BIOACTIVITY-GUIDED ISOLATION OF *AILANTHUS MALABARICA* FOR *TOXOPLASMA GONDII* AND CHOLINESTERASE INHIBITORY ACTIVITIES

by

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Thesis submission in fulfillment of the requirements

for the Degree of

Master of Science (Pharmacy)

March 2017

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my main supervisor Prof. Chan Kit Lam for the continuous support of my master study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my master study.

Besides that, I would like to express my special thanks to my co-supervisor, Dr. Vikneswaran Murugaiyah, who has given me generous amount of guidance despite his busy schedule. Without his support, I would not have fulfilled this dissertation. I would also like to show my deepest gratitude to Prof. Rahmah Noordin, who as my co-supervisor has given me full support in completing my research. I would like to also thank the members of Prof Rahmah's laboratory (Pn. Ezzati Zahidah and En. Hafiz) who have given me generous help during my research at INFORMM.

I would also like to acknowledge School of Pharmaceutical Sciences and Institute of Postgraduate Studies (IPS) for giving me the opportunities to pursue my graduate studies. Besides, I would like to express my gratitude to Graduate Assistance Scheme (GA), MyBrain 15 from ministry of higher education and BIDP programme from IPHARM for funding me throughout this research.

Last but not the least, I would like to thank my family: my parents and to my brothers and friends for supporting me spiritually throughout writing this thesis and my life in general.

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LIST OF SYMBOLS AND ABBREVATION

%	Percentage
°C	Degree celcius
α	Alpha
β	Beta
µ	Micro
Aβ	Amyloid beta
ACh	Acetylcholine
AChE	Acetylcholinesterase
Abs	Absorbance
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
BuChE	Butyrylcholinesterase
DEPTQ	Distorsionless enhancement by polarization transfer including the detection of quaternary nuclei
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
ESI	Electrospray ionization

FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	Gram
GM	Growth medium
h	Hour
H	Hydrogen
HMBC	Heteronuclear Multiple Bond Coherence
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Inhibitory concentration of 50 %
IR	Infrared
mg	Milligram
mg/mL	Milligram per millilitre
mins	Minutes
mL	Millilitre
MS	Mass spectrometry
MTS	3-(4,5-Dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-sulfophenyl)-2H-tetrazolium
nm	Nanometer
NMR	Nuclear magnetic resonance

PBS	Phosphate buffered saline
R_f	Retention factor
SEM	Standard error of means
SI	Selectivity index
TLC	Thin layer chromatography
WHO	World Health Organization

PEMENCILAN BERPANDU BIO-AKTIVITI *AILANTHUS MALABARICA* UNTUK AKTIVITI PERENCATAN *TOXOPLASMA GONDII* DAN KOLINESTERASE

ABSTRAK

Tumbuh-tumbuhan daripada *Ailanthus* genus diedarkan secara meluas di seluruh Asia dan memainkan peranan penting sebagai tumbuhan perubatan. Aktiviti-aktiviti biologi spesies *Ailanthus* telah dikaji secara meluas dan didapati mempunyai aktiviti seperti anti-protozoa, anti-virus, anti-bakteria dan anti-fungal. Kajian ini bertujuan untuk menilai perencatan aktiviti *Toxoplasma gondii* dan enzim kolinesterase, serta analisis fitokimia untuk ekstrak metanol *Ailanthus malabarica*. Dalam kajian ini, ekstrak metanol *Ailanthus malabarica* disiasat bagi aktiviti-aktiviti anti-parasit terhadap *Toxoplasma gondii*. IC_{50} daripada anti-parasit aktiviti *Ailanthus malabarica* adalah 11.12 μg/mL. *Ailanthus malabarica* diperingkatkan dengan mengguna kromatografi resin untuk menghasilkan lima fraksi. Fraksi yang paling aktif ialah AM F5 dengan IC50 9.98 μg/mL. Selepas itu, AM F5 diperingkatkan menggunai kromatografi silika gel untuk menghasilkan ocotillone. IC_{50} untuk ocotillone adalah 16.18 μg/mL. *Ailanthus malabarica* kemudian diuji pada sel Vero dalam keadaan *in vitro* dengan menggunakan MTS assay untuk mengaji tahap toksik. Ekstrak dan fraksi telah didapati tidak toksik kepada sel-sel (LC50> 20 μg / mL). AM F3, AM F4 dan ocotillone menunjukkan tahap toksik yang rendah pada nilai LC_{50} 247 μg/mL, 238 μg/mL dan 223 μg/mL masing-masing. Tahap toksik AM F5 adalah tinggi berbanding dengan fraksi lain dengan nilai 49.87 μg/mL. Indeks pemilihan (SI) daripada ocotillone menunjukkan pemilihan tertinggi iaitu 13.83.

