ACETYLCHOLINESTERASE INHIBITORY AND ANTIOXIDANT PROPERTIES OF *Ampelocissus* sp. (ISI NYARU) EXTRACT

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FACULTY OF SCIENCE
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ACETYLCHOLINESTERASE INHIBITORY AND ANTIOXIDANT PROPERTIES OF *Ampelocissus* sp. (ISI NYARU) EXTRACT

ABSTRACT

Ampelocissus sp. (Isi Nyaru) is a genus of Vitaceae has been used in traditional medicine for the treatment of inflammation in tissue damage, bruises and after childbirth. Alzheimer's disease (AD) is neurodegenerative disease characterized by deficiency in more than an area of cognition which involving mood and behavior changes, sporadic memory, language, and attention. In this study the antioxidant and acetylcholinesterase (AChE) inhibitory activity of *Ampelocissus* sp. extract was evaluated. The chemical compounds of *Ampelocissus* sp. were separated by thin layer chromatography (TLC) and detected with Vanillin-sulphuric and Dragendroff's spray reagent. Saponin, alkaloids, terpenoids and flavonoids were detected in the extracts. The phytochemical compounds were analyzed using Liquid Chromatography Mass Spectrometry tandem with Mass Spectrometry (LCMS/MS). The methanol aqueous extract showed the presence of 12 compounds namely 5-aminopentanoic acid, 11-amino-undecanoic acid, C16 sphinganine, (+)-eudesmin, his lys cys, lys his met, his met lys, ethephon, L-arginine, sulpho NONOate, gln gln trp and dichloroacetate5-aminopentanoic acid, 11-amino-undecanoic acid, C16 sphinganine, (+)-eudesmin, his lys cys, lys his met, his met lys, ethephon, L-arginine, sulpho NONOate, gln gln trp and dichloroacetate. The highest content of phenol and flavonoids were detected in chloroform extracts at 0.42 ± 0.012 mg GAE/g and 2.007 ± 0.001 mg QE/g, respectively. The ethyl acetate extract showed the highest DPPH scavenging activity at IC_{50} 1.49 mg/ml, highest metal chelating activity at IC_{50} 0.07 mg/ml. Meanwhile chloroform extract showed the highest reducing power at 6.03 mmol Fe^{+}/g in FRAP, highest IC_{50} 0.49 mg/ml in NORSA and the highest superoxide radical scavenging activity at 26.8 %. Among 17 compounds isolated from TLC plates of *Ampelocissus* sp.

the highest percentage of AChE inhibition activity was shown by methanol aqueous extract at 55.56%. Thus, the findings of this research showed that the extract of *Ampelocissus* sp. possessed good antioxidant activity and AChE inhibitors potential to be used for AD treatment.

Keywords: *Ampelocissus* sp. (Isi Nyaru), Alzheimer's disease, neurodegenerative, antioxidant, acetylcholinesterase (AChE) inhibitory

RENCATAN ASETILKOLINESTERASE DAN SIFAT ANTIOKSIDAN DARI EKSTRAK *Ampelocissus* sp. (ISI NYARU)

ABSTRAK

Ampelocissus sp. (Isi Nyaru) adalah dari genus Vitaceae telah digunakan secara tradisional untuk merawat keradangan dalam kerosakan tisu, lebam dan selepas melahirkan anak. Penyakit Alzheimer (AD) adalah penyakit neurodegeneratif yang dicirikan oleh kekurangan pada lebih daripada satu kawasan kognisi yang melibatkan perubahan mood dan tingkah laku, memori sporadis, bahasa dan perhatian. Dalam kajian ini, aktiviti antioksidan dan perencatan asetilkolinesterase dari ekstrak *Ampelocissus* sp. dinilaikan. Sebatian kimia *Ampelocissus* sp. telah dipisahkan oleh kromatografi lapisan nipis (TLC) dibantu oleh reagen semburan Vanillin-sulfur dan Dragendroff. Saponin, alkaloid, terpenoid dan flavonoid dikesan dalam ekstrak. Pemisahan sebatian fitokimia juga dilakukan menggunakan tandan spektrometri Massa Kromatografi Cecair dengan Spektrometri Massa (LCMS / MS). Analisis ekstrak akues metanol menunjukkan kehadiran 12 sebatian iaitu 5-aminopentanoik asid, 11-amino-undekanoik asid, C16 sphinganin, (+)-eudesmin, his lys cys, lys his met, his met lys, ethephon, L-arginin, sulfo NONOate, gln gln trp dan dikloroasetat. Kandungan fenolik dan flavonoid yang tertinggi dikesan dalam ekstrak kloroform yang masing-masing pada 0.42 ± 0.012 mg GAE / g dan 2.007 ± 0.001 mg QE / g. Ekstrak etil asetat menunjukkan IC_{50} aktiviti penyahan DPPH pada 1.49 mg / ml, aktiviti pengkelat logam tertinggi pada IC_{50} 0.07 mg / ml. Sementara itu, kloroform menunjukkan kuasa penurunan FRAP tertinggi pada 6.03 mmol Fe + / g, IC_{50} tertinggi pada 0.49 mg / ml di dalam NORSA dan aktiviti penyahan radikal superoksida terbaik pada 26.8 %. Di antara 17 sebatian yang diasingkan dari plat TLC *Ampelocissus* sp., peratusan tertinggi aktiviti perencatan AChE oleh ekstrak metanol akueus pada 55.56 %. Oleh itu, hasil

daripada kajian ini menunjukkan bahawa ekstrak *Ampelocissus* sp. mempunyai aktiviti antioksidan dan perencatan AChE berpotensi untuk digunakan bagi rawatan AD.

Kata kunci: *Ampelocissus* sp., penyakit Alzheimer, neurodegeneratif, antioksidan, perencatan asetilkolinesterase

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This work is dedicated to the memory of my parents for always believed in my ability to be successful in academic. You both are gone but your belief in me has made this journey possible. Most importantly, this dissertation could not have happened without my family. They always there, stood by me through my ups and downs. This dissertation stands as evidence to your unconditional love and inspiration.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
U	Unit
L	Liter
M	Molar
mm	Millimeter
ml	Milliliter
cm	Centimeter
nm	Nanometer
g	Gram
°C	Degree Celcius
mg	Milligram
µl	Microliter
µM	Micromolar
mmol	Millimolar
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer's disease
$AlCl_3$	Aluminium Chloride
aMCI	Amnestic mild cognitive impairment
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATCI	Acetylthiocholine iodide
ATP	Adenosine triphosphate
Aβ	Amyloid-β
BuChE	Butyrylcholinesterase
$C_2H_9NaO_5$	Sodium acetate trihydrate
Ca^{2+}	Calcium
CAS	Catalytic anionic site
CH_3CN	Acetonitrile
CH_3COOH	Acetic acid
ChAT	Choline acetyltransferase

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2diphenyl -1- picrylhydrazyl
DTNB	5,5'-dithiobis [2nitrobenzoic acid]
$EDTANa_2 \cdot 2H_2O$	Ethylenediaminetetraacetic acid disodium dehydrate
ELISA	Enzyme linked immunosorbent assay
EOAD	Early onset Alzheimer's disease
FC	Folin-Ciocalteu
FDA	Food and Drug Administration
Fe^{2+}	Ferrous
$FeCl_2$	Ferum chloride
$FeCl_3$	Ferric Chloride Solution
$FeCl_3 \cdot 6H_2O$	Ferric chloride hexahydrate
$FeSO_4$	Ferrous sulfate
$FeSO_4$	Ferrous sulphate
FZ	Ferrozine
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric Acid
HCO_2H	Formic acid
IC_{50}	Half maximal inhibitory
IWG	International working group
K_2HPO_4	Potassium phosphate (dibasic)
KH_2PO_4	Potassium phosphate (monobasic)
LCMS	Liquid Chromatography Mass Spectrometry
LOAD	Late- onset Alzheimer's disease
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
Na_2	Disodium
$Na_2[Fe(CN)_5NO].2H_2O$	Sodium nitroferricyanide
NBT	Nitro Blue Tetrazolium
Na_2CO_3	Sodium Carbonate
NaCl	Sodium chloride

NADH	Nicotinamide Adenine Dinucleotide
naMCI	Non-amnestic mild cognitive impairment
$NaNO_2$	Sodium Nitrite
NaOH	Sodium Hydroxide
NFTs	Neurofibrillary tangles
NH_4HCO_2	Ammonium formate
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NORSA	Nitric Oxide Radical Scavenging Assay
NP	Neuritic plaques
NT	Neuropil thread
O_2^-	Superoxide radical anion
OH	Hydroxyl radical
ONOO−	Peroxynitri
OS	Oxidative stress
PAS	Peripheral anionic site
PCAD	Preclinical alzheimer's disease
PET	Positron emission tomography
PMS	Phenazine Methosulphate
QAE	Quercetin equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolution per minute
SOD	Superoxide dismutase
SP	Senile plaques
TCA	Tricarboxylic acid cycle
TLC	Thin layer chromatoghraphy
TPTZ	2,4,6- tripyridyl-s-triazine
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Ampelocissus is a genus of Vitaceae having 90 or more species found variously in tropical Africa, Asia, Central America, and Oceania. In Malaysia, it is known as Isi nyaru and commonly found in rural area such as in aboriginal people settlements. It has been used in traditional medicine for the treatment of inflammation in tissue damage, bruises and after childbirth. In this study the antioxidant and acetylcholinesterase (AChE) inhibitory activity of *Ampelocissus* sp. (Isi Nyaru) extract was evaluated.

Alzheimer‘s disease (AD) was first discovered more than 100 years ago, but study into its causes, symptoms, risk factors, and treatment has increased only in the past 30 years (Alzheimer‘s Association Update, 2014). It is the most common type of dementia in the elderly population and has become the fourth main cause of death in the industrialized countries (Thiratmatrakul *et al*., 2014). It is a progressive disease which means that more parts of the brain are damaged gradually over time. As this happens, more symptoms develop and become more severe. Recent estimations state that there are approximately 5.4 million people of all age ranges diagnosed with AD in the United States in 2016. The figure possibly will closely triple by 2050, from 5.2 million to an estimated 13.8 million, with a projected 2 trillion US dollars related cost (Tramutola *et al*., 2017). The growth is a consequence of the global aging populace which represents the point that AD is a disease of aging and thus aging signifies the only largest threat for AD (Wang *et al*., 2014).

AD is identified by the widespread spreading of senile plaques (SP), development of neurofibrillary tangles (NFTs), chronic neuroinflammation, synapse loss and neuronal damage (Tramutola *et al*., 2017). It is also involved with a damage of presynaptic cholinergic function in the areas of the brain that associated with memory

and learning. Clinically, AD is characterized by memory loss and progressive deficits in different cognitive domains related to a distinct degradation of the cholinergic system and change in other neurotransmitter system (Minarini *et al*., 2012). The most optimistic approach for the symptomatic cure of AD is to improve the synaptic levels of acetylcholine (ACh) in the brain by inhibiting the acetylcholinesterase enzyme, which is predominantly accountable for its hydrolysis and termination of action (Anand *et al*., 2012). AChE inhibitors are chemical agents used for symptomatic treatment of AD. Acetylcholinesterase is an enzyme belongs to the α/β hydrolase fold protein super family; a group defined by common structural homology and includes the cholinesterases, carboxylesterases and lipases. Its principal physiological function is the rapid hydrolysis of acetylcholine in the synapse and neuromuscular junction due to its protease activity, resulting in the termination of the nerve impulse (Singh *et al.*, 2013) The acetylcholinesterase inhibitor (AChEI) such as donepezil, galanthamine and rivastigmine are designated for the treatment of AD from the mild stages onwards by inhibiting the action of the ACh-hydrolysing enzyme AChE. ACh levels were boost and thus disease symptoms associated with the progressive loss of cholinergic function in AD are alleviating (Parson *et al*., 2013).

Many oxidative stress related diseases occur as a result of accumulation of free radicals in the body. One of the diseases is AD. As our brain is extensively susceptible to free radical damage, brain aging seems like to be nearly connected with reactive oxygen species (ROS) (Müller *et al*., 2010). Recently, numerous researches in transgenic animals, post-mortem brains, and biological fluids from subjects afflicted with AD or mild cognitive impairment prove the solid connection between mitochondrial dysfunction and oxidative stress in AD and the primary association of these two factors in the pathology of AD (Müller *et al*., 2010). Free radicals are constantly produced within the human body in response to both internal and external

stimuli. In small amount these products play an important role as growth regulator, signal transducers, and as part of the immune defence system. However, excess generation of free radicals and other oxidants will cause oxidative stress (Jindal & Mohamad, 2012). On a molecular basis, cell cycle changes and oxidative stress resulting from increases in ROS and reactive nitrogen species (RNS) have also been shown to play a detrimental role in AD (Swomley *et al*., 2014).

Several natural substances with effective antioxidant properties, such as spices, green tea, resveratrol, and vitamins, have been appraised as therapeutic agents for AD. A polyphenol such as curcumin can perform as a free radical scavenger and antioxidant that prevents lipid peroxidation and oxidative deoxyribonucleic acid (DNA) damage (Dumont & Beal, 2011).

1.1 Research Objectives

1. To separate and detection of the bioactive compounds of *Ampelocissus* sp. extracts using TLC and LCMS.
2. To determine the antioxidant activity of *Ampilocissus* sp.
3. To determine *in vitro* Acetylcholinesterase inhibitory effects of *Ampelocissus* sp.

CHAPTER 2

LITERATURE REVIEW

2.1 Alzheimer's Disease

2.1.1 Global Scenario and Background of Alzheimer's Disease

Dementia can be best described as a clinical disorder indicated by a group of signs and symptoms expressed by troubles in memory, disruption in language and other cognitive functions, alterations of behaviours, as well as weakening in daily living activities (Qiu *et al*., 2009). Alzheimer's disease (AD) was first discovered by a German physician, Alois Alzheimer in 1906 who noted alterations in the brain tissue of a woman who had died due to an unfamiliar mental disease (Parihar & Hemnani, 2004). AD is the most frequent reason of dementia and up to 75 % of all dementia cases are cause by Alzheimer (Qiu *et al*., 2009).

The clinical signs of AD are progressive onset and deficiency in more than an area of cognition which involving mood and behaviour changes, sporadic memory, language, praxis and attention. In addition, typical early symptom noticed is difficulty in remembering newly learned information (Singh *et al*., 2013). AD brain is distinguished by serious neurodegenerative modifications, for example the loss of synapses and neurons, atrophy, and the selective reduction of neurotransmitter systems such as acetylcholine in the hippocampus and cerebral cortex. These defects are generally observed in the late stage of the disease (Müller *et al*., 2010).

AD is categorized into early onset AD (EOAD, onset < 65 years) which accounting for 1–5% of total cases, and late-onset AD (LOAD, onset ≥ 65 years) accounting for >95% of afflicters. EOAD is commonly related to a faster rate of progression and a Mendelian pattern of inheritance. Three genes;

APP, *PSEN1* and *PSEN2* are encode proteins engaged in APP breakdown as well as Aβ generation. They have been strongly associated with the pathophysiology of EOAD. On the contrary, the genes involved in LOAD give rise to disease possibility in a non-Mendelian fashion. First-degree families of patients with LOAD have doubled the probable life-time risk of people without an AD-affected first-degree relative (Qiu *et al*., 2009).

Discovering the cause of a neurodegenerative disorder, particularly; AD is important to the advance of effective managements and treatments as well as the discovery of an eventual cure for these patients. Moreover, AD has upsetting consequences on the value of life of the suffering individual, and caretakers have to endure a heavy financial and emotional affliction (Craig *et al*., 2011).

2.1.2 Epidemiology of Alzheimer's Disease

Alzheimer's disease is an acute neurodegenerative disorder that impact on many people aged from 65 years or older and approximately half of those of age 85 are suffered from this disorder. Since the main causative reason of AD is age, the number of AD cases is rising at an ever-growing pace as the world population grows and life anticipation increase (Swomley *et al*., 2014). By 2050, the number of new cases is expected to reach approximately a million cases every year with the overall expected prevalence is to be 13.8 million (Alzheimer's Association Update, 2014). The World Alzheimer Report 2010 expected that ageing of the worldwide population will cause the economic effect of dementia superior than that of cancer, heart illness, and stroke mixed (Chan *et al*., 2013). Of all the variety of dementias, AD is the most widespread subtype which accounting for about 60% from all dementias (Yiannopoulou & Papageorgiou, 2012). In 2010, official death certificates documented 83,494 deaths from AD, thus making AD is the sixth most prominent cause of death in the United States and the fifth

top cause of death in Americans aged 65 years or older (Alzheimer‘s Association Update, 2014).

Within regional populations of 60 year-olds, individuals from North America and Western Europe are suspected to show the highest prevalence and incidence rate of dementia, followed by those from Latin America and China and its western-Pacific neighbours. For all these people, the incidence rate for dementia rises as age increase, with the most prominent growth taking place during the 7^{th} and 8^{th} decades of life. Identical figures were seen for the prevalence and incidence of AD (Reitz & Mayeux, 2014).

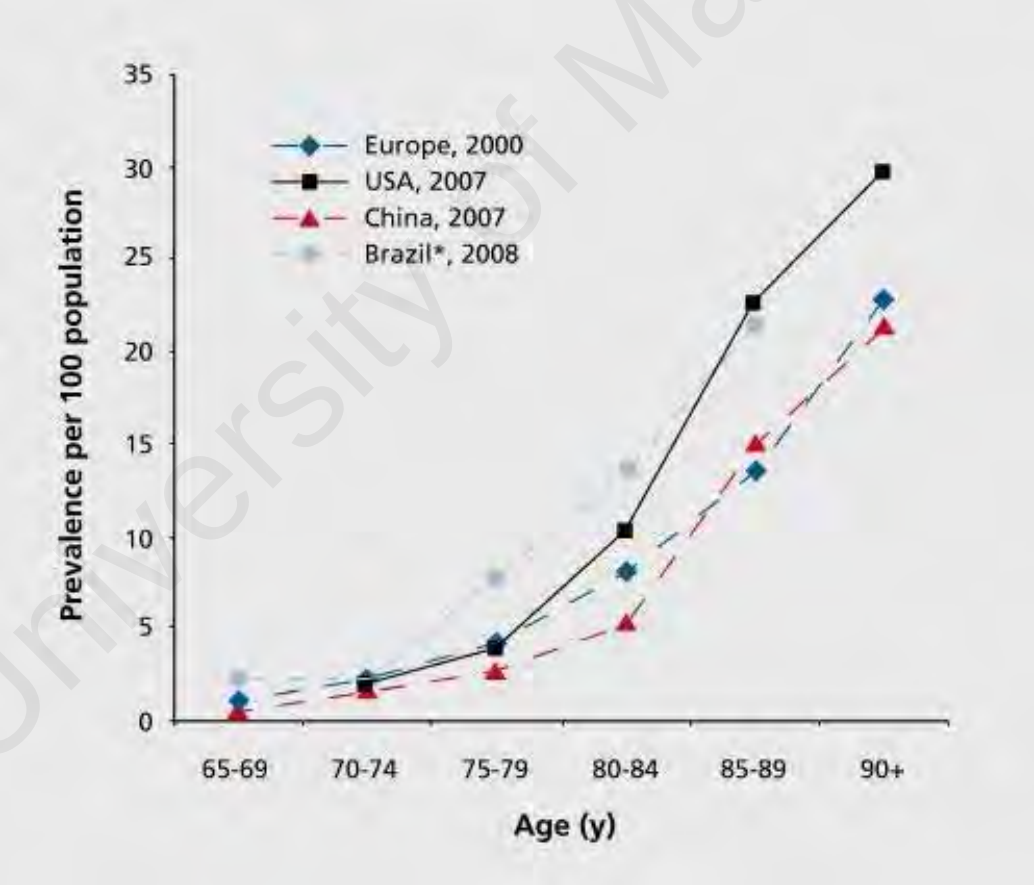


Figure 2.1: Prevalence of Alzheimer's disease (per 1 000 person years) across continents and countries (Qiu *et al*., 2009). Reprinted permission granted by Qiu.

