MONASH University

CHEMICAL CONSTITUENTS AND BIOACTIVITY OF SELECTED MALAYSIAN PLANTS

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[redacted]

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Name: Yong Mei Mun

Date: 1st December 2017

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ABSTRACT

The main plants of interest are *Aquilaria malaccensis* and three plants from the *Fagraea* spp. genus which are *Fagraea elliptica*, *Fagraea gardenioides* and *Fagraea racemosa*. To date, there has been lack of literature on the chemical constituents and bioactivity of the leaves of these plants. Leaf extracts were screened for antioxidant and antimicrobial properties and the plant with the best bioactivity was selected for further investigation. This is the first study on antioxidant and antimicrobial properties of the three *Fagraea* plants.

A. malaccensis was found to have the highest antioxidant activity among all four plants and was chosen as the primary plant of interest. *A.malaccensis* is a large evergreen tree native to Southeast Asia rainforests where its resinous heartwood is traditionally used to relieve pain, fever, asthma, and rheumatism. Five solvents of different polarity were used to extract the bioactive compounds within the leaves. Among these, the methanol crude extract was found to have the highest activity for all antioxidant assays and the most promising with regards to cytotoxicity testing against MCF-7 breast cancer cell lines and antimicrobial activity against five gram positive bacteria.

Therefore, the methanol extract was subjected to further separation using column chromatography. Sub-fractions with better bioactivity and sufficient yield were focused upon for isolation and purification in hopes of obtaining purified extracts with potentially improved bioactivity. Purity of sub-fractions were preliminarily determined using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) before being sent for analysis such as LC-MS/MS and NMR to help in identification.

Compound 22-6-2 is a yellowish needle-like crystal isolated out of the methanol extract of *A. malaccensis* leaves. This compound is identified as violanthin using LC-MS/MS matching to database and comparison of NMR data obtained to literature values. This is the first time that violanthin has been reported in *Aquilaria* species. The IC_{50} of DPPH activity could not be determined due to insufficient sample for screening, but a concentration of 2.5mg/ml of violanthin exhibits approximately 20% scavenging activity.

Keywords: *Aquilaria malaccensis*, *Fagraea elliptica*, *Fagraea gardenioides*, *Fagraea racemosa*, antioxidant, antimicrobial, cytotoxicity, MCF-7, violanthin.

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ABBREVIATIONS

^{13}C	Carbon-13
^{1}H	Proton
AA	Ascorbic acid
AEAC	Ascorbic acid equivalent antioxidant capacity
ANOVA	Analysis of variance
AOA	Antioxidant activity
ATCC	American Type Culture Collection
BCB	β-carotene bleaching
BHA	Butylated hydroxyanisole
CLSI	Clinical and Laboratory Standards Institute
d	Doublet
dd	Doublet of doublet
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediamine tetraacetic acid
EI	Electron impact ionization
ESI	Electrospray ionization
FBS	Fetal bovine serum
FRP	Ferric reducing power
GAE	Gallic acid equivalents
GC	Gas chromatography
HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration at 50%
J	Coupling constant
LC	Liquid chromatography
m	Multiplet
m/z	mass-to-charge ratio
MBC	Minimum bactericidal concentration
MCA	Metal chelating assay
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth

MHz	Megahertz
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NA	Nutrient agar
NB	Nutrient broth
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
ppm	parts per million
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RP-HPLC	Reverse phase high performance liquid chromatography
s	Singlet
spp.	Species
SD	Standard deviation
t	triplet
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TPC	Total phenolic content
UV	Ultraviolet spectroscopy
δ	Chemical shift in ppm

1.0 GENERAL INTRODUCTION

1.1 Role of plant-derived natural products in drug discovery

Since early civilizations, mankind has relied upon nature for treatment of diseases sourcing them from animals and plants. Medical records from ancient empires of Mesopotamian, Egyptian, Chinese, and Indian origin have extensively documented thousands of plant derived substances used as traditional medicine for ailments ranging from colds to infections as early as 1000 BCE (Cragg and Newman, 2013). The Greeks, Romans and Arabs further contributed via records of collection, storage, use and complex prescriptions of medicinal herbs. Till now, plants continue to play an essential role in healthcare. The World Health Organization (WHO) estimates that 80% of the world's population still relies on plant-derived traditional medicines as their primary healthcare (Cragg and Newman, 2013).

However, rather than direct consumption of plants as therapeutic agents, the most important role of plants in modern medicine is in guiding drug discovery and development. Plant-derived compounds with bioactivity often become starting materials and models for the production of pharmacologically active compounds and synthesis of drug analogs (Li and Vederas, 2009; Schmidt *et al.*, 2008). The best example would be antimalarial drugs. In 1820, quinine was isolated from the bark of *Cinchona officinalis* and subsequently forms the basis of synthesis of chloroquine and mefloquine. Following development of resistance to these drugs, a search for better drugs to treat Malaria commenced. In 1971, Chinese scientists using data from ancient texts in Traditional Chinese Medicine discovered artemisinin from sweet wormwood, *Artemisia annua* (Klayman *et al.*, 1985). Current malarial treatments predominantly use artemisinin analogues.

Other notable examples include Galegine, from Goat's Rue (*Galega officinalis L),* being the model for the synthesis of bisguanidine-type of antidiabetic drugs; papaverine, from poppy (*Papaver somniferum),* which leads to synthesis of verapamil and hypertension drugs; and salicylic acid from willow bark (*Salix spp*.) being the basis of non-steroidal anti-inflammatory drugs (Cordell and Colvard, 2012; Li and Vederas, 2009; Schmidt *et al.*, 2008). In cancer treatment, paclitaxel (Taxol) from the bark of the Pacific yew (*Taxus brevifolia*) has led to the synthesis of analogues like Docetaxel and Cabazitaxel (Cragg and Newman, 2013).

1.2 Renaissance of interest in natural products research

An estimated 50% of prescription drugs in Europe and USA and 34% of new medicines approved by the US Food and Drug Administration (FDA) between 1981 and 2010 were based on natural products and their derivatives (Harvey *et al.*, 2015; Newman and Cragg, 2012). These natural products are compounds isolated from sources such as plants, animals, bacteria and fungi. In the last century, major pharmaceutical companies have favored chemically-synthesized drugs due to their higher potency against diseases as well as ability of modification of structure to target certain aspects of diseases. An analysis into approximately a thousand small molecules, new chemical entities (NCEs), from 1981 to 2012 revealed that 66% of the drugs are formally synthetic. Of these, 65% are naturally-derived and inspired and only 36% being truly synthetic (not inspired by natural products) (Newman *et al*., 2015). The partiality on combinatorial chemical techniques for drug development leads to a decline in interest on natural products as chemotherapeutic agents.

However, several factors have rekindled this interest into natural products-derived lead compounds. Firstly, the declining number of novel NCEs in R&D of pharmaceutical industries has been worrying. From 2004 to 2012, only 26 NCEs were approved (FDA, 2013). There is need for new inspiration for drug design. Secondly, natural products offer several advantages over synthetic compounds. As opposed to synthetic drugs which are usually single chemical entities, natural products are highly complex involving multiple active compounds and high degree of stereochemistry (Fugh-Berman and Ernst, 2001). This structural diversity means unique bioactive constituents. Most importantly, highly specific biological activities based mechanisms of action have been discovered in natural compounds, such as, paclitaxel that promotes tubulin-assembly activity (Cragg and Newman, 2013). Being natural metabolites, they are also likely to be substrates of transporter systems which can help deliver the compounds to intracellular action sites. In other words, they have receptor-binding ability. This is interesting as compounds which are successful as drugs usually have 'metabolite-likeness' properties (Harvey *et al.*, 2015; Zaid et al., 2010).

Thirdly, there is increasing reports of antimicrobial drug resistance such as the emergence of bacterial strains with growing resistance to antibiotics like methicillin-resistant bacteria (Moloney, 2016). One of the contributing factors to increased antibiotic resistance in bacteria has been postulated to involve signaling molecules and their binding to specific receptor proteins which alter cellular activity and function (Corrigan *et al.*, 2013). Natural products include secondary metabolites, and interestingly, they are known to 'evolve' along and against the complex dimensionality and chirality of biological receptors (Nicolaou, 2014).

This may account for their selective, potent and diverse nature whereby incorporation of these structural features may be useful in the development of new active pharmaceuticals.

Fourthly, adverse side effects of certain synthetic drugs limit their usage. Although plant derived natural products have side effects such as allergy and unknown toxicity, they are often perceived to be less toxic and relatively safer substitutes to synthetic drugs by the general public (Singh *et al.*, 2018). New lead compounds will hopefully provide an alternative to current drugs with horrendous side effects particularly pertaining to treatment of cancer and infectious diseases. Lastly, strong associations among free radicals and oxidative stress to aging and various chronic diseases contribute to the growing interest in compounds with antioxidant properties (Balsano and Alisi, 2009).

1.3 Plant-based drugs

Early usage of medicinal herbs involves the consumption of the plant itself, as concoction or ground to powder. However, usage of whole plant (crude extract) may be undesirable. Plants are chemically complex organisms consisting of hundreds of compounds. Not all of these compounds are pharmacologically active, and they may influence the effect of active compounds via synergistic, antagonistic or modulation effect. Presence of undesirable compounds in the plant may lower its therapeutic potential. Active constituents present in a plant also vary depending on the geographical area, climate and ecological condition of the plant's source, amount and parts of plants utilized, collection, storage conditions and preparation (Colegate and Molyneux, 2008). Hence, the amount of plants required to be consumed for treatment is not consistent and the medicinal effects would vary depending on plant's quality.

This consequently led to methodologies to isolate and purify biologically active compounds from plants where only 'active' compounds of interest are extracted. Purified active constituents confer advantages over crude plant extracts. The therapeutic potential of purified compounds are unhindered by other inactive compounds present, better storage stability, and can be delivered in more accurate and reproducible doses with confirmed pharmacological effects (Colegate and Molyneux, 2008).

In natural product research, multiple samples from parts of a plant are taken and extracted to bring out compounds of interest. A sequential extraction system which involves usage of solvents of increasing polarity is commonly employed. Plant samples are selected either via an ethnopharmacological approach or random approach (Newman *et al.*, 2015). The former method involves selection of plants based on prior knowledge such as medicinal herbs

documented to be used by native people while the later involves random screening of several plants in a laboratory. Simple bioactivity screens based on antioxidant, antimicrobial or cytotoxic activities focus upon isolation of extracts of therapeutic importance and enables easier selection of which compounds to purify further. This is known as bioassay-guided isolation (Colegate and Molyneux, 2008). A purified compound with activity of interest is then identified using a combination of spectroscopic techniques such as nuclear magnetic resonance (NMR) and mass spectroscopy (MS) to solve the structures of the purified constituent. Structural elucidation is important as the probability that an isolated compound can directly become a drug is relatively low with exception to antimicrobial activity. If the compound is not potent enough or slightly problematic such as having solubility issues, with knowledge of the structure, synthetic chemistry may be used to generate analogues that have more desirable therapeutic characteristics. Lastly the purified compound is subjected to further bioassays to assess their potency and efficacy (Newman *et al.*, 2015).

1.4 Phytochemicals

Most plant-derived pharmacologically active compounds used in drug research are attributed to their secondary metabolites which are divided into several classes based on their biosynthetic origin such as phenolics, terpenoids, alkaloids, steroids and so on (Wink *et al.*, 2012). Also known as phytochemicals, these bioactive compounds are produced by plants to interact with specific environmental stimuli and increase their survival ability against external factors such as production of defensive substances against pathogens, predators and stress due to their sessile nature (Kennedy and Wightman, 2011). Secondary metabolites have a wide range of physiological properties such as anti-allergenic, anti-inflammatory, antimicrobial, antioxidant, antiproliferative, anticarcinogenic, anti-artherogenic, cardioprotective and vasodilatory effects (Daglia, 2012; Lane *et al.* 2015).

1.4.1 Phenolic compounds

Phenolic compounds comprise of an aromatic ring with one or more hydroxyl substituents. Depending on their structural complexity, they are divided into several classes as in Table 1 (Balasundram *et al.*, 2006; Garcia-Salas *et al.*, 2010). Flavonoids and phenolic acids are the most abundant polyphenols in diet, 60% and 30% respectively (Nichenametla *et al.*, 2006). Flavonoids consist of two benzene rings linked by a linear three-carbon chain that usually form a closed central pyran ring with one of the benzene rings. Based on the oxidation state of the central pyran ring, flavonoids can be further subdivided into chalcones,

dihydrochalcone, auron, flavones, flavonols, dihydroflavonol, flavanones, flavanols (catechins), flavandioles or leucoanthocyanidins, anthocyanidins, isoflavononas, flavonoids, and condensed tannins or proanthocyanidins. Phenolic acids are present in plant tissues in different forms, including aglycones (free phenolic acids), esters, glycosides or bound to complex polymerized molecules (Garcia-Salas *et al.*, 2010).

Phenolic compounds have been reported to be potent antioxidants in which increased consumption has been linked to a lower risk of various diseases including coronary heart disease, cancer, diabetes mellitus and neurodegenerative diseases (Costa *et al.*, 2009; Fresco *et al.*, 2010). Phenolic compounds act as antioxidants via several mechanisms such as free-radical scavenging, pro-oxidant metal ions chelation, and protection and regeneration of other dietary antioxidants such as vitamin E depending on their structural properties (Garcia-Salas *et al.*, 2010). For example, the numbers and positions of hydroxyl groups in relation to carboxyl groups in a phenolic acid affects the degree of hydroxylation and hence the antioxidant activity of the compound (Naczk and Shahidi, 2006).

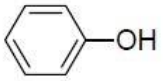
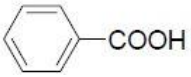
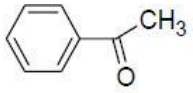
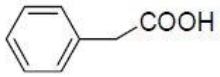
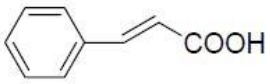
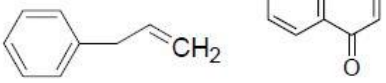
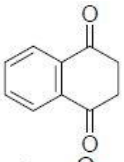
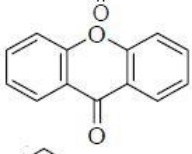
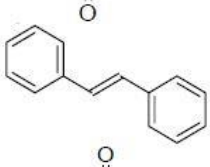
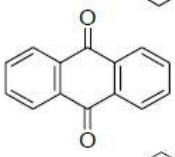
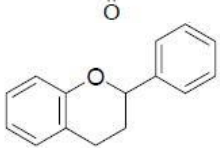
Polyphenols, especially flavonols and tannins, exhibit antimicrobial activity with mechanisms of microbial suppression includes inhibition of biofilm formation, reduction of host ligand adhesion and bacterial toxin neutralization (Daglia, 2012). Similarly, the antibacterial, antiviral, and antifungal properties of polyphenols are structurally dependent. For example, presence of a galloyl group increases antimicrobial activity (Taguri *et al.*, 2004).

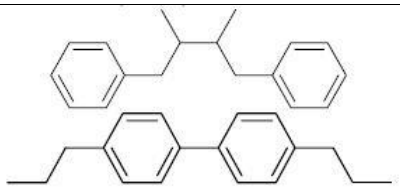
Polyphenols through cancer cell lines and animal tumor models studies also display chemopreventive activity which includes antimutagenesis, antimetastatic, inhibition of carcinogen activation or detoxification of carcinogen. They are able to influence cellular and molecular mechanisms related to carcinogenesis such as inhibition of key proteins in signal transduction pathways like MAP-kinases, inhibition of nuclear factor-κB (NF-κB) and related activities, activation or deactivation of enzyme systems, alter immune responses, and modulate cell-cycle including cell differentiation, proliferation and apoptosis (Nichenametla *et al.*, 2006; Singh *et al.*, 2018). Examples are the polyphenols curcumin and resveratrol (Shukla and Singh, 2011). As free radicals are one of the contributors to cancer, the antioxidant activity of polyphenols particularly against reactive radical scavenging is also significant in cancer prevention.

Other therapeutic benefits have been attributed to the capability of polyphenols to (1) inhibit and reduce enzymes such as telomerase, cyclooxygenase and lipoxygenase; (2) exert physiological effects such as tannins being able to reduce blood pressure and modulate immunoresponses; and (3) interact with signal transduction pathways and cell receptors

(Muchuweti *et al.*, 2006; Naasani *et al.*, 2003; O'Leary *et al.*, 2004; Sadik *et al.*, 2003;; Wiseman *et al.*, 2001).

Table 1. Classification of phenolic compounds (Garcia-Salas *et al.*, 2010)

Carbon no.	**Class**	**Basic structure**	**Sources**
C_6	Simple phenols		
	Benzoquinones		
C_6–C_1	Benzoic acids		Cranberry, cereals
C_6–C_2	Acethophenones		Apple, apricot, banana, cauliflower
	Phenylacetic acids		
C_6–C_3	Cinnamic acids		Carrot, citrus, tomato. spinach, peaches, pears, eggplant
	Phenylpropanoids (coumarins, chromones)		Carrot, celery, citrus, parsley
C_6–C_4	Napthoquinones		Nuts
C_6–C_1–C_6	Xanthones		Mango, mangosteen
C_6–C_2–C_6	Stilbenes		Grapes
	Anthraquinones		
C_6–C_3–C_6	Flavonoids, isoflavonoids		Widely distributed

$(C_6–C_3)_2$	Lignans, neolignans		Sesame, rye, wheat, flax
$(C_6–C_1)_n$	Hydrolysable tannins	Heterogeneous polymer composed of phenolic acids and simple sugars	Pomegranate, raspberry
$(C_6–C_3)_n$	Lignins	Highly crosslinked aromatic polymer	

1.4.2 Terpenes and terpenoids

Terpenes share an isoprene unit: isopentenyl (3-methyl-3-en-1-yl) pyrophosphate. These C_5 units are linked together in a head-to-tail manner. When the compounds contain oxygen, they are called terpenoids. Depending on the number of isoprene units, terpenes and terpenoids are categorized into several classes with examples as in Table 2 (Gonzalez-Burgos and Gomez-Serranillos, 2012). Terpenoids are active against bacteria, fungi, viruses, and protozoa (Souza *et al.*, 2011). Terpenoids from bark of *Acacia nilotica* have antimicrobial activity against *S. aureus, E. coli, B. subtilis and S. viridans* (Chandra *et al.*, 2017). Similar to polyphenols, terpenoids possess inflammatory and anticancer properties; acting via inhibition of transcription factor NF-κB signaling, changing enzymatic activity and various other mechanisms (Salminen *et al.*, 2008).

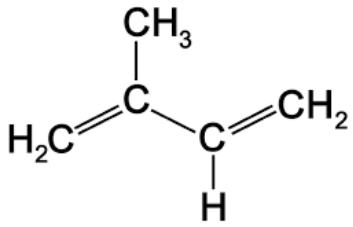


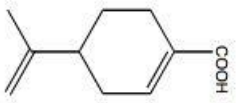
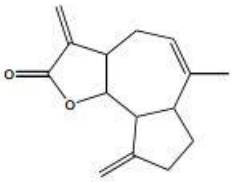
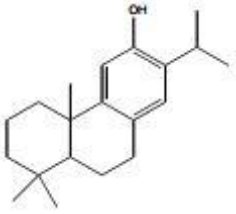
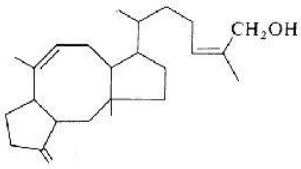
Figure 1. Isoprene unit: isopentenyl pyrophosphate of terpenes

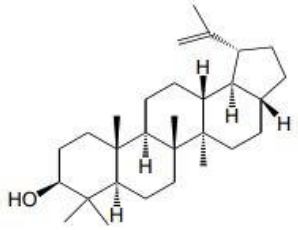
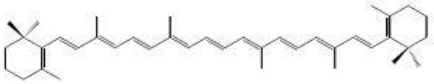
In oxidative stress conditions, terpenes that possess antioxidant properties provide protection against liver, renal, neurodegenerative and cardiovascular diseases, ageing, diabetes and cancer (Gonzalez-Burgos and Gomez-Serranillos, 2012). Mechanisms involved include direct reactive oxygen species (ROS) scavenging pathway and modulation of endogenous antioxidant systems. Antioxidant potency of terpenoids is similarly affected by their structure such as the higher number of conjugated systems, number of hydroxyl groups,

and type of functional side groups attached to the ring structure. For example, xanthophylls which have high radical scavenging activity has a hydroxyl group coupled to the conjugated double bonds in the terminal rings (Gonzalez-Burgos and Gomez-Serranillos, 2012).

Another influencing factor is the concentration of the terpenes. They act as antioxidants at low concentrations but as pro-oxidant compounds at high concentrations (Maleknia and Adams, 2007). In contrast to antioxidants which lower cellular levels of ROS, pro-oxidants increase ROS levels. An increase in ROS level means increased oxidative stress which in turn induces carcinogenic effects and other oxidative stress associated diseases. Surprisingly, pro-oxidant agents can be manipulated to be beneficial in cancer therapy. This is by using pro-oxidants to increase the cellular ROS to cytotoxic levels and therefore induce selective killing of cancer cells (Martin-Cordero *et al.,* 2012). Examples of terpenes with both pro-oxidant and anticancer activities include artemisinin (lactone sesquiterpenoid), lupeol (triterpenoid), Taxol (triterpenoid) and β-carotene (carotenoid) (Martin-Cordero *et al.*, 2012).

Table 2. Classification of terpenes (Gonzalez-Burgos & Gomez-Serranillos, 2012)

Carbon no.	**Isoprene units**	**Class**	**Examples**
C_5	1	Hemiterpenes	
C_{10}	2	Monoterpenes	 Perillic acid
C_{15}	3	Sesquiterpenes	 Eremanthin
C_{20}	4	Diterpenes	 Ferruginol
C_{25}	5	Sesterterpenes	 Ceroplastol

C_{30}	6	Triterpenes	 Lupeol
C_{40}	8	Tetraterpenes	 β-carotene
$C_{5n\ (n < 30,000)}$	8	Polyterpenes	

1.4.3 Alkaloids

Alkaloids are heterocyclic nitrogen containing compounds. Alkaloids are mutagenic and toxic with a marked therapeutic effect in small dosage and hence rarely used in traditional medicine. Nevertheless, anticancer and antimicrobial activities have been reported (Chandra *et al.*, 2017; Singh *et al.*, 2018). Examples of alkaloids of medicinal value include morphine and papaverine from *Papaver somniferum* which is used as analgesic or pain relief and antispasmodic respectively, berberine from *Berberis* spp. with antimicrobial activity against *Streptococcus agalactiae*, and yohimbine from *Pausinystalia yohimbe* bark with antiviral activity (Chandra *et al.*, 2017; Ozcelik *et al.*, 2011).

1.5 Antioxidants and their importance

Oxidative stress caused by free radicals had been implicated in the pathogenesis of various disorders including cancer, cardiovascular disease and neurodegenerative diseases. Free radicals are unstable and highly reactive as they contain unpaired electrons in their outermost shell which predisposes them to react with other compounds such as deoxyribonucleic acid (DNA), proteins and lipid to obtain electrons to neutralize themselves and gain stability. This loss of electrons causes the biological molecules to become radicals themselves. They would then oxidize others to neutralize themselves, initiating a chain reaction of oxidation. In normal metabolism, free radicals play a role in oxidative reactions important to enzymatic reactions, phagocytosis and cell signaling (Ames *et al.*, 1993). However in cases of extensive oxidation, it causes necrotic or apoptotic cell death leading to severe tissue damage (Kaur and Kapoor, 2001). Examples include reactive oxygen species

(ROS): singlet oxygen, hydrogen peroxide, superoxide, hydroxyl, and peroxyl radical and reactive nitrogen species (RNS): nitric oxide and peroxynitrite (Devasahayam *et al.*, 2004).

Endogenous free radicals are mainly produced in aerobic respiration though mitochondrial reduction of molecular oxygen, cytochrome P450 enzymes for chemical detoxification, peroxisomes to degrade fatty acids and other molecules and by phagocytic cells to destroy cells infected by bacteria, parasites or virus (Ames *et al.*, 1993; Blokhina and Fagerstedt, 2006). Exogenous sources include pollution, ultraviolet radiation, cigarette smoke and herbicides (Scheibmeir *et al.*, 2005).

Antioxidants are any substance that can significantly delay or inhibit the oxidation reaction (Tachakittirungrod *et al.*, 2007). Based on their mechanism of action, they can be classified as primary, secondary or multi-functional (Chaiyasit *et al.*, 2007). Primary antioxidants such as catechins and β-carotene mainly interfere with the initiation or propagation of the oxidative chain reaction and β-scission reactions. They achieved this by donating electrons to free radicals to stabilize them and terminate the electron-stealing chain reaction (Chaiyasit *et al.*, 2007; Kaur and Kapoor, 2001). In contrast, secondary or preventive antioxidants such as flavonoids decrease the rate of oxidation by scavenging radicals before they initiate the oxidation chain (Scheibmeir *et al.*, 2005). They accomplish this by a number of mechanisms including acting as reducing agents, free-radical scavengers, singlet oxygen quenchers as well as chelators to bind and inactivate pro-oxidant metals that can catalyse oxidation (Chaiyasit *et al.*, 2007). Multi-functional antioxidants such as propyl gallate and proanthocyanidins can interfere with oxidation reactions using more than one mechanism such as a combination of metal chelation and free radical scavenging (Chaiyasit *et al.*, 2007).

Endogenous antioxidant defenses in the body include antioxidant enzymes like glutathione peroxidase, superoxide dismutase and catalase, structural defenses like sequestrations of oxidant producing enzymes in peroxisomes and chelation of free metals as in transferrin and ferritin (Ames *et al.*, 1993). Nevertheless, oxidative stress may occur when free radicals generation exceeds the capacity of the body's antioxidant defenses and repair mechanisms to neutralize them (Antolovich *et al.*, 2002). Exogenous antioxidants can be synthetic or natural. Synthetic antioxidants which are generally phenolic compounds with various degrees of alkyl substitutions are commonly used as additives and preservatives in the food industry to retard food deterioration and discoloration caused by oxidation (Kaur and Kapoor, 2001). Although synthetic antioxidants are efficient and relatively cheap, concerns on their potential toxic side effects lead to the preference of natural antioxidants via fruits and

vegetables consumption. Natural antioxidants had been reported to possess comparable or even higher antioxidant activity than that of synthetic antioxidants (Balasundram *et al.*, 2006).

1.6 Antioxidant assays

Antioxidant activity cannot be directly measured. Indirect measurement involves the ability of antioxidants to control the extent of oxidation and determination of amount of phytochemicals present such as phenolic content and anthocyanin as they had been reported to be positively correlated to antioxidant activity. Essential features of antioxidant assays include an oxidation initiator, a substrate and a suitable end-point for measurement (Antolovich *et al.*, 2002). As antioxidant capacity differs when measured against different oxidants, a single assay system cannot accurately determine antioxidant activity (MacDonald-Wicks *et al.*, 2006). Hence, there is a need for combination of assays to be carried out.

1.6.1 Total phenolic content (TPC) assay

Total phenolic content generally shows a positive correlation with antioxidant activity. The Folin-Ciocalteu's reagent consists of the strong oxidants phosphotungstic and phosphomolybdic acids in the +6 states which form the acidic compound hetero-polyphosphotungstates-molbdenum, a hydrated octahedral complex coordinated with phosphate. At alkaline conditions, the proton of the phenolic compound dissociates forming a phenolate anion that reduce the reagent to yield a stable blue complex $(PMoW_{11}O_{40})^{4-}$. This complex exhibits strong absorbance at 765 nm (Roginsky and Lissi, 2005; Singleton *et al.*, 1999).

This method is simple, rapid, reproducible, sensitive and convenient (Singleton *et al.*, 1999). However, it is not specific for phenolic compounds and does not accurately determine the quantity or quality of phenolic constituents. It actually measures the reducing capacity of the sample, that is, any compound able to reduce the Folin-Ciocalteu's reagents (MacDonalds-Wicks *et al.*, 2006). This includes non-phenolics like proteins, ascorbic acid and sugar. In addition, different phenolic compounds responds differently in this method depending on chemical structure (Naczk and Shahidi, 2006). For example, phenolics with more hydroxyl groups would show better activity.

1.6.2 DPPH free radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is a free nitrogen radical that absorbs strongly at 517 nm. This radical when reduced changes color from violet to pale orange. The discoloration depends on the ability of antioxidants to donate hydrogen atom to the DPPH radical and hence terminates the oxidation chain reaction. The antioxidant itself becomes a stable free radical after hydrogen donation whereas the DPPH radical is converted into α, α-diphenyl-β-picrylhydrazine which is a stable diamagnetic molecule (Antolovich *et al.*, 2002).

This assay measures the radical capture ability of the extract rather than monitor the actual oxidation as it assumes that capture of initiating or propagating free radicals largely inhibits oxidation (Antolovich *et al.*, 2002). A recent hypothesis proposed a rapid initial electron transfer reaction and a subsequent slower hydrogen transfer (Dangles, 2012; MacDonald-Wicks *et al.*, 2006). The rate constants and whether electron or hydrogen atom transfer occurred during the reaction are influenced by the hydrogen-bond accepting solvent and presence of acids or bases in the solvent as they may affect the ionization equilibrium of phenols. Other influencing factors are concentration of radicals as well as molecular structure, kinetic behavior and concentration of antioxidants (Brand-Willams *et al.*, 1995; Juntachote and Berghofer, 2005).

Results are reported as IC_{50} which is the concentration of antioxidant necessary to reduce half of the initial DPPH radicals and ascorbic acid equivalent antioxidant capacity (AEAC). This method is simple, rapid, reproducible and accurate (MacDonald-Wicks *et al.*, 2006). Drawbacks include problems in measurement of IC_{50} as reaction kinetics between DPPH and antioxidants is not linear to DPPH concentration, the presence of other reducing agents able to decolorize the solution and less sensitive compared to other methods for hydrophilic antioxidants (Brand-Willams *et al.*, 1995; MacDonald-Wicks *et al.*, 2006; Prior *et al.*, 2005). Moreover, the structural conformation of the antioxidants influences its interaction with the free radicals, for instance, the degree and type of substitutions like glycosylation (Kaur and Kapoor, 2001). It had also been reported that most phenolic antioxidants react slowly with DPPH and requires 1-6 hours to reach a steady state and that light, oxygen and pH of the reaction mixture can affect the absorbance of DPPH (Bondet *et al.*, 1997).

1.6.3 Ferric reducing power (FRP) assay

In FRP assay, antioxidants present in the extract first reduce the Fe^{3+} ferricyanide ion in the potassium ferricyanide reagent to ferrocyanide ion Fe^{2+}, the later then reacts with excess ferric ion to form the blue color ferric ferrocyanide which absorbs maximally at 700 nm (Graham, 1992). Although this method is simple, rapid, inexpensive, robust, reproducible and highly sensitive, it is nonspecific as it measures reducing ability (Antolovich *et al.*, 2002). The measured reducing capacity may not necessarily be due entirely to antioxidant activity and antioxidants that do not act this way will not be detected (MacDonald-Wicks *et al.*, 2006). Several drawbacks are that the absorbance increases as incubation time lengthens and presence of other Fe (III) species in the mixture may affect the results (Graham, 1992; MacDonald-Wicks *et al.*, 2006).

1.6.4 Metal chelating assay (MCA)

Metal chelating assay measures the capacity of extracts to chelate iron using ferrozine. This is important as iron catalyzes Fenton and Haber-Weiss reactions which can produce ROS (Denisov and Afanas'ev, 2005). Ferrozine is a water soluble disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine which is able to react with Fe^{2+} to form a stable magenta complex that absorbs maximally at 562nm. Antioxidants able to chelate iron will compete with ferrozine to bind iron resulting in less intense color as less of the Fe^{2+}-ferroxine complex is produced (Stookey, 1970). This assay is easy, sensitive and reproducible with stable color change.

1.6.5 β-carotene bleaching (BCB) assay

The β-carotene bleaching (BCB) assay measures the capability of an antioxidant to protect β-carotene from oxidation. It mimics the naturally occurring lipid peroxidation process. Free radicals such as hydroxyl radicals produced by linoleic acid can cause the loss of double bonds in β-carotene leading to the loss of its chromophore and hence bleaching of its orange color at 470nm. The presence of antioxidants can reduce the oxidation and hence slow down the bleaching of β-carotene (Ismail and Tan, 2002).

This assay is sensitive and fast but may not be reproducible as it highly depends on experimental conditions. A disadvantage of this method is that non-polar antioxidants would bring about a stronger inhibition of the bleaching activity compared to polar antioxidants. This 'polar paradox' is because non-polar compounds will form a layer at the lipid-air surface and hence provides protection for the emulsion itself whereas polar compounds remain in the

aqueous phase. Although this assay is sensitive and fast, it is limited to less polar compound and may not be reproducible as it is highly dependent on the experimental variables (Koleva *et al.*, 2002).

1.7 Antimicrobial agents and their importance

The development of antibiotics has substantially reduced the threat of infectious diseases. Over the last decade, the emergence of microbes with reduced susceptibility to existing antimicrobial agents has accelerated markedly methicillin-resistant, vancomycin-resistant, multidrug-ressistant and the more current colistin-resistant bacteria in China (Moloney, 2016). Together with lack of new antibacterial drug development in the 21st century, there is an urgent need for novel antimicrobial agents. This has led to renewed interest in natural products. Plant extracts and essential oils containing phenolics, flavonoids, alkaloids, tannins, and terpenes have been found to have antimicrobial activity including inhibitory activity against bacteria growth, bacterial quorum-sensing, efflux and biofilm production (Savola, 2012). Examples include resveratrol, berberine and carvacrol respectively. Although natural product research is often criticized for re-isolation of known substances, novel chemotypes have been discovered via this approach in recent years and inspire the synthesis of analogues (Brown *et al.*, 2014).

1.8 Antimicrobial assays

The most basic *in vitro* methods to evaluate antimicrobial activity of an extract are the disk diffusion and broth dilution method with standards as published by the Clinical and Laboratory Standards Institute (CLSI). More in depth studies may include time-kill test and flow cytofluorometric methods if the compound showed promising antimicrobial activity.

1.8.1 Kirby-Bauer disc diffusion susceptibility method

In the Kirby-Bauer disc diffusion susceptibility method, agar plates are inoculated with a standardized inoculum of test microorganism. The plant extract or test compound at desired concentration is loaded into filter paper discs of approximately 6 mm in diameter and placed on the agar surface followed by incubation under suitable conditions for a set time period. Generally, the test compound diffuses into the agar and will inhibit the germination and growth of the test microorganism if it has antimicrobial activity. This will be indicated by a clear zone around the disc where diffusion is proportional to the algorithms of their concentration (Balouiri *et al.*, 2016). Measurement of the diameter of the inhibition growth

zone provides qualitative results categorizing the activity as susceptible, intermediate or resistant.

This method is simple, low cost, easy results interpretation and provides rapid screening of large numbers of microorganisms and antimicrobial agents. However, this method cannot distinguish bactericidal and bacteriostatic effects as bacterial growth inhibition does not mean bacterial death. It also cannot quantitate the amount of extract diffused into the agar medium and hence cannot determine the minimum inhibitory concentration (MIC) (Balouiri *et al.*, 2016). Nevertheless, repeated experiments with usage of varying concentration of the antimicrobial agent loaded in the disc can provide a rough estimation of the MIC.

1.8.2 Broth microdilution method

Broth microdilution technique involves preparation of two-fold dilutions of the antimicrobial agent (e. g. 1, 2, 4, 8, 16 and 32µg/mL) in a liquid growth medium dispensed in 96-well microtitre plates. Each well is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. After incubation under suitable conditions, growth of the test microorganism in the wells is observed and MIC can be determined. MIC is defined as the lowest concentration of the test antimicrobial agent that inhibits the visible growth (clear wells) of the microorganism tested as detected by the unaided eye (Balouiri *et al.*, 2016). As MIC can be affected by inoculum size, type of growth medium, incubation time and inoculum preparation method, CLSI standards are used.

Samples from wells showing a negative microbial growth are sub-cultured into agar plates to determine the number of surviving cells (CFU/ml) after 24 hours of incubation to determine minimum bactericidal concentration (MBC). MBC is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the final inoculum (Balouiri *et al.*, 2016). Although broth microdilution is more costly and tedious compared to disc diffusion, it provides quantitative determination of both MIC and MBC. It also circumvents the issue of diffusion of test compound into agar in disc diffusion which can be affected by the compound's polarity (Balouiri *et al.*, 2016).

1.9 Anticancer agents and their importance

Cancer is the second leading cause of death worldwide and the fourth in Malaysia (Nurhanan *et al.*, 2008). Cancer begins when cells start to grow out of control and keep on dividing, forming immortal and abnormal cells (George and Abrahamse, 2016). Extensive cell proliferation forms a mass called tumor which can become malignant if it spreads and invades nearby tissues. Primary cancer prevention involves the practice of healthy lifestyle, avoiding environmental carcinogens, removal of prophylactic organs, and use of chemopreventive drugs and substances (Sanders *et al.*, 2016). In regards to this, natural products are commonly used in oral form for their chemopreventive potential to reduce the risk of cancer development. As free radicals and oxidative stress is noted to be a contributor in the pathogenesis of cancer, most chemopreventive natural products consumed consist of antioxidants. In 2012, an estimated 18% adults in America reported use of natural products beyond multivitamins (Sanders *et al.*, 2016). A notable example is the catechin (-)-epigallocatechin-3-gallate (EGCG) in green tea (*Camellia sinensis*) reported to prevent cancer by inhibition of enzymes, elimination of carcinogen and reduction of oxidative stress. Completed trials have found potential benefits in oral, skin, cervical, breast, stomach and prostate cancer prevention. However, most natural products have unknown efficacy and toxicity due to lack of clinical trials and should be used with caution as there is erroneous perception that natural products are 'safe'. In fact, some have been reported to be neurotoxic such as laetrile extracted from apricot kernel and have contraindications with medications (Sanders *et al.*, 2016).

