ISOLATION AND CHARACTERIZATION OF ACETYLCHOLINESTRASE INHIBITORS FROM *AQUILARIA* SPECIES FOR TREATMENT OF ALZHIMER DISEASE (AD)

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ABSTRACT

Aquilaria subintegra locally known as “Gaharu” belongs to the Thymelaeceae family. Its leaves have been claimed by Malay traditional practitioner in Malaysia were effective for the treatment of Alzheimer’s disease (AD). However, there is no scientific evidence available to support these claims. The AD is one of the most common neurodegenerative disorders and it causes dementia with aging. The acetylcholesterase (AChE) inhibitors were used for the treatment of AD. In this research the leaves and stem chloroform extract of *A. subintegra* were tested for AChE inhibitory activity. The TLC results of the leaves and stem of *A. subintegra* showed the presence of phenols, flavonoids, terpenoids, and alkaloids compounds in the extracts. The total phenol contents in the leaves and stem chloroform extracts were 164mgGAE/g and 210mgGAE/g respectively. Whereas, the total flavonoids contents in leaves and stem chloroform extracts were 414mgQE/g and 645mgQE/g respectively. The analysis of the stem chloroform extracts with LCMS/MS displayed that it contain kaempferol 3,4,7-trimethyl ether. The AChE inhibitory activity of leaves, stem chloroform extracts and kaempferol were 80%, 93% and 85.8% respectively. Brine Shrimp Lethality Assay (BSLA) exhibited LC_{50}=11.64µg/ml of leaves chloroform extract, LC_{50}=3.11µg/ml of stem chloroform extract, and LC_{50}=9.59µg/ml for kaempferol. The response of leaves, stem chloroform extracts and kaempferol were perceived in Radial Arm Maze (RAM) through oral gavage to ICR male and female mice with impaired memory by valium. Administration of kaempferol to the mice significantly reduced the number of repeated entries to arms of maze in male and females rats. In conclusion, it could be postulated that the inhibition of AChE by leaves and stem chloroform extracts of *A. subintegra* could be due to the presence of kaempferol. Thus, it is safe to be used as a natural AChE inhibitor instead of berberine for the treatment of AD.

ABSTRAK

Aquilaria subintegra dikenali sebagai "Gaharu" adalah dari keluarga Thymelaeceae. Daunnya telah didakwa oleh pengamal tradisional Melayu di Malaysia berkesan untuk rawatan penyakit Alzheimer (AD). Walau bagaimanapun, tiada bukti saintifik yang menyokong dakwaan itu. AD adalah salah satu penyakit neurodegenaratif yang paling biasa dan ia menyebabkan demensia dengan penuaan. Perancat Acetylcholesterase (AChE) telah digunakan untuk rawatan AD. Dalam kajian ini ekstrak batang dan daun kloroform telah diuji untuk aktiviti perencatan AChE. Keputusan dari ekstrak TLC daun dan batang *A. subintegra* menunjukkan kehadiran sebatan fenol, flavonoid, terpenoid dan alkaloid. Jumlah kandungan fenol dalam ekstrak daun dan ekstrak batang adalah 164 mgGAE/g dan 210 mgGAE/g masing-masing. Manakala, jumlah kandungan flavonoid dalam ekstrak daun dan ekstrak batang adalah 414 mgQE /g dan 645 mgQE/g masing-masing. Analisis ekstrak batang dengan LCMS/MS memaparkan bahawa ia mengandungi kaempferol 3,4,7-trimethyl eter. Aktiviti perencatan AChE daripada ekstrak daun, batang dan kaempferol adalah 80%, 93% dan 85.8% masing-masing BSLA memberikan nilai LC_{50} = 11.64 µg/ml bagi ekstrak daun, LC_{50} = 3.11 µg/ml bagi ekstrak batang, dan LC_{50} = 9.59 µg/ml bagi kaempferol. Kesan ekstrak daun, batang dan kaempferol diperhatikan dalam kajian RAM (lengan radial maze) secasa oral gavage pada tikus jantan dan betina ICR dengan dengan menjejaskan ingatan menggunakan valium. Penganbilan kaempferol pada tikus mengurangkan dengan signifikan bilangan entri berulang ke RAM maze dalam tikus jantan dan betina. Kesimpulannya, maka boleh dipostilasikan bahawa perencatan AChE oleh ekstrak daun dan batang *A. subintegra* adalah disebabkan kehadiran kaempferol dan dengan itu ia boleh digunakan sebagai perencat AChE semulajadi selain daripada berberine untuk rawatan AD.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ATCI	Acetylthiocholine iodide
Aβ	Amyloid beta
BDNF	Brain Derived Neurotrophic Factor
BSLA	Brine Shrimp Lethality Assay
CNS	Central Nervous System
DTNB	5,5'-Dithiobis [2-Nitrobenzoic Acid]
EGGG	Epigallocatechin-3-Gallate
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
GABA	Gamma-Aminobutyric Acid
HPLC	High Pressure Liquid Chromatography
ICR	Imprinting Control Region
LCAS	Leaves Chloroform Extract of *Aquilaria subintegra*
LCMS	Liquid Chromatography Mass Spectrometry
LC_{50}	Lethal Concentration, 50%
MRI	Magnetic Resonance Imaging
NEF	Number of Entries Until the First Error Occurs
NMDA	N-methyl-D-aspartate
NRE	Number of Repeat Entries to Arms of the Maze
PNS	Peripheral Nerves System
RAM	Radial Arm Maze
SCAS	Stem Chloroform Extract of *Aquilaria subintegra*
TLC	Thin Layer Chromatography
TNB	5 thio-2-nitrobenzoate

CHAPTER 1 INTRODUCTION

AD is one of the most common neurodegenerative disorders that cause dementia with aging. Brookmeyer et al. (2007) predicted that AD would affect 1 in 85 people globally by 2050. The AChE inhibitors are chemical agents used for symptomatic treatment of AD. AChE could hydrolyze the neurotransmitter acetylcholine due to its protease activity. Golan et al. (2011) declared that AChE has high catalytic activity and it is able to hydrolyze 4×105 molecules of acetylcholine in one minute. The most common view in the treatment of AD is the effectiveness of AChE inhibitors to increase acetylcholine medicated neuron to neuron transmission. However, Tabet (2006) broadcasted that AChE inhibitors could have anti-inflammatory role by protecting cells from free radical toxicity and amyloid beta peptide injury. In 1999, McGleenon et al. proposed that Aβ and abnormal tau protein could be main factors in the development of AD. The influence of hyperphosphorylated tau protein on the expression of AChE was observed in another study by Garcia-Ayllon et al. (2011). They also found out the important role of AChE in AD by decreasing acetylcholine. Later, Anand et al. (2012) reported the activity of AChE inhibitors in AD management through decreasing Amyloid beta (Aβ) production and aggregation, or by increasing the removal of Aβ.

Aquilaria subintegra is locally known as "Gaharu", is a plant which belongs to Thymelaeceae family. Recently, Malays traditional practitioners claim that *A. subintegra* leaves are effective for the treatment of AD. In South-East Asians traditional medicine the usage of agarwood was as a component in Kampo-formulae, such as 'kiogan' and 'rokushingan,' for making sedatives. However, there is no scientific evidence available to support these claimed effects. Ueda et al. (2006) considered that the effects of agarwood (*Aquilaria*) as a traditional sedative, is due to its induction effects on central nervous system. Additionally, their study in rat cultured neuronal cells displayed that a new compound (4R, 5R, 7R)-1(10)-spirovetiven-11-o1-2-one and a 2-(2-phenylethyl) chromone derivative which were isolated from methanol extract of agarwood had significant succession effect on mRNA expression of brain derived neurotrophic factor (BDNF). Furthermore, some studies approved presence of natural AChE inhibitors in other plants. Yang et al. (2011) reported strong AChE inhibitory activity of an alkaloid skimminaine extracted from *Zanthoxylum nitidum* roots. They indicated that the anti-AChE activity of skimmianine may be impressive in the treatment of AD. In 2010, Pereira et al. found out that water extract of alkaloids from leaves of *Catharanthus roseus* strongly inhibited AChE.

It was suggested by Kim et al. (2004) that kaempferol has anti-inflammatory properties like other flavonoids. Likewise, Serhan et al. (2008) illustrated that chronic inflammation occurs when acute inflammation is not resolved. Kaempferol has a detrimental effect in numerous disease including atherosclerosis, cancer, asthma, and some neurological disorders, such as AD. In 2012 Song et al. demonstrated that the kaempferol isolated from butterbur (*Petasites japonicas*) leaves might prevent the effects of Aβ that can

cause AD by inducing neurotoxicity and by producing free radicals that lead to cellular death.

Using animal (ICR mice) might be beneficial to improve AChE inhibitors in treatment of AD. Dong et al. (2005) investigated two AChE inhibitors physostigmine and donepezil on memory related behaviors of mice. They suggested that AChE inhibitors could improve memory deficits in mice. Ikarashi et al. (2004) examined mice memory disturbance by measuring AChE concentrations in their brains. They found out that memory impairment in cholinergic system of the mice is due to Aβ accumulation which happens when AChE increased. Figueiró et al. (2010) extracted AChE inhibitors from *Ptychopetalum olacoides* and orally administrate these compounds to the mice. They improved meaningful AChE inhibitor activity of these extracts for treating neurodegenerative conditions which is useful for AD treatment. The Radial Arm Maze (RAM) is a practicable memory testing for displaying the effect of drugs. Foti et al. (2011) considered that the RAM can be analyzed via movement most aspects of the spatial function, as the analytical memory and the appropriate working memory.

In this study the determination AChE of inhibitory activity of the chloroform leaves and stem extract of *A. subintegra* were evaluated in enzymatic bioassay system. The AChE inhibitory activity of the leaves, stem extracts and kaempferol detected in the stem chloroform extract of *A. subintegra*, was determined using Radial Arm Maze (RAM) through oral gavage to the ICR male and female mice with impaired memory by valium.

CHAPTER 2 LITRETURE REVIEW

2.1 Plant studied-*Aquilaria subintegra*

2.1.1 Scientific classification of *Aquilaria subintegra*

Kingdom	: Plantae
Subkingdom	: Angiosperms
Division	: Eudicots
Class	: Rosids
Subclass	: Malvales
Order	: Thymelaeaceae
Family	: Thymelaeaceae
Genus	: *Aquilaria*
Species	: *Subintegra*

Figure 2.1: *Aquilaria Subintegra*

2.1.2 Scientific and common names

Aquilaria subintegra is a species of plant in the family of Thymelaeaceae. Common names: agarwood, aloeswood, eaglewood. Vernacular names: Alas, Calambac, Ching karas, Gaharu, Galoop, Garu, Gharu, Karas, Kayu gaharu, Kekaras, Kepang, Laroo, Mengkaras, Ngalas, Sigi-sigi, Tabak, Taras gharu, Tengkaras.

2.1.3 Distribution

The phytogeographical region for *Aquilaria subintegra* comprises India, Burma, Thailand, Peninsular Malaysia, Sumatra, Borneo (Sabah, East-Kalimantan) and Philippines.

2.1.4 Description

Aquilaria subintegra is a tree that grows 6 to 20 m tall, its bole up to 60 cm in diameter, consistently straight however sometimes fluted. The buttresses are up to 2 m high. The bark is plane and whitish. The branchlets protected with the soft short hairs, they are slender and pale brown.

The leaves of *Aquilaria subintegra* fortified by 4 to 6 mm long stalk, they are simple and arranged alternate. The area of the blade is between 7.5-12 × 2.5-5.5 cm, it shaped elliptical oblong to oblong lance. Generally the leaves are hairless but sometimes covered by soft short hairs. They are shiny on both surfaces. The apex is acuminate with acumen up to 2 cm long with rather aberrant veins in 12 to 16 pairs. Their bases are acute, attenuate or obtuse. The veins are distributed plane or obscure on the top surface and curving upward to the margin of leaves (Chua, 2008).

Aquilaria subintegra has terminal inflorescence, which is arising from axils, consistently from two nodes umbel. Each umbel has 10 flowers uphold by 5 to 15 mm long stalk. The pedicel is 3 to 6 mm long and slender. The flowers of this tree have 5 merous with the shape like a bell, 5 to 6 mm long and the soft short hairs on its outside. The color of the flowers is green or dirty yellow (Chua, 2008).

The fruit of *Aquilaria subintegra* is a dorsal suture capsule, it has reverse egg shaped. It is protected with the soft short hairs. The fruit size is 3-4 cm × 2.5 cm. The seed of this tree has red hair. The seed size is 10 mm × 6 mm (Chua, 2008).

2.1.5 Medicinal and other uses of *Aquilaria subintegra*

The first appearance of *Aquilaria* plant was appreciated in the ceremonies of Buddhism or Islam since early ages, due to its ethereal pleasant scent. In South-East Asians traditional medicine the usage of agarwood was as a component in Kampo-formulae, such as ‘kiogan’ and ‘rokushingan,’ for making sedatives.

Ueda et al. (2006) declared that agarwood extract could be effective on central nervous system (CNS) for instance it leads to excessive brain function. Additionally, their study in rat cultured neuronal cells displayed that a new compound (4R, 5R, 7R)-1(10)-spirovetiven-11-o1-2-one and a 2-(2-phenylethyl) chromone derivative which were isolated from methanol extract of agarwood had significant succession effect on mRNA expression of brain derived neurotrophic factor (BDNF).

2.2 Flavonoids

2.2.1 Introduction

One of the largest groups of secondary metabolites which is extensively scattered in plant kingdom are flavonoids. More than 4000 structurally exclusive flavonoids have been perceived, in plant sources (Harbone et al., 1975). Flavonoids almost entirely act as photoreceptors (Moore, 1989), antifeedants, visual attractors (Hedin and Wangea, 1986), antioxidants (Pietta, 2000), bactericides and fungicides (Tomas-Barberan et al., 1988). In all vascular plants, water soluble polyphenolic compounds are exist, they are biosynthesized from the phenylpropanoid metabolic pathway. As shown in Figure 2.2 displayed flavonoid fundamentally acquire 15 carbon atoms, through which a linear three carbon chain correlates two fused six member rings, a hetrocyclic C ring and an aromatic A ring to an aromatic B ring (Patel, 2008). The skeleton of flavonoids delineated as C_6-C_3-C_6.

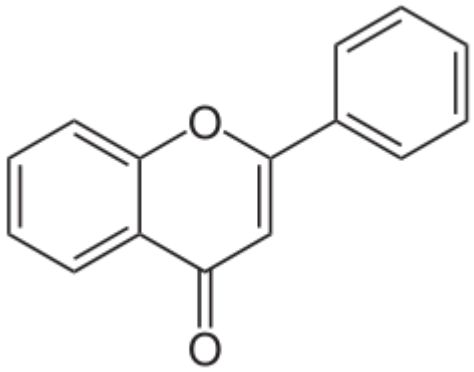


Figure 2.2: General structure of flavonoid

2.2.2 Main categories and food sources of flavonoids

Based on chemical structure flavonoids can be classified into six different major groups including flavonols, flavones, flavanones, isoflavones, flavanols and anthocyanidins (Beecher, 2003). Flavonoids appear consistently in assorted food sources that are exhausted frequently in human regime such as vegetables, fruits, beverages and herbs.

Table 2.1: Flavonoid classes, chemical characteristics, prominent food and common food sources.

Flavonoid classes	C ring unsaturation	C ring functional group	Prominent food flavonoids	Common food sources
Flavonols	2-3 double bond	3-Hydroxy, 4-Oxy	Kaempferol Quercetin Myricetin	Broccoli, Apples, Yellow onions, Cherry, Tomato
Flavones	2-3 double bond	4-Oxy	Apigenin Luteolin	Green leafy spices, Parsley
Flavanones	None	4-Oxy	Naringenin Hesperetin Eriodictyol	Citrus fruits: Orange, Lemon
Isoflavones	2-3 double bond	4-Oxy	Genistein Daidzein Glycitein	Legumes, Soybeans
Flavanols	2-3 double bond	3-Hydroxy 3-O-gallate	Epicathechin Catechin Epicathechin-3-gallate	Tea, Chocolate, Red grapes, Cocoa
Anthocyanidins	1-2, 3-4 double bonds	3-Hydroxy	Cyanidin Delphinidin	Berries

2.3 Kaempferol

2.3.1 Introduction

Kaempferol is a type of flavonoid, a natural flavonol. The melting point of kaemferol is 276-278°C with the molecular weight of 286.2 g/mol. It's a yellow crystalline solid which is soluble in hot ethanol and diethyl ether. However, it is hardly soluble in water. Winkel-Shirley (2002) considered that kaempferol has a diphenylpropane structure (C_6-C_3-C_6) like other flavonoids. Condensation of 4-coumaroyl-CoA (C_6-C_3) with three molecules of malonyl-CoA leads to synthesis of kaempferol.

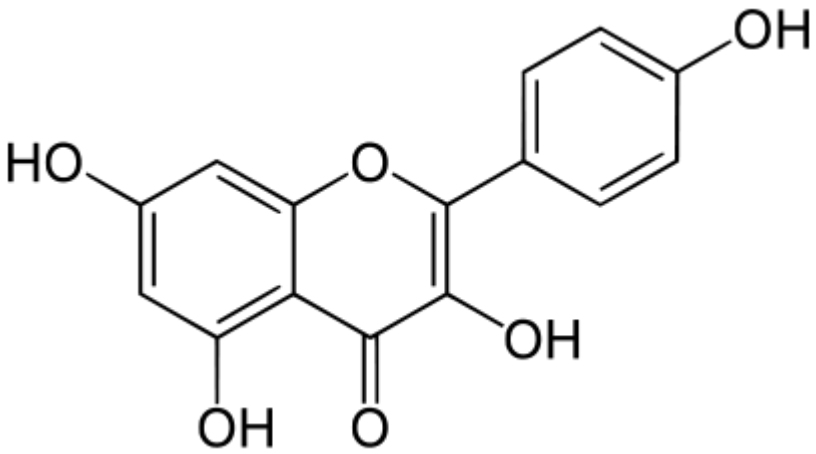


Figure 2.3: General structure of kaempferol

2.3.2 Natural sources

It can be isolated from apples, citrus fruits, onions and leeks, grapes, tea, red beans and pinto beans, broccoli, delphinium and other plant sources.

2.3.3 Biological activities of kaempferol

Cui et al. (2008) reported that high consumption of foods containing kaempferol may diminish the risk of developing several types of cancers such as lung, gastric, pancreatic and ovarian cancer. Likewise, it has the same effect on cardiovascular diseases. They suggested that further studies need to prove protective effect of kaempferol in these diseases.

(i) Antioxidant activity

Several studies have displayed that kaempferol, some glycosides of kaempferol and numerous kaempferol containing in plants have antioxidant activity. Rice-Evans (2001) mentioned that the important structural features involved in the antioxidant activity of kaempferol are the presence of a double bond at C_2-C_3 in conjugation with an oxo group at C_4 and the presence of hydroxyl groups at C_3.

In another study Wang et al. (2006) found that kaempferol can be a superoxide scavenger, with an IC_{50} of 0.5 μM. Besides, they mentioned that kaempferol antioxidant activity is due to its ability to decrease superoxide levels at low concentrations.

(ii) Anti-inflammatory activity

Kim et al. (2004) displayed that kaempferol has anti-inflammatory properties like other flavonoids. Furthermore, Serhan et al. (2008) reported that chronic inflammation occurs, when acute inflammation is not resolved. It has a detrimental effect in numerous disease including atherosclerosis, cancer, asthma, and some neurological disorders, such as Alzheimer's disease.

Several mechanisms of action play role in the anti-inflammatory activity of kaempferol. One of the most important mechanisms is activation of the nuclear factor kappa B which is increase the expression of pro-inflammatory cytokines, chemokines and enzymes like TNF-α, IL-1, IL-6, IL-8, COX-2 and iNOS (Kim et al., 2010).

(iii) Anticancer activity

Cemeli et al. (2004) suggested that low concentrations of kaempferol may preserve DNA from damage induced by different carcinogens. DNA alteration considered as the most accepted view of carcinogenesis that caused cancer.

