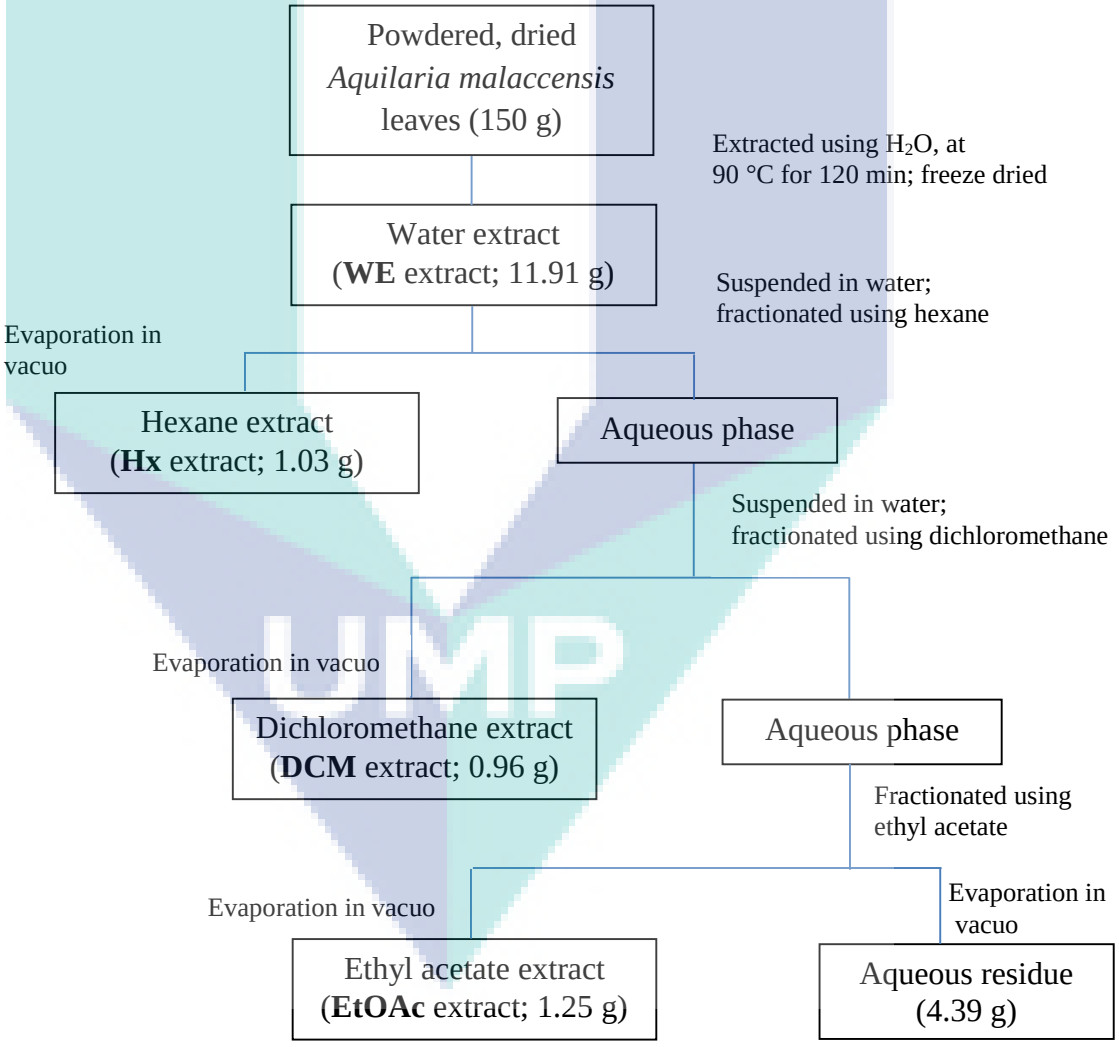


Figure 3.1 Extraction of *A. malaccensis* leaves and the extract fractionation by reflux extraction

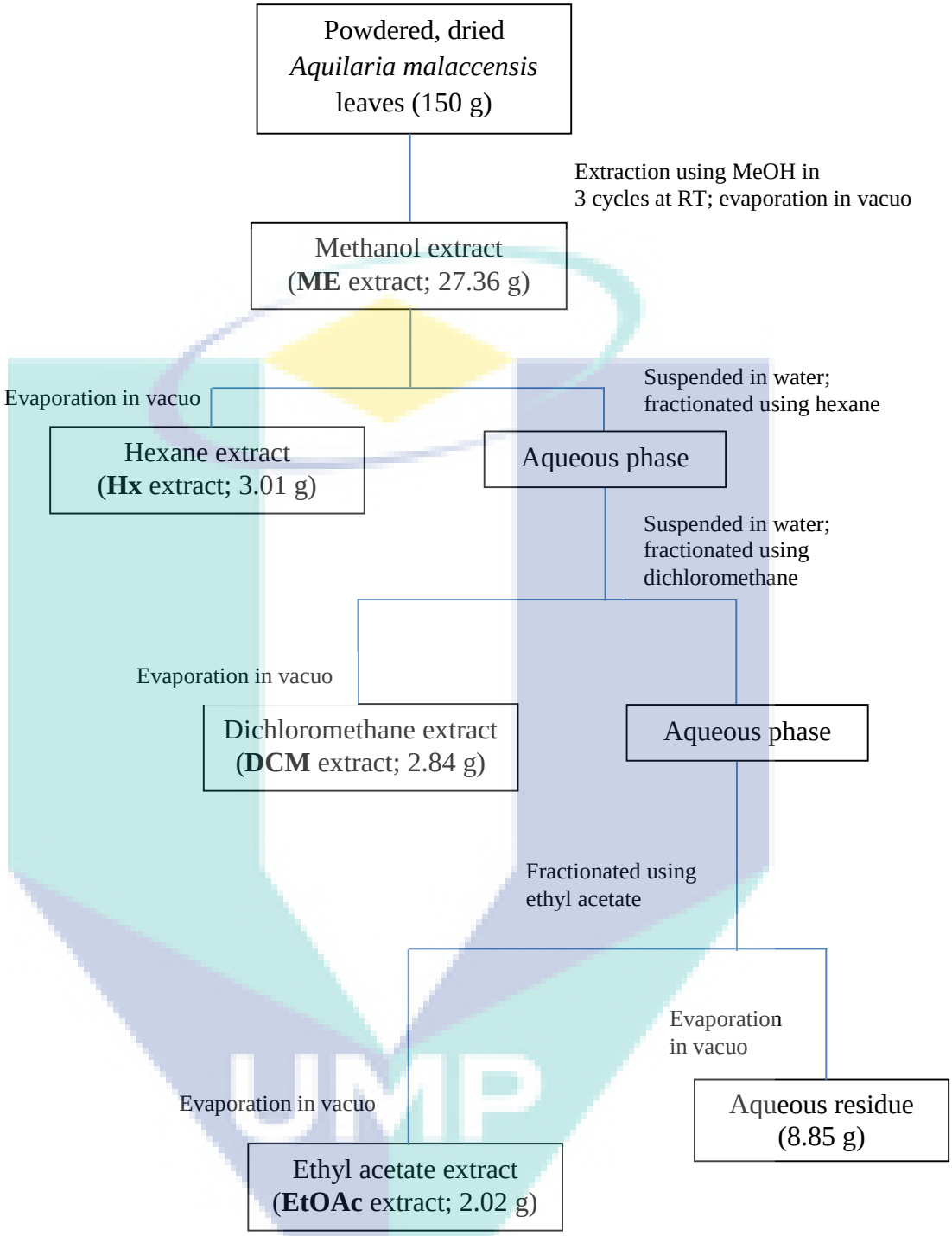


Figure 3.2 Extraction of *A. malaccensis* leaves and the extract fractionation by maceration

3.3.4 Solid-phase Microextraction (SPME)

The aroma components from the WE and ME were extracted using solid-phase microextraction (SPME). For each extraction, 1 g of samples was transferred into a 4 mL flat-bottom headspace vial. The arrangement of SPME apparatus is shown in Figure 3.3. The equipment used in SPME was purchased from Supelco Inc., Bellefonte, PA, USA. A manual SPME holder and 65 μm polydimethylsiloxane-divinylbenzene (PDMS/DVB/CAR) fiber were used; these were preconditioned at 230 °C for 60 min prior to being injected to GC injection port, which was cleaned before use and in between of analysis to prevent contamination (Taskin *et al*., 2013). Then, the extraction was performed manually by using SPME holder (Figure 3.4). The extraction was conducted by exposing 1 cm SPME fiber inside the sample vial, and heated at 45 ^{o}C for 30 min.

Figure 3.3 Arrangement of apparatus in SPME sampling technique

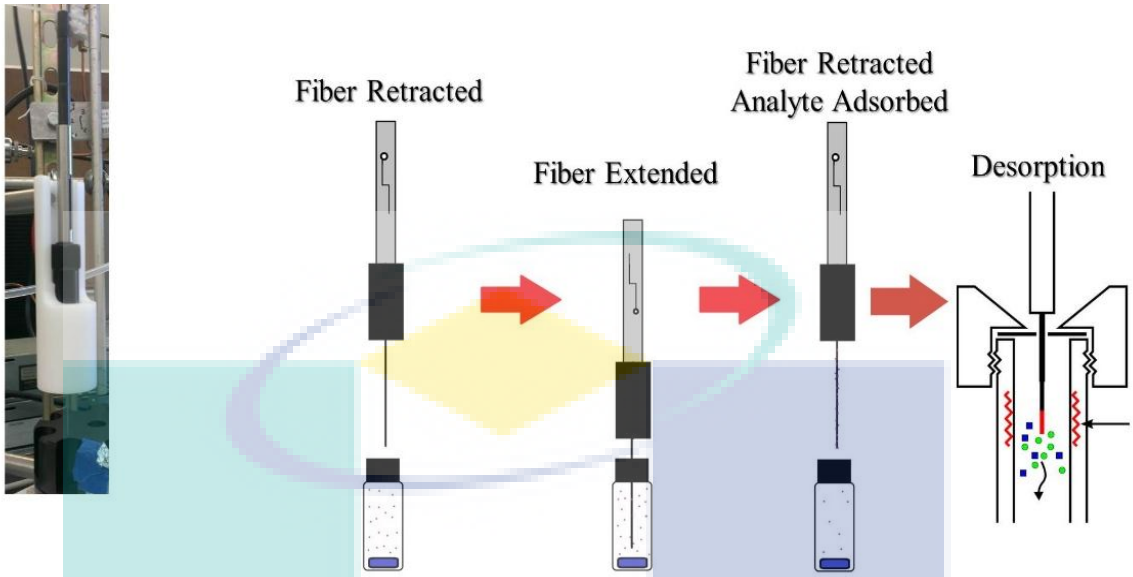


Figure 3.4 Schematic diagram of SPME extraction method

3.3.5 Direct Thermal Desorption (DTD)

This is another extraction technique chose for the purpose of this study due to its capacity to allow for analysis of solid sample devoid of any extraction or sample preparation. Direct thermal desorption (DTD) technique using Thermal Separation Probe (TSP) coupled with gas chromatography (GC) was used for rapid and cost-effective analysis rendered by ability to analyze the sample at the same time. A granule of the sample was introduced into a micro ultra-inert vial (Agilent Technologies, U.S.A).

Collected components were subsequently thermal desorbed (TD) and volatile components were transferred to GC for separation, identification and quantification of chemical compositions in the sample. The sample vial was subjected to GC injector by using the thermal desorption tool. Figure 3.5. shows the adapter used in the set-up of direct thermal desorption.

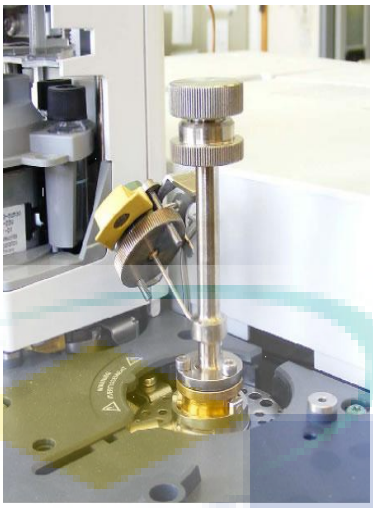


Figure 3.5 Direct thermal desorption (DTD) adaptor

3.4 Phytochemical Analysis

3.4.1 Gas Chromatography-Mass Spectrometry (GC-MS)

GC/MS of the model Agilent 7890A Network System equipped with DB-1 column was used. Samples were injected into the GC in splitless mode using a narrow SPME inlet liner. Helium (1.0 mL/min) was used as carrier gas. The initial temperature of the oven was programmed and hold at 60°C, and ramped at 3°C/min to 100°C before raised to 180°C at 3°C/min for 15 min. A final hold for 1 min was allowed for a complete column cleanup. The injector and detector temperatures were set at 230°C. The compounds from the samples (extracts) were identified by comparing their mass spectral fragmentation patterns with that of similar compounds from database (Wiley/NBS library), as well as by comparing their retention indices and mass spectra with the published data (Konig & Hochmuth, 2004).

3.4.2 Gas Chromatography-Flame Ionisation Detector (GC-FID)

WE and ME were analysed by using Agilent 7890A gas chromatography coupled with the flame ionisation detector (FID) and DB-1 capillary column (30 m x 0.25 mm and film thickness 0.25 µm). The injector and detector temperatures were set at 230°C. Oven temperature was kept at 60°C, then gradually raised to 100°C at 3°C/min before finally raised to 180°C at 3°C/min and held for 15 min. Data were obtained electronically in the form of retention times and percentage of the area. Based on the data obtained, estimates

of chemical composition was calculated using Kovats Indices. The Kovats Indices was calculated using the equation 3.1 and 3.2 (Kovats, 1958).

For isothermal chromatography, the Kovats index is given by the Equation 3.1:

$$I = 100 \text{ x } \left[n + (N - n) \frac{\log\left(t'_{r(unknown)}\right) - \log(t'_{r(n)})}{\log\left(t'_{r(N)}\right) - \log(t'_{r(n)})} \right] \quad 3.1$$

For temperature programmed chromatography, the Kovats index is given by the Equation 3.2:

$$I = \left[\frac{t_{r\,(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right] * (100 \text{ X } z) + (100 \text{ X } n) \quad 3.2$$

I = Kovats retention index; n = the number of carbon atoms in the smaller alkane; N = the number of carbon atoms in the larger alkane; z = the difference in the number of carbon atoms in the smaller and larger alkane; tr = the retention time.

3.4.3 Quadrupole Time-of-Flight Liquid Chromatography-Mass Spectrometry (QTOF-LCMS)

1 g of each WE and ME were dissolved in 1 mL water and methanol, respectively, and subjected to ultra-high performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-QToF/MS) for determination of active compounds present in leaves of *A. malaccensis*. The samples were analyzed by using HSS BEH C_{18} column (1.8 µm, 2.1 mm × 100 mm column, Waters Corp.). The HPLC data was obtained from Waters Xevo G2-S QToF. The mobile phase was composed of solvent A (0.1 % formic acid aqueous, v/v) and solvent B (acetonitrile). Injection volume was 2 µL, and the flow rate was set at 0.6 mL/min. The QToF/MS was operated within the range of 10 - 1500 Da, in positive modes.

3.4.4 Fourier Transform Infrared Spectroscopy (FTIR)

Infra-red spectra were recorded by using Perkin Elmer Spectrum 100 FT-IR/FT-NIR Spectrometer, using chloroform as a solvent. FTIR was used to give information regarding functional groups present in the molecules.

3.4.5 UV-Vis Spectrophotometer (UV-Vis)

The UV spectra were measured by using the UV-Vis Scanning Spectrophotometer (Thermo Scientific), using methanol as a solvent. UV-Vis was used to measure the absorption of UV by the samples at different wavelengths.

3.4.6 Nuclear Magnetic Resonance (NMR)

NMR spectra were obtained using the Bruker Ultra Shield Plus 500MHz. The structures of the isolated compounds were elucidated with the aid of spectroscopic methods such as 1D (^{1}H,^{13}C, DEPTQ) and 2D NMR (COSY, HMBC, HSQC). Deuterated chloroform ($CDCl_3$) was used as NMR solvent. Chemical shifts were reported in ppm, and coupling constants were given in Hz.

3.5 Total Phenolic Content (TPC)

Samples were measured in triplicates for total phenolic content according to the method of (Singleton *et al.*, 1998) with some modifications. A stock solution of plant extracts was prepared in different volume of aliquots (31.25, 62.5, 125, 250, 500 and 1000 µg/mL). An aliquot of 50 µL of each extract and fractions was mixed with 150 µL of Folin-Ciocalteu phenol reagent and allowed to react at room temperature for 5 minutes. Then, about 150 µl of 7.5 % sodium carbonate solution was added and the mixtures were incubated in the dark for 2 h at room temperature. The absorbance of the reaction mixture was read at 765 nm against the blank using microplate reader. The TPC of the extracts was expressed as mg gallic acid equivalent (GAE) per gram dry weight of plant and all determinations were performed in triplicates by using the following Equation 3.3 (Singleton *et al.*, 1998):

$$A = (C \times V) / m \qquad 3.3$$

where;

A: The total content of phenolic compounds, mg/g plant extract

C: The concentration of gallic acid established from the calibration curve, mg/mL

V: The volume of extract in mL

m: The weight of plant extract in g

3.6 Bioactivity Assay

3.6.1 DPPH radical-scavenging activity

The scavenging activity of the extracts was determined using DPPH-scavenging assay (Brand-Williams *et al*., 1995) with slight modification. A volume of 100 µL of each extract and standard was allowed to react with 200 µL of DPPH solution (0.004 % w/v) in 95% methanol. The concentration of the samples were 31.25, 62.5, 125, 250, 500 and 1000 µg/ml. The mixtures were then incubated in the dark at room temperature for 30 min, and the absorbance (A) was measured at 517 nm using a microplate reader against a blank. All measurements were performed in triplicates. Ascorbic acid was used as a positive control. Absorbance of DPPH solution in the absence of the extract or reference agent and methanol were used as negative controls. The scavenging activity was calculated as a percentage of DPPH decolouration relative to a negative control using the following Equation 3.4 (Brand-Williams *et al*., 1995):

$$\% \text{ DPPH scavenging activity} = \{(A_0 - A_1)/A_0\} \times 100 \qquad 3.4$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard.

3.6.2 Cupric Reducing Antioxidant Capacity (CUPRAC)

The capacity to reduce cupric ions was determined using the Cupric Reducing Antioxidant Power (CUPRAC) assay as described by (Apak *et al.*, 2004). A volume of 50 µl was drawn from the extracts and standard to prepare mixtures of different concentrations (31.25, 62.5, 125, 250, 1000 µg/mL), and mixed with 100 µL of copper (II) chloride ($CuCl_2$) solution (1.0×10^{-2} M), 100 µL of neocuproine alcoholic solution (7.5×10^{-3} M), 100 µL of ammonium acetate (NH_4Ac) buffer solution and 30 µl distilled water. All mixtures were allowed to stand for 30 min at room temperature. Absorbance against a blank was measured at 450 nm using Tecan Infinite M 200 PRO microplate reader. The percentage of reducing capacity of the extracts and the standards were calculated using the equation (Apak *et al.*, 2004): % reducing capacity = $\{(A_0 - A_1) / A_1\} \times 100$, where A_0 is the absorbance of the reaction mixture and A_1 is the absorbance of the blank.

3.7 Separation and Purification

The crude hexane and dichloromethane fractions of WE and ME were subjected to separation on the column chromatography over silica gel (Kiesel gel 60H). A slurry of silica gel in hexane system was poured into a glass column of appropriate size with gentle tapping to remove trapped bubbles. The isolation was conducted based on gradient elution method. The solvent systems used were hexane-ethyl acetate and ethylacetate-methanol. The eluent was let to run through the column until components of the mixture had been eluted. Vials of each fraction were collected and evaporated to remove solvents.

Fractions with similar compound were then combined by under TLC monitoring. Aluminum supported silica gel 60 F 254 plates were used in TLC. Analysis of the fractions of crude extract from the column chromatography and the isolated compounds. The sample was spotted on the TLC plates by using a piece of micro-hematocrit capillary tube, the TLC plates were spotted and placed in developing tanks saturated with the developing solvent system. The spots developed on the TLC plates were visualized under UV lamp at 254 nm and 365 nm, followed by spraying with 6 % sulfuric acid.

3.7.1 Compound I

A. malaccensis hexane extract was chromatographed on silica gel column in gradient systems of hexane-ethyl acetate and ethyl acetate-methanol. Fractions showing similar TLC profile were pooled to give 11 fractions (A1 – A11) as shown in Table 3.1. Repeated run of fraction A3 (115.20 mg) on silica gel column chromatography in gradient system of hexane-ethyl acetate yielded 12 fractions (A3.1 - A3.12). A3.2 (35.0 mg) was chromatographed in gradient system of hexane-ethyl acetate to affordcompound (**I**) (3.1 mg).

Table 3.1 Fractions from column chromatography of *A. malaccensis* hexane extract

Eluent	**Ratio (%)**	**Fraction**	**Weight (mg)**
Hx	100	A1	112.37
Hx : EtOAc	98 : 2	A2	57.43
Hx : EtOAc	93 : 7	A3	115.20
Hx : EtOAc	90 : 15	A4	81.97
Hx : EtOAc	80 : 20	A5	57.60
Hx : EtOAc	60 : 40	A6	30.93
Hx : EtOAc	50 : 50	A7	24.55
EtOAc	100	A8	28.42

Table 3.1 Continued

Eluent	Ratio (%)	Fraction	Weight (mg)
EtOAc : MeOH	90 : 10	A9	54.28
EtOAc : MeOH	50 : 50	A10	66.17
MeOH	100	A11	220.15

3.7.2 Compound I

Dichloromethane extract from *A. malaccensis* was chromatographed on silica gel column in gradient systems of hexane-ethyl acetate and ethyl acetate-methanol to give 12 fractions. Fractions which have spots with same R_f values and stains on the TLCs were combined to give 11 fractions (B1 – B11) as shown in Table 3.2. Repeated run of fraction B3 (65.80 mg) on silica gel column chromatography in gradient system of hexane-ethylacetate yielded 7 fractions (A3.1 - A3.7). A3.3 (10.39 mg) was chromatographed in gradient system of hexane-ethyl acetate to yield compound (**II**) (0.8 mg).

Table 3.2 Fractions from column chromatography of *A. malaccensis* dichloromethane extract

Eluent	Ratio (%)	Fraction	Weight (mg)
Hx	100	B1	86.43
Hx : EtOAc	98 : 2	B2	69.71
Hx : EtOAc	95: 5	B3	65.80
Hx : EtOAc	90 : 10	B4	89.72
Hx : EtOAc	80 : 20	B5	14.87
Hx : EtOAc	60 : 40	B6	16.55
Hx : EtOAc	50 : 50	B7	37.83
EtOAc	100	B8	76.34
EtOAc : MeOH	90 : 10	B9	45.27
EtOAc : MeOH	50 : 50	B10	59.40
MeOH	100	B11	109.38

3.8 Statistical Analyses

All analytical values shown represent the means of triplicates. The data are expressed as mean ± standard deviation. Statistical significant difference was determined using One-way ANOVA and differences were considered to be significant if $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

Identification of chemical compounds and biological activity of *A. malaccensis* leaves have been the main focus of this study. In order to obtain the crude extracts, water and methanol were used as solvent. Identification of volatile and non-volatile chemical compounds present in the *A. malaccensis* extracts were carried out via analysis using GC and UPLC-QToF/MS. Preliminary screening using bioactivity assays on different leaves extracts of *A. malaccensis* showed the presence of antioxidative activity. Column chromatography of Hx and DCM fractions from the leaves of *A. malaccensis* has led to the isolation of friedelanol (**I**) and friedelin (**II**). The structures of the isolated compounds were determined by analyzing the spectral data in reference to the literature.

