MONASH University

NEUROPROTECTIVE EFFECT OF EMBELIN IN EXPERIMENTAL MODELS OF ALZHEIMER'S DISEASE

By

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Abstract

Alzheimer's disease (AD) is a severe progressive neurodegenerative brain disorder which displays global cognitive decline involving memory, orientation, judgment, and reasoning. It affects millions of people and it is estimated that by 2050, 1 in 85 individuals worldwide will be living with the disease. Embelin (2, 5dihydroxy-3-undecyl-1,4 benzoquinone) is the major active constituent in the fruits of *Embelia ribes* Burm. The first part of our thesis results revealed that embelin is a potent acetylcholinesterase (AChE) inhibitor when compared with donepezil. Besides that, both nootropic and anti-amnesic effects of embelin were evaluated using the scopolamine model of amnesia in rats. These research findings suggested that embelin is a potent nootropic and anti-amnesic phytocompound. When tested in experimental models of amnesia, embelin significantly improved recognition index and memory retention in both novel object recognition (NOR) and elevated plus maze (EPM) tests. The hippocampal tissues from scopolamine-induced amnesia model were extracted for gene expression, neurotransmitter, and immunocytochemistry studies. The results revealed that embelin elevated the mRNA expression of brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB1), and scavenger enzymes superoxide dismutase 1 (SOD1) and catalase (CAT). Rats pre-treated with embelin could mitigate scopolamine-induced neurochemical and histological changes in a manner comparable to donepezil. The second part of this research investigated the neuroprotective effect of embelin in permanent bilateral common carotid artery occlusion (PBOCCA) model of AD. PBOCCA and embelin (0.3, 0.6, and 1.2mg/kg) treated rats were subjected to behavioural analysis to assess learning and memory functions. The hippocampal tissues from PBOCCA model were extracted for gene expression and neurotransmitter studies. Overall, embelin improved cognitive dysfunction in an animal model of chronic cerebral hypoperfusion. We reported downregulation of amyloid precursor protein (APP) and microtubule-associated protein tau (MAPT) mRNA expression, upregulation of synaptic plasticity-related genes, reduced oxidative stress and the inflammatory response which might have contributed to the neuroprotective effects of embelin. In the third part of our thesis, we investigated whether embelin would confer protection against streptozotocin (STZ) mediated neuronal damage in rat primary hippocampal neuronal cells. In this study, embelin demonstrated protective effect against STZ induced neurotoxicity. Pre-treatment with embelin reduced the mRNA expression levels of APP, MAPT, glycogen synthase kinase 3 alpha (GSK-3α) and GSK-

3β mRNA indicating that this compound could revert the STZ-induced insulin signalling (IR) dysfunction. Notably, embelin pre-treatment increases SOD1 and reduces nuclear factor kappa B (NF-κB) mRNA levels, which is responsible for oxidative stress and neuroinflammation respectively. Finally, we found that embelin inhibited STZ-induced amyloid beta protein expression. In addition, the results from porcine brain endothelial cells permeability assay indicated that embelin could cross the blood-brain barrier. Using molecular docking, we could predict that embelin has favourable binding mode within the AChE and Aβ peptide active sites. Based on the studies so far, it is postulated that embelin could be a promising molecule as a memory enhancer and neuroprotective in Alzheimer's disease condition. Embelin exerts its effect via cholinergic pathway, amyloid cascade and vascular pathway by protecting the neurons against AD like condition.

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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Date: 18th November 2018

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 (1) original paper published in peer-reviewed journal and (3) currently submitted for publications. The core theme of the thesis is neuroprotective effect of embelin in experimental models of Alzheimer's disease. 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 under the supervision of Dr. Mohd Farooq Shaikh.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 3-7, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status *(published, in press, accepted or returned for revision)*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
4	Amelioration of Cognitive Deficit by Embelin in a Scopolamine-Induced Alzheimer's Disease-Like Condition in a Rat Model	Published	Performed all the experiments, data collection, analysis and writing the manuscript in its entirety. 60%	1) Yatinesh Kumari, input into manuscript 10% 2) Iekhsan Othman, input into manuscript. 10% 3) Mohd Farooq Shaikh, critical revision of the manuscript for important intellectual content. 20%	No
5	Embelin Improves the Spatial Memory and Hippocampal Long-Term 1	Submitted	Performed experiments, data collection, analysis and drafting the manuscript. 50%	1) Siti Najmi Syuhadaa Bakar, performed Morris water maze. 5% 2) Yatinesh Kumari, input into manuscript 5% 3) Iekhsan Othman, input	No

	Potentiation in a Rat Model of Chronic Cerebral Hypoperfusion			into manuscript. 5% 3) Mohd Farooq Shaikh, critical revision of the manuscript for important intellectual content. 15% 4) Zurina Hasaan, performed LTP and critical revision of the manuscript for important intellectual content. 20%	
6	Embelin prevents amyloid beta accumulation via GSK-3 pathway in an in vitro model of streptozotocin-induced AD like condition	Submitted	Performed all the experiments, data collection, analysis and writing the manuscript in its entirety. 60%	1) Yatinesh Kumari, input into manuscript 10% 2) Iekhsan Othman, input into manuscript. 10% 3) Mohd Farooq Shaikh, critical revision of the manuscript for important intellectual content. 20%	No
7	Embelin, a potent molecule for Alzheimer's disease: A proof of concept from BBB permeability, Ache inhibition and molecular docking studies	Submitted	Performed experiments, data collection, analysis and drafting the manuscript. 50%	1) Nur Aziah Hanapi, performed BBB permeability assay. 10% 2) Nafees Ahmed, docking studies and input into manuscript 10% 3) Iekhsan Othman, input into manuscript. 5% 3) Mohd Farooq Shaikh, critical revision of the manuscript for important intellectual content. 10% 4) Siti Rafidah Yusof, critical revision of the manuscript for important intellectual content. 15%	No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  **Date:** 18th **December** 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the

responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:  **Date:** 18th **December** 2018

Other PhD-related publications during the PhD period

1. Uday Kundap, **Saatheeyavaane Bhuvanendran**, Yatinesh Kumari, Iekshan Othman and Mohd Farooq Shaikh. Plant derived phytocompound, Embelin in CNS disorders: A systematic review. Front. Pharmacol. 2017; 8(76). doi: 10.3389/fphar.2017.00076

2. Alina Arulsamy, Yogini S Jaiswal, Bey Hing Goh, **Saatheeyavaane Bhuvanendran**, Thaarvena Retinasamy, Yatinesh Kumari, Iekhsan Othman, Leonard L Williams, Mohd Farooq Shaikh. Neuroactive drugs–A perspective on drugs of synthetic and medicinal plants origin. Pharm Pharmacol Int J. 2018; 6(6):422–430.

PhD-related presentations during PhD period

1. **Poster:** Pharmacological evaluation of phytocompound Embelin, derived from Embelia ribes in Alzheimer's disease using chronic cerebral hyperfusion rats. **Saatheeyavaane Bhuvanendran**, Tharveena Retinasamy, Mohd Farooq Shaikh, Yatinesh Kumari, Iekshan Othman, Zurina Hassan. CDR Mini Symposium on Drug Addiction and Neurodegeneration, USM, Penang November 2015.

2. **Poster:** Memory enhancing effect of embelin against scopolamine-induced amnesia in Sprague Dawley rats. **Saatheeyavaane Bhuvanendran**, Yatinesh Kumari, Iekshan Othman, Mohd Farooq Shaikh. 14th Meeting of Asian-Pacific Society for Neurochemistry (APSN 2016), Kuala Lumpur, Malaysia.

3. **Poster:** Neuroprotective effect of Embelin in primary rat hippocampal culture model of neurotoxicity. **Saatheeyavaane Bhuvanendran**, Yatinesh Kumari, Iekshan Othman, Mohd Farooq Shaikh. EMBO Workshop on Neural Development, 02-06 March 2018, Taipei, Taiwan

Award during the PhD period

1. Received **travel grant award** and selected as APSN-ISN Neuroscience School 2016 delegate for APSN-ISN School 2016, UPM, Malaysia (Basic Techniques in in Vitro Neural Differentiation from Stem Cells).

2. Received **travel grant award** (NTD 17 500) for EMBO Workshop on Neural Development, 02-06 March 2018, Taipei, Taiwan

3. **Bronze winner**: NP-A as a memory enhancer and potential molecule for neurodegenerative diseases. Mohd Farooq Shaikh, **Saatheeyavaane Bhuvanendran**, Yatinesh Kumari, Iekshan Othman, Nafees Ahemad Mahammed Yunus. International Conference and Exhibition on Inventions by Institutions of Higher Learning (PECIPTA 2017), 7-9 October 2017, Kuala Terengganu, Malaysia

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Chapter 1

1.0 Introduction

Alzheimer's disease (AD) is a permanent and progressive neurodegenerative brain disorder of elderly humans with loss of cognitive functioning (1). The term AD was first coined by German psychiatrist, Alois Alzheimer's following the admission of a 50 year old woman with progressive memory loss, sleep disturbance, aggression, and confusion (2). In AD patients, these symptoms occur due to damage of neurons in the brain part involved in cognitive function and motor coordination (3). The patient died after 5 years and from her brain autopsies, Dr. Alzheimer detected the presence of distinctive deposits, which are now known as amyloid plaques and neurofibrillary tangles (4, 5).

Currently about 50 million people are living with dementia globally and this figure will be more than triple by 2050 (6). Furthermore, 60 percent to 80 percent of the cases involve solely Alzheimer's pathology (3). World health organization (WHO) reported that dementia is the seventh leading cause of death. On the other hand, 4.9 million cases of dementia were recorded in Asian continents (7). This shows that Asia comprised of low-income and middle-income countries will face financial burden (8) as their economic sources are not enough for long-term care of AD patients by family or at nursing homes. In short, it can be said that AD is one of the major public health problems in the world. In Malaysia, the prevalence of dementia is estimated at 0.126% in 2020 and will increase to 0.454% by 2050 (9-11). The mentioned low prevalence rate is due to the fact that family members perceive AD and other related dementia as a normal part of aging and therefore the patients are missed to be diagnosed (9).

AD is typically categorized into three general stages known as mild (early stage), moderate (middle stage) and severe (late stage) (12). However, AD begins with mild cognitive impairment (MCI) with amnesic symptoms without affecting other cognitive area (13, 14). The patients may fail to recall the latest activity or become confused with input they recently received (15). At this mild Alzheimer's stage, patients may function independently as language, motor, and sensory functions are not affected. Interestingly, not all patients with MCI develop into AD (12) and according to Ward, Tardiff (16) an average of 32% of MCI patients developed into AD within 5 years of follow up. Then followed by the mild AD in which the symptoms include poor object recognition, poor direction sense and reading problem. Even though the patients may function independently, but family and friends may be able to notice difficulties faced by these patients. In the moderate stage,

which for some is the longest and can last for many years, the patients will have personality changes and having difficulty in performing routine tasks (3). Thus, as the disease progress, these individuals require greater level of care. In the severe stage, the brain part involve in cognition and movement become damaged and therefore they become bed-bound (3). Thus, at this stage patients require help in basic activity of daily living.

Current drug therapy for AD is ineffective as the treatment does not prevent, slow or stop the progression of the disease. Treatment of patients diagnosed with AD is restricted to treat the cognitive symptoms of AD by helping to lessen or stabilize the symptoms for a limited time (17). Therefore, there is a pressing need for new and better treatments for AD as this disease condition is so disabling, and social and economic burden continue to grow on. Currently, there are 26 agents in phase III clinical trial of the 2018 AD pipeline (Figure 1). Among those treatments, 14 addressed amyloid targets, one involved a tau-related target, eight drugs for behavioral symptoms, one cognitive-enhancing agent, one involved neuroprotection, and one had a metabolic mechanism of action (18). Thus, search for a potential drug that might reverse the disease progression or even stop it from developing is at top priority.

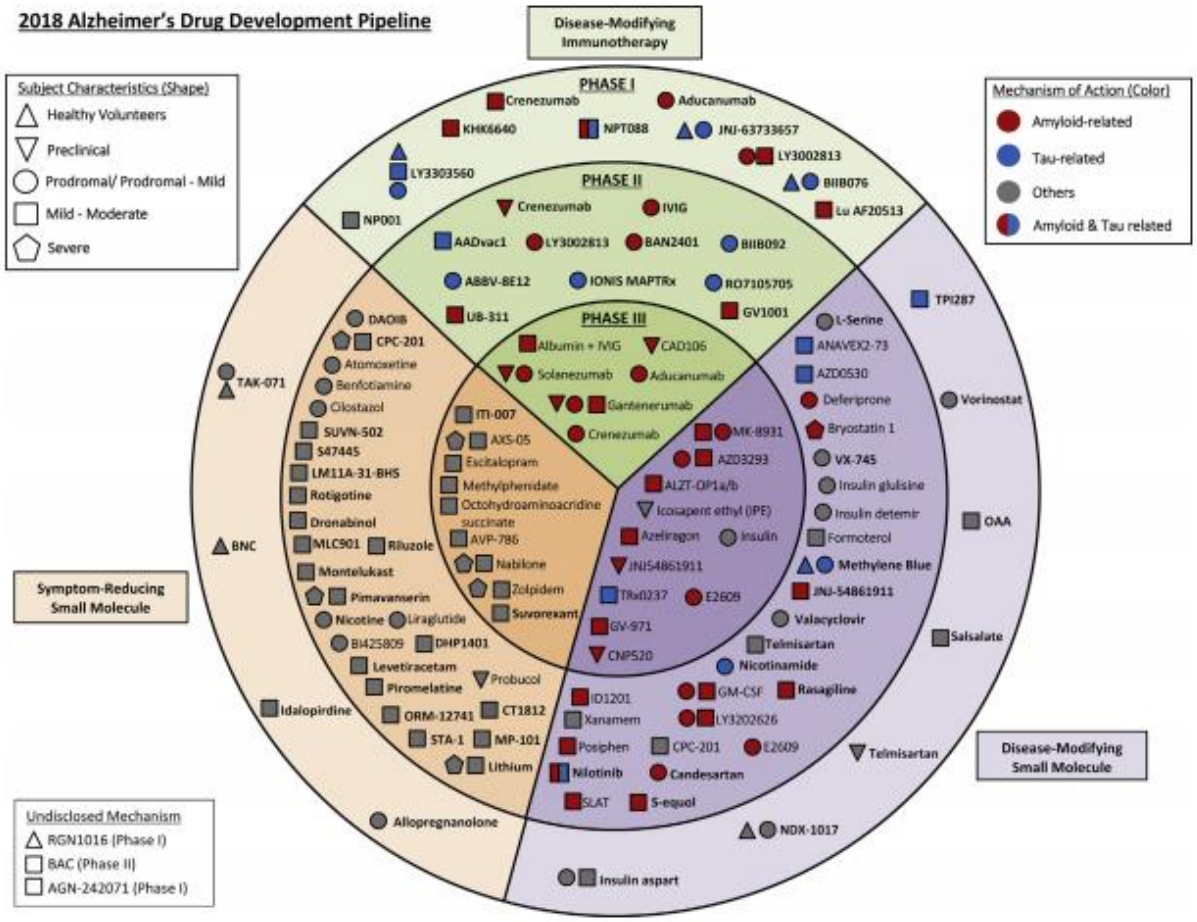

Figure 1: Agents in clinical trials for treatment of Alzheimer's disease in 2018 ; adapted from Cummings, Lee (18)

Chapter 2

2.0 Research Questions

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder. The current AD drugs that are available in the market are purely symptomatic, with little or no beneficial effect on the disease progression. AD is not a result of a single factor but instead is a multifactorial condition. Thus, the failure of current anti-AD drugs justifies the need towards alternative novel drug candidates with no to minimum side effects. Earlier studies have proven that embelin from *Embelia ribes* Burm has antioxidant, anti-inflammatory, anticonvulsant and neuroprotective properties. Thus, we hypothesized that embelin is neuroprotective against AD by targeting the multifactorial condition and can be further developed into drug for treatment and/or prevention of AD. To that end, the specific aims of this thesis were:

(i) To evaluate the neuroprotective effect of embelin on nootropics condition as well as in scopolamine induced amnesia rats **(Paper I)**

(ii) To study the neuroprotective effect of embelin in permanent bilateral common carotid artery occlusion (PBOCCA) model of AD **(Paper II)**

(iii) To investigate the neuroprotective effects of embelin as well as mRNA expression of targeted genes in STZ-induced sporadic AD like condition in an *in-vitro* model **(Paper III)**

(iv) To study the effect of embelin on blood-brain barrier integrity and permeability **(Paper IV)**

Chapter 3

3.1 Introduction

AD is a multifactorial neurodegenerative disease involving many risk factors etiology (19). However, the pathogenesis of AD is still ambiguous due to the complex nature of human brain (20). Many hypotheses about AD have been developed, including amyloid beta (Aβ), Tau, cholinergic and vascular dysfunction and so forth. Thus, many efforts have been made to develop anti-AD drugs based on these hypotheses to stop or revert the progression of AD. This literature review will summarize the pathological hypotheses of AD; limitations of the current therapy and role of embelin in central nervous system (CNS) related disorders.

3.2 Pathology of AD

3.2.1 The Amyloid Hypothesis

The amyloid hypothesis was first proposed by Hardy and Allsop in 1991 and become one of the main etiologic hypothesis in AD which serve as the top explanation on how disease develop until now (21). This hypothesis proposed that imbalance between production and clearance of both intracellular and extracellular amyloid beta is the triggering event for progressive neuronal damage which fully characterizes the AD (22-24). Amyloid beta is a small peptide (~4.5 kDa) with resistance to proteolytic degradation (24, 25). It comprises up to 43 amino acids, in which most of amyloid beta is in the form of 1-40 and less than 5% are generated in isoform 1-42 (25, 26). Since the long form of 1–42 amyloid beta peptide is known to be the most hydrophobic (24), it has the greater tendency to aggregate and initiate the pathological oligomers, fibril and plaques (26). Oligomers and fibrils are considered to have the greatest toxicity while the final stage of senile plaques are relatively inactive (26). Amyloid beta peptide is generated through sequential proteolytic breaks of the amyloid precursor protein (APP) (24). APP is a transmembrane glycoprotein of type I which is highly expressed in the brain (27) and associated with neuronal development, neurite outgrowth, and axonal transport (28). Pathogenetic mutations of APP at β-secretase or γ-secretase cleavage sites are associated with an increase in amyloid beta 1-42 production (29). The increase in APP amount 1.5 times more compared to normal group proportionally give rise in amyloid beta which leads to AD-like pathology (30). According to Šalković-Petrišić (26) these potent amyloid beta neurotoxicants initiate a whole range of pathological cascade including microgliosis and astrocytosis, oxidative stress, inflammatory

response, neuronal dysfunction, cell death, neurotransmitter deficits, and finally cognitive dysfunction.

3.2.2. Tau Hypothesis

Tau is encoded by a single gene, microtubule-associated protein tau (MAPT), which is located on human chromosome 17 (31). Localization of tau mRNA has been found in the soma and dendrites of both normal and AD neurons of human post-mortem brains based on in situ hybridization techniques (32). In CNS, the low molecular weight tau which is highly soluble is responsible for assembly and stabilization of microtubules required for morphogenesis, axonal transport and neuronal growth (33, 34). The microtubules formation activity is known to be regulated by certain degree of tau phosphorylation whereas the hyperphosphorylation of tau suppresses this activity (35, 36).

Abnormal hyperphosphorylated tau undergoes conformational changes, aggregates, and eventually leads to formation of neurofibrillary tangles (NFTs) (37). The accumulation of NFT inhibit the formation of microtubule which eventually causes malfunction of axonal transport (38). In pathological condition, NFT trigger neurotoxic actions that affect neurons by synaptic dysfunction (39) and by transmission from affected neurons to connecting naive neurons (40) and finally leads to neuronal degeneration. On the other hand, according to Dickey, Dunmore (41) tau haplotypes that rise tau expression increase AD risk and therefore reducing tau levels might be an alternative approach to protect against AD (24, 42).

3.2.3. Cholinergic Hypothesis

One of the most common symptoms in AD is cognitive impairment which has been linked to a deficiency of brain neurotransmitter known as acetylcholine (43, 44). Acetylcholine (ACh) is used by cholinergic neurons for sending signal or message for attention, learning, memory, sensory, wakefulness and sleep related physiological processes (20, 45, 46). In AD pathological condition, damaged cholinergic neurons generate a downregulation of acetyltransferase and increase in acetylcholinesterase activity (24). Acetylcholinesterase metabolizes acetylcholine to choline and acetic acid. Thus, this will eventually reduce ACh release, impaired binding of Ach to nicotinic and muscarinic receptors, dysfunctional neurotrophin support, and deficits in axonal transport (47). The cholinergic system plays an important role in the regulation of cognitive functions, as

evidenced by the extensive loss of cholinergic neurons observed in Alzheimer's patients (48) which finally led to the "cholinergic hypothesis". Furthermore, AChE inhibitors have become important alternatives in the treatment of AD (49).

3.2.4. Vascular hypothesis

The vascular hypothesis for AD was first proposed by De la Torre and Mussivan (50) identifying vascular risk factors for involving brain, the heart and the circulation (51). According to vascular hypothesis, reduction in cerebral blood flow (CBF), glucose metabolism and oxygen utilization is the primary factor driving to AD pathogenesis (50). This condition appear to be inversely proportional to the disease severity which is consistent with the pathogenesis and progression of AD. Chronic cerebral hypoperfusion (CCH) is one of the major mechanisms of cerebral vascular disorders (52). CCH can affect the cerebral vascular system and eventually cause decreased blood supply to the brain (53). This condition will finally lead to progressive neuronal damage, transmission failure and brain tissue death (50) with cognitive impairment (54).

3.3 Limitations of current therapy

Unfortunately, there is no cure has been found for AD. The pharmacological treatments available today for AD have not been shown to slow, reverse or prevent the disease progression (19). This is likely due to many factors which are involved in initiation and progression of AD based on the hypotheses discussed above. So far the US Food and Drug Administration (FDA) approved two drug classes for the treatments of AD which are known as AChE inhibitors (rivastigmine, galantamine, donepezil, tacrine) and not competitive N-methyl-D-aspartate (NMDA) receptor antagonist (memantine) (55). The aforementioned AChE inhibitors drugs raise ACh levels and enhance cholinergic functions in the brain (56). Likewise, memantine stabilizes dysfunctional glutamatergic neurotransmission and protect neurons from glutamate toxicity (55). Both these drugs; AChE inhibitors and NMDA receptor antagonists can only improve symptoms and are mainly used for mild to moderate AD and moderate to severe AD respectively (57). Besides, these drugs cause undesired side effects. For instance, tacrine caused liver problem and loss of appetite whereas for NMDA receptor antagonist, it causes hallucination, confusion, and mood swings following medication (58). Thus, due to its adverse side effects, tacrine is now discontinued in the United States (3) and memantine is only licensed in several countries (57). Furthermore, both

effectiveness and tolerability of these drugs have an important interpatient variability and these represent a limitation to current treatment for AD.

3.4 Role of embelin in CNS related disorders

Embelin (2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone) is a naturally occurring alkyl substituted hydroxyl benzoquinone which identified as a major constituent of the fruits of *Embelia ribes* Burm (Family: Myrsinaceae). Traditionally, this plant has been utilized as brain tonic for treating mental disorders (59). In this chapter we feature our published literature review on the role of embelin in CNS related disorders. In the publication, we did a literature search on embelin in a systematic way using appropriate electronic database from January 2000 to February 2017. As a result of this systematic search, we then presented the efficacy of embelin against CNS related complications.

frontiers in Pharmacology

REVIEW
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Plant Derived Phytocompound, Embelin in CNS Disorders: A Systematic Review

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A Central nervous system (CNS) disease is the one which affects either the spinal cord or brain and causing neurological or psychiatric complications. During the nineteenth century, modern medicines have occupied the therapy for many ailments and are widely used these days. Herbal medicines have often maintained popularity for historical and cultural reasons and also considered safer as they originate from natural sources. Embelin is a plant-based benzoquinone which is the major active constituent of the fruits of *Embelia ribes* Burm. It is an Indo-Malaysian species, extensively used in various traditional medicine systems for treating various diseases. Several natural products including quinone derivatives, which are considered to possess better safety and efficacy profile, are known for their CNS related activity. The bright orange hydroxybenzoquinone embelin-rich fruits of *E. ribes* have become popular in ethnomedicine. The present systematic review summarizes the effects of embelin on central nervous system and related diseases. A PRISMA model for systematic review was utilized for search. Various electronic databases such as Pubmed, Springer, Scopus, ScienceDirect, and Google Scholar were searched between January 2000 and February 2016. Based on the search criteria for the literature, 13 qualified articles were selected and discussed in this review. The results of the report showed that there is a lack of translational research and not a single study was found in human. This report gives embelin a further way to be explored in clinical trials for its safety and efficacy.

Keywords: embelin, CNS disorders, neuropharmacology, neurodegenerative diseases, natural product

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INTRODUCTION

Central nervous system (CNS) is an integral part of the nervous system. It consists of the brain and spinal cord, and are associated with a number of important actions of the body. A CNS disease can be defined as one which affects either the spinal cord (myelopathy) or brain (encephalopathy) or both. The etiology of CNS involves a number of factors, for example, structural defects, infections, trauma, autoimmune disorders, tumors, neurodegeneration, and others, which may lead to neurological or neuropsychiatric or neurodegenerative or neurodevelopment disorders (Cannas et al., 2002; Upadhyay, 2014). The prevalence of CNS diseases is at least two times higher in developing countries than developed countries. According to World Health Organization (WHO), traditional medicines have become a topic of global importance. In many developing countries, a large proportion of the population relies heavily on traditional healers and phytomedicine for

primary health care requirements. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (World Health Organization, 1999; Saraf, 2012).

Embelin is chemically known as 2,5-dihydroxy-3-undecyl-1,4-benzoquinone, which is the major active constituent of the fruits from *Embelia ribes* Burm (Family: Myrsinaceae), commonly known as "False Black Pepper" (**Figure 1**). It is an Indo-Malaysian species, reported from India, Sri Lanka, Singapore, Malaysia, and South China. *Embelia ribes* Burm is extensively used in Indian, Folk, Homeopathy, Tibetian, Unani, and Siddha traditional medicinal systems for treating various ailments like chronic inflammatory disorders, heart and urinary conditions, snake and insect bites, and tumor (Radhakrishnan et al., 2012). The dried fruit is considered anthelmintic, astringent, carminative, alterative, and stimulant (Nadkarni, 1996). Embelin is already studied for its safety and toxicity profile in rodents and non-rodents. It is reported that embelin is safe up to 3 g/kg orally when tested in rodents after acute exposure. Another report on subacute toxicity after repeated administration of embelin at 10 mg/kg dose found to be safe in rats (Poojari, 2014).

Fruits of *E. ribes* have been used for the treatment of central nervous system (CNS) disorders, mental disorders and as a brain tonic in the traditional systems of medicine. Embelin was found to be useful in decreased cerebral infarction area and histopathological alteration, such as normal glial density, decreased edema, absence of lymphocytes, congestion of blood vessels, and necrosis. These reports suggest that embelin would be useful as an adjunct therapy for cerebral stroke and as a potent neuroprotective agent (Thippeswamy et al., 2011). Embelin posses all the characteristics of a compound which can cross the blood-brain barrier (BBB) and elicit an effect on the CNS (Pathan et al., 2009). Embelin reported for its CNS effect by diverse mechanisms, namely by scavenging free radicals and antioxidant effect, by inhibiting pro-inflammatory cytokines like NF-κB and p53, by modulating sodium channel, chloride conductance, and $GABA_A$ receptor, by inhibiting STAT3, XIAP, and PPARγ pathways (**Figure 2**).

Embelin has been explored and reported for various CNS disorders using cell lines and animal models. There is no single study which summarizes the effectiveness of embelin in CNS associated disorders. Although embelin proved to be effective in laboratories against various CNS disorders, but it is not being translated to humans yet. In the present systematic review, an effort is being made to systematically review all the literature available with embelin in animal and clinical research.

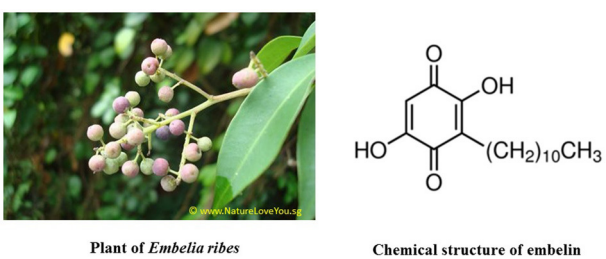

FIGURE 1 | Image of the plant *Embelia ribes* and chemical structure of embelin.

MATERIALS AND METHODS

Search Technique

The extensive literature search was done to conduct a systematic review summarizing the effects of embelin on central nervous system and related diseases. Various electronic databases were used, namely Pubmed, Springer, Scopus, ScienceDirect, and Google Scholar between the period January 2000 and February 2016. The following keywords were searched individually and in combination with the embelin: brain, trauma, CNS, neurological disorder, neurodegenerative disease, and psychological disorder.

Study Selection and Exclusion/Inclusion Criteria

The search was limited to, articles published in English language and original research articles only. Abstracts of symposiums and conferences, review articles, books, and patents were excluded due to insufficient information for evaluation and comparison. Articles which were not related to CNS diseases were excluded. Any clinical, pre-clinical, *ex-vivo*, and *in-vitro* studies were also the part of the inclusion criteria.

Data Extraction

Two separate researchers obtain data independently, and then the titles and abstracts of each article were compared to delete duplication of the data. Based on the mentioned eligibility criteria for the literature search, 14 articles were excluded and 13 qualified articles were evaluated in this study. The aim of using PRISMA statement is to help authors to understand and improve the reporting of systematic reviews and meta-analyses related to use of embelin in CNS related disorders (Moher et al., 2015). Flow diagram was prepared according to the guidelines of PRISMA-Transparent reporting of systematic reviews and meta-analyses (Moher et al., 2009).

RESULTS AND DISCUSSION

The search based on the keywords mentioned in the methodology yielded 6,448 records. After applying exclusion criteria, total articles removed were 6,435, which includes; (a) 3,470 reviews, book and patents, (b) 2,090 did not meet review criteria, (c) 402 abstracts, (d) 459 duplicates, and (e) 14 not relevant to the aim of the review based as they deal with formulations of the embelin (**Figure 2**). Thirteen eligible articles were included, compiled in **Table 1** and discussed in the present systematic review (**Figure 3**).

Anticonvulsant Activity

Mahendran et al. (2011b) isolated embelin from the berries of *E. ribes* and reported on the anticonvulsant activity of the embelin using maximal electroshock (MES) and pentylenetetrazole (PTZ). MES cause the spread of seizure similar to grandmal epilepsy. In MES method, brief high-intensity shock is applied to the head through corneal or ear electrodes with a stimulator

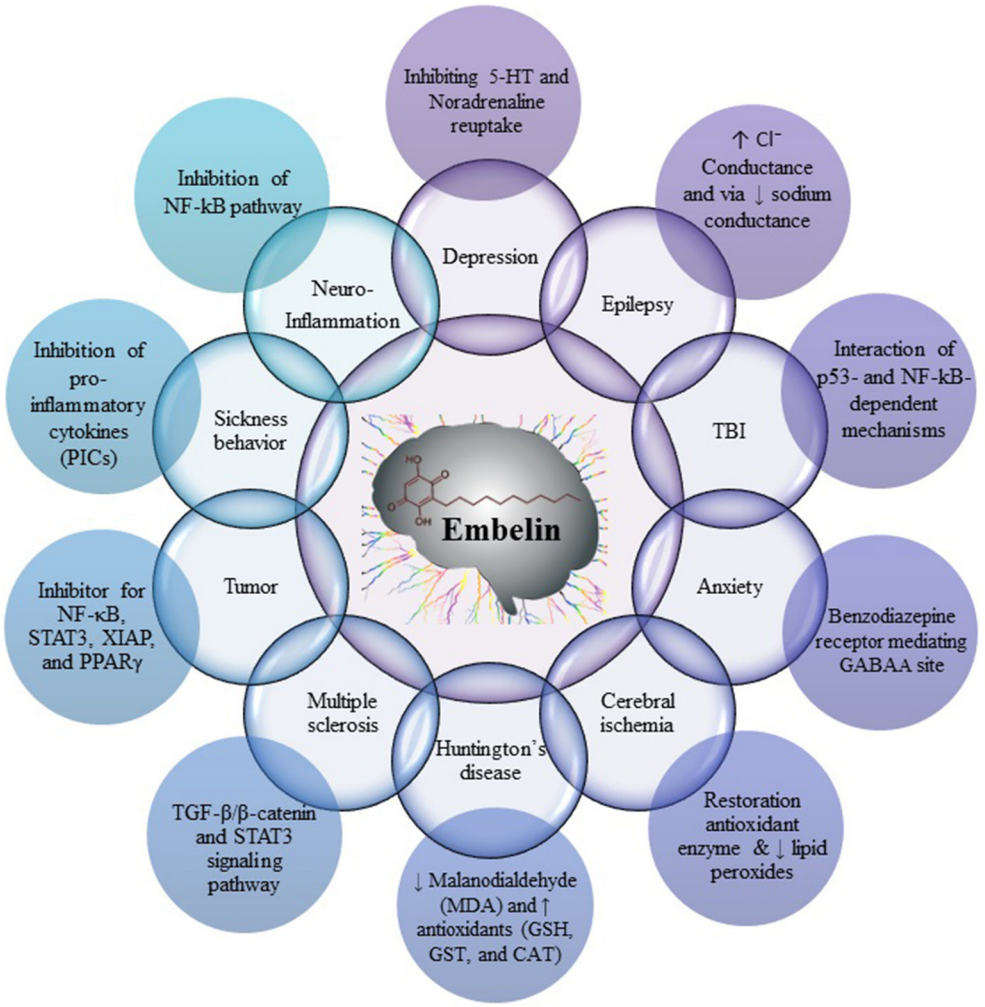

FIGURE 2 | Mechanism of action of embelin on various CNS disorders.

that either delivers a constant current to constant voltage at a frequency of 50–60/s. The MES convulsions are divided into five phases such as the phase of tonic limb flexion, the phase of tonic limb extension, the phase of clonic convulsions, stupor, and recovery or death (Castel-Branco et al., 2009). Protection against hind leg tonic extension (HLTE) in MES predicts the ability of embelin to prevent the spread of seizure discharge from the epileptic focus in the brain and suppressing generalized tonic–clonic and partial seizures. Phenytoin is said to protect against seizures by causing blockage of voltage-dependent, voltage-gated sodium channels. This block sustains repetitive high-frequency firing of action potentials. The results show that there is a complete absence of HLTE in MES model when treated with embelin 10 mg/kg dose in comparison with phenytoin. It shows that embelin at 10 mg/kg dose might act via blockade of voltage-gated sodium channels. Embelin at 2.5, 5, and 10 mg/kg shows, dose-dependent activity against MES model which act of sustaining repetitive high-frequency firing of action potential exhibiting anticonvulsant activity. Pentylenetetrazole (PTZ) is used to induce clones seizure-like behavior with increased locomotor activity. The increased chloride conductance drives the membrane potential toward the reversal potential of the Cl↓ ion which is about −65 mV in neurons, inhibiting the firing of new action potentials. This mechanism is responsible for the anti-epileptic effects of $GABA_A$ allosteric agonists. Embelin might increase chloride conductance which drives membrane potential, inhibiting the firing of action potential delaying onset of a clonic-tonic seizure in PTZ induced epilepsy. PTZ basically acts at the picrotoxin site of the $GABA_A$ receptor and reduces chloride conductance which further leads to glutamate excitation (Desmond et al., 2012).

For each seizure model, embelin was administered intraperitoneally 30 min prior to the induction of MES and PTZ. Administration of embelin (2.5 and 5 mg/kg, i.p.) showed significant ($P < 0.001$) reduction in the duration of HLTE compared to the control. Based on the results, the study

TABLE 1 | Pharmacological activities reported with embelin in central nervous system related disorders.