Hahn et al. (2002) found that apoptosis resistance, increased angiogenesis, capacity of invasion and metastasis leads to formation of a malignant tumor. Additionally, Huang et al. (2010) perceived that kaempferol can be inducing apoptosis. The formation of a cancer requires that tumor cells develop apoptosis resistance.

(iv) Antimicrobial activity

Cushnie and Lamb (2005) noted that kaempferol and its glycosides which are isolated from plants, used in medicine due to their antimicrobial properties such as antibacterial, antiviral, antifungal and antiprotozoal.

Habbu et al. (2009) perceived that after 7 days oral administration of kaempferol-7-O-methyl-3-sulphate, the number of survived mice which were infected with the bacteria *Klebsiella pneumonia* significantly increased. Some investigations displayed that kaempferol has anti-viral activity against numerous viruses, including herpes simplex virus (Lyu et al., 2005), cytomegalovirus (Mitrocotsa et al., 2000), influenza virus (Jeong et al.,

2009) and human immunodeficiency virus (HIV) (Min et al., 2001). Barbosa et al. (2007) described that kaempferol has antiprotozoal activity. Furthermore, Marin et al. (2009) showed that this activity is against *Leismania* spp, *Entamoeba histolytica* and *Giardia lamblia*.

(v) Other biological activates

Consumption of kaempferol containing foods is associated with a decreasing in mortality from coronary heart disease. It can be reduce risk of coronary heart disease cause to death, incidence of myocardial infarction and incidence of cerebrovascular disease (Lin et al., 2007; Marniemi et al ., 2005). Other studies by Belguith-Hadriche et al. (2010) suggested that kaempferol and some of its glycosides may decline triglycerides levels, cholesterol levels and body weight. Ghaffari and Mojab (2007) broadcasted that kaempferol glycosides and numerous kaempferol containing in plants have antidiabetic activity, it can be inhibit diabetic complications.

2.4 AChE inhibitory activity and other accessible health advantages of flavonoids

One of the various group of phytonutrients that have been extensively studied for human health are flavonoids, due to their instrumental medicinal properties, such as antioxidant (Pietta, 2000), anti-inflammatory (Jung and park, 2007) and AChE inhibitory activity (Tim Cushnie and Andrew, 2005).

During a study Jung and park (2007) reported that flavonoids from *Agrimonia pilosa ledeb* possess significant AChE inhibitory activity. Exclusively, AChE inhibitory activity of quercetin was twice as dehydroevodiamine. Further research by Khan et al.

(2009) proved that flavonoid derivatives, quercetin and macluraxanthone have AChE inhibitory activity with different deals of characteristics.

Flavonoids due to their estrogenic activities have been considered as successors for estrogen (Miksicek, 1993), beside that some flavonoids such as apigenin and kaempferol illustrated strong neuroprotective effects in the brain versus Aβ association and Aβ impel cell death (Zhu et al., 2007). Thus, one of the most beneficial natural compounds could act important role against Alzheimer's disease are flavonoids (Ji and Zhang, 2006).

Besides the biological actions noted above, flavonoids have been prevalent to exploit many other effects including antitumor, anticancer, anticarcinogenic, antihepatotoxic, antiallergic, antiatherosclerotic, antithrombotic, antihypertensive and antiarrhythmic activity (Formica and Regelson, 1995). Flavonoids have the quiescent to be an admirable candidate in pharmaceutical drug augmentation against AD due to their discrete explorations on the biochemical and pharmacological properties.

2.5 Alzheimer's disease (AD)

2.5.1 Introduction

Alzheimer's disease is a chronic neurodegenerative disorder. Many investigations demonstrated that it causes degradation in cognitive functions for instance in concentration, language skills, learning, reading and writing. This is associated with the deterioration in non-cognitive competence which induces personal changes, behavioral abnormalities, biological disability, neurological disturbances and ultimately death (Tariot, 1994; McGleenon et al., 1999; Jung and park, 2007). AD is one of the major causes of death, especially for the people over 65 years old in the world (Launer et al, 1999). It is a

neurodegenerative brain disease that insidiously robs patients of their cognition, function, independence identity and results in morbidity and eventual mortality (Henley et al., 2012).

In 2007 Brookmeyer and colleagues broadcasted that AD is the most dominant cause of dementia and reports for roughly 60 to 80 % of cases in our aging population. They assessment by linear extrapolation there is 33.9 million AD patient worldwide in 2011. Barnes and Yaffe (2011) predicted that AD would be increase exceedingly in the next future. At the age of 60 AD pervasiveness is around 1% and its contingency doubles every 5 years after age 65.

Extensively AD is classified into several clinical forms such as sporadic AD includes 85 to 90 % of cases with late onset, AD affiliated with Down's syndrome and familial AD indicates approximately 5% of AD patients with early and late onset (Khachaturian, 1985).

2.5.2 The physiopathology and neuropathology of AD

The pathogenesis of AD has not been absolutely ascertained due to its complexity. Benzi and Moretti (1998) demonstrated that AD was evidently delineated pathologically by the severe loss of neuronal and synaptic cells, the presence of neurofibrillary tangles and cerebral Aβ.

(i) Synaptic and neuronal cell death

The cholinergic hypothesis of Alzheimer's disease was endowed hence early 1970's. Silvestrelli and colleagues (2006) mentioned that the features, cognitive dysfunction, behavioral and functional annoyances in AD patient may due to discerning impairment on cholinergic neurotransmitter systems. The depletion of acetylcholine mainly

caused cholinergic inadequacy, which could be overflow AChE activity and resulted cell death in cholinergic basal forebrain system.

In other researches indicated by Beeri et al. (1995) exuberance of AChE were adapted the structural features of neuromuscular junctions in transgenic *Xinopus* tadpoles. Additionally they displayed memory and learning impairment in transgenic mice. Otherwise, Hohmann and colleagues (1988) discovered that high affinity choline uptake in cortical areas and significant deficiency in AChE activity was persistent with the extensive neuronal loss especially in nucleus basalis of AD patient. Whitehouse et al. (1986) recognized that progressive memory and cognitive deterioration in AD were due to severe and consistent loss of nicotinic receptors.

(ii) Neurofibrillary tangles (NFT)

Abnormal microtubule associated tau protein made by neurofibrillary tangles which are double helical filaments. Hernandez and Avila (2007) reported that phosphorylated tau proteins by stabilizing microtubules help to growth and development of axon in normal cells. Nonetheless, inside the soma of neurons hyperphosphorylated tau proteins begin to pair and link with other tau protein threads to assemble neurofibrillary tangles. They consistently placed in the parietotemporal region, hippocampus, the frontal affiliation cortices and medial temporal lobe. Some studies illustrated that by collapsing the neuronal transport system, the microtubules become degenerate and ultimately leads to neuronal cell annihilation (Iqbal et al ., 2005; Cummings, 2004).

Silvestrlli et al. (2006) displayed that the density of NFT within neurons in brain precisely related with the dangerousness of dementia. Furthermore, Hardy and Selkoe

(2002) during a study hypnotized that different alleles of a gene may generate forms of tau protein that prone to become tangles, although the role of tau protein and NFT formation in the access and progression of AD are still ambiguous.

(iii) Neuritic plaques (Amyloid plaques)

Benzi and Mretti (1998) declared that dense and insoluble amyloid beta peptide protein (Aβ), glial elements and dystrophic neuritis are neuritic plaques. Furthermore, Silvestrelli et al. (2006) considered that Aβ is a peptide fragment borrowed from sequential proteolysis of the larger Aβ precursor protein (APP) by β-secretase and γ-secretase. Selkoe and Schenk (2003) discussed that APP is encoded by a gene located on chromosome 21, it's a transmembrane glycoprotein that concentrates in neuronal synapses. Some investigations suggested that Aβ fibers which produce Aβ fragments, formerly combined and figure Aβ plaques and drop between nerve cells within extracellular spaces (Ohnishi and Takano, 2004: Tiraboschi et al., 2004).

In keeping with Hensley et al. (1994), in vitro studies notify that Aβ leading to the excessive membrane lipid peroxidation and oxidative damage to the brain cells by stimulating the generation of free radicals. The experiments exhibiting Aβ deposition, cholinergic disorder, memory impairment, neurotoxicity and formation of fibrillary tangles, have been supported by the idea of Aβ is one of the culprits leading AD (Giovannelli et al., 1995; Frautschy et al., 1996). Recent researches by Alzheimer's Association in 2011 including that Aβ plaques and tangles may form in old generations, however, incline to develop far more in AD patient.

Farlow (1998) broadcasted that numerous other prominent factors contribute to pathogenic process of AD, including mild cognitive impairment, inflammation, oxidative stress, cardiovascular disease risk factors, traumatic brain injury and estrogen hormone deficiency.

2.5.3 Warning signs of AD

In 2009 a list of ten warning signs of Alzheimer's disease that can serve as a guideline to help distinguish AD sufferers created by the Alzheimer's Association of United States and Alzheimer's disease Foundation of Malaysia.

I. Changes in characteristic and attitude.

   AD sufferers can become calmly suspicious, confused, nervous or depressed with people and their surrounding environment.

II. Short term memory impairment.

   AD patients regularly lose track of dates, events and information that they learned recently. Frequently, they may ask for the same counsel and wait for memory assistants or their relatives to handle the things they used.

III. Poor judgments and decision making.

   People with AD may incompetent to manage their current budget, for instance they pay wrong amounts of money. They may have less responsibility about grooming and self-cleanliness.

IV. Problem in recognizing visual image and spatial relationships.

Vision problems, feeing hard in judging distance, reading and identifying colors noticed in AD patients.

V. Misplacing things and being unable to reiterate steps.

A person with AD not be able to fetch the things again, they may put things in improper places or lose things.

VI. Problems in vocabulary and language in writing or speaking.

Forgetting simple words or use wrong words, making incomprehensible sentence and unable to continue or follow a conversation properly are signs of AD.

VII. Complexity in solving problems or planning.

Some AD patient cannot contemplate and take longer time to do things than they did before. They may have problems to deal with numbers, develop or follow a plan.

VIII. Dilemma in achieving familiar tasks.

It's difficult for AD sufferers to fulfill daily tasks for instance walking to a familiar place, turning on the television or remembering a favorite game's rules.

IX. Bewilderment with place or time.

People with AD perplexed with date and time. They may become lost readily by forgetting where they are, how they got here and how to get home.

X. Departure from social activities or work.

AD patient may begin to avert social activities, hobbies or sports due to the changes they have expert.

2.5.4 Treatments for AD

Alzheimer's Association of United States in 2007 demonstrated that AD cannot be cured by any definitive drug treatments. Nonetheless, United States Food and Drug Administration (FDA) have been approved two main types of medication to ameliorate symptoms of AD, AChE inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists.

Memantine (Namenda) was the first drug of the NMDA receptor antagonist type certified by FDA for treatment of moderate to severe AD. Alzheimer's Association of United States in 2007 broadcasted that Memantine protect brain's nerve cells against excess glutamate, it acts by adjusting the activity of glutamate. This is because excess glutamate can arouse NMDA receptor and leads to the excess entry of calcium into nerve cells. This can causes degeneration of nerve cells. Memantine could prevent such destructive sequence by locking NMDA receptor. Generally, Memantine is well tolerated however, some common side effects such as headache, confusion, dizziness and constipation repots by Alzheimer's Association of United States.

Recently, many studies released that could act as potential future treatments of AD. For instance, Craft et al. (2011) declared that Aβ could be reducing by administrating of intranasal insulin which helps to improve brain glucose metabolism. Moreover, Krohn et al. (2011) asserted that Aβ formation can reduce by activating of membrane transport protein $ABCC_1$. This leads to blocking neurodegeneration that causes dementia in AD patient.

2.6 Acetylcholine (ACh)

Ekman (2001) considered that an important neurotransmitter that serves both peripheral nervous system (PNS) and central nervous system (CNS) of most organisms is ACh. Regularly, ACh involves in memory, learning and mood, in CNS (Silvestrelli et al., 2006). In PNS, ACh function is division of somatic nervous system and it's a major neurotransmitter in autonomic nervous system. Therefore, smooth muscle, cardiac muscle and skeletal muscle movement regulates by ACh (Ekman, 2001). Despite of that, it has inhibitory effect in heart however, with excitative effect at neuromuscular junction.

In presynaptic cholinergic nervous ACh is synthesized from acetyl coenzyme A and choline via the reaction of choline acetyltransferase. Rosenfeld and Loose (2007) suggested that ACh packaged into synaptic vesicles and discharged into synaptic cleft through calcium dependent exocytosis upon the arrival of nerve impulse at presynaptic axon terminal. Consequently, ACh diffuses across synaptic cleft to bind, activate or inhibit acetylcholine receptors found on another adjacent muscle cell or neuron.

Muscarinic and nicotinic receptors are the two main families of cholinergic receptors. As noted by Golan (2008) muscarinic receptors are G-protein coupled receptors and mainly located in the CNS, autonomic ganglia, terminal synapses of all parasympathetic postganglionic fibers and a few sympathetic postganglionic fibers. Effect on cardiac muscles, smooth muscle and secretory exocrine glands due to the binding of ACh on tissue muscarinic receptor excites the parasympathetic or cholinergic. During a study Okpako (1991) discussed that parasympathetic effects include lower heart rate, bronchi contraction, lower blood pressure, stimulation of gastrointestinal motility and

secretion. In another research Purves et al. (2012) reported that nicotinic receptors are ligand gated ionotropic receptors that are largely concentrated at CNS, autonomic ganglia, adrenal medulla and neuromuscular junctions. Ekman (2001) observed that the voluntary skeletal muscle concentration was due to binding of ACh to nicotinic receptor at neuromuscular junctions and autonomic ganglia.

Cholinesterase like AChE and butyrylcholinesterase can be hastily degrading ACh. Hydrolysis of ACh leads to nerve impulse signaling at postsynaptic cells manifests in convenient timing and prevent excessive stimulation adjacent to the muscle cell or neuron.

2.7 Acetylcholinesterase (AChE)

(i) Introduction

ACh promptly break down into choline and acetic acid by AChE. Foye et al. (2007) reported that the resulting choline is recycled through reuptake back into presynaptic nerve terminal for use in producing new ACh, since acetic acid hastily diffuses into the surrounding medium. AChE could hydrolyze the neurotransmitter acetylcholine due to its protease activity. AChE has high catalytic activity it could hydrolyzed 4×105 molecules of acetylcholine in one minute (Golan et al., 2011).

Conforming to Zimmerberg (1998) variety chemical structure of AChE leads to exists in hydrophilic and hydrophobic types. The hydrophilic species of AChE that frequently works within the cell break down the excess intracellular ACh. Quick inactivation of ACh at synaptic cleft or neuromuscular junction accomplished with the hydrophobic species of AChE is concentrated within post synaptic membrane adjacent to post synaptic receptor. Otherwise, Soreq and Seidman (2001) broadcasted that

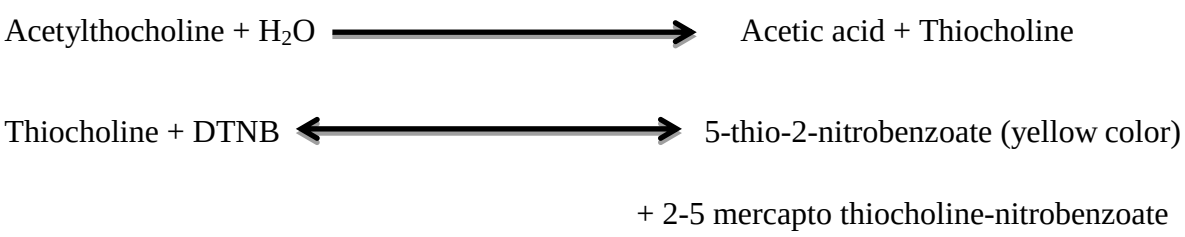
administration of AChE in red blood cells leads to releasing the ACh and cholinoceptive neurons in brain.

Appleyard (1992) mentioned that AChE is an important key in cholinergic transmission, additionally, he proved that it has multifarious other 'non-classical' activities. Besides that Soreq and Seidman (2001) suggested AChE role includes in neuritogenesis, thrombopoiesis and promotion of amyloid fiber assembly. In another study Att-ur Rahman (2001) declared that AChE has capability in degrading ACh, accordingly, it is often regarded as an attractive target for the discovery of mechanism based inhibitors and rational drug design for the treatment of diseases that involve impaired of ACh mediated neurotransmission, such as AD and dementia.

(ii) AChE activity

AChE activity was used to examine by several methods. One of them is Michel's method which needs sensitive pH meter and large samples (Michel, 1949). Another one is Ellmam's method the most simplest and popular technique was developed by George Ellman in 1961. This is the method needs micro size samples and well functioned laboratory spectrophotometer (Ellman et al., 1961) or ELISA.

In Ellmam's method, the solution prepared by adding test sample, AChE, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide (ATCHi). The, AChE breaks down ATCHi into thiocholine and acetic acid, primarily. Then, yellow colored compound known as 5-thio-2-nitrobenzoate (TNB) produce due to reacting thiocholine with DTNB which the maximum absorbance is at 412nm (Yang et al., 2011).



(iii) AChE inhibitors

Carbamates, tertiary amino compounds and organophosphates are three major categories of AChE inhibitors. Based on their mechanism of inhibition actions and chemical structures AChE inhibitors divided to 3 categories (Benzi and Moretti, 1998). Metcalf (1971) reported that carbamats are pseudo irreversible inhibitors which form a carbamoylated enzyme inhibitor complex with serine residue of AChE, hence inhibiting AChE activity such as eptastigmine. Benzi and Moretti (1998) declared that tertiary amino compounds are reversible inhibitors ether non-competitive or mixed type that can cause allosteric inhibition of AChE such as donepezil and tacrine, respectively. Fukuto (1990) mentioned that irreversible inhibitors that can form a stable phosphorylated enzyme inhibitor compound are organophosphates such as malathion. This group has serine residue of AChE via covalent bonds, it prohibit hydrolyze of ACh by AChE.

Yang and colleagues (2011) has reported that an alkaloid skimmianine from *Zanthoxylum nitidum* roots has strong AChE inhibitory activity. They indicated that anti acetylcholinestrase activity of skimmianine may be effective in the treatment of Alzheimer disease. Kim and colleagues (2002) reported that methanol extract from the roots of *Angelica dahurica* has significant AChE inhibitory activity.

Another study carried out by Kim and colleagues (2004) showed that alkaloid of corynoxidine, protopine, palmatine, and berberine which have been isolated from aerial

parts of *Corydalis speciosa* have possessed AChE inhibitory activity. Similarly, Lee and colleagues (2004) have found that taraxerol and scopoletin from methanol extract of *Vaccinium oldhami* have AChE inhibitory activity.

Liu and colleagues (2011) investigated on extract of *Styrax agrestis* fruits showed that 3 new egonoltype benzofurans: egonol-9(Z), 12(Z) linoleate, 7-demethoxyegonol-9(Z),12(Z) linoleate , and 7-demethoxyegonol oleate exhibited significant AChE inhibitory activity. Pereira and colleagues (2010) have determined the water extract of alkaloids from leaves of *Catharanthus roseus* strongly inhibited AchE. They have also reported that the roots extract of *C. roseus* may constitute a promising source of compounds with pharmaceutical interest.

In another study by Wang and colleagues (2007) have found that strong anti-AChE activity compound (hydroquinone) in dried rhizome chloroform extract of *Rhodiola rosea*. A similar strong AChE inhibiters reported by Mukherjee and colleagues (2007) known as huperzine-A, galantamine, a-viniferin and ursolic acid was obtained from *Huperzia serrata*, *Galanthus nivalis* and *Narcissus* sp, *Caragana chamlague* and *Origanum majorana*, respectively.

The amount of ACh which is available to deliver nerve messages in axonal transport is reduced by the destruction of brain cells that produce and use ACh in AD. Stahl (2000) suggested that AChE inhibitors are extensively employed to avoid AChE from hydrolyzing the released ACh, thereby the ACh level available at cholinergic synapse and neuromuscular junction enhance and it leads to increasing cholinergic transmission.