4.2 Extraction

Samples of *A. malaccensis* were extracted using two different methods of extraction: reflux extraction by water and maceration using methanol to obtain water extract (WE) and methanol extract (ME), respectively. The percentage yield of crude water and methanol extract was calculated based on dry weight of the ground plant materials. The yield of ME (27.36 g; 18.24 %) was higher than WE (11.91 g; 7.94 %) on dry weight basis. The difference in the yields may be attributed to the presence of different groups of chemical compounds in the extracts, which extractability depends on the type of solvent and method of extraction used (Turkmen *et al.*, 2006). Both crude extracts were further fractionated to obtain hexane (Hx), dichloromethane (DCM) and ethyl acetate (EtOAc) fractions to allow for extraction and separation of a wide range of components that were present in the samples. Extracts of different colors were observed. It was found that WE formed dark brown gum; Hx extract formed dark green gum; DCM extract

formed dark yellowish-green gum; EtOAc extract formed dark brown gum; and ME formed dark green gum. Evaporation of WE extracts produced 1.03 g, 0.96 g and 1.25 g of extracts using Hx, DCM and EtOAc, respectively. On the other hand, evaporation of ME produces 3.01 g of Hx, 2.84 g of DCM and 2.02 g of EtOAc extracts, respectively. All fractions from WE and ME of the leaves of *A. malaccensis* were subjected to determination of antioxidant properties.

4.3 Profiling of *A. malaccensis* leaves extracts using GC and UPLC-QToF/MS

Application of mass spectrometry in combination with chromatographic techniques is considered as one of the most powerful approach for analysis of organic substances in complex mixtures. In this work, WE and ME of the leaves of *A. malaccensis* were obtained and subjected to identification of chemical constituents; solvent with high polarity have high affinity towards phenolic compounds in the plant leaves extract compared to the solvent with low polarity (Roby *et al.*, 2013). The volatile aroma compounds were analyzed using two common analytical techniques which combined the use of SPME and DTD with GC-MS and GC-FID, whereas non-volatile compounds were identified by using UPLC-QToF/MS.

4.3.1 Profiling of Volatile Chemical Constituents by using SPME/DTD using GCMS/FID

Analysis of WE and ME using SPME/DTD chromatographed with GC-MS/FID had identified fouty-eight compounds, composed of three compounds from the group of carboxylic acid derivatives, twenty-one compounds of sesquiterpene hydrocarbons and twenty-four compounds of oxygenated sesquiterpenes. Generally, similar chemical profiles were observed in all samples of leaves extracts obtained from different extraction procedures using different methods of analysis. Overall, the study had identified higher number of chemical compounds in ME than those present in WE. Profiles of volatile compounds in WE and ME obtained using SPME and DTD techiques are listed in Table 4.1 and Table 4.2, respectively, according to retention time on a fused silica capillary column. The use of SPME to extract volatile and semi-volatile compounds from crude leaves extracts have been in practice for years (Constant & Collier, 1997). Previously, a study reported that DVB/CAR/PDMS was able to generate a more complete profile compared to other methods, due to the wider range of chemical compounds detected and higher signal intensity it renders (Rodrigues et al., 2008).

Table 4.1 Tentative identification of volatile chemical compounds in WE and ME obtained from the leaves of *A. malaccensis* by using SPME

No	Compound	RI	Percentage Area (%)		Identification
			WE	ME	
1	benzaldehyde	935	1.34	0.60	RI
2	α-pinene	940	3.34	0.41	RI
3	sabinene	970	0.34	0.42	RI
4	limonene	1025	0.79	0.43	RI
5	acetophenone	1066	2.51	2.91	RI, MS
6	nonanal	1085	7.83	0.43	RI
7	linalool	1087	0.34	10.54	RI
8	4-phenyl-2-butanone	1210	11.11	10.25	RI
9	α-copaene	1369	0.54	0.22	RI
10	β-cubebene	1390	0.44	0.41	RI
11	β-maaliene	1414	2.57	0.55	RI
12	β-selinene	1419	-	0.61	RI
13	caryophellene	1421	-	6.24	RI
14	α-guaiene	1440	1.16	0.37	RI
15	α-humulene	1457	0.70	1.01	RI
16	ϒ-gurjunene	1472	0.79	2.80	RI
17	β-agarofuran	1474	16.55	0.84	RI
18	germacrene D	1480	-	21.16	RI, MS
19	β-selinene	1486	0.81	1.15	RI
20	ϒ-muurolene	1488	-	0.73	RI
21	α-muurolene	1496	1.00	1.22	RI
22	ϒ-guaiene	1499	-	0.37	RI
23	α-selinene	1501	-	1.79	RI
24	δ-cadinene	1516	6.01	0.73	RI
25	δ-elemol	1530	0.53	0.56	RI
26	β-elemol	1535	-	0.65	RI
27	germacrene B	1554	1.85	1.61	RI
28	spathulenol	1566	0.26	0.29	RI
29	epoxybulnesene	1572	0.75	0.44	RI
30	isoaromadendrene epoxide	1590	0.33	0.47	RI
31	caryophellene oxide	1600	1.98	0.75	RI
32	10-epi-γ-eudesmol	1619	1.09	0.37	RI
33	α-cadinol	1641	-	0.55	RI
34	jinkoh-eremol	1643	0.62	0.58	RI
35	α-eudesmol	1652	0.63	0.35	RI
36	bulnesol	1664	1.54	0.49	RI
37	dehydrojinkoh-eremol	1673	-	0.21	RI
38	α-bisabolol	1683	-	0.21	RI
39	selina-3,11-dien-9-one	1687	14.48	6.92	RI
40	pentadecanal	1691	0.75	0.24	RI
41	rotundone	1703	0.86	3.79	RI
42	selina-3,11-dien-14-ol	1750	0.67	2.44	RI
43	hexadecanal	1794	-	0.33	RI
44	guaia-1(10),11-dien-15-al	1806	-	1.68	RI
45	karanone	1812	0.91	0.29	RI

Table 4.1 Continued

No	Compound	RI	Percentage Area (%)		Identification
			WE	ME	
46	oxo-agarospirol	1822	0.51	2.14	RI
47	eudesmol	1880	1.41	0.26	RI
48	*n*-hexadecanoic acid	1950	12.72	8.18	RI, MS

RI = indicates the retention indices which were calculated against C_7–C_{20} *n*-alkanes on the DB-1 column and compared with those reported in the literature (Adam *et al*., 2018) ; MS = comparison of the MS with those of the NIST library (> 90%).

As Table 4.1 shows, even if these samples seem to be quite similar, they are different considering only their number of chemical components and percentage peak identified in the two samples. Thirty-six compounds were identified in WE. Meanwhile, forty-eight compounds were identified in ME. Observation showed that the major compounds in WE were tentatively identified as β-agarofuran (16.55 %), selina-3,11-dien-9-one (14.48 %), *n*-hexadecanoic acid (12.72 %), 4-phenyl-2-butanone (11.11%), nonanal (7.83 %) and δ-cadinene (6.01 %). On the other hand, the main compounds found in ME were germacrene D (21.16 %), linalool (10.54 %), 4-phenyl-2-butanone (10.25 %), *n*-hexadecanoic acid (8.18 %), selina-3,11-dien-9-one (6.92 %) and caryophellene (6.24 %). From the results, 4-phenyl-2-butanone, *n*-hexadecanoic acid, and selina-3,11-dien-9-were the major compounds present in both leaves extracts.

Among the major compounds present in ME that had not been identified in WE were germacrene D and caryophellene; other compounds include β-selinene, ϒ-guaiene, α-selinene, β-elemol, α-cadinol, dehydrojinkoh-eremol, α-bisabolol, hexadecanal, guaia-1(10),11-dien-15-al were also not present in WE. A study by Manuhara *et al.* (2018) revealed that the different temperature and time of extraction affected the content of compounds in aqueous extracts. The germacrene D was present in the result of 55°C - 30 minutes extraction but not present at 75°C and 95°C. These difference are probably due to the degradation of some compound (with contact in water) during the extraction process.

Table 4.2 Tentative identification of volatile chemical compounds in WE and ME obtained from the leaves of *A. malaccensis* by using DTD

No	Compound	RI	Percentage Area (%)		Identification
			WE	ME	
1	benzaldehyde	935	1.09	0.42	RI
2	α-pinene	940	0.26	2.07	RI
3	sabinene	970	1.35	0.33	RI
4	limonene	1025	4.29	1.29	RI
5	acetophenone	1066	1.80	1.59	RI, MS
6	nonanal	1085	0.50	2.05	RI
7	linalool	1087	0.40	3.66	RI
8	4-phenyl-2-butanone	1210	2.06	0.40	RI
9	α-copaene	1369	0.38	0.32	RI
10	β-cubebene	1390	28.06	0.33	RI
11	β-maaliene	1414	0.65	1.34	RI
12	β-selinene	1419	1.08	0.55	RI
13	caryophellene	1421	0.53	0.43	RI
14	α-guaiene	1440	0.32	0.51	RI
15	α-humulene	1457	0.74	13.72	RI
16	ϒ-gurjunene	1472	0.30	2.22	RI
17	β-agarofuran	1474	0.34	0.75	RI
18	germacrene D	1480	-	0.78	RI, MS
19	β-selinene	1486	0.43	3.59	RI
20	ϒ-muurolene	1488	1.41	0.29	RI
21	α-muurolene	1496	-	0.34	RI
22	ϒ-guaiene	1499	-	0.43	RI
23	α-selinene	1501	0.77	0.51	RI
24	δ-cadinene	1516	0.28	-	RI
25	δ-elemol	1530	0.29	-	RI
26	β-elemol	1535	0.29	0.72	RI
27	germacrene B	1554	0.27	0.40	RI
28	spathulenol	1566	0.27	0.30	RI
29	epoxybulnesene	1572	0.08	1.32	RI
30	isoaromadendrene epoxide	1590	0.25	0.31	RI
31	caryophellene oxide	1600	1.86	0.39	RI
32	10-epi-γ-eudesmol	1619	0.44	0.66	RI
33	α-cadinol	1641	3.68	-	RI
34	jinkoh-eremol	1643	0.70	-	RI
35	α-eudesmol	1652	0.29	-	RI
36	bulnesol	1664	-	1.44	RI
37	dehydrojinkoh-eremol	1673	0.68	0.30	RI
38	α-bisabolol	1683	0.38	1.17	RI
39	selina-3,11-dien-9-one	1687	2.08	1.03	RI
40	pentadecanal	1691	0.26	1.52	RI
41	rotundone	1703	0.57	2.15	RI
42	selina-3,11-dien-14-ol	1750	6.72	0.29	RI

Table 4.2 Continued

No	Compound	RI	Percentage Area (%)		Identification
			WE	ME	
43	hexadecanal	1794	0.26	1.80	RI
44	guaia-1(10),11-dien-15-al	1806	0.52	0.30	RI
45	karanone	1812	0.27	3.35	RI
46	oxo-agarospirol	1822	1.43	16.96	RI
47	eudesmol	1880	0.83	0.58	RI
48	*n*-hexadecanoic acid	1950	30.52	27.06	RI, MS

RI = indicates the retention indices which were calculated against C_7–C_{20} *n*-alkanes on the DB-1 column and compared with those reported in the literature (Adam *et al*., 2018) ; MS = comparison of the MS with those of the NIST library (> 90%).

Volatile constituents of WE and ME obtained from the leaves of *A. malaccensis* by using DTD are shown in Table 4.2. Both extracts were analyzed based on the volatile organic compounds released during the automated process by using DTD. In general, the volatile compounds detected in both WE and ME were mainly from the group of terpenes, of which sesquiterpenes constituted the major components. A total of forty-four compounds were tentatively identified in WE and ME. Four compounds, namely *n*-hexadecanoic acid (30.52 %), β-cubebene (28.06 %), and selina-3,11-dien-14-ol (6.72 %) constituted the highest percentage of WE based on the calculated percentage area in GC analysis. On the other hand, *n*-hexadecanoic acid (27.06 %), oxo-agarospirol (16.96 %) and α-humulene (13.72 %) were the major compounds identified in ME. Germacrene D, α-muurolene, ϒ-guaiene and bulnesol were found present only in the ME.

From the result, forty-eight components were tentatively identified based on the calculated retention indices, in reference to the credible database of substances from past literature and library search of the known mass spectral databases. The identified compounds were classified into different groups such as non-terpenes, monoterpenes and sesquiterpenes. Their compositions have similar components but vary in the concentration of the lesser compounds. Table 4.3 summarizes the chemical constituents of the leaves extract according to classification of the compounds. According to a research done by Khalil *et al.* (2013), *n*-hexadecanoic acid or palmitic acid were identified as one of the main chemical component in the leaves of *A. malaccensis*. These compounds were reportedly exhibited anti-inflammatory activity (Aparna *et al.*, 2012) and could be potentially acted as larvicidal agent (Rahuman *et al*., (2000), which findings may provide the rationale behind the traditional use of plant from this species.

Table 4.3 Percentage of peak area of chemical compounds in WE and ME extract obtained from the leaves of *A. malaccensis*

Compounds	RI	Percentage Area (%)			
		SPME		DTD	
		WE	ME	WE	ME
Carboxylic acid derivatives					
benzaldehyde	935	1.34	0.60	1.09	0.42
4-phenyl-2-butanone	1210	11.11	10.25	2.06	0.40
acetophenone	1066	2.51	2.91	1.80	1.59
Sesquiterpene hydrocarbons					
α-pinene	940	3.34	0.41	0.26	2.07
sabinene	970	0.34	0.42	1.35	0.33
limonene	1025	0.79	0.43	4.29	1.29
nonanal	1085	7.83	0.43	0.50	2.05
linalool	1087	0.34	10.54	0.40	3.66
α-copaene	1369	0.54	0.22	0.38	0.32
β-cubebene	1390	0.44	0.41	28.06	0.33
β-maaliene	1414	2.57	0.55	0.65	1.34
β-selinene	1419	-	0.61	1.08	0.55
caryophellene	1421	-	6.24	0.53	0.43
α-guaiene	1440	1.16	0.37	0.32	0.51
α-humulene	1457	0.70	1.01	0.74	13.72
ϒ-gurjunene	1472	0.79	2.80	0.30	2.22
β-agarofuran	1474	16.55	0.84	0.34	0.75
germacrene D	1480	-	21.16	-	0.78
β-selinene	1486	0.81	1.15	0.43	3.59
ϒ-muurolene	1488	-	0.73	1.41	0.29
α-muurolene	1496	1.00	1.22	-	0.34
ϒ-guaiene	1499	-	0.37	-	0.43
α-Selinene	1501	-	1.79	0.77	0.51
δ-Cadinene	1516	6.01	0.73	0.28	-
Oxygenated sesquiterpenes					
δ-elemol	1530	0.53	0.56	0.29	-
β-elemol	1535	-	0.65	0.29	0.72
germacrene b	1554	1.85	1.61	0.27	0.40
spathulenol	1566	0.26	0.29	0.27	0.30
epoxybulnesene	1572	0.75	0.44	0.08	1.32
isoaromadendrene epoxide	1590	0.33	0.47	0.25	0.31
caryophellene oxide	1600	1.98	0.75	1.86	0.39
10-epi-γ-eudesmol	1619	1.09	0.37	0.44	0.66
α-cadinol	1641	-	0.55	3.68	-

Table 4.3 Continued

Compounds	RI	Percentage Area (%)			
		SPME		DTD	
		WE	ME	WE	ME
Oxygenated sesquiterpenes					
jinkoh-eremol	1643	0.62	0.58	0.70	-
α-eudesmol	1652	0.63	0.35	0.29	-
bulnesol	1664	1.54	0.49	-	1.44
dehydrojinkoh-eremol	1673	-	0.21	0.68	0.30
α-bisabolol	1683	-	0.21	0.38	1.17
selina-3,11-dien-9-one	1687	14.48	6.92	2.08	1.03
pentadecanal	1691	0.75	0.24	0.26	1.52
rotundone	1703	0.86	3.79	0.57	2.15
selina-3,11-dien-14-ol	1750	0.67	2.44	6.72	0.29
hexadecanal	1794	-	0.33	0.26	1.80
guaia-1(10),11-dien-15-al	1806	-	1.68	0.52	0.30
karanone	1812	0.91	0.29	0.27	3.35
oxo-agarospirol	1822	0.51	2.14	1.43	16.96
eudesmol	1880	1.41	0.26	0.83	0.58
n-hexadecanoic acid	1950	12.72	8.18	30.52	27.06

RI = indicates the retention indices which were calculated against C_7–C_{20} *n*-alkanes on the DB-1 column and compared with those reported in the literature (Adam *et al.*, 2018); MS = comparison of the MS with those of the NIST library (> 90%).

Several chemical compounds identified in the leaves such as benzaldehyde, caryophyllene, *n*-hexadecanoic acid, eudesmol and oxo-agarospirol were also found in other parts of *Aquilaria* trees, such as the stem and resinous part of the wood. Previous study showed that these compounds were also detected in agarwood oils (Tajuddin & Yusoff, 2010). The presence of benzaldehyde, caryophyllene, hexadecanoic acid and oxo-agarospirol in the methanol extract of agarwood oils were also reported. In addition, the compounds such as (-)-guaia-1(10),11-dien-15-al and (-)-selina- 3,11-dien, 9-one which were isolated from high-quality agarwood were also found present in both WE and ME of the leaves of *A. malaccensis* (Ishihara *et al.*, 1991). The compound, (-)-guaia-1(10),11-dien-15-al which emits a pleasant note is the most important component that contributes to the characteristic odor of agarwood.

4.3.2 Profiling of *A. malaccensis* Compounds through UPLC-QToF/MS

In this study, LC-MS based profiling was used to analyze the chemical compounds in the leaves extract of *A. malaccensis*. In an effort to investigate the

relationship between the composition of the extracts and its biological activities, ultrahigh-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QToF/MS) was used to determine the content of the active components in the samples obtained from *A. malaccensis*. A rapid, sensitive and reliable UPLC-QToF/MS is an established chromatographic technique used in plants analysis, to detect chemical compounds on high resolution. The compounds in WE and ME were tentatively identified or characterized based on the data obtained from MS.

Chemical compound identification was performed by determining the retention time (RT), mass error, ion response, and fragmentation pattern of the peak in chromatogram. Since all fragment ions are automatically elucidated by using Mass Fragment, verifying the identity of the components has become easier.

Based on the data processed on UNIFI software, the component matching were classified as good match when the value is ± 5 mDa error and poor match when the value is ± 10 mDa error. In order to verify if the matching was reasonable, adduct ions and the fragmentation ions were observed. The compositions of an element were determined based on the light emitted from high-accuracy detection of energy level of molecular ions [M - H]+, [M - Na]+ and [M - K]+, within the mass accuracy of 5 mDa. The results were verified by assessing the MS/MS fragmentation ions in reference to relevant literatures. The results may provide useful information for further study on pharmacological aspects of the chemical compounds.

Figure 4.1 shows the base peak ion (BPI) in the chromatogram generated from the analysis of WE. *A. malaccensis* using UPLC-QToF/MS. Based on the plotted components, the major compounds found in WE of *A. malaccensis* were 7-Hydroxy-5,3',4'-trimethoxy flavone, 2,4,4′,6′-tetrahydroxy-benzophenone, genkwanin, gallocatechin(4α→8)-epicatechin, loganic acid-6'-O-β-D-glucoside, (-)-epigallocatechin, epicatechin gallate (epicatechin-3-O-gallate), quercetagetin-6,7,3',4'-tetramethyl ether, rhamnetin and stigmasta-4,22-dien-3-one. Among them, compound 7-hydroxy-5,3',4'-trimethoxy flavone is the most abundant present in WE (Figure 4.2 and Appendix E1).

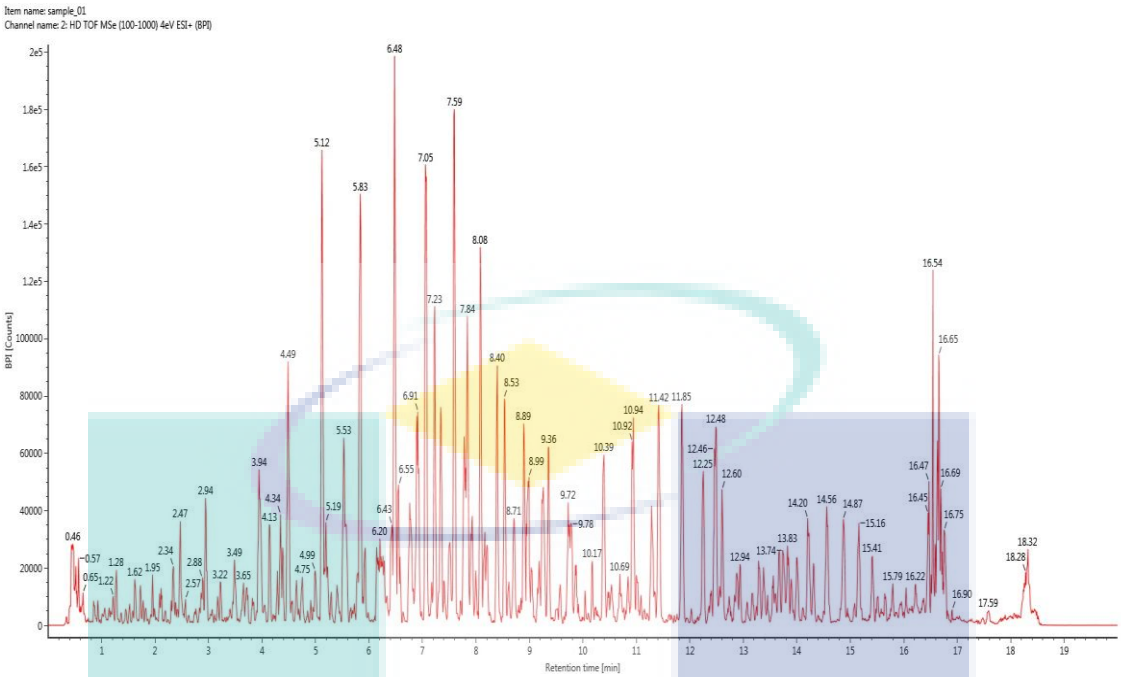


Figure 4.1 UPLC-QToF/MS positive BPI chromatogram of WE from the leaves of *A. malaccensis*

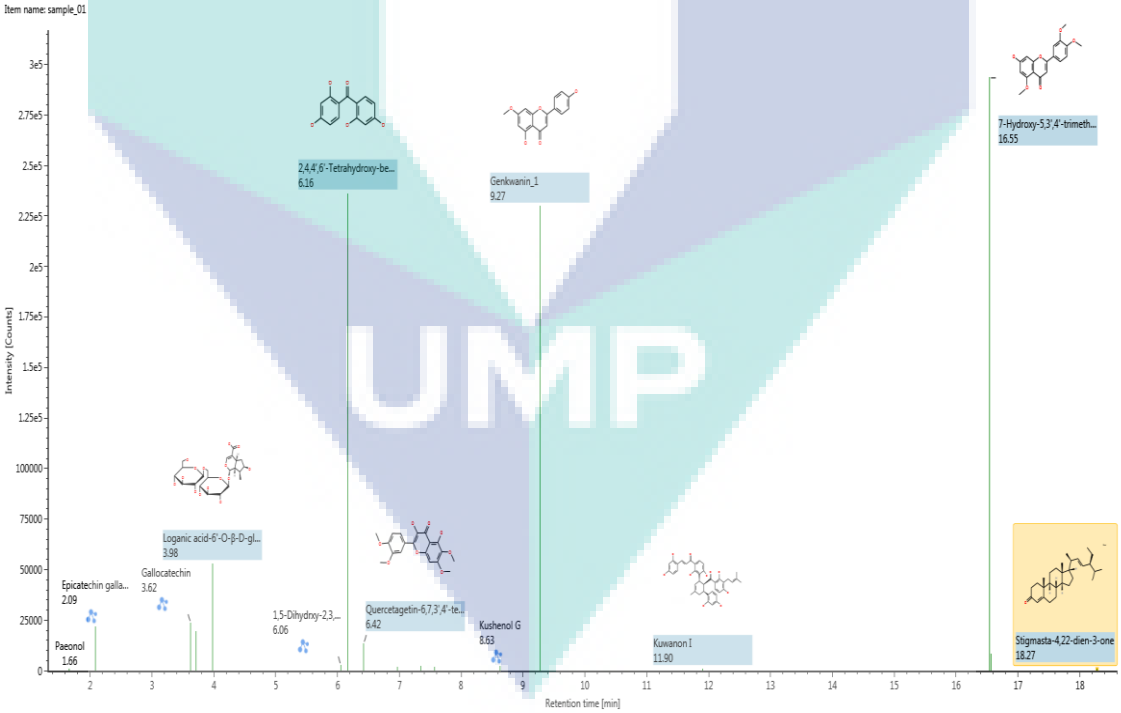


Figure 4.2 Major compounds plotted on (+) ESI-MS of WE

Figure 4.3 shows the base peak ion (BPI) in a chromatogram generated from the analysis of ME of *A. malaccensis* by using UPLC-QToF/MS. The compounds identified as genkwanin, 3′,5-dihydroxy-7,4'-dimethoxy flavone, 7-hydroxy-5,3',4'-trimethoxy flavone, 2,4,4′,6′-tetrahydroxy-benzophenone, stigmasta-4,22-dien-3-one, loganic acid-6'-O-β-D-glucoside and quercetagetin-6,7,3',4'-tetramethyl ether were found in the ME (Figure 4.4). Among them, compound genkwanin are the most abundant present in ME (Appendix E2).

