PHYTOCHEMICAL, BIOASSAY AND *IN SILICO* STUDIES ON ANTI - CHOLINESTERASE ACTIVITIES OF *CASSIA TIMORIENSIS* DC. AND *CASSIA GRANDIS* L.F.

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by

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celcius
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
IC_{50}	Half maximal inhibitory concentration
M	Molar
mg	Milligram
mg/mL	Milligram per milliliter
MHz	Megahertz
min	Minute
mL	Milliliter
mm	Millimeter
N	Normality
nm	Nanometer
pH	Scale of basicity and acidity
ppm	Parts per million

LIST OF ABBREVIATIONS

Aβ	Amyloid βeta protein
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
ACTI	Acetylthiocholine iodide
AD	Alzheimer's Disease
BTCI	Butyrylcholine iodide
BuChE	Butyrylcholinesterase
BuChEI	Butyrylcholinesterase inhibitor
CAT	Choline acetyltransferase
CAT	Choline Acetyl Transferase
$CDCl_3$	Deuterated chloroform solvent
CNS	Central nervous system
COSY	^1H-^1H Correlation Spectroscopy
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	Ethyl acetate
GC-MS	Gas chromatography-Mass spectrometry
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear Multiple-Bond Connectivity
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High performance liquid chromatography
*Hs*BChE	*Homo sapiens* butyrylcholinesterase
HSQC	Heteronuclear Single Quantum Coherence

Ki	Inhibition constant
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MRI	Magnetic resonance imaging
MRI	Magnetic resonance imaging
n-Hex	Hexane
NINCDS/ADRDA	National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's disease and Related Disorders Association
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NTF	Neurofibrillary tangles
PET	Positron emission tomography
PET	Positron Emission Tomography
Q-TOF	Quadrupole- time of flight detectors
RMSD	Root mean square deviation
ROS	Reactive oxygen species
RSD	Relative standard deviation
Rt	Retention time
SOD	Superoxide dismutase
*Tc*AChE	*Torpedo californica* acetylcholinesterase
TLC	Thin layer chromatography
UPLC	Ultraperformance liquid chromatography
UV	Ultra-violet spectroscopy

LIST OF APPENDICES

KAJIAN FITOKIMIA, BIOASSAI DAN IN SILICO TERHADAP AKTIVITI ANTI-KOLINESTERASE *CASSIA TIMORIENSIS* DC. DAN *CASSIA GRANDIS* L.F.

ABSTRAK

Dari segi sejarah, spesies *Cassia* telah ditunjukkan mempunyai beberapa aktiviti biologi. Walau bagaimanapun, penyelidikan saintifik mengenai *Cassia timoriensis* dan *Cassia grandis* masih terhad. Dalam kajian ini, matlamatnya adalah untuk menemui perencat kolinesterase (ChE) baharu untuk mengurangkan kekurangan asetilkolin (ACh) dalam penyakit Alzheimer (AD). Oleh itu, terhadap asetilkolinesterase (AChE) dan butirilkolinesterase (BChE), keupayaan penghapusan radikal, dan sifat anti-radang ekstrak berbeza *C. timoriensis* dan *C. grandis* telah dijalankan menggunakan ujian-ujian Ellman, DPPH dan denaturasi albumin. Analisis fitokimia mengesahkan kewujudan tanin, flavonoid, terpenoid, dan steroid dalam ekstrak *C. timoriensis*. Manakala flavonoid dan kuinon hanya terdapat dalam ekstrak etil asetat dan metanol *C. grandis*. Ekstrak etil asetat *C. timoriensis* dan *C. grandis* mempunyai kandungan fenolik tertinggi (masing-masing 527.43 ± 5.83 dan 187.74 ± 2.11 mg GAE/g DW) dan flavonoid (masing-masing 851.83 ± 10.08 dan 143.29 ± QEg/DW) berbanding dengan ekstrak-ekstrak lain. Selain itu, ekstrak etil asetat dan metanol kedua-dua tumbuhan mempamerkan aktiviti antioksidan, anti-radang dan anti-AChE yang tertinggi. Lapan sebatian telah diasingkan daripada *C. timoriensis* dan *C. grandis*. Lima daripadanya dilaporkan buat kali pertama dalam *C. timoriensis*: octadekanol (**1**), arakidil arkidat (**2**), β-sitosterol (**3a**), stigmasterol (**3b**), dan luteolin (**4**). Selain itu, **3a**, **3b**, asid sinamat (**5**), asid 4-hidroksisinamik (**6**), dan hidroksimetilfurfural (**7**) telah dikenal pasti daripada *C. grandis*. Sebatian **4**

menunjukkan perencatan yang ketara terhadap AChE (IC_{50}: 5.86 ± 0.31 µg/mL) dan BChE (IC_{50}: 13.21 ± 0.63 µg/mL), diikuti oleh **5** dan **6**. Manakala sebatian-sebatian lain menunjukkan aktiviti anti-ChE yang lemah dan sederhana. Pendokan molekul mendedahkan bahawa **4** menunjukkan pertalian pengikatan yang baik terhadap TcAChE (PDB ID: 1W6R) dan HsBChE (PDB ID: 4BDS), di mana ia membentuk ikatan hidrogen dengan TYR121 di tapak anionik periferi (PAS, 2.04 Å), bersama-sama dengan hidrofobik interaksi dengan tapak anionik dan PAS (TRP84 dan TYR121). Selain itu, **4** juga membentuk tiga ikatan hidrogen dengan sisi tapak pengikat. Ini mungkin menerangkan aktiviti perencatan **4** terhadap AChE dan BChE, secara *in vitro*. Akhir sekali, kajian perbandingan profil fitokimia (HPLC dan UPLC/QTOF-MS) dan aktiviti anti-ChE bagi ekstrak etanol *C. timoriensis*, *C. grandis*, *C. longa*, *C. asiatica*, *Z. officinale*, dan *P ginseng* mengenal pasti kemungkinan metabolit fitokimia yang bertanggungjawab untuk aktiviti anti-AChE yang kuat di kalangan *C. timoriensis*, *C. longa*, dan *Z. officinale* di mana *C. timoriensis* mempamerkan aktiviti perencatan tertinggi terhadap kedua-dua enzim, dengan IC_{50} sebanyak 12.89 ± 0.65 µg/mL untuk AChE dan 9.70 ± 0.70 µg/mL untuk BChE.

PHYTOCHEMICAL, BIOASSAY AND *IN SILICO* STUDIES ON ANTI-CHOLINESTERASE ACTIVITIES OF *CASSIA TIMORIENSIS* DC. AND *CASSIA GRANDIS* L.F.