(iv) AChE inhibitors for the treatment of AD

Wang and colleagues (2009) has also investigated on huperzine-A, an alkaloid isolated from the Chinese herb *Huperzia serrate*. It has high AChE inhibitory activity, with 300–500 μg per day of huperzine A could be effective in treatment of AD. Luttmann et al (2002) reported that in cholinergic nervous system, neuromuscular junction and AChE by swift hydrolysis of neurotransmitter acetylcholine in the synaptic cleft is due to termination of nerve impulse transmission.

One of the strategies could reverse memory impairment of AD patients is treatment with AChE inhibitors (Kasa et al., 2000). The bioassay results broadcasted by Hong and colleagues (2010) showed that novel compounds of phenyl N-methylcarbamate have high AChE inhibitory activity at the concentration of 100 mg/L. Bruhlmann and colleagues (2001) have tested monoamine oxidase (MAO) as AChE inhibitors and they found that these compounds might decrease Aβ deposition causes to treatment of AD. Finkel (2004) mentioned acetylcholine dysfunction is one of the consequences of AD and it makes AChE inhibitors effective for AD treatment. García-Ayllón and colleagues (2011) have reported that AChE is due to decreasing in acetylcholine has important role in AD treatment.

Silvestrelli et al. (2006) broadcasted that the cholinergic hypothesis of AD has act as a powerful encouragement to pharmacotherapeutic strategies of AD, which focus on improving the cholinergic inadequacy and potentiating residual activity in affected neuronal circuits by reforming cholinergic transmission.

For amplifying cholinergic activity in brain by maintain ACh level, AChE inhibitors has been widely pursued as the most powerful approach. Loizzo et al. (2008) discussed that in AD patients, AChE inhibitors could minimize the formation of neurotoxic fibrils and

clumping of Aβ, hence AChE inhibitors might be useful in treatment cognitive symptoms of AD (Kalauni et al., 2002; Atta-ur-Rahman et al., 2004).

FDA certified three types of AChE inhibitors for treatment of AD, including Donepezil (Aricept), accepted in 1996, Rivastigmine (Exelon), accepted in 2000 and Galantamine (Reminyl), accepted in 2001. The first AChE inhibitor approved was Tacrine (Cognex) in 1993 however, due to its unfavorable side effects, like liver damage it occasionally prescribed nowadays (Alzheimer's Association of United States, 2007).

On the other hand benefits of AChE inhibitors were mentioned by publications of Alzheimer's Association of United States (2007). For instance, block the activity of another enzyme participating in degradation of ACh, by rivastigmine, galantamine capable of promoting ACh release and thereby support the response of certain message receiving nerve cells to ACh. However, in plentiful clinical trials AChE inhibitors have been studied on AD patients and results displayed that taking these drugs as medications achieved feeble improvement in thinking and memory. AChE inhibitors may postpone the progression of AD approximately six months to a year.

Sung et al, (2002) considered that due to short half- lives or unfavorable side effects of AChE inhibitors they have small degree of benefits and limited clinical uses, either. According to Alzheimer's Association of United States (2007) AChE inhibitors unable to terminate the underlying damage of nerve cells and reverse AD. Therefore, their capability in improving AD is reducing brain cell destruction progresses.

2.8 Animal study

(i) Introduction

Using animal (ICR mice) might be beneficial to improve AChE inhibitors in treatment of AD. Dong and colleagues (2005) investigated two AChE inhibitors physostigmine and donepezil on memory related behaviors of mice. They suggested that AChE inhibitors could improve memory deficits in mice. In another study by Ikarashi and colleagues (2004) have examined mice memory disturbance by measuring AChE concentrations in their brains. They founded that memory impairment in cholinergic system of the mice is due to Aβ accumulation when AChE increased. Figueiró and colleagues (2010) have extracted AChE inhibitors from *Ptychopetalum olacoides* and orally gave these compounds to the mice. They improved meaningful AChE inhibitor activity of these extracts for treating neurodegenerative conditions which is useful for AD treatment.

(ii) Radial arm maize (RAM)

Olton and Samuelson (1976) constructed the radial arm maze to assess memory and spatial learning in mice. The archetype apparatus has eight equal arms, and all of them branching from a petite round central platform. There is a food section at the end of each arm, which is not noticeable from the basic platform. Furthermore, they illustrated that mice have peachy memories for visited and unvisited arms; on average the mice had 88% correct entries, in their first 8 decision they had approximately 7 new entries.

Levin (1988) suggested that radial arm maze is a practicable memory testing for displaying the effect of drugs. In another study Foti et al, (2011) considered that the radial

arm maze can be analyzed via movement most aspects of the spatial function, as the analytical memory and the appropriate working memory.

Figure 2.4: The mice in the radial arm maze

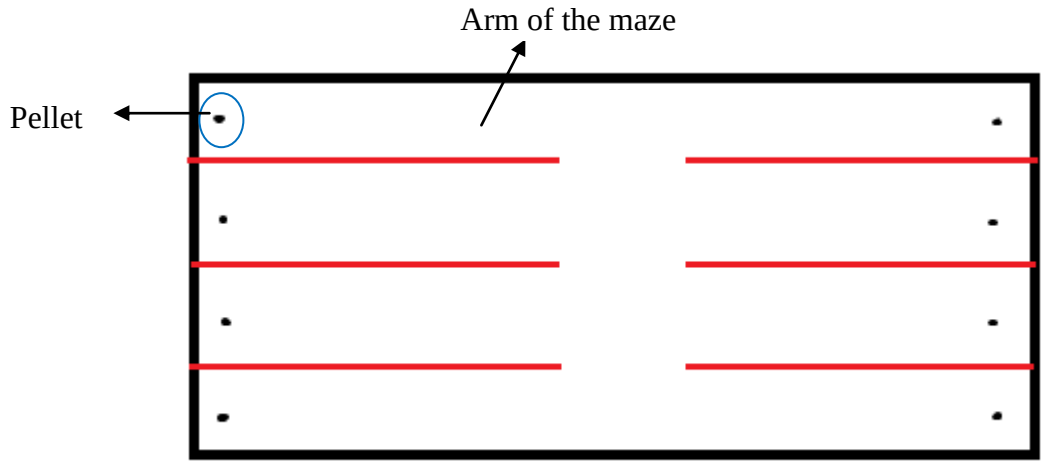


Figure 2.5: Radial arm maze

Objectives of the study

1. To determine AChE inhibitor activity in *Aquilaria subintegra* for treatment of Alzheimer's disease (AD).

2. To isolate and characterize the chemical compounds present in *Aquilaria subintegra* that inhibits AChE.

3. To observe the effect of AChE inhibitors in mice.

CHAPTER 3 METHODOLOGY

3.1 Plant materials

The leaves and stem of *Aquilaria subintegra* were collected from RAL plantations farm in, Kuala Kangsar, Perak, Malaysia. The authentic of the plant sample was verified by plant taxonomist Professor Dr. Ong Hean Chooi from the Institute of Biological Science, Faculty of Science, University of Malaya.

3.2 Chemicals

Acetylcholinesterase (EC3.1.1.7, Sigma product no C2888), acetylthiocholine iodide (ATCI), 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), and Berberine were purchased from Sigma (St. Louis, MO, USA). Standard kaempferol purchased from Calibiochem (Japan). TLC Silica gel 60 F254 (Merck KGaA, Germany).

3.3 Extraction and isolation of chemical compounds

The leaves, stem and fruit of *Aquilaria subintegra* were powdered and extracted with chloroform, methanol and distilled water. 1 L of chloroform, methanol and water was used to extracted leaves and stem powder, for 2 hours in water bath at 40°C. Then each sample extract was filtered and the filtrate was concentrated to 10 ml using rotary evaporator with medium speed at 40°C. Then 1 ml of each sample was evaporated to dryness for crude extract preparation.

3.4 Detection of the chemical compounds

(i) Visible light

The separated bands occurred on TLC plates were determined under visible light. Then Retention Factor (R_f) value of each band calculated by using the formula:

R_f = Distance traveled by the substance (cm) / Distance traveled by the solvent (cm)

(ii) UV Light

For detecting ultraviolet active absorbing spots TLC plates were perceived under ultraviolet (UV) light.

(iii) Chemical reagents

a) Folin-Phenol

50 ml of Folin-Ciocalteu mixed with 50 ml of distilled water for preparing Folin-Phenol. It used to detect phenols on TLC plates after spraying with the manifestation of blue bands.

b) Vanilin-H_2SO_4

For preparing Vanilin-H_2SO_4 mixture of 1g of Vanilin and 1.5 ml of H_2SO_4 added to 100 ml of ethanol. Then the mixture has smoothly shaken to dissolve all to become a solution. After spraying Vanilin-H_2SO_4 on TLC plates they need to be heated on a hot plate at 100°C for 5 minutes. The exhibition of blue or purple bands announced the presence of terpenoids.

c) Dragendroff's

5 ml of solution A and 5 ml of solution B with 20g of acetic acid and 70 ml of distilled water were mixed for preparing Dragendroff's. Solution A was prepared by dissolving 1.6g of Bismuth Nitrate into 100 ml solution of water acetic acid. Solution B

was made by dissolving 40g of Potassium Iodide into 100 ml of distilled water. Dragendroff's used to detect alkaloids on TLC plates after spraying with presence of orange bands.

3.5 Determination of bioactive compound by TLC and LCMS/MS

TLC plates with height and wide of 20 cm were used. The crude extract was spotted as a thin line on the sample line by using a tapered capillary. Then the TLC plate was transferred to well covered tank with using chloroform and 10% methanol in chloroform as a solvent respectively. When the sample was raised up the plate, the separated bands appeared on each plate were identified under visible light and UV light. Then R_f value of each band was calculated. Finally reagents of Dragendroff's, Vanillin-H_2SO_4 and Folin-Phenol used to detect alkaloids, terpenoids and phenols respectively. LCMS/MS (Perkin Elmer Flexar FX-15 ultra-high performance liquid chromatography (USA) with AB Sciex 3200Qtrap tandem mass spectrometry (Singapore) used to detect the chemicals presence in the leaves and stem of chloroform crude extract with concentration of 5mg/ml. Column: Phenomenex Aqua C18 – 50mm x 2.0mm x 5uM; Buffer: A: Water with 0.1% formic acid and 5mM ammonium formate, Buffer B: Acetonitrile with 0.1% formic acid and 5mM ammonium formate. Gradient run program: 10% A to 90% B from 0.01min to 8.0min, hold for 2 min and back to 10% B in 0.1min and re-equilibrated for 5min. The sample extracts were diluted two times with appropriate solvents, and filtered with 0.2uM nylon filter prior to analyses. MS/MS method: Negative ESI, with -4500kV, temperature at 500°C, purified nitrogen gas as nebulization and collision gas 99.9995% purity, fragmentation energy: Collision energy spread -35eV +/- 15eV. The data analysis was performed using AB Sciex

Analyst 1.5 software with internally built MS/MS library search and further interpretation and processing via ACD/Labs MS fragmenter software.

3.6 Determination of total phenols and total flavonoids contents

(i) Total phenols contents

The total of the phenolic content was measured according to the method as described by Thind et al. (2011). Briefly, 500µl of each crude extract (5mg/ml) was mixed with 5 ml Folin ciocalteu (1:10, V/V diluted with distilled water) and 4 ml of 1M sodium carbonate. The mixture was incubated in water bath at 45°C for 15 min, and then its absorbance was determined at 765 nm by using spectrophotometer. The standard calibration curve was prepared by using 0, 50, 100, 150, 200, 250 and 500 mg/L solution of gallic acid in methanol: water (50:50, V/V). The experiments were done in triplicate.

(ii) Total flavonoids contents

The total of the flavonoids content was measured according to the method as described by Zhang et al. (2009). Briefly, 500µl of each crude extract (5mg/ml) was added to 0.3ml of 5% sodium nitrate. The mixture was incubated at room temperature for 5 min. Then, 0.3 ml of 10% aluminum chloride was mixed into the solution before incubated again at room temperature for 6 min. Subsequently, the mixture was added with 2 ml of 1 M sodium hydroxide and 10 ml distilled water. Absorbance was read at 510 nm using spectrophotometer. The standard calibration curve was performed by using 0, 200, 400, 600, 800, 1000 and 2000 mg/L quercetin solution in methanol. The experiments were done in triplicate.

3.7 Toxicity assay

3.7.1 Brine shrimp lethality assay (BSLA)

The toxicity test of crude extract was carried out using brine shrimp lethality assay (BSLA). The artificial sea water was prepared by dissolving 38 g sea salt in 1 L of distilled water. After filtering it, the brine shrimps (*Artemia salina*) eggs were added and keep in dark environment. They were allowed to hatch by incubation at room temperature for 48 h. three concentrations of each crude extract (1000µg/ml, 100µg/ml, 10µg/ml) were transferred to each vial of microplate with 5 ml of sea water and 10 brine shrimps in each vial. The control was also conducted simultaneously and prepared by replacing the crude extract with sea water (Atta-ur Rahman et al., 2001). After 24 h the number of surviving shrimps was counted and data were analysed using probit analysis program to determine the median lethal concentration (LC_{50}). The experiments were done in triplicate.

3.7.2 Toxicity test on mice

(i) Mean body weight

Mice were randomly divided into 8 groups. Each group had 3 male and female mice. Group 1: control; Group 2, 3 and 4 leaves and stem crud extracts were given by oral gavage at doses of 0.1ml/g body weight, 0.5 ml/g body weight and 1 ml/g body weight respectively, for a period of at least 28 days (Kushwaha et al., 2010). The mean body weight of each group was measured each day during the study. The mean body weight of mice was analyzed by ANOVA, p values of <0.05 were used to indicate a significant group differences.

(ii) In-life observations

Once in a day from cage-side mortality and general condition included: mice appearance, activity, behavior, respiration, skin, fur, eyes, nose, oral activity, abdomen and external genitalia were perceived for any sign of toxicity. During the study, once in a week mice were removed from their cages for observation of mortality and general condition following the method as described by DeMerlis et al. (2005).

3.8 AChE inhibitory activity assay

Ellman's method as described by Yang et al. (2011) was used for AChE inhibitory activity of the crude extract and isolated compound of kaempferol 3,4,7-trimethyl ether from stem chloroform extract. Concisely, 140 µl of 0.1 M sodium phosphate buffer, with pH 8 and 20µl of 1 mg/ml sample solution mixed with enzyme (AChE). The Amount of AChE used was 15 µl with activity of 0.25 U/ml. They were pre incubated at 4°C for 20 min. The reaction was started by adding 10 µl of 0.01 M DTNB and 10 µl of 0.075 M of ATCI. Then the extract was incubated at room temperature for 20 min. After that the absorbance at 405 was measured with ELISA. The percentage of inhibitory activity was calculated by using formula:

% of AChE inhibitor = $A_c - A_t / A_c \times 100$

Berberine was used as positive control. The experiments were done in triplicate.

3.9 Animal study

The male and female adult (2 months old, 35–45 g) ICR mice were obtained from the Animal House, University of Malaya. The mice were sustained under controlled

environmental conditions with a temperature of 22±1 °C, 12-h light/dark cycle and food and water accessible ad libitum. During the 3 months study, the mice were divided to 12 groups. Each group had 3 male and female. Group 1: control; Group 2: stem extract; Group 3: leaves extract; Group 4: leaves plus stem extract; Group 5: kaempferol and Group 6: berberine. Each extract and standard was given by oral gavage to the mice group respectively. Valium was used to impair the memory of the mice. All drugs were given at doses of 0.1 ml/g body weight. The animal test was conducted in an eight-arm radial maze apparatus with eight arms a radial maze (Figure 2.4). The central hub is consisted of a white polypropylene octagonal base (28.6 cm in diameter, 11.4 cm sides). The arms radiated from the center hub, with equal spacing between each arm. Each arm was 76.2 cm long and 8.9 cm wide with clear polycarbonate walls (17.5 cm high). Each baited arm had only one pellet. The working memory was assessed by measuring the number of repeat entries to arms of the maze already visited (NRE). For instance if NRE is 5 it means that the mouse eats all 8 pellet with 5 times comes back to the arms of the maze which is the pellet was eaten before (5 errors). The number of entries until the first error occurs (NEF) is commonly used as an additional measure of performance in radial arm maze. For instance if NEF is 6 it means that the mouse eats 6 pellet without any repeated entries to the arms of the maze that the pellet was eaten before. However, the mouse eats the 7^{th} pellet after some repeated entries to empty arms. Each group was first given valium. After 30 min it was placed in the radial arm maze. Then after 1 hour the mice was given the test sample and was placed again on radial arm maze. Each mouse was kept in radial arm maze for 10 min. The results of the memory testing data were analyzed with ANOVA, p values of <0.05 were used to indicate a significant group difference in all analyses.

CHAPTER 4 RESULTS

4.1 TLC and LCMS/MS

The thin layer chromatography (TLC) of chloroform and methanol extract of leaves and stems showed the presence of phenols, flavonoids and alkaloids compounds (Tables 4.1; 4.2; 4.3; 4.4; 4.5; 4.6; 4.7; 4.8; 4.9; 4.10; 4.11; 4.12; 4.12; 4.13; 4.14; 4.15; 4.16; 4.17; 4.18). The analysis of the chloroform leaves extract with LCMS/MS showed that it contain Delphinidin-3-glucoside (Figure 4.2), 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid (Figure 4.3) and benzenepropanoic acid conjugate (Figure 4.4). Whereas, 5-Hydroxy-7,4-dimethoxyflavone (Figure 4.6), kaempferol 3,4,7-trimethyl ether (Figure 4.7) and 5.7-Dihydroxyl-4'-dimethoxyflavone (Figure 4.8) was presence in the stem chloroform extract.