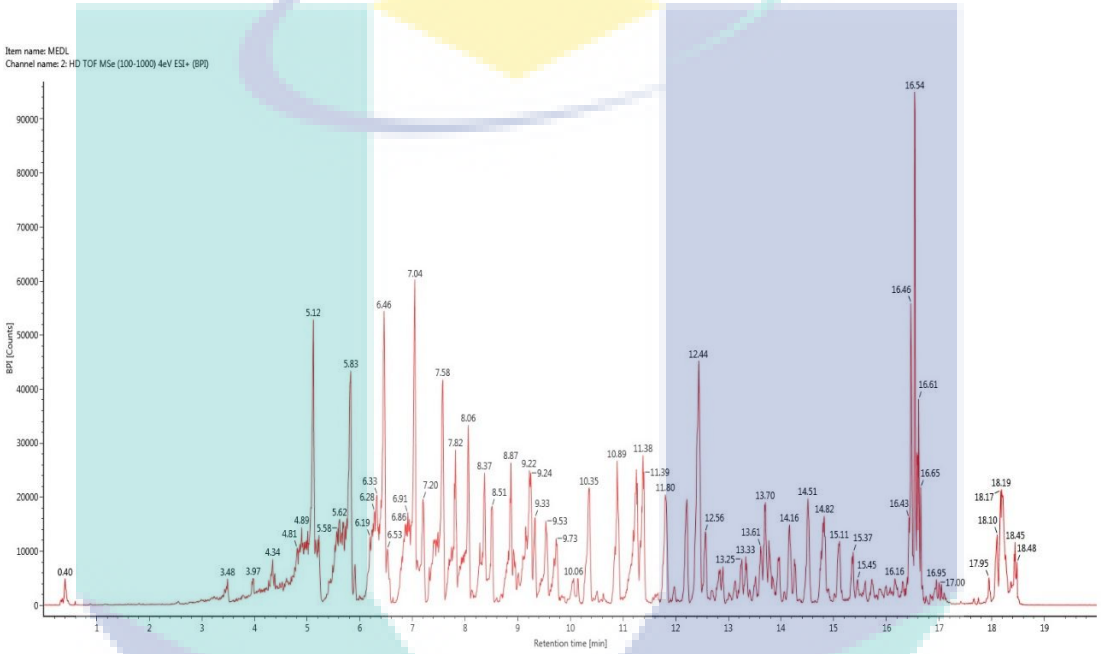


Figure 4.3 UPLC-QToF/MS positive BPI chromatogram of ME from the leaves of *A. malaccensis*

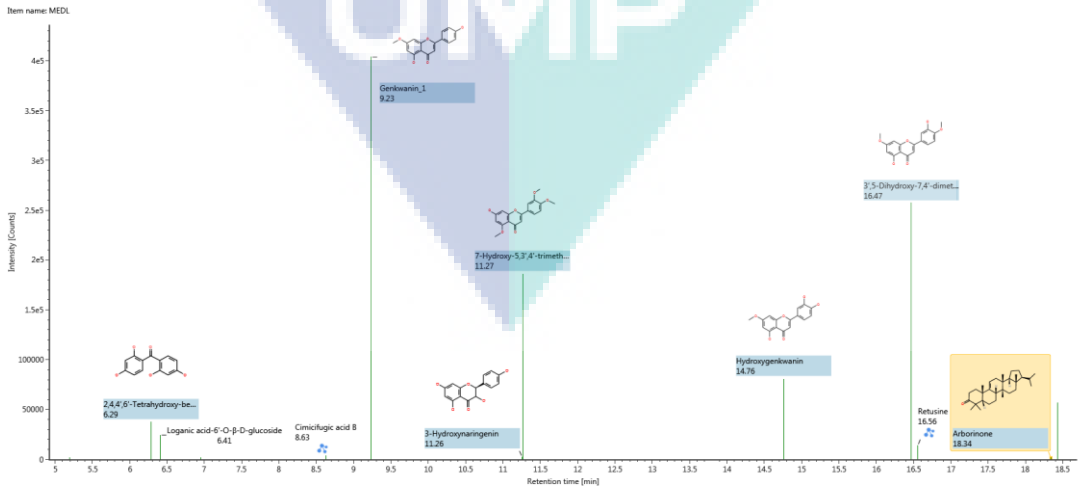


Figure 4.4 Major compounds plotted on (+) ESI-MS of ME

4.4 Total Phenolic Contents (TPC)

The Folin-Ciocalteu method is classified as electron transfer-based assay to determine the total phenolic contents based on the assessment of reducing capacity, which is expressed as mg/g gallic acid equivalents (GAE) (Prior et al., 2005). The amount of phenolic content was calculated based on the absorbance of the extract, reacted with Folin-Ciocalteu reagent, which was measured against the standard solutions of gallic acid equivalents. The phenolic content of different extracts were evaluated.

4.4.1 TPC of WE and its fraction

The total phenolic contents in the WE of the leaves of *A. Malaccensis* and its fractions are presented in Table 4.4. The preliminary analysis showed that the highest TPC (191.005 ± 0.002 mg GAEs/ g) was measured in WE, followed by EtOAc extract (82.779 ± 0.004 mg GAE/g) and DCM extract (40.130 ± 0.004 mg GAE/g). Hexane extract showed the lowest value of TPC (31.164 ± 0.002 mg GAEs/ g). From the result, it was observed that the total phenolic content varied in different extracts, might be due to the presence of phenols compounds.

Table 4.4 Quantitative analysis of phenolic contents in WE and its fractions obtained from the leaves of *A. malaccensis*

Sample	Total phenolic content, (mg GAE/g)
WE	191.005 ± 0.002^{d}
Hx	31.164 ± 0.002^{a}
DCM	40.130 ± 0.004^{b}
EtOAc	82.779 ± 0.004^{c}
Gallic acid	260.397 ± 0.002^{e}

Each value is expressed as mean ± SD (n=3). Mean values in the same column with different letters are significantly different ($p < 0.05$).

4.4.2 TPC of ME and its fraction

In this study, the various fractions of ME obtained from the leaves of *A. malaccensis* were screened via total phenolic content assay (Table 4.5). Phenolic content in ME was the highest with TPC value of 177.927 ± 0.001 mg GAEs/ g compared to that of other samples. Similar to WE, the lowest TPC was measured in Hx extract, following that of DCM and EtOAc extracts.

Table 4.5 Quantitative analysis of phenolic contents in the ME and its fractions obtained from the leaves of *A. malaccensis*

Sample	Total phenolic content, (mg GAE/g)
ME	177.927 ± 0.001^{d}
Hx	26.562 ± 0.003^{a}
DCM	34.486 ± 0.004^{b}
EA	70.365 ± 0.005^{c}
Gallic acid	260.397 ± 0.002^{e}

Each value is expressed as mean ± SD (n=3). Mean values in the same column with different letters are significantly different ($p < 0.05$).

The results revealed that water and methanol are the solvents suitable for extracting the phenolic compounds from plant extract due to; the polarity of the solvents allows for the binding and solubilization of phenolic components (Do *et al.*, 2014). However, it was observed that ME contained lower TPC than WE despite the higher yield obtained from the former. This may be attributable to the presence of other non-phenol compounds such as terpenes in ME.

In addition, the results also was in agreement with the previous study by Lee *et al.*, (2007), which showed that water is the most suitable solvent for extraction of phenolic compounds from *Pleurotus citrinopileatus.* Different TPC values were also observed in Hx, DCM and EtOAc extracts obtained from different methods of extraction. The content of total phenols in the descending order is presented as EtOAc > DCM > Hx. According to this result, solvent with relatively low polarity, particularly hexane and dichloromethane, showed relatively less ability to bind with phenolic compounds.

Phenolic compounds, including phenolic, phenolic acids, hydroxycinnamic acid derivatives and flavonoids showed considerable antioxidative activity (Khan & Mukhtar, 2008; Pandey & Rizvi, 2009). The large group of plant phenolic is known as flavonoids (Panche *et al*., 2016). Flavonoids produce beneficial effects on human health, which include antioxidative activity (Heim *et al*., 2002; Pietta, 2000) and have been widely used for medicinal purposes, as bioactive compounds (Havsteen, 2002; Kumar & Pandey, 2013). Previous studies have reported the phytochemical screening of methanol and water extracts, which identified the presence of flavonoids in the leaves of *A. malaccensis* (Khalil *et al*., 2013; Wil *et al.*, 2014).

4.5 Bioactivities

Different extracts of *A. malaccensis* were tested for their antioxidative activity. Two assays were used: DPPH free radical scavenging and reducing power (CUPRAC). The EC_{50} value (50 % Efficient concentration) or known as IC_{50} (50 % inhibition correlation) is defined as the concentration of antioxidant required to scavenge 50 % of DPPH activity. The crude extract with the IC_{50} values of more than 50 % in DPPH radical scavenging assay was identified as positive for scavenging activity. The higher the content of antioxidants in a sample, the higher the reducing capacity over DPPH. DPPH is scavenged by antioxidant through donation of hydrogen radical to form a stable DPPH diamagnetic molecule (Shimada *et al.,* 1992). DPPH radical scavenging methods is widely used because of the stability of organic nitrogen free radical, which allows for strong absorption band at 517 nm, characterized by changes in color from dark purple to slightly yellow or transparent, following reduction of DPPH by antioxidants.

According to Maisuthisakul *et al*., (2007), the small IC_{50} value of a plant extract or fraction corresponds to the high radical scavenging activity and high antioxidant activity. The IC_{50} value of the extracts in DPPH radical scavenging assay was obtained by extrapolation of the graph. The percentage of inhibition rendered by WE and ME, and their fractions (viz. hexane, dichloromethane, ethyl acetate) were plotted out as dose response curves and compared with that of ascorbic acid standard (Table 4.5).

Reducing assay is a simple method that gives fast and reproducible result. In this assay, the antioxidant activity was determined on the basis of the ability of the extracts to reduce cupric copper (Cu^{2+}) into cuprous (Cu^{1+}) ion. The change in the color of the reaction from yellow to blue depended on the reducing power of crude extracts. The high absorbance reading indicated the increase in reducing capacity.

4.5.1 DPPH and CUPRAC assay of WE and its fraction

Figure 4.5 depicts the ability of leaves extracts of *A. malaccensis* to scavenge DPPH radicals in comparison to that of ascorbic acid at different concentrations ranging from 31.25 to 1000 μg/mL. Increasing the concentrations of *A. malaccenis* extracts resulted in the increase of radical scavenging activities. The inhibition of DPPH radical by the different fractions of WE of *A. malaccensis* in descending order is represented as Ascorbic acid > WE > EtOAc > DCM > Hx.

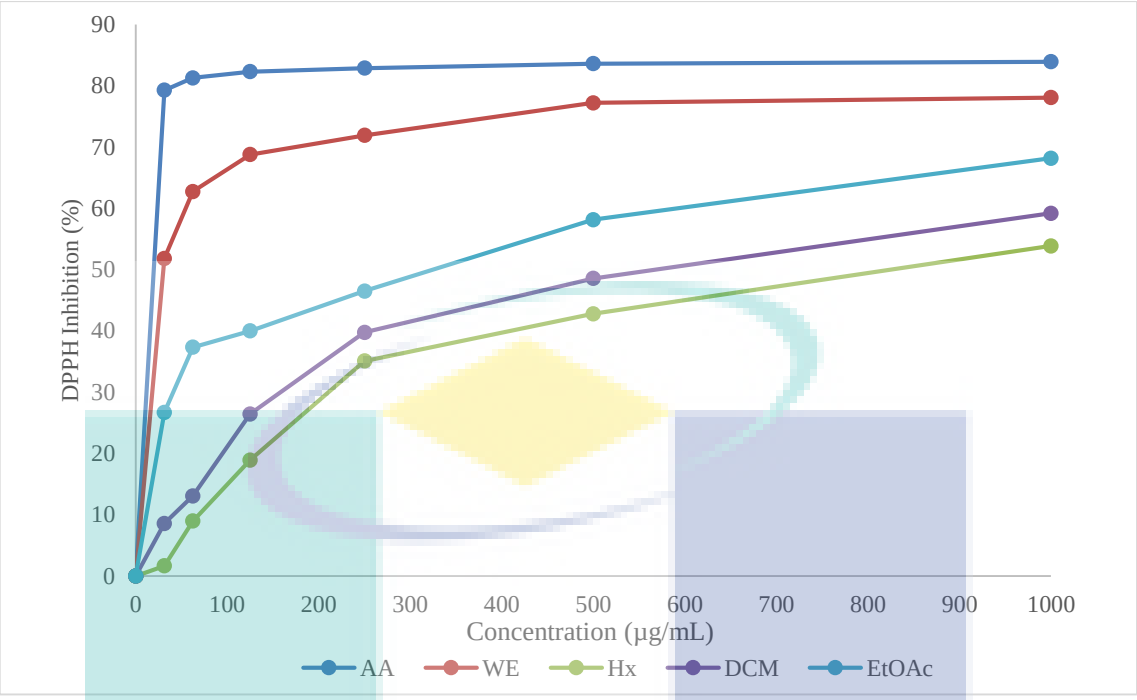


Figure 4.5 The DPPH free radical scavenging activity of *A. malaccensis* from WE and its fractions

The results from antioxidant assays showed that the IC_{50} values of 30.15 ± 0.002, 825.98 ± 0.003, 568.33 ± 0.006 and 273.19 ± 0.002 µg/mL were recorded in WE, Hx, DCM and EtOAc extracts, respectively. The results were compared with that of ascorbic acid, which recorded an IC_{50} value of 19.71 ± 0.002 µg/mL. The IC_{50} values obtained from DPPH assays are summarized in the Table 4.6.

The difference in slope observed on the plotted graph of DPPH scavenging activity of different extracts were due to capability of different solvents used to extract different types of compounds. Different concentration of compounds present in the extracts may have affected the ability to donate hydrogen atom and reaction with the free radicals. The highest free radical scavenging activity in WE could be due to the high content of phenolic components such as loganic acid-6'-O-β-D-glucoside, identified via HPLC analysis (Figure 4.2). The presence of polyphenols such as epicatechin gallate and epigallocatechin in *Aquilaria* leaves may have contributed to the high antioxidative activity (Tay *et al.*, 2014). In a study done by Kang *et al.*, (2014), extract from *Aquilaria* leaves also yielded phenolic acids group such as vanillic acid and syringic acid.

Table 4.6 IC_{50} in DPPH assay for WE and its fractions

Sample	IC_{50} (µg/mL)
Ascorbic acid (AA)	19.71 ± 0.002^{a}
WE	30.15 ± 0.002^{b}
Hx	825.98 ± 0.003^{e}
DCM	568.33 ± 0.006^{d}
EtOAc	273.19 ± 0.002^{c}

Each value is expressed as mean ± SD (n=3). Mean values in the same column with different letters are significantly different ($p < 0.05$).

Reduction of Cu^{2+} ion to Cu^{1+} by WE and its fractions increased steadily with increased concentration (Figure 4.6). The graph shows that the absorbance readings of ascorbic acid at all concentrations tested were higher than all samples of the extracts. In this assay, the reducing capacity of WE and its fractions in descending order is represented as Ascorbic acid>WE> EtOAc> DCM>Hx.

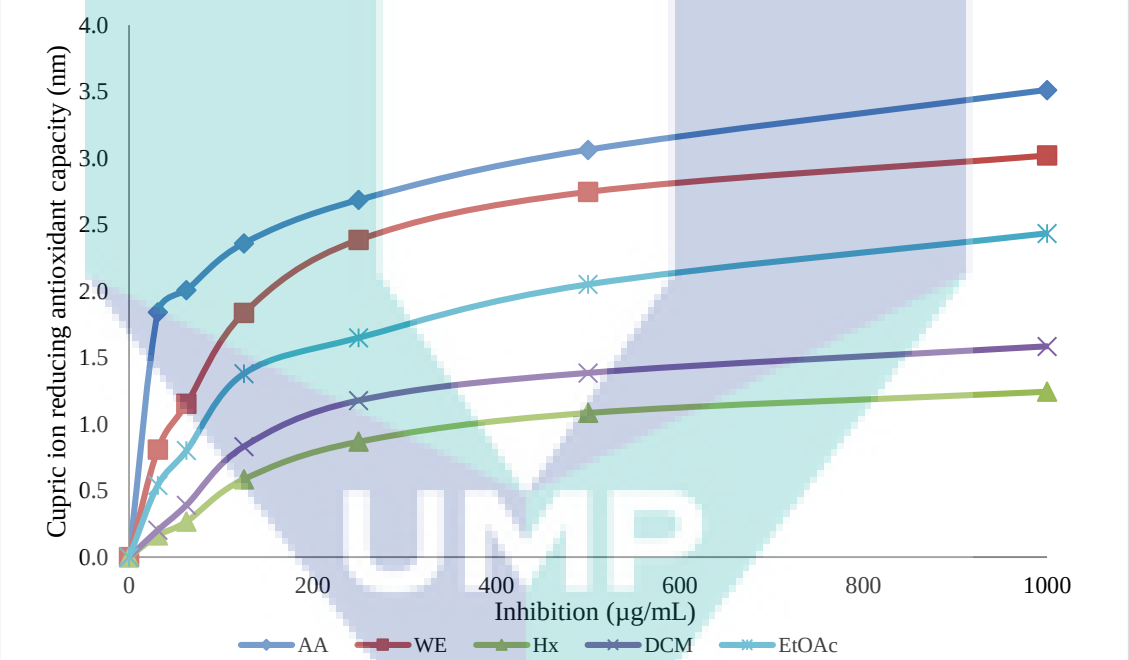


Figure 4.6 Cupric ion reducing capacity of WE and its fractions of *A. malaccensis*

4.5.2 DPPH and CUPRAC assays of ME and its fraction

As presented in Figure 4.7, ME of the leaves of *A. malaccensis* demonstrated effective scavenging activity against DPPH radical, in a concentration-dependent manner. The scavenging activity of ME compared to its fractions and positive controls in descending manner is presented as follows: Ascorbic acid > ME> EtOAc > DCM > Hx. This result shows similar trend with observed in WE.

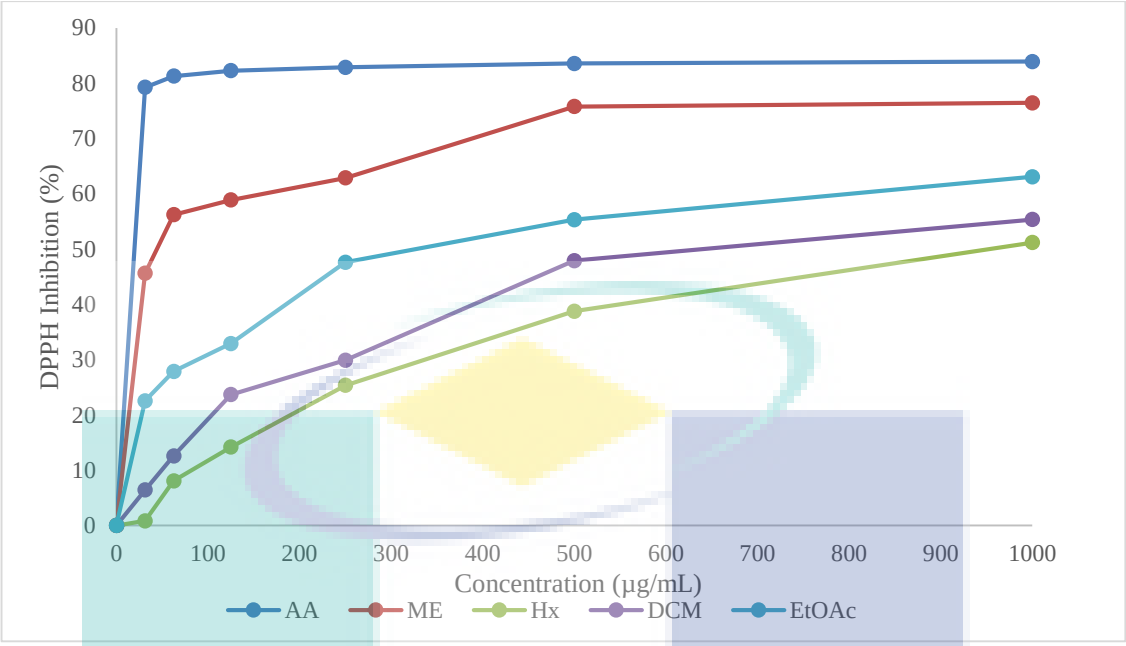


Figure 4.7 The DPPH free radical scavenging activity of *A. malaccensis* from ME and its fractions

A lower IC_{50} indicates higher antioxidant activity of a compound. Table 4.7 shows the IC_{50} values of the extracts representing the radical scavenging activity against DPPH. It was found that the ME showed the strongest reducing power against DPPH radical (IC_{50} = 44.18 ± 0.001 µg/mL), followed by EtOAc (327.68 ± 0.001 µg/mL), DCM (640.21 ± 0.004 µg/mL) and Hx (952.14 ± 0.003 µg/mL) extracts.

Table 4.7 IC_{50} in DPPH assay for ME and its fractions

Sample	**IC_{50} (µg/mL)**
Ascorbic acid	19.71 ± 0.001^{a}
ME	44.18 ± 0.001^{b}
Hx	952.14 ± 0.003^{e}
DCM	640.21 ± 0.003^{d}
EtOAc	327.68 ± 0.001^{c}

Each value is expressed as mean ± SD (n=3). Mean values in the same column with different letters are significantly different ($p < 0.05$).

In Figure 4.8, all extracts showed some degrees of electron-donating capacity in a concentration-dependent manner. The ME exhibited the highest reducing power compared to other extracts at all concentrations tested, followed by EtOAc extract, DCM extract and Hx extract.

