

**ACETYLCHOLINESTERASE INHIBITORY AND ANTIOXIDANT PROPERTIES
OF ROOT EXTRACT FROM *Pueraria mirifica***

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**FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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**DISSERTATION SUBMITTED IN FULFILMENT OF
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**DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE UNIVERSITY OF MALAYA
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ABSTRACT

Pueraria mirifica or commonly known as Kwao Krua and Thai Kudzu is originated from north Thailand and known as a popular traditional medicine for woman. The Alzheimer's disease (AD) is progressive inexorable loss of cognitive function associated with the presence of senile plaques in the hippocampal area of the brain. The objective of this study is to determine the antioxidant and acetylcholinesterase inhibition activity of *Pueraria mirifica* root extracts. The thin layer chromatography was carried out to detect the chemical compounds present in the root extracts of *Pueraria mirifica*. Alkaloids were detected using Dragendroff's reagent and terpenoids with Vanillin reagent. The total phenolic and flavonoid contents was highest in methanol aqueous extracts at 10.35 mg GAE/g and chloroform extract at 1.182 mg QE/g dry extract respectively. The antioxidant activity of DPPH showed high percentage of inhibition where all extracts achieved IC₅₀ inhibition percentage and the highest percentage of inhibition was IC₅₀ = 1.145 in n-butanol extracts. However the percentage inhibition of metal chelating, NORSA, superoxide radical scavenging activity and FRAP are relatively low. The highest percentage inhibition of metal chelating is IC₅₀ = 1.768 in ethyl acetate extracts, 21.54 % also in ethyl acetate extracts in NORSA and 43.167 % in n-hexane extracts in superoxide radical scavenging activity. In FRAP, methanol aqueous extract showed the highest reducing power at 0.18 mmol Fe⁺/g. Based on the result above, there is potential for *Pueraria mirifica* extract to be used as antioxidant and acetylcholinesterase inhibition treatment.

Keywords : *Pueraria mirifica*, acetylcholinesterase inhibition, Alzheimer's disease

RENCATAN ASETILKOLINESTERASE DAN SIFAT ANTIOKSIDAN DARI EKSTRAK AKAR *Pueraria mirifica*

ABSTRAK

Pueraria mirifica juga dikenali sebagai Kwao Krua dan Thai Kudzu berasal daripada utara Thailand dan terkenal sebagai ubatan tradisional untuk kaum wanita. Penyakit Alzheimer adalah kehilangan fungsi kognitif berterusan berkaitan dengan kehadiran plak senil di bahagian hippocampus otak. Objektif utama dalam kajian ini ialah menentukan aktiviti antioksidan dan untuk menilai cerakinan rencatan asetilkolinesterase dari ekstrak akar *Pueraria mirifica*. Ujian kromatografi lapisan nipis dijalankan untuk mengenalpasti komponen kimia yang terdapat dalam ekstrak akar *Pueraria mirifica*. Alkaloid telah dikenal pasti menggunakan reagen Dragendroff's dan terpenoid telah dikenalpasti menggunakan reagen Vanillin. Bagi jumlah fenolik dan jumlah flavonoid, nilai kandungan jumlah fenolik dan jumlah flavonoid tertinggi ialah dalam ekstrak akueus metanol iaitu 10.35 mg GAE/g dan ekstrak klorofom iaitu 1.182 mg QE/g secara berturutan. Untuk ujian antioksidan, DPPH menunjukkan peratusan rencatan paling tinggi di mana kesemua ekstrak mencapai peratusan rencatan IC_{50} dan peratusan rencatan tertinggi ialah $IC_{50} = 1.145$ dalam ekstrak n-butanol. Walaubagaimanapun, peratusan rencatan untuk ujian pengkelat logam, NORSA, pengurangan kuasa ferric (FRAP) dan hapus-sisa radikal superoksida, peratusan rencatan adalah agak rendah. Peratusan rencatan tertinggi untuk ujian pengkelat logam ialah $IC_{50} = 1.768$ bagi ekstrak etil asetat, 21.54 % juga bagi ekstrak etil asetat untuk NORSA dan 43.167 % bagi ekstrak n-heksana dalam ujian hapus sisa radikal superoksida. Bagi FRAP, ekstrak metanol akueus menunjukkan kuasa penurunan tertinggi iaitu 0.18 mmol Fe^{+}/g . Berdasarkan keputusan, terdapat potensi pada ekstrak akar *Pueraria mirifica* untuk digunakan sebagai antioksidan dan rawatan rencatan asetilkolinesterase.

Kata kunci : *Pueraria mirifica*, rencatan asetilkolinesterase, penyakit Alzheimer

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree celcius
g	Gram
kg	Kilogram
mg	Miligram
mL	Mililiter
Mmol	Milimolar
mU	Miliunit
Nm	Nanometer
v/v	Volume / volume
w/v	Weight / volume
µg	Microgram
µL	Microliter
ACH	Amyloid cascade hypothesis
-OH	Hydroxide
8OHdG	8-hydroxydeoxyguanosine
8OHG	8-hydroxyguanosine
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChRs	Acetylcholine receptors
AD	Alzheimer Disease
AlCl ₃	Aluminium Chloride
APH-1	Anterior pharynx-defective 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	β-amyloid protein
BACE1	Beta-site APP cleaving enzyme 1
BuChE	Pseudocholinesterase
ChAt	Choline acetyltransferase
DMSO	Dimethyl sulfoxide
DPPH	2,2diphenyl-1-picrylhydrazyl (DPPH)
DTNB	5,5'-Dithio-Bis -2-Nitrobenzoic Acid
EDTANa ₂ .2H ₂ O	Ethylenediaminetetraacetic acid disodium dehydrate
ETC	Electron transport chain

FAD	Familial AD
FC	Folin-Ciocalteu
FDA	Food and Drug administration
Fe ²⁺	Ferrous ion
FeCl ₃	Ferric chloride solution
FeCl ₃ .6H ₂ O)	Ferric chloride hexahydrate
FeSO ₄)	Ferrous sulfate
FRAP	Ferric reducing antioxidant power
FZ	Ferrozine
GPx	Glutathione peroxidase
GSK-3 beta	Glycogen synthase kinase-3 beta
GSSG	Oxidised glutathione
GST	Cytosolic glutathione S-transferase
H ₂ O ₂	Hydrogen peroxide
HACHT	High affinity choline transporter
HHE	4-hydroxyhexenal
IC ₅₀	Half maximal inhibitory
K ₂ HPO ₄ ⁴	Potassium phosphate (dibasic)
KH ₂ PO ₄	Potassium phosphate (monobasic)
LCMS	Liquid Chromatography Mass Spectrometry
LDL	Low-density lipoprotein (LDL
MAPK	Mitogen-activated protein kinase
MMSE	Mini Mental Status examination
Na ₂ [Fe(CN) ₅ NO].2H ₂ O	Sodium nitroferricyanide
Na ₂ CO ₃	Sodium Carbonate
NADH	Nicotinamide adenine dinucleotide
NaNO ₂)	Sodium nitrite
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NCT	Nicestrin
NFT	Neurofibrillary tangles
NORSA	Nitric Oxide Radical Scavenging Activity
O ₂ ⁻	Superoxide anions
OH ⁻	Hydroxyl ions
OH [·]	Hydroxyl radicals

PEN-2	Presenilin enhancer protein 2
PMS	Phenazine methosulphate
PS	Multiprotein complex composed of presenilin
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SP	Senile plaques
TLC	Thin layer chromatography
TPTZ	2,4,6-tripyridyl-s-tirazine
VACht	Vesicular acetylcholine transporter
WHO	World health organisation

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CHAPTER 1

INTRODUCTION

Pueraria mirifica also known as *Kwao Krua* or *Kwao Krua Kao* (white *Kwao Krua*) is a native herb of Thailand. It is classified in the family of Leguminosae, subfamily Papilionodeae, also known as the soy, bean and pea subfamily, and have several phytoestrogens compounds, like phenol miroestrol and deoxymiroestrol (Manonai et al., 2007). The most active part of this plant is in the tuberous roots that is interconnected with each other throughout the entire roots and it comes in different sizes forming a chain of bulbs. Due to its beneficial properties based on its rich phytoestrogenic content, *Pueraria mirifica* has been widely used in cosmetics products such as breast cream, skin moisturiser, eye gel and hair tonic (Siangcham et al., 2010).

According to Chomchalow (2013), a study was done to find out the preliminary benefits of *Pueraria mirifica* to cure Alzheimer's disease, whether it could inhibit the damage of the brain cells or not, including the promotion of the brain cells to grow and develop successfully. In the study, when the *Pueraria mirifica* extracts was given to the injured and malfunction brain cells, the rate of death of the brain cells decrease by 30-40 % which is considered satisfactory.

Alzheimer's disease (AD) is a common cause of dementia and affecting over 40 million people worldwide and it is expected to grow to 65 million by the year 2030. Dr. Alois Alzheimer is the one responsible for the first description of a dementing condition which later become known as Alzheimer's Disease (AD) (Korolev, 2014). Dementia is a clinical syndrome that involves progressive deterioration of intellectual function including memory, language, reasoning, decision making, visuospatial function, attention and orientation. The study of hypotheses, concepts and theories of AD have

been done including hypothesis related to oxidative imbalance, the loss of cholinergic neuron, calcium, microtubule instability and amyloid cascade, the concept about mild cognitive impairment and the disruption and adjustment of original molecules. Studies on glutamate neurotoxicity and nitric oxide theories has also been done (An et al., 2008).

The main objective of this study is to determine the function of acetylcholinesterase inhibitors as a drug for the treatment of AD. Based on Sims et al. (1981), a term cholinergic hypothesis had been introduced and they suggested that in the brain of AD patients, the synthesis of a neurotransmitter known as acetylcholine in the neocortex of the brain was low. It also found that the level of choline acetyltransferase was clearly found downregulated in the hippocampus and frontal cortex, and cholinergic neuron counts in the nucleus basalis was generally lowered in AD condition (An et al., 2008) therefore acetylcholinesterase inhibitors are used as a drug in AD patients.

Acetylcholinesterase (AChE) is the predominant cholinesterase in the brain. It hydrolyses acetylcholine to choline and acetate. This process resulted in terminating the effect of this neurotransmitter at cholinergic synapses. As a result, AChE became the target of cholinesterase inhibitors used for addressing the cholinergic deficit in AD patients (Hlila et al., 2015).

Treatment with AChE inhibitors is required by AD patients and most of the specialist will have holistic approach where pharmacological treatments and multidisciplinary team assessments of needs must be done together without leaving behind the necessity of community support. Initiation of an AChE inhibitor is recommended as early as possible upon full assessment and full diagnosis of AD. It is

due to only 30-40 % patients respond to AChE inhibitors treatment (McGleenon et al., 1999)

Another contributing factor of AD is oxidative stress. A collection of evidence suggests that as the disease developing, the brain tissues in AD patients are exposed to oxidative stress. Oxidative stress is normally distinguished by an imbalance in the reactive oxygen species (ROS) production and antioxidative defence system which are responsible for the removal of ROS, these two systems play a huge role in the cognitive decline and age related neurodegeneration process (Feng & Wang, 2012). There are a few types of oxidative stress includes protein oxidation, lipid oxidation, DNA oxidation, and glycooxidation. There are a few antioxidants namely, glutathione, α -tocopherol (vitamin E), carotenoids, ascorbic acid, antioxidant enzymes such as catalase and glutathione peroxidases that can detoxify H_2O_2 by converting it to O_2 and H_2O under physiological conditions. Unfortunately, oxidative stress will occur when ROS levels exceeds the removal capacity of antioxidant system under pathological conditions or by aging and metabolic demand thus causing biological dysfunction (Feng & Wang., 2012).

1.1 Research Objectives

The objectives of this study are:

1. To analyze the chemical constituents of *Pueraria mirifica* root extracts.
2. To determine the antioxidant activity of *Pueraria mirifica* root extracts using antioxidant assay.
3. To evaluate the Acetylcholinesterase inhibition assay of *Pueraria mirifica* root extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 Alzheimer's Disease

Dementia is a chronic disease relating to the mental processes due to injury of the brain and it is shown by memory disorders, personality changes and impaired reasoning. One of the most common cause of dementia is Alzheimer's disease and this disease is thought of as untreatable degenerative condition (McGleenon et al., 1999). Alzheimer's disease was first described as a dementing condition by the German psychiatrist and neuropathologist Dr. Alois Alzheimer. Alzheimer describe the case of Auguste D, a 51 years old woman with a 'peculiar disease of the cerebral cortex' where this woman was presented with progressive memory and language impairment, disorientation and behavioural symptom also psychosocial impairment in his 1906 conference lecture followed by 1907 article (Korolev *et al.* 2014). The most common misconception about AD is that it is deemed to be normal and expected as human is aging. It is also thought to be as part of typical trajectory of age related cognitive decline (DeFina et al., 2013).

There are several etiological factors that can cause AD including genetics, environmental factors, and general lifestyles (Feng & Wang, 2012). From the early classification of AD, there are two particular hallmark lesions found in the brain of the patient which are extracellular β -amyloid protein ($A\beta$) deposition in a form of senile plaques and intracellular deposits of microtubule-associated protein tau as neurofibrillary tangles (NFTs) (Aliev et al., 2008 ; Feng & Wang, 2012).

2.1.1 Epidemiology of AD

World health organisation (WHO) has reported that the incidence of AD is drastically increasing along as human aging. It is estimated that 60% of over-60-year-old population will be affected with AD. Based on statistic, the incidence of AD is 25 million in 2000 and it is expected that it will reach 63 and 114 million in year 2030 and 2050, respectively (An et al., 2008). Several meta-analysis and nationwide surveys have resulted in almost similar age-specific prevalence of AD across regions (Figure 2.1). After the age of 65, the age-specific prevalence of AD almost doubles every 5 years. In comparison of prevalence between developed and developing countries, in developed nations, approximately 1 in 10 (10 %) older people (65+ years) is affected by dementia and 1 in 3 very old people (85+ years) is experiencing dementia related symptoms and signs. However, in developing countries the overall prevalence of dementia was 3.4 %. Seven developing nations show prevalence, ranging widely from less than 0.5 % to more than 6 % in people aged 65+ years, which is noticeably lower than in developed country (Qiu et al., 2009).

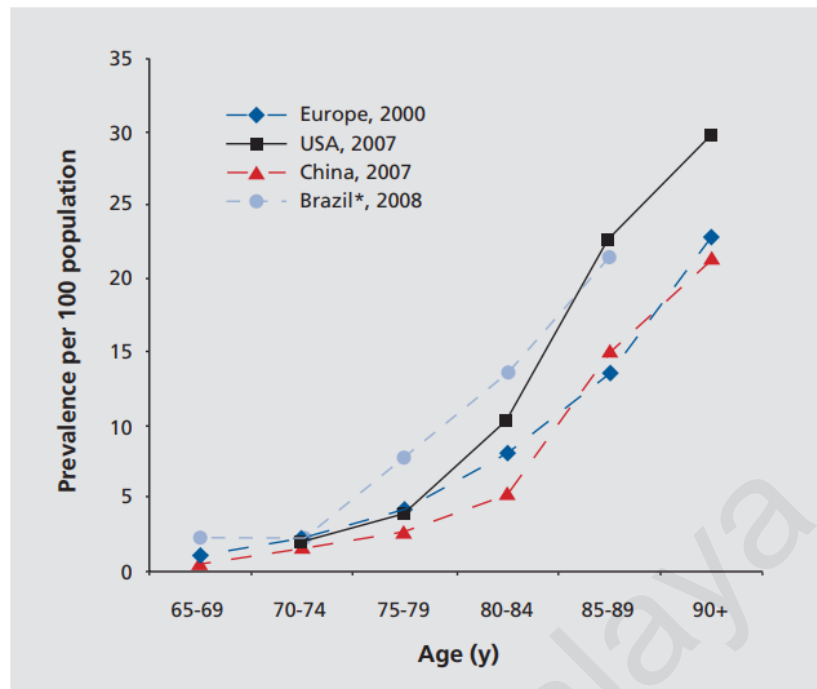


Figure 2.1: Age specific prevalence of Alzheimer's disease (per 100 population) across continents and countries (Qiu et al., 2009). Reprinted permission granted by Qiu

There are two common case of AD which are 'late onset' also known as 'sporadic' and also 'early onset' also known as 'familial' AD. The majority cases of AD are aged 65 or older therefore it falls below the 'late onset' category (> 95 % of all cases). Even though the cause of this type have not been discovered by the researches, they have determined a few similar risk factor involved including age, prior head injury, low educational and occupational attainment, female gender, sleep disorders (e.g., sleep apnea), estrogen replacement therapy and vascular risk factor, such as diabetes, hypercholesterolemia, and hypertension. Apolipoprotein E (APOE) also has been discovered to have the ability to increase the likelihood of developing late-onset AD (DeFina et al., 2013). The development of early onset AD is associated with rare genetic mutations and it happens to patients below 65 years old. Patients with familial forms of AD have an autosomal dominant mutation in either one of the presenillin genes located chromosome 1 and 14

in the amyloid precursor protein (APP) gene located on chromosome 21. Usually, individuals with Down's Syndrome (trisomy 21) have a higher risk of developing early onset AD (Korolev, 2014).

2.1.2 Pathology and Pathogenesis of AD

Since the early classification and explanation of presenile dementia by Alzheimer in 1907, senile plaques (SPs) and neurofibrillary tangles have become the "signature" or hallmark lesions of Alzheimer's disease (Armstrong, 2011). There are also other markers associated which includes neuronal and dendritic loss, neurophil threads, dystrophic neurites, granulovacuolar degeneration, Hirano bodies, cerebrovascular amyloid and atrophy of the brain (Aliev et al., 2008). AD is a progressive neurodegenerative brain disorder that cause notable damage of normal brain structure and function. The AD-related degeneration begins at the medial temporal lobe, specifically in the entorhinal cortex and hippocampus causing memory and learning deficits (Korolev, 2014). The next step is spreading to the frontal, temporal cortex and parietal area with relative sparing of the motor and sensory cortical regions and subcortical regions (DeFina et al., 2013).

NFTs are known as the major intracellular protein accumulation in brains of AD patients and it is made up of an abnormal form of the intraneuronal protein Tau which usually plays a part in structural support and cellular communication. It is located mainly in cerebral cortex, particularly in the large pyramidal neurons in the hippocampal and frontotemporal region (Aliev et al., 2010). Tau is one of the microtubule-associated proteins that promote assembly of tubulin to microtubules and stabilize them. The abnormal form of intraneuronal protein Tau will form NFT. Few abnormal processes that happens cause the Tau protein to miss fold and aggregate into

NFTs and further leads to a collapse in communication and neuronal function and finally cell death (Hasegawa, 2016).

The most powerful theory used to explain pathogenesis process of AD is amyloid cascade hypothesis (ACH) that was first introduced in 1992. This theory suggested that the primary and initial triggering event is deposition of A β where formation of amyloid plaques throughout the medial temporal lobe and cerebral cortex takes place (Figure 2.2). This is known as an initial pathological event in the disease development thus these cascades of events occur, including neuronal distortion, damaged neuronal communication, and the initiation of a second abnormal protein process leading to formation of NFTs, cell death and lastly resulting in dementia (Armstrong, 2011).