ABSTRACT

Historically, *Cassia* species have been demonstrated to possess several biological activities. However, scientific research on *Cassia timoriensis* and *Cassia grandis* remains limited. In this study, the aim was to discover new cholinesterase (ChE) inhibitors to alleviate acetylcholine (ACh) depletion in Alzheimer's disease (AD). Hence, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory potentials, radical scavenging ability, and anti-inflammatory properties of various extracts of *C. timoriensis* and *C. grandis* were carried out using Ellman's assay, DPPH assay, and an albumin denaturation assay, respectively. The phytochemical analysis confirmed the existence of tannins, flavonoids, terpenoids, and steroids in *C. timoriensis* extracts. While flavonoids and quinones are present only in ethyl acetate and methanol extracts of *C. grandis*. Ethyl acetate extracts of *C. timoriensis* and *C. grandis* possessed the highest phenolic (527.43 ± 5.83 and 187.74 ± 2.11 mg GAE/g DW, respectively) and flavonoid (851.83 ± 10.08 and 143.29 ± 1.78 mg QE/g DW, respectively) contents as compared to the other extracts. In addition, the ethyl acetate and methanol extracts of both plants exhibited the highest antioxidant (34.74 μg/L, 21.03 μg/L for *C. timoriensis* and 38.92 μg/L, 37.79 μg/L for *C. grandis*), anti-inflammatory (92.22%, 92.50% for *C. timoriensis* and 66.37%, 64.80% for *C. grandis*), and anti-AChE activities (47 μg/L, 6.95 μg/L for *C. timoriensis* and 72.66 μg/L, 84.47 μg/L for *C. grandis*). Eight compounds were isolated from *C. timoriensis*

and *C. grandis*. Five of which were reported for the first time in *C. timoriensis*: octadecanol (**1**), arachidyl arachidate (**2**), β-sitosterol (**3a**), stigmasterol (**3b**), and luteolin (**4**). In addition, **3a**, **3b**, cinnamic acid (**5**), 4-hydroxycinnamic acid (**6**), and hydroxymethylfurfural (**7**) were identified from *C. grandis*. Compound **4** showed significant inhibition towards AChE (IC_{50}: 5.86 ± 0.31 µg/mL) and BChE (IC_{50}: 13.21 ± 0.63 µg/mL), followed by compounds **5** and **6**. Whilst, the other compounds exhibited poor to moderate anti-ChE activity. Molecular docking revealed that **4** showed a good binding affinity toward *Tc*AChE (PDB ID: 1W6R) and *Hs*BChE (PDB ID: 4BDS), where it formed a hydrogen bond with TYR121 at the peripheral anionic site (PAS, 2.04 Å), along with hydrophobic interactions with anionic site and PAS (TRP84 and TYR121, respectively). In addition, it also formed three H-bonds with the binding site residues. This possibly explains the inhibitory activity of **4** against AChE and BChE, *in vitro*. Finally, in order to comprehend more about the cholinesterase activity of our plant of study, a comparative study on the phytochemical profiles (HPLC and UPLC/QTOF-MS) and anti-ChE activity of the ethanolic extracts of *C. timoriensis*, *C. grandis*, *C. longa*, *C. asiatica*, *Z. officinale*, and *P. ginseng* identified the possible phytochemical metabolites responsible for the potent anti-AChE activity among *C. timoriensis*, *C. longa*, and *Z. officinale*; where *C. timoriensis* exhibited the highest inhibitory activity against both enzymes, with an IC_{50} of 12.89 ± 0.65 µg/mL for AChE and 9.70 ± 0.70 µg/mL for BChE.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders associated with aging-related dementia (Anand *et al.*, 2017). By 2050, it is anticipated that AD will afflict 1 in every 85 people worldwide (Rocca *et al.*, 2011). AD is characterized by the loss of cholinergic neurons as well as a decrease in the neurotransmitter acetylcholine (ACh) (Ferreira-Vieira *et al.*, 2016). ACh is a neurotransmitter that helps in the improvement of memory in the cortex, basal ganglia, and basal forebrain (Maurer *et al.*, 2017). After leaving the presynaptic neuron, ACh migrates to the postsynaptic cell by calcium influx. It then binds to nicotinic or muscarinic ACh receptors associated with neuronal or tissue responses (Nathanson, 2018). Cholinesterase inhibitors (acetylcholinesterase inhibitor (AChEI) and butyrylcholinesterase inhibitor (BChEI)) improve cognition and memory loss in AD patients by preventing ACh from being degraded by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Sharma, 2019). AChE, the more prevalent enzyme, is more strongly associated with cognitive function compared to BChE (Zagórska *et al.*, 2020). AChE is a powerful catalyzer capable of hydrolyzing around 250000 molecules of ACh per second (Norouzi *et al.*, 2010). The most frequently held belief in AD therapy is that AChE inhibitors can improve cognitive function through increasing ACh-mediated neuronal transmission (Zaki *et al.*, 2020). As a result, AChE inhibition, which had previously been used to treat myasthenia gravis, was utilized to be the first FDA approved treatment for AD (Crismon, 1994; Mehta *et al.*, 2012). In contrast, BChE is present in much lower concentrations with more restricted distribution in the

brain and is referred to as a "pseudo-cholinesterase" (Colovic *et al.*, 2013). As dementia symptoms advance, BChE activity increases, while AChE activity declines (Lane *et al.*, 2006). Therefore, BChE inhibition may also be advantageous in the late stages of AD (Arendt *et al.*, 1984; Zhou *et al.*, 2019).

Cholinergic effects on the neuroimmune system have also focused on the nicotinic ACh receptor (α7nAChR) (Maurer *et al.*, 2017). The activation of α7nAchR can reverse many of the detrimental consequences of immune responses, which are known as "non-neuronal cholinergic effects" (Maurer *et al.*, 2017). ACh binds to α7nAChR on macrophages and dendritic cells, restricting the inflammatory regulating receptor's function and causing a decrease in the generation and secretion of pro-inflammatory mediators (Báez-Pagán *et al.*, 2015). On the other hand, ACh inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNFα) released via α7nAChR. Likewise, AChE inhibitors have been shown to reduce glial activation and inflammatory cytokine production in a cerebral hypoperfusion model in the rat-hypoxia model (Gnatek *et al.*, 2012; Vaknine *et al.*, 2020). The hydrolysis of ACh by AChE can thereby cause an increase in the generation of pro-inflammatory cytokines (Vaknine *et al.*, 2020). These findings prompted the establishment of the "cholinergic hypothesis" for AD, as well as the use of AChE inhibitors (AChEIs) in the treatment of patients with AD. Thus, AChEIs contribute to the cholinergic-related memory loss associated with AD by raising ACh postsynaptic activity (Hoskin *et al.*, 2019) .

1.2 Problem Statement

Cholinesterase inhibitors are among the most commonly prescribed treatments for alzheimer's disease (Santos *et al.*, 2018). However, the effectiveness of these medications is limited, as they may cause undesirable side effect (nausea, vomiting, diarrhea, anorexia, and headache) and are consider as a symptomatic treatment for AD (Colovic *et al.*, 2013). However, recent research has shown that cholinesterase inhibitors do not only produce short-term symptomatic effects; they can also play a role in other pathological mechanisms of the disease, such as the delay the formation of amyloid-beta plaques (Jin *et al.*, 2020). This has resulted in a renewed interest in the discovery of new cholinesterase inhibitors with minimal side effects.