Sr. no.	CNS activity	Study sample	Source of embelin	Dose	Result	Number of citations	Author (year)
1	Anticonvulsant activity	Swiss albino rats (150–200 g; $n = 6$) Swiss albino mice (20–25 g; $n = 6$)	Embelin isolated from berries of *Embelia ribes*	2.5, 5, and 10 mg/kg	• ↓ in the duration of HLTE in MES (2.5 and 5 mg/kg, i.p.). • Electroshock—100% protection against mortality. • ↑ Clonic and tonic onsets at all dose.	54	Mahendran et al., 2011b
2	Antidepressant activity	Swiss albino mice (20–25 g; $n = 6$)	Embelin isolated from berries of *Embelia ribes*	2.5 and 5 mg/kg	• Antidepressant-like effect in Tail suspension test (TST). • ↓ Immobility in the Forced swimming test (FST). • Exhibited significant activity in mice TST and FST experimental models.	6	Gupta et al., 2013
3	Anxiolytic activity	Swiss albino mice ($n = 6$)	Embelin isolated from berries of *Embelia ribes*	2.5 and 5 mg/kg	• ↑ Time spent and number of entries in open arm (elevated plus maze). • ↓Decrease in the duration of immobility in light box (light and dark model). • ↑Increase rearing assisted rearing and number of square crossed (open field test). • Embelin showed its anxiolytic effect in dose-dependent manner.	6	Afzal et al., 2012
4	Sickness behavior	Male Swiss albino mice (25–30 g; $n = 8$)	Embelin isolated from berries of *Embelia ribes*	10 and 20 mg/kg	• Embelin prevented anhedonia, anorexia. • Ameliorated brain oxidative stress markers. • Protective effect of embelin in LPS-induced sickness behavior in mice.	0	Shaikh et al., 2016
5	Huntington's disease	Adult Wistar rats (190–220 g; $n = 8$)	Embelin isolated from berries of *Embelia ribes*	10 and 20 mg/kg/day	• Loss of body weight. • Decreased the oxidative stress. • Decrease of 69–76% brain lesion. • Protect the neurons from 3-NP toxicity.	1	Dhadde et al., 2016
6	Multiple sclerosis (Autoimmune encephalomyelitis, CNS inflammation)	Female C57BL/6 mice, aged 6–8 weeks ($n = 6$)	Embelin pure form	25 and 50 mg/kg	• ·↓ Human CD14+ monocyte-derived dendritic cell differentiation. • ·↓Duction in the EAE (experimental autoimmune encephalomyelitis) clinical score. • ·↓ Inflammatory Th1 and Th17 cells in EAE.	9	Xue et al., 2014
7	Traumatic brain injury	Female Sprague–Dawley rats, male C57BL/6 mice($n = 10$)	Embelin pure form	200 nM	• Inhibition of NF-κB expression of XIAP increases in PFT-treated animals. • p53 and NF-κB dependent mechanisms delayed neurodegeneration	62	Plesnila et al., 2007
8	Hypoxia-ischemia (HI) induced neurological injury	Female and male Wistar rats pups ($n = 15$)	Embelin pure form (Sigma-Aldrich, USA)	20 mg/kg embelin	• Confirm sex differences in behavioral and anatomical outcome. • XIAP acts to protect the female brain from the early HI injury.	18	Hill et al., 2011

(Continued)

TABLE 1 | Continued

Sr. no.	CNS activity	Study sample	Source of embelin	Dose	Result	Number of citations	Author (year)
9	Global ischemia/reperfusion-induced brain injury	Male Wistar rats (200–260g; $n = 6$)	Extraction of embelin from *Embelia ribes*	25 and 50 mg/kg	• ↑ Locomotor activity and hanging latency time. • ↓ Beam walking latency. • ↓ Lipid peroxidation. • ↑ Total thiol content and glutathione-S-transferase neuroprotective agent and useful in the treatment of stroke.	22	Thippeswamy et al., 2011
10	Focal cerebral ischemia brain	Male Wistar rats (200-250 g; $n = 6$)	Embelin isolated from berries of *Embelia ribes*	50, 75, 100 mg/kg	• Decreased the infarction and edema (100 mg/kg). • Decreased MDA level (75 and 100 mg/kg). • ↑ SOD and CAT (100 mg/kg).	0	Patel and Gohil, 2014
11	Cerebral ischemia	C57BL/6 male, GI female, and Ovx female mice($n = 7$)	Embelin pure form (Sigma-Aldrich, USA)	20 mg/kg	• Inhibitor of XIAP exacerbated stroke-induced injury in females but had no effect in males.	97	Siegel et al., 2011
12	Apoptosis in human glioma cells via NF-κB inhibition	Human glioma cell lines T98G, U87MG, and H4. Immortalized primary human fetal astrocytes (IM-PHFA)	Embelin pure form (Sigma-Aldrich, USA)	(0–50 μM)	• Embelin suppressed proliferation of human glioma cells. • Apoptosis in human glioma cells by inhibiting NF-κB. • ↓ NF-κB activity by reducing nuclear translocation of p65.	20	Park et al., 2013
13	Apoptosis in human glioma cells via the mitochondrial pathway	Human brain glioma U87 cells	Embelin pure form (Sigma-Aldrich, USA)	(0, 50, and 100 μg/ml)	• Time- and dose-dependent apoptosis of brain glioma cells. • Arrest the cell cycle in the G0/G1 phase. • Changes in brain glioma cell mitochondrial membrane potential. • Shifting of Bax and Bcl-2 to cause apoptosis.	6	Wang et al., 2013

demonstrated that embelin at 10 mg/kg dose could significantly reduce the duration of the HLTE in MES model. Embelin at all the three doses significantly ($P < 0.001$) decreased the onset of stupor when compared to the control. Eventually, the percentage protection reported was 100% as no mortality was observed in all the embelin treated rats when challenged with maximum electroshock. On the other hand, embelin significantly delayed the onset of clonic and tonic seizures with an increased in a survival rate in a dose-dependent manner when checked against PTZ. Embelin also exhibited significant and dose-dependent delayed the onset of clonic-tonic actions and protection from PTZ induced mortality. At 5 and 10 mg/kg doses, it exhibited 50 and 83.33% protection against mortality. This study does not include the effectiveness of embelin in chronic models of epilepsy like lithium-Pilocarpine, kindling, or intracerebroventricular kainic acid. Based on the preliminary results, Mahendran et al. (2011a) postulated that embelin is a potent anticonvulsant phytocompound and the plausible mechanism is through GABAergic modulation. But, there was no supporting information like brain GABA estimation or GABA receptor expression were included in the published report.

Antidepressant Activity

Depression is one of the common neuropsychiatric disorder which contribute to the global burden of the diseases affects about 1 in 20 people across the world (Currie and World Health Organization Regional Office for Europe, 2000). Gupta et al. (2013) reported the anti-depressant effect of embelin in experimental animals using two universally accepted experimental models: mice tail suspension test (TST) and forced swimming test (FST). Embelin was isolated from fresh fruits of *E. ribes*. The TST is used as an experimental method in scientific research to measure stress in rodents. The FST—in a rodent is used for evaluation of the antidepressant efficacy of new compounds, antidepressant drugs, and experimental development that are aimed at translating or preventing depressive-like states. It is based on the observation that if an animal is subjected to short term inescapable stress then it will become immobile. It has been described as rendering

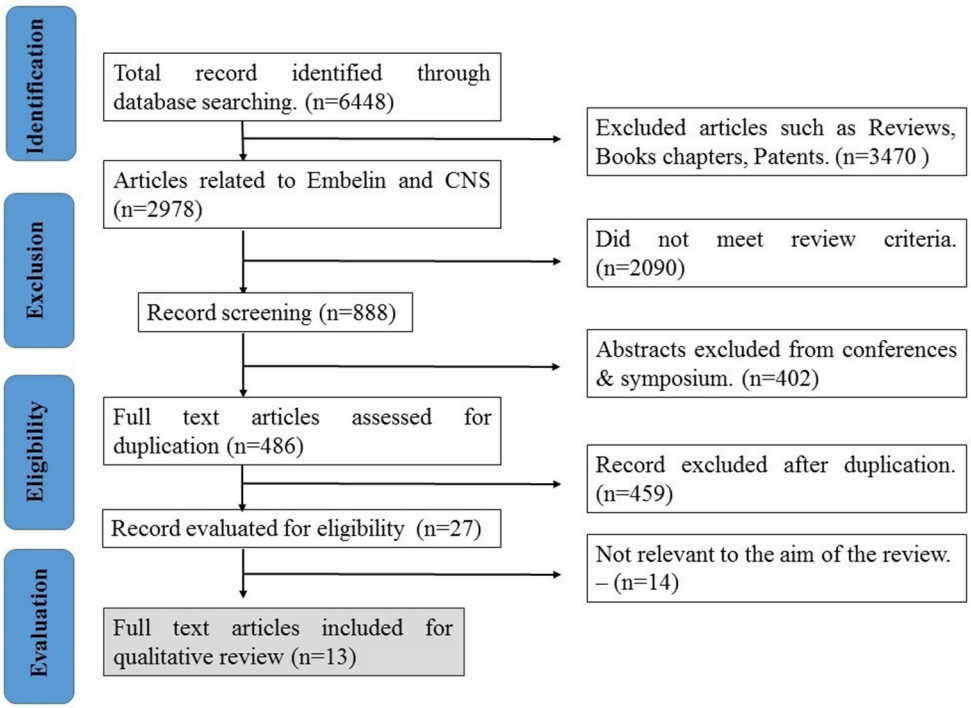

FIGURE 3 | Flow chart of study selection procedure.

a situation in which "behavioral despair" is induced; that is, the animal loses hope to escape the stressful environment. It is well-known that compounds which selectively bind to high-affinity benzodiazepine receptors possess both anxiolytic and antidepressant effects. The anxiolytic effect of embelin was shown to be mediated through the effect on the GABA system. The similar mechanism of antidepressant action cannot be ruled out (Afzal et al., 2012). It is, therefore, reasonable to assume that the observed antidepressant-like activity of embelin could be attributed to its known antioxidant effect. $GABA_A$ receptors are allosteric modulatory sites for benzodiazepine. They are probably composed of five protein subunits, at least some of which belong to different subunit classes. So far $GABA_A$ receptors have been identified as six alpha–four beta–three gamma-, and delta- and two-rho $= p$ subunits. A 3D-structural model similarity, further shows that embelin is closely related with the well-known antioxidant alpha-tocopherol (AT, vitamin E), especially in the long-chain non-polar tails and polar phenolic heads (Lobato et al., 2010). Embelin at 2.5 and 5 mg/kg dose administered orally and tested using tail suspension test (TST) and forced swimming test (FST) in mice. It was found to effectively reduce immobile time in both experimental models suggesting its antidepressant potential. Embelin (5 mg/kg) was reported to be comparably better than the standard antidepressant drug, imipramine (15 mg/kg), which is a tricyclic antidepressant drug (Gupta et al., 2013). As a criticism, Gupta et al. (2013) fail to mention about actual activity of embelin as an anti-depressant, which may be via inhibiting 5-hydroxytryptamine receptors (5-HT) and noradrenaline (NA) reuptake. In their study, imipramine was used as a positive control which has the known antidepressant activity and it acts by inhibiting NA and 5-HT reuptake into neurons.

Anxiolytic Activity

Anxiety is a feeling of discontent, such as fear or worry that can be intense or gentle. Everyone has feelings of anxiety at some point in their life for example, you may feel worried and anxious about a job interview or having a medical examination or sitting an exam. An anxiolytic is a medication or other intervention that inhibits anxiety. This effect is in contrast to anxiogenic agents, which increases anxiety. Afzal et al. (2012) revealed the anxiolytic potential of embelin using behavioral models of anxiety. The elevated plus maze (EPM) is a test used to measure anxiety in laboratory animals. The test uses two open and two enclosed arm apparatus with an elevated, plus-shaped (+). The behavioral model is based on the general disinclination of rodents to open spaces. This disinclination leads to the behavior termed thigmotaxis, a greater liking to remain in enclosed spaces or close to the edges of a bounded space. Reduction in anxiety is indicated in the plus-maze by an increase in the amount of time spent or entries in the open arms (time or entries in open arms/total time or total entries in open or closed arms; Walf and Frye, 2007). Open field test (OFT) is an experiment used to assay general locomotor activity levels and anxiety in rodents. Rodents display a natural aversion to brightly light areas. They also have an urge to explore a perceived threatening stimulus. The result of these two conflicting drives is anxiety. The increase in exploratory behavior leads to decreased anxiety. Increased

anxiety will result in less locomotor motion and the animal will have a preference to remain at the edges of the field (Ramos, 2008). The light/dark test is based on the natural version of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stress, that is, novel environment and light. The test apparatus consists of a small dark safe compartment (one-third) and a large illuminated preference compartment (two-thirds; Bourin and Hascoët, 2003). Embelin at 5 mg/kg dose significantly increased the percentage of time spent and the number of entries in open arm in EPM apparatus. Percentage of time spent in the open arms and number of open arm entries was significantly ($P < 0.01$ and $P < 0.001$) increased by embelin (2.5 and 5 mg/kg) and diazepam. Time spent in the open arm by animal treated with embelin 2.5 and 5 mg/kg dose was 47.92 ± 1.25 and 66.17 ± 1.93 and no. of entries in the open arm by animal treated with embelin 2.5 and 5 mg/kg dose was 5.61 ± 0.47 and 7.90 ± 0.45 significant. The result shows that embelin exhibited dose-dependent activity as an anxiolytic in mice EPM-test. In the open field test, embelin exhibited a significant increase in a number of rearing, assisted rearing and number of the crossing. A number of rearing in open field test by animal treated with embelin 2.5 and 5 mg/kg dose was 17.68 ± 0.52 and 20.33 ± 0.59 and number of assisted rearing in open field test by animal treated with embelin 2.5 and 5 mg/kg dose was 16.20 ± 1.00 and 21.12 ± 1.2. In light and dark model, embelin produced a significant increase in time spent, the number of crossing and decrease in the duration of immobility in a light box. The animals treated with diazepam (1 mg/kg) and embelin (2.5 and 5 mg/kg) showed significant ($P < 0.05$ and $P < 0.001$) increase in the time spent in the lighted box and decrease in the time spent in the dark box. Time spent in the lighted box (s) by animal treated with embelin 2.5 and 5 mg/kg dose was 94.92 ± 1.73 and 116.8 ± 4.24 and time spent in a dark box (s) by animal treated with embelin 2.5 and 5 mg/kg dose was 167.0 ± 4.59 and 148.3 ± 1.26. Embelin at 2.5 mg/kg dose, failed to produce any significant change in the number of crossing and duration of immobility. Afzal et al. (2012) concluded that embelin exhibits significant anxiolytic activity in a dose dependant manner. They proposed that the observed activity could be due to an antagonistc effect on GABA receptor complex as most of the anxiolytic and antidepressant molecules selectively bind to high-affinity benzodiazepine binding site, present on GABA receptor. Both Gupta et al. (2013) and Afzal et al. (2012) contradicted about vehicle used to dissolve embelin, they mentioned two different vehicles, olive oil and 1% Tween 80 (v/v) as embelin has poor water solubility.

Sickness Behavior

During the course of an infection, the adaptive behavioral changes that develop in ill individuals is known as sickness behavior. It is relevant to understand depression and some aspects of the suffering in any disease. Sickness behavior is like a complex behavior induced by infections and immune trauma and mediated by pro-inflammatory cytokines. Some of the evidence state that sickness behavior is mediated through the effects of pro-inflammatory cytokines (PICs), such as IL-1, TNFα, and IL-6 (Maes et al., 2012). Embelin has been reported to possess neuroprotective, anxiolytic and antiinflammatory assets and has been shown to inhibit Nf-κB pathway and cytokine production (Mahendran et al., 2011a). Few characteristics of the behavioral pattern including malaise, hyperalgesia, pyrexia, listlessness, and disinterest in social interactions with the environment, lethargy, behavioral inhibition, exploration and grooming, reduction of reproductive performance, anorexia and weight loss, failure to concentrate, and anxiety (Maes et al., 2012). The effect of embelin was evaluated in sickness behavior in mice by Shaikh et al. (2016). Adult male Swiss albino mice were pre-treated with embelin (10 and 20 mg/kg per oral) for 3 days and then challenged with lipopolysaccharide (LPS; 400 μg/kg intraperitoneal). In EPM-test, pre-treatment with embelin (10 and 20 mg/kg) and dexamethasone (1 mg/kg) significantly reversed LPS-mediated effects and increased both the number of open arm entries (3.00 ± 0.53, 3.12 ± 0.58 and 3.12 ± 0.47, respectively) and time spent in open arm (15.38 ± 3.19, 14.00 ± 2.67 and 13.63 ± 1.94 s, respectively) when compared with LPS-alone. In light–dark box test, Pre-treatment with both the tested doses of embelin and dexamethasone (1 mg/kg) prior to LPS-shot significantly increased the time spent in the light compartment (33.88 ± 2.11, 43.75 ± 6.81 and 34.13 ± 4.38 s, respectively). In the forced swim test, embelin (10 and 20 mg/kg) prior to LPS-injection significantly decreased the floating time (78.75 ± 5.03 and 62.88 ± 5.03 s, respectively) when compared with LPS-alone-administered group. In social behavior tests, social exploration was measured just before the administration of LPS and again 2, 4, 8, and 24 h later. LPS-associated reduction in social behavior was attenuated by pre-treatment with embelin 10 mg/kg (20.61 ± 4.15%, 29.24 ± 8.45% and 56.61 ± 5.44%, respectively) and 20 mg/kg (38.41 ± 5.90%, 44.78 ± 5.17% and 63.55 ± 5.95%, respectively), dexamethasone 1 mg/kg (43.84 ± 5.31% 49.12 ± 2.95% and 64.87 ± 4.42%, respectively) when compared with LPS-alone-treated animals. In the open field test, pre-treatment with embelin (10 and 20 mg/kg) and dexamethasone (1 mg/kg) significantly attenuated LPS-induced changes and increased the peripheral, central and total number of line crossings and a number of climbs rear when compared with LPS-alone-treated group. Food and water intake test, pre-treatment of LPS-challenged mice with embelin (10 and 20 mg/kg) and dexamethasone (1 mg/kg) significantly reversed LPS-induced anorexia and adipsia in comparison to animals with LPS-alone-treated group. This all comparative finding eventually concluded that embelin is neuroprotective against LPS-induced sickness behavior in mice (Shaikh et al., 2016).

Huntington's Disease

Huntington's disease (HD) is a progressive neurodegenerative disorder associated with severe degeneration of basal ganglia neurons, which affects muscle coordination and leads to mental decline and behavioral symptoms. Systemic administration of 3-nitropropionic acid (3-NP), an inhibitor of the mitochondrial citric acid cycle, results in a progressive locomotor deterioration resembling that of HD. It differs mechanistically from excitotoxic lesions in that 3-NP irreversibly inhibits the mitochondrial citric acid cycle and leads to depressed ATP levels and elevated lactate concentrations (Borlongan et al., 1997; Brouillet, 2014).

The study carried out by Dhadde et al. (2016) evaluated the neuroprotective potential of embelin against 3-nitropropionic acid (NP) induced experimental HD in rats. 3-NP significantly altered the behavioral and neuronal antioxidant status and caused significant neuronal damage in the striatal region. Elevated levels of malondialdehyde (MDA) and decreased levels of antioxidants (GSH, GST, and CAT) in the 3-NP treated rat brains supports the increased oxidative stress in HD. Behavioral tests were carried in the following order: neurological scoring, locomotor activity, EPM-test, beam walking test and hanging wire test. Biochemical estimation and brain lesion measurement were carried out in order to explore the molecular and structural differences of embelin in the brain. Administration of 3-NP alone shows motor abnormalities, decreased locomotor counts, loss of memory in EPM, decreased motor coordination in beam walking test, decreased hanging latency on hanging wire test and even 3-NP alone treatment resulted in highly significant ($p < 0.001$) reduction in body weight. In neurological scoring, none of the rats in embelin treated groups (10 and 20 mg/kg) showed hind limb paralysis and inability to move indicating its potent activity in reversing 3-NP induced motor abnormalities. The treatment with embelin at both the doses (10 and 20 mg/kg) reversed the decrease in locomotor counts induced by 3-NP toward the normal, and it was found to be 129.2 ± 5.58 and 160 ± 11.14, thus both the doses of embelin showed improvement in the locomotor count. In the EPM-test, embelin treatment at 10 mg/kg body weight significantly ($p < 0.01$) Reversed the memory loss ($27.73 \pm 3.92\%$) induced by 3-NP toward the normal, when compared with 3-NP alone treated animals. However, embelin at 20 mg/kg body weight dose showed a complete reversal of 3-NP induced memory loss same as a normal control group. At beam walking test, treatment dose of embelin (10 and 20 mg/kg) to 3-NP treated rats significantly ($p < 0.001$) improved the motor coordination and body balance. These animals traversed the beam in 5.11 ± 0.66 and 5.51 ± 0.72 s, respectively. In hanging wire test, embelin at doses of 10 and 20 mg/kg increased 3-NP induced decrease in hanging latency period, with values 36.66 ± 1.78 ($p < 0.05$) and 49.34 ± 2.62 s. The percentage decrease in the brain lesion area in both these groups was 69.59 and 76.21%, respectively. Embelin at 10 and 20 mg/kg to 3-NP treated animals significantly ($p < 0.01$) reduced the brain lesion area to 4.32 ± 0.44 and $3.38 \pm 0.17\%$, respectively. Embelin treatment significantly protected neurons against 3-NP induced toxicity and reduced brain lesion up to 76%. It also exhibited a significant antioxidant and improved behavioral alterations induced by 3-NP. It is postulated that effectiveness of embelin could be due to its antioxidant potential and ability of embelin to modulate Ca^{2+} influx associated with increased brain glutamate levels (Dhadde et al., 2016). In 3-NP induced HD like condition model in rats, embelin found to be effective neuroprotectant.

Multiple Sclerosis (MS)

A chronic, typically progressive damage to the sheaths of nerve cells in the brain and spinal cord is termed as multiple sclerosis (MS). Symptoms may include numbness, impairment of speech and muscular coordination, blurred vision, and severe fatigue (Loma and Heyman, 2011). Animal models of brain inflammation are used to study autoimmune encephalomyelitis, or experimental allergic encephalomyelitis (EAE). Dendritic cells (DCs) have a pivotal role in the immune response and in stimulating naïve T-lymphocytes. Induction and maintenance of self-tolerance is a critical role of DCs and the failure of which can lead to autoimmune/inflammatory diseases. Embelin concentrations of 10, 30, and 60 μM, inhibits the differentiation and endocytosis of Human Monocyte-Derived dendritic cell (DCs). Compared with the day 5 untreated iDCs, a significant dose-dependent reduction in cell surface marker expression was observed in EB-treated cells. These results indicate that embelin inhibited the differentiation of human $CD14^+$ monocytes into DCs in a dose-dependent manner. DC-derived cytokines are required for the polarization of the adaptive immune response. Therefore, Xue et al. (2014) investigated the potential effects of embelin on the regulation of the expression of the cell-polarizing cytokines. The production of the inflammatory cytokine tumor necrosis factor-alpha (TNF-α), the Th1 cell polarizing cytokine IL-12p35, the Th17 cell-polarizing cytokines IL-6 and IL-12/23p40, and the Th1 cytokine IFN-γ is substantially inhibited by embelin. Embelin suppressed the DC-mediated polarization of Th1 and Th17 cells and that it may be useful for the treatment of autoimmune inflammatory diseases that are mediated by Th1 and Th17 cells. Embelin ameliorates the clinical severity of experimental autoimmune encephalomyelitis (EAE). Compared with PBS-treated mice, the incidence of clinical symptoms in the 25 and 50 mg/kg/day EB-treated mice were reduced. These data suggest that embelin significantly ameliorates the clinical outcome of EAE. TGF-β/β-catenin and STAT3 signaling pathway are used by embelin to inhibit DC function, which leads to a reduction in the EAE clinical score and in CNS inflammation and demyelination. The novel finding of this study is that the anti-inflammatory effect of embelin appears to require the presence of functional TGF-β/β-catenin and the absence of activated STAT3 in DCs. It was also found that embelin-induced inhibition of the differentiation of Th1 and Th17 cells was associated with a down regulation of the production of Th1-polarizing and Th17-polarizing. Embelin, a novel XIAP inhibitor, significantly increased TGF-β/β-catenin signaling and decreased STAT3 phosphorylation in DCs (Xue et al., 2014).

Embelin is a potent inhibitor of the activation of pro-inflammatory transcription factors, such as nuclear factor kappa B and signal transducer and activator of transcription 3 (STAT3; Heo et al., 2011). Embelin has been shown to inhibit the X-linked inhibitor of apoptosis protein and various inflammatory pathways (Ahn et al., 2007). In one of the study, Xue et al. (2014) demonstrated that embelin possess a strong therapeutic potential for autoimmune inflammatory conditions in MS. The study revealed the role of embelin in modulating newer regulatory mechanisms and molecular targets essential for the effectiveness in EAE. Therefore, these reports suggest that embelin could be used as a therapeutic agent to control pathological conditions, such as MS and other inflammatory autoimmune diseases, that are induced by the functional expansion of Th1 and Th17 cells.

Traumatic Brain Injury

Traumatic brain injury (TBI) is one of the common causes of mortality in both children and young adults. Survivors have many complications like brain edema and programmed death of

neuronal cells following acute and chronic neurodegeneration. The study carried out by a team from five European institutes addresses the role and interaction of p53 and NF-κB-dependent mechanisms in TBI induced delayed neurodegeneration (Plesnila et al., 2007). Neuroprotection mediated by PFT is reversed by embelin in three different *in-vitro* models of neuronal cell death induced by camptothecin, glutamate, or oxygen-glucose deprivation (OGD). Embelin was used to evaluate whether enhanced X-chromosomal linked inhibitor of apoptosis (XIAP) levels is indeed involved in neuroprotection by pifithrin-a (PFT). Hence, they strongly suggest the involvement of NF-κB dependent regulation of XIAP in the observed neuroprotective effect (Plesnila et al., 2007).

Hypoxia-Ischemia (HI) Induced Neurological Injury

Hypoxia-ischemia (HI) occurs when there is a deficiency in both oxygen and blood supply, which results in neonatal neurological impairment. Hill et al. (2011) tested on the caspase-dependent progression of apoptosis using embelin which is known as potent XIAP inhibitors in order to prove that sexes influences in differing pathways of cell death due to HI. So they found out that embelin inhibits XIAP by binding to BIR3 domain and thus eventually increase in cell death through a caspase-dependent pathway. Similarly, the behavioral outcomes showed that through XIAP inhibition, HI induced female rats possess severe behavioral deficits compared to HI males. These *in-vivo* data revealed that there were significant differences in severity of cognitive deficits in male infants compared to female infants with HI. This phenomenon supports the evidence of activation of caspase-independent cell death in males compared to females that activate caspase-dependent cascade following neonatal ischemia. By using embelin as XIAP inhibitor, they could conclude that gender influences cell death mechanism following HI injuries and suggest that it is very important to develop a sex-specific neuroprotection to cure HI.

Ischemic Stroke

The majority of strokes occur when blood vessels to the brain become narrowed or clogged with fatty deposits called plaque, This cuts off blood flow to brain cells. A stroke caused by lack of blood reaching part of the brain is called an ischemic stroke. Stroke is the third major cause of mortality and the leading cause of long-term disability. Ischemic stroke accounts for ∼80% of all strokes (Jauch et al., 2013). Ischemic stroke can be divided into two main types: thrombotic and embolic. Deprived of oxygen and other nutrients, the brain suffers damage as a result of the stroke. A thrombotic stroke occurs when diseased or damaged cerebral arteries become blocked by the formation of a blood clot within the brain (Rha and Saver, 2007). In order to investigate the mechanisms underlying injury after ischemic stroke as well as to develop effective therapeutic approaches to the disease, several ischemic stroke models have been developed in a variety of species. Models of stroke that can be used in rodents are becoming increasingly popular at the bench because (1) genetically-engineered animals; (2) a number of neurosensory and motor behavior outcomes; (3) fewer animal welfare concerns. In general, there are four major types of animal models of ischemic stroke: (1) complete global cerebral ischemia; (2) incomplete global ischemia; (3) focal cerebral ischemia and (4) Multifocal cerebral ischemia (Liu and McCullough, 2011, **Figure 4**).

Global Ischemia

Brain tissue and cells require oxygen and nutrients to sustain survival and contribute to standard neural operating procedures which are deprived in the a global ischemic event. This leads to the death of brain tissue or cerebral infarction/ischemic stroke which is due to poor oxygen supply or cerebral hypoxia. Siegel et al. (2011) also hypothesized that caspase dependent mechanism of ischemic cell death is also influenced by gender differences. They found that XIAP mRNA level was higher in the normal female mice brain compared to stroke induced female mice whereas no differences were observed in the male brain. They reported that embelin decreased the association between XIAP and Caspase-3 in both sexes and was acting as an XIAP inhibitor. Based on the results, larger brain infarcts were seen in embelin treated ovariectomized (Ovx) females compared to gonadally intact (GI) females. The effects of embelin on infarct exacerbation may be due to independent of circulating estrogen levels. Siegel et al. (2011) concluded that embelin treatment significantly increases stroke-induced injury in females but had no effect in males. This shows that XIAP is an important mediator of sex-specific responses after stroke.

Thippeswamy et al. (2011) investigated the protective role of embelin in transient global ischemia induced by occluding bilateral common carotid arteries followed by reperfusion. Embelin pre-treated rats significantly improved locomotion. Vestibulomotor function was assessed by beam walking test and pre-treatment with embelin significantly decreased beam walking latency when compared with ischemic control animals. Grip strength was measured using Hanging Wire test and embelin treated animals had better and longer hanging time compared to ischemic control. The behavioral observations were very well-supported by biochemical estimations, where embelin found to be modulating the lipid peroxidation, the total thiol content and glutathione-S-transferase activity in brain homogenates. Histopathological studies confirmed, decrease in the infarct size in embelin-treated animals.

Focal Cerebral Ischemia

Cerebral ischemia is characterized by the inadequate oxygenated blood supply to the brain leads to the death of brain tissue It has been well-studied that reactive oxygen species play a key role in the pathogenesis of cerebral ischemia (Woodruff et al., 2011). Embelin is reported to have potent antioxidant activity and its chemical structure is similar to antioxidant coenzyme Q_{10} (Matthews et al., 1998). Patel and Gohil, investigated the effect of embelin against focal cerebral ischemia using the middle cerebral artery occlusion (MCAO) model. The model of MCAO involves the insertion of a surgical filament into the external carotid artery and threading it forward into the internal carotid artery (ICA) until the tip occludes the origin of the MCA, resulting in a cessation of blood flow and subsequent brain

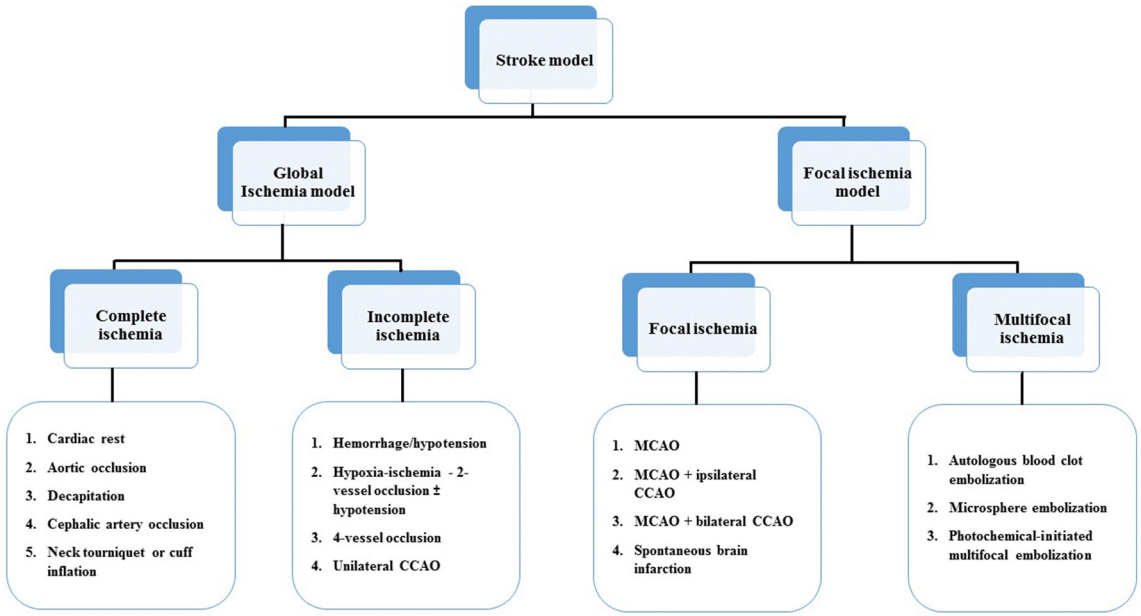

FIGURE 4 | Animal models of ischemic stroke model.

infarction in the MCA territory. Male Wistar rats were treated with embelin (50, 75, and 100 mg/kg, p.o.) for 20 days, followed by MCAO-induced focal cerebral ischemia and the parameters evaluated were infarct size and score. The antioxidant evaluation includes MDA, superoxide dismutase (SOD), and catalase (CAT) in brain homogenates. Embelin significantly decreased infarct size and improved infarct score. Embelin also decreased the MDA level whereas increased SOD and CAT level as compared to ischemic control group. The probable mechanisms by which embelin could be effective in cerebral ischemic condition is by the restoration of altered antioxidant enzyme activity as well as decreasing the production of lipid peroxides (Patel and Gohil, 2014). Some herbal medicines or their products having anti-oxidant activity have been suggested to protect against ischemic reperfusion injury, and thus justifying their use in cerebral ischemic patients.

Brain Cancer/Apoptosis in Human Cancer Cells

NF-κB Inhibition

Glioblastoma is known to be the most aggressive primary brain malignancy with median survival rates (Chou et al., 2015). Embelin is an active compound that acts an inhibitor for NF-κB, STAT3, XIAP, and PPARγ to induce growth suppression and apoptosis in human cancer cells (Park et al., 2013). Embelin is a small-molecule inhibitor of an XIAP, which has the ability to specifically inhibit XIAP of various types of a tumor cell to control and regulate the apoptosis (Wang et al., 2013). A recent finding shows that embelin also enhanced TRAIL-mediated apoptosis Allensworth et al. (2012) and thus on the basis of above reference study, Park et al. (2013) suggested that embelin may be a good anti-cancer agent with less toxicity in normal cells. IκBs regulate nuclear translocation and activation of NF-κB, embelin decreases phosphorylation of IκBα in a dose- and a time-dependent manner, which indicates that embelin activates IκBα that is a negative regulator of NF-κB. In addition, furthermore decreased NF-κB activity as a transcriptional activator and they found that embelin reduced nuclear translocation of NF-κB. Embelin suppressed proliferation of human glioma cells without affecting the normal, immortalized human astrocytes. It also has been reported to induce apoptosis in human glioma cells by inhibiting NF-κB which plays an important role in cell proliferation and survival of tumor. However, embelin has found to show no inhibitory effect on XIAP in glioma cells, although this active compound was discovered as an XIAP inhibitor. Besides that, overexpression of p65 was decreased in embelin induced apoptosis glioma cells. So, they concluded that embelin could be a potent novel therapeutic compound by blocking cancer cell proliferation and inducing apoptosis through NF-κB inhibition.

Mitochondrial Pathway

On the other hand, in support of the above finding, Wang et al. (2013) investigated the role of mitochondrial pathway played in embelin-induced brain glioma cell apoptosis and the effect of embelin on the cell cycle. Expression of apoptosis-associated proteins, Bcl-2, Bcl-xL, Bax, and Bak, as well as cytochrome-*c* levels, were determined by performing western blot analysis. Embelin was found to be apoptotic to brain glioma cells in a time and dose-dependent manner. The observed effect could be due to arrest of the cell cycle in the G0/G1 phase. Changes in mitochondrial membrane potential were caused by embelin in brain glioma cell. Additionally, embelin regulated the shifting of Bax and Bcl-2 to promote the mitochondrial release

of cytochrome *c*, thus activating the caspase proteins to cause apoptosis. Thus, embelin induces apoptosis in brain glioma cells is closely associated with the mitochondrial pathway (Wang et al., 2013).