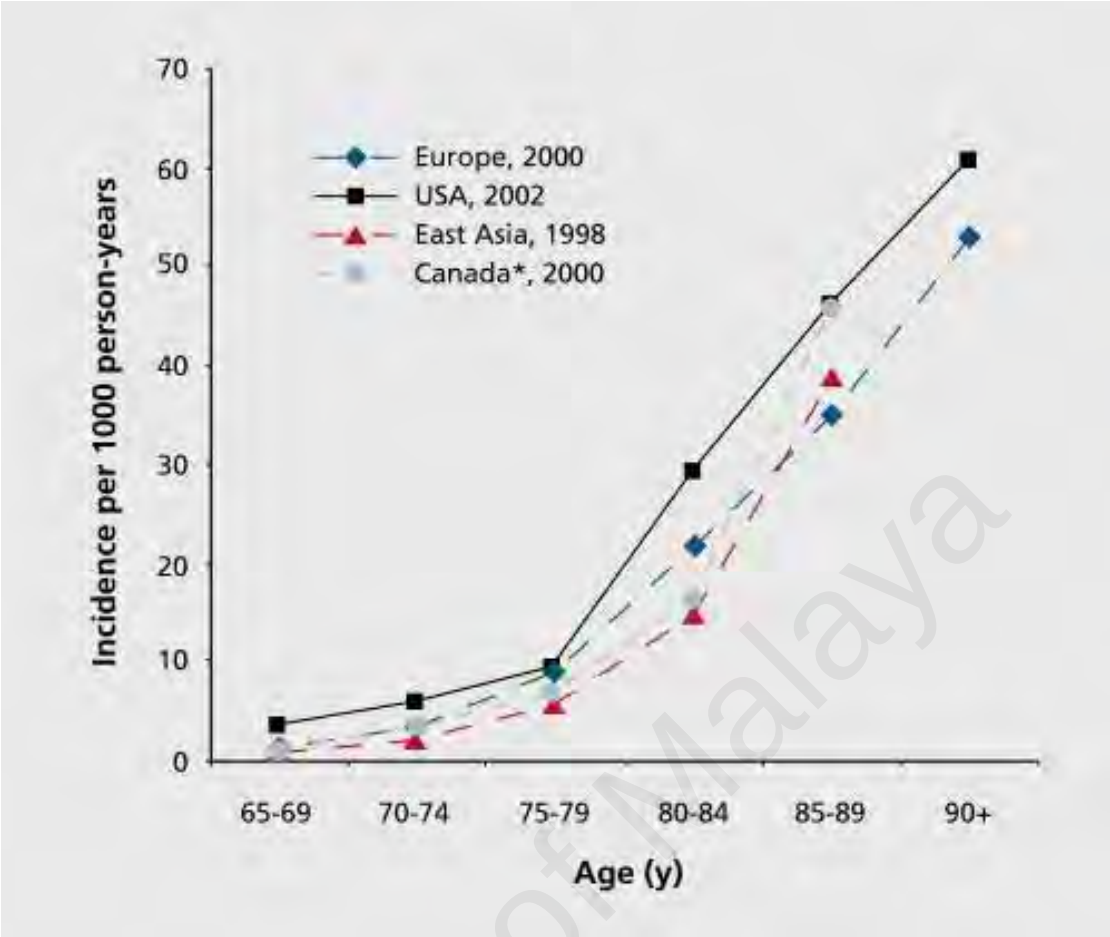


Figure 2.2: Incidence of Alzheimer's disease (per 1 000 person years) across continents and countries (Qiu *et al.*, 2009). Reprinted permission granted by Qiu.

2.1.3 Factors of AD

Age is the most significant risk factor. In a recent analysis of 1246 subjects aged 30 to 95 years, the risk of developing AD increased with age typically after 70 years (Dubois *et al*., 2016). Other than that, having a close blood relative who has AD increases the risk of getting this disease. Women also are more prone to develop AD as they are usually live longer than men.

According to Dubois *et al*. (2016) some somatic and lifestyle factors for AD are including diabetes, physical inactivity, smoking, depression, midlife hypertension, midlife obesity, and low education level. In estimation, about one-third of worldwide AD dementia cases are likely occurred as effect to the mentioned modifiable risk

factors. In addition to these factors, Singh *et al*. (2013) summarized the factors affecting disease progression of AD are including smoking, aging, head injury, ApoE4 genotype, alcoholism, depression, menopause, hemorheologic abnormalities, diabetes mellitus as well as stroke.

2.1.4 Staging of Alzheimer 's Disease

Theoretically, preclinical AD would be defined as period from the initial neuropathologic brain lesions to the onset of the first clinical symptoms of AD. The international working group (IWG) has considered preclinical states into two types: the presymptomatic and the asymptomatic at risk state. The presymptomatic AD known that certain individuals are virtually meant to have full clinical AD as they are identifies to carry an autosomal dominant monogenic mutation and the disease can be diagnosed at all stages with the identification of the mutation. In contrary, "asymptomatic at risk" state is more controversial because to be classified as asymptomatic at risk individuals must not possess clinical evidence of prodromal AD. However as stated by the recent IWG revision, both typical and atypical phenotypes of preclinical states of AD call for the absence of clinical signs and symptoms of AD as well as the presence of at least one biomarker of Alzheimer's pathology (Dubois *et al*., 2016). Additionally, current reports from autosomal dominant forms of Alzheimer's disease (AD) propose that amyloid-β (Aβ) build-up may be evident 20 years before the phase of dementia, and that there is already significant (Sperling *et al*., 2014).

The growth of intraneuronal lesions on particular at risk brain sites is dominant to the pathological development in AD. The lesions comprise mainly of hyperphosphorylated tau protein including pretangle material, NFTs in cell bodies, neuropil threads (NTs) in neuronal processes, as well as material in dystrophic nerve cell processes of neuritic plaques (NPs). AD-linked pathological progression extents in

decades where the dispersal form of the lesions progresses based on a predictable sequence. A staging system for the intraneuronal lesions established in 1991 distinguished initial, intermediate, and late phases of the disease process in both non-symptomatic and symptomatic individuals. Later in 1997, this system was integrated into the NIH-Reagan standards for the neuropathological diagnosis of AD (Braak *et al.*, 2006)

Conventional AD progression is classified into four stages which are preclinical AD (PCAD), mild cognitive impairment (MCI), EOAD, LOAD. AD patients are commonly analysed based on the severances of symptoms throughout the progression into each stage (Tramutola *et al*., 2017). Numerous persons with PCAD have a high amyloid plaque burden, however function normally. MCI is the transition phase between normal cognition and EOAD/AD and can be further sub-categorized into amnestic MCI (aMCI) and non-amnestic MCI (naMCI). By using imaging procedures such as magnetic resonance imaging (MRI), many degrees of deterioration could be observed for all phases of clinical AD. Other than that, positron emission tomography (PET) technology which is used to probe regional glucose consumption inside the brain proposes acute energy deficit for PCAD and MCI patients. Bearing in mind that glucose is the core energy source for brain; it shows that the brain is under energy deficiency, consistent with the progression of AD (Swomley *et al*., 2014). Alterations in amyloid precursor protein (APP), which speed up Aβ production, were seen in some cases of a monogenic type of the disease, EOAD (Small & Duff, 2008).

2.1.5 Pathology and Pathogenesis of AD

In history, a diagnosis of AD was made by a post-mortem autopsy that discloses the occurrence of senile plaques and neurofibrillary tangles (Craig *et al*., 2011). The common histopathological distinctive feature of AD can be summarized as the growth

of extracellular Aβ-rich senile plaques that are resulted from the cleavage of APP, the build-up of intracellular NFTs, which are mainly consisted of the accumulated form of hyperphosphorylated tau, and synapse loss (Swomley *et al*., 2014). Additionally, anatomical researches among AD patients revealed a massive loss of brain white matter and a certain decline of cholinergic neurons (Lombardo & Mascos, 2015). The neuronal loss is greatly present throughout the basal forebrain, amygdala, hippocampus, and cortical area (Borlongan, 2012) which are related with higher mental roles (Francis *et al*., 1999). Though these abnormalities arise to some point in all brains with age, there are extra more of them in the brains of people with AD. In addition, cell cycle alterations and oxidative stress as a result in increasing of ROS and RNS have also been proven to play a damaging role in AD (Swomley *et al*., 2014). While Aβ plaques and tau tangles were seen in the late stage of AD, in contrast mitochondrial dysfunction and oxidative stress are take place in early incidences in the pathology of AD (Müller *et al*., 2010)

Generally, the subsequent hypothesis has been suggested on the basis of the several contributing factors such as amyloid cascade hypothesis, cholinergic hypothesis, tau hypothesis, and mitochondrial dysfunction.

2.1.5.1 Cholinergic hypothesis

The cholinergic hypothesis was the first theory suggested to describe AD and ever since, it has led to the invention of the only drugs currently permitted to cure mild to moderate AD (Craig *et al*., 2011). Insufficiency of acetylcholine, an important neurotransmitter in brain was detected either due to lessened production of neurotransmitter or increased of acetylcholinesterase activity. The reduced level of the neurotransmitter leads to impairment of the cholinergic neurotransmission and eventually will cause the loss of intellectual abilities (Singh *et al*., 2013). Moreover,

several authors detected a decrease in the activity of AChE which is the enzyme that metabolises Ach after its release in the synaptic cleavage. The function of the cholinergic system in cognition and the modification detected in neurodegenerative diseases such as in AD, directed to the origination of the "cholinergic hypothesis" of geriatric disorders; in which the reduction of cholinergic innervation is answerable for the cognitive decrease found in AD patients (Lombardo & Maskos, 2015). A particular cholinergic deficit which concerning the cholinergic projection from a basal forebrain neuronal population, the nucleus basalis magnocellularis of Meynert, to the cortex and hippocampus was constantly seen in autoptic substance of Alzheimer's patients. In addition, based on the observation through pathological samples from the cortex and hippocampus of Alzheimer's patients, the action of choline acetyltransferase; the enzyme in control for the synthesis of acetylcholine was learnt to be extraordinarily decreased and at times in a rather severe way (Contestabile, 2011). Comprehensive literature from animal experiments verifies the human data described and in actual fact, the significance of cholinergic function in the brain to learning and memory was first documented more than 30 years ago after cholinergic antagonists (specially antimuscarinic agents) were detected to impair memory in rats. Cholinergic defects may also cause noncognitive behavioural irregularities in addition to the deposition of toxic neuritic plaques in AD patients (Terry, 2003).

2.1.5.2 Amyloid hypothesis

The hypothesis suggests that amplified levels of both soluble and insoluble Aβ peptides initiate memory deficits. These peptides are originated from the larger APP by sequential proteolytic processing (Borlongan, 2012).

Histological researches of the brain of the AD patient showed the incidence of plaque that provides a route to a special study of these objects. In 1984, building block

of amyloidogenic peptide was established to be amyloid beta protein that forms the amyloid fibrils in the neuritic plaques. In the amyloid hypothesis, a misfolded amyloid beta, an oligomeric species, mostly toroidal or star-shaped deposited in the brain may possibly stimulate apoptosis by physically piercing the cell membrane. Then, plaque amyloid depositions as well as partially aggregated soluble b-amyloid will start the neurotoxic cascade and induces neurodegeneration that leads to AD (Singh *et al*., 2013).

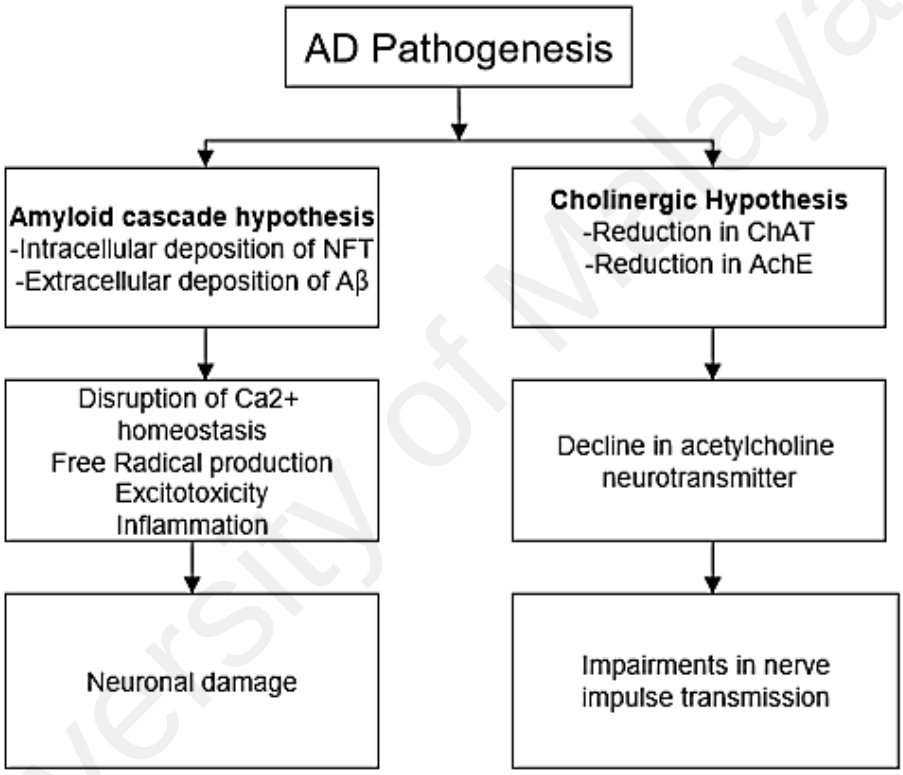


Figure 2.3: Amyloid cascade hypothesis and cholinergic hypothesis of AD (Parihar & Hemnani, 2004). Reprinted permission granted by Parihar.

Figure 2.3 above describes a simplified description for both amyloid cascade hypothesis and cholinergic hypothesis. Explaining to amyloid cascade hypothesis, the pathogenesis of AD is started by the overproduction and extracelluar degradation of Aβ and intracellular degradation of NFT. These degradations become the starting reasons for several neurotoxic pathways which might include excitotoxicity, Ca^{2+} homeostatic interference, free radical productivity and inflammation in neurons. Meanwhile, the

cholinergic theory reveals damage of cholinergic markers like choline acetyltransferase (ChAT) and AchE and degeneration of acetyl choline neurotransmitter causing the cognitive and memory functions to diminish (Parihar & Hemnani, 2003).

2.1.5.3 Tau hypothesis

Tau proteins which richly exist in neurons of the central nervous system stabilize the microtubules. In this progression, the altered hyperphosphorylated protein tau starts to pair with other threads of tau and produce neurofibrillary tangles inside nerve cell bodies. The formation of neurofibrillary tangles will cause the breakdown of microtubules and collapsing the neuron‘s transport system. Eventually, this can lead to failures in biochemical communication between neurons and results to cells death (Singh *et al*., 2013).

2.1.5.4 Mitochondrial dysfunction

Since malfunctioning energy metabolism is a fundamental component of AD, mitochondrial dysfunction is monitored in AD brain and has been suggested as an underlying mechanism of the disease pathogenesis. Additionally, early defects in glucose utilization in the brain of AD patients propose feasible abnormalities in mitochondrial function (Müller *et al*., 2010). Mitochondria are motile and vital organelles. The build-up of mitochondria in synapses is based on mitochondrial transport to neuronal terminals. Motility alteration of mitochondria has been detected in patients with AD. As a main contributing factor of AD, Aβ interrupts mitochondrial motility and vitality in neurites, hence causing of disordered synaptic mitochondrial dispersal (Du *et al*., 2012).

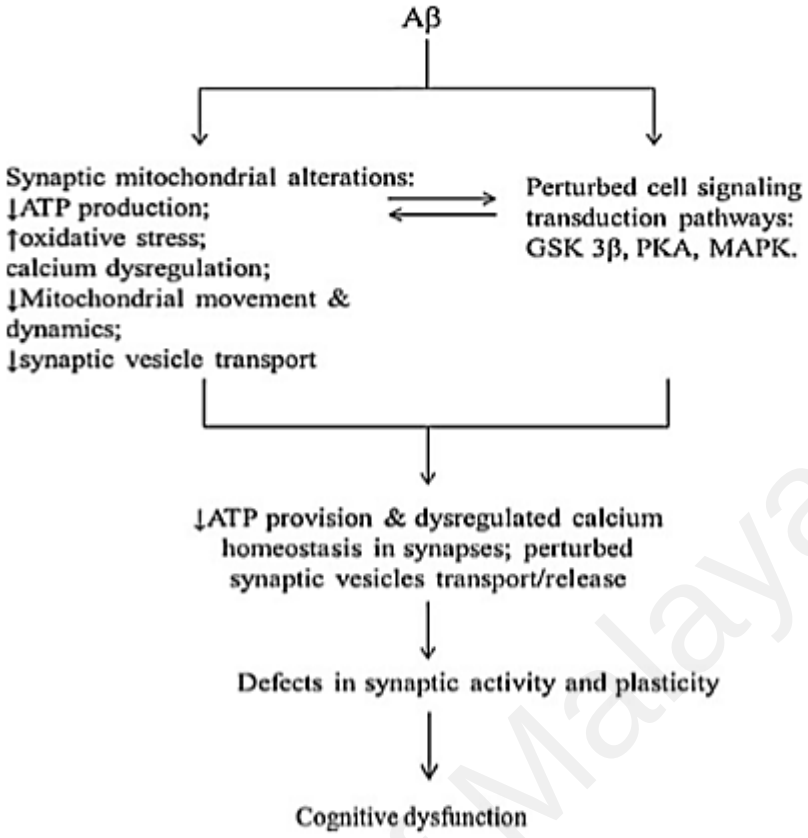


Figure 2.4: Mitochondrial dysfunction (Du *et al*., 2012). Reprinted permission granted by Du.

In the existence of Aβ, mitochondrial transport and vitality are damaged with injured synaptic mitochondrial structure and function, thus leading to the reduced of energy metabolism, decontrolled calcium homeostasis, and disturbed cell signalling cascades, finally leading to synaptic injury and cognitive dysfunction (Du *et al*., 2012).

2.2 Acetylcholinesterase inhibition in Alzheimer's Disease

2.2.1 Choline, Acetylcholine and Acetylcholinesterase

Choline and its derivatives are components of structural lipoproteins, blood and membrane lipids (Ueland, 2011). It is an intermediate in the construction of acetylcholine, a neurotransmitter that is crucial to many processes of the central and peripheral nervous systems, including motor, arousal, as well as cognitive functioning specifically, memory (Arenth *et al.,* 2011). In cholinergic neurons, choline is acetylated

to form the acetylcholine (Ueland, 2011). Choline is an important nutrient in humans and several researches has proved its role in neurodevelopment of rodents (Ueland, 2011).

Three varieties of neurotransmitters frequently affected by AD are acetylcholine, serotonin, and norepinephrine. Among three of these, acetylcholine is affected the most. ACh was found in the 1920s, thus making ACh is the earliest known neurotransmitter. It can be found in the brain, neuromuscular junctions, spinal cord, as well as in the postganglionic terminal buttons of the parasympathetic division of the autonomic nervous system and the ganglia of the autonomic nervous system (Alzheimer's, Memory, And Acetylcholine, 2015).

Acetylcholinesterase is one of the α/β hydrolase protein super family which possessed an important role in acetylcholine-mediated neurotransmission (Singh *et al*., 2013). AChE hydrolyses ACh in which when released from synaptic vesicles shortly depolarises the postsynaptic cell membrane. ACh is then hydrolysed by acetylcholinesterase to choline and acetate (López & Pascual-Villalobos 2010).

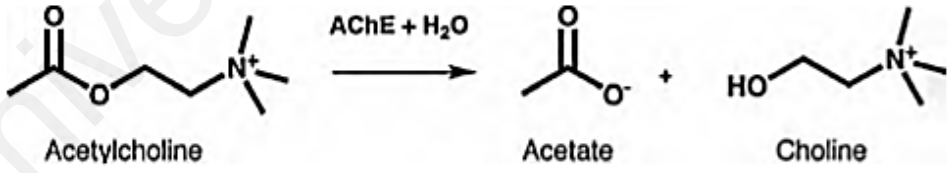


Figure 2.5: Enzymatic hydrolysis of ACh by AChE (Dvir *et al*., 2010). Reprinted permission granted by Dvir.

Past researches on the function and structure of AChE showed that the enzyme consist of two binding sites which are catalytic anionic site (CAS) and peripheral anionic site (PAS). It was suggested that PAS could encourage the removal and accumulation of Aβ in the brain (Akrami *et al*., 2014).

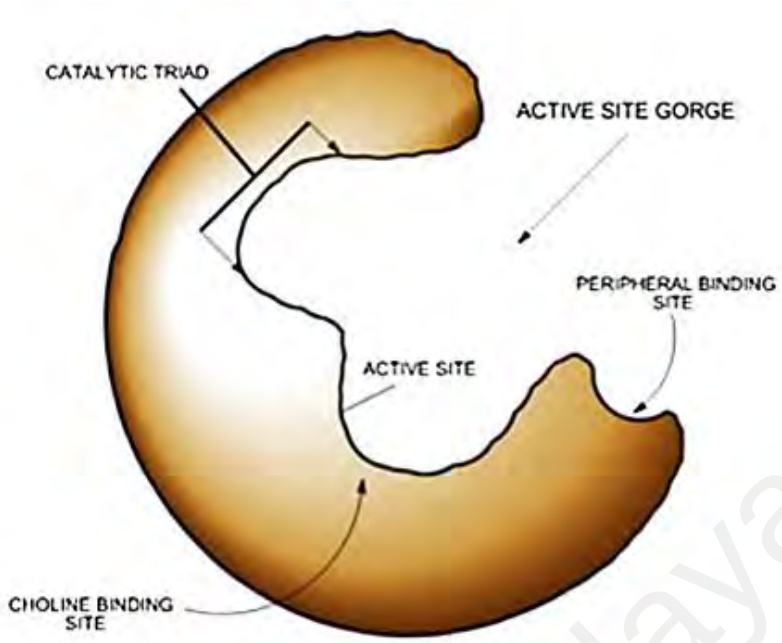


Figure 2.6: Diagrammatic representation of active site of cholinesterase (Singh *et al*., 2013). Reprinted permission granted by Singh.