Throughout history, medicinal plants have gained widespread acceptance due to their lower side effects when compared to synthetic medicine. Furthermore, natural products have immensely contributed to the discovery of many cholinesterase inhibitors, such as physostigmine, huperzine A, and galantamine, as well as semisynthetic rivastigmine (Santos *et al.*, 2018). As a result, the current study was designed to investigate the potential bioactive phytochemicals of *Cassia timoriensis* DC. and *Cassia grandis* L.f. against cholinesterase enzymes through *in vitro* and *in silico* studies. Previously, our research group screened seventeen methanol extracts from different parts of five *Cassia* species for AChE inhibitory potentials (Azman *et al.*, 2020). The results revealed that *Cassia timoriensis* DC. and *Cassia grandis* L.f. have potent AChE inhibitory activity, indicating that these two plants could be good candidates for further phytochemical exploration in order to identify potential ChEIs for AD management.

1.3 Objectives of Study

The major aim of this research project is to discover new naturally occurring AChEIs derived from *Cassia timoriensis* DC. and *Cassia grandis* L.f. via the following objectives:

1. To investigate the phytochemical constituents, antioxidant, anti-inflammatory, and anti-AChE activities of various *C. timoriensis* and *C. grandis* extracts.
2. To isolate and identify potential AChE inhibitors from the active fraction(s) of *C. timoriensis* and *C. grandis* by various chromatographic and spectroscopic techniques, including MS, 1D-NMR (^{1}H and ^{13}C), and 2D-NMR (COSY, HSQC, and HMBC).
3. To evaluate the *in vitro* and *in silico* cholinesterase activity (AChE and BChE) of the identified compounds (objective #2) using Ellman's method and molecular docking, respectively.
4. To compare the HPLC and LC-MS/MS phytochemical profiles and anti-cholinesterase activities of the aqueous ethanolic extracts of *C. grandis* and *C. timoriensis* to other plants well-known to boost cognitive and memory functions in the literature (*Panax ginseng* C.A. Mey., *Curcuma longa* L., *Centella asiatica* (L.) UrB., and *Zingiber officinale* Roscoe).

1.4 General Structure of Study

This study investigates the phytochemical constituents of *C. timoriensis* flowers and *C. grandis* pods as well as their *in vitro* and *in silico* anti-AChE and anti-BChE activities. This study was divided into three major parts as described in Figure

1.1. Part one will be a qualitative and quantitative phytochemical analysis of *C. timoriensis* and *C. grandis*. Part two will be the isolation and structural characterization of the bioactive compounds from *C. timoriensis* and *C. grandis*. Finally, part three will be a comparative study on the phytochemical constituents and cholinesterase activity of the aqueous ethanolic extracts of two *Cassia* species studied, with the ethanolic extracts of the most well-known plant as a memory booster.

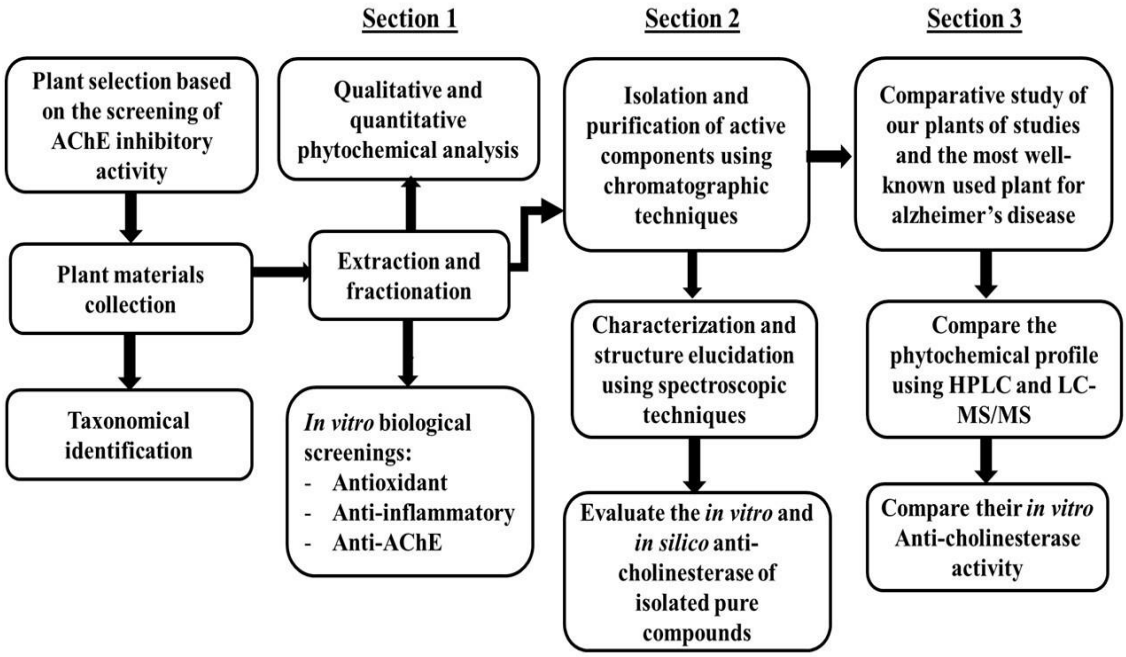

Figure 1.1 Descriptive scheme for the flow of the research study.

CHAPTER 2

LITERATURE REVIEW

2.1 Alzheimer's Disease Overview

Dementia is a broad term used to describe memory loss and at least one other cognitive ability impairment in elderly people over the age of 65 years old, such as perception, spatial ability, language, or executive performance (Farina *et al.*, 2017; Musa *et al.*, 2020). It is one of the most important causes of disability and loss of independence for elderly people to perform their daily activities (Tucker-Drob, 2019). It is more than just a medical condition; dementia has physical, psychological, social, and economic consequences for families and caregivers of patients with dementia (Farina *et al.*, 2017). AD is the most frequent cause of dementia, accounting for 60–80% of all dementia cases globally (Anand *et al.*, 2017).