Disebabkan *Toxoplasma gondii* adalah parasit neurotropic, jangkitan telah dibukti akan menyebabkan perubahan tingkah laku dan gejala neurocognitive dalam mamalia. Dalam kajian berikutnya, *Ailanthus malabarica* telah diuji untuk perencatan enzim kolinesterase. Pemeringkatan berulang AM F5 menghasilkan sebatian lain, AM S4 (hydroxydammarenone-II). Ekstra dan fraksi telah diuji untuk perencatan kedua-dua acetylcholinesterase (AChE) dan butyrylcholinesterase (BuChE). Pada 100 μg/mL, AM S4 (hydroxydammarenone-II), AM S6 dan AM S8 daripada AM F5 menunjukkan perencatan melebihi 50 %. IC_{50} daripada ketiga-tiga pecahan ini adalah 12.54 μg/mL, 10.00 μg/mL dan 76.05 μg/mL masing-masing. Sebaliknya, untuk perencatan BuChE aktiviti, AM S10 dan AM S14 menunjukkan perencatan yang melebihi 50% apabila diuji di kepekatan 100 μg/mL. IC_{50} AM S10 dan AM S14 pada BuChE adalah 2.98 μg/mL dan 25.31 μg/mL masing-masing.

AM F5 yang memiliki kesan anti- toxoplasmicidal tertinggi dinilai kepada aktiviti anti- cholinesterase . Tetapi, perencatan enzim kolinesterase AM F5 adalah kira-kira 13 %. Ocotillone yang didapati merencat pertumbuhan *Toxoplasma gondii* menunjukkan perencatan di acetylcholinesterase pada 34 % dan 18 % pada butyrylcholinesterase . Peratusan perencatan adalah agak rendah berbanding dengan lain-lain pecahan. Struktur AM S4 dan AM S7 telah ditentukan sebagai hydroxydammarenone-II dan ocotillone masing-masing dengan menggunakan NMR , IR dan spektroskopi jisim. Sebagai kesimpulan, hanya AM S4 (hydroxydammarenone -II) menunjukkan aktiviti anti-acetylcholinesterase yang aktif pada 12 μg/mL. Bagaimanapun , sub-fraksi ini perlu diuji ke atas aktiviti anti-toxoplasma untuk menentukan kesan terhadap *Toxoplasma gondii*.

BIOACTIVITY-GUIDED ISOLATION OF *AILANTHUS MALABARICA* FOR *TOXOPLASMA GONDII* AND CHOLINESTERASE INHIBITORY ACTIVITY

ABSTRACT

The plants of the genus *Ailanthus* are distributed widely over Asia and play a significant role as medicinal plant. Biological activities of *Ailanthus* species has been studied extensively and found to possess activities like anti-protozoal, anti-viral, anti-bacterial and anti-fungal. The present study aimed to evaluate the anti-toxoplasma and cholinesterase inhibitory activity, as well as phytochemical analysis of methanolic extract of *Ailanthus malabarica*. In this work, the methanolic extract of *Ailanthus malabarica* was investigated for the anti-parasitic activities against *Toxoplasma gondii*. The IC_{50} of *Ailanthus malabarica* on the anti-parasitic activity was 11.12 µg/mL. *Ailanthus malabarica* was further fractionated using resin column chromatography to yield five fractions. The most active fraction was AM F5 with IC_{50} of 9.98 µg/mL. AM F5 was then further subjected to silica gel column chromatography to yield ocotillone. The IC_{50} of ocotillone was 16.18 µg/mL. *Ailanthus malabarica* was then tested on Vero cell line in an *in vitro* MTS assay to study for the cytotoxicity of the plant. The crude extract as well as the sub-fractions were not toxic to the cells (TD_{50} > 20 µg/mL). AM F3, AM F4 and ocotillone showed little cytotoxicity at TD_{50} value of 247 µg/mL, 238 µg/mL and 223 µg/mL respectively. AM F5 was the most toxic at TD_{50} of 49.87 µg/mL. The selectivity index (SI) of ocotillone showing the highest selectivity was 13.83. As *Toxoplasma gondii* was a neurotropic parasite, the infection has been shown to cause behavioral

changes and neurocognitive symptoms in mamalian hosts. In subsequent study, *Ailanthus malabarica* was tested for cholinesterase enzyme inhibition. Fraction AM F5 which was found active in anti-toxoplasmic activity was subjected to anti-cholinesterase inhibitory assay. Repeated fractionation of AM F5 yielded another compound, AM S4 (hydroxydammarenone-II). The crude extract and the sub-fractions were tested for inhibition of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). At 100 µg/mL, AM S4 (hydroxydammarenone-II), AM S6 and AM S8 from AM F5 showed above 50 % of inhibition on AChE. The IC_{50} of these three sub-fractions were 12.54 µg/mL, 10.00 µg/mL and 76.05 µg/mL respectively. On the other hand, for inhibition of BuChE, AM S10 and AM S14 showed inhibition above 50 % when tested at the concentration of 100 µg/mL. The IC_{50} of AM S10 and AM S14 on BuChE were 2.98 µg/mL and 25.31 µg/mL respectively. AM F5 which possessed highest anti-toxoplasmicidal effect was evaluated on the anti-cholinesterase activity. However, the inhibition on cholinesterase enzymes of AM F5 was about 13 %. Ocotillone which was found to inhibit the growth of *Toxoplasma gondii* showed inhibition at acetylcholinesterase at 34 % and 18 % on butyrylcholinesterase. The percentage of inhibition was comparatively lower when compared to other sub-fractions. AM S4 and AM S7 were structurally elucidated as hydroxydammarenone-II and ocotillone respectively by using NMR, IR and mass spectrometer. Only AM S4 (hydroxydammarenone-II) showed potent anti-acetylcholinesterase activity at 12 µg/mL. However, this sub-fraction was not tested on anti-toxoplasmicidal activity due to laboratory limitations. In conclusion, AM F5 is potent against anti-toxoplasmic and cholinesterase inhibitory activity, however further study should be evaluated on the potency of hydroxydamarenone-II on anti-toxoplasmic activity to further confirm that it is the

compound which is responsible to inhibit both toxoplasma and cholinesterase activities.

