ANTI-CHOLINESTERASE ACTIVITY OF MALAYSIAN *CASSIA* SPP. AND ITS BIOACTIVE COMPOUND

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ANTI-CHOLINESTERASE ACTIVITY OF MALAYSIAN *CASSIA* SPP. AND ITS BIOACTIVE COMPOUND

by

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
˚C	Degree Celsius
^{13}C NMR	Carbon nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
2-D	Two dimensions
3-D	Three dimensions
Å	Angstrom
Acetone-D_6	Deuterated acetone
ACh	Acetylcholine
AChE	Acetylcholinesterase enzyme
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
ADT	AutoDock tools
ALA	Alanine
APP	Amyloid precursor protein
ARG	Arginine
ASP	Aspartic acid
ATCI	Acetylthiocholine iodide
AutoDock	Automated docking
Aβ	Alpha beta
BBB	Blood brain barrier
C	Carbon
CADD	Computer aided drug design
CH_3	Methyl group
ChAT	choline acetyltransferase
$CHCl_3$	Chloroform
COSY	Homonuclear Correlation Spectroscopy
CT-SF	*Cassia timorensis* sub fraction
DMSO	Dimethyl sulfoxide
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
EtOAC	Ethyl acetate

FDA	Food Drug and Administration
FEB	Free energy binding
g	Gram
GA	Genetic algorithm
GLN	Glutamine
GLU	Glutamic acid
GLY	Glycine
GNT	Galanthamine derivatives
GNT1	Galanthamine
H-C	Hydrogen carbon correlation
H-H	Hydrogen hydrogen correlation
HIS	Histidine
HMBC	Heteronuclear Multiple-Bond Correlation
HSQC	Heteronuclear Single – Quantum Correlation
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
J	Coupling constant
kcal/mol	Kilo calories per mole
K	Kelvin
Kg	Kilogram
L	Litre
M	Molarity
m/z	Mass to charge ratio
mAChR	Muscarinic receptor
MeOH	Methanol
mg/mL	Milligram per microliter
MHz	Mega hertz
mL	Milliliter
mM	Millimolar
MQUE	3-methoxyquercetin
n	Number of data
nAChR	Nicotinic receptor
NFTs	Neurofibrillary tangles

nM	Nanomolar
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
OCH_3	Methoxy group
OH	Hydroxyl group
PAS	Peripheral binding site
PDB	Protein Data Bank
PHE	Phenylalanine
PHY	Physostigmine
ppm	Part per million
PRO	Proline
QUE	Quercetin
R^2	Relative coefficient
Rf	Retention factor
RMSD	Root Mean Square Deviation
ROS	Reactive oxygen species
sd	Standard deviation
SER	Serine
spp.	Species
TcAChE	*Torpedo californica* acetylcholinesterase enzyme
TLC	Thin layer chromatography
TRP	Tryptophan
TYR	Tyrosine
U/mg	Unit per milligram
U/mL	Unit per milliliter
UV	Ultraviolet
γ	Gamma
λ	Lambda
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
π	Pi
τ	Tau

AKTIVITI ANTI-KOLINESTERASE DARIPADA *CASSIA* SPP. DI MALAYSIA DAN SEBATIAN BIOAKTIFNYA

ABSTRAK

Penyakit Alzheimer menjadi perhatian utama dunia kerana sehingga kini masih tiada penawar untuk merawat penyakit ini. Alzheimer dikategorikan sebagai penyakit neurodegeneratif di mana sel-sel otak dan saraf pada sistem kognitif berkurang secara konsisten. Pendekatan hipotesis kolinergik merupakan pendekatan yang paling efektif dalam merawat penyakit Alzheimer iaitu dengan merencatkan aktiviti enzim asetilkolinesterase (AChE). Berdasarkan penemuan terbaru kami yang telah diterbitkan berkenaan saringan anti-AChE terhadap pelbagai keluarga pokok di Malaysia. Pokok daripada genus *Cassia* menunjukkan keputusan yang memberangsangkan dalam merencatkan AChE. Oleh itu, ujian saringan bagi mengenal pasti perencatan aktiviti asetilkolinesterase menggunakan kaedah Ellman telah dilakukan terhadap lima spesies *Cassia* iaitu *Cassia timorensis, Cassia grandis, Cassia fistula, Cassia spectabilis,* dan *Cassia alata* dengan kepekatan 200 ppm. Melalui pendekatan bio-fraksinasi, proses pengasingan sebatian ditumpukan kepada ekstrak daun *C. timorensis* daripada fraksi etil asetat oleh kerana capaian aktiviti perencatan yang tinggi dan nilai IC_{50} yang rendah. Pengasingan kompaun daripada *C. timorensis* menemukan satu sebatian bioaktif iaitu 3-metoksikuersetin dengan mencatatkan aktiviti perencatan pada kepekatan 100 ppm sebanyak 83.81 peratus dengan nilai IC_{50} 83.71 mikromolar. Aktiviti perencatan 3-metoksikuersetin ini dibandingkan dengan kuersetin dan peratus perencatan kuersetin hanya memberi aktiviti perencatan sederhana sebanyak 52.31 peratus dengan nilai IC_{50} 249.10 mikromolar. Perbandingan ini dilakukan adalah untuk memahami dengan lebih

mendalam terhadap perbezaan hubungan struktur berbanding struktur induk kuersetin dan struktur derivatifnya terhadap aktiviti perencatan. Lanjutan daripada itu, kedua-dua sebatian tersebut disimulasikan menggunakan kaedah molekular doking terhadap protein TcAChE untuk meramal interaksi yang terlibat di dalam tapak aktif. Interaksi 3-metoksikuersetin khususnya berlaku pada asid amino di bahagian periferal (PAS) dan poket asil manakala interaksi antara kuercetin dan tapak aktif protein berlaku pada PAS dan juga asid amino di bahagian poket kolin. Perbezaan struktur kedua-dua kompaun adalah pada kumpulan gantian pada kedudukan C-3, di mana kumpulan metoksi pada sebatian 3-metoksikuercetin memberi impak terhadap perencatan aktiviti asetilkolinesterase.