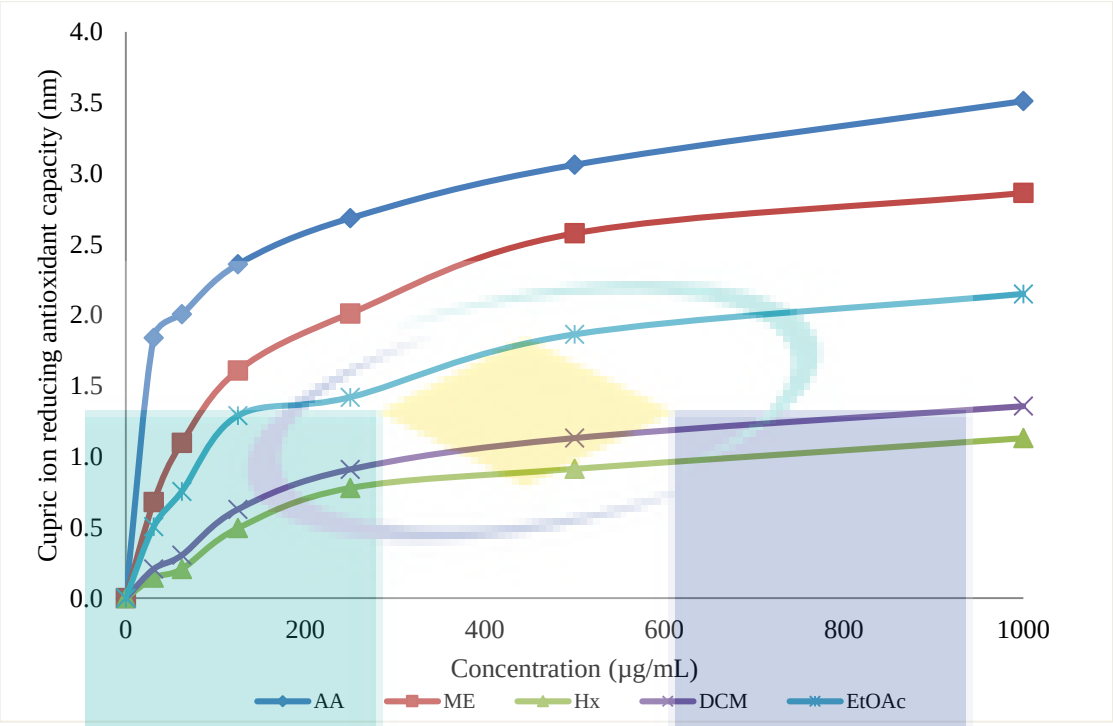


Figure 4.8 Cupric ion reducing antioxidant capacity of ME and its fractions of *A. malaccensis*

4.6 Correlation of bioactivities with total phenol content

Water and methanol extracts of a plant are equally important as both of extracts are rich in phenol although the use of water allows for maximum extraction. Water extraction involves the boiling of sample to enhance extraction and inactivate the enzymes present in the plant tissue. The phenolic compounds, which bear the hydroxyl group (–OH) in their structures have been associated with the antioxidative activity and therefore, may play an important role as a powerful antioxidant.

From the findings, there was a direct correlation between total phenol content and antioxidant activity. WE demonstrated the strongest antioxidative activity in all the tested conditions (Table 4.8). The IC_{50} values measured in the WE were excellent, representing strong reducing power; the phenolic content in WE was higher than that of ME. It could be due to lower recovery of polyphenols in ME following maceration of samples for 24 h; during this period, some unknown e.g. enzymatic reactions, especially oxidation could occur, causing decomposition of these compounds (Biesaga, 2011).

Table 4.8 Total phenolic content (TPC) and antioxidant activities (DPPH and CUPRAC) of *A. malaccensis*

Extract	TPC (mg GAE/g)	DPPH *(IC_{50}) (µg/mL)	CUPRAC *(IC_{50}) (µg/mL)
Water extract			
WE	191.005 ± 0.002[f]	30.15 ± 0.002[a]	23.28 ± 0.001[a]
Hx	31.164 ± 0.002[b]	825.98 ± 0.003[g]	549.22 ± 0.005[g]
DCM	40.130 ± 0.004[b]	568.33 ± 0.006[e]	241.14 ± 0.003[e]
EtOAc	82.779 ± 0.004[d]	273.19 ± 0.002[c]	119.48 ± 0.002[c]
Methanol extract			
ME	177.927 ± 0.001[e]	44.18 ± 0.001[b]	62.13 ± 0.003[b]
Hx	26.562 ± 0.003[a]	952.14 ± 0.003[h]	609.58 ± 0.001[h]
DCM	34.486 ± 0.004[b]	640.21 ± 0.004[f]	412.53 ± 0.007[f]
EtOAc	70.365 ± 0.005[c]	327.68 ± 0.001[d]	231.33 ± 0.002[d]

Each value is expressed as mean ± SD (n=3). Mean values in the same column with different letters are significantly different ($p < 0.05$).

The profiles generated from analysis using UPLC-QToF/MS also supported the presence of phenolic compounds in water and methanol extracts. The phenolic compounds act as a reductant by donating a hydrogen atom, and thus breaking the free radical chain. The result proved the positive correlation between TPC and antioxidative activity. Based on the confirmed component plotted via UPLC-QToF/MS, catechin found in WE was reported to exhibit antioxidative activity in this extract. In addition, the antioxidative activity of phenolic compounds could also be due to these compounds behaving as hydrogen donors and singlet oxygen quenchers. According to Yu *et al.*, (2013) the major active compounds in the leaves of *A. sinensis* were phenolic compounds including glycosides of xanthones, benzophenones and flavones. Meanwhile, mangiferin and genkwanin have been identified as the main phenolic compounds in the leaves of *A. crassna* (Hara *et al.*, 2008; Ito *et al.*, 2012; Kakino *et al.*, 2010). Therefore, the high phenolic content in the leaves of *A. malaccensis* observed in the present study is not surprising.

4.7 Compound isolation

Fractionation and chromatographic analysis of hexane and dichloromethane extracts from *A. malaccensis* had led to the isolation of friedelanol and friedelin. The isolated compounds were characterized by using spectroscopic techniques. The structures of the compounds were elucidated via references made to the previous work. The structures were obtained from the analysis using 500 MHz Varian NMR at Central

Laboratory, Universiti Malaysia Pahang. Previously study by Nie et al. (2009) reported both of this compounds were present in *A. sinensis*.

4.6.1 Compound I (Friedelanol)

Compound (**I**) (3.1 mg) isolated as white needle crystal indicating a triterpene-like compound. The GC-MS spectrum of compound (I) was relatively simple with a molecular ion peak, $[M+H]^+$ observed at m/z 428, which correlated to the molecular formula of $C_{30}H_{52}O$. The UV spectrum displayed the absorption bands at 294 nm. The spectrum of infrared (IR) indicated the presence of the –OH stretching vibration at 3468 cm^{-1} region. 2931 cm^{-1} region showed CH stretching vibration while CH bending vibration was shown at 1387 cm^{-1} region.

The results from the analysis using ^{H}NMR ($CDCl_3$, 500 MHz) (Table 4.9) showed some multiplicity. The existence of broad singlet at δ 3.74 indicated an H atom on the OH group. CH_3 was reflected in some of them with the multiplicity of δH 0.95, 0.99, 0.88, 0.99, 1.02, 1.19. 1.01. 0.96. The result indicated the presence of eight groups of -CH_3 on the analyzed compounds. The ^{13}C NMR spectrum showed the presence of 30 carbon atoms including six quaternary carbons, five CH carbons, eleven CH_2 carbons and eight CH_3 carbons. The presence of a hydroxyl group was evident in the ^{13}C NMR spectrum from the appearance of a hydroxyl carbon signal at δ 72.76. From COSY spectrum, the H-3 proton showed a correlation with two hydrogen atoms of H-2 (δH 1.45 and 0.96) and H-4 (δH 1.26). The ^{1}H, ^{13}C, COSY and HMBC spectral data of this compound indicated that it belongs to the friedelane group; the compound was identified as friedelanol (Figure 4.9) based on the ^{1}H and ^{13}C NMR data and physical properties reported in the literature (Monkodkaew *et al.*, 2009).

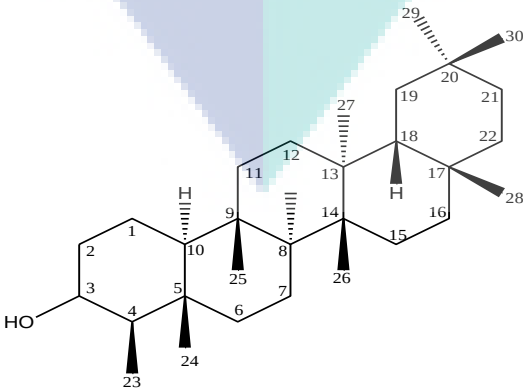


Figure 4.9 Structure of friedelanol

Table 4.9 ^{1}H-NMR and ^{13}C-NMR data of friedelanol in $CDCl_3$

Carbon Number	**Friedelanol isolated compound**				**Literature** (Monkodkaew *et al.* (2009)	
	δ_C	**δ_H**	**COSY**	**HMBC**	**δ_C**	**δ_H**
1	17.55	1.39			17.50	1.39
2	35.09	1.45, 0.96			35.03	1.45, 0.96
3	72.76	3.74	H2, H4	C-23	72.77	3.73
4	49.19	1.26		C-24	49.17	1.26
5	37.09	-			37.10	-
6	41.74	1.73, 1.00			41.72	1.73, 1.00
7	15.80	1.42			15.79	1.42
8	53.21	1.29		C-6b, C-25	53.20	1.29
9	37.85	-			37.83	-
10	61.37	0.92		C-24, C-25	61.34	0.92
11	35.34	1.91			35.34	1.91
12	30.64	1.30			30.64	1.30
13	38.38	-			38.37	-
14	39.68	-			39.67	-
15	32.34	1.15			32.33	1.14
16	36.09	1.20			36.08	1.20
17	30.03	-			30.03	-
18	42.74	1.57		C-16, C-28	42.81	1.57
19	35.20	1.88			35.18	1.88
20	28.17	-			28.18	-
21	32.83	1.52			32.81	1.52
22	39.29	1.47		C-28	39.28	1.47
23	11.60	0.95			11.63	0.94
24	16.39	0.99			16.40	0.98
25	18.24	0.88			18.25	0.88
26	20.11	0.99		C-8	20.13	0.99
27	18.63	1.02		C-18	18.66	1.00
28	32.08	1.19			32.09	1.19
29	31.79	1.01			31.80	1.00
30	35.01	0.96			35.00	0.96

4.6.2 Compound II (Friedelin)

Compound (**II**) (0.8 mg) was isolated from dichloromethane extracts of ME obtained from the leaves of *A. malaccensis*. The compound gave R_f value of 0.33 with the use of a relatively non-polar solvent system, indicating its low polarity nature. The MS spectrum displayed a molecular ion peak at m/z 426, with the molecular formula of $C_{30}H_{50}O$. Its IR spectrum revealed absorption bands at 1635 cm^{-1} thus indicating the presence of carbonyl group, probably that of a ketone; absorptions at 1387 cm^{-1} indicated the presence of methyl groups.

The integration of ^1H NMR spectrum corresponded with eight methyl groups was observed at δ 0.90 (H-23), 0.71 (H-24), 0.86 (H-25), 0.97 (H-26), 1.07 (H-27), 1.28 (H-28), 0.94 (H-29) and 1.01 (H-30). Based on spectral received and previous reported (Monkodkaew et al., 2009) as shown in Table 4.10, led to proposing of structural compound (II) as friedelin (Figure 4.10).

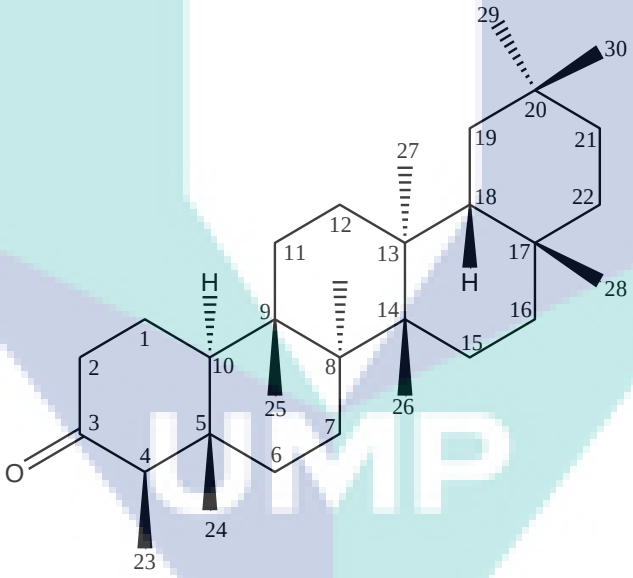


Figure 4.10: Structure of friedelin

Table 4.10 ^{1}H-NMR data of friedelin in $CDCl_3$

Carbon number	Friedelin isolated compound	Reference (Monkodkaew *et al.* (2009)
	δ_H	δ_H
1	-	1.99
2	2.33	2.42
3	-	-
4	2.25	2.25
5	-	-
6	1.79	1.77
7	-	1.52
8	1.39	1.41
9	-	-
10	-	1.55
11	-	1.25
12	-	1.35
13	-	-
14	-	-
15	-	1.18
16	-	0.98
17	-	-
18	1.59	1.58
19	-	0.98
20	-	-
21	-	1.20
22	-	0.95
23	0.90	0.89
24	0.71	0.73
25	0.86	0.85
26	0.97	1.01
27	1.07	1.05
28	1.28	1.18
29	0.98	0.95
30	1.01	1.01

The molecular ion peak of this compound was observed at m/z 426 (**1**). The loss of - CH_3 was indicated by the presence of a peak at m/z 411 (**2**). In the mass spectrum, peak at m/z 341 (**3**) was formed by the loss of ring A and the peak at m/z 273 (**4**) by the loss of ring A and B. Other fragmentized ions were represented as peaks at m/z 205 (**5**) and 123 (**6**) due to the loss of ring C and D, respectively. Possible structures of the selected ion fragments in the mass spectrum and fragmentation of friedelin is given below (Figure 4.11 and Figure 4.12).

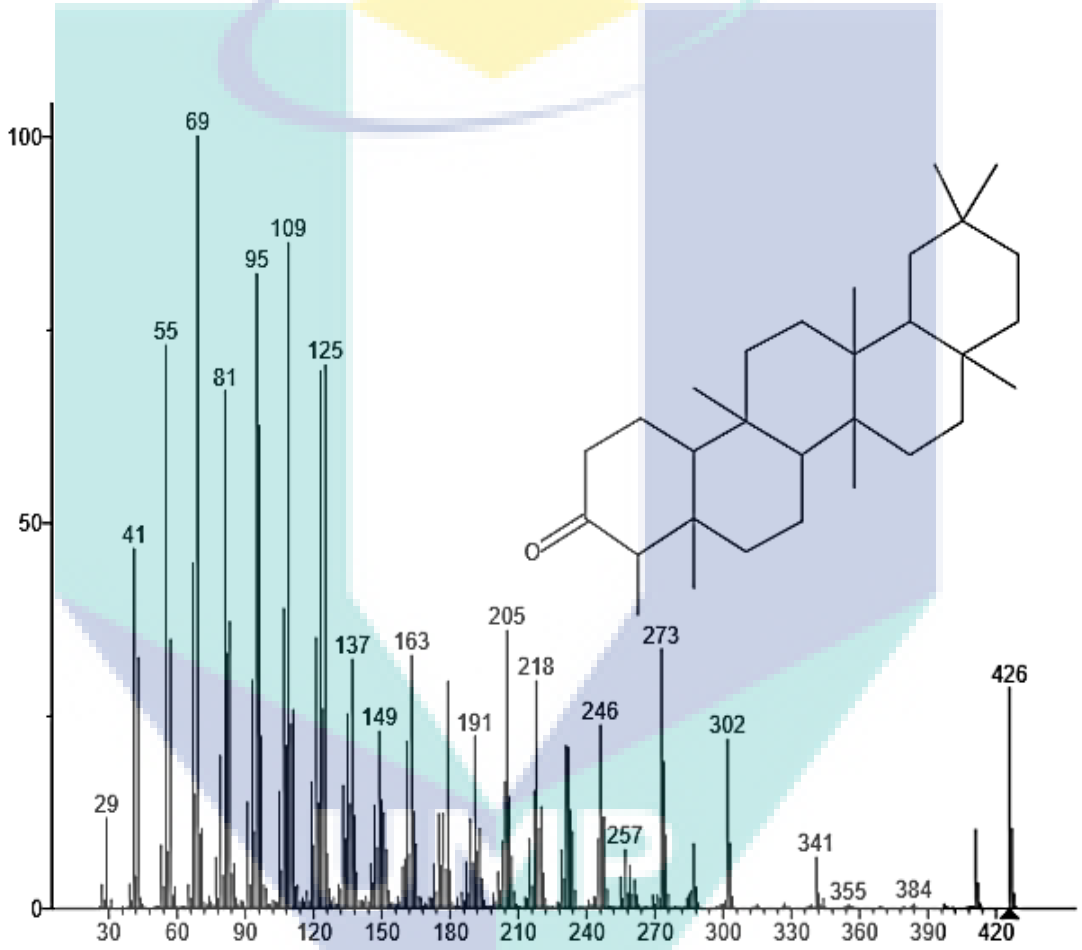


Figure 4.11 EIMS spectrum data of friedelin

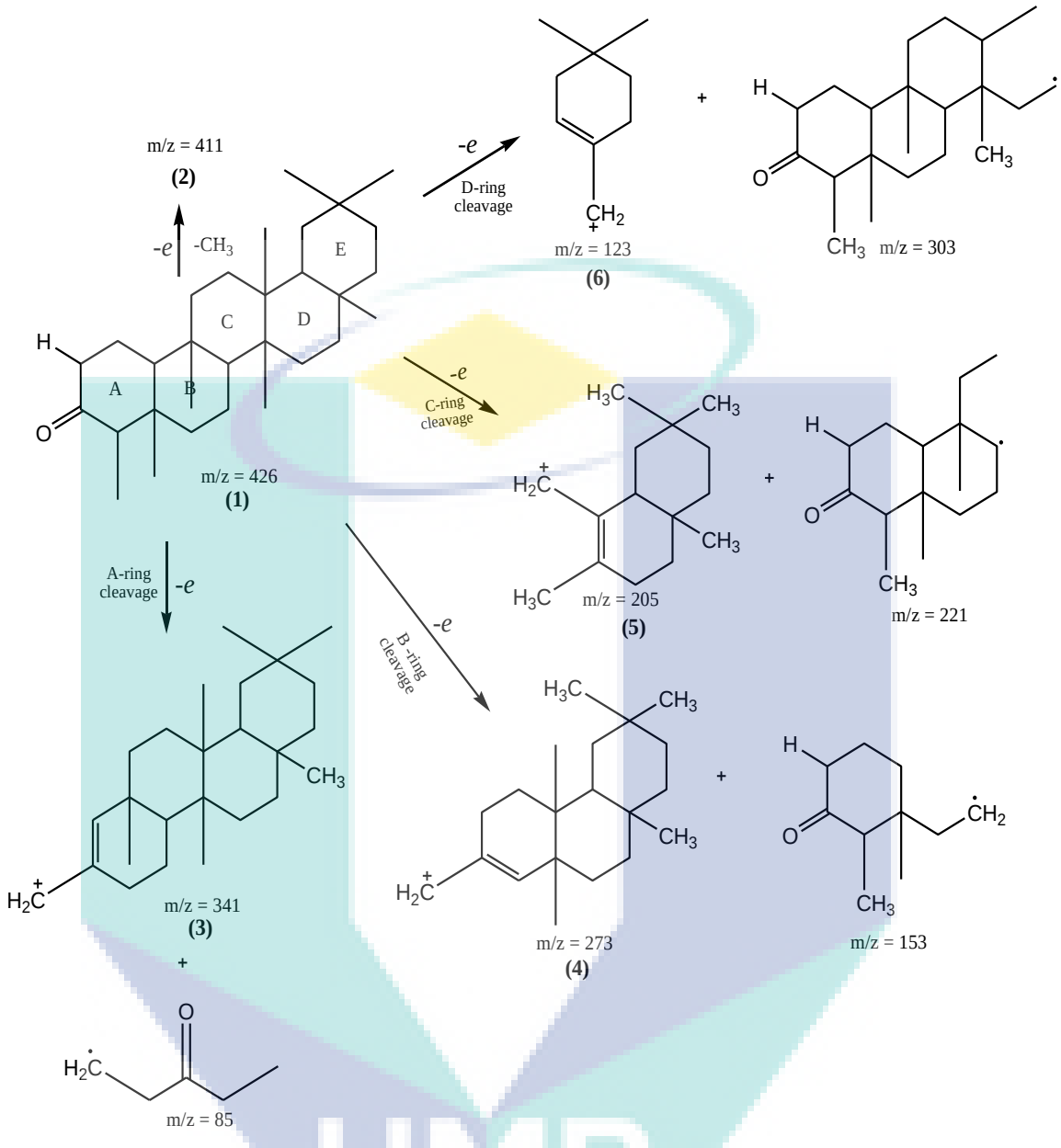


Figure 4.12 Mass fragmentation patterns of friedelin

4.7 Bioactivities of Isolated Compound

The isolated compounds were evaluated for their antioxidant activities via DPPH radical scavenging assays. The bioactivity was only focusing on DPPH assays due to the limited sample volumes to examine other antioxidant activities. The results of DPPH assay were summarised in Table 4.11.

Table 4.11 Results of DPPH assay for isolated compounds

Sample Tested	**Antioxidant Activity, IC_{50} (µg/mL)**
Friedelanol (**I**)	>1000
Friedelin (**II**)	>1000

The DPPH scavenging activity of the isolated compounds **I** and **II** from the leaves showed IC_{50} more than 1000 µg/mL, which the highest concentration tested in this study was at 1000 µg/ml. From the result, compound **I** and **II** were observed as inactive antioxidant agent as negative results obtained. As this assay is very sensitive to other solvents and little environmental variations, there are some precaution steps that should be taken into consideration while doing this assay. High concentrations of DPPH in the reaction mixture will give the absorbance beyond the accuracy of spectrophotometric measurement.

CHAPTER 5

CONCLUSION

5.1 Conclusion

In this study, volatile aroma compounds in WE and ME obtained from the leaves of *A. malaccensis* were analyzed by using SPME and DTD coupled with chromatographic techniques using GC-MS and GC-FID. Several commonly found compounds were tentatively identified as oxo-agarospirol, eudesmol and *n*-hexadecanoic acid. Among these compounds, *n*-hexadecanoic acid was the principal volatile constituents in both extracts.

The relationship between the compositions of the extracts and biological activities was determined by using UPLC-QToF/MS. The major flavonoid compounds found in *A. malaccenis* were 7-Hydroxy-5,3',4'-trimethoxy flavone, 2,4,4′,6′-tetrahydroxy-benzophenone, genkwanin, gallocatechin(4α→8)-epicatechin, loganic acid-6'-O-β-D-glucoside, (-)-epigallocatechin, epicatechin gallate (epicatechin-3-O-gallate), quercetagetin-6,7,3',4'-tetramethyl ether, rhamnetin and stigmasta-4,22-dien-3-one. Bioactivity studies of the total phenolic content and antioxidant properties were carried out on the different leaves extracts of *A. malaccesis*. Antioxidant properties of the extracts were measured based on the radical scavenging activities against DPPH and reducing power in CUPRAC assays. From the results, the isolated compounds showed no activity, whereas the WE and ME exhibited significant values comparable to amount of synthetic antioxidants.

The present investigation on the different leaves extracts of *A. malaccensis* also resulted in isolation of friedelanol and friedelin. Both friedelanol and friedelin belong to the class of terpenoids. friedelanol (**I**) (3.1 mg) was present in Hx extract while friedelin (**II**) (0.8 mg) was found in dichloromethane extract.

5.2 Recommendations

In order to develop chemical profile of agarwood leaves, several difficulties were encountered in this study. This includes inability to match the Kovats index of a particular compound present in the leaves of *A. malaccensis* with the mass spectrum generated from GC-MS analysis. Therefore, the use of advanced chromatographic technique such as MS/MS-QTOF is recommended to improve the separation efficiency and isolation of minor compounds, as well as to speed up the purification process.

Current studies of *A. malaccensis* mostly focus on the leaves and their rich bioactive secondary metabolites, but these studies are still unclear and insufficient. Pharmacological studies using different chemical constituents from the bark, roots and other parts of *A. malaccensis* is required. It is recommended to be directed towards the bioassay-guided isolation of bioactive compounds with proper chemical characterization and investigations of the underlying mechanism towards drug discovery. A systematic phytochemical investigation of *A. malaccensis* and its pharmacological properties, to illustrate its ethnomedicinal use, and support further healthcare product development will undoubtedly be the focus of further research.