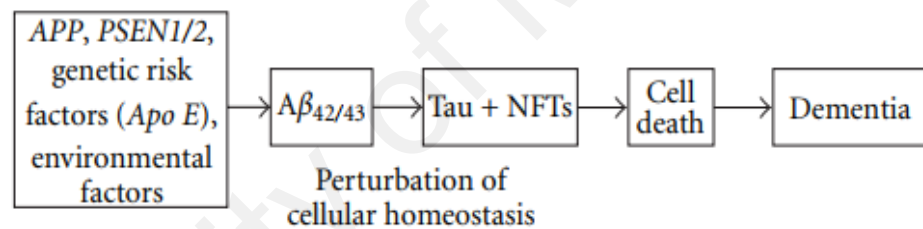


Figure 2.2: The original amyloid cascade hypothesis (ACH). A β : β -amyloid, APOE: Apolipoprotein E. APP: Amyloid precursor protein, PSEN 1/2: Presenilin genes 1 and 2, NFT: Neurofibrillary tangles (Armstrong, 2011). Reprinted permission granted by Armstrong

Unfortunately, the initial ACH formulated faces two major limitations. First, rather than being the cause of neurodegeneration, SPs and NFT may be the reactive product resulting it. Second, there is lacking of accepted mechanism explaining the deposition of A β leads to the formation of NFTs. A modified version from the original ACH including those concerns is presented in Figure 2.3. There are two main observation resulted in the formulation of this version of ACH, the first on is the identification of A β as the main component of the SPs and second, mutations of the APP, PSEN1 and PSEN2 genes. The second observation was found in families with early onset AD

(Familial AD (FAD), disease onset < 60 years). As outcome of these observation, the presence A β within SPs was elucidated as an impact of these mutations thus finally leads to cell death and dementia. FAD possesses a similar phenotype to sporadic (late onset) AD except that it has earlier onset, therefore it was presumed that the pathogenesis of all types of AD can be explained by this amyloid deposition (Reitz, 2012).

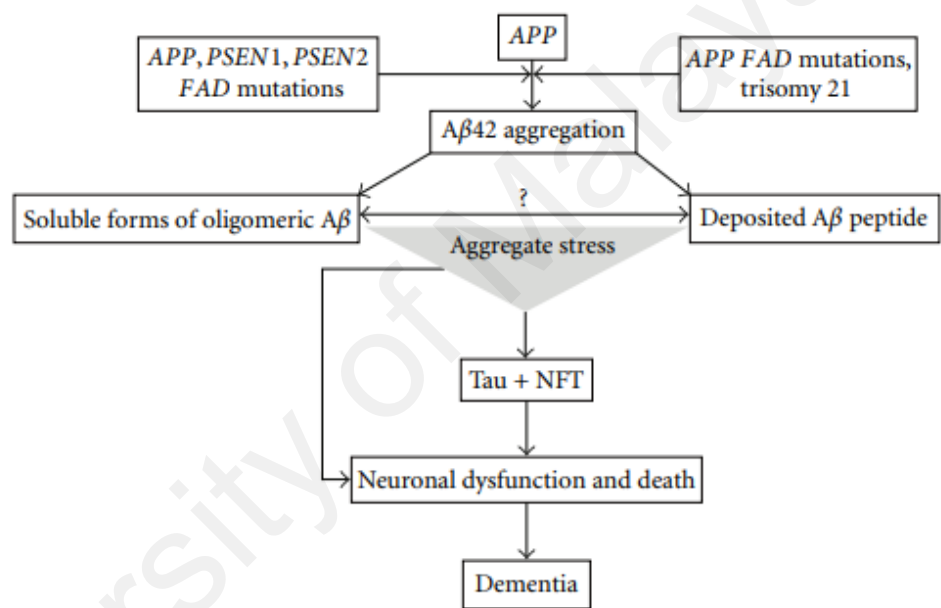


Figure 2.3: Amyloid Cascade Hypothesis (ACH) (Reitz, 2012). Reprinted permission granted by Reitz

2.2 Acetylcholinesterase Inhibition in Alzheimer Disease

2.2.1 Choline

According to Zeisel (2004), choline is a dietary component that is crucial to all cells to function normally. The National Academy of Sciences, USA, in 1998 reported that choline was identified as a required nutrient for humans and the amount of daily intake was recommended. Choline plays a major role in the human metabolism from cell structure to neurotransmitter synthesis and lack of choline in the body may lead to diseases such as liver disease, neurological disorder and atherosclerosis. Choline has a complex role in the body where it is crucial for neurotransmitter synthesis (acetylcholine), cell membrane signalling (phospholipids), lipid transport (lipoproteins) and methyl group metabolism. It is also important in brain and memory development in fetus and it also help lower the risk of the development of neural tube defects. Another role of choline is to make phospholipids phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen and sphingomyelin which is essential components for all membranes (Zeisel & da Costa, 2009).

2.2.2 Acetylcholine and Acetylcholinesterase

Acetylcholine (ACh) is a point-to-point neurotransmitter that is fast-acting at the neuromuscular junction and in the autonomic ganglia. There are few suggestions of similar actions happens in the brain. However, central cholinergic neurotransmission predominantly changes neuronal excitability, thus alters presynaptic release of neurotransmitter and coordinates the firing of groups of neurons therefore resulting that instead of ACh role as the primary excitatory neurotransmitter in the periphery, it appears to act as neuromodulator in the brain instead (Picciotto et al., 2012).

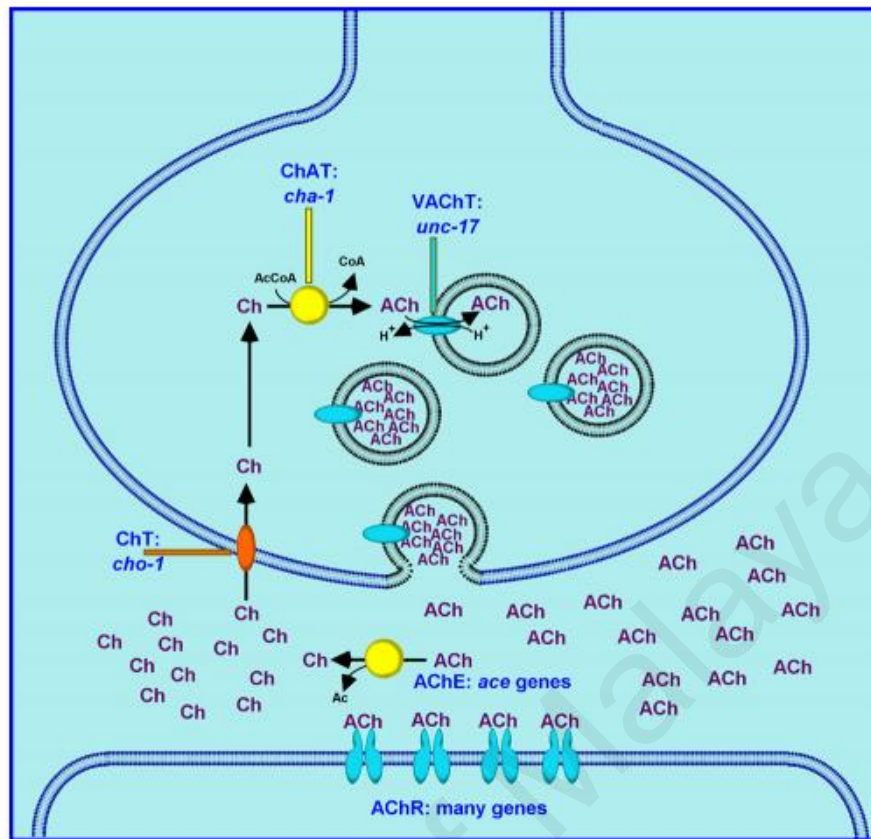


Figure 2.4: Cholinergic enzymes and transporters. (Rand, 2007). Reprinted permission granted by Rand

The first substance proven to be a neurotransmitter was acetylcholine. Henry Dale and Otto Leowi had won a nobel prize in 1936 for their pioneering research on chemical neurotransmission especially for the discovery and functional characterization of the first identified neurotransmitter, acetylcholine (Contestabile, 2010). Based on Figure 2.4, synthesis of acetylcholine is initiated by choline acetyltransferase (ChAt) and then the vesicular acetylcholine transporter (VAcHT) loads them into synaptic vesicles by the action of ATP-dependent proton pump located in the synaptic vesicle membrane will acidify the synaptic vesicle lumen. The vesicle lumen and the cytoplasm entice the driving force for ACh transport due to the pH gradient where the VAcHT essentially “exchange” ACh for proton. The general process of docking and priming of synaptic vesicles, and their calcium-stimulated fusion with the cell membrane are independent of

the neurotransmitter contained in the vesicles. After the synaptic vesicle fusion and transmitter release, ACh then diffuses within the synaptic cleft and activates acetylcholine receptors (AChRs) that is normally located on post synaptic cells. The action of acetylcholine is terminated by direct enzymatic hydrolysis of the neurotransmitter in the synaptic cleft by acetylcholinesterase unlike most other neurotransmitter (e.g., GABA, dopamine, serotonin), where the termination happens by transporter mediated removal of the transmitter from the synaptic cleft. Choline synthesized is then transported back into the presynaptic neuron via a high affinity choline transporter (HAChT, or ChT) and it will be available for the synthesis of additional ACh (Rand, 2007).

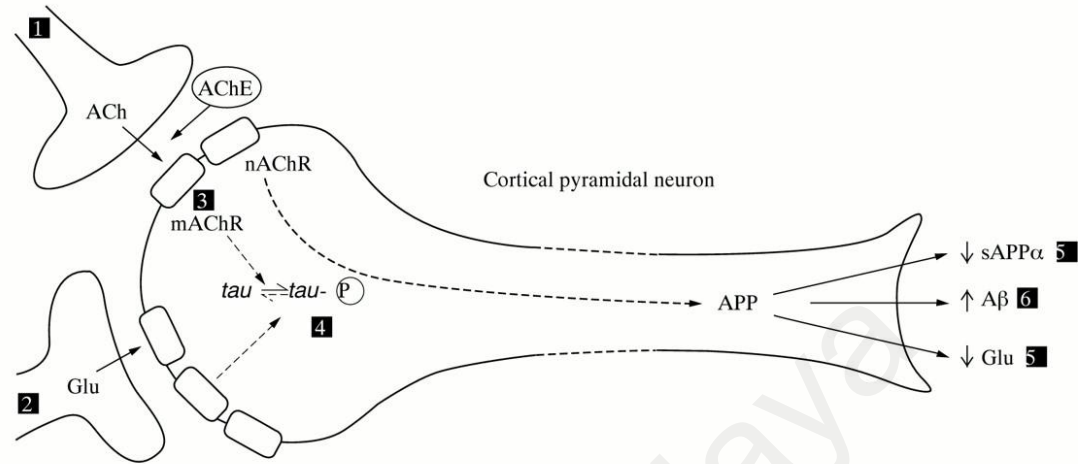
According to Garcia-Ayllon et al. (2009), acetylcholinesterase (AChE) is an enzyme responsible for the inactivation of cholinergic neurotransmission and it is consistently decreased in AD brain however, despite the overall decrease, level of AChE is increased around β -amyloid plaques and it is suggested that AChE could play a potential role in β -amyloid fibrillogenesis. AChE enzymes will catalyse the hydrolysis of the ester bound of ACh to terminate the impulse transmitted action of ACh through cholinergic synapses (Filho et al., 2006). This condition will lead to AChE become the target of cholinesterase inhibitors used to address the cholinergic deficit in AD patients. AChE inhibitors treatment will help increase acetylcholine concentration in synaptic cleft and thus improve the cholinergic transmission (Hlila et al., 2015).

2.2.3 Cholinergic Hypothesis

Cholinergic hypothesis was introduced over 20 years ago and suggested that a failure of acetylcholine containing neurons to function properly in the brain significantly contributes to the cognitive decline observed in those with advanced age and Alzheimer's disease (AD). The core point of cholinergic hypothesis is that the cognitive decline associated with advanced age and AD is associated with the loss of cholinergic function in the central nervous system (Jr & Buccafusco, 2003). Neuropathologists started to examine samples from AD's patients during the seventies and eighties and cholinergic hypothesis was revealed and gained momentum. A specific cholinergic deficit in the cholinergic projection from a basal forebrain neuronal population, the nucleus basalis magnocellularis of Meynert, to the cortex and hippocampus was found abundantly in AD's patient autopsic material. The enzyme activity important for synthesis of acetylcholine, choline acetyltransferase, important markers of cholinergic synapse and neurons was discovered to be dramatically decreased severely in pathological samples from the cortex and hippocampus of AD patients. Depolarization-induced acetylcholine release and choline uptake in nerve terminals to replenish the acetylcholine synthetic machine were two other specific markers of the function of cholinergic synapse that were reduced in the same tissue (Contestabile, 2010).

There are also pharmacological research and histological analysis of brain pathology in AD patients that supports this hypothesis. The scopolamine model was used to demonstrate a link between cholinergic dysfunction and age associated memory impairment. In short, the evidence shows that decline of the cholinergic system leads to the cognitive decline in aging and AD however the specific role of the cholinergic system remains controversial (Araujo et al., 2005).

A Proposed neurochemical changes in Alzheimer's disease



B Rectification of neurotransmission with cholinesterase inhibitors

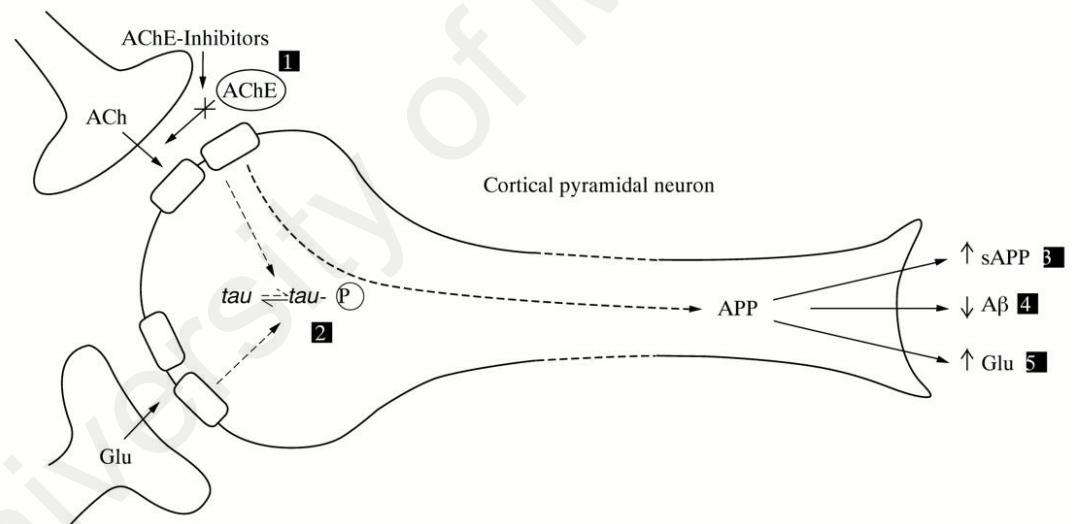


Figure 2.5: Figure shows schematic diagram of neuron. Diagram (A) represent alteration in AD and in (B) the hypothetical mode of action of AChE inhibitors. Reprinted permission granted by Francis

Based on Figure 2.5, in A, the cholinergic innervation and corticocortical glutamatergic neurotransmission is reduced due to neuron or synapse loss. This leads to reduced coupling of muscarinic M1 receptors to second messenger system. Tau protein will shift to the hyperphosphorylated state and become the precursor for neurofibrillary tangles.

This process reduces secretion of soluble APP therefore increase the production of β -amyloid protein. This will eventually decrease glutamate production. In Figure B, AChE inhibitors reduce the breakdown of endogenously release ACh, resulting in greater activation of presynaptic ACh receptors therefore phosphorylation of Tau is reduced to normal. This is probably due to activation of muscarinic and nicotinic receptors (Francis et al., 2017).

The cholinergic hypothesis is still extensively studied in the recent years to further investigate the conceptual consistency and its therapeutic potential. Many researches have done and contributed novel findings regarding the topic. The extension of studies on cholinergic deficits in early and prodromal stages of the disease have been one of the most interesting findings. This study was made possible due to the participation of various aged nuns, brothers and priest all over the US convents, monasteries and churches under funding from the National Institute of Aging (Contestabile, 2010).

2.2.4 Acetylcholinesterase Inhibition Treatment in AD

AChE inhibitors also known as anti-cholinesterase plays a role to inhibit the cholinesterase enzyme from breaking down ACh, therefore the duration and level of neurotransmission action will be increased. Based on its mechanism of action, AChE inhibitors can be divided into two groups which are irreversible and reversible. Reversible inhibitors usually have therapeutic applications while irreversible inhibitors usually associated with toxic effects (Colovic et al., 2013). According to Mehrpouya et al. (2016), reduced cerebral production of choline acetyl transferase in AD's patients resulted to a decrease in acetylcholine synthesis therefore disturbed the cortical cholinergic function. Due to that factor, AChE inhibitors became the first medication approved by the Food and Drug administration (FDA) for the treatment of cognitive deficits in AD.

There are three AChE inhibitors that is currently approved by the FDA which are Donepezil, Rivastigmine and Galantamine (Figure 2.6). These AChE inhibitors are indicated for the individuals with mild to moderate stage of AD. Memantine is another drug approved by FDA and it works by increasing the levels of glutamate, another transmitter involves in learning and memory. This drug is prescribed to moderate to severe AD patients and it's proven that it provides added benefit for individuals already taking Donepezil (De Fina et al.,2013). The first AChE inhibitors that made it to proceed to large scale commercial trials and commercial launch in USA and parts of Europe is Tacrine. It is an aminotacridine and their most prominent action is as a centrally active reversible AChE inhibitor and tacrine is rapidly absorbed and cleared by the liver (McGleenon et al., 1999). However, the use of tacrine as AChE inhibitors has been abandoned due to its high incidence of side effects and hepatotoxicity (Colovic et al., 2013).

Donepezil is especially designed piperidine derivatives with reversible AChE inhibitors activity. It's cholinesterase inhibition's specificity is higher compared to tacrine and its central nervous system (CNS) selectivity is highlighted by the lack of activity in peripheral tissue like cardiac tissue or gut smooth muscle (Mc Gleenon et al., 1999). Rivastigmine is a slow-reversible carbamate inhibitor that inhibits cholinesterase activity through binding at the esteratic part of the active site. It is a powerful inhibitor and it can inhibit both pseudocholinesterase (BuChE) and AChE unlike donepezil that inhibits just AChE (Colovic et al., 2013).