More importantly, plants are the major source of cancer therapeutic drugs with 77.8% drugs in cancer treatment being naturally derived or inspired (Newman *et al.*, 2015). Examples include paclitaxel (Taxol) from *Taxus* spp. to treat ovary and breast cancer; vincristine from Madagascar periwinkle *Catharanthus roseus* to treat leukemias and lymphomas; and camptothecin from *Camptotheca acuminate* to treat ovarian cancer and recurrent small-cell lung cancer. These natural compounds inspire the synthesis of analogues with improved efficacy including doxetazel, ortataxel, vindesine, vinflunine, belotecan and irinotecan (Newman and Cragg, 2016). Nevertheless, recent occurrence of tumor cells gaining single- and multi-drug resistance as well as adverse side effects of current drugs necessitates a search for new drugs (Levitsky and Dembitsky, 2015).

1.10 Cytotoxicity assays

Many methods have been developed to evaluate the viability and proliferation of cancer cells. The simplest method is trypan blue dye exclusion assay which is the direct counting of viable cells using a hemacytometer. It is simple and expensive but time consuming and sometimes inaccurate (Kanemura *et al.*, 2002). Other methods include MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium))-based assays and direct measurement of enzymes, ATP or DNA.

1.10.1 MTT assay

Screening of the anti-proliferative activity of the plant extracts against MCF-7 breast cancer cells was determined using MTT assay. MCF-7 breast cancer cells were chosen as breast cancer is the most common cancer in the world and in Malaysia. The Malaysian National Cancer Registry reported that breast cancer has the the highest percentage of cancer incidence from 2007 to 2011 being 17.7% among all cancers of residents with the highest age-standardized rate (ASR) which was 31.1 per 100,000 population (Manan *et al.*, 2016). The highest incidence occurs in females of 25-59 accounting for 40.7% of most common cancers in females, followed by cervix uteri (8.9%) and colorectal cancer (7.6%).

The MTT assay is a colorimetric method to determine cell viability. The tetrazolium MTT dye is reduced to insoluble purple formazan crystals by mitochondrial dehydrogenase enzymes in the succinate-tetrazolium reductase systems (Wu *et al.*, 2008). The formazan is then solubilizes using an organic solvent. With assumption that the enzymes are only active in viable cells, the color intensity or the amount of formazan generated is theoretically directly proportional to the number of living cells. The absorbance of the plant extract treated cells is compared to their respective control with a lower absorbance from that of control denotes a decrease in cell viability. The results are expressed in IC_{50}, the concentration of compound where 50% cells are viable (Wu *et al.*, 2008).

MTT assay was chosen as prior testing of the crude extracts using MTS assay was noted to be inaccurate whereby absorbance reading did not correlate with observations under the microscope. It is also inconsistent with direct cell counting using trypan blue assay. It was found that the plant extract itself interacts with the MTS reagent directly given erroneous results. This is circumvented in MTT, where the extracts were removed prior to addition of the MTT reagent.

The assay is simple and fast for screening of potential anticancer compounds. As MTT measures mitochondrial metabolic rate directly, different conditions and chemical treatments that affect the metabolic activity of dehydrogenase enzymes can cause variation in results. For instance, polyphenols may interfere with the formazan formation in MTT possibly by increasing mitochondrial enzyme activity or an intrinsic potential to reduce MTT reagent as in the case of EGCG from green tea (Wang *et al.*, 2010). MTT is also less sensitive compared to ATP or DNA quantification assays. It underestimates the anti-proliferative effect of compounds by approximately twofold. Moreover, the MTT-formazan solubilization process prior to quantification is not only troublesome but also error-prone (Goodwin *et al.*, 1995). Results can also be affected if substances that cause cell death are present such as heavy metals, sodium and calcium channel poisons making it difficult to distinguish between toxic versus anti-proliferative substances (Olivieri *et al.*, 2000). Lastly, results of *in vitro* cytotoxicity assays cannot be extrapolated to *in vivo* studies due to numerous issues. For instance, a 50% decrease in MTT absorbance indicates fewer cells but does not necessary mean cells have lost their viability. The drug metabolism, bioavailability, and treatment parameters are also dissimilar to that of real life treatment (Eastman, 2016).

1.11 Natural products research

The strategies employed in natural product research continued to evolve over time. Nevertheless, they can be divided into two broad categories (Sarker & Nahar, 2012). Older strategies focus on chemistry of compounds but not activity. Compounds from natural sources are directly isolated and identified before biological activity testing. Selection of organisms is based on ethnopharmacological information, folklore, or traditional uses as well as chemotaxonomic investigation. On the other hand, modern strategies utilize bioassay-guided isolation and identification of active 'lead' compounds from natural sources with the focus predominantly on bioactivity. Figure 2 shows a generic protocol. Selection of organisms could be random or based on ethnopharmacological information, folklore, or traditional uses. It can involve production of active compounds in cell or tissue culture, natural combinatorial chemisty, genetic manipulation and dereplicated "natural product libraries" for high throughput screening (HTS) (Sarker & Nahar, 2012).

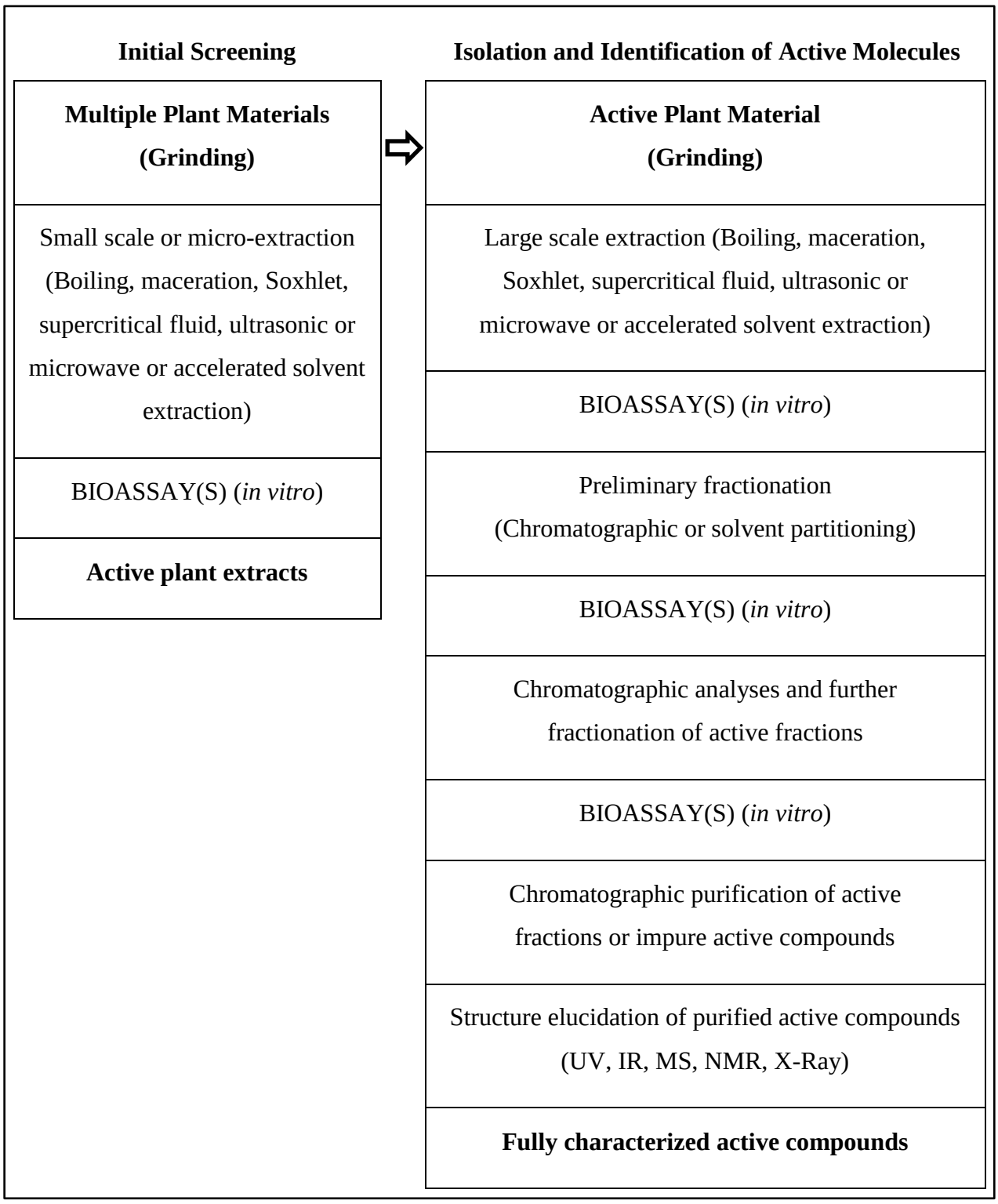


Figure 2. A generic protocol of bioassay-guided approach using plants (Sarker & Nahar, 2012 with modifications).

Two major issues are commonly associated with the isolation and purification of compounds from natural products. Poor yield and poor recovery due to active compound present in low concentration. For example, 15 tons of dried leaves of *Vinca rosea* produce

only 30g of vincristine (Farnsworth, 1990). The second is reduced or loss of bioactivity after the isolation process. Possible reasons could be: (a) active compound retained in column chromatography; (b) compound unstable or degraded during isolation; (c) active components spread over too many fractions leading to concentration too low to be detected by bioassay; (d) compound extracted using incompatible solvent leading to active components precipitated out during loading of column; and (e) activity of extract is due to synergistic effect of a number of compounds and when separated, individual compounds are inactive (Sarker & Nahar, 2012).

This is the reason natural product research focuses on identification of 'lead' compounds rather than being simply large scale-extraction of active compounds. Knowledge of their structure enables problems of poor yield to be solved easily using semisynthesis or synthesis of the compound by combinatorial chemistry and that of poor activity through alteration of the chemical structure to produce more stable and active compounds. Genetic manipulation of source and usage of tissue or cell culture production may also help to increase yield (Sarker & Nahar, 2012). The various steps in natural product research: extraction, isolation, and structural identification with their associated techniques are reviewed in detail in the following sub-sections.

1.12 Extraction of source materials

Natural products have to be released from their surrounding biological matrix before isolation and purification work. Typically, a small amount of material is first extracted to conduct preliminary studies to detect the phyrochemicals it contains or measure its bioactivity. If the extracted material seems promising, bulk extraction is performed (Seidel, 2012).

A typical extraction process involves several steps. The first is the drying and grinding of plant material or homogenizing or maceration of plants with a solvent. The second is choosing the extraction procedures to be undertaken. Solvent-based procedures include boiling, maceration, percolation, Soxhlet extraction, supercritical fluid, reflux, steam distillation, and ultrasound-assisted solvent extraction (Seidel, 2012). Although these procedures lack reproducibility as well as time- and solvent-consuming, they are widely used due to convenience as they only require basic glassware. The last step is to choose the solvents to be used for extraction. This depends on the nature of the compounds of interest. The aim is usually to maximize the yield of compounds obtained and to remove undesirable compounds. Solvents are chosen based on their polarity: water, ethanol and methanol for polar compounds extraction (e.g. flavonoids); ethyl acetate and dichloromethane for medium

polarity extraction (e.g. alkaloids); and hexane, petroleum ether or chloroform for nonpolar compounds extraction (e.g. steroids) (Seidel, 2012).

Extraction of plants using water provides useful information particularly if the investigated plant is used in traditional medicine or commercially marketed as tea. This is because herbal medicines made from plants are often infusions (steeping in water) or decoctions (extract in boiling water) (Jones & Kinghorn, 2012). *A. malaccensis* leaves are commercially sold as 'gaharu' tea purportedly possessing numerous therapeutic benefits. Scientific investigation into aqueous extracts could help to support or invalidate these claims of beneficial consumption.

However, in natural products research, organic solvents are generally preferred. They offer several advantages. Firstly, organic solvents usage prevents microbial growth, a common problem associated with aqueous extraction. Secondly, they are easier to evaporate than water (Jones & Kinghorn, 2012). Thirdly, employing organic solvents of varying polarities enable targeted extraction of compounds based on their polarity. Sequential extraction using solvents of different polarity can ensure a more complete extraction and also removal or undesirable components (Seidel, 2012). Lastly, organic solvents such as alcohols can cause cell membrane instability facilitating extraction of phenolic compounds and inactivate enzymes that oxidize phenolic compounds resulting in improved stability of the extracted compounds (Hurst, 2008).

1.13 Isolation of compounds

Crude plant extracts consist of a complicated mix of numerous compounds. A single separation technique is insufficient to isolate a pure single compound as many compounds have similar properties. Therefore, the crude extract was initially fractionated based on polarity or molecular sizes using techniques such as liquid-liquid extraction or column chromatography (Sarker & Nahar, 2012). Although collecting large number of very small fractions means each fraction is likely to contain a pure compound, there are disadvantages. More work is required given the large number of fractions to be analyzed. More importantly, there is the risk of target compound being spread over too many fractions. Fractions containing target compounds in low concentration may not showed detectable activity in bioassays (Cannell, 1998). Therefore, a few large relatively crude fractions are collected and bioassays performed to single out fractions with bioactivity in initial fractionation. Active fractions are then separated again where larger numbers of fractions are collected.

Isolation protocol depends on target compounds' properties such as solubility, charge, polarity, molecular size and acid-base properties. When compounds are unknown, preliminary profiling using qualitative phytochemical tests to test for the presence of classes of compounds such as alkaloids, flavonoids, and steroids may help in choosing an appropriate protocol. Monitoring the bioactivity after each stage of fractionation or purification in bioassay-guided isolation enables one to focus on extracting bioactive compounds as well as detect whether activity is due to single or multiple components (Sarker & Nahar, 2012).

1.13.1 Chromatography

Chromatographic techniques are often used in the isolation of compounds. Older techniques include flash chromatography, column chromatography, size exclusion chromatography, ion-exchange chromatography, thin layer chromatography (TLC) and preparative thin layer chromatography. Chromatotron, solid-phase extraction, high performance thin layer chromatography, high performance liquid chromatography (HPLC), and liquid chromatography coupled with mass spectrometer (LC-MS) or nuclear magnetic resonance (LC-NMR) are more modern techniques (Sarker & Nahar, 2012).

The separation in chromatography is based on the difference in distribution of a compound or solute between two phases: a moving mobile phase passed over an immobile stationary phase (Cannell, 1998). Analytes can be introduced by direct application on stationary phase or carried by mobile phase. As the mobile phase passes through the stationary phase, compounds migrate in different rates through the system depending on their strength of interaction with both phases and are eluted from the column at different times called retention time (Gibbons, 2012). For example, when the system employs a stationary phase with higher polarity than mobile phase, polar compounds in the analytes migrates a shorter distance as they are retained by the stationary phase, resulting in a longer retention time compared to less polar compounds.

Column chromatography involves packing of column with stationary phase of choice such as silica with particle sizes ranging from 40-200µm. Sample is applied to the top of the column and then covered with a layer of sand before addition of solvent. As the solvent flows through the column under gravity, fractions are collected by volume or bands (Reid & Sarker, 2012). A solvent step gradient method was employed where solvents mixture of increasing polarity was used to elute out the sample.

TLC was used to analyze individual fractions collected from column chromatography. Fractions with similar TLC migration patterns are pooled together. TLC consists of thin-layer

of adsorbents coated on glass or aluminium sheets. Plant extracts are spotted at the bottom of TLC plates and placed inside a tank containing solvent. As the solvent moves up the plate through the sorbent by capillary action, the extract is separated into components according to their relative polarity. Polar compounds move slower than nonpolar compound in silica TLC (Sarker & Nahar, 2012). This can be visualized under UV light or spraying the plate with suitable stains that give a color reaction such as sulfuric acid and ninhydrin (Gibbons, 2012). This method is easy, rapid and inexpensive (Sarker & Nahar, 2012).

1.13.2 High performance liquid chromatography

A high performance liquid chromatography (HPLC) system (Figure 3) consists of several components: mobile phase reservoir, autosampler or injection system, degaser, pumps, column, detectors, fraction collector and a system controller or computer. Detectors are usually ultraviolet/visible (UV-vis), refractive index (RI) and evaporative light scattering (ELS). This technique is simple, fast, sensitive, specific, reproducible, and compounds can be recovered. There is also high versality where stationary phase, mobile phase and detectors can be varied depending on requirements (Latif & Sarker, 2012).

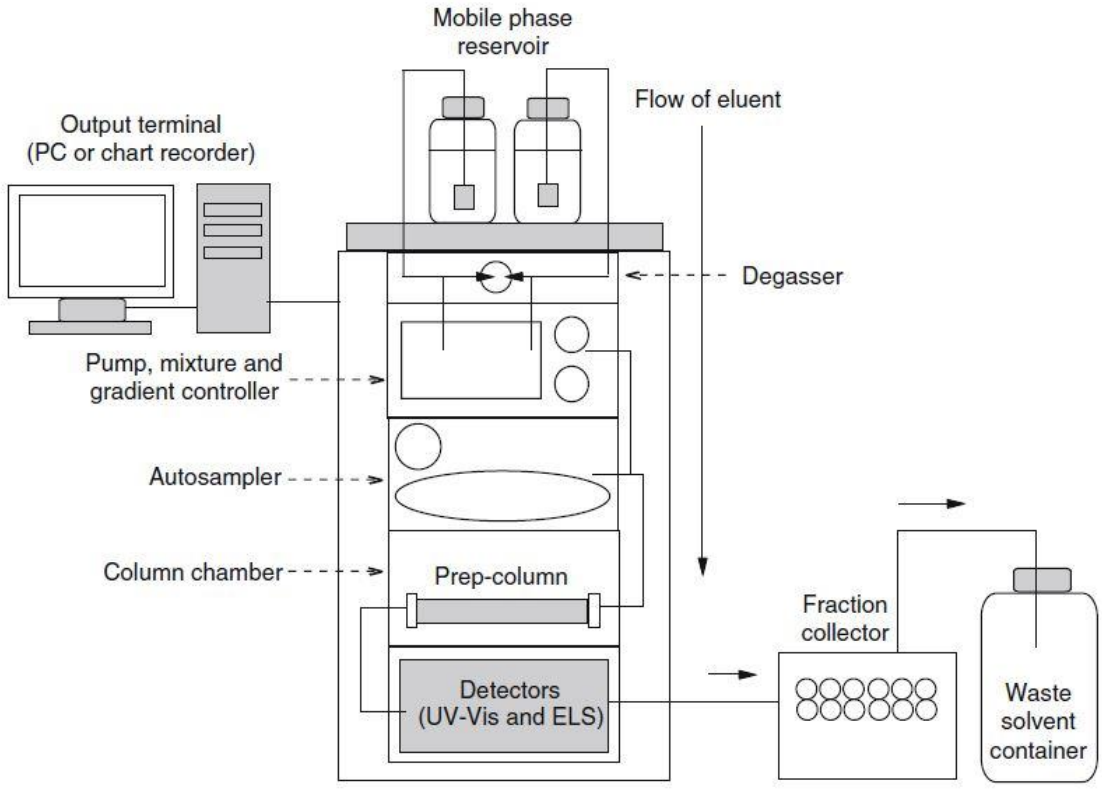


Figure 3. A typical HPLC system (Latif & Sarker, 2012).

HPLC affords better separation compared to column chromatography mainly due to the smaller particle size of stationary phase typically 3-10µm. A smaller particle size means greater surface area available and more number of interactions between solutes and the stationary phase. This leads to better separating power or higher resolutions between two compounds in a sample mixture. In HPLC columns, the synthesized particles are spherical with narrow size distribution allowing it to be tightly packed in a highly uniform and reproducible manner. This minimizes the occurance of voids that can disrupt mobile phase from travelling uniformly through the stationary phase and lead to inefficient separation. Due to the small particle size, a high pressure is required to push the mobile phase through the system (Latif & Sarker, 2012).

Deciding the type of column to be used depends on the quantity of natural product extracts to be isolated and purified as well as the nature of the target compound. Analytical columns (internal diameter 4.6mm) are used for microgram quantities while greater quantities necessitate the use of preparative HPLC (prep-HPLC) (internal diameter 10-100mm) (Latif & Sarker, 2012). Analytical HPLC are used for initial analysis of the compound mixture and to establish a suitable solvent system and method before scaling up to prep-HPLC. Prep-HPLC was used to separate compounds and purify. This is crucial as large quantities of purified compound are required for repeated bioassays testing and to carry out analysis for structure elucidation like LC-MS/MS and NMR (Latif & Sarker, 2012).

The choice of column packing or stationary phase whether normal phase, reversed-phase, ion exchange or gel permeation chromatography (GPC) was decided based on compatibility with the extracts and solvents of choice. Normal phase columns have a polar stationary phase and less polar eluting solvents. More polar compounds have stronger adsorption to stationary phase and will be eluted after less polar solvents, having a longer retention time. It is suitable for isolation of lipohilic compounds, long chain alkanes, and when extract mixture is only slightly soluble in aqueous conditions (Latif & Sarker, 2012).

Reversed-phase HPLC (RP-HPLC) is the opposite of normal phase where stationary phase is more nonpolar than mobile phase. The most common is C18 octadecyl derivatised silica which is able to separate most classes of compounds. Eluants are commonly mixture or water with miscible organic solvents like acetonitrile and methanol (MeOH). Buffers, acids and bases can be added to reduce peak tailing and improve chromatogram as they are able to control the degree of ionization of compounds and free unreacted silanol groups (Latif & Sarker, 2012).

GPC is predominantly used for proteins and oligosaccharides purification. It has a hydrophobic and inert stationary phase like macroporous polystyrene/divinylbenzene copolymers. Also known as size exclusion chromatography, compounds are separated by their ability to enter pores. Smaller molecules enter the pores and are temporarily 'trapped', thus having longer retention times than larger molecules (Latif & Sarker, 2012). Ion exchange chromatography separates acids and amines using anionic or cationic stationary phase. Compounds with net charge bind reversibly to ionizable groups on stationary phase. They are then eluted through displacement of a stronger ionized species in the eluent. As both ion exchange and GPC requires prior knowledge of chemical nature of sample mixture, normal phase and reverse-phase are commonly chosen as the "first line method" to isolate unknown natural products. Reverse-phase columns are often preferred over normal phase columns as they give more stable performance and better compound recovery (Latif & Sarker, 2012).

To identify and isolate a compound, an analytical analysis is first carried out to access the number of compounds present in the extract fraction. A gradient analysis was conducted where sample was injected into mobile phase that is highly aqueous (e.g. 5% MeOH in H_2O) and the organic proportion was increased over time (until 100% MeOH) to elute out all compounds (Latif & Sarker, 2012). This is advantageous as complex mixtures over a range of polarites can be separated in a single separation and gradient analysis sharpens the chromatographic bands giving better separation of closely eluting solutes (Cannell, 1998). Based on gradient analysis and the solvent composition where the peak of the compound of interest appears, the best isocratic elution method was developed. Solvent mixture is kept constant throughout the run in isocratic conditions. Isocratic runs are not suitable if compounds elute over a broad range. In this case, truncated gradient were used where the solvent system for elution is adjusted to when the first peak appears till the last peak is eluted (e.g. 5-40% MeOH) (Latif & Sarker, 2012). Fractions are collected by peak.

Optimization of HPLC to obtain optimal peak shape and good separation involves changing the solvent system, solvent proportions, type or length of column, flow rate, runtime, amount of sample loaded, and addition of buffers, bases or acids to sharpen peaks. Each variable was adjusted one at a time to achieve the best separation profile. In cases of broad peaks (Figure 13, sub-fraction 22-3), where several compounds have close retention times and could not be separated further, the leading and tail end of the peak and the center merged portions are collected as different fractions and individually analysed (Latif & Sarker, 2012). After fractions have been collected from HPLC, the solvents are removed by rotary evaporator with further freeze drying to remove the aqueous part of the solvent mixture.

1.14 Structural elucidation and identification

Structural elucidation of natural products is a time-consuming process and the 'bottleneck' in natural product research as interpretation of spectra requires special skills and knowledge (Sarker & Nahar, 2012). A known compound can be identified by comparing spectroscopic data with literature data or direct chromatographic comparison with the standard sample. In contrast, a combination of physical, chemical and spectroscopic data analyses is necessary to determine the structure of an unknown compound. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are spectroscopic techniques often used to analyse and determine compound structure.

1.14.1 Nuclear magnetic resonance

A NMR spectrometer consists of several components: sample compartment, a powerful magnet, a radio-frequency (RF) source, a detector, and a system controller (Figure 4). Sample is prepared in a glass tube using deuterated solvents and inserted in the external magnetic field, B_0. A RF coil or transmitter produces radiofrequency radiation, spins are excited and the stimulated signal is detected by the receiver. The resonance signal is then amplified and transmitted to a computer where spectrum can be recorded and resonance frequencies can be measured (Gunther, 2013). There are two methods: continuous wave (CW) and fourier-transform (FT). Currently, FT method is used exclusively in NMR where the RF pulse which has a broad frequency spectrum generates a strong RF field (50W) of short duration (typically 10-50 μs) to excite the nuclei.

NMR can be one-dimensional (^{1}H NMR, ^{13}C NMR and ^{13}C DEPT) or two-dimensional (COSY, HMBC, HSQC). NMR provides information on the number and types of protons, carbons and other elements like nitrogen and fluorine present in the compound and the relationship between these atoms (Sarker & Nahar, 2012). This technique is simple, fast and compounds can be recovered. However, it is less sensitive compared to other spectroscopic methods as the most abundant isotope such as ^{12}C is not NMR active and ^{13}C which is active is only 1% of the sample (Gunther, 2013). Other issues associated with NMR are that adequate material is required for generation of NMR data and the lack of ready access to NMR-based database (Gunther, 2013).

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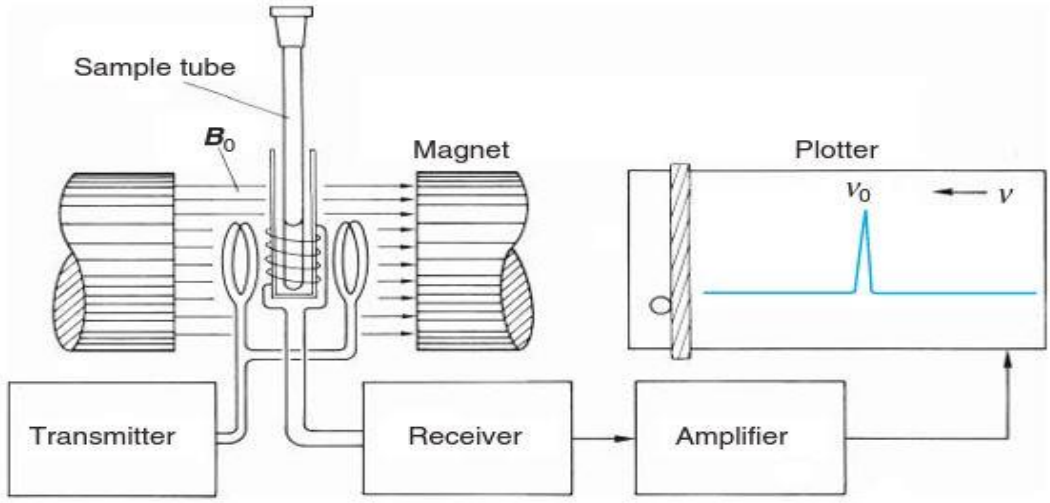


Figure 4. Schematic diagram of NMR spectrometer (Gunther, 2013).

NMR spectroscopy probes the magnetic properties of atomic nuclei induced by their spin states using powerful magnets (Gunther, 2013). Magnetically active atomic nuclei are usually nuclei with odd numbers of protons or neutrons. The overall spin of atomic nuclei with magnetic moments is determined by the spin quantum number, *I*, of the nuclei. Generally, nuclei with even atomic mass and atomic number, the even-even nuclei, has $I = 0$ and all other nuclei $I \geq ½$; *I* is an integral multiple of 1 for even–odd nuclei and for odd–odd and odd–even nuclei it is an integral multiple of ½. The total number of possible eigenstates (spin states) of energy levels is equal to $2I + 1$. For example, compounds containing proton (^{1}H), carbon-13 (^{13}C), nitrogen-15 (^{15}N), fluorine-19 (19^{F}) and phosphorus-31 (^{31}P) have nuclear spins of ½ (Gunther, 2013). Acting as tiny magnets, their magnetic vectors align in an external field into only two eigenstates, either parallel or antiparallel to the field. There is a small energy difference between the parallel and antiparallel orientations which can be visualized by irradiation with proper radiofrequencies (Vogler & Setzer, 2006).

Besides strength of magnet, the frequency of nuclei transitions also depends on chemical environment of the nuclei and their molecular structure. Electrons which form the chemical bonds of molecules produce different local magnetic fields, and hence, resonate at different frequencies. This is called chemical shift δ and is measured in ppm. This scale is frequency-independent as they are calculated against the proton signal of a reference compound, tetramethylsilane (TMS) which was selected as an artificial start point. A typical NMR spectrum gives several informations. Firstly, the type of protons present. Magnetically equivalent protons (^{1}H) will have the same chemical shift or position in the spectrum. As different functional groups have different range of ^{1}H chemical shift (Figure 5), the type of

compound can be inferred. Second, number of protons of a compound can be estimated from area integration as signal intensities (peak area) is proportional to the number of protons giving rise to the signal. Lastly, the splitting pattern of a signal called spin-spin coupling provides information on the number of chemically equivalent neighboring proton. Neighbouring nuclei two or three bonds away spins and produces local magnetic fields which influences the nucleus in question and splits their signal (Gunther, 2013; Vogler & Setzer, 2006).

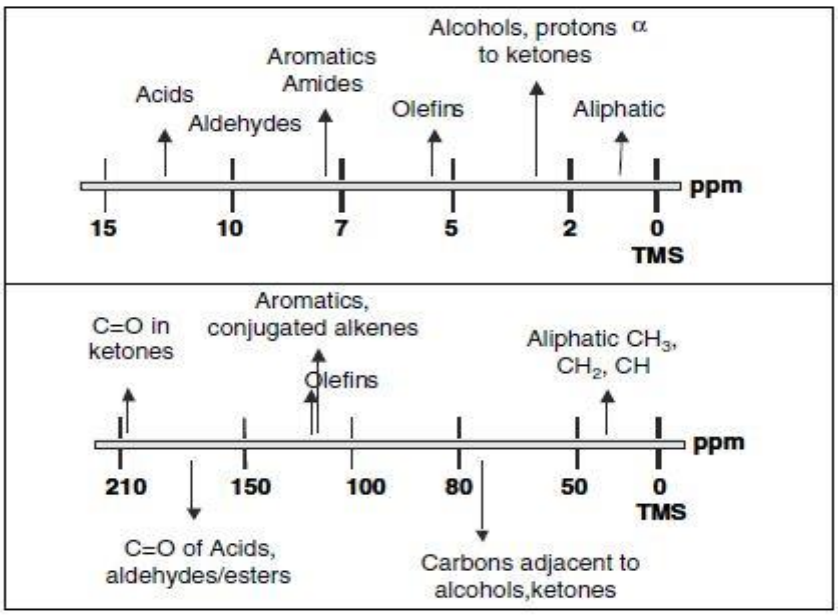


Figure 5. Typical ^{1}H shift ranges for various functional groups (Vogler & Setzer, 2006).

NMR analyses of ^{13}C spectra are much easier than ^{1}H spectra, being only single lines due to proton decoupling. The number of carbon can be deduced by the number of signals, usually one carbon per signal. However, equivalent carbons will give overlapping signals. The second information is the type of carbon as different carbons have different chemical shifts (Vogler & Setzer, 2006). Distortionless enhancement through polarization transfer (DEPT) is a ^{13}C NMR technique that provides extra information beyond normal ^{13}C spectra. Depending on the number of protons attached, signals are viewed as positive or negative depending on the flip angle (45°, 90° and 135°) of the pulse. All signals CH, CH_2 and CH_3 are positive in DEPT-45, only CH signals appear in DEPT-90, and CH_2 negative while CH and CH_3 positive in DEPT-135 (Vogler & Setzer, 2006).

Two-dimensional (2D) NMR technique was developed as the relatively small chemical shift differences in ^{1}H-NMR spectroscopy causes signal overlap and difficulties in spectra analysis of large and complex molecules (Vogler & Setzer, 2006). Correlation

spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC) and heteronuclear multibond correlation (HMBC) are the most common 2D techniques. In ^{1}H-^{1}H-COSY, different protons in spectrum that are coupled to each other are correlated. COSY 2D spectrum has one axis displaying chemical shifts of proton plotted against a second axis of chemical shifts of proton where "cross peaks" appear when there is coupling. HSQC detects correlations between proton and carbon (^{13}C-^{1}H) separated by one bond whereas HMBC detects ^{13}C-^{1}H couplings over more than one bond. Their 2D spectrum has one axis displaying proton against a second axis displaying carbon chemical shifts. They offer the advantage of less overlap between signals as the large chemical shift range of carbon spreads out the proton spectroscopic information (Vogler & Setzer, 2006).

1.14.2 Mass spectrometry

A mass spectrometer consists of several components: ion source, analyzer, detector and system controller. A mass spectrometer produces charged particles (ions) from the chemical substances that are to be analysed by electron ionization. These ions become fragmented due to their high energy. The fragmentation is controlled by the different stabilities of cations and anions produced, and thus, provide information on the structure and chemical properties of the compound. The mass (weight) of these newly generated ions are then measured using electric and magnetic fields by the analyzer. The ions are separated according to their mass-to charge ratio (*m/z*) and detected in proportion to their abundance (Vogler & Setzer, 2006).

A problem associated with MS is that molecular ions are hard to produce in many cases leading to difficulty in determining molecular formulas of unknown compounds. Interpretation of MS spectra is more complex than NMR spectra despite MS being more sensitive than NMR. Nevertheless, MS is a powerful technique and versatile. They can be combined with chromatographic techniques such as liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS) giving both qualitative and quantitative results (Vogler & Setzer, 2006).

There are many techniques on process of ion generation but only electron impact ionization (EI) and electrospray ionization (ESI) will be discussed in detail. Typically, the method of choice is EI for volatile compounds and ESI for polar and non-volatiles. In electron impact ionization (EI), a beam of electrons passes through the gas-phase sample and collides with the neutral analyte molecule. This collision can knock off an electron from the analyte molecule, resulting in a positively charged radical ion. The ionization process can produce

molecular ions with the same molecular weight and elemental composition as the starting analyte or produce fragment ions which are smaller pieces of the analyte. EI mainly uses energy of 70 electron volts (eV). Reducing the electron energy reduces fragmentation and the number of ions formed. EI mass spectra are reproducible. Thus, searching libraries of mass spectra using fragmentation patterns gives structural information of a compound and aids in identification. The mass range of sample is typically less than 1000 Da as the samples must be volatile and higher molecular weight compounds can not generate ions (Vogler & Setzer, 2006).

In electrospray ionization (ESI), solution containing the analyte is sprayed at atmospheric pressure into an interface to the vacuum of the mass spectrometer ion source. As they enter the ion source, the sample is desolvated to ions using heat and gas flows (e.g. nitrogen). ESI produces multiply charged ions. Typically, increases in molecular weight increases the number of charges. The mass range of sample can be up to 200,000 Da (Vogler & Setzer, 2006). There are two modes of analysis. The negative ion mode and positive ion mode produces deprotonated molecular ions ($[M-H]^-$) and protonated molecular ions ($[MH]^+$) respectively (Banerjee & Mazumdar, 2012). ESI are most widely used in flow injection and LC-MS techniques for analyses of polar and non-volatile molecules. It is not a good method for uncharged, non-basic, and low-polarity compounds such as steroids. In the case where less polar compounds are present, atmospheric pressure chemical ionization (ACPI) which has a similar interface to ESI but with gas-phase ionization should be the method of choice (Vogler & Setzer, 2006).

The mass analyser component in MS sorts the fragmented ions according to their *m/z*. Most commonly used are quadrupole and time-of-flight (TOF) mass analyzers. A quadrupole mass analyser 'filters mass'. It consists of a pair of rods with a positive potential and a pair of rods with a negative potential. One pair selects molecular weight higher than a threshold, whereas the other selects for a mass lower than a certain threshold. In other words, the analyser only permits ions of certain mass-to-charge (*m/z*) ratios to pass through these quadrupole rods to reach the detector. On the other hand, TOF measures the time it takes ions of different *m/z* to move from the ion source (pulsed ionization) to the detector. Ions with similar charges have similar velocity when electric field is applied, and thus, the time taken to reach the detector is mass-dependant (Vogler & Setzer, 2006).

Tandem mass spectrometry (MS/MS) uses two mass spectrometers to provide additional structural information. In the first mass spectrometer, ions are generated by the ion source and separated according to their *m/z*. Ions of a particular *m/z* are then selected to be

investigated. The selected ions are passed into a collision chamber where collisions with a gas results in further fragmentation generating 'daughter ions' which are analysed in a second mass analyser (Vogler & Setzer, 2006).

Although fragmentation patterns can be diagnostic and helpful in recognizing a compound by comparing spectra to reference libraries, MS data with regards to structure is not conclusive. This is because these techniques involve soft ionization which can yield adductions (e.g. $[M + Na]^+$ and $[M + K]^+$ ions) (Blunt *et al*., 2016). In summary, MS approach provides information on the mass of fragments produced but is not as helpful as the direct structural information that can be extracted from NMR data. Therefore, both NMR and MS are usually carried out to elucidate the structure compound rather than only performing either analysis alone (Blunt *et al*., 2016).

1.15 Rationale and significance of research

Plants play a crucial role in the search for new pharmacologically active drugs. Identification and knowledge of the structure of these compounds and their association with any therapeutic benefits will be useful as potential leads for production of synthetic drugs. Interestingly, only 6% and 15% of 300,000 species of terrestrial flora have been investigated pharmacologically and phytochemically respectively representing a great untapped potential of new source materials (Cragg & Newman, 2013). Malaysia is particularly promising in this aspect with 85% of more than 250,000 species of higher plants remaining unexplored with regards to their therapeutic potential (Chew *et al.*, 2011). Plants of Malaysian rainforests are particularly promising as anticancer agents. In one study, 143 crude extracts from 32 were screened. Out of these, 13 crude extracts from 11 plant species showed anti-proliferative activity of IC_{50} < 0.1mg/ml against MCF-7 breast cancer lines (Nurhanan *et al.*, 2008). Similarly, this study also focuses upon breast cancer which is among the top ten cancer-related deaths in Malaysia. In this research, several Malaysian plants used in traditional medicine were examined to investigate their pharmacological properties. The plant with the best bioactivity was selected followed by separation, isolation and extraction of biologically active compounds. These compounds were then characterized and identified.

