COMPARATIVE ANALYSIS OF *IN VITRO* BIOACTIVITIES AND PHENOLIC CONTENT OF LEAF EXTRACTS FROM SIX SPECIES OF *Aquilaria*

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by

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ATCC	American Type Culture Collection
COX	Cyclooxygenase
cm	Centimeter
DMSO	dimethyl sulphoxide
DPPH	2,2-diphenyl picryl-hydrazyl
EDTA	ethylene diamine tetracetic acid
EC_{50}	effective concentration at 50% of activity
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalent
g	Gram
HCl	hydrochloride acid
MIC	minimum inhibition concentration
M	Molar
mg	milligram (10^{-3} g)
ml	Milliliter (10^{-3} litre)
mM	millimolar (10^{-3} M)
NA	nutrient agar
NB	nutrient broth
nm	Nanometer
NSAIDs	nonsteroidal anti-inflammatory drugs
PDA	photo diode array
PDA	potato dextrose agar
PDB	potato dextrose broth
PG	Postraglandin
Rt	retention time
SD	standard deviation
TPC	total phenolic content
TPTZ	2,4,6-tri (2-pyridyl)-s-triozine
UPLC	ultra performance liquid chromatography

UV	Ultraviolet
v/v	volume to volume
w/v	weight to volume
λmax	lambda maximum

ANALISIS PERBANDINGAN BIOAKTIVITI *IN VITRO* DAN KANDUNGAN FENOLIK EKSTRAK DAUN DARIPADA ENAM SPESIES *Aquilaria*

ABSTRAK

Kajian ini dijalankan untuk membandingkan aktiviti antioksida, antibakteria, anti-diabetes dan anti-radang daripada ekstrak enam spesies daripada genus *Aquilaria* iaitu *Aquilaria beccariana*, *Aquilaria hirta*, *Aquilaria malaccensis*, *Aquilaria rostrata*, *Aquilaria sinensis* dan *Aquilaria subintegra*. Sebatian fenolik mereka juga telah dikenalpasti dan dikuantifikasi. Daripada penilaian awal menggunakan lima jenis ekstrak (etil asetat, aseton, etanol, metanol dan air suling) daripada setiap spesies, ekstrak metanol menunjukkan aktiviti antioksidan yang terbaik. Pelarut ini kemudiannya dipilih untuk pengekstrakan lanjut dan separuh daripada ekstrak ini juga dihidrolisiskan. *A. sinensis* mencatatkan peratusan tertinggi bagi aktiviti 2,2-difenil-1-pikrilhidrazil (DPPH) (ekstrak terhidrolisis; 94.33±4.89%) dan aktiviti keupayaan aktioksida penurunan ferik (FRAP) (ekstrak metanol dan ekstrak terhidrolisis; 96.00±0.55% dan 95.88±0.16%), dan ekstrak metanol *A. hirta* memberikan peratusan tertinggi bagi aktiviti pengkelat logam (90.05±3.47%). Hanya bakteria gastrousus telah dipilih untuk kajian antibakteria. Ekstrak terhidrolisis *A. hirta* menunjukkan aktiviti yang baik dengan nilai minimum kepekatan perencatan (MIC) dalam linkungan 250 to 500 µg/mL. Dalam ujian perencatan α-glukosidase, 2.5 mg/mL ekstrak terhidrolisis *A. sinensis* mempamerkan peratusan tertinggi perencatan pada 59.00±8.07%. Dalam ujian perencatan α-amilase, aktiviti perencatan tertinggi adalah hanya pada 23.35±1.30% dikesan dari 10 mg/mL ekstrak terhidrolisis *A. hirta*. Aktiviti perencatan siklooksigenase tertinggi hanyalah pada 25.89±2.59% untuk ekstrak metanol *A. subintegra.* Ekstrak ini juga mempunyai

jumlah kandungan fenolik yang tertinggi (127.80±2.57 µg GAE/mg ekstrak). Daripada analisis kuantitatif menggunakan kromatografi cecair berprestasi ultra (UPLC), kandungan tertinggi mangiferin dikuantifikasikan daripada ekstrak metanol *A. hirta*, *A. malaccensis*, *A. sinensis* dan *A. subintegra* dan ekstrak terhidrolisis *A. hirta*. Iriflofenon 2-*O*-α-rhamnosida didapati merupakan sebatian fenolik utama dalam *A. rostrata* dan iriflofenon 3-*C*-β-glukosida ialah sebatian utama dalam *A. beccariana*. 7,4'-di-*O*-metilapigenin adalah sebatian utama bagi ekstrak terhidrolisis untuk *A. beccariana*, *A. malaccensis*, *A. rostrata* dan *A. subintegra*. Manakala 7,3'-di-*O*-metilluteolin ialah sebatian utama dalam ekstrak terhidrolisis *A. sinensis*.

