MONASH University

Evaluation of neuroprotective activity of standardized ethanolic *Orthosiphon stamineus* extract to ameliorate cognitive alterations in Alzheimer's disease

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Notice 1

Abstract

Alzheimer's disease (AD) is the most common form of dementia and a chronic neurodegenerative disease. It is characterized by impairment in cognitive functioning whereby patients diagnosed with AD lose the ability to encode new memories. *Orthosiphon stamineus* Benth. (Lamiaceae), a medicinal herb widely distributed in South East Asia, is an important plant used in traditional folk medicine for various diseases. Previous studies have shown that *O. stamineus* leaves extracts possess strong antioxidant, anti-inflammatory and anti-bacterial properties with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid. and nitric oxide inhibitory isopimarane-diterpenes. For instance, rosmarinic acid, the main flavonoid component of *O. stamineus* has been shown to have various pharmacological properties. In vitro studies have demonstrated neuroprotective as well as choline esterase inhibitory effects of this compound. In this study, some of the key hypotheses pertaining to Alzheimer's disease were explored. The vascular hypothesis was addressed using the permanent bilateral occlusion of the common carotid artery (PBOCCA) model whereby based on the results obtained, it was observed that the vehicle-treated PBOCCA rats demonstrated hippocampus dependent spatial learning and memory deficits as evaluated via the morris water maze test and contextual memory as evaluated using the step-through passive avoidance task. Interestingly, O. stamineus extract treatment was observed to ameliorate all these cognitive deficits in PBOCCA rats. The cholinergic hypothesis was explored using the scopolamine-induced model whereby based on the results obtained for the behavioral analyses, the spatial memory was observed to be improved in both the acute and chronic scopolamine model and this improved performance was attributed to its enhanced cholinergic neuronal transmission. However, a celing effect was observed with higher doses. Additionally, induction of scopolamine-induced amnesia demonstrated decreased expression of BDNF and TrkB in the hippocampus and when treated with OS extract, both the BDNF and TrKB expression levels in the hippocampus region were observed to be increased thus showing maximum protection. Scopolamine was also observed to reduce the expression of the CREB1 gene and pre-treatment with OS extract markedly increased the CREB1 mRNA levels. Lastly the amyloid-tau hypothesis was investigated using the streptozotocin-induced model. Based on the results obtained, memory retention was improved in the groups treated with OS extract and this result were supported by the biochemical study results where the induction of STZ demonstrated overexpression of all the key genes namely APP, MAPT, GSK3-α and GSK3-β in both the

hippocampus and the prefrontal cortex region. However, when treated with OS extract, the expressions of all these genes were observed to be suppressed indicating maximum protection and hence reducing AD pathology. All these models mimicked the Alzheimer's condition and served as apposite models in insinuating the effectiveness of OS extract in curbing cognitive dysfunction. These research findings suggest that the OS ethanolic extract demonstrated an improving effect on memory and hence could serve as a potential therapeutic target for the treatment of Alzheimer's disease.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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List of Achievement

Publications in Progress/Submitted

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2. **Retinasamy, T**., Hassan, Z**,** Bakar, S. N. S, Shaikh, M. F., Kumari, Y., Shah, A. M., Shah, A., Othman, I., Standardized ethanolic extract of *O. stamineus* ameliorates cognitive dysfunctions in chronic cerebral hypoperfused rats, Frontiers in Pharmacology (2018) (In preparation)
3. **Retinasamy, T.,** Kumari, Y., Othman, I., Shaikh, M. F., Ethanolic extract of *O. stamineus improves* memory in scopolamine-induced amnesia model, Frontiers in Pharmacology (2018) (In preparation)
4. **Retinasamy, T.,** Kumari, Y., Othman, I., Shaikh, M. F., *O. stamineus* extract reverses Alzheimer's disease-like condition in Streptozotocin model, Frontiers in Pharmacology (2018) (In preparation)

Abstracts

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1. Oral presentation at the CDR Mini Symposium on Drug Addiction & Neurodegeneration 2015
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3. 29th International Invention, Innovation & Technology Exhibition, Malaysia (ITEX) Competition (May 10 – 12, 2018): Silver Award Winner

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 4 submitted publications. The core theme of the thesis is pharmacological evaluation of *Orthosiphon stamineus* in Alzheimer's disease induced cognitive impairment in rats. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia under the supervision of Dr. Mohd. Farooq Shaikh.

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
3	*Neuroprotective effects of ethanolic extract of Orthosiphon stamineus in an in-vitro model.*	*Submitted*	*60%. Involved in design of the study, performed experiment, data analysis, manuscript writing and responded to reviewers' comments.*	*1) 25%, Mohd. Farooq Shaikh, conceptualised the idea, designed the study, manuscript editing, proofreading and responded to reviewer's comments.* *2) 10%, Yatinesh Kumari input into designing the study and result analysis.* *3) 5%, Iekhsan Othman conceptualised the idea, and helped in designing the study,*	*No* *No* *No*

4	*Ethanolic extract of O. stamineus improves memory in scopolamine-induced amnesia model.*	*Submitted*	*60%. Involved in design of the study, performed experiment, data analysis, manuscript writing and responded to reviewers' comments.*	*1) 20%, Mohd. Farooq Shaikh, conceptualised the idea, designed the study, proofreading responded to reviewer's comments.* *2) 15%, Yatinesh Kumari input into designing gene expression study and ICC study and result analysis.* *3) 5%, Iekhsan Othman conceptualised the idea, and helped in designing the study*	*No* *No* *No*
5	*O. stamineus Extract Reverses Alzheimer's Disease-Like Condition in Streptozotocin Model*	*Submitted*	*60%. Involved in designing, performed experiment, data analysis, manuscript writing and responded to reviewers' comments.*	*1) 20%, Mohd. Farooq Shaikh, conceptualised the idea, designed the study, proofreading responded to reviewer's comments.* *2) 15%, Yatinesh Kumari input into designing gene expression study and ICC study and result analysis.* *3) 5%, Iekhsan Othman conceptualised the idea, and helped in designing the study*	*No* *No* *No*
6	*Standardized ethanolic extract of O. stamineus ameliorates cognitive dysfunction in chronic cerebral hypoperfused rats*	*Submitted*	*60%. Involved in designing, performed experiment, data analysis, manuscript writing and responded to reviewers' comments.*	*1) 20%, Zurina Hassan, designed the study, helped to perform behavioural experiments and proofreading.* *2) 5%, Siti Najmi Syuhadaa Bakar helped to perform behavioural experiments* *3) 5%, Mohd. Farooq Shaikh, conceptualised the idea,supervised the study and proofreading* *4) 5%, Iekhsan Othman conceptualised the idea,supervised the*	*No* *No* *No* *No* *No*

				study and proofreading *5) 5%, Amin Malik Abdul Majeed conceptualised the idea,supervised the study and proofreading*	

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I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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Dedicated to my parents, Mr Retina & Mrs Neermila

Table of Contents

List of abbreviations and acronyms

Aβ: Beta amyloid

µl: Microliter

°C: Degree Celsius

i.e.: That is

e.g: Example

mg: Milligram

min: Minute

ml: Milliliter

Ach: Acetylcholine

AChE: Acetylcholinesterase enzyme

AD: Alzheimer's disease

APP: Amyloid Precursor Protein

BDNF: Brain-derived neurotrophic factor

CCH: Chronic Cerebral Hypoperfusion

CNS: Central nervous system

CREB: cAMP response element-binding protein

DCX: Doublecortin

EPM: Elevated plus maze

FAD: Familial Alzheimer's Disease

GSK3α: Glycogen synthase kinase-3 alpha

GSK3β: Glycogen synthase kinase-3 beta

ICC: Immunocytochemistry

ICV: Intracerebroventricular

IR: Inflexion Ratio

LTP: Long Term Potentiation

MAPT: Microtubule Associated Protein Tau

MARP: Monash Animal Research Platform

MWM: Morris Water Maze

NFκB: Nuclear factor kappa-light-chain-enhancer of activated B

NFTs: Neurofibrillary tangles

NOR: Novel Object Recognition

OS: *Orthosiphon stamineus*

PA: Passive Avoidance

PBOCCA: Permanent Bilateral Occlusion of Common Carotid Artery

PFA: Paraformaldehyde

ROS: Reactive Oxygen Species

TL: Transfer latency

TrKB: tropomyosin receptor kinase B

SAD: Sporadic Alzheimer’s Disease

SD: Standard deviation

SOD: Superoxide dismutase

STZ: Streptozotocin

WHO: World Health Organization

Chapter 1

1.0 GENERAL INTRODUCTION

In the 21st century, cognitive dysfunction has been deemed as one of the key health problems and also one of the most demoralizing feature of many neurodegenerative diseases like Parkinson's, Alzheimer's and dementia (Pena, Yoon et al. 2014, Lee, Jeon et al. 2016). Dementia is a brain disease that impacts one's ability to perform daily activities and serves as a major cause of dependence, incapability and mortality (Hanumanthachar, Navneet et al. 2007). Alzheimer's disease serve as the most common form of dementia particularly among the older generation which primarily involves parts of the brain that regulates memory, learning, language and contemplation that eventually leads to brain impairment (Yang, Yoon et al. 2009, Pena, Yoon et al. 2014).

Currently about 44 million people have been estimated to be living with dementia worldwide and it is predicted that as the population ages, it could triple by the year 2050 (Lane, Hardy et al. 2018). The prevalence of dementia is expected to be increased in low and middle income countries and Alzheimer's has been deemed as the single largest cause of dementia, accounting for 50 – 75% with its prevalence doubling every 5 years after the age of 65 (Lane, Hardy et al. 2018).

Alzheimer's disease has been acknowledged by the World Health Organization as a significant global public health concern (Lane, Hardy et al. 2018). Alzheimer's disease primarily impacts older adults and the growth in the number of people suffering from the disease is unprecedented. At present about a total of 50 million people were found to be living with Alzheimer's globally and it is expected to rise to about 98 million by 2060 (Lane, Hardy et al. 2018). The mean onset age for Alzheimer's is said to be around 75 years of age and a rapid surge in Alzheimer's prevalence from 1% within the 60 – 64 year age group to more than 25% prevalence in those aged more than 85 years of age (Sun, Xie et al. 2017). The rates of Alzheimer's disease deaths have been observed to escalate between 1999 to 2014 (Lane, Hardy et al. 2018). This irreversible, progressive brain disorder is the sixth leading cause of death among all adults and the fifth leading cause for those aged 65 and above (Lane, Hardy et al. 2018). Alzheimer's disease doesn't precisely cause death, but it prompts most patients to pneumonia, sepsis and ultimately trauma.

Alzheimer's disease is characterized by a perturbing onset of memory impairment followed by a relentless development of cognitive decline (Pluta, Ulamek et al. 2009). Those diagnosed with this disease is said to be unable to encode new memories which in turn damage both declarative and non-declarative memory thus gradually reducing the ability for reasoning, abstraction and language (Pluta, Ulamek et al. 2009). Neuropathological

examination of an Alzheimer's patient brain demonstrated massive atrophy as well as the presence of intraneuronal neurofibrillary tangles (NFTs) and extracellular β-amyloid fibrillar deposits (Aβ Plaques) in the brain (Zhao, Gu et al. 2014, Ghumatkar, Patil et al. 2015).

Alzheimer's is an intricate disorder that could be potentially triggered by a combination of features like infection or reduced circulation and genetic propensity. However the sole contributing factors may never be distinguished, some of the common threads include age, genetic history, lifestyle, vascular risk factors and diet (Lane, Hardy et al. 2018). As of now, the cure for cognitive deficit diseases like amnesia and Alzheimer's is still a nightmare in the field of drug discovery.

In recent times, there has been a developing interest in using complimentary therapy and phytochemicals from medicinal herbs to augment the quality of life and prevent therapy induced side-effects, particularly in using phytochemicals from medicinal herbs as they possess anti-inflammatory and antioxidant activities that may potentially hinder neurodegeneration and improve memory and cognitive functioning (Essa, Vijayan et al. 2012). Some of the medicinal herbs that hold a potential in treating Alzheimer's include German chamomile, Ginseng, licorice, turmeric, and white willow bark as they contain anti-inflammatory properties that aid in reducing inflammation of the brain tissue in Alzheimer's (Singhal, Naithani et al. 2012). Additionally, certain Ayurveda herbs like *Guduchi, Yashtimadhuk, Padma (Nelumbo nucifera), Vacha, Convolvulus pluricaulis, Shankhpushpi, Pancha-Tikta-Ghruta Gugguli, Amalaki, Musta Arjun, Amalaki, Ashwagandha, Galo Satva, Kutaj*, and others were also found to exhibit the ability to reduce brain cell degeneration triggered by AD and enhance the brain's ability to function appropriately when used consistently (Singhal, Naithani et al. 2012). Besides that, medicinal plants were also found to demonstrate potential anticholinesterase and neuroprotective actions in both *in vitro* model using Ellman method and in *in vivo* AD rat model thus underlining a potential for safer drugs for Alzheimer's (Majlessi, Choopani et al. 2012, Ali, Hamed et al. 2013, Ibrahim, Farooq et al. 2013).

Orthosiphon stamineus Benth. (Lamiaceae), is a medicinal herb that is extensively distributed around South East Asia. This medicinal herb is typically employed in traditional folk medicine for the treatment of a variety of angiogenesis related diseases (Jaganath and Ng, 2000). It is commonly referred as cat's whiskers and is also known as misai kuching and kumis kuching in Malaysia and Indonesia respectively (George, Chinnappan et al. 2015). Various in vitro and in vivo models have addressed the presence of different types of phytochemicals present in this plant like flavonoids, terpenoids, and essential oils. Earlier studies have demonstrated that O. stamineus (OS) leaves extracts possess strong antioxidant, anti-

inflammatory and anti-bacterial properties with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid and nitric oxide inhibitory isopimarane-diterpenes (Sumaryono, Proksch et al. 1991, Awale, Tezuka et al. 2003, Awale, Tezuka et al. 2003, Yam, Lim et al. 2010). For instance, Rosmarinic acid, a major flavonoid component of O. stamineus has been shown to have various pharmacological properties. Flavonoids which are the principal group of polyphenols are also reported to be efficacious in decreasing oxidative stress and are said to promote various physiological benefits, particularly in learning and memory, scavenging free radicals and cognitive impairment (Bhullar and Rupasinghe 2013, Ghumatkar, Patil et al. 2015). Besides that, standardized ethanolic extract of O. stamineus were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration (George, Chinnappan et al. 2015). In addition, in vitro studies have demonstrated that O. stamineus enhanced H2O2 induced oxidative stress by antioxidant mechanisms in SH-SY5Y human neuroblastoma cells (N. Vijaya Sree et al. 2015).

2.0 AIM AND SCOPE OF STUDY

There has not been much advances in the understanding of Alzheimer's disease (AD) and distinguishing a suitable mode of treatment for it. Memory loss is one of the characteristic features of AD and hippocampus is the principal region associated with learning and memory. There have not been any available reports on CNS mediated activities of OS except that it contains various active phytocompounds that have been previously reported to be antioxidant and anti-inflammatory.

Considering the adverse effects of synthetic drugs, there is a curiosity among scientific community to search a safer and effective remedy from natural sources. As natural products may provide a new source of beneficial therapeutic agents, provided they are adequately tested, and their mechanisms are properly deciphered. Hence, the current project focusses on firstly investigating the effectiveness of OS standardized extract in inhibiting acetylcholinesterase enzyme followed by establishing the effect of the OS extract on learning & memory in Alzheimer's disease using various established disease models. This also helps in addressing the key hypothesises pertaining to the development of AD. Additionally, the molecular mechanisms and pathways involved in the OS mediated anti-Alzheimer's potential were elucidated. Based on the pathophysiology of AD and the possible usefulness of OS, the objectives of the present investigation were:

1. To determine the neuroprotective ability of the OS extract
2. To study the effect and mechanism of action of OS extract on nootropic and anti-amnesic effect using the Scopolamine-induced disease Model
3. To study the neuroprotective effect of OS on Streptozotocin (STZ)-induced neurotoxicity in a rat model of AD.
4. To study the effect of OS extract on cognitive functions using the permanent bilateral occlusion of the common carotid arteries (PBOCCA) model

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Chapter 2

2.0 LITERATURE REVIEW

2.1 LEARNING AND MEMORY

The brain serves as an important organ that is responsible for the mind and transpires as the basis for thinking, learning and memory, perceiving and behaviour (Okano, Hirano et al. 2000). Memory is a central mental process by which organisms document their experiences and retain them either over a short or long period of time (Besche 2013). Since learning and memory play a key role in human life, learning and memory has deemed as of the most intensively studied subject in the neuroscience field.

Memory is the ability to store and form of sensations, impressions and ideas whereas learning is the ability to acquire new information about the events occurring in the given surrounds (White and McDonald 2002). For an experience to become part of a memory, it must produce persistent structural and functional changes that represent the experience in the brain. Learning and memory are complex phenomenon requiring the synchronized interaction of multiple brain structures. The parts of the brain involved with memory include the association between the cortex of the frontal, parietal, occipital and temporal lobes, parts of the limbic system, especially the hippocampus, amygdala and diencephalon, which are all extremely important for different types of memory (Markowitsch 1988, Squire 1992, White and McDonald 2002).

Memory can be broadly classified into declarative and non-declarative memory whereby memory concerning events and facts are examples of declarative memory. Declarative memory can be further classified into episodic and semantic memory. Episodic memory is memory of the past and personally encountered events. The

knowledge for the meaning of words and how to use them is called as semantic memory (White and McDonald 2002, Vorhees and Williams 2014). Non-declarative memory on the other hand does not depend on the time and place and a particular skill or habit are examples of non-declarative memory. Memory tend to occur in stages over a period of time. Immediate memory is the ability to recall ongoing experiences for a few seconds and it provides a perspective to the present time that enables people to ascertain their whereabout deeds. Short term memory is the temporary ability to recall pieces of information for seconds to minutes and are vulnerable to disruption (A, rajan et al. 2015). The brain areas involved in immediate and short-term memory include the hippocampus, the mammillary bodies and two nuclei of the thalamus (anterior and medial nuclei) (A, rajan et al. 2015). Short term memory may later be transformed into a more permanent type of memory called long term memory, which lasts from days to years. Information in long term memory usually can be retrieved for usage whenever needed and frequent retrieval of a piece of information is termed memory consolidation (Scharff, Friederici et al. 2013, A, rajan et al. 2015).

The memory consolidation hypothesis postulates that memory storage proceeds via temporally distinct phases, each destructible by a subset of agents. The retrieval hypothesis postulates that amnesic agents disrupt memory recall rather than storage as the effect of some agents tend to diminish over time resulting in the reappearances of normal memory retention (Dudai, Karni et al. 2015). The consolidation of information is mediated by limbic structures with hippocampal formation playing a central role in memory processing.

2.2 DEMENTIA

Dementia refers to an acquired global impairment of intellect, memory and personality (cognitive function) and is not a disease per say but simply as group of symptoms caused by the impact of a diseased brain (Vrish, Ashwlayan et al. 2011). Dementia is usually chronic and progressive in nature and their symptoms typically include memory problems, disturbances in speech and perception, instabilities in multiple higher cortical functioning including memory, thinking, orientation, comprehension, calculation, learning capacity, language and judgement. In dementia, performances of learned motor skills, social skills and control of emotions are primarily affected but not the vents from long term memory (Gustafson 1996, Vrish, Ashwlayan et al. 2011).

Dementia can be produced by numerous pathological states that impacts the brain causing a progressive deterioration of certain brain parts that are essential for learning and memory. This in turn causes the brain to shrink eventually as the gaps develop causing the disease to initially begin with lapses in memory followed by swinging of mood and difficulty in finding the right words to articulate (Gustafson 1996). This neurological disorder is of several types and could be reversible when it occurs due to drugs, alcohol, hormones, vitamin imbalances or depression but however is irreversible when it is caused due to a disease or injury and invariably involves with impairment of memory (Shively, Scher et al. 2012). One of the most common cause of dementia is Alzheimer's disease, which is a progressive neurodegenerative disorder associated with the loss of neuronal functions in distinct areas of the brain.

2.3 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder that is characterized by the progressive deterioration of certain parts of the brain that are essential for learning and memory. The disease progresses in stages over months or years, gradually affecting memory, reasoning, judgement, language and eventually even simple tasks (Berchtold and Cotman 1998). AD was first described by a German psychiatrist, Alois Alzheimer in 1906, while performing an autopsy on a woman with memory and language impairment, where abnormal structures called senile plaques and neurofibrillary tangles were found throughout the cerebral cortex of her brain (Hardy 2006). These AD pathological hallmarks trigger neuronal dysfunction, neurotoxicity, and inflammation that lead to cognitive dysfunction (Eikelenboom, Veerhuis et al. 2006). During the initial stages of the disease, the neuronal and synaptic impairment occurs within the para-hippocampal regions, which are the regions of the brain responsible for the formation of new memories. However, as the disease progresses, the neuropathology continues to spread triggering cortical atrophy and ventricular enlargement which in turn causes the total brain mass to reduce up to 35%, as depicted in **Figure 1** (Farfara, Lifshitz et al. 2008, Alves, Correia et al. 2012).

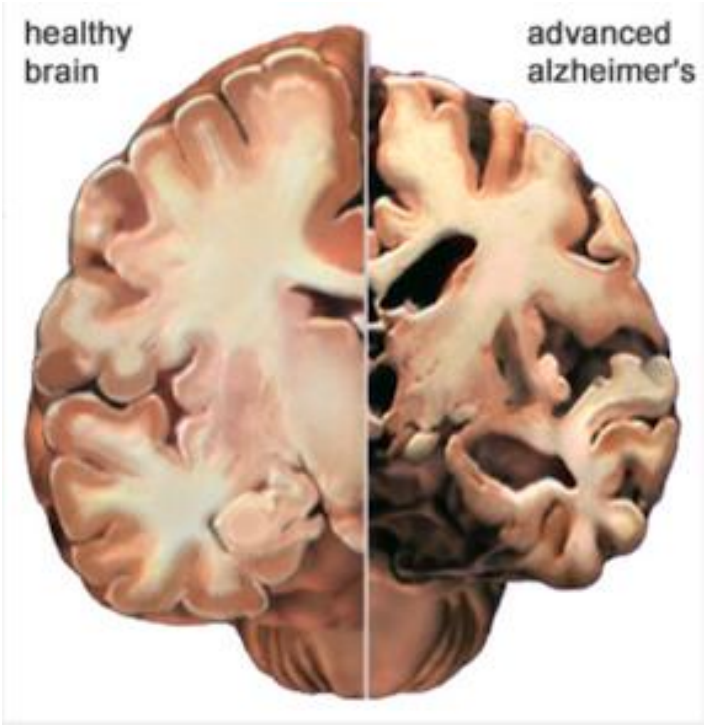

Figure 1: Comparison between the brain of a healthy individual and the brain of an Alzheimer's patient. Shrinkage of the cortex, particularly in the hippocampal region which is responsible for new memory formation is observed in AD. Taken from https://www.alz.org braintour images alzheimer brain.jpg

Currently an estimated 44 million people have been said to be living with dementia worldwide and as the population ages, the amount is predicted to be tripled by the year 2050 (Lane, Hardy et al. 2018). The prevalence of dementia is expected to be increased in low and middle income countries and Alzheimer's has been deemed as the single largest cause of dementia, accounting for 50 – 75% with its prevalence doubling every 5 years after the age of 65 (Lane, Hardy et al. 2018). The risk of AD developing over the age of 85 was found to be 11% in males and 17% in females respectively (Genin, Hannequin et al. 2011), and for those diagnosed at the age of 65 experienced a 67% reduction in lifespan while when diagnosed later at the age of 90, a reduction in lifespan of 39% was observed (Brookmeyer, Corrada et al. 2002). The

number of mortality cases for AD has been observed to double, demonstrating an increase of 123 percent between 2000 and 2015 in comparison to deaths from other major diseases, like heart disease that demonstrated a decrease in 11%. Despite these alarming numbers, AD is still observed to have the lowest number of compounds progressing to therapeutic trials thereby exhibiting the absence in pharmaceutical quest for treating this disease (Alzheimer's Association 2018 (Lewis and Torgerson 2017).

It is often a challenge to clinically distinguish AD from other types of dementia and the disease is usually difficult to be conclusively diagnosed until post-mortem where amyloid plaques and tau neurofibrillary tangles within the brain are identified. Thus, a diagnosis is only feasible based on the symptoms and cognitive assessments, thereby making diagnosing, treating and managing AD extremely demanding. This in turn has given rise to emphasis on research pertaining to image markers or biomarkers in live patients to aid in a smooth diagnosis process. One of the most crucial features across most neurodegenerative diseases is progressive accumulation of specific protein aggregates in the brain with a regional pattern specific to each disease (Lee, Lim et al. 2011). These accumulated proteins typically form intracellular inclusions or extracellular aggregates in specific brain areas which are specific pathological hallmarks for certain diseases (Takalo, Salminen et al. 2013). AD is characterized by extracellular deposition of amyloid-β (Aβ) protein in the form of senile plaques and by intraneuronal accumulation of hyperphosphorylated tau as neurofibirillary tangles as depicted in **Figure 2** (Lee, Lim et al. 2011).