Blood-Brain Barrier (BBB)—Cerebral Ischemia

Blood-brain barrier plays an important role in drug delivery to the CNS. Blood-brain barrier restricts, facilitates and regulates many substances from entering the CNS. It also secretes substances into the blood and the CNS (Banks, 2009). The entry of compounds across the BBB depends on their lipid solubility based on the estimation of oil/water partition coefficient (Laterra and Betz, 1999). Besides that, molecular weight, charge, tertiary structure and degree of protein binding are also among the factors in addition to lipid solubility affecting the ability of a drug to cross the BBB (Banks, 2009).

According to Pathan et al. (2009) a drug is likely to be able to transport across the BBB, if it possesses some important properties like, the compound should be in un-ionized form, partition coefficient (log P)-value should be near 2, molecular weight must be <400 Da and cumulative number of hydrogen bonds should not go beyond 8–10. According to this embelin is un-ionized molecule with log P-value of 4.83, the molecular weight is 294.38 and cumulative H bonds are 6. These properties of embelin make it permeable to BBB. So far, not a single study reported BBB permeability of embelin in *in-vitro* model. However, Siegel et al. (2011) performed *in-vivo* BBB permeability study and reported that embelin could cross the BBB. They performed liquid chromatography/tandem mass spectrometry (LC-MS/MS) on male and female sham and stroke brains. Embelin (20 mg/kg s.c.) was dosed for 3 days and it was found that the brain concentrations were elevated in both the sham and stroke mice, but the level was significantly higher in stroke mice which were close to reported IC_{50} for embelin (4.1 ± 1.1 μM).

Safety and Toxicity

Acute toxicity studies in mice treated with embelin 50 and 100 mg/kg oral dose showed no significant body weight change, mortality or apparent toxic effects, signifying its safety profile. This study suggests that embelin is safe on acute administration (Gupta et al., 1976). The LD50-value of embelin was reported as 44 mg/kg by i.p. route. Embelin in doses of 10 mg to 3 g/kg given orally to rats and mice did not show any toxic effects. Subacute toxicity on 10 weeks administration of 10 mg/kg of embelin to rats also indicated the drug to be free from toxic effects on heart, liver, kidney, and bone marrow, thereby having a high margin of safety in acute toxicity studies (Rathinam et al., 1976).

The toxicity of emblin has been assessed in female cyclic rats. Its administration at a dose of 120 mg/kg body weight did not cause any changes in the weight of liver, kidney, and spleen, however, the wet weight of the adrenals showed a remarkable increase. Biochemical constituents such as protein and glycogen did not show any change in these organs except in the adrenal where a significant increase was observed. The activity of acid and alkaline phosphatase was increased in the kidney and adrenal. These toxic effects seem to be due to exposure of a very high dose i.e., 120 mg/kg, whereas LD_{50} reported was around 44 mg/kg.

Administration of embelin for 6 weeks caused severe pathological changes in the liver and kidney which mainly included disintegration, necrotic changes, and perinuclear vacuolation. Marked tubular damage was observed in the kidneys. The adrenals showed hypertrophy and the histological features of the spleen remained unchanged (Prakash, 1994). In chronic toxicity study, the administration of embelin to Wistar rats at a dose of 50 mg/kg/day for 14 weeks did not cause any extreme drop in the blood counts but showed toxic effects on the hematopoietic cells (Sreepriya and Bali, 2006). Previous studies had also reported the non-toxic nature of embelin on hematopoietic cells when administered for 6 months in mice, rats, and monkeys (Radhakrishnan and Gnanamani, 2014).

For *in-vitro* cytotoxicity studies, embelin showed the toxic effect at 217 μg /ml to lung fibroblasts (Feresin et al., 2003). IC_{50} of 16.85 and 27.52 μM of embelin was calculated against mouse lymphocytes and mouse macrophages, respectively (Sreepriya and Bali, 2006). Isolated ovarian cells were directly challenged with embelin and showed a direct effect on isolated ovarian cells (Simukoko, 2000). It did not show the toxic effect on human fibroblasts at 20 μg/ml for 72 h in an *in-vitro* setting. Embelin was most active against sarcoma (XC) cells after 72 h of incubation (ED50 8 μg/ml) and slightly less active against Murine melanoma (B16) cells (ED50 13 μg/ml). An encouraging observation is a fact, that at these concentrations, embelin did not affect normal cells (HSF; Podolak et al., 2005).

Overall toxicity studies revealed that embelin at therapeutic doses found to be non-toxic and safe to use. Higher doses of embelin exhibit some sort of toxicity, but these doses are well above LD_{50}-value and toxic effects are very much expected. There is also a need to carry out detailed toxicity study of embelin as per the International Council for Harmonization (ICH) safety guidelines.

CONCLUSION AND FUTURE DIRECTIONS

Embelin is the main constituent found in the plant *E. ribes.* Embelin posses favorable physical and chemical properties and its ability to cross the blood brain barrier make it a suitable candidate for the treatment of CNS disorders. In the present systematic review, an attempt was made to compile and discuss the efficacy of embelin against CNS complications. Embelin had been studied using various *in-vitro* prototypes and *in-vivo* animal models. It is well-reported that embelin exhibit strong anticonvulsant, anxiolytic, antidepressant properties and also improve conditions like sickness behavior, Huntington's disease, multiple sclerosis, cerebral ischemia and TBI.

Although a vast number of activities have been reported with embelin in experimental settings, there is not a single human study found on embelin related to CNS activity. None of the animal experimental outcomes was translated into human clinical research. One of the potential reasons for the non-translational research could be a lack of detailed safety and toxicity profile. Future pre-clinical and clinical trials are required

to support the safety and efficacy of this active compound. Once safety profile is established, embelin should be taken up for clinical trials. As embelin is being studied for a rich number of CNS activities, a controlled human clinical trial will open up a new horizon for this promising molecule.

AUTHOR CONTRIBUTIONS

UK and SB has equal contribution for first author. MS, UK, and SB contributed in perceiving and designing the study. UK and SB equally contributed with literature search and collection of data for the study. Data analysis and draft of the manuscript were completed by all authors. All the authors approved the content of the manuscript.

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Chapter 4

4.1 Introduction

Cholinergic deficit is a neuropathological condition associated with memory loss and it is interconnected with the severity of Alzheimer's disease (60). Therefore, restoration of cholinergic function by increasing the availability of acetylcholine remains a rational target for treating AD symptoms (61). Based on the cholinergic hypothesis, brain cholinergic function can be enhanced by inhibiting AChE, which will increase ACh activity (62). Scopolamine, a muscarinic antagonist had been extensively used to induce experimental models of Alzheimer's disease that affect learning and memory functions (63). A clinical observation on scopolamine treatment as a premedication for anesthesia has been reported to cause amnesia, (64) most likely caused by a blockade of cholinergic signaling. Studies have reported that scopolamine-induced amnesia model could be used to evaluate the efficacy of compounds that have the potential to be developed as therapeutic agents for AD (65).

In our study, we evaluated the neuroprotective effect of embelin on nootropics condition, as well as in scopolamine-induced amnesia rat model. We described the findings of this study in the following publication entitled ‘Amelioration of Cognitive Deficit by Embelin in a Scopolamine-Induced Alzheimer’s Disease-Like Condition in a Rat Model’.

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Amelioration of Cognitive Deficit by Embelin in a Scopolamine-Induced Alzheimer's Disease-Like Condition in a Rat Model

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Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is one of the active components (2.3%) found in *Embelia ribes* Burm fruits. As determined via *in vitro* AChE inhibition assay, embelin can inhibit the acetylcholinesterase enzyme. Therefore, embelin can be utilized as a therapeutic compound, after further screening has been conducted for its use in the treatment of Alzheimer's disease (AD). In this study, the nootropic and anti-amnesic effects of embelin on scopolamine-induced amnesia in rats were evaluated. Rats were treated once daily with embelin (0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg) and donepezil (1 mg/kg) intraperitoneally (i.p.) for 17 days. During the final 9 days of treatment, a daily injection of scopolamine (1 mg/kg) was administered to induce cognitive deficits. Besides that, behavioral analysis was carried out to assess the rats' learning and memory functions. Meanwhile, hippocampal tissues were extracted for gene expression, neurotransmitter, and immunocytochemistry studies. Embelin was found to significantly improve the recognition index and memory retention in the novel object recognition (NOR) and elevated plus maze (EPM) tests, respectively. Furthermore, embelin at certain doses (0.3 mg/kg, 0.6 mg/kg, and 1.2 mg/kg) significantly exhibited a memory-enhancing effect in the absence of scopolamine, besides improving the recognition index when challenged with chronic scopolamine treatment. Moreover, in the EPM test, embelin treated rats (0.6 mg/kg) showed an increase in inflection ratio in nootropic activity. However, the increase was not significant in chronic scopolamine model. In addition, embelin contributed toward the elevated expression of BDNF, CREB1, and scavengers enzymes (SOD1 and CAT) mRNA levels. Next, pretreatment of rats with embelin mitigated scopolamine-induced neurochemical and histological changes in a manner comparable to donepezil. These research findings suggest that embelin is a nootropic compound, which also possesses an anti-amnesic ability that is displayed against scopolamine-induced memory impairment in rats. Hence, embelin could be a promising compound to treat AD.

Keywords: embelin, Alzheimer's disease, cognition, neuroprotective, anti-amnesic effect

INTRODUCTION

Alzheimer's disease (AD) is known as the leading cause of dementia amongst people aged 65 and older (Ghumatkar et al., 2015). This age-related disease affects millions of individuals, and it is estimated that by 2050, 1 in 85 people worldwide will be suffering from AD (Brookmeyer et al., 2007). According to Tanzi and Bertram (2005), AD is a progressive and chronic neurodegenerative disorder which displays global cognitive decline involving memory, orientation, judgment, and reasoning. The key features of AD's pathogenesis are the gradual amassing of the protein fragment beta-amyloid (plaques) and twisted fibers of the protein tau (tangles), outside and inside neurons in the brain, respectively (Alzheimer's Association, 2017). Beta-amyloid plaques function as a neurotoxin by intervening in neuron-to-neuron communication at synapses. On the other hand, tau tangles prevent the passage of essential molecules and nutrients inside neurons, which causes axonal transport dysfunction and neuronal loss (Ali et al., 2015; Alzheimer's Association, 2017).

Apart from that, memory impairment is associated with cholinergic system dysfunction, which involves cholinergic neurons, neurotransmitters, and their receptors (Bartus et al., 1982; Lee et al., 2015). Cholinergic system dysfunction results from a loss of cholinergic neurons in the basal forebrain and hippocampus, which diminishes cognitive capability (Bartus et al., 1982; Lee et al., 2015). In healthy individuals, activation of the central cholinergic system enhances hippocampal neurogenesis through the cAMP response element-binding protein/brain-derived neurotrophic factor (CREB/BDNF) pathway (Lee et al., 2015). At present, one of the treatments for AD is a dispensation of acetylcholinesterase (AChE) inhibitors like tacrine or donepezil that increase the availability of acetylcholine at cholinergic synapses (Pandareesh et al., 2016). Moreover, oxidative stress plays an important role in AD, with some studies suggesting that beta-amyloid toxicity is linked to an increment in reactive oxygen species (ROS), including H_2O_2 (Butterfield and Lauderback, 2002), and lipid peroxidation in neuronal cultures (Yatin et al., 1999). High oxidative stress can cause memory deficits via impairment of hippocampal synaptic plasticity (Serrano and Klann, 2004) and oxidative damage in neurodegenerative diseases (Ding et al., 2007).

Current pharmacological options for AD, only have a partial effect and poor control over the disease-causing neurons linked with Alzheimer's symptoms and lethal complications (Alzheimer's Association, 2017). As such, the available drugs in the market mainly focus on the improving memory by inhibiting the AChE enzyme (Ghumatkar et al., 2015). However, AD is not a result of a single factor like AChE, but rather is a multifactorial condition and this needs to be considered when designing a drug. Other factors such as oxidative stress and synaptic dysfunction play a significant role in the cognitive deficits in AD. Natural products could be a source of neuroprotective drugs as they can maintain normal cellular interaction in the brain and reduce the loss of neuronal functions in pathological circumstances (Hritcu et al., 2014). Presently, many AD research groups have already explored the potential of using natural products as neuroprotective agents.

One such potential natural product is embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), which is the main active constituent in the fruits of *Embelia ribes* Burm (Family: Myrsinaceae), commonly known as "False Black Pepper" (Kundap et al., 2017a). The bright orange fruits of *E. ribes* have been utilized in traditional medicinal practice for treating central nervous system (CNS) disorders such as mental disorders and as a brain tonic (Poojari, 2014). Moreover, embelin has displayed anti-inflammatory, antioxidant, analgesic, antifertility, antitumor, wound healing, hepatoprotective, and antibacterial activities (Mahendran et al., 2011). Additionally, it has been reported that embelin is neuroprotective and possesses anticonvulsant ability when tested using animal models (Mahendran et al., 2011).

Embelin possesses all the features of a compound that can traverse the blood-brain barrier (BBB) and prompt a reaction in the CNS (Pathan et al., 2009; Kundap et al., 2017a). Even though embelin has various uses, there have been no studies of its neuropharmacological activities against AD-like conditions. Thus, in the present study, the anti-amnesic potential of embelin on memory deficits in a rat model of cognitive impairment caused by scopolamine was examined.

MATERIALS AND METHODS

Animal Care

In-house bred Sprague Dawley rats weighing between 180–200 g and between 6–8 weeks old were housed in the animal facility of the Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia. The rats were kept in cages and maintained under standard husbandry conditions (12:12 h light/dark cycle, controlled room temperature ($23 \pm 2°C$), stress-free, *ad libitum* water, standard diets, and sanitary conditions). Before commencing the experiment, the rats were allowed to acclimatize for a period of 1 week to reduce stress. The Monash Animal Research Platform (MARP) Animal Ethics Committee in Australia approved all the animal experimentations conducted in this study.

Experimental Design

Drug Treatment

Embelin (98%) batch number (Yucca/EM/2015/01/01) was purchased from Yucca Enterprises, Mumbai, India. The range of doses for embelin was determined based on pre-screening results. Embelin was solubilized in DMSO and then dissolved in saline. Donepezil and scopolamine were prepared in saline. Normal control rats were administered saline throughout the experiment. The treatments were given intraperitoneally (i.p) at a volume corresponding to 0.1 ml/100 g of body weight.

All experiments were performed in a balanced design (9 animals/group) to avoid being influenced by order and time. The behavioral studies were divided into two categories namely the nootropic and scopolamine models.

Nootropic Model

(i) **Group 1**: Control (Saline) ($n = 9$);
(ii) **Group 2**: Positive control (donepezil (DPZ) 1 mg/kg);
(iii) **Group 3**: Low dose of embelin (EMB) 0.3 mg/kg;
(iv) **Group 4**: Medium dose of EMB 0.6 mg/kg;
(v) **Group 5**: High dose of EMB 1.2 mg/kg

For nootropic activity, all the groups received pretreatment via the intraperitoneal route, for 8 days. All these rats were subjected to a battery of behavioral tests from day six onward until day eight for NOR and EPM (**Figure 1**).

Scopolamine Model

(i) **Group 1**: Control (Saline) ($n = 9$);
(ii) **Group 2**: Negative control [scopolamine (SCP) 1 mg/kg] ($n = 9$);
(iii) **Group 3**: Positive control [donepezil (DPZ) 1 mg/kg] + (SCP 1 mg/kg) ($n = 9$);
(iv) **Group 4**: Low dose of embelin (EMB) 0.3 mg/kg + (SCP 1 mg/kg) ($n = 9$);
(v) **Group 5**: Medium dose of EMB 0.6 mg/kg + (SCP 1 mg/kg) ($n = 9$);
(vi) **Group 6**: High dose of EMB 1.2 mg/kg + (SCP 1 mg/kg) ($n = 9$)

For scopolamine, amnesia was induced in all the groups except the control group by daily intraperitoneal injections of scopolamine (1 mg/kg) for 9 days after embelin pre-treatment (day nine to day 17). Half an hour after scopolamine administration, NOR was conducted on day 15, and EPM was carried out on day 16 and 17 of the study. At the end of the experiment, the rats were sacrificed, and their brains were isolated for further biochemical and immunohistochemistry analysis.

Novel Object Recognition (NOR)

For the object recognition task, an open field box (40 × 40 × 20 cm) composed of black acrylic material was utilized as the experimental apparatus. This method is similar to that used by Ennaceur and Delacour (1988), with minor modifications. Besides that, behavioral testing was carried out between 9:00 am and 6:00 pm under red light illumination. The scrutinized objects were two similar transparent culture flasks containing water and a Lego toy of similar height as that of the flask (new object). Both objects types presented during the test session varied in texture, color, and size. This assessment has three phases: (i) habituation; (ii) training, and (iii) test. On the first day, each rat was allowed to become familiarized with the open field box without the presence of an object for about 10 min. On the second day, each rat was placed in the open field for 5 min and allowed to freely explore the two identical objects (transparent cultured flask with water). After an interval of 90 min post-training session, one of the old objects used was substituted with a new object and the rats were subjected to a 2 min test run. The time spent with each object was recorded and evaluated using SMART software version 3.0. The open field box was cleaned with 70% ethanol between runs to minimize scent trails. The recognition index was calculated using the formula [TB/(TA + TB)*100] where TA and TB are time spent exploring familiar object A and novel object B respectively (Batool et al., 2016). Exploration of an object was noted when a rat sniffed or touched the object with its nose and/or forepaws.

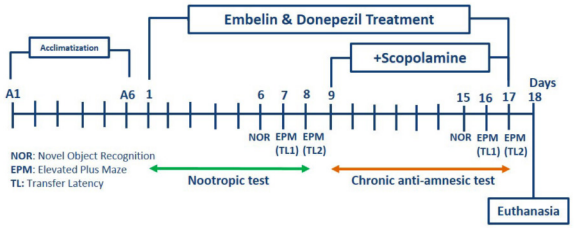

FIGURE 1 | Schematic representation of the experimental procedure.

Elevated Plus Maze (EPM)

The EPM device was comprised of four arms sharing the same dimensions, i.e., two open arms (50 × 10 cm) that crossed over two closed arms with 40 cm high walls. These arms were connected using a central square (10 × 10 cm), thus giving the apparatus plus sign look. Furthermore, the EPM was elevated 50 cm above floor level. This technique is almost similar to one reported by Halder et al. (2011). The behavioral testing was conducted between 9:00 am and 6:00 pm under dim red light illumination. Assessment of memory via EPM was done in two sessions. During the training phase, each rat was placed at the end of an open arm and by using a stopwatch, transfer latency time (s), which is the time each rat took to enter (with all four paws) into either closed arm, was noted. The maze was cleaned with 70% ethanol between runs to minimize scent trails. To evaluate memory retention, a test phase was conducted 24 h (retention) after a training session. The cut-off time for each rat to explore the maze in both the phases (training and test) was 90 s. A drop in transfer latency time during test sessions was taken as an index of memory improvement.

Tissue Processing

All the rats were sacrificed under ketamine and xylazine anesthesia 1 h after completing the behavioral test. In each group, five rat brains were fixed in 4% paraformaldehyde, and hippocampi of remaining four rats were used for real-time PCR and neurotransmitter analysis. One part of the hippocampus was used for isolation of RNA and another part of the hippocampus was homogenized on ice using methanol containing formic acid.

Total RNA Extraction and Real-Time PCR

Total RNA was extracted from the rat brain's hippocampal region and was similar to the method used by Kundap et al. (2017b), with some minor modifications. One part of the hippocampus tissue was momentarily homogenized in Trizole solution. The mixture was extracted using chloroform and centrifuged at 13,500 rpm at 4°C. Then, the aqueous phase was precipitated with isopropanol and followed by centrifugation at 13,500 rpm at 4°C. The volume of isopropanol added was same as the volume

of the supernatant from the aqueous phase. After that, the alcohol was removed. The pellet on the other hand, was rinsed twice with 70% ethanol and resuspended in 20 μL of RNase free water. RNA concentration was ascertained via absorbance at 260 nm using a Nanodrop machine. The total RNA (500 ng) was then reverse transcribed to synthesize cDNA using a QuantiTect® Reverse Transcription Kit, according to the manufacturer's protocol. Next, the mRNA expression of genes encoding cAMP response element-binding protein (CREB1), brain-derived neurotrophic factor (BDNF), superoxide dismutase 1 (SOD1), catalase (CAT), and IMPDH2 in the hippocampus, was measured by real-time PCR using the StepOne Real-Time PCR system. Subsequently, cDNA from the reverse transcription reaction was subjected to real-time PCR using a QuantiNova™ SYBR® Green PCR kit according to manufacturer's protocol. A comparative threshold (C_T) cycle method was applied to normalize cDNA content of samples, which involves of normalization of a number of target gene copies against the endogenous reference gene, IMPDH2.

Neurotransmitter Analysis Using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The brain levels of neurotransmitters like dopamine (DA), glutamate (Glu), norepinephrine (NE), and acetylcholine (ACh) were estimated using LC-MS/MS in a similar manner to that used by Kundap et al. (2017b), with some modifications. For all these standard neurotransmitters, stock solutions of 1 mg/ml were prepared in methanol (0.1% formic acid) and then stored at 4°C until use. Four calibration standards with the concentration ranges of 0.25–200.00, 250.00–20,000.00, 0.50–200.00, and 0.25–200.00 ng/mL were used for validation of DA, Glu, NE, and ACh respectively. In brief, hippocampal tissue was homogenized in ice-cold methanol containing formic acid. Then, the homogenate was vortex-mixed followed by centrifugation at 14,000 rpm for 10 min at 4°C. Finally, the supernatant was subjected to LC-MS/MS analysis, which was run on an Agilent 1290 Infinity UHPLC, coupled with an auto-sampler system comprising of Agilent 6410 Triple Quad LC/MS, ZORBAXEclipse plus C18 RRHD 2.1 × 150.0 mm and 1.8-micron (P/N959759-902) column (Agilent Technologies, Santa Clara, CA, United States). The mobile phase consisted of 0.1% formic acid in (i) water (Solvent A) and (ii) acetonitrile (Solvent B). It was used with a gradient elution: (i) 0–3 min, 50% B; (ii) 3–6 min, 95% B; (iii) 6–7 min, 95% B at a flow rate of 0.1 mL/min.

Immunohistochemical Stain Analysis

Immunohistochemical stain analysis was conducted via assessment of neurogenesis using Doublecortin (DCX) and lipid peroxidation with 4-hydroxy-2-nonenal (4HNE) staining in the hippocampus. Five brain samples from each group were immersed in 4% paraformaldehyde overnight. The samples were methodically cryoprotected in 10, 20, and 30% sucrose for 24 h. Next, the brains were embedded in 15% polyvinylpyrrolidone (PVP), frozen using dry ice, and cut into 40 μm frozen coronal sections using a Leica CM3050 cryostat. All sections were then stored in an anti-freeze buffer. Endogenous quenching using 1% H_2O_2 in methanol for 30 min was performed on the free-floating sections. After washing with phosphate buffered saline (PBS), the tissues were treated with blocking buffer (1.0% bovine serum albumin in PBS and 0.3% Triton X-100) for 1 h, followed by incubation with primary DCX (1:500, Abcam) and 4HNE (1:250, Abcam) antibodies overnight at 4°C. The tissues were then incubated with a biotinylated goat anti-rabbit secondary antibody (Abcam) for 2 h after being washed with PBS. Subsequently, the tissues were exposed for 2 h to an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector). Peroxidase activity was visualized using a stable diaminobenzidine solution (DAB, Sigma). All immunoreactions were monitored via a microscope (BX41, Olympus) and using the DigiAcquis 2.0 software, results were calculated.

Statistical Analysis

All findings were expressed as mean ± standard error of the mean (SEM). These data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's tests. 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.

RESULTS

Nootropic effect of Embelin

Findings obtained from the NOR test for embelin nootropic activity are illustrated in **Figure 2A**. The effect of different embelin doses on memory function were assessed following 7 days of pretreatment. The results were expressed as recognition index (%) for the novel object. Based on the outcomes, the pretreated groups of embelin showed an increase in recognition index for novel object compared with the control group and donepezil groups. Only 0.6 mg/kg of embelin showed statistically significant results with p value of <0.05. In EPM, the inflection ratio was significantly increased in 0.6 mg/kg embelin treated groups when compared with the control (**Figure 2B**). There was no significant difference in other treated groups.

Anti-amnesic Effect of Embelin in Rats With Scopolamine-Induced Amnesia

The NOR test showed a reduction in recognition index percentage for the negative group (SCP 1 mg/kg) in the chronic scopolamine model (**Figure 3A**). Moreover, the recognition index percentage for all the embelin treated groups were high and comparable with donepezil (1 mg/kg) group. A significant difference in the recognition index percentage was observed between all embelin treated and the negative group ($P < 0.05$). In EPM, inflection ratio analysis showed that there was an increase in retention memory in all embelin treated groups compared with the negative control group; however, it was statistically not significant (**Figure 3B**).

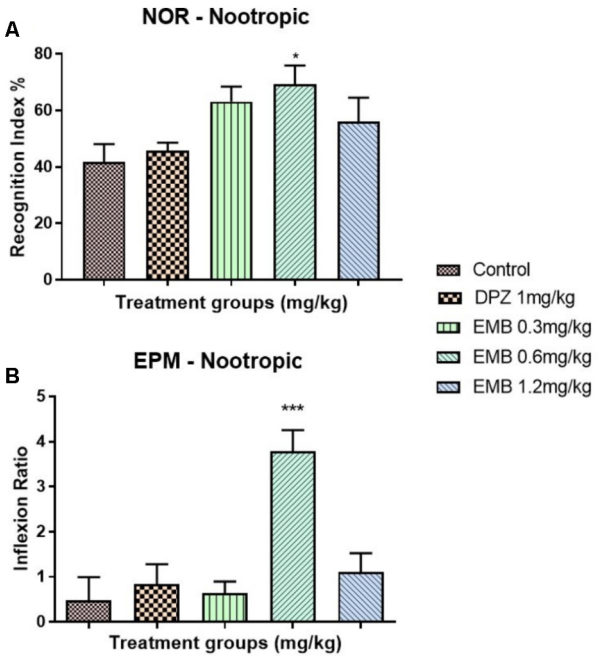

FIGURE 2 | Behavioral analysis for novel object recognition (NOR) and elevated plus maze (EPM). **(A)** represent the graph plot for the recognition indices in NOR for the nootropic model **(B)** represents the graph plot for the inflection ratios in EPM for the nootropic model. The behavioral analysis for **(A,B)** were compared to control group. Data are expressed as Mean ± SEM, $n = 9$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

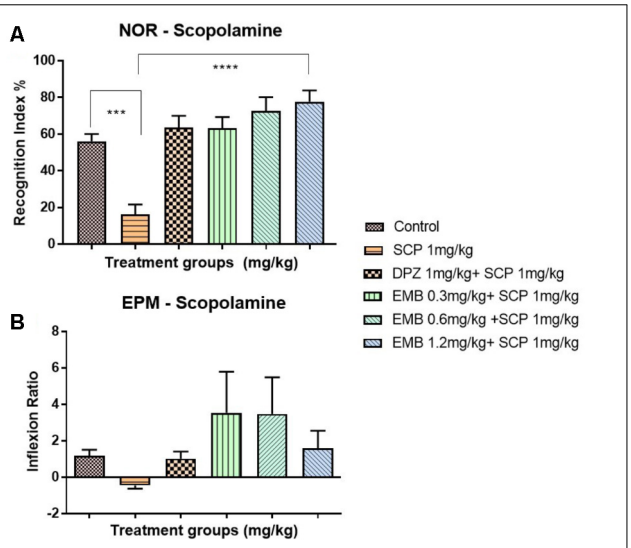

FIGURE 3 | Behavioral analysis for NOR and EPM. **(A)** represents the graph plot for the recognition indices in NOR for the scopolamine model **(B)** represents the graph plot for inflection ratios in EPM for the scopolamine model. The behavioral analysis for **(A,B)** were compared to negative control (SCP 1mg/kg). Data are expressed as Mean ± SEM, $n = 9$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Changes in mRNA Levels in the Hippocampus

BDNF mRNA levels were significantly down-regulated, approximately twofold in the hippocampus of the scopolamine group, compared with the positive control, $P < 0.01$. This down-regulation was ameliorated by embelin, in a dose-dependent manner, in comparison with the negative control, and a significant difference was revealed for the 1.2 mg/kg dose of embelin (**Figure 4A**). In addition, multiple exposures to scopolamine significantly down-regulated (twofold) the mRNA expression level of CREB1 in the negative control, compared with the positive control ($P < 0.001$). Embelin treatment increased CREB1 expression level in a dose-dependent manner, compared with the negative control, and it was significant for the 1.2 mg/kg embelin dose (**Figure 4B**). Furthermore, scopolamine depleted antioxidant mRNA in hippocampal tissues, including (CAT) (**Figure 4C**) and SOD1 gene expression (**Figure 4D**). The down-regulation of CAT mRNA was significantly ameliorated through embelin treatment compared to the negative control for the 1.2 mg/kg embelin group ($P < 0.05$). In SOD1, these changes were reversed by embelin pretreatment for all embelin treated groups, and the result was significant in the 0.6 mg/kg embelin group, with approximately a 1.5-fold change in comparison with the scopolamine treated group.

Estimation of Neurotransmitters by LC-MS/MS

Administration of scopolamine significantly altered the levels of ACh, DA, NE, and Glu in the rat brain's hippocampus. Specifically, the level of ACh ($P < 0.05$) decreased substantially whereas other neurotransmitters' levels increased significantly ($P < 0.05$ for DA and Glu; $P < 0.01$ for NE). Nevertheless, embelin treatment significantly normalized the level of all these neurotransmitters, and it was in a dose-dependent manner for ACh and DA (**Figures 5A–D**) ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Neurogenesis and Lipid Peroxidation in the Hippocampus

Scopolamine significantly inhibited adult neurogenesis via a reduction in the distribution of dendrites and neuron bodies in the dentate gyrus (DG) region, as shown by DCX staining in the subgranular zone (SGZ) (**Figure 6A**). Pretreatment with embelin totally ameliorated adult neurogenesis by enhancing immature neurons in the SGZ in a dose-dependent approach in comparison with the negative control ($P < 0.05$ for 0.3 mg/kg, $P < 0.01$ for 0.6 mg/kg and $P < 0.001$ for 1.2 mg/kg; **Figure 6B**). On the other hand, scopolamine injection significantly induced lipid peroxidation in the hippocampus, as represented by a deep brown color in the cornu ammonis 3 (CA3) regions through 4HNE staining. Pretreatment with embelin significantly lowered 4HNE-positive staining in the CA3 (threefold change) compared with the negative group ($P < 0.0001$ for all embelin groups; **Figures 7A,B**). Besides that, donepezil ameliorated these

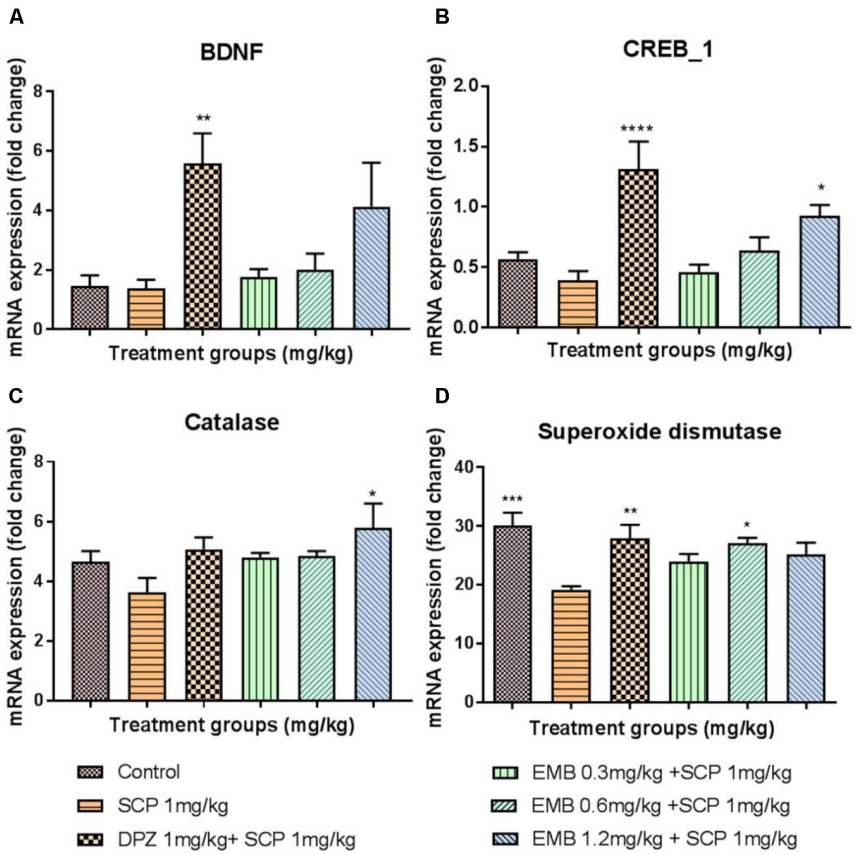

FIGURE 4 | Gene expression in the rat hippocampi determined by real time-PCR. The genes included are **(A)** BDNF, **(B)** CREB1, **(C)** Catalase, and **(D)** Superoxide Dismutase. All changes in the expressions levels were compared to the negative control group (SCP 1 mg/kg). Data are expressed as 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 $^{****}P < 0.0001$.

alterations triggered by scopolamine, as displayed through both DCX and 4-HNE staining.

DISCUSSION

This work aims to determine whether embelin has an anti-amnesic effect by modulating the cholinergic pathway. An animal model of hippocampal memory damage due to intraperitoneal injection of scopolamine was adopted to verify this hypothesis. The experiments comprised of two parts: Experiment 1 (pretreatment with embelin without scopolamine injection during training) to test embelin's nootropic effects on learning and memory process, and Experiment 2 (multiple exposures of scopolamine injection) to assess the effect of embelin on anti-amnesic activities and biochemical aspects during learning and memory process.

At the beginning of this experiment, we conducted a dose deciding study to find the therapeutic dose of embelin. A prior literature search determined that the range of embelin dose was between 2.5 mg/kg to 10 mg/kg for the intraperitoneal route in CNS related animal models (Mahendran et al., 2011; Afzal et al., 2012). However, our preliminary study using these range of embelin doses resulted in a neurobehavioral effect on coordination and motor activity whereby the treated rats were immobile and kept falling from the behavioral apparatus. Thus, we decided 1.2 mg/kg as the highest dose as the LD_{50} value for embelin was 44 mg/kg for intraperitoneal administration reported by Poojari (2014). Furthermore, we decided 0.3 mg/kg and 0.6 mg/kg would be 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.