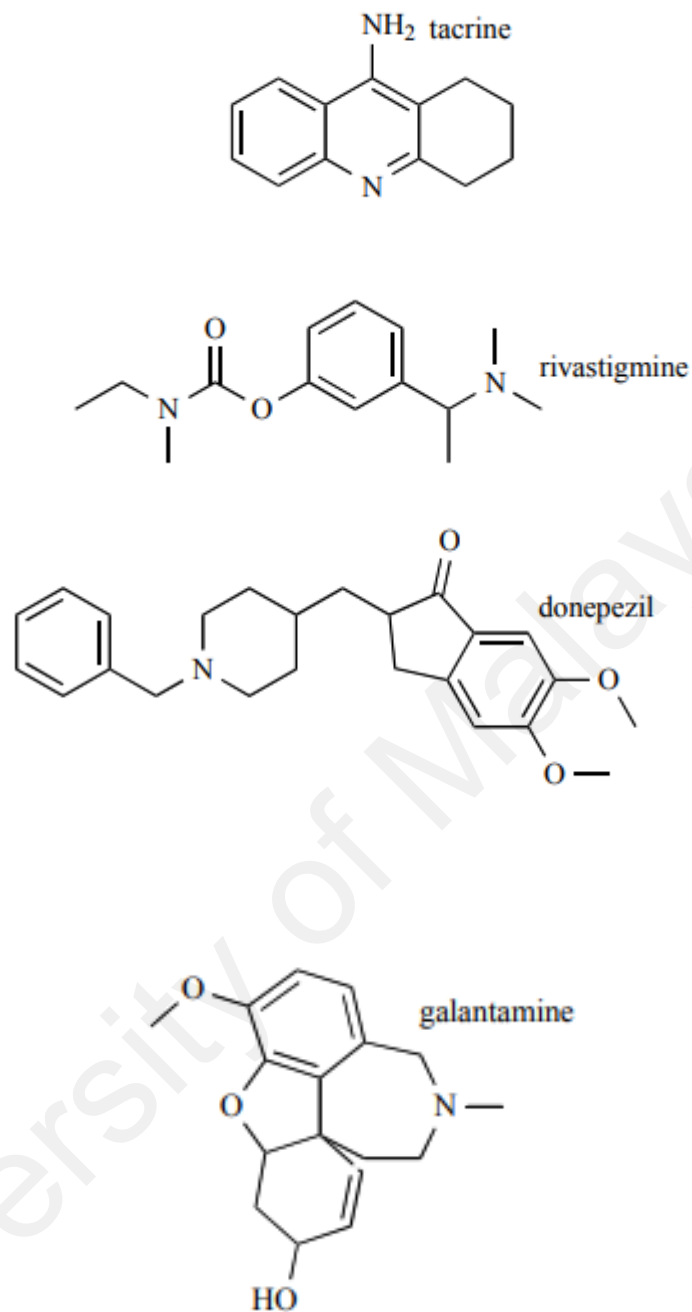


Figure 2.6: Selected reversible AChE inhibitors in pharmacotherapy of AD.

Galantamine was isolated from plant *Galanthus woronowii* and it is applied for mild to moderate AD. It is competitive, selective and highly reversible AChE inhibitors and can react with the anionic subsite as well as with the aromatic gorge. Galantamine is also an allosteric ligand at nicotinic cholinergic receptors promoting their modulation and interacts with the nicotinic receptor at binding sites separate from those for ACh and nicotinic agonists. Its specific action is to enhance the activity of nicotinic receptors in the presence of ACh. Severity of cognitive impairment in AD correlates with loss of nicotinic receptors therefore this effect is important for AD treatment (Colovic et al., 2013).

Table 2.1: Characteristic of three main AChE Inhibitors (Colovic et al., 2013; Mc Gleenon et al., 1999; Mannens et al., 2002).

Drug	Type	Half-life (plasma)	Dose	Elimination
Tacrine	Aminoacridine	1.4-3.6 h	Four times daily	Hepatic Hydroxylation. Was abandoned due to negative side effects and hepatotoxicity.
Donepezil	Piperidine	70 h	Once Daily	Dual excretion-cytochrome P450
Rivastigmine	Carbamate	1 h	Twice Daily	Renal
Galantamine	Alkaloid	7	Twice Daily	Urine

2.3 Oxidative Stress in Alzheimer Disease

Oxidative stress happens when the production of reactive oxygen species (ROS) or free radicals and antioxidative defence systems that are responsible for the removal of the ROS are imbalance. These two systems have major role and function in the process of age-related neurodegeneration and cognitive decline (Feng & Wang, 2012). The capacity for neurons to compensate for redox imbalance is decreasing with increased age, therefore minor cellular stresses can potentially lead to irreversible injury and able to initiate the pathogenesis of neurodegenerative disease (Aliev et al., 2008).

Mitochondrial electron transport chain (ETC) is the main source of ROS where the energy is generated from ATP. During the process of ETC, the electrons are transferred from NADH to FADH₂ through four membrane bound complexes (complex I to IV), to oxygen that at the end produces water. However, naturally there will be some leakage from the inner membrane and it will react with oxygen to form superoxide anions (O₂⁻). Other ROS can be generated due to the reaction of the superoxide anions such as hydrogen peroxide (H₂O₂), hydroxyl radicals (OH[·]) and hydroxyl ions (OH⁻) (Persson et al., 2014). Unfortunately, when superoxide anions and hydrogen peroxide is produced more than it needs, it can result in tissue damage in the presence of catalytic ions and copper ions. One of the major antioxidant defences, since metal is responsible to catalyse redox reaction, includes storing and transporting the irons in forms that do not catalyse the formation of reactive radicals, like during tissue injury where the iron availability is increased to accelerate free radical reaction (Huang et al., 2016).

Free radicals are very reactive compounds usually associated with an odd or unpaired electron and formed when oxygen interacts with certain molecules. To achieve a stable configuration, it needs to pair with odd electrons therefore they had to be neutral, short lived and highly reactive. Once a stable configuration formed, they can start a chain

reaction and they are capable to attack the healthy cells of the body, causing the cells to lose its structure and function (Singh et al, 2012).

The effect of oxygen radicals is a total damage to the structure and function of the brain cells and neurons. Damage includes advance glycation end products, lipid peroxidation adduction products, nitration, also carbonyl-modified neurofilament protein and free carbonyls. These damages, prominently includes all neurons vulnerable to death in AD, not particularly just those containing neurofibrillary tangles. The biology and chemistry of each modification was truly a representation of the spatio-temporal distribution of specific types of damage (Perry et al., 2002).

Based on the following collection of evidence it is proven that the presence of extensive oxidative stress is a characteristic of AD brains other than the established pathology of senile plaques and NFT. As the matter of fact, oxidative damage was discovered to be the first observable event in the AD disease progression among all AD hallmark (Persson et al., 2014) The levels of protein carbonyls and 3-nitrosine, which are a product of protein oxidation was shown to be increased in AD brains (Zhao & Zhao, 2013) moreover, the amount of 8-hydroxyguanosine (8OHG) and 8-hydroxydeoxyguanosine (8OHdG), a nucleic acid modification that predominantly derived from hydroxide (-OH) attack of guanidine, is elevated in cytoplasmic RNA in vulnerable neuronal population (Perry et al., 2002). Another study of brains in different disease stage demonstrates that already in the early stage of the disease, the level of 4-hydroxyhexenal (HHE), a marker of lipid peroxidation has increase. The same thing was also observed for other markers like F₂-isoprostane and F₄-neuroprostane, when comparing levels in frontal, parietal, and occipal lobes between controls, individuals with mild cognitive impairment and late AD patients. When comparison is made in frontal poles from Parkinson's Disease patients and schizophrenia patients and controls,

the level of F₂-isoprostane has shown no difference while the level were increased in AD patients (Persson et al., 2014).

2.3.1 Contributing Factors of Oxidative Stress in Alzheimer Disease

According to Persson et al (2014), amyloid beta (A β) also known as abeta is a product of sequential proteolytic cleavages of amyloid -beta precursor protein (APP) by two membrane bound proteases, beta (β)-secretase that is also known as beta-site APP cleaving enzyme 1 (BACE1), and gama (γ)-secretase, a multiprotein complex composed of presenilin (PS), nicastrin (NCT), anterior pharynx-defective 1 (APH-1) and presenilin enhancer protein 2 (PEN-2). The amino terminus of A β is generated by protease enzyme β -secretase while the γ secretase cleavage at the carboxy-terminus determines its length. There are two types of abeta which are A β ₄₀, the common species and A β ₄₂, the more fibrillogenic and neurotoxic species possibly because it's self-aggregate into oligomers much faster. (Gotz et al., 2008). The clumping of A β fragments will form a senile plaque (SP) that is prominently found in the Alzheimer patient's brain. Many researches have been done that associates oxidative stress in abeta-induced toxicity. *In vitro* experiments using cell models showed that the level of hydrogen peroxide and lipid peroxidase elevates due to abeta treatment. Constantly, in various AD transgenic mouse models carrying mutants of APP and PS-1, increase in hydrogen peroxide and nitric oxide production also raised oxidative modification of proteins and lipids were parallel with the age-associated Abeta accumulation, thus verifies that abeta promotes oxidative stress (Zhao & Zhao, 2013).

Neurofibrillary lesions that are found in cell bodies and in apical dendrites are known as neurofibrillary tangles, it is also known as neutrophil threads in distal dendrites and it is associated with some β -amyloid plaques (SP) in the abnormal neurites. The neurofibrillary tangles can be found greatly in the absence of overt plaques, in FTD and

other so called tauopathies. (Gotz et al., 2008). Tau protein that has been abnormally phosphorylated can impair its binding with tubulin and its capacity to promote microtubule assembly, therefore the tau protein will self-aggregate into filaments. Glycogen synthase kinase-3 beta (GSK-3 beta), cyclin-dependent kinase 5, mitogen-activated protein kinase (MAPK), calcium-calmodulin kinase and protein kinase C are the products of abnormal phosphorylation of tau. It has been proposed that the accumulation of abeta may appear before the tau pathology and the aggregation of abeta may be the sequence of molecular events that leads to hyperphosphorylation. On another note, overexpression of tau reportedly inhibits kinesin-dependent transport of peroxisome, neurofilaments and golgi derived vesicles into neurites, thus leading to transport defect in primary neuronal cells including the trafficking of APP. This will cause the transport of APP into axons and dendrites was blocked resulting its accumulation in the body (Zhao & Zhao, 2013).

Based on the evidence above, it is proven that oxidative stress is well connected with tau pathology. It was also shown that the cells overexpressing tau protein had increased susceptibility against oxidative stress, most probably due to depletion of peroxisome.

2.3.2 Antioxidant and Antioxidant Treatments in Alzheimer disease

An antioxidant can simply be explained as “any substance that, when present in low concentrations compared to that of a substrate that can be oxidised, significantly delays or inhibits the oxidation of that substrate” (Young & Woodside, 2001). There are two types of antioxidants system which are endogenous and exogenous systems for example catalase and vitamin antioxidants, respectively (Boora et al., 2013).

Endogenous antioxidant includes enzymatic antioxidant systems and cellular molecules and it helps protect against free radical-induced cellular damage. There are three primary enzymes involve in elimination of active oxygen species which are superoxide

dismutase (SOD), catalase, and glutathione peroxidase (GPx). Secondary enzymes like glutathione reductase (GR), glucose-6-phosphate dehydrogenase, and cytosolic glutathione S-transferase (GST) is needed to decrease peroxide levels or to frequently supplies metabolic intermediates like glutathione (GSH) and NADPH to ensure the primary antioxidant enzymes works at optimum level (Aliev et al., 2008). GSH is the most prevalent antioxidant in the brain and it is found in millimolar concentration in most cells. GSH consists of the amino acid glutamate, glycine and cysteine. Glutamate and cysteine are found in millimolar concentration and free cysteine is also limited with non-protein cysteine being stored within GSH. γ -glutamylcysteine ligase (also known as γ -glutamylcysteine synthetase) and glutathione synthase are two enzymes involved in the synthesis of GSH (Lu, 2013).

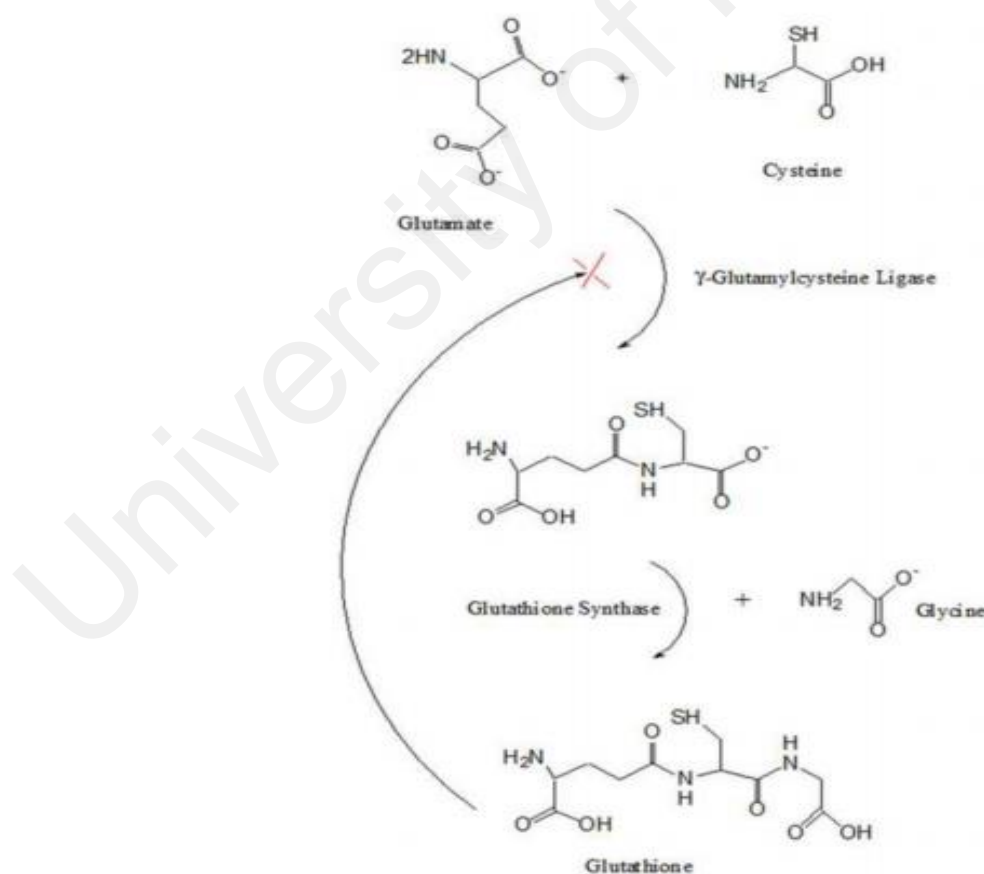


Figure 2.7: Synthesis of Glutathione (Pocernich & Butterfield, 2012). Reprinted permission granted by Pocernich & Butterfield

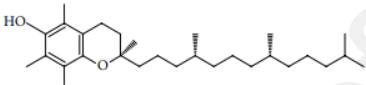
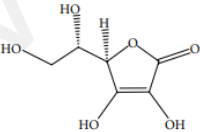
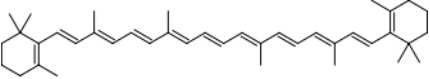
In AD, GSH levels are decreased. In AD peripheral lymphocytes, GSH levels are decreased and oxidised glutathione (GSSG) are increased which are consistent with increased oxidative stress. The ratio of GSSG to GSH is used as a marker of oxidative stress and redox thiol status. As AD progress, the level of GSSG and GSSG/GSH levels are found to be elevated. Discovery by Lloret and colleagues demonstrates the linear correlation between increased GSSG levels and decreased cognitive status of AD using the Mini Mental Status examination (MMSE) (Pocernich & Butterfield, 2012).

Exogenous antioxidants are naturally obtained from the diet. The most widely studied dietary antioxidants are vitamin E (α -tocopherol), vitamin C, and β -carotene. Table 2.2 demonstrates the summary of chemical structure and potential function of these three antioxidants. These antioxidants decrease free-radical-mediated damage caused by toxic chain reaction in neuronal cells and contributes to stop dementia pathogenesis in mammalian cells. α -tocopherol plays a major role as a lipid-phase antioxidant and this type of antioxidant is powerful, lipid-soluble chain breaking antioxidant and can be found in lipid membranes, circulating lipoproteins and low-density lipoprotein (LDL) particles (Feng & Wang, 2012). Based on a study by Grundman. (2017) vitamin E can inhibit hydrogen peroxide production induced by β -amyloid that can caused cytotoxicity. Vitamin E also reduces β -amyloid induced cell death in rat hippocampal cell cultures and PC12 cells. It also deflates excitatory amino acid-induced toxicity in neuroblastoma cells.

Vitamin C plays a major role as water soluble antioxidant in extracellular fluids as it has the capability to neutralize ROS in the aqueous phase before lipid peroxidation begins (Aliev et al., 2008). Carotenoid on the other hand is a fat soluble and it has a natural dark green or red and yellow colour in fruits and vegetable. Carotenoid contains poly-saturated hydrocarbons consisting of 40 atoms and it has many double bonds. They can quench single oxygen in physical way and it also has three ways of reacting with

oxygen free radicals such as electron transfer, hydrogen atom transfer, and radical coupling. β -carotene, γ -carotene and lycopene are three most common carotenoids in plants and astaxanthin in animals. According to research on nutrition and health and corresponding analysis of 6,658 elderly people with the age over 50 years old presented a significantly negative correlation between lycopene and lutein in serum and the risk of AD which means high intake of foods rich in lycopene and lutein can reduce the risk of AD (Li et al., 2014).

Table 2.2: Chemical structure and potential function of three main antioxidant (Feng & Wang, 2012)

Antioxidant	Chemical structures	Potential Function
Vitamin E (α -tocopherol)		A powerful, lipid-soluble chain breaking antioxidant and can be found in lipid membranes, circulating lipoproteins and low-density lipoprotein (LDL) particles and shown to decrease free radical mediated damage caused by toxic reactions in neuronal cells and helps inhibit dementia pathogenesis in mammalian cells.
Vitamin C		A water-soluble antioxidant, and an inhibitor of lipid peroxidation, acts as a major defence against free radicals in whole blood and plasma.
β -carotene		A lipid-soluble antioxidant which may reduce lipid peroxidation, improve antioxidant status, and quench singlet oxygen rapidly.

2.4 Studied Plant - *Pueraria Mirifica*

2.4.1 Family Fabaceae and Genus *Pueraria*

Fabaceae is also known as Leguminosae is an economically important and one of the largest families of flowering plant. It is commonly known as the legume family, pea family, bean family or pulse family. It has about 440 genera and 12 000 species all widely distributed in most part of the world. However, it has the greatest diversity in tropical and subtropical regions (Hawshabi et al., 2013). Fabaceae family grows in all sorts of soil and climate and the members of the family range from forest giants to tiny ephemerals. They extend in all terrestrial climates including dry to cold deserts and it is mostly centered in varied topography area influenced by seasonal climates. 'Fabaceae' name comes from the *Faba* defunct genus and now included into *Vicia*. The old name for Fabaceae family is Leguminosae is still considered valid and it refers to the typical fruits of these plants known as legumes (Sharma & Kumar, 2013). High secondary metabolites are produced by legumes and serve as defense compound against herbivores and microbes and serves as a signaling compound to attract fruit-dispersing and pollinating animals. Legumes are also a nitrogen-fixing organisms, and it produce more nitrogen containing secondary metabolites compared to other families. Compounds with nitrogen includes alkaloids and amines (Wink., 2013).

Pueraria is a plant native to Asia and it has 15-20 species. Kudzu is known as *P. montana* (Lour.) Merr. and *P. phaseoloides* (Roxb.) Benth also known as Tropical Kudzu are two species that have been widely introduced outside their native range. De Candolle was responsible of establishing the genus *Pueraria* in his memoir sur la des famille des Legumineuses. *Pueraria* commemorate Mark Nicolas Puerari, who was a Professor at the University of Copenhagen, a swiss national and a personal friend of De Condolle. The species of the *Pueraria* genus are widely distributed over China, Japan, South and South-east Asia, and also parts of Oceania. The distribution of several

species of *Pueraria* genus are narrow, however Kudzu is widely spread over China, Japan and has been brought a long time ago to the highlands of New Guinea and New Caledonia. (Keung., 2002). The morphology of Kudzu and it's relative is they are strong climbers, rarely shrubs and abundant in growth. They usually widely spread over the road and most of the species have short-hairy stem. Some of them also wind on support such as shrubs or trees. Several species have tuberous roots including *P. tuberosum*, *P. edulis* and *P.mirifica*. Chinese and Japanese literature recorded uses of *Pueraria* in food, medicine, paper, clothing and also construction materials (Lindgren et al., 2012).