CHAPTER ONE

INTRODUCTION

1.1 Toxoplasmosis: Overview

Toxoplasmosis is a disease caused by an obligate intracellular parasite, *Toxoplasma gondii.* The discovery of *Toxoplasma gondii* can be dated back to 100 years ago, by scientists in North Africa and in Brazil. *Toxoplasma gondii* is capable of infecting all warm-blooded animals including humans. This protozoan parasite infects up to a third of the world's population and mainly through consumption of undercooked food containing tissue cysts or by ingestion of food and water that is contaminated with oocysts shed by cats (Bowie *et al*., 1997). Most of the infections of *Toxoplasma* in humans are asymptomatic, however, there are times where the infections lead to devastating disease. Encephalitis causes severe damage, making it the most important manifestation of toxoplasmosis in immune suppressed patients. The infection may be found in any organs, causing symptom-like headache, disorientation and drowsiness. Apart from that, toxoplasmosis can be acquired congenitally and post-natally. Sero-negative pregnant women may be at risk of contracting *Toxoplasma gondii* infection. This congenital infection may cause the fetuses to be infected and develop severe complications and sometimes causing death. The prevalence varied from place to place where it is estimated that 16-40 % of the population was infected in the United States and the United Kingdom (Hill & Dubey, 2002). In Malaysia, the epidemiological surveys indicate that *Toxoplasma* antibody is found in all ethnic groups with Malays having the highest percentage at 33 %, followed by Indians at 29 % and Chinese at 18 % (Tee *et al*., 1998).

1.1.1 Apicomplexan parasites

Parasites refer to organisms that depend on the hosts to survive and at the same time causing diseases to the hosts. These pathogenic parasites can be harmful to human as well as some economically important animal species. One of the most important groups of parasites is the apicomplexan parasites (Table 1) that include *Plasmodium spp.,* the causative agent of malaria, *Cryptosporidium*, an opportunistic intestinal pathogen and also *Toxoplasma gondii*. Infection by protozoan parasites of the phylum Apicomplexa sometimes leads to mortality of human and livestock.

Table 2.1 Taxonomic classification of *Toxoplasma gondii*

Taxonomic Classification	
Kingdom	Chromalveolata
Phylum	Apicomplexa
Class	Conoidasida
Order	Eucoccidiorida
Family	Sarcosystidae
Genus	*Toxoplasm*a
Species	*gondii*

There are some morphological traits that are shared among the parasites in the phylum apicomplexa. These protists are elongated in shape and have a specialised apical region. The apical complex consists of a collection of unique organelles with unique functions (Morrissette & Sibley, 2002). Apart from the apical complex, a highly conserved DNA of 35 kb has been discovered from several genera within the

phylum Apicomplexa including *Toxoplasma* (Beckers *et al*., 1995). This DNA resides in an organelle called the apicoplast. This plastid-like organelle is found in nearly all members of the phylum except for *Cryptosporidium* spp. (Zhu *et al*., 2000). The apicoplast is believed to have its origins in a secondary endosymbiotic event. This organelle has been investigated extensively since its discovery. It was found that in *Toxoplasma gondii*, the inhibition of apicoplast DNA synthesis blocked the replication of parasite. This suggests that apicoplast plays an essential role in the survival of the parasites (Fichera & Roos, 1997). Apart from that, the chloroplast-derived organelles were not found in humans as well as other mammals, thus suggesting that the apicoplast might be useful in the study of parasite-specific targets in the drug development against Apicomplexa. *Toxoplasma gondii* has been an important model in the studies of apicomplast development and function.