Table 4.1: Thin layer chromatography of methanol extract from *Aquilaria subintegra* fruit in methanol solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
A1	0.58	Yellow(+)	Purple(+)	-	-	-	-
A2	0.65	-	Yellow(+)	-	-	-	-
A3	0.74	-	Yellow(+)	-	-	-	-
A4	0.79	Yellow(+)	Purple(+)	-	-	-	-
A5	0.88	Yellow(+)	Purple(+)	-	Purple($^{++}$)	-	Terpenoid
A6	0.97	Yellow(+)	Yellow(+)	-	-	-	-

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.2: Thin layer chromatography of methanol extract from *Aquilaria subintegra* fruit in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
B1	0.61	-	Yellow(+)*	-	Blue(+)	-	Terpenoid
B2	0.68	-	Purple(++)	-	Blue(+)	-	Terpenoid
B3	0.82	-	Yellow(+)	-	Purple(+)	-	Terpenoid
B4	0.85	-	Yellow(+)	-	-	-	-
B5	0.89	-	Yellow(+)	-	-	-	-
B6	0.95	-	Yellow(+)	-	Blue(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.3: Thin layer chromatography of methanol extract from *Aquilaria subintegra* fruit in 10% methanol in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
C1	0.046	Yellow(++)	-	Blue(+)	Blue(++)	-	Phenolic/terpenoid
C2	0.153	Yellow(+)	Purple(+)	-	-	Orange(+)	alkaloid
C3	0.215	-	-	-	Purple(+)	-	terpenoid
C4	0.338	-	-	-	Purple(+)	-	terpenoid
C5	0.400	-	-	-	Purple(+)	-	terpenoid
C6	1.000	-	-	-	Purple(+)	-	terpenoid
C7	0.765	-	-	Blue(+)	-	-	phenolic

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.4: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* fruit in methanol solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
A1	0.45	Yellow(+)	Yellow(+*)	-	Purple(+)	-	Terpenoid
A2	0.49	Yellow(+)	Yellow(+)	-	Purple(+)	-	Terpenoid
A3	0.69	Yellow(+)	Purple(+)	-	Purple(++)	Orange(+)	Terpenoid/Alkaloid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.5: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* fruit in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
B1	0.06	Green(+)	Yellow(+)	-	Blue(+)	-	Terpenoid
B2	0.18	Green(+)	Yellow(+)	-	Blue(+)	-	Terpenoid
B3	0.26	-	Purple(+)	-	Purple(++)	-	Terpenoid
B4	0.35	-	Purple(++)	-	Blue(+)	Orange(+)	Terpenoid/Alkaloid
B5	0.94	-	Purple(+)	-	Blue(+)	-	Terpenoid
B6	0.50	-	-	-	Blue(+)	-	Terpenoid
B7	0.72	-	-	-	Blue(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.6: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* fruit in 10% methanol in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
C1	0.26	-	Yellow(+)	-	Purple(+)	Orange(+)	Terpenoid/Alkaloid
C2	0.41	-	Yellow(+)	-	Blue(+)	-	Terpenoid
C3	0.45	-	Purple(+)	-	Blue(+)	-	Terpenoid
C4	0.54	Yellow(+)	Purple(++)	-	Blue(+)	-	Terpenoid
C5	0.76	-	Purple(+++)	-	Blue(+++)	Orange(++)	Terpenoid/Alkaloid
C6	0.93	Green(++)	Purple(++)	-	Blue(+++)	Orange(+)	Terpenoid/Alkaloid
C7	0.63	-	-	-	Blue(++)	-	Terpenoid
C8	0.87	-	-	-	Blue(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.7: Thin layer chromatography of methanol extract from *Aquilaria subintegra* leaves in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
A1	0.046	Yellow(++)	-	Blue(+)	Blue(++)	-	Phenolic/terpenoid
A2	0.153	Yellow(+)	Purple(+)	-	-	Orange(+)	alkaloid
A3	0.215	-	-	-	Purple(+)	-	terpenoid
A4	0.338	-	-	-	Purple(+)	-	terpenoid
A5	0.400	-	-	-	Purple(+)	-	terpenoid
A6	1.000	-	-	-	Purple(+)	-	terpenoid
A7	0.765	-	-	Blue(+)	-	-	phenolic

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.8: Thin layer chromatography of methanol extract from *Aquilaria subintegra* leaves in methanol solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
B1	0.15	Yellow(+)	Purple(++)	-	Purple(+++)	-	Terpenoid
B2	0.83	Yellow(++)	Purple(+++)	-	Purple(+++)	-	Terpenoid
B3	0.96	-	-	-	Purple(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.9: Thin layer chromatography of methanol extract from *Aquilaria subintegra* leaves in 10% methanol in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
C1	0.125	Yellow(+)	Purple(+++)	-	Purple(+++)	-	Terpenoid
C2	0.250	Yellow(+)	Purple(+)	-	Purple(+)	-	Terpenoid
C3	0.296	Yellow(+)	Purple(+)	-	Purple(+)	-	Terpenoid
C4	0.343	Yellow(+)	Purple(+)	-	Purple(++)	-	Terpenoid
C5	0.453	-	Purple(+)	-	Blue(+)	-	Terpenoid
C6	0.500	Yellow(+)	-	-	Purple(+)	Orange(+)	Terpenoid/Alkaloid
C7	0.625	-	Purple(+)	-	Blue(+)	-	Terpenoid
C8	0.718	Yellow(+)	-	Blue(+)	Blue(++)	-	Phenol/Terpenoid
C9	0.890	Yellow(+)	-	-	Purple(++)	Orange(+)	Terpenoid/Alkaloid
C10	0.968	Yellow(+)	Purple(++)	Blue(+)	Purple(++)	-	Phenol/Terpenoid
C11	0.461	-	-	-	Purple(+)	-	Terpenoid

Table 4.9, continued

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin- H_2SO_4**	**Dragendroff's**	
C12	0.661	-	-	-	Purple(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.10: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* leaves in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff**	
A1	0.045	Yellow(+++)	-	Blue(++)	Blue(+++)	-	Terpenoid
A2	0.136	Green(++)	-	-	Green(+++)	-	Terpenoid
A3	0.242	Yellow(+)	Purple(+)	-	Purple(+)	-	Terpenoid
A4	0.363	Yellow(+)	Purple(+)	Blue(+)	Purple(+)	-	Terpenoid
A5	0.484	Yellow(+)	Purple(+)	Blue(+)	Purple(++)	-	Terpenoid
A6	0.636	Yellow(+)	Purple(+)	Blue(+)	Purple(+)	-	Terpenoid
A7	0.696	Yellow(+)	Purple(+)	-	Purple(++)	-	Terpenoid
A8	0.893	Yellow(+)	Purple(++)	-	Purple(++)	-	Terpenoid
A9	0.939	Yellow(+++)	-	-	Purple(++)	Orange(+)	Terpenoid/Alkaloid
A10	0.090	-	-	-	Blue(++)	-	Terpenoid
A11	0.212	-	-	-	Purple(++)	-	Terpenoid

Table 4.10, continued

Labelled compounds	R_f value	Observation					comments
		Visible light	UV light	Reagents			
				Folin-Phenol	Vanilin-H_2SO_4	Dragendroff	
A12	0.318	-	-	-	Purple(++)	-	Terpenoid
A13	0.560	-	-	-	Blue(+)	-	Terpenoid
A14	0.779	-	-	-	Blue(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.11: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* leaves in 10% methanol in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
B1	0.220	-	Purple(+)	-	Purple(+)	-	Terpenoid
B 2	0.441	Green(+)	-	Blue(+)	Purple(++)	-	Phenol/Terpenoid
B 3	0.500	-	Purple(+)	-	Purple(+)	-	Terpenoid
B 4	0.573	-	Purple(+)	Blue(++)	Purple(+)	-	Phenol/Terpenoid
B 5	0.676	Yellow(++)	-	Blue(++)	Blue(++)	-	Phenol/Terpenoid
B 6	0.779	Yellow(+)	Purple(+)	-	Purple(++)	-	Terpenoid
B 7	0.911	Green(+++)	-	Blue(++)	Purple(+++)	-	Phenol/Terpenoid
B8	0.073	-	-	-	Purple(+)	-	Terpenoid
B9	0.147	-	-	-	Purple(+)	-	Terpenoid

Table 4.11, continued

Labelled compounds	R_f value	Observation					Comments
		Visible light	UV light	Reagents			
				Folin-Phenol	Vanilin-H_2SO_4	Dragendroff's	
B10	0.308	-	-	-	Purple(++)	-	Terpenoid
B11	0.367	-	-	-	Purple(+)	-	Terpenoid
B12	0.405	-	-	-	-	Orange(+)	Alkaloid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.12: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* leaves in methanol solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
C1	0.66	Yellow(++)	-	Blue(++)	Purple(+++)	-	Phenol/Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.13: Thin layer chromatography of methanol extract from *Aquilaria subintegra* stem in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
A1	0.076	Yellow(+)	-	-	Blue(++)	-	Terpenoid
A2	0.369	Yellow(+)	-	Purple(+)	Purple(+)	-	Phenol/Terpenoid
A3	0.461	-	Purple(+)	Purple(+)	Purple(+)	-	Phenol/Terpenoid
A4	0.184	-	-	-	Purple(+)	-	Terpenoid
A5	0.292	-	-	-	Purple(++)	-	Terpenoid
A6	0.953	-	-	-	Purple(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.14: Thin layer chromatography of methanol extract from *Aquilaria subintegra* stem in methanol solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
B1	0.641	Yellow(++)	Purple(+)	-	Purple(+)	-	Terpenoid
B2	0.746	-	Purple(++)	Purple(++)	Purple(++)	-	Phenol/Terpenoid
B3	0.567	-	-	-	Purple(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.15: Thin layer chromatography of methanol extract from *Aquilaria subintegra* stem in 10% methanol in chloroform solvent

Labelled compounds	R_f value	Observation					Comments
		Visible light	UV light	Reagents			
				Folin-Phenol	Vanilin-H_2SO_4	Dragendroff's	
C1	0.104	-	Purple(+)	-	Purple(+)	-	Terpenoid
C2	0.164	-	Blue(++)	-	Purple(+)	-	Terpenoid
C3	0.208	-	Purple(+)	-	Purple(+)	Orange(+)	Terpenoid/Alkaloid
C4	0.268	-	Purple(+)	-	Purple(+)	-	Terpenoid
C5	0.313	-	Purple(+)	-	Purple(+)	-	Terpenoid
C6	0.358	-	Blue(+)	-	Purple(+)	-	Terpenoid
C7	0.447	-	Purple(++)	-	Purple(+)	-	Terpenoid
C8	0.611	-	Purple(+)	-	Purple(+)	-	Terpenoid
C9	0.791	-	Purple(+)	-	Purple(+)	-	Terpenoid
C10	0.910	-	Purple(++)	-	Purple(+)	-	Terpenoid
C11	0.979	Yellow(+)	-	Purple(++)	Purple(+)	Orange(+)	Phenol/Terpenoid/Alkaloid
C12	0.298	-	-	-	Blue(+)	-	Terpenoid

Table 4.15, continued

Labelled compounds	R_f value	Observation					Comments
		Visible light	UV light	Reagents			
				Folin-Phenol	Vanilin-H_2SO_4	Dragendroff's	
C13	0.492	-	-	-	Purple(++)	-	Terpenoid
C14	0.686	-	-	-	Purple(++)	-	Terpenoid
C15	0.850	-	-	-	Purple(++)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.16: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* stem in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
A1	0.068	Yellow(+)	-	-	Purple(++)	-	Terpenoid
A2	0.191	-	Purple(++)	-	Purple(++)	Orange(+)	Terpenoid/Alkaloid
A3	0.287	-	-	-	Purple(++)	-	Terpenoid
A4	0.616	-	-	-	Blue(+)	-	Terpenoid
A5	0.945	-	-	-	Purple(++)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.17: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* stem in methanol solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
B1	0.416	Yellow(+)	-	-	Purple(+)	-	Terpenoid
B2	0.805	Yellow(+)	Purple(+)	Purple(+)	Purple(+)	-	Phenol/Terpenoid
B3	0.666	-	+	+	Purple(+)	-	Terpenoid
B4	0.736	-	+	+	Purple(+)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

Table 4.18: Thin layer chromatography of chloroform extract from *Aquilaria subintegra* stem in 10% methanol in chloroform solvent

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
C1	0.077	-	Purple(+)	Purple(+)	Purple(+)	-	Phenol/Terpenoid
C2	0.181	-	Purple(+)	Purple(+)	Purple(+)	-	Phenol/Terpenoid
C3	0.246	-	Purple(+)	Purple(+)	Purple(+)	-	Phenol/Terpenoid
C4	0.363	Yellow(+)	-	-	Purple(+)	-	Terpenoid
C5	0.881	-	Purple(+)	-	Purple(+)	-	Terpenoid
C6	0.948	Green(++)	-	-	Purple(++)	-	Terpenoid
C7	0.051	-	-	-	Purple(++)	-	Terpenoid
C8	0.142	-	-	Purple(+)	Purple(++)	-	Phenol/Terpenoid

Table 4.18, continued

Labelled compounds	**R_f value**	**Observation**					**Comments**
		Visible light	**UV light**	**Reagents**			
				Folin-Phenol	**Vanilin-H_2SO_4**	**Dragendroff's**	
C9	0.285	-	-	-	Blue(++)	-	Terpenoid
C10	0.636	-	-	-	Purple(++)	-	Terpenoid
C11	0.792	-	-	-	Purple(++)	-	Terpenoid

Indication for intensity of color: +++ = strong + = weak ++ = medium - = no color observed

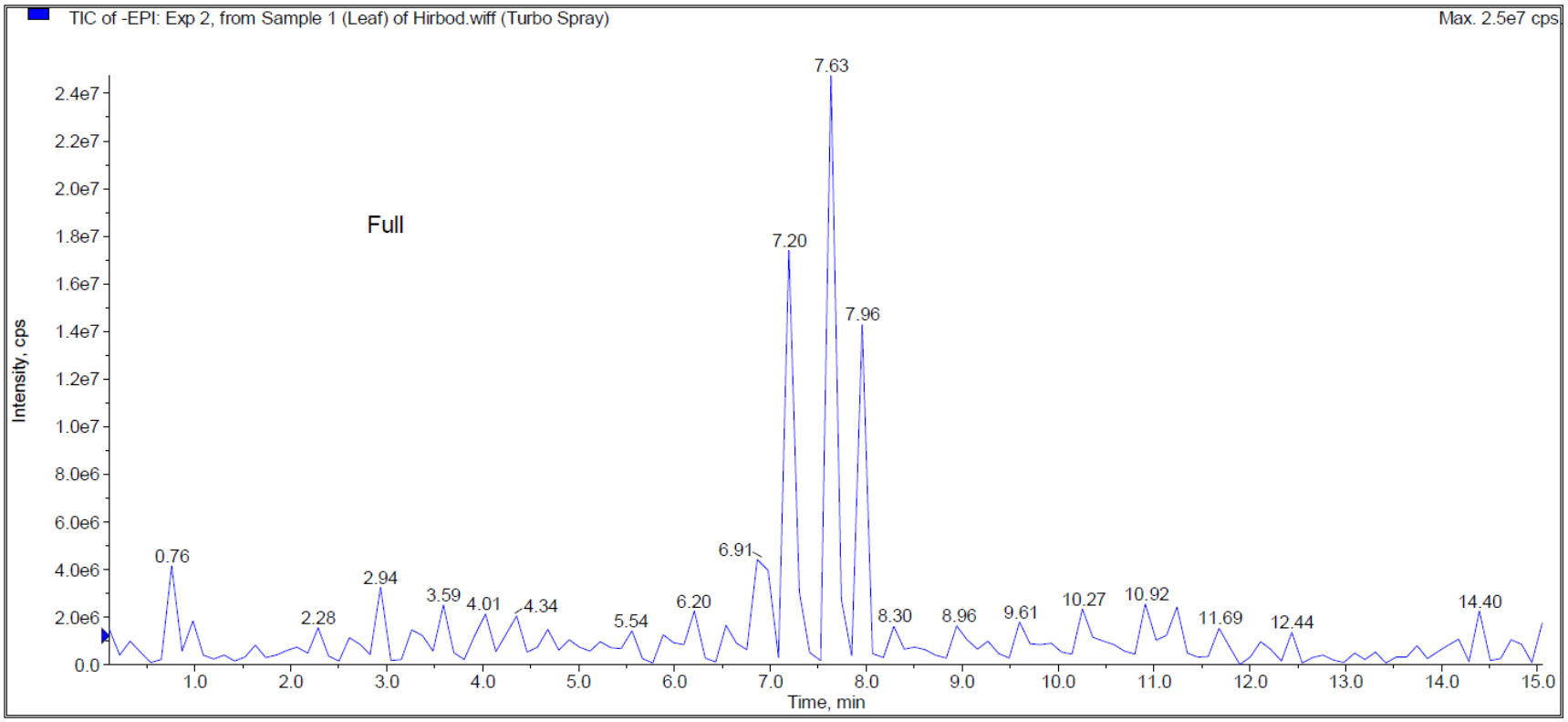


Figure 4.1: HPLC/MS profile of chloroform leaves crude extracts from *Aquilaria subintegra.*

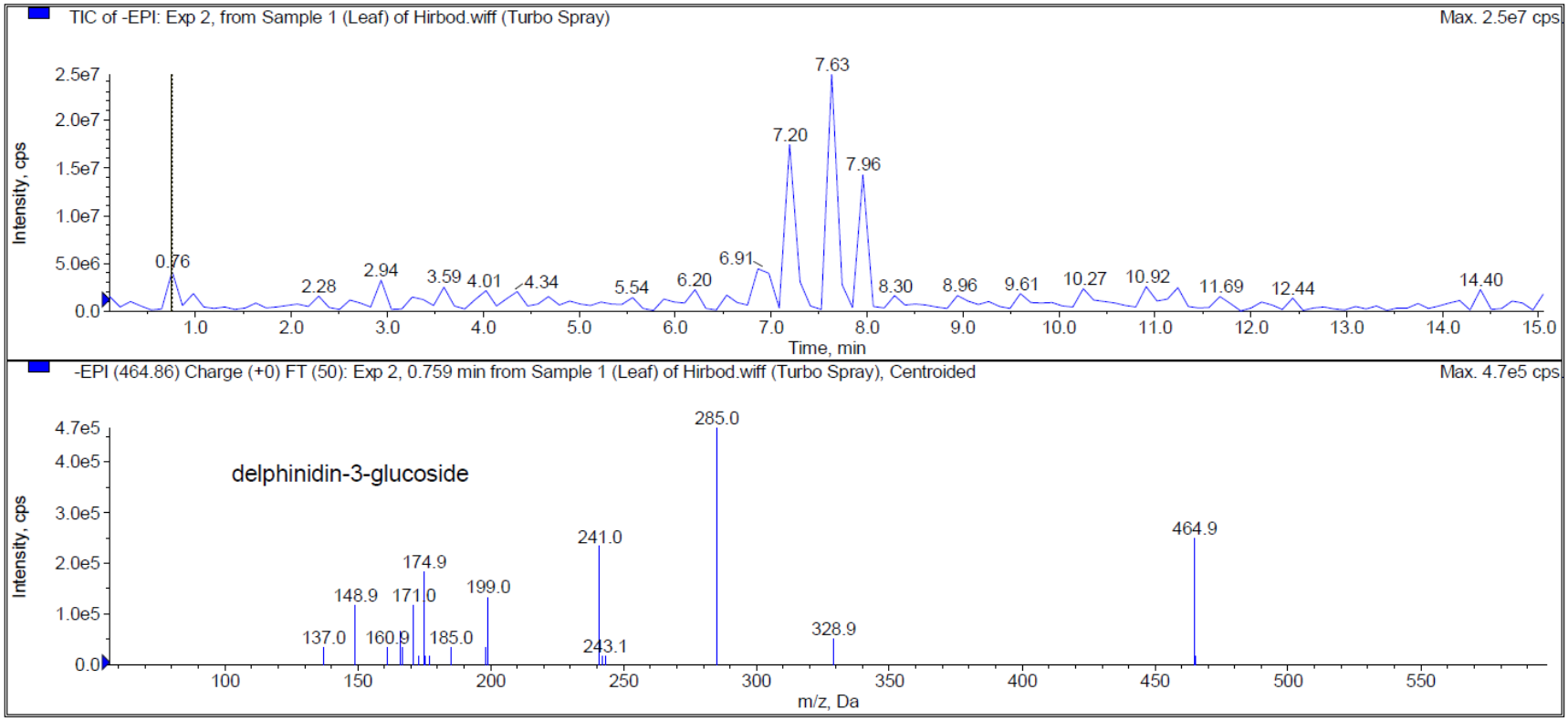


Figure 4.2: The LCMS/MS profile of Delphinidin-3-glucoside from chloroform leaves crude extract from *Aquilaria subintegra*

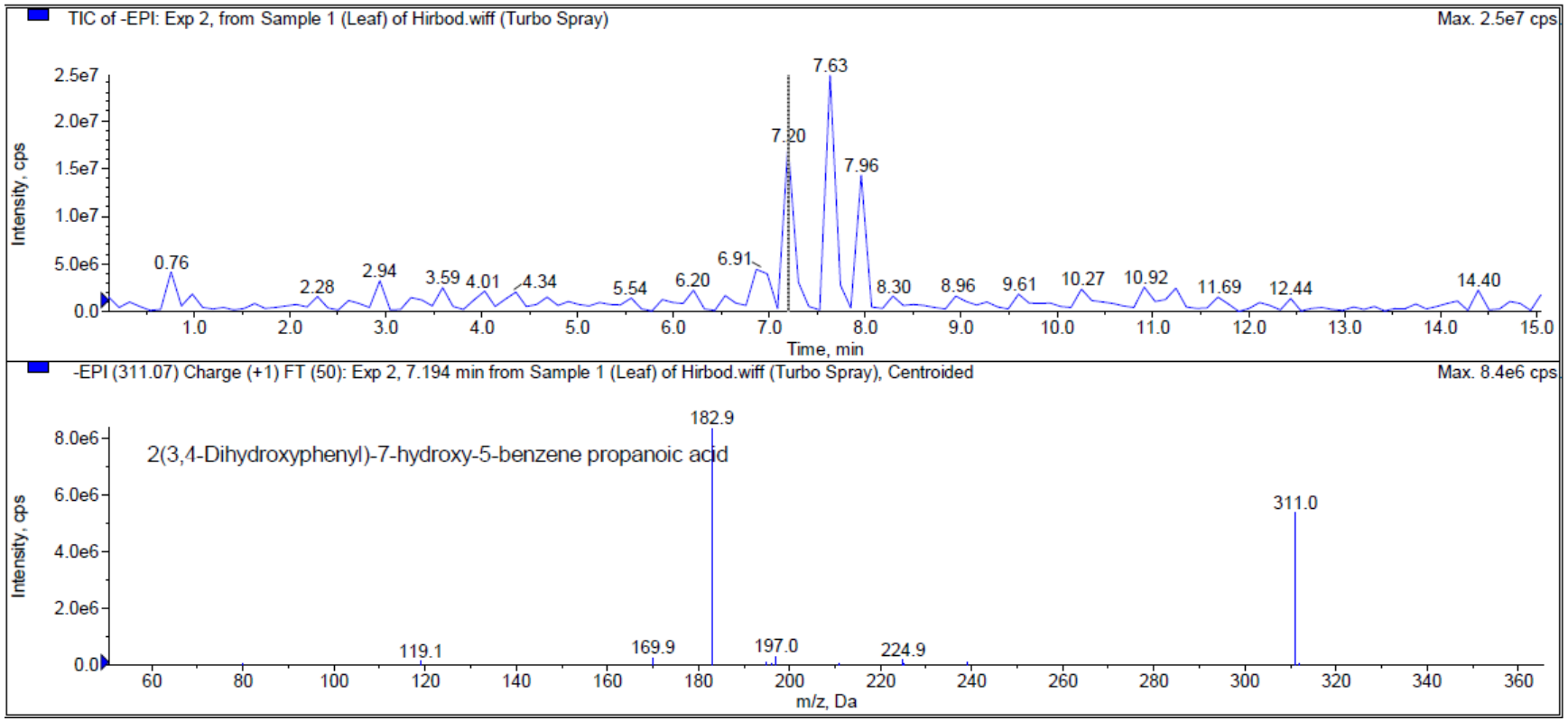


Figure 4.3: The LCMS/MS profile of 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid from chloroform leaves crude extract from *Aquilaria subintegra*.