Several relevant justifications underlie the need to identify chemical components present in a plant. Interactions between herb-herb or herb-drug can occur and may lead to undesirable side effects. This is because herbal medicinal prescriptions generally comprise of a mixture of medicinal plants and medicinal plants as dietary supplements are often used concomitantly with prescribed drugs (Laird, 2011). Therefore, knowledge of the chemical

constituents of medicinal plants may enable one to ascertain the level of safety and efficacy of the medicinal concoction when used in combination with other herbs or concurrently with therapeutic drugs as well as enable the prediction of possible alteration in drug effects.

Understanding of the chemical composition can also lead to discovering how a therapeutic effect could be achieved and their underlying mechanism. For instance, multiple active compounds are present in plants. Hence, their therapeutic benefit might be attributed to a potentiating or synergistic effect between various components (Fugh-Berman and Ernst, 2001). Separating out each component enables determination of which compound gives rise to their bioactive properties. On the other hand, it is also possible that a single bioactive compound can give more potent effects compared to whole plant extracts. After all, most drugs usually contain single chemical entities (Fugh-Berman and Ernst, 2001). Identification of important pharmaceutically active compounds may be informative and useful in the development of newer drugs though combinatorial chemistry (Koehn and Carter, 2005). In addition, the active compound can be used as a possible marker to assess and evaluate the quality of plants in terms of therapeutic benefit. In other words, quantification of the active components in the same plants may enable grading and classification of the plants based on their quality, efficacy, side effects and toxicity (Liu *et al.*, 2017). This is particularly important as in traditional medicine; plants are generally used without consideration of quality or quantity unlike dose-dependent commercial drugs.

1.16 Objectives and scope of research

The main objectives of the present study were as follows:

i. To screen the antioxidant, antimicrobial and cytotoxic activities of *Aquilaria malaccensis*, *Fagraea elliptica*, *Fagraea gardeniodes*, and *Fagraea racemosa* extracts.
ii. To identify the plant with the best bioactive extract in which this extract would then be selected as the primary fraction for isolation and purification work.
iii. To isolate and purify the chemical constituents from the chosen plant through bioassay-guided fractionation technique.
iv. To identify the chemical structures of bioactive compounds using spectroscopic methods including HPLC, LC-MS and NMR

2.0 LITERATURE REVIEW ON PLANTS OF INTEREST

Initial screening of the bioactive properties will be focused on four plants, whereby three plants are from the same *Fagraea* genus. Each plant, as listed below, is accompanied by a description of its purported health benefits and biological properties based on existing literature. It should be noted that for plants where background literature is significantly lacking or absent, chemotaxonomical data is described instead. Chemotaxonomical data are important as they enable the deduction of compounds contained in the plants of interest by investigating compounds contained in plants related to it by species or genera.

2.1 *Fagraea* species

Fagraea is a genus of plant in the family Gentianaceae, formerly controversially classified as Loganiaceae (Sugumaran and Wong, 2012). They are paleotropical, distributed from Sri Lanka and India, southern China, South East Asia to the archipelagos of the Pacific Ocean and Northern Australia. The genus with over 70 species is centred in tropical Malesia with 50 species found in Peninsula Malaysia and Borneo (Sugumaran and Wong, 2012). They are shrubs or small trees mostly found at sea level to altitudes of 3000m in moist montane conditions such as along stream beds in wet tropical forests, rocky outcrops and forest gaps and edges (Motley, 2004).

Most *Fagraea* species have coriaceous, fleshy, and entire oblong-shaped leaves; funnel-shaped and fleshy flower corollas which turn yellow to orange with age; and pale green fruits that turn orange to red at maturity and contain black seeds (shown in Figure 6). Reknown for their hard durable wood and showy flowers, their wood is often used as timber, flowers used in perfumes and to make flower leis, and the sticky latex beneath the skin of the fruits are used as adhesive (Fern, 2014a; Motley, 2004).

The three plants examined in this study are *Fagraea elliptica*, *Fagraea gardenioides and Fagraea racemosa. F. racemosa* has many local Malesian names including sepuleh (restorer), setebal (referring to their thick leaf), lidah rusa (deer's tongue), kopi hutan (woodland coffee), kahawa hutan (woodland coffee), tahi musang (civet cat's droppings) and false coffee tree due to their resemblance to the Indian mulberry coffee family (Motley, 2004).

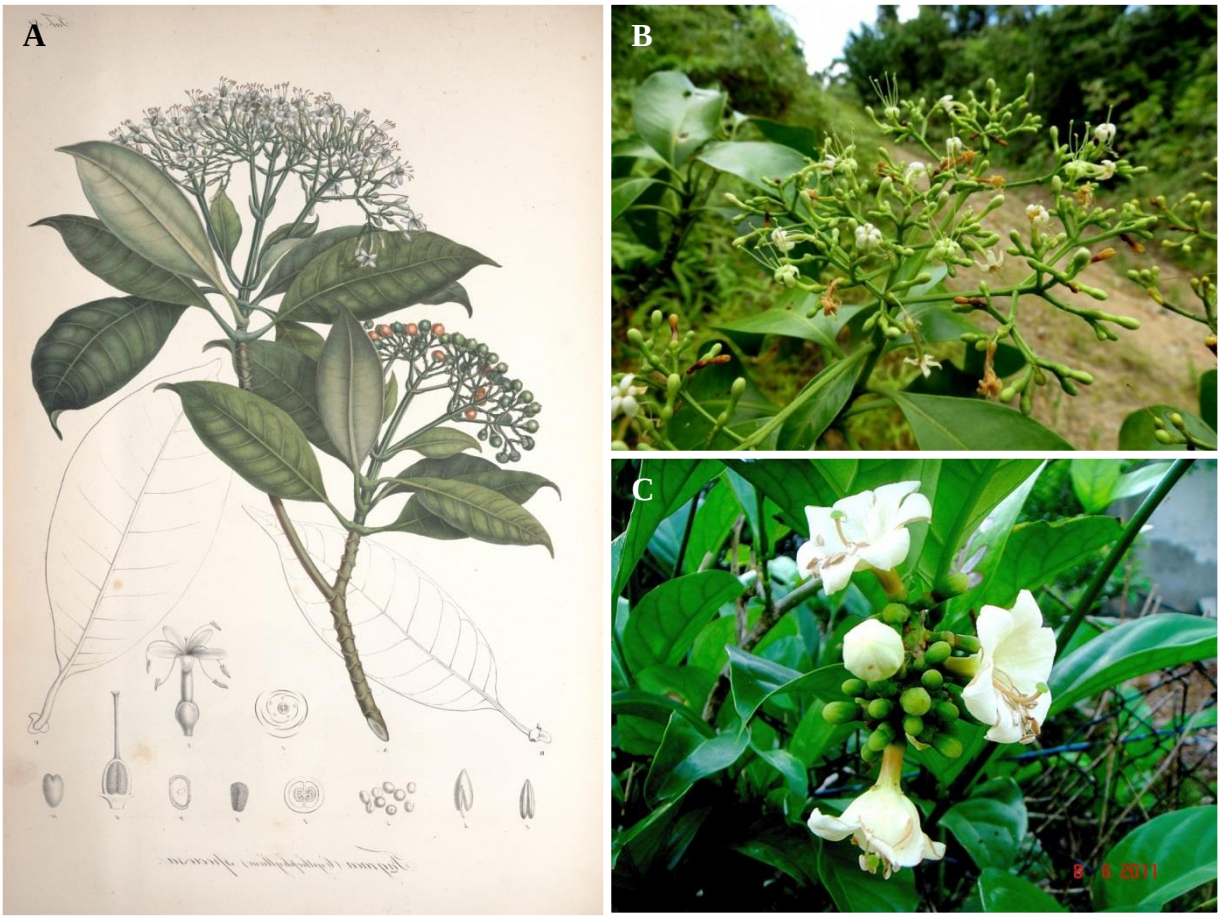


Figure 6. Selected species of *Fagraea.* **A.** Drawing of leaves and fruits of *F. elliptica*; **B.** *F. elliptica* inflorescences; **C.** *F. racemosa* inflorescences (Fern, 2014a).

2.1.1 Traditional medicine

Several *Fagraea* species are used in traditional medicine by indigenous people mainly to treat fever, pain, malaria and splenomegaly. Table 3 provides a detail compilation of the usage of *Fagraea* in traditional medicine based on regional culture and folklore (Grosvenor *et al.*, 1995; Leaman *et al.*, 1995; Motley, 2004; Suciati *et al.*, 2011). Their widespread usage by native people to treat various illnesses suggests great potential as therapeutic agents and consequently *Fagraea* plants are selected for this research study.

Table 3. *Fagraea* species used in traditional medicine

Location	Species	Folklore and traditional medicine
Asia		
Cambodia	*F. fragrans*	Bark infusion drunk by elderly to prolong life
China	*F. fragrans*	Pounded bark infusion used to wash scabies
India	*F. fragrans*	Plant emission of volatiles purportedly cleanse surrounding air by neutralizing the effects of carbon monoxide or ammonia gases
	F. racemosa	Bark and root infusion used to reduce fever
Malesia		
Borneo	*F. fragrans*	Leaves and fruits used to treat fever
Java	*F. blumei* & *F. obovata*	Leaves infusion used to reduce fever and headaches
Malaysia	*F. fragrans*	Leaves and twigs decoction used to treat dysentery, associated bleeding, and malarial fever
	F. racemosa	Root decoction to treat fever, coughs, sciatica and pain of the loins
		Pounded root to treat ulcerated noses
		Leaves to treat dropsy, rheumatism and fever
		Leaves grounded and mixed with tobacco to relieve head colds
		Bark to relieve pain and discomfort from miscarriage
	F. elliptica	Leaves to treat stomachaches
Phillipines	*F. fragrans*	Bark used to treat malarial fever
	F. racemosa	Bark and flowers applied on snakebites as antidote
Brunei	*F. racemosa*	Leaves and bark decoctions consumed as tonic
Sumatra	*F. auriculata*	Bark used to treat ulcers
		Leaves decoctions as rinse for mouth ulcers and to treat fever

Melanesia		
Fiji	*F. berteroana*	Leaves, inner bark and roots infusion to treat asthma and diabetes Root decoction used to reduce genital irritation and inflammation
New Caledonia	*F. schlechteri*	Heated leaves applied to wounds, bruises and boils Macerated leaves used as purgative and to stop menstrual bleeding Leaf infusion to treat ear infection and orally administered to children to stimulate growth
New Guinea	*F. maingayi*	Leaves applied to boil
	F. bodenii	Levaes chewed with salt to reduce spleen swelling caused by malarial infections
Polynesia		
Kingdom of Tonga	*F. berteroana*	Bark infusion drunk for pain relief from broken bones and internal injuries
Samoa	*F. berteroana*	Fruit decoction to relief fevers
Society Islands	*F. berteroana*	Berries used to treat bronchitis Bark extract to heal bone fractures

2.1.2 Chemical composition and biological activities

Another aspect considered prior to plant selection is that there are currently no publications on both *F. elliptica* and *F. gardenioides* regardless of parts of plants. There is only one study on leaves of *F. racemosa* which was found to have weak antimalarial activity in the *Plasmodium falciparum* viability test (Leaman *et al.*, 1995). There are no other studies on chemical constituents or bioactive properties to date. However, there are several investigations on their bark and roots. Okuyama *et al.* (1995) isolated lignans including (+)-pinoresinol, (+)-epipinoresinol, (+)-lariciresinol, and (+)-isolariciresinol, and phenols such as syringaldehyde and 7, 8-dihydro-7-oxy-coniferyl alcohol from root of *F. racemosa* from Sabah. The extract, especially the lignan fraction, exhibited analgesic properties in mice subjected to acetic acid-induced writhing and tail pressure tests. It also produced local

anesthesia in guinea pigs and showed relaxation effects in rat aortic strips (Okuyama *et al.*, 1995). Suciati *et al.* (2011) isolated alkaloid fagraeosides and iridoid glycoside secologanosides from stem bark *F. racemosa* from Java with the compounds having weak anti-inflammatory activity.

2.2 *Aquilaria malaccensis*

Aquilaria malaccensis is one of fifteen tree species in the genus *Aquilaria* belonging to the Thymelaeaceae family. Although *A. agallocha* distributed in India and Myanmar is treated as a synonym to *A. malaccensis* in certain research papers, they are oftentimes considered to be distinct species (Borris *et al.*, 1988). Indeed, investigations into their chemical profile indicate that there is a difference between *A. agallocha* and *A. malaccensis* attributed to their geographical difference. Local names include agarwood, eaglewood, aloeswood, gaharu in Malay and chénxiāng (沉香) in Chinese language (Barden *et al.*, 2003).

Aquilaria are native to the rainforests of Malaysia and southern part of continental South East Asia and are mostly found in dense primary forest and open secondary formations such as plains and hillsides up to 750m (Barden *et al.*, 2003; Hou, 1960). The distribution of *A. malaccensis* as denoted in Barden *et al.* (2003) was plotted using mapchart.net in Figure 7. To date, its presence in Iran is still questioned.

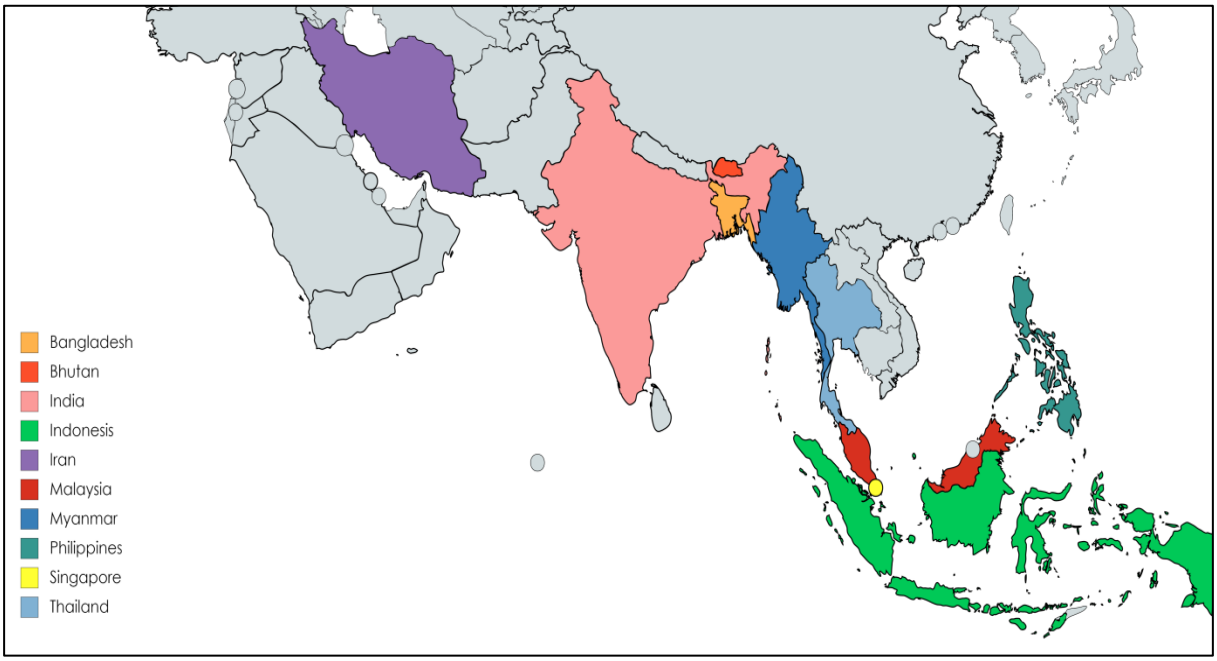


Figure 7. Distribution of *Aquilaria malaccensis*

A. malaccensis is an evergreen slow-growing tree 15-40m tall and 1.5-2m in diameter, smooth whitish pale brown bark, green and dirty-yellow ovate-oblong flowers, and obovoid-oblong fruits. Their leaves are chartaceous, subcoriaceous, glabrous, sometimes pubescent beneath, glabrescent, and shiny on both surfaces, elliptic-oblong to oblong-lanceolate with acute or obtuse base and acuminate apex (Hou, 1960). The wood is used as fuel and carved into religious objects like praying beads, idols and boxes; bark used to make writing material, clothes and ropes; and leaves consumed as tea (Barden *et al.*, 2003; Borris *et al.*, 1988).

Figure 8. *A. malaccensis* LAMK. **A.** Drawing of leaves, flowers and fruits; **B.** Tree trunk; **C.** Fruiting branch; **D.** Flowers and surface of leaves; **E.** Bell-shaped yellowish inflorescences (Fern, 2014b; Mohamed, 2016).

Aquilaria trees can produce agarwood, dark and dense resinous heartwood formed within the trunk and roots, in response to infection by a parasitic ascomycetous mould, *Phaeoacremonium parasiticum* (Wu *et al.*, 2012b). This aromatic resin of agarwood makes it one of the most expensive trees on earth. Its fragrance is widely sought after for joss sticks and incense-making and its extracted essential oil highly prized in perfumery, cosmetics and Taiwanese wine (Barden *et al.*, 2003). This has led to overexploitation, illegal harvesting and trade, causing a drastic decline in the number of *Aquilaria* in natural forests that it is listed as 'threatened' in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. In most countries, they are now commercially cultivated at sustainable levels (Barden *et al.*, 2003).

2.2.1 Traditional medicine

Since the 5th century, agarwood was used in more than 1500 Chinese medical materials preparations (Liu *et al.*, 2017). *A. malaccensis* have been prescribed to promote the flow of *qi*, relieve pain, fever, asthma, rheumatism, arrest vomiting and as stimulant, for heart palpitations (Barden *et al.*, 2003). Grated agarwood is used to cure smallpox, abdominal pains, rheumatism, disease involving female genital organs and as tonic to be taken during pregnancy and after childbirth (Chakrabarty *et al.*, 1994). In Malaysia, uninfected *A. malaccensis* wood decoction is used to treat jaundice, ulcer, oedema and body pains (Islam *et al.*, 2014). In Ayurvedic practices, it is used in fumigation therapy for sterilization for personal hygiene and in treatment of diseases such as epilepsy and schizophrenia (Vishnuprasad *et al.*, 2013). Agarwood powder sprinkled on clothes and skin acts as insect repellent against fleas and lice (Barden *et al.*, 2003). There is lack of literature on the usage of *A. malaccensis* leaves in traditional medicine. Leaves of *A. sinensis* have been used to treat fractures and bruises whereas leaves of *A. crassna* used to treat high blood pressure, constipation, headache and diabetes (Adam *et al.*, 2017). Despite no scientific evidence, the leaves of *A. malaccensis* are believed to have health benefits. This has given rise to commercial cultivation of the trees in China and South East Asia to produce agarwood tea called 'teh gaharu' in Malaysia.

2.2.2 Chemical composition of agarwood

Alkaloids, flavonoids, tannins, phenols, quinones, saponins, steroids, terpenoids, triterpenoids, and chromenones had been discovered in *Aquilaria malaccensis* (Huda *et al.*, 2009; Huo *et al.*, 2017; Wu *et al.*, 2012a; Wu *et al.*, 2012b). Of all plant parts, agarwood extracts and essential oil had been the most extensively studied primarily due to their pleasant spicy, woody, and sweet odor and attempts to artificially manufacture it. Agarwood has two predominant constituents which are sesquiterpenes and 2-(2-phenylethyl) chromone derivatives with structural examples in Figures 9 and 10.

Based on their skeleton backbone, sesquiterpenoids can be divided into several categories: agarofurans, cadinanes, eudesmanes, valencanes and eremophilanes, guaianes, prezizanes and vetispiranes (Chen *et al.*, 2012). Oxidized forms of sesquiterpenes include jinkoh-eremol and agarospirol. Chromones identified are mainly 2-(2-phenylethyl)-chromones, tetrahydro-2-(2-phenylethyl)-chromones and diepoxytetrahydro-2-(2-phenylethyl)-chromones. Seventeen chromones were noted to be agarwood specific and can be used as markers for quality control, for example, 5,6,7,8-tetrahydro-2-(2-phenylethyl)-chromone (Liu *et al.*, 2017). Other compounds present include phenols, fatty acids and simple volatiles such as alkenes and aldehydes.

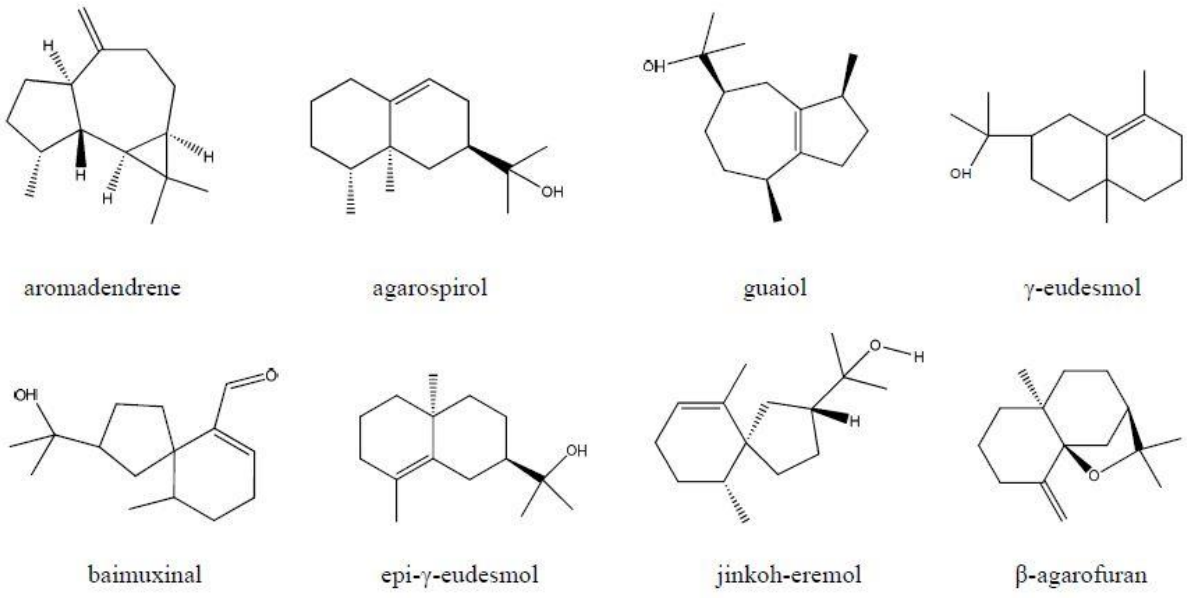


Figure 9. General structures of sesquiterpenoids (Liu *et al.*, 2017)

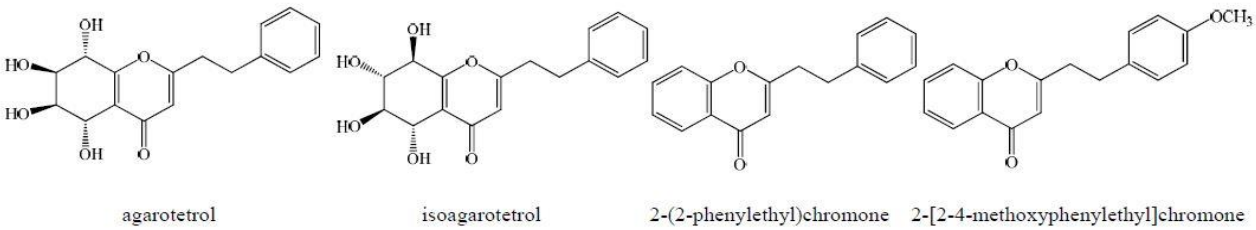
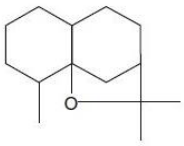
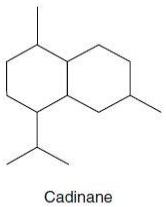
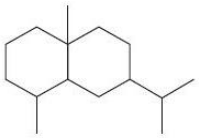
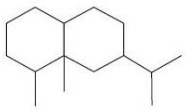
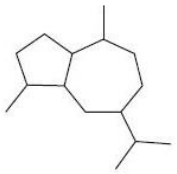
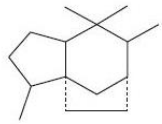
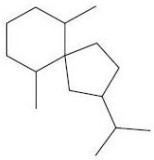


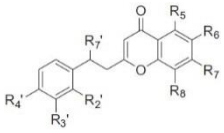
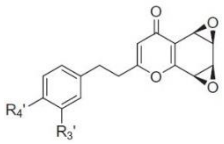
Figure 10. General structures of 2-(2-phenylethyl)chromones (Liu *et al.*, 2017).

So far, 70 sesquiterpenoids and 40 phenylethylchromones have been identified (Liu *et al.*, 2017; Mohamed, 2016). Table 4 shows only a few examples of the compounds present in barks, agarwood and essential oil of *A. malaccensis*. Several papers on chemical constituents are on *A. agallocha*. In the olden days, it is considered a synonym of *A. malaccensis*. A more comprehensive review on agarwood constituents can be found in papers by Naef (2011) and Chen *et al.* (2012). Given that they are too many reports on chemical composition from various plant parts of *A. malaccensis*, literature pertaining to leaves will be separated into section 2.2.4. Since there are too little studies on chemical constituents of *A. malaccensis* leaves, all leaves of the *Aquilaria* genus will be included.

Table 4. Chemical constituents in *A. malaccensis* agarwood and essential oils

Category/Class	Chemical constituents	References
Sesquiterpenes		
1) Agarofuran  Agarofuran	α-agarofuran	Yoneda *et al.* (1984), Nakanishi *et al.* (1984)
	β-agarofuran	Yoneda *et al.* (1984)
	nor-ketoagarofuran	Yoneda *et al.* (1984)
	Baimuxinal	Wu *et al.* (2012b)
	Isobaimuxinol	Naf *et al.* (1993)
	Epoxy-β-agarofuran	Naf *et al.* (1993)
	(1R,6S,9R)-6,10,10-Trimethyl-11-oxatricyclo[7.2.1.01,6]dodecane	Naf *et al.* (1992)
	(1R,2R,6S,9R)-6,10,10-Trimethyl-11-oxatricyclo[7.2.1.01,6] dodecan-2-ol	Naf *et al.* (1992)
2) Cadinanes  Cadinane	Agarol	Pant & Rastogi (1980)
	Gmelofuran	Pant & Rastogi (1980)

3) Selinanes	(5S,7S,10S)-(-)-Selina-3,11-dien-9-one	Ishihara *et al.* (1991)
	(5S,7S,9S,10S)-(+)-Selina-3,11-dien-9-ol	Ishihara *et al.* (1991)
	(−)-selina-3,11-dien-14-ol	Ishihara *et al.* (1993)
	(−)-selina-3,11-dien-14-al	Ishihara *et al.* (1993)
	(−)-methyl selina-3,11-dien-14-oate	Ishihara *et al.* (1993)
	(+)-selina-4,11-dien-14-al	Ishihara *et al.* (1993)
Eudesmane/ Selinane	Selina-4,11-diene-12,15-dial	Wu *et al.* (2012b)
	11(13)-Eudesmen-12-ol	Jain & Bhattacharyya (1959)
	(-)-10-epi-γ-eudesmol	Yoneda *et al.* (1984), Nakanishi *et al.* (1984)
	Eudesm-4-ene-11,15-diol	Wu *et al.* (2012a)
	(S)-4a-Methyl-2-(1-methylethylidene)-1,2,3,4,4a,5,6,7-octahydronaphthalene	Naf *et al.* (1992)
4) Valencanes/ Eremophilanes	(+)-(4S,5R)-Dihydrokaranone	Yoneda *et al.* (1984),
	(+)-(4S,5R)-Karanone	Naf *et al.* (1995)
	Eremophila-9,11-dien-8-one (Neopetasane)	Ishihara *et al.* (1993), Wu *et al.* (2012a)
	(-)-(4R,5S,7R)-Jinkoh-eremol	Yoneda *et al.* (1984)
Eremophilane/ valencane	Dehydro-jinkoh-eremol	Ishihara *et al.* (1993)
	38 (+)-(4R,5S,7R)-Kusunol	Yoneda *et al.* (1984)
5) Guaianes	α-Guaiene	Ishihara *et al.* (1991); Tajuddin *et al.* (2013)
	(−)-Guaia-1(10),11-dien-15-ol,	Ishihara *et al.* (1991)
	Methyl guaia-1(10),11-diene-15-carboxylate,	Ishihara *et al.* (1991)
	(+)-Guaia-1(10),11-dien-9-one,	Ishihara *et al.* (1991)
	(−)-1,10-epoxyguai-11-ene,	Ishihara *et al.* (1991)
	(+)-1,5-Epoxy-nor-ketoguaiene	Ishihara *et al.* (1991)
Guaianes	(−)-Rotundone	Ishihara *et al.* (1991)
	α -Bulnesene	Tajuddin *et al*. (2013)
6) Prezizanes	Jinkohol	Yoneda *et al.* (1984)
	Jinkohol II	Yoneda *et al.* (1984)
Prezizane		
7) Vetispiranes		
	Agarospirol	Yoneda *et al.* (1984), Tajuddin *et al.* (2013)
	Oxo-agarospirol	Yoneda *et al.* (1984), Nakanishi *et al.* (1984)
	Neopetasane	Wu *et al.* (2012a), Wu *et al.* (2012b)
	Vetispira-2(11),6-dien-14-al	Naf *et al.* (1995)
Agarospirane/ vetispirane		

Phenylethylchromones		
	6,8-dihydroxy-2-(2-phenylethyl)chromone	Konishi *et al.* (2002)
	6-hydroxy-2-[2-(4-hydroxyphenyl)ethyl]chromone	Konishi *et al.* (2002)
	6-hydroxy-2-[2-(2-hydroxyphenyl)ethyl]chromone	Konishi *et al.* (2002), Wu *et al.* (2012a)
	7-hydroxy-2-(2-phenylethyl)chromone	Konishi *et al.* (2002)
	6-methoxy-2-(2-phenylethyl)-chromone,	Wu *et al.* (2012), Wu *et al.* (2012b)
	6,7-dimethoxy-2-(2-phenylethyl)chromone,	Wu *et al.* (2012a)
	7-methoxy-2-(2-phenylethyl)-4H-chromen-4-one	Wu *et al.* (2012b)
	5,6 : 7,8-diepoxy-5,6,7,8-tetrahydro-2-(2-phenylethyl)chromone,	Wu *et al.* (2012a)
	5,6 : 7,8-diepoxy-5,6,7,8-tetrahydro-2-[2-(4-methoxyphenyl)ethyl]chromone,	Wu *et al.* (2012a)
	5,6 : 7,8-diepoxy-5,6,7,8-tetrahydro-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]chromone,	Wu *et al.* (2012a)
Others		
Diterpenes	12-*O*-*n*-Deca-2,4,6-trienoyIphorbol 13-acetate	Gunasekera *et al.* (1981)
Fatty acids	1,3-dibehenyl-2-ferulyl glyceride	Gunasekera *et al.* (1981)
Simple volatiles	Anisol	Ishihara *et al.* (1991)
	Acetophenone	Ishihara *et al.* (1991)
	Toluene	Ishihara *et al.* (1991)
	Benzaldehyde	Ishihara *et al.* (1991)
	Furfural	Ishihara *et al.* (1991)

* *A. agallocha* is used as a synonym for *A. malaccensis* in older studies by Pant and Rastogi (1980), Jain and Bhattacharyya (1959); Ishihara *et al.* (1991/1993), and Naf *et al.* (1992/1993/1995).

2.2.3 Biological activities of agarwood

Antimicrobial activity and antioxidant activity of *A. malaccensis* agarwood had yet to be reported. Nevertheless, agarwood from *A. agallocha* has strong antioxidant effect whereas *A. sinensis* and *A. crassna* is known to have significant antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* (Chen *et al.*, 2011b; Chen *et al.*, 2012).

Agarwood has anticancer activity. Stem bark oil extracts exhibits strong cytotoxic activity toward human colon (HCT-116) cancer cells with an IC_{50} of 4µg/ml (Ibrahim *et al.*, 2011). Moreover, ethanol extracts of stem bark has significant cytotoxic activity against P-388 lymphocytic leukemia system *in vitro* and in Eagles' carcinoma of the nasopharynx test system (Gunasekera *et al.*, 1981). Essential oil extracts exhibit mosquito repelling properties and strong larvicidal activity (Zaridah *et al.*, 2006).

Agarwood can be used as sedative. Inhalation of agarwood oil vapor which contains benzylacetone and calarene are able to sedate mice (Takemoto *et al.*, 2008). Jinkoh-eremol and agarospirol from benzene extract of agarwood is neuroleptic, inducing a depressant action on the central nervous system. It prolongs hexobarbital-induced sleeping time, hypothermia, suppress acetic acid-writhing and reduces spontaneous motility in mice (Okugawa *et al.*, 1996). Phenylethylchromone derivatives possess anti-allergic activity and benzylacetone can be used to relief cough (Chen *et al.*, 2012).

2.2.4 Chemical composition of *Aquilaria* leaves

As detailed in Table 5, chemical composition of leaves include 2-(2-phenylethyl) chromones, phenolic acids, steroids, fatty acids, benzophenones, xanthonoids, flavonoids, terpenoids, nucleosides and alkanes (Adam *et al.*, 2017; Huo *et al.*, 2017). Mangiferin, polyphenolic glycosides and flavonoids are most widely reported due to their biological properties with constituents such as genkwanin and its derivatives being specific to *Aquilaria* species (see Figure 11).