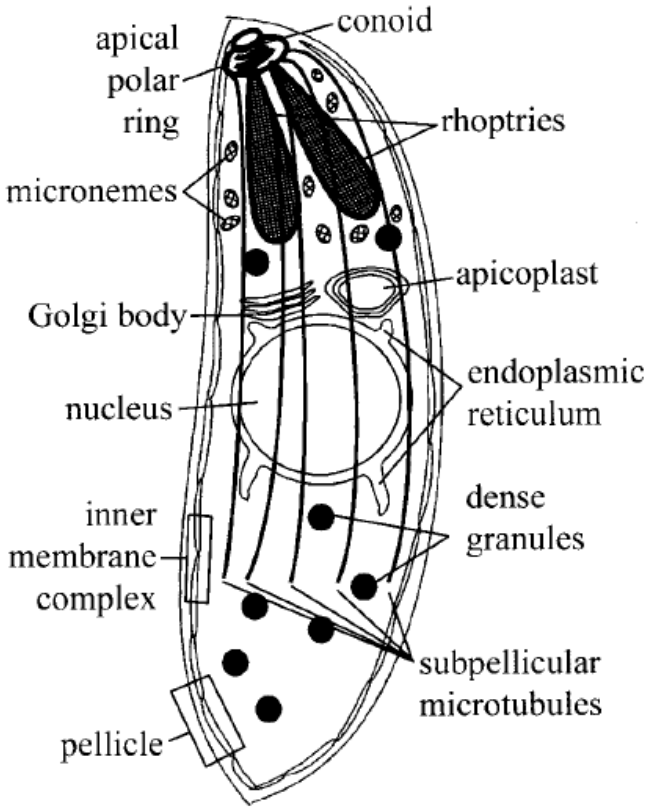

Figure 1.1 The morphology of apicomplexan parasites. There are a collection of organelles found specifically in this phylum. The apicoplast is located next to the Golgi body. (Figure is adapted from Morrissette & Sibley, 2002).

In the phylum Apicomplexa, *Toxoplasma gondii* appears to be used the most as experimental models for the study of apicomplexan biology (Roos *et al*., 1999). The intracellular morphology of *Toxoplasma gondii* is distinct (Figure 1). Besides, the organelles can be visualised readily using light microscope or advance electron microscope, despite the small size of tachyzoites, $2 \times 7\ \mu m^2$ (Swedlow *et al*., 2002). Furthermore, there are various experimental advantages such as the pathogenic stages of *Toxoplasma gondii* are easily propagated and quantified in the laboratory; the mouse animal model is well-established; and the availability of the reagents used in the study of the parasites, have played a major part in making the parasites being studied widely.

1.1.2 Life cycle and transmission of *Toxoplasma gondii*

Toxoplasma gondii has a complex life cycle. Figure 2 illustrated the life cycle and transmission route of *Toxoplasma gondii.* This parasite has a heteroxenous life cycle and it can infect a wide range of warm-blooded host which includes human. An example of a definitive host is the domestic cats. The infectious stages of the parasite can be divided into three stages, namely the tachyzoites, bradyzoites and sporozoites. Tachyzoites and bradyzoites are contained in tissue cysts while sporozoites are in sporulated oocysts. All these three stages are infectious. (Hill & Dubey, 2002)

The sexual cycle occurs only in the intestine of cats. Replication occurs which leads to the production of oocysts. This will then result in the shedding of oocysts in the faeces of cats for 7-21 days. Cats are able to shed millions of oocysts even if only one bradyzoite is ingested. Three infectious stages of *Toxoplasma gondii* ingested will lead to shedding of oocysts in cats (Dubey *et al*., 1970). Sporulation will take place and these infectious oocysts containing sporozoites will lead to tachyzoite stage upon ingestion by mammals including human. Infection of the parasites on human might also occur through the oral ingestion of tissue cysts found in raw or undercooked meat.

Tachyzoites are oval or crescent in shape and are about 2-4 μm wide and 4-8 μm long (Montoya & Liesenfeld, 2004). Parasites in this stage multiply rapidly and enter the nucleated cells by active penetration to form cytoplasmic vacuole (Dobrowolski & Sibley, 1996). Repeated replication of the tachyzoites will cause the disruption of host cells. The tachyzoites will then enter and infect the central nervous system, the

eye, placental and skeletal tissues through the bloodstream. Replication of the parasites will cause cell death and neighbouring cells will be invaded rapidly. Inflammatory response and tissue destruction will be triggered in the tachyzoite stage, which lead to clinical manifestations of diseases. Immune response triggered will act as a pressure to transform tachyzoites into bradyzoites to form cysts.

The difference between the structure of bradyzoites and tachyzoites is that bradyzoites have a nucleus situated toward the posterior end, while tachyzoites has a nucleus found in the central (Dubey *et al*., 1998). Cysts are the infective stages for both intermediate and definitive hosts. They are similar to tachyzoites morphologically but multiply slowly and express their function differently. Thousands of bradyzoites can be found in tissue cysts from brain cells, skeletal and heart muscles. After ingestion of tissue cysts by the host, proteolytic enzymes in the stomach and small intestine will dissolve the cyst wall. Bradyzoites released from cysts will then enter the small intestine and initiate the generation of the parasites (Dubey *et al*., 1998).

Infection of *Toxoplasma gondii* in human (Figure 2) often occurs through oral route. Inappropriate handling of cat litters, eating undercooked meat and drinking of contaminated water are a few common routes of transmission of the parasites to human. Apart from that, infection might also occur congenitally. Congenital infection occurs when a woman becomes infected during her pregnancy. Transmission of *Toxoplasma gondii* might occur through blood transfusions and organ transplants.