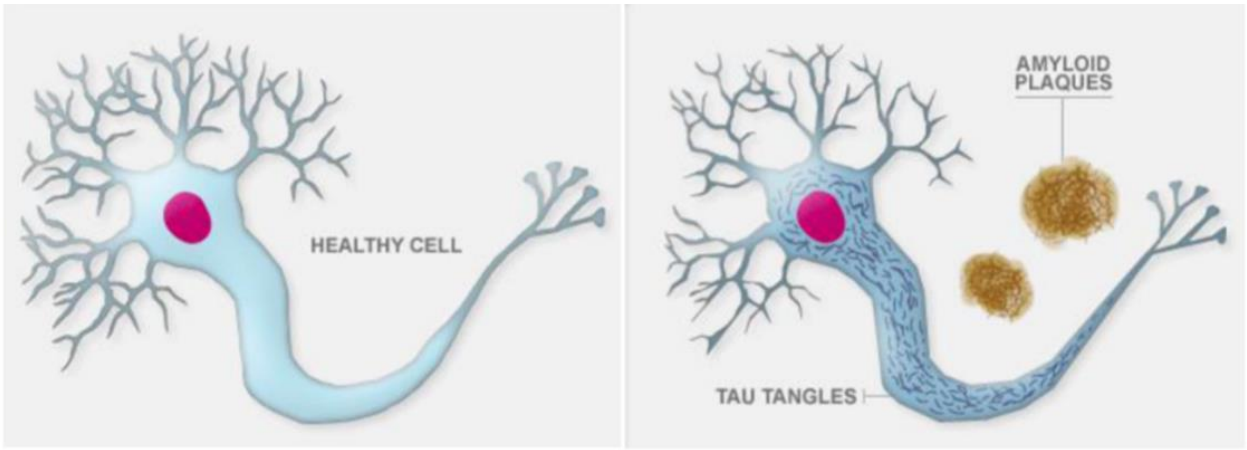

Figure 2: A healthy neuronal cell (left) and a neuronal cell from an AD patient (right), depicting the intracellular tau neurofibrillary tangles and the extracellular amyloid plaques. Taken from http://www.alzheimersresearchuk.org brain-tour

2.4 PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE

Our insight on AD has since developed to distinguish the notion that AD is indeed a multifactorial disease. A cascade of pathophysiological events are triggered in AD that ultimately involves common cellular signalling pathways that lead to cellular and neural networks dysfunction, failure of neurotransmission and cell death (Selkoe 2000). These events triggered a quest for a key regulator and comprehensive hypotheses to elucidate these occurrences that led to multiple hypotheses for AD being proposed. A few key hypotheses are discussed briefly in this section.

2.4.1 AMYLOID HYPOTHESIS

Some of the key pathological features of AD are the extracellular deposits of amyloid β peptides, intraneuronal neurofibrillary tangles (NFTs), and large-scale neuronal loss. Thus, for a long period of time, new drug research development has been focusing on amyloid β peptides. In AD, plaques develop in regions of brain that are related to learning and memory whereby these plaques contain beta-amyloid

protein predominantly. Beta-amyloid is a protein fragment that are cleaved from larger proteins called amyloid precursor protein, APP during metabolism. Normally, soluble beta-amyloid proteins are catabolized but in AD, the beta-amyloid protein is formed inside the cell from the cleavage of the APP and is then deposited outside the cell. However, the mechanism that leads to the polymerization process where normal soluble cellular protein is converted into an insoluble aggregate remains unclear (Du, Wang et al. 2018).

Neuropathological studies are inconclusive as to the pathogenic role of amyloid in AD. Quantitative radioimmunoassay have demonstrated equal amounts of soluble APP found in the brains of Alzheimer's patients and age matched individuals with no Alzheimer's, thus doubting the role of APP. Additionally, since dense amyloid which is made up of another peptide-β/A4 is also insoluble, it in turns makes it harder to quantitate as dense plaque also accumulate with age in individuals who have no cognitive impairment (Selkoe and Hardy 2016).

The strongest evidence for a key role of amyloid in the pathogenesis of AD is the genetic evidence from families with APP mutations. However, the association of a mutation in APP with AD in the relatively few families in which such mutations have been found does not imply that APP is the initiating factor in most forms of AD. Patients with Down syndrome are also predisposed to developing AD whereby the amyloid accumulation is sufficient to cause the neuropathology of AD (Selkoe and Hardy 2016, Du, Wang et al. 2018).

The interaction of several molecules with beta-amyloid in the inflammatory cascade suggests that inflammation may play a role in the pathogenesis of AD. Inflammatory proteins found in the brain that are affected by AD suggest the possibility

that an acute-phase response in AD affected tissue could occur (Zotova, Nicoll et al. 2010).

2.4.2 CHOLINERGIC HYPOTHESIS

Neuropathological changes in AD include cerebral atrophy. Neuritic plaques and neurofibrillary tangles (Du, Wang et al. 2018). Neurons that use acetylcholine are critical to memory and learning and it is primarily cholinergic neurons that show changes and degeneration in AD (Du, Wang et al. 2018). The decrease in cholinergic function correlates closely with cognitive deficits in patients. However, the primary pathologic process that causes AD is still unknown (Du, Wang et al. 2018). The major neurotransmitter change observed in the brains of the patients with AD is the decrease in the biosynthetic enzyme choline acetyltransferase in the cerebral cortex and hippocampus. Biopsy analyses have suggested that this cholinergic marker is reduced even in the first years of the symptoms (Haam and Yakel 2017, Du, Wang et al. 2018).

The basal forebrain which is the major source of cholinergic innervation to the neocortex and hippocampus show progressive neuronal loss in AD. Relative preservation of postsynaptic muscarinic receptors suggests that cholinergic stimulation may be effective in restoring function. Increased levels of acetylcholine with the use of acetylcholinesterase inhibitors produce a modest improvement in cognitive function in some patients (Haam and Yakel 2017).

2.4.3 TAU HYPOTHESIS

Another key neuropathological hallmark of AD is the neurofibrillary tangle, which is one of the cytoskeletal abnormal characteristics of the disease. Neurofibrillary tangles contain paired helical filaments which appear to be formed primarily from abnormal aggregations of tau protein (Maccioni, Farias et al. 2010, Du, Wang et al.

2018). Tau is a microtubule-associated protein (MAP), which is an important component of the neurofibrillary tangles. Neurofibrillary tangles are abnormal collections of twisted threads of protein found inside nerve cell bodies that may interfere with nerve cell functioning by impairing the axonal transport (Brier, Gordon et al. 2016).

The distribution of neurofibrillary tangles spread as the severity of AD increases. During the early stages of the disease, neurofibrillary tangles occur predominantly in the entorhinal region and subsequently appear in the hippocampus and nearby regions of the cortex and throughout the cortex. These regions possess a concentration of neurons that receive cholinergic input and are also the ones that demonstrate the greatest degree of degeneration (Maccioni, Farias et al. 2010, Brier, Gordon et al. 2016). Neuronal death is also a pathological hallmark in AD whereby certain neurons tend to be selectively lost and it has been proposed that the loss of synaptic density is more likely to have a more immediate relationship to dementia in AD than the accumulation of amyloid (Ghoshal, Garcia-Sierra et al. 2002, Brier, Gordon et al. 2016, Du, Wang et al. 2018).

2.4.4 OTHER HYPOTHESES

Additionally, oxidative stress plays a significant role in the pathogenesis of neurodegenerative disorder including AD whereby imbalances between free radicals and antioxidant defence system contributes to the aetiology of conditions like AD (Markesbery 1997, Manoharan, Guillemin et al. 2016). Brain cells are particularly vulnerable to oxidative damage because of their high consumption of oxygen and the significant amount of polyunsaturated fatty acid content and its ability to combat oxidative stress (Halliwell and Gutteridge 1995, Halliwell 2001). Besides that,

increased lipid peroxidation and decreased polyunsaturated fatty acids in the AD brain, and increased 4-hydroxynonenal, an aldehyde product of lipid peroxidation in AD ventricular fluid has also been said to trigger AD whereby these oxidative damage lead to the disruption of cell membrane, inactivation of enzymes, and finally cell death thus triggering AD (Markesbery 1997). Another pathogenesis of AD is inflammation whereby a vigorous elevation in inflammatory mediators are observable with the release of increased levels of inflammatory factors including nitric oxide (NO), inducble nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNFα), interleukins (IL-1β, IL-6,) (Grammas, Samany et al. 2006, Zhang, Mao et al. 2008). The activation of Nuclear factor kappa B (NF-κB) has also been considered as a common feature of many neurodegenerative diseases, particularly AD and activated NF-κB has been predominantly found in neurons and glial cells in β amyloid plaque found in AD patients (Mattson and Camandola 2001, Dresselhaus and Meffert 2019).

2.5 CURRENT TREATMENTS

The currently available pharmacological treatment for AD (donepezil, rivastigmine, galantamine and memantine) exert beneficial effects on the cognitive, functional, and behavioural symptoms of the disease (Birks 2006). However, their effects are predominantly symptomatic and is not able to prevent or reduce the progression of the disease (Herrmann, Chau et al. 2011, Yiannopoulou and Papageorgiou 2013).The quest for a disease-modifying intervention firstly focussed on compounds targeting the Aβ pathway like tarenflurbil, tramiprosate and semagacestat which were unsuccessful in demonstrating efficacy in the final clinical stages of testing (Gauthier, Aisen et al. 2009, Imbimbo and Giardina 2011). Additionally, other neuronal mechanism that play a vital role in the pathophysiology of

AD like neuroinflammation, hyperphosphorylation, the deposition of tau and oxidative stress are now being explored as promising therapeutic targets.

2.6 *ORTHOSIPHON STAMINEUS*

Orthosiphon Stamineus (OS) is a medicinal perennial herb plant that hails from the *Lamiaceae* family. It is locally called “Misai Kucing’ or “Cats whiskers” because of its pale purple flowers with long wispy stamens that are shaped similar to the cats whiskers (Indubala and Ng, 2000). OS is found to be widely distributed around tropical areas and have emerged to become one of the most important medicinal plants due to its effectiveness in traditionally treating various diseases like rheumatism, influenza, diuresis, jaundice, oedema and hypertension (Sumaryono, Proksch et al. 1991, Ohashi, Bohgaki et al. 2000, Tezuka, Stampoulis et al. 2000).

Common names of *Orthosiphon stamineus*

Malaysia: Misai kucing

Thailand: Yaa Nuat Maeo

Indonesia: Kumis kucing

Phillipines: Balbas-pusa

India: Poonai meesai

The genus name *Orthosiphon* originated from a Latin word “lortho”, that mean “straight” and “siphon” that mean “tube-like structure” which matches the characteristics of the Lamiaceae family (Lai Keng and Poay Siong 2006). This plant is usually cultivated around open areas like the roadsides and wastelands in either lowlands or highlands.

2.6.1 MORPHOLOGY

In Malaysia, OS exist as two varieties whereby they are both classified following the colour of their flowers, which is white and purple respectively, as depicted in **Figure 3** (Lai Keng and Poay Siong 2006).

Figure 3: White *O. stamineus* (left) and purple *O. stamineus* (right)

The purple variety has randomly scattered yellowish spots on the leaf surface and it tends to generate light purple flowers. On the other hand, the white variety possesses green leaves but lacks the yellowish spots on the leaf surface and tend to form white flowers (Keng and Siong, 2006). The other parts of the plant like the stems, stigma and pollen grain are same for both varieties. OS is an herbaceous shrub that grows up to a height of 1.5 metre (Keng and Siong, 2006).

2.6.2 PHARMACOLOGICAL USES

In Malaysia, OS are mostly used as herbal teas and as diuretics and is said to be highly effective in treating kidney problems, hypertension, oedema, rheumatism, abdominal pain, jaundice, diabetes, gout and jaundice (Han, Hj Abas et al. 2008, Shafaei, Esmailli et al. 2015,). OS is also found to be useful for conditions related to renal cleaning that includes diseases like cystitis, nephritis and urethritis. Additionally,

OS extract are also famous as tonics for liver and gall bladder problem as well as kidney and bladder stones conditions. It is also employed in controlling blood pressure and cholesterol levels. Besides that, emerging researches have proven the effectiveness and efficiency of OS using animal models to treat various conditions including diabetes and lipid profiling (Sriplang, Adisakwattana et al. 2007), hypertension (Ohashi, Bohgaki et al. 2000), kidney conditions (Arafat, Tham et al. 2008) and inflammatory conditions (Yam, Vuanghao et al. 2010). OS has also demonstrated strong antioxidant properties that has caused many researches to focus on exploring it due to its range of properties. OS has also been scientifically shown to demonstrate a range of pharmacological properties like antioxidant, antibacterial, cytotoxic, diuretic, antihypertensive and anti-angiogenesis (Sahib, Aisha et al. 2009, Ho, Noryati et al. 2010).

2.6.3 PHYTOCHEMICAL CONSTITUENTS

OS largely comprises of phenolic compounds and flavonoids. Earlier studies have showed that OS contains more than twenty phenolic compounds and the most important constituents are nine lipophilic flavones, two flavanol glycosides and nine caffeic acid derivatives (Sumaryono, Proksch et al. 1991). The key chemical constituents of OS, as depicted in **Figure 5**, are caffeic acid, diterpenes, orthosiphols, cirrchoric acid, saponins, organic acids, monoterpenes, triterpenes, sinensetin, euphatorin, rosmarinic acid and 3-hydroxyl-5,6,7,4-tetramethoxyflavone (TMF) (Tezuka, Stampoulis et al. 2000, Awale, Tezuka et al. 2003, Akowuah, Zhari et al. 2004, Olah, Hanganu et al. 2004)

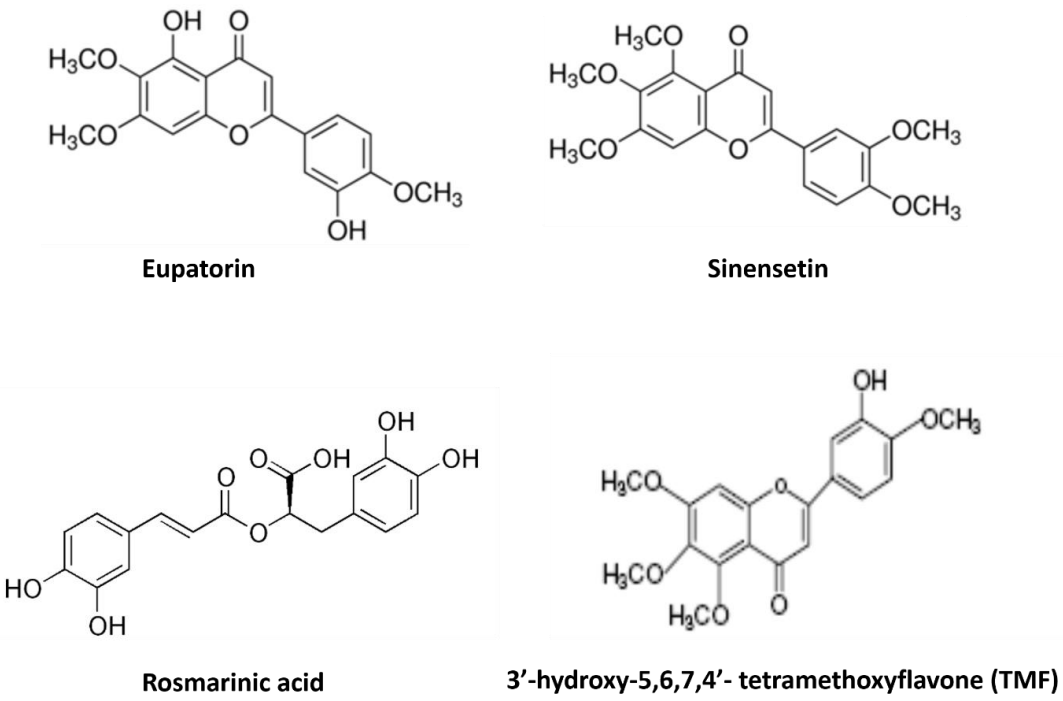

Figure 5: Chemical components of the key phenolic compounds present in OS plants.

The leaves of the OS plant have been identified to be most important part as it contains most useful bioactive compounds that exhibit the therapeutic properties observable with OS. Rosmarinic acid, a major flavonoid component of OS has been shown to have various pharmacological properties. Flavonoids which are the principal group of polyphenols are also reported to be efficacious in decreasing oxidative stress and are said to promote various physiological benefits, particularly in learning and memory, scavenging free radicals and cognitive impairment (Bhullar and Rupasinghe 2015, Ghumatkar, Patil et al. 2015). Besides that, standardized ethanolic extract of OS were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration (George, Chinnappan et al. 2015).

In recent times, extensive researches are being focussed on employing plant extracts in treating diseases as plant extract have a relatively higher therapeutic window with reduced side effects which are also economical. This in turn has given rise to the potential of exploring OS plant in this research scope.

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Chapter 3

BRIEF INTRODUCTION

In Alzheimer's disease (AD), the dysregulation of the amyloid-beta (Aβ) level results in the formation of senile plaques that contain Aβ depositions. Aβ is a complex biological molecule which interacts with many types of receptors forming insoluble assemblies thereby affecting its normal neuronal conditions. Aβ triggers neurotoxicity in AD by evoking a cascade of oxidative damage to the neurons. During this situation, signs of AD begin to appear and the patients experience marked cognitive disabilities (Sadigh-Eteghad, Sabermarouf et al. 2015). On the other hand, acetylcholinesterase (AChE) serves as an important enzyme in the cholinergic nervous system. During the progression of AD, in addition to the deterioration of various neurons, a profound loss of forebrain cholinergic neurons as well as a decrease in acetylcholine levels are observed (Davies and Maloney 1976, García-Ayllón, Small et al. 2011). Both the acetylcholine synthesizing enzymes, choline acetyltransferase (ChAT) and acetylcholine-hydrolysing enzyme, AChE is affected in AD. However, the current available treatment for AD, tend to focus on inhibiting AChE, which enhances cholinergic transmission, but have modest and transient therapeutic effects (Giacobini 2002, Kaduszkiewicz, Zimmermann et al. 2005).

The available drugs in the market are only able to compensate for the death of the cholinergic neurons and therefore only offer symptomatic relief by inhibiting acetylcholine (ACh) turnover and restoring synaptic levels of this neurotransmitter. However, AChE have recently been found to play a larger role in the pathogenesis of AD whereby AChE appears to directly interact with amyloid-beta in a manner that increases the deposition of this peptide into insoluble plaques (Rees and Brimijoin 2003). Thus, a properly designed AChE inhibitors might be able to act as disease-modifying agents rather than as mere palliatives. *O. stamineus* possesses various phytochemicals that can protect neurons against a variety of insults and would hence serve as a promising candidate for future Alzheimer's disease therapy.

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Neuroprotective effects of ethanolic extract of *Orthosiphon stamineus* in an *in-vitro* model

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Abstract: Alzheimer disease (AD) are one of the most common types of dementia, which is characterized by chronic and progressive neurodegeneration that triggers, advanced cognitive impairment. In recent times, there has been a growing interest in using complimentary therapy and phytochemicals from medicinal herbs to enhance the quality of life and prevent therapy induced side-effects, particularly in using phytochemicals from medicinal herbs as they possess anti-inflammatory and antioxidant activities that may potentially hinder neurodegeneration and improve memory and cognitive functioning. In this study, the in vitro neuroprotective activity of the *Orthosiphon stamineus* extract was investigated via inhibition of acetylcholinesterase (AChE) enzyme as well as rosmarinic acid, a major flavonoid component of *O. stamineus* extract. Additionally, the *in-vitro* evaluation of neurotoxicity & neuroprotective property of *O. stamineus* standardized extract was further evaluated using SH-SY5Y human neuroblastoma cell that was challenged with β-amyloid, a key component in Alzheimer's disease. Based on the results attained from the AChE enzyme inhibition activity, both *O. stamineus* and Rosmarinic acid are found to be potent AChE inhibitors with an IC50 value of 3.13mg/ml and 0.164 mg/ml respectively. *O. stamineus* extract was also found to be neuroprotective when challenged with β-amyloid. Thus, present study further validated the use of *O. stamineus* plant extract as a strong candidate for the development of potential therapeutic target for the treatment of neurodegenerative diseases like Alzheimer's disease.

Keywords: Rosmarinic acid; acetylcholinesterase enzyme; Alzheimer's disease

1. Introduction

As the population ages, the incumbrance of neurodegenerative diseases have risen to be a global indispensable concern. Approximately 46.8 million people have been diagnosed with Alzheimer's disease (AD) worldwide as of 2015 and this numbers are expected to increase over time [1,2]. During the development of AD,

the brain of the patient shrinks due to the death of the nerve cells in the cerebral cortex [3]. This in turn leads to gaps being formed between the temporal lobe and the hippocampus where new information is usually stored and retrieved, thus affecting the patient's ability in decision making and reasoning [1,2]. AD is characterised by the accumulation of beta amyloid peptide (Aβ) plaque and intracellular neurofibrillary tangles [3]. Several studies have insinuated that Aβ-induced oxidative stress plays a key role in the pathogenesis of AD whereby oxidative stress is stimulated by Aβ which in turn trigger the build-up of amyloid plaques [3]. These build-ups impose toxicity on neurons thus causing the development of many neurodegenerative diseases. Given the association of Aβ-induced oxidative stress in the aetiology and pathology of AD, a promising approach will be to include the use of natural plant extracts containing many phytochemicals as a potential candidate to protect the neurons by attenuating the oxidative stress-dependent, Aβ-mediated cytotoxicity.

Additionally, the neurotransmitter acetylcholine plays an important role in determining brain processing and memory whereby when there is a decrease in acetylcholine levels; learning and memory are found to be impaired [1,4]. Additionally, acetylcholine deficiency has also been observed in the brain of Alzheimer's patient, thereby correlating acetylcholine shortage with AD [5]. Acetylcholine deficiency occurs due to the inability to synthesize adequate amount of acetylcholine for information transmission [1,5]. Acetylcholinesterase (AChE) are vital for the breakdown of acetylcholine in the synaptic cleft. Therefore, by inhibiting AChE in the brain, more acetylcholine will be available to interact with the acetylcholine receptors thus enhancing cognitive functions [1,5,6]. The AChE enzyme belongs to the hydrolase of the carboxylesterase family that primarily involves in the breakdown of acetylcholine neurotransmitter [7,8,9]. It is mainly present within the neuromuscular junctions and cholinergic brain synapses and plays an important role in synaptic transmission termination [9]. During neurotransmission, acetylcholine, which is released into the synaptic, cleft binds to its respective receptors on the post-synaptic membrane, relaying the nerve signal [9,10]. AChE, which is also present on the post-synaptic membrane, inhibits the signal transmission by breaking down acetylcholine [9,10]. The choline, which is released as a result of acetylcholine breakdown, is taken up by the pre-synaptic nerve thus aiding in the synthesis of acetylcholine by reacting with the acetyl-CoA via the choline acetyltransferase action [9,11]. Since acetylcholine is actively linked with synapse signal transmission and its pharmacological action is predominantly terminated by AChE, a promising strategy for AD treatment would be active inhibitors of these metabolizing enzymes.

In recent times, many plant-derived compounds have been explored as potential treatment for AD where almost more than 150 different plant species have been used in age related CNS diseases [12,13,14]. *Orthosiphon stamineus* Benth (Lamiaceae) commonly called "Misai Kuching" is a well-distributed medicinal plant in South East Asian countries. Previous studies have demonstrated that the plant contains many bioactive compounds like diterpenes, orthosiphols A-E, monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene and myoinositol [15,16]. Additionally, the plant has also been shown to have antioxidant, anti-inflammatory, hepatoprotective, gastroprotective, antihypertensive, antidiabetic, antihyperlipidemic and antimicrobial activities [17,18]. However, as of now *O. stamineus* have yet being explored for neuroprotective activities. Therefore, the present study was carried out to examine the acetylcholinesterase activity as well as neuroprotective activity of *O. stamineus* extract against beta amyloid induced neuronal cell damage

2. Materials and Methods

2.1 Plant Extract Preparation

The leaf extract of *O. stamineus* (OS) was prepared under GMP-based environment using Digmaz technology by Natureceuticals Sdn. Bhd. Briefly, the powdered material (100 g) of the air-dried leaves was extracted with 1 L of 50% ethanol at 50 °C for 24 h with continuous stirring counter current technology. The extract was filtered via a filter paper (Whatman No. 1) using a Buchner filter under vacuum and concentrated to dryness in a rotary evaporator and refrigerated at −20 °C until further use.

2.2 Acetylcholinesterase (AChE) Inhibitory Assay

The AChE activity was measured by the micro-plate assay based on Ellman's method [19] with some modifications. This method is based on gaging production of thiocholine when acetylthiocholine is hydrolyzed. In brief, 140 µL of sodium phosphate buffer (pH 7.8) was added to each well of the 96-well microplate followed by 20µL of OS plant extracts (0.3125, 0.625, 0.125, 2.5 and 5 mg/mL) and Rosmarinic acid (0.625, 0.125, 0.25, 0.5 and 1 mg/mL). The absorbance was than measured at 412 nm and this reading served as the blank. After that, 20µL of the enzyme AChE (0.2 U/ml) was added and was incubated at 25°C for 15 minutes. Subsequently, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (10 µL, 3 mM) and acetylthiocholine iodide (ATCI) (10 µL, 15 mM) were added respectively. The absorbance was measured again at 412 nm for 30 minutes at 5 minutes interval. Donepezil (0.015mM) was used as the positive control and the experiment was carried out in triplicates. The percentage of inhibition was calculated using the equation: -

$$\textbf{(\%) Inhibition} = 1 - [(A_{Sample})/(A_{Control})] \times 100$$

where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank (distilled water). Extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the percentage inhibition against extract concentration.