The key biological role of AChE is the termination of impulse communication at cholinergic synapses by abrupt hydrolysis of the acetylcholine. Acetylcholinesterase is a very rapid enzyme which functioning at a speed approaching that of a diffusion-controlled reaction (Dvir *et al*., 2010).

2.2.2 Acetylcholinesterase Inhibitors (AChEI)

Levels of acetylcholine which is the central chemical messenger in the brain are depressed in AD. Treatments that serve to proliferate its levels by obstructing a substance called cholinesterase can enhance behaviour and thinking in patients with Alzheimer's disease. To reimburse the decreased of synthesis and synaptic availability of acetylcholine, researchers‘ attention was focused on inhibitors of cholinesterase; the enzymes hydrolysing acetylcholine released in the synaptic cleft (Contestabile, 2011). The functions of AChEI are to prevent acetylcholinesterase activity and thus enhance the levels of ACh available for postsynaptic stimulation. AChEI is considered as

symptomatic management for AD because it only improves the symptoms of AD without modifying its natural clinical course (Massoud & Léger 2011).

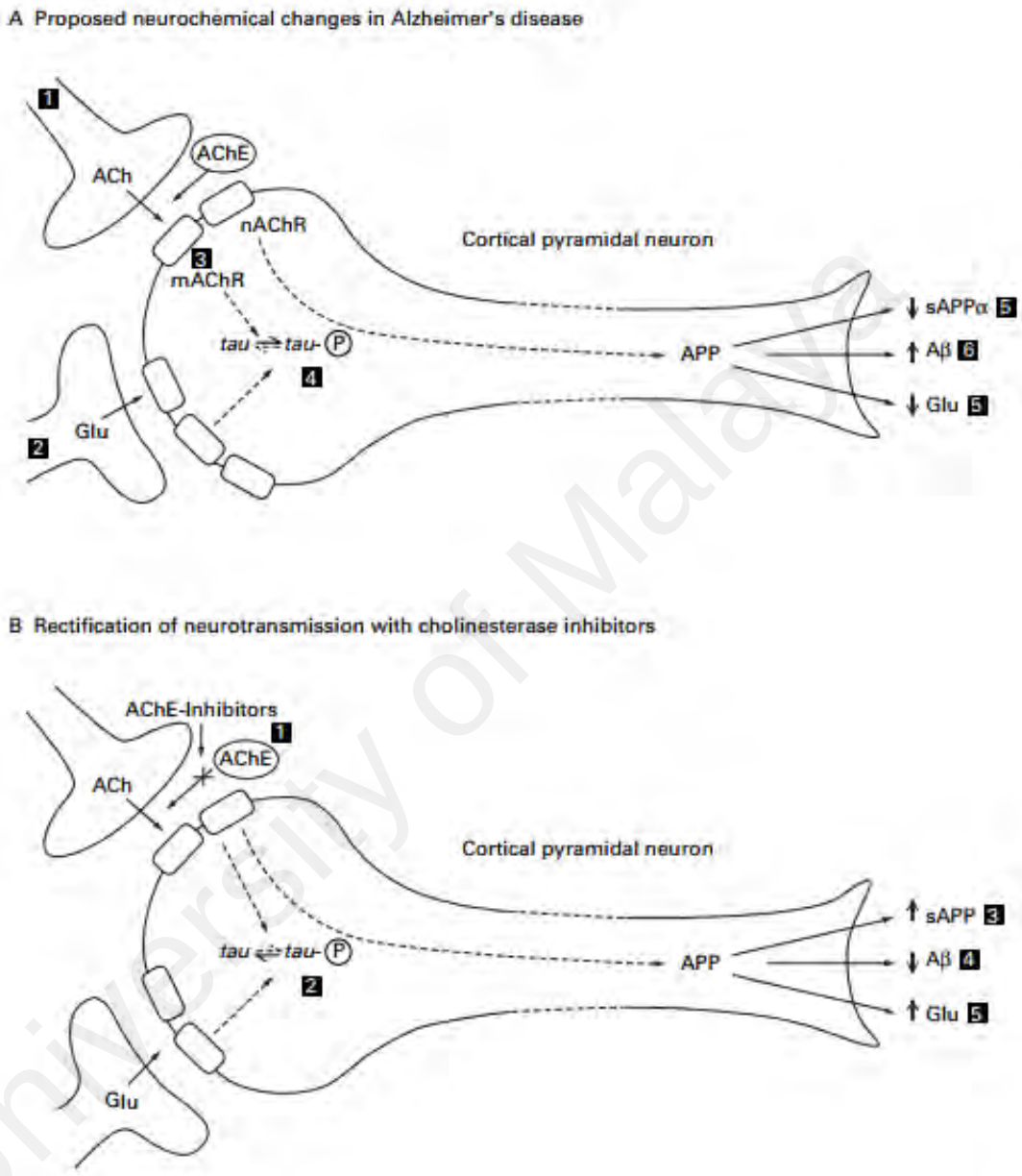


Figure 2.7: Diagram of a neuron demonstrating (A) changes in neurotransmission in Alzheimer's disease and (B) the hypothetical mode of action of AChE inhibitor (Francis *et al*., 1999). Reprinted permission granted by Francis.

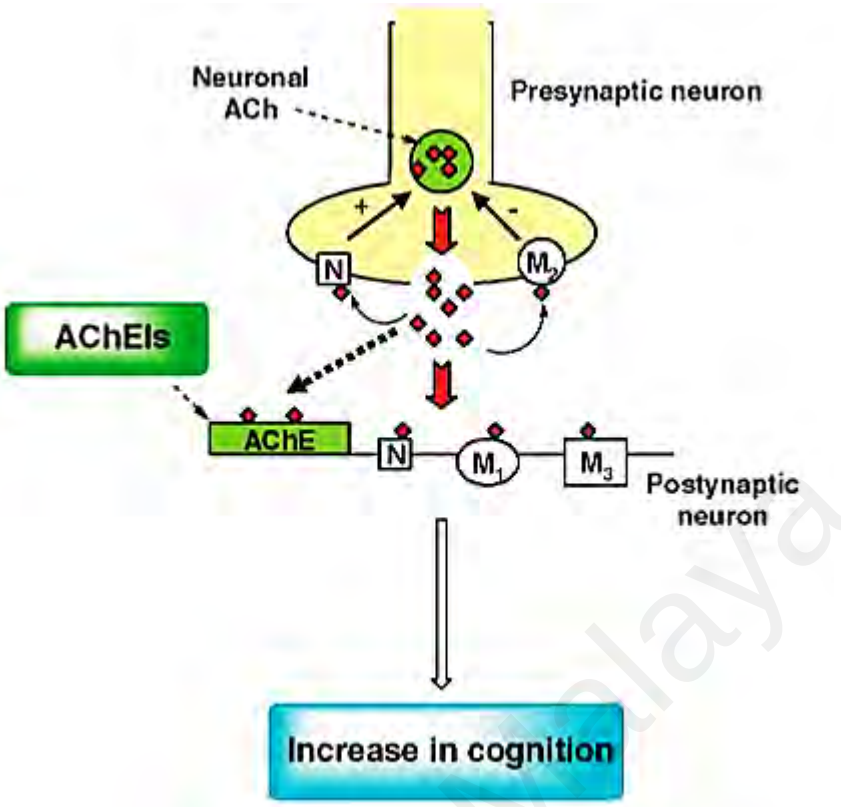


Figure 2.8: Mechanism of action of ACh at a cholinergic synapse (Cavalli *et al*., 2008). Reprinted permission granted by Cavalli.

According to the figure above, ACh is released in the synaptic cleft and leads to activation of both postsynaptic and presynaptic cholinergic receptors which are nicotinic (N) and muscarinic (M). It will cause the increasing in cholinergic transmission, which effects in cognition enhancement. ACh is removed from the synapse by the act of the enzyme AChE, which is the objective of the available AChEIs for AD treatment (Cavalli *et al*., 2008).

Throughout the last twenty years, professionals have completed extensive investigation on minimizing or clearing the related AD pathological occurrences with different strategies. By means of the cholinergic hypothesis, two important strategies of evolving AChE inhibitors and designing ACh receptor agonists were used to stabilize the Ach level (Sun *et al*., 2014). The cholinesterase inhibitors have been

comprehensively studied in treatment of AD and their effectiveness along with limitations are well-known (Nygaard, 2013). From all the hypotheses of the AD pathogenesis, the cholinergic hypothesis is the earliest and gave the strongest impact on the advance of clinical treatment plans. Acetylcholinesterase inhibitors are used in the management of numerous neurological disorders and are the primary medications permitted thus far by The Food and Drug Administration (FDA) for treatments of Alzheimer‘s disease (Dvir *et al*., 2010). Among six varieties of drugs that have been approved by FDA for AD treatment, five of them are AChE inhibitors (Sun *et al*., 2014). In 1993, it directed to the establishment of the AChEI tacrine which is the first drug to be permitted for the cure of AD. Then, three other AChEIs which are donepezil, rivastigmine, as well as galantamine entered the market and becoming the standard for AD treatment. Later the treatment was supplemented by memantine, a non-competitive N-methyl-D-aspartate (NMDA) antagonist (Cavalli *et al*., 2008).

Table 2.1: Drugs currently being used for the treatment of Alzheimer's disease (Ferris, 2001).

Drug	Percentage (%)
Donepezil	50
Risperidone	9
Vitamin E	3
Olanzapine	3
Haloperidol	3
Lorazepam	3
Aspirin	2
Rivastigmine	6
Sertraline	2
Divalproex	1
Others	18

As stated by Ferris (2001), from all the medications shown in the figure above, only donepezil and rivastigmine are accepted for the management of Alzheimer's disease. Tacrine, which was permitted by the US Food and Drug Administration in 1993, is infrequently used now for the reason of its high potential for hepatotoxicity.

Donepezil, which was accepted in 1996, is the most prescribed currently (50% of all prescriptions for the treatment of AD). Rivastigmine, permitted in April 2000, currently represents 28% of prescriptions that were formerly dispensed for donepezil and 6% of all prescriptions for AD. The current endorsement of galanthamine in February 2001 has providing doctors with another alternative for treating their patients.

Individuals afflicted with mild to moderate AD prescribed with donepezil since diagnosis exhibited remarkably better cognitive results up to 3 years, compared to those in whom therapy was on hold for 1 year (Molinuevo *et al*., 2011). Tacrine is the first AChE inhibitor approved by the FDA to enter the medical marketplace. Even though it showed some side effects after a long duration of practical using, it is still of attraction because of its conventional pharmacophore for effective AChE inhibition and its familiar action mode (Sun *et al*., 2014). However, treatments with donepezil showed acceptable antagonistic side effects and usages on for more than 2 years did not exhibit remarkable rise of mortality risk (Contestabile, 2011).

Other than that is rivastigmine. It is a carbamate derived that reversibly inhibits both AChE and butyrylcholinesterase (BuChE). A Cochrane review which comprising 9 trials and 4775 patients, evaluated the advantages of rivastigmine in mild-to-moderate stage of AD. Pooled analyses indicated advantages on global, cognitive, and functional result measures (Massoud & Léger 2011). Galantamine is a tertiary alkaloid which reversibly inhibits AChE and allosterically binds to nicotinic receptors increasing cholinergic transmission. It is extracted from bulbs of the common snowdrop and several *Amaryllidaceae* plants and has been used in anaesthetics to inverse neuromuscular paralysis caused by turbocurarine-like muscle relaxants. In recent times, it has been revealed to reduce drug- and lesion-induced cognitive deficits in animal models of learning and memory (Sramek *et al*., 2000).

A Cochrane review evaluated collective data from 7298 AD patients prescribed with rivastigmine, donepezil, or galantamine at suggested doses. The finding was parallel to earlier occurrence, and treatment with any of these medications in patients with AD is linked with small but statistically expressive progressions in cognitive function, activities of daily living, behavioural disorders as well as general clinical condition (Nygaard, 2013).

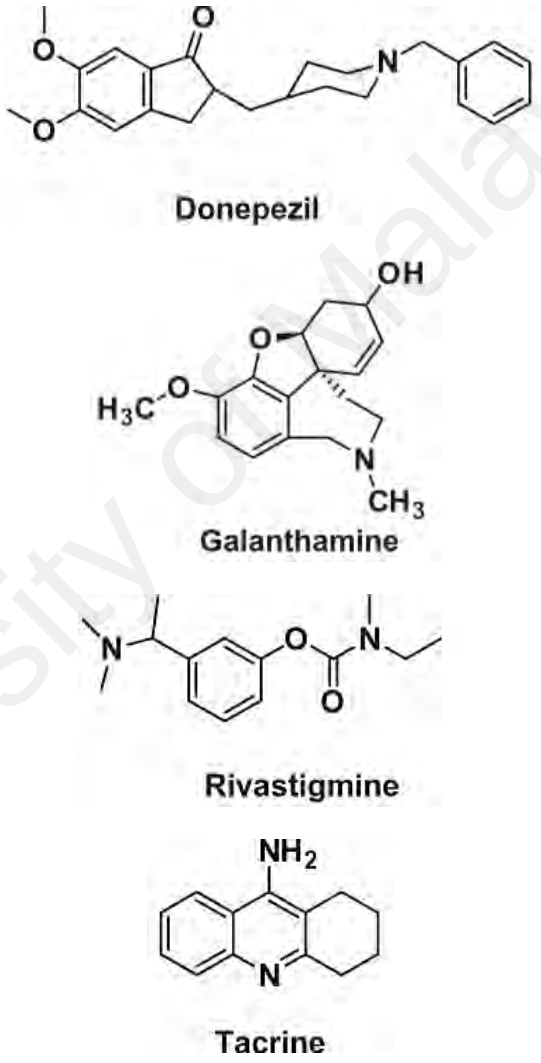


Figure 2.9: Chemical structures of the commercial AChE inhibitors for AD treatment (Sun *et al*., 2014). Reprinted permission granted by Sun.

2.2.3 Acetylcholinesterase Inhibitory Assay

AChE inhibitory assay is vital for in vitro classification of drugs such as possible cures for AD. It has become a significant implementation in designing and discovering drug besides in toxicology and medicine. A wide selection of techniques has been established over the previous years for AChE inhibitory activity determination. The mostly used assay is based on Ellman‘s method using the5, 5‘dithio-bis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide (ACTI) substrate. The enzyme hydrolyses the substrate into thiocholine and acetic acid. Thiocholine is react with DTNB and caused in the formation of a yellow color. The color concentration of the product is then measured at 405 nm, and it is proportionate to the enzyme activity. (Ali-Shtayeh *et al*., 2014). Test was repeated for a minimum of three times and the average of concentration values was calculated (Sun *et al*., 2014).

Today, the method is still generally used with substantial modifications. Although the method can potentially cause large interference of some compounds, this method has several advantages such are fast processing of large amounts of samples, simple, rapid conversion of ACTI comparing to other synthetic substrates such as naphthyle acetate and reasonably inexpensive (Ali-Shtayeh *et al*., 2014).

In previous paper, Rosini *et al*. (2005) applied the Ellman‘s method to determine the AChE inhibitory activity of potential substances of interest which are human recombinant AChE for the treatment of AD. Besides, they also determine that the study has revealed that it is potential to obtain multipotent drugs for the treatment of AD: lipocrine developed in in vitro models as a convincing prospect to be studied in vivo for its numerous biological properties which are inhibition of AChE-induced αβ aggregation, inhibition of AChE and BChE activities as well as ability to protect cells against ROS.

2.3 Oxidative stress

Oxidative stress (OS) has been proposed as a pathogenic mechanism in Alzheimer's disease. It has long been accepted that the role of oxidative stress in AD is critical which leads to the destruction of essential cellular components such as proteins, lipids, and nucleic acids. If left uncontrolled, the damage will be the main reason for the ultimate degeneration of neurons, feasibly through apoptotic manners (Swomley *et al*., 2014).

Degenerative disease is triggered by free radicals in our body that are capable to destruct living tissues and eventually will lead to death of cells (Ahmat *et al*., 2012). Currently, several researches have concentrated into the function of free radical formation and oxidative cell destruction in the pathogenesis of AD. Recent study has also proved that oxidative stress acts as a crucial role in commencing the accumulation of Aβ and tau protein hyperphosphorylation, associated in the early phase of the pathologic cascade. Therefore, oxidative stress has become the significant objective for AD treatment and some antioxidants have been tried in clinical trials (Thiratmatrakul *et al*., 2014).

Oxidants comprise of ROS and RNS. The commencement and proliferation of ROS and RNS production have been revealed to play a key role in the pathogenesis of AD. Therefore, it is significant to know the basis of these oxidants, besides their modes of action. Example of ROS and RNS are including superoxide radical anion (O2 −•), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), nitric oxide (•NO), and peroxynitri (ONOO−), most of which are free radicals (Swomley *et al*., 2014).

In any functional aerobic cell, the activities take part in respiration unescapably produce ROS .In precise, the oxidation-reduction reactions is essential for the production of ATP through the establishment of a proton gradient in oxidative

phosphorylation that create free radical intermediates such as electrons are moved from one molecule to another molecule (Bonda *et al.*, 2010).

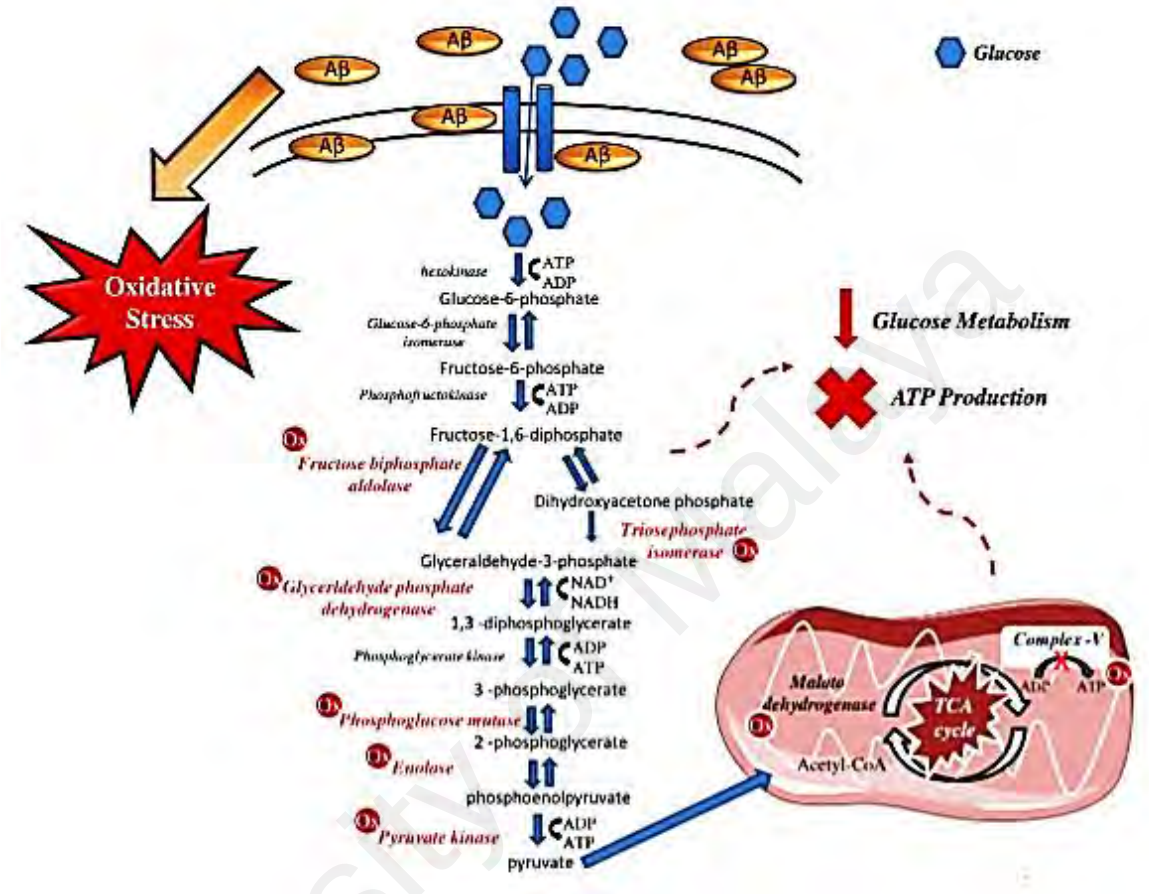


Figure 2.10: Protein oxidation of enzymes involved in energy metabolism (Tramutola *et al*., 2017). Reprinted permission granted by Tramutola.