COMPARATIVE ANALYSIS OF *IN VITRO* BIOACTIVITIES AND PHENOLIC CONTENT OF LEAF EXTRACTS FROM SIX SPECIES OF *Aquilaria*

ABSTRACT

This study was carried out to compare the *in vitro* antioxidant, antibacterial, anti-diabetic and anti-inflammatory properties of the leaf extracts of six species from genus *Aquilaria* which are; *Aquilaria beccariana*, *Aquilaria hirta*, *Aquilaria malaccensis*, *Aquilaria rostrata*, *Aquilaria sinensis* and *Aquilaria subintegra*. Their phenolic compounds were also identified and quantified. From the preliminary screening using five fresh extracts (ethyl acetate, acetone, ethanol, methanol and distilled water) from each species, the methanolic extracts exhibited the best antioxidant activities. This solvent was then selected for further extraction and half of the extracts were also hydrolyzed. *A. sinensis* recorded the highest percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity (hydrolyzed extract; 94.33±4.89%) and ferric-reducing antioxidant power (FRAP) activity (methanolic extract and hydrolyzed extract; 96.00±0.55% and 95.88±0.16%), and the methanolic extract of *A. hirta* gave the highest percentage of metal chelating activity (90.05±3.47%). Only gastrointestinal bacteria were selected for antibacterial study. The hydrolyzed extract of *A. hirta* performed better activity with minimum inhibitory concentration (MIC) values ranging from 250 to 500 µg/mL. In the α-glucosidase inhibition test, the 2.5 mg/mL hydrolyzed extract of *A. sinensis* exhibited the highest percentage of inhibition at 59.00±8.07%. In α-amylase inhibition test, the highest inhibitory activity was only 23.35±1.30% detected from the 10 mg/mL hydrolyzed extract of *A.*

hirta. The highest cyclooxygenase inhibitory activity was only 25.89±2.59% for the methanolic extract of *A. subintegra*. This extract also has the highest total phenolic content (127.80±2.57 µg GAE/mg extract). From the quantitative analysis using ultra performance liquid chromatography (UPLC), major content of mangiferin was quantified from the methanol extracts of *A. hirta*, *A. malaccensis*, *A. sinensis* and *A. subintegra* and the hydrolyzed extract of *A. hirta*. Iriflophenone 2-*O*-α-rhamnoside was found to be the major compound of *A. rostrata* and iriflophenone 3-*C*-β-glucoside is the major compound in *A. beccariana*. 7,4'-di-*O*-methylapigenin was the major compound of the hydrolyzed extracts of *A. beccariana, A. malaccensis, A. rostrata* and *A. subintegra*, while 7,3'-di-*O*-methylluteolin is the major compound in the hydrolyzed extract of *A. sinensis.*

CHAPTER 1

INTRODUCTION

1.1 Background of study

Aquilaria is an aromatic evergreen tree that belongs to the family of Thymelaeaceae, which is popularly known as "Gaharu" in the Southeast Asian countries. *Aquilaria* is also known under many names all around the globe such as agarwood, aloeswood, eaglewood, oud, kalambac, chen-xiang, jinkoh, khi-nam and others depend on their localities (Baharuddin, 2014).

In Malaysia, there are several native species of *Aquilaria* that are *A. malaccensis*, *A. hirta, A. microcarpa, A. beccariana* and *A. rostrata*. Three exotic *Aquilaria* species, that are also cultivated in Malaysia are *A. crassna* and *A. subintegra* that are native to Thailand and *A. sinensis*, which originate from China (Hashim and Ahmad Zuhaidi, 2011).

Agarwood plantations with a total area of 232.8 hectares (ha) had been established in Malaysia since the beginning of year 2000 (Ismail and Mohd Zin, 2011). In year 2014, the total area for *Aquilaria* plantations was reported to be 1119 ha (Ismail, 2014). Sabah is the major producing state for agarwood with 311.0 ha of plantation areas. This is followed by Perak (298.2 ha) and Pahang (144.1 ha).

Aquilaria malaccensis that is locally known as karas is randomly scattered throughout Peninsular Malaysia except in Perlis and Kedah. *A. hirta* is known by locals as chandan is mainly distributed in the east coast of the Peninsular Malaysia especially in the states of Terengganu, Pahang and Johor. Other *Aquilaria* species such as *A. microcarpa* and *A. beccariana* are confined to Sarawak and Johor (Whitmore, 1972). Moreover, *A. rostrata* that is known as chandan gunung was recently found in Terengganu as well as Gunung Tahan, Pahang (Lee and Mohamed, 2016).

Agarwood that is derived from the resin produced after pathological process at the injured stem is utilized and practised by mankind since the ancient time for various purposes. This high-priced agarwood is highly sought-after especially in the manufacturing of fragrances, medicines and beauty products (Chung and Purwaningsih, 1999; Baharuddin, 2014). In traditional Malay medicine, it is also used to treat various disorders such as fatigue, pain in the stomach or chest, edema and as tonic for men and women as well as a post partum medicine (Gimlett and Burkill, 1930).