2.3 In-vitro evaluation of neurotoxicity & neuroprotective property of O. stamineus

Human neuroblastoma SH-SY5Y cell line (ATCC No: CRL-2266) used in this study. SH-SY5Y cells were cultured in DMEM medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin and maintained at 37 °C in 5% CO2 incubator. To distinguish the protective effects of OS against beta amyloid cytotoxicity, the SH-SY5Y cells were first pre-treated with OS at concentrations ranging from 9.76×10-3 to 5ng/mL for 24 hours upon which the pre-treated cells were then exposed to 1µm beta amyloid to distinguish its neuroprotective effect. All experiments were done in triplicates.

Cell viability was determined by a mitochondria enzyme-dependent reaction of 3 -(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In this assay, metabolic active cells deactivate the yellow tetrazolium salt MTT to purple formazan crystals. MTT assay was carried out as described previously with minor modifications. The SH-SY5Y cells, in the exponential phase were employed whereby they were seeded onto the 96 well plates (10×104 cells/well). They were left to adhere for 24 hours and were then treated with the range of OS concentrations, vehicle and beta amyloid (1µm) and incubated for 24 hours. After the incubation time, the cells were washed with PBS, followed by the addition of MTT and were incubated for 4 hours. After the incubation time, the MTT solution was removed and 100µL of DMSO was added. After 10 minutes, the absorbance was recorded at 540nm. Results were expressed as percentage of control.

2.4 Statistical analysis

Data was expressed as mean ± standard deviation (std) of minimum of six independent experiments. Statistical differences between control and target groups for all experiments were determined using Student's t-test. The comparison between the groups were considered significant if $p \leq 0.05$.

3. Results

3.1 Plant Extract Preparation

The OS extract was purchased from Natureceuticals Sdn. Bhd and the percentage yield of the extract obtained was 9.8%. The extract was found to be water-soluble.

3.2 In-vitro AChE Inhibition Assay

In this study, the OS extract was tested for their inhibitory activity against AChE at concentrations of 0.3125, 0.625, 0.125, 2.5 and 5 mg/mL by in vitro Ellman's method. Results of the AChE inhibitory activities (%) of the OS extracts are presented in **Figure 1**. It can be observed that at 30 minutes, OS demonstrated weak AChE inhibition at all concentration but 5mg/ml on the other hand exhibited strong inhibition (80-90%) at all the time points.
The IC_{50} value of the plant extract indicating AChE inhibitory activity is demonstrated in **Figure 2**. The 5mg/mL OS extract was found to possess potent AChE inhibition with an IC_{50} value of 3.13mg/mL. Additionally, Rosmarinic acid was also found to possess potent AChE inhibition as demonstrated in **Figure 3**. The IC_{50} value for Rosmarinic acid was calculated as 0.164mg/ml.

3.3 In-vitro evaluation of neurotoxicity & neuroprotective property of O. stamineus

When the SH-SY5Y cells were exposed to various concentrations of OS (9.76×10-3 - 5ng/mL) alone for 24 hours, the cell viability was not altered as demonstrated in **Figure 4** where the OS extract was found to be non-toxic up to the concentration of 0.1563µg/ml and the IC_{50} value was found to be 0.127µg/mL. When the cells were exposed to 1µm beta amyloid, significant oxidative stress and cell death were observed where following pre-treatment of cells with various concentrations of OS, when exposed to beta amyloid, cell viability of the cell was not drastically affected. As demonstrated in **Figure 5**, OS pre-treatment dose dependently prevented beta amyloid induced cell death. OS extract was found to be neuroprotective at 0.0391, 0.0781 and 0.1563µg/ml concentrations.

4. Discussion

The free radical theory advocates that augmented lipid peroxide and reactive oxygen species (ROS) production, which are generated with free radicals in membrane lipids, trigger a range of cellular enzymes deterioration, thereby impairing the neurodegenerative process [20,21]. Oxidative stress has been long known to be involved in the causation and development of various diseases like cardiovascular diseases, cancer, diabetes and neurodegenerative diseases. Since free radicals are generally very reactive, they tend to impair vital biomolecules like DNA, proteins, RNA and lipids, thus triggering cell death. Similarly, brain aging also cause augmented oxidative impairment during both normal aging and AD [21].

The use of medicinal herbs as alternative treatments has been increasing worldwide and gaining popularity in developing countries [21]. Earlier studies have demonstrated that OS leaves extracts possess strong antioxidant, anti-inflammatory and anti-bacterial properties with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid and nitric oxide inhibitory isopimarane-diterpenes [22,23,24,25]. For instance, Rosmarinic acid, a major flavonoid component of OS has been shown to have various pharmacological properties. Flavonoids, which are the principal

group of polyphenols, are also reported to be efficacious in decreasing oxidative stress and are said to promote various physiological benefits, particularly in learning and memory, scavenging free radicals and cognitive impairment [26,27]. Besides that, standardized ethanolic extract of OS were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration [28]. These reported pharmacological and therapeutic properties of OS further corroborate that this plant extract has a potential to act as a source of secondary metabolites that serve as natural antioxidants.

Numerous studies have exhibited that formation of toxic fibrillar deposits from the excess deposition of Aβ plaques plays a key role in Alzheimer's disease aetiology [3,29]. Several in vitro and in vivo studies have demonstrated the neurotoxic effects of the Aβ-related fragments in neurons. The exact mechanism facilitating the toxic Aβ properties are yet to be comprehended, however it has been insinuated that it could be due to oxidative-stress dependent apoptosis [30]. Based on the results attained, as expected, OS extract was found to be able to protect the human neuroblastoma SH-SY5Y cells against beta amyloid induced neurotoxicity thus further endorsing the neuroprotective ability of OS extract.

Acetylcholinesterase (AChE) enzyme serve as a suitable target for drug designing and for determining mechanism-based inhibitors due to its ability in catalysing the hydrolysis of neurotransmitter acetylcholine. Based on the results attained from the AChE enzyme inhibition activity for both the OS extract and rosmarinic acid, they were both found to be potent AChE inhibitors. It is known that preventing breakdown of acetylcholine (ACh) is responsible for the elevation of ACh level in the synaptic cleft by acetylcholinesterase (AChE) inhibition is the most significant changes observed in AD. Since OS extract was found to demonstrate significant AChE inhibitory activity, it can be assumed that the OS ethanolic extract presented substantial amount of polyphenols and flavonoids like rosmarinic acid that exhibited potential antioxidant and radical scavenging activities thus indicating that this plant possesses a wide margin of medicinal value and could potentially play a crucial role in AD therapy.

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Author Contributions: TR performed all the experiments and was responsible for the writing of the manuscript in its entirety. YK helped in designing of study and result analysis. MS & IO were involved in conceptualizing, designing of the study, result analysis, and manuscript editing. All authors gave their final approval for the submission of the manuscript.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FIGURES

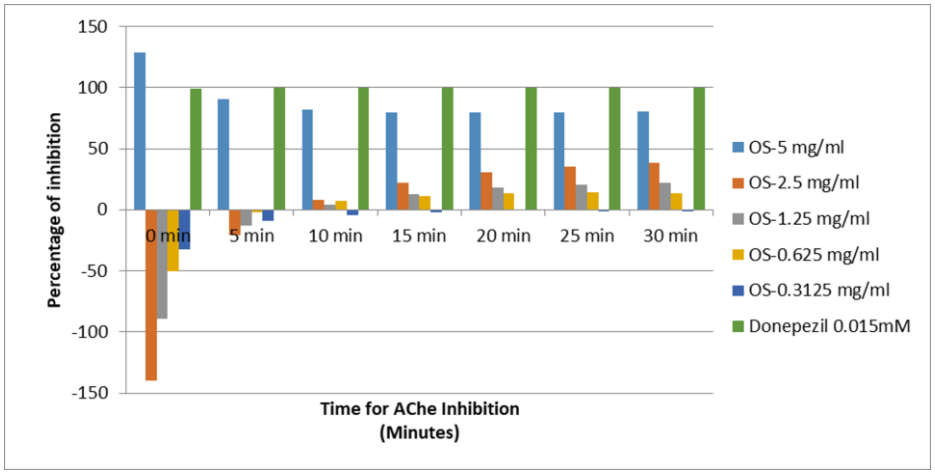

Figure 1. Percentage of AchE inhibition of different *O. stamineus* concentrations

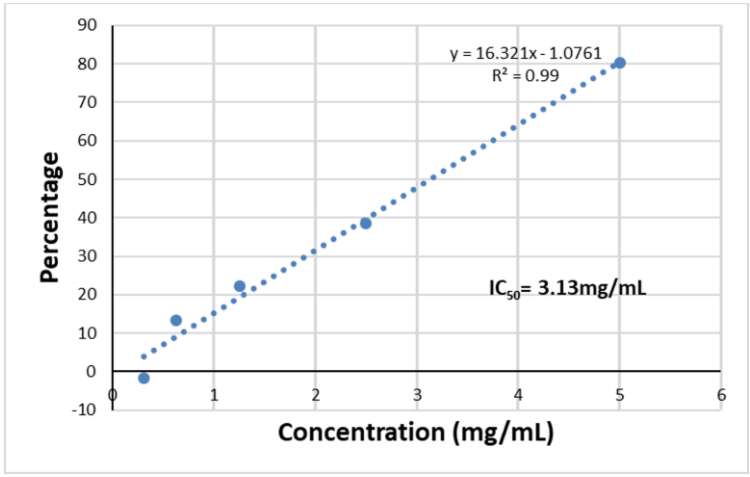

Figure 2. AChE inhibition assay: IC_{50} value for *O. stamineus*

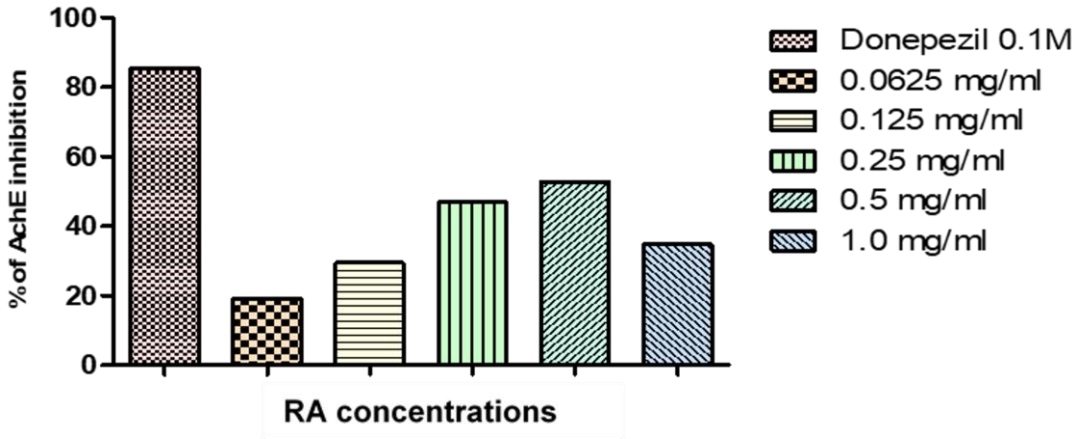

Figure 3. Percentage of AChE inhibition of different Rosmarinic acid concentrations. The data are represented as mean ± SD of three independent experiments.

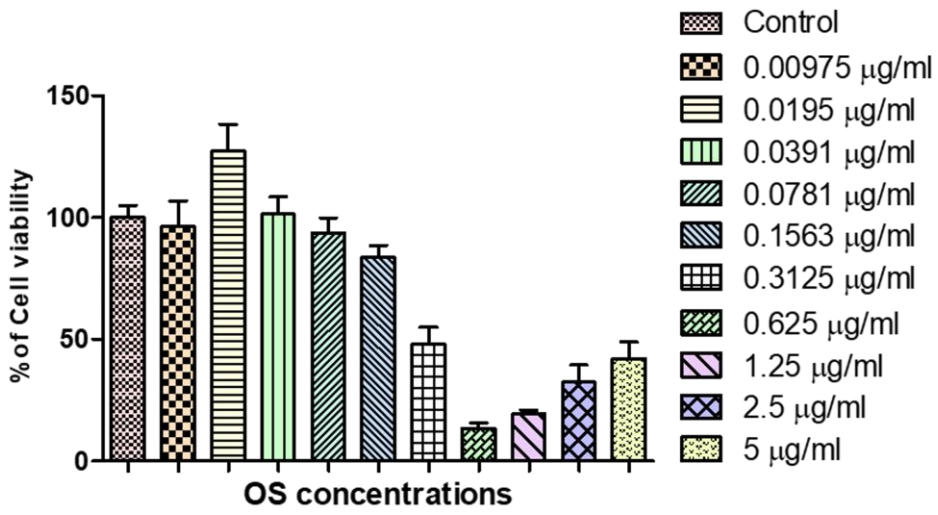

Figure 4. Effects of *O. stamineus* on SH-SY5Y cell viability using MTT assay. The data are represented as mean ± SD of three independent experiments.

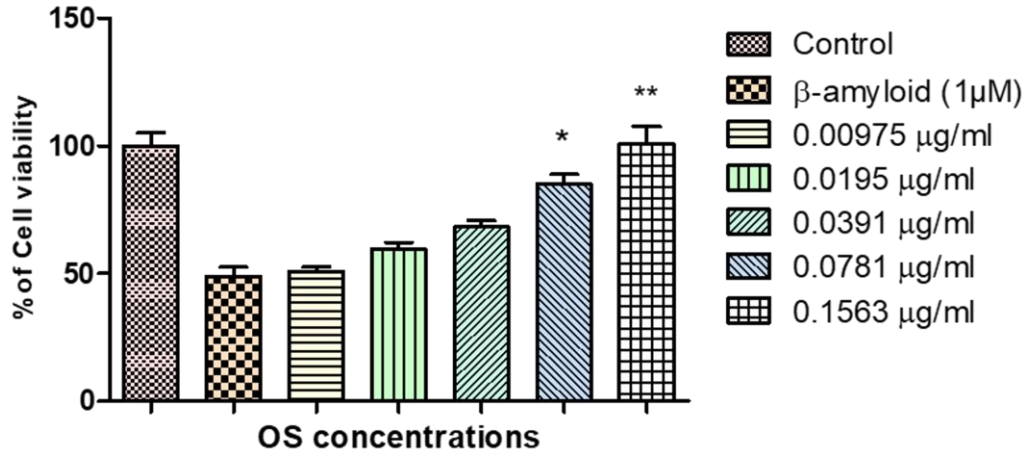

Figure 5. Neuroprotective effect of OS on β-amyloid induced neurotoxicity. The data are represented as mean ± SD of three independent experiments, $^{**}P < 0.01$, $^{*}P < 0.05$

Chapter 4

BRIEF INTRODUCTION

There have been many hypotheses pertaining to the pathogenesis of Alzheimer's disease. One of the key hypotheses that was first explored in this study was the cholinergic hypothesis. The cholinergic hypothesis suggests that a dysfunction of acetylcholine containing neurons in the brain contributes substantially to the cognitive decline observed in those with advanced age and Alzheimer's disease (AD) (Mufson, Counts et al. 2008). Previous research has implied that degeneration of cholinergic neurons in the basal forebrain as well as decreased cholinergic neurotransmission in the cerebral cortex and other areas triggered the deterioration in cognitive function observed in an Alzheimer's condition (Mufson, Counts et al. 2008, Ferreira-Vieira, Guimaraes et al. 2016). Acetylcholine is hydrolytically damaged in the brain by acetylcholinesterase (AChE) which is largely involved in the development of amyloid plaques characteristic for Alzheimer's (Colović, Krstić et al. 2013). Hence, treatments are largely designed to reverse the cholinergic deficit due to the importance of cholinergic functioning in cognition. This led to the development of acetyl cholinesterase (AChE) inhibitors to treat the dementia associated with Alzheimer's as decreased cholinergic activity are predominantly observed in brains of Alzheimer's patients (Bond, Rogers et al. 2012, Hyde, Peters et al. 2013). However, the commonly used AChE inhibitors like Rivastigmine and Donepezil exerted various side effects, which in turn led to the quest for a safer drug treatment option.

As the cholinergic system and neuronal damage in Alzheimer's disease progression are linked to each other, the present study focussed on distinguishing the anti-amnesic potential of *O. stamineus* using the scopolamine-induced Alzheimer's disease model which explores the cholinergic hypothesis.

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Ethanolic extract of O. stamineus improves memory in scopolamine-induced amnesia model

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Keywords: Alzheimer's disease, Learning & memory, scopolamine, *Orthosiphon stamineus* animal model

Abstract

Alzheimer's disease (AD) is a chronic neurodegenerative brain disease which is characterized by impairment in cognitive functioning. *Orthosiphon stamineus (OS)* Benth. (Lamiaceae) is a medicinal plant found around Southeast Asia that has been employed as treatments for various diseases. OS extract contains many active compounds that have been shown to possess various pharmacological properties whereby *in vitro* studies have demonstrated neuroprotective as well as cholinesterase inhibitory effects. This study, therefore aimed at determining whether this Malaysian plant derived flavonoid can reverse scopolamine induced learning and memory dysfunction in the novel object recognition (NOR) test and the elevated plus maze (EPM) test. In the present study, rats were treated once daily with OS 50mg/kg, 100mg/kg, 200mg/kg and donepezil 1mg/kg via oral dosing and were given intraperitoneal (ip) injection of scopolamine 1mg/kg daily to induce cognitive deficits. Rats were subjected to behavioral analysis to assess learning and memory functions and hippocampal tissues were extracted for gene expression and immunohistochemistry studies. All the three doses demonstrated improved scopolamine-induced impairment by showing shortened transfer latency as well as the higher inflexion ratio when compared to the negative control group. OS extract also exhibited memory-enhancing activity against chronic scopolamine-induced memory deficits in the long-term memory novel object recognition performance as indicated by an increase in the recognition index. OS extract was observed to have modulated the mRNA expression of CREB1, BDNF and TRKB genes and pretreatment with OS extract were observed to have increased the immature neurons against hippocampal neurogenesis suppressed by scopolamine, which was confirmed by the DCX-positive stained cells. These research findings suggest that the OS ethanolic extract demonstrated an improving effect on memory and hence could serve as a potential therapeutic target for the treatment of neurodegenerative diseases like AD.

1 Introduction

Neurodegenerative diseases have emerged to become a globally critical burden with the aging population. The number of Alzheimer's patients have steadily increased over the years with currently more than 46 million people living worldwide with the disease and the number is expected to increase to 131.5 million by 2050 (Mat Nuri, Hong et al. 2017). Alzheimer disease (AD) is the most common cause of cognitive impairment in the elderly population and is characterized by various

symptoms that include learning and memory impairment, cognitive dysfunction, language impairment and behavioral dysfunction like depression, agitation and psychosis that continue to become more severe with the disease progression (Brookmeyer, Johnson et al. 2007, Mahdy, Shaker et al. 2012). Thus, due to the debilitating nature of the disease, it continues to exist as a huge societal social and economic burden.

One of the key neuropathological features underlying the symptoms associated with Alzheimer's is neuronal loss and when examined microscopically, the presence of senile plaques and neurofibrillary tangles (NFTs) serve as the main features of the disease. A number of mechanisms have been conjectured to further elucidate the pathogenesis of Alzheimer's like cholinergic dysfunction, oxidative damage, beta toxicity, hyperphosphorylation of tau protein and inflammation of senile plaques (Mahdy, Shaker et al. 2012, von Bernhardi, Eugenín-von Bernhardi et al. 2015). The cholinergic system that comprises of the cholinergic neurotransmitters play a vital role in memory processing whereby since acetylcholine serves as the central neurotransmitter in the cholinergic system, loss of the cholinergic neurons and its subsequent decrease results in learning and memory dysfunction characteristic of Alzheimer's (Watanabe, Yamagata et al. 2009, El-Marasy, El-Shenawy et al. 2012, Blake, Krawczyk et al. 2014). Thus far there are yet to be disease-modifying drugs approved for Alzheimer's. The medications available are only capable of temporarily alleviating the symptoms of cognitive impairment, however, they do not halt the inevitable progression of the disease. Besides that, a class of drug called "acetylcholineesterase (ACE) inhibitors are used in early stages of Alzheimer's but their use are limited due to severe side effects (Birks 2006). Thus, major therapeutic research is underway to explore the memory enhancing activities of natural products.

The pharmacological and therapeutic effects of traditional medicinal plants have been associated with the various chemical constituents isolated from their crude extract whereby in particular, active constraints that demonstrate antioxidant activity have been linked to play a central role in various neurodegenerative diseases (Silva, Herdeiro et al. 2005, Mahdy, Shaker et al. 2012). *Orthosiphon stamineus* Benth. (Lamiaceae) is an Asian folklore medicinal plant that has been employed as treatments for various diseases like influenza, inflammation, urinary tract infections and angiogenesis related conditions like cancer (Geng, Chaudhuri et al. 2013, Yehya, Asif et al. 2018). *Orthosiphon stamineus* (OS) have been reported to demonstrate anti-inflammatory, antioxidant, antibacterial and hypoglycemic effects (Awale, Tezuka et al. 2003, Akowuah, Zhari et al. 2004, Ho, Noryati et al. 2010, Abdelwahab, Mohan et al. 2011, George, Chinnappan et al. 2015). Additionally, several scientific studies have also reported the safety profile of 50% ethanol extract of OS in *in vivo* rat models and the LD_{50} have been said to be more than 5000 mg/kg (Han, Hj Abas et al. 2008, Mohamed, Lim et al. 2011, Yehya, Asif et al. 2018). Phytochemical studies have demonstrated that OS leaves extracts contain more than 20 phenolic bioactive compounds like rosmarinic acid, 2,3-dicaffeoyltartaric acid, eupatorine, sinesitin, oleanolic acid, ursolic acid, pentacyclic triterpenes and b-sitosterol (Awale, Tezuka et al. 2003, Shin, Kang et al. 2015, Yehya, Asif et al. 2018) Among these active compounds, rosmarinic acid has been reported to be the main flavonoid present in the 50% ethanol extract of OS extract and plays a central role for the various pharmacological activities exerted by the OS extract. Flavonoids which are the principal group of polyphenols are also reported to be efficacious in decreasing oxidative stress and are said to promote various physiological benefits, particularly in learning and memory, scavenging free radicals and cognitive impairment (Bhullar and Rupasinghe 2013, Bhullar and Rupasinghe 2015, Ghumatkar, Patil et al. 2015). Besides that, standardized ethanolic extract of OS were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration (George, Chinnappan et al. 2015).

Additionally, preliminary studies of OS extract have also demonstrated neuroprotective and choline esterase inhibitory effects, this in turn further indicate OS extract's potential in prompting CNS related reactions. Although OS extract possesses various uses, there are yet no studies on its neuropharmacological activities against AD-like conditions. Therefore, this present study aimed at distinguishing the anti-amnesic potential of this plant derived flavonoid memory deficits in a rat model of cognitive impairment caused by scopolamine.

2 Materials & Method

2.1 Plant Materials

The 50% ethanolic OS extract was procured from NatureCeuticals Sendirian Berhad, Kedah DA, Malaysia. The extract from leaves of Orthosiphon stamineus was prepared under GMP-based environment using Digmaz technology by Natureceuticals Sdn. Bhd., Malaysia. The extract was kept in an airtight container until further experimentations. The OS extract was dissolved in distilled water and filtered using a membrane filter unit (0.22 lm) before being administered to the rats for the study.

2.2 Experimental Animals

In house bred adult male Sprague Dawley (SD) rats weighing between 200 – 300g and between 6 – 8 weeks old were acquired from the animal facility of School of Medicine and Health Sciences of Monash University Malaysia. The rats were kept and maintained in cages under standard husbandry conditions (12:12 h light/dark cycle, at controlled room temperature (22 ± 2°C), stress free, water ad libitum, standard diet and sanitary conditions). Prior to the experiment, the rats were allowed to acclimatize for a period of 1 week to reduce environmental stress. The experiment protocols were approved and conducted according to the approval of the Monash Animal Research Platform (MARP) animal ethics committee in Australia. The experiment protocols were approved and conducted according to the approval of the Animal Ethics Committee Monash University with the reference number MARP/2016/028.

2.3 Experimental Design

The range of OS extract doses was determined following the pre-screening results. OS extract, donepezil and scopolamine were prepared by dissolving it in saline. Normal control rats were administered with saline throughout the experiment. The treatments were given both orally and intraperitoneally (i.p) at a volume corresponding to 0.1ml/100g of bodyweight. All experiments were performed in a balanced design (8 animals/group) to avoid being influenced by order and time. The behavioral studies were divided into two categories namely the nootropic and the scopolamine models.

Nootropic Model

Group 1: Normal Contol (saline)

Group 2: Positive control (Donepezil (DPZ) 1mg/kg)

Group 3: Low dose of Orthosiphon stamineus (OS) (50 mg/kg OS)

Group 4: Medium dose of Orthosiphon stamineus (OS) (100mg/kg OS)

Group 5: High dose of Orthosiphon stamineus (OS) (200mg/kg OS)

Scopolamine Model

Group 1: Normal Contol (saline)

Group 2: Positive control (Donepezil (DPZ) 1mg/kg)

Group 3: Negative control (Scopolamine 1mg/kg)

Group 4: Low dose of Orthosiphon stamineus (OS) (50mg/kg OS + Scopolamine;1mg/kg)

Group 5: Medium dose of Orthosiphon stamineus (OS) (100mg/kg OS + Scopolamine;1mg/kg)

Group 6: High dose of Orthosiphon stamineus (OS) (200mg/kg OS + Scopolamine;1mg/kg)

For the nootropic activity, all the groups received pre-treatment orally for 6 consecutive days before being subjected to a battery of behavioral tests from day six until day eight for NOR and EPM respectively as observed in **Figure1**. For the scopolamine model, amnesia was induced in all the groups except the control group by daily intraperitoneal injections of scopolamine (1 mg/kg) for 9 days after OS extract pre-treatment (day nine to day 17). 30 minutes prior to the administration of scopolamine, NOR was conducted on day 10 and day 15, and EPM was carried out on day 11 & 12 and day 16 & 17 of the study as seen in Figure 1. At the end of the experiment, the rats were sacrificed, and their brains were isolated for further biochemical and immunohistochemistry analysis.