ANTI-CHOLINESTERASE ACTIVITY OF MALAYSIAN *CASSIA* SPP. AND ITS BIOACTIVE COMPOUND

ABSTRACT

Alzheimer's disease (AD) has become a major concern worldwide as no has cure yet to be found to treat this disease. AD is classified as neurodegenerative disorder with consistently loss of neurons in the cognitive system. To date, cholinergic hypothesis has become the most successful approach to treat AD by inhibiting the acetylcholinesterase enzyme (AChE). Based on our recent published work on the anti-AChE screening of diverse botanical plant family from Malaysia, plants from *Cassia* spp. showed promising result in inhibiting AChE at high percentage. This project is the continuous study from the previous research to focus on the potential of several *Cassia* spp. in inhibiting AChE. Five *Cassia* species including *Cassia timorensis, Cassia grandis, Cassia fistula, Cassia spectabilis,* and *Cassia alata* were screened at 200 ppm for *in vitro* AChE inhibition assay by using the Ellman's method. Through bio-guided fractionation, the isolation process was focused on the ethyl acetate fraction of *C. timorensis* leaves extract due to high inhibition activity and low IC_{50}. The isolation led to the discovery of a bioactive compound, 3-methoxyquercetin that exhibit 83.81% inhibition activity with an IC_{50} of 83.71 μM. The inhibition activity of 3-methoxyquercetin was then compared with the quercetin which manifested only moderate percentage inhibition of 52.31% and IC_{50} value of 249.10 μM. The comparison was done to understand more on the structural relationship between parent structure quercetin with its derivative against AChE. Subsequently, molecular docking was performed on both compounds against the target protein (TcAChE). The mode of interaction of 3-methoxyquercetin mainly occurs at peripheral binding site (PAS) and

acyl pocket of the active sites while the interaction of quercetin occurs at the peripheral binding site and choline binding site. The substitution of methoxy group at position 3 of 3-methoxyquercetin is likely to impact the inhibition activity and binding orientation towards AChE.

CHAPTER 1

INTRODUCTION

1.1 Statement of Problem

The world population is ageing due to drastic decrease in birth rate and mortality in tandem with increase in life expectancy. Within two decades, statistics showed that the number of older population increased from 540 million in 1995 to 900 million in 2015, forming 12% of the global population (Abeykoon *et al*., 2017). It is of no surprise that the number of the older population is projected to increase to one billion by 2020.

According to the definition by the United Nation, the term 'older population' includes those who are 60 years or older (World Health Organization, 2017). Worldwide, Asia recorded the highest number of older population. Based on official population statistics in Malaysia by the Department of Statistics, Malaysia (DOSM) in 2018, 2.8 million people in the country are 60 years old and above, from the 32.3 million population (Department of Statistics Malaysia Official Portal, 2019). This number is expected to increase to 5.7 million or 15% of the population in the country in 2030 (Suhaimi, 2013). With the increasing number of the older population, the morbidity rates will also increase (Mafauzy, 2000). This issue has been a major concern for governments and health care providers as the cost of providing health care to the elderly can be very high.

Among the most common disabling illnesses concomitant with old age is dementia. Dementia worldwide accounts for approximately 48 million people which comprises of different types including Alzheimer's disease, vascular dementia, dementia with Lewy bodies, and Parkinson disease (Livingston *et al*., 2017). All dementia types are related to the abnormalities of the brains that interfere with the

cortical functions including memories, language and communication, the ability to solve daily problems and can be severe enough to interrupt daily activities.

Alzheimer's disease (AD) is a silent global epidemic that approaches almost 48 million patients worldwide along with other dementia and this number is expected to increase to 76 million people by the year 2030 (Alzheimer's Association, 2019). According to Alzheimer disease facts and figures 2018 report by Alzheimer's Association, nearly 5.7 million Americans of all ages suffer from AD in 2018. From this number, 5.5 million are the elderly and surprisingly, the younger generations are no exception in getting younger onset AD. To make it worse, in every 65 seconds, an American start to develop AD (Alzheimer Association, 2018). AD has become the sixth leading cause of death and now it is being considered as one of the top 10 diseases that could not be cured or prevented. AD has always been taken lightly by the normal communities because many believe that AD is a typical process of ageing.

Based on the statistic from the Alzheimer Disease Foundation Malaysia, the number of AD patients has already reached 50,000 people and will double its figure in the coming decade, as the baby boom generation catches up (Alzheimer Disease Foundation Malaysia, 2019). The stigma on AD in Malaysia still remains strong because as mentioned earlier, Malaysians also do have a strong believe that the symptoms associated with AD are a normal part of aging and hence they normally do not seek for medical attention.

The pathology hallmark of AD is the accumulation of neurofibrillary tau tangles (τ) inside neurons and the formation of β-amyloid plaques on the outer side of the neurons. Formation of tau tangles unfortunately block the transportation of nutrients

and other essential molecules from entering the neurons while β-amyloid plaques may cause neurons death as it is interfering with neuron-neuron communications.

Several hypotheses such as Aβ-amyloid hypothesis, tau hypothesis, and cholinergic hypothesis have been suggested to aid in the discovery of therapeutics for the treatment of this disease. However, none of these hypotheses have given the solution to the unanswered question on the exact causes of AD and hence the pathogenesis of AD remains unclear, although it does seem to be a chronic and progressive disorder (Wang & Zhang, 2018).

The first hypothesis proposed in treating AD, i.e. the cholinergic hypothesis has been successful by far in alleviating the AD symptoms. It emphasises on three aspects observed from the brain of AD patients; (1) significant degeneration of neurons in the nucleus basalis of Meynert at the basal forebrain, to the cortex and to the hippocampus, (2) depletion of cholinergic neuron markers and synapses, and (3) the impairment of memories by antagonist and the alleviation of cognitive deficit by agonist (Contestabile, 2011). The findings from this hypothesis has directed the development of acetylcholinesterase inhibitors (AChEIs) that increase the level of acetylcholine by inhibiting the AChE enzyme.

Acetylcholinesterase enzyme (AChE) is a serine hydrolase enzyme that is responsible in breaking down the acetylcholine neurotransmitter into choline and acetate. However, superfluous hydrolysis of AChE in AD patients will worsen the condition of the patients. The treatment of AD patients by using AChEIs have been effective to counteract the deficit level of AChE.