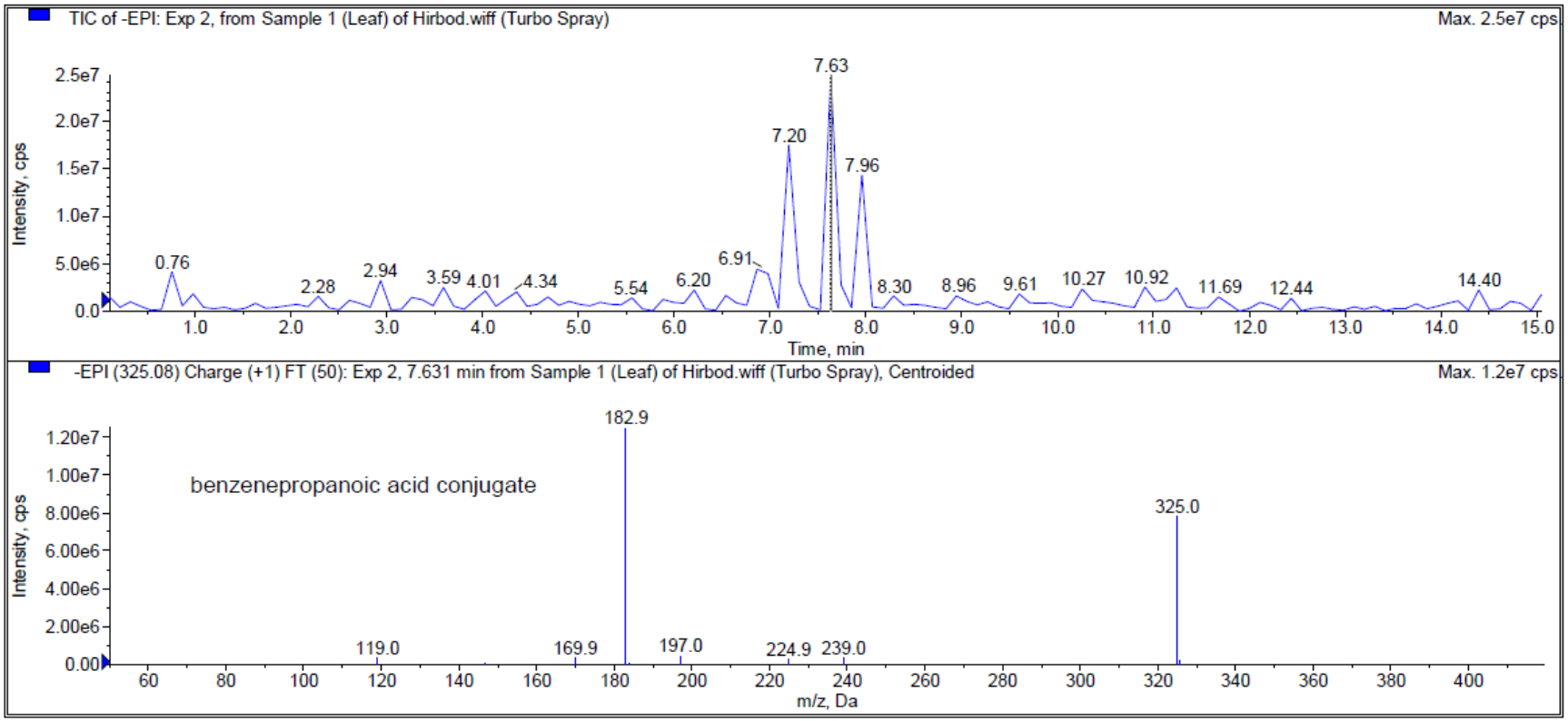


Figure 4.4: The LCMS/MS profile of benzenepropanoic acid conjugate from chloroform leaves crude extract from *Aquilaria subintegra*

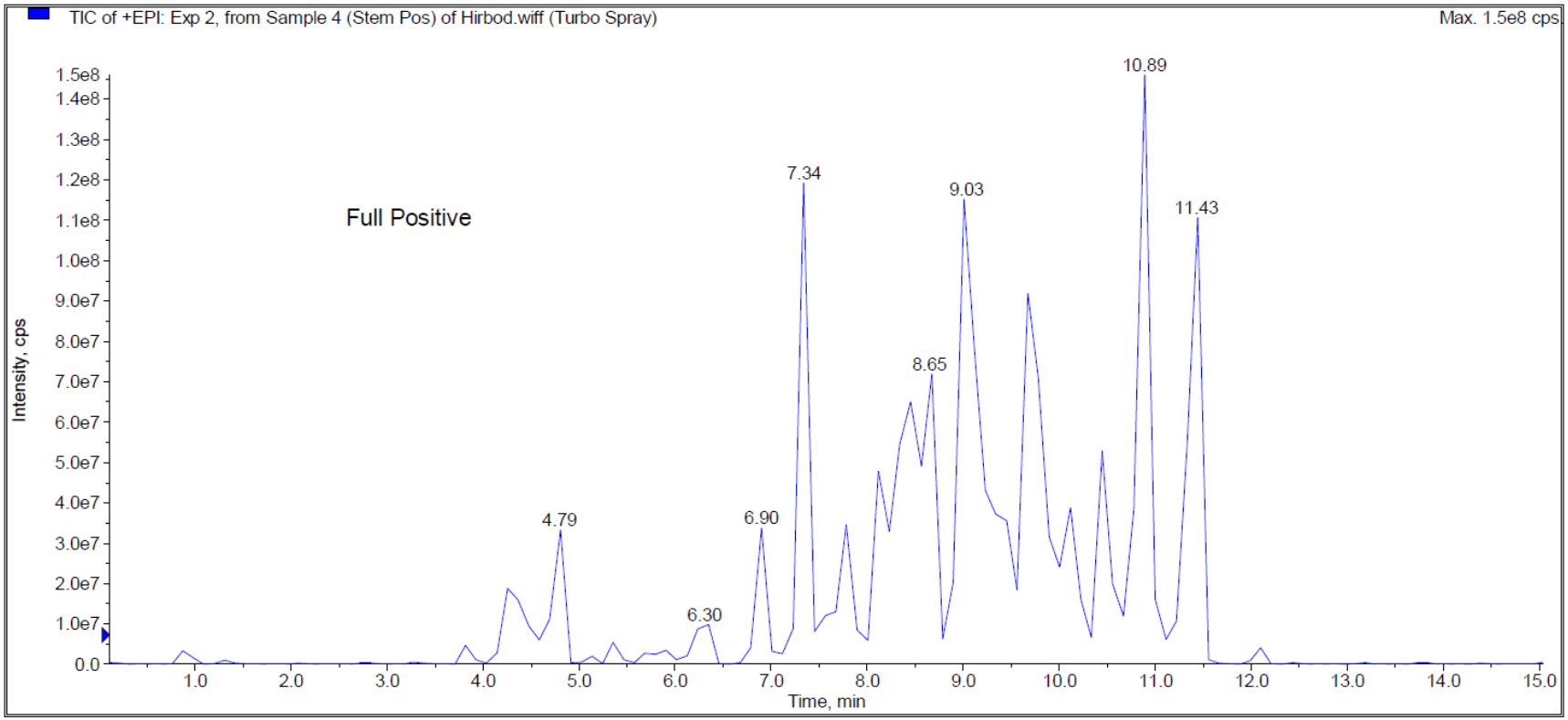


Figure 4.5: HPLC/MS profile of chloroform stem crude extracts from *Aquilaria subintegra.*

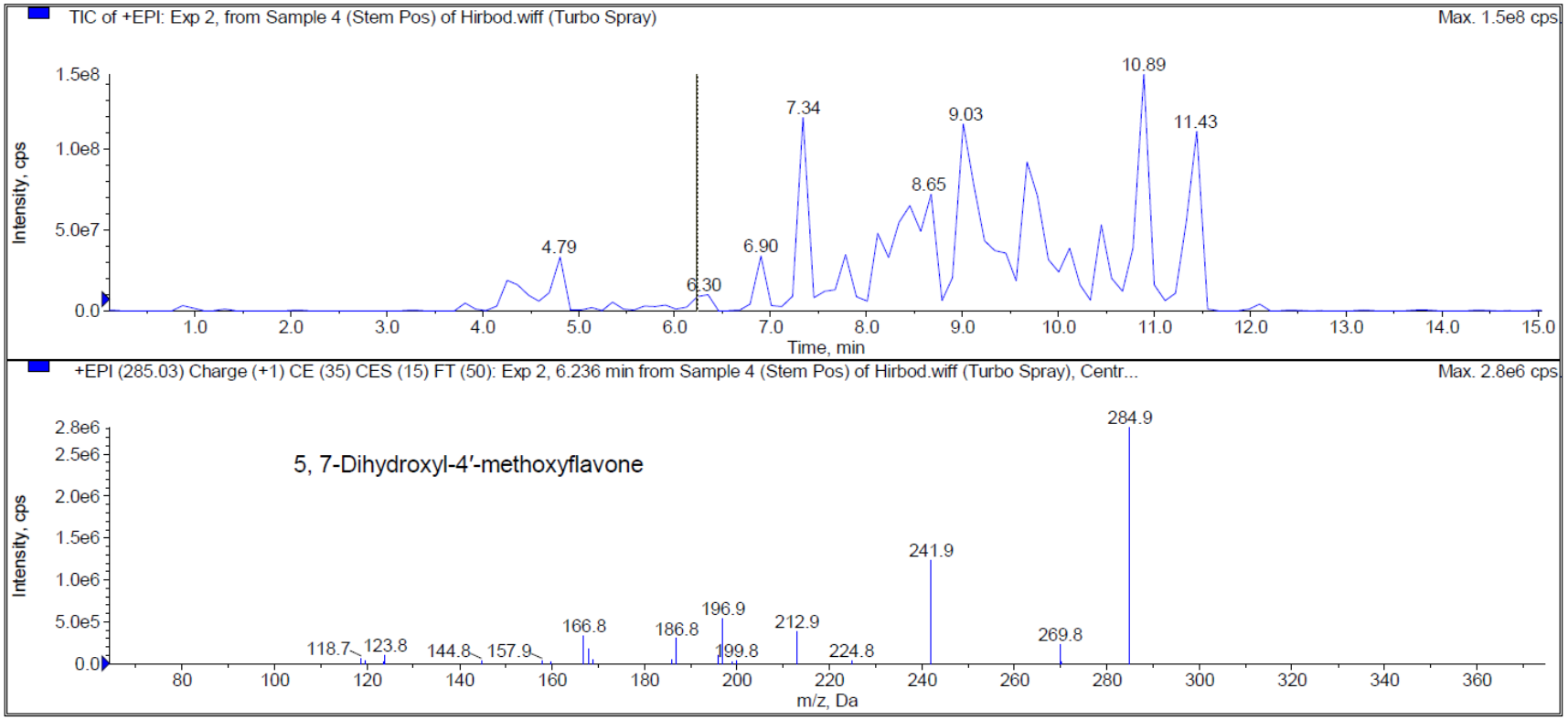


Figure 4.6: The LCMS/MS profile of 5.7-Dihydroxyl-4’-dimethoxyflavone from chloroform stem crude extract from *Aquilaria subintegra*

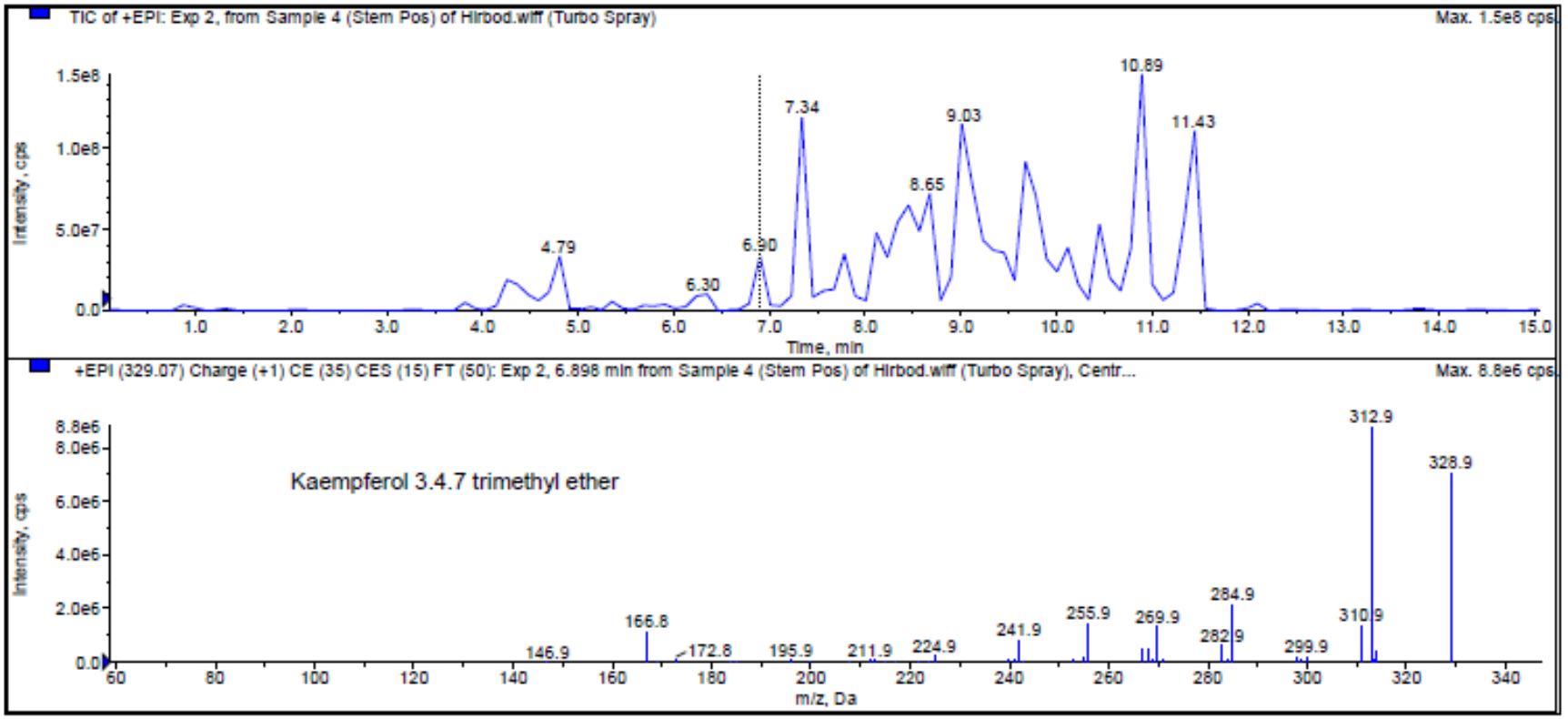


Figure 4.7: The LCMS/MS profile of Kaempferol 3,4,7-trimethyl from chloroform stem crude extract from *Aquilaria subintegra.*

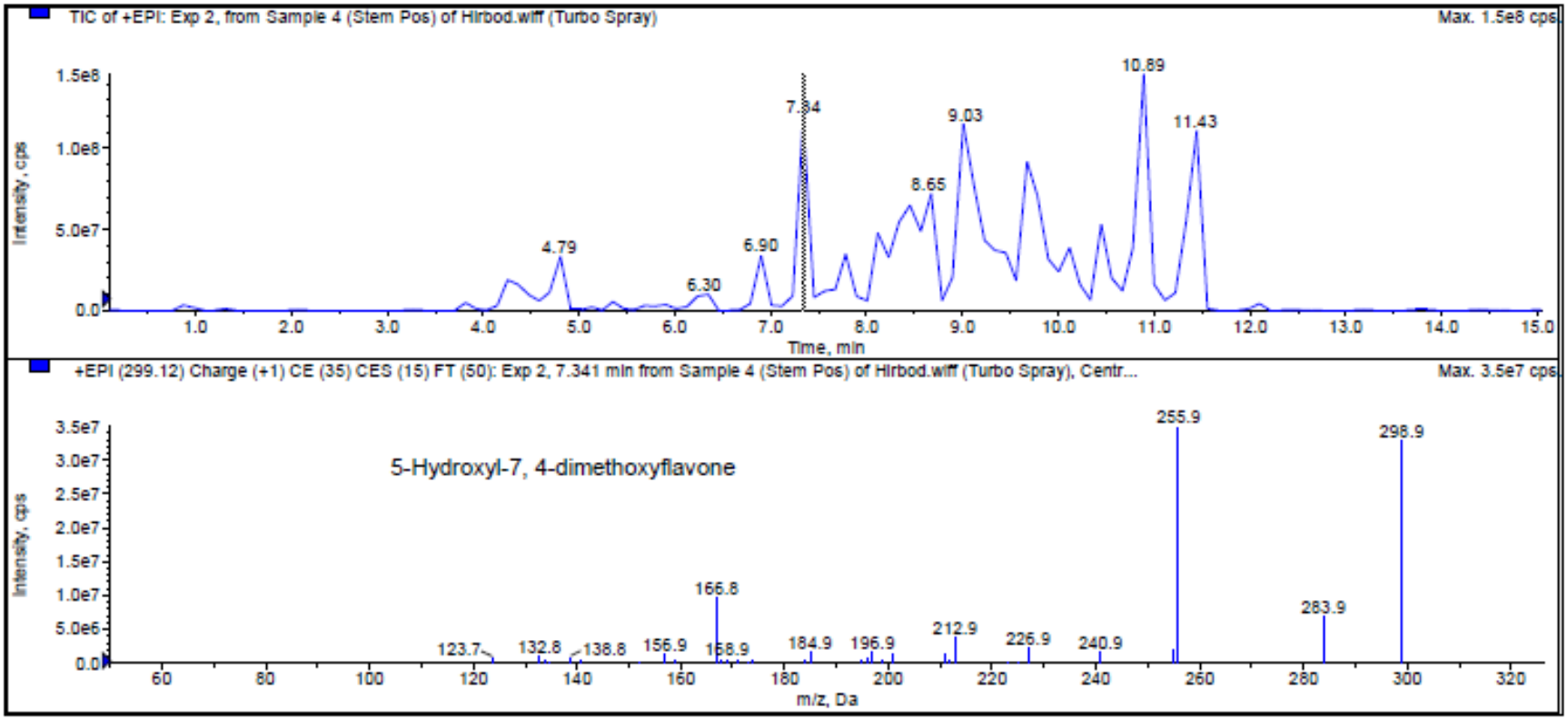


Figure 4.8: The LCMS/MS profile of 5-Hydroxy-7,4-dimethoxyflavone from chloroform stem crude extract from *Aquilaria subintegra*.