REFERENCES

Abkenar, D., ShivaYamini, Y., Shemirani, F., & Assadi, Y. (2006). Solid phase microextraction with gas chromatography-mass spectrometry: A very rapid method for identification of volatile organic compounds emitted by *Carum copticum*. *Natural Product Research*, *20*(9), 850–859.

Adam, A. Z., Tajuddin, S. N., Sudmoon, R., Chaveerach, A., Abdullah, U. H., Mahat, M. N., & Mohamed, R. (2018). Chemical constituents and toxicity effects of leaves from several agarwood tree species (*Aquilaria*). *Journal of Tropical Forest Science*, *30*(3), 342–353.

Akter, S., Islam, M. T., Zulkefeli, M., & Khan, S. I. (2013). Agarwood Production - A Multidisciplinary Field to be Explored in Bangladesh. *International Journal of Pharmaceutical and Life Sciences*, *2*(1), 22–32.

Alam, J., Mujahid, M., Badruddeen, Jahan, Y., Bagga, P., & Rahman, M. A. (2017). Hepatoprotective potential of ethanolic extract of *Aquilaria agallocha* leaves against paracetamol induced hepatotoxicity in SD rats. *Journal of Traditional and Complementary Medicine*, *7*(1), 9–13.

Ali, N. A. M., Jin, C. B., & Jamil, M. (2015). Agarwood *(Aquilaria malaccensis)* oils. In Victor R. Preedy (Eds).., *Essential Oils in Food Preservation, Flavor and Safety*. 173-180.

Antonopoulou, M., Compton, J., Perry, L. S., & Al-Mubarak, R. (2010). The Trade and Use of Agarwood (Oudh) in the United Arab Emirates. *TRAFFIC Southeast Asia*.

Apak, R., Guclu, K., Ozyurek, M., & Karademir, S. E. (2004). Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E, Using Their Cupric Ion Reducing Capability in the Presence of Neocuproine: CUPRAC Method. *Journal of Agricultural and Food Chemistry*, *52*(26), 7970–7981.

Aparna, V., Dileep, K. V., Mandal, P. K., Karthe, P., Sadasivan, C., & Haridas, M. (2012). Anti-Inflammatory Property of n-Hexadecanoic Acid: Structural Evidence and Kinetic Assessment. *Chemical Biology & Drug Design*, *80*(3), 434–439.

Aromdee, C., Pannangpetch, P., & Pranakhon, R. (2015). Effects of iriflophenone 3-C-β-glucoside on fasting blood glucose level and glucose uptake. *Pharmacognosy Magazine*, *11*(41), 82-89.

Azziz, S. S. S. A., Arriffin, N. M., Alimon, H., Sukari, M. A., & Naz, H. (2013). Chemical study of *Aquilaria crassna*. *Chemistry of Natural Compounds*, *49*(3), 575–576.

Bahrani, H., Mohamad, J., Paydar, M., & Rothan, H. (2014). Isolation and characterisation of acetylcholinesterase inhibitors from *Aquilaria subintegra* for the treatment of Alzheimer's disease (AD). *Curr Alzheimer Res*, *11*(2), 206–214.

Barden, A., Anak, N., Mulliken, T., & Song, M. (2000). Heart of the Matter: Agarwood use and trade and CITES implementation for *Aquilaria malaccensis*. Cambridge: TRAFFIC International, 1–52.

Biesaga, M. (2011). Influence of extraction methods on stability of flavonoids. *Journal of Chromatography A*, *1218*(18), 2505–2512.

Blanchette, R. A., Heuveling, V. B (2009). Cultivated agarwood. *U.S. Patent No. 7,638,145. Washington, DC. U.S. Patent and Trademark Office.*

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, *28*(1), 25–30.

Chakrabarty, K., Kumar, A., & Menon, V. (1994). Trade in Agarwood: A Study by TRAFFIC India.

Chanda, S., & Dave, R. (2009). In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties : An overview. *African Journal of Microbiology Research*, *3*(13), 981–996.

Chua, L. S. L. (2018). Agarwood (*Aquilaria malaccensis*) in malaysia. *A Paper Presented during NDF Workshop Case Studies, Mexico*.

CITES. (2003). Annex 2. *Review of Significant Trade Aquilaria Malaccensis*, (November), 45–76. Retrieved from https://www.cites.org/sites/default/files/eng/com/pc/14/E-PC14-09-02-02-A2.pdf

Constant, M., & Collier, J. (1997). Headspace gas chromatography profiles of fruit-flavored malt beverages using solid-phase microextraction. *Journal of the American Society of Brewing Chemists*, *55*(3), 112–118.

Cui, J., Guo, S., & Xiao, P. (2011). Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. *Journal of Zhejiang University Science B*, *12*(5), 385–392.

Dahham, S. S., Ahamed, M., B, K., Saghir, S., M., Alsuede, F., S., Iqbal, A., M., & Majid, A., M., S., A. (1997). Bioactive essential oils from *Aquilaria crassna* for cancer prevention and treatment. *Rev. Ecuat. Cancerol*, *4*(1), 14–19.

Dahham, S. S., Majid, A., Saghir, S. M., & Al suede, F. S. (2014). Antibacterial , Antifungal and Antioxidant Activities of *Aquilaria crassna*. *International Journal of Advances in Science and Technology*, 196–200.

Dahham, S. S., Tabana, Y. M., Iqbal, M. A., Ahamed, M. B. K., Ezzat, M. O., Majid, A. S. A., & Majid, A. M. S. A. (2015). The anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. *Molecules*, *20*(7), 11808–11829.

Dash, M., Patra, J. K., & Panda, P. P. (2008). Phytochemical and antimicrobial screening of extracts of *Aquilaria agallocha* Roxb ., *7*(20), 3531–3534.

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis*, *22*(3), 296–302.

Donovan, D. G., & Puri, R. K. (2004). Learning from Traditional Knowledge of Non-timber Forest Products: Penan Benalui and the Autecology of *Aquilaria* in Indonesian Borneo. *Indonesian Borneo. Ecology and Society*, *9*(3), 1–5.

Duan, Z., Li, W., Dou, Z., Xie, H., He, A., & Shi, M. (2015). Extraction and antioxidant activity of flavonoids from *Aquilaria sinensis* (Lour.) Gilg leaves. *Food Science*, *36*(6), 45–50.

Escobedo-Martínez, C., Concepciõn Lozada, M., Hernández-Ortega, S., Villarreal, M. L., Gnecco, D., Enríquez, R. G., & Reynolds, W. (2012). 1H and 13C NMR characterization of new cycloartane triterpenes from *Mangifera indica*. *Magnetic Resonance in Chemistry*, *50*(1), 52–57.

Falkovich, A. H., & Rudich, Y. (2001). Analysis of semivolatile organic compounds in atmospheric aerosols by direct sample introduction thermal desorption GC/MS. *Environmental Science and Technology*, *35*(11), 2326–2333.

Feng, J., & Yang, X. (2011). Liposolubility constituents from leaves of *Aquilaria sinensis*]. *Journal of Chinese Materia Medica*, *36*(15), 2092–2095.

Feng, J., & Yang, X. (2012). Constituents from the leaves of *Aquilaria sinensis*. *Journal of Chinese Materia Medica*, *37*(2), 230–234.

Gao, Y., Liu, J., Lu, H., & Wei, Z. (2012). Two New 2- (2-Phenylethyl) chromen-4-ones from *Aquilaria sinensis* (Lour.) Gilg as sedative, analgesic, and digestive medicine in traditional Chinese medicine, *95*, 951–954.

Ghasemzadeh, A., & Ghasemzadeh, N. (2011). Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *Journal of medicinal plants research*, *5*(31), 6697-6703.

Gunasekera, S. P., Kinghorn, A. D., Cordell, G. A., & Farnsworth, N. R. (1981). Plant anticancer agents. xix. constituents of *Aquilaria malaccensis*. *Journal of Natural Products*, *44*(5), 569–572.

Hara, H., Ise, Y., Morimoto, N., Shimazawa, M., Ichihashi, K., Ohyama, M., & Iinuma, M. (2008). Laxative Effect of Agarwood Leaves and Its Mechanism. *Bioscience, Biotechnology, and Biochemistry*, *72*(2), 335–345.

Hashim, Y. Z. H. Y., Kerr, G. P., Abbas, P., & Salleh, M. H. (2016). *Aquilaria* spp. (agarwood) as source of health beneficial compounds: A review of traditional use, phytochemistry and pharmacology. *Journal of Ethnopharmacology*, *189*, 331–360.

Hashim, Y.Z.H.Y., Abbas, P., & Awale, R. (2012). Antimicrobial Activities of Agarwood *A. subintegra* Leaves Extracts International Institute for Halal Research and Training (INHART), Ground Floor , Block, (July), 394–397.

Hendra, H., Moeljopawiro, S., & Nuringtyas, T. R. (2016). Antioxidant and antibacterial activities of agarwood (*Aquilaria malaccensis* Lamk.) leaves. *AIP Conference Proceedings*, *1755*.

Huda, A., Munira, M., Fitrya, S., & Salmah, M. (2009). Antioxidant activity of *Aquilaria malaccensis* (thymelaeaceae) leaves. *Pharmacognosy Research*, *1*(5), 270–273.

Ishihara, M., Tsuneya, T., & Uneyama, K. (1991). Guaiane sesquiterpenes from agarwood. *Phytochemistry*, *30*(10), 3343–3347.

Ishihara, M., Tsuneya, T., & Uneyama, K. (1993). Components of the volatile concentrate of agarwood. *Journal of Essential Oil Research*, *5*(3), 283–289.

Ito, T., Kakino, M., Tazawa, S., Oyama, M., Maruyama, H., Araki, Y., … Iinuma, M. (2012). Identification of Phenolic Compounds in *Aquilaria crassna* Leaves Via Liquid Chromatography-Electrospray Ionization Mass Spectroscopy. *Food Science and Technology Research*, *18*(2), 259–262.

Ito, T., Kakino, M., Tazawa, S., Watarai, T., Oyama, M., Maruyama, H., … Iinuma, M. (2012). Quantification of polyphenols and pharmacological analysis of water and ethanol-based extracts of cultivated agarwood leaves. *J Nutr Sci Vitaminol (Tokyo)*, *58*(2), 136–142.

IUCN. (2019). *The IUCN Red List of Threatened Species 2018*. International Union for Conservation of Nature - IUCN. Retrieved from https://www.iucnredlist.org/species/32056/2810130. Accessed 7 Apr, 2019.

Jiang, S., Yong Jiang, Guan, Y.-F., Tu, P.-F., Wang, K.-Y., & Chen, J.-M. (2011). Effects of 95% ethanol extract of *Aquilaria sinensis* leaves on hyperglycemia in diabetic db/db mice. *Journal of Chinese Pharmaceutical Sciences*, *20*(6), 609–614.

Kakino, M., Izuta, H., Ito, T., Tsuruma, K., Araki, Y., Shimazawa, M., … Hara, H. (2010). Agarwood Induced Laxative Effects via Acetylcholine Receptors on Loperamide-Induced Constipation in Mice. *Bioscience, Biotechnology, and Biochemistry*, *74*(8), 1550–1555.

Kakino, M., Tazawa, S., Maruyama, H., Tsuruma, K., Araki, Y., Shimazawa, M., & Hara, H. (2010). Laxative effects of agarwood on low-fiber diet-induced constipation in rats. *BMC Complementary and Alternative Medicine*, *10* (1), 68.

Kalia, K., Sharma, K., Singh, H. P., & Singh, B. (2008). Effects of Extraction Methods on Phenolic Contents and Antioxidant Activity in Aerial Parts of *Potentilla atrosanguinea Lodd*. and Quantification of Its Phenolic Constituents by RP-HPLC. *Journal of Agricultural and Food Chemistry*, *56*(21), 10129–10134.

Kamonwannasit, S., Nantapong, N., Kumkrai, P., Luecha, P., Kupittayanant, S., & Chudapongse, N. (2013). Antibacterial activity of *Aquilaria crassna* leaf extract against Staphylococcus epidermidis by disruption of cell wall. *Annals of Clinical Microbiology and Antimicrobials*, *12*(1), 1–7.

Kang, Y. F., Chien, S. L., Wu, H. M., Li, W. J., Chen, C. T., Li, H. T., … Chen, C. Y. (2014). Secondary metabolites from the leaves of *Aquilaria sinensis*. *Chemistry of Natural Compounds*, *50*(6), 1110–1112.

Khalil, A. S., Rahim, A. A. ., Taha, K. K. ., & Abdallah, K. B. (2013). Characterization of Methanolic Extracts of Agarwood Leaves. *Journal of Applied and Industrial Sciences*, *1*(3), 78–88.

Khan, N., & Mukhtar, H. (2008). Multitargeted therapy of cancer by green tea polyphenols. *Cancer Letters*, *269*(2), 269–280.

Khoddami, A., Wilkes, M., Roberts, T., Khoddami, A., Wilkes, M. A., & Roberts, T. H. (2013). Techniques for Analysis of Plant Phenolic Compounds. *Molecules*, *18*(2), 2328–2375.

Kim, S. J., Kim, J. A., Kim, D. H., Kwak, S. H., Yu, K. Y., Jang, S. Il, … Jeong, S. Il. (2015). Bio-assay guided isolation and identification of α-glucosidase inhibitors

from the leaves of *Diospyros lotus*. *Korean Journal of Pharmacognosy*, *46*(2), 105–108.

Konig, W. A., & Hochmuth, D. H. (2004). Enantioselective gas chromatography in flavor and fragrance analysis: Strategies for the identification of known and unknown plant volatiles. *Journal of Chromatographic Science*, *42*(8), 423–439.

Kovats, E. (1958). Gas chromatographic characterization of organic compounds. I. Retention indexes of aliphatic halides, alcohol, aldehyde and ketones. Helv. Chim. Acta 41: 1915–32.

Kulip, J., Fan, L. N., Nurhuda Manshoor, Julius, A., Idris Said, Gisil, J., … Tukip, W. F. (2010). Medicinal plants in Maliau Basin, Sabah, Malaysia. *Journal Of Tropical Biology And Conservation*, *6*(January), 21–33.

Kumar, S., & Pandey, A. K. (2013). Chemistry and Biological Activities of Flavonoids: An Overview. *The Scientif World J*, *4*(2), 32–48.

Lafrankie, J. V. (1994). Population Dynamics of Some Tropical Trees That Yield Non-Timber Forest Products. *Economic Botany*, *48*(3), 301–309.

Lee, N. Y., Yunus, M. A. C., Idham, Z., Ruslan, M. S. H., Aziz, A. H. A., & Irwansyah, N. (2016). Extraction and identification of bioactive compounds from agarwood leaves. *IOP Conference Series: Materials Science and Engineering*, *162*(1).

Lee, S. Y., & Mohamed, R. (2016). The Origin and Domestication of *Aquilaria*, an Important Agarwood-Producing Genus (pp. 1–20). Springer, Singapore.

Lee, Y.-L., Huang, G.-W., Liang, Z.-C., & Mau, J.-L. (2007). Antioxidant properties of three extracts from *Pleurotus citrinopileatus*. *LWT - Food Science and Technology*, *40*(5), 823–833.

List, T. P. (2013). Thymelaeaceae. Retrieved January 12, 2019, from http://www.theplantlist.org/1.1/browse/A/Thymelaeaceae/. Accessed 7 Apr, 2019.

Liu, Y., Chen, H., Yang, Y., Zhang, Z., Wei, J., Meng, H., … Chen, H. (2013). Whole-tree agarwood-inducing technique: An efficient novel technique for producing high-quality agarwood in cultivated *Aquilaria sinensis* trees. *Molecules*, *18*(3), 3086–3106.

Liu, Y., Yang, X., & Liu, T. (2007). Yufeng, L., Xiuwei, Y., & Tonghua, L. (2007). GC-MS Analysis of Essential Oil from the Leaves of *Aquilaria sinensis* [J]. *Modern Chinese Medicine*, *8*, 004.

López-Sampson, A., & Page, T. (2018). History of Use and Trade of Agarwood. *Economic Botany*, *72*(1), 107–129.

Maisuthisakul, P., Suttajit, M., & Pongsawatmanit, R. (2007). Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chemistry*, *100*(4), 1409–1418.

Manuhara, G. J., Mentari, G. P., Khasanah, L. U., & Utami, R. (2018). Aqueous Extract Composition of Spent Ginger (*Zingiber officinale var. Amarum*) from Essential Oil Distillation. In IOP Conference Series: *Materials Science and Engineering*, *333* (1), IOP Publishing.

Miniyar, P. B., Chitre, T. S., Karve, S. S., Deuskar, H. J., & Jain, K. S. (2008). Anti - oxidant activity of ethyl acetate extract of *Aquilaria agallocha* on nitrite - induced methemoglobin formation. *Int J Green Pharm*, *2*(2), 116–117.

Mohamed, R., Jong, P. L., & Kamziah, A. K. (2014). Fungal inoculation induces agarwood in young *Aquilaria malaccensis* trees in the nursery. *Journal of Forestry Research*, *25*(1), 201–204.

Monkodkaew, S., Loetchutinat, C., Nuntasaen, N., & Pompimon, W. (2009). Identification and antiproliferative activity evaluation of a series of triterpenoids isolated from *Flueggea virosa* (Roxb. ex Willd.). *American Journal of Applied Sciences*, 6(10), 1800-1806.

Molyneux, P. (2004). The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology*, *26*(2), 211–219.

Moosa, S. (2010). Phytochemical and anti oxidant screening of *Aquilaria malaccensis* leaves. *In Proceedings of the Nuclear Malaysia R&D Seminar Proceedings of the Nuclear Malaysia R&D Seminar 12- Oct 2010. Bangi (Malaysia).*

Naef, R. (2011). The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: A review. *Flavour and Fragrance Journal*, *26*(2), 73–87.

Ng, L. T., Chang, Y. S., & Kadir, A. A. (1997). A review on agar (gaharu) producing Aquilaria species. *Journal of Tropical Forest Products*, *2*(2), 272–285.

Nie, C., Song, Y., Chen, D., Xue, P., Tu, P., Wang, K., & Chen, J. (2009). Studies on chemical constituents of leaves of *Aquilaria sinensis*. *China Journal of Chinese Materia Medica*, *34*(7), 858–860.

Oktay, M. uni., Gulein, I., & Kufrevioglu, I. O. (2003). Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *LWT - Food Science and Technology*, *36*(2), 263–271.

Okudera, Y., & Ito, M. (2009). Production of agarwood fragrant constituents in *Aquilaria* calli and cell suspension cultures. *Plant Biotechnology*, *26*(3), 307–315.

Oyen, L. P. A. . N. X. D. (1999). *Plant Resources of South-East Asia No. 19 : Essential-oil plants*. the Netherlands: Leiden : Backhuys Publishers.

Pandey, K. B., & Rizvi, S. I. (2009). Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–278.

Pawliszyn, J. (2007). *Applications of solid phase microextraction*. Royal Society of Chemistry. Cambridge, UK. The Royal Society of Chemistry.

PerezCoello, M. S., Sanz, J., & Cabezudo, M. D. (1997). Analysis of volatile components of oak wood by solvent extraction and direct thermal desorption gas chromatography mass spectrometry. *Journal of Chromatography A*, *778*(1–2), 427–434.

Persoon, G. A., & van Beek, H. H. (2008). Growing 'The Wood of The Gods': Agarwood Production in Southeast Asia (pp. 245–262). Springer, Dordrecht.

Pranakhon, R., Pannangpetch, P., & Aromdee, C. (2011). Antihyperglycemic activity of agarwood leaf extracts in STZ-induced diabetic rats and glucose uptake enhancement activity in rat adipocytes. *Songklanakarin Journal of Science and Technology*, *33*(4), 405–410.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E. *Analytical Biochemistry*, *269*(2), 337–341.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standarized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry*, *53*(10), 4290–4302.

Prosen, H., & Zupancic-Kralj, L. (1999). Solid-phase microextraction. *TrAC Trends in Analytical Chemistry*, *18*(4), 272–282.

Qi, J., Lu, J., Liu, J., & Yu, B.-Y. (2009). Flavonoid and a rare benzophenone glycoside from the leaves of *Aquilaria sinensis*. *Chemical & Pharmaceutical Bulletin*, *57*(2), 134–137.

Rahman, H., Vakati, K., & Eswaraiah, M. C. (2012). In-vivo and in-vitro anti-inflammatory activity of *Aquilaria agallocha* oil. *International Journal of Basic Medical Sciences and Pharmacy*, *2*(1), 7–10.

Rahuman, A. A., Gopalakrishnan, G., Ghouse, B. S., Arumugam, S., & Himalayan, B. (2000). Effect of Feronia limonia on mosquito larvae. *Fitoterapia*, *71*(5), 553–555.

Ray, G., Leelamanit, W., Sithisarn, P., & Jiratchariyakul, W. (2014). Antioxidative Compounds from *Aquilaria crassna* Leaf. *Mahidol University Journal of Pharmaceutical Sciences*, *41*(4), 54–58.

Reiter, R. J. (1995). Oxidative processes and antioxidative defense mechanisms in the aging brain. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *9*(7), 526–533.

Richter, J., & Schellenberg, I. (2007). Comparison of different extraction methods for the determination of essential oils and related compounds from aromatic plants and optimization of solid-phase microextraction/gas chromatography. *Analytical and Bioanalytical Chemistry*, *387*(6), 2207–2217.

Roby, M. H. H., Sarhan, M. A., Selim, K. A.-H., & Khalel, K. I. (2013). Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. *Industrial Crops and Products*, *43*, 827–831.

Rodrigues, F., Caldeira, M., & Câmara, J. S. (2008). Development of a dynamic headspace solid-phase microextraction procedure coupled to GC–qMSD for evaluation the chemical profile in alcoholic beverages. *Analytica Chimica Acta*, *609*(1), 82–104.

Samadi, M., Abidin, Z. Z., Yunus, R., Awang Biak, D. R., Yoshida, H., & Lok, E. H. (2017). Assessing the kinetic model of hydro-distillation and chemical composition of *Aquilaria malaccensis* leaves essential oil. *Chinese Journal of Chemical*

Engineering, *25*(2), 216–222.

Sattayasai, J., Bantadkit, J., Aromdee, C., Lattmann, E., & Airara, W. (2012). Antipyretic, analgesic and anti-oxidative activities of *Aquilaria crassna* leaves extract in rodents. *Journal of Ayurveda and Integrative Medicine*, *3*(4), 175-179.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry*, *113*(4), 1202–1205.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, *40*(6), 945–948.

Shirey, R. E. (2000). Optimization of extraction conditions and fiber selection for semivolatile analytes using solid-phase microextraction. *Journal of chromatographic science*, *38*(7), 279-288.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1998). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, *299*(1974), 152–178.

Singl Sushil Chandra Sati, Nitin Sati, U. R. and O. P. S. (2010). Medicinal Plants as a Source of Antioxidants. *Research Journal of Phytochemistry*, *4*(4), 213–224.

Tajuddin, S. N., & Yusoff, M. M. (2010). Chemical composition of volatile oils of *Aquilaria malaccensis* (Thymelaeaceae) from Malaysia. *Natural Product Communications*, *5*(12), 1965–1968.

Taskin, H., Baktemur, G., Kafkas, E., & Buyukalaca, S. (2013). Determinaton of Volatile Aroma Components of Gyromitra Mushroom Using Headspace Gas Dietary intake, Chromatography Mass Spectrometry (HS-GC/MS) Target hazard quotients, Ready-to-eat-foods,. *Turkish Journal of Agriculture - Food Science and Technology*, *1*(1), 8–11.