Currently, there are three AD drugs that are approved by the U.S Food and Drug Administration (U.S FDA) which are donepezil, rivastigmine, and galanthamine for

the treatment of mild to moderate AD. Even though the three drugs have shown acceptable effectiveness, they could only provide short-term relief from AD symptoms and are only effective in certain patients and not beneficial for the rest. The certainty of who response and who did not still remain indistinct (Craig *et al*, 2011). In addition to their pharmacological effects, these drugs have also shown some unwanted side effects that mainly involve the gastrointestinal tract. Rapid escalating dose of AChEIs have proven to induce nausea, vomiting, anorexia, and diarrhoea for several days (Kavirajan & Schneider, 2007; Raskind, 2003).

To date, several potent AChE inhibitors from naturally-occuring alkaloid such as physostigmine, galanthamine, huperzine A, and huperzine B have been isolated from *Physostigma venenosum, Galanthus nivalis,* and *Huperzia serrata*, respectively (López *et al*., 2002; Mukherjee *et al*., 2007). These natural-based products are either being abandoned or still in the various stages of clinical trial.

Since then, many studies conducted to find new AChEIs have been focusing on plant-based alkaloid compounds. Galanthamine, an Amaryllidaceae alkaloid has provided the impetus for scientists to screen for alkaloid compounds in the search for potent AChEIs. For example, sanguinine that contains the galanthamine scaffold is reported to be a more potent inhibitor than galanthamine (López *et al*., 2002).

Cassia species (synonym *Senna*) from the family of Fabaceaea is known as "gelenggang" in Malaysia. *Cassia* is native throughout Asia; Malaysia, India, and China are among the countries that cultivate this plant species. They are also available in the other continents including East Africa, South Africa, America, Mexico, and Brazil. There are more than 600 species in this family including trees, herbs, and

shrubs. Traditionally, they have been used to treat wounds and skin diseases such as eczema, scabies, and ringworms (Deshpande & Bhalsing, 2013).

Cassia is also widely known for its laxative effects and is consumed as remedial herbal tea (Balasankar *et al*., 2013). Throughout decades, scientists have discovered many pharmacological activities of *Cassia* species including anti-oxidant, anti-inflammatory, hepatoprotective, anti-mutagenic, anti-bacterial, anti-fungal, and recently on anti-cholinesterase activity (Jung *et al*., 2016; Mehta *et al*., 2017; Shivjeet *et al*., 2013). There are a broad range of *Cassia* species' secondary metabolites, which are mainly the anthraquinones and flavonoids and they have been shown to be accountable for its pharmacological values (Kolar *et al*., 2018; Mortada *et al*., 2013; Yadav *et al*., 2010).

1.2 Scopes of Study

Cholinergic hypothesis approach is still relevant and regarded as the safest way to alleviate AD symptoms. However, the limitations of finding new AChEIs to treat AD has become the main concern worldwide, as finding the cure for AD is still a far-fetched effort. Adverse effects and efficacy of the current AChEIs have also been the issues whether the drugs themselves are safe to be used and tolerable towards the potential adverse effects.

To date, scientists have shifted their focus on finding safer natural-based compounds in inhibiting AChE to combat AD. Natural products, specifically medicinal plants that were used in the ancient times have consistently given promising results in treating certain diseases. Based on our recent published work on the anti-cholinesterase potential of diverse botanical families from Malaysia, the plants from *Cassia* species were found to inhibit AChE at greater than 80% inhibition (Rawa *et*

al., 2019) . For this reason, plants belonging to *Cassia* species were chosen in this study to be further investigated on their inhibitory potential through *in vitro* cholinesterase bioactivity guided approach and to explore the modes of enzyme inhibition through molecular docking simulations.

1.3 Objectives

1. To screen AChE enzyme inhibition activity from *C. timorensis*, *C. grandis, C. fistula*, *C. spectabilis,* and *C alata* methanol extract using different plant parts including leaves, stems, fruits, and flowers.
2. To perform bio-guided isolation from the most active fractions of the most active *Cassia* spp.
3. To isolate, characterize, and elucidate the bioactive compounds from the most active fractions of the most active *Cassia* spp.
4. To explain the mode of interaction between the AChE enzyme (protein) and the active isolated compound using molecular docking simulation study.

CHAPTER 2

LITERATURE REVIEW

2.1 Alzheimer's Disease

Alzheimer's disease (AD) is a type of prolonged and progressive neurodegenerative disorder. It is associated with consistent loss of neurons in cognitive system which leads to the development of dementia several years after (Wilson *et al*., 2012). Based on previous population studies, AD patients were found to develop a rather slow cognitive impairment – about twelve years before dementia symptoms appear (Amieva *et al*., 2008). AD usually affect the elderly of 65 years and older, resulting in memory impairment as well as difficulties in doing daily activities.

The Alzheimer Association estimated that in 2019, there are about 5.7 million AD patients in the United States of America alone, in which 5.6 million of them are the elderly (≥ 65 years) and approximately 200,000 are those below 65 years old. The association has also reported that AD is one of the top five diseases and is the top sixth leading cause of death among the Americans based on the latest available data (Alzheimer Association, 2017). The summary of Alzheimer disease in America was illustrate in Figure 2.1.

In the context of local population in Malaysia, statistics have shown that the number of dementia cases has surged tremendously from 2009 with 60,000 patients to 123,000 patients in 2015. The number is estimated to double itself to 261,000 by 2030 and will continually to increase to 590,000 patients in the following years (Alzheimer's Disease Foundation Malaysia, 2018). This statistic and its estimation was made based on the number of diagnosed AD patients. However, in reality there are still a substantial number of undiagnosed patients due to common belief that the symptoms

of AD are part of the normal ageing process and hence do not pursue any medical attention. This is a common situation in Malaysia and more awareness campaign would be needed to improve the medical seeking behaviour among AD patients and their family members.

Figure 2.1: Summary of Alzheimer's disease in United States of America (Alzheimer Association, 2018).

2.2 History of Alzheimer's Disease

AD was first discovered in 1901 by Dr. Alois Alzheimer who was a neurologist and psychiatrist, and the name of this disease was given in his honour. His passion in psychiatric study through microscopic has led to the first exploitation in histology study of human brains. In 1901, Auguste D., a 50-year old woman was first noticed to have personality and behavioural changes by her family member and was brought to Dr. Alois Alzheimer's psychiatry clinics in Frankfurt. Dr. Alzheimer diagnosed her with chronic dementia that causes memory impairments, deficits in language, and behavioural disorder. Auguste D. died on April, 1906.