In this experiment, NOR and EPM were applied as behavioral models to evaluate learning and memory. The NOR test is particularly relevant in AD research as it allows the assessment of visual recognition memory, which is affected early in AD progression, involving brain regions similar to those affected by this devastating and debilitating neurodegenerative disease (Grayson et al., 2015). On the other hand, EPM is a behavioral test employed to study long-term spatial memory (Uddin et al., 2016b). Certain EPM parameters like retention transfer latency are utilized for the evaluation of memory. A decrease in transfer latency on the second day, which is after 24 h, indicates an improvement of memory and vice-versa (Dhingra and Kumar, 2012). The findings of this study showed that embelin at 0.6 mg/kg displayed nootropic activity in both the recognition

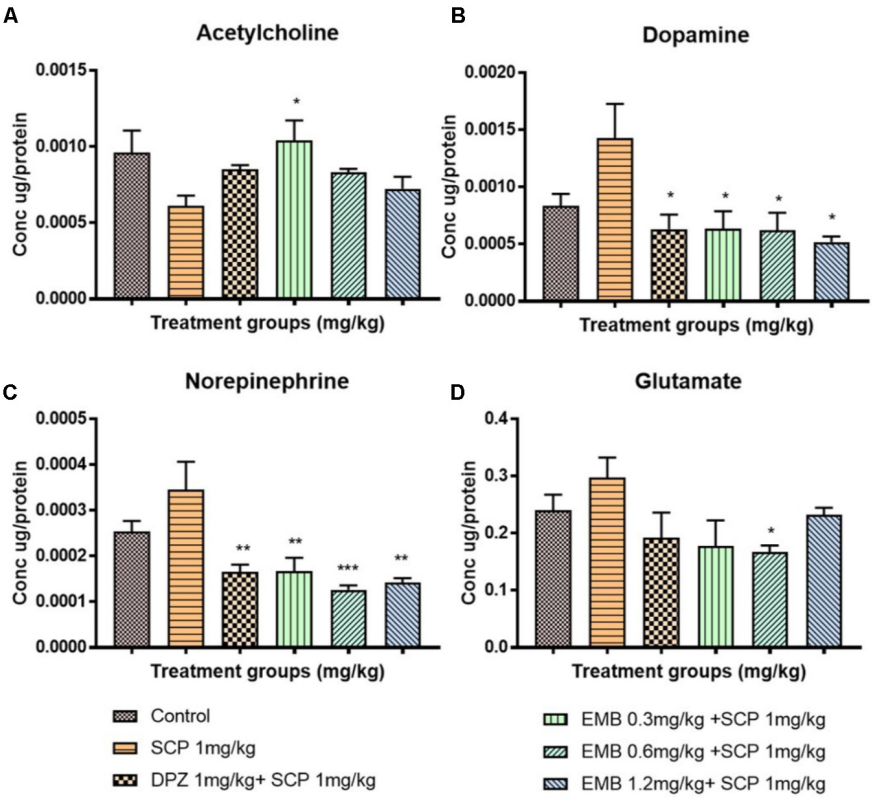

FIGURE 5 | The concentration of neurotransmitters in the rat hippocampi after chronic scopolamine. The figure represents the rat hippocampal neurotransmitter levels of **(A)** Acetylcholine, **(B)** Dopamine, **(C)** Norepinephrine, and **(D)** Glutamate. All changes in the neurotransmitter levels were 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 $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

index and inflection ratio in the NOR and EPM tests, respectively (**Figures 2A,B**). However, the nootropic activity of embelin in both behavioral paradigms was found to be dose independent. This could be explained that at a higher dose, the drug reaches its maximum effect so increasing the drug dosage does not increase its effectiveness, but on the contrary, effectiveness decreases. This theory is supported by the fact that CNS drugs such as antipsychotic drugs produce maximum dopaminergic blockage at high doses. However, further dose increments will not produce any dopamine blockage but eventually lead to other side effects such as anticholinergic activity (Bridges, 1981). It is possible that in this experiment, the 1.2 mg/kg embelin group has reached its maximum effect and therefore cognitive ability has declined. Based on the behavioral results obtained, it can be suggested that embelin is a nootropic drug that acts as a natural cognitive enhancer. These findings show that supplementation of embelin significantly amplified the rats' memory function and 0.6 mg/kg of embelin demonstrated significant nootropics effects. Nootropic drugs are used to treat cognition deficits in patients with AD, schizophrenia, stroke, attention deficit hyperactivity disorder (ADHD), and vascular dementia (VaD) (Birks and Grimley Evans, 2009; Froestl et al., 2012).

Scopolamine-induced dementia has been used extensively to assess potential therapeutic agents for treating AD (Kwon et al., 2009). Scopolamine is a nonselective muscarinic cholinergic receptor antagonist associated with cholinergic dysfunction, which causes performance deficits in learning and memory (Heo et al., 2014). Therefore, in this study, scopolamine was administered to rodents for 1 week to induce cholinergic neurodegeneration along with cognitive deficits. Following 6 days of scopolamine administration, the scopolamine treated group had less than 20% of the recognition index of other groups. Pretreatment with embelin ameliorated memory impairment caused by scopolamine (**Figure 3A**), with the recognition index being twofold more, in comparison with scopolamine treated group in a dose-dependent manner. These results exposed that embelin was as effective as the donepezil-treated group. Moreover, the findings showed that embelin treatment attenuated amnesic behavior in EPM, but it was insignificant (**Figure 3B**). Hence, these outcomes suggest that embelin had an anti-amnesic effect in the scopolamine model.

The brain is susceptible to oxidative stress because it consumes huge amounts of oxygen, has an abundant lipid content, and a low antioxidant level compared than other organs (Serrano and Klann, 2004). Furthermore, it is well known that the hippocampus region in the brain is crucial for learning and memory, and the formation of spatial memory (Huang et al., 2015; Lee et al., 2016). The scopolamine-induced memory

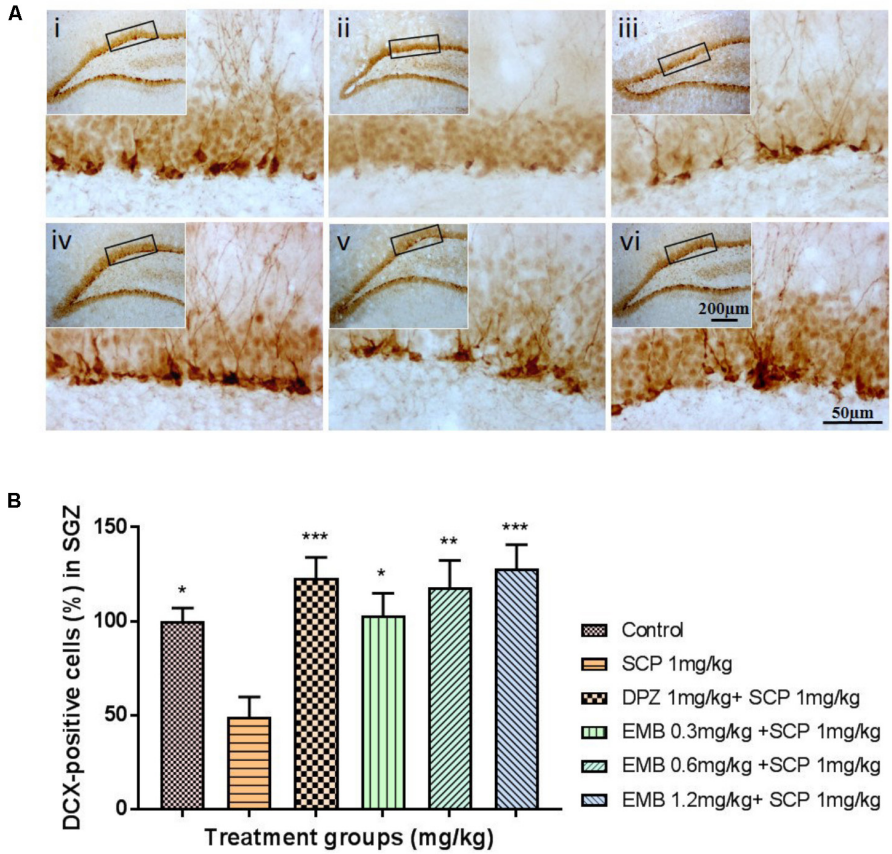

FIGURE 6 | DCX immunohistochemical analysis of the effects of embelin in improving scopolamine-induced suppression of neurogenesis in the dentate gyrus. **(A)** DCX-positive staining in immature neurons is shown in the subgranular zone of the dentate gyrus. Photomicrographs of the hippocampal section of treatment groups was (i) Control (ii) SCP 1 mg/kg alone (iii) DPZ 1 mg/kg + SCP 1 mg/kg (iv) EMB 0.3 mg/kg + SCP 1 mg/kg (v) EMB 0.6 mg/kg + SCP 1 mg/kg (vi) EMB 1.2 mg/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 $^{***}P < 0.001$.

deficit model demonstrated that prominent oxidative stress and memory deficits in a rodent model is similar to that in AD patients, even though the mechanism of action remains unclear (Lee et al., 2015). The change in the mRNA levels of antioxidants in the hippocampus after embelin pretreatment was examined using the scopolamine model in this present study. Scopolamine injection induced oxidative stress in the hippocampus, as evident by the decreased levels of CAT and SOD1 mRNA levels in the scopolamine alone treated negative group. To prevent or slow down the progression of free radical-mediated oxidative stress, brain antioxidant defense enzymes such as CAT and SOD play a vital role in protecting tissues against oxidative damage (Uddin et al., 2016a). Antioxidant mRNA alteration caused by scopolamine injection was significantly ameliorated for SOD1 via pretreatment with embelin. However, CAT mRNA level was decreased by scopolamine induction, but it was not significant (**Figures 4C,D**). Additionally, scopolamine-induced lipid peroxidation in the hippocampus's CA3 was shown as positively stained 4HNE cells. Nonetheless, pretreatment with embelin completely attenuated the over-production of 4HNE cells (**Figures 7A,B**). These results propose that the protective antioxidant gene response by embelin pretreatment reduced lipid peroxidation induced by scopolamine. An increase in 4HNE cells is a key histopathological feature of neurodegenerative diseases like AD (Serrano and Klann, 2004).

Expression of BDNF and CREB1 mRNA levels in scopolamine-induced hippocampal tissue were examined to investigate the role of embelin in neurogenesis and synaptic plasticity. In this study, hippocampal BDNF and CREB1 were markedly reduced due to scopolamine injection, and pretreatment with embelin increased the mRNA expression level of both BDNF and CREB1. A high dose of embelin at 1.2 mg/kg exhibited maximum protection by increasing the levels of BDNF and CREB1. Other than that, cAMP response element binding protein (CREB) plays a crucial role in neuronal growth, proliferation, differentiation, and survival (Lee et al., 2016). In our results, the explanation for the increased in dose dependency for both BDNF and CREB 1 could possibly be that embelin may be responsible for visual recognition memory in NOR through this BDNF/CREB pathway. We noticed that at the 1.2 mg/kg dose, embelin expressed high mRNA levels of BDNF and CREB1 and this could be the reason for a 60%

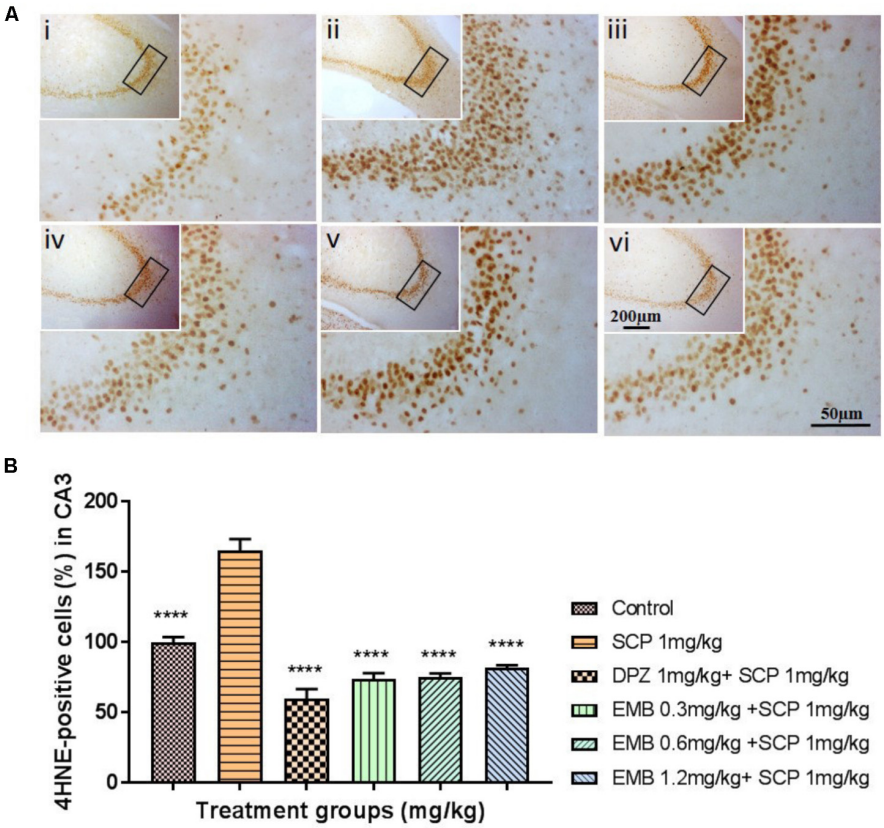

FIGURE 7 | 4HNE immunohistochemical analysis illustrating the inhibitory effects of embelin against scopolamine-induced lipid peroxidation in the hippocampus. **(A)** The 4HNE-positive stained cells in the CA3 region of the hippocampus are indicative of lipid peroxidation. Photomicrographs hippocampal section of treatment groups was (i) Control (ii) SCP 1 mg/kg alone (iii) DPZ 1 mg/kg + SCP 1 mg/kg (iv) EMB 0.3 mg/kg + SCP 1 mg/kg (v) EMB 0.6 mg/kg + SCP 1 mg/kg (vi) EMB 1.2 mg/kg + SCP 1 mg/kg. Representative photomicrographs were taken at magnifications of 40X, and 200X. **(B)** Quantification of 4HNE protein in CA3. Data are expressed as means Mean ± SEM, $n = 5$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

increase in visual recognition index in NOR when compared with the scopolamine treated group. Thus, this validates the role of BDNF-CREB signaling in visual recognition memory, particularly for hippocampus-dependent learning.

Likewise, adult hippocampal neurogenesis plays a key role in hippocampal memory function (Mu and Gage, 2011). Altman and Das (1965) first reported on the continual production of new neurons in the adult hippocampus. These new neurons originated from adult neural stem cells (NSCs) residing in the SGZ of DG (Bonaguidi et al., 2011). In this present research, a significantly reduced level of immature neurons, revealed through DCX staining of scopolamine-induced rat hippocampus was determined, while pretreatment with embelin distinctly ameliorated repression of the SGZ region's neuronal precursor cells in a dose-dependent manner (**Figures 6A,B**).

Numerous studies have reported that most classical neurotransmitter systems such as ACh, NE, Glu, and DA, influence learning and memory (Myhrer, 2003). We adopted LC-MS/MS method as it is a simple, sensitive and simultaneously able to quantify the four major neurotransmitters from rat hippocampal tissue in a single run (Zheng et al., 2012). The extraction of the neurotransmitters from rat hippocampus was done with utmost care and prior to LC-MS/MS analysis to avoid any possibilities of sample degradation and oxidation as described by He et al. (2013). The neurotransmitters' concentrations were expressed as a ratio of total protein concentration in order to get correct value and to avoid possible variation in sample when subjected to LC-MS/MS. In AD patients, pathological changes affecting glutamatergic, cholinergic, noradrenergic, and serotonergic systems have been revealed (Francis et al., 1999). In this study, the effect of embelin on brain neurotransmitter levels in rats administered scopolamine was investigated. ACh plays an essential role in learning process and memory as a key transmitter in the cholinergic system (Chen et al., 2016). A decrease in ACh levels is reported in this study as a biomarker of scopolamine-induced cognitive impairment in the rat hippocampus. Embelin administered at a dose of 0.3 mg/kg significantly increased ACh levels, subsequently improving cholinergic function. Interestingly, Arora and Deshmukh (2017) reported that embelin treatment in a streptozotocin-induced rat model decreased AChE activity, which is the enzyme that metabolizes ACh

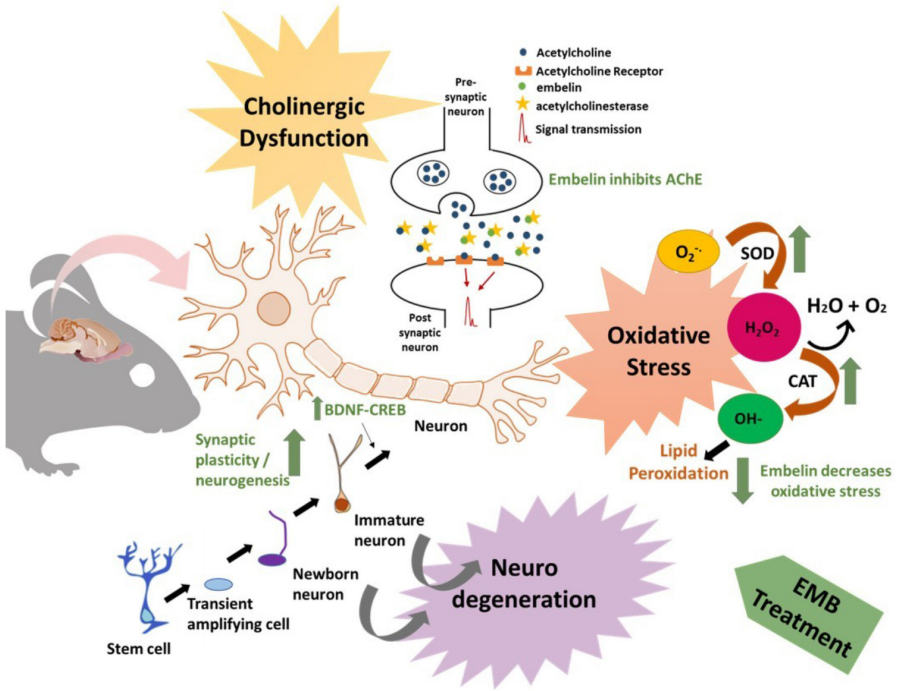

FIGURE 8 | Schematic diagram showing the effect of embelin in a diseased condition. The figure depicts cholinergic dysfunction, oxidative stress, and neurodegeneration which contributes to neuronal loss, synaptic dysfunction, and an AD-like condition. Embelin could act as memory enhancer by stimulating the cholinergic systems via acetylcholine release and inhibition of AChE. In the oxidative defense pathway, embelin reduces oxidative stress by increasing SOD and CAT mRNA levels and eventually reducing lipid peroxidation. In addition to these effects, a rise in DCX expression by embelin treatment contributes to neurogenesis and an increase in BDNF-CREB levels may contribute to synaptic plasticity.

into choline and acetate. Therefore, a reduction in AChE level indicates a high level of ACh as a result of embelin treatment, which is similar to our results. In the current research, Glu levels were raised after being treated with scopolamine. Similar outcomes were reported by Pandareesh et al. (2016) and Arora and Deshmukh (2017). Administration of 0.6 mg/kg embelin significantly lowered the level of Glu. A rise in Glu level has been reported to cause excitotoxic neuronal damage and loss of cognitive function (Arora and Deshmukh, 2017) and also associated with excitotoxicity in AD brains (Jackson, 2014). Scopolamine treatment also caused an increment in the levels of DA and NE in the hippocampus. Earlier reports suggests that an increase in DA and NE levels leads to amnesia and memory deficits. Wu et al.'s (2014) study, demonstrated that donepezil treatment can modulate the increase levels of DA and NE in disease control group. Interestingly, a similar protective effect was observed with embelin pre-treatment in amnesia condition.

In this scopolamine model, our results are unusual, with embelin causing different dose dependency in the behavioral model and neurotransmitters, particularly ACh when compared to other reported studies that utilized embelin. This could be explained by embelin being neuroprotective in a scopolamine-induced amnesia model via visual recognition memory but not in long-term spatial memory. This theory is supported by our results as there was a dose dependency in embelin treatment in NOR and the result of embelin is comparable with the donepezil group. However, we could not see this pattern in EPM. Whilst embelin improved visual recognition in dose dependency manner, it also reduced the level of ACh in a dose-dependent manner as well. This discrepancy could be because at a dose of 0.3 mg/kg, embelin might be effectively increasing the level of ACh but stops further production of ACh at 1.2 mg/kg. At this particular dose, embelin probably plays a different role in inhibiting the enzyme AChE. This could be the reason that at 1.2 mg/kg of embelin, we observed a high recognition index in NOR of scopolamine-induced amnesia rats.

CONCLUSION

In conclusion, the results from this study have demonstrated that embelin displays nootropic and neuroprotective abilities in scopolamine-induced amnesia in rats. Nootropic effects may be attributed to an increase in visual recognition and spatial memory in both NOR and EPM. Embelin possesses anti-amnesic effects, which could be mediated by an antioxidant gene response particularly though SOD1, the CREB-BDNF pathway, hippocampal neurogenesis, and cholinergic activity. The anti-amnesic effect of embelin is also comparable to that of donepezil at a specific concentration even though it is not in a dose-dependent manner in certain cases. Therefore, embelin could be a promising treatment for patients suffering from neurodegenerative diseases. **Figure 8** shows the potential

mechanism of action of embelin in scopolamine-induced memory impairment in rodents.

ETHICS STATEMENT

The experimental protocol was approved by the Monash Animal Research Platform (MARP) Animal Ethics Committee, Monash University, Australia (MARP/2016/054).

AUTHOR CONTRIBUTIONS

SB performed all the experiments and was responsible for the writing of the manuscript in its entirety. YK helped in designing gene expression study, result analysis and figures in the manuscript. IO helped in LC-MS/MS method. MS helped in conceptualizing, designing the study, result analysis, and manuscript writing. All authors gave their final approval for the submission of the manuscript.

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Conflict of Interest Statement: 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.

Chapter 5

5.1 Introduction

Neurovascular dysfunction had been linked to cognitive impairment and neurodegeneration especially in the early stages of AD (66). Chronic cerebral hypoperfusion (CCH) is a pathogenic factor in cerebrovascular diseases and neurodegenerative disorders, such as vascular dementia (67). CCH is characterized as a condition where there is insufficient blood flow to the brain, which aggravates AD-associated cognitive dysfunction (68). Rodent models of CCH were first established in experimental studies using occlusion or ligation of both common carotid arteries in rats, which allowed exploration on the early pathological events that may lead to vascular dementia (51). Moreover, studies had shown that this animal model is suitable for the further development of potential neuroprotective targets in neurodegenerative related disorders (69). Therefore in the second part of our study, we evaluated the neuroprotective effect of embelin in permanent bilateral common carotid artery occlusion (PBOCCA) model of AD.

Detailed Status Information

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Authorship	Yes
Abstract	Alzheimer's disease (AD) is the second most occurring neurological disorder after stroke and is associated with cerebral hypoperfusion, possibly contributing to cognitive impairment. In the present study, neuroprotective and anti-AD effects of embelin were evaluated in chronic cerebral hypoperfusion (CCH) rat model using permanent bilateral occlusion of common carotid artery (PBOCCA) method. Rats were administered with embelin at doses of 0.3, 0.6 or 1.2 mg/kg (i.p) on day 14 post-surgery and tested for Morris water maze (MWM) followed by electrophysiological recordings to access cognitive abilities and synaptic plasticity. The hippocampal brain regions were extracted for gene expression and neurotransmitters analysis. Treatment with embelin at the doses of 0.3 and 0.6 mg/kg significantly reversed the spatial memory impairment induced by CCH in rats. Embelin treatment has significantly protected synaptic plasticity impairment as assessed by hippocampal long-term potentiation (LTP) test. The mechanism of this study demonstrated that embelin treatment alleviated the decreased expression of BDNF, CREB1, APP, Mapt, SOD1 and NF⊐B mRNA levels caused by CCH rats. Furthermore, treatment with embelin demonstrated neuromodulatory activity by its ability to restore hippocampal neurotransmitters. Overall these data suggest that embelin improve memory and synaptic plasticity impairment in CCH rats and can be a potential drug target for neurodegenerative disease-related cognitive disorders.
Techniques	Life sciences techniques, Gene expression analysis [PCR-based techniques]; Life sciences techniques [Experimental organisms]; Life sciences techniques, Experimental organisms [Rats]; Life sciences techniques, Biophysical methods [Field potential recordings];
Subject Terms	Biological sciences/Neuroscience/Diseases of the nervous system/Alzheimer's disease Health sciences/Molecular medicine
Competing Interests Policy	There is **NO** Competing Interest.

Embelin Improves the Spatial Memory and Hippocampal Long-Term Potentiation in a Rat Model of Chronic Cerebral Hypoperfusion

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Abstract

Alzheimer's disease (AD) is the second most occurring neurological disorder after stroke and is associated with cerebral hypoperfusion, possibly contributing to cognitive impairment. In the present study, neuroprotective and anti-AD effects of embelin were evaluated in chronic cerebral hypoperfusion (CCH) rat model using permanent bilateral occlusion of common carotid artery (PBOCCA) method. Rats were administered with embelin at doses of 0.3, 0.6 or 1.2 mg/kg (i.p) on day 14 post-surgery and tested for Morris water maze (MWM) followed by electrophysiological recordings to access cognitive abilities and synaptic plasticity. The hippocampal brain regions were extracted for gene expression and neurotransmitters analysis. Treatment with embelin at the doses of 0.3 and 0.6 mg/kg significantly reversed the spatial memory impairment induced by CCH in rats. Embelin treatment has significantly protected synaptic plasticity impairment as assessed by hippocampal long-term potentiation (LTP) test. The mechanism of this study demonstrated that embelin treatment alleviated the decreased expression of BDNF, CREB1, APP, Mapt, SOD1 and NFκB mRNA levels caused by CCH rats. Furthermore, treatment with embelin demonstrated neuromodulatory activity by its ability to restore hippocampal neurotransmitters. Overall these data suggest that embelin improve memory and synaptic plasticity impairment in CCH rats and can be a potential drug target for neurodegenerative disease-related cognitive disorders.

Keyword: embelin, PBOCCA, CCH, LTP, Vascular Dementia, cognitive dysfunction

Introduction

Recognised as the second cause of age related cognitive deficits after Alzheimer's disease(AD), vascular dementia (VD) is generally a neurological disorder [1]. The hypothesis on vascular dementia proposed that the reduction in blood flow to the brain affects the glial and neuronal cells energy demands, thus causing neurodegeneration and brain dysfunction [2]. It has been discovered that this age-related neurodegenerative disorder contributed to 20% of all dementia patients, which is foreseen to be tripled by 2050 [3,4]. The decrease in cerebral blood flow namely chronic cerebral hypoperfusion (CCH) has been detected in cerebrovascular patients who later develop marked cognitive deficiency [5].

In this study, CCH was induced in rodent using permanent bilateral occlusion of common carotid arteries (PBOCCA) method. According to Damodaran, et al. [6], significant decrease in cerebral blood flow by 32% in the hippocampus and by 21% in the cortex was reported in PBOCCA rats which made it a suitable model for CCH [7]. For the duration of four days to three months (chronic phase), these rats demonstrated learning and memory impairments [6] followed by neuronal damage

and oxidative stress, which resemble the deficiencies that occur during dementia in humans [8,9]. Studies based on this animal model revealed the potential strategies to thwart, slow down and reverse the neurodegenerative disease progression associated with imbalance of cerebral blood flow [10].

Synaptic integrity and plasticity are crucial for a healthy brain function especially in learning and memory [11]. Contrary, cognitive decline symptoms in neurological disorders including AD and dementia have been associated with synaptic plasticity impairment [12] by evidence of loss of synapse numbers and functions in the hippocampus [13,14]. Recently, numerous studies on long-term potentiation (LTP) have emerged as it is considered an indicator of synaptic plasticity at the cellular level that correlates with changes in cognitive function [13]. Experimental evidences have reported that by assessing long-term potentiation (LTP), neural plasticity dysfunction can be directly detected [11] as LTP inhibition has been observed in the CA1 region of the hippocampal in CCH rat models [15,16].

Embelin is a promising benzoquinone compound ($C_{17}H_{26}O_4$) with a molecular weight of 294.39 g/mol belonging to the fruits of *Embelia ribes* Burm (Family: Myrsinaceae) [17]. In Indian traditional medicinal practice, the fruits of *Embelia ribes* are consumed as a brain tonic to cure disorders related to central nervous system (CNS) [18]. Kundap, et al. [19], stated that the anticonvulsant, antidepressant and anxiolytic activities possessed by embelin have been demonstrated by many studies with the ability to improve neurological-related disorders such as Huntington's disease, multiple sclerosis, sickness behaviour, ischemia and traumatic brain injury. Recently, the neuroprotective effect of embelin in Alzheimer's disease-like condition model has been reported [17,20], but specific neuroprotective mechanism of action against memory impairments in CCH-induced PBOCCA rats remain elusive.

Thus, this study aims at identifying whether the administration of embelin can pharmacologically ameliorate the memory impairment in PBOCCA rats. The first part of the experiment involved the behavioural effect of embelin on spatial cognitive performance using the Morris water maze (MWM), which was followed by the assessment on the effects of embelin on LTP in the CA1 (Cornu ammonis 1) region of the hippocampus using *in vivo* electrophysiological recording. LTP of embelin in this study is the first to be reported, which may reveal the synaptic plasticity properties of this yellowish-orange compound as a potential therapy for Alzheimer's disease-like conditions. On the other hand, the last part of this experiment involved the extraction of rat's brain for studying their gene expression and neurotransmitter to aid in determining the potential mechanism responsible for the neuroprotective effect of embelin in vascular cognitive impairment and dementia conditions.

Results

Effects of embelin on memory performance in MWM test in CCH-induced rats

The MWM test was utilised in this study to evaluate the effects of embelin on the spatial memory impairment induced by CCH rats. Embelin was administered after each training session to study the post-training effect of this compound on learning and memory functions among PBOCCA rats. Figure 1A displays the swim traces of 5 groups on each test day where it was apparent that the PBOCCA rats treated with only vehicle (negative control) demonstrated longer latency to reach the submerged platform than that of the sham rats ($p < 0.05$), thus indicating a poor learning disability following the PBOCCA surgery. Nevertheless, significant differences were confirmed

by two-way ANOVA analysis between escape latency and post-training administration of embelin due to treatment (post-training $F_{4,86} = 5.92$; $p < 0.001$) and test day (post-training $F_{3,86} = 20.34$; $p < 0.0001$). The relationship between embelin post-training treatment and test day was statistically significant ($F_{12,86} = 2.121$; $p < 0.05$). Besides, a gradual shortening of latency during the training stage in PBOCCA was noticed on rats with embelin, which showed significant enhancement with 0.3 mg/kg embelin on training day 3 ($p < 0.01$) and day 4 ($p < 0.001$). Moreover, a significant decrease in escape latency was recorded in PBOCCA rats when receiving 0.6 mg/kg embelin on day 3 and 4 ($p < 0.05$). However, the group with 1.2 mg/kg embelin displayed a decrease in escape latency on day two, but it was not significant as the performance to find the hidden platform remained constant for day 3 and 4 even after the training.

On the other hand, the untreated PBOCCA rats were seen unable to recall the location of platform in the probe trial on the fifth day, thus spending significantly less time in target quadrant compared to that of sham rats ($p < 0.001$) (Figure 1B). A significant difference in post-training treatments with embelin ($F_{4,19} = 8.477$; $p < 0.001$) was noted between the groups in the percentage time spent in the target quadrant during the probe trial. PBOCCA rats with embelin of 0.3 mg/kg ($p < 0.05$) and 0.6 mg/kg ($p < 0.01$) after the training session significantly spent longer time in the target quadrant compared to untreated PBOCCA rats, which indicates memory improvement. However, there was no significant difference in probe trial task on rats receiving 1.2 mg/kg embelin than those with PBOCCA ($p > 0.05$).

LTP in the CA3-CA1 region of the hippocampus

LTP recording was performed to investigate the synaptic plasticity in the CA3-CA1 region of the hippocampus. The normalised time course changes of fEPSP amplitude to one hr baseline period are presented in Figure 2A showing that the fEPSP amplitude of all the five groups was increased after TBS and stabilised to different levels above the baseline period. Meanwhile, two-way ANOVA analysis confirmed a significant difference in the synaptic activity in hippocampus due to treatment ($F_{4, 288} = 57.14$; $p < 0.0001$) and time ($F_{17, 288} = 60.64$; $p < 0.0001$) as well as the interaction between treatment and time ($F_{4, 288} = 57.14$; $p = 0.0148$). The results indicated reduced LTP in the PBOCCA group treated with vehicle alone compared to that of the sham group ($p < 0.0001$) with enhanced LTP in the PBOCCA rats treated with embelin 0.3 and 0.6 mg/kg groups ($p < 0.0001$). Statistical mean values of the fEPSP amplitude for the last 2 hrs after TBS are graphically represented in Figure 2B. It was discovered that after high-frequency stimulation, LTP formation in the hippocampus has significantly inhibited PBOCCA group treated with vehicle (1.57 ± 0.02) than those in the sham group (1.96 ± 0.04; $p < 0.0001$). In addition, one-way ANOVA showed significant restoration of the LTP inhibition in the vehicle group with 0.3 mg/kg (2.01 ± 0.04; $p < 0.0001$) and 0.6 mg/kg (1.83 ± 0.06; $p = 0.001$) embelin in PBOCCA rats. Interestingly, 1.2 mg/kg embelin treated PBOCCA (1.38 ± 0.04; $p < 0.05$) group displayed a significant decrease in fEPSP amplitude after TBS compared to the PBOCCA group.

Changes in the mRNA level in the hippocampus by Real-time PCR

The expression of mRNA in PBOCCA rat hippocampal tissues after embelin treatment was studied by Real-time PCR analysis showing a significant increase in APP mRNA expression of PBOCCA group ($p < 0.0001$) than sham rats. In addition, a significant reduction in APP mRNA expression was observed in PBOCCA rats treated with 0.3 mg/kg ($p < 0.001$) and 0.6 mg/kg ($p < 0.01$) embelin compared to PBOCCA group. Meanwhile, group with 1.2 mg/kg embelin also demonstrated a significant reduction but a slight increase in APP mRNA expression compared to other embelin

groups ($p < 0.05$) (Figure 3A). Moreover, the Mapt mRNA expression was seen increasing significantly for PBOCCA rats treated with vehicle alone compared to sham-vehicle treated group ($p < 0.01$) along with a significant reduction in a dose-dependent manner for embelin treated groups (*$p < 0.05$ and ** $p < 0.01$). Figure 3B graphically displays the expression level of Mapt mRNA for each rat group.

The expression of CREB 1 mRNA level was observed significantly decreasing in PBOCCA rats than sham-vehicle treated rats ($p < 0.01$). However, statistically insignificant change in the gene expression level of CREB 1 for all the embelin treated groups was recorded compared to PBOCCA group at a level of $*\alpha = 0.05$. Nevertheless, 0.3 and 0.6 mg/kg embelin treated groups had visibly increased in the expression level of CREB 1 compared to the PBOCCA-vehicle treated alone group (Figure 4B). On the other hand, insignificant downregulation of BDNF mRNA level was noted in PBOCCA group compared with sham groups where only 0.3 mg/kg of embelin treated PBOCCA rats presented a significant increase in BDNF mRNA expression ($p < 0.05$). Besides, there was an insignificant increase in BDNF mRNA expression level in 0.6 and 1.2 mg/kg of embelin groups ($p > 0.05$) when compared to PBOCCA group as shown in Figure 4A. Furthermore, Figure 5A depicts a significant depletion of the gene level of SOD1 in terms of antioxidant gene expression for the PBOCCA group compared to the healthy control group ($p < 0.0001$). Additionally, all embelin treated PBOCCA rats demonstrated a significant increase in SOD1 expression with 3-fold change ($p < 0.0001$) compared to PBOCCA group. On the other hand, there was a substantial rise in the gene expression level of NFκB for PBOCCA group compared to that of sham-vehicle treated rats ($p < 0.05$). The mRNA level of NFκB was significantly ameliorated by embelin treatment ($p < 0.05$) as presented in Figure 5B.