AD is a more specific term for the most prevalent form of dementia to characterize a neurodegenerative condition that causes memory loss, cognitive impairment, and learning disability in the affected individuals (Gallaway *et al.*, 2017; Zvěřová, 2019). Alois Alzheimer, a German psychiatrist, described Alzheimer's disease for the first time in 1906 (Anand *et al.*, 2017; Möller *et al.*, 1998). Alzheimer's disease was named after him in 1910 by Dr. Kraeplin (Möller *et al.*, 1998). Statistically, about 150 million people around the world are estimated to suffer from AD by 2050 (Patterson, 2018). Moreover, AD-induced mortality increased by 66 % between 2000 and 2008 (Gallaway *et al.*, 2017). Among the elderly, AD is the fifth leading cause of death, with a mean duration of around eight to nine years between the onset of clinical symptoms and death (Gallaway *et al.*, 2017; Wong, 2020). Therefore, maintaining and improving our brain health is one of the main goals for most countries

around the world. This is particularly important for countries that have a longer life expectancy than the birth rate, since AD affects mainly elderly people over 65 years old (O'caoimh *et al.*, 2015).

Clinically, impaired memory is the first and primary feature of AD, such as misplaced items, skipped conversations, trouble recalling addresses, and missing appointments (Longhe, 2020). According to the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA), the confirmation of dementia syndrome with cognitive impairment is the main criteria for the classification of definite, probable, possible, or unlikely AD cases (van der Flier, 2021). Cognitive and memory impairments in AD patients are linked directly to the neuropathological features of AD and brain changes (van der Flier, 2021). The accumulation of amyloid protein (Aβ) in the parietal cerebral cortex is reported to be the earliest pathological evidence of AD (Hampel *et al.*, 2021). Later, tau protein accumulation was seen in the hippocampal region of the brain's medial temporal lobe (Chandra *et al.*, 2019). Typically, the pathological process starts prior to the observation of clinical signs of AD, such as short-term memory impairment, word finding difficulties, and communication disabilities, which progresses to global cognitive dysfunction (Longhe, 2020). Identification of the clinical features and signs is important for proper diagnosis and treatment, particularly for those who have a more advanced disease stage (Morris *et al.*, 2018).

In the past, the diagnosis of AD was made by a post-mortem autopsy that revealed the presence of senile plaques and neurofibrillary tangles (Chandra *et al.*, 2019; Grandal *et al.*, 2018). Nowadays, the two aggregated proteins implicated in the pathogenesis of AD (Aβ and tau) can be visualized with positron emission tomography

(PET), and the proposed downstream consequences of neurodegeneration can be examined with structural magnetic resonance imaging (MRI), functional MRI, and glucose metabolism PET (Chandra *et al.*, 2019). Moreover, MRI detects brain atrophy, as well as diffusion and perfusion abnormalities, which are most prominent in the vulnerable hippocampal and cortical regions (Park *et al.*, 2016). AD also causes a significant deterioration of the cortical grey matter, which represents the beginning of the neuronal loss (Wu *et al.*, 2021).

Despite the huge amount of research on the etiology of AD, it has been difficult to confirm the etiology and pathogenesis of AD. There is no consensus on the genetic, immunological, and toxic factors' relative etiological roles. Therefore, AD is still a fruitful path for conducting research.

2.2 Pathophysiology and Clinical Features

The key pathologic characteristics of AD are brain atrophy as a result of regional neuronal and synaptic failure, extracellular amyloid deposition in the form of neuritic plaques, and intraneuronal tau protein deposition in the form of neurofibrillary tangles (NFT) (Jellinger, 2020). Studies suggest that many enzymes such as α-secretase, β-secretase, and γ-secretases play a crucial role in causing an imbalance between the clearance and production of Aβ, which results in its accumulation as extracellular plaques (Kumar *et al.*, 2015; Rajmohan *et al.*, 2017). The impairment of amyloid and its resultant oligomers have a toxic impact on the pathological pathway of tau phosphorylation, leading to the formation of NFT (Rajmohan *et al.*, 2017). Amyloid deposition occurs throughout the early stages of the disease, and some scientists hypothesize that the malfunction of amyloid metabolism and its deposition began 20 years prior to the development of clinical signs of AD (Jellinger, 2020;

Morris *et al.*, 2018; Sadigh-Eteghad *et al.*, 2015). Meanwhile, brain atrophy, neuropathological and synaptic failure of AD are described as significant neuronal loss, impairment of the cholinergic, serotonergic, and noradrenergic systems, as well as glutamatergic dysfunction, all of which contribute to cognitive and behavioral symptoms (Jellinger, 2020).

2.3 Proposed Hypothesis

The etiology and pathogenesis of AD are still unknown (DeBay *et al.*, 2020; Fan *et al.*, 2020). However, in the last few decades, many studies have proposed that the pathogenesis of AD may be linked to different hypotheses, including cholinergic hypothesis, oxidative stress hypothesis, neuroinflammatory hypothesis, Aβ hypothesis, and tau phosphorylation hypothesis (Kumar *et al.*, 2015; Majdi *et al.*, 2020).

2.3.1 Oxidative Stress Hypothesis

Recent research indicates that the AD brain has a high degree of oxidative stress, which may contribute to neuronal degeneration and death (Anand *et al.*, 2017; Tramutola *et al.*, 2017). The central nervous system is particularly prone to oxidative damage by free radicals due to its high brain oxygen consumption rate, large lipid content, and low number of antioxidant enzymes compared to other peripheral tissues (Lee *et al.*, 2020). The oxidative stress hypothesis of AD is characterized by the potential for neurodegenerative and neural loss due to accumulated oxidative damage over time (Buccellato *et al.*, 2021). Free radicals have been demonstrated to play a major part in the development of oxidative stress in AD's brain (Wojtunik-Kulesza *et al.*, 2016). Increased active free radicals in the brain, such as some metallic elements,

especially aluminum (Al), mercury (Hg), and iron (Fe), support the oxidative stress hypothesis in AD (Farina *et al.*, 2013). The presence of Aβ plaques in the AD brain also showed an additional source of free radicals which facilitates their penetration into the vascular endothelium (Lee *et al.*, 2020; Reddy, 2006). Elevation of lipid peroxidation and reduced levels of the most important antioxidant enzymes in the body, glutathione peroxidase and dismutase, have also been observed in AD brain patients (Casado *et al.*, 2008; Lee *et al.*, 2020). *In vitro* and *in vivo* neuroprotective effects of natural and synthetic antioxidants were tested, demonstrating the protective role of antioxidant therapy in the treatment of AD (Sereia *et al.*, 2019; Song *et al.*, 2020). As a result, antioxidants are becoming a global concern in the prevention and delay of disease progression by combating oxidative stress, which plays an important role in the neurodegeneration of AD (Singh *et al.*, 2019).

2.3.2 Neuroinflammatory Hypothesis

There is significant evidence that inflammatory mediators in the central nervous system contribute to cognitive decline through cytokine mediated reactions among glial cells and neuronal cells in the brain (Kinney *et al.*, 2018; Sochocka *et al.*, 2017). AD has also been linked to an increase in proinflammatory cytokines, which can initiate plaque formation and accelerate nerve cell degeneration (Kinney *et al.*, 2018; Rubio-Perez *et al.*, 2012). Brain inflammation appears to be helpful in the early stages of AD. However, it becomes detrimental in the late stage when it stimulates microglia cells, resulting in the release of toxic proinflammatory mediators such as reactive oxygen species, cytokines, and nitric oxide (Kinney *et al.*, 2018; Rakic *et al.*, 2018). Recent clinical studies indicate that neuroinflammation is a prevalent pathological feature of AD, emphasizing the importance of anti-inflammatory therapy

in delaying or reversing disease onset (Guzman-Martinez *et al.*, 2019; Kinney *et al.*, 2018; Olajide *et al.*, 2020).