For example, the decoction of the leaves of *A. malaccensis* is applied externally to treat swelling and consumed for treating vomiting (Ong, 2004). The decoction of its root is consumed to heal edema. The bark juice is useful in treating diarrhea, and to stop vomiting (Quattrocchi, 2012). The incense wood is used to treat thyroid gland cancer, as a sedative against abdominal complaints, asthma and diarrhea, and as an aphrodisiac agent. Grated wood is added into various herbal formulations especially for women during and after childbirth, and to treat

rheumatism. Thus, many medicinal and health supplementary products can be derived from different parts of this agarwood species (Chung and Purwaningsih, 1999; Ong, 2004).

Due to over-exploitation of wild *Aquilaria* species, all six have been listed under Appendix II of CITES (Convention of International Trade of Endangered Species) since 1994. According to Lau and Chua (2011), *A. hirta* and *A. malaccensis* were classified as Vulnerable (VU) for their conservation in Peninsular Malaysia while the other three species (*A. rostrata*, *A. microcarpa* and *A. beccariana*) were listed in Data Deficient (DD) list (Table 1.1). The IUCN Red List of Threatened Species (IUCN, 2015) had classified *A. beccariana, A. hirta, A. malaccensis* and *A. microcarpa* as Vulnerable (VU), while *A. rostrata* is categorized as Critically Endangered (CR). Therefore, the cultivation of *Aquilaria* species was encouraged to conserve the endangered *Aquilaria* (Baharuddin, 2014).

Table 1.1 *Aquilaria* conservation status in Peninsular Malaysia and the IUCN Red List of Threatened Species (Lau and Chua, 2011; IUCN, 2015).

Species	Peninsular Malaysia (CITES)	IUCN
A. beccariana	Data deficient (DD)	Vulnerable
A. hirta	Vulnerable	Vulnerable
A. malaccensis	Vulnerable	Vulnerable
A. microcarpa	Data deficient (DD)	Vulnerable
A. rostrata	Data deficient (DD)	Critically Endangered

Since it takes many years for the wood to produce the aromatic and valuable resin, the leaves of *A. crassna, A. malaccensis* and *A. sinensis* were harvested for tea production in some Asian countries. The agarwood tea is popular as daily healthy drink for some communities, especially in China. Besides, the herbal tea of agarwood is commonly consumed for the treatment of many health disorders such as diabetes, headache, constipation and high blood pressure (Pranakhon *et al*., 2011; Kakino *et al*., 2012).

Up until now, *A. crassna, A. malaccensis* and *A. sinensis* were previously reported to possess many beneficial biological activities and pharmacological properties such as antioxidant, anti-diabetic, anti-inflammatory, antibacterial, antidepressant and antiviral activities (Dash *et al*., 2008; Zhou *et al*., 2008; Huda *et al*., 2009; Pranakhon *et al*., 2011; Kamonwannasit *et al*., 2013). In addition, the extraction of *A. malaccensis* leaves using methanol was reported to contain a lot of chemical constituents such as flavonoids, terpenoids, alkaloids and tannins (Khalil *et al*., 2013).

1.2 Problem statements

Recently, instead of focusing only in producing agarwood resin, leaves of *A. malaccensis* are used to make health products such as tea (Baharuddin, 2014). To date, not all leaves of *Aquilaria* have been studied for their bioactivities. The identification of phenolic compounds in the leaf extracts was limited to a few findings from *A. crassna, A. malaccensis* and *A. sinensis* (Chen *et al*., 2012).

Besides, there is no comparison of the bioactivities among the *Aquilaria* leaf extracts. Therefore, this project was proposed to comparatively evaluate the bioactivities of the leaf extracts. This study also may provide the scientific evidence to prove the medicinal values of each *Aquilaria* species. The composition of phenolic compounds present in these extracts and their contribution to the bioactivities were also be evaluated.

1.3 Objectives of the study

The purpose of this study is to identify the leaf extract with the best bioactivity from six different *Aquilaria* species and to quantify their phenolic compounds. For those reasons, the following objectives were drawn for this study:

1. To determine the best solvent to extract antioxidants from the leaf part of six species of *Aquilaria*.
2. To screen the *in vitro* antioxidant activities of the effective extracts using three different colorimetric assays.
3. To determine anti-diabetic activity using the enzyme inhibitory assays of α-glucosidase and α-amylase.
4. To determine anti-inflammatory activity using cyclooxygenase inhibitory assay.
5. To determine antibacterial activity against gastroenteritis bacteria.
6. To identify and quantify the major phenolic compounds from the methanolic and hydrolyzed extracts and correlate with the bioactivities.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus *Aquilaria*

Aquilaria is one of the genus of family Thymelaeaceae from the order Malvales. This genus is native to the Asian continent and widely distributed in India and from southern China to the countries in Southeast Asia region (Whitmore, 1972; Allaby, 2012). It is also found mostly in Malaysia, Indonesia, Singapore, Myammar, Philliphine and Thailand (Huda *et al.*, 2009). The genus *Aquilaria* comprises of slow growing trees, medium sized up to 40 meter in height. There are 15 species of *Aquilaria* namely *A. acuminata*, *A. baillonii*, *A. banaensis*, *A. brachyantha*, *A. crassna*, *A. cumingiana*, *A. filaria*, *A. rostrata*, *A. rugosa*, *A. sinensis*, *A. subintegra,* including the Malaysian gaharu which comes from *A. hirta*, *A. malaccensis*, *A. beccariana* and *A. microcarpa* (Barden *et al.*, 2000; Hashim and Ahmad Zuhaidi, 2011).