2.4 Novel Object Recognition (NOR)

In the object recognition task, the experimental apparatus consisted of an open field box (40 × 40 × 40 cm) made of black acrylic material. The method used was the same as described by (Ennaceur and Delacour 1988), with slight modifications. The behavior test was conducted between 9:00 AM and 6:00 PM under dim red-light illumination conditions. The objects to be discriminated was two similar transparent cultured flasks filled with water and a toy Lego of the same height (new object). One day prior to the experiment, each rat was habituated to the open field box without any object for 10min. On the experiment day, during the first trial, each rat was placed in the open field for 5 min and allowed to freely explore the two identical objects (transparent cultured flasks with water). After 90 min of post-training session, one old object used during the training session was replaced by a novel object and the rat was left to explore the objects for 2 min. The time spent with each object was recorded and evaluated using SMART software version 3.0 (Panlab, Harvard Apparatus). Both objects presented during the test session were different in texture, color, and size. The open field box was cleaned with 70% ethanol between runs to minimize scent trails. The recognition index was computed using the formula [TB/(TA + TB)∗100] where TA and TB are time spent exploring familiar object A and novel object B respectively (Batool, Sadir et al. 2016). Exploration of an object was deemed when a rat sniffed or touched the object with its nose and/or forepaws.

2.5 Elevated Plus Maze (EPM) Test

The EPM apparatus consists of four arms of equal dimensions, i.e., two open arms (50 × 10 cm) that are crossed with two closed arms, enclosed by high walls of 40cm high. These arms are connected with the help of a central square (10 × 10 cm) that gives an appearance of a plus sign to the maze. This maze is elevated from the ground by 50cm. The method used was the same as described by (Vasudevan and Parle 2006). The behavior test was conducted between 9:00 AM and

6:00 PM under dim red-light illumination conditions. The memory was assessed in EPM in two sessions, 24 hours apart. During the training session, the rats was placed at the end of the open arm, facing away from the central platform. With the help of the stopwatch, the transfer latency (TL1) was noted i.e. the time taken by rat with all its four legs to move into any one of the enclosed arms. If the rat failed to enter any one of the enclosed arms within 90 s, it was gently pushed into one of the two enclosed arms and the TL was assigned as 90 s. The rat was allowed to explore the maze for the next 10 s and then returned to its home cage. The maze was cleaned with 70% ethanol between runs to minimize scent trails. To assess memory, the retention test phase was carried out 24 hours after the training session whereby a decrease in time latency (TL2) during the test session was deemed as an index of memory improvement. The cut-off time for each rat to explore the maze in both the phases (training and test) was 90 s.

The transfer latency was expressed as inflexion ratio, calculated using the formula:-

$$\text{IR} = \frac{(L1-L0)}{L0}$$

L0: Initial TL (s) on the 1st day and

L1: TL (s) on the 2nd day.

2.6 Tissue Processing

All the rats were sacrificed 1 hour after the behavioral test under ketamine-xylazine anesthesia. In each group half the rats (n=4/group) were fixed with 4% paraformaldehyde (PFA) for immunohistochemistry analysis while the remaining half of the rats (n=4/group) were used for gene expression analysis. The hippocampal region from the whole brain was isolated immediately and were homogenized on ice cold 200µL Trizol and stored at -80°C for real-time PCR analysis.

2.7 Gene Expression

Total RNA from the rat brain's hippocampal region was extracted following the method employed by (Bhuvanendran, Kumari et al. 2018), with some minor modifications. The single-step method, phenol-chloroform extraction and Trizol reagent (Invitrogen) was used to isolate the total RNA from the hippocampal region. Briefly, the tissues were homogenized in 200µL of Trizol solution. The mixture was then extracted using chloroform and centrifuged at 135, 000 rpm at 4°C. The alcohol was removed, and the pellet was washed twice with 70% ethanol and resuspended in 20µL of RNase free water. RNA concentration was determined by reading absorbance at 260 nm using Nanodrop. A 500ng amount of total RNA was reverse transcribed to synthesize cDNA using Quantitect® Reverse Transcription Kit according to the manufacturer's protocol. Then the mRNA expression of genes encoding cAMP response element-binding protein (CREB1), brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase B (TrkB) and IMPDH2 in the hippocampus was measured via real-time PCR using the StepOne Real-Time PCR system. Subsequently, the cDNA from the reverse transcription reaction was subjected to Real-Time PCR using QuantiNova™ SYBR® Green PCR kit according to manufacturer's protocol. The comparative threshold (C_T) cycle method was used to normalize the content of the cDNA samples, which consists of the normalization of the number of target gene copies versus the endogenous reference gene, IMPDH2.

2.8 Immunohistochemistry

Immunohistochemical analysis was performed by assessing neurogenesis using Doublecortin (DCX) in the hippocampus. Four brain tissues from each group were immersed in the fixative solution, 4% paraformaldehyde overnight and were methodically cryoprotected in 10, 20, and 30% sucrose solution respectively for 24 hours. The brains were then embedded in 15% Polyvinypyrrolidone (PVP), frozen using dry ice and cut into coronal frozen sections (40µm) using a Leica CM3050 cryostat. The sections were stored in an anti-freeze buffer. The free-floating sections were subjected to endogenous peroxidase quenching with 1% H_2O_2 in methanol for 30 minutes. After washing with phosphate buffered saline, PBS, the tissues were treated with blocking buffer (1% Bovine Serum Albumin (BSA) and 0.3% Triton X-100) for 1 hour followed by incubation with primary DCX (1:250, Abcam) antibodies overnight at 4°C. After washing with PBS, the tissues were then biotinylated with goat anti-rabbit secondary antibody (Abcam) for 2 hours. The tissues were then subsequently washed with PBS and exposed to an avidin biotin peroxidase complex (Vectastain ABC kit, Vector) for another 2 hours. The peroxidase activity was then visualized using a stable diaminobenzidine solution (DAB, Sigma). All immunoreactions were observed under a microscope (BX41, Olympus) and these results were quantified using DigiAcquis 2.0 software.

2.9 Statistical Analysis

Data obtained from all studies were expressed as mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett post hoc test. The P-values of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered as statistically significant. All the experimental groups were compared with the SCP 1 mg/kg group except for the nootropic model where the experimental groups were compared with the control group.

3 Results

3.1 Scopolamine Induced Model Behavioral Analysis

3.1.1 Effect of OS Extract in Nootropic Model

The NOR test was used to evaluate whether OS treatment could reverse scopolamine-induced recognition impairment whereby the effect of OS extract at different doses were assessed following 7 days of pretreatment. Based on the results obtained in **Figure 2A**, pretreated group of OS extract demonstrated an increase in recognition index for novel object particularly observable in animals treated with dose of 50mg/kg and 200mg/kg OS extract. Overall, it can be said that the preference for the novel object was more or less the same among the OS treated groups when compared to the controls.

The memory function was also assessed using the EPM test to gauge the spatial long-term memory. Based on the results obtained in **Figure 2B**, the time taken for each rat to move from the open arm to either of the enclosed arms on the first trial (familiarization session), termed transfer latency 1, did not significantly differ between groups. However, during the test session, termed transfer latency 2, a decrease in time for transfer latency 2 was observed within the groups, thus resulting in improvement in inflexion ratio observed among all groups. Significant results were observed in rats administered with 100mg/kg and 200mg/kg OS extract. These results demonstrate that supplementation of OS extract significantly improved memory function in rats thus demonstrating optimal nootropic effects.

3.1.2 Effect of OS Extract in Acute Scopolamine Model

In the acute scopolamine-induced memory impairment in rats as depicted in **Figure 3 (A1),** the percentage of recognition index for the positive control (donepezil 1mg/kg), negative control (scopolamine 1mg/kg) and the OS extract treated groups, were found to be unchanged, indicating that the acute scopolamine-induced memory was not impaired.

For the EPM test, the scopolamine administered group (negative group) demonstrated a decrease in inflexion ratio when compared to the control and the other treated groups as depicted in **Figure 3 (B1)**. When donepezil (positive group), a well-established standard drug for Alzheimer's disease was administered, a significant increase in inflexion ratio was observed when compared to the scopolamine treated rats. Similarly, significant improvement in inflexion ratio was observed in all the 3 doses of OS extract with both 50mg/kg and 100mg/kg demonstrating a notable increase in inflexion ratio when compared to the scopolamine treated group indicating that the memory impairment induced by scopolamine was reversed. These results further reiterate that OS extract were able to improve retention memory.

3.1.3 Effect of OS Extract in Chronic Scopolamine Model

In the chronic scopolamine model, the NOR test showed that there was a relatively large drop in the percentage of recognition index for the scopolamine treated group as shown in **Figure 3 (A2)**. The percentage of recognition index for all the OS extract groups were observed to have a significant increase when compared to the scopolamine treated group indicating improved memory retention.

For the EPM test as observed in **Figure 3 (B2)**, when chronic exposure of scopolamine was given to the rats, a notable decrease in inflexion ratio was observed in the scopolamine treated group whereas both 50mg/kg and 100mg/kg dose of OS extract demonstrated a significant improved inflexion ratio when compared to the scopolamine treated group indicating that the increase could be due to the repeated exposure of scopolamine. However, the 200mg/kg OS extract demonstrated an increase in inflexion ratio when compared to the negative group, but when compared between the OS extract doses, it was much lower compared to the other two doses. Based on these results, we can say that OS extract does improved memory retention when exposed repeatedly to scopolamine.

3.2 Scopolamine Induced Model Gene Expression Analysis

3.2.1 Gene Expression in the Hippocampal Region

In the hippocampal region, the BNDF mRNA levels were significantly down regulated when injected with scopolamine as compared to the control group, as depicted in **Figure 4A**. Similarly, even the CREB1 and TRKB mRNA levels were observed to be down regulated when given scopolamine as shown in **Figure 4B & 4C** respectively. This down regulation was ameliorated significantly by OS extract pretreatment as compared with the negative (scopolamine 1mg/kg) group. Moreover, in all the three mRNA expression levels, namely CREB1, BDNF and TRKB were observed to be significantly higher when the rats were treated with 100mg/kg OS extract. Similar up regulation in the mRNA levels were also observed in the positive group rats that were treated with donepezil.

3.3 Scopolamine Induced Model Immunohistochemistry Analysis

In the immunohistochemical studies, scopolamine injection was observed to have suppressed adult neurogenesis, shown as distributed dendrites and neuron bodies in the dentate gyrus (DG) region by DCX staining, particularly in the subgranular zone (SGZ). Additionally, pretreatment with

OS extract were observed to have ameliorated the adult neurogenesis by enhancing immature neurons in the SGZ compared to the scopolamine treated group, as depicted in **Figure 5**.

4 Discussion

Alzheimer's disease (AD) is one of the most common neurodegenerative disease and has been said to induce progressive loss of learning ability and memory function (Moosavi, Khales et al. 2012). Scopolamine is a non-selective muscarinic cholinergic receptor antagonist that inhibits the central cholinergic neuronal activity which in turn leads to impairment in spatial learning and memory in rodents and humans (Konar, Shah et al. 2011). The central cholinergic system is also found to be closely associated with neurogenesis and/or cell proliferation in the hippocampus (Yoo, Kim et al. 2011). In this study, we illustrated the nootropic and neuropretective effects of OS extract in a scopolamine induced amnesia model. The medicinal value of OS extract has been well recognized, particularly in regard to its anti-oxidant and anti-inflammatory activities (Arafat, Tham et al. 2008) In particular, Rosmarinic acid which is the main flavonoid component of OS extract has demonstrated various pharmacological properties that may potentially hinder neurodegeneration and improve memory and cognitive functioning (Essa, Vijayan et al. 2012). This in turn conjectured that OS extract serves as a promising therapeutic candidate and could be further explored in the scopolamine induced amnesia model.

The present study demonstrated that pretreatment with OS extract improved memory retention as evident by the improved inflexion ratio observed in the EPM test as well as the increase in the recognition index observed in the OS treated rats. Previous studies have demonstrated scopolamine to show profound amnesic effects in various learning paradigms through the disruption of the cholinergic neurotransmission whereby when given acutely, scopolamine was said to produce spatial memory deficit (Goverdhan, Sravanthi et al. 2012, Ghumatkar, Patil et al. 2015). In our study, similar results were observed whereby the scopolamine treated group in both the acute and chronic model for the EPM test demonstrated decreased inflexion ratio indicating impairment of spatial memory. Similarly, in the NOR test, the recognition index was decreased in the scopolamine treated group in the chronic model, but similar results were not observed in the acute model. This showed that since the NOR test primarily measures the working memory and was hence not influenced by the acute scopolamine treatment. However, when a more prolonged exposure to scopolamine was given via the chronic scopolamine treatment, a decrease in recognition index was observed indicative of cognitive deficits. Donepezil is a well-established drug used to treat dementia associated with Alzheimer's disease (Sumanth, Sowmya et al. 2010) and was hence used as the positive control in our *in vivo* study as it was said to be able to reverse scopolamine induced memory impairment in previous studies (Cachard-Chastel, Devers et al. 2008, Ghumatkar, Patil et al. 2015). The positive effects of OS extract was evident with the improved inflexion ratio observed in the EPM test as well as the increased recognition index observed in the NOR test indicating memory retention. The rats that were treated with 50mg/kg OS extract showed a decrease in transfer latency in which the rats were able to remember and enter the closed arm quickly compared to the training session, which was observable by the improved inflexion ratio, however the rats that were treated with 200mg/kg did show improved memory retention as compared to the scopolamine treated group but not as that observed in the 50mg/kg OS treated group. A celing effect was observed with higher doses. Therefore, based on the behavioral analyses, we can conclude that the spatial memory was improved in both the acute and chronic scopolamine model and this improved performance may be attributed to its enhanced cholinergic neuronal transmission. Thus, OS extract might have an effect on spatial memory retention, which needs to be further explored.

The underlying mechanism for the improvement in memory retention observed in the behavioral studies were further explored by evaluating the biochemical parameters like expression of CREB1, BDNF and TrKB genes in rats treated with OS extract and scopolamine. Adult hippocampal neurogenesis and neuroplasticity are modulated by many neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Begni, Riva et al. 2016). BDNF is a small dimeric protein which is one of the neurotrophic factors that play a vital role in regulating not only the neuronal development, maintenance and survival, but also in the cognition, formation and storage of memory. In 1991, reduced expression of BDNF were first seen in hippocampus samples from AD donors suggesting that this decrease may contribute to the progressive cell death characteristic of AD (Phillips, Hains et al. 1991). Furthermore, BDNF was also found to promote the survival of all major types of neurons related to functional changes in AD, and has been suggested as an essential contributor of the etiology of neurodegenerative disorders (Schindowski, Belarbi et al. 2008). As stated earlier, BDNF is involved in neuronal survival and plasticity that binds to high-affinity receptors, tyrosine kinase B (TrkB) (tropomyosin receptor kinase B) (Givalois, Arancibia et al. 2004). Previous studies have also demonstrated that both BDNF and TrkB play a critical role in long-term synaptic plasticity in the adult brain (Schinder and Poo 2000, Dwivedi 2009). BDNF-TrkB interaction promotes the survival and differentiation of neurons and synaptic plasticity of the central nervous systems (Lu, Christian et al. 2008, Kim, Ko et al. 2010). Thus a decrease in BDNF and its receptor, TrkB may lead to synaptic and cellular loss and memory deficits characteristic of AD. In the present study, the induction of scopolamine-induced amnesia showed suppression of BDNF and TrkB expressions in the hippocampus. Similar results were also observed in the prefrontal cortex whereby scopolamine reduced mRNAs of BDNF and TrKB. OS extract was found to have increased both the BDNF and TrKB stained cells in the hippocampus and the prefrontal cortex region. In the hippocampus, all the OS extract doses were found to be effective and showed maximum protection by increasing the BDNF and TrKB levels.

On the other hand, CREB1 is a co-factor of CREB and is essential for memory and synaptic plasticity in the central nervous system whereby disruption of phosphorylated CREB within the hippocampal region triggers the progression of neurodegenerative diseases like Alzheimer's disease, Parkinson's disease and Huntington's disease (Lee, Kim et al. 2015). Previous studies have demonstrated that the activation of CREB ameliorated cognitive impairment via the cholinergic system (Kotani, Yamauchi et al. 2006, Lee, Kim et al. 2015). This was congruent with our results whereby the expression of the CREB1 gene was reduced by scopolamine and pretreatment with OS extract markedly increased the CREB1 mRNA levels. So, it can be suggested that OS extract could be a potent treatment for neurodegenerative diseases and its possible mechanism might be modulating the cholinergic activity via the CREB-BDNF pathway.

The hippocampus is a pivotal region of the brain that is critical for learning and memory function and is highly susceptible to neuronal injury produced by scopolamine-induced cholinergic activity dysregulation, which can in turn trigger impairment of synaptic plasticity and loss of spatial learning memory (Mattson, Chan et al. 2002, Heo, Shin et al. 2014). DCX is a marker of neuroblasts, neuronal precursor cells, and immature neurons. It is associated with structural plasticity in the adult mammalian brain, and has been used as a marker of newly formed neurons in the dentate gyrus of the adult hippocampus (Bonfanti 2006, Heo, Shin et al. 2014). DCX is involved in neuronal migration and development, and it is continuously expressed during adult neurogenesis thus enabling it to be used to measure neurogenesis (Knoth, Singec et al. 2010, Heo, Shin et al. 2014). Previous studies have reported decreased DCX expression during aging and thus decrease in neurogenesis (Brown, Couillard-Despres et al. 2003, Hwang, Yoo et al. 2008, Heo, Shin et al. 2014). In our study, similar results were observed whereby the number of DCX-positive cells in the hippocampal dentate gyrus

were decreased in scopolamine induced rats, whereas the OS treated rats were observed to have increased number of DCX-positive cells. However, further research is necessary to verify its mechanism. Based on the behavior results for EPM, improved inflexion index was observed for both 50 and 100 mg/kg which was equivalent to the positive group, Donepezil indicating that OS at these doses were able to completely reverse the scopolamine induced memory impairment. This was further reiterated by the increase in dendrites and neuron bodies observed. For the 200mg/kg dose, behavioral studies did show a slight improvement in inflexion index compared to the only scopolamine induced group but when compared to the positive group, Donepezil and the other 2 doses, 50 and 100mg/kg groups, the improvement was not that convincing. This result was further supported as there were dendrites and neuron bodies observed during the cell counting but not as much as the other 2 doses.

5 Conclusion

In conclusion, the present work demonstrated that OS extract was able to revert the scopolamine induced amnesia in the rats thus further distinguishing its anti-amnesic effects. Additionally, we also established that the positive effects of OS extract could be mediated via the BDNF-TrKB pathway, CREB-BDNF pathway and also the hippocampal neurogenesis. This suggests that the OS extract could be a promising candidate as a memory enhancer or as a therapeutic treatment for neurodegenerative diseases like Alzheimer's disease.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

TR performed all the experiments and was responsible for the writing of the manuscript in its entirety. YK helped in designing of gene expression and immunohistochemistry study. MS & IO were involved in conceptualizing, designing of the study, result analysis, and manuscript editing. All authors gave their final approval for the submission of the manuscript.

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10 Reference styles

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List of Figures

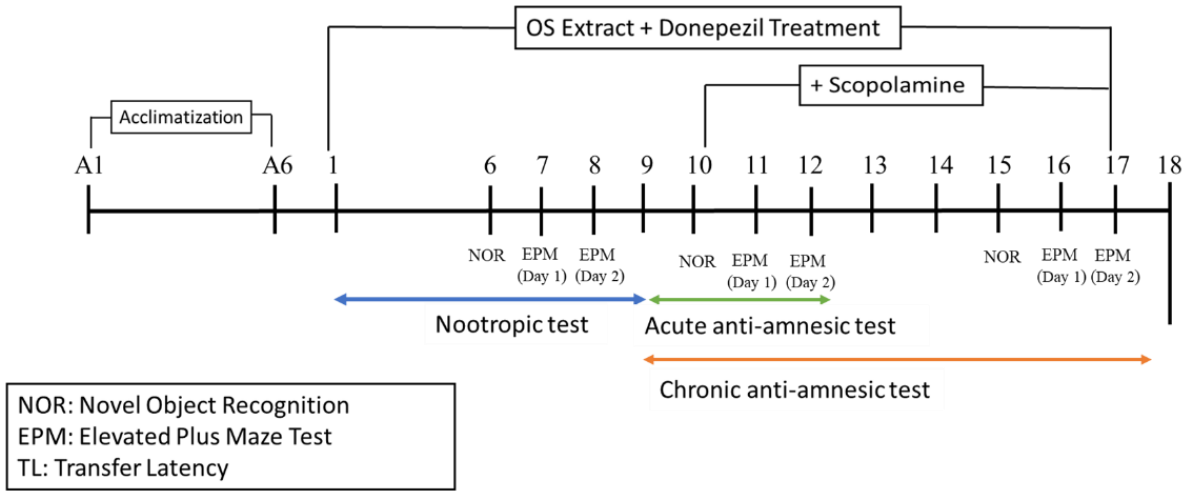


FIGURE 1. Schematic representation of the experimental procedure.

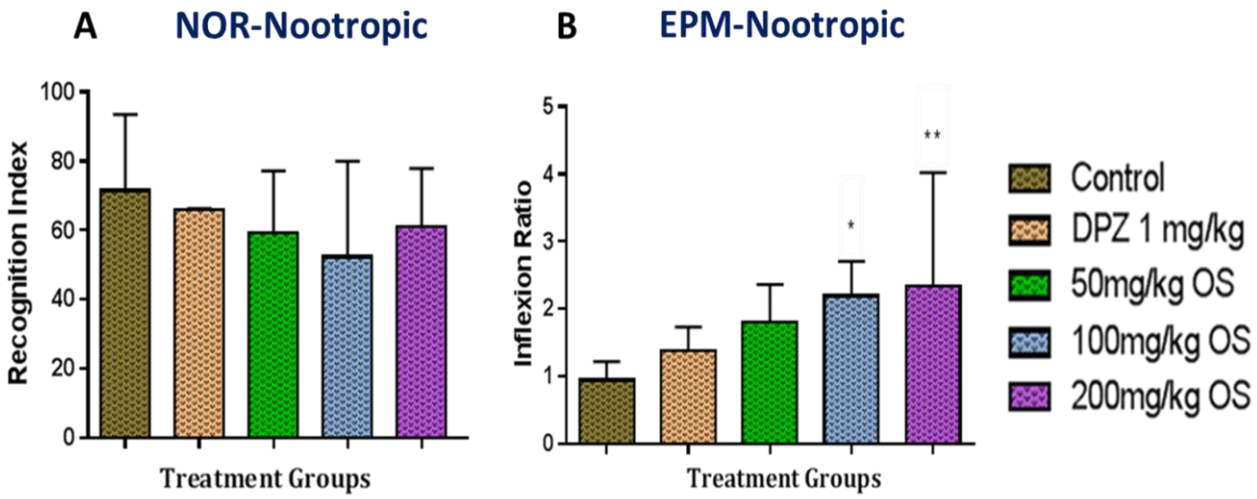
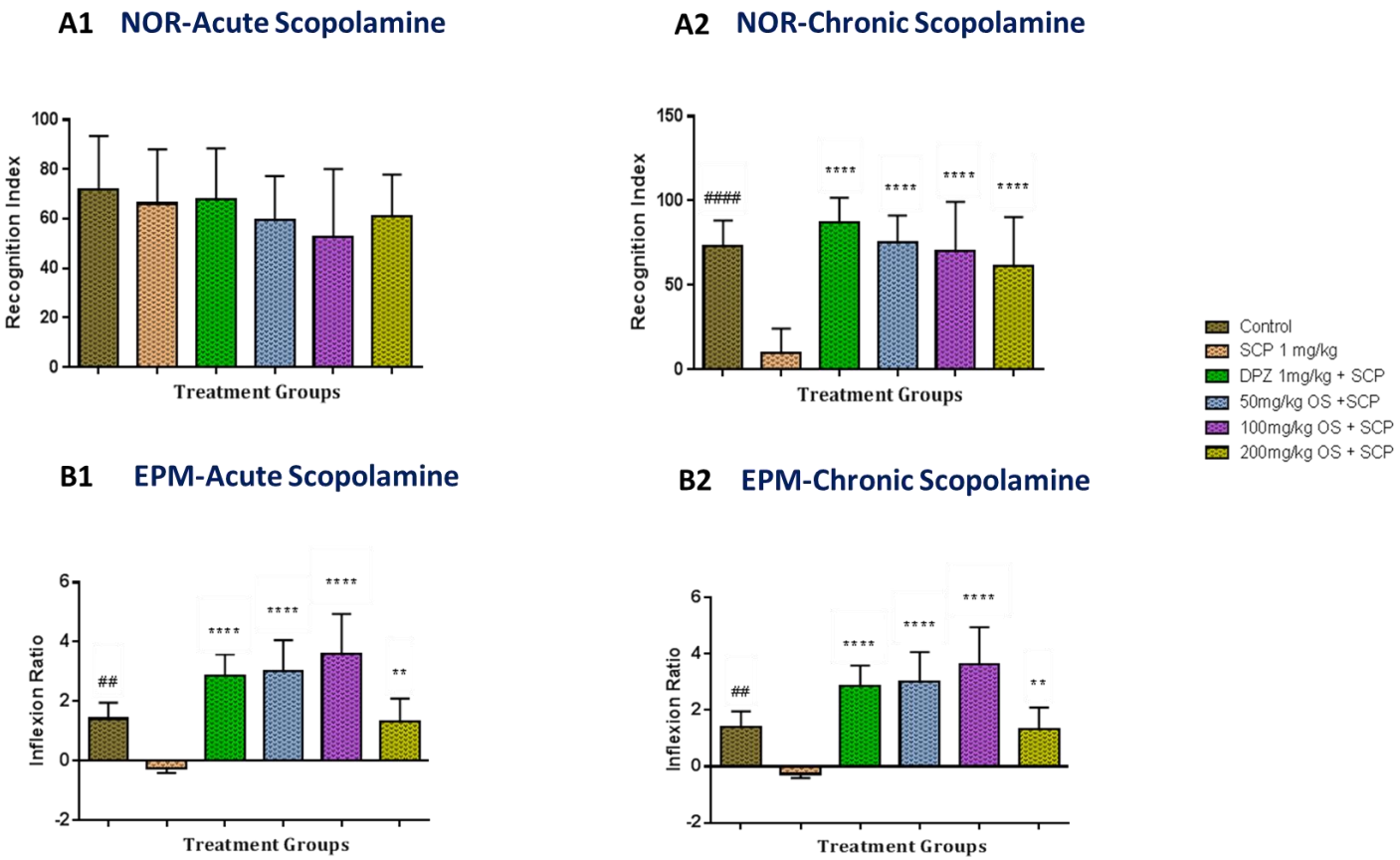


FIGURE 2. Behavioral analysis for novel object recognition (NOR) and elevated plus maze (EPM). **(A)** represent the graph plot for the recognition indices in NOR for the nootropic model **(B)** represents the graph plot for the inflection ratios in EPM for the nootropic model. The behavioral analysis for **(A,B)** were compared to control group. Data are expressed as Mean ± SEM, $n = 8$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.