2.3.3 Cholinergic Hypothesis

ACh is a neurotransmitter present in the autonomic nervous system at neuromuscular junctions, ganglia, and synapses (Nathanson, 2018). Acetyl-CoA (derived from the glucose-pyruvate pathway) and choline are used to synthesize ACh in nerve endings (Kucherenko *et al.*, 2019; Nathanson, 2018). Choline is concentrated in plasma and rapidly transferred into cholinergic neurons through sodium/choline transporter (Ferreira-Vieira *et al.*, 2016). The reaction of acetyl-CoA with choline is catalyzed by choline acetyltransferase enzyme (CAT) (Ferreira-Vieira *et al.*, 2016). The release of ACh into the synaptic cleft is promoted by Ca^{2+}dependent synaptic vesicle exocytosis (Leitz *et al.*, 2016). ACh binds to the postsynaptic receptors for a short period of time (Colovic *et al.*, 2013). Following dissociation from the receptor, ACh is quickly hydrolyzed by AChE to produce acetic acid and choline, which is then taken up into the nerve ending (presynaptic) and used in the synthesis of ACh again (Colovic *et al.*, 2013; Nathanson, 2018).

Early studies found a close association between ACh-mediated neurotransmission and cognitive ability (Albanus, 1970; Pradhan *et al.*, 1968). In 1976, Davis *et al.* suggested for the first time a cholinergic explanation for the underlying etiology of cognitive dysfunction in AD (Davies *et al.*, 1976). The investigation was done by performing a biochemical study of numerous enzymes responsible for the main brain neurotransmitters, such as ACh, γ-aminobutyric acid, dopamine, noradrenaline, and 5-hydroxytryptamine, in twenty brain's regions of a group of patients who died of AD complications. CAT levels in AD brains were

significantly reduced compared to control, while glutamic acid decarboxylase showed a normal level in all tested regions (Davies *et al.*, 1976). Thus, the first definition of the cholinergic hypothesis was linked specifically to the significant reduction in the activity of the CAT enzyme, which is the key enzyme for the synthesis of ACh neurotransmitter. A massive reduction of CAT was observed, mainly in the amygdala, hippocampus, and cortex regions (Davies *et al.*, 1976). Thus, a reduction of ACh neurotransmitter was noticed in the same regions, that play an important role in the regulation of emotions and memory (Tyng *et al.*, 2017). Later, the cholinergic theory has been found to involve other parameters' impairments, including reduction of choline transport activity in hippocampus and cortex (Rylett *et al.*, 1983), impaired ACh exocytosis process (Nilsson *et al.*, 1986), and the loss of the muscarinic receptor on the cholinergic neurons (Whitehouse *et al.*, 1986).

The cholinergic hypothesis is considered the most well-known theory in neuroscience history for cognitive and learning decline and the main mechanistic theory for AD management. Prior to the establishment of the AChE function in AD treatment, AChE inhibitors (AChEIs) were a viable therapy option (Mehta *et al.*, 2012). AChEIs were discovered for the first time for the treatment of glaucoma and myasthenia gravis (Khan *et al.*, 2018). Therefore, as candidates for the symptomatic treatment of AD, a number of AChEIs, including natural compounds such as physostigmine, huperzine A and galantamine as well as various synthetic compounds such as tacrine and donepezil with semisynthetic rivastigmine have been discovered (Mehta *et al.*, 2012). However, only four drugs are currently approved worldwide: one N-methyl-D-aspartate (NMDA) antagonist (memantine) and three AChEIs (donepezil, galantamine, and rivastigmine) (Folch *et al.*, 2016).

2.4 Available Medications for AD

Treatment options for AD are confined to symptomatic medications including anti-cholinesterase medicatons such as donepezil, galantamine, and rivastigmine, as well as N-methyl-D-aspartate receptor antagonists (memantine) (Nazam *et al.*, 2021). A significant proportion of AD patients were demonstrated to benefit from these drugs owing to improvements in their functional and cognitive outcomes (Nazam *et al.*, 2021).

2.4.1 Exelon (Rivastigmine tartrate)

Rivastigmine is a phenyl-carbamate ester compound that acts as a reversible inhibitor of both cholinesterase enzymes (AChE and BChE) (Zhang *et al.*, 2022). Rivastigmine has an oral bioavailability of 35.5% with 0.8 to 1.2 hour to reach maximum concentration (T_{max}) of rivastigmine, low binding affinity for plasma proteins of 40% and a half-life of approximately two hours (Nguyen *et al.*, 2021). Rivastigmine has a prolonged duration of action and easily enters the CNS. The duration of rivastigmine's inhibition of cholinesterase is nearly 10 hours (Nguyen *et al.*, 2021). In human volunteer trials, central AChE inhibition was much larger than inhibition of peripheral AChE or BChE (Darreh–Shori *et al.*, 2002). Moreover, animal studies reveal that rivastigmine is a more effective inhibitor of AChE in the cortex and hippocampus which is the most affected parts of the brain by AD (Darreh–Shori *et al.*, 2002).

Rivastigmine is sold under the brand name Exelon (rivastigmine tartrate), which available in the form of pills, liquid, or a recently designed transdermal patch and it is FDA-approved for dementia and cognitive impairment associated with mild-moderate stage of AD and Parkinson's disease (Worley, 2014). Despite the fact that

rivastigmine is regarded as a symptomatic treatment for mild to moderate stage AD. However, rivastigmine transdermal patch with a daily dose of 13.3 mg was approved for the severe stage of AD by FDA (Sadowsky *et al.*, 2014). Each capsule contains rivastigmine tartrate equivalent to 1.5, 3, 4.5, or 6 mg of rivastigmine (Nguyen *et al.*, 2021). Exelon oral solution is a solution containing a concentration of 2 mg/mL rivastigmine tartrate. Based on pharmacokinetic principles and randomized, controlled clinical trial (Farlow *et al.*, 2013), it was concluded that the transdermal patch has advantages of less gastrointestinal side effects over the oral formulation and that a once-daily dose would promote adherence because the patch delivers a more constant concentration of rivastigmine to the body and has an equivalent exposure to high dose of the oral form (9.5 mg as a transdermal patch is equivalent to 12 mg daily in the oral form) (Birks *et al.*, 2015; Farlow *et al.*, 2013). It is believed to exert its therapeutic effect through increasing cholinergic activity by preventing cholinesterase breakdown of acetylcholine to increase its concentration. Consequently, the effectiveness of Exelon may diminish as the disease progresses and fewer cholinergic neurons remain functionally intact (Worley, 2014).