Estimation of neurotransmitter levels in the hippocampus by LC-MS/MS

CCH-induced PBOCCA rats administered with vehicle caused a significant increase in the level of Glu ($p < 0.0001$) compared to sham rats. PBOCCA rats treated with embelin (0.3, 0.6, and 1.2 mg/kg, i.p.) significantly ameliorated the levels of Glu compared to PBOCCA vehicle-treated group (**$p < 0.01$ and ***$p < 0.001$). On the other hand, levels of GABA, ACh, and 5HT in the hippocampal of PBOCCA rats were reduced in comparison to normal control group. However, only 5HT levels were detected to be significantly less in PBOCCA rats at a p-value < 0.0001. Nevertheless, embelin treatment has restored the decreased levels of GABA and 5HT compared to PBOCCA vehicle-treated rats, but were not significant. Interestingly, PBOCCA rats treated with embelin at dose 0.3 mg/kg and 0.6 mg/kg significantly attenuated the decrease in the level of ACh ($p < 0.01$) compared to that of PBOCCA group. Meanwhile, embelin at dose 1.2 mg/kg group failed to restore the decreased level of ACh caused by CCH-induced PBOCCA rats as can be seen in Figure 6.

Discussion

The therapeutic effects of embelin were found promising in many neurological-related disorders using various animal models [19]. Recently, embelin treatment in rodents has successfully reversed scopolamine [17] and streptozotocin-induced cognitive deficits [20] by modulating the antioxidant pathway, cholinergic activity, hippocampal neurogenesis and neuroinflammatory cytokines. Even though embelin has been found as a potential molecule in the previous findings against AD-like conditions, to date, no studies have reported the memory-improving effects of embelin in a CCH animal model. Thus, this paper is the first to report the acute effects of embelin in cognitive

impairment and pathophysiological transformation following two weeks of carotid arteries occlusion. The selection of dose range for embelin treatment used in this current study was determined based on previously published study [17].

According to Farkas, et al. [9], the cerebral blood flow to hippocampus was reduced by ~60% compared to control level, which progressively continued for a week with their effects remained for several months resulting from the PBOCCA surgery. Hence, PBOCCA-induced CCH in rodents could be extrapolated to human cerebral hypoperfusion resembling ageing or demented people [21]. Those with AD tend to have deficits in spatial abilities as they become lost in familiar places and unable to relocate the place [22]. Therefore, MWM has been considered as a standard method to study the spatial cognitive function in rats [23]. In the current study, it was discovered that the PBOCCA group showed longer escape latency during the 4-days training period, as well as during probe trials when the platform was removed from the pool. These data suggest that the PBOCCA rats had significantly impaired spatial learning and reference memory that were in line with previous studies [6,24]. In contrast to PBOCCA group, rats treated with embelin 0.3 and 0.6 mg/kg groups were able to locate the platform much easier and faster and spent more time in the target quadrant. For the case of higher concentration, embelin at 1.2 mg/kg dose showed a longer escape latency similar to PBOCCA group. These results indicate that embelin administration with 0.3 and 0.6 mg/kg doses has effectively alleviated the cognitive deficiency caused by chronic cerebral hypoperfusion.

Synaptic transmission and plasticity are the basis of learning and memory; thus, this present study examined whether or not the embelin administration can affect the long-term potentiation (LTP) in hippocampal of PBOCCA model. Based on results, synaptic plasticity was found significantly impaired in PBOCCA rats, which was in agreement with those of previous studies [12,24,25]. Interestingly, the adverse effect of chronic cerebral hypoperfusion on LTP was significantly attenuated after treatment with 0.3 mg/kg embelin. According to Rong, et al. [26], enhanced LTP in the CA1 and CA3 fields of the dorsal hippocampus were due to a presynaptic mechanism of action. The result of LTP for 0.3 and 0.6 mg/kg was seen parallel with MWM performance supported by the idea that changes in synaptic efficacy underlie learning and memory processes [27]. Meanwhile, in the case of higher concentration, the present results are unusual with embelin 1.2 mg/kg dose displaying lower LTP than that of the PBOCCA model. The explanation for the decrease in the LTP for 1.2 mg/kg dose is similar to the previous study using scopolamine-induced memory impairment [17] in which the compound reached its maximum effect with the decline in cognitive ability at this dose for novel object recognition (NOR) and elevated plus maze (EPM). This experimental result is also supported by the fact that particular drug in which higher doses produce no effect or the opposite effect compared to intermediate doses [28].

In the case of APP mRNA expression, it was found that chronic cerebral hypoperfused rats displayed significant upregulation due to occlusion of the common carotid arteries. Literature has reported that the chronic cerebral hypoperfusion resulted in an increase in protein level of APP in the hippocampus of rats [29,30]. Meanwhile, the APP mRNA expression was significantly downregulated by the embelin treatment in a dose-dependent manner. Moreover, significant increase in Mapt mRNA level was found in PBOCCA rats, which is consistent with the results reported by other studies [31,32]. Although the Mapt mRNA expression level was significantly decreased by all embelin treatments, the highest dose of embelin at 1.2 mg/kg group showed maximum protection against Mapt in CCH rats. On the other hand, the lowest dose of embelin at 0.3 mg/kg group demonstrated maximum protection against APP. It can be explained from the

discrepancy that the downregulation of APP and Mapt by embelin maybe mediated through a different mechanism. This is another interesting outcome as this is the first time that embelin has been reported to downregulate the expression of APP and Mapt, which was directly linked to the AD.

To study the molecular basis of synaptic plasticity impairment, the expression of BDNF and CREB in the hippocampus of PBOCCA induced CCH rats was examined. BDNF is a crucial mediator involved in neuronal survival, development and synaptic plasticity [33]. Chronic cerebral hypoperfusion caused by blood flow insufficiency can cause progressive cognitive dysfunction with BDNF and CREB down-regulation. The cAMP-responsive element binding protein (CREB)-mediated transcription is needed in CNS for neuronal survival [34]. The transcription of the BDNF gene was CREB-regulated in an activity-dependent manner as demonstrated by several studies whereby its expression was involved in neuronal development, synaptic plasticity and neuroprotection [35]. In this present study, PBOCCA rats treated with vehicle alone showed a decrease in the BDNF level, which was significantly restored after treated with embelin. Moreover, it was reported that embelin at 0.3 mg/kg was able to enhance BDNF levels of the normal group in CCH rats. Besides, a similar pattern was recorded in CREB expression levels, but was insignificant compared to PBOCCA rats treated with vehicle alone. Furthermore, these outcomes were in accordance to MWM and LTP results whereby embelin at 0.3 mg/kg revealed BDNF and CREB1 upregulation as the reason for maximum improvement of spatial memory which is related to hippocampal and enhance the synaptic strength within the CA1 and CA3 neurons. Thus, it can be postulated based upon BDNF and CREB results that embelin is able to activate CREB pathway and improve BDNF mediated synaptic plasticity and neurogenesis.

There are several studies conducted emphasising the pathogenesis of chronic cerebral hypoperfusion and oxidative stress are caused by the implication of oxygen-derived free radicals, which play a significant role in cognitive dysfunction [36]. It has been proven by previous study that embelin has antioxidant properties in the hippocampus of the scopolamine model [17]. Therefore, it can be said that oxidative stress mechanism might have the potential to cause cognitive impairment. SOD in the mitochondrial matrix has been known as the prime line of antioxidant defence system. Hence, the mRNA expression level of SOD1 in the hippocampus was measured to assess whether or not the oxidative stress mechanism is involved in the effect of embelin. Data from this study displayed a good agreement with Zhang, et al. [37] from the observation of a significant decrease in SOD1 mRNA expression level of the PBOCCA rats' hippocampus. Interestingly, administrating embelin has significantly increased the SOD1 mRNA expression level, which indicates the antioxidant action of embelin that might be attributed to a direct receptor-mediated mechanism activating the downstream protein kinase signalling pathways and intracellular antioxidant enzyme systems [25].

The NFκB was the last gene studied displaying a huge upregulation in the expression level following the CCH, which is in accordance to study reported by Fu, et al. [38]. Nuclear factor NFκB is a transcription factor serving a vital role in gene regulation and is applied in inflammation and oxidative stress [39]. In this study, the NFκB expression level in all groups was significantly reduced by embelin treatment.

The glutamatergic, GABAergic, cholinergic and monoaminergic neurotransmitters were observed to highly regulate the hippocampal activity, which play an imperative role in memory acquisition, consolidation and storage in the brain [40,41]. Kaundal, et al. [42] explained that significant alterations

in hippocampal neurotransmitters can be observed following PBOCCA surgery in rats in addition to cognitive dysfunction. In the present study, PBOCCA-induced CCH rats caused a significant elevation in hippocampal glutamate levels, which is in agreement with the results obtained in other AD-like conditions including scopolamine [17,43] and streptozotocin rat model [20]. On the contrary, the GABA levels in CCH rats were noticed to be slightly decreased but it was statistically insignificant. Moreover, previous studies recorded that imbalance levels in glutamate/GABA have caused excitotoxic neuronal damage and cognitive dysfunction in related neurological disorder [41], which was similar to the present results. In the current study, embelin treatment significantly reduced hippocampal glutamate level and restored GABA level (insignificant) in CCH rats. It was also discovered that PBOCCA rats have caused a reduction in ACh level, thus indicating cognitive impairment due to the metabolism of ACh into acetate and choline by acetylcholinesterase (AChE) [20]. Interestingly, PBOCCA rats treated with embelin 0.3 and 0.6 mg/kg significantly attenuated ACh level denoting the acetylcholinesterase inhibitor action of embelin. Moreover, these results are also comparable to probe trial performance in MWM. Furthermore, studies have revealed that a rise in the extracellular level of endogenous ACh by AChE inhibitor treatment can contribute to an increase in cerebral blood flow [12,44,45]. Hence, it can be explained that embelin is neuroprotective in PBOCCA-induced CCH rats via the up-regulation of cholinergic function by restoring cerebral blood flow.

Many studies conducted on knockout animals reported an increase in serotonin level, which improved memory performance, whereas the reduction in this neurotransmitter led to impairment in spatial memory [46]. Therefore, the primary function of serotonin in spatial learning and memory could be due to its involvement in cortical-hippocampal synaptic connections [47]. In this CCH rat model, significant decrement in serotonin level was found in the hippocampal of PBOCCA rats compared to sham rats. However, the present results showed that PBOCCA rats treated with embelin could not significantly ameliorate the serotonin levels to normal even though there was a slight increment in serotonin level when compared to that of PBOCCA rats. Hence, these outcomes suggest that embelin might modulate spatial memory through different mechanisms other than serotonin. Although the exact molecular mechanism of embelin on the expression of neurotransmitters level remained unclear, the results from this study are convincing in postulating that embelin might play a role as a neuromodulator. Thus, the improvement in cognitive functions based on MWM and LTP in this PBOCCA-induced CCH rats could be linked to the ability of embelin to modulate hippocampal neurotransmitters to a normal state. In conclusion, this study has provided novel finding where embelin treatment has mitigated the spatial memory and LTP impairment in a PBOCCA-induced CCH rat model. Embelin possessed neuroprotective and anti-AD effects that could be mediated by synaptic plasticity, antioxidant, anti-inflammatory, APP and Mapt gene response, cholinergic activity, neurochemical modulation and BDNF-CREB pathway. Thus, this current study suggests that embelin could become a potential therapeutics compound for treating cognitive disorders including VaD and AD. Figure 7 displays the potential mechanism of embelin action in CCH-induced memory impairment in rodents.

Materials And Methods

Animals

In-house bred male Sprague Dawley rats weighing 200-300 g and 6–8 weeks old were obtained from the Animal Research and Service Centre, Universiti Sains Malaysia (USM), Penang, Malaysia. The rodents were kept in cages maintained under standard husbandry conditions (12:12

h light/dark cycle, constant room temperature with no restriction on food and water supply). Prior to the employment of this study, the rats were allowed to acclimatise for one week in the transit house to reduce stress. We confirm that all animal experimentations were performed in accordance with relevant guidelines and regulations that have been approved by USM Animal Ethics Committee with the reference number USM/Animal Ethics Approval/2015/ (97) (707).

Surgery

Permanent bilateral common carotid arteries occlusion (PBOCCA) in rats was performed as previously described [6]. Briefly, a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) was utilised to intraperitoneally anaesthetise all the rats. For PBOCCA surgery, the common carotid arteries were exposed by a surgical cut at the ventral midline. Both common carotid arteries were permanently ligated using a 5/0 silk suture. The skin incision was then closed, while the rats were kept in a well-ventilated room at a temperature of 25^{o}C. The sham group was subjected to the same method without PBOCCA. All the rats were left for two weeks recovery period before being subjected to the Morris water maze and *in vivo* electrophysiology. Rats that subjected to PBOCCA surgery are susceptible to seizures, impaired vision and drastic weight loss. Thus, rats that showed any of these characteristics after PBOCCA surgery were excluded from this study. The mortality rate for rats that underwent PBOCCA surgery in this study was 20%.

Experimental design and treatment

Embelin preparation and the selection of doses for treatment were determined using the data from our previous study [17]. PBOCCA rats were divided into four groups. Group 1: Negative Control (PBOCCA + Vehicle) Saline with DMSO (n=6-7); Group 2: (PBOCCA + EMB 0.3mg/kg) low dose of EMB (n=6-7); Group 3: (PBOCCA + EMB 0.6mg/kg) medium dose of EMB (n=6-7); Group 4: (PBOCCA + EMB 1.2 mg/kg) high dose of EMB (n=6-7). The sham rats were labelled as Group 5 (n=6-7) and treated with the same vehicle as the negative control. Embelin and vehicle were given intraperitoneally (i.p.) at a volume corresponding to 0.1 ml/100 g of body weight from day 14 onwards. Figure 8 illustrates a schematic representation of the experimental procedure for CCH-induced PBOCCA rats.

Behavioural Assessment

Morris Water Maze Test

The experiment protocol for the Morris water maze was conducted adopting from Damodaran, et al. [12] with slight modifications. This study selected a black circular pool with 160 cm in diameter and 70 cm in height, which was placed in a test room surrounded by several visual cues. This was then followed by the addition of water into the pool to a depth of 39 cm. Besides, the pool was made opaque by the white paint added to it. The pool was divided into four quadrants, with a platform (10 cm diameter) situated 2 cm below the surface of the water in a fixed position in one quadrant while the opaque water was kept constant temperature at 25 ± 1°C. Furthermore, the rats were given a pre-training session on the habituation day where they were allowed to swim freely in the pool for 60 s without a platform. All rats were put in the water at four starting points during the training session, respectively, and 60 s was set as the limit for the latency of escaping onto the platform to be recorded as a trial. Four trial were conducted daily within four consecutive days for each rat. Treatments were given intraperitoneally after each training session. It was observed on the fifth day that no platform was present; thus, each rat was subjected to a probe trial. The

percentage time spent in the target quadrant was used for obtaining the spatial reference memory for each rat.

Long-Term Potentiation (LTP)

The *in vivo* electrophysiological recording was conducted following the protocol proposed by Damodaran, et al. [12]. Briefly, urethane was utilised to anaesthetise the rats (2.0 g/kg, i.p., divided into four, 0.5 g/ kg doses every 20 min). An incision line was made to expose the skull of the rat on a stereotaxic frame. Standard stereotaxic measurements relative to bregma were utilised as the reference in drilling small holes in their skull to implant the recording electrode in the hippocampus CA1 region (AP: − 4.2 mm, mL: − 3.0 mm, V: − 3.0 mm). The bipolar stimulating electrode was placed into the Schaffer collaterals CA3 region of the hippocampus (AP: − 4.2mm, mL: + 3.0mm, V: − 4.0mm). Meanwhile, to find the stimulating intensity that could evoke 50-60 % response of its maximum extracellular field excitatory postsynaptic potentials (fEPSPs) amplitude, stimuli intensities between 0.1 and 1.0 mA with increment of 0.1 mA were delivered to the Schaffer collaterals. The stable baseline was recorded every 30 s for 1 hour. Then, single theta burst stimulation (TBS) comprising of ten bursts (each burst consisting of 5 pulses at 100 Hz) with bursts repeated every 200ms was delivered to induce LTP. The recording of fEPSPs were recorded every 30 s for 2 hrs.

Tissue Processing

After the completion of the *in vivo* electrophysiological recording, all the rats were sacrificed under urethane anaesthesia with their brains extracted. Following this, the hippocampi isolation and the samples were stored at −80 °C until further analysis. For gene expression, one part of the hippocampus was transferred into 200μL ice-cold TRIzol®, whereas for neurotransmitter study, the other one was put into 200 μL ice-cold methanol with formic acid.

Total RNA extraction and Real-Time PCR

The method of Bhuvanendran, et al. [17] was seen similar to that of this study where the total RNA was extracted with identical Real-time PCR for the CCH- induced rat brain's hippocampal. The mRNA expression level of genes encoding brain-derived neurotrophic factor (BDNF), superoxide dismutase 1 (SOD1), amyloid precursor protein (APP), microtubule-associated protein tau (Mapt), nuclear factor kappa B (NF-κB) and IMPDH2 (Inosine Monophosphate Dehydrogenase 2) housekeeping gene was measured utilising Applied Biosystem real-time PCR. Threshold cycle (Ct) values of genes of interest was used against the Ct value of housekeeping gene to measure the expression level of five genes of interest using the formula: 2^ (Ct value of housekeeping gene – Ct value of gene of interest).

Neurotransmitter analysis using LC-MS/MS

LC-MS/MS was used to estimate the level of neurotransmitters such as glutamate (Glu), γ-aminobutyric acid (GABA), acetylcholine (ACh) and serotonin (5HT) of CCH-induced rats. The protocol for neurotransmitter analysis has been described in details in the previous studies [17]. For validating Glu, GABA, ACh and 5HT, four calibration standards for neurotransmitters in concentration ranges of 250.00–20,000.00, 250.00–20,000.00, 0.25–600.00 ng/mL and 0.05–5.00 ng/mL were used, respectively. The stock for the standards was prepared with methanol (0.1% formic acid) and kept at 4 ^{o}C until use. In short, one part of the hippocampus was homogenised in ice-cold methanol containing 1% formic acid followed by vortex-mixed for 60 s. The resulting

homogenate was then centrifuged at 14 000 rpm for 10 min at 4 °C. Finally, the resulting supernatant was put into vial inserts for the analysis of LC-MS/MS. ZORBAXE clipse plus C18 RRHD 2.1 × 150.0 mm and 1.8-micron (P/N959759-902) column was utilised to separate the samples, which was then placed on Agilent 6410 Triple Quad (Agilent Technologies, Santa Clara, CA, United States) at 30 °C. The mobile phase comprising 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B) was used with a gradient elution of 0–3 min, 50% B; 3–6 min, 95% B; 06–07 min and 95% B at a flow rate of 0.1 ml/min. MS acquisition of Glu, GABA, ACh, and 5HT was done in a positive electrospray ionisation multiple reaction monitoring (MRM) mode.

Statistical Analysis

Mean ± standard errors of the mean (SEM) were used to express the results obtained. Two-way ANOVA was used to analyse the results of acquisition trial of MWM and differences in fEPSP amplitude after TBS, which was then followed by Bonferroni's post hoc test. Statistical analysis for probe trial in MWM demonstrated the average fEPSP amplitude for 2 h recording, whereas mRNA expression and neurotransmitters levels were analysed using one-way ANOVA followed by Dunnett's post hoc test. Comparison was made for all the groups with the negative control group (PBOCCA) and found that *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ were statistically significant. Additionally, the statistical analysis was carried out using GraphPad Prism software (version 7.0).

Data Availability Statement

The dataset generated from this study is available from the corresponding authors on reasonable request.

Ethics Statement

All animal experimentations conducted in this study have been approved by USM Animal Ethics Committee with the reference number USM/Animal Ethics Approval/2015/ (97) (707).

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Contributions

MFS, SB and ZH were involved in conceptualizing, designing the study, data analysis, and manuscript writing. SB performed all the experiments and supported by SNSB and ZH. IO and YK were involved in LC-MS/MS and gene expression study respectively. All authors gave their final approval for the submission of the manuscript.

Competing interest

The authors declare no competing interests

Figure 1. The effect of post-training administration of embelin on the performance of MWM in PBOCCA- induced CCH rats. (a) Escape latency in the MWM test of each training day. (b) Mean time spent on the platform zone in the MWM test. The behavioral analysis for (a) and (b) were compared to negative control group (P + Veh). Data are expressed as mean ± S.E.M. from (n=7) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. The effect of embelin on LTP in the CA1 hippocampus. (a) Time course changes in normalized fEPSP amplitude after TBS (b) The mean fEPSP amplitude during the 2-h interval after TBS. The analysis for (a) and (b) were compared to negative control group (P + Veh). Data are expressed as the mean ± S.E.M. (n=6) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3. The effect of embelin on amyloid-beta (a) APP and Tau (b) Mapt mRNA expression level in the rat hippocampus using real-time PCR. All changes in the expression levels were compared to negative control group (P + Veh). Data are expressed as the mean ± S.E.M. (n=6) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$

Figure 4. The effect of embelin on synaptic plasticity mRNA expression level (a) BDNF and (b) CREB1 in the rat hippocampus using real-time PCR. All changes in the expression levels were compared to negative control group (P + Veh). Data are expressed as the mean ± S.E.M. (n=6) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

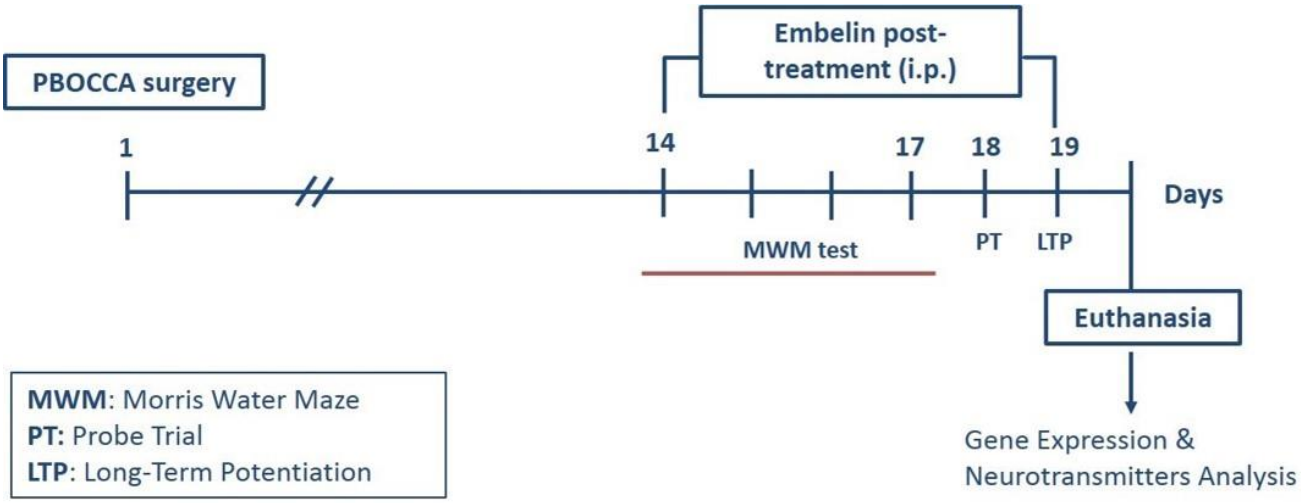
Figure 5. The effect of embelin on oxidative stress (a) SOD1 and neuroinflammation (b) NF-κB mRNA expression level in the rat hippocampus using real-time PCR. All changes in the expression levels were compared to negative control group (P + Veh). Data are expressed as the mean ± S.E.M. (n=6) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$

Figure 6. The effect of embelin on hippocampus neurotransmitters level of CCH-induced PBOCCA rats. The neurotransmitters included are (a) Glutamate (b) GABA (c) Acetylcholine (d) Serotonin. All changes in the expression levels were compared to negative control group (P + Veh). Data are expressed as the mean ± S.E.M. (n=6) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$

Figure 7. Mechanism of action of embelin in a Rat Model of Chronic Cerebral Hypoperfusion

Figure 8. Schematic representation of the experimental procedure and treatment schedule for CCH-induced PBOCCA rats

Figure 1



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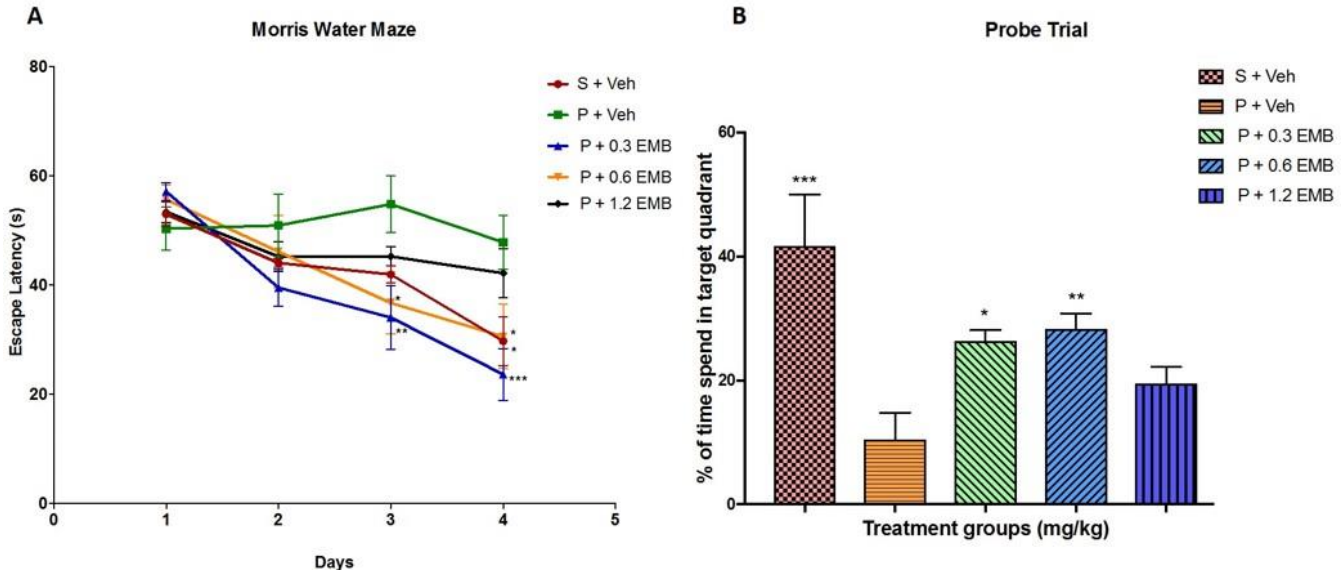

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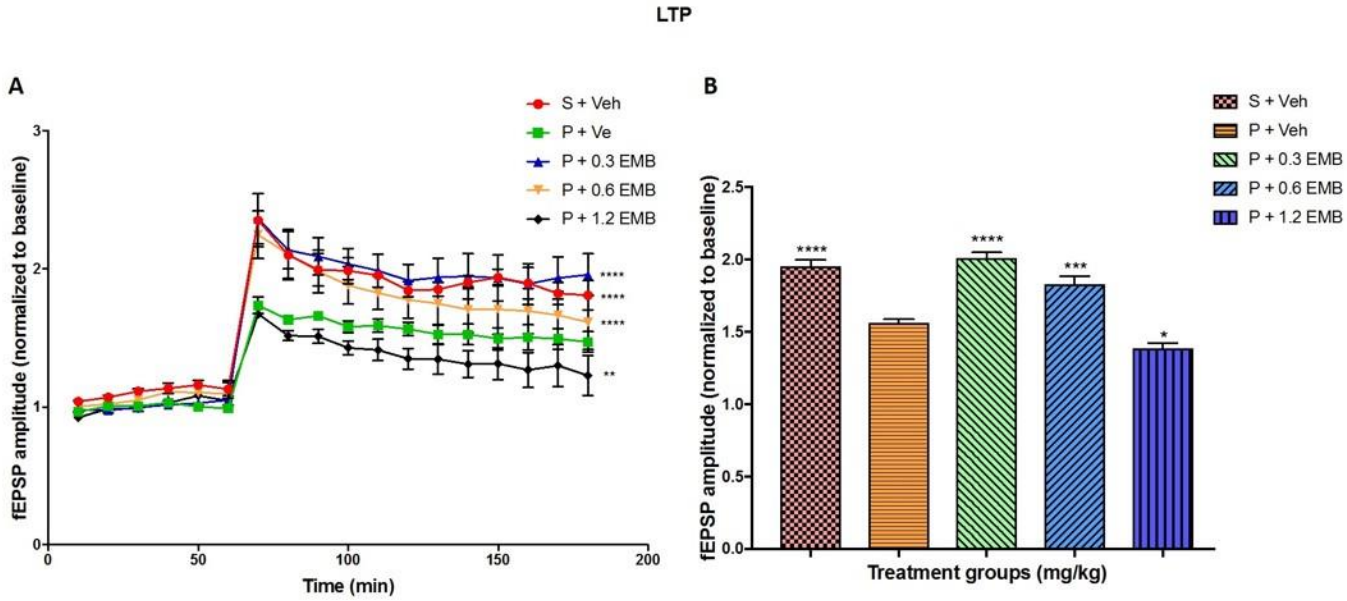

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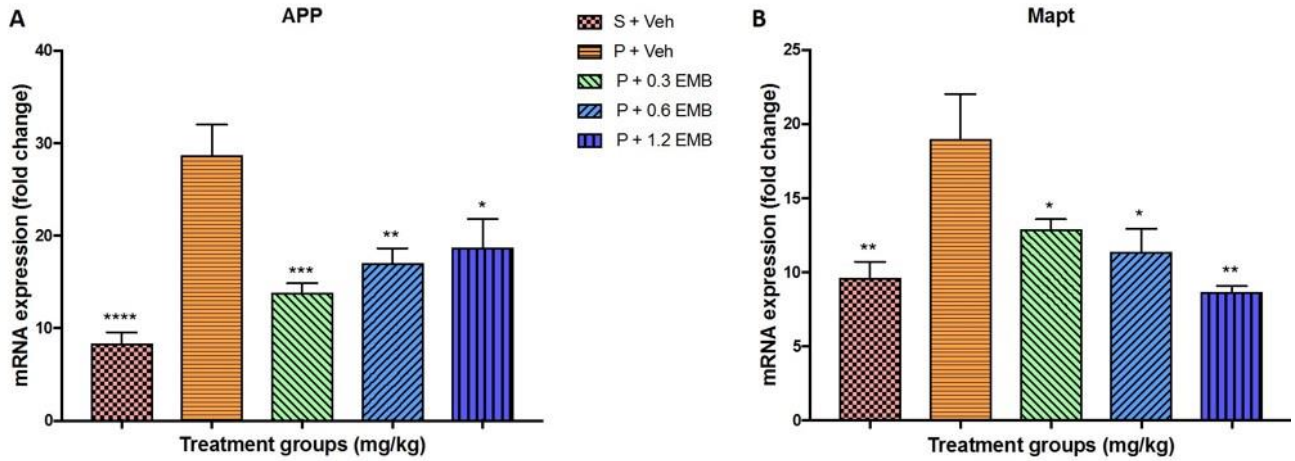

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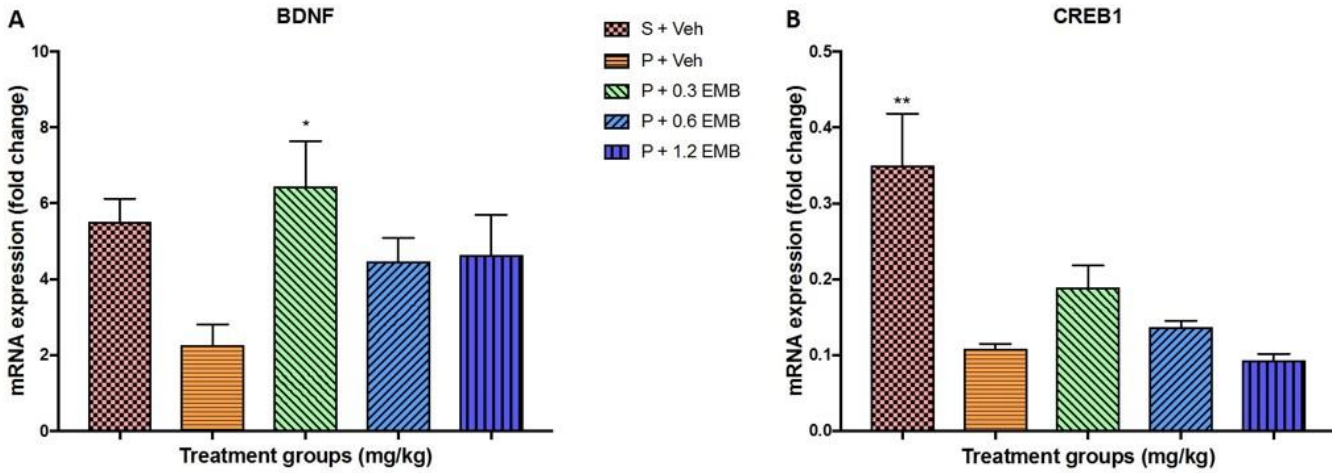


Figure 6

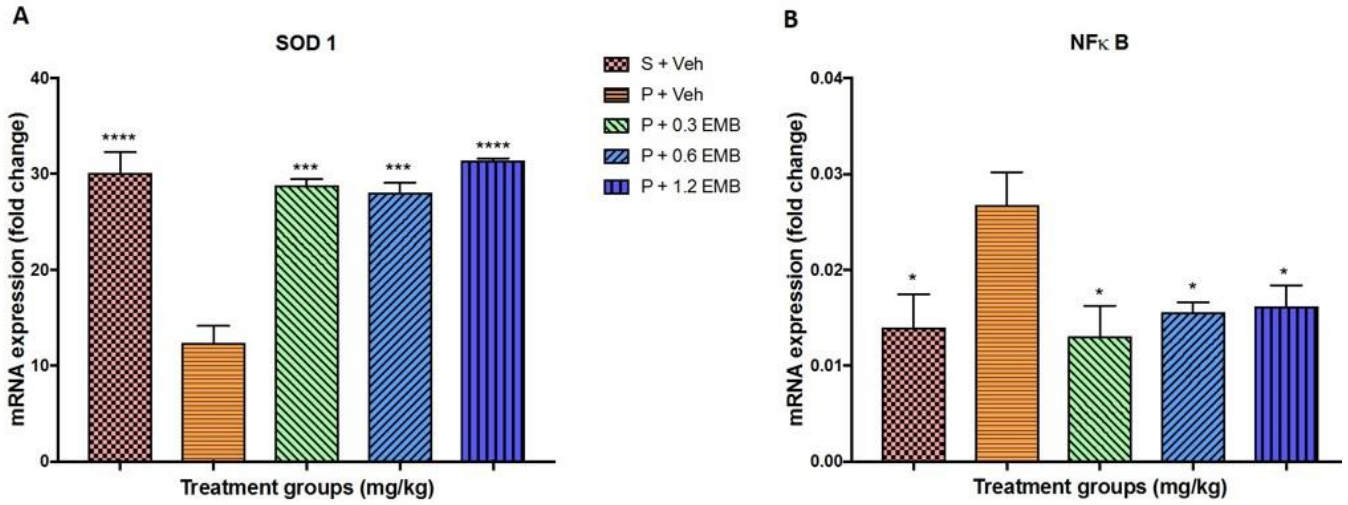
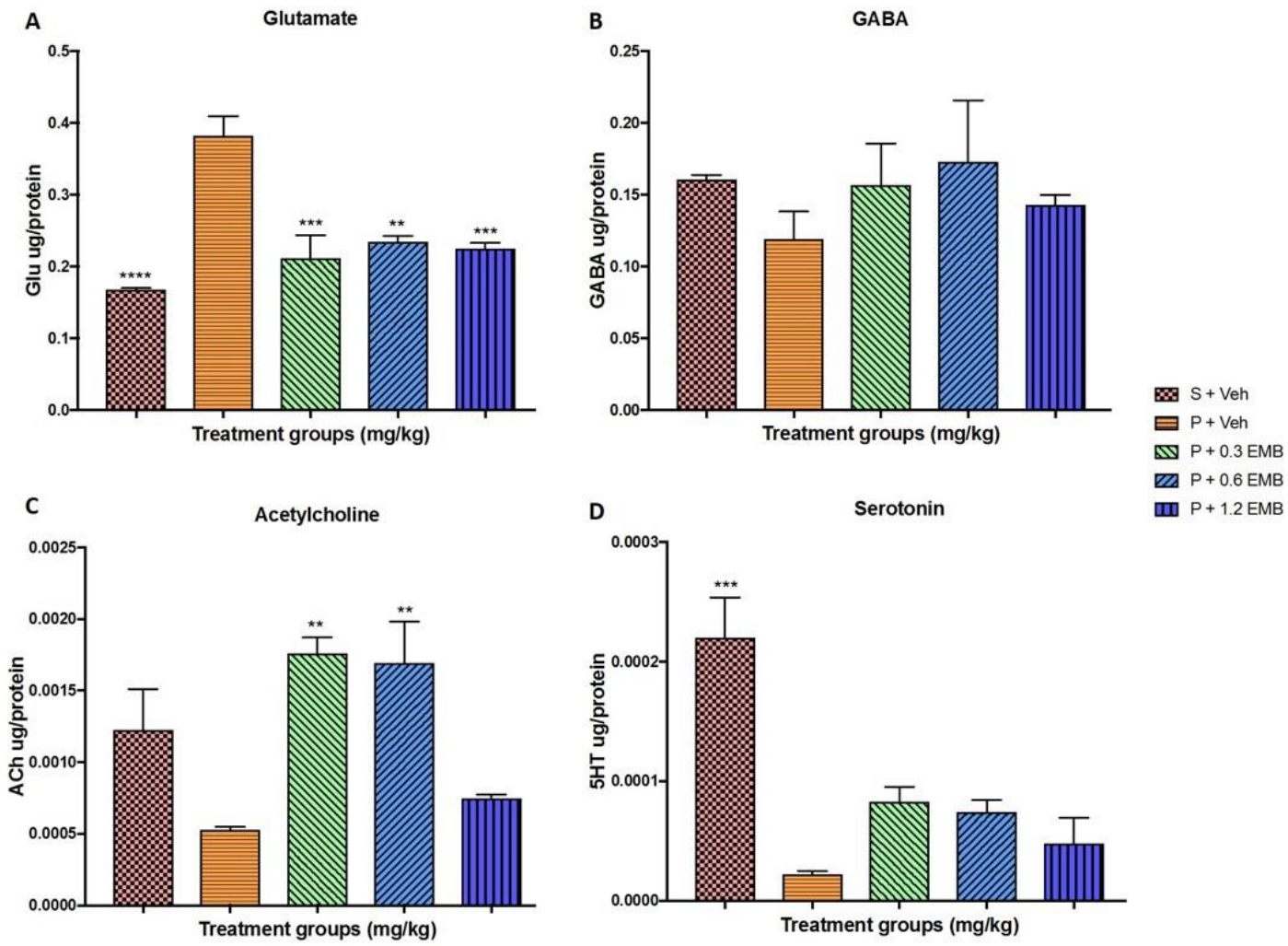