In particular, amplified yield of Aβ will brings OS that causes the oxidation of glycolytic enzymes (highlighted in red) and tricarboxylic acid cycle (TCA) enzymes, (highlighted in black). The oxidative alterations of the targeted enzymes showed in the picture lead up to reduce of glucose metabolism and lessened the synthesis of Adenosine triphosphate (ATP) in AD brain. Defeat of ATP synthase activity will crucially cause ATP levels to decrease, feasibly evolving in electron leakage and accumulated ROS production, proposing an alternative reasoning for the OS detected in AD (Tramutola *et al*., 2017).

ROS are produced under normal environments and their volumes are kept fairly low by the slight balance between the rate of their productivity and the rate of their clearance by antioxidant and corresponding enzymes. Therefore, either boosted ROS production or reduced antioxidant system will end the cellular redox balance to oxidative disproportion and lead to ROS overproduction. ROS are regularly very sensitive, unstable and have an exceptionally short half-life, hence making them complicated to estimate directly. Oxidized biomolecule products produced by ROS are considerably more stable and usually used as ROS markers. Moreover, ROS could also be measured indirectly by assessing either antioxidant levels or antioxidant enzyme activity (Wang *et al.*, 2014).

In addition, Wang *et al*. (2014) stated that ROS are inevitable physiological side effects which function as a double-edged sword in the biological system. They can aid critical roles such as signalling molecules under cautiously organized conditions, but can do destruction to the biological system when exist in overabundance volume since they are able to oxidize all important. The brain is greatly prone to oxidative imbalance because of its high energy demand, high oxygen consumption, high abundance of easy peroxidizable polyunsaturated fatty acids, high level of potent ROS catalyst iron, and also relative paucity of antioxidants and associated enzymes.

The free radical concepts of aging and mitochondrial deterioration hypothesis are most common amongst the different theories of aging. Post-mortem tissue gives a solid proof for amplified levels of cellular oxidative stress in susceptible regions of AD brains compared to aged controls. Rise of protein oxidation, protein nitration, and lipid peroxidation were discovered in brain areas showing neurofibrillary tangles and amyloid plaques (Müller *et al*., 2010)**.**

It is acknowledged that AD has a prolonged dormant time span before symptoms are become noticeable and a diagnosis can be establish. Current analysis revealed that the onset of AD is usually begin by an interval stage known as MCI which there is no remarkable proliferation of senile plaques and NFTs. Certainly, MCI subjects showed significant oxidative imbalance when compared with age-matched controls. Prior and more recent studies exhibited significant reduced levels of non-enzymatic antioxidants for example vitamin C, vitamin E, uric acid, vitamin A, lutein, zeaxanthin, β-cryptoxanthin, as well as α-carotene, and declined activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione reductase in MCI patients. As MCI patients are on high risk to develop to early AD and the extensive oxidative damage in MCI might lead to the prominent AD neuropathological changes, these evidences clearly suggest that oxidative imbalance is noticeable at the very early stage of AD and is possibly a primary hallmark of the pathogenesis of AD (Wang *et al*., 2014).

Mitochondria are the main cause of oxidative stress since the inevitable electron leak during electron transfer culminates to the continuous yield of superoxide anion which, regardless of the existence of an effective mitochondrial/cellular antioxidant system, is responsible for 90% of the endogenous ROS. It is also proposed that dysfunctional mitochondria are less effective producers of ATP but more effective growers of ROS, which might signify a main source of oxidative imbalance detected in AD. Certainly, mitochondrial dysfunction is a prominent and early hallmark of AD, and nearly all aspects of mitochondrial function have been described to be diminished in AD (Wang *et al*., 2014). The pyruvate dehydrogenase complex, cytochrome oxidase, and the α-ketoglutarate dehydrogenase complex demonstrate reduced activity as a consequence of oxidative damage. Other outcomes of cellular oxidative damage are

including cell cycle aberration and tau hyperphosphorylation, which leading to the development of NFTs (Bonda *et al*., 2010).

Besides to impaired mitochondrial function, a peptides and the incidence of trace metal ions for instance copper and iron, have been determined as possible sources of OS. Aβ attachment as oligomers into the bilayer can cause the ROS production and commencing lipid peroxidation of membranes followed by intracellular protein and nucleic acids oxidation (Tramutola *et al*., 2017).

2.4 Antioxidant and its Roles in AD

The term ―antioxidant" is increasingly prevalent in current world as it increases exposure via abundance of media publicities of its health advantages. The dictionary definition of antioxidant is describe as ―a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances (as the tocopherols) being used as preservatives in various products (as in fats, oils, food products, and soaps for retarding the development of rancidity, in gasoline and other petroleum products for retarding gum formation and other undesirable changes, and in rubber for retarding aging)". Another biologically significant explanation of antioxidants is ―synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air". Additionally, in biochemistry and medicine, antioxidants are defined as enzymes or other organic materials, for example vitamin E or β-carotene which are able to neutralize the harmful consequences of oxidation in animal tissues. In nutrition science, antioxidants hold a wider range, in which they comprise elements that prevent fats in food from becoming rotten while the Institute of Medicine defined dietary antioxidants as an element in foods that remarkably declines the adversative effects of reactive species, for instance reactive oxygen and nitrogen species, on usual physiological role in human being. A dietary

antioxidant can sacrificially scavenge reactive oxygen species as well as reactive nitrogen species to halt radical chain reactions, or it can prevent the reactive oxidants from being formed primarily. Dietary antioxidants normally comprise of metal chelators, oxidative enzyme inhibitors, radical chain reaction inhibitors and antioxidant enzyme cofactors (Huang *et al.,* 2005).

In addition to the extensive growth of oxidative biomolecule products, remarkable reduction of antioxidant levels or antioxidant enzyme activity has been repeatedly recorded. The plasma levels of antioxidants such as bilirubin, uric acid, albumin, lycopene, vitamin C, vitamin A, and vitamin E were found to be reduced in AD patients (Wang *et al*., 2014).

An efficient antioxidant treatment program might possibly diminish the consequences of in vivo ROS such that cellular injury remains negligible. Ahmat *et al*. (2012) stated that the best approach to combat degenerative diseases is to improve antioxidant activity in our body system and that could be accomplished by intake of vegetables, fruits or edible plants. One of these includes the practice of using naturally occurring antioxidants, and findings actually show certain consistent potential. For example, RRR-α-tocopherol (vitamin E) has been proven to be a chain breaking antioxidant, lipid soluble, and random tests have revealed the vitamin to efficiently deliberate the development of AD (Bonda *et al*., 2010).

Also, natural agents of food supplements which could have various properties such as antioxidant, improving mitochondrial energetics, anti-inflammatory, and cross blood–brain barrier might seemingly inhibit or slow down or else sustain the individuals at their greater level of functioning. Besides, there is an increasing concern in using the polyphenolic antioxidants to inverse age-associated deterioration in neuronal signal transduction as well as in cognitive and motor activities deficiencies. For instance,

extract of *Ginkgo biloba* has been revealed to give positive reaction on cognitive function. It is further proved by an investigation on neuroprotective properties of extracts of *Asparagus racemosus, Convolvulus pleuricauas* and *Withania somnifera* against free radicals induced destruction in distinctive brain sections in experimental animals (Parihar & Hemnani, 2004).

In order to avoid oxidative destruction, the cell has developed an amount of synergistic defence systems. Antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase react in concert to catabolize ROS or RNS. Another antioxidant care comes from endogenous radical scavengers such as Glutathione (GSH) or uric acid and exogenous radical scavengers like vitamin C or secondary plant metabolites (Müller *et al*., 2010).

2.4.1 Measurement of Antioxidant Assay

Various assay techniques to measure antioxidant activity in vitro and in vivo have been established, but only limited fast and steadfast techniques is relevant to antioxidant activity assay for a massive quantity of plant extract samples available (Cai *et al*., 2004). Usually, in these techniques a radical is produced and the antioxidant ability of a sample counter to the radical is assessed (Jindal & Mohamad, 2012).

One of the method of measurement was determined by the free radical 1,1 diphenly-2-picrylhydrazyl-hydrate (DPPH) .DPPH is a free radical and receives an electron or hydrogen radical to turn into a stable diamagnetic molecule. Then, DPPH responds with an antioxidant compound that can give hydrogen and becomes reduced. The reaction will cause of change in colour from deep violet to light yellow. The strength of the colour change is relational to the antioxidant concentrations (Dhanasekaran *et al*., 2015). Originally, a reaction time of 30 minutes was suggested and has been followed in more recent studies. Shorter times also have been used in

other studies such as 5 or 10 minutes. Though, in view of the fact that the rate of reaction differs broadly amongst substrates, the best practice appears to be to follow the reaction until it has reach completion (–plateau") (Molyneux, 2004). The reaction end point is achieved when colour change stops. Then, IC_{50} is calculated. The IC_{50} value is the concentration that causes the initial amount of DPPH radicals decrease by 50%. It is the point where the active crude extract will exhibit 50% of antioxidant activity (Ahmat *et al*., 2012).

Besides, the ferric reducing antioxidant power (FRAP) assay too is based on electron-transfer reactions. This assay is established on the ability of antioxidants to reduce Fe3+ to Fe2+ in the presence of tripyridyltriazine (TPTZ) that cause the formation of an intense blue Fe2+–TPTZ complex with an absorbance maximum at 593 nm. The increase in absorbance shows an increase in reductive capability (Riaz *et al*., 2012).

Metal chelating activity is important by way of it reduces the concentration of the catalysing conversion metal in lipid peroxidation through the Fenton reaction (Jindal & Mohamad, 2012). Metal chelation activity is also an instance of a complexation reaction. Ferrozine [disodium salt of 3-(2-pyridyl)-5,6-bis(4phenylsulfonic acid)-1,2,4-triazine] is a complex-forming agent of Fe(II) and will form a magenta complex Fe(II)(Ferrozine)(III) with maximum absorbance at 562nm. When reducing agent is available, the complex formation is inhibited causing the decrease in the colour of the complex and therefore a decrease of the absorbance. The metal chelating activity of the coexisting chelators can be estimated by measuring the absorbance (Sen Gupta & Ghosh, 2013).

Other than that, the common antioxidant assay is nitric oxide radical scavenging activity (NORSA). Sodium nitroprusside in aqueous solution at physiological pH

impulsively produces nitric oxide (NO) that reacts with oxygen to create nitrite ions, which can be assessed using Griess-Illosvosy reaction. Scavengers of NO compete with oxygen hence reduced the production of NO and produce a pink coloured chromophore (Nishaa *et al*., 2012).

2.5 Alternative treatments of AD

Recently, the idea of mitochondrial defence as a treatment approach for dementia has been further reinforced by not yet final issued data on significant clinical improvement in AD patients cured with methylene blue. In some animal studies, methylene blue improves cognitive functions related with raised up oxygen consumption. Other than that, flavonoids also improve mitochondrial dysfunction and seem like to have therapeutic advantage for long term management of age-related cognitive weakening in animals and human. For example, the substantial reduction of the risk in getting AD by practicing Mediterranean diet is very possibly clarified by the high daily intake of flavonoids (Müller *et al*., 2010).

As ChE and oxidative stress are the main targets for treatment of AD, several researches have been directed to look for multifunctional substances that act on both ChE and oxidative stress, such as tacrineelipoic acid hybrid and tacrineemelatonin hybrid. These discoveries support that the combination of AChEIs and antioxidant is a potent approach for creating new multifunctional medications for AD treatment (Thiratmatrakul *et al*., 2014).

2.6 *Ampelocissus* sp.

The medicinal plants played a very significant role and have been used ever since the ancient times. The ancient ayurvedic, homeopathic, unani and siddha structure of medicines which are still prevalent primarily use raw materials from plant in nearly all

of their preparations and formulations (Anand & Patni, 2016). Natural products are recommended as a therapeutic substitute to traditional antimicrobial drugs whose efficiency is often constrained by the resistance that the infectious agents have developed against drugs (Zongo *et al*., 2010). On top of that, potent drugs are not generally affordable in developing countries. Hence, most of the people use medicinal plants to cure their health conditions. Once used only in conventional medical systems, natural products which have potential antioxidant properties are now proposed as the prevention of numerous pathological disorders for instance cancer, cardiovascular and neurodegenerative diseases (Zongo *et al*., 2010).

Kingdom -	Plantae
Order -	Vitales
Family -	Vitaceae
Subfamily -	Vitoideae
Genus -	*Ampelocissus*
Species -	*Ampelocissus* sp.

The genus *Ampelocissus* from family Vitaceae is usually used in traditional medicines to cure a variety of pathological disorders (Chaudhuri & Ray, 2014). Vitaceae is a grape family amongst commercial fruits and belongs to order Rhamnale. This family contains about 700 species and allocated into 15 genera. They are mostly climbing plants dispersed in tropical and temperate regions throughout the world (Chen & Manchester, 2007). They are woody plants with unisexual apetalous flowers. Almost all members of the family possessed characteristics as stamens opposite to petals, leaf opposite tendril and berry type of fruits (Karkamkar *et al*., 2010).

The genus *Ampelocissus* is identified by tendril-associated inflorescences, a projecting floral disc typically with 10 linear marks on its side, and also the common link of rusty arachnoid hair in young parts of the plants. It has 94 species which are habitually scattered in Malaysia, southern Asia, and Africa while five species found in Central America (Chen & Manchester, 2007).

The root decoction and fresh stem node paste of *Ampelocissus latifolia*; native herb to Indian subcontinent is very frequently used in traditional medicine to heal a variety of illnesses (Chaudhuri & Ray, 2014). The extract from tuber is also used to cure fractured bone, dyspepsia, indigestion and tuberculosis. Recently the anti-inflammatory, antioxidant and antibacterial activities of Philippines *Ampelocissus* have been reported. They have numerous phytochemicals such as alkaloids, fixed oils and fats, flavonoids, saponins, tannins, carbohydrates and glycosides (Chaudhuri & Ray, 2014). The initial chemical analyses by Anand and Patni (2016) on *Ampelocissus latifolia* (Roxb.) Planch show the existence of fairly higher amounts of tannins, saponins, terpenoids, flavonoids, carbohydrates and anthraquinones and a trace quantity of alkaloids and glycosides in the aqueous extract of its aerial parts. *Ampelocissus grantii* (Baker) Planch.; usually available in Africa are widely used in traditional medicine to treat diseases such as bacterial, parasitic, viral, protozoa and fungal diseases (Zongo *et al*., 2010).

The plant is also traded as isolated species of medicinal plant and it is the direct or indirect source of earnings for aboriginal people (Anand & Patni, 2016). Isi Nyaru is a type of the *Ampelocissus* sp. that is still not well-studied in Malaysia. It has tuber that develop underground and they are purple color in dried form. In Malaysia, this species is commonly found in mangrove wetland forests, peat wetland forests, limestone hill forests as well as at the lowland, hill dipterocarp forests and mixed dipterocarp forests. As they are photophilous plant, they are commonly found in forest gaps, along forest edges and other environments with enormous light.

The extract of *Ampelocissus* sp. is believed to have the antioxidant and acetylcholinesterase inhibitory properties as they are commonly being used as health supplement by aboriginal people in rural area of Malaysia. Therefore, this study was carried out to explore the advantages and economical value of this unexplored plant which could be commercialized in future.

Figure 2.11: Sliced and dried tuber of *Ampelocissus* sp.

Figure 2.12: Freshly harvested *Ampelocissus* sp.

CHAPTER 3

METHODOLOGY

3.1 Plant Materials

Ampelocissus sp. (*isi nyaru*) was collected from Endau Rompin, Pahang reserved forest. The material was dried and grounded into fine powder form.

3.2 Instruments and Chemical & Reagents

ELISA microplate reader Tecan Sunrise (Austria), LCMS Flexar FX-15 UHPLC, USA, Spectrophotometry UV – 1700 Shimadzu, Japan, 2,2diphenyl -1-picrylhydrazyl (DPPH), 2,4,6- tripyridyl-s-triazine (TPTZ), gallic acid monohydrate, ferrozine, sodium nitroferricyanide(III)dehydrate, hydrogen peroxide, sodium hydroxide, aluminium chloride, sodium nitrite, Griess reagent, curcumin, sodium phosphate monobasic and dibasic, 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 ($FeCl_3 \cdot 6H_2O$), ethylenediaminetetraacetic acid disodium dehydrate ($EDTANa_2 \cdot 2H_2O$), Folin-Ciocalteu phenol reagent, and sodium carbonate were purchased from Merck Chemical C0. (Malaysia). Acetylcholinesterase (EC3.1.1.7, Sigma product no C2888), acetylthiocholine iodide (ATCI), 5,5‘-dithiobis [2nitrobenzoic acid] (DTNB), and Berberine were purchased from Sigma (St. Louis, MO, USA). All chemicals used are of analytical grade and were used without further purifications.

3. 3 Chromatographic Media

Silica gel 60 F254 – precoated TLC plates (Merck, Germany) were purchased from Merck Chemical Co. (Malaysia).

3.4 Preparation of Plant Extract

The pieces of *Ampelocissus* sp. was powdered and extracted using 10 % methanol. 100 g of powdered *Ampelocissus* sp. was macerated in 500ml of 10 % methanol for 48 hours. The extract was filtered and was fractionated by liquid-liquid extraction using 250 ml of n-hexane, chloroform and ethyl acetate. The extract was collected as methanol aqueous solution. All the filtrates collected from each fractionation were concentrated to 10 ml using a rotary evaporator at medium speed at 40 °C. The extracts were kept in airtight containers until further used.

3.5 Detection of Phytochemical Compounds

3.5.1 Thin Layer Chromatography (TLC)

Silica gel 60 F_{254} – precoated TLC plates with 8 cm height and 2 cm width were used. 1cm was measured from the base of the TLC plate to mark the origin and labelled with pencil. The sample solutions were placed on the plates as bands with capillary tube. Chloroform and 10 % methanol in chloroform solution were used as mobile phase. Then, plates with the sample solutions were placed inside the developing chamber and covered, ensuring the mobile phase solvent was just below the bands. The plates were allowed to develop a separation chromatography. The plate was removed when the solvent had risen close to the top edge. The distance travelled by solvent was immediately marked using pencil and was then dried at room temperature before viewed under ultraviolet (UV) light at 254 nm.

The retardation factors (R_f) of the samples which is the distance of compound moved in chromatography relative to the solvent front were calculated. The detection of chemical compound of TLC plates were carried out by observing under visible light and UV light for any colour presence and then TLC plates were dried in fume cupboard before were sprayed with Dragendroff_s and Vanillin-sulphuric acid reagents.

a) Dragendroff's Reagent

Dragendroff's reagent was prepared according to Wagner *et al*. (2001). Two types of solutions were prepared. To prepare solution A, 0.85 g bismuth nitrate was dissolved in 10 ml glacial acetic acid and 40 ml distilled water. While solution B was prepared by dissolving 8 g potassium iodide in 30 ml distilled water. Then, stock solution was prepared by mixing 30 ml solution A and 30 ml solution B to give 60 ml stock solution. Spray reagent was prepared by mixing of 50 ml stock solution with 100 ml glacial acetic acid and 500 ml distilled water. Dragendroff's reagent was used to detect alkaloids.

b) Vanillin-sulphuric Acid Reagent

Vanillin-sulphuric acid reagent was prepared according to Wagner *et al*. (2009). First, 1 ml of concentrated sulphuric acid was added to 1 g of vanillin, and then this solution was mixed with 100 ml of ethanol. The TLC plates sprayed with this solution were then heated at 110 °C for 5-10 minutes. The reagent was used for detection of terpenoids and phenolic compounds.

3.5.2 Liquid Chromatography Mass Spectrometry/ Mass Spectrometry (LCMS/MS)

The methanol aqueous of *Ampelocissus* sp. extracts was analysed with Liquid Chromatography Mass Spectrometry (LCMS) to determine the chemical compounds

exist. Extracts was screened with AB Sciex 3200Q Trap LCMS/MS 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 a rapid screening at 15 minutes run time. Water with 0.1 % formic acid (HCO_2H) and 5 mM 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 was run with gradient mode; 10 % A to 90 % B from 0.01 minutes to 8 minute and was held for 3 minutes and back to 10 % A in 0.1 minutes and re-equilibrated for 4 minutes. Pre-run equilibration time was 1.0 minute.1 ml of concentrated sample of methanol aqueous extracts was diluted 5 times with methanol and filtered with 0.2 µM nylon filter before being analysed. Injection volume for sample was 20 µl.