An early Phase II experiment found that up to 12 mg/day of rivastigmine was well tolerated and effective in mild to moderate AD patients (Forette *et al.*, 1999). Participants were randomly assigned into three groups: placebo, rivastigmine twice daily (b.i.d.) or three times daily (t.i.d.) Each participant received a daily dose of 10 mg rivastigmine, which was administered twice or three times daily, depending on their preference. The study has stated that the most frequent side effects were gastrointestinal effect such as nausea and vomiting. However, most patients who couldn't handle this side effect (b.i.d. or t.i.d.) were able to reach higher maximum tolerated doses of 10 mg/day with antiemetic medicine (Forette *et al.*, 1999).

2.4.2 Razadyne (Galantamine hydrobromide)

Galantamine is a tertiary alkaloid with a structural similarity to codeine which isolated from the *Galanthus* species such as *Galanthus caucasicus* (Baker) Grossh. and *Galanthus woronowii* Losinsk. (Kong et al., 2021). afterward, In the 1950s, Mashkovsky and Kruglikova-Lvova synthesized galantamine in the Soviet Union for the first time (Lei *et al.*, 2018). Then in 1959, it was manufactured by Paskov in Bulgaria (Lei *et al.*, 2018). It was originally used to treat a number of neurological, myopathies and paralytic illnesses under the name Nivalin (Lei *et al.*, 2018; Mucke, 2015). Early 1980s, the acceptance of the cholinergic hypothesis of AD generated interest in using cholinesterase inhibitors to treat symptoms of dementia associated with AD (Mucke, 2015). The development of galantamine as a therapy for AD has been hindered by patent issues and galantamine was approved by FDA in 2001 for the treatment of mild to moderate stage of AD in USA (Mehta *et al.*, 2012).

Galantamine hydrobromide is a prescribed drug for dementia symptoms related to cholinergic deficiency and it is now marketed as Razadyne, formerly Reminyl (Berkov *et al.*, 2012). There are two types of dosage forms available on the market: immediate release and extended release (Kalola *et al.*, 2021). The recommended starting dosage for the extended-release formulation is 8 mg/day in the morning, with an increase to the initial maintenance dose of 16 mg/day after at least 4 weeks (Seltzer, 2010). After a minimum of 4 weeks at 16 mg/day, the dosage may be increased to 24 mg/day based on clinical benefit and tolerability. In contrast, the suggested starting dosage of immediate tablet is 4 mg twice day, with an increase to the initial maintenance dosage of 8 mg twice daily after at least 4 weeks. After a minimum of four weeks at 8 mg twice daily, the dosage may be increased to 12 mg twice daily based on clinical benefit and tolerability (Seltzer, 2010).

2.4.3 Aricept (Donepezil HCl)

Donepezil is a new class of AChE inhibitor with an N-benzylpiperidine and an indanone moiety which shows longer and more selective action with minimal side effects (Sugimoto, 1999). it was authorized by USA-FDA for the treatment of mild to moderate AD in 1996 and is currently marketed under the trade name Aricept (Hara *et al.*, 2019; Mehta *et al.*, 2012). Moreover, Donepezil was authorized by the FDA for the treatment of severe AD cases at a maximum daily dosage of 23 mg (Mehta *et al.*, 2012). However, high-dose patients often had intermittent nausea, diarrhea, and drowsiness (Mehta *et al.*, 2012). Moreover, there are few FDA off label uses for donepezil such as Lewy body dementia, traumatic brain injury, vascular dementia, dementia associated with Parkinson's disease (Kumar *et al.*, 2021). The relative oral bioavailability of donepezil is 100%, and it reaches peak plasma concentrations in three to four hours (Kumar *et al.*, 2021). Donepezil has a half-life of about 70 hours and is extensively bound to plasma protein, with a total binding ratio of approximately 96 %, including 75 % binding to albumin and 21 % binding to alpha-1-glycoprotein (Kumar *et al.*, 2021). Rogers and colleagues conducted a double-blind, 12-week trial. 468 patients diagnosed with AD were assigned into three groups: placebo, low dose (5 mg/day), and high dose (10 mg/day). Improvements were seen as early as three weeks; clinically significant outcomes were observed nine weeks later; and side effects were equivalent to those of the placebo (Rogers *et al.*, 1998).

2.5 Natural Product Role in AD

Plants provide a significant source of bioactive compounds such as phenolics, terpenoids, essential oils, sterols, alkaloids, polysaccharides, tannins, and anthocyanins (Zhao *et al.*, 2015). Investigation of the biological activities of medicinal

plants, particularly antioxidants, anti-inflammatory, and anticholinesterase activities have attracted considerable interest in AD field (Apetz *et al.*, 2014; Patel *et al.*, 2018). These biological activities of medicinal plant products have been shown to be primarily attributable to the phytochemical groups mentioned above (Stagos, 2020). The majority of medicinal plants have a natural antioxidants that prevent the destructive effects induced by oxidative damage of the free reactive oxygen species (ROS) and the reactive nitrogen species (RNS), implicated in neurodegenerative diseases, such as AD (Hunyadi, 2019).

Between 1981 and 2019, approximately 50% of all approved drugs worldwide, were produced or inspired by natural products (Newman *et al.*, 2020). The known cholinesterase inhibitor rivastigmine (Figure 2.1) used for AD treatment is an example of a semi-synthetic drug developed based on the naturally occurring cholinesterase inhibitor physostigmine scaffold (Lima *et al.*, 2020). Physostigmine (Figure 2.1), an alkaloid isolated from *Physostigma venenosum* Balf. is indicated for glaucoma and myasthenia gravis, but its use for the AD treatment is restricted in certain countries due to the serious hepatic and cardiac side effects (Mehta *et al.*, 2012; Sahoo *et al.*, 2018). Nonetheless, galantamine (Figure 2.1), a pure natural product isolated from the bulbs and flowers of *Galanthus caucasicus* (Baker) Grossh. and *Galanthus woronowii* losinsk., is currently in the market for the treatment of cognitive decline in mild to moderate AD (Berkov *et al.*, 2012; Heinrich *et al.*, 2004).

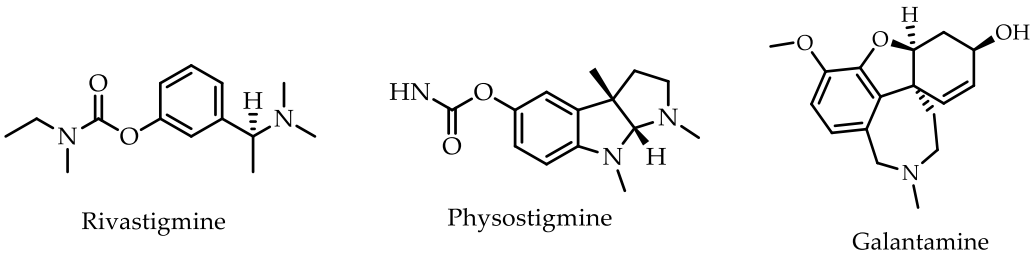

Figure 2.1 The chemical structure of naturally derived acetylcholinesterase inhibitors.