Figure 7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Treatment groups (mg/kg)
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P + 0.6 EMB
P + 1.2 EMB
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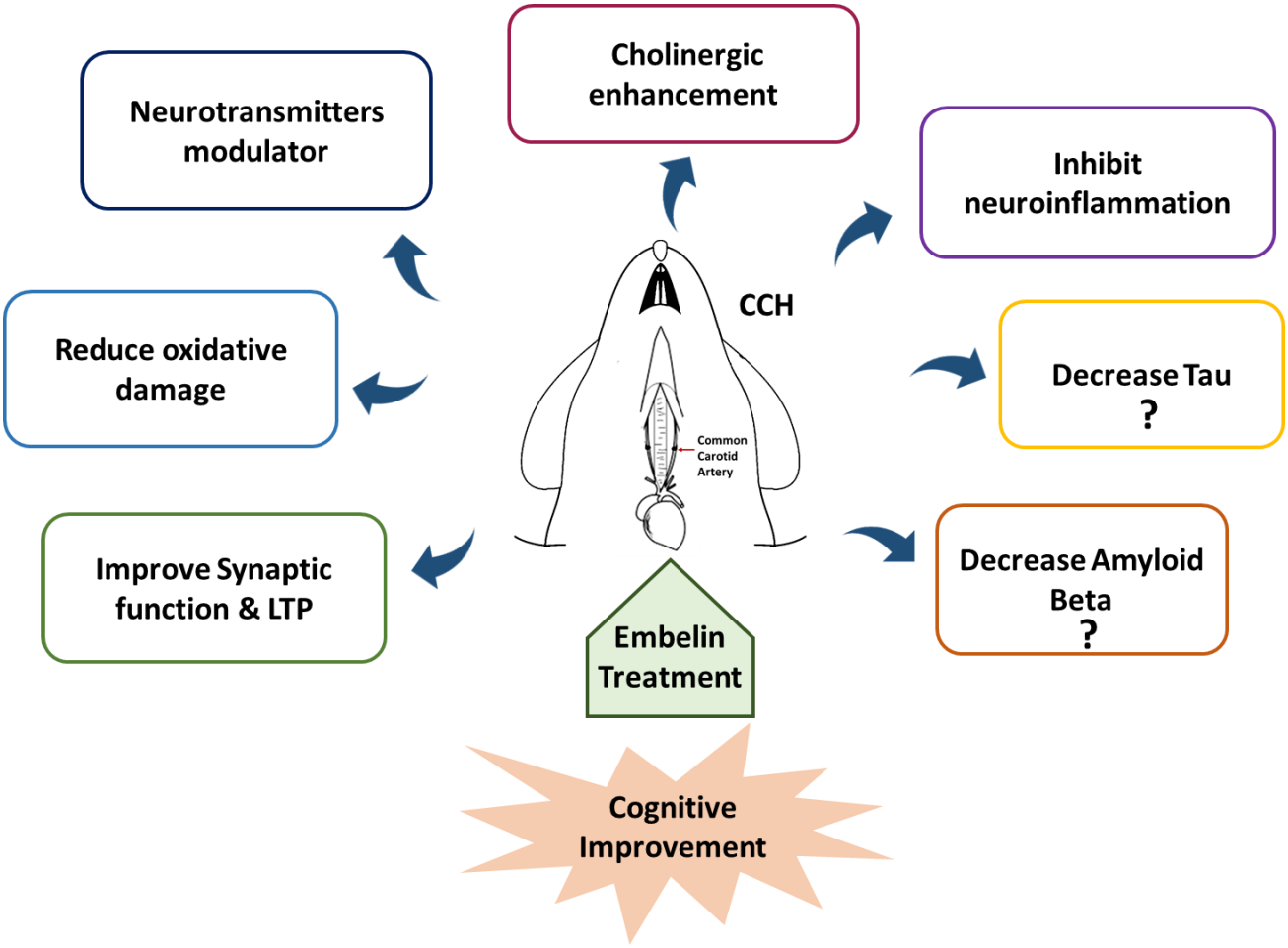
Treatment groups (mg/kg)

Figure 8


Cholinergic enhancement
Neurotransmitters modulator
Inhibit neuroinflammation
CCH
Reduce oxidative damage
Decrease Tau ?
Common Carotid Artery
Improve Synaptic function & LTP
Decrease Amyloid Beta ?
Embelin Treatment
Cognitive Improvement

Chapter 6

6.1 Introduction

Streptozotocin (STZ) (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose) is also known as glucosamine-nitrosourea. This compound is generally used to induce experimental diabetes in animals (70). On the other hand, administration of STZ through intracebroventricular (icv) or intraperitoneal (ip) routes is able to induce sporadic AD-like condition with brain insulin resistance, accumulation of Aβ, tau, oxidative stress and progressive decline in cognitive function (71). According to Šalković-Petrišić (26), similar pathological features can be observed between sporadic AD in humans and STZ-icv treated rats. Accumulation of laboratory evidences demonstrated that STZ-icv animal models had been extensively used to assess the therapeutic potential of both old and novel compounds and drugs for the development of potential anti-AD drug (72). Herein, the third part of our study, we evaluated the neuroprotective effects of embelin in STZ-induced sporadic AD like condition in an *in-vitro* model using rat primary hippocampal neuronal cultures.

Elsevier Editorial System(tm) for European Journal of Pharmacology

Manuscript Draft

Manuscript Number: EJP-49604

Title: Embelin prevents amyloid beta accumulation via GSK-3 pathway in an in vitro model of streptozotocin-induced AD like condition

Article Type: Research Paper

Section/Category: Molecular and cellular pharmacology

Keywords: embelin, streptozotocin, Alzheimer's disease, neuroprotection, hippocampal neuronal culture

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Abstract: Previous studies have shown that embelin has beneficial effects in scopolamine-induced amnesia rat model. However, the neuroprotective role of embelin and the mechanism of action in Alzheimer's disease (AD) are remain unclear. Therefore, in this study the neuroprotective effect of embelin against neuronal damage induced by streptozotocin (STZ) in rat hippocampal neuronal culture were evaluated. STZ had been proven to cause imbalance in glycogen synthase kinase 3 (GSK-3) along with amyloidogenesis, oxidative stress and neuroinflammation mimicking the condition of human AD. Present findings demonstrated that embelin (2.5-10μM) has efficiently protected hippocampal neurons against STZ-induced neurotoxicity. An increase in amyloid precursor protein (APP), microtubule-associated protein tau (Mpat), glycogen synthase kinase 3 alpha (GSK-3α) and glycogen synthase kinase 3 beta (GSK-3β) expression levels was observed when STZ (8mM) stimulation was done for 24 hours in the hippocampal neurons. Meanwhile, a huge decrement was showed in APP, Mapt, GSK-3α and GSK-3β mRNA expression levels suggesting that the pre-treatment with embelin has attenuated STZ-induced insulin signalling (IR) dysfunction. Furthermore, embelin demonstrated protective effect against STZ-induced oxidative stress and neuroinflammation, which was made apparent by the elevated expression of scavenger enzyme (SOD1) and reduction in neuroinflammatory markers (NF-κB) of mRNA levels. Moreover, the histological changes of amyloid beta protein expression induced by STZ were mitigated in a manner comparable to the control group by the pre-treatment of embelin. In conclusion, embelin can improve STZ induced sporadic AD-like condition and can be further developed into drug target to treat and prevent neuronal damages related to AD.

Cover Letter

To,

F. A. Redegeld

The Chief Editor,

European Journal of Pharmacology (Elsevier)

Subject: Submission of a manuscript entitled **"Embelin prevents amyloid beta accumulation via GSK3 pathway in an in-vitro model of streptozotocin-induced AD like condition"**

Dear Editor,

I would like to submit the article entitled above to your esteemed European Journal of Pharmacology (Elsevier). This original research article is regarding an investigation into the anti-AD potential of embelin, a plant derived bezoquinone for Alzheimer's disease using an *in vitro* model of primary cultured rat hippocampal neurons. This research is important as embelin has been experimentally proven to possess many properties such as being anti-oxidant and anti-inflammatory, but no study so far has determined the mechanism of action of embelin against STZ induced neurotoxicity which mimics pathology of AD. Neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

Kindly consider this article for publication in your esteemed journal.

Thanking you.

Regards,

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Embelin prevents amyloid beta accumulation via GSK-3 pathway in an *in vitro* model of streptozotocin-induced AD like condition

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Abstract

Previous studies have shown that embelin has beneficial effects in scopolamine-induced amnesia rat model. Therefore, in this study the neuroprotective effect of embelin against neuronal damage induced by streptozotocin (STZ) in rat hippocampal neuronal culture were evaluated. STZ had been proven to cause imbalance in glycogen synthase kinase 3 (GSK-3) along with amyloidogenesis, oxidative stress and neuroinflammation mimicking the condition of human AD. Present findings demonstrated that embelin (2.5-10μM) has efficiently protected hippocampal neurons against STZ-induced neurotoxicity. An increase in amyloid precursor protein (APP), microtubule-associated protein tau (Mpat), glycogen synthase kinase 3 alpha (GSK-3α) and glycogen synthase kinase 3 beta (GSK-3β) expression levels was observed when STZ (8mM) stimulation was done for 24 hours in the hippocampal neurons. A huge decrement was showed in APP, Mapt, GSK-3α and GSK-3β mRNA expression levels suggesting that the pre-treatment with embelin has attenuated STZ-induced insulin signalling (IR) dysfunction. Furthermore, embelin has protective effect against STZ-induced oxidative stress and neuroinflammation, which was made apparent by the elevated expression of scavenger enzyme (SOD1) and reduction in neuroinflammatory markers (NF-κB) of mRNA levels. Moreover, the histological changes of amyloid beta protein expression induced by STZ were mitigated in a manner comparable to the control group by the pre-treatment of embelin. In conclusion, embelin can improve STZ induced sporadic AD-like condition and can be further developed into drug target to treat and prevent neuronal damages related to AD.

Keywords: embelin, streptozotocin, Alzheimer's disease, neuroprotection, hippocampal neuronal culture

1. Introduction

One of the most common brain neurodegenerative disorders is Alzheimer's Disease (AD), which is irreversible with clinical symptoms of severe cognitive and memory impairment (Bertram and Tanzi, 2005; Ferri et al., 2005; Qu et al., 2012; Zhao et al., 2016). The late onset sporadic AD condition can be represented by the range of people affected by this disease, which is ranging from 65 years old and above (Ghumatkar et al., 2015). On the contrary, very rare cases have been reported related to early onset familial AD on people age between 30 and 60 years (Sherrington et al., 1995) caused by missense mutation or inheritance (Šalković-Petrišić, 2008). Nonetheless, these two AD conditions have been reported to have a relationship with common pathological hallmarks with extracellular amyloid beta plaques and intracellular neurofibrillary tau tangles that disrupt synaptic connections, leading to neuronal death (Butterfield et al., 2006).

Recently, Plaschke and Kopitz (2015) showed a pathogenetic link between sporadic AD and type 2 diabetes mellitus (T2DM). Both disorders share similar features associated with glucose metabolism dysfunction and insulin signalling impairment that results in neuronal damage and cognitive deficits (Candeias et al., 2012). High expression of insulin mRNA in the hippocampus and rise in insulin receptor (IR) at hippocampal synaptic membrane are functionally associated with improved learning and memory (Zhao et al., 2004; Zhao et al., 1999). Nonetheless, the exact mechanisms of insulin influencing learning and memory still remain vague. However, growing evidence from *in vitro* and *in vivo* studies revealed that impairment in IR signalling leads to the activation of glycogen synthase kinase 3 (GSK-3) through kinase/phosphatase imbalance state (Cross et al., 1995; Hooper et al., 2008; Šalković-Petrišić, 2008). Furthermore, studies have demonstrated that GSK-3α and GSK-3β are the two key isoforms derived from GSK-3 regulating the production of amyloid beta (Aβ) (Hooper et al., 2008; Phiel et al., 2003) and tau phosphorylation (Ishiguro et al., 1993), respectively.

Streptozotocin (STZ) is chemically known as (2-deoxy-2-[3-methyl-3-nitrosourea] 1-D-glucopyranose), which is also popular as a diabetic inducing agent in animals (Goud et al., 2015). In AD related studies, STZ administration induced IR signalling impairment along with neuroinflammation and oxidative stress in *in vivo* and *in vitro* model, which mimic the human AD pathology (Calvo-Ochoa and Arias, 2015; Rajasekar et al., 2014; Salkovic-Petrisic et al., 2006).

Embelin, an active ingredient isolated from the fruits of *Embelia ribes* Burm has been traditionally used as brain tonic for the treatment of neurological related disorders (Kundap et al., 2017). Recently, this compound has been studied for its neuroprotective effects in AD like conditions in animals (Arora and Deshmukh, 2017; Bhuvanendran et al., 2018). In this study, the primary culture of hippocampal neurons was utilised as a model to study the neuroprotective effect of embelin in STZ-induced neurotoxicity. Using this model, it was aimed to develop a better understanding the factors that can reverse AD through IR signalling pathways mimicking the sporadic AD pathology by embelin derived benzoquinone.

2. Materials and Methods

2.1 Primary neuronal culture

Newborn P4 Sprague Dawley (SD) rat pups were acquired from the Animal Facility of Jeffery Cheah School of Medicine and Health Sciences, Monash University Malaysia. All animal experimentation was approved and performed in agreement with Monash Animal Research Platform (MARP) Animal Ethics Committee with reference number MARP/2017/032. Primary cultures of hippocampal neurons were prepared as described by Kaech and Banker (2006) and Qu et al. (2012) with a minor modification. After treatment with 5 mL of trypsin for 15 min at 37 ^{o}C, the hippocampal neurons were washed in Krebs buffer for 5 min to stop the trypsin reaction. Then, the hippocampal neurons were suspended in fresh 2.5 mL Krebs buffer with 5 µL DNase and slowly triturated. Neurons were plated in poly-D-lysine-coated well, followed by 2 hours incubation at 37 ^{o}C in a humidified atmosphere comprising 95% air and 5% CO_2. Once the neurons adhered to the coated well, the medium was substituted with serum-free Neurobasal A added with 2% B27, 1.34mM glutamine, 28mM Glucose and 1.34 mL antibiotic-antimycotic solution (100X) followed by 8– 10 days incubation period with half of the medium being changed each 2 days to guarantee neuronal growth (Chen et al., 2008; Chen et al., 2009).

2.2 *In vitro* neuroprotection test

To evaluate the protective role of embelin against STZ instigated neurotoxicity on primary hippocampal neuronal culture, three independent experiments were directed to examine (i) the effect of various concentrations of embelin on neuronal cultures, (ii) the impact of various

concentrations of STZ on neuronal cultures viability and lastly (iii) the defensive role of various embelin concentrations against the toxicity caused by STZ on the primary hippocampal neuronal cultures. Poly-D-lysine-coated 96 well flat-bottomed plates were used for seeding the neurons at $3x10^4$ neurons per well. STZ and embelin were dissolved in DMEM at the chosen concentrations before adding them to neuronal culture.

2.3 Effect of different concentrations of embelin on primary hippocampal neuronal culture

Hippocampal neuronal cultures were treated with embelin at concentrations of 2.5, 5, 10, 20, 40, 80, and 160µM for 24 hours. The untreated neuronal cultures served as control. Each sample were screened in triplicates and each test was repeated 3 times in order to confirm the accuracy of the results.

i. Effects of different concentrations of STZ on primary hippocampal neuronal culture

Hippocampal neuronal cultures were treated with STZ for 24 hours at varied concentrations of 0.39, 0.78, 1.56, 3.125, 6.25 and 12.5mM. The control neurons did not receive any treatment. Each sample test was run in triplicate. The half maximal inhibitory concentration (IC_{50}) of STZ was determined using following formula as described by Qu et al. (2012).

$$Lg\ (IC_{50}) = Lg\ (X_m) - Lg\ (I) \times [P_s - (3 - P_m - P_n]/4]$$

in which, $\mathbf{X_m}$ is the maximal concentration; **I** is the dilution factor; $\mathbf{P_s}$ is the sum of inhibition ratio; $\mathbf{P_m}$ is the maximal inhibition ratio; $\mathbf{P_n}$ is the minimal inhibition ratio and **Lg** is the common logarithm.

ii. Protective effects of different embelin concentrations against STZ induced toxicity

Hippocampal neuronal cultures were pre-incubated with embelin for 2 hours at concentrations of 0.04, 0.08, 0.16, 0.63, 1.25, 2.5, 5, 10 and 20µM before exposure to STZ for 24 hours at the IC_{50} concentration of 8mM. Untreated neuronal cultures served as control.

2.4 MTT Viability Assay

The hippocampal neuronal culture viability was analysed using the standard MTT assay (Chen et al., 2009). After embelin and STZ treatment, 20 µL MTT (5mg/mL) was added to the culture and

incubated for 4 hours. Then, 100 µL DMSO was replaced with the culture medium and absorbance was read at 570 nm using a Microplate reader. The data was presented as a percent of control value. The percentage of hippocampal neuronal culture viability was ascertained using the formula as follows;

Percentage of neurons viability = (absorbance of treated neurons / absorbance of untreated neurons) x 100

2.5 Grouping and Drug Treatment for Gene expression and Immunofluorescence Studies

The hippocampal neurons were cultured on 24 well flat-bottomed plates coated with poly-D-lysine at $5x10^5$ neurons per well. The treatments were divided into 5 groups as below with triplicate well for each group.

(i) Group 1: Normal Control, Basic Culture Medium only;

(ii) Group 2: Negative control, Basic Culture Medium + STZ (8mM);

(iii) Group 3: Embelin (Low dose) 2.5µM + STZ (8mM);

(iv) Group 4: Embelin (Medium dose) 5µM + STZ (8mM);

(v) Group 5: Embelin (High dose) 10µM + STZ (8mM)

The treatment began once the neurons reached confluence. Further, the hippocampal neuronal cultures were pre-treated with embelin or basic media for 2 hours and then were induced with STZ 8mM for 24 hours. At the end of the experiment, the hippocampal neurons were extracted for gene expression and immunohistochemistry studies.

2.6 Total RNA extraction and Real-Time PCR

Total RNA was extracted from hippocampal neurons using Trizol reagent and phenol-chloroform extraction as described by Bhuvanendran et al. (2018) with a minor adjustments. The hippocampal neurons were homogenised in 200 µL Trizol solution. Then by adding 40 µL of chloroform, the homogenate was centrifuged at 13,500 rpm for 15 min at 4 °C. After that, the supernatant was deliberately removed and then precipitated with same volume of isopropanol in another Eppendorf tube. This was then pursued by centrifugation at 13,500 rpm for 10 min at 4 °C. Then, the supernatant was delicately removed, and the pellet was washed twice with 70%

ethanol. Lastly, the pellet was suspended in 20 µL of RNase free water. Nanodrop Spectrophotometer was utilised to obtain the total RNA concentration and purity. Later, 300 ng of total RNA from each sample was reverse transcribed to complementary DNA (cDNA) by using QuantiTect® Reverse Transcription Kit as described by the manufacturer's instruction. Next, the mRNA gene expression for encoding amyloid precursor protein (APP), microtubule-associated protein tau (Mpat), (GSK3α), (GSK3β), nuclear factor kappa B (NF-κB), superoxide dismutase 1 (SOD1) and IMPDH2 in the treated neuronal cultures were estimated by using the below primer sets procured from Qiagen.

APP: Rn_App_1_SG QuantiTect Primer Assay (Cat no: QT00177408)

Mapt: Rn_Mapt_1_SG QuantiTect Primer Assay (Cat no: QT00174797)

GSK3α: Rn_RGD:620351_1_SG QuantiTect Primer Assay (Cat no: QT00187453)

GSK3β: Rn_ Gsk3b_1_SG QuantiTect Primer Assay (Cat no: QT00182406)

Sod1: Rn_Sod1_1_SG QuantiTect Primer Assay (Cat no: QT00174888)

NF-κB: Rn_Nkapl_va.1_SG QuantiTect Primer Assay (Cat no: QT02476803)

IMPDH2: Rn_ Impdh2_1_SG QuantiTect Primer Assay (Cat no: QT01576036)

The resulting cDNA and the primer sets for gene of interest were subjected to StepOne Real-Time PCR using QuantiNova^{TM} SYBR® Green PCR kit. The thermal cycling conditions were set using a similar protocol that was used by Choo et al. (2018). Lastly, the level of expression for six genes of interest according to fold change was measured by normalising the comparative threshold (CT) cycle of target gene against reference gene, IMPDH2 utilising the equation: 2 ^ (CT IMPDH2 - CT target gene).

2.7 Amyloid beta immunofluorescent staining

The hippocampal neurons that underwent treatment were fixed with 4% paraformaldehyde (PFA) for 1 hour and rinsed in TBS (50 mM Tris, 150 mM NaCl) followed by 30 mins incubation in 1% BSA (Sigma) to block non-specific binding sites. Following this, incubation with the anti-beta amyloid primary antibody (1:500; Abcam; ab68896) was carried out at 4 ^{o}C

and left overnight. After being washed in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20), the neurons were incubated with secondary goat anti-rabbit conjugated with IgG-H&L Alexa Fluor ® 488 (1:2000, Abcam; ab150077) at room temperature for 1 hour followed by 3 times washing in TBST. The neurons were then counterstained with mounting media ProLong Gold antifade Reagent with DAPI (Invitrogen). Image from the samples were photographed using fluorescence microscope (BX41, Olympus) and neurons were quantified using DigiAcquis 2.0 software. Data were expressed as the percentage ratio of amyloid beta positive neurons (green fluorescence; excitation 495 nm, emission 519 nm) to total neurons (blue fluorescence; excitation 358 nm, emission 461 nm) (Zhao et al., 2016).

2.8 Statistical Analysis

All results were expressed as mean ± standard errors of the mean (SEM). One-way analysis of variance (ANOVA) with Dunnett's post hoc test were used to calculate the significance difference between treatment groups. Meanwhile, $^{*}P < 0.05$ was set as threshold of significance. Analysis were done using GraphPad Prism version 7.02 software (La Jolla, CA, USA).

3. Results

3.1 Embelin pre-treatment ameliorated STZ-induced neuronal damage in rat primary hippocampal neuronal culture

The primary hippocampal neuronal culture was treated with increasing embelin concentrations ranging from 2.5 to 160µM to examine the neurotoxicity effect of embelin to neuronal culture. Findings presented that cell viability significantly decreased when primary hippocampal neurons treated with embelin at concentrations from 20µM and above displayed (Fig. 1A). The IC_{50} of embelin for primary hippocampal neurons was found to be 37.5 µM. Thus, the concentration of embelin from 10µM and below was selected for the next neuroprotection assay. Further, the primary hippocampal neuronal cultures were treated with increasing concentrations of STZ for 24 hours and assessed by MTT assay (Fig. 1B) to obtain an optimal dose of STZ as an inducer for neuroprotection assay. The neurotoxicity effect of STZ on the primary hippocampal neurons was dose dependent and the IC_{50} of STZ was found to be 8mM. Therefore, 8mM of STZ concentration was selected for further neuroprotection assay.

In the neuroprotection assay, the primary hippocampal neurons were pre-incubated with embelin at concentration ranging from 0.04 to 10μM for 2 hours, which was then followed by the stimulation of STZ at 8mM for 24 hours. For the neuroprotection assay, neurons viability were seen decreasing to 38.92% ± 3.06% after exposure to 8mM STZ for 24 hours, while the pre-treatment with embelin (2.5, 5, and 10μM) for 24 hours significantly enhance neurons viability to 68.74% ± 2.96% ($P < 0.0001$), 60.09% ± 2.77% (***$P < 0.001$) and 56.98% ± 3.76% (**$P < 0.01$) (Fig. 2), respectively. Therefore, embelin at concentration of 2.5, 5 and 10μM were chosen for further gene expression and immunohistochemistry studies.

3.2 Changes in mRNA levels in the primary rat hippocampal neuronal culture

To observe the impact of STZ-induced neurotoxicity on gene expression, the mRNA expression level was studied using the real time PCR analysis. The expression levels of APP and Mapt mRNA were up-regulated by STZ (8mM) exposure in primary rat hippocampal neuronal culture when contrasted with the control. This up-regulation was reduced by embelin pre-treatment in contrast with the STZ treated group alone (Fig. 3A and B). However, elevated expression of Mapt mRNA level was seen in hippocampal neuronal culture with high dose of embelin at 10μM. On the other hand, upregulation of GSK3α and GSK3β mRNA levels were noted in STZ treated group compared to that of the control group. Moreover, 2.5μM embelin was seen to significantly decrease the expression of both mRNA levels. Nevertheless, there was a significant increment in GSK3β mRNA level by embelin treatment at 10μM (Fig. 4A and B).

Besides, STZ was observed to deplete SOD1 antioxidant mRNA in the hippocampal neuronal culture (Fig 5A). Meanwhile, embelin pre-treatment at 2.5μM and 5μM concentrations significantly up-regulated the SOD1 expression level with a significant value of ***$P < 0.001$ and **$P < 0.01$, respectively. Furthermore, an increment in inflammatory mRNA expression of NFκB was noted in the hippocampal neuronal culture of STZ treated group. However, pre-treatment of embelin suppressed the expression level of NFκB mRNA in a dose-dependent manner (*$P < 0.05$) with 1-fold changes in correlation with STZ treated group (Fig 5B).

3.3 Embelin pre-treatment inhibited amyloid beta protein expression level in the primary rat hippocampal neuronal culture

To examine whether embelin can inhibit amyloid beta via its neuroprotective effects, the protein expression of amyloid beta was studied using immunofluorescence staining method. As shown in Fig. 6A, the green amyloid beta-positive neurons were significantly increased in STZ-treated neuronal culture, whereas few amyloid beta-positive neurons were identified in the control group. Besides, the quantity of amyloid beta-positive neurons were seen to be reduced in a dose dependent manner when treated with embelin. Statistical result on the ratio of amyloid beta staining to total neuron number as in Fig. 6B suggested that STZ had significantly increase the expression of amyloid beta (25.63% + 2.73%, ****$P < 0.0001$). Contrary, embelin pre-treatment (2.5, 5 and 10μM) has reversed the Aβ-induced neuronal death (4.94% + 0.58%, ****$P < 0.0001$; 7.64% + 0.60%, ****$P < 0.0001$; 11.76% + 1.53%, **$P < 0.01$).

4. Discussion

An *in vitro* AD model was established by inducing the rat primary hippocampal neuronal cultures with streptozotocin (STZ). Streptozotocin (STZ) is a glucosamine-nitrosourea compound utilised as an experimental tool to induce AD-like condition. The key features of this model mimicking the clinical AD are Aβ fragments, Aβ deposits and total tau protein (Kamat, 2015). The present results demonstrated that embelin possess neuroprotective potential against STZ induced neurotoxicity in rat primary hippocampal neurons. This study is first of its kind demonstrating the neuroprotective effect of embelin on STZ induced AD-like condition in rat primary hippocampal neuronal culture. As of late, it has been demonstrated by Arora and Deshmukh (2017) that embelin administration had improved intracerebroventricular (icv) STZ-induced memory deficit in rats. The mechanism of action supporting the potential benefit of embelin on STZ induced AD-like condition was not studied in great detail. Therefore, this study was aimed to investigate the anti-AD effect of embelin in rat primary hippocampal neuronal cultures exposed to STZ. The exposure of cultured neurons to STZ resulted in neuronal death. The present findings demonstrated that pre-treatment with embelin (2.5-10μM) has significantly thwarted STZ-induced neurotoxicity and subsequent neuronal death as shown by MTT assay. These results suggest the potential of embelin as a neuroprotective drug against STZ-induced neurotoxicity without side effects. However based on our results, we found that embelin does not fully restore the neuronal viability. This is because more than 50% of neuronal death occurred

due to exposure of 8mM STZ during the treatment assay. Thus, pre-treatment with embelin can only lead to 70% increase in neuronal viability and it was significant when compared to STZ treated group alone.

In icv-STZ treated rats, alterations of brain insulin system lead to the insulin receptor (IR) and phosphatidylinositol-3 kinase (PI3K) signalling cascade dysfunction that induces insulin-resistant brain state (Šalković-Petrišić, 2008). This further leads to the activation of glycogen synthase kinase-3 (GSK-3) in which isoforms alpha and beta subsequently induce Aβ accumulation and tau hyperphosphorylation (Kamat, 2015), which share many features with a sporadic AD in humans. *In vitro* experiments of this study revealed a significant increase in APP and GSK-3α mRNA when neurons isolated from the primary rat hippocampus were exposed to STZ for 24 hours. This is supported by Phiel et al. (2003) clarifying the close relationship between the increase in GSK-3α activity and the processing of APP as well as generation of Aβ. Furthermore, according to Rajasekar et al. (2016) there was increased in APP expression level in STZ induced astrocytes for 24 hours. In this study, it was demonstrated that pre-treating the rat hippocampal neurons with embelin for 2 hours has significantly decreased the APP and GSK-3α mRNA expression level. However in the case of APP, our results are unusual with embelin treated groups demonstrated significant decreased below the baseline vehicle control level. Generally APP play an important role in synaptic maintenance as well as neuronal migration during early embryogenesis (van der Kant and Goldstein, 2015). Yet in this study, it is still unclear whether the reduction in APP mRNA level is due to embelin alone or some other factors that may involve in neuroprotection.

On the other hand, STZ treatment significantly increased the GSK-3β mRNA level. As the GSK-3β regulates the expression level of Mapt in AD condition, it can be said that the Mapt expression level should mimic that of GSK-3β. In this study, there was an increment of Mapt mRNA expression similar to GSK-3β (Mendes et al., 2009), but not significant compared to the control group. The present results showed that only rat hippocampal neurons pre-treated with embelin 2.5μM concentration had significantly reduced the mRNA expression level of Mapt and GSK-3β. In the case of higher concentration, the results were unusual with embelin at 10μM dose having a significant rise in GSK-3β mRNA level and a similar pattern that can be observed in Mapt mRNA expression even though it was not significant. One plausible reason for this is

that once a drug had reached the plateau of the dose-effect curve, there is a very little benefit but a significantly greater risk for toxicity at higher doses (Lowe and Lertora, 2013). This theory is supported by the neuroprotection results in this study showing a bell shape dose-response curve whereby the neuroprotective effect of embelin at 5 and 10µM dose started to reduce in comparison to 2.5µM dose (Fig. 3).

The present study investigated whether the STZ-induced AD-like pathology is associated with alteration in the level of SOD1 in primary rat hippocampal neuronal culture. It was found that the induction of STZ decreased the expression of SOD1 mRNA expression neuronal culture. Besides, Qu et al. (2012) stated that STZ can cause oxidative stress in the brain and cognitive disabilities in rats. In this study, embelin with 2.5 and 5µM dose significantly attenuated STZ-induced SOD1 downregulation in neuronal culture. According to literature, embelin possesses potent antioxidative activities (Mahendran et al., 2011a; Poojari, 2014). Hence embelin might exert its neuroprotective effects through antioxidant activity with upregulation of SOD1 mRNA expression.

In the case of NFκB, it was discovered that there was an increment in NFκB expression due to STZ-induced neurotoxicity, which is predictable with the outcomes found in literature (Choi et al., 2014; Rajasekar et al., 2016) with somewhat higher expression level than that of the baseline vehicle control. Pre-treatment of embelin has significantly ameliorated the NFκB mRNA expression caused by STZ in a dose-dependent manner. Besides, previous literature yielded the anti-inflammatory abilities of embelin (Mahendran et al., 2011a; Mahendran et al., 2011b; Poojari, 2014) whereby the bright orange hydroxybenzoquinone embelin-rich from *Embelia ribes* had been utilised traditionally to treat inflammation. However, all embelin pre-treatments had a slightly lower expression level of NFκB mRNA when compared to baseline vehicle control. This suggests that embelin may at least partially exert its anti-AD effect by acting as anti-inflammatory agent as NFκB involved in activating systemic inflammation.

The protective effect of embelin was further verified through immunofluorescence staining technique in which the effect of embelin on amyloid beta expression was explored. Laboratory evidences have revealed that accumulation of amyloid beta found in STZ-treated rats (El Halawany et al., 2017; Wang et al., 2017). The present study showed that pre-treatment of embelin significantly diminished the amyloid beta expression level induced by STZ treatment in

a dose dependent manner similar to APP mRNA level. Thus, this result further supported the gene expression study as APP gene generates amyloid beta through enzyme secretases known as alpha, beta and gamma (Zhang et al., 2011). It can be concluded that this study is the first to report the protective effects of embelin against amyloid beta induced by STZ treatment on primary rat hippocampal neuronal culture. Figure 7 summarize the potential mechanism of action of embelin in STZ-induced neurotoxicity in primary rat hippocampal neuronal culture.

In conclusion, embelin showed a promising anti-AD like effect in *in vitro* model. Embelin exerts its neuroprotective effect through GSK-3 pathway, reducing oxidative stress and neuroinflammation and preventing amyloidogenesis in STZ-induced neurotoxicity in rat hippocampal neuronal culture.

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Author contributions

MS and SB were involved in designing, conceptualising of the research project and data analysis. SB performed all the experiments and manuscript writing in its entirety. YS helped in data analysis and figures in the manuscript. IO gave critical feedback for this study. All authors gave their final approval for the submission of this manuscript.