During the five years under the care of Dr. Alzheimer, Auguste was described to have abnormal symptoms such as hallucination, delusion, confusion, paranoia, and speech struggle. Following her death, Dr. Alzheimer performed an autopsy in her brain and found the neurofibrillary tangles in the nerve cells and military deposits known as senile plaque in the cerebral cortex of her brain (Jellinger, 2006). Shortly after the discovery, Dr. Alzheimer brought the issue to the 37th reunion of Southwest German psychiatrists in 1906 where he briefly described AD without any illustrations. However, the early report of AD did not draw any attention from the psychiatric community that attend the reunion until the publishing of "The senile and presenile dementia" as one of the chapters in Emil Kraeplin's Textbook of Psychiatry in 1910.

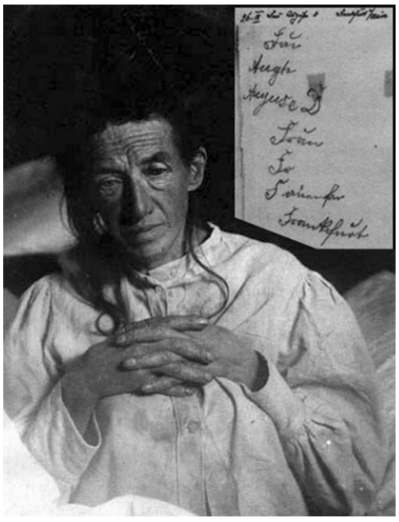

Figure 2.2: Auguste D. in 1902 with her hand writing; she gradually forgot to write her name (Jellinger, 2006).

2.3 Pathophysiology of Alzheimer's Disease

AD is characterized neurochemically as consistent deficit in cholinergic neurons in basal brains that develop with the appearance of amyloidal plaques, intercellular hyper-phosphorylated neurofibrillary tangles (NFTs), and inadequate level of acetylcholine (ACh) neurotransmitter in the patient's brain (Di Giovanni *et al*., 2008). It is also related to the accretion of abnormal tau (τ) tangles and Aβ amyloids plaque that formed inside as well as outside the neurons. Tau proteins was reported to hinder the absorption of essential nutrients by neurons and hence causes slow degeneration of neurons. The inter-correlation between all these factors have given various hypotheses on explaining this multifactorial malady such as amyloid (Aβ) hypothesis, tau (τ) hypothesis, and cholinergic hypothesis (Farooqui *et al*., 2016).

In the initial years following the discovery of AD by Dr. Alzheimer, AD was known as Dementia of the Alzheimer's Type (DAT) that are categorized as a classic presenile dementia. Throughout the years, AD was further categorized into several subtypes; (1) Senile dementia of the Alzheimer's type (SDAT), which is associated with the just-getting-older kind of patients with the senility diagnosis and hardening

of the arteries in the brain, (2) Late-onset Alzheimer's disease (LOAD), which generally occurs in patients who develop AD symptoms at later age (65 or older), (3) Early onset Alzheimer's disease, which refer to patients who develop AD symptoms at an early age (before 55 years old), and (4) Familial Alzheimer's disease (FAD), which generally refers to patients with positive family history of AD (Swerdlow, 2007).

2.3.1 Amyloid (Aβ) Cascade Hypothesis

Amyloid (Aβ) aggregation usually appears in the frontal cortex before spreading to the intact cortical region and it is associated with the amyloid precursor protein (APP). The mutation of the APP genes encoding at chromosomes 21 will largely influences the APP processing, often ensuing in higher production of Aβ 42 and increases the ratio of Aβ 42 to Aβ 40. Aβ 42 peptide is recognized as toxic to the cells and the increase level of this peptides will usually lead to the accumulation and aggregation of Aβ 42 with increasing of plaques formation, consequently triggering the cascade of events that causes neuronal death (Swerdlow, 2007).

In the case of FAD, the disease progression is closely related to the gene mutation of preseniline 1 (PS1) and preseniline 2 (PS2) which are located at chromosome 14 and 1, respectively. The mutations at these genes will cause the alteration of the APP cleavages and activate a higher production of longer Aβ 42 peptide. There is also another view which describe that AD is not caused by the absolute increase in Aβ 42 level alone but rather the imbalance in Aβ 42/40 ratio. This imbalance will trigger the cascade of deleterious changes in AD patients (Pimplikar, 2009).

The causes of high Aβ production in the brain of AD patients are varied and this hypothesis has been modified over the years as the Aβ accumulation is non-linear,

even in humans (Karran *et al*, 2011). Figure 2.3 shows the cause of amyloid cascade hypothesis modification over times.

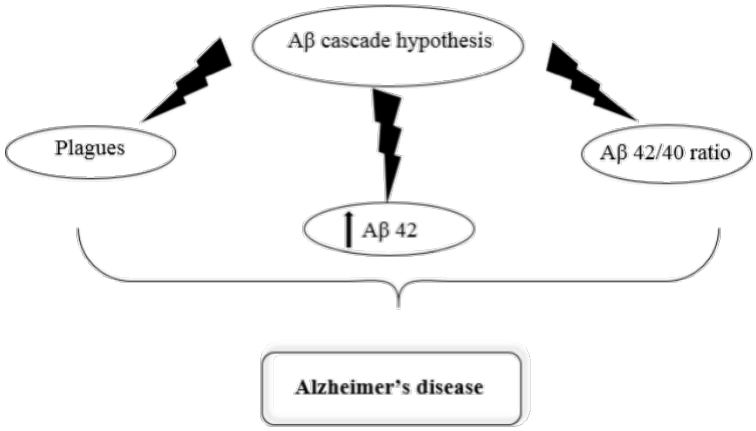

Figure 2.3 :Modifications of Aβ cascade hypothesis over the years.

2.3.2 Tau Hypothesis

Tau protein is low molecular weight microtubule-associated protein (MAP) localized in the chromosome 17 that is responsible in stabilizing the microtubules (Maccioni *et al*., 2010). In a normal condition, tau protein appears to be water-soluble and could be found abundantly in axons. However, mutations causes alteration to the tau protein which result in reduced solubility and affinity of the tau protein towards the axon microtubules (Mohandas *et al*., 2009). The accumulation of tau lesion may occur earlier than Aβ plaque formation and it is more severe as compared to the plaque load, correlating with disease progression at a faster rate.