4.2 Total phenol and total flavonoids contents

(i) Total flavonoids contents

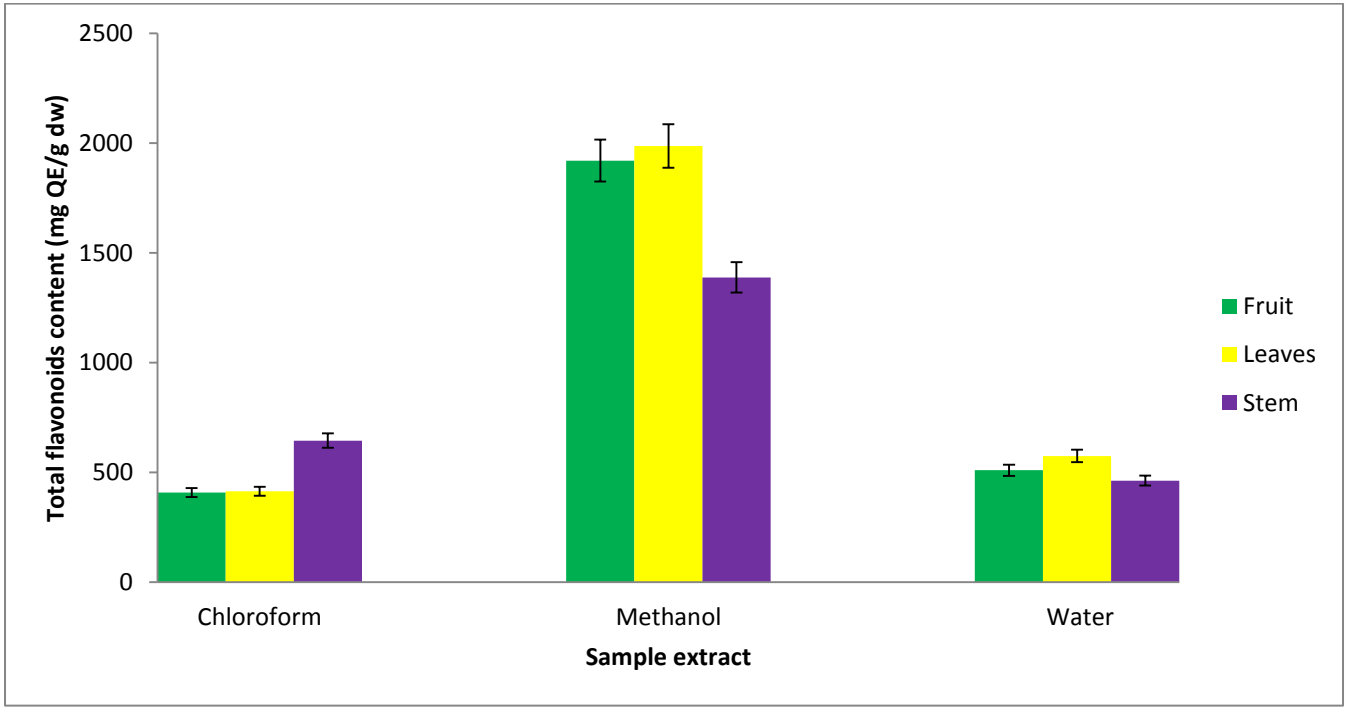


Figure 4.9: The total flavonoids contents of fruit, leaves and stem of *Aquilaria subintegra* from chloroform, methanol and water extract.

(ii) Total phenols contents

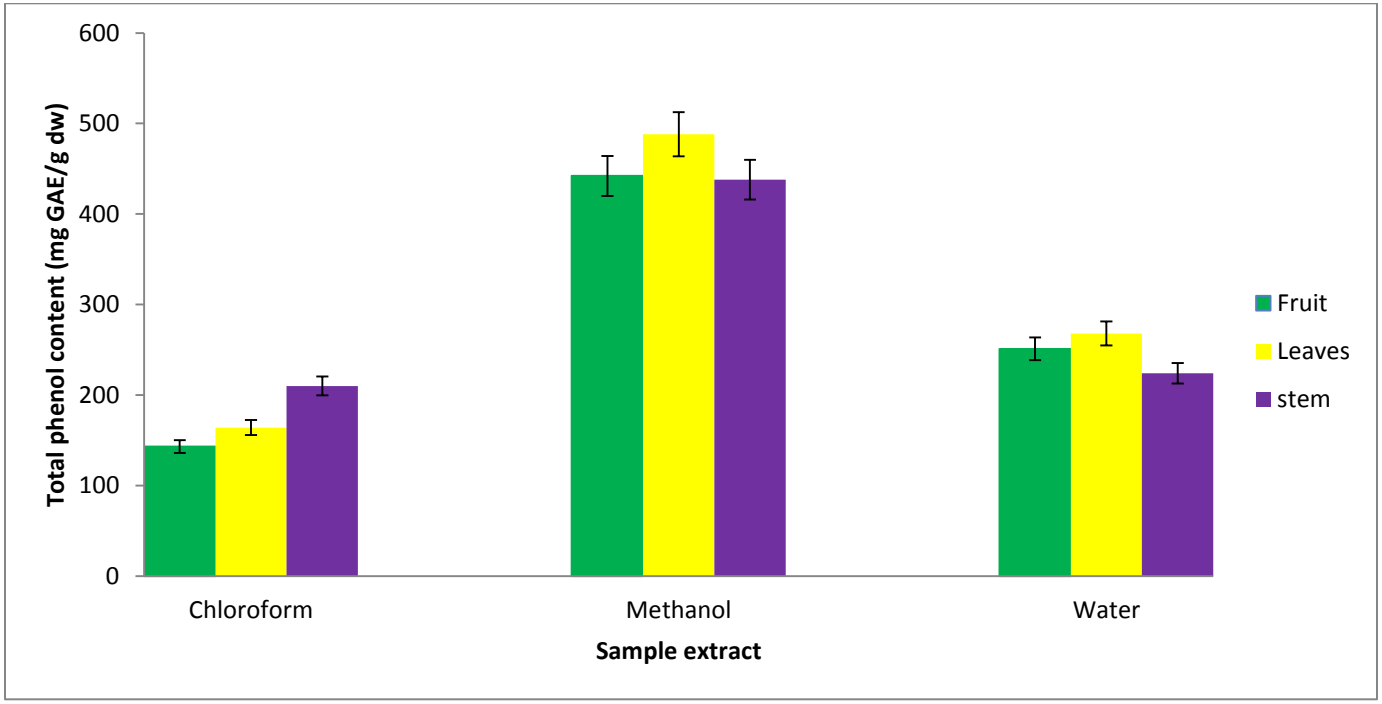


Figure 4.10: The total phenol contents of fruit, leaves and stem of *Aquilaria subintegra* from chloroform, methanol and water extract

4.3 Toxicity assay

4.3.1 Brine shrimp lethality assay (BSLA)

Table 4.19: Brine Shrimp Lethality Assay (BSLA)

Extract	Sample	LC_{50} value (μg/ml)±SD
Chloroform	Stem	3.11±0.09
	Leaves	11.64±0.19
	Fruit	68.76±0.28
Methanol	Stem	49.42±0.55
	Leaves	17.71±0.85
	Fruit	61.57±0.38
Water	Stem	32.39±0.39
	Leaves	17.58±0.26
	Fruit	61.32±0.34
Test sample	Kaempferol	9.59±0.20
Standard	Berberine	12.57±0.14

4.3.2 Toxicity test on mice

(i) Mortality

No mortality figured out during study

(ii) Physical observations

Daily observations recorded during the study showed that test article administrations had no side effects on mice.

(iii) Body weights

There were no statistically significant differences found between 3 groups of mice which were orally gavage at doses of 0.1, 0.5 and 1 ml/g body weight compared with control group (0 ml/g body weight).

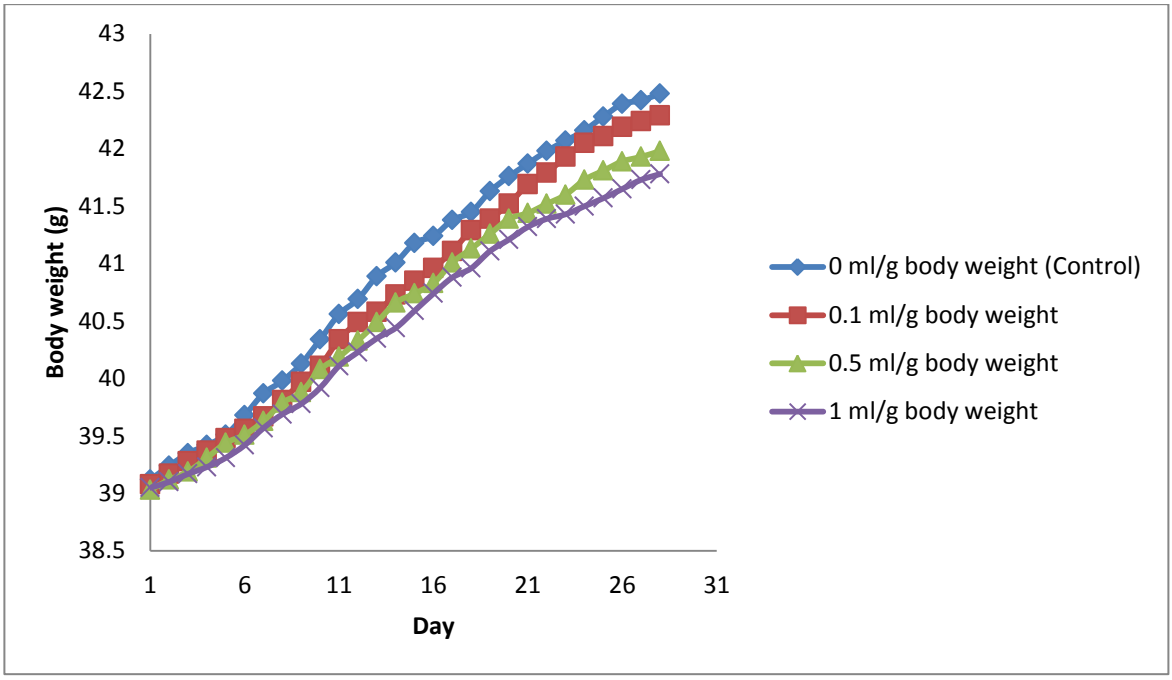


Figure 4.11: Mean body weights in male mice

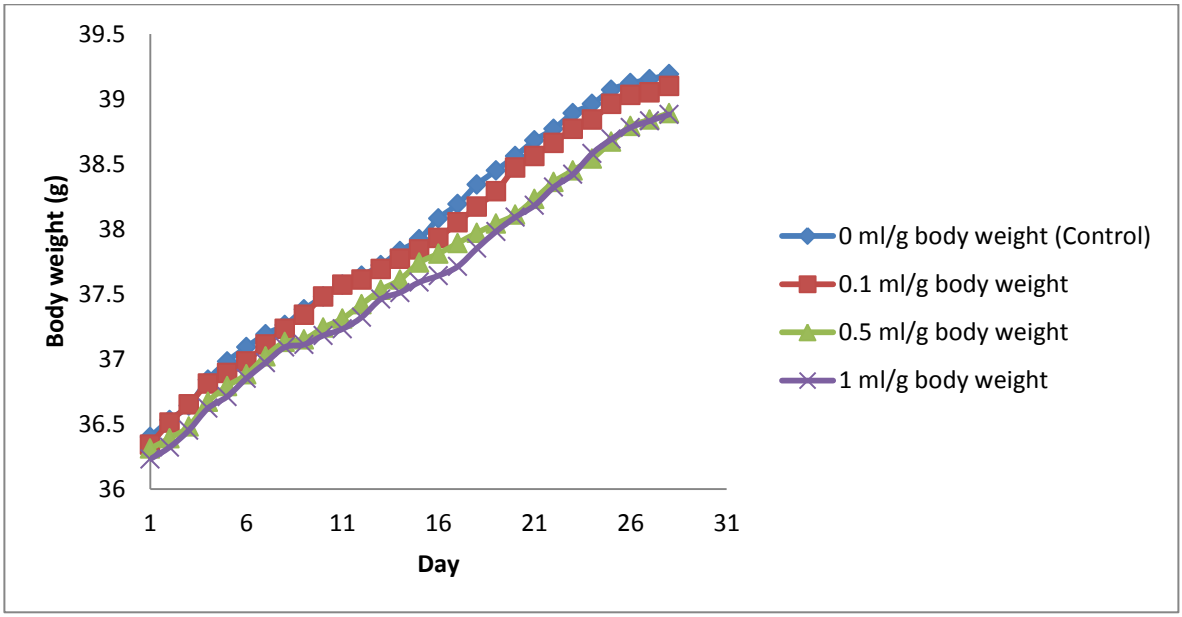


Figure 4.12: Mean body weights in female mice

4.4 AChE activity assay

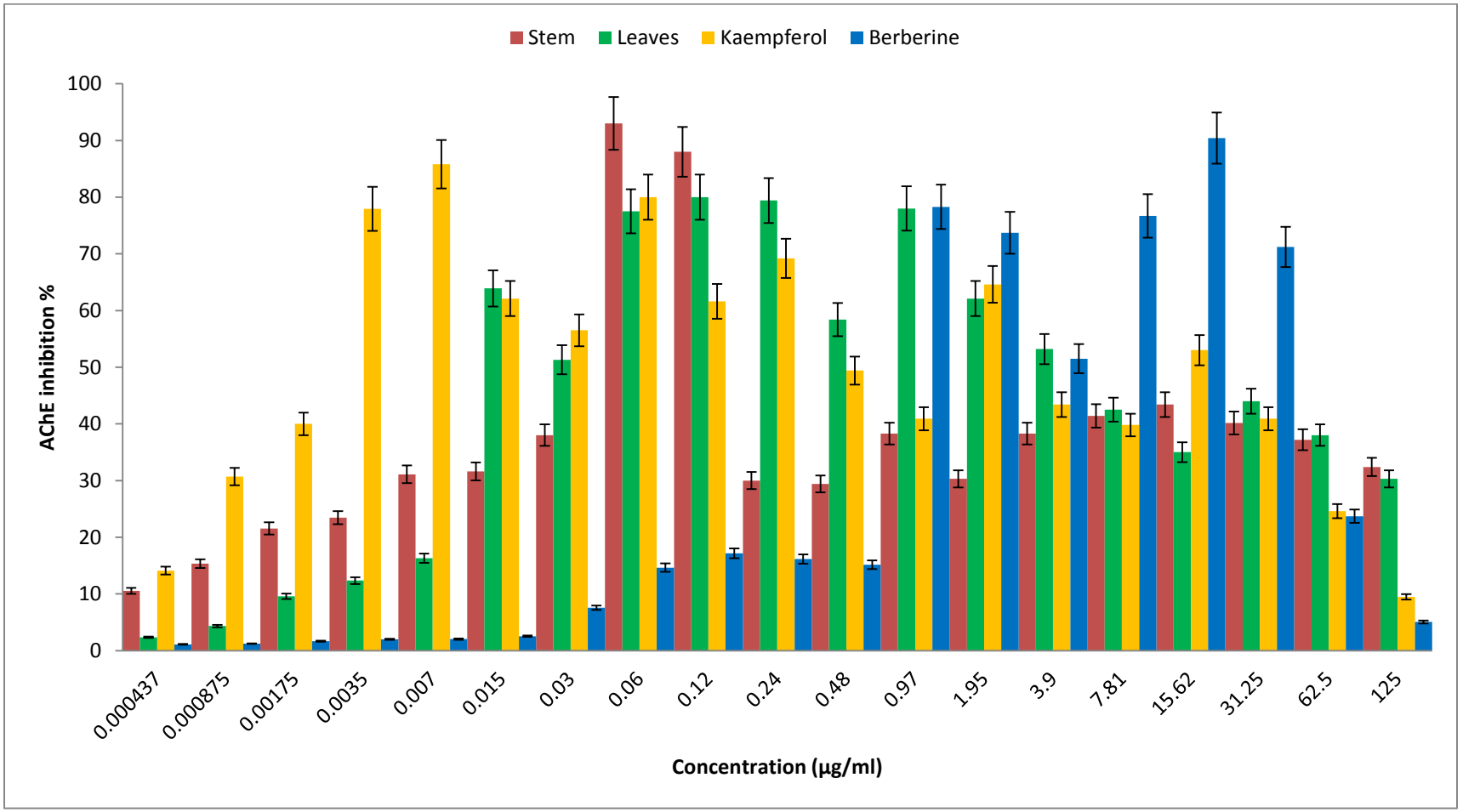


Figure 4.13: Percentage of AChE inhibition of chloroform leaves and stem crude extracts of *Aquilaria subintegra*, kaempferol and berberine.