Figure 2.8: Kudzu flower

2.4.1 *Pueraria Mirifica*

Pueraria mirifica also known as Thai Kudzu or White Kwao Krua is a tropical perennial climbing herbal plant. *Pueraria mirifica* grow and distributed in dry and deciduous forest as well as in sandy soiled mountain forest at roughly 80-800 meters above the sea level. Fascinatingly, these different Thai cultivars exhibit morphometric diversity within and between them. For example, cultivars from Kanchanaburi possess darker blue flowers than those from Chiang Mai (Suwanvijitr et al., 2010). Table 2.3 shows the taxonomy of *Pueraria mirifica* and Figure 2.9 depicts the *Pueraria mirifica* tree.

Division -	Plantae
Class -	Magnoliopsida
Subclass -	Rosidae
Order -	Fabales
Family -	Fabaceae (alt: Leguminosae)
Genus -	<i>Pueraria</i>
Species -	<i>P.mirifica</i>

Table 2.3: Taxonomy of *Pueraria mirifica*



Figure 2.9: *Pueraria mirifica* tree

Pueraria mirifica which belongs to Fabaceae family is a leguminous medical plant that grows widely in Thailand and possess rejuvenating qualities in aged women and men for nearly one hundred years. *Pueraria mirifica* is said to contain active phytoestrogens therefore a test using high performance liquid chromatography have shown that at least 17 phytoestrogens, mainly isoflavones were isolated (Malaivijitnond, 2012). *Pueraria mirifica* belongs in the same family as soy and contains estrogen like compounds consists of genistein and daidzein and modern scientific studies has discovered the chemical components found in *Pueraria mirifica* which is phytoestrogen. This phytoestrogen, such as Miroestrol and Deoxymiroestrol is more likely to be powerful than those found in soy (Stansbury et al., 2012).

The estrogenic activity of miroestrol was previously estimated to be about 2.5×10^{-1} times that of 17β - estradiol in a rat vaginal cornification model and miroestrol was considered to be the compound with the highest estrogenic potency among the known phytoestrogens (Manonai et al., 2007).



(A)



(B)

Figure 2.10: *Pueraria mirifica* (A) Bulb and (B) Leaves

CHAPTER 3

METHODOLOGY

3.1 Plant Sample

Pueraria mirifica roots was purchased in 2013 from Xianjiang area in China. The distributor has confirmed the authenticity of the plant sample. The roots obtained were in a powder form and kept in airtight container prior to further study.

3.2 General Chemicals and Reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium nitroferricyanide(III)dehydrate, 2,4,6-tripyridyl-s-tirazine (TPTZ), gallic acid monohydrate, hydrogen peroxide, sodium hydroxide, ferrozine, aluminium chloride, sodium nitrite, Griess reagent, curcumin, sodium phosphate, tris base and quercetin dihydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid, acetic acid glacial, hydrochloric acid, sodium chloride, dimethyl sulfoxide (DMSO) ferrous sulfate (FeSO_4), nitro blue tetrazolium, nicotinamide adenine dinucleotide, phenazine methosulphate, ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ethylenediaminetetraacetic acid disodium dehydrate ($\text{EDTANa}_2 \cdot 2\text{H}_2\text{O}$), DTNB (5,5'-Dithio-Bis -2-Nitrobenzoic Acid), ATCI, berberine, sodium phosphate buffer, AChE enzyme. Folin-Ciocalteu phenol reagent, and sodium carbonate were purchased from Merck Chemical Co. (Malaysia). Silica gel 60 F₂₅₄ – pre-coated TLC plates (Merck, Germany) were purchased from Merck Chemical Co. (Malaysia). All chemicals used were of analytical grade and were used without further purification.

3.3 Preparation of Plant Extracts

The plant sample of *Pueraria mirifica* was air dried for two weeks and was grinded to fine powder using domestic blender. Next 50 g of *Pueraria mirifica* sample was soaked with 300 ml of 10 % methanol solution for 72 hours at room temperature. Then the solvent was filtered with Whatman filter paper. The solvent collected was subjected to liquid to liquid partition using 250 ml of four different chemicals. The first chemical to be used was n-hexane, followed by chloroform, ethyl acetate, n- butanol and the product is aqueous solution. The samples were evaporated using rotary evaporator. After being evaporated, the crude extracts were air dried and the dry extracts were kept in sample bottle at 2-8 °C for further use.

3.4 Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out to detect the chemical compounds present in the extracts of *Pueraria mirifica*. TLC was performed on silica gel, 60 F254 aluminium backed plates (size 8 cm x 2 cm). 1cm was measured from the base of the TLC plate and labelled with pencil to mark the origin. The process begins by placing sample solutions placed as bands with capillary tube by dipping it in the solution. The mobile phase solution used is chloroform and 10 % methanol in chloroform solution. Plates with the sample solutions were then placed inside the developing chamber and separation was developed. The apparatus was placed on level surface for the solvent to rise. After being removed, the position of solvent line was labeled and plates were left to dry. The plates then subjected to UV light at 254 nm.

The distance travelled, and the final spots were measured and labelled in centimetre with a ruler. Next, the retention factors (R_f) of the samples were calculated. Retention factor is the ratio of the distance travelled by the centre of a spot to the distance travelled by the solvent front. Next, the dried plates were sprayed with Dragendroff's

and Vanillin-sulphuric acid. Dragendroff's and vanillin-sulphuric acid reagents were prepared according to Wagner et al., (2009)

a) Dragendroff's Reagent

Dragendroff's reagent is used to detect alkaloid. Two solutions, A and B were prepared. Solution A was prepared by dissolving 0.85 g bismuth nitrate in 10 ml glacial acetic acid and 40 ml of distilled water. Solution B was prepared by dissolving 8 g potassium iodide in 30 ml distilled water. Stock solution was prepared by mixing 30ml solution A and 30 ml solution B to give 60 ml stock solution. Spray reagent was prepared by mixing 50 ml of stock solution with 100 ml glacial acetic acid and 500 ml distilled water.

b) Vanillin Sulphuric Acid Reagent

Vanillin sulphuric acid reagent is used to detect terpenoids and phenolic compounds. Vanillin sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 1g of vanillin, and then this solution was mixed with 100 ml of ethanol and stirred. The TLC plates were heated at 110 °C for 5-10 minutes until the band appeared.

3.5 Liquid Chromatography Mass Spectrometry (LCMS)

LCMS test was done for methanol aqueous extract sample to determine the chemical compound present in the extract. AB sciex 3200 QTrap LCMS/MS was used to screen the extract and was fully scanned with MS/MS data collection with negative ionization mode. Phenomenex Aqua C18 (50 mm x 2.0 mm x 5 µM) was used as a column in rapid screening at 15 minutes run time. Water with 0.1 % formic acid (HCO_2H) and 5mM ammonium formate (NH_4HCO_2) was used as buffer A and a mixture of acetonitrile (CH_3CN) with 0.1 % HCO_2H and 5 mM ammonium formate as buffer B.

Sample were run with gradient mode; 10 % A to 90% B from 0.01 min to 8.0 min and were held for 3 min and back to 10 % A in 0.1 min and re-equilibrated for 4 min. Pre-run equilibration time was 0.1 min. 1 mL of concentrated sample of methanol aqueous was diluted five times with methanol and filtered with 0.2 µM nylon filter prior to being analyzed. Injection volumes for both were 20 µL.

3.6 Determination of Total Phenolic Compound

For total phenolic content, Folin-Ciocalteu method was used. In a 96-well microplate, 20 µL sample extract was mixed with 100 µL Folin Ciocalteu reagent (10-fold dilution with distilled water). The mixture was incubated for 5 minutes which later 75 µL sodium carbonate solution (7.5 %) was added. It was incubated again for 2 hours in darkness at room temperature. The absorbance was measured at 740 nm with a microplate reader (Sunrise, Switzerland). Total phenolic contents were estimated from Gallic acid (2.0- 1.0 mM) standard curve. Water was used as blank. The results were expressed as mg gallic acid equivalent (mg GAE)/g of dry extract.

Preparation of Folin-Ciocalteu (FC) Reagent

Folin-ciocalteu was prepared by dissolving 20 ml of the folin-ciocalteu reagent with 200 ml of distilled water.

Preparation of 7.5% Sodium Carbonate (Na₂CO₃)

Sodium carbonate was prepared by dissolving 7.5 g of Na₂CO₃ stock in 100 mL distilled water

Preparation of Gallic acid 1mg/mL Standard Solution

Gallic acid was prepared by dissolving 0.01 g of gallic acid in 10 mL distilled water and used as positive control.

3.7 Determination of Total Flavonoid Compound

Total Flavonoid compound was determined following the method described by Ablat et al, (2014). For total flavonoid content, 50 μ L sample extract was mixed with 70 μ L distilled water and 15 μ L sodium nitrite solution in 96-well microplate. The mixture was incubated at room temperature for 5 minutes. After that, 15 μ L of 10% aluminium chloride solution was added and incubated again for 6 minutes. Next, 100 μ L of 1 M sodium hydroxide solution was added. The absorbance was measured at 510 nm with microplate reader (Sunrise, Switzerland). Quercetin (0.2- 1.0 mg/mL) was used for standard calibration curve. Results were expressed as mg quercetin equivalent (mg QE)/g of dry extract

Preparation of 5 % Sodium Nitrite (NaNO_2) Solution

5 % of NaNO_2 was prepared by dissolving 5 g NaNO_2 in 100 mL distilled water.

Preparation of 10 % Aluminium Chloride (AlCl_3)

10 % AlCl_3 was prepared by dissolving 10 g AlCl_3 in 100 mL distilled water.

Preparation of 1M Sodium Hydroxide (NaOH) Solution

1M NaOH solution was prepared by dissolving 1.599 g NaOH in 60 mL distilled water.

3.8 Determination of Antioxidant Activity

3.8.1 DPPH

The free radical scavenging activity was determined using DPPH assay that measures hydrogen donating ability of the extracts. 40 μL sample extract of different concentration (0.1- 2.0 mg/mL) was added into 96-well microplate with 200 μL of 50 μM ethanolic DPPH solution. The mixture was then shaken immediately and incubated for 15 minutes in darkness at room temperature. The absorbance at 517 nm was measured with microplate reader (Tecan Sunrise, Austria) along with ascorbic acid (0.1- 2.0 mg/mL) as standard. Ethanol was used as a control. The formula of DPPH free radical scavenging activity was calculated as follows:

DPPH free radical scavenging activity (%) :

$$\frac{A \text{ control} - A \text{ sample/standard}}{A \text{ control}} \times 100$$

The concentration of extracts needed to scavenge 50 % of DPPH radical was estimated from graph plotted against the percentage inhibition and compared to standard. All tests were done in triplicate and results expressed as $\mu\text{g/mL}$ (Ablat et al., 2014).

Preparation of 50 μM DPPH solution in methanol.

0.00197 g DPPH was dissolved in 100 mL methanol. The solution was stored in a flask wrapped with aluminium foil because DPPH is light sensitive. It was stored in refrigerator at 4°C where it is most stable for several days.

3.8.2 FRAP

Ferric reducing antioxidant power (FRAP) assay determines the ferric reducing ability of the plant extracts in acidic medium that the pH is 3.6. The medium created deep blue colour when the ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex undergoes reduction into ferrous (Fe^{2+}) form. This assay was carried out according to method described in Ablat, (2014). FRAP reagent that consists of 5mL 10mM TPTZ in 40mM HCl, 5mL 20mM FeCl_3 , and 50mL 0.3M acetate buffer (pH 3.6) was prepared fresh before the analysis. In 96-well microplate, 20 μL extracts were mixed with 200 μL FRAP reagent. The mixture was incubated for 8 minutes and absorbance was measured at 595nm with microplate reader (Texan Sunrise, Austria). Ethanol was used as blank. Ferrous sulphate (FeSO_4) solution (0.2 -1.0 mM) was applied as standard for construction of calibration curve. The results expressed as mmol Fe^{2+} /g of dry extract from triplicated tests (Ablat et al., 2014).

Preparation of Acetate Buffer 0.3M

16 mL of glacial acetic acid was added to 3.1 g sodium acetate trihydrate. Then, solution was made up to 1 L using distilled water. The pH of solution was adjusted using pH meter.

Preparation of 2,4,6-Tripyridyl-s-Triazine (TPTZ) Solution

0.031 g of TPTZ was added to 10 mL of 40 mM GCl and dissolved at 50 °C in water bath. TPTZ solution was freshly prepared each time the assay was performed.

Preparation of Ferric Chloride Solution (FeCl_3)

0.054 g of FeCl_3 was dissolved in 10 mL of distilled water. FeCl_3 solution was freshly prepared each time the assay was performed.

3.8.3 Metal Chelating

The ferrous ions chelation was studied by measuring the formation of Fe²⁺-ferrozine complex in the assay. The assay was performed to measure chelating ability of ferrous ion to chelate ion Fe²⁺ with ferrozine to form ferrous ferrozine complex that can be detected at 562 nm. 100 µL sample extracts (0.1-2.0 mg/mL) were mixed with 120 µL distilled water and 10 µL 2 mM FeCl₂ in 96-well microplate. 20 µL 5 mM ferrozine was added for reaction initiation. After incubation of 20 minutes, absorbance at 562 nm was measured. EDTA-Na₂ (5-160 mg/mL) was used as standard. 100 µL of ethanol was used as control and blank was prepared with plant extract without ferrozine. The percentage of Fe²⁺-ferrozine complex inhibition was calculated using the following formula:

Ferrous ion chelating activity (%):

$$\frac{A \text{ control} - A \text{ sample/standard}}{A \text{ control}} \times 100$$

The concentration of extracts needed to chelate 50 % of Fe²⁺ ion (IC 50) was estimated from graph plotted against the percentage inhibition and compared to standard. All tests were done in triplicate and results expressed as mg/mL (Ablat et al., 2014).

Preparation of 5 mM of Ferrozine (FZ)

0.0246 g of FZ was dissolved in 10 mL deionized water. The stock solution was kept in centrifuge tube and wrapped with aluminium foil.

Preparation of 2 mM Ferum Chloride (FeCl₂)

A stock of 0.0025 g of FeCl₂ was dissolved in 10 ml deionized water. The stock solution was kept in centrifuge tube and wrapped with aluminium foil.

3.8.4 Nitric Oxide Radical Scavenging Activity (NORSA)

Nitric oxide radical scavenging activity (NORSA) of fractions was determined according to Hlila et al (2015) by measuring the formation of the nitrite ions in the reaction mixture that can be detected by Griess reagent. Fifty microliters of sample solutions at different concentrations (0.1–2.0 mg/mL) and an equal amount of sodium nitroferricyanide (10 mM) in phosphate-buffered saline (20 mM, pH 7.4) were mixed well in a 96-well microplate. The mixture was incubated at room temperature for 150 min and 125 μ L of Griess reagent was added. After 10 min, the absorbance was measured at 546 nm with a microplate reader (Tecan Sunrise, Austria). Curcumin (0.1–2.0 mg/mL) and ethanol were used as a standard and control. The reaction mixture without Griess reagent was served as blank. The percentage of inhibition of nitric oxide was calculated using the formula:

Nitric oxide radical scavenging activity (%) =

$$\frac{A \text{ control} - A \text{ sample/standard}}{A \text{ control}} \times 100$$

The concentration of extracts needed to scavenge 50 % of the nitric oxide (IC₅₀) was estimated from the graph against the percentage of inhibition. All the tests were performed in triplicate, and the results were expressed as mg/mL.

Preparation of 20 mM Phosphate Buffered Saline (pH 7.4)

0.1 M Phosphate buffer was prepared according to phosphate buffered saline system and they are consist of the following reagents, potassium phosphate (monobasic) (KH₂PO₄), potassium phosphate (dibasic) (K₂HPO₄), and sodium chloride (NaCl).

Solution A: 27.6 g of KH₂PO₄ (monobasic) was dissolved in 19 mL distilled water

Solution B: 28.4 g of K_2HPO_4 (dibasic) was dissolved in 19 mL distilled water

Solution C: 4.68 g of NaCl was dissolved in 80 mL distilled water.

Solution A, Solution B and solution C were mixed and diluted with 100 mL of distilled water, then, the pH of buffer was adjusted to 7.4. To prepare 20 mM phosphate buffer, 10 mL 0.1 M phosphate buffer were diluted with 40 mL distilled water. The pH of the buffer was adjusted to 7.4

Preparation of 10 mM of Sodium Nitroferricyanide ($Na_2[Fe(CN)_5NO].2H_2O$)

A stock of 0.149 g sodium nitroferricyanide was dissolved in 50 mL phosphate buffer.

Preparation of Griess Reagent.

0.2 % naphthylethylenediamine dihydrochloride and 2 % sulphanilamide in 5 % phosphoric acid were prepared separately in an amber colored bottle and it was labelled and stored in 4 °C. Both solutions was mixed before assay in equal volume.

3.8.4 Determination of Superoxide Radical Scavenging Activity.

Superoxide radicals are generated in phenazine methosulphate-nicotinamide adenine (PMS-NADH) systems by the oxidation of NADH and assayed by the reduction of NBT which can be measured at 570 nm. The reaction mixture consisted of 1ml of nitroblue tetrazolium (NBT) solution (1 M NBT in 100 mM phosphate buffer, pH7.4), 1 ml NADH solution (1 M NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of different fractions and ascorbic acid (50 mM phosphate buffer, pH7.4) was mixed. The reaction began by adding 100 μ l of (PMS) solution (60 μ M PMS in 100 mM phosphate buffer, pH7.4) in the mixture. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes

The ability to scavenge the superoxide radical were calculated by using the following formula

$$\% \text{ scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

Preparation of 0.1M Tris-HCl Buffer.

121.11 g Tris base was dissolved in 800 mL distilled water to prepare the stock solution. The desired pH was adjusted using concentrated 1 M HCl. The volume was adjusted to 1 L with distilled water. 0.1 M was prepared by dilution of 5 mL of 1 M Tris-HCl buffer with 54 mL of distilled water. The desired pH (pH8) was adjusted with concentrated 1M HCl

Preparation of 0.2 mM Nitro Blue Tetrazolium (NBT) solution

0.0016 g NBT from stock was dissolved in 10 mL distilled water.

Preparation of 0.5 mM Nicotinamide adenine dinucleotide (NADH) solution

0.0038 g NADH from stock was dissolved in 10 mL Tris-HCl buffer

Preparation of 25 µM phenazine methosulphate (PMS) solution

0.0007 g PMS from stock was dissolved in 10 mL Tris-HCl buffer.

3.9 AChE Inhibitory Activity Assay

Preparation of Extract

First, thin layer chromatography was conducted as per the method described in section 3.3.1. After the visualisation using UV and visible light is done and the band appeared on each TLC plates are labelled, the silica powder on the TLC plate was scraped carefully to take out the compound present in the band. Next, the silica powder was

transferred into the pre-weight eppendorf tube and suspended with distilled water. Then, it was centrifuged at 5000 rpm for 5 minutes. The supernatant was taken out and transferred to another Eppendorf tube. The remaining pellet was dried for 2 days and weighed. The compound and extract concentration were calculated.

AChE Inhibition Assay

The samples obtained were used for acetylcholinesterase inhibition assay. Ellman's method, as described by Yang et al. (2011) was used to determine the AChE inhibitory activity of the plant extract. Briefly, 140 μ l of 0.1 M sodium phosphate buffer, pH 8, and 20 μ l of 1 mg/ml sample solution were mixed with enzyme (AChE). The amount of AChE used was 15 μ l, with an activity of 0.25 U/ml. The mixtures were pre-incubated at 4°C for 20 min. The reaction was started by adding 10 μ l 0.01 M DTNB and 10 μ l 0.075 M ATCI. The extract was incubated at room temperature for 20 min. The absorbance at 405 nm was measured with an ELISA reader.