In normal adult brain, six tau isoforms are expressed that contain a range of 352-441 amino acid residues. Longer tau isoform consists of repetition of four R1, R2, R3, and R4 (4R) with the insertion of exon 10 while the shortest tau isoforms consists of three times repetition of R1, R3 and R4 (3R) with no exon insertion (Kametani & Hasegawa, 2018).

In the brain of AD patients, the mutations that alter the function and isoforms leads to the 3R and 4R accumulations in a hyper-phosphorylated manner with unique twisted fibrils appeared as paired helical filament and intracellular neurofibrillary tangles (NFTs). The toxic forms of insoluble tau protein eventually damage the cytoplasm structure of microtubules and interrupts the axonal transmission transport (Lansdall, 2014). This will lead to neuronal death as could be observed in AD brains. The overview of Tau hypothesis was shown in Figure 2.4.

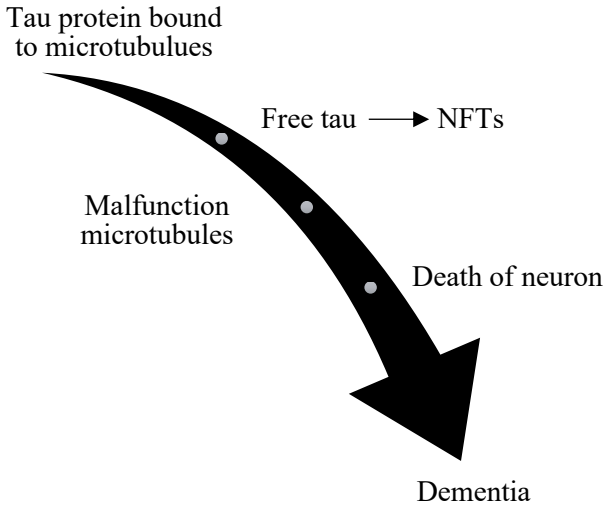

Figure 2.4: Overview of Tau hypothesis.

2.3.3 Cholinergic Hypothesis

Acetylcholine (ACh) neurotransmitter plays a major role in the transmission signals within neurons and neuromuscular junction. ACh is significantly contributed to the memory and learning. The cholinergic hypothesis imposed the reduction level of ACh in the brain specifically in the nucleus basalis of Meynert will worsen AD symptoms (Craig *et al*, 2011). The level of ACh in the brain is correlated with muscarinic receptor (mAChR), nicotinic receptor (nAChR), choline acetyltransferase (ChAT), and AChE. The ACh neurotransmitter is synthesized by ChAT and the cholinergic transmission within neurons occurs through the mAChR and nAChR that consist of several subtypes

and subunit each with 5 mAChR subtypes and 17 subunits nAchR, respectively. The transmissions occurred at these receptors were then terminated by the action of AChE (Piciotto *et al*., 2002).

The ChAT enzyme is responsible in synthesizing ACh and a reduction in ChAT activity strongly relates to the degree of cognitive impairment in AD patient's brains. However, several studies have reported that the ChAT activity in AD patients did not show any large decrement (Francis *et al*., 1999). Thus, although the reduction of ChAT activity correlates with AD, other factors also are likely to be involved in the cognitive impairment. Further studies conducted to understand the cause of ACh depletion has shown that the reduction in mAChR and nAchR level in AD patient's brains are significant, showing that these receptors do play a major role in memory, motor control, and also learning (Dannenberg *et al*., 2017).

mAChR is a G-protein couple receptor, functioning as coupling and activator for the heterometric G proteins. There are five subtypes of mAChR, which are classified as M1, M2, M3, M4, and M5 with M1 as the primary subtypes that is responsible for AD (Dannenberg *et al*., 2017). In AD patient's brain, the reduction of M1-M4 level was observed in cortex and hippocampus region. In addition, the M1/M3 receptors have been proven to involve in the effect of acetylcholine-based cognition. The stimulation of M1/M3 also increases the γ-secretase thus decreases the production of Aβ42 peptide (Flynn *et al*., 1995; Verma *et al*., 2018).

On the other hand, nAChR is the ionotropic receptor that is responsible as non-selective cation channel. nAChR is a receptor that is activated by the nicotine drug. Several subtypes of nAChR are present at the presynaptic membrane with α7 as the primary receptor involve in AD. α7-nAchR is dominantly expressed in hippocampus region and this appears to be the most affected area in AD. The presence of this subtype

controls the ACh neurotransmitter release, neuron activity, and the generation of the postsynaptic current at the junction, therefore the reduction of this subtype will highly affect AD patients (Verma *et al*., 2018).

The ACh neurotransmitters that are produced by ChAT and released by mAchR, and nAchR are hydrolysed by the AChE. Excessive degradation of ACh neurotransmitter will affect the ACh level in the brain. Blocking the hydrolysis of ACh into choline and acetate by AChE have proven to repair the memory impairments in AD patients (Terry & Buccafusco, 2003). Figure 2.5 briefly described the cholinergic hypothesis.