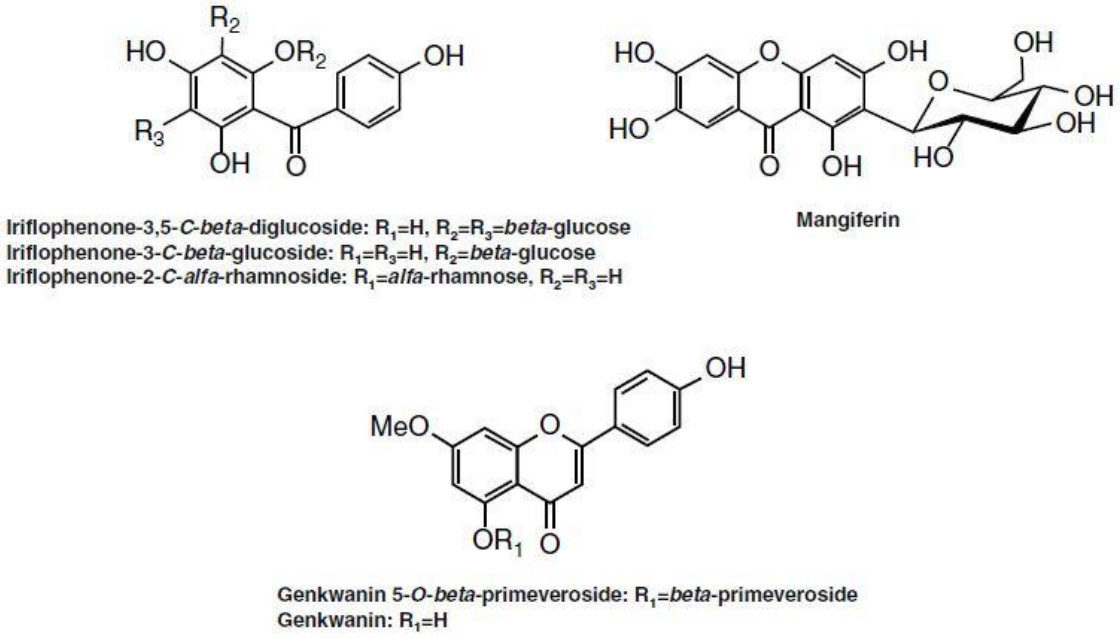


Figure 11. Main chemical constituents in *Aquilaria* leaves (Mohamed, 2016)

Table 5. Chemical constituents in *Aquilaria* leaves (Adam *et al.*, 2017 with modifications)

Category/Class	Chemical constituents	Species	References
2-(2-Phenylethyl) chromones	5-hydroxy-6-methoxy-2-(2-phenylethyl)chromone	AS	Wang *et al.* (2015b)
	6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone	AS	Wang *et al.* (2015b)
	6-hydroxy-2-[2-(4-hydroxyphenyl)ethyl]chromone	AS	Wang *et al.* (2015b)
Phenolic acids	p-hydroxybenzoic acid	AS	Wang *et al.* (2008),
		AS	Feng *et al.* (2011),
		AS	Kang *et al.* (2014)
	Vanillic acid	AS	Kang *et al.* (2014)
	Isovanillic acid	AS	Kang *et al.* (2014)
	Methylparaben	AS	Kang *et al.* (2014)
	Syringic acid	AS	Kang *et al.* (2014)
	Protocatechuic acid	AS	Pranakhon *et al.*(2015)
Phytosterols/ Steroids	Ergosterol	AS	Yang *et al.* (2014)
	β-sitosterol	AS	Kang *et al.* (2014)
	7α-hydroxy-β-sitosterol	AS	Wang *et al.* (2008)
	β-sitostenone	AS	Kang *et al.* (2014)
	Stigmasterol Stigmasta-4,22-dien-3-one	AS	Kang *et al.* (2014)
	β- daucosterol	AS	Wang *et al.* (2008)
Fatty acids	N- hexadecanoic acid	AM	Khalil *et al.* (2013),
		AS	Liu *et al.* (2007)
	Nonanoic acid	ASu	Bahrani *et al.* (2014),
		AS	Liu *et al.* (2007)
	Pentadecanoic acid	ASu	Bahrani *et al.* (2014),
		AS	Liu *et al.* (2007)
	1,2,3-propanetriol, monoacetate	AM	Khalil *et al.* (2013)
	(E)-9-octadecenoic acid	AS	Liu *et al.* (2007)
	9,12,15-Octadecatrienoic acid, (z,z,z)- \	AM	Khalil *et al.* (2013)
	Dodecyl acrylate	AM	Khalil *et al.* (2013)
	1-Tetradecanol	AM	Khalil *et al.* (2013),
		AS	Liu *et al.* (2007)
Pyranones	2,3-Dihydro-3,5-Dihydroxy-6-methyl-4H-pyran-4-one	AM	Khalil *et al.* (2013)
Quinones	6-ethyl-5-hydroxy-2,3n,7-trimethoxynaphthoquinone	AM	Khalil *et al.* (2013)
Carbohydrates/ carbohydrates conjugates	Glycerine	AM	Khalil *et al.* (2013)
	1,3 dihydroxy	AM	Khalil *et al.* (2013)
	Phenyl-β-D-glucoside	AM	Khalil *et al.* (2013)
Benzophenones	Aquilarisinin	AS	Feng *et al.* (2011)
	Aquilarinoside	AS	Feng *et al.* (2011),
		AS	Qi *et al.* (2009)
	Aquilarinensides	AS	Sun *et al.* (2014)
	Iriflophenone 2-O-α-L-rhamnopyranoside	AS	Feng *et al.* (2011),
		AS	Yu *et al.* (2013)

Benzophenones	Iriflophenone 3,5-C-β-D-diglucopyranoside	AS	Feng *et al.* (2011),
		AS	Yu *et al.* (2013)
	Iriflophenone 3-C-β-D-glucoside	AC	Ito *et al.* (2012),
		AS	Feng *et al.* (2011),
		AS	Yu *et al.* (2013)
Xanthonoids	Aquilarixanthone	AS	Yu *et al.* (2013)
	Mangiferin	AC	Ito *et al.* (2012),
		AS	Yu *et al.* (2013)
	Neomangiferin	AS	Yu *et al.* (2013)
	Homomangiferin	AS	Yu *et al.* (2013)
	Isomangiferin	AS	Yu *et al.* (2013)
Flavonoids	Apigenin −7, 4′-dimethyl ether	AS	Wang *et al.* (2008),
		AS	Kang *et al.* (2014),
		AC	Azziz *et al.* (2013)
	Genkwanin	AS	Wang *et al.* (2008),
		AC	Ito *et al.* (2012),
		AS	Yu *et al.* (2013),
		AS	Qi *et al.* (2009)
	Hydroxygenkwanin	AS	Wang *et al.* (2008),
		AS	Yu *et al.* (2013)
	Luteolin	AS	Wang *et al.* (2008),
		AS	Qi *et al.* (2009)
	Luteolin-7, 3 ', 4′-methyl ether	AS	Kang *et al.* (2014)
	Luteolin-7, 4′-dimethyl ether	AS	Kang *et al.* (2014)
	5-hydroxy-4′,7-dimethoxyflavonoid	AS	Kang *et al.* (2014)
	5,3′-dihydroxy-7,4′-dimethoxyflavone	AS	Kang *et al.* (2014)
	Delphinidin-3-glucoside	ASu	Bahrani *et al.* (2014)
	Hypolaetin 5-O-β-D-glucuronopyranoside	AS	Feng *et al.* (2011),
		AS	Yu *et al.* (2013)
	Epicatechin gallate	AC	Tay *et al.* (2014)
	Epigallocatechin gallate (ECGC)	AC	Tay *et al.* (2014)
	Vitexin	AS	Nie *et al.* (2009)
Cucurbitacin	2-O-β-D-glucopyranosyl cucurbitacin I	AS	Sun *et al.* (2015)
glycosides	Cucurbitacin	AS	Feng *et al.* (2011)
	Bryoamaride	AS	Sun *et al.* (2015)
Megastigmane	Citroside B	AS	Sun *et al.* (2015)
glycosides	Corchoionoside C	AS	Sun *et al.* (2015)
	Macarangloside D	AS	Sun *et al.* (2015)
	Staphylionoside H	AS	Sun *et al.* (2015)
	(9S) megastigma-4,7-diene-2,3,9-triol 9-O-β-D-glucopyranoside	AS	Sun *et al.* (2015)
	(9S) megastigma-4(13),7-diene-3,6,9-triol 9-O-β-D-glucopyranoside	AS	Sun *et al.* (2015)
	(+) 3-oxo-α-ionol-β-D-glucopyranoside	AS	Sun *et al.* (2015)
	(–) 3-oxo-α-ionol-β-D-glucopyranoside	AS	Sun *et al.* (2015)

Terpenoids	Squalene	AC	Azziz *et al.* (2013),
		AM	Khalil *et al.* (2013)
	Phytol	AM	Khalil *et al.* (2013),
		ASu	Bahrani *et al.* (2014),
		AS	Liu *et al.* (2007)
	Isophytol	AS	Liu *et al.* (2007)
	Friedelan-3-one	AS	Wei and Bin (2011)
	Epifriedelanol	AC	Azziz *et al.* (2013)
	Friedelin	AS	Nie *et al.* (2009)
Nucleosides	Adenosine	AS	Wang *et al.* (2015b)
	Cytidine	AS	Wang *et al.* (2015b)
	Guanosine	AS	Wang *et al.* (2015b)
	Inosine	AS	Wang *et al.* (2015b)
	Thymidine	AS	Wang *et al.* (2015b)
	Uridine	AS	Wang *et al.* (2015b)
Alkanes	Tetracosane	AS	Wei and Bin (2011)
	Docosane	AS	Wei and Bin (2011)
	Dodecane	AS	Wei and Bin (2011)
	9-Hexacosene	AS	Wei and Bin (2011)
	Octacosane	AS	Wei and Bin (2011)
	z-14-Nonacosane	AS	Wei and Bin (2011)
	1-Bromodocosane	AS	Wei and Bin (2011)
	Hexadecane,1-iodo	AS	Wei and Bin (2011)
	Hexadecane,7,9- dimethyl-	AS	Wei and Bin (2011)
	Heptadecane	AS	Wei and Bin (2011)
	Heneicosane	AS	Wei and Bin (2011)
	1-hexacosene	AS	Wei and Bin (2011)
	Triacontane	AS	Wei and Bin (2011)
Alkaloids	Isocorydine	AS	Nie *et al.* (2009)
Ketones	6, 10, 14-trimethy l-2-pentadecanone	AS	Liu *et al.* (2007)
	4, 8, 12, 16-tetramethylheptadecan-4-olide	AS	Liu *et al.* (2007)
Others	Vitamin E	AS	Xia *et al.* (2013)

Abbreviations used in species - AC: *A. crassna*; AM*: A. malaccensis*; AS: *A. sinensis*; and ASu: *A. subintegra*.

The extraction method such as solvent system and techniques employed affects the chemical composition. For example, methanol extracts of agarwood of *A. malaccensis* consist mainly of chromenones whereas sesquiterpenes dominates in benzene extracts (Nakanishi *et al.*, 1984; Wu *et al.*, 2012a; Wu *et al.*, 2012b). Likewise in leaves, phenolic acids such as vanillic acid, p-hydroxybenzoic acid, syringic acid and isovanillic acid were reported in methanol extracts of *A. sinensis* leaves (Kang *et al.*, 2014). In contrast, sterols β-daucosterol and 7α-hydroxy-β-sitosterol are reported in exthanol extracts (Wang *et al.*, 2008).

As for influence of techniques, liquid chromatography-mass spectrometry (LC-MS) together with silica gel column chromatography was able to detect mangiferin, genkwanin and iriflophenone glycosides in *A. sinensis* leaves (Yu *et al.*, 2013). On the other hand, reversed-phase high performance liquid chromatography coupled with UV detector (HPLC-UV) revealed the presence of several major constituents like iriflophenone, 3-C-β-D-glucoside, iriflophenone 3,5-C-β-D-diglucoside, mangiferin, and iriflophenone 2-O-α-L-rhamnoside (Xia *et al.*, 2015).

2.2.5 Biological activities of *Aquilaria* leaves

A. malaccensis leaves have been reported to be antioxidant, antibacterial, antidiabetic, and anticancer. Huda (2009) and Kshirsagar and Upadhyay (2009) reported strong DPPH radical scavenging activity for leaves, stem and aerial parts. Antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* has been reported (Hendra *et al.*, 2016). Khalil *et al.* (2013) noted that one of the major compounds in methanolic extracts of leaves is hexadecanoic acid. This saturated fatty acid has antibacterial activity against Gram-positive and Gram-negative bacteria, comparable to ampicillin antibiotic in *Pentanisia prunelloides* (Saidana *et al.*, 2008).

Callus and shoot of *A. malaccensis* leaves was found to have cucurbitacin I that is cytotoxic against MDA-MB-468 human breast cancer cells by indirectly interrupting dynamics of actin (Knecht *et al.*, 2010). Cucurbitacin has been noted to be antibacterial and antifungal (Chen *et al.*, 2014). Ethanol, water and ethyl acetate extracts improve glucose uptake by elevating levels of glucose transporter type 4 (GLUT4), a regulator of whole-body glucose homeostasis. The increased in GLUT4 level was higher compared to the diabetic drug pioglitazone (Said *et al.*, 2016).

The biological properties of other *Aquilaria* leaves are listed in Table 6. A look into *A. agallocha* leaves oftentimes considered similar to *A. malaccensis* leaves reveals hepatoprotective and antiarthritic activity. The hepatoprotective activity against paracetamol induced hepatotoxicity in Sprague-Dawley rats was comparable to the drug silymarin (Alam *et al.*, 2017). Ethanolic extracts exhibited both *in-vitro* and *in-vivo* anti-arthritic activity via inhibition of protein denaturation and chemical mediators involved in chronic development of rheumatoid arthritis which is further supported by hematological and radiological analysis (Rahman *et al.*, 2016).

Table 6. Biological activities of *Aquilaria* leaves

***Aquilaria* Species**	**Biological Activities**	**References**
A. malaccensis	Antibacterial activity	Chen *et al.* (2014)
		Hendra *et al.* (2016)
	Anticancer activity (callus and shoots)	Knecht *et al.* (2010)
	Antioxidant activity	Huda *et al.* (2009)
		Kshirsagar and Upadhyay (2009)
	Antidiabetic activity	Said *et al.* (2016)
A. agallocha	Anti-arthritic activity	Rahman *et al.* (2016)
	Hepatoprotective effect	Alam *et al.* (2017)
A. sinensis	Anticancer	Nie *et al.* (2009),
		Zhong *et al.* (2014),
		Sun *et al.* (2015)
	Antihistaminic activity	Wu *et al.* (2013)
	Anti hyperglycemic activity/Anti diabetic	Pranakhon *et al.* (2011),
		Mei *et al.* (2013)
	Anti-inflammatory activities	Zhou *et al.* (2008),
		Lin *et al.* (2013),
		Huo *et al.* (2017)
	Antioxidant activities	Han and Li (2012),
		Duan *et al.* (2015)
	Antitumor activities	Wang *et al.* (2008),
		Wei and Bin (2011)
	AMP-activated protein kinase (AMPK) activating effect	Jiang *et al.* (2011)
	Analgesics activities	Lin *et al.* (2013)
	Laxative effect	Kakino *et al.* (2010a)
	Lipid-lowering effect	Wu *et al.* (2012)
	α-glucosidase inhibitory activity	Feng *et al.* (2011)
A.crassna	Analgesics activities	Sattayasai *et al.* (2012)
	Antibacterial activities	Kamonwannasit *et al.* (2013),
		Chen *et al.* (2014b)
	Anticancer	Dahham *et al.* (2015)
	Anti-inflammatory activities	Kumphune *et al.* (2011)
	Antioxidant activities	Dahham *et al.* (2015)
	Antipyretic activities	Sattayasai *et al.* (2012)
	Laxative effect	Kakino *et al.* (2010a)
		Kakino *et al.* (2012)
A.subintegra	Acetylcholinesterase (AChE) inhibitory activity	Bahrani *et al.* (2014)

Similarly, antioxidant, antibacterial, antidiabetic and anticancer activity had been reported in other *Aquilaria* leaves. The mechanism of antidiabetic effects had been investigated in *A. sinensis* leaves. Their ability to activate AMP-activated protein kinase (AMPK) activity and inhibit alpha-glucosidase activity plays a major role. AMPK activation reduces fasting blood glucose and glycosylated hemoglobin levels in mice through control of carbohydrate absorption from the intestine resulting in an ameliorating effect on insulin resistance (Jiang *et al.*, 2011). Alpha-glucosidase inhibitors identified include aquilarisinin, aquilarisin, mangiferin, iriflophenone 2-O-α-L-rhamnopyranoside, iriflophenone 3-C-β-D-glucoside and iriflophenone 3,5-C-β-D-diglucopyranoside (Feng *et al.*, 2011). In anticancer, *A. sinensis* leaves shows cytotoxicity against human lung, gastric, and liver cells. Anticancer compounds identified includes cucurbitacin glycosides, isocorydine and their derivatives whereas antitumor compounds include squalene, acosanol and glycosides moieties (Nie *et al.*, 2009; Reddy and Couvreur, 2009; Wei and Bin, 2011).

The flavonoids of *A. sinensis* leaves inhibit nitric oxide release in macrophages and stabilize cell membranes resulting in anti-inflammatory activity (Yang *et al.*, 2012). Mangiferin and genkwanin-5-O-β-premeveroside in *A. crassna* and *sinensis* leaves showed laxative effect in constipated rats and increase contraction tension of intestines (Kakino *et al.* 2010a; Kakino *et al.*, 2010b, Kakino *et al.*, 2012). *A. subintegra* leaves containing kaempferol 3,4,7-trimethylether shows potential in Alzheimer's disease treatment as they are able to inhibit acetylcholinesterase (AChE) activity and reduce repeated entries into arms of radial arm maze in valium-impaired memory model (Bahrani *et al.*, 2014).

3.0 METHODS AND MATERIALS

3.1 Plant materials and drying treatment

All the leaves of the plants of interests were obtained from University of Malaya. Identity of plants through morphological examination was confirmed by Dr Sugumaran Manickam of University of Malaya. Leaves were washed thoroughly to remove debris, soil and dust and then oven-dried at 30°C until constant weight is achieved. The leaves of *A. malaccensis* were collected in August 2013 from Rimba Ilmu, Institute of Biological Sciences, University of Malaya, Malaysia. The leaves are mature in age and a voucher specimen (MUM-THYME-001) was deposited in the herbarium of School of Science, Monash University, Malaysia. The leaves of *Fagraea* plants are collected in August 2013 from Genting Highlands. These leaves are mature in age and voucher numbers (SM274, SM257) were deposited in the herbarium of Institute of Biological Sciences, University of Malaya.

3.2 Sample extraction (sequential)

Sequential extraction method as described by Bbosa *et al.* (2007) was carried out. The dried leaves were cut and blended into fine stringy material for *A. malaccensis* or coarse powder for *Fagraea spp.* using a kitchen grinder. The finely ground leaves (1 kg) were extracted by sequential extraction using five different solvents in increasing polarity. The order is hexane, dichloromethane, ethyl acetate, methanol and distilled water. Leaves now in powder form are placed in jars, soaked in solvent and placed on orbital shaker at room temperature (27 ± 1°C). Solvents were decanted and extract filtered using Buchner funnel connected to the vacuum pump. The remaining plant residues in the jar were replenished with fresh solvents. The filtered extracts were evaporated under reduced pressure using rotary vacuum evaporator to obtain concentrated crude extracts which are in turn stored at -20°C until further analysis. Extraction is repeated three times per solvent which takes up to five days each or till the newly added solvents remained colorless.

3.3 Determination of antioxidant properties of crude extracts

3.3.1 Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the extracts was determined according to the Folin-Ciocalteu method described by Kahkonen *et al.* (1999) adapted to the 96-well plate assay. Sample extracts were diluted to a suitable concentration using their respective

extraction solvent. Aliquots (30µl) of the extract were added to a 96-well plate in triplicates followed by the addition of 150µl Folin-Ciocalteu's reagent (10% v/v) and 120µl sodium carbonate (7.5% w/v). The reaction mixtures were thoroughly mixed and incubated for ten minutes in the dark before measurement of absorbance at 765 nm. For blank control, 30µl of extraction solvent was used instead of the extract. Gallic acid is used as standard. with the calibration equation of gallic acid obtained $y = 0.0065x + 0.0113$ ($R^2 = 0.99$) with y representing absorbance at 765 nm and x representing concentration of gallic acid in mg/L. TPC values are quantified based on the gallic acid standard curve and expressed as mg gallic acid equivalents (GAE) per 100 g of sample (mg GAE/100 g).

3.3.2 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH free radical scavenging assay was carried out according to the method described by Brand-Williams *et al.* (1995). Briefly, extracts were diluted to five different concentrations using their respective extraction solvent. An aliquot (50µl) of each diluted extract was added to 150µl of 0.3mM DPPH solution in each well of a 96-well microtitre plate. For blank control, 50µl of extraction solvent was used instead of the extract. Absorbance was measured at 515nm after 30 minutes incubation in the dark at room temperature using a microplate reader. All tests were performed in triplicate.

The antioxidant activity (AOA) was expressed as the percentage decrease of DPPH based on the equation as follows:

$$\text{AOA (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{sample} and A_{control} represent the absorbance values of the sample extract and control respectively. Free radical scavenging activity was expressed as IC_{50} which is the inhibitory concentration in mg/ml of extracts required to reduce the absorbance of DPPH by 50% whereby the lower the IC_{50}, the higher the radical scavenging activity. IC_{50} was determined by plotting a graph of DPPH scavenging activity (%) against the concentration of extract (mg/ml). As the standard used is ascorbic acid, results were also expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg of ascorbic acid (AA) per 100 g of material. Given that, $IC_{50\,(\text{ascorbic})}$ was determined to be 0.015 ± 0.001 mg/ml, AEAC was calculated as follows:

$$\text{AEAC (mg AA/100g)} = [IC_{50\,(\text{ascorbic acid})} / IC_{50\,(\text{sample})}] \times 100000$$

3.3.3 Ferric reducing power (FRP) assay

The total reducing power (FRP) of extracts was determined based on the methodology described by Ashawat *et al.* (2007) and Zubia *et al.* (2009). Briefly, crude extracts or standard ascorbic acid was diluted to five different concentrations using their respective extraction solvent. Aliquots (200µl) of the diluted extracts were mixed with 200µl phosphate buffer (0.2M, pH 6.6) and 200µl 1% potassium ferricyanide. For blank control, 200µl of extraction solvent was used instead of extract. After incubation at 50°C for 30min, the mixture was allowed to cool down for a few minutes followed by addition of 200µl of 10% trichloroacetic acid. Aliquots (125µl) of the above mixture were transferred to a 96-well microplate where 20µl of 0.1% ferric chloride solution are added to each well. Absorbance were read at 620nm using a microplate reader. All tests were performed in triplicate for each sample.

The total reducing power activity (TRP) was expressed as the percentage increase of absorbance based on the equation as follows:

$$TRP\ (\%) = [1- (A_{control} - A_{sample})] \times 100$$

where A_{sample} and $A_{control}$ represent the absorbance values of the sample extract and control respectively. IC_{50}, the concentration of extract in mg/ml require to increase absorbance by 50%, was determined by plotting a graph of TRP activity (%) against the concentration of extract (mg/ml). Results were also expressed in reference to the reducing power of an equal amount of standard ascorbic acid. Given that, $IC_{50\ (ascorbic)}$ was determined to be 0.022 ± 0.000 mg/ml, it was calculated as follows:

$$AEAC\ (mg\ AA/g) = [IC_{50\ (ascorbic\ acid)} / IC_{50\ (sample)}] \times 1000$$

3.3.4 Metal chelating assay (MCA)

The capacity of extracts to bind ferrous ions was determined as described by Dinis *et al.* (1994) with modifications. Firstly, crude extracts or standard ethylenediaminetetraacetic acid (EDTA) or gallic acid were diluted to five different concentrations using their respective extraction solvent. An aliquot (100µl) of each diluted extract was added to a 96-well plate followed by addition of 100µl of 0.1mM iron (II) sulphate heptahydrate and 100µl of 0.25mM ferrozine. For blank control, 100µl of extraction solvent was used instead. After 10 minutes of incubation, absorbance was measures at 562nm.

Results were expressed as metal chelating ability (%) using the following equation:

$$Metal\ chelating\ ability\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where A_{sample} and $A_{control}$ represent the absorbance values of the sample extract and control respectively.

3.3.5 β-carotene bleaching assay (BCBA)

The β-carotene bleaching assay was based on the method described in Koleva *et al.* (2002) with slight modifications. All solutions were freshly prepared prior to each experiment. β-carotene (1 mg) was first dissolved in 5ml of chloroform. Linoleic acid (25µl) and Tween 40 (200mg) were then added to 1ml of the solution. The chloroform was then evaporated under vacuum at 40°C. This is followed by addition of 50ml oxygenated distilled water (obtained by bubbling air through the water for 24 hours). The mixture is then vigorously shaken. An aliquot (200µl) of the obtained emulsion was added into wells of 96-well plate. Aliquot (50µl) of extracts diluted to different concentrations are then added with extraction solvent replacing extracts for negative blank control. Dilutions of 11 different concentrations were carried out to enable comparison due to IC_{50} of extracts and control having a wide spread of effective concentration. The plates are incubated at 50°C with readings at 450nm performed immediately (t = 0 min) and after 2.5 hr of incubation. The 2.5 hr time period was chosen as a decrease in absorbance were no longer significant and plateaus after this time period. This was determined by a preliminary study whereupon absorbance readings were taken every 30 min up to 3 hrs. Butylated hydroxyanisole (BHA) was used as a positive control.

Results were expressed as the percentage decrease of absorbance based on the following equation:

$$\text{β-carotene bleaching inhibition (\%)} = [1 - [(A_{t=0h} - A_{t=2.5h\ sample})/ (A_{t=0h} - A_{t=2.5h\ control})]] \times 100$$

where $A_{t=0h}$ and $A_{t=2.5h}$ represent the absorbance values of extract (A_{sample}) or blank ($A_{control}$) at 0 min and 150min reading respectively. It is also expressed in IC_{50} which is the concentration in mg/ml of extracts required to reduce the absorbance by 50%. IC_{50} was determined by plotting a graph of β-carotene bleaching inhibition (%) against the concentration of extract (mg/ml). Given that $IC_{50\,(BHA)}$ was determined to be 0.079 ± 0.004 mg/ml, results can be expressed in percentage relative to BHA's bleaching capability:

$$\text{Percentage bleaching relative to BHA} = [IC_{50\,(BHA)} / IC_{50\,(sample)}] \times 100$$

3.4 Determination of antimicrobial properties

3.4.1 Microorganisms

Ten strains of bacteria were used in this study, five Gram positive and five Gram negative bacteria as listed in Table 7. The microorganisms were identified by CAB International (CABI) located at Serdang. The bacteria were grown on nutrient agar and kept at 4°C.

Table 7. Microorganisms used for antimicrobial susceptibility testing.

	Microorganisms	ATCC No.	Remarks
Gram positive			
1	*Micrococcus luteus*	4698	Deposited by A. Fleming into ATCC.
2	*Bacillus cereus*	14579	Deposited by R.E. Gordon into ATCC.
3	*Methicillin-sensitive Staphylococcus aureus* (MSSA)	25923	Isolated by F. Schoenknecht. Deposited by FDA into ATCC.
4	*Methicillin-resistant Staphylococcus aureus* (MRSA)	33591	Isolated from Elmhurst hospital (USA) by S. Schaefler. Deposited by S. Schaefler into ATCC.
5	*Methicillin-resistant Staphylococcus aureus* (MRSA) *clinical strain*		Hospital isolate obtained from microbiology lab UPM (2008)[a].
Gram negative			
6	*Escherichia coli*	25922	Isolated by F. Schoenknecht. Deposited by FDA into ATCC.
7	*Klebsiella pneumonia*	10031	Deposited by FDA into ATCC.
8	*Pseudomonas aeruginosa*	10145	Isolated by Merck Sharp & Dohme. Deposited by F. Kavanagh into ATCC.
9	*Enterobacter aerogenes*	13048	Obtained from Institute for Medical Research (IMR).
10	*Salmonella choleraesuis*	10708	Deposited Walter Reed Army Medical Center (AMC) into ATCC.

[a] Isolated from patients in Kuala Lumpur General Hospital.

3.4.2 Preparation of media

Nutrient agar and Mueller Hinton agar (MHA) were prepared according to manufacturer's instructions. The medium were autoclaved at 121°C for 20 minutes and cooled down to approximately 50°C before pouring into 15 x 100 mm plastic Petri dishes with uniform depth of 4mm (25.0 – 30.0ml of medium). Medium was left to solidify and incubated overnight at 37°C. Nutrient broth was also prepared according to manufacturer instruction. After that, 10ml of the broth was transferred into universal bottle to be autoclaved at 121 °C for 20 minutes. All sterile nutrient broth was kept at 4°C until use.

3.4.3 Preparation and standardization of inocula

A single colony of each type of bacteria from the stock cultured on nutrient agar was transferred into 10ml of sterile nutrient broth using an inoculating loop. After incubation in a shaking incubator at 37°C for 16-20 hours at 100rpm, 10ml of sterile distilled water was added to 1ml of the inoculated nutrient broth of each type of bacteria. Bacterial density was standardized using McFarland 0.5 turbidity standard, 1 X 10^8 cfu/ml. This was prepared by mixing 0.5ml of 1.175% (w/v) barium chloride dihydrate with 99.5ml of 1% (v/v) sulfuric acid. Each bacteria suspension was diluted in 10ml of sterile distilled water to reach the proper density. Adjustment of suspension turbidity was done by either increasing microorganism growth or adding more sterile distilled water to dilute it.

3.4.4 Disc diffusion method

The disc diffusion method was performed as described by Jantan *et al.* (2003). The standardized bacterial inoculums were swabbed on MHA agar plates using sterile cotton swabs. To ensure even distribution of the inoculums, swabbing was done twice by rotating the plate and swabbing approximately 60° to the first application. When the agar surface was dried, 6.0mm antimicrobial susceptibility discs containing the plant extract (1mg/100µl per disc) were placed aseptically on the agar using a needle. All samples were done in triplicates. For negative control, the extraction solvent (100% w/v methanol) was loaded onto the blank disk. Vancomycin (30µg) and streptomycin (10µg) antibiotic disc are used as positive control for methicillin-resistant bacteria and methicillin-sensitive bacteria respectively. Streptomycin antibiotic disc was also used as positive control for the other strains of bacteria tested. The agar plates were incubated at 37°C for 16-20 hours and the diameter of inhibition zone was measured and recorded in mm.

3.4.5 Broth microdilution

Minimum inhibitory concentration (MIC) were determined using broth microdilution in 96 wells microtiter plates as described by the Clinical and Laboratory Standards Institute (CLSI). The bacterium of interest was cultured for 24 hours on a nutrient agar plate. A well-isolated colony was transferred and inoculated into 10ml of Mueller-Hinton broth in universal bottle. The broth culture is incubated at 37°C for 18 hours. The turbidity of the broth culture is adjusted to 0.5 McFarland standard using sterile broth and verified using a spectrophotometer. The resulting suspension contains approximately 1 to 2 X 10^8 CFU/ml. All procedures were carried out in triplicates. A two-fold serial dilution of the plant extracts were pipetted into the 96 wells. Seven concentrations were tested ranging from 1 to 0.0156mg/ml. This is followed by addition of 100µl of the adjusted bacterial suspension. Three controls were included in each microtiter plate. The positive control comprises the addition of 0.25mg/ml of the antibiotic chloramphenicol. The negative control contained both bacterial suspension and solvent used to dissolve the plant extract whereas the sterile control contains only sterile broth and plant extract. The plates were incubated for 24 hours at 37°C. MIC is determined as the lowest concentration of extract tested that completely inhibits growth of the organism in the microtiter wells as detected as clear wells by the unaided eye. All clear wells in the microtiter plate were subcultured on Mueller-Hinton agar and incubated at 37°C overnight. The lowest concentration with both a clear solution and no growth observed on the agar plate was recorded as MBC.

3.5 Determination of cytotoxic activity

3.5.1 Cell culture and media

MCF-7 human breast cancer cell line was obtained from ATCC (American Type Culture Collection). The cell line was maintained using RPMI 1640 media with 10% of heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin with growing conditions of 37 °C and 5% CO_2 in humidified atmosphere. Subculture was performed when cells in the flask are 60-70% confluent. The medium in the flask was discarded and 5 ml of phosphate buffered saline (PBS) was added to wash away dead cells and debris that were not attached to the flask. PBS was discarded followed by addition of 2 ml of trypsin-EDTA to detach the cells from the flask surface. After 5 minutes incubation at 37°C, the cells were observed under the microscope to ensure complete detachment of cells. To neutralize the typsin, 10 ml of media was added into the flask. All the cells were then pipetted out into a falcon tube and centrifuged at 1000 rpm for 10 minutes at 24°C. The supernatant was discarded and the

remaining pellet was re-suspended in 12 ml of RPMI 1640 media, 10% of heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Based on the number of cells present after re-suspension counted using a haematocytometer, an appropriate volume was transferred to a new T75 flask containing 10 ml of RPMI 1640 media, 10% of heat-inactivated FBS and 1% penicillin-streptomycin for subculturing.

3.5.2 Preparation of media

The cell density of in the suspension was determined *via* counting using haemacytometer under the microscope at x10 magnification. Each well of a flat bottomed, 96-well tissue culture plate was seeded with 2000 cells in 100 µl media. For blank controls, the wells are not seeded and left empty. All plates were incubated for 24 hours at 37 °C and 5% CO_2 to allow the cells to attach to the wells.

3.5.3 Treatment with plant extracts

Plant extracts were diluted using RPMI 1640 media to concentrations of 12.5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml and 400µg/ml. After the 24 hours incubation, the cells in the microplate were treated with these concentrations of plant extracts with 100µl of the mixture pipetted into each well. All treatments were done in triplicates. The percentage volume of DMSO used in the various concentrations was used for vehicle controls while the various diluted media were pipetted into the blank controls. Plates were incubated at 37°C and 5% CO_2 at various intervals for up to 24 hours.

3.5.4 Preparation of MTT

A stock solution of 5 mg/ml of 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) was prepared by dissolving 5 mg of MTT powder in 1 ml sterile PBS. The stock solution was filtered using a 0.22µm sterile syringe filter and stored at -20°C until further use. Prior to usage, the stock MTT was diluted 1:10 with RPMI medium.

3.5.5 MTT cell viability assay

After the various intervals of incubation up to 24 hours, 100 µl of the MTT solution diluted with RPMI medium was added into each well. This was followed by incubation at 37 °C and 5% CO_2 for 3 hours. After incubation, all media was pipetted out and 100µl of DMSO was added to each well. DMSO was added to solubilize the purple formazan needle-shaped crystals as formazan is insoluble in water. The plates were left for 5 minutes for the solvent to

thoroughly mix with the formazan. For blanks, DMSO was added to the empty wells. Optical density was then measured at 570nm using a microplate reader.

3.6 Qualitative phytochemical analysis

3.6.1 Flavonoids

Testing of the presence of flavonoids was modified from the method described by Parekh and Chanda (2007). Extract (0.1 g) was dissolved in 1ml distilled water and then filtered. This is followed by addition of 10 mg of magnesium turnings and 0.05 ml concentrated sulfuric acid. Development of magenta red color within three minute indicates the presence of flavonoids.

3.6.2 Tannins

Testing of the presence of tannins was modified from the method described by Parekh and Chanda (2007). Extract (0.1 g) was dissolved in 1ml distilled water and then filtered. This is followed by addition of 0.01g/ml ferric chloride reagent. Formation of blue-black precipitates indicates the presence of tannins.

3.6.3 Terpenoids

Presence of terpenoids was determined using the method described by Kumar *et al.* (2009). Extract (0.01 g) was dissolved in 1ml chloroform and then filtered. This is followed by addition of 1 ml of acetic anhydride and 2 ml of concentrated sulfuric acid. The presence of reddish brown on the interface indicates the presence of terpenoids.

3.6.4 Steroids

The presence of steroids was modified from Kumar *et al.* (2009). Extract (0.02 g) was dissolved in 1ml methanol and then filtered. This is followed by addition of 1 ml of chloroform and 1 ml of concentrated sulfuric acid. The presence of yellow with green fluorescence at the sulfuric acid layer indicates the presence of steroids.

3.6.5 Alkaloids

The presence of alkaloids was determined as described by Parekh and Chanda (2007). Extract (0.02 g) was dissolved in 1 ml methanol and then filtered. This is followed by addition of 2 ml of 1% hydrochloric acid and then boiled for 5 minutes. Mixture is then cooled and 4-6

drops of Mayer/Dragendroff reagent was added. Formation of cream or orange precipitates indicates the presence of alkaloids.

3.6.6 Saponins

The presence of saponins was determined as described by Parekh and Chanda (2007). Extract (1.0 g) was dissolved in 5 ml deionized water and shaken vigorously for 4 minutes. Formation of honey-comb froth that persists for 45 minutes indicates presence of saponins.

3.7 Isolation and purification

3.7.1 Column chromatography

Methanol crude extract was dissolved with minimal amount of methanol and mixed with silica gel 60 until fine powder slurry was formed. The slurry is left to dry in the fume hood. A suitable column size was chosen for separation of compounds depending on the amount of the powder mixture. Column was first washed with acetone before packing with silica gel 60. The silica column was packed by mixing the silica powder with hexane and then pouring the suspension mixture into the column. The silica was allowed to settle with the eluent being allowed to drip into a flask. After silica has settled, the mixture of silica with extract was added to the column. For a uniform band, column was tapped on the side. After that, a layer of sand was added to prevent any addition of solvent from disrupting the surface of the extract mixture. The solvent was allowed to run through the column and 250 ml of eluent were collected for each fraction. The solvent systems used were solvent mixtures in increasing polarity which is hexane, ethyl acetate, chloroform, and methanol with elution gradient in 20% increment. The eluent was transferred into a round bottom flask and subjected to rotary evaporation to concentrate the mixture. These were transferred into sample vials and allowed to dry in the fume hood.

3.7.2 Thin layer chromatography

Thin layer chromatography was done for all fractions collected. Firstly, capillary tubes were used to spot each sample onto the TLC plate. During this process, the TLC plate was examined under ultraviolet (UV) light to ensure that sufficient amount of each sample is spotted onto the plate. The plate is then placed in a developing chamber containing 5 ml of eluting solvent. The solvent system used depends on the polarity of the fractions. The plate was removed from the chamber when solvent has reached the top of the plate and solvent front was marked with a pencil. The plate was examined under UV light for fluorescence

compounds, and fractions with similar spot patterns were combined. In cases where UV visualization is not suitable, iodine vapour is used. The TLC plate was placed in a chamber where few iodine crystals are added. Another method is spraying the plate with 1.0M sulphuric acid and then dried on top of a hot plate.

3.7.3 Fraction screening and further purification

All fractions of interest of sufficient amount were subjected to screening with TPC, DPPH and cytotoxicity MTT assay against MCF-7 breast cancer cell lines. The fraction with the best bioactivity will be chosen for further isolation and purification work using repeated column chromatography.

3.7.4 Crystallization

Crystallization was done using liquid/liquid diffusion. Extracts were dissolved in methanol. This was followed by addition of 2-3 drops of the diethyl ether solvent using a Pasteur pipette to the side of the vial every two days. The vials were placed in the fume hood undisturbed until crystals start to form. Excess solvent was removed using pipette and with further drying under the fume hood and freeze-dryer. Filtration of crystals was not performed due to their fine needle-like nature.

3.8 Reversed-phase high performance liquid chromatography (RP-HPLC)

3.8.1 Sample preparation

Fractions of interest were dissolved in methanol and filtered using a syringe and filter disc (PTFE, 0.45µm) before injection into the HPLC.

3.8.2 HPLC analysis

Separation of compounds was further carried out using Agilent Technologies HPLC and analyzed with the ChemStation for LC 3D Systems. A reversed phase C18 silica packing column was used which is Cosmosil 5C 18-MS-II (4.6 mm i.d. x 250mm, 5µm particle size). Solvents selected as mobile phase were acetonitrile and methanol. Preliminary testing shows that methanol solvent gives a better elution profile compared to acetonitrile due to sample tested being highly polar. Hence, subsequently the two mobile phase primarily used was water (Milli-Q, sonicated) and 100% methanol. The mobile phase was delivered through the column with a flow rate of 1.0 ml/min. The initial elution profile was of gradient elution from 100% mobile phase A (water) to 100% mobile phase B (100% methanol) in a linear gradient for 45

minutes. Based on the results from each sample where peaks of interest are eluted, an isocratic solvent system was chosen. This was generally narrowed down to isocratic 40% or 50% methanol. The samples were analyzed at 210nm, 245nm, 280nm and 365 nm. Sample was injected only when baseline is stable with no peaks observed. Injection of sample was automated with a volume of 20µl to 100µl. The runtime is optimized based on initial runs depending on where the peak of the last compound was observed.

3.9 LC-MS/MS

Polar samples were outsourced for liquid chromatography-mass spectrometry (LC-MS/MS) analysis to Advanced Chemistry Solutions Sdn. Bhd. (ACS) and Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia (MUM). The parameters for each analysis were as listed in Table 8 and 9 with the parameters of the second set of analysis being further optimized based on results from the first analysis.

Table 8. Parameters of LC-MS/MS analysis (Advanced Chemistry Solutions)

Parameters	**Details**
Model system	Agilent 1290 Infinity LC system coupled to AB Sciex QTRAP 5500
Column	Agilent Zorbax C18, 4.6x150mm, 5 µm
Column temperature	25°C
Flow rate	0.25ml/min
Solvents	A. Water (with 0.1% formic acid and 5mM ammonium formate B. Acetonitrile (with 0.1% formic acid and 5nM ammonium formate)
Run program	10% B to 90% B from 0.01min to 8.0min, hold for 3 min and back to 10% B in 0.1min and re-equilibrated for 5min (Sub-fraction 22-6) 5% B to 95% B from 0.01min to 15.0min, hold for 2 min and back to 10% B in 0.1min and re-equilibrated for 3min (Sub-fraction 22-6-2)
Mode MS	Positive ionization Voltage: 5500V, Source temperature: 500°C, 40 psi, mass range (m/z) minimun 50 to maximum 1200. Negative ionization Voltage: -4500V, Source temperature: 500°C, 40 psi, mass range (m/z) minimun 50 to maximum 1200.