The percentage of inhibitory activity was calculated using the following formula:

$$\% \text{ AChE inhibition} = (A_c - A_t / A_c) \times 100.$$

20 μ l of 1mg/mL Berberine was used as positive control. The experiments were performed in triplicate (Hirboid et al., 2014)

3.10 Statistical Analysis

Data collected are in triplicate and was presented as average \pm standard error (SEM). The differences between extracts were analysed using one way and two way analysis of variance (ANOVA) followed by Duncan post hoc multiple comparison test at the 5 % level ($p < 0.05$). The statistical program (SPSS 22.0 version, Chicago, IL, USA) was used in the entire test.

CHAPTER 4

RESULTS

4.1 Preparation of *Pueraria mirifica* Root Extracts

The n-hexane extract gives 0.006 % yield, chloroform extract gives 0.02 % yield, ethyl acetate gives 0.026 % yield, n-butanol gives 0.012 % yield and finally methanol aqueous gives 0.014 % yield.

Table 4.1: Yields of *Pueraria mirifica* extracts

Extract	Yield (%) (w/v)
n-hexane	0.006
Chloroform	0.02
Ethyl Acetate	0.026
n-butanol	0.012
Methanol aqueous	0.014

4.2 Detection of chemical compound

4.2.1 Thin layer chromatography

Alkaloids were detected using Dragendroff's reagent for both chloroform and 10% methanol in chloroform solvent (Table 4.2 and 4.3). In Table 4.2, using 100% chloroform solution as solvent, all extracts showed no visible colours under visible light and UV light. However, after spraying with Dragendroff's reagent, chloroform and butanol extracts showed visible orange colours that indicates the presence of alkaloid. In table 4.3, using 10 % methanol in chloroform solution as solvent, all extracts showed no visible colours under visible light and chloroform and ethyl acetate showed the

presence of grey colour under UV light. After spraying with Dragendorff's reagent, all five extracts showed visible orange colours that indicates the presence of alkaloid.

Meanwhile terpenoid were detected using Vanillin-sulphuric acid reagent both for chloroform and 10 % methanol in chloroform solvent (Table 4.4 and 4.5). In Table 4.4, using chloroform solution as solvent, all extracts showed no visible colours under visible light and UV light. However, after spraying with Vanillin reagent, n-hexane extracts showed visible orange and yellow colours that indicates the presence of terpenoid. Chloroform and n-butanol on the other hand, showed purple colour which also indicate the presence of terpenoid. In Table 4.5, 10 % methanol in chloroform solvents were used and all extracts showed no visible colours under visible light and UV light except for ethyl acetate that displayed grey colour under UV light. After spraying with vanillin, only n-hexane and chloroform extracts displayed yellow colour that indicates the presence of terpenoid.

Table 4.2: Thin Layer Chromatography of *Pueraria mirifica* extract with chloroform solution

Plant Extract	Label Compounds	Rf Value	Observation			Remarks
			Visible light	UV light	Spraying Reagent Dragendroffs	
n-hexane	A1	0.0	-ve	-ve	-ve	-
Chloroform	A2	0.1	-ve	-ve	Orange (++)	Alkaloid
Ethyl Acetate	A3	0.0	-ve	-ve	-ve	-
n-Butanol	A4	0.7	-ve	-ve	Orange (+)	Alkaloid
Methanol	A5	0.0	-ve	-ve	-ve	-
Aqueous						

Indication for intensity of colour

- +++ = Strong
- ++ = Moderate
- + = Weak
- ve = No colour Observed

Table 4.3: Thin Layer Chromatography of *Pueraria mirifica* extract with 10 % methanol in chloroform solution

Plant Extract	Label Compound	Rf Value	Observation			Remarks
			Visible Light	UV	Spraying Reagent Dragendorff	
n-hexane	A6	0.30	-ve	-ve	Orange (++)	Alkaloid
Chloroform	A7	0.20	-ve	Gray (++)	Orange (+++)	Alkaloid
Ethyl Acetate	A8	0.18	-ve	Gray (++)	Orange (++)	Alkaloid
n-Butanol	A9	0.16	-ve	-ve	Orange (+)	Alkaloid
Methanol	A10	0.27	-ve	-ve	Orange (++)	Alkaloid
Aqueous						

Indication for intensity of colour

+++ = Strong

++ = Moderate

+ = Weak

-ve = No colour Observed

Table 4.4: Thin Layer Chromatography of *Pueraria mirifica* with chloroform solution

Plant Extract	Label Compound	Rf Value	Observation			
			Visible Light	UV	Spraying Reagent Vanillin	Remarks
n-hexane	B1	0.43	-ve	-ve	Yellow (++)	Terpenoids
	B2	0.38			Orange (+)	
Chloroform	B3	0.38	-ve	-ve	Purple (+++)	Terpenoids
Ethyl Acetate	B4	0.00	-ve	-ve	-ve	-
n-Butanol	B5	0.83	-ve	-ve	Purple (++)	Terpenoids
Methanol	B6	0.00	-ve	-ve	-ve	-
Aqueous						

Indication for intensity of colour

+++ = Strong

++ = Moderate

+ = Weak

-ve = No colour Observed

Table 4.5: Thin Layer Chromatography of *Pueraria mirifica* with 10 % methanol in chloroform solution

Plant Extract	Label Compound	Rf Value	Observation			
			Visible Light	UV	Spraying Reagent Vanillin	Remarks
n-hexane	B7	0.84	-ve	-ve	Yellow (++)	Terpenoids
	B8	0.90			Yellow (+)	
Chloroform	B9	0.76	-ve	-ve	Yellow (+)	Terpenoids
	B10	0.83			Yellow (+)	
Ethyl Acetate	B11	0.33	-ve	Grey (++)	-ve	-
n-butanol	B12	0.00	-ve	-ve	-ve	-
Methanol aqueous	B13	0.16	-ve	-ve	-ve	-

Indication for intensity of colour

+++ = Strong

++ = Moderate

+ = Weak

-ve = no colour observed

4.2.2 Liquid Chromatography Mass Spectrometry (LCMS/MS)

The chemical compounds present in methanol aqueous extract of *Pueraria mirifica* was determined using Liquid Chromatography Mass Spectrometry combined with Mass Spectrometry (LCMS/MS). Methanol aqueous extracts was fully screened with AB Sciex 320oQTrap LCMS/MS followed scanning by MS/MS data collection.

Full LCMS/MS analysis presented 14 peaks separated and different retention time (RT); 0.791', 0.867', 0.918', 0.986', 1.088', 1.134', 1.206', 1.223', 1.282', 1.325', 1.46', 1.503', 1.994', 2.231', 3.624', and 9.049'. Eleven phytochemical compound detected were as followed; 26,27-Dinor-3alpha,6alpha,12alpha-trihydroxy-5beta-cholestan-24-one, 3alpha,7alpha,12alpha,24-tetrahydroxy-5beta-cholestan-26-al, C16 Sphinganine, 4-Fluoro-L-threonine, N6-Methyl-2'-deoxyadenosine, Ulexone B, Cys Lys His, p-Aminobenzoic acid, Eplerenone, 5-Aminopentanoic acid, L-Arginine, Oleolyl Ethyl Amide, Ethephon and lastly, Bufexamac.

The chemical structure, RT, mass and name of compounds detected in methanol aqueous extracts using LCMS/MS are shown in Table 4.6.

Table 4.6: Chemical structure, RT, Mass and name of compounds detected in methanol aqueous extracts with LCMS/MS

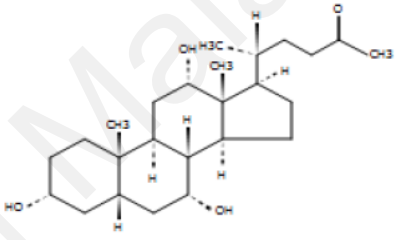
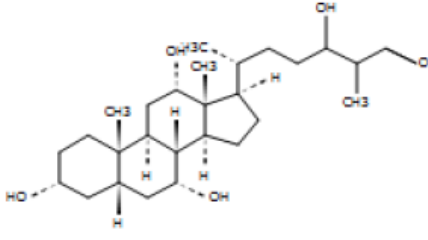
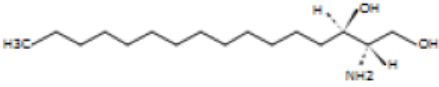
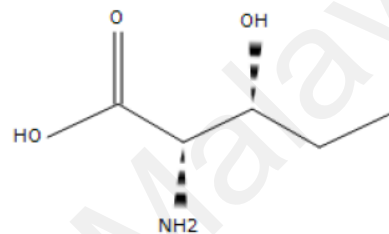
Compound formula	RT	Mass	Name	Structure	Group
$C_{25}H_{42}O_4$	0.791	406.30	26,27-Dinor-3 α ,6 α ,12 α -trihydroxy-5 β -cholestan-24-one		Terpenoid
$C_{27}H_{46}O_5$	0.867	450.33	3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-al		Terpenoid
$C_{16}H_{35}NO_2$	0.986	273.26	C16 Sphinganine		Sphingosine

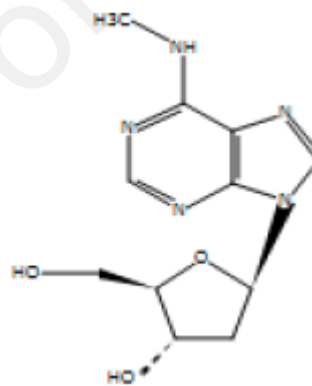
Table 4.6, Continued.

$C_4H_8FNO_3$ 1.088 137.04 4-Fluoro-L-threonine



Amino acid

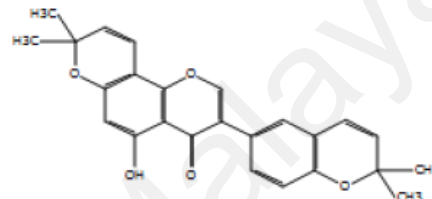
$C_{11}H_{15}N_5O_3$ 1.134 265.11 N6-Methyl-2'-
deoxyadenosine



Purine derivatives

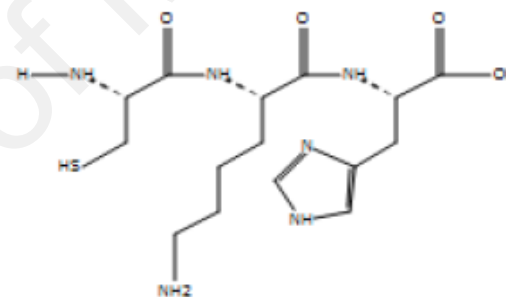
Table 4.6, Continued.

$C_{25}H_{22}O_5$ 1.206 402.14 ulexone B



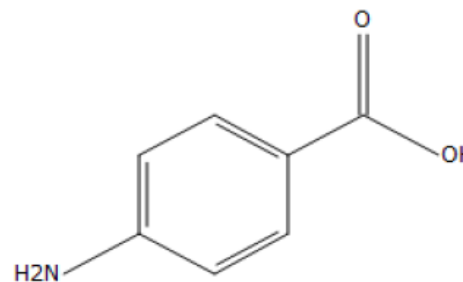
Flavonoid

$C_{15}H_{26}N_6O_4S$ 1.223 386.17 Cys Lys His



Amino acid

$C_7H_7NO_2$ 1.282 137.04 p-aminobenzoic acid



Phenol

Table 4.6, Continued.

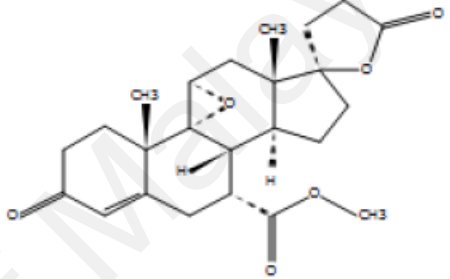
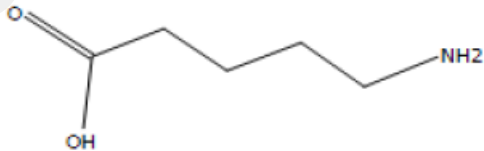
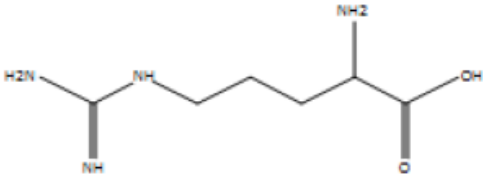
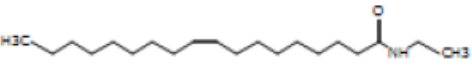
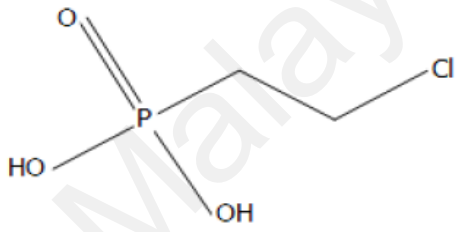
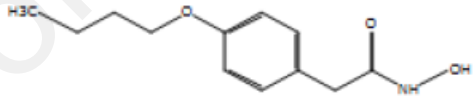
$C_{24}H_{30}O_6$	1.325	414.20	eplerenone		Phenol
$C_5H_{11}NO_2$	1.503	117.08	5-aminopentanoic acid		Amino acid
$C_6H_{14}N_4O_2$	1.994	174.11	L-arginine		Amino acid
$C_{20}H_{39}NO$	2.231	309.30	Oleoyl Ethyl Amide		Amino acid

Table 4.6, Continued.

$C_2H_6ClO_3P$	3.624	143.97	ethephon		Not classified
$C_{12}H_{17}NO_3$	9.049	223.12	bufexamac		Not classified

4.3 Determination of Total Phenolic and Total Flavonoid Contents

The total phenolic contents of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts were determined by Folin-Ciocalteu method using standard curve of gallic acid. The standard curve is constructed by the absorbance values obtained at different concentration of gallic acid. This standard curve was used as positive reference. The total phenolic content was calculated using the standard equation ($y = 3.6772x$, $r^2 = 0.8503$) obtained from Gallic acid standard curve. It is expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). At (1.0 mg/ml) concentration, methanol aqueous extracts showed the highest concentration of phenolic compound (10.35 ± 0.007 mg GAE/g) followed by chloroform (1.84 ± 0.006 mg GAE/g), ethyl acetate (1.69 ± 0.003 mg GAE/g), N-butanol (1.66 ± 0.004 mg GAE/g) and n-hexane aqueous (1.26 ± 0.001 mg GAE/g).

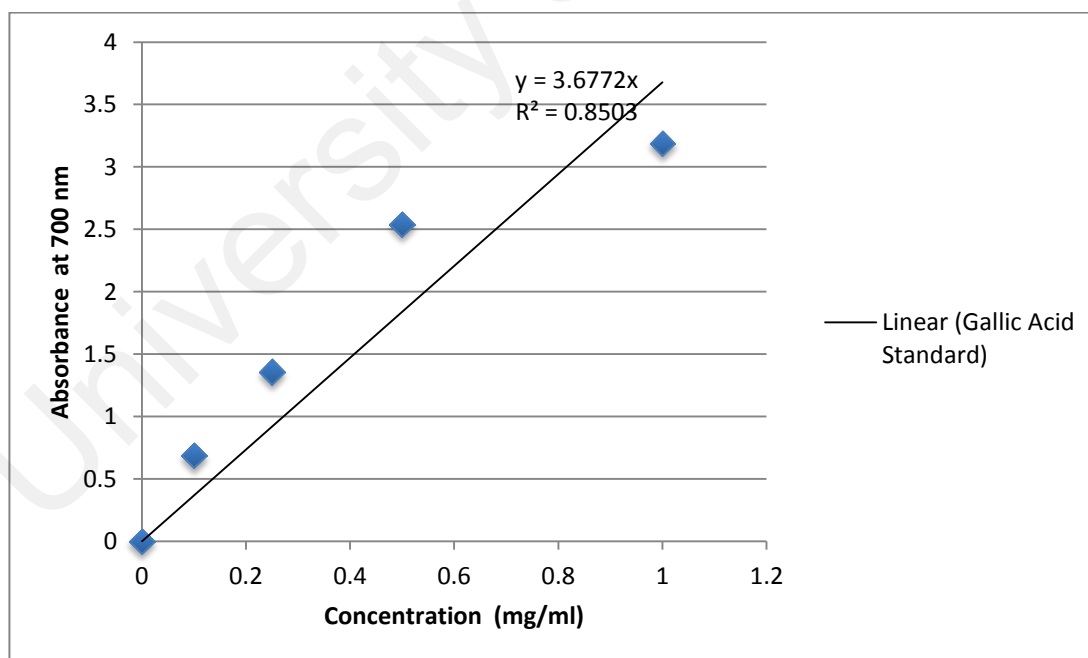


Figure 4.1: Standard curve of gallic acid

The total flavonoid contents of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts were determined by aluminium chloride method using standard curve of Quercetin. The standard curve is constructed by the absorbance values obtained at different concentration of quercetin. This standard curve was used as positive reference. The total flavonoid content was calculated using the standard equation ($y=0.5634x$, $r^2=0.837$) obtained from Quercetin standard curve. At (1.0 mg/ml) concentration, chloroform extracts showed the highest concentration of flavonoids compound (1.182 ± 0.003 mg QE/g), followed by n-hexane (0.98 ± 0.005 mg QE/g), ethyl acetate (0.98 ± 0.002 mg QE/g), n-butanol (0.70 ± 0.001 mg QE/g) and methanol aqueous (0.40 ± 0.05 mg QE/g).

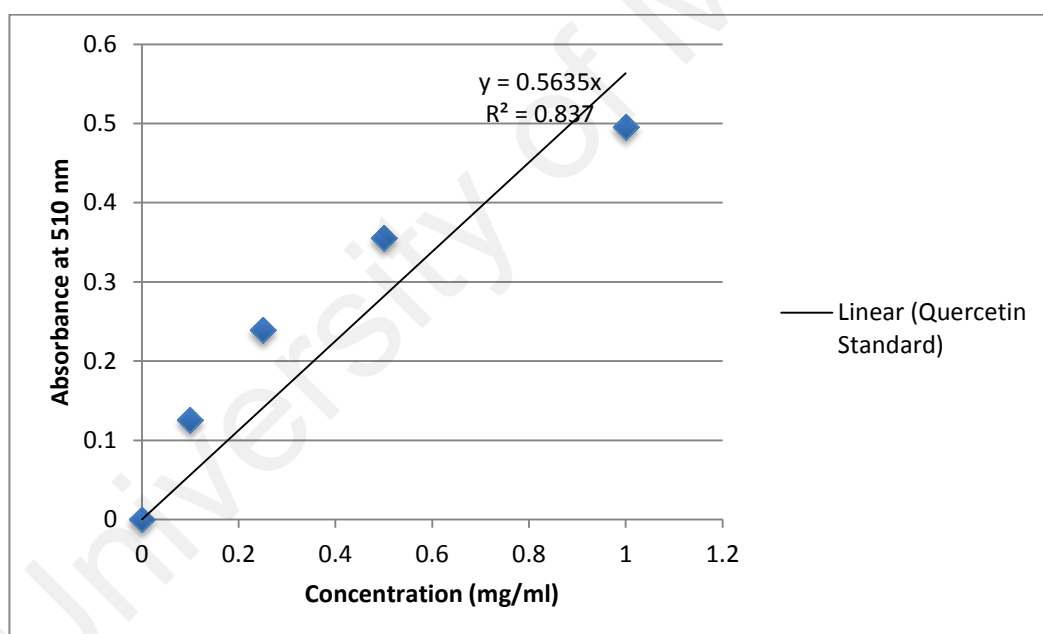


Figure 4.2: Standard curve of quercetin

Table 4.7: TPC and TFC values of *Pueraria mirifica* root extracts. Each value is represented as mean \pm SE (n = 3). The means with different lower case letters (a,b,c and d) in the same column are significantly different at P < 0.05 (ANOVA, followed by Duncan's multiple comparison test)

Extracts of <i>P. mirifica</i>	Total Phenolic Content (mg	Total Flavonoid Content
	GAE)/g dry extract	(mg QE)/g dry extract
n-hexane	1.26 \pm 0.001 _a	0.98 \pm 0.005 _a
Chloroform	1.84 \pm 0.006 _b	1.182 \pm 0.003 _a
Ethyl Acetate	1.69 \pm 0.003 _b	0.98 \pm 0.002 _a
n-butanol	1.66 \pm 0.004 _c	0.70 \pm 0.001 _b
Methanol aqueous	10.35 \pm 0.007 _d	0.40 \pm 0.05 _b

4.4 Antioxidant Activity Assays

4.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay.