Tay, P. Y., Tan, C. P., Abas, F., Yim, H. S., & Ho, C. W. (2014). Assessment of extraction parameters on antioxidant capacity, polyphenol content, epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and iriflophenone 3-C-β-glucoside of agarwood (*Aquilaria crassna*) young leaves. *Molecules*, *19*(8), 12304–12319.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chemistry*, *99*(4), 835–841.

Ueda, J. ya, Imamura, L., Tezuka, Y., Tran, Q. L., Tsuda, M., & Kadota, S. (2006). New sesquiterpene from Vietnamese agarwood and its induction effect on brain-derived neurotrophic factor mRNA expression in vitro. *Bioorganic and Medicinal Chemistry*, *14*(10), 3571–3574.

Vernon H. Heywood, Richard K. Brummitt, A. C. and O. S. (2007). Flowering Plant Families of the World. Royal Botanic Gardens, Kew.

Wardencki, W., Michulec, M., & Curyło, J. (2004). A review of theoretical and practical aspects of solid-phase microextraction in food analysis. *International Journal of Food Science and Technology*, *39*(7), 703–717.

Wetwitayaklung, P., Thavanapong, N., & Charoenteeraboon, J. (2009). Chemical Constituents and Antimicrobial Activity of Essential Oil and Extracts of Heartwood of *Aquilaria crassna* Obtained from Water Distillation and Supercritical Fluid Carbon Dioxide Extraction. *Silpakorn U Science & Technology Journal*, *3*(1), 25–33.

Wil, N. N. A., Mohd Omar, N. A., Awang@Ibrahim, N., & Tajuddin, S. N. (2014). In vitro antioxidant activity and phytochemical screening of *Aquilaria malaccensis* leaf extracts. *Journal of Chemical and Pharmaceutical Research*, *6*(12), 688–693.

Yang, X. B., Feng, J., Yang, X. W., Zhao, B., & Liu, J. X. (2012). Aquisiflavoside, a new nitric oxide production inhibitor from the leaves of *Aquilaria sinensis*. *Journal of Asian Natural Products Research*, *14*(9), 867–872.

Yu, Q., Qi, J., Yu, H. X., Chen, L. L., Kou, J. P., Liu, S. J., & Yu, B. Y. (2013). Qualitative and quantitative analysis of phenolic compounds in the leaves of *Aquilaria sinensis* using liquid chromatography-mass spectrometry. *Phytochemical Analysis*, *24*(4), 349–356.

Zhang, L., Brockelman, W. Y., & Allen, M. A. (2008). Matrix analysis to evaluate sustainability: The tropical tree *Aquilaria crassna*, a heavily poached source of agarwood. *Biological Conservation*, *141*(6), 1676–1686.

Zhang, W., & Ni, B. (2011). GC-MS analysis of the chemical components of the volatile oil from leaves of *Aquilaria sinensis* (Lour.) Gilg. *Medicinal Plant*, *2*(8), 34-36.

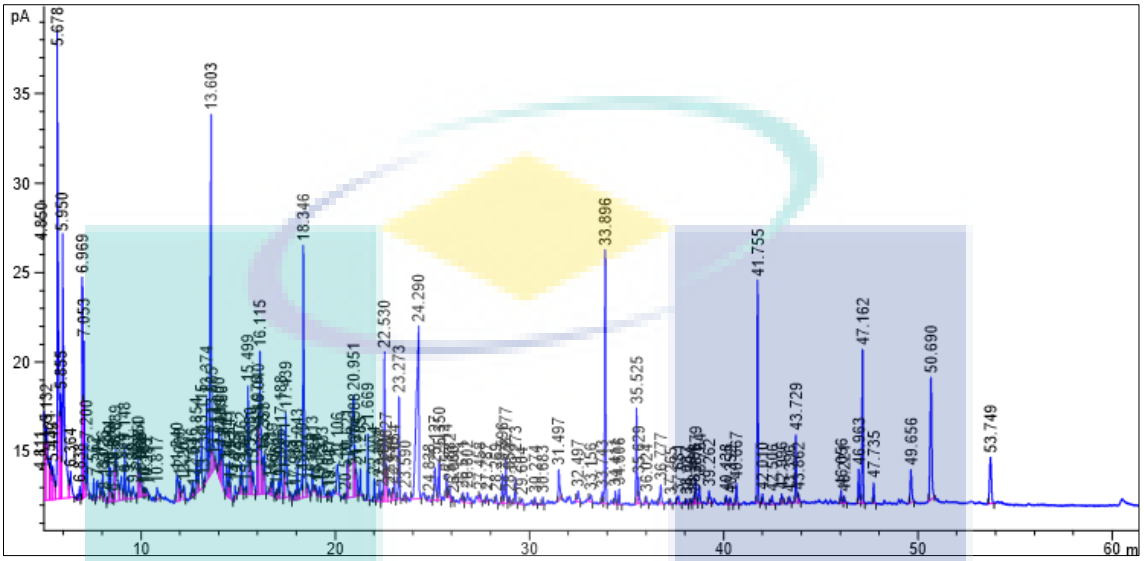
Zhou, M., Wang, H., Suolangjiba, Kou, J., & Yu, B. (2008). Antinociceptive and anti-inflammatory activities of *Aquilaria sinensis* (Lour.) Gilg. Leaves extract. *Journal of Ethnopharmacology*, *117*(2), 345–350.

Zich, F., & Compton, J. (2002). The Final Frontier: Towards Sustainable Management of Papua New Guinea's Agarwood Resource. *A TRAFFIC Oceania Report in Conjunction with WWF South Pacific Programme*.

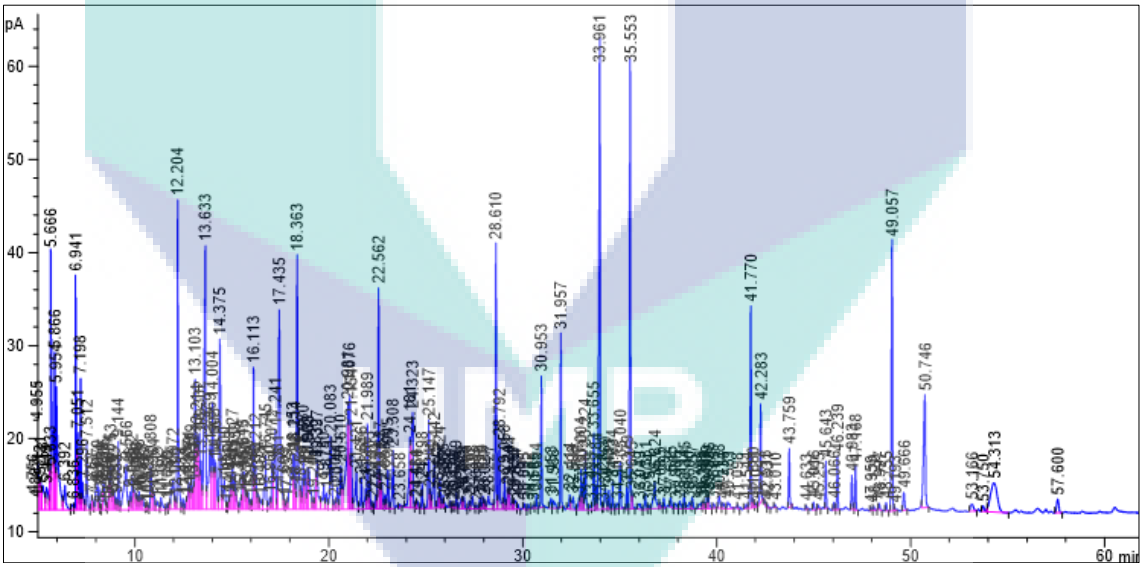
Zulkifle, N. L., Omar, N. A. M., Tajudin, S. N., & Shaari, M. R. (2013). Antidiabetic Activities of Malaysian Agarwood (*Aquilaria spp*.) Leaves Extract. *Conference on Industry-Academia Joint Initiatives in Biotechnology CIA*.

APPENDIX A

ANALYSIS GC-FID OF *A. MALACCENSIS* LEAVES EXTRACT BY SPME



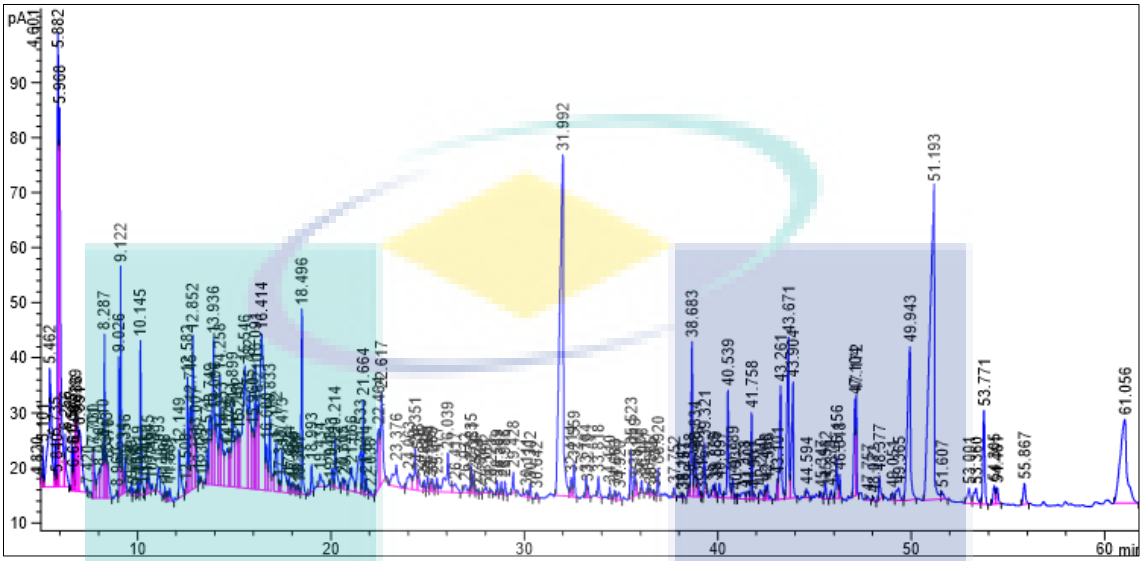
A.1 GC-FID Chromatograms of WE coupled with SPME analysis



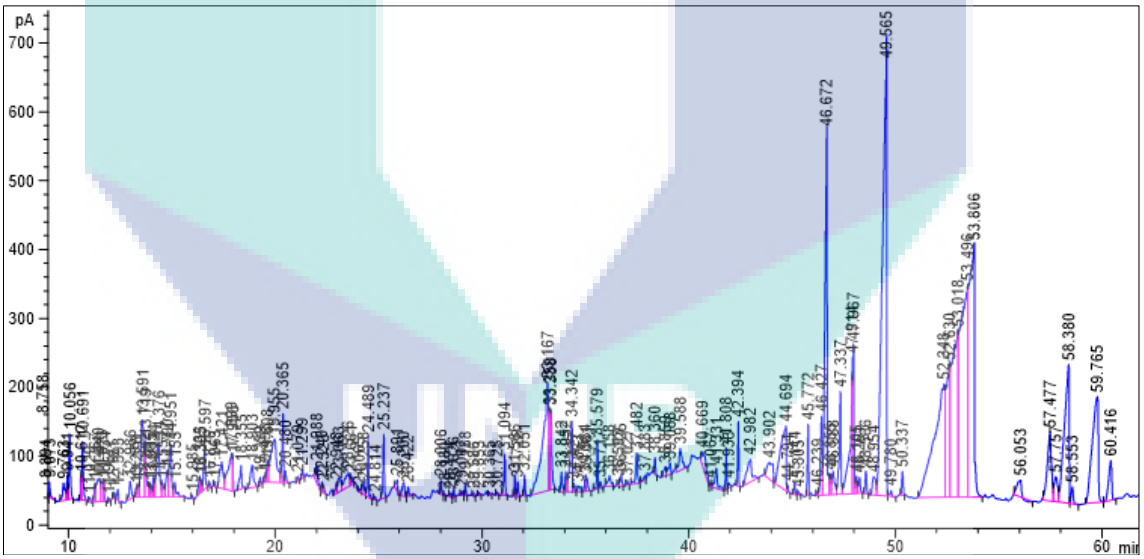
A.2 GC-FID Chromatograms of ME coupled with SPME analysis

APPENDIX B

ANALYSIS GC-FID OF *A. MALACCENSIS* LEAVES EXTRACT BY DTD



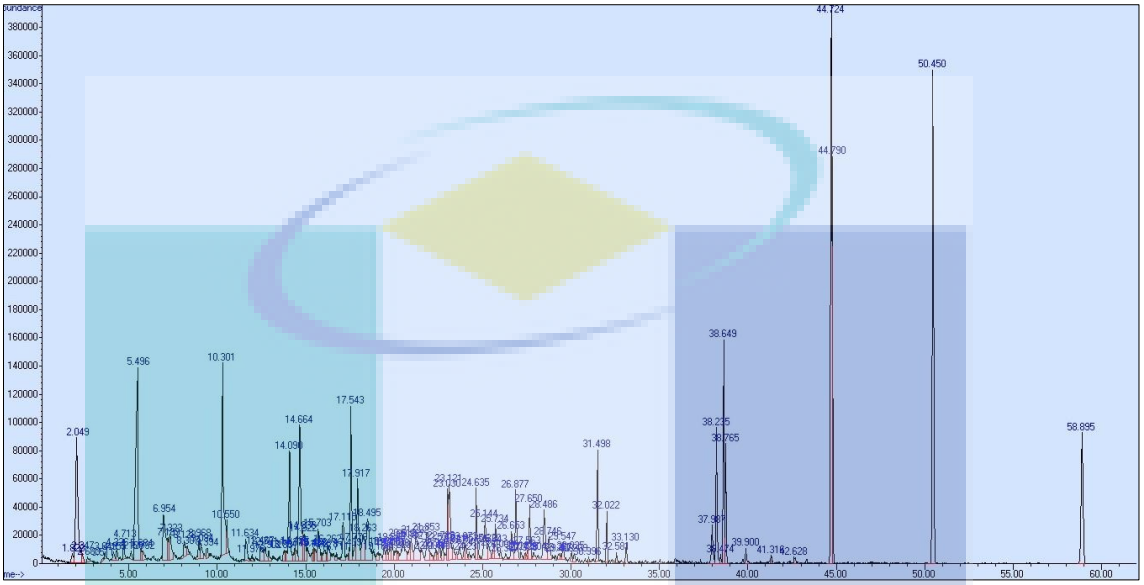
B.1 GC-FID Chromatograms of WE coupled with DTD analysis



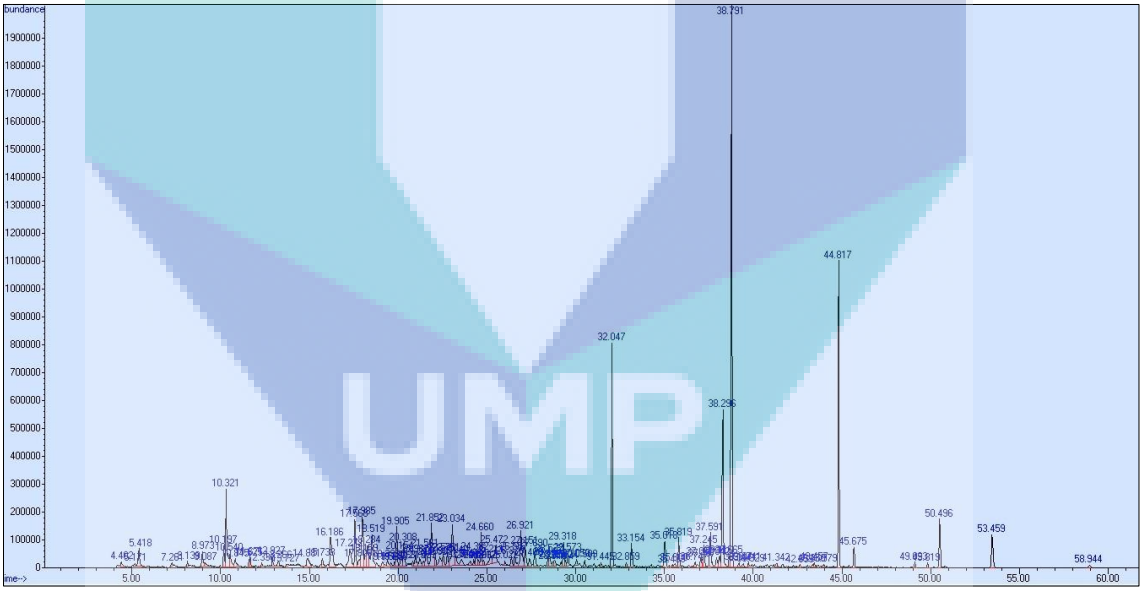
B.2 GC-FID Chromatograms of ME coupled with DTD analysis

APPENDIX C

ANALYSIS GC-MS OF *A. MALACCENSIS* LEAVES EXTRACT BY SPME



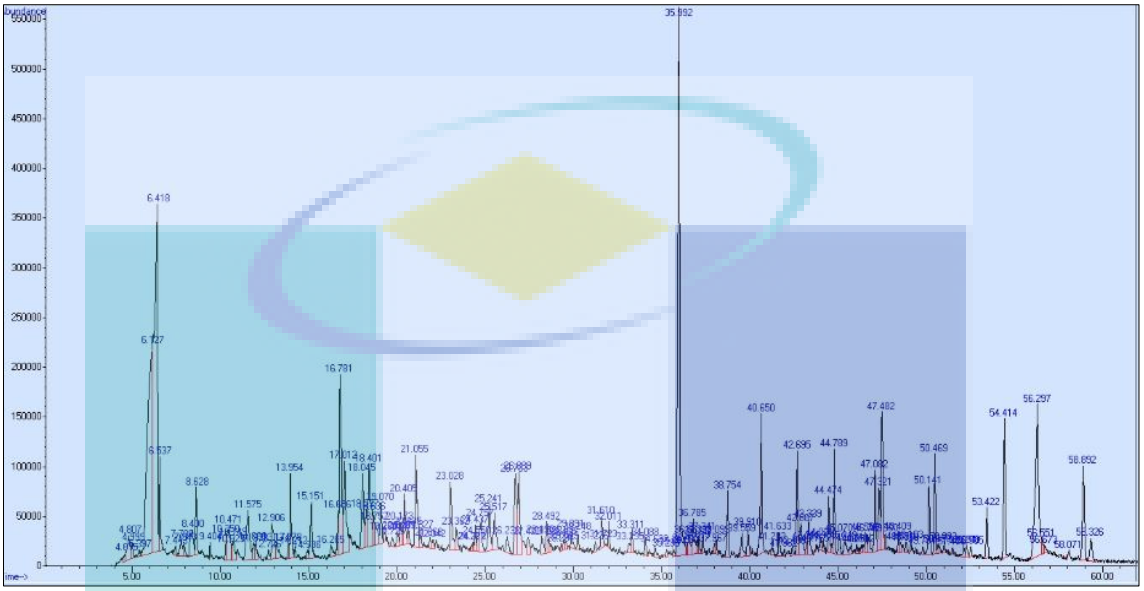
C.1 GC-MS Chromatograms of WE coupled with SPME analysis



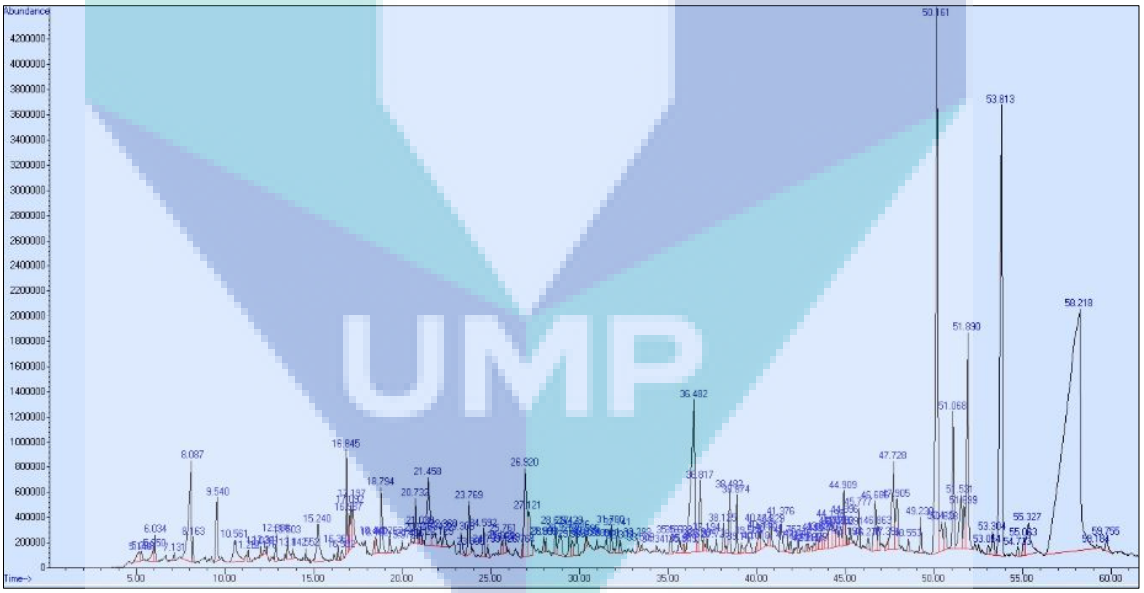
C.2 GC-MS Chromatograms of ME coupled with SPME analysis

APPENDIX D

ANALYSIS GC-MS OF *A. MALACCENSIS* LEAVES EXTRACT BY DTD



D.1 GC-MS Chromatograms of WE coupled with DTD analysis



D.2 GC-MS Chromatograms of ME coupled with DTD analysis

APPENDIX E1

DATA OF COMPOUNDS ON (+) ESI-MS OF *A. MALACCENSIS* FROM WE

No	Component name	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
1	7-Hydroxy-5,3',4'-trimethoxy flavone	328.09469	328.0949	329.1022	0.3	0.8	16.55	5.87	180.1	293489	+H
2	2,4,4′,6′-Tetrahydroxy-benzophenone	246.05282	246.0528	247.06	-0.1	-0.2	6.16	4.8	155.38	236210	+H, +Na
3	Genkwanin_1	284.06847	284.0684	285.0756	-0.1	-0.4	9.27	8.44	248.71	229885	+H
4	Gallocatechin(4α→8)-epicatechin	594.13734	594.1379	617.1271	0.6	1	3.71	8.07	237.05	190826	+Na, +H, +K
5	Loganic acid-6'-O-β-D-glucoside	538.18977	538.1895	577.1527	-0.2	-0.4	3.98	7.92	233	52815	+K
6	(-)-Epigallocatechin	306.07395	306.0736	329.0628	-0.3	-1	3.62	8.01	236.02	23888	+Na, +H
7	Epicatechin gallate (Epicatechin-3-O-gallate)	442.09	442.0903	443.0976	0.3	0.6	2.09	8.12	238.29	21737	+H
8	Quercetagetin-6,7,3',4'-tetramethyl ether	374.10017	374.1002	375.1075	0	0.1	6.42	8.14	239.18	13495	+H
10	1,5-Dihydrxy-2,3,4,7-tetramethoxyxanthone	348.08452	348.0857	371.075	1.2	3.3	6.06	6.05	184.33	2678	+Na
	3′,5-Dihydroxy-7,4'-dimethoxy flavone	314.07904	314.079	315.0863	0	-0.1	7.34	5.68	175.47	2426	+H
11	Rhamnetin	316.0583	316.0581	317.0654	-0.2	-0.5	6.97	5.4	168.7	1754	+H
12	Stigmasta-4,22-dien-3-one	410.35487	410.3549	411.3622	0	0.1	18.27	7.6	224.35	1193	+H