3.6 Determination of total phenolic content

Total phenolic content were determined using Folin-Ciocalteu (FC) method.

a) Preparation of Folin-Ciocalteu (FC) Reagent

The reagent was prepared by mixing 20 ml of the Folin-Ciocalteu reagent with 200 mL of distilled water.

b) Preparation of 7.5% Sodium Carbonate (Na_2CO_3)

Na_2CO_3 was prepared by dissolving 7.5 g of Na_2CO_3 in 100 mlof distilled water.

c) Preparation of Gallic Acid 1mg/mL Standard Solution

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

d) Total Phenolic Assay

In a 96-wells microplate, 20 µL of sample extract was mixed with 100 µl of FC reagent. It was then incubated for 5 minutes and 75 µl of 7.5 % Na_2CO_3 solution was added. It was incubated again for 2 hours in darkness at room temperature before was measured at 740 nm using microplate reader (Tecan Sunrise, Austria). 1mg/ 1ml gallic acid was used as standard for calibration and construction of a linear regression line and water was used as blank. Total phenolic content of the extract was calculated as mg gallic acid equivalent (GAE) mg/g of dry weight extract and experiment were done in triplicate.

3.7 Determination of Total Flavonoid Content

Total flavonoid content was determined using the method described by Ablat *et al*., (2014).

a) Preparation of 5 % Sodium Nitrite ($NaNO_2$) solution

5 g of $NaNO_2$ was dissolved in 100 ml of distilled water.

b) Preparation of 10 % Aluminium Chloride ($AlCl_3$)

10 g of Aluminium Chloride ($AlCl_3$) was dissolved in 100 ml of distilled water.

c) Preparation of 1M Sodium Hydroxide (NaOH) Solution

1.599 g of Sodium Hydroxide (NaOH) was dissolved in 60 ml of distilled water.

d) Total Flavonoid Assay

Firstly, 50 µl of extract was mixed with 70 µl of distilled water and 15 µl of 5 % $NaNO_2$ solution in a 96-wells microplate. The mixture was incubated for 5 minutes at room temperature. Then, 15 µl of 10 % $AlCl_3$ solution was added. In was incubated

again for 6 minutes before 100 µl of 1 M NaOH solution was added. Then, the absorbance was measured at 510 nm using a microplate reader (Tecan Sunrise, Austria). Methanol was used as blank and the absorbance of each extracts were compared with a standard curve plotted of quercetin. The total flavonoid content of the extracts was expressed in mg quercetin equivalent (QAE) mg/g of dry weight extract.

3.8 Determination of Antioxidant Activity

3.8.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging Activity Assay

Quantitative measurements of radical scavenging assay were determined using the method described by Ablat *et al*. (2014).

a) Preparation of 50 µM DPPH Solution in Methanol

0.00197 g of DPPH was dissolved in 100 ml methanol. The solution was kept in flask wrapped in aluminium foil. The reagent is stable up to several days when stored at 4 °C in a refrigerator.

b) DPPH Scavenging Assay

The free radical scavenging activity was determined using DPPH assay by measuring hydrogen donating ability of the extracts. First, 40 µl sample extract of different concentration (0.1-2.0 mg/ml) was mixed with 200 µl of 50 µM ethanolic DPPH solution in 96-well microplate. The mixture was immediately shaken and incubated for 15 minutes in darkness at room temperature. The absorbance was measured at 517 nm using a microplate reader (Texan Sunrise, Austria). Ascorbic acid (0.1-2.0 mg/ml) and ethanol was used as standard and control respectively. The formula of DPPH free radical scavenging activity was calculated as follows:

DPPH free radical scavenging activity (%):

$$= \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} \times 100$$

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 μg/mL (Ablat *et al.*, 2014).

3.8.2 Ferric Reducing Anti-oxidant Power (FRAP)

The FRAP assay was carried out by using method described by Ablat *et al*., (2014).

a) Preparation of Acetate Buffer 0.3M

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

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

0.031 g of TPTZ was added to 10 mL of 40 mM HCl and dissolved in water bath at 50 °C. TPTZ was freshly prepared each time assay was carried out.

c) 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 before assay was carried out.

d) FRAP Assay

FRAP reagent which consists of 5mL 10mM TPTZ in 40 mM HCl, 5 ml 20 mM $FeCl_3$, and 50 ml 0.3 M acetate buffer (pH 3.6) was freshly prepared before the experiment. First, 20 μl extracts were mixed with 200 μl FRAP reagent in 96-well

microplate. Then the mixture was incubated for 8 minutes and absorbance was measured at 595 nm using microplate reader (Texan Sunrise, Austria). Ethanol and ferrous sulphate ($FeSO_4$) solution (0.1-1.0 mM) was applied as blank and standard respectively. The results were expressed as mmol Fe^{2+} /g of dry extract from triplicated tests (Ablat *et al.,* 2014).

3.8.3 Metal Chelating Activity Assay

The ferrous ion chelating activity of the *Ampelocissus sp.* extracts was determined according to the procedure by Ablat *et al.* (2014) by measuring the formation of the Fe^{2+}-Ferrozine complex.

a) Preparation of 5mM of Ferrozine (FZ)

A stock of 0.0264 g of FZ was dissolved in 10 ml deionized water. The stock solution was kept in a centrifuge tube and wrapped with aluminium foil.

b) Preparation of 2 mM Ferum Chloride ($FeCl_2$)

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

c) Metal Chelating Assay

100 μl sample extracts (10-160 μg/ml) were mixed with 120 μl distilled water and 10 μl 2 mM $FeCl_2$ in 96-well microplate. 20 μl 5mM ferrozine was added to initiate the reaction. After 20 minutes of incubation, absorbance at 562 nm was measured. EDTA-Na_2 (0.025-1.0 mg/ml) was used as standard. Control was 100 μl ethanol and blank was without ferrozine (20 μl distilled water instead of ferrozine). The percentage of Fe^{2+}-ferrozine complex inhibition was calculated using the following formula:

Ferrous ion chelating activity (%):

$$= \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} \times 100$$

The concentration of extracts needed to chelate 50 % of Fe^{2+} ion (IC_{50}) was estimated from graph plotted against the percentage inhibition and compared to standard. All tests were done in duplicate and results expressed as mg/ml (Ablat *et al.*, 2014).

3.8.4 Nitric Oxide Radical Scavenging Assay (NORSA)

The nitric oxide radical scavenging activity assay of *Ampelocissus* sp. extracts were done according to the procedure by Srivastava *et al*. (2012) by measuring the formation of the nitrite ions in the reaction mixture that can be detected by Griess reagent.

a) Preparation of 20 mM Phosphate Buffered Saline (pH 7.4)

0.1 M Phosphate buffer was prepared following to phosphate buffered saline system. They are contain of the following reagents: potassium phosphate (monobasic) (KH_2PO_4), potassium phosphate (dibasic) (K_2HPO_4), and Sodium chloride (NaCl). Solution A: 27.6 g of KH_2PO_4 (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. 20 mM phosphate buffer was prepared by diluting 10 ml 0.1 M phosphate buffer with 40 ml distilled water. The pH of the buffer was then adjusted to 7.4.

b) 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.

c) Preparation of Griess reagent

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

d) Nitric Oxide Radical Scavenging Activity

First, 50 µl 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-wells microplate. Then, the mixture was incubated at room temperature for 150 minutes and 125 μl of Griess reagent was added. After 10 minutes, the absorbance was measured at 546 nm using a microplate reader (Tecan Sunrise, Austria). Curcumin (0.1-2.0 mg/ml) and ethanol were used as a standard and control, respectively. The reaction mixture without Griess reagent was served as blank. The percent inhibition of nitric oxide was calculated using the formula:

$$\text{Nitric oxide radical scavenging activity (\%)} = \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} \times 100$$

The concentration of extracts needed to scavenge 50 % of the nitric oxide (IC_{50}) was estimated from the graph against the percentage of inhibition. All the tests were performed in duplicate, and the results were expressed as mg/ml (Ablat *et al.*, 2014).

3.8.5 Superoxide Scavenging Activity Assay

The superoxide scavenging activity was done by phenazine methosulphate-nicotinamide adenine (PMS-NADH) systems as described by Shukla *et al.* (2012).

a) Preparation of 0.1M Tris-HCl Buffer

The stock buffer solution was prepared by dissolving 121.11 g Tris base in 800 ml of distilled water. The desired pH was adjusted with concentrated 1 M HCl. The volume was adjusted to 1 L with distilled water. 0.1 M tris-HCl buffer was prepared by diluting 5 ml of 1 M Tris-HCl buffer with 45 ml of distilled water. Then, the pH was adjusted to 8 with concentrated 1 M HCl.

b) Preparation of 0.2 mM Nitro Blue Tetrazolium (NBT) solution

0.0016 g of stock NBT was dissolved in 10 ml of distilled water.

c) Preparation of 0.5 mM Nicotinamide Adenine Dinucleotide (NADH) solution

0.0038 g of stock NADH was dissolved in 10 ml Tris-HCl buffer.

d) Preparation of 25 µM Phenazine Methosulphate (PMS) solution

0.0007 g of stock PMS was dissolved in 10 ml Tris-HCl buffer.

e) Superoxide Radical Scavenging Assay

Firstly, 50 µl of NBT solution (0.2 mM in distilled water) with 50 µl NADH solution (0.5 mmol/L in 0.1 M Tris-HCl, pH 8.0) and 100 µl of extract at each concentrations were mixed and treated with 50 µl of PMS solution (25 µM PMS in distilled water). The mixture was then incubated at room temperature for 10 minutes and the absorbance at 570 nm was measured. Quercetin and ascorbic acid were used as positive control.

The percentage of scavenging was calculated using the formula:

$$\text{Superoxide Scavenging Activity} = \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} \times 100$$

3.9 AChE Inhibitory Activity Assay

3.9.1 Preparation of Extract

Thin layer chromatography was carried out according to the method described in 3.2.2.1. Once the visualisation using UV and visible light is completed and the bands on each TLC plates were labelled, the silica layer on the TLC plate was scraped to get the compound present in the band. The silica powder obtained was transferred into respective pre-weight Eppendorf tubes and suspended with distilled water.

After that, they were centrifuged at 5000 rpm for 5 minutes. The supernatant was taken out and transferred to another Eppendorf tube. The remaining pellet was dried for two days and later, they were weighted. The compound and extract concentration were calculated. The samples obtained were used for acetylcholinesterase inhibition assay.

3.9.2 AChE Inhibition Assay

As described by Yang *et al*. (2011), Ellman's method was used to determine the AChE inhibitory activity of *Ampelocissus* sp. extract. Briefly, 140 µl of 0.1 M sodium phosphate buffer, pH 8, and 20 µl of 1 mg/ml sample solution were mixed with 15 µl AChE with an activity of 0.25 U/ml. The mixtures were then pre-incubated at 4 °C for 20 min. 10 µl of 0.01 M DTNB and 10 µl of 0.075 M acetylthiocholine iodide (ATCI) were added to start the reaction. The extracts were incubated at room temperature for 20 min and later, the absorbance at 405 nm was measured with an enzyme linked

immunosorbent assay (ELISA) reader. Berberine was used as positive control. The experiments were performed in triplicate.

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

$$\text{Percentage of AChE inhibition} = \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} \times 100$$

3.10 Statistical Analysis

All results were expressed as the mean ± standard error (SEM) for the three independent experiments. Differences between extracts were analysed by one way analysis of variance (ANOVA) followed by Duncan's post hoc multiple comparison test at the 5 % level ($P < 0.05$). The statistical program (SPSS 20.0 version, Chicago, IL, USA) was performed in the entire test.

CHAPTER 4

RESULT

4.1 Preparation of *Ampelocissus* sp. (*isi nyaru*) extracts

The dry tubers of *Ampelocissus* sp. was grounded to fine powder and extracted using 10 % methanol. 100 g of powdered *Ampelocissus sp*. was macerated in 500 ml of 10 % methanol for 48 hours. The extract was filtered and fractionated with 250 ml of n-hexane, chloroform and ethyl acetate solvent. The remaining fraction was collected as methanol aqueous solution. All the filtrates collected from each fractionation were concentrated using a vacuum rotary evaporator. Each 15 ml of the concentrated extract was dried to obtain dry weight and the yield percentage was determined. The result in Table 4.1 showed that in descending order, the yields of methanol aqueous extract > ethyl acetate extract > n-hexane extract > chloroform extract.

Table 4.1: Yields of *Ampelocissus* sp. (*isi nyaru*) extracts.

Extract	Yield from the dry sample (%)
n- Hexane	0.362
Chloroform	0.047
Ethyl Acetate	0.593
Methanol aqueous	0.6

4.2 Detection of chemical compounds

4.2.1 Thin layer chromatography (TLC)

Thin layer chromatography was carried out to detect the presence of chemical compounds in *Ampelocissus* sp. (*isi nyaru*) extracts. Silica gel 60 F_{254} – precoated TLC plates with 8 cm height and 2 cm width were used with two different solvent systems

which are 100 % chloroform and 10 % methanol in chloroform. When the separation chromatography was developed, the chemical compound was visualised by spraying the TLC plates with reagents which are Dragendroff‘s and vanillin-sulphuric acid reagent.

The separation of TLC with solvent 100 % chloroform, the saponin and alkaloids were detected in ethyl acetate extract of *Ampelocissus* sp. The alkaloids and terpenoids were detected in both n-hexane and methanol aqueous extract after TLC plate was sprayed with Dragendroff‘s and Vanillin reagent, respectively.

The TLC separation with solvent of 10 % methanol in chloroform, the saponin and alkaloids were detected in ethyl acetate extract. The alkaloids, terpenoids and saponin were found in chloroform extract, while ethyl acetate extract showed the presence of alkaloids, saponin, terpenoids and flavonoids. The methanol aqueous extract showed no visible band under visible light and UV light. However, medium intensity orange band color was spotted after sprayed with Dragendroff‘s reagent which indicates the presence of alkaloids.

Table 4.2: Thin Layer Chromatography of *Ampelocissus* sp. (*isi nyaru*) extracts with 100 % chloroform solvent.

Label Compounds	Plant extract	R_f Value	Observation				Remarks
			Visible light	UV light	Reagent Vanillin	Reagent Dragendroff`s	
AMP-CHL1	Ethyl acetate	0.89	-ve	Blue (+)	-ve	-ve	Saponin
AMP-CHL2	Ethyl acetate	0.35	-ve	-ve	Blue (+)	-ve	Saponin
AMP-CHL3	Ethyl acetate	0.46	-ve	-ve	-ve	Orange (+++)	Alkaloids
AMP-CHL4	N-hexane	0.37	-ve	-ve	Orange (++)	-ve	Alkaloids
AMP-CHL5	N-hexane	0.92	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-CHL6	Chloroform	0.37	-ve	-ve	Orange (+)	-ve	Alkaloids
AMP-CHL7	Methanol aqueous	0.91	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-CHL8	Methanol aqueous	0.03	-ve	-ve	-ve	Orange (+++)	Alkaloids

Indication for intensity of colour

+++ = Strong; ++ = medium; + = Weak; -ve = No color observed

Table 4.3: Thin Layer Chromatography of *Ampelocissus* sp. (*isi nyaru*) extracts with 10 % methanol in chloroform solvent.

Label Compounds	Plant extract	R_f Value	Observation				
			Visible light	UV light	Reagent Vanillin	Reagent Dragendroffs	Remarks
AMP-Met1	n-hexane	0.95	-ve	-ve	Orange (+)	-ve	Alkaloids
AMP-Met2	n-hexane	0.77	-ve	-ve	Orange (++)	-ve	Alkaloids
AMP-Met3	n-hexane	0.94	-ve	Blue (+)		-ve	Saponin
AMP-Met4	n-hexane	0.26	-ve	-ve	-ve	Orange (++)	Alkaloids
AMP-Met5	Chloroform	0.28	Orange (+)	-ve	-ve	-ve	Alkaloids
AMP-Met6	Chroloform	0.75	-ve	-ve	Purple (++)	-ve	Terpenoids
AMP-Met7	Chloroform	0.46	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-Met8	Chroloform	0.88	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-Met9	Chloroform	0.94	-ve	Blue (+)	-ve	-ve	Saponin
AMP-Met10	Chloroform	0.26	-ve	-ve	-ve	Orange (++)	Alkaloids
AMP-Met11	Ethyl acetate	0.26	Orange (++)	-ve	-ve	-ve	Alkaloids
AMP-Met12	Ethyl acetate	0.11	Orange (+++)	Orange (+++)	-ve	-ve	Alkaloids
AMP-Met13	Ethyl acetate	0.03	Orange (++)	-ve	-ve	-ve	Alkaloids

Indication for intensity of colour

+++ = Strong; ++ = medium; + = Weak; -ve = No color observed

Table 4.3, continued.

Label Compounds	Plant extract	R_f Value	Observation				
			Visible light	UV light	Reagent Vanillin	Reagent Dragendroffs	Remarks
AMP-Met14	Ethyl acetate	0.97	-ve	Blue (+)	-ve	-ve	Saponin
AMP-Met15	Ethyl acetate	0.72	-ve	-ve	Purple (++)	-ve	Terpenoids
AMP-Met16	Ethyl acetate	0.05	-ve	-ve	Orange (+++)	-ve	Alkaloids
AMP-Met17	Ethyl acetate	0.62	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-Met18	Ethyl acetate	0.51	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-Met19	Ethyl acetate	0.45	-ve	-ve	Purple (+)	-ve	Terpenoids
AMP-Met20	Ethyl acetate	0.15	-ve	-ve	Orange (+)	-ve	Alkaloids
AMP-Met21	Ethyl acetate	0.23	Orange (+)	Orange (+++)	-ve	-ve	Alkaloids
AMP-Met22	Ethyl acetate	0.37	-ve	Yellow (+++)	-ve	-ve	Flavonoids
AMP-Met23	Ethyl acetate	0.32	-ve	Blue (++)	-ve	-ve	Saponin
AMP-Met24	Ethyl acetate	0.28	-ve	Orange (+++)	-ve	-ve	Alkaloids
AMP-Met25	Ethyl acetate	0.25	-ve	Orange (+)	-ve	-ve	Alkaloids

Indication for intensity of colour

+++ = Strong; ++ = medium; + = Weak; -ve = No color observed

Table 4.3, continued.

Label Compounds	Plant extract	R_f Value	Observation				
			Visible light	UV light	Reagent Vanillin	Reagent Dragendroffs	Remarks
AMP-Met26	Ethyl acetate	0.28	-ve	Blue (+++)	-ve	-ve	Saponin
AMP-Met27	Ethyl acetate	0.09	Orange (++)	-ve	-ve	-ve	Alkaloids
AMP-Met28	Ethyl acetate	0.38	-ve	Blue (+)	-ve	-ve	Saponin
AMP-Met29	Ethyl acetate	0.34	-ve	Yellow (+++)	-ve	-ve	Flavonoids
AMP-Met30	Ethyl acetate	0.09	-ve	Yellow (++)	-ve	-ve	Flavonoids
AMP-Met31	Ethyl acetate	0.17	-ve	-ve	-ve	Orange (++)	Alkaloids
AMP-Met32	Methanol aqueous	0.18	-ve	-ve	-ve	Orange (++)	Alkaloids

Indication for intensity of colour

+++ = Strong; ++ = medium; + = Weak; -ve = No color observed

4.2.2 Liquid Chromatography Mass Spectrometry (LCMS/MS)

Liquid Chromatography Mass Spectrometry combined with Mass Spectrometry (LCMS/MS) was used to detect the chemical compounds presence in *Ampelocissus* sp. extracts. The methanol aqueous extract was analysed with AB Sciex 3200Qtrap LCMS/MS and followed by MS/MS data collection.

The full LCMS chromatogram showed 12 peaks were separated at different retention time (RT) of 0.495‘, 0.8‘, 1.105‘, 1.207‘, 1.223‘, 1.283‘, 1.296‘, 3.328‘, 3.587‘, 5.061‘, 5.107‘, 6.145‘. 12 phytochemical compounds were identified in the extract which are 5-aminopentanoic acid, 11-amino-undecanoic acid, C16 sphinganine, (+)-eudesmin, his lys cys, lys his met, his met lys, ethephon, L-arginine, sulpho NONOate, gln gln trp and dichloroacetate.

Table 4.4 summarized the chemical structure, retention time, mass and name of the compounds spotted in methanol aqueous extracts using LCMS/MS.

Table 4.4: Chemical structure, retention time (RT), mass and name of compounds detected in methanol aqueous extracts using LCMS/MS.