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Legends

Fig 1. Viability of primary rat hippocampal neurons at various doses of embelin and STZ assessed by MTT assay. (A) Neuronal cultures were incubated with various concentrations of embelin for 24 hours. Embelin at a concentration up to 10μM for 24 hours shows no toxic effect on primary rat hippocampal neurons. (B) Neuronal cultures were incubated with various concentrations of STZ for 24 hours. STZ shows a dose-dependent cytotoxic effect on primary rat hippocampal neurons. Data are expressed as Mean ± SEM of three independent experiment (n = 3)

Fig 2. Neuroprotection effect of embelin on STZ-induced neurotoxicity in primary rat hippocampal neuronal culture. Hippocampal neuronal cultures were pre-incubated with various concentrations of embelin for 2 hours before stimulation of 8mM STZ. embelin at 2.5, 5 and 10μM exhibits significant protection against STZ-induced neurotoxocity. Data are expressed as Mean ± SEM of three independent experiment (n = 3) and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Fig 3. The effect of embelin treatment on STZ-induced neurotoxicity in primary rat hippocampal neuronal culture. A) APP and B) Mapt mRNA expression by real time-PCR. The expressions were normalized with IMPDH2. Data are expressed as Mean ± SEM of three independent experiment (n = 3) and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

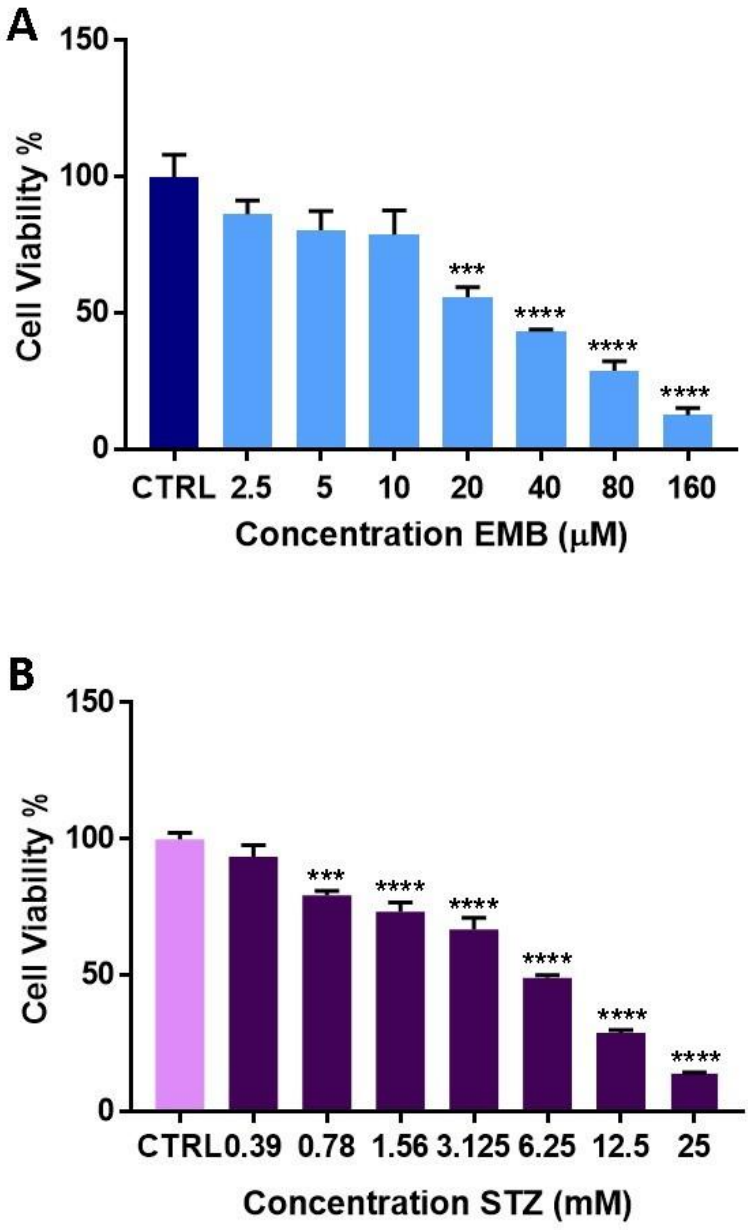
Fig 4. The effect of embelin treatment on STZ-induced neurotoxicity in primary rat hippocampal neuronal culture. A) GSK-3α and B) GSK-3β mRNA expression by real time-PCR. The expressions were normalized with IMPDH2. Data are expressed as Mean ± SEM of three independent experiment (n = 3) and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Fig 5. The effect of embelin treatment on STZ-induced neurotoxicity in primary rat hippocampal neuronal culture. A) SOD1 and B) NFκB mRNA expression by real time-PCR. The expressions were normalized with IMPDH2. Data are expressed as Mean ± SEM of three independent experiment (n = 3) and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Fig 6. The effect of embelin on STZ-induced amyloidogenesis. A) Photomicrographs of hippocampal neuronal culture treatment for 24 hr was (A1-3) Control (B1-3) STZ 8mM (C1-3) EMB 2.5μM + STZ 8mM (D1-3) EMB 5μM + STZ 8mM (E1-3) EMB 10μM + STZ 8mM Amyloid beta-positive neurons were stained in green while cells' nuclei were stained in blue. Representative photomicrographs were taken at magnifications of 40X B) Quantitative analysis for the ratio of Amyloid-beta neurons to total neuron number. Data are expressed as Mean ± SEM of three independent experiment ($n = 3$) and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Fig 7. Schematic diagram represents the mechanism of action of embelin in STZ-induced AD-like condition. The figure depicts STZ induced GSK-3 activation of both alpha and beta isoforms which further lead to amyloid pathology and tau pathology that mimics sporadic Alzheimer's disease. In addition, STZ increased NFκB mRNA and reduced SOD1 mRNA expression levels which contributed to neuroinflammation and oxidative stress respectively. Embelin treatment act as neuroprotectant agent by deactivating GSK-3 that cause reduction in APP and Mapt mRNA expression levels. Besides, embelin act as antioxidant by increasing SOD1 levels and in the case of inflammatory pathway, embelin reduces NFκB mRNA. With these effects, embelin treatment inhibit neurodegeneration and maintain healthy hippocampal neurons in the brain

Figure 1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Concentration EMB (μM)
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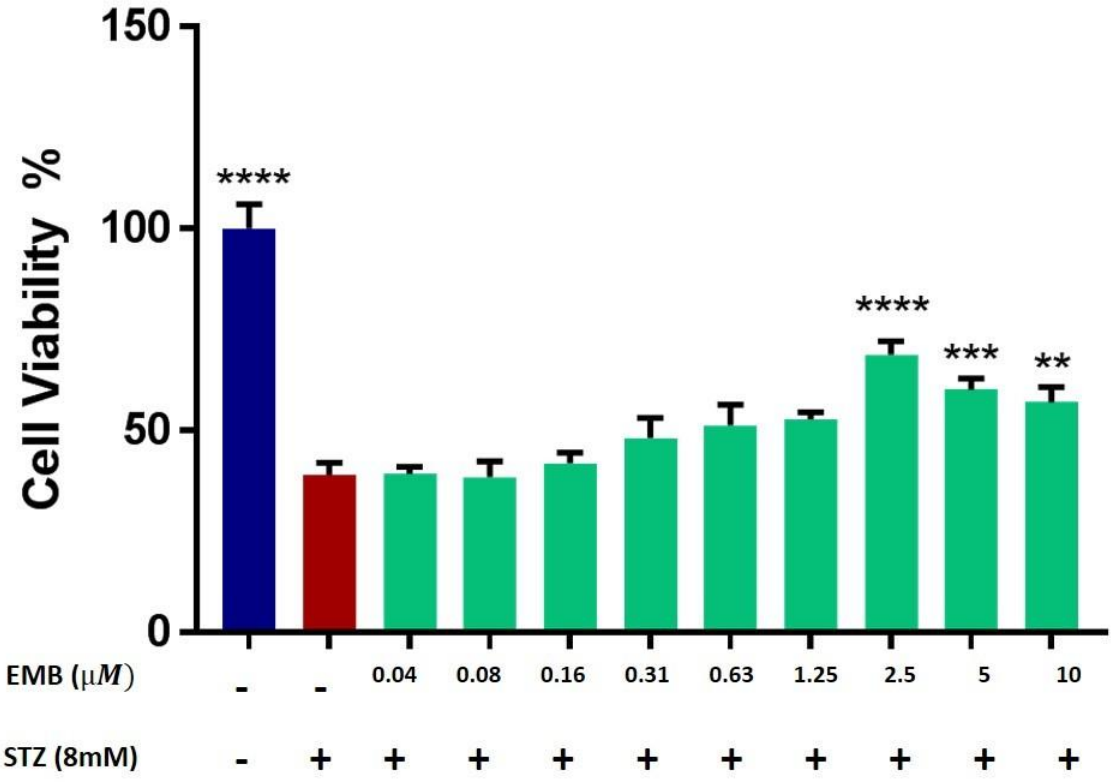
Concentration STZ (mM)

Figure 2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+

Figure 3

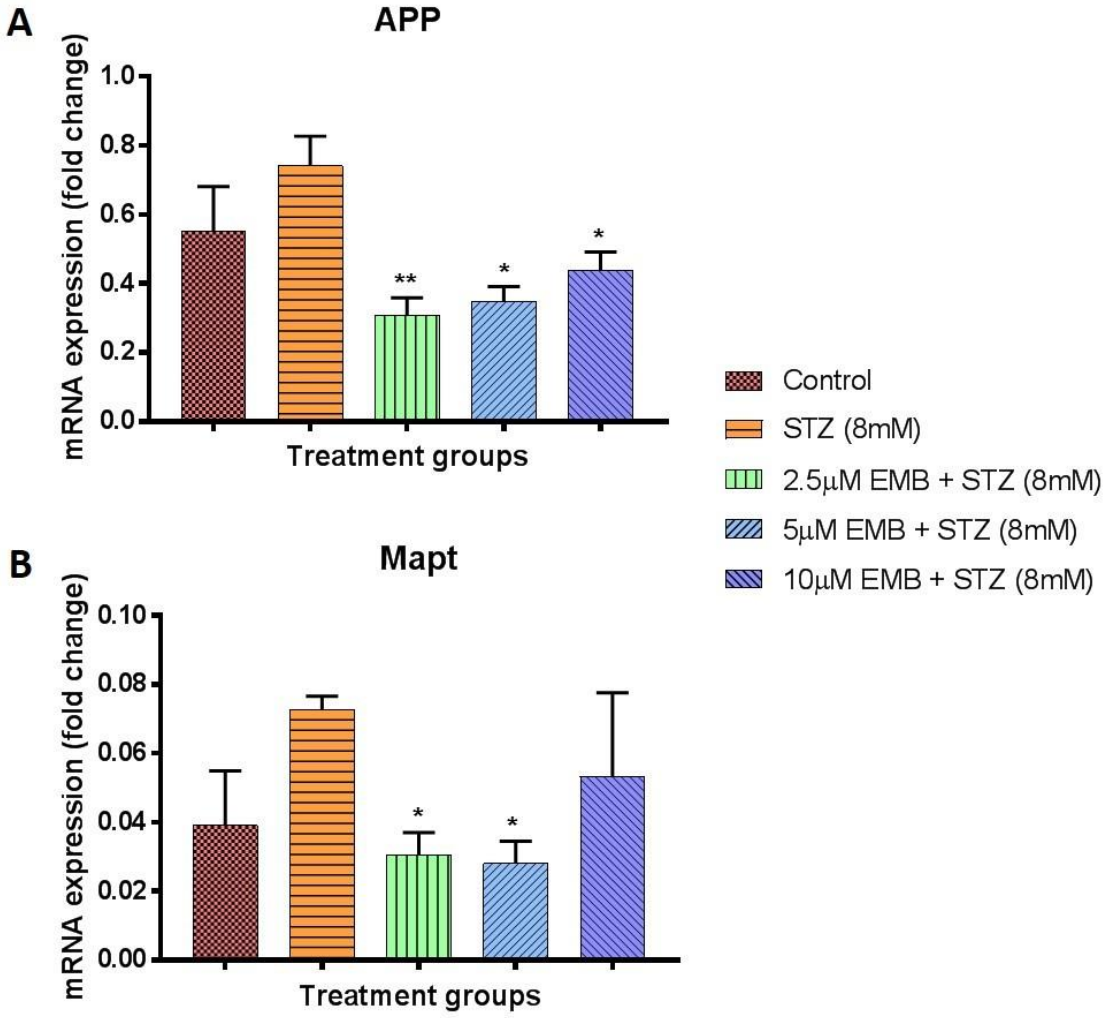

A
APP
mRNA expression (fold change)
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
Treatment groups
B
Mapt
mRNA expression (fold change)
0.10
0.08
0.06
0.04
0.02
0.00
*
*
Treatment groups
Control
STZ (8mM)
2.5μM EMB + STZ (8mM)
5μM EMB + STZ (8mM)
10μM EMB + STZ (8mM)

Figure 4

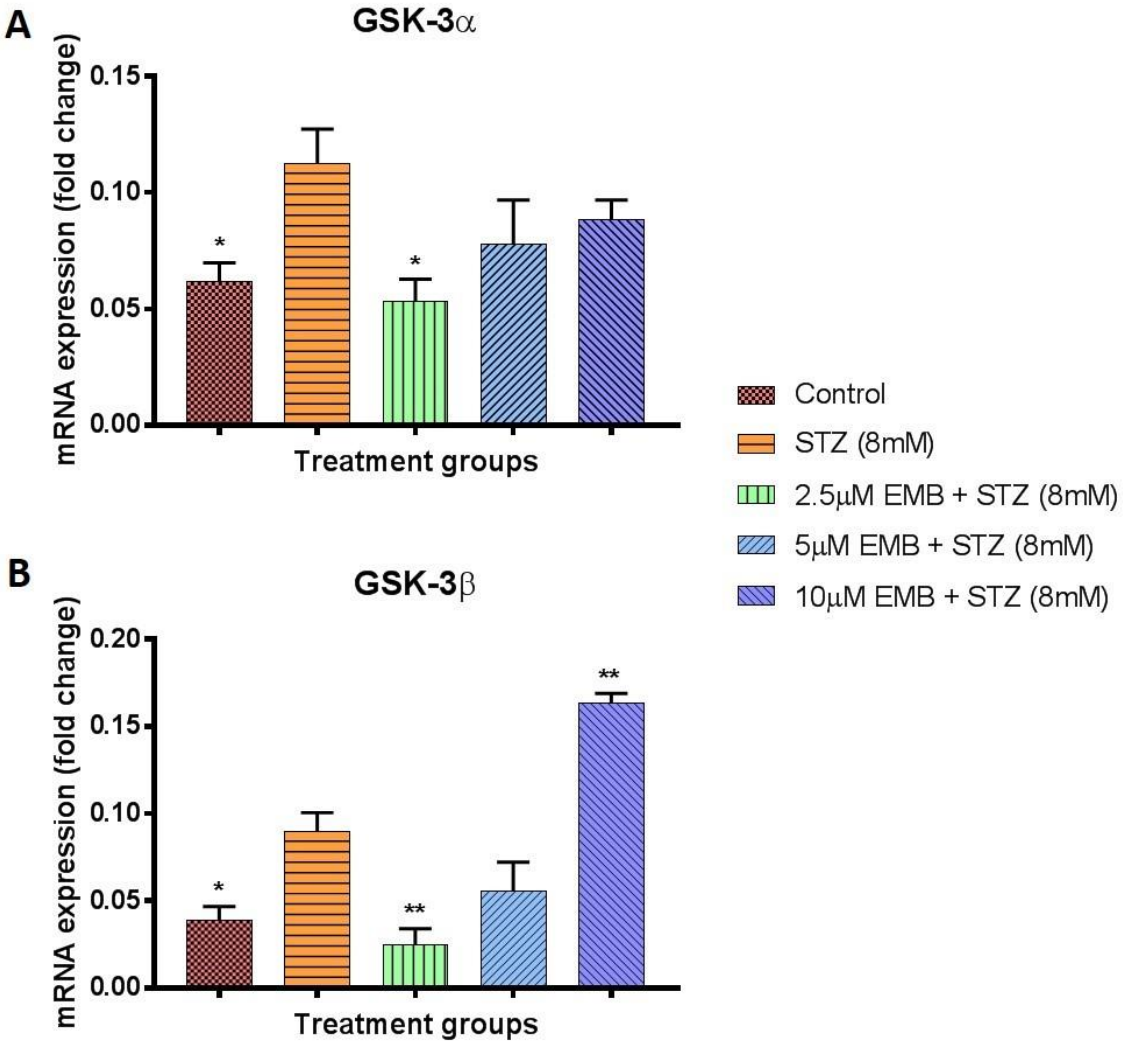

A
GSK-3α
mRNA expression (fold change)
0.15
0.10
0.05
0.00
*
*
Treatment groups
B
GSK-3β
mRNA expression (fold change)
0.20
0.15
0.10
0.05
0.00
*
**
**
Treatment groups
Control
STZ (8mM)
2.5μM EMB + STZ (8mM)
5μM EMB + STZ (8mM)
10μM EMB + STZ (8mM)

Figure 5

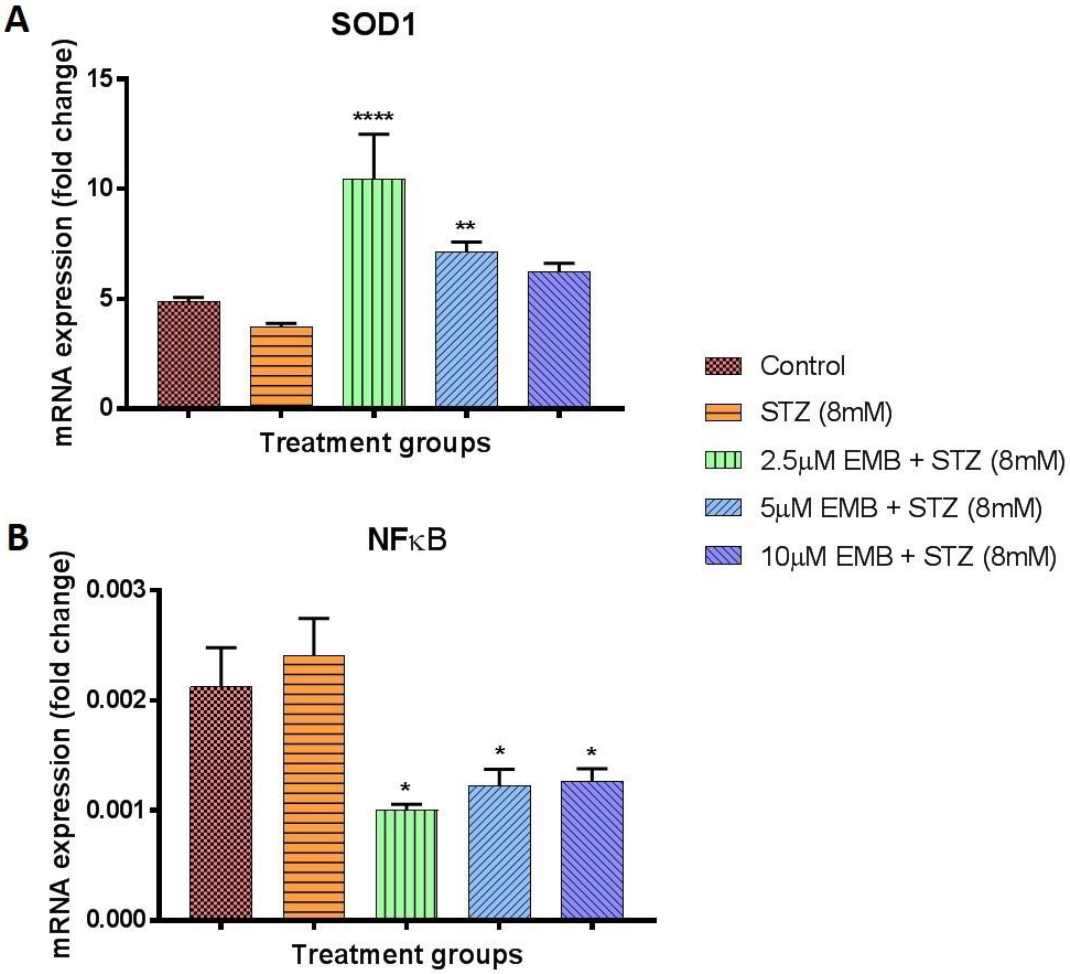

A
SOD1
mRNA expression (fold change)
15
10
5
0
****
**
Treatment groups
B
NFκB
mRNA expression (fold change)
0.003
0.002
0.001
0.000
*
*
*
Treatment groups
Control
STZ (8mM)
2.5μM EMB + STZ (8mM)
5μM EMB + STZ (8mM)
10μM EMB + STZ (8mM)

Figure 6

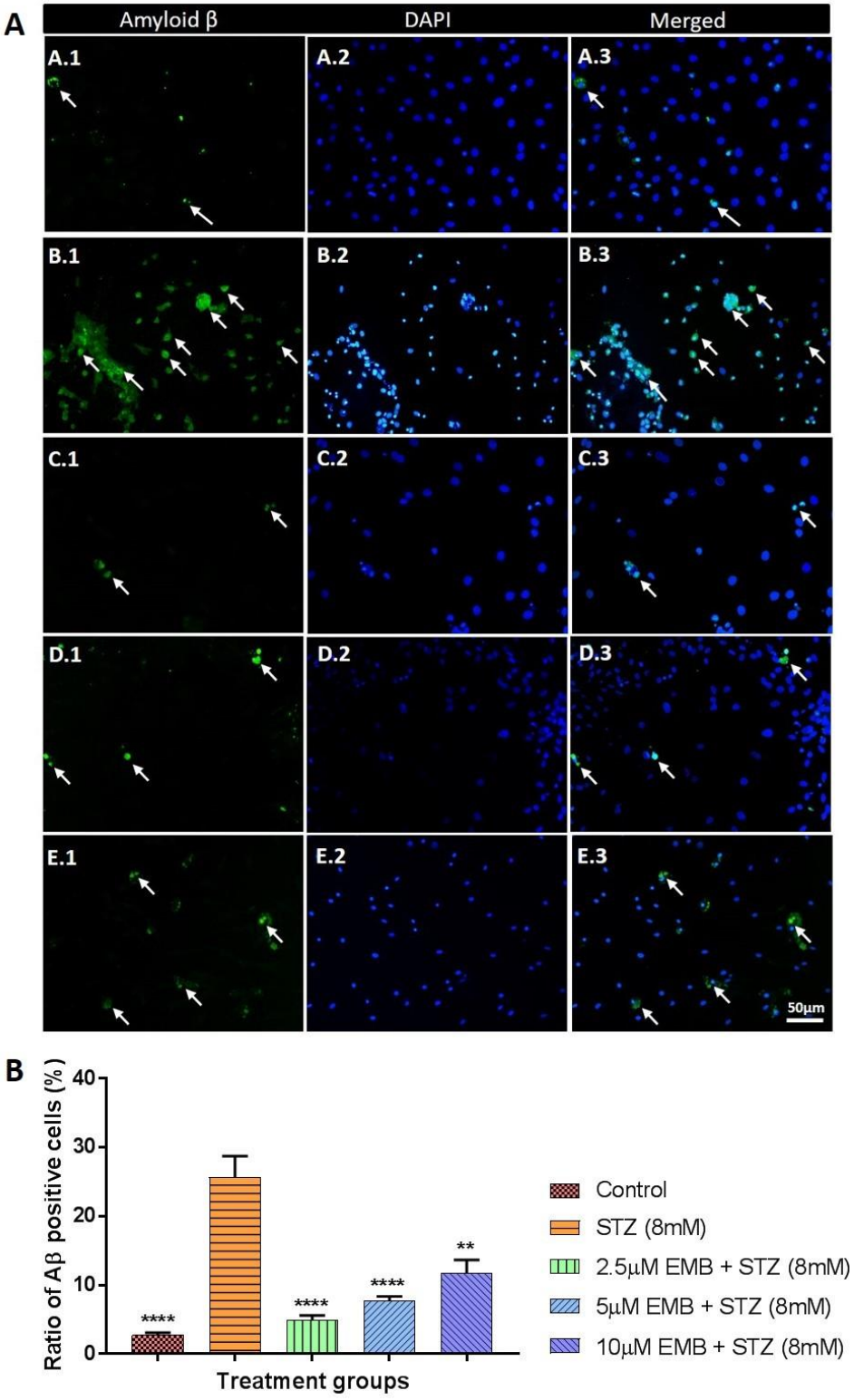
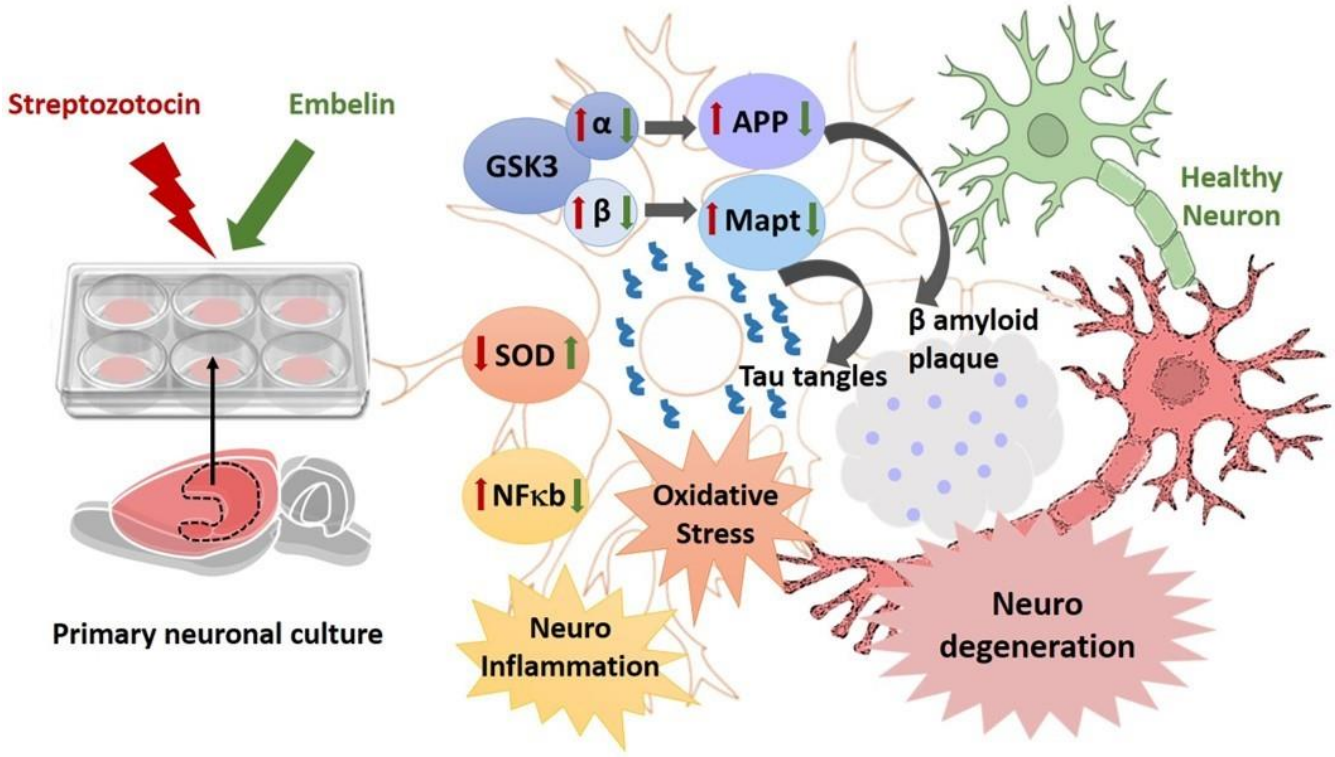

A
Amyloid β
DAPI
Merged
A.1
A.2
A.3
B.1
B.2
B.3
C.1
C.2
C.3
D.1
D.2
D.3
E.1
E.2
E.3
50µm
B
Ratio of Aβ positive cells (%)
40
30
20
10
0
****
****
****
**
Treatment groups
Control
STZ (8mM)
2.5µM EMB + STZ (8mM)
5µM EMB + STZ (8mM)
10µM EMB + STZ (8mM)

Figure 7


Streptozotocin
Embelin
Primary neuronal culture
GSK3
α
β
APP
Mapt
SOD
NFκb
Oxidative Stress
Neuro Inflammation
Tau tangles
β amyloid plaque
Healthy Neuron
Neuro degeneration

Chapter 7

7.1 Introduction

The blood–brain barrier (BBB) permeability is an important factor to be considered during drug designing targeting central nervous system (73). This is due to the complexity nature of the BBB that restrict the entry of most therapeutics agents into the brain causing the compound to be pharmacologically ineffective (74). Thus, an *in vitro* BBB model become a useful tool as a permeability assay to measure the ability of compounds of interest to cross the BBB (75). Porcine brain endothelial cells (PBECs) model tend to retain most of their *in vivo* BBB characteristics when compared to *in vitro* BBB model (76). In this last part of our study, we reported BBB permeability of the embelin using the PBEC models. In addition to that, drug-like properties of embelin were evaluated using molecular docking studies in order to identify binding sites for AChE and Aβ with embelin to further develop this compound into anti-AD drug.

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Embelin, a potent molecule for Alzheimer's disease: A proof of concept from BBB permeability, AChE inhibition and molecular docking studies

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Abstract: Embelin is well known in ethnomedicine and reported to have central nervous system activities. However, there is no report on blood-brain barrier (BBB) permeability of embelin. Here the BBB permeability of embelin was evaluated using *in vitro* primary porcine brain endothelial cell (PBEC) model of the BBB. Embelin was also evaluated for acetylcholinesterase (AChE) inhibitory activity and docking prediction for interaction with AChE and amyloid beta (Aβ) binding sites. Embelin was found to be non-toxic to the PBECs and did not disturb the PBEC tight junction function. The PBECs showed restrictive tight junctions with average transendothelial electrical resistance of 365.37±37.67 Ω.cm^2. Permeability assays were conducted from apical-to-basolateral direction (blood-to-brain side). Embelin showed apparent permeability (P_{app}) value of 35.46±9.09 × 10^{-6} cm/s with 85.53% recovery. *In vitro* AChE inhibitory assay demonstrated that embelin could inhibit the enzyme. Molecular docking study showed that embelin binds well to active site of AChE with CDOCKER interaction energy of -65.75 kcal/mol which correlates with the *in vitro* results. Docking of embelin with Aβ peptides also revealed the promising binding with low CDOCKER interaction energy. Thus, findings from this study indicate that embelin could be a suitable molecule to be further developed as therapeutic molecule to treat neurological disorders particularly Alzheimer's disease.

Keywords: embelin, blood-brain barrier, permeability, acetylcholinesterase inhibitor, molecular docking, amyloid beta peptides

1. Introduction

The blood brain barrier (BBB) is highly selective interface that separates the brain and the central nervous system (CNS) from the bloodstream [1, 2]. The BBB is composed of brain endothelial cells that formed the cerebral microvasculature which are interconnected by tight junctions [2, 3]. The endothelium facilitates and regulates substance entry between the blood and the CNS, as well as protecting the brain from harmful toxins and pathogens. Unfortunately, the protective nature of the BBB becomes a disadvantage as it also restricts the entry of many potential therapeutic agents [4]. Newly developed drugs targeting CNS disorders have the poorest success rate and often failed in clinical trial [5]. Around 98% of the potential drugs do not cross the BBB [6]. Due to their inability or

poor ability to cross BBB, they cannot be utilized for CNS related disorders [7] and this imposed major hurdles in pharmacological treatment of CNS disorders [8]. Therefore, it is very crucial to know whether a compound can cross the BBB and utilize this information during drug development before proceeding to clinical trial.

In vivo BBB methods provide the most reliable measurement for drug permeation due to the complex nature of the BBB, but with limitations of a low throughput and being labor intensive [9, 10]. Thus, good *in vitro* BBB model which demonstrates restrictive tight junctions reflected by high transendothelial electrical resistance (TEER) [11] and resembles the *in vivo* conditions is very important for effective screening for BBB permeability in drug discoveries [10, 12]. Several studies have reported on *in vitro* BBB models from variety of species including from mice, rats, cows, pigs, and human [12-15]. However, some of the reported BBB models suffered from low TEER indicating leaky tight junctions [12]. For instance, the human cerebral microvascular endothelial cell line (hCMEC/D3) which showed TEER values of less than 50 $\Omega.cm^2$ is probably not suitable for BBB permeability studies of small molecules even though it is of human origin [16-18]. *In vitro* BBB model from the PBECs has been reported to show well-developed tight junctions, polarized expression of functional transporters [19], which features comparable to that of human BBB. Additionally, the larger size of porcine brain compared to rodent brain enables higher cell yield, and it is relatively cheaper and more convenient to set up as porcine brains are by-product of the meat industry, and therefore do not require ethical approval [15, 20].

On the other hand, *in silico* modelling also allows for prediction of BBB permeation of compounds particularly for passive diffusion [9]. Modelling based on absorption, distribution, metabolism, excretion, and toxicity **(**ADMET)-related descriptors predicts the effectiveness and bioavailability of compounds based on pharmacokinetic properties [21]. Docking studies predict interaction between the compounds to their protein targets [22] which is also very crucial in drug designing. Recent reports indicated that embelin alleviates scopolamine-induced amnesia in rats and reversed memory impairment caused by streptozotocin (STZ) [23, 24]. However the BBB permeability of embelin and its mechanism of action are unknown. Here, assessment of embelin cytotoxicity, its effect on the BBB tight junction function and BBB permeability were performed using *in vitro* PBEC BBB model; its mechanism of action was determined using AChE inhibitory assay and docking studies, to investigate its potential as a new candidate for CNS therapeutic molecule particularly for the treatment of Alzheimer's disease (AD).

2. Results

2.1. Cytotoxicity of embelin towards the PBECs

Prior to the BBB permeability assay, viability of the PBECs in presence of embelin was determined. One-way ANOVA analysis shows a significant difference between the treatment groups and the cell viability ($F=6.134$; $P<0.001$). As shown in **Figure 1**, the PBECs treated with embelin from 10 to 90 μg/mL did not show reduction in viability when compared to the untreated cells. However, embelin at 100 μg/mL caused reduction in PBEC viability ($P < 0.01$) compared to the untreated cells.

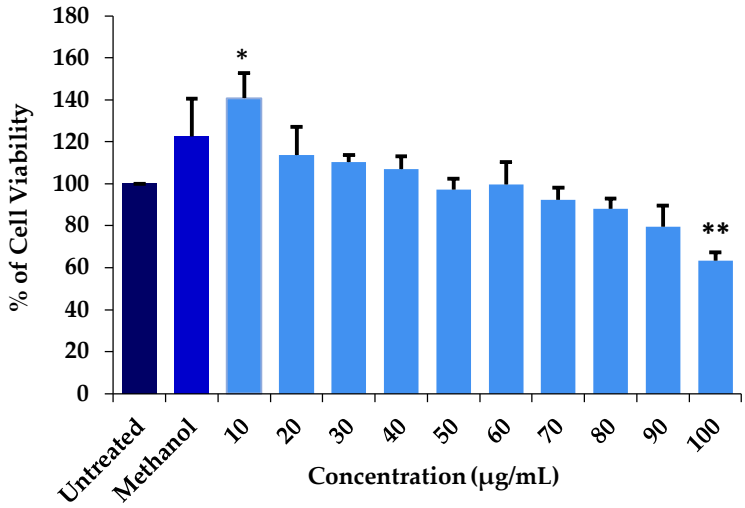


Figure 1: Effect of embelin at concentrations ranging from 10 - 100 µg/mL on PBECs viability, tested using (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay. Data are mean ± SEM, n = 3 independent experiments. * $P < 0.05$ ** $P < 0.01$, as tested using One-way ANOVA followed by Dunnett test.