The genus *Aquilaria* is often recognized with a smooth, stringy hard and pale grey to dark with dense foliage of outer bark of wood. While the inner bark or sapwood is white and soft. The leaves of *Aquilaria* are simple, alternately arranged with acuminate tips and undulate margins. The flowers are hermaphroditic and arranged in umbel inflorescences on the short stalks at the terminal twigs where the fruits are produced. The fruits are woody capsules, drupes or berries and develop from the calyx lobe (Whitmore, 1972; Hashim and Ahmad Zuhaidi, 2011). This

genus is well adapted to grow in various habitats that are sandy or rocky environments, well-drained slopes and is commonly found in primary and secondary forest depending on the species, mainly in lowland and hillsides at altitudes up to 850 m with an average daily temperature of 20-22°C (Jantan, 1990, Keller and Sidiyasa, 1994).

Agarwood is classified as non wood forest product (Lata, 2007). From this genus, there are four known species that yield high-grade gaharu, which are *A. malaccensis*, *A. crassna*, *A. sinensis* and *A. subintegra* (Hashim and Ahmad Zuhaidi, 2011). According to Chua (2011), *A. malaccensis* is the major source of high-quality traded gaharu resin in Peninsular Malaysia.

2.1.1 Morphological description of *Aquilaria* used in this study

Each species of the genus *Aquilaria* is known under various vernacular names by the locals. *A. hirta, A. beccariana, A. malaccensis, A. rostrata, A. sinensis* and *A. subintegra* are known by the local people in Malaysia as chandan, gaharu tanduk, tengkaras or karas, chandan gunung, "Pak Muk Heung" and karas, respectively. The morphological characteristics of the six *Aquilaria* species were differentiated and compared as shown in Table 2.1.

Species in the genus *Aquilaria* are slow growing trees. The size of the tree of the genus is different in each species. Most of them are medium in size and can grow between 5 m to 40 m high. *A. rostrata* was the shortest with a height of 5 m and has a relatively the thinnest diameter at breast height (dbh) of 3 to 4 cm. The greatest size of the species was *A. malaccensis* which can grow up to 40 m in height with the widest dbh of 60 cm.

The leaves of all species in the genus *Aquilaria* comprising similar morphological features except the size of leaves. *A. beccariana* has the broadest leaf area (11-27 cm x 6-8.5 cm), followed by *A. subintegra* with leaf size (7-27 cm x 3-8 cm) while *A. sinensis* has the smallest leaf size (5-11 cm x 2-4 cm) with elliptic-oblong shape. *Aquilaria* tree barks is smooth, soft and pale in colour. Reproductive organs of *Aquilaria* species are hermaphroditic. The flowers are arranged in axillary umbel inflorescence. Fruits productions from the flowers vary between species.

Aquilaria species has a medium size fruit excluding *A. rostrata. A. malaccensis* is one of the species that produces the largest fruit measuring about, 3-4 cm x 2.5 cm. Whereas, *A. rostrata* produces the smallest fruit measuring 1.5 cm x 0.75 cm. Depending on the species of *Aquilaria*, the shapes of fruit capsules are different for each species. Some have obovoid-oblong shaped capsule and some have flattened egg-shaped. However, every fruit for each species in the genus *Aquilaria* contains two seeds per fruit. In addition, there are various shapes, sizes and colours of the seeds of the *Aquilaria* species.

Table 2.1 Morphological characteristics of *Aquilaria* species

Plant part	**Description**					
	***Aquilaria hirta* Ridl.**	***Aquilaria beccariana* Tiegh.**	***Aquilaria malaccensis* Lam.**	***Aquilaria rostrata* Ridl.**	***Aquilaria sinensis* (Lour.) Gilg**	***Aquilaria subintegra* Ding Hou**
Size of tree	Medium size[3,5]	Medium size[5,6,7]	Medium size[1,5]	Small size[5,8]	Medium size[2]	Medium size[5,7]
Height	< 15 m[5]	< 20 m[5,6,7]	< 40 m[1,5]	< 5 m[5,8]	< 20 m[2]	< 20 m[5,7]
Diameter breast height (dbh)	50 cm[5]	36 cm[5,6,7]	60 cm[1,5]	3 to 4 cm[5,8]	20 m[2]	36 cm[5,7]