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556 **FIGURE 3.** Behavioral analysis for NOR and EPM. **(A1)** & **(A2)** represents the graph plot for the
557 recognition indices in NOR for both the acute and chronic scopolamine model respectively **(B1)** &
558 **(B2)** represents the graph plot for inflection ratios in EPM for both the acute and chronic
559 scopolamine model. All the behavioral analysis were compared to the negative control (SCP
560 1mg/kg). Data are expressed as Mean ± SEM, $n = 9$ and statistical analysis by one-way ANOVA
561 followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

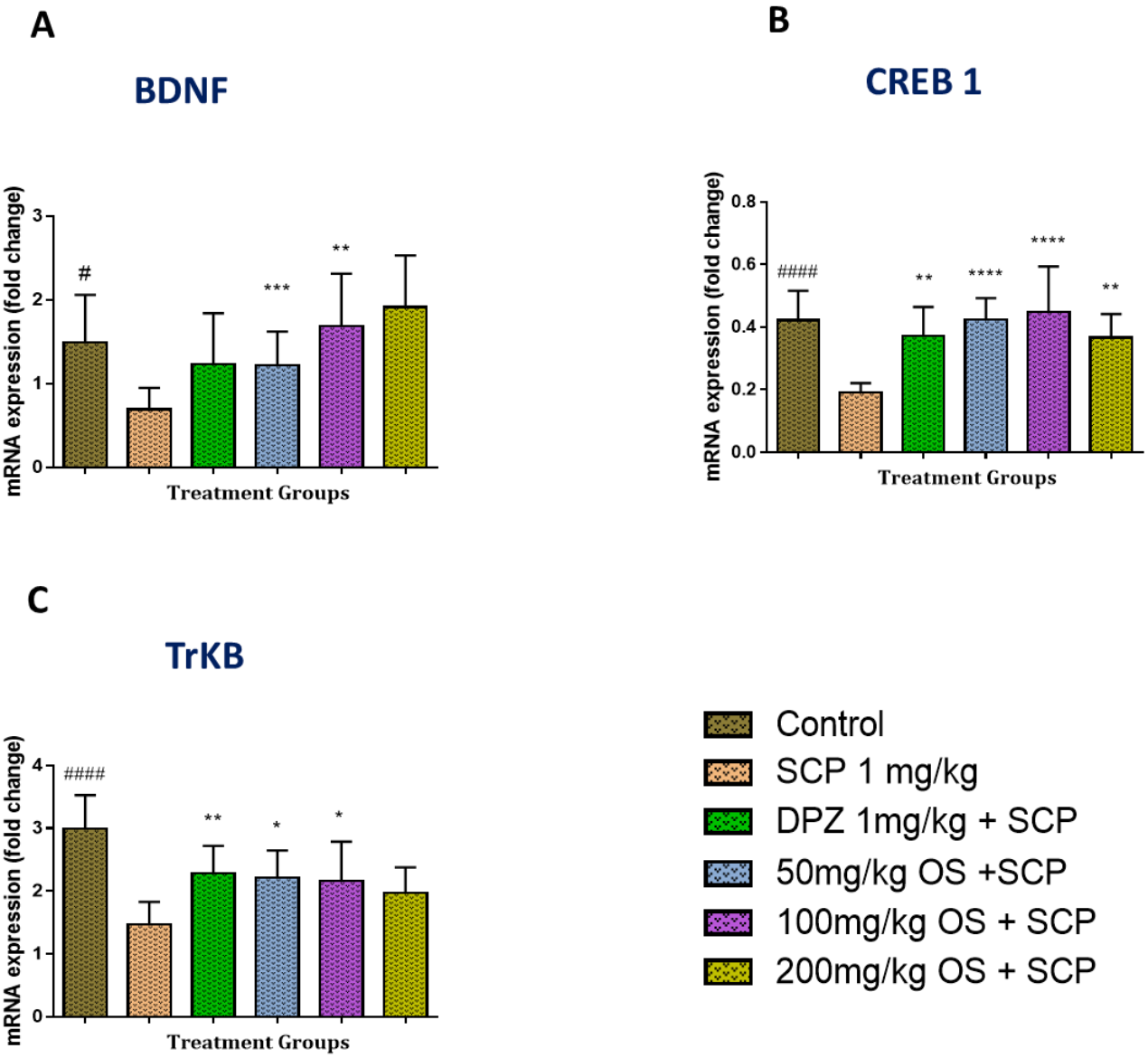
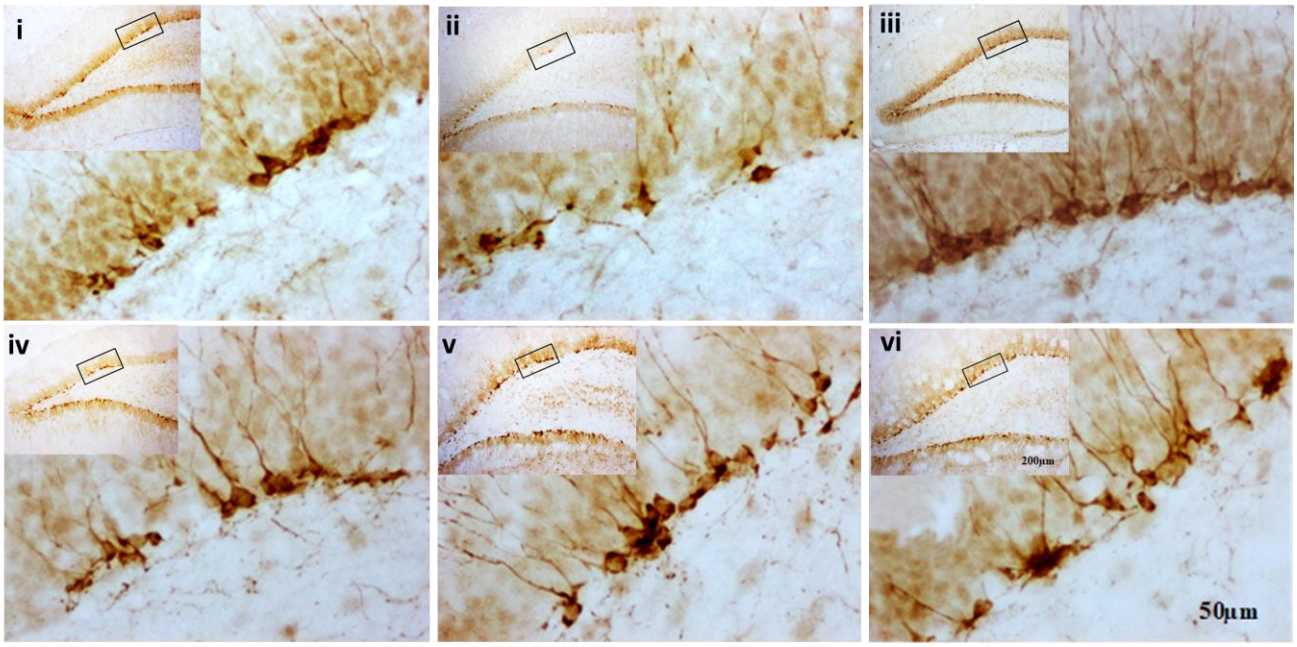


FIGURE 4. Gene expression in the rat hippocampi determined by real time-PCR. The genes included are **(A)** BDNF, **(B)** CREB1, and **(C)** TrKB. All changes in the expression levels were compared to the negative control group (SCP 1 mg/kg). Data are expressed as Mean ± SEM, $n = 4$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.



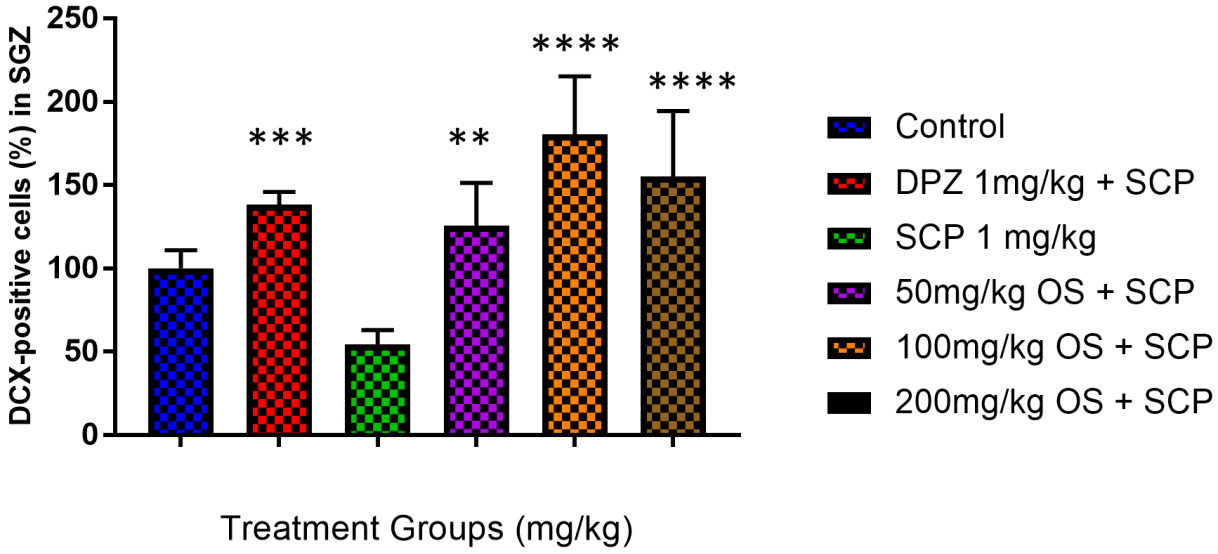


FIGURE 5. DCX immunohistochemical analysis of the effects of OS extract in improving scopolamine-induced suppression of neurogenesis in the dentate gyrus. **(A)** DCX-positive staining in immature neurons is shown in the subgranular zone of the dentate gyrus. Photomicrographs of the hippocampal section of treatment groups was (i) Control (ii) SCP 1 mg/kg alone (iii) DPZ 1 mg/kg + SCP 1 mg/kg (iv) 50mg/kg OS + SCP 1 mg/kg (v) 100mg/kg + SCP 1 mg/kg (vi) 200mg/kg + SCP 1 mg/kg. Representative photomicrographs were taken at magnifications of 40X and 200X. **(B)** Quantification of DCX population. Data are expressed as means Mean ± SEM, $n = 5$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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Chapter 5

BRIEF INTRODUCTION

In the following part of the study, the beta amyloid-tau hypothesis was explored. The two key neuropathological hallmarks of Alzheimer's disease (AD) are the extracellular β-amyloid (amyloid precursor protein (APP) deposits and intracellular neurofibrillary tangles (NFT) (Hardy and Selkoe 2002). The mis-metabolism of APP and the inability for β amyloid to clear trigger a cascade of events that includes the hyperphosphorylated tau mediated breakdown of microtubular that causes synaptic failure leading to AD (Mohandas, Rajmohan et al. 2009). According to the amyloid hypothesis, the extracellular amyloid plaques that is produced due to the aggregates of Aβ peptide generated by the proteolytic cleavages of APP are extremely vital to the pathology of AD (Hardy and Selkoe 2002, Mohandas, Rajmohan et al. 2009). On the other hand, the tau hypothesis advocates that excessive or abnormal phosphorylation of tau causes the transformation of normal adult tau into PHF-tau (paired helical filament) and NFTs (Mohandas, Rajmohan et al. 2009) which than leads to the development of AD.

In recent times, the need for anti-Alzheimer's drug that are both able to decrease beta amyloid levels as well as production of neurofibrillary tangles are gaining more attention which led to the quest seeking for drug treatments that are both versatile to address both these hypotheses. This present study therefore focussed on ascertaining if *O. stamineus* could exert positive effects in the streptozotocin-induced Alzheimer's model which explores both the beta amyloid as well as the tau hypothesis.

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O. stamineus Extract Reverses Alzheimer's Disease-Like Condition in Streptozotocin Model

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Keywords: Alzheimer's disease, cognitive function, streptozotocin, orthosiphon stamineus, oxidative stress

Abstract

AD is largely characterized histopathologically via the presence of intraneuronal neurofibrillary tangles (NFTs) and extracellular senile plaques together with neurodegeneration in the brain. There is a growing interest in the potential of phytochemicals to improve memory, learning and general cognitive abilities. *Orthosiphon stamineus* (OS) has been shown to have various pharmacological properties. In vitro studies have demonstrated neuroprotective as well as cholinesterase inhibitory effects of this compound. Streptozotocin (STZ), a glucosamine-nitrosourea compound, produces a cytotoxic agent that specifically damages the β cells in pancreatic islet and upon being metabolized tend to create a diabetes mellitus condition. Previous studies have reported that intracerebroventricular administration of streptozotocin (STZ) causes cholinergic dysfunction, hyperphosphorylation of tau protein, insulin receptor dysfunction, oxidative stress, mitochondrial dysfunction, impaired energy metabolism and activation of proapoptotic signaling pathways which are similar to those observed in AD. This study, therefore aimed at determining whether this Malaysian plant derived flavonoid can reverse STZ- induced learning and memory dysfunction. In the present study, STZ was injected ICV bilaterally at a dose of 3mg/kg. One week after the ICV-STZ injection, the rats were treated with OS extract through oral dosing for 10 days before being subjected to a series of behavioral studies. Rats were subjected to behavioural analysis to assess learning and memory functions and hippocampal as well as the prefrontal cortical tissues were extracted for gene expression analysis. All the three doses demonstrated improved STZ-induced impairment by showing increased step-through latency as well as higher inflexion ratio when compared to the negative control group. OS extract was observed to have increased the mRNA expression of APP, MAPT, NFκB, GSK3α and GSK3β genes. These research findings suggest that the OS ethanolic extract demonstrated an improving effect on memory and hence could serve as a potential therapeutic target for the treatment of neurodegenerative diseases like AD.

1 Introduction

Alzheimer disease (AD) is an age-related brain disease and one of the most common types of dementia. AD is characterized by chronic and progressive neurodegeneration that triggers advanced cognitive impairment, thus ultimately leading to death (Zlokovic 2002, Zhao, Gu et al. 2014). The pervasiveness of this disease is expected to quadruple from 26.6 million cases (1

in 253 people) to 1 in 85 people living with the disease by 2050 (Brookmeyer, Johnson et al. 2007, 2017). Those diagnosed with this disease are unable to encode new memories which in turn damages both declarative and non-declarative memory thus gradually reducing the ability for reasoning, abstraction and language (Pluta, Ulamek et al. 2009). Elderly people are most predispose to developing AD and the risk increases with age. There are namely two forms of the disease, sporadic (SAD) and familial (FAD). It has been predicted that the pervasiveness of SAD is projected to be increased to 131.5 million in 2050 which in turn could lead to serious socioeconomic burden (Arora and Deshmukh 2017).

AD is largely characterized by the presence of intraneuronal neurofibrillary tangles (NFTs) and extracellular senile plaques together with neurodegeneration in the brain (Zhao, Gu et al. 2014, Ghumatkar, Patil et al. 2015). The key aetiological component of AD includes a causative protein, amyloid β protein whereby the aggregation and deposition of the amyloid β protein activate an inflammatory immune reaction that in turn obliterates the brain neurons (Harman 2006). The synthesis of amyloid is regulated by the secretase enzyme. Thus, it can be further said that the damage to the amyloid β peptide cerebral clearance causes abnormal increase in its brain level during late onset of AD, which in turn accounts for most of the AD cases (Hardy and Selkoe 2002). Another key hallmark of AD is the decrease in the production of neurotransmitter acetylcholine which is vital in controlling various memory related functions (Muir 1997). AD predominantly affects cholinergic neurons in the cerebral cortex of the brain whereby the neuronal activity are largely controlled by the neurotransmitter "acetylcholine" (Contestabile 2011, Craig, Hong et al. 2011). In AD, memory decline occurs because the enzyme that produces acetylcholine becomes defective resulting in shortage of this neurotransmitter at the neuronal synapse (Craig, Hong et al. 2011). Additionally, recent studies have shown that neuronal degeneration linked with AD has been triggered largely by neuroinflammation, oxidative stress, neurotransmitter imbalance and neurotoxicity ((Salkovic-Petrisic, Osmanovic-Barilar et al. 2011, Salkovic-Petrisic, Knezovic et al. 2013, Arora and Deshmukh 2017).

Streptozotocin (STZ) is a glucosamine-nitrosourea compound which produces a cytotoxic agent that particularly affects the β cells in pancreatic islet, impairing the brain biochemistry, cholinergic transmission as well as increasing the generation of free radicals (Lannert and Hoyer 1998, Sharma and Gupta 2001, Singh, Kakalij et al. 2015). Intracereberoventricular administration of STZ has been shown to resemble the similar neuropathology and biochemical alterations observed during an AD condition thus resulting in STZ-induced models playing a vital role in the pathophysiology of sporadic Alzheimer's.

In recent times, there has been a growing interest in using complimentary therapy and phytochemicals from medicinal herbs to enhance the quality of life and prevent therapy induced side-effects, particularly in using phytochemicals from medicinal herbs as they possess anti-inflammatory and antioxidant activities that may potentially hinder neurodegeneration and improve memory and cognitive functioning (Essa, Vijayan et al. 2012). *Orthosiphon stamineus* Benth. (Lamiaceae), is a medicinal herb that is extensively distributed in South East Asia. Various in vitro and in vivo models have addressed the presence of different types of phytochemicals in this plant like flavonoids, terpenoids, and essential oils. Earlier studies have demonstrated that *O. stamineus* (OS) leaves extracts possess strong antioxidant, anti-inflammatory and anti-bacterial properties with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid and nitric oxide inhibitory isopimarane-diterpenes

(Sumaryono, Proksch et al. 1991, Awale, Tezuka et al. 2003, Awale, Tezuka et al. 2003, Yam, Lim et al. 2010). For instance, Rosmarinic acid, a major flavonoid component of O. stamineus has been shown to have various pharmacological properties. Flavonoids which are the principal group of polyphenols are also reported to be efficacious in decreasing oxidative stress and are said to promote various physiological benefits, particularly in learning and memory, scavenging free radicals and improving cognition (Bhullar and Rupasinghe 2013, Ghumatkar, Patil et al. 2015). Besides that, standardized ethanolic extract of O. stamineus were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration (George, Chinnappan et al. 2015). In addition, in vitro studies have demonstrated that *O. stamineus* enhanced H2O2 induced oxidative stress by antioxidant mechanisms in SH-SY5Y human neuroblastoma cells (N. Vijaya Sree * 2015).

Therefore, since the OS extract has been observed to demonstrate strong antioxidant and anti-inflammatory properties, these reports on the OS extract further support its neuroprotective potential in combating neurodegenerative diseases like AD. Based on the activity profile of OS extract, it can be hypothesized that OS could serve to halt or modify intricate neurodegenerative diseases like AD. Thus, the aim for this study was to investigate the protective potential of OS against STZ-induced AD like condition, using two established behavioral paradigms for learning and memory as well as to observe the hippocampal alterations associated. Since preliminary studies have demonstrated neuroprotective as well as cholinesterase inhibitory effect, hence it is hypothesized that *O. stamineus* can be establish as an effective and safer potential therapeutic agent to combat cognitive alterations in AD.

2 Materials & Method

Animals

Locally bred adult male Sprague Dawley (SD) rats weighing between 200 – 300g, were acquired from the animal facility of Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia. The rats were maintained under standard husbandry conditions (12:12 h light/dark cycle, at controlled room temperature (22 ± 2°C), stress free, water ad libitum, standard diet and sanitary conditions). The experiment protocols were approved and conducted according to the approval of the Animal Ethics Committee Monash University Animal Research Platform (MARP/2016/028).

Intracerebroventricular (ICV) Infusion of Streptozotocin

Streptozotocin (STZ) was injected ICV bilaterally at a dose of 3mg/kg as described previously (Sharma and Gupta 2001). Briefly, the rats were firstly anaesthetized using a combination of ketamine hydrochloride (75mg/kg, i.p.) and xylazine (10mg/kg, i.p.). The head was positioned and fixed on the stereotaxic frame. A midline sagittal incision was done on the scalp and a burr hole were drilled through the skull on both sides over the lateral ventricles. The coordinates employed was: 0.8 mm posterior to bregma, 1.5 mm lateral to sagittal suture, 3.6 mm beneath the surface of the brain (Paxinos and Watson 1982). An injection cannula was lowered very slowly into the lateral ventricles to deliver STZ (3mg/kg, 10µL/injection site) or saline (10µL/injection site) through the skull holes. STZ was prepared freshly before each injection. The injection cannula was connected to a Hamilton syringe and the injection was done using a micro-injector unit. The cannula was left *in situ* for a further 5 minutes following the injection to allow passive diffusion from the cannula tip and to minimize spread into the injection tract.

The cannula was then removed slowly from the scalp and the cut skin was closed with sutures. Following the surgery, postoperative care was made by applying the betadine povidone-iodine solution on the wound. The rats were also placed on thermal sheets to maintain body temperature and were kept under close observation for the next 4 days.

Experimental Design

Prior to the experiment, animals were acclimatized to the surrounding and were handled for a period of 1 week to reduce the stress. The animals were than randomly divided into 5 groups (n=8/group). The ethanolic extract of OS was freshly prepared in distilled water.

Group 1: Sham-control (Saline)

Group 2: Negative control (Saline+STZ; 3mg/kg)

Group 3: STZ (3mg/kg) + *Orthosiphon stamineus* Low dose (50 mg/kg OS)

Group 4: STZ (3mg/kg) + *Orthosiphon stamineus* Medium dose (100 mg/kg OS)

Group 5: STZ (3mg/kg) + *Orthosiphon stamineus* High dose (200 mg/kg OS)

After 7 days of acclimatization period, the animals were subjected to ICV injection where Group 1 rats were sham operated where only the surgery was done, and the brain was injected with saline. Groups 2-4 received STZ (3mg/kg, single injection bilateraaly). One week after the ICV-STZ injection, the rats were treated with OS extract through oral dosing for 10 days before being subjected to a series of behavioral studies.The behavioral parameters were conducted on day 18, where elevated plus maze test was conducted on day (18 & 19) and passive avoidance test was conducted on day (20 & 21). At the end of the study, the animals were sacrificed, and their brains were isolated for gene expression analysis. The treatment schedule is presented in **Figure 1.**

Elevated Plus Maze (EPM)

The elevated plus maze test was employed to evaluate acquisition and retention memory following the procedure described by (Sharma and Gupta 2001). Briefly, after being treated with the OS extract, the rats were put on to the end of the open arm, facing away from the central platform. With the help of the stopwatch, the transfer latency (TL_1) was noted i.e. the time taken by rat with all its four legs to move into any one of the enclosed arms. If the rat failed to enter any one of the enclosed arms within 90 s, it was gently pushed into one of the two enclosed arms and the TL was assigned as 90 s. The rat was allowed to explore the maze for the next 10 s and then returned to its home cage. The maze was cleaned with 70% ethanol between runs to minimize scent trails. The retention test phase was carried out 24 hours after the training session to assess memory, whereby a decrease in time latency (TL_2) during the test session was deemed as an index of memory improvement. The transfer latency was expressed as inflexion ratio, calculated using the formula: -

$$\mathbf{IR} = \frac{(L1-L0)}{L0}$$

L_0: Initial TL (s) on the 1st day and

L_1: TL (s) on the 2nd day.