Table 9. Parameters of LC-MS/MS analysis (School of Medicine, Monash)

Parameters	**Details**
Model system	Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI
Column	Agilent Zorbax Eclipse XDB-C18, 4.6x150mm, 5 µm
Column temperature	25°C
Flow rate	0.5ml/min
Solvents	A. Water (with 0.1% formic acid) B. Methanol (with 0.1% formic acid)
Run program	5% B to 40% B for 0.01min to 30.0 min, hold at 40% B for 5 min
Mode MS	Positive ionization Voltage: 4000V, Source temperature: 300°C, 45 psi, mass range (m/z) minimum 100 to maximum 3200.

3.10 NMR

Methanol compounds were sent to School of Pharmacy, Monash University Malaysia for nuclear magnetic resonance (NMR) analysis with Bruker Ultrashield 300MHz. Crystals were dissolved in deuterated methanol-d4 (CD_3OD) and ^{1}H NMR and ^{13}C NMR were run. A second run was carried out up to 35 hours to obtain better spectra. All NMR spectras were personally analyzed using MestreNova program version 6.0.2-5475.

3.11 Statistical analysis

All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, version 16.0). All data were presented as mean ± standard deviation (SD) of three replicates. Data were analyzed using one-way analysis of variance (ANOVA) and post hoc T-test where differences with a p-value ≤ 0.05 were considered significant.

4.0 RESULTS AND DISCUSSION

4.1 Bioactivity screening of selected Malaysian plants

4.1.1 Introduction

An initial screening for their bioactivity screening was carried out to screen four plants with purported medicinal benefits: *A. malaccensis*, *F. elliptica*, *F. gardeniodes*, and *F. racemosa*. This is to narrow down which plant to focus on for more extensive studies.

4.1.2 Antimicrobial activity

In this preliminary screening using disk diffusion assay, leaves of plants were extracted using 1:1 dichloromethane: methanol. The solvent combination is chosen for extraction due to their capability to extract most of the compounds present in plants. Dichloromethane facilitates the extraction of non-polar compounds whereas methanol facilitates the extraction of polar compounds. Discs were loaded with 1 mg/100µl of extract. Results of the Kirby-Bauer disc diffusion susceptibility method are summarized in Table 10.

All the extracts screened do not exhibit antimicrobial activity against all five strains of gram negative bacteria tested as denoted by the visual absence of an inhibitory zone around the disc loaded with extract. This could be due to the inability of the plant extracts to penetrate the lipopolysaccharide layer encompassing Gram negative bacteria which has been noted to restrict the diffusion of hydrophilic compounds (Burt, 2004).

The leaves of four plants showed varying degree of antimicrobial activity against Gram positive bacteria. All plants from the *Fagraea* genus are not active against *Micrococcus luteus* and only *F. gardenioidea* being active against MSSA. Although *A. malaccensis* manages to inhibit the growth of all five Gram postive bacteria indicating a broad spectrum antimicrobial effect, this activity is very weak. Overall, *F. gardeniodes* showed the best antimicrobial activity against MSSA, MRSA ATCC and MRSA clinical strains. Interestingly, this activity was approximately half that of the positive control which is streptomycin for MSSA and vancomycin for MRSA denoting promising potential for antibiotic development against these bacteria.

Table 10. Antimicrobial activity of *A. malaccensis* and *Fagraea spp.*

Microorganism	Measurements of zone of inhibition diameter (mm)				
	Positive control	***Aquilaria malaccensis***	***Fagraea elliptica***	***Fagraea gardenioides***	***Fagraea racemosa***
Gram positive					
Bacillus cereus	20.50 ± 0.71	7.00 ± 0.00	7.00 ± 0.00	8.50 ± 0.00	7.17 ± 0.29
Micrococcus luteus	20.50 ± 0.71	8.50 ± 0.00	-	-	-
Staphylococcus aureus(MSSA)	14.50 ± 0.00	8.00 ± 0.00	-	9.33 ± 0.58	-
MRSA ATCC (33591)	20.00 ± 0.00	10.17 ± 0.29	7.83 ± 0.29	13.33 ± 1.15	9.83 ± 0.29
MRSA clinical strain	18.75 ± 0.35	6.83 ± 0.58	7.00 ± 0.00	10.83 ± 0.29	8.67 ± 0.29
Gram negative					
Pseudomonas aeruginosa	16.0 ± 0.00	-	-	-	-
Enterobacter aerogenes	14.0 ± 0.00	-	-	-	-
Salmonella choleraesuis	15.0 ± 0.00	-	-	-	-
Escherichia coli	15.0 ± 0.00	-	-	-	-
Klebsiella pneumoniae	15.0 ± 0.00	-	-	-	-

Each value corresponds to the mean ± standard deviation (n = 3) with (-) no inhibition observed. Positive control used was vancomycin for MRSA ATCC and clinical strain and streptomycin for all other bacteria.

4.1.3 Antioxidant activity

As antioxidant activity varies depending on assays, for ease of comparison, only two antioxidant assays were selected for initial screening of plants. The total phenolic content (TPC) assay was chosen as a higher amount of polyphenolic compounds are known to be positively correlated with higher antioxidant activity (Kaur and Kapoor, 2002). The total phenolic content was expressed as gallic acid equivalents (GAE) whereby a higher value indicates a higher content of phenolics in the plant extract. There is insufficient ethyl acetate extract for both *F. elliptica* and *F. racemosa* for TPC screening.

Table 11. The total phenolic content of *A. malaccensis* and *Fagraea spp.*

Extract	**Total phenolic content (g GAE/100g)**			
	A. malaccensis	***F. elliptica***	***F. gardenioides***	***F. racemosa***
Hexane	2.64 ± 0.01	0.42 ± 0.02	2.19 ± 0.03	1.61 ± 0.02
Dichloromethane	5.91 ± 0.04	1.15 ± 0.02	4.25 ± 0.06	1.83 ± 0.03
Ethyl acetate	12.66 ± 0.01	-	4.83 ± 0.13	-
Methanol	20.01 ± 0.09[a]	8.04 ± 0.07[b]	5.17 ± 0.08[c]	5.85 ± 0.11[d]
Water	9.05 ± 0.03	8.01 ± 0.02	7.99 ± 0.04	2.39 ± 0.05

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters where comparisons were made of methanol crude extracts from different plants. Equation of the gallic acid standard curve used was $y = 0.0065x + 0.0113$.

Results in Table 11 shows that the total phenolic content vary with different plants, different extraction solvents and tends to be higher in more polar extracts. An overall trend was noted whereby the methanol crude extract of each plant shows the highest phenolic content except *F. gardenioides* where water shows the best activity. Nevertheless, for ease of comparison, only statistical analysis comparing methanol extracts for all plants was carried out as they generally gives the best activity. *A. malaccensis* was noted to have significantly higher phenolic content compared to the other three plants. In contrast, *F. gardenioides* has the lowest phenolic content.

On the other hand, the DPPH assay was chosen to provide a rough measure of the primary antioxidant ability. The extent of discoloration observed reflects the ability of the antioxidant to donate hydrogen or electron transfer to stabilize and scavenge DPPH nitrogen radicals. A lower IC_{50} (the concentration of extract required to reduce half of the initial DPPH

radicals) or higher AEAC (scavenging ability relative to that of ascorbic acid) denotes a better radical scavenging ability. As the ethyl acetate extract obtained from each plant is of little amount, only four of the extraction solvents are screened for DPPH.

Table 12. The DPPH scavenging activity of *A. malaccensis* and *Fagraea spp.*

Extract	***A. malaccensis***	***F. elliptica***	***F. gardenioides***	***F. racemosa***
IC_{50} (mg/ml)				
Hexane	7.95 ± 0.11	> 50.00	6.18 ± 0.19	29.03 ± 0.20
Dichloromethane	6.54 ± 0.11	21.29 ± 0.13	9.57 ± 0.11	31.02 ± 0.02
Methanol	0.10 ± 0.00^{a}	0.79 ± 0.01^{b}	0.81 ± 0.00^{b}	3.03 ± 0.02^{c}
Water	0.43 ± 0.01	2.32 ± 0.01	9.17 ± 0.06	14.65 ± 0.05
AEAC (g AA/100g)				
Hexane	0.19 ± 0.00	NA	0.24 ± 0.00	0.05 ± 0.00
Dichloromethane	0.23 ± 0.00	0.07 ± 0.00	0.16 ± 0.00	0.05 ± 0.00
Methanol	14.80 ± 0.19^{a}	1.90 ± 0.02^{b}	1.86 ± 0.00^{b}	0.49 ± 0.00^{c}
Water	3.48 ± 0.06	0.65 ± 0.00	0.16 ± 0.00	0.10 ± 0.00

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters where small letters denote comparison between different plants of similar extraction solvent. NA indicates that values of IC_{50} and AEAC could not be determined. The IC_{50} of L-ascorbic acid (positive control) was 0.02 ± 0.00 mg/ml. Based on that, AEAC was calculated as follows: AEAC (g AA/100g) = [IC_{50} (ascorbic acid) / IC_{50} (sample)] × 100.

The IC_{50} for *F. elliptica* could not be determined. The highest concentration tested was 50mg/ml of extract resulting in approximately 20% radical scavenging ability. A further increase in concentration of extract caused cloudiness of the reaction mixture which interferes with absorbance reading. Similar to TPC, the DPPH scavenging ability was higher in more polar extracts with the highest activity in methanol extracts. *A. malaccensis* has the highest DPPH radical scavenging ability, up to seven times higher than that of the other plants. Overall, *F. racemosa* has the weakest DPPH activity.

4.1.4 Summary

Generally, antimicrobial activity of all plants was considerably weak against Gram positive bacteria with no activity against the five Gram negative bacteria tested. Consequently, antimicrobial activity was not used as a factor to select which plant to focus upon for further work. *A. malaccensis* was noted to have significantly higher phenolic content and DPPH radical scavenging ability, approximately up to twice and seven times higher than the other plants respectively. This superior antioxidant activity was the deciding factor in choosing *A. malaccensis* as the primary plant of study. Following this, a more in depth study on their biological properties was carried out before isolation and purification work.

4.2 Bioactivity screening of *A. malaccensis*

4.2.1 Introduction

Sequential extraction using five solvents of increasing polarity; hexane, dichloromethane, ethyl acetate, methanol and water, was performed on *A. malaccensis* leaves. As the solvents are of different polarity, the compounds extracted by each solvent in each fraction would be of different polarity and have different bioactivity. Thus, it is necessary to subject these fractions again to antioxidant, antimicrobial and cytotoxicity assay to determine the best solvent fraction to be focused on for isolation and purification work. The yield obtained from ethyl acetate was considerably poor. Hence, it is not chosen for future work and bioactivity screening was not carried out.

4.2.2 Phytochemical analysis

Secondary metabolites have been associated with important therapeutic benefits such as antioxidant, antimicrobial and anticancer activity. Hence, a quick phytochemical analysis was carried out to screen for the type of secondary metabolites present in *A. malaccensis* leaves with results as presented in Table 13.

The qualitative screening of *A. malaccensis* leaves reveals the presence of alkaloids, saponins, terpenoids, steroids and phenolic compounds (flavonoids and tannins). The chemical composition of the leaves of *A. malaccensis* is affected by the extraction solvent. It is seen that with increasing polarity of solvents, more phytochemicals are present. Tannins, alkaloids and saponins are notably absent in both the hexane and dichloromethane crude extract. This might be due to the polar nature of these phytochemicals which requires a more polar solvent to be extracted (Koruthu *et al.*, 2011). Polar solvents such as methanol and water seem to be better solvents at extraction of phytochemicals.

Table 13. Phytochemical screening on *A. malaccensis* extracts

Phytochemicals	**Extraction solvent**			
	Hexane	***Dichloromethane***	***Methanol***	***Water***
Flavonoids	+	+	+	+
Tannins	-	-	+	+
Terpenoids	+	+	+	+
Steroids	+	+	+	+
Alkaloids	-	-	+	+
Saponins	-	-	+	+

* (-) indicates absence and (+) indicates presence

This is the first report regarding the testing on presence of tannins in various extracts of *A. malaccensis* leaves as well as the phytochemicals found in their water extract. Water extract is important as *A. malaccensis* leaves have been widely commercialized as gaharu tea leaves. However, the leaves in this study are extracted using room temperature distilled water unlike tea preparation which usually involves boiling water. Nevertheless, water extract was discovered to contain flavonoids, tannins, terpenoids, steroids, alkaloids and saponins. The presence of these phytochemicals in the leaves suggest health benefits in consumption of the gaharu tea. Likewise, all phytochemicals tested are also present in methanol extracts. The presence of alkaloid, terpenoid, steroid, saponin and flavonoid are consistent with that reported by Huda *et al.* (2009).

Identification of the phytochemicals is useful as they may indicate potential pharmacological benefits. Flavonoids which are polyphenols have strong antioxidant activity, antimicrobial, anticancer, anti-diabetic activity and anti-inflammatory activity in *A. sinensis* leaves (Pranakhon *et al.*, 2011; Wei and Bin, 2011; Zhou *et al.*, 2008). The anti-inflammatory activity of flavonoids is due to stabilization of cell membrane and reduction in the release of inflammatory mediators. Tannins, terpenoids and phenolics were also reported to possess anti-inflammatory and anti-arthritic activity as observable in *A. agallocha* by inhibition of protein denaturation (Rahman *et al.*, 2016). Terpenoids in *Aquilaria* leaves was noted to have anticancer and antitumor activity such as cucurbitacins in *A. malaccensis* and 1-hexacosanol in *A. sinensis*. Cucurbitacins and tannins are also active antibacterial and antifungal agent (Chen *et al.*, 2014). Alkaloids have the potential to treat toothache, colic, headache,

rheumatism and pregnancy pains as well as antimalaria and antispasmodic activity (Khalil *et al.*, 2013). Hence, the presence of alkaloids suggests usage of leaves extracts as potential analgesic agents. The alkaloid isocorydine and its derivatives in *A. sinensis* leaves also display anticancer properties (Zhong *et al.*, 2014). Presence of glycoside moieties like saponins and flavonoids can also inhibit tumour growth and prevent gastrointestinal infections (Khalil *et al.*, 2013). Saponins also can precipitate and coagulate red blood cells, bind cholesterol and treat mycotic infections (Khalil *et al.*, 2013).

4.2.3 Antimicrobial activity

The results from Kirby-Bauer disc diffusion susceptibility method tested with extract of different solvents are shown in Table 14. The results differ from that of preliminary screening which utilizes a 1:1 ratio of dichloromethanol:methanol as extraction solvent. Most notable is that the methanol extract of *A. malaccensis* was able to inhibit the growth of one gram negative bacteria, *Pseudomonas aeruginosa*. Other differences include a weaker antimicrobial activity against MRSA ATCC.

Majority of the antimicrobial activity in *A. malaccensis* leaves is contained in the methanol fraction, being able to inhibit the growth of all five gram positive bacteria and *P. aeruginosa*. The hexane extract is totally inactive, dichloromethane extract only active against *Bacillus cereus* and MRSA clinical strain whereas the water extract showed activity against *Staphylococcus aureus*. Overall, methanol extract was found to be the best among all extracts but this antimicrobial activity is considerably weak, being less than half than that of the positive controls.

Although the results suggest that polar compounds have better antimicrobial activity, this postulation might be erroneous and could be due to limitations in the disc diffusion method. For instance, non-polar extracts like hexane might not be suitable for this test as poorly soluble compounds might not be able to diffuse into the agar plates, rate of diffusion affected by the molecular weight of compounds and volatile compounds present might evaporate faster leading to reduced or zero inhibition zones.

To circumvent the limitations of disc diffusion, broth microdilution technique was performed to further evaluate the antimicrobial activity. Non-polar extracts such as hexane extract would be problematic as they have poor solubility in broth media. Hence, an emulsifier which is 5% Tween was used to dissolve the hexane extract. Seven concentrations were tested ranging from 1 to 0.0156mg/ml of extract and the quantitative result obtained for minimum inhibitory concentration (MIC) is shown in Table 15.

Table 14. Antimicrobial activity of extracts of *A.malaccensis*

Microorganism	**Measurements of zone of inhibition diameter (mm)**				
	Positive control	***Hexane***	***Dichloromethane***	***Methanol***	***Water***
Gram positive					
Bacillus cereus	23.33 ± 0.58	-	6.83 ± 0.29	7.67 ± 0.29	-
Micrococcus luteus	19.67 ± 0.58	-	-	7.33 ± 0.29	-
Staphylococcus aureus (MSSA)	19.00 ± 0.00	-	-	8.83 ± 0.29	6.83 ± 0.29
MRSA ATCC (33591)	21.00 ± 1.00	-	-	8.33 ± 0.58	-
MRSA clinical strain	20.67 ± 0.58	-	8.67 ± 1.15	7.83 ± 0.29	-
Gram negative					
Escherichia coli	17.67 ± 0.58	-	-	-	-
Klebsiella pneumonia	18.00 ± 0.00	-	-	-	-
Pseudomonas aeruginosa	18.67 ± 0.58	-	-	7.17 ± 0.29	-
Enterobacter aerogenes	16.67 ±0.58	-	-	-	-
Salmonella choleraesuis	13.33 ± 0.58	-	-	-	-

* (-) no inhibition observed. Each value corresponds to the mean ± standard deviation (n = 3)

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Table 15. MIC (mg/ml) of extracts of *A.malaccensis*

Microorganism	**Minimum Inhibitory Concentration (mg/ml) (MIC)**			
	Hexane	***Dichloromethane***	***Methanol***	***Water***
Gram positive				
Bacillus cereus	>1.0	>1.0	>1.0	>1.0
Micrococcus luteus	1.0	0.5	0.5	0.5
Staphylococcus aureus (MSSA)	1.0	0.5	1.0	1.0
MRSA ATCC (33591)	1.0	0.5	0.5	1.0
MRSA clinical strain	>1.0	1.0	>1.0	1.0
Gram negative				
Escherichia coli	1.0	0.5	0.5	0.5
Klebsiella pneumonia	>1.0	1.0	>1.0	>1.0
Pseudomonas aeruginosa	1.0	0.5	0.5	0.5
Enterobacter aerogenes	>1.0	1.0	>1.0	1.0
Salmonella choleraesuis	>1.0	0.5	0.5	0.5

In disk diffusion assay, dichloromethane and water extracts barely exhibit antimicrobial activity towards the ten bacterial strains tested but this is not so for MIC. Certain gram negative bacteria which are unaffected in disk diffusion were seen to be susceptible in the broth microdilution test. This difference in results confirms the unreliability of disk diffusion testing particularly with respect to the possibility of poor diffusion of extract into the agar. According to King *et al.* (2008), a difference in results between disc diffusion and broth microdilution technique is fairly common. Among all extracts, hexane has the highest MIC denoting that it is the weakest in antimicrobial activity as it required higher concentration of extract to inhibit visible growth of bacteria. The dichloromethane and methanol extracts were the most promising for MIC.

Table 16. MBC (mg/ml) of extracts of *A.malaccensis*

Microorganism	**Minimum Bacteriocidal Concentration (mg/ml) (MBC)**			
	Hexane	***Dichloromethane***	***Methanol***	***Water***
Gram positive				
Bacillus cereus	>1.0	>1.0	>1.0	>1.0
Micrococcus luteus	1.0	0.3	0.5	0.5
Staphylococcus aureus (MSSA)	1.0	1.0	1.0	>1.0
MRSA ATCC (33591)	1.0	>1.0	>1.0	>1.0
MRSA clinical strain	>1.0	>1.0	1.0	>1.0
Gram negative				
Escherichia coli	>1.0	>1.0	>1.0	>1.0
Klebsiella pneumonia	>1.0	>1.0	>1.0	>1.0
Pseudomonas aeruginosa	1.0	1.0	1.0	1.0
Enterobacter aerogenes	>1.0	>1.0	>1.0	>1.0
Salmonella choleraesuis	>1.0	>1.0	>1.0	>1.0

Minimum bactericidal concentration (MBC) was also determined in order to investigate whether the extract is able to kill 99.9% of the bacteria after 24 hours incubation (Table 16). *Micrococcus luteus* shows the highest susceptibility to the extracts with the dichloromethane extract requiring only 0.25 mg/ml to kill the bacteria and methanol and water requiring 0.5 mg/ml. *B. cereus* was not affected by the extracts, MRSA ATCC was only susceptible to hexane and MRSA clinical strain to methanol extract. Of the five gram negative bacteria, *A. malaccensis* extracts are only bacteriocidal against *P. aeruginosa*.

Both MIC and MBC results indicate that the extracts of *A. malaccensis* leaves have the ability to inhibit bacterial growth and kill certain bacteria tested. There is only one study on

antimicrobial activity of *A. malaccensis* leaves. Hendra *et al.* (2009) reported that methanol extracts (150-300 mg/ml) have antibacterial activity against *S. aureus* and *E.coli* using disc diffusion with increasing concentrations of extracts resulting in higher diameter of inhibition zone. In contrast, all extracts (1 mg/100µl) did not showed inhibitory activity against *E. coli* when disc diffusion was carried out suggesting that a higher concentration should be tested. Nevertheless, the results of MIC where visible growth of *E. coli* was not observed at 1.0 mg/ml hexane extract and 0.5 mg/ml dichloromethane, methanol and water extract supports the study by Hendra *et al.* (2009).

4.2.4 Antioxidant activity

4.2.4.1 Total phenolic content and DPPH scavenging ability

Table 17 shows a brief summary of the statistical comparison of total phenolic content and DPPH scavenging activity among the different extraction solvents. Generally, an increase in solvent polarity is accompanied by an increase in phenolic content and DPPH scavenging activity. Methanol crude extracts of *A. malaccensis* has the highest total phenolic content and DPPH scavenging ability. The total phenolic content was approximately double that of hexane, dichloromethane and water extracts and the DPPH activity (AEAC) quadruples that of others. Methanol extracts contains polar compounds such as phenolics, glycosides, alcohols and anthocyanins. These compounds may account for its high antioxidant activity.

Table 17. Total phenolic content and DPPH activity of *A. malaccensis* extracts

Extraction solvent	**Total phenolic content (g GAE/100g)**	**DPPH radical scavenging ability**	
		IC_{50} (mg/ml)	**AEAC (mg AA/100g)**
Hexane	2.64 ± 0.01^{d}	7.95 ± 0.11^{d}	0.19 ± 0.00^{d}
Dichloromethane	5.91 ± 0.04^{c}	6.54 ± 0.11^{c}	0.23 ± 0.00^{c}
Methanol	20.01 ± 0.09^{a}	0.10 ± 0.00^{a}	14.80 ± 0.19^{a}
Water	9.05 ± 0.03^{b}	0.43 ± 0.01^{b}	3.48 ± 0.06^{b}

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) between different solvent fractions are indicated by different superscript letters.

The findings that methanol extract in *A. malaccensis* has the highest antioxidant activity compared to other solvents are similar to that of several studies. Kshirsagar and Upadhyay (2009) found that 92.62% antioxidant activity was found in methanol extracts of aerial parts. Likewise, Huda *et al.* (2009) reported DPPH activity with IC_{50} values of 0.80, 0.16, 0.14 and 0.03 mg/ml for hexane, dichloromethane, ethyl acetate and methanol extracts respectively for *A. malaccensis* leaves. The DPPH scavenging activity obtained was considerably lower than that of Huda *et al.* (2009) and this could be due to different extraction conditions and plant samples. Another contributing factor is possibly the age of the leaves. Hendra *et al.* (2016) has found that old leaves shows higher antioxidant activity compared to young leaves and postulates that this might be due to a higher content of secondary metabolites in old leaves as their longer growth period means more resources produced for defense than younger leaves.

There is no study on phenolic content of *A. malaccensis* leaves. However, a comparison of total phenolic contents to that of commonly consumed vegetables and fruits (68-400 mg GAE/100 g) indicate that *A. malaccensis* leaves (2638-200005 mg GAE/100 g) have very high phenolic content which could be beneficial to health (Kaur and Kapoor, 2002). This is exciting as many studies have demonstrated a linear relationship between antioxidant activity and total phenolic content of plant extracts (Kaur and Kapoor, 2002).

4.2.4.2 Ferric reducing power (FRP)

The FRP assay determines the primary antioxidant ability of plant extracts, more specifically the reducing ability of an antioxidant to terminate the oxidation chain reaction via electron transfer. A higher reducing power is denoted by a low IC_{50} or high AEAC with results as in Table 18.

It is seen that polar extracts such as methanol and water extract shows better ferric reducing power. The methanol extract was found to have the highest reducing power, more than double that of others. The ranking of the different solvent extracts for total reducing power was observed to be similar to that of TPC and DPPH activity with the methanol fraction having the highest antioxidant ability, followed by the water, dichloromethane and lastly hexane fractions.

Table 18. Total reducing activity of *A. malaccensis* extracts

Extraction solvent	**IC_{50} (mg/ml)**	**AEAC (mg AA/g)**
Hexane	0.29 ± 0.00^{a}	75.04 ± 0.85^{a}
Dichloromethane	0.13 ± 0.00^{b}	163.86 ± 3.28^{b}
Methanol	0.05 ± 0.00^{c}	492.97 ± 3.94^{c}
Water	0.09 ± 0.00^{d}	238.49 ± 1.17^{d}

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) within a column are indicated by different superscript letters. The IC_{50} of L-ascorbic acid (positive control) was 0.02 ± 0.00 mg/ml.

4.2.4.3 Metal chelating ability

The metal chelating assay provides a measure of the secondary antioxidant ability. Secondary antioxidants are often preventive where they hinder the initiation of the oxidation chain. Metal chelation is an important aspect of antioxidant function as transition metals can cause oxyradicals production and lipid peroxidation in the biological system (Dinis *et al.*, 1994). As usage of a higher concentration of plant extracts tends to result in cloudiness of solution, 1 mg/ml was fixed as the maximum concentration where all solutions of crude extracts tested remain clear at this concentration.

Figure 12 shows the percentage metal chelating ability of *A. malaccensis* extracts. As IC_{50} could not be determined for most of the extract, percentage metal chelating activity was compared at the same concentration for all extracts and to the gallic acid standard to give a rough measure of their metal chelating activity. At 1 mg/ml, the percentage metal chelating ability was 13.33%, 30.20%, 33.47%, 54.89% and 47.66% for gallic acid, hexane, dichloromethane, methanol and water respectively. This indicates that more polar extracts has a higher metal chelating ability with the methanol extract of *A. malaccensis* having the highest ability. The ranking of solvent extracts in terms of antioxidant potential was similar to that of TPC, DPPH and FRP. Of notable mention, the metal chelating ability for all extracts are higher than that of gallic acid suggesting that *A. malaccensis* leaves has strong secondary antioxidant capabilities.

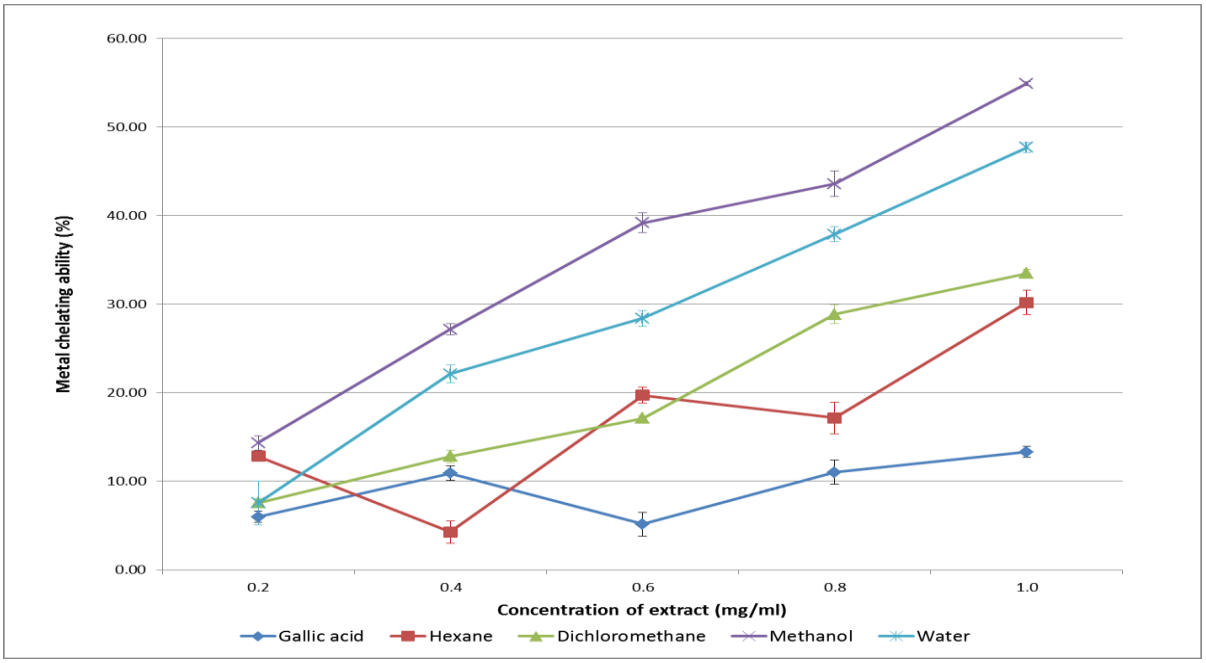


Figure 12. Percentage metal chelating ability of *A. malaccensis* extracts. Plots are of mean ± standard deviation (n = 3).

4.2.4.4 β-carotene bleaching (BCB) inhibition activity

The BCB assay measures the ability of plant extracts to inhibit the bleaching of β-carotene. This is important as it mimics the lipid peroxidation process that naturally occurs in food and biological processes (Koleva *et al.*, 2002). Results are reported in Table 19.

Table 19. β-carotene bleaching inhibition activity of *A. malaccensis* extracts.

Extraction solvent	**IC_{50} (mg/ml)**	**BCBA (%) relative to BHA activity**
Hexane	0.14 ± 0.00^{a}	43.71 ± 1.00^{a}
Dichloromethane	0.09 ± 0.00^{b}	67.19 ± 1.45^{b}
Methanol	0.05 ± 0.00^{c}	126.41 ± 2.28^{c}
Water	0.06 ± 0.00^{d}	98.31 ± 0.18^{d}

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters. The IC_{50} of BHA (positive control) was 0.06 ± 0.00 mg/ml.

Methanol extract has the highest BCB inhibition activity, followed by water extract, dichloromethane extract and hexane extract. Butylated hydroanisole (BHA) which is used as a positive control in the test is a synthetic antioxidant commonly used as food preservatives. Interestingly, both methanol and water extracts has comparable activity to that of BHA, 126% and 98% respectively, indicating their potential to be used to retard oxidative deterioration of lipid-rich foods.

4.2.5 Cytotoxicity testing using MTT assay

The anti-proliferative activity of *A. malaccensis* leaves extracts was screened using MTT assay. MTT assay was chosen as prior testing of the crude extracts using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was noted to be inaccurate whereby observations under the microscope do not correlate with that of absorbance reading. It was found that the extract itself interacts with the MTS reagent giving erroneous results. This is circumvented in MTT, where the extracts were removed prior to addition of the MTT reagent. MCF-7 or breast cancer cells were chosen for cytotoxicity testing as breast cancer is the most common cancer in the world and in Malaysia with the National Cancer Registry reporting an age-standardized rate (ASR) of 31.1 per 100,000 population in Malaysia (Manan *et al.*, 2016).

The MTT assay is a colorimetric method to determine cell viability (Wu *et al.*, 2008). The absorbance of the extract-treated cells was compared to their respective control. A lower absorbance from that of control denotes a decrease in cell viability. The results of MTT assay after 24 hours of treatment with plant extracts are expressed in IC_{50}, the concentration of compound where 50% cells are viable. This is summarized in Table 20.

Table 20. IC_{50} of *A. malaccensis* extracts against MCF-7 cell line

Extraction solvent	**IC_{50} (µg/ml)**
Hexane	> 400.00
Dichloromethane	NA
Methanol	160.99
Water	> 400.00

NA indicates that values of IC_{50} could not be determined while > 400 indicates that after 24 hours, samples did not reach IC_{50} even at a high concentration of 400µg/ml.

Methanol extract shows the highest anti-proliferative activity against MCF-7 breast cancer cell lines. The IC_{50} for dichloromethane crude extract was not determined as clumping was observed under the microscope despite filtration of samples using a 0.22µm syringe filter before cell treatment. Both hexane and water extract did not reach IC_{50} even at a high concentration of 400µg/ml.

The criteria of promising cytotoxic activity for plant extracts are $IC_{50} < 30$ µg/ml by the American National Cancer Institute (NCI) (Talib and Mahasneh, 2010). In other words, any extract that requires more than 30µg/ml is considered weak in terms of cytotoxicity ability. Although the methanol crude extract of *A. malaccensis* shows anti-proliferative activity, its IC_{50} was 160.99 µg/ml denotes weak activity.

4.2.6 Summary

The methanol extract of *A. malaccensis* leaves was chosen for further isolation and purification work based on several justifications. Although the presence of flavonoids, tannins, terpenoids, steroids, alkaloids and saponins are detected in both methanol and water extracts, methanol extract is preferable as this compound has higher stability at room temperature. The water extract were powdery after freeze-drying but when left at room temperature for prolonged durations turned into a brown viscous liquid. In contrast, the methanol extracts remain in powder form regardless of temperature differences. The high polar nature of water extract might also be problematic in compound separation through column chromatography.

Further bioactivity testing supports the selection of methanol extract. Antimicrobial activity of *A. malaccensis* leaves is weak. Nevertheless, methanol extract has the best activity in disc diffusion as it is able to inhibit the growth of all five gram positive bacteria tested and *Pseudomonas aeruginosa*. In MIC and MBC, methanol is the second most potent antimicrobial after dichloromethane extract. Although methanol crude extract shows the best anti-proliferative activity against MCF-7 breast cancer cell lines among all extracts in the MTT assay, it does not meet the NCI's IC_{50} criteria. Considering the weak antimicrobial and anticancer activity, antioxidant activity becomes the main criteria for selection of the best solvent extract.

Methanol extract also gives the highest antioxidant activity, followed by water extract, dichloromethane extract and lastly hexane extract. Remarkably, all the five antioxidant assays tested which are TPC, DPPH, FRP, MCA, and BCB displays exactly the same trend indicating that methanol has both strong primary and secondary antioxidant capabilities. This is of great significance as increased consumption of plants rich in phenolic compounds with

potent antioxidant activity has been linked to a lower risk of chronic diseases caused by oxidative stress (Fresco *et al.*, 2010).

There are many advantages in the usage of methanol as extraction solvent. The polarity index for hexane, dichloromethane, methanol and water are 0.1, 3.1, 5.1 and 10.2 respectively. Methanol being of intermediate polarity seems to be the best solvent for extraction of bioactive compounds from *A. malaccensis* leaves due to its wider range of solubility. It can extract both polar and moderately polar or slightly non-polar compounds particularly phenolic compounds. This differs from hexane and dichloromethane which tends to extract non-polar compounds whereas water extracts being specific for very polar compounds. The results of the phytochemical tests support this where more compounds were extracted by methanol which might have accounted for the higher biological activity in antimicrobial, antioxidant and cytotoxicity testing. In addition, methanol can cause cell membrane instability facilitating extraction of phenolic compounds. It can also inactivate enzymes like polyphenol oxidase which oxidizes phenolic compounds resulting in improved stability of the extracted compounds (Hurst, 2008).

4.3 Isolation and purification of methanol extract of *A. malaccensis*

4.3.1 Introduction

Isolation and purification work is essential to remove undesirable compounds and target bioactive compounds. As methanol extract of *A. malaccensis* leaves shows strong antioxidant activity, it was subjected to column chromatography using silica gel 60 to isolate out compounds of interest. Bioassay-guided fractionation was carried out where fractions of interest with high bioactive properties are subjected to repeated rounds of column chromatography until fractions are sufficiently pure.

4.3.2 Fractions collected from column chromatography

The methanol extract was subjected to column chromatography using silica and eluted with increasing polarity of solvent mixtures from hexane to chloroform to ethyl acetate to methanol. Fractions were collected based on the bands observable during separation and by volume of 250ml when bands are not present. The collected fractions are then subjected to rotary evaporation to concentrate them. Thin layer chromatography (TLC) was then used to determine fraction similarity. Fractions with similar migrating pattern on TLC plates as observed under short-wave and long-wave UV light were pooled together. This yields 26 pooled fractions. However, only four sub-fractions are of sufficient amount for bioactivity

screening and further purification work. Bioactivity-guided fractionation was carried out where the pooled fractions were screened for antioxidant activity and cytotoxicity against MCF-7 cell lines.

4.3.3 Antioxidant activity of sub-fractions of methanol extract

4.3.3.1 Selection of antioxidant assays

Prior screening of *A. malaccensis* extracts in this study involves a panel of antioxidant assays in the belief that each assay reflects a different aspect of antioxidant behavior of the extract and together they can provide a better estimate on overall antioxidant capacity. Results of the screening revealed that TPC, DPPH, FRP, MCA and BCB displayed the same trend suggesting that less assays may be used for antioxidant screening of methanol sub-fractions. Regression analysis shows positive correlation (R^2) between TPC and AEAC of DPPH (R^2 = 0.96); TPC and AEAC of FRP (R^2 = 0.99); and TPC and percentage BCBA (R^2 = 0.90). Therefore, to reduce the waste of potentially valuable extracts particularly with regards to significantly lower yield obtained after each column separation, only two antioxidant assays TPC and DPPH were chosen to screen the sub-fractions obtained from methanol extract of *A. malaccensis* leaves.