Natural products have contributed immensely to AD by providing many natural compounds and medicinal plant extracts with anti-AChE, anti-oxidant, and anti-inflammatory properties (Mehta *et al.*, 2012; Noori *et al.*, 2021). In addition, literature provides a wide range of crude extracts of medicinal plants that have the potentials to alleviate AChE-induced cognitive impairment (da Silva Oliveira *et al.*, 2019; Patel *et al.*, 2018).

In this context, *Nigella sativa* L. extract enhance the memory and learning ability in rat model through antioxidant and neuroprotective activity (Beheshti *et al.*, 2016; Sahak *et al.*, 2016). Pomegranate has also been shown to be effective in preventing and delaying the onset of neurodegenerative disorders due to its high antioxidant polyphenol content (Akbar *et al.*, 2015). Long-term dietary supplementation with 4% pomegranate was revealed to diminish the oxidized byproducts in the brain (hippocampus and cortex) using a transgenic mouse model compared to the control group, as evidenced by decreased malondialdehyde levels and protein carbonyl accumulation levels (Subash *et al.*, 2014). The present finding suggests that pomegranate aids in the AD-like pathology due to the direct antioxidant effect and the regeneration of antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione S-transferase (GST), as well as glutathione (GSH) levels in the AD mouse model (Subash *et al.*, 2014). Moreover, oral supplementation of grape seed extract (100 mg/kg/bw, 30 days) in a rat model prevents and improves memory impairment induced by intracerebroventricular injection of streptozotocin (STZ) (Farbood *et al.*, 2016). In addition, grape leaf extract showed a similar neuroprotective effect in the $AlCl_3$ induced-AD rat model by restoring ACh neurotransmitter (Ach) levels in the brain and resisting the hemostasis change in brain modulators (Borai *et al.*, 2017).

An i*n vivo* study confirmed the effect of fig (*Ficus carica* L.), pomegranate (*Punica grantum* L.), and date palm (*Phoenix dactylifera* L.) extracts on cognitive and behavioral deficits of AD through neuroprotective activity (Essa *et al.*, 2015). Administration of 4% diet of fig, pomegranate and date palm demonstrated beneficial properties on proinflammatory cytokines and tumor necrosis factor (TNF-α) level in transgenic mouse model of AD (Essa *et al.*, 2015). However, the pomegranate diet demonstrated the highest protective impact, attenuating interleukin 1β (IL-1β) pro-inflammatory mediators by 1.21 and 1.50-fold in the cortex and hippocampus, respectively, compared to the control group (Essa *et al.*, 2015).

2.5.1 *Cassia* Genus

Cassia belongs to the Leguminosae (Fabaceae) family, which comprises more than 700 genera and 18000 species (Hu *et al.*, 2000; Khurm *et al.*, 2020). Plant biologists estimate that the Leguminosae family is the third-largest family of flowering plants (Khurm *et al.*, 2020). This plant family is predominantly distributed across tropical and subtropical Asian areas (Raes *et al.*, 2013). Leguminosae family is subdivided into three subfamilies: Caesalpinioideae, Faboideae, and Mimosoideae (Khurm *et al.*, 2020). *Cassia* is a major genus of Caesalpinioideae subfamily, with around 600 species of flowering trees, shrubs, and herbaceous plants (Abdel Hakim *et al.*, 2019). It is widely distributed in West India, China, Malaysia, Indonesia, Brazil, Mexico, America, and East Africa (Abdel Hakim *et al.*, 2019).

Historically, *Cassia* species have long been employed as diuretics and purgatives (Abdel Hakim *et al.*, 2019). Traditionally, *Cassia* species are utilized in to treat headaches, fever, skin conditions, constipation, anthelmintic, and urinary disorders (Abdel Hakim *et al.*, 2019; Khurm *et al.*, 2020). Further studies have

indicated that *Cassia* species have numerous pharmacological benefits, including antimicrobial (VijayaSekhar *et al.*, 2016), antioxidant (Kolar *et al.*, 2018), antidiabetic (Jani *et al.*, 2020), antihepatotoxicity (Chaerunisa *et al.*, 2018), and antimutagenic properties (Hofileña *et al.*, 2000). A wide range of secondary metabolites have been discovered in *Cassia* species, including anthraquinones, alkaloids, flavonoids, phenolic acid, sterols, fatty acids, and polysaccharides (Abdel Hakim *et al.*, 2019). Anthraquinones are abundant in many *Cassia* species and are responsible for a great number of their traditional uses, most notably their laxative and purgative properties (Abdel Hakim *et al.*, 2019; Dave *et al.*, 2012). Emodin, aloe-emodin, rhein, chrysophanol, and physcion are the most prevalent anthraquinones identified in different *Cassia* species (Hafez *et al.*, 2019). Furthermore, among *Cassia* species studied in the literature, kaempferol, quercetin, luteolin, and their glycosides are the most abundant flavonoids (Hafez *et al.*, 2019).

In the context of AD, various *Cassia* species have been investigated as potential adjuvant therapies for AD. *Cassia tora* L. was among the most investigated species that were reported to have multifunctional anti-AD potential (Chethana *et al.*, 2017; Malabade *et al.*, 2015; Ravi *et al.*, 2020; Ravi *et al.*, 2019). *In vitro* studies revealed that *Cassia tora* extract inhibits aggregation of Aβ(1-42) oligomers and helps in the dissociation of the pre-formed Aβ fibrils (Chethana *et al.*, 2017; Ravi *et al.*, 2019). Another *in vivo* study confirmed *Cassia tora* extract's beneficial effects in a rat model by significantly lowering oxidative stress in the hippocampus and cortex as measured by lipid peroxidation, inhibition of AChE, proinflammatory mediators, and primarily cytokines (Ravi *et al.*, 2020). In addition, several studies evaluated the possible therapeutic application of *Cassia obtusifolia* L. in the treatment of neurodegenerative disorders including Parkinson’s disease and AD (Drever *et al.*,

2008; Ju *et al.*, 2010; Kim *et al.*, 2007). The neuroprotective characteristics of the seed of Cassia obtusifolia in mouse primary hippocampal cultures revealed that treatment with C. obtusifolia decreased cell death and calcium dysregulation produced by N-methyl-D-aspartate and 3-Nitropropionic (Drever *et al.*, 2008). *Cassia obtusifolia* seed extract also improved memory impairment in a mouse model via anti-AChE action (Kim *et al.*, 2007). It is clear that many plants belonging to *Cassia* spp are potential sources for AChEI, thus, in this study, two relatively less known species of *Cassia* such as *C. timoriensis* and *C. grandis* are chosen for the discovery of potential AChEIs.