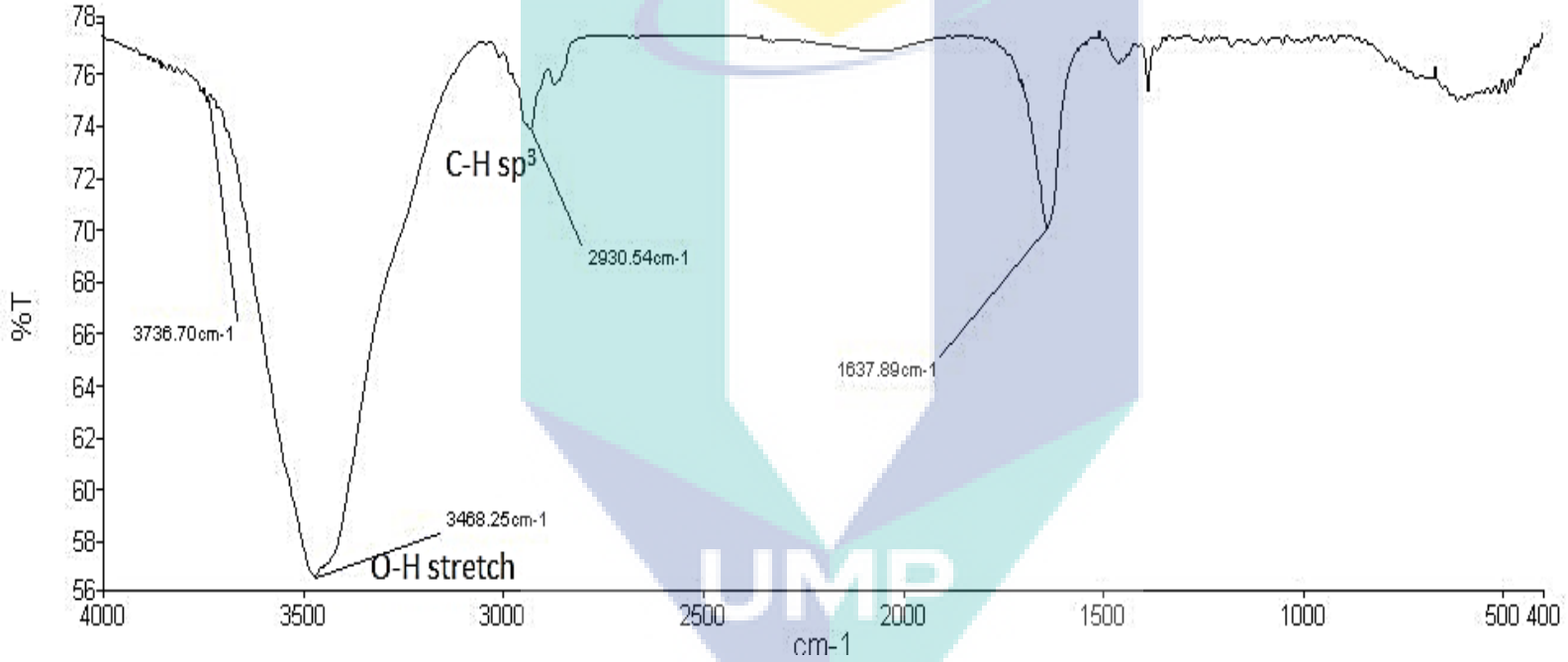
APPENDIX E2

DATA OF COMPOUNDS ON (+) ESI-MS OF *A. MALACCENSIS* FROM ME

No	Component name	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
1	Genkwanin_1	284.06847	284.0684	285.0756	-0.1	-0.4	9.23	8.33	245.68	403963	+H
2	3′,5-Dihydroxy-7,4'-dimethoxy flavone	314.07904	314.0789	315.0862	-0.1	-0.3	16.47	5.6	173.66	257608	+H
3	7-Hydroxy-5,3',4'-trimethoxy flavone	328.09469	328.0946	329.1019	-0.1	-0.2	11.27	8.95	262.73	186468	+H
4	Hydroxygenkwanin	300.06339	300.0638	301.0711	0.4	1.3	14.76	5.39	168.56	80876	+H
5	Stigmasta-4,22-dien-3-one	410.35487	410.3552	411.3624	0.3	0.7	18.42	7.64	225.34	57189	+H
6	2,4,4′,6′-Tetrahydroxy-benzophenone	246.05282	246.0532	247.0604	0.3	1.4	6.29	4.77	154.67	37732	+H, +Na
7	Loganic acid-6'-O-β-D-glucoside	538.18977	538.1879	577.151	-1.9	-3.3	6.41	8.2	240.5	24189	+K
8	5-Hydroxy-3,6,7,4'-tetramethoxy falvone	358.10525	358.105	359.1123	-0.2	-0.7	16.56	6.25	189.43	14164	+H
10	Quercetagetin-6,7,3',4'-tetramethyl ether	374.10017	374.1001	375.1074	0	-0.1	6.29	6.24	189.02	4157	+H
11	Dihydrokaempferol	288.06339	288.0627	289.07	-0.6	-2.2	11.26	8.32	245.36	2385	+H