The antioxidant activity of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts were determined by scavenging activity against the DPPH radicals. The radicals were identified by the typical deep purple colour and the maximum absorbance range is between 515-520 nm. In this test, ascorbic acid was used as the standard due to its powerful antioxidant ability. The scavenging activity of ascorbic acid against DPPH was presented in Table 4.8. The standard curve of ascorbic acid standard is shown in Figure 4.3 to compare the percentage of inhibition of ascorbic acid with all the extracts.

Table 4.8: Percentage inhibition of DPPH radical by standard reference, ascorbic acid. Percentage of inhibition are expressed as mean \pm SE, (n=3)

Concentration of ascorbic acid (mg/mL)	Percentage of DPPH inhibition (%)
0.1	97.009 \pm 0.000
0.5	96.856 \pm 0.001
1.0	96.741 \pm 0.000
1.5	96.664 \pm 0.002
2.0	96.281 \pm 0.003

The radical scavenging activity of ascorbic acid is higher than all of *Pueraria mirifica* root extracts however the percentage of inhibition of ascorbic acid decreases slightly with increasing concentration. The percentage of inhibition of ascorbic acid ranged between 97.009 % at 0.1 mg/mL to 96.281 % at 2.0 mg/mL and at concentration 0.5, 1.0, and 1.5 mg/mL the percentage of inhibition of ascorbic acid is 96.856 %, 96.741 % and 96.664 % respectively. The ascorbic acid standard have an $IC_{50} = 1.03$ mg/mL.

The results of DPPH assay for n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root DPPH scavenging assay are shown in Table 4.9 and the concentrations used were 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL. Statistical analysis was done to determine the significant difference amongst all extracts at different concentration to percentage of inhibition of DPPH and mean different are statistically significant different at $P < 0.05$. Two way ANOVA was done followed by Duncan's post hoc test. Tests of between subject effects shows that interaction between independent variables which is extracts and concentration give statistically significant effect on dependant variable which percentage of inhibition where $p = 0.007$. However,

when independent variable is analysed directly, one variable which is concentration have no statistically significant different effect on percentage of inhibition at $P = 0.077$. Another variable that is extracts gives statistically significant different effect on percentage of inhibition where $P = 0.015$.

Table 4.9: DPPH activities of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root in metal chelating assay. All percentage of inhibition (%) are expressed as mean \pm SE, (n=3). The means with different lower case (a and b) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Concentration of Extracts ($\mu\text{g/mL}$)	Percentage Inhibition of DPPH (%)				
	n-hexane	Chloroform	Ethyl acetate	n-butanol	Methanol Aqueous
0.1	89.264 \pm 0.002 _a	93.021 \pm 0.035 _a	87.922 \pm 0.004 _b	89.187 \pm 0.001 _{a,b}	88.804 \pm 0.001 _b
0.5	89.801 \pm 0.002 _a	87.960 \pm 0.004 _a	87.730 \pm 0.002 _b	87.692 \pm 0.000 _{a,b}	88.382 \pm 0.002 _b
1.0	89.187 \pm 0.001 _a	87.193 \pm 0.000 _a	89.302 \pm 0.012 _b	89.187 \pm 0.001 _{a,b}	88.229 \pm 0.001 _b
1.5	89.532 \pm 0.005 _a	87.883 \pm 0.003 _a	83.627 \pm 0.044 _b	87.577 \pm 0.000 _{a,b}	87.960 \pm 0.000 _b
2.0	89.225 \pm 0.004 _a	87.768 \pm 0.005 _a	88.305 \pm 0.020 _b	87.270 \pm 0.005 _{a,b}	92.983 \pm 0.023 _b

Based on Table 4.9, percentage of inhibition of n-hexane, chloroform and n-butanol extracts are not significantly different with each other, and percentage of inhibition of n-butanol, ethyl acetate and methanol aqueous extracts are also not significantly different with each other but percentage of inhibition between n-hexane and chloroform extracts with ethyl acetate and methanol aqueous extracts are significantly different amongst each other.

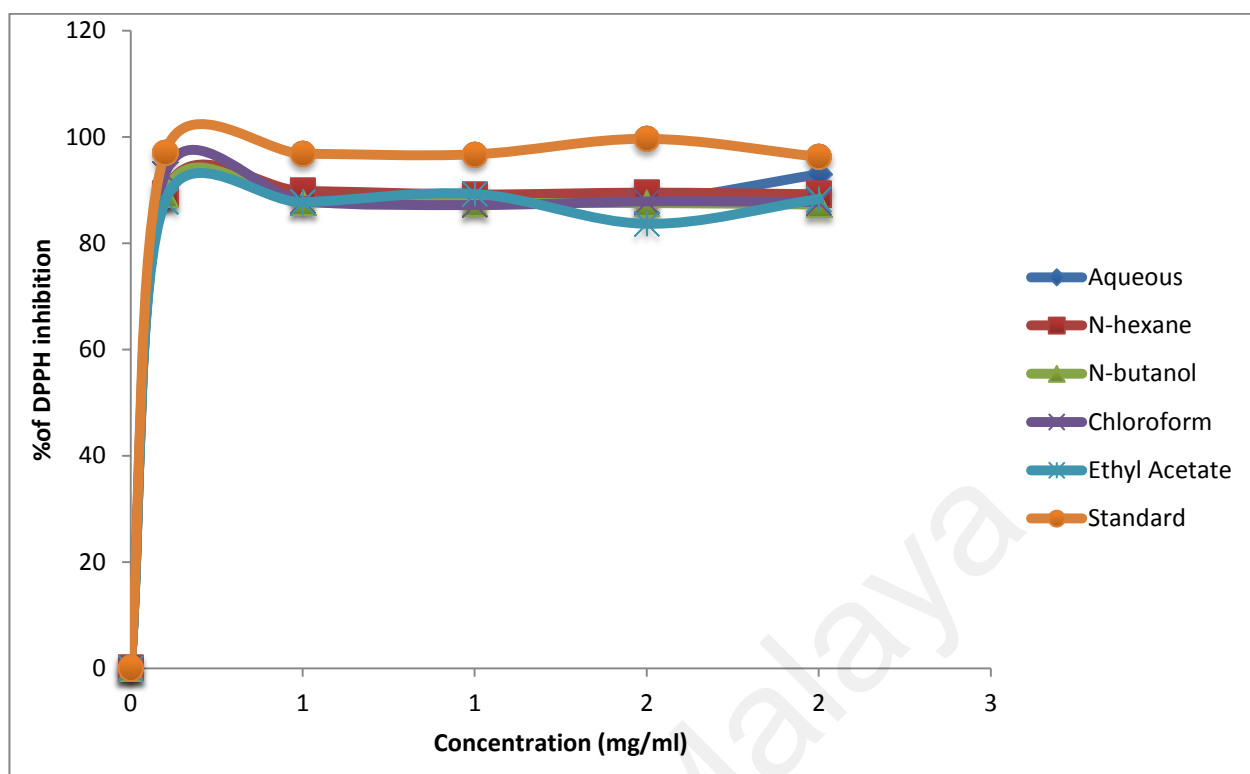


Figure 4.3: DPPH inhibition percentage of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous

Figure 4.3 showed the DPPH scavenging activities of five different extracts namely n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous. In comparison with the positive reference standard ascorbic acid, the percentage inhibitions of all extracts are lower than the ascorbic acid standard. However, all extracts showed high percentage inhibition and achieved IC_{50} . The scavenging activity of N-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts showed in the Figure 4.3. Ascorbic acid was used as the standard. The results showed that the n-butanol extracts $IC_{50} = 1.145$ mg/ml, ethyl acetate $IC_{50} = 0.896$ mg/ml, chloroform $IC_{50} = 0.573$ mg/ml, methanol aqueous $IC_{50} = 0.556$ mg/ml and n-hexane $IC_{50} = 0.5602$ mg/ml.

4.4.2 Metal Chelating Assay.

Metal chelating assay was done to evaluate the ability of *Pueraria mirifica* root extract to chelate ferrous ion and prevent the formation of ferrozine-Fe²⁺ complex. Ethylenediaminetetra acetic acid (EDTA) was selected as the standard in the metal chelating assay. Table 4.10 presented the percentage of inhibition of Ferrozine-Fe²⁺ complex formation by standard EDTA. The standard curve of EDTA is shown in Figure 4.4 to compare the percentage of inhibition of EDTA standard with all extracts.

Table 4.10: The percentage inhibition Ferrozine- complex formation by EDTA in metal chelating assay

Concentration of EDTA (mg/mL)	Percentage of inhibition Ferrozine – Ferum ²⁺ complex (%)
0.1	29.754 ± 0.08
0.5	52.283 ± 0.01
1.0	65.805 ± 0.04
1.5	79.328 ± 0.05
2.0	96.136 ± 0.00

The percentage of inhibition of Ferrozine-Fe²⁺ complex formation by EDTA is increasing as the concentrations increase. The highest chelating activity was observed at the highest concentration 2.0 mg/mL with 96.136 % chelating activity. At 0.1, 0.5, 1.0, and 1.5 mg/mL concentration the chelating activity was 29.754 %, 52.283 %, 65.805 % and 79.328 % respectively. The EDTA standard has an IC₅₀ = 1.02 mg/mL.

The result of metal chelating assay for n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root in metal chelating assay are shown in Table 4.11 and the concentrations used were 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL.

Statistical analysis was done to determine the significant difference amongst all extracts at different concentration to percentage of inhibition of metal chelating and mean different are statistically significant different at $P < 0.05$. Two way ANOVA was done followed by Duncan's post hoc test. Tests of between subject effects shows that interaction between independent variables which is extracts and concentration give statistically significant effect on dependant variable which percentage of inhibition where $p = 0.022$. However, independent variable, extracts have no statistically significant different effect on percentage of inhibition at $P = 0.215$ meanwhile concentration gives statistically significant different effect on percentage of inhibition where $P = 0.016$.

Table 4.11: Metal chelating activities of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root in metal chelating assay. All percentage of inhibition (%) are expressed as mean \pm SE, (n=3). The means with different lower case letters (a,b,c and d) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Concentration of extracts (mg/mL)	Percentage Inhibition of metal chelating (%)				
	n-hexane	Chloroform	Ethyl acetate	n-butanol	Methanol Aqueous
0.1	31.05 \pm 0.095 _a	44.90 \pm 0.049 _a	41.24 \pm 0.059 _a	36.65 \pm 0.070 _a	45.58 \pm 0.190 _a
0.5	33.91 \pm 0.036 _a	50.17 \pm 0.033 _a	43.45 \pm 0.004 _a	37.13 \pm 0.093 _a	34.44 \pm 0.066 _a
1.0	34.34 \pm 0.007 _a	41.77 \pm 0.022 _a	39.31 \pm 0.120 _a	46.43 \pm 0.082 _a	34.17 \pm 0.022 _a
1.5	39.46 \pm 0.031 _a	35.39 \pm 0.074 _a	17.66 \pm 0.336 _a	29.10 \pm 0.101 _a	34.57 \pm 0.029 _a
2.0	47.24 \pm 0.180 _a	38.56 \pm 0.070 _a	56.55 \pm 0.180 _a	36.77 \pm 0.020 _a	34.37 \pm 0.100 _a

Based on Table 4.11, percentage inhibition of metal chelating for all extracts, n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts exhibit no significant different amongst the extracts.

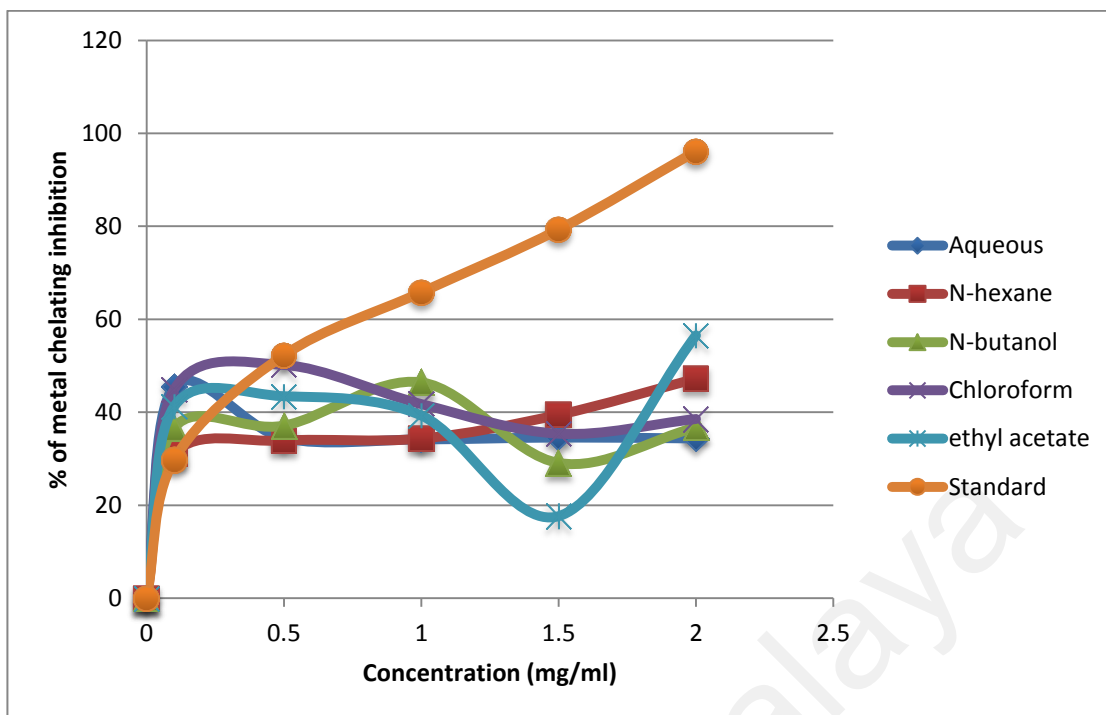


Figure 4.4: Metal chelating inhibition percentage of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous.

Figure 4.4 showed the metal chelating activities of five different extracts namely n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous. The concentration range used was 0.1 mg/ml – 2.0 mg/ml. Only chloroform and ethyl acetate extracts manage to reach IC_{50} inhibition where the $IC_{50} = 0.489$ and $IC_{50} = 1.768$ respectively. The other three extracts n-hexane, n-butanol and methanol aqueous showed low chelating activity where at the highest 2.0mg/ml concentration, the percentage of inhibition were 47.24 %, 36.77 % and 34.37 % respectively.

4.4.3 Nitric Oxide Radical Scavenging Assay (NORSA)

The aim of NORSA test is to determine the ability of *Pueraria mirifica* root extracts to scavenge the nitric oxide radical and compete with oxygen resulting to reduced production of nitrite ions. The scavenging ability of nitric oxide was determined by the decrease in the absorbance at 546 nm. The standard reference used in this test is curcumin and the percentage of inhibition of nitric oxide radical was presented in the table 4.12

Table 4.12: The percentage of inhibition of nitric oxide radical by curcumin in NORSA

Concentration of curcumin (mg/mL)	Percentage of inhibition Nitric Oxide radical (%)
0.1	18.366 ± 0.063
0.5	26.623 ± 0.000
1.0	33.422 ± 0.003
1.5	30.993 ± 0.010
2.0	32.274 ± 0.039

Based on Table 4.12, the percentage of inhibition of nitric oxide radical by curcumin is increasing as the concentrations increase from 0.1 mg/mL until 1.0 mg/mL with 18.36 %, 26.62 % and 33.42 %. At the concentration of 1.5 mg/mL and 2.0 mg/mL, the percentage of inhibition is plateauing with the percentage of inhibition 30.99 % and 32.27 % at 1.5 mg/mL and 2.0 mg/mL respectively. However, the highest concentration of 2.0 mg/mL is used to ensure that scavenging activity of nitric oxide is at maximum level.

Nitric Oxide Radical Scavenging Assay assay was done on n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous. The concentration used was from 0.1 mg/mL

– 2.0 mg/mL. The result of nitric oxide scavenging assay for n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root in nitric oxide scavenging assay are shown in Table 4.13 and the concentrations used were 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL. Statistical analysis was done to determine the significant difference amongst all extracts at different concentration to percentage of inhibition of nitric oxide and mean different are statistically significant different at $P < 0.05$. Two way ANOVA was done followed by Duncan's post hoc test. Tests of between subject effects shows that interaction between independent variables which is extracts and concentration give statistically significant effect on dependant variable which percentage of inhibition where $p = 0.008$. Independent variable is also analysed directly to see the effects on dependant variable, which is concentration have statistically significant different effect on percentage of inhibition at $P = 0.000$. Another variable that is extracts also gives statistically significant different effect on percentage of inhibition where $P = 0.000$.

Table 4.13: Nitric oxide scavenging activities of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root in nitric oxide scavenging assay. All percentage of inhibition (%) are expressed as mean \pm SE, (n=3). The means with different lower case letters (a and b) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Concentration of extracts (mg/mL)	Percentage inhibition of nitric oxide radical (%)				
	n-hexane	Chloroform	Ethyl Acetate	n-butanol	Methanol Aqueous
0.1	6.979 \pm 0.016 _b	10.949 \pm 0.016 _b	21.943 \pm 0.014 _a	26.225 \pm 0.024 _a	21.501 \pm 0.025 _a
	0.5	12.671 \pm 0.002 _b	16.336 \pm 0.007 _b	29.139 \pm 0.006 _a	28.521 \pm 0.003 _a
1.0		10.993 \pm 0.012 _b	11.744 \pm 0.018 _b	8.124 \pm 0.147 _a	28.168 \pm 0.009 _a
	1.5	1.987 \pm 0.007 _b	10.243 \pm 0.014 _b	26.490 \pm 0.012 _a	26.137 \pm 0.020 _a
2.0		-2.428 \pm 0.028 _b	-10.419 \pm 0.050 _b	21.545 \pm 0.031 _a	15.232 \pm 0.054 _a

As depicted in Table 4.13, percentage of inhibition in n-butanol, ethyl acetate and methanol aqueous extracts are not significantly different with each other and percentage of inhibition of n-hexane and chloroform extracts are also not significantly different with each other but percentage of inhibition between n-butanol, ethyl acetate and methanol aqueous extracts with percentage of inhibition of n-hexane and chloroform extracts are significantly different amongst each other.