Table 2.1 Continued

Plant part	Description					
	***Aquilaria hirta* Ridl.**	***Aquilaria beccariana* Tiegh.**	***Aquilaria malaccensis* Lam.**	***Aquilaria rostrata* Ridl.**	***Aquilaria sinensis* (Lour.) Gilg**	***Aquilaria subintegra* Ding Hou**
Leaf	Simple, alternately arranged, shining and glabrous on both surfaces and elliptic oblong in shape					
Size						
Width	3 to 6 cm[5]	6 to 8.5 cm[5,7]	2.5 to 5.5 cm[1,5]	2.5 to 5 cm[5,8]	2 to 4 cm[2]	3 to 8 cm[7]
Length	6 to 16 cm[5]	11 to 27 cm[5,7]	7.5 to 12 cm[1,5]	6.5 to 14 cm[5,8]	5 to 11 cm[2]	7 to 27 cm[7]
Bark	Smooth and whitish grey in colour					

Table 2.1 Continued

Plant part	Description					
	***Aquilaria hirta* Ridl.**	***Aquilaria beccariana* Tiegh.**	***Aquilaria malaccensis* Lam.**	***Aquilaria rostrata* Ridl.**	***Aquilaria sinensis* (Lour.) Gilg**	***Aquilaria subintegra* Ding Hou**
Inflorescence						
Arrangement	Axillary umbel					
Colour	White greenish[3]	Green to yellowish[5]	Pale yellowish green[1]	Yellowish white[5]	Yellowish green[2]	White[7]
Pedicel length	3 to 6 mm[3]	6 to 12 mm[5]	3 to 6 mm[1]	5 to 6 mm[5]	3 to 6 mm[2]	6 to 13 mm[7]
Calyx tube length	1 to 2 mm[3]	3 to 6 mm[5]	2 to 3 mm[1]	5 to 6 mm[5]	2 to 3 mm[2]	5 to 12 mm[7]

Table 2.1 Continued

Plant part	**Description**					
	***Aquilaria hirta* Ridl.**	***Aquilaria beccariana* Tiegh.**	***Aquilaria malaccensis* Lam.**	***Aquilaria rostrata* Ridl.**	***Aquilaria sinensis* (Lour.) Gilg**	***Aquilaria subintegra* Ding Hou**
Fruit						
Size	Medium size[5]	Medium size[7]	Medium size[1]	Small size[5,8]	Medium size[2]	Medium size[7]
Width	1.1 cm[5]	0.7 cm[7]	2.5 cm[1]	1.0 cm[5,8]	2.5 cm[2]	2.5 cm[7]
Length	3.2 cm[5]	3.5 cm[7]	3 to 4 cm[1]	1.5 cm[5,8]	3 cm[2]	3 cm[7]
Shape of capsule	The fruit is an obovoid-shaped capsule					
Number of seeds	There are two seeds per fruit					

Table 2.1 Continued

Plant part	Description					
	***Aquilaria hirta* Ridl.**	***Aquilaria beccariana* Tiegh.**	***Aquilaria malaccensis* Lam.**	***Aquilaria rostrata* Ridl.**	***Aquilaria sinensis* (Lour.) Gilg**	***Aquilaria subintegra* Ding Hou**
Seed						
Colour	Brown[3]	Black[7]	Blackish brown[1]	Brown[5,8]	Brown[2]	Blackish brown[7]
Shape	Ovoid[5]	Ovoid[5,7]	Ovoid[1,5]	Ellipsoid-oblong[8]	Ovoid[2]	Narrowly elliptic[7]
Diameter	1 x 0.6 cm[5]	1 x 0.6 cm[7]	1 x 0.6 cm[1]	1 x 0.4 to 0.7 cm[8]	1 x 0.6 cm[2]	1 x 0.6 cm[7]

References [1] Corner (1988), [2] Huang (2009), [3] Hutchinson (1959), [4] Lee *et al*. (2013), [5] Lee and Mohamed (2016), [6] Soehartono and Newton (2001), [7] Tawan (2004), [8] Ridley (1924)

2.1.2 Uses of *Aquilaria* leaves

In Malaysia, the decoction of the leaves of *A. malaccensis* is applied externally to treat swelling and consumed for treating vomiting (Ong, 2004). In China, the leaves of *A. sinensis* are prepared as tea to treat fractures and bruising (Zhou *et al*., 2008; Yu *et al*., 2013). In Thailand and Vietnam, the leaves of *A. crassna* have been used as a healthy tea food additive (Sattayasai *et al.*, 2012).

Herbal teas of *A. malaccensis*, *A. sinensis* and *A. crasnna* are found to be beneficial for health to treat various kind of diseases. The teas can be used as anti-depressant and anti-aging skin agents. Besides, by consuming agarwood tea from its leaves also provide energy and soothe sleep disorders (Health benefits, 2015).