This decision was based on several considerations. Many studies have noted that high phenolic content is associated with high antioxidant activity supporting the choice of inclusion of TPC assay. Metal chelating assay was not chosen as IC_{50} could not be determined. BCB was not selected as it is slow, tedious, and reproducibility of results severely affected by emulsion preparation and antioxidant portioning into different phases of the emulsion (Koleva *et al.*, 2002). Clarke *et al.* (2013) has stated that usage of TPC, DPPH and FRAP assays in parallel is redundant as the assays displayed a high ($R^2 \geq 0.82$) and significant correlation with each other in the screening of 92 extracts from 27 Malaysian plants. DPPH and FRP detect similar mechanism which is the transfer of electron from the antioxidant to reduce an oxidant which means both DPPH and FRP essentially give identical results. Indeed, regression analysis displays positive correlation of R^2 = 0.95 between AEAC of DPPH and AEAC of FRP. DPPH was chosen instead of FRP as color of extracts can affect FRP results (Clarke *et al.*, 2013).

4.3.3.2 Total phenolic content

The total phenolic content of the four sub-fractions of interest were screened with results as in Table 21. Statistical analysis shows that sub-fraction 22 has the highest total phenolic content. This is followed by sub-fraction 24 and lastly 21 and 23 which have comparable phenolic content. The total phenolic content of all four sub-fractions adds up to approximately 41.17g GAE/100g which is double that of the phenolic content in the methanol extract (20.01g GAE/100g) before column chromatography. This indicates that there were presence of undesirable compounds in the methanol extract and their removal can gives rise to increased ability to reduce the Folin-Ciocalteu's reagent and, hence, detection of higher phenolic content.

Table 21. Total phenolic content of sub-fractions of methanol extract

Fraction number	**Total phenolic content (g GAE/100g)**
21	6.90 ± 0.04^{a}
22	17.73 ± 0.21^{b}
23	6.68 ± 0.42^{a}
24	9.86 ± 0.56^{c}

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters. Equation of the gallic acid standard curve used was $y = 0.0065x + 0.0113$.

4.3.3.3 DPPH radical scavenging activity

Sub-fraction 22 has found to have the highest DPPH radical scavenging activity (see Table 22). This is followed by sub-fraction 23, 21 and lastly 24. The difference in activity of sub-fraction 22 is particularly profound with an AEAC value that is seven times that of the second highest value in sub-fraction 23. The total DPPH activity detected in the sub-fractions (29.65g AA/100g) were higher compared to the methanol extract (14.80g AA/100g) suggesting that some compounds that interfere with reducing activity has been removed by column chromatography.

Table 22. DPPH scavenging activity of sub-fractions of methanol extract

Fraction number	IC_{50} (mg/ml)	AEAC (g AA/100g)
21	0.54 ± 0.00^{a}	2.78 ± 0.01
22	0.07 ± 0.00^{b}	23.15 ± 0.52
23	0.48 ± 0.00^{c}	3.14 ± 0.01
24	2.62 ± 0.06^{d}	0.58 ± 0.01

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters. The IC_{50} of L-ascorbic acid (positive control) was 0.02 ± 0.00 mg/ml. Based on that, AEAC was calculated as follows: AEAC (g AA/100g) = [IC_{50} (ascorbic acid) / IC_{50} (sample)] × 100.

4.3.4 Cytotoxicity testing using MTT assay

All sub-fractions showed very poor or non-existent anti-proliferative activity against MCF-7 breast cancer cells after 24 hours of treatment as seen in Table 23. Sub-fraction 24 was found to be the best with an IC_{50} of 342.16µg/ml whereas the other fractions do not reach IC_{50} even when high concentrations of 400µg/ml extract were used. Given that NCI's criteria of promising cytotoxic activity for plant extracts are $IC_{50} < 30$ µg/ml, sub-fraction 24 is not a good anticancer agent.

Table 23. IC_{50} of methanol sub-fractions against MCF-7 cell line

Fraction number	IC_{50} (µg/ml)
21	> 400
22	> 400
23	> 400
24	342.16

IC_{50} > 400 indicates that after 24 hours, samples did not reach IC_{50} even when at 400µg/ml concentration.

The anti-proliferative activity were considerably worse compared to the crude methanol extract ($IC_{50:}$ 160.99µg/ml) before column chromatography implying that compounds removed in the separation and purification process were the ones that have

cytotoxicity or provide synergistic and modulative effects that are essential for anti-proliferative activity against breast cancer cell lines.

4.3.5 Selection of sub-fraction for further work

Sub-fractions 21 and 23 have poor antioxidant activity and no anti-proliferative activity and were not considered for further work. Sub-fraction 24 has the second highest phenolic content. Intriguingly, sub-fraction 24 which had higher phenolic content compared to sub-fractions 21 and 23 had a significantly lower DPPH activity, being only one-fifth of the later. The poor correlation between phenolic content and DPPH activity differs from previous screenings which had always indicated good correlation. Nevertheless, several studies did report that there is no correlation between scavenging activity and TPC (Othman *et al.,* 2014; Sulaiman *et al.,* 2011).

A possible reason for the low DPPH despite high phenolic content could be that sub-fraction 24 was observed to have lower solubility in methanol solvent compared to the other fractions. It could also be due to the lack of specificity of the Folin-Ciocalteu reagent which measures reducing ability and is unable to differentiate between phenol type and non-phenolic substances, giving an inaccurate and overestimation of TPC (Prior *et al.*, 2005). In addition, a high yield of phenolic compounds may not necessarily have a high antioxidant activity as antioxidant activity might depend on synergistic effects of the extracted phenolic compounds as well as polarities of the chemical structure of the compounds (Singleton *et al.*, 1999; Thoo *et al.*, 2010). Sub-fraction 24 has the best anti-proliferative activity against breast cancer cells. However, the IC_{50} of 342.26µg/ml is very weak compared to the 30µg/ml criteria and the anti-proliferative activity is also significantly worse after column chromatography. Henceforth, MTT assay will no longer be carried out in screening of bioactivity to conserve amount of plant extracts.

Sub-fraction 22 has the highest TPC and DPPH scavenging activity among all fractions but no discernible anti-proliferative activity against MCF-7 cells. The antioxidant activity was notably higher than that of the methanol crude extract before column chromatography. Overall, future bioactive screening will focus primarily on antioxidant activity and sub-fraction 22 was chosen for subsequent purification work using column chromatography.

4.4 Isolation and purification of sub-fraction 22 of *A. malaccensis*

4.4.1 Introduction

Sub-fraction 22 which had the most promising antioxidant activity was further purified with another round of gravity column chromatography using silica. Sub-fractions were screened for antioxidant activity and ones with higher potential selected for further purification. HPLC was also used to narrow down fractions with fewer compounds present and of higher purity.

4.4.2 Sub-fractions collected from column chromatography

Sub-fraction 22 was subjected to column chromatography and eluted with increasing polarity of solvent mixtures from hexane to chloroform to ethyl acetate to methanol. Fractions were collected based on the bands observable during separation and by volume of 100ml when bands are not present. Sub-fractions with similar pattern on TLC were pooled together yielding 11 different sub-fractions. After freeze drying, sub-fraction 8 and 11 was not soluble in methanol even with sonication.

4.4.3 DPPH radical scavenging activity

The yield collected from column chromatography of sub-fraction 22 was quite low. In order to conserve the quantity of sample for other spectrometric analysis for identification purposes, only DPPH screening was done with results as found in Table 24. The DPPH activity of sub-fraction 22-2 could not be determined as IC_{50} was not reached even when 50 mg of sample was tested which was more than half of the yield obtained. Likewise, DPPH could not be determined for sub-fractions 22-8 and 22-11 which have poor solubility in methanol solvent.

The DPPH activity of each sub-fraction was found to be significantly different from each other. Sub-fraction 22-3 has the highest DPPH scavenging activity, followed by sub-fractions 22-4, 22-6, 22-7, 22-9, 22-1, 22-5 and lastly sub-fraction 22-10. The total sum of AEAC of all sub-fractions (22-1 to 22-10) were approximately 4.26 g AA/100g which is significantly inferior to the AEAC of 23.15 g AA/100 g of the sub-fraction 22. There is a loss of nearly 81.60% DPPH activity after this round of column chromatography.

Table 24. DPPH scavenging activity of sub-fractions 22-1 to 22-10

Fraction number	Mass collected (mg)	DPPH	
		IC_{50} (mg/ml)	**AEAC (mg AA/100g)**
22-1	121.87	10.67 ± 1.15^{a}	311.75 ± 34.35
22-2	72.96	-	-
22-3	240.01	3.28 ± 0.09^{b}	1006.32 ± 27.10
22-4	28.61	3.61 ± 0.13^{c}	915.56 ± 32.38
22-5	138.37	13.65 ± 1.11^{d}	242.76 ± 19.67
22-6	632.71	4.65 ± 0.24^{e}	711.46 ± 35.41
22-7	416.12	5.96 ± 0.23^{f}	554.05 ± 21.46
22-8	235.68	-	-
22-9	102.40	7.77 ± 0.39^{g}	425.46 ± 21.57
22-10	159.63	35.32 ± 5.22^{h}	94.81 ± 14.03
22-11	154.25	-	-

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters. The IC_{50} of L-ascorbic acid (positive control) was 0.03 ± 0.00 mg/ml. Based on that, AEAC was calculated as follows: AEAC (mg AA/100g) = [IC_{50} (ascorbic acid) / IC_{50} (sample)] × 100000.

4.4.4 Selection of sub-fractions for further work

Three sub-fractions of fraction 22 which are 22-3, 22-4 and 22-6 were selected for further work. This is mainly due to their high DPPH scavenging activity. However, the yield obtained and TLC pattern were also taken into account as criteria for selection and the choice of technique for further purification. Sub-fractions 22-3 (240.01 mg) and 22-4 (28.61 mg) exhibit one spot and two spots on TLC plates respectively. Given the lower yield and TLC pattern, these two sub-fractions are directly analyzed using reverse-phase HPLC to determine their purity with preparative HPLC to attempt to separate the compounds present. Sub-fraction 22-6 which has the highest yield of 632.71 mg showed four spots on TLC plate and will be subjected to column chromatography to further isolate out compounds of interest.

4.5 Sub-fractions 22-3 and 22-4

4.5.1 HPLC analysis of sub-fraction 22-3

Sub-fraction 22-3 was analyzed using reverse phase HPLC using a C18 analytical column and methanol mobile phase at four different wavelengths. A preliminary gradient run was done from 10% to 90% methanol for 45 minutes. Based on their elution profile, further optimization was made.

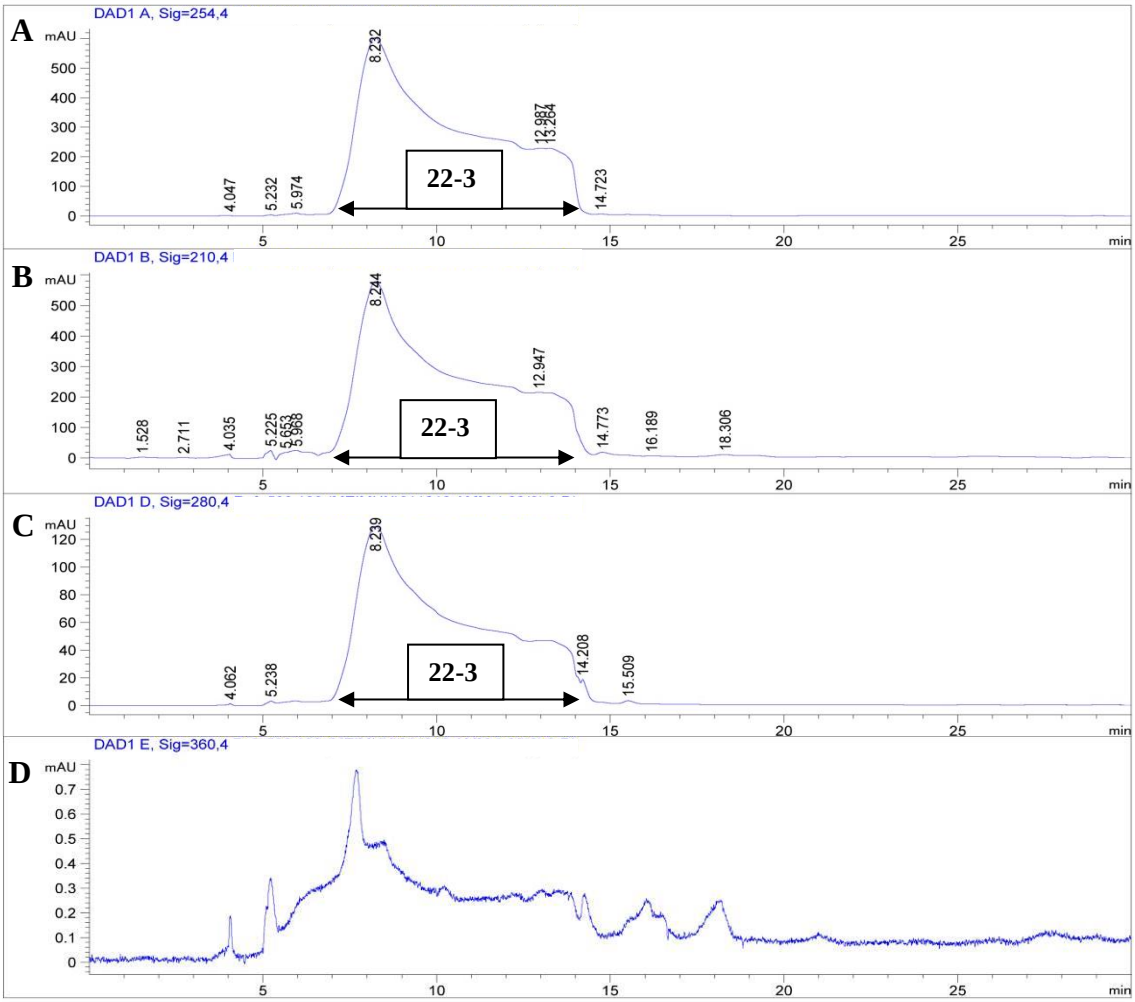


Figure 13. HPLC chromatogram of sub-fraction 22-3. Wavelengths detected at **A.** 254nm; **B.** 210nm; **C.** 280nm; **D.** 360nm.

A very broad peak was detected in sub-fraction 22-3 at isocratic 50% methanol with a runtime of 30 minutes as shown in Figure 13. It can be deduced that there are two or more compounds present but they are poorly resolved. In attempts for better separation, flow rate was reduced to 0.5ml/min, lesser volume of sample injected to avoid overloading and change

of mobile phase was done. However, the compounds could not be separated. Preparative HPLC was not carried out as the compounds elution time were close to each other.

4.5.2 HPLC analysis of sub-fraction 22-4

Sub-fraction 22-4 was analyzed using reverse phase HPLC using a C18 analytical column and methanol mobile phase at four different wavelengths. A gradient run was done from 10% to 90% methanol for 30 minutes with the elution profile as in Figure 14.

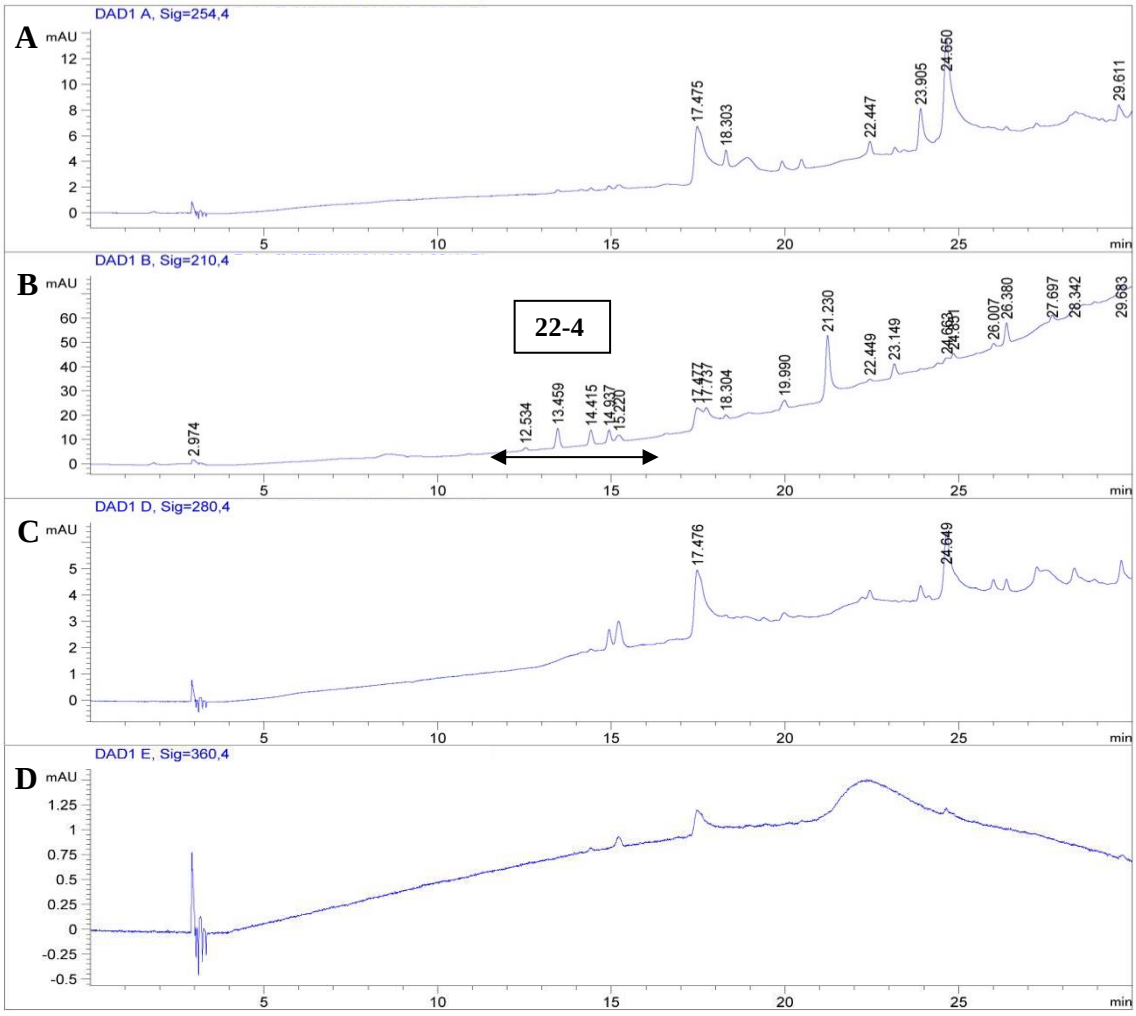


Figure 14. HPLC chromatogram of sub-fraction 22-4. Wavelengths detected at **A.** 254nm; **B.** 210nm; **C.** 280nm; **D.** 360nm.

Five peaks were observed from retention time 12-15 minutes at wavelength 210nm. Attempts to isolate out these compounds using preparative HPLC column (Agilent Zorbax SB-C18, 21.2 x 100mm, 5 µm), gradient 10-90% methanol, 45 mins at flow rate of 5ml/min using 20mg of the sub-fraction was not successful.

4.5.3 Summary

Sub-fraction 22-3 showed one broad peak under HPLC which could consist of several compounds. Optimization of HPLC procedures was not able to properly resolve the compounds and hence preparative work was not carried out. Sub-fraction 22-4 was observed to contain five compounds of interest in HPLC but attempts to isolate out the compounds using preparative HPLC were not successful. Proton NMR (Appendix II) was done too; results showed that the compounds were not of sufficient purity for identification. With regards to this, sub-fraction 22-6 will be the primary focus to isolate out pure compounds.

4.6 Sub-fraction 22-6

4.6.1 LC-MS/MS analysis

Sub-fraction 22-6 is the primary sub-fraction of interest from methanol crude extract due to having the highest mass collected (632.71 mg) after column chromatography as well as good antioxidant activity. This fraction was sent out for LC-MS/MS analysis to Advanced Chemistry Solutions to evaluate the type of phytochemicals present in the extract. Both positive ionization and negative ionization using electrospray ionization (ESI)-MS were carried out to detect the presence of both protonated and deprotonated compounds which can also provide complementary structural information to each other (Banerjee & Mazumdar, 2012). For instance, the positive ion mode is appropriate for compounds with amino groups, amides or carbonyls which have strong proton affinity. On the other hand, negative ion mode is suitable for compounds with carboxyl or phenolic hydroxyl groups. Both chromatograms are as in Figure 15.

Numerous broad peaks were observed in both of the liquid chromatography (LC) chromatograms. The positive ionization mode detected up to 13 compounds whereas the negative ionization mode detected up to 14 compounds. However, matching of the compounds detected to NIST11 library using mass spectrum pattern only managed to identify two compounds for positive mode and three compounds for negative mode. A summary of the chemical structures of the compounds identified and their bioactivity based on existing literature is provided in Table 25 with detailed MS chromatograms in the Appendix III.

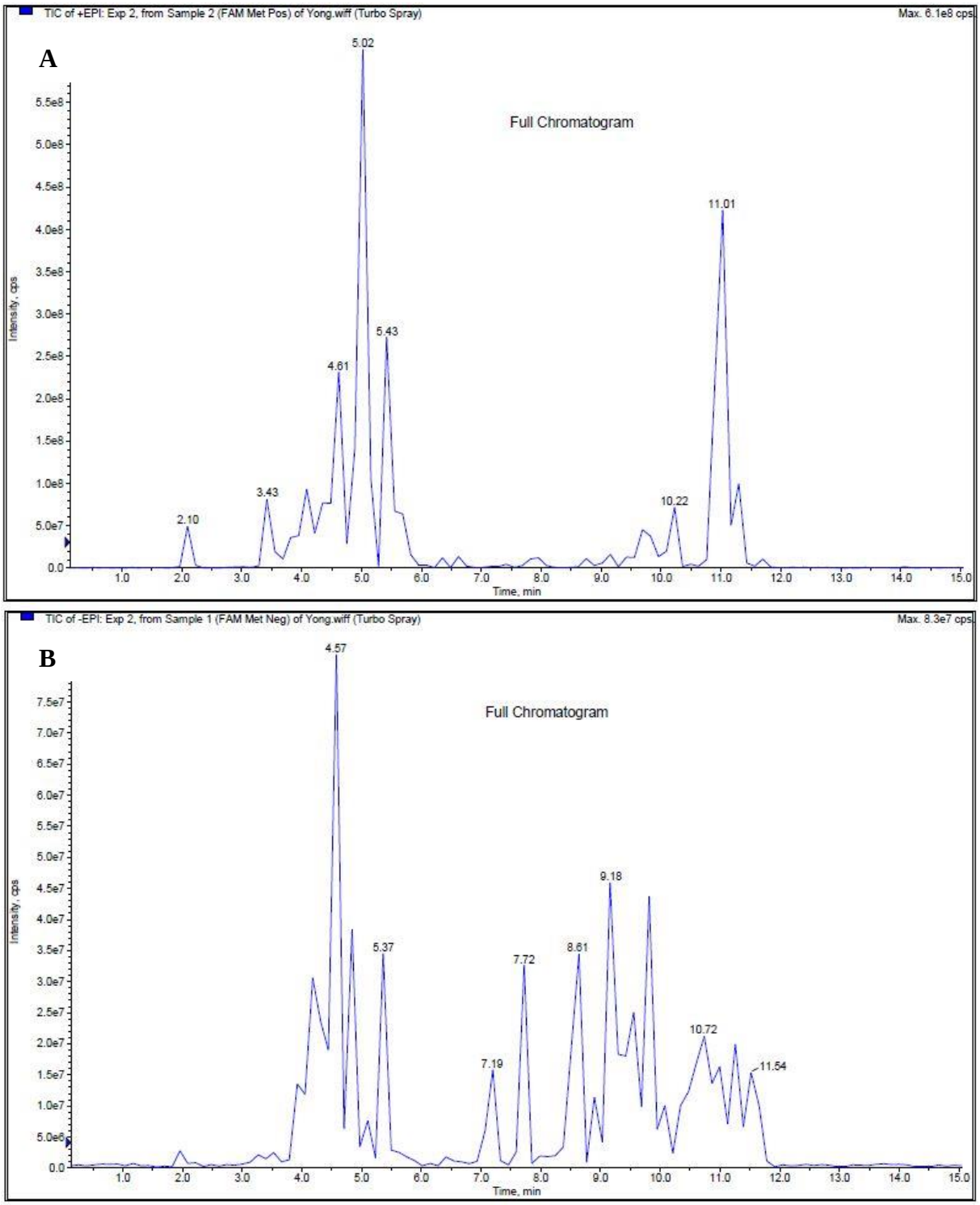
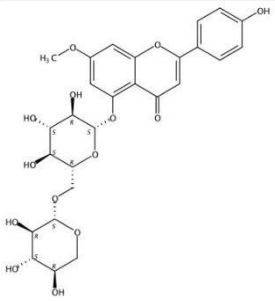
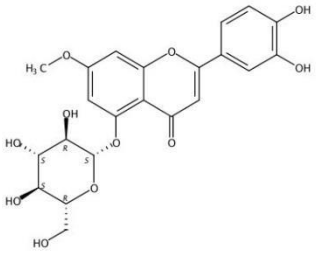
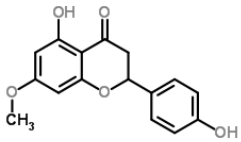
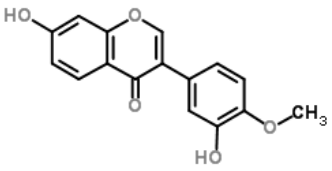


Figure 15. LC chromatograms of *A. malaccensis* methanol fraction. A: Positive ionization mode; B. Positive ionization mode.

Table 25. Compounds identified in sub-fraction 22-6 by LC-MS.

Retention Time (RT)	**Library/ID Chemical**	***m/z***	**Structure**	**Reported bioactivity**
Negative ionization				
4.184	Genkwanin-5-O-β-D-primeveroside (yuankwanin)	576.99		None
5.757	Luteolin-7-methylether-5-O-β-D-glucopyranoside	461.00		None
7.722	Genkwanin	282.94		Anti-inflammatory (Gao et al., 2014)
Positive ionization				
3.420	Calycosin-o-pentosyl-o hexoside	579.25	None	None
3.953	Calycosin	285.37		Angiogenesis (Tang *et al.*, 2010), Neuroprotective (Guo *et al.*, 2012), Anti-tumor MCF-7 (Chen *et al.*, 2011; Chen *et al.*, 2014a)

*Structures derived from www.chemspider.com and that of genkwanin and luteolin derivatives from Chen *et al.* (2013).

In LC-MS positive ionization $(M+H)^+$ mode, the highest peak at retention time of 5.02 with over 20 *m/z* fragment patterns could not be identified. Calycosin and calycosin-o-pentosyl-o hexoside were identified from peaks of very low intensity approximately 15% of that of the highest peak. Calycosin is a phytoestrogen that can be found in plants of the *Fabaceae* family such as the herb *Radix astragali* (Chen *et al.*, 2011a). There is no previous report on calycosin in plants of the *Aquilaria* genus.

Similarly, in LC-MS negative ionization $(M-H)^-$ mode, the most intense peak was observed at retention time 4.57 with mass-to-charge ratio (*m/z*) of fragments at 298, 313, 607. However, the compounds for this peak could not be identified by library matching nor did a search of literature yield compounds of similar mass spectrum pattern. Only three compounds were identified which were genkwanin, genkwanin-5-O-β-D-primeveroside and luteolin-7-methylether-5-O-β-D-glucopyranoside but these were at peaks of lower intensity being approximately less than 35% of the height of the highest peak. Both genkwanin and genkwanin-5-O-β-D-primeveroside have been previously reported by Feng and Yang (2012) in the leaves of *Aquilaria sinensis*. There has yet to be any reports on luteolin-7-methylether-5-O-β-D-glucopyranoside isolated from the *Aquilaria* genus although luteolin and several of its derivatives have been discovered in leaves of *A. sinensis* (Qi *et al.*, 2009). Nevertheless, luteolin-7-methylether-5-O-β-D-glucopyranoside has been identified in plants of the *Daphne* genus (Su *et al.*, 2008; Chen *et al.*, 2013). Both plants of the *Daphne* and *Aquilaria* genera are of the same Thymelaeaceae family.

The presence of approximately 27 compounds in the extract detected by LC-MS was contrary to its TLC pattern indicating that further work needs to be done. The compounds identified are mainly polyphenols and flavonoids. They have been known to have strong antioxidant properties and may account for the high DPPH activity found in this fraction.

4.6.2 Sub-fractions collected from column chromatography

Sub-fraction 22-6 was again separated using silica gravity column chromatography. The fractions obtained are pooled into sub-fractions 22-6-1 and 22-6-2. DPPH assay was carried out to determine which sub-fraction to focus upon with activity as reported in Table 26.

Interestingly, the total DPPH activity of both sub-fractions 22-6-1 and 22-6-2 after column chromatography were higher than sub-fraction 22-6 (711.46 mgAA/100g). Of the two fractions, the DPPH scavenging activity is mainly found in 22-6-2. Hence, sub-fraction 22-6-2 with only one spot on the TLC (3:2 dichloromethane: methanol; R_f 0.86) was primarily of interest for further isolation work.

Table 26. DPPH scavenging activity of sub-fractions 22-6-1 and 22-6-2

Fraction number	Mass collected (mg)	DPPH	
		IC_{50} (mg/ml)	**AEAC (mg AA/100g)**
22-6-1	70.19	10.56 ± 0.52^{a}	312.89 ± 15.64
22-6-2	96.96	1.76 ± 0.05^{b}	1875.71 ± 56.11

Each value corresponds to the mean ± standard deviation (n = 3). Significant differences ($p < 0.05$) are indicated by different superscript letters. The IC_{50} of L-ascorbic acid (positive control) was 0.03 ± 0.00 mg/ml. Based on that, AEAC was calculated as follows: AEAC (mg AA/100g) = [IC_{50} (ascorbic acid) / IC_{50} (sample)] × 100000.

4.6.3 Sub-fraction 22-6-2

The extract of sub-fraction 22-6-2 was noted to be able to form fine needle-like yellowish crystals when it is in highly concentrated form. Following this observation, crystallization was performed. Sub-fraction 22-6-2 was first freeze-dried to a powdery extract which is re-dissolved in methanol solvent. Drops of diethyl ether were then added to the side of the trident vial every two days allowing the solution to flow into the methanol solvent. After a few months with the vials left undisturbed, crystals were noted to form. Excess solvent was removed using Pasteur pipette and followed by freeze-drying. The yield obtained was 61.21mg in total. It should be noted that the fine nature of the needle-like compound means that they are relatively weightless and that the mass was calculated based on vial weight before and after crystallization and freeze-drying. In other words, the mass is an estimated value.

This compound was analyzed using reverse phase HPLC C18 column. Best separation was optimized at isocratic 40% methanol, 1 ml/min for 30 minutes with the elution profile in Figure 16. Only one peak was detected at retention time of 24.36 min indicating that the compound was of sufficient purity. As a result, the sample was sent for LC-MS/MS analysis.

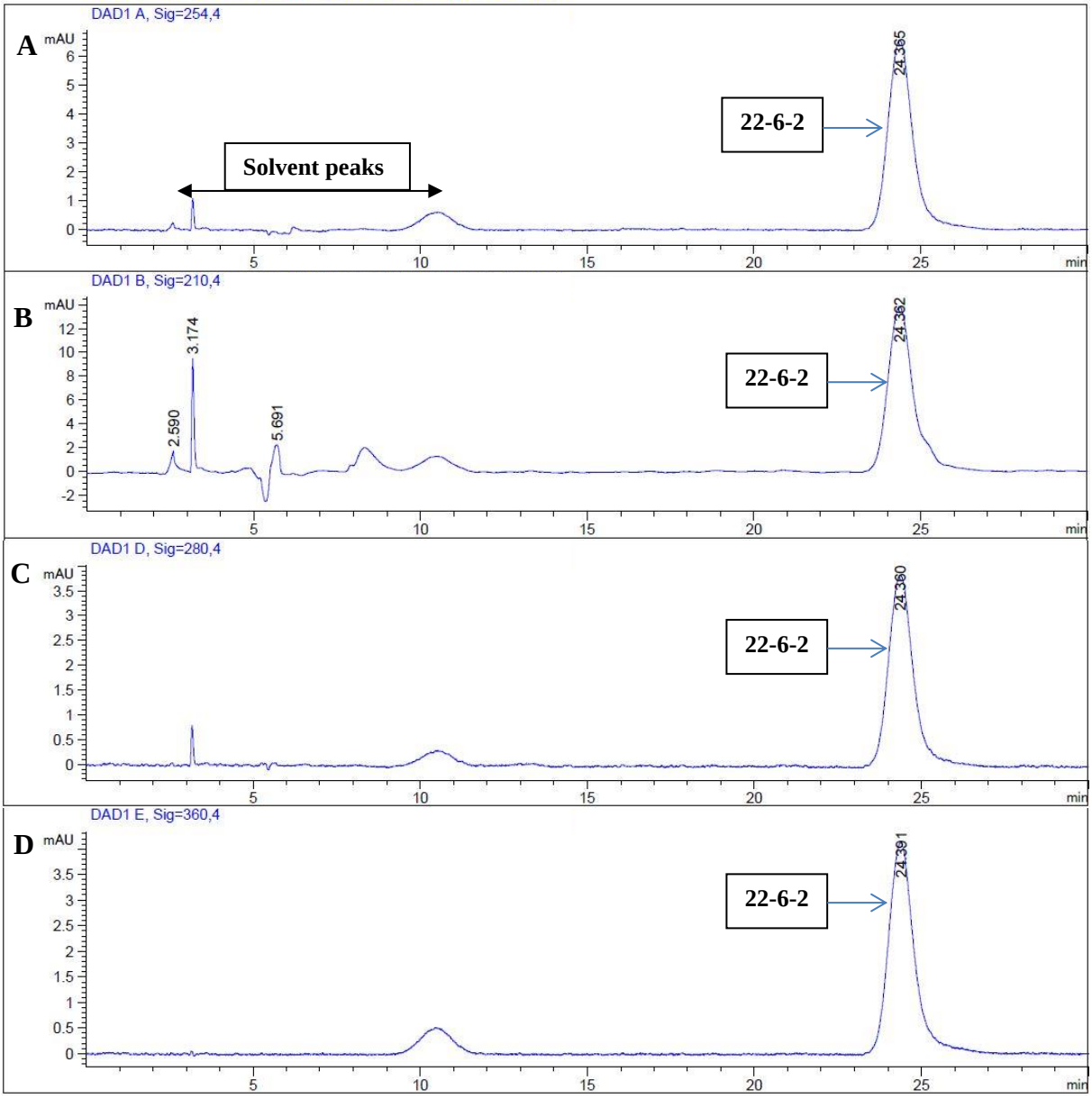


Figure 16. HPLC chromatogram of sub-fraction 22-6-2. Wavelengths detected at **A.** 254nm; **B.** 210nm; **C.** 280nm; **D.** 360nm.

4.6.4 LC-MS/MS of sub-fraction 22-6-2

Sub-fraction 22-6-2 was sent out for LC-MS/MS analysis to Advanced Chemistry Solutions in hopes of identifying the compound observed in HPLC. Based on the results of the previous LC-MS/MS scan, negative ionization mode was selected this time. Numerous peaks were observed and 13 compounds detected from LC-MS/MS (Figure 17) despite the HPLC profile only showing the presence of one peak. Matching to NIST11 library identified three compounds summarized in Table 27. Detailed LC-MS chromatograms can be found in Appendix IV.

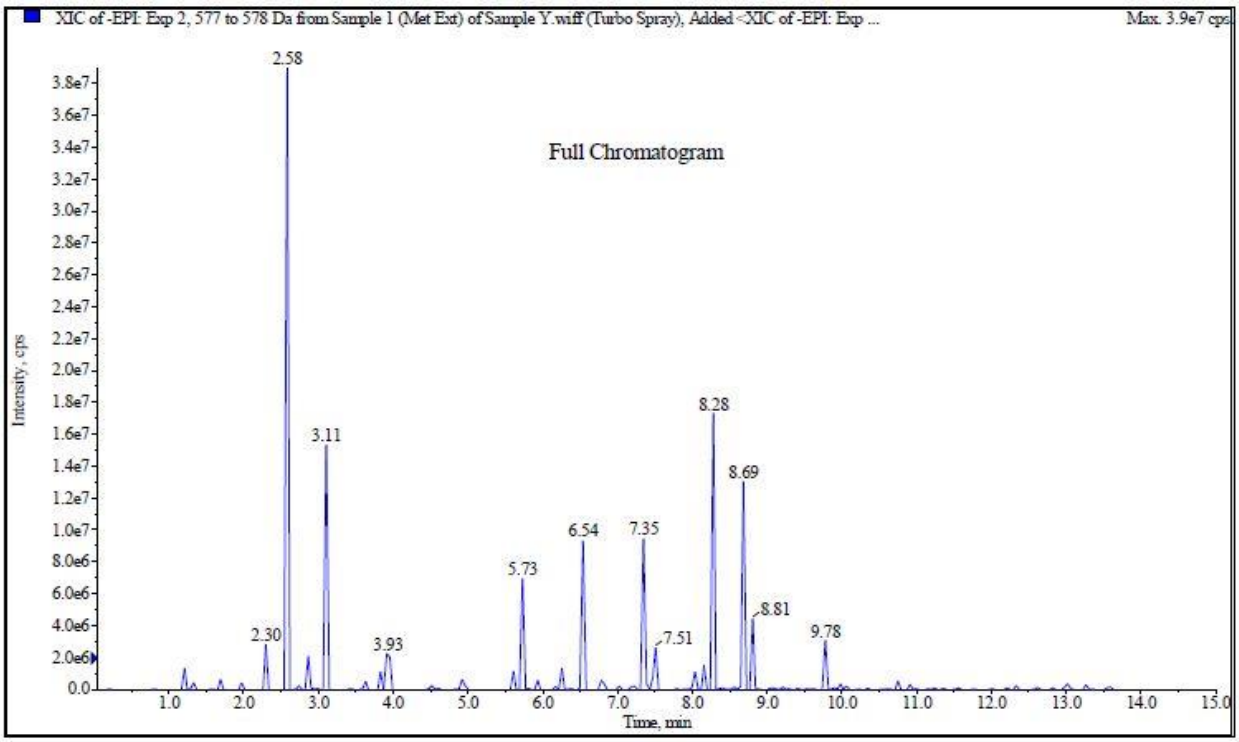


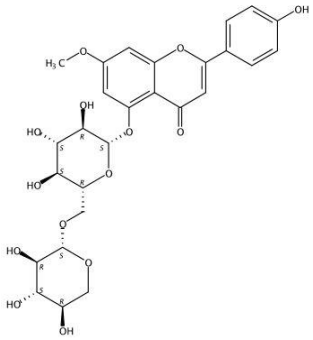
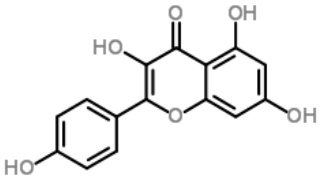
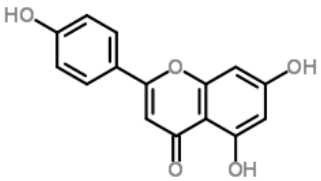
Figure 17. LC-MS profile of sub-fraction 22-6-2 (negative ESI)

Four polyphenols were putatively identified using LC-MS negative ionization $(M-H)^-$ mode. They include apigenin, kaempferol and two genkwanin-5-O-β-D primeveroside isomers with different fragmentation patterns at 1.209 and 3.106 retention time respectively. The presence of these polyphenols has been previously reported in several literature of *Aquilaria* extracts; genkwanin-5-O-β-D primeveroside in the leaves of *A. sinensis* (Feng and Yang, 2012), kaempferol 3,4,7-trimethyl ether in chloroform extract of leaves of *A. subintegra* (Bahrani *et al.*, 2014) and apigenin-7,4'-dimethylethers in *A. sinensis* stem barks and leaves (Wang *et al.*, 2015a). Most of the peaks with high intensity could not be identified. A search of previously reported fragmentation patterns on compounds isolated from *Aquilaria* plants such as that reported by Yu *et al.* (2013) did not yield any potential clues on the identity of these compounds.