4.5 Animal study

(i) The number of repeat entries to arms of the maze

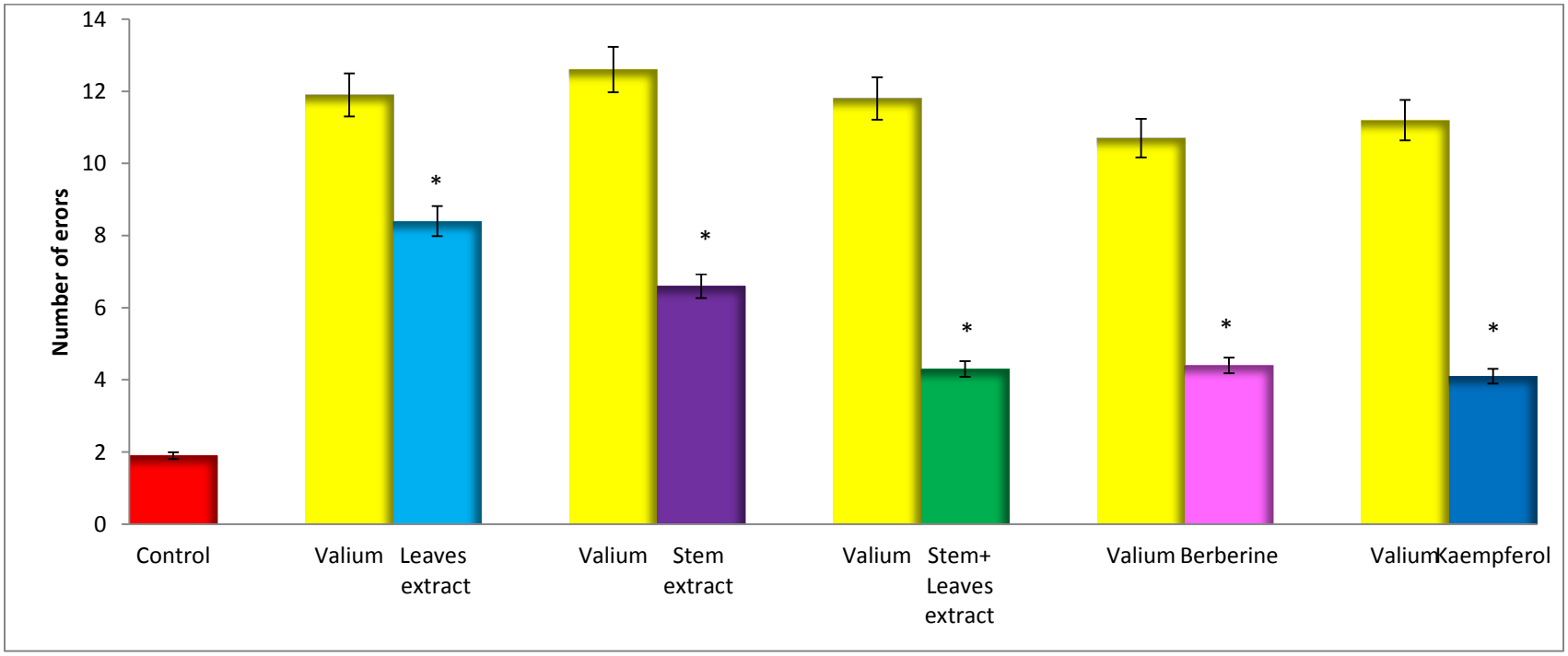


Figure 4.14: The number of repeat entries to arms of the maze (NRE) in male mice

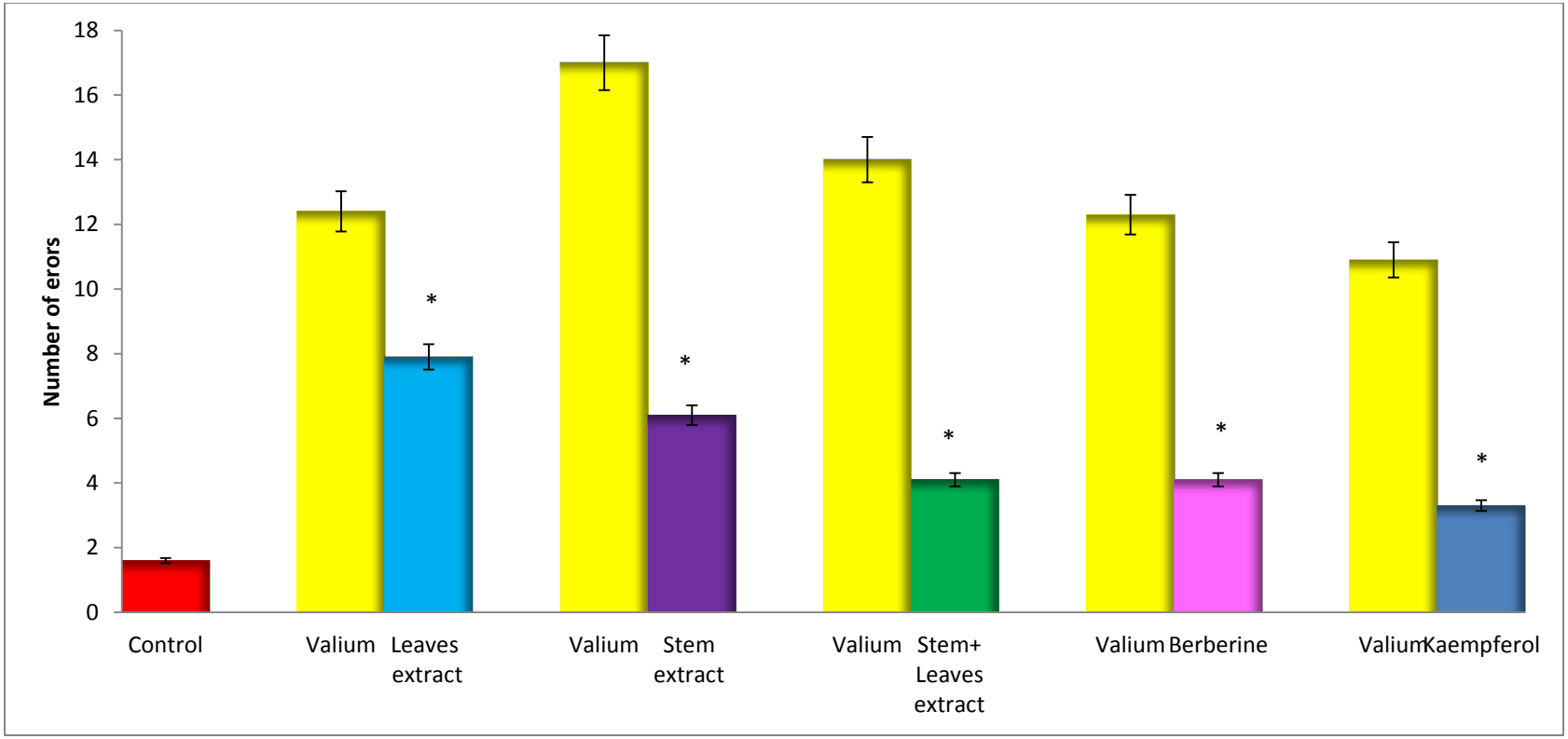


Figure 4.15: The number of repeat entries to arms of the maze (NRE) in female mice

(ii) The number of entries to arms of maze until the first error occurs

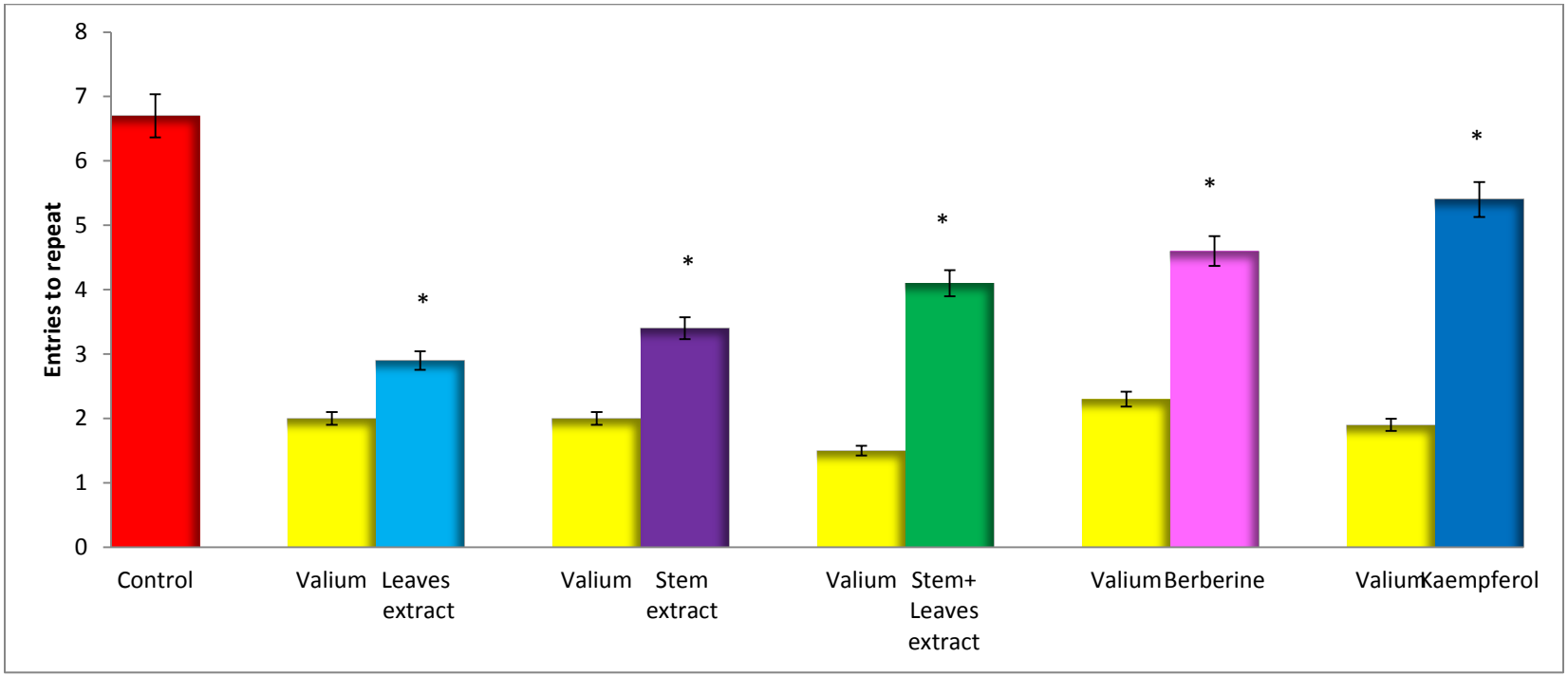


Figure 4.16: The number of entries to arms of maze until the first error occurs (NEF) in male mice

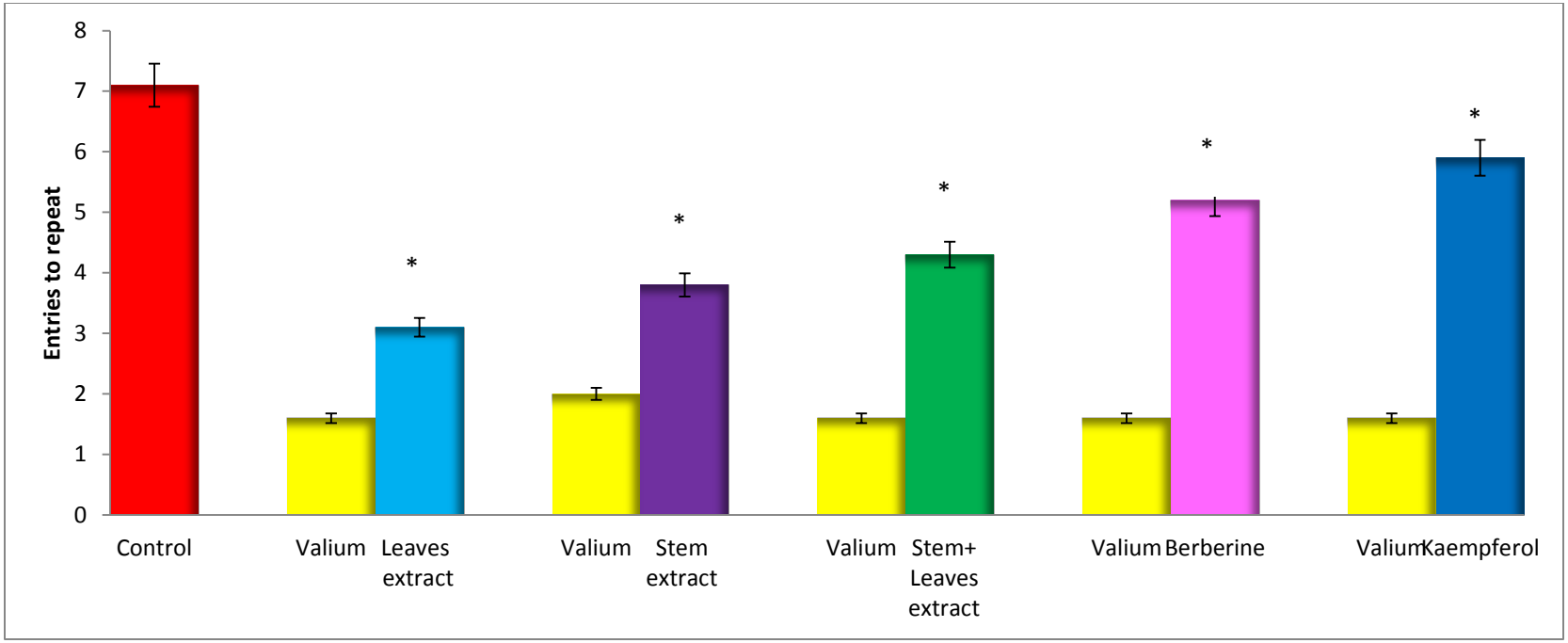


Figure 4.17: The number of entries to arms of maze until the first error occurs (NEF) in female mice

(iii) The number of repeat entries to arms of the maze without administration of valium

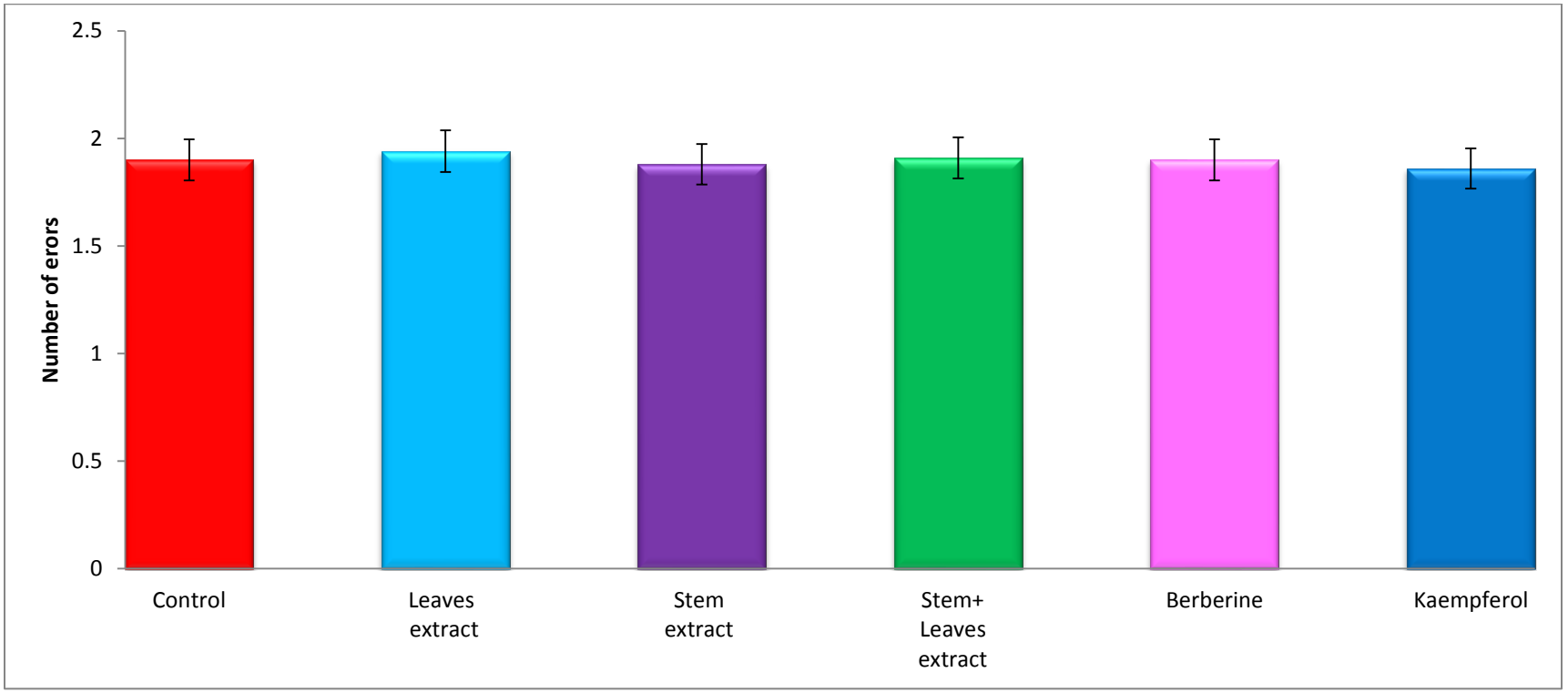


Figure 4.18: The number of repeat entries to arms of the maze (NRE) without administration of valium in male mice

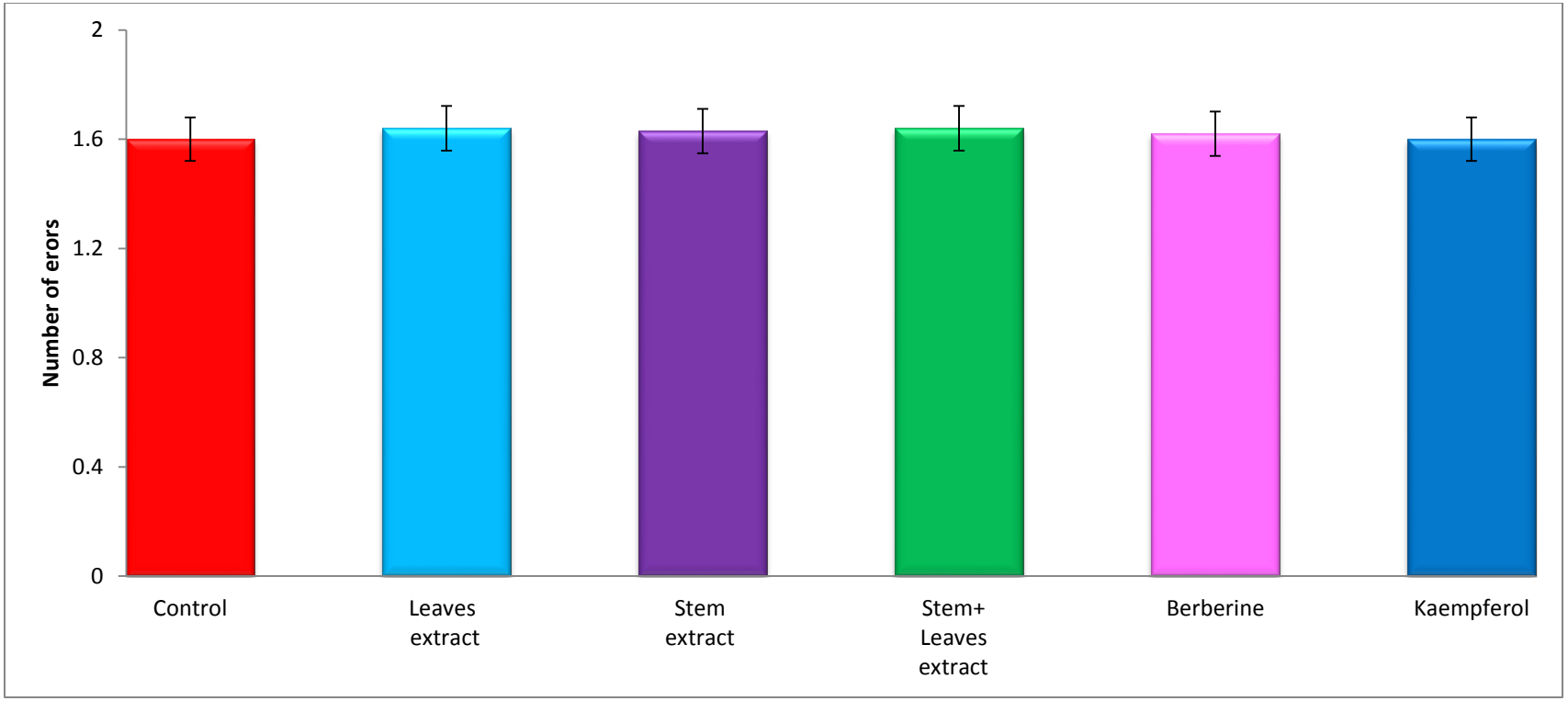


Figure 4.19: The number of repeat entries to arms of the maze (NRE) without administration of valium in female mice

(iv) The number of entries to arms of maze until the first error occurs without administration of valium

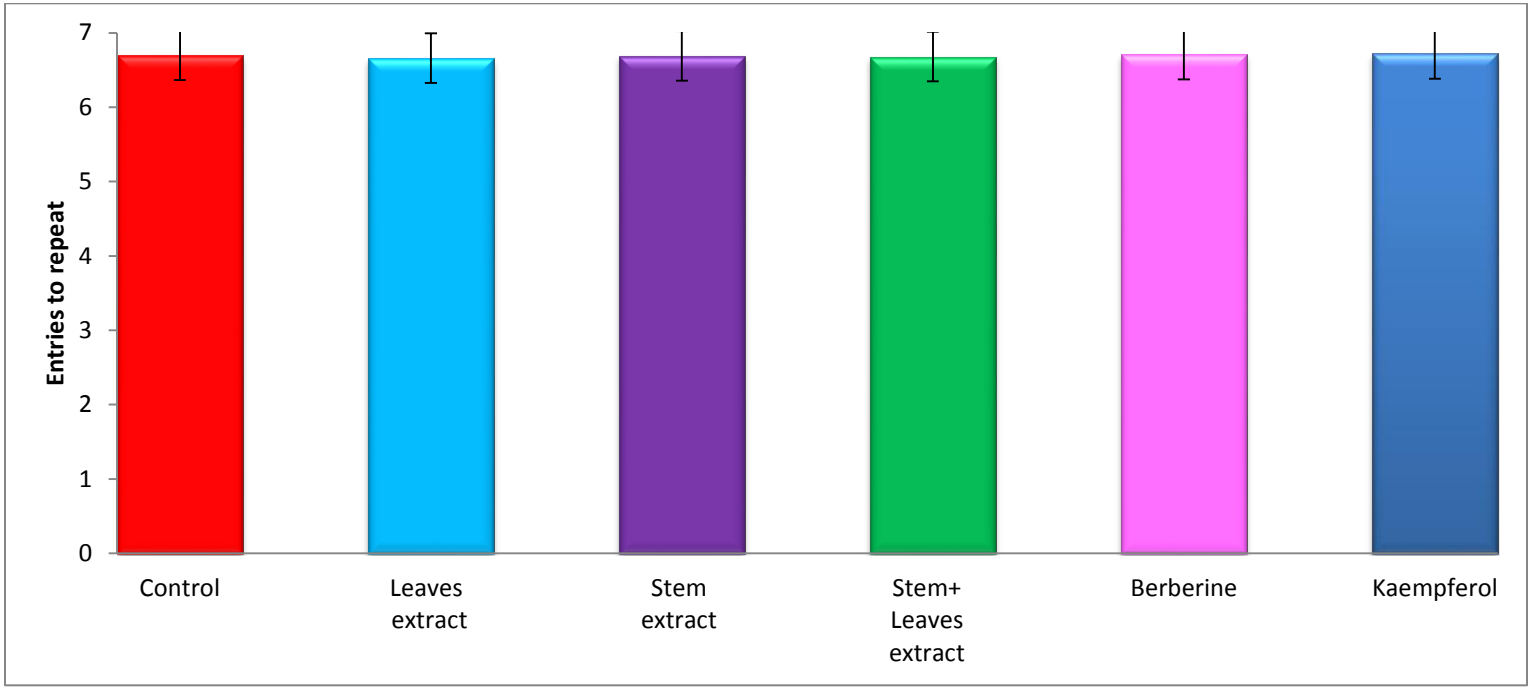


Figure 4.20: The number of entries to arms of maze until the first error occurs (NEF) without administration of valium in male mice