The leaves of *A. sinensis* is also consumed as a laxative agent (Hara *et al*., 2008). Preedy (2015) reported that the root and leaves of *A. malaccensis* were used as a prescription for dropsy. According to Chung and Parwaningsih (1999), the leaves of several *Aquilaria* species were burnt throughout the world for incense purposes.

2.2 Bioactivities of *Aquilaria* leaves

The term “bioactivity” can be defined as a reaction in or the specific effect on the living tissues upon exposure to a substance (Carbonell-Capella *et al*., 2014). Scientifically, the term “bioactivity” also refers to an alternative term for “biological activity” (Cammack, 2006). The concept of bioactivity including events relating to the transportation and movement of bioactive compounds and reach the target tissue, their interaction with biomolecules, biotransformation or metabolism they may undergo, and the generation of biomarkers and the physiological responses they cause (Fernández-García *et al.,* 2009). Bioactivity is measured primarily based on events that occurs during the interaction between bioactive components with biomolecules. This interaction can provide health benefits through the achievement of systemic physiological responses (such as antioxidant and anti-inflammatory) (Fernández-García *et al.,* 2009; Carbonell-Capella *et al*., 2014).

The evaluation of the bioactivities of an extract or a pure substance from a living organism can be done through *in vivo* and *in vitro* experimental models (Colegate and Molyneux, 2007; Fernández-García *et al.,* 2009; Carbonell-Capella *et al*., 2014). *In vitro* methods have been developed to determine bioactivity, including the screening of various activities such as antioxidant, anti-diabetic, anti-inflammatory, anti-tumor, and others (Fernández-García *et al.,* 2009). *In vitro* test is the most desirable as it is more simple, specific and rapid in data generation. Meanwhile, *in vivo* tests in mammals are often variable and highly constrained by ethical considerations of animal welfare (Colegate and Molyneux, 2007).

2.2.1 Antioxidant activity

Antioxidant activity is among the most frequently reported bioactivity properties from the medicinal plants including *Aquilaria* species. In addition, there are many antioxidant studies conducted on leaf samples and other parts of *Aquilaria*. Antioxidants can be defined as substances that are present in low concentrations which could inhibit the oxidation process of substrate and neutralize the action of free radicals (Li, 1999). From previous studies on the leaf samples of *Aquilaria*, Ray *et al.* (2014) had compared the *in vitro* antioxidant activity of petroleum ether, dichloromethane and 95% ethanol extracts of dried leaves of *A. subintegra* and found the highest radical scavenging activity from the 95% ethanolic extract. Huda *et al*. (2009) had extracted the dried leaves sample of *A. malaccensis* using ethyl acetate, dichloromethane, methanol and hexane and found the highest potential of antioxidant properties from the methanol extract.

Another *in vitro* study for antioxidant activity by Miniyar *et al.* (2008), who had revealed the ethyl acetate extract of *A. malaccensis* gives a strong antioxidant for inhibitory effect on nitrite-induced oxidation of haemoglobin in human blood haemolysate. Han and Li (2012) found the strong antioxidant potential of the methanol dried leaf extract of *A. sinensis* after testing it using different *in vitro* antioxidant assays; 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity, ferric ion (Fe^{3+}) reducing power, cupric ion (Cu^{2+}) reducing power, superoxide anion

($O_2^{\cdot-}$ **)** scavenging activity, hydroxyl radical (·OH) scavenging activity, metal chelating assays (Fe^{2+} and Cu^{2+}) and lipid peroxidation.

Tay *et al.* (2014) had extracted the dried leaves of *A. crassna* using different percentages of ethanol in water (0% to 100%) and reported that extraction using 60% ethanol gave the highest yield of polyphenols and extraction using 100% ethanol gave the highest DPPH radical scavenging activity with low yield of flavonoids. The essential oil from the stem bark of *A. crassna,* which was obtained from hydrodistillation method also exhibited a significant DPPH free radical scavenging and ferric reducing antioxidant power **(**FRAP) activities (Dahham *et al*., 2015). Rattanama *et al*. (2014) had compared *in vitro* antioxidant activity of deionise water and ethyl acetate extracts of *A. subintegra* tea leaves and found ethyl acetate extract gave a better antioxidant activity, which possesed higher extraction yield and total flavonoid content.

2.2.2 Anti-diabetic activity

Recently, researchers became interested in investigating the plant polyphenols that are capable in inhibiting carbohydrate-hydrolyzing enzymes such as α-glucosidase and α-amylase to prevent hyperglycemia. Control of postprandial hyperglycemia after a meal is the most effective approach for treating Type 2 diabetic patients by preventing the absorption of glucose in the small intestine and

enhance glucose disposal in the cells (Yao *et al.*, 2009). Dietary carbohydrates such as starch are the main source of glucose that is hydrolyzed by α-glucosidase and pancreatic α-amylase enzymes (Kim *et al*., 2005). Therefore, inhibiting the activity of these carbohydrate digesting enzymes are the best way to retard or delay the absorption of glucose and maintaining the postprandial glucose level in the blood (Watanabe *et al*., 1997). The synthetic inhibitors that have been commercialized as drugs to treat Type 2 diabetic are acarbose, miglitol, and voglibose (Yoshikawa *et al*., 1998; Yao *et al.*, 2009).