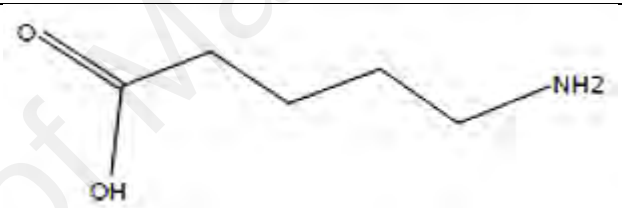
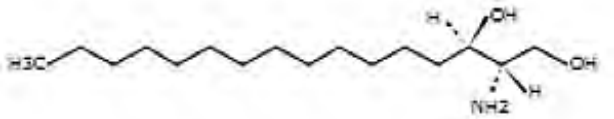
No	Name	Compound formula	RT	Mass	Chemical structure	Reference
1	5-aminopentanoic acid	C5 H11 N O2	0.495	117.0788		Figure 8.1 (Appendix A)
2	11-amino-undecanoic acid	C11 H23 N O2	0.8	201.1739		Figure 8.2 (Appendix A)
3	C16 sphinganine	C16 H35 N O2	1.105	273.2668		Figure 8.3 (Appendix A)

Table 4.4, continued.

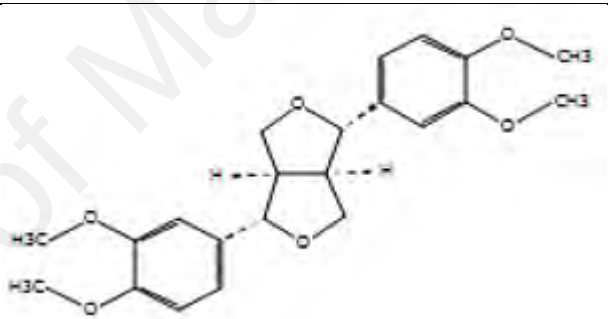
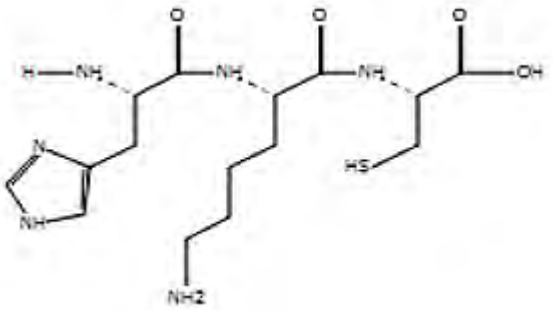
No	Name	Compound formula	RT	Mass	Chemical structure	Reference
4	(+)-eudesmin	C22 H26 O6	1.207	386.1739		Figure 8.4 (Appendix A)
5	his lys cys	C15 H26 N6 O4 S	1.223	386.1737		Figure 8.5 (Appendix A)

Table 4.4, continued.

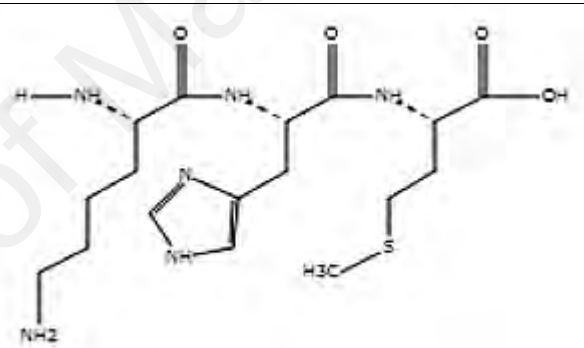
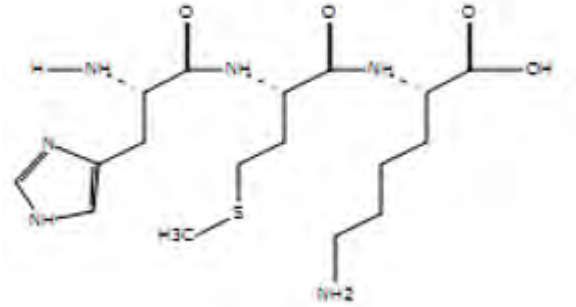
No	Name	Compound formula	RT	Mass	Chemical structure	Reference
6	Lys his met	C17 H30 N6 O4 S	1.283	414.2055		Figure 8.6 (Appendix A)
7	His met lys	C17 H30 N6 O4 S	1.296	414.2055		Figure 8.7 (Appendix A)

Table 4.4, continued.

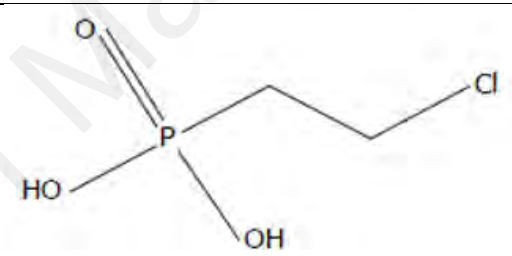
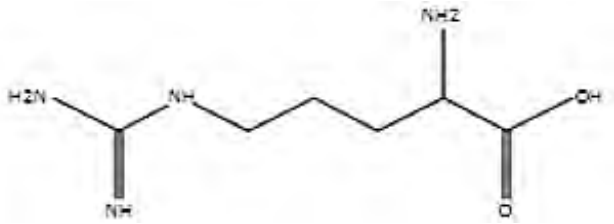
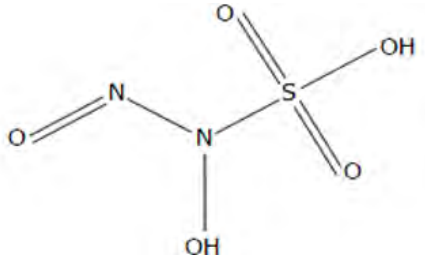
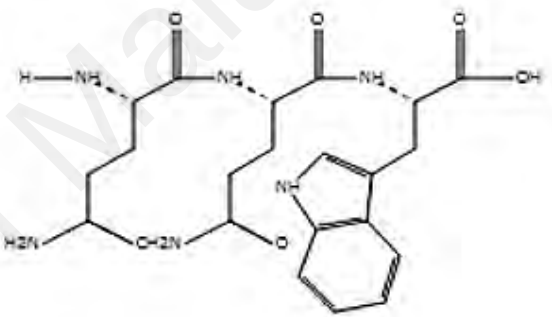
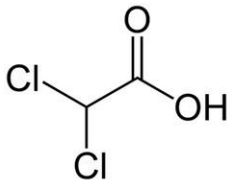
No	Name	Compound formula	RT	Mass	Chemical structure	Reference
8	Ethephon	C2 H6 Cl O3 P	3.328	143.9754		Figure 8.8 (Appendix A)
9	L- arginine	C6 H14 N4 O2	3.587	174.1125		Figure 8.9 (Appendix A)
10	Sulpho NONOate	H2 N2 O5 S	5.061	141.971		Figure 8.10 (Appendix A)

Table 4.4, continued.

No	Name	Compound formula	RT	Mass	Chemical structure	Reference
11	Gln Gln Trp	C21 H28 N6 O6	5.107	460.2073		Figure 8.11 (Appendix A)
12	Dichloroacetate	C2 H2 Cl2 O2	6.145	127.9442		Figure 8.12 (Appendix A)

4.3 Determination of Total Phenolic and Total Flavonoid content

4.3.1 Total phenolic content

Total phenol content in n-hexane, chloroform, ethyl acetate and methanol aqueous extract of *Ampelocissus* sp. was determined by Folin-Ciocalteu (FC) method as described by Ablat *et al*. (2014) using standard curve of Gallic acid as positive reference standard. The absorbance values at different concentrations of gallic acid were used to form the standard curve. The total phenol content of each extracts was measured by using equation (y= 3.427x, r^2 = 0.769) obtained from gallic acid standard curve and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). At concentration of 1.0 mg/ml, chloroform extract showed the highest concentration of phenolic compounds at 0.42 ± 0.012 mg GAE/g followed by ethyl acetate extract at 0.196 ± 0.042 mg GAE/g, methanol aqueous extracts at 0.103 ± 0.024 mg GAE/g and n-hexane extracts at 0.033 ± 0.002 mg GAE/g.

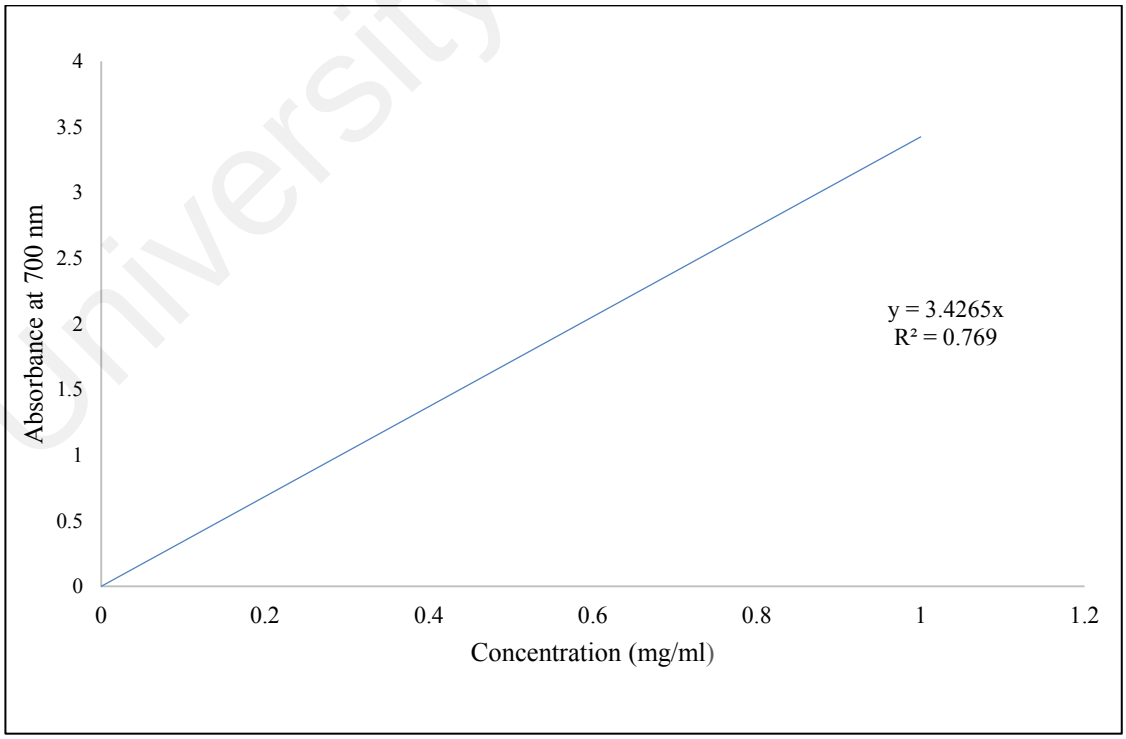


Figure 4.1: Standard curve of gallic acid.

4.3.2 Total flavonoid compound

Total flavonoid contents in n-hexane, chloroform, ethyl acetate and methanol aqueous extract from *Ampelocissus* sp. were determined by aluminium chloride method using standard curve of Quercetin as positive reference standard as described by Ablat *et al*., (2014). The absorbance values at different concentrations of quercetin were used to form the standard curve. The total flavonoid content of each extracts was measured by using equation ($y = 0.456x$, $r^2 = 0.9014$) which obtained from the Quercetin standard curve. The results were expressed as mg of quercetin equivalents (QE) per gram of sample in dry weight (mg/g). Chloroform extract showed the highest concentration of flavonoid compounds at 2.007 ± 0.001 mg QE/g followed by ethyl acetate extract at 1.455 ± 0.120 mg QE/g, methanol aqueous extracts at 0.574 ± 0.012 mg QE/g and n-hexane extracts at 0.095 ± 0.001 mg QE/g.

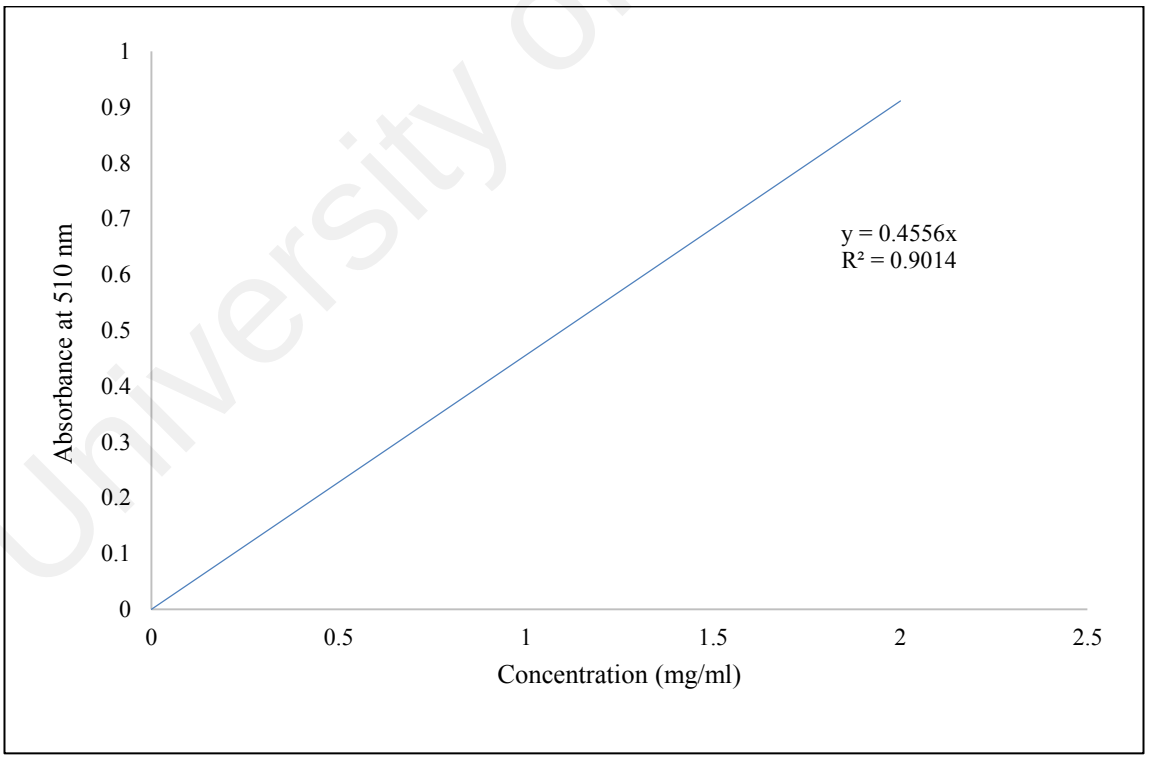


Figure 4.2: Standard curve of Quercetin.

Table 4.5: TPC and TFC of *Ampelocissus* sp. extracts. Each value is represented as mean ± 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).

Extract of *Ampelocissus* sp.	Total phenolic content (mg GAE)/g dry extract	Total Flavonoid Content (mg QE)/ g dry extract
N-hexane	$0.033 \pm 0.002_{a}$	$0.095 \pm 0.001_{a}$
Chloroform	$0.42 \pm 0.012_{b}$	$2.007 \pm 0.001_{a}$
Ethyl acetate	$0.196 \pm 0.042_{d}$	$1.455 \pm 0.120_{c}$
Methanol aqueous	$0.103 \pm 0.024_{c}$	$0.574 \pm 0.012_{b}$

4.4 Antioxidant Activity Assay

In this study the antioxidant of *Ampelocissus* sp. extracts was evaluated by using five different assays.

4.4.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The antioxidant activity of n-hexane, chloroform, ethyl acetate and methanol aqueous extract of *Ampelocissus* sp. was evaluated based on their scavenging activity against the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The radicals were characterised by a deep purple colour and maximum absorbance at 517 nm. Since ascorbic acid is a potent antioxidant, it was used as a standard.

The scavenging effect of the ascorbic acid on DPPH radicals was showed in Table 4.6.

Table 4.6: Percentage inhibition of DPPH radical by standard reference of ascorbic acid. Percentage of inhibition are expressed as mean ± SE, (n=3).

Concentration of Ascorbic acid (mg/ml)	Percentage of DPPH inhibition (%)
0.1	82.69 ± 0.022
0.5	82.13 ± 0.001
1.0	83.24 ± 0.001
1.5	83.5 ± 0.000
2.0	83.8 ± 0.001

The radical scavenging activity of ascorbic acid was increased with increasing concentrations. The percentage inhibition of ascorbic acid ranged between 82.69 % at 0.1 mg/ml to 83.80 % at 2.0 mg/ml. At concentration 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml the percentage of inhibition were 82.13 %, 83.24 % and 83.52 %, respectively.

In comparison with ascorbic acid, the percentage inhibitions of all extracts of *Ampelocissus* sp. (*isi nyaru*) were lower than the percentage inhibition of ascorbic acid. DPPH scavenging activity of n-hexane, chloroform, ethyl acetate and methanol aqueous extracts of *Ampelocissus* sp. (*isi nyaru*) were shown in Figure 4.3. Based on the percentage inhibition of DPPH, the chloroform extract has IC_{50} = 0.40 mg/ml, ethyl acetate extract IC_{50} = 1.49 mg/ml and methanol aqueous extract IC_{50}= 1.46 mg/ml. However, the n-hexane extract showed low scavenging activity lower than 50 %.

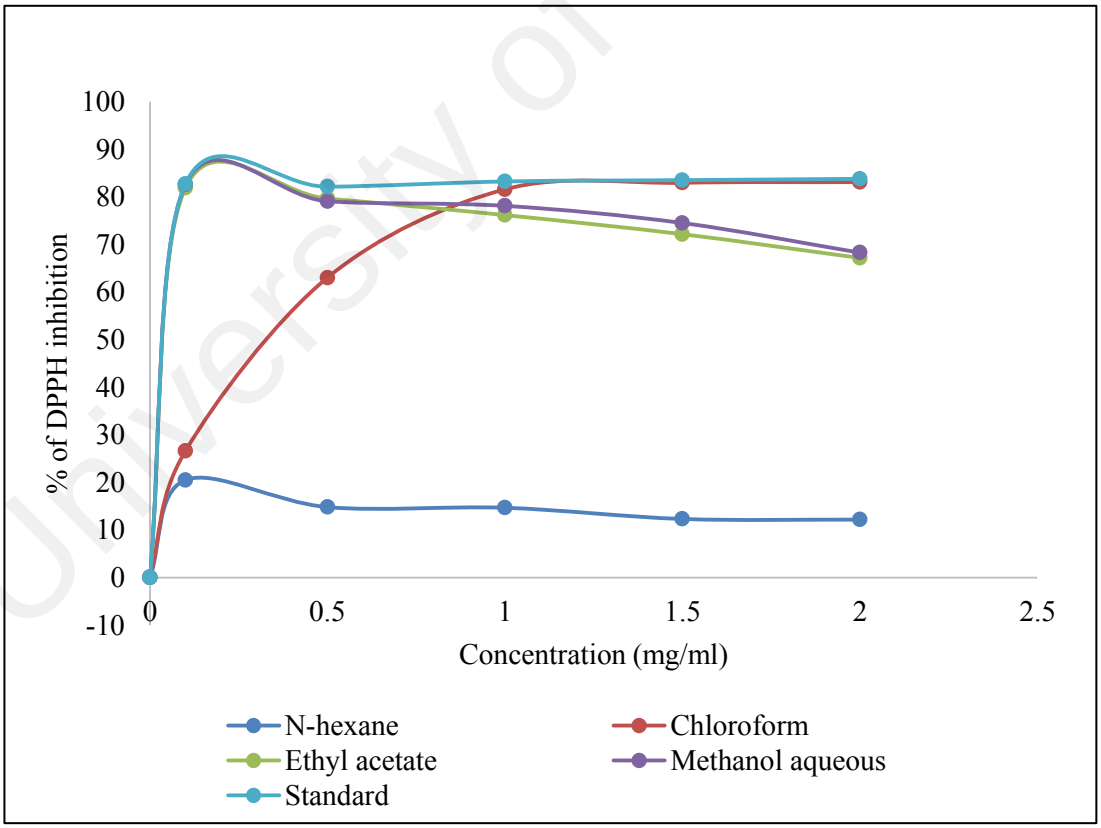


Figure 4.3: DPPH scavenging activity of n-hexane, chloroform, ethyl acetate, methanol aqueous extracts of *Ampelocissus* sp.

4.4.2 Metal Chelating Activity Assay

Metal chelating assay was carried out to estimate the ability of *Ampelocissus* sp. extracts to chelate ferrous ion and preventing the formation ferrozine- Fe^{2+} complex. Ethylenediaminetetraacetic acid (EDTA) was used as a reference standard. Table 4.9 showed the percentage of inhibition of ferrozine- Fe^{2+} complex formation by EDTA at different concentration.

Table 4.7: The percentage of inhibition Ferrozine - Fe^{2+} complex formation by EDTA. Percentage of inhibition are expressed as mean ± SE, (n=3).