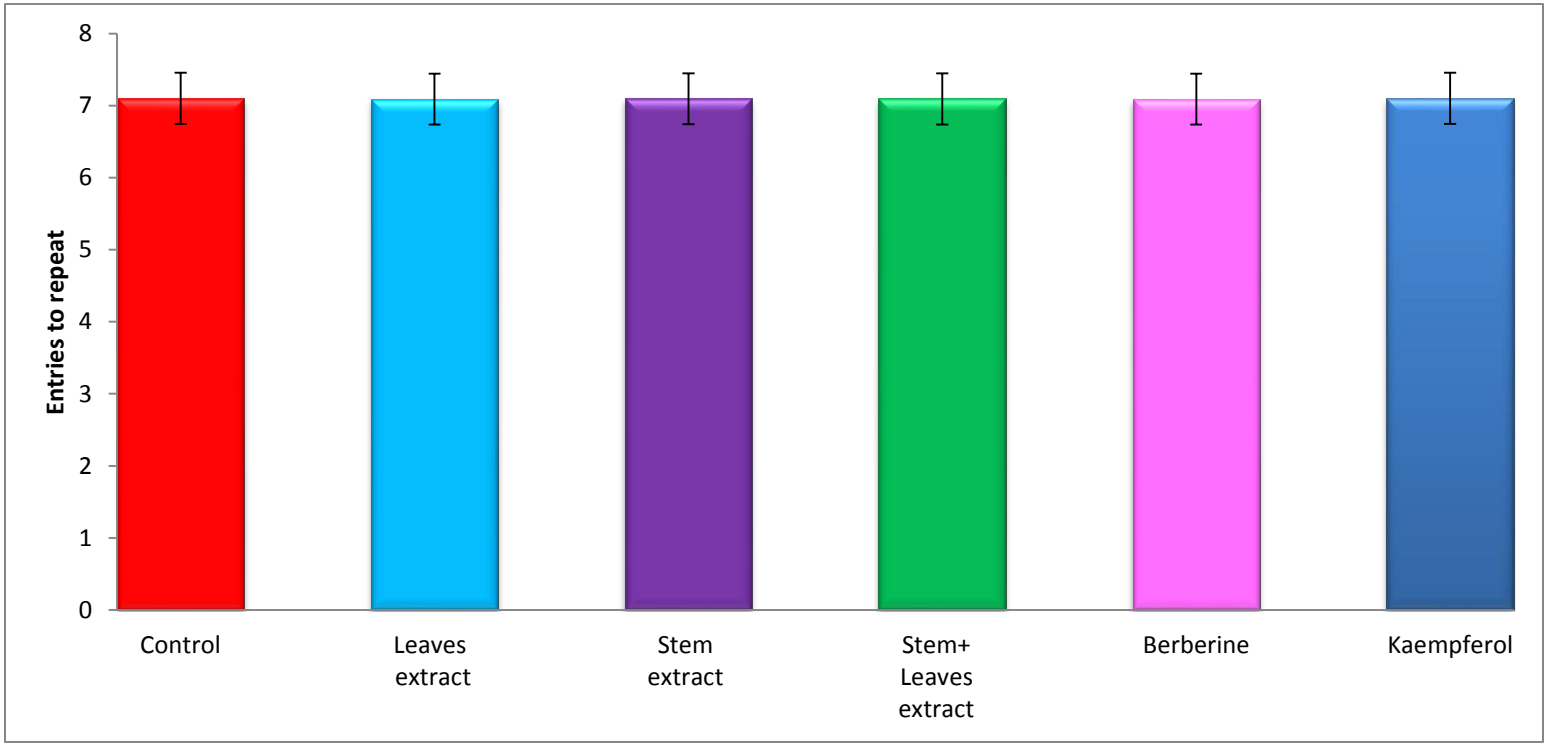


Figure 4.21: The number of entries to arms of maze until the first error occurs (NEF) without administration of valium in female mice

CHAPTER 5 DISCUSSIONS

The AChE inhibitory activity for leaves chloroform extract of *Aquilaria subintegra* (LCAS), stem chloroform extract of *Aquilaria subintegra* (SCAS) and kaempferol were 80%, 93% and 85.8%, respectively (Figure 4.13). Acetylcholine (ACh) is hydrolyzed by AChE through a reaction between the thiocholine and DTNB, to generate the yellow anion of 5-thio-2-nitrobanzoic acid (Atta-ur Rahman et al., 2001). The inhibition activity of AChE was measured by this reaction. LCAS and SCAS displayed high inhibitory activity at low concentrations, which was comparable to kaempferol, whereas the inhibitory activity of berberine was observed at higher doses only. The LC_{50} of LCAS and SCAS, kaempferol were 3.11μg/ml, 11.64μg/ml and 9.59μg/ml respectively and there were not toxics (Table 4.19). The results of toxicity test on mice exhibited no mortality during the study. Daily observations showed that administration of the articles had no side effects on mice. Additionally, there were no statistically significant differences between 3 groups of mice which were orally gavage at doses of 0.1, 0.5 and 1 ml/g body weight compared with control group (Figures 4.11; 4.12). These results displayed the insignificant toxicity effect of LCAS and SCAS, kaempferol and the standard drug. For LCAS and SCAS the total flavonoid contents were 414 mg QE/g dw and 645 mg QE/g dw (Figure 4.9) and the total phenol contents were 164 mg GAE/g dw and 210 mg GAE/g (Figure 4.10), respectively.

The results from TLC of LCAS displayed that the existence of alkaloids and phenols (Tables 4.16; 4.17). Phenols cover the existence of flavonoids. Numerous studies broadcasted that some of the alkaloids and flavonoids have AChE inhibitor activity (Yung et al., 2011; Kim et al., 2004: Jung and park, 2007). Besides, TLC of SCAS exhibited the presence of alkaloids and phenols (Tables 4.10; 4.11).

The analysis of the LCAS with LCMS/MS displayed that it contain Delphinidin-3-glucoside (Figure 4.2), 2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid (Figure 4.3) and benzenepropanoic acid conjugate (Figure 4.4). The same analysis on SCAS, indicated the presence of 5-Hydroxy-7,4-dimethoxyflavone (Figure 4.6), kaempferol 3,4,7-trimethyl ether (Figure 4.7) and 5.7-Dihydroxyl-4'-dimethoxyflavone (Figure 4.8).

Dong et al. (2005) investigated two AChE inhibitors physostigmine and donepezil on memory related behaviors of mice. They suggested that AChE inhibitors could improve memory deficits in mice. Ikarashi et al. (2004) examined mice memory disturbance by measuring AChE concentrations in their brains. They found out that memory impairment in cholinergic system of the mice is due to Aβ accumulation which happens when AChE increased. Figueiró et al. (2010) extracted AChE inhibitors from *Ptychopetalum olacoides* and orally administrate these compounds to the mice. They improved meaningful AChE inhibitor activity of these extracts for treating neurodegenerative conditions which is useful for AD treatment. In 2011, Foti and colleagues considered that the Radial Arm Maze (RAM) can be analyzed via movement most aspects of the spatial function, as the analytical memory and the appropriate working memory. RAM is a practicable memory testing for displaying the effect of drugs. ICR mice (male and female) were used to evaluate the effectiveness of LCAS and SCAS. The working memory was assessed by measuring the number of repeat entries to arms of the maze already visited (NRE). For instance if NRE is 2 it means that the mouse eats all 8 pellet with 2 times comes back to the arms of the maze which is the pellet was eaten before (2 errors). Together with the kaempferol and berbrine the results of testing memories with RAM displayed that the lowest number of NRE was related to the control group (Figures 4.14; 4.15).

Valium possesses sedative intensifies the effect of the neurotransmitter Gamma-Aminobutyric Acid (GABA) leading to Central Nervous System (CNS) depression, it utilized to cause memory impairment on mice. During a study in 2012 taking the valium causes side effects in 18583 people. Among them, in 23 people (69.57% in females and 30.43% in males) taking the valium leads to AD (http://www.ehealthme.com). Regular valium taking causes 77.27% AD among the people with more than 60 years of age and 69.57% in females. As figure 4.14 and 4.15 noticed repeated entries to arms of the maze in control group were 1.9 in male and 1.6 in female mice. The NRE was significantly increased when the mice were administrated with valium at 11.2 and 13.1 in male and female mice, respectively (Figures 4.14; 4.15).

Then, the plant extracts and kaempferol were administrated to the valium-treated mice. Lowest NRE was observed in mice which were administrated with kaempferol 3.3 and 4.1 in female and male nice, respectively (Figures 4.14; 4.15). In 2008, Serhan and colleagues reports that chronic inflammation has a detrimental effect on AD. Besides that, in 2010 Kim and colleagues broadcasted that administration of kaempferol in ICR mice significantly inversed the effect of Aβ. They utilized the Y-maze test to prove the ability of kaempferol to meliorate the memory impairment in mice caused by Aβ. One of the outstanding features perceived in AD patients is deficiency of ACh, a neurotransmitter in the synapses of the cerebral cortex. AChE is ACh hydrolyzing enzyme, which can promulgate the assembly of Aβ into fibrils. In 2006, Tabet suggested that AChE inhibitors could be effective in treatment of AD by increasing the acetylcholine mediated neuron to neuron transmission. García-Ayllón and colleagues (2011) have reported that AChE is due to decreasing in acetylcholine has important role in AD treatment. Silvestrelli et al. (2006) broadcasted that the cholinergic hypothesis of AD has act as a powerful encouragement to

pharmacotherapeutic strategies of AD, which focus on improving the cholinergic inadequacy and potentiating residual activity in affected neuronal circuits by reforming cholinergic transmission. For amplifying cholinergic activity in brain by maintain ACh level, AChE inhibitors has been widely pursued as the most powerful approach. Loizzo et al. (2008) discussed that in AD patients, AChE inhibitors could minimize the formation of neurotoxic fibrils and clumping of Aβ, hence AChE inhibitors might be useful in treatment cognitive symptoms of AD (Kalauni et al., 2002; Atta-ur-Rahman et al., 2004). Since AChE inhibitors have the anti-inflammatory activity they could protect neuron cells from the injuries caused by free radicals and Aβ. The significant activity of kaempferol as a natural AChE inhibitor (Figure 4.13) confirms its potential to be applied as an alternative drug in the treatment of AD.

Berberine is an alkaloid with anti-inflammatory activity and is utilized as a drug for treatment of AD. Some investigations suggested that berberine may have AChE inhibitory effects and it could be useful for the prevention of AD as well (Xiang et al., 2009 and Huang et al., 2009). The NRE for the berberine administrated mice was 4.4 and 4.1, for male and female mice, respectively (Figures 4.14; 4.15). Results obtained from the mice administrated with berberine, LCAS and SCAS, were approximately the same (Figures 4.14; 4.15). The decline in NRE might be due to the presence of delphinidin in LCAS and kaempferol in SCAS, which is a flavonoid with anti-inflammatory effect and support the finding made by Takasawa et al. (2010). In 2012, Inoue and colleagues found that delphinidin declined the amount of epigallocatechin-3-gallate (EGGG), which causes cytotoxicity and enhanced the endoplasmic reticulum (ER) stress. The ER stress, which is one of the AD pathogenesis effects on Aβ generation (Kudo et al., 2006). The group of mice, administrated with LCAS, had higher NRE compare to SCAS administrated one. The

NRE in mice which administrated LCAS was 8.4 and 7.9 in male and female mice, respectively (Figures 4.14; 4.15). The group of mice which administrated SCAS had 6.6 and 6.1 in male and female (Figures 4.14; 4.15). The results obtained from the group of mice administrated with valium indicated a remarkable increase in NRE. Contrarily, LCAS, SCAS and kaempferol-treated animals exhibited a drastic decrease in NRE (Figures 4.14; 4.15).

The ANOVA results demonstrated the significant effects of LCAS and SCAS on the memory of mice (Appendix A.1; A.2). The findings of the RAM clearly showed higher effects of LCAS, SCAS and kaempferol in female mice compare with the males. Likewise, there was a decreasing trend in NRE. After administration of valium, no statistically significant effects were perceived in NRE between the male mice administrated with LCAS plus SCAS, keampferol and berberine (Appendix A.5). On the other hand, the difference in NRE administrated with kaempferol compared was severely significant in female mice to the ones administrated with LCAS plus SCAS and berberine. It displayed that LCAS plus SCAS, kaempferol and berberine were more effective than LCAS and SCAS in male and female mice. Kaempferol was the most effective treatment in the female mice and significantly declined NRE (Appendix A.6).

Comparison of the ANOVA results of the NRE, between male and female mice, demonstrates that the kaempferol has been significantly more effective in females (Appendix A.7). It exhibited that female mice got a better response to the AChE inhibitors than males. Spampinato et al. (2011) focused on the analysis of Magnetic Resonance Imaging (MRI) images which displayed that AD tends to harm women in advance, however it is more aggressive in men.

In another measurement for memory testing on mice the number of entries until the first error occurs (NEF), was counted. As it is observable in Figure 4.16 and 4.17, control group had the highest NEF 6.7 and 7.1 in male and female mice, respectively. The lowest NEF was perceived when the mice were administrated with valium (1.94 in male and 1.68 in female mice). The mice administrated with kaempferol had 5.4 and 5.9 entries in male and female mice, respectively. The group of mice administrated berberine had 4.6 and 5.2 entries in male and female mice, respectively. NEF significantly decreased when the mice was treated with valium (Appendix A.3; A.4). The group of mice administrated with kaempferol had the highest NEF. NEF in the mice administrated with LCAS plus SCAS was more than 2 other groups of mice administrated with LCAS and SCAS. The lowest NEF was noticed in the group of mice administrated with LCAS. In group of mice administrated with LCAS plus SCAS, NEF were 4.1 and 4.3 in male and females, respectively (Figures 4.16; 4.17). The lowest NEF was noticed in the group of mice administrated with LCAS, 2.9 and 3.1 in male and female mice, respectively (Figures 4.16; 4.17). The group of mice administrated with SCAS had 3.4 and 3.8 NEF in male and female, respectively (Figures 4.16; 4.17).

The results obtained from the group of mice, treated with LCAS, SCAS and kaempferol, without administration of valium, indicated statistically insignificant effects in NRE and NEF in all groups, male and female (Figures 4.18; 4.19; 4.20; 4.21) . It illustrated that LCAS, SCAS and kaempferol were effective after administration of valium to the mice. Administration of LCAS, SCAS and kaempferol after treating with valium, caused a marked reduction in NRE. This finding indicates that without impairment of mice memory LCAS, SCAS and kaempferol exhibited insignificant effects.

CHAPTER 6 CONCLUSION

The results from TLC of leaves and stem chloroform extract of *A.subintegra* displayed that the existence of alkaloids and phenols. The analysis of the stem chloroform extract with LCMS/MS displayed that it contain kaempferol. A flavonoid kaempferol has the AChE inhibitor activity of 85.8% and LC_{50} = 9.59µg/ml. Leaves and stem chloroform extract significantly reduced the number of repeated entries to the arms of the maze in male and female ICR mice with impaired memory by valium in Radial Arm Maze (RAM). The decline in the number of repeated entries to the arms of the maze might be due to the presence of kaempferol. Furthermore, the highest number of entries until the first error occurs was perceived in kaempferol administrated mice. The kaempferol could improve memory impairment in mice. It can be a potent natural AChE inhibitor and could be used as an alternative to the chemical drug instead of berberine for the treatment of Alzheimer's disease. However, there is a definite requirement for further mechanistic investigations to confirm and validate the effects for the treatment of Alzheimer's disease.

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APPENDIX

Appendix A.1: The ANOVA results of Number of repeated entries to arms of maze (NRE) in male mice

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
valium vs stem	4.11	58.76	Yes	***	3.877 to 4.343
valium vs leaves	6.625	94.71	Yes	***	6.392 to 6.858
valium vs stem+leaves	8.805	125.9	Yes	***	8.572 to 9.038
valium vs kaempferol	9	128.7	Yes	***	8.767 to 9.233
valium vs berberine	8.72	124.7	Yes	***	8.487 to 8.953
valium vs control	11.23	160.5	Yes	***	11.00 to 11.46

Appendix A.2: The ANOVA results of Number of repeated entries to arms of maze (NRE) in female mice

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
valium vs stem	3.025	54.84	Yes	***	2.841 to 3.209
valium vs leaves	6.895	125	Yes	***	6.711 to 7.079
valium vs stem+leaves	8.875	160.9	Yes	***	8.691 to 9.059
valium vs kaempferol	9.675	175.4	Yes	***	9.491 to 9.859
valium vs berberine	9.08	164.6	Yes	***	8.896 to 9.264
valium vs control	11.38	206.2	Yes	***	11.19 to 11.56

Appendix A.3: The ANOVA results of the number of entries until the first error occurs (NEF) in male mice

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
control vs valium	4.795	224.3	Yes	***	4.722 to 4.868
control vs stem	3.835	179.4	Yes	***	3.762 to 3.908
control vs leaves	3.305	154.6	Yes	***	3.232 to 3.378
control vs stem+leaves	2.700	126.3	Yes	***	2.627 to 2.773
control vs kaempferol	1.300	60.80	Yes	***	1.227 to 1.373
control vs berberine	2.110	98.69	Yes	***	2.037 to 2.183

Appendix A.4: The ANOVA results of the number of entries until the first error occurs (NEF) in female mice

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
control vs valium	5.425	196.2	Yes	***	5.333 to 5.517
control vs stem	4.000	144.7	Yes	***	3.908 to 4.092
control vs leaves	3.310	119.7	Yes	***	3.218 to 3.402
control vs stem+leaves	2.825	102.2	Yes	***	2.733 to 2.917
control vs kaempferol	1.210	43.77	Yes	***	1.118 to 1.302
control vs berberine	1.875	67.82	Yes	***	1.783 to 1.967

Appendix A.5: The ANOVA results of comparison of LCAS plus SCAS, kaempferol and berberine in male mice

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
stem+leaves vs kaempferol	0.195	2.788	No	ns	-0.1293 to 0.5193
stem+leaves vs berberine	-0.085	1.215	No	ns	-0.4093 to 0.2393
kaempferol vs berberine	-0.28	4.003	No	ns	-0.6043 to 0.04428

Appendix A.6: The ANOVA results of comparison of LCAS plus SCAS, kaempferol and berberine in female mice

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
stem+leaves vs kaempferol	0.8	14.5	Yes	***	0.5443 to 1.056
stem+leaves vs berberine	0.205	3.716	No	ns	-0.05073 to 0.4607
kaempferol vs berberine	-0.595	10.79	Yes	***	-0.8507 to -0.3393

Appendix A.7: The ANOVA results of comparison of LCAS plus SCAS, kaempferol and berberine in male and female mice

Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
berberine (male) vs berberine (female)	0.54	13.68	Yes	***	0.3545 to 0.7255
kaempferol (male) vs kaempferol (female)	0.855	21.66	Yes	***	0.6695 to 1.040
stem+leaves (male) vs stem+leaves (female)	0.25	6.333	Yes	*	0.06455 to 0.4355