Feng *et al.* (2011) used α-glucosidase inhibition assay to isolate the compounds from 70% aqueous ethanolic extract of *A. sinensis* leaves. Some potent phenolic compounds were isolated which are mangiferin, iriflophenone 2-*O*-α-L-rhamnopyranoside, iriflophenone 3-*C*-β-D-glucoside and iriflophenone 3,5-*C*-β-D-diglucopyranoside.

Nur Liyana *et al.* (2013) had compared α-glucosidase and α-amylase inhibitory activity of *A. malaccensis* and *A. hirta* and found the highest α-glucosidase inhibitory activity of the methanol extract of *A. hirta* while methanol extract of *A. malaccensis* for α-amylase inhibitory activity. Another comparative study by Yunus *et al*. (2015) on the drying effects of 70% ethanol extracts of *A. malaccensis* and *A. subintegra* leaves using microwave power intensity (50W-150W) and found that both of these exhibited the highest α-amylase inhibition and high yield of polyphenols at low power intensity (50W). The ethanol extract of *A.*

subintegra gave higher α-amylase inhibitory activity than ethanol extract of *A. malaccensis*.

The leaves of *A. sinensis* has been reported to have *in vitro* and *in vivo* anti-diabetic activities. The methanolic extract was found to have a significant effect in lowering fasting blood glucose level in Steptozotocin-induced (STZ) diabetic rats *in vivo* as compared to hexane and ethyl acetate extract. The same extract also was the most active in enhancing the uptake of glucose into rat adipocytes *in vitro* (Pranakhon *et al*., 2011).

Further *in vitro* and *in vivo* anti-diabetic studies by Pranakhon *et al.* (2015), found the presence of iriflophenone 3-*C*-β-glucoside (IPG) in the methanol extract of *A. sinensis*, which was active in reducing fasting blood glucose compared to insulin effectively.

2.2.3 Anti-inflammatory activity

Inflammation can be defined as the physical condition of body that appear redness, warmth, swollen and pain caused by the body's response to noxious stimuli, injury or infection by pathogens (Rahman *et al*., 2012). Nowadays, non-steroidal anti-inflammatory drugs (NSAIDs) play an important role as anti-inflammatory

drugs to reduce the pain of inflammation in the body by inhibiting prostaglandin synthesis as well as cyclooxygenase inhibitory activity (Olivier, 2001). However, long-term consumption of synthetic NSAIDs drugs may cause severe side effects and carry risk to cardiovascular disorders, gastrointestinal toxicity and others (Hawkey, 2001; Andreas and Oliver, 2012).

Therefore, in recent time, several studies have been carried out to discover a novel, effective, less toxic or safe anti-inflammatory drugs from natural sources that may provide alternative ways to treat the inflammation disorders. Different parts of several *Aquilaria* species including leaves have been studied to discover anti-inflammatory agents within this genus. Kumphune *et al.* (2011) reported that the ethyl acetate extract of *A. crassna* heartwood had anti-inflammatory potential by inhibiting the tumour necrosis factor-alpha gene expression and secretion in lipopolysaccharides (LPS)-induced human peripheral blood mononuclear cells. Zhou *et al*. (2008) revealed that the ethanolic extract of *A. sinensis* leaves potentially inhibited the elevated nitric oxide (NO) level in lipopolysaccharides (LPS)-stimulated nitric oxide (NO) release from macrophages *in vitro*.

Wu *et al*. (2012) reported that the peel extract of *A. sinensis* had significantly suppressed inflammation in RAW 264.7 cells by lipopolysaccharides (LPS), which can be reached by suppressing the protein level of cyclooxygenase (COX-2) isozymes *in vitro*. Chen *et al.* (2014) revealed that the ethyl acetate-soluble fraction from the pericarp of *A. sinensis* in the presence bioactive compounds (velutin, pillion and β-sitostenone) exhibited potent inhibition against lipopolysaccharide (LPS)-

induced NF-kB production by macrophages *in vitro*. Wang *et al*. (2015) had isolated twenty-one bioactive compounds including two new flavones **(**4'-*O*-geranyltricin and 3'-*O*-geranylpolloin) from the stem bark of *A. sinensis* and stated that these compounds have potential for the treatment and prevention of inflammatory.