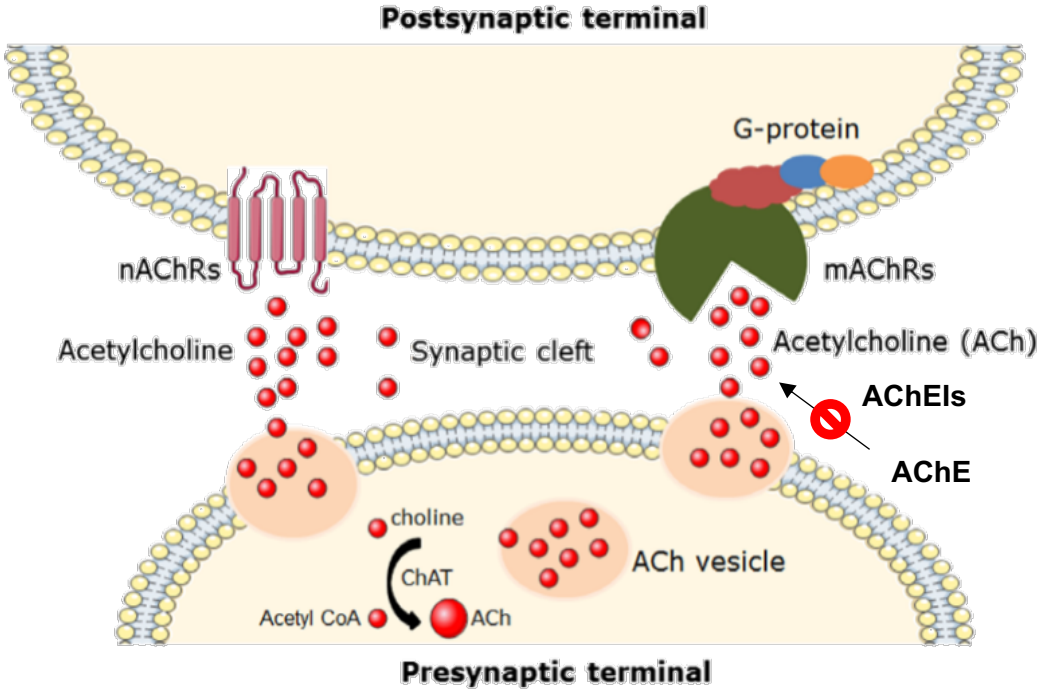

Figure 2.5: Overview of cholinergic hypothesis (Verma *et al*., 2018).

2.4 Current Approaches for the Treatment of Alzheimer's Disease

Currently, there are no known cures available for AD despite the significant advancement in medical technology in the recent years. A previously reported therapeutic approaches through immunization to clear the Aβ amyloid was found to be a major failure and was reported lethal towards the AD patients (Citron, 2010). The fluctuations of ACh neurotransmitter in hippocampus and cerebral cortex was the most notable factor that causes the acceleration of AD symptoms. In order to slow down the symptom and alleviate distress, cholinergic hypothesis focusing on the inhibition of AChE was taken as the main key to combat AD.

The AChE controls the metabolic breakdown of ACh by metabolic hydrolysis of neurotransmitter ACh. However, excessive hydrolysis of the said neurotransmitter will lead to the development of AD. AChE inhibition has hence become an ideal choice as a pharmacotherapeutic approach for the mild to moderate AD. Currently there are several cholinergic-enhancing drugs available in the market for AD treatment. The first AChEIs drug approved by Food and Drug Administration (FDA) was tacrine (THA, Cognex®) and later the use of this drug was halted due to severe side effects. The discovery of tacrine was followed by other drugs including donepezil (Aricept®), rivastigmine (Exelon®), and galanthamine (Reminyl®) (Viegas *et al*., 2005). These compounds were synthesized from small molecules of natural-based phytochemicals and their therapeutic activity for AD treatment was successfully demonstrated. To date, several other natural compounds from alkaloid are under investigation for their potential to inhibit AChE such as Huperzine A and Huperzine B (Swanberg, 2016; Xing *et al*., 2014).

2.5 Structure and Catalytic Function of Acetylcholinesterase Enzyme

The AChE is a serine hydrolase enzyme, which usually accumulates at neuromuscular junctions and cholinergic brain synapse. AChE terminates impulse transmission by catalysing the hydrolysis of neurotransmitter ACh to choline and acetate. AChE hydrolyses approximately 25000 molecules of ACh per second. AChE also involved in the non-cholinergic functions, which involves the peripheral anionic site and partake in the process of maturation and deposition of β-amyloid at the outer neurons (Rouleau *et al*., 2011).

An ellipsoidal shape of AChE with the dimension of 45 Å length, 60 Å width and 65 Å depth will contain the most notable features of AChE structure characterized by a deep and narrow gorge approximately at 20 Å penetrating half AChE and widens out at the base (Colovic *et al*., 2013). The active site of AChE consists of two sub sites; anionic sub site and esteratic sub-site corresponding to the choline-binding pocket and catalytic mechanism, respectively. In addition to the two sub sites, the peripheral anionic site (PAS) located at the top of the enzyme acts as an entry gate. PAS consist of several important amino acids including TYR70, ASP72, TYR121, and TRP279. A recent study reported that the inhibition of PAS can prevent the deposition of amyloidogenic protein on neurons (Khaw *et al*., 2014).

Anionic sub site is where the quaternary amine of ACh binds during the chemical reaction, consist of important aromatic amino acid namely TRP84 and is highly conserved in all species. Among all aromatic residues, aromatic TRP84 is the most significant amino acids for the hydrophobic cationic-π interactions in enzymatic activity of AChE. The active site of AChE is shown in Figure 2.6.

The esteratic sub site has two binding pockets; the oxyanion hole and the acyl pocket. The oxyanion hole consists of 3 important amino acids (GLY116, GLY117, and ALA199) whilst the acyl pocket (PHE330 and PHE331) are important in the stabilisation of substrate and in rotating the substrate into horizontal position to prepare for hydrolysis process. The esterase sub site is responsible for the hydrolysis of ACh to choline and acetate at its catalytic triad by three significant amino acids; SER200, HIS440, and GLU327.

Based on a previous study, additional three anionic residues within the active site gorge GLU327, ASP443 and GLU199 are crucial to interact with cationic substrate and inhibitors for the catalytic activity.