Concentration of EDTA (mg/ml)	Percentage of ferrozine- Fe2+ complex (%)
0.1	94.37 ± 0.002
0.5	95.18 ± 0.003
1.0	95.85 ± 0.002
1.5	92.46 ±0.003
2.0	96.14 ± 0.001

The percentage of inhibition Ferrozine - Fe^{2+} complex formation by EDTA was increased with increasing concentrations. The percentage inhibition of EDTA ranged between 94.37 % at 0.1 mg/ml to 96.14 % at 2.0 mg/ml. At concentration 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml the percentage of inhibition were 95.18 %, 95.85 % and 92.46 %, respectively.

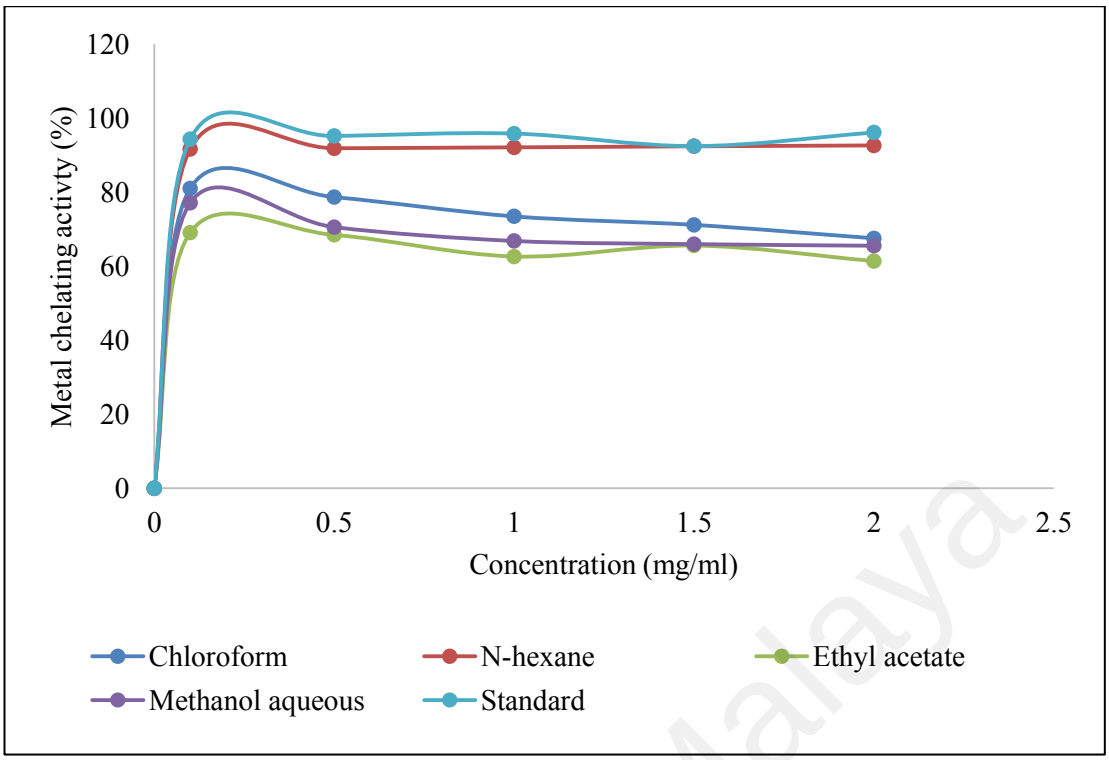


Figure 4.4: Metal chelating activity of n-hexane, chloroform, ethyl acetate, methanol aqueous extracts of *Ampelocissus* sp.

Figure 4.4 showed metal chelating activities of n-hexane, chloroform, ethyl acetate and methanol aqueous extracts of *Ampelocissus* sp. (*isi nyaru*). The ethyl acetate extract showed the highest IC_{50} which is 0.07 mg/ml. Meanwhile, chloroform extract has IC_{50} = 0.06 mg/ml, n-hexane IC_{50} = 0.05 mg/ml and methanol aqueous extract IC_{50} = 0.06 mg/ml.

4.4.3 Nitric Oxide Radical Scavenging Assay (NORSA)

NORSA was carried out to evaluate the capability of *Ampelocissus* sp. to scavenge the nitric oxide radical and compete with oxygen that leading to reduction of the nitrite ions production. Nitric oxide scavenging activity is defined by the decrease in the absorbance at 546 nm. In this assay, curcumin was use as the standard. Table 4.10 below shows the percentage of nitric oxide radical inhibition by standard curcumin at different concentration.

Table 4.8: The percentage of nitric oxide inhibition by curcumin in NORSA. Percentage of inhibition are expressed as mean ± SE, (n=3).

Concentration of curcumin (mg/ml)	Percentage of inhibition nitric oxide radical (%)
0.1	49.14 ± 0.034
0.5	61.67 ± 0.012
1.0	62.03 ± 0.007
1.5	63.67 ± 0.000
2.0	54.26 ± 0.018

The percentage of inhibition nitric oxide radical by curcumin was increased with increasing concentrations except at concentrations of 2.0 mg/ml. The percentage inhibition of curcumin ranged between 49.14 % at 0.1 mg/ml to 63.67 % at 1.5 mg/ml. At concentration 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml the percentage of inhibition were 61.67 %, 62.03 % and 54.26 %, respectively.

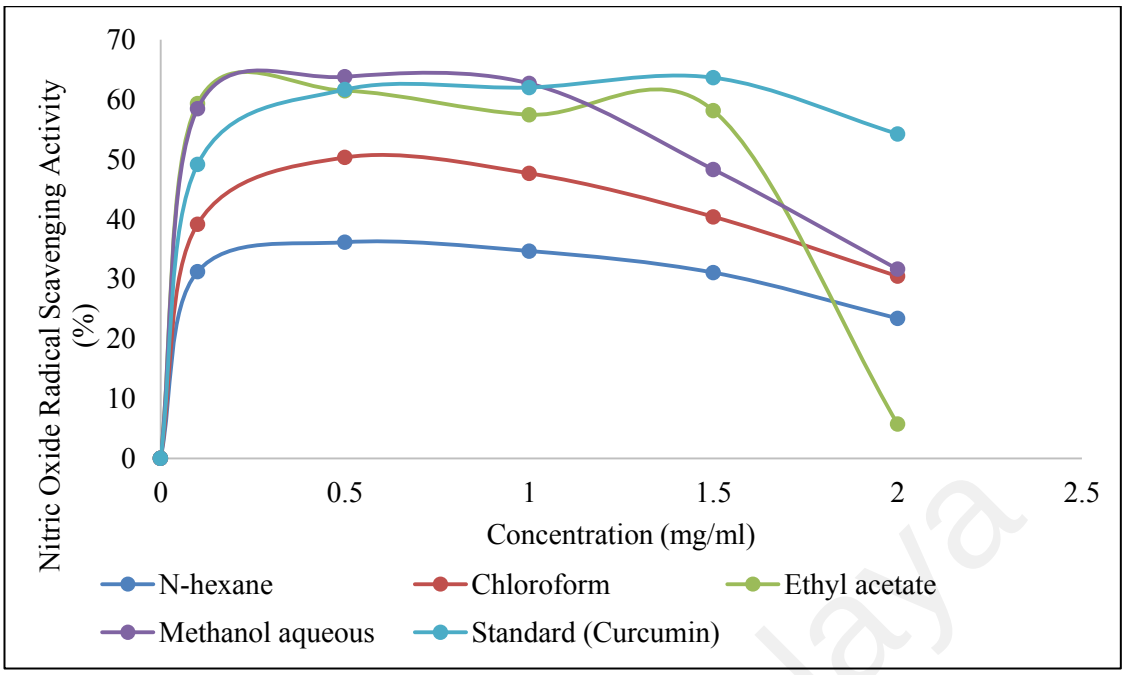


Figure 4.5: Nitric oxide radical scavenging inhibition percentage of N-hexane, chloroform, ethyl acetate and methanol aqueous extracts of *Ampelocissus* sp.

Figure 4.5 showed nitric oxide inhibition of n-hexane, chloroform, ethyl acetate and methanol aqueous extracts of *Ampelocissus* sp. (*isi nyaru*). It shows that chloroform extract has IC_{50} = 0.49 mg/ml, ethyl acetate extract IC_{50} = 0.08 mg/ml and methanol aqueous extract IC_{50} of 0.10 mg/ml. However, the n-hexane extract showed scavenging activity less than 50 %.

4.4.4 Superoxide radical scavenging Assay

Superoxide radical scavenging assay was carried out to determine the ability of *Ampelocissus* sp. (*isi nyaru*) extracts to scavenge superoxide anion from dissolved oxygen by PMS- NADH coupling reaction reduces NBT in the system. In this assay, ascorbic acid was used as a standard and the percentage of inhibition of superoxide radical by ascorbic acid at different concentration was shown in the Table 4.9.

Table 4.9: The percentage of superoxide inhibition by ascorbic acid. Percentage of inhibition are expressed as mean ± SE, (n=3).

Concentration of ascorbic acid (mg/ml)	Percentage of superoxide inhibition (%)
0.1	18.55 ± 0.003
0.5	18.55 ± 0.003
1.0	24.19 ± 0.001
1.5	29.03 ± 0.001
2.0	19.36 ± 0.003

The percentage of superoxide radical inhibition by ascorbic acid was increased with increasing concentrations. The percentage inhibition of ascorbic acid ranged between 18.55 % at 0.1 mg/ml to 29.03 % at 1.5 mg/ml. At concentration 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml the percentage of inhibition were 18.55 %, 24.19 % and 19.36 %, respectively.

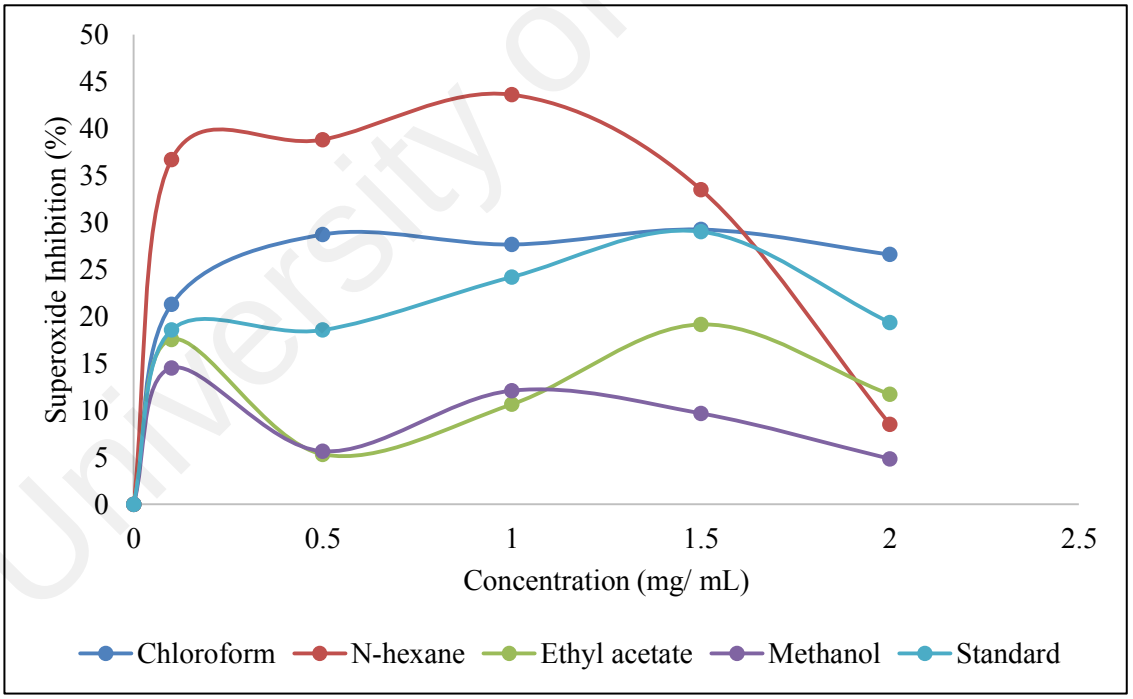


Figure 4.6: Superoxide scavenging activity of N-hexane, chloroform, ethyl acetate, methanol aqueous extracts of *Ampelocissus* sp. extracts.

Figure 4.6 shows the superoxide scavenging activity of chloroform, n-hexane, ethyl acetate, and methanol aqueous extracts of *Ampelocissus* sp. All extracts showed percentage of scavenging activity less than 50 %. At concentration of 2.0 mg/ml,

chloroform showed higher percentage of inhibition than the standard at 26.8 %. The percentage of inhibition for n-hexane extract, ethyl acetate extract and methanol aqueous extract were 8.51 %, 11.70 % and 4.84 %, respectively.

4.4.5 Ferric Reducing Antioxidant Power Assay (FRAP)

The basis of this assay is to check the capability of *Ampelocissus* sp. extracts to reduce Fe^{3+} to Fe^{2+}. At low pH, colourless ferric complex (Fe^{3+} - tripydyltriazine) was reduced to a blue coloured ferrous complex (Fe^{2+} - tripydyltriazine) by the action of electron-donating antioxidants. The reduction is measured at the absorbance of 595 nm and ferrous sulphate ($FeSO_4$) was used a reference standard. The FRAP value was calculated based on the linear regression between standard and absorbance at 595 nm. The results were estimated as mmol Fe^{2+} / g of dry extract. Figure 4.7 showed the standard curve of ferrous sulphate. The reducing ability of *Ampelocissus* sp. extracts was calculated based on the reaction signal given by a Fe^{2+} solution.

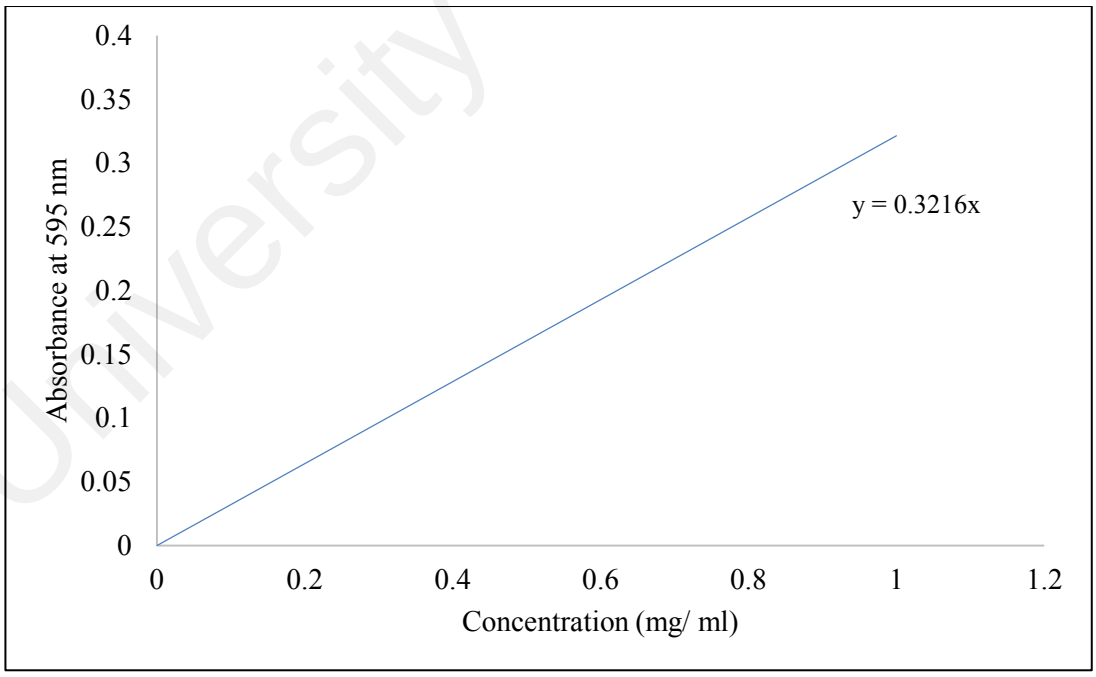


Figure 4.7: Standard curve of ferrous sulphate.

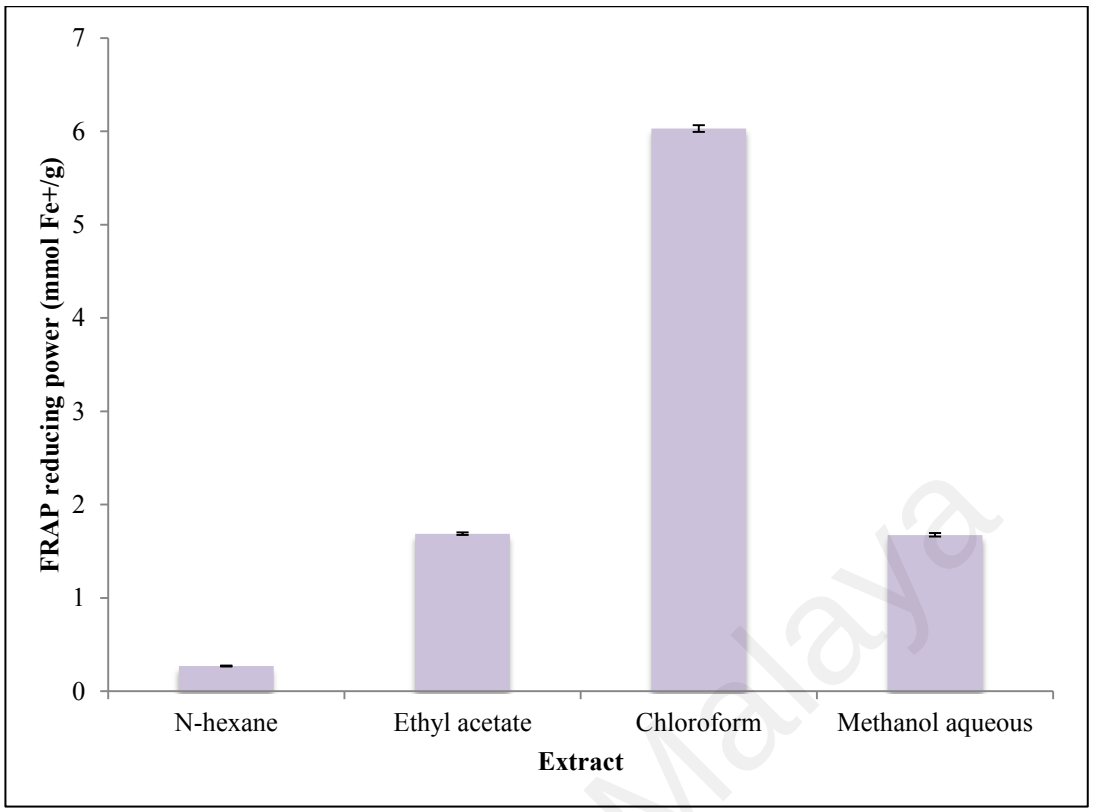


Figure 4.8: Ferric reducing antioxidant power assay.

Figure 4.8 shows the ferric reducing antioxidant power assay. The chloroform extract showed the highest reducing power at 6.03 mmol Fe^{+}/g, followed by ethyl acetate extract at 1.69 mmol Fe^{+}/g, methanol aqueous extract at 1.68 mmol Fe^{+}/g, n-hexane extract at 0.27 mmol Fe^{+}/g and N-hexane extract at 0.06 mmol Fe^{+}/g.

4.5 Acetylcholinesterase Inhibition Assay

Acetylcholinesterase inhibition was carried out according to Ellman's method as described by Yang *et al*. (2011). In this assay, the percentage inhibition of n- hexane, chloroform, ethyl acetate, methanol aqueous and compounds isolated from TLC were evaluated. The results were shown in Table 4.10 and 4.11.

Table 4.10: Acetylcholinesterase inhibition of N-hexane, chloroform, ethyl acetate, and methanol aqueous extracts of *Ampelocissus* sp. at 1 mg/ml.

Extracts	Percentage of Acetylcholinesterase Inhibition (%)
N-Hexane	$2.84 \pm 0.023_{cde}$
Chloroform	$-2.67 \pm 0.006_{e}$
Ethyl Acetate	$37.69 \pm 0.005_{b}$
Methanol Aqueous	$55.56 \pm 0.014_{a}$
Berberine	60.66 ± 0.003

Each value is represented as mean ± SE (n = 3). The means with different lower case letters (a, b, c, d and e) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Table 4.10 shows acetylcholinesterase inhibition of N-hexane, chloroform, ethyl acetate, and methanol aqueous extracts of *Ampelocissus* sp. at 1 mg/ml. Methanol aqueous extract showed the highest percentage of inhibition which is 55.56 %. Percentage inhibition for ethyl acetate extract and n-hexane extract was 37.69 % and 2.84 %, respectively. However, chloroform extract of *Ampelocissus* sp. showed no inhibition activity (negative value indicated no inhibition of AChE enzyme).