Passive Avoidance (PA)

A step through passive avoidance (PA) test was carried out to measure memory retention deficit follwoing the method of (Nakahara, Iga et al. 1988, Elcioglu, Aslan et al. 2016). Briefly, during the acquisition trial each rat was placed in the light chamber. Following a 60s of habituation, the guillotine door separating the light and dark chamber was opened and the initial latency time for the rat to enter the dark chamber was recorded. The rats with the initial latency time of more than 60 s were excluded from the study. Once the rat entered the dark chamber, the guillotine door was closed and an electric foot shock (75V, 0.2mA, 50Hz) was delivered to the floor grids for 3 s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. After twenty-four hours, the retention latency was measured in the same way as the acquisition trial, but the foot shock was not delivered, and the latency time was recorded to a maximum of 300 s.

Gene Expression

Total RNA from the rat brain's hippocampal and pre-frontal cortical region was extracted following the method employed by (Bhuvanendran, Kumari et al. 2018), with some minor modifications. The single-step method, phenol-chloroform extraction and Trizol reagent (Invitrogen) was used to isolate the total RNA from both the pre-frontal cortical and hippocampal region. Briefly, the tissues were homogenized in 200µL of Trizol solution. The mixture was then extracted using chloroform and centrifuged at 135, 000 rpm at 4°C. The alcohol was removed, and the pellet was washed twice with 70% ethanol and resuspended in 20µL of RNase free water. RNA concentration was determined by reading absorbance at 260 nm using Nanodrop. A 500ng amount of total RNA was reverse transcribed to synthesize cDNA using Quantitect® Reverse Transcription Kit according to the manufacturer's protocol. Then the mRNA expression of genes encoding amyloid precursor protein (APP), Microtubule Associated Protein Tau (MAPT), Nuclear factor kappa-light-chain-enhancer of activated B (NFκB), Glycogen synthase kinase-3 alpha (GSK3α), Glycogen synthase kinase-3 beta (GSK3β) and IMPDH2 in the hippocampus was measured via real-time PCR using the StepOne Real-Time PCR system. Subsequently, the cDNA from the reverse transcription reaction was subjected to Real-Time PCR using QuantiNova™ SYBR® Green PCR kit according to manufacturer's protocol. The comparative threshold (C_T) cycle method was used to normalize the content of the cDNA samples, which consists of the normalization of the number of target gene copies versus the endogenous reference gene, IMPDH2.

Statistical Analysis

All findings were expressed as mean ± standard error of the mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's tests. All the experimental groups were compared with the SCP 1 mg/kg group and the *P*-values of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered to be statistically significant.

Results

Effect of OS Extract on memory performance in EPM and PA task in ICV-STZ infused Rats

Both the EPM and PA task was carried out to assess spatial long-term memory retention. As observed in Figure 2 (A), the negative (only STZ-induced) group demonstrated a notable decrease in inflexion ratio whereas the OS treated groups demonstrated a significant increase in inflexion ratio when compared to the negative group. In the PA task, memory performance was assessed by determining the latencies to enter the dark (shock-paired) compartment during the post 24-hour retention trial. All the groups did not demonstrate any differences in latency during the learning trial (data not shown), signifying that all rats showed similar responses to the testing environment and electric shocks. On the other hand, the retention test which was performed 24 hours following the initial training demonstrated a significant decrease in step-through latency in the negative (only STZ-induced) group as compared to the sham operated and the other treated groups as depicted in Figure 2 (B). However, when the STZ-induced rats were treated with all the 3 doses (50, 100 and 200mg/kg) of OS extract, a significant increase in step-through latency was observed indicating improved memory retention. Based on these results obtained, it can be said that OS extract does improve memory retention.

Effect of OS extract on the gene expression in the rat hippocampal and prefrontal cortical region

In the hippocampal region, the APP mRNA levels were significantly up regulated when administered with STZ as compared to the control group, depicted in Figure 3A. Similarly, even the MAPT, NFκB, GSK3α and GSK3β were observed to be up regulated when administered with STZ as demonstrated in Figure 3B, C, D and E respectively. This up regulation was decreased significantly by OS extract treatment as compared with the negative (STZ 3mg/kg) group. All the five mRNA expression levels, namely APP, MAPT, NFκB, GSK3α and GSK3β were observed to be significantly lower when treated with OS extract. The expression of APP mRNA was observed to be down regulated in all the three doses of OS extract and similarly, even the expression levels of MAPT, NFκB, GSK3α and GSK3β mRNA were observed to be decreased in all the three doses of OS extract. On the other hand, in the pre-frontal cortical region, similar results were also observed whereby, the APP mRNA levels were observed to be significantly augmented when administered with STZ as compared to the control group, as shown in Figure 4A. Likewise, even the MAPT, NFκB, GSK3α and GSK3β were observed to be increased when administered with STZ as demonstrated in Figure 4B, C, D and E respectively. This up regulation was decreased significantly by OS extract treatment whereby all the five mRNA expression levels, namely APP, MAPT, NFκB, GSK3α and GSK3β were observed to be significantly lowered when treated with OS extract.

3 Discussion

Alzheimer's disease (AD) serves as one of the most common neurodegenerative disease and has been said to induce progressive loss in the ability of learning as well as memory function (Moosavi, Khales et al. 2012). Streptozotocin (STZ), a glucosamine-nitrosourea compound, produces a cytotoxic agent that specifically damages the β cells in pancreatic islet and upon being metabolized tend to create a diabetes mellitus condition (Sharma and Gupta 2001, Rani, Deshmukh et al. 2016). Previous studies have reported that intracerebroventricular administration of streptozotocin (STZ) causes impaired energy metabolism, cholinergic dysfunction, oxidative stress, hyperphosphorylation of tau protein, mitochondrial dysfunction, insulin receptor dysfunction and activation of proapoptotic

signaling pathways which are also observable in AD (Lannert and Hoyer 1998, Park 2011, Salkovic-Petrisic, Knezovic et al. 2013). In this study, we illustrated the neuroprotective role of OS extract against streptozotocin-induced neurotoxicity in rats. The medicinal value of OS extract has been well recognized, particularly in regard to its anti-oxidant and anti-inflammatory activities (Arafat, Tham et al. 2008). In particular, OS extract contains many phytochemicals that contribute to its overall neuroprotective effect where for instance, rosmarinic acid which is one of the main flavonoid component of OS extract has demonstrated various pharmacological properties that may potentially hinder neurodegeneration and improve memory and cognitive functioning (Essa, Vijayan et al. 2012).

ICV injection of STZ has been established to be characterized by a progressive decline in learning and memory. In this present study, a dose of 3mg/kg STZ was employed as it has been shown to not interfere with the changes in the peripheral blood glucose level but induce a significant cognitive impairment in all animals (Sharma and Gupta 2001, Balouchnejadmojarad 2009). Additionally, central administration of low STZ doses triggers an insulin-resistance brain state that produces a similar neuropathology and biochemical alterations observed during AD enabling the pathophysiology of sporadic Alzheimer's disease (sAD) to be further comprehended. Thus the expression of increased phosphorylated tau protein in the hippocampus as well as the accumulation of β amyloid in the meningeal capillaries suggest that the ICV-STZ model recapitulates most of the sAD pathological feature and hence can serves as an apt experimental model of developing the AD hallmarks (Grunblatt, Salkovic-Petrisic et al. 2007, Salkovic-Petrisic, Knezovic et al. 2013).

The performance of animals during the behavioral assessments for spatial memory acquisition and retention using elevated plus maze and passive avoidance test are well documented to estimate the extend of neuronal injury. The present study demonstrated that that treatment with OS extract improved memory retention as evident by the improved inflexion ratio observed in the EPM test as well as the increase in the step-through latency observed in the OS treated rats. Previous studies demonstrated significant cognitive impairment in the ICV-STZ treated group (Sharma and Gupta 2001, Veerendra Kumar and Gupta 2003, Tota, Kamat et al. 2009, Agrawal, Mishra et al. 2010, Mehla, Pahuja et al. 2013). In our study, similar results were observed whereby bilateral ICV administration of STZ resulted in spatial memory deficit, as observed by the decrease in step through latency in the passive avoidance test and decrease in inflexion ratio observed in the elevated plus maze test indicating memory impairment. However, when the STZ-injected rats were treated with OS extract, improved performances in both tests were observed. The positive effects of OS extract were evident with the rats that were treated with 50mg/kg and 100mg/kg OS extract namely where a decrease in transfer latency was observed in which the rats were able to remember and enter the closed arm quickly compared to the training session, which was observable by the improved inflexion ratio. The rats that were treated with 200mg/kg did show improved memory retention as compared to the STZ treated group but not as that observed in the 50mg/kg and 100mg/kg OS treated group. A celling effect was observed with higher doses. Therefore, based on the behavioral analyses, we can conclude that the spatial memory was improved. Thus, OS extract might influence spatial memory retention, which needs to be further explored.

The underlying mechanism for the improvement in memory retention observed in the behavioral studies were further explored by evaluating the biochemical parameters like expression of amyloid precursor protein (APP), microtubule associated protein tau (MAPT), nuclear factor kappa-light-chain-enhancer of activated B (NFκB), glycogen synthase kinase-3 alpha (GSK3-α) and glycogen synthase kinase 3 beta (GSK3-β) genes in rats treated with OS extract and STZ. Beta amyloid (Aβ) and tau are some of the key aspects of AD and are undeniably crucial in comprehending the

pathogenesis of AD. The AD amyloid cascade hypothesis postulates that, up regulation of Aβ triggers the pathogenic hyper-phosphorylation of tau which in turn leads to the formation of neurofibrillary tangles (NFTs) thus causing neurodegeneration. Furthermore, dysregulation of GSK3 has been implicated in numerous neurodegenerative diseases, including AD (Martinez and Perez 2008, Kremer, Louis et al. 2011, DaRocha-Souto, Coma et al. 2012). Additionally, GSK3 also plays a key role in AD as its deregulation accounts for most of the pathological hallmarks of the disease observed in both the sporadic and familial AD. Both GSK3β and GSK3α stimulates tau hyper-phosphorylation at both primed and non-primed phosphorylation sites, in both cell culture models as well as *in vitro* models of neurodegeneration, further implicating GSK3 as a vital factor in AD (Cho and Johnson 2003, Asuni, Hooper et al. 2006). GSK3β has been said to have an effect on the abnormal tau hyperphosphorylation, a key component of neurofibrillary tangles observed in AD brain, which enhances tau aggregation and neurotoxicity (Lucas, Hernandez et al. 2001, Engel, Goni-Oliver et al. 2006). On the other hand, GSK3α, has been shown to monitor APP cleavage resulting in the augmented Aβ production (Sun, Sato et al. 2002, Phiel, Wilson et al. 2003). Although increased expression of GSK3 is not the main cause of the disease, augmented GSK3 could serve to enhance production of Aβ that in turn also trigger tau hyper-phosphorylation and neuronal degeneration in both FAD and sAD which is in line with the amyloid cascade hypothesis of AD. The NF-κB transcription factor is one of the proteins that detect and react to oxidative stress (Songin, Jesko et al. 2007). NF-κB regulates transcription of pro- and antiapoptotic proteins as well as antioxidant enzymes. It is also a key player in the regulation of inflammation, a process that can indirectly change the fate of neural cells (Songin, Jesko et al. 2007). In the present study, the induction of STZ demonstrated overexpression of all the key genes namely APP, MAPT, GSK3-α and GSK3-β in both the hippocampus and the prefrontal cortex region. However, when treated with OS extract, the expressions of all these genes were observed to be suppressed indicating maximum protection and hence reducing AD pathology. Thus, it can be inferred that OS extract is able to restore the immune response, produce anti-inflammatory effects, reduce neurotoxicity and improve learning and memory.

4 Conclusion

In summary, the present study demonstrated that OS extract is effective in ameliorating ICV streptozotocin induced behavior alterations. Additionally, we also established that the GSK3α-GSK3β pathway could serve as the potential target for beta amyloid and tau accumulation characteristically observed in AD condition and OS extract could potentially inactivate this pathway and hence serve as a promising therapeutic treatment for neurodegenerative diseases like Alzheimer's disease.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

TR performed all the experiments and was responsible for the writing of the manuscript in its entirety. YK helped in designing of gene expression study and result analysis. MS & IO were involved in conceptualizing, designing of the study, result analysis, and manuscript editing. All authors gave their final approval for the submission of the manuscript.

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9 Reference styles

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Figures

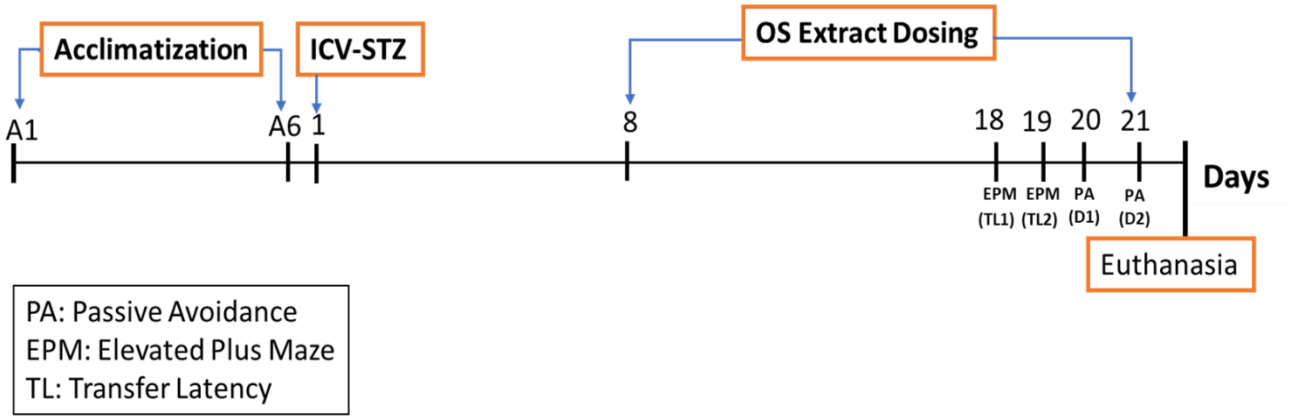

FIGURE 1. Schematic representation of the experimental procedure

FIGURE 2. Behavioral analysis for elevated plus maze (EPM) and passive avoidance (PA). (A) represent the graph plot for the inflection ratio (B) represents the graph plot for the step-through latency in PA. The behavioral analysis for (A,B) were compared to the control group. Data are expressed as Mean ± SEM, *n* = 8 and statistical analysis by one-way ANOVA followed by Dunnett test **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

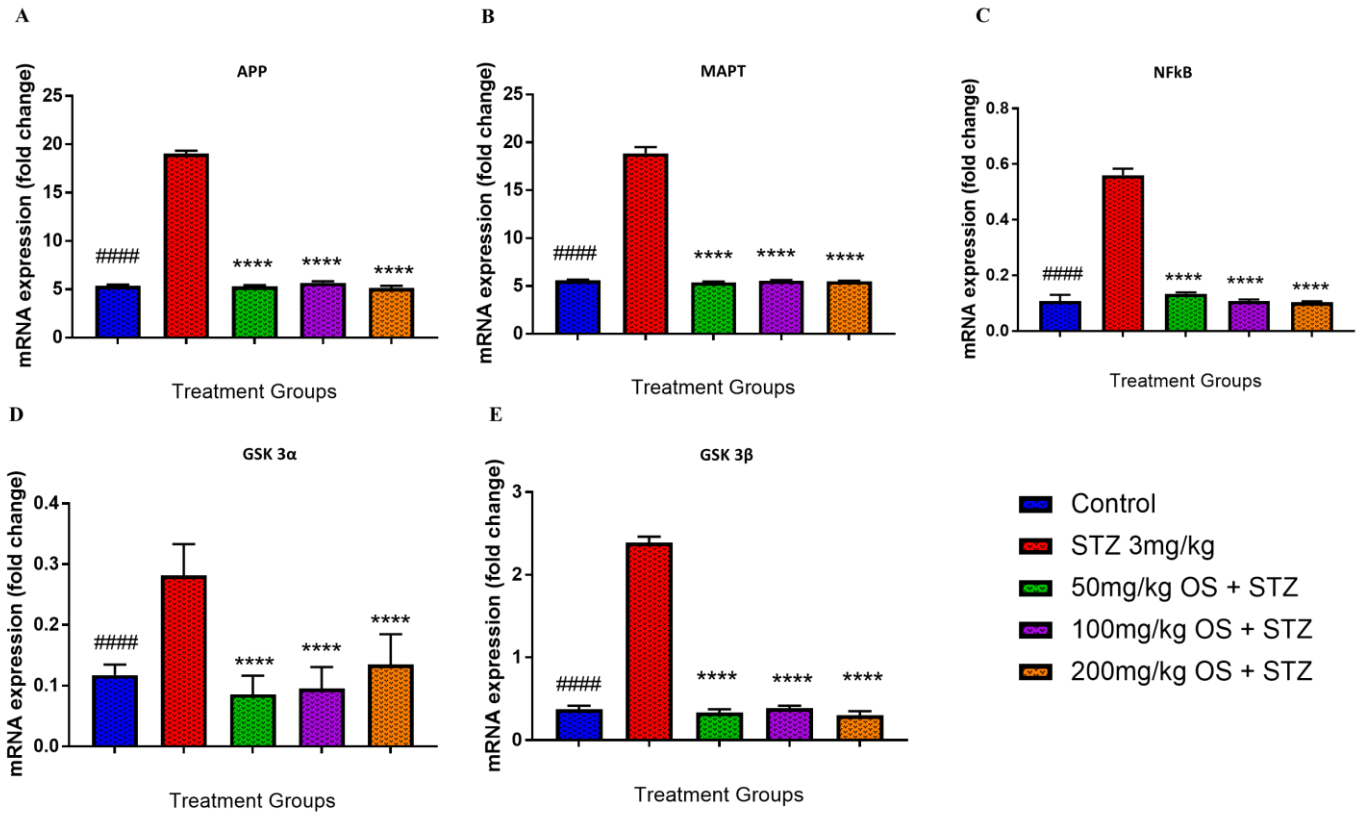

FIGURE 3. Gene expression in the rat hippocampi determined by real time-PCR. The genes included are **(A)** APP, **(B)** MAPT, **(C)** NFkB, **(C)** GSK 3α and **(D)** GSK 3β. All changes in the expressions levels were compared to the negative control group (STZ 3 mg/kg). Data are expressed as Mean ± SEM, $n = 4$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

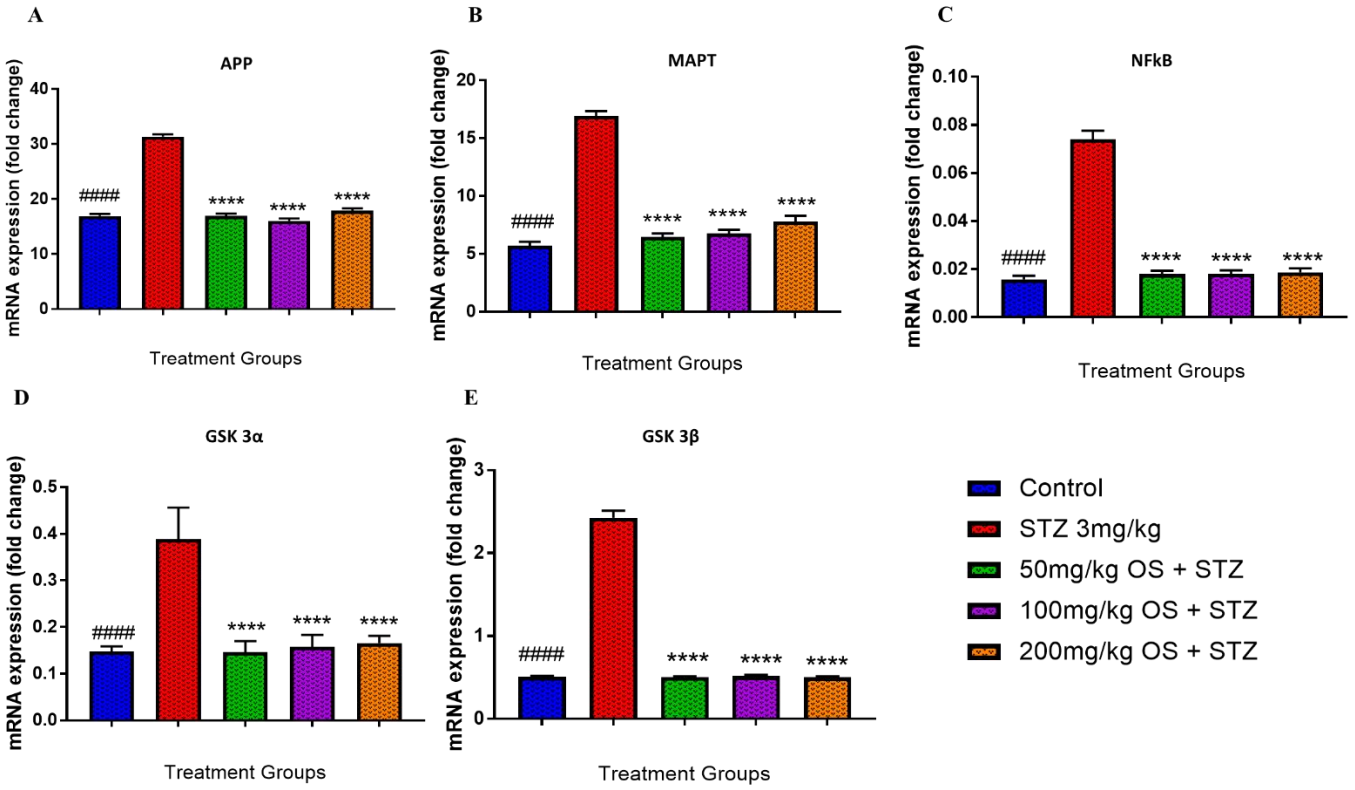

FIGURE 4. Gene expression in the rat pre-frontal cortical determined by real time-PCR. The genes included are (A) APP, (B) MAPT, (C) NFkB, (C) GSK 3α and (D) GSK 3β. All changes in the expressions levels were compared to the negative control group (STZ 3 mg/kg). Data are expressed as Mean ± SEM, *n* = 4 and statistical analysis by one-way ANOVA followed by Dunnett test **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Chapter 6

BRIEF INTRODUCTION

In this study, the relationship between long term reduction of blood flow and the development of Alzheimer's was explored. One of the important parameters in vascular hypothesis is the reduction in cerebral blood flow. Chronic cerebral hypoperfusion has been linked with cognitive dysfunction in Alzheimer's disease. A persistent decrease in cerebral blood flow can compromise memory processes and contribute to the development and progression of dementia (Farkas, Luiten et al. 2007, Zhao, Zhang et al. 2013). The association of reduced cerebral blood flow, particularly in the temporal and parietal cortices, with Alzheimer's disease (AD) has been firmly established (Farkas, Luiten et al. 2007, Zhao, Zhang et al. 2013). The occlusion of the bilateral common carotid artery is an established model for investigating chronic cerebral hypoperfusion-related neurodegenerative diseases. Chronic cerebral hypoperfusion promote neurodegeneration by triggering the production of reactive oxygen species, and pro-inflammatory cytokines via activated microglial cells that in turn damage the neurons and contribute to white matter lesions. This promotes Alzheimer-like brain pathology and the development of Alzheimer's disease (Farkas, Luiten et al. 2007, Adibhatla and Hatcher 2008, Bang, Jeon et al. 2013).

In summary, the present study focussed on distinguishing if *O. stamineus* possesses potent neuroprotective activity against brain damage induced by a chronic hypoperfused condition. The ability of *O. stamineus* to alleviate learning and memory deficits infer that it is a capable of intersecting multifactorial mechanisms to alleviate brain damage.

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Standardized ethanolic extract of *O. stamineus* ameliorates cognitive dysfunction in chronic cerebral hypoperfused rats

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder and is the leading cause of dementia in the elderly. Studies have demonstrated that medicinal plants have the potential to improve memory in animal models of AD. *Orthosiphon stamineus* is a Malaysian plant belongs to the *Lamiaceae* family and is rich in rosmarinic acid. *O. stamineus* has wide traditional and pharmacological uses in various pathological conditions. Leaf extract has been shown to have potent neuroprotective, anti-oxidant and anti-inflammatory activities. These properties may help to revert or prevent the pathological cascade that occurs during AD. Chronic cerebral hypoperfusion has been proved to be a valuable model to study neurodegenerative diseases like AD and vascular dementia. The study aims to assess the effects of standardized *O. stamineus* extract on cognitive functions in chronic cerebral hypoperfused rats. Male Sprague Dawley rats (200-300g) underwent a two-vessel occlusion with permanent ligation of bilateral common carotid arteries (PBOCCA) or sham surgery. Acute treatment with *O. stamineus* at doses of 100, 200 and 400 mg/kg, respectively were given orally prior to the experiment. Two-week post-surgery, these rats were subject to behavioral tasks. *O. stamineus* significantly increased step-through latency in passive avoidance task when compared to the PBOCCA vehicle. The results also showed a significant decrease in escape latency during training in the Morris Water Maze test.

Additionally, the initial magnitude of potentiation was much lower in treated-PBOCCA compared to sham and no changes were observed at the higher doses of *O. stamineus*, exhibiting protective effects. These results strongly support the effectiveness of *O. stamineus* in a neurodegenerative disease like Alzheimer's and have the ability to prevent pathological like vascular dementia developed by cerebral hypoperfusion.