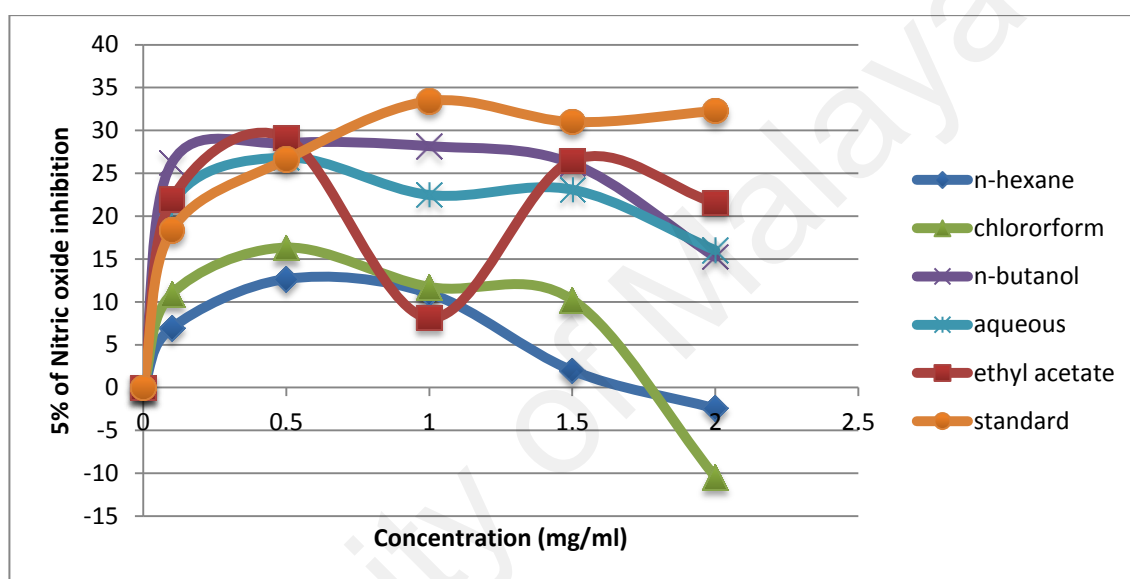


Figure 4.5: Nitric oxide radical scavenging inhibition percentage of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous.

Figure 4.5 illustrated the curve of nitric oxide radical scavenging activity of the extracts. However, the IC_{50} of the assay cannot be obtained because all the extracts displayed low scavenging activity. Ethyl acetate extract displayed the highest percentage of inhibition at 2.0 mg/ml concentration (21.54 %) followed by methanol aqueous (16.02 %), n-butanol (15.23 %), n-hexane (-2.42 %) and chloroform (-10.41). The negative results simply showed that there are no scavenging activities in n-hexane and chloroform extracts.

4.4.4 Superoxide radical scavenging Assay

Superoxide radical scavenging assay was done to determine the ability of *Pueraria mirifica* root extract to scavenge superoxide anion obtained from dissolved oxygen by PMS-NADH coupling reaction leads to reduced NBT in the system. The reaction process was measured at 570 nm absorbance.

Ascorbic acid was used as a standard reference in this test. Table 4.14 displayed the percentage of inhibition of superoxide radical by ascorbic acid standard in superoxide radical test using varying concentration of 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL.

Table 4.14: The percentage of superoxide inhibition by Ascorbic acid standard in Superoxide Radical Scavenging assay

Concentration of Ascorbic Acid (mg/mL)	Percentage of superoxide inhibition (%)
0.1	5.83 ± 0.001
0.5	7.50 ± 0.003
1.0	20.00 ± 0.001
1.5	19.17 ± 0.00
2.0	22.51 ± 0.002

Based on Table 4.14, the percentage of inhibition of superoxide radical by ascorbic acid is increasing as the concentrations increase from 0.1 mg/mL until 1.0 mg/mL with 5.83 %, 7.50 % and 20.00 %. At the concentration of 1.5 mg/mL and 2.0 mg/mL, the percentage of inhibition is plateauing with the percentage of inhibition 19.17 % and 22.51 % at 1.5 mg/mL and 2.0 mg/mL respectively. However, the highest concentration of 2.0 mg/mL is used to ensure that scavenging activity of superoxide is at maximum.

Superoxide radical scavenging assay was done on n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts. The concentration used was from 0.1 mg/mL

– 2.0 mg/mL. The result of superoxide scavenging assay for n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* root in nitric oxide scavenging assay are shown in Table 4.15 and the concentrations used were 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL. Statistical analysis was done to determine the significant difference amongst all extracts at different concentration to percentage of inhibition of superoxide and mean different are statistically significant different at $P < 0.05$. Two way ANOVA was done followed by Duncan's post hoc test. Tests of between subject effects shows that interaction between independent variables which is extracts and concentration is not statistically significant on dependant variable which is percentage of inhibition where $p = 0.477$. Independent variable is also analysed directly to see the effects on dependant variable, which is concentration have statistically significant different effect on percentage of inhibition at $P = 0.000$. Another variable that is extracts gives no statistically significant different effect on percentage of inhibition where $P = 0.228$.

Table 4.15: Superoxide scavenging activities of n-Hexane, chloroform, ethyl acetate, n-Butanol and methanol aqueous extracts of *Pueraria mirifica* root in Superoxide scavenging assay. All percentage of inhibition (%) are expressed as mean \pm SE, (n=3). The means with different lower case letters (a and b) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Concentration of extracts (mg/mL)	Percentage Inhibition of superoxide (%)				
	n-Hexane	Chloroform	Ethyl Acetate	n-Butanol	Methanol Aqueous
0.1	13.33 \pm 0.003 _a	9.17 \pm 0.004 _a	11.67 \pm 0.003 _a	14.17 \pm 0.004 _a	8.33 \pm 0.003 _a
0.5	16.67 \pm 0.003 _a	15.83 \pm 0.003 _a	12.50 \pm 0.003 _a	10.00 \pm 0.003 _a	9.17 \pm 0.004 _a
1.0	25.00 \pm 0.001 _a	19.17 \pm 0.000 _a	23.33 \pm 0.001 _a	19.17 \pm 0.000 _a	23.33 \pm 0.001 _a
1.5	28.33 \pm 0.001 _a	30.00 \pm 0.001 _a	26.67 \pm 0.001 _a	25.83 \pm 0.000 _a	26.67 \pm 0.001 _a
2.0	34.17 \pm 0.002 _a	23.33 \pm 0.001 _a	25.00 \pm 0.002 _a	24.17 \pm 0.002 _a	26.67 \pm 0.002 _a

Based on Table 4.15, percentage inhibition of metal chelating for all extracts, n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous exhibit no significant different amongst the extracts.

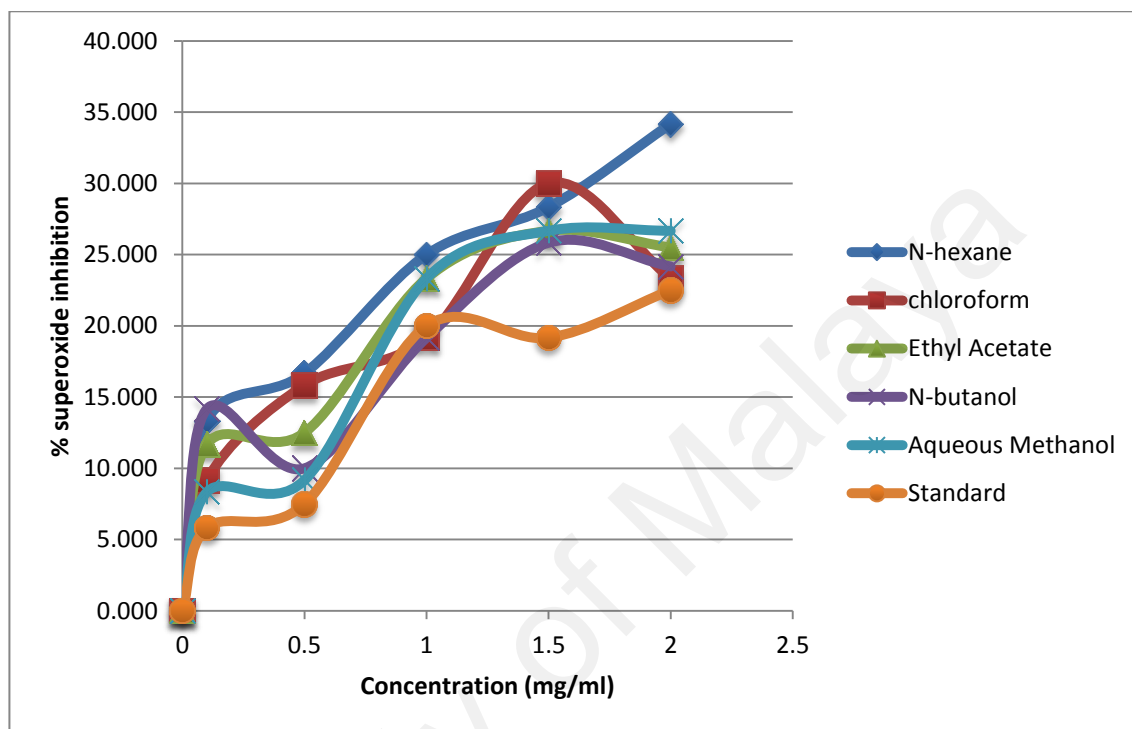


Figure 4.6: Superoxide radical scavenging inhibition percentage of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous.

Figure 4.6 illustrated the curve of superoxide radical scavenging activity of the extracts. Unfortunately, the IC_{50} for all extracts was unsuccessfully obtained. At 2.0 mg/mL concentration, N-hexane showed the highest percentage of superoxide inhibition at 43.167% followed by methanol aqueous at 26.667 %, ethyl acetate at 25.000%, butanol at 24.167 % and lastly chloroform at 23.333%. The result of superoxide radical scavenging assay for all extracts was presented in the appendix.

4.4.5 Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP assay was carried out to evaluate the ability of *Pueraria mirifica* root extracts to reduce Fe^{3+} to Fe^{2+} . The principle of this test is based on the reduction of a colourless ferric complex to a blue coloured ferrous complex, at a low pH. This reduction is monitored by measuring the absorbance value at 595 nm. The standard used for this test is ferrous sulphate (FeSO_4) standard and the results were estimated as $\text{mmol Fe}^{2+}/\text{g}$ of dry extract. The Figure 4.7 showed the standard calibration curve of Ferrous Sulfate (FeSO_4). The equation $y = 0.409x$ was used to measure the ferric reducing power. In this assay, the reducing capacity of the root extracts of *Pueraria mirifica* was calculated with reference to reaction signal given by a Fe^{2+} solution.

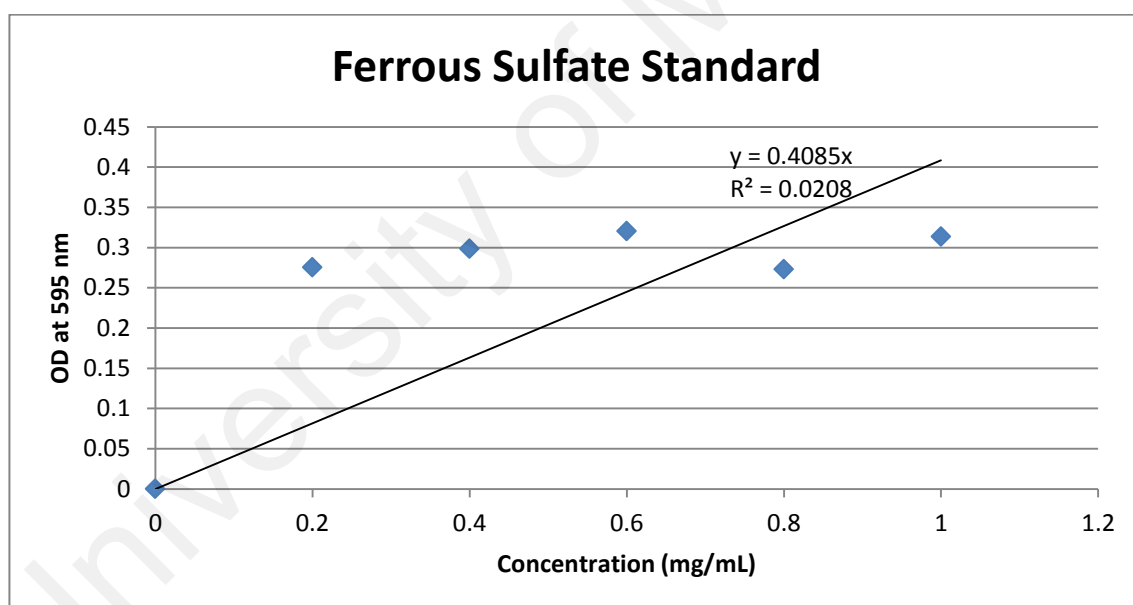


Figure 4.7: Standard linear curve of Ferrous Sulfate.

Figure 4.8 showed the ferric reducing power antioxidant assay. The methanol aqueous extracts showed the highest reducing power at $0.220 \text{ mmol Fe}^{+}/\text{g}$, followed by ethyl acetate extract at $0.215 \text{ mmol Fe}^{+}/\text{g}$, n-butanol extract at $0.211 \text{ mmol Fe}^{+}/\text{g}$, chloroform extract at $0.205 \text{ mmol Fe}^{+}/\text{g}$ and n-hexane extract at $0.200 \text{ mmol Fe}^{+}/\text{g}$.

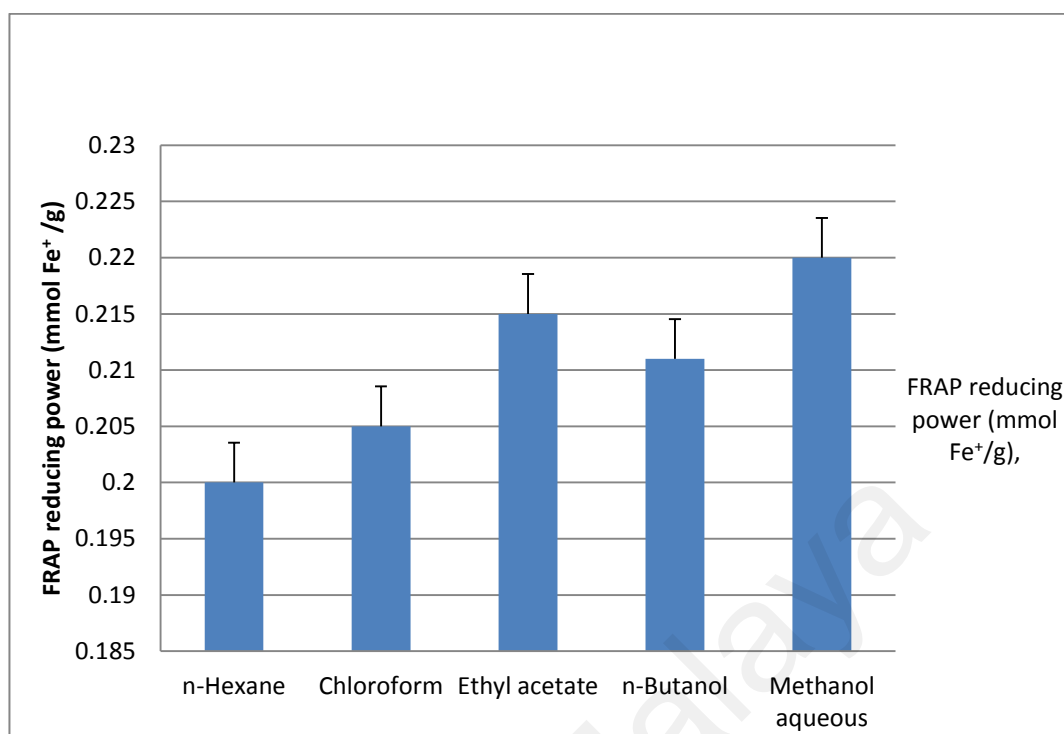


Figure 4.8: Ferric reducing antioxidant power assay

4.5 Acetylcholinesterase Inhibition (AChE) Assay

The basic mechanism of acetylcholinesterase inhibition (AChE) assay is the production of thiocholine by the action of acetylcholinesterase forming a yellow colour with 5,5'-dithiobis (2-nitrobenzoic acid). The intensity of the product colour was measured using spectrophotometer at 405 nm, which is proportionate to the enzyme activity in the sample.

Berberine was used as a standard because of its known inhibitors for acetylcholinesterase. It also has significantly low cytotoxicity. In this assay, the isolated compounds of TLC and the extracts of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous were assayed for the acetylcholinesterase inhibition. The result in Table 4.16 showed that the acetylcholinesterase inhibition for n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* at a concentration of 1 mg/mL. Table 4.17 showed the acetylcholinesterase inhibition of

the compound isolated from n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* with chloroform as solvent at concentration of 1 mg/mL. Table 4.18 showed the acetylcholinesterase inhibition of TLC compound isolated from n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* with 10% methanol in chloroform as solvent at concentration of 1 mg/mL.

Table 4.16: Acetylcholinesterase inhibition of n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* at concentration of 1 mg/mL. Each value is represented as mean \pm SE (n = 3). The means with different lower case letters (a and b) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Extracts	Percentage of Acetylcholinesterase Inhibition (%)
N-Hexane	15.137 \pm 0.029 _a
Chloroform	1.530 \pm 0.004 _a
Ethyl Acetate	-31.723 \pm 0.073 _b
N-Butanol	-36.957 \pm 0.026 _b
Methanol Aqueous	7.488 \pm 0.004 _a
Berberine	68.357 \pm 0.003

Table 4.16 showed the the acetylcholinesterase inhibition for n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* at concentration of 1 mg/mL. The highest percentage of AChE inhibition is in n-hexane extract at 15.137 % followed by methanol aqueous at 7.488 % and chloroform at 1.530 %. Ethyl acetate and n-butanol extract showed no inhibition activity.

Table 4.17: Acetylcholinesterase inhibition of TLC compounds isolated from n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* with chloroform as solvent at concentration of 1 mg/mL. Each value is represented as mean \pm SE (n = 3). The means with different lower case letters (a and b) in the same column are significantly different at P < 0.05 (ANOVA, followed by Duncan's multiple comparison test).

Isolated Label Compound	Percentage of Acetylcholinesterase inhibition (%)
PM-HE1	10.709 \pm 0.025 _a
PM-CH1	6.924 \pm 0.020 _a
PM-CH2	0.403 \pm 0.013 _a
PM-ET1	-0.161 \pm 0.016 _a
PM-NB1	13.607 \pm 0.027 _a
PM-NB2	5.636 \pm 0.007 _a
PM-AQ1	21.739 \pm 0.033 _a
Berberine	68.357 \pm 0.003

Table 4.17 showed the acetylcholinesterase inhibition of TLC compounds isolated from n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* with chloroform as solvent at concentration of 1 mg/mL. The highest percentage of AChE inhibition is in isolated compound PM-AQ1 at 21.739 % and the lowest is in isolate compound PM-CH2 at 0.403 %. PM-ET1 isolated from ethyl acetate extracts showed no inhibition activity.

Table 4.18: Acetylcholinesterase inhibition of TLC compounds isolated from n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* with 10 % methanol in chloroform solvent at concentration of 1mg/mL. Each value is represented as mean \pm SE (n = 3). The means with different lower case letters (a and b) in the same column are significantly different at P < 0.05 (ANOVA, followed by Duncan's multiple comparison test).