The LC-MS/MS results were contrary to the HPLC results where only one major peak was found. Despite HPLC parameters given to the company before the analysis, the technician utilizes an acetonitrile mobile phase which might have affected the results. Preliminary gradient solvent runs of HPLC using acetonitrile mobile phase has found that the peak detected in Figure 16 around 24 mins is eluted before 3 mins suggesting that the peak at 2.58 might be the compound of interest. This peak has a fragmentation pattern of *m/z* 180.9, 240.9, 263.0 and 349.0 under MS/MS with no match to any compounds. In view of the LC-

MS/MS results which detected more than one compound, further crystallization and repeated washing with organic solvents was carried out to obtain crystals of higher purity. In future LC-MS/MS analysis, the mobile phase parameters would also be emphasized on to better replicate the HPLC profile.

Table 27. Compounds identified in 22-6-2 by LC-MS (Negative ESI).

Retention Time (RT)	**Fragment**	**Library/ID Chemical**	**Structure**	**Reported bioactivity**
1.209 3.106	268.0, 284.0, 578.0 268.0, 283.0, 578.0	Genkwanin-5-O-β-D primeveroside isomer ($C_{17}H_{15}O_5$) (yuankwanin)		Laxative effects (Kakino *et al.*, 2010)
3.913	165.0, 183.0, 268.0, 285.1, 579.0	Kaempferol conjugate ($C_{15}H_{10}O_6$)		Acetylcholinesterase inhibitor (Bahrani *et al.*, 2014)
8.280	155.0, 171.1, 207.1, 225.1, 269.2	Apigenin ($C_{15}H_{10}O_5$)		Antidiabetic (Coman *et al.*, 2012)

*Structures except genkwanin isomer (Chen *et al.*, 2011) are derived from chemspider.

4.7 Compound 22-6-2

4.7.1 Description

The crystals isolated from sub-fraction 22-6-2 will be referred to as compound 22-6-2. After repeated crystallization and washing, finally 37.38 mg of 22-6-2 were obtained. The crystals are yellowish, fine needle-like as observed under microscope at x10 objective (Figure 18).

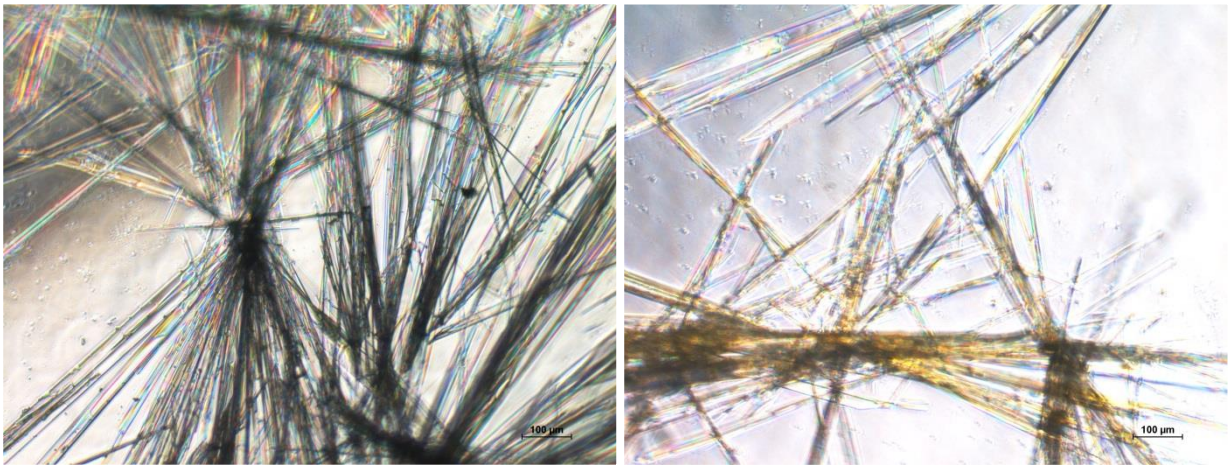


Figure 18. Compound 22-6-2 viewed under microscope (x10 objective)

Melting point was determined using a melting point apparatus. Crystals were placed into a capillary tube which was then inserted into the apparatus and temperature was increased in 5°C increments. The compound was observed to melt at 245°C. Analysis using HPLC revealed a similar profile of one peak around 24 minutes (see Figure 16). The compound was sent out for LC-MS/MS and NMR analysis to confirm its purity and aid in compound and structural identification.

4.7.2 LC-MS/MS of compound 22-6-2

Compound 22-6-2 was sent to School of Medicine, Monash University for LC-MS/MS analysis. As aforementioned, in order to replicate the results of HPLC, the parameters on the conditions of the analysis is strictly adhere to that of the HPLC analysis wherever possible such as details of column used and mobile phase. Given that previous negative ionization did not help in compound identification, positive ESI mode was chosen for this analysis. The general scan profile for LC-MS in Figure 19 showed one peak around 12-13mins which is similar to that of the HPLC results. Overall, 14 compounds were detected by LC-MS compounds with 8 compounds identified by matching to database. All the compounds

listed showed >95% matching to the database with exception of dimethyl trisufide (72%). The compounds are summarized in Table 28 with detailed MS chromatograms in Appendix IV.

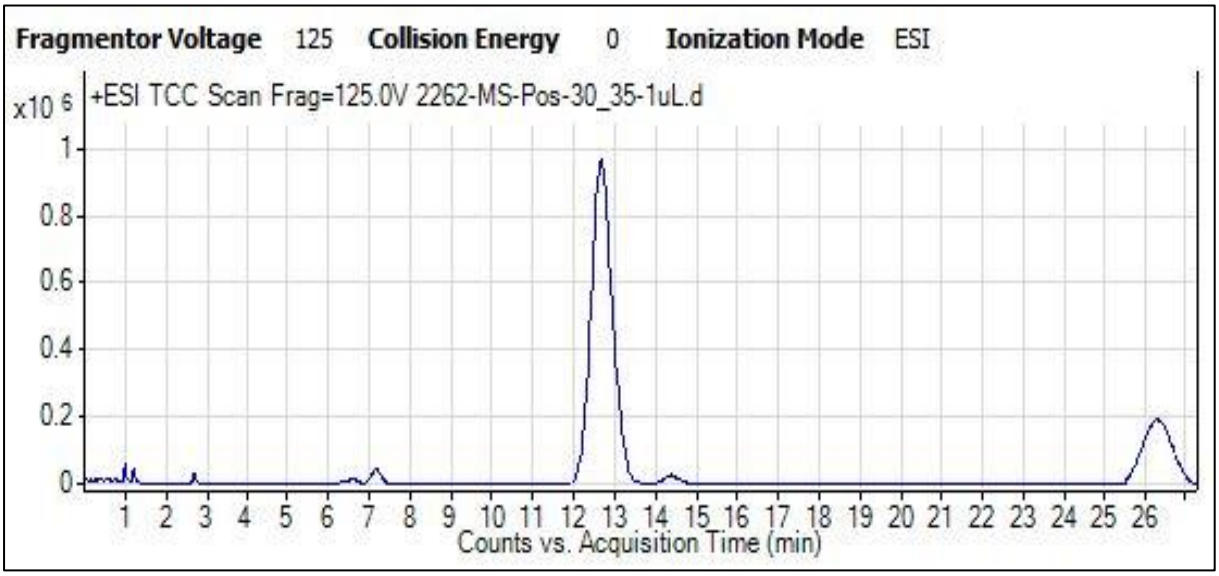
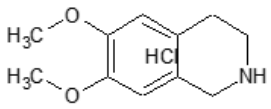
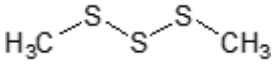
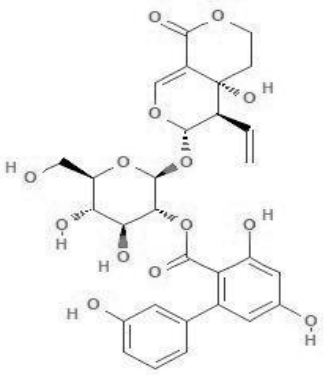
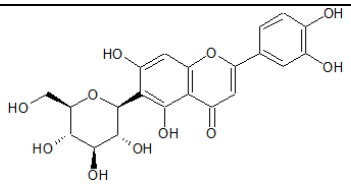
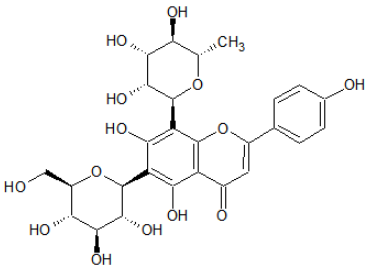
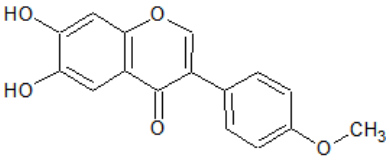
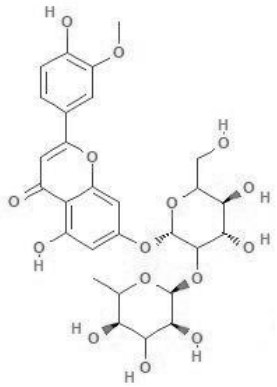
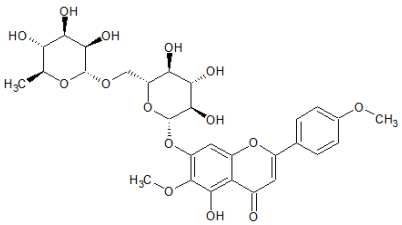


Figure 19. LC-MS profile of compound 22-6-2 (positive ESI).

Table 28. Compounds identified in 22-6-2 by LC-MS (Positive ESI).

Retention Time (RT)	Library/ID Chemical	m/z	Structure	Reported bioactivity
0.973	Heliamine	194.12		Narcotic (Temerdashev *et al.*, 2014)
1.202	Dimethyl trisulfide	144.00		Antifungal, larvicidal, cyanide antidote, pro-algesic (Kiss *et al.*, 2017)
6.510	Amaroswerin	603.17		Gastroprotective effect (Niiho *et al.*, 2006)

7.126	Isoorientin 7-O-rhamnoside	617.15		Antidiabetic (Chakroun *et al.*, 2016); Anti-inflammatory (Wedler *et al.*, 2014); Inhibits hyperlidemia, antioxidant (Yuan *et al.*, 2016)
12.605	Violanthin	579.17		Anti-cholinessterase activity (Dung *et al.*, 2015), Antioxidant (Vukics *et al.*, 2008a)
12.629	Texasin	285.08		5-Lipo-Oxygenase Inhibitor (Mascayano *et al.*, 2015)
12.917 14.368	Chrysoeriol 7-neohesperidoside	609.18 631.16		None
26.267	Pectolinarin	645.18		Anti-inflammatory (Lim *et al.*, 2008); Antioxidant, hepatoprotective (Yoo *et al.*, 2008); Sedative effects (Nugroho *et al.*, 2011).

*Structures except amaroswerin and chrysoeriol (Pubchem) are derived from chemspider.

LC-MS indicated that the compounds present in 22-6-2 consist mainly of flavonoids and alkaloids. All of the compounds have not been associated to Aquilaria plants so far. Nevertheless, the flavone glycoside isoorientin has been isolated from *Daphne gnidium* which belongs to the Thymelaeaceae family similar to *Aquilaria* (Cottiglia *et al.*, 2001).

However, only compounds within the major peak at acquisition time 12.0-13.5 minutes were of interest and further MS-MS were performed. The five possible compounds detected within the retention time range with their fragmentation pattern are as listed in Table 29. Three of the five possible compounds have fragmentation patterns matched to the database being compound 1 (violanthin), compound 3 (texasin) and compound 5 (chrysoeriol 7-neohesperidoside) with percentage matching of 98.33%, 94.87% and 99.66%. All three compounds are phenolics which have been isolated from plants. As aforementioned, current literature search do not link these compounds to that of the *Aquilaria* species.

Table 29. Possible compounds of 22-6-2 by LC-MS/MS

No.	**RT**	**Library/ID chemical**	**Formula**	**Mass**	**Major fragments (MS/MS)**
1	12.605	Violanthin	C27 H30 O14	578.16	285.08, 579.17, 601.15
2	12.617	Unknown	-	1178.30	285.07, 579.17
3	12.629	Texasin	C16 H12 O5	284.07	245.06, 285.07, 579.17
4	12.917	Chrysoeriol 7-neohesperidoside	C28 H32 O15	608.17	285.07, 315.08, 579.17
5	13.031	Unknown	C27 H22 N10 O9	630.16	285.07, 609.18. 631.16

4.7.3 NMR results of compound 22-6-2

The crystals of 22-6-2 solvated in deuterated methanol-d4 (CD_3OD) were sent for proton (^{1}H) and carbon (^{13}C) analysis with Bruker 300MHz. The NMR spectra are provided in Appendix V. Despite freeze-drying for two days prior to sending of sample for analysis, a large water peak was noted at 4.8 ppm (> 3E+08 intensity) whereas the peaks of the compound are relatively weak in intensity (Figure 20). To circumvent this problem, NMR data accumulation time was carried out for longer durations up to 35 hours. A possible reason was that the compound is hygroscopic in nature and absorbs moisture from the air giving an overestimation of sample weight and the low concentration of sample leads to poor resolution of peaks. In addition, further NMR analysis such as 2D NMR could not be performed due to the low concentration.

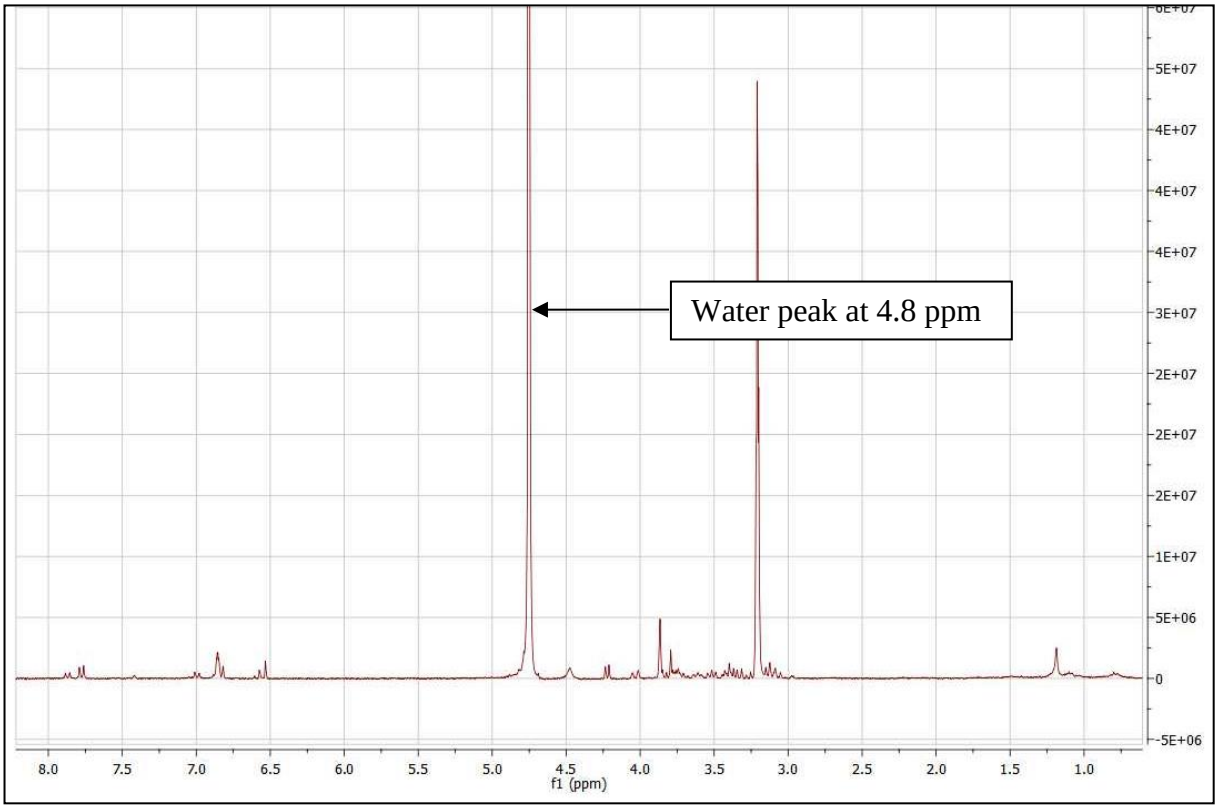


Figure 20. The ^{1}H spectrum (300 MHz) of sample 22-6-2.

Analysis of the NMR data gives an estimate of approximately 39 carbons and 43 protons with the results as follows:

^{13}C NMR (300 MHz, CD_3OD) δ 223.40, 178.88, 174.49, 171.86, 164.71, 163.20, 161.55, 159.31, 158.18, 127.94, 127.79, 121.40, 115.72, 114.23, 108.98, 105.92, 105.31, 104.33, 103.28, 102.95, 95.81, 76.35, 76.07, 75.86, 73.58, 73.29, 70.14, 69.73, 69.10, 65.48, 56.35, 56.05, 55.77, 55.38, 54.66, 48.92, 16.11, 15.85, 15.60.

^{1}H NMR (300 MHz, CD_3OD) δ 7.97 (d, *J* = 8.9 Hz, 1H), 7.88 (d, *J* = 8.9 Hz, 2H), 7.10 (d, *J* = 8.8 Hz, 1H), 6.96 (m, 5H), 6.92 (s, 1H), 6.63 (s, 1H), 4.56 (s, 1H), 4.33 (d, *J* = 7.3 Hz, 2H), 4.12 (d, *J* = 1.9 Hz, 1H), 3.96 (d, *J* = 4.7 Hz, 7H), 3.86 (m, 6H), 3.72 (dd, *J* = 9.0, 7.2 Hz, 2H), 3.62 (t, *J* = 8.3 Hz, 2H), 3.47 (m, 8H), 3.23 (s, 1H), 3.20 (m, 1H), 1.29 (s, 1H)

4.7.4 Conclusion based on LC-MS/MS and NMR

As reported in Table 29, the five postulated compounds based on LC-MS/MS analysis have approximately 16-28 carbons whereas approximately 39 carbons was detected in the NMR results. This indicates that there are impurities present in the sample. In order to identify the yellowish crystals of compound 22-6-2, the NMR data of the LC-MS/MS predicted compounds which are texasine, violanthin and chrysoeriol-7-neohesperidoside from literature were compared with the NMR data of 22-6-2 in hopes of a match between both data.

4.7.5 Identification of compound 22-6-2

The NMR data of compound 22-6-2 was compared to NMR values of texasine, violanthin and chrysoeriol 7-neohesperidoside reported by Vdovitchenko *et al.* (2007), Carnat *et al.* (1998) and Cimanga *et al.* (1995) respectively. The compound was found to have the best match with violanthin. The comparison of NMR data obtained for 22-6-2 to the literature values reported by Carnat *et al.* (1998) is shown in Table 30 with the structure of violanthin in Figure 21.

Violanthin has been reported by Carnat *et al.* (1998) as a yellow amorphous powder. The melting point determined in this study was 245°C which might be an overestimation of violanthin's at 229°C. Melting point determination is after all subjective to observation of when the melting of the compound occurred. The LC-MS reported $[M+H]^+$ signal at *m/z* 579.17 (see Appendix V) is in agreement with the molecular weight of violanthin (578.2 g/mol) reported in literature (Vukics *et al.*, 2008b).

Table 30. Comparison of ^{1}H and ^{13}C NMR chemical shift data of compound 22-6-2 with literature values by Carnat *et al.* (1998)

Position	Literature δC	Experimental δC	Literature δH	Literature Multiplicity	Experimental δH	Experimental Multiplicity
Apigenin						
2	163.3	164.71				
3	102.7	102.95	6.81	s	6.71	s
4	182.2	178.88				
5	160.0	159.31				
6	109.1	108.98				
7	162.5	163.20				
8	102.7	102.95				
9	153.1	158.18				
10	103.0	103.28				
1’	121.2	121.40				
2’	128.5	127.94	7.91	d (8.5)	7.97	d (8.9)
3’	116.0	115.72	6.95	d (8.5)	6.96	m
4’	161.3	161.55				
5’	116.0	115.72	6.95	d (8.5)	6.96	m
6’	128.5	127.94	7.91	d (8.5)	7.88	d (8.9)
6-C-glucosyl						
1”	73.1	73.29	4.62	d (9.8)	4.56	s
2”	70.1	69.10	4.08	dd (9.8; 9.0)	4.13	m
3”	79.0	76.35	3.21	dd (9.0; 9.0)	3.20	m
4”	70.7	69.73	3.12	dd (9.0; 9.0)	3.08	m
5”	81.7	-	3.16	ddd (9.0; 4.0; 1.2)	3.20	m
6”	61.5	65.48	3.70	dd (11.2; 1.2)	3.72	dd (7.17, 8.97)
			3.42	dd (11.2; 4.0)	3.47	m
8-C-rhamnosyl						
1”’	75.0	75.86	5.26	s	-	
2”’	72.3	73.29	3.90	d (2.6)	3.96	d (4.68)
3”’	74.2	73.58	3.60	dd (2.6;9.0)	3.62	t (8.29, 8.29)
4”’	71.8	70.14	3.40	dd (9.0; 9.0)	3.47	m
5”’	77.3	76.07	3.45	dq (9.0, 6.0)	3.47	m
6”’	18.2	16.11	1.28	d (6.0)	1.29	s

*Coupling constants *J* (in Hz) are in parenthesis

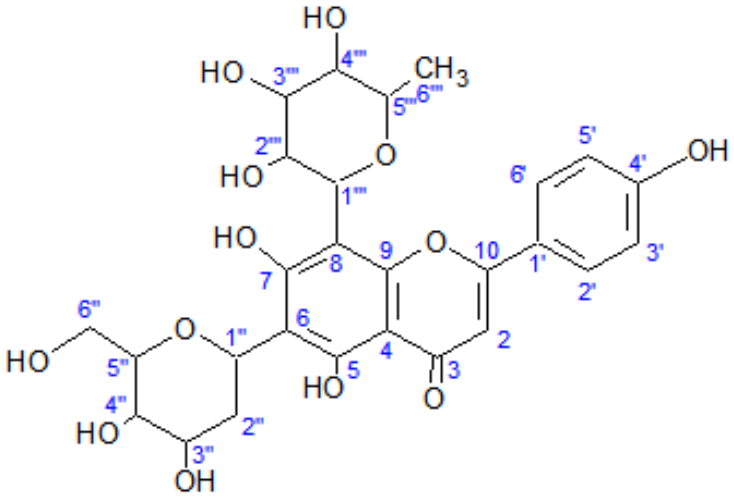


Figure 21. Structure of violanthin

For carbon-13 NMR, more carbons were obtained in experimentation compared to the 27 carbons reported by Carnat *et al.* (1998) indicating the presence of impurities. Nevertheless, based on the structure and table above, comparison of the chemical shifts of C-13 matched those reported by Carnat *et al.* (1998) for violanthin with the exception being the absence of the 5" carbon (δ 81.7) in the experimental results. The absence of peaks in the NMR data could be largely attributed to the low amount of compound 22-6-2 obtained after isolation which was insufficient for a better NMR analysis. There is also quite a difference in chemicals shifts between carbons 4, 9 and 6". This could be due to CD_3OD-d_4 being used as solvent in experimentation whereas that of Carnat *et al.* (1998) employed the use of DMSO-d_6 as solvent. Flamini (2007) which uses a similar solvent of CD_3OD-d_4 reported δ 180.6 and 161.5 for carbons 4 and 9 respectively which are comparatively closer to the experimental values obtained which was δ 178.9 and 158.2.

The proton values obtained in experimentation closely matches that of literature for the apigenin structure of the compound. The ^{1}H-NMR data confirmed the presence of the flavone skeleton characterized by two doublet signals assigned to H-3'and H-5' (δ 6.96) as well as H-2'and H-6' (δ 7.88/7.97, similar coupling constant) with a singlet at δ 6.71 (H-3). Interestingly, Flamini (2007) reported δ 6.73 for H-3 which is closer to the experimental value obtained of δ 6.71 compared to Carnat *et al.* at δ 6.81. Both studies used DMSO-d_6 for proton NMR.

However, there is quite a difference in chemical shifts of protons with respect to the glucosyl and rhamnosyl groups particularly in terms of multiplicity as well as the absence of the protons at δ5.26 which corresponds to 1"' carbon of rhamnosyl. The difference in proton NMR could be due to multiple factors. Firstly, impurities are present in the isolated

compound. Secondly, the low concentration of sample was insufficient for good NMR resolution as evident by the domination of peaks of contaminants particularly the water peak in the NMR spectra. A suggested improvement would be analyzing the sample using NMR machine with higher sensitivity such as Bruker 700 mHz.

Although other derivatives of apigenin has been reported in *Aquilaria* such as apigenin 7,4′-dimethyl ether in *A. sinensis* (Wang *et al.*, 2015), as of current, violanthin (apigenin-6-C-β-D-glucopyranosyl-8-C-α-L-6-rhamnopyranoside) have yet to be reported in literature for the *Aquilaria* species.

4.7.6 Bioactivity of compound 22-6-2 (violanthin)

There are two studies on the biological properties of violanthin. Vukics *et al.* (2008a) reported that the antioxidant capacity of herb and leaf samples of heartease and garden pansies could be attributed to violanthin. Dung *et al.* (2015) found that violanthin exhibit acetylcholinesterase inhibitory activity which might be helpful in Alzheimer's disease.

Compound 22-6-2 identified as violanthin was screened for its DPPH radical scavenging activity. The entire compound remaining (2.5mg) after all analysis were performed was used in this assay. However, it only reached 19.92% DPPH scavenging activity at this concentration (2.5mg/ml) and IC_{50} was not reached. It is estimated that approximately 8-9 mg of compound is required to achieve IC_{50}. Although IC_{50} could not be determined, a rough comparison made with the DPPH activity of sub-fraction 22-6-2 (IC_{50} 1.76 mg/ml) indicate that most of the antioxidant activity of *A. malaccensis* leaves have been lost in the purification process.

5.0 CONCLUSION AND FUTURE WORK

5.1 Conclusion

A. malaccensis was selected over *F. elliptica*, *F. gardenioides* and *F. racemosa* as the primary plant of interest due to their stronger antioxidant activity. Screening of the extracts obtained from sequential extraction using the solvents hexane, dichloromethane, methanol and water shows that more polar fractions have higher bioactivity. Phytochemical analysis also reveals that both methanol and water is able to extract more compounds compared to their less polar counterparts.

The methanol extract of *A. malaccensis* leaves was chosen for more detailed study for isolation of bioactive compounds. This is because this extract exhibits the strongest antioxidant activity regardless of antioxidant assays employed. It had the highest phenolic content, DPPH radical scavenging activity, ferric reducing power, metal chelating activity and β-carotene bleaching inhibitory activity. This is the first study that investigates the antioxidant activity of *A. malaccensis* leaves besides DPPH and the effect of extraction solvent used. In disc diffusion, it shows broad spectrum antimicrobial activity being able to inhibit all five gram positive bacteria tested and *P. aeruginosa*. This is confirmed by broth microdilution where it is the second most potent antimicrobial after dichloromethane extract. Although, methanol extract also shows the best anti-proliferative activity against MCF-7 breast cancer cells compared to others, this activity is very weak and does not meet the NCI's criteria.

Compound 22-6-2 was isolated out from the methanol extract using bioassay-guided fractionation where active fractions are favored for isolation of compounds. This yellowish compound was identified as violanthin based on the results of LC-MS/MS and comparison of the NMR data with literature. This is the first report on violanthin in plants of *Aquilaria* family. Due to insufficient amount of compound 22-6-2, the IC_{50} of DPPH activity could not be determined. Nevertheless, it shows 20% scavenging activity at a concentration of 2.5mg/ml.

5.2 Future work

The low yield obtained after isolation and purification work has heavily influenced the number of bioactivity assays screened in this study as well as quality of the spectroscopic and HPLC analysis. Future work should focus on employing different extraction methods using various solvent combinations to increase the yield. As methanol fraction was found to have the best bioactivity, methanol solvent or solvent of similar polarity is recommended.

The water extract shows good antioxidant activity. As *A. malaccensis* leaves is commercially marketed as 'gaharu' tea, it would be interesting to investigate the antioxidant and antimicrobial properties of the leaves extracted using boiling water to mimic tea preparation.

There is lack of studies on chemical constituents of *A. malaccensis* leaves. This study only managed to isolate out one compound. Nevertheless, LC-MS/MS investigation in this study has managed to putatively identify compounds that have yet to be reported in *Aquilaria*. Future studies should involve further isolation and purification of compounds in order to identify other bioactive compounds that are possibly present.

Bioactivity studies on *A. malaccensis* leaves are also scarce with most research on *Aquilaria* being centered on *A. sinensis* leaves. Given that *A. sinensis* had been associated with a wide range of activities from anti-inflammatory, anti-arthritic, anticancer, antidiabetic to analgesics, it is possible that the bioactive compounds accounting for these therapeutic benefits is present in *A. malaccensis* leaves. Hence, it would be of benefit to look into other bioactivity properties of *A. malaccensis* leaves such as anti-inflammatory and antidiabetic.

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APPENDIX I. CHEMICALS AND EQUIPMENTS

A) Chemicals

Solvents

Acetone (JT Baker, Analytical grade, ≥ 99.8%)

Acetonitrile (JT Baker, Liquid chromatography grade, ≥ 99.9%)

Chloroform (JT Baker, Analytical grade, ≥ 99.8%)

Dichloromethane (Merck, Analytical grade, ≥ 99.9%)

Diethyl ether (Friendemann Schmidt, Analytical grade, ≥ 99.5%)

Dimethyl sulfoxide (Fisher Scientific, Analytical grade, ≥ 99.7%)

Ethyl acetate (JT Baker, Analytical grade, ≥ 99.9%)

Hexane (JT Baker, Analytical grade, ≥ 98.5%)

Liquid nitrogen (Malaysia Oxygen (MOX) Sdn. Bhd., Malaysia)

Methanol (Merck, Analytical grade, ≥ 99.9%)

Methanol (Fisher Scientific, Liquid chromatography grade, ≥ 99.9%)

Phytochemical assays

Acetic anhydride $(CH_3CO)_2O$ (Sigma Aldrich, ≥ 98%)

Concentrated hydrochloric acid (R&M Chemicals, 23%)

Concentrated sulfuric acid H_2SO_4 (HmbG Chemicals)

Dragendorff reagent (Merck)

Iron (III) chloride-6-hydrate $FeCl_3.6H_2O$ (Fisher Scientific)

Magnesium ribbon

Antioxidant assays

Total phenolic content assay

Folin-Ciocalteu's phenol reagent (Sigma)

Gallic acid $C_7H_6O_5$ (Fluka, >98.0 %)

Sodium carbonate anhydrous Na_2CO_3 (Fluka, 99.0 %)

DPPH scavenging activity assay

DPPH $C_{18}H_{12}N_5O_6$ (Sigma, 90 %)

L (+) Ascorbic acid $C_6H_8O_6$ (Merck, 99.7 %)

Ferric reducing power (FRP) assay

Dipotassium hydrogen phosphate $K_2HPO_4.3H_2O$ (Merck)

Iron (III) chloride-6-hydrate $FeCl_3.6H_2O$ (Fisher Scientific, 99.8%)

Potassium dihydrogen phosphate KH_2PO_4 (Fisher Chemicals)

Potassium ferricyanide $K_3Fe(CN)_6$ (Unilab, 99%)

Trichloroacetic acid CCl_3COOH (HmbG Chemicals)

Metal chelating assay (MCA)

Ferrozine (Acros Organics)

Iron (II) sulfate-7-hydrate $FeSO_4.7H_2O$ (HmbG Chemicals)

β-carotene bleaching (BCB) assay

β-carotene $C_{40}H_{56}$ (Sigma, Type I: synthetic)

Chloroform (Fisher Scientific, 99.9%)

L (+) Ascorbic acid $C_6H_8O_6$ (Merck, 99.7 %)

Linoleic acid $C_{18}H_{32}O_2$ (Sigma)

Tween 40 (Fluka)

Antimicrobial activity assays

Disc-diffusion technique

Blank discs 6.0 mm (Whatman® Schleicher & Schuell, Germany)

Muller Hinton agar (OXOID, England)

Nutrient agar (Merck, Germany)

Nutrient broth (OXOID, England)

Petri dishes 100 x 15 mm (Brandon)

Sterile collection swab (Copan, USA)

Streptomycin 10µg/disc (OXOID, England)

Vancomycin 30µg/disc (OXOID, England)

Broth microdilution technique

96-well microplate (TPP, Switzerland)

Chloramphenicol (Sigma)

Muller Hinton agar (OXOID, England)

Muller Hinton broth (OXOID, England)

Nutrient agar (Merck, Germany)

Nutrient broth (OXOID, England)

Antiproliferative activity

96-well microplate (TPP, Switzerland)

Cryovials (Corning, Canada)

Culture flask 25 cm^3 x 75 cm^3 (TPP, Switzerland)

Dimethyl sulfoxide (Sigma, USA)

Dulbecco's Modified Eagle's Medium (Sigma, USA)

Fetal bovine serum (Gibco, Canada)

MCF-7 human breast cancer cell line (ATCC; LA, USA)

MTS (Sigma, USA)

MTT dye (Sigma, USA)

Penicillin-streptomycin (Gibco, Canada)

Phosphate buffer saline (PBS) (Sigma, USA)

PMS (Sigma, USA)

RPMI 1640 medium with L-glutamine (Invitrogen Corporation, California, USA)

Trypsin-EDTA 0.25% 1X (Gibco, Canada)

Column chromatography

Silica gel 60, 7749 (Merck, Germany)

Silica gel, 230-400 Mesh (Merck, Germany)

Sephadex gel LH-20 (Sigma, USA)

Glass columns (Favorit); Length x I.D.: 600mm x 80mm and 500mm x 25mm

Thin Layer Chromatography

Silica gel 60TLC aluminium sheets 20x20 cm (Merck, Germany)

Reversed-phase HPLC analysis

Cosmosil 5C18-MS-II, 4.6 x 250 mm, 5µm

Agilent Zorbax SB-C18, 21.2 x 100mm, 5 µm

Acetonitrile HPLC grade (Fisher Scientific)

Methanol HPLC grade (Merck)

PTFE membrane filter 0.45µm (Whatman)

B) Instruments

Biosafety cabinet (ESCO Class II Type A2)

Centrifuge machine (Hettich Zentrifuge, Universal 32R)

Freeze dryer Christ Alpha 1-4, Salm en Kipp (Netherlands)

Freeze dryer Christ Alpha 1-4, Salm en Kipp (Netherlands)

Hot plate (Favorit H50707V2)

HPLC (Agilent Technologies series 1260, Germany)

Incubator (Memmert, Germany)

Orbital shaker (Protech, Model 1719)

Oven (Memmert, Germany)

pH meter (Cyberscan 1100 Ph)

Rotary vacuum evaporator N-N series (Eyela Tokyo Rikakikai Co. Ltd., Japan)

Tecan microplate scanning spectrophotometer (Tecan, Switzerland)

U-1800 Hitachi spectrophotometer (Japan)

UV light 254/365 nm (Upland, USA)

Vacuum pump aspirator A-35 (Eyela Tokyo Rikaikai Co. Ltd., Japan)

Water bath (Memmert, Germany)

Weighing balance (Mettler Toledo XP205)

APPENDIX II. SUB-FRACTIONS 22-3 and 22-4.