2.5.1(a) Limestone Cassia (*Cassia timoriensis* DC.)

Cassia timoriensis DC. is a perennial tree or shrub, usually about 2-6 meters tall (Figure 2.2) . The plant is widely spread in tropical areas, particularly in East Asia, such as India, Sri Lanka, Thailand, Malaysia, and Indonesia (Lim, 2014). A flowering plant with yellow blooms and shiny brown seedpods, *C. timoriensis*, is also sometimes valued as an ornamental plant (Monkheang *et al.*, 2011). According to the plant list website (www.theplantlist.org), *Senna timoriensis* (DC.) is the accepted used name with the following synonyms: *Cassia timoriensis* (DC.), *Cassia arayatensis* Litv., *Cassia exaltata* Blume, *Cassia goensis* Dalzell, *Cassia montana* Naves and Villar, and *Senna glauca* Roxb.

Figure 2.2 Images of *Cassia timoriensis* DC. trees depicting the flowering stage of the plant with both flowers and pods.

Traditionally, *Cassia timoriensis* has been used for treating toxins, scabies, itching, skin diseases, and as an anthelmintic medicine (Lim, 2014; Palasuwan *et al.*, 2005). It is also used as a general tonic, antitumor, and for blood disorders, in particular its heartwood component, which is commonly used for menstrual blood disorders (Lim, 2014; Monkheang *et al.*, 2011; Palasuwan *et al.*, 2005). In 2004, a screening of 20 Thai medicinal plants, aqueous extract of *C. timoriensis***,** demonstrated powerful antioxidant activity through the inhibition of Heinz bodies induction (Palasuwan *et al.*, 2005). Despite its wide range of traditional uses, *C. timoriensis* has hardly been studied for its phytochemical constituents and biological activities. The first compound identified from this plant is barakol, by a Thai group in 1984 (Gritsanapan *et al.*, 1984). Previously, barakol was reported to be a significant component of *Cassia siamea* Lam. with almost 0.40% w/w barakol content (Monton *et al.*, 2015). Bycroft *et al.* isolated and characterized barakol for the first time from *Cassia siamea* leaves in 1970 (Bycroft *et al.*, 1970). Barakol is a tricyclic ring structure that is unstable and transforms to anhydrobarakol by the elimination of one molecule of water (Figure 2.3) (Monton *et al.*, 2015). Barakol has been employed as a therapeutic agent for insomnia due to its

hypnotic and anxiolytic effects (Deachapunya *et al.*, 2009; Thongsaard *et al.*, 1997). In Thailand, *Cassia siamea* leaves were originally promoted as an herbal sleep aid, available in capsule form containing 400 mg of leaf powder per capsule (10 mg of anhydrobarakol, a prodrug of barakol) (Padumanonda *et al.*, 2006). 2-4 pills before bedtime were the recommended dosage for this natural medication (Monton *et al.*, 2015). However, due to its hepatotoxicity, Thailand's Food and Drug Administration withdrew products containing *C. siamea* leaves from the market (Monton *et al.*, 2015).

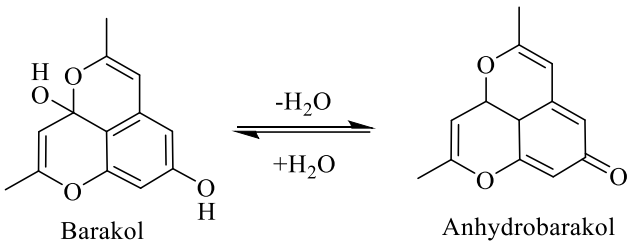

Figure 2.3 The chemical structure and conversion of barakol and anhydrobarakol.

Several studies have been conducted to investigate more about the toxicological effect of *C. siamea* extract as well as the pure barakol compound (Chavalittumrong *et al.*, 2003; Pumpaisalchai *et al.*, 2003; Wiam *et al.*, 2005). An *in vivo* study of the chronic toxicity of *C. siamea* leaves powder on rats found that a daily dosage of 200-2,000 mg/kg of powder resulted in dose-dependent hepatic cell degeneration and necrosis. Furthermore, following 14 days of powder removal, the hepatotoxic impact was determined to be reversible. (Chavalittumrong *et al.*, 2003). However, another *in vivo* investigation studied the hepatotoxicity of barakol in rats and found that oral administration of barakol at a dose of 60-120 mg/kg was safe and showed no hepatotoxicity (Pumpaisalchai *et al.*, 2003). While higher dose at 240 mg/kg resulted in pathological changes in the liver, including fatty liver changes and a disruption in liver function with an increase in bilirubin (Pumpaisalchai *et al.*, 2003). This led to the conclusion that the toxicity of crude *C. siamea* leaves may be caused

by other chemical constituents that may have more toxic effects on the liver and blood than barakol. Toxicological study of *C. siamea* revealed that there was a poisonous alkaloid, 3β-acetoxy-4α-hydroxy-2β(p-methylbenzyl)pyrrolidine (C14H19NO3), as previously described in the branch, leaf, and pod of *C. siamea*. Intraperitoneal injection of 1 ml of 5% solution of this alkaloid into animals caused toxicity and fatality (Pumpaisalchai *et al.*, 2003). In addition to the previous toxicological findings, lethal dose of *C. siamea* extract and pure barakol were found to be 9600 mg/kg and 2.3 mg/kg in rats, respectively (Pumpaisalchai *et al.*, 2003; Wiam *et al.*, 2005). Thus, according to toxicity classification scheme barakol has been classified as slightly toxic while *C. siamea* classified as practically non-toxic (Hodge *et al.*, 1949). Moreover, the traditional Thailand curry food from Khi Lek (*Cassia siamea*) leaves remains popular without any reports of hepatotoxicity after hundreds of years (Teangpook *et al.*, 2011).

In the light of previous findings, the presence of barakol in *C. timoriensis* may slightly contribute to the hepatotoxic impact; nonetheless, it is not possible to draw the conclusion that the whole plant may be toxic and cause damage to the liver based on presence of barakol in *C. timoriensis*. Moreover, the toxicology of *C. timoriensis* requires more study to confirm its potentially toxic dosage and the possibility to cause hepatotoxicity. likewise, it is crucial to assess the link between its pharmacology and toxicity, as therapeutic effectiveness often occurs at a lower dosage, but an overdose may result in severe side effects or poisoning.

2.5.1(b) Pink Shower (*Cassia grandis* L.f.)

Cassia grandis L.f. is a tree with shiny pink flowers and brown pods containing hard flat seeds (Albuquerque *et al.*, 2014) (figure 2.4). It is a member of Leguminosae family and subfamily of Caesalpinioideae (Albuquerque *et al.*, 2014). *C. grandis* is