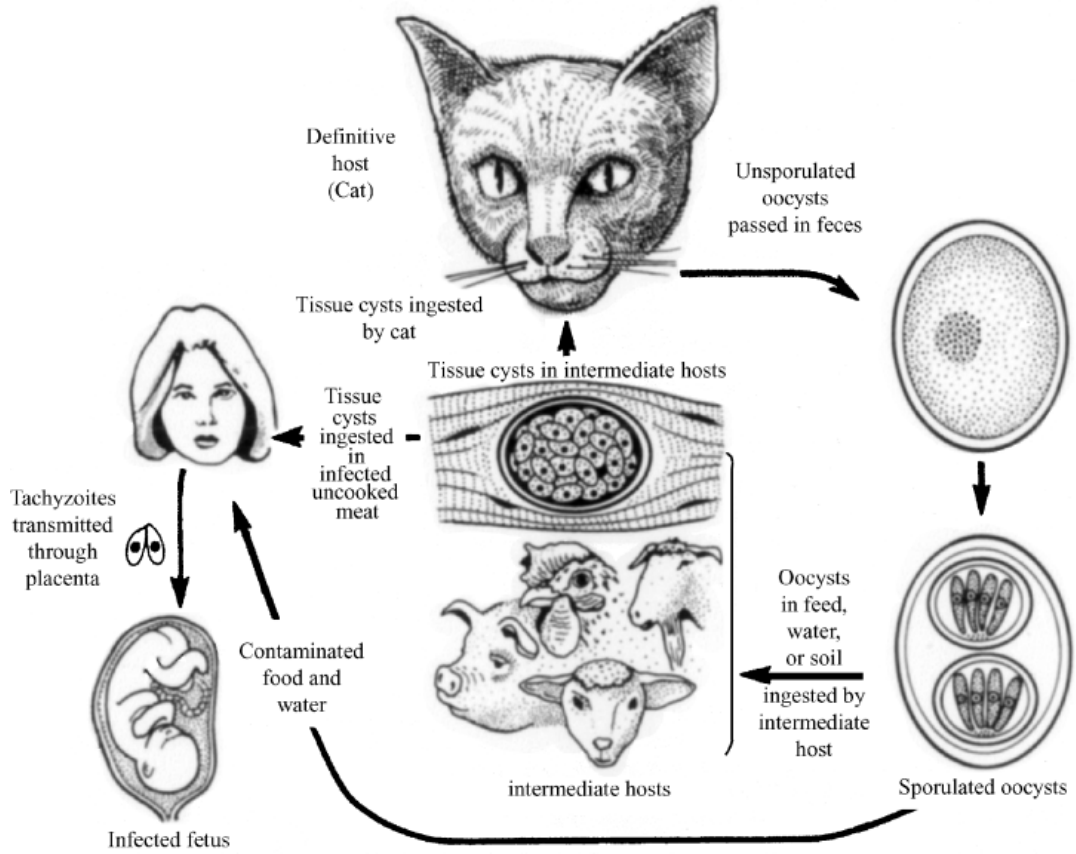

Figure 1.2 Life cycle of *Toxoplasma gondii.* The figure was adapted from (Hill & Dubey, 2002)

1.1.3 Treatment of toxoplasmosis

Immunocompetent patients infected with toxoplasmosis are usually not treated unless the symptoms are severe or persistent. The combination of pyrimethamine and sulfadiazine has remained as the standard treatment for this disease (Petersen *et al*., 2003). Pyrimethamine and sulfadiazine work by inhibiting the enzyme dihydrofolate reductase that is involved in the tetrahydrofolic acid synthesis (Kadri *et al*., 2014). This frequently used therapy is successful. However, many side effects like bone marrow suppression are observed (Bosch-Driessen *et al*., 2002).

Toxoplasma gondii infection can also be acquired by pregnant women. This may happen during gestation and the infection can be transmitted to the foetus (Goldstein, 2008). Management of maternal and foetal infection varies between different countries. Spiramycin or combination of pyrimethamine and sulfadiazine are often used depending on the trimester of pregnancy (Rajapakse *et al*., 2013). Spiramycin is a macrolide antibiotic and is often used during the first 18 weeks of gestation. It was reported that this treatment is able to reduce the vertical transmission of the parasite (Couvreur *et al*., 1988). However, spiramycin is not able to treat the infection in the foetus due to the inability of the drug to cross the placenta. Combination of pyrimethamine, sulfadiazine and folinic acid is used to treat pregnant women who are infected after 18 weeks of gestation and also for those confirmed foetal infection. Pyrimethamine should not be used during the first trimester of pregnancy due to the teratogenic potential. The drug produces dose-related depression of the bone marrow (Goldstein, 2008). This anti-toxoplasma treatment should be continued throughout the pregnancy period. Consequently, the patients who received pyrimethamine treatment should have their complete blood counts closely monitored.

1.1.4 Role of natural products in toxoplasmosis

Natural products continued to play a significant role in the drug discovery for the treatment of various human diseases. Natural products are thought to contain the desired properties of potency and often considered to have low toxicity. The use of medicinal plants for the treatment of parasitic diseases is well documented since ancient times. For example, *Cinchona succiruba* has been used to treat the infection of malaria for centuries (Kaur *et al*., 2009).

Numerous natural products have been studied extensively for potential source of new agents to treat toxoplasmosis. Among them, *Artemisia* annua L., an annual herb belonging to the family Asteraceae, has been reported to inhibit *Toxoplasma* replication *in vitro* (Jones-Brando, *et al*. 2006; Ke *et al*., 1990). The active compound, artemisinin isolated from the plant was initially developed for anti-malaria activity (de Ridder *et al*., 2008).