Keywords: *O. stamineus*, passive avoidance task, morris-water-maze, cognitive function, cerebral hypoperfused rats

1.0 Introduction

Alzheimer's disease (AD) and vascular dementia (VD) are the two most common types of dementia (Zlokovic, 2002). AD is characterized by chronic and progressive neurodegeneration that triggers advanced cognitive impairment, thus ultimately leading to death (Zhao et al., 2014). Alzheimer disease is typically categorized as a neurodegenerative disease but it also serves as a vascular disorder (Zlokovic, 2002), particularly in the initial stages, when chronic cerebral hypoperfusion escalates the AD-associated cognitive decline. AD is typically sporadic and triggered by various factors. AD is largely characterized histopathologically via the presence of intraneuronal neurofibrillary tangles (NFTs) and extracellular senile plaques together with neurodegeneration in the brain (Ghumatkar, Patil, Jain, Tambe, & Sathaye, 2015; Zhao et al., 2014). This age-related disease is one of the most common form of dementia and it is expected that by 2050, the pervasiveness will quadruple from 26.6 million cases (1 in 253 people) as of 2006 to 1 in 85 persons worldwide living with the disease (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007).

Chronic cerebral hypoperfusion (CCH) serves as a key contributor to cognitive decline and is crucial determining factor for dementia. The persistent drop in cerebral blood flow (CBF) correlates with the severity of memory disruptions (X. Yan et al., 2010). Bilateral permanent occlusion of the common carotid arteries (2VO) in rats causes relatively significant decrease in cerebral blood flow, and serves as a well-characterized animal model employed in investigating the cognitive magnitudes of chronic cerebral hypoperfusion (Thenmoly Damodaran et al., 2014; Z.-Q. Yan et al., 2015). These animals demonstrate learning and memory deficiencies, accompanied by neuronal degeneration and microvascular abnormalities, mimicking those established in human subjects having vascular dementia (Farkas et al., 2004). Studies based on this rat model have tested potentially beneficial

strategies to prevent, delay or reverse the progression of dementia associated with reduced cerebral blood flow (Jin et al., 2014; Z.-Q. Yan et al., 2015).

Orthosiphon stamineus Benth. (Lamiaceae), is a medicinal herb extensively distributed in South East Asia. This medicinal herb is typically employed in traditional folk medicine for the treatment of a variety of angiogenesis related diseases (Jaganath and Ng, 2000). It is commonly referred as cat's whiskers and is also known as "misai kucing" and "kumis kucing" in Malaysia and Indonesia respectively. Various *in vitro* and *in vivo* models have addressed the presence of different types of phytochemicals present in this plant like flavonoids, terpenoids, and essential oils. Earlier studies have demonstrated that *O. stamineus* leaves extracts possess strong antioxidant, anti-inflammatory and anti-bacterial properties with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid (Sumaryono, Proksch, Wray, Witte, & Hartmann, 1991; M. Yam et al., 2010), and nitric oxide inhibitory isopimarane-diterpenes (S. Awale, Tezuka, Banskota, & Kadota, 2003). For instance, rosmarinic acid, the main flavonoid component of *O. stamineus* has been shown to have various pharmacological properties. Flavonoids which are the principal group of polyphenols are also reported to be efficacious in decreasing oxidative stress and are said to promote various physiological benefits, particularly in learning and memory, scavenging free radicals and cognitive impairment (Bhullar & Rupasinghe, 2015). Besides that, standardized ethanolic extract of *O. stamineus* were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration (George et al., 2015). In addition, *in vitro* studies have demonstrated that *O. stamineus* enhanced H_2O_2 induced oxidative stress by antioxidant mechanisms in SH-SY5Y human neuroblastoma cells (Sree et al., 2015). Therefore, since preliminary studies have demonstrated neuroprotective as well as choline esterase inhibitory effects of this compound, it can hence be hypothesized that *O. stamineus* can be used as adjuvant therapy for neurodegenerative diseases like AD.

As summarized above, there is a lack of information regarding its effects on behavior and cognition in chronic cerebral hypoperfusion rats. Consequently, we conducted the present experiments to further characterize the behavioral effects of *O. stamineus* treatment in rodents assessing spatial performance (by Morris water maze testing), as well as fear-aggravated test (by passive avoidance test).

2.0 Materials and Method

2.1 Plant material & extraction

The leaf extract of *O. stamineus* was prepared under GMP-based environment using Digmaz technology by Natureceuticals Sdn. Bhd. Briefly, the powdered material (100 g) of the air-dried leaves was extracted with 1 L of 50% ethanol at 50 °C for 24 h with continuous stirring counter current technology. The extract was filtered via a filter paper (Whatman No. 1) using a Buchner filter under vacuum and concentrated to dryness in a rotary evaporator and refrigerated at −20 °C until further use

2.2 Standardization of O. stamineus 50% ethanolic extract

The extract was standardized for the active marker compounds, namely sinensetin, eupatorin, 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone, acid, orthosiphol A, rosmarinic betulinic acid, oleanolic acid and ursolic acid using HPLC fingerprint method (Akowuah & Zhari, 2008). The protocol for the standardization is demonstrated using HPLC. The standardization of the extract was carried out against 4 bioactive standard markers; rosmarinic acid (RA), sinensetin (SIN), eupatorin (EUP) and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF). The quantification of the markers was analyzed using HPLC. The chromatographic conditions were already validated by (Saidan, Aisha, Hamil, Majid, & Ismail, 2015). Briefly, using Agilent 1260 Infinity HPLC system, USA, the chromatographic analysis was carried out using a reverse phase C18 column (Aclaim Polar Advantage II, USA). The DAD detector was used at 320 nm at 40 °C. The gradient elution was used with the mobile phase A (0.1% formic acid) and by (acetonitrile) with a flow rate (1 ml/min), injection volume (5 µL) with 18 min run time (**Table 1**).

Time	**Flow rate (mL/min)**	**Solvent ratio**	
		A	**B**
		(0.1% formic acid)	**(ACN)**
0	1	85	15
1	1	85	15
12	1	35	65
15	1	85	15
18	1	85	15

Table 1 Gradient elution of *O. stamineus*

2.3 Fourier-transform infrared spectroscopy (FTIR) Analysis of raw material (leaves) and 50% ethanol extract of *O. stamineus*

The phytochemical quality of raw material and the extract was confirmed by Infrared (IR) spectroscopy. Briefly, FTIR analysis of powdered leaves and 50% ethanolic extract was recorded in the region from 4000 to 600 cm-1 and 16 scans were accumulated for each spectra using attenuated total reflection (ATR) device (Nicolet iS10, Thermo Scientific, USA). The spectral data was processed using Omnic software (Thermo Scientific, USA). The IR measurement was made at a resolution of 4 cm-1.

2.4 Experimental Animals

Adult male Sprague Dawley (SD) rats weighing between 200 – 300g were acquired from the Animal Research and Service Centre (ARASC), University Sains Malaysia (USM). The rats were housed 5 per cage on a standard 12:12 light/dark cycle with lights on at 7.00 am and were maintained at room temperature (25±2°C). Food and water were given ad libitum. The experimental protocols were approved and conducted according to the approval of the Animal Ethics Committee University Sains Malaysia with the reference number USM/Animal Ethics Approval/2015 (677).

2.5 Permanent Bilateral Occlusion Common Carotid Artery (PBOCCA) Surgery

The permanent bilateral occlusion common carotid artery (PBOCCA) surgery was performed as described in Damodaran et al. (2014). Briefly, all rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (80mg/kg) and xylazine hydrochloride (10mg/kg). For PBOCCA surgery (4 groups x 6 rats, n=24 rats), the common carotid arteries were exposed via a ventral midline incision whereby the common carotid arteries were then gently separated from their sheaths and vagus nerve and permanently doubly ligated with a 5/0 silk suture approximately 8 – 10 mm below the origin of the external carotid artery. The skin incision was then closed, and the rat was left in a room at 25°C. On the other hand, the sham group (n=6 rats) was subjected to the same procedure without PBOCCA. All rats were left for recovery 2 weeks before being subjected to a series of behavioral studies.

2.6 Experimental Groups

Prior to the experiment, animals were acclimatized to the surrounding and were handled for a period of 1 week to reduce the stress. The animals were than randomly divided into 5 groups (n=6/group). The ethanolic extract of OS was freshly prepared in distilled water.

Group 1: Sham-operated group (Sham + Vehicle)

Group 2: PBOCCA group (PBOCCA+Vehicle)

Group 3: Orthosiphon stamineus Low dose (PBOCCA+ 100 mg/kg OS)

Group 4: Orthosiphon stamineus Medium dose (PBOCCA+ 200 mg/kg OS)

Group 5: Orthosiphon stamineus High dose (PBOCCA+ 400 mg/kg OS)

After 7 days of acclimatization period, the animals were subjected to PBOCCA surgery where Group 1 rats were sham operated where the rats was subjected to the same procedure without PBOCCA. Groups 2-5 underwent the PBOCCA surgery. Two weeks after the surgery, the rats were treated with OS extract through oral dosing for 10 days before being subjected to passive avoidance behavioral test and Morris water maze behavioral test.

2.7 Behavioral Study

2.7.1 Passive Avoidance Test

The passive avoidance apparatus consists of two sections: a lighted box (plexiglass) and a dark box (black), both of the same size (20×20×40 cm each) divided by a guillotine door (8×8 cm). The lighted box was well-lit with a lamp (60W located above the apparatus and the floor was composed of stainless-steel grids 2mm in diameter and at 8mm intervals. The grid floor in the dark compartment delivered intermittent electric shock (50Hz, 10s, 0.5mA intensity) via an isolated stimulator. At 2 weeks following the BOCCA surgery, an acquisition trial was performed. During the acquisition trial, the rat was permitted to freely explore both the compartments for 3 minutes. During the training session, each rat was first placed in the light compartment whereby 10s later, the door between the compartments was opened and the latency to enter the dark compartment with all four paws was timed. Once the rat has entered into the dark compartment with all four paws, the door was closed and the 0.5mA foot shock was administered for 10s. The rat was then removed and placed back in its cage. A retention trial was then executed 24 hours after the training session whereby it was conducted in a similar manner as the training session except that the shock was not supplied to the grid floor in the dark compartment. If the rat failed to enter into the dark compartment

within 300s, the retention trial was completed and the maximal step through latency of 300s was recorded.

2.7.2 Morris Water Maze

The Morris water maze consists of a black circular pool (160 cm diameter, with a depth of 70 cm of water). The pool was positioned in a large test room and surrounded by several visual cues. Besides that, the pool was filled to a depth of 39 cm with water and were maintained at 25 ± 1°C and made opaque by adding white paint. The pool was divided into four quadrants, with a platform (10cm diameter), situated 2 cm below the surface of the water in a fixed position in one quadrant. One day before the start of the formal training, the rats were given a pre-training session where they were allowed to swim freely in the pool for 60s without an escape platform. During the formal training, there were daily training consisted of four trials whereby during the trials, the rat was placed in the water from four different starting points and the latency of escaping the platform was recorded. This training was conducted for four consecutive days where each rat was given a maximum of 60s to find the platform and climb on to it and inability to perform the task within the 60s resulted in the trial being terminated and a maximum score of 60s been given. The rat was then guided to the hidden platform by hand and was allowed to stay on the platform for an additional 10 s before it was removed from the water. On the fifth day, each rat was subjected to a probe trial in which there was no platform present. The time taken for the rat to cross the former platform quadrant as well as the total time taken to cross all the quadrants in 1 minute was recorded. The percentage of crossing the quadrant of the former platform represented the ratio between the time of crossing the quadrant of the former platform and the total time of crossing all quadrants. The percentage was calculated as a measure of spatial reference memory.

2.8 Electrophysiology

Electrophysiological procedures were performed on day 28 after induction of CCH. The rats were anaesthetized with urethane (Sigma; 2.0 g/kg, administered in four doses 0.5 g/kg 20 min apart; supplements of 0.5 g/kg were given when necessary) intraperitoneally before placing on the stereotaxic frame. Local analgesic xylocaine (5mg/kg; AstraZeneca, Australia) was administered subcutaneously on the head. The rats were placed on the stereotaxic apparatus and the ear bar fixed into the auditory canal of the rat's right and left ears. The rat's body temperature was maintained between 36 ºC and 37ºC by using

homeothermic blanket (Harvard Apparatus). After that, small incision on the head was made and the connective tissue adhered to the bone was scraped and removed. The bone surface was cleared by using 30% hydrogen peroxide (Sigma) until the bregma and lambda were clearly visible. Four small holes were drilled on the skull overlying the hippocampus CA1 (AP: -4.2. ML: +3, V: -3) and hippocampus CA3 (AP: -4.2, ML: -3, V: -4) and other two small holes were drilled overlying the frontal region for the placement of ground and reference electrodes. All stereotaxic placements were based on the anatomical rat brain atlas of Paxinos and Watson (X. Yan et al., 2010).

After the drilling, a recording electrode (Perfluoroalkoxy [PFA] Insulated Steel Wire, A-M Systems, USA) was lowered ventrally into the CA1 and a concentric, bipolar stimulation electrode (SNE 100, MicroProbes, USA) was used to stimulate CA3 region. The final ventral placement of the CA3 stimulating electrode and recording electrode were adjusted to yield maximum field excitatory postsynaptic potential (fEPSP) amplitude in CA1 in response to CA3 stimulation. The CA3 stimulation electrode was connected to a stimulus isolator providing constant current output (ML 180 Stimulus Isolator, ADInstruments, Australia). The recording, ground and reference electrodes were connected to an amplifier (Model 1800, A-M Systems Inc., Sequim, WA; half amplitude filters set at 0.1 Hz to 500 Hz) which digitized at 100 Hz and stored the recorded signal for offline analysis (PowerLab 16/s System, ADInstruments, Australia).

For each rat, the intensity required to elicit the maximal fEPSP amplitude was determined via input-output curves. The stimulation was given between 0.1 mA until 1.0 mA (0.1 mA increments). The stimulation intensity eliciting 50-60% of maximal fEPSP amplitude was used for the remainder of the experiment. Paired-pulse facilitation was measured by delivering pairs of stimulation pulses at interstimulus interval of 20, 50, 100, 200, 500 and 1000 ms. For long-term potentiation (LTP) experiments, a baseline of the fEPSP was recorded over a 60-minute period. Baseline recording were considered stable when fEPSP amplitude stayed within a range of 90-100% of the average fEPSP amplitude over the 60 min baseline period. Once a stable baseline wa established, theta burst stimulation (TBS) was applied as a train of ten bursts (each burst consists of 5 pulses at 100 Hz) with bursts repeated every 200ms for a single train. Following the TBS, single pulse stimulation of the CA3 continued every 30 secs for 3 hours recording period.

2.9 Statistical Analysis

Behavioral performance in the water maze task was analyzed using a two-way ANOVA, followed by Bonferroni post hoc test. Statistical analysis for passive avoidance latencies, and probe trial performance were performed by unpaired Student t-test. Data were expressed as means ± S.E.M. Probability values less than 5% ($p < 0.05$) were considered significant. Statistical analysis was performed using the software Graph Pad Prism.

3.0 Results

3.1 Standardization of *O. stamineus* 50% ethanol extract

The powdered material of the air-dried *O. stamineus* leaves was extracted with 50% ethanol with continuous stirring counter current technology. The percentage yield of the extract obtained was 9.8%. The FTIR spectra of powdered leaves and 50% ethanolic extract were recorded in the region from 4000 to 600 cm-1 with 16 scans. **Figure 1** showed the spectra for both samples.

The quantification of the 4 markers including RA, SIN, EUP and TMF was analyzed using HPLC and the result obtained was shown in **Table 2**. The chromatographic profile of *O. stamineus* ethanolic extract are shown in **Figure 2**. Based on **Table 2**, it is known that TMF is present in very low amount as compared to the other markers while the RA is present in abundance in the 50% ethanolic extract.

Samples	**Percentage of markers (%)**			
	RA	**TMF**	**SIN**	**EUP**
50% ethanolic extract	**5.02±0.02**	**ND**	**0.21±0.01**	**0.17±0.01**

Table 2 Quantification of four markers in *O. stamineus* 50% ethanolic extract. ND: Not detected

3.2 Effect of *O. stamineus* extract on memory performances

3.2.1 Passive avoidance task

Figure 3 depicts the results obtained from the passive avoidance task where memory performance of the three doses of *O. stamineus* extract was assessed 24 hours following the administration of *O. stamineus* in the post-training consolidation phase. Memory performance was assessed by determining the latency to enter the dark (shock-paired) compartment during

the post 24-hour retention trial. *O. stamineus* dose-dependently improved memory consolidation in a passive avoidance task to a similar degree as sham ($F_{4,29} = 21.44$, $P<0.001$ versus PBOCCA vehicle). The PBOCCA vehicle significantly showed impaired memory consolidation in a passive avoidance task ($P<0.05$).

3.2.2 Morris water maze

Figure 4 shows the results attained from the post-training administration of 100, 200 and 400mg/kg *O. stamineus* extract during the acquisition training on the PBOCCA+ vehicle induced spatial learning and reference memory deficit in the Morris water maze test. The two-way ANOVA demonstrated a statistically significant between the treatment ($F_{4,100} = 10.11$, $P<0.0001$ versus sham) and day ($F_{3,100} = 10.91$, $P<0.0001$ versus sham) but not with the interaction between the groups ($F_{12,100} = 0.4864$, $P=0.9185$). Based on the performance during acquisition training as demonstrated in Figure 4A, PBOCCA+100 (the lowest dose) learned to locate the hidden platform with progressively shorter latencies over the four days training period similar as sham rats.

The probe trial performance was assessed by determining the effect of the 100, 200 and 400mg/kg *O. stamineus* extract on the time spent in target quadrant (Figure 4B). This experiment was performed after acquisition training and reflect a spatial bias of animals toward the previous location of the hidden platform. The pool was divided into four quadrants of equal area, a chance level of performance i.e. the percent of time swam in the previous target quadrant would generally approximate 25%. As indicated in Figure 4B, there was no significant effects on percentage of time spent in the previous target quadrant ($P>0.05$).

3.3 Electrophysiology

The input-output relationship for electrical stimulation of the CA3 region of the hippocampus and the resulting amplitude of the fEPSP recorded in the CA1 region of the hippocampus are shown in **Figure 5**. Based on the results obtained in Figure 5A, it was observed that PBOCCA +VHL and PBOCCA +100mg/kg OS resulted in a decrease in the maximum fEPSP amplitude ($P<0.05$). The change could be the result of the persistent loss of hippocampal CA1 neurons or decrease in stimulated glutamate release or both. However, there were no changes of synaptic transmission between PBOCCA +400mg/kg OS and sham were observed.

The initial magnitude of potentiation after TBS was much lower in treated-PBOCCA compared to sham. All PBOCCA treatments showed depression of the fEPSP amplitude throughout 2 hours recording, as observed in Figure 5B. At this cellular level of the hippocampus, all three doses did not show any significant enhancement of the LTP ($P>0.05$) compared to sham. It can be concluded that single administration of OS did not enhance LTP.

4.0 Discussion

Permanent bilateral occlusion of the common carotid arteries (PBOCCA) of rats has been found to be capable of mimicking the chronic cerebral hypoperfusion condition and is therefore considered as a model for learning the pathophysiology of learning and memory deficits related with cerebral circulation impairments as well as for gaging therapeutic potential and mechanism of putative anti-Alzheimer drugs (Farkas, Luiten, & Bari, 2007; Liu et al., 2007). Following the PBOCCA surgery, the cerebral blood flow has been observed to be reduced by 25–50%, decreasing progressively for a week, with their effects remaining for several months, resembling the decreased cerebral blood flow observed with human aging and dementia (Farkas et al., 2007; Ni, Ohta, Matsumoto, & Watanabe, 1994). We investigated the cognitive impairment and pathophysiological transformation observed following 2 weeks of carotid arteries occlusion. Previous studies have demonstrated that chronic cerebral hypoperfusion following PBOCCA surgery prompted deficits in spatial learning and memory (Cechetti et al., 2012; Vicente et al., 2009;) and non-spatial memory dysfunction (Sarti, Pantoni, Bartolini, & Inzitari, 2002). However, despite knowing that chronic cerebral hypoperfusion triggers cognitive deficits, the key underlying mechanisms are not completely comprehended (Farkas et al., 2007).

The medicinal value of *O. stamineus* has been well recognized, particularly in regard to its antioxidant and anti-inflammatory activities (Arafat et al., 2008). The results obtained from the chromatographic profile of *O. stamineus* ethanolic extract as well as the quantification of the four markers used are demonstrated in Figure 2 and Table 2 respectively. The chromatographic profile shows that the *O. stamineus* extract contains methoxylated flavones, like sinensetin and eupatorin and high content of phenolic compounds, like rosmarinic acid which tend to exhibit high antioxidant activity that can prevent those free radical-mediated oxidative damage. Additionally, *O. stamineus* leaves extracts have been found to contain strong antioxidant, anti-inflammatory and anti-bacterial properties with more than 20 phenolic compounds, two flavonol glycosides, nine lipophilic

flavones, nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid (Sumaryono et al., 1991; M. F. Yam et al., 2010), and nitric oxide inhibitory isopimarane-diterpenes (Suresh Awale, Tezuka, Banskota, Adnyana, & Kadota, 2003). In particular, rosmarinic acid which is the main flavonoid component of *O. stamineus* has demonstrated various pharmacological properties that may potentially hinder neurodegeneration and improve memory and cognitive functions (Essa et al., 2012). Additionally, various *in vitro* and *in vivo* models have also been explored along with all phytochemicals identified in this plant, including essential oils, flavonoids and terpenoids.

The passive avoidance task is primarily utilized to assess the suitable treatment on the three stages of memory, typically the learning acquisition, memory retention and the retrieval stage (Rabiei, Rafieian-Kopaei, Mokhtari, Alibabaei, & Shahrani, 2014). Thus, the passive avoidance task was employed to gage the 24 hour memory retention of an aversive event whereby the rat acquires to escape the dark compartment that was supplied with mild electric foot shock (0.5 mA, 10 s) during training (T. Damodaran et al., 2014). Our results demonstrated that PBOCCA rats significantly decreased the ability in retaining avoidance memories in contrast to the sham-operated group following the PBOCCA surgery. This indicated that the PBOCCA rats failed to learn or retrieve the memory of the previous unpleasant experience of foot shock experienced in the dark compartment, thus signifying poor retention memory as observed by reduced step-through latency to enter the dark compartment during the test. It can also be assumed that PBOCCA triggered rats to have reduced emotional reactiveness hence reducing their avoidance response in this aversively motivated task. Irrespective of the defined nature of the underlying deficit, it is distinct that PBOCCA causes an enduring behavioral impairment in the expression of an avoidance in response to an environment linked with a noxious sensory stimulus (T. Damodaran et al., 2014). On the other hand, PBOCCA rats treated with three doses of *O. stamineus* extract increase step through latencies 24 h after training. This finding indicates that the rats treated with *O. stamineus* extracts exhibit acquisition of avoidance learning.

On the other hand, the morris water maze has been regularly employed to determine the deficits in spatial learning and memory in rats (D'Hooge & De Deyn, 2001). In accordance with the findings (T. Damodaran et al., 2014), PBOCCA rats displayed impaired spatial learning and memory, as demonstrated by prolonged escape latency and briefer time spent in the target quadrant in the morris water maze, as opposed to the sham-operated rats.

When the rats treated with *O. stamineus* extract, cognitive functions were observed to be improved. Spatial learning and memory were significantly improved only by the dose 100 mg/kg *O. stamineus* extract suggesting that this dose can ameliorate impairment in PBOCCA rats. Coherent with the findings, it was observed that the vehicle-treated PBOCCA rats demonstrated hippocampus dependent spatial learning and memory deficits as evaluated via the morris water maze test and contextual memory as evaluated using the step-through passive avoidance task. Interestingly, *O. stamineus* extract treatment was observed to ameliorate all these cognitive deficits in PBOCCA rats.