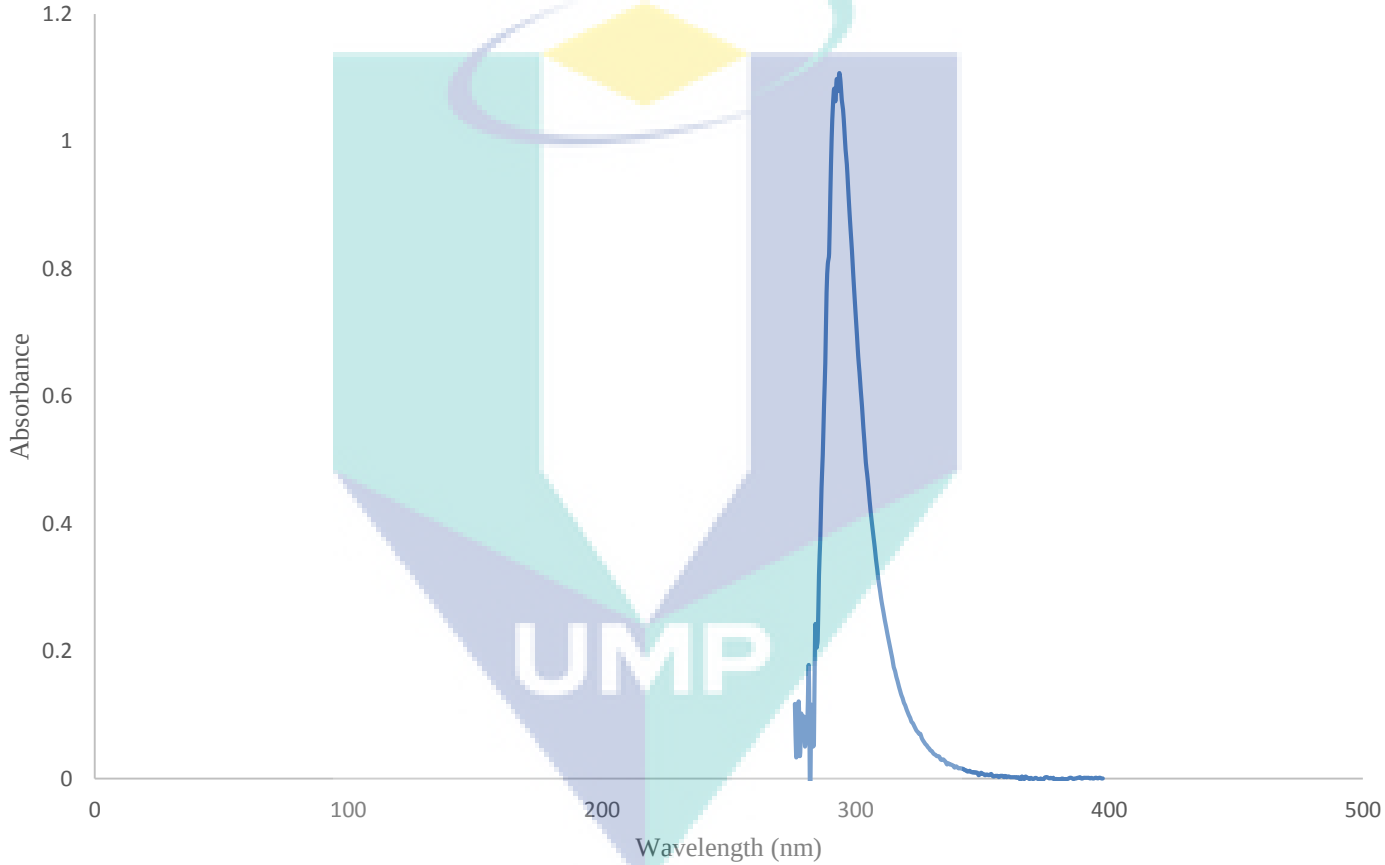
APPENDIX F1

IR SPECTRA OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



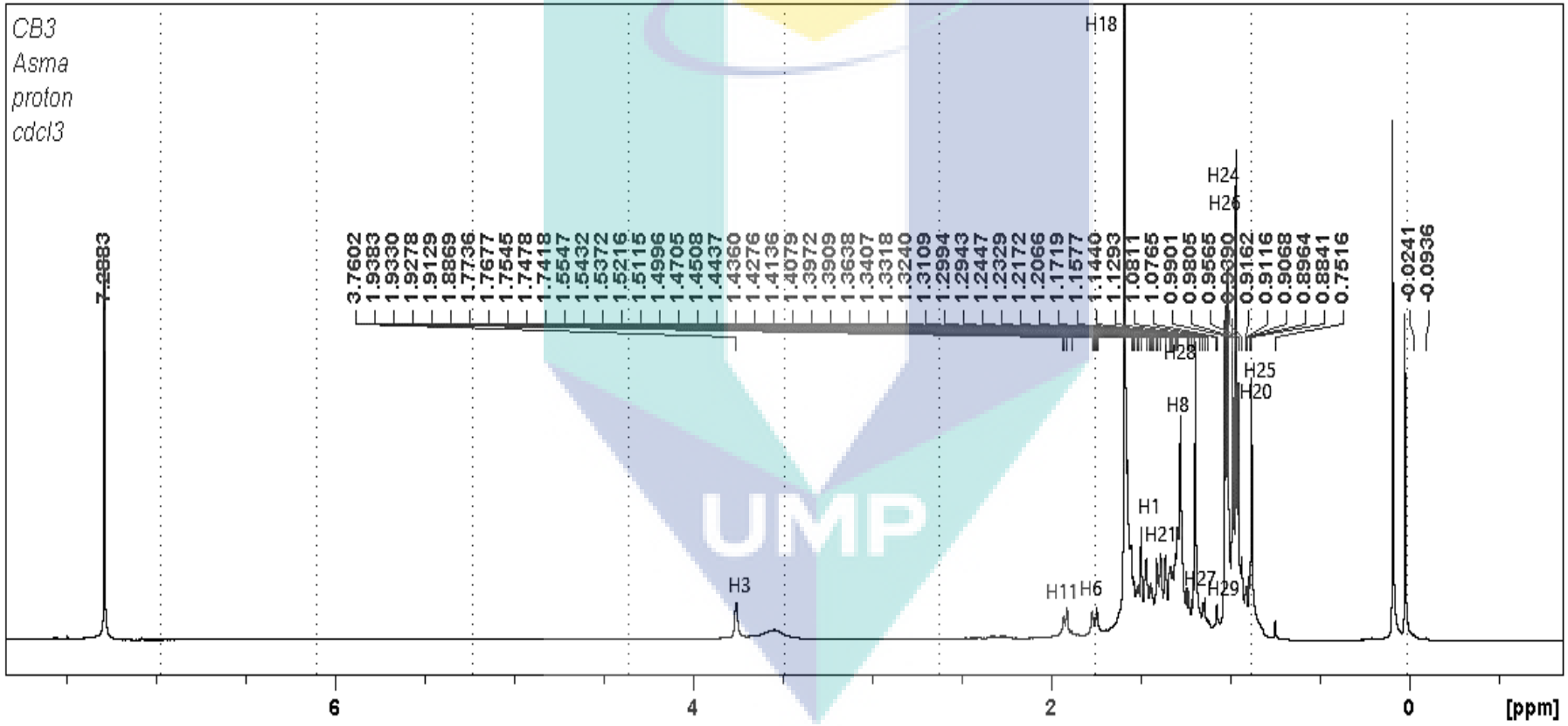
APPENDIX F2

SPECTRA OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



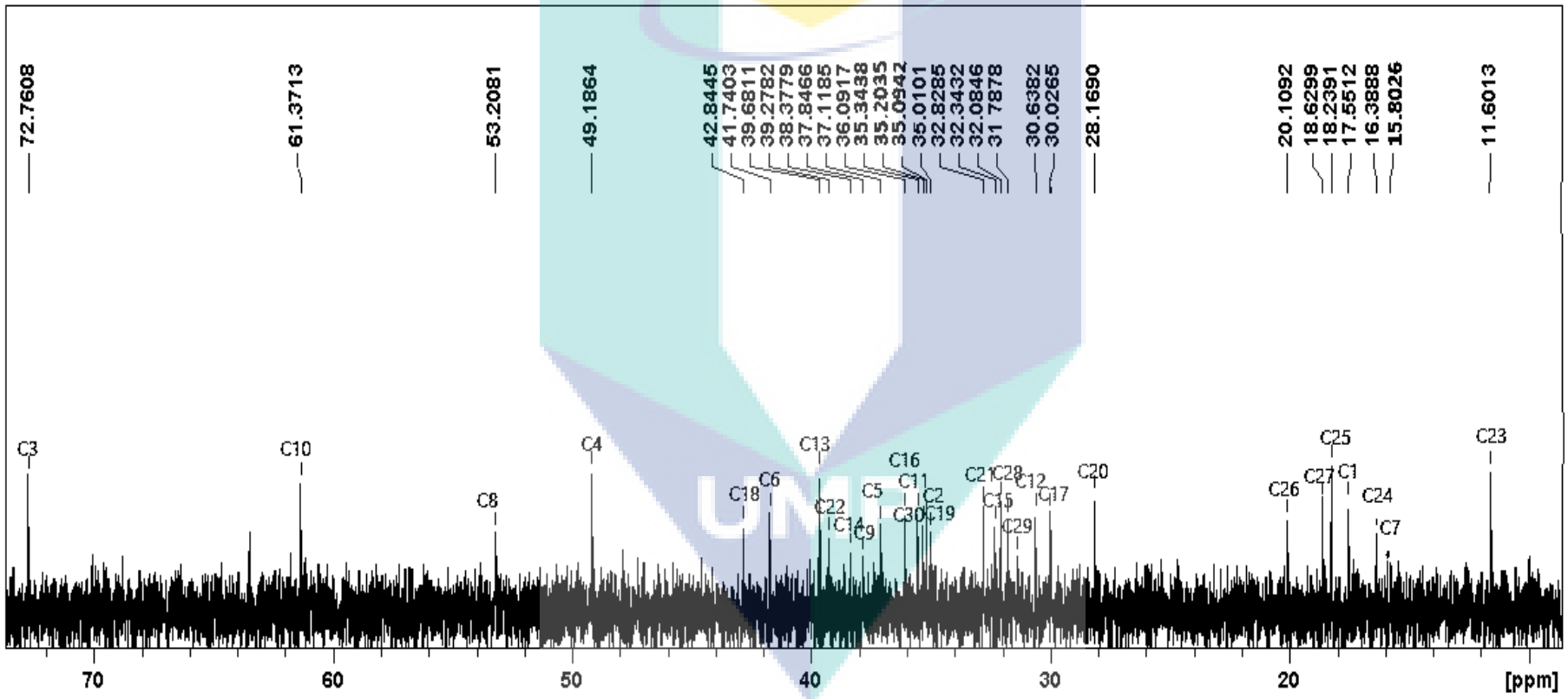
APPENDIX F3

^{1}H NMR SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



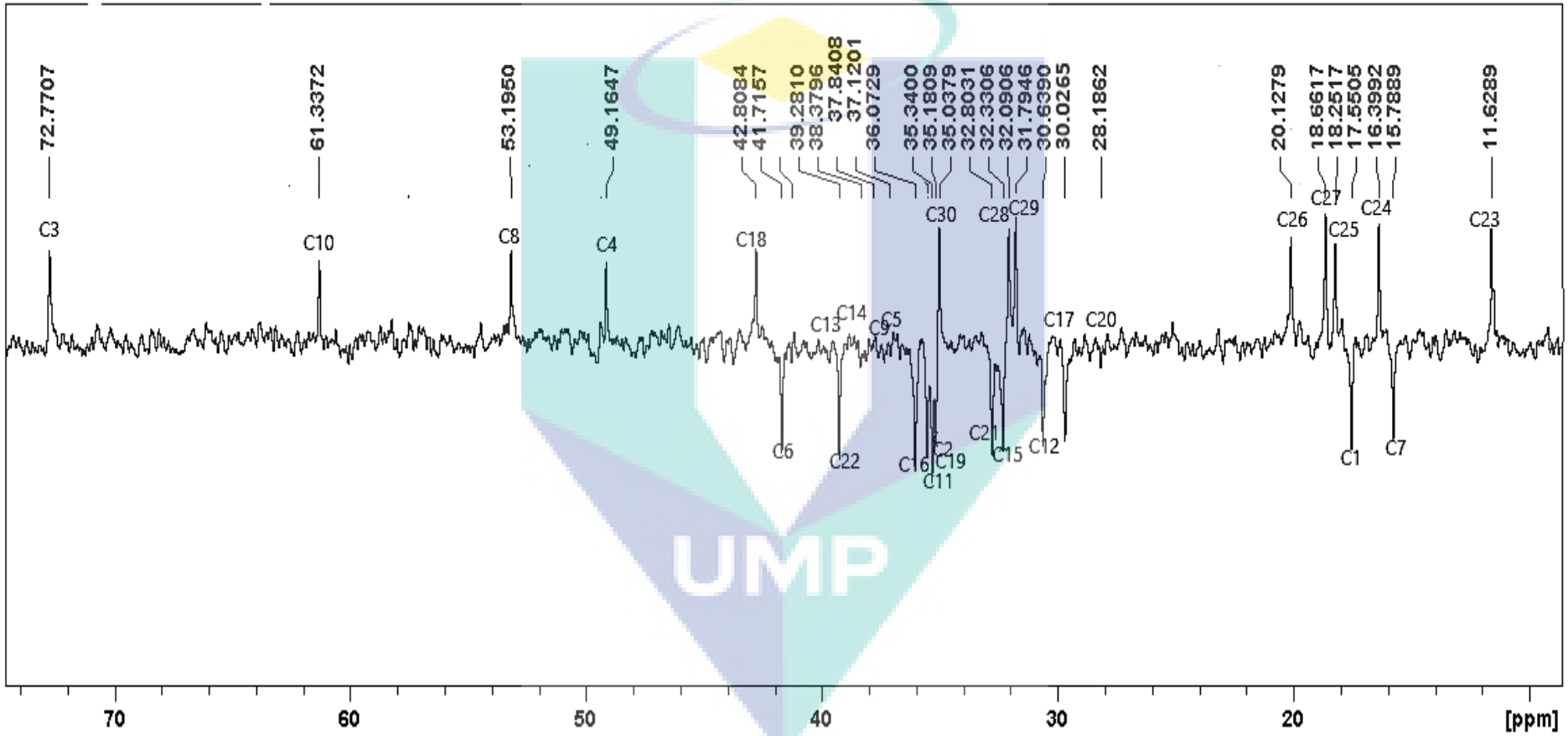
APPENDIX F4

^{13}C NMR SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



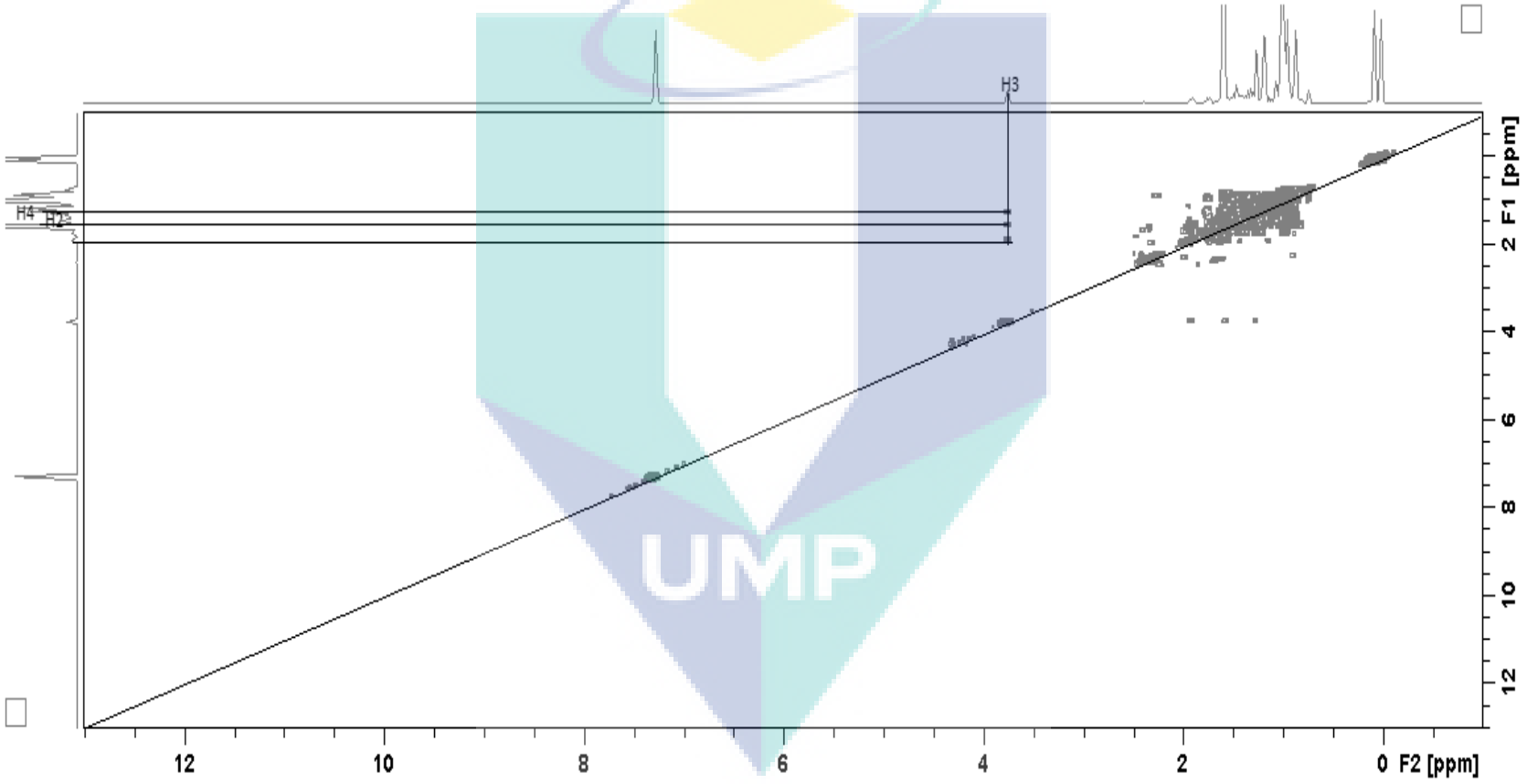
APPENDIX F5

DEPTQ SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



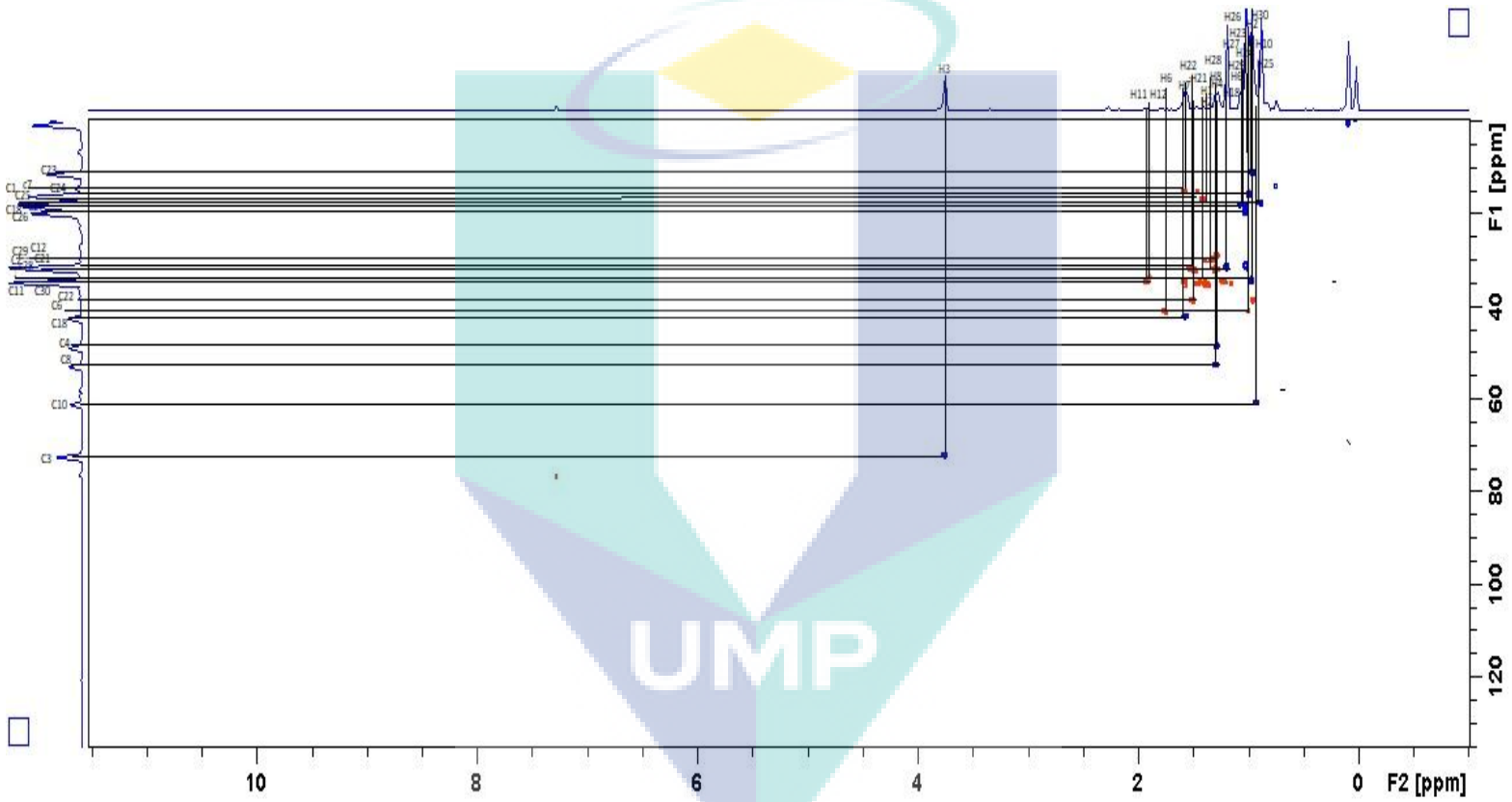
APPENDIX F6

COSY SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



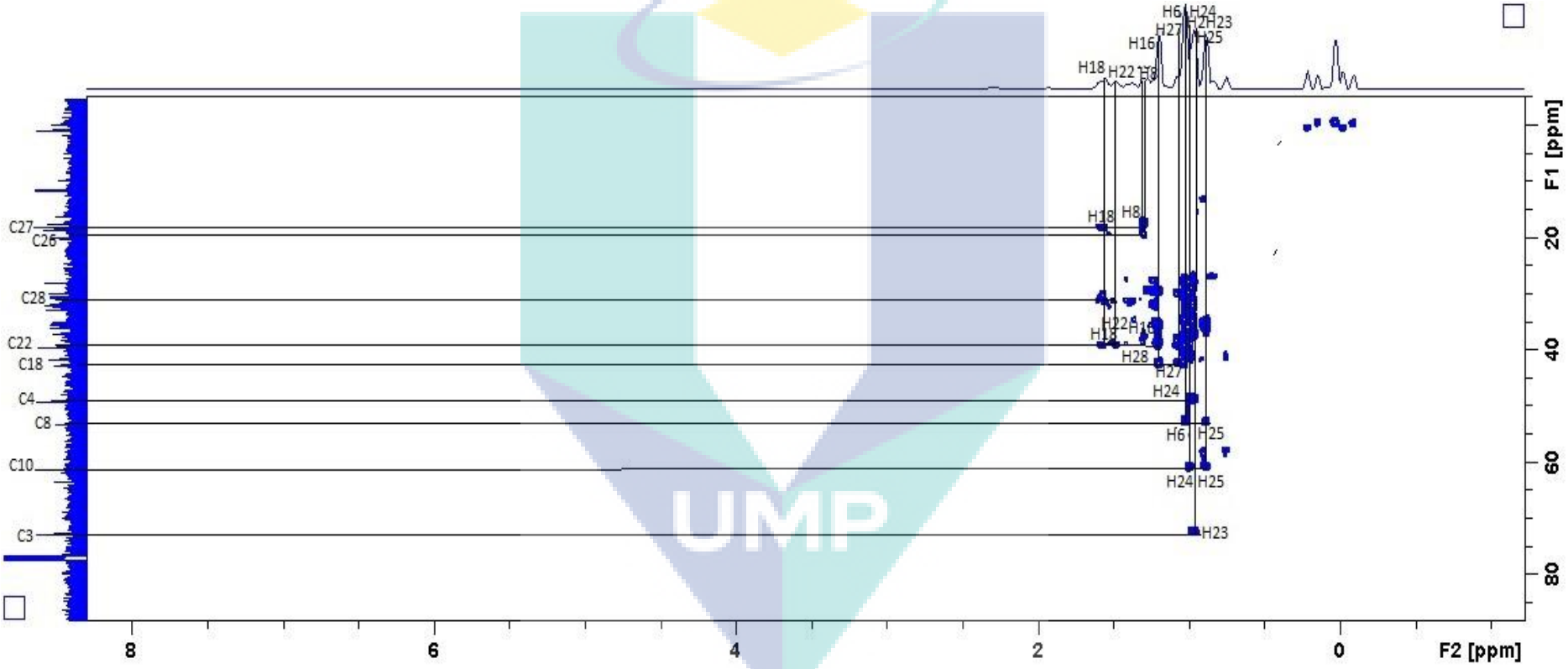
APPENDIX F7

HSQC SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



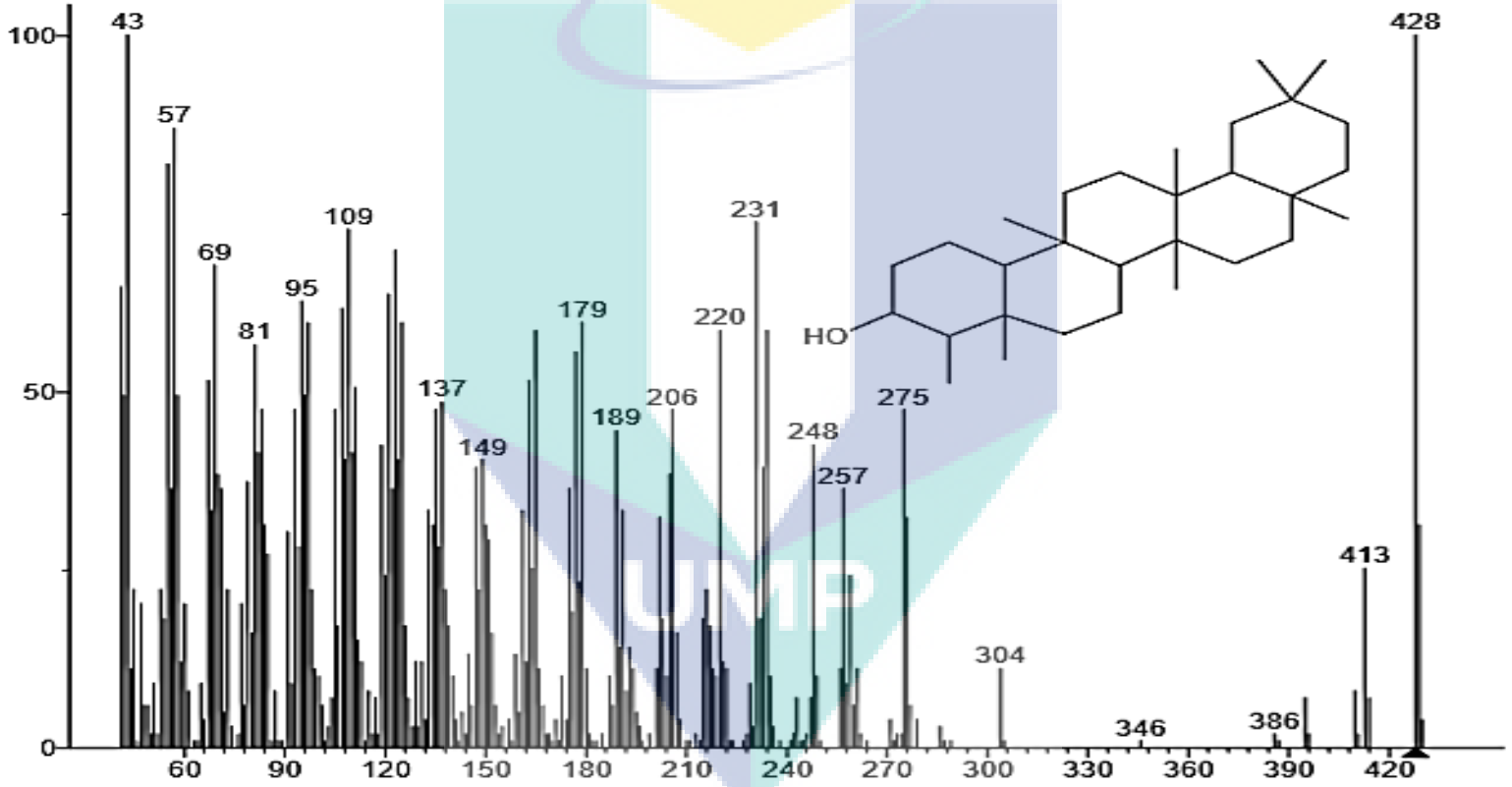
APPENDIX F8

HMBC SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



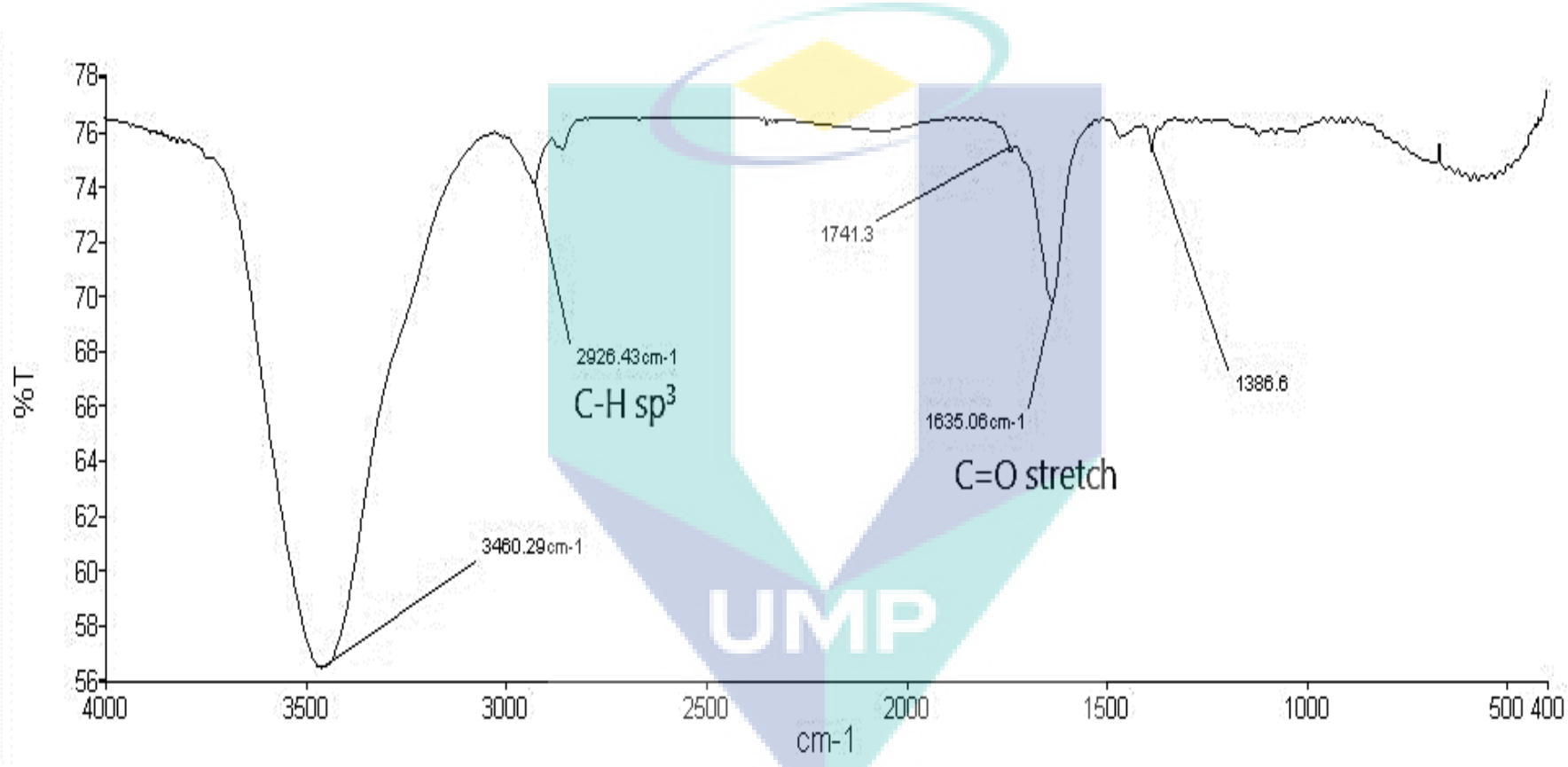
APPENDIX F9

EIMS SPECTRUM OF OF FRIEDELANOL (I) FROM *A. MALACCENSIS*



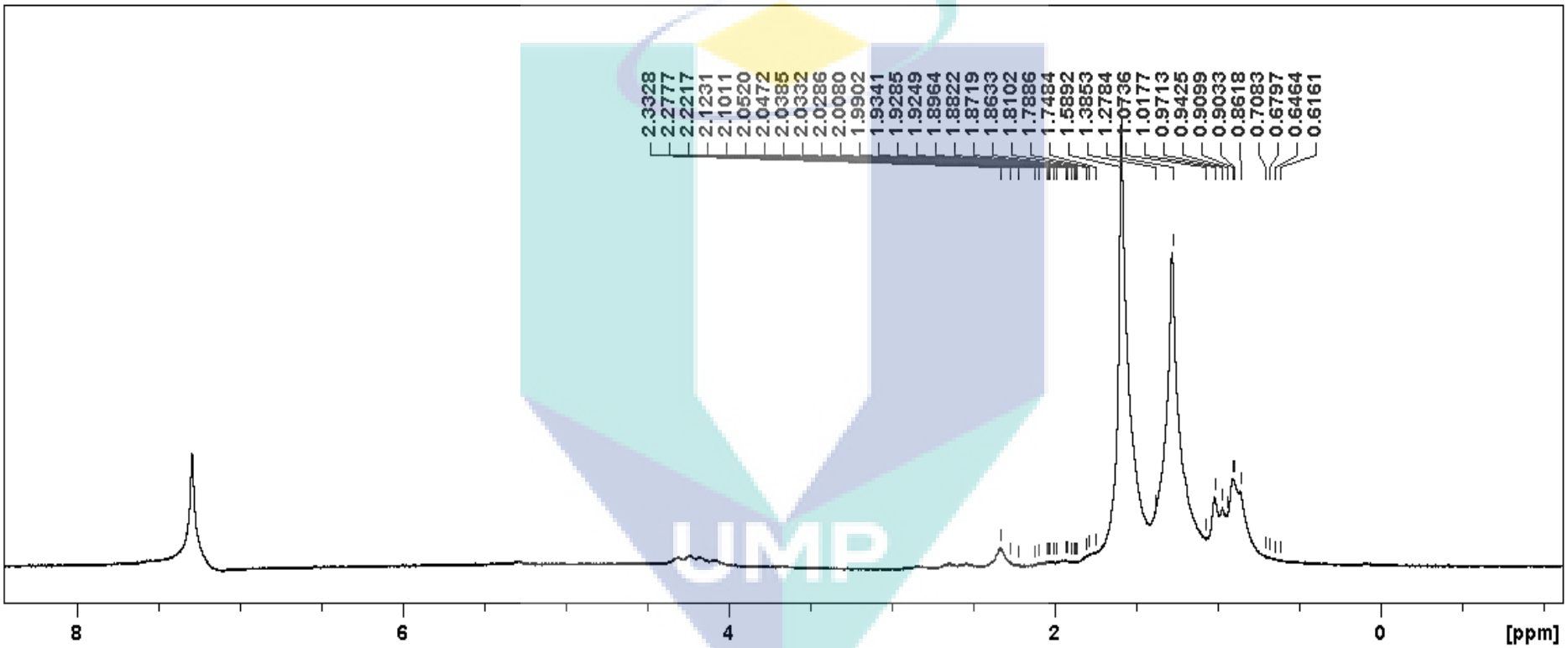
APPENDIX G1

IR SPECTRUM OF OF FRIEDELIN (II) FROM *A. MALACCENSIS*



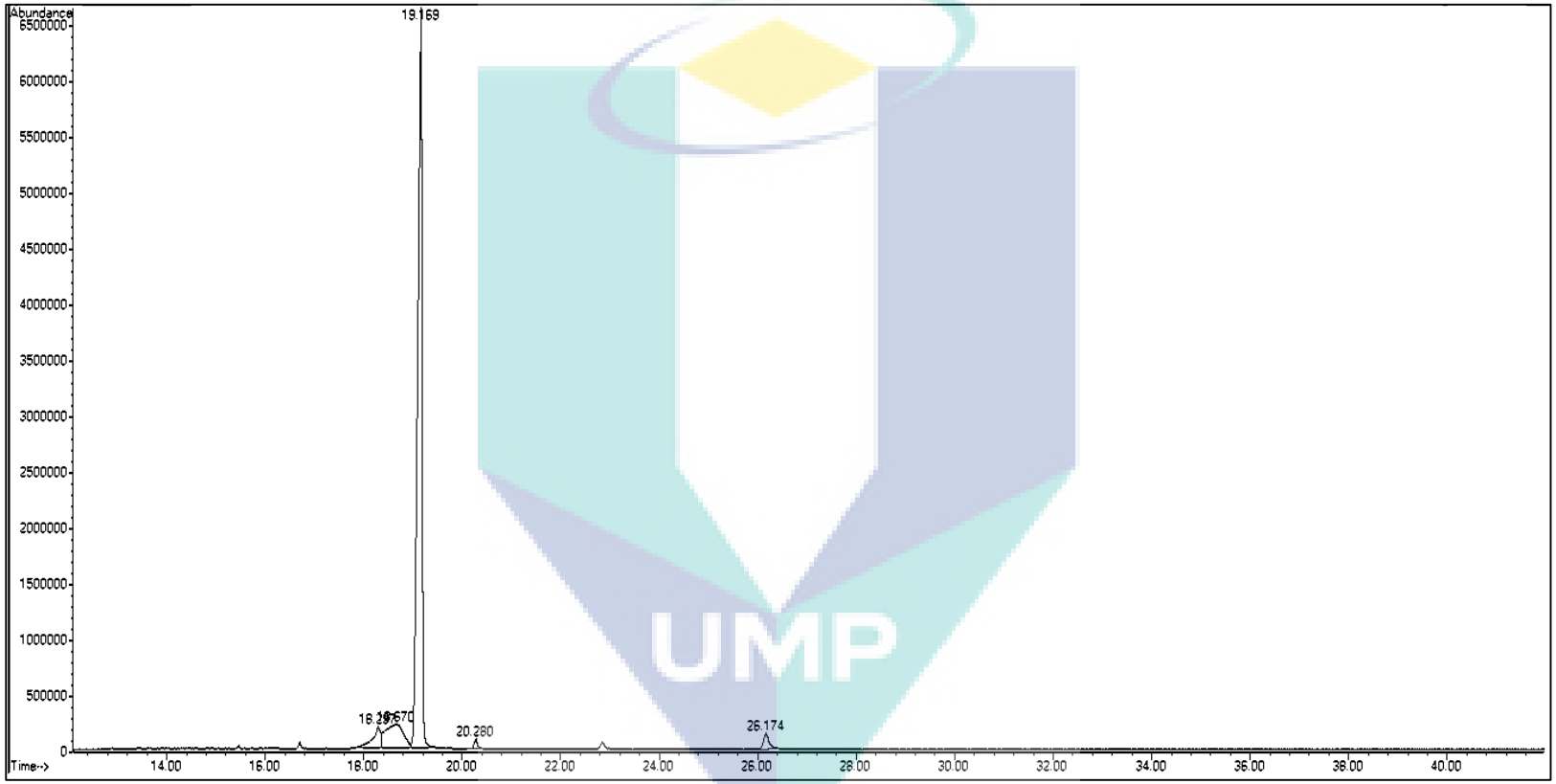
APPENDIX G2

^{1}H NMR SPECTRUM OF OF FRIEDELIN (II) FROM *A. MALACCENSIS*



APPENDIX G3

GC-MS CHROMATOGRAM OF FRIEDELIN (II) FROM *A. MALACCENSIS*



APPENDIX H
LIST OF PUBLICATION

E.1: Award:

Creation, Innovation, Technology, Research & Exploration Competition (CITREX) (Research & Innovation Centre,UMP). March 2014. (Gold Medal).

BioInnovation Awards 2014. Nov 2014. (Bronze Medal).

E.2: Journal:

Nik Noor Asma Nik Wil, Nor Adila Mhd Omar, Noorhuda Awang@Ibrahim and Saiful Nizam Tajuddin. 2014. In vitro antioxidant activity and phytochemical screening of *Aquilaria malaccensis* leaf extracts. Journal of Chemical and Pharmaceutical Research, 6(12), 688-693.

D.3: Presentation:

Composition and Antioxidant Activity of *Aquilaria Malaccensis* Leaf Extract. In: 18th Malaysian International Chemical Congress (18MICC) 2014, 3-5 November 2014, Kuala Lumpur. pp. 1-5.


BioInnovation Awards 2014
Bronze Award
presented to
PROFESSOR MADYA DR. SAIFUL NIZAM BIN TAJUDDIN,
NOORHUDA BINTI AWANG @ IBRAHIM,
NIK NOOR ASMA BINTI NIK WIL,
PROFESSOR DR. MASHITAH BINTI MOHD YUSOFF
HIGH QUALITY OF AGARWOOD TEA EXTRACTION
For your outstanding contribution
in the research & development of biotechnology
RONALD CHIEW
Group Managing Director
PROTEMP Group of Companies
DR WAN MANSHOL BIN W. ZIN
Organising Chairman of BioInnovation Awards 2014
Malaysian Association of Research Scientists, MARS
Brought to you by
protemp
MARS


Universiti Malaysia PAHANG
ITREX
Certificate of Award
Gold Medal
This Certificate of Award is presented to
Dr. Saiful Nizam Bin Tajuddin, Nik Noor Asma Binti Nik Wil, Noorhuda Binti Awang @ Ibrahim,
Profesor Dr. Mashitah Binti Mohd Yusoff
For the invention / innovation of
High Quality Agarwood Tea Extraction
CREATION, INNOVATION, TECHNOLOGY & RESEARCH EXPOSITION 2014
5th – 6th March 2014, Universiti Malaysia Pahang
PROFESSOR DR. MASHITAH MOHD. YUSOFF
DEPUTY VICE CHANCELLOR (RESEARCH & INNOVATION)
UNIVERSITI MALAYSIA PAHANG