Medicinal herbs from South Korea were also tested for the anti-protozoal activity against cultures of *Toxoplasma gondii*. In the study, it was found that the alcohol extracts of *Torilis japonica* and *Sophora flavescens* demonstrated the best inhibition of *Toxoplasma gondii* among all the medicinal herbs tested (Youn *et al*., 2003). Oleuropein isolated from *Fraxinus rhychophylla* was investigated for its activity against *Toxoplasma gondii* both *in vitro* and *in vivo*. It was reported that oleuropein showed anti-toxoplasma activity in the infected mice (Jiang *et al*., 2008). Meanwhile 15 methanolic extracts of Korean traditional medicine were tested on the effect on *Toxoplasma gondii*. Among them, it was reported that *Zingiber officinale* possesses high anti-toxoplasma activity as well as low cytotoxicity (Choi *et al*., 2008).

Apart from that, maslinic acid (2α,3β-dihydroxyolean-12-en-28-oic acid) found in the fruits of the olive (*Olea europaea)* was reported to have the ability of blocking the entry of *Toxoplasma gondii* into the cells. This compound is present in many plants and is found mainly in the leaves and fruits of *Olea europaea* (De Pablos *et al*., 2010). Secrurinine produced by *Securinega suffructicosa* was evaluated against *Toxoplasma gondii*. This widely used traditional Chinese medicine was found to

inhibit the growth of the parasite *in vitro*. This compound was also less toxic when compared to pyrimethamine (Holmes *et al*., 2011). Another compound isolated from the Chinese medicine was ginkgolic acid. This compound is found in *Ginkgo biloba*, a chinese herb which was reported to possess anti-microbial properties and anti-tumor properties (Yang *et al*., 2004). In the study, it was reported that ginkgolic acids significantly inhibit *Toxoplasma gondii* DNA and protein synthesis at low concentrations (Chen *et al*., 2008).

In Malaysia, crude methanolic extract and sub-fractions of *Tinospora crispa* were investigated on against *Toxoplasma gondii*. This alkaloid-rich plant was reported to show potential anti-toxoplasma activity (Lee *et al*., 2012). *Eurycoma longifolia* Jack from the Simaroubaceae family has also been tested on the activity against *Toxoplasma gondii*. It was reported that its extracts and sub-fractions significantly inhibit the growth of *Toxoplasma gondii* at low concentrations (Kavitha *et al*., 2012).

1.1.5 *Toxoplasma gondii* and neurodegenerative diseases

Toxoplasma gondii, a neurotropic parasite, when infecting immunocompromised hosts, often leads to severe encephalitis and neurocognitive sequelae (Daniels *et al*., 2014). In year 1979, it was reported that infection of toxoplasmosis affects the learning performance and memory of laboratory rats and mice. It was found that, in the maze experiments and memory tests, infected rats and mice showed poor learning performance and their memory was severely affected (Witting, 1979).

Antipsychotic medications have been shown to have anti-protozoal activities. *In vitro* studies have evaluated the effect of antipsychotic medicines like phenothiazines, which was found to inhibit the growth of *Leishmania donovani* (Pearson *et al*., 1984), *Plasmodium falciparum* (Kristiansen & Jepsen, 1985) as well as *Toxoplasma gondii* (Pezzella *et al*., 1997).

Various investigations evaluated the effects of drugs used in the treatment of schizophrenia on the *Toxoplasma gondii*. Schizophrenia is known to cause deficits in learning, memory as well as other cognitive functions. It still remain unclear how the infection contributes to cognitive deficits in schizophrenia patients. Thus, with the understanding of how latent infection cause deficits in learning and memory, new therapeutic treatments can be developed (Daniels *et al*., 2014).

In year 2011, the possible association between toxoplasma infection and Alzheimer's disease was investigated. The findings suggested that toxoplasma infection may be involved in the pathogenesis mechanisms of Alzheimer's disease. This can serve as a new approach for the management of this neurodegenerative disease (Kusbeci *et al*., 2011).

1.2 Alzheimer's disease: Overview

Alzheimer's disease (AD) was first described more than 100 years ago. It is the most common cause of dementia and was first reported by a German psychiatrist named Alois Alzheimer in his study of a woman who suffered from progressive dementia. This patient had suffered from a rapid loss of memory and she had become disoriented in time and space. At the end of her life, she became bedridden and incontinent and died four and a half years later. Post-mortem examination done on her revealed the presence of amyloid plaques in her brain (Small & Cappai, 2006). AD patients will present a gradual onset, showing a decline in cognition, behavioural as well as motor functions. Patients suffer from AD will have their daily functioning and quality of life affected greatly (Geldmacher & Whitehouse, 1996). At the early stage of AD, symptoms of depression might be observed. Other cognitive symptoms include loss of short-term memory, disorientation to time, place and people, and language impairment.