Using animal models to investigate the anti-inflammatory activity in the genus *Aquilaria*, Rahman *et al.* (2012) studied the essential oil of *A. malaccensis* wood that was obtained from hydrodistillation method for *in vivo* and *in vitro* anti-inflammatory activity and found this agarwood oil has anti-inflammatory potential with a significant reduction of edema in carrageenan induced rat paw edema model *in vivo* and strong membrane stabilizing on human red blood cell *in vitro* as compared with the standard diclofenac. Another *in vivo* anti-inflammatory study by Huanze *et al*. (2013), on *A. sinensis* leaves, found that the leaves originating from two different places have similar inhibitory effect when tested on mice induced by xylene.

2.2.4 Antibacterial activity

Numerous studies of the "gaharu" tree of the genus *Aquilaria* with antibacterial properties have been carried out. By definition, antibacterial agent is an action of substance that kill bacteria and/or inhibits their growth or replication. It will destroy the pathogen without affecting the infected patient. Some antimicrobial

agents are antibiotics and antibiotics can be defined as a substance that is produced by a microorganism that is effective in killing and suspressing the growth of the other microorganism. The antibacterial agents usually target pathogen membrane structure and metabolic processes to accomplish their actions (Paul and Janet, 2008).

Dash *et al*. (2008) had investigated the inhibitory capacity of aqueous and methanol extracts from *A. malaccensis* leaf and bark against *Shigella flexneri, Bacillus brevis, Pseudomonas aeruginosa* and *Bacillus subtilis* and found all of the extracts had moderate activity against all tested bacteria while the methanol extract of the leaf gave the highest inhibition against *B. subtilis*. Kamonwannasit *et al.* (2013) reported the effect of aqueous extract of *A. crassna* leaves that was found to disrupt the bacterial cell wall and inhibit bacterial biofilm formation against *Staphylococcus epidermis*.

Essential oils from different chemical stimulation methods of *A. sinensis* were tested against Gram-positive bacterial strains such as *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacterial strain *Escherichia coli* and found that all the essential oils, which are containing sesquiterpenes and aromatic compounds actively inhibited all the tested bacteria except *E. coli* (Chen *et al*., 2011). In agreement with a study by Wen-Jian *et al*. (2014), their results revealed that the antibacterial activity of *A. sinensis* was due to the presence of sesquiterpenes and 4-hydroxyphenylacetic acid.

Remarkable results using the disc diffusion methodology were established using twenty-eight fungal endophytes of the stem of *A. sinensis* against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Aspergillus fumigatus.* Thirteen endophytic fungi associated with *A. sinensis* exhibited high antibacterial activity to at least one of the bacterial tested (Cui *et al*., 2011). Similarly, the ethyl acetate extract of endophytic fungus of *A. malaccensis* had been reported by Shoeb *et al*. (2010) to have mild antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus*.

Rahman *et al*. (2013) studied the antibacterial activity of *A. malaccensis* oil and *Citrullus lanatus* seed oil by agar well diffusion method and compared with standard ciprofloxacin, against *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus* and *Pseudomonas aeruginosa*. It was found that both oils have antibacterial activity against the selected bacteria and the *Citrullus* oil possesed stronger antibacterial activity than *A. malaccensis* oil.

2.2.4(a) Antibacterial activity of herbal teas from *Aquilaria* species

Nowadays, tea from *Camelia sinensis* has been recognized as a tonic and remains a kind of medicine particularly in traditional Chinese medicine to treat a variety of disorders (Chen, 2003). Lee *et al*. (2006) examined the ethyl acetate and water polyphenols tea extracts with different strains of intestinal bacteria and found that pathogenic bacteria such as *Clostridium perfringens, Clostridium difficile* and

Bacteroides spp. were suppressed significantly by tea phenolics and their derivatives while *Clostridium* spp. and *Bifidobacterium* spp. which are anaerobic bacteria and probiotics such as *Lactobacillus* sp. were poorly affected by tea phenolics. This showed that the tea phenolics substantially affected the implementation of the gastrointestinal tract. The presence of caffeic acid in the tea extracts provided strong inhibition against *E. coli* and *Salmonella* sp. which are usually causing food-borne diseases such as diarrhea and nausea and also pathogenic bacteria such as *Staphylococcus aureus, Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Vibrio chlorae* and others (Chen, 2003; Lee *et al.*, 2006).

In this study, the *Aquilaria* leaves were tested for their antibacterial activities against gastrointestinal pathogenic bacteria. Previous studies reported that herbal teas from bark and stem of *A. malaccensis* are consumed traditionally to treat gastrointestinal disorders such as diarrhea and abdominal pain and these teas have been practised for many years in folk medicine in certain countries (Ong, 2004). Previous studies by Kamonwannasit *et al.* (2011) revealed that the aqueous extract of *A. crassna* leaves is beneficial for treating diarrhea caused by *Staphylococcus aureus* and skin infection associated with *Staphylococcus epidermis*. The disc-diffusion methodology was established on aqueous extract of *A. malaccensis* leaves against various pathogenic microbes including *Bacillus cereus*, *Candida albicans*, *Streptococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Aspergillus niger*. It was found that the extract suppressed the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Manasi *et al*., 2008).