Isolated Label Compound	Percentage of Acetylcholinesterase Inhibition (%)
PM-HE2	15.700 \pm 0.021 _a
PM-CH3	1.530 \pm 0.046 _a
PM-ET2	1.771 \pm 0.012 _a
PM-ET3	4.670 \pm 0.025 _a
PM-ET4	1.530 \pm 0.010 _a
PM-NB3	4.992 \pm 0.006 _a
PM-NB4	8.937 \pm 0.016 _a
PM-AQ2	0.966 \pm 0.025
PM-AQ3	8.374 \pm 0.010
PM-AQ4	5.878 \pm 0.018
Berberine	68.357 \pm 0.003

Table 4.18 showed the acetylcholinesterase inhibition of TLC compounds isolated from n-hexane, chloroform, ethyl acetate, n-butanol and methanol aqueous extracts of *Pueraria mirifica* with 10 % methanol in chloroform solvent at concentration of 1 mg/mL. The highest percentage of AChE inhibition is in isolated compound PM-HE2 at 15.700 % and the lowest is in isolated compound PM-AQ2 at 0.966 %.

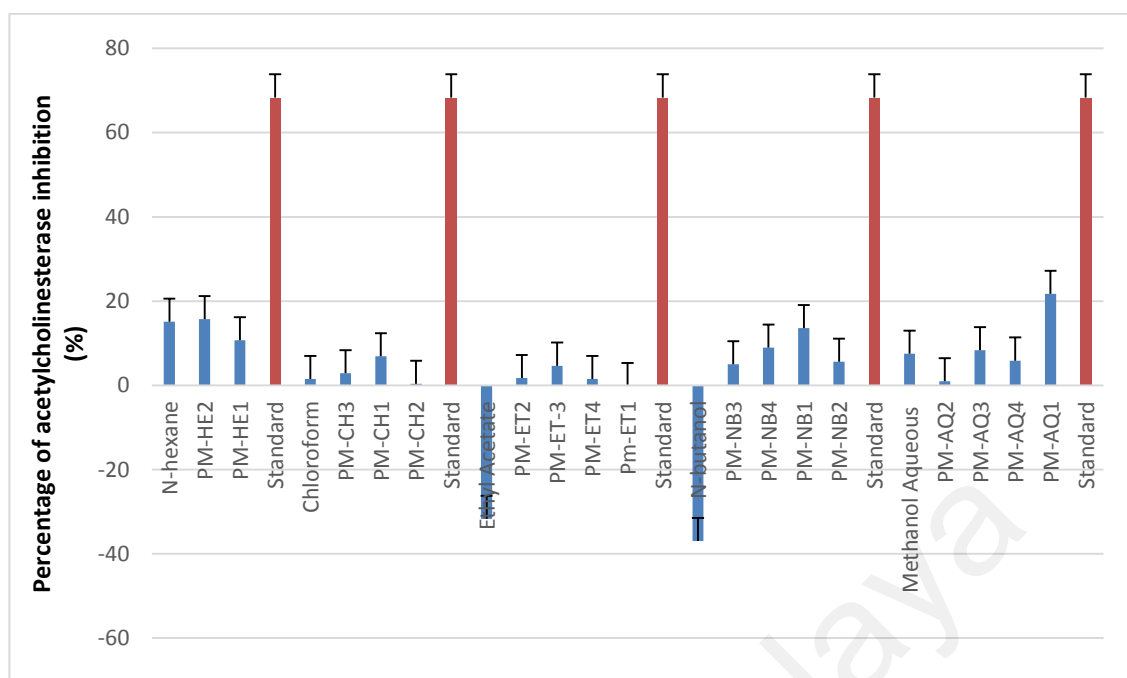


Figure 4.9: Percentage of acetylcholinesterase (AChE) inhibitory activity of different extracts at concentration of 1mg/mL from *Pueraria mirifica*. Each value is represented as mean \pm S.E. of three separate experiments in triplicate measurements.

Figure 4.9 showed the percentage acetylcholinesterase (AChE) inhibitory of all extracts and isolated compound at concentration of 1 mg/mL from *Pueraria mirifica*. In hexane extracts, the highest percentage obtained is PM-HE2 compound at 15.7 % inhibition, and in chloroform extracts the highest percentage inhibition PM-CH1 compound at 6.924 %. The PM-ET3 showed the highest percentage of inhibition for ethyl acetate extracts at 4.670 % while for butanol extract, the highest percentage is the PM-NB1 compound at 13.607% and methanol aqueous extract, the highest percentage is the PM-AQ1 compound at 21.739%. Ethyl acetate and N-butanol extracts showed no acetylcholinesterase inhibition.

CHAPTER 5

DISCUSSION

The dried *Pueraria mirifica* root was grounded to a fine powder with a domestic grinder, soaked with 10 % methanol and extracted using fractionation extraction method involving four different solvents which are n-hexane, followed by chloroform, ethyl acetate, n-butanol and the final solvent is methanol aqueous. The methanol was used as a solvent to soak the plant sample because it has a good ability of extracting primary and secondary metabolites from plant extracts. The fractionation extraction was carried out using chemical from lower polarity to higher polarity. The extracts were dried, and the yields obtained were presented in Table 4.1.

The thin layer chromatography (TLC) was carried out to further separates the chemical compounds and the results was presented in Table 4.2, Table 4.3, Table 4.4 and Table 4.5. The spraying reagents were used to determine different types of phytochemical compound present in the extracts with the formation of a specific colour. The TLC demonstrates the presence of alkaloids and terpenoids in the *Pueraria mirifica* extracts. The presence of alkaloids was detected using spraying reagents of Dragendroff's with the development of orange colour and the presence of terpenoids were detected using spraying reagent of Vanillin-H₂SO₄ reagent. According to Kumar et al. (2010), as the polarity of the solvent system increase, all the components of the mixture move faster, and the ideal solvent system is the one that gives the best separation. The two different solvent systems were used in the TLC which is 10 % methanol in chloroform and also chloroform solvent. In chloroform solvent, alkaloids were detected in chloroform and n-butanol extracts while alkaloids were detected in all extracts when using spraying reagent Dragendroff's in 10 % methanol in chloroform. This may due to increase polarity of the solvent when 10 % methanol is added to the

solvent system. In chloroform solvent, the presence of terpenoids were detected in N-hexane, chloroform and N-butanol extracts while in 10 % methanol in chloroform were detected in N-hexane and chloroform extracts.

Based on Bacciottini et al (2007), the most widely distributed and the largest category of phytochemical is phenolic phytochemicals or polyphenols which are in a group of phytoestrogen. *Pueraria mirifica* is a rich source of phytoestrogen including mirestrol, deoxymirestrol and isoflavonoids (Boonchird et al., 2010). Phytochemical compound present in *Pueraria mirifica* root extracts was detected by separation of the phytochemical compound using Liquid Chromatography Mass Spectrometry tandem with mass spectrometry (LCMS/MS). The methanol aqueous extract sample was analysed using LCMS/MS extracts showed the presence of 14 different compounds namely 26,27-Dinor-3 α ,6 α ,12 α -trihydroxy-5 β -cholestan-24-one, 3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-al, C16 Sphinganine, 4-Fluoro-L-threonine, N6-Methyl-2'-deoxyadenosine, Ulexone B, Cys Lys His, p-Aminobenzoic acid, Eplerenone, 5-Aminopentanoic acid, L-Arginine, Oleoyl Ethyl Amide, Ethepon and lastly, Bufexamac. These compounds are classified in the group of amino acid, terpenoid, phenol and flavonoid. The inability to detect compound in alkaloid group may be due to the low amount of compound present in the plant extract and the different approach to extraction method in this study.

According to Hossein et al. (2011), polar compounds of lipids, phenolic compound and flavonoids can be extracted using polar solvent such as chloroform and methanol. Plant phenolic and flavonoids plays a major role in biological activity which outline the necessity of their determination. The phenolic content was determined using Folin-Ciocalteu assay. The total flavonoids were measured spectrophotometrically by using the aluminium chloride colorimetric assay. Phenolic compounds are plant substances that possess common aromatic ring bearing one or more hydroxyl groups.

(Sulaiman & Balachandran, 2012). Since the total phenolic was qualitatively detected, the phenolic compounds were quantified in extracts of *Pueraria mirifica*. In total phenolic assay, methanol aqueous extracts showed the highest concentration of phenolic compound while in total flavonoid assay, chloroform showed the highest flavonoid compound. The high phenolic content could be due to the presence of p-Aminobenzoic acid and Eplerenone and high flavonoid content due to the presence of Ulexone-B compound in the methanol extracts of *Pueraria mirifica* extract. The result obtained suggests that extractability of polyphenols from plant is influenced by the type of solvent, their solubility in the extraction solvent, the degree of polymerization of phenols and the interaction of phenols with other plant constituents also the insoluble complexes formation (Medini et al., 2014).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Ferric Reducing Power Assay (FRAP), Metal Chelating Assay, Nitric Oxide radical scavenging assay and Superoxide radical scavenging assay were carried out to evaluate the antioxidant activities of *Pueraria mirifica* root extract. The DPPH assay was done to evaluate the ability of antioxidant to scavenge free radicals. It is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. The violet colour of DPPH was changed to pale yellow colour due to the abstraction of hydrogen atom from the antioxidant compound. Therefore, the higher level of antioxidant compound present in the extracts, the more DPPH will reduce (Garcia et al., 2012). In this study, the scavenging activity of *Pueraria mirifica* root extract was compared with ascorbic acid as standard which is known as a good natural antioxidant. Based on Figure 4.3, the scavenging activity of *Pueraria mirifica* extracts is slightly lower than that of ascorbic acid. However, all extracts managed to achieve IC_{50} which is the half maximal inhibitory concentrations of ligands on their protein targets (Kalliokoski et al., 2013). As shown on Figure 4.3, n-butanol extracts has $IC_{50} = 1.145$ mg/ml, ethyl acetate has an

IC₅₀ = 0.896 mg/ml, chloroform has an IC₅₀ = 0.573 mg/ml, methanol aqueous has an IC₅₀ = 0.556 mg/ml and n-hexane has an IC₅₀ = 0.5602 mg/ml. All extracts expressed good scavenging activity that indicate it as good antioxidants since antioxidant activity in plant is related to their phenolic content. n-butanol showed the highest scavenging activity might due to the presence of terpenoids detected thin layer chromatography with chloroform solution as stated in Table 4.4.

Metal chelating assay was carried out to determine the antioxidant activity of *Pueraria mirifica* extracts. Based on Rohman et al. (2010) the higher metal chelating activity was indicated by the smaller IC₅₀ value. The chelation therapy can be defined as the administration of chelating agents to remove heavy metals from the body. The oxidative stress can be caused by the disruption of iron ions homeostasis where formation of reactive oxygen species (ROS) overwhelm body antioxidant protection therefore resulting in DNA damage, lipid peroxidation, protein modification and can cause numerous diseases namely cancer, cardiovascular disease, diabetes and neurological disorders and others (Naithani et al., 2011). In this study, the chloroform and ethyl acetate extracts showed IC₅₀ value of IC₅₀ = 0.489 and IC₅₀ = 1.768 respectively. Chloroform showed the highest percentage of inhibition might due to the presence of alkaloids and terpenoids compound in the extracts. The alkaloids structure consists of hydroxyl group and this indicated that it's potential to be a good antioxidant agent. The other three extracts n-hexane, n-butanol and methanol aqueous showed low chelating activity where at the highest 2.0 mg/ml concentration, the percentage of inhibitions were 47.24 %, 36.77 % and 34.37 % respectively. A good metal chelator needs to have high affinity for the toxic metal and low affinity for essential metals, minimal toxicity and also lipid solubility therefore chloroform and ethyl acetate are suggested to possess either one of these qualities. These results showed that the extract can interfere during

the formation of ferrous and ferrozine complex, thus suggesting the potential of the extract to chelate and capture ferrous ion before ferrozine.

The Nitric Oxide (NO) radical scavenging activity was carried out to evaluate the ability of *Pueraria mirifica* extract to scavenge nitric oxide radical. Nitric oxide is free radicals that are derived from the interaction of NO with oxygen or reactive oxygen species. NO is classified as a free radical because of it is unpaired electron and displays important reactivity with certain type of protein and other free radicals such as superoxide. Low concentration of NO is enough to affect the physiological function of the body (Boora et al., 2014). In this study, ethyl acetate extract displayed the highest percentage of inhibition at 2.0 mg/ml concentration (21.54 %) followed by methanol aqueous (16.02%), n-butanol (15.23 %), n-hexane (-2.42 %) and chloroform (-10.41 %). Based on the result, it is suggested that alkaloids that is present in the ethyl acetate extracts detected in TLC, could potentially be the reason why it displayed the highest percentage. The nitrite formation was inhibited due to alkaloid manage to compete with oxygen in the reaction. Negative results in n-hexane and chloroform extracts suggest that there are no scavenging activity for these extracts however the negative result does not necessarily means free radicals are formed. There are also possibility that nitrite is already present in n-hexane and chloroform plant samples.

Superoxide Radical Scavenging Activity of *Pueraria mirifica* extract was done to determine the ability of *Pueraria mirifica* extracts to scavenge superoxide radicals. Although superoxide can be considered as weak antioxidant, it is responsible for formation of other reactive species. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT) (Elmastas et al., 2003). The generation of four-electron reduction of molecular oxygen into water forming superoxide anion radical ($O_2^{\cdot-}$). This radical also formed in aerobic cells due to electron leakage from the electron transport chain. In this study, at

2.0 mg/mL concentration, n-hexane extract showed the highest percentage of superoxide inhibition at 43.167 % followed by methanol aqueous at 26.667 %, ethyl acetate at 25.00 %, n- butanol at 24.167 % and lastly chloroform at 23.333 %. n-hexane extract contains terpenoid compound TLC with chloroform solution as depicted in Table 4.4 and alkaloid compound detected in the TLC with 10% methanol in chloroform solution as shown in Table 4.3. This could potentially be the antioxidant agents. Interestingly, all the percentage of superoxide inhibition in all extracts is higher than the percentage of inhibition of ascorbic acid as a standard. This suggests that the extracts can be an excellent antioxidant.

The Ferric Reducing Antioxidant Power (FRAP) assay was carried out to assess the ability of extracts from *Pueraria mirifica* to reduce the ferum ion due to its antioxidant activity. The analysis of ferric reducing activity was based on the reduction of ferric-TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) to blue ferrous TPTZ. Antioxidant compounds will act as a reducing agent displaying their effect by donating hydrogen atom to ferric complex therefore breaking the radical chain reaction. In this study, the extracts that shows the highest reducing power was methanol aqueous (0.18 ± 0.00 mmol Fe^+ /g) followed by ethyl acetate (0.10 ± 0.014 mmol Fe^+ /g), n-butanol (0.07 ± 0.007 mmol Fe^+ /g), chloroform (0.07 mmol Fe^+ /g) and n-hexane (0.06 ± 0.007 mmol Fe^+ /g). The methanol aqueous extracts displayed the highest reducing power might due to the high polarity therefore it was able to donate electron making the radicals stable and unreactive. Methanol aqueous extracts also showed the highest reducing power due to the presence of phenolic compound detected in LCMS/MS analysis which are p-aminobenzoic acid and eplerenone and flavonoid compound which is ulexone B.

AChE inhibition assay was carried out to determine the *Pueraria mirifica* extracts inhibitory activities *in vitro* using Ellman's method. In AChE inhibition assay, acetylcholine (ACh) is hydrolysed by AChE through a reaction between the thiocholine and DTNB to generate the yellow anion of 5-thio-2-nitrobenzoic acid thus measuring the inhibition activity (Bahrani et al., 2014) According to Vinutha et al. (2007), the extracts inhibitory activity (%) was classified as potent when it's more than 50%, moderate within the range of 20-50% inhibition and low when less than 30% inhibition. In hexane extracts, the highest percentage obtained is in PM-HE1 which is 15.7 % inhibition, and in chloroform extracts the highest percentage inhibition is in PM-CH1 which is 6.924 %, PM-ET3 showed the highest percentage of inhibition for ethyl acetate extracts at 4.670 % meanwhile for butanol extract, the highest percentage is in PM-NB1 giving 13.607 % and lastly for methanol aqueous extract, the highest percentage is in PM-AQ1 giving 21.739 %. The overall result of AChE inhibition assay showed the highest percentage of AChE inhibition is in isolated compound of methanol aqueous extracts. The high percentage of AChE inhibition is due to the presence of phenolic compound which are p-Aminobenzoic acid and Eplerenone and flavonoid compound which is Ulexone B. However, in acetylcholinesterase inhibition assay of extracts, ethyl acetate and n-butanol showed the negative results. This may be due to interference in the assay. Since AChE inhibition is a chromogenic assay, the inaccuracy of AChE enzyme or extracts concentration, can interfere with yellow colour formation thus giving the negative result. The inhibitors which are ethyl acetate and n-butanol are also probably not soluble at the tested concentration and precipitates, thus the turbidity of the precipitate can masquerade as absorbance and affected the result. The synergistic effect of plant compounds in n-butanol extracts could also possibly interfere with acetylcholinesterase inhibition compared to positive result in compounds isolated from n-butanol which are PM-NB1 and PM-NB2. In acetylcholinesterase inhibition assay of

TLC compound isolated from extracts with chloroform solvent, the negative results of PM-ET1 compound from ethyl acetate extract is probably because of degradation of PM-ET1 compound during the isolation process using TLC method therefore this compound didn't manage to inhibit acetylcholinesterase.

The presented results strongly suggest that methanol aqueous extract from *Pueraria mirifica* possess good antioxidant activity based on few different mechanism namely hydrogen atom donation, ferric ion reducing ability, metal chelating effect, superoxide anion scavenging ability and nitric oxide scavenging ability. The main attributor of these antioxidant properties in methanol aqueous extract is the presence of phenolic and flavonoids compound detected in LCMS/MS. Alkaloid compound was also detected in TLC for methanol aqueous extracts. Furthermore, an isolated compound from methanol aqueous extracts also showed the highest percentage of AChE inhibition thus suggesting it to be good AChE inhibitors for AD treatment.

CONCLUSION

Thin Layer Chromatography was done to detect the phytochemical bioactive compounds of *Pueraria mirifica* extracts and two phytochemical groups, alkaloids and terpenoids were detected. Liquid Chromatography Mass Spectrometer tandem mass spectrometer was done to methanol aqueous extracts and displayed the presence of 11 compounds as followed, 26,27-Dinor-3 α ,6 α ,12 α -trihydroxy-5 β -cholestan-24-one, 3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-al, C16 Sphinganine, 4-Fluoro-L-threonine, N6-Methyl-2'-deoxyadenosine, Ulexone B, Cys Lys His, p-Aminobenzoic acid, Eplerenone, 5-Aminopentanoic acid, L-Arginine, Oleoyl Ethyl Amide, Ethephon and lastly, Bufexamac. In Total Phenolic compound and Total Flavonoid Compound tests, the highest phenol and flavonoid contents was in methanol aqueous and chloroform extracts respectively. For antioxidant test, DPPH showed high percent of inhibition where all extracts achieved IC₅₀ inhibition percentage. However, for metal chelating, NORSA, FRAP and superoxide radical scavenging activity the percentage of inhibitions are relatively low and was not able to obtain IC₅₀ inhibition percentage. AChE assay demonstrates relatively low inhibitory activities in all extracts where none of them achieve 50% inhibition percentage. In hexane extracts, the highest percentage obtained is in Hx-1 extracts and in chloroform extracts the highest percentage inhibition is in chlo-a, EtAc-2 showed the highest percentage of inhibition for ethyl acetate extracts meanwhile for butanol extract, the highest percentage is in n-but-a and lastly for methanol aqueous extract, the highest percentage is in Aq-a. It can be concluded that *Pueraria mirifica* roots extracts can be a good antioxidants and may potentially be a potent natural AChE inhibitors and could be used as an alternative in the treatment of Alzheimer disease however further investigation is required.

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