Early diagnosis of AD can be done using improved diagnosis techniques and criteria. Patients will can evaluated through some physical examinations, the detailed history of patients and their mental state will be examined by using specific cognitive and psychological tests (Alloul *et al*., 1998). However, at present, the definitive diagnostic can only be done by histological examination of the brain tissue.

1.2.1 Hypothesis of Alzheimer's disease

Two major hypotheses have been proposed to explain the underlying molecular mechanism of Alzheimer's disease. One of the hypotheses is the amyloid cascade hypothesis. This hypothesis remains to be the best defined and most studied framework for Alzheimer's disease even though the exact cause of the disease is still being strongly debated. As described by Alois Alzheimer in his finding in year 1907, the detection of amyloid plaques in patients were defined as the characteristic of Alzheimer's disease. This hypothesis suggests that the deposition of amyloid beta (Aβ) will lead to neuronal dysfunction and death in the brain (Ballard *et al*., 2011). This extracellular deposits are found mostly in the neocortex and are comprised of 4-kDa polypeptides (Glenner & Wong, 1984). Aβ is produced by the enzyme β-secretase and subsequently by γ-secretase from a larger precursor known as the β-amyloid protein precursor (APP) (Small & Cappai, 2006). This pathway is termed as amyloidogenic pathway and produces both less toxic or more toxic and highly aggregating Aβ depending on the cleavage sites. It was suggested that genetic and environmental factors might contribute to the production of more toxic Aβ, of which the mechanisms are not known (Tayeb *et al*., 2012). Accumulation of toxic Aβ leads to a cascade of events including an inflammatory response, free radical formation, oxidative stress and hyperphosphorylation of tau protein (Cummings, 2011).

On the other hand, intracellular neurofibrillary tangles (NFTs), according to amyloid cascade hypothesis, is also another major component found in Alzheimer's disease. It was said that the spread of NFTs from the hippocampus and enthorhinal cortex,

slowly to the rest of the brain matches the clinical profile of Alzheimer's disease (Thal *et al*., 2000). NFTs are made up of hyperphosphorylated microtubule-associated protein, tau. Tau is found widely in the brain and it belongs to the microtubule associated protein family. The main function of this phosphoprotein is to maintain the stability of microtubule (Guela *et al*., 1998). In Alzheimer's disease, this function of stabilizing the microtubules is impaired and consequently, tangles will be formed, leading to neurons dystrophy (Parihar & Hemnani, 2004).

The second hypothesis, known as the "cholinergic hypothesis" has gained attention since the 1980's. Many studies supported the theory that age-related deficit in acetylcholine (ACh) neurotransmission leads to cognitive impairments, which is similar to the pathology of Alzheimer's disease. After conducting the post-mortem and biopsy studies, it was found that acetylcholine synthesis and activity of choline acetyltransferase were greatly reduced in Alzheimer's diseases patients. (Bowen *et al.*, 1977). In addition, the release and metabolism of acetylcholine were also reduced. The cholinergic deficits were caused by the extensive cell loss in neocortex, amygdala and hippocampus. This hypothesis was further supported by the findings that cholinergic neurotransmission alters memory, learning and attention where conditions such as senile plaques, choline acetyltransferase deficit and cognitive impairment were found in Alzheimer's disease patients (Geula & Mesulam, 1995). It was also found that when the activities of choline acetyltransferase and acetylcholinesterase were reduced in the cortex, the amount of senile plaques increased. Studies were done since the early 1980s to improve the cognitive abilities of animals by manipulating the cholinergic systems and inducing learning and memory disorders in hope to seek for potential drugs that are able to reverse this

alterations (Benzi & Moretti, 1998). This was done by prolonging the availability of ACh released by inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity, which are enzymes that hydrolyze ACh in the brain.

1.2.2 Role of cholinesterases in Aizheimer's Disease

In patients of Alzheimer's disease, a decline in choline acetyltransferase and acetylcholinesterase (ACh) has been reported in the cerebral cortex of the brain. Both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are involved in the breakdown of acetylcholine to choline and acetate and hence, terminating the neurotransmitter's function in the brain. Both enzymes are found in neurons and glial and also neuritic plaques in Alzheimer's disease patients. Studies have suggested that AChE plays the role of promoting the formation of amyloid beta plaques which will lead to aggregation of this peptide into insoluble plaques (Rees *et al*., 2003). In the brain of Alzheimer's disease patient, BuChE activity increases whereas AChE activity remained unchanged or declines (Greig *et al*., 2002). BuChE may replace AChE to hydrolyze brain ACh in advanced stage of Alzheimer's disease. With the involvement of both AChE and BuChE in the breakdown of ACh in the brain, dual inhibition of these enzymes might increase the efficacy of the treatment of Alzheimer's disease.

1.2.3 Pharmacological aspects of Alzheimer's Disease

The major therapeutic approach to Alzheimer's disease is based on the cholinergic hypothesis where the main action is to affect the cognitive decline by improving the

cholinergic neurotransmission (Benzi & Moretti, 1998). There are many available strategies to enhance the cholinergic activity in the brain. However, cholinesterase inhibitors (ChEIs) are the best-developed therapy and are used in most mild to moderate stages of the disease. ChEIs work by blocking the breakdown of neurotransmitter acetylcholine (ACh) by acetylcholinesterase (AChE) and hence, prolonging the cholinergic transmission. Among many compounds developed, tacrine and physostigmine were evaluated most extensively on their ability to inhibit cholinesterase.