Table 4.11: Acetylcholinesterase inhibitions of TLC compounds isolated from chloroform and ethyl acetate extracts of *Ampelocissus* sp. using 100 % chloroform solution as solvent at concentration of 1 mg/ml.

Isolated Label Compound	Percentage of Acetylcholinesterase inhibition (%)
AMP-ch1	$11.2 \pm 0.019_{cde}$
AMP-ch2	$12.53 \pm 0.031_{cde}$
AMP-ch3	$6.76 \pm 0.007_{cde}$
AMP-EA1	$15.29 \pm 0.026_{cde}$
Berberine	60.66 ± 0.003

Each value is represented as mean ± SE (n = 3). The means with different lower case letters (c, d and e) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Table 4.11 shows acetylcholinesterase inhibitions of TLC compounds isolated from chloroform and ethyl acetate extracts of *Ampelocissus* sp. using 100 % chloroform as solvent at concentration of 1 mg/ml. The compound AMP-EA1 showed the highest percentage of inhibition at 15.29 % followed by AMP-ch2, AMP-ch1 and AMP-ch3.

Table 4.12: Acetylcholinesterase inhibitions of TLC compounds isolated from chloroform and ethyl acetate extracts of *Ampelocissus* sp. using 10 % methanol in chloroform as solvent at concentration of 1mg/ml.

Isolated Label Compound	Percentage of Acetylcholinesterase Inhibition (%)
AMP- ch4	$4.18 \pm 0.019_{cde}$
AMP- ch5	$1.16 \pm 0.041_{de}$
AMP- ch6	$6.84 \pm 0.003_{cde}$
AMP-EA2	$21.51 \pm 0.016_{c}$
AMP-EA3	$15.02 \pm 0.016_{cde}$
AMP-EA4	$18.49 \pm 0.019_{cd}$
AMP-EA5	$17.16 \pm 0.027_{cd}$
AMP-EA6	$6.04 \pm 0.012_{cde}$
AMP-EA7	$15.29 \pm 0.010_{cde}$
Berberine	60.66 ± 0.003

Each value is represented as mean ± SE (n = 3). The means with different lower case letters (c, d and e) in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Duncan's multiple comparison test).

Table 4.12 shows the acetylcholinesterase inhibition of TLC compounds isolated from chloroform and ethyl acetate extracts of *Ampelocissus* sp. by using 10 % methanol in chloroform solvent at concentration of 1mg/ml. There were nine compounds isolated

and the compound AMP-EA2 showed the highest percentage of inhibition at 21.51 %. The lowest percentage of inhibition was shown by AMP-ch5 at 1.16 %.

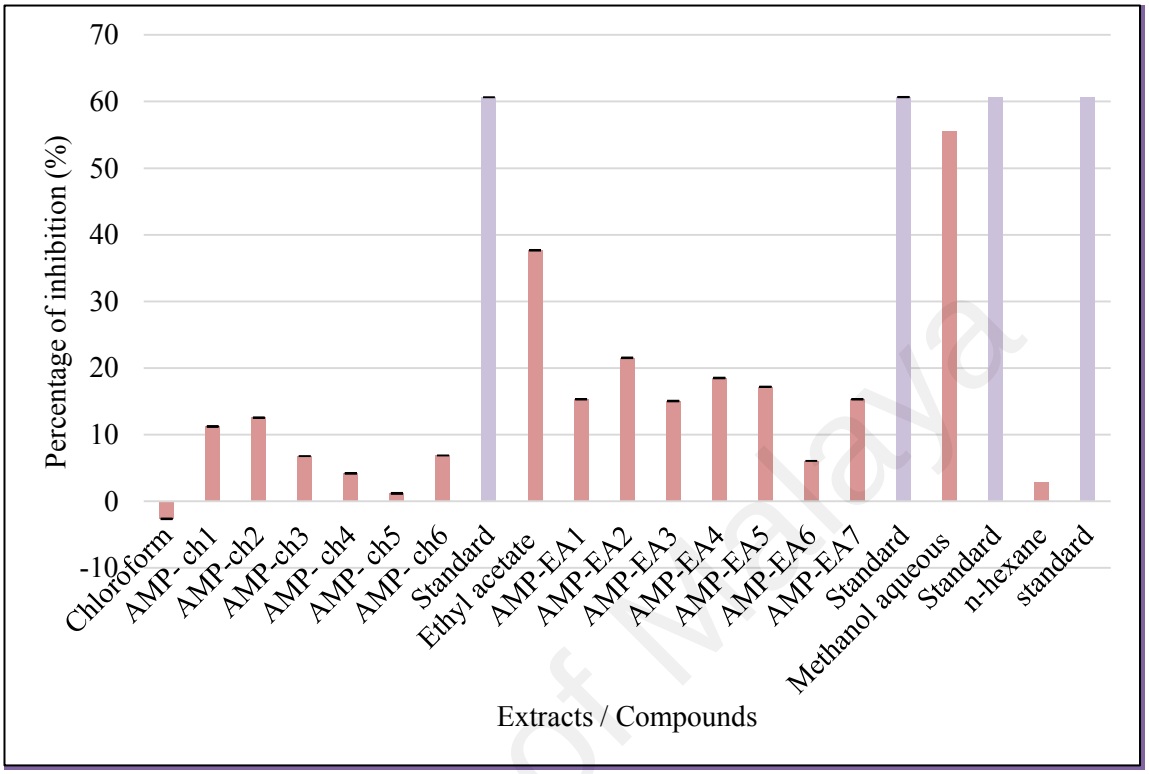


Figure 4.9: Percentage of acetylcholinesterase (AChE) inhibitory activity of extracts and compounds isolated from TLC plates from extracts of *Ampelocissus* sp. at concentration of 1mg/ml. Each value is represented as mean ± S.E. of three separate experiments in triplicate measurements.

Figure 4.9 shows percentage of acetylcholinesterase (AChE) inhibitory activity of extracts and compounds separated from TLC plates from extracts of *Ampelocissus* sp. at concentration of 1mg/ml. The highest percentage of inhibition was expressed by methanol aqueous extract which is 55.56 % and the lowest percentage of inhibition was possessed by AMP-ch5 compound which is 1.16 %. However, chloroform extract of *Ampelocissus* sp. showed low acetylcholinesterase inhibitory activity.

CHAPTER 5

DISCUSSION

Ampelocissus sp. was powdered to fine powder form to maximize the extraction yield and was extracted by macerating in 10 % methanol for 48 hours. Although Ablat *et al*. (2014) reported that ethanol is less toxic and broadly used in pharmaceutical and cosmetic industry; methanol was used in this study instead of ethanol. It is because methanol has lower boiling point than ethanol. Thus, methanol extracts required lower temperature to evaporate the solvent in the rotary evaporator and less damage was occurred to the extract. Moreover, methanol is also a great option for the plant extraction because it could extract the diverse chemical groups of the plant material. The extract was further fractionated using n-hexane, chloroform, ethyl acetate following the polarity of the solvents and collected as methanol aqueous extract. The yield obtained was differed subjecting to extraction solvents used. Methanol aqueous extraction gave highest yield of 0.6 %, followed by ethyl acetate extraction which yielded 0.593 %, n-hexane (0.362 %) and chloroform extract (0.047 %). The variation of the attained yields may have been cause by the variation of polarities as well as solubility of the solvents and the extracted compounds (Zahradníková *et al*., 2018). In other study, Nakamura *et al.*, (2017) extracted the leaves of *Sasa quelpaertensis* Nakai with 80% ethanol and further partitioned with n-hexane, chloroform, ethyl acetate, n-butanol, and aqueous fractions to assess the biological activity through evaluation via various in vitro assays. Every plant yields and stores a specific amount of active compounds in their tissues and thus, the attraction to the solvents varies subjecting to its polarity. Hence, best extraction methods must be exclusively invented for optimization. In their study, they partitioned five fractions which are n-hexane, chloroform, ethyl acetate, n-butanol, and aqueous from 80% ethanol extract to assess the extraction yield of *S. quelpaertensis Nakai* leaves. As a result, they concluded that a high polarity

solvent such as the aqueous and n-butanol fractions generated higher yields than the chloroform and ethyl acetate fractions.

The chemical compounds of *Ampelocissus* sp. were separated by thin layer chromatography (TLC) and the result was presented in Table 4.2 and Table 4.3. In this study, two different solvents were used which are 100 % chloroform and 10 % methanol in chloroform. In detection of alkaloids, Dragendroff's spraying reagent was used while vanillin-sulphuric acid reagent was sprayed to detect terpenoids, phenols and flavonoids. For 100 % chloroform as solvent, orange colour band confirmed the presence of alkaloids in methanol aqueous extract and ethyl acetate extract. When sprayed with vanillin-sulphuric acid reagent- blue, purple, orange and yellow indicated the presence of saponins, terpenoids, alkaloids and flavonoids; respectively. In 100 % chloroform, saponin was detected in ethyl acetate extract and terpenoids was detected in n-hexane as well as in methanol aqueous. Meanwhile alkaloids were successfully detected in all extracts of *Ampelocissus* sp. In 10 % methanol in chloroform, terpenoids were presented in chloroform and ethyl acetate extract, flavonoids were detected in ethyl acetate extract, saponins were detected in all extract except methanol aqueous extract and alkaloids were detected in all extracts. Alkaloids are abundantly and diversely group of secondary metabolites and contribute about 10-15 % concentration in nearly all floras. Flavonoids on the other hand are capable to inhibit particular enzymes, to act as neurotransmitters as well as to excite some hormones. Most of flavonoids serve as antioxidant in human and to cure inflammation (Paulsamy & Karthika, 2015).

The analysis of the phytochemical compounds in *Ampelocissus* sp. extracts was carried out using Liquid Chromatography Mass Spectrometry tandem with Mass Spectrometry (LCMS/MS). The analysis of methanol aqueous extract showed the presence of 12 compounds namely 5-aminopentanoic acid, 11-amino-undecanoic acid, C16 sphinganine, (+)-eudesmin, his lys cys, lys his met, his met lys, ethephon, L-

arginine, sulpho NONOate, gln gln trp and dichloroacetate. All of these compounds were classified into amino acid group.

Phenolic compounds can generally found in living organisms, and plants retain an adequate volume of them inside their extended bodies. Usually, phenolic compounds occur in different structures, for example hydrolysable and condensed tannins (polyphenol), hydroxycinnamic acids (phenolic acids), as well as flavonoids (Nakamura *et al.*, 2017). It is notable that phenolic compounds associate with the bioactive elements of plant products and possess beneficial health-promoting activities (Lu *et al*., 2017). Total phenol content of different extracts of *Ampelocissus* sp. was determined by Folin-Ciocalteu (FC) method. The total phenol content of each extracts was measured by using equation (y= 3.427x, r^2 = 0.769) obtained from Gallic acid standard curve. The result showed that chloroform extract showed the highest concentration of phenolic compounds which is 0.42 ± 0.012 mg GAE/g followed by ethyl acetate extract (0.196 ± 0.042 mg GAE/g), methanol aqueous extracts (0.103 ± 0.024 mg GAE/g) and n-hexane extracts (0.033 ± 0.002 mg GAE/g). The amount of total phenolic were contrary according to the extracts as a result of the different solubility and polarity of the extracts, the variation of polarity among the compounds contained in the fractions as well as the solvent used for extraction (Zahradníková *et al*., 2018). Meanwhile total flavonoid contents from *Ampelocissus* sp. were determined by aluminium chloride method using standard curve of Quercetin as positive reference standard. The total flavonoid content of each extracts was measured by using equation (y = 0.456x, r^2= 0.9014) which obtained from the Quercetin standard curve. The highest contents of flavonoid was recorded in chloroform extract which is 2.007 ± 0.001 mg QE/g followed by ethyl acetate extract (1.455 ± 0.120 mg QE/g), methanol aqueous extracts (0.574 ± 0.012 mg QE/g) and n-hexane extracts (0.095 ± 0.001 mg QE/g). The high content of phenolic and flavonoids compound is might due to the terpenoids, saponin and alkaloids

as detected in TLC.

The antioxidant activity of *Ampelocissus* sp. was assessed by five assays which are 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, ferric reducing power assay, metal chelating assay, nitric oxide radical scavenging assay and superoxide radical scavenging assay. The data obtain showed small range of standard error (SE). To describe variation, SE was chose instead of standard deviation (SD) because SE gives the smallest of the error bars. The DPPH scavenging activity was done to analyse the capability of antioxidant to scavenge free radicals due to their hydrogen-donating capability. DPPH is a free radical compound which is widely used to investigate the free radical-scavenging competency of numerous samples. Antioxidants respond with DPPH and lowering a quantity of DPPH molecules equivalent to the number of free hydroxyl groups (Boutennoun *et al*., 2017). Based on the result showed in Figure 4.3, all the percentage inhibitions of all extracts of *Ampelocissus* sp. (*isi nyaru*) were lower than the percentage inhibition of ascorbic acid. However, nearly all extract exhibited a good scavenging activity. The highest was recorded by ethyl acetate extract which has an IC_{50} of 1.49 mg/ml, followed methanol aqueous extract (IC_{50}= 1.46 mg/ml) and chloroform extract (IC_{50} = 0.40 mg/ml). N-hexane extract displayed low scavenging activity thus IC_{50} was not achieved. The high scavenging activity of ethyl acetate may due to the presence of saponins, terpenoids, alkaloids and flavonoids as found in TLC.

Metal chelating activity assay was carried out to estimate the ability of *Ampelocissus* sp. extracts to chelate ferrous ion and preventing the formation ferrozine-Fe^{2+} complex which was detected at 562 nm (Ablat *et al*., 2014). Ethylenediaminetetraacetic acid (EDTA) was used as a reference standard in the assay. Even though chelating activity of all extracts was lower than the reference standard, all of these extracts were managed to achieve IC_{50}. Ethyl acetate extract showed the highest

IC_{50} which is 0.07 mg/ml. Meanwhile, chloroform extract has an IC_{50} = 0.06 mg/ml, n-hexane has an IC_{50}= 0.05 mg/ml and methanol aqueous extract has an IC_{50}= 0.06 mg/ml. Similar to DPPH the high chelating activity of ethyl acetate extract might due to the presence of saponins, terpenoids, alkaloids as well as flavonoids.

The ferric reducing antioxidant power (FRAP) assay was done to assess the capability of *Ampelocissus* sp. extract to decrease the ferum ion in relation to its antioxidant activity. Then, the ferric reducing activity were evaluated according to the decline of ferric-TPTZ (2, 4, 6-tri (2-pyridyl)-l, 3, 5-triazine) to blue ferrous-TPTZ. Antioxidant compounds donated hydrogen atom to ferric complex hence the radical chain reaction was break. The FRAP value was calculated based on the linear regression between standard of ferrous sulphate ($FeSO_4$) and absorbance at 595 nm. The chloroform extract showed the highest reducing power at 6.03 mmol Fe^+/g, followed by ethyl acetate extract at 1.69 mmol Fe^+/g, methanol aqueous extract at 1.68 mmol Fe^+/g, n-hexane extract at 0.27 mmol Fe^+/g and N-hexane extract at 0.06 mmol Fe^+/g. High reducing power of reported by chloroform might have been contributed by alkaloids, terpenoids and saponins detected in chloroform extract.

Nitric oxide radical scavenging activity (NORSA) of extracts was done by evaluating the development of the nitrite ions in the mixture of reaction which can be spotted by Griess reagent (Ablat *et al*., 2014). In this study, for extracts of *Ampelocissus* sp. which are N-hexane, chloroform, ethyl acetate and methanol aqueous were assessed with curcumin as a reference standard. According to Chanda and Dave (2009), sodium nitrite, ascorbic acid, caffeic acid, rutin, BHT as well as α-tocopherol also could be applied as a positive control. Based on result presented on table 4.5, chloroform extract showed the highest IC_{50} at 0.49 mg/ml and followed by methanol aqueous extract (IC_{50} 0.10 mg/ml) and ethyl acetate extract (IC_{50} = 0.08 mg/ml). Dissimilar with these three extracts, n-hexane extract displayed low scavenging activity than that of standard as

IC_{50} was not achieved**.** It can be conclude that the chloroform possessed the highest scavenging activity as it contains the highest content of phenolic and flavonoid as described in table 4.7. This result was agreed by the finding of Ablat *et al*. (2014) which stated that polyphenol-rich EAF displayed the strongest effect, proposing that polyphenol was powerfully contributed in scavenging Nitric Oxide on *Brucea javanica* seed.

Even though superoxide anion is not a powerful oxidant, it could generate potent and vulnerable hydroxyl radicals and singlet oxygen which cause the oxidative stress. The superoxide anions resulting from dissolved oxygen from PMS/NADH integrating reaction decreases NBT in PMS/NADH-NBT system. The decline of absorbance at 560 nm with antioxidants shows the ingestion of superoxide anion in the mixture of the reaction (Bahrani *et al*., 2014). A strong antioxidant, ascorbic acid was used as a reference standard of the superoxide radical scavenging activity assay. As shown in Figure 4.6, the scavenging activity of chloroform, n-hexane, ethyl acetate and methanol aqueous extracts of *Ampelocissus* sp. displayed low percentage of scavenging activity consequently IC_{50} was not achieved. However, at concentration of 2.0 mg/ml, chloroform showed higher percentage of inhibition than the standard which is 26.8 %. The finding was might due to the effect of superoxide scavenging activity from alkaloids, terpenoids and saponins in chloroform extract as found in TLC. Percentage of inhibition for n-hexane extract, ethyl acetate extract and methanol aqueous extract were 8.51 %, 11.70 % and 4.84 %, respectively.

The enzyme hydrolyses the substrate acetylthiocholine and produced thiocholine. It then reacted with Ellman‘s reagent (DTNB) to create 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2- nitrobenzoate (Dhanasekaran *et al.,* 2015). As a result of this activity, a yellow colour was developed. The absorbance of the product is measured at 405 nm which is equivalent to the enzyme activity (Jamous *et al.,* 2015).

Berberine is an alkaloid which has anti-inflammatory property that is applied as a drug to cure AD (Bahrani *et al*., 2014). In this assay, berberine was chosen as a standard because it has been well known as an acetylcholinesterase (AChE) inhibitor. It produces notably low cytotoxicity, has higher value with less side effects (Xiang *et al.,* 2009). Figure 4.9 presented the percentage of acetylcholinesterase (AChE) inhibitory activity of extracts and compounds isolated from TLC plates from extracts of *Ampelocissus* sp. There are 17 compounds isolated and at concentration of 1mg/ml, the highest percentage of inhibition was expressed by methanol aqueous extract which is 55.56 % whereas the lowest percentage of inhibition was possessed by AMP-ch5 compound which is 1.16 %. The high AChE inhibition might due to terpenoids and alkaloids detected in methanol aqueous extract. Although further analysis is required to confirm the finding, this present study show that *Ampelocissus* sp. could be a potential option of AChE inhibitors which can be used to inhibit the effect of acetylcholinesterase in AD.

Based on several mechanisms such as metal chelating effect, hydrogen atom donation, capability to reduce ferric ions, nitric oxide scavenging ability and superoxide anion scavenging capability, the findings suggested that chloroform extract and ethyl acetate extract of *Ampelocissus* sp. possess good antioxidant activity. Apart from that, both extracts also contain high flavonoid and phenolic compounds. On the other hand, methanol aqueous extract showed a good inhibitory activity in AChE inhibitory activity assay. Hence, the overall finding of this research has indicated that the bioactive compounds detected in the extracts of *Ampelocissus* sp. are capable to be a good antioxidant as well as AChE inhibitors.

CHAPTER 6

CONCLUSION

The phytochemical bioactive compounds of *Ampelocissus* sp. was identified using Thin Layer Chromatography and showed the existence of alkaloids, terpenoids, flavonoids and saponins. The examination on the methanol aqueous extract of *Ampelocissus* sp. with liquid chromatography mass spectrometer tandem mass spectrometer (LCMS/MS) displayed the occurrence of 12 phytochemical compounds which are 5-aminopentanoic acid, 11-amino-undecanoic acid, C16 sphinganine, (+)-eudesmin, his lys cys, lys his met, his met lys, ethephon, L-arginine, sulpho NONOate, gln gln trp and dichloroacetate. Chloroform extracts of *Ampelocissus* sp. showed the highest content of phenols and flavonoids content. For antioxidant test, a good percentage of activity was achieved in2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, ferric reducing power assay, metal chelating assay and nitric oxide radical scavenging assay. However, superoxide radical scavenging assay showed relatively low percentage of inhibitions for all the fractional extracts. For AChE inhibitory assay, overall result exhibits quite low inhibitory activities in almost all extracts except for methanol aqueous which showed the notably high percentage of inhibition at 55.56 %. As a final conclusion, this research has showed that the extract of *Ampelocissus* sp. can be a prospect of a good antioxidant as well as potent AChE inhibitors to cure AD.

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