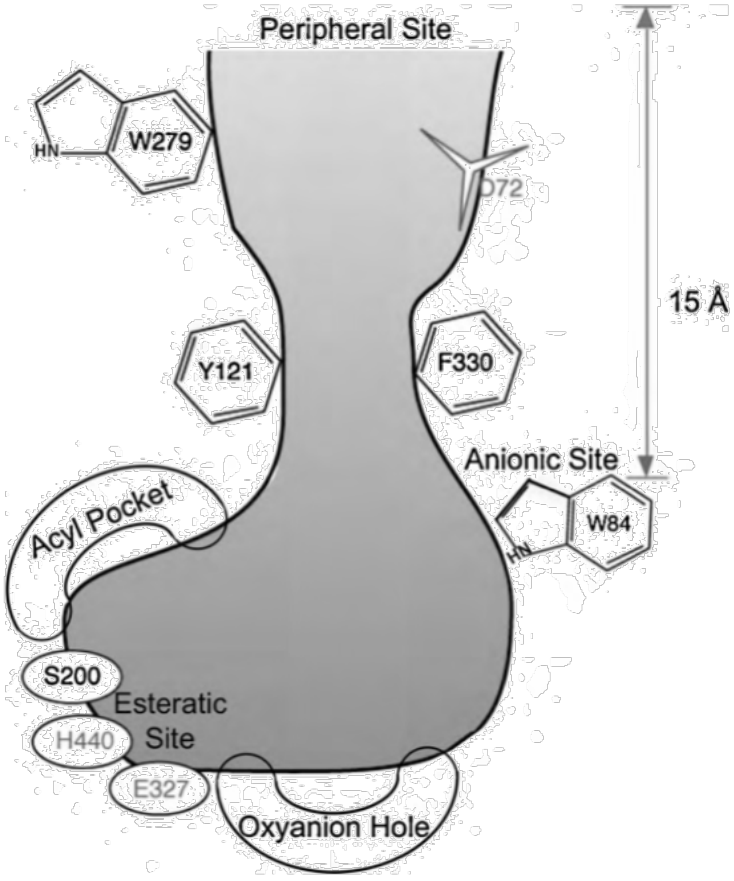

Figure 2.6: Schematic illustration of the active sites of AChE (Dvir *et al*., 2010).

2.6 Acetylcholinesterase Inhibitors from Natural-Based Compounds

AChEIs have become the therapy of choice for AD patients to slow down the metabolic breakdown of ACh and hence increasing the lifetime and concentration of neurotransmitter ACh at the cholinergic synapse in the brain of AD patients. Natural compounds manifested great pharmacological potential for many diseases including AD. Fallarero and co-workers found one compound named as coumarin 106 to have good inhibitory effect at 5 and 30 μM as compared to the other 28 coumarin compounds tested (Fallarero *et al*., 2008).

In another study, flavonoids from secondary metabolites, which include quercetin, rutin, kaempferol-3-O-β-D-galactosides, and macluraxanthone have shown great potential as inhibitors. Macluraxanthone showed the most potent inhibitor in a non-competitive manner with IC_{50} value of 8.47 μM. On the other hand, quercetin was reported to be as a competitive inhibitor against AChE with 353.86 μM whilst two flavonoids with sugar moiety; rutin and kaempferol-3-O-β-D-galactosides were ineffective against AChE (Khan *et al*., 2009). Another study reported by Ademosun *et al*., (2016) showed quercetin and rutin both inhibited AChE with quercetin having a lower IC_{50} value of 0.181 mM as compared to its glycosylated form, rutin which has an IC_{50} of 0.219 mM (Ademosun *et al*., 2016).

Alkaloids have also shown a strong activity as AChEIs. An indole alkaloid, physostigmine isolated from *Physostigma venenosum* has managed to improve cognitive function but only for a short term, showing a reversible AChE inhibition (Mukherjee *et al*., 2007). In another study, galanthamine-derived alkaloids isolated from amaryllidaceae family including sanguinine, 11-hydroxygalanthamine, and epinorgalanthamine showed positive inhibitory activity on the AChE (López *et al*.,

2002). It is hence postulated that plant-derived alkaloids can be a useful template for AChEIs and can be useful for the development of more drugs for AD treatment.

The result of AChE inhibition assay could also be supported by computational studies through molecular docking simulations. Computational studies will allow reciprocal evaluation of the experimental result by observing the ligand-protein interactions and its binding energy. As an example, coumarin 106 was observed to form a hydrogen bond with the catalytic triad and PAS. Carbonyl group (C-26) of the coumarin 106 formed a hydrogen bond with the catalytic triad SER200 and π-π stacking with PHE330 at the active gorge sites. C-26 also interacts with the amide group from ARG289 at PAS (Fallarero *et al*., 2008).

The AChE inhibition of quercetin and macluraxanthone was also reported to show positive results in molecular docking simulations. Quercetin showed lower binding energy with the formation of more hydrogen bonds as compared to macluraxanthone. The hydrogen bonds were reported between the oxygen atoms from quercetin with amino acids TYR133, TRP86, TYR72, GLN71, and ASP74 from AChE, with additional hydrophobic interaction with HIS447, SER125, and GLU202. Macluraxanthone on the other hand showed tight binding with higher energy and hydrogen bond interaction with only two amino acid residues; TYR124 and TYR72 at PAS. However, macluraxanthone showed stronger hydrophobic interaction with amino acid residues in the AChE active sites making it a potent inhibitor as compared to quercetin (Ademosun *et al*., 2016; Khan *et al*., 2009).

Recently, a molecular docking study of rutin on AChE was reported with a low free energy of binding (FEB) of -12.34 kcal/mol. It was found to form hydrogen bonds with amino acid residues ASN564, HIS436, ARG327, PRO399, and GLU344.

Hydrophobic interaction was also recorded between rutin and the HIS436 residue (Subramaniyan *et al*., 2017).

2.6.1 Acetylcholinesterase inhibitors from Traditional Herbal

Since the olden days, people rely greatly on traditional medicine and herbal remedies to treat various diseases and science has proven that herbal remedies do have certain pharmacological properties. Scientists continue to explore the effectiveness of herbal plants and remedies to treat various diseases and this includes AD. Among the approaches taken is to explore the possibility of increasing the ACh level in the brain by using certain plants' extracts.

The screening of medicinal herbs and remedies from various countries including the European countries, Egypt, Portugal, India, and Thailand as AChEIs have been reported in the literature. In 2013, Ali and his team reported about a study conducted on 23 Egyptian plants. From these tested plants, *Adhatoda vasica* and *Peganum harmala* exhibited inhibitory effect on AChE with an IC_{50} value of 294 and 68 µg/mL, respectively (Ali *et al*., 2013). Wszelaki and co-workers reported on a screening done on 24 European-based herbal medicines. From the various extracts produced, two most potent AChEIs were the hexane extracts of *Arnica chamissonis* and *Ruta graveolens* with an IC_{50} value of 29 and 34 µg/mL, respectively. *R. graveolens* was previously reported to contain 2-5% flavonoids comprising of rutin, furanocoumarins, and 0.4-1.4% alkaloids consisting of furoquinoline alkaloid, gamma-fagarine, acridone alkaloids, arborinine, and skimmianine. The inhibitory activity shown by the extract may due to the presences of these chemical compounds especially those from the alkaloid group (Wszelaki *et al*., 2010).