As advocated by the free radical theory, the increased production of lipid peroxide and reactive oxygen species (ROS) trigger the deterioration of various cellular enzymes causing neurodegeneration (Rabiei et al., 2014; Yatin, Aksenov, & Butterfield, 1999). Oxidative stress is involved in the causation and development of various diseases like cardiovascular diseases, cancer, diabetes and neurodegenerative diseases. Since free radicals are generally very reactive, they tend to damage vital biomolecules like DNA, proteins, RNA and lipids, thus triggering cell death. Similarly, during both normal aging and Alzheimer's disease increased oxidative impairment are observable (Rabiei et al., 2014). The levels of superoxide, DNA oxidation and neuronal death in the CA1 subfield of the hippocampus were found to be increased in PBOCCA rats. This lead to the development of cognitive impairment (Choi et al., 2014). Rosmarinic acid, the major content of *O. stamineus* 50% ethanolic extract (Figure 2) was found to be very efficient in preventing the alteration of lipid membranes by oxidative stress and efficiently against hydrophilic radicals (Fadel et a., 2011). Based on these research findings, it can be said that, *O. stamineus* 50% ethanolic extract may act by reversing the neuronal death in the CA1 subfield of the hippocampus and improve cognitive impairment induced by PBOCCA rats. Thus, it can be further corroborated that the *O. stamineus* extract could be a promising candidate for future therapeutic treatment for anti-Alzheimer drugs.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

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Figures

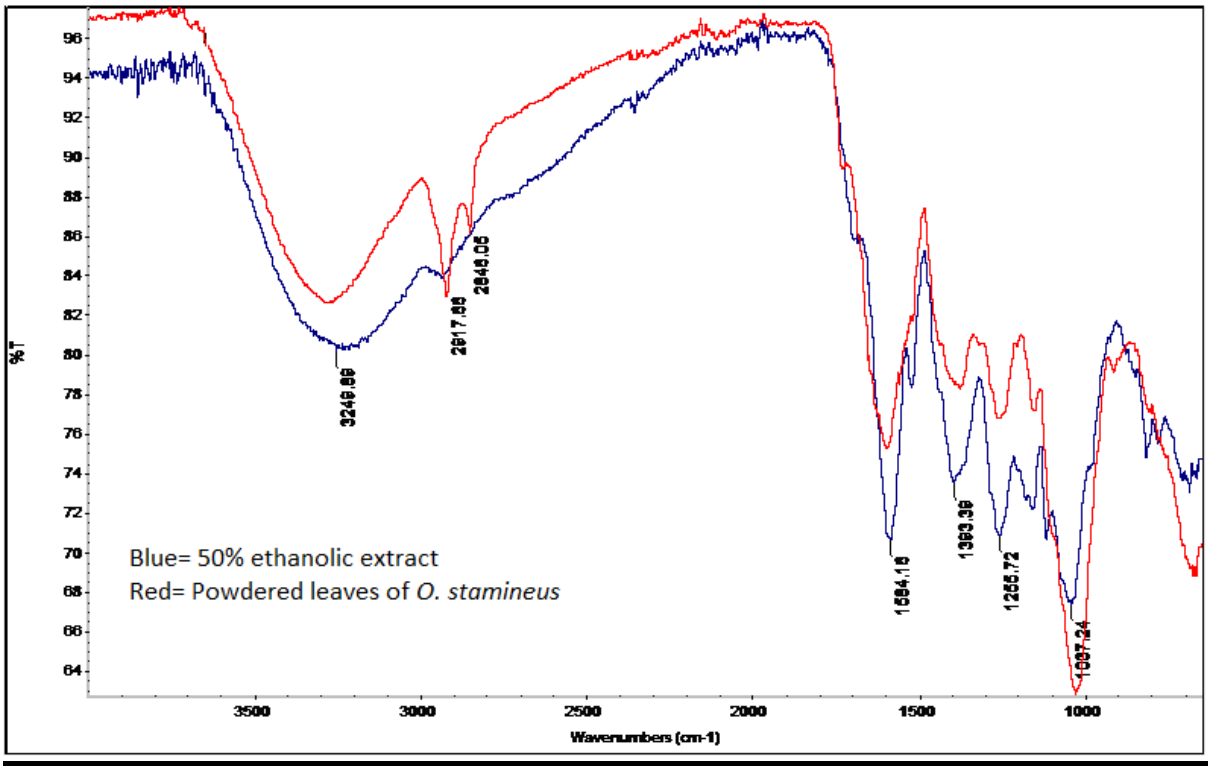

Figure 1: FTIR spectra of powdered leaves and 50% ethanolic extract of *O. stamineus*

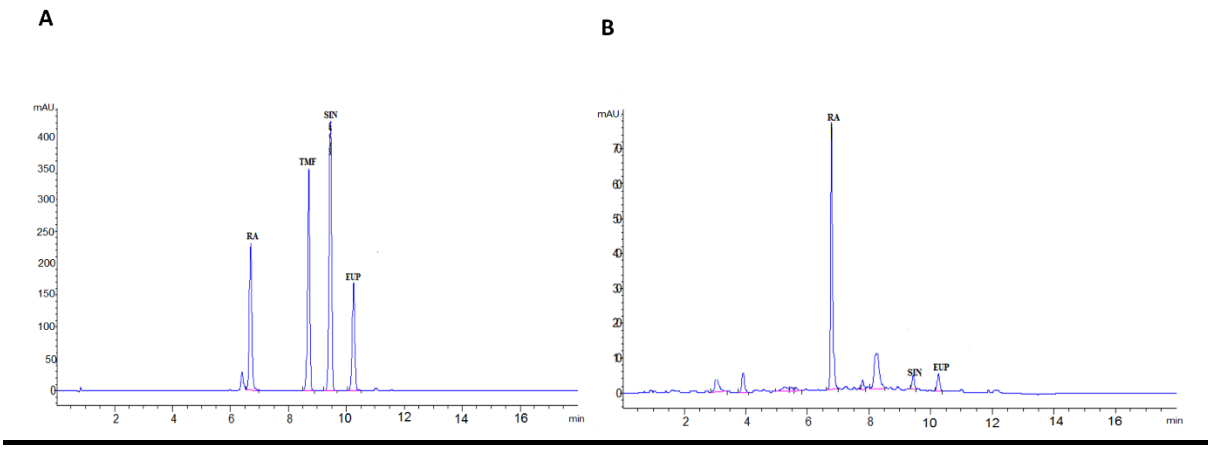

Figure 2: (a) Chromatogram of HPLC profile of standard markers and (b)*O. stamineus* 50% ethanolic extract.

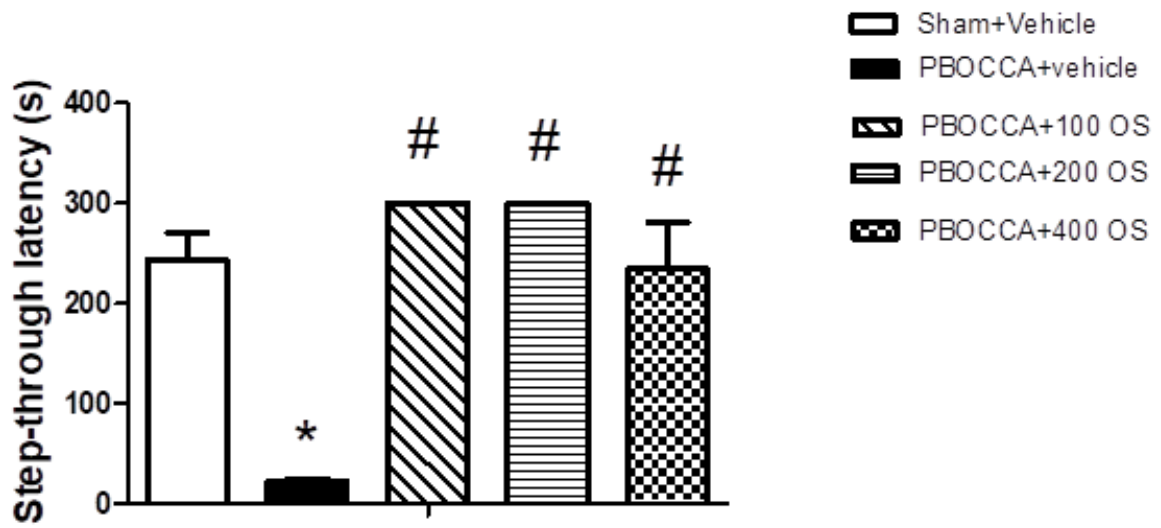

Figure 3: The effects of 100, 200 and 400mg/kg *O. stamineus* extracts on step-through latencies during consolidation (administered post-trial) of a passive avoidance task in rats. Data are expressed as mean ± S.E.M. n = 6. *p<0.001 vs. sham group and #p<0.001 vs. PBOCCA+ vehicle.

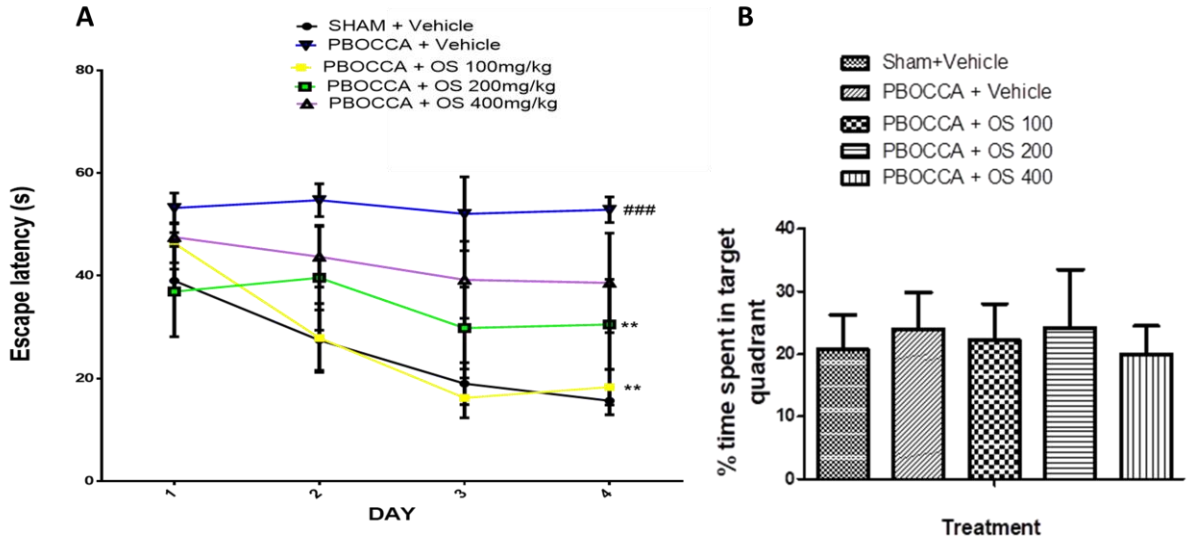

Figure 4: The effects of 100, 200 and 400mg/kg *O. stamineus* extract administration on (a) the escape latency to the hidden platform and learning patterns of animals during days 1 – 4 of training (b) the percentage time spent in the target quadrant during probe trial of the morris water maze test. Data are represented as mean ±S.E.M. n=6. *p<0.05 vs. sham group.

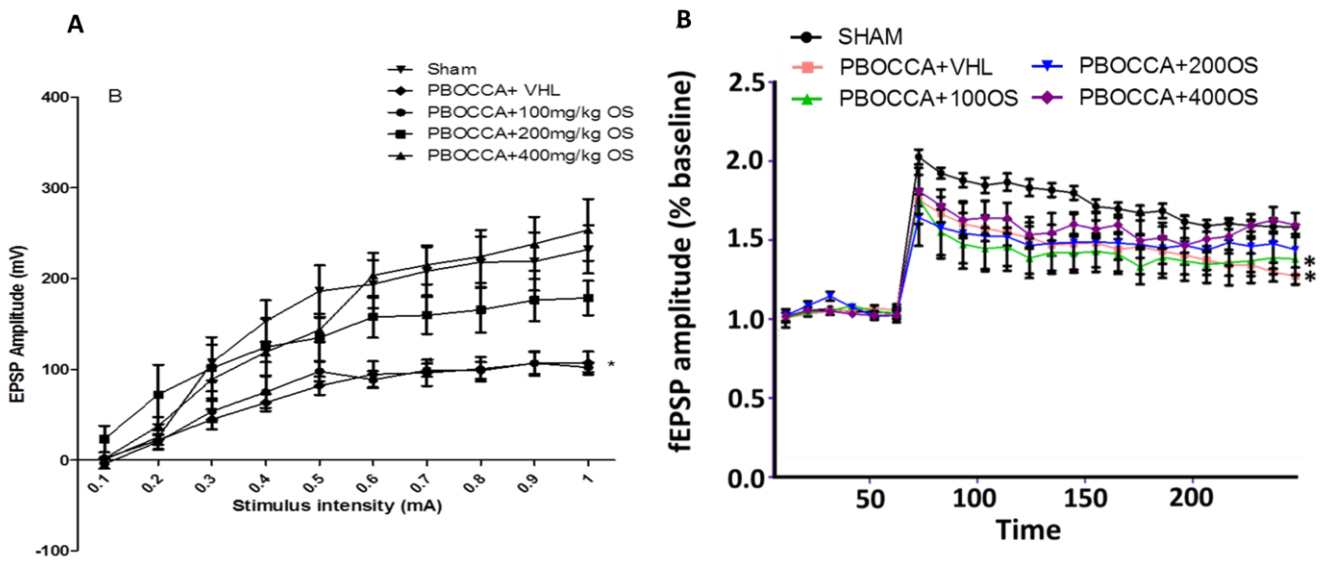

Figure 5: Effect of OS extract on LTP in PBOCCA rats

Chapter 7

7.0 GENERAL DISCUSSION

Alzheimer's disease (AD) is a progressive neurodegenerative condition that is characterised by cognitive impairment and memory loss (Mehla, Pahuja et al. 2013). Some of the key neuropathological hallmarks of AD are the formation of beta-amyloid (Aβ) plaques, neurofibrillary tangles (NFT) and degeneration of cholinergic neurons (Johnson, Storandt et al. 2008, Weiss, Kohler et al. 2008). Aβ plaques and NFT are responsible for the generation of free radicals, activation of astrocytes and cholinergic dysfunction observed in AD. The resulting oxidative stress and inflammation play a central role in the pathogenesis of AD (Huang, Zhang et al. 2016). Additionally, augmented breakdown of acetylcholine by cholinesterase enzyme causes deficiency in the hippocampus and frontal cortex which are responsible for cognitive deficits observed in AD (Khan, Khan et al. 2012).

In this study, OS extract was employed whereby in the first part of the study, the acetylcholinesterase activity as well as neuroprotective activity of OS extract was explored. Based on the results attained it was observed that OS extract was found to be a potent AChE inhibitor and was also found to be able to protect the human neuroblastoma SH-SY5Y cells against beta amyloid induced neurotoxicity further endorsing the neuroprotective ability of OS extract. Thus, it can be inferred that the OS extract contained substantial number of polyphenols and flavonoids like rosmarinic acid that demonstrated potential antioxidant and radical scavenging activities which further indicate OS extract possesses a wide margin of medicinal value and could potentially play a crucial role in AD therapy. Once this was established, three experimental models were employed to explore some of the key hypotheses pertaining to AD. The first hypothesis explored was the amyloid-tau hypothesis using the streptozotocin (STZ) model. Intracerebroventricular (ICV) injection of streptozotocin (STZ) has been associated to multiple pathological conditions of AD like oxidative stress, neuroinflammation and impaired brain glucose and energy metabolism (Nitsch and Hoyer 1991, Tota, Awasthi et al. 2010), which in turn lead to cognitive impairment (Sharma and Gupta 2001). These findings were further confirmed based on the results we observed whereby based on the behavioural studies, memory retention was improved in the groups treated with OS extract and this results were supported by the biochemical study results where the induction of STZ demonstrated overexpression of all the key genes namely APP, MAPT, GSK3-α and GSK3-β in both the hippocampus

and the prefrontal cortex region. However, when treated with OS extract, the expressions of all these genes were observed to be suppressed indicating maximum protection and hence reducing AD pathology. Thus, it can be corroborated that OS extract is able to restore the immune response, produce anti-inflammatory effects, reduce neurotoxicity and improve learning and memory upon the induction of an Alzheimer's like condition

The next hypothesis explored in this study was the cholinergic hypothesis using the established scopolamine model. Scopolamine induced amnesia rodent model is one of a highly established animal model for cognitive dysfunction (Blokland 2005). Scopolamine is a non-selective muscarinic receptor antagonist that impedes the central cholinergic neuronal activity causing learning and memory impairment. Scopolamine affects the expression of various genes including those associated with muscarinic receptor signalling pathways, reconstruction of cytoskeleton, protein trafficking and cell differentiation in the rat brain (Hsieh, Hsieh et al. 2003, Konar, Shah et al. 2011). Based on the behavioral analyses, the spatial memory was observed to be improved in both the acute and chronic scopolamine model and this improved performance was attributed to its enhanced cholinergic neuronal transmission. However, a celing effect was observed with higher doses. BDNF and TrkB have been observed to play a critical role in long-term synaptic plasticity in the adult brain whereby BDNF-TrkB interaction promotes the survival and differentiation of neurons and synaptic plasticity of the central nervous systems (Schinder and Poo 2000, Dwivedi 2009, Lu, Christian et al. 2008, Kim, Ko et al. 2010). Thus, reduction in the levels of BDNF and its receptor, TrkB may trigger synaptic and cellular loss and memory deficits characteristic of AD. These findings were parallel with the results obtained whereby induction of scopolamine-induced amnesia demonstrated decreased expression of BDNF and TrkB in the hippocampus and when treated with OS extract, both the BDNF and TrKB expression levels in the hippocampus region were observed to be increased thus showing maximum protection. CREB1 is a co-factor of CREB and plays a crucial role in memory and synaptic plasticity in the central nervous system whereby neurodegenerative diseases like AD triggers disruption of phosphorylated CREB within the hippocampal region (Lee, Kim et al. 2015). Activation of CREB has also been observed to ameliorated cognitive impairment via the cholinergic system (Kotani, Yamauchi et al. 2006, Lee, Kim et al. 2015), which was congruent with the results

obtained whereby scopolamine reduced the expression of the CREB1 gene and pre-treatment with OS extract markedly increased the CREB1 mRNA levels. Additionally, previous studies have reported decreased expression of DCX during aging and thus decrease in neurogenesis (Brown, Couillard-Despres et al. 2003, Hwang, Yoo et al. 2008, Heo, Shin et al. 2014). Similar results were also observed in our study whereby the number of DCX-positive cells in the hippocampal dentate gyrus were decreased in scopolamine induced rats and when treated with OS extract, the number of DCX-positive cells were observed to be increased.

Lastly, the vascular hypothesis of AD was explored using the chronic cerebral hypoperfusion (CCH) model. CCH is one of the key cause of vascular dementia, which is the second most common form of dementia after AD in the older population (Battistin and Cagnin 2010). Previous studies have distinguished that CCH promotes neurodegeneration by triggering reactive oxygen species and proinflammatory cytokines via activated microglial cells that in turn deteriorate the neuronal cells contributing to cognitive impairment (Farkas, Luiten et al. 2007, Adibhatla and Hatcher 2008, Bang, Jeon et al. 2013). Based on the results obtained, it was observed that the vehicle-treated PBOCCA rats demonstrated hippocampus dependent spatial learning and memory deficits as evaluated via the morris water maze test and contextual memory as evaluated using the step-through passive avoidance task. Interestingly, O. stamineus extract treatment was observed to ameliorate all these cognitive deficits in PBOCCA rats.

There have been multiple studies carried out over a few decades to combat this appalling neurodegenerative disorder. Although there are a few available drugs in the market to manage AD, there is yet to be a substantial drug or plant extract that is capable to reverse the symptoms of AD. The use of medicinal plant extract may be a potential corner stone in this quest and it is extremely tangible that there has been an immerse need for such therapeutic intervention. One such suitable potential candidate will be *Orthosiphon stamineus* (OS). OS contains various bioactive compounds that have been proven to exhibit various pharmacological properties that could aid in treating and reversing AD. Flavonoids which are the principal group of polyphenols have been reported to be exerting anti-oxidant properties thus promoting various physiological benefits, particularly in learning and memory, scavenging free radicals and cognitive impairment (Bhullar and Rupasinghe 2015, Ghumatkar, Patil et al.

2015). Besides that, standardized ethanolic extract of OS were also found to be able to reverse age-related deficits in short-term memory as well as prevent and reduce the rate of neurodegeneration (George, Chinnappan et al. 2015).

In a nutshell, present finding revealed that treatment with OS extract demonstrated improvement of memory, largely due to its antioxidant and anti-inflammatory properties. OS also prevented cognitive impairment by ameliorating the oxidative burdens and neuroinflammation via its potent antioxidant and anti-inflammatory properties and protected the rats brain injury. Thus, it can be said that OS serves to be a promising source of treatment for AD as an apt pharmacological agent for AD would be one that are acetylcholinesterase inhibitors as well as able to exert antioxidant activity. Additionally, OS contains phenols and flavonoids which are key natural products that are involved in inhibiting acetylcholinesterase and restoring the levels of acetylcholine which is extremely crucial for normal brain functioning. Therefore, based on these research findings it can be corroborated that OS is indeed a promising candidate in reversing cognitive impairment in AD and it would be highly recommended to extend the research on OS to translate it into clinical studies to further verify the research attained here.

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Supplementary Information

What was the rationale behind choosing 50mg/kg, 100mg/kg, 200mg/kg doses of OS for scopolamine induced amnesia model as well as STZ induced model?

Based on our preliminary study using the acute oral toxicity results, the LD50 value for OS was found to be more than 2000 mg/kg and 1/20th, 1/10th and 1/5th was chosen as therapeutic doses. So, for our first disease model, the PBOCCA study, a dose range of 100mg/kg, 200mg/kg and 400mg/kg was used but the 400mg/kg group were exhibiting neurobehavioral effect on coordination and motor activity and were not able to demonstrate any results. Thus, for the second and third disease model, which was the Scopolamine and STZ model respectively, the new range of doses were used. We decided to use the 200mg/kg as the highest dose and 50mg/kg as well as 100mg/kg were used as the low dose and medium dose respectively, and all these 3 doses were effective therapeutic doses for our study as we noticed no side effects.

What is the rationale for choosing 100, 200, and 400mg/kg of OS in PBOCCA model of dementia? These are different from previous studies.

At the beginning of this experiment, we conducted a dose deciding study to find the therapeutic dose of OS extract. Based on our preliminary study using the acute oral toxicity results, the LD_{50} value for OS was found to be more than 2000 mg/kg. Since PBOCCA study was our first disease model, a dose range of 100mg/kg, 200mg/kg and 400mg/kg was used but the 400mg/kg group were exhibiting neurobehavioral effect on coordination and motor activity and were not able to demonstrate any results. Thus, for the second and third disease model, which was the Scopolamine and STZ model respectively, the new range of doses were used. We decided to use the 200mg/kg as the highest dose and 50mg/kg as well as 100mg/kg were used as the low dose and medium dose respectively, and all these 3 doses were effective therapeutic doses for our study as we noticed no side effects.

How were the animals taken care of infection/pain after the PBOCCA surgery?

Following the surgery, postoperative care was made by applying the betadine povidone-iodine solution on the wound. The rats were also placed on thermal sheets to maintain body temperature and were kept under close observation for the next 4 days.

In the PBOCCA chapter, standardization of OS is described. This should be described in the beginning.

The PBOCCA study was the first study that was carried out, hence why the standardization of OS was described in this study. This study therefore should have been included as the earlier chapters.

Where was the leaf extract of OS prepared? Was it outsourced or made in the Monash Unversity?

The leaf extract of O. stamineus (OS) was outsourced. It was prepared under GMP-based environment using Digmaz technology by Natureceuticals Sdn.

Why wasn't positive control added in this part of the study?

A positive control was not included in this study because there is no established anti-Alzheimer's drug in the market. Thus, comparison can only be made between control and treatment groups.

In the last paragraph oxidative stress is extensively discussed. Why wasn't oxidative stress measured in this study?

In the PBOCCA study, we did not perform any molecular or gene studies. We focussed on firstly standardizing OS extract followed by Alzheimer's model development and behavioural studies. Additionally, as behavioural results were not positive hence downstream processing was not done.

Page 71, line 283: Was statistical analysis carried out to compare between the doses of OS to support the statement " the rats were treated with 200 mg/kg did show improved memory retention as compared to STZ treated group but not as that observed with 50 mg/kg and 100 mg/kg OS treated group"?

The Dunnett's multiple comparison test was carried to compare between the doses of OS and they were all found to be significant. Thus based on the results it can be inferred that the 200mg/kg group did show an improvement in memory retention but not as good as that

observed in the 50mg/kg and 100mg/kg group. This could possibly due to the OS extract reaching a ceiling effect.

The rosmarinic acid used is a pure compound. As the candidate rosmarinic acid as a key chemical constituent of OS, if the % of rosmarinic acid of the extract is available, it would help to support the contribution in the AChE activity.

Based on our preliminary studies, the content of rosmarinic acid is the highest in the OS extract, as supported by the chromatogram profile highlighted below.

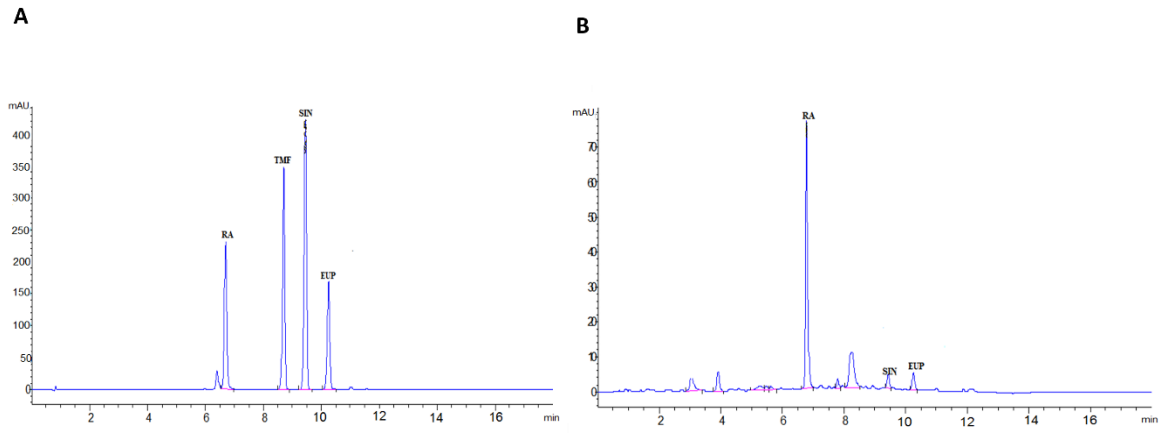

Figure 2: (a) Chromatogram of HPLC profile of standard markers and (b)*O. stamineus* 50% ethanolic extract.

Source of B amyloid? How was it added to SHSY5Y cells in culture

B amyloid chemical was purchased and the lypholized peptide was dissolved in 10% DMSO, followed by vortexing and stored immediately at -80˚C