2.2 Real-time TEER assay

Tight junction integrity of the PBEC monolayer was determined by measuring the TEER at 1 minute interval up to 60 minutes. Embelin was tested at 30 µg/mL, Dulbecco's modified Eagle's medium (DMEM) and dimethyl sulfoxide **(**DMSO) were used as negative and positive control respectively. As shown in **Figure 2**, embelin at 30 µg/mL did not disrupt the tight junction integrity and it was significant at $P< 0.001$ when compared to 100% DMSO.

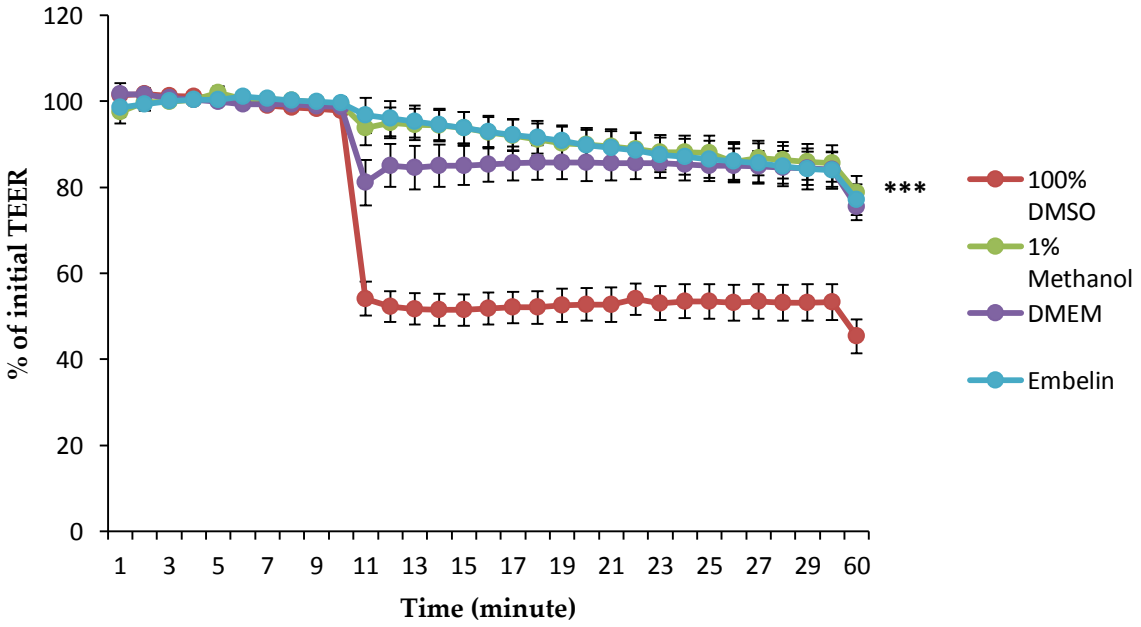


Figure 2: TEER across the PBEC monolayer was measured for 60 minutes at 1-minute interval using WPI STX-100C chopstick electrode pair connected to EVOM meter. Embelin (30 µg/mL), DMEM (negative control) and DMSO (positive control) were added separately to the inserts after minute 10 TEER was recorded. Data are mean ± SEM, n = 3 independent experiments.

2.3 *In vitro* BBB permeability assay

Permeability assay is conducted to measure the rate of BBB crossing for compounds. In this study, the rate at which embelin transverse across the PBEC monolayer from apical to basolateral direction (blood to brain side) was measured and reported as apparent permeability (P_{app}, cm/s). As shown in **Table 1**, embelin demonstrated P_{app} value of 35.46±9.09 x 10^{-6} cm/s with 83.53% recovery. Sodium fluorescein (NaF) as paracellular permeability marker compound showed low P_{app} of 2.47±0.82 x 10^{-6} cm/s, indicating that the tight junctional integrity was preserved during the assay.

Table 1: P_{app} values and % recovery of embelin and NaF

Insert	P_{app} (10^{-6}cm/s)	% recovery
Embelin	35.46±9.09	83.53±6.58
NaF	2.47±0.82	78.16±1.81

2.4 AChE inhibitory assay

Embelin was evaluated for its inhibitory activity of AChE from electric eel (*Electrophorus electricus*). Donepezil was used as a positive control and to validate the assay by comparing IC_{50} value obtained in this study with reported values [25]. Embelin was tested at a series of concentration from 3.68 to 58.9 µg/mL in order to determine the IC_{50} value using standard curve generated using Microsoft Excel. As shown in Figure 3, IC_{50} value obtained for embelin against AChE is 4974 µg/mL.

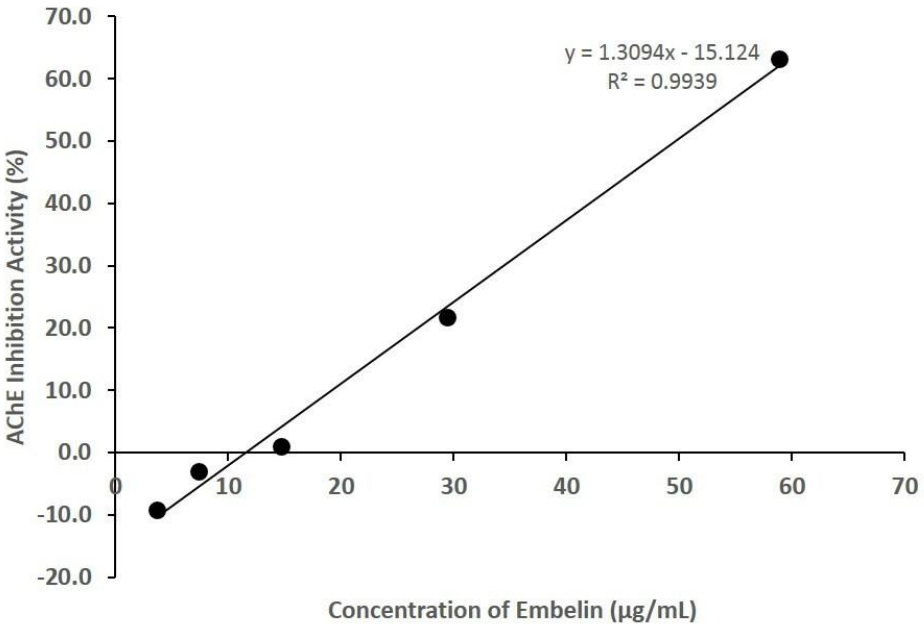
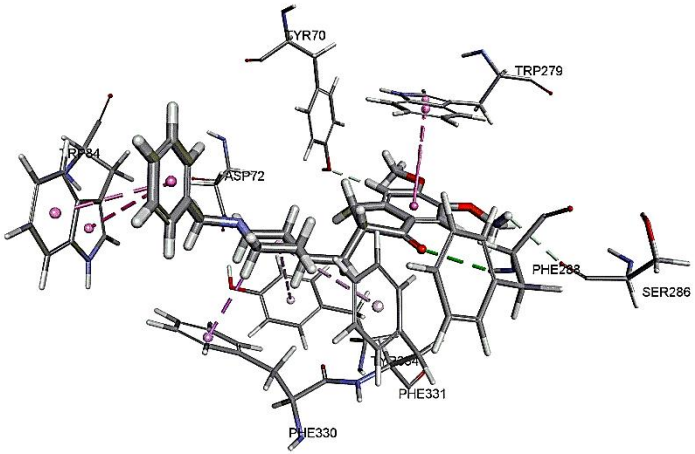


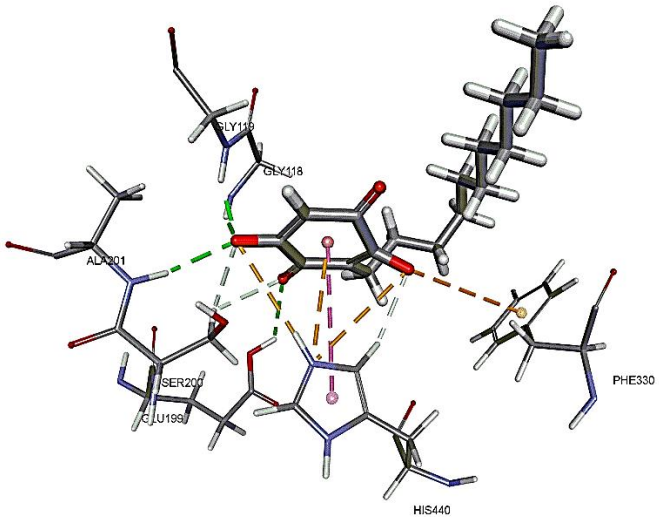
Figure 3: The anti-cholinesterase activity of embelin (3.68 - 58.9 µg/mL) using an *in-vitro* AChE inhibitory assay. The graph was plotted by keepin embelin concentration on X-axis against AChE inhibition activity (%) on Y-axis. IC_{50} values was calculated using standard curve generated using Microsoft Excel.

120 2.5 Molecular docking

121 The results for docking studies are expressed as interaction energy in -kcal/mol. The docked conformations of donepezil and embelin and key interactions are
122 summarized in **Table 2**. Based on the results, embelin has better binding to the AChE active site with the interaction energy of -65.75 kcal/mol compared to E2020.
123 Likewise, the docked conformations of embelin and Aβ and key interactions are summarized in **Table 3**. Binding to fibril 6Aβ $_{9–40}$ and 5Aβ $_{17–42}$ display high
124 interaction energy of -54.01 and -38.77 respectively when compared with Aβ monomers.

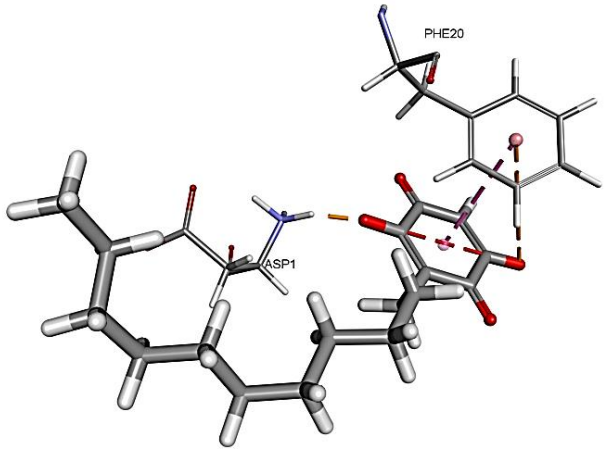
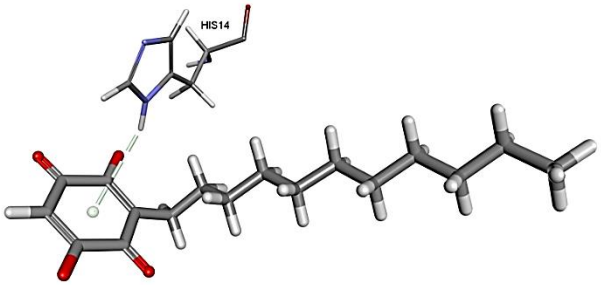
125 **Table 2:** Binding modes of embelin docked to AChE active sites

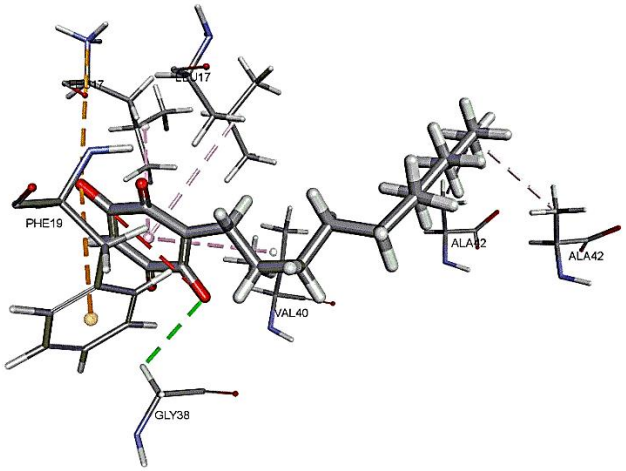
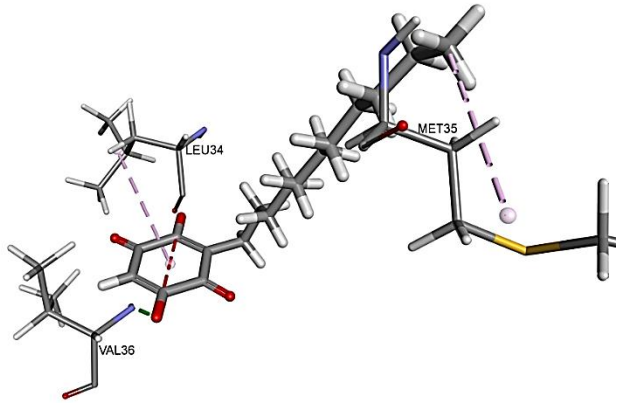
Compounds	Docked pose	CDocker Interaction Energy (-kcal/mol)	Non-bond Interactions
E2020 (reference)		48.5319	Hydrogen Bonds E2020 to PHE288 E2020 to ASP72 E2020 to SER286 E2020 to TYR70 Hydrophobic interactions -Pi-Sigma E2020 to PHE330 E2020 to TRP84 E2020 to TRP279 E2020 to PHE331 E2020 to TYR334

Embelin		65.7525	Hydrogen Bonds Embelin to GLY118 Embelin to GLY119 Embelin to GLY199 Embelin to ALA201 Embelin to SER200 Embelin to HIS440 Hydrophobic interactions -Pi-Pi Embelin to HIS440 Electrostatic interactions HIS440 to Embelin Embelin to PHE330

132

Table 3: Binding modes of embelin docked to Aβ active sites

PDB ID	Docked pose	CDocker Interaction Energy (-kcal/mol)	Non-bond interactions
1BA4 (Aβ 1-40)		34.1594	Hydrogen Bonds Embelin to ASP1 Hydrophobic interactions Embelin to PHE20 Electrostatic interactions Embelin to PHE20
1Z0Q (Aβ 1-42)		24.2574	Hydrogen Bonds Embelin to HIS14

2BEG **(5Aβ 17-42)**		38.7666	Hydrogen Bonds Embelin to GLY38 Hydrophobic interactions Embelin to ALA42 Embelin to ALA42 Embelin to LEU17 Embelin to LEU17 Embelin to VAL40 Electrostatic interactions Embelin to PHE19
2LMN **(6Aβ 9-40)**		54.0122	Hydrogen Bond Embelin to VAL36 Hydrophobic interactions Embelin to MET35 Embelin to LEU34

133

3. Discussion

To date, not a single study reported on BBB permeability of embelin [26]. According to Pathan et al. in order to cross the BBB, a compound should be in unionized form, molecular weight of less than 400 Da, log P value near to 2 and around 8-10 hydrogen bonds [8]. Embelin has all these characteristics, hence high possibility to permeate the BBB. Cell culture models are the most favored tools for assessing BBB permeation of compounds, giving information on passive permeability across cell membranes and also on carrier-mediated transport [27]. Therefore, we conducted *in vitro* BBB permeability assay of embelin using PBEC BBB model. Prior to the permeability assay, we established that embelin does not cause any toxicity to the PBECs up to 90 μg/mL, and embelin at 30 μg/mL does not affect BBB tight junctional integrity compared to the 100% DMSO. Based on our results, cells treated with methanol and embelin at 10 μg/mL have higher cell viability when compared to other groups. The increase in cell viability is due to stochastic effect which occurs when small stress (low concentration) is applied to the cells. However, stronger stress will disrupt the tight junctions and eventually will lead to cell death. Thus, this supported our results that cell viability decreased on cells treated with higher concentration of embelin. For the permeability assay, cell monolayer with TEER values exceeding 200 $\Omega.cm^2$ was used as the cells were considered to have minimal tight junction leakiness [28]. From the results, embelin demonstrated high P_{app} value of $35.46 \pm 9.09 \times 10^{-6}$ cm/s. The P_{app} value is comparable to that of donepezil ($30.6 \pm 9.09 \times 10^{-6}$ cm/s), reported by Liew et al. [11].

The high P_{app} value of embelin could consists of one or a combination of routes used by the compound to cross the BBB. Embelin could permeate via passive transcellular route across the cell membrane only or at the same time facilitated by membrane transporter expressed on cell membranes. To further dissect the mechanisms involved, bidirectional permeability assay could be conducted [11]. Additionally, P_{app} value of embelin is much higher than P_{app} of the paracellular marker compound used in this study i.e. NaF with P_{app} of $2.47 \pm 0.82 \times 10^{-6}$ cm/s. This could indicate that embelin largely cross the BBB via transcellular route and not paracellular route (via the tight junction) *in vitro*. This is further supported by the outcome of ADMET for BBB penetration for embelin which is level 1. According to Ponnan et al. [21], ADMET BBB penetration level 1 indicates high penetration of a compound across the BBB after an oral administration.

The CDOCKER was used for docking of all compounds. The CDOCKER is CHARMm-based docking algorithm that uses the CHARMm family of force fields and offers all the advantages of full ligand flexibility (including bonds, angles, and dihedrals) and reasonable computation times [29]. The CDOCKER algorithm adopts a strategy involving the generation of several initial ligand orientations in the active site of target protein followed by molecular dynamics based simulated annealing and final refinement by energy [30].

The molecular docking study was carried out to understand the binding mode of embelin within the active site of AChE using Discovery Studio suit 4.5 software. The x-ray crystal structure of AChE complexed with donepezil (or E2020) was retrieved from Protein Data Bank (PDB code: 1EVE). To validate the docking protocol, donepezil was first docked into AChE active site. As revealed by Kryger, et al. [31] phenyl ring of E2020 form π-stacking with Trup 84 and Phe 330 while another aromatic ring stacked with Trp279. Further, hydrogen bond was observed between Phe288 and ketone oxygen. The root mean square deviation (RMSD) and CDOCKER interaction energy (CDIE) were found to be 1.28Å and -48.53 kcal/mol respectively [31]. Embelin showed a promising favorable interaction with AChE binding site with CDOCKER interaction energy of -65.75 kcal/mol. This finding is consistent with AChE inhibitory activity for of embelin. Higher binding interaction energy indicating embelin may bound to the AChE active site which likely to trigger the catalytic site for its inhibitory activity for AChE [25].

Accumulation of research evidence over the last 20 years revealed that Aβ oligomers is associated with AD pathogenesis [32]. Therefore, there is a pressing need to find compounds that are able to promote anti-Aβ aggregation and clearance [33]. There are studies reported the potential of small molecules in converting toxic oligomers into non-toxic amorphous aggregates [34, 35]. Furthermore, small molecules could also contribute in morphological changes of amyloid fibrils to inert form [36, 37]. Since Aβ peptides are located in the brain, an efficient drug should be able to cross the BBB to interfere with their activities [33]. Similar to AChE docking study, embelin also interacted favourably with Aβ peptides as evident from CDOCKER interaction energy as shown in **Table 3**. These results revealed that embelin has potential to bind with Aβ peptides which may then slow down or degrade mature fibrils of Aβ peptides.

4. Materials and Methods

4.1 Materials

Iscove's modified Dulbecco's medium (IMDM 1X), Dulbecco's modified Eagle's medium (DMEM) without Phenol Red, Hank's Balanced Salt Solution (HBSS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}) and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, USA). Phosphodiesterase inhibitor (RO-20-1724) was obtained from Merck Chemicals Ltd. (Nottingham, UK). Corning Transwell® translucent polycarbonate filter inserts (product no. 3401, 12 mm diameter, 0.4 μm pore size, 1 x 10^8 pores/cm^2, 1.12 cm^2 growth area) were obtained from Corning (New York, USA). All other chemicals were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

4.2 Isolation of porcine brain microvessels and culture of the PBECs

The porcine brain microvessels were isolated using published method [10, 20] with slight modifications. Porcine brains from Department of Veterinary Services Penang abattoir (Sungai Pinang, Penang, Malaysia) were transported to the lab in ice-cold IMDM supplemented with FBS (10% v/v), penicillin (100 U/mL) and streptomycin (100 μg/mL) on ice. The brains were stored at 4°C overnight prior to the isolation of microvessels due to schedule of animal slaughter at the abattoir. Microvessels obtained were stored in liquid nitrogen until further use. Here, the cryopreserved microvessels were thawed and cultured in flasks according to previous studies [10, 19] to obtain the PBECs. The PBECs were then passaged onto plates or Transwell® after 4 days in culture. For cytotoxicity assay, the PBECs were cultured in 96-well plates at a seeding density of 3.2x10^4 cells/well, while for TEER measurement and permeability assay, the PBECs were cultured on the Transwell® inserts at a density of 1x10^5 cells/insert.

When culturing in wells and inserts, culture medium used was DMEM (with Phenol Red; Sigma D5546) supplemented with 10% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM) and heparin (125 μg/mL). To further induce BBB differentiation of the PBECs cultured on the inserts, at confluency, the culture medium was replaced by serum-free medium with added hydrocortisone (550 nM). The PBECs were also treated with 8-(4-chlorophenylthio-cAMP) (250 μM) and phosphodiesterase inhibitor (RO-20-7024) (17.5 μM) to increase tight junction tightness. Cell culture was conducted at 37°C in a humidified atmosphere with 5% CO_2 in air.

4.3 Cytotoxicity of embelin towards the PBECs

MTT assay was conducted as described by Mosmann [38] with slight modifications. Confluent PBECs in 96-well plate were incubated with embelin prepared in the culture medium at concentrations ranging from 10 to 100 μg/mL, for 1 hour at 37°C. After 1 hour, embelin solution was discarded and the PBECs were incubated with 100 μL MTT solution (1 mg/mL) prepared in DMEM without Phenol Red for 4 hours at 37°C. Untreated cells were used as control to represent total viable

cells. After 4 hours, the MTT solution was removed and replaced with 100 µL of propan-2-ol to dissolve formazan crystals formed. Absorbance was measured at 560 nm and 690 nm using Multiskan Go Microplate Reader (Thermo Fisher Scientific, MA, USA). The experiment was conducted in triplicate, in three independent experiments. Percentage of cell viability was calculated using following equation:

$$\%\ \boldsymbol{of\ cell\ viability} = \frac{(\boldsymbol{Absorbance_{560} - Absorbance_{690}})\ \boldsymbol{of\ treated\ cells}}{(\boldsymbol{Absorbance_{560} - Absorbance_{690}})\ \boldsymbol{of\ untreated\ cells}} \times \mathbf{100} \quad \textbf{(1)}$$

4.4 Real-time TEER assay

The assay was conducted to assess effect of embelin on the PBEC tight junction function. Approximately 24 hours after the serum-free medium change and treatment with 8-CPT-cAMP and RO-20-1724, TEER of the PBEC monolayer was measured using WPI STX-100C chopstick electrode pair connected to EVOM meter (World Precision Instruments Inc., Sarasota, FL, USA) for 1 hour at 1 minute interval. After minute 10 TEER was recorded, embelin (30 µg/mL), DMEM (negative control) and DMSO (positive control) were added to inserts separately and the TEER measurement was resumed until minute 60. TEER values of the cell monolayer were subtracted from value recorded for blank insert (without cells) and multiplied by growth surface area as shown by the following equation:

$$\boldsymbol{TEER}\ (\Omega.\boldsymbol{cm^2}) = \left(\boldsymbol{R_{cell\ monolayer} - R_{blank}}\right) \times \boldsymbol{A} \quad \textbf{(2)}$$

in which, $R_{cell\ monolayer}$ is the resistance (Ω) of insert with cells, R_{blank} is the resistance (Ω) of blank insert without cells and A is the surface area of insert (1.12 cm^2). For each insert, the TEER values obtained at the different time points were then normalized to initial measurement at t = 0 minute, and results are reported as percentage of initial TEER.

4.5 *In vitro* BBB permeability assay

Cell monolayers with TEER values more than 200 Ω.cm^2 were selected for permeability assay. Briefly, DMEM without Phenol Red supplemented with HEPES (25 mM) at pH 7.4 was used as buffer. Embelin was dissolved in DMSO at 2 mg/mL and diluted in the buffer to obtain a concentration of 30 µg/mL. NaF, a paracellular permeability marker compound was added to the embelin solution at concentration of 5 µM. To start the assay, the culture medium in the apical (filter insert) and the basolateral (well) compartments was aspirated and the filter inserts containing the PBECs were transferred to a 12-well plate containing the pre-warmed buffer on a shaker-incubator (THERMOstar, BMG Labtech, Germany). To start the experiment, 500 µL of the embelin solution was added to the apical compartment. The assay was carried out at 37°C for 60 minutes under stirring condition at 150 rpm. At the end of the assay, samples were taken from each compartment (400 µL from the apical and 1200 µL from the basolateral) for analysis.

The samples were processed using liquid-liquid extraction method using chloroform (organic phase) to extract embelin from the buffer (aqueous phase), followed by drying using nitrogen gas. When dried, methanol was added to tubes to re-dissolve embelin and the samples were analyzed using liquid chromatography tandem mass spectrophotometry (LC-MS/MS). Fluorescence of NaF was measured at 485 nm excitation and 535 nm emission using a fluorescence intensity plate reader (CHAMELEON™ V, Hidex, Finland). Apparent permeability (P_{app}, cm/s) of embelin was calculated using the following equation:

$$\boldsymbol{P_{app}}(\boldsymbol{x\ 10^{-6} cm/s}) = \frac{\boldsymbol{C_R.V_R}}{\boldsymbol{C_D.V_D.t.A}}\ \boldsymbol{V_D} \quad \textbf{(3)}$$

in which C_R and C_D are embelin concentrations (mol/cm^3) in the receiver and donor compartments i.e. basolateral and apical compartment respectively, V_R and V_D are the volumes in the receiver compartment (1500 μL) and the donor compartment (500 μL), t is the incubation time (60 minutes), and A is the surface area of the filter insert (1.12 cm^2). Values obtained were divided by 60 to express results in cm/s.

4.6 LC-MS/MS for quantification of embelin

The concentrations of embelin in the apical and basolateral compartments from the BBB permeability assay were quantified using LC-MS/MS. Standard solutions of embelinwere prepared in methanol with concentrations of 1, 2, 5, 7.5, and 10 μg/mL. The standard solutions and samples from the assay were injected at 10 μL into Agilent 6410 Triple Quad LC/MS comprising ZORBAX Eclipse plus C18 RRHD 2.1 x 150 mm and 1.8 μm column at a flow rate of 0.5 mL/min. The mobile phase was consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with a total run time of 4 minutes. The gradient elution was set as (i) 0-1 minute, 75% B; (ii) 1-2 minutes, 90% B; (iii) 2-3 minutes, 95% B; (iv) 3-4 minutes, 100% B. Electrospray ionization mass spectrometry condition was programmed with gas temperature of 300°C, nebulizer pressure of 40 psi, capillary voltage of 4000 V and drying gas flow at 10.0 L/minute. The MS scan parameters had a dwell time of 250 s with two products of 122.9 and 96 Da, performed in negative polarity mode.

4.7 *In vitro* AChE inhibitory assay

AChE inhibition of embelin was evaluated using the Ellman's method [25, 39] with slight modifications. A serial dilution of embelin which highest concentration lesser than 200 μM was prepared using DMSO and 0.1 M sodium phosphate buffer (pH 7.8), with DMSO final concentration of less than 1% (v/v). Sodium phosphate buffer (140 μL) was added to 96-well plate followed by sample solution (20 μL) and absorbance was measured at 412 nm. This reading served as blank. Then, AChE enzyme from electric eel (0.2 U/mL, 20 μL) was added to the wells and incubated for 15 minutes at room temperature. Finally, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (3mM, 10 μL) was added, followed by addition of acetylthiocholine iodide (ATCI) (15 mM, 10 μL). The rate of absorbance change was measured at 412 nm for 30 minutes with a Multiskan Go Microplate Reader (Thermo Fisher Scientific, MA, USA). Each assay was carried out with donepezil as positive control (0.015 μM). The reactions were performed in three independent runs. Each run of a sample was performed in triplicates and the IC_{50} values were determined from inhibition versus concentration plot. Below is the equation to calculate AChE inhibition.

$$\textbf{Percentage inhibition}\ (\%) = \left[\mathbf{1} - \left(\frac{\textbf{Sample}}{\textbf{Control}}\right)\right] \times \mathbf{100} \quad \textbf{(4)}$$

4.8 Molecular docking

All molecular docking studies were performed on Biovia Discovery Studio (BDS) 4.5 (www.3dsbiovia.com). For AChE, the x-ray crystal structure of AChE complexed with anti-Alzheimer drug (donepezil or E2020) was retrieved from the Protein Data Bank (PDB code: 1EVE) [31]. The water molecules were deleted, and hydrogen atoms were added. Finally, protein was refined with CHARMm at physiological pH. To validate the docking reliability, co-crystalized ligand donepezil was first re-docked to the binding site of AChE. Consequently, embelin was docked into same active site; 30 conformations of the compound were obtained through CDOCKER. The conformations with lowest energy were selected as the most probable binding conformation for each ligand. Docking studies was further carried out with Aβ peptide. Four receptors were chosen for Aβ peptide docking including monomers Aβ $_{1-40}$, Aβ $_{1-42}$ and fibril fragments 6Aβ $_{9-40}$ and 5Aβ $_{17-42}$ [33, 40-42]. The structures of Aβ were retrieved from Protein Data Bank and respective PDB ID are shown in **Table 3**. Embelin was docked by using CDOCKER program. The BBB prediction for embelin was also calculated using BDS 4.5.

4.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (La Jolla, CA). All data are presented as mean ± SEM and the samples were analyzed using One-way ANOVA followed y Dunnett test. Statistical significance was reported as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

5. Conclusions

This study for the first time has demonstrated the use of *in vitro* PBEC BBB model in the evaluation of embelin BBB permeability. This cell based model showed that embelin is able to cross the BBB which further supported by *in silico* results. Besides that, this study has found embelin as a promising AChE inhibitor as evidence from the AChE inhibition assay. Using molecular docking, we could predict that embelin has favourable binding mode within the AChE and Aβ peptide active sites. Hence, based from this study we discovered that embelin is a favorable compound which can be further developed into a potential therapeutic multipotent agent for AD.

Author Contributions: S.B conceived, performed experiments and wrote the manuscript. N.A.H helped in PBEC *in vitro* studies and gave valuable input in writing the paper. N.A. performed molecular docking and helped in writing of manuscript. I.O. was involved in LC-MS/MS and gave critical feedback for this study. S.R.Y and M.F.S. were involved in conceptualization, designing the study, interpreted, supervised the study and contributed in writing of the manuscript. All the authors read and approved the final manuscript.

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Abbreviations

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
AChE	Acetylcholinesterase
Aβ	Amyloid beta
ATCI	Acetylthiocholine iodide
BBB	Blood brain barrier
CNS	Central nervous system
CDIE	CDOCKER interaction energy
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTNB	5,5′-dithiobis (2-nitrobenzoic acid)
FBS	Fetal bovine serum
HBSS	Hank's Balanced Salt Solution
hCMEC/D3	Human cerebral microvascular endothelial cell line
IMDM	Iscove's modified Dulbecco's medium
LC-MS/MS	Liquid chromatography tandem mass spectrophotometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaF	Sodium fluorescein
P_{app}	Apparent permeability
PBECs	Primary porcine brain endothelial cells
PDB	Protein Data Bank
RMSD	Root mean square deviation
STZ	Streptozotocin

TEER Transendothelial electrical resistance

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Chapter 8

8.1 Strength and Limitations

Till date, there is no cure or effective treatment for AD. Currently available therapies do not completely eradicate all the symptoms of AD but show no to little improvements in cognitive functions which are greatly affected in AD (77). Majority of the potential anti-AD drug candidates failed at early phase of the clinical trials due to their ability to target a single mechanism of AD pathogenesis. This is one of the major problem in the drug development phase as existing preclinical approaches does not allow to study the complexity of the disease using one single disease model. Presently, researchers focus on the existing hypotheses which emphasize on a single factor at a time that contributes to AD (78). As mentioned in the previous chapters, AD is a multifactorial condition caused by disparity of a collective action of several factors (19). Thus, it is impossible to halt or restore AD back to normal state by just aiming at a single target rather than a multi-targeted approach.

Therefore, in the current project, the neuroprotective potential of embelin was explored by using varied experimental models of AD. Animal models representing different hypotheses were deployed to discover whether embelin has the ability to work through multiple mechanisms and affect positively to prevent the genesis of the key hallmarks of the AD. Moreover, this project also focused on the understanding the BBB permeability of embelin. BBB permeability is another key factor, which failed many potential drug candidates in the translational phase from basic screening to preclinical phase in the anti-AD drug development. To the best of our knowledge, this is the first study of embelin's BBB permeability using an *in-vitro* PBECs cell model.

The approach for the assessment of anti-AD effect of embelin was comprehensive ranging from biochemical, behavioral, pharmacological and molecular docking studies. The outcomes demonstrated excellent research findings as embelin has a remarkable improvement in learning and memory in both cholinergic and vascular dysfunction rat models. Likewise, this study also revealed that embelin has the potential to down-regulate the APP and MAPT mRNA expression as well as prevented over expression of amyloid beta protein induced by STZ in an *in-vitro* model of AD. As mentioned in the previous chapters, STZ induced sporadic AD-like condition, which reflects amyloid and tau hypothesis of AD. Furthermore, the molecular docking study predicted that embelin has a favorable binding at the active sites namely AChE and amyloid beta, which are among key hallmarks of AD. Besides that, based on docking study using ADMET, we found that

BBB penetration level for embelin was at level 1. This result predicted the ability of embelin to cross BBB, which are very much in-line with the *in vitro* results using PBECs cell model.

Thus, it is found that embelin could act through multiple mechanisms in AD pathogenesis. Relevant to this PhD thesis, we reported our findings and submitted to four different peer-reviewed journals. Out of that four, one has been successfully published in Frontiers in Pharmacology and the other three are currently under review.

The main limitation on the current work is that we designed the study based on the different experimental model to test each hypothesis instead of just using a single experimental model. As AD is a complex neurodegenerative disorder that is affected by the multifactorial mechanism in the pathogenesis of the disease. In the past few years, transgenic rodent became a promising model as many studies have been carried out to describe the disease progression as it can mimics various characteristic at a time which closely resemble human AD (79). However, most of the transgenic AD models created by genetic modifications rely on the early onset familial AD forms which only represent 5% of AD cases (26). Whereas the majority of AD cases are sporadic in nature for which no ideal animal model has been developed so far. Therefore, it would be insignificant to evaluate the effectiveness of potential drug candidates using a transgenic model as it can mimic only the early onset type familial AD form.

Another limitation in this study is that the vascular dementia and experiments using PBOCCA rats are not completely linked to AD but it is more general to dementia. Based on our results, embelin is neuroprotective on CCH-induced PBOCCA rats. Therefore, this compound may be promising for other types of dementia as well since it possesses several effects on the brain. On the other hand, we used STZ induced *in-vitro* rat primary hippocampal neuronal cultures as a model to address the amyloid hypothesis. However, it would have been good if we also use *in vivo* STZ model to link the *in vitro* results with the animal behavior to further support the effectiveness of embelin in AD.

Another limitation to the current study is uni-directional permeability assay using PBECs cell model, which is from apical to basolateral (blood to the brain). Although it is reported that embelin can cross the blood brain barrier, but the mechanism involved in BBB permeation of embelin was not explained. The bi-directional permeability assay which involved both apical to basolateral and

vice versa could clarify the potential involvement of any carrier-mediated or efflux protein in the BBB permeation of embelin (80).

8.2 Future work

The following is recommended as important and promising research directions for developing embelin as a potential anti-AD therapeutic drug.

a) Whilst embelin has been studied in various CNS related disorders, no study have been done on human due to lack of knowledge on safety and toxicity profiles. Therefore, establishing safety profiles for embelin is very crucial because it helps to minimize risk before moving forward to clinical trials.
b) Evaluating embelin in transgenic model could be another option to confirm its effectiveness.
c) Bidirectional permeability assay could be conducted in future to further explore the mechanisms involved in embelin BBB permeation.

8.3 Conclusion

This study has demonstrated the use of various experimental models addressing AD-related hypothesis for the evaluation of embelin as a therapeutic compound. In conclusion, the outcomes of this study give credence to embelin as a potential multi-targeted drug candidate for AD.

Chapter 9

9.0 References

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