NMR spectra

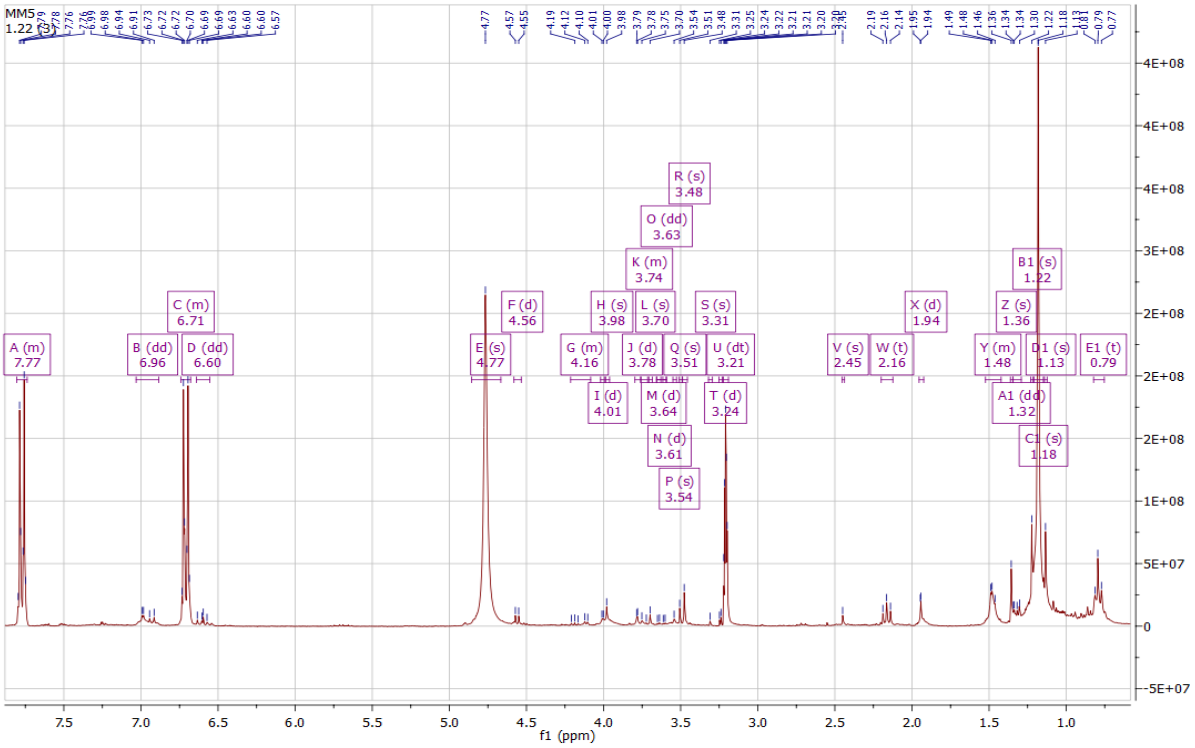


Figure I. Proton NMR of sub-fraction 22-3.

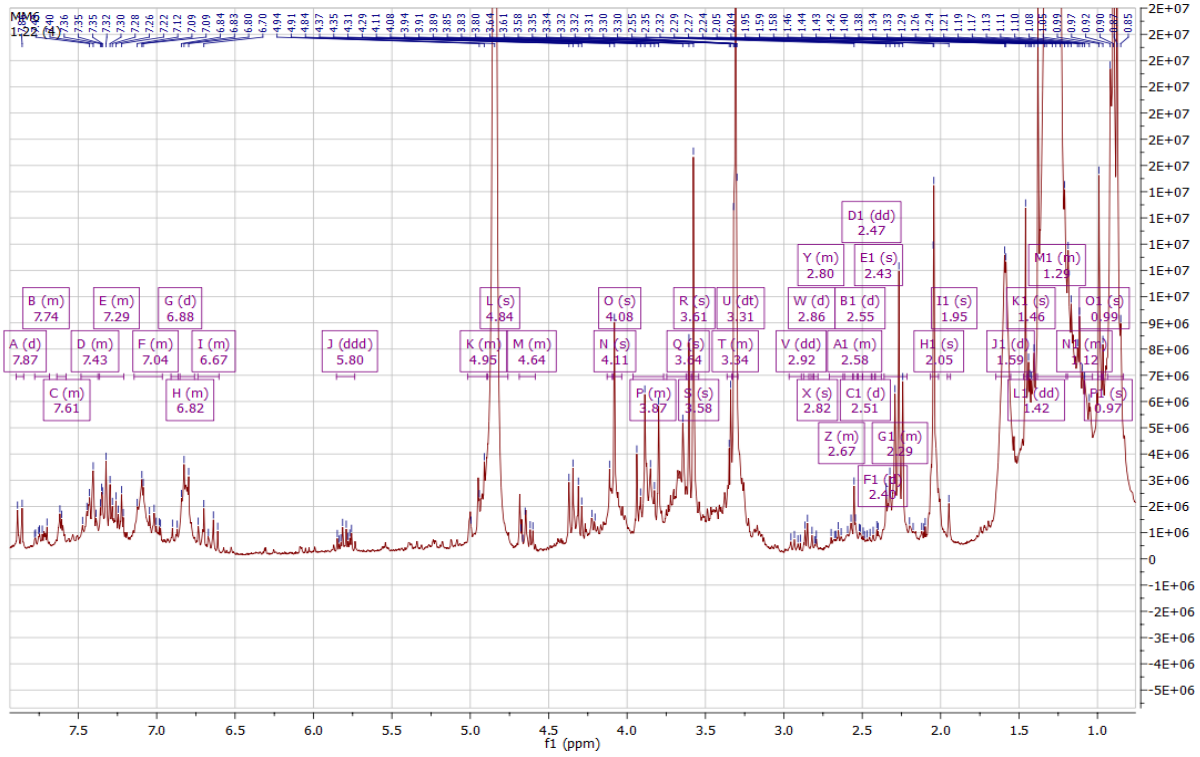


Figure II. Proton NMR of sub-fraction 22-4.

APPENDIX III. Sub-fraction 22-6.

LC-MS

A) Negative ionization

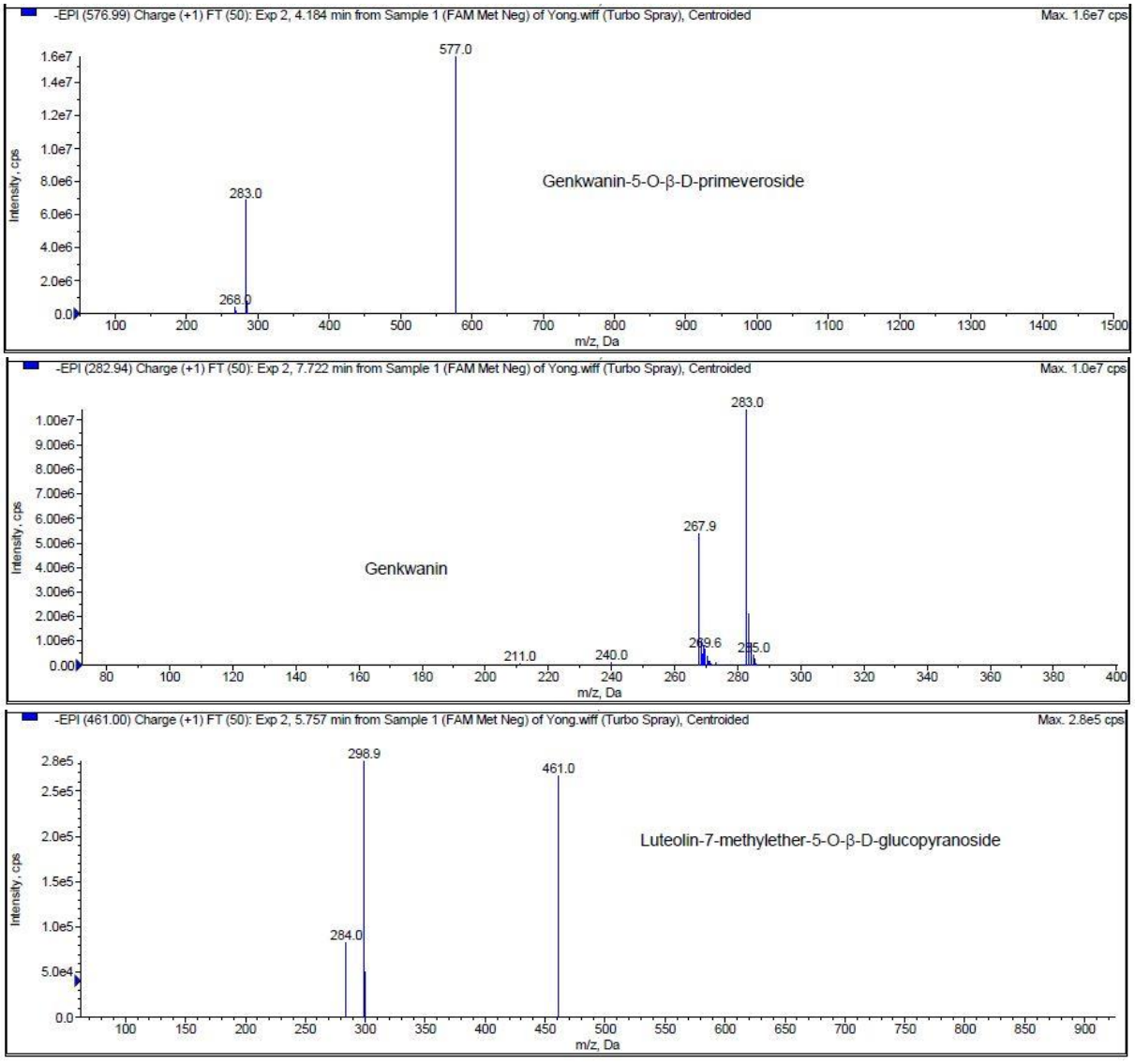


Figure III. LC-MS of sub-fraction 22-6 (Negative ionization)

B) Positive ionization

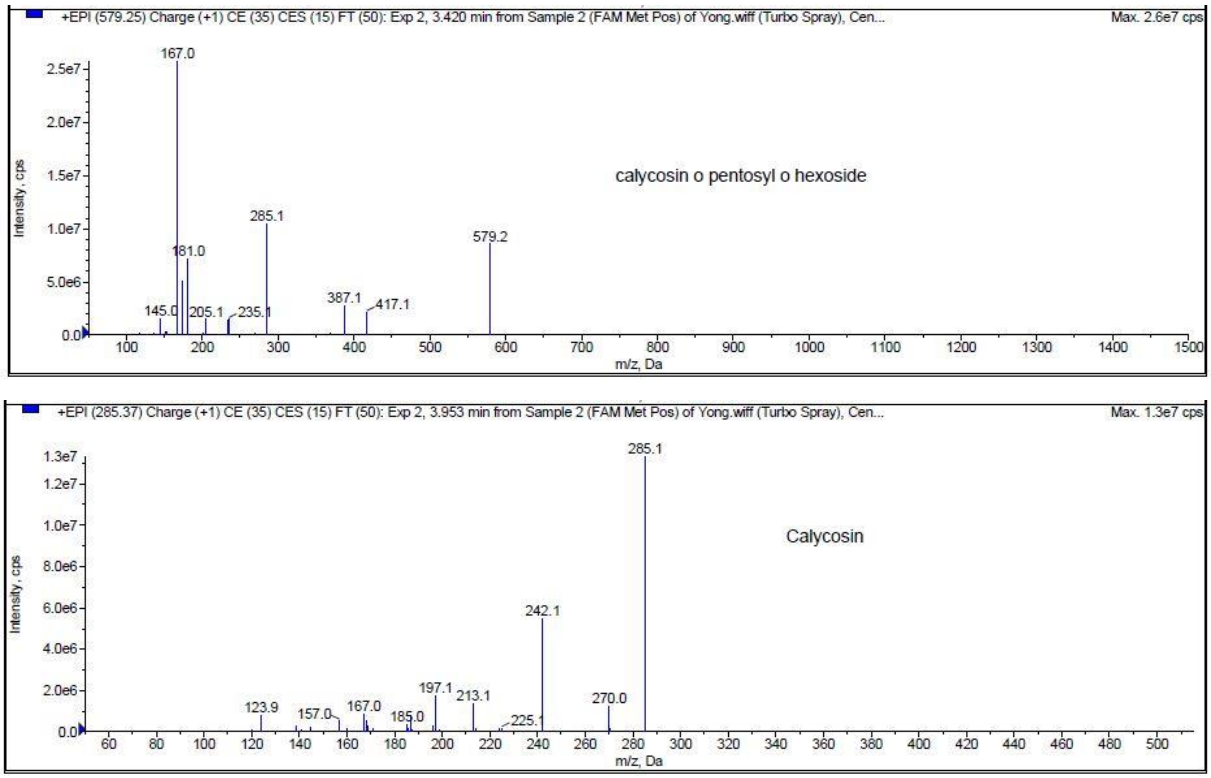


Figure IV. LC-MS of sub-fraction 22-6 (Positive ionization)

APPENDIX IV: Sub-fraction 22-6-2

LC-MS

A) Negative ionization

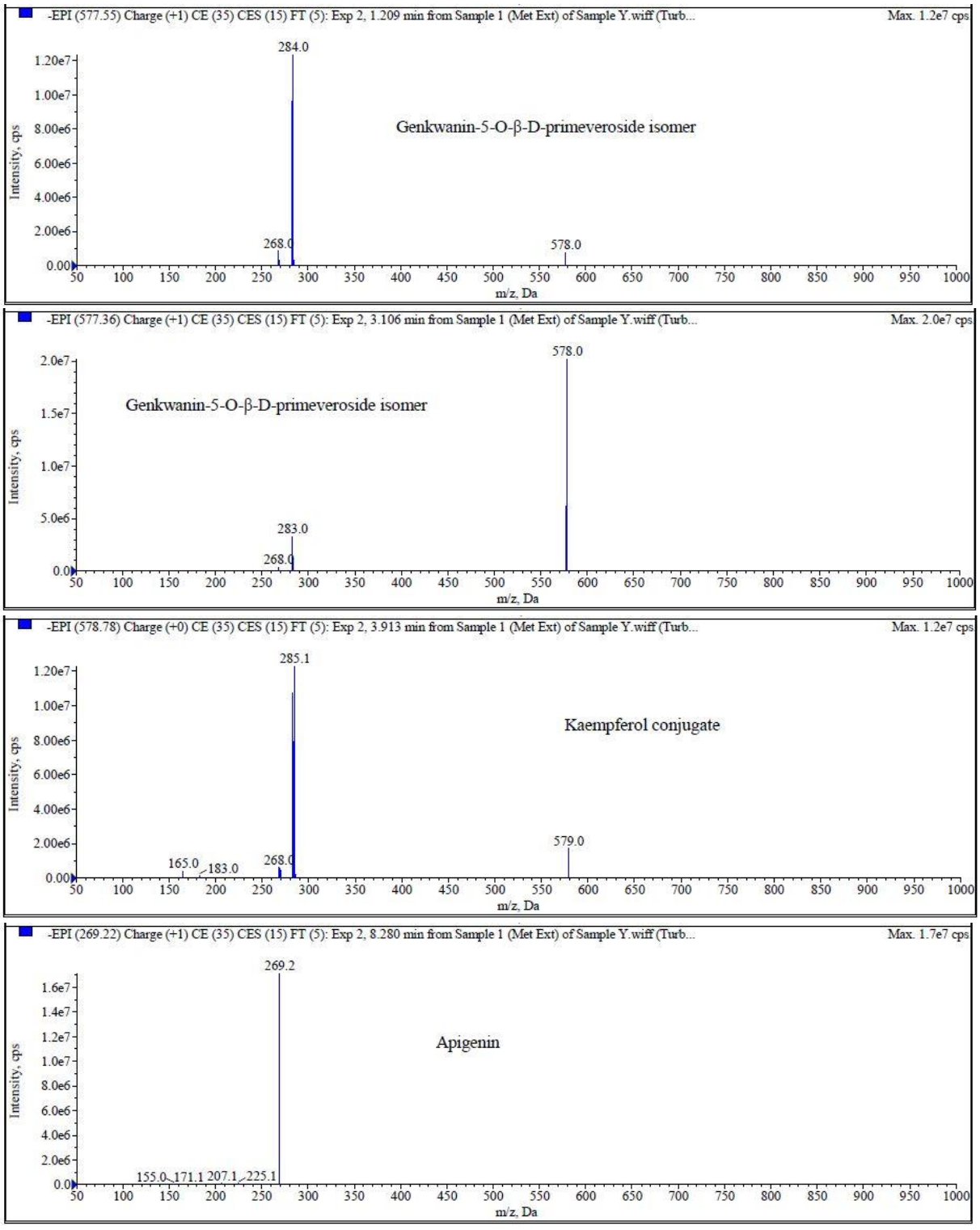


Figure V. LC-MS of sub-fraction 22-6-2 (Negative ionization)

B) Positive ionization

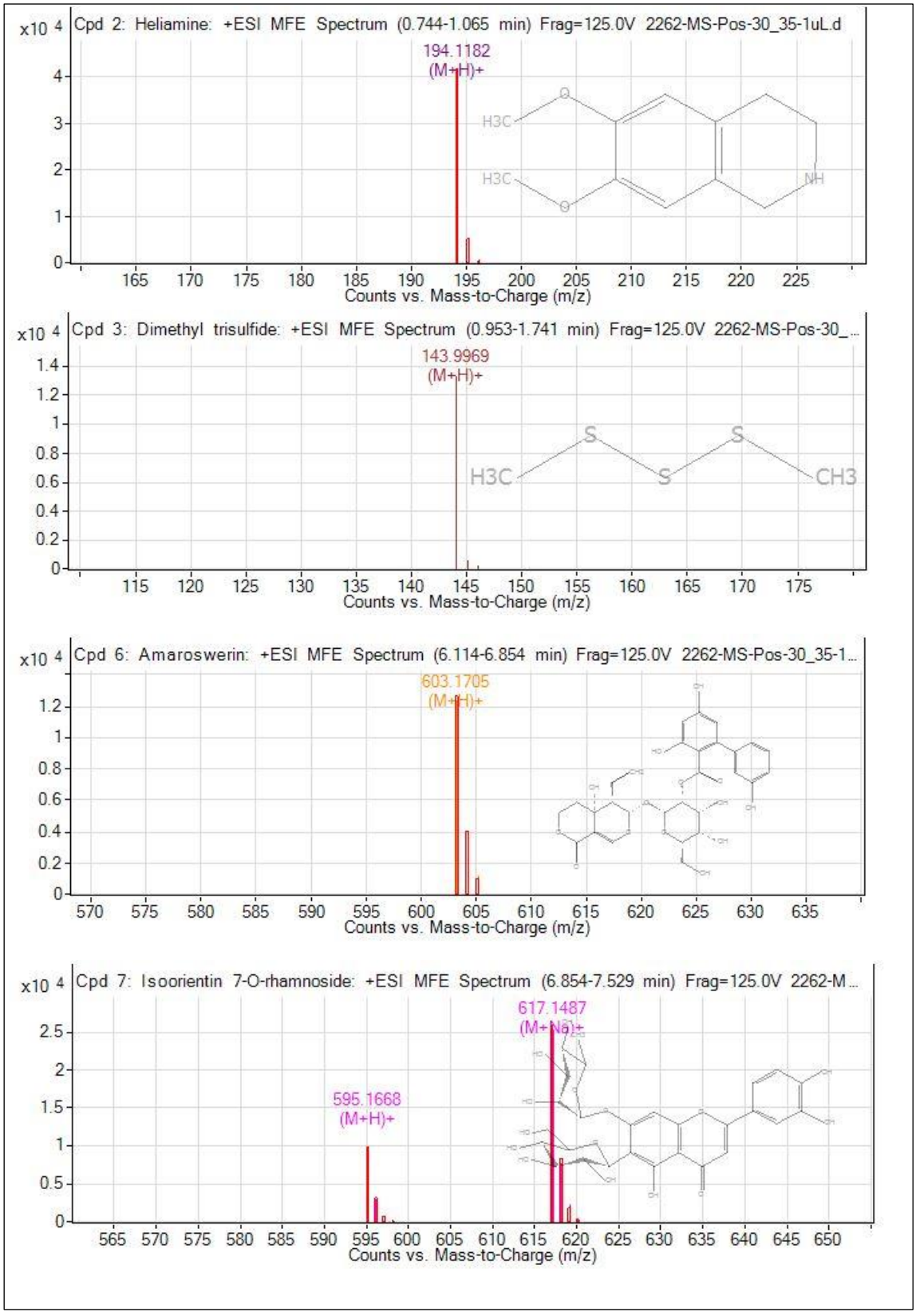


Figure VIa. LC-MS of sub-fraction 22-6-2 (Positive ionization)

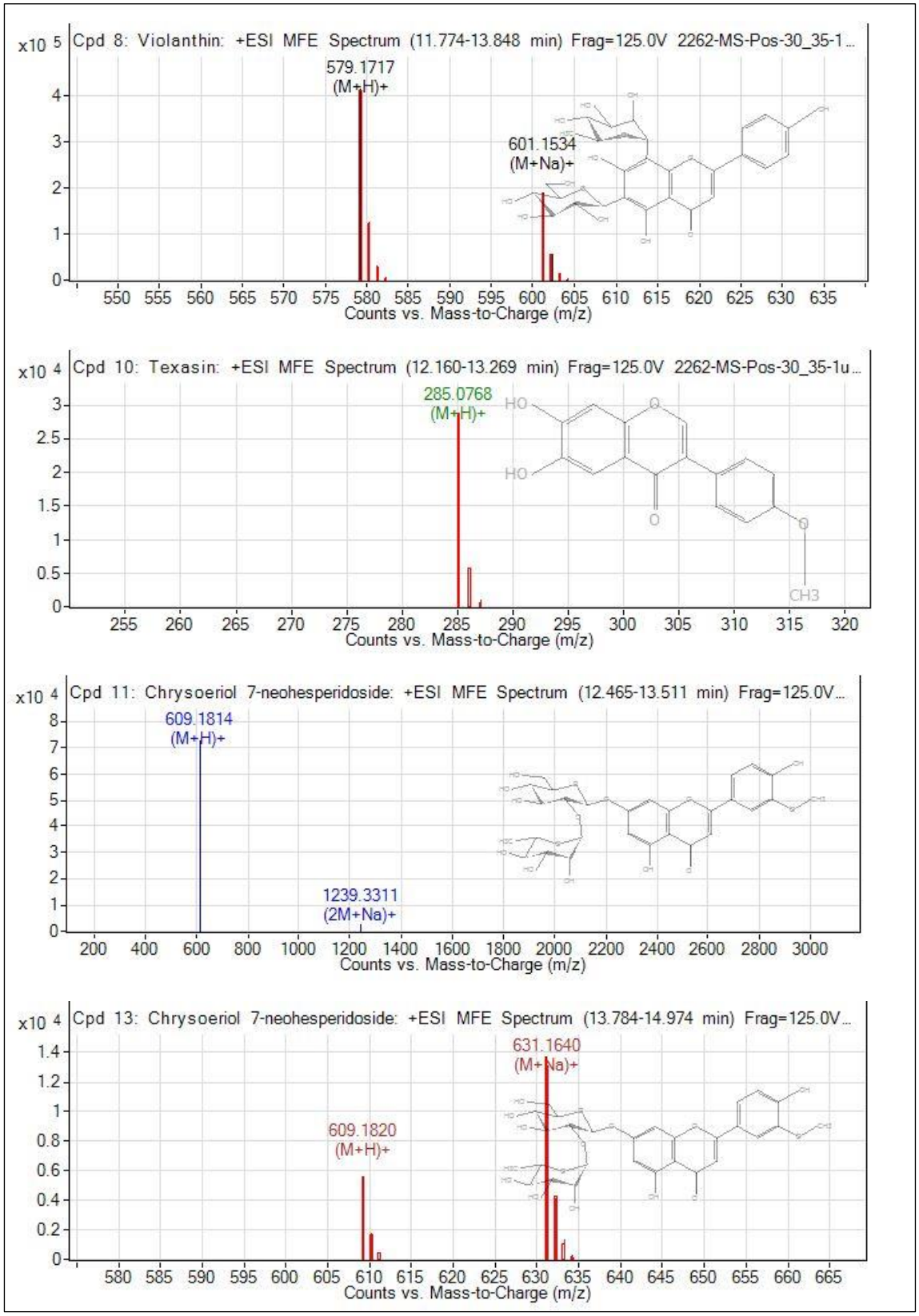


Figure VIb. LC-MS of sub-fraction 22-6-2 (Positive ionization)

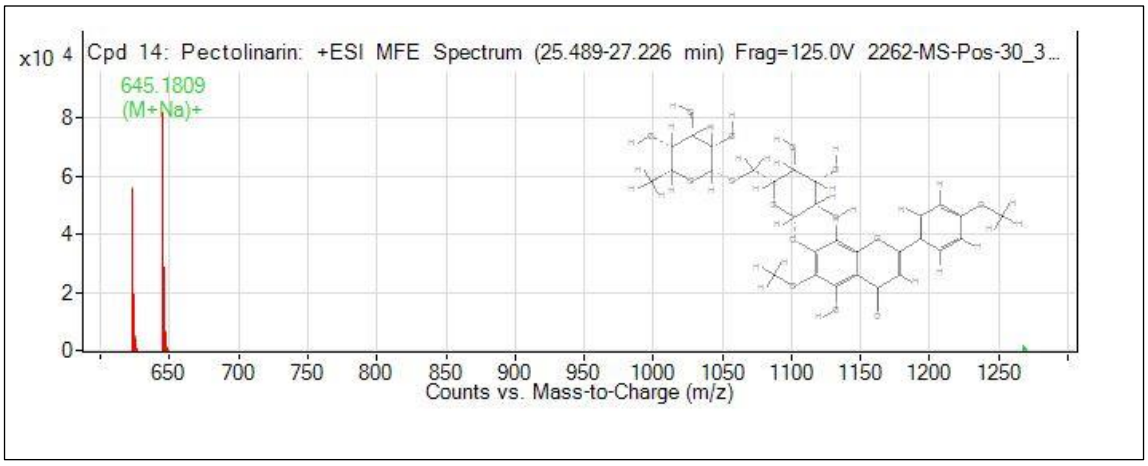


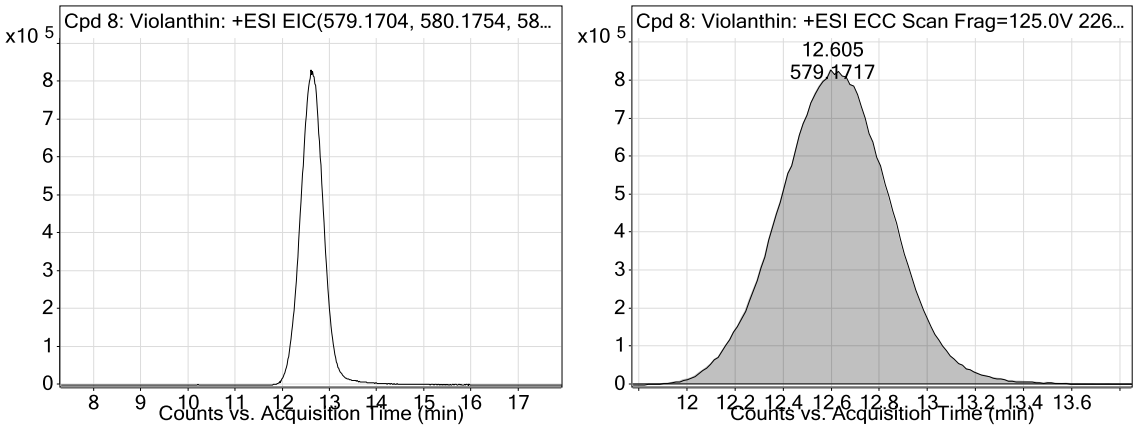
Figure VIc. LC-MS of sub-fraction 22-6-2 (Positive ionization)

Appendix V: Compound 22-6-2 (Violanthin)

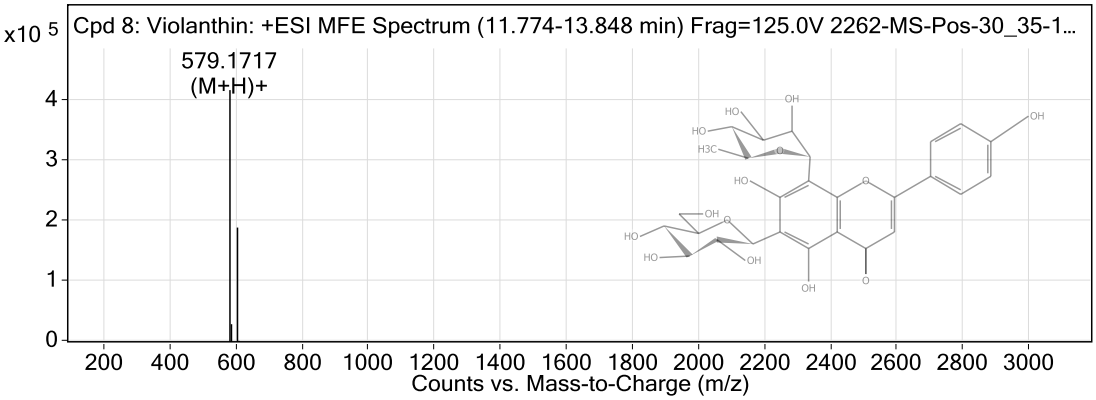
LC-MS/MS

Compound Label	Name	*m/z*	RT	Mass
Cpd 8: Violanthin	**Violanthin**	579.1717	12.61	578.1643

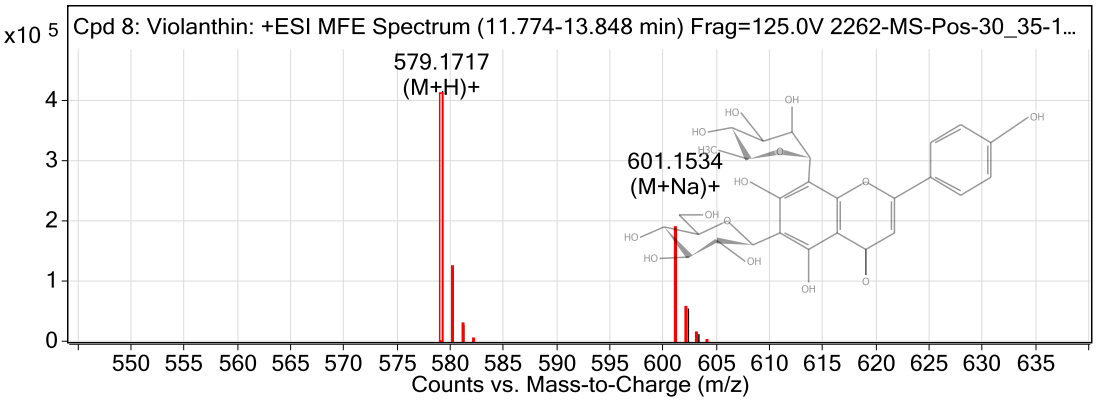
Compound Chromatograms



MFE MS Spectrum



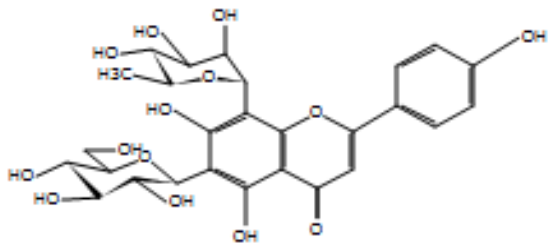
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund
579.1717	1	418793.31
580.1745	1	119510.63
581.1765	1	28842.33
582.1795	1	4991.73
583.1814	1	1027.52
601.1534	1	190455.97
602.1563	1	57007.12
603.1584	1	13864.95
604.1608	1	2475.1
605.1625	1	685.6

Compound Structure



ID Source	Name	Formula	RT	m/z	Mass	Score (DB)	Polarity	Ions	Height	Vol
DBSearch-MFG	Violanthin	C27 H30 O14	12.605	579.1717	578.1643	98.33	Positive	11	418793	27399066

Figure VII. LC-MS/MS data of violanthin

NMR spectra

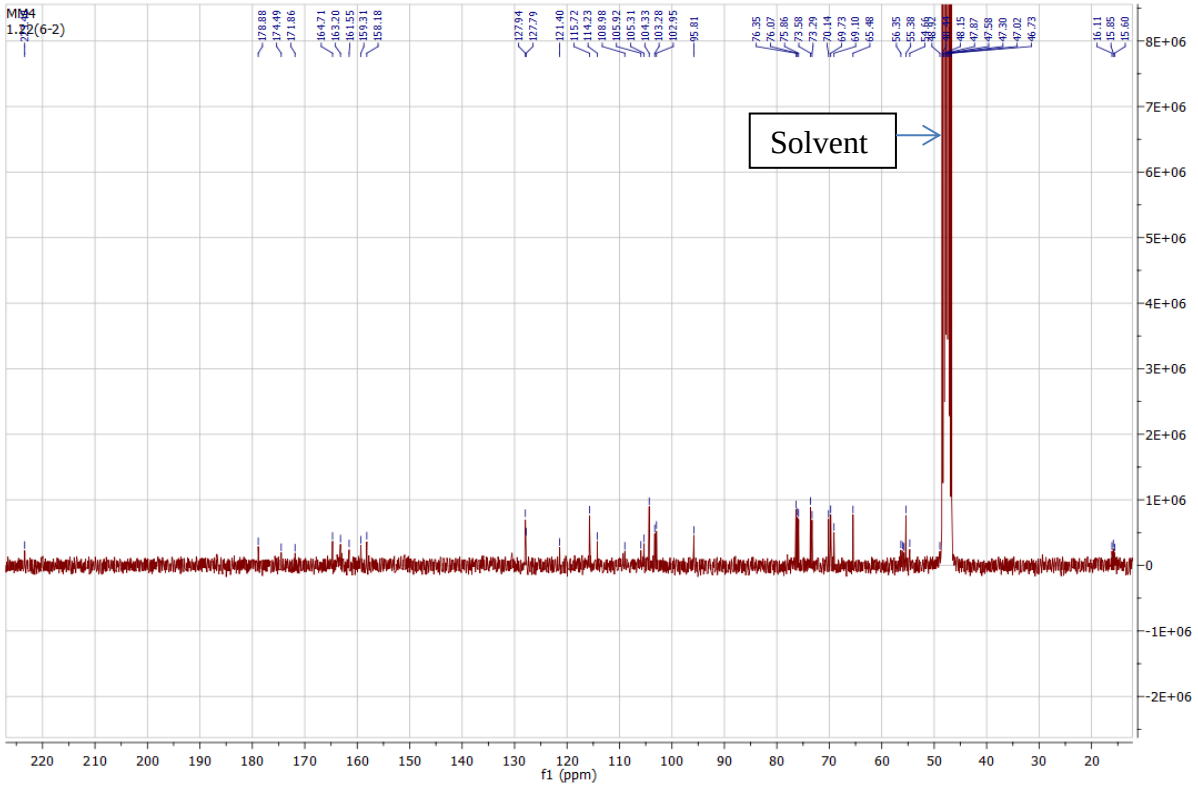


Figure VIII. Carbon-13 NMR of compound 22-6-2.

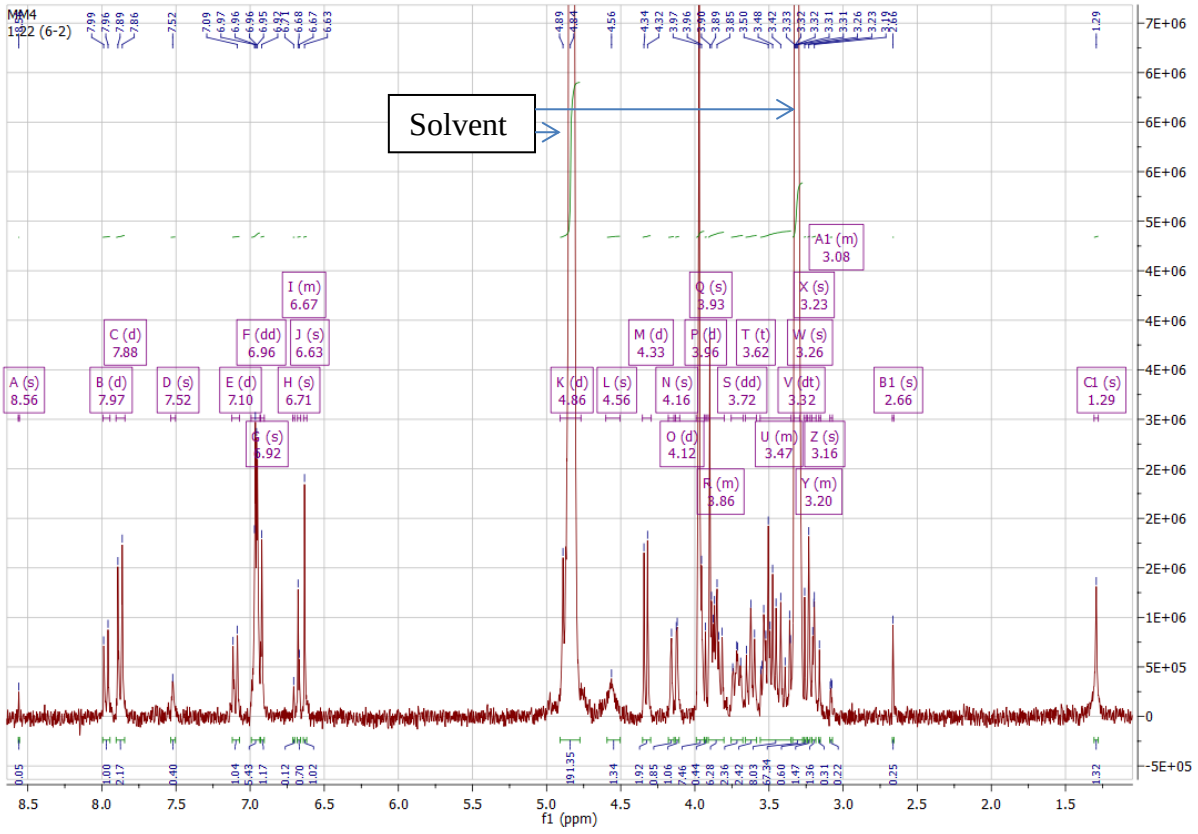


Figure IX. Proton NMR of compound 22-6-2.