Generally, the available cholinesterase inhibitors can be divided into three main classes based on the structure and mechanism. Tertiary amine compounds are the reversible inhibitors where this class of compounds bind to the hydrophobic region near the anionic α or β sites to trigger the inhibition. Drugs in this class are donepezil (non-competitive inhibitor) and tacrine (mixed type). Second type of the compounds are carbamates such as eptastigmine, which are pseudo-irreversible inhibitors and third type are organophosphates such as dichlorvos that are irreversible inhibitors (Benzi & Moretti, 1998).

Tacrine was the first drug approved by the Food and Drug Administration (FDA) for the treatment of Alzheimer's disease in year 1993. However, adverse effects such as hepatotoxicity and gastrointestinal upset were reported (Camps & Achab, 2000), thus undermining its use as a drug. Galanthamine is a long acting, selective and reversible AChE inhibitor. Another drug approved by FDA for the treatment of Alzheimer's disease is the donepezil which is highly selective for AChE and lower affinity for

BuChE (Racchi *et al*., 2004). Recently, it was reported that combination therapy of cholinesterase inhibitors with memantine has shown positive effect on the treatment of Alzheimer's disease of moderate to severe cases (Matsunaga *et al*., 2014).

1.2.4 Role of natural products in Alzheimer's Disease

Natural products play a highly significant role in the drug discovery and development process. In traditional practices, various plants have been used to treat cognitive disorders. In Indian and Chinese traditional medicine system, *Bacopa monniera* and *Ginkgo biloba* were used to improve cognitive disorders (Das *et al*., 2002). In Korea, methanolic extracts of traditional herbs were studied for the improvement of memory and cognition. It was found that *Acorus calamus* and *Epimedium koreanum* possess cholinesterase inhibitory properties (Oh *et al*., 2004). Several plants used in the traditional medicine remedies to treat forgetfulness and to improve memory were studied on their AChE inhibitory activities. It was found that the extracts from *Stephania suberosa* and *Tabernaemontana divaricata* showed AChE inhibitory activity (Ingkaninan *et al.,* 2003). In 2010, plants that are used in traditional European medicine to treat different central nervous system disorders or to improve memory were tested for their AChE as well as BuChE inhibitory effects. The study showed that hexane or methanolic extracts of *Arnica chamissonis, Ruta graveolens* and *Hypericum perforatum* showed inhibitory activity on cholinesterase enzymes (Wszelaki *et al*., 2010).

Bioactive compounds were isolated from medicinal plants which contributed greatly to the discovery of effective drugs to treat Alzheimer's disease. Physostigmine is an

indole alkaloid AChE inhibitor. It was isolated from *Physostigma venenosum*, which was traditionally used as a ritual poison in Africa (Da-Yuan *et al*., 1996). The discovery of physostigmine has led to the discovery of rivastigmine. Rivastigmine is used in the UK for the treatment of mild to moderate Alzheimer's disease. This AChE inhibitor works by inhibiting AChE in the cortex and hippocampus of the brain (Farlow *et al*., 2000). Rivastigmine was tested in a group of clinically characterised patients with Lewy-body dementia and results showed that patients taking rivastigmine showed a 30% of improvement (McKeith *et al*., 2000). *Galanthus nivalis* was traditionally used to treat neurological conditions. An alkaloid, galantamine, isolated from this plant was reported to possess selective activity against AChE than BuChE. The drug was well-tolerated when administered to patients affected by Alzheimer's disease (López *et al*., 2002). In China, a moss which is used traditionally to treat dementia was tested on the cholinesterase inhibitory activity. The isolated huperzine A from this plant, *Huperzia serrata* was found to improve memory retention processes in studies that used aged and adult rats (Raves *et al*., 1997).

Tropical forest has a large repository of the medicinal plant species. This provides an invaluable amount of materials for potential drug discovery. The search for cholinesterase inhibitors from plant source has been the main focus since the last decade. Natural-product based treatment on Alzheimer's disease has gained its popularity due to the positive outcome of previous research studies. This serves as a platform for future research and study to obtain effective cholinesterase inhibitors from plant resources.

1.3 *Ailanthus malabarica*

The plants of the genus *Ailanthus* (Table 2), which belong to family of Simaroubaceae are distributed widely over Asia as well as north Australia. The Simaroubaceae family consists of 150 species of trees and shrubs. Among them, the genus *Ailanthus* plays a significant role as medicinal plant. Several species from this genus have been studied extensively due to their economical and medicinal importance. In Indian villages, majority of *Ailanthus* varieties have been used as traditional medicine. The word *Ailanthus* comes from ailanto. It is an Ambonese word that means "tree of heaven". These plants can grow up to 25-45m tall, with spreading branches and large pinnate leaves (Laskar, 2010).

Table 1.2 Taxonomic classification of *Ailanthus malabarica*

Taxonomic Classification	
Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Sapindales
Family	Simaroubaceae
Genus	*Ailanthus*
Species	*malabarica*