Ferreira and co-workers tested the inhibitory activity of Portugal-based herbal plants on AChE. Two extracts which are *Hypericum undulatum* and *Sanguisorba minor* showed positive results in inhibiting AChE (Ferreira *et al*., 2006). Vinutha *et al*., (2007) reported on the screening work conducted on 34 herbal plants from India, in which the methanolic extracts from six plants showed moderate activity as AChEIs - *Embelia ribes*, *Ficus religiosa*, *Nardostachys jatamansi*, *Semecarpus anacardium*, *Tinospora cordifolia*, and *Withania somnifera* (Vinutha *et al*., 2007).

In addition, a study on the methanolic extract of *Zingiber officinale* Roscoe, a common spice worldwide showed that the methanolic extract have inhibitory activity on the AChE and it was confirmed that among the major compounds present in the extract were gallic acid and quercetin (Mathew & Subramanian, 2014). Another study on the leaves extracts of *Rauvolfia reflexa* reported that the herb exhibited promising inhibitory effect with an IC_{50} ranging from 14.65 to 52.23 μg/mL from three different solvent extracts; dicholoromethane, ethanol, and methanol. Traditionally, *R. reflexa* has been used to treat aging-related brain diseases, malaria, and as remedy for poisons (Fadaeinasab *et al*., 2013).

2.7 *Cassia* species

2.7.1 General Description

The Genus of *Cassia* was belong to the family of Fabaceae and sub family of Caesalpiniaceae (Hennebelle *et al*., 2009). The taxonomic classification of genus *Cassia* are presented in Table 2.1 below.

Table 2.1: Taxonomy classification of *Cassia* spp.

Category	Classification
Kingdom	Plantae
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Subfamily	Caesalpiniaceae
Genus	*Cassia*

Cassia species encompasse of more than 600 species with various herbs, shrubs, and trees. It is widely distributed all over the world and are usually native to tropical countries; India, China, Malaysia, tropical American, African countries, Brazil, and Mexico. Each of the plant in *Cassia* spp. has its own medicinal values that may differ from one another. *Cassia* spp. is easy to spot with the existence of its bright yellow flower and some have pink and white flowers. *Cassia* spp.; *C. timorensis, C. grandis, C. fistula, C. spectabilis*, and *C. alata* used in this study were shown in Figure 2.7.

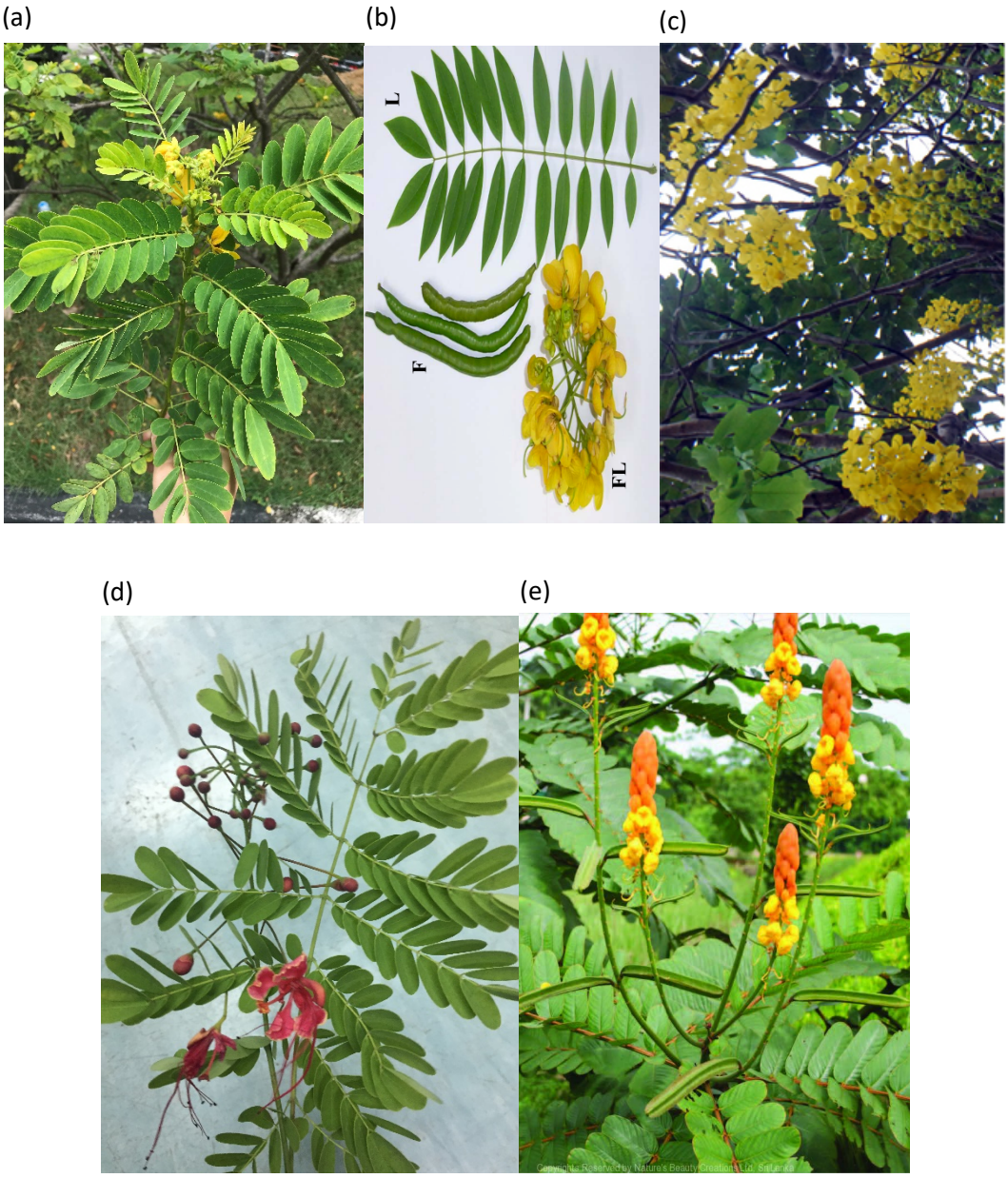

Figure 2.7: *Cassia* spp. used in this study (a) *C. timorensis*, (b) *C. spectabilis*, (c) *C. fistula*, (d) *C. grandis*, and